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KINETIC STUDIES ON DNA POLYMERASE

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SUMMARY

Pyrophosphorolysis and hydrolysis of DNA by DNA polymerase (deoxynucleosidetriphosphate DNA deoxynucleotydyl-transferase, EC 2.7.7 7) from *Escherichia coli* were studied Competition between pyrophosphate and hydroxyl ions is postulated The K_m values of pyrophosphate in pyrophosphorolysis and of hydroxyl ions in hydrolysis equaled the corresponding K_i values in hydrolysis and pyrophosphorolysis Furthermore both reactions were inhibited competitively to the same degree by ATP and dATP It is concluded that one active site is responsible for both pyrophosphorolysis and hydrolysis These results, together with additional studies on the specificity of the polycondensation, lead to a model for the three functions of the enzyme

INTRODUCTION

The DNA polymerase (deoxynucleosidetriphosphate DNA deoxynucleotidyltransferase, EC 2 7.7 7) isolated from *Escherichia coli* catalyzes the polycondensation of deoxynucleoside triphosphates and the reverse reaction, the pyrophosphorolysis of DNA from the 3'-end[1]. The same enzyme preparation also catalyzes the hydrolysis of DNA from the 3'-end This exonuclease II activity could not be separated from the polymerase activity and both are destroyed at the same rate by urea or heat[1]

$$\text{DNA-3'-OH} + \text{pppN} \rightleftharpoons \text{DNA-3'-pN} + \text{PP}_i$$
$$\text{DNA-3'-OH} + \text{pN} \quad \leftarrow \text{DNA-3'-pN} + \text{H}_2\text{O}$$

This paper presents evidence that there is one active site on one enzyme that is responsible for both the pyrophosphorolysis and hydrolysis It is demonstrated that probably not water, but hydroxyl ions represent the reactant in the hydrolytic action and that pyrophosphate and hydroxyl ions seem to compete for the same active site and both reactions are inhibited by the same inhibitors to the same extent

MATERIALS AND METHODS

DNA polymerase was purified following the procedure of RICHARDSON *et al*.[1]

Abbreviations pppN or NTP, nucleoside 5'-triphosphate, pN or NMP, nucleoside 5'-monophosphate.

including Step IX, *i e*, chromatography on hydroxyapatite The final specific activity was determined with activated thymus DNA as a primer to be 9200 units of polymerase per mg of protein Endonuclease from *Micrococcus lysodeikticus* was purchased from Worthington Biochemical Corp.

Deoxyribonucleoside triphosphates were prepared by the method of SMITH AND KHORANA[2] 3'-Acetyl-TTP was prepared by acetylation of thymidine 5'-monophosphate and subsequent phosphorylation according to the method mentioned above [^{3}H]dATP was purchased from Schwarz, Bio-Research, Inc ; ATP, UTP, CTP, GTP, ADP and AMP from Zellstoffwerke, Waldhof, deoxyadenosine from Calbiochem A.G

Calf thymus DNA and salmon sperm DNA were purchased from Sigma Chemical Company, herring sperm DNA from Serva, Entwicklungslabor. Activated thymus DNA was prepared by treatment with bovine pancreas deoxyribonuclease as described by APOSHIAN AND KORNBERG[3]. DNA from bacteriophage T7 was prepared by the procedure of THOMAS AND ABELSON[4]. [^{32}P]DNA was prepared from *E. coli* by the method of THOMAS *et al.*[5]. [^{3}H]DNA from *E coli* and ΦX174 were kind gifts of Dr. H SCHALLER

Assay of polycondensation

Polycondensation was determined by a modification of the method of RICHARDSON *et al*[1]. The assay measures the conversion of ^{3}H-labelled deoxyribonucleoside triphosphates into an acid-insoluble product. The incubation mixture (0.3 ml) contained: 20 μmoles of glycine buffer, pH 9 2, 2 μmoles of $MgCl_2$, 0.01 μmole each of TTP, dCTP, dGTP and [^{3}H]dATP (20 μC/μmole), 12 μg of activated thymus DNA and 0.02 to 0.5 polycondensation units of the enzyme The incubation period was 30 min at 37° The reaction was terminated by chilling to 0°. 0.5 ml of a solution of herring sperm DNA (5 mg/ml) and 1 ml of 1 M perchloric acid were added. After 5 min at 0°, 3 ml of water and 0.5 ml of saturated $Na_4P_2O_7$ solution were added and the mixture filtered through a membrane filter (MF 50), which had been washed previously with a solution saturated with $Na_4P_2O_7$ After washing with 25 ml of 1% trichloroacetic acid the filter was dried, placed in a liquid scintillation solution and the radioactivity determined in a Packard Tri-Carb liquid scintillation counter.

One unit of enzymatic activity in the polycondensation reaction caused the incorporation of 10 mμmoles of total nucleotide into the acid-insoluble product in 30 min. All determinations were done in duplicate and the mean values taken The standard deviation of a single observation was 5%

Assay of hydrolysis

Hydrolysis was determined by the method of LEHMAN AND RICHARDSON[6]. The assay measures the conversion of ^{32}P-labelled DNA to acid-soluble fragments. The incubation mixture (0.3 ml) contained· 20 μmoles of glycine buffer, pH 9.2; 2 μmoles of $MgCl_2$, 0 3 μmole of 2-mercaptoethanol; 6 μg of [^{32}P]DNA from *E coli* (20–75 μC/μmole phosphate) 0.01–0 2 hydrolysing units of the enzyme For hydrolyses carried out at pH 7 7, 8.0, 8.3 and 8.7, appropriate mixtures of Tris buffer and glycine buffer were prepared at 37° The incubation time was 30 min at 37° The reaction was stopped by chilling to 0°. Then 0.2 ml of a solution of denatured salmon sperm DNA (1 mg/ml) and 0 5 ml of a solution of 1 M trichloroacetic acid were added After 5 min at 0° the mixture was centrifuged for 10 min at 3000 rev./min at 0–2° 0 2 ml of the

supernatant was transferred into a liquid scintillation solution and the radioactivity measured 1 unit of enzymatic activity in the hydrolysis caused the release of 10 mμmoles of acid-soluble material in 30 min Determinations were made in triplicate and the mean values taken The standard deviation of the mean was 7%

Assay of pyrophosphorolysis

Pyrophosphorolysis was determined by converting ^{32}P-labelled DNA to acid soluble fragments and then chromatographically analyzing the nucleoside triphosphates in the mononucleotide fraction The incubation mixture (0 3 ml) contained 20 μmoles of potassium phosphate buffer, pH 7 0, 2 μmoles of $MgCl_2$, 0 3 μmole of 2-mercaptoethanol, 1 0 μmole of $Na_4P_2O_7$, 6 μg of [^{32}P]DNA (20–75 μC/μmole of P) and 0 01 to 0 1 pyrophosphorolysing unit of enzyme For pyrophosphorolyses at the pH values 7 0, 7.3 and 7 7 appropriate mixtures of potassium phosphate buffer and Tris buffer were prepared at 37° The incubation period was 30 min at 37°. 10% of the solution was treated as described for the assay of hydrolysis To 90% of the solution (0.27 ml) 25 μl of 1 M formic acid and a suspension of 5 mg of activated charcoal in 0 1 ml of water were added After shaking for 5 min the charcoal was filtered and washed with 25 ml of water. The charcoal was shaken with a solution of 50% ethanol and 0 6% NH_3 in water for 5 min at 30°, cooled to 0° and centrifuged for 10 min at 3000 rev./min The supernatant was evaporated at reduced pressure, redissolved in 10 μl of water and applied to a thin layer of polyethyleneimine cellulose according to RANDERATH[7] It was eluted with 0 7 M NaCl–1 M formic acid at 0° The nucleoside triphosphate (NTP) and nucleoside monophosphate (NMP) spots were identified by autoradiography, scraped from the plate, introduced into a liquid scintillation solution and the radioactivity measured Nucleoside diphosphates were not observed unless the elution was done at room temperature From the proportion of NTP/(NTP+NMP) and the amount of acid-soluble radioactivity, the amount of nucleoside triphosphate was calculated. 1 unit of enzymatic activity in pyrophosphorolysis was the amount that caused the release of 10 mμmoles of nucleoside triphosphate in 30 min Determinations were made in triplicate and the mean values taken The standard deviation of the mean was 7%

The same assay was used to measure pyrophosphorolysis and hydrolysis simultaneously Under the conditions of this assay, the adsorption of [^3H]thymidine monophosphate and [^3H]TTP on charcoal was almost complete The filtrate contained less than 1% of the radioactivity The elution from the charcoal was incomplete, but the ratio of NMP to NTP was always 1.

Assay of endonuclease

This assay measures the biological inactivation of circular single stranded DNA of bacteriophage ΦX174 To compare the endo-with the exonuclease activity, [^3H]DNA from *E coli* was incubated in the same samples The incubation mixture (0 3 ml) contained 20 μmoles of Tris buffer, pH 8 0, 2 μmoles of $MgCl_2$, 0.3 μmole of 2-mercaptoethanol; 1 25 μg of [^3H]DNA from *E coli* (40 μC/μmole of phosphate); 6 15 μg of DNA from ΦX174, and 0.5 μg of enzyme Incubation was performed at 37° and samples taken periodically. The acid-soluble radioactivity was determined as described above Spheroblasts of *E coli* were used to measure the biological activity of ΦX174 DNA 2 5% of the [^3H]DNA were degraded in 30 min In the same period the biological

activity of ΦX174 was reduced by 40%. Since this DNA contains 5500 nucleotides per ring, approximately 0.0075% of the internucleotide bonds of ΦX174 DNA present in the incubation mixture were split in 30 min. This corresponds to about 0.3% endonuclease of the total nuclease.

RESULTS

Purity of DNA polymerase

A low content of other nucleases was crucial for these studies. According to LEHMAN AND NUSSBAUM[8] exonuclease I is eliminated by the fractionation with ammonium sulphate, *i.e.*, Step IV of the purification of DNA polymerase. Therefore the presence of exonuclease I was not checked. DNA-phosphatase-exonuclease (exonuclease III) was assayed by the release of P_i from DNA terminating in 3'-phosphate according to the method of RICHARDSON AND KORNBERG[9]. It was present and amounted to 0.6% of the total exonucleolytic activity. Endonuclease was assayed by a very sensitive test, the biological inactivation of circular single-stranded DNA of the bacteriophage ΦX 174. 0.3% of the nucleolytic activity of the preparation was due to endonucleases. In comparison with exonuclease II activity the contamination by endonucleases and exonuclease III was so low that it did not obscure the kinetic studies on DNA polymerase and exonuclease II.

After the final step of purification of the enzyme, the proportion of the unit activities of polycondensation to hydrolysis to pyrophosphorolysis was 10 to 2 to 1. Each reaction was assayed under its standard conditions.

Binding of DNA to the enzyme

Fig. 1 shows the dependence of the rates of hydrolysis and pyrophosphorolysis on the concentration of DNA. The maximal velocity v_{max} and the Michaelis constant K_m were determined from the reciprocal plots according to LINEWEAVER AND BURK[10]. For both reactions the same K_m value of 7 mg DNA/l is obtained. Since the molecular weight of the DNA was not well defined in that case the concentration can only be given in mg/l. To obtain an exact K_m value, homogeneous DNA was prepared from T7 bacteriophage which has a molecular weight of $25 \cdot 10^6$ (ref. 4). By the same method K_m for polycondensation was determined to be $3.2 \cdot 10^{-10}$ mole DNA/l.

Fragments of DNA obtained by treatment with endonuclease from *Micrococcus lysodeikticus* have 3'-phosphoryl end groups. LEHMAN AND RICHARDSON[6] have shown that this product inhibits the polycondensation by DNA polymerase, but not the hydrolysis by exonuclease II. This result argues against the identity of both enzymes. Therefore, the effect of pretreatment of the DNA on hydrolysis was compared to that on pyrophosphorolysis. Fig. 2 shows that the DNA is degraded by micrococcal nuclease as measured by loss of viscosity, but that the reaction rates of pyrophosphorolysis and hydrolysis remain unchanged. This is in agreement with the assumption that one enzyme might catalyze both reactions and that a free 3'-OH end is necessary for the condensation, but not for the degrading reactions.

Competition between pyrophosphate and hydroxyl ions for the enzyme

If PP_i and OH^- do compete for the same active site of the enzyme, the rate of pyrophosphorolysis should decrease to the same extent as the rate of hydrolysis

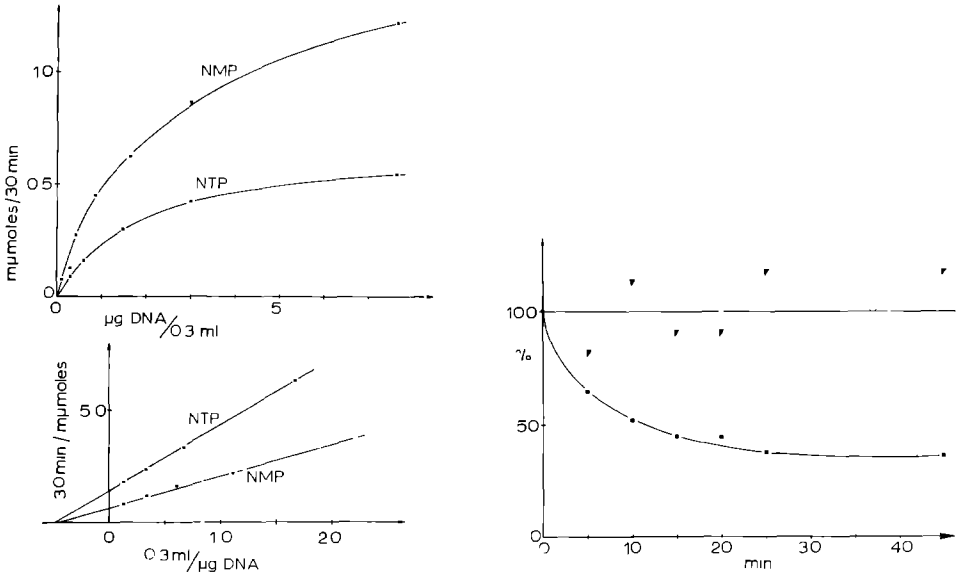

Fig 1 Dependence of pyrophosphorolysis and hydrolysis on the concentration of DNA (standard assays with 0 05 μg of enzyme)

Fig 2 Effect of pretreatment of DNA with micrococcal nuclease on the rates of pyrophosphorolysis and hydrolysis ●, specific viscosity, •, relative rate of hydrolysis, ▲, relative rate of pyrophosphorolysis The reaction mixture (10 ml) contained 1 mmole of glycine buffer (pH 9 2), 0 1 mmole of $CaCl_2$, 2 μmoles of [^{32}P]DNA (4 μC/μmole of P_i) and 0 2 unit[9] (○) of micrococcal nuclease The decrease of the specific viscosity was followed in an Ostwald viscosimeter maintained at 37° Samples of 1 ml were drawn at the indicated intervals and dialyzed against 0 1 M KCl at 0° for 15 h The samples were tested as described under standard conditions except that 2 mμmoles of DNA and 0 05 μg of enzyme were added

increases The increase of hydrolytic activity with increasing OH^- concentration and the simultaneous decrease of pyrophosphorolysis (Fig. 3) suggests that this assumption might be true Certainly, the pH has other effects on the enzyme, which might explain

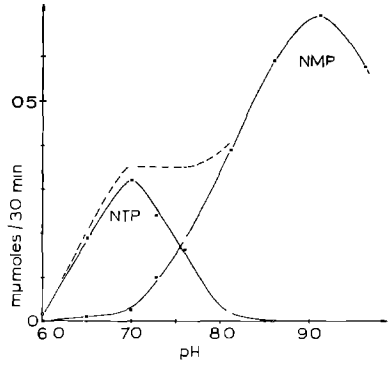

Fig 3 Effect of pH on the degradation of DNA by DNA polymerase Total degradation and shares of NMP and NTP are shown The standard assay of pyrophosphorolysis was used with 0 05 μg of enzyme

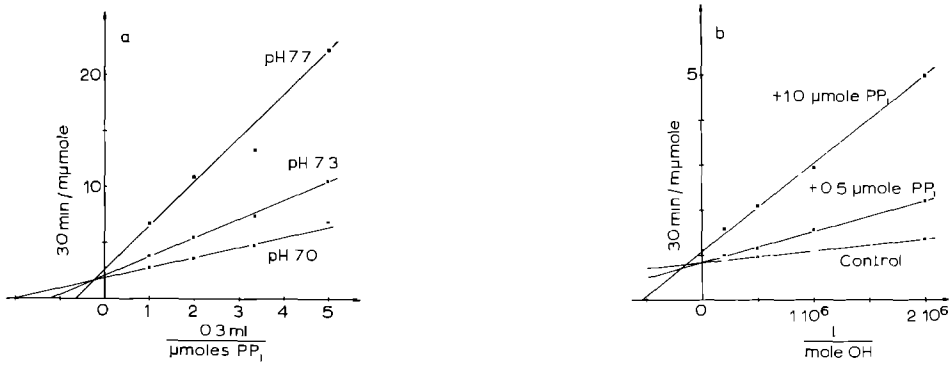

Fig. 4 Inhibition of (a) pyrophosphorolysis by OH^-, (b) hydrolysis by pyrophosphate (standard assays with 0 05 μg of enzyme)

the decrease of both reaction rates below pH 7 and above pH 9 In order to test these assumptions, the pyrophosphorolysis was measured as a function of pyrophosphate concentration at three OH^- concentrations and the hydrolysis was determined as a function of OH^- at 3 concentrations of pyrophosphate For both reactions reciprocal plots according to LINEWEAVER AND BURK were made. Figs. 4a and b show that approximately the same intercept ($1/v_{max}$) was found for all three OH^- concentrations in pyrophosphorolysis and for all three pyrophosphate concentrations in hydrolysis Therefore, in the investigated pH range (7 0–7 7), OH^- act like competitive inhibitors in pyrophosphorolysis, and, *vice versa*, pyrophosphate ions compete for OH^- in hydrolysis between pH 7.7 and 8 7 From Fig 4a the Michaelis constant of pyrophosphate was derived as $K_m = 1\ 10^{-3}$ mole/l and the inhibition constant of OH^- as $K_i = 3 \cdot 10^{-7}$ mole/l Reciprocally, from Fig 4b the Michaelis constant of OH^- was determined as $K_m = 3 \cdot 10^{-7}$ mole/l, and the inhibition constant of pyrophosphate as $K_i = 1 \cdot 10^{-3}$ mole/l. These results demonstrate a competition of pyrophosphate and OH^- for the DNA polymerase and a much lower Michaelis constant for OH^- than for pyrophosphate ions The Michaelis constant for OH^- is a very rough figure because

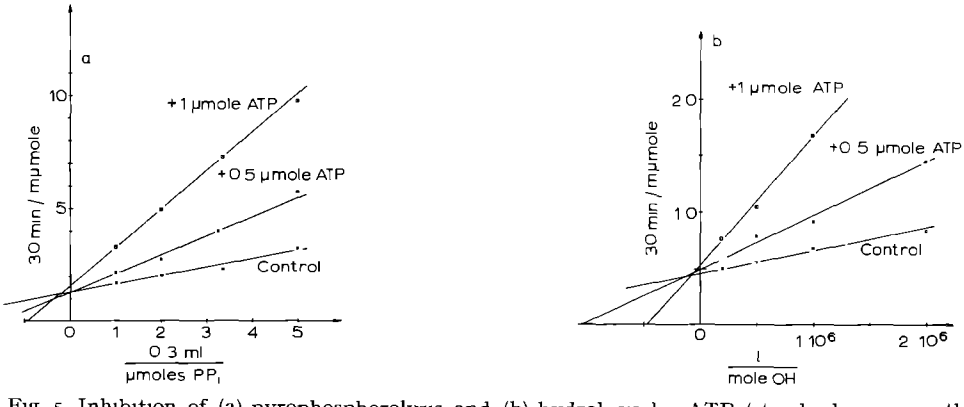

Fig. 5 Inhibition of (a) pyrophosphorolysis and (b) hydrolysis by ATP (standard assays with 0.1 μg of enzyme)

only the activities of the OH^- were used and no attempt was made to correct these values by appropriate activity coefficients

Inhibition by specific inhibitors

If the active site of the enzyme for pyrophosphorolysis and hydrolysis is identical, specific inhibitors should inhibit both reactions and should have the same inhibitor constant in both reactions As analogues to pyrophosphate, ribonucleoside triphosphates were chosen because they contain a pyrophosphate moiety Ribonucleoside triphosphates cannot be condensed with DNA under the standard conditions[11] Pyrophosphorolysis and hydrolysis were studied without and in the presence of two concentrations of ATP Fig. 5a and 5b show the reciprocal plots of the results, which indicate a competition of ATP for pyrophosphate as well as for OH^- In both reactions the same value of $K_i = 2 \cdot 10^{-3}$ mole ATP/l was determined from the plots. The good agreement of the K_i values indicates the identity of the enzymatic site in both reactions In the same way dATP was studied dATP is not incorporated into DNA in the absence of the other 3 dNTPs (ref 11) Therefore the inhibition observed by this substrate is not due to the reversal of the pyrophosphorolysis It can be seen from

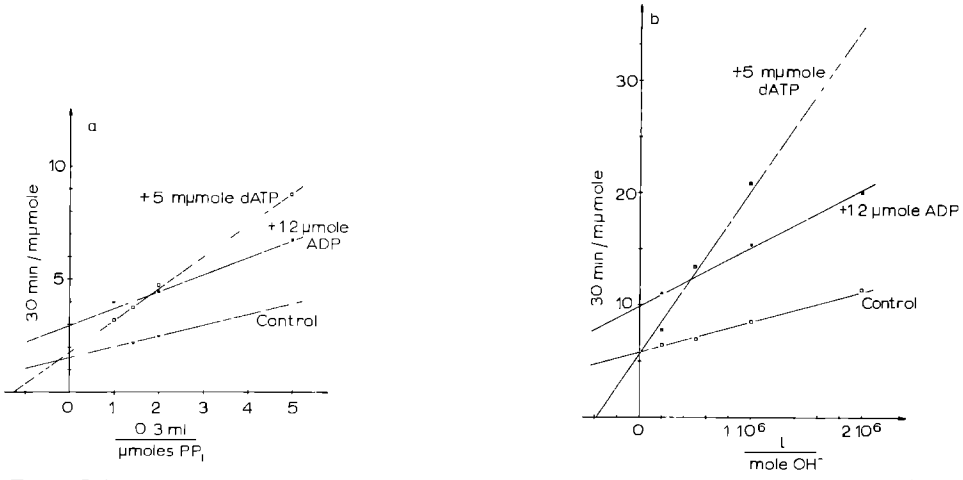

Fig 6 Inhibition of (a) pyrophosphorolysis and (b) hydrolysis by dATP and ADP (standard assay of pyrophosphorolysis with 0 05 μg of enzyme, standard assay of hydrolysis with 0 01 μg of enzyme)

Figs 6a and 6b that pyrophosphorolysis and hydrolysis are competitively inhibited by dATP In the pyrophosphorolysis K_i was $1 \cdot 10^{-5}$ mole dATP/l, whereas in hydrolysis K_i was $3 \cdot 10^{-5}$ mole dATP/l Although these values are different they are of the same order of magnitude which is about 100 times lower than those of rATP This can be interpreted that dATP has a higher affinity for the enzyme than has rATP

In Figs 6a and b the results with ADP are included Both reactions are inhibited, but the inhibition is neither competitive for pyrophosphate nor for hydroxyl ions The inhibitory effect of ADP is neither reversed by increasing amounts of DNA Therefore the inhibition by ADP is not due to the displacement of DNA Adenosine monophosphate and deoxyadenosine had no significant inhibitory effect in pyro-

phosphorolysis or hydrolysis Each compound was added in an amount of 2 μmoles to the standard assays.

Inhibition of the polycondensation

In order to compare the specificity of the enzyme in condensation and degradation, the effect of the four dNTP's, each of the four ribo-NTP's, ADP, adenosine monophosphate and deoxyadenosine on the polycondensation was studied.

The K_m value for the condensation with the four dNTP's was determined as $K_m = 2 \cdot 10^{-5}$ mole dNTP/l This is nearly the same value as the inhibitor constants of dATP in pyrophosphorolysis and hydrolysis

The ribonucleoside triphosphates inhibited the incorporation of the deoxyribonucleoside triphosphates competitively Fig 7 shows the results of inhibition experiments with ATP. The inhibition constants were found as. $K_{i(ATP)} = 1\,4\ 10^{-2}$ mole ATP/l; $K_{i(CTP)} = 1\,4\ 10^{-2}$ mole CTP/l, $K_{i(GTP)} = 0\,6\ 10^{-2}$ mole GTP/l, $K_{i(UTP)} = 0\,7\ 10^{-2}$ mole UTP/l These results show that the ribo-NTP's have a lower affinity to the enzyme than the deoxy-NTP's. It is remarkable that the K_i values of the ribo-NTP's are different for the condensing and the degrading reactions.

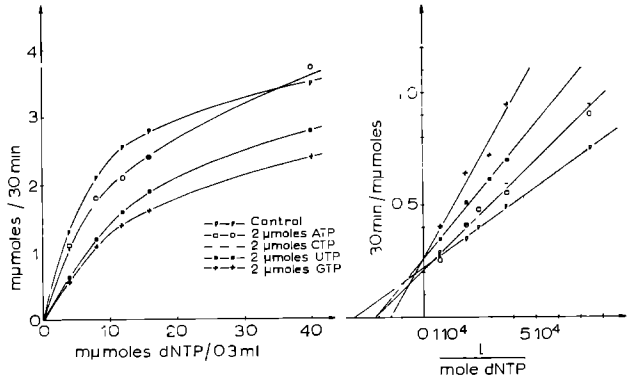

Fig 7 Inhibition of polycondensation by ribonucleoside triphosphates (standard assay with 0 05 μg of enzyme)

The addition of 2 μmoles each of ADP, adenosine monophosphate and deoxyadenosine had no significant inhibitory effect on the polycondensation It is remarkable that ADP inhibited pyrophosphorolysis and hydrolysis, but not polycondensation. For inhibition of the condensation the presence of a γ-phosphate in the nucleoside phosphate seems to be necessary

The difference in the Michaelis constants and inhibitor constants between ribo- and deoxy-NTP's indicates that the structure of the sugar moiety is important. To prove this, the 3'-OH in TTP was blocked by acetylation It was found that the polycondensation is competitively inhibited by 3'-acetyl-TTP. In an experiment where 1 μmole of this inhibitor was added to the standard assay of polycondensation, an inhibitor constant of 2 10^{-4} mole of 3'-acetyl-TTP/l was found Obviously, a free 3'-OH of the mononucleotide is recognized by the enzyme, although it does not participate in the formation of the diester linkage However, it cannot be excluded that 3'-acetyl-TTP can substitute TTP to a certain extent, since in a control experi-

ment the initial velocity of polycondensation with 3'-acetyl-TTP was 7% of that found with TTP

DISCUSSION

In Table I the effect of substrates and inhibitors are summarized with the corresponding K_m and K_i values. Pyrophosphate and hydroxyl ions seem to compete for the same site of the enzyme in the degradation of DNA, since for each substrate the K_m values equal the inhibitor constants K_i. Both reactions are inhibited by ATP and dATP and the inhibition is approximately competitive for pyrophosphate and for hydroxyl ions The inhibition of both reactions by ADP is not competitive Pyrophosphorolysis and hydrolysis and thus polymerase and exonuclease II activity seem to be two functions of the same enzyme.

TABLE I

EFFECTS OF SUBSTRATES AND INHIBITORS
All values are given in moles/l

Substance	*Polycondensation*	*Pyrophosphorolysis*	*Hydrolysis*
T7-DNA	$K_m = 3\ 10^{-10}$		
3'-p-DNA	Inhibition	No inhibition	No inhibition
OH^-		$K_i = 3 \cdot 10^{-7}$	$K_m\ 3\ 10^{-7}$
PP_i		$K_m = 1\ 10^{-3}$	$K_i = 1\ 10^{-3}$
dATP		$K_i = 1\ 10^{-5}$	$K_i = 3\ 10^{-5}$
+dGTP			
+dTTP			
+dCTP	$K_m = 2\ 10^{-5}$		
ATP	$K_i = 1\ 10^{-2}$	$K_i = 2\ 10^{-3}$	$K_i = 2\ 10^{-3}$
3'-Acetyl-TTP	Competitive inhibition		
ADP	No inhibition	Inhibition	Inhibition
dAMP	No inhibition	No inhibition	No inhibition
Deoxyadenosine	No inhibition	No inhibition	No inhibition

In polycondensation the enzyme exhibits a higher specificity than in the degrading reactions (1) DNA with a 3'-phosphoryl- end inhibits condensation, but permits pyrophosphorolysis and hydrolysis, (2) ADP inhibits the degrading, but not the condensing reaction, (3) ribo-NTP's show a lower inhibition of the condensing than of the degrading reactions The reason may be that the deoxyribose moiety is essential for the condensation, whereas for the inhibition or propagation of the degrading reactions the pyrophosphate moiety is sufficient

These results lead to some conclusions about the active site of the enzyme. Apparently a site A exists for which OH^-, PP_i and NTP compete (Fig 8a) Evidently, the γ-phosphate of the triphosphates competes for this site, because ADP is not a competitive inhibitor for OH^- and PP_i in the degradation of DNA, nor for dNTP's in the condensation At least two further combining sites B and C may be assumed for the dNTP's B might be necessary for the α-phosphate, since this group has to be activated for the nucleophilic attack by the terminal 3'-OH of DNA by removal of the negative charge. A combining site C for the sugar moiety is assumed because dNTP's

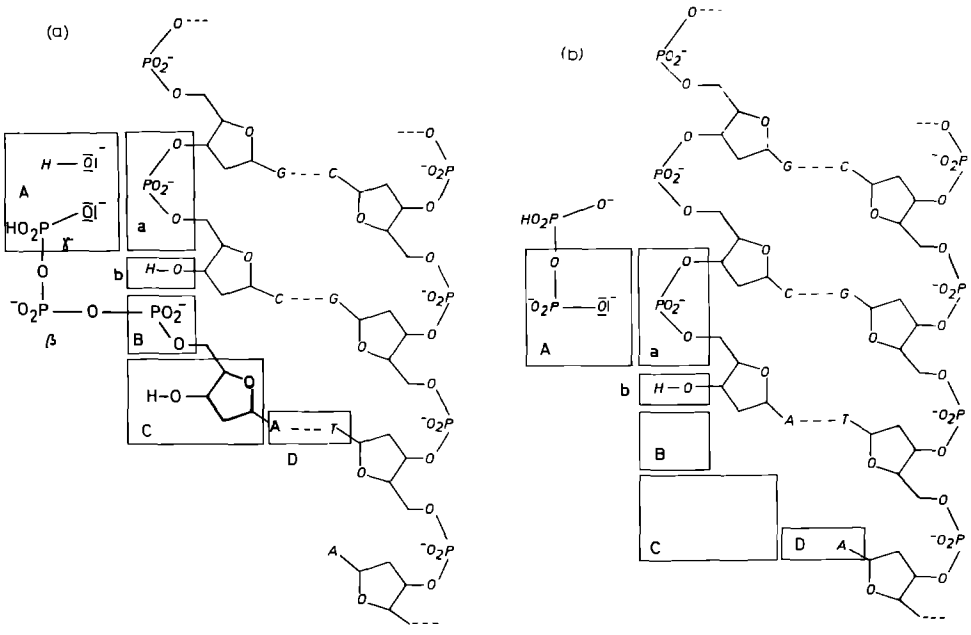

Fig 8 Model of the active site

are much stronger inhibitors of the degradation reactions than the rNTP's and because only derivatives of deoxyribose having a free 3'-OH are accepted in condensation It is not certain whether a fourth combining site D exists which recognizes correct base pairs Genetic experiments by SPEYER[12] indicate that a DNA polymerase is involved in the selection of the complementary base.

The second substrate DNA is probably bound to at least two sites of the enzyme One site a close to A might be necessary for the binding and activation of the terminal phosphodiester in hydrolysis and pyrophosphorolysis A nucleophilic attack of OH^- and PP_i would be very difficult unless the negative charge of the phosphodiester is transferred to the enzyme Another combining site b close to B is required for the 3'-OH terminus of DNA Probably other points of attachment exist, since the K_m values for DNA with exonuclease II are extremely low and are strongly dependent on its molecular weight and structure LEHMAN found $K_m = 1.7 \cdot 10^{-10}$ mole/l for the reaction with native *E. coli* DNA and $1.5 \cdot 10^{-4}$ with $(pT)_5$ (ref 6) After the substrates have been bound, reactions can occur between the groups bound to A and a (degradation of DNA) or between those bound to B and b (condensation) If the terminal phosphodiester bond is broken, the site a of the enzyme may be shifted backward to the adjacent diester group.

On the other hand, if a new phosphodiester bond is formed by the reactions between the groups attached to b and B, these sites are released and a forward shift of the enzyme is possible. Thus site a is transferred from the old to the new diester group, b is bound to the newly formed 3'-OH end of the growing chain B and C are free to bind the α-phosphate and the sugar moiety of a new dNTP, whose γ-phosphate displaces the split-off pyrophosphate from site A (Fig 8b)

This tentative model explains how polycondensation, pyrophosphorolysis and

hydrolysis can be performed by the same active centre of the enzyme It may be useful for more detailed studies of the reaction mechanism

ACKNOWLEDGEMENT

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