Urocanic Acid Production by Sporogenous Bacteria

Part II. Cultural Conditions and Mechanisms of Urocanic Acid Accumulation

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As the results of a screening of several type cultures of bacteria, *Bacillus subtilis* var. *thermophilus* was revealed to be the most powerful strain for urocanic acid production. The accumulation of urocanic acid by this bacteria is caused by deamination of L-histidine, and is particularly accelerated in the presence of a component (X-factor) in meat extract. In the decomposition of urocanic acid the optimal pH of urocanase activity is markedly inhibited by the deviation of pH of the culture medium. The histidase of this bacteria is supposed to be a new exo-type enzyme.

INTRODUCTION

In the previous paper¹⁾, it was reported that sporogenous bacteria, which the author named *Bacillus subtilis* var. *thermophilus*, produced a significant amount of urocanic acid in culture media. Several authors²⁻⁵⁾ have reported the isolation of urocanic acid from urine and some other biological sources. Sera⁶⁾ and others^{7,8)} stated that histidine could be converted to urocanic acid in good yields using purified liver preparation, and this conversion was also successfully made using histidine-adapted cell of *Pseudomonas fluorescens* by Tabor and Hayashi⁹⁾. However, the information on the accumulation of

urocanic acid in culture media of microorganisms could not hitherto be found. The present paper concerns the studies on cultural conditions and the mechanisms of urocanic acid production.

EXPERIMENTAL METHODS

1) Strains.

Bacillus subtilis var. *thermophilus* N-10 stored on bouillon agar slant was used as inoculum. *Ps. fluorescens* and other bacteria used in this experiment were supplied from the culture collection of our laboratory.

2) Cultivation.

The compositions of medium used in this experiment are shown in Table I. Each 100 ml of medium poured into 500 ml shaking flasks or 10 ml in 25 ml. T flasks, cotton plugged and sterilized in autoclave under 1.5 atm pressure for 15 minutes. Incubation was carried out on reciprocating machines at 37°C.

3) Analytical Methods.

a) L-Histidine. L-Histidine was separated by paper chromatography using mixture of *n*-butanol, acetic acid and water (4:1:2) as developing solvent and the spot was detected by ninhydrin reagent. The fraction of L-histidine on paper chromatography was

1) H. Imanaka, T. Miyoshi and H. Sakai, This Journal, **25**, 680 (1961).

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3) S. Ota, *Osaka Daigaku Igaku Zasshi*, **8**, 439 (1956).

4) B. Magasanik, *J. Biol. Chem.*, **205**, 1019 (1953).

5) H. Tristman, *J. Gen. Microbiol.*, **23**, 425 (1960).

6) Y. Sera, *Osaka Univ. Med. J.*, **4**, 1 (1951).

7) Y. Oyamada, *J. Biochem. (Japan)*, **36**, 227 (1944).

8) D.A. Hall, *Biochem. J.*, **51**, 499 (1952).

9) H. Tabor, and O. Hayashi, *J. Biol. Chem.*, **194**, 171 (1952).

TABLE I. COMPOSITION OF MEDIA

Bouillon medium	
meat extract	1(%)
polypeptone	1
NaCl	0.5
glucose	1
pH 7.2	
Basal medium	
glucose	1%
NH ₄ Cl	0.25
NH ₄ NO ₃	0.5
MgSO ₄ ·7H ₂ O	0.05
CaCl ₂ ·2H ₂ O	0.0025
K ₂ HPO ₄	0.3
KH ₂ PO ₄	0.15
Na ₂ SO ₄	0.05
asparagine	0.07
trace element solution	0.25 ml/1000 ml.
pH 7.0	

cut off and eluted with 0.1 N hydrogen chloride. L-histidine in this eluted solution was determined colorimetrically¹⁰⁾ at 480 μ by Pauly's¹¹⁾ reagent.

b) Urocanic acid. Urocanic acid was separated by the same technique and was detected under ultraviolet lamp. A fraction of urocanic acid on a paper strip was cut off and eluted with 0.1 N sodium hydroxide. Urocanic acid in this eluted solution was determined by spectrophotometer at 282.5 μ .

4) Determination of Growth Rate.

The growth rate of the culture was determined by weight of dried cell or sometimes by turbidimetric method.

RESULTS AND DISCUSSIONS

1) Screening of Urocanic Acid Production by Several Species of Bacteria.

Some type cultures of bacteria were tested of production of urocanic acid in culture media, according to the methods described above. The results were shown in Table II. Although several workers^{3,4,9)} had demonstrated that resting cells or cell-free preparations of *Pseudomonas* and *Aerobacter* converted histidine to urocanic acid, the present data pointed out that these bacteria could produce only a little urocanic acid compared with *B.*

TABLE II. AMOUNT OF UROCANIC ACID PRODUCED BY SOME TYPICAL BACTERIA

	max. amount of UA*
<i>Escherichia coli</i>	0
<i>Proteus vulgaris</i>	0
<i>Pseudomonas aeruginosa</i>	0
<i>Pseudomonas fluorescens</i>	150
<i>Aerobacter aerogenes</i>	70
<i>Serratia marcesens</i>	0
<i>Bacillus subtilis</i>	100
<i>Staphylococcus aureus</i>	0
<i>Bacillus cereus</i>	100
<i>Bacillus brevis</i>	110
<i>Sarcina lutea</i>	0
<i>Micrococcus flavus</i>	0
<i>Flavobacterium fuscum</i>	0
<i>Brevibacterium saccharolyticum</i>	0
<i>Bacillus Subtilis</i> var. <i>thermophilus</i>	750

* UA: urocanic acid.

subtilis var. *thermophilus*.

2) Studies on Nitrogen Sources.

As nitrogen sources, 20 g of corn steep liquor, soy bean meal extract (extracted with 0.1 N sodium hydroxide in boiling water for 60 minutes from 20 g of soy bean meal), dried

FIG 1. Effects of Various Nitrogen Sources.

●—● Bouillon. □—□ Casamino acid.
 △—△ Soy bean meal. ●—● Corn Steep Liquor.
 ○—○ Dried yeast.

10) H.T. Macpherson, *Biochem. J.*, **40**, 470 (1946).

11) H. Pauly, *Z. Physiol. Chem.*, **42**, 508 (1904).

FIG 2. Effects of Bouillon Medium Components.

Δ—Δ 1% meat extract + 0.5% NaCl
 ○—○ " " + " " + 1% polypeptone + 1% glucose
 ●—● 1% polypeptone + " "

yeast extract (extracted with boiling water for 60 minutes from 10 g of dried yeast), and 10 g of casamino acid (Difco) were added to 1000 ml of basal medium (Table I) for screening of composition of medium. And, as shown in Fig. 1, the best result was obtained using bouillon medium. Then, the three substances which compose the bouillon medium were examined of their influences on urocanic acid production. The result in Fig. 2 indicated that meat extract was the most important component for urocanic acid production and its optimal concentration was proved to be 5% (Fig. 3). The advantage of meat extract for urocanic acid production would be understood by the presence of con-

siderable amount of histidine in meat extract.

3) Studies on the Synthetic Media for Urocanic Acid Production.

To elucidate the mechanism of urocanic acid production, a synthetic medium containing L-histidine or urocanic acid was examined using *B. subtilis* N-10 and *Ps. fluorescens*. The results were shown in Fig. 4A~C. *Ps. fluorescens* metabolized L-histidine and urocanic acid very rapidly, though *B. subtilis* degraded urocanic acid rather slowly. And the accumulation of urocanic acid is markedly less in the synthetic medium than in the bouillon medium. From these observations, the author assumed the presence of a factor in meat extract by which the accumulation of urocanic

FIG. 3. Effects of Concentration of Meat Extract. Incubation was carried out in meat extract containing 0.5% NaCl at 37°C.

●—● 5% meat extract. ○—○ 2% meat extract.
 △—△ 7% " " ●—● 1% " "

acid from histidine is markedly accelerated. This factor, tentatively named X-factor, was isolated from meat extract according to the fractionating method shown in Fig. 5. The crude concentrated X-factor solution was revealed to have specific activity to *B. subtilis* but not to *Pseudomonas* (Table III). As the X-factor concentrate showed ninhydrin positive reaction, the author tried to find out replacement activity of many sorts of amino acids and related compounds. Here, *B. subtilis* was incubated in the basal medium containing 0.1% histidine and 0.05% of test samples, and the results were shown in Table IV. Although the compound which is able to replace completely X-factor could not be de-

FIG. 4—A. L-Histidine Consumption Curve in the Synthetic Media Containing L-Histidine.

FIG. 4—B. Urocanic Acid Production Curve in the Synthetic Media Containing L-Histidine.

tected, the following compounds—L-glutamine, L-aspartic acid, DL-ornithine, L-arginine, L-alanine, and L-histamine—were found to be effective to some degree. It is an interesting fact that ornithine cycle members showed good effects for urocanic acid production. This favorable effect was confirmed not to be the results of growth promoting action.

4) Urocanic Acid Production by Resting Cell.

The resting cell of *B. subtilis* N-10 was harvested from fermented broth of basal medium containing either 0.1% L-histidine or 0.1% urocanic acid. Each resting cell was incubated in phosphate buffer and tris buffer solution of various pH containing L-histidine or urocanic acid as substrate. As shown in Fig. 6,

FIG. 4—C. Urocanic Acid Consumption Curve in the Synthetic Media Containing Urocanic Acid.

FIG. 5. Fractionation of X-factor.

the cells cultured in presence of L-histidine possessed both histidase and urocanase activity, but the cells cultured in presence of urocanic acid possessed urocanase but not histidase. These results suggested histidase and urocanase were produced by adaptation to histidine. The fact agreed with the theory of Magasanik¹²⁾.

12) B. Magasanik, *J. Bact.*, **75**, 253 (1957).

TABLE III. EFFECTS OF X-FACTOR UROCANIC ACID PRODUCTION

(A) <i>B. subtilis</i> . N-10				
Addition of X-factoe	Incubation periods	24(hrs)	48	72
	UA produced (γ/ml.)	150	400	700
	Histidine consumed (γ/ml.)	170	460	860
	Rate* (%)	88	87	82
	UA produced (γ/ml.)	130	200	270
Control	Histidine consumed (γ/ml.)	250	530	880
	Rate* (%)	52	38	30
(B) <i>Ps. fluorescens</i>				
Addition of X-factor	Incubation periods	24(hrs)	48	72
	UA produced (γ/ml.)	110	80	50
	Histidine consumed (γ/ml.)	650	800	930
	Rate* (%)	18	10	5.4
	UA produced (γ/ml.)	150	70	50
Control	Histidine consumed (γ/ml.)	700	810	930
	Rate* (%)	21	7.6	5.4

* Rate is urocanic acid produced per histidine consumed. Incubation is carried out in basal medium contg. 0.1% L-histidine monohydrochloride at 37°C..

1 ml. of X-factor solution is added to 100 ml. of synthetic medium.

The optimal pH of histidase and urocanase are 8.2~8.8 and 7.0~7.5, respectively. This difference of optimal pH value may explain the accumulation of urocanic acid in broth of *B. subtilis*, since the pH value of the culture media of this bacteria rises up to pH 8.5 or sometimes more (Fig. 2). When filtrates of culture broth (fermentation periods 24 hrs) were saturated with ammonium sulfate, the precipitated fraction had very strong histidase (optimal pH 8.5~8.8) but not urocanase. From this fact, histidase of this organism was assumed to be a new exo-type enzyme, as the amino acid deaminases so far described were all known as endo-enzymes. The detailed studies of this new enzyme will be reported in the subsequent paper.

SUMMARY

1) In consequence of screening of several type cultures of bacteria, *B. subtilis* var. *thermophilus* was revealed to be the most powerful strain for urocanic acid production.

TABLE IV. EFFECTS OF VARIOUS AMINO ACIDS ON UA PRODUCTION IN THE SYNTHETIC MEDIUM

	UA. produced	max growth rate*
L-phenylalanine	170 (γ /ml)	0.33
DL-ornithine	270	0.31
DL-methionine	160	0.28
L-tryptophane	160	0.27
L-tyrosine	210	0.30
L-hydroxyproline	170	0.26
DL-threonine	160	0.30
DL-leucine	140	0.10
DL-isoleucine	220	0.28
L-lysine	190	0.25
L-arginine	260	0.31
L-proline	200	0.30
glycine	170	0.18
L-serine	220	0.25
L-valine	200	0.25
L-cysteine	110	0.16
L-cystine	130	0.22
L-glutamic acid	300	0.33
L-asparatic acid	250	0.32
DL-alanine	220	0.28
L-glutamine	540	0.31
L-histamine	380	0.32
X-factor (0.1 ml.)	750	0.32
Control	205	0.26

* Growth rate is measured with turbidimeter at 590 m μ . Incubation is carried out with T flask.

2) The results of studies on composition of culture media, showed that meat extract was essential for urocanic acid production and its optimal concentration was 5%.

3) From the studies on synthetic media containing histidine, a factor (X-factor) in meat extract was assumed to play an important role for urocanic acid accumulation of *B. subtilis*.

4) X-Factor is a ninhydrin positive substance and can be replaced partly by some amino acid like compounds such as L-glutamine or ornithine cycle members.

5) Histidase of *B. subtilis* var. is a new exo-type enzyme (opt. pH 8.5~8.8), and urocanase is a endo-enzyme (opt. pH 7.0~7.5)

FIG. 6. Incubation of Resting Cell in Various pH.

Resting cells (as dried cell 5 mg) were incubated in 10 ml buffer solution containing 0.2% L-histidine (A) or 0.2% urocanic acid (B) at 37°C.

●—● Resting cell of cultivation under presence of L-histidine.
 △—△ Resting cell of cultivation under presence of urocanic acid.

which is rather weak in activity.

6) The noticeable accumulation of urocanic acid in bouillon medium by *B. subtilis* can be explained as the combined results of the following reasons; a) the optimal pH of histidase produced by *B. subtilis* lies between 8.5 and 8.8 and the pH of the broth rises to this range, b) urocanase produced by this organism is weak and the urocanic acid is scarcely degraded, c) X-factor presented in meat extract stimulates the action of histidase.

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