THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 244, No. 11, Issue of June 10, pp. 3009-3018, 1969
Printed in U.S.A.

Enzymatic Synthesis of Deoxyribonucleic Acid

XXVII. CHEMICAL MODIFICATIONS OF DEOXYRIBONUCLEIC ACID POLYMERASE*

(Received for publication, November 6, 1968)

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SUMMARY

The purified *Escherichia coli* DNA polymerase contains a single sulfhydryl group which reacts with Hg(II) to produce either a monomer containing a single mercury atom or a dimer of two protein molecules joined by a mercury atom. Both forms retain full polymerase and exonuclease activities, implying that the sulfhydryl group is relatively exposed and not essential for enzymatic activity.

The acylating agent, *N*-carboxymethylisatoic anhydride, reacts with an observed maximum of 11 sites on the enzyme to form a highly fluorescent derivative with altered functional properties. Only 0.2% of the original polymerase activity but 920% of the exonuclease activity are observed at pH 7.4 with DNA as primer or substrate. Measurements of release of inorganic pyrophosphate and binding of triphosphates indicate that the predominant effect of this acylating agent is to cause a marked reduction in the affinity for deoxyribonucleoside triphosphate substrates, but concomitant changes in the interaction of the enzyme with polynucleotides are probably also involved. The data are discussed in terms of a model in which the several activities of the enzyme are catalyzed at the same active center, which is differentiated into physically distinct sites.

In the preceding paper of this series (1) some of the physical and chemical characteristics of the purified *Escherichia coli* DNA polymerase were presented. This communication will describe the effects of two chemical modifications on the physical and chemical properties of the enzyme. One of these, the addition of a mercury atom to the single sulfhydryl group of the enzyme, does not affect any of its activities, and with the radioisotope, ^{203}Hg, provides an extremely sensitive tracer for physical studies. The other modification is the production of a fluorescent derivative with the acylating agent, *N*-carboxymethylisatoic anhydride. This derivative has useful properties for physical studies, and is the first example of a differential modification of the enzymatic activities associated with the DNA polymerase molecule. The virtual abolition of polymerase function with retention or even enhancement of the exonuclease activity, coupled with the indications that a single enzyme center encompasses both functions, provides additional information about the active sites of the enzyme.

* This study was supported in part by grants from the National Institutes of Health (United States Public Health Service), the National Science Foundation, and the National Aeronautics and Space Administration.

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EXPERIMENTAL PROCEDURE

Materials

$^{203}Hg(NO_3)_2$ (in 2 N HNO_3), obtained from New England Nuclear, was diluted to about 2 mM and neutralized with Tris. The Hg(II) concentration was determined by a titration of reduced glutathione based on the 5,5′-dithiobis(2-nitrobenzoic acid) sulfhydryl method of Ellman (2); 0.5 mole of Hg(II) neutralized the reactivity of 1.0 mole of glutathione for 5,5′-dithiobis(2-nitrobenzoic acid). The specific radioactivity, determined with a scintillation counter set for ^{14}C counting, was 1.6×10^8 cpm μmole^{-1}. The synthesis and properties of NCMIA,[1] a gift of Dr. Lubert Stryer, will be described elsewhere.[2] 5,5′-Dithiobis(2-nitrobenzoic acid) was obtained commercially. DEAE paper was Whatman DE-81. Darco G-60 was a product of Atlas Chemical Industries. DNA polymerase was the homogeneous Fraction 7; its concentration was based on a molecular weight of 109,000 (1). The sources of other materials and reagents are given in the previous paper (1).

Methods

Sucrose Gradient Centrifugation—Sucrose gradient centrifugation was performed according to the method of Martin and Ames (3). Gradients (3.6 ml) of 5 to 20% sucrose in 0.2 M potassium phosphate buffer, pH 7.4, were centrifuged at 60,000 rpm in an International model B-60 centrifuge (SB 405 rotor) for 11 hours at 3°. Fractions were analyzed for enzymatic activity, radio-

[1] The abbreviations used are: NCMIA, *N*-carboxymethylisatoic anhydride (*N*-carboxymethyl-*N*-carboxyanthranilic acid, cyclic anhydride); poly d(A-T), the alternating copolymer of deoxyriboadenylate and deoxyribothymidylate previously designated dAT.

[2] R. P. Haugland and L. Stryer, manuscript in preparation.

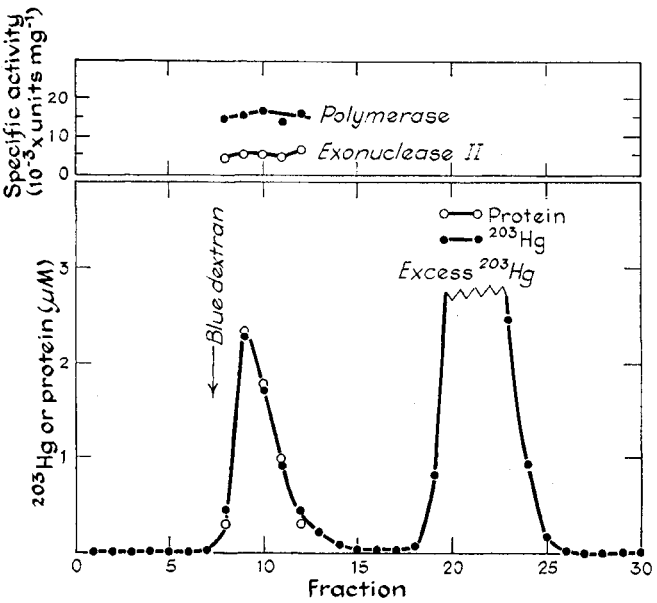


FIG. 1. Gel filtration of polymerase allowed to react with excess ^{203}Hg(II). The 50-μl sample contained 1.8 nmoles of polymerase and 8.5 nmoles of ^{203}Hg(II) in 0.1 M potassium phosphate, pH 7.0. The protein had been incubated with the mercury for 15 min at 0°. The column of Sephadex G-150 (74 cm × 0.07 cm^2) was eluted with 0.2 M Tris-0.1 M HCl. Fractions of 0.19 ml were collected at a rate of 2.5 ml per hour at 4°. The column was calibrated with blue dextran in a separate experiment. The polymerase and exonuclease assays were run without 2-mercaptoethanol. Recovery of protein from the column was 62%.

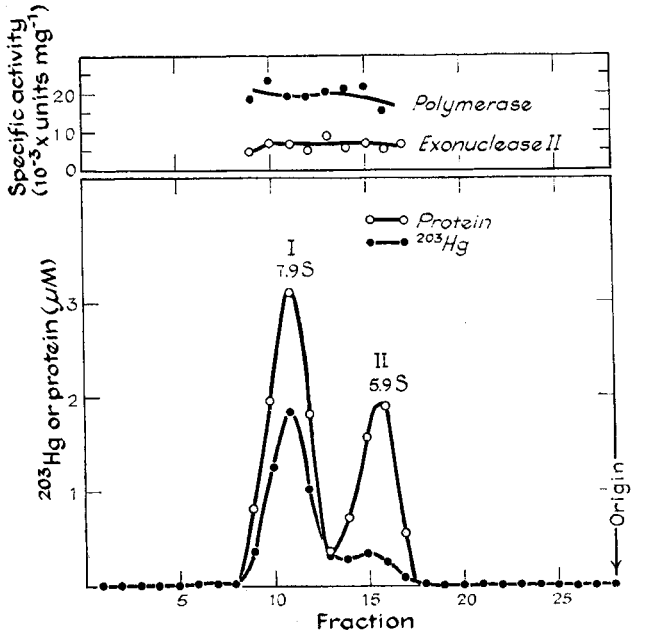


FIG. 2. Sucrose density gradient of ^{203}Hg(II) allowed to react with excess polymerase. The 70-μl sample contained 2.2 nmoles of polymerase and 1.06 nmoles of ^{203}Hg(II) in 0.1 M potassium phosphate, pH 7.0. The protein had been incubated with the mercury for 4½ hours at 0°. Fractions of 0.14 ml were collected for radioactivity determinations, enzymatic assay, and protein determination. The sedimentation coefficients were determined by comparison with a hemoglobin marker in a separate tube. The polymerase and exonuclease assays were run without mercaptoethanol. The recovery of protein from the gradient was 82%.

activity, and protein. Sedimentation coefficients were estimated with human hemoglobin as a marker (4.6 S) at concentrations above 0.1 mg ml^{-1} in order to preserve the tetrameric state (4).

Fluorescence Spectra—Fluorescence emission and excitation spectra of NCMIA and its derivatives were made with an Aminco-Bowman spectrophotofluorometer equipped with an RCA 1P21 photomultiplier. The emission, excitation, and polarization measurements of the protein conjugates were performed with the recording spectrofluorometer described by Stryer (5). Microcuvette assemblies accommodating quartz tubes with an internal diameter of 3 mm were used. The emission spectra are uncorrected for variations in sensitivity of the detection system with wavelength. Calibrations for the system have been given by Haugland and Stryer (6). Corrected fluorescence excitation spectra and polarization measurements were recorded directly with the use of a ratio amplifier (5). In the case of polarization measurements, the exciting light was isolated with a Corning 7-31 filter. All measurements were made at 5° ± 1°.

Other methods have already been described (1) or are given in the table and figure legends. Exonuclease assays were under conditions described for exonuclease II (1), except where other conditions are defined.

RESULTS

Reaction of DNA Polymerase with ^{203}Hg(II)

With excess ^{203}Hg(II), the enzyme, after isolation by gel filtration, contained 1 mole of ^{203}Hg per mole of protein (Fig. 1). The sedimentation coefficient of this derivative, as estimated by sucrose density gradient centrifugation, was identical with that of the unmodified enzyme (5.9 S), and agrees with the value of 5.6 S previously obtained by analytical centrifugation (1).

TABLE I

Stability of polymerase-mercury linkage

The monomer and dimer were isolated by gel filtration and sucrose density centrifugation, respectively (Figs. 1 and 2). The 0.1-ml reaction mixtures, containing either 3.7 pmoles of monomer or 6.2 pmoles of dimer, in 0.05 M potassium phosphate, pH 7.2, were incubated for 10 min at 37°. They were then precipitated with 0.2 ml of cold 10% trichloracetic acid and centrifuged for 10 min at 12,000 × *g*. The supernatant fluid (0.25 ml) was removed and neutralized with 0.7 ml of 0.8 M Tris. Mercury released from the protein was measured in a scintillation counter with the use of 10 ml of the dioxane fluid.

Treatment	Mercury liberated from	
	Monomer	Dimer
	%	%
None	0	2
Glutathione, 0.02 M	87	98
dATP + dTTP, 0.05 mM each	8	4
Poly d(A-T), 0.1 mM[a]	4	3

[a] In a separate experiment, 0.1 mM poly d(A-T) was incubated with 1.08 μM ^{203}Hg(II). Any ^{203}Hg-poly d(A-T) complex formed must have been dissociated by the trichloracetic acid precipitation, because the mercury was recovered quantitatively in the supernatant fluid. Had mercury been removed by poly d(A-T) from the polymerase monomer or dimer, it would have appeared in the supernatant fluid after the acid precipitation.

Since Hg(II) is divalent, it might be expected to link polymerase molecules in dimers, as it does with serum albumin (7). This possibility was realized in a reaction mixture with a 2-fold excess of polymerase over Hg(II) (Fig. 2). *Peak I* was designated as dimer on the basis of a sedimentation coefficient of 7.9 S, compared to 5.9 S for the native enzyme, and the molar ratio of mercury to protein of 0.55. *Peak II* was a mixture of unmodified polymerase and mercury-polymerase which was still in the monomer state. Had the time of reaction been increased, all of the mercury might have been found in *Peak I*. In another experiment in which polymerase was incubated with mercury for only 20 min before the centrifugation, less than 25% of the ^{203}Hg(II) was incorporated into dimer.

The specific activities of the mercury-containing monomer and dimer for both polymerase and exonuclease were the same as those of the unmodified enzyme in each experiment. It was necessary to establish, however, that components of the mixture used for assay of these activities, such as DNA, poly d(A-T), or triphosphates, did not remove the mercury and regenerate the native enzyme. Englund, Kelly, and Kornberg have shown elsewhere that mercury is not removed by DNA or poly d(A-T) (8). Furthermore, the results in Table I also indicate that the mercury-polymerase linkage is stable in the presence of poly d(A-T) and triphosphates, but that the mercury is quantitatively removed by glutathione. Since care was taken to omit a sulfhydryl reagent from the assay solution and from the enzyme diluent, it is likely that the mercury-polymerase linkage was intact during the assay. Jovin, Englund, and Bertsch have previously shown that carboxymethylation of the single sulfhydryl group also does not affect either enzymatic activity (1).

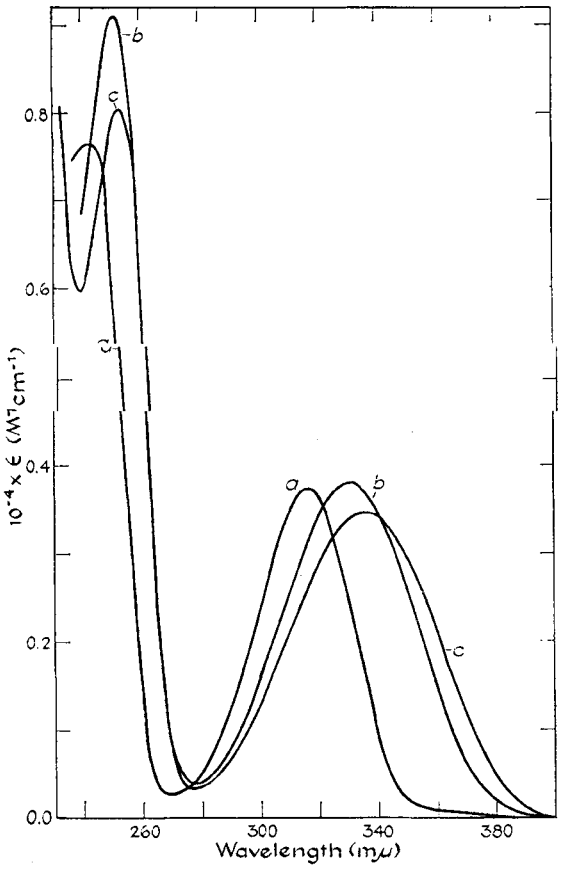


FIG. 3. Absorption spectra of NCMIA and derivatives. *a*, NCMIA in 0.1 M potassium phosphate, pH 7.4; *b*, reaction product of NCMIA in 0.1 M potassium phosphate, pH 7.4, after 23 hours at 37° (no further spectral change was observed after 15 additional hours); *c*, reaction product of NCMIA after ammonolysis in 13.3 M NH_3 for 21 hours at room temperature, in 0.1 M potassium phosphate, pH 7.4. All solutions were 0.1 mM by reference to an original NCMIA solution prepared by weight with the use of a molecular weight of 239 (this value includes 1 water molecule of crystallization). The spectra were recorded on a Cary 14 spectrophotometer at 4–10°. The absorption maxima, in millimicrons, of the three compounds were *a*, 243 and 317; *b*, 251 and 331; and *c*, 253 and 334.

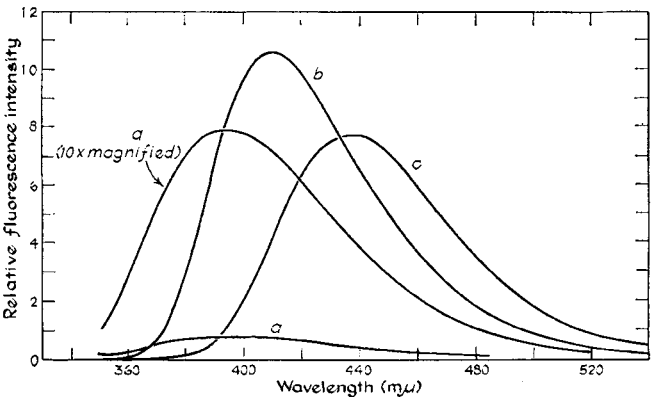


FIG. 4. Fluorescence emission spectra of NCMIA and derivatives. Spectra *a*, *b*, and *c* are of the compounds described in the legend to Fig. 3. Excitation was at 330 mμ. Relative quantum yields were calculated by dividing the peak emission intensity by the absorbance at 330 mμ. The emission maxima, in millimicrons, of the three compounds were *a*, 395; *b*, 412; and *c*, 438.

Electrophoresis of mercury-polymerase preparations on polyacrylamide gels showed that in Buffer System A (1), the monomeric form migrated with the same mobility as the unmodified enzyme. However, a mercury-polymerase preparation in which the formation of dimers was favored showed, in 9% polyacrylamide gels, a new zone with a mobility of 0.75 relative to that of the more prominent monomer zone. The presence of a diffuse zone of protein between the zones suggested that the equilibrium between the two states was under constant readjustment during the electrophoretic separation, possibly because of dissociation and counter migration of Hg(II).

Reactions of DNA Polymerase with NCMIA

Properties of NCMIA—The absorption and fluorescence emission spectra of NCMIA (I) and some derivatives are shown in Figs. 3 and 4, respectively. Hydrolysis at 37° in 0.1 M potassium phosphate, pH 7.4, caused a 9-fold increase in quantum yield and red shifts of 14 mμ in the absorption spectrum and 17 mμ in the emission spectrum. The half-life in the same buffer at 0° was about 30 hours when measured by changes in absorbance at 260 mμ, in contrast with the reported half-life of 13 min for isatoic anhydride at 25° (9). The observation of time-dependent isosbestic and isoemissive points during the course of the reaction at 0° indicated the presence of more than two species and suggested that a sequence of hydrolytic reactions was involved.

Ammonolysis of NCMIA also caused an 8-fold increase in quantum yield and red shifts of 17 mμ in the absorption and 43 mμ in the emission spectra (Figs. 3 and 4). NCMIA in 13.3 M NH_3 had a half-life of 2 hours at approximately 20° and 1.15 hours at 28°. This relative stability is surprising in view of the re-

TABLE II

Properties of DNA polymerase derivatives prepared with NCMIA

Property	Derivative			
	1	2	3	4
Preparation[a]				
NCMIA (mM)	0.50	0.47	0.089	0.077
dATP (mM)		0.70		0.72
Polymerase (mM)	0.043	0.039	0.044	0.040
Stoichiometry of conjugation				
Moles dye per mole enzyme[b]	6.6	6.1	2.3	2.2
NCMIA reacted (%)	55	51	100	92
Absorption spectra				
$A_{280}:A_{260}$	0.98	1.02	1.40	1.44
$A_{332}:A_{280}$	0.26	0.25	0.13[c]	0.13[c]
Fluorescence spectra				
$E_{427}:E_{335}$[d]	1.77	1.65	0.47	0.41
Energy transfer (%)[e]	47	43	24	20
Emission anisotropy[f]	0.166	0.166	0.155	0.155
Quantum yield of conjugated dye[g]	0.6	0.5	0.4	0.4
Enzymatic activities[h]				
Polymerase (poly d(A-T) primer)	0.006	0.013	0.10	0.30
Polymerase (DNA primer)		0.08	0.27	0.45
Polymerase:[i] ratio DNA primer to poly d(A-T) primer		6	2.7	1.5
Exonuclease	0.42	0.51	1.18	1.10

[a] The incubations, in 40 μl, were for 40 hours at 0°. The buffer was 0.05 M potassium phosphate, pH 7.5. After reaction, the solutions were diluted 6-fold and dialyzed extensively against the same buffer. The spectra were recorded without further dilutions.

[b] Calculated from difference spectra (*e.g.* Fig. 5) using an $\epsilon_{254\ m\mu}$ value of 8×10^3 M^{-1} cm^{-1} for the conjugated dye (Fig. 3*c*). Owing to many uncertainties, the true values might differ by about 30%.

[c] These values are subject to potentially large error from light scattering.

[d] Ratio of emission intensities at the indicated wave lengths (millimicrons), with excitation at 290 mμ.

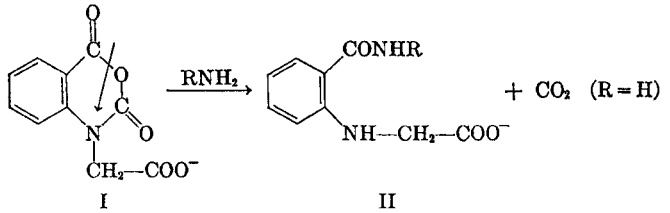
[e] Calculated from the excitation spectra (*e.g.* Fig. 6*B*) as described in the text.

[f] The emission anisotropy, A, is given by $(F_x - F_y)/(F_x + 2F_y)$, where F_x and F_y are the emission intensities with the analyzer set parallel and perpendicular to the polarizer, respectively.

[g] Quantum yield of fluorescence emission is defined as the ratio of emitted to absorbed quanta. An emission spectrum in which energy transfer from tryptophan to a conjugated dye is observed can be used to calculate quantum yield, Q, according to the formula $Q = K\ (E_{dye}/E_{trp})\ (1 - T)/T$, where E_{dye} and E_{trp} are the intensities of the two peaks in the spectrum representing emission by the dye (due to excitation by energy transfer) and the protein emission (directly excited), respectively; T is the efficiency of energy transfer, measured from an excitation spectrum; and K is an instrument constant (generally wave length-dependent). The term $E_{trp}\ T/(1 - T)$ is a measure of the exciting energy because it represents the *change* in the tryptophan emission intensity due to energy transfer. The value of K for the particular instrument used in this study was calculated by use of the above equation with the data for anthraniloylchymotrypsin (Fig. 1 of Haugland and Stryer (6)), a system in which Q was measured directly. This value of K (0.29) was then used to calculate the quantum yields for the polymerase-NCMIA derivatives using the data given in this table. Because the anthraniloylchymotrypsin and NCMIA-polymerase spectra had peaks with similar half-widths and maxima (within 5 mμ), peak fluorescence intensities rather than the integrated emission curves could be used.

[h] Expressed as ratios relative to unmodified polymerase. Exonuclease III was not used in the polymerase assays (1).

[i] The scale is normalized so that the value for unmodified polymerase is 1.



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port that isatoic anhydride and *N*-methylisatoic anhydride (10, 11) react within 5 min under similar conditions. Assuming the same reaction pathway as observed with *N*-methylisatoic anhydride (10, 11), the most probable product of the ammonolysis of NCMIA is *N*-carboxylmethylanthranilamide (II).

Fluorescence excitation spectra of the parent compound and the products of its reaction with water and ammonia were compatible with the absorption spectra, although the peaks were shifted to longer wave lengths by an average of 8 mμ, presumably because of a varying emission intensity in this region of the spectrum.

Spectral Properties of NCMIA-DNA Polymerase Conjugates—

DNA polymerase was allowed to react with NCMIA at neutral pH and 0°. The reisolated protein had spectral properties consistent with the covalent attachment of the dye. In one experiment, polymerase was incubated with two levels of NCMIA in the presence and in the absence of dATP (Table II). The absorption spectra of two of these derivatives are shown in Fig. 5. The difference absorption spectra had peaks at 254 and 334 mμ, and were similar to the spectra for the reaction product of NCMIA and ammonia (Fig. 3). The degree of conjugation was a function of the input concentration of NCMIA and of the time of incubation; the kinetics of conjugation will be described below. The number of dye molecules per protein molecule was calculated by using a molar extinction coefficient for the conjugated NCMIA of 8×10^3 M^{-1} cm^{-1} at 254 mμ (Fig. 3c). These values and other data for the derivatives are given in Table II. The extent of reaction of NCMIA at the lower concentration was 90 to 100%; at the higher concentration it was 50 to 60%. As a test for the covalent nature of the attachment, the products were subjected to electrophoresis on a 9% polyacrylamide gel with Buffer System A. Fluorescent zones were detected which corresponded with the positions of the protein zones. Derivatives 3 and 4 showed somewhat skewed distributions favoring the less mobile protein; the center of mass had a mobility about 5% greater than that of the control enzyme. Derivative 2 had a broader, more symmetrical protein distribution, the center of which migrated about 16% faster than unmodified polymerase. This qualitative effect was expected, since each conjugated dye molecule contributes at least one negative charge in the form of a carboxyl group

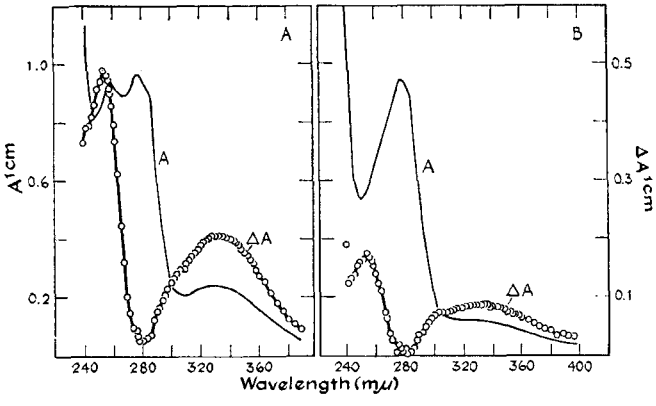

FIG. 5. Absorption spectra of DNA polymerase allowed to react with NCMIA. *A*, Derivative 2; *B*, Derivative 4. The preparation of both derivatives is described in Table II. The *solid lines* are the absorption spectra of the dye-protein conjugate. A^{1cm} is the absorbance measured with a 1-cm light path. The *curves* formed from individual *points* are difference spectra for the conjugated dye obtained by pointwise subtraction of the unmodified polymerase spectrum from that of each conjugate. All spectra were normalized to a protein concentration of 1.09 mg ml^{-1} (10^{-5} M) by first estimating the conjugation stoichiometry from the absorbances at 280 and 260 mμ, applying a correction factor to A_{280} for the dye contribution (less than 3%), and adjusting the spectra to a corrected A_{280} of 0.926 (actual absorbances were within 14% of this value). Protein concentrations measured by the method of Lowry *et al.* (12) gave values within experimental error of those determined spectrophotometrically. The data were recorded on a Zeiss PMQ II spectrophotometer with 1-cm light path cuvettes at a temperature of 2–10°. Corrections for light scattering were not applied.

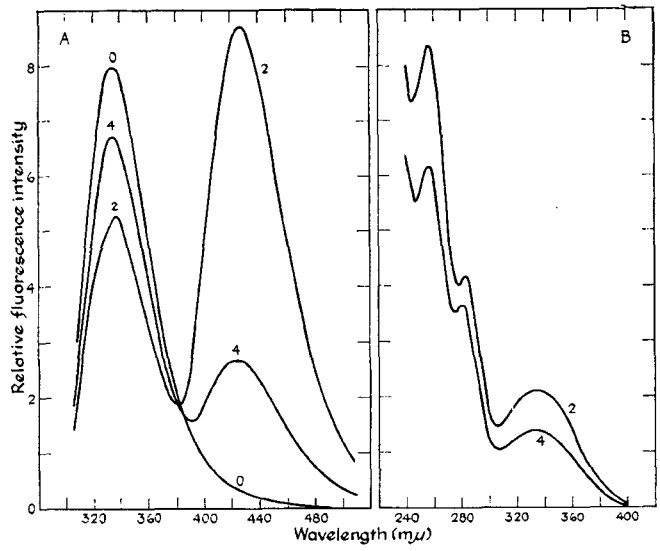

FIG. 6. Fluorescence emission spectra (*A*) and excitation spectra (*B*) of DNA polymerase allowed to react with NCMIA. The numbers *2* and *4* refer to the corresponding derivatives of Table II and the number *0* to unreacted polymerase. Excitation for the emission spectra was at 290 mμ. In the case of the excitation spectra, Corning CS 3-73 filters were used to isolate the emission.

and, if conjugated with an amino group of lysine, results in the removal of a fractional positive charge. We calculated that a DNA polymerase molecule has 50 to 70 negative charges under the conditions of electrophoresis (pH 9.8 at 0°), based on its amino acid composition, the assumption that the ammonia is derived from amide groups, and the reported values for the intrinsic dissociation constants of the amino acid side groups (13). An increase of mobility of 16% for Derivative 2 would therefore correspond to a change of about 8 to 11 negative charges as compared with the conjugation stoichiometry of 6.

Fluorescence emission spectra of Derivatives 2 and 4 are shown in Fig. 6*A*; corresponding data for the other derivatives are given in Table II. Native DNA polymerase had a tryptophan emission at 335 mμ when excited at 290 mμ. Reaction with NCMIA reduced the 335 mμ emission, and a new peak appeared at 427 mμ. The effect was more pronounced in the case of the more highly conjugated derivatives. The emission spectrum of the conjugated dye, excited directly at 365 mμ, also had a peak at 427 mμ and thus resembled the spectrum of the presumed amide derivative of NCMIA (Fig. 3). Together with the similarity in the absorption spectra, this finding suggests that a large fraction of the dye may have been conjugated with amino groups on the protein. If so, the probable structure would be the *N*-carboxyanthranilamide (II), with R the side group of lysine. The single nonessential sulfhydryl group and NH_2-terminal methionine (1), if involved in the reaction with NCMIA, cannot account stoichiometrically for the extent of reaction.

The excitation spectra of Derivatives 2 and 4 (Fig. 6*B*) confirmed that the tryptophan emission was quenched because of energy transfer to the dye chromophore. The three peaks in the spectra were in good correspondence with the absorption peaks (Fig. 5). The efficiency of energy transfer, *T*, was obtained

from the excitation spectra by the following relation (6)

$$T = n \cdot (E_{280}/E_{334} - \epsilon_{280}/\epsilon_{334})\ \epsilon_{280}/\epsilon^{p}_{280}$$

where E_{280} and E_{334} are the magnitudes of the excitation spectra at 280 and 334 mμ, respectively; ϵ_{280} and ϵ_{334} are the molar extinction coefficients of the conjugated dye at 280 and 334 mμ with values of 360 and 3480 M^{-1} cm^{-1} (Fig. 3), respectively; ϵ^{p}_{280} is the molar extinction coefficient of the protein at 280 mμ and is equal to 9.26×10^4 M^{-1} cm^{-1}; and n is the number of dye molecules per protein molecule. The calculated efficiencies of energy transfer ranged from 20 to 47% (Table II). Since these values were necessarily averages for mixed populations of different molecules, quantitative deductions as to the probable situation of dye molecules relative to the nine tryptophan residues in the enzyme are not justified.

The polarization of the fluorescence emission of the conjugated dye was measured; values are given in Table II in the form of emission anisotropies. The excited state lifetime of the conjugated chromophore is not yet known; therefore an evaluation of the rotational relaxation time was not attempted.

The quantum yield of the conjugated dye was not measured directly; estimated values of 0.4 to 0.6 were obtained by an indirect method (Table II).

Enzymatic Properties of NCMIA-DNA Polymerase Conjugates—NCMIA had pronounced effects upon the enzymatic properties of DNA polymerase, most notably a marked depression of polymerase activity (Table II). The specific activity of Derivative 1, having about 6.6 dye molecules per protein molecule, was reduced by more than 99% (poly d(A-T)-primed assay). Derivative 3, with only about 2.3 dye molecules per protein molecule, showed 10% residual activity. When measured in the DNA-primed polymerase assay, the apparent reduction of activity was less (Table II). The derivatives formed in the presence of dATP retained somewhat more polymerase activity despite only a slight reduction in conjugation.[3] The presence of Mg^{++}, which has been found recently to facilitate binding of deoxynucleoside triphosphates (14), did not make the conjugation less pronounced in preliminary experiments.

The effects on exonuclease activity produced by reaction with NCMIA were strikingly different from those on polymerase activity. Low levels of conjugation (Derivatives 3 and 4, Table II) actually led to an increase in specific activity of 10 to 20%, whereas higher degrees of reaction (Derivatives 1 and 2) depressed the activity but only by 20 to 50%.

The relatively greater destruction of the polymerase activity compared to exonuclease activity suggested the possibility that a modification of the enzyme in the site for triphosphate binding and reactions might be responsible for this effect. To test this possibility, an NCMIA derivative of the enzyme was prepared in which polymerase activity was reduced to 0.02% in a poly d(A-T)-primed assay (Table III). The exonuclease activity was 20% of that of the unmodified enzyme when tested with a poly d(A-T) substrate under the usual conditions of assay but 58% at pH 6.5 and 920% with a DNA substrate at pH 7.4. With this derivative, the functions involving triphosphates, such as PP_i exchange, stimulation of exonuclease activity, and binding, were either markedly depressed or undetectable. However, pyrophosphorolysis, in which triphosphates appear as products, was catalyzed at the relatively high level of 2%.

If the triphosphate site was altered in the reaction with NCMIA, an increase in K_m for triphosphate in the synthetic reaction might be anticipated. In order to differentiate between the rates of phosphodiester bond formation and net synthesis, simultaneous measurements of both nucleotide incorporation into product and PP_i release were made as a function of triphosphate concentration (Fig. 7). The unmodified enzyme showed a typical Michaelis-Menten substrate saturation curve with the same kinetic parameters for the two functions (K_m of 8 μM and v_{max} of 24,000 units mg^{-1}). The NCMIA derivative showed a strikingly different behavior, with a clear dissociation between synthesis and PP_i release. Whereas synthesis remained at a constant and low level, the rate of PP_i release increased linearly with substrate concentration, suggesting that the K_m for triphosphates was in fact much greater. These results are all consistent with a profound distortion of the triphosphate site in the modified enzyme.

In another experiment, the specific activity of synthesis was shown to be independent of enzyme concentration over a 25-fold range and was equivalent to the incorporation of only about two nucleotide units at the end of the polynucleotide chain bound to a given polymerase molecule. This calculation assumes the existence of a uniform population of enzyme molecules. An alternative possibility is that *all* the residual synthetic activity resided in a small fraction of relatively unaltered enzyme and that the PP_i release represented a catalytic function aborted before actual phosphodiester bond formation. The postulate of a limited synthesis by most or all molecules, however, is consistent with the high level of residual nuclease activity. It would appear that the rate of synthesis is so reduced that the product is rapidly degraded leaving only a small steady state amount at the end of the chain. There is effectively, therefore, a turnover of substrate without a net prolongation.

Assuming the validity of the proposed model, the linear dependence of PP_i release on substrate concentration in the case of the modified enzyme would be consistent with a much increased K_m and thus conditions of limiting substrate for which $v = S \cdot v_{max}/K_m$. The slope of 2.6×10^2 therefore represents the ratio of the altered kinetic parameters, v_{max}/K_m. If pyrophosphorolysis is the reversal of phosphodiester bond formation, then thermodynamic considerations stipulate that the ratio of these activities should not change in the modified enzyme when measured *at equilibrium* or even under the nonequilibrium assay conditions, assuming additionally that the catalytic mechanisms are not altered, and that the assay for the reverse reaction conforms to the same limiting rate law, as stated above. Although these conditions have not been established, it is instructive to consider their possible validity in the light of the experimental results. The ratio of the specific activities for synthesis (or PP_i release) and pyrophosphorolysis for the unmodified enzyme is 14.9 from Table III. The modified enzyme showed 24 units mg^{-1} for pyrophosphorolysis, which would correspond to a v_{max} of 360 units mg^{-1} for PP_i release assuming the invariance of the ratio of activities. It follows from the observed value of v_{max}/K_m that K_m should be on the order of 2 mM. In other experiments,

[3] In another experiment, performed in parallel with that of Table III, an NCMIA derivative of the enzyme was formed in the presence of a saturating concentration of poly d(A-T) oligomer (8). This enzyme had 25 times more polymerase activity and 3 times more exonuclease activity than enzyme modified in the absence of poly d(A-T) oligomer. The conjugation stoichiometry of this derivative was not measured.

TABLE III

Altered enzymatic activities of an NCMIA derivative of polymerase

The NCMIA derivative was prepared in a reaction mixture containing 22 μM enzyme, 30 μM ^{203}Hg(II), and 1.6 mM NCMIA, in 0.1 M potassium phosphate, pH 7.4. After 25 hours at 0° the solution was extensively dialyzed against the same buffer. The isolated material had a protein concentration of 1.93 mg ml^{-1} both by the method of Lowry *et al.*, and by corrected spectral determinations. The absorbance ratio, A_{280}:A_{260}, was 0.794 and the number of conjugated dye molecules per protein molecule was calculated to be 10.7 by the method described in Table II. The mercury was present in the incubation in order to provide a convenient determinant of concentration. It had no apparent effect on the course of the reaction or on the properties of the derivative, although this point was not systematically investigated. All assays were performed at 37° for 30 min under conditions for which activity was proportional to enzyme concentration. One unit of activity corresponds to the incorporation, release, or exchange of 10 nmoles of nucleotide per 30 min. The polymerase and nuclease assays were carried out as previously described (1), except for the indicated changes. Pyrophosphorolysis and PP_i exchange were measured according to the method of Deutscher and Kornberg (15), except that the PP_i concentration was 1.3 mM. PP_i exchange rates were calculated assuming that, for the unmodified enzyme, pyrophosphorolysis is 50% inhibited by triphosphates under the conditions used (15) but that for the modified enzyme the activities are additive. The binding of dTTP to the enzyme was measured by equilibrium dialysis at 5° in 0.05 M potassium phosphate, pH 7.4-7 mM $MgCl_2$; the method will be described elsewhere (14). For primer or substrate, ^{3}H-DNA from *E. coli* was prepared according to the procedure of Deutscher and Kornberg (15); the specific activity was 1,140 cpm nmole^{-1}. Single strand breaks were introduced by incubation with pancreatic DNase (Worthington) at a level of 0.1 μg per μmole of DNA for 100 min at 37° before addition of polymerase and triphosphates. In the case of the poly d(A-T) as primer, the triphosphates dATP and dTTP were added at the indicated concentrations. With DNA as primer or substrate, dGTP and dCTP were also supplied. Except where indicated, pH values were at 25°.

Activity	Primer or substrate	Triphosphate	pH	Buffer[a]	Unmodified enzyme	Modified enzyme	Residual activity
		μM			units mg^{-1}		%
Polymerase	Poly d(A-T)	33	7.4	P_i	18,000[b]	3.2	0.018
	DNA (activated, calf thymus)	33	7.4	P_i	4,800	12	0.25
Exonuclease	Poly d(A-T)		6.50[c]	P_i	450	260	58
			6.98[c]	P_i	1,500	260	17
			7.32[c]	P_i	2,680	320	12
			7.90[c]	P_i	4,740	360	8
			7.69[c]	Tris	6,640	1,170	18
			8.16[c]	Tris	6,370	1,410	22
			8.28[c]	Gly	5,460	1,440	26
			8.65[c]	Gly	3,600	940	26
			8.76[c]	Gly	5,020	990	20
	DNA (activated *E. coli*)	—[d]	7.4	P_i	13	120	920
		333[d]	7.4	P_i	58	120	210
Pyrophosphorolysis	Poly d(A-T)		7.4	P_i	1,210	24	2.0
Pyrophosphorolysis + PP_i exchange	Poly d(A-T)	33	7.4	P_i	6,050	25	0.41
PP_i exchange	Poly d(A-T)	33	7.4	P_i	~5,400	~1	~0.018
					$K_{dissociation}$ (M)		
Binding of dTTP			7.4	P_i	4.5×10^{-5}	$>5 \times 10^{-4}$	<10

[a] P_i refers to potassium phosphate, Tris to Tris-HCl, and Gly to sodium glycinate. The concentration in all assays was 67 mM.

[b] Exonuclease III was not required with this particular poly d(A-T) primer for the full expression of polymerase activity (1).

[c] pH at 37°.

[d] The activation by triphosphates of the exonuclease function of the unmodified enzyme is described by Lehman (16).

saturation of the PP_i release function was not achieved at a 0.8 mM triphosphate concentration, a result consistent with the predicted K_m.

Extent of Conjugation of Enzyme—We wished to determine the minimum number of dye molecules required for alterations of enzymatic activity and to assess possible specificity for sites on the protein. The maximum stoichiometry of conjugation observed was about 11 dye molecules per protein molecule (Table III). One simple model postulates the existence of N equally reactive sites, of which m are important for enzymatic function, so that reaction at any one or more virtually abolishes activity. The fraction, f, of residual activity in a population with an average of p dye molecules per protein molecule would then be given by $(1 - p/N)^m$. Assuming $N = 11$, then a p of 2.3 and f of 0.1 (Table II) lead to a value for m of about 10. The data for Derivative 1 of Table II yield a value for m of 6.

Kinetics of Conjugation of Enzyme—This model was evaluated further by studying the kinetics of reaction with DNA polymerase at several concentrations of NCMIA (Fig. 8). Depression of polymerase function proceeded from zero time with neither a lag nor a transient activation (Fig. 8*A*). This finding is consistent with "one-hit" kinetics, an effect dependent on the reaction of a single NCMIA molecule.

The time course of exonuclease activity (Fig. 8*B*) was quite different from that of the polymerase. At the highest NCMIA concentration, the activity at the first time point (0.4 hour) was

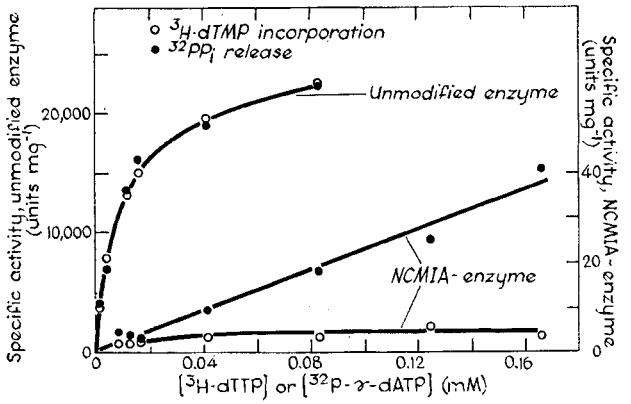

FIG. 7. Dependence of synthesis and PP_i release kinetics on substrate concentration for unmodified and NCMIA-conjugated enzyme. The derivative described in Table III was used. Synthesis and PP_i release were measured concurrently in a reaction volume of 0.6 ml containing 67 mM potassium phosphate, pH 7.4, 6.7 mM $MgCl_2$, 1 mM mercaptoethanol, 31 nmoles of poly d(A-T), the indicated concentrations of ^{3}H-dTTP (specific activity, 3.7×10^4 cpm nmole^{-1}) and γ-^{32}P-dATP (specific activity, 7.7×10^3 cpm nmole^{-1}), and either 4 ng of unmodified enzyme, 1.5 μg of the modified enzyme, or polymerase diluent alone. The poly d(A-T) was incubated for 30 min at 37° prior to the addition of triphosphates and enzyme with 0.1 unit of exonuclease III per nmole of polynucleotide in order to increase its priming efficiency (1); the exonuclease III was inactivated by heating for 5 min at 65°. After all additions, incubation was resumed for 30 min. The degree of incorporation of ^{3}H-dTMP into polymer was measured by a modification of the procedure of Falaschi, Adler, and Khorana (17). A 0.1-ml aliquot of each incubation mixture was plated on a 2-cm square of DEAE-cellulose paper and dropped into 500 ml of 0.3 M ammonium formate (brought to pH 8 with NH_3). The papers were shaken occasionally for 10 min at room temperature and drained. This process was repeated four times, followed by two washes each with 50 ml of ethanol and ether. The air dried papers were counted in 5 ml of a standard toluene scintillation fluid. The blanks were linearly related to the input of triphosphate, and the proportionality factor was 0.03%. PP_i release was measured by a combination of adsorption to charcoal to remove nonutilized dATP and precipitation of interfering $^{32}P_i$ from the supernatant by formation of the insoluble triethylammonium-phosphomolybdate complex according to the method of Sugino and Miyoshi (18). To the remainder of the chilled incubation mixture (0.5 ml) were added 1 ml of 3 mM sodium pyrophosphate-0.15 N HCl and 6 drops (0.15 ml) of a 20% suspension of Darco G-60. After 5 min at 0° the suspension was centrifuged and 0.5 ml of the supernatant fluid was placed in a polyethylene scintillation vial with 0.05 ml of 2 M Tris base and 10 ml of the naphthalene-dioxane scintillation fluid. Blanks at this step were proportional to substrate input, with a factor of 1.6%. Controls indicated that the Darco was capable of adsorbing 99.6% of a pure triphosphate. Thus about 1% of the γ-^{32}P-dATP appeared to have been degraded during the incubation at 37°. The $^{32}P_i$ so produced was precipitated by treatment of an additional 0.8 ml of the Darco supernatant with 0.1 ml of 4 M $HClO_4$, 0.7 ml of 0.08 M ammonium molybdate, and 0.14 ml of 0.8 M triethylammonium-HCl (pH 5). The dense, yellow precipitate was removed by centrifugation and 1.3 ml of the supernatant containing the $^{32}PP_i$ were placed in a polyethylene scintillation vial with 0.2 ml of 2 M Tris base and 10 ml of scintillation fluid. This procedure reduced the blank to 0.5% of the input triphosphate, thus permitting this experiment with the modified enzyme when the maximal utilization of substrate was only 0.7%.

The data for the unmodified enzyme were analyzed by plotting the observed incorporation, ΔS, against $-\ln(1 - \Delta S/S_0)$, where S_0 is the initial concentration of each triphosphate. This integrated form of the rate equation was used in order to correct for the 1 to 8% utilization of substrate in the 30-min incubation period. A straight line was obtained for both PP_i release and synthesis, yielding a K_m of 8.1 μM and a v_{max} in the form of a specific activity of 24,000 units mg^{-1}. The rate corresponding to the usual substrate concentration of 33 μM was 19,300, within experimental error of usually observed values.

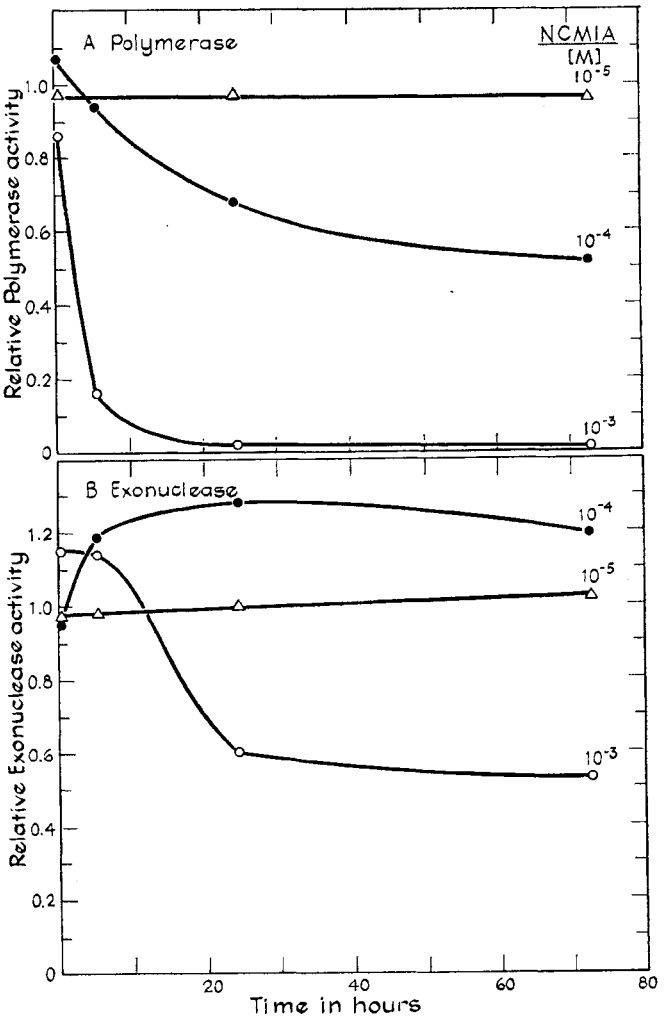

FIG. 8. Kinetics of NCMIA reaction with DNA polymerase. *A*, Polymerase activity (poly d(A-T)-primed); *B*, exonuclease activity. Incubation was at 0° in volumes of 12 μl containing 0.98 mg of polymerase ml^{-1} in 0.1 M potassium phosphate buffer, pH 7.1, and the indicated concentrations of NCMIA. The values are relative to unmodified polymerase (set at 1.0). Aliquots of 2 μl were removed at the indicated times for assay.

actually *above* the control level and remained so even at 5.5 hours, when 84% of the polymerase activity had been lost. Only upon further reaction was a decrease in exonuclease function observed. The limiting value of 48% to which exonuclease activity decreased agreed within experimental error with the values for Derivatives 1 and 2 (Table II). At the intermediate NCMIA concentration, only enhancement of exonuclease activity was observed and the time course was also compatible with a single hit process. The maximum observed increase was about 30% (at 24 hours), with some indication of an incipient decline at about 72 hours.

In order to determine rate constants, the logarithm of the

residual polymerase activity in Fig. 8 was plotted against a time axis corrected for NCMIA hydrolysis. Reasonably straight lines were obtained; they yielded an apparent second order rate constant of 5 M^{-1} min^{-1}. Assuming a value for m of 10, the rate constant expressed with respect to sites instead of molecules would be 10 times less, or 0.5 M^{-1} min^{-1}. The limited data do not permit a more detailed analysis, but it is of interest that the apparent second order rate constant for the reaction of NCMIA with NH_3 (20°, 13.3 M NH_3) was 3.6×10^{-4} M^{-1} min^{-1}, or three orders of magnitude less.[4]

The incubation mixtures of Fig. 8 were analyzed electrophoretically 2 weeks after the last indicated time point. The three proteins, in order of increasing initial NCMIA concentration, migrated faster than unmodified polymerase by factors of 3%, 5%, and 23%. By comparison with the data for Derivative 2 (Table II), it was calculated that the conjugates (Fig. 8) formed at 0.1 and 1 mM NCMIA, respectively, had an average of about 2 and 8 dye molecules per polymerase molecule. Thus the initial increase in exonuclease activity observed in Fig. 8*B* occurred under conditions where on the average less than one NCMIA molecule had reacted and suggests that attachment of a single molecule affects both polymerase and exonuclease activities concurrently. From the data of Fig. 8 it was estimated that the quantitative effect of a single substitution is to increase the nuclease function of the enzyme by a factor of about 2. Subsequent reaction reduces activity, and thus in a mixed population the full potentiation is not seen.

DISCUSSION

The reactions of DNA polymerase with Hg(II) confirm the observation from alkylation experiments that the single sulfhydryl group of the enzyme is not required for activity (1). The formation of a polymerase dimer bridged by a mercury atom indicates that the cysteine side chains are located in a relatively exposed site on the surface of the molecule. It is of interest that both members of the dimer retain full enzymatic activity. The possibility of forming dimeric structures through a direct disulfide bond, as is possible with serum albumin (19), was not explored. If such dimers or related forms exist in the cell, they would not have been detected because of the use of reducing agents in our purification procedure.

DNA polymerase exhibits a quite different behavior in its reaction with the fluorescent acylating agent, *N*-carboxymethylisatoic anhydride. A product of limited acylation containing an average of 2.3 moles of dye per mole of protein showed a 20% increase in exonuclease, but its polymerase activity was reduced by 90%. In a more extensive reaction in which approximately 11 dye molecules were conjugated to the enzyme, only 0.02% of the polymerase activity remained while 20% or more of the exonuclease activity was preserved with poly d(A-T) as a primer or substrate. These are the first instances of selective inactivation of the polymerase function, excluding the effects seen after treatment with guanidine-HCl and mercaptoethanol and described in the preceding paper (1). Prior studies have shown the constancy of the polymerase to nuclease ratio throughout purification and during heat and urea inactivation (20). These and related observations (8, 14, 15) suggest, therefore, that the two catalytic functions are carried out in the same general active center of the molecule but that the sites do not correspond exactly.

Inactivation of an enzyme by a modifying agent may often involve an *alteration* rather than abolition of function, as through shifts in pH maxima and relative affinities to substrates and cofactors. This point is supported by studies relating the various activities of polymerization, hydrolysis, pyrophosphate exchange, and pyrophosphorolysis. The finding of approximately 11 reactive sites is of interest because there are potentially a much greater number of suitable groups on the protein, *e.g.* 61 ϵ-amino groups of lysine. It is possible that sites of unusual reactivity are involved; in this regard the much lower reaction rate of NCMIA with ammonia is noteworthy.

The functional properties of the modified enzyme and direct binding measurements indicate a large reduction in the affinity of the enzyme for the deoxyribonucleoside triphosphates. However, other sites and effects must be involved. The protection from inactivation by poly d(A-T) suggests some capacity to redirect the course of reaction but probably not through direct interaction with NCMIA, since isatoic anhydride has been reported to be nonreactive toward phosphate and phosphate esters, including nucleotides (21). It will be of interest to determine the effect of structure of various nucleic acids and nucleotides on this protective function.

The altered pattern of nuclease function also indicates involvement of sites other than that for the triphosphates. Shifts in the pH spectrum of activity were observed, along with indications of altered influences of ionic strength. More remarkable was the fact that at pH 7.4 the extensively conjugated enzyme was almost 10 times more effective with a DNA substrate but 10 times less effective with poly d(A-T) as compared with the original exonuclease, although in terms of absolute rates the poly d(A-T) was still the better substrate. Such an effect may reflect subtle changes in the influence of polynucleotide secondary structure upon the interaction with the enzyme.

To what extent the exonuclease measurements reflect the activity on the 3′ terminus as compared with the activity directed toward the 5′ terminus (22) has not been determined. In view of the recent evidence that the regions in the active center of the enzyme responsible for these two exonuclease activities of DNA polymerase are distinctly different,[5] it becomes of great interest to analyze the modified enzyme for each of these activities.

The kinetic model proposed for the inactivation of the enzyme is not unique, owing to the probable existence of sites with varying reactivity and functional importance. Clarification of this problem requires the preparation and isolation, as by ion exchange chromatography, of molecules with known and homogeneous extents of conjugation. In order to refine the spectral studies, additional information about NCMIA and its derivatives is required. Model compounds would be of help in the evaluation of the nature of the substitution and the polarity of the modified site. Better quantitative determination of conjugation stoichiometry would follow from the use of radioactive reagent. It remains to be seen whether energy transfer, quantum yield, and polarization measurements are sensitive to the interaction of the enzyme with DNA and substrates. Finally, similar

[4] This calculation does not take into account the probable nature of the reactive species in concentrated ammonia solutions. In reactions of isatoic anhydride and *N*-methylisatoic anhydride with *n*-butylamine at 1.3°, rate constants of 180 M^{-1} sec^{-1} and 86 M^{-1} sec^{-1}, respectively, have been reported (9).

[5] N. R. Cozzarelli, R. B. Kelly, and A. Kornberg, manuscript in preparation.

studies with other polymerases and reagents should indicate whether the phenomenon is a general one or specific to NCMIA and the *E. coli* enzyme.

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