APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1977, p. 512-517

Vol. 34, No. 5
Printed in U.S.A.

Determination of Pools of Tricarboxylic Acid Cycle and Related Acids in Bacteria

W. H. SIEGEL,† T. DONOHUE,[1] AND R. W. BERNLOHR[2]*

Departments of Biochemistry and Biophysics[2] and Microbiology and Cell Biology,[1] Pennsylvania State University, University Park, Pennsylvania 16802

Received for publication 1 June 1977

Methods for sampling, extracting, and quantitating the metabolic pools of organic acids from bacteria have been developed. The concentration of these metabolites was determined by a new gas chromatographic method that can quantitatively determine the levels of lactate, pyruvate, fumarate, succinate, malate, α-ketoglutarate, and citrate. Values obtained were confirmed by fluorimetric analyses of five of the individual acids. In *Escherichia coli*, pools range from about 1 to 5 μmol/g of dry weight, with a variation in replicate samples of 5 to 15%. Under similar conditions, these pools in *Bacillus licheniformis* are in the same range, although the pyruvic acid pool is significantly larger.

Although gas chromatographic methods for measuring the more common organic acids of biological importance exist (1, 7-10, 17, 18, 21), no reports have appeared that describe the methodology needed to determine the intracellular pools of these compounds in bacteria with an accuracy comparable to alternate procedures. Rosenqvist et al. (17) and Andersson et al. (1), using the gas chromatograph, have detected some tricarboxylic acid cycle intermediates, but the levels reported are different from those reported by Lowry et al. (11). These latter authors show that enzymatic methods that use fluorimetric analysis yield reproducible data for four of these acids: pyruvate, citrate, α-ketoglutarate, and malate. They also use rapid filtration as a method of harvesting cells (11), a method that we have confirmed to be preferable to centrifugation (5). In addition, Weibel et al. (20) have devised a rapid sampling technique for yeast and have measured pools of several metabolites, including pyruvate and citrate, by automated fluorimetric analysis. This technique, however, does not involve the washing of the yeast cells, but simply pumps samples of culture into precooled perchloric acid. Cook et al. (6) have used similar techniques with a concentrated cell suspension of *Alcaligenes eutrophus* H16.

The measurement of the intracellular pools of the tricarboxylic acid cycle and related acids in bacteria has been an elusive problem, because the levels of these acids are relatively low and exist in pools that are rapidly turning over (5, 18). Therefore, large quantities of cells must be harvested rapidly by a method that ensures the complete removal of the extracellular medium, since significant quantities of metabolic intermediates have been found in the extracellular medium of growing bacteria (5, 14). By filtration on large membrane filters (Millipore Corp., Bedford, Mass.), cells from about 100 ml of culture can be harvested, washed, and placed in acid within 30 s. When extracts from 10 such filters are combined, sufficient amounts of the tricarboxylic acid cycle acids are available for analysis by conventional gas chromatographic techniques. We describe this complete method and report on pool sizes of seven acids from two different bacteria.

† Present address: Medical School, Virginia Commonwealth University, Richmond, VA 23298.

MATERIALS AND METHODS

Cell growth conditions. *Bacillus licheniformis* strain A-5, type I (19), and *Escherichia coli* K-12 were used as the experimental organisms. *B. licheniformis* was grown in three different media. The first (glucose medium) consisted of minimal salts (14) supplemented with 20 mM glucose and 10 mM NH_4Cl. The second (glutamate medium) was composed of minimal salts (15) together with 10 mM NH_4Cl and 30 mM glutamate. Extracts of cells grown in these two media were analyzed by gas chromatography. The third (A) medium consisted of 250 mg of $MgCl_2 \cdot 6H_2O$, 50 mg of $CaCl_2 \cdot 2H_2O$, 2 mg of $MnCl_2 \cdot 4H_2O$, 40 mg of NaCl, and 10 mg of $FeCl_3 \cdot 6H_2O$ per liter supplemented with 65 mM potassium phosphate, pH 7.1, 15 mM glucose, and 5 mM $(NH_4)_2SO_4$. Extracts of cells grown in this medium were analyzed by fluorimetric techniques. The composition of the medium used to culture *E. coli* was that of Makman and Sutherland (13).

All cells were grown in 1-liter lots except those grown in the glutamate medium, which was 800 ml. Cultures were grown in 2.8-liter Fernbach flasks shaken on a gyratory shaker (New Brunswick Scien-

tific Co., New Brunswick, N.J.) set at 240 rpm at 37°C. Growth was monitored turbidimetrically by using a Klett-Summerson colorimeter equipped with a no. 54 filter. The dry weight of cells in a culture of *B. licheniformis* (5) or *E. coli* was determined by construction of individual standard curves.

Sample collection. Cells were removed from a culture flask with a 100-ml glass syringe or poured into a graduated cylinder and were filtered under vacuum on a 90-mm membrane filter apparatus (Millipore Corp.). A cylinder of glass tubing (90 by 160 mm) ground flat at both ends was used as a chimney. Before filtering, the filter paper, either a 0.45-μm-pore membrane filter (Millipore Corp.) or a Whatman GF/C glass-fiber filter, was moistened with either prewarmed (37°C) distilled water or medium (lacking the carbon and nitrogen sources) to ensure a good vacuum at the initiation of filtration. Immediately after filtration, the filter was washed with 10 ml of prewarmed medium lacking the carbon and nitrogen sources. The filters were then placed into cold acid to begin the extraction procedure. Total time elapsed from the start of sample collection to immersion of the filter paper in acid was less than 30 s.

We found that it was necessary to wash the cells on the filter paper immediately after filtration. Extracts prepared from unwashed *B. licheniformis* cells had significantly higher levels of pyruvate, fumarate, succinate, and α-ketoglutarate, acids that we have found to be present in relatively high concentrations in the extracellular medium. Lowry et al. (11) did not wash their filtered *E. coli* cells, but subtracted a predetermined value from reported levels. However, since larger quantities of these acids are present in the extracellular medium of a *B. licheniformis* culture than in a comparable *E. coli* culture, washing of cells before acid extraction is necessary.

Identical data were obtained whether the cells were washed once or three times or whether washed with growth medium lacking or containing the carbon and nitrogen sources. Therefore, a single wash with a buffered salt solution appeared to be sufficient to rid the filter paper of contaminating extracellular acids as efficiently as possible without adverse effects. In all cases, samples were taken when the cultures reached a density of 150 to 200 μg of dry weight per ml, a time in the growth cycle that is approximately two generation times before the end of exponential growth.

Preparation of cell extracts for gas chromatography. After the cells had been filtered and washed, the filters were incubated in cold 0.1 M HCl (70 ml) in a petri dish for 30 min. The filters were then lifted above the 0.1 M HCl solution, rinsed with distilled water, and discarded, and the contents of the petri dish (including the rinse) were centrifuged at 30,000 × *g*. The supernatant fluid (approximately 100 ml) was evaporated to dryness in a 1-liter round-bottom flask on a rotary evaporator (Rinco Inst. Co., Inc., Greenville, Ill.). Evaporation was initiated with the supernatant fluid at 4°C and water bath at 37°C. The residue was dissolved in water, quantitively transferred to a 250-ml round-bottom flask, and evaporated to dryness again. Further drying was accomplished by washing the residue twice with 3 ml of absolute ethanol, each wash being followed by drying on the rotary evaporator for 2 min, using a 34°C water bath. A final drying of 3 min at 32°C was done with a dry condenser flask to prevent water vapor from being swept into the dry flask when the vacuum was released. It is imperative that all samples be completely dry before addition of BF_3, because if not, the results will be widely variant and unpredictable. Preparation of methyl esters of the intracellular acids was done by a modification of the method of Hautala and Weaver (9). After the final drying, 4 ml of 14% BF_3 in methanol was added, and the mixture was incubated at room temperature overnight, after which 4 ml of 50% saturated ammonium sulfate and 2 ml of spectrophotometric grade chloroform were added. Methyl esters of the acids were extracted into the chloroform phase by 1 min of shaking, followed by a 45-min equilibation period. The chloroform phase was removed and stored over anhydrous sodium sulfate in a screw-cap vial with a Teflon-lined cap, from which samples were injected into the gas chromatograph.

Preparation of cell extracts for fluorimetric analysis. Many metabolites can be easily assayed fluorimetrically in protein-free cell extracts prepared with either $HClO_4$ or trichloroacetic acid. $HClO_4$ is usually preferred because most of the perchlorate can be removed by precipitation of the potassium salt at 0 to 2°C and because trichloroacetic acid has been found to be an inhibitor of several enzymes used in metabolite assays (12). The procedure used for the preparation of perchloric acid extracts is based on the one described by Lowry et al. (11) with minor modifications. After the cells had been filtered and washed, the filters were placed into 15 ml of 0.3 N $HClO_4$ on ice. The filter paper was incubated in the cold acid for 20 min with periodic agitation with a glass stirring rod. The filter paper was then squeezed dry with the glass rod, and the solution was centrifuged at 12,000 × *g* for 10 min at 4°C. The supernatant liquid was decanted and neutralized to a pH of approximately 6 with a calculated amount of 0.2 M K_2CO_3. After standing on ice for 5 min to facilitate the precipitation of the $KClO_4$, the precipitated $KClO_4$ was removed by centrifugation at 12,000 × *g* for 10 min at 4°C. The supernatant fluid was decanted and stored at −20°C until analysis.

Gas chromatography. The instrument used in this study was a Packard 409 gas chromatograph, equipped with temperature programming, dual flame ionization detectors, and an automatic attenuator. Signals were recorded with a 1-mV Honeywell electronic 194 recorder, and hydrogen was supplied by an Elhygen hydrogen generator (Milton Roy Co., St. Petersburg, Fla.). Dual stainless-steel columns (8 feet by 0.125-inch [ca. 2.4 m by 2.86-mm] outer diameter) packed with 15% Hi-Eff-2BP (an ethylene glycol succinate polyester) on 80- to 100-mesh Chromosorb W were purchased from Applied Science Laboratories (State College, Pa.).

Temperature programming was necessary due to the broad spectrum of volatilities exhibited by the methyl esters of the acids being investigated. Injection volume was 10 μl, with an attenuation of 128 and a range of 1. Initially the columns were kept at 100°C for 4 min, after which the temperature was increased

to 190°C at 10°C/min. The temperature remained at 190°C for 22 min. Between runs, the oven and columns cooled for 10 min. The temperatures of the injection ports and detector chamber were maintained at 200 and 250°C, respectively.

Flow rates for the sample column were 37.5 ml of He (carrier) per min, 375 ml of air per min, and 33.3 ml of H_2 per min. Rates for the reference column were 26.8 ml of He per min, 231 ml of air per min, and 70.6 ml of H_2 per min. Differences between these flow rates to the two columns resulted from different sensitivities of the two detectors and were necessary to adjust the output signal to zero.

Quantitation of the acids was done using peak height. For the purpose of providing a standard curve, a cell sample was taken, and, after centrifugation, the extract was divided in half. To one half, known amounts of the acids were added from a standard solution, while the other half served as a blank. Esters of the two samples were prepared as described above and analyzed. By plotting the extra peak height against the amount of standard acid injected, standard curves were constructed. There are several reasons for preparing standard curves in cell extracts. It is well known that extraction of organic acids from aqueous solutions is affected by pH and salt concentration. Since bacterial cells are cultured in various media, extracts will contain tricarboxylic acid cycle and related acids in the presence of a wide variety of buffering salts of differing concentrations. The efficiency of extraction of these acids will vary with each medium and bacterial strain used. Consequently, a standard curve must be prepared for each culture. By the use of this procedure, the problem of the efficiency of recovery of standard or unknown acids is circumvented and occasional internal standards are not necessary. The range of variation among replicate samples of standard acids in extracts appears to be somewhat dependent on the bacterial extract used. With *E. coli* extracts, the average variation among standard samples was ±7.2%, with a range of ±3 to 11.5% for individual acids. When standard acids were measured in *B. licheniformis* extracts, the average variation was ±12.7%, with a range of ±7.1 to 25.4%.

Fluorimetric analyses. Fluorimetric assays of metabolites were performed in a Turner model 111 fluorimeter set at 30× sensitivity and equipped with a 7-37 filter on the primary filter side and the combination of no. 4 and 48 filters on the secondary filter side. All assays were conducted at room temperature in selected fluorimeter cuvettes (12 by 75 mm) with a total volume of 4 ml. All solutions used in the fluorimetric assays were filtered through 0.45-μm membrane filters (Millipore Corp.) to remove any particulate matter that would interfere with the stability of the fluorimeter signal. Changes in fluorescence were recorded on a Sargent-Welch model SRLG recorder. The amount of a metabolite present in a cell extract was calculated by reference to a standard curve of change in fluorescence versus picomoles of metabolite for each particular metabolite assayed. Once individual standard curves were obtained for each metabolite, the reproducibility of these was checked periodically with freshly standardized solutions.

Pyruvate was assayed according to the direct assay procedure of Lowry and Passonneau (12) except that 50 mM phosphate buffer was used rather than imidazole-HCl buffer. The amount of pyruvate present was quantitated by measuring the loss of reduced nicotinamide adenine dinucleotide (NADH) fluorescence after the addition of lactate dehydrogenase (EC 1.1.1.27). Citrate was assayed according to the method of Passonneau and Brown (16). The amount of citrate present was quantitated by measuring the loss of NADH fluorescence due to the conversion of citrate to malate by the action of citrate lyase (EC 4.1.3.6) and malate dehydrogenase (EC 1.1.1.37).

The conditions used for the measurement of α-ketoglutarate were those of Narins and Passonneau (15). The amount of α-ketoglutarate present was quantitated by measuring the loss of NADH fluorescence after the addition of glutamate dehydrogenase (EC 1.4.1.3). Malate was assayed according to Method I of Lowry and Passonneau (12). The amount of malate present was quantitated by measuring the formation of NADH fluorescence due to the conversion of malate to oxaloacetate by malate dehydrogenase in the presence of glutamate-oxaloacetate transaminase (EC 2.6.1.1) at pH 9.9. To prevent changes in the pH of the reaction mixture caused by the presence of NH_4^+ in the enzyme preparations, the enzymes used in this assay were treated to remove most of the NH_4^+ from the enzyme preparations. Enzyme suspensions (for each day's assays) were layered on columns (0.9 by 10 cm) packed to a bed volume of 8 ml with Amberlite IR-120 ion-exchange resin (Mallinckrodt Chemical Works, St. Louis, Mo.) that had been converted to the Na^+ form. The enzymes were then eluted from this column with sufficient 20 mM tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 8) containing 0.02% bovine serum albumin to give a final protein concentration of approximately 2 mg/ml for the malate dehydrogenase and 1 mg/ml for the glutamate-oxaloacetate transaminase preparation.

Fumarate was assayed according to the method of Lowry and Passonneau (12). The amount of fumarate present was quantitated by measuring the formation of NADH due to the conversion of fumarate to oxaloacetate by fumarase (EC 4.2.1.2) and malate dehydrogenase in the presence of glutamate-oxaloacetate transaminase at pH 9.9. Since the assay conditions for the determination of fumarate were identical to those for the determination of malate and involved only the addition of fumarase to a cuvette after the determination of malate was completed, it was necessary to remove most of the NH_4^+ from the fumarase suspension by the above procedure. The concentration of fumarase after it was eluted from the ion-exchange column was approximately 2 mg/ml.

Materials. The following chemicals were obtained in grade A form from Calbiochem: lithium L-lactate, disodium fumarate, malic acid, and α-ketoglutaric acid. Lyophilized citrate lyase and fluorimetric-grade NADH were purchased from Boehringer-Mannheim Corp. Citric acid monohydrate was a product of Allied Chemical, and succinic acid was furnished by Fisher Scientific Co. Sodium pyruvate (type II and dimer free, anhydrous), 14% boron trifluoride in methanol, and all other enzymes and biochemicals were purchased from Sigma Chemical Co. Spectrophotometric-

grade Insta-Analyzed chloroform was obtained from Baker. All other chemicals used were of reagent grade. The selected fluorimeter cuvettes (12 by 75 mm) were obtained from VWR Scientific. Double-distilled water was used to prepare all solutions.

RESULTS AND DISCUSSION

Figure 1A shows the elution profile of the methyl esters of lactate, pyruvate, fumarate, succinate, malate, α-ketoglutarate, and citrate from the Hi-Eff-2BP column. In contrast to the reports of others (8, 21) who used alternate liquid phases, dual peaks for the elution of pyruvate and α-ketoglutarate were not observed. In a variety of experiments using other liquid phases on Chromosorb W and either methyl (9) or trimethylsilyl esters (7), we have found the procedure outlined in Materials and Methods to be preferable. Attention was directed toward the seven acids listed above because preliminary results showed that these are the prime organic acids found in extracts of both *E. coli* and *B. licheniformis* cells.

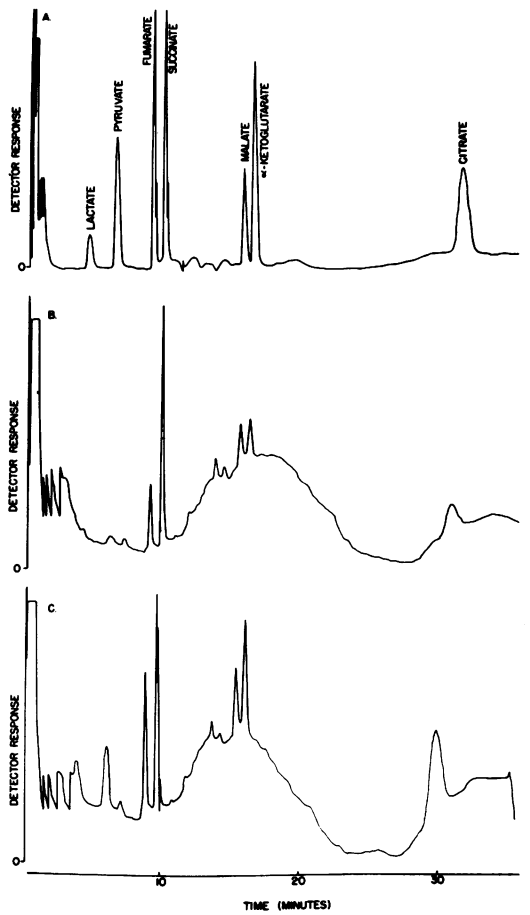

FIG. 1. *(A) Elution profile of the gas chromatographic separation of the methyl esters of seven standard acids. All conditions are given in Materials and Methods. The automatic attenuator of the gas chromatograph was activated for the solvent (1-min rentention time) and for the dimethylfumarate (8.5 min) and dimethyl succinate (9.5 min) fractions. (B) A typical chromatogram of an extract from E. coli. (C) Same extract as in (B) to which 6 nmol of lactate, 23 nmol of pyruvate, 3 nmol of fumarate, 7 nmol of succinate, 6 nmol of malate, 5 nmol of α-ketoglutarate, and 13 nmol of citrate have been added. The automatic attenuator was activated by the dimethyl succinate fraction.*

When the esters from an extract of *E. coli* cells are chromatographed, the typical profile shown in part B (Fig. 1) is seen. The elution pattern of acids from bacteria is much less complex than that observed from urine or liver (7). The base line drift observed in the 10- to 22-min and 30-min areas is usually present and leads, on occasion, to some difficulty in drawing base lines under elution peaks. Since this drift is not significant in the initial analysis of each day, it is assumed that it is caused by the breakdown of the liquid phase of the column during analysis. Our columns were normally kept at 100°C overnight with carrier gas flowing at the rates given in Materials and Methods.

Part C (Fig. 1) was produced by adding known quantities of acids to a fraction of the same extract shown on part B. In this way, standard curves for each of the seven acids were prepared; an example of one set of standard curves is shown in Fig. 2. It was necessary to prepare the esters used in constructing standard curves in crude cell extracts, because the yields of individual esters varied considerably and because of differential loss of some of the acids during the original concentration of the extracts.

We determined the pool sizes of the seven acids in extracts of log-phase *E. coli,* grown on glucose, and in *B. licheniformis* grown on either glucose or glutamic acid; Table 1 compiles the results of these studies. The first column represents data resulting from analyses of four *E. coli* extracts, each assayed in duplicate. The values shown for pyruvate, malate, and α-ketoglutarate are very similar to those reported by Lowry et al. (11) in *E. coli,* whereas Rosenqvist et al. (17) reported values for lactate, succinate, and malate that are significantly different, and did not report values for α-ketoglutarate or citrate.

In *B. licheniformis* cells growing logarithmically on glucose, the pool sizes were unique but in the same range as in *E. coli.* Pools of several of the acids differed significantly (pyruvate, succinate, malate) from those of *E. coli* and liver (11, 18). In confirmation of the work of Lowry

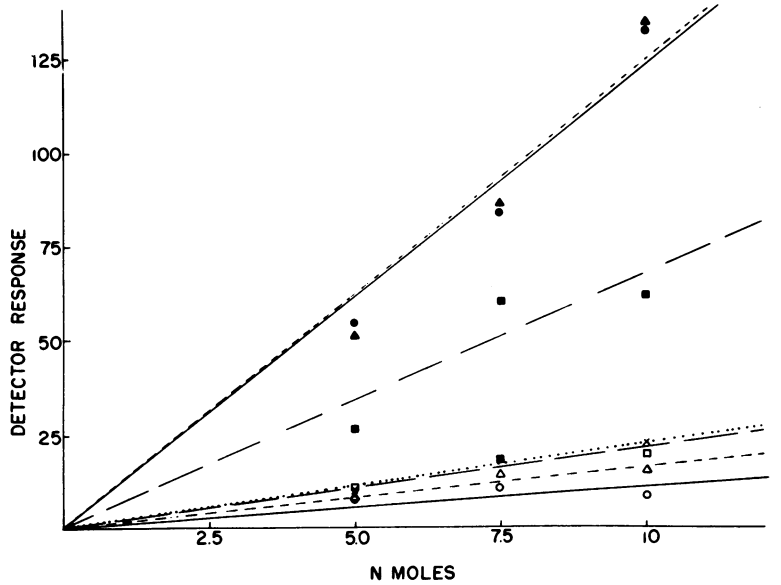

FIG. 2. *Typical standard curves plotting the detector response as a function of fumarate (●), succinate (▲), α-ketoglutarate (■), malate (×), citrate (□), lactate (△), and pyruvate (○) concentration. Each point represents the average of four analyses.*

TABLE 1. *Pool sizes of carboxylic acids from log-phase cells*

Acid determination	Microbial source (μmol/g of dry wt)			
	E. coli[a]	*B. licheniformis* grown on:		Fluorimetric analysis of acids from glucose-grown cells[c]
		Glutamate[a]	Glucose[b]	
Lactate	0.9 ± 0.1	4.5 ± 1.6	0.8 ± 0.2	
Pyruvate	2.5 ± 0.2	15.0 ± 2.4	14.7 ± 1.0	(13.7 ± 1.1)
Fumarate	0.9 ± 0.04	0.8 ± 0.2	0.22 ± 0.05	(0.12 ± 0.02)
Succinate	4.9 ± 0.1	4.5 ± 0.3	0.98 ± 0.2	
Malate	3.4 ± 0.4	1.8 ± 0.3	1.0 ± 0.3	(1.1 ± 0.2)
Ketoglutarate	1.3 ± 0.05	3.7 ± 0.6	1.8 ± 0.4	(2.0 ± 0.2)
Citrate	1.6 ± 0.2		2.4 ± 0.5	(2.9 ± 0.1)

[a] Average of four analyses.
[b] Average of eight analyses.
[c] Average of four fluorimetric analyses.

et al. (11) reporting on the pools of several of these acids, growth of cells on alternate carbon sources led to altered pools of at least some tricarboxylic acid cycle intermediates. When grown on glutamate, *B. licheniformis* cells accumulated larger pools of lactate, succinate, and α-ketoglutarate than cells grown on glucose (Table 1). Therefore, pool sizes of these acids are not constant in different biological systems, necessitating the measurement of pool sizes and flux rates in many isotope studies.

Values obtained from analyses of *B. licheniformis* show a greater variability (up to 30%) than those from *E. coli*, and we attribute this to inefficient washing of the cells on the filters. We found much larger quantities of most of the organic acids in the culture filtrate of *B. licheniformis* cells as compared with *E. coli* cultures. Also, the amounts of these acids changed with the stage of the life cycle of the cells and with the nutrient medium used. Similar changes were observed for pyruvate and citrate pools in yeast (20), which, interestingly, are very similar to the pyruvate and citrate pools of *B. licheniformis*. These facts underscore the necessity of thorough washing of cells when measuring the levels of intracellular pools. Lowry et al. (11) extensively discuss the importance of filtering rather than centrifuging cells as the method of harvesting and have shown that their pool sizes represent

an accurate picture of real intracellular pools. Previous work from our laboratory (5) on the measurement of amino acid pools supports this thesis.

The reliability of the method was additionally confirmed by fluorimetric analysis of five of the organic acids in extracts of *B. licheniformis* cells grown on glucose. These data are shown in parentheses in the last column of Table 1 and indicate a significant concordance of the two methods of analysis. We have also measured the pyruvate pool in *B. licheniformis* cells (grown on glutamate) by the spectrophotometric method of Bücher and Pfleiderer (4) and found similar values to those reported in Table 1. Using fluorimetric methods, Lowry et al. (11) report values of pyruvate, citrate, α-ketoglutarate, and malate in *E. coli* extracts that are similar to ours. Thus the results obtained using the new gas chromatographic procedure are similar to those obtained by fluorimetry.

The method presented here is convenient and reliable, although variation among samples can be somewhat large in *B. licheniformis*. Some of this variability must be due to the nature of the biological system (i.e., the presence of relatively large amounts of many of these acids in the extracellular medium), as values from logarithmically grown *E. coli* cells were quite precise. In cases in which the pool size of more than one acid is desired, this method is more rapid than fluorimetric methods. The detection of as little as 1 nmol of acid is common, and the sensitivity could be increased by concentration of the final chloroform solution. When taken as a whole, we believe that this method of sampling, extraction, and gas chromatographic analysis provides reliable values for the intracellular pool sizes of most of the intermediates of the tricarboxylic acid cycle.

ACKNOWLEDGMENTS

We thank V. Clark and D. Chard for technical assistance.

This investigation was supported by Public Health Service grant AI 05096 from the National Institute of Allergy and Infectious Diseases and grants GB 35377X and PCM 74-22671 from the National Science Foundation. This work was initiated in the Department of Microbiology at the University of Minnesota.

LITERATURE CITED

1. **Andersson, I., B. Norkrans, and G. Odham.** 1973. Determination of the Krebs cycle related keto acids of microorganisms by capillary gas chromatography on glass columns. Anal. Biochem. **53**:629–638.
2. **Bernlohr, R. W.** 1967. Changes in amino acid permeation during sporulation. J. Bacteriol. **93**:1031–1044.
3. **Bernlohr, R. W., and V. Clark.** 1971. Characterization and regulation of protease synthesis and activity in *Bacillus licheniformis*. J. Bacteriol. **105**:276–283.
4. **Bücher, T., and G. Pfleiderer.** 1955. Pyruvate kinase from muscle. Methods Enzymol. **1**:435–436.
5. **Clark, V. L., D. E. Peterson, and R. W. Bernlohr.** 1972. Changes in free amino acid production and intracellular amino acid pools of *Bacillus licheniformis* as a function of culture age and growth medum. J. Bacteriol. **112**:715–725.
6. **Cook, A. M., E. Urban, and H. G. Schlegel.** 1976. Measuring the concentrations of metabolites in bacteria. Anal. Biochem. **72**:191–201.
7. **Dalgliesh, C. E., E. C. Horning, M. G. Horning, K. L. Knox, and K. Yarger.** 1966. A gas-liquid chromatographic procedure for separating a wide range of metabolites occurring in urine or tissue extracts. Biochem. J. **101**:792–810.
8. **Harmon, M. A., and H. W. Doelle.** 1969. Gas chromatographic separation and determination of micro quantities of the esters of the tricarboxylic acid cycle and related compounds. J. Chromatogr. **42**:157–169.
9. **Hautala, E., and M. L. Weaver.** 1969. Separation and quantitative determination of lactic, pyruvic, fumaric, succinic, malic, and citric acids by gas chromatography. Anal. Biochem. **30**:32–39.
10. **Ishitoya, Y., C. Itoh, N. Osawa, I. Hashimoto, T. Iwanaga, and T. Nambara.** 1970. Gas chromatographic separation of tricarboxylic acid cycle components. Clin. Chim. Acta **27**:233–240.
11. **Lowry, O. H., J. Carter, J. Ward, and L. Glaser.** 1971. The effect of carbon and nitrogen sources on the level of metabolic intermediates in *E. coli*. J. Biol. Chem. **246**:6511–6521.
12. **Lowry, O. H., and J. V. Passonneau.** 1972. A flexible system of enzymatic analysis. Academic Press Inc., New York.
13. **Makman, R. S., and E. W. Sutherland.** 1965. Adenosine 3',5'-phosphate in *Escherichia coli*. J. Biol. Chem. **240**:1309–1314.
14. **Moses, V., and P. Sharp.** 1972. Intermediary metabolite levels in *E. coli*. J. Gen Microbiol. **71**:181–190.
15. **Narins, R. G., and J. V. Passonneau.** 1974. Alpha-ketoglutarate: fluorimetric determination, p. 1580–1584. *In* H. U. Bergmeyer (ed.), Methods of enzymatic analysis, 2nd Engl. ed., vol. 3. Academic Press Inc., New York.
16. **Passonneau, J. V., and J. G. Brown.** 1974. Citrate: fluorimetric determination, p. 1565–1569. *In* H. U. Bergmeyer (ed.), Methods of enzymatic analysis, 2nd Engl. ed., vol. 3. Academic Press Inc., New York.
17. **Rosenqvist, H., H. Kallio, and V. Nurmikko.** 1972. Gas chromatographic analysis of citric acid cycle and related compounds from *E. coli* as their trimethylsilyl derivatives. Anal. Biochem. **46**:224–231.
18. **Sauer, F., J. D. Erfle, and M. R. Binns.** 1970. Turnover rates and intracellular pool size distribution of citrate cycle intermediates in normal, diabetic and fat-fed rats estimated by computer analysis from specific activity decay data of ^{14}C-labeled citrate cycle acids. Eur. J. Biochem. **17**:350–363.
19. **Tuominen, F. W., and R. W. Bernlohr.** 1971. Pyruvate kinase of the spore-forming bacterium *Bacillus licheniformis*. I. Purification, stability, regulation of synthesis and evidence for multiple molecular states. J. Biol. Chem. **240**:1732–1745.
20. **Weibel, K. E., J.-R. Mor, and A. Fiechter.** 1974. Rapid sampling of yeast cells and automated assays of adenylate, citrate, pyruvate and glucose-6-phosphate pools. Anal. Biochem. **58**:208–216.
21. **Zaura, D. S., and J. Metcoff.** 1969. Quantification of seven tricarboxylic acid cycle and related acids in human urine by gas-liquid chromatography. Anal. Chem. **41**:1781–1787.