BBA 66228

INHIBITION OF *ESCHERICHIA COLI* DNA POLYMERASE BY MONOVALENT CATIONS

C. C. BISHOP, JR. AND J. E. GILL

Bio-Medical Division, Lawrence Radiation Laboratory, University of California, Livermore, Calif. (U.S.A.)

(Received July 21st, 1970)

SUMMARY

1. The polymerase and nuclease activities of *Escherichia coli* DNA polymerase are markedly affected by monovalent cations. Inhibition of polymerase activity by Li^+ and Na^+ appears to be competitive with Mg^{2+}, whereas inhibition of the nuclease activity appears to be noncompetitive.

2. The hyperbolic inhibition curves produced by Li^+ and Na^+ in the polymerase reactions, together with their linear inhibition curves produced in the nuclease reactions, indicate that the enzyme contains at least two sites for Mg^{2+}, one for polymerase function and the other for nuclease function.

3. In connection with the two sites for Mg^{2+}, we offer evidence suggesting that the site for nuclease function may be better protected sterically by the enzyme from Li^+ and Na^+ than is the corresponding site for polymerase function.

4. There seems to be an absolute requirement for the monovalent cation in the *E. coli* DNA polymerase reactions.

INTRODUCTION

The DNA polymerase enzyme from *Escherichia coli* has been shown to catalyze at least four different reactions[1], including polymerization of deoxyribonucleoside triphosphates with the release of PP_i (ref. 2), exchange of the β, γ-phosphates of deoxyribonucleoside triphosphates with inorganic pyrophosphate[2,3], pyrophosphorolysis of DNA to form deoxyribonucleoside triphosphates[2,3], and hydrolysis of DNA to form deoxyribonucleoside monophosphates[4]. This report is concerned with the inhibition of DNA polymerase by Li^+, Na^+, K^+, Rb^+, and Cs^+ under conditions in which either the polymerization or hydrolysis is predominant.

Monovalent cations are reported to exert profound effects on the activity of DNA polymerase from several sources[5–10]. The only detailed studies, however, are those of KLENOW AND HENNINGSEN[5] and WALWICK AND MAIN[7]. KLENOW AND

Abbreviations: poly d(A–T), poly deoxyadenylate–deoxythymidylate; dATP, deoxyadenosine 5′-triphosphate; TTP, thymidine 5′-triphosphate.

HENNINGSEN[5], who studied stimulation by monovalent cations, concluded (1) that DNA polymerase activity from *E. coli* was stimulated 3–15-fold in the presence of chloride salts containing K^+, Rb^+, Cs^+, or NH_4^+, whereas Li^+ and Na^+ had little or no stimulating effect, (2) that monovalent cations are indispensible activators of DNA polymersase under certain conditions, (3) that the stimulation of the enzyme activity by K^+ seems to be due not to an effect on the binding of substrates but rather to an effect on the catalytic activity of the enzyme, and (4) the DNA-degrading activity of the enzyme was stimulated by K^+ but inhibited by Li^+. Our results are consistent with conclusions (2) and (3) and confirm their observation (4), but differ somewhat from conclusion (1).

MATERIALS AND METHODS

Chemicals

LiCl, NaCl, KCl and $MgSO_4$ were purchased from J. T. Baker Co., RbCl and CsCl from Fisher Scientific Co., deoxyadenosine and thymidine 5'-triphosphates (TTP) and Tris from Sigma Chemical Co., ^{3}H-labeled TTP from Schwarz BioResearch, Inc., and *E. coli* B for the preparation of DNA polymerase enzyme was obtained from the Grain Processing Corp.

Preparation of enzyme

The DNA polymerase from *E. coli* was prepared essentially by the procedure of RICHARDSON *et al.*[3], except that Steps IV, V, and VI were eliminated and the material from the DEAE-cellulose step was passed through Sephadex G-100 to remove endonuclease III (A. KORNBERG, personal communication and ref. 11). Dithiothreitol was used throughout the purification procedures instead of mercaptoethanol. The purified enzyme had a specific activity of 11000 units/mg when measured in the poly d(A–T)-primed standard assay mixture. One unit of DNA polymerase activity corresponds to the incorporation in 30 min of 10 nmoles of total deoxynucleoside monophosphates into an acid-insoluble product. The enzyme was stored frozen at −20° in 0.02 M potassium phosphate buffer (pH 7.2).

Assay procedure for DNA polymerase

The standard assay mixture contained per 0.3 ml: 50 μmoles Tris–acetate buffer (pH 7.25), 1.2 μmoles $MgSO_4$, 30 nmoles dATP, 30 nmoles [^{3}H]TTP, 5 μg poly d(A–T), 1.0 μmole dithiothreitol and 1.5 units enzyme. The reaction mixture was incubated at 37° for 20 min, then the reaction was stopped by the addition of 1.0 ml of 10% trichloroacetic acid. After 10 min the trichloroacetic acid precipitate was collected on a Whatman glass filter (GF/C) and washed 5 times with 2-ml portions of 5% trichloroacetic acid followed by several washes with 70% isopropanol. The filter discs were counted in a Beckman scintillation counter in 5.0 ml of BRAY'S[12] aqueous counting solution.

The inhibition curves were recorded from reactions in which the monovalent salt solution was added to the standard mixture or to a similar mixture with varying amounts of Mg^{2+} or buffer solutions. Variations from the standard assay procedure are described under the results for the experiment involved. Linear reaction kinetics were observed for the first 30 min under the standard conditions and also in the

presence of Li^+ and K^+ at concentrations that caused 50% inhibition of polymerase activity (10 and 140 mM, respectively). Polymerase activities are expressed in units of nmoles of total nucleoside incorporated in 20 min.

Preparation of poly d(A–T)

The poly d(A–T) used in these experiments was prepared as described by SCHACHMAN *et al.*[13]. The poly d(A–T) preparation was heated at 70° in 0.3 M KCl, passed through Sephadex G-25 equilibrated with distilled water and then dialyzed against several changes of distilled water. The poly [^{3}H]d(A–T) was prepared in the same manner with the addition of [^{3}H]TTP to the reaction mixture.

Assay for nuclease activity

The nuclease reactions were performed under the same conditions as for polymerase except that poly [^{3}H]d(A–T) replaced unlabeled poly d(A–T) and no deoxynucleoside triphosphates were present in the assay mixture. Nuclease activities are expressed as counts/min released in 20 min.

RESULTS

Reaction rates as a function of Mg^{2+} concentration

Under the standard reaction conditions described under MATERIALS AND METHODS, DNA polymerase activity was measured with Mg^{2+} concentrations ranging from 1.0 to 56.0 mM, and with buffer concentrations ranging from 16.6 to 200 mM, corresponding to 13.1–158 mM $Tris^+$ at pH 7.2. The results are summarized in Fig. 1. At low buffer concentrations (16.6–66.6 mM), the polymerase activity decreased as the Mg^{2+} concentration increased from 1 to 56 mM. However, at 132 or 200 mM Tris activity reached a maximum at about 4.0 mM Mg^{2+} and then decreased hyperbolically with increasing Mg^{2+} concentration. Note that all the curves intersect at about 30 mM Mg^{2+}.

The effects of Mg^{2+} concentration on the polymerase and the nuclease reactions under our standard conditions are compared in Fig. 2. The Mg^{2+} optimum for the nuclease reaction occurred at about 16–18 mM, which is about four times the optimum for polymerase activity.

Inhibition of polymerase activity by Li^+ and Na^+

Fig. 3 shows the inhibition by Li^+ (1.0–60.0 mM) at different Mg^{2+} concentrations. The reaction rates were determined in the standard assay mixture with 166 mM Tris–acetate buffer (131 mM $Tris^+$; pH 7.2). The control for each series of reactions contained the indicated amount of $MgSO_4$ but no LiCl or NaCl. This procedure in effect subtracts the effect of Mg^{2+} on the reaction rates. In other words, the true reaction rate of the 8 mM $MgSO_4$ control was less than the 2.0 mM $MgSO_4$ control, since 8 mM Mg^{2+} inhibits the polymerase activity, but the relative rates were higher in the 8.0 mM $MgSO_4$ mixture after LiCl was added.

Li^+, as Mg^{2+}, inhibited the polymerase activity in a manner such that the activity curves decreased hyperbolically as the ion concentrations were increased.

In the inset in Fig. 3 the Li^+ concentrations that give 50% inhibition of activity are plotted against Mg^{2+} concentration. The data were taken directly from the inhi-

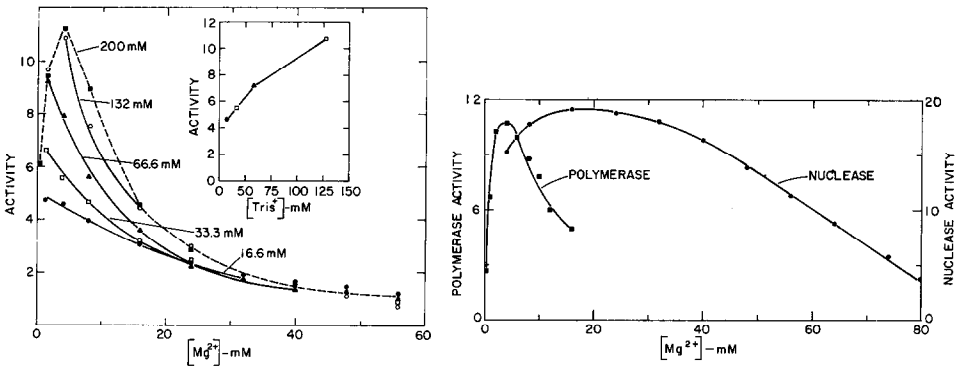


Fig. 1. Effect of Mg^{2+} concentration on polymerase activity with $Tris^+$ concentrations varied from 16.6 to 200 mM. The standard assay conditions were used except that the concentration of Tris–acetate buffer and $MgSO_4$ were varied. The $Tris^+$ concentration values were as marked on the curves (mM in each case). Each reaction tube was incubated at 37° for 20 min. Inset: Activity at 4.0 mM Mg^{2+} *versus* $Tris^+$ concentration.

Fig. 2. Dependence of polymerase and nuclease activities on Mg^{2+} concentration. The polymerase reaction mixtures contained, in 0.3 ml: 50 mmoles Tris–acetate buffer (pH 7.2); 5.0 μg poly d(A–T); 30 μmoles dATP; 30 μmoles [^{3}H]dTTP; 1.0 μmole dithiothreitol; and 1.5 units enzyme. $MgSO_4$ was added in the amounts indicated, the final volume was adjusted to 0.3 ml with distilled water ,and the mixtures were incubated at 37° for 20 min. The nuclease reaction mixtures contained the same concentration buffer, dithiothreitol and enzyme; poly[^{3}H]d(A–T) (213 μg, specific activity 10 000 counts/min per μg) was included but nucleoside triphosphates were not. The samples were incubated for 20 min.

bition curves. The plots show as Mg^{2+} concentration is increased, more Li^+ is required to cause 50% inhibition, and the relationship is linear.

When the experiments described by Fig. 3 were reversed so that the $MgSO_4$ concentration was increased at different LiCl concentrations, the activity curves of Fig. 4 were obtained. As LiCl concentration was increased, the maxima of the activity curves decreased, and the maxima shifted to higher Mg^{2+} concentrations (about 4.0, 6.0, and 8.0 mM for 0.0, 5.0, and 10 mM LiCl, respectively). Thus the converse of the result shown in Fig. 3 is also true: when LiCl concentration was increased, more $MgSO_4$ was required by the polymerase enzyme for maximum activity.

Inhibition of polymerase activity by K^+, Rb^+, and Cs^+

Inhibition of polymerase activity by K^+, Rb^+, and Cs^+ was quite different from inhibition by Li^+ and Na^+ (Fig. 5). The larger ions stimulated polymerase activity at concentrations up to about 30 mM and then inhibited the reaction rates linearly. The concentrations for 50% inhibition of polymerase activity were 140 mM for KCl, 155 mM for RbCl, and 150 mM for CsCl. The variation among the three ions is probably due to some specific ion effect. The degree of stimulation by these ions was 1–19% under the conditions of the assays where the $Tris^+$ concentration was 131 mM. In other experiments, not reported in detail here, we have observed greater stimulation by K^+, Rb^+, and Cs^+ when the buffer concentration was lower.

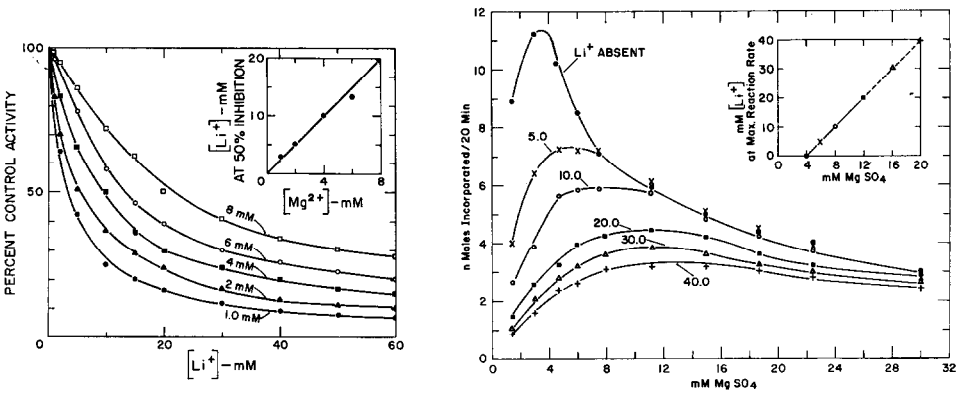


Fig. 3. Inhibition of polymerase activity by Li^+; competition between Li^+ and Mg^{2+}. Reaction conditions were standard except that Mg^{2+} concentrations were as indicated on the curves. Controls (no LiCl present) were run for each $MgSO_4$ concentration and the points on the curves represent the percent of incorporation relative to the respective controls. The inset graph shows the relation between LiCl concentration and $MgSO_4$ concentration such that the polymerase activity is inhibited by 50%.

Fig. 4. The effect of $MgSO_4$ on the inhibition of polymerase by LiCl. The reaction conditions were standard except that Li^+ concentrations had the values (mM) indicated on the curves. The inset is a plot of Li^+ concentration *versus* Mg^{2+}concentration at the maximum reaction rates; the dotted portion suggests that the maximum rates at 30.0 and 40.0 mM Li^+ should have occurred at the Mg^{2+} concentration values indicated; inhibition by Mg^{2+} is so great above 12.0 mM that the maxima could not be determined accurately.

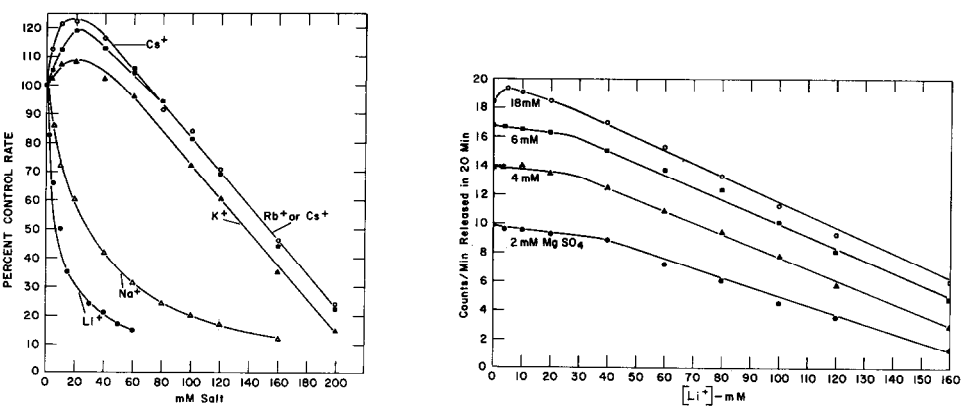
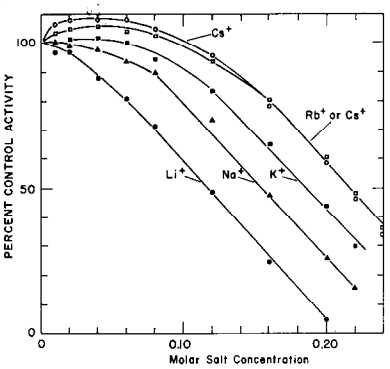


Fig. 5. Inhibition of polymerase activity by KCl (▲—▲), RbCl (○—○) and CsCl (■—■). The curves for LiCl (●—●) and NaCl (△—△) (4.0 mM $MgSO_4$) are included for comparison. Reaction mixtures contained the standard concentrations of components (4.0 mM $MgSO_4$); after addition of salt solutions, final volumes were adjusted to 0.3 ml with distilled water. The samples were reacted for 20 min at 37° and incorporation was determined by the standard procedure. Activity is expressed as percentage of control, based on nmoles of [^{3}H]TTP incorporated per 20 min.

Fig. 6. Inhibition of the DNA nuclease reaction by LiCl and the effects of $MgSO_4$ concentration on reaction rate. The reaction mixtures contained 50 μmoles Tris–acetate buffer, 2.3 μg poly[^{3}H]-d(A-T) (specific activity 10 000 counts/min per μg), 1.0 μmole dithiothreitol, and 2.0 units enzyme. The LiCl and $MgSO_4$ were added in the appropriate amounts to a final volume of 0.3 ml. The control for each concentration of $MgSO_4$ (LiCl absent) is represented by points on the vertical axis. All samples were incubated for 20 min at 37°.

Inhibition of nuclease activity by Li^+

Fig. 6 shows how LiCl inhibited nuclease activity at Mg^{2+} concentrations between 2.0 and 18.0 mM; Mg^{2+} concentrations 10.0, 14.0, and 16.0 mM were also tested but are not shown because the differences were small. The points on the ordinate axis are the controls (no LiCl). In general, activity rates increased as Mg^{2+} concentration increased up to 18.0 mM. At 18.0 mM a slight stimulation occurred between 5.0 and 20 mM LiCl. Polymerase activity required only 10.0 mM LiCl to cause 50% inhibition with 4.0 mM $MgSO_4$ (Fig. 3), whereas nuclease activity (18.0 mM $MgSO_4$)



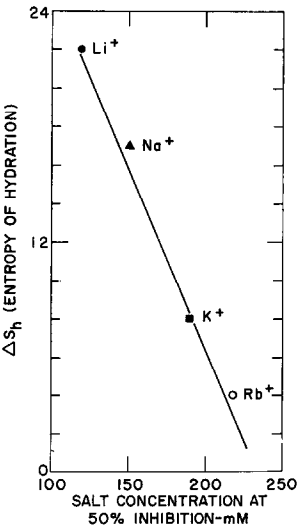


Fig. 7. Inhibition of nuclease activity by monovalent cations. The final concentration of the reaction components were: 100 mM Tris–acetate buffer (pH 7.2); 10 mM $MgSO_4$; poly [^{3}H]d(A–T) (8.1 μg/ml); 3.3 mM dithiothreitol; and the appropriate salt solution in a final volume of 0.3 ml. The same reaction mixture with no salt solution added served as the control and all rates were expressed as a percentage of that obtained for the control. Activity is based on counts/min released.

Fig. 8. Inhibition of nuclease activity by monovalent cations. The relation between the concentration of monovalent ion which gave 50% inhibition of nuclease activity and the entropy of hydration (ΔS_h) for the respective ion. The concentrations of the monovalent ions causing 50% inhibitions were taken directly from Fig. 8 and the values for ΔS_h were obtained from BULL[18].

required 125 mM LiCl, more than 10 times as much, to produce the same degree of inhibition.

Fig. 7 compares the effects on nuclease activity of all the monovalent ions studied. Reaction conditions varied from the standard procedure in that 100 mM Tris–acetate buffer (79 mM $Tris^+$) and 10.0 mM $MgSO_4$ were used. All of the ions inhibited activity linearly and the curves had about the same slope. Stimulation was apparent with the larger ions but not with Li^+ and Na^+. The concentration of salt solution necessary to cause 50% inhibition increased in the order, LiCl < NaCl < < KCl < RbCl ≈ CsCl.

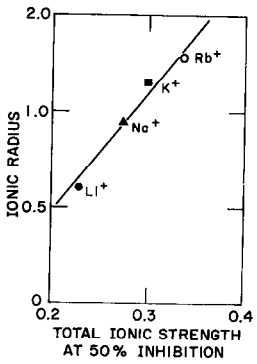


Fig. 9. Inhibition of nuclease activity by monovalent cations. The relation between the total ionic strength of the reaction mixture and the ionic radius of the respective ions. The ionic radii are values for the naked ions and were obtained from BULL[18].

Relationship between nuclease inhibition and the thermodynamic properties of monovalent ions

Fig. 8 and 9 show how the inhibition of nuclease activity by monovalent cations may be related to the entropy of hydration and to ionic radius. The concentration of monovalent ion for 50% inhibition is linearly increased as its entropy of hydration is decreased. The total ionic strength of the reaction mixture at 50% inhibition for the four ions is similarly related to ionic radius (naked ion). An approximately linear relation (not shown) also exists between free energy of hydration, heat of hydration, and concentration of ions for 50% inhibition of nuclease.

DISCUSSION

Mg^{2+} is required for both polymerase and nuclease activities, but the requirements are quite different. Polymerase activity is greatly reduced by Mg^{2+} above 4.0 mM under standard reaction conditions. ENGLUND *et al.*[14] showed triphosphate binding requires Mg^{2+} but template binding does not[15]. Nothing is yet known about the mechanism of Mg^{2+} participation in triphosphate binding, but it is possible that the activation resembles that of ATPases. WOLF AND ADOLPH[16] and PHILLIPS[17] suggest that Mg^{2+} and ATP form the complex Mg–ATP^{2-}, which is the active substrate, and in the presence of excess Mg^{2+}, other complexes are formed that inhibit ATPase. A similar complex (Mg–$dATP^{2-}$) in triphosphate binding could explain why Mg^{2+} greatly inhibits polymerase activity. Nuclease is less sensitive to magnesium concentration, as Fig. 2 shows. Therefore, its catalytic properties must involve a different association between enzyme and Mg^{2+}. The Mg^{2+} concentration for 50% inhibition of nuclease activity was about 60 mM compared to 15 mM for polymerase activity, and the total ionic strength at 50% inhibition was 0.371 for nuclease and 0.191 for polymerase. These results indicate that there are at least two sites for magnesium activation of the DNA polymerase enzyme, one for polymerase and one for the nuclease.

The idea of distinct Mg^{2+} sites for polymerase and nuclease activities is further supported by study of the effects of monovalent cations. The inhibition of polymerase activity by Li^+ and Na^+ was very similar to its inhibition by Mg^{2+}, suggesting a

closely related mechanism. Li^+ has about the same ionic radius as Mg^{2+} (0.6 and 0.65 Å, respectively), but only half the charge density.

By contrast, ions of the size of K^+ or larger (Rb^+ and Cs^+) appear to inhibit polymerase activity by a mechanism other than direct competition with Mg^{2+} for some common site. In ions of larger radius, the ion charge acts through a greater distance and thus the electrostatic force is weaker. These ions probably stimulate polymerase activity at concentrations below 30 mM because optimal ratio of charge concentration, about 38, is established between monovalent and divalent ions (+/++). This ratio points to the indispensibility of the monovalent ion in polymerase activity.

The effects of Li^+ and Na^+ on nuclease activity are quite different from their effects on polymerase activity. These ions in fact inhibit nuclease in very much the same way as K^+, Rb^+, and Cs^+ inhibit polymerase. In the standard reaction mixture (10 mM $MgSO_4$, 131 mM $Tris^+$), Li^+ and Na^+ never stimulated nuclease activity but inhibited it linearly with increasing salt concentration.

Perhaps the most significant feature of the inhibition of nuclease by monovalent ions is the linear relation between the ion concentration for 50% inhibition of activity and the ionic radius. The concentration of ions for 50% inhibition increases in the order $Li^+ < Na^+ < K^+ < Rb^+ \approx Cs^+$. These concentrations are also related to the change in free energy and enthalpy due to hydration, which suggests that the inhibition of nuclease activity by monovalent ions is related to the extent of ion hydration and to ionic strength. At high ionic strength, the enzyme could unfold and/or the binding of template and Mg^{2+} could be increased.

In the polymerase reaction, one must also consider the possibility that ionic strength is involved in the effects of the K^+, Rb^+, and Cs^+. ENGLUND *et al.*[14] observed that the dissociation constant for triphosphate binding to polymerase changed from $1.6 \cdot 10^{-5}$ to $3.2 \cdot 10^{-5}$ M when the total ionic strength was raised to 0.277 with KCl. We observed a 50% decrease in polymerase activity at an ionic strength of 0.287 in the presence of KCl. Such high ionic strengths could be expected to decrease the association or binding of Mg–dATP complex to polymerase enzyme or to interfere with the formation of the Mg–dATP complex.

ACKNOWLEDGEMENT

This work was performed under the auspices of the U.S. Atomic Energy Commission.

REFERENCES

1 M. P. DEUTSCHER AND A. KORNBERG, *J. Biol. Chem.*, 244 (1969) 3019.
2 M. J. BESSMAN, I. R. LEHMAN, E. S. SIMMS AND A. KORNBERG, *J. Biol. Chem.*, 233 (1958) 171.
3 C. C. RICHARDSON, C. L. SCHILDKRAUT, H. V. APOSHIAN AND A. KORNBERG, *J. Biol. Chem.*, 239 (1964) 222.
4 I. R. LEHMAN AND C. C. RICHARDSON, *J. Biol. Chem.*, 239 (1964) 233.
5 H. KLENOW AND I. HENNINGSEN, *European J. Biochem.*, 9 (1969) 133.
6 I. R. LEHMAN, M. J. BESSMAN, E. S. SIMMS AND A. KORNBERG, *J. Biol. Chem.*, 233 (1958) 163.
7 E. R. WALWICK AND R. K. MAIN, *Biochim. Biophys. Acta*, 61 (1964) 876.
8 M. GOULIAN, Z. J. LUCAS AND A. KORNBERG, *J. Biol. Chem.*, 243 (1968) 627.
9 C. W. M. ORR, S. T. HERRIOTT AND M. J. BESSMAN, *J. Biol. Chem.*, 240 (1965) 4652.

10 T. Y. HUH AND C. W. HELLEINER, *Biochim. Biophys. Acta*, 155 (1968) 107.
11 T. M. JOVIN, P. T. ENGLUND AND L. L. BERTSCH, *J. Biol. Chem.*, 244 (1969) 2996.
12 G. A. BRAY, *Anal. Biochem.*, 1 (1969) 279.
13 H. K. SCHACHMAN, J. ADLER, C. M. RADDING, I. R. LEHMAN AND A. KORNBERG, *J. Biol. Chem.*, 235 (1960) 3242.
14 P. T. ENGLUND, J. A. HUBERMAN, T. M. JOVIN AND A. KORNBERG, *J. Biol. Chem.*, 244 (1969) 3038.
15 P. T. ENGLUND, R. B. KELLEY AND A. KORNBERG, *J. Biol. Chem.*, 244 (1969) 3045.
16 H. U. WOLF AND L. ADOLPH, *Europ. J. Biochem.*, 8 (1969) 68.
17 R. PHILLIPS, *Chem. Rev.*, 66 (1966) 501.
18 H. B. BULL, *Introduction to Physical Biochemistry*, F. A. Davis Co., Philadelphia, Pa., 1964, p. 65.