Stabilization of Neocarzinostatin Nonprotein Chromophore Activity by Interaction with Apoprotein and with HeLa Cells†

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ABSTRACT: The methanol-extracted, nonprotein chromophore of the protein antibiotic neocarzinostatin (NCS), which possesses the full in vitro and in vivo deoxyribonucleic acid (DNA) strand-breaking activities and the ability to inhibit DNA synthesis and growth in HeLa cells of the holoantibiotic, is much more labile to inactivation by heat, 2-mercaptoethanol, long-wavelength UV light, and pH values above 4.8. Inactivation is inversely related to the methanol concentration. The pH activity profile of the isolated chromophore extends to pH values below 7.0. Chromophore inactivation is specifically blocked by the apoprotein of NCS; 100-fold higher concentrations of the apoprotein of another protein antibiotic, auromomycin, gave similar protection, whereas bovine serum albumin is even less effective. The chromophore, and not the apoprotein, is inactivated by heat or light (360 nm) as determined by both activity and isoelectric focusing experiments. In contrast to other chromophoric antibiotic substances (daunorubicin and the extracted chromophore of auromomycin), the NCS chromophore interacts irreversibly with HeLa cells at 0 °C in serum-free medium so as to inhibit subsequent DNA synthesis at 37 °C. Such interaction at 0 °C is very rapid, reaching 50% completion in about 15 s, and is not found with native NCS or when apo-NCS is added before the chromophore or when serum is included in the preincubation at 0 °C. Washing with apo-NCS or serum-containing (or -free) medium after preincubation of the cells with the chromophore at 0 °C fails to reverse the subsequent inhibition of DNA synthesis.

Earlier work from this laboratory has provided indirect evidence for the role of a nonprotein chromophore (Napier et al., 1979) in the action of the antitumor protein antibiotic neocarzinostatin (NCS)[1] (Kappen & Goldberg, 1979; Napier et al., 1980). Recently we reported that the isolated, purified chromophore possesses the in vitro and cytotoxic properties of the parent compound and that the apoprotein serves as a carrier for the chromophore and acts to stabilize it and to control its release for interaction with target DNA (Kappen et al., 1980a). In a reaction markedly stimulated by a mercaptan, the free chromophore induces single-strand breaks in supercoiled DNA at a much faster rate than does either native or reconstituted NCS. Evidence that the chromophore binds to DNA by an intercalative mechanism has been presented (Povirk & Goldberg, 1980; L. F. Povirk, N. Dattagupta, and I. H. Goldberg, unpublished data). Chemical characterization and a partial structure of the NCS chromophore (M_r 661) has recently been reported (Albers-Schönberg et al., 1980).

In this paper we characterize the stability properties of the isolated chromophore and the biological consequences of its interaction with its apoprotein and with the HeLa cell. Evidence is presented for the rapid and irreversible association of the chromophore with HeLa cells at low temperatures.

Materials and Methods

NCS (clinical form, Kayaku Antibiotics) was kindly provided by Dr. W. T. Bradner of Bristol Laboratories and was stored frozen in 0.015 M sodium acetate buffer, pH 5.0. Procedures for the preparation of the chromophore and apoprotein of NCS have been previously described (Kappen et al., 1980a). In all experiments the chromophore concentration is expressed in terms of the equivalent of native NCS (μg/mL) from which it was extracted. Purified macromomycin was a gift from Dr. T. S. A. Samy. It was further chromatographed on nonionic Amberlite XAD-7 to remove any remaining chromophore. The conditions of the chromatography were identical with those described earlier (Napier et al., 1980) for NCS. The H_2O-eluted fraction was used.

Drug-Induced DNA Scission. Assays were carried out with [^{3}H]thymidine-labeled λDNA or supercoiled pMB9 DNA as the substrate. Standard incubation solutions (100 μL) contained 50 mM Tris (pH 8.0), 10 mM 2-mercaptoethanol, 0.35 μg of λDNA (3.7×10^4 cpm/μg) or 0.25–0.5 μg of pMB9 DNA (4×10^4 cpm/μg), and the drug at levels given in the legends. The concentration of chromophore is expressed in terms of the starting NCS from which it was extracted. Activity was assessed by measuring the amount of trichloroacetic acid soluble radioactivity (λDNA) or the percent of nicked (form II pMB9) DNA formed. Details of these procedures have been described elsewhere (Kappen & Goldberg, 1979).

Reconstitution of NCS and Isoelectric Focusing. Reconstitution of NCS by colyophilization of solutions of chromophore and apoprotein has been described previously (Kappen et al., 1980a) and was assessed by isoelectric focusing on polyacrylamide gels. Where indicated the chromophore (in methanol) or apo-NCS (in H_2O) was subjected to heat (65 °C, 2 h) or UV treatment (long-wavelength UV, mineralight lamp, Model UV SL-58, 366 nm, and 1 h at a 15-cm distance) prior to reconstitution. Isoelectric focusing was carried out as before (Kappen et al., 1980a; Napier et al., 1980) and as detailed in the legend to Figure 6. Native NCS and apo-NCS have p*I* values of 3.3 and 3.2, respectively (Napier et al., 1980).

Drug Treatment of Cells and DNA Synthesis. HeLa cells were prelabeled with low levels of [^{14}C]thymidine (1.5 nCi/mL, 48 mCi/mmol) for 18 h in order to correct for cell recovery at the end of the experiment. The cells were suspended in serum-containing or serum-free minimum essential medium during drug treatment. In the latter case the cells were spun down and the cell pellet was washed twice either with phosphate-buffered saline (PBS; 10 mM sodium phosphate and

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[1] Abbreviations used: NCS, neocarzinostatin; apo-NCS, apoprotein of neocarzinostatin; PBS, phosphate-buffered saline.

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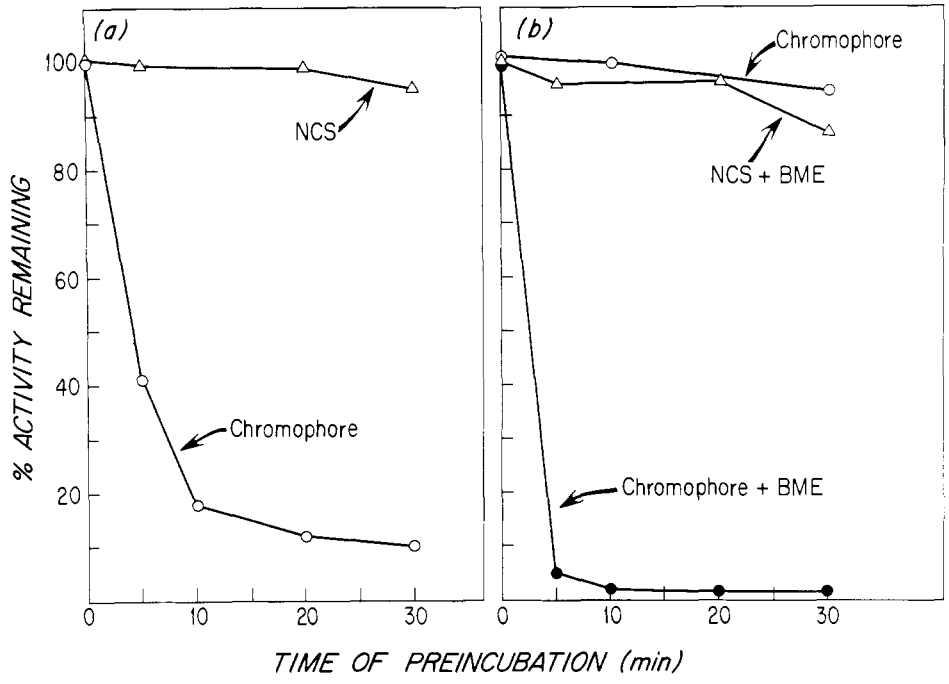

FIGURE 1: (a) Effect of preincubation at 37 °C on drug activity. Chromophore (1.2 mg/mL) in methanol or NCS (0.6 mg/mL) in 15 mM sodium acetate, pH 5.0, was incubated at 37 °C. At various times 8 μL of chromophore or 16 μL of NCS was withdrawn and was added to the reaction mixture containing λDNA to make a final volume of 100 μL. The reaction with NCS also contained 8% methanol. The complete reaction was incubated at 37 °C for 35 min, and the amount of trichloroacetic acid solubilized radioactivity was determined. (b) Effect of preincubation of drug with 2-mercaptoethanol (BME). The reaction conditions are similar to those in (a) except that the drugs preincubated at 0 °C contained 10 mM 2-mercaptoethanol. At various times portions of the drug were assayed for production of acid-solubilized radioactivity. In both (a) and (b) the final concentrations of chromophore and NCS were 96 μg/mL. With no preincubation chromophore and NCS produced respectively 17% and 10% of acid-soluble radioactivity.

150 mM NaCl, pH 7.2) or with serum-free medium before resuspension in serum-free medium at a cell density of 5×10^5 cells/mL. The cell suspension (usually 2 mL, in triplicate) was precooled to 0 °C or prewarmed to 37 °C before addition of the drug. After drug treatment, the cells were collected by centrifugation and were washed twice by resuspension in 2 mL each of cold PBS. The washed cells were resuspended in 2 mL of serum-free medium. Unless otherwise stated, the cells were then preincubated for 40 min at 37 °C on a shaker bath prior to the addition of 0.5 μCi/mL [*methyl*-^{3}H]thymidine (50 Ci/mmol). Thymidine incorporation for 40 min was determined. Similar results were obtained if serum was included in the incubations following drug treatment. The details of the assay have been given elsewhere (Kappen et al., 1979). In experiments where the cells were treated with the drug in serum-containing medium (10% serum), the washing procedure after the drug treatment was similar. Chromophore addition was in methanol. An equal volume of methanol was added to the controls and to the other samples not receiving chromophore. The final methanol concentration did not exceed 1%.

Results and Discussion

Stability of the Nonprotein Chromophore. The extracted chromophore is relatively stable when stored in 100% methanol at −70 °C for weeks or at 0 °C for hours. Unlike native NCS the chromophore in 100% methanol at 37 °C (Figure 1a) or at 0 °C when treated with 2-mercaptoethanol (Figure 1b) rapidly loses its DNA strand-cutting activity. Activity of the chromophore is lost still more rapidly in aqueous solutions; this is inversely related to the concentration of methanol (Figure 2) and may be in part due to the lowered solubility of the chromophore in aqueous solutions. The stability of the chromophore in aqueous solutions is very pH dependent; at pH values >4.8 there is a rapid loss of biological activity

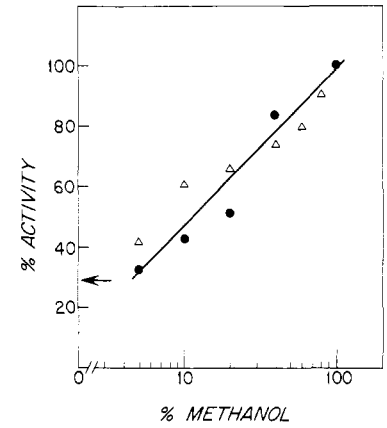

FIGURE 2: Effect of dilution of chromophore at different methanol levels at 0 °C on subsequent activity. The stock chromophore was diluted to 1.25 μg/mL in 10 mM sodium acetate, pH 5.0, at 0 °C containing various levels of methanol. The diluted drug (10 μL) was assayed in a 250-μL incubation (20 min at 37 °C) containing pMB9 form I DNA. Methanol was adjusted to the same final level of 4% in the subsequent incubation at 37 °C. The amount of form II DNA produced by the drug (0.05 μg/mL) was quantitated. 100% activity represents 64% of form II DNA. The two symbols represent two series of experiments. The arrow indicates the activity after dilution at the lowest level of methanol used (0.05%).

(Figure 3). The small loss of activity of the native NCS at pH values >6 is mainly due to the inclusion of 16% methanol in the preincubation (Kappen & Goldberg, 1979) in order to have equivalent conditions. This has been attributed to the alcohol-induced unfolding of NCS and release of chromophore (Kappen & Goldberg, 1979; Kappen et al., 1980a). Inasmuch as the chromophore is active at the same pH values (Figure 4) where it is rapidly inactivated in the absence of the substrate (Figure 3), the active form of the drug must interact very rapidly with the DNA. The chromophore is much more active

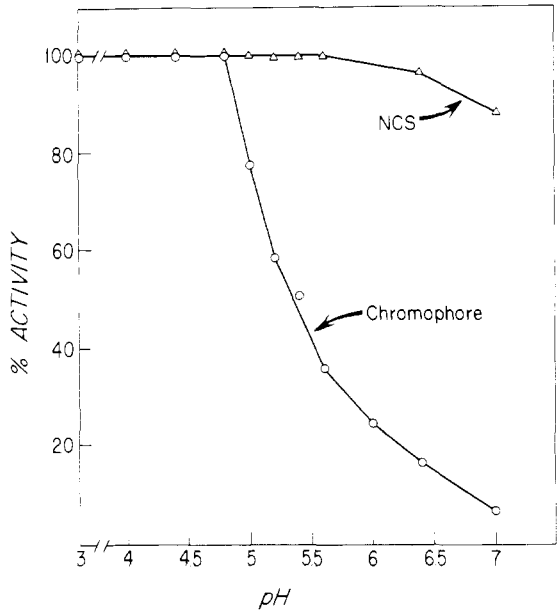

FIGURE 3: Effect of preincubation of drugs at different pH values. The drugs (192 μg/mL) were preincubated (10 min at room temperature) in 10 mM sodium phosphate–citrate buffer of various pH values containing 16% methanol in a total volume of 50 μL. The remaining standard reaction components were added to the preincubated drug in 50-μL volume, and the complete reaction was incubated at 37 °C for 30 min before determination of acid-solubilized radioactivity. NCS made 15% of radioactivity acid soluble (100%). Chromophore preincubated in methanol for 10 min at room temperature produced 14% acid-soluble radioactivity (50% of original activity) and this was taken as 100%.

than holo-NCS at pH 7 and below (Figure 4), probably reflecting the effect of pH on its stability and on its binding to the apoprotein.

Specificity of Protection of the Chromophore by Its Apoprotein. The protection by NCS apoprotein against chromophore inactivation is highly specific. One hundred-fold higher concentrations of macromomycin, the apoprotein form of auromomycin (Yamashita et al., 1979), provide the same degree of protection as does NCS apoprotein (Figure 5). The shapes of the dose–response curves are similar, however, and may reflect the sequence homologies detected in the two proteins (Sawyer et al., 1979). Bovine serum albumin is even less effective and its dose–response curve is much flatter, suggesting a different mechanism for the protection. Similar results were obtained with pepsinogen which has a p*I* (3.7) close to that of NCS (3.3).

Table I: Effect of UV and Heat Treatment of Chromophore and Apo-NCS on Their Subsequent Activity after Reconstitution[a]

	form II DNA (%)
apo-NCS plus chromophore	60
apo-NCS plus UV chromophore	3
UV apo-NCS plus chromophore	63
heated apo-NCS plus chromophore	59
heated chromophore plus apo-NCS	0

[a] Details for heat and UV treatment of the samples and their reconstitution are given under Materials and Methods. The reconstituted drug (0.04 μg/mL) was assayed for activity with form I pMB9 DNA as the substrate. Incubation was for 15 min at 37 °C.

Comparison of Stabilities of the Chromophore and Apoprotein. NCS is inactivated at elevated temperatures (Beerman et al., 1977), especially in the presence of a mercaptan at neutral or alkaline pH (Kappen & Goldberg, 1978; Ishida & Takahashi, 1978), or by treatment with UV–visible light (Kohno et al., 1974; Burger et al., 1978; Kappen et al., 1979; Kappen & Goldberg, 1979; Napier et al., 1980; Iseki et al., 1980). Drug inactivation by light has been attributed to destruction and loss of the nonprotein chromophore from the complex with the protein (Kapper & Goldberg, 1979; Napier et al., 1980; Iseki et al., 1980). Since we treated NCS with either long-wavelength UV light (mineralight lamp, Model UV SL-58, 366 nm) (Kappen & Goldberg, 1979) or narrow-band monochromatic light at 360 nm (Napier et al.,

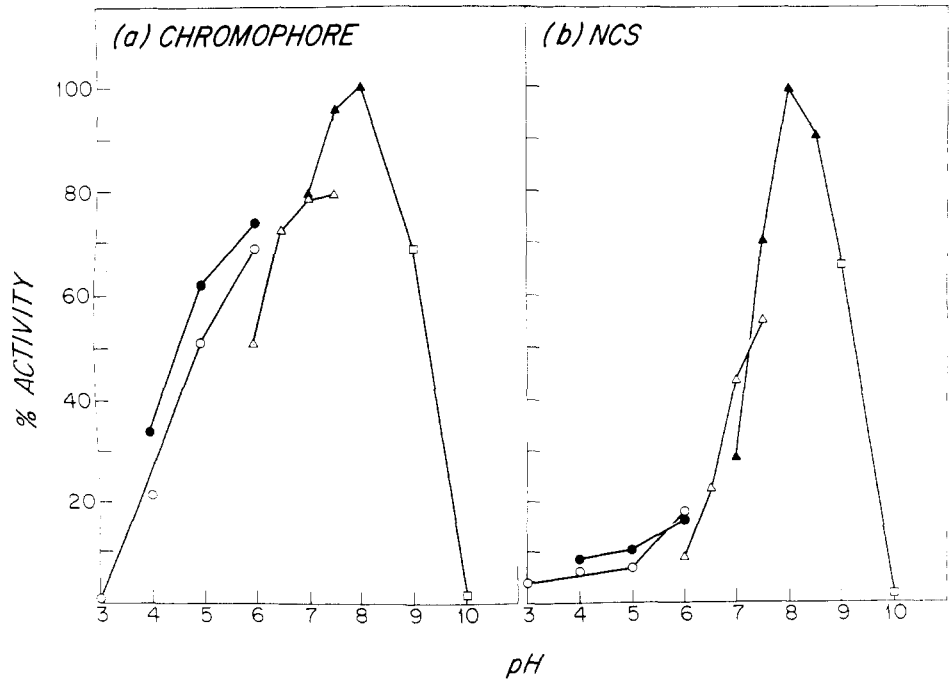

FIGURE 4: Effect of pH during the incubation on drug activity. Standard incubations (100 μL) in 50 mM buffers at different pH values were carried out at 37 °C for 20 min. The final drug level was 96 μg/mL. Only chromophore samples contained 8% methanol. At the optimum pH of 8.0, chromophore and NCS produced respectively 30% and 8.5% of acid-soluble radioactivity. Buffers used: (○) sodium phosphate–citrate; (●) sodium acetate; (△) sodium phosphate; (▲) Tris-HCl; (□) glycine–NaOH.

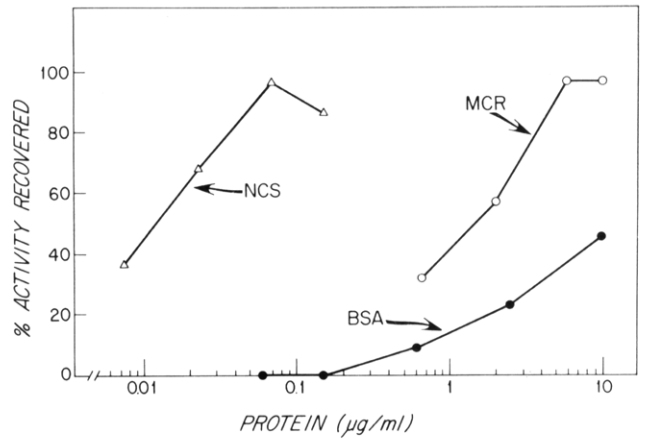

FIGURE 5: Protection of chromophore by proteins against inactivation. Chromophore (0.75 μg/mL) was preincubated at room temperature for 15 min in 10 mM sodium acetate, pH 5, and 2.5% methanol in the presence and absence of the various proteins at concentrations shown. Portions (10 μL) of the drug samples were assayed for activity at 37 °C for 15 min in 100-μL incubations containing pMB9 DNA. 100% activity represents the formation of 75% form II DNA. On preincubation, with no added protein, chromophore lost 67% of its activity. MCR, macromomycin; BSA, bovine serum albumin; NCS, apoprotein of NCS.

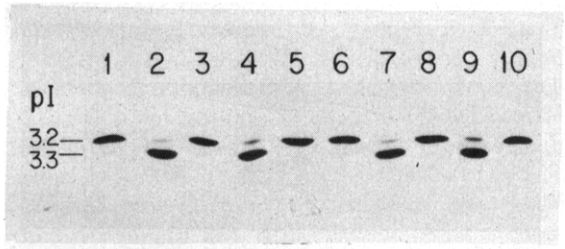

FIGURE 6: Isoelectric focusing of reconstituted NCS on polyacrylamide gel. The gel (5%, pH 2.5–4.2) was prefocused at 10 W for 45 min. Reconstitution of NCS from chromophore and apoprotein (2:1 ratio in μg/mL) was carried out as described previously (Kappen et al., 1980a). After application of the sample (24 μg of protein) electrofocusing was done at 25 W (70 mA) for 3 h at 2 °C in the dark. (1) Apo-NCS, (2) apo-NCS plus chromophore, (3) apo-NCS plus UV-treated chromophore, (4) UV-treated apo-NCS plus chromophore, (5) UV-treated apo-NCS, (6) apo-NCS plus heated chromophore, (7) heated apo-NCS plus chromophore, (8) heated apo-NCS, (9) native NCS, and (10) UV-treated apo-NCS plus UV-treated chromophore.

1980), it seemed likely that the nonprotein chromophore was selectively inactivated. As shown in Table I and Figure 6, the chromophore and not the apoprotein is labile to both heat and light treatment as determined by its ability to reconstitute holo-NCS with a p*I* of 3.3 and DNA scission capability. Furthermore, the chromophore in NCS is considerably more resistant to inactivation by long-wavelength UV light than is the free chromophore. The observed stability of the apoprotein to heat treatment is consistent with the stability of the native β-pleated sheet conformation of NCS in aqueous solution (Samy et al., 1974).

Irreversible Interaction between Chromophore and HeLa Cells at 0 °C. Given the extreme lability of the free chromophore at neutral pH in aqueous solution, our earlier finding that the chromophore was as active as holo-NCS in inducing DNA strand breaks and in inhibiting DNA synthesis and growth in HeLa cells in culture (Kappen et al., 1980a) at first seemed surprising. Even though chromophore binding to serum albumin is very poor, because of the high concentration of serum in the medium, it is possible that the chromophore is protected under these conditions until it interacts with the cell. The question then arose as to whether HeLa cells

Table II: Effect of Exposure of HeLa Cells to NCS or Its Extracted Chromophore at 0 or 37 °C on Subsequent DNA Synthesis[a]

	% inhibn at pretreatment temp	
	0 °C	37 °C
expt 1 (with serum)		
chromophore (2 μg/mL)	6	63
NCS (2 μg/mL)	0	72
expt 2 (without serum)		
chromophore (2 μg/mL)	53	56
NCS (2 μg/mL)	0	65

[a] In all experiments the cells were pretreated with NCS or its extracted chromophore for 10 min in either medium containing 10% fetal calf serum (experiment 1) or medium lacking serum (experiment 2). In the latter case the cells were washed twice with serum-free medium before drug treatment. In the absence of any drug, the cells in experiments 1 and 2 incorporated respectively 1.4×10^4 and 1.3×10^4 cpm into DNA.

Table III: Comparison of Effects of Exposure of Cells to Drugs at 37 or 0 °C on Subsequent DNA Synthesis[a]

	% inhibn of DNA synthesis after treatment of cells at	
	0 °C	37 °C
NCS chromophore (5 μg/mL)	44	44
NCS chromophore (10 μg/mL)	70	77
AUR chromophore (2 μg/mL)	11	48
AUR chromophore (5 μg/mL)	13	78
daunorubicin (3 μg/mL)	19	38
daunorubicin (10 μg/mL)	20	80

[a] The cells were treated with the drugs in serum-free medium for 10 min at 0 or 37 °C. In the absence of any drug, 1.1×10^4 cpm ^3H was incorporated. AUR, auromomycin.

themselves might provide a protected environment, possibly on the cell surface, for the chromophore. In fact, if cellular binding is very rapid, serum proteins might not be required during drug treatment.

Holo-NCS does not form a stable association with HeLa cells at low temperatures, as shown by the finding that cells preincubated with NCS at 0 °C in serum-containing growth medium can be washed free of the drug and not suffer any of the cytotoxic effects when the temperature is subsequently raised to 37 °C (Beerman & Goldberg, 1977). Similar results are found with the nonprotein chromophore (Table II, experiment 1). When serum is omitted from the preincubation at 0 °C, however, the chromophore, but not the holoantibiotic, inhibits subsequent DNA synthesis at 37 °C (Table II, experiment 2). By contrast, neither the anthracycline antibiotic, daunorubicin, nor the active chromophore extracted from the antibiotic auromomycin (Kappen et al., 1980a,b) shows evidence of comparable cellular binding at 0 °C (Table III). The binding of NCS chromophore to the cell at 0 °C is very rapid (50% by 15 s) (Figure 7). Addition of a low concentration of NCS apoprotein (or a high level of serum albumin) at the beginning of the preincubation at 0 °C, but not at the end, blocks the subsequent inhibition of DNA synthesis by the NCS chromophore (Table IV). The interaction between the chromophore and the cells appears to be essentially irreversible since washing of the cells with apoprotein or serum albumin containing phosphate-buffered saline after the preincubation fails to reverse the inhibitory action (Table IV).

It is possible that the chromophore was taken up into the cell cytoplasm at the low temperature or was merely "solubilized" in the hydrophobic environment of the cell

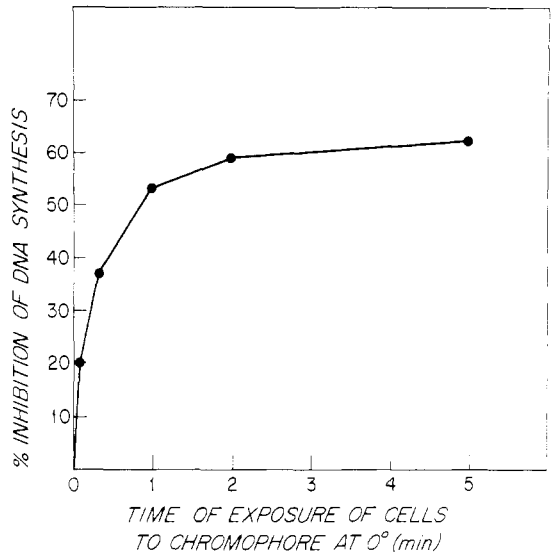

FIGURE 7: Effect on DNA synthesis of exposure of HeLa cells for various times to NCS chromophore at 0 °C. Chromophore was added to the cells in serum-free medium at 0 °C. At the times shown, apo-NCS was added to bind any free chromophore remaining in the medium. After the drug treatment, the cells were washed and DNA synthesis was measured as described under Materials and Methods. The final concentrations of chromophore and apo-NCS were 10 μg/mL and 13 μg/mL, respectively. In the absence of any drug, the cells incorporated 1.2×10^4 cpm into DNA.

Table IV: Effect of Apo-NCS on Chromophore Action on Cells at 0 °C[a]

	% inhibn of DNA synthesis
chromophore (10 μg/mL)	77
apo-NCS (13 μg/mL) and then chromophore (10 μg/mL)	8
chromophore (10 μg/mL) and then cells washed with PBS–apo-NCS (11 μg/mL)	75
chromophore (10 μg/mL) and then cells washed with PBS–serum (1%)	78

[a] The cells were treated with chromophore at 0 °C for 15 min in serum-free medium. Following drug treatment, the cells were washed twice with the cold PBS. Apo-NCS or 1% serum was included in the wash solutions as indicated. Thymidine incorporation was measured as described under Materials and Methods. The control cells incorporated 9×10^3 cpm.

membrane. Recent preliminary data show that cellular DNA breakage occurs under these conditions at 0 °C, indicating that drug was taken up into the cell nucleus. In any case, it appears that the chromophore, once associated with the cell, is maintained in a stable state until it interacts with the DNA. Furthermore, either because of cellular inaccessibility or extremely tight binding to a cell surface component, once bound to the cell the chromophore cannot be removed by washing with the apoprotein. Whether normal drug uptake first involves release of the chromophore from the apoprotein at the cell outer surface remains to be shown, although such a mechanism presumably exists in the case of active drug bound to an insoluble support (Nakamura & Ono, 1974; Lazarus et al., 1977). This mechanism requires that either the apoprotein of soluble holo-NCS be degraded at or near the cell surface or that conditions favoring chromophore release from the apoprotein prevail, since the chromophore binds very tightly to its apoprotein ($K_D \sim 10^{-10}$ M; Povirk & Goldberg, 1980). The lack of generation of a stable association between chromophore and the cell during preincubation at 0 °C in the presence of apoprotein is due to the inability of the apoprotein to release the chromophore at this temperature (Kappen et al., 1980a). There is no evidence that specific binding sites for apo-NCS exist on the HeLa cell surface (M. A. Napier and I. H. Goldberg, unpublished data). On the other hand, using NCS labeled in the protein with radioactivity or fluorescein isothiocyanate, Maeda and his co-workers (Maeda et al., 1975; Sakamoto et al., 1979) have presented evidence for the uptake of the protein into mammalian cells and into the nuclei of such cells. Whether the chromophore remains complexed with the apoprotein during the uptake and intracellular transport process is not known. If it is, then chromophore may not be released from the carrier protein until it reaches its target, DNA, in the nucleus. In this case, chromophore release could be effected concomitantly with activation by a mercaptan (Kappen & Goldberg, 1979; Povirk & Goldberg, 1980).

Acknowledgments

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