STUDIES ON
TRANSGLYCOSIDATION TO
VITAMIN B_6 AND PANTOTHENIC ACID
BY MICROORGANISMS

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ABBREVIATIONS

PIN	Pyridoxine
PAL	Pyridoxal
PAM	Pyridoxamine
PAL-P	Pyridoxal-5'-phosphate
PIN-G	Pyridoxine glucoside
PIN-4'-G	Pyridoxine-4'-α-D-glucoside
PIN-5'-G	Pyridoxine-5'-α-D-glucoside
PaA	Pantothenic acid
PaA-G	Pantothenic acid glucoside
PaA-α-G	Pantothenic acid-α-D-glucoside
PaA-β-G	Pantothenic acid-β-D-glucoside

INTRODUCTION

In 1934, vitamin B_6 was first demonstrated by György as a rat pellagra preventive factor.[1] The apparent and specific chemical properties of vitamin B_6 (PIN) were definitely established by Birch and György.[2] Within approximately two years of their publication, five independent groups reported, announcing the isolation of PIN. Lepkovsky,[3] Keresztesy and Stevens,[4] György,[5] Kuhn and Wendt,[6] and Ichiba and Michi[7] had succeeded in isolating PIN as a crystalline form from various natural materials. Subsequently, its chemical constitution was recognized by two independent groups, Stiller *et al.*[8] and Kuhn *et al.*[9-11] The synthesis of the vitamin was accomplished without delay in the same year by Kuhn *et al.*,[12] Harris and Folkers,[13,14] and Morii and Makino.[15]

It was considered that all the vitamin B_6 activity of natural products could be attributed to PIN before the existence of other forms of vitamin B_6 was recognized by Snell *et al.*[16] as a result of microbiological assay. These compounds were confirmed as PAL and PAM.[17-21] Thereafter, it was established that the biological activity of the vitamin B_6 group was displayed by PIN, PAL, and PAM, and their 5'-phosphate esters.[22]

An unidentified phosphorylation product of PAL, which was later identified as PAL-5'-P,[23] was discovered as a coenzyme of tyrosine decarboxylase by Gunsalus *et al.*[24] It has now been made evident that PAL-P is the principal biocatalytically active form of the vitamin as a coenzyme for various enzyme systems, associated almost entirely with

nitrogen metabolism.[25-30] A number of vitamin B_6-dependent enzymes

have now been found, and the mechanisms of many PAL-dependent reactions

in amino acid metabolism have been interpreted by Braunstein and

Shemyakin,[31] and independently by Metzler, Ikawa, and Snell with non-

enzymatic model systems.[32-34]

All vitamin B_6-related compounds owed their vitamin activity to

their conversion to PAL-P. The enzymatic interconversion of various

forms of vitamin B_6 and their conversion to PAL-P have also been

investigated by many workers.[35-44] Snell *et al.*[35,45-47] investigated

the degradation pathway of vitamin B_6 and isolated some degradation

products from a culture of certain soil pseudomonads grown on PIN

or PAM as the sole source of carbon and nitrogen. Recently, Contractor

and Shane have identified a new metabolite of PIN in rat.[48]

However, little has been reported on the biosynthetic pathway of

vitamin B_6. It has been indicated that common metabolites such as

glycerol, aspartate, leucine, and serine are useful for the biosynthe-

sis of the precursors of vitamin B_6,[49-54] but no immediate precursors

have yet been characterized.

Many derivatives of vitamin B_6 have hitherto been chemically

synthesized and have also been found to occur naturally. For the

purpose of the development of medical drugs or food supplements, many

derivatives like vitamin B_6-fatty acid esters,[55-57] PIN-nicotinic

acid esters,[58,59] PIN-thiamine disulfide,[60] PAL-homocysteine,[61]

and vitamin B_6 disulfides[62,63] have been chemically synthesized.

Naturally occurring derivatives of vitamin B_6, such as the PAL-indole

complex,[64] PIN- or PAL-3-sulfate,[65] PIN glucuronide,[66] and pyri-

doxilidene-β-phenylethylamine[67] have hitherto been identified as

metabolites. However, the glycosides of vitamin B_6 were neither

synthesized nor isolated from natural sources until PIN-G was first

discovered by Ogata et $al.$[68] during investigations on the microbial

metabolic route of vitamin B_6. In this thesis, the author deals with

the microbial formation of PIN-G, the mechanism of its enzymatic

formation, and some properties of PIN-G in the following sections.

With regard to sugar derivatives of vitamins, many compounds

have already been reported. Thiamine glycosides,[69] the glucose ether

of vitamin A,[70] D-glucose pentanicotinate,[71] and vitamin D_2 glucoside

tetraacetate[72] are chemically synthesized. Whitby first discovered

the formation of riboflavinylglucoside by rat liver in 1950.[73]

Katagiri et $al.$ investigated the microbial formation of various ribo-

flavinylglycosides, including riboflavinyl-glucoside,[74] -galactoside,

[75] -fructoside,[76] and some oligosaccharides.[77] Suzuki et $al.$ also

studied the formation of riboflavinylglycosides[78-81] by various

microorganisms and by various enzyme preparations from plants and

microorganisms. It has been suggested that these compounds may play

a physiological role in catalyzing the successive transfer of glucosyl

groups via the formation of riboflavinyl compounds of oligosaccharides.

[77,82] Recently, Kasai et $al.$ identified riboflavinyl-α-glucoside in

cat liver.[83] Miyake and Suzuki have studied the enzymatic formation

of the sugar derivative of ascorbic acid.[84] Takagi and Shiraishi

have suggested the formation of a sugar derivative of carotinoids in

spinach.[85]

4'-O-(β-D-Glucopyranosyl)-D-PaA has lately been isolated from tomato juice as a growth factor of certain malo-lactic fermentation bacteria in wine fermentation.[86-88] The compound showed a microbial activity 100 times as great as that of free D-PaA for a certain lactic acid bacterium.[87] D-PaA is widely distributed in animals, plants, and microorganisms as a component of coenzyme A and plays an important role in many metabolic pathways.[89] It has been clarified by Vagelos et al.[90,91] and Wakil et al.[92] that 4'-phosphopantetheine is another active form of D-PaA and functions as a prosthetic group in an acyl carrier protein. D-PaA is required by Saccharomyces carls-bergensis 4228 ATCC 9080,[93] Lactobacillus plantarum ATCC 8014,[94] and L. casei ATCC 7469.[95] Recently, bifidus factors, which are growth factors for Bifidobacterium bifidum N4, were isolated from carrot root and identified as 4'-phosphopantetheine-S-sulfonic acid and 3'-dephospho-coenzyme A-S-sulfonic acid by Yoshioka et al.[96,97] It was suggested that the activity of these derivatives was due to the specific membrane transport of the organisms.

In this thesis, the author describes the microbial formation of PIN-G and PaA-G. In the first chapter, the distribution of PIN-G-forming activity in culture collections and isolated strains of bacteria, yeasts, molds and actinomycetes is discussed. With an isolated strain, Micrococcus sp. No. 431, having the highest activity, several conditions affecting the fermentative production of PIN-G are described. The PIN-G-synthesizing enzyme was also purified from the

organism and the enzymatic properties have been investigated; the biological activity and some properties of PIN-G are discussed in the last section. In the second chapter, the formation of PaA-G was checked in yeasts. PaA-β-G could scarcely be detected at all, but PaA-α-G was found in the culture filtrate of several strains. *Sporobolomyces coralliformis* IFO 1032 showed the highest activity for PaA-α-G formation. PaA-α-G was isolated from the reaction mixture of *S. coralliformis* and characterized.

Chapter I. Microbial Formation of Pyridoxine Glucoside

Section I. Formation of PIN-G by Microorganisms

INTRODUCTION

During the investigation on the metabolism of PIN in microorganisms, PIN-G was first detected in the culture filtrate of *Sarcina lutea* IFO 3232, grown on a sucrose-peptone medium containing PIN.[68] This compound was isolated from a reaction mixture incubated with intact cells of the organism by paper-pile chromatography, and active charcoal, Dowex 1X2 (Cl⁻ type) and Sephadex G-15 column chromatography. The compound was characterized and identified as a mixture of PIN-4'-G and PIN-5'-G by various analytical methods.[98,99] The chemical structures of PIN-4'-G and PIN-5'-G are shown in Fig. 1.
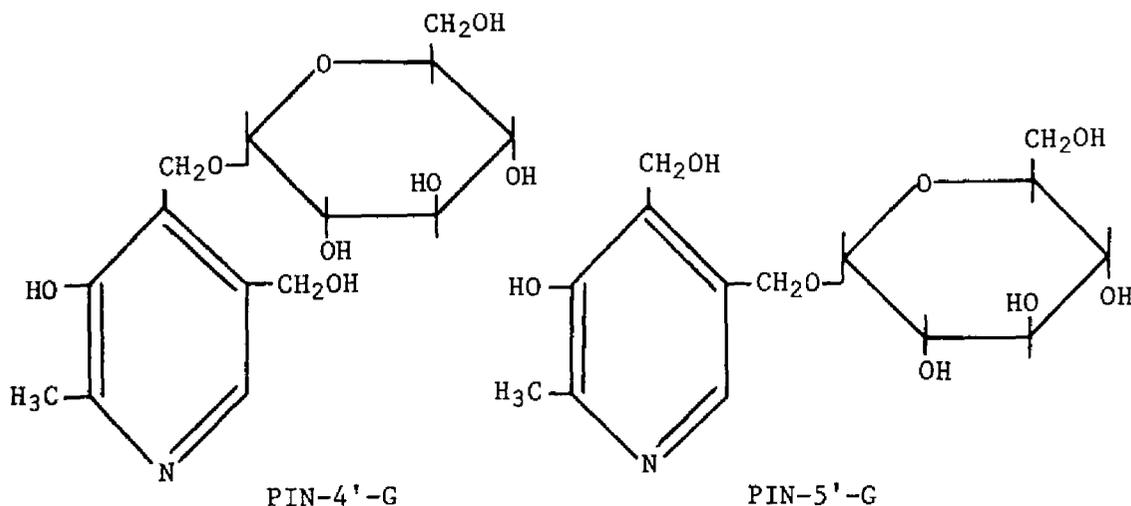

Fig. 1. Chemical structures of PIN-4'-G and PIN-5'-G.

The distribution of PIN-G-forming activity in microorganisms is an interesting problem in regard to its biological significance. The screening of more producible strains of the compound is useful for the convenient isolation.

This section describes the distribution of PIN-G-forming activity in microorganisms including bacteria, molds, yeasts and actinomycetes. The screening was also performed with isolated strains from soil. An isolated bacterium, No. 431, which had the highest activity for the formation of PIN-G, was identified as *Micrococcus* sp. No. 431 according to the Bergey's Manual of Determinative Bacteriology *etc.*[100-103)

EXPERIMENTAL

Materials. All chemicals used in this section were obtained from commercial sources.

Microorganisms. Microorganisms preserved in the Laboratory of Physiology of Fermentation and Applied Microbiology, Department of Agricultural Chemistry, Kyoto University (AKU), were used for the screening of PIN-G formation. It was also carried out with the isolated strains from soil in Kyoto Prefecture. All strains used were preserved on a nutrient agar slant by a monthly transfer at 5°C.

Cultivation. The composition of culture media for PIN-G formation by microorganisms is summarized in Table I. All cultivations were carried out in a test tube with 5 ml of the corresponding media. Bacteria except lactic acid bacteria and *Clostridium* were grown at 28°C for 3 days with shaking. Cultures of *Clostridium* were grown statically

TABLE I. COMPOSITION OF CULTURE MEDIA FOR PIN-G FORMATION

Glucose was used in place of sucrose or maltose as a blank.

Component	Bacteria	Lactic acid bacteria	Yeasts	Molds	Actinomycetes
Sucrose	2.0 %	10.0 %	5.0 %	4.0 %	2.0 %
Maltose	–	–	–	(4.0)	–
Soluble starch	–	–	–	–	0.5
Peptone	1.0	–	0.5	–	2.0
Polypeptone	–	0.05	–	–	–
NH_4NO_3	–	–	–	0.1	–
$NaNO_3$	–	–	–	0.1	–
KNO_3	–	–	–	–	0.2
$(NH_4)_2SO_4$	–	0.03	–	–	–
K_2HPO_4	0.5	–	0.4	0.1	0.02
KH_2PO_4	0.1	0.5	0.2	–	0.07
NaCl	0.2	0.1	–	–	0.3
KCl	–	–	–	0.05	–
$MgSO_4 \cdot 7H_2O$	0.02	0.02	0.02	0.05	0.01
Yeast extract	0.2	0.15	0.5	–	0.2
PIN·HCl	1.22 mg/ml	1.22 mg/ml	1.22 mg/ml	1.22 mg/ml	1.22 mg/ml
$CaCO_3$	–	–	–	+	–
pH	7.0	7.0	6.0	not adjusted	7.0

at 37°C for 4 days. Lactic acid bacteria were cultivated statically at 20-37°C for 2 days. Yeasts were cultivated at 28°C for 2 days with shaking. Molds were cultivated at 28°C for 4 days with shaking and actinomycetes were at 28°C for 3 days with shaking. The cultivations were stopped by immersing culture tubes in boiling water for 5 min. After cooling them in ice-cold water, the cells were removed by centrifugation and the broth was supplied to the assay of PIN-G.

Assay of PIN-G. A suitable amount of the broth was employed for paper chromatography on Toyo filter paper No. 53 with a solvent system of *n*-butanol:acetic acid:water (4:1:1, v/v). The product on a paper chromatogram was detected by Manaslu lamp (2536 A), cut off and eluted from the paper with a mixture of 2.5 ml of 99% ethanol and 2.5 ml of 25% sodium acetate solution at 37°C for 90 min under occasional stirring in a test tube. To the extract was added 0.2 ml of the diazotized *p*-aminoacetophenone reagent [104,105] and the absorbancy at 470 mμ was measured with a Hitachi Perkin-Elmer spectrophotometer model 139. PIN-G was measured as free PIN with a calibration curve prepared from authentic PIN. The *p*-aminoacetophenone reagent was prepared by mixing 1 ml of *p*-aminoacetophenone solution (318 mg of *p*-aminoacetophenone was dissolved in 4.5 ml of conc. HCl and then diluted to 50 ml with deionized water) and 5 ml of 4.5% sodium nitrite solution.

Identification of bacteria. Classification and identification of an isolated bacterium was performed by standard methods according to the Bergey's Manual of Determinative Bacteriology *etc.* [100-103]

RESULTS

Distribution of PIN-G-forming Activity in Microorganisms

The screening of PIN-G-forming activity was carried out with 100

strains of bacteria from culture collections. The result is shown

in Table II. The high PIN-G-forming activities (more than 10-150

μg/ml of PIN-G·as PIN) were distributed specifically in some strains

of bacteria belonging to *Micrococcus* and *Sarcina*, but the majority

of the bacteria accumulated little amounts of PIN-G in their culture

media. *Pseudomonas fragi* formed a PIN-G-like compound in the medium,

which was different from PIN-G in *Rf* values on paper chromatography.

This compound still remains to be elucidated.

About 130 strains of yeasts were searched for the accumulation

of PIN-G with the screening medium containing sucrose and PIN. As

shown in Table III, only several strains among *Endomycopsis*, *Hansenula*,

Sporobolomyces, *Brettanomyces*, *Kloeckera* and *Trichosporon* had the

relatively higher activities. The highest activity was found in

Torulopsis sake IFO 0435, which accumulated maximally 260 μg/ml of

PIN-G as PIN.

It has been reported that mold α-glucosidases readily hydrolyze

maltose rather than aryl-α-glucosides, and are not capable of hydro-

lyzing sucrose.[106,107] The screening was carried out on a maltose-

peptone medium and a sucrose-peptone medium. The results are shown

in Tables IV-a and IV-b, respectively. Only 6 strains of molds among

40 strains accumulated 10-150 μg/ml of PIN-G on a sucrose medium,

while 14 strains among 34 strains tested accumulated about the same

TABLE II. DISTRIBUTION OF PIN-G-FORMING ACTIVITY IN BACTERIA

Bacteria were grown on the screening medium described in Table I.
The cultivation was carried out as described in the EXPERIMENTAL.

Family Genus	No. of strains tested	PIN-G formed (µg/ml as PIN)		
		10>	10 - 150	150<
Enterobacteriaceae				
Escherichia	6	6	0	0
Aerobacter	4	4	0	0
Erwinia	1	1	0	0
Serratia	3	3	0	0
Proteus	4	4	0	0
Achromobacteraceae				
Alcaligenes	2	2	0	0
Achromobacter	5	5	0	0
Flavobacterium	4	4	0	0
Rhizobiaceae				
Agrobacterium	1	1	0	0
Micrococcaceae				
Micrococcus	7	4	2	1*
Staphylococcus	5	5	0	0
Sarcina	5	1	2	2**
Brevibacteriaceae				
Brevibacterium	2	2	0	0
Corynebacteriaceae				
Corynebacterium	2	2	0	0
Bacillaceae				
Bacillus	21	21	0	0
Clostridium	6	6	0	0
Pseudomonadaceae				
Pseudomonas	9	8	1	0
Xanthomonas	1	1	0	0
Lactobacillaceae				
Streptococcus	5	5	0	0
Leuconostoc	4	4	0	0
Lactobacillus	2	2	0	0
Pediococcus	1	1	0	0
Sum total:	100	92	5	3

*) *Micrococcus flavus* IFO 3242   226 μg/ml as.PIN
**) *Sarcina lutea* IAM 1099   231 "   "
    "   *marginata* IFO 3066   205 "   "

extent of PIN-G when cultured on a maltose medium. However, the

amounts of PIN-G formed in a maltose medium were higher than those in

a sucrose medium. Especially, *Penicillium rubrum* Stoll IFO 6580

accumulated 210 μg/ml of PIN-G as PIN on a maltose medium. Table V

shows that 15 strains out of 45 strains of actinomycetes tested had

the ability to form considerable amounts of PIN-G. Among them,

Streptomyces hygroscopicus IFO 3934 had the highest activity, which

accumulated 260 μg/ml of PIN-G as PIN.

Bacteria (324 strains), molds (12 strains) and actinomycetes

(6 strains) were isolated from soil in Kyoto Prefecture for a screening

of PIN-G formation. The result is shown in Table VI. Neither molds

nor actinomycetes had the low activity, but higher activities were found

in 6 strains of bacteria, in which a strain No. 431 had the highest

activity, accumulating 290 μg/ml of PIN-G as PIN. Thus, the strain

No. 431 was selected and used as the PIN-G-forming strain in the

following experiment.

Identification of the Strain No. 431

The isolated bacterial strain No. 431 described above, was classi-

fied and identified as follows, according to the method of the Bergey's

Manual of Determinative Bacteriology *etc.*[100-103)

(a) Morphological: Spheres, 1 micron in diameter in average size,

occurring in pairs. Non-motile. Gram-positive.

TABLE III. DISTRIBUTION OF PIN-G-FORMING ACTIVITY IN YEASTS

Yeasts were grown on the screening medium described in Table I.
The cultivation was carried out as described in the EXPERIMENTAL.

Faminly Genus	No. of strains tested	PIN-G formed (μg/ml as PIN)		
		10>	10 - 150	150<
Endomycetaceae				
Saccharomyces	35	35	0	0
baker's yeast	2	2	0	0
brewer's yeast	3	3	0	0
shoyu yeast	1	1	0	0
Endomyces	3	3	0	0
Endomycopsis	2	1	1	0
Schizosaccharomyces	2	2	0	0
Zygosaccharomyces	3	3	0	0
Pichia	4	4	0	0
Hansenula	9	7	2	0
Schwanniomyces	1	1	0	0
Debaryomyces	2	2	0	0
Saccharomycodes	2	2	0	0
Hanseniaspora	1	1	.0	0
Nadsonia	1	1	0	0
Lipomyces	2	2	0	0
Sporobolomycetaceae				
Sporobolomyces	5	3·	2	0
Cryptococcaceae				
Cryptococcus	3	3	0	0
Torulopsis	7	6	0	1*
Brettanomyces	3	2	1	0
Candida	21	21	0	0
Kloeckera	3	2	1	0
Wickerhamia	1	1	0	0
Rhodotorula	10	10	0	0
Trichosporon	3	2	1	0
Sum total:	129	120	8	1

*) *Torulopsis sake* IFO 0435 260 μg/ml as PIN

TABLE IV-a. DISTRIBUTION OF PIN-G-FORMING ACTIVITY IN MOLDS

Molds were grown on the sucrose medium described in Table I. The cultivation was carried out as described in the EXPERIMENTAL.

Genus	No. of strains tested	PIN-G formed (µg/ml as PIN)		
		10>	10 ‑ 150	150<
Mucor	9	7	2	0
Rhizopus	3	2	1	0
Aspergillus	13	12	1	0
Penicillium	6	6	0	0
Monascus	1	1	0	0
Neurospora	3	3	0	0
Pullularia	1	1	0	0
Fusarium	4	2	2	0
Sum total:	40	34	6	0

TABLE IV-b. DISTRIBUTION OF PIN-G-FORMING ACTIVITY IN MOLDS

Molds were grown on the maltose medium described in Table I. The cultivation was carried out as described in the EXPERIMENTAL.

Genus	No. of strains tested	PIN-G formed (µg/ml as PIN)		
		10>	10 ‑ 150	150<
Mucor	8	5	3	0
Rhizopus	8	7	1	0
Aspergillus	10	4	6	0
Penicillium	3	0	2	1*
Neurospora	3	3	0	0
Pullularia	1	1	0	0
Fusarium	1	0	1	0
Sum total:	34	20	13	1

*) *Penicillium rubrum* IFO 6580   210 µg/ml as PIN

TABLE V. DISTRIBUTION OF PIN-G-FORMING ACTIVITY IN ACTINOMYCETES

Actinomycetes were grown on the screening medium described in Table I.
The cultivation was carried out as described in the EXPERIMENTAL.

Genus	No. of strains tested	PIN-G formed (µg/ml as PIN)		
		10>	10 - 150	150<
Mycobacterium	3	3	0	0
Nocardia	4	3	1	0
Streptomyces	24	13	10	1*
Unidentified	14	11	3	0
Sum total:	45	30	14	1

*) *Streptomyces hygroscopicus* var. *angustomyceticus* IFO 3934 260
µg/ml as PIN

TABLE VI. DISTRIBUTION OF PIN-G-FORMING ACTIVITY IN MICROORGANISMS
ISOLATED FROM SOIL

Microorganisms isolated from soil were grown on the screening medium
described in Table I. The cultivation was carried out as described in
the EXPERIMENTAL.

Microorganisms	No. of strains tested	PIN-G formed (µg/ml as PIN)		
		10>	10 - 150	150<
Bacteria	324	290	28	6*
Molds	12	12	0	0
Actinomycetes	6	5	0	1
Sum total:	342	307	28	7

*) Strain No. 431   289 µg/ml as PIN

(b) Cultural:

   Gelatin stab: No liquefaction.

   Agar slant: Yellow, smooth growth.

   Nutrient broth: Turbid, with yellowish sediment.

   Potato: Abundant, yellow growth.

(c) Physiological:

   Litmus milk: Alkaline, not peptonized and no coagulation; litmus not reduced.

   Indole not produced.

   Hydrogen sulfide produced.

   Ammonia produced from peptone.

   Acid from fructose, galactose, xylose, and mannitol. No acid and alkaline from glucose, maltose, dextrin, starch, glycerol, sucrose, arabinose, raffinose, lactose, inulin, mannose and sorbitol. No gas from all carbohydrates.

   Citric acid utilized as a sole source of carbon.

   Nitrites produced from nitrates.

   Catalase positive.

   Methyl red test negative.

   Voges-Proskauer test negative.

   Optimum temperature between 28 and 37°C. Killed at 60°C for 10 min.

   Optimum pH between 5.5 and 10.0.

   Aerobic.

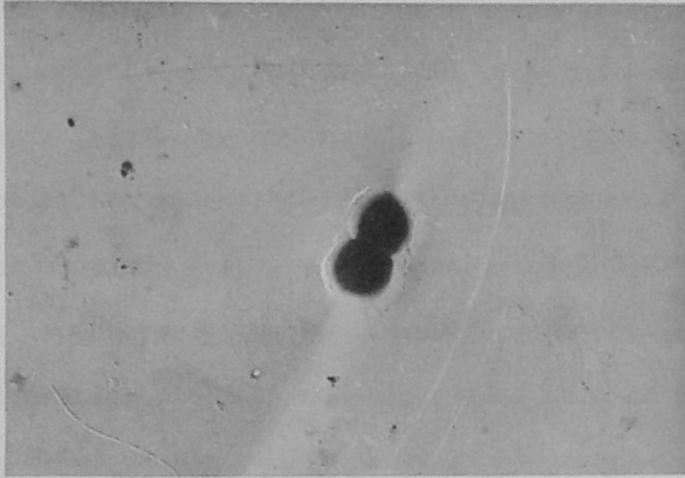(d) Source: Isolated from soil in Kyoto Prefecture.

Fig. 2.   Electron Micrograph of the Strain No. 431.

From the data described above, the strain No. 431 may be classi-
fied into genus *Micrococcus*.   However, the isolated strain was not
applicable to any known species in *Micrococcus* described in the
manual, and so it was tentatively designated as *Micrococcus* sp. No.
431.   The electron micrograph of the organism is shown in Fig. 2.

DISCUSSION

The relatively higher activities for PIN-G formation were parti-
cularly observed in several strains of bacteria belonging to the
genera *Sarcina* and *Micrococcus*.   It is an interesting fact that these
coccidal strains belonging to the same family, *Micrococcaceae*, have
the higher activities.   On the other hand, other cocci such as *Staphylo-
coccus*, which is also classified into *Micrococcaceae*, *Leuconostoc*,
Pediococcus and *Streptococcus* had no activity.   Although the trans-
glucosidation to riboflavin to form riboflavinyl glucoside has been

demonstrated in various bacterial strains[108] including *Escherichia
coli, Aerobacter aerogenes, Bacillus subtilis, Clostridium acetobuty-
licum, Lactobacillus casei* and *Streptococcus lactis,* these strains did
not form PIN-G from PIN under the conditions as described in this
section. The PIN-G-forming activity was also observed in some strains
of yeasts, molds and actinomycetes. When 34 strains of various molds
were cultivated on a maltose-peptone medium, 14 strains including
Mucor, Rhizopus, Aspergillus, Penicillium, and *Fusarium* accumulated
10-150 μg/ml or more than 150 μg/ml of PIN-G. On the other hand, only
several strains were found to form a slight amount of PIN-G on a
sucrose-peptone medium. These results coincide with the fact that
transglucosidase of molds generally hydrolyzes maltose, but not sucrose.

The PIN-G-forming activity in microorganisms which were isolated
from soil was distributed mainly among bacteria (34 strains of bacteria,
one strain of actinomycetes), in which 7 strains accumulated relatively
large amounts of PIN-G (more than 150 μg/ml of PIN-G as PIN). Best of
all, an isolated bacterium strain No. 431, which was classified into
Micrococcaceae, had the highest activity (289 μg/ml of PIN-G as PIN).
The various conditions for PIN-G formation by this organism will be
discussed in the next section.

SUMMARY

The distribution of PIN-G-forming activity was investigated with
various kinds of microorganisms under the conditions of their growth
on the media containing PIN and sucrose or maltose. The formation of

PIN-G was searched in the culture broth of organisms including bacteria (100 strains), yeats (129 strains), molds (34 strains) and actinomycetes (45 strains). The screening was also performed with isolated strains of bacteria, molds and actinomycetes (total 342 strains) from soil. The results of screening showed that the higher activities were specifically found in several strains of the bacteria belonging to the genera *Sarcina* and *Micrococcus*. The activity was also shown to be distributed in yeasts, molds and actinomycetes. The activity was found in the isolated strains. A bacterium No. 431, which was identified as *Micrococcus* sp. No. 431 according to the Bergey's Manual *etc.*, showed the highest activity (289 μg/ml of PIN-G as PIN) among the tested organisms and selected to be the most suitable strain for PIN-G formation.

Section II. PIN-G Formation by *Micrococcus* sp. No. 431

INTRODUCTION

In the preceding section, a bacterium was isolated and identified
as *Micrococcus* sp. No. 431, which accumulated approximately 300 µg/ml
of PIN-G as PIN in the culture medium composed of PIN and sucrose.
In order to get a large amount of PIN-G, various factors on the forma-
tion of PIN-G are studied in this section with the growing cultures
of this organism. PIN-G was first detected in the culture filtrate
of *Sarcina lutea* IFO 3232,[68] and was identified as a mixture of PIN-
4'-G and PIN-5'-G.[99] In this section, PIN-4'-G and PIN-5'-G were
isolated by various separating methods from the culture filtrate of
Micrococcus sp. No. 431, which was grown under the optimal conditions.
The products were also identified by various analytical methods inclu-
ding IR and NMR spectroscopy. Moreover, PIN-diglucoside (possibly
PIN-4'-, PIN-5'-, and PIN-4',5'-diglucosides) was isolated from the
culture filtrate and identified by the same methods.

EXPERIMENTAL

Materials. Cellulase (Type II, from *Aspergillus niger*), β-gluco-
sidase (from almonds), maltase (Grade II, from *Aspergillus niger*)
were purchased from Sigma Chem. Co., and α-glucosidase (from yeast)
was from Boehringer Mannheim Japan K.K. Other chemicals used in this
work were obtained from commercial sources.

Microorganism and cultivation. *Micrococcus* sp. No. 431, which

was isolated from soil and identified in the preceding section, was used for the formation of PIN-G. The basal medium contained 2.0% sucrose, 1.0% peptone, 0.2% yeast extract. 0.5% K_2HPO_4, 0.1% KH_2PO_4, 0.2% NaCl, 0.02% $MgSO_4 \cdot 7H_2O$ and 1 mg/ml of PIN (pH 7.0). The cultivation was carried out at 28°C for 40 hr with reciprocal shaking.

Analyses. After cultivation, the cultural broth was heated for 5 min in a boiling water bath. The supernatant solution obtained by centrifugation was followed to the assay. PIN-G was assayed by paper chromatography as described in the preceding section.

Absorption spectra were recorded on a Shimazu multi-purpose spectrophotometer model MPS-50L or a Hitachi double-beam spectrophotometer type 124.

Paper chromatography of vitamin B_6 was carried out with the solvent systems of I. *n*-butanol:acetic acid:water (4:1:1, v/v), II. *n*-amylalcohol:acetone:water (2:1:1, v/v, upper layer), III. water: acetone:*t*-butanol:diethylamine (20:35:40:5, v/v) and IV. water: acetone:*t*-butanol:acetic acid (20:35:40:5, v/v). The diazotized *p*-aminoacetophenone reagent,[104,105] a mixture of 1 ml of *p*-aminoacetophenone solution, 5 ml of 4.5% sodium nitrite and 5 ml of 25% sodium acetate, was sprayed for vitamin B_6 detection.

Paper chromatography of sugars was carried out with the solvent systems of I. *n*-butanol:benzene:pyridine:water (5:1:3:3, v/v, upper layer), II. *n*-butanol:pyridine:water (6:4:3, v/v) and III. isopropanol:pyridine:water:acetic acid (8:8:4:1, v/v), and the silver nitrate reagent[109] was sprayed for sugar detection.

A PIN moiety was determined by the diazotized *p*-aminoacetophenone reagent[104,105] as described in the preceding section.

An anthrone method[110] was used for the assay of total sugars and a glucostat method[111] was used for the assay of a glucose moiety. Reducing sugar was determined by the method of Somogyi.[112]

The reaction by several glucosidases was carried out on a reaction mixture containing 212 µg of PIN-G, 20 µmoles of 2-mercaptoethanol, 20 µmoles of buffer and an appropriate amount of enzyme in a total volume of 1.0 ml. The reaction was carried out at 37°C overnight. Phosphate buffer (pH 6.5) was used in the incubations with mold maltase and yeast α-glucosidase, and acetate buffer (pH 4.4) in those with cellulase and β-glucosidase. The reaction mixture was submitted to paper chromatographic analyses for PIN and glucose as described above.

Measurement of IR and NMR spectra. IR spectrum of PIN-G was recorded in a micro KBr tablet with a Hitachi infrared spectrometer type EPI-G3.

NMR spectra of PIN-G and PIN-diglucoside were recorded in D_2O on a Hitachi Perkin-Elmer high resolution NMR spectrometer type R-22 at 90 MHz with DSS as an internal standard.

RESULTS

Cultural Conditions for PIN-G Formation

Carbon sources

The effect of various carbohydrates as the glucosyl donors on

TABLE I. EFFECT OF CARBON SOURCES

Micrococcus sp. No. 431 was cultivated on the basal medium described
in the EXPERIMENTAL except that various carbohydrates were used as shown
in the Table. The cultivation was performed aerobically at 28°C for 40 hr.

Carbon sources	Growth	Final pH	PIN-G formed (μg/ml as PIN)
Glycerin	+++	7.6	0
Arabinose	++	7.6	0
Xylose	++	6.6	0
Glucose	++++	6.4	0
Fructose	++++	6.4	0
Galactose	+++	6.4	0
Mannose	++++	6.0	0
Mannit	+++	7.0	0
Methyl-α-D-glucoside	++	8.6	0
Sucrose	+++	7.0	313
Maltose	++++	6.6	32
Trehalose	++	8.8	0
Lactose	++	8.2	0
Raffinose	++	7.8	0
Dextrin	++	8.6	0
Starch	++	8.6	0
Inulin	++	8.0	0

the formation of PIN-G was studied with *Micrococcus* sp. No. 431.

As shown in Table I, sucrose and maltose, having the α-glucosidic

linkage, were effective for the PIN-G formation. Maltose was one-

tenth as effective as sucrose, and trehalose was not effective, though

it has an α(1→1) glucosidic linkage. Polysaccharides such as dextrin

TABLE II. EFFECT OF NITROGEN SOURCES

Micrococcus sp. No. 431 was cultivated on the basal medium described
in the EXPERIMENTAL except that various nitrogen sources were used as
shown in the Table. The cultivation was performed at 28°C for 40 hr.

Nitrogen sources	Growth	Final pH	PIN-G formed (μg/ml as PIN)
None	+	6.8	83
Peptone	++++	8.2	269
Casamino acid	++++	8.2	170
Urea	+	8.2	41
$(NH_4)_2SO_4$	+	6.6	108
NH_4Cl	++	6.2	104
$NaNO_3$	++	7.2	88
$NaNO_2$	+	7.2	29
Asparagine	++	9.2	36
Glutamate	+++	9.2	91

and starch also had no effect on the formation of PIN-G. No deriva-
tive of PIN other than PIN-G was formed with lactose, raffinose and
inulin.

Nitrogen sources

    The incubation was undertaken on a sucrose medium with various
inorganic and organic nitrogen compounds. The result is shown in
Table II. The organic nitrogen compounds such as peptone and casamino
acid were useful for the formation of PIN-G and the growth of the
organism. Ammonium sulfate and ammonium chloride were fairly effec-
tive on the formation of PIN-G. A considerable amount of PIN-G was
formed in a medium containing no nitrogen compounds except 0.2% yeast

extract as a growth factor.

Surfactants

The effect of various surfactants on the formation of PIN-G was investigated with the addition of 0.1%, the result being shown in Table III. Nonionic surfactants, Span and Tween series showed a stimulating effect on the formation of PIN-G. Among them, Span 80 was found to be most effective. On the other hand, anionic and cationic surfactants were rather inhibitory on both of the growth and the formation. With the addition of 0.7% Span 80, the amount of PIN-G accumulated reached to a maximum of about 600 µg/ml as PIN from 1 mg/ml of PIN.
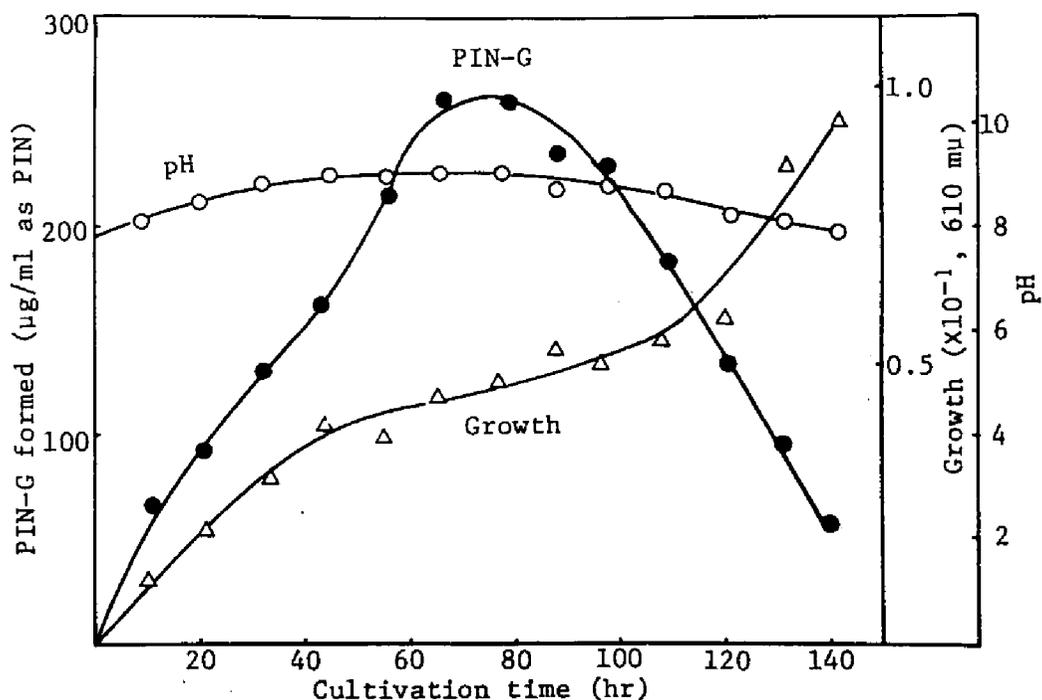
Fig. 1. Effect of Cultivation Time.

The organism was grown on a medium composed of 5.0% sucrose, 1.5% peptone, 0.2% yeast extract, 0.5% K_2HPO_4, 0.1% KH_2PO_4, 0.2% NaCl, 0.02% $Na_2MoO_4 \cdot 2H_2O$, 0.1% Tween 60 and 1 mg/ml of PIN (pH 7.0). The cultivation was carried out at 28°C with reciprocal shaking.

TABLE III.   EFFECT OF VARIOUS SURFACTANTS

Micrococcus sp. No. 431 was cultivated on the basal medium described in the EXPERIMENTAL except that 0.1 % of a final concentration of various surfactants were added as shown in the Table. The cultivation was performed at 28°C for 40 hr with shaking.

Surfactant		Growth	Final pH	PIN-G formed (μg/ml as PIN)
None	−	++++	7.4	212
CTAB	cationic	+	6.4	22
CPC	"	+	6.4	33
Aerosol OT	anionic	+	6.4	19
Na-Laurate	"	+++	7.0	92
Na-Myristate	"	+	6.8	81
Na-Palmitate	"	++++	7.4	248
Na-Stearate	"	++++	7.2	243
Na-Oleate	"	+	6.6	98
Tween 20	nonionic	++++	7.2	388
" 40	"	++++	7.2	353
" 60	"	++++	7.2	376
" 80	"	++++	7.0	407
" 85	"	++++	6.8	359
Span 20	"	+	6.4	94
" 40	"	+++	7.0	86
" 60	"	++++	7.0	243
" 80	"	++++	6.2	524
" 85	"	++++	6.4	335

Abbreviations:
CTAB;Cetyltrimethyl ammonium bromide, CPC;Cetylpyridinium chloride, Aerosol OT;Na-dioctylsulfosuccinic acid, Tween 20, 40, 60, 80 and 85; Polyoxyethylene sorbitan monolaurate, monopalmitate, monostearate, monooleate and trioleate, Span 20, 40, 60, 80 and 85;Sorbitan mono-laurate, monopalmitate, monostearate, monooleate and trioleate.

TABLE IV. EFFECT OF METAL IONS

Micrococcus sp. No. 431 was grown on the basal medium described in the EXPERIMENTAL except that 5.0% sucrose, 1.5% peptone, 0.7% Span 80, and 0.02% various metal ions were added. The cultivation was carried out at 28°C for 60 hr with shaking.

Metal ions	Growth $(\times 50^{-1}, 610 \text{ m}\mu)$	Final pH	PIN-G formed (μg/ml as PIN)
None	.193	6.6	695
EDTA*	.268	6.2	542
$MgSO_4 \cdot 7H_2O$	.308	6.2	528
$Na_2MoO_4 \cdot 2H_2O$	.188	6.4	704
$FeSO_4 \cdot 7H_2O$	.234	6.4	651
$Fe_2(SO_4)_3 \cdot xH_2O$	.184	6.6	649
$ZnSO_4 \cdot 7H_2O$	.086	7.0	435
$CoCl_2 \cdot 6H_2O$	.122	7.0	386
$MnSO_4 \cdot 4-6H_2O$	.133	8.4	150
$CuSO_4 \cdot 5H_2O$	.188	6.8	297
$AgNO_3$	.013	6.4	–
$HgCl_2$	.005	6.4	–

*) Ethylenediamine tetra-acetic acid

Incubation time

A time course of PIN-G formation and growth of the organism is shown in Fig. 1. The amount of PIN-G increased in an earlier stage of growth and reached to a maximum in 60 to 80 hr culture. A prolonged cultivation caused a rapid degradation of PIN-G. It may be suggested that the organism has a relatively strong α-glucosidase activity which hydrolyzes PIN-G to liberate PIN.
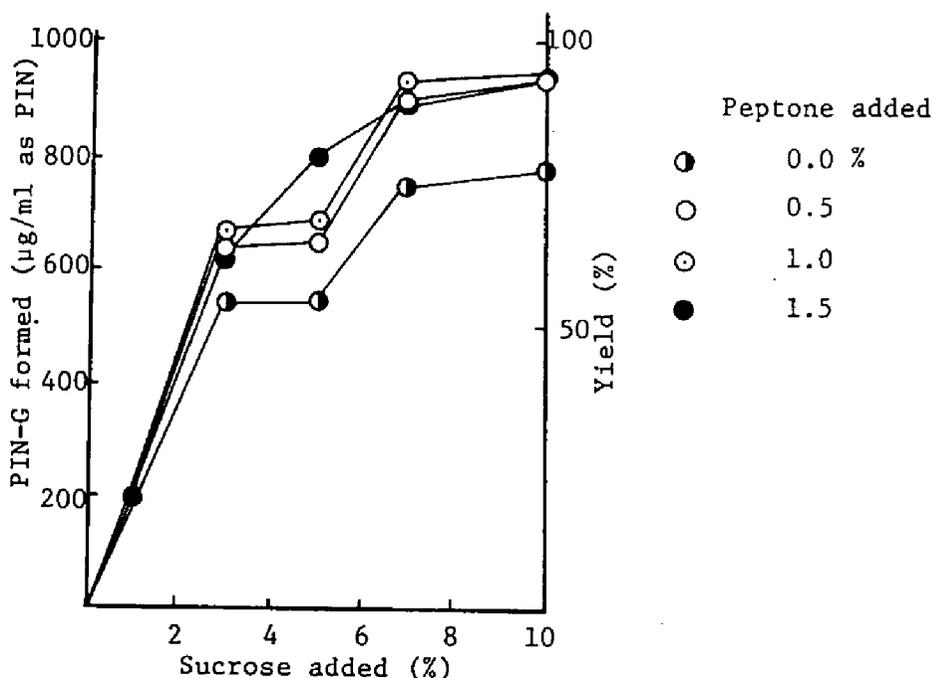
Metal ions

Fig. 2. Effect of Sucrose and Peptone Concentration.

The organism was grown on a medium composed of various concentrations of sucrose and peptone, 0.2% yeast extract, 0.5% K_2HPO_4, 0.1% KH_2PO_4, 0.2% NaCl, 0.7% Span 80 and 1 mg/ml of PIN (pH 7.0). The cultivation was carried out at 28°C for 60 hr with reciprocal shaking.

The effect of metal ions was examined. As shown in Table IV, the presence of metal ions did not affect the formation or excretion of PIN-G by the organism.

Sucrose and peptone concentrations

The effect of sucrose and peptone concentrations in culture media was investigated. The cultivation was performed at 28°C for 60 hr with shaking by the addition of 0.7% Span 80. As shown in Fig. 2, the amount of PIN-G increased proportionately to the concentration of sucrose added and reached to a maximum in the presence of 10% sucrose.
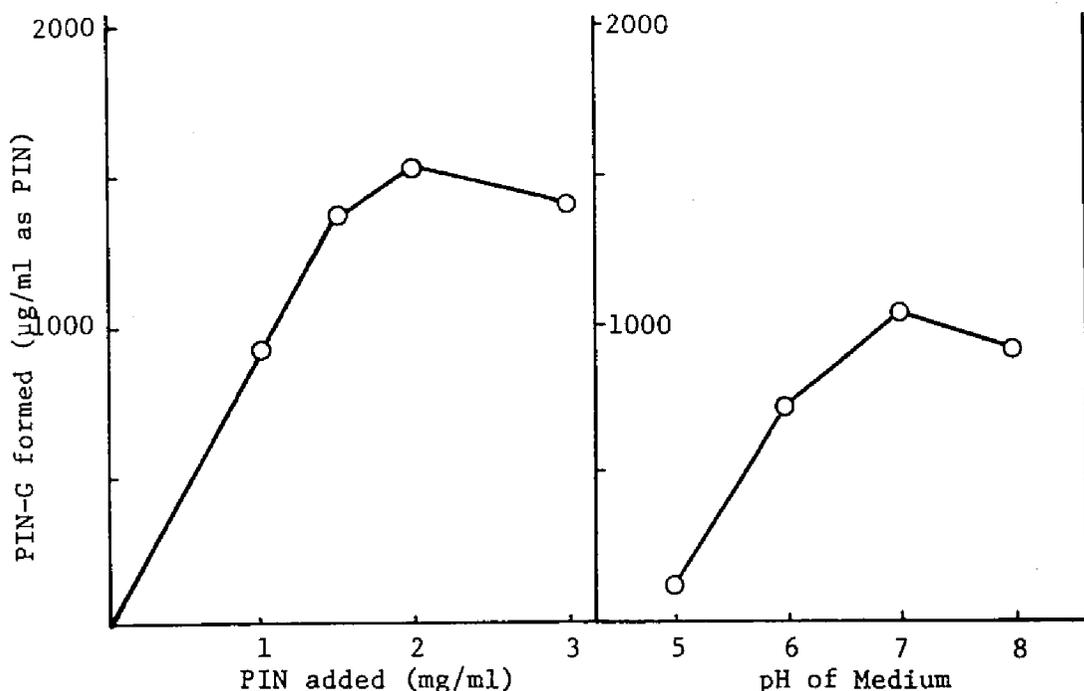
Fig. 3.   Effect of PIN Concentration.

Fig. 4.   Effect of pH of Culture.

The organism was grown on a medium composed of 10% sucrose, 0.5% peptone, 0.2% yeast extract, 0.5% K_2H-PO_4, 0.1% KH_2PO_4, 0.2% NaCl, 0.7% Span 80 and various concentrations of PIN (pH 7.0). The cultivation was carried out at 28°C for 60 hr with reciprocal shaking.

The organism was grown on the same medium with 2 mg/ml of PIN added as described in Fig. 3 except that pH of the medium was changed as shown in the Fig. The cultivation was carried out at 28°C for 60 hr with reciprocal shaking.

On the other hand, the concentration of peptone gave no effect on the formation of PIN-G. By the addition of 10% sucrose and 0.5-1.5% peptone, the yield of PIN-G attained to 90% against PIN added.

PIN Concentration

The effect of PIN concentration was examined in the presence of 10% sucrose and 0.5% peptone. The incubation was carried out at 28°C for 60 hr with shaking. Figure 3 shows that PIN-G is increased proportionately to the concentration of PIN added and attained to a maximam at

2 mg/ml of PIN.

pH of culture

As shown in Fig. 4, a maximal formation of PIN-G occurred at pH 7.0. At pH 5.0, PIN-G was scarcely formed.

From the results described above, the optimal incubation conditions were determined as shown in Table V.

TABLE V. OPTIMAL CULTURE CONDITIONS
FOR PIN-G FORMATION

Sucrose	10.0	%
Peptone	1.5	
Yeast extract	0.2	
K_2HPO_4	0.5	
KH_2PO_4	0.1	
NaCl	0.2	
Span 80	0.7	
PIN·HCl	2.44	mg/ml

pH 7.0, tap water

The cultivation was performed aerobically at 28°C for 60 hr.

Isolation and Identification of PIN-G and PIN-diglucoside

From the results described above, optimal culture conditions of *Micrococcus* sp. No. 431 for PIN-G formation were settled as shown in Table V. In order to isolate PIN-G on a laboratory scale, the bacterium was cultured with 1000 ml of the optimal medium (each 500 ml of the medium in a 2 liter flask) under the optimal conditions as indi-

cated in Table V. The cultivation was stopped by heating the flask
in a boiling water bath for 5 min. After cooling the broth in an
ice bath, the cells were removed by centrifugation at 10,000 rpm for
30 min. The supernatant solution was adjusted to pH 2.0 and stored
at 4°C overnight. Any material precipitating was removed by filtra-
tion. Sixty grams of active carbon was added to the supernatant
solution. After stirring the mixture for 1 hr, the charcoal was
collected by filtration, washed with 0.01N HCl and water, and then the
adsorbed material was eluted with 28% ammonia:99% ethanol:water (5:
50:45, v/v). The eluate was concentrated to a small volume *in vacuo.*
The concentrated solution was applied on a Dowex 1X2 (Cl⁻ type, 3 X
10 cm) column, and water was passed through. The effluent was shaken
vigorously with chloroform to remove lipophilic impurities. The
water layer was concentrated to a small volume to contain 30-50 mg of
materials/ml as PIN. Two or three milliliters of the concentrate
was repeatedly applied on a Sephadex G-10 (1 X 105 cm), followed by
elution with water by 3 ml portions. The absorbancy at 325 mμ of
each fraction was measured, and an aliquot of the ultraviolet
absorbing fractions was chromatographed on Toyo filter paper No. 53
with a solvent system of *n*-butanol:acetic acid:water (4:1:1, v/v).
The elution pattern on a Sephadex column and the paper chromatogram
of each fraction are shown in Fig. 5. The fractions (No. 14-16)
containing PIN-G were collected, concentrated and applied on a
Sephadex column. This procedure was repeated until PIN and unknown
compounds were separated from PIN-G fractions. PIN-G fractions were
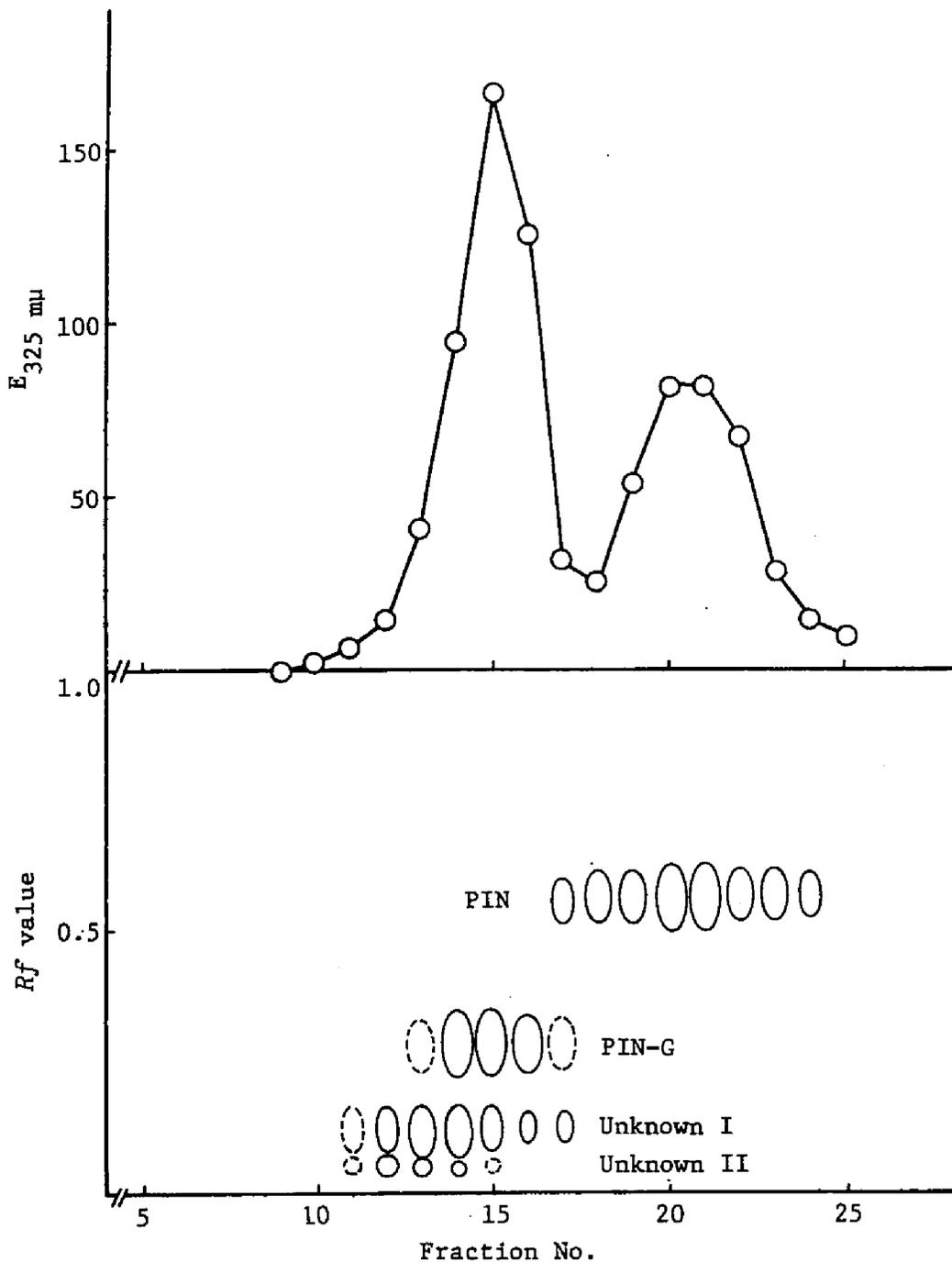
Fig. 5. Sephadex G-10 Gel Filtration and Paper Chromatogram of Each Fraction.

Column size; 1 X 104 cm, Flow rate; 9 ml/hr, One fraction; 3 ml/hr.
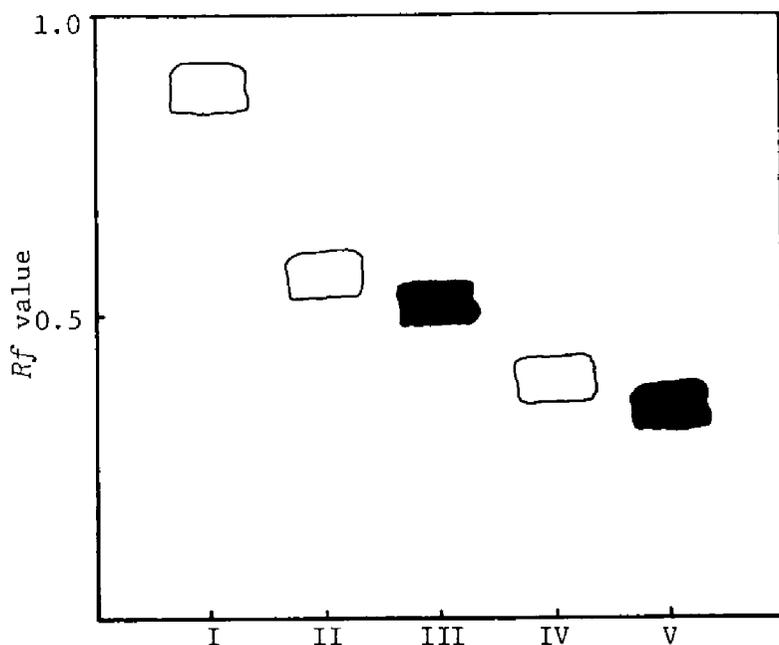Solvent system: *n*-butanol:acetic acid:water (4:1:1, v/v)

Fig. 6. Paper Chromatogram of Isolated Compounds.

Solvent system: n-butanol:acetic acid:water (4:1:1,v/v)

The diazotized p-aminoacetophenone reagent was sprayed, followed by respraying 0.2M borate buffer (pH 9.0).

☐ yellow spot, ■ orange spot

   I. PIN

  II. PIN-5'-G

 III. PIN-4'-G

  IV. PIN-diglucoside (5'-)

   V. PIN-diglucoside (4'- and 4',5'-)

followed by paper chromatography with a solvent system of n-butanol: benzene:pyridine:water (5:1:3:3, v/v, upper layer). Two fluorescent zones were detected on the paper chromatogram. Each zone was separately cut off and eluted with 50% ethanol. The first compound with a large Rf value formed a borate complex and was not colored with the diazotized p-aminoacetophenone reagent in the presence of borate

buffer. On the other hand, the second compound with a small *Rf* value, did not form a borate complex and was colored with the diazotized *p*-aminoacetophenone reagent. It has been reported that 3- and 4-hydroxyl groups of PIN combine with borate.[113] PIN is colored with the diazotized *p*-aminoacetophenone reagent when 3-hydroxyl group is free. Thus, these two compounds were assumed to be PIN-5'-G and PIN-4'-G, respectively, in which PIN-4'-G was slightly dominant. One of the unknown compounds (I) which were obtained by repeated chromatographies on a Sephadex column was followed to paper chromatography and two fluorescent zones were also confirmed. Each zone was cut off and eluted with 50% ethanol. These compounds were also followed to the identification. Figure 6 shows a paper chromatogram of these compounds.

The identification of PIN-G was carried out with a mixture of

in 0.1N HCl

in 0.1M phosphate buffer (pH 6.9)
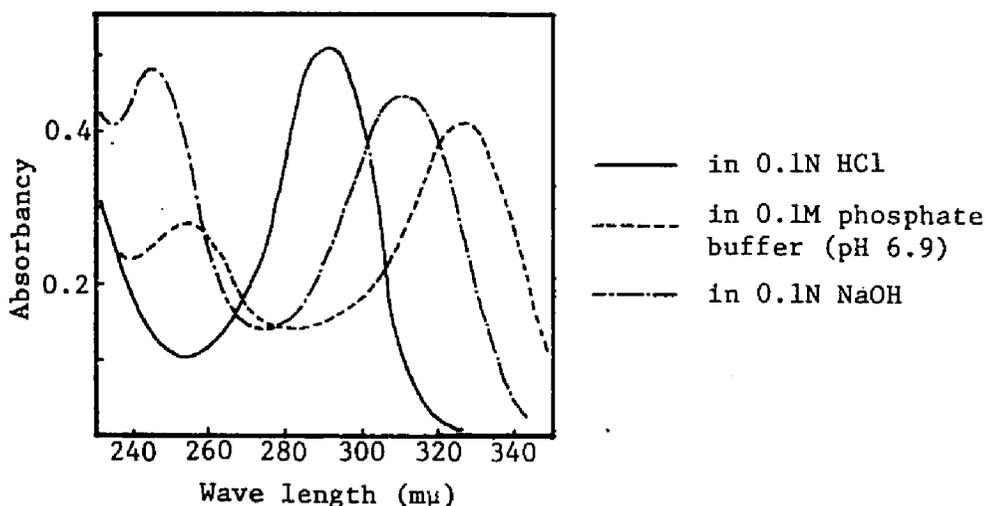
in 0.1N NaOH

Fig. 7. Ultraviolet Absorption Spectra
of Isolated PIN-G.

The absorption spectra were recorded on a Shimazu multi-purpose spectrophotometer model MPS-50L.

PIN-4'-G and PIN-5'-G except NMR spectroscopy. The ultraviolet

absorption spectra of the compound at acidic, neutral and alkaline

pH were shown in Fig. 7. Absorption maxima of the compound were at

292 mµ in 0.1N HCl, at 254 and 327 mµ in 0.1M potassium phosphate

buffer (pH 7.0), and at 245 and 312 mµ in 0.1N NaOH, respectively.

These spectra coincided with those of PIN. The compound was hydrolyzed

in 0.055N H_2SO_4 at 120°C and 15 lb for 3 hr in an autoclave. As

shown in Table VI, the Rf values of a vitamin B_6 component liberated

by acid-hydrolysis were consistent with those of PIN in three solvent

systems. The Rf values of a sugar component in the hydrolyzate

TABLE VI. PAPER CHROMATOGRAPHY OF ISOLATED PIN-G AND ITS ACID-
HYDROLYZATE (DIAZOTIZED P-AMINOACETOPHENONE POSITIVE)

| Compound | Solvent | | | |
	I	II	III	IV
	Rf values			
PIN-G	.39	.07	.27	.37
Acid-hydrolyzate*	.72	.40	.50	.55
PIN	.71	.41	.53	.58
PAL	.68	.39	.76	.60
PAM	.45	.35	.76	.27

*) hydrolyzed in 0.055N H_2SO_4 at 120°C and 15 lb for 3 hr.

Solvent system:
  I. n-butanol:acetic acid:water (4:1:1, v/v)
 II. n-amylalcohol:acetone:water (2:1:1, v/v, upper layer)
III. water:acetone:t-butanol:diethylamine (20:35:40:5, v/v)
 IV. water:acetone:t-butanol:acetic acid (20:35:40:5, v/v)

TABLE VII.   PAPER CHROMATOGRAPHY OF ISOLATED PIN-G AND ITS ACID-
HYDROLYZATE (SILVER NITRATE POSITIVE)

Compound	Solvent		
	I	II	III
	Rf values		
PIN-G	.38	.49	.67
Acid-hydrolyzate*	.23	.34	.54
Glucose	.23	.34	.54
Fructose	.28	.37	.56
Maltose	.13	.22	.47
Sucrose	.17	.28	.53

*) hydrolyzed in 0.055N H_2SO_4 at 120°C and 15 lb for 3 hr.

Solvent system:

 I. n-butanol:benzene:pyridine:water (5:1:3:3, v/v, upper layer)

 II. n-butanol:pyridine:water (6:4:3, v/v)

III. isopropanol:pyridine:water:acetic acid (8:8:4:1, v/v)

TABLE VIII.   MOLAR RATIO OF PIN TO GLUCOSE

	PIN found (μmole)	Glucose found (μmole)	Molar ratio
Diazotized p-aminoaceto-phenone method	2.50	–	1.00
Anthrone method	––	2.38	0.95
Glucostat method*	––	2.33	0.93

*) PIN-G was hydrolyzed in 0.055N H_2SO_4, at 120°C and 15 lb for
   3 hr.

Reducing activity was not found by Somogyi-Nelson method.

TABLE IX. HYDROLYSIS BY α- AND β-GLUCOSIDASES

The isolated compound was hydrolyzed under the conditions described in the EXPERIMENTAL. PIN and glucose moieties were confirmed by paper chromatography.

Glucosidases	PIN liberated	Glucose liberated
Yeast α-glucosidase	+	+
Mold maltase	+	+
β-Glucosidase (Emulsin)	−	−
Cellulase	−	−

coincided with those of glucose, as shown in Table VII. The molar ratio of PIN to glucose was confirmed to be 1:1 by the diazotized p-aminoacetophenone method,[104,105] the anthrone method[110] and the glucostat method[111] (Table VIII). The reducing activity of the compound was not found with the method of Somogyi.[112] Table IX shows the enzymatic hydrolysis of the isolated compound. The compound was hydrolyzed by yeast α-glucosidase and mold maltase, but not by β-glucosidase and cellulase. All these results indicated that the product of *Micrococcus* sp. No. 431 was PIN-α-glucoside. An IR spectrum of the compound supported this assumption (Fig. 8). As shown in Table X, NMR signals of PIN-G showed no difference between PIN-4'-G and PIN-5'-G.

PIN-diglucoside was also identified by various analytical methods. The ultraviolet absorption spectrum of the compound coincided with that of PIN. On hydrolysis in 0.055N H_2SO_4 at 120°C and 15 lb for 3 hr, the isolated compound gave two components corresponding to glucose
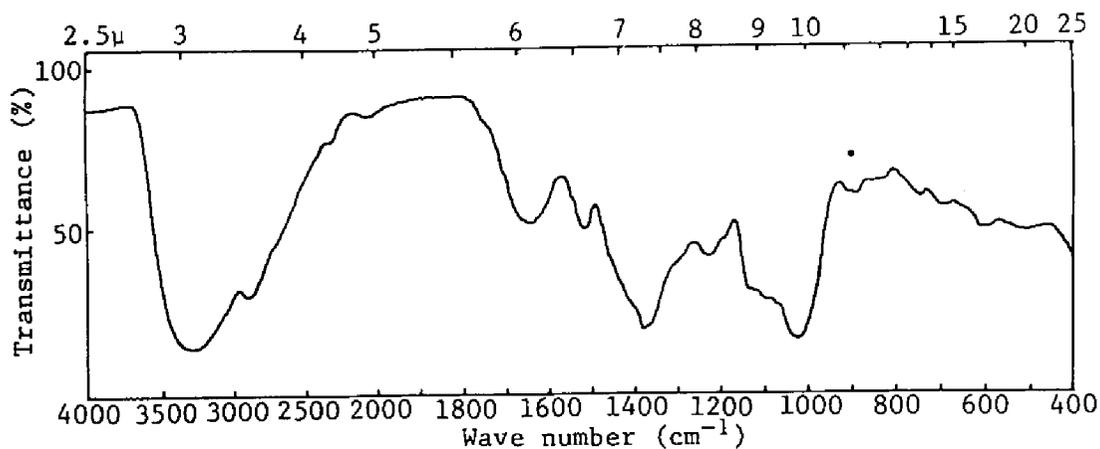
Fig. 8.   IR Spectrum of Isolated PIN-G (Micro KBr Tablet).

TABLE X.   NMR SIGNALS OF PIN-4'-G AND PIN-5'-G

PIN-4'-G	PIN-5'-G	
δ 2.49 ppm	δ 2.47 ppm	(C-2', -CH$_3$, single)
δ 3.2-3.9 ppm	δ 3.3-3.9 ppm	(ring protons of sugar, multiple)
δ 4.87 ppm	δ 4.89 ppm	(C-5', -CH$_2$-, single)
δ 5.07 ppm	δ 5.07 ppm	(C-4'. -CH$_2$-, single)
δ about 5 ppm	δ about 5 ppm	(α-anomeric proton)
δ 7.82 ppm	δ 7.85 ppm	(H-6', single)

NMR spectra were recorded on a Hitachi Perkin-Elmer high resolution NMR spectrometer type R-22 at 90 MHz in D$_2$O with DSS as an internal standard.
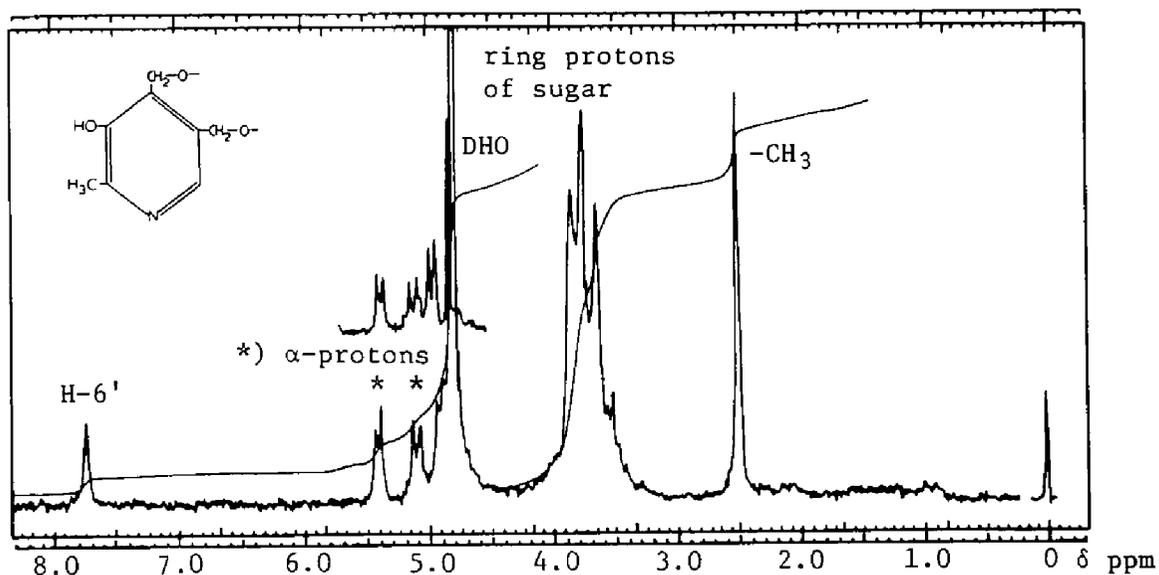
Fig. 9. NMR Spectrum of Isolated PIN-diglucoside.

The spectrum was recorded on a Varian HA-100D spectrometer at 100 MHz in D_2O with DSS-D_6 as an internal standard.

and PIN, the molar ratio being confirmed to be 2:1 by the glucostat method [111] and the diazotized p-aminoacetophenone method. [104,105] NMR spectrum showed the existence of PIN and sugar moieties and two α-protons (Fig. 9). It was suggested that PIN-4'-diglucoside, PIN-5'-diglucoside and PIN-4',5'-diglucoside might be existent. PIN-4'-diglucoside and PIN-4',5'-diglucoside may be colored with the diazotized p-aminoacetophenone reagent in the presence of borate buffer. On the other hand, PIN-5'-diglucoside may not be colored with the reagent in the presence of borate buffer. The two colored spots on a paper

chromatogram may support this assumption, as shown in Fig. 6.

DISCUSSION

The cultural conditions of the formation of PIN-G were investigated with the isolated strain, *Micrococcus* sp. No. 431, for the convenient isolation of the compound. Sucrose was the most effective glucosyl donor. It seems that the PIN-G-synthesizing enzyme may be a kind of glucosido-invertase like those from yeasts,[114] honey,[115] and some aphids.[116] The amount of PIN-G increased proportionately to the concentration of added sucrose, and was remarkably accerelated by the addition of several nonionic surfactants. The effects of surfactants on the metabolism of microorganisms have been reported in several microbial processes such as glutamic acid fermentation[117] and respiration of yeast.[118] It is not clear whether the stimulative effect of nonionic surfactants on the formation of PIN-G is caused by the accerelation of the permeability of the product or of the enzyme responsible for the transglucosidation reaction. Under the optimal conditions of culture, more than 90% of PIN added was converted to PIN-G by *Micrococcus* sp. No. 431. PIN-G was isolated from the culture broth by the treatment with active carbon, column chromatography on Dowex 1X2 (Cl⁻ type) and Sephadex G-10, and paper chromatography. PIN-4'-G and PIN-5'-G could be separated by paper chromatography with a solvent of *n*-butanol:benzene:pyridine:water (5:1:3:3, v/v). The two isomers were identified by various analytical methods including absorption spectra, paper chromatography, the color development,

enzymatic hydrolysis, and IR and NMR spectra. Contrary to the case

of *Sarcina lutea* IFO 3232, PIN-4'-G was dominant in *Micrococcus*.

In addition to PIN-G, PIN-diglucoside was detected in the culture

broth, the isolation and identification of the compound being carried

out.

It is already reported that riboflavinylglycosides (mono-, di-,

tri-, and tetraglycosides)[77] are formed from riboflavin and sugar

by transglucosidation. The mechanism of transglucosidation to PIN

to form PIN-G and PIN-diglucoside may probably be similar to the

case of riboflavinylglycosides.

SUMMARY

With the isolated and identified strain, *Micrococcus* sp. No. 431,

several conditions of the formation of PIN-G were investigated by the

growing culture. Sucrose and peptone were the most proper compounds

as carbon and nitrogen sources. A few nonionic surfactants exhibited

a stimulative effect on the formation of PIN-G, in which Span 80 was

the most effective. The formation of PIN-G reached to the maximum

after 60 to 80 hr of cultivation. Metal ions had no effect. The

amount of PIN-G increased proportionately to the concentration of

added sucrose. Under the optimal culture conditions, the amount of

PIN-G attained to maximally 1800 to 1900 μg/ml (90-95% yield based on

PIN added) at 2 mg/ml of PIN. The yield was maximal at pH 7.0. PIN-G

was isolated from the culture broth and identified by several methods

including IR and NMR analyses. PIN-diglucoside was also produced in

the broth, and the isolation and identification of the compound were performed.

Section III. Purification and Some Properties of PIN-G-

synthesizing Enzyme of *Micrococcus* sp. No. 431

INTRODUCTION

In the previous section, the PIN-G-accumulating activity of

bacteria was found to be distributed specifically in genera *Sarcina*

and *Micrococcus*. An isolated bacterium No. 431, which was classified

into a *Micrococcus* sp., had the highest activity of accumulating

PIN-G in the culture broth. Various factors affecting the PIN-G

formation were examined with this organism and over than 90% of PIN

added was converted to PIN-G under the optimal cultural conditions.

However, the enzyme catalyzing the synthesis of PIN-G has not yet been

investigated and the mechanism of its formation remains to be eluci-

dated. The present section describes a purification procedure of the

enzyme from *Micrococcus* sp. No. 431, which catalyzes a glucosyl

transfer from sucrose to PIN to form PIN-G. The author also reports

some properties of PIN-G-synthesizing enzyme purified from *Micrococcus*

sp. No. 431.

EXPERIMENTAL

Materials. Uridine diphosphate glucose was kindly given by the

Laboratory of Industrial Microbiology, Department of Food Science and

Technology, Kyoto University. Other chemicals used in this section

were purchased from commercial sources.

Microorganisms and cultivation. The bacteria except *Micrococcus*

sp. No. 431 were from laboratory collections. *Micrococcus* sp. No. 431 was isolated from soil in Kyoto Prefecture as reported in the previous section.

The bacteria were grown on a medium containing 3.0% sucrose, 1.0 % peptone, 0.2% yeast extract, 0.5% K_2HPO_4, 0.1% KH_2PO_4, 0.2% NaCl, and 0.02% $MgSO_4 \cdot 7H_2O$. The pH was adjusted to 7.0. The cultivation was carried out aerobically at 28°C for 24 hr in a 2 liter-shaking flask containing 500 ml of the medium. Cultures were also performed at 28°C for 15 hr in a 20 liter jar fermentor (Marubishi Laboratory Equipment Co., Ltd.) with the rotation of impeller at 225 rpm and the aeration at 15 liters/min.

The cells harvested by centrifugation were washed once with 0.85 % saline solution. The washed cells were suspended in 0.01M potassium phosphate buffer (pH 7.0) containing 3×10^{-3}M 2-mercaptoethanol and followed to the sonication.

Preparation of cell extract. The washed cells obtained as described above, were disrupted with a Kaijo-Denki 19 Hz ultrasonic oscillator (20 kc) at 0-15°C. The cell debris was removed by centrifugation at 10,000 rpm for 20 min and the supernatant solution was used as cell extract.

Assay of enzyme activity. Through the purification of PIN-G-synthesizing enzyme of *Micrococcus* sp. No. 431, both α-glucosidase and transglucosidase activities were assayed. In the assay of α-glucosidase activity, the standard reaction mixture contained 25 μmoles of sucrose, 100 μmoles of potassium phosphate buffer (pH 8.0),

-44-

5 μmoles of 2-mercaptoethanol and a suitable amount of enzyme in a total volume of 2.5 ml. After incubation at 30°C for 30 min, the reaction was stopped by heating the tube in a boiling water bath for 5 min. The α-glucosidase activity was determined by measuring the liberated reducing sugars. The standard reaction mixture for the assay of transglucosidase activity contained 25 μmoles of PIN, 25 μmoles of sucrose, 100 μmoles of potasssium phosphate buffer (pH 8.0), 5 μmoles of 2-mercaptoethanol and a suitable amount of enzyme in a total volume of 2.5 ml. After incubation at 30°C for 60 min, the reaction was stopped by heating the tube in a boiling water bath for 5 min. PIN-G was determined by paper chromatography as described in the previous section. One unit of both enzyme activities was defined as the amount of enzyme which produced 1mμmole of products per min under the conditions described above. Specific activity was shown as units per $E_{280 \, m\mu}$. Protein was estimated by measuring the optical density at 280 mμ with a Hitachi Perkin-Elmer 139 spectrophotometer.

Analytical methods. Reducing sugars were determined by Somogyi-Nelson method.[112] PIN and PIN-G were determined by paper chromatography and the method described in the previous section. For the convenience of time, the ascending development was carried out for 3 hr with running by approximately 10 cm from the spotted line.

Electrophoresis. Polyacrylamide-gel electrophoresis was carried out by a modification of Davis method.[119] Stacking and running gels were polymerized in a Pyrex tube (5 X 65 mm). After the run, the gel was stained with 1% naphthol blue-black, destained electro-

phoretically and stored in 7% acetic acid.

Ultracentrifugal analysis. Sedimentation velocities were measured with the Beckman-Spinco analytical centrifuge model E operating at 59,780 rpm.[120)]

Measurement of ultraviolet absorption spectrum. The ultraviolet absorption spectrum was recorded on a Shimazu multi-purpose spectrophotometer model MPS-50L.

RESULTS

Enzyme Activity in Cell Extracts

Five species of *Sarcina* and *Micrococcus* were examined for PIN-G-forming activity using cell extracts. The result is shown in Table I. Relatively high transglucosidase activity (PIN-G-forming activity)

TABLE I. PIN-G-SYNTHESIZING ENZYME ACTIVITY IN CELL EXTRACT

Enzyme activities were assayed with the extracts prepared from the cells grown in the basal medium at 28°C for 15 hr on a reciprocal shaker under the standard conditions.

Strains	Total absorbancy at 280 mμ	α-Glucosidase		Transglucosidase	
		S.A.	T.U.	S.A.	T.U.
Micrococcus sp. No. 431	2700	10.2	18300	1.0	1800
Micrococcus flavus IFO 3242	2580	4.3	7470	0.0	0
Micrococcus roseus IFO 3764	0	–	–	–	–
Sarcina lutea IFO 1099	762	0.2	100	0.0	0
Sarcina marginata IFO 3066	123	20.1	1650	3.8	311

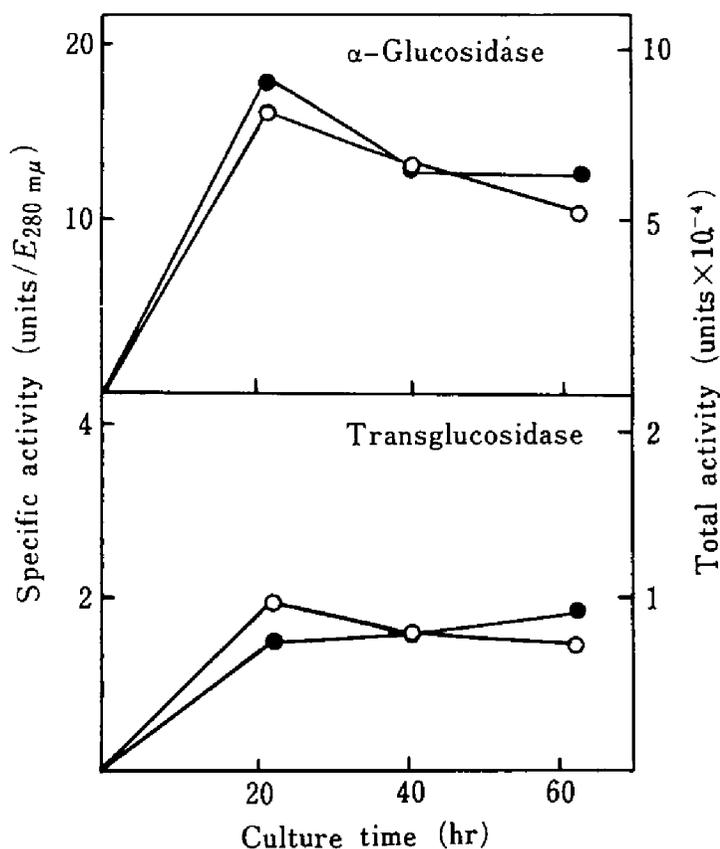S.A.: Specific activity (units/$E_{280 \, m\mu}$)
T.U.: Total units

Fig. 1. Effect of Cultivation Time.

Both α-glucosidase and transglucosidase activities were estimated using cell extract of *Micrococcus* sp. No. 431 under the standard assay conditions. Incubation was carried out at 30°C.

○ , Specific activity; ● , Total activity.

was found in two organisms, *Sarcina marginata* IFO 3066 and *Micrococcus* sp. No. 431. Total enzyme activity in cell extract of the latter organism was higher than that of the former. Therefore, it seems that *Micrococcus* sp. No. 431 is an appropriate enzyme source for the purification of PIN-G-synthesizing enzyme.

The effect of culturing time on the formation of both enzymes

of *Micrococcus* sp. No. 431 is shown in Fig. 1. The specific activities reached to maxima after about 20 hr of incubation. Total activities of α-glucosidase and transglucosidase also reached to maxima after about 20 hr of incubation and remained constant until about 60 hr.

Based on these data, *Micrococcus* sp. No. 431 was selected to purify PIN-G-synthesizing enzyme.

Purification of Enzyme

All operations for the purification of the enzyme were performed at 0-15°C.

Routine enzyme assays in the course of purification were carried out by determining an α-glucosidase activity unless otherwise noted. α-Glucosidase fractions were subjected to the assay of transglucosidase activity.

1) Sonication. The washed cells were suspended in 0.01M potassium phosphate buffer (pH 7.0) containing 3×10^{-3}M 2-mercaptoethanol, and were disrupted with a Kaijo-Denki 19 Hz ultrasonic oscillator (20 kc) at 0-15°C for 8 hr. Then, the cell debris was centrifuged off at 10,000 rpm for 20 min. The supernatant solution was used as a starting material of the purification.

2) 1st Ammonium sulfate fractionation. To the cell extract (1425 ml, total $E_{280 m\mu}$ = 53,000) was added solid ammonium sulfate to make 0.30 saturation, followed by standing for 30 min. After removal of the resulting precipitate by centrifugation, solid ammonium sulfate was added to the supernatant solution to make 0.80 saturation.
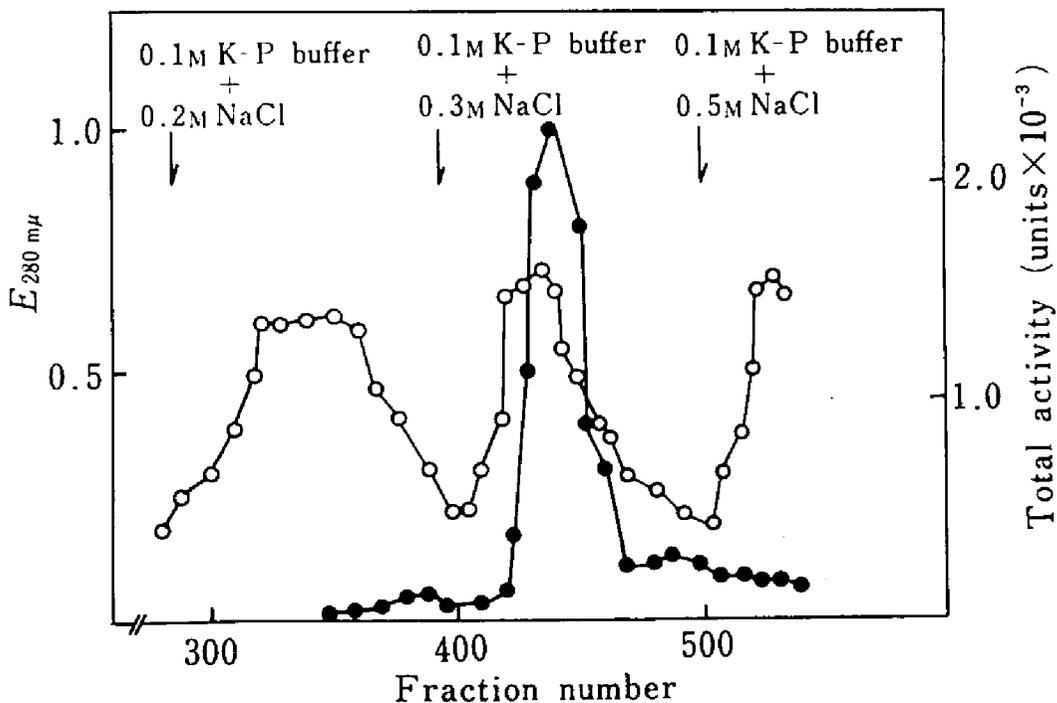
Fig. 2. DEAE-Sephadex Column Chromatography.

Column size 5.6 X 53 cm, Flow rate 70 ml/hr.

One fraction 10 ml.

○ , Protein; ● , PIN-G-synthesizing enzyme activity.

The mixture was left to stand overnight, and the precipitate formed was collected by centrifugation at 10,000 rpm for 20 min and dissolved in 0.01M potassium phosphate buffer (pH 7.0) containing 3×10^{-3}M 2-mercaptoethanol. The solution was dialyzed overnight against the same buffer.

3) DEAE-Sephadex column chromatography. The dialyzed enzyme solution (386 ml, total $E_{280\ m\mu}$ = 27,200) was subjected to DEAE-

Sephadex column chromatography. A DEAE-Sephadex column (5.6 X 53 cm) was preliminarily equilibrated with 0.01M potassium phosphate buffer (pH 7.0) containing 3×10^{-3}M 2-mercaptoethanol. The enzyme solution was placed on the column and was washed with the same buffer. Subsequently, it was treated with 0.1M potassium phosphate buffer (pH 7.0) containing 3×10^{-3}M 2-mercaptoethanol and 0.2M sodium chloride. Ten milliliters of fractions were collected and the protein was followed by measurement of the absorbancy at 280 mµ. Much of the inactive proteins were removed in this step. The enzyme was subsequently eluted with 0.1M potassium phosphate buffer (pH 7.0) containing 3×10^{-3}M 2-mercaptoethanol and 0.3M sodium chloride. The elution pattern of the enzyme is shown in Fig. 2. The active fractions (tube No. 424-466) were combined and the enzyme was precipitated by the addition of solid ammonium sulfate (0.65 saturation). The precipitate was collected by centrifugation and dissolved in 0.01M potassium phosphate buffer (pH 7.0) containing 3×10^{-3}M 2-mercaptoethanol, and then dialyzed overnight against 2 liters of the same buffer with three changes.

4) 2nd Ammonium sulfate fractionation. The dialyzed enzyme solution was applied to 2nd ammonium sulfate fractionation. The precipitate obtained between 0.45-0.60 saturation of ammonium sulfate was dissolved in 0.01M potassium phosphate buffer (pH 7.0) containing 3×10^{-3}M 2-mercaptoethanol and dialyzed for 10 hr against 2 liters of the same buffer with three changes.

5) Hydroxylapatite column chromatography. The dialyzed enzyme solution (24 ml, total $E_{280 \, m\mu}$ = 342) was applied to hydroxylapatite
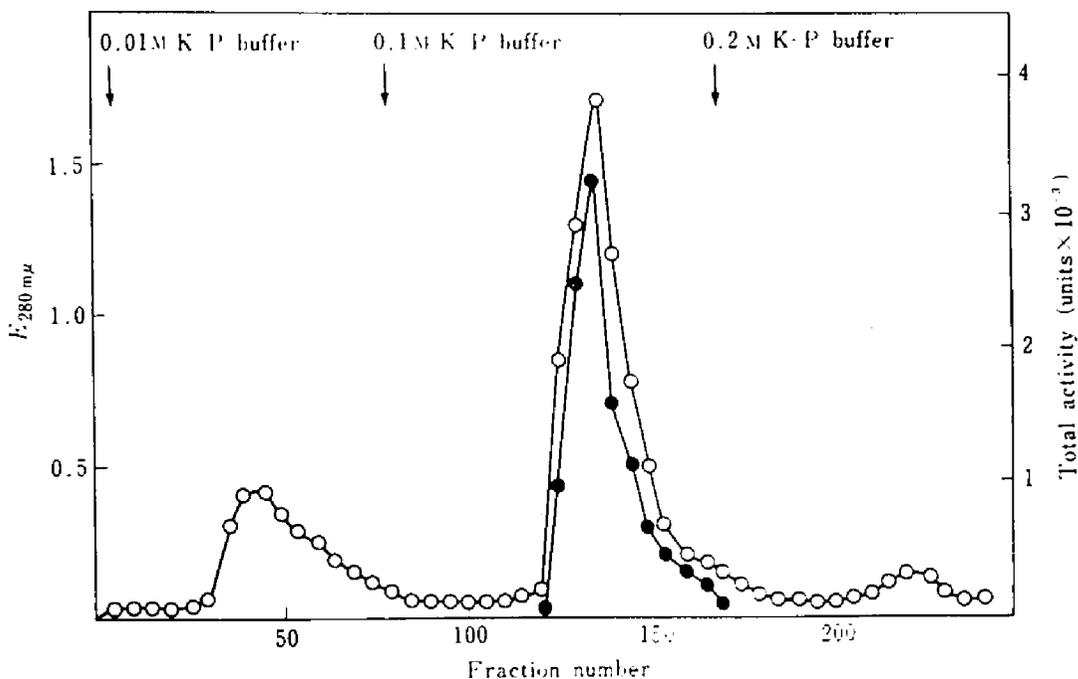
Fig. 3. Hydroxylapatite Column Chromatography.

Column size 3.6 X 13.5 cm, Flow rate 18 ml/hr, One fraction 3 ml.
O , Protein; ● , PIN-G-synthesizing enzyme activity.

column chromatography. The column (3.6 X 13.5 cm) was preliminarily

bufferized with 0.01M potassium phosphate buffer (pH 7.0) containing

$3 \times 10^{-3}M$ 2-mercaptoethanol. After the enzyme solution was charged,

the column was washed with the same buffer and eluted with 0.1M

potassium phosphate buffer (pH 7.0) containing $3 \times 10^{-3}M$ 2-mercapto-

ethanol. The elution pattern is shown in Fig. 3. Active fractions

were combined and brought to 0.65 saturation by ammonium sulfate.

The precipitate was taken by centrifugation and dissolved in 0.01M
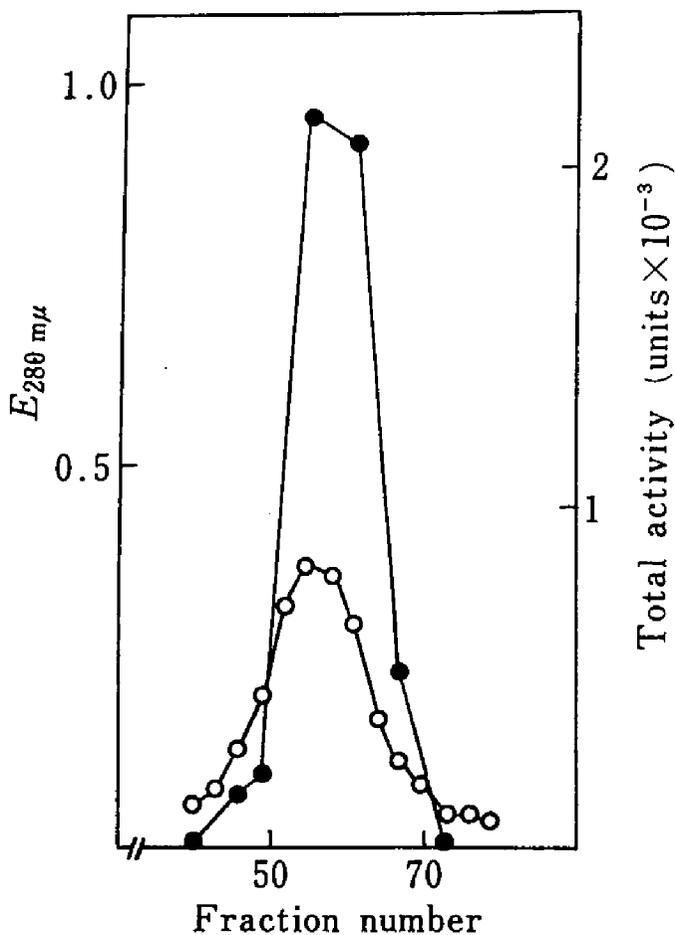
Fig. 4.   Second Sephadex G-100 Gel Filtration.

Column size 2.4 x 88 cm, Flow rate 24 ml/hr, One fraction 3ml.

O , Protein; ● , PIN-G-synthesizing enzyme activity.

potassium phosphate buffer (pH 7.0) containing 3 X 10^{-3}M 2-mercapto-ethanol.

6) Sepahdex G-100 column chromatography.   The enzyme solution (1 ml, total $E_{280 m\mu}$ = 60) was applied to a column of Sephadex G-100 (2.4 x 88 cm). The protein was eluted with 0.01M potassium phosphate

TABLE II. PURIFICATION OF PIN-G-SYNTHESIZING ENZYME

Fraction and step	Total absorbancy at 280 mμ	α-Glucosidase		Transglucosidase	
		S.A.	T.U.	S.A.	T.U.
1. Cell extract	53000	8.5	302000	1.1	39500
2. 1st AmSO₄ (30-80%)	27200	15.9	289000	1.5	26500
3. DEAE-Sephadex A-50	342	365	82700	20.2	4610
4. Hydroxylapatite	134	635	56700	33.1	2950
5. 2nd AmSO₄ (45-65%)	60	1040	41800	66.2	2650
6. Sephadex G-100 (I)	23	2450	38000	163	2520
7. Sephadex G-100 (II)	11	3020	22500	249	1850

buffer (pH 7.0) containing 3×10^{-3}M 2-mercaptoethanol. The active

fractions (fraction No. 46-60) were collected, concentrated by ammo-

nium sulfate at 0.65 saturation, and dissolved in 0.01M potassium

phosphate buffer (pH 7.0) containing 3×10^{-3}M 2-mercaptoethanol.

The enzyme solution was rechromatographed on Sephadex G-100. By the

2nd Sephadex G-100 column chromatography, a single peak was obtained

as shown in Fig. 4. The fractions from No. 52 to No. 65 were used

as the purified enzyme. The enzyme was purified approximately 354-

fold with 7.4% recovery. A summary of the purification procedure is

presented in Table II.

Purity and Properties of Enzyme

Disc electrophoresis

Disc electrophoresis (polyacrylamide-gel) was attempted with the

purified enzyme. The electrophoretic pattern (Fig. 5) showed a single

-53-

band, supporting that the obtained enzyme was homogeneous.

Ultracentrifugal analysis

The ultracentrifugal analysis of the purified enzyme was performed with a Beckman-Spinco analytical centrifuge model E. As shown in Fig. 6, the Schlieren patterns indicated the presence of a single component.

The sedimentation coefficient, $s_{20,w}^0$, was estimated for the enzyme $(E_{280 \ m\mu} = 5.4)$ to be 3.68 S.

These results show that the purified enzyme is a single protein.

Properties of enzyme

PIN-G-synthesizing enzyme was purified according to the method
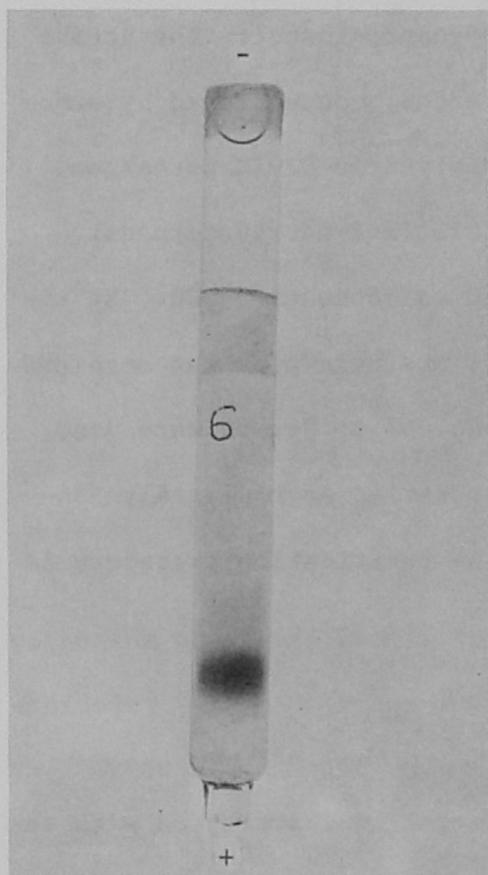
Fig. 5. Acrylamide-gel Electrophoresis of PIN-G-Synthesizing Enzyme.

The purified enzyme (31.5 µg) was applied to the stacking gel and subjected to the electrophoresis at a current of 2.0 mA for 50 min in Tris-glycine buffer (pH 8.3) containing 3×10^{-3}M 2-mercapto-ethanol. The direction of migration is from the cathode to the anode.
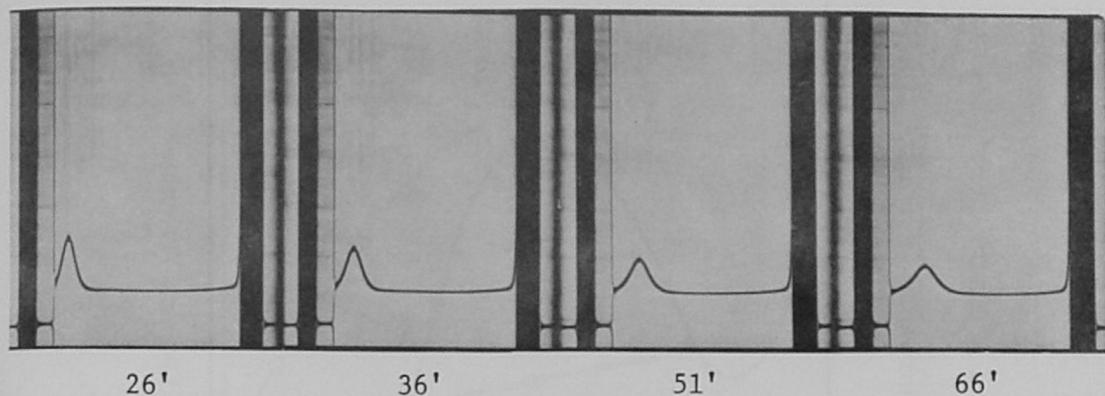
Fig. 6.   Sedimentation Patterns of PIN-G-synthesizing Enzyme.

The experiment was carried out at a concentration of 0.36% enzyme in 0.01M potassium phosphate buffer (pH 7.0) containing 3×10^{-3}M 2-mercaptoethanol.   The photographs were taken at 26, 36, 51 and 66 min after reaching 59,780 rpm.   The sedimentation is left to right.

described above.   The first ammonium sulfate fraction (the partially purified sample) and the 2nd Sephadex G-100-treated fraction (the purified sample), homogeneous on ultracentrifugation and polyacryl-amide-gel electrophoresis, were used throughout this section.

Ultraviolet absorption spectrum

The ultraviolet absorption spectrum of the purified enzyme in 0.01M potassium phosphate buffer (pH 7.0) containing 3×10^{-3}M 2-mercaptoethanol was measured with a Shimazu multi-purpose recording spectrophotometer model MPS-50L, as shown in Fig. 7.   The spectrum showed a typical protein spectrum and the absorption maximum and minimum were found to be at 280 mμ and 253 mμ, respectively. $E_{280 \ m\mu}/E_{260 \ m\mu}$ was 1.6.
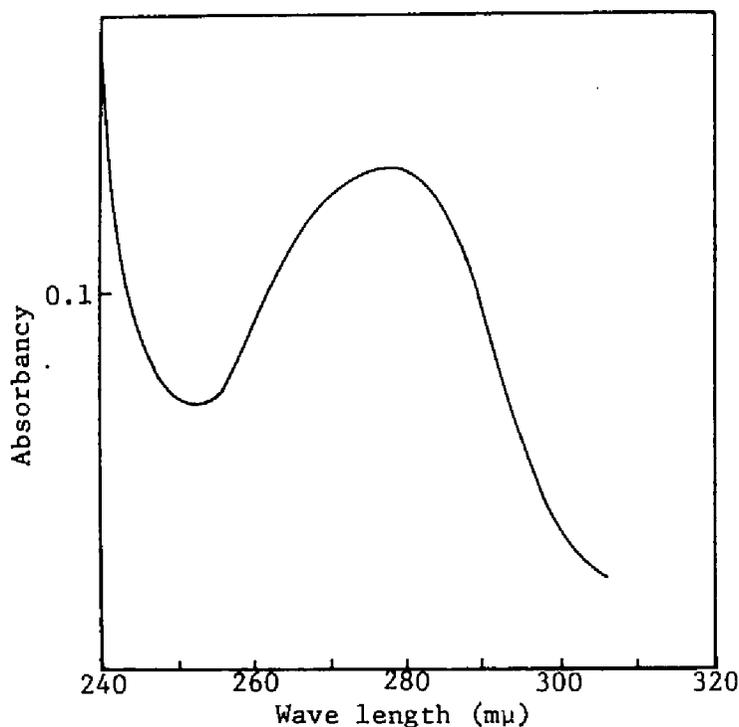
Fig. 7. Ultraviolet Absorption Spectrum of Enzyme.

The purified enzyme was dissolved in 0.01M potassium phosphate buffer (pH 7.0) containing 3 X 10^{-3}M 2-mercaptoethanol. The spectrum of the solution was measured by a Shimazu multi-purpose spectrophoro-meter model MPS-50L.

Linearity of enzyme activity

A linear relationship was obtained between enzyme activity and reaction time, up to 30 min for α-glucosidase activity and up to 120 min for transglucosidase activity, as shown in Fig. 8. A linearity between enzyme activity and enzyme concentration was observed, up to 150 units for α-glucosidase activity and up to 12 units for trans-glucosidase activity, as shown in Fig. 9.

Stability of enzyme

After an aliquot of enzyme solution was kept at various pH at 40°C for 10 min, the remaining enzyme activity was estimated. As shown
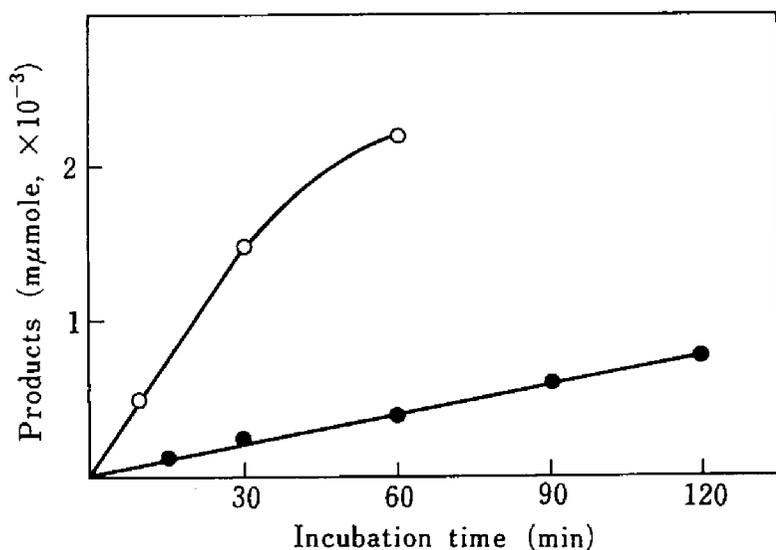
Fig. 8. Effect of Incubation Time.

Both α-glucosidase and transglucosidase activities were estimated using 1st ammonium sulfate fraction under the standard assay conditions. Incubations were carried out at 30°C.

    O α-Glucosidase activity (48.0 units)
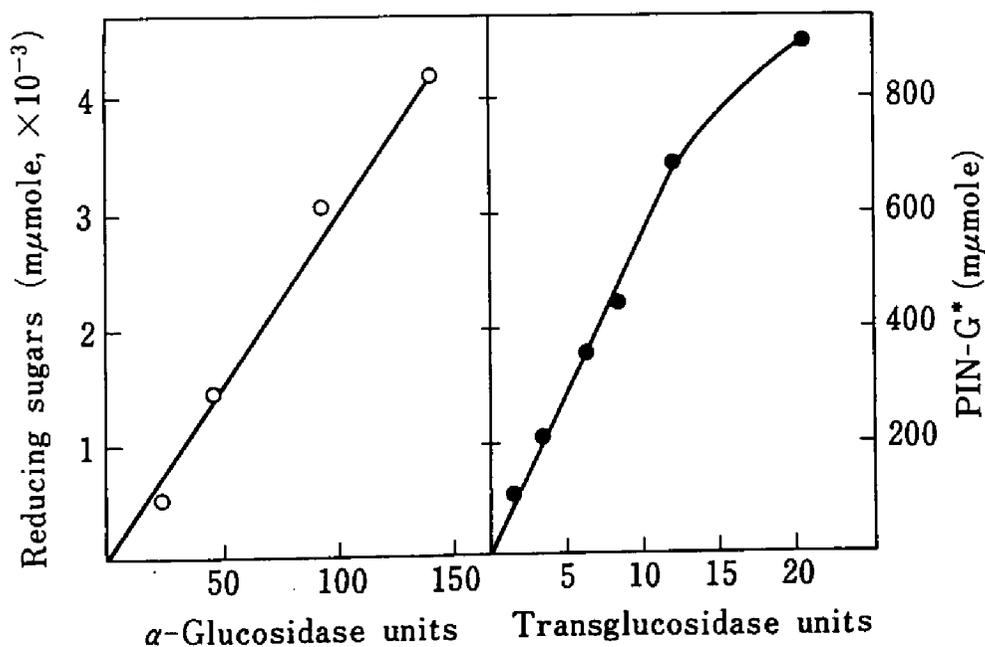   ● Transglucosidase activity (7.3 units)

Fig. 9. Effect of Enzyme Concentration.

Both α-glucosidase and transglucosidase activities were estimated using 1st ammonium sulfate fraction under the standard assay conditions. Incubations were carried out for 30 min (α-glucosidase activity) and 60 min (transglucosidase activity) at 30°C.
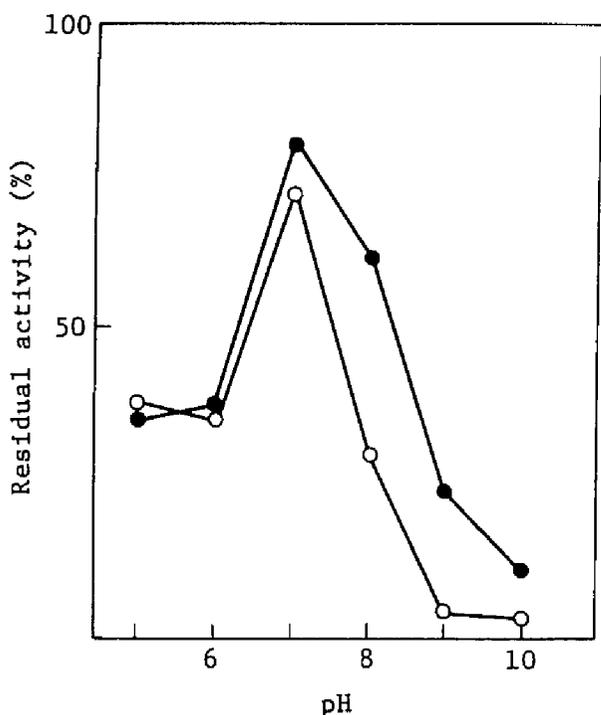
-57-

Fig. 10. pH Stability of Enzyme.

Fig. 11. Heat Stability. of Enzyme.

An aliquot of enzyme solution (1st ammonium sulfate fraction) was kept at various pH at 40°C for 10 min. After adjustment of pH 8.0, the residual enzyme activity was measured under the standard assay conditions. Incubations were made for 30 min (α-glucosidase activity) and 60 min (transglucosidase activity) at 30°C.

 ○ α-Glucosidase activity (19.0 units)
 ● Transglucosidase activity (10.4 units)

After an aliquot of enzyme solution (1st ammonium sulfate fraction) was kept at various temperature for 15 min. The residual enzyme activity was measured under the standard assay conditions. Incubations were made for 30 min (α-glucosidase activity) and 60 min (transglucosidase activity) at 30°C.

 ○ α-Glucosidase activity (46.0 units)
 ● Transglucosidase activity (11.0 units)

in Fig. 10, both α-glucosidase and transglucosidase activities were

stable at pH 7.0, where 70-80% of initial activities remained.

Figure 11 shows the stability of the enzyme activity against

heating. The enzyme was heated at various temperature at pH 8.0 for

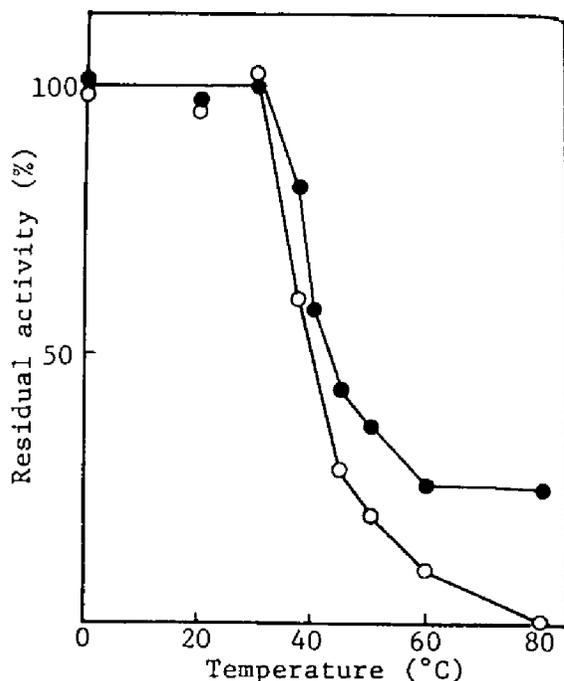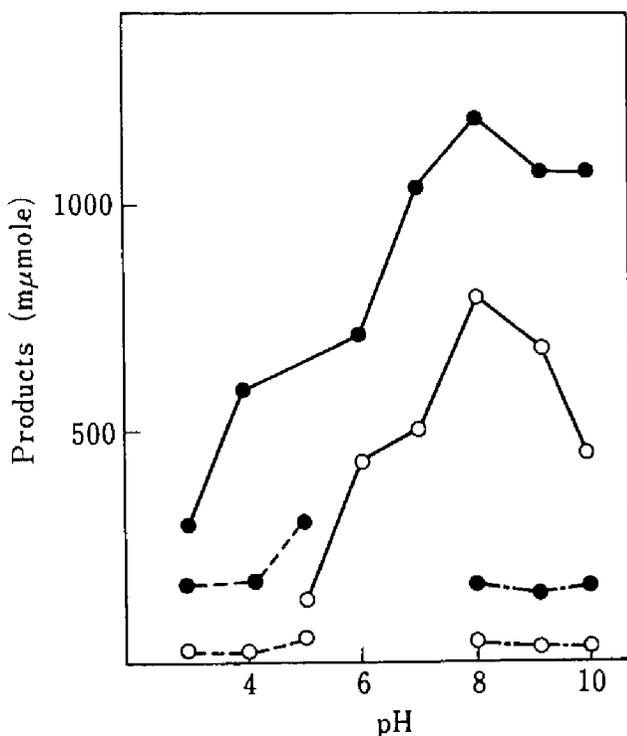15 min. The enzyme was stable in a range from 0°C to 30°C. However,

Fig. 12. Effect of pH on Enzyme Activity.

Both α-glucosidase and transglucosidase activities were estimated using 1st ammonium sulfate fraction under the standard assay conditions. Incubations were carried out for 30 min (α-glucosidase activity) and 60 min (transglucosidase activity) at 30°C at various pH.

        ○ α-Glucosidase activity (26.0 units)
        ● Transglucosidase activity (20.0 units)
— — Acetate buffer   ——— Phosphate buffer   —·— Tris buffer

it was shown that transglucosidase activity was more stable than α-glucosidase activity against heating; at 80°C, almost all α-glucosidase activity were lost, but 25% of transglucosidase activity remained.

It seems that transglucosidase activity is stabilized against heating in the presence of PIN.

Effect of pH

The activity curves of the enzyme are shown in Fig. 12. Higher enzyme activities were found at pH of alkaline side, and optimal pH
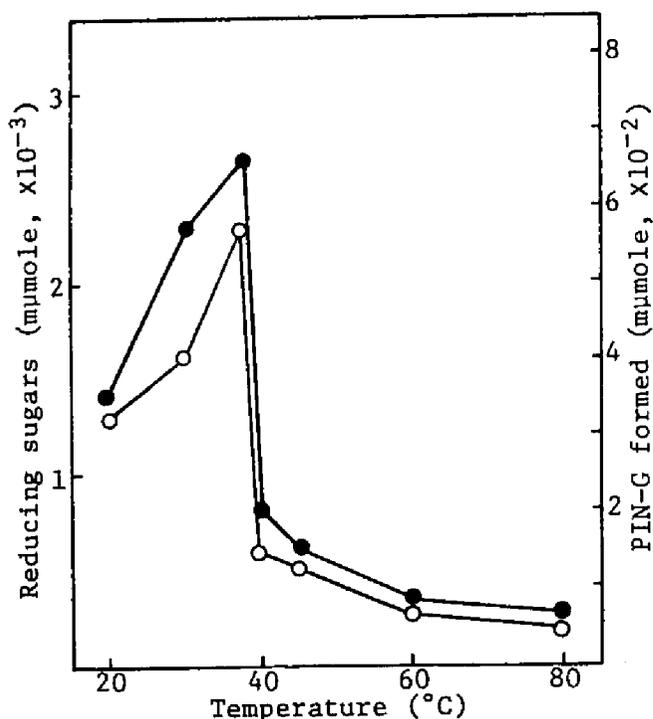
Fig. 13. Effect of Temperature on Enzyme Activity.

Both α-glucosidase and transglucosidase activities were estimated
using 1st ammonium sulfate fraction under the standard assay condi-
tions. Incubations were carried out for 30 min (α-glucosidase acti-
vity) and 60 min (transglucosidase activity) at various temperature.
      ──○──   α-Glucosidase activity (52.7 units)
      ──●──   Transglucosidase activity (9.5 units)

was 8.0 with both enzyme activities in phosphate buffer. However,

when Tris-HCl buffer was used in place of phosphate buffer, the both

activities decreased extremely even at pH 8.0.

Effect of temperature

Optimal temperature on the enzyme activities was observed around

37°C, but the enzyme activities markedly decreased above 40°C under

the standard assay conditions, as shown in Fig. 13.

Substrate specificity

TABLE III. EFFECT OF GLUCOSYL DONORS

The reaction was performed using the purified enzyme preparation under the standard reaction conditions except 25 µmoles of glucosyl donor.

Glucosyl donor	α-Glucosidase activity[b] (mµmole)	Transglucosidase activity[b] (mµmole)
D-Glucose	–	0
D-Fructose	–	0
Sucrose	362	599
Maltose	444	892
Trehalose	0	0
Lactose	0	0
Starch[a]	0	0
Glycogen[a]	0	0
Phenyl-α-glucoside	624	2070
Methyl-α-glucoside	0	0
Glucose-6-phosphate	–	0
α-Glucose-1-phosphate		
– NaF	–	0
+ NaF 10^{-2}M	–	0
UDPG[c]	–	0

  a) 10 mg in the standard reaction mixture except 25 µmoles of sucrose.

  b) α-Glucosidase activity 24.1 units, Transglucosidase activity 10.0 units.

  c) Uridine diphosphate glucose.

The effect of various glucosides on the enzyme activity was examined with mono-, di-, and poly-saccharides, methyl- and phenyl-α-D-glucosides, sugar phosphate esters and uridine diphosphate glucose. As shown in Table III, both α-glucosidase and transglucosidase activi-

TABLE IV.   EFFECT OF GLUCOSYL ACCEPTORS

The reaction was performed using the purified enzyme preparation
(7.6 units) under the standard reaction conditions except 25 μmoles
of glucosyl acceptor.

The formation of glucoside was determined by paper chromatography
using Toyo filter paper No. 53. The ascending development was carried
out in the solvent systems (1), (2) and (3). Glucoside on paper was
detected by Manaslu lamp (2536 A filter) and confirmed by spraying
with the diazotized p-aminoacetophenone reagent.[104,105] The ultra-
violet absorbing spot of PIN-G was extracted with a mixture of 2.5 ml
of 99% ethanol and 2.5 ml of 25% sodium acetate for 90 min at 37°C.
The extracted solution was assayed colorimetrically at 470 mμ accor-
ding to the diazotized p-aminoacetophenone method[104,105] with a
Hitachi photoelectric photometer EPO-B.

Solvent system:

  (1) acetone:t-butanol:diethylamine:water (35:40:5:20, v/v)

  (2) t-butanol:formate:water (70:15:15, v/v)

  (3) t-amylalcohol:acetone:diethylamine:water (40:35:5:20, v/v)

Glucosyl acceptor	Transglucosidase activity (mμmole)
PIN	453
PAL	0
PAM	0
4-Pyridoxic acid	0

ties were revealed in the presence of sucrose, maltose and phenyl-α-
D-glucoside. The reaction rates of enzyme decreased in the following
order: phenyl-α-D-glucoside, maltose and sucrose. Essentially no enzyme
activity was observed with methyl-α-D-glucoside. α-Glucose-1-phosphate
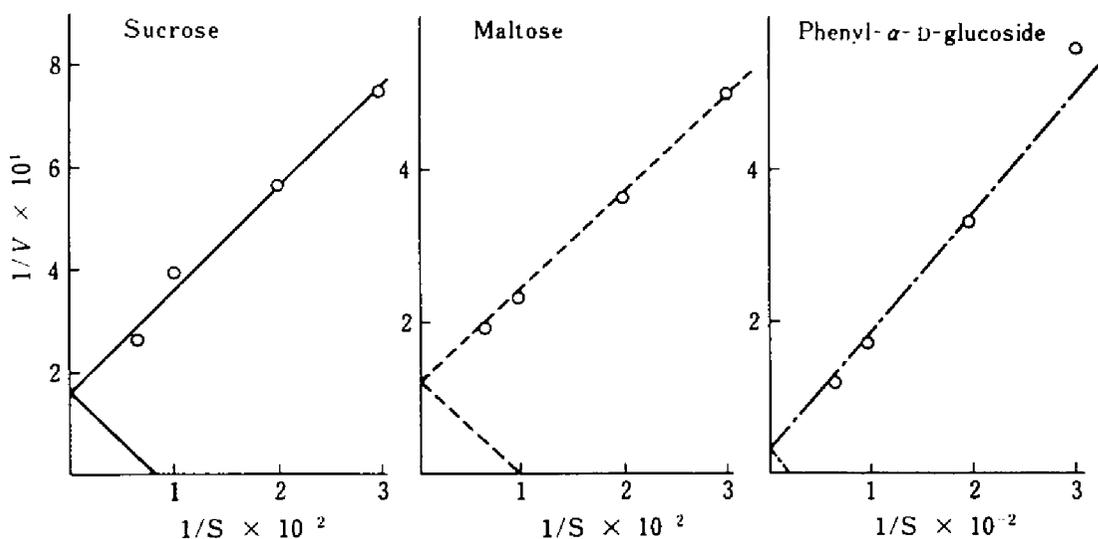and uridine diphosphate glucose were also inert.

Fig. 14. Effect of Concentrations of Glucosyl Donors
on Enzyme Activity.

The reactions were carried out at 30°C for 60 min in reaction mixtures
containing 42.5 units of the enzyme (1st ammonium sulfate fraction),
25 μmoles of PIN, 100 μmoles of potassium phosphate buffer (pH 8.0),
5 μmoles of 2-mercaptoethanol and indicated amounts of sucrose, maltose,
or phenyl-α-D-glucoside as a substrate in a total volume of 2.5 ml.
Velocity (V) was expressed as mμmoles of PIN-G formed/min/unit, and
substrate concentration (S) as moles per liter.

From these observations, the enzyme seems to belong to one of

α-glucosidase, but not to α-methylglucosidase,[121] phosphorylase or

uridine diphosphate glucosyl transferase.

The effect of vitamin B_6 compounds on the enzyme activity was

examined with PIN, PAL, PAM and 4-pyridoxic acid. As shown in Table

IV, only PIN served as glucosyl acceptor.

Kinetics

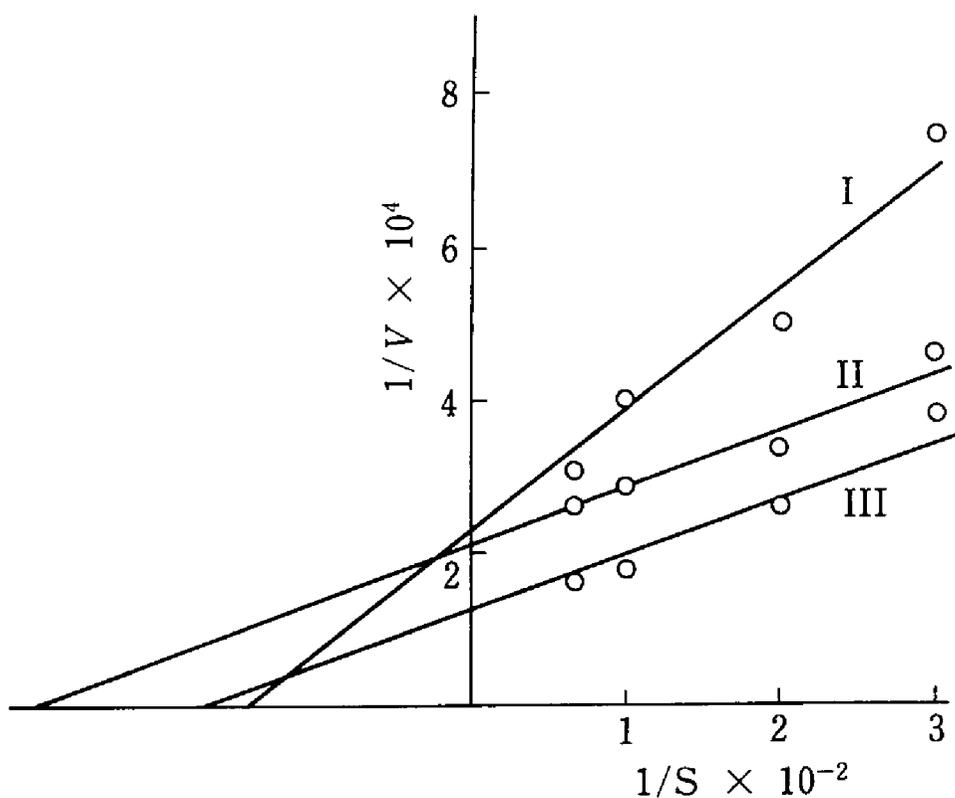Figure 14 shows the effect of concentrations of sucrose, maltose

Fig. 15. Effect of PIN Concentrations on Enzyme Activity.

The reactions were carried out at 30°C for 60 min in reaction mixtures containing 40.5 units of the enzyme (1st ammonium sulfate fraction), 25 μmoles of sucrose (I), maltose (II), or phenyl-α-D-glucoside (III), 100 μmoles of potassium phosphate buffer (pH 8.0), 5 μmoles of 2-mercaptoethanol and indicated amounts of PIN, in a total volume of 2.5 ml. Velocity (V) was expressed as mμmoles of PIN-G formed/min/unit, and substrate concentration (S) as moles per liter.

and phenyl-α-D-glucoside on the formation of PIN-G.

The effect of concentrations of PIN on the formation of PIN-G is shown in Fig. 15.

Kms for sucrose, maltose and phenyl-α-D-glucoside were calculated from Lineweaver-Burk's plots to be 1.25 x 10^{-2}M, 1.00·x 10^{-2}M and 5.26

TABLE V. KINETIC PARAMETERS FOR SUBSTRATES OF ENZYME

Substrate	Km (M)	V_{max} (mμmole/min/unit)
Sucrose	1.3×10^{-2}	2.5
Maltose	1.0×10^{-2}	3.3
Phenyl-α-D-glucoside	5.3×10^{-2}	13.1
PIN (Sucrose)	7.1×10^{-3}	1.8
(Maltose)	3.6×10^{-3}	2.0
(Phenyl-α-D-glucoside)	5.7×10^{-3}	3.3

$\times 10^{-2}$M, respectively. Kms for PIN in the coexistence of sucrose,
maltose or phenyl-α-D-glucoside were also calculated to be 7.14×10^{-3}
M, 3.57×10^{-3}M and 5.71×10^{-3}M, respectively. These Km values are
summarized in Table V.

Effect of various agents

As shown in Table VI, the enzyme activity was affected by sulf-
hydryl reagents and heavy metal ions. The activity was almost comple-
tely inhibited by p-chloromercuric benzoate at a concentration of 10^{-4}
M. Iodoacetate was a less effective inhibitor at a concentration of
10^{-4}M. However, the inhibition was nearly overcome when 2-mercapto-
ethanol (10^{-3}M) coexisted with p-chloromercuric benzoate (10^{-4}M) or
monoiodoacetate (10^{-4}M). Heavy metal ions, which are known as the
inhibitors of the sulfhydryl enzymes, also showed an inhibitory effect
on the enzyme activity. At a concentration of 10^{-4}M of metal ion,
the activity was almost or completely inhibited by Cu^{2+}, Hg^{2+}, Ag^{+}
and Pb^{2+}, and moderately inhibited by Zn^{2+}. Similar sensitivities to

TABLE VI. EFFECT OF SULFHYDRYL INHIBITORS

Reaction mixture contained sucrose 25 μmoles, PIN 25 μmoles, potassium phosphate buffer (pH 8.0) 100 μmoles, the dialyzed enzyme solution (the purified enzyme preparation, 13.8 units) and each reagent in a total volume of 3.0 ml. Incubation was made for 60 min at 30°C.

Agent	Concentration (M)	Transglucosidase inhibition (%)
None	-	0
PCMB*	10^{-4}	96
PCMB + 2-Mercaptoethanol	$10^{-4} + 2 \times 10^{-3}$	0
Monoiodoacetate	10^{-4}	19
Monoiodoacetate + 2-Mercaptoethanol	$10^{-4} + 2 \times 10^{-3}$	0
$AgNO_3$	10^{-4}	100
$HgCl_2$	10^{-4}	89
$CuSO_4$	10^{-4}	99
$2(PbCO_3)Pb(OH)_2$	10^{-4}	82
$Zn(CH_3COO)_2$	10^{-4}	36

*) p-Chloromercuric benzoate

sulfhydryl reagents or heavy metals have been reported with intestine [122] and yeast[123]. α-glucosidases. Thus, the enzyme seems to be a sulfhydryl enzyme.

Table VII shows the effect of various agents on the enzyme activity. The activity was not appreciably affected by Na_2HAsO_4, NaF, KCN, carbonyl reagents and chelating agents. But, Tris caused a 56% inhibition at the concentration of 10^{-2}M, which is a well-known

TABLE VII. EFFECT OF INHIBITORS

Reaction mixture contained sucrose 25 μmoles, PIN 25 μmoles, potassium phosphate buffer (pH 8.0) 100 μmoles, 2-mercaptoethanol 5 μmoles, the enzyme solution (the purified enzyme preparation, 15.0 units) and each reagent in a total volume of 3.0 ml. Incubation was made for 60 min at 30°C.

Agent	Concentration (M)	Transglucosidase inhibition (%)
None	–	0
NaF	5×10^{-4}	17
KCN	"	9
Na_2HAsO_4	"	12
Phenylhydrazine·HCl	"	13
Semicarbazide·HCl	"	4
α,α'-Dipyridyl	10^{-4}	1
o-Phenanthroline	"	1
EDTA[a)	"	5
Tris[b)	"	2
	10^{-3}	2
	10^{-2}	56

a) Ethylenediamine tetra-acetic acid

b) Tris-HCl buffer (pH 8.0)

inhibitor of intestinal carbohydrases[122,124)] and yeast α-glucosidase.[123)]

Effect of metal ions

The effect of metal ions at 10^{-3}M on the enzyme activity was examined in the presence of 2-mercaptoethanol at 1.7×10^{-3}M and the

TABLE VIII. EFFECT OF METAL IONS

Reaction mixture contained sucrose 25 μmoles, PIN 25 μmoles, potassium phosphate buffer (pH 8.0) 100 μmoles, 2-mercaptoethanol 5 μmoles, the enzyme solution (the purified enzyme preparation, 5.3 units) and each metal ion (10^{-3}M) in a total volume of 3.0 ml. Incubation was made for 75 min at 30°C.

Metal ion	Transglucosidase activity (%)
None	100
NaCl	94
KCl	100
LiCl	99
$BaCl_2$	113
$CaCl_2$	113
$FeSO_4$	114
$FeCl_3$	106
$MgSO_4$	105
$NiSO_4$	38
$CoCl_2$	61
$MnSO_4$	104
$Cr_2(SO_4)_3$	104
Na_2MoO_4	95

results are shown in Table VIII. The enzyme was dialyzed overnight against 0.01M potassium phosphate buffer (pH 7.0) containing 5×10^{-3} M 2-mercaptoethanol and then tested. Ni^{2+} caused a 60% inhibition and Co^{2+} a 40% inhibition. The activity was practically unaffected by other metal ions tested.
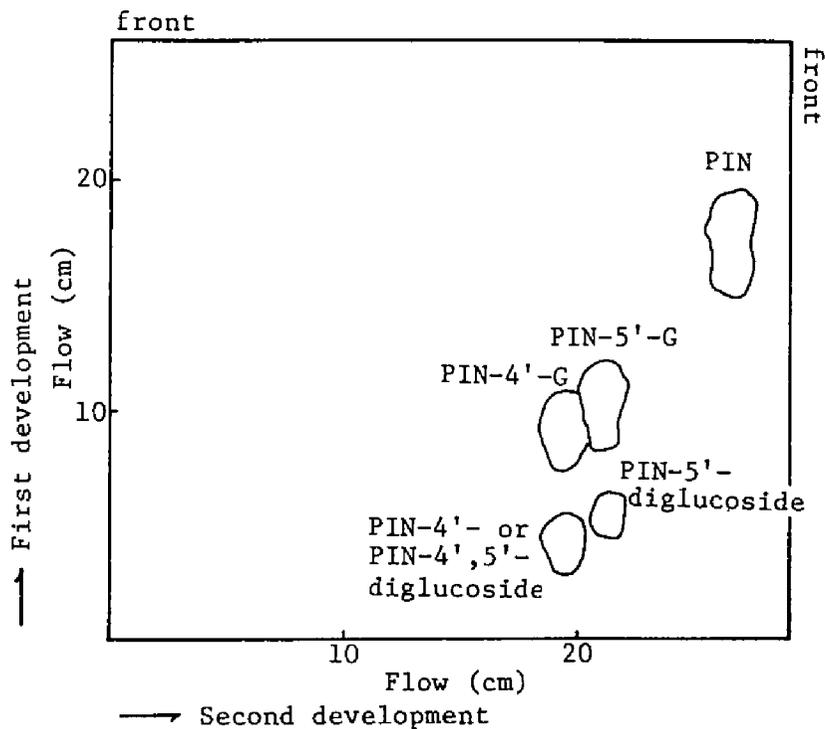
Fig. 16. Determination of Reaction Products
by Two-dimensional Paper Chromatography.

Solvent system:

First development; *n*-butanol:acetic acid:water (4:1:1, v/v).

Second development; *n*-butanol:pyridine:water (6:4:3, v/v).

Identification of Reaction Products by Two-dimensional Paper Chromatohraphy

The reaction products of PIN-G-synthesizing enzyme were confirmed by two-dimensional paper chromatography. The result is shown in Fig. 16. An appropriate amount of the reaction mixture using the purified enzyme preparation was spotted on Toyo filter paper No. 53. The ascending development was carried out in the direction of one-dimension with running by about 26 cm from the spotted line in the following solvent system; *n*-butanol:acetic acid:water (4:1:1, v/v).

The ascending development was also carried out in the direction of two-dimension with running by 27 cm from the base line in the following solvent system: n-butanol:pyridine:water (6:4:3, v/v). The products on a paper chromatogram were detected with a Manaslu lamp and qualitatively identified by spraying the diazotized p-aminoaceto-phenone reagent.[104,105] PIN-G gave two orange spots. Thereafter 0.2M borate buffer (pH 9.0) was sprayed on a paper, followed by drying at 60°C for 10 min. Then, one of the orange spots changed to yellow. These two compounds were identified as PIN-4'-G and PIN-5'-G, as described in the preceding section. Other two spots with smaller Rf values than those of PIN-G were also detected as shown in Fig. 16. One spot gave an orange color in the presence of borate and another orange spot disappeared in the presence of borate. These compounds also seemed to be PIN-4'-G and PIN-4',5'-diglucoside, and PIN-5'-diglucoside, respectively.

DISCUSSION

Throughout the purification of PIN-G-synthesizing enzyme from *Micrococcus* sp. No. 431, transglucosidase activity to form PIN-G was always associated with α-glucosidase activity to hydrolyze sucrose. It may be indicated that both activities are due to a single enzyme and that the synthesis of PIN-G is catalyzed by a glucosido-invertase which transfers a glucosyl residue from sucrose to PIN.

A number of α-glucosidases with different substrate specificities have been purified from microorganisms, plants and animals. Most of

invertase preparations from various sources[125-127] catalyze the transfer of fructosyl residue of sucrose. α-Glucosidases which catalyze the transglucosidation from sucrose to certain alcoholic groups are found in brewer's yeasts,[114,128,129] honey,[115] insects,[116] and bacteria.[130] It is of interest to note whether or not these α-glucosidases catalyze the synthesis of PIN-G from PIN and sucrose.

The purified PIN-G-synthesizing enzyme utilized phenyl-α-D-glucoside and maltose as well as sucrose as glucosyl donors. It showed no transglucosidase activity with α-glucose-1-phosphate, which is an intermediate in the reaction of sucrose phosphorylase. These results are compatible to the assumption that the enzyme belongs to a glucosido-invertase (α-glucosidase).

It is known that yeast α-glucosidase has a low specificity for glucosides of holoside and heteroside while mold α-glucosidase has a high one. PIN-G-synthesizing enzyme purified from *Micrococcus* sp. No. 431 had a specificity for both holoside and heteroside.

Among vitamin B_6 compounds tested, only PIN was effective as glucosyl acceptor for the formation of PIN-G. Yet, further investigations are required on the specificity of the enzyme for other glucosyl acceptors. It was also shown that this enzyme examined here, like many other α-glucosidases, was characteristic of the sulfhydryl nature.

SUMMARY

An enzyme catalyzing the synthesis of PIN-G was demonstrated in cell extracts of *Sarcina* and *Micrococcus* incubated on a medium contai-

ning PIN and sucrose. *Micrococcus* sp. No. 431 was found to have a high activity of the enzyme and the enzyme activity reached to a maximum after 20 hr of cultivation.

The enzyme which synthesized PIN-G from sucrose and PIN by trans-glucosidation was purified from *Micrococcus* sp. No. 431 by means of ammonium sulfate fractionation, and DEAE-Sephadex, hydroxylapatite and Sephadex G-100 column chromatography. The enzyme was purified about 354-fold and confirmed to be homogeneous on polyacrylamide-gel electrophoresis and ultracentrifugation.

Some properties of PIN-G-synthesizing enzyme were investigated with purified enzyme preparations from *Micrococcus* sp. No. 431.

The enzyme was stable at pH 7.0 and between 0°C and 30°C. The maximal activity was obtained at pH 8.0 and 37°C. Besides sucrose, phenyl-α-D-glucoside and maltose served as glucosyl donors. Of vitamin B_6 compounds tested, only PIN served as glucosyl acceptor. The enzyme activity was inhibited by PCMB and heavy metal ions, and the inhibition was prevented by 2-mercaptoethanol, indicating the enzyme would be a sulfhydryl enzyme. The activity was not affected by chelating agents and not activated by metal ions.

The reaction products of PIN-G-synthesizing enzyme were confirmed by two-dimensional paper chromatography. Besides PIN-G (PIN-4'-G and PIN-5'-G), PIN-diglucoside was detected on a paper chromatogram.

Section IV. Stability and Biological Activities of PIN-G

INTRODUCTION

In the previous sections, it has been reported that PIN-G, PIN-4'-G or PIN-5'-G, is synthesized from PIN and glucosyl donors *e.g.* sucrose, maltose or phenyl-α-D-glucoside by various microorganisms including bacteria, yeasts, molds and actinomycetes. *Micrococcus* sp. No. 431, having the highest activity for PIN-G formation, accumulated maximally 1800 to 1900 µg/ml of PIN-G (90-95% yield based on PIN added) at 2 mg/ml of PIN. PIN-G-synthesizing enzyme was purified from *Micrococcus* sp. No. 431 to a homogeneous state. It was suggested that the enzyme might be a glucosido-invertase (α-glucosidase) which catalyzed the transfer of glucosyl residue from sucrose to PIN to form PIN-G.

However, the properties and biological activity of PIN-G have not been investigated as yet. The present paper describes the stability of PIN-G against irradiation of ultraviolet light and heating in comparison with that of PIN and PIN-borate complex. The microbiological activity of PIN-G is also examined in this section. As to the investigations on the transport of vitamin B_6 into human erythrocytes, Yamada *et al.*[131-133] have reported that free forms of vitamin B_6 are actively transported into the erythrocytes against a concentration gradient, and Suzue *et al.*[134] have reported that PAL-P is transported into cells without hydrolysis to PAL. It is of interest to note whether PIN-G is also transported into erythrocytes. In this section, the cellular transport of PIN-G with rabbit erythrocytes was investi-

gated *in vitro*, comparing with that of PIN as a standard. The various factors affecting the transport of vitamin B_6 compounds were also investigated.

EXPERIMENTAL

Materials. PIN-G was prepared from PIN and sucrose by intact cells of *Micrococcus* sp. No. 431. A reaction mixture containing 2 g of PIN, 5 g of sucrose, 35 mmoles of potassium phosphate buffer (pH 8.0) and 2.2 g (as dry wt.) of the intact cells in a total volume of 350 ml was incubated aerobically at 28°C for 11 hr. After removal of cells by centrifugation, the supernatant solution was adjusted to pH 2.0, and 50 g of active charcoal was added, and stirred at room temperature for 2 hr. Active charcoal collected by filtration was washed with 100 ml of 0.01N HCl and 200 ml of deionized water, followed by elution with ammoniacal ethanol (99% ethanol 50:28% ammonia 5:water 45, v/v). The eluate containing PIN and PIN-G was concentrated under reduced pressure at 30°C and subjected to Sephadex G-10 column chromatography (1 X 120 cm). PIN-G was eluted with deionized water at a flow rate of 7 ml per hr. The PIN-G fractions were collected and concentrated under reduced pressure at 30°C to about 5 ml and applied to the preparative paper chromatography on Toyo filter paper No. 526 with a solvent system of *n*-butanol:acetic acid:water (4:1:1, v/v). A small amount of concomitant PIN could be removed from PIN-G in this procedure. The fluorescent zone of PIN-G (*Rf* = 0.4) was cut off and extracted with deionized water. The extract was concentrated under

reduced pressure and used in this section. The amount of PIN-G was
indicated as that of PIN moiety.

The Alsevers-treated rabbit erythrocytes were purchased from
commercial sources. Other materials used in this work were commercial
products.

*Degradation of vitamin B_6 compounds by ultraviolet irradiation
and heating.* Ultraviolet light by a sterilizing lamp GL-15 (National
Electric Co., Ltd.) was irradiated directly upon 5 ml of a sample
solution in a test tube (1.5 X 11 cm) from a distance of about 30 cm.
Five milliliters of the sample was repeatedly heated in a test tube
(1.6 X 160 cm) five times at 120°C and 15 lb for 1 hr in an autoclave.

The degradation of vitamin B_6 compounds was followed by measuring
the decrease in the absorbancy at 254 mμ and 325 mμ for PIN-G and PIN,
and at 295 mμ for PIN-borate complex with a Shimazu multi-purpose
spectrophotometer model MPS-50L.

Microbiological assay. Microbiological activity of vitamin B_6
was assayed by the method of Atkin *et al.*,[135] using *Saccharomyces
carlsbergensis* 4228 ATCC 9080.

Transport of vitamin B_6 into rabbit erythrocytes. Experiments
were performed according to the method of Yamada *et al.*[131-133]

1. Preparation of rabbit erythrocytes. The commercial rabbit
erythrocytes were washed three times at room temperature with an ice-
cold 0.9% saline solution by centrifugation and resuspension. The
fluffy layer of white blood cells was removed as much as possible.
The packed cells were finally suspended in an equal volume of 0.9%

saline solution, and equilibrated to the desired temperature in a
water bath for 10 min. Aliquots of this suspension were used in the
experiment.

2. *Incubation and analysis.* The incubation mixture contained an
aliquot of the erythrocyte suspension having a hematocrit value of
0.50, 6 mM glucose and a suitable amount of vitamin B_6 in total 5 ml
of 1/15M Krebs-Ringer phosphate buffer (pH 7.4). The incubation was
carried out at 37°C for the indicated time intervals. After incuba-
tion, the cells were collected by centrifugation at 3,000 rpm for 10
min, and washed three times with an ice-cold 0.9% saline solution.
The cells were then hemolyzed in 8 ml of 1/12N H_2SO_4 to liberate
intracellular vitamin B_6 compounds. The hemolyzate was hydrolyzed
at 120°C and 15 lb for 210 min in an autoclave, and was deproteinized
by adding 0.5 ml of 10% sodium tungstate. The supernatant solution
was used for determining the total vitamin B_6 by microbioassay with
Saccharomyces carlsbergensis 4228 ATCC 9080.[135] PIN-G could be
assayed as PIN according to this procedure.

3. *Preparation of ghosts (erythrocyte membranes).* According to
the method of Post,[136] 20 ml of erythrocytes were washed three times
with 70 ml of an ice-cold 0.9% saline solution at room temperature.
All the further procedures were carried out at 2°C. The cells were
packed tightly by centrifugation, and the supernatant solution was
discarded. Sixty milliliters of distilled water was added to the
packed cells by forceful injection from a syringe. The hemolyzate
mixed by inversion was centrifuged at 15,000 X g for 30 min. The

transparent dark red supernatant solution was carefully sucked

and discarded. The residue was suspended into 80 ml of 5×10^{-4}M

histidine-imidazole buffer (pH 7.1), followed by centrifugation at

15,000 X g for 10 min. The treatment was repeated eight times until

the supernatant solution became colorless. A large volume of fluffy

pink precipitate, called ghosts, was separated by decantation and was

stored at pH 7.

   4. Determination of vitamin B_6 compounds adsorbed on ghost cells.

An appropriate amount of PIN or PIN-G was incubated with histidine-

imidazole buffer (histidine 7 μmoles and imidazole 13 μmoles, pH 7.1),

5 μmoles of MgCl , 200 μmoles of NaCl, 200 μmoles of KCl, 4 μmoles of

adenosine triphosphate (ATP), and the ghosts in a final volume of 4.0

ml. The reaction was carried out at 37°C for 60 min. After removal

of the ghosts by centrifugation, the amounts of PIN or PIN-G in the

supernatant fluid were determined by microbioassay. PIN-G was assayed

as PIN after hydrolysis to PIN and glucose by heating at 120°C and

15 lb for 210 min in 0.055N H_2SO_4.

RESULTS

Stability of PIN-G against Ultraviolet Light

   The stability of PIN-G against ultraviolet light was investigated

in comparison with that of PIN or PIN-borate. PIN-G and PIN were

adjusted to pH 6.8 with NaOH. PIN-borate complex was prepared by

mixing PIN with borate buffer, pH 6.8, in a final concentration of

0.1M. Changes in absorption spectra of PIN-G, PIN, and PIN-borate

TABLE I. CHANGES IN ULTRAVIOLET ABSORBANCY OF VITAMIN B_6 COMPOUNDS DURING IRRADIATION

Irradiation	PIN-G E_{254} mμ	PIN-G E_{325} mμ	PIN E_{254} mμ	PIN E_{325} mμ	PIN-borate E_{295} mμ
			per cent		
0 hr	1.62 (100)	1.18 (100)	1.07 (100)	1.78 (100)	1.35 (100)
6	1.58 (98)	1.02 (86)	0.99 (93)	1.42 (80)	1.33 (99)
9	1.55 (96)	0.97 (82)	0.98 (92)	1.40 (79)	1.31 (97)
22	1.52 (94)	0.81 (69)	0.84 (79)	0.96 (54)	1.22 (90)
47	1.47 (91)	0.64 (54)	0.58 (54)	0.55 (31)	0.81 (60)

complex on irradiation of ultraviolet light are shown in Fig. 1.
As shown in Table I, PIN-borate complex is the most stable against ultraviolet light, for 60% of the initial absorbancy at 295 mμ remains even after 47 hr of irradiation. The higher stability of PIN-G was observed, 54% of the original absorbancy at 325 mμ being retained after 47 hr of irradiation. On the other hand, the absorbancy of PIN decreased to 30% of the original.

Stability of PIN-G against Heating

PIN and PIN-G neutralized to pH 6.8 with NaOH were heated five times at 120°C and 15 lb for 1 hr in an autoclave, cooled, and followed by standing in an ice box overnight. After centrifugation, the absorbancy of the supernatant solution was measured. The result is shown in Table II. About 90% of the initial absorbancy of PIN-G was retained. PIN-borate complex was not degraded at all, while PIN was decomposed to about 80% of the initial absorbancy.
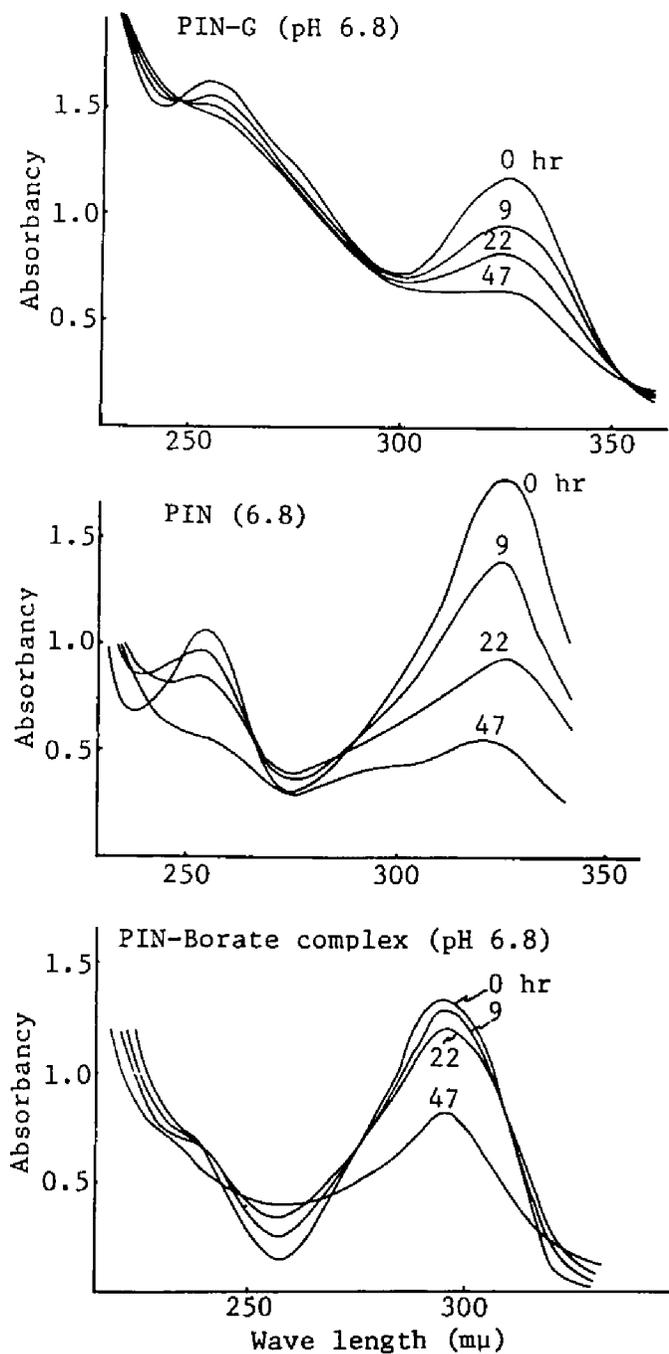
Fig. 1. Changes in Absorption Spectra of PIN, PIN-G
and PIN-borate Complex during Irradiation.

The irradiation of ultraviolet light was performed as described
in the text. Ultraviolet absorption spectra were recorded on a
Shimazu multi-purpose spectrophotometer.

TABLE II. CHANGES IN ULTRAVIOLET ABSORBANCY OF VITAMIN B$_6$ COMPOUNDS BY HEATING

Heating was performed as described in the text. Ultraviolet absorbancy was measured by a Shimazu multi-purpose spectrophotometer.

compound	Wave length		Absorbancy Heating time	
			0 hr	5 hr
PIN-G	$E_{254\ m\mu}$ (%)		1.62 (100)	1.61 (99)
	$E_{325\ m\mu}$ (%)		1.18 (100)	1.06 (90)
PIN	$E_{254\ m\mu}$ (%)		0.77 (100)	0.67 (87)
	$E_{325\ m\mu}$ (%)		1.40 (100) ,	1.15 (82)
PIN-borate	$E_{295\ m\mu}$ (%)		1.03 (100)	1.10 (106)

From these results, it seems that PIN-G is more stable than PIN against irradiation of ultraviolet light and heating.

Effect of Heating Time on Hydrolysis of PIN-G

PIN-G could be bioassayed as PIN, after hydrolysis to PIN and glucose, by heating at 120°C and 15 lb in 0.055N H$_2$SO$_4$. The effect of heating time on hydrolysis of PIN-G was investigated. The result is shown in Fig. 2. Thirty-three per cent of PIN-G was hydrolyzed by heating for 1 hr, and 100% hydrolysis was achieved for 3 hr by the heat treatment.

Microbiological Activity of PIN-G for Saccharomyces carlsbergensis 4228 ATCC 9080

As shown in Fig. 3, PIN-G showed about 20% of microbiological
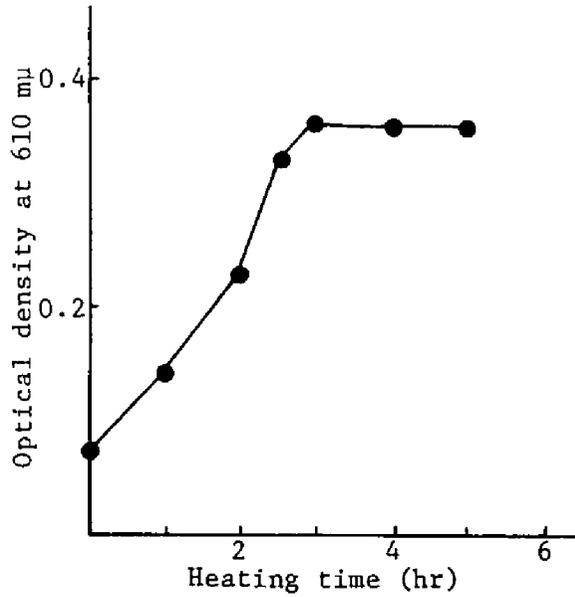
Fig. 2. Effect of Heating Time on Hydrolysis of PIN-G.

The growth of *S. carlsbergensis* 4228 ATCC 9080 on the Atkin's medium containing each hydrolyzed sample (PIN-G 30.4 mμg as PIN) was measured from optical density at 610 mμ after 23 hr of incubation.
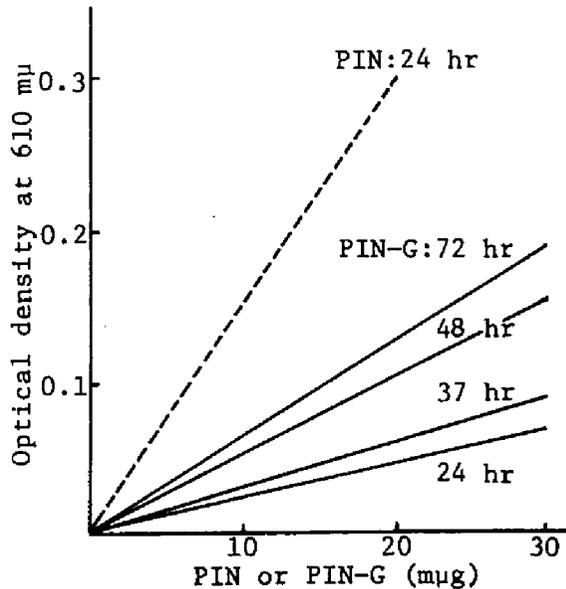

Fig. 3. Microbiological Activity of PIN-G.

Microbiological activity was assayed as described in the text. Optical density at 610 mμ was measured after each time of incubation as shown in the figure.

activity of equivalent mole of PIN after incubation for 24 hr. The microbiological activity of PIN-G was increased with the prolonged incubation and reached to above 50% of activity of equivalent mole of PIN.

Transport of PIN-G into Rabbit Erythrocytes

Effect of incubation time on the transport of PIN and PIN-G into erythrocytes

The cells were incubated with 5 µg of PIN and PIN-G for 10, 30 and 60 min under the conditions described in the EXPERIMENTAL. The results are given in Table III. The amount of vitamin B_6 in the

TABLE III. EFFECT OF INCUBATION TIME ON THE TRANSPORT OF PIN AND
PIN-G INTO ERYTHROCYTES

Erythrocytes (0.8 ml of cell suspension) were incubated at 37°C for indicated time intervals in 1/15M phosphate buffer (pH 7.4) containing 6 mM glucose and 5 µg of PIN or PIN-G.

Substrate added	Incubation time	Vitamin B_6 concentration in cells	in medium	Concentration ratio
	min	*mµg/ml*	*mµg/ml*	*cell/medium*
PIN	10	1150	987	1.2
	30	1840	927	2.0
	60	2420	877	2.8
PIN-G	10	265	1060	0.3
	30	365	1060	0.3
	60	385	1050	0.4

erythrocytes incorporated was increased with incubation time. The concentration ratio of intracellular PIN to extracellular PIN was 2.8 after 60 min. However, the amount of transported PIN-G was about 20% of that of transported PIN.

Effect of concentrations of PIN and PIN-G on the transport into erythrocytes

The cells were incubated with 1 to 20 μg of PIN and PIN-G for 60 min. As shown in Fig. 4, the amount of intracellular vitamin B_6
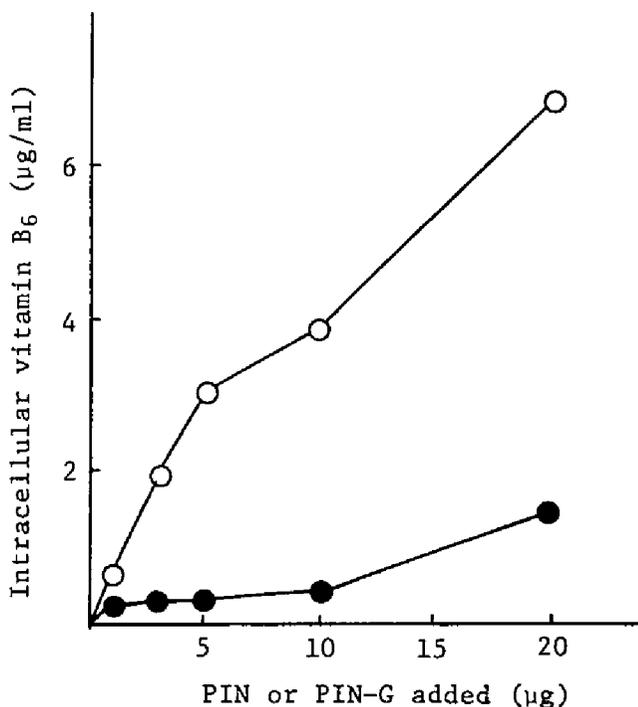
Fig. 4. Effect of Concentrations of PIN and PIN-G
on the Transport into Erythrocytes.

Erythrocytes (2.0 ml of cellssuspension) were
incubated at 37°C for 60 min in 1/15M phosphate
buffer (pH 7.4) containing 6 mM glucose and various
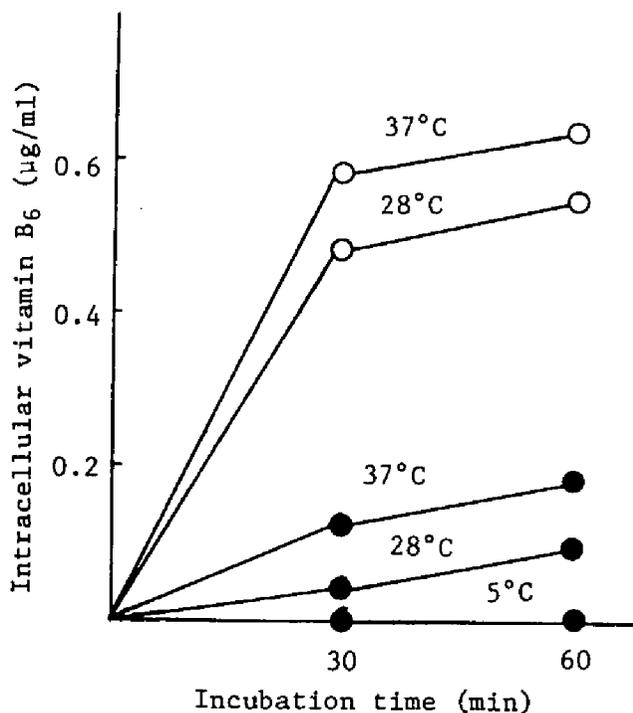concentrations of PIN or PIN-G
—○— PIN, —●— PIN-G

Fig. 5. Effect of Incubation Temperature on the
Transport of PIN and PIN-G into Erythrocytes.

Erythrocytes (1.2 ml of cell suspension) were
incubated at various temperature in 1/15M phosphate
buffer (pH 7.4) containing 6 mM glucose and 1 µg
of PIN or PIN-G.

————O—— PIN, ————●——— PIN-G

was increased proportionately to that of PIN or PIN-G added. However,

the amount of intracellular vitamin B_6 transported from PIN-G was very

small, as compared with that from PIN. The result suggests that a

barrier may be present for the transport of PIN-G.

Effect of incubation temperature on the transport of PIN and

PIN-G into erythrocytes

The cells were incubated with 1 µg of PIN or PIN-G at different

temperature. The result is shown in Fig. 5. Neither PIN nor PIN-G

TABLE IV. EFFECT OF GLYCOLYTIC INHIBITORS ON THE TRANSPORT OF PIN AND
PIN-G INTO ERYTHROCYTES

Erythrocytes (2.0 ml of cell suspension) were incubated at 37°C for
60 min in 1/15M phosphate buffer (pH 7.4) containing 6 mM glucose and
1 µg of PIN or PIN-G.

	Inhibitor	Concentration	Intracellular vitamin B_6
PIN	None		611 mµg/ml
	NaF	5×10^{-3} M	500
	CH_2ICOOH	"	528
PIN-G	None		86
	NaF	5×10^{-3} M	54
	CH_2ICOOH	"	55

was incorporated into the cells at 5°C. But, the incorporation of

vitamin B_6 was accelerated at 28°C and 37°C.

Effect of glycolytic inhibitors on the transport of PIN and PIN-G

into erythrocytes.

Sodium fluoride and monoiodoacetate are known as glycolytic

inhibitors which depress the active transport. Effect of sodium

fluoride or monoiodoacetate (5×10^{-3}M) on the transport of PIN and

PIN-G is shown in Table IV. The incorporation of PIN and PIN-G was

fairly inhibited in the presence of the inhibitors. This result would

suggest that the transport of PIN and PIN-G might be dependent upon

the energy supplying system.

Effect of ATP and glucose on the transport of PIN-G into erythro-

cytes

As shown in Table V, the transport of PIN-G was evidently enhanced by the addition of glucose or ATP. This result leads to the suggestion that the transport of PIN-G may be related to the energy supplying system.

Adsorption of PIN and PIN-G on ghost cells

From the results described above, it was shown that PIN and PIN-G was found in erythrocytes after incubation. But it should be noted whether these vitamin B_6 compounds are really transported through erythrocyte membranes or merely combined or adsorbed on membranes (ghost cells). The author examined to clarify whether PIN and PIN-G were combined to ghost cells. As shown in Table VI, the vitamin B_6 compounds are not combined to ghost cells, but remain uncharged in

TABLE V. EFFECT OF ATP AND GLUCOSE ON THE TRANSPORT
OF PIN-G INTO ERYTHROCYTES

Erythrocytes (1.5 ml of cell suspension) was incubated at 37°C for 60 min in 1/15M phosphate buffer (pH 7.4) containing 6 mM glucose, 20 µmoles of ATP and 5 µg of PIN-G.

Addition	Intracellular vitamin B_6
None	84 mµg/ml
+ Glucose	99
+ ATP	132
+ Glucose, + ATP	144

TABLE VI. ADSORPTION OF PIN AND PIN-G ON GHOST CELLS

The ghost cells (prepared from 0.75 ml of erythrocytes) were incu-
bated at 37°C for 60 min with PIN or PIN-G under the conditions
described in the EXPERIMENTAL.

	Concentration		Incubation	
			0 min	60 min
PIN	40	mµg	41.3 mµg	40.2 mµg
	80	"	81.5 "	83.6 "
PIN-G	50	"	51.7 "	51.7 "
	100	"	100.0 "	98.7 "

the medium. Therefore, it seems to be concluded that a large part of
PIN and PIN-G incubated with rabbit erythrocytes was actively trans-
ported into the cytoplasm.

Retention of PIN and PIN-G in the erythrocytes

After incubation at 37°C for 60 min with various concentrations
of PIN and PIN-G, the cells were separated from the suspending medium
by centrifugation and washed three times with a 0.9% saline solution.
The result is shown in Table VII. A great part of PIN and PIN-G in
the first washing is considered to be the contamination of the medium.
The amount of PIN and PIN-G eluted in the second and third washings is
smaller than the intracellular vitamin B_6, showing the retention of
PIN and PIN-G in the erythrocytes.

TABLE VII. RETENTION OF PIN AND PIN-G IN THE ERYTHROCYTES

Erythrocytes (2.0 ml of cell suspension) were incubated at 37°C for 60 min in 1/15M phosphate buffer (pH 7.4) containing 6 mM glucose and the indicated amounts of PIN or PIN-G. After incubation, the cells were separated by centrifugation and washed three times with 5 ml of an ice-cold 0.9% saline solution.

Added amount		in Washings			in Erythrocytes
		1st	2nd	3rd	
PIN	1 μg	118	99	67 mμg	610 mμg
	5	354	199	171	3040
	10	1320	682	251	3890
PIN-G	1	158	31	15	172
	5	410	111	81	319
	10	451	260	76	403

DISCUSSION

It has been reported that the dipolar form of PIN in a neutral solution is very labile to heat and light.[137,138] On the other hand, PIN-borate complex is reported to be thermostable.[113] It seems that the stability of PIN-borate complex depends on the monopolar form in which 3' and 4' positions of PIN are masked by borate. It was also reported that 4' or 5' position of PIN was first attacked by photooxidation[138] and that by heating at 120°C for 30 min in a neutral solution, PIN formed dimer or polymer,[139] in which 4' hydroxymethyl group of PIN was combined to nitrogen atom of 1' position of another PIN. In PIN-G, the dipolar form is maintained, but 4' or 5' position of PIN is

masked by glucosyl residue. The higher stability of PIN-G seems to be based on that 4' or 5' position of PIN is masked by glucosyl residue.

PIN-G served as vitamin B_6 (PIN) vitamer, which showed 20% of microbiological activity of equivalent mole of PIN for *S. carlsbergensis* 4228 ATCC 9080. However, the activity of PIN-G was gradually increased accompanied with the prolonged incubation.

It was suggested that PIN-G was hydrolyzed to PIN and glucose by α-glucosidase of *S. carlsbergensis* 4228 ATCC 9080 and then functioned as PIN.

The cellular transport of vitamin B_6 compounds has been investigated by Yamada *et al.*[131-133] and Suzue *et al.*[134] with human erythrocytes. Yamada *et al.* reported that free forms of vitamin B_6 were actively transported into the cells, while their phosphate esters were hardly transported. They also reported that PAL was most rapidly and abundantly transported into the erythrocytes. Suzue *et al.* found out that PAL-P was also incorporated into red blood cells without being hydrolyzed.

As described in this paper, PIN and PIN-G were transported into rabbit erythrocytes. The concentration of intracellular vitamin B_6 incorporated was 2.8 times as high as that found in the medium of PIN, and 0.4 times in the medium of PIN-G. The cellular transport of PIN and PIN-G was increased with the incubation time and the concentration of them added. At low temperature (5°C), the transfer of PIN and PIN-G into the cells was remarkably depressed. Sodium fluoride and mono-iodoacetate considerably inhibited the transport of PIN and PIN-G.

By the addition of ATP and glucose, the transport of PIN-G was accelerated. All these results suggest that both of PIN and PIN-G are actively transported through erythrocyte membranes. No PIN and PIN-G was combined to ghost cells, which would show that these vitamins were truly incorporated into erythrocytes. A small amount of PIN and PIN-G was leaked out from the cells into saline washings, but a great part of them were retained in the cells. This result also supports the above suggestion. However, the amount of PIN-G transported was about 10-20% of that of equal mole of PIN. This is probably due to the existence of a permeability barrier for PIN-G. It remains to be investigated whether PIN-G is incorporated directly into the cells or it is incorporated in the form of PIN after PIN-G is degraded by a membrane α-glucosidase.

SUMMARY

PIN-G was found to be as stable as PIN-borate complex against irradiation of ultraviolet light and heating, while PIN was less stable.

Intact PIN-G showed about 20% of microbiological activity of equivalent mole of PIN for *Saccharomyces carlsbergensis* 4228 ATCC 9080. The activity was gradually increased accompanied with the prolonged incubation. It seems that PIN-G is hydrolyzed to PIN and glucose by α-glucosidase of the organism and can be bioassayed as PIN. Hydrolysis of PIN-G by heating in 0.055N H_2SO_4 yielded PIN quantitatively.

PIN and PIN-G are transported into rabbit erythrocytes *in vitro*. The amounts of vitamin B_6 compounds transported into the erythrocytes were bioassayed with *Saccharomyces carlsbergensis* 4228 ATCC 9080 after hydrolyzing them to free forms of vitamin B_6 by heating for 210 min in $1/12N$ H_2SO_4. The transport of PIN and PIN-G was affected by incubation time, incubation temperature and concentration of PIN and PIN-G added. The transport of PIN-G increased with the coexistense of ATP or glucose. The transport of PIN and PIN-G was decreased by the addition of sodium fluoride or monoiodoacetate. The adsorption of PIN and PIN-G on ghost cells was not found. Small amounts of transported PIN and PIN-G were leaked out by washings, indicating that large parts of them were retained in the erythrocytes.

Chapter II. Microbial Formation of a New Derivative of
Pantothenic Acid: Pantothenic Acid-α-Glucoside

INTRODUCTION

PaA was first recognized from natural sources with the aid of a
microbiological assay employing the Gebrüder Mayer strain of yeast
Saccharomyces cerevisiae, growing in a synthetic medium.[140] The
compound also showed the biological activity for chicks, designated
as a "filtrate factor" or a "chick antidermatitis factor". Jukes[141]
and Wooley *et al.*[142] in 1939 simultaneously announced that PaA,
isolated in pure form from liver by Williams *et al.*,[143] and the
filtrate factor were apparently identical. Lipmann and coworkers[144]
demonstrated that coenzyme A contained a bound PaA which could be
released effectively by the combined actions of a liver enzyme and
alkaline phosphatases.[145]

PaA is widely distributed in animals, plants and microorganisms
as a component of coenzyme A. However, other complexes derived from
coenzyme A, as well as phosphorylated forms of both PaA and pantethine,
have also been demonstrated to occur widely.[146,147] Many workers
have investigated the biosynthetic[148,149] and degradative[150-153] meta-
bolisms of PaA. A large number of derivatives of PaA except metabolites
described above, have been found in nature[96,97] and have also been
chemically synthesized.[154-157] Recently a β-glucoside of PaA was
isolated from tomato juice as a growth factor for malo-lactic fermen-
tation bacteria in wine fermentation.[86-88] However, an α-glucoside

of PaA has not been synthesized chemically and biologically.

The present chapter describes the microbial formation of the glucoside of D-PaA. A considerable amount of α-glucoside of D-PaA was formed by intact cells of *Sporobolomyces coralliformis* IFO 1032, when incubated with maltose and D-PaA. The isolation and identification of the compound is presented.

EXPERIMENTAL

Materials. DEAE-Cellulose was kindly given by the Green Cross Co., Osaka. Cellulase (Type II, from *Aspergillus niger*), β-glucosidase (from almonds), maltase (Grade II, from *A. niger*) were purchased from Sigma Chem. Co., and α-glucosidase (from yeast) was from Boehringer Mannheim Japan K.K. Takadiastase B was purchased from Sankyo Co., Ltd. Other chemicals used in this work were obtained from commercial sources.

Microorganisms and cultivation. Yeasts preserved in the Laboratory of Physiology of Fermentation and Applied Microbiology, Department of Agricultural Chemistry, Kyoto University were used for a screening. All strains used were preserved on a nutrient agar slant by a monthly transfer at 5°C.

Yeasts were grown on a medium composed of 5% glucose, 0.5% peptone, 0.5% yeast extract, 0.5% K_2HPO_4, 0.2% KH_2PO_4, and 0.02% $MgSO_4 \cdot 7H_2O$, adjusted to pH 6.0. The cultivation was carried out at 28°C for 48 hr on a reciprocal shaker with 50 ml of the medium in a 300 ml shaking flask. After cultivation, the cells were harvested by centrifugation at 5000 rpm, and washed once with 0.9% saline solution. The washed

cells were suspended in 2-5 ml of 0.01M potassium phosphate buffer
(pH 7.0) containing 5 x 10^{-3}M 2-mercaptoethanol and subjected to the
reaction.

Reaction conditions. The standard reaction mixture contained
50 µmoles of D-PaA, 100 µmoles of maltose or cellobiose, 100 µmoles
of potassium phosphate buffer (pH 7.0), 3 µmoles of 2-mercaptoethanol,
a final concentration of 0.1% of Span 80, and 0.5 ml of intact cell
suspension in a total volume of 1.5 ml. The incubation was performed
at 28°C for 24 hr aerobically. Glucose was used as a blank in place
of maltose or cellobiose. The reaction was stopped by immersing a
tube in a boiling water bath for 5 min. After cooling the reaction
mixture in an ice bath, the cells were removed by centrifugation and
the supernatant fluid was assayed for a derivative of D-PaA.

Analyses. PaA was bioassayed by the paper disc method using
Saccharomyces carlsbergensis 4228 ATCC 9080.[93,158] PaA glucoside
was identified as follows. A suitable amount of reaction mixture
(20-50 µl) was spotted on Toyo filter paper No. 53 (2 cm width) and
developed with a solvent system of *n*-butanol:acetic acid:water (4:1:1,
v/v). After development, the dried paper strip was cut off in 0.5 cm
widths from the 2 cm width and subjected to bioautography by *S. carls-
bergensis* 4228 ATCC 9080[93,158] and *Lactobacillus plantarum* ATCC 8014.
[94,158,159] An appropriate amount of Takadiastase B or mold maltase
solution (4 mg/ml of 0.01M acetate buffer, pH 5.0) was daubed on a
paper strip with a pipette after it was placed on a culture plate in
order to get a clearer active zone of PaA-α-G with a maltose-PaA

medium. An appropriate amount of cellulase (2 mg/ml of 0.01M acetate buffer, pH 5.0) was similarly used for the detection of PaA-β-G with a cellobiose-PaA medium.

Paper chromatography of PaA-G and its acid-hydrolyzate was carried out on Toyo filter paper No. 53 with the solvent systems as follows; n-butanol:acetic acid:water (4:1:1, v/v), n-propanol:28% ammonia:water (6:3:1, v/v), n-butanol:benzene:pyridine:water (5:1:3:3, v/v, upper layer) and isopropanol:pyridine:water:acetic acid (8:8:4:1, v/v). The PaA moiety was confirmed by bioautography and the glucose moiety was determined by the color development with the silver nitrate reagent.[109]

Glucose was determined by the glucostat method[111] and reducing sugar was estimated by the Somogyi-Nelson method.[112]

The reaction by several glucosidases was carried out on a reaction mixture containing 2.6 μmoles of PaA-G, 10 μmoles of 2-mercaptoethanol, 50 μmoles of buffer and an appropriate amount of enzyme in a total volume of 1.0 ml. The reaction was carried out at 37°C for 3 hr. Phosphate buffer (pH 7.0) was used in the incubation with yeast α-glucosidase and acetate buffer (pH 5.0) in that with mold maltase, β-glucosidase and cellulase. The reaction mixture was submitted to bioautographic and paper-chromatographic analyses for PaA and glucose as described above.

Measurement of IR and NMR spectra. IR spectrum of PaA-G was measured in a micro KBr tablet with a Hitachi infrared spectrometer type EPI-G3. NMR spectrum was measured in D_2O on a Varian HA-100D spectrometer at 100 MHz with DSS-D_6 as an internal standard.

RESULTS

PaA-G Formation by Yeasts

PaA-G-forming activity was checked with yeasts. Maltose was used

for PaA-α-G formation and cellobiose was used for PaA-β-G formation.

Glucose was also used in place of maltose or cellobiose as a blank.

A considerable amount of α-glucoside of PaA was found in the reaction

mixture of some strains of yeasts belonging to *Saccharomyces*, and

Sporobolomyces coralliformis (Table I). *S. coralliformis* IFO 1032

seemed to have the highest activity for PaA-α-G formation. A β-gluco-

side was scarcely detected in all strains. No derivative was found

with a glucose medium, as shown in Fig. 1. Thus, *S. coralliformis*

IFO 1032 was selected and used as the PaA-α-G-forming strain in the

following experiment. The formation of PaA-α-G at 48 hr of incubation

was larger than at 24 hr of incubation.
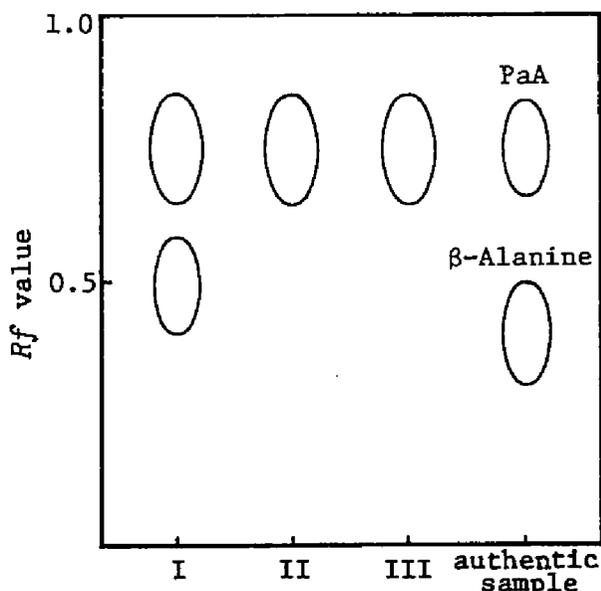
Fig. 1. Bioautogram of PaA-G.

Solvent system: *n*-butanol:acetic acid:water (4:1:1, v/v)
I. maltose medium, II. cellobiose medium, III. glucose medium

TABLE I.   DISTRIBUTION OF PaA-G-FORMING ACTIVITY IN YEASTS

Intact cells of yeasts were incubated in the reaction mixture described in the EXPERIMENTAL.   The incubation was performed at 28°C for 24 hr aerobically.

Strains	PaA-α-G formed	PaA-β-G formed
Saccharomyces cerevisiae AKU 4100	−	−
" " AKU 4103	−	−
" *delbrueckii* IFO 0285	++	−
" *carlsbergensis* IFO 0641	−	−
" *rouxii* IAM 4369	−	−
" *sake* Kyokai No. 7 AKU 4111	++	−
" *bayanus* IFO 0206	−	±
" *sake* Fukumusume AKU 4017	+	−
" *rouxii* IFO 0686	−	−
" *cerevisiae* AKU 4104	−	−
" *sake* Unryu AKU 4019	−	−
" " Shokkomasamune AKU 4015	−	−
" " Hozan AKU 4013	−	−
Bass Bier Hefe (Burton on Trent No. 1) AKU 4002	−	−
Kagawakenshi (Shoyu) AKU 4033	+	−
Fukumoto 3 AKU 4034	++	−
Beer yeast (Nippon) AKU 4038	−	−
Brennerei Hefe Rasse 12 AKU 4010	−	−
Sporobolomyces salmonicolor IFO 0374	−	±
" " IFO 0375	−	−
" *coprophilus* IFO 1422	−	−
" *coralliformis* IFO 1032	++	±
" *pararoseus* IFO 1103	−	−
" *salmonicolor* IFO 1038	−	−
" *odorus* IFO 1035	−	−

++; good, +; fair, ±; slight, −; nil.

Isolation of PaA-α-G

For the purpose of good formation of PaA-α-G-synthesizing enzyme, *S. coralliformis* was grown on a maltose medium with 500 ml of the medium in a 2 liter shaking flask. The cultivation was carried out at 28°C for 48 hr on a reciprocal shaker. The cells were collected by continuous centrifugation at 5000 rpm, washed once with 0.9% saline solution and suspended in the same solution. Twenty-one grams (calculated as dried cells) of intact cells of the organism were incubated with 40 g of maltose, 4 g of D-PaA, 8 mmoles of potassium phosphate buffer (pH 7.0), and 0.4 g of cetyltrimethyl ammonium bromide (surfactant) in a total volume of 400 ml. The reaction was carried out aerobically at 28°C for 40 hr on a 2 liter shaking flask. The reaction was stopped by immersing the incubation flask (adjusted to pH 7.0) in boiling water for 5 min, and the cells were removed by centrifugation at 10,000 rpm for 20 min. Isolation of a product was made from the supernatant solution as a starting material. The supernatant solution was adjusted to pH 4.0 and left at 4°C overnight. Any material precipitating was removed by filtration. Active charcoal (60 g) was added to the filtrate, followed by stirring for 1 hr. Then, the charcoal was collected by filtration, washed with 0.01N HCl and deionized water and eluted with five volumes of acetone and an equal volume of 50% acetone. The eluate was concentrated by evaporation *in vacuo*. The concentrated solution was submitted to paper chromatography with Toyo filter paper No. 526 in a solvent system of *n*-butanol:acetic acid:water (4:1:1, v/v), followed by bioautography with *Saccharomyces carlsber-*

gensis 4228 ATCC 9080.

Two different bioactive zones were detected, one of which with a higher *Rf* value was identical to D-PaA. The other bioactive zone with a *Rf* of 0.5, was cut off and extracted with 50% ethanol. The extract was concentrated *in vacuo* and desalted with Amberlite CG-50 (H$^+$ type). The concentrated effluent was applied to a Sephadex G-10 column (1 x 105 cm), and eluted with water. The active fractions were adsorbed on a DEAE-cellulose column (OH$^-$ type), and eluted with 0.02M LiCl. The eluted fraction was desalted again with Amberlite CG-50 (H$^+$ type), and adjusted to pH 5.0 with HCl. Saturated Ba(OH)$_2$ solution was added to the fraction until the pH became to 7.0-7.2. Excess Ba(OH)$_2$ was removed repeatedly by concentration and filtration. The completely dried Ba-salt of the compound was dissolved in methanol, followed by adding ether. A resultant fluffy precipitate was collected and dissolved in water, and subjected to paper chromatography in the solvent system as described above. The active zone was extracted with 50% ethanol, and the extract was evaporated, lyophilized and dried *in vacuo* on P$_2$O$_5$ and KOH. The lyophilized powder (yield 21.9 mg) was used for the characterization.

Identification of the Compound

The *Rf* values of the isolated compound on bioautography in several solvent systems were examined, comparing with known metabolites of PaA. The result is shown in Table II. The *Rf* values of the isolated compound differed from PaA, β-alanine and phospho-PaA. Pantenol, pantethine and pantoyl lactone had no biological response for *Saccharomyces*

carlsbergensis 4228 ATCC 9080. The compound was also responsible for

Lactobacillus plantarum ATCC 8014. The organism utilizes PaA, phospho-

PaA, and panthethine, but not pantenol, β-alanine and pantoyl lactone.

The activity for both strains increased with the prolonged incubation.

It seems that the compound is hydrolyzed to D-PaA and glucose by α-

glucosidases of *S. carlsbergensis* and *L. plantarum* and can be bioassayed

as PaA.

The isolated compound had no reducing activity for Somogyi-Nelson

reagent[112]. However, on hydrolysis in 0.055N H_2SO_4 at 120°C and 15

lb for 3 hr, or on treatment with yeast α-glucosidase at 30°C and pH

TABLE II. *Rf* VALUES FROM BIOAUTOGRAPHY

Compounds	Solvent				
	I	II	III	IV	V
Isolated compound	.49	.36	.63	.52	.48
D-PaA	.76	.50	.76	.85	.69
β-Alanine	.29	.13	.54	.39	.62
4'-Phospho-PaA[160]	.45	.19	.28	.40	.38
Panthenol	no response				
Pantethine	no response				
Pantoyl lactone	no response				

Solvent system:
  I. *n*-butanol:acetic acid:water (4:1:1, v/v)
 II. *n*-butanol:pyridine:water (6:4:3, v/v)
III. *n*-propanol:28% ammonia:water (6:3:1, v/v)
 IV. *n*-butanol:ethanol:water (5:1:4, v/v, upper layer)
  V. isobutyric acid:0.5N ammonia:0.1M EDTA (100:80:1.6, v/v)

TABLE III.   IDENTIFICATION OF PaA AND GLUCOSE MOIETIES OF
THE ISOLATED COMPOUND

The PaA moiety was confirmed by bioautography using *S. carlsbergen-sis* 4228 ATCC 9080 and the glucose moiety was identified by the color development with the silver nitrate reagent.[109]

| Compounds | Solvent | | | |
	I	II	III	IV
		Rf values		
Isolated compound	.46	.58	.24	.73
Acid hydrolyzate*	.80	.76	.33	.57
Enzymatic hydrolyzate**	.80	.73	.31	.55
PaA	.79	.75	–	–
β–Alanine	.32	.55	–	–
Glucose	–	–	.33	.57
Maltose	–	–	.22	.49

* hydrolyzed in 0.055N H_2SO_4 at 120°C and 15 lb for 3 hr.

** hydrolyzed by yeast α-glucosidase.

Solvent system:
   I. *n*-butanol:acetic acid:water (4:1:1, v/v)
  II. *n*-propanol:28% ammonia:water (6:3:1, v/v)
 III. *n*-butanol:benzene:pyridine:water (5:1:3:3, v/v, upper layer)
  IV. isopropanol:pyridine:water:acetic acid (8:8:4:1, v/v)

7.0, the isolated compound gave two components corresponding to glucose and PaA.   The identification of the components by paper chromatography and bioautography is shown in Table III.   The molar ratio of glucose and PaA was determined to be 1:1 by the glucostat method and bioassay (Table IV).   Enzymatic hydrolysis of the isolated compound by various

TABLE IV. MOLAR RATIO OF PaA AND GLUCOSE

	PaA found (mμmole)	Glucose found (mμmole)	Molar ratio
Microbioassay by paper disc method* (*S. carlsbergensis*)	195	–	1.00
Glucostat method*	–	211	1.08

*) The isolated compound was hydrolyzed at 37°C overnight by yeast
   α-glucosidase.
Reducing activity of the compound was not found by Somogyi-Nelson
method.

glucosidases was investigated. Figure 2 shows that the compound is

hydrolyzed to glucose and PaA by yeast α-glucosidase and mold maltase,

but not by cellulase and β-glucosidase. The data suggest that glucose

and PaA are bound through an α-glucosidic linkage in the isolated

compound, that is, the compound is α-glucopyranosyl-D-PaA. The IR

spectrum of the compound showed that the compound contained PaA (chela-

ting with Ba^{2+}) and sugar moiety (Fig. 3). The NMR spectrum of the

compound is shown in Fig. 4. The signals shows the presence of two

methyl groups (C-3', δ 0.98 and 0.99 ppm, 6H, single), a methylene

group (C-2, δ 2.42 ppm, 2H, triplet, j=13 Hz), a methylene group (C-3,

δ 3.45 ppm, 2H, triplet, j=11.5 Hz), a methylene group of C-4' and ring

protons of the sugar moiety (δ 3.55-3.90 ppm, multiple), a methyne

group (C-2', δ 4.12 ppm, 1H, single), and one anomeric proton of the

α-glucosidic linkage (δ 4.89 ppm, 1H, doublet, j=3.5 Hz). The compa-

rison of this spectrum with that of authentic PaA suggests that a PaA

Solvent I
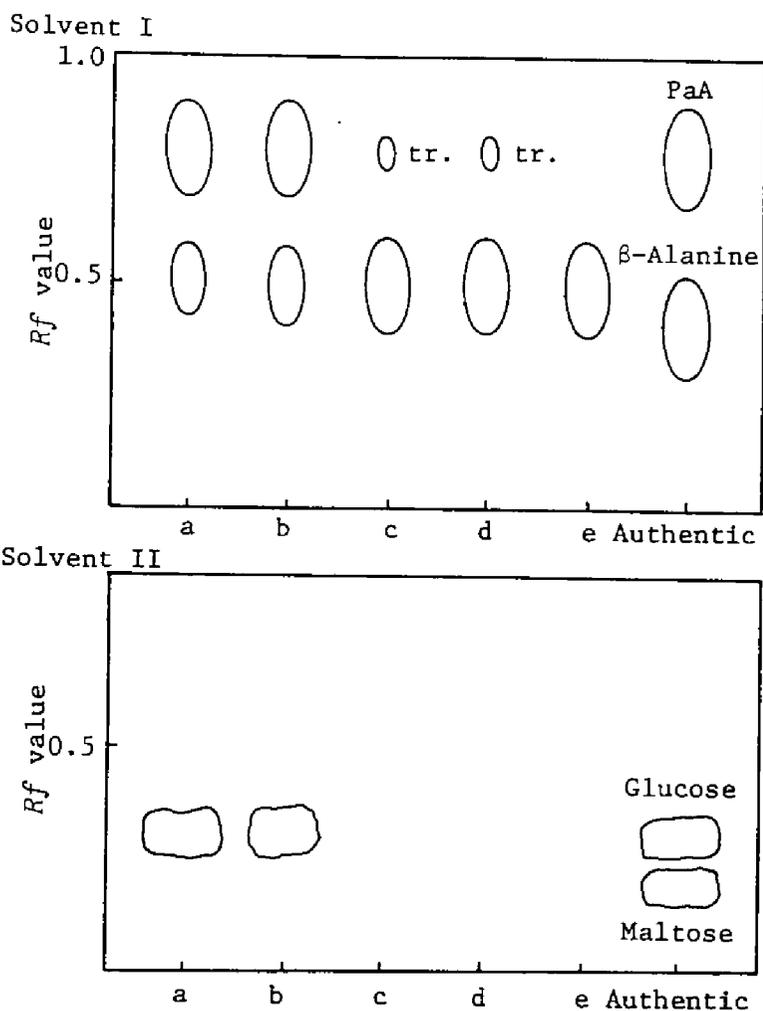
Solvent II

Fig. 2. Enzymatic Hydrolysis of the Isolated
Compound by Glucosidases.

The PaA moiety was confirmed by bioautography and the
glucose moiety was determined by the color development with
the silver nitrate reagent.

Solvent system:
 I. *n*-butanol:acetic acid:water (4:1:1, v/v)
II. *n*-butanol:benzene:pyridine:water (5:1:3:3, v/v)

a. mold maltase
b. yeast α-glucosidase
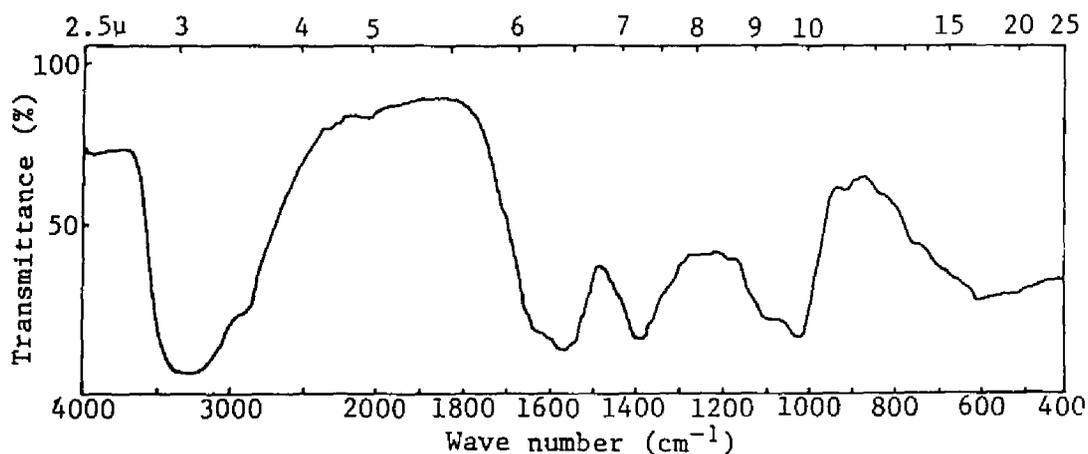c. β-glucosidase (emulsin)
d. cellulase
e. isolated compound

Fig. 3. IR Spectrum of the Isolated Compound

(Micro KBr Tablet)

The spectrum was recorded on a Hitachi infrared spectrometer type EPI-G3 in a micro KBr tablet.

moiety is contained in this compound. The ratio of the numbers of protons attributed to two methyl groups in PaA moiety and glucose residue is approximately 1:1. The proposed structures of the isolated compound are shown in Fig. 5. Comparing the methyne signal (δ 4.12 ppm) of the proposed α-D-glucopyranosyl-D-PaA with that of 2'-O-(β-D-glucopyranosyl)-D-PaA and that of 4'-O-(β-D-glucopyranosyl)-D-PaA which were reported by Amachi *et al.*,[86-88] it is suggested that the glucosidic linkage of the compound may be present at C-4' position. From the results described above, it is concluded that the isolated compound is possibly 4'-O-(α-D-glucopyranosyl)-D-PaA.
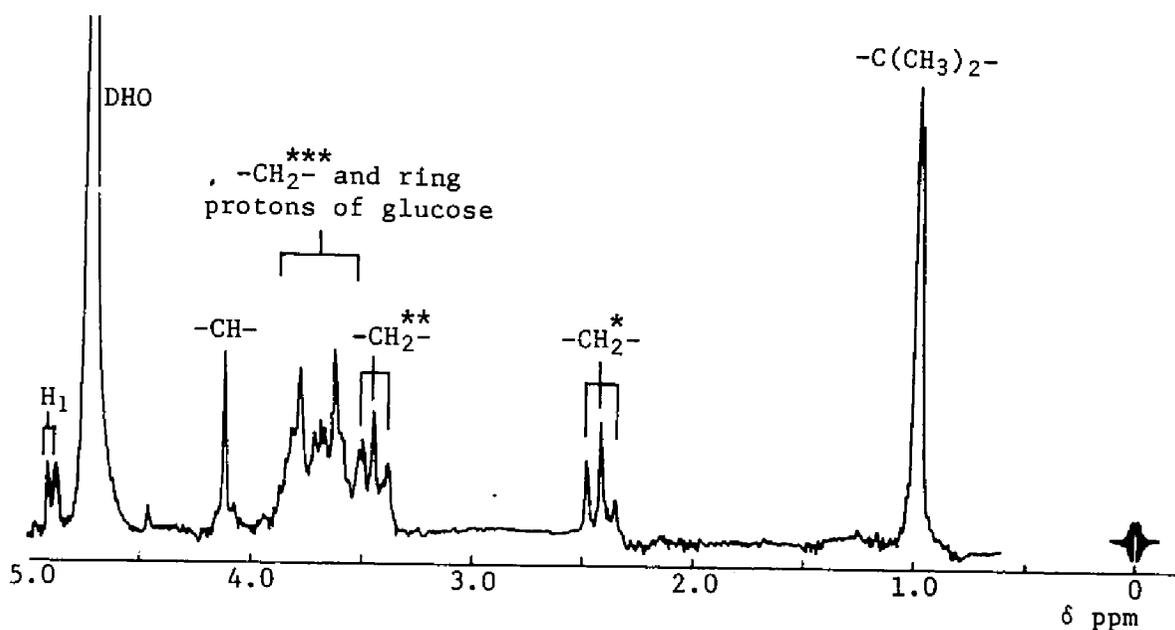
Fig. 4.   NMR Spectrum of the Isolated Compound.

The spectrum was recorded on a Varian HA-100D spectrometer at 100 MHz in D_2O with DSS-D_6 as an internal standard.
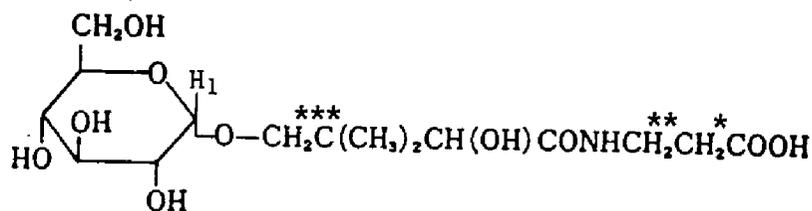
4'-O-(α-D-glucopyranosyl)-D-pantothenic acid
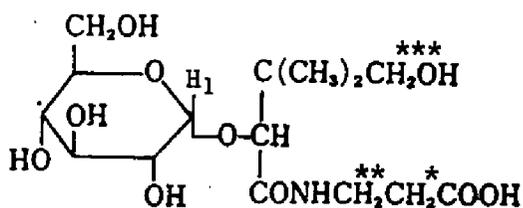
2'-O-(α-D-glucopyranosyl)-D-pantothenic acid

Fig. 5.   Proposed Structures for α-Glucosyl-D-pantothenic Acid.

DISCUSSION

PaA-β-G has recently been isolated from tomato juice as a growth factor of malo-lactic fermentation bacteria in wine fermentation.[86-88] In this chapter, the author has first described the formation of PaA-α-G, a new sugar derivative of PaA, by yeast *Sporobolomyces coralliformis* IFO 1032.

Many sugar derivatives of vitamins have been isolated from nature, and synthesized by enzymatic and chemical methods. Since riboflavinyl-α-glucoside has been found in rat liver by Whitby in 1950,[73] the formation of various riboflavinylglycosides containing glucoside, galactoside, fructoside and oligosaccharides is reported in microorganisms, plants, and animals by many investigators.[74-81,83] A sugar derivative of ascorbic acid has been prepared enzymatically by Miyake *et al.*[84] PIN-G, the first sugar derivative of PIN to be recognized, has been isolated by Ogata *et al.*[68] from bacterial cultures during investigations on the metabolism of vitamin B_6 by microorganisms. Microbial distribution of PIN-G-forming activity, purification and properties of the PIN-G-synthesizing enzyme of *Micrococcus* sp. No. 431 and some physico-chemical and biological properties of PIN-G were investigated in detail by the author in the preceding chapter.

In a preliminary experiment on microbial formation of PaA-G, the author examined the distribution of PaA-G-forming activity in microorganisms under cultural conditions. The cultivations were performed on a PaA-maltose medium and a PaA-cellobiose medium in expect of the formation of PaA-α-G and PaA-β-G in the respective media. Very few

organisms produced an appreciable amount of PaA-β-G in the PaA-cello-biose medium, but some strains of yeast accumulated a considerable amount of PaA-α-G in the PaA-maltose medium. *Sporobolomyces coralli-formis* IFO 1032 had a relatively high activity for the formation of PaA-α-G among those tested. It was also found that a large amount of PaA-α-G was formed when PaA and maltose were incubated with intact cells of *S. coralliformis* grown on a maltose medium. Therefore, the large scale reaction with the intact cells was carried out to isolate and identify PaA-α-G for the simplicity of reaction system.

The author isolated a new compound from a reaction mixture of the organism by the treatment with active charcoal, Amberlite CG-50, Sephadex G-10 and DEAE-cellulose column chromatography and paper chromatography. The compound was characterized as α-glucopyranosyl-D-PaA. It was suggested that the glucosidic linkage of the compound might be present at C'-4 from NMR spectrum and the facillity of reac-tivity of C'-2 or C'-4 alcoholic groups. The compound showed the slight microbiological activity for *Saccharomyces carlsbergensis* and *Lactobacillus plantarum*. The activity increased with the incubation period. It seemed that the compound might be gradually hydrolyzed by α-glucosidases of these strains and could be utilized as PaA. The transglucosidation seems to be catalyzed by α-glucosidase, react-ing on maltose. However, *Micrococcus* and *Sarcina*, having the high activity of PIN-G formation did not form the compound. These α-gluco-sidases differ from each other on the substrate specificity for glucosyl acceptor.

SUMMARY

The formation of PaA-G was checked with intact cells of yeasts. PaA-β-G-forming activity was scarcely found, but PaA-α-G-forming activity was found with several strains. Among them, *Sporobolomyces coralliformis* IFO 1032 showed the highest activity for PaA-α-G formation. The compound was isolated from a reaction mixture of the organism incubated on a medium containing maltose and D-PaA, by the treatment with active charcoal, Amberlite CG-50, Sephadex G-10 and DEAE-cellulose column chromatography and paper chromatography. The isolated compound was characterized as 4'(2')-O-(α-D-glucopyranosyl)-D-PaA by various analytical methods including bioautography, paper chromatography, and IR and NMR spectroscopy.

The intact compound showed the slight microbiological activity for *Saccharomyces carlsbergensis* 4228 ATCC 9080 and *Lactobacillus plantarum* ATCC 8014, which are used as strains for bioassay of PaA. The activity increased gradually with the prolonged incubation.

CONCLUSION

In this thesis, the author has investigated the formation of PIN-G and PaA-G by microorganisms.

In the first chapter, the distribution of PIN-G-forming activity was investigated in various microorganisms. Various factors influencing on the formation, the enzymatic metabolism, the stability and the biological activity of PIN-G were also examined. The distribution of PIN-G-forming activity was searched in culture collections of bacteria, yeasts, molds and actinomycetes together with isolated strains. A relatively high activity of PIN-G formation was found in bacteria belonging to *Micrococcus* and *Sarcina*. The activity was also widely distributed in yeasts, molds and actinomycetes. A bacterium strain No. 431, which was isolated from soil and identified as *Micrococcus* sp. No. 431, had the highest activity among the tested organisms and selected as the most suitable strain for PIN-G formation. With this strain, several conditions on the formation of PIN-G were investigated by the growing culture. Sucrose and peptone were the most excellent substrates as carbon and nitrogen sources. A few nonionic surfactants showed the stimulative effect on the formation of PIN-G, in which Span 80 was the most effective. Metal ions had no effect. The accumulation of PIN-G reached to a maximum after 60 to 80 hr of cultivation and decreased after then. The formation of PIN-G increased proportionately with the concentrations of sucrose and PIN. Optimal pH of the culture was 7.0. Under the optimal conditions, the amount of PIN-G reached to a maximum of 1800 to 1900 µg/ml as PIN moiety (yield over 90%) at 2

mg/ml of PIN added.

PIN-G was isolated from the culture filtrate of *Micrococcus* sp. No. 431 grown under the optimal conditions, and identified by various analytical methods including IR and NMR spectroscopy. PIN-4'-G and PIN-5'-G were separately confirmed by paper chromatography. PIN-diglucoside was also found in the filtrate, which was isolated and identified by the same methods as PIN-G.

The enzyme activity to synthesize PIN-G from PIN and sucrose was demonstrated in intact cells and cell extracts of *Sarcina* and *Micrococcus*. *Micrococcus* sp. No. 431, an isolated bacterium, was found to have the high activity of this enzyme in its cell extract and used as the enzyme source. The enzyme activity of the microorganism reached to a maximum after 20 hr of cultivation. The enzyme which catalyzes the synthesis of PIN-G *via* transglucosidation from sucrose to PIN was purified from the organism by means of ammonium sulfate fractionation, and DEAE-Sephadex, hydroxylapatite and Sephadex G-100 column chromatography. The enzyme was purified about 354-fold and confirmed to be homogeneous on polyacrylamide-gel electrophoresis and ultracentrifugation.

Some properties of PIN-G-synthesizing enzyme of the organism were studied using the partially and highly purified enzyme preparations. The enzyme was stable at pH 7.0 and between 0°C and 30°C. The maximal activity was obtained at pH 8.0 and 37°C. Besides sucrose, phenyl-α-D-glucoside and maltose served as glucosyl donor. Of vitamin B_6 compounds tested, only PIN served as glucosyl acceptor. The enzyme acti-

vity was inhibited by PCMB and heavy metal ions, and the inhibition was prevented by 2-mercaptoethanol, indicating that the enzyme was a sulfhydryl enzyme like many α-glucosidases. The activity was not affected by chelating agents and not activated by metal ions.

Intact PIN-G showed about 20% of microbiological activity of equivalent mole of PIN for *Saccharomyces carlsbergensis* 4228 ATCC 9080. The biological activity increased gradually accompanied with the prolonged incubation. It seems that PIN-G is hydrolyzed to PIN and glucose by α-glucosidase of the yeast and can be bioassayed as PIN. Hydrolysis of PIN-G by heating in 0.055N H_2SO_4 at 120°C and 15 lb for 3 hr yielded PIN quantitatively.

PIN-G was transported into rabbit erythrocytes *in vitro*. The cellular transport was affected by the incubation time, the incubation temperature and the concentration of PIN-G added. The transport was increased with the coexistense of ATP and glucose and was decreased by the addition of sodium fluoride or monoiodoacetate. These results suggest that PIN-G is actively transported into cells through erythrocyte membranes. Adsorption of PIN-G on ghost cells was not found. This fact shows that PIN-G is truly incorporated into erythrocytes. A small amount of PIN-G was leaked out from the cells in the saline washings, but a great part of it was retained in the cells. This result also supports the above suggestion. However, the amount of PIN-G incorporated was about 10 to 20% of that of equal mole of PIN, showing the existence of a permeability barrier for PIN-G. It remains to be investigated whether PIN-G is incorporated into the cells with

the whole form of PIN-G or with the form of PIN after PIN-G is hydrolyzed by a membrane α-glucosidase of erythrocyte cells.

PIN-G was found to be as stable as PIN-borate complex against irradiation of ultraviolet light and heating, while PIN was less stable.

In the second chapter, the formation of the glucoside of D-PaA was investigated.

The formation of the glucoside of D-PaA was checked with intact cells of yeasts incubated on a medium containing maltose or cellobiose and D-PaA. A β-glucoside of D-PaA was scarcely formed, but an α-glucoside of D-PaA was found with several strains. *Sporobolomyces coralliformis* IFO 1032 showed the highest activity. Then, this new compound was isolated from a reaction mixture of the strain incubated in a medium containing maltose and D-PaA by the treatment with active charcoal, Amberlite CG-50, Sephadex G-10 and DEAE-cellulose column chromatography and paper chromatography. The isolated compound was characterized as 4'(2')-O-(α-D-glucopyranosyl)-D-PaA by various analytical methods including bioautography, paper chromatography, and IR and NMR spectroscopy.

The intact compound showed the slight microbiological activities for *Saccharomyces carlsbergensis* 4228 ATCC 9080 and *Lactobacillus plantarum* ATCC 8014. The activity increased gradually with the prolonged incubation. It seems that the compound is hydrolyzed to D-PaA and glucose by α-glucosidases of *S. carlsbergensis* and *L. plantarum* and can be bioassayed as D-PaA.

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