Deamination of 4-Aminopyrimidine Nucleosides by Extracts of Rye Grass (*Lolium perenne*)[1]

David M. Frisch and M. Arthur Charles

Department of Chemistry, California State College at Los Angeles

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Summary. A deaminase specific for 4-aminopyrimidine nucleosides has been found in rye grass, *Lolium perenne*. The pH optimum, temperature stability, Km values and specificity was determined.

A new and more accurate method for following the course of deamination is reported. This method depends on the shift in maximum absorbance wavelength of the mixture containing the 4-aminopyrimidine nucleoside and its deaminated product.

Enzymatic deamination of naturally occurring 4-aminopyrimidine nucleosides has been reported in a wide variety of organisms. Detailed studies of such enzymes have been made with preparations from mouse kidney (5), *Tetrahymena pyriformis* (13) and *Escherichia coli* (3). In the higher plants this enzyme has been reported in the growing anthers of *Lilium longiflorum* (8), and a brief abstract has appeared indicating the presence of this enzyme in barley seed (9). This report describes distribution and properties of a 4-aminopyrimidine nucleoside deaminase present in rye grass, *Lolium perenne*. A comparable deaminase for the purine nucleosides, the purine and pyrimidine bases and their corresponding nucleotides was not found.

An improved method for following the course of deamination is also described.

Materials and Methods

Growth Conditions. Rye grass seeds were germinated for 6 to 7 days at room temperature on gauze stretched over petri dishes filled with distilled water. This technique afforded a simple means of separating the blades and roots.

Enzyme Extraction. Preparations were made from blades, roots, seeds or whole seedlings. The material was homogenized in a Waring blendor with 0.6 M Tris-maleate buffer (pH 7.6) containing 0.01 M EDTA. All operations for the preparation of the enzyme were carried out at 5°. The homogenate was filtered through gauze and the filtrate centrifuged at 17,000 × *g* for 1 hour. Lipid material remaining on the surface after centrifugation was removed by straining through glass wool. When $(NH_4)_2SO_4$ fractions were collected, the suspensions were kept at pH 7.6 by the addition of small amounts of solid Tris. The protein fractions were dissolved in a small amount of the Tris-maleate buffer and dialyzed against 5 liters of distilled water for 15 hours. The enzymatic activity of these preparations was stable for several weeks when stored at −10°.

Protein Determination. Protein was determined by the method of Lowry et al. (10).

Ammonia Determination. Ammonia was determined colorimetrically after micro-diffusion (4) from incubation mixtures using Nessler's reagent prepared by the method of Bock and Benedict (7).

Chromatography. All paper chromatograms were developed by the descending method using Whatman No. 1 paper.

Deaminase Assay. The usual reaction mixture consisted of 6.11 μmoles of the appropriate substrate, 0.14 mmole of Tris-maleate buffer (pH 8.0) and 20 mg of protein, in a final volme of 1.60 ml. When a nucleotide was utilized as the substrate, NaF to a final concentration of 0.1 M was included in the reaction mixture to inhibit a potent phosphatase present in the extract. After incubation at 30° for zero to 40 minutes, a 0.2 ml aliquot was removed and added to an equal volume of cold 10 % (v/v) perchloric acid to terminate the reaction and precipitate the protein. After centrifugation a 0.2 ml aliquot of the supernatant fraction was added to 4.8 ml of 0.1 M HCl and its UV spectrum recorded by means of a Cary Model 14 spectrophotometer.

The percent deamination was determined by comparing the shift in the recorded UV wavelength maximum (λ max) of the perchloric acid supernatant against a standard curve (fig 1). The latter was obtained by mixing known concentrations of the 4-aminopyrimidine nucleoside and its deaminated prod-

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[2] According to the Chemical Abstracts numbering system.

[3] The following abbreviations have been used: CR: cytidine; CdR: 2′-deoxy-cytidine; MCdR: 5-methyl-2′-deoxycytidine; UR: uridine; UdR: 2′-deoxyuridine; and TdR: 2′-deoxythymidine.

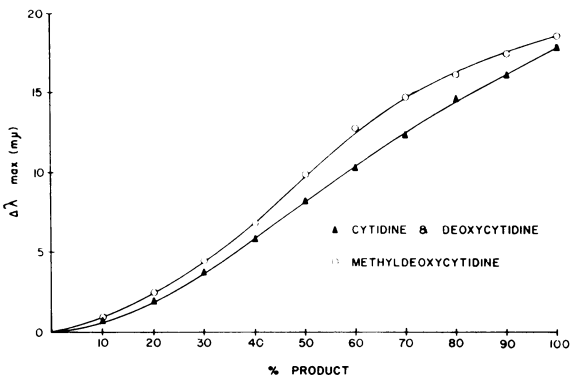

FIG. 1. Standard curves representing shift of the absorption peak in mμ of mixtures of 4-aminopyrimidine nucleoside and its deaminated product.

uct, e.g. a given tube might contain 10 mole percent cytidine and 90 mole percent uridine. Each tube would therefore represent a specific percentage of deamination. When the λ max of the 4-aminopyrimidine nucleoside was compared to the λ max of its deaminated product, a difference of 17 mμ was obtained between cytidine and uridine since the λ max of cytidine is 279.5 mμ and of uridine is 262.5 mμ at pH 1. The same difference was obtained for deoxycytidine and deoxyuridine. In the case of 5-methyldeoxytidine and thymidine, the difference in UV maximum was 19 mμ. Therefore, the shift in the recorded λ max was used as a measure of the percent deamination that occurred. This method has an advantage over measuring the decrease in absorbance at some particular wavelength (12) because in the latter method small transfering errors results in a change of absorbance hence an error in determining the percent deamination. When the λ max method described above is utilized, the volume of solution used does not affect the wavelength at which the maximum absorption occurs so there is less chance of error in determining the percent deamination.

Materials. All substrates were purchased from the California Corporation for Biochemical Research. Rye grass seed was obtained from local commercial sources.

Results

Activity Distribution. The highest deaminase activity was found in the 25 to 55 % saturated $(NH_4)_2SO_4$ fraction. No activity was found below 25 % saturation. The 55 to 85 % fraction was only one-fifth as active as the 25 to 55 % fraction. On the basis of the amount of deamination per mg of protein, the root extract was 5 times as active as the blades and 9 times as active as the seeds.

Substrate Specificity. The enzyme preparation from the whole seedlings, roots and blades, was specific for deamination of the 4-aminopyrimidine nucleosides, cytidine, deoxycytidine and 5-methyldeoxycytidine. No deaminase activity was observed when the following pyrimidine bases or nucleotides were tested: cytosine, 5-methylcytosine, cytidine-5-P, cytidine -2′(3′)-P, 2′-deoxycytidine-5′-P and 5-methyl-2′deoxycytidine-5′-P. Also, no deamination occurred with any purine substrate tested. These included adenine, guanine, adenosine, guanosine, 2′-deoxyadenosine, 2′-deoxyguanosine, adenosine-5′-P, adenosine-2′(3′)-P, 2′-deoxyadenosine-5′-P, guanosine-5′-P and 2′-deoxyguanosine-5′-P.

Stoichiometry and Reaction Products. Paper chromatography of the incubated mixture, after addition of perchloric acid to precipitate proteins and neutralization with KOH to remove the excess perchloric acid, indicated that one of the reaction products was the deaminated nucleoside of the corresponding 4-aminopyrimidine nucleoside (table I).

Table I. *R_f Values Demonstrating the Presence of the Deaminated Nucleoside*

Nucleoside	*n*-Butanol : water (86 : 14)	*n*-Butanol : water : formic acid (77 : 13 : 10)
CR	0.08	0.06
UR	0.16	0.16
Reaction product	...	0.17
CdR	0.19	0.19
UdR	0.30	0.36
Reaction product	0.30	0.33
MCdR	0.23	0.25
TdR	0.42	0.46
Reaction product	0.44	0.49

Table II. *Stoichiometry of Pyrimidine Nucleoside Deaminase Reaction*

Substrate	Time of incubation (min)	μmoles deaminated substrate (UV method)	μmoles NH_3 formed (micro-diffusion)
CR	70	5.4	5.6
CR	100	6.7	6.3
CdR	70	5.5	4.2
CdR	100	5.4	5.6
MCdR	70	5.3	5.3
MCdR	100	6.5	6.8

The μmoles of ammonia released were equal to the μmoles of deaminated product formed as determined by the shift in max (table II).

Dependence of Deamination on pH. The enzyme has a broad activity peak from pH 7–10 using Tris-maleate or phosphate buffer (fig 2).

Heat Stabilitity. The enzyme preparation exhibited a high degree of stability. Heating up to 70° for 20 minutes caused practically no loss in deaminase activity (fig 3).

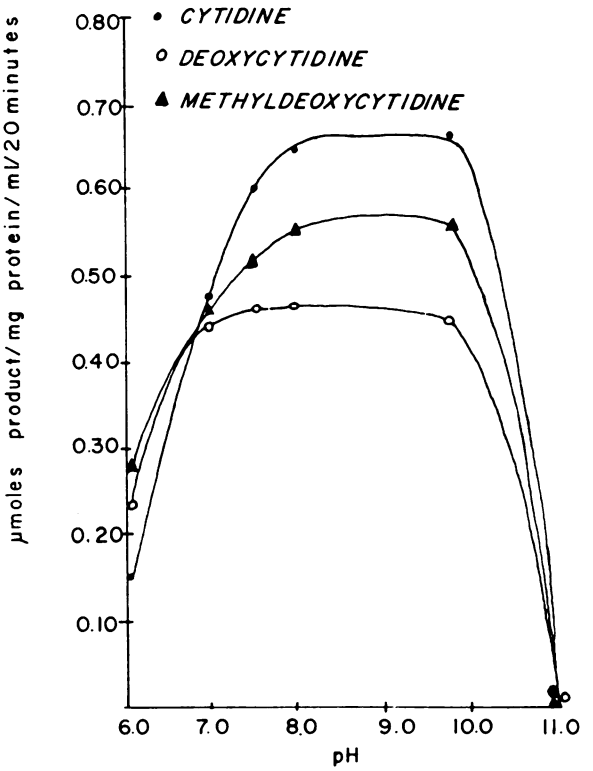

FIG. 2. pH Dependence of 4-aminopyrimidine nucleoside deaminase.

Determination of Km, Effect of Enzyme Concentration. A graphic determination of Km of the reaction with CR, CdR and MCdR was made. The Km values were 2.6×10^{-3} M, 2.6×10^{-3} M and 3.0×10^{-3} M, respectively for the 3 nucleoside substrates, using the 25 % to 55 % ammonium sulfate fraction obtained from the root proteins (fig 4). These determinations were made as described in the deaminase assay. The deamination of the 4-aminopyrimidine nucleoside was linear for at least 30 minutes.

Discussion

The degree of purification of the enzymic preparation from rye grass does not permit any definite conclusions as to whether one or more deaminases are present in the extract. However, the similarity in the rates of deamination and Km values of CR, CdR and MCdR suggests that only 1 enzyme is present. A similar pyrimidine nucleoside deaminase has been reported in the higher plants, barley (9) and *Lilium* (8) but no mention was made of the presence or absence of pyrimedine or purine nucleotide or free base deaminases. It would be of interest to examine other higher plants to determine if the pyrimidine nucleoside deaminase is ubiquitous to the higher plant kingdom with the exclusion of deaminases for purine nucleosides, purine and pyrimidine nucleotides or the free pyrimidine and purine bases.

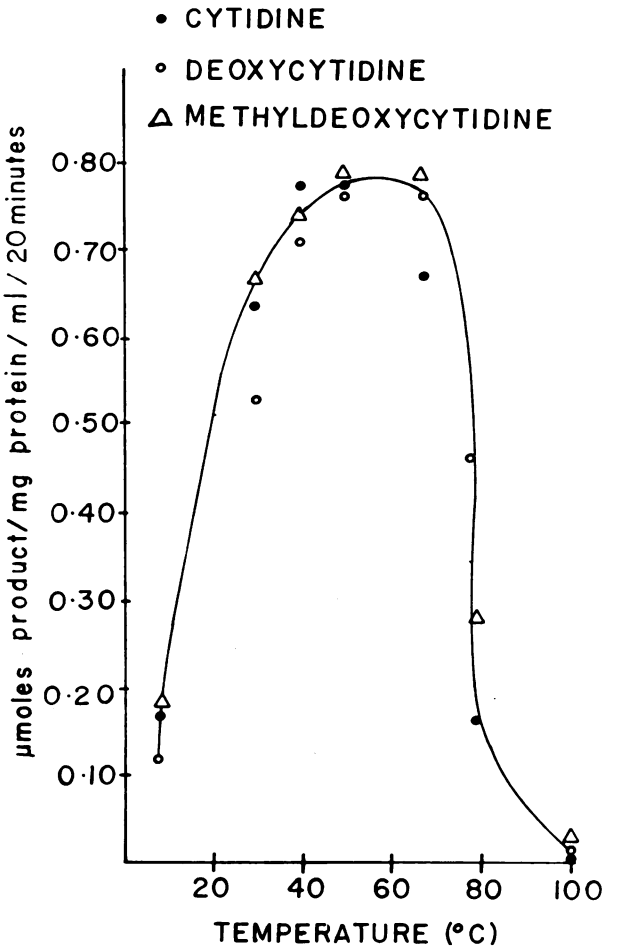

FIG. 3. Heat stability of 4-aminopyrimidine nucleoside deaminase (see text).

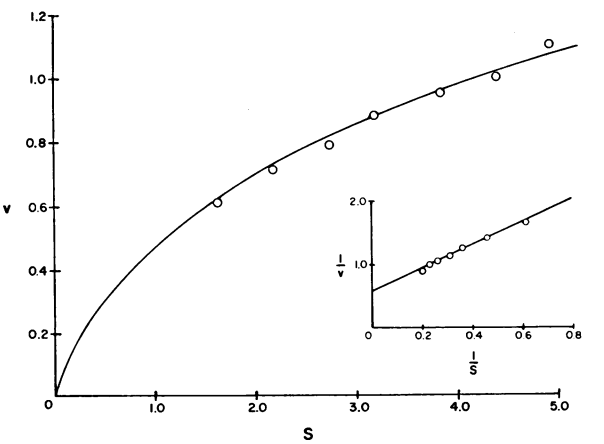

FIG. 4. Determination of Km, effect of enzyme concentration. Velocity (v) represents the μmoles of deaminated product formed in 20 minutes. S is the substrate concentration $\times 10^3$ (M).

At present no biological function can be assigned to the 4-aminopyrimidine deaminase. Holta and Stern (8) could find no effect of this enzyme on DNA synthesis. However, the corresponding pyrimidine nucleotide deaminases (11) exert control over the relative amounts of the pyrimidine deoxynucleotides needed for DNA formation. It is also of interest that sea urchin eggs, which contain the pyrimidine nucleotide deaminase, show little phosphatase activity (11), whereas rye grass with its pyrimidine nucleoside deaminase contains active phosphatase. Whether this deaminase acts as a salvage reaction or exerts a more profound effect on the control of nucleic acid metabolism remains to be investigated.

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