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Studies on the Degradation of Pterine and Pterine-6-carboxylic Acid by *Pseudomonas fluorescens* UK-1

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Pseudomonas fluorescens UK-1 has been incubated in basal mineral medium which was 0.5 mM in pterine or pterine-6-carboxylic acid. During incubation the test organism splits the pteridine ring by liberating carbon dioxide from position 2. Glucose added to the medium greatly enhances both the growth of the organism and the carbon dioxide formation. Despite the structural similarities between pterine and pterine-6-carboxylic acid, only the degradation products derived from pterine are fluorescent in UV-light. Among the degradation products lumazine, pyrazine-2-carboxylic acid, and pyrazine-2-carboxamide have been identified. Also the activities of pterine deaminase and a carbon dioxide-liberating enzyme have been determined.

In a previous report we have shown that during incubation of *Pseudomonas fluorescens* UK-1 in a medium containing pterine-6-carboxylic acid, compounds as yet unidentified accumulate in the growth medium.[1] In preliminary experiments we have found that pterine-6-carboxylic acid and pterine (2-amino-4-hydroxypteridine) give rise to slightly different degradation products in the growth medium, despite their structural similarity. In this report we describe the nature and amounts of compounds accumulating in the growth medium when pterine--6-carboxylic acid and pterine are used as carbon and nitrogen sources. The effect of glucose on the degradation of these compounds and appearance of the degradation products has also been studied.

In addition to studying the degradation, we also determined the activities of two enzymes that are operative during the first stages of the degradation of the pteridines, namely a carbon dioxide-liberating enzyme and pterine deaminase. The studies of the last-mentioned enzyme are based on the findings of Levenberg and Hayaishi.[2] They showed that *Alcaligenes metalcaligenes*, which uses pterine-6-carboxylic acid as a source of carbon and nitrogen, possesses a pterine deaminase activity.

METHODS

Reagents. All chemicals used in experiments were guaranteed reagents from Sigma Chemical Co. or AnalaR reagents from The British Drug Houses Ltd., except folic acid-2-^{14}C which was a product of Radiochemical Centre, Amersham, England.

Culture conditions. Ps. fluorescens UK-1 was preserved and cultivated in 5 mM pantothenate medium as described earlier.[3] The main cultivation in degradation studies was carried out in a medium which was 0.5 mM in pterine-6-carboxylic acid or pterine, these providing the only source of carbon and nitrogen. The pH of the medium was 8.0 to facilitate the dissolving of pterine-6-carboxylic acid.

In the studies of enzyme activities we used, for purposes of comparison, a medium which was 10 mM in sodium glutamate.

Determination of degradation products. During the growth of the test organism, 5 ml samples were withdrawn and the cells centrifuged off. A 200 μl sample of clear supernatant was pipetted onto Whatman No. 3 MM paper and paper chromatograms were run in a 1:2 1% NH_3-propanol mixture as solvent.[4] After drying, the chromatograms were photographed in ultraviolet light with a Desaga MinUVIS lamp at a wave length of 254 nm. Spots visible in UV-light were cut off and eluted with 4 ml of 0.1 M potassium phosphate buffer, pH 8.0. The absorbances of the eluates were measured with a Unicam SP-800 spectrophotometer over the range 200 – 400 nm.

In preliminary experiments, we used glucose in concentrations of 0.1, 0.2, and 0.5 % to

determine its effect on the uptake and degradation of pteridines. In other experiments a concentration of 0.2 % was used.

For the study of CO_2 liberation from pterine and pterine-6-carboxylic acid by *Ps. fluorescens* UK-1, folic acid labelled with ^{14}C at position 2 (total activity 250 000 dpm) was added to 100 ml of culture medium. Inlet air was conducted to the culture vessel through three washbottles containing successively 5 N KOH, 1 N H_2SO_4, and distilled water. The liberated CO_2 was collected in 0.1 % $Ca(OH)_2$ solution, which was changed at intervals of 6 to 12 h. The precipitated calcium carbonate was filtered through a Millipore membrane filter and dried. The filter with precipitate was weighed and then counted in a Wallac NTL-314 liquid scintillation counter. Permablend III (Packard Instrument Company Inc.) was used as the scintillator in a concentration of 5.5 g to 1000 ml of toluene.

To study the uptake velocities of the pteridines used, the cells were grown for 25 – 30 h in basal medium supplemented with either pterine-6-carboxylic acid or pterine in a concentration of 0.5 mM. The washed cells (*ca.* 20 mg dry weight) were suspended in 10 ml of a reaction mixture which was similar to the growth medium but supplemented with folic acid-2-^{14}C, 2×10^5 dpm/ml. Specimens (1 ml) taken at intervals from 30 s to 40 min were cooled rapidly, filtered through a Millipore membrane filter, and washed twice with cold 2 % Na_2CO_3 solution. The filter with cells was dried and counted.

Determination of enzyme activities

(a) *Carbon dioxide-liberating enzyme. Ps. fluorescens* UK-1 was grown to the middle of the logarithmic phase in 100 ml of the pterine-glucose medium and the glutamate medium. The cells were centrifuged off, washed twice with cold 0.9 % NaCl solution, and ruptured in 2 ml of 0.05 M potassium phosphate buffer pH 8.0 using a MSE ultrasonic disintegrator 60 W, 18 000 kc, 1.5 A. The rupturing time was 7 min. The disintegrating vessel was kept in an ice bath during handling. After the disintegration the suspension was centrifuged and the protein content of the clear supernatant was determined by the sulfosalicylic acid method.[5] The enzymatic reaction was carried out in a Warburg apparatus at 30 °C. Two millilitres of reaction mixture was pipetted into the main cup. The reaction mixture was the same as the growth medium but supplemented with folic

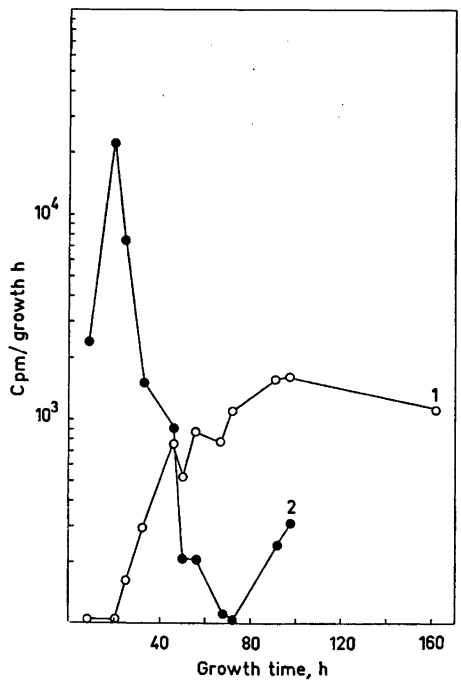

Fig. 1. CO_2 liberation during cultivation of *Ps. fluorescens* UK-1 in pterine medium. Curves: (1) without glucose; (2) with glucose.

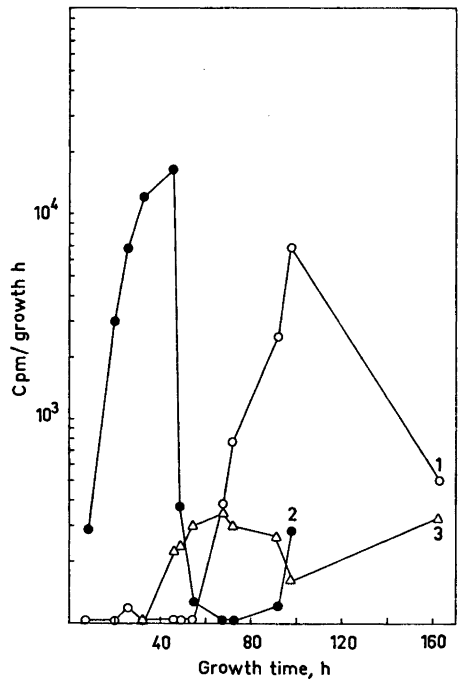

Fig. 2. CO_2 liberation during cultivation of *Ps. fluorescens* UK-1 in pterine-6-carboxylic acid medium. Curves: (1) without glucose; (2) with glucose; (3) CO_2 liberation due to spontaneous degradation.

acid-2-^{14}C, 2×10^5 dpm/ml. The side arm contained 0.5 ml of cell extract, and the middle well a filter paper strip which had been moistened with 0.5 M KOH. The reaction was started by pouring the cell extract into the main cup. The reaction time was 60 min. Thereafter the strip was removed, dried, and its radioactivity counted.

(b) *Pterine deaminase.* The cell extract for pterine deaminase activity determination was made as in the case of the carbon dioxide-liberating enzyme. The amount of ammonia which is liberated in the course of deaminase reaction at 30 °C was measured according to Conway's micro-diffusion technique.[6] Several attempts were made to purify the enzyme and to determine its activity spectrophotometrically according to Levenberg and Hayaishi,[2] but they were unsuccessful perhaps due to a very rapid inactivation of the enzyme.

RESULTS AND DISCUSSION

Carbon dioxide liberation from pterine and pterine-6-carboxylic acid during the growth of *Ps. fluorescens* UK-1 is shown in Figs. 1 and 2. The carbon dioxide originated from carbon atom 2 in the pteridine ring, which was labelled with ^{14}C in that position. Instead of labelled pterine or pterine-6-carboxylic acid we used folic acid-2-^{14}C, for it has earlier been shown that *Ps. fluorescens* UK-1 splits the bond between C-9 and N-10 in the folic acid molecule.[3] In addition, the amount of radioactive folic acid is so small (0.85 μg) that it could not serve as an effective carbon and nitrogen source. When pterine was used as the carbon and nitrogen source the $^{14}CO_2$ formation started after 20 h of incubation and reached its maximum at 95 h (Fig. 1). In the case of pterine-6-carboxylic acid the $^{14}CO_2$ formation started only after 55 h of incubation but reached its maximum about the same time as with pterine (Fig. 2). In both experiments only 50 % of the added activity was recovered as $^{14}CO_2$. About 10 % of the pteridine was degraded spontaneously, as can be seen in Fig. 2, curve 3.

When the culture medium contained 0.2 % glucose the carbon dioxide formation from pterine and pterine-6-carboxylic acid was greatly increased. With pterine in the growth medium, maximal carbon dioxide formation was noted after 20 h of growth, whereas with pterine-6-carboxylic acid the formation was at maximum at 45 h. In both cases almost the entire ^{14}C activity was liberated in the form of $^{14}CO_2$ during 70 h of growth.

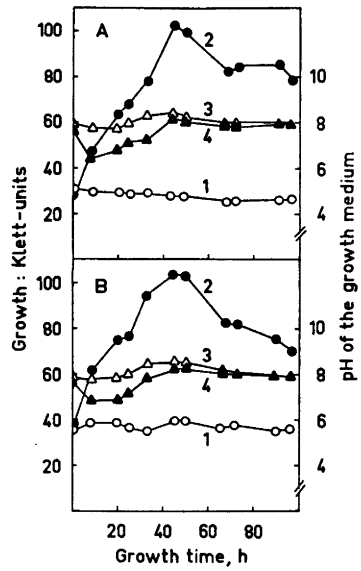

Fig. 3. Effect of glucose on growth rate and pH during cultivation of *Ps. fluorescens* UK-1. Part A: conditions in pterine medium. Part B: conditions in pterine-6-carboxylic acid medium. Curves: (1) growth without glucose; (2) growth with glucose; (3) changes in pH when the growth medium was lacking glucose; (4) changes in pH when the growth medium contained glucose.

When testing the uptake of pterine and pterine-6-carboxylic acid into cells we found that the maximal uptake velocities were reached after 30 sec and 2 min with pterine and pterine-6-carboxylic acid, respectively. The amounts of these compounds transported were about 50 % higher when glucose was present in the reaction mixture.

In the experiments described here glucose plays a remarkable role. As mentioned above, the degradation of the pteridine ring at position 2 is a quick and complete process when glucose is present in the growth medium. In the absence of glucose only one-half of the added pteridine is degraded. Obviously the transport of pterine or pterine-6-carboxylic acid into the cell is enhanced by the energy supplied by glucose. Glucose has a growth promoting effect, too, as can be seen in Fig. 3. When pterine or pterine-6-carboxylic acid was the only carbon and nitrogen source, no growth occurred. However, the cells survived, as is indicated by carbon dioxide formation (Figs. 1 and 2). In

Table 1. Some properties of compounds which can be detected chromatographically in the culture medium of *Ps. fluorescens* UK-1 during incubation. Pterine was the source of carbon and nitrogen. The medium did not contain glucose.

Compound	Appearance time, h	R_F-value	Colour	Absorption maxima, nm
(Pterine)		0.37	light blue	274, 340
I	20	0.30	yellow-green	255, 360
II	20	0.50	dark red-violet	269
III	20	0.78	dark red-violet	269
IV	44	0.27	blue-green	274, 328
V	15	0.17	blue-green	230, 255, 280, 335

Table 2. Activity of the carbon dioxide-liberating enzyme in cells grown in different media to the middle of the logarithmic phase.

Medium	Specific activity mU/mg of protein
Glutamate	1.2
Pterine-glucose	1.25×10^3

Table 3. Pterine deaminase activity of cells grown in glutamate medium to the late logarithmic phase. Specificity of pterine deaminase towards different substrates.

Substrate	Specific activity mU/mg of protein
Pterine-6-carboxylic acid	0.7
Pterine	2.9

the presence of glucose, the growth reaches its maximum within 50 h. From this we can conclude that *Ps. fluorescens* UK-1 easily utilizes the nitrogen of the pteridines, but the actual carbon source in these circumstances is glucose.

The pteridine ring is also degraded by *Ps. fluorescens* UK-1 without glucose and the degradation products accumulate in the medium. Five degradation products could be detected in chromatograms illuminated with UV-light when pterine was used as the carbon and nitrogen source. The appearance time from the beginning of the growth, the R_F-value, absorption maxima, and colour of different spots are presented in Table 1. The presence of glucose accelerates the disappearance of pterine from the culture medium. Also the number of spots of the degradation products is smaller since the degradation products are metabolized more rapidly in the presence than in the absence of glucose.

By using several standards and identification tables for substituted pteridines, and by comparing R_F-values and colours of the spots, we

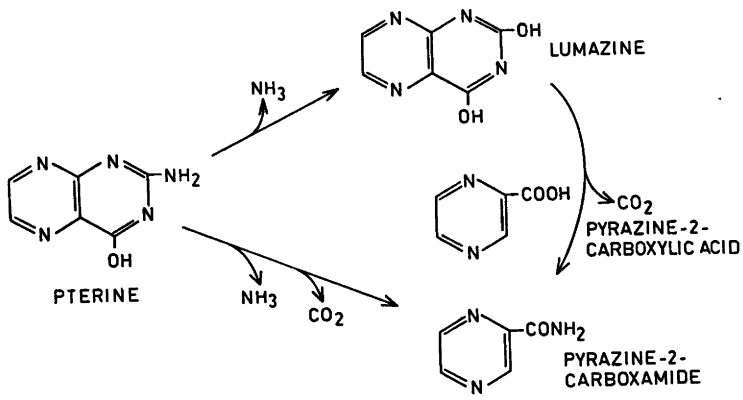

Fig. 4. Hypothetical model of the first stages during the degradation of pterine by *Ps. fluorescens* UK-1.

have identified three compounds which originate from pterine during the incubation of *Ps. fluorescens* UK-1. Compounds I, II, and III are lumazine, pyrazine-2-carboxylic acid, and pyrazine-2-carboxamide, respectively. Compounds IV and V are still unidentified, but there is strong evidence that they are hydroxylated lumazines.

Lumazine, pyrazine-2-carboxylic acid, and pyrazine-2-carboxamide appear in the growth medium after 20 h from the beginning of the incubation. The formation of pyrazine-2-carboxylic acid and pyrazine-2-carboxamide is in good agreement with the liberation of carbon dioxide, the formation of which also starts after 20 h from the beginning of the incubation (Fig. 1). The formation of lumazine, which is the deamination product of pterine, indicates the presence of pterine deaminase.

On the basis of the results described above it can be concluded that the first stages in the catabolism of pterine in *Ps. fluorescens* UK-1 occur as shown in Fig. 4. By the action of pterine deaminase, lumazine is formed which, catalyzed by the carbon dioxide-liberating enzyme, forms pyrazine-2-carboxylic acid and pyrazine-2-carboxamide. The last-mentioned compounds can also be formed directly from pterine. It must be pointed out that the mechanism is presented here only as a hypothesis. In further studies we will attempt to explain the mechanism in more detail.

To determine the activity of the carbon dioxide-liberating enzyme we cultivated *Ps. fluorescens* UK-1 in pterine-glucose medium and, for comparison, in glutamate medium. The cells were harvested in the middle of the logarithmic phase (after 20 h of growth), treated as described above, and their activity was measured. The results are presented in Table 2. The enzyme activity in cells grown in pterine-glucose medium was about 1000 times greater than in cells grown in glutamate medium, which possibly indicates that this enzyme is inducible.

For the determination of pterine deaminase activity we cultivated *Ps. fluorescens* UK-1 in glutamate medium to the late logarithmic phase (cells grew 40 h) and treated it as described above. In activity determinations we used both pterine-6-carboxylic acid and pterine as substrates. The pterine deaminase activity in the presence of these substrates is presented in Table 3. With pterine, the activity was about four times as great as with pterine-6-carboxylic acid. According to Levenberg and Hayaishi,[2] the pterine deaminase activity in *Alcaligenes metalcaligenes* in sonic extract is 0.1 mU/mg protein when pterine-6-carboxylic acid is the substrate. However, their measurements were made at 23 °C.

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