Reed, K., Vandlen, R., Bode, J., Duguid, J., & Raftery, M. (1975) *Arch. Biochem. Biophys. 167*, 138–144.
Reynolds, J. A., & Karlin, A. (1978) *Biochemistry 17*, 2035–2038.
Ross, M. J., Klymkowsky, M. W., Agard, D. A., & Stroud, R. M. (1977) *J. Mol. Biol. 116*, 635–659.
Saitoh, T., Oswald, R., Wennogle, L., & Changeux, J. P. (1980) *FEBS Lett. 116*, 30–36.
Schiebler, W., & Hucho, F. (1978) *Eur. J. Biochem. 85*, 55–63.
Shorr, R., Dolly, J., & Barnard, E. (1978) *Nature (London) 274*, 283–284.
Sobel, A., Weber, M., & Changeux, J. P. (1977) *Eur. J. Biochem. 80*, 215–224.
Vandlen, R., Wu, W., Eisenach, J., & Raftery, M. (1979) *Biochemistry 18*, 1845–1854.
Weill, C., McNamee, M., & Karlin, A. (1974) *Biochem. Biophys. Res. Commun. 61*, 997–1003.
Wu, W., & Raftery, M. (1979) *Biochem. Biophys. Res. Commun. 89*, 26–35.

Proton Nuclear Magnetic Resonance Study of the Self-Complementary Hexanucleotide $d(pTpA)_3$ and Its Interaction with Daunomycin†

Don R. Phillips* and Gordon C. K. Roberts

ABSTRACT: The helix–coil transition of the self-complementary hexanucleotide $d(pTpA)_3$ has been studied in 1 M NaCl by high-resolution proton nuclear magnetic resonance spectroscopy. Almost all of the 12 resonances deriving from the three environments of the four nucleotide protons have been assigned to the central, internal, or terminal nucleotides. At 5 °C, the effect of extensive fraying is evident since the central base pairs exhibit only 20% of the chemical shifts observed for poly(dA-dT)·poly(dA-dT) accompanying denaturation. Daunomycin interacts with the hexanucleotide duplex at 5 °C and stabilizes it by 21 °C at a drug/nucleotide ratio of 0.063 (i.e., drug/hexanucleotide duplex ratio of 0.75). The chemical shifts of the drug protons suggest that ring D of daunomycin does not overlap significantly with the central base pairs of the hexanucleotide and that it extends out from the "helix". This information, together with studies of space-filling models of the complex, suggests that rings B and C of daunomycin overlap with adjacent base pairs and are skewed with respect to the base pairs.

Daunomycin and its analogue, adriamycin, are both used extensively for the treatment of a variety of forms of cancer (Di Marco et al., 1975) with an increasing emphasis on their clinical use in combination therapy (Keiser & Capizzi, 1977). The chemical, biochemical, and physicochemical aspects of these drugs have been extensively reviewed by Arcamone (1978). Although several modes of action of these drugs have been proposed and have been reviewed by Neidle (1978), the dominant mode of action is still thought to be due to their ability to intercalate into DNA, resulting in the inhibition of both DNA polymerase and RNA polymerase (Neidle, 1978). Unfortunately, the use of these drugs for cancer chemotherapy remains limited by their associated cardiotoxicity (Lefrak et al., 1973; Gilladoga et al., 1976) and adverse side effects common to all cytotoxic drugs (Di Marco et al., 1975). Several attempts have been made to alleviate the cardiotoxicity and other side effects by encapsulating the drugs in various ways (Gregoriadis, 1977) and by administering a drug–macromolecule complex (Cornu et al., 1974). An alternative approach has been to modify the existing drugs in an attempt to produce derivatives with decreased side effects. However, this approach requires a detailed knowledge of the nature of the receptor site. Although the DNA intercalation mechanism is acknowledged as the dominant mode of action, there has been a surprising lack of detailed information about the exact geometry of the DNA–daunomycin complex. Pigram et al. (1972) have used X-ray diffraction to obtain information about the complex in the solid state. Recently, proton NMR studies by Patel & Canuel (1978) and Patel (1979) have provided insight into the geometry of the complex in solution by using dG-dC-dG-dC and poly(dA-dT) as model DNA compounds.

We have attempted to obtain details of the geometry of the complex formed between daunomycin and the self-complementary hexanucleotide $d(pTpA)_3$ by using high-resolution proton NMR spectroscopy. This hexanucleotide was selected as a model DNA compound, suitable for NMR studies, on the basis of a compromise between the desire to have a low molecular weight duplex (with a short correlation time) and the requirement that the duplex be stable above 0 °C. Solubility problems precluded the use of the comparable G-C containing hexanucleotide, $d(pGpC)_3$. Since the hexanucleotide $d(pTpA)_3$ has not previously been studied by high-resolution proton NMR, it was first necessary to assign all of the resonances of the hexanucleotide, since each nucleotide exists in three

† From the Biochemistry Department, La Trobe University, Bundoora, Victoria, Australia 3083 (D.R.P.), and the National Institute for Medical Research, The Ridgeway, Mill Hill, London, NW7 1AA England (G.C.K.R.). *Received December 31, 1979.* This work was carried out with the support of the Australian Research Grants Committee.

0006-2960/80/0419-4795$01.00/0

environments. This has been achieved by using the sequential melting procedure that has been applied to other self-complementary oligonucleotides (Patel, 1975, 1977).

Experimental Section

Materials. The $d(pTpA)_3$ was purchased from Collaborative Research, Waltham, MA. The concentration was estimated by using an extinction coefficient of 8900 M^{-1} cm^{-1} at 262 nm and 20 °C, based on the molar extinction coefficient of poly(dA-dT) in the coil form (Inman & Baldwin, 1962).

Daunomycin hydrochloride was a gift from Farmitalia, Milan. Concentrations were determined from the absorbance at 480 nm by using an extinction coefficient of 11 500 M^{-1} cm^{-1}.

The oligonucleotide and the daunomycin were each dissolved in 1 M NaCl, 10 mM phosphate buffer, and 0.1 mM EDTA in D_2O, pH (meter) 6.4. Both solutions were twice exchanged with D_2O by lyophilizing.

Methods. A Bruker WH-270 Fourier Transform NMR spectrometer, interfaced to a Nicolet 1180 computer, was used for all high-resolution NMR studies. Selective saturation of water was performed by using the procedure of Campbell et al. (1974) (0.5-s presaturation pulse, 1-ms delay before the 90° observation pulse and 1-s data acquisition). Transients (15 000, each of 8K data points) were averaged for all spectra. The internl reference standard was 4,4-dimethyl-4-silapentane-5-sulfonate (DSS).

Results

Baldwin (1971) has studied the helix–coil transition of d(T-A) oligomers as a function of ionic strength and chain length (9–22 base pairs). From these studies it is possible to estimate the melting temperature of the hexanucleotide duplex in 1 M salt at between 15 and 25 °C. Since the duplex was required to be stable above 0 °C for the present work, 1 M NaCl was used for all studies. The high salt concentration has the additional advantage of screening the phosphate charges on the hexanucleotide, thereby minimizing weak electrostatic interactions between the $d(pTpA)_3$ and the drug.

Helix–Coil Transition of $d(pTpA)_3$. The NMR spectra of $d(pTpA)_3$ are shown in Figure 1 at temperatures from 5 to 35 °C. The combined effect of line broadening and base-line variations effectively precluded studies below 5 °C. The sharpening that accompanies the increase of temperature is characteristic of enhanced mobility of oligonucleotide chains as they progress from a duplex at low temperatures to single strands at higher temperatures (Patel, 1977). The resonances have been partially assigned by analogy with the resonances of poly(dA-dT)·poly(dA-dT) at denaturing temperatures (Patel & Canuel, 1976). At 5 °C three resonances are observed for adenine H(2) and H(8) protons and for thymine H(6) protons in the three distinct environments they occupy in the hexanucleotide duplex. These three environments are most clearly illustrated in the spectrum at 35 °C. The thymine $CH_3(5)$ group appears to indicate four environments in the duplex at 5 °C, but only the expected three resonances are observed in the single-strand form at 35 °C. With increasing temperature, the $CH_3(5)$ peak marked with an asterisk disappears underneath the larger peak moving downfield (see also Figure 6). The chemical shifts of each of these resonances as a function of temperature are presented in Figures 4–8 and are discussed later in relation to the effect of daunomycin.

Effect of Daunomycin. The effect of adding increasing amounts of daunomycin to the hexanucleotide duplex at 5 °C is shown in Figure 2. At a daunomycin/hexanucleotide duplex ratio of only 0.1, extensive line broadening of all three adenine

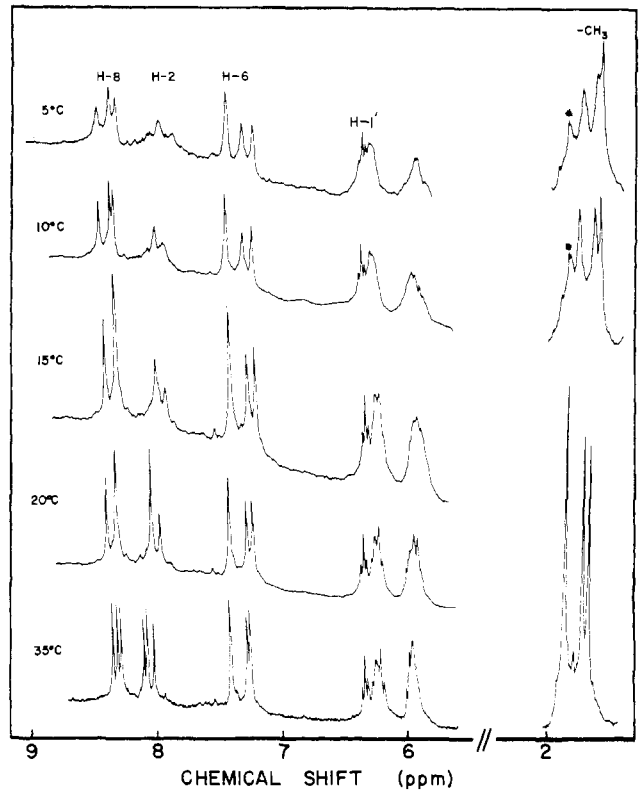

FIGURE 1: Temperature dependence of the 270-MHz Fourier transform proton NMR spectra of $d(pTpA)_3$ in 1 M NaCl, 10 mM phosphate, and 1 mM EDTA, pH 6.8. The nucleotide concentration was 5.0 mM.

H(2) resonances is observed. Line broadening of all resonances is clearly apparent when there is approximately one daunomycin present for each hexanucleotide duplex. The reason for the preferential broadening of the H(2) resonances remains unknown but could well be an exchange effect (this resonance has the largest helix–coil shift) with daunomycin decreasing the helix–coil exchange rate.

The thymine $CH_3(5)$ resonances are also broadened by the presence of daunomycin. The additional peak (marked with an asterisk) which merges with another $CH_3(5)$ resonance accompanying the helix–coil transition, remains as the most dominant methyl resonance at the maximum daunomycin/nucleotide ratio studied.

Helix–Coil Transition of Daunomycin–$d(pTpA)_3$ Complex. When the hexanucleotide duplex–daunomycin complex is heated, sharpening of all the nucleotide proton resonances is observed (Figure 3), accompanying the appearance of daunomycin resonances (indicated by parentheses). (H-1,3) has been used to denote the H(1) and H(3) protons of daunomycin, whose resonances have yet to be specifically assigned.

Only two resonances are observed for thymine H(6) protons at 60 °C. This merely reflects the slight temperature dependence of the chemical shifts of these protons in the denatured state, the two resonances at 7.25 ppm (35 °C) coalescing at 65 °C.

That the "fourth" $CH_3(5)$ resonance is related to the coil form of the hexanucleotide is suggested by its coalescence (with increasing temperature) with one of the three resonances which exist at higher temperatures.

Hexanucleotide Chemical Shifts Accompanying the Helix–Coil Transition. The adenine H(2) and H(8) chemical shifts are presented as a function of temperature in the 5–60 °C range in Figures 4 and 5, respectively. It was expected that the three resonances for each proton could be assigned

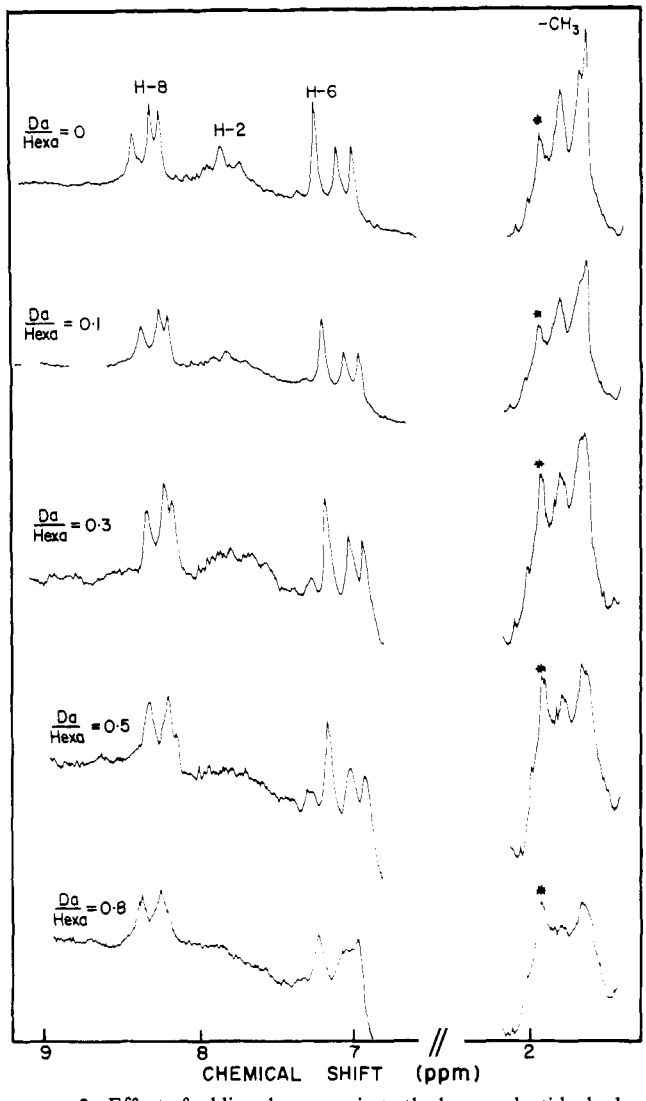

FIGURE 2: Effect of adding daunomycin to the hexanucleotide duplex at 5 °C. All conditions were as indicated in Figure 1. The maximum daunomycin/nucleotide ratio was 0.06, corresponding to a daunomycin/hexanucleotide duplex ratio of 0.75.

in the same manner as employed for dApTpGpCpApT by Patel (1975). The hexanucleotide would therefore be represented as t–i–c–c–i–t, where t, i, and c represent the terminal, internal, and central residues, respectively.

The high-field adenine H(2) resonance (7.88 ppm at 10 °C, Figure 4) is tentatively assigned as the central adenine nucleotide solely because of its occurrence at higher field than the other two resonances. The assignment cannot be confirmed by the melting behavior in the presence of daunomycin because of the limited information available. The 8.1-ppm shifts for the $d(pTpA)_3$ H(2) protons of adenine (Figure 4), when in the *coil* form, are, as expected, identical with those of the coil form of poly(dA-dT) under similar conditions (Patel & Canuel, 1976).

The assignment of the adenine H(8) resonances (Figure 5) is much more straightforward. The highest field resonance of the helical form is expected to be from the central nucleotide (because of maximal ring-current effects), and this is confirmed by the relative melting temperatures (T_m) of 40 °C when in the presence of daunomycin, compared to 30 °C for the internal nucleotide and a T_m of <20 °C for the terminal nucleotide. The internal and terminal adenine H(8) resonances exhibit upfield shifts accompanying the helix–coil transition. The mechanism producing this upfield shift remains obscure,

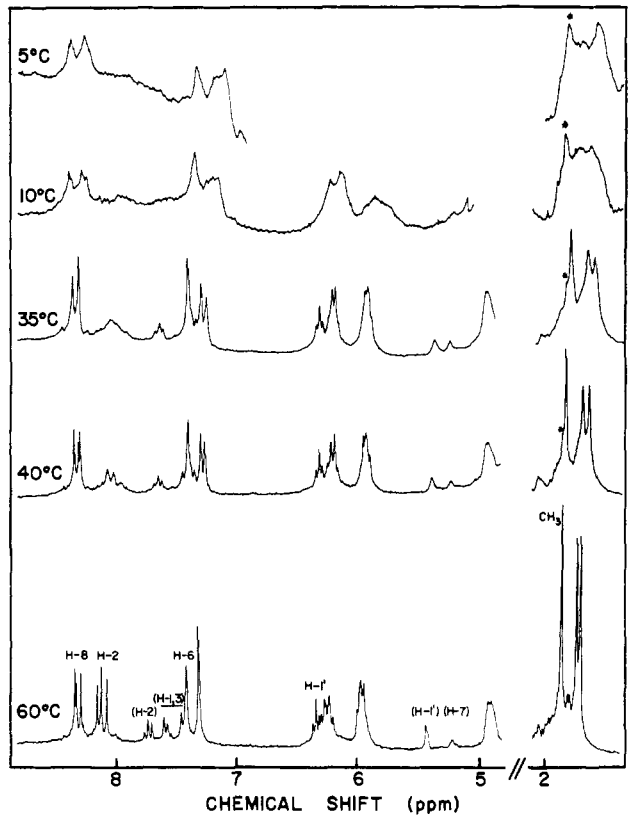

FIGURE 3: Temperature dependence of the proton NMR spectra of daunomycin–$d(pTpA)_3$. The daunomycin/hexanucleotide duplex ratio was 0.75. All other conditions were as indicated in Figure 1.

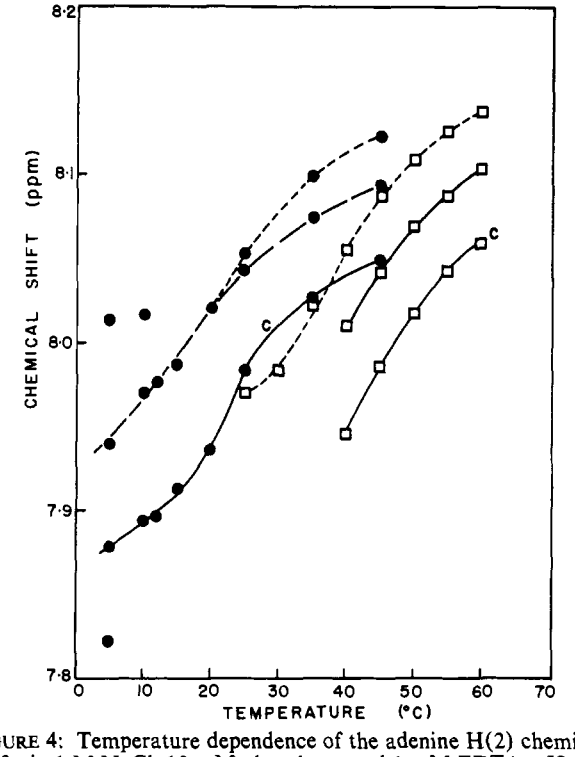

FIGURE 4: Temperature dependence of the adenine H(2) chemical shifts in 1 M NaCl, 10 mM phosphate, and 1 mM EDTA, pH 6.8, in the absence (●) and presence (□) of daunomycin (daunomycin/nucleotide ratio of 0.06).

but such shifts have been reported for a variety of oligonucleotides (Borer et al., 1975; Patel & Canuel, 1977; Patel, 1977, 1979) and polynucleotides (Patel & Canel, 1978).

It is interesting to note that the H(8) resonances of poly(dA-dT)·poly(dA-dT) occur at 8.15 ppm (Patel & Canuel,

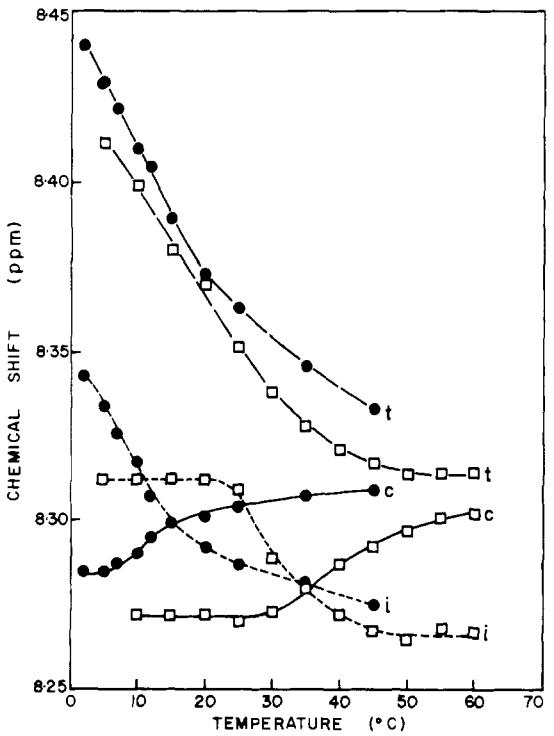

FIGURE 5: Temperature dependence of the adenine H(8) chemical shifts in the absence (●) and presence (□) of daunomycin. Experimental conditions were as described for Figure 4.

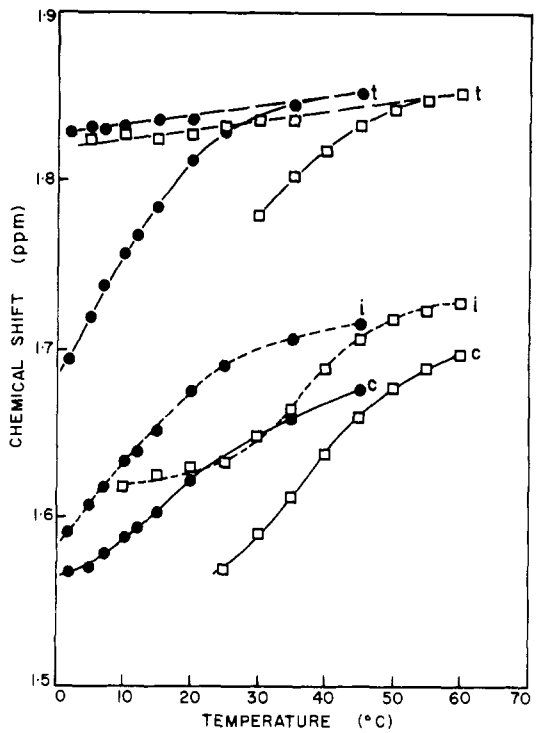

FIGURE 6: Temperature dependence of the thymine $CH_3(5)$ chemical shifts in the absence (●) and presence (□) of daunomycin. Experimental conditions were as described for Figure 4.

1976) [cf. 8.17 for $d(pTpA)_3$] and at 8.3 ppm for the denatured form [cf. 8.32 for $d(pTpA)_3$]. These shifts therefore indicate that the low temperature duplex of $d(pTpA)_3$ is similar to that of poly(dA-dT)·poly(dA-dT). It is important to note, however, that Patel & Canuel (1975) have shown that adenine H(2) resonance is a much more sensitive probe of the secondary structure, because it changes by almost 1 ppm accompanying the helix–coil transition of poly(dA-dT)·poly(dA-dT).

The thymine $CH_3(5)$ and H(6) chemical shifts are presented in Figures 6 and 7, respectively, as a function of temperature. The $CH_3(5)$ resonances (Figure 6) at 1.7 ppm for the denatured form are definitely due to the central and internal nucleotides because of their high T_m values when in the presence of daunomycin. This is confirmed by the 1.7-ppm $CH_3(5)$ resonances of the *coil* form of poly(dA-dT) (Patel, 1978) under similar conditions (1 M NaCl and 1 mM EDTA in D_2O, pH 6.5). The lowest field resonance at 2 °C in the absence of daunomycin appears to be due to the central nucleotide since little denaturation is apparent at 2 °C. When in the presence of daunomycin, this resonance exhibits a T_m of ~40 °C, whereas the internal residue has a T_m of 36 °C. Since these T_m values are so close, the assignment of these resonances cannot be taken as conclusive at this stage.

The terminal thymine $CH_3(5)$ resonances (Figure 6) quite clearly indicate the presence in solution of two distinct environments. The linear dependence of the chemical shifts with temperature, both in the absence and in the presence of daunomycin, is attributed to completely non-base-paired terminal thymine residues. These resonances coincide at high temperatures with the terminal methyl residue resonances involved in the helix–coil transition (Figure 6) and are therefore positively assigned as also arising from terminal thymine residues. It is possible that this additional environment of the terminal thymine methyl residues may be due to staggered helix formation of some of the duplexes, such that these thymine residues exist as single-strand ends and would

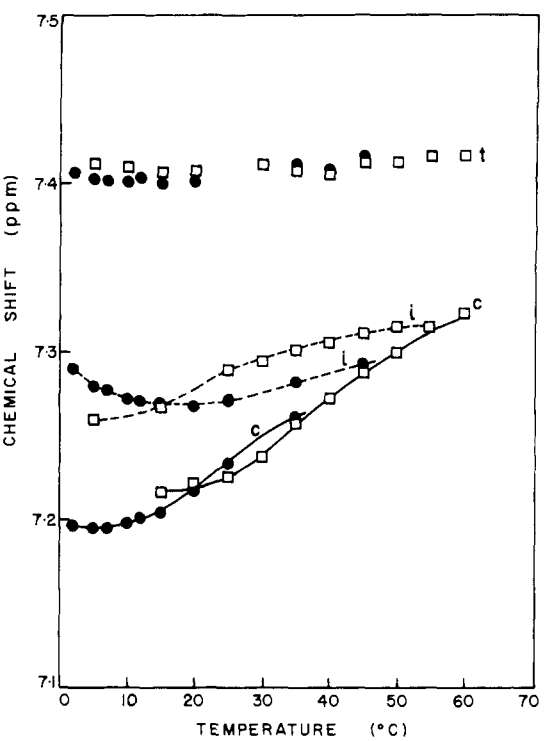

FIGURE 7: Temperature dependence of the thymine H(6) chemical shifts in the absence (●) and presence (□) of daunomycin. Experimental conditions were as described for Figure 4.

therefore be completely independent of the influence of the helix–coil transition (Figure 6). This observation therefore indicates that imperfect base pairing, previously detected for $d(pApT)_2$ in the solid state by X-ray crystallography (Viswamitra et al., 1978), also exists in solution. The reason that this resonance (marked with an asterisk in Figure 2) appears as the most dominant of the methyl resonances upon maximal addition of daunomycin is probably because it is broadened

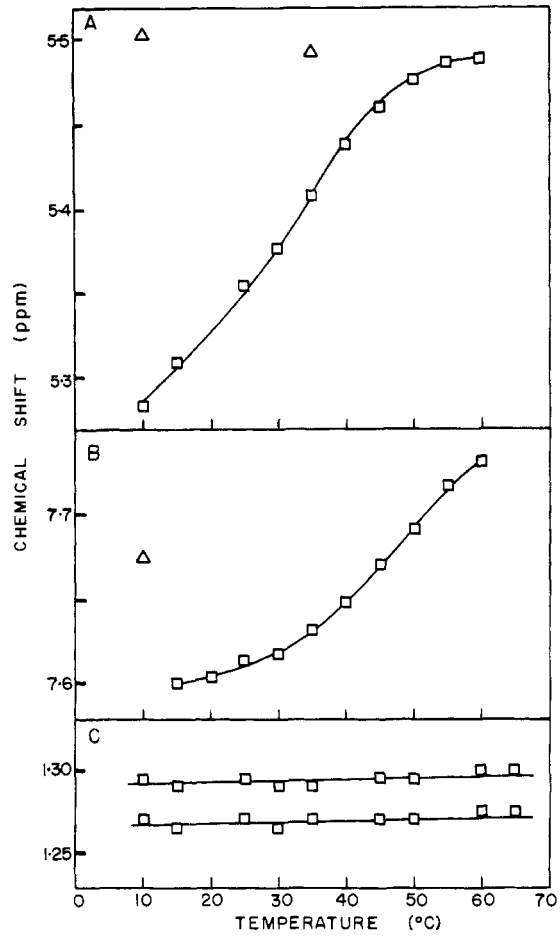

FIGURE 8: Temperature dependence of daunomycin proton chemical shifts in the absence of hexanucleotide (●) and when associated with the $d(pTpA)_3$ hexanucleotide (□). (A) H(1'), (B) most downfield of the aromatic protons [H(1) or H(3)], (C) $CH_3(5')$. Experimental conditions were as described for Figure 4.

less than the other methyl resonances, since it is not affected by the helix–coil transition.

The thymine H(6) resonances (Figure 7) can again be assigned on the basis of their relative chemical shifts in the absence of daunomycin, their relative T_m values when in the presence and absence of daunomycin, and their chemical shifts when in the denatured state. The assignments are indicated in Figure 7. The chemical shifts of the central and internal residues in the denatured state (7.32 ppm) are consistent with the shift of 7.3 ppm observed (Patel & Canuel, 1976) for the denatured state of poly(dA-dT). Two resonances for the terminal thymine residues were not observed (cf. Figure 6) because the terminal H(6) resonance was completely insensitive to the structure of the hexanucleotide.

Daunomycin Chemical Shifts Accompanying the Helix–Coil Transition. When the hexanucleotide–daunomycin complex was heated, daunomycin was released (Figure 8), consistent with the known properties of DNA–daunomycin complexes (Zunino et al., 1972; Doskocil & Fric, 1973). Since only one daunomycin resonance was observed for each proton monitored, the daunomycin exchange rate must be rapid. This has also been noted for other synthetic nucleotide duplex–drug systems (Patel & Canuel, 1977; Patel, 1979). The daunomycin chemical shifts observed therefore represent an average of the chemical shifts of the free drug and the complexed drug. The cooperative release of daunomycin from the hexanucleotide duplex can therefore be monitored by observing the change of chemical shifts of daunomycin protons at increasing temperature. This is shown in Figure 8 for protons [H(1') and $CH_3(5')$] on the sugar moiety of daunomycin and an aromatic proton [either H(1) or H(3)] on ring D of the aglycon.

Table I: Downfield Shifts Obtained Experimentally for the Helix–Coil Transition of $d(pTpA)_3$[a] and Poly(dA-dT)·Poly(dA-dT)[b]

resonance	exptl (ppm)		calcd (ppm)	
	$d(pTpA)_3$	poly-(dA-dT)	B-DNA	D-DNA
adenine H(2)	0.17	0.93	0.85	1.0
adenine H(8)	0.03	0.17	0.0	0.0
thymine $CH_3(5)$	0.11	0.36	0.25	0.25
thymine H(6)	0.06	0.27	0.15	0.05

[a] This work, central nucleotides. [b] The calculated values are from Patel & Canuel (1978) and take into account only ring-current effects.

On melting the helix, the H(1') and aromatic proton signals shift downfield by ≥0.21 and 0.14 ppm, respectively. These shifts are similar to those seen for the daunomycin–poly(dA-dT)·poly(dA-dT) complex (Patel & Canuel, 1978) and similar in magnitude to the upfield shifts seen when daunomycin binds to the duplex at 10 °C (0.22 and 0.08 ppm, respectively).

The chemical shift of the daunomycin sugar $CH_3(5')$ protons was independent of temperature and the state of the hexanucleotide (Figure 8C), consistent with the results obtained by others (Patel & Canuel, 1978; Patel, 1979).

Discussion

For all four groups of nucleotide proton resonances studied, one resonance in each group has been assigned to the central residue of the hexanucleotide. In each case, the central residue resonance is characterized by (1) the highest field resonance present in the duplex (i.e., at 5 °C), because of maximum ring-current effects, (2) the highest melting transition in the absence of daunomycin, because of maximal base-stacking contributions, and (3) the highest melting transition in the presence of daunomycin. In contrast, the terminal residue is characterized by (1) the lowest field resonance present in the duplex (i.e., at 5 °C), (2) little or no indication of cooperative melting, either in the absence or presence of daunomycin, and (3) the lowest field resonance present in the denatured state (i.e., at 60 °C) because of minimal base-stacking, hence minimal ring-current effects.

The helix–coil transitions are generally more clearly defined when in the presence of daunomycin. The assignment of the central, internal, and terminal resonances was therefore based on these transitions wherever possible, together with the knowledge that the shifts of these nucleotide resonances must extrapolate to the same value at high temperature whether in the presence or absence of daunomycin.

The downfield shifts of the hexanucleotide duplex accompanying thermal denaturation are presented in Table I. Also shown are comparable experimental values for the denaturation of poly(dA-dT)·poly(dA-dT) and calculated values for B-DNA and D-DNA. X-ray diffraction studies have shown that poly(dA-dT)·poly(dA-dT) exists in the D-DNA form (Arnott et al., 1974). However, its structure in solution remains less defined (Patel & Canuel, 1976). All four central base-pair downfield chemical shifts accompanying denaturation of the hexanucleotide duplex are 20–25% of the values obtained for the alternating copolymer (Table I). We have interpreted this on the basis of fraying of the ends of the duplex.

Fraying of the internal and terminal base pairs has been demonstrated for the self-complementary hexanucleotide, dApTpGpCpApT at 0 °C, in 0.1 M NaCl and 2.5 mM $MgCl_2$,

Table II: Melting Temperatures of $d(pTpA)_3$ Hexanucleotide in the Absence and Presence of Daunomycin[a]

resonance	T_m (°C) $d(pTpA)_3$ + daunomycin	T_m (°C) $d(pTpA)_3$	ΔT_m (°C)
thymine H(6)	~40	~22	~18
thymine methyl	40	15	25
adenine H(2)		22	
adenine H(8)	40	12	28
daunomycin H(1')	~35		
daunomycin aromatic proton	~42		
average	39	18	21

[a] Daunomycin/nucleotide ratio was 0.06 in 1 M NaCl, 10 mM phosphate, and 1 mM EDTA, pH 6.8.

pH 7.3 (Patel, 1975; Patel & Hilbers, 1975). This fraying is estimated to extend down to near −20 °C for the internal AT base pair and down to and perhaps below −30 °C for the terminal AT base pair (Patel & Hilbers, 1975). The central GC base pairs were stable up to 25 °C under these conditions. Rapid exchange on the NMR time scale was observed for the helix–coil transition of this oligomer. In comparison, the central AT base pairs of the $d(pTpA)_3$ duplex will be considerably less stable than the central GC base pairs in the dApTpGpCpApT duplex (Levitt, 1972). It therefore seems quite likely that the reason the chemical shifts of the central base pairs are only 20–25% of the value of comparable shifts for the polymer (Table I) is because these protons experience only a fraction of the maximal ring-current effects possible as a result of the internal base pairs not existing with optimal base stacking, from neighboring base pairs, as in the polymer. All of the base pairs are therefore affected by this fraying. The picture of $d(pTpA)_3$ at 5 °C is thus that of hexanucleotides being present as a duplex with the two strands held mainly by the central two base pairs. That all of the hexanucleotide is present as two strands held together to some degree is indicated by the complete denaturation profiles obtained for some resonances (Figures 5 and 7). The structure of this minihelix is unknown but is assumed to be similar to that of the polymeric material. The internal base pairs are only partially paired and therefore provide only a fractional ring-current contribution to the chemical shift of the central residue protons. The presence of "mismatched" duplexes, suggested by the observation of four thymidine methyl signals, will also contribute to the smaller chemical shifts in the hexamer than in the polymer.

The melting temperatures presented in Table II show the T_m values of $d(pTpA)_3$ in the absence and presence of daunomycin, as determined by different resonances. The stabilization of the melting temperature of 21 °C for a daunomycin/nucleotide ratio of 0.06 is entirely consistent with that observed for daunomycin intercalated into poly(dA-dT)·poly(dA-dT) (Phillips et al., 1978). This indicates that the drug is bound to both AT sequences in the same manner and to the same extent. The large deviations noted for the T_m, as detected by the chemical shifts of different nucleotide protons, is due largely to the inability to obtain accurate limiting values of the temperature dependence of the chemical shifts for the hexanucleotide at low temperatures.

When the drug–hexanucleotide duplex is heated from 10 to 60 °C, the drug is released accompanying denaturation of the hexanucleotide and therefore exhibits downfield shifts of the H(1') (0.22 ppm) and aromatic (0.14 ppm) resonances (Figure 8). These downfield shifts are essentially identical with those detected by Patel & Canuel (1978) for the displacement of daunomycin from poly(dA-dT)·poly(dA-dT) over the 80–100 °C range [0.25 and 0.14 ppm for the H(1') and aromatic resonances, respectively]. The similarity of these shifts indicates that essentially all of the daunomycin is intercalated into the $d(pTpA)_3$ duplex at 5–10 °C since it is known that almost all of the drug is bound to the polymeric material under similar conditions (Phillips et al., 1978).

The 0.22-ppm upfield shift of the daunomycin anomeric sugar proton H(1') on binding to $d(pTpA)_3$ could be due to intermolecular or intramolecular ring-current shielding or to non-ring-current effects (intermolecular and intramolecular). Calculations based on the crystal coordinates of daunomycin (Neidle & Taylor, 1977) showed clearly that the relative location of the anomeric proton with respect to the planar aromatic aglycon cannot account for the chemical shift for the H(1') proton accompanying its release from the hexanucleotide duplex. Although non-ring-current effects arising from the hexanucleotide could account for the observed shift, it seems more likely, in view of the magnitude, that it is caused by ring-current shielding by the base pairs when the drug is bound to the hexanucleotide duplex.

The small chemical shift of the aromatic protons of daunomycin accompanying denaturation of the drug–hexanucleotide complex (Figure 8B) is somewhat unexpected. Aromatic protons of other drugs have typically shown up to 0.8-ppm upfield shifts accompanying intercalation (Patel & Canuel, 1977), and this has been attributed largely to ring-current contributions from adjacent base pairs (Giessner-Prettre & Pullman, 1976). However the low-field aromatic proton of daunomycin exhibits only a 0.08-ppm upfield shift. The chemical shift data of both the sugar [H(1')] and aromatic protons of daunomycin indicate that all the drugs present are intercalated. Therefore, although the drug intercalates in some manner between adjacent central base pairs of the hexanucleotide duplex, the aromatic protons of the drug are located sufficiently far away from the base pairs to avoid ring-current shielding effects. This implies that the planar aromatic portion of the drug (rings B and C) is located between adjacent base pairs, with ring D protruding outside the helix and on the opposite side of the sugar moiety. However, from solution studies of the complex with DNA, the amino group of daunomycin is thought to be associated with the phosphate groups two nucleotides away (Phillips et al., 1978). In addition, for steric reasons, ring A is thought to protrude right out of the intercalation site (Neidle, 1978). These facts suggest that the aglycon cannot form a complex with $d(pTpA)_3$ with maximal hydrophobic interaction. Rather, the aglycon must be angled with respect to the base pairs to enable ring D protons to extend away from the helix. This has been confirmed by studies of space-filling models of the complex and is essentially the same conclusion reached by others from high-resolution NMR studies of the daunomycin complex with poly(dA-dT)·poly(dA-dT) and with dG-dC-dG-dC (Patel & Canuel, 1978; Patel, 1979). It should be noted that the stereochemistry of the complex is very similar to that suggested by Neidle (1978), based on the X-ray diffraction model of Pigram et al. (1972). However, it would be necessary to propose somewhat more skewing of the drug relative to the adjacent base pairs to explain the lack of ring-current effects on the ring D protons.

This model is entirely consistent with the daunomycin H(1') shifts accompanying the formation of a complex with the hexanucleotide duplex. Space-filling models show that the H(1') of the drug is located between adjacent base pairs and will therefore experience large ring-current effects.

Extrapolation of this model to that of a DNA–daunomycin complex shows several potentially interesting features. Since ring D of the drug protrudes out from the helix, substituents attached to C(1) or C(2) [and possibly even C(3)] would not be expected to alter significantly the binding of such drugs to DNA. Specific probes could therefore be attached to these sites without altering the drug–DNA interaction. Attempts to fluorinate daunomycin at these sites are in progress, since the fluorine can be monitored by ^{19}F NMR, which is especially sensitive to the polarity of its immediate environment.

Acknowledgments

The gift of daunomycin from Farmitalia, Milan, is gratefully acknowledged.

References

Arcamone, F. (1978) *Top. Antibiot. Chem. 2*, 99–239.
Arnott, S., Chandrasekaran, R., Hukins, D., Smith, P., & Watts, L. (1974) *J. Mol. Biol. 88*, 523–533.
Baldwin, R. L. (1971) *Acc. Chem. Res. 4*, 265–272.
Borer, P. N., Kan, L. S., & Ts'O, P. O. P. (1975) *Biochemistry 14*, 4847–4863.
Campbell, I. D., Dobson, C. M., Jeminet, G., & Williams, R. J. P. (1974) *FEBS Lett. 49*, 115–119.
Cornu, G., Michaux, J., Sokal, G., & Trouet, A. (1974) *Eur. J. Cancer 10*, 695–700.
Di Marco, A. D., Arcamone, F., & Zunino, F. (1975) in *Mechanism of Action of Antimicrobial and Anti-tumor Agents* (Corcoran, J. W., & Hahn, F. E., Eds.) pp 101–128, Springer-Verlag, Berlin.
Doskocil, J., & Fric, I. (1973) *FEBS Lett. 37*, 55–58.
Giessner-Prettre, C., & Pullman, B. (1976) *Biochem. Biophys. Res. Commun. 70*, 578–581.
Gilladoga, A. C., Manuel, C., Tan, C. T. C., Wollner, N., Sternberg, S. S., & Murphy, M. L. (1976) *Cancer (Philadelphia) 37*, 1070–1078.
Gregoriadis, G. (1977) *Nature (London) 265*, 407–409.
Inman, R. B., & Baldwin, R. L. (1962) *J. Mol. Biol. 5*, 172–184.
Keiser, L. W., & Capizzi, R. L. (1977) *Cancer: Compr. Treatise 5*, 163.
Lefrak, E. A., Pitha, J., Rosenheim, S., & Gottlieb, J. A. (1973) *Cancer (Philadelphia) 32*, 302–304.
Levitt, M. (1972) *Ciba Found. Symp. 7*, 147.
Neidle, S. (1978) *Top. Antibiot. Chem. 2*, 240–278.
Neidle, S., & Taylor, G. (1977) *Biochim. Biophys. Acta 479*, 450–459.
Patel, D. J. (1975) *Biochemistry 14*, 3984–3989.
Patel, D. J. (1977) *Biopolymers 16*, 1635–1656.
Patel, D. J. (1979) *Biopolymers 18*, 553–569.
Patel, D. J., & Hilbers, C. W. (1975) *Biochemistry 14*, 2651–2660.
Patel, D. J., & Canuel, L. L. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 674–678.
Patel, D. J., & Canuel, L. L. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2624–2628.
Patel, D. J., & Canuel, L. L. (1978) *Eur. J. Biochem. 90*, 247–254.
Phillips, D. R., Di Marco, A., & Zunino, F. (1978) *Eur. J. Biochem. 85*, 487–492.
Pigram, W. J., Fuller, W., & Hamilton, L. D. (1972) *Nature (London), New Biol. 235*, 17–19.
Viswamitra, M. A., Kennard, O., Jones, P. G., Sheldrick, G. M., Salisbury, S., Falvello, L., & Shakked, Z. (1978) *Nature (London) 273*, 687–688.
Zunino, F., Gambetta, R., Di Marco, A., & Zaccara, A. (1972) *Biochim. Biophys. Acta 277*, 489–498.

Binding of *Clostridium perfringens* [^{125}I]Enterotoxin to Rabbit Intestinal Cells†

James L. McDonel

ABSTRACT: ^{125}I-Labeled enterotoxin from *Clostridium perfringens* was utilized to characterize the association of the enterotoxin with cells isolated from rabbit intestine and tissue homogenates from liver, kidney, and brain. The enterotoxin was found to bind in a specific and saturable manner to cells from intestine and to tissue homogenates from liver and kidney but not the brain. Detailed studies of the binding were carried out with the ileal epithelial intestinal cells. The rate and amount of binding of enterotoxin to cells appeared to be temperature dependent. Apparent affinity and association and dissociation rate constants were calculated for what appeared to be two classes of saturable binding sites. The amount of enterotoxin molecules that bound per milligram of cell protein was similar in tissue of intestinal, liver, and kidney origin (approximately 10^{13} molecules/mg of cell protein). Spontaneous dissociation into the supernatant medium was observed to be much slower than expected from calculations based on the rate of association. Chaotropic ions did not enhance dissociation of the enterotoxin from cells. Enterotoxin binding was demonstrated to be heat labile (binding ability was lost after the enterotoxin was heated for 10 min at 60 °C). A mechanism is described whereby the enterotoxin binds and then is inserted into the membrane where it becomes trapped.

Recent studies on the mechanism of action of *Clostridium perfringens* enterotoxin have indicated that it acts through destructive interaction with cellular membranes in susceptible cells (McDonel, 1979). These effects have been noted in rat (McDonel, 1974; McDonel & Duncan, 1975) and rabbit (McDonel & Duncan, 1977; McDonel et al., 1978) intestinal models and in Vero (African green monkey kidney) cells grown in culture (McClane & McDonel, 1979, 1980; McDonel & McClane, 1979). The results of preliminary studies with [^{125}I]enterotoxin in Vero cells (McDonel & McClane, 1979)

† From the Department of Microbiology, Cell Biology, Biochemistry, and Biophysics, The Pennsylvania State University, University Park, Pennsylvania 16802. *Received April 7, 1980.* This research was supported in part by U.S. Public Health Service Grant AI14161-03 from the National Institute of Allergy and Infectious Diseases and by funds from the Agricultural Experiment Station, The Pennsylvania State University.

0006-2960/80/0419-4801$01.00/0