A Nuclear Magnetic Resonance Study of the Interaction of Inhibitory Nucleosides with *Escherichia coli* Aspartate Transcarbamylase and Its Regulatory Subunit†

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ABSTRACT: Aspartate transcarbamylase from *Escherichia coli* and the zinc derivative of the regulatory subunit affect the proton magnetic relaxation times of several pyrimidine nucleosides. These effects are shown to arise because of rapid chemical exchange of the nucleosides between the solution and allosteric binding sites on the enzyme. The transverse relaxation rates of the C-6 proton of the native enzyme-bound nucleosides are 830 and 190–370 sec^{-1} for 5-methylcytidine and cytidine, respectively, while the corresponding values with the regulatory subunit are 38 and 22 sec^{-1}. For each nucleoside the ratio of the value determined with the native enzyme to that with the regulatory subunit is close to the expected ratio of the protein rotational correlation times. Addition of saturating levels of carbamyl phosphate and succinate to samples containing the native enzyme and 5-methylcytidine greatly reduces the observed relaxation rate of the C-6 proton. The transverse relaxation rates of the *methyl* protons on the native enzyme-bound nucleosides are 770 and 110 sec^{-1} for 5-methylcytidine and thymidine, respectively. From these values, and assuming rapid internal rotation of the methyl group, rotational correlation times of the bound pyrimidine moiety are estimated to be 2×10^{-7} sec for 5-methylcytidine and 3×10^{-8} sec for thymidine. Comparison of these values with the expected rotational correlation time of the native enzyme indicates that 5-methylcytidine is bound rigidly and thymidine is not. Nuclear magnetic resonance was used to study the competition between cytidine and the nucleotides CTP and ATP on binding sites on both native aspartate transcarbamylase and the zinc containing regulatory subunit. With the native enzyme the ratios of the nucleotide/cytidine dissociation constants are 2.8×10^{-3} and 4.8×10^{-3} for CTP and ATP, respectively. Comparison of these values with data obtained from previous experiments indicates that these ratios are dependent on the buffer used, being larger in phosphate than in imidazole–acetate buffer. The fact that a good fit of the data can be made assuming a single value for the ratio of the nucleotide/cytidine dissociation constants leads to the conclusion that the cytidine binding is inhomogeneous, as has been observed for CTP. The results are also shown to be more consistent with a model which assumes a negative interaction (direct or indirect) between pairs of regulatory sites, rather than two sets of structurally inequivalent sites. Similar experiments with the zinc regulatory subunit gave for the ratios of the nucleotide/cytidine dissociation constants 6.5×10^{-3} and 2.7×10^{-2} for CTP and ATP, respectively.

Nuclear magnetic resonance (nmr) has been applied with considerable success to the study of the interaction of inhibitors with enzymes (Sykes and Scott, 1972; Mildvan and Cohn, 1970). As an extension of this technique, the interaction of the enzyme aspartate transcarbamylase with several of its allosteric effectors is the object of the present study. Although changes in the proton relaxation times of the effectors are relatively small, the technique of adiabatic demagnetization in the rotating frame (the $T_{1\rho}$ experiment) provides sufficient sensitivity to detect these effects. A further advantage of the $T_{1\rho}$ technique is that it can be used to obtain both T_1 and T_2 (Sykes, 1969). A knowledge of both relaxation times is necessary to separate the contribution produced by exchange effects from the nonspecific viscosity effect produced by the enzyme (Sykes *et al.*, 1970).

Pyrimidine nucleosides are the major subjects of the work presented below because they provide a unique probe for the study of regulatory sites of aspartate transcarbamylase. While CTP is most likely the species involved in feedback inhibition of enzyme activity *in vivo* (Gerhart and Pardee, 1962), cytidine binding leads to the same maximum level of inhibition seen with CTP (London and Schmidt, 1972) implying that the interaction of the nucleoside moiety is similar for both molecules. Furthermore a great difficulty in experiments performed with CTP arises from nonspecific binding to the carbamyl phosphate site (Porter *et al.*, 1969; Hammes *et al.*, 1970; Evans *et al.*, 1973). Finally, in addition to having no affinity for carbamyl phosphate sites, cytidine would also not be expected to interact with any sites having affinity for only ATP, if such sites exist (Buckman, 1970). Based on these arguments, nucleosides should show enhanced specificity in their interactions with the regulatory sites.

Nmr relaxation measurements provide the means to determine rotational correlation times of molecules bound to proteins and enzymes. In the study presented below the technique is used to gauge the specific interaction of allosteric effectors with their sites from the standpoint of motional restriction of the bound nucleosides. The results obtained indicate that weak allosteric inhibitors experience greater motional freedom when bound than do strong inhibitors. The modes of nucleoside binding to the regulatory subunit and native enzyme appear to be similar when the difference in molecular weight is taken into account.

In spite of the fact that aspartate transcarbamylase has been shown to contain six regulatory chains, there have been several indications that these chains, perhaps as a result of their association with the catalytic subunits, do not all function

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equivalently. Information regarding this question has been obtained in the present study by examining the competitive effects of the allosteric nucleotides CTP and ATP on the magnetic relaxation times of the H-6 resonance of cytidine. Nucleotide–cytidine competition was also observed with a preparation of the zinc derivative of the regulatory subunit.

Materials and Methods

Enzyme. Native aspartate transcarbamylase was prepared as described by Gerhart and Holoubek (1967). The regulatory subunit was obtained by reacting the native enzyme with neohydrin [1-(3-chloromercuri-2-methoxypropyl)urea] and separated by chromatography on DEAE-cellulose (Kirschner, 1971). The zinc derivative of the regulatory subunit was then prepared in a manner similar to that described by Nelbach *et al.* (1972). A concentrated phosphate buffer was added initially to bring the pH to 7.0, followed by the addition of 2-mercaptoethanol to 0.2 M and the addition of zinc acetate to 2 mM. An Amicon filtration apparatus with a UM20E filter was then used to concentrate the regulatory subunit. The concentrated regulatory subunit was dialyzed into D_2O buffer for nmr studies using heat treated dialysis tubing, the preparation of which is described by Nelbach *et al.* (1972).

Chemicals. All nucleosides used were purchased from Sigma Chemical Co., with the exception of cytidine, which was purchased from Pierce Chemical Co.

Nmr Studies. Nmr studies were performed on Varian HA-100 and HR-220 spectrometers. The longitudinal and transverse relaxation times were determined by using the $T_{1\rho}$ method described by Sykes (1969). This required modification of the HA-100 spectrometer for observation in the dispersion mode, as described by Sykes and Wright (1970). An HR-220 spectrometer has not been previously used for relaxation studies by adiabatic half-passage ($T_{1\rho}$). In fact the instrument is exceptionally well suited for the technique because of its good signal-to-noise characteristics and magnetic field stability. No internal modifications are required for the experiment. The "external" sweep mode is used, the phase is tuned 90° from absorption, and simple circuitry similar to that described by Sykes and Wright (1970) is used to voltage sweep into resonance. For experiments at both 220 and 100 MHz sufficient radiofrequency power was used to collapse multiplets and provide single exponential decay times.

The $T_{1\rho}$ method offers a number of advantages for the type of study described here. It is fast, unaffected by magnetic field inhomogeneity, and is sufficiently sensitive to measure the small effects produced by proteins lacking paramagnetic ions. The last condition is frequently not satisfied by line-width measurements. In all of the experiments reported here, an exponential sweep into resonance was used. Care must be taken to be sure that the time constant for the sweep into resonance is much less than the value of $T_{1\rho}$ being measured. Failure to meet this condition can result in erroneous results. Although this problem is easier to overcome when a linear sweep is used, it then becomes difficult to satisfy the adiabatic condition (Abragam, 1961). The data were recorded either on a Midwest Instrument Corp. high-speed light oscillograph chart recorder or on the x–y plotter of the spectrometer.

A method for the successive addition of nucleotide solutions was developed for the cytidine–nucleotide competition study. Small volumes of concentrated stock solution were added to the nmr sample with a calibrated syringe. The sample was then mixed with a small plunger moved through the solution several times.

Buffers. In all studies with the native enzyme, the D_2O buffer used included 0.01 M imidazole acetate (pH* 7.0), 2×10^{-3} M 2-mercaptoethanol, and 2×10^{-4} M EDTA. (pH* is the uncorrected meter reading obtained with a glass electrode standardized in H_2O buffers.) In all studies with the regulatory subunit, the buffer used was 0.04 M phosphate (pH* 7.7)–2 mM 2-mercaptoethanol. Attempts to dialyze the zinc-containing regulatory subunit into imidazole acetate pH* 7.0 buffer resulted in extensive precipitation of the protein.

Theory

The ability of a relatively low protein concentration to measurably affect the nuclear relaxation times of inhibitors or, in the present case, allosteric effectors, is a consequence of chemical exchange. Results originally obtained by Swift and Connick (1962) and Luz and Meiboom (1964) have been applied by Sykes *et al.* (1970) to the case of the reversible binding of a relatively large concentration of small molecules, I, to enzymes. A simple bimolecular kinetic scheme is assumed

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \tag{1}$$

and the observed values of $1/T_2$ and $1/T_1$ are given by

$$\frac{1}{T_2} = \frac{1}{T_2(I)} + \frac{P}{\tau}\frac{\left[\frac{1}{T_2(EI)}\left(\frac{1}{\tau} + \frac{1}{T_2(EI)}\right) + \Delta^2\right]}{\left[\left(\frac{1}{\tau} + \frac{1}{T_2(EI)}\right)^2 + \Delta^2\right]} \tag{2}$$

$$\frac{1}{T_1} = \frac{1}{T_1(I)} + \frac{P}{\tau + T_1(EI)} \tag{3}$$

In the above equations, I is the species being observed, $T_1(I)$ and $T_2(I)$ are the relaxation times for the free species, $T_1(EI)$ and $T_2(EI)$ refer to the bound species, $P = [EI]/[I]$, Δ is the chemical-shift difference in rads/second between the free and bound species, and $\tau = 1/k_{-1}$. The exchange contribution to $1/T_2$ can frequently be simplified if the relative magnitudes of the terms Δ, $1/T_2(EI)$, and $1/\tau$ can be determined. In the slow exchange limit, defined by the conditions $\Delta^2 \gg 1/T_2(EI)^2$, $1/\tau^2$ or $1/T_2(EI)^2 \gg \Delta^2$, $1/\tau^2$, the exchange term reduces to P/τ. In the fast exchange limit, defined by the condition $1/\tau^2 \gg \Delta^2$, $1/T_2(EI)^2$, the form of the exchange term depends on the relative magnitude of Δ and $1/T_2(EI)$. If $1/T_2(EI)^2 \gg \Delta^2$, the exchange term becomes $P/T_2(EI)$; if $\Delta^2 \gg 1/\tau T_2(EI)$, the exchange term becomes $P\tau\Delta^2$; if $1/T_2(EI) \simeq \tau\Delta^2$, the exchange term can be approximated by $P(\tau\Delta^2 + 1/T_2(EI))$. The slow and fast exchange limits can be distinguished by the temperature dependence; the term P/τ should increase with increasing temperature, whereas all of the fast exchange terms should decrease with increasing temperature (Sykes *et al.*, 1970). Since Δ is proportional to the frequency, the term $P\tau\Delta^2$ will be proportional to the frequency squared; none of the other limits is expected to have a frequency dependence unless $T_2(EI)$ is frequency dependent. This possibility is discussed below.

The frequency dependence of both $T_1(EI)$ and $T_2(EI)$ depends on the relaxation mechanism which dominates for the bound species. In the case of aspartate transcarbamylase, nuclear–nuclear dipolar relaxation modulated by rotational diffusion is the dominant mechanism. Based on this mechanism, the bound relaxation times can be expressed in terms of the interaction parameters and the correlation time by the

Solomon–Bloembergen equations (Carrington and McLachlan, 1967)

$$\frac{1}{T_2(\mathrm{EI})} = \sum_{ij} \frac{3}{40}\left(\frac{\hbar^2\gamma^4}{r_{ij}^6}\right) \times \left\{6\tau_R + \frac{10\tau_R}{1+(\omega_0\tau_R)^2} + \frac{4\tau_R}{1+4(\omega_0\tau_R)^2}\right\} \quad (4)$$

$$\frac{1}{T_1(\mathrm{EI})} = \sum_{ij} \frac{3}{40}\left(\frac{\hbar^2\lambda^4}{r_{ij}^6}\right)\left\{\frac{4\tau_R}{1+(\omega_0\tau_R)^2} + \frac{16\tau_R}{1+4(\omega_0\tau_R)^2}\right\} \quad (5)$$

In the above equations, τ_R refers to the rotational correlation time of the proton(s) under observation when bound to the enzyme, r_{ij} is the internuclear distance, and ω_0 is the resonance frequency in rads/second. Although an enzyme the size of aspartate transcarbamylase (mol wt 310,000) is expected to have a long correlation time ($\sim 3 \times 10^{-7}$ sec), molecules bound to it may experience a degree of motional freedom, for example the rapid rotation of a methyl group. Marshall *et al.* (1972) have shown that the effect of such motion is to reduce the effective magnitude of the interaction causing relaxation; however, in the limit of very rapid internal rotation the effective rotational correlation time of the proton remains equal to that of the entire macromolecule. A value of 7×10^{-8} sec has been found for the correlation time of succinate bound to the catalytic subunit (mol wt 100,000) (Beard and Schmidt, 1973) and a value of 5×10^{-8} sec was reported for acetyl phosphate when bound to native aspartate transcarbamylase (McMurray *et al.*, 1972). Based on these rotational correlation times $(\omega_0\tau_R)^2 \gg 1$ at 100 and 220 MHz and it can be concluded that: (1) $T_1(\mathrm{EI}) \gg T_2(\mathrm{EI})$ and (2) the frequency dependence of $T_2(\mathrm{EI})$ is negligible. These findings are consistent with the data obtained in the present study.

The use of nmr to quantitatively study ligand competition for enzyme binding sites is based on the proportionality between the exchange contribution to the relaxation rate and the fraction of bound ligand, $P = [\mathrm{EI}]/[\mathrm{I}]$. The addition of a second ligand, S, reduces the number of available binding sites and consequently the fraction of I which is bound at a given time. Although previous studies (Lanir and Navon, 1971; Taylor *et al.*, 1971) have used changes in line width as a measure of the exchange contribution to relaxation, linewidth changes reflect changes in viscosity produced by the enzyme, as well as the effects of chemical exchange. Such viscosity effects can represent an appreciable fraction of the total change in $1/T_2$ for enzymes lacking a paramagnetic ion near the ligand binding site. The experimental method used in the present study was the serial addition of a stock solution of S to a sample initially containing E and I with I at a concentration sufficient to saturate all binding sites on E. Although the percentage change in volume can be kept small, the decrease in viscosity which results from this dilution can alter the results. One solution to this problem is to consider the quantity $1/T_2 - 1/T_1$. It can readily be demonstrated, using eq 2 and 3, that this quantity is proportional to P and is insensitive to changes in viscosity if the relaxation times $T_1(\mathrm{I})$ and $T_2(\mathrm{I})$ of the free species are assumed to be equal.

However, the relaxation times of cytidine at pH* 7.0 were not equal even in the absence of enzyme. The difference is very small (less than 10% of T_1), probably constant regardless of enzyme concentration, and does not affect calculations of relaxation times of bound species.[1] But in the competition experiments even a slight inequality of T_1 and T_2 must be corrected. Letting δ_0 represent the difference between $1/T_2$ and $1/T_1$ arising from effects not produced by the enzyme, the exchange contributon due to an interaction with the enzyme may be set equal to $1/T_2 - 1/T_1 - \delta_0$. An expression giving the fraction of sites occupied by I may be obtained by dividing $1/T_2 - 1/T_1 - \delta_0$ by its initial value, measured prior to the addition of S

$$\frac{\frac{1}{T_2} - \left(\frac{1}{T_1} + \delta_0\right)}{\frac{1}{T_{2_i}} - \left(\frac{1}{T_{1_i}} + \delta_0\right)} = \frac{[\mathrm{EI}]/[\mathrm{I}]}{[\mathrm{EI}]_i/[\mathrm{I}]_i}$$

where the subscript i indicates initial values. Then, assuming the initial concentration of I is sufficient to saturate n sites on E, $[\mathrm{EI}]_i = n[\mathrm{E}]_{0,i}$. Furthermore, if the initial volume ΔV of the solution containing S is added, the right-hand side of the above equation reduces to

$$\frac{[\mathrm{EI}]/[\mathrm{I}]}{[\mathrm{EI}]_i/[\mathrm{I}]_i} = \frac{[\mathrm{EI}]}{n[\mathrm{E}]_{0,i}\frac{[\mathrm{I}]}{[\mathrm{I}]_i}} = \frac{[\mathrm{EI}]}{n[\mathrm{E}]_{0,i}\frac{V_i}{V_i+\Delta V}\frac{[\mathrm{I}]}{[\mathrm{I}]_i}} = \frac{[\mathrm{EI}]}{n[\mathrm{E}]_0}$$

Combining these results gives

$$\frac{\frac{1}{T_2} - \left(\frac{1}{T_1} + \delta_0\right)}{\frac{1}{T_{2_i}} - \left(\frac{1}{T_{1_i}} + \delta_0\right)} = \frac{[\mathrm{EI}]}{n[\mathrm{E}]_0} \quad (6)$$

In the above equation, the relaxation times refer to species I, and the total concentration of enzyme sites, $n[\mathrm{E}]_0$, must be recalculated with each addition of S due to the change in volume. It should be noted that since the exchange contribution is considered proportional to the number of sites occupied, eq 6 implicitly assumes that the contribution to the relaxation rate resulting from the binding of I to each of the n sites is equivalent. The fraction of sites occupied by I, which can be obtained from the data using eq 6, can be related to the ratio of the dissociation constants K_I and K_S of I and S from E, respectively[2]

$$\frac{[\mathrm{EI}]}{n[\mathrm{E}]_0} = \frac{n[\mathrm{E}]_0 - [\mathrm{S}]_0 - R[\mathrm{I}]_0 + \sqrt{([\mathrm{S}]_0 + R[\mathrm{I}]_0 - n[\mathrm{E}]_0)^2 + 4R[\mathrm{I}]_0 n[\mathrm{E}]_0}}{2n[\mathrm{E}]_0} \quad (7)$$

In the above equations, the subscript 0 indicates total concentrations, $R \equiv K_S/K_I$, and the total concentration of enzyme sites is $n[\mathrm{E}]_0$. Equation 7 is derived under the assumptions that all enzyme sites are occupied by either I or S, so that $n[\mathrm{E}]_0 = [\mathrm{EI}] + [\mathrm{ES}]$, and that $[\mathrm{I}]_0 \gg [\mathrm{EI}]$. A computer program was developed to vary n and R for a least-squares fit of eq 7 to the data as analyzed using eq 6. It must be emphasized, however, that a reliable value for n can be obtained only if the condition $n[\mathrm{E}]_0 \gg R[\mathrm{I}]_0$ is satisfied. In general, this was not true for the experiments reported here. Since the molar concentration of enzyme is necessarily very low and I_0 must be present at a sufficient concentration to be easily observable, the nmr method can be used to determine n only when R is sufficiently small.

Results and Discussion

Proton Relaxation of Nucleosides in Solution. The C-6 protons of the pyrimidine nucleosides cytidine, uridine,

[1] The origin of this difference is unclear. It is extremely small and might reflect a chemical exchange process involving labile protons of the nucleoside, for example.

[2] There is a sign error in the corresponding equation given in the reference by Taylor *et al.* (1971).

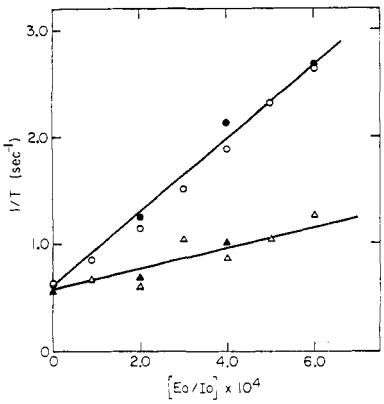

FIGURE 1: Relaxation rates of the C-6 proton of 5-methylcytidine as a function of the concentration ratio of total enzyme to total 5-methylcytidine, $[E]_0/[I]_0$ (no correction for the number of sites has been made): (○) $1/T_2$ at 220 MHz; (●) $1/T_2$ at 100 MHz; (△) $1/T_1$ at 220 MHz; (▲) $1/T_1$ and 100 MHz. Each sample contained 0.05 M 5-methylcytidine. Data were taken at 28° in a pH* 7.0 D_2O buffer containing 0.01 M imidazole acetate, 2 mM 2-mercaptoethanol, and 0.2 mM EDTA.

thymidine, and 5-methylcytidine have been the primary objects of the study reported here. As can be seen directly from molecular models of any of these nucleosides, in the predominant anti conformation the C-6 proton is close to the ribose protons H-2′, H-3′, H-5′, and H-5″. These protons can be expected to contribute substantially to the relaxation rate. In imidazole acetate pH* 7.0 buffer, H-6 of 0.1 M cytidine has a spin–spin relaxation time of 1.8 sec, compared with 5.4 sec for the T_2 of cytosine H-6. In order to assess the importance of *inter*molecular dipolar relaxation, the relaxation times of cytidine H-6 were measured as a function of concentration. Although at high concentrations this relaxation time begins to decrease, the change in T_2 is negligible over the concentration range at which the experiments with aspartate transcarbamylase were carried out (~100–25 mM).

Effect of Aspartate Transcarbamylase on Proton Relaxation of Several Pyrimidine Nucleosides. C-6 PROTON RELAXATION. The effect of the native enzyme on relaxation times of the C-6 proton of 5-methylcytidine is shown in Figure 1. Qualitatively similar results are obtained from the C-6 proton of cytidine, uridine, and thymidine. Table I includes data for all nucleosides studied in the form of the slope of relaxation rates *vs.* the enzyme–inhibitor concentration ratio.

As predicted by eq 2 and 3, the effect is directly proportional to the fraction of [bound nucleoside]/[total nucleoside] in the limit that the nucleoside concentration is sufficient to saturate the enzyme sites. These results indicate that a specific nucleoside–enzyme interaction is being observed; the magnitude of the effects observed roughly parallels the inhibitory strength of the nucleosides. An analysis of the relaxation rates using the chemical exchange equations 2 and 3 is given below.

In order to obtain more quantitative information from the results summarized in Table I, it is first necessary to subtract from the observed relaxation rate, $1/T_2$, the relaxation rate of the free inhibitor, $1/T_2(I)$. This latter term does not necessarily remain unchanged as enzyme is added and may be increased by the viscosity increase produced by the enzyme (Marshall *et al.*, 1972). As discussed by Sykes *et al.* (1970) and Marshall *et al.* (1972) under certain rather general conditions an indication of the importance of the viscosity and other solution effects is given by the change in $1/T_1$. If (1) the bound ligand I is relaxed by a dipolar mechanism, (2) the protein is large enough so that it has a long correlation time and consequently $T_1(EI) \gg T_2(EI)$, and (3) the exchange rate is sufficiently rapid so that $\tau \ll T_1(EI)$, then the term $P/[\tau + T_1(EI)]$ in eq 2 is negligible and $T_1 \simeq T_1(I) = T_2(I)$. This appears to be true for the cases studied here. Thus, the exchange contribution to the transverse relaxation rate is given by $1/T_2 - 1/T_1(I) \simeq 1/T_2 - 1/T_1$.

TABLE I: Nuclear Relaxation of Nucleoside C-6 Protons.[a]

Nucleoside	Aspartate Transcarbamylase[b] $1/PT_2$ at 220 MHz	Aspartate Transcarbamylase[b] $1/PT_2$ at 100 MHz	Aspartate Transcarbamylase[b] $1/PT_1$ at 220 MHz	Aspartate Transcarbamylase[b] $1/PT_1$ at 100 MHz	Zinc Regulatory Subunit[c] $1/PT_2$ at 220 MHz	Zinc Regulatory Subunit[c] $1/PT_1$ at 200 MHz
5-Methylcytidine	1100	1100	300	300	52	14
Cytidine	920	680	440	440	38	16
Uridine	300	300	230	180		
Thymidine	240		80			

[a] Slopes of plots of $1/T_2$ and $1/T_1$ as functions of enzyme concentration (*e.g.*, Figures 1 and 3). Values reported are best fits of the data to a straight line. [b] Conditions as in Figure 1 except values are calculated on the basis of three sites per 310,000 daltons (see text). [c] 0.04 M phosphate–2 mM 2-mercaptoethanol (pH* 7.7). Assumes 1 site per 17,000 daltons.

The results obtained for the C-6 proton of 5-methylcytidine are considered first. Since no frequency dependence is observed, any approximations of the exchange term which involve Δ are inapplicable. A study of the temperature dependence of $1/T_2$ and $1/T_1$ (Figure 2) indicates that the fast exchange approximation is applicable, since the exchange contribution to $1/T_2$ decreases with increasing temperature. This conclusion makes possible a calculation of $P/T_2(EI)$, where P is the ratio of bound to total inhibitor. In order to obtain $1/T_2(EI)$, however, it is necessary to know the number of enzyme sites to which the nucleoside can bind and to determine whether each of these sites is characterized by the same $1/T_2(EI)$. A number of studies of the binding of CTP by aspartate transcarbamylase indicate that the nucleotide binding sites are not all equivalent (Winlund and Chamberlin, 1970; Buckman, 1970; Cook, 1972; Matsumoto and Hammes,

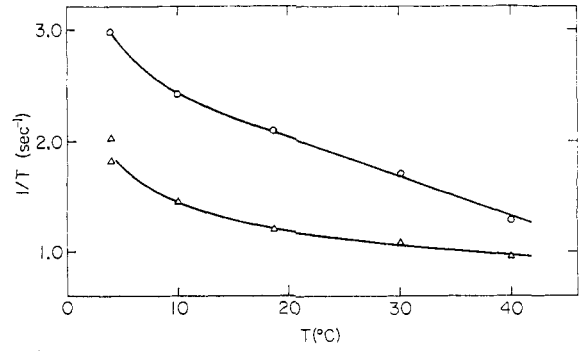

FIGURE 2: Relaxation rates of the C-6 proton of 5-methylcytidine as a function of temperature: (○) $1/T_2$ at 220 MHz; (△) $1/T_1$ at 220 MHz. The sample contained 0.05 M 5-methylcytidine and 7.75 mg/ml of asparate transcarbamylase. The buffer was as described in Figure 1.

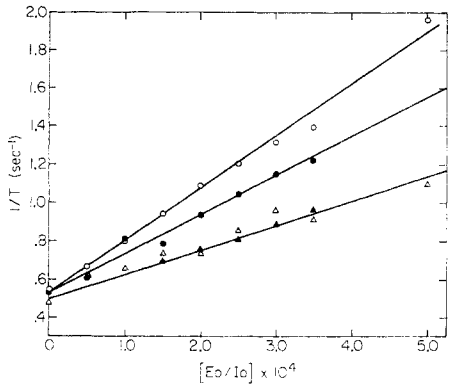

FIGURE 3: Relaxation rates of the C-6 proton of cytidine as a function of the concentration ratio of total enzyme to total cytidine, $[E]_0/[I]_0$ (no correction has been made for the number of sites): (○) $1/T_2$ at 220 MHz; (●) $1/T_2$ at 100 MHz; (△) $1/T_1$ at 220 MHz; (▲) $1/T_1$ at 100 MHz. Data were taken at 28° in a pH* 7.0 D_2O buffer containing 0.01 M imidazole acetate, 2 mM 2-mercaptoethanol, and 0.2 mM EDTA. Successive additions of enzyme were made to a solution initially containing 0.1 M cytidine.

1973). Data obtained in the nucleoside–nucleotide competition studies presented below suggest that a similar inequivalence may characterize cytidine binding. If the cytidine binding sites are inequivalent, they may not contribute equally to the observed relaxation. Specifically, $1/T_2$(EI) for each class of sites will reflect the conformation and the motional freedom of the bound nucleoside and an intermolecular contribution due to dipolar coupling with enzyme protons. It is probable that weaker binding sites would be characterized by smaller values of $1/T_2$(EI) due to greater motional freedom of the bound nucleoside and fewer contacts with the protein residues leading to a smaller intermolecular contribution. In addition, the concentration of 5-methylcytidine used may not be sufficient to saturate weak binding sites. It is therefore assumed for the remaining calculations that the data reflect primarily a contribution from three strong binding sites. This assumption introduces a possible error into the values obtained for T_2(EI). Since the relaxation data are accurate to within ~10%, the error imposed by the assumption of the number of binding sites is potentially much larger. However, it is unlikely that there is much variation in the number of sites with which the various nucleosides studied can interact since they have in common a large number of potential points of interaction with the enzyme. Thus, in spite of the uncertainty in the values of T_2(EI), a comparison of the values obtained with the different nucleosides can yield significant differences. Letting $T_2(I) = T_1$, a value of 1.2×10^{-3} sec is obtained for T_2(EI).

The cytidine C-6 proton relaxation data are qualitatively similar to the data obtained for the C-6 proton of 5-methylcytidine; however, there are some significant quantitative differences. The T_2 values exhibit a dependence on the nmr frequency while the T_1 values do not (Figure 3). The temperature-dependence study at 220 MHz showed results similar to those obtained with 5-methylcytidine; however, $1/T_1$ decreases with increasing temperature almost as rapidly as $1/T_2$. Thus, the exchange term has only a slightly negative temperature dependence. Measurements made at two frequencies are insufficient to determine τ, Δ, and T_2(EI). Although attempts were made to measure Δ directly, reliable data could not be obtained since (1) only the fraction $P\Delta$ can be measured and this is probably less than 1 Hz for the available range of concentrations and (2) chemical shifts due to intermolecular association of nucleosides interfere with the measurements (Schweizer *et al.*, 1968; Raszka and Kaplan 1972). If the small frequency dependence is neglected, a value of 2.7×10^{-3} sec is obtained for T_2(EI). Alternatively, if Δ is sufficiently large so that $1/T_2(EI) \simeq \tau\Delta^2$ and the exchange rate is fast enough so that $1/\tau^2 \gg [1/T_2(EI)^2]^2, \Delta^2$ then the exchange contribution is given by $P(1/T_2(EI) + \tau\Delta^2)$ and we find $T_2(EI) = 5.3 \times 10^{-3}$ sec. Both results are obtained assuming the same number of binding sites as in the 5-methylcytidine case, $n = 3$. The two values obtained probably represent upper and lower limits to the value of T_2(EI) in this case. It should be noted that it is impossible to describe the cytidine relaxation data in any exchange approximation if the value of T_2(EI) is assumed to be the same as that determined for the C-6 proton of 5-methylcytidine. Thus, there is a significant difference in the T_2(EI) values for the methylated and unmethylated nucleosides.

A larger value of $1/T_2$(EI) for the C-6 proton of 5-methylcytidine is expected due to the greater intramolecular dipolar contribution of the C-6–methyl interaction as compared with the C-6–C-5 interaction in cytidine. Additional differences may arise from differences in the bound orientation of the molecule resulting from the steric effects of the methyl group. Factors in addition to the difference between the values of $1/T_2$(EI) which could lead to differences in the data for cytidine and 5-methylcytidine are differences in Δ caused by the presence of the methyl group and a difference in τ. The methyl group may sterically interfere with the binding causing the mean residence time of the nucleoside on the protein, τ, to be shorter so that the inequality $1/T_2(EI) \gg \tau\Delta^2$ is true for 5-methylcytidine but is not as valid for cytidine.

The cytidine relaxation data can also be used to explore some of the underlying assumptions about the relative importance of the terms P/T_1(EI) and $1/T_1$(I) in determining the observed T_1. If the change in the observed T_1 arises from a contribution of the P/T_1(EI) term, and assuming that T_1(EI) results from a dipolar mechanism as described by eq 5, a correlation time for the bound species can be calculated by setting the ratio of the slopes of $(1/T_2)/(1/T_1)$ *vs.* P equal to the ratio of eq 4/eq 5. Using the values obtained at 220 MHz for cytidine, $\omega_0\tau_R = 1.01$ and $\tau_R = 7.3 \times 10^{-10}$ sec. This value of τ_R cannot be correct, however, since it predicts a frequency dependence of T_1 which is not observed. The ratio for $T_1(EI)^{100}/T_1(EI)^{220}$ is calculated to be 2.35 using the value of τ_R given above; the observed ratio is 1, *i.e.*, no frequency dependence. Therefore, most of the change in T_1, if not all of it, reflects a change in T_1(I) which results from the change in viscosity accompanying the addition of protein. Furthermore, the absence of a frequency dependence in T_1 and the result that $T_1(EI) > T_2(EI)$ lead to the conclusion that the correlation time for the bound C-6 proton is sufficiently long so that $(\omega_0\tau_R)^2 \gg 1$ for both frequencies, which is consistent with a value of τ_R expected for the entire macromolecule.

THYMIDINE AND URIDINE RELAXATION. As seen in Table I, the relaxation times of the C-6 proton of uridine and thymidine are affected much less than the relaxation times of the C-6 proton of cytidine and 5-methylcytidine. The slopes of $1/T_2$ and $1/T_1$ for the C-6 proton of uridine were found to be nearly equal, so most of the effect of the enzyme in this case is probably the result of an increase in viscosity. However, there is a significant difference between the slopes of $1/T_2$ and $1/T_1$ for the C-6 proton of thymidine. A somewhat larger effect is expected for thymidine than for uridine since (1) thymidine triphosphate is a slightly stronger inhibitor than uridine triphosphate (Gerhart and Pardee, 1962) and (2) as in the case of 5-methylcytidine, the dipolar interaction of

the methyl group with the C-6 proton should enhance the relaxation rate for thymidine. Since thymidine triphosphate is a weak allosteric inhibitor, it is probable that thymidine has a mean residence time, τ, no larger than cytidine or 5-methylcytidine. Assuming that τ is sufficiently short so that $1/\tau^2 \gg 1/\tau_2(\mathrm{EI})^2$ and Δ^2, and $1/T_2(\mathrm{EI}) \gg \tau\Delta^2$, the exchange contribution to $1/T_2$ is given by $P/T_2(\mathrm{EI})$. Assuming as before that thymidine is binding to three sites gives a value of 170 sec^{-1} for $1/T_2(\mathrm{EI})$.

It is interesting to compare the bound relaxation times of the 5-methylcytidine and thymidine C-6 protons since in both cases the methyl C-6 contribution to $1/T_2(\mathrm{EI})$ is present. The large difference could result from differences in both the intra- and intermolecular dipolar interactions. Lanir and Navon (1971) have found a significant intermolecular contribution to the aromatic protons of several sulfonamides when bound to carbonic anhydrase. However, if the bound nucleoside has a conformation similar to that in solution (the anti conformation), the proximity of the ribose protons will lead to a large intramolecular contribution to $1/T_2(\mathrm{EI})$. Thus, since the methyl protons and very possibly the ribose protons are likely to make an important contribution to the relaxation, the large difference may reflect differences in the intramolecular contribution resulting from greater motional freedom of bound thymidine. This question is addressed more quantitatively in the following section.

METHYL PROTON RELAXATION. In addition to studies of the pyrimidine C-6 proton relaxation times, the effect of the native enzyme on the relaxation times of the methyl protons of thymidine and 5-methylcytidine was determined (Table II). As discussed above, the thymidine exchange rate is probably sufficiently rapid so that the exchange contribution to $1/T_2$ is given by $1/T_2(\mathrm{EI})$; the absence of a frequency dependence of the 5-methylcytidine relaxation rates and the fact that the chemical exchange of the C-6 proton of 5-methylcytidine can also be treated by the $P/T_2(\mathrm{EI})$ approximation indicate that the methyl exchange parameters satisfy similar limiting conditions. Bound relaxation rates are then calculated to be 110 and 770 sec^{-1} for the methyl protons of thymidine and 5-methylcytidine, respectively. Assuming that the rotation about the methyl axis is rapid relative to the rotational correlation time of the enzyme-bound nucleoside, and assuming that the relaxation process reflects primarily the intramolecular dipolar coupling between the methyl protons (Lanir and Navon, 1971), rotational correlation times for the nucleoside base are calculated to be 3×10^{-8} sec for thymidine and 2×10^{-7} sec for 5-methylcytidine, where the equations of Marshall *et al.* (1972) have been used to describe relaxation in the presence of rapid internal motion. The rotational correlation time of native aspartate transcarbamylase can be estimated from the correlation times reported for other proteins; Shimshick and McConnell (1972) find a correlation time of 1.2×10^{-8} sec for chymotrypsin, and Allerhand *et al.* (1971) calculate a τ_R of 3×10^{-8} sec for ribonuclease. Assuming that the correlation time is proportional to molecular weight, the τ_R of aspartate transcarbamylase (mol wt 310,000) would be at least 10^{-7} sec. Long correlation times of this order are predicted from the Stokes–Einstein equation when the viscosity of a concentrated protein solution is taken into account (Koenig and Schillinger, 1969). Therefore, the hypothesis of rapid rotation about the methyl axis leads to a correlation time for the bound 5-methylcytidine which is close to that expected for the entire enzyme molecule, and a correlation time for bound thymidine which is considerably shorter. These results suggest that the bonds formed between the cytosine base and the enzyme, which are important for the regulatory mechanism (Gerhart, 1970; London and Schmidt, 1972), immobilize the cytosine base; the absence of the corresponding thymidine–enzyme bonds leads to greatly decreased inhibitory strength and increased motional freedom of the base when the nucleoside is bound to the enzyme.

TABLE II: Nuclear Relaxation of Nucleoside Methyl Protons in the Presence of Aspartate Transcarbamylase.[a]

Nucleoside	$1/PT_2$	$1/PT_1$
5-Methylcytidine	970	200
Thymidine	190	80

[a] Slopes of plots of $1/T_2$ and $1/T_1$ as a function of enzyme concentration. Calculated assuming three sites per 310,000 daltons (see text). Nucleoside (0.05 M) and buffer as in Figure 1.

SENSITIVITY OF THE C-6 PROTON OF 5-METHYLCYTIDINE TO THE ALLOSTERIC TRANSITION. Following the measurements of the relaxation times of the C-6 proton of 5-methylcytidine reported in Figure 1, saturating levels of carbamyl phosphate (0.02 M) and succinate (0.05 M) were added and the relaxation times remeasured. The $1/T_1$ curve was nearly unaffected but the slope of $1/T_2$ decreased by a factor of 1.6. The results were frequency independent, as was the case prior to the addition of carbamyl phosphate and succinate.

The presence of the substrate analogs would be expected to affect the exchange of the allosteric inhibitor in several ways. (1) The enzyme would have a lower affinity for the inhibitor so that τ, the mean residence time of the nucleoside on the enzyme, would be shortened. (2) The binding site might be altered so that the inhibitor would bind very poorly with much freedom of motion, or so that there would be a decreased interaction between inhibitor and protein residues. Either effect would decrease $1/T_2(\mathrm{EI})$. (3) Some of the allosteric sites might become completely closed to inhibitor binding. This effect would be equivalent to decreasing the enzyme concentration. The first effect mentioned above would not alter the measured value of T_2 since prior to the addition of carbamyl phosphate and succinate the C-6 proton is already in the fast exchange limit so that the exchange lifetime does not affect relaxation. That the fast exchange limit remains applicable after the addition of carbamyl phosphate and succinate is supported by the absence of any frequency dependence. Either of the other two effects mentioned above could produce the observed decrease in the effect on $1/T_2$. The fact that $1/T_1$ remains unchanged supports the conclusion that the longitudinal relaxation rate in this case is affected primarily by viscosity or other effects not related to the specific binding of the nucleoside by the enzyme.

Nucleoside Interactions with the Zinc Regulatory Subunit. Experiments similar to those carried out with the native enzyme were performed with the zinc derivative of the regulatory subunit. A phosphate pH* 7.7 buffer containing 2 mM 2-mercaptoethanol but no EDTA was used. The effect of the regulatory subunit on the relaxation rates of the C-6 proton of cytidine and 5-methylcytidine differed only in magnitude from the effect of the native enzyme; the effect produced by the regulatory subunit is considerably less than that produced by the same concentration of native enzyme sites. This suggests that the nucleoside exchange rate is fast and that the difference reflects a difference in the rotational correlation time and correspondingly $1/T_2(\mathrm{EI})$. It is also probable that

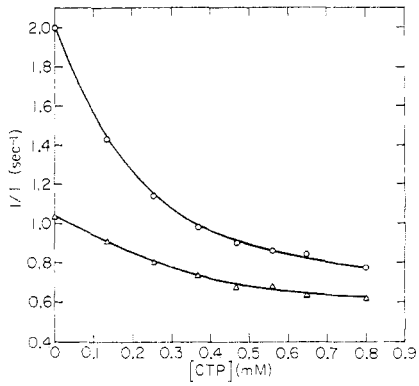

FIGURE 4: Relaxation rates $1/T_2$ (○) and $1/T_1$ (△) of the C-6 proton of cytidine measured at 220 MHz, 28°, as a function of added CTP. Initially, the sample contained 0.05 M cytidine and 2.5×10^{-5} M native aspartate transcarbamylase; the concentration of the stock CTP solution prior to addition to the sample was 2.8×10^{-3} M. Buffer was 0.01 M imidazole acetate (pH* 7.0), 2×10^{-3} M 2-mercaptoethanol, and 2×10^{-4} M EDTA.

the values of τ are similar to or less than the values for the mean residence times of the nucleosides on the native enzyme. Assuming fast exchange with $1/T_2(\mathrm{EI}) \gg \tau\Delta^2$, and one binding site per 17,000 daltons (two sites per regulatory dimer), the value obtained for $1/T_2(\mathrm{EI})$ for the C-6 proton of 5-methylcytidine is 88 sec^{-1} and, for the same proton on cytidine, $1/T_2(\mathrm{EI}) = 22$ sec^{-1}. As in the studies with native aspartate transcarbamylase, $1/T_2(\mathrm{EI})$ is smaller for the cytidine C-6 proton than for the same proton of 5-methylcytidine.

It is interesting to compare the values obtained for $1/T_2(\mathrm{EI})$ with the regulatory subunit to the values obtained with the native enzyme. Equation 4 predicts that if the rotational correlation time is sufficiently long so that $(\omega_0\tau_R)^2 \gg 1$, $1/T_2(\mathrm{EI})$ will be proportional to the molecular weights of the proteins, and using the fact that the zinc derivative of the regulatory subunit exists primarily as a dimer of mol wt 34,000 in solution (Cohlberg *et al.*, 1972), the predicted ratio of the bound transverse relaxation times determined for the regulatory subunit to that determined for the native enzyme should be ~9. The value of this ratio for the C-6 proton of 5-methylcytidine is 22; the value for the C-6 proton of cytidine is in the range 9–17 (due to the uncertainty in the value obtained with the native enzyme).

The difference between the calculated and experimental ratios may reflect several factors unrelated to the mode of binding including viscosity differences (which are small) and differences in shape between the macromolecules. X-Ray diffraction data suggest that the native enzyme is virtually spherical (Evans *et al.*, 1973) and that the regulatory dimer is nearly spherical although other data support a more cylindrical shape for the regulatory subunit (Cohlberg *et al.*, 1972). It is thus probable that differences in shape between regulatory subunit and native enzyme would contribute only a small perturbation to the ratios of relaxation times. Naturally, the calculated ratio is also affected by the choice of number of binding sites. If, for example, the nucleosides interact strongly with only one site on the regulatory dimer, the experimental ratios would become 11 and 4–8 for 5-methylcytidine and cytidine, respectively.

Small structural differences between nucleotide binding sites on native enzyme and regulatory subunit could have very large effects on bound relaxation times due to the r_{ij}^{-6} dependence on the dipolar interaction (eq 4). Inasmuch as the experimental ratios for cytidine and 5-methylcytidine are within approximately a factor of 2 of the calculated ratio of 9, we conclude that the mode of nucleoside binding to zinc regulatory subunit is not greatly different from the mode of binding at sites to which nucleosides bind on native enzyme.

Displacement of Cytidine from Native Aspartate Transcarbamylase by CTP. The effect of the serial addition of CTP on both T_1 and T_2 of the cytidine C-6 proton in the presence of native aspartate transcarbamylase is shown in Figure 4. From these data, the fraction of sites occupied by cytidine ($[\mathrm{EI}]/n[E]_0$) can be computed as function of CTP concentration using eq 6 with a value of 0.03 sec^{-1} for δ_0 as determined from the cytidine relaxation data. A fit of these data can be made to eq 7, or to a sum of terms having the form of eq 7, each representing n_i sites with dissociation constant ratio R_i. In principle, even if each site contributed equally to the cytidine relaxation, it would be necessary to solve for six values of R_i. Such a six-parameter fit would extract more information than can reliably be obtained from the data. However, a number of conclusions concerning the cytidine binding can be obtained by considering several specific cases.

(1) Six equivalent cytidine binding sites are assumed, *i.e.*, each contributes equally to the observed relaxation and is characterized by the same cytidine dissociation constant. There are a number of studies (Winlund and Chamberlin, 1970; Buckman, 1970; Cook, 1972; Matsumoto and Hammes, 1973) which indicate that the affinities of the regulatory sites for CTP are not all equivalent. Winlund and Chamberlin (1970) find a 44-fold difference in the dissociation constants for two classes of three sites each, at 4°, and Matsumoto and Hammes (1973) report a 36-fold difference at 23° and pH 7. Thus, if we assume that there are six equivalent cytidine binding sites, then the cytidine–CTP competition would be characterized by two classes of three sites with values of R differing by at least an order of magnitude. This conclusion is inconsistent with the data obtained. Assuming two classes of three sites and two values of R differing by a factor of 10 or more gives a very poor fit to the data. Alternatively, assuming two classes of three sites and using a computer program for a least-squares fit of the data give for the ratio of each class of sites $R_1 = 1.6 \times 10^{-3}$ and $R_2 = 4.2 \times 10^{-3}$, which differ by considerably less than the amount predicted using the assumption given above. Therefore, the data are inconsistent with the hypothesis of six equivalent cytidine binding sites. This result eliminates the possibility that the inequivalence of the CTP binding sites arises from a direct interference of the phosphate portion of the nucleotides at binding sites located close together.

(2) It is assumed that there are n equivalent sites contributing to the cytidine relaxation, with n and R to be determined by a computer fit of the data to eq 7. This assumption leads to an excellent fit of the data, as shown in Figure 5. The values obtained are $n = 2.4$ sites/moledule and $R = K_D^{CTP}/K_D^{Cyt} = 2.8 \times 10^{-3}$. This value for n is uncertain by at least ± 2 sites and is therefore not significant. The value of R is considerably more reliable. For example, if n is doubled, the least-squares fit gives a value of R which differs by only 20%. The fact that the assumption of a single class of sites leads to so good a description of the data is compatible with either of two conclusions. (a) Most of the contribution to the observed relaxation comes from a single class of equivalent sites. Since the data have already been shown to be inconsistent with the existence of six equivalent cytidine binding sites, the most probable alternative is an interaction with only the three strong binding sites. (b) There are two sets of sites which contribute equivalently to the cytidine relaxation and which

have nearly the same values of R. This implies that the difference in the cytidine dissociation constants for the two sets of sites is roughly equal to the difference found for CTP. If this is true, the dissociation constant for cytidine from the weaker binding sites can be estimated using the value of R determined above along with the K_D for the weak CTP sites found by Matsumoto and Hammes (1973). They find $K_D = 7.1 \times 10^{-4}$ M for CTP binding to the weaker binding sites under conditions (23°, 0.1 M imidazole–acetate (pH 7.0)) similar to those employed here. Using $R = 2.8 \times 10^{-3}$, the estimated value for cytidine interaction with the weak sites is $K_D = 0.2$–0.3 M. This represents very weak binding indeed, and at the concentration of cytidine used (0.05 M) less than 20% of those sites would be saturated. This again leads to the conclusion that most of the observed effect reflects only the interaction with the strong binding sites.

(3) It is assumed that there are two sets of three sites, each having a different affinity for cytidine and making a different contribution to the relaxation rate. In this case, eq 6 is not valid so that the fraction of sites occupied is not given correctly in Figure 5. As disussed earlier in the paper, it is probable that the higher affinity sites would make a proportionately larger contribution to the relaxation rate. Thus, most of the observed effect would reflect only the binding to the strong sites and the values of n and R obtained would refer primarily to the interaction at these sites.

To summarize, the CTP–cytidine competition data indicate that cytidine does not bind with equal affinity to six enzyme sites. It is also probable that most of the observed effect reflects an interaction with only three strong binding sites.

It is interesting to compare the result obtained above for R with the value obtained from steady-state kinetic data. Gerhart (1970) has reported that, in the series of cytidine nucleotides cytidine, CMP, CDP, and CTP, a fourfold increase in concentration is required for each phosphate group removed in order to produce equivalent inhibition. This leads to a value of 1/64 for R, considerably different than the result obtained above.

However, Gerhart's results were obtained in a phosphate (pH 7.0) buffer. We have found that the replacement of 0.04 M phosphate by 0.05 M imidazole acetate considerably reduces the nucleotide concentration needed to produce a given degree of inhibition. Specifically, at 4.5 mM aspartate, 3.6 mM carbamyl phosphate, and 0.75 μg/ml of enzyme, the buffer change mentioned above decreased the nucleotide concentration needed to produce a 10% inhibition by a factor of *ca.* 5 (see Figure 3 of London and Schmidt, 1972). Assuming that the phosphate in the buffer competes for binding sites with the phosphate portion of the nucleotide, but not with cytidine, the result obtained by Gerhart would become $R = 3.1 \times 10^{-3}$ in 0.05 M imidazole acetate, a value close to the nmr result obtained in 0.01 M imidazole acetate. It should be noted, however, that, as discussed above, the value of R obtained probably refers primarily to the competition for the strong binding sites. Matsumoto and Hammes (1973) have shown that the degree of inhibition and activation involves binding to all six sites. Thus, the kinetic data may reflect the average value of R for all of the six sites to a greater extent than the nmr data.

ATP-Induced Displacement of Cytidine from Native Aspartate Transcarbamylase. ATP is also capable of reversing the effect of native aspartate transcarbamylase on the relaxation times of cytidine. Treating the data as in the CTP experiment, $[EI]/n[E]_0$ *vs.* ATP concentration was calculated

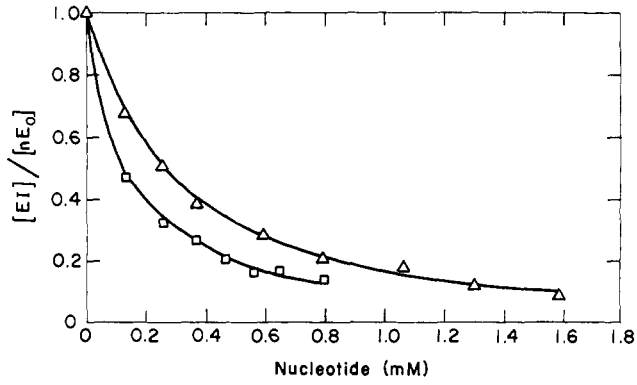

FIGURE 5: The fraction of sites occupied by cytidine, $[EI]/n[E]_0$, as a function of added CTP (□) or ATP (△) as determined from the data in Figure 4 for CTP and analogous data for ATP using eq 6 with a value of 0.03 sec^{-1} for δ_0. The curves represent a least-squares fit of eq 7 to the data assuming n equivalent sites all characterized by the same value of R.

using eq 6. As in the CTP–cytidine experiment, an excellent fit to the data can be made assuming a single class of n equivalent sites with a dissociation constant ratio R (Figure 5). The values obtained for this fit are $n = 2.9$ sites/molecule and $R = K_D^{ATP}/K_D^{Cyt} = 4.8 \times 10^{-3}$. As in the CTP–cytidine experiment, this value of n is uncertain by at least ±2 sites and is therefore not significant. However, for the reasons discussed above, the cytidine relaxation probably reflects an interaction with only three strong binding sites, and the observed effect therefore results from the displacement of cytidine from these sites by ATP. The value obtained for R is estimated to be accurate to within ~40%.

It is interesting to use the CTP–cytidine and ATP–cytidine studies to compute the dissociation constant ratio of ATP to CTP: $K_D^{ATP}/K_D^{CTP} = 1.7$. This value is considerably less than the ratio of ten found by Changeux *et al.* (1968) using equilibrium dialysis. It is probable that most of this difference reflects the different buffers used in the experiments. The data of Changeux *et al.* were obtained in 0.04 M phosphate (pH 7.0); the nmr data were obtained using 0.01 M imidazole acetate (pH* 7.0). As discussed above, the presence of inorganic phosphate reduces the affinity of the enzyme for the phosphate moiety of the nucleotides. In the nmr experiments, the combination of higher values for the nucleotide/nucleoside affinity ratio and less variation between the values of R obtained with ATP and CTP are consistent with a greater relative contribution of the phosphate moiety to the nucleotide binding energy. This conclusion is supported by the fact that the dissociation constant ratio found by Matsumoto and Hammes under conditions similar to those of the nmr study (0.1 M imidazole acetate (pH 7.0) buffer with carbamyl phosphate and succinate present to suppress effects of nonspecific binding) is four.

Another factor which may affect this ratio is the indirect allosteric effect of ATP. A 0.25 mM concentration which, according to our data, has displaced ~48% of the bound cytidine, produces a 41% inhibition of the native enzyme (Matsumoto and Hammes, 1973). It should be noted, however, that the conformational effect of 0.25 mM ATP in the absence of cytidine is probably considerably greater than in the presence of 0.05 M cytidine. Such an indirect effect could involve both structural and affinity changes of the cytidine binding sites.

Since the six regulatory sites have been shown to bind the nucleotides inhomogeneously, a central question has been whether the three low affinity CTP sites are identical with the

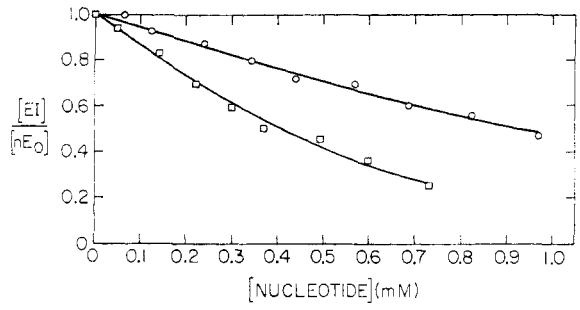

FIGURE 6: The fraction of sites occupied by cytidine, $[EI]/n[E_0]$, as a function of added CTP (□) and ATP (○) in the presence of zinc regulatory subunit. Relaxation time measurements were made at 220 MHz, 28°, in samples initially containing 0.047 M cytidine and 4.04×10^{-4} M regulatory subunit (assuming a mol wt of 17,000). The buffer used was 0.04 M phosphate (pH* 7.7) and 2 mM 2-mercaptoethanol. Stock solutions of nucleotides prior to addition were 2.02×10^{-3} M CTP and 2.74×10^{-3} M ATP. The data were computed using eq 6 with a value of 0.08 sec^{-1} for δ_0. The curves represent a least-squares fit of eq 7 to the data setting $n = 1.0$ site/17,000 daltons.

high affinity ATP sites. The existence of ATP specific sites as a possible basis for the activation mechanism was suggested by Buckman (1970). If the CTP–cytidine competition observed reflects a competition for the strong CTP binding sites, and the ATP–cytidine competition observed reflects a competition for the weak ATP binding sites, then ATP should be far less effective than CTP at displacing cytidine. Using the values obtained by Matsumoto and Hammes (1973) for the dissociation constant of CTP from the strong CTP binding sites and for the dissociation constant of ATP from the weak ATP binding sites, the ATP/CTP dissociation constant ratio obtained is 145. This leads to the conclusion that an extremely large indirect effect of the ATP binding would have to be present to account for the measured ratio of 1.7. Alternatively, if the strong and weak sites are not structurally different but reflect only an indirect negative interaction between pairs of regulatory nucleotides, then the ability of CTP or ATP to displace cytidine reflects primarily the dissociation constants from the strong sites in each case, which leads to a prediction of an ATP/CTP dissociation constant ratio of four. Our data are clearly more consistent with the latter interpretation.

The strong competition observed between ATP and cytidine is consistent with a proposal by Changeux *et al.* (1968) as well as with a more recently proposed model by the authors (London and Schmidt, 1972) that activation and inhibition can be produced by the interaction of the appropriate nucleotide with the *same* enzyme sites. Nevertheless, the inhomogeneity of CTP binding and, as the current study suggests, cytidine binding, indicates that in spite of the hexameric structure of the enzyme, the six regulatory sites are not all equivalent. These two conclusions are not incompatible and can both be incorporated into a more complete description of the allosteric mechanism. As noted in the preceding paragraph, the ATP–cytidine competition data appear to be incompatible with a model which assumes structural differences between three ATP specific activating sites and three CTP specific inhibitory sites.

Nucleotide Displacement of Cytidine from the Zinc Regulatory Subunit of Aspartate Transcarbamylase. Experiments similar to those described with native aspartate transcarbamylase were performed using the zinc regulatory subunit, the preparation of which has been described by Nelbach *et al.* (1972). The results obtained with CTP and ATP are qualitatively similar to the results obtained with the native enzyme. In addition to experiments similar to those previously described, the effect of ATP on a solution containing cytidine but no enzyme was also studied. It was found that the presence of the nucleotide in the concentration range tested has no effect on the cytidine C-6 proton relaxation times. This eliminates the possibility that a direct interaction or an indirect effect of the nucleotides, such as the formation of complexes with metal ions in solution, contributes to the observed effect.

Interpretation of the data obtained with the regulatory subunits is more difficult than in the case of the native enzyme for two reasons: (1) the size of the effect is considerably smaller so that the error, particularly in the measurement of T_1, is relatively large and (2) there is a relatively large difference between T_1 and T_2 in the absence of enzyme. In order to minimize the first source of error, a smooth curve was drawn through the T_1 data, and points from the curve were used. This procedure was not necessary for the T_2 data which can be determined with excellent precision using the $T_{1\rho}$ method. A value of 0.08 sec^{-1} was used for δ_0 based on the data for cytidine relaxation in the 0.04 M phosphate pH* 7.7 buffer used for studies of the regulatory subunit. Although attempts were made to fit the data by varying n and R, the values obtained for n were extremely sensitive to the value chosen for δ_0, varying from 0.3 to 2.0 sites/mol wt 17,000 as δ_0 is varied by 0.01 sec^{-1}. Since the values of R show considerably less variation, n was set at 1.0/17,000 mol wt and the best R was then determined. This choice of n is based on the CTP binding data of Rosenbusch and Weber (1971). The calculated values of $[EI]/n[E]_0$, shown in Figure 6, gave $R = 6.5 \times 10^{-3}$ in the CTP–cytidine experiment, and $R = 2.7 \times 10^{-2}$ in the ATP–cytidine experiment. As indicated in the figure, the buffer used was 0.04 M phosphate (pH* 7.7) including 2 mM 2-mercaptoethanol (a pH* 7.0 imidazole acetate buffer was found to precipitate the regulatory subunit). Due to the different buffers, the results obtained with the regulatory subunit cannot be compared readily with the data obtained usng the native enzyme. The larger values of R are expected as a result of using the phosphate buffer.

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References

Abragam, A. (1961), The Principles of Nuclear Magnetism, Oxford, Clarendon Press, p 34.

Allerhand, A., Doddrell, D., Glushko, V., Cochran, D. W., Wenkert, E., Lawson, P. J., and Gurd, F. R. N. (1971), *J. Amer. Chem. Soc. 93*, 544.

Beard, C. B., and Schmidt, P. G. (1973), *Biochemistry 12*, 2255.

Buckman, T. (1970), *Biochemistry 9*, 3255.

Carrington, A., and McLachlan, A. D. (1967), Introduction to Magnetic Resonance, New York, N. Y., Harper and Row, p 192.

Changeux, J.-P., Gerhart, J. C., and Schachman, H. K. (1968), *Biochemistry 7*, 531.

Cohen, R. M., and Wolfenden, R. (1971a), *J. Biol. Chem. 246*, 7561.

Cohen, R. M., and Wolfenden, R. (1971b), *J. Biol. Chem. 246*, 7566.

Cohlberg, J. A., Pigiet, V. P., Jr., and Schachman, H. K. (1972), *Biochemistry 11*, 3396.
Cook, R. A. (1972), *Biochemistry 11*, 3792.
Evans, D. R., Warren, S. G., Edwards, B. F. P., McMurray, C. H., Bethge, P. H., Wiley, D. C., and Lipscomb, W. N. (1973), *Science 179*, 683.
Gerhart, J. C. (1970), *Curr. Top. Cell. Regul. 2*, 275.
Gerhart, J. C., and Holoubek, H. (1967), *J. Biol. Chem. 242*, 2886.
Gerhart, J. C., and Pardee, A. B. (1962), *J. Biol. Chem. 237*, 891.
Hammes, G. G., Porter, R. W., and Wu, C. W. (1970), *Biochemistry 9*, 2992.
Kirschner, M. W. (1971), Ph.D. Thesis, University of California, Berkeley, Calif.
Koenig, S. H., and Schillinger, W. E. (1969), *J. Biol. Chem. 244*, 3283.
Lanir, A., and Navon, G. (1971), *Biochemistry 10*, 1024.
London, R. E., and Schmidt, P. G. (1972), *Biochemistry 11*, 3136.
Luz, Z., and Meiboom, S. (1964), *J. Chem. Phys. 40*, 2686.
Marshall, A. G., Schmidt, P. G., and Sykes, B. D. (1972), *Biochemistry 11*, 3875.
Matsumoto, S., and Hammes, G. G. (1973), *Biochemistry 12*, 1388.
McMurray, C. H., Evans, D. R., and Sykes, B. D. (1972), *Biochem. Biophys. Res. Commun. 48*, 572.
Mildvan, A. S., and Cohn, M. (1970), *Advan. Enzymol. Relat. Areas Mol. Biol. 33*, 1.
Nelbach, M. E., Pigiet, V. P., Jr., Gerhart, J. C., and Schachman, H. K. (1972), *Biochemistry 11*, 315.
Porter, R. W., Modebe, M. O., and Stark, G. R. (1969), *J. Biol. Chem. 244*, 1846.
Raszka, M., and Kaplan, N. O. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 2025.
Rosenbusch, J. P., and Weber, K. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 1019.
Schweizer, M. P., Broom, A. D., Ts'o, P. O. P., and Hollis, D. P. (1968), *J. Amer. Chem. Soc. 90*, 1042.
Shimshick, E. G., and McConnell, H. M. (1972), *Biochem. Biophys. Res. Commun. 46*, 321.
Swift, T. J., and Connick, R. E. (1962), *J. Chem. Phys. 37*, 307.
Sykes, B. D. (1969), *J. Amer. Chem. Soc. 91*, 949.
Sykes, B. D., Schmidt, P. G., and Stark, G. R. (1970), *J. Biol. Chem. 245*, 1180.
Sykes, B. D., and Scott, M. D. (1972), *Annu. Rev. Biophys. Bioeng. 1*, 251.
Sykes, B. D., and Wright, J. M. (1970), *Rev. Sci. Instrum. 41*, 876.
Taylor, P. W., Feeney, J., and Burgen, A. S. V. (1971), *Biochemistry 10*, 3866.
Winlund, C. C., and Chamberlin, J. J. (1970), *Biochem. Biophys. Res. Commun. 40*, 43.

Mouse Ascites Sarcoma 180 Deoxythymidine Kinase. General Properties and Inhibition Studies†

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ABSTRACT: Thymidine kinase derived from mouse Sarcoma 180 cells has been partially purified. Although only a ninefold purification has been achieved, the enzyme preparation is devoid of adenosine triphosphatase, deoxythymidine triphosphatase, thymidylate phosphatase, nucleoside diphosphokinase, thymidylate kinase, phosphodiesterase, nucleoside phosphotransferase, and DNA polymerase. The stability of the enzyme decreased as purity increased. Thymidine or rATP partially prevented inactivation; however, mercaptoethanol, in contrast to studies with this enzyme derived from other sources, markedly inactivated the enzyme. When the molar ratio of magnesium to rATP exceeded unity, inhibition of thymidine kinase activity resulted. Various nucleoside triphosphates were evaluated for phosphate donor capabilities and dTTP and 5-iodo- and 5-bromo-2′-deoxyuridine 5′-triphosphate were not only inactive but also prevented utilization of rATP. A comparison of rATP, dATP, and araATP showed no effect of the sugar moiety on their binding ability since the K_m for all three are identical; however, araATP relative to rATP and dATP was a poor phosphate donor. Various 5′ derivatives of thymidine were evaluated as inhibitors of thymidine kinase and the order of inhibition is $NH_2 > Br > H = Cl > F = I > PO_4 > CO_2H = NHCOCH_2Br$. Kinetic analysis showed 5′-amino-, fluoro-, or chlorothymidine to be competitive inhibitors of thymidine, the K_I for 5′-aminothymidine being 3 μM. Although subtle changes in the 2′ position of thymidine alter the binding affinity to the enzyme, considerable bulk tolerance is permitted in the 3′ but not the 5′ position.

Thymidine kinase (dThd-kinase) catalyzes the phosphorylation of dThd[1] to form dTMP in the presence of a nucleoside 5′-triphosphate donor such as ATP and a divalent cation such as Mg^{2+} (Ives *et al.*, 1963; Okazaki and Kornberg, 1964a,b). The activity of dThd-kinase appears in general to be closely related to the proliferative ability of the cell. Thus, the catalytic activity is elevated in regenerating liver (Bresnick *et al.*, 1967), DNA viral infected cells (Kit *et al.*, 1966; Kara and Weil, 1967; Sheinin, 1966), neoplastic tissues (Bresnick and Thompson, 1965; Hashimoto *et al.*, 1972), and cells entering the S phase of the cell cycle (Brent, 1971). Some

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[1] Abbreviations used are: dThd, deoxyribosylthymine (thymidine); dThd-kinase, thymidine kinase; dTMPase, deoxythymidylate phosphatase; aradThd, thymine arabinoside; 3′-NH_2-dThd, 2′-deoxy-3′-aminothymidine.