ethanol solution containing a linear gradient of 0.1–1.0 M ammonium chloride (pH 8.5) at a flow rate of 17 ml/hr. All the products of these addition reactions were desalted on columns of Bio-Gel P-2 polyacrylamide gel, as above described.

*Addition of Deoxycytidine Phosphate to pdT-dT$_2$-A.* The reaction mixture consisted of the buffer, 1 M sodium tris(hydroxymethyl)methylaminopropanesulfonate, pH 9 at 20° (10 $\mu$l), 0.1 M manganese chloride (10 $\mu$l), 0.05 M deoxycytidine 5′-diphosphate (10 $\mu$l), pdT-dT$_2$-A (4.0 ODU$_{260nm}$) in water (50 $\mu$l), and 20 $\mu$l of *M. luteus* polynucleotide phosphorylase (10 units/ml). After incubation at 37° for 20 hr the mixture was separated on a column (40 × 0.4 cm) of Dowex 1-X2 (−400 mesh) using 200 ml of a 20% ethanol solution containing a linear gradient of 0.1–1.0 M ammonium chloride (pH 8.5). The elution pattern showed that all of the starting material had been converted to products and that the ratio of products pdT-dT$_2$-A-dC-dC:pdT-dT$_2$-A-dC was 4:1. The retention volume of the hexanucleotide product is listed in Table I.

References


Bennett, G. N., Mackey, J. K., Wiebers, J. L., and Gilham, P. T. (1973), *Biochemistry 12*, 3956.

Bon, S., Godefroy, T., and Grunberg-Manago, M. (1970), *Eur. J. Biochem. 16*, 363.

Canellakis, E. S., and Canellakis, Z. N. (1963), Informational Macromolecules, New York, N. Y., Academic Press, p 107.

Chou, J. Y., and Singer, M. F. (1971), *J. Biol. Chem. 246*, 7505.

Feix, G. (1972), *Biochem. Biophys. Res. Commun. 46*, 2141.

Garen, A., and Levinthal, C. (1960), *Biochim. Biophys. Acta 38*, 470.

Ho, N. W. Y., and Gilham, P. T. (1973), *Biochim. Biophys. Acta 308*, 53.

Kaufmann, G., and Littauer, U. Z. (1969), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 4*, 79.

Khorana, H. G., and Vizsolyi, J. P. (1961), *J. Amer. Chem. Soc. 83*, 675.

Khorana, H. G., Vizsolyi, J. P., and Ralph, R. K. (1962), *J. Amer. Chem. Soc. 84*, 414.

Kondo, N. S., Fang, K. N., Miller, P. S., and Ts'o, P. O. P. (1972), *Biochemistry 11*, 1991.

Mackey, J. K., and Gilham, P. T. (1971), *Nature (London) 233*, 551.

Moon, M. W., Nishimura, S., and Khorana, H. G. (1966), *Biochemistry 5*, 937.

Rammler, D. H., and Khorana, H. G. (1962), *J. Amer. Chem. Soc. 84*, 3112.

Rosenberg, M., and Gilham, P. T. (1971), *Biochim. Biophys. Acta 246*, 337.

Rosenberg, M., Wiebers, J. L., and Gilham, P. T. (1972), *Biochemistry 11*, 3623.

Schott, H., Rudloff, E., Schmidt, P., Roychoudhury, R., and Kössel, H. (1973), *Biochemistry 12*, 932.

Singer, M. F. (1966), *Procedures Nucl. Acid Res.*, 245.

Sulkowski, E., and Laskowski, M. (1971), *Biochim. Biophys. Acta 240*, 443.

Thach, R. E., and Doty, P. (1965a), *Science 147*, 1310.

Thach, R. E., and Doty, P. (1965b), *Science 148*, 632.

Tomlinson, R. V., and Tener, G. M. (1963), *Biochemistry 2*, 697.

Volkin, E., and Cohn, W. E. (1953), *J. Biol. Chem. 205*, 767.

Weith, H. L., Wiebers, J. L., and Gilham, P. T. (1970), *Biochemistry 9*, 4396.


# Interaction of Guanine Ligands with Ribonuclease T$_1$†

Frederick G. Walz, Jr.,* and Lois L. Hooverman

ABSTRACT: Difference spectral measurements of the binding of ribonuclease T$_1$ with a variety of guanine ligands conducted at pH 5.0, 0.2 M ionic strength, and 25° were used to determine association constants and maximal difference molar extinction coefficients, $\Delta\epsilon$, at 290 nm. The following order of affinities for ribonuclease T$_1$ binding were found: Guo-2′-P > Guo-3′-P > dGuo-3′-P > Guo-5′-P = dGuo-5′-P > Guo > dGuo. In all cases the binding stoichiometry was found to be 1:1 within experimental error. The qualitative nature of the difference spectra was similar for all the ligands tested except for a minor variation found with dGuo-3′-P. Values of $\Delta\epsilon$ showed a considerable variation and were greater than those found for the acid pH-induced difference spectra of Guo-3′-P and dGuo-3′-P. The results are compared with those of previous studies and are contrasted with results from similar studies on ribonuclease A with emphasis on the interaction of the ligand 2′-hydroxyl group with these enzymes.

It is an interesting fact that ribonuclease T$_1$ (EC 2.7.7.26) is an unusually acidic protein (Egami *et al.*, 1964) because its substrate, RNA, is a negatively charged polymer. Since polyanionic inhibitors of ribonuclease A (a basic protein) do not inhibit ribonuclease T$_1$ (Egami *et al.*, 1964) it appears likely that there are few, if any, nonspecific electrostatic interactions contributing to the negative free energy of binding for this enzyme with RNA. This apparent lack of utilization of electrostatic binding in the formation of the ribonuclease T$_1$–RNA complex presents an interesting problem regarding protein–nucleic acid interactions.

In an effort to elucidate the specific enzyme–substrate interactions of ribonuclease T$_1$ many investigations have been

† From the Department of Biological Sciences, State University of New York at Albany, Albany, New York 12222. *Received June 27, 1973.* This work was supported by National Science Foundation Grant B033468.

reported on the binding of the enzyme with different low molecular weight substrate analogs using a variety of techniques including inhibition kinetics (Irie, 1967, 1968) difference spectroscopy (Sato and Egami, 1965; Epinatjeff and Pongs, 1972; Oshima and Imahori, 1971a), circular dichroism (Sander and Ts'o, 1971; Oshima and Imahori, 1971b), gel filtration (Sato and Egami, 1965; Takahashi, 1970; Cambell and Ts'o, 1971), nuclear magnetic resonance (nmr) (Rüterjans *et al.*, 1969; Rüterjans and Pongs, 1971), and fluorescence (Pongs, 1970). The results of these studies appeared to corroborate a 1:1 enzyme–ligand binding stoichiometry and the following relative affinities of the substrate analogs with ribonuclease $T_1$: Guo-2′-P[1] > Guo-3′-P > Guo-5′-P > Guo. Unfortunately, the reported association constants for ribonuclease $T_1$ binding with these ligands are not in good agreement, in some cases varying over an order of magnitude which appears to be beyond the deviations expected for differences in experimental conditions and technique. Nevertheless, the relationship of affinities of the guanine ligands with ribonuclease $T_1$ was the same as that for ribonuclease A binding with analogous pyrimidine nucleotides and nucleosides (Anderson *et al.*, 1968; Richards and Wyckoff, 1971); in view of the similar reactions catalyzed by these enzymes (Egami *et al.*, 1964) perhaps this similarity is not particularly surprising.

The present report concerns the binding of Guo-2′-P, Guo-3′-P, dGuo-3′-P, Guo-5′-P, dGuo-5′-P, Guo, and dGuo with ribonuclease $T_1$. Difference spectroscopy was the technique employed and experiments were conducted under conditions where results could readily be compared with those from other studies. In addition to the determination of association constants, difference extinction coefficients were determined to further characterize the enzyme–substrate analog interaction. In two of the cases studied (Guo-3′-P and Guo) the presence of a 2′-hydroxyl group on the ligand is shown to considerably enhance binding when compared to binding the 2′-deoxy analogs; interestingly, this result is the reverse of that obtained for ribonuclease A with Urd-3′-P (Walz, 1971). Although the proposal that the difference spectra of guanine derivatives binding with ribonuclease $T_1$ results from ring protonation of the bound guanine group (Epinatjeff and Pongs, 1972; Oshima and Imahori, 1971a) is again corroborated in the present study, it is shown that additional factors are needed to account for the observed spectral perturbations.

## Experimental Procedure

*Materials.* RIBONUCLEASE $T_1$. The preparation described by Fields *et al.* (1971) was used to purify ribonuclease $T_1$ from 500 g of Sanzyme-R powder (Calbiochem). The final DEAE-cellulose chromatographic fractions containing the purified enzyme were desalted using a Bio-Rad hollow fiber dialyzer. After lyophilization the material was chromatographed on a Sephadex G-50 column (2.6 × 75 cm) using 0.025 M ammonium acetate as an eluent at a flow rate of 1.5 ml/min. In this way traces of an unanalyzed yellow-brown impurity could be separated from the enzyme since ribonuclease $T_1$ had a smaller elution volume. The colorless ribonuclease $T_1$ fractions were then pooled and lyophilized. This material showed a single band (*i.e.*, >98%) on 10 and 15% polyacrylamide gels and 7.5% sodium dodecyl sulfate–polyacrylamide gels. At high concentrations of the purified enzyme (~3 mg/ml) there was evidence of deoxyribonuclease activity which was specific for single-stranded DNA and completely digested sonicated, denatured calf thymus DNA to its component nucleotides. This activity probably results from a minor contamination by nuclease $S_1$ (Ando, 1966) and could be removed by heating concentrated enzyme solutions at pH 6.5 to 100° for 5 min; a slight precipitate formed during this treatment which was removed by filtration. The specific activity of all the ribonuclease $T_1$ preparations agreed with that determined previously (Fields *et al.*, 1971) and was not affected by the heat treatment. The concentration of ribonuclease $T_1$ was determined spectrophotometrically using an extinction coefficient of $2.1 \times 10^4$ $M^{-1}$ $cm^{-1}$ at 278 nm (Egami *et al.*, 1964).

SUBSTRATE ANALOGS. All guanine nucleosides and nucleotides including Guo-2′-P, Guo-3′-P, Guo-5′-P, dGuo-3′-P, dGuo-5′-P, Guo, and dGuo were obtained from P-L Laboratories; Guo-3′-P and Guo-2′-P gave single spots on thin-layer chromatography (Kumar *et al.*, 1972). The concentrations of all guanine nucleosides and nucleotides were determined spectrophotometrically using an extinction coefficient at 253 nm of 13,700 $M^{-1}$ $cm^{-1}$ at pH 7.0.

MISCELLANEOUS REAGENTS. All other chemicals were reagent grade. Distilled deionized water having a specific resistance of at least 5 Mohms was used to prepare all solutions.

*Methods.* The technique used for the difference spectroscopic measurements of ribonuclease $T_1$–ligand binding was similar to that described previously (Walz, 1971) except that a Cary 118B spectrophotometer was employed. All spectra were determined manually at each unit wavelength from 310 to 240 nm at a constant slit width of 0.2 mm. A standard buffer of 0.2 M ionic strength containing 0.1 M KCl, 0.05 M sodium acetate, and 0.05 M Tris was used. The final pH of this buffer was adjusted by the addition of acetic acid. All ribonuclease $T_1$ binding experiments were conducted at pH 5.0 and 25° at enzyme concentrations of $\sim 4 \times 10^{-5}$ M and ligand concentrations ranging from $4 \times 10^{-5}$ to $8 \times 10^{-4}$ M. For the determination of the pH-induced difference spectra, a standard buffer at pH 7.0 and a solution of 0.1 M HCl and 0.1 M KCl (pH 1.10) were prepared. Identical small volumes of concentrated Guo-3′-P or dGuo-3′-P in distilled water were added to both solutions and the difference spectra was determined at 25° using the pH 7.0 solution as a blank.

## Results and Treatment of the Data

The difference spectra observed for ribonuclease $T_1$ binding with Guo-2′-P, Guo-3′-P, Guo-5′-P, Guo, dGuo-3′-P, dGuo-5′-P, and dGuo were remarkably similar. In all cases the difference absorbance, $\Delta A$, had a miximum at 290 nm and a minimum at 246 nm, generally with crossovers at 276, 265, and 260 nm. These features of the difference spectrum for a given ligand were verified by at least eight separate experiments and were independent of the enzyme and ligand concentrations tested. For dGuo the difference spectrum was not as pronounced (under a given set of experimental conditions) as those of the other ligands and the persistance of the crossover points in this difference spectrum is less certain. In order to obtain a more detailed comparison of the ribonuclease $T_1$ induced difference spectra, the data for some of the ligands were normalized to the minimum value of $\Delta A$ (*i.e.*, $\Delta A$ at 246 nm). As shown in Figure 1, it was found that the normalized difference spectra for Guo-2′-P and Guo-3′-P were the same, within experimental error, whereas that for dGuo-3′-P

[1] The abbreviations used for nucleotides follow the recommendations of the IUPAC–IUB Commission as reported in *Biochemistry* 9, 4025 (1970).

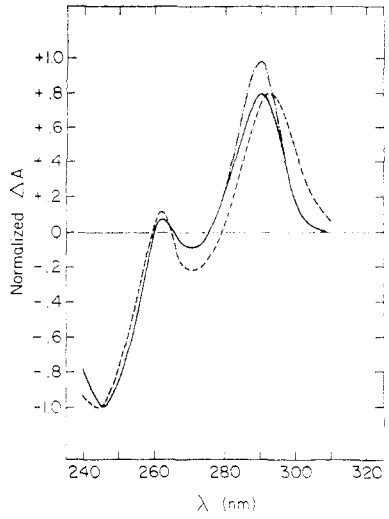


FIGURE 1: The normalized difference spectra for Guo-3′-P, Guo-2′-P, and dGuo-3′-P. Difference spectra were normalized to the minimum value of $\Delta A$: (—) ribonuclease $T_1$ difference spectra for Guo-3′-P and Guo-2′-P; (-·-) dGuo-3′-P–ribonuclease $T_1$ difference spectrum; (- - -) Guo-3′-P–acid pH differencd spectrum. Conditions were as described under Experimental Procedure.

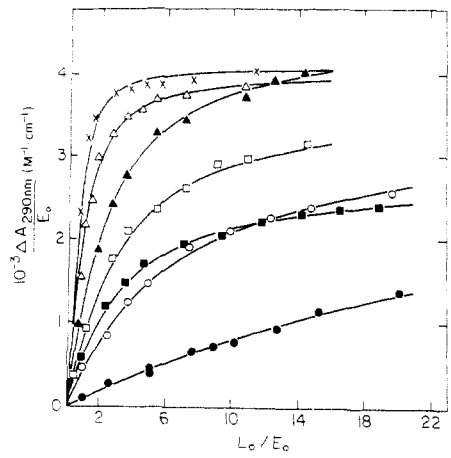


FIGURE 2: A plot of the difference absorbance at 290 nm divided by the total enzyme concentration *vs.* the ratio of the total ligand and enzyme concentrations: (X) Guo-2′-P ($[E]_0 = 4.09 \times 10^{-5}$ M); (△) Guo-3′-P ($[E]_0 = 4.09 \times 10^{-5}$ M); (▲) dGuo-3′-P ($[E]_0 = 4.34 \times 10^{-5}$ M); (□) Guo-5′-P ($[E]_0 = 4.39 \times 10^{-5}$ M); (■) dGuo-5′-P ($[E_0] = 4.57 \times 10^{-5}$ M); (○) Guo ($[E]_0 = 4.94 \times 10^{-5}$ M); (●) dGuo ($[E]_0 = 4.72 \times 10^{-5}$ M). Conditions were as described under Experimental Procedure. Theoretical curves were calculated as described in the text.

showed significant deviations in the wavelength range from 280 to 300 nm. The characteristics of the difference spectra for Guo-3′-P, Guo-2′-P, and dGuo-3′-P were quantitated by determining the ratio of $\Delta A$ at the minimum (246 nm) to $\Delta A$ at the maximum (290 nm) in at least eight experiments for each ligand. As shown in Table I this ratio is significantly lower for dGuo-3′-P when compared with that for Guo-3′-P and Guo-2′-P. The data for the other ligands were not sufficiently precise to distinguish between these two cases. Also shown in Figure 1 is the acid pH difference spectrum (conducted at pH 1.1 using a pH 7.0 blank) for Guo-3′-P which was normalized to the minimum value of $\Delta A$ (*i.e.*, $\Delta A$ at 244 nm). As noted previously (Oshima and Imahori, 1971a), the ribonuclease $T_1$ and acid pH difference spectra are similar; nevertheless, there are minor differences: for example, the acid pH difference spectra exhibit a maximum at 293 nm and a minimum at 244 nm with crossovers at 279, 265, and 260 nm. These features of the acid pH difference spectra of Guo-3′-P were also observed for dGuo-3′-P. However, the experiments using dGuo-3′-P were difficult to perform due to a slow reaction of this nucleotide at pH 1.1; nevertheless, quantitative measurements of $\Delta A$ were performed at 293 and 244 nm for this nucleotide prior to any significant time-dependent change in absorbance. By determining the ratios of $\Delta A$ at the minimum (244 nm) to $\Delta A$ at the maximum (293 nm) for the acid pH difference spectra of Guo-3′-P and dGuo-3′-P in five experiments, it was found that these nucleotides could be distinguished in the same characteristic manner that was found for the ribonuclease $T_1$ induced difference spectra (see Table I). The difference molar extinction coefficients at the minima (244 nm) of the acid pH difference spectra for Guo-3′-P and dGuo-3′-P were 3350 and 3020 $M^{-1}$ $cm^{-1}$, respectively, and those at the maxima (293 nm) were 2710 and 2680 $M^{-1}$ $cm^{-1}$, respectively.

In order to determine the association constant, $K$, and the difference molar extinction coefficient, $\Delta\epsilon$, for ribonuclease $T_1$ binding with each guanine nucleoside and nucleotide, $\Delta A$ was measured at 290 nm at a constant total enzyme concentration, $[E]_0$, while varying the total ligand concentration, $[L]_0$. The resulting binding isotherms for all the ligands could be described by the equilibrium expression

$$K = \frac{[EL]}{[E][L]} = \frac{\Delta A/\Delta\epsilon}{([E_0] - \Delta A/\Delta\epsilon)([L]_0 - \Delta A/\Delta\epsilon)} \quad (1)$$

where [EL], [E], and [L] are the concentrations of the enzyme–ligand complex, the free enzyme, and the free ligand, respectively (Anderson *et al.*, 1968). All experiments were conducted at an ionic strength of 0.2 M at pH 5.0 and 25° at 290 nm and the results for ribonuclease $T_1$ binding with Guo-2′-P, Guo-3′-P, Guo-5′-P, Guo, dGuo-3′-P, dGuo-5′-P, and dGuo are shown in Figure 2. The best value of $K$ and $\Delta\epsilon_{max}$ ($\Delta\epsilon_{max} \equiv \Delta\epsilon$ at 290 nm) for a given binding isotherm were determined using the minimization procedure described previously (Anderson *et al.*, 1968). For the binding of each ligand a discrete minimum in the per cent standard deviation of $K$ plotted *vs.* assumed values of $\Delta\epsilon_{max}$ was observed (Anderson *et al.*, 1968). The values of $K$ and $\Delta\epsilon_{max}$ for the binding of all the substrate analogs studied are presented in Table II. The errors in the values of $K$ and $\Delta\epsilon_{max}$ are estimated to be ±15 and ±10%, respectively. These values of $K$ and $\Delta\epsilon_{max}$ were used with eq 1 to generate the theoretical curves in Figure 2.

TABLE I: Ratios of $\Delta A_{min}$:$\Delta A_{max}$ for the pH and Ribonuclease $T_1$ Induced Difference Spectra of Different Substrate Analogs.[a]

| Difference Spectra | Substrate Analog | $\Delta A_{min}/\Delta A_{max}$[b] |
|---|---|---|
| pH | Guo-3′-P | 1.24 ± 0.01 |
| | dGuo-3′-P | 1.13 ± 0.02 |
| Ribonuclease $T_1$ | Guo-3′-P | 1.27 ± 0.07 |
| | dGuo-3′-P | 1.09 ± 0.06 |
| | Guo-2′-P | 1.25 ± 0.07 |

[a] Experiments were conducted as described under Experimental Procedure. [b] For the ribonuclease $T_1$ difference spectra $\Delta A_{min}$ and $\Delta A_{max}$ were at 246 and 290 nm, respectively; for the acid pH induced difference spectra $\Delta A_{min}$ and $\Delta A_{max}$ were at 244 and 293 min, respectively.

TABLE II: Values of the Binding Parameters for Ribonuclease $T_1$ Binding with Substrate Analogs.[a]

| Substrate Analog | $\Delta\epsilon_{max}$[b] (M × cm)$^{-1}$ | $10^{-3}K$[b] ($M^{-1}$) | $n$[b] | $-\Delta G°$[b] (kcal/mol) |
|---|---|---|---|---|
| Guo-2′-P | 4100 | 145 | 1.05 ± 0.45 | 7.04 |
| Guo-3′-P | 4050 | 52 | 0.86 ± 0.19 | 6.44 |
| dGuo-3′-P | 4550 | 12.0 | 1.34 ± 0.57 | 5.56 |
| Guo-5′-P | 3700 | 8.66 | 1.29 ± 0.56 | 5.37 |
| dGuo-5′-P | 2750 | 8.10 | 0.79 ± 0.31 | 5.33 |
| Guo | 3400 | 3.42 | 0.6 ± 0.8 | 4.82 |
| dGuo | 4050 | 0.528 | 2 ± 10 | 3.71 |

[a] Experiments were conducted as described under Experimental Procedure. [b] See text for details.

In all cases there was a good fit between the experimental points and the theoretical curves. The fact that all of the binding isotherms are in the form of a rectangular hyperbola indicates, for a given ligand, that there is either a single binding site on the enzyme or that there are multiple sites having the same intrinsic association constant.

It had been suggested previously (Sato and Egami, 1965; Takahashi, 1970; Cambell and Ts'o, 1971) that the stoichiometry of ribonuclease $T_1$ binding with a variety of nucleotides was 1:1. In the present study the data obtained were used to determine the number of ligand binding sites, $n$, on ribonuclease $T_1$. For this purpose, eq 1 can be expressed as

$$K = \frac{n\Delta A/\Delta\epsilon}{([L]_0 - n\Delta A/\Delta\epsilon)(n[E]_0 - n\Delta A/\Delta\epsilon)} = \frac{nP}{([L]_0 - nP)(n[E]_0 - nP)} \quad (2)$$

where $P \equiv \Delta A/\Delta\epsilon$ (Murphy and Morales, 1970; Wu and Hammes, 1973) which can be rearranged to

$$[L]_0/P = 1/K([E]_0 - P) + n \quad (3)$$

Thus for the data in Figure 2 a plot of $[L]_0/P$ *vs.* $([E]_0 - P)^{-1}$ should yield a straight line having an intercept equal to $n$ and a slope equal to $K^{-1}$. When plotted in the form of eq 3 all of the data gave reasonable straight lines and some of these plots are shown in Figure 3. When the data were analyzed by a least-squares linear regression of eq 3, every ligand gave an intercept that encompassed a value of one within the standard deviation. The values of $n$ with their standard deviations are shown in Table II. It is clear that more accurate values of $n$ required very good data obtained over a broad concentration range (*e.g.*, $n$ determined for dGuo is obviously meaningless (see Table II); the maximal saturation of the enzyme with this ligand was ~33%). Nevertheless, the nearest integral value of $n$ for all ligands is one, and none of the determined values of $n$ were significantly different. The values of $K$ for each ligand were also determined using eq 3 and were not different from those presented in Table II.

The standard free energy of binding, $\Delta G°$, was calculated for all the ligands using eq 4. See Table II for a listing.

$$\Delta G° = -RT \ln K \quad (4)$$

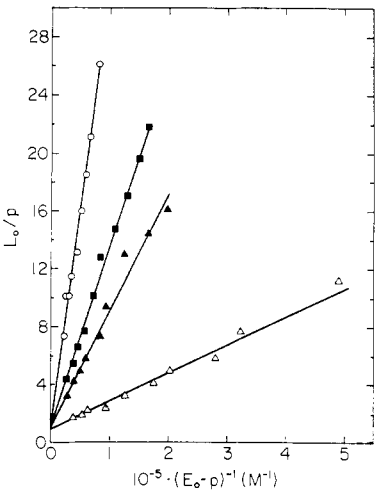


FIGURE 3: Graphical determination of the number of binding sites for ribonuclease $T_1$–ligand binding. The symbols are the same as those in Figure 2. In this plot $P$ is $\Delta A/\Delta\epsilon_{max}$ and $[E]_0$ and $[L]_0$ are the total enzyme and ligand concentrations, respectively. The lines represent a least-squares fit to the data. See text for details.

## Discussion

In general, the qualitative nature of the difference spectra resulting from ribonuclease $T_1$ binding with guanine nucleosides and nucleotides is in agreement with those reported previously (Sato and Egami, 1965; Epinatjeff and Pongs, 1972; Oshima and Imahori, 1971a). However, in some cases there is disagreement in the region of 258–280 nm regarding crossover points. In the present work a sufficient number of experiments were performed to verify these crossover points whereas previously reported difference spectra (Sato and Egami, 1965; Epinatjeff and Pongs, 1972; Oshima and Imahori, 1971a) apparently represent single experiments. However, the ribonuclease $T_1$ induced difference spectrum of dGuo-3′-P was significantly different from that of Guo-3′-P and Guo-2′-P as indicated by the values of $\Delta A_{min}/\Delta A_{max}$ in Table I. It is also possible that the ribonuclease $T_1$ induced difference spectra of dGuo and dGuo-5′-P show a similar deviation as that observed for dGuo-3′-P; however, this could not be statistically demonstrated with the present data.

It has been suggested that the gross similarity of the low pH and ribonuclease $T_1$ induced difference spectra for Guo-3′-P indicates that protonation of the guanine ring occurs in the enzyme–ligand complex (Epinatjeff and Pongs, 1972; Oshima and Imahori, 1971a). In the present investigation the similarity of $\Delta A_{min}/\Delta A_{max}$ ratios for the pH and ribonuclease $T_1$ induced difference spectra of dGuo-3′-P and the similarity of these ratios for the low pH and enzyme induced difference spectra of Guo-3′-P (see Table I) give further circumstantial support for this hypothesis. In any event, the similarity of the acid pH and ribonuclease $T_1$ difference spectra suggests that the enzyme-induced spectral perturbations are confined for the most part to the guanine base group, and not to chromophores on the enzyme. However, in contrast to an earlier suggestion (Oshima and Imahori, 1971a) it is clear from the present results that additional factors, other than guanine ring protonation, are involved in generating the enzyme–ligand difference spectra. This was indicated by the lack of congruency of the acid pH and ribonuclease $T_1$ induced difference spectra as shown in Figure 1. Furthermore, the values of $\Delta\epsilon_{max}$ for ribonuclease $T_1$ binding of Guo-2′-P and Guo-3′-P were at least 50% greater than the corresponding values (*i.e.*,

TABLE III: Difference in $\Delta G^\circ$ Values for Ribonuclease $T_1$ Binding with Substrate Analogs.[a]

| Comparison | $-\Delta\Delta G^\circ$ (kcal/mol) |
|---|---|
| 2′-Hydroxyl and 2′-Hydrogen | |
| Guo–dGuo | 1.11 |
| Guo-3′-P–dGuo-3′-P | 0.88 |
| Guo-5′-P–dGuo-5′-P | 0.04 |
| Phosphoester on Guanosine | |
| Guo-2′-P–Guo | 2.22 |
| Guo-3′-P–Guo | 1.62 |
| Guo-5′-P–Guo | 0.55 |
| Phosphoester on Deoxyguanosine | |
| dGuo-3′-P–dGuo | 1.85 |
| dGuo-5′-P–dGuo | 1.62 |

[a] Values of $\Delta G^\circ$ were taken from Table I.

$\Delta\epsilon$ at 293 nm) characterizing the acid pH induced difference spectra of these nucleotides (see Table I).

The values of $\Delta\epsilon_{max}$ for ribonuclease $T_1$–substrate analog binding which are summarized in Table II vary considerably and do not directly correlate with the corresponding values of $K$. This finding suggests that the interaction of the guanine group with the enzyme is somehow dependent on the presence of other interacting chemical groups of the substrate analog. In the absence of a firm theoretical basis regarding the origin of the difference spectra, no further interpretation of these results is possible at this time. Nevertheless, the fact that the ribonuclease $T_1$ induced difference spectra are qualitatively similar for *all* the ligands tested suggests that the guanine moiety occupies the same general site in the different enzyme–ligand complexes.

There is considerable confusion in the literature regarding the values of the association constants for ribonuclease $T_1$ binding with a variety of substrate analogs; for example, under similar conditions of pH, ionic strength, and temperature the association constants determined by gel filtration techniques for Guo, Guo-5′-P, and Guo-3′-P differed by factors of 166, 147, and 45, respectively, when reports from two different laboratories (Takahashi, 1970; Cambell and Ts'o, 1971) were compared.[2] The association constants determined in the present study agree in some cases with those from studies employing different techniques; *e.g.*, the association constant of ribonuclease $T_1$ binding with Guo-2′-P determined by inhibition kinetics at pH 5.0, 0.01 M ionic strength, and 23° was $\sim 10^5$ $M^{-1}$ (Irie, 1967, 1968) which is in reasonable agreement with the comparable value in Table II. In two previous reports on difference spectral measurements of ribonuclease $T_1$–ligand binding the values of the association constant and/or $\Delta\epsilon_{max}$ were not independently determined using the difference spectral data obtained. In one report a value of $\Delta\epsilon_{max}$ was assumed to be 3100 $M^{-1}$ $cm^{-1}$ and was used to calculate the association constants for ribonuclease $T_1$ binding with Guo and Guo-3′-P (Oshima and Imahori, 1971a). In another report, an unpublished value of the association constant for ribonuclease $T_1$ binding with Guo-2′-P (determined by microcalorimetry) was used to determine the equivalent of $\Delta\epsilon_{max}$ ($\sim$15,000 $M^{-1}$ $cm^{-1}$) for this ligand; in turn, this value of $\Delta\epsilon_{max}$ was used to calculate the corresponding association constants for Guo-3′-P, Guo-5′-P, and Guo (Epinatjeff and Pongs, 1972). In the present work where values of $\Delta\epsilon_{max}$ were independently determined for ribonuclease $T_1$ binding with each ligand, it is clear that the assumptions regarding $\Delta\epsilon_{max}$ made in these two reports are not valid (see Table II). Nevertheless, the experimental results reported in these studies (Oshima and Imahori, 1971a; Epinatjeff and Pongs, 1972) are compatible with those shown in Figure 2. In any event, most of the reported binding studies using a variety of ligands gave an order of relative affinities with ribonuclease $T_1$ that does not differ from that indicated in Table II; *i.e.*, Guo-2′-P > Guo-3′-P > dGuo-3′-P > Guo-5′-P = dGuo-5′-P > Guo > dGuo.

Selected differences in the values of $\Delta G^\circ$ for enzyme–ligand binding ($\Delta\Delta G^\circ$) were calculated to quantify the specific binding energy contributions regarding the nature and position of the various chemical groups on the sugar moiety of the substrate analogs. As shown in Table III the free-energy contribution of the 2′-hydroxyl group (when compared with the analogous 2′-hydrogen compounds) is largely dependent on the presence and position of the phosphoester group: for Guo and Guo-3′-P the presence of a 2′-hydroxyl group clearly favors binding, whereas for Guo-5′-P binding appears to be independent of this substitution. These results suggest that the binding interaction of the enzyme at one chemical locus of the ligand strongly affects the binding interaction of other loci. In addition to interactions with the 2′-hydroxyl another major binding free-energy contribution results from phosphomonoester substitutions for hydroxyl groups at every available position on the ribose moiety of Guo. As indicated in Table III, the binding energy contribution is significantly dependent on the position of the phosphomonoester group. In addition, the presence of a phosphomonoester group substituted for a hydroxyl group on dGuo is more significant in promoting binding than the corresponding substitutions on Guo (*e.g.*, compare $-\Delta\Delta G^\circ$ values for dGuo-3′-P–dGuo and dGuo-5′-P–dGuo with those for Guo-3′-P–Guo and Guo-5′-P–Guo, respectively). A possible interpretation of this result is that the bound deoxyribonucleotides have a greater degree of freedom (presumably due to the absence of interactions at the 2′ position) which serves to maximize enzyme–phosphomonoester group interactions. This interpretation is consistent with the finding that both 9-(2′-hydroxyethyl)-Gua-2′-P and 9-(4′-hydroxybutyl)-Gua-2′-P bind well with ribonuclease $T_1$ (Cambell and Ts'o, 1971). In any event, the full significance of the individual free-energy contributions of the different chemical groups comprising the ligands must await further details of their binding mechanisms.[3]

It is interesting to note that ribonuclease A binding with uridine nucleotides gave the same order of relative affinities for phosphomonoester isomers as that shown for ribonuclease $T_1$; *i.e.*, Urd-2′-P > Urd-3′-P > Urd-5′-P (Anderson *et al.*, 1968; Richards and Wyckoff, 1971). However, these ribonucleases also exhibit a critical difference in their relative

[2] The values of $K$ derived from the present data are in good agreement with those from gel filtration experiments run under equilibrium conditions (Takahashi, 1970). Other gel filtration experiments (Cambell and Ts'o, 1971) were performed under conditions that assumed an enzyme–ligand equilibration half-life in excess of 6 min. Since the equilibration half-life found for ribonuclease $T_1$–ligand binding is in the submillisecond time range (unpublished temperature-jump kinetic experiments from our laboratory) it appears that this assumption is not valid and that the resulting binding constants are in error.

[3] Preliminary evidence from temperature-jump experiments in our laboratory conducted at pH 5, 0.2 M ionic strength, and 25° indicates that Guo-3′-P binding with ribonuclease $T_1$ is characterized by a simple on–off equilibrium, whereas Guo-2′-P bonding involves an isomerization of the initial enzyme–ligand complex.

affinities for 3′-nucleotide substrates and 2′-deoxy-3′-nucleotide substrate analogs; for ribonuclease $T_1$ Guo-2′-P > dGuo-3′-P, whereas for ribonuclease A dUrd-3′-P > Urd-3′-P (Walz, 1971). The significance that binding (or not binding) of the 2′-hydroxyl group has regarding the catalytic mechanisms of the two ribonucleases remains to be determined. It could be speculated that ribonuclease $T_1$ utilizes binding at the 2′-hydroxyl position of guanosine moieties in RNA as a significant contribution to the binding energy of the enzyme–substrate complexes in the absence of gross coulombic interactions which probably contribute to ribonuclease A–RNA binding (Richards and Wyckoff, 1971). Nevertheless, preliminary difference spectral experiments in our laboratory have indicated that ribonuclease $T_1$ can bind to single-stranded calf thymus DNA.

Future kinetic and equilibrium binding studies will consider the interactions of ribonuclease $T_1$ with diphosphate substrates and substrate analogs including P-5′-Guo-2′:3′-P, P-5′-Guo-3′-P, and P-5′-dGuo-3′-P. These planned studies were prompted by steady-state kinetic studies of the ribonuclease A catalyzed hydrolysis of P-5′-Urd-2′:3′-P which indicated a specific interaction of the substrate 5′-phosphomonoester group with the enzyme (J. Li and F. G. Walz, submitted for publication).

References


Anderson, D. G., Hammes, G. G., and Walz, Jr., F. G. (1968), *Biochemistry 7*, 1637.

Ando, T. (1966), *Biochim. Biophys. Acta 114*, 158.

Cambell, M. K., and Ts'o, P. O. P. (1971), *Biochim. Biophys. Acta 232*, 427.

Egami, F., Takahashi, K., and Uchida, T. (1964), *Progr. Nucl. Acid. Res. Mol. Biol. 3*, 59.

Epinatjeff, C., and Pongs, O. (1972), *Eur. J. Biochem. 26*, 434.

Fields, R., Dixon, H. B. F., Law, G. R., and Yui, C. (1971), *Biochem. J. 121*, 591.

Irie, M. (1967), *J. Biochem. (Tokyo) 61*, 550.

Irie, M. (1968), *J. Biochem. (Tokyo) 63*, 649.

Kumar, D. S. S., Pinck, L., and Hirth, L. (1972), *Anal. Biochem. 48*, 497.

Murphy, A. J., and Morales, M. F. (1970), *Biochemistry 9*, 1528.

Oshima, T., and Imahori, K. (1971a), *J. Biochem. (Tokyo) 69*, 987.

Oshima, T., and Imahori, K. (1971b), *J. Biochem. (Tokyo) 70*, 197.

Pongs, O. (1970), *Biochemistry 9*, 2316.

Richards, F. M., and Wyckoff, H. W. (1971), *Enzymes, 3rd Ed., 4*, 647.

Rüterjans, H., and Pongs, O. (1971), *Eur. J. Biochem. 18*, 313.

Rüterjans, H., Witzel, H., and Pongs, O. (1969), *Biochem. Biophys. Res. Commun. 37*, 247.

Sander, C., and Ts'o, P. O. P. (1971), *Biochemistry 10*, 1953.

Sato, S., and Egami, F. (1965), *Biochem. Z. 342*, 437.

Takahashi, K. (1970), *J. Biochem. (Tokyo) 68*, 941.

Walz, Jr., F. G. (1971), *Biochemistry 10*, 2156.

Wu, C., and Hammes, G. G. (1973), *Biochemistry 12*, 1400.


# 24,25-Dihydroxyvitamin $D_3$. Synthesis and Biological Activity†

Hing-Yat Lam, Heinrich K. Schnoes, Hector F. DeLuca,* and Tai C. Chen‡

ABSTRACT: A kidney metabolite of vitamin $D_3$, 24,25-dihydroxyvitamin $D_3$, has been synthesized by an unambiguous route. Like the natural metabolite, the synthetic product elicits a pronounced and long-lasting intestinal calcium transport response, but has no effect on the bone calcium mobilization process.

The isolation and characterization of 24,25-dihydroxyvitamin $D_3$ (24,25-$(OH)_2D_3$)[1] (**1**) (Figure 1) was reported by us recently (Holick *et al.*, 1972). Although the function of this naturally occurring vitamin D metabolite is not completely understood at present, several findings suggest that the compound may play a significant role in vitamin D metabolism and its control: (a) 24,25-$(OH)_2D_3$, like the very potent 1$\alpha$,25-dihydroxyvitamin $D_3$ (1$\alpha$,25-$(OH)_2D_3$ (**2**) (Figure 1), is made in kidney tissue from 25-hydroxyvitamin $D_3$ (25-OH-$D_3$) (Holick *et al.*, 1972); (b) it is specifically active in promoting calcium transport in intestine and shows little or no effect on bone mineral mobilization (Boyle *et al.*, 1973); (c) its biological synthesis in kidney occurs at normal or high levels of serum calcium whereas low calcium levels induce production of 1$\alpha$,25-$(OH)_2D_3$ (Boyle *et al.*, 1971), an effect which can be shown to be mediated through parathyroid hormone (Garabedian *et al.*, 1972); and (d) it is further metabolized in the kidney to a more polar product (Boyle *et al.*, 1973) recently characterized (Holick *et al.*, 1973) as 1,24,25-trihydroxyvitamin $D_3$ (1,24,25-$(OH)_3D_3$) (**3**), the metabolite believed to be responsible for the intestinal calcium transport activity of **1**, since 24,25-$(OH)_2D_3$ itself can be shown to be inactive in anephric animals. These facts, which suggested promising further investigations into possible biological roles of metabolite **1**, and the need to confirm its structure, led us to undertake a synthesis of this compound.

† From the Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin-Madison, Madison, Wisconsin 53706. *Received July 16, 1973.* Supported by grants from the U. S. Public Health Service, Nos. AM-14881 and AM-15512, and the Wisconsin Alumni Research Foundation.

‡ Present address: Veterans Administration Hospital, LaJolla, Calif. 92161.

[1] Abbreviations used are: 24,25-$(OH)_2D_3$, 24,25-dihydroxyvitamin $D_3$; 1$\alpha$,25-$(OH)_2D_3$, 1$\alpha$,25-dihydroxyvitamin $D_3$; 25-OH-$D_3$, 25-hydroxyvitamin $D_3$; 1,24,25-$(OH)_3D_3$, 1,24,25-trihydroxyvitamin $D_3$.