Studies on the Production-Conditions of Iodinin by *Brevibacterium stationis var. indininofaciens*

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Introduction

Since CLEMO and MCILWAIN first reported an antibiotic phenazine pigment, iodinin [1], a great many reports have been presented about phenazines and phenazine-producing bacteria, but only a few on biosynthesis and antibiotic action of iodinin.

Gram-negative phenazine-producing bacteria, excepting *Pseudcmonas iodina* IFO 3558, produce water-soluble phenazines, such as pyocyanin, phenazine-l-carboxylic acid, and oxychlororaphine ; while gram-positive phenazine-producing bacteria produce a water-insoluble form, iodinin.

A marine bacterium, *Brevibacterium stationis var. iodininofaciens*, isolated from the sea-water-sample in the Sea to the East of Japan in the Pacific Ocean, produced a large amount of iodinin extracellularly. [2, 3, 4]

This report describes an approach to the biosynthetic mechanism of iodinin through the investigation of production-conditions of iodinin and through that of relation between production of iodinin and bacterial amino acid pool.

Materials and Methods

Organisms. *Br. stationis var. iodininofaciens* was isolated from the sea water sample at a depth of 99 m at the station (145°00'E, 39°03'N) in the Sea to the East of Japan in the Pacific Ocean [4]. The strain Po-363 is one of the many strains obtained from an original strain Po-36 by plating method [2].

Culture medium. Culture medium for collecting bacterial cells consists of glucose, 5 gm, polypepton, 5 gm, and 75 per cent aged sea water, 1 *l*, pH 7.6 to 7.8 initial (G 55). [3]

Intact cells. A 300-m*l* Erlenmeyer flask, containing 50 m*l* of a culture medium and inoculated with 0.5 m*l* of 2-day preculture, was incubated on a reciprocal shaker

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operating at 95 times per min. with a width of motion of 7.5cm, usually for 5 days at 30°C. Cells were centrifuged at 3500rpm (ca. 2000×G) for 15min., washed twice with a sterile water or a sterile sea water, and suspended in a sterile water or a sterile sea water to give one fiftieth optical density at 0.11 to 0.13 at 660nm. Care was taken not to add crystals of iodinin in a pellet to a cell suspension, if possible. Mass culture was employed, according to experiments. Three liter of the medium in a 5*l* of Erlenmeyer flask was inoculated with 300m*l* of 2-day preculture, and then incubated at an air flow of approximately 1*l* per min. for 48 hours at 30°C, being stirred with a magnetic stirrer. One fiftieth optical density of mass culture reached 0.08 to 0.10 at 660nm, when a wet weight of pellet is approximately 30gm, and a larger amount of 1,6-phenazine-diol was produced in addition to iodinin by insufficient air-supply for cultivation. In a certain instance, the amounts of iodinin and 1,6-phenazine-diol produced in 200m*l* of culture for 48 hours, were 12.80mg and 0.68mg, respectively.

Effects of various kinds of culture media. Sea water, sea water concentrated to one third volume, mineral salts solution, 0.01M phosphate buffer, and pure water were employed.[3] Twenty milliliters of cell suspension in a culture medium was brought up to 21m*l* with addition of glucose and ammonium chloride at concentrations of 0.03M and 0.01M, respectively. A culture was incubated at 30°C on a reciprocal shaker, and served for analyses of iodinin, and measurements of one fiftieth optical density and final pH.

Effects of various concentrations of phosphate. Culture media were employed, to which phosphate was added to give concentrations of 0, 10^{-3}, 5×10^{-3}, or 5×10^{-2}M in sea water, 3.5 percent sodium chloride solution, mineral salts solution, or pure water. Carbon and nitrogen-free media also were employed, to which phosphate was added to give concentrations of 0, or 10^{-2}M.

Effect of an amino acid as a nitrogen source on iodinin production. An alcohol-sterilized amino acid was added to 20m*l* of a cell suspension in a sterile 75 per cent sea water to give a concentration of 0.05M, of which pH was adjusted to 7.0 with sterile 1N sodium carbonate solution and 1N hydrogen chloride solution. Incubation was carried out on a reciprocal shaker operating at 100 times per min. with a width of motion of 2cm at 30°C for 72 hours. Amino acids employed were shown in Table 1.

Effect of glucose as a substrate of respiration. Glucose was added as a substrate of respiration to cell-suspension to give a concentration of 0.05M.

Relation between consumption of sugar and iodinin production. Glucose, and L-leucine or ammonium sulfate were added to a cell-suspension to give concentrations of 0.01M and 0.005M, respectively. Quantitative analysis of sugar was carried out by means of a modification of Somogyi method.[5] In order to investigate a time-course of consumption of sugar and iodinin production, analyses of amounts of remained sugar, one fiftieth optical density, and amounts of produced iodinin were done by the period of an hour.

Transformation between iodinin and 1,6-phenazine-diol, under aerobic condition and under anaerobic condition. A flask contained 30m*l* of cell-suspension, to which 2mg of iodinin, or 1,6-phenazine-diol and glucose was added, or not. Aerobic condition was prepared with air in a flask, and anaerobic condition with nitrogen gas, replacing air in a flask.

Extraction of pool amino acids. Investigation of extraction of pool amino acids was carried out with cells in a stationary phase, cultivated for 48 hours in the medium

G 55, and the methods of extraction are as follows. 1) Extraction with boiling water: Cell suspension, of which one part of boiling water was added to one, was kept at 100°C for 10 min., and then centrifuged at 8500 rpm for 10 min. A supernatant was filtered through a Seitz filter, and a filtrate was served for analysis of amino acids. 2) Extraction with ice-cold 5 per cent trichloroacetic acid: Cell suspension, of which one part of ice-cold 10 per cent trichloroacetic acid was added to one, to give a concentration of 5 per cent, was kept at 0°C for 10 min., and then filtered through a Seitz filter. A filtrate was served for analysis of amino acids. 3) Extraction with ice-cold 0.25 N perchloric acid: Cell suspension, of which one part of ice-cold 0.5 N perchloric acid was added to one, to give a concentration of 0.25 N, was kept at 0°C for 10 min., and then filtered through a Seitz filter. A filtrate was served for analysis of amino acids. 4) Extraction from a sonicate with ice-cold 5 per cent trichloroacetic acid: Cell-suspension was treated with a sonic oscillator at 10,000 cps for 30 min. Sonicate, of which one part of ice-cold 10 per cent trichloroacetic acid was added to one, to give a concentration of 5 per cent, was centrifuged at 20,000 rpm for 20 min. A supernatant was filtered through a Seitz filter, and a filtrate was served for analysis of amino acids. Yanagimoto amino acid autoanalyzer SLC-5N was served for amino acid analyses.

Effects of penicillin and chloramphenicol on iodinin production and pool amino acids. To cell suspension was added glucose and sodium L-glutamate to give concentrations of 0.03 M and 0.01 M, respectively. Penicillin G was employed at concentrations of 0, 10^{-2}, 10^{-3}, and 10^{-4} M, and chloramphenicol at a concentration of 10^{-2} M. Incubation was carried out at 30°C for 72 hours.

Effect of feed back control with an excess of 1,6-phenazine-diol on amino acid pool. Cell-suspension at one fiftieth optical density of 0.129 was employed, to which glucose and sodium L-glutamate were added to give concentrations of 0.03 M and 0.01 M respectively. Ten milligrams of 1,6-phenazine-diol was added to a flask, containing 30 m*l* of cell suspension, and nitrogen gas replaced air in a flask to obtain anaerobic condition. Incubation was carried out at 30°C for 2 hours.

Separation of peak 1 compound. In order to separate peak 1 compound, one-dimensional paperchromatography was carried out on Tôyô No. 50 filter paper, using n-butanol-acetic acid and water (4 : 2 : 1) as a solvent. Peak 1 compound was extracted with hot water from a piece of filter paper, corresponding to Rf value of peak 1 compound. Hydrolysis of peak 1 compound was carried out with hydrogen chloride at a concentration of 0.57 N at 105°C for 12 hours, or for 24 hours.

Preparation of L-glutamic decarboxylase. In order to determine a configuration of glutamic acid in peak 1 compound, L-glutamic decarboxylase was prepared by usual method from a pumpkin.[6] Peak 1 compound was hydrolyzed with hydrogen chloride, and after the removal of hydrogen chloride, diluted with 0.1 M phosphate buffer, pH 5.7. To 2 m*l* of the sample solution was added 0.5 m*l*, or 1 m*l* of the enzyme solution, and to 2 m*l* of 10^{-2} M L-glutamic acid solution as a control and to 2 m*l* of 0.1 M phosphate buffer as a blank, respectively. Incubation was carried out at 37°C for 30 min. After incubation, these samples were chromatographed on a filter paper, using a solvent system, n-butanol-acetic acid and water, to detect disappearance of L-glutamic acid and appearance of γ-aminobutyric acid, into which L-glutamic acid was decarboxylized.

Separation of L-methionyl-L-glutamic acid. Extract with hot water was decolorized with activated charcoal, and fractionated through ion exchange columns.

Effect of L-methionyl-L-glutamic acid on iodinin production. Investigation was carried out by the same method, as that of amino acids. Dipeptide was added to cell-suspension at a concentration of 0.005 M as methionine, according to ninhydrin method.

Results and Discussion

Effects of various kinds of culture media

As shown in Table 1, neither sea water, nor sodium chloride solution was required for iodinin production, and phosphate might take part in iodinin production.

Effects of various concentrations of phosphate

As shown in Fig. 1, the more amount of phosphate was added to culture media, the more amount of iodinin would be produced.

Effect of an amino acid as a nitrogen source on iodinin production

As shown in Table 2, one fiftieth optical density and the amounts of iodinin produced increased, only when L-glutamic acid was added to a cell-suspension. All amino

Table 1. Effects of various kinds of culture media on iodinin production.

medium	dry weight g/ml	iodinin produced, mg/21 ml	
		24 hr	72 hr
concentrated sea water	0.1080	0.230	0.390
sea water	0.0360	1.170	1.440
NaCl solution	0.0350	0.530	0.630
mineral salts solution	0.0022	1.764	1.550
phosphate buffer, 0.01 M, pH 7.4	0.0014	2.010	1.570
pure water	0.0000	0.766	0.940

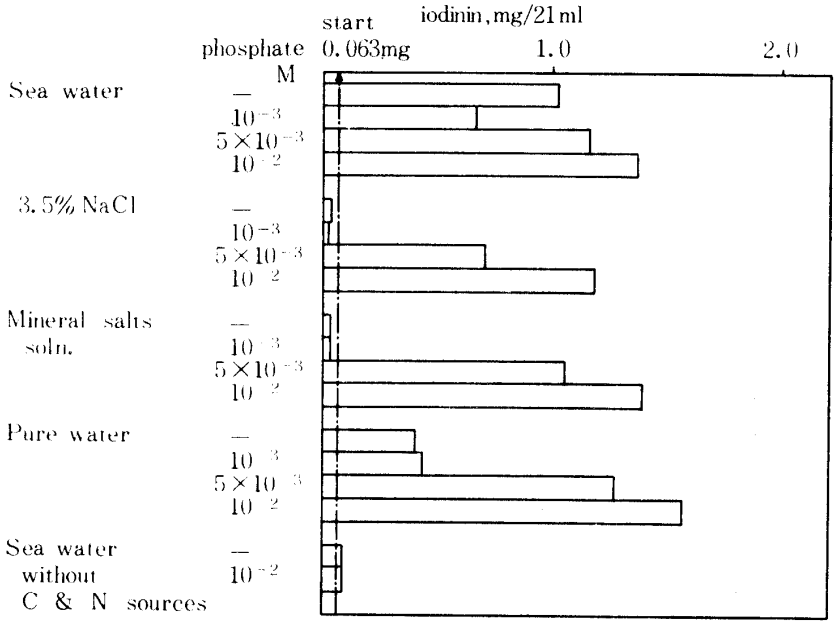

Fig. 1. Effects of various concentrations of phosphate.

Table 2. Effects of an amino acid as a nitrogen source and glucose as a substrate of respiration.

amino acids	no addition of glucose		addition of glucose	
	1/50 O. D.	iodinin mg/20m*l*	1/50 O. D.	iodinin mg/20m*l*
L-aspartic acid	0. 122	0. 006	0. 153	1. 616
L-glutamic acid	0. 185	0. 328	0. 238	1. 624
D-glutamic acid	0. 132	0. 012	0. 127	0. 022
o-amino benzoic acid (OABA)	0. 137	0. 011	0. 115	0. 034
L-histidine	0. 154	0. 010	0. 232	0. 688
L-tyrosine	0. 189	0. 084	0. 201	0. 464
L-cystine	0. 121	0. 008	0. 117	0. 014
DL-valine	0. 145	0. 022	0. 151	0. 452
L-leucine	0. 131	0. 030	0. 227	1. 480
DL-isoleucine	0. 158	0. 016	0. 170	1. 440
L-phenylalanine	0. 192	0. 096	0. 153	0. 124
DL-methionine	0. 131	0. 004	0. 145	0. 246
L-lysine-HCl	0. 202	0. 098	0. 303	1. 064
DL-alanine	0. 156	0. 096	0. 252	1. 592
$(NH_4)_2 SO_4$			0. 147	2. 85
no addition	0. 111	0. 012	0. 175	0. 436
starting culture	0. 157	0. 011	0. 137	0. 007

acids employed, excepting L-glutamic acid, seemed not to take part in iodinin production, but it was considered to have resulted from a slow permeation of such amino acids into cell, as L-aspartic acid, D-glutamic acid, o-aminobenzoic acid, L-histidine, DL-valine, L-leucine, L-phenylalanine, L-lysine, DL-isoleucine, and DL-methionine. In 3 days, L-glutamic acid and DL-alanine, added at a concentration of 0.05M, were not detected in a cell-suspension by means of paper chromatography.

Effect of glucose as a substrate of respiration

When such amino acids, as L-aspartic acid, DL-alanine, DL-leucine, and DL-isoleucine, were employed, addition of glucose resulted in increase of iodinin produced. Production of large amount of iodinin was observed, using ammonium sulfate as a nitrogen source. Employment of o-aminobenzoic acid, or sulfur-containing amino acids did not bring about good results.

Relation between consumption of sugar and iodinin production

As shown in Fig. 2, consumption of sugar preceded iodinin production to some extent.

Ratio of a carbon source to a nitrogen source

Effect of a ratio of a carbon source to a nitrogen source on iodinin production was investigated as follows: L-leucine, or ammonium sulfate, employed a nitrogen source at a concentration of 0.01M; glucose, employed as a carbon source at a concentration of 0.00 to 0.08M; incubation at 30°C for 72 hours. As shown in Table 3, L-leucine employed, amount of iodinin produced increased with increasing amount of glucose added, and ammonium sulfate employed, an amount of iodinin produced almost reached the ceiling at glucose concentration of 0.03M, and no longer increased with the increas-

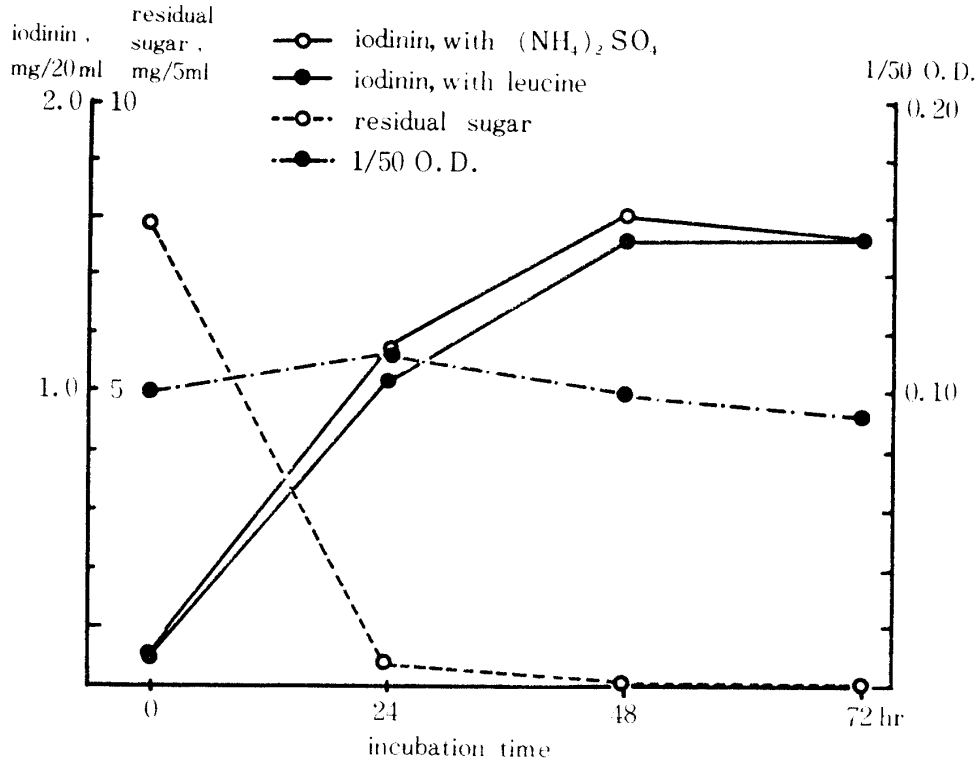

Fig. 2. Relation between consumption of sugar and iodinin production.

Table 3. Effect of a ratio of a carbon source to a nitrogen source on iodinin production.

N-source	glucose	1/50 O. D.	iodinin mg/20ml
L-leucine 0.01M	0 M	0.075	0.242
	0.03	0.123	5.250
	0.05	0.164	6.650
	0.08	0.155	6.890
$(NH_4)_2SO_4$ 0.01M	0 M	0.077	0.246
	0.03	0.120	4.180
	0.05	0.152	4.220
	0.08	0.140	4.663
starting culture		0.112	0.180

ing amount of glucose added.

Relation between iodinin production and various concentrations of amino acid

A considerable amount of amino acid added at a concentration of 0.05M remained in a broth after 72 hour-incubation. As shown in Table 4, amino acids that presented good iodinin production were L-proline, L-arginine, L-ornithine, L-glutamic acid, L-aspartic acid, L-leucine, DL-isoleucine, L-serine, and DL-norleucine. With amino acids, other than these, iodinin production almost reached the ceilings at a certain concentration of amino acid, and no longer increased, or decreased with the increasing concentration of amino acids. o-Aminobenzoic acid and L-tyrosine presented a low

Table 4. Relation between iodinin production and various concentrations of amino acid.

amino acids		iodinin mg/20m*l*	amino acids		iodinin mg/20m*l*	amino acids		iodinin mg/20m*l*
L-lys	0.001	0.680	L-tyr	0.001	0.210	D-glu	0.001	0.451
	0.005	2.108		0.005	0.265		0.005	0.820
	0.01	2.080		0.01	0.450		0.01	1.640
	0.03	1.500		0.03	0.450		0.03	1.500
OABA	0.001	0.309	L-phe	0.001	0.146	L-glu	0.001	0.347
	0.005	0.294		0.005	0.555		0.005	1.150
	0.01	0.216		0.01	1.140		0.01	1.920
	0.03	0.173		0.03	1.250		0.03	2.910
L-ala	0.001	0.595	DL-try	0.001	0.126	DL-met	0.001	0.440
	0.005	1.207		0.005	0.186		0.005	0.800
	0.01	1.725		0.01	0.320		0.01	0.800
	0.03	1.080		0.03	0.300		0.03	0.830
L-leu	0.001	0.500	L-nleu	0.001	0.340	L-cys	0.001	0.340
	0.005	1.560		0.005	1.260		0.005	0.424
	0.01	2.678		0.01	1.810		0.01	0.500
	0.03	2.775		0.03	2.010		0.03	0.136
no amino acid		0.302	no amino acid		0.150	no amino acid		0.228
starting culture		0.175	starting culture		0.026	starting culture		0.054
L-arg	0.001	1.300	L-pro	0.001	0.232	L-ornithine	0.001	0.238
	0.005	3.010		0.005	1.020		0.005	2.660
	0.01	3.330		0.01	2.850		0.01	2.910
	0.03	3.480		0.03	4.813		0.03	2.970
L-asp	0.001	0.415	DL-ser	0.001	0.326	DL-citrulline	0.001	0.380
	0.005	0.890		0.005	1.175		0.005	1.823
	0.01	2.190		0.01	2.080		0.01	1.840
	0.03	2.890		0.03	2.360		0.03	1.840
DL-ileu	0.001	0.585	DL-thr	0.001	0.375	gly	0.001	0.284
	0.005	0.955		0.005	0.705		0.005	0.550
	0.01	1.540		0.01	1.310		0.01	1.245
	0.03	2.580		0.03	1.870		0.03	1.090
L-his	0.001	0.310	DL-val	0.001	0.284	NH_4Cl	0.001	0.314
	0.005	0.715		0.005	1.010		0.005	1.270
	0.01	1.020		0.01	1.590		0.01	2.360
	0.03	1.020		0.03	1.850		0.03	2.260
no amino acid		0.245	no amino acid		0.153	no amino acid		0.184
starting culture		0.079	starting culture		0.079	starting culture		0.089

Table 5. Effects of carbon sources, other than glucose, on iodinin production.

C-source	N-source		iodinin mg/20ml
p-hydroxybenzoate	leu	G*	3.120
		—	1.590
	$(NH_4)_2So_4$	G**	2.830
		—	1.445
OABA	leu	G	0.915
		—	0.252
	$(NH_4)_2SO_4$	G	0.383
		—	0.205
Na-salicylate	leu	G	0.950
		—	0.194
	$(NH_4)_2SO_4$	G	0.980
		—	0.240
leucine+glucose	0.01M		1.960
$(NM_4)_2$+glucose	0.01M		1.790
glucose	0.03M		0.464
starting culture			0.125

C-source		iodinin mg/20ml
arabinose	G	0.978
	—	0.141
xylose	G	1.048
	—	0.166
fructose	G	2.128
	—	1.458
galactose	G	2.148
	—	0.988
capric acid	G	0.154
	—	0.140
lauric acid	G	2.088
	—	1.596
shikimic acid	G	1.268
	—	0.197
control	G 0.01M	1.240
control	G 0.03M	2.818
starting culture		0.103

* G : Glucose, 0.01M ; a carbon source, other than glucose, 0.02M.
** — : No addition of glucose.

fatty acid		iodinin mg/20ml
Na-acetate	G	0.830
	—	0.239
n-valeric acid	G	1.250
	—	0.202
iso-valeric acid	G	1.780
	—	0.205
n-capric acid	G	1.370
	—	0.530
n-caprylic acid	G	3.645
	—	0.067
glucose	0.01M	1.100
glucose	0.03M	3.280
starting culture		

C-source		iodinin mg/20ml
n-butyric acid	G	0.750
	—	0.360
4-Me-valeric acid	G	0.860
	—	0.222
palmitic acid	G	0.640
	—	0.076
gluconate	G	1.200
	—	0.500
citrate	G	1.120
	—	0.500
adipic acid	G	0.920
	—	0.080
succinic acid	G	1.050
	—	0.440
diacetyl	G	0.550
	—	0.099
glycerol	G	0.850
	—	0.640
kerosene	G	0.850
	—	0.013
glucose	0.01M	0.660
glucose	0.03M	2.300
starting culture		0.012

Table 6. Effect of various metabolic inhibitors.

inhibitor	conc., M	iodinin, mg/20m*l*	rate of inhibition, %
NaN_3	10^{-2}	0.157	98
	10^{-3}	0.612	84
	10^{-4}	1.460	46
NaF	10^{-2}	2.780	0
	10^{-3}	2.780	0
	10^{-4}	2.960	0
Na_2-arsenate	10^{-2}	0.225	95
	10^{-2}	2.340	12
	10^{-4}	2.700	0
KCN	10^{-2}	0.568	82
	10^{-3}	0.795	100
	10^{-4}	0.091	100
control+ethanol		0.190	97
control		2.630	—
starting culture		0.105	
inhibitor	conc., M	iodinin, mg/20m*l*	rate of inhibition, %
EDTA	10^{-2}	2.890	9
	10^{-3}	2.760	14
	10^{-4}	3.530	0
iodoacetic acid	10^{-2}	0.202	100
	10^{-3}	0.226	100
	10^{-4}	0.518	90
PCMB	10^{-2}	0.200	100
	10^{-3}	0.164	100
	10^{-4}	0.480	91
DNP	10^{-2}	0.164	100
	10^{-3}	2.140	35
	10^{-4}	3.040	0
Na-arsenite	10^{-2}	0.214	100
	10^{-3}	1.840	45
	10^{-4}	3.140	0
semicarbazide-HCl	10^{-2}	1.900	16
	10^{-3}	3.100	0
	10^{-4}	3.240	0
control		3.050	—
starting culture		0.215	—

* EDTA : ethylenediaminetetraacetic acid, Na_4-salt.
PCMB : p-chloromercuribenzoic acid.
DNP : 2, 4-dinitrophenol.

iodinin production. This fact indicates that an intact benzene-ring of these amino acids might not be incorporated to iodinin. An adequate concentration of a nitrogen source for iodinin production is 0.01 M, with some exceptions.

Carbon sources as a substrate of respiration in place of glucose

Effects of sugars, organic acids, and fatty acids at a concentration of 0.03 M on iodinin production was investigated. L-leucine or ammonium sulfate was employed at a concentration of 0.01 M. In addition, glucose at a concentration of 0.01 M and other carbon source at a concentration of 0.02 M was employed for carbon source. As shown in Table 5, caebon sources which showed good iodinin production were fructose, galactose, lauric acid, isovaleric acid, n-caprylic acid, p-hydroxybenzoic acid, gluconic acid, citric acid, and succinic acid, in the presence of glucose. Only p-hydroxybenzoic acid, of three aromatic compounds, seemed to take part in iodinin production, while the others might suppress iodinin production. Shikimic acid, an important intermediate in the pathway from glucose to aromatic compounds, seemed not to take part in iodinin production. The fact indicates that iodinin biosynthesis through the shikimic acid pathway is improbable, although permeation-problem still remains.

Effect of various metabolic inhibitors

Glucose and ammonium sulfate were employed at concentrations of 0.03 M and 0.01 M, respectively, Metabolic inhibitors were employed at concentrations of 10^{-2}, 10^{-3}, and 10^{-4} M, respectively, and ethanol, at a concentration of 2 per cent. As shown in Table 6, it was only sodium fluoride that showed no inhibitory effect on iodinin production at any concentration, and potassium cyanide, iodoacetic acid, p-chloromercuribenzoic acid and ethanol that showed a strong inhibition.

Transformation between iodinin and 1,6-phenazine-diol, under aerobic condition and under anaerobic condition

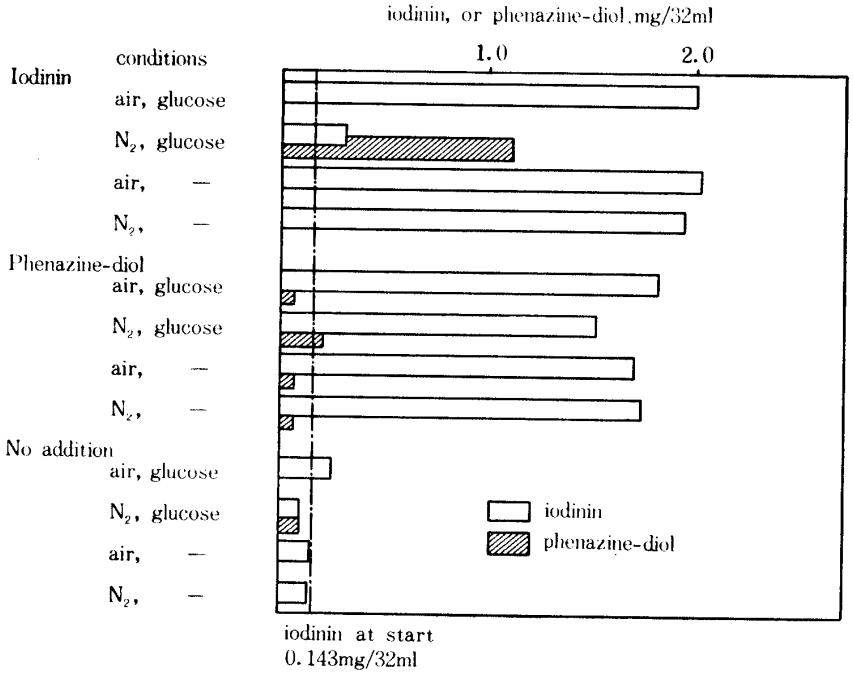

Fig. 3. Transformation between iodinin and 1,6 phenazine diol under aerobic and anaerobic conditions.

As shown in Fig. 3, under aerobic condition, iodinin did'nt change with brevibacterial cells, and under anaerdbic condition, approximately 55 per cent of iodinin was reduced into 1, 6-phenazine-diol, but in the absence of glucose, iodinin was not reduced. The fact indicates that glucose is required as an energy source for transformation of iodinin to 1, 6-phenazine-diol. Transformation of 1, 6-phenazine-diol to iodinin proceeded completely under aerobic condition, not requiring an hydrogen donor.

Effect of redox pigments on transformation of 1, 6-phenazine-diol to iodinin

To 20 ml of cell-suspension was added 0. 175 mg of 1, 6-phenazine-diol, and a redox pigment was added to give a concentration of 10^{-4} M. After one hour incubation under aerobic condition, the amounts of pigment were determined according to the usual method[3], or calculated from absorption spectra of supernatant. Excepting triphenyltetrazolium chloride, all redox pigments employed seemed to suppress the transformation, and especially pigments, such as thionine, toluidine blue, Janus green B, and 1, 4-naphthoquinone, seemed even to reverse the transformation, as shown in Fig. 4.

Iodinin production under anaerobic condition

Glucose and ammonium sulfate were employed at concentrations of 0. 03 M and 0. 01 M, respectively, and nitrogen gas or carbon dioxide gas, in place of air. As shown in Table 7, iodinin production and sugar consumption were completely suppressed in nitrogen gas, or carbon dioxide gas.

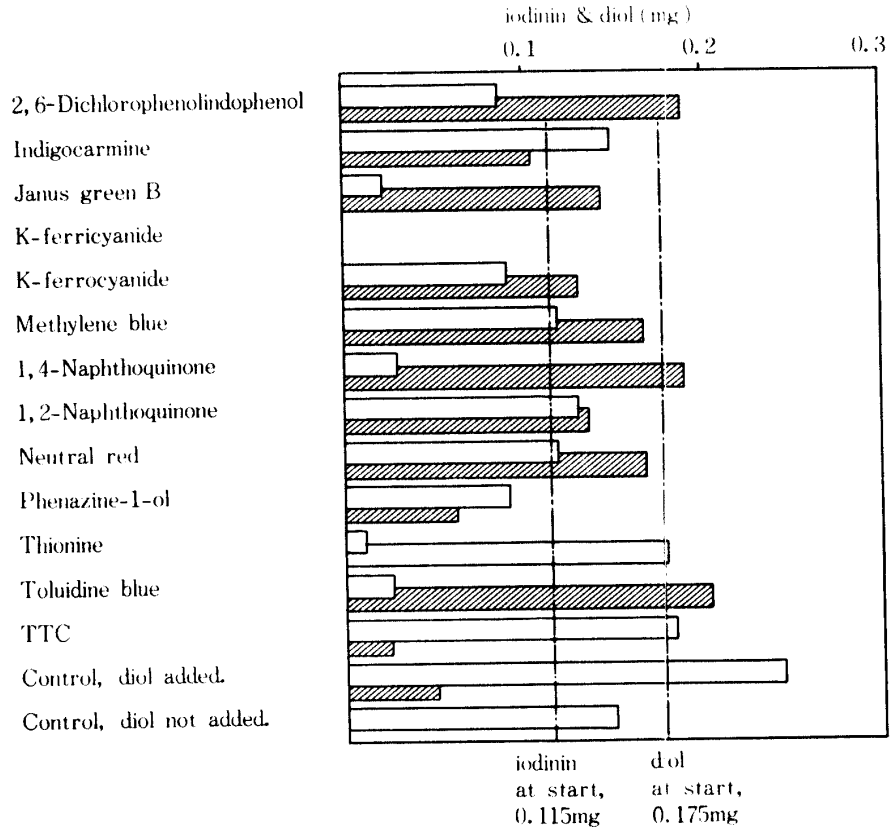

Fig. 4. Effect of redox pigments on transformation of 1, 6-phenazine-diol to iodinin.
TTC : 2, 3, 5-triphenyl-2, 1, 3, 4-tetrazolium chloride.
diol : 1, 6-phenazine diol

Table 7. Iodinin production under anaerobic conditions.

	1/50 O. D.	dr. w. mg/20m*l*	consumed sugar, mg/20m*l*	iodinin mg/20m*l*
air	.183	65.6	101.62	2.650
N_2	.1045	45.0	3.90	.171
CO_2	.097	43.4	8.35	.174
start	.132		108.00	.215

Extraction of pool amino acids

As shown in Fig. 5, a large peak preceded that of aspartic acid, and this unknown compound was called peak 1 compound. As shown in Table 8, kinds and amounts of amino acid did'nt vary with the methods of extraction of amino acid pool. Accordingly, hot water extraction was adopted for a further extraction.

Analyses of pool amino acids

As shown in Table 8, L-glutamic acid was in the largest amount, agreeing with the result of gram-positive bacteria of TEMPEST and MEERS.[7] It was characteristic that amino acid pool of this brevibacteria would not contain L-proline, and large amounts of peak 1 compound, ninhydrin-positive, were found.

Pool amino acids of *Br. stationis var. iodininofaciens* were compared with those of the following bacteria: *Pseudomonas aureofaciens*, IFO 3521, phenazine-1-carboxylic

Table 8. Analyses of pool amino acids.

	Extraction with:		(μmole/25 m*l* of extract)		Extraction with boiling water μmole/100 mg dry cell
	boiling water	5% TCA	0.25N PCA	sonic and TCA	
lys	0.424	0.534	0.765	1.84	0.252
his	17.70	13.35	13.59	19.02	9.950
arg	trace	0.082	0.144	0.515	nd
asp	0.072	0.654	0.069	2.705	0.043
thr	2.775	2.665	2.490	3.50	1.640
ser	0.275	trace	nd	0.425	0.163
glu	38.87	33.93	35.3	36.81	23.90
pro	nd	nd	nd	nd	nd
gly	0.024	0.454	0.330	1.075	0.013
ala	10.825	9.890	9.975	10.95	6.460
cys	nd	0.285	0.400	1.290	nd
val	0.004	nd	0.080	0.845	0.002
met	0.029	0.038	0.330	0.096	0.017
ileu	0.023	0.187	0.135	0.322	0.013
leu	trace	0.052	nd	0.734	nd
tyr	nd	nd	nd	0.091	nd
phe	0.038	nd	nd	0.253	0.022
peak 1*	35.20	42.42	32.25	48.75	20.80

* Peak 1 compound, calculated as aspartic acid. nd: not detected.

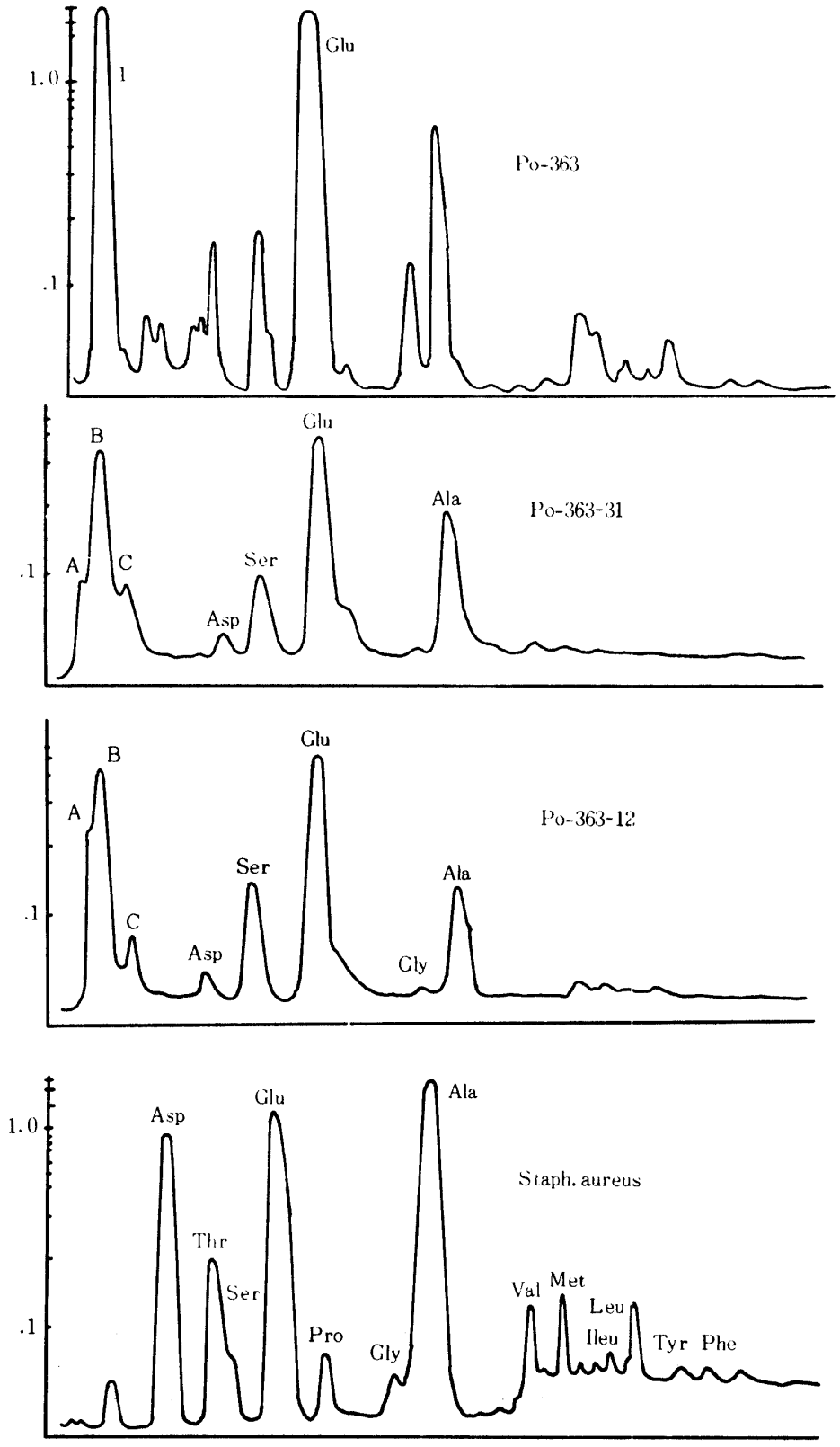

Fig. 5. Amino acid pools of bacteria.

Table 9. Pool amino acids of *Ps. aureofaciens* and *B. megaterium*.

	Ps. aureofaciens	*B. megaterium*
	μmole/100 mg dry cell	μmole/100 mg dry cell
asp	0.365	0.197
thr	0.045	0.067
ser	0.069	0.049
glu	0.236	1.062
pro	nd	1.570
gly	0.183	0.069
ala	0.146	0.381
cys	nd	nd
val	0.052	0.075
het	0.108	0.212
ileu	trace	0.052
leu	0.044	0.063
tyr	nd	0.046
phe	nd	0.105
peak 1	nd	nd

Table 10. Effecfs of penicillin on iodinin production.

Penicillin	1/50 O. D.	iodinin, mg/20m*l*
10^{-2}M	0.128	0.72
10^{-3}M	0.159	4.28
10^{-4}M	0.129	3.80
no addition	0.165	2.14
starting culture	0.104	

acid-producing bacterium; *Bacillus megaterium*, IAM 1030; *Staphylococcus aureus*, IAM 1011; *Br. st. var. iodininofaciens*, Po-363-12, and Po-363-31, mutants of *Br. st. var. iodininofaciens*, Po-363, iodinin-producing bacterium, losing the ability of iodinin production partially. As shown in Fig. 5, and Table 9, in the amino acid pool of *Staph. aureus*, a peak of unknown compound appeared before that of aspartic acid, but differed from that of peak 1 compound in the retention time. As shown in Fig. 5, in amino acid pools of strains Po-363-12, and Po-363-31, three peaks, A, B, and C, appeared before that of aspartic acid, of which peak B agreed with that of peak 1 compound, judging from a retention time.

Effects of penicillin and chloramphenicol on iodinin production and pool amino acids

In the presence of penicillin at a concentration of 10^{-2}M, iodinin production was suppressed, while at concentrations of 10^{-3} and 10^{-4}M, amounts of iodinin produced were nearly twice as much as that in no addition, as shown in Table 10. As shown in Fig. 6, a certain difference was observed in the proportion of peak 1 compound to glutamic acid between in the presence of penicillin at concentrations of 10^{-2}, and 10^{-4}.

In the presence of chloramphenicol, iodinin production was stopped, and amounts

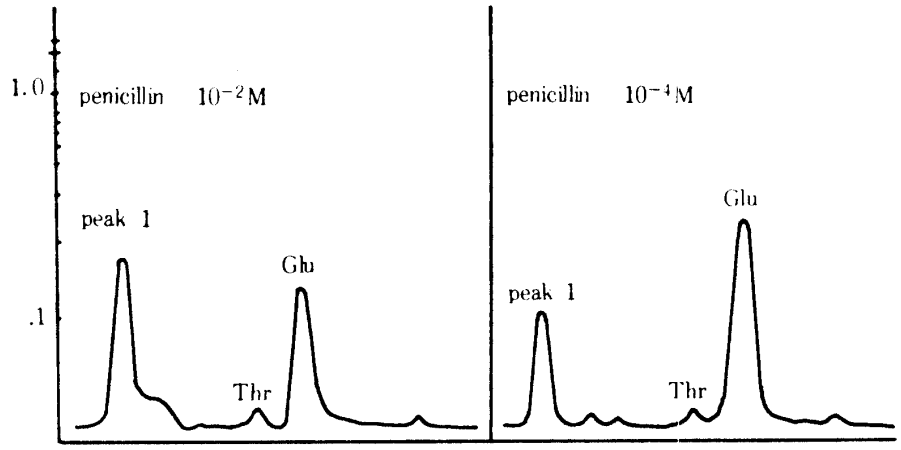

Fig. 6. Amino acid pools in the presence of penicillin.

Table 11. Effect of chloramphenicol on iodinin production and pool amino acids.

1) Pool amino acids

	In the presence of chloramphenicol	
	24hr μmole/100 mg dry cell	48hr μmole/100 mg dry cell
asp	1.09	nd
thr	nd	1.6
ser	0.01	nd
glu	19.40	14.05
pro	nd	nd
gly	nd	nd
ala	4.0	3.02
cys	nd	trace
val	0.38	nd
met	trace	0.11
ileu	nd	0.16
leu	trace	0.13
tyr	nd	nd
phe	nd	nd
peak 1	9.20	4.28

2) Iodinin production

chloramphenicol		24hr	48hr
addition	1/50 O. D.	0.150	0.143
	cell dry weight, mg/m*l*	25.5	22.9
	iodinin, mg/20m*l*	0.124	0.124
no addition	cell dry weight, mg/m*l*	30.1	31.9
	iodinin, mg/20m*l*	0.54	1.44

of pool glutamic acid increased markedly, as compared with that in no addition, as shown in Table 11.

Effect of feed back control with an excess of 1, 6-phenazine-diol on amino acid pool

As already reported, 1, 6-phenazine-diol may be a precursor of iodinin, and rather a final product in anaerobic condition. In anaerobic condition, a considerable change in amounts of pool amino acids was brought about, 2 hours after addition of an excess of 1, 6-phenazine-diol. As shown in Table 12-1, alanine, histidine, and peak 1 compound in pool increased remarkably, as compared with glutamic acid. As shown in Table 12-2, a difference between 1, 6-phenazine-diol added and recovered after 2 hours, was 4.233 mg, a considerable amount of which seemed to be incorporated to bacterial cells. Alanine, glutamic acid, histidine, and peak 1 compound might take part in the biosyn thesis of iodinin.

Separation of peak 1 compound

Peak 1 compound showed the same Rf value as that of aspartic acid, using a solvent system, n-butanol-acetic acid-water, and as that of glutamic acid, using a solvent

Table 12. Effects of feed back control with an excess of 1, 6 phenazine diol.

1) Pool amino acids.

amino acid	feed back μmole/100 mg dry cell	a control μmole/100 mg dry cell
try	nd	nd
lys	nd	0.25
his	13.86	9.95
arg	nd	nd
asp	nd	0.04
thr	1.87	1.64
ser	nd	0.16
glu	7.48	23.9
pro	nd	nd
gly	nd	0.01
ala	12.9	6.46
cys	nd	nd
val	nd	trace
met	nd	0.02
ileu	nd	0.01
leu	nd	nd
tyr	nd	nd
phe	nd	0.02
peak 1.	12.3	30.0

2) Pigment production

	1/50 O. D.	iodinin (mg)	diol (mg)
starting culture	0.129	0.42	9.70
feed back	0.131	0.35	5.47

system, phenol-ammonia. A spot of peak 1 compound was separated into two spots by hydrolysis, which proved to be glutamic acid and methionine. According to negative biuret reaction, peak 1 compound was dipeptide, in which glutamic acid combined with methionine in the ratio 1 : 1.

System of bonding of the dipeptide

A spot of DNP-derivatives of hydrolysate agreed with that of DNP-methionine. This indicates that the dipeptide is methionyl-glutamic acid, $H_3CSCH_2CH_2CH(NH_2)CONHCH(COOH)CH_2CH_2COOH$.

Configuration of glutamic acid in the peptide

When a hydrolysate of dipeptide was treated by L-glutamic decarboxylase, a spot of glutamic acid in a hydrolysate disappeared on a paper chromasogram, and that of γ-aminobutyric acid appeared, showing that glutamic acid in the dipeptide has L-configuration. The dipeptide was determined as L-methionyl-L-glutamic acid.

Effect of L-methionyl-L-glutamic acid on iodinin production

As shown in Table 13, the amounts of iodinin produced with L-methionyl-L-glutamic acid were a little smaller than that with L-methionine and L-glutamic acid, a little larger than that with L-methionine only, and about equal to that with L-glutamic acid only. The best growth was given with L-methionyl-L-glutamic acid. These facts indicate that L-methionyl-L-glutamic acid might play an important role in biosynthesis of iodinin.

Table 13. Effect of L-methionyl-L-glutamic acid on iodinin production.

N-source	1/50 O. D.	iodinin, mg/10 m*l*
met−glu	0. 181	0. 824
glu	0. 163	0. 828
met	0. 147	0. 660
met+glu	0. 153	1. 216
no addition	0. 142	0. 018
starting culture	0. 103	0. 050

Summary

Production conditions for iodinin production with resting cells of *Brevibacterium stationis var. iodininofaciens*, a marine bacterium, were investigated.

Good iodinin production was given with L-glutamic acid, L-leucine, L-alanine, and L-proline, and also with ammonium sulfate, though not supporting bacterial growth. Neither sea water, nor sodium chloride solution was required for iodinin production with this marine bacterium, and phosphate at a concentration of more than 10^{-3} M was required. Transformation of 1, 6-phenazine-diol to iodinin proceeded smoothly without hydrogen donor under aerobic condition, but anaerobic condition and hydrogen donor were required for the reverse to take place. Of redox pigments employed, thionin, toluidine blue, Janus green B, and 1, 4-naphthoquinone seemed to reverse the transformation of 1, 6-phenazine-diol to iodinin under aerobic condition.

In amino acid pool of this bacterium, L-glutamic acid, L-alanine, L-histidine, and peak 1 compound (L-methionyl-L-glutamic acid) were found in large amounts. Peak 1

compound was characteristic of this bacterium. When an excess of 1, 6-phenazine-diol was added to cell-suspension under anaerobic condition, accumulation of L-alanine and peak 1 compound was observed in amino acid pool of this bacterium. Peak 1 compound proved to be L-methionyl-L-glutamic acid, and might play an important role in biosynthesis of iodinin.

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