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Mechanism of the Effect of Organic Solvents and Other Protein Denaturants on Neocarzinostatin Activity†

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ABSTRACT: Several alcohols and other organic solvents enhance the in vitro DNA cutting activity of neocarzinostatin (NCS). Hexamethylphosphoramide is the most potent of the stimulators tested. Kinetic analysis of the DNA scission by NCS in the presence of 2-propanol shows that the solvent effect is on the V_{max} and not on the K_m. All the solvents, above their optimal levels, inhibit NCS activity, and they also increase the rate of its inactivation on preincubation in the absence of deoxyribonucleic acid (DNA). Among the other protein denaturants, urea stimulates the activity of NCS over a wide range of concentrations. In contrast, guanidine hydrochloride at subdenaturing levels strongly inhibits the reaction. One possible explanation for the stimulation of NCS activity by organic solvents and urea is that by breaking down water-cooperative structures they allow increased motility of the antibiotic to a conformation favorable for reduction of its disulfide bonds to generate an active species of the drug for its interaction with DNA. The possible involvement of the nonprotein chromophore of NCS in the activation process is also discussed. In addition, NCS rendered inactive by various treatments has a lower isoelectric point and specifically blocks the activity of native NCS. These results indicate that there are a limited number of DNA binding sites for which the two forms of NCS compete and provide further evidence that direct interaction between drug and DNA is responsible for its biological effects. Since several of the treatments generating the inhibitory species of NCS also cause chromophore release and since auromomycin-induced scission of DNA is blocked by its nonchromophore-containing form (macromomycin), the possibility is raised that loss of chromophore from the protein generates the inhibitory species.

Neocarzinostatin (NCS[1]), an antitumor antibiotic, is a single-chain, acidic protein of molecular weight 10700 with two disulfide bonds (Ishida et al., 1965; Meienhofer et al., 1972a). Recently, we have shown that NCS possesses a nonprotein chromophore that can be removed by methanol extraction and other techniques (Napier et al., 1979). In addition to its ability to inhibit DNA synthesis (Ono et al., 1966; Homma et al., 1970; Sawada et al., 1974; Beerman & Goldberg, 1977), cause DNA strand breakage (Beerman & Goldberg, 1974; Sawada et al., 1974; Tatsumi et al., 1974; Ohtsuki & Ishida, 1975; Beerman & Goldberg, 1977), and induce DNA repair synthesis in whole cells (Tatsumi et al., 1975; Hatayama & Goldberg, 1979) and in isolated nuclei (Kappen & Goldberg, 1978a), NCS introduces single-strand breaks almost exclusively at thymidylate and adenylate residues in DNA (Poon et al., 1977; Hatayama et al., 1978; D'Andrea & Haseltine, 1978) in vitro in a reaction greatly stimulated by a sulfhydryl compound (Beerman & Goldberg, 1974; Poon et al., 1977; Beerman et al., 1977; Kappen & Goldberg, 1977, 1978b). We have further shown that while mercaptans activate the DNA cutting by NCS, they also inhibit it at high concentrations and rapidly inactivate NCS on preincubation in the absence of DNA (Kappen & Goldberg, 1978c). In addition, several DNA-intercalating drugs (Kappen et al., 1979) and α-tocopherol strongly inhibit the in vitro reaction, but alcohols and other organic solvents greatly stimulate the in vitro reaction (Kappen & Goldberg, 1978c). In an attempt to understand the mechanism of alcohol stimulation of NCS activity, we have studied the effects of several alcohols, other organic solvents, and denaturants on the activity of NCS under different conditions by using linear duplex (λ) or superhelical (pMB9) DNA as the substrate. In addition, we find that NCS rendered inactive by various treatments is able to block the DNA scission reaction of native drug.

Materials and Methods

[*methyl*-^{3}H]Thymidine-labeled λDNA was prepared as described (Kappen & Goldberg, 1977). The plasmid DNA (pMB9) was isolated by gentle lysis of the cells (*Escherichia coli* HMS 174pMB9) with lysozyme and detergent, followed by centrifugation to remove chromosomal DNA (Clewell & Helinski, 1969). Further treatment included banding in a CsCl gradient containing ethidium bromide at 200 μg/mL; the ethidium bromide was subsequently removed with Dowex 50

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[1] Abbreviations used: NCS, neocarzinostatin; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate.

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resin. The DNA preparation contained 85–90% supercoiled (form I) and the rest open circular (form II) forms. NCS (clinical form) was generously provided by Dr. W. T. Bradner of Bristol Laboratories. This material gave a single band on polyacrylamide gel electrophoresis, and on isoelectric focusing about 80% had an isoelectric point (p*I*) of 3.3 and 20% focused at a p*I* of 3.2 (pepsin marker, p*I* 2.9), corresponding to biologically active NCS and biologically inactive pre-NCS, respectively (Kikuchi et al., 1974; Maeda & Kuromizu, 1977; Beerman et al., 1977). The 89 amino acid peptide of NCS (lacking the first 20 amino acids from the NH_2 terminus) and the arginine-blocked NCS (arginines at positions 66, 67, and 78 are blocked with cyclohexanedione) were generous gifts from Dr. T. S. A. Samy of the Sidney Farber Cancer Institute.

The standard in vitro DNA cutting reaction contained 50 mM Tris (pH 8.0) (100 mM at high drug levels), 10 mM 2-mercaptoethanol when present, and the drugs at levels indicated in the figure legends. After incubation at 37 °C, the reaction mixture was analyzed on 5–20% alkaline sucrose gradients containing 0.7 M NaCl, 0.3 M NaOH, and 1 mM EDTA. The samples with pMB9 DNA were centrifuged at 20 °C in an SW 50.1 rotor for 2 h at 40000 rpm. The gradient fractions after neutralization with HCl were mixed with 10 mL of Scintiverse, and the radioactivity was determined in a liquid scintillation spectrometer. The reaction products with pMB9 DNA were also separated electrophoretically on 1% agarose gels in a buffer containing 40 mM Tris, 5 mM sodium acetate, and 1 mM EDTA, pH 7.9. The radioactivity in gel slices was determined after digestion with 10 mL of a mixture of protosol–liquifluor–toluene (5:4:91 v/v). Since in both sucrose gradient and gel analysis pMB9 (form I) DNA is well separated from the nicked forms, the extent of the reaction was measured from the amount of form I remaining after treatment. Strand breaks (μ) were calculated from the equation $\mu = -\log$ [form I]. Measurement of trichloroacetic acid soluble radioactivity produced from DNA by the drug was determined as reported earlier (Kappen & Goldberg, 1978c).

Inactivation of the drug was carried out by the following treatments in 50 mM Tris buffer, pH 8.0: (a) heating for 2 h at 73 °C (Beerman et al., 1977); (b) exposure to long-wavelength ultraviolet light (Mineralight Lamp, Model UVSL-58, 366 nm) for 45 min at 15-cm distance; (c) incubation with 45 mM 2-mercaptoethanol at 37 °C for 30 min; (d) treating with 6 M guanidine hydrochloride for 12 h. In (c) and (d) the samples, after treatment, were dialyzed against 50 mM Tris buffer, pH 7.5, to remove the inactivating agents. Chromophore-free NCS was obtained by two methods. (1) Extraction of lyophilized NCS with methanol was as described by Napier et al. (1979). The residual protein was redissolved in 0.015 M sodium acetate buffer, pH 5.0. (2) Chromatography of NCS on Amberlite XAD-7 and elution with distilled H_2O lead to the recovery of a protein essentially free of chromophore as judged by UV and fluorescence spectral criteria. Details of the separation procedure and characterization of the chromophore-free protein will be reported elsewhere.

All of the above methods of inactivation result in a lowering of the p*I* of the protein, as determined by isoelectric focusing in a pH gradient from 2.5 to 4.0 on polyacrylamide, from 3.3 to 3.2. Chromophore removal was monitored by absorption and fluorescence measurements as previously described (Napier et al., 1979).

All solvents were of analytical grade and, in some cases, were redistilled before use.

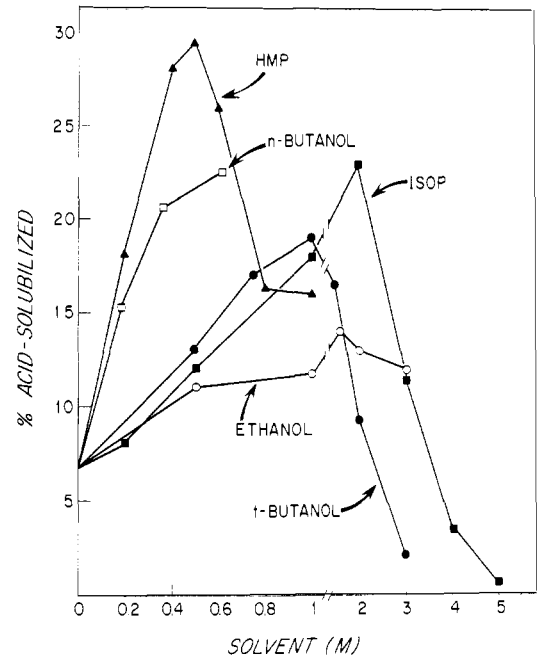

FIGURE 1: Effect of organic solvents on NCS activity. Reaction mixtures (100 μL) contained 0.8 μg of λDNA (2.4×10^4 cpm), 100 μg/mL NCS, and varying amounts of solvents. After incubation for 30 minutes, the amount of trichloroacetic acid soluble radioactivity was determined as described under Materials and Methods. Hexamethylphosphoramide (HMP); 2-propanol (ISOP).

Results

Figure 1 shows the dose-dependent effect of four alcohols and hexamethylphosphoramide on the activity of NCS as measured by the production of trichloroacetic acid soluble radioactivity from ^{3}H-labeled λDNA. All the alcohols enhance the activity of NCS; their ability to stimulate the reaction at comparable concentrations increases with increasing chain length. Above their optimal levels these solvents inhibit the reaction. Hexamethylphosphoramide with the lowest optimum concentration of 0.5 M is the most potent stimulator of the reaction (Figure 1). A time course of the DNA cutting by NCS (Figure 2) shows that the reaction is linear for at least 15 min, by which time 5.4% of the DNA is solubilized. In the presence of 0.5 M hexamethylphosphoramide the reaction is considerably enhanced from the start but levels off while the unstimulated reaction remains linear. Thus, at 1-min incubation in the presence of hexamethylphosphoramide NCS solubilizes 44 times more DNA than by the drug alone. Excess DNA does not eliminate the solvent effect. Similarly, the stimulation remains the same at increasing levels of drug or 2-mercaptoethanol. While hexamethylphosphoramide stimulates the reaction when it is present in the complete reaction mixture, it increases the rate of inactivation of NCS when preincubated with or without 2-mercaptoethanol in the absence of DNA (Figure 3). The rate of inactivation is not affected by the inclusion of EDTA or radical scavengers during the preincubation.

Among the other solvents tested, dimethyl sulfoxide at its maximally effective level of 1.5 M is only 65% as good as 1 M 2-propanol. With ethylene glycol and glycerol there is a small enhancement of activity at 1 M levels, but even at concentrations as high as 3 M the increase in activity is only 76% and 65%, respectively, of that given by 1 M 2-propanol (data not shown).

In order to get a general idea of where the organic solvent has its principal effect on the drug's mechanism, we studied the kinetics of the reaction and constructed the double-re-

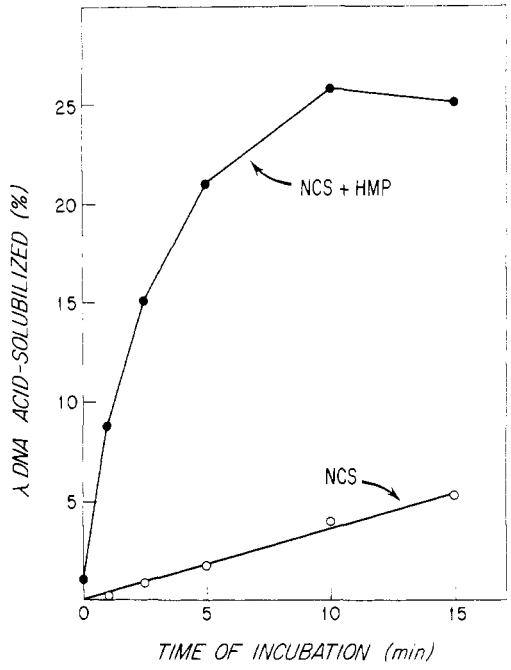

FIGURE 2: Stimulation of NCS activity by hexamethylphosphoramide. Incubations (1 mL) were as in Figure 1. One hundred microliter portions were withdrawn at times indicated for the determination of acid-solubilized radioactivity.

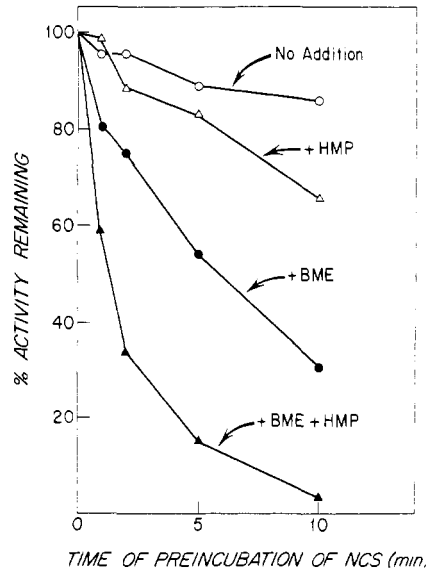

FIGURE 3: Rate of inactivation of NCS in the presence of hexamethylphosphoramide. NCS (125 μg/mL) was preincubated at 37 °C in 62.5 mM Tris buffer, pH 8.0, alone or with 0.5 M hexamethylphosphoramide in the presence and absence of 5 mM 2-mercaptoethanol (BME). At various times shown, 80-μL aliquots were taken out and added to tubes containing λDNA (0.8 μg, 3.6×10^4 cpm). The complete reaction mixture had 100 μg/mL NCS and 10 mM 2-mercaptoethanol. After 30 min of incubation, the amount of trichloroacetic acid solubilized radioactivity was determined. Without preincubation the percentage of the DNA made acid soluble was 8.2% and 31% without and with hexamethylphosphoramide, respectively.

ciprocal plots with 2-propanol as the stimulant. The data show (Figure 4) that the alcohol affects the V_{max} but not the K_m.

Since many potential denaturing organic solvents were found to stimulate the activity of NCS, it was of interest to test the effect of two other protein denaturants, urea and guanidine hydrochloride, on the drug's activity. As shown in Figure 5, urea enhances the DNA cutting activity of NCS over a wide range of concentration. In contrast, there is a steady decrease

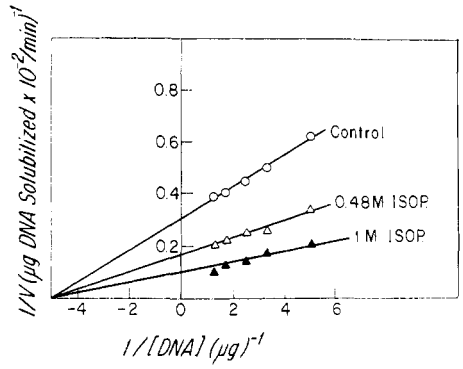

FIGURE 4: Double-reciprocal plot of the effect of 2-propanol on NCS activity. A series of triplicate incubations (100 μL) contained varying amounts of λDNA (0.1–0.5 μg) and 400 μg/mL NCS. One set of tubes (control) received water while 2-propanol was added to the other two sets at the levels shown. The reaction was started by the addition of NCS to the rest of the components. All the components were prewarmed at 37 °C before the start of the reaction. The extent of trichloroacetic acid solubilization of DNA at various times was determined. The data plotted were taken from the linear part (2 min) of the curves.

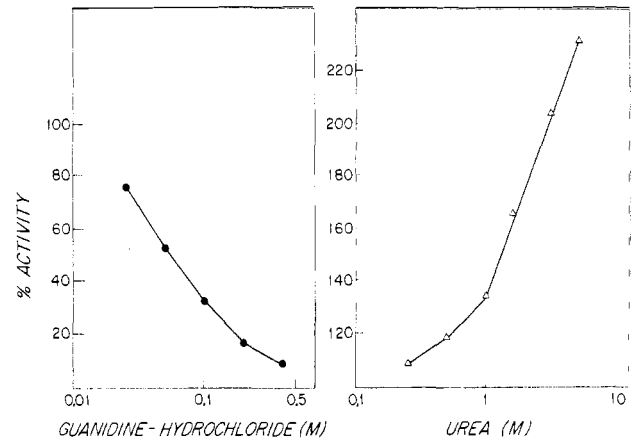

FIGURE 5: Effect of urea and guanidine hydrochloride on NCS activity. In standard incubations similar to those in Figure 1, varying amounts of urea or guanidine hydrochloride were added. Activity was calculated based on the amount of acid-solubilized radioactivity. In the reaction lacking urea or guanidine hydrochloride, NCS made 10.5% of the DNA acid soluble.

in activity on addition of increasing amounts of guanidine hydrochloride. Fifty percent inhibition of the reaction is obtained at 0.05 M guanidine hydrochloride. The inhibition could be completely reversed by dialyzing out the guanidine hydrochloride after its treatment of NCS at levels up to 1 M. On the other hand, if NCS is preincubated with urea (4.8 M) or guanidine hydrochloride (0.05 M) in Tris buffer, pH 8.0, before the addition of DNA, there is appreciable decrease in the activity of the drug, as has also been observed with organic solvents.

Since denaturing agents are found in most cases to enhance the activity of NCS, it appeared that they might alter in some way the interaction of the drug with DNA. Commonly employed methods involving chromatography and centrifugation techniques with a radioactive form of the drug have failed to give evidence for any direct binding of NCS to DNA (Maeda et al., 1975; L. S. Kappen and I. H. Goldberg, unpublished experiments). We have used an indirect method involving the ability of NCS rendered inactive by denaturing agents or other treatments to block the activity of native NCS. Acid solubilization of λDNA by native NCS was measured in the presence of increasing amounts of inactivated NCS. As shown in Figure 6, both heat-denatured and 2-mercaptoethanol-in-

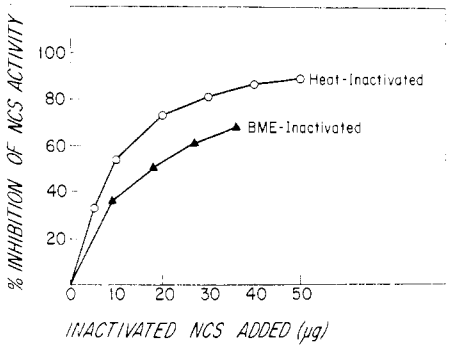

FIGURE 6: Effect of inactivated NCS on NCS activity. Reaction conditions are similar to those in Figure 5, except that varying amounts of NCS inactivated by pretreatment with heat or 2-mercaptoethanol, as described under Materials and Methods, were added to incubations containing 100 μg/mL active NCS.

Table I: Effect of Inactivated NCS and Other Proteins on the Activity of Native NCS (100 μg/mL)[a]

addn (μg/mL)	% inhibn
none	
heat-inactivated NCS (200)	70
UV-inactivated NCS (180)	63
6 M guanidine-treated NCS (80)	59
chromophore-free NCS[b] (100)	75
arginine-blocked NCS (200)	none
arginine-blocked NCS after heat treatment (200)	none
heat-inactivated 89 amino acid peptide of NCS (50)	53
heat-inactivated auromomycin (200)	none
bovine serum albumin (500)	none
ovalbumin (500)	none
pepsinogen (500)	none

[a] The effect of addition of the proteins listed to incubations similar to those in Figure 6 was determined by measuring the amount of trichloroacetic acid soluble radioactivity. 8.7% of the DNA was made acid soluble by NCS (100 μg/mL) without addition of other proteins. [b] Prepared by methanol extraction as described under Materials and Methods.

activated NCS block the activity of native NCS; the former is somewhat more inhibitory than the latter. With heat-treated NCS 50% inhibition was obtained at equimolar levels with native drug. Inhibition is less at higher ratios of DNA/native drug, presumably because of the existence of excess binding sites for the two drug forms. In order to establish the specificity of this assay, we have compared the effect of NCS treated by different agents to that of other acidic proteins and the protein antibiotics macromomycin and its chromophore-containing form, auromomycin (Yamashita et al., 1979) (Table I). Auromomycin is somewhat similar to NCS in some of its in vitro and in vivo activities, but highly purified macromomycin has essentially no in vitro DNA scission activity (Kappen et al., 1979). It can be seen that NCS inactivated by different methods inhibits the activity of native NCS. It is interesting to note that arginine-blocked NCS, which is not active in vitro or in vivo (T. S. A. Samy, L. S. Kappen, and I. H. Goldberg, unpublished experiments), does not inhibit the reaction of NCS with or without heat treatment. On the other hand, the 89 amino acid peptide derivative of NCS, which is as active as NCS in vitro and in vivo (T. S. A. Samy, L. S. Kappen, and I. H. Goldberg, unpublished experiments), inhibits NCS activity after heat inactivation. NCS from which the chromophore has been removed by methanol extraction retains very little (<1%), if any, in vitro or in vivo biological activity (L. S. Kappen, M. A. Napier, and I. H. Goldberg, unpublished experiments) and, as seen in Table I, is a potent

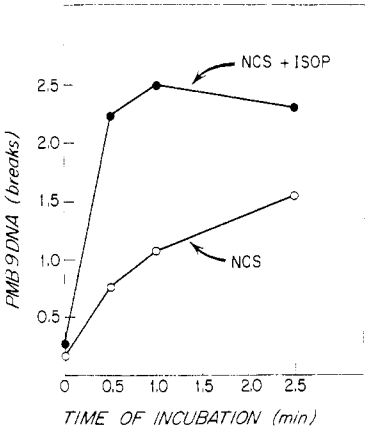

FIGURE 7: Effect of 2-propanol on the NCS-induced conversion of superhelical form I pMB9 DNA to the open circular form II. The reaction mixture (100 μL) contained 1 μg of form I DNA (3×10^4 cpm) and 0.75 μg/mL NCS. At various times of incubation, 20 μL of the mixture was withdrawn to tubes containing 6 μL of 5 mM α-tocopherol which inhibits any further reaction. Twenty microliters of this mixture was analyzed on agarose gels as described under Materials and Methods. 2-Propanol, when present, was at 1 M.

inhibitor of native drug in cell-free DNA scission. Similar results were obtained with chromophore-free NCS prepared by Amberlite XAD chromatography. Auromomycin, macromomycin (data not shown), bovine serum albumin, and pepsinogen (all with or without heat denaturation) do not have any effect on the activity of NCS. Similar results were obtained in experiments in which DNA strand breakage was followed on alkaline and neutral sucrose gradients, although due to the relatively high levels of DNA much more inactive drug was required for comparable inhibition. Similarly, we have found that in vitro DNA strand scission by auromomycin is blocked specifically by heated auromomycin or by macromomycin, heated or unheated in a dose-dependent way. Auromomycin differs from macromomycin only in possessing a nonprotein chromophore (Yamashita et al., 1979).

The data so far presented were derived from assays in which linear λDNA and high levels of NCS were used. It is important to establish that the effects we observe are not peculiar to the experimental conditions employed. Hence, we have examined the effect of alcohols on NCS activity in sensitive assays with supercoiled pMB9 DNA as the substrate. Introduction of a single nick converts the superhelical pMB9 DNA (form I) molecule to the open circular form (form II), and a double-strand break produces the linear (form III) DNA. The different forms can be separated electrophoretically on agarose gels. On incubation with 0.75 μg/mL NCS, form I pMB9 DNA is converted to form II (Figure 7) and the initial rate of this reaction is markedly enhanced by the presence of 2-propanol. At this and lower levels of NCS (where DNA is not limiting) the reaction without 2-propanol eventually reaches the same extent of strand scission as in the presence of the solvent. Reaction mixtures similar to those used in Figure 7 were also analyzed on alkaline sucrose gradients which separate form I DNA and the nicked forms into two distinct peaks. 2-Propanol stimulates about fourfold the DNA cutting by NCS in incubations carried out at 24 °C for 45 s or in ice for 15 min (data not shown). It should be emphasized that when the reaction products of a long incubation (e.g., 30 min at 37 °C) were analyzed on gel or on alkaline sucrose gradients, the stimulation of cutting by 2-propanol was much less pronounced than at early times.

At low levels of NCS (<0.8 μg/mL) which convert 70–90% of form I molecules to form II, no significant amount of linear

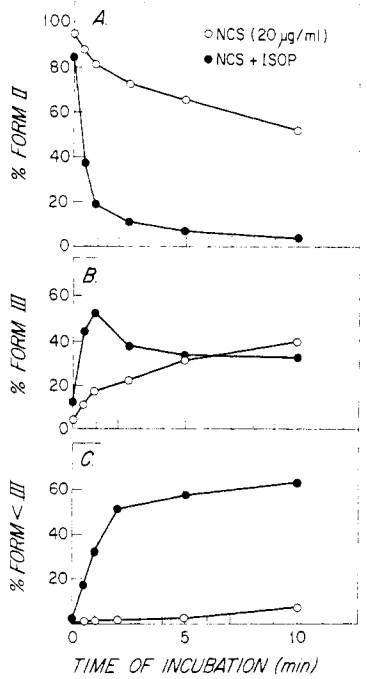

FIGURE 8: Time course of the effect of 2-propanol on the formation of various forms of pMB9 DNA in the presence of NCS. The reaction conditions are essentially similar to those in Figure 7, except that 20 μg/mL NCS was used. The radioactivity corresponding to the peaks to forms I, II, and III was quantitated. For an estimation of the fragmented DNA forms, all the radioactivity present in the area of the gel ahead of form I was summated.

DNA molecules is produced, suggesting that the double-strand breaks produced at high drug levels are the result of the random placement of single-strand breaks on opposite strands and not due to single events. In the next two figures evidence is presented to show that 2-propanol does not alter this basic process, as also suggested by DNA sequencing studies (Hatayama et al., 1978). In Figure 8 is shown the effect of high levels of NCS (20 μg/mL) on pMB9 DNA with and without 2-propanol, as a function of time. At this level of the drug, ~96% of the DNA is instantaneously converted to form II. Upon incubation, form II decreases (Figure 8A) with a concomitant increase mainly in form III (Figure 8B) and small amounts (<10% of the total) of degraded forms. In the presence of 2-propanol form II disappears much faster; the amount of form II is reduced to 37% in 30 s and to 4% in 10 min. On the other hand, in a 10-min incubation with NCS alone, 50% of form II remains unconverted. The most striking difference in the presence of 2-propanol is the production of small fragments of DNA (Figure 8C) which amount to 65% of the total in 10 min.

A similar effect was observed when 2-propanol was included in a reaction containing varying amounts of NCS. As shown in Figure 9, in the presence of increasing amounts of NCS alone (5–40 μg/mL) there is a linear decrease of form II and a corresponding increase in form III with no significant amounts of small fragments up to 20 μg/mL drug. In the presence of 2-propanol there is a much greater decrease in form II; at 15 μg/mL NCS and 2-propanol only 5% of form II remains in contrast to 70% with NCS alone. The level of form III in the presence of 2-propanol is much higher than that in its absence up to 20 μg/mL NCS. At levels of NCS above 20 μg/mL there is a sharp drop in form III associated with a quantitatively similar rise in small fragments. At 40 μg/mL NCS and 2-propanol ~96% of the total DNA is in small fragments, in contrast to 10% with the drug alone.

Discussion

In an attempt to understand the mechanism whereby alcohols stimulate the mercaptan-dependent scission of DNA by NCS, we have examined in detail the effects of several alcohols, other organic solvents, and two commonly used protein denaturants, urea and guanidine hydrochloride. NCS is more active in an aqueous–alcohol cosolvent binary mixture than it is in water. Thus, we would expect (Tan & Lovrien, 1972), as was found, the higher alcohols to be more effective than the lower ones and the di- and trihydric alcohols. The stimulatory effect, however, is not restricted to alcohols since other solvents such as dimethyl sulfoxide and hexamethylphosphoramide also enhance the activity of NCS; these results rule out the involvement of alcoholysis in the reaction. In fact, hexamethylphosphoramide is the most potent stimulator of the reaction. The solvent effect does not appear to be due to a slow, time-dependent activation of the drug since the reaction proceeds linearly with time in the absence of the stimulator and the change in rate caused by the solvent is immediate (Figure 2). The kinetics of the reaction of NCS with and without 2-propanol show that the effect of the solvent is exclusively on the V_{max} and not on the K_m (Figure 4). None of the stimulating solvents substitutes for 2-mercaptoethanol or alters the basic mechanism whereby single- or double-strand breaks are produced. On the other hand, the small amount of DNA cutting by very high levels of NCS in the absence of 2-mercaptoethanol is not affected by the presence of 2-propanol or hexamethylphosphoramide (Kappen et al., 1979).

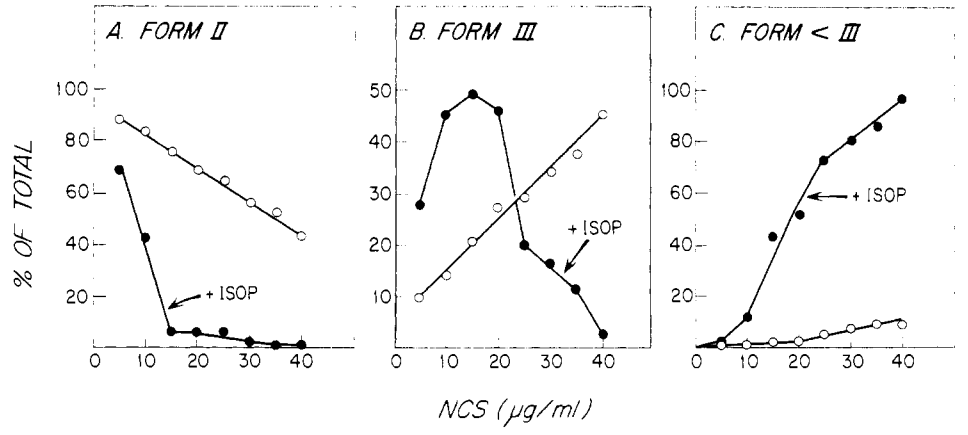

FIGURE 9: Formation of different nicked forms of pMB9 DNA by varying the levels of NCS in the presence and absence of 2-propanol (1 M). In a 3-min incubation similar to those in Figure 7, the levels of NCS were varied. The different forms of the DNA present after the treatment were quantitated by electrophoretic analysis on agarose gels.

It is thus possible that the solvent effect is linked to the action of 2-mercaptoethanol. For example, the solvents might change the conformation of the antibiotic protein so as to expose its disulfide bonds for facile reduction to generate an active species of the drug. In fact, the undenatured drug is known to be very resistant to reduction of its disulfides by reducing compounds (Meienhofer et al., 1972b). It should be noted, however, that gross changes in native NCS conformation due to 1 M 2-propanol have not been detected by circular dichroism measurements (M. A. Napier and I. H. Goldberg, unpublished experiments). If unfolding is involved in generating an active, labile form of the drug, then the same agents that stimulate the reaction might be expected to inactivate the drug. This has been found to be the case. Other possibilities also exist as an explanation for the solvent-stimulation phenomenon. Thus, organic solvents can inhibit ionization of groups in the active site of the molecule (Schuster, 1979), facilitate charge interaction with the substrate by lowering the dielectric constant of the medium (Uhing et al., 1979) (see following discussion), or change the conformation of a macromolecular substrate (i.e., DNA). The last possibility seems unlikely since excess DNA does not eliminate the solvent-induced stimulation of DNA strand scission. Further, at low drug levels with pMB9 DNA the solvent increases the initial rate of DNA cutting (Figure 7) but not the final extent. In addition, the discovery of a tightly but noncovalently bound chromophore in NCS (Napier et al., 1979) offers another component that is possibly involved in the solvent effect, either indirectly through protein conformational changes or by direct interaction. The enhancement of NCS activity by organic solvents is similar to that reported for several enzyme systems. The increased activities of a variety of enzymes in aqueous–organic cosolvent binary mixtures have been attributed to the breakdown of the water-cooperative structure so as to permit increased conformation motility to active forms of the enzymes (Tan & Lovrein, 1972). Also, the catalytic activity of biotin carboxylase was enhanced by ethanol (11 times at 15% v/v) and the kinetic effect was exclusively on the maximal velocity of the reaction (Dimroth et al., 1970). The possibility was raised that biotin carboxylase undergoes a productive conformational change by a solvent-induced increase in the hydrophobicity of the environment. Similarly, phosphorylase *b* (Uhing et al., 1979) and mitochondrial ATPase (Schuster, 1979) were also stimulated by organic solvents. Hexamethylphosphoramide was shown to protect several proteins against denaturation (Asakura et al., 1978) and may act to stabilize the active form of NCS during the DNA cutting reaction.

The contrast between the effects of urea and guanidine hydrochloride on NCS activity is striking. Both of these denaturants cause complete unfolding of many proteins under appropriate conditions. In our experiments, addition of increasing amounts of urea gave increasing DNA cutting activity, possibly due to an unfolding of the antibiotic to a conformation favorable for reduction and interaction with the substrate. In contrast, the inhibition of NCS activity obtained with low levels of guanidine hydrochloride suggests that interactions other than protein denaturation occur with this compound. Indeed, this finding simulates the inhibition of activity of *Streptomyces griseus* trypsin by subdenaturing levels of guanidine hydrochloride (Russin et al., 1974). In the latter case the inhibition of enzyme activity is due to guanidine binding at its specificity site which interacts with the arginylguanidino groups of the substrate molecules. An analogy of a reversed nature can be drawn for NCS. When the arginines in NCS and the 89 amino acid peptide (lacking the NH_2-terminal 20 amino acid residues of NCS) were chemically blocked, their in vitro and in vivo activities were completely lost. Removal of the blocking groups restored the activity (T. S. A. Samy, L. S. Kappen, and I. H. Goldberg, unpublished experiments). This indicates that unblocked arginine residues are essential for the activity of NCS, as is also evidenced by the failure of arginine-blocked NCS to compete with NCS. It is thus possible that guanidinium ions compete with the arginine residues in NCS for interaction at specific sites on DNA. Compatible with this formulation is our finding that arginine also blocks the NCS reaction but at higher concentrations (0.2 M for 50% inhibition). On the other hand, it is also possible that there is a direct interaction between the NCS protein and/or its chromophore with guanidine hydrochloride leading to the generation of an inactive structure. Similarly, the increase in acidity of NCS on blocking the arginine residues could be responsible for its change in activities, although the marked conformational changes noted on CD of this compound (T. S. A. Samy, L. S. Kappen, and I. H. Goldberg, unpublished experiments) likely explain this effect.

Another aspect of NCS–DNA interaction that emerges from this study is the finding that NCS, rendered inactive by various treatments, can compete with active NCS and block its activity. These experiments demonstrate that there are a limited number of DNA binding sites for which the two forms of NCS can compete and provide additional evidence for DNA as a target for the drug. Further, nonfunctional derivatives of NCS (e.g., arginine-blocked NCS), with or without heat treatment, will not block NCS action. In addition, the finding that other acidic proteins or inactivated auromycin and macromomycin do not compete with NCS further points to the specificity of the interaction of NCS with its substrate. This specificity appears not to reside in the chromophore since the chromophore-free protein is at least as inhibitory as inactivated NCS. In fact, it is of particular interest that several of the methods used to generate the inhibitory species of NCS (i.e., heating, incubation with 2-mercaptoethanol, treating with guanidine hydrochloride, exposure to long-wavelength ultraviolet light, methanol extraction, and Amberlite XAD chromatography) lead to loss of biological activity and chromophore release or removal from the protein (Napier et al., 1979; unpublished experiments). It is perhaps this form of the protein that competes for DNA binding. It is also of interest that these several different treatments produce a protein with a lower p*I*, as revealed by polyacrylamide gel isoelectric focusing. A more acidic form of NCS (p*I* 3.2) which is biologically inactive itself (Kikuchi et al., 1974; Beerman et al., 1977) but can block NCS action in vivo has been identified as a possible biosynthetic precursor of active drug and has been called pre-NCS (Kikuchi et al., 1974). Maeda & Kuromizu (1977) have proposed that this form of the drug, as well as that generated from native NCS by treatment at an acidic pH, differs from native NCS in having an aspartic acid instead of an asparagine in position 83 of the protein. We are currently studying the possible role of chromophore removal in generating the species with the lower p*I*. Finally, the ability of macromomycin to block the in vitro DNA scission activity of auromomycin is another instance where the chromophore-free protein competes with the active species for DNA interaction.

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