DNA polymerase catalyzes the formation of a phosphodiester bond between the 3'-hydroxyl terminus of a DNA chain and the α-phosphate of a deoxyribonucleoside 5'-triphosphate (2). There is a single binding site in the *Escherichia coli* enzyme for which all four common deoxyribonucleoside triphosphates compete (3). A wide variety of analogues of the deoxyribonucleoside triphosphates can also bind to this site, but it is essential that they contain the triphosphate grouping (3). In addition, deoxyribonucleoside mono- or diphosphates bind tightly to the enzyme, but in another, distinct site. Binding in this second site, as the studies in this report will show, requires that the nucleotide sugar have a free 3'-hydroxyl group in the ribose configuration and that a phosphate (or pyrophosphate) group be esterified to the sugar have a free 3'-hydroxyl group in the ribose configuration and a 5'-phosphate linkage suggest a relation ship of this site to the one that binds the primer terminus of a DNA chain.

**EXPERIMENTAL PROCEDURE**

**Materials**

*E. coli* DNA polymerase (Fraction 7) isolated as previously described (4) was dialyzed against 50 mM potassium phosphate, pH 7.4, for use in equilibrium dialysis measurements. A molar extinction coefficient of 9.26 × 10^4 M^-1 cm^-1 at 250 nm (4) was used to determine the concentration.

1 The abbreviations used are: pTTP, thymidylyl-(5', 3')-thymidine 5'-monophosphate; TTP, thymidylyl-(5', 3')-thymidine triphosphate; d(T)_{1000}, polydeoxythymidylic acid of length 1000 nucleotides; pppTTP, thymidylyl-(5', 3')-thymidine 5'-triphosphate; poly d(A), alternating copolymer of deoxyadenylate and deoxythy midylate; d(A)_{4000}, polydeoxyadenylate of length 4000 nucleotides.

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**SUMMARY**

A site distinct from that for binding a deoxyribonucleoside triphosphate is available on *Escherichia coli* DNA polymerase for binding a 3'-hydroxyribonucleotide. Strict specificity for nucleotides with both a 3'-hydroxyl group in the ribose configuration and a 5'-phosphate linkage suggest a relation ship of this site to the one that binds the primer terminus of a DNA chain.
brane and DNA polymerase (20 to 70 PM) was placed on the other as previously (3), by the equation

$$K_{\text{dAMP}} = \frac{\text{[dAMP]}}{\text{[dAMP][E] - [E]}}$$

The value for $K_{\text{dAMP}}$ was determined directly. Several dAMP levels were used; $K_{\text{dAMP}}$ was estimated for each dAMP level by substituting experimental values of $\text{[dAMP]}$ and [E] obtained in the absence of a competitive analogue into the mass action equation which assumes 1 dAMP binding site

$$K_{\text{dAMP}} = \frac{\text{[dAMP][E]}(1 - \text{[dAMP][E]})}{\text{[dAMP][E]}}$$

**Methods**

Equilibrium Dialysis—Experiments, performed as previously described (3), were at 22° in 50 mM potassium phosphate, 7 mM MgCl$_2$ at pH 7.4. HgCl$_2$ was always present in a 1.2- to 1.7-fold excess over polymerase in order to inhibit contaminating hydrolytic activities (3). At the start of the experiments, nucleotides were placed in the chamber on one side of the dialysis membrane and DNA polymerase (20 to 70 PM) was placed on the other side. At the conclusion of some experiments, small samples were subjected to high voltage electrophoresis on orange ribbon No. 559 paper in 20 mM sodium citrate, pH 3.5, to determine whether any hydrolysis of nucleotides had taken place. No hydrolysis of dAMP, dADP, or dATP was ever detected. Some hydrolysis of PP$_i$, to P$_i$, did take place during equilibrium dialysis and was taken into account in experiments with PP$_i$.

Calculation of Binding Constants—Data were plotted using Scatchard’s equation (10)

$$\frac{\text{[dAMP]}(1 - \text{[dAMP][E]})}{\text{[dAMP][E]}}$$

$$\psi = -\frac{1}{K} (\psi - n)$$

$\psi$ is the average number of ligand molecules bound per polymerase molecule, [L] is the free ligand concentration, $K$ is the dissociation constant of the complex, and $n$ is the number of identical, independent binding sites on a polymerase molecule. The plot of $\psi/[L]$ versus $\psi$ yields a line with slope of $-1/K$ and an intercept on the absissa of $n$. Straight lines were fitted to the experimental points by least squares analysis.

In experiments designed to determine whether an analogue was bound competitively in the 3'-hydroxylribonucleotide site or the deoxyribonucleoside triphosphate site, $^{32}$P-dAMP and $^{32}$P-dATP were both present along with the unlabeled analogue or the deoxyribonucleoside triphosphate site. "C-dAMP and "C-dATP were both present along with the unlabeled analogue or the deoxyribonucleoside triphosphate site. "C-dAMP and "C-dATP were both present along with the unlabeled analogue or the deoxyribonucleoside triphosphate site.

**RESULTS**

Deoxyribonucleoside 5'-monophosphates Bind to Single and Distinctive Site on Enzyme—Equilibrium dialysis measurements of $^3$H-dAMP binding to DNA polymerase plotted in Fig. 1 indicate by least squares analysis a dissociation constant ($K$) of 0.016 mM and a binding site value ($n$) of 0.78. Close inspection of the data reveals that there is considerable curvature in the points. The measurements at lower dAMP concentrations give lower values of $K$ and $n$ than do measurements at higher dAMP concentrations. However, there is no suggestion that any extrapolations could yield a binding site value greater than 1.0. This departure from linearity for dAMP binding is in sharp contrast with the data obtained in the binding of deoxyribonucleoside triphosphates (3) in which there was no curvature in the line and where values for $n$ were much closer to 1.0.

We interpret the dAMP binding data to indicate heterogeneity among DNA polymerase molecules in their capacity to bind dAMP. The possibility that the heterogeneity might be due to lower molecular weight enzyme fragments generated by proteolysis (13, 19) was ruled out by electrophoretic analysis (11, 12) of enzyme preparations in several experiments (see Fig. 1). The influence of other agents, such as trace metals, as another possible source of heterogeneity in the binding behavior of the enzyme, remains to be investigated.

To determine whether the other deoxyribonucleoside 5'-monophosphates bind to the dAMP site, competition experiments were carried out between $^3$H-dAMP and each of these nucleotides. In every case, with dCMP, dGMP and dTMP, there was strong competition (Table I). The purine nucleotides were bound more strongly than the pyrimidine nucleotides as
FIG. 1. Scatchard plot of the binding of dAMP to DNA polymerase. DNA polymerase concentrations ranged from 0.02 to 0.07 mM. The line was determined by the least squares method. Points indicated by • were obtained with polymerase preparations which were checked for possible degradation by acrylamide gel electrophoresis (11, 12); no degradation was detected.

TABLE I

Binding of deoxyribonucleoside 5'-monophosphates to DNA polymerase

These competition binding experiments were performed as described under "Methods." 14C-dAMP at concentrations of 0.01 to 0.06 mM and unlabeled nucleotides at the indicated concentrations were present on one side of the dialysis membrane at the beginning of the experiments.

<table>
<thead>
<tr>
<th>Competing ligand</th>
<th>Concentration (mM)</th>
<th>Dissociation constant (K) (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dCMP</td>
<td>0.85</td>
<td>0.134</td>
</tr>
<tr>
<td>dGMP</td>
<td>0.23</td>
<td>0.031</td>
</tr>
<tr>
<td>dTMP</td>
<td>0.11</td>
<td>0.027</td>
</tr>
<tr>
<td>dAMP</td>
<td>0.89</td>
<td>0.088</td>
</tr>
</tbody>
</table>

has also been observed for binding of triphosphates in their site (3). However these quantitative estimates of binding, calculated as dissociation constants for a competing ligand (Table I), must be regarded as only gross estimates in view of the acknowledged heterogeneity in the dAMP binding site.

We reported previously that deoxyribonucleoside monophosphates (and diphosphates) at concentrations of 1 mM show no binding to the deoxyribonucleoside triphosphate site (3). Similarly we now find no competition by dATP at 0.1 mM with 14C-dAMP binding. In the extensive survey of analogues, to be described below, each analogue was tested in the same dialysis chamber for its capacity to compete with 14C-dAMP and 32P-dATP. In no instance did an analogue show competition with analogues which bind to 5'-hydroxylribonucleotide site

Experiments were performed as described in the legend to Table I. In the structure diagrams, P represents an orthophosphate group and A represents adenine.

<table>
<thead>
<tr>
<th>No.</th>
<th>Structure</th>
<th>Concentration (mM)</th>
<th>Dissociation constant (K) (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><img src="image1.png" alt="Structure 1" /></td>
<td>0.37</td>
<td>0.035</td>
</tr>
<tr>
<td>2</td>
<td><img src="image2.png" alt="Structure 2" /></td>
<td>0.32</td>
<td>0.144</td>
</tr>
<tr>
<td>3</td>
<td><img src="image3.png" alt="Structure 3" /></td>
<td>0.68</td>
<td>0.069</td>
</tr>
<tr>
<td>4</td>
<td><img src="image4.png" alt="Structure 4" /></td>
<td>1.62</td>
<td>0.015</td>
</tr>
<tr>
<td>5</td>
<td><img src="image5.png" alt="Structure 5" /></td>
<td>0.22</td>
<td>0.023</td>
</tr>
<tr>
<td>6</td>
<td><img src="image6.png" alt="Structure 6" /></td>
<td>0.83</td>
<td>0.062</td>
</tr>
<tr>
<td>7</td>
<td><img src="image7.png" alt="Structure 7" /></td>
<td>0.03</td>
<td>0.087</td>
</tr>
</tbody>
</table>

*Analogue names: 1, AMP; 2, 6-mercaptopurine ribonucleoside 5'-phosphate; 3, 6-methylmercaptapurine ribonucleoside 5'-phosphate; 4, 4'-deoxy-4'-thioadenosine ribonucleoside 5'-phosphate; 5, aristeromycin 6'-phosphate; 6, 5-fluorouracil deoxyribonucleoside 5'-phosphate; 7, purine deoxyribonucleoside 5'-phosphate.
both of these compounds. There appears therefore to be no significant overlap in specificity between the triphosphate site and that which binds dAMP.

**Analogue with 3'-Hydroxyl Group in Ribose Configuration Bind in Nucleotide Site**—Certain analogues of the deoxyribonucleotides compete effectively for the dAMP binding site (Table II). These include sugar analogues such as a ribonucleotide (AMP), a 4'-deoxy-4'-thioribonucleotide, and a cyclopentane ring compound (aristeromycin 6'-phosphate). Each of these analogues

**TABLE III**—Continued

<table>
<thead>
<tr>
<th>No.</th>
<th>Analoguea</th>
<th>Structure</th>
<th>Concentration (mM)</th>
<th>Dissociation constant (K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td></td>
<td></td>
<td>1.15</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td></td>
<td></td>
<td>1.46</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
<td>0.52</td>
<td></td>
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<tr>
<td>13</td>
<td></td>
<td></td>
<td>1.50</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td></td>
<td></td>
<td>1.50</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td></td>
<td></td>
<td>1.30</td>
<td></td>
</tr>
</tbody>
</table>

*a Analogue names: 1, 2',3'-dideoxyadenosine 5'-phosphate; 2, 2',3'-dideoxythymidine 5'-phosphate; 3, 2',3'-dideoxy-2',3'-didehydroadenosine 5'-phosphate; 4, uracil xylonucleoside 5'-phosphate; 5, adenine xylonucleoside 5'-phosphate; 6, uracil xylonucleoside 5'-phosphate; 7, adenine-2'-deoxyxylonucleoside 5'-phosphate; 8, adenine-3'-deoxyxylonucleoside 5'-phosphate; 9, 3'-O-methyl-5-fluouracil deoxyribonucleoside 5'-phosphate; 10, 3'-O-methyl AMP; 11, thymidine 3',5'-diphosphate; 12, deoxyguanosine 3'-phosphate; 13, deoxyadenosine 3'-phosphate; 14, deoxythymidine 3'-phosphate; 15, deoxyguanosine 3'-phosphate.

Analogues lacking a free 3'-hydroxyl group in ribose configuration fail to bind in 3'-hydroxyribonucleotide site

Experiments were performed as described in the legend to Table I. In all cases, no inhibition (<15%) of the binding of labeled dAMP was detected. Using Equation 2 (see "Methods") and assuming a reduction in the value of v for dAMP of 15%, one can calculate that the dissociation constants for the analogues listed were at least as high as those shown below. In the structure diagrams, A, T, U, G, and C stand for the bases adenine, thymine, uracil, guanine, and cytosine, and P represents an orthophosphate group.

...has a furanose-like ring structure with a 3'-hydroxyl group in the ribose configuration; the presence of the 2'-hydroxyl group in the ribose configuration appears to have little influence. Base analogues such as 6-mercaptopurine, 6-methylmercaptothymine, purine, and fluorouracil, some as part of ribonucleotides and others as part of deoxyribonucleotides, were bound in the nucleotide site.

**Analogue Which Lack Free 3'-Hydroxyl Group in Ribose Configuration Fail to Bind in Nucleotide Site**—Replacement of the 3'-hydroxyl group as in 2',3'-dideoxyribonucleotides, removal as in a 2',3'-dideoxy-2',3'-didehydrobocynucleotide, inversion of configuration as in xylose or lyxose nucleotides, blockage by a methyl group as in 3'-O-methyl nucleotides or blockage by a phosphate group as in several deoxyribonucleoside 3'-phosphates prevented nucleotides from competing with dAMP for binding to the enzyme (Table III). In all of these instances the analogue was present at a concentration near 1 mM and reduction of 3H-dAMP binding to the enzyme was not detectable (<15%).
**TABLE IV**

Importance of phosphate group for binding to 3'-hydroxylribonucleotide site

Experiments were performed as described in the legend to Table III. In the structure diagrams, A, T, and G represent the bases adenine, thymine, and guanine.

<table>
<thead>
<tr>
<th>Analogue</th>
<th>Dissociation constant (K)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mM</td>
</tr>
<tr>
<td>1</td>
<td>1.44</td>
</tr>
<tr>
<td>2</td>
<td>1.68</td>
</tr>
<tr>
<td>3</td>
<td>1.27</td>
</tr>
<tr>
<td>4</td>
<td>1.56</td>
</tr>
<tr>
<td>5</td>
<td>8.60</td>
</tr>
<tr>
<td></td>
<td>2.00</td>
</tr>
</tbody>
</table>

Analoguea: 1, adenosine 5'-phosphite; 2, guanosine 5'-methane phosphonate; 3, thymidine 5'-phosphonate; 4, thymidyl(5',3')-thymidine 5'-triphosphate; 5, 2'-deoxyadenosine.

The striking specificity of the dAMP binding site for nucleotides with a free 3'-hydroxyl in the ribose configuration has led us to name this site the 3'-hydroxylribonucleotide binding site.

**Influence of Modifications of Phosphate Group in Binding to the 3'-Hydroxylribonucleotide Site**—Replacement of phosphate by a phosphite, a methyl phosphonate, or a phosphonate linked directly to the sugar carbon prevented binding of each of these analogues (Table IV). Complete absence of the phosphate group did not entirely prevent binding of the remaining nucleoside (deoxyadenosine), although very high concentrations were required for detection (Table IV).

Addition of two phosphate groups as in the case of nucleoside triphosphates resulted, as already described, in a failure to bind to the 3'-hydroxylribonucleotide site. Esterification with a deoxyribonucleoside triphosphate, to form a dinucleoside tetraphosphate, also prevented binding in this site (Table IV). Addition of one phosphate group, as in the nucleoside diphosphates, permitted binding in the site, as shown in the following experiments.

**Fig. 2.** Scatchard plot of the binding of dADP to DNA polymerase. Conditions were as in Fig. 1; the polymerase preparations were known to be intact.

**TABLE V**

Identity of dAMP and dADP binding sites

These competition binding experiments were performed as described under “Methods.” 14C-Labeled nucleotides at 0.01 to 0.06 mM and unlabeled nucleotides at the indicated concentrations were present on one side of the dialysis membrane at the beginning of the experiments. DNA polymerase at concentrations of 0.02 to 0.07 mM was present on the other side.

<table>
<thead>
<tr>
<th>Labeled nucleotide</th>
<th>Unlabeled competing nucleotide</th>
<th>Initial concentration of unlabeled nucleotide (mM)</th>
<th>Dissociation constant for unlabeled nucleotide (K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dAMP</td>
<td>dADP</td>
<td>0.76</td>
<td>0.108</td>
</tr>
<tr>
<td>dAMP</td>
<td>dADP</td>
<td>0.66</td>
<td>0.080</td>
</tr>
<tr>
<td>dADP</td>
<td>dAMP</td>
<td>0.003</td>
<td>0.018</td>
</tr>
<tr>
<td>dADP</td>
<td>dAMP</td>
<td>0.019</td>
<td>0.020</td>
</tr>
</tbody>
</table>

Measurements of 14C-dADP binding, as plotted in Fig. 2, indicated a dissociation constant of 0.004 mM and a binding site value of 0.68. As in the case of dAMP binding, the plot is non-linear and therefore suggestive of heterogeneity in the enzyme in its binding of dADP. Evidence that dADP and dAMP compete for the same site is supplied by experiments in which one or the other nucleotide was labeled (Table V). The dissociation constants in Table V, calculated from competition between dAMP and dADP (see Equation 2 in “Methods”), are in fair agreement with the values determined by direct binding measurements (Figs. 1 and 2).

In comparable competition experiments with the other deoxyribonucleoside diphosphates, binding was evident in each case (Table VI), although the affinity of each diphosphate was mark-
TABLE VI
Binding of deoxyribonucleoside diphosphates to DNA polymerase

Experiments were performed as in the legend to Table I.

<table>
<thead>
<tr>
<th>Competing ligand</th>
<th>Concentration (mM)</th>
<th>Dissociation constant (Kd)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dCDP</td>
<td>1.82</td>
<td>1.3</td>
</tr>
<tr>
<td>dGDP</td>
<td>0.93</td>
<td>0.10</td>
</tr>
<tr>
<td>dGDP</td>
<td>0.76</td>
<td>0.21</td>
</tr>
<tr>
<td>dTDP</td>
<td>1.92</td>
<td>0.23</td>
</tr>
<tr>
<td>dTDP</td>
<td>0.76</td>
<td>0.25</td>
</tr>
</tbody>
</table>

ably less than that of the corresponding monophosphate (Table I).

The capacity of the 3'-hydroxylribonucleotide site to bind a nucleoside diphosphate is consistent with its ability to bind inorganic pyrophosphate. In two experiments, at PPi concentrations of 2 and 8 mM, inhibition of dAMP binding indicated dissociation constants for PPi at this enzyme site of 1.0 and 0.7 mM, respectively. At these levels of PPi, no binding to the triphosphate site was observed (3).

Arabinosyl Nucleotides and Charged Base Analogues Fail to Bind to 3'-Hydroxylribonucleotide Site—Failure of arabinosyl nucleotides to bind (Table VII) demonstrates that availability of a 3'-hydroxyl group in the ribose configuration of a nucleotide is not enough to overcome the presence of an obstructive substituent. In this case the 2'-hydroxyl group in an inverted position (compared with that in the ribose sugar) prevents binding.

Although a wide variation in structure of the purine and pyrimidine base can be tolerated (Tables I and II), the failure of barbituric acid ribonucleotide and nicotinamide ribonucleotide to be bound (Table VII) suggests that the strong charge which these bases carry is incompatible with a site which may be hydrophobic in character.

Effect of Temperature, Buffer Concentration, EDTA, and Enzyme Modification on Binding—The binding of dAMP, measured at 5.5°, 22°, and 39.5°, decreased as a function of temperature (Fig 3), a dependence similar to that previously observed for dATP binding in the triphosphate site (3). Unlike the effect of salt on binding in the triphosphate site, an increase in phosphate buffer concentration from 25 to 75 mM did not significantly inhibit the binding of dAMP. Likewise, omission of MgCl2 did not significantly alter dAMP binding. However, in the absence of MgCl2, the presence of 0.9 mM EDTA completely abolished dAMP binding. Since 14C-EDTA was itself not bound to the enzyme, a role for some divalent metal in binding to the 3'-hydroxylribonucleotide site is suggested and warrants further study.

Denaturation of DNA polymerase by heating at 65° for 5 min in 50 mM potassium phosphate buffer (pH 7.4), reduced dAMP binding to an undetectable level (Kdiss > 0.3 mM). Acylation of the enzyme by N-carboxymethylisatoic anhydride (11 residues per molecule of enzyme), a treatment which virtually eliminates binding in the triphosphate site (14), produced no detectable decrease of dAMP binding in its site. This observation further emphasizes the distinction between these two sites on the enzyme.

Effect of Nucleoside Monophosphates on Several Polymerase Functions—Although nucleoside monophosphates are not substrates for reactions catalyzed by DNA polymerase, they are the sole products of the 3'→5'-exonuclease activity and the principal products of the 5'→3'-nuclease activity (15). Thus it seemed that the 3'-hydroxylribonucleotide binding site might represent the product binding site for one of these exonuclease functions.

Accordingly the influence of dGMP was tested as a competitive inhibitor of cleavage of pTpT; cleavage of pTpT is a measure of 3'→5'-exonuclease activity (16). Assays were performed with levels of pTpT well below its Km (about 15 mM). Nevertheless only concentrations of dGMP

* Analogs are: 1, 5-fluorouracil arabinonucleoside 5'-phosphate; 2, adenine arabinonucleoside 5'-phosphate; 3, thymine arabinonucleoside 5'-phosphate; 4, uracil arabinonucleoside 5'-phosphate; 5, barbituric acid ribonucleoside 5'-phosphate; 6, nicotinamide ribonucleoside 5'-phosphate.

* D. Brutlag and A. Kornberg, unpublished results.

* J. Huberman and A. Kornberg, unpublished results.
FIG. 3. Effect of temperature on the binding of dAMP to DNA polymerase. Measurements were at 5.5° (O), 22° (C), and 39.5° (Q). Lines were determined by the least squares method.

TABLE VIII

Effect of dGMP on cleavage of pTpT

<table>
<thead>
<tr>
<th>dGMP (mM)</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With 0.5 mM pTpT</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>0.028</td>
<td>100</td>
</tr>
<tr>
<td>0.28</td>
<td>95</td>
</tr>
<tr>
<td>0.71</td>
<td></td>
</tr>
<tr>
<td>1.42</td>
<td></td>
</tr>
<tr>
<td>2.8</td>
<td></td>
</tr>
</tbody>
</table>

As an assay for 5'→3'-exonuclease activity, the liberation of dTMP and pTpT from p(T)100, labeled at the 5'-end with 32P, was used. In the presence of complementary chains of d(A)100, 32P was rapidly released, as previously described (6), mostly in the form of dTMP and pTpT. In the presence of 0.1 mM dGMP, no inhibition was observed; with 1 mM dGMP the inhibition was 58%. Thus the effect of the nucleotide on the 5'→3'-nuclease function was no more striking than on the 3'→5'-activity.

The effects of dGMP and dAMP were also tested on the polymerizing activity of the enzyme in the primed synthesis of poly d(A-T). Again inhibitory effects were observed only at very high nucleotide concentrations (22 and 90% inhibition by dGMP at 2 and 10 mM, respectively; 55% inhibition by dAMP at 15 mM).

DISCUSSION

The results presented in this paper demonstrate that E. coli DNA polymerase possesses a single site capable of binding 3'-hydroxylribonucleotides and showing much greater specificity than was found for the deoxyribonucleoside 5'-triphosphate binding site (3). The common deoxyribonucleoside 5'-mononucleotides bind to the 3'-hydroxylribonucleotide site as do certain other nucleotide analogues which have a free 3'-hydroxyl group in the ribose configuration. Analogues with modified, inverted, or missing 3'-hydroxyl groups do not bind. Additional specificity of the 3'-hydroxylribonucleotide binding site is shown by the fact that analogues with modified phosphate groups, with 2'-hydroxyl groups in the arabinose configuration, or with charged bases also do not bind.

What is the significance of the 3'-hydroxylribonucleotide site? There are persuasive reasons for believing that it is related to the site occupied by the primer terminus of the DNA chain but yet the evidence which we can advance at this time is far from conclusive. To begin with, we are dealing with an enzyme which is involved in formation of a 3', 5'-phosphodiester bond through catalysis of nucleophilic attack by the 3'-hydroxyl moiety of the deoxyribonucleotide residue at the primer terminus of a DNA chain. We might therefore expect that the enzyme would specifically recognize and bind a 3'-hydroxydeoxyribonucleotide. Beyond this theoretical argument, there are the following correlative findings between the properties of this binding site and various functions of the primer terminus.

Certain analogues of the normal deoxyribonucleotides have been tested for their capacity to be incorporated into DNA, or to be excised from it, by DNA polymerase. As outlined in Table IX, there is a striking correspondence between these capacities and the capacities of the same analogues to bind to the 3'-hydroxyribonucleotide site. In the presence of Mn++ and a DNA primer, DNA polymerase can synthesize polymers containing both ribonucleotides and deoxyribonucleotides in the same chain (17). Under certain conditions the rate of incorporation of ribonucleotides approaches that of the corresponding deoxyribonucleotides (17). Correspondingly, the ribonucleotides bind as well as the deoxyribonucleotides to the 3'-hydroxyribonucleotide site.

Tests of the susceptibility of 3'-phosphate-terminated oligonucleotides to 3'→5'-hydrolysis catalyzed by DNA polymerase have shown these structures to be resistant (16, 18). If 3'→5'-hydrolysis were to occur, one would expect the 3'-terminal nucleotide to be released as a nucleoside 3',5'-diphosphate, and,
TMP is about a thousand-fold less than the initial rate of in-
alteration of the site during catalysis might be the result, for
3'-hydroxylribonucleotides, we favor the second alternative.
the theoretical expectation of a site on DNA polymerase specific
for dGMP comparable to its K_{dis} for binding at
3'-hydroxylribonucleotide binding site, we have failed to show that
specificities of DNA polymerase and the specificities of the 3'-
hydroxylribonucleotide site, does not require added Mg^{2+} ion.
Along these lines, Atkinson and Kornberg have recently shown that
arabinose AMP residues behave like 2',3'-dideoxyribose
TMP residues: a single arabinose AMP is added, at one-thou-
sandth the normal rate, to a DNA chain, and, once incorporated,
is released at a similarly reduced rate. Once again, these
properties correlate with the inability of arabinonucleotides to
bind to the 3'-hydroxylribonucleotide site.

There is also a spatial correlation between those activities of
DNA polymerase related to the primer terminus (polymeriza-
tion and 3'-5'hydrolysis) and the 3'-hydroxylribonucleotidase
site. A fragment of DNA polymerase produced by proteolytic
cleavage of the native enzyme and having a molecular weight
about two-thirds of the native enzyme (13, 19) retains both the
3'-hydroxylribonucleotide site and the deoxynucleoside tri-
phosphate site.

This fragment also retains full polymerase and
3'-5'-exonuclease activity, but not 5'-3'-3'-exonuclease activity
(13).

An additional correlation between binding at the 3'-hydroxyl-
ribonucleotide site and 3'-5'-exonuclease activity is provided
by the fact that 3'-5'-exonuclease activity, like binding at the
3'-hydroxylribonucleotide site, does not require added Mg^{2+}
ion.

This property of the 3'-5'-exonuclease has prevented us from
being able to measure the binding of small oligonucleotides
such as pTPt at the 3'-hydroxylribonucleotide site; such oligonucleotides are too rapidly degraded by the 3'-5'-exonuclease,
even in the absence of Mg^{2+} ion.

In contrast to these positive correlations between the catalytic
specificities of DNA polymerase and the specificities of the 3'-
hydroxylribonucleotide binding site, we have failed to show that
concentrations of dGMP comparable to its K_{dis} for binding at
the 3'-hydroxylribonucleotide site have a significant effect on the
rate of DNA-polymerase-catalyzed synthesis or hydrolysis.
This lack of direct effect of dGMP on catalysis by DNA poly-
merase suggests either that the 3'-hydroxylribonucleotide site
plays no role in catalysis, or that the site is altered during cataly-
sis so that it no longer is able to bind nucleotides such as dGMP.

Because of the correlations listed in Table IX and because of
the theoretical expectation of a site on DNA polymerase specific
for 3'-hydroxylribonucleotides, we favor the second alternative.
Alteration of the site during catalysis might be the result, for
instance, of a conformational change induced by binding to DNA
or to oligonucleotides. This interpretation suggests that a
search for alterations in the properties of the 3'-hydroxylribonu-

5 P. Setlow and A. Kornberg, unpublished results.

### Table IX

<table>
<thead>
<tr>
<th>Type of nucleotide</th>
<th>Rate of synthesis</th>
<th>Rate of 3'→5' hydrolysis</th>
<th>Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>2'-Deoxyribose</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Ribose</td>
<td>&gt;25^a</td>
<td>&lt;4^a</td>
<td>&lt;3^a</td>
</tr>
<tr>
<td>3'O-Phosphoryl-2'-deoxyribose</td>
<td>&lt;1^a</td>
<td>&lt;1^a</td>
<td>&lt;2^a</td>
</tr>
<tr>
<td>2',3'-Dideoxyribose</td>
<td>&lt;1^a</td>
<td>&lt;1^a</td>
<td>&lt;3^a</td>
</tr>
<tr>
<td>Arabinose</td>
<td>&lt;1^a</td>
<td>&lt;1^a</td>
<td>&lt;3^a</td>
</tr>
</tbody>
</table>

* Based on results of Berg, Fancher, and Chamberlin (17) for
  effect of substitution of GMP for dGMP in polynucleotide synthe-
  sis.

1 Table II.
2 Lehman and Richardson, 1964 (16) and Deutscher and Korn-
  berg, 1960 (18).
3 Table III.
4 Atkinson et al., 1970 (1).
5 Table III.
6 Footnote 4.
7 Table VII.

### Acknowledgment

We are especially grateful to Dr. Alexander Nussbaum for making much of this study possible by converting
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Joel A. Huberman and Arthur Kornberg


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