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Purification and Partial Characterization of a Daunorubicin-Binding Protein from Rat Liver

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SUMMARY

A daunorubicin-binding protein has been isolated from the soluble fraction of normal rat liver by affinity chromatography on daunorubicin-Sepharose columns. This protein, which apparently binds anthracyclines specifically, appears to be composed of four polypeptides of 54,000 M_r, is different from tubulin, and is not involved in anthracycline metabolism. Its physiological function remains to be determined.

INTRODUCTION

Anthracycline antibiotics are effective anticancer chemotherapeutic agents (1). Various lines of evidence strongly suggest that most of the biological effects of anthracyclines can be ascribed to their binding to DNA with consequent impairment of nucleic acid synthesis (1, 2). However, structurally these drugs have the potential for many molecular interactions; indeed, they have been found to act at several biochemical levels. Thus, they interfere with the electron transport system (3, 4) and affect membrane organization and integrity (5, 6). It is likely that the latter effect may be due to the known ability of these drugs to bind phospholipids (7–9). The interaction of anthracyclines with phospholipids and other negatively charged macromolecules (10) and proteins (11) is expected, since the drugs contain a charged amino group in the sugar moiety. In general, protein binding (with particular reference to plasma protein) is a phenomenon common to several drugs and may represent an important factor that influences the degree to which the active drug acts on its primary biochemical receptor (12).

In the present study, we explore the interaction of daunorubicin with proteins by affinity chromatography. Using this technique, we have isolated a DNR-BP 54[1] from the soluble fraction of rat liver.

MATERIALS AND METHODS

Drugs. Daunorubicin, doxorubicin, and *N*-acetyl-doxorubicin were kindly supplied by Farmitalia-Carlo Erba (Milan, Italy). Colchicine was obtained from Sigma Chemical Company (St. Louis, Mo.), actinomycin D from Serva (Heidelberg, West Germany), and vincristine sulfate from Eli Lilly Italia (Florence, Italy). Tilorone was a gift of Merrell-National Laboratories (Cincinnati, Ohio).

Preparation of daunorubicin-Sepharose and aniline-Sepharose. The affinity adsorbent was prepared by coupling daunorubicin to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden). After 15 g of dry activated Sepharose had been swollen as suggested by the manufacturer, 25 mg of daunorubicin, dissolved in 50 ml of 0.2 M $NaHCO_3$ (pH 8.5), were added. The slurry was stirred at 4° for 24 hr. The excess reactive sites were saturated by reaction with 0.1 M ethanolamine HCl (pH 8.5) for 4 hr at 4°. Unbound daunorubicin was removed by washing with 8 M urea and 0.5 M NaCl, and this procedure was routinely adopted to regenerate the adsorbent after use. The amount of daunorubicin bound to Sepharose was determined to be 300 μg/ml of packed gel. The same procedure was used to couple aniline to Sepharose. Thirty micromoles of redistilled aniline were used per milliliter of Sepharose. The actual amount bound was not determined.

Purification of DNR-BP 54. The purification scheme is summarized in Table 1. All operations were carried out at 4°. One hundred grams of liver from outbred male albino Wistar rats (Charles River Laboratories, Calco, Italy) were homogenized with an Ultra-Turrax homogenizer in 300 ml of 50 mM Tris-HCl (pH 8.0) and centrifuged for 60 min at 100,000 × *g*. After removal of the lipid layer, the supernatant fluid was collected and fractionated by ammonium sulfate precipitation (30–65% saturation). The precipitate was suspended in 150 ml of Buffer A (10 mM Tris-HCl, pH 8.6) and dialyzed overnight against two 5-liter changes of the same buffer. The solution was then filtered through a column (5 × 20 cm) of DEAE-cellulose (Whatman DE 23), equilibrated with Buffer A and washed with the same buffer. The nonadsorbed material was adjusted to 50 mM Tris-HCl (pH 8.0) and filtered through a 40-ml column of daunorubicin-Sepharose. The column was washed with 0.5 M NaCl and 50 mM Tris-HCl (pH 8.0) until no UV-adsorbing material could be detected in the effluent. DNR-BP 54 was eluted with 50 mM Tris-HCl (pH 8.0) and 0.5 M NaCl in 50% (v/v) ethylene glycol. This fraction was subjected to ultrafiltration under reduced pressure in dialysis bags suspended in 20 mM Tris-HCl, 0.15 M NaCl, and 1 mM EDTA (pH 7.5) to bring the protein concentration up to 1 mg/ml and to remove the ethylene glycol completely. In a parallel experiment, aniline-binding proteins were obtained with the same procedure, except that aniline-Sepharose was used instead of daunorubicin-Sepharose.

Coupling of DNR-BP 54 to agarose. DNR-BP 54 was coupled to *N*-hydroxysuccinimide-activated, cross-linked agarose (Affi-Gel 10, Bio-Rad Laboratories, Richmond, Calif.) at pH 8.0. Approximately 2 ml of

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[1] The abbreviations used are: DNR-BP 54, daunorubicin-binding protein of 54,000 M_r; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin.

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TABLE 1

Purification scheme for DNR-BP 54

Homogenate	0.05 M Tris-HCl, pH 8.0
High-speed supernatant	100,000 × *g*, 1 hr
$(NH_4)_2SO_4$ precipitation	0.30–0.65 saturation
Dialysis	10 mM Tris-HCl, pH 8.6
DEAE-cellulose	10 mM Tris-HCl, pH 8.6
Daunorubicin-Sepharose	0.05 M Tris-HCl, pH 8.0
Elution	0.05 M Tris-HCl, 0.5 M NaCl, and 50% (v/v) ethylene glycol or daunorubicin, 50 μg/ml

Affi-Gel 10, washed with distilled water, were added to 2.32 mg of DNR-BP 54 dissolved in 2 ml of 0.2 M $NaHCO_3$ and 0.5 M NaCl (pH 8.0), and the suspension was stirred overnight at 4°. The excess reactive groups were saturated as described for daunorubicin-Sepharose. The amount of protein bound to agarose was 0.81 mg/ml. Control Affi-Gel 10 was subjected to the same treatment except that DNR-BP 54 was omitted.

Binding of drugs to DNR-BP 54. The binding of drugs to DNR-BP 54 was qualitatively evaluated by their elution volumes from a column of DNR-BP 54-Affi-Gel. Forty microliters of each drug (0.212 mM) were applied to a column (0.7 × 5.5 cm) equilibrated with 20 mM Tris-HCl, 0.15 M NaCl, and 1 mM EDTA. The elution of the drug was monitored at the absorption maximum with a Zeiss spectrophotometer equipped with a recorder. The flow rate was kept constant by a peristaltic pump, and the elution volume (V) was calculated from the flow rate and the elution time of the drug peak. The dead volume was subtracted. The elution volumes of the drugs (V_0) from control Affi-Gel were obtained from an identical column of control Affi-Gel. V is expected to be similar to V_0 in the absence of detectable binding (i.e., $V/V_0 = 1$) or higher than V_0 ($V/V_0 > 1$) when interaction between drug and immobilized protein occurs.

Protein determination. Protein concentration was measured by absorbance at 280 nm, using $E^{1\%} = 16$ at 280 nm. This value was calculated by the plot of A_{280} of different preparations of DNR-BP 54 versus their protein content, determined by the method of Lowry *et al.* (13), using BSA as the standard.

Electrophoresis. SDS/polyacrylamide gel electrophoresis was performed as described by Laemmli (14) using molecular weight protein standards purchased from Bio-Rad Laboratories. Electrophoresis under non-denaturing conditions was run on a linear 5–20% polyacrylamide gradient (acrylamide to bisacrylamide ratio 19:1, w/w) in the presence of a 5–20% ethylene glycol gradient. Gel buffer and running buffer were the same as for SDS gels, except that SDS was omitted. The gradient was run for 64 hr at about 10 V/cm and at a temperature of 8°. Isoelectric focusing was performed on precast 5% polyacrylamide gel plates (LKB, Stockholm, Sweden), pH range 3.5–9.5. About 10 μg of DNR-BP 54, spotted on filter paper, were applied to the plate. The gels were run at a constant power of 80 mW/cm² (limiting voltage 1500 V) at 4° for different times up to 3 hr. A bovine red blood cell lysate was used as a marker. For glycoprotein staining on SDS gels, the periodic acid-Schiff reagent was used. Lipoprotein staining was performed, with Oil Red O, on preparations subjected to electrophoresis on cellulose acetate strips in barbital buffer (pH 8.2). The composition of the affinity fraction was quantitated by densitometric scanning of the stained gel with a Helena Quick Scan photodensitometer.

In vitro metabolism of daunorubicin. Five micrograms of DNR-BP 54 were incubated in a total volume of 20 μl with 0.18 mM daunorubicin and 100 μM NADPH in 0.2 M Tris-Hcl (pH 7.4) (15). Anaerobic conditions were obtained with a nitrogen atmosphere. After a 1-hr incubation at 37°, 15 μl of the reaction mixture were applied to a silica gel thin-layer chromatography plate and developed with $CHCl_3$/methanol/CH_3COOH (80:20:4, v/v).

Sedimentation. Sucrose gradient centrifugations were performed as

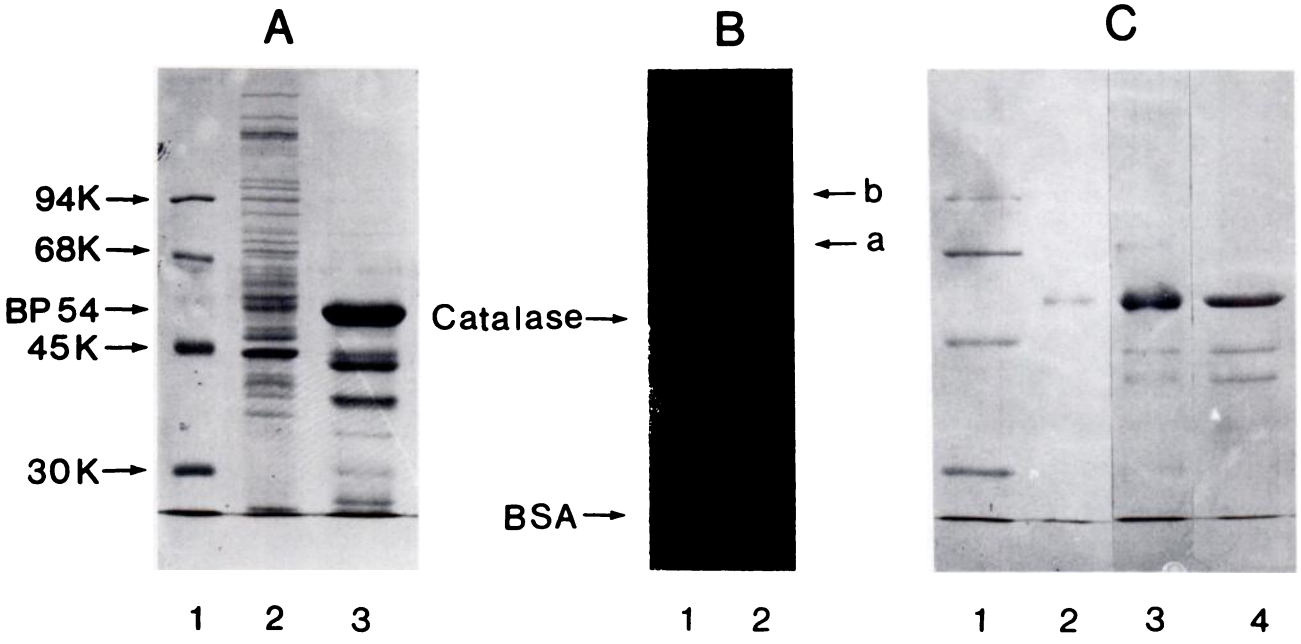

FIG. 1. *SDS gel electrophoresis*

A. SDS gel electrophoresis of the affinity fractions from aniline-Sepharose (Lane *2*) and daunorubicin-Sepharose (Lane *3*). Protein standards (Lane *1*) were as follows: phosphorylase *b* (94,000 M_r), BSA (68,000 M_r), ovalbumin (45,000) M_r), and carbonic anhydrase (30,000 M_r).

B. Gradient gel electrophoresis under non-denaturing conditions: Lane *1*, catalase and BSA; Lane *2*, affinity fraction from daunorubicin-Sepharose.

C. Rerun of bands *b* (Lane *2*) and *a* (Lane *3*) on SDS gel; lane 4, starting sample. Protein standards are as in A.

The samples run on gels A and B were from different preparations.

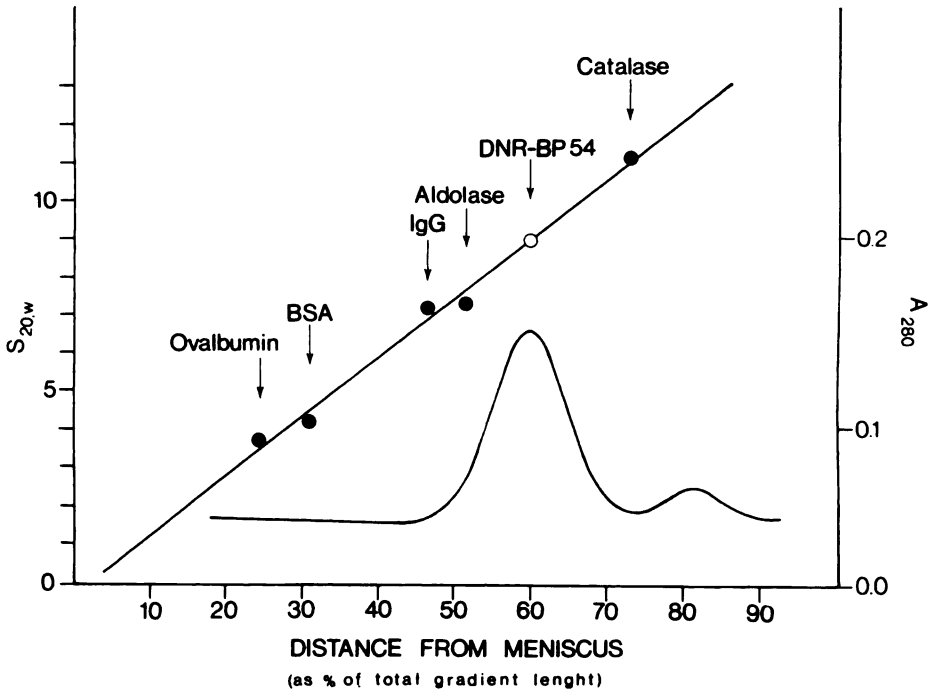

FIG. 2. *Sucrose gradient centrifugation of the affinity fraction from daunorubicin-Sepharose at high ionic strength (0.5 M NaCl)*
The gradients were fractionated with an ISCO Model 640 density fractionator, and the absorbance at 280 nm was continuously monitored with an ISCO UA-5 absorbance monitor equipped with a Type 6 optical unit. See text for details.

described by Martin and Ames (16). Sucrose gradients (5–20%) in 20 mM Tris-HCl, 0.5 M NaCl (0.15 M NaCl for binding experiments), and 1 mM EDTA (pH 7.5) were centrifuged at 114,000 × *g* at 4° for 16 hr with a Beckman SW 50.1 rotor. When binding studies were performed, drug (1 μg/ml) was added to the gradient. The drug concentration in gradient fractions was estimated by reading the fluorescence of the drug in a Perkin Elmer MPF-44A fluorescence spectrophotometer equipped with microcuvettes. The excitation wavelength was 500 nm, and the emission was monitored at 590 nm.

RESULTS AND DISCUSSION

The amount of protein recovered in the affinity fraction from 100 g of liver ranged from 2 to 4 mg. The polypeptide composition of average preparations is shown in Fig. 1A (Lane *3*) and C (Lane *4*). Although affinity fractions composed of a single polypeptide of 54,000 M_r were occasionally obtained, most preparations contained more components. The 54,000 M_r chain always represented more than 70% of the total protein content. The minor components had a lower M_r, and their relative amounts were different in each preparation. Although affinity chromatography theoretically could allow a one-step purification, contamination from nucleic acids, which bind daunorubicin, and protein-protein interactions, which could mask the binding sites of the protein, made the use of this technique on partially purified fractions more convenient. With the prepurification steps adopted, we obtained a fraction from which most of the non-daunorubicin-binding proteins, besides nucleic acids, had been removed. Elution of protein from the affinity column under non-denaturing conditions was formerly afforded by a daunorubicin solution (50 μg/ml in 50 mM Tris-HCl/0.5 M NaCl, pH 8) to compete out the binding of the adsorbed protein to daunorubicin-Sepharose. We obtained an almost complete elution, since very little material was recovered by further washing with 8 M urea. The elution by competition had some disadvantages. Because of the strong daunorubicin-daunorubicin interaction, the elution required a large amount of drug. In addition, the removal of daunorubicin from the eluted fraction required additional chromatography on Dowex 1. Therefore, a quantitative elution under non-denaturing conditions was routinely obtained using 50% ethylene glycol, which was readily removed by dialysis. Because of the lack of a specific and quantitative assay for this protein in crude fractions, the procedure adopted is purely qualitative, although we found only traces of daunorubicin/agarose-binding proteins in side fractions. Protein solutions formed precipitates during the concentration step. The same tendency was observed upon standing. This phenomenon was reduced but not eliminated by 0.5 M NaCl and low daunorubicin concentrations. The precipitate is probably due to formation of large aggregates and not to selective precipitation of different components, because the polypeptide composition of the precipitate was identical with the soluble fraction in SDS gel electrophoresis (data not shown).

The affinity fraction was further fractionated by electrophoresis on a polyacrylamide gradient gel under non-denaturing conditions in an attempt to define its protein composition. The stained gels (Fig. 1B, Lane *2*) showed a main band (*a*), migrating more slowly than catalase, followed by a thinner band (*b*). These bands were eluted from acrylamide and rerun by SDS gel electrophoresis (Fig. 1C, Lanes *2* and *3*), which showed that both contained the 54,000 M_r polypeptide and that the polypeptide composition of protein *a* (and also of protein *b*, although the lower molecular weight polypeptides did

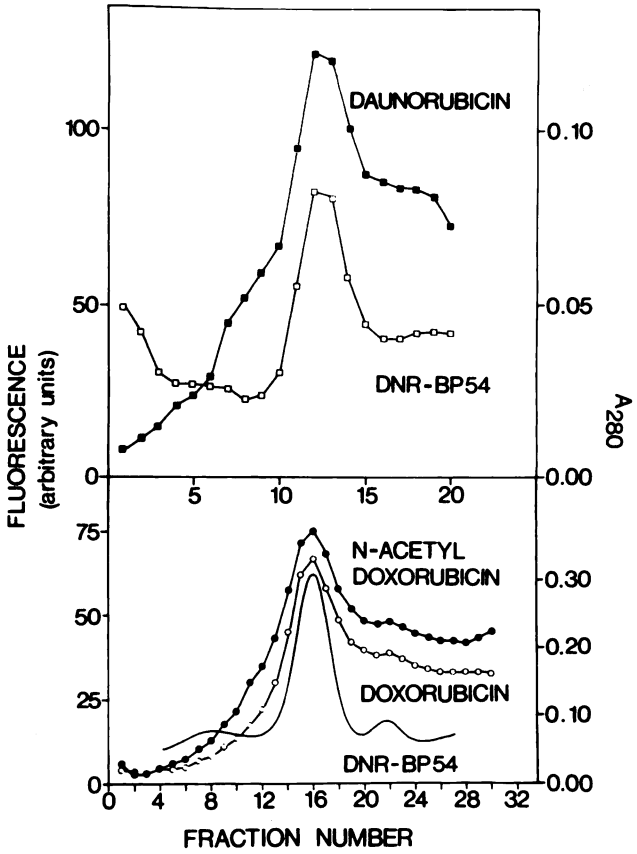

FIG. 3. *Cosedimentation of anthracyclines and DNR-BP 54*
Each gradient contained drug (1 μg/ml).
Top. Approximately 90 μg of DNR-BP 54 were loaded. On each fraction of the gradient the drug fluorescence and the A_{280} were measured. Bottom. Approximately 180 μg of DNR-BP 54 were loaded. The gradients were fractionated as described in Fig. 2. The two sedimentation patterns of DNR-BP 54 in the two gradients were exactly overlapping.

not show up in the picture) was identical with that of the starting sample; thus band *b* might well represent an aggregate and not a different protein. These data indicate that daunorubicin-agarose binds a single protein made up of subunits of 54,000 M_r, the lower molecular weight polypeptides probably being proteolytic fragments or tightly bound contaminants.

The molecular size of the native protein was estimated by sucrose gradient centrifugation at high ionic strength in order to minimize aggregation (Fig. 2). A symmetrical peak at about 9 S and a smaller fraction sedimenting at higher Svedberg values were observed. Although the sedimentation coefficient does not correlate directly with the molecular weight, it is reasonable to assume that DNR-BP 54 is composed of four polypeptides for a total of 216,000 M_r. The subunits are kept together by noncovalent bonds, since the dissociation did not require reduction with mecaptoethanol. Despite the smaller molecular size, the electrophoretic migration of DNR-BP 54 on a polyacrylamide gradient gel under non-denaturing conditions was slower than that of catalase. However, the finding that DNR-BP 54 did not bind to DEAE-cellulose indicated a low electric charge for this protein. A high basicity, which also would result in a low electrophoretic mobility at alkaline pH, had been excluded because of the isoelectric point of the protein, which fell between 7 and 7.5. A more precise value for the isoelectric point could not be obtained because the protein did not focus in 5% polyacrylamide gel, probably because of the low electrophoretic mobility.

DNR-BP 54 does not appear to have a relevant carbohydrate or lipid content, since it was negative to specific stains for carbohydrate and lipid (see Materials and Methods).

Evidence of the binding in solution between anthracyclines and DNR-BP 54 was obtained by gradient centrifugation. In these experiments, 90–180 μg of DNR-BP 54 in 150 μl were loaded on top of sucrose gradients containing daunorubicin (1 μg/ml), doxorubicin, and *N*-acetyl-doxorubicin in separate tubes (Fig. 3). All three drugs bound to DNR-BP 54 under these conditions. Both daunorubicin and doxorubicin have a charged amino group at pH 7.5; in contrast, *N*-acetyl-doxorubicin is uncharged. This means that the interaction between drug and protein is not of an electrostatic nature. Exact binding parameters for this interaction are not yet available.

To investigate the possibility that the binding to daunorubicin-agarose was due only to hydrophobic interaction through the antibiotic chromophore, a DEAE fraction was split, and one half was subjected to affinity chromatography and the other half to hydrophobic chromatography on aniline-agarose. The very different polypeptide pattern, shown in Fig. 1A, and the absence of the 54,000 M_r component in the fraction eluting from the aniline-agarose column indicated for this protein an actual affinity for daunorubicin and not only a simple hydrophobic interaction.

The binding specificity of DNR-BP 54 was investigated using some drugs with different molecular structures. Their binding to DNR-BP 54 was examined by affinity chromatography on agarose columns containing DNR-BP 54 covalently bound. The results are shown in Table 2. Although the binding parameters cannot be calculated from the presented data, it is apparent that anthracy-

TABLE 2
Binding of drugs to DNR-BP 54-agarose
Forty microliters of a solution containing each drug at the indicated concentration were loaded on a 2.1-ml column of DNR-BP 54-agarose. *V*, Elution volume (milliliters) from the affinity column; V_0, elution volume from control column. V_0 values at 0.21 mM and 0.42 mM were found to be identical. Although the V_0 values were different for the various drugs, thus suggesting a possible interaction between agarose and drugs, this would not impair the drug's binding to the immobilized protein.

Drug	V_0	V		V/V_0	
		0.21 mM	0.42 mM	0.21 mM	0.42 mM
Daunorubicin	2.8	4.8	3.9	1.7	1.4
Doxorubicin	2.9	4.6	3.7	1.6	1.3
N-Acetyl-doxorubicin	2.7	5.5	3.9	2.0	1.4
Tilorone	2.3	2.4	ND[a]	1.0	
Vincristine	1.8	2.0	2.0	1.1	1.1
Actinomycin D	1.4	1.6	1.6	1.1	1.1
Colchicine	1.7	1.9	ND	1.1	

[a] ND, Not determined.

clines were retarded on the DNR-BP 54 column in a concentration-dependent fashion, whereas the other drugs were eluted unretarded from the same column. This clearly indicated that only anthracyclines were capable of interacting with DNR-BP 54.

The isolation from rat liver extracts of an anthracycline-binding protein raises the question of whether this protein is one already known or one that we expect to bind to daunorubicin. To our knowledge, the only well-characterized protein that binds daunorubicin is tubulin (17), whose monomer has a molecular weight very similar to that of the DNR-BP 54 monomer. However, the sedimentation coefficients of native tubulin (5.8 S) (18) and native DNR-BP 54 are quite different, and, as shown in Table 2, DNR-BP 54 did not bind colchicine. In addition, binding experiments, performed by gel filtration as described (18), showed that DNR-BP 54 does not bind GTP and confirmed the lack of interaction with colchicine (data not shown). Other proteins that we would expect to bind anthracyclines are the enzymes involved in drug metabolism. Our attempts to reveal a transformation in daunorubicin incubated with DNR-BP 54 under the conditions described for *in vitro* metabolism (15) were unsuccessful. In addition, affinity chromatography of rat liver extracts containing daunorubicin reductase (19) showed that this enzyme does not bind to daunorubicin-Sepharose.

The possibility that DNR-BP 54 is involved in intracellular drug transport was also considered. It has been shown (20) that anthracyclines enter the cells by passive diffusion but are pumped out by an active transport mechanism. The same mechanism also appears to be involved in the extrusion of vinca alkaloids and seems to be responsible for the cellular cross-resistance between anthracyclines and vinca alkaloids. If DNR-BP 54 is part of this mechanism, then a competition of a vinca alkaloid for daunorubicin binding sites on the protein is to be expected. However, DNR-BP 54 did not bind vincristine, and the sedimentation pattern of daunorubicin in the presence of DNR-BP 54 was not modified by a 5-fold molar excess of this drug (data not shown).

Although the possibility of a function in drug transport or metabolism has not been completely ruled out by our experiments, DNR-BP 54 could have other functions as yet unknown which could shed more light on the molecular pharmacology of anthracyclines. Interest for drug targets other than nucleic acids has been raised by findings that antimitotic activity of anthracyclines is evident at doses that do not affect nucleic acid synthesis (21). The antimitotic activity is maintained by anthracyclines (22) characterized by a low affinity for DNA (i.e., $K_{app} \sim 10^4\ M^{-1}$, for *N*-acetyl derivatives) as compared with daunorubicin and doxorubicin ($K_{app} \sim 10^6\ M^{-1}$) (23), and it has been shown recently that *N*-acetyl derivatives of doxorubicin exhibit significant antitumor activity even though they do not intercalate into DNA (24). These observations suggest that part of the action of anthracycline might be mediated by interaction at different cellular levels. Since the interaction between tubulin and daunorubicin is mainly electrostatic (17), uncharged anthracyclines probably will not interfere with microtubule formation; thus their effects on cell proliferation remain unexplained. DNR-BP 54 binds both charged and uncharged anthracyclines, and this could be relevant if an action of this protein on mechanisms of cell proliferation could be demonstrated. Studies are in progress to elucidate the physiological role of the DNR-BP 54.

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