Makita, A., & Shimojo, H. (1973) *Biochim. Biophys. Acta 304*, 571–574.
Muto, M., Yoshimura, M., Okayama, M., & Kaji, A. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4173–4177.
Roblin, R., Albert, S. O., Gelb, N. A., & Black, P. H. (1975) *Biochemistry 14*, 347–357.
Russell, W. C., Newman, C., & Williamson, D. H. (1975) *Nature (London) 253*, 461–462.
Saito, H., & Uzman, B. G. (1971) *Biochem. Biophys. Res. Commun. 43*, 723–728.
Saito, H., Yamagata, T., & Suzuki, S. (1968) *J. Biol. Chem. 243*, 1536–1542.
Satoh, C., Duff, R., Rapp, F., & Davidson, E. A. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 54–56.
Satoh, C., Banks, J., Horst, P., Kreider, J., & Davidson, E. A. (1974) *Biochemistry 13*, 1233–1241.
Takeuchi, J., Sobue, M., Shamoto, M., Yoshida, M., Sato, E., & Leighton, J. (1977) *Cancer Res. 37*, 1507–1512.
Temin, H. M. (1965) *J. Natl. Cancer Inst. (U.S.) 35*, 679–693.
Terry, A. H., & Culp, L. A. (1974) *Biochemistry 13*, 414–425.
Turley, E. A. & Roth, S. (1980) *Nature (London) 283*, 268–271.
Umemoto, J., Bhavanandan, V. P., & Davidson, E. A. (1977) *J. Biol. Chem., 252*, 8609–8614.
Vannucchi, S., Rosso, M. D., Cella, C. Urbano, P., & Chiarugi, V. (1978) *Biochem. J. 170*, 185–187.
Winterbourne, D. J. & Mora, P. T. (1978) *J. Biol. Chem. 253*, 5109–5120.

Neocarzinostatin Chromophore: Purification of the Major Active Form and Characterization of Its Spectral and Biological Properties†

Mary A. Napier, Barton Holmquist, Daniel J. Strydom, and Irving H. Goldberg*

ABSTRACT: The nonprotein chromophore of neocarzinostatin (NCS-Chrom), which possesses the full biological activity of native NCS, can be separated by high-performance liquid chromatography into two components, A and B, with 90% and 10%, respectively, of the total UV-absorption and in vitro DNA scission activity. Each component possesses in vitro DNA scission activity equal to NCS, as does a third component, C, which is present only when NCS is extracted with nonacidic methanol and appears to be derived from A. NCS-Chrom A possesses in vivo activity equal to NCS; however, B and C have about 5% and 80%, respectively, of the activity of NCS for inhibition of HeLa cell growth. NCS-Chrom A possesses characteristic absorption, fluorescence, circular dichroism (CD), and magnetic circular dichroism (MCD) spectra. The spectral properties of B and C are similar to those of A. These spectral properties cannot be attributed solely to the presence of the known naphthoate component of NCS but are due to the presence of an additional conjugated component (as yet unidentified) in the chromophore. NCS-Chrom D, an inactive minor component of NCS-Chrom preparations, can be generated by hydrolysis of each of the three active components at pH 8. It possesses a very different CD spectrum and, in contrast to NCS-Chrom, possesses no MCD, indicating a major change in or loss of the naphthoic acid residue. NCS-Chrom D is the 490-nm fluorescent product generated when NCS-Chrom spontaneously looses biological activity in aqueoous pH 8 buffers. It is the source of the 490-nm fluorescence in clinical NCS. The optical activity of the biologically active NCS-Chrom accounts for the Cotton effects previously observed for native NCS. Changes in both the absorption and circular dichroism spectra of NCS-Chrom A (and B and C) occur on binding to apo-NCS, generating spectra identical with those of native NCS and providing stoichiometry of the association process. Complex formation between NCS-Chrom and DNA is also detected by CD, with the maximal effect being observed at low molar ratios of NCS-Chrom to DNA. Furthermore, hypochromicity between 290 and 330 nm and a bathochromic shift centered near 365 nm occur upon binding of NCS-Chrom to apo-NCS and to DNA, raising the possibility that similar modifications in the electronic structure of the NCS-Chrom result from both interactions.

Neocarzinostatin (NCS),[1] an antitumor antibiotic, causes single-strand breaks in linear duplex or superhelical DNA in vitro in an oxygen-dependent reaction which is greatly stimulated by mercaptans. NCS also causes DNA strand scission in vivo, and considerable evidence exists indicating that DNA damage is the primary result of its activity in vivo [reviewed in Goldberg et al. (1981)].

We have shown that NCS contains a methanol-soluble, nonprotein chromophoric component (Napier et al., 1979) in addition to the sequenced acidic protein (M_r 10700) (Meienhofer et al., 1972). The isolated chromophore, possessing the full biological activity of NCS (Kappen et al., 1980a; and data reported here), exhibits a characteristic absorption spectrum between 300 and 400 nm and an intense blue

† From the Department of Pharmacology, Harvard Medical School (M.A.N. and I.H.G.), and the Center for Biochemical and Biophysical Sciences and Medicine, Harvard Medical School and Brigham and Women's Hospital (B.H. and D.J.S.), Boston, Massachusetts 02115. *Received February 26, 1981.* This work was supported by U.S. Public Health Service Research Grants GM 12573 (I.H.G. and M.A.N.) and GM 15003 (B.H. and D.J.S.) from the National Institutes of Health. M.A.N. is partially supported by a fellowship from the Interdisciplinary Programs in Health, Harvard School of Public Health, funded by the Andrew W. Mellon Foundation.

[1] Abbreviations used: NCS, neocarzinostatin; NCS-Chrom, nonprotein chromophoric component extracted from NCS; apo-NCS, protein component of NCS; HPLC, high-performance liquid chromatography; MCD, magnetic circular dichroism; CD, circular dichroism.

0006-2960/81/0420-5602$01.25/0

fluorescence emission at 420 nm when excited at 340 nm. The isolated chromophore is chemically labile but is stable at pH ≤4 (Kappen et al., 1980a; Albers-Schönberg et al., 1980; Kappen & Goldberg, 1980), facilitating the purification by reverse-phase HPLC (Kappen et al., 1980a; Napier et al., 1980a). The presence of this extractable, active chromophore in NCS has recently been confirmed by others (Ohtsuki & Ishida, 1980; Suzuki et al., 1980). The NCS chromophore (NCS-Chrom), with an elemental composition $C_{35}H_{35}NO_{12}$ (M_r 661), contains 2,6-dideoxy-2-(methylamino)galactose and 2-hydroxy-5-methoxy-7-methylnaphthoate covalently linked to a highly unsaturated $C_{15}H_{10}O_4$ unit (Albers-Schönberg et al., 1980) which possesses a five-membered cyclic carbonate ring (1,3-dioxolan-2-one) (Napier et al., 1981). Derivatives of the naphthoic acid have been isolated (Edo et al., 1980). The chromophore-free protein (apo-NCS) possesses none of the biological activities of NCS (Kappen et al., 1980a; Napier et al., 1980b; Ohtsuki & Ishida, 1980; Suzuki et al., 1980) but stabilizes the labile chromophore and appears to control its release for interaction with its target DNA (Kappen et al., 1980a; Napier et al., 1980b; Kappen & Goldberg, 1980; Povirk & Goldberg, 1980).

In our ongoing study of the mechanism of NCS action, we have now purified the active chromophore, characterized a number of its spectral properties, and employed these properties to study the association of the chromophore both to apo-NCS, regenerating native NCS, and to DNA.

Materials and Methods

NCS (clinical form) was stored frozen in 0.015 M sodium acetate buffer, pH 5. Sonicated calf thymus DNA (150 base pairs long) and [*methyl*-^{3}H]thymine–DNA (0.24 mM, 2.0×10^4 cpm/mmol) from Chinese hamster ovary (CHO) cells (^{3}H-labeled CHO DNA) (Povirk & Goldberg, 1980) were a generous gift of L. F. Povirk. All chemicals were of reagent grade. Absorption spectra were recorded on a Cary 118 or 219 spectrophotometer, and uncorrected fluorescence spectra were obtained on a Perkin-Elmer 512 or MPF-3 spectrofluorometer. All spectra were recorded at 4 °C and with a path length of 1 cm unless otherwise noted. MCD spectra were measured at ambient temperature on a Cary Model 61 CD instrument equipped with a Varian Model V4145 superconducting solenoid at a field strength of 40 kG. The slit width was adjusted to permit spectral dispersion of 2 nm or less. CD spectra were measured under identical experimental conditions as those for MCD but in the absence of the magnetic field. Metal analyses were performed on a Perkin-Elmer Model 5000 by standard methods. HPLC was performed with a Waters Associates Model A2C/GPC-204 chromatograph equipped with a Model 660 solvent-flow programmer, a μBondapak C_{18} column (3.9 mm × 30 cm), a 254-nm absorption detector, and a Schoeffel Model SF 970 fluorescence detector (340-nm excitation with a 418-nm emission cutoff filter). Care was taken to avoid exposure to light for all manipulations prior to spectral measurements.

Preparation of NCS-Chrom and Apo-NCS. NCS-Chrom was extracted from lyophilized clinical NCS with 0.1 M acetic acid in methanol at 4 °C (Albers-Schönberg et al., 1980), leaving a virtually chromophore-free protein. The extracted chromophore and the residual protein were stored at −20 °C. The concentrations of NCS-Chrom and apo-NCS were calculated on the basis of the absorbance, using $\epsilon_{340nm} = 8000$ and $\epsilon_{278nm} = 14000$, respectively, as determined for native NCS (Napier et al., 1979).

Chromatography of NCS-Chrom. The methanol-soluble NCS-Chrom was injected onto the HPLC column in a small volume (50–100 μL) and eluted at ambient temperature into cold amber tubes in dim light with a concave gradient of increasing percent solvent B (methanol containing 0.5% water and 0.01 M ammonium acetate, pH 4) in solvent A (20% aqueous methanol containing 0.01 M ammonium acetate, pH 4). Fractions were stored at −20 °C in the dark in the elution buffer. No change was observed in the number or the relative amounts of components by flushing the elution buffers with argon or by cooling the solvents and column to 4 °C.

Bioactivity Assays. Inhibition of cell growth, of in vivo DNA synthesis, and of DNA strand scission in HeLa cells was measured as reported (Beerman & Goldberg, 1977). The assays for drug-induced scission of [*methyl*-^{3}H]thymine-labeled supercoiled pMB9 DNA have been published (Beerman et al., 1977; Kappen et al., 1980a). Growth inhibition of *Escherichia coli* TA85rfa by the drug has been described (Napier et al., 1980b). NCS-Chrom-induced release of [^{3}H]thymine from ^{3}H-labeled CHO DNA in vitro was determined as reported (Povirk & Goldberg, 1980).

Metal Analysis. NCS, dialyzed against double-distilled H_2O and lyophilized, was extracted with 50% acetic acid in methanol, and the protein was removed by centrifugation. The supernatant containing NCS-Chrom (0.15 μM) was analyzed by atomic absorption spectroscopy. The metals analyzed for and the amounts (micromole) found per micromole of NCS-Chrom are as follows: Mg, 0.035; Ca, 0.108; V, 0; Cr, 0.02×10^{-3}; Mn, 0.006; Fe, 0; Co, 0.5×10^{-3}; Ni, 0.002; Cu, 0.002; Zn, 0.006; Cd, 0; Pb, 0. These data indicate the absence of stoichiometric amounts of metals associated with NCS-Chrom, consistent with the evidence that various metal chelators do not inhibit the in vitro activity of NCS or NCS-Chrom (Kappen & Goldberg, 1978; Kappen et al., 1980a).

Results

Purification of NCS-Chrom by HPLC. Reverse-phase HPLC on μBondapak C_{18} of NCS-Chrom extracted from NCS with 0.1 M acetic acid in methanol with elution by a gradient of 56–84% methanol in aqueous 0.01 M ammonium acetate, pH 4, results in a clear separation of two active UV-absorbing components, the major peak A (NCS-Chrom A) and the minor peak B (Figure 1). Similar results are obtained by extraction with either 0.1 M hydrochloric acid in methanol, 50% acetic acid in methanol, or glacial acetic acid (Albers-Schönberg et al., 1980). The ratio of both absorption and in vitro DNA scission activity (assayed in a 30-min incubation at 37 °C) of fraction A to fraction B is 9 to 1. Partially solubilized protein and minor chromophoric components including peak D (see below and Figure 2) elute in the first 5 min and are well separated from the biologically active fractions. No biological activity is present in fractions 1–20.

The total recovery of the injected in vitro DNA scission activity and absorption in A and B is greater than 95%. NCS-Chrom A possesses activity at least equivalent to that of native NCS both in vitro, as measured by the mercaptan-dependent generation of single-strand breaks in pMB9 DNA and the release of [^{3}H]thymine from ^{3}H-labeled CHO DNA, and in vivo, as assayed by the inhibition of cell growth, the inhibition of DNA synthesis and the induction of DNA strand scission in HeLa cells and by the inhibition of growth of the lipopolysaccharide-defective mutant of *Escherichia coli* (TA85rfa). NCS-Chrom is no more effective than NCS for inhibition of growth of TA85, the nondefective parent strain of TA85rfa, requiring in each case 100-fold more drug in the parent strain. NCS-Chrom B possesses in vitro DNA scission activity equal to A but only about 5% or less of the in vivo activity of NCS for inhibition of cell growth and DNA syn-

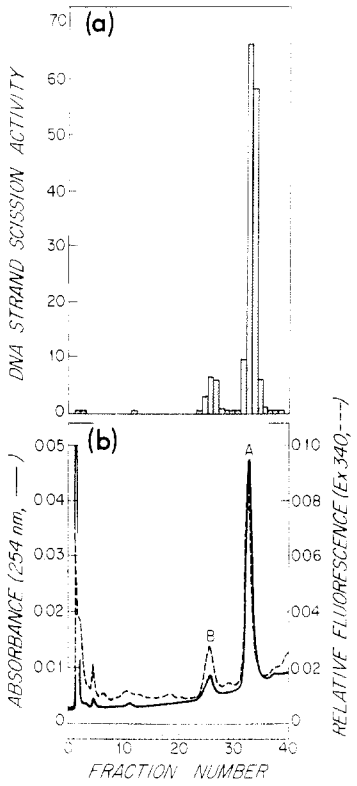

FIGURE 1: HPLC of NCS-Chrom extracted from lyophilized NCS with 0.1 M acetic acid in methanol at 4 °C and distribution of in vitro pMB9 DNA scission activity. Extracted NCS-Chrom (50 μL) (1.4 mg equiv of NCS per mL) was injected, and 1-min fractions were collected. A concave gradient of 56–84% methanol in 0.01 M ammonium acetate, pH 4, for 50 min at a rate of 2 mL/min was employed for elution. (b) HPLC elution profile. (a) Relative in vitro DNA strand scission activity (incubation at 37 °C for 30 min) calculated as dilution factor times n, where n = number of breaks in pMB9 DNA calculated from n = log [(percent form I DNA without drug)(percent form I DNA with drug)]. Total recovery of the 254-nm absorbing material and in vitro DNA scission activity was greater than 95%. The absorption eluting at the solvent front is due to acetic acid, methanol, and trace-solubilized protein present in the injected sample.

Table I: Relative Distribution of 254-nm Absorbance among the HPLC Components of NCS-Chrom with in Vitro DNA Scission Activity from Different Extraction Procedures[a]

extraction solvent	% of total A_{254} in active peaks		
	A	B	C
0.1 M acetic acid in methanol	89	11	0
0.1 M HCl in methanol	87	13	0
100% methanol, 2 h after extraction	70	10	20
100% methanol, stored 3 months at −70 °C	14	14	72
native NCS in 0.015 M sodium acetate, pH 5[b]	85	15	0

[a] NCS-Chrom extracted by the indicated solvents at 4 °C from lyophilized clinical NCS and separated by HPLC with the conditions described under Materials and Methods. [b] Clinical NCS in 0.015 M sodium acetate, pH 5, chromatographed directly without prior extraction of NCS-Chrom.

thesis in HeLa cells and for growth inhibition in *Escherichia coli* TA85rfa. No evidence of NCS-Chrom A was present in samples of B at a level of <1% B by HPLC.

An additional active component can be isolated by extraction of NCS with 100% methanol. Chromatography of this methanol extract (Figure 2a) results in the separation of a third active component, peak C, in addition to peaks A and B (Kappen et al., 1980a; Napier et al., 1980a). Peak C, originally reported as a shoulder of peak A (Kappen et al., 1980a), is separated by use of a more shallow gradient than

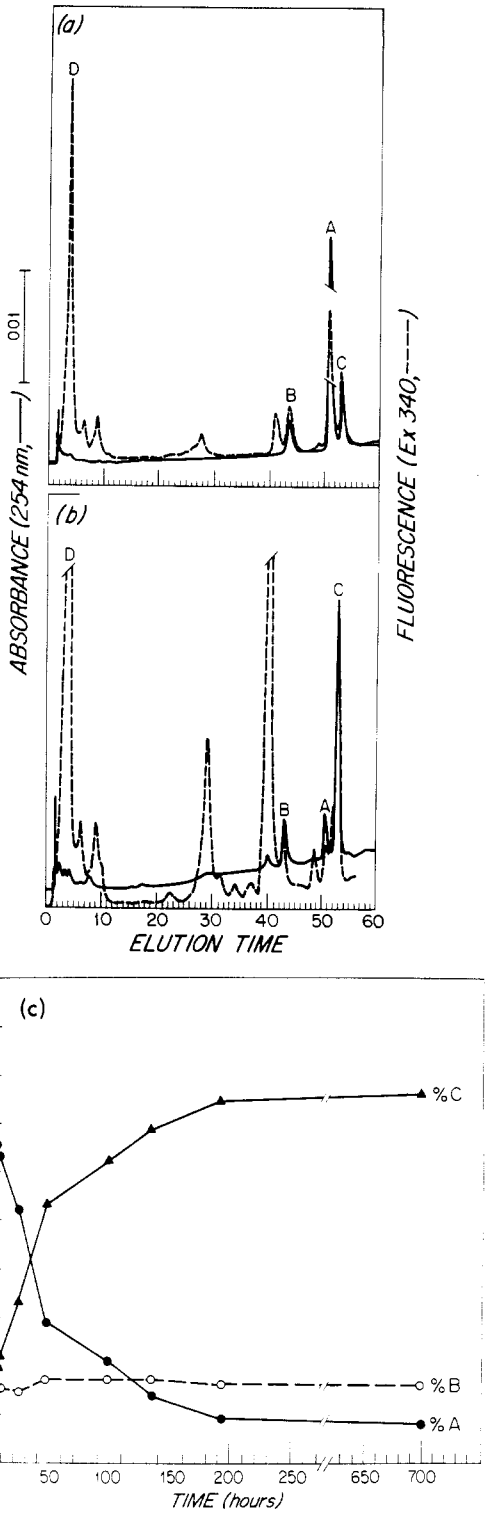

FIGURE 2: HPLC of NCS-Chrom extracted from lyophilized NCS with methanol at 4 °C (a) 2 h after extraction and (b) after 3 months stored at −70 °C in the dark. A profile equivalent to (b) was observed after 5 days at −20 °C. Chromatographic conditions were identical with those in Figure 1, except that the gradient of 56–84% methanol in aqueous 0.01 M ammonium acetate, pH 4, was extended to 70 min. Total recovery of the 254-nm absorbing material and in vitro DNA scission activity was greater than 95%. The components A and B correspond to the identical peaks in Figure 1. (c) Change in the percent of the total drug active for in vitro DNA strand scission present as NCS-Chrom A (percent A), NCS-Chrom B (percent B), and NCS-Chrom C (percent C) when stored in methanol at −20 °C in the dark. An aliquot of methanol-extracted chromophore was separated by HPLC, and the percent of total active drug (calculated from the peak area of the A_{254} profile) eluting as peaks A, B, and C was determined.

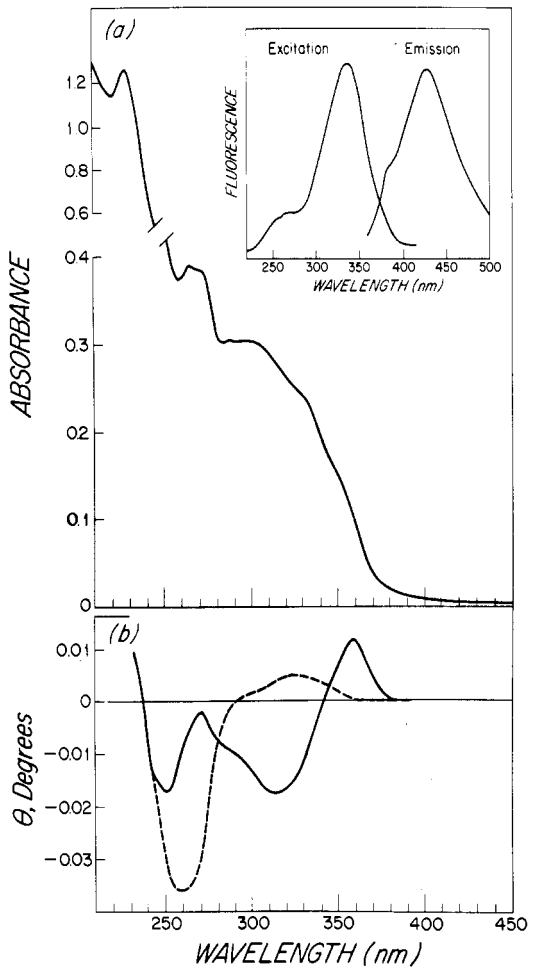

FIGURE 3: Absorption, fluorescence, MCD, and CD spectra of purified NCS-Chrom. (a) Absorption spectrum of HPLC-purified NCS-Chrom A (fraction A, Figure 1) 0.025 mM, in 0.01 M ammonium acetate, pH 4, and 70% methanol. Inset: Fluorescence excitation (emission wavelength at 420 nm) and emission (excitation wavelength at 340 nm) spectra. (b) MCD (—) and CD (---) spectra of NCS-Chrom A, 0.06 mM, in 0.01 M ammonium acetate, pH 4, and 70%, methanol.

that reported earlier. This distribution of the in vitro DNA scission activity and absorption among A, B, and C 2 h after extraction at 4 °C is 70, 10, and 20%, respectively (Table I). Although the relative fluorescence intensity per A_{254} of B and C is greater than that for A (2.1- and 1.7-fold, respectively), as seen on the fluorescence trace (Figure 2), the absorption, fluorescence, CD, and MCD spectra of B and C are qualitatively and quantitatively identical with that of A (Figure 3). Furthermore, the in vitro activities of NCS-Chrom C are equal to those of NCS-Chrom A and B under the conditions tested (incubation for 30 min at 37 °C). NCS-Chrom C possesses 80% of the activity of NCS for inhibition of growth of HeLa cells. It appears that C is a direct product of A, generated in nonacidic methanol. Thus, in an NCS-Chrom sample stored at −70 °C for 3 months, there is a 3.6-fold increase in UV-absorption and in vitro DNA scission activity in peak C at the expense of peak A (Figures 2b, Table I) with no loss in the total activity of the preparation. A study of the kinetics of this conversion at −20 °C (Figure 2c) revealed that in 133 h the percentage of peak A decreases from 80 to 13% as peak C increases from 6 to 69%. Peak B is essentially unchanged. Also present are increased quantities of the highly fluorescent, but biologically inactive, peaks present in trace amounts in the original sample (Figure 2, see below). The conversion of A to C appears to be irreversible since addition of acid after extraction with 100% methanol does not decrease the amount of C. Each of the three active components is stable when stored in the presence of pH 4 buffer, and rechromatography of the isolated peaks results, in each case, in recovery of greater than 95% of the injected in vitro DNA scission activity in a single peak, with the original elution characteristics. Peak C, but not peak B, appears to be generated by the extraction process since HPLC of the native NCS at pH 4 by direct injection of a clinical drug without prior extraction produces only components A (85%) and B (15%). Furthermore, HPLC of the chromophore extracted with acidic methanol from NCS reconstituted from NCS-Chrom A and apo-NCS reveals only NCS-Chrom A. There is no evidence of interconversion between A and B, or between B and C. All subsequent studies with active NCS-Chrom were done by using HPLC-purified NCS-Chrom fraction A.

Spectral Properties of Purified NCS-Chrom A. NCS-Chrom A has considerable absorption fine structure with maxima or shoulders at wavelengths of 228, 265, 272, 287, 300, 330, and 350 nm (log ϵ = 4.75, 4.23, 4.11, 4.11, 4.04, and 3.80, respectively) and a single blue fluorescence emission maximum at 430 nm with excitation maxima at 270 and 340 nm (Figure 3a). All in vitro and in vivo activity studies of NCS-Chrom have been carried out in aqueous buffers near neutral pH, where optimal activity is observed. The spectral properties deteriorate under these conditions in parallel with the rapid loss of biological activity observed when NCS-Chrom A is added to aqueous pH 8 buffer in the absence of substrate (Kappen et al., 1980a; Povirk & Goldberg, 1980; Kappen & Goldberg, 1980). However, in water at pH ≤4, the absorption spectrum and the biological activity are both stable.

Circular dichroism studies show that NCS-Chrom is optically active. The CD spectrum (Figure 3b) shows optically active bands with a negative extremum at 255 nm of molar ellipticity, $[\theta]$, ~−900 deg.cm^2/dmol, and positive extrema at 290 (sh) and 325 nm. Magnetically induced optical activity is also present with negative extrema at 250, 280–290 (sh), and 315 nm and positive extrema at 270 and 355 nm. The overall shape of the MCD spectrum above 300 nm is similar to that of native NCS (Napier et al., 1979), except that the maximum is at 355 nm instead of 365 nm. The latter maximum reflects the red shift in absorption found when NCS-Chrom A binds to apo-NCS (see Figure 5).

Spectral Characterization of HPLC Peak D. Clinical NCS and NCS-Chrom extracted with 100% methanol exhibit relatively intense yellow fluorescence (emission at 490 nm with excitation at 380 nm) (Napier et al., 1979; Kappen et al., 1980a) in addition to the blue fluorescence exhibited by the active NCS-Chrom components A, B, and C. The activity of methanol-extracted NCS-Chrom when diluted into aqueous pH 8 buffer decreases spontaneously in parallel with the increase of the 490-nm fluorescence (Povirk & Goldberg, 1980), suggesting that the 490-nm fluorescent material is a decomposition product of active NCS-Chrom. Under these conditions, fluorescence at 490 nm is also generated from the purified NCS-Chrom components A, B, and C, confirming the fluorescent material as a decomposition product of the active NCS-Chrom. HPLC of the decomposition products of either A, B, or C, using a shallow gradient of increasing methanol concentration (conditions described in Figure 2), separates a major UV-absorbing material at 4 min which is intensely fluorescent, peak D, as well as several minor components with emission maxima at 420 and 490 nm which also lack biological activity and elute at positions different from those of peaks A, B, or C.

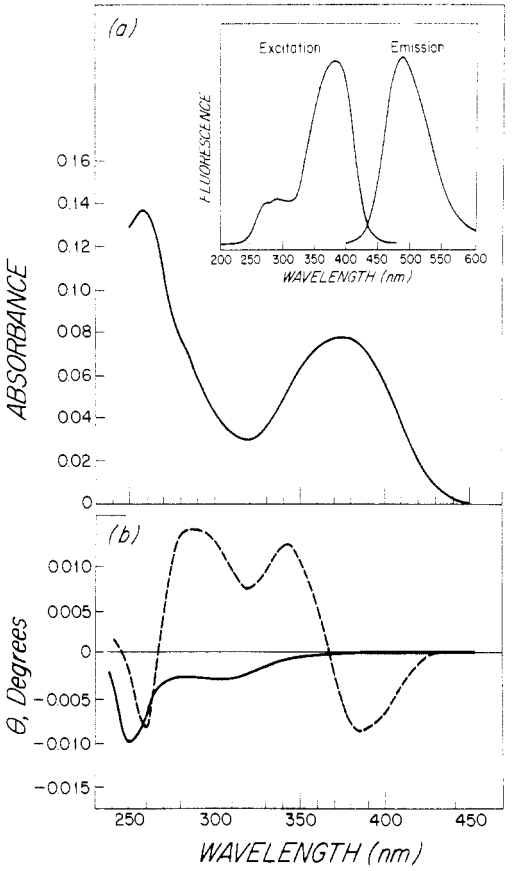

FIGURE 4: Absorption, fluorescence, MCD, and CD spectra of HPLC fraction D. (a) Absorption spectrum of HPLC component D in methanol. Inset: Fluorescence excitation (emission wavelength at 490 nm) and emission (excitation wavelength at 380 nm) spectra. (b) MCD (—) and CD (- - -) spectra of component D, $A_{380} = 0.075$, in methanol.

NCS-Chrom D in methanol exhibits a smooth broad absorption spectrum with none of the fine structure observed for NCS-Chrom, with absorption maxima at 260 and 280 (sh) nm and a broad maximum at 380 nm (Figure 4a). The fluorescence emission spectrum consists of a broad band with a maximum at 490 nm with excitation maxima at 270, 290, and 380 nm, reflecting the absorption maxima. Peak D exhibits strong optical activity with CD extrema at 285, 340, and 385 nm. However, unlike NCS-Chrom A, it possesses only weak MCD activity above 275 nm (Figure 4b).

Reconstitution of NCS: Binding of NCS-Chrom A to Apo-NCS. The CD spectrum of isolated NCS-Chrom is different from that of native NCS both in the intensity of the Cotton effect at 255 nm and in the position of extrema above 300 nm. Addition of apo-NCS to NCS-Chrom A (or to B or C) regenerates both the absorption and the CD spectra identical with those of native NCS (Napier et al., 1979). As apo-NCS is added to NCS-Chrom A (Figure 5), absorption changes consisting of hypochromicity from 295 to 345 nm and a bathochromic shift above 345 nm are observed until a ratio of apo-NCS to NCS-Chrom of 1 to 1 is achieved. Further additions cause no further spectral changes.

The CD spectrum also reflects this association. Titration of NCS-Chrom A with apo-NCS by monitoring the ellipticity of the 255-nm CD extremum (Figure 6) and the absorption at 360 nm (Figure 5) shows the formation of a one-to-one complex with the apoprotein. The CD spectrum of reconstituted NCS (the 1:1 complex) exhibits a large negative extremum at 255 nm, a positive extremum at 290 nm, and negative extrema at 320 and 365 nm, with $[\theta]_{255nm} = -2000$ deg·cm^2/dmol, twice that for chromophore alone and identical with that of native NCS (Samy et al., 1974; Napier et al., 1979).

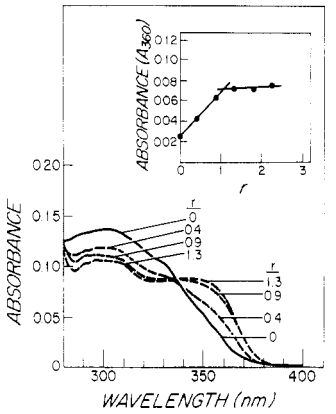

FIGURE 5: Effect of apo-NCS on the absorption spectrum of NCS-Chrom. The absorption spectrum of NCS-Chrom A, 9 μM, in 0.02 M sodium citrate, pH 4, and 20% methanol (—), and its modification with successive additions of apo-NCS (in 0.01 M sodium citrate, pH 4) (- - -) are shown. The reference cuvette contained equivalent concentrations of apo-NCS in the buffer solution. The molar ratio of apo-NCS to NCS-Chrom A (r) is indicated. Maximum dilution at $r = 1.35$ is 4%. Inset: Variation of A_{360} (corrected for dilution) as a function of r.

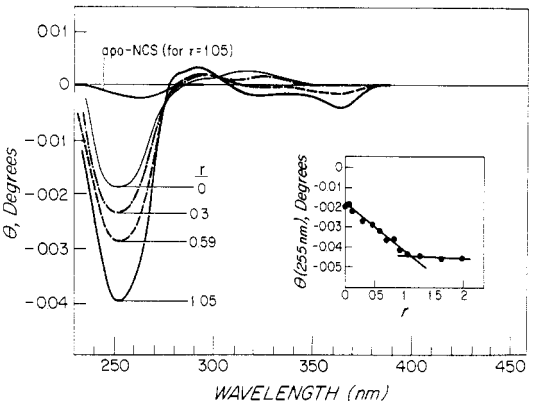

FIGURE 6: Effect of apo-NCS on the CD spectrum of NCS-Chrom. Increasing amounts of apo-NCS in 0.01 M sodium citrate, pH 4, were added to NCS-Chrom A, 0.02 mM, in 0.02 mM sodium citrate, pH 4, and 20% methanol (—). The molar ratio of apo-NCS to NCS-Chrom (r) is indicated. The CD spectrum of apo-NCS at the maximum protein concentration (at $r = 1.05$) is shown for reference. Apo-NCS has no CD spectrum above 280 nm. Maximum dilution at $r = 1.05$ is 11%. Optical path length is 0.3 cm. Inset: Variation of the ellipticity, θ, at 255 nm (corrected for dilution) as a function of r.

Binding of NCS-Chrom A to DNA. Interaction of NCS-Chrom A with calf thymus DNA in 0–20% methanol and 10 mM sodium citrate, pH 4, has been indicated by both fluorescence (Povirk & Goldberg, 1980) and absorption methods (Povirk et al., 1981; M. A. Napier and I. H. Goldberg, unpublished experiments). CD spectra also reflect this association. Absorption hypochromicity is observed from 290 to 345 nm (extremum at 315 nm) and a bathochromic shift occurs above 345 nm (extremum at 363 nm), similar to the changes noted above (Figure 6) for the addition of apo-NCS to NCS-Chrom A. Increased ellipticity in the ultraviolet near 250 nm is observed on addition of DNA to NCS-Chrom A, but little or no change is observed in the visible region (Figure 7). The change in the CD spectrum of NCS-Chrom A which occurs on binding to DNA is shown by the difference CD spectrum (Figure 7) and is maximal at the negative extremum near 250 nm. In contrast to the binding to apo-NCS, a Cotton

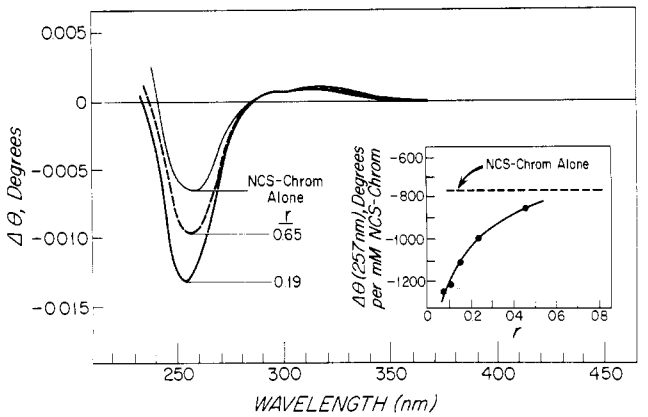

FIGURE 7: Difference CD spectra of the NCS-Chrom A/DNA complex. All spectra were obtained on a single sample of NCS-Chrom A in 0.02 M sodium citrate, pH 4, in 20% methanol, with stepwise addition of DNA. The CD spectrum from parallel samples of DNA without NCS-Chrom is subtracted from the appropriate NCS-Chrom plus DNA sample. CD spectrum of NCS-Chrom alone (0.04 mM) (—); difference CD spectra of NCS-Chrom plus DNA [0.062 mM nucleotide, 3% dilution (---); 0.210 mM nucleotide, 7% dilution (—)]. Optical path length is 0.3 cm. Inset: Variation of the ellipticity difference, $\Delta\theta$, with the molar ratio of NCS-Chrom nucleotide (r). The molar ellipticity (corrected for dilution) is calculated relative to NCS-Chrom concentration.

effect is not observed at 365 nm although a similar red shift of the absorption is observed. The ellipticity changes are measured at 257 nm, where the contribution to the CD spectrum from DNA is minimal. These ellipticity changes per aliquot of added NCS-Chrom are maximal at low NCS-Chrom to DNA ratios, i.e., at $r \leq 0.2$. This titration is complicated by a major overlap of the Cotton effect of the free chromophore with that of the NCS-Chrom/DNA complex, precluding the determination of the stoichiometry of the process. Nonetheless, the changes of the slope of the titration curve (Figure 7, inset), with the change in slope maximal at low r, clearly indicate an association between DNA and NCS-Chrom.

Discussion

The recent appreciation that neocarzinostatin (Napier et al., 1979) and the related protein antibiotic auromomycin (Yamashita et al., 1979) are specific complexes (shown to be 1:1 for NCS) between a labile chromophoric ligand and a protein (Kappen et al., 1980a,b; Kappen & Goldberg, 1980) and that the isolated chromophore from both possesses the full biological activity of the complex (Kappen et al., 1980a,b; data reported here; Ohtsuki & Ishida, 1980; Suzuki et al., 1980; Woynarowski & Beerman, 1980) suggests that they are representative of a novel class of macromolecular antitumor antibiotics with unusual mechanisms for achieving base-specific DNA damage [see Goldberg et al. (1981)]. The requirement for molecular oxygen and the sensitivity of this reaction to inhibition by low levels of α-tocopherol (Kappen & Goldberg, 1978), as well as the fact that the activity of both antibiotic chromophores is temperature independent (Kappen et al., 1980a,b), suggest that free radicals may be involved. Furthermore, evidence that fragments of the deoxyribose moiety of thymidylate in DNA are generated in the strand-breakage reaction (Hatayama & Goldberg, 1980) is compatible with such a mechanism. Our findings that NCS-Chrom is essentially metal free and that various metal-chelating agents fail to block the DNA scission reaction argue against the role of a metal in this reaction.

The absorption spectrum of NCS-Chrom A, which accounts for 90% of the total UV-absorbing and bioactive material in NCS, has considerable fine structure with band maxima in spectral regions characteristic of various hydroxynaphthoic acids (Schulman & Kovi, 1973): specifically in the regions 230–270 nm and 320–340 nm, where the isolated hydroxynaphthoic acid derivative of NCS-Chrom and its methyl ester (Edo et al., 1980; M. A. Napier and I. H. Goldberg, unpublished experiments) absorb. However, NCS-Chrom A also has substantial absorption, primarily that near 300 nm, which cannot be attributed to the hydroxynaphthoic acid moiety alone. Since the galactose moiety is expected to be transparent in the near-UV–visible region, the intense absorption from 280 to 310 nm of NCS-Chrom A is most likely contributed by the $C_{15}H_{10}O_4$ unit. It is in this region of the spectrum where hypochromicity is found when NCS-Chrom A binds to apo-NCS or to DNA, in addition to the shift of the maximum to higher wavelengths. Furthermore, these absorption changes resemble those observed upon the treatment of NCS-Chrom with mercaptans (M. A. Napier and I. H. Goldberg, unpublished experiments), which is known to add to the C_{15} substructure (Albers-Schönberg et al., 1980). Thus, the similarity of these absorption changes raises the possibility that the electronic structure of the highly unsaturated $C_{15}H_{10}O_4$ unit of NCS-Chrom is affected in a similar manner by its binding to apo-NCS and to DNA and that a mercaptan-activated form of this unit is responsible for inducing the DNA damage, although other explanations are also possible.

HPLC of crude NCS-Chrom yields several biologically active peaks. Minor peak B, containing 10–15% of the total in vitro DNA scission activity but with an activity equal to that of NCS-Chrom A (incubation for 30 min at 37 °C), is found whether or not the extracting methanol is acidic (Figures 1 and 2). A second minor component, peak C (Figure 2), also possessing in vitro DNA scission activity equal to that of A (incubation for 30 min at 37 °C), is found in 100% methanol-extracted chromophore. NCS-Chrom B and C, however, possess 5% and 80%, respectively, of the activity of A for growth or DNA synthesis inhibition of HeLa cells. Peak C appears to be generated from A in the presence of 100% methanol and is not present in native NCS. Components A, B, and C possess essentially identical absorption, CD, MCD, and fluorescence spectra. We have recently determined (Napier et al., 1981) that NCS-Chrom A possesses a five-membered cyclic carbonate ring (1,3-dioxolan-2-one) within the incompletely defined C_{15} substructure. NCS-Chrom B and NCS-Chrom C appear to be related to A by modification of this cyclic carbonate moiety. NCS-Chrom B is formally equivalent to a hydrolysis product of A, followed by decarboxylation. Whether B is less active than A or C in vivo (although as active in vitro) because of reduced cellular uptake or other factors remains to be assessed. NCS-Chrom C is a methanolysis product of A. Several fractions with intense blue or yellow fluorescence which have no biological activity are also present. Some of the inactive materials have been identified as decomposition products of NCS-Chrom obtained by various chemical treatments. The most obvious one on the HPLC profile (Figure 2) is the highly fluorescent fraction D, which appears to be a hydrolysis product of A, as well as of B and C. NCS-Chrom B and NCS-Chrom C have been found to generate 490-nm fluorescence 4 and 2 times, respectively, more slowly at room temperature in aqueous pH 8 buffer than does NCS-Chrom A (L. F. Povirk, M. A. Napier, and I. H. Goldberg, unpublished experiments), presumably accounting for the earlier observed (Povirk & Goldberg, 1980) biphasic nature of the rate of 490-nm fluorescence generation by crude NCS-Chrom under these conditions. Peak D exhibits very

different spectral properties from the parent compound (compare Figures 3 and 4) and from the isolated naphthoic acid derivative of NCS, including longer wavelength visible absorption and fluorescence maxima and the absence of long-wavelength MCD activity. These spectral properties indicate either that D does not contain the naphthoic acid moiety or, rather, that it contains it in a chemically modified form which has little MCD activity. The long-wavelength absorption (380 nm) and intense fluorescence (490 nm) indicate that extensive unsaturation still remains. Asymmetry induced perhaps by the vicinal effect of an attached sugar residue or by a new asymmetric center is indicated by its strong CD activity. The generation of D from NCS-Chrom has been shown to correlate with the loss of NCS-Chrom bioactivity in aqueous buffers, pH 8 (Povirk & Goldberg, 1980), and its spectral properties are similar to those of a product generated by treatment of NCS with guanidine (Napier et al., 1979). It can be detected in clinical preparations of NCS by its fluorescence, and not by its absorption.

NCS-Chrom A is optically active and from CD and MCD data is unambiguously identified as the source of the unusual CD bands at 365 and 297 nm previously observed in NCS, as well as the strong negative Cotton effect at 255 nm. Importantly, this CD activity serves as an extrinsic probe of the association of NCS-Chrom to apo-NCS. A 2-fold increase in the ellipticity at 255 nm and striking changes in the CD spectrum above 300 nm, including a new band at 365 nm, accompany the reassociation. The 365-nm Cotton effect is absent in the isolated NCS-Chrom A and arises by perturbation of the optical transition clearly observable by both absorption and MCD in the free NCS-Chrom A. Both absorption and CD studies (Figures 5 and 6) reveal the formation of a 1:1 complex between purified NCS-Chrom A and apo-NCS. The product appears identical with native NCS.

The binding of NCS-Chrom to DNA has been shown by the ability of DNA to block the inactivation of the chromophore in aqueous buffers and by the ability of NCS-Chrom to unwind supercoiled pMB9 DNA and lengthen linear duplex DNA by intercalation (Povirk & Goldberg, 1980; Povirk et al., 1981). The CD studies (Figure 7) provide direct physical evidence of the formation of an NCS-Chrom A DNA complex. The NCS-Chrom DNA complex exhibits optical activity in the ultraviolet. Binding of NCS-Chrom to DNA by intercalation or by outside binding could cause an alteration of the conformation of the nucleic acid and/or the chromophore molecule, either of which could be manifested as ultraviolet CD perturbations. However, the CD spectrum of the NCS-Chrom DNA complex exhibits a minimum near 255 nm, similar to that of NCS-Chrom, indicating that it is probably the conformation of the chromophore that is altered. The small alteration in the CD spectrum and the decrease in the magnitude of the change as r increases are effects similar to those reported in the visible region for DNA complexes of the intercalating molecules, 1-, 2-, and 9-aminoacridine (Dalgleish et al., 1969, 1971). Furthermore, for NCS-Chrom, as for 9-aminoacridine, the ellipticity tends to finite limits as r tends to zero, suggesting that the induced optical activity is a property of singly bound molecules and not a cooperative phenomenon between bound molecules as observed with proflavin (Dalgleish et al., 1969, 1971), 3-aminoacridine (Dalgleish et al., 1969, 1971), and actinomycin (Yamaoka & Ziffer, 1968).

Acknowledgments

We thank Dr. T. Uchida for metal analyses, Dr. W. T. Bradner for the generous gift of clinical NCS, Dr. L. Kappen and J. Thivierge for the in vivo DNA strand scission analysis and the gift of ^{3}H-labeled pMB9 DNA, Dr. L. F. Povirk for the ^{3}H-labeled CHO DNA and sonicated calf thymus DNA, and Dr. E. Eisenstadt for the *E. coli* TA85rfa culture.

References

Albers-Schönberg, G., Dewey, R. S., Hensens, O. D., Liesch, J. M., Napier, M. A., & Goldberg, I. H. (1980) *Biochem. Biophys. Res. Commun.* **95**, 1351–1356.

Beerman, T. A., & Goldberg, I. H. (1977) *Biochim. Biophys. Acta 475*, 281–293.

Beerman, T. A., Poon, R., & Goldberg, I. H. (1977) *Biochim. Biophys. Acta 475*, 294–306.

Dalgleish, D. G., Fujita, H., & Peacocke, A. R. (1969) *Biopolymers 8*, 633–645.

Dalgleish, D. G., Peacocke, A. R., Fey, G., & Harvey, C. (1971) *Biopolymers 10*, 1853–1863.

Edo, K., Katamine, S., Kitame, F., Ishida, N., Koide, Y., Kusano, G., & Nozoe, S. (1980) *J. Antibiot. 33*, 347–351.

Goldberg, I. H., Hatayama, T., Kappen, L. S., Napier, M. A., & Povirk, L. F. (1981) *Molecular Actions and Targets for Cancer Chemotherapeutic Agents, Bristol-Myers Cancer Symposium in Cancer Research* (Sartorelli, A. C., Bertino, J. R., & Lazo, J. S., Eds.) pp 163–191, Academic Press, New York.

Hatayama, T., & Goldberg, I. H. (1980) *Biochemistry 19*, 5890–5898.

Kappen, L. S., & Goldberg, I. H. (1978) *Nucleic Acids Res. 5*, 2959–2967.

Kappen, L. S., & Goldberg, I. H. (1980) *Biochemistry 19*, 4786–4790.

Kappen, L. S., Napier, M. A., & Goldberg, I. H. (1980a) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1970–1974.

Kappen, L. S., Napier, M. A., Goldberg, I. H., & Samy, T. S. A. (1980b) *Biochemistry 21*, 4780–4785.

Meienhofer, J., Maeda, H., Glaser, C. B., Czombos, J., & Kuromizu, K. (1972) *Science* (Washington, *D.C.*) *178*, 875–876.

Napier, M. A., Goldberg, I. H., Hensens, O. D., Dewey, R. S., Liesch, J. M., & Albers-Schönberg, G. (1981) *Biochem. Biophys. Res. Commun. 100*, 1703–1712.

Napier, M. A., Holmquist, B., Strydom, D. J., & Goldberg, I. H. (1979) *Biochem. Biophys. Res. Commun. 89*, 635–642.

Napier, M. A., Kappen, L. S., Holmquist, B., Strydom, D. J., & Goldberg, I. H. (1980a) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 39*, 1609.

Napier, M. A., Kappen, L. S., & Goldberg, I. H. (1980b) *Biochemistry 19*, 1767–1773.

Ohtsuki, K., & Ishida, N. (1980) *J. Antibiot. 33*, 744–750.

Povirk, L. F., Dattagupta, N., Warf, B. C., & Goldberg, I. H. (1981) *Biochemistry 20*, 4007–4014.

Povirk, L. F., & Goldberg, I. H. (1980) *Biochemistry 19*, 4773–4780.

Samy, T. S. A., Atreyi, M., Maeda, H., & Meienhofer, J. (1974) *Biochemistry 13*, 1007–1014.

Schulman, S. G., & Kovi, P. J. (1973) *Anal. Chim. Acta 67*, 259–267.

Suzuki, H., Miura, K., Kumada, Y., Takeuchi, T., & Tanaka, N. (1980) *Biochem. Biophys. Res. Commun. 94*, 255–261.

Woynarowski, J. M., & Beerman, T. A. (1980) *Biochem. Biophys. Res. Commun. 94*, 769–776.

Yamaoka, K., & Ziffer, H. (1968) *Biochemistry 7*, 1001–1008.

Yamashita, T., Naoi, N., Hidaka, T., Watanabe, K., Kumada, Y., Takeuchi, T., & Umezawa, H. (1979) *J. Antibiot. 32*, 330–339.