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Baker's Yeast Cytosine Deaminase. Some Enzymic Properties and Allosteric Inhibition by Nucleosides and Nucleotides*

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ABSTRACT: Enzymological studies on partially purified preparations of baker's yeast cytosine deaminase are described. The enzyme has a molecular weight of about 34,000, shows a strict specificity for cytosine and 5-methylcytosine (K_m = 2.5 mM for both substrates), and is inhibited by a number of nucleosides and nucleotides, including cytidine, CMP, CDP, CTP, thymidine, TTP, guanosine, GMP, GDP, and GTP. The concentrations required for 50% inhibition range between 0.385 mM for GMP and 0.690 mM for thymidine. Plots of kinetic data for most of the inhibitors take the form of sigmoidal inhibition curves; however, first-order kinetics are observed in substrate saturation curves, suggesting the absence of interaction between substrate binding sites. The inhibition is markedly dependent on hydrogen ion concentration: thus the per cent inhibition, which is maximal below pH 6, decreases to a minimum near pH 7. The inhibition data, together with other observations showing that crude yeast extracts catalyze the biosynthesis of UMP in the presence of cytosine and 5-phosphoribosyl 1-pryophosphate, suggest an anabolic, rather than catabolic role of yeast cytosine deaminase. It is postulated that the enzyme is involved in a "salvage pathway" for pyrimidine nucleotide biosynthesis in yeast, leading to the formation of UMP from cytosine, *via* the combined action of cytosine deaminase and UMP pyrophosphorylase.

Cytosine deaminase (cytosine aminohydrolase, EC 3.5.4.1) was originally found by Hahn and Schäfer (1925) in yeast and *Escherichia coli*, and has since been identified in other microorganisms (see O'Donovan and Neuhard, 1970, for review).

In yeast, the hydrolytic deamination of cytosine and 5-methylcytosine, catalyzed by a fraction precipitated with ammonium sulfate from crude extracts, has been described by Kream and Chargaff (1952). The properties of the enzyme, however, have never been reported in purified systems. Yeast cytosine deaminase appears to be distinct from cytidine deaminase, since after gel filtration on G-100 Sephadex, two separate peaks are obtained for the two enzyme activities (Ipata *et al.*, 1970).

The present paper describes a procedure for the purification of cytosine deaminase from yeast plasmolysates. The final preparation shows a strict specificity for cytosine and 5-methylcytosine.

The kinetic data show that the enzyme is allosterically inhibited by a number of nucleosides and nucleotides, when tested with cytosine or 5-methylcytosine as substrate.

Experimental Section

Materials. Purine and pyrimidine bases, nucleosides, and nucleotides were obtained from Sigma Chemical Co. Whale skeletal myoglobin was obtained from Seravac Laboratories. Pancreatic ribonuclease was obtained from Sigma Chemical Co. Adenosine deaminase from calf intestinal mucosa was obtained from Boehringer and Soehne. Tris (Sigma) was used as a buffer. Other chemicals were of reagent grade or of the highest quality available.

Cytosine Deaminase Assay Procedure. Cytosine deaminase was assayed according to Ipata *et al.* (1970), by a spectrophotometric method based on the differential absorption of cytosine and uracil and of 5-methylcytosine and thymine

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TABLE I: Purification of Cytosine Deaminase from Baker's Yeast.

Fraction	Volume (ml)	Protein (mg)	Specific activity (units/mg of protein)[a]	% recov of total activity
1	4230	36,400	9.2	100
2	154	12,370	14.3	53
3	183	311	233	21
4	102	128	494	19
5	102	23.7	1973	14
6	111	11.4	3128	11

[a] One unit equals an activity equivalent to 1 mμmole of cytosine deaminated per min per mg of protein.

at 286 mμ where the molar differential extinction coefficients are 937 and 1520, respectively. The assay was conducted in microcuvets with 1-cm light path and was monitored at 286 mμ with a Beckman DB absorbance recording spectrophotometer at 27°. The assay mixture contained, in a final volume of 2.2 ml, 0.03 M Tris-Cl buffer (pH 7.0), different amounts of enzyme preparation, and substrate solution; the reaction was started by addition of the enzyme preparation, and the decrease in optical density at 286 mμ was recorded against a reference cuvet in which substrate was substituted by water. The velocity of the reaction was strictly proportional to the amount of cytosine deaminase up to rates higher than 0.150 absorbance unit/min. All rate studies reported here have been conducted at rates of less than 0.080 absorbance unit/min. The molarities of all substrate and inhibitor solutions were measured spectrophotometrically

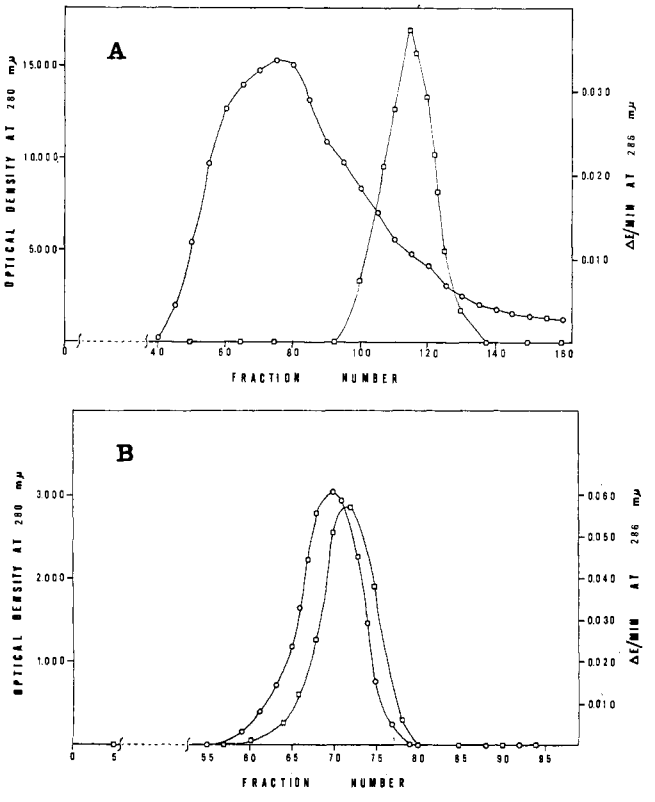


FIGURE 1: Elution profile on G-100 Sephadex columns for fraction 2 (A) and fraction 3 (B). (—O—O—O—O—) Optical density at 280 mμ and (—□—□—□—□—) cytidine deaminase activity. Enzyme activity was assayed as described under Experimental Section, and is expressed as Δ absorbance per minute at 286 mμ. Each fraction (200 μl) was used in the assay.

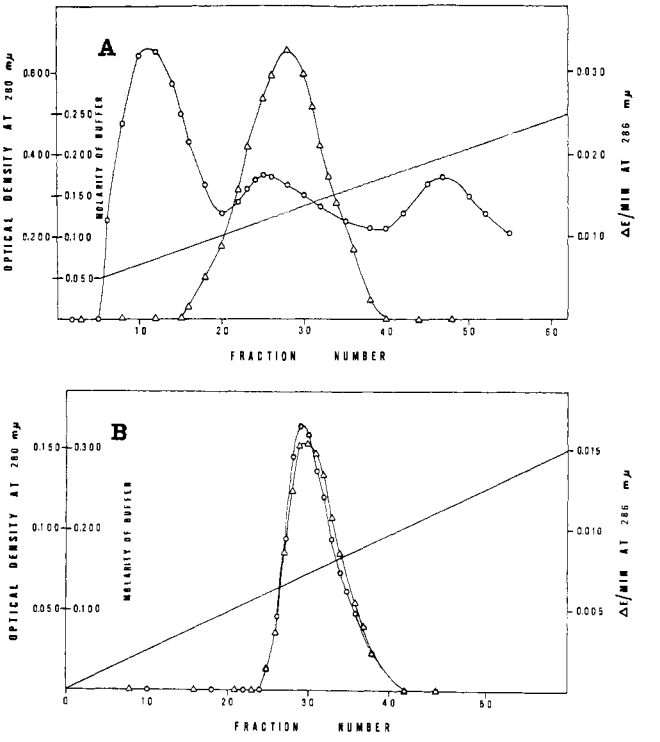


FIGURE 2: Elution profiles on DEAE-cellulose for fraction 4 (A) and fraction 5 (B). (—O—O—O—O—) Optical density at 280 mμ and (—Δ—Δ—Δ—) cytosine deaminase activity. Enzyme activity was assayed and expressed as in Figure 1.

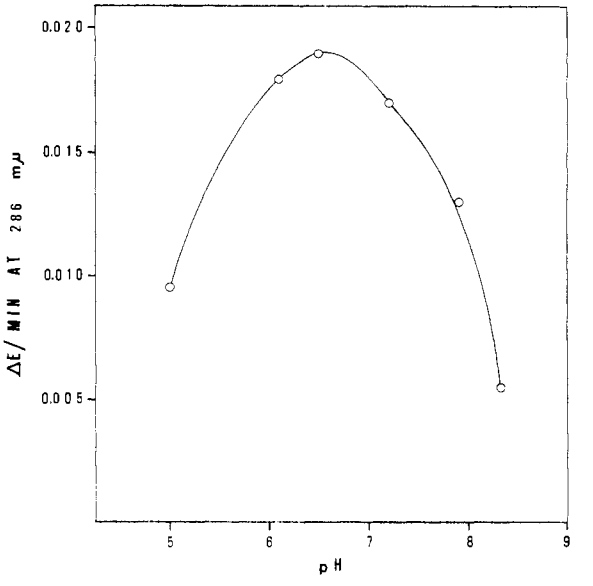


FIGURE 3: Effect of hydrogen ion concentration on initial velocity of baker's yeast cytosine deaminase. The velocity is expressed as in Figure 1.

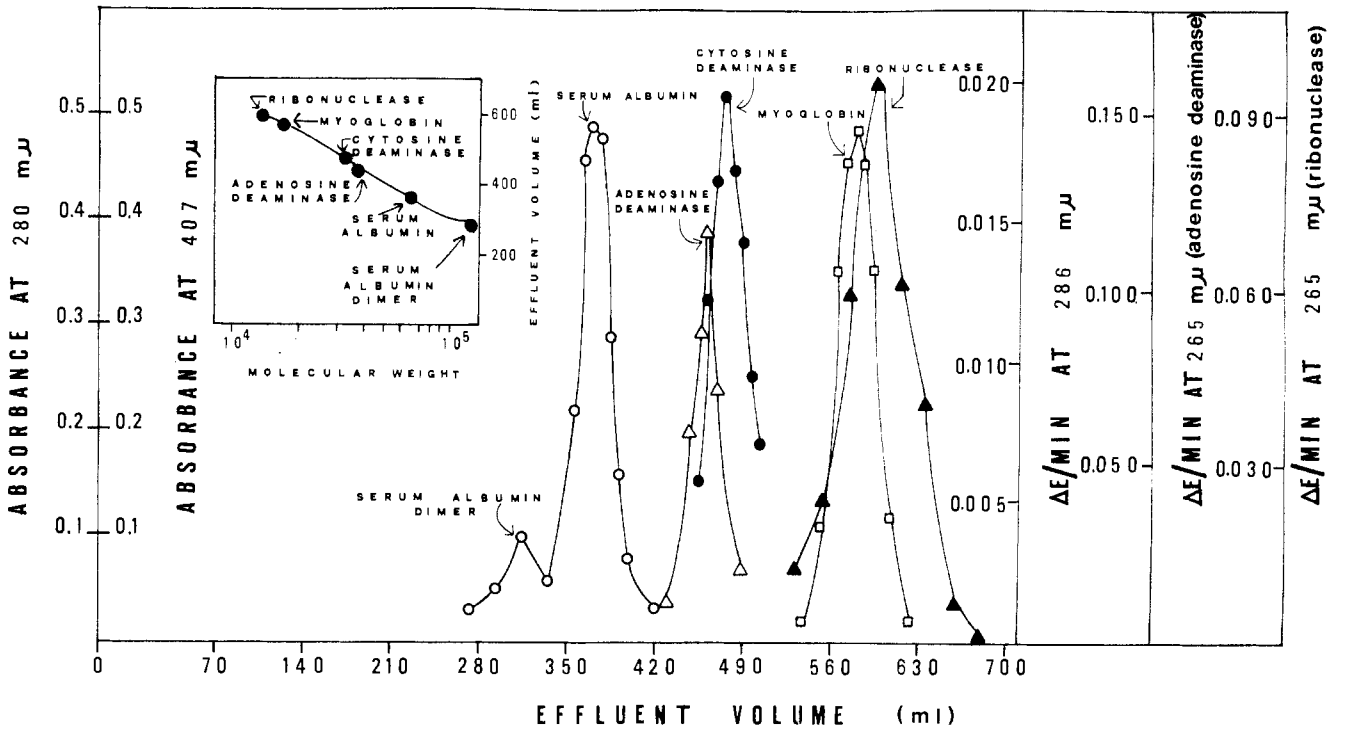


FIGURE 4: Elution pattern of cytosine deaminase (-●-●-●-) serum albumin (-O-O-O-), adenosine deaminase (-Δ-Δ-Δ-), myoglobin (-□-□-□-) and pancreatic ribonuclease (-▲-▲-▲-). Elution volume for myoglobin was estimated by absorption at 407 mμ; elution volumes for adenosine deaminase and ribonuclease were estimated by assaying the effluent by the methods of Kalckar (1947) and of Ipata and Felicioli (1968), respectively. Cytosine deaminase was located by assaying the effluent as in Figure 1. The inset shows the plot of elution volume against log (molecular weight).

from the extinction coefficients at 260 mμ at pH 7 (Cohn, 1955). Modifications of the standard conditions are described in the presentation of experimental data.

Preparation of Cytosine Deaminase. In the fractionation procedure reported below, the temperature was maintained between 2 and 6° unless otherwise stated. The purification scheme is summarized in Table I.

PREPARATION OF YEAST AUTOLYSATE. Baker's yeast Vulcania (3.5 kg) was plasmolyzed with toluene according to the method of Kunitz (1947) to obtain fraction 1.

AMMONIUM SULFATE FRACTIONATION. Fraction 1 was precipitated with ammonium sulfate between 60 and 70% saturation at pH 7. The pellet was solubilized in the minimal amount of 0.05 M Tris-Cl buffer, pH 7.2 (fraction 2).

G-100 SEPHADEX FRACTIONATIONS. Fraction 2 (150 ml) was applied to the top of a G-100 Sephadex column (120 × 7 cm), equilibrated with 0.05 M Tris-Cl buffer (pH 7.2), and eluted with the same buffer in 18-ml fractions at a flow rate of approximately 50 ml/hr. The elution profile is shown in Figure 1A; the most active fracions (113–122) were pooled and are referred to as fraction 3.

Fraction 3 was precipitated with ammonium sulfate between 65 and 80% saturation at pH 7, solubilized in 0.05 M Tris-Cl buffer (pH 7.2), and then passed through a second G-100 Sephadex column (60 × 3 cm), eluted as before in 5.5-ml fractions, at a flow rate of about 10 ml/hr (Figure 1B); the pool of the active fractions (64–78) is referred to as fraction 4.

DEAE-CELLULOSE CHROMATOGRAPHY. Fraction 4 was absorbed on a DEAE-cellulose (acid form) column (18 × 2.5 cm), which was then eluted in 8-ml fractions with the following buffers; 50 ml of 0.05 M Tris-Cl (pH 7.2); and a linear gradient between 0.05 and 0.25 M Tris-Cl (pH 7.2). A flow rate of approximately 30 ml/hr was maintained. The enzyme protein was eluted around a concentration of 0.13 M Tris buffer. The most active fractions (22–32) were pooled and are referred to as fraction 5 (Figure 2A).

Fraction 5 was dialyzed 5 hr against H_2O (adjusted at pH 7.2), absorbed on a DEAE-cellulose column as before, and eluted in 8-ml fractions with a linear gradient between 0 and 0.3 M Tris-Cl buffer (pH 7.2). The elution profile is shown in Figure 2B (fraction 6). Examination of samples of concentrated enzyme from this fraction by polyacrylamide gel electrophoresis performed at pH 7.2 and at pH 9.5 showed two sharp protein bands, after staining with Amido Black. The two bands were very close to each other, and appeared to be of about the same intensity. No minor protein bands were present.

Proteins were determined with a biuret method (Gornall and Hunter, 1943) in fraction 1, and spectrophotometrically (Warburg and Christian, 1942) in the other fractions.

The enzyme preparation was divided into small portions and stored at −20°. No appreciable loss of activity was detected in the stored preparation after 5 months. The final preparation was tested for adenosine deaminase (Kalckar, 1947), AMP deaminase (Setlow and Lowenstein, 1967), cytidine deaminase (Ipata *et al.*, 1970), 5′-nucleotidase (Ipata, 1967), ribonuclease (Ipata and Felicioli, 1968), guanase (Roush and Norris, 1950), and RNA phosphodiesterase (Ipata and Felicioli, 1969), which were found to be undetectable.

Results

Effect of pH. Figure 3 shows the variation of reaction rate as a function of hydrogen ion concentration. As can be seen,

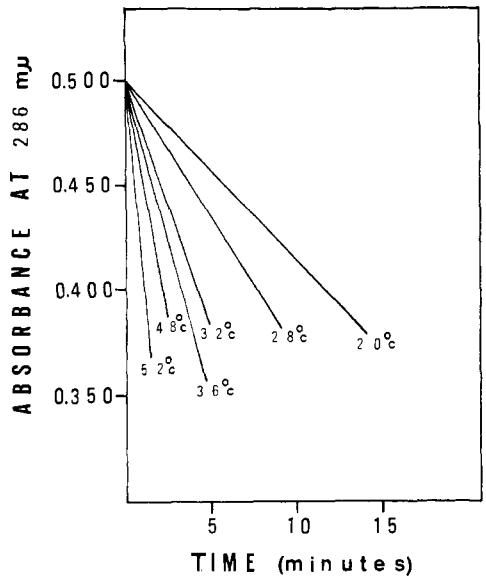


FIGURE 5: Plots of velocities (expressed as absorbance at 286 mμ as a function of time) at different temperatures. The protein concentration was 33 μg/reaction mixture. The final cytosine concentration was 4.8 mM.

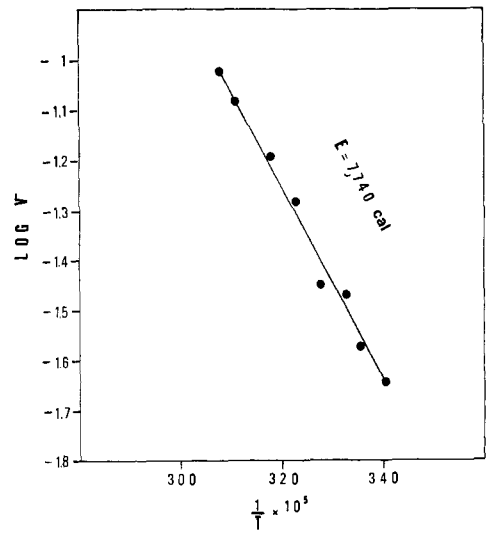


FIGURE 6: Arrhenius plot. The data include initial velocity determinations at temperatures between 20.5° and 52°, expressed as Δ absorbance at 286 mμ/min, plotted against reciprocal temperatures (Kelvin scale). Cytosine deaminase was not inactivated during the 10-min incubation period up to the maximal temperature tested. *E* equals activation energy.

a single activity optimum is found near pH 7. It must be noted that the difference molar absorptivities for cytosine and uracil and for 5-methylcytosine and thymine are practically independent of hydrogen ion concentration in the pH range studied. The enzyme is stable at 4° in the pH range 5–9 for at least 48 hr.

Molecular Weight. This determination was carried out according to Andrews (1964) by gel filtration on a G-100 Sephadex column (140 × 3 cm), equilibrated with 0.050 M Tris-Cl buffer (pH 7.2). A mixture of enzyme preparation and marker proteins of known molecular weight was applied to the top of the column, which was then eluted with the same buffer, at a flow rate of about 10 ml/hr. The elution volume corresponding to cytosine deaminase led to a calculation of 34,000 as its molecular weight (Figure 4).

Reaction Kinetics. Cytosine deaminase from baker's yeast displays uncomplicated reaction kinetics. The reaction rate is proportional to enzyme concentration up to about 70 μg of protein/ml of reaction mixture and the time course of the reaction is linear for about 15 min at 27°. Figure 5 shows that the linearity of the reaction rate is maintained through a fairly wide range of temperature.

Figure 6 is a standard Arrhenius plot, the logarithm of enzyme velocity plotted against the reciprocal of absolute temperature. The activation energy calculated from the negative slope, $-E/R$, has a value of 7740 cal/mole. The evaluation of the K_m for cytosine and 5-methylcytosine by the use of a double-reciprocal plot is shown in Figure 7. A value of 2.5 mM was obtained for both substrates.

Sensitivity to p-Mercuribenzoate. In contrast with cytidine deaminase (Ipata *et al.*, 1970) cytosine deaminase from baker's yeast is relatively insensitive to mercurial agents. With both cytosine and 5-methylcytosine as substrates no reduction of reaction rate was observed at concentration as high as 0.1 mM *p*-mercuribenzoate, added simultaneously with substrate, before the addition of the enzyme preparation. At 0.3 mM *p*-mercuribenzoate, the enzyme was inactivated about 50%.

Inhibitors. Cytosine deaminase from baker's yeast is inhibited by a number of nucleosides and nucleotides, including cytidine, CMP, CDP, CTP, thymidine, TTP, guanosine, GMP, GDP, and GTP. The concentrations required to produce 50% inhibition ranged between 0.385 mM for GMP and 0.690 mM for thymidine (Table II). The following compounds, at 0.5 mM concentration, had no inhibitory effect on enzyme activity, when tested with both cytosine and 5-methylcytosine as substrates: adenine, adenosine, uracil, uridine, guanine, xanthine, hypoxanthine, thymine, AMP, ADP, ATP, UTP, IMP, and ITP.

Double-reciprocal plots obtained in the absence and in the

TABLE II: Effect of Some Inhibitors on Cytosine Deaminase from Baker's Yeast.[a]

Inhibitor	Concentration (mM) required for 50% inhibition	Hill coefficients (n')
Cytidine	0.517	4.0
Thymidine	0.690	4.4
Guanosine	0.400	5.53
CMP	0.450	3.8
GMP	0.385	4.55
GDP	0.412	4.7
GTP	0.405	4.31
TTP	0.580	5.16
CDP	0.520	
CTP	0.442	

[a] The enzyme activity was assayed as described under Experimental Procedure in the presence of 4.8 mM cytosine. Essentially the same figures were obtained with 5-methylcytosine as substrate.

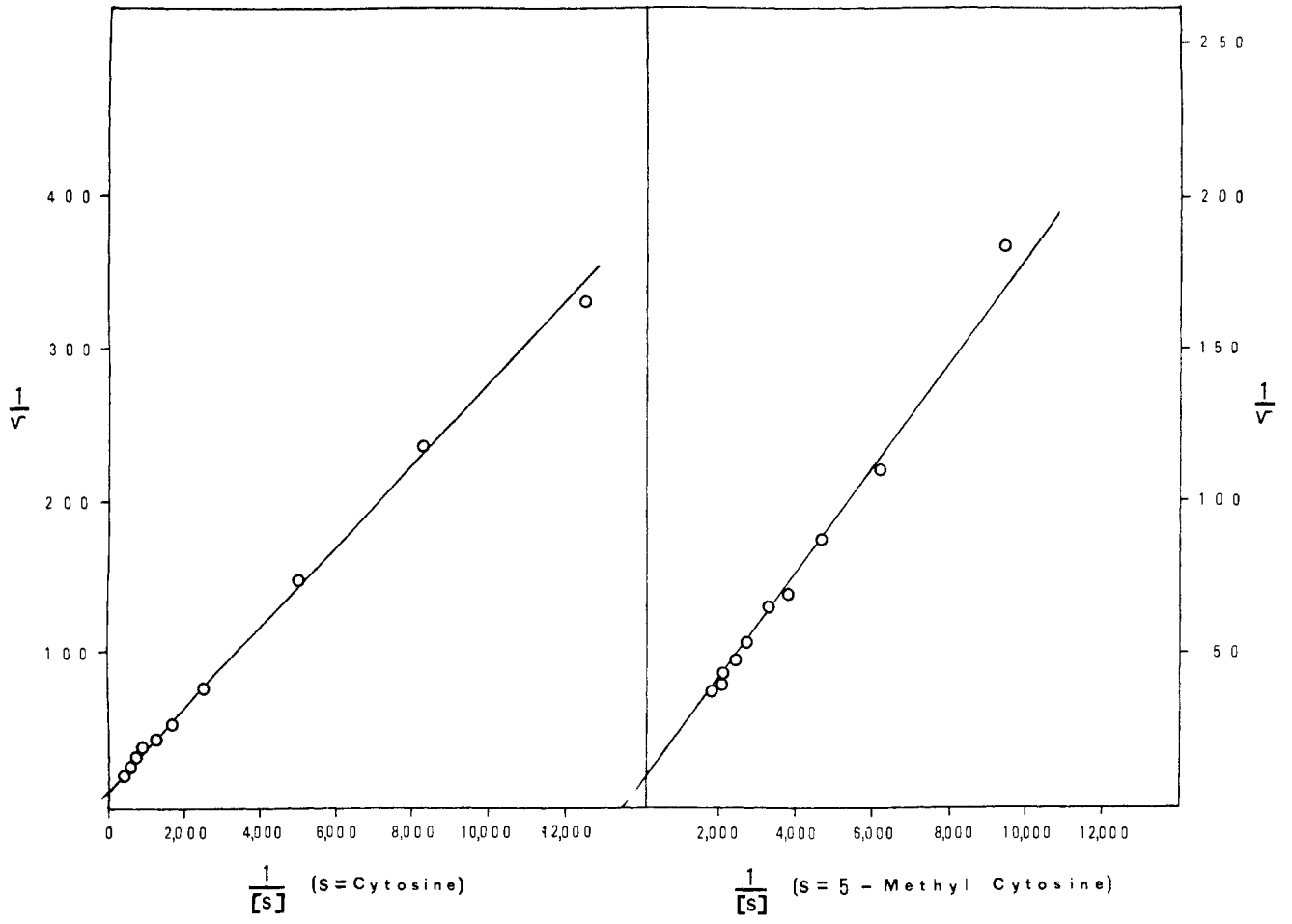


FIGURE 7: Double-reciprocal plots for cytosine (left) and 5-methylcytosine (right) concentration and initial reaction velocities. The initial velocity is expressed as Δ absorbance per minute at 286 mμ under the standard assay conditions.

presence of most of the inhibitors showed that the inhibition is of the mixed competitive and noncompetitive type with respect to both substrates.

When cytosine deaminase was assayed in the presence of increasing concentration of the inhibitors, highly sigmoidal inhibition curves are obtained. Figure 8 shows the shape of the inhibition curve, obtained with CMP, GMP, GDP, GTP, guanosine, thymidine, and cytidine; the values of interaction coefficients, calculated from the Hill plots are listed in Table II.

Effect of pH on the Enzyme Inhibition. Figure 9 shows the effect of hydrogen ion concentration on the enzyme activity and on the inhibition exerted by GMP, thymidine, cytidine, and GTP. The per cent inhition is referred to initial velocity observed in the absence of inhibitors at each pH value tested. It can be seen that the inhibition is markedly pH dependent. For instance, at pH 5 the enzyme is inhibited 100% by 0.55 mM cytidine, and this inhibition is reduced to about 50% at pH 7.2; at pH 5 the enzyme is inhibited 95% by 0.56 mM thymidine and the inhibition is reduced to about 35% at pH 7.2.

Discussion

The data presented above indicate the presence in yeast plasmolysates of a cytosine deaminase with a molecular weight of about 34,000, characterized by inhibition by cytidine, thymidine, guanosine, and their nucleotides.

Interaction of more than one inhibitor molecule with the enzyme at noncatalytic sites is suggested by the strongly sigmoidal shape of the inhibition curves. Furthermore, any direct interaction at the catalytic site between inhibitor molecules could be excluded "*a priori*," on the basis of kinetic data showing that these nucleotides do not behave as competitive inhibitors.

Whether cytosine and 5-methylcytosine are attacked by the same enzyme cannot be definitely stated, but the identical K_m values for the two substrates and the parallel sensitivity to *p*-mercuribenzoate and inhibitors are in favor of this concept.

Variation of enzyme inhibition as a function of pH has been observed with several regulated enzymes (Mansour, 1963; Mansour and Ahlfors, 1968; Ipata and Cercignani, 1970) and has been interpreted in terms of a conformational alternation of the enzyme molecule, leading to a variation of accessibility of the binding sites to inhibitor molecules. This seems to be the case for cytosine deaminase, as shown by the data reported in Figure 9.

Furthermore, in the alkaline pH region, where nucleotides exist as fully deprotonized molecules, the marked decrease of the inhibition observed by us cannot be a consequence of a change in protonation of the inhibitor molecules. Similarly,

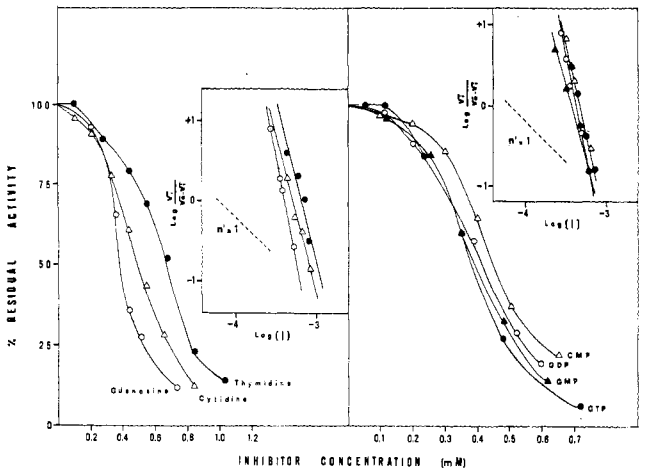


FIGURE 8: Effect of varying concentrations of some inhibitors on the initial velocities of cytosine deaminase. The insets show the Hill plots for the determination of the interaction coefficient, n', between inhibitor binding sites. The calculated values for n' are listed in Table II.

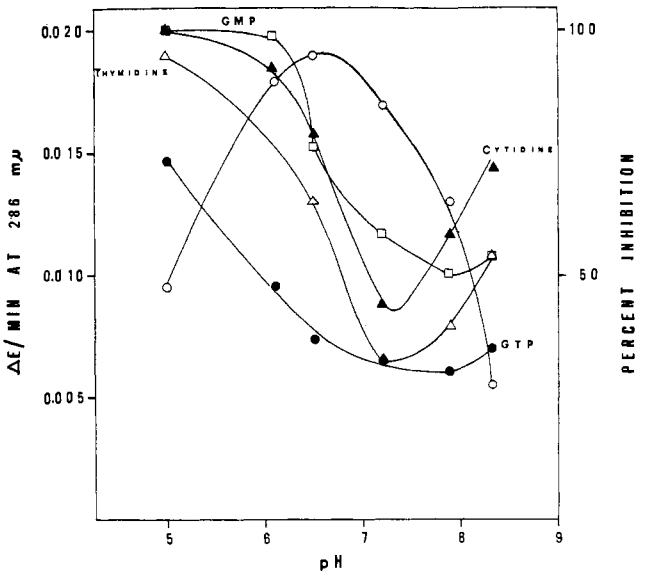


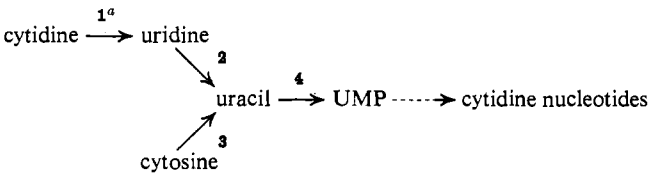
FIGURE 9: The effect of pH on the activity of baker's yeast cytosine deaminase in the absence and in the presence of some inhibitors. (—O—O—O—) Initial velocity as a function of pH. The other curves refer to the per cent inhibition at each pH tested in the presence of 0.55 mM cytidine, 0.56 mM thymidine, 0.30 mM GMP, and 0.350 mM GTP.

no change in protonation occurs in the pH range studied in the inhibitory nucleosides.

It is conceivable that cytosine deaminase may play an anabolic, rather than a catabolic role in yeast. Yeast possesses a UMP pyrophosphorylase, catalyzing the formation of UMP from uracil and 5-phosphoribosyl 1-pyrophosphate; cytosine does not seem to be a substrate for this enzyme (unpublished results). The combined action of cytosine deaminase and UMP pyrophosphorylase may therefore represent a "salvage pathway" for pyrimidine nucleotide biosynthesis in yeast leading to the formation of UMP from cytosine. This view is substantiated by the observation (unpublished results) that when crude yeast extracts are incubated in the presence of cytosine and 5-phosphoribosyl 1-pyrophosphate, UMP, but *not* CMP, can be readily detected on paper chromatograms of the reaction mixtures, after formation of uracil. Similarly, with cytidine and 5-phosphoribosyl 1-pyrophosphate as substrates, UMP can be easily detected after the sequential appearance of uridine and uracil, which are probably formed *via* the combined action of cytidine deaminase (Ipata *et al.*, 1970) and a specific uridine nucleosidase (Carter, 1951; Felicioli *et al.*, 1970).

These concepts are summarized in Scheme I.

SCHEME I



[a] **1** = cytidine deaminase; **2** = uridine nucleosidase; **3** = cytosine deaminase; **4** = UMP pyrophosphorylase.

Since the only known pathway for the formation of cytidine nucleotides involves the amination of UTP formed from UMP by successive kinase reactions (Mahler and Cordes, 1967), the inhibition by cytidyl compounds on cytosine deaminase described above and that on cytidine deaminase reported previously (Ipata *et al.*, 1970) may play a role in the regulation of pyrimidine nucleotide biosynthesis in yeast.

The inhibition exerted on cytosine deaminase by thymidine and thymidyl compounds is also relevant in this respect, since 5-methylcytosine is deaminated by the action of cytosine deaminase, to give thymine.

The inhibition by guanyl compounds is difficult to interpret. Molloy and Finch (1969) have observed that UMP pyrophosphorylase from *E. coli* is strongly activated by GTP and inhibited by UMP and UTP. It is therefore likely that the levels of guanyl compounds could also contribute in regulating the "salvage pathway" for pyrimidine nucleotide formation from cytidine and cytosine.

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Evidence for the Binding of Pyridoxal 5′-Phosphate in a Hydrophobic Region of Glycogen Phosphorylase *b* Dimer*

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ABSTRACT: Although pyridoxal 5′-phosphate (PLP) is known to be required for the catalytic activity of glycogen phosphorylase, the role of this cofactor in the catalytic process is uncertain. Pyridoxal 5′-phosphate does appear to stabilize the dimeric form of phosphorylase *b*, and several kinds of evidence suggested that this cofactor is buried in a hydrophobic region in the dimer, and perhaps lies between the two monomers. The purpose of this study was to examine the fluorescent properties of phosphorylase *b* and its reduced form for additional evidence that this cofactor is indeed buried in the dimer. An estimate of the rigidity of binding of pyridoxal 5′-phosphate to the apoenzyme also was investigated by polarization of fluorescence. One suggestion of the inaccessibility of pyridoxal 5′-phosphate on the dimer came from the effect of hydroxylamine on the fluorescence of the cofactor. Hydroxylamine reacted directly with bound pyridoxal 5′-phosphate to form a unique fluorescent species only in the presence of "deforming agents," which are known to dissociate phosphorylase *b* to monomers. Another suggestion that pyridoxal 5′-phosphate is buried in the hydrophobic interior of the dimer came from studies on phosphorylase *b* which had been reduced with sodium borohydride. The low fluorescence efficiency of this reduced enzyme was similar to that of pyridoxamine 5′-phosphate in nonpolar solvents. When the reduced phosphorylase *b* dimer was placed in a solution which was known to dissociate dimers to monomers, the fluorescence intensity of reduced phosphorylase *b* increased to that characteristic of pyridoxamine 5′-phosphate free in aqueous solution. Two kinds of observations indicate that pyridoxal 5′-phosphate is firmly bound to phosphorylase *b*. The polarization of fluorescence was near the maximum value for pyridoxal 5′-phosphate and reduced pyridoxal 5′-phosphate bound to both the dimer and monomer of phosphorylase *b*, which suggested that more than one binding interaction exists between the cofactor and the apoenzyme. Also, in connection with the hydroxylamine studies, it was observed that there was energy transfer from tryptophan residues to the pyridoxal 5′-phosphate–hydroxylamine complex. This demonstrated that the complex was still bound to the protein and suggested a binding site in addition to that of the 4′-aldehyde group of pyridoxal 5′-phosphate and the enzyme. A polarization of fluorescence study of reduced phosphorylase *b* in imidazole–citrate suggested that the PLP binding site on the monomer did not change upon dimer dissociation. This implies PLP may be bound to the surface of the dissociated monomer and not buried in a hydrophobic pocket within each monomer. In summary, these fluorescence studies strengthen the hypothesis that pyridoxal 5′-phosphate is firmly bound in a hydrophobic environment between the monomers of the phosphorylase *b* dimer.

The function of PLP[1] on phosphorylase *b* has remained elusive for many years. A catalytic role for the 4′-aldehyde group of PLP has been eliminated (Fischer *et al.*, 1958; Hedrick and Fischer, 1965; Strausbauch *et al.*, 1967), and there is only indirect evidence that the 5′-phosphate (Fischer and Krebs, 1966; Shaltiel *et al.*, 1969) or the ring nitrogen (Bresler and Firsov, 1968) of PLP may be involved in the catalytic process. There is clear evidence that PLP stabilizes the dimeric form of phosphorylase *b*, and perhaps that is its role. PLP could not be removed from the dimer of phosphorylase *b* by carbonyl reagents unless a "deforming buffer" was present to dissociate the dimers to monomers (Shaltiel *et al.*, 1966). Once the PLP was removed, the apoenzyme monomers did not reaggregate to enzymatically active dimers, but to a random assortment of aggregates. Furthermore, PLP or PLP analogs which restored enzymatic activity to apophosphorylase also restored phosphorylase to its dimeric form (Shaltiel *et al.*, 1969).

How PLP stabilizes the dimeric structure, however, remains unknown. The location of PLP may have an important bear-

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[1] Abbreviations used are: PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; AMP, adenosine 5′-monophosphate; *A* : *E*, activation : emission wavelength maxima, in nanometers.