New Affinity Adsorbents Containing Deoxycytidine, Deoxyadenosine, or Deoxyguanosine and Their Interactions with Deoxynucleoside-Metabolizing Enzymes†

Seiichiro Ikeda, Inshik Park, Paul Gardner, and David H. Ives*

ABSTRACT: 3′-(4-Aminophenyl phosphate) derivatives of deoxycytidine (dCyd), deoxyadenosine (dAdo), and deoxyguanosine (dGuo) were synthesized. The inhibitory effects of these compounds on mammalian and bacterial deoxynucleoside kinases and several other deoxynucleoside-metabolizing enzymes were examined. The same derivatives were coupled to carboxyl-terminal Sepharose CL-6B (3–8 μmol of ligand/mL of gel), and each of the resulting affinity adsorbents was tested with various partially purified enzymes. Reasonable correlation between the inhibitory effect of a soluble deoxynucleoside 3′-phosphate diester and affinity of the corresponding Sepharose adsorbent for the enzyme was observed. Among the three dCyd kinases examined, only the bovine mitochondrial enzyme was adsorbed onto the dCyd–Sepharose column and eluted biospecifically by 1 mM dCyd (1400-fold purification). Its K_i toward the dCyd derivative was relatively low (1.1 mM), whereas no measurable inhibition was seen with mammalian cytosol or bacterial enzymes that did not stick to the column. The K_i of the dAdo derivative toward three dAdo kinases was more than 5 mM in each case, and none of these were retained by dAdo–Sepharose. Among the other dAdo-metabolizing enzymes examined, nucleoside phosphotransferase from barley (K_i = 1.2 mM) was adsorbed to dAdo–Sepharose at pH 5.0 and was biospecifically eluted with dAdo or AMP after suppressing ionic binding by adjusting the pH to 6.0 (480-fold purification to homogeneity). Mammalian mitochondrial dGuo kinase (beef liver) showed the lowest K_i (0.16 mM) among the enzymes tested and was biospecifically purified with dGuo–Sepharose (2800-fold purification). Bacterial dGuo kinase, with an intermediate K_i value (0.73 mM), exhibited only slight retardation upon chromatography through a long column of dGuo–Sepharose. Mammalian cytosol dGuo kinase, on the other hand, was not inhibited by the soluble dGuo analogue, nor did it show any affinity for the dGuo–Sepharose column. Among the various factors that reinforce the relatively weak biospecific binding of these immobilized substrate analogues by the active sites of enzymes, controlled ionic interactions seem particularly important.

The most critical factor in successful affinity chromatography is undoubtedly the strength of the interaction between the immobilized ligand and the macromolecule. Molecules with intrinsically high affinity for the enzymes are generally chosen as ligands. However, the ligand has to be derivatized in some way to be immobilized on the matrix, and this derivatization may well impair biospecific binding of enzyme sterically or electrostatically. Therefore, the site of derivatization of the ligand and the nature of the spacer arm inserted between ligand and matrix are crucial.

In spite of the many enzymes involving deoxynucleosides as substrates or products, only a few cases of affinity chromatography on adsorbents containing deoxynucleosides have been reported so far, and all of these have used thymidine derivatives. An affinity column containing 5′-amino-2′,5′-dideoxythymidine coupled directly to cyanogen bromide activated agarose was prepared by Rhode & Lezius (1971). Thymidine kinase from *Escherichia coli* retained by the column was eluted only with 0.5 M NaCl (200-fold purification). When a hexyl group was inserted between ligand and matrix, the enzyme was eluted biospecifically by thymidine, but the capacity of the column was drastically reduced. The authors suggest that in the former case ionic interactions between protein and supporting material may have increased the stability of the enzyme–ligand complex. Unfortunately, attempts to apply this column to the purification of thymidine kinase from mouse Sarcoma 180 (Cheng & Prusoff, 1974) or human (Lee & Cheng, 1976) cells were unsuccessful. Inhibition studies with various thymidine analogues revealed that considerable tolerance of bulky groups is allowed in the 3′- but not the 5′-position of thymidine as the substrate of the Sarcoma 180 enzyme (Cheng & Prusoff, 1974). On the basis of this observation, Kowal & Marcus (1976) have prepared thymidine 3′-(4-aminophenyl phosphate) coupled to carboxyhexyl-Sepharose. Although both a negatively charged phosphate and a bulky phenyl group were inserted right next to the 3′-position of thymidine, this affinity adsorbent proved useful in the purification of thymidine kinase from rat adenocarcinoma. It has also been used by Cheng's group to purify thymidine kinase from human cytosol and mitochondrial extracts, as well as enzyme expressed by Herpes simplex virus (Lee & Cheng, 1976; Cheng et al., 1977; Cheng & Ostrander, 1976). Most recently, this medium has been used in series with Blue Sepharose in the preparation of homogeneous thymidine kinase from human placenta (Gan et al., 1983). In each case of affinity chromatography with this thymidine derivative, a certain concentration of electrolyte was required along with thymidine to elute the adsorbed enzyme. These studies suggest several interesting practical questions to us: (1) Are all of the deoxynucleoside kinases as tolerant of bulky substituents at the 3′-position as the various thymidine kinases are? (2) Are these 3′-deoxynucleoside derivatives coupled to Sepharose generally useful for purifying other enzymes of deoxynucleoside metabolism? (3) To what extent do ionic interactions between the negatively charged 3′-phosphate group and cationic groups on the enzyme contribute to or interfere with enzyme retention and biospecific elution?

† From the Department of Biochemistry, The Ohio State University, Columbus, Ohio 43210. *Received October 5, 1983.* This work was supported by Grants PCM82-3003 from the National Science Foundation and GM26900 from the U.S. Public Health Service.

0006-2960/84/0423-1914$01.50/0

To answer these questions, we have prepared dCyd,[1] dAdo, and dGuo 3′-derivatives and have examined the interactions of the soluble or immobilized derivatives with 17 different deoxynucleoside-metabolizing enzymes.

Materials and Methods

5′-*O*-(4,4′-Dimethoxytrityl)-N^6-benzoyl-2′-deoxyadenosine and 5′-*O*-(4,4′-dimethoxytrityl)-N^4-benzoyl-2′-deoxycytidine were purchased from Boehringer Mannheim Biochemicals. Di-*p*-methoxytrityl chloride, *p*-nitrophenyl phosphorodichloridate, palladium on activated carbon (palladium content 1% or 10%), and 1,1′-carbonyldiimidazole were obtained from Aldrich Chemical Co. Sepharose CL-6B was from Pharmacia Fine Chemicals. 1-Ethyl-3-[3-(dimethylamino)propyl]-carbodiimide hydrochloride was obtained from Calbiochem. Tritiated deoxynucleosides were supplied by ICN and Amersham Corp. All other chemicals used were of reagent grade.

Bacterial deoxynucleoside kinases were from extracts of *Lactobacillus acidophilus* R-26 (ATCC 11506), partially purified through the ammonium sulfate fractionation step (Deibel & Ives, 1978). This fraction contains two deoxynucleoside kinases, one being dCyd/dAdo kinase and the other being dGuo/dAdo kinase. Each of these kinases is believed to have separate binding sites for its respective two substrates (Deibel et al., 1977). Mammalian cytosol dCyd kinase was partially purified from calf thymus extract, through the ammonium sulfate step (Ives & Wang, 1978). This enzyme also phosphorylates dAdo and dGuo but with higher K_m's than for dCyd. Beef liver mitochondrial extract was prepared as described for the calf thymus enzyme (Gower et al., 1979) and provided a source of mitochondrial dCyd and dGuo kinases. Nucleoside phosphotransferase was isolated from barley shoots and partially purified by ammonium sulfate and phosphocellulose fractionation (Prasher et al., 1982). Crude adenosine deaminase from calf intestinal mucosa was obtained from Sigma Chemical Co.

Enzyme Assays and Inhibition Studies. Deoxynucleoside kinases were assayed radiometrically under conditions described previously for *L. acidophilus* (Deibel & Ives, 1978), calf thymus cytosol (Ives & Wang, 1978) and bovine mitochondria (Gower, 1980). Nucleoside phosphotransferase was also assayed radiometrically (Prasher et al., 1982). Phosphodiesterase from *L. acidophilus* (Fiers & Khorana, 1963), nucleoside deoxyribosyltransferase from *L. acidophilus* (Cardinaud, 1978), and adenosine deaminase (Nygaard, 1978) were all assayed spectrophotometrically. In each case 1 enzyme unit is the amount of enzyme forming 1 nmol of product/min under the conditions specified. Proteins were assayed by the Bradford method (1976).

Determination of K_i values with dCyd-, dAdo-, or dGuo-3′-(4-aminophenyl phosphate) as competitive inhibitors was carried out at optimal pH for each enzyme, that is, at pH 8.0 for bacterial and mammalian cytosol kinases and for mitochondrial dCyd and dAdo kinases and at pH 5.0 for mitochondrial dGuo kinase and barley phosphotransferase. Two inhibitor concentrations, 0.2 and 1.0 mM, were used for each kinetic determination. When no measurable inhibition was observed with 1 mM inhibitor, the K_i was assumed to be more than 10 mM.

Synthesis of dCyd-, dAdo-, and dGuo-3′-(4-aminophenyl phosphates). The corresponding nitro compounds were prepared according to Fiers & Khorana (1963). In the case of the dCyd and dAdo derivatives, commercially available protected intermediates 5′-*O*-(4,4′-dimethoxytrityl)-N^4-benzoyl-2′-deoxycytidine and 5′-*O*-(4,4′-dimethoxytrityl)-N^6-benzoyl-2′-deoxyadenosine were used as starting materials. Deoxynucleoside 3′-(4-nitrophenyl phosphates) were then reduced catalytically to the 4-amino derivatives: 50 μmol of dAdo- or dGuo-3′-(4-nitrophenyl phosphate) was dissolved in 50 mL of anhydrous methanol, and 50 mg of 10% palladium/activated carbon was added. The mixture was hydrogenated in a Parr apparatus for 2 h at room temperature with 35 psi of hydrogen pressure. With the dCyd derivative, the hydrogenation was carried out at 50 psi for 30 min with 5 mg of 1% palladium/activated carbon per μmol of nitro compound. The modified conditions were necessary to avoid formation of side products. In either case, the nitro compound was quantitatively converted to the amino compound, judging from cellulose TLC with methyl ethyl ketone–acetone–water (65:20:15 v/v) as the solvent.

Preparation of Affinity Adsorbents. A carboxyl-terminal spacer arm was introduced into a matrix as follows: first ethylenediamine was coupled to Sepharose CL-6B activated with carbonyldiimidazole by the method of Bethell et al. (1979). Moderate substitution was achieved by using 3.6 mg of carbonyldiimidazole/mL of Sepharose. The concentration of amino groups, estimated by ninhydrin assay, was 8.2 μmol/mL of Sepharose. The aminoethyl-Sepharose was quantitatively succinylated according to the method of Cuatrecasas & Parikh (1972). When an affinity adsorbent with a relatively high ligand concentration was to be prepared, 18 mg of carbonyldiimidazole/mL of Sepharose was used to activate the gel (amine content: 49 μmol/mL of gel).

Coupling of dCyd-, dAdo- and dGuo-3′-(4-aminophenyl phosphate) to carboxy-derivatized Sepharose was accomplished by using 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC). To 5 mL of carboxy-Sepharose suspended in 10 mL of water, stoichiometric amounts of aminophenyl phosphate was added. EDC totaling a 4-fold molar excess was added in increments over a period of 2 h at room temperature, maintaining the pH at 4.75 by adding 0.1 M HCl. After an additional hour of stirring, the gel was washed with excess water and 1 M NaCl. Any remaining carboxyl groups left underivatized by nucleoside were masked by treating with 0.1 M ethanolamine and EDC, as above. After extensive washing, the gel was equilibrated with 0.1 M potassium phosphate buffer, pH 7.0, containing 0.02% sodium azide and stored at 4 °C.

The ligand concentrations of deoxynucleoside–Sepharose preparations were estimated by determination of inorganic phosphate content after total hydrolysis with $Mg(NO_3)_2$. The UV absorption spectra of the deoxynucleoside–Sepharose samples were measured directly, with underivatized Sepharose as a blank, by suspending the gel in 0.1 M potassium phosphate buffer, pH 7.0, containing 75% glycerol.

Screening experiments were conducted with each deoxynucleoside–Sepharose to determine whether it has any affinity for various deoxynucleoside-metabolizing enzymes. Bio-Rad minicolumns containing 0.5 mL of gel were equilibrated with various equilibration buffers (EB) containing 10% glycerol, as well as 5 mM 2-mercaptoethanol when mammalian enzymes were to be applied. Samples (0.1 mL) of each crude enzyme fraction dialyzed against EB were applied at 4 °C. The column was washed successively with 10 mL of EB, 5 mL

[1] Abbreviations: dCyd, deoxycytidine; dAdo, deoxyadenosine; dGuo, deoxyguanosine; C, cytosine; A, adenine; Hx, hypoxanthine; AMP, adenosine 5′-monophosphate; UMP, uridine 5′-monophosphate; EDTA, ethylenediaminetetraacetate; HPLC, high-pressure liquid chromatography; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane.

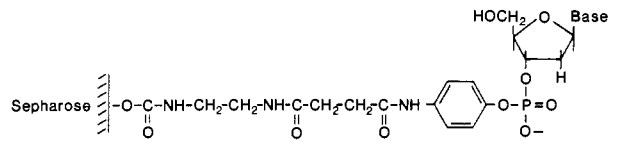

FIGURE 1: Structure of dCyd–, dAdo–, or dGuo–Sepharose.

of 1 mM deoxynucleoside in EB, and 5 mL of 1 M NaCl plus 1 mM deoxynucleoside in EB. After use, the gels were washed extensively with 1 M NaCl in 20 mM Tris-HCl (pH 7.5), 1% Triton X-100 in buffer, and 6 M guanidine hydrochloride.

Results

Characterization and Stability of the Affinity Adsorbents. Carbonyldiimidazole was chosen as the means of attaching spacer arms to Sepharose because the linkage is both stable and nonionic. The concentration of carboxyl termini was easily controlled between 8.2 and 49 μmol/mL of gel by varying the amount of carbonyldiimidazole in the activation step. The hydrophilic character of the spacer arm (Figure 1) was achieved by coupling ethylenediamine to the activated gel, followed by succinylation. The coupling of dCyd-, dAdo-, or dGuo-3′-(4-aminophenyl phosphate) to the carboxyl-derivatized Sepharose was almost stoichiometric, judging from the disappearance of the deoxynucleoside derivative from the supernatant of the reaction suspension. After thorough washing, total phosphate analyses of aliquots of gel were carried out to provide more accurate estimates of ligand concentrations. For the three preparations with moderately low concentrations of dCyd, dAdo, and dGuo, the phosphate contents were found to be 7.4, 7.8, and 3.0 μmol/mL of gel, respectively. These were the preparations used in most of the experiments reported in this paper. For certain experiments, however, high ligand concentrations of dCyd, dAdo, and dGuo were required, and the corresponding phosphate contents were 41.8, 49.2, and 38.2 μmol/mL of gel, respectively.

The UV absorption spectrum of dGuo–Sepharose (moderate substitution) is shown in Figure 2. A weighed sample of dGuo-3′-(4-aminophenyl phosphate) gave an estimated ϵ_{max} = 27 700 at 240 nm. Using this, in turn, to estimate the dGuo concentration on the gel gave a value of 1.1 μmol/mL of gel, which is considerably lower than the results of phosphate analysis. Attachment to Sepharose particles produced a 10-nm shift in the wavelength maximum, so the ϵ_{max} of the soluble derivative may not be an accurate standard for determining absolute substituent concentrations. An alternative explanation for the discrepancy might be that purine, but not phosphate, was lost during the coupling reaction, but we regard this as quite unlikely.

It is known that crude extract of *L. acidophilus* R-26 contains significant amounts of phosphodiesterase activity and that dCyd-, dAdo-, and dGuo-3′-(4-nitrophenyl phosphates) are all good substrates (Fiers & Khorana, 1963). Since there is the possibility that this, or some other enzyme, activity might destroy the Sepharose-bound deoxynucleoside, the UV spectrum of the gel was examined before and after the chromatography with each crude enzyme preparation. In no case was a detectable change in absorbance observed, indicating that the base moiety of the immobilized ligand was not released by phosphodiesterase, nucleoside deoxyribosyltransferase, or hydrolytic nucleosidase activities. Although dAdo is a good substrate for adenosine deaminase from calf intestinal mucosa, no deamination of dAdo-3′-(4-aminophenyl phosphate) could be detected. We infer from this that dAdo–Sepharose should be stable to deaminase activities found in mammalian or bacterial extracts, and this inference is also supported by the absence of spectral changes in the bound ligand after chromatography.

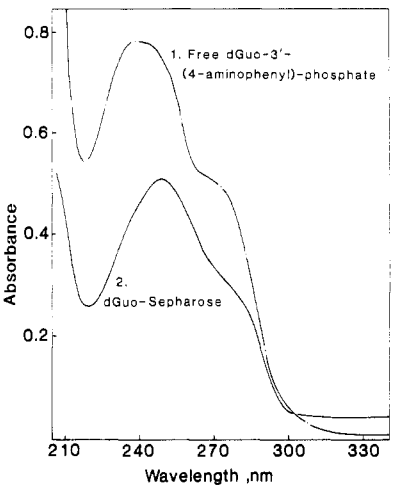

FIGURE 2: UV absorption spectra of free and Sepharose-bound dGuo-3′-(4-aminophenyl phosphate). Curve 1: 27.8 μM free compound in 0.05 M sodium phosphate buffer, pH 7.0. Curve 2: 20 mg of moist dGuo–Sepharose suspended in 2 mL of 0.05 M sodium phosphate buffer, pH 7.0, containing 75% glycerol.

These affinity adsorbents were found to be stable upon storage at 4 °C and have been used repeatedly for more than 1 year.

Correlation of Competitive Inhibition by Soluble Deoxynucleoside Derivatives and Binding to Affinity Columns. Seventeen different deoxynucleotide-metabolizing enzyme activities were tested for competitive inhibition by the soluble dCyd-, dAdo-, or dGuo-3′-(4-aminophenyl phosphates) at optimal pH for each enzyme. Each enzyme activity was also tested for ability to bind to the appropriate affinity adsorbent at two pHs, 8.0 (or 7.0) and 5.5 (or 5.0). This was a means of screening for the possible importance of ionic interactions or pH dependence of biospecific interactions between immobilized ligand and enzyme. The results are summarized in Table I, along with K_m values determined in the course of these studies or obtained from the literature where noted.

Crude extract of *L. acidophilus* R-26 contains, in relatively high specific activities, phosphodiesterase [1.5, 2.1, and 3.9 nmol min^{-1} mg^{-1} for dCyd-, dAdo-, and dGuo-3′-(4-nitrophenyl phosphates), respectively] and nucleoside deoxyribosyltransferase (5.5 and 1.6 nmol min^{-1} mg^{-1} for dCyd to adenine and dIno to adenine transfer, respectively), compared to deoxynucleoside kinase activities (0.65, 0.18, and 1.36 nmol min^{-1} mg^{-1} for dCyd, dAdo, and dGuo, respectively). It was found, however, that these two enzymes (five activities) ran through any of our affinity adsorbents under the conditions employed. This is consistent with the fact that none of the three immobilized ligands were attacked by any of these enzyme activities. A similar result was obtained with adenosine deaminase. This enzyme neither deaminates nor binds to dAdo–Sepharose, in spite of its relatively low K_m (0.023 mM) for dAdo. Although K_i's were not determined for these enzymes, the derivatization of the deoxynucleosides and insertion of the spacer arm appear to have weakened the interaction between ligands and enzymes. Holguin & Cardinaud (1975) have successfully purified two nucleoside deoxyribosyltransferases of different specificities from *Lactobacillus helveticus* by means of affinity chromatography on purine or pyrimidine derivatives attached to Sepharose. In any of the deoxyribosyl transfer reactions catalyzed by these enzymes, bases were shown to have lower K_m's than their deoxynucleoside counterparts (Cardinaud, 1978), suggesting that bases can be expected to be the better ligands

Table I: Inhibition of Various Enzymes by Deoxynucleoside Derivatives and Results of Affinity Chromatography

substrate	enzyme[i]	source[j]	K_m (μM)	K_i (μM)[a]	K_i/K_m	interaction with adsorbent: retention	interaction with adsorbent: biospec elution	purifcn factor, x-fold
dCyd	kinase	*L. acidophilus*	2.9	NI[b]	>4500	−	−	
	kinase	calf thymus cyto	3.7	NI	>2700	−	−	
	kinase	beef liver mito	13	1100	85	+	+	1400
	phosphodiesterase	*L. acidophilus*	(630)[c]	n.d.[d]		−	−	
	dR-transferase	*L. acidophilus*	(90)[e]	n.d.		−	−	
dAdo	kinase	*L. acidophilus*	2.8	NI	>3500	−	−	
	kinase	calf thymus cyto	330	5000	15	−	−	
	kinase	beef liver mito	430	8900	21	−	−	
	phosphodiesterase	*L. acidophilus*	(590)[c]	n.d.		−	−	
	dR-transferase	*L. acidophilus*	(450, 120)[f]	n.d.		−	−	
	deaminase	calf intestine	(28)[g]	n.d.		−	−	
	phosphotransferase	barley epicotyls	130	1200	9.2	+[h]	+	480
dGuo	kinase	*L. acidophilus*	6.1	730	120	±[h]		
	kinase	calf thymus cyto	310	NI	>32	−	−	
	kinase	beef liver mito	30	160	5.3	+	+	2800
	phosphodiesterase	*L. acidophilus*	(280)[c]	n.d.		−	−	
	dR-transferase	*L. acidophilus*		n.d.		−	−	

[a] Inhibitor dissociation constant for corresponding deoxynucleoside 3′-(4-aminophenyl phosphate). [b] No inhibition observed with the 1.0 mM derivative (K_i > 10 mM). [c] K_m for corresponding deoxynucleoside 3′-(4-nitrophenyl phosphate), obtained by Fiers & Khorana (1963). [d] n.d. = not determined. [e] Data for *L. helveticus* deoxyribosyltransferase, dCyd → A (Cardinaud, 1978). [f] Data for *L. helveticus* deoxyribosyltransferase, dAdo → Hx, dAdo → C (Cardinaud, 1978). [g] From Coddington (1965). [h] Enzyme retarded by column but not retained. [i] dR = deoxyribosyl. [j] cyto = cytosol; mito = mitochondrial.

for affinity chromatography of this enzyme.

In more than half of the cases examined in our inhibition studies, some degree of competitive inhibition by soluble deoxynucleoside derivatives was observed. By comparing the K_i value with the K_m for the normal substrate and with the results of attempted affinity chromatography, the enzymes tested can be divided into three categories. In the first, the derivatization dramatically lowered the affinity of the nucleoside for enzyme (K_i/K_m > 2000; K_i > 10 mM). Due to the high K_i values, no affinity was observed between enzymes and adsorbent in spite of extremely low K_m's for substrates. Bacterial dCyd kinase and dAdo kinase and mammalian cytosol dCyd kinase belong to this group. Their active sites may surround substrate so tightly, especially at the 3′-position, that deoxynucleoside derivatives carrying a bulky group at this position are not tolerated. Another possible explanation for failure of enzyme to bind is charge repulsion between the ionized phosphate group of the nucleoside derivative and a negatively charged amino acid residue near the active site.

The second group of enzymes exhibited relatively large K_m's (>0.3 mM) for the natural substrates and very large K_i's (>5 mM) for the nucleoside derivatives. Although the impact of derivatization (K_i/K_m = 20–50) is much smaller than in the first group, these enzymes showed no significant affinity for the adsorbents because of the large values of K_i. Mammalian cytosol dAdo and dGuo kinase and mitochondrial dAdo kinase fall into this category.

Enzymes in the third group have relatively low K_m's toward their natural substrates (ca. 0.1 mM), and the effects of derivatization are moderate (K_i/K_m = 10–100). As a consequence, the K_i's within this group are the lowest among all of the enzymes tested. Mammalian mitochondrial dCyd kinase and dGuo kinase and barley nucleoside phosphotransferase belong in this category. All three enzymes were selectively retained on one of the affinity adsorbents and biospecifically eluted, resulting in large purification factors; the details of these purifications will be presented below. Clearly, a relatively low K_i value for a 3′-nucleoside phosphate derivative is a necessary prerequisite for retention of an enzyme on a column substituted with the same nucleoside. However, inasmuch as

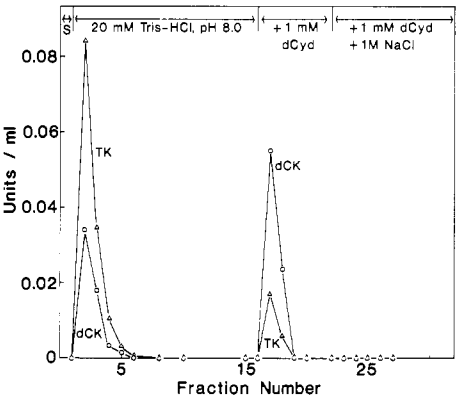

FIGURE 3: Affinity chromatography of dCyd kinase from beef liver mitochondria on dCyd–Sepharose. A 1 cm diameter column containing 4 mL of dAdo–Sepharose was equilibrated with 20 mM Tris-HCl (pH 8.0) containing 5 mM 2-mercaptoethanol and 15% glycerol. A 1-mL sample of crude beef liver mitochondrial extract, containing 0.26 unit of dCyd kinase and 0.66 unit of dThd kinase, was dialyzed against the equilibration buffer and applied to the column; 1.5-mL fractions were collected.

bacterial dGuo kinase, which has a fairly low K_i toward the dGuo derivative (0.73 mM), was not retained on dGuo–Sepharose, low K_i alone is not sufficient to ensure binding. Additional steric or electrostatic effects may be introduced by attachment of the spacer arm.

Affinity Chromatography of Mitochondrial Deoxycytidine Kinase on dCyd–Sepharose. Figure 3 shows the elution profile of beef liver mitochondrial dCyd kinase at pH 8.0. About 70% of the total dCyd kinase activity in crude extract applied was retained on the column. Upon addition of 1 mM dCyd to the washing buffer, 45% of the total activity was eluted with a 1400-fold purification factor. Addition of 1 M NaCl to the washing buffer failed to release any more activity, indicating that the column interaction with enzyme is entirely biospecific under these conditions, with a minimum of ionic forces being involved. The dCyd kinase eluted by 1 mM dCyd also phosphorylated thymidine. After extensive dialysis of this fraction, the dCK/dTK ratio remained similar to that of crude extract or the run-through fraction (ca. 3.0). These results

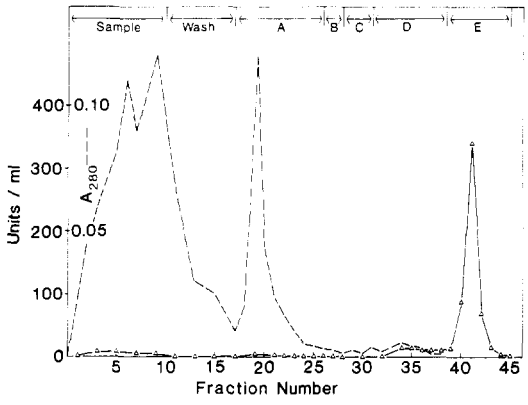

FIGURE 4: Affinity chromatography of nucleoside phosphotransferase from barley on dAdo–Sepharose. A 1-mL column of dAdo–Sepharose was equilibrated with 50 mM sodium acetate, pH 5.0. A sample of an ammonium sulfate fraction of barley epicotyl extract, containing 4.4 mg of protein (1500 units) and dissolved in the same buffer, was applied. Washing and elution were carried out as follows: Wash with 0.05 M sodium acetate, pH 5.0, and (A) 0.05 M sodium acetate (pH 5.0) plus 0.2 M NaCl, (B) 0.05 M sodium acetate, pH 5.0, (C) 0.05 M potassium phosphate, pH 6.0, (D) 0.05 M potassium phosphate (pH 6.0) plus 0.2 M NaCl, or (E) 15 mM AMP in 0.05 M potassium phosphate (pH 6.0) plus 0.2 M NaCl. The enzyme assay mixture contained [^{3}H]dAdo (1 mM), UMP (50 mM), sodium acetate (pH 5.0, 0.025 M), $MgCl_2$ (0.5 mM), and Triton X-100 (0.001%). In addition to these final assay concentrations were the contributions of individual fractions, which comprised half of the assay volume. The specific activity of pooled fractions 40–42 was 480 times that of the sample.

are precisely congruent with the observations of Cheng's group (Cheng et al., 1977; Lee & Cheng, 1976), who found that thymidine eluted both dCyd and dThd kinases of human mitochondria from a column of dThd-3′-(4-aminophenyl phosphate) bound to Sepharose. Taken together, these parallel experiments on exactly analogous media provide very convincing evidence that a single mammalian mitochondrial enzyme phosphorylates both dCyd and dThd as suggested by Leung et al. (1975).

When affinity chromatography of mitochondrial dCyd kinase on dCyd–Sepharose was carried out at lower pH (5.5), almost 90% of the dCK activity was retained (results not shown). However, very little activity was released upon addition of dCyd, whereas 1 M NaCl eluted 85% of the total activity. These results suggest that at the lower pH, ionic interaction with the column (i.e., ion exchange) is too strong to allow elution of enzyme by substrate competition alone, and reduced biospecificity of elution can be expected with salt as the elutrient. A similar situation was encountered when another dCyd–Sepharose preparation with a very high ligand concentration (42 μmol/mL of gel) was used at pH 8.0. Most of the dCK activity was retained but was eluted only when 1 M NaCl was added to the washing buffer.

Affinity Chromatography of Nucleoside Phosphotransferase on dAdo–Sepharose. Barley seedling nucleoside phosphotransferase has been purified to homogeneity in this laboratory by conventional procedures (Prasher et al., 1982). Although it phosphorylates all the normal nucleosides, the transferase shows the lowest K_m for dAdo (0.087 mM). Its pH–activity profile is very broad, spanning the range from pH 4.5 to pH 8.5.

Figure 4 shows the results of affinity chromatography of nucleoside phosphotransferase on dAdo–Sepharose, under what appear to be optimal conditions. The fraction precipitated by ammonium sulfate between 40 and 80% saturation (1520 units, 4.4 mg) was applied to dAdo–Sepharose equilibrated with 50 mM sodium acetate, pH 5.0. All of the phosphotransferase activity was retained by the column, whereas most of the contaminating protein was washed out with equilibration buffer. Then the buffer was changed to 50 mM potassium phosphate, pH 6.0, and, with the addition of 0.2 M NaCl to this buffer, additional protein impurities were removed. Finally, 65% of the total phosphotransferase activity was eluted biospecifically by adding 15 mM AMP to the buffered NaCl solution. Either nucleoside or nucleotide can be used to elute the nucleoside phosphotransferase, owing to the single-site Ping Pong kinetic mechanism of the enzyme (Prasher et al., 1982), but as a practical consideration, the nucleotide causes less interference with the radiochemical assay. A 480-fold increase in specific activity, resulting in a single band of protein and activity upon nondenaturing acidic polyacrylamide gel electrophoresis. Sodium dodecyl sulfate gel electrophoresis resulted in a major band of about 25 000 daltons and a leading minor band several thousand daltons smaller. This is exactly the type of pattern that has been obtained with conventionally purified fractions, probably due to variations in glycosylation (Prasher et al., 1982).

When this affinity chromatography was carried out on dAdo–Sepharose equilibrated with 50 mM potassium phosphate buffer at pH 6.0 or 7.0, the retention of enzyme by the column was poor in comparison with its performance at pH 5.0, although biospecific elution by 10 mM AMP in the equilibration buffer was equally efficient. Apparently, at pH 5.0, stronger electrostatic interaction between this rather basic enzyme and the negatively charged affinity column takes place in addition to the biospecific binding. It should be noted here that the soluble dAdo-3′-(4-aminophenyl phosphate) produced a noncompetitive inhibition pattern vs. dAdo, in contrast to the competitive patterns seen with the kinases, and the value of the apparent K_i increases in proportion to the phosphate donor concentration. This is because of the ordered nature of the Ping-Pong kinetic mechanism, with nucleoside and nucleotide binding to different forms of the enzyme, and the ligand is being treated here as a nucleotide. It is interesting, however, that the K_i is independent of pH between 5 and 7 (1.3 mM at 2 mM AMP). Thus, it seems clear that the biospecific interaction of ligand and active site is constant, but it can be supported to any desired degree through electrostatic binding by changing the pH, so as to achieve maximum biospecificity.

When the elution by AMP was attempted at pH 5, in the absence of additional salt in the equilibration buffer, no enzyme activity was eluted. This suggests that at this pH, the electrostatic interaction between enzyme and column must be suppressed if biospecific elution is to take place. This could be accomplished in two ways: (1) increasing the concentration of competing ions by raising the concentration of salt and (2) by raising the pH to reduce the charge on the protein, since it would be closer to its isoelectric pH. At pH 5, 0.2 M NaCl added to the equilibration buffer removed most of the protein but none of the enzyme activity. However, the enzyme did not elute sharply from the column at this pH when AMP concentrations as high as 30 mM were added to the 0.2 M buffered salt solution. On the other hand, 0.4 M salt caused half of the enzyme to leak from the column before AMP was added. Both methods provide satisfactory purification factors, but the protocol shown in Figure 4 provided optimal recovery of enzyme in the smallest volume and concentration of AMP.

Affinity Chromatography of Beef Liver Mitochondrial Deoxyguanosine Kinase on dGuo–Sepharose. Mitochondrial deoxyguanosine kinase was first identified in calf thymus tissue in our laboratory (Gower et al., 1979). However, a much more

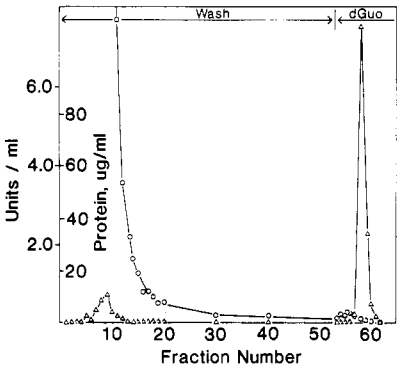

FIGURE 5: Affinity chromatography of beef liver mitochondrial dGuo kinase on dGuo–Sepharose. A 30-mL column (1.5-cm diameter) of dGuo–Sepharose was equilibrated with equilibration buffer (50 mM sodium acetate, pH 5.5, 20% glycerol, 10 mM 2-mercaptoethanol, 1.0 mM EDTA, and 0.05 mM phenylmethanesulfonyl fluoride). A sample of crude mitochondrial supernatant extract (58 units, 48 mg) was dialyzed against equilibration buffer and applied to the column. After washing with 10 volumes of equilibration buffer, the enzyme was eluted with 1 mM dGuo in the same buffer, with a purification factor of 2800-fold over the crude extract and recovery of 93%. (○) Protein; (△) dGuo kinase.

abundant source is found in beef liver mitochondria, which have been used for extensive purification and characterization of the enzyme (Gower, 1980). Unlike the broadly specific dCyd/dAdo/dGuo kinase of cytosol, the mitochondrial enzyme phosphorylates neither dCyd nor dAdo. It exhibits a sharp optimum at pH 5.0, in contrast to the broad activity plateau of the thymus cytosol enzyme, which ranges from pH 6 to pH 10.

Initially, the affinity chromatography of the mitochondrial dGuo kinase was carried out at pH 7.5 because of the lability of purified enzyme at more acidic pH values. More than 60% of the total dGuo kinase activity applied ran through the column, but the retained activity was eluted biospecifically by 1 mM dGuo with a 690-fold purification factor. Reapplication of the run-through fraction to the regenerated column resulted in a very similar pattern of run through and retention, indicating that incomplete retention was due simply to unfavorable equilibrium conditions or else to limited capacity of the adsorbent.

The discovery that 20% glycerol increases the stability of the enzyme dramatically (at 4 °C $T_{1/2}$ = 6 days) impelled us to attempt affinity chromatography at a pH closer to the optimum. As may be seen in Figure 5, most of the activity was retained at pH 5.5. Washing with 10 column volumes of equilibration buffer brought off a large peak of protein, and the enzyme activity was then eluted quantitatively with 1 mM dGuo. A small peak of protein partially overlapped the activity peak, suggesting the presence of another protein, besides dGuo kinase, which can be eluted biospecifically from dGuo–Sepharose. Obviously, bringing the pH closer to the optimum resulted in an enhancement of the interactions between enzyme active sites and immobilized substrate. Nonspecific electrostatic bonds do not seem to play a major role here, since no additional electrolyte was required prior to enzyme elution by substrate.

The specific activity increment of the peak fraction was about 2800. Polyacrylamide gel electrophoresis of this fraction revealed that the activity was coincident with the one of the several protein bands that could be detected by silver staining the gel.

Other Variables Affecting Affinity Chromatography. In those cases where affinity chromatography was unsuccessful in this study, the problem seemed to be that specific interaction between an enzyme and immobilized ligand was too weak, as reflected in a high K_i value for the derivatized deoxynucleoside. In an attempt to compensate for these unfavorable binding equilibria, adsorbents with much higher concentrations of each substituent (35–50 μmol/mL of gel) were prepared and tested with the same group of enzymes. Unexpectedly, none of these three media showed significant enhancement in biospecific binding of any of the enzymes. Instead, ionic interaction between enzyme and adsorbent became predominant.

Another attempt to strengthen the affinity of an enzyme for immobilized ligand was by adding ATP or MgATP to the equilibration buffer. This was based on kinetic analysis of bacterial dCyd kinase, which showed that the Michaelis constant for dCyd in the presence of saturating ATP is some 40 times smaller than the dissociation constant in the absence of ATP (Deibel et al., 1977). Surprisingly, in no case did the ATP or MgATP improve the affinity.

Discussion

Theoretical guidelines for effective affinity chromatography based on simple kinetic and equilibration models of the affinity adsorption and desorption events have been proposed by Graves & Wu (1974). They predict that adequate affinity would be produced if the ligand concentration (L_0) during the adsorption step is more than 10 times the ligand–enzyme dissociation constant (K_i'). If the ligand concentration on the adsorbent is, say, 10 mM, the critical K_i' would have to be about 1 mM or less.

In this study we have examined the apparent dissociation constants (K_i) of several derivatized substrates relative to a variety of deoxynucleoside-metabolizing enzymes. These K_i values will not necessarily be equal to the theoretical K_i' values mentioned above, because the insertion of a spacer arm and immobilization could change the effective equilibrium constants. Moreover, the K_i values were determined at 25 °C, while chromatography was necessarily carried out at 4 °C because of the lability of most of these enzymes. Lowe & Dean (1974b) have pointed out that adsorption generally decreases with increasing temperature when there is a decrease in enthalpy during the adsorptive process. In some cases, however, where a favorable free energy of binding is due primarily to an increase in entropy, binding may actually improve with increasing temperatures. Therefore, the effects of temperature would have to be determined for individual cases. We have compared the behavior of dGuo kinase (*L. acidophilus*) at 4 and 20 °C and observed no change in the tendency of the activity to lag slightly behind bulk protein as it emerges from the dGuo–Sepharose column. These limitations notwithstanding, our results indicate that there is reasonable correlation between the apparent K_i for a deoxynucleoside 3′-phosphate derivative and the affinity of the immobilized ligand for enzyme. In all six cases where the apparent K_i was found to be more than 5 mM, no affinity was observed between enzyme and adsorbent (L_0 = 3–8 mM). On the other hand, practical retention by, and biospecific elution from, the affinity adsorbent was observed in the three cases where the apparent K_i was relatively low (≤1 mM).

Our attempts to compensate for weak affinity between enzyme and immobilized ligand by increasing L_0 up to 50 mM failed. If we assume that immobilized ligands are uniformly distributed in space on the affinity column and located at each corner of units in a cubic lattice (Lowe & Dean, 1974a), the distance between neighboring ligands is calculated to be 69.2 or 32.1 Å at 5 mM or 50 mM ligand concentrations, respectively. Most globular proteins fall into this range of diameters (ca. 50 Å). At a 50 mM ligand concentration, the enzyme

might interact with multiple charged ligands. This situation may well explain why ionic interactions became dominant over biospecific forces when affinity adsorbents with high ligand concentrations (30–50 mM) were used. From this point of view, the derivatization of ligands with an ionic linker chain may not be desirable when making affinity adsorbents with high ligand concentrations. About 5 mM seems to be the highest practical ligand concentration for these affinity adsorbents bearing ion-exchange groups.

In one group of enzymes we tested (bacterial dCyd and dAdo kinases and mammalian cytosol dCyd kinase) the K_i's for the 3′-hydroxyl derivatives were drastically increased over the K_m's of the unaltered substrates. Apparently bulk tolerance is limited at this position of the substrate, or the negative charge on the phosphate group of the derivatized nucleosides may have caused electrostatic interference with binding to the active sites of the enzymes. Krenitsky et al. (1976) examined the K_m or K_i values of various nucleoside analogues as they interacted with calf thymus cytosol dCyd kinase. Nucleosides containing bulky hydrophobic substituents on either the base or the pentose moiety were shown to be relatively potent competitive inhibitors. Derivatization of deoxynucleoside substrates at the base or insertion of an uncharged spacer arm at the 3′-position of deoxynucleoside substrates might give rise to more efficient affinity adsorbents for this group of enzymes. In view of the effective use of the immobilized 3′-phosphate derivative of thymidine in the purification of thymidine kinase from mammalian cytosol and mitochondria, as well as the Herpes virus enzyme, it is surprising that the structurally analogous dCyd- and dGuo–Sepharose retained only the mitochondrial dCyd and dGuo kinases. The reasons why the mammalian cytosol or bacterial enzymes are not retained are unclear at present.

We have noticed that enzymes tend to stick to our affinity adsorbents more efficiently at pH 5–5.5 than at pH 7–8. It would be difficult, however, to ascribe this to a general effect of lower pH on the affinity of enzymes for immobilized ligands. In one case (mammalian mitochondrial dCyd kinase on dCyd–Sepharose), the increased retention of the enzyme by the affinity adsorbent at pH 5.5 was due mainly to enhanced nonspecific electrostatic interaction. The bulk of the retained enzyme was eluted by NaCl, whereas the portion eluted biospecifically was unchanged. In another case (mammalian mitochondrial dGuo kinase on dGuo–Sepharose), a change in pH from 7.5 to 5.0 apparently strengthened biospecific forces but not ionic interaction, since the retained enzyme was eluted at either pH with only 1 mM dGuo without any additional salt. However, the amount of enzyme retained was increased at the acidic pH. We infer that this is due to pH dependence of the K_m, because of a very sharp pH optimum at pH 5 when determined under nonsaturating substrate concentrations. In the third case (nucleoside phosphotransferase on dAdo–Sepharose), biospecific interaction between enzyme and ligand is apparently constant between pH 5 and pH 7, judging from the constant K_i, whereas electrostatic binding increases as the pH is lowered. These electrostatic forces may supplement the relatively weak biospecific binding when the oppositely charged groups are sterically positioned to reinforce association of the ligand with the active site. As Scopes (1982) has suggested, these nonbiospecific interactions seem to be a necessary evil if an enzyme exhibiting such weak binding at the active site is to be adsorbed. It may seem curious that all of the activity is retained when added to the affinity column at pH 5 and also with subsequent adjustment to pH 6.0, whereas substantial leakage of enzyme is observed when passed onto the column at pH 6.0. This anomaly may be explained by noting that we have evidence from cation-exchange HPLC suggesting strong protein–protein interaction between the phosphotransferase and another protein at pH 6 and above but not at pH 5.0 (P. Gardner and D. H. Ives, unpublished results). Thus, this interaction may partially block the active site or cationic groups, preventing normal affinity binding seen at pH 5.

It is apparent from these studies that some degree of ionic interaction is essential to support relatively weak biospecific forces (K_i values of the order of 1 mM), whereas ionic forces are unnecessary, or even undesirable, when the K_i value is an order of magnitude smaller.

References

Bethell, G. S., Ayers, J. S., Hancock, W. S., & Hearn, M. T. W. (1979) *J. Biol. Chem. 254*, 2572–2574.

Bradford, M. (1976) *Anal. Biochem. 72*, 248–254.

Cardinaud, R. (1978) *Methods Enzymol. 41*, 446–455.

Cheng, Y.-C., & Prusoff, W. H. (1974) *Biochemistry 13*, 1179–1185.

Cheng, Y.-C., & Ostrander, M. (1976) *J. Biol. Chem. 251*, 2605–2610.

Cheng, Y.-C., Domin, B., & Lee, L.-S. (1977) *Biochim. Biophys. Acta 481*, 481–492.

Coddington, A. (1965) *Biochim. Biophys. Acta 99*, 442–451.

Cuatrecasas, P., & Parikh, I. (1972) *Biochemistry 11*, 2291–2299.

Deibel, M. R., Jr., & Ives, D. H. (1978) *Methods Enzymol. 41*, 346–354.

Deibel, M. R., Jr., Reznik, R. B., & Ives, D. H. (1977) *J. Biol. Chem. 252*, 8240–8244.

Fiers, W., & Khorana, H. G. (1963) *J. Biol. Chem. 238*, 2780–2788.

Gan, T. E., Brumley, J. L., & Van Der Weyden, M. B. (1983) *J. Biol. Chem. 258*, 7000–7004.

Gower, W. R., Jr. (1980) Ph.D. Dissertation, The Ohio State University.

Gower, W. R., Jr., Carr, M. C., & Ives, D. H. (1979) *J. Biol. Chem. 254*, 2180–2183.

Graves, D. J., & Wu, Y.-T. (1974) *Methods Enzymol. 34*, 140–163.

Holguin, J., & Cardinaud, R. (1975) *Eur. J. Biochem. 54*, 505–514.

Ives, D. H., & Wang, S.-M. (1978) *Methods Enzymol. 41*, 337–345.

Kowal, E. P., & Markus, G. (1976) *Prep. Biochem. 6*, 369–385.

Krenitsky, T. A., Tuttle, J. V., Koszalka, G. W., Chen, I. S., Beacham, L. M., Rideout, J. L., & Elion, G. B. (1976) *J. Biol. Chem. 251*, 4055–4061.

Lee, L.-S., & Cheng, Y.-C. (1976a) *J. Biol. Chem. 251*, 2600–2604.

Lee, L.-S., & Cheng, Y.-C. (1976b) *Biochemistry 15*, 3686–3690.

Leung, W.-C., Dubbs, D. R., Trkula, D., & Kit, S. (1975) *J. Virol. 16*, 486–497.

Lowe, C. R., & Dean, P. D. G. (1974a) in *Affinity Chromatography*, pp 40–43, Wiley, London, New York, Sydney, and Toronto.

Lowe, C. R., & Dean, P. D. G. (1974b) in *Affinity Chromatography*, p 50, Wiley, London, New York, Sydney, and Toronto.

Nygaard, P. (1978) *Methods Enzymol. 41*, 508–512.
Prasher, D. C., Carr, M. C., Ives, D. H., Tsai, T.-C., & Frey, P. A. (1982) *J. Biol. Chem. 257*, 4931–4939.
Rhode, W., & Lezius, A. G. (1971) *Hoppe-Seyler's Z. Physiol. Chem. 352*, 1507–1516.
Scopes, R. (1982) in *Protein Purification—Principles and Practice*, pp 111–124, Springer-Verlag, New York, Heidelberg, and Berlin.

cis- and *trans*-Diamminedichloroplatinum(II)-Mediated Cross-Linking of Chromosomal Non-Histone Proteins to DNA in HeLa Cells†

Zainy M. Banjar,‡ Lubomir S. Hnilica,* Robert C. Briggs, Janet Stein, and Gary Stein

ABSTRACT: The cross-linking of chromosomal non-histone proteins to DNA in isolated nuclei or intact HeLa cells exposed to different concentrations of *cis*- and *trans*-diamminedichloroplatinum(II) (*cis*- and *trans*-DDP) for various time intervals was investigated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunochemical methods. Both the *cis*- and the *trans*-DDP cross-linked significant numbers of chromosomal non-histone proteins to the DNA. The quantity and the types of the cross-linked proteins depended on the time of incubation as well as on the concentrations of the drugs. The immunochemical techniques revealed that both the 0.35 M sodium chloride insoluble and soluble chromosomal non-histone proteins were cross-linked to the DNA by both isomers. The action of *cis*- and *trans*-DDP was reversed and/or blocked by thiourea or 2-mercaptoethanol. Pretreatment of isolated nuclei or chromatin with iodoacetamide or *N*-ethylmaleimide did not prevent the DNA–protein cross-linking.

Since Rosenberg et al. (1965) reported the growth-inhibitory effects of the platinum complexes in bacteria, several investigators have tested this class of compounds for their antitumor activity (Roberts & Thomson, 1979). Because of its clinical applications in human cancer chemotherapy, *cis*-diamminedichloroplatinum(II) (*cis*-DDP)[1] and its biologically inactive isomer *trans*-diamminedichloroplatinum(II) (*trans*-DDP) have been subjected to intensive investigations. Cellular DNA is the principal target of this drug, and the interaction is of a bifunctional nature in a manner analogous to bifunctional alkylating agents. However, there is disagreement as to whether the critical lesion is an inter- or intrastrand DNA cross-linking (Roberts & Thomson, 1979; Shooter et al., 1972; Pascoe & Roberts, 1974). While extensive work has been done on the interaction of *cis*-DDP with DNA (Zwelling & Kohn, 1980), very little is known about the interaction of *cis*-DDP with proteins. Recent literature indicates that in addition to intra- and interstrand cross-linking of DNA, the platinum coordination complexes could also form covalent protein–DNA cross-links (Zwelling et al., 1979; Lippard, 1982; Filipski et al., 1983; Banjar et al., 1983). In this paper, we used SDS–polyacrylamide gel electrophoresis and immunochemical methods to investigate the nature of the protein–DNA cross-links in isolated nuclei or intact live HeLa cells exposed for various times and to different concentrations of *cis*- as well as *trans*-DDP. Our results show that both isomers cross-linked significant numbers of chromosomal non-histone proteins to DNA in isolated nuclei or intact cells and that the quantity and the quality of the cross-linked proteins is time as well as concentration dependent. Furthermore, the immunochemical methods showed that both the 0.35 M NaCl insoluble and soluble chromosomal non-histone proteins cross-linked to the DNA, suggesting that both types of proteins are closely associated with the DNA and within the cross-linking distance of the two isomers. Thus, in addition to its importance in human cancer chemotherapy, *cis*-DDP and its biologically inactive isomer can be used to probe chromatin structure.

Materials and Methods

Cell Culture. HeLa cells (S3 strain) were maintained in suspension cultures in Eagle's (1959) minimum essential medium as modified by W. Joklik and supplemented with 3.5% each of calf and fetal calf serum.

Nuclei Isolation. Cells in cultures were harvested by centrifugation at 1200 rpm for 10 min. Nuclei were isolated by homogenizing the cells in 0.25 M sucrose/0.01 M Tris-HCl, pH 7.5, and centrifuging at 660*g* for 10 min. The crude nuclei were further purified by homogenization in 2.2 M sucrose/0.01 M Tris-HCl/5 mM $MgCl_2$, pH 7.5, and centrifugation at 110000*g* for 1 h. Finally, nuclear pellets were homogenized in 0.25 M sucrose/0.01 M Tris-HCl 0.5% (v/v) Triton X-100, pH 7.5, until dispersed, and nuclei were sedimented by centrifugation at 1000*g* for 10 min. Triton X-100 was removed by washing the nuclei in 0.25 M sucrose/0.01 Tris-HCl, pH 7.5, followed by centrifugation at 1000*g* for 10 min. All solutions used for the isolation of nuclei contained 0.1 mM phenylmethanesulfonyl fluoride (PMSF) and were used at 0–4 °C.

Drug Treatment. cis-DDP and *trans*-DDP (Sigma) were dissolved in water with gentle heating and stirring and used within 2 h. Suspension of cells (1×10^8 cells) in fresh serum-free medium or corresponding amounts of nuclei in 1 mM

† From the Department of Biochemistry and the A. B. Hancock, Jr. Laboratory of the Vanderbilt University Cancer Center, Vanderbilt University School of Medicine, Nashville, Tennessee 37232 (Z.M.B., L.S.H., and R.C.B.), and the Department of Biochemistry and Molecular Biology, University of Florida School of Medicine, Gainesville, Florida 32610 (J.S. and G.S.). *Received August 3, 1983; revised manuscript received November 29, 1983.* This work was supported by a project funded through NIEHS Grant ES 00267, NSF Grant PCM-18075 (to G.S.), and a grant from King Abdulaziz University (to Z.M.B.).

‡ Present address: School of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia.

[1] Abbreviations: *cis*- and *trans*-DDP, *cis*- and *trans*-diamminedichloroplatinum(II); SDS, sodium dodecyl sulfate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PAP, peroxidase–antiperoxidase; PMSF, phenylmethanesulfonyl fluoride; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/84/0423-1921$01.50/0