Effect of Neocarzinostatin-Induced Strand Scission on the Template Activity of DNA for DNA Polymerase I†

Lizzy S. Kappen and Irving H. Goldberg*

ABSTRACT: Neocarzinostatin (NCS), an antitumor protein antibiotic that causes strand scissions of DNA both in vitro and in vivo, is shown to lower the template activity of DNA for DNA polymerase I in vitro. There is a correlation between the extent of strand scission and the degree of inhibition, maximal inhibition of the polymerase reaction being obtained under conditions promoting maximal strand scission. These effects can be related to the concentrations of NCS and of 2-mercaptoethanol and are maximized by pretreatment of the DNA with drug. Results from polymerase assays in which the amount of drug-treated DNA template was varied at a constant level of the enzyme suggest that the sites associated with NCS-induced breaks are nonfunctional in DNA synthesis but bind DNA polymerase I. The binding of the enzyme to the inactive sites is further confirmed using [^{203}Hg]polymerase. It is shown that the lowering of the template activity of DNA by NCS under conditions of strand scission is due to the generation of a large number of inactive sites that block, competitively, the binding of DNA polymerase to the active sites on the template. Furthermore, the inhibition of DNA synthesis, which depends on the extent of strand breakage and on the relative amounts of template and enzyme, can be reversed by increasing the levels of template or polymerase. The finding that DNA synthesis directed by poly[d(A-T)] is much more sensitive to NCS than that primed by poly[d(G-C)] suggests that the drug preferentially interacts at regions containing adenine and/or thymine residues.

Neocarzinostatin (NCS[1]), an acidic protein antibiotic produced by *Streptomyces carcinostaticus*, is active against gram-positive organisms (Ishida et al., 1965) and a variety of experimental tumor cells (Ishida et al., 1965; Kumagai et al., 1966; Bradner and Hutchison, 1966). There have been reports of its effectiveness in the treatment of solid tumors and acute leukemia in man (Takahashi et al., 1969; Hiraki et al., 1973). NCS selectively inhibits DNA synthesis in *Sarcina lutea* (Ono et al., 1966), HeLa cells (Ono et al., 1966; Homma et al., 1970), and L1210 cells (Sawada et al., 1974). Previous studies have demonstrated that NCS causes strand scissions in HeLa cell (Beerman and Goldberg, 1974; Ohtsuki and Ishida, 1975) and L1210 cell (Sawada et al., 1974; Tatsumi et al., 1974) DNA in vivo. NCS was further shown to cut DNA in a system containing only 2-mercaptoethanol and Tris buffer (Beerman and Goldberg, 1974). Recently, it has been reported that DNA strand breaks are produced when a rat-liver nuclear suspension was incubated with NCS (Sarma et al., 1976).

Since NCS causes strand scissions in DNA both in vivo and in vitro, a study of the template activity of NCS-nicked DNA might be of value in understanding the role of strand breakage in its mode of action. Therefore, we tested the template activity of DNA for the *Escherichia coli* DNA polymerase I under conditions where NCS produces strand breaks in vitro. The data presented in this paper show that the sites associated with NCS-induced nicks on the template are not active points for replication but bind DNA polymerase I, and at limiting enzyme levels this nonproductive binding of the polymerase to the large number of inactive sites results in a lowering of the template activity of DNA. Furthermore, the inhibition of polymerase, the degree of which depends on the extent of strand breakage and on the relative amounts of template and enzyme, can be reversed by increasing the levels of template or polymerase.

Materials and Methods

[^{3}H]dTTP and $^{203}Hg(NO_3)_2$ were obtained from New England Nuclear and unlabeled deoxyribonucleotides were from Schwarz/Mann. *E. coli* DNA polymerase I, a homogeneous and endonuclease-free preparation purified by the method of Jovin et al. (1969a), was used in most of the experiments and was a generous gift from Dr. C. C. Richardson. In a few experiments, DNA polymerase I, Grade I (5000 units/mg) from Boehringer Mannheim Corp., was used. Deoxyribonuclease I was a product of Worthington Biochemical Corp. Poly[d(A-T)] and poly[d(G-C)] were purchased from P-L Biochemicals. All other chemicals were analytical grade.

Preparation of HeLa Cell and λ DNA. Nuclei were prepared from HeLa S_3 cells according to the procedure of Hershey et al. (1973). The washed nuclei were lysed with 1% sodium dodecyl sulfate–10 mM EDTA and the chromatin was gently shaken with an equal volume of phenol saturated with 10 mM Tris-HCl, (pH 8)–1 mM EDTA. The layers were separated by centrifugation. The aqueous layer, after a second extraction with phenol, was dialyzed for 2 days against four changes of buffer (200-fold) containing 10 mM Tris-HCl, pH 7.5, and 0.1 mM EDTA. DNA concentration was estimated from its absorbance at 260 nm.

[*methyl*-^{3}H]Thymidine-labeled λ DNA was prepared essentially by the procedure of Hedgpeth et al. (1972).

Assay of DNA Synthesis. The standard incubation (total volume 0.1 ml) contained 0.035 mM of each of the deoxynucleoside triphosphates (dTTP carried a tritium label of 2×10^6 counts/min), 70 mM Tris-HCl (pH 7.5), 7 mM $MgCl_2$, 1 mM 2-mercaptoethanol, DNA template, and DNA polymerase in amounts specified in the legends. In experiments involving

† From the Department of Pharmacology, Harvard Medical School, Boston, Massachusetts 02115. *Received July 27, 1976.* This work was supported by United States Public Health Service Research Grant GM 12573 from the National Institute of General Medical Sciences.

[1] Abbreviations used are: NCS, neocarzinostatin; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid.

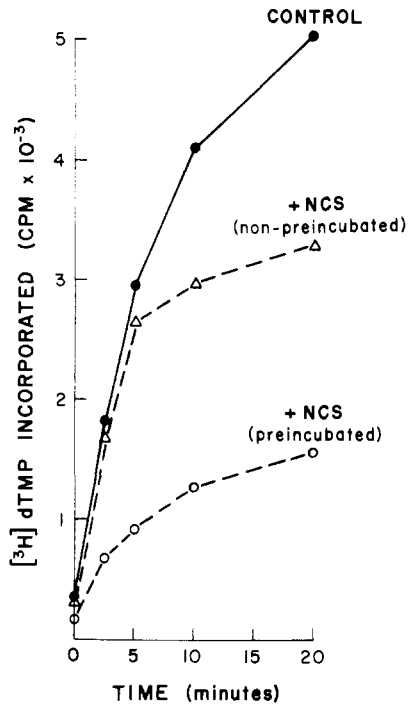

FIGURE 1: Effect of preincubation of the DNA template with NCS on in vitro DNA synthesis. Three 100-μl portions of the activated HeLa DNA (7 μg) were made 10 mM in 2-mercaptoethanol and 100 mM in Tris-HCl, pH 7.5. NCS (13.6 μg/ml) was added to one of the tubes and all three were incubated at 37 °C for 30 min. After cooling in ice, NCS was added to the second tube while the third received an equal volume of water. The polymerase assay (total volume 0.1 ml) contained 80 μl of the preincubated template, 0.96 unit of DNA polymerase I and the rest of the components mentioned under Materials and Methods. The reaction was started by addition of the enzyme. Fifteen-microliter aliquots were withdrawn at times indicated for the determination of [^{3}H]dTTP incorporated into trichloroacetic acid insoluble material.

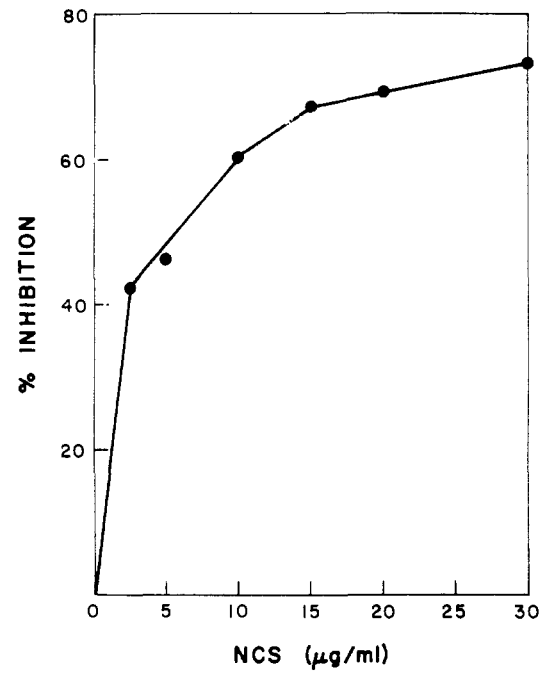

FIGURE 2: Effect of varying concentrations of NCS on DNA synthesis. Twenty-five-microliters contained 1 μg of activated HeLa DNA, 10 mM 2-mercaptoethanol and varying concentrations of NCS. After incubation at 37 °C for 30 min, 25 μl of the mix containing 0.4 unit of polymerase and the rest of the components was added and the incubation was continued to 15 min. [^{3}H]dTTP incorporated in the control was 2.2×10^4 cpm.

preincubation of the template with NCS and 2-mercaptoethanol, the final concentration of 2-mercaptoethanol in the DNA synthesis assay was higher than 1 mM. After incubation at 37 °C for 15 min (unless otherwise stated), the acid-insoluble radioactivity was determined by addition of 0.2 ml of an ice-cold mixture of 80% (w/v) trichloroacetic acid, saturated $Na_2HPO_4 \cdot 12H_2O$, saturated $Na_4P_2O_7 \cdot 10H_2O$ (1:1:1, v/v). The precipitate collected on glass-fiber filters (GF/A) was washed with cold 5% trichloroacetic acid and the filters were dried. Radioactivity on the filters was determined using a toluene-based scintillator. In experiments where the DNA concentration was varied, the filters were digested with hyamine prior to the addition of the scintillator as described by Silverman and Mirsky (1973).

Activation and NCS Treatment of DNA. DNA was activated with pancreatic DNase according to the procedure of Richardson (1966). The activated DNA was further incubated with NCS in the presence of 10 mM 2-mercaptoethanol for 30 min at 37 °C. The amount of drug varied with the concentration of DNA; in most cases, a DNA–drug ratio of 4:1 (w/w) or higher was maintained. Since the volume change due to the addition of NCS and 2-mercaptoethanol was small (<1%), the concentration of the components present from the activation of DNA, i.e., 70 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, and 50–100 μg/ml of bovine serum albumin, did not change appreciably. Magnesium and bovine serum albumin have no effect on NCS action. In some cases after drug treatment, the samples were heated at 80 °C for 10 min to inactivate the drug (Beerman et al., 1977). Incubation of λ DNA with NCS (30 min at 37 °C) contained 50 mM Tris-HCl (pH 7.5), 10 mM 2-mercaptoethanol and the drug at levels given in the legends.

In cases where the NCS-treated λ [^{3}H]DNA was to be used for the binding of [^{203}Hg]polymerase, the samples after incubation were dialyzed against several changes of 10 mM Tris-HCl (pH 7.5) for 2 days to remove 2-mercaptoethanol. After dialysis, aliquots were checked for radioactivity to ensure there was no appreciable change in the concentration of DNA during the procedure.

Estimation of DNA Strand Breaks. DNA was sedimented on alkaline sucrose (5–20%) gradients as described by Beerman and Goldberg (1974). Strand breaks were calculated using the equation of Abelson and Thomas (1966).

Assay for Endonuclease Contaminant in DNA Polymerase I. An incubation of 0.1 ml (15 min at 37 °C) contained 60 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 1 μg of λ [^{3}H]DNA, and 0.5–2 units of DNA polymerase I. At the end of the incubation, the mixture was made 14 mM in EDTA and was centrifuged on alkaline sucrose gradients as mentioned above.

Preparation of the Mercury Derivative of DNA Polymerase I and Its Binding to DNA. The mercury derivative of DNA polymerase I was prepared using $^{203}Hg(NO_3)_2$ (3.4 Ci/mmol), as described by Jovin et al. (1969b). The mercury polymerase was isolated by gel filtration on a Sephadex G-150 column. The fractions were assayed for polymerase activity in a poly[d(A-T)]-primed assay under conditions described above. In the binding experiments performed by the method of Englund et al. (1969), [^{203}Hg]polymerase and DNA were mixed and kept in ice for 15 min. The mixture was then layered onto 5-ml sucrose gradients (5–20%) containing 20 mM potassium phosphate (pH 7.4), 7 mM $MgCl_2$ and the gradients were centrifuged for 2 h in a Spinco L265B centrifuge with an SW 50.1 rotor. Fractions of five drops were collected into scintillation vials. After addition of 1 ml of water and 10 ml of Bray's so-

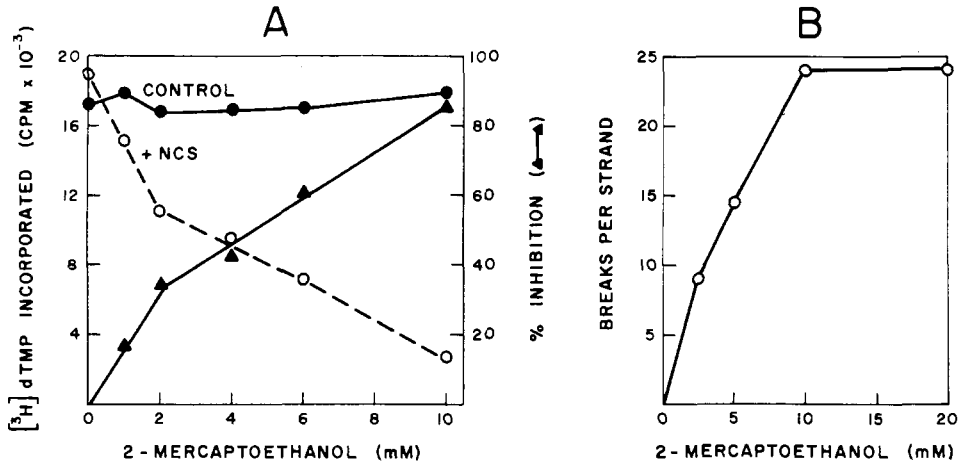

FIGURE 3: Influence of 2-mercaptoethanol on NCS-induced inhibition of DNA polymerase I and breakage of DNA. (A) HeLa DNA was preincubated with or without NCS under conditions identical to those described in Figure 1, except that the concentration of 2-mercaptoethanol was varied. Since in the absence of 2-mercaptoethanol NCS was found to give a small nonspecific stimulation in the polymerase assay, bovine serum albumin equivalent to the amount of NCS was added to all the control tubes to minimize the effect. Polymerase assay (50 μl) contained 1.4 μg of preincubated DNA, 0.4 unit of enzyme, and other components. (B) Effect of 2-mercaptoethanol on NCS-induced strand breaks in λ DNA. Fifty-microliter incubation (30 min at 37 °C) contained 1 μg of λ [^{3}H]DNA (1×10^4 cpm), 5 μg/ml of NCS, and varying concentrations of 2-mercaptoethanol. Forty-microliter aliquots were analyzed on alkaline sucrose gradients and the number of breaks per strand of DNA was determined from the equation of Abelson and Thomas (1966).

lution, the radioactivity due to ^{3}H and ^{203}Hg was determined in a liquid scintillation counter using the ^{14}C setting for ^{203}Hg.

Results

When DNA template was preincubated with NCS in the presence of 10 mM 2-mercaptoethanol, the incorporation of [^{3}H]dTTP into DNA is inhibited by 70% (Figure 1), the inhibition being apparent from the beginning of the reaction. In contrast, when NCS was added only at the start of the reaction, the onset of inhibition is delayed until 5 min, but thereafter the degree of inhibition of incorporation is the same as for the preincubated sample. Preincubation of none of the other components of the reaction with the drug enhanced the inhibition. This suggests that NCS inhibits DNA polymerase I by interaction with the template and is in accord with the observations made by other workers (Tsuruo et al., 1971; Sawada et al., 1974; Beerman and Goldberg, 1977).

The degree of inhibition of DNA synthesis by NCS depends on the concentrations of NCS (Figure 2) and of 2-mercaptoethanol during preincubation (Figure 3A). In the control containing no drug, [^{3}H]dTTP incorporation is not affected to any significant extent by concentrations of 2-mercaptoethanol up to 10 mM in the preincubation reaction (4 mM in the final polymerization reaction), whereas in the presence of NCS the inhibition increases with increasing concentrations of 2-mercaptoethanol with maximal inhibition being obtained at 10 mM. It should be pointed out that in the absence of 2-mercaptoethanol a slight stimulation of in vitro DNA synthesis by NCS has been observed but appears to be nonspecific inasmuch as similar stimulation can be induced by bovine serum albumin.

Earlier studies (Beerman and Goldberg, 1974) have shown that low concentrations of NCS in the presence of 2-mercaptoethanol cut DNA in vitro into large but not acid-soluble fragments. Using λ [^{3}H]DNA to estimate the number of strand breaks caused by the drug, it was found that, under the preincubation conditions used for DNA template in Figure 1 (10 mM 2-mercaptoethanol, 13.6 μg/ml of NCS), NCS introduced 20–35 breaks/strand of λ DNA in addition to 6–8 breaks made by DNase during the initial activation. On the other hand, in the absence of 2-mercaptoethanol there is no strand breakage and no inhibition of polymerase activity. An effort was made to relate the NCS-induced inhibition of DNA polymerase I activity, as determined by the concentration of 2-mercaptoethanol to the number of single-strand breaks in the DNA. As shown in Figure 3A, the maximal inhibition of dTTP incorporation by NCS occurs under conditions of maximal strand scission (Figure 3B), suggesting that there is a connection between the two effects of the drug. Hence, we sought to establish that NCS-induced strand breakage generated inactive binding sites for DNA polymerase I.

It is known from the work of Kornberg and his group (Kornberg, 1969; Englund et al., 1969) that sites that are nonfunctional as well as functional in DNA synthesis bind DNA polymerase I and by using conditions of either excess enzyme or excess template it is possible to distinguish between these sites on the template. Thus, in the experiments shown in Figure 4, increasing amounts of DNA, control or drug-treated, were added to incubations containing a constant level of DNA polymerase I, and [^{3}H]dTTP incorporation into DNA was measured. It can be seen that the template activity of control DNA increased nearly linearly with the amount of DNA. Under these conditions, there is an excess of enzyme over the binding sites on the DNA. With the template exposed to NCS, however, the pattern of dTTP incorporation is different. At levels of DNA less than 1.6 μg (excess of enzyme), the incorporation of dTTP remained at almost the same level as the control (not shown), but, at the higher levels of drug-treated DNA, incorporation levels off, indicating that among the new sites produced by NCS there are no active sites for DNA synthesis. It appears that at low levels of template, despite the presence of inactive sites binding polymerase molecules, there is still enough enzyme available for the existing active sites; hence, dTTP incorporation is not significantly affected. Addition of increasing amounts of treated DNA results in a corresponding increase in inactive sites and, once the DNA is sufficient to provide excess of such inactive enzyme binding sites, there is no further increase in template activity. These results are similar to those obtained by Saffhill et al. (1974)

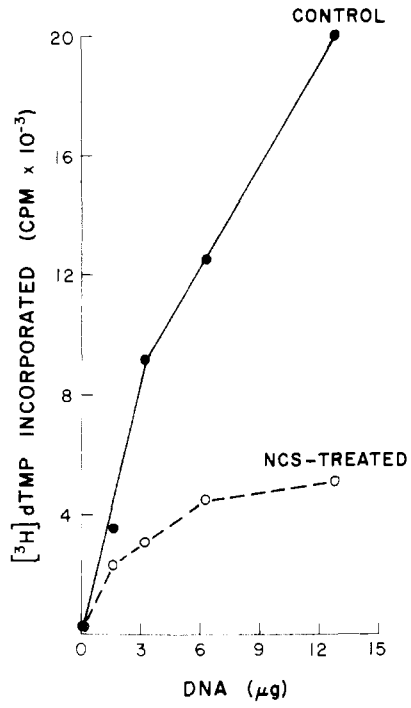

FIGURE 4: DNA synthesis with increasing amounts of NCS-treated DNA. Activated HeLa DNA (162 μg/ml) was incubated with NCS (37 μg/ml) at 10 mM 2-mercaptoethanol for 20 min at 37 °C. The mixture was then transferred to a water bath at 80 °C for 5 min before cooling in ice. Control DNA containing no drug but an equal volume of water was treated similarly. In the dTTP incorporation assay (0.1 ml), the amount of the control or treated template was varied keeping the polymerase level constant at 0.05 unit.

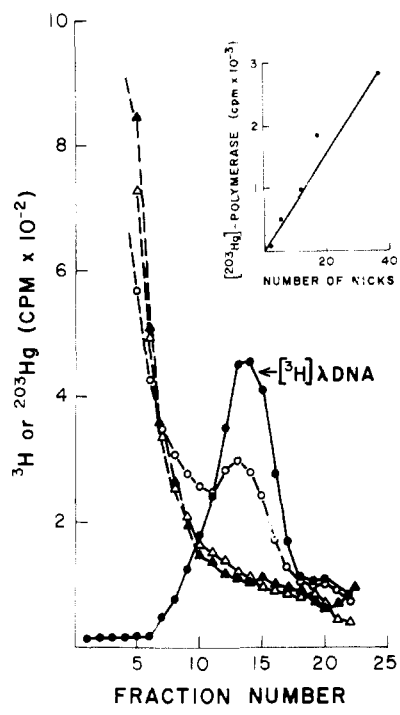

FIGURE 5: Effect of NCS treatment of λ [^{3}H]DNA on [^{203}Hg]polymese binding. λ [^{3}H]DNA was preincubated with NCS in the presence or absence of 10 mM 2-mercaptoethanol as described under Materials and Methods. One-hundred-microliter incubation contained 4 μg of DNA (4 × 10^4 cpm) and 20 μg/ml of NCS. A control incubation containing DNA but no drug also received 2-mercaptoethanol. Fifty-microliter aliquots of the samples (after dialysis) were mixed with 150 μl of [^{203}Hg]polymerase (5.4 × 10^4 cpm, 6 μg of protein) and the amount of enzyme bound to DNA was determined by layering 150-μl aliquots of the mixture onto neutral sucrose gradients. Strand breaks were estimated with 8-μl aliquots of DNA, as described under Materials and Methods. (In the presence of 2-mercaptoethanol, the drug made 23 breaks/strand and none in its absence.) The radioactive profile of λ [^{3}H]DNA shown is the one obtained with NCS in the presence of 2-mercaptoethanol and it was nearly identical to those obtained in the other two cases. ^{3}H did not give any crossover radioactivity in the ^{203}Hg channel. Crossover from ^{203}Hg to ^{3}H (5–10%) has been subtracted. Experiments shown in the insert were identical, except for the variation of drug levels as indicated in Table I. In these experiments, ^{203}Hg radioactivity in the region of the λ [^{3}H]DNA peak was quantitated in each case and the background radioactivity found in this region of the gradient with only [^{203}Hg]polymerase (900 cpm) has been subtracted from the ^{203}Hg radioactivity obtained with DNA. (△) Control; (▲) +NCS in the absence of 2-mercaptoethanol; (○) +NCS in the presence of 2-mercaptoethanol. Sedimentation is from left to right.

with rat liver chromatin in which inactive strand breaks had been introduced in vivo by methylmethanesulfonate.

Further support for the binding of DNA polymerase to the NCS-generated sites comes from experiments using [^{203}Hg]polymerase and λ [^{3}H]DNA. In these experiments, the enzyme and DNA (control, DNase nicked or NCS treated) were mixed and the amount of enzyme bound to DNA was determined by analyzing the mixture on neutral sucrose gradients. As shown in Figure 5, there is no significant binding of [^{203}Hg]polymerase to control DNA or to DNA treated with NCS in the absence of 2-mercaptoethanol. On the other hand, with DNA treated with NCS in the presence of 2-mercaptoethanol there is a significant increase in ^{203}Hg radioactivity coincident with [^{3}H]DNA peak. Parallel control gradients containing (1) only [^{203}Hg]polymerase but no DNA and (2) equivalent radioactivity in the form of $^{203}Hg(NO_3)_2$ with λ [^{3}H]DNA did not give any peak of ^{203}Hg radioactivity in the region of DNA. This shows that ^{203}Hg radioactivity associated with DNA is due to the formation of [^{203}Hg]polymerase–DNA complex and is not the result of any free mercury bound to DNA. The above results thus confirm that the sites on the DNA template associated with NCS nicks, though not active in DNA synthesis, bind DNA polymerase I. It is of interest to know how the variation in strand breakage affects polymerase binding. In another set of experiments, 1 μg of DNA was incubated in the presence of 10 mM 2-mercaptoethanol and varying concentrations of NCS. In each case, the number of strand scissions in the template and the amount of [^{203}Hg]polymerase bound to it were determined. It can be seen from Table I that, with an increase in concentration of the drug, there is an increase in the number of strand breaks and a corresponding linear increase in the amount of [^{203}Hg]-polymerase bound to DNA (insert, Figure 5 and Table I). It has been shown that DNase-induced nicks in plasmid DNA bind one enzyme molecule per nick (Englund et al., 1969; Kornberg, 1969). We further found (not shown) that DNA having the same number of strand scissions made either by DNase or NCS bind almost the same amount of [^{203}Hg]-polymerase. Hence, it is reasonable to assume that there is one molecule of enzyme bound per nick introduced by NCS.

The foregoing results on the inhibition of DNA polymerase I by NCS, coupled with the finding that the enzyme binds to the inactive sites on the template, suggest that addition of DNA not exposed to the drug (more active sites) might influence the inhibition. As shown in Table II, addition of control DNA does reverse the inhibition and a Lineweaver–Burk plot (not shown) of these data is consistent with a competitive relationship between the drug-modified and control DNA in binding the polymerase.

If the inhibition of DNA synthesis by NCS under the conditions described is due to the binding of polymerase molecules to the large number of inactive sites associated with strand

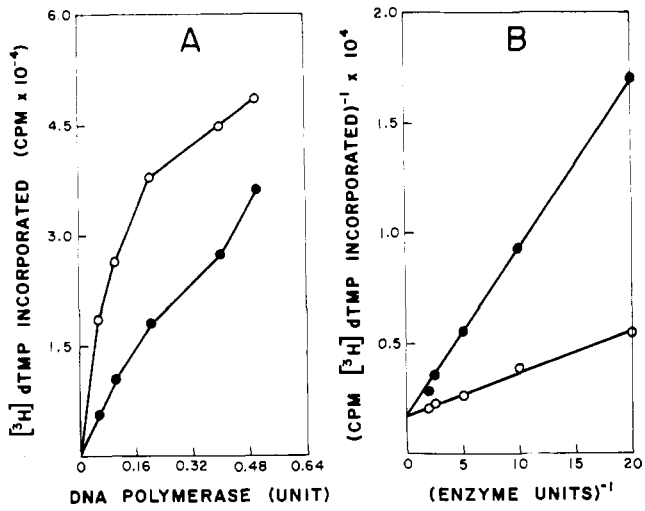

FIGURE 6: Influence of DNA polymerase level on NCS inhibition. (A) Activated HeLa DNA (40 μg/ml) was incubated with NCS (25 μg/ml) in the presence of 10 mM 2-mercaptoethanol for 20 min. The mixture was then kept in an 80 °C water bath for 10 min before cooling in ice. Polymerase assay (50 μl) contained 0.8 μg of the template and varying amounts of enzyme. Tubes containing volumes of enzyme solution lower than the maximum received enzyme buffer to bring the total volume to the same level. Incorporation of dTTP at 10 min was determined. (B) Lineweaver-Burk plot of the data in A. In parallel experiments involving alkaline sucrose gradient centrifugation (not shown), 0.8 μg of λ [^{3}H]DNA and 0.5 unit of polymerase I were used to detect contaminating endonuclease in the DNA polymerase preparation. The DNA polymerase I was found to be free of such activity. (○) Control DNA; (●) NCS-treated DNA.

TABLE I: Effect of NCS Concentration on DNA Strand Scission and Polymerase Binding.[a]

NCS (μg/ml)	No. of Breaks/Strand	[^{203}Hg]Polymerase Bound to λ DNA (cpm)
0	0	0
1	2	85
2	5	512
5	12	985
10	17	1855
20	37	2801

[a] Experimental conditions are the same as given in Figure 5.

scissions and if the already existing functional sites on the template remain unaffected, one would expect that addition of large excess of enzyme at a given template level would overcome the inhibition. We, therefore, examined the effect of polymerase concentration on the inhibition of template activity by NCS. As shown in Figure 6A, with the addition of increasing amounts of enzyme to incubations containing 0.8 μg of control or NCS-treated DNA, there is a steady decrease in inhibition. A Lineweaver-Burk plot of the data also shows a competitive relationship (Figure 6B). Since DNA polymerase I used in these experiments is a highly purified, homogeneous preparation free of any detectable endonuclease, one can exclude the possibility that the overcoming effect is due to introduction of more active sites on the template by endonucleolytic nicking.

If NCS cuts at a preferred base sequence, the base composition of the template will influence the polymerase inhibition by the drug. To test for this, we used two alternating copolymers poly[d(A-T)] and poly[d(G-C)] as templates. The results, presented in Figure 7, demonstrate that, at all NCS levels shown, synthesis primed by poly[d(A-T)] is much more sensitive to the drug than poly[d(G-C)]-dependent synthesis, suggesting that the drug preferentially interacts at regions containing adenine and/or thymine residues.

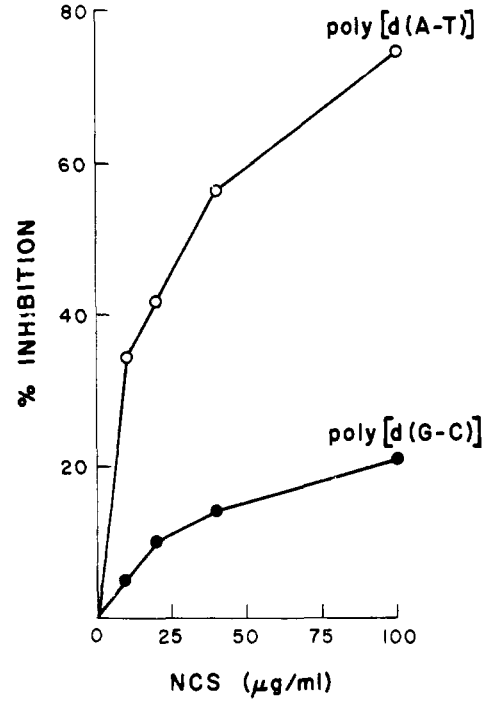

FIGURE 7: Influence of base composition of the DNA template on NCS inhibition. The templates (67 μg/ml) were incubated with varying levels of the drug at 10 mM 2-mercaptoethanol for 30 min at 37 °C. Assay conditions for the incorporation of [^{3}H]dTTP or [^{3}H]dGTP were the same as given under Materials and Methods. Fifty-microliter incubation contained 1.7 μg of either poly[d(A-T)] or poly[d(G-C)], 0.02 unit of polymerase, and other components. Incubation was for 30 min. Incorporation of radioactivity in the controls with poly[d(A-T)] and poly[d(G-C)] were 6.2×10^4 cpm ([^{3}H]dTTP) and 6.7×10^4 cpm ([^{3}H]dGTP), respectively.

TABLE II: Reversal of NCS Inhibition of DNA Polymerase I by Increasing DNA Not Treated with NCS.[a]

DNA Added (μg)	Control (cpm)	+NCS (cpm)	% Inhibition
0	3209	689	79
1.9	6764	3292	51
5.6	8661	5770	34
7.5	9735	6345	35
11.2	9391	7227	23

[a] Preincubation of activated HeLa DNA with the drug was done as given in Figure 4, except the drug concentration was increased twofold. Polymerase assay (50 μl) contained 1.9 μg of control or drug-treated template and 0.4 unit of enzyme. Increasing amounts of control DNA were added to identical tubes. Incorporation of dTTP into DNA at 10 min was determined.

Discussion

Earlier studies have shown that NCS causes strand breaks in DNA in vitro in the presence of a sulfhydryl agent (Beerman and Goldberg, 1974). The results reported in this paper demonstrate a correlation between the extent of strand scission of the template and the in vitro inhibition of DNA polymerase I by the drug. Thus, there is an absolute requirement for 2-

mercaptoethanol in both the inhibition of polymerase and in the generation of DNA nicks. Furthermore, maximal inhibition of the polymerase reaction is obtained under conditions favoring maximal strand scission, i.e., preincubation of the template in the presence of 10 mM 2-mercaptoethanol (Figures 1 and 3). We show that the sites associated with NCS-induced breaks are all inactive sites for DNA synthesis but bind DNA polymerase I (Figures 4 and 5). These inactive sites, which far exceed the active sites in number, competitively block the binding of the polymerase to the active sites on the template and, by so doing, lower the template activity of the DNA. As expected, increasing the number of active sites by addition of activated DNA unexposed to drug reverses the inhibition (Table II). Furthermore, the extent of inhibition is also dependent on the enzyme level (Figure 6). Once the enzyme concentration is sufficient to saturate all the binding sites, the template activity of the treated DNA reaches the same level as the control. Thus, it should be emphasized that the degree of inhibition obtained depends on the extent of strand breakage, the amount of template, and the enzyme level. Using crude DNA polymerase from L1210 cells and calf thymus DNA as template, Sawada et al. (1974) obtained, at best, a relatively small degree of inhibition (34% at 80 μg/ml of NCS) of DNA polymerase by NCS at a concentration of 1 mM 2-mercaptoethanol. They found, in agreement with our results, a reversal of the inhibition by increasing amounts of DNA. They did not report, however, any significant effect of the enzyme level on inhibition, but they did not use as wide a range of enzyme concentrations as used here. The different experimental conditions employed likely account for the difference in the results.

The nonproductive binding of DNA polymerase I to NCS-nick sites is not incompatible with the finding that the drug stimulates DNA repair in lymphocytes (Tatsumi et al., 1975), since the action of DNA polymerase I in the intact cell would have presumably been preceded by the excision of the damaged region of the DNA. Kornberg and his group established (Kornberg, 1969; Englund et al., 1969) that DNA polymerase binds to nicked regions of the DNA containing 3′-OH, as well as 3′-phosphate groups, but only binding to the former results in chain extension. Our data on the inactive binding of polymerase to NCS-induced breaks, taken together with the observations of Poon et al. (1977) that nicks caused by NCS contain 5′-phosphate-ended groups but are not sealed by polynucleotide ligase, appear to indicate the presence of a defect at the 3′-end of the break. This might result from the presence of a blocked -OH group, opening of the deoxyribose ring, or hydrolysis of the N-glycosidic bond with base release. The latter possibility is supported by the finding that thymine release (Ishida and Takahashi, 1976; Poon et al., 1977) (and a small amount of adenine release, Poon et al., 1977) is observed when DNA is treated with high levels of NCS. The involvement of thymine and/or adenine residues in the cutting reaction is also suggested by our finding that DNA synthesis directed by poly[d(A-T)] is much more sensitive to NCS than that by poly[d(G-C)]. These data are also in agreement with our finding that the ability of a DNA to protect against the cutting of λ DNA by NCS was related to its thymine and adenine content (Poon et al., 1977). That base release may be accompanied by β-elimination reactions producing 3′- as well as 5′-phosphate ended polynucleotide fragments is suggested by preliminary experiments in which the activity of the NCS-treated DNA for DNA polymerase I was markedly increased by prior treatment with nuclease-free alkaline phosphatase.

NCS resembles the copper-chelating peptide antibiotics phleomycin and bleomycin in its selective inhibition of DNA synthesis and in causing DNA strand scission in vivo and in vitro (Tanaka et al., 1963; Suzuki et al., 1970; Umezawa, 1974). The action of both agents in cutting DNA is markedly stimulated by a reducing agent (Suzuki et al., 1970; Stern et al., 1974). Phleomycin has been shown to be a potent inhibitor of DNA polymerase I in vitro (Falaschi and Kornberg, 1964). We found that in poly[d(A-T)]-primed DNA synthesis, preincubation of the template with phleomycin in the presence of 10 mM 2-mercaptoethanol markedly increased the inhibition of dTTP incorporation (unpublished data). A requirement for a sulfhydryl agent in the inhibition of DNA polymerases by bleomycin has also been reported (Yamazaki et al., 1973; Dicioccio and Srivastava, 1976). This raises the possibility that these drugs, like NCS, produce inactive polymerase-binding sites on the template and at limiting enzyme levels; this will result in a lowering of the template activity of DNA. Since vastly different experimental conditions have been used to study this possibility, it is difficult to reach a conclusion on this point from the published literature. There is also a close similarity in the influence of the base composition of the template on the inhibition of DNA polymerases by these antibiotics. As in the case of phleomycin (Falaschi and Kornberg, 1964) and bleomycin (Yamazaki et al., 1973), NCS has a preference for A-T residues in the template. On the other hand, while bleomycin stimulates (Yamazaki et al., 1973), NCS slightly inhibits poly[d(G-C)] directed synthesis (Figure 7).

Other studies on the molecular mechanism of action of NCS have shown DNA to be its principal target (Beerman et al., 1976; Beerman and Goldberg, 1977), but the precise mechanism by which NCS exerts its action in the intact cell is not known. It is not yet known whether the strand scissions produced by the drug in vitro are similar to those made in vivo or whether the latter are even a direct effect of the drug and not secondary to induction of endogenous nuclease. Furthermore, the role of the sulfhydryl compound in the in vitro cutting reaction of NCS is unclear, although it has been speculated that reduction of the two cystines of NCS might be involved (Beerman et al., 1976). It may be of general importance to point out that for an increasing number of protein toxins (e.g., diphtheria (Pappenheimer and Gill, 1973) and cholera (Bitensky et al., 1975; Gill, 1976) toxins, colicins E2 (Saxe, 1975a,b), and E3 (Boon, 1971; Bowman et al., 1971), and the plant toxins abrin and ricin (Olsnes et al., 1974)) evidence has been accumulating that, contrary to earlier views, they possess inherent activities (usually enzymatic) that can be demonstrated in cell-free systems and can be related to their cytotoxic effects. Also, for several of these agents a reducing sulfhydryl compound can be shown to be involved in their activation. Of particular interest, with regard to the proposed mechanism of action of NCS, are the findings that colcins E2 and E3 cause the breakdown of DNA (Saxe, 1975a,b) and ribosomal RNA (Boon, 1971; Bowman et al., 1971), respectively, both in vivo and in vitro and must penetrate the susceptible cell to do so (Lau and Richards, 1976).

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