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Effect of Nonprotein Chromophore Removal on Neocarzinostatin Action†

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ABSTRACT: Evidence is presented for the possible biologic role of the nonprotein chromophore associated with the antitumor, protein antibiotic neocarzinostatin (NCS). Thus, irradiation of NCS at 360 nm, where the chromophore absorbs, is as effective in inactivating its ability to block the growth of *Escherichia coli* as irradiation in the region of protein (tryptophan and tyrosine) absorption. Further, nonprotein chromophore removal by chromatography on Amberlite XAD-7 results in chromophore-free and chromophore-poor fractions whose activities in inhibiting DNA synthesis and cell growth in HeLa cells or in inducing single-strand breaks in supercoiled pMB9 DNA correlated well with their chromophore contents, as measured by their UV–visible absorption and fluorescence spectra. The chromophore-free form of NCS blocks in a dose-dependent and specific manner the in vitro DNA strand scission activity of native NCS. Similarly, the chromophore-free form (macromomycin) of the antibiotic auromomycin inhibits DNA scission by the latter. Chromophore removal from NCS (p*I* 3.3) leads to formation of a protein with a more acidic p*I* (3.2) and a CD spectrum characteristic of a possible biosynthetic precursor form of NCS, referred to as pre-NCS. Inactive, chromophore-free forms of NCS (p*I* 3.2) have also been isolated from clinical NCS and from previously purified and stored NCS by rechromatography on CM-cellulose. Further, a protein-free fraction eluting at a pH of 3.8–4.0 has the spectral characteristics of the nonprotein chromophore. These data raise the possibility that pre-NCS is a chromophore-free (apoprotein) form of NCS.

Neocarzinostatin (NCS)[1] is an acidic antitumor, protein antibiotic whose primary 109 amino acid sequence has been determined (Meienhofer et al., 1972). Work from several laboratories indicates that cellular DNA is a target in the action of NCS and that DNA strand scission is a consequence of NCS action both in vivo and in vitro [reviewed in Goldberg et al. (1980)]. Evidence for the existence of an active, labile form of NCS that places single-strand breaks in linear and supercoiled DNA has been presented (Kappen & Goldberg, 1978a, 1979). It has only recently been appreciated that NCS

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[1] Abbreviations used: NCS, neocarzinostatin; Tris, tris(hydroxymethyl)aminomethane; CM-cellulose, carboxymethylcellulose (CM-23; Whatman).

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contains a nonprotein chromophore that can be separated from the protein (Napier et al., 1979). In this paper we present experiments designed to determine the possible role of the chromophore in NCS action.

Materials and Methods

Except where indicated, most experiments used the clinical form of NCS (Kayaku Antibiotics, lot no. 761165) stored frozen in 0.015 M sodium acetate buffer, pH 5.0, and generously provided by Dr. W. T. Bradner of Bristol Laboratories. NCS purified to homogeneity (Samy et al., 1977) 2 years before and stored as a solid in the deep freeze was the generous gift of Dr. T. S. A. Samy of the Sidney Farber Cancer Institute. Samples of purified auromomycin and macromomycin were provided by Dr. T. S. A. Samy. The assays for NCS-induced scission of linear λDNA and supercoiled pMB9 DNA and for macromomycin-induced scission of supercoiled pMB9 DNA were described previously (Kappen et al., 1979). DNA synthesis in HeLa cells was measured as reported (Kappen et al., 1979). HeLa cell growth was as described (Beerman & Goldberg, 1977).

NCS was chromatographed on nonionic Amberlite XAD-7 to remove the nonprotein chromophore. Amberlite XAD-7 (Rohm Haas) resin was thoroughly washed with H_2O, methanol, and acetone and again with H_2O before packing it in the column. Clinical NCS (5.4 mg) was applied to the column (0.8 × 10 cm) that was previously equilibrated with 8% $(NH_4)_2SO_4$. After sample application, the column was washed with 8% $(NH_4)_2SO_4$ until the washings were free of any UV-absorbing (A_{280}) material. Elution (1.2-mL fractions) was started with H_2O, followed by 30% ethanol. The protein peaks were dialyzed against 0.1 mM sodium acetate, pH 5.0, before use.

The ability of NCS and its light-inactivated derivatives to inhibit the growth of *Escherichia coli* TA 85rfa (lipopolysaccharide defective) was determined by measuring the A_{660} of cultures incubated for 4 h at 37 °C with and without added drug (Eisenstadt et al., 1979).

All manipulations and incubations involving the protein antibiotics were carried out in the dark.

Results

Inactivation of NCS by Monochromatic Light. It has been known that NCS is sensitive to ultraviolet light and sunlight (Kohno et al., 1974; Burger et al., 1978). Since NCS possesses considerable absorption at 360 nm, as well as at 280 nm, the region of tryptophan absorption, we irradiated the drug at both wavelenghts (20-nm slit width) and determined the residual antibacterial activity. As shown in Figure 1, when correction is made for the energy of the light source and the absorbance of the NCS at the two wavelenghts, NCS is inactivated equally well at 280 and 360 nm.

Chromophore Removal by Chromatography on Amberlite XAD-7. In order to determine the effect of chromophore removal on NCS activity, we sought a gentle method of chromophore removal that would leave the protein in as native a state as possible. For this purpose we employed chromatography of NCS on Amberlite XAD-7. We modified the method recently reported to convert the protein antibiotic auromomycin into its chromophore-lacking form, macromomycin (Yamashita et al., 1979), by eluting with distilled H_2O before elution with 30% ethanol (Figure 2). Two protein fractions were obtained, the one eluting with H_2O comprising 65% of the original protein and the one eluting with 30% ethanol comprising 24% of the original protein (total recovery of 89%). Both fractions had the amino acid composition of

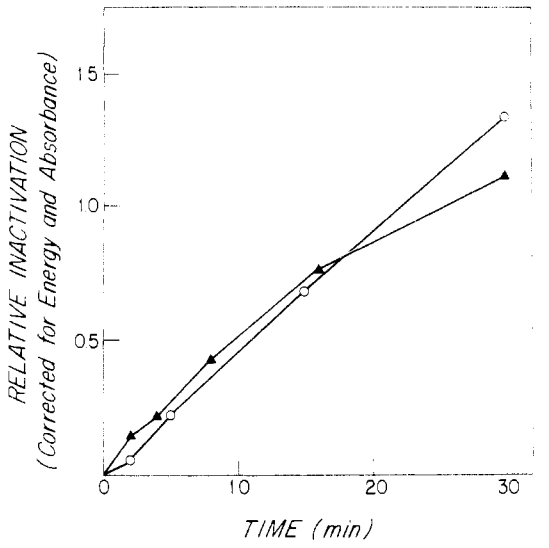

FIGURE 1: Light inactivation of NCS. NCS (30 μg/mL in 50 mM Tris, pH 8.0) was irradiated in quartz cuvettes at 280 (▲) and 360 nm (○) (20-nm slit width) in a Perkin-Elmer 512 spectrofluorometer. Inhibition of growth of *E. coli* TA 85rfa by 2 μg/mL drug measured as described under Materials and Methods was as follows: untreated NCS, 76%; NCS irradiated at 280 nm for 30 min, 15.2%; NCS irradiated at 360 nm for 30 min, 43.5%. Results are expressed as relative inactivation so as to normalize residual drug activity for the energy flux of the lamp (84 erg s^{-1} at 280 nm; 161 erg s^{-1} at 360 nm), as measured by potassium ferrioxalate actinometry (Hatchard & Parker, 1956), and for the relative absorbance at the respective irradiation wavelengths (A_{280}/A_{360} = 5.3).

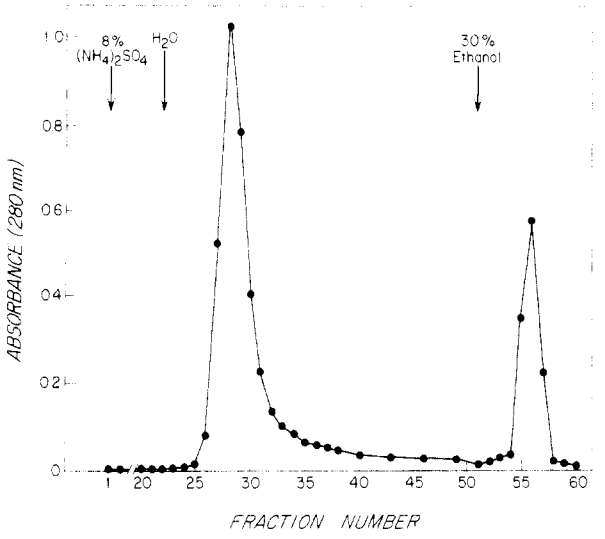

FIGURE 2: Amberlite XAD-7 chromatography of clinical NCS. NCS (5.4 mg) was applied to a column of Amberlite XAD-7, and chromatography was carried out as described under Materials and Methods. The H_2O-eluted protein and the 30% ethanol eluted protein constitute 65 and 24%, respectively, of the total protein applied.

NCS. The H_2O eluted fraction was essentially free of absorbance above 310 nm, while the 30% ethanol eluted material possessed a small portion of the absorbance above 310 nm of native NCS (Figure 3). The exact amount of material absorbing above 310 nm in the latter case was difficult to determine because of the presence of end absorption. Fluorescence spectra of the original NCS and the two column fractions are shown in Figure 4. When relative fluorescence of the three samples is compared, it should be noted that all three solutions contain the same A_{280} of 0.25, although the ϵ_{270} for the original NCS is taken as 23 000 and the ϵ_{277} for the chromophore-free material (based on methanol-extracted

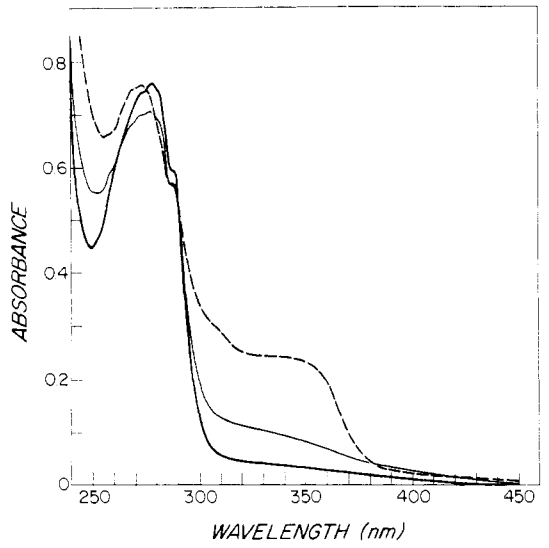

FIGURE 3: Ultraviolet–visible absorption spectra of proteins eluted from Amberlite XAD-7 chromatography of clinical NCS. H_2O-eluted protein (0.57 mg/mL in H_2O; ϵ 14000) (—); 30% ethanol eluted protein (0.55 mg/mL in H_2O; assuming ϵ 14000) (—); native NCS (0.34 mg/mL in 0.01 M sodium acetate, pH 5.0; ϵ 23000) (---). Spectra were recorded on a Cary 219 spectrophotometer.

protein) is taken as 14000 (Napier et al., 1979). Although the concentration of the H_2O-eluted material is the highest, it has the least fluorescence emitting at 420 and 490 nm but the highest tryptophan fluorescence, as previously described for the methanol-extracted protein (Napier et al., 1979).

Biological Activity of NCS after Chromophore Removal. The ability of the fractions from the Amberlite XAD-7 chromatography to inhibit DNA synthesis and cell growth in HeLa cells and to convert form I pMB9 DNA to form II in the presence of 2-mercaptoethanol was assayed. In each assay the H_2O-eluted fraction had less than 1% (0.1–0.2%) and the 30% ethanol fraction had ~3–4%, respectively, of the activity of native NCS. Similarly, the H_2O-eluted fraction was found to be nonmutagenic for *E. coli* and *Salmonella typhimurium* (M. Wolf, E. Eisenstadt, and I. H. Goldberg, unpublished results), in contrast to the holoprotein (Eisenstadt et al., 1979). Since inactivated forms of NCS can specifically block the interaction of native NCS with DNA in vitro (Kappen & Goldberg, 1979), we tested the ability of the H_2O-eluted protein from XAD-7 to inhibit DNA scission (Figure 5). Increasing concentrations of the chromophore-free protein inhibited this reaction in a dose-dependent manner. Similarly, macromomycin, the chromophore-free form of auromomycin, which does not cause DNA strand breaks in vitro (Kappen et al., 1979; Suzuki et al., 1979), blocked the in vitro auromomycin scission of pMB9 DNA (Figure 6), and this inhibitory action is specific for the protein of macromomycin (auromomycin). Further, we have also carried out Amberlite XAD-7 chromatography on "purified" macromomycin and have found that the material eluted by water has only ~10% of the DNA synthesis inhibitory activity of the starting material, raising the possibility that the in vivo activity attributed to macromomycin is due to contaminating auromomycin.

CD Spectrum of Chromophore-Free NCS. Chromophore-free NCS possesses no optical activity above 310 nm, in contrast to native NCS (Figure 7). Further, the negative peak at 255 nm found in native NCS is markedly diminished and the trough is shifted to 270 nm. These optical properties are very similar to those reported for an inactive form of NCS (Maeda & Kuromizu, 1977) that is possibly related to a biosynthetic precursor of NCS (pre-NCS) found in culture filtrates of *Streptomyces carzinostaticus* variant F-41 (Kikuchi et al., 1974).

Isoelectric Focusing on Polyacrylamide Gels of NCS Fractions. Both fractions from the XAD-7 chromatography electrofocused at a p*I* of ~3.2, similar to that of the minor component (30%) of the original clinical material (Figure 8) and the same as that of pre-NCS (Kikuchi et al., 1974; Beerman et al., 1977). The major component (70%) of the clinical NCS had a p*I* of ~3.3, in agreement with published values (Kikuchi et al., 1974; Beerman et al., 1977). A preparation of previously purified and homogeneous NCS (Samy et al., 1977) that was stored frozen in a solid form for 2 years contained about 60% of the form with the lower p*I*. Assay of the ability of the clinical and the stored, purified NCS to convert pMB9 form I DNA to form II revealed the former to be about twice as active as the latter, in good agreement with their relative contents of drug with a p*I* of 3.3.

Rechromatography of NCS on CM-cellulose. Since both the clinical and the stored, purified samples of NCS contained material with a p*I* of 3.2 in proportion to their in vitro biological activities and since chromophore-free NCS also has a p*I* of 3.2, it seemed possible that the former material might also be chromophore free. Accordingly, we rechromatographed both the clinical (Figure 9) and the stored, purified (Figure 10) NCS preparations on CM-cellulose using a very gradual pH gradient from 3.2 to 4.7. Both materials had been originally chromatographed on CM-cellulose in their initial purification (Kikuchi et al., 1974; Samy et al., 1977). As seen

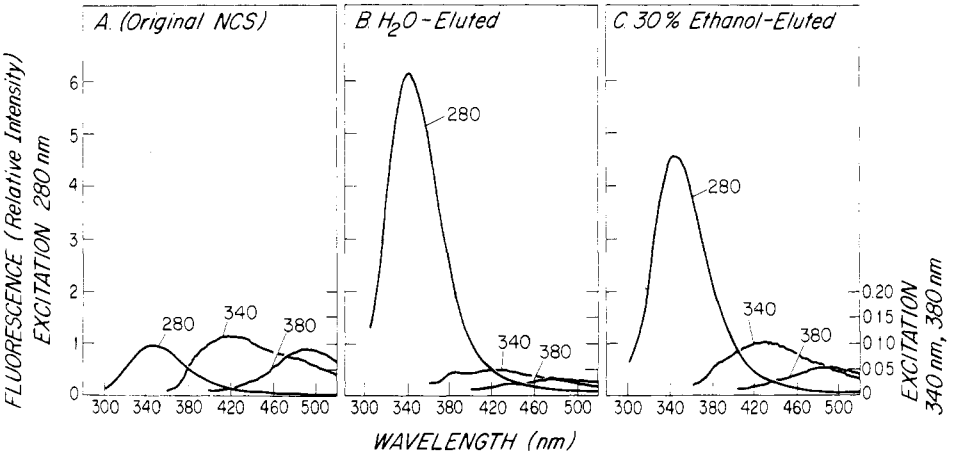

FIGURE 4: Fluorescence emission spectra of proteins eluted from Amberlite XAD-7 chromatography of clinical NCS. Uncorrected fluorescence spectra of aqueous solutions (A_{280} = 0.25) were recorded with a Perkin-Elmer 512 spectrofluorometer.

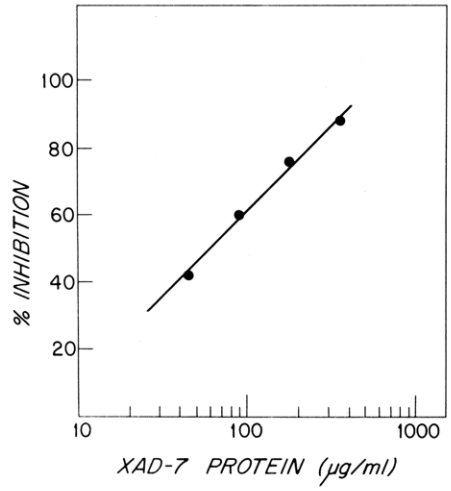

FIGURE 5: Inhibition of NCS activity by H_2O-eluted protein from Amberlite XAD-7 chromatography of clinical NCS. Reaction mixtures (100 μL) contained 100 mM Tris, pH 8.0, 10 mM 2-mercaptoethanol, 0.4 μg of λDNA (1.8×10^4 cpm), 95 μg/mL active NCS, and varying amounts of protein obtained by Amberlite XAD-7 chromatography of NCS. After 30 min of incubation at 37 °C, the amount of trichloroacetic acid solubilized radioactivity was determined. With NCS alone, 863 cpm was made acid soluble.

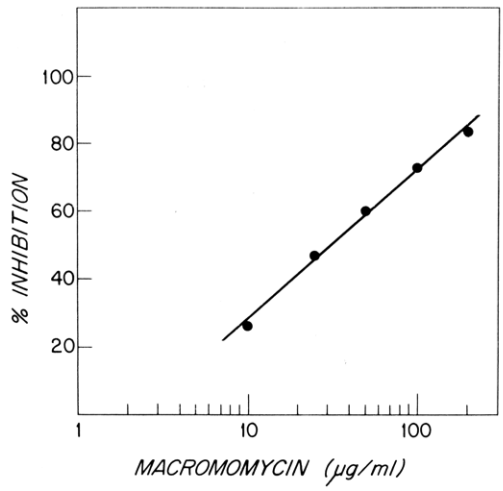

FIGURE 6: Inhibition of auromomycin scission of pMB9 DNA by macromomycin. Incubations (100 μL) contained 100 mM Tris, pH 8.0, 0.38 μg of superhelical DNA (1×10^4 cpm), and varying amounts of macromomycin at a constant level of auromomycin (10 μg/mL). Incubation was for 20 min at 37 °C. The reaction mixture was then analyzed on 5–20% alkaline sucrose gradients. The radioactivity present in the peaks of the supercoiled (form I) and the nicked forms of DNA was quantitated. 68% of form I DNA was nicked by auromomycin.

in Figure 9, when a dialyzed sample of clinical NCS was chromatographed on CM-cellulose, there eluted at a pH of 3.4 (0.1 M sodium acetate buffer) a fraction absorbing at 280 nm but differing from the main peak eluting at a pH of 3.6 by lacking absorption at 340 nm. Further, the early eluting fraction also possessed a much higher tryptophan fluorescence (280-nm excitation; 340-nm emission), as noted previously for the chromophore-lacking protein (Napier et al., 1979). A small amount of material with both 280- and 340-nm absorption was found to elute at a pH of 3.8–4.0. The first two peaks possessed amino acid compositions identical with that of native NCS, and their UV–visible absorption spectra are shown in Figure 11, confirming that the material eluting at a pH of 3.4 was essentially free of chromophore absorbing above 310 nm. Similar chromatography of the stored, purified NCS revealed a larger proportion of the chromophore-free

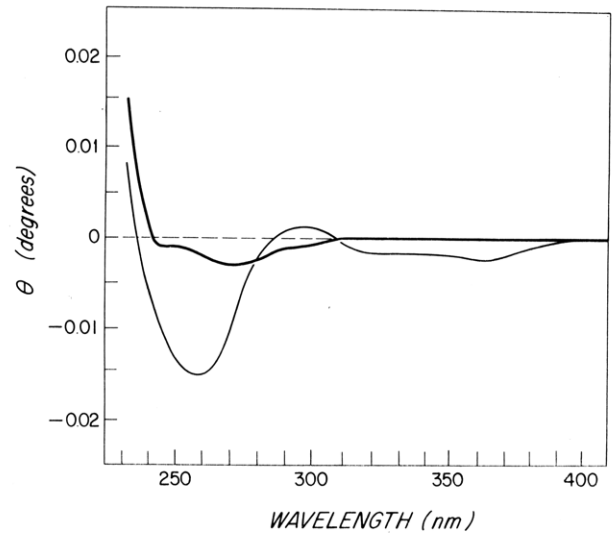

FIGURE 7: Circular dichroic spectra of H_2O-eluted NCS protein (0.434 mg/mL in 0.01 M NaOAc, pH 5.0) from Amberlite XAD-7 (—) and of clinical NCS (0.434 mg/mL in 0.01 M NaOAc, pH 5.0) (—). Spectra were recorded on a Cary 61 circular dichroism instrument in a 0.3-cm path length cell. θ represents ellipticity.

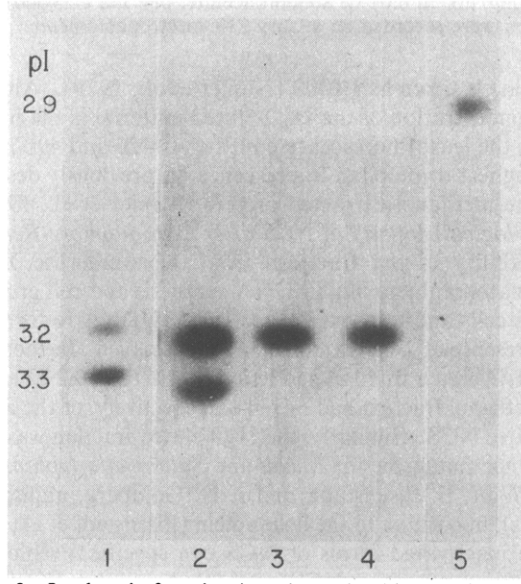

FIGURE 8: Isoelectric focusing in polyacrylamide gel of NCS and fractions from Amberlite XAD-7 chromatography of NCS. Polyacrylamide gel (5%) containing 1% ampholine, pH 2.5–4, was prepared as per specifications given in the LKB manual. The gel was prefocused at 10 W for 45 min before application of the sample. Electrofocusing was at 2 °C in the dark for 4 h at 25 W. In order to determine the pH gradient of the gel at the end of the experiment, we made 0.5-cm wide slices from strips (1 cm) cut out from either end of the gel. The gel slices were homogenized in 1 mL of distilled H_2O, and the pH of the supernatant was measured. The p*I* values of the Coomassie Blue stained protein bands are indicated on the left of the figure. The samples and the approximate amounts (micrograms) of material spotted are as follows: (1) clinical NCS (12); (2) stored, purified NCS (40); (3) H_2O-eluted fraction (12); (4) 30% ethanol eluted fraction (12); (5) pepsin (48).

material (Figures 10 and 12). In addition, material absorbing at both 280 and 340 nm but lacking significant tryptophan fluorescence (and free of protein as determined by amino acid analysis) eluted at a pH of 3.8–4.0. There was much more of this nonprotein material in the stored, purified sample than in the clinical NCS in large part because the latter had been thoroughly dialyzed before chromatography. The UV–visible absorption spectrum of this material resembled that of the nonprotein chromophore extracted from NCS by methanol (Napier et al., 1979). Assay of the various fractions for DNA

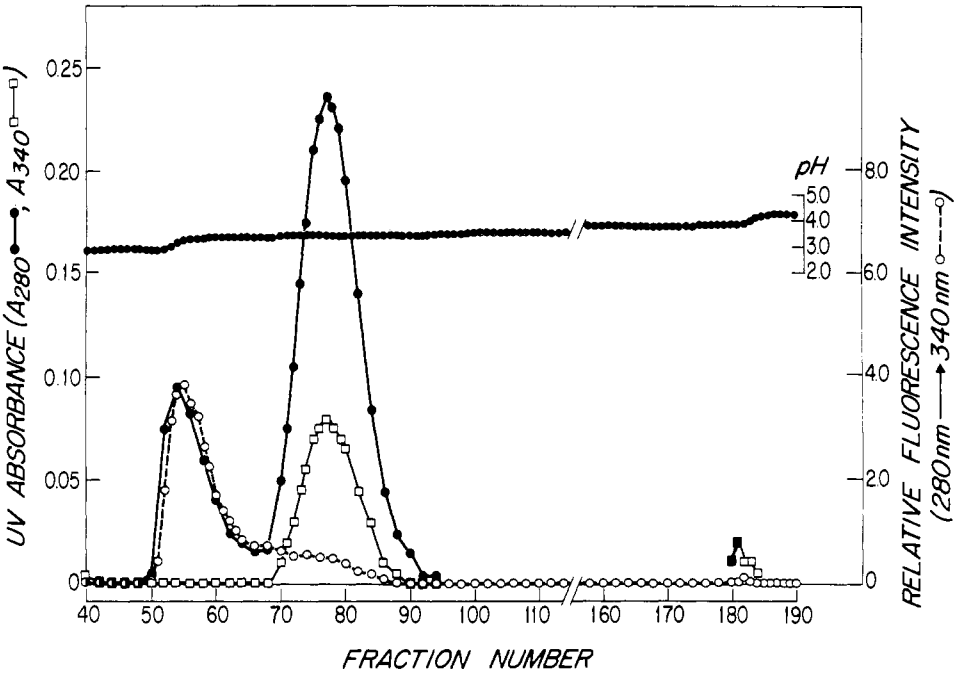

FIGURE 9: Chromatography of clinical NCS (3.0 mg) on a CM-cellulose column (1.5 × 75 cm). The sample was first dialyzed against water, lyophilized, and redissolved in 0.1 N acetic acid. After sample addition, the column was washed with 0.05 M acetic acid and eluted with a pH gradient of 0.1 M sodium acetate, pH 3.2–3.7 (500 mL) and pH 3.7–4.7 (500 mL). Fractions (5 mL) were collected at 43 mL/h. Chromatography was at 4 °C in the dark. Absorbance at 280 (●) and 340 nm (□); fluorescence emission at 340 nm (excitation 280 nm) (○).

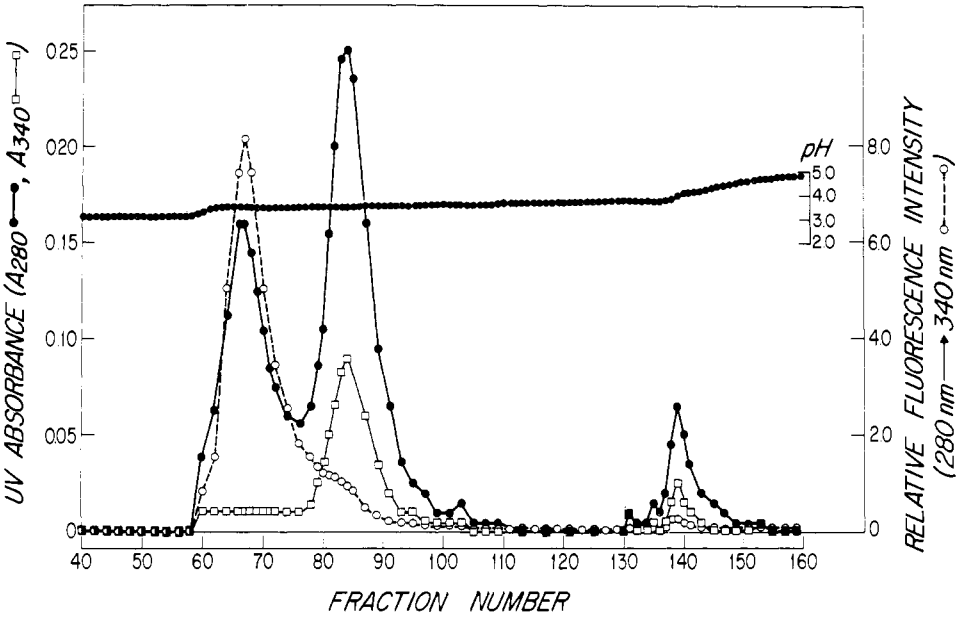

FIGURE 10: Chromatography of stored, purified NCS (3.7 mg) on a CM-cellulose column (1.5 × 75 cm). The column was washed with 0.05 M acetic acid after sample addition and eluted with a pH gradient of 0.1 M sodium acetate, pH 3.2–3.7 (400 mL) and pH 3.7–4.7 (200 mL). Fractions (5 mL) were collected at 43 mL/h. Chromatography was at 4 °C in the dark. Absorbance at 280 (●) and 340 nm (□); fluorescence emission at 340 nm (excitation 280 nm) (○).

synthesis inhibition in HeLa cells showed that only the material eluting at pH 3.6 and having absorption at 340 nm was active.

Discussion

Considerable evidence has accumulated implicating DNA as the main target and DNA single-strand breakage as the primary event in the cytotoxic action of NCS [reviewed in Goldberg et al. (1980)]. Further, the requirement for a reducing compound (Beerman & Goldberg, 1974; Beerman et al., 1977; Kappen & Goldberg, 1978a) and oxygen (Kappen & Goldberg, 1978a; Burger et al., 1978) and inhibition of the DNA single-strand scission reaction in vitro by α-tocopherol (Kappen & Goldberg, 1978a) suggest that an oxidative reaction, possibly involving free radicals, is involved in the DNA damage. In addition, evidence has been presented that NCS does not cause simple splitting of phosphodiester bonds (Kappen & Goldberg, 1978b) but induces direct damage to the deoxyribose of thymidylic acid (and to a lesser degree deoxyadenylic acid) residues in DNA, resulting in sugar cleavage and sugar fragment formation and base release (Poon et al., 1977; Hatayama et al., 1978; d'Andrea & Haseltine, 1978; Goldberg et al., 1980). Such results and our finding that NCS can be inactivated by irradiation at 360 nm are difficult to rationalize on the basis of the action of a simple

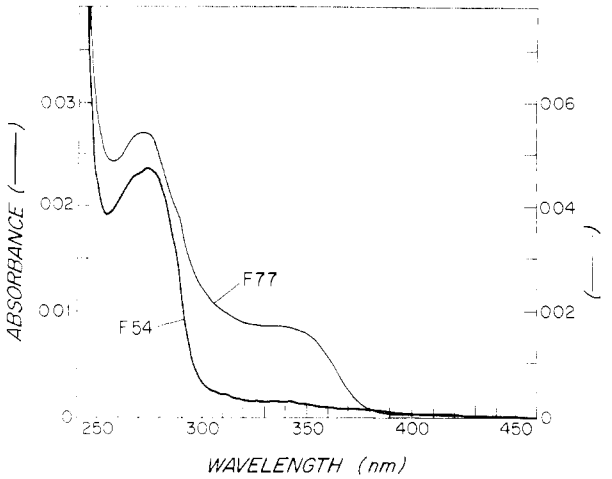

FIGURE 11: Ultraviolet–visible absorption spectra of fractions F54 (—) and F77 (—) eluted from CM-cellulose chromatography of clinical NCS (Figure 9). Spectra were recorded on a Cary 219 spectrophotometer.

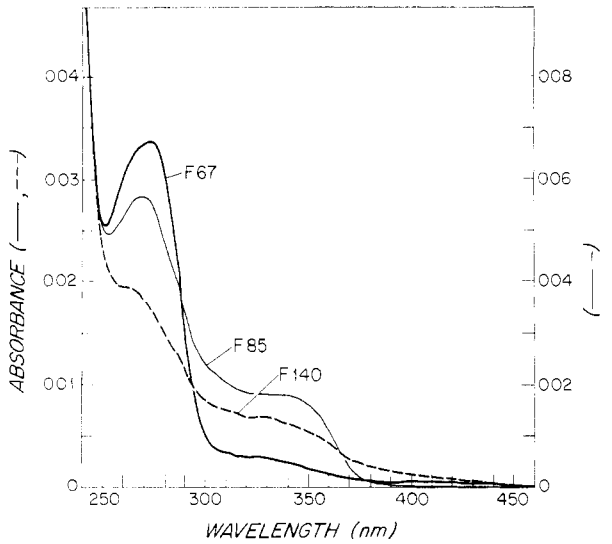

FIGURE 12: Ultraviolet–visible absorption spectra of fractions F67 (—), F85 (—), and F140 (- - -) eluted from CM-cellulose chromatography of stored, purified NCS (Figure 10). Spectra were recorded on a Cary 219 spectrophotometer.

protein molecule. The discovery of the existence of a nonprotein chromophore in association with the single-chain polypeptide has raised the possibility that it is the chromophore that is directly involved in these reactions. Efforts are under way to purify and characterize the chromophore. Until this is done and attempts at reconstitution of full activity by combining the protein and its chromophore are successful, we shall not be certain what role the chromophore plays in NCS action. The results presented in this paper, however, although indirect, suggest that the chromophore is required for both the cytotoxic and in vitro DNA damaging activities of NCS and agree with earlier results that the two types of activities go hand in hand (Beerman & Goldberg, 1977; Beerman et al., 1977; Samy et al., 1980). Thus, biological activity, whether measured as ability to inhibit DNA synthesis or cell growth in HeLa cells or to place breaks in superhelical DNA, correlated well with the presence of chromophore in NCS. This was true both for drug from which chromophore was removed by chromatography on Amberlite XAD-7 and for previously purified drug which presumably was partially inactivated and converted to a chromophore-free form on purification and storage. We assume that no other chemical modification of the NCS protein accompanied chromophore loss; at least the amino acid composition remains the same. Deamidation of a single asparagine in NCS, however, would not have been detected. Finally, consistent with these results is our recent finding that tryptic removal of the first 20 amino acids from the NH_2 terminus of NCS results in an 89 amino acid fragment that retains full biological activity (in vitro and in vivo) and contains the nonprotein chromophore (Samy et al., 1980).

Since the chromophore-lacking form of NCS has the same p*I* and CD spectrum as reported for pre-NCS, the possibility arises that pre-NCS is a chromophore-free form of the drug. Further, since very different treatments (Napier et al., 1979; Goldberg et al., 1980) of NCS, such as Amberlite XAD-7 chromatography, methanol extraction, or treatment with guanidine hydrochloride, 2-mercaptoethanol, 0.1 N NaOH, heat, or long-wavelength UV light, all lead to chromophore release or destruction and generate a protein with a p*I* of 3.2 that interferes with the interaction of native NCS (or, more specifically, of its chromophore) with DNA (Kappen & Goldberg, 1979), it appears that it is the chromophore-lacking form of NCS that competes in vitro, as does pre-NCS in vivo (Kikuchi et al., 1974). In preliminary experiments we have also found that preincubation of HeLa cells with high levels of H_2O-eluted, chromophore-free protein from Amberlite XAD-7 chromatography partially blocks the inhibition of DNA synthesis caused by native drug. Further, the ability of macromomycin to block the in vitro DNA scission activity of auromomycin is another example where the chromophore-free protein competes with DNA for interaction with the active form (probably chromophore) of the drug. On the basis of its identical amino acid composition and its biosynthetic appearance before that of active NCS and its disappearance coinciding with the early synthesis of NCS, pre-NCS is possibly a biosynthetic precursor of NCS (Kikuchi et al., 1974; Maeda & Kuromizu, 1977). Maeda & Kuromizu (1977) have proposed that pre-NCS found in culture filtrates, as well as the material 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. In order to explain the apparent precursor–product relationship between pre-NCS and NCS, it was suggested that amidation of aspartic acid-83 may take place after ribosomal protein synthesis and release of the precursor form (Maeda & Kuromizu, 1977). This would be highly unusual for protein biosynthesis since asparagine has its own triplet codon. Our data, rather, raise the possibility that active antibiotic is generated by the addition of the chromophore to the apoprotein precursor. It should be noted, however, that we do not have information as to whether or not chromophore removal was also associated with a minor chemical change in the protein, such as deamidation. Given the diversity of procedures that lead to chromophore removal and formation of a protein with a p*I* of 3.2, it seems unlikely that deamidation was a common feature in all cases. In this regard, it is of particular interest that both original preparations of NCS, in particular the stored, purified material, contain free chromophore which elutes on CM-cellulose chromatography at pH 3.8–4.0. Since the chromophore is less negatively charged than the chromophore-free protein (p*I* 3.2) or NCS (p*I* 3.3), it is conceivable that it is the combination of the chromophore with the apoprotein that determines the characteristic intermediate p*I* of native NCS.

Finally, the finding that the apoprotein of NCS blocks the in vitro effect of active drug (Kappen & Goldberg, 1979; this paper) suggests the possibility that it competes with target

DNA for the active component, the nonprotein chromophore. A similar explanation may exist for the observation that pre-NCS antagonizes NCS action in vivo (Kikuchi et al., 1974), although it is also possible that cellular uptake of released chromophore is interfered with by its binding to added apoprotein (pre-NCS). In any case, it is clear that the inhibitory phenomenon is highly specific for the apoprotein of NCS. Thus, only chromophore-free and/or inactivated forms of NCS block the action of NCS; other proteins and other protein antibiotics, such as macromomycin, auromomycin, and actinoxanthin, do not interfere with the in vitro action of native NCS. Similarly, only macromomycin or inactivated auromomycin blocks the DNA strand scission activity of auromomycin. Taken together, the data suggest that the antibiotic protein acts as a carrier for the nonprotein chromophore and by controlling its release determines its availability for interaction with the DNA to produce DNA sugar damage.

Added in Proof

Recently we have found that the extracted nonprotein chromophore possesses the cytotoxic and DNA strand scission activities of NCS and that the apoprotein stabilizes the chromophore and controls its release (Kappen et al., 1980).

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