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FORMIMINOGLYCINE TRANSPORT SYSTEMS IN *CLOSTRIDIUM ACIDI-URICI* [1,2]

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Abstract

The formiminoglycine (FIG) transport in ***Clostridium acidi-urici*** required metabolic energy and Na. Either uric acid or xanthine could be employed as energy source for the FIG transport. Other compounds such as D-glucose, sucrose, glycerol, pyruvic acid, succinic acid, and D-lactic acid were not able to support the transport of FIG. Ions of Li^+, Rb^+, and NH_4^+ could not substitute for the role of Na^+. Optimal temperature for the transport of FIG was 37^0C.

The transport systems were highly specific for FIG. Among the forty eight common compounds examined, including formiminoaspartic acid, formiminoglutamic acid and glycine, no compound other than FIG could be taken up by the FIG transport systems.

Introduction

The soil anaerobic bacterium, *Clostridium acidi-urici*, can only grow on uric acid, xanthine, guanine and hypoxanthine. To investigate the metabolism of this organism by means of tracer techniques, the fermentable purines labeled with ^{14}C at either C-4 or C-5 would be especially useful as known from the breakdown of these purines by the organism (Robinowitz, 1963). Unfortunately, such kinds of labeled compounds are not commercially available at the present time.

Formiminoglycine (FIG) is a key intermediate metabolite in the purine fermentation by *C. acidi-urici* and *C. cylindrosporum* (Rabinowitz, 1956). C-1, C-2, and C-3 of FIG come from C-4, C-5 and C-8 of the fermentable purines, respectively. Therefore, if the purine-fermenting *Clostridia* can take up and utilize FIG, it is obvious that [^{14}C]FIG can be employed as tracer in the metabolic studies of these two organisms. Previous results have shown that

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in *C. acidi-urici* cells FIG could be taken up by two kinetically distinguishable transport systems (Chen and Robinowitz, 1972). In this report, the properties of FIG transport systems in *C. acidi-urici* are described.

Materials and Methods

Growth of C. acidi-urici

Media and conditions for growth of *C. acidi-urici* (ATCC 7906) have been described by Rabinowitz (1963). The procedures were modified as follows for the purpose of transport assay. Lyophilized stock culture of the organism was transferred to 10 ml of media in a tube containing per liter: uric acid, 2 g; 10 N KOH, 1.2 ml; 70% $K_2HPO_4 \cdot 3H_2O$, 1.3 ml; 5% $MgSO_4 \cdot 7H_2O$, 0.7 ml; 0.2% $FeSO_4 \cdot 7H_2O$, 1.3 ml; 0.6% $CaCl_2 \cdot 2H_2O$, 0.7 ml; yeast extract, 1 g; agar, 1.5 g; mercaptoacetic acid, 2 ml and were incubated at 37° for 1 to 2 days. Four ml of the 10 ml culture then was inoculated to a 50 ml bottle stoppered with a serum cap containing urate minimal medium whose composition was the same as that of 10 ml culture except that agar and Difco yeast extract were omitted. Twenty five ml of the 50 ml culture was transferred to 25 ml of urate minimal medium. The culture was incubated at 37° and was used for transport assay immediately.

Synthesis and purification of [1-^{14}C]FIG and [2-^{14}C]FIG

[1-^{14}C]FIG and [2-^{14}C]FIG were chemically synthesized by the method of Tabor and Rabinowitz (1957). Thirty one mg of unlabeled glycine (0.413 mmole), 2 mg of [1-^{14}C]glycine or [2-^{14}C]glycine (0.027 mmole, specific activity =56 mCi/mmole), 70.9 mg of formamidine hydrochloride (0.88 mmole), 0.1 ml of redistilled pyridine, and 1 ml of redistilled formamide were placed in a flask equipped with a magnetic stirrer and a condenser stoppered with a $CaCl_2$ tube. The mixture was stirred for 24 hours at 50° in an oil bath, the precipitates formed were removed from the mixture by filtration and were redissolved in small volume of 5% formic acid, pH 1.7. The filtrate was adjusted with 97–100% formic acid to pH 1.7. These two solutions were applied to a Dowex-50 column, X12, 200–400 mesh (1.2×45 cm) which had been equilibrated with 1 M $HCOONH_4$, pH 3.4. The column was washed with distilled water until the effluent was free of radioactivity, [^{14}C]FIG was eluted with a linear gradient by running 100 ml of 1 M ammonium formate, pH 3.4 into 100 ml of H_2O at a flow rate of 0.2 ml per minute. [^{14}C]FIG and unreacted [^{14}C]glycine have been separated completely by this method. Fractions in the [^{14}C]FIG peak were pooled. Water and ammonium formate were removed by evaporation. [^{14}C]FIG was redissolved in small volume of water, transferred to a vial and lyophilized overnight. Recoveries of [^{14}C]FIG ranged

from 45–57%. The specific radioactivity was 2.1 mCi/mmole as determined colorimetrically. The [^{14}C]FIG thus made was 99% radiochemically pure as examined by radioautography.

Transport assay

The method of Ames (1964) was modified for uptake assays of anaerobes. Tubes with serum caps containing labeled substrate, chloramphenicol (final concentration=100 μg/ml), and 5 mM NaCl in 0.05–0.1 ml water were flushed with nitrogen gas for 10 min. After preincubating the tubes at 37° for 5 min. 0.9–0.95 ml of the clostridial culture was injected into each tube to start the transport assay. The tubes were incubated at 37° for the desired length of time. The transport was stopped by rapid filtration of the content on a Millipore filter or Selection filter (pore size: 0.45 μ, 25 mm diameter). The filtration was finished in a few seconds. The tubes were rinsed once with 3 ml of washing buffer (0.15 M Tris HCl buffer, pH 7, containing 0.4 M NaCl). The filter was washed two more times with 3 ml of the washing buffer. The Millipore filter without being dried was transferred into a vial containing 10 ml Bray's solution for scintillation counting. Transport rate was defined as the number of μmole of substrate taken up by cells equivalent to one gram dry weight per minute.

Paper chromatography

The following solvent systems were employed for paper chromatography. All of these solvent systems are acidic because FIG is labile in alkaline solvent. Solvent 1 is *t*-butanol-formic acid-H_2O (70:15:15, V/V/V); Solvent 2, methanol-chloroform-90% formate (3:3:1, V/V/V); Solvent 3, *n*-butanol-acetic acid-H_2O (12:3:5, V/V/V). A decending paper chromatography was employed.

Determination of the dry weight of ***C. acidi-urici*** *cells*

The dry weight of *C. acidi-urici* cells was determined by the method of Hardman and Stadtman (1963) with modification. The following relationships were obtained: dry weight of 10^8 *C. acidi-urici* cells=0.0799 mg; 0.1 scale of A_{660} of the cell suspension was equivalent to 0.0286 mg dry weight per ml.

Fractionation of yeast extract powder

Four grams of yeast extract powder (NBC) was dissolved in 20 ml H_2O and adjusted to pH 3.0 with 6 N HCl. The solution was then applied to a Dowex-50, X8 colomn (H^+ form, 100–200 mesh, 2.6×15 cm). The column was washed with H_2O until A_{260} of the effluent was below 0.1. The effluent (Effluent 1) which containing neutral and acidic compounds was kept for further fractionation.

Compounds adsorbed on the Dowex-50 column were eluted with 1 N NH_4OH. The eluate was neutralized with formic acid and concentrated by flash evaporator and lyophilizer. This fraction was called "Cationic Fraction".

Effluent 1 was concentrated with a flash evaporator at 40°C under reduced pressure to a small volumn. The solution was adjusted to pH 9.5 with NH_4OH and introduced to a Dowex-2, X8 column (OH^- form, 100-200 mesh, 2.6×15 cm). The column was washed with H_2O. The effuent was evaporated to dryness at 40° under reduced pressure. This fraction was called "Neutral Fraction".

Compounds adsorbed on the Dowex-2 column was eluted with 1 N formic acid. The eluate was neutralized with NH_4OH and concentrated with a flash evaporator. This fraction was called "Acidic Fraction".

Results

Requirement for energy source

Uric acid, Xanthine and some compounds which have been commonly used as energy source were examined for their ability to support the transport of FIG by *C. acidi-urici*. The results, shown in Table 1, indicated that only uric acid and xanthine could effectively serve as energy source. D-fructose and D-galactose were also able to support the FIG transport to a lesser extent (about 34% that of uric acid). D-glucose, sucrose, glycerol, pyruvic acid, and succine acid apparently could not serve as energy source for this anaerobe.

Table 1. *Energy Sources for FIG Transport by C. acidi-urici*

Cells were grown in urate minimal medium until almost all of uric acid was utilized. 0.68 ml of this culture was used for assay of FIG transport with or without various possible energy source added at 5 mM.

Energy source	Rate of FIG transport (μmole/g dry wt/min)	Energy source	Rate of FIG transport (μmole/g dry wt/min)
None	0.62	Pyruvate	0.43
Urate	3.23	Succinate	0.72
Xanthine	3.50	D-lactate	1.03
D-glucose	0.43	D-fructose	1.73
Sucrose	0.64	D-galactose	1.31
Glycerol	0.63		

Optimal Temperature

The optimal temperature for FIG transport is shown in Fig. 1. The temperature-activity profile showed a characteristic bell shape curve. The optimal temperature, in the range of 35-40°C, closely agreed with the optimal temperature for growth.

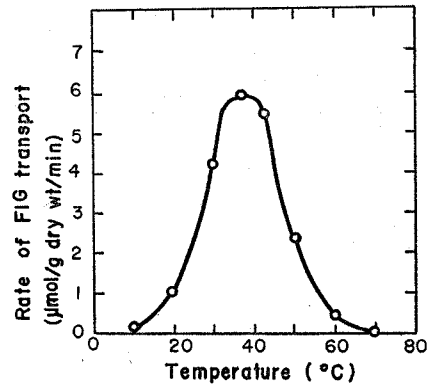

Fig. 1. Effect of temperature on FIG transport by *C. acidi-urici*

Effect of Na on the transport of FIG

The results shown in Table 2 indicated that 2.55 mM of either sodium formate or sodium pyruvate stimulated FIG uptake and NaCl at the same concentration also had the same extent of stimulation. Since neither NH_4Cl, LiCl nor RbCl at 2.5 mM affected the FIG transport, it was concluded that the active ion was the cation, Na^+, rather than the anions, chloride, formate and pyruvate. It was found that the maximal transport of FIG occurred at a Na concentration range from 2.5 to 10 mM. The concentration of Na which gave half maximal stimulation was 1.9×10^{-1} mM.

Table 2. *Effect of Sodium Ion on FIG Transport by C. acidi-urici*

Various sodium salts were added at 2.55 mM.

Addition	Rate of FIG Transport (μmol/g dry weight/min)
None	0.78
Sodium chloride	1.57
Sodium formate	1.50
Sodium pyruvate	1.46

Substrate specificity

The substrate specificity of the FIG transport systems was determined by means of competitive inhibition experiments. Fig. 2 showed that glycine at concentration five fold higher than that of FIG did not inhibit the FIG transport, whereas in the presence of a five-fold excess of unlabled FIG transport of [2-^{14}C]FIG was reduced to about one seventeenth. The results eliminate the possibility that [2-^{14}C]glycine derived from [2-^{14}C]FIG is taken up by the organism or that the glycine transport system is responsible for FIG transport. It was found that the following compounds at indicated con-

centration had neither inhibitory nor stimulatory effect on [2-^{14}C]FIG transport: L-alanine, L-aspartic acid, L-asparagine, L-glutamic acid, L-glutamine, L-lysine, L-histidine, L-phenylalanine, L-tryptophan, L-tyrosine, L-hydroxyproline, L-valine, L-leucine, L-isoleucine, L-serine, L-threonine, L-methionine, L-cysteine, L-cystine, CoA, L-formiminoaspartic acid and L-formiminoglutamic acid at concentration 3-fold that of [2-^{14}C]FIG; α-ketoglutaric acid, maleic acid, DL-ethionine, uridine, inosine and cytidine at concentration 29-fold that of [2-^{14}C]FIG; glycine, L-proline, L-arginine, urea, ethanol, fumaric acid, citric acid, DL-malic acid, D-malic acid, acrylamide, malonic acid, allantoin, α-D-glucose 1-phosphate, DL-glyceraldehyde 3-phosphate, dihydroxyacetone phosphate, ascorbic acid, glutathione, N-acetylglucosamine, glycoaldehyde and FMN at concentration 50-fold that of [2-^{14}C]FIG.

The results demonstrated that the FIG transport systems were highly specific for FIG.

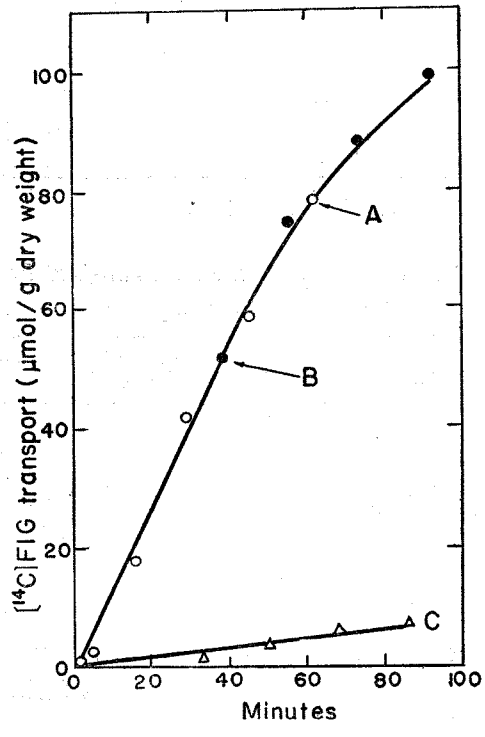

Fig. 2. Time course of [2-^{14}C]FIG transport by *C. acidi-urici* in the presence of unlabeled FIG or glycine.

The reaction mixture of final volume 1 ml contained: 1.7 μmoles (0.68 μCi) of [2-^{14}C]-FIG; 100 μg of chloramphenicol; 0.9 ml of the clostridial culture containing 7.7×10^{7} cells. A: Control (○——○). B: 8.5 μmoles of unlabeled glycine added (●——●). C: 8.5 μmoles of unlabeled FIG added (△——△).

Effects of two components of yeast extract on the transport of FIG

The data in Table 3 showed that addition of 8 mg yeast extract (Nutritional Biochemical Corporation) to the assay mixture caused a 42.7% decrease in FIG transport rate. In order to determine what component of the yeast extract was responsible for the inhibitory activity, the yeast extract was fractionated into cationic, neutral and anionic fractions. Each fraction was assayed for its inhibition on FIG transport. It was found that the cationic and anionic fractions showed inhibitory effect on FIG uptake whereas the neutral fraction did not exhibit any effect. Two fractions, tentatively called C4 and A5, were further isolated from the cationic and anionic fractions, respectively, by means of preparative paper chromatography. The inhibitory activities of C4 and A5 on the FIG transport are shown in Table 4. The data indicated that A5 is a more potent inhibitor than C4. The nature of these two fractions awaits further investigation.

Table 3. *Inhibition of FIG Transport by Yeast Extract*

The reaction mixture in a final volume of 1 ml contained: [2-^{14}C]FIG (specific activity=0.56 μCi/μmol), 0.7 μmole; NaCl, 10 μmole; chloramphenicol, 100 μg; yeast extract, 8 mg when added; and 0.88 ml of the clostridial culture containing 4.48×10^7 cells.

Addition	Rate of FIG Transport (μmole/g dry weight/min)
None	0.908
Yeast extract	0.520

Table 4. *Inhibition of FIG Transport by Two Components of Yeast Extract*

The conditions were similar to those described in Table 2. C4 and A5 were isolated by means of paper chromatography from the cationic and anionic fractions of yeast extract, respectively.

Addition	Rate of FIG Transport (μmole/g dry weight/min)
None	0.955
C4 (2 mg)	0.539
A5 (1 mg)	0.131

Discussion

The incapability of *C. acidi-urici* cells to utilize energy sources other than the fermentable purines (Barker and Beck, 1942) may account for the finding that among the nine compounds tested only uric acid and xanthine could effectively support the FIG transport in this organism. The question of why D-fructose and D-galactose could also support the FIG transport to a lesser extent is not clear at the present time. It is speculated that these two sugars

might interact with the cell membrane in such a manner that the membrane become more permeable to FIG.

Na dependent transport processes appear to be widespread among the vertebrates (Schultz and Curran, 1970), however, relatively few cases of such Na effect have been reported to occur in bacteria. Pseudomonad B-16 had a Na-dependent uptake of α-aminoisobutyric acid (Drapeau, Matula and MacLeod, 1966). A Na-dependent thiomethyl α-D-galactopyranoside transport system in *Salmonella typhimurium* has been described by Stock and Roseman (1971). Na-stimulated transport of glutamic acid in *E. coli* has also been reported (Miner and Frank, 1974; Halpern *et al*, 1973). All of these transport systems had one thing in common, namely, sodium correlated with only one transport system. On the contrary, in *C. acidi-urici* Na not only stimulated the transport of FIG but also stimulated the transport of glycine, L-serine, L-valine, L-leucine, L-phenyalanine, and L-tyrosine. Therefore, *C. acidi-urici* seems to be a good material to study the role of Na in bacterial transport systems.

Since the FIG transport in *C. acidi-urici* was mediated by two carriers (Chen and Rabinowitz, 1972), and required energy source and Na, it was concluded that in this organism FIG was taken up by two active transport systems. This is in agreement with the observation that all Na-dependent amino acid transport processes appear to be active transport systems (Schultz and Curran, 1970).

During the course of FIG uptake, the cells of *C. acidi-urici* were still not saturated with [2-^{14}C]FIG after 90 minute incubation (Fig. 2). This finding demonstrated that this organism could take up and metabolize FIG.

Literature Cited

AMES, G. F. 1964. Uptake of amino acids by *Salmonella typhimurium*. Arch. Biochem. Biophys. **104**: 1-18.

BARKER, H. A. and J. V. BECK. 1942. *Clostridium acidi-urici* and *Clostridium cylindrosporum*, organisms fermenting uric acid and some other purines. J. Bacteriol. **43**: 291-304.

CHEN, C. S. and J. C. RABINOWITZ. 1972. Unpublished.

DRAPEAU, G. R., T. I. MATULA and R. A. MACLEOD. 1966. Relation of Na^+-activated transport to the Na^+ requirement of a marine Pseudomonad for growth. J. Bacteriol. **92**: 63-71.

HALPERN, Y. S., H. BARASH, S. DOVER and K. DRUCK. 1973. Sodium and potassium requirement for active transport of glutamate by *E. coli* K-12. J. Bacteriol. **114**: 53-58.

HARDMAN, J. K. and STADTMAN, T. C. 1963. Energetics of the γ-aminobutyrate fermentation by *Clostridium aminobutyricum*. J. Bacteriol. **85**: 1326-1333.

MINER, K. M. and L. FRANK. 1974. Sodium-stimulated glutamate transport in osmotically shocked cells and membrane vesicles of *E. coli*. J. Bacteriol. **117**: 1093-1098.

RABINOWITZ, J. C. 1963. Intermediates in purine breakdown. *In:* Methods in Enzymology, Vol. 6. (S. P. Colowick and N. O. Kaplan, eds.), pp. 703-713. Academic Press, New York.

RABINOWITZ, J. C. and W. E. Jr. PRICER. 1956. Purine fermentation by *Clostridium cylindrosporum*. V. Formiminoglycine. J. Biol. Chem. **222**: 537-554.

SCHULTZ, S. G. and P. F. CURRAN. 1970. Couple transport of sodium and organic solutes. Physiological Reviews. **50**: 637-718.

STOCK, J. and S. ROSEMAN. 1971. A sodium-dependent sugar co-transport system in bacteria. Biophys. Biochem. Res. Comm. **44**: 132-138.

TABOR, H. and J. C. RABINOWITZ. 1957. Formiminoglycine, formiminoaspartic acid, formiminoglutamic acid. *In:* Biochemical Preparations. Vol. 5. (D. Shemin, ed.), pp. 100-105. John Wiley and Sons, Inc., New York.

Clostridium acidi-urici 之 formiminoglycine 運輸系統

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C. acidi-urici 的二組 FIG 運輸系統需要代謝能和鈉離子。脲酸和 2,6-二氧嘌呤都可作爲 FIG 運輸的能源。葡萄糖，蔗糖，甘油，焦葡萄酸，琥珀酸，乳酸都不是此項運輸的能源。鋰，鉀，氨等離子不能取代鈉離子的作用。運輸最適溫度是 37°C。

這二運輸系統對 FIG 有很高的特異性。試驗過的四十八種常見化合物中，只有 FIG 是這二組運輸系統的基質。