Lin, S., and Riggs, A. D. (1976), *Nature (London) 263,* 748.
Kleppe, K., van de Sande, J. H., and Khorana, H. G. (1970), *Proc. Natl. Acad. Sci. U.S.A. 67,* 68.
Lehman, I. R. (1974), *Science 186,* 790.
Maniatis, T., Jeffrey, A., and van de Sande, J. H. (1975), *Biochemistry 14,* 3787.
Modrich, P., and Lehman, I. R. (1973), *J. Biol. Chem. 248,* 7502.
Olivera, B. M., and Lehman, I. R. (1967), *Proc. Natl. Acad. Sci. U.S.A. 57,* 1700.
Panet, A., van de Sande, J. H., Loewen, P. C., Khorana, H. G., Raae, A. J., Lillehaug, J. R., and Kleppe, K. (1973), *Biochemistry 12,* 5045.
Peacock, A. C., and Dingman, C. W. (1969), *Biochemistry 8,* 608.
Pike, L. M., and Rottman, F. (1974), *Anal. Biochem. 61,* 362.
Sanger, F., Donelson, J. E., Coulson, A. R., Kossel, H., and Fisher, D. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 1209.
Sano, H., and Feix, G. (1974), *Biochemistry 13,* 5110.
Schaller, H., and Khorana, H. G. (1963), *J. Am. Chem. Soc. 85,* 3841.
Sgaramella, V. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 3389.
Sgaramella, V., and Khorana, H. G. (1972), *J. Mol. Biol. 72,* 493.
Sgaramella, V., van de Sande, J. H., and Khorana, H. G. (1970), *Proc. Natl. Acad. Sci. U.S.A. 67,* 1468.
Smith, M., and Khorana, H. G. (1963), *Methods Enzymol. 6,* 645.
Sugino, A., Goodman, H. M., Heynecker, H. L., Shine, J., Boyer, H. W., and Cozzarelli, N. R. (1977), *J. Biol. Chem. 252,* 3987.
Tomlinson, R. V., and Tener, G. M. (1963), *Biochemistry 2,* 697.
Ullrich, A., Shine, J., Chirgwin, J., Pictet, R., Tischer, E., Rutter, W. J., and Goodman, H. M. (1977), *Science 196,*
van de Sande, J. H., Caruthers, M. H., Sgaramella, V., Yamada, T., and Khorana, H. G. (1972), *J. Mol. Biol. 72,* 457.
Weber, H., and Khorana, H. G. (1972), *J. Mol. Biol. 72,* 219.
Weiss, B., and Richardson, C. C. (1967), *J. Biol. Chem. 242,* 4270.
Weiss, B., Jaquemin-Sablon, A., Live, T. R., Fareed, G. C., and Richardson, C. C. (1968), *J. Biol. Chem. 243,* 4543.

Gaps in DNA Induced by Neocarzinostatin Bear 3′- and 5′-Phosphoryl Termini†

Lizzy S. Kappen* and Irving H. Goldberg

ABSTRACT: Neocarzinostatin (NCS)-induced strand breakage of DNA generates nonfunctional binding sites for the *E. coli* DNA polymerase I. Treatment of the NCS-nicked DNA with alkaline phosphatase at 65 °C prior to the polymerase reaction results in 60–100-fold stimulation of dTMP incorporation, whereas in a control not treated with the drug there is only a 2-fold increase. Sites of strand scission on the NCS-treated DNA bear phosphate at the 3′ termini. This conclusion is supported by the kinetics of release of inorganic phosphate from NCS-cut DNA by exonuclease III. Since our earlier work has shown that virtually all the 5′ ends of the nicks caused by NCS bear phosphomonoester groupings, the 3′- and 5′-phosphoryl termini could be quantitated using alkaline phosphatase and exonuclease III. Over a wide range of drug levels the amount of inorganic phosphate released by alkaline phosphatase is approximately twice as much as that removed by exonuclease III, indicating the presence of equal amounts of 3′- and 5′-phosphoryl termini. This, taken together with other previously demonstrated effects of NCS on DNA, such as the introduction of nicks not sealable by polynucleotide ligase, the release of thymine, and the formation of a malonaldehyde type compound, suggests that NCS-induced strand breakage involves base release accompanied by opening of the sugar ring with the destruction of one or more nucleosides and results in a gap bounded by 3′- and 5′-phosphoryl termini.

Neocarzinostatin (NCS[1]), an acidic protein antibiotic of molecular weight 10 700, is cytotoxic to gram positive organisms (Ishida et al., 1965) and a variety of tumor cells (Ishida et al., 1965; Ono et al., 1966; Kumagai et al., 1966; Bradner & Hutchison, 1966). Studies on the molecular mechanism of action of NCS revealed DNA to be a principal target of action. Thus, NCS primarily inhibits DNA synthesis in certain gram positive bacteria (Ono et al., 1966) and in a variety of mammalian cell lines (Ono et al., 1966; Homma et al., 1970; Sawada et al., 1974; Beerman & Goldberg, 1977). It induces degradation of DNA in sensitive bacteria (Ohtsuki & Ishida, 1975a) and causes DNA single-strand scissions in mammalian cell lines (Beerman & Goldberg, 1974; Sawada et al., 1974; Tasumi et al., 1974; Ohtsuki & Ishida, 1975b; Beerman & Goldberg, 1977). Strong support for DNA damage being involved in NCS action comes from the recent finding that the drug is a mutagen for *E.* coli and from genetic studies in *E. coli* suggesting that a nonexcisable misrepair type of damage to the DNA is produced (Tatsumi & Nishioka, 1977). Further, we

† From the Department of Pharmacology, Harvard Medical School, Boston, Massachusetts 02115. *Received August 29, 1977.* This work was supported by U.S. Public Health Service Research Grant GM 12573 from the National Institute of General Medical Sciences.

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

0006-2960/78/0417-0729$01.00/0

have shown that the drug's ability to induce breakage of DNA in vitro and cellular DNA in HeLa S_3 cells correlates with its inhibition of DNA replication and growth inhibiting action (Beerman & Goldberg, 1977; Beerman et al., 1977).

Previous studies have shown that NCS causes single-strand nicks in DNA in vitro in the presence of 2-mercaptoethanol (Beerman & Goldberg, 1974), and that virtually all 5'-hydroxyl groups at the point of break bear phosphoryl end groups (Poon et al., 1977). We have also shown (Kappen & Goldberg, 1977) that the sites associated with NCS-induced breaks are not functional in DNA synthesis by *E. coli* DNA polymerase I but bind the enzyme in an inactive form. This result raised the possibility that the 3'-hydroxyl also carried a phosphomonoester grouping.

In this paper we present evidence that NCS action on DNA results in the formation of gaps bearing 3'- and 5'-phosphoryl moieties at each end of the break.

Materials and Methods

[^{3}H]dTTP and $H_3{}^{32}PO_4$ were obtained from New England Nuclear and unlabeled deoxyribonucleotides were from Schwarz/Mann. *E. coli* DNA polymerase I, grade 1 (5000 units/mg), was purchased from Boehringer Mannheim Corp. Pancreatic DNase I, micrococcal nuclease, and bacterial alkaline phosphatase were products of Worthington Biochemical Corp. Alkaline phosphatase was further purified by the methods described by Weiss et al. (1968). Highly purified exonuclease III was a kind gift of Dr. C. C. Richardson. NCS was generously provided by either Dr. T. S. A. Samy (highly purified form) or by Dr. S. Schepartz (clinical form). Bleomycin A2 was obtained from Bristol Laboratories.

HeLa cell DNA was prepared as described previously (Kappen & Goldberg, 1977). ^{32}P-labeled λ DNA was prepared essentially by the procedure of Hedgepeth et al. (1972) using a phosphate deficient medium to which 40 μmol of potassium phosphate and 2 mCi of $H_3{}^{32}PO_4$ had been added per 250 mL of culture. The specific activity of the λ DNA preparation varied between 1.2 and 2.5 × 10^5 cpm per μg.

Enzyme Assays. Template activity of HeLa DNA for DNA polymerase I was measured as described earlier (Kappen & Goldberg, 1977). To prepare nicked DNA, λ DNA was treated with micrococcal nuclease and pancreatic DNase by methods previously described (Richardson & Kornberg, 1964; Richardson, 1966). In the case of the pancreatic DNase-treated DNA, however, the reaction was stopped by shaking gently with one-tenth the volume of a mixture of chloroform and isoamyl alcohol (5:1 v/v) and the aqueous layer was collected after centrifugation at 3000*g*. The aqueous layer, after a second treatment with chloroform–isoamyl alcohol, was dialyzed for 24 h against two changes of a 200-fold excess of 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA. The DNA solution was stored frozen until use.

Reaction conditions for exonuclease III assays were similar to those described by Richardson & Kornberg (1964) with a slight modification. In a typical assay, 0.3-mL incubation contained 83 mM potassium phosphate (pH 7.0), 6.6 mM $MgCl_2$, 7 mM 2-mercaptoethanol, 2–6 μg of ^{32}P-labeled λ DNA and 0.2 unit of exonuclease III. Unless otherwise specified, the incubation was for 20 min at 37 °C. At the end of the incubation 0.2 mL of calf thymus DNA (2 mg/mL) was added followed by 0.5 mL of 10% trichloroacetic acid. After keeping in ice for 10 min, the supernatant fluid was collected after centrifugation at 10 000*g* for 10 min. Aliquots of 50 μL were used for determination of total acid-soluble radioactivity. Supernatant fluid (0.8 mL) was treated with Norit under the conditions described (Richardson & Kornberg, 1964) except that a 50% Norit suspension was used and the treatment was repeated three times. The Norit supernatant fluid (1 mL) was mixed with 10 mL of Bray's solution and the Norit nonadsorbable radioactivity was determined in a Packard liquid scintillation counter at a counting efficiency of 80%. The Norit nonadsorbable ^{32}P-labeled product was identified as inorganic phosphate by chromatography on Dowex I-Cl column (Richardson & Kornberg, 1964) and also by chromatography on Whatman I paper using the solvent system methanol–formic acid–H_2O (80:15:5) (Benson, 1957). Inorganic phosphate ($H_3{}^{32}PO_4$) and glucose 6-phosphate were run as markers.

Treatment of DNA with alkaline phosphatase was carried out as described by Weiss et al. (1968) either at 65 or at 37 °C after denaturation of the DNA under alkaline conditions. Incubations (0.3 mL) contained in addition to the other components 2–6 μg of ^{32}P-labeled λ DNA and 0.5–1 unit of alkaline phosphatase. At the end of the incubation (30 min, 37 °C), the reaction mixture was processed as in the exonuclease III assays.

Drug Treatment of DNA. DNA was incubated with NCS in 50 mM Tris-HCl (pH 7.5) and 10 mM 2-mercaptoethanol at 37 °C for 45 min. The amounts of the DNA and the drug varied in different experiments as given in the legends. Except in a few experiments the drug was subsequently inactivated either by heating in a water bath at 65 °C for 45–60 min or by extracting with an equal volume of phenol saturated with 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA. The aqueous layer, after extraction with an equal volume of chloroform, was dialyzed extensively against 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA. Alternatively, the drug was inactivated by extraction of the mixture with one-tenth the volume of chloroform:isoamyl alcohol (5:1 v/v), and the DNA was dialyzed first against 200-fold excess of 100 mM KCl, 10 mM Tris-HCl (pH 7.5) for 12 h and then against 10 mM Tris-HCl (pH 7.5) for 24 h.

Incubation of DNA with bleomycin was done under conditions described for NCS.

Denaturation of NCS-Treated DNA with Dimethyl Sulfoxide or Formamide. Denaturation under nonalkaline conditions was done by a modification of the procedure described by Helmkamp & Ts'o (1961). DNA was treated with NCS and the drug was removed by chloroform–isoamyl alcohol as described above. The NCS-treated DNA samples (1.5 mL) were dialyzed against 300 mL of 85% formamide or 75% dimethyl sulfoxide for 24 h at 4 °C. The dialysis bags were then transferred to a liter of 10 mM Tris-HCl (pH 7.5) and dialysis was continued for 48 h with two changes of the same buffer. Finally the samples were dialyzed for 12 h against 400 mL of 10 mM Tris-HCl (pH 8.0).

Results

In order to test the possibility that the inactive binding sites for DNA polymerase I generated in DNA by NCS treatment possessed 3'-hydroxyl groups blocked by a phosphomonoester, we examined the effect of prior alkaline phosphatase treatment on the template activity of DNA (Figure 1). In the absence of phosphatase treatment increasing levels of the drug inhibited the incorporation of [^{3}H]dTMP into DNA up to 80%, as had been found earlier (Kappen & Goldberg, 1977). Whereas treatment of the control DNA with phosphatase results in only 1.5-fold stimulation of dTMP incorporation, with NCS-treated DNA, the incorporation is enhanced many fold and this effect is related to the dose of NCS up to 20 μg/mL. At 20 μg/mL of NCS the template activity is increased 65-fold. These results suggest that NCS-induced nicks bear 3'-phosphoryl termini and that the removal of phosphate by phosphatase results in

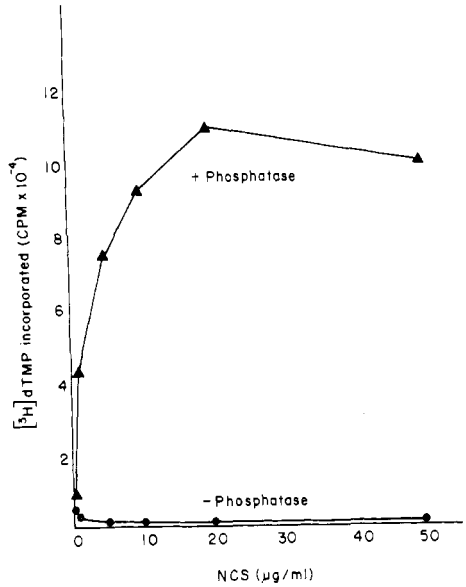

FIGURE 1: Effect of phosphatase treatment on the template activity of NCS-nicked DNA. HeLa DNA (17.5 μg) was first treated with varying levels of NCS in a total incubation volume of 200 μL under the conditions given in Materials and Methods. Aliquots of DNA (40 μL) were distributed for phosphatase treatment in a final volume of 50 μL. One set of tubes received 0.2 unit of alkaline phosphatase while an equal volume of buffer was added to the rest. After incubation at 65 °C for 30 min, the tubes were cooled in ice. Twelve microliters of a mix containing 0.08 unit of DNA polymerase I and the rest of the components was subsequently added. [^{3}H]dTMP incorporated into DNA for 15 min at 37 °C was measured.

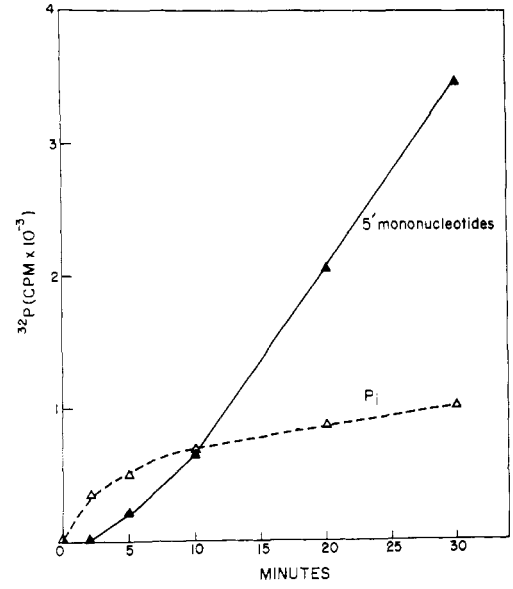

FIGURE 2: Exonuclease III induced release of radioactivity from NCS-treated DNA. ^{32}P-labeled λ DNA (32 μg, 2.4 × 10^5 cpm per μg) was treated with 200 μg/mL of NCS in a final volume of 1 mL. The drug was subsequently heat inactivated. Exonuclease III assay, in a total volume of 2 mL, contained 1 mL of NCS-treated DNA, 0.5 unit of exonuclease III, and the other components. Aliquots of 300 μL were withdrawn at times indicated and processed for total acid-soluble and Norit nonadsorbable radioactivity.

TABLE I: Effect of Phosphatase Treatment on the Template Activity of DNA Treated with NCS or Bleomycin for DNA Polymerase I.[a]

DNA	Temp of phosphatase incubation (°C)	[^{3}H]dTMP incorp into DNA (cpm) −phosphatase	[^{3}H]dTMP incorp into DNA (cpm) +phosphatase
Control	37	4612	5 425
	65	6015	12 500
NCS-treated (27 μg/mL)	37	1213	9 406
	65	1147	128 300
Bleomycin-treated (27 μg/mL)	37	1156	988
	65	1018	1 619

[a] Experimental conditions are similar to those in Figure 1 except that alkaline phosphatase incubation was done at 37 and 65 °C. The drugs were not removed prior to the enzyme assays.

the generation of 3′-OH termini which are active points for DNA polymerase action.

Weiss et al. (1968) established that alkaline phosphatase at 65 °C hydrolyzes both the external and the internal phosphomonoester groups and that at 37 °C primarily those at the ends of the chain are hydrolyzed. We, therefore, compared the effect of temperature of alkaline phosphatase treatment on the template activity of NCS-treated DNA. As shown in Table I in a control containing 2-mercaptoethanol but no drug phosphatase treatment at 37 °C does not significantly affect the template activity, but at 65 °C there is a twofold stimulation in the dTMP incorporation. On the other hand with NCS-treated DNA alkaline phosphatase treatment at 37 °C enhances the template activity eightfold and at 65 °C, the incorporation of dTMP is increased 100 times that in the absence of phosphatase. It must be pointed out that the small stimulation of dTMP incorporation obtained in the control at 65 °C was not observed when 2-mercaptoethanol was removed by dialysis after the NCS cutting reaction but prior to the phosphatase treatment. The increased incorporation with the NCS-treated DNA, however, remained the same under these conditions. These results clearly demonstrate that the stimulation of the template activity of NCS-cut DNA upon alkaline phosphatase treatment is due to the removal of phosphate groups mainly from the nicks and not from the ends of the DNA chain. Under similar conditions we compared the effect of bleomycin, another antibiotic known to cause strand scissions of DNA and to inhibit DNA polymerase systems under certain experimental conditions (Umezawa, 1974). Bleomycin inhibits DNA synthesis 75% and, unlike in the case of NCS-treated template, the incorporation is virtually unaffected by treatment of the bleomycin-treated DNA with phosphatase at either temperature (Table I). That the lack of stimulation is not due to the direct inhibition of phosphatase or polymerase by the drug is evident from experiments in which a reaction containing bleomycin-treated DNA was mixed with a reaction containing NCS-treated DNA. In this case the extent of stimulation of dTMP incorporation was the same as that obtained with the same amount of NCS-cut template alone (data not shown).

Conclusive evidence for the presence of phosphate blocked 3′ termini at the NCS-induced nicks comes from experiments using ^{32}P-labeled λ DNA and exonuclease III which degrades DNA from the 3′ end. As shown in Figure 2, exonuclease III treatment of NCS-cut DNA sequentially releases inorganic phosphate and 5′-mononucleotides. As expected of a 3′-phosphate-ended DNA, the release of P_i is immediate and reaches a limit in about 20 min. The release of mononucleotides occurs after an initial lag and is then linear with time. In agreement with these results, exonuclease III treatment of NCS-treated DNA results in marked stimulation of its template activity for DNA polymerase I (data not shown).

TABLE II: Enzymatic Release of Inorganic Phosphate from Nuclease- and NCS-Treated DNA.[a]

DNA treatment	$^{32}P_i$ (cpm)	
	Alkaline phosphatase	Exonuclease III
DNase	5117	4
Micrococcal nuclease	5271	4453
NCS	1347	763

[a] Incubation (300 μL) contains 5 μg of NCS-treated (100 μg/mL of NCS under the conditions described in Figure 2) or nuclease-treated ^{32}P-labeled λ DNA (1.5×10^5 cpm per μg). The drug had been heat-inactivated prior to the enzyme assays. In the absence of any treatment less than 50 cpm was released with either alkaline phosphatase or exonuclease III.

TABLE III: Enzymatic Release of Inorganic Phosphate from NCS-Treated DNA.[a]

NCS (μg/mL)	$^{32}P_i$ (cpm)		
	A alkaline phosphatase	B exonuclease III	A/B
0	20	6	
25	405	231	1.8
50	750	384	2.0
100	1200	553	2.2
200	2034	761	2.6
400	2915	972	3.0

[a] Incubation of the DNA with NCS contained 32 μg/mL of ^{32}P-labeled λ DNA (2.4×10^5 cpm per μg) and varying concentrations of the drug. After heat inactivation of the drug, the samples were dialyzed for 24 h against a 200-fold excess of 1 M KCl, and then for 12 h each against 0.02 M KCl and 0.025 M Tris-HCl (pH 7.5). The incubation reaction of either enzyme assay contained 3.2 μg of DNA in 300 μL.

TABLE IV: Alkaline Phosphatase Induced P_i Release from NCS-Cut DNA after Various Denaturation Treatments.[a]

Original denaturation procedure	$^{32}P_i$ (cpm)	
	Without alkali treatment	After alkali treatment
No denaturation	945	2146
	822	2054
Formamide	1926	2167
	1958	2108
Dimethyl sulfoxide	2014	2173
	1957	2148

[a] The NCS-cutting incubation contained, in a total volume of 6 mL, 120 μg of ^{32}P-labeled λ DNA (1.2×10^5 cpm per μg) and 200 μg/mL of NCS. After the incubation, the drug was removed by chloroform–isoamyl alcohol treatment. Of the three 1.5-mL aliquots of the NCS-treated DNA, two were denatured by dimethyl sulfoxide or formamide as described in Materials and Methods. The third aliquot was dialyzed as a control against 10 mM Tris-HCl (pH 7.5) until the transfer of all three bags to 10 mM Tris-HCl (pH 8.0) at the final step. After dialysis, radioactivity in 25 μL was determined to check the recovery of DNA. Where indicated, the various samples of DNA were further denatured with alkali in order to assess the completeness of the prior denaturation for the phosphatase reaction. The release of P_i by phosphatase was measured in an assay volume of 0.5 mL containing 4 μg of the dialyzed DNA, 1 unit of phosphatase, and the other components. Correction has been applied to account for the dilution occurring during dialysis so that the amount of P_i shown above is that released from the same amount of DNA.

While these data indicate the presence of 3′-phosphoryl termini at the NCS-generated nicks, we have earlier shown that they also bear 5′-phosphoryl termini (Poon et al., 1977). If each site of scission contains a 3′- and a 5′-phosphate on the fragmented ends of the DNA, the alkaline phosphatase should release twice as much inorganic phosphate as does exonuclease III (Tables II and III). DNase-cut DNA which is known to bear 5′-phosphoryl termini and micrococcal nuclease treated DNA with 3′-phosphoryl termini were included for comparison (Table II). As expected, exonuclease III does not release any P_i from DNase-cut DNA but does remove from micrococcal nuclease cut-DNA nearly as much P_i as is released by alkaline phosphatase. In contrast, exonuclease III releases from NCS-treated DNA about half as much P_i as that removed by alkaline phosphatase. The results obtained with bleomycin-treated DNA are similar to those with DNase-treated DNA (data not shown). Similar results were obtained whether the NCS was inactivated by heat, as in the above experiments, or by phenol treatment. Except at the highest concentrations of NCS, the ratio of the amount of P_i produced by phosphatase to that removed by exonuclease III is approximately two (Table III).

Similar results were obtained whether the drug-treated DNA was denatured in alkali prior to phosphatase treatment at 37 °C or whether the phosphatase reaction was carried out at 65 °C without prior denaturation of the DNA. In order to establish that the increased amount of P_i release by phosphatase is not due to additional strand breakage caused by incubation at 65 °C or by alkali, drug-treated DNA was also denatured with dimethyl sulfoxide or formamide prior to phosphatase treatment at 37 °C. The data presented in Table IV show that the amount of P_i released by phosphatase after denaturation under alkaline or nonalkaline conditions is the same and that alkali treatment subsequent to denaturation with dimethyl sulfoxide or formamide does not increase phosphatase-releasable inorganic phosphate significantly, indicating that these agents denatured the DNA maximally for subsequent phosphatase action.

Discussion

Our earlier finding (Poon et al., 1977) that nicks caused by NCS contain 5′-phosphate ended groups but are not sealed by polynucleotide ligase indicated that the strand scission caused by NCS is more than a simple splitting of the phosphodiester bond and probably involves damage to a nucleoside with possible opening of the deoxyribose ring and/or hydrolysis of the *N*-glycoside bond with base release. The latter possibility was further supported by the demonstrated release of thymine from DNA by higher levels of NCS (Ishida & Takahashi, 1976; Poon et al., 1977). In fact, single-strand scissions as analyzed by alkaline sucrose gradients could be equated with the amount of thymine released (Poon et al., 1977). Furthermore, the findings reported here that the NCS-generated, nonfunctional binding sites for DNA polymerase I can be activated by treatment with alkaline phosphatase and that there is an exonuclease III dependent release of inorganic phosphate from NCS-cut DNA indicate that the 3′ end of the scission may contain a phosphomonoester group.

Base release accompanied by a series of β-elimination reactions would lead to the simultaneous generation of equal

amounts of 3′- and 5′-phosphoryl ends. In experiments designed to quantitate 3′- and 5′-phosphoryl ends in the NCS-treated DNA, we found over a wide range of drug levels (25–100 μg/mL) that the amount of P_i released by phosphatase is about twice that removed by exonuclease III, indicating that there are equal amounts of 3′- and 5′-phosphoryl ends. At very high levels of the drug, however, this ratio appears to increase, suggesting, as might be expected, that extensive cutting of DNA by the drug renders it a poor substrate for exonuclease III but not for alkaline phosphatase. Since the incubation conditions for the two enzyme assays are vastly different, it is important to ascertain that no additional strand breakage occurred during one assay but not the other. The release of the same amount of P_i from NCS-treated DNA after denaturation by dimethyl sulfoxide or formamide as that after heat or alkali rules out the possibility of significant additional strand breakage due to these latter denaturation procedures and further supports the earlier observation (Beerman & Goldberg, 1974) that NCS-induced breaks are mainly true strand scissions and not due to alkali-labile bonds. In experiments exploring the possible role of alkali-labile bonds in NCS action, it has been determined that about 30% of the breaks found on alkaline gradients can be so ascribed (Beerman & Goldberg, unpublished results, 1977). More direct evidence for drug-induced sugar damage is the detection of a malonaldehyde-like compound in an acid-soluble fraction of NCS-treated DNA by its reaction with thiobarbituric acid (L. S. Kappen and T. Hatayama, preliminary data). Whether base release (followed by β-elimination reactions) or opening of the sugar ring is the primary event in NCS-induced DNA strand scission remains to be shown. In either case, gaps of one or more nucleoside residues, rather than simple nicks due to phosphodiester bond splitting, would be produced. Each gap appears to be bounded by a 3′- *and* a 5′-phosphate since there are no, or very few, 5′-hydroxyl groups generated by NCS treatment (Poon et al., 1977), and there are approximately equal numbers of 3′- and 5′-phosphate ended fragments. Furthermore, studies with NCS-treated DNA as a template for DNA polymerase I fail to show that any new *active* (3′-hydroxyl) sites for the enzyme are generated by drug treatment, in contrast to the findings with x-irradiation (Saffhill & Weiss, 1973). Finally, the production of gaps in the DNA by NCS treatment in vitro is consistent with that presumed to occur in vivo as revealed by the genetic analysis of different DNA repair mechanisms (Tatsumi & Nishioka, 1977).

The glycopeptide antibiotic bleomycin is functionally similar to NCS in its inhibition of DNA synthesis and in inducing DNA strand scission in vivo and in vitro (Suzuki et al., 1970; Umezawa, 1974; Haidle, 1972; Clarkson & Humphrey, 1976). Reports on the action of bleomycin in DNA polymerase systems have been contradictory. It has been shown to inhibit (Muller et al., 1975; Muller et al., 1972; Dicioccio & Srivastava, 1976), to stimulate (Yamazaki et al., 1973) and to have no effect (Muller et al., 1972; Ross & Moses, 1976). Bleomycin, under our experimental conditions, markedly lowers the template activity of HeLa DNA. The lack of any stimulation in the template activity of bleomycin-cut DNA upon alkaline phosphatase treatment is in agreement with the report by Kuo & Haidle (1973) and confirmed here that the nicks produced by bleomycin do not bear 3′-phosphoryl termini. It is of interest that, although bleomycin resembles NCS in causing base release (Muller et al., 1972) and in the stimulation of its cutting reaction by a sulfhydryl compound, there appears to be a distinct difference in the product of strand cleavage, as well as in certain other properties of the reaction (Umezawa, 1974; Ishida & Takahashi, 1975; Sausville et al., 1976).

Acknowledgments

The excellent technical assistance of Jeanne Thivierge is greatly appreciated.

References

Beerman, T. A., & Goldberg, I. H. (1974) *Biochem. Biophys. Res. Commun. 59,* 1254.

Beerman, T. A., & Goldberg, I. H. (1977) *Biochim. Biophys. Acta 475,* 281.

Beerman, T. A., Poon, R., & Goldberg, I. H. (1977) *Biochim. Biophys. Acta 475,* 294.

Benson, A. A. (1957) *Methods Enzymol. 3,* 163.

Bradner, W. T., & Hutchison, D. J. (1966) *Cancer Chemother. Rep. 50,* 79.

Clarkson, J. M., & Humphrey, R. M. (1976) *Cancer Res. 36,* 2345.

Dicioccio, R., & Srivastava, B. I. S. (1976) *Cancer Res. 36,* 1664.

Haidle, C. W., Weiss, K. K., & Kuo, M. T. (1972) *Mol. Pharmacol. 8,* 531.

Hedgpeth, J., Goodman, H. M., & Boyer, H. W. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69,* 3448.

Helmkamp, G. K., & Ts'o, P. O. P. (1961) *J. Am. Chem. Soc. 83,* 138.

Homma, M., Koida, T., Saito-Koide, T., Kamo, I., Seto, M., Kumagai, K., & Ishida, N. (1970) *Prog. Antimicrob. Anticancer Chemother., Proc. 6th Int. Congr. Chemother., 6th, 1969, 2,* 410.

Ishida, N., Miyazaki, K., Kumagai, K., & Rikimaru, M. (1965) *J. Antibiot. 18,* 68.

Ishida, R., & Takahashi, T. (1975) *Biochem. Biophys. Res. Commun. 66,* 1432.

Ishida, R., & Takashashi, T. (1976) *Biochem. Biophys. Res. Commun. 68,* 256.

Kappen, L. S., & Goldberg, I. H. (1977) *Biochemistry 16,* 479.

Kumagai, K., Ono, Y., Nishikawa, T., & Ishida, N. (1966) *J. Antibiot. 19,* 50.

Kuo, M. T., & Haidle, C. W. (1973) *Biochim. Biophys. Acta 335,* 109.

Muller, W. E. G., Yamazaki, Z., & Zahn, R. K. (1972) *Biochem. Biophys. Res. Commun. 46,* 1667.

Muller, W. E. G., Totsuku, A., Nusser, I., Zahn, R. K., & Umezawa, H. (1975) *Biochem. Pharmacol. 24,* 911.

Ohtsuki, K., & Ishida, N. (1975a) *J. Antibiot. 28,* 229.

Ohtsuki, K., & Ishida, N. (1975b) *J. Antibiot. 28,* 143.

Ono, Y., Watanabe, Y., & Ishida, N. (1966) *Biochim. Biophys. Acta 119,* 46.

Poon, R., Beerman, T. A., & Goldberg, I. H. (1977) *Biochemistry 16,* 486.

Richardson, C. C. (1966) *Proc. Nucleic Acid Res. 1,* 263.

Richardson, C. C., & Kornberg, A. (1964) *J. Biol. Chem. 239,* 242.

Ross, S. L., & Moses, R. E. (1976) *Antimicrob. Agents Chemother. 9,* 239.

Saffhill, R., & Weiss, J. J. (1973), *Nature (London) 241,* 69.

Sausville, E. A., Peisach, J., & Horwitz, S. B. (1976) *Biochem. Biophys. Res. Commun. 73,* 814.

Sawada, H., Tatsumi, K., Sasada, M., Shirakawa, S., Kakamura, T., & Wahishaka, G. (1974) *Cancer Res. 34,* 3341.

Suzuki, H., Nagai, K., Akutsu, E., Yamaki, H., Tanaka, N., & Umezawa, H. (1970) *J. Antibiot. 23,* 473.

Tatsumi, K., & Nishioka, H. (1977) *Mutat. Res. 48,* 195.

Tatsumi, K., Nakamura, T., & Wakisaka, G. (1974) *Gann 65*, 459.
Umezawa, H. (1974) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 33*, 2296.
Weiss, B., Live, T. R., & Richardson, C. C. (1968) *J. Biol. Chem. 243*, 4530.
Yamazaki, Z., Muller, W. E. G., & Zahn, R. (1973) *Biochim. Biophys. Acta 308*, 412.

Nucleotide Specificity in Microtubule Assembly in Vitro†

Stephen M. Penningroth‡ and Marc W. Kirschner*

ABSTRACT: A procedure is described for removing most of the GDP bound at the exchangeable GTP binding site (E site) of tubulin. Microtubule protein containing substoichiometric amounts of GDP at the E site is found to polymerize in response to: (a) two nonhydrolyzable ATP analogues, adenylyl imidodiphosphate (AMP-PNP) and adenylyl β,γ-methylenediphosphonate (AMP-PCP); and (b) substoichiometric levels of GTP or dGTP. The results are interpreted as suggesting that: (1) when GDP is removed from tubulin, the E site shows broad specificity for nucleoside triphosphates; (2) microtubule assembly can be induced by the binding of substoichiometric amounts of nucleoside triphosphate to the E site.

Tubulin, the 110 000 molecular weight subunit of microtubules (Shelanski & Taylor, 1967; Weisenberg, 1972), binds 2 mol of GTP, one at an exchangeable or E site and one at a nonexchangeable or N site (Weisenberg et al., 1968). Exchangeably bound GTP is readily displaced by GTP or GDP in the medium, and the K_{diss} for GTP binding at the E site has been estimated at 10^{-6} to 10^{-7} mol L^{-1} (Jacobs & Caplow, 1976; Levi et al., 1974). At the N site no exchange of GTP has been detected in vitro; however, the release of N-site GTP on denaturation of the tubulin molecule indicates that it is noncovalently bound to tublin.

Recent work in several laboratories has led to an increased understanding of nucleotide requirements in microtubule assembly in vitro (Arai & Kaziro, 1976; Kobayashi & Simizu, 1976; Penningroth et al., 1976; Weisenberg et al., 1976; Penningroth & Kirschner, 1977). Two nonhydrolyzable GTP analogues, guanylyl β,γ-methylenediphosphonate (GMP-PCP) and guanylyl imidodiphosphate (GMP-PNP), as well as GTP have been found to induce microtubule assembly by binding to the E site. Other nucleoside triphosphates such as ATP, UTP, and CTP appear to act indirectly by phosphorylating exchangeably bound GDP in situ at the E site by means of a nucleoside diphosphate kinase (NDP-kinase) activity, which is not intrinsic to tubulin, and binding of the resulting GTP at the E site then induces microtubule assembly. GTP at the N site has been shown not to turn over during the polymerization reaction in vitro.

In previous examinations of the nucleotide specificity of the E site (Jacobs et al., 1974; Arai et al., 1975; Penningroth & Kirschner, 1977), no nucleotides other than GTP, GDP, GMP-PCP, and GMP-PNP were found to interact demonstrably with the E site, suggesting that the E-site may be absolutely specific for guanosine nucleotides. However, an absence of binding of weak nucleotide ligands to tubulin could be due to inhibition by GDP, which is present in most tubulin preparations. As a strong ligand, GDP could block the binding of weak ligands to the E site. GDP in the medium has been shown to inhibit microtubule polymerization with GMP-PCP and GMP-PNP (Penningroth et al., 1976). In this paper a method is described which removes approximately 60% of the GDP from the E site of tubulin. When the concentration of GDP at the E site is reduced, two weakly binding, nonhydrolyzable ATP analogues, adenylyl imidodiphosphate (AMP-PNP) and adenylyl β,γ-methylenediphosphonate (AMP-PCP) will induce microtubule assembly comparable in rate and extent to the polymerization reaction observed with GTP. Polymerization is also induced by weakly binding, hydrolyzable nucleoside triphosphates such as UTP and ATP, suggesting that the E site of tubulin possesses broad specificity for nucleoside triphosphates in the microtubule polymerization reaction. In addition, microtubule polymerization is shown to be promoted by substoichiometric concentrations of GTP and dGTP. The results are interpreted as suggesting that nucleoside triphosphates and GDP may act allosterically as antagonists in the microtubule polymerization reaction.

Materials and Methods

Preparation of Microtubule Protein. Microtubule protein was purified from porcine brain by alternate cycles of polymerization/depolymerization according to the procedure of Shelanski et al. (1973) as modified by Weingarten et al. (1974) and stored in purification buffer (0.1 M Mes, 0.5 mM $MgCl_2$, 1.0 mM β-mercaptoethanol, 2.0 mM EGTA, 0.1 mM EDTA (pH 6.4) with NaOH) containing 8 M glycerol plus 1 mM GTP at −20 °C. Protein was used within 4 weeks of purification. Prior to each experiment, microtubule protein was diluted 1:1 with purification buffer and repolymerized with 0.1 mM GTP for 30 min at 37 °C. Microtubules were centrifuged at 180 000*g* for 35 min at 25 °C in a Ti 50 fixed angle rotor (Beckman) using 10-mL polycarbonate tubes. The supernatant was discarded, and the surface of the pellet and the walls of the centrifuge tube were washed with a 1.0-mL aliquot of purification buffer to ensure the complete removal of the superna-

† From the Department of Biochemical Sciences, Princeton University, Princeton, New Jersey 08540. *Received June 7, 1977; revised manuscript received November 3, 1977.* Supported by Public Health Service Grant 19667 to M.W.K., the American Cancer Society Grant VC213, and U.S. Public Health Service Training Grant GM 962 for S.M.P. M.W.K. thanks the Dreyfus Foundation and the U.S. Public Health Service Research Career Development Award for their support.

‡ Current address: Department of Biology, Princeton University, Princeton, N.J. 08540.

0006-2960/78/0417-0734$01.00/0