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A Qualitative Demonstration of the Degradation of Folic Acid by *Pseudomonas fluorescens* UK-1

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The degradation of folic acid *in vitro* has been studied by using a cell free extract obtained from *Pseudomonas fluorescens* UK-1 after ultrasonic treatment and streptomycin sulphate precipitation. The degradation products have been separated chromatographically. With folic acid as the substrate *p*-aminobenzoylglutamic acid and a substituted pteridine were formed. The degradation increased as a function of time and protein concentration. A weak spontaneous degradation occurred without cell extract. The pteridine moiety formed in the reaction was eluted from paper after chromatographic run and examined both spectrophotometrically and fluorometrically. On the basis of these studies it is obvious that pterin-6-carboxylic acid is formed during incubation. When *p*-aminobenzoyl-L-glutamic acid was the substrate the appearance of *p*-aminobenzoic acid was noted. However, the degradation of *p*-aminobenzoyl-L-glutamic acid was slower than that of folic acid.

It has earlier been shown[1,2] that some pseudomonads isolated from soil are capable of degrading methotrexate and folate to 4-{*N*[(2,4-diamino-6-pteridyl)methyl]-*N*-methylamino} benzoic acid and pteroic acid, respectively. The enzyme carboxypeptidase G_1 which catalyzes the degradation has been isolated and purified 1050-fold.[2] We have found that still another splitting of the folic acid molecule occurs, resulting in a substituted pteridine, *p*-aminobenzoylglutamic acid, and *p*-aminobenzoic acid.* In our studies we have used *Pseudomonas fluorescens* strain UK-1, which was isolated from sea water in Hietalahti harbour, Helsinki.

EXPERIMENTAL

Reagents. Folic acid and PABA were purchased from British Drug Houses Ltd., and PABGA, pterin, and pterin-6-carboxylic acid from Sigma Chemical Co.

Culture conditions. Ps. fluorescens UK-1 was grown in a basal mineral medium containing (per litre) 20 mmol KH_2PO_4, 1 mmol $MgSO_4.7H_2O$ and 10 μmol $FeSO_4.7H_2O$.

* In this publication the following abbreviations are used: PABA=*p*-aminobenzoic acid; PABGA=*p*-aminobenzoyl-L-glutamic acid.

The medium contained 10 mmol of folic acid as a source of carbon and nitrogen and the pH was adjusted to 6.8. The precultivation of the organism was carried out in glutamate (20 mM) or pantothenate (5 mM) media. The culture was aerated during several days with an aquarium pump and incubated at 30°.

Preparation of cell extract and determination of degradation products. The cells were preserved on an agar slant whose salt composition was the same as above. It contained 5 mmol of pantothenate per litre and 1.5 % agar. The culture was kept at 2°C. From the agar slant the cells were transferred aseptically to 200 ml of sterile pantothenate medium in which they grew for 24 h under aeration at 30°C. The whole culture was poured into 500 ml of the same medium where it continued to grow for 12 h in similar conditions. The cells were separated by centrifugation (4000 *g*, 10 min), washed with cold saline, and transferred to 2000 – 5000 ml of medium which was 10 mM in folic acid. In this final medium the culture grew for 4 – 7 days at 30° under aeration. During this time the culture medium became yellow and turbid due to the precipitated pteroic acid.[1] The cells, together with the precipitate, were separated by centrifugation (4000 *g*, 15 min). A 2 % Na_2CO_3 solution was added in order to dissolve the yellow material, 100 ml per litre of medium being required. The remaining cells were washed with cold saline and ruptured for 15 min in 15 ml of 0.05 M TRIS–HCl buffer, pH 7.3, using the Raytheon Sonic Oscillator 10 kc, 250 W. The suspension was centrifuged at 17 300 *g* for 30 min, after which was added 0.5 ml of 5 % streptomycin sulphate solution per 3 ml of clear supernatant. After standing for 30 min, the precipitate was centrifuged at 17 300 *g* for 30 min and discarded. The protein content of the clear supernatant was estimated with sulphosalicylic acid,[3] employing crystalline bovine serum albumin as standard.

The reaction mixture contained 10 mg of folic acid or PABGA in 10 ml of 0.05 M TRIS–HCl buffer, pH 7.3. The reaction, which took place at 30°, was started by adding the enzyme preparation and stopped by putting the reaction tube into an ice bath. 200 µl of reaction mixture was pipetted onto a Whatman No. 3 paper. The spots were dried in an air stream and the chromatogram was run using the ascending technique. The solvent used was a 1:2 1 % NH_3 – 1-propanol mixture.[4]

After drying, the chromatograms were photographed in ultraviolet light employing a Desaga MinUVIS lamp at a wave lenght of 254 nm. Parallel chromatograms were diazo-stained.[5]

The spots that exhibited a blue fluorescence at 254 nm and whose R_F-value was 0.2 were cut off and eluted with 0.1 N NaOH or 0.1 M KH_2PO_4-KOH buffer, pH 8, for determination of their spectra spectrophotometrically or fluorometrically, respectively.

RESULTS AND DISCUSSION

Fig. 1 shows the degradation of folic acid as a function of time and enzyme preparation concentration. It can be seen that the amount of folic acid (dark spots) decreases when the enzyme concentration increases. The decrease has reached a constant level after 30 min when the protein concentration is 0.9 mg/10 ml. The decrease of folic acid is slower when lowest protein concentration (0.225 mg/10 ml) is used. In every reaction spot in Fig. 1, a white spot above the dark spot can be seen (in the original chromatogram the spot, in UV-light, is light blue). We believe that this spot is due to a substituted pteridine moiety (designated as Compound P) derived from the folic acid molecule. The spots cannot be seen in reaction mixtures that lack the protein (blank). PABGA is represented in the uppermost light spots, which in the original chromatogram in the UV-light appear as red-violet. Using pure PABGA and 1 % NH_3 – 1-propanol 1:2 as solvent, we obtained an R_F-value of 0.43 for PABGA. It is obvious that, during the reaction, the PABGA moiety is split off by the action of the enzyme preparation. As can be seen from the blank spots, a weak spontaneous splitting increasing as a function of time also occurs. Commercial folic acid always contains a little PABGA.

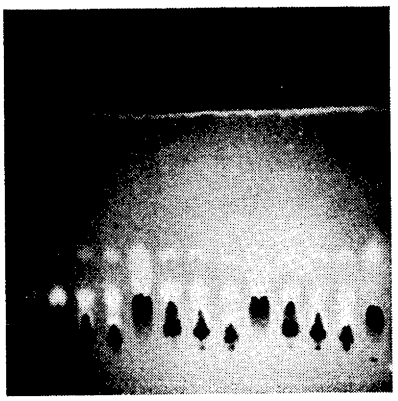

Fig. 1. Degradation of folic acid with time and protein concentration. Reaction times in spots 1–4, 5–8, and 9–12 were 30, 60, and 90 min, respectively. Protein concentration in spots 2, 6, and 10 was 0.09 mg/ml; in spots 3, 7, and 11, 0.045 mg/ml; and in 4, 8, and 12, 0.023 mg/ml. No protein was used in 1, 5, and 9. Each spot contained 200 µl of reaction mixture. As a reference, spot 13 is folic acid. The chromatogram was illuminated with UV-light.

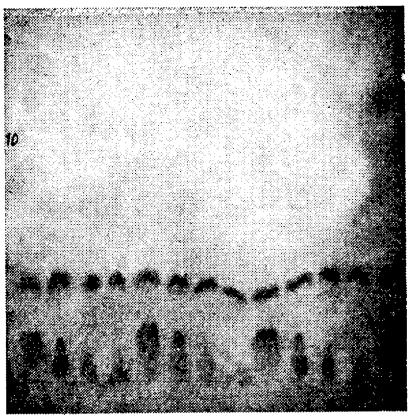

Fig. 2. Conditions are as in Fig. 1 except that the chromatogram has been diazo-stained.

The formation of PABGA during the course of the reaction is shown in Fig. 2, which represents a diazo-stained chromatogram. The strenght of the colour increases during 60 min as the protein concentration increases. The weakest spots belong to the blank mixtures and the pure standard.

The degradation of PABGA was also studied, with results shown in Figs. 3 and 4. The splitting of PABGA to PABA and glutamic acid increases as a function of time and a weak spontaneous splitting during the incubation occurs here, too. In the photographs, the lower dark spots represent PABGA and the spots above them PABA (in the original chromatogram the spots are dark red). For pure PABA we obtained an R_F-value of 0.56. The weak spots at the top of the chromatogram were originally greenish-brown and they probably represent non-diazotizable compounds.

In chromatographic runs we obtained an R_F-value of 0.2 both for Compound P and for a pure pterin-6-carboxylic acid standard. However, when the chromatograms, after drying, were run a second time for a better separation, the spot of Compound P moved faster than that of the pterin-6-carboxylic acid standard.

In preliminary spectrophotometric and fluorometric studies, Compound P behaved in a manner like pterin-6-carboxylic acid (Figs. 5–8). In Figs. 5 and 6 the absorption maxima for Compound P are 365 nm and 260 nm and those for pterin-6-carboxylic acid, 366 nm and 263 nm (lit.[6] 365 nm and 262

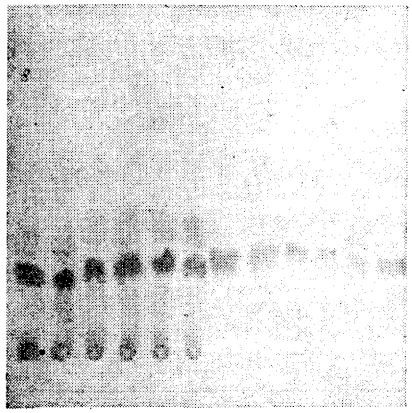

Fig. 3. Degradation of *p*-aminobenzoylglutamic acid with time by the action of cell protein. The reaction mixture contained 5 mg/10 ml of protein. 200 µl specimens were taken from the reaction mixture at intervals of 30 min and applied onto paper. Spot 1 represents the specimen taken at the beginning of reaction, whereas spots 7 – 12 represent those taken at intervals of 30 min from a reaction mixture that did not contain protein.

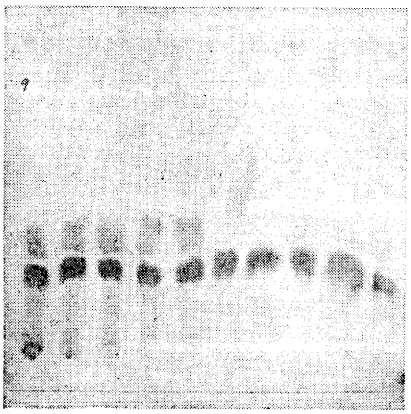

Fig. 4. Continuation of Fig. 3. Spots 1 – 5 represent specimens taken from the reaction mixture between 3 and 5 h after the reaction was initiated. Spots 6 – 10 are specimens taken from a reaction mixture not containing protein.

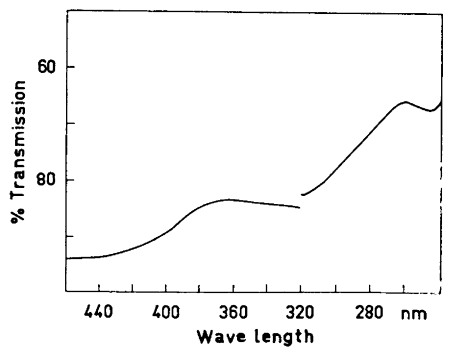

Fig. 5. Absorption spectrum of Compound P.

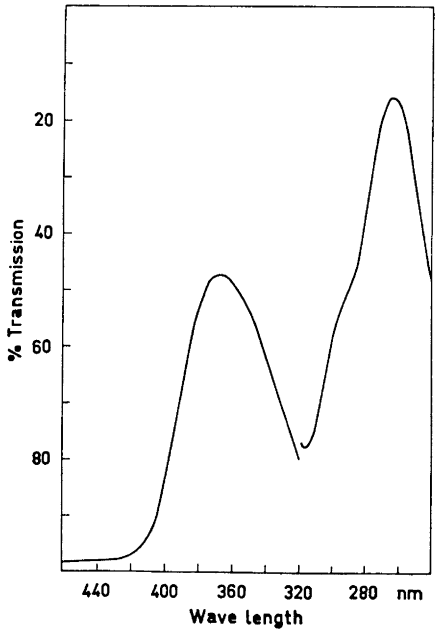

Fig. 6. Absorption spectrum of pterin-6-carboxylic acid.

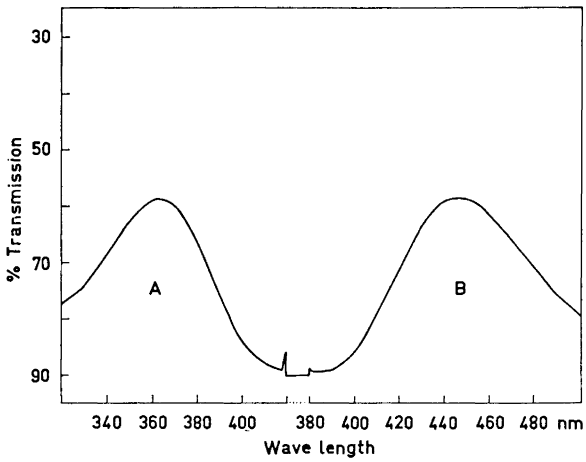

Fig. 7. Excitation spectrum A and fluorescence spectrum B of Compound P.

nm). For pure pterin we got values of 363 nm and 252 nm which agree with the literature values.[6] A third compound that could represent Compound P is 6-methylpterin. It was not available, but the literature values for it at pH 13 are 365 nm and 253 nm.[7]

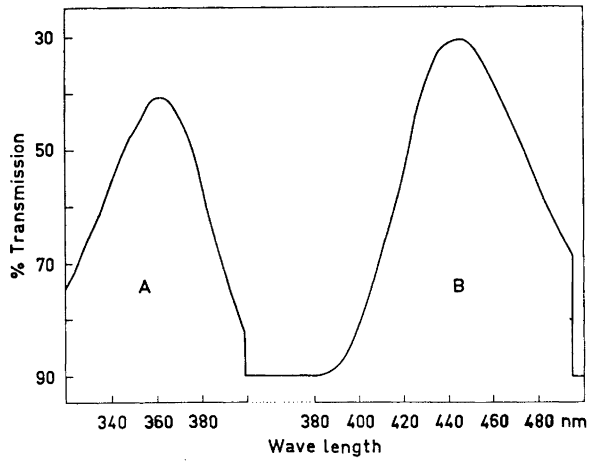

Fig. 8. Excitation spectrum A and fluorescence spectrum B of pterin-6-carboxylic acid.

Figs. 7 and 8 show the results of fluorometric measurements. Both Compound P and pterin-6-carboxylic acid exhibited the same excitation and fluorescence maxima, 363 nm and 447 nm. The literature values are 360 nm and 450 nm.[8]

The appearance of Compound P was noted only when *Ps. fluorescens* UK-1 had grown in the presence of folic acid. When glutamate or pantothenate were the only sources of carbon and nitrogen, Compound P did not form in the reaction mixture. We intend to investigate the nature of Compound P in future studies.

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