Biochemical Pharmacology, Vol. 27, pp. 1571–1576.

0006-2952/78/0601-1571/$02.00/0

NATURE AND REACTIVITY OF PLATINUM–PYRIMIDINE BLUES WITH BIOMACROMOLECULES

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(*Received* 22 *August* 1977; *accepted* 14 *November* 1977)

Abstract—The reaction of platinum–pyrimidine complexes with a range of biomacromolecules was studied using circular dichroic (CD) and viscometric techniques. The reaction of platinum–uracil blue with DNA is characterized by a small increase in the positive CD transition at 275 nm and a large decrease in the amplitude of the negative band at 245 nm; these data are consistent with a change to a conformational state characteristic of the premelt stage as well as of partial denaturation respectively. Concomitant with denaturation, the viscosity of DNA changes from 74 to 45 dl/g. Part of this interaction with DNA may involve intercalation of the platinum–uracil complex since it will competitively displace ethidium bromide from the DNA. Alternatively, the displacement of ethidium bromide may be the result of denaturing the nucleic acid. The platinum complex also reacts instantaneously with single and double stranded synthetic nucleic acids, RNA, whole ribosomes and proteins. Platinum–uracil causes nearly complete destruction of the secondary structure of bovine serum albumin which was used as a model for protein interaction. Using computer analysis of the CD data, the helical content of serum albumin decreased from 70 to 10 per cent. Carbon-13 nuclear magnetic resonance (^{13}C-n.m.r.) spectra of the platinum–thymine blue complex revealed that this complex consists of a multiplicity of isomers. Because of this and the complex nature of its reactions with selected biomacromolecules, it is doubtful that a precise mechanism of action for antitumor activity can be established.

There is now convincing evidence that coordination complexes involving platinum-group metals represent a new class of potent antitumor agents [1]. Several years ago Rosenberg *et al.* [2] discovered the antitumor activity of *cis*-dichlorodiammine platinum (II) and a number of structural analogues. Succeeding work by many laboratories [3] has advanced the development of this first drug to the degree that it is currently used as a chemotherapeutic agent. The general features of its mechanism of action are characterized by the following considerations as outlined by Davidson *et al.* [1]: (1) the complexes exchange only some of their ligands; (2) the complexes are initially neutral; (3) the geometries of the complexes are either square planar or octahedral; (4) two *cis*-monodentate leaving groups are required whose atomic centers are separated by some 3.3 Å; and finally (5) the ligands *trans* to the leaving groups should be strongly bonded and exchange inert.

Some difficulties arise with the use of *cis*-dichlorodiammine Pt (II) since it is cytotoxic at low concentrations particularly with respect to renal toxicity [1]. In contrast to *cis*-dichlorodiammine platinum (II), platinum–pyrimidine complexes are minimally toxic and display high water solubility while still possessing potent antitumor activity [1]. The mechanism of their selective action is not understood. *In vitro* studies have pointed to a specific interaction of the platinum–pyrimidine complexes with nucleic acids, their reaction with protein being slower by a factor of at least 100 [4]. This specificity has been further demonstrated through the use of these complexes as nucleic acid stains for electron microscopy [5–7]. It has been postulated [4] that the drug increases host immunity by reacting with DNA associated with the cytoplasmic membrane of the tumor cell. This DNA, which is thought to be weakly antigenic and associated only with cells which are supposedly tumorigenic, in some way masks strong antigenic sites, thus protecting the cell against a host immune response. The reaction of the drug with this surface DNA is thought to remove the masking effect. The precise chemical structure of these pharmacologic agents is also unknown. Elemental analysis is consistent with the presence of one pyrimidine and two ammonia molecules per platinum [1].

We present evidence in this communication that platinum–uracil blue undergoes an extensive interaction with nucleic acids and proteins as well. The result is a dramatic disruption of the structure of both types of macromolecules. In addition, the platinum–thymine complex is shown to exist as a mixture of isomeric forms.

MATERIALS AND METHODS

All compounds were kindly analyzed for C, H, N and PtO by Merrell National Laboratories (Cincinnati, OH). Elemental analysis gave the following percentages: Pt–thymine blue, C:H:N:PtO (14.72:2.74:14.73:47.65); Pt–uracil blue, C:H:N:PtO (10.99:2.35:13.95:50.84).

Platinum–uracil blue and platinum–thymine blue were prepared according to the method of Davidson *et al.* [1]. These compounds were stored prior to use in powdered form under vacuum desiccation in the dark at 4°C.

Calf thymus DNA was obtained from Worthington (Freehold, New Jersey); single and double stranded synthetic nucleic acids, tRNA and ethidium bromide were purchased from Sigma (St. Louis, Missouri). Ribosomes were isolated from the blood of anaemic mice according to the method of Lingrel, as described in [8]. All other chemicals were of the highest purity commercially available.

Circular dichroic (CD) studies. CD spectra were obtained at 25° with a Cary 61 spectropolarimeter. Standardization of the spectropolarimeter was accomplished using a 1 mg/ml aqueous solution of camphor-sulfonic-d-10 acid as specified by Varian Associates (Palo Alto, California). All spectra were recorded in cylindrical cells of 10-mm path length, using a full scale deflection of 0.05 deg. Ellipticity is expressed as molecular ellipticity: $[\theta]_\lambda^{25} = (\theta/10)(M/lc)$ with units of deg-cm²/dmole, where θ is the observed ellipticity in degrees, M is the molecular weight (average nucleotide molecular weight), l is the path length in cm and c is the concentration in g/ml. Molecular ellipticities are not corrected for the refractive index of the solvent.

All CD spectra were performed in 0.01 M sodium phosphate buffer, pH 7.4. Nucleic acid concentrations were determined by measuring the u.v. absorbance on a Cary 15 spectrophotometer at 25°. Molar extinction coefficients used for DNA, poly A and poly I:poly C were 6050, 8600 and 4900 respectively. The extinction coefficient used for ribosomes was 10 A_{260} units/mg and for tRNA was 18 A_{260} units/mg. All CD spectra were recorded immediately after addition of the platinum–uracil.

Carbon-13 nuclear magnetic resonance. ^{13}C proton-decoupled spectra, $^{13}C[^1H]$, were obtained on a Varian CFT-20 n.m.r. spectrometer operating at an observed frequency of 20.0 MHz and employing deuterium as internal lock. All chemical shifts are reported relative to tetramethylsilane (TMS). Samples of thymine and platinum–thymine blue were dissolved in 99.7% deuterium oxide (Merck, St. Louis, Missouri) to yield solutions of approximately 0.1 M. After dissolution, Pt–thymine was centrifuged in order to remove residual undissolved compound.

Viscosity studies. Viscosity measurements were made with a three-bulb viscometer of the Ubbelohde type with average shear gradients, $\bar{G}$, from 70 to 20 sec^{-1}. Viscosities were observed at 25° in 0.01 M sodium phosphate buffer, pH 7.4, and then extrapolated to both zero concentration and zero rate of shear ($\bar{G}$). Extrapolation to zero rate of shear was made by plotting relative viscosity (η_{rel}) vs $\bar{G}$. Intrinsic viscosity, $[\eta]$, defined by $\lim_{c \to 0} \left(\ln \frac{\eta_{rel}}{c}\right)$, was provided by a plot of η_{rel}/c vs c of DNA in g/dl.

RESULTS

The interaction of platinum–uracil blue with nucleic acids was studied by circular dichroism. CD spectra were observed in the region from 210 to 300 nm where nucleic acids exhibit intrinsic Cotton effects mainly due to exciton coupling of neighboring bases [9]. Any disturbance to the DNA helical structure will be reflected in an altered CD. In all cases any ellipticity contributed by platinum–uracil has been subtracted from the spectra.

Figure 1 shows the CD spectrum of calf thymus DNA in the presence and absence of platinum–uracil. Platinum–uracil undergoes an instantaneous reaction with DNA causing a small increase in the positive peak at 275 nm and a large decrease in the amplitude of the negative peak at 245 nm using a Pt–uracil to phosphate ratio of 1.3:1. Since thermal denaturation results in a decreased ellipticity throughout this region, the decrease seen here in the 245 nm peak is consistent with denaturation of the DNA. The small increase in the positive peak is in the opposite direction of a complete denaturation of the DNA. However, the increase is in the direction of and about the same magnitude as a DNA premelt stage [10].

This conclusion was further supported by a shear experiment in which the viscosity of DNA was

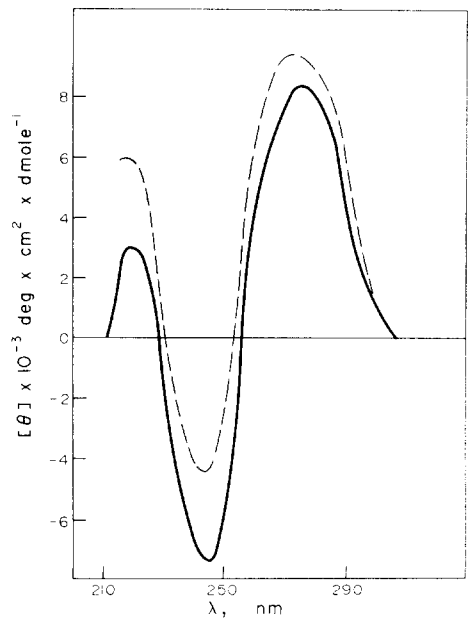

Fig. 1. Ultraviolet CD spectrum of DNA before (solid line) and after (dashed line) reaction with Pt–uracil. Conditions were: 0.01 M sodium phosphate, pH 7.4; [DNA] = 2.5×10^{-4} M in phosphorus; [Pt–uracil] = 3.2×10^{-4} M; Pt/P = 1.3. Not shown in the figure was a separate experiment in which ethidium bromide was added to the DNA before reaction with Pt–uracil. Conditions were the same except: [ethidium bromide] = 6.3×10^{-5} M; EB/P = 0.25. Ellipticity was observed at 310 nm.

measured with and without Pt–uracil at a Pt–uracil: P ratio of 1:1. The intrinsic viscosity of native DNA was found to be 74 dl/g and decreased to 45 dl/g in the presence of Pt–uracil. Thermally denatured DNA has an intrinsic viscosity of 4.5 dl/g [11].

Because of its aromatic ligand, Pt–uracil must be considered as a candidate for intercalative binding to nucleic acids. To examine this possibility, ethidium bromide was added to DNA and the CD observed at 310 nm. Ethidium bromide itself has no optical activity but acquires several bands, including one at 310 nm, when bound to DNA [12]. The molar ellipticity at 310 nm, at a concentration of one ethidium bromide per four nucleotide residues, was 13,000. Adding platinum–uracil to the solution (2 Pt: 1 P) results in a decrease in ellipticity to 9500.

Platinum–uracil was also shown to react with RNA. As indicated in Fig. 2, a marked decrease in the ellipticity of transfer RNA occurs in the presence of platinum–uracil, clearly in the direction of denaturation.

Cis-dichlorodiammine platinum (II) has been shown [13, 14] to exhibit G-C specificity in its interactions with DNA. In order to determine if platinum–uracil has any base specificity in its interactions with nucleic acids, identical CD experiments were performed on a number of synthetic compounds. Included in the study were the single stranded nucleic acids, poly A, poly U, poly C and poly G. Figure 3 shows the results with poly A (others not shown). With poly A, as well as with the other three single stranded polymers, there appears to be a dramatic decrease in helical structure. No significant differences were observed in the degree of decreased ellipticity among the homopolymers used indicating that no preferential interactions had occurred with Pt–uracil. Two double-stranded nucleic acids were also included. Poly I: poly C, shown in Fig. 4, also resulted in a decreased structure,

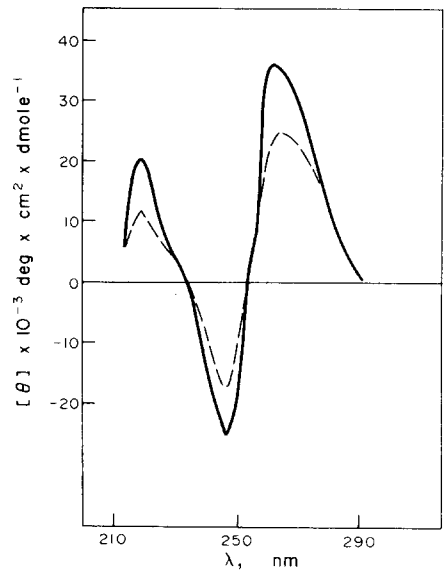

Fig. 3. Ultraviolet CD spectrum of poly A before (solid line) and after (dashed line) reaction with Pt–uracil. Conditions were: 0.01 M sodium phosphate, pH 7.4; [poly A] = 7.2×10^{-5} M; [Pt–uracil] = 6.5×10^{-5} M; Pt/P = 0.9.

although not of the same magnitude as that observed for the single stranded polymers. Qualitatively similar results were obtained with poly A: poly U (data not shown).

Pt–uracil has been shown to stain ribosomes intensely when examined by electron microscopy. For this reason, ribosomes were isolated and their interaction with Pt–uracil was studied by CD. The results are given in Fig. 5. The positive CD peak of the ribosomes is primarily due to its RNA while the negative peak is primarily due to the backbone of ribosomal proteins [15]. Therefore, the results sug-

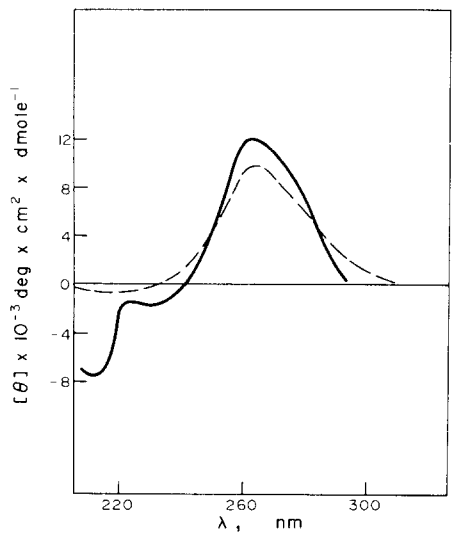

Fig. 2. Ultraviolet CD spectrum of RNA before (solid line) and after (dashed line) reaction with Pt–uracil. Conditions were 0.01 M sodium phosphate, pH 7.4; [tRNA] = 5.5×10^{-5} M; [Pt–uracil] = 8.1×10^{-5} M; Pt/P = 1.5.

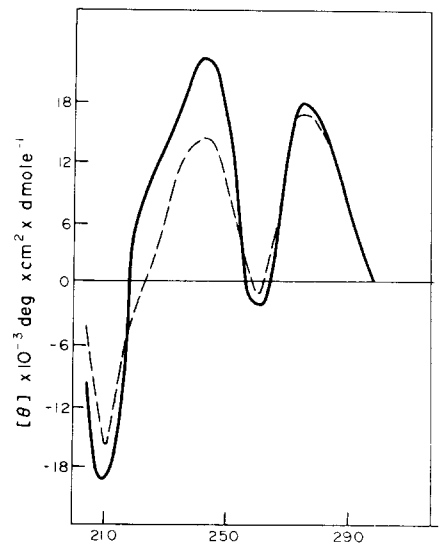

Fig. 4. Ultraviolet CD spectrum of poly I: poly C before (solid line) and after (dashed line) reaction with Pt–uracil. Conditions were: 0.01 M sodium phosphate, pH 7.4; [poly I: poly C] = 5.7×10^{-5} M; [Pt–uracil] = 8.1×10^{-5} M; Pt/P = 1.4.

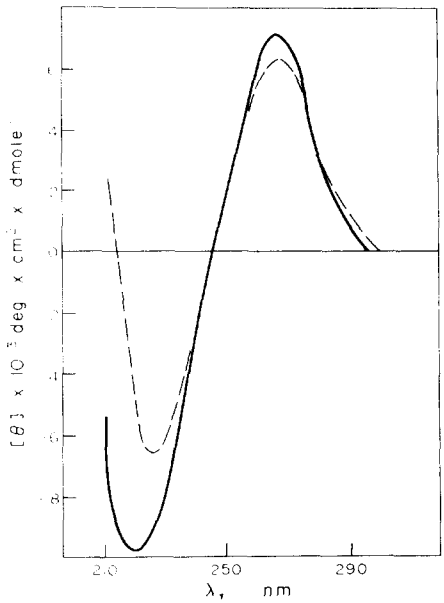

Fig. 5. Ultraviolet CD spectrum of whole ribosomes before (solid line) and after (dashed line) reaction with Pt–uracil. Conditions were: 0.01 M sodium phosphate, pH 7.4; [ribosomes] = 0.064 mg/ml; [Pt–uracil] = 8.1×10^{-5} M; Pt/P = 0.8 assuming 50 per cent of the ribosomes consist of nucleic acid.

gest a greater breakdown in the secondary structure of the protein than of the RNA. However RNA–protein interactions existing in ribosomes make drawing such conclusions difficult. In order to overcome this ambiguity, ribosomal RNA and ribosomal protein were individually purified, and the experiment was repeated separately on each (data not shown). The ribosomal protein, which has a CD minimum at 224 nm, underwent a 30 per cent loss in ellipticity at the same relative concentrations while the ribosomal RNA CD changed very little.

To examine more closely the reaction of Pt–uracil with protein, the experiment was performed using bovine serum albumin (Fig. 6). Pt–uracil causes an almost complete destruction of the protein secondary structure. Changes in secondary structure were quantitated by applying the CD data of Fig. 6 to computer analysis [16] in order to determine the amount of helical and β-structure. The results, shown in Fig. 6, reveal a reduction in the α-helical content from 70 to 10 per cent at a platinum to amino acid residue ratio of 2:1 (no β-structure was present in any of the spectra). No difference in rate could be detected by the present method; the reaction with both nucleic acids and proteins was complete in a matter of seconds.

In order to determine the nature of the chemical structure of the platinum–pyrimidine, an analysis of the ^{13}C-n.m.r. of platinum–uracil blue was attempted. This compound, however, showed a complex envelope of carbon resonances in the C-5 and C-6 positions which were very poorly resolved. Moreover, the carbonyl resonances were even less informative. For these reasons, plantinum–thymine blue was synthesized and the methyl region subsequently analyzed using ^{13}C-n.m.r. Platinum–thymine blue was shown to have similar reactivity as platinum–uracil blue toward biomacromolecules as studied by CD.

Panels A and B of Fig. 7 represent the ^{13}C[^{1}H] n.m.r. spectra of 0.1 M thymine and Pt–thymine blue compounds in D_2O respectively. The methyl resonance of 0.1 M thymine is centered at 13.17 ppm whereas the Pt–thymine methyl region is resolved into a minimum of two distinct resonances centered at 13.55 and 12.48 ppm. The absence of a discernible C-6 resonance in the Pt–thymine complex indicates that neither of the two resolved methyl resonances are due to the presence of free thymine. This is in accord with the elemental analyses as well as with previous results of the ^{1}H-n.m.r. spectrum of the methyl region of Pt–thymine which shows a broadened ^{1}H methyl resonance relative to that observed for free thymine [1].

DISCUSSION

The data presented here are consistent with a nonselective reaction of Pt–pyrimidine complexes with a variety of biomacromolecules. ^{13}C-n.m.r. of the platinum complex itself is consistent with the presence of multiple chemical species. These observations confirm previous reports involving the heterogeneous nature of platinum–pyrimidine reaction products [17].

Pt–pyrimidine reactions with DNA result in a partial denaturation of DNA as judged by both CD and viscometric techniques. It is clear that Pt–uracil causes some degree of destabilization of DNA but generally not as extreme as a complete helix → coli

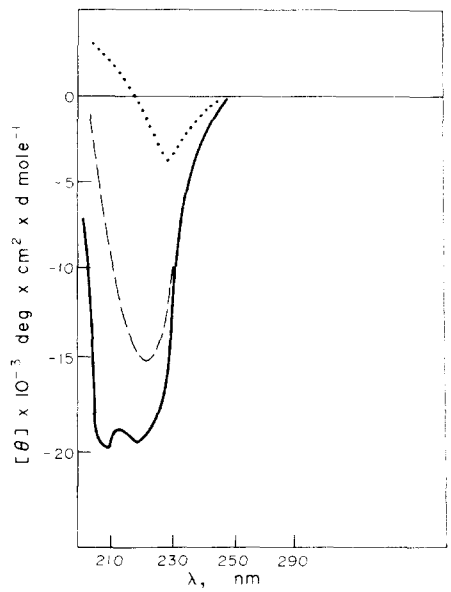

Fig. 6. Far u.v. CD spectrum of bovine serum albumin (BSA) before (solid line) and after reaction with Pt–uracil. Conditions were: 0.01 M sodium phosphate, pH 7.4; [BSA] = 2.8×10^{-7} M; [Pt–uracil] = 8.1×10^{-5} M (dashed line), and 3.2×10^{-4} M (dotted line). BSA in the absence of Pt–uracil (solid line) was estimated to have 70% α-helix and no β-pleated sheet. Adding Pt–uracil to a Pt/residue ratio of 0.5 (dashed line) and 2.0 (dotted line) resulted in a decrease in the α-helix to 55 and 10 per cent respectively.

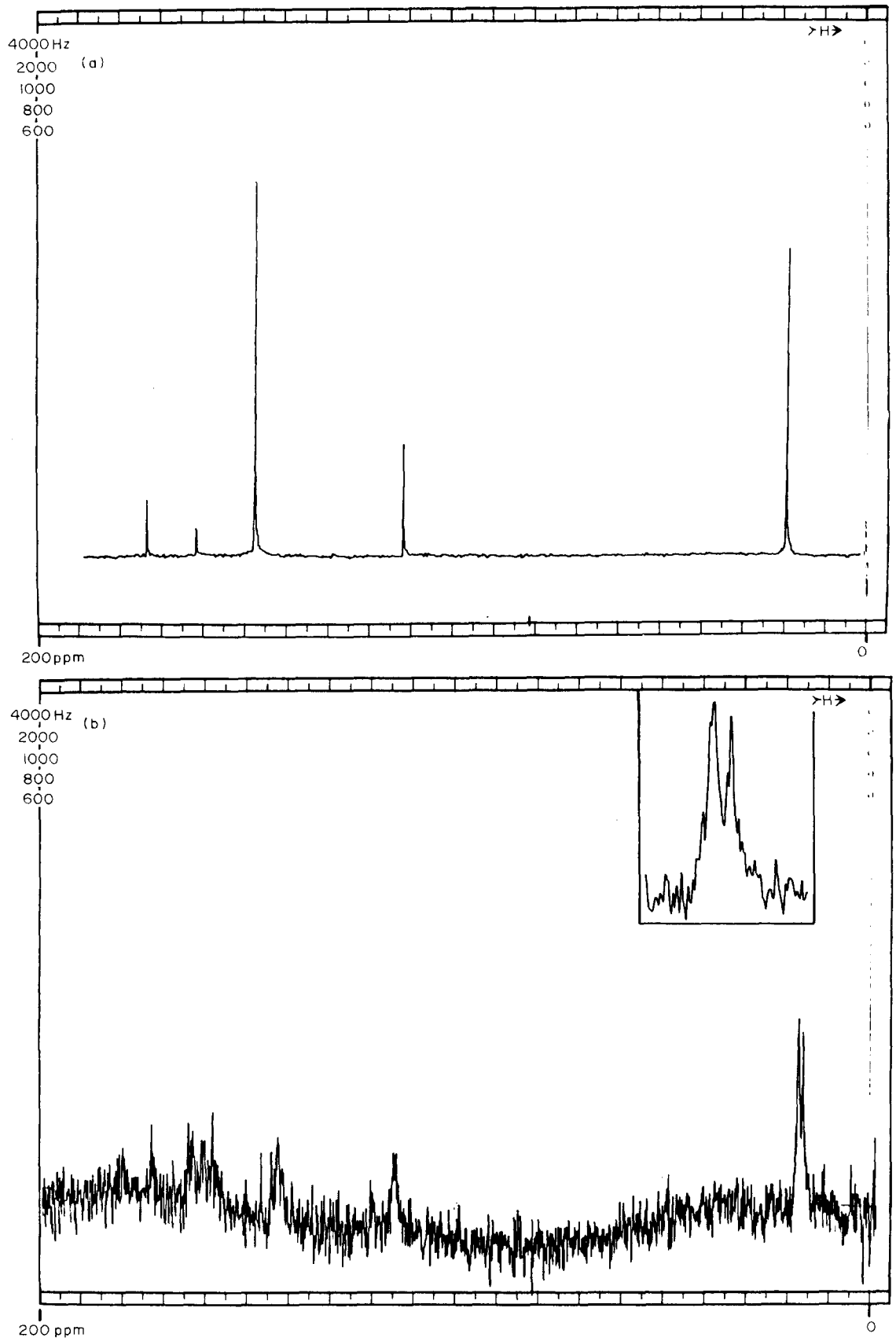

Fig. 7. $^{13}C[^1H]$ n.m.r. spectrum of approximately (A) 0.1 M thymine and (B) platinum–thymine blue in D_2O. Each spectrum represents a 4000 Hz sweep width, 4096 data points, 8 μsec pulse width and a 0.511 sec data acquisition time. Each spectrum represents about 28 hr of data accumulation. The inset of spectrum B represents a 4-fold expansion of the methyl region for platinum–thymine blue.

transition. The CD results, in fact, are indicative of DNA in a premelt stage.

Pt–uracil was shown to interact with several types of biomacromolecules resulting in every case in a destabilization of secondary structure. Lower Pt/P ratios exhibited qualitatively similar results but the amplitudes were lower in magnitude.

Pt–uracil was shown to remove part but not all of the ethidium bromide bound to calf thymus DNA. One explanation for this, in part, is that platinum–uracil is also a DNA intercalating agent and competes with ethidium bromide for some of the sites on DNA. However, this is unlikely since an appreciable percentage of the ethidium bromide is not displaced at high Pt–uracil concentrations (unpublished results). Base specificity cannot explain the results since, from model compound studies presented here, none seems to exist for Pt–uracil interactions. An alternate explanation would be that the Pt–uracil exerts its effect indirectly through conformational forces in such a way as to decrease the affinity of ethidium bromide for its DNA binding sites. This explanation seems more plausible in the light of the partial denaturing effects presented here.

Pt–uracil was found to be less selective in its reaction with biomacromolecules than *cis*-dichlorodiammine platinum (II) as it reacted extensively with synthetic nucleic acids, DNA and protein. This is probably due to the hydrophobic nature of the pyrimidine ring in the former. In addition, platinum–pyrimidine complexes are thermally unstable as indicated by ultraviolet-visible spectrophotometry (unpublished results). Such considerations may preclude an exact molecular understanding of their action; it seems unlikely that one can separate and evaluate the critical effect on cellular function with compounds that display such a range of activity and molecular dispersity.

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