Deoxycytidylate Aminohydrolase. III. Modifications of the Substrate Sites Caused by Allosteric Effectors*

Mosè Rossi, Giuseppe Geraci, and Eduardo Scarano

ABSTRACT: Binding of allosteric ligands to deoxycytidylate aminohydrolase causes specific changes of the enzyme affinity for competitive inhibitors. Three groups of competitive inhibitors were demonstrated: (1) competitive inhibitors with the highest affinity for the deoxythymidine triphosphate (dTTP)–magnesium–enzyme complex, (2) competitive inhibitors with the highest affinity for the deoxycytidine triphosphate (dCTP)–Mg–enzyme complex, and (3) competitive inhibitors with equal affinity for the dCTP–magnesium–enzyme and for the dTTP–magnesium–enzyme complexes. Some competitive inhibitors, at low substrate concentrations, can activate the enzyme while others cannot.

The competitive inhibitors which activate the enzyme at low substrate concentrations have the highest affinity for the dCTP–magnesium–enzyme complex. The competitive inhibitors which cannot activate the enzyme at low substrate concentrations have the highest affinity for the dTTP–magnesium–enzyme complex. The physiological significance of the regulation of deoxycytidine monophosphate aminohydrolase activity by allosteric and by isosteric ligands is discussed.

The hypothesis of the occurrence of multiple substrate sites and of multiple regulatory sites on the molecule of deoxycytidylate aminohydrolase was advanced in previous papers (Scarano *et al.*, 1962, 1963, 1967a).

Two classes of inhibitors of deoxycytidylate aminohydrolase were found: competitive as dUMP[1] and dTMP, and allosteric as dTTP–Mg and dUTP–Mg (Scarano *et al.*, 1964, 1967a). The competitive inhibitors inhibit the enzyme with kinetics of order one at high substrate concentrations (Scarano *et al.*, 1967a; Geraci *et al.*, 1967). The allosteric inhibitors inhibit the enzyme with kinetics of order higher than one, decrease the affinity of the enzyme for the substrate, and increase the cooperative interaction of the substrate sites. In addition to regulation by allosteric inhibitors, the activity of dCMP aminohydrolase is regulated also by the allosteric activators dCTP–Mg and CH_3-dCTP–Mg (Scarano *et al.*, 1967a). The allosteric activators activate the enzyme at low substrate concentrations, eliminate the cooperative interaction of the substrate sites, and revert the inhibition of the enzyme by allosteric inhibitors, without reverting that by competitive inhibitors. Furthermore, it was demonstrated that no monomer–polymer conversion of the enzyme molecule occurs on binding of the allosteric effectors (Geraci *et al.*, 1967). Modifications of the substrate sites of dCMP aminohydrolase caused by binding of the allosteric effectors to specific sites of the enzyme molecule could explain the regulation of the activity. These modifications might cause also changes of the affinity of the enzyme for competitive inhibitors. Moreover, the changes of the affinity of the enzyme for competitive inhibitors might differ according to the inhibitor. A possible example is the partial reversion by dCTP of the inhibition of dCMP aminohydrolase by dGMP, which has already been reported (Scarano *et al.*, 1963). This effect can be interpreted by the assumption that the dCTP–Mg–enzyme has a lower affinity for dGMP than the enzyme which has not bound dCTP–Mg.

The present paper reports evidence that specific changes of the affinity of the substrate sites of deoxycytidylate aminohydrolase for competitive inhibitors occur on binding of dTTP–Mg or dCTP–Mg to the enzyme. The correlation between these changes and the activatory effect of some competitive inhibitors at low substrate concentration is discussed.

Material and Methods

Homogeneous dCMP aminohydrolase was prepared from donkey spleen (Geraci *et al.*, 1967). All reagents, of analytical reagent grade, were crystallized from 10 mM Versene and recrystallized from quartz-redistilled

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[1] Abbreviations used: dUMP, deoxyuridine monophosphate; dTMP, deoxythymidine monophosphate; dTTP, deoxythymidine triphosphate; dUTP, deoxyuridine triphosphate; dCTP, deoxycytidine triphosphate; dGMP, deoxyguanosine monophosphate; dAMP, deoxyadenosine monophosphate; dCMP, deoxycytidine monophosphate; CH_3-dCTP, 5-methyldeoxycytidine triphosphate.

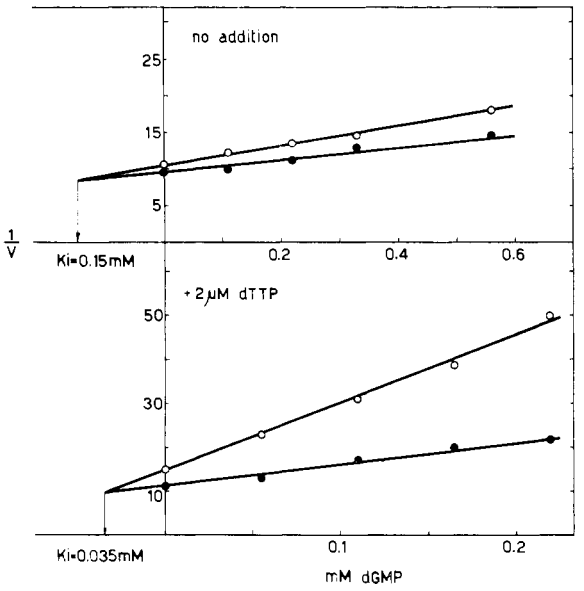

FIGURE 1: Effect of dTTP on the inhibition of dCMP aminohydrolase by dGMP. Substrate: (O——O) 2 mM dCMP and (●——●) 3 mM dCMP; 0.75 μg of enzyme/ml of incubation mixture.

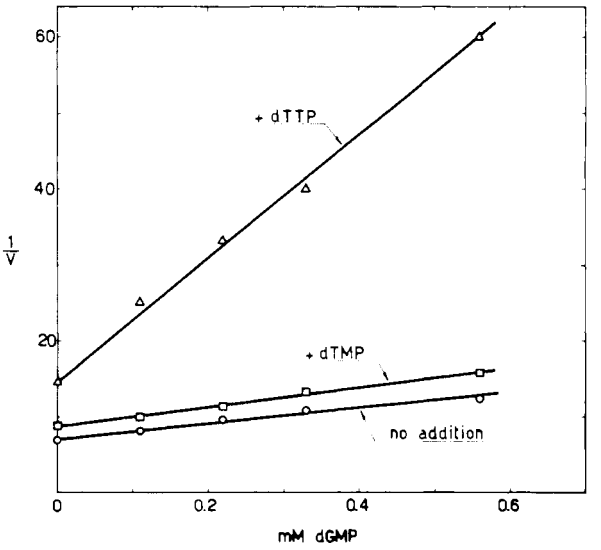

FIGURE 2: Effect of dTTP and dTMP on the inhibition of dCMP aminohydrolase by dGMP. Substrate, 3 mM dCMP; (□——□) 0.15 mM dTMP, and (△——△) 3 μM dTTP; 1.85 μg of enzyme/ml of incubation mixture.

water. Tris was recrystallized from 85% ethanol. Nucleotides were purchased from Calbiochem; the deoxyribonucleoside triphosphates were purified by thin layer chromatography (Grippo *et al.*, 1965).

A Beckman DK2A spectrophotometer, equipped with a thermostatic chamber at 38°, was used for the spectrophotometric determinations. The decrease of absorbancy at 285 mμ as a function of time was recorded by a special device for the DK2A spectrophotometer obtained from Terzano, Milan, Italy. The incubation mixture (0.3-ml final volume) contained 0.05 M phosphate, 0.05 M Tris, 2 mM $MgCl_2$ (pH 7.5), and the nucleotides as specified. Addition of the enzyme to the incubation mixture started the reaction. The enzyme activity was measured by extrapolation of the linear part of the automatic record and was expressed as $\Delta_{absorbancy}$ at 285 mμ/30 sec (Scarano *et al.*, 1967a). The recording was always started 15 sec after the addition of the enzyme to the incubation mixture and was followed for the next 30 sec. All samples were preincubated for 5 min in a water bath at 38°. Cuvets of 0.10- and 1.0-cm light paths were used.

Results

Effect of dTTP–Mg and of dCTP–Mg on the Affinity of dCMP Aminohydrolase for Competitive Inhibitors. The experiments described in Figure 1 demonstrate that dGMP is a competitive inhibitor of dCMP aminohydrolase. In the presence of a nonsaturating concentration of dTTP–Mg the value of the K_i of dGMP decreases from 0.15 to 0.035 mM. It appears that the binding of dTTP–Mg to the enzyme molecule increases the affinity of the substrate sites for dGMP while it does not affect the competitive nature of the inhibition. In Figure 2 it is shown that the inhibition of the enzyme obtained in the presence of both dGMP and dTMP is as expected for inhibitors competing for the same site. This finding strengthens the hypothesis that dGMP binds to the substrate sites since dTMP is the product

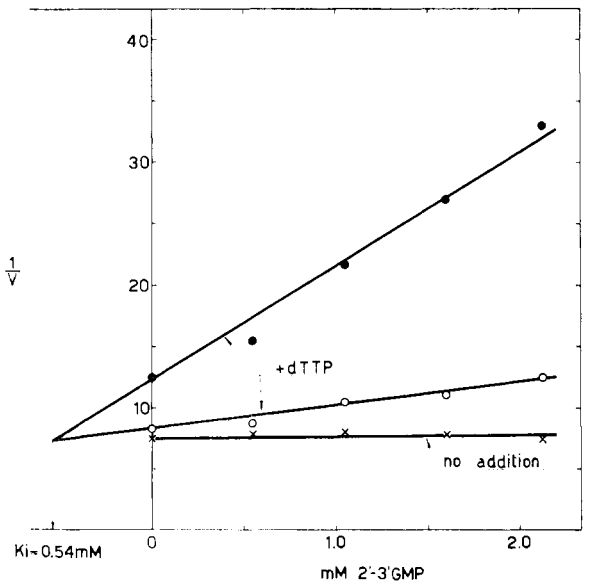

FIGURE 3: Inhibition of dCMP aminohydrolase by 2′,3′-GMP in the presence of dTTP. (×——×) 2 mM dCMP substrate, (O——O) 3 mM dCMP substrate and 2 μM dTTP, and (●——●) 2 mM dCMP substrate and 2 μM dTTP; 0.75 μg of enzyme/ml of incubation mixture.

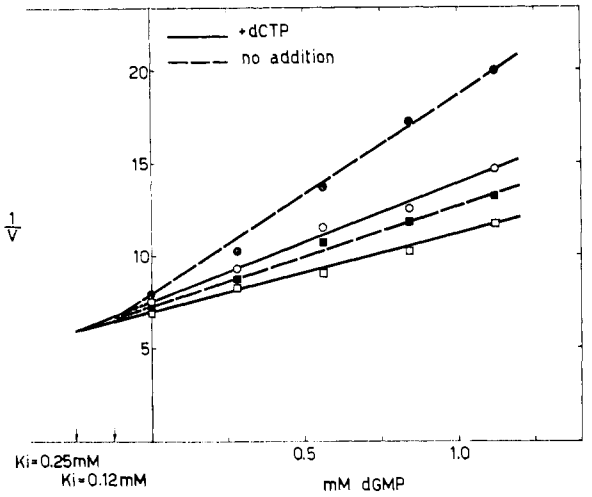

FIGURE 4: Effect of dCTP on the inhibition of dCMP aminohydrolase by dGMP. (■---■) 3 mM dCMP substrate, (●---●) 2 mM dCMP substrate, (□——□) 3 mM dCMP substrate and 20 μM dCTP, (○——○) 2 mM dCMP substrate and 20 μM dCTP; 0.75 μg of enzyme/ml of incubation mixture.

of the enzyme reaction when CH_3-dCMP is used as substrate. For comparison in the same figure the enhancement of dGMP inhibition by dTTP–Mg is shown. Nonsaturating levels of dTTP–Mg also increase the affinity of the enzyme for dGTP and 5′-GMP, which,

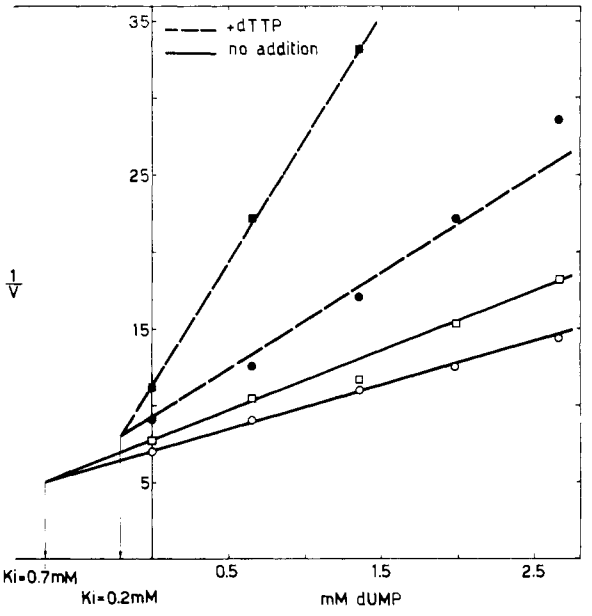

FIGURE 5: Effect of dTTP on the inhibition of dCMP aminohydrolase by dUMP. (○——○) 3 mM dCMP substrate, (□——□) 2 mM dCMP substrate, (●---●) 3 mM dCMP substrate and 2 μM dTTP, and (■---■) 2 mM dCMP substrate and 2 μM dTTP; 0.75 μg of enzyme/ml of incubation mixture.

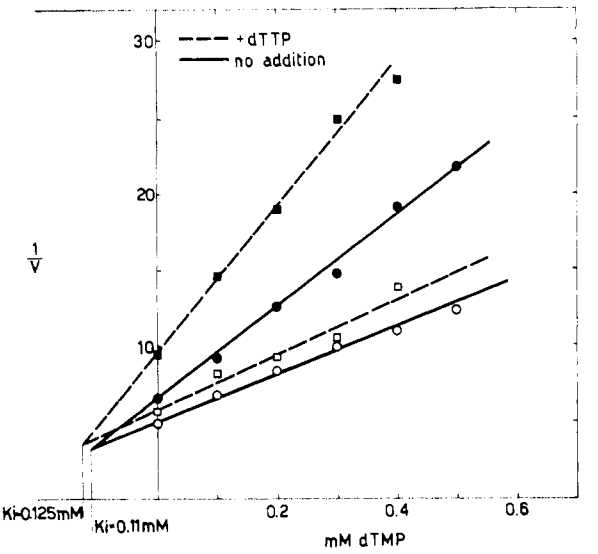

FIGURE 6: Effect of dTTP on the inhibition of dCMP aminohydrolase by dTMP. (○——○) 2 mM dCMP substrate, (●——●) 1 mM dCMP substrate, (□---□) 2 mM dCMP substrate and 2 μM dTTP, (■---■) 1 mM dCMP substrate and 2 μM dTTP; 0.95 μg of enzyme/ml of incubation mixture.

although less active than dGMP, are competitive inhibitors of the enzyme. Moreover, 2′,3′-GMP, which up to a value of 2.2 mM has no inhibitory effect on dCMP aminohydrolase, in the presence of dTTP–Mg becomes a competitive inhibitor of the enzyme with a K_i of 0.54 mM (Figure 3). In contrast to the effect of dTTP–Mg, dCTP–Mg decreases the affinity of the substrate sites of dCMP aminohydrolase for dGMP (Figure 4).

The effect of dTTP–Mg on the inhibition by dUMP is shown in Figure 5. As in the case for the guanine nucleotides an increase of the affinity of the substrate sites for dUMP in the presence of dTTP–Mg occurs. On the other hand, dCTP–Mg has no effect on the kinetics of dUMP inhibition.

In Figure 6 are reported data on the effect of dTTP–Mg on the inhibition of dCMP aminohydrolase by dTMP. It appears that the two nucleotides do not affect each other, nor is the affinity of the enzyme for dTMP affected by dCTP–Mg.

The data of Figure 7a demonstrate that the inhibition of dCMP aminohydrolase by dAMP can be described by the Dixon equation for a competitive inhibitor. In the presence of nonsaturating concentration of dTTP–Mg, as shown in Figure 7b, the affinity of the enzyme for dAMP is decreased. The inhibition of the enzyme by dAMP was not affected by dCTP–Mg at the substrate concentrations indicated in Figure 7a,b nor by higher concentrations.

Effect of Competitive Inhibitors on the Enzyme Activity at Low Substrate Concentration. Three types of effect, caused by competitive inhibitors, on the activity of dCMP aminohydrolase at low substrate

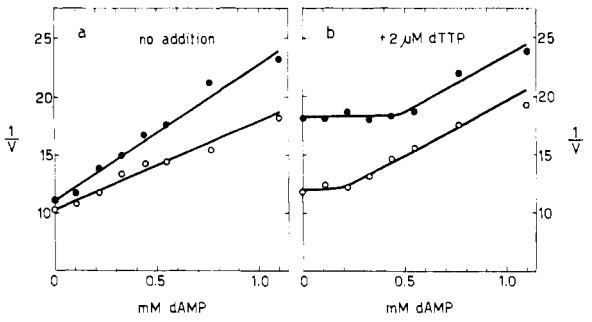

FIGURE 7: Effect of dTTP on the inhibition of dCMP aminohydrolase by dAMP. (●——●) 2 mM dCMP substrate and (○——○) 4 mM dCMP substrate; 0.72 μg of enzyme/ml of incubation mixture.

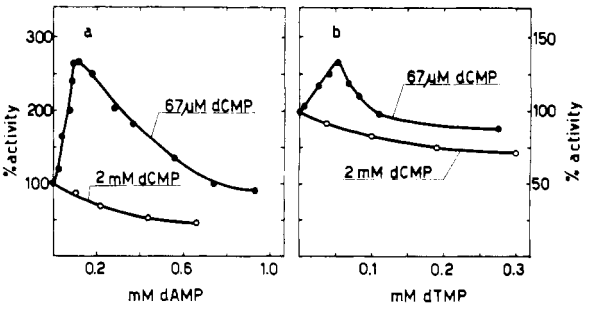

FIGURE 8: Effect of dAMP and of dTMP on the activity of dCMP aminohydrolase at low and at high substrate concentration; 0.63 μg of enzyme/ml of incubation mixture.

concentration have been observed: (1) strong activation, (2) slight activation, and (3) no activation. The competitive inhibitor dAMP, at low substrate concentration, strongly activates dCMP aminohydrolase (Figure 8a). The slight activation of the enzyme by a competitive inhibitor is demonstrated by the effect of dTMP (Figure 8b). The lack of any activation by competitive inhibitors is demonstrated by the effect of dGMP and dUMP, which behave as enzyme inhibitors at substrate concentrations as low as 60 μM dCMP.

The activation of the enzyme at low substrate concentration of dAMP and that by dCTP–Mg are not additive. In the presence of saturating concentrations of dCTP–Mg, dAMP does not activate the enzyme but behaves as a competitive inhibitor with a K_i of 0.035 mM (Figure 9).

Discussion

The data reported in the present paper permit one to classify the competitive inhibitors of dCMP aminohydrolase into three groups on the basis of the following criteria. (1) The change of the enzyme affinity for competitive inhibitors caused by nonsaturating concentrations of dTTP–Mg; (2) the change of the enzyme affinity for competitive inhibitors caused by saturating concentration of dCTP–Mg; and (3) the effect of competitive inhibitors on the enzyme activity at low substrate concentrations. By the first criterion three groups of competitive inhibitors result: (a) inhibitors with highest affinity for the dTTP–Mg–enzyme complex (dGMP and dUMP); (b) inhibitors with lowest affinity for the dTTP–Mg–enzyme complex (dAMP); and (c) inhibitors with identical affinity for the enzyme and for the dTTP–Mg–enzyme complex (dTMP). By the second criterion three groups of competitive inhibitors result: (a) inhibitors with lowest affinity for dCTP–Mg–enzyme (dGMP); (b) inhibitors with highest affinity for dCTP–Mg–enzyme (dAMP); and (c) inhibitors with identical affinity for the enzyme and for dCTP–Mg–enzyme complex (dTMP). Likewise, by the third criterion three groups of competitive inhibitors result: (a) inhibitors effective at all substrate concentrations (dGMP and dUMP); (b) inhibitors that at low substrate concentrations cause a powerful activation (dAMP); and (c) inhibitors that at low substrate concentrations cause a slight activatory effect (dTMP). By all three criteria the same three groups of nucleotides are found.

The assumption that dAMP and dGMP bind to the substrate sites of dCMP aminohydrolase is supported by three lines of evidence. First, both dAMP and dGMP inhibit the enzyme with kinetics typical of competitive inhibitors. Second, the inhibition resulting in the presence of a mixture of dAMP or dGMP with dTMP or dUMP, products of the enzyme reaction, is as expected for compounds competing for the same sites. Third, dAMP and dGMP are more effective competitive inhibitors than dATP and dGTP, the corresponding triphosphates; yet only dCTP and dTTP

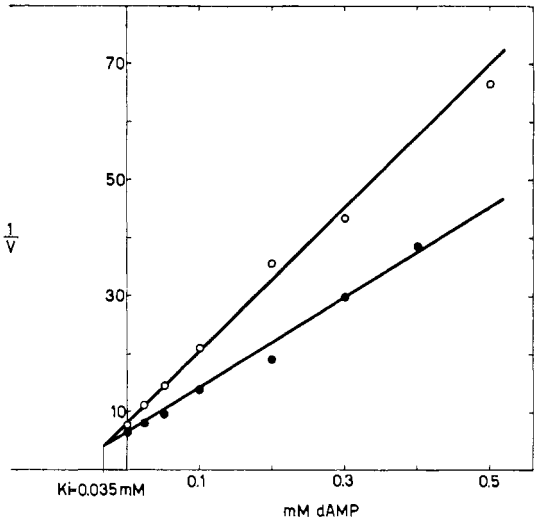

FIGURE 9: Inhibition of dCMP aminohydrolase at low substrate concentrations by dAMP in the presence of saturating concentrations of dCTP. (●——●) 60 μM dCMP, (○——○) 120 μM dCMP, and 7 μM dCTP; 0.72 μg of enzyme/ml of incubation mixture.

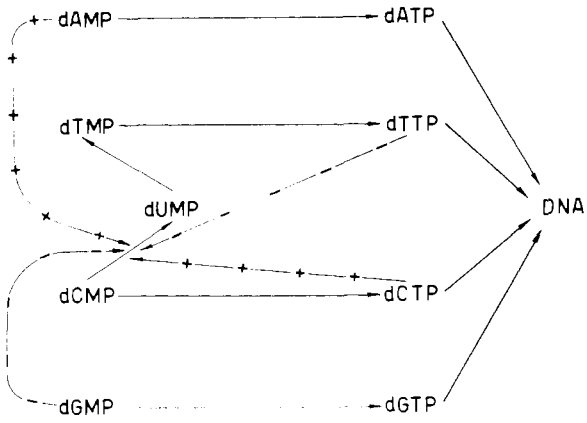

FIGURE 10: Hypothetical control of the deoxyribonucleotide pool by deoxycytidylate aminohydrolase.

are the effective allosteric regulators. Moreover, the enzyme can bind at the same time four molecules of dTTP–Mg and four molecules of dGMP, or four molecules of dCTP–Mg and four molecules of dAMP (Scarano *et al.*, 1967b). Thus the binding sites for dGMP and for dAMP are different from those for dTTP–Mg and for dCTP–Mg.

The changes of the enzyme affinity for dAMP and for the substrate dCMP, caused by the allosteric regulators, are of the same type. Moreover, while at high substrate concentration dAMP is a competitive inhibitor, at low substrate concentrations it is an activator. dAMP behaves as a competitive inhibitor also at low substrate concentrations in the presence of dCTP–Mg. The activatory effect of dAMP can be explained by assuming that, when the concentration of the substrate dCMP is not sufficient to induce the activated form of the enzyme, the addition of dAMP can do so. On the other hand, when the activated form of the enzyme is induced by the binding of dCTP–Mg, the addition of dAMP gives rise to competitive inhibition (Figure 9) because only competition between dAMP and the substrate for the catalytic site can occur if the enzyme is in the activated form. Activation of an allosteric enzyme by a competitive inhibitor at low substrate concentration has been previously reported by Gerhart and Pardee (1963) for aspartate transcarbamylase and by Changeux (1963) for threonine deaminase.

The changes of affinity of the enzyme–substrate sites for dGMP caused by the allosteric regulators are opposite to those found for dAMP. In fact, the affinity of the dCTP–Mg–enzyme complex for dGMP is lower, and the affinity of the dTTP–Mg–enzyme complex for dGMP is higher than that of the enzyme. At low substrate concentrations dGMP does not activate the enzyme as dAMP does. This indicates that dGMP cannot induce the activated form of the enzyme as dAMP does.

The results presented in the present paper can be described in terms of the definitions by Monod *et al.* (1965). The allosteric effectors of dCMP aminohydrolase exhibit homotropic and heterotropic effects. The homotropic effects for dCTP and dTTP are demonstrated by the corresponding values n of the Hill equation (Scarano *et al.*, 1967a). The heterotropic effects of dCTP and dTTP are seen not only in the activation and inhibition of the enzyme activity but also in the changes of the affinity of the enzyme for competitive inhibitors. dCTP causes a positive heterotropic effect for dAMP and a negative one for dGMP. dTTP causes a positive heterotropic effect for dGMP and a negative one for dAMP. The substrates of dCMP aminohydrolase, dCMP, and CH_3-dCMP (Scarano *et al.*, 1967a), as well as some competitive inhibitors (present paper), show homotropic effects.

In Figure 10 the regulatory effects of the allosteric ligands (dCTP and dTTP) and the isosteric ligands (dAMP and dGMP) are summarized. dTTP–Mg, by inhibiting the dCMP aminohydrolase step, favors the formation of dCTP, while dCTP–Mg, by reversing the dTTP inhibition and activating the enzyme, promotes the formation of dTTP. Thus, the concentrations of dCTP and of dTTP are controlled. dAMP and dGMP may also play a role in controlling the pools of the precursors of DNA biosynthesis. dAMP, by activating the dCMP aminohydrolase step, promotes the formation of dTTP, and dGMP, by inhibiting the same step, promotes the formation of dCTP. These effects are as expected from the Watson and Crick base-pairing rules.

Another reaction sequence which gives rise to dTMP from UDP has been reported by Bertani *et al.* (1963). The final product of the pathway, dTTP, activates the UDP reduction (Larson and Reichard, 1966; Moore and Hulbert, 1966). The synthesis of dTMP from dCMP *via* dUMP might be an alternative pathway of limited biosynthetic importance. Thus, the most important physiological function of dCMP aminohydrolase could be the regulation of the deoxynucleotide pool. Some enzymes catalyzing alternative biosynthetic pathways may play a more important role on the control of concentration of metabolites than in biosynthetic reactions.

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Deoxycytidylate Aminohydrolase. IV. Stoichiometry of Binding of Isosteric and Allosteric Effectors*

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ABSTRACT: The technique by Hummel and Dreyer (Hummel, J. R., and Dreyer, W. J. (1962), *Biochim. Biophys. Acta 95*, 1) of gel filtration was used to measure the number of isosteric and allosteric sites of deoxycytidylate aminohydrolase from donkey spleen. The enzyme binds at saturation four molecules of deoxycytidine triphosphate (dCTP) or of deoxythymidine triphosphate (dTTP) per molecule, but cannot bind at the same time both regulatory nucleotides. Magnesium ions are required for the binding of the allosteric effectors. Four molecules of deoxyadenosine monophosphate (dAMP) and four molecules of dCTP, or four molecules of deoxyguanosine monophosphate (dGMP) and four molecules of dTTP, bind at the same time per molecule of enzyme. We conclude that the molecule of dCMP aminohydrolase has four catalytic sites and either four or eight regulatory sites. It is possible that the enzyme has four regulatory sites which can bind either dCTP or dTTP, but it is also possible that dCTP and dTTP have independent binding sites on the enzyme and consequently the enzyme would possess four catalytic sites, four regulatory sites for the activator nucleotide and four regulatory sites for the inhibitor nucleotide.

The kinetic properties of homogeneous deoxycytidylate aminohydrolase (EC 3.5.4.5) (Geraci *et al.*, 1967; Scarano *et al.*, 1967; Rossi *et al.*, 1967) are compatible with a model of the enzyme molecule having multiple substrate (isosteric) sites and multiple regulatory (allosteric) sites, but no molecular mechanism can be demonstrated by kinetic experiments, and other interpretations of the kinetic data could be advanced, as for instance one based on relaxation effects of the enzyme molecule with one binding site for each ligand (Weber, 1965; Anderson and Weber, 1965). We have attempted to measure by a direct method the number of allosteric and isosteric sites of dCMP[1] aminohydrolase.

Hummel and Dreyer (1962) have described a method for measuring the binding of small molecules to proteins by gel filtration. Fairclough and Fruton (1966) have discussed the advantages of gel filtration over dialysis equilibrium in investigating complexes of bovine serum albumin with tryptophan and tryptophan derivatives.

In the present paper the method of Hummel and Dreyer was used to measure the number of allosteric and isosteric sites of dCMP aminohydrolase. For the allosteric sites no technical problems arise because of the high affinity of the enzyme for the allosteric effectors. For the measurements of the number of the substrate sites the competitive inhibitors of dCMP

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[1] Abbreviations used: dCMP, deoxycytidine monophosphate; dAMP, deoxyadenosine monophosphate; dGMP, deoxyguanosine monophosphate; dTTP, deoxythymidine triphosphate; dCTP, deoxycytidine triphosphate; dTMP, deoxythymidine monophosphate; PPO, 2,5-diphenyloxazole; dimethyl-POPOP, 1,4-bis[2-(4-methyl-5-phenyloxazolyl)].