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Salvage Fermentation and Enzymatic Synthesis of Bredinin*

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Abstract

Bredinin is a novel imidazole nucleoside (4-carbamoyl-1-β-D-ribofuranosylimidazolium-5-olate) produced by *Eupenicillium brefeldianum*. It was salvage-synthesized by a large number of growing microorganisms including bacteria, fungi and actinomycetes in media containing synthetic aglycone. *Eupenicillium brefeldianum* M-2166 utilized the aglycone effectively and showed the best conversion rate. The conditions for the salvage fermentation were investigated using *Eupc. brefeldianum* and *Escherichia coli*.

Bredinin was also synthesized enzymatically in a cell free system of *E. coli*. This enzymatic synthesis was inhibited by adenine but not guanine. The enzyme was remarkably induced by an addition of adenine or guanine to the culture medium.

Introduction

Various types of nucleosides have been isolated from microbial sources, and large numbers of them are known to have biological activities *e.g.* antimicrobial or antitumor actions.[1)]

5-Amino-4-imidazolecarboxamide riboside (AICA-R), an intermediate of purine nucleotides, was the first imidazole nucleoside to be isolated from a sulfonamide-inhibited *Escherichia coli* culture,[2)] however, it has no notable activity.

Recently we isolated a novel imidazole nucleoside with potent immunosuppressive activity, named bredinin, from a culture filtrate of *Eupenicillium brefeldianum* M-2166.[3)] Its chemical structure was determined by X-ray crystalographic analysis as 4-carbamoyl-1-β-D-ribofuranosylimidazolium-5-olate.[4)] We then chemically synthesized bredinin.[5)]

The conversion of purine to the corresponding nucleoside or nucleotide by living cells has been called salvage synthesis rather than *de novo* synthesis. Many investigations concerning these conversions by growing microorganisms have been published. For example, adenosine,[6)] guanosine,[7)] xanthosine,[8)] orotidine,[9)] AICA-R[9)] and 6-azauracyl riboside[10)] have been salvage-synthesized from their respective bases by different growing bacteria.

Mammalian purine nucleoside phosphorylase has been shown to catalyze the synthesis of inosine, guanosine, xanthosine, nicotinamide riboside and AICA-R from their corresponding bases and ribose-1-phosphate.[11-12)]

We hare report some results of the salvage fermentation of bredinin by various microorganisms and its enzymatic synthesis by a cell-free extract of *E. coli*.

Materials and Methods

Reagents The aglycone, 4(5)-carbamoylimidazolium-5(4)-olate, was synthesized in this laboratory from aminomalonamide and ethyl orthoformate according to the modified procedure described by Schipper

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Table 1. Screening program for salvage fermentation.

	Temperature (°C)	Aglycone		Cultivation (hr)
		Concentration (μg/ml)	Addition period (hr)	
Bacteria	25, 37	200	4	20
Molds	26	500	48	96
Yeasts	26	500	24	72
Actinomycetes	30, 45	500	48	96

and Day.[13] Ribose-1-phosphate was obtained from the Sigma Chemical Company, U.S.A. All other chemicals were commercial and of analytical reagent grades.

Microorganisms Microorganisms comprised of 50 strains (50 genera) of bacteria, 174 strains (174 genera) of molds, 19 strains (19 genera) of yeasts and 49 strains (24 genera) of actinomycetes from our collection were used in the screening of the salvage synthesis of bredinin.

Cultivation Bacteria were cultured on a Monoshin shaker in a medium containing 4.0% glucose, 0.5% peptone, 0.5% $CaCO_3$, 0.2% $(NH_4)_2SO_4$, 0.2% K_2HPO_4 and 0.075% $MgSO_4 \cdot 7H_2O$ (pH 7.5). Molds, yeasts and actinomycetes were cultured on a rotary shaker in a medium containing 4.0% glucose, 2.0% peptone, 0.2% KH_2PO_4, and 0.02% $MgSO_4 \cdot 7H_2O$ (pH 6.5 for molds and yeasts, pH 7.3 for actinomycetes). Synthetic aglycone was added during the early stage of culture; then once a day after this addition a test for bredinin activity was made. For each culture there was control culture run without the aglycone. General culture conditions are shown in Table 1.

Assay method for bredinin activity Bredinin was bioassayed by the conventional cup-plate diffusion procedure using *Candida albicans*, as described in a previous paper.[3]

Identification procedure Each culture was first tested for anticandida activity and compared to the control culture without aglycone. The anticandida activity of the aglycone was about one three-hundredth that of bredinin, therefore, the error in bioassay was practically negligible. When the control culture showed bio-activity, the culture was subjected to ethyl acetate-extraction to remove all the solvent extractable antibiotics. The resulting aqueous layer was treated with an anion exchange resin (Amberlite IRA-411, OH) then eluted with aqueous acetic acid. The active eluate was applied to a thin-layer chromatogram (TLC, Eastman's CHROMAGRAM No. 6060) as was the authentic bredinin. The solvent systems and the respective R_f values are given in Table 2.

To detect bredinin, the chromatogram was exposed to uv light at 280 nm and sprayed with 1.0% $FeCl_3$ solution. For further confirmation, the band on the thin-layer chromatogram was scraped off, then after the water-extraction, the uv spectrum of the extract was measured.

Isolation of salvaged bredinin The bredinin in the filtrate of the salvage culture was absorbed on a column of Amberlite IRA-411 (OH cycle) and eluted with 2.0% aqueous acetic acid. The active eluate was concentrated under reduced pressure, and was subjected to Dowex-50×2 (H cycle) column chromatography developed with distilled water. The excess aglycone was not eluted in this chromatography. The bredinin-containing fractions monitored by the $FeCl_3$ reaction were collected and concentrated to a syrupy

Table 2. Thin-layer chromatography.

Solvent systems	R_f values	
	Bredinin	Aglycone
a) *n*-Butanol-acetic acid-water, 3:1:1	0.56	0.60
b) *n*-Propanol-14% ammonia water, 10:2	0.16	0.21
c) Chloroform-methanol-acetic acid, 10:1:1	0.14	0.62

mass. Ethanol was added to this concentrate and the alcoholic solution was kept overnight at 5°C to give bredinin monohydrate crystals.

Preparation of the cell-free extract *E. coli* (ATCC 4157) was used. The organism was grown at 37°C in 100 ml of a medium which consisted of the same constituents as used in the screening. A 15 to 20 hr culture was harvested by centrifugation for 10 min at 5,000 $\times g$, then washed twice in 0.05 M phosphate buffer solution containing 5 mM of 2-mercaptoethanol (pH 6.0). The washed cells were then suspended in 10 ml of the buffer and were sonicated at 4°C (80 W, 4 min). The sonicates were centrifuged for 60 min at 10,000 $\times g$ and the supernatant was recentrifuged for 60 min at 105,000 $\times g$. This supernatant was used as the enzyme solution.

Assay for enzyme activity A mixture of 50 μg of ribose-1-phosphate, 0.3 ml of the enzyme solution and 0.5 ml of 0.2 M phosphate buffer (pH 6.0) in a total volume of 1 ml was incubated at 30°C for 60 min. The reaction was stopped by placing the vessel in a boiling water-bath for 1 min. The reaction mixture was directly tested for bredinin formation by the bioassay procedure described above. One unit of enzyme is defined as that amount which will catalyze the synthesis of one micromole of bredinin under the conditions given.

Results

Screening for the microorganisms A large number of microorganisms, including bacteria, fungi and actinomycetes, in our collection were widely screened for the salvage synthesis of bredinin. This involved the ribofuranosylation of the aglycone by growing organisms. In this experiment, specific and sensitive identification procedures for detecting the synthesized bredinin were the greatest problems. Thus the bioassay method was supplemented with TLC and measurement of the uv spectrum and compared to results of similar measurements of the control culture without the aglycone.

The result of the screening is summarized in Table 3. Microorganisms marked bredinin accumulation over 11 μg per ml of culture broth are listed in Table 4.

All the organisms used could convert the aglycone to bredinin, though there were differences in their conversion rates. Among the test strains, *Eupenicillium brefeldianum* was a bredinin-producing organism which could be utilized effectively and which showed outstanding result.

Salvage synthesis by *Eupenicillium brefeldianum* M-2166 In the screening, *Eupc. brefeldianum* exhibited the best activity for the conversion of the aglycone. The significant increase in this activity during salvage synthesis suggested that the organism would be useful for the bredinin production. Bredinin was produced by both the *de novo* and salvage syntheses routes.

The effect of the concentration of aglycone in the culture medium was examined. The aglycone was initially added in varying concentrations, and the seed broth which had been

Table 3. Salvage synthesis of bredinin by various microorganisms.

Organisms	Accumulation of bredinin (μg/ml)			
	<5	6-10	11-20	>21
Bacteria	39	8	2	1
Molds	74	53	35	12
Yeasts	6	9	4	0
Actinomycetes	7	23	13	6

Numerals show the number of strains.

Table 4. Microorganisms showing relatively high accumulations of bredinin.

Strains	Accumulation of bredinin (μg/ml)	Strains	Accumulation of bredinin (μg/ml)
(a) Bacteria		*Sporormiella minima*	15
Escherichia coli	23**	*Sordaria fimicola*	25
Microbacterium lacticum	18*	*Stachybotrys atra*	13
Micrococcus lysodeikticus	19*	*Stemphylium astragali*	15
(b) Molds		*Talaromyces helicus*	24
Aspergillus aculeatus	22	*Thermoascus crustaceus*	15
Botryotrichum piluliferum	14	*Thielavia terricola*	18
Cephaliophora tropica	11	*Trichocladium asperum*	14
Chloridium chlamydosporis	11	*Trichoderma viride*	30
Chrysosporium keratinophilum	18	*Trichophyton mentagrophytes*	15
Circinella minor	23	*Trichothecium roseum*	15
Coemansia brasiliensis	14	*Wardomyces anomala*	14
Cordana pauciseptata	30	*Zygorhynchus moelleri*	20
Delitschia marchalii	12	(c) Yeasts	
Diaporthe phaseolorum	34	*Hansenula anomala*	15
Dichotomomyces albus	14	*Kluyveromyces lactis*	11
Emericella nidulans	11	*Rhodotorula rubra*	13
Endothia parasitica	30	*Saccharomyces cerevisiae*	15
Eupenicillium brefeldianum	300	(d) Actinomycetes	
Fomes fomentarius	11	*Actinobifida dichotomica*	15***
Fusidium coccineum	12	*Chainia antibiotica*	11*
Gelasinospora cerealis	12	*C. flava*	32*
Gilmaniella humicola	19	*C. minutisclerotica*	20*
Glomerella cingulate	13	*C. ochracea*	15*
Gonytrichum macrocladum	12	*C. poonensis*	29*
Gymnoascus umbrinus	11	*Elytrosporangium brasiliense*	15*
Hamigera avelanea	11	*Microbispora aerata*	15***
Kabatiella caulivora	11	*Micropolyspora caesia*	15***
Leptographium kitajimana	20	*Nocardia asteroides*	20*
Melanospora zamiae	11	*N. collarina*	30*
Microascus trigonosporus	11	*Streptomyces afghaniensis*	15*
Microthecium retisporum	20	*S. bikiniensis*	17*
Mucor mucedo	22	*S. griseus*	12*
Neosartorya fischeri	20	*S. halstedii*	14*
Neurospora tetrasperma	46	*Streptoverticillium griseocarneum*	34*
Phamo citricarpa	12	*Stv. kentuchense*	22*
Phomopsis oblonga	18	*Stv. netropsis*	43*
Sepedonium chrysospermum	12	*Thermomonospora curvata*	20***
Shanorella spirotricha	41		

Temperature for cultivation * 30°C
** 37°C
*** 45°C

Table 5. Effect of the concentration of the aglycone.

Concentration of aglycone (μg/ml)	Bredinin activity (μg/ml)		Conversion rate (%)*
	Maximum	Increased	
0	243	—	
10	264	21	100
100	366	93	46
1,000	949	706	35

* The conversion rate was calculated as follows:

$$\frac{\text{Increased activity } (\mu\text{g/ml})}{\text{Added aglycone } (\mu\text{g/ml}) \times 2.03} \times 100$$

precultured for 48 hr was transferred to the respective medium in about a 2% inoculam. As seen in Table 5, bredinin activity increased with the rise in the concentration of the added aglycone, whereas the conversion rate decreased.

Table 6 shows the effect of the addition time of the aglycone. Since it was scarcely soluble in water, its hot aqueous solution (*ca.* 50°C, 10 mg/ml) was added at different culture stages. The cultivation was carried out for 120 hr. When the aglycone was added at an early stage of culture, good results were obtained.

The conversion of the aglycone by resting cells was also examined. A 3-day old culture was harvested by centrifugation and washed three times in 0.1 M phosphate buffer solution (pH 6.0). The washed cells were suspended in phosphate buffer containing 1.0% glucose, 0.01% $MgSO_4 \cdot 7H_2O$ and 0.05% of the aglycone in the same volume as the original culture. Then the suspension was shaken at 30°C for 72 hr. As shown in Fig. 1, bredinin was obviously synthesized from the aglycone by the resting cells though slight activity, probably due to *de novo* synthesis, was observed in the control group without the aglycone.

The effect of purines on salvage fermentation was examined. Results demonstrated that adenine completely inhibited *de novo* synthesis at a high concentration but did not inhibit salvage synthesis (Table 7). The other bases used had no significant effect. Bredinin was actually isolated from the salvage-cultured broth in a yield proportional to the increased activity. For exaple, 5 l of the salvage-cultured broth, to which 500 μg/ml of the aglycone had been added 24 hr after the inoculation, was filtered to give 4.1 l of the filtrate (bredinin potency: 500 μg/ml). In the parallel run, the control culture without the

Table 6. Effect of the addition time of the aglycone.

Addition time (hr)	Maximum activity (μg/ml)	Increased activity (μg/ml)	Conversion rate (%)
0	378	261	26
24	420	303	30
48	375	258	25
72	263	146	14
Control	117	—	—

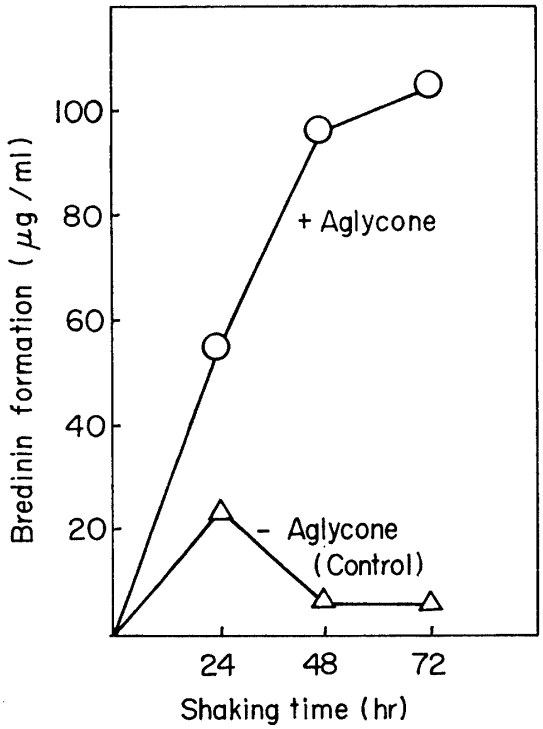

Fig. 1. Salvage synthesis of bredinin by resting cells of *Eupc. brefeldianum.*

Table 7. Effect of purines on salvage fermentation by *Eupc. brefeldianum.*

Purines (μg/ml)	Aglycone (500 μg/ml)	Total bredinin (μg/ml)	Salvaged bredinin (μg/ml)	Conversion rate (%)
Adenine (1,000)	Yes	513	513	51
〃 (〃)	No	Trace		
〃 (100)	Yes	643	379	27
〃 (〃)	No	264		
Guanine (1,000)	Yes	675	458	47
〃 (〃)	No	217		
〃 (100)	Yes	678	438	43
〃 (〃)	No	240		
Xanthine (1,000)	Yes	733	459	45
〃 (〃)	No	274		
〃 (100)	Yes	808	545	54
〃 (〃)	No	263		
Hypoxanthine (1,000)	Yes	618	429	42
〃 (〃)	No	189		
〃 (100)	Yes	665	350	35
〃 (〃)	No	315		
Control	Yes	763	482	48
〃	No	281		

A 48 hour-old culture was transferred to each medium containing purine. The basal medium composition and the culture conditions are described in the text. The aglycone was added at 24 hr after inoculation. The amount of salvaged bredinin was calculated by subtracting the value without the aglycone from the value with the aglycone.

aglycone showed a potency of 200 μg/ml. After treatment of the filtrate with ion-exchange resins, 960 mg of the crystals were obtained. The crystals were identical with the authentic bredinin monohydrate in all respects including their physico-chemical and biological properties.

Salvage synthesis by *E. coli* To investigate salvage synthesis in detail, *E. coli* was employed as the test organism. Various conditions influencing salvage synthesis were tested. Results are given in Fig. 2. *E. coli* was shake-cultured at 37°C for 20 hr in the same medium as that used in the screening. When the aglycone was added at 4 hr after inoculation, the maximum accumulation of bredinin was observed (Fig. 2-A).

Bredinin accumulation increased with an increase in the concentration of the added aglycone, but there was no significant difference in the conversion rate. The concentration of glucose or phosphate in the medium markedly influenced conversion; the optima concentrations being 4.0% and 0.5%, respectively (Fig. 2-C and 2-D). The initial pH of the medium was also an important factor, the value of pH 6.0 was optimum as indicated in Fig. 2-E.

250 μg/ml aliquots of purines or pyrimidines were added to the medium and their effects were tested. The addition of guanine produced about a two fold increase in activity, but adenine inhibited the conversion by about one fourth, in comparison to the control. Adenosine and adenosine-5′-monophosphate were slightly inhibitory while guanosine and guanosine-5′-monophosphate were slightly promotive (Fig. 2-F). However, no other bases, nucleosides or nucleotides, *i.e.* xanthine, hypoxantine, xanthosine, inosine, xanthosine-5′-monophosphate and pyrimidines had any remarkable effect.

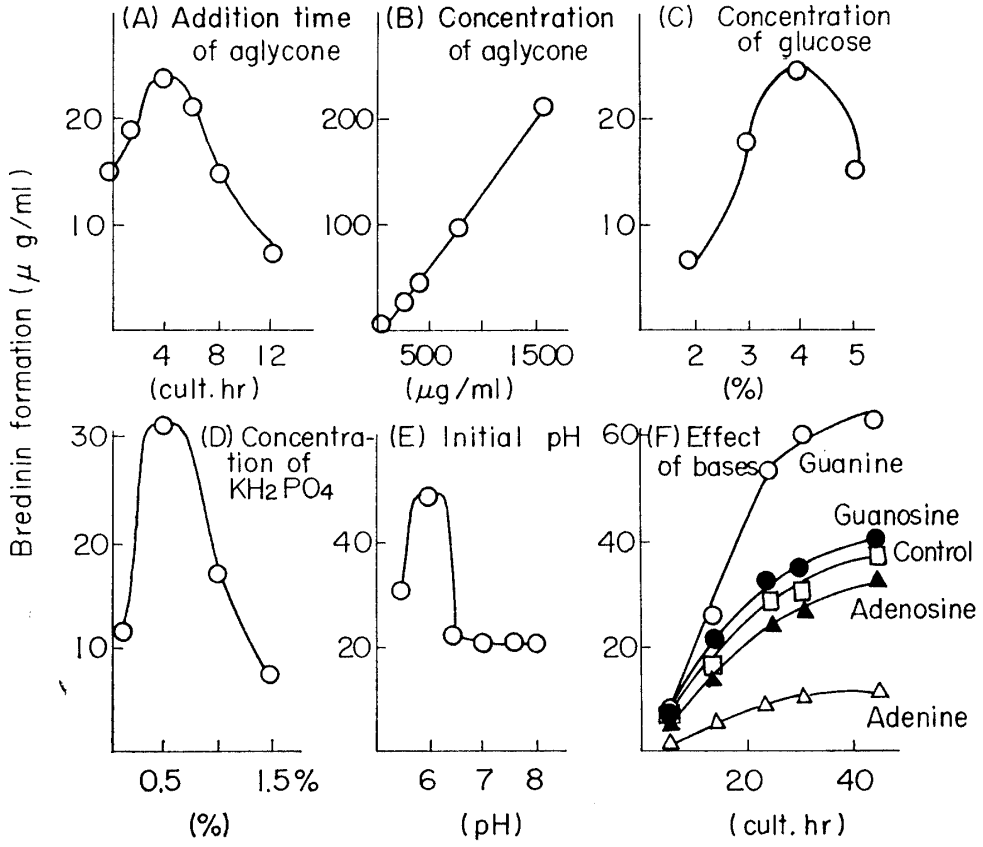

Fig. 2. Conditions for salvage synthesis by *E. coli*.

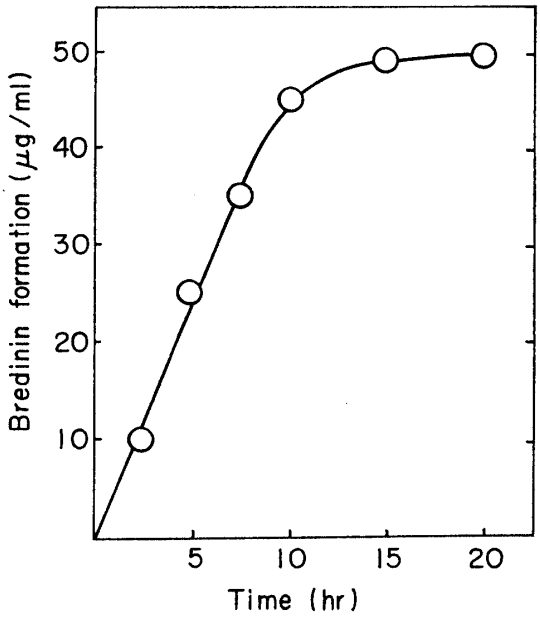

Fig. 3. Enzymatic synthesis of bredinin.
A mixture of the aglycone (50 μg), ribose-1-phosphate (50 μg) and the enzyme (5.9×10^{-3} u) in a total volume of 1.0 ml of 0.1 M phosphate buffer (pH 6.0) was incubated at 30°C.

Table 8. Effect of adenine or guanine on the enzymatic reaction.

Reaction systems	Enzyme activity ($\times 10^{-3}$ U/mg protein)
AG+R-1-P+ENZ	5.9
AG+R-1-P+ENZ+AD	0.0
AG+R-1-P+ENZ+GU	5.7

AG: synthetic aglycone, R-1-P: ribose-1-phosphate, ENZ: enzyme solution, AD: adenine, GU: guanine. Adenine or guanine was added to make final concentrations of 50 μg/ml.

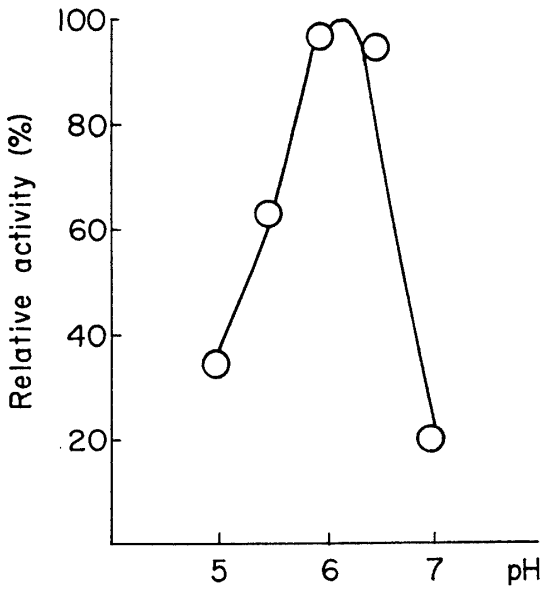

Fig. 4. Optimum pH of the enzyme from *E. coli* which catalyzes the synthesis of bredinin from the aglycone and ribose-1-phosphate (purine nucleoside phosphorylase).

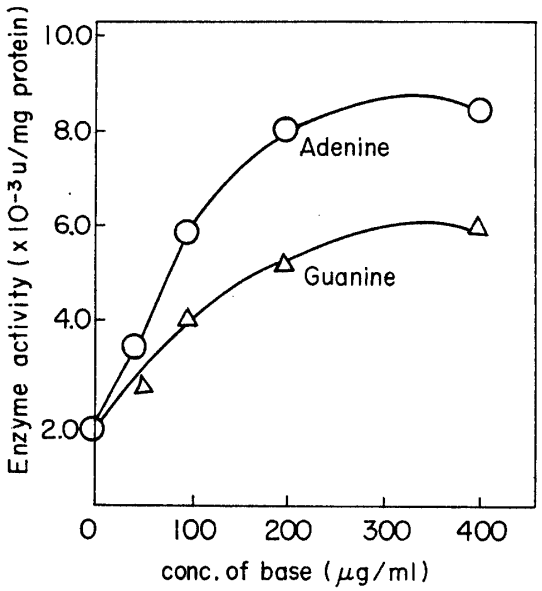

Fig. 5. Effect of adenine or guanine on enzyme-induction.
E. coli was shake-cultured at 37°C for 20 hr in media containing adenine or guanine in addition to the basal constituents. Harvested cells were immediately sonicated, then the enzyme was prepared as described in the text.

Enzymatic synthesis of bredinin A mixture of 50 μg of the aglycone, 0.5 ml of the sonicated homogenate from *E. coli* and 0.4 ml of 0.2 M phosphate buffer (pH 6.0) in a total volume of 1 ml was incubated at 30°C for 3 hr. After the reaction mixture had been treated at 100°C for one min it was bioassayed and showed 4 μg/ml of bredinin formation. In a similar reaction system using the dialyzed homogenate, no bredinin activity was observed. This suggested that the homogenate probably contained ribose-1-phosphate as the substrate. Actually bredinin formation was significantly increased when ribose-1-phosphate was added to the reaction system. Fig. 3 shows the results of the enzymatic synthesis of bredinin using an enzyme solution from *E. coli*.

Enzymatic synthesis was completely inhibited by the addition of adenine, however, the addition of guanine had not influence on the synthesis (Table 8).

The optimum pH of this enzyme was about 6.0 as shown in Fig. 4. The enzyme was markedly induced by adding adenine or guanine to the medium in the *E. coli* culture. Adenine was a more effective inducer than guanine as shown in Fig. 5.

Discussion

In the present study, bredinin was synthesized by microbial and enzymatic reactions and various conditions which might effect these reactions were examined. Results are summarized in Fig. 6. In salvage synthesis by growing microorganisms, all the organisms used were capable of converting aglycone to bredinin, though they differed widely in their conversion rates. Mammalian cells also converted the aglycone. When the aglycone was intraperioneally administered to a rat, bredinin activity was recognized in its blood and in its urine. This was confirmed on the isolation of crystalline bredinin from the rat's urine (Tsujino M. *et al.*, 194th Meeting of Japan Antibiotics Research Association, held in Tokyo, July 26, 1974). These facts suggest that the ability to perform salvage synthesis is very common and normal in living cells.

Koaze *et al.* widely screened a large number of strains of microorganisms (75 bacteria, 38 yeasts, 48 molds, and 49 actinomycetes) capable of converting adenine to adenosine but they found that no microorganisms, except for two bacterial strains, could accumulate adenosine.[7] Their results do not negate the ability of microorganisms to carry out salvage

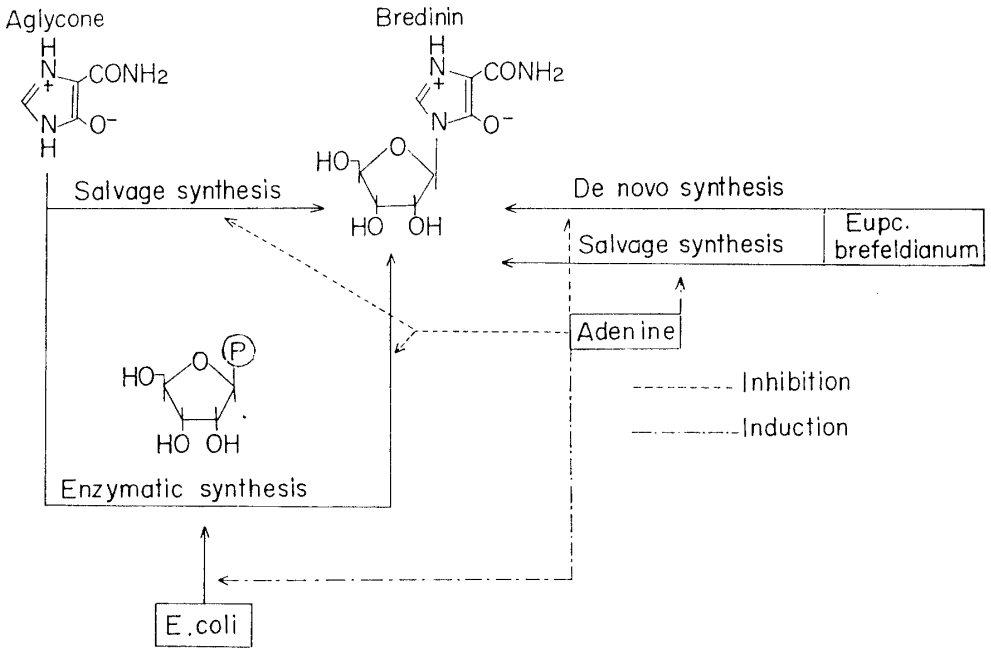

Fig. 6. Bredinin formation by various routes and the effect of adenine.

synthesis, because salvage synthesis would be catalyzed by purine nucleoside phosphorylase which is widely distributed in microorganisms. Their negative results are probably due to problems concerning detection or culture conditions. Therefore, specific and sensitive detecting procedures are exceedingly important.

For the enzymatic synthesis of bredinin when using a cell-free extract from *E. coli*, purine nucleoside phosphorylase might act as a catalyst. Although the aglycone is a imidazole, it is somewhat related to the purine precursor in its structure and would, therefore, be a sufficient substrate for purine nucleoside phosphorylase. In this synthetic reaction, bredinin-formation was approximately 50% that of the theoretical yield. This is thought to be due to the reversibility of the enzyme.

The optimum pH value of 6.0 for the synthesis coincides well with the initial pH value for cultivation in salvage synthesis by growing cells of *E. coli*.

Adenine played interesting roles in this study; as an inducer of enzyme formation and as an inhibitor of the synthetic reaction and the *de novo* fermentation of bredinin. Adenine is considered to serve as a competitive inhibitor in the inhibition of the synthetic reaction. The reason why the *de novo* fermentation is inhibited by adenine is not clear, since the biogenesis of bredinin is not yet known. However, salvage synthesis was not influenced by adenine, as seen in Table 7. This is probably due to the induction of the enzyme by adenine. For improved bredinin production, salvage fermentation using *Eupenicillium brefeldianum* is very useful.

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