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Detection of the Sites of Alkylation in DNA and Polynucleotides by Laser Raman Spectroscopy†

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ABSTRACT: A laser Raman study of the alkylation of calf thymus DNA, poly(dG)·poly(dC) and poly(dA)·(dT) has been made using two water soluble alkylating agents: an antitumor drug, the difunctional methyl nitrogen mustard (HN2), which forms interstrand cross-links, and the dimethyl nitrogen half mustard (HN1). When an excess of the alkylating agent was used, the observed Raman frequencies due to the guanine ring modes in DNA and poly(dG)·poly(dC) changed virtually quantitatively to those of 7-methylguanosine (7-Me-Guo) showing that essentially all of the guanine bases were alkylated in the N-7 position. Furthermore, this alkylated DNA formed a stable double helical complex at neutral pH in which the alkylated guanine residues are in the keto form. No changes in the Raman bands of any of the other bases were observed in alkylated DNA. The DNA double helix, completely alkylated in at the N-7 position of guanine, melts about 35 °C below that of the native DNA. Upon melting, the alkylated guanine changes from the keto to the zwitterionic form.

Alkylation of nucleic acids and nucleic acid components has been of considerable interest in recent years (Michelson and Pochon, 1966; Pochon and Michelson, 1967; Ramstein et al., 1971; Engle and von Hippel, 1974; Lawley, 1966; Kohn et al., 1966). The interest in these experiments comes from the fact that nucleic acids sometimes possess alkylated bases, and alkylating agents are often either tumor-producing or tumor-inhibiting drugs. The interest in the alkylation of DNA using mustard derivatives arises from the fact that the nitrogen mustards, such as HN2,[1] *N*-methylbis(2-chloroethyl)amine hydrochloride, which are difunctional, are known to be potent tumor-inhibiting agents (Goldacre et al., 1949; Haddow et al., 1948; Loveless, 1951). Often in studies of alkylation by the difunctional alkylating agent HN2, the monofunctional alkylating agent HN1 (*N,N*-dimethyl-2-chloroethylamine hydrochloride) is also studied to obtain differences between mono- and difunctional alkylation (Kohn et al., 1966). In this paper we wish to show that alkylation of DNA and double helical polynucleotides by HN1 and HN2 leads to specific changes in their Raman spectra. From the changes in the bands it is possible to show the site of attack of the alkylating agents and the structure of the alkylated guanine residues in the DNA double helix.

Experimental Section

N,N-Dimethyl-2-chloroethylamine hydrochloride (HN1) and *N*-methylbis(2-chloroethyl)amine hydrochloride (HN2) were purchased from Aldrich Chemical Company, Milwaukee, Wisconsin. DNA calf thymus was purchased from Sigma Chemical Co., St. Louis, Missouri. The polydeoxynucleotides were purchased from Miles Laboratories, Indiana. GMP and

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[1] Abbreviations used: HN1, *N,N*-dimethyl-2-chloroethylamine hydrochloride; HN2, *N*-methylbis(2-chloroethyl)amine hydrochloride; 7-Me-Guo, 7-methylguanosine.

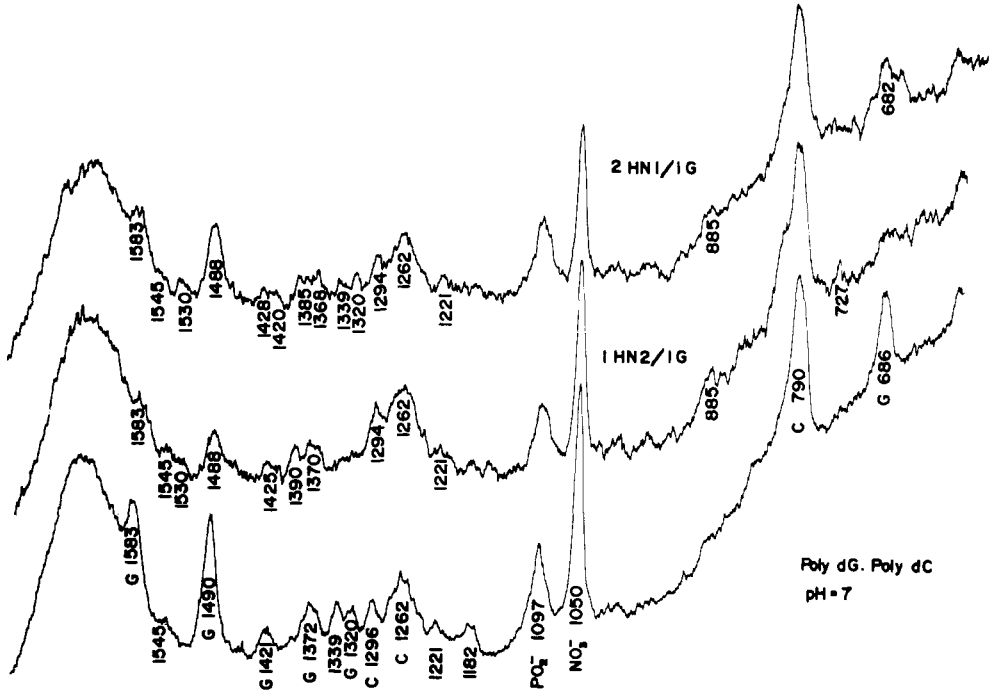


FIGURE 1: Laser Raman spectrum of (bottom) poly(dG)·poly(dC); (middle) poly(dG)·poly(dC) + one equiv of HN2 per guanine base; (top) poly(dG)·poly(dC) + 2 equiv of HN1 per guanine base. In each case, the concentration of the helical complex is about 20 mg/ml and the pH 7.0.

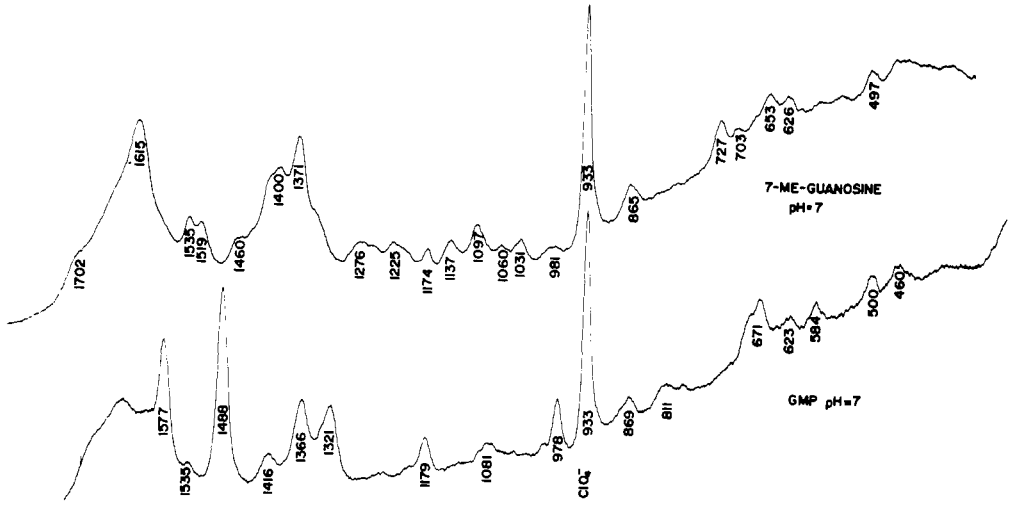


FIGURE 2: A comparison of the Raman spectra of GMP (bottom) and 7-Me-Guo (top) at pH 7.0. Note the absence of the strong band at 1488 cm^{-1} in the methylated derivative.

7-Me-Guo were purchased from Cyclo Chemical, Los Angeles, California. The materials were assumed to meet the purity criteria stated by the manufacturer and were used without further purification.

In order to alkylate the DNA and polynucleotide double helices, the following procedure was developed. Ten milligrams of DNA or a polynucleotide double helix was dissolved in 10 ml of water and adjusted to pH 7.0. This solution was placed in a pH-stat manufactured by the Radiometer Co. of Copenhagen. To the 10 mg of DNA in the pH-stat was added the desired moles of alkylating agent freshly dissolved in 1 ml of water, and the pH of the solution was held constant at pH 7.0 ± 0.1 by automatic addition of 0.1 N NaOH solution.

Upon completion of alkylation, the solutions were concentrated by means of an Amicon ultrafiltration apparatus until the final DNA concentration was about 20 mg/ml. Raman spectrum of this solution was then taken as described previously (Small and Peticolas, 1971a,b).

Results and Discussion

Since earlier work has shown that alkylating agents attack DNA primarily at the N-7 position of guanine (for a review, see Lawley, 1966), we began by examining the effect of HN1 and HN2 on the spectrum of poly(dG)·poly(dC). The Raman spectrum of poly(dG)·poly(dC) has been reported earlier (Pohl et al., 1974), but the spectrum of its alkylated derivative has not been reported previously. Figure 1 shows the Raman spectrum of an aqueous solution of double helical poly(dG)·poly(dC) at pH 7.0 and compares it with that of the alkylated derivative under the same conditions. The Raman spectrum of poly(dG)·poly(dC) shows strong bands at 686 and at 1490 cm^{-1} which have been assigned to the guanine ring modes

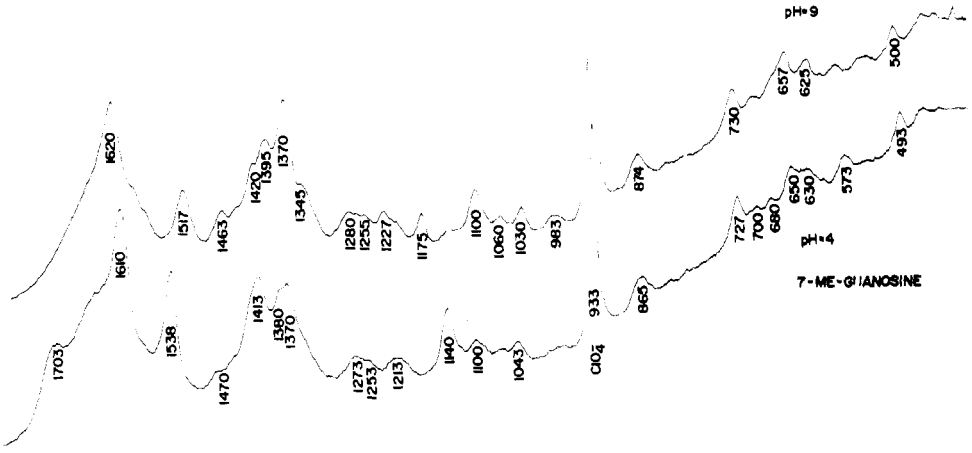


FIGURE 3: Comparison of the Raman spectrum of 7-Me-Guo at pH 4 (keto form) and pH 9 (zwitterion). The concentration in each case is 20 mg/ml.

TABLE I: Raman Bands of Guanine in GMP, Poly(dG)·Poly(dC), 7-Me-Guo, and Alkylated Poly(dG)·Poly(dC)[a] at pH 7.

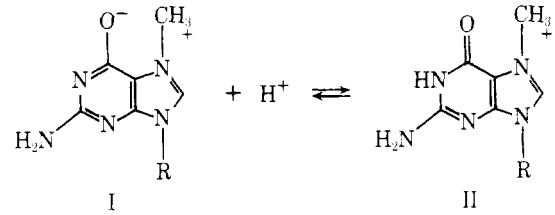
GMP	Poly(dG)·Poly(dC)	7-Me-Guo	Alkylated Poly(dG)·Poly(dC)[a]
		653	640
671	686		*b*
		727	727
1320	1320		*b*
1366	1372	1371	1372
		1400 (S)	1400
1488 (S)	1490 (S)		*b*
		1535 (W)	1535
1577 (S)	1583 (S)		*b*
		1615 (M)	*c*

[a] Alkylation with both HN1 and HN2 gave similar changes in DNA Raman spectra (see Figure 1). However, the changes with HN2 are much larger for a given concentration of alkylating agent. [b] Decrease upon incomplete alkylation of poly(dG)·poly(dC) sufficient to show that the band would disappear upon complete alkylation of all guanine bases. [c] At low concentration, one cannot see the 1615-cm^{-1} band under the water band.

(Lord and Thomas, 1967; Brown et al., 1972; LaFleur et al., 1972). In order to observe a significant change in the Raman spectrum, we reacted 1 mol of HN2 to 1 mol of guanine base in the poly(dG)·poly(dC) solution. This is a twofold excess of alkylating agent since each HN2 molecule is bifunctional. In alkylated poly(dG)·poly(dC), the 686-cm^{-1} Raman band disappears completely and the 1488-cm^{-1} Raman band shows a large decrease in intensity. In addition to these changes, there are two new bands which appear at 1390 and 1530 cm^{-1}. The 1583-cm^{-1} Raman band of the guanine base also shows decrease in intensity and probable shift to higher wave number. Upon reacting the monofunctional agent HN1 with poly(dG)·poly(dC), the changes of the Raman bands of the guanine base are similar to that brought up by the HN2 agent. The reactivity of NH1 appears to be much less than that of HN2.

In order to determine the site of alkylation in poly(dG)·poly(dC), we have taken 7-methylguanosine as a model compound. The Raman spectrum of 7-Me-Guo is shown in Figure 2 compared with the Raman spectrum of GMP. First of all we notice the absence of the strong 1488-cm^{-1} guanine band in 7-Me-Guo. From this observation we can now make a more accurate assignment of this band. The 1488-cm^{-1} band in guanine has been shown to change to 1465 cm^{-1} upon replacement of the C(8)–H with C(8)–D (Livramenot and Thomas, 1974; Erfurth and Peticolas, 1975). As we see in Figure 2, this band disappears upon N-7 methylation. We may also deduce that this band disappears upon N-7 protonation since it is not found in the Raman spectrum of guanosine at pH 1.0 (Lord and Thomas, 1967) and the N-7 position has been identified as the protonation site in guanosine (Miles et al., 1963). In view of these facts, we may assign this 1488-cm^{-1} band to a guanine ring mode which strongly involves the C(8)–H bond stretch and the N(7)=C(8) double bond stretch.

Table I lists the important guanine ring frequencies in GMP, poly(dG)·poly(dC), 7-Me-Guo, and alkylated poly(dG)·poly(dC). The complete correspondence between the Raman bands in 7-Me-Guo and alkylated poly(dG)·poly(dC) shows conclusively that alkylation of this double helical polymer occurs at the N-7 position of the ring in guanine, confirming the earlier chemical work.



I II

The molecule, 7-Me-Guo, has been shown (Lawley and Brookes, 1961) to exist as an equilibrium between a zwitterionic (I) and keto (II) form. Figure 3 shows 7-Me-Guo at pH 4 and 9. The keto form (II) at low pH has a strong band at 1538 cm^{-1} and none at 1517 cm^{-1}, while at the high pH zwitterion (I) we see a strong band at 1517 cm^{-1} and none at 1538 cm^{-1}. In Figure 2 we see that the 1519-cm^{-1} band due to the zwitterion form is almost as strong as the keto form band at 1535 cm^{-1}. Hence we conclude from the Raman intensities that, at pH 7.0, the two structures are present in approximately equal amounts in agreement with the reported pK of 7.2.

In Figure 1 neither of the alkylated poly(dG)·poly(dC) samples shows a Raman band at 1517 cm^{-1}, although a band at 1538 cm^{-1} is present which was absent in the native poly(dG)·poly(dC) helix. Consequently we conclude that, in the double helical poly(dG)·poly(dC) complex, the alkylated guanine bases are held in the keto form. This is to be expected because the keto form is stabilized by the Crick–Watson hy-

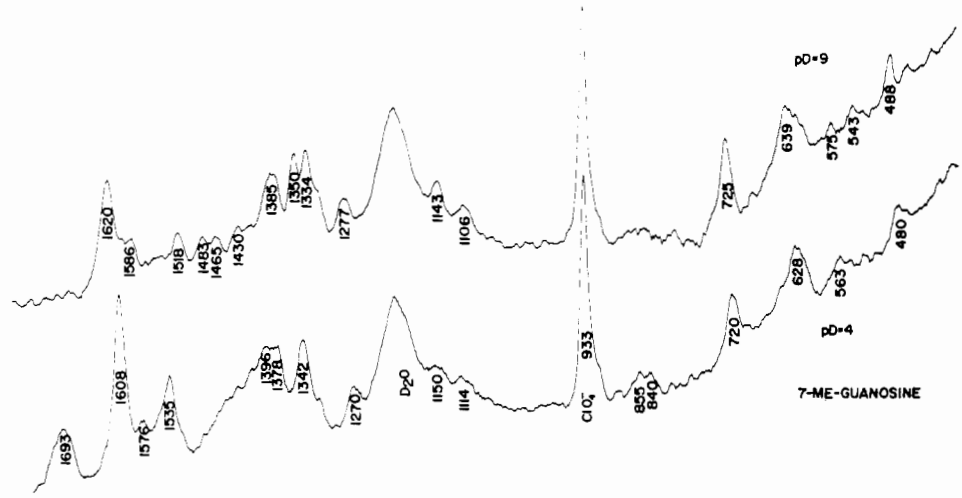


FIGURE 4: The Raman spectrum of 7-Me-Guo in D_2O at pD 4 (keto form) and pD 9 (zwitterion).

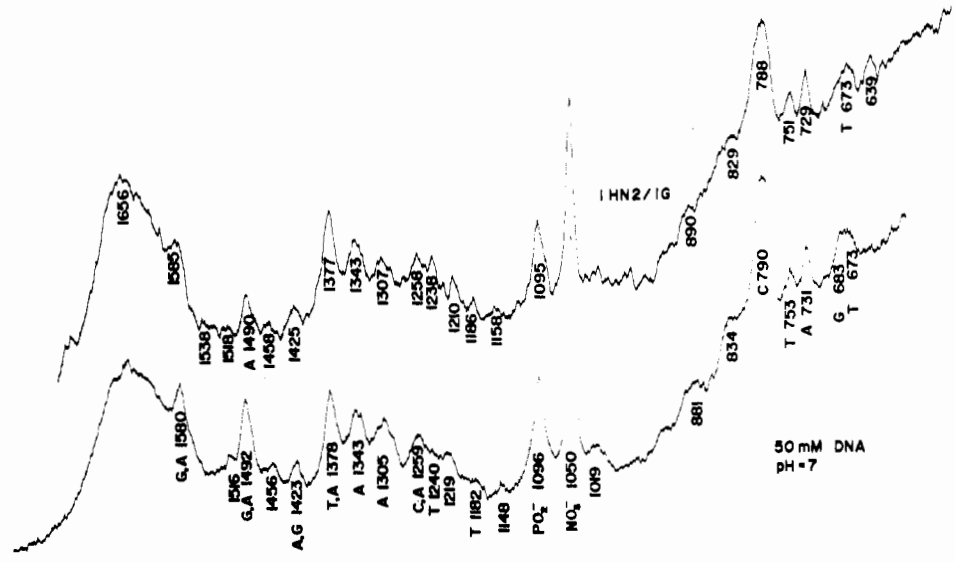


FIGURE 5: The Raman spectra of DNA at pH 7.0 (20 mg/ml) in aqueous solution before and after alkylation reaction with 1 mol of HN2 per mol of guanine base.

TABLE II: Raman Bands of DNA Bases of DNA and Alkylated DNA at pH 7 at 25 °C.

DNA	Alkylated DNA
	641
673 (T)	669
683 (G)	
834	PO_2 diester antisymmetric stretch
1240 (T)	1241 (T)[a]
1378 (T, A)	1378 (T, A)[a]
	1400 alkylated G
1492 (G, A)	1489 (A)
	1530 alkylated G
	1656

[a] Increase in intensity.

TABLE III: Raman Bands of DNA and Alkylated DNA in D_2O at pD 7, 25 °C.

DNA	Alkylated DNA
677 (G)	
765 (C)	777 (C)
785	792
828	833 PO_2 diester antisymmetric stretch
1484 (G, A)	1484 (A)
1575 (G, A)	1579 (A) decrease in intensity
1673 (T)	1667 (T)

drogen bonding of the keto form guanine to the cytosine.

In order to look at the carbonyl vibration in the keto form of 7-Me-Guo at low pH and to confirm its absence at high pH, the spectrum of 7-Me-Guo was taken in D_2O at pD 4.0 and pD 9.0. These spectra are shown in Figure 4. The spectrum at low pD reveals a band at 1693 cm^{-1} which is exactly the frequency of the C=O stretching vibration in D_2O solution of poly(rG) under comparable salt conditions (Rice et al., 1973). At the high pD 9.0 this band disappears. A band at 1620 cm^{-1} appears and is tentatively assigned to the $C-O^-$ stretch of the zwitterion structure.

Figure 5 shows the Raman spectrum of DNA and DNA after reaction with HN2 so that there is 1 mol of HN2 per mol of guanine base in solution. The large decrease in intensity of

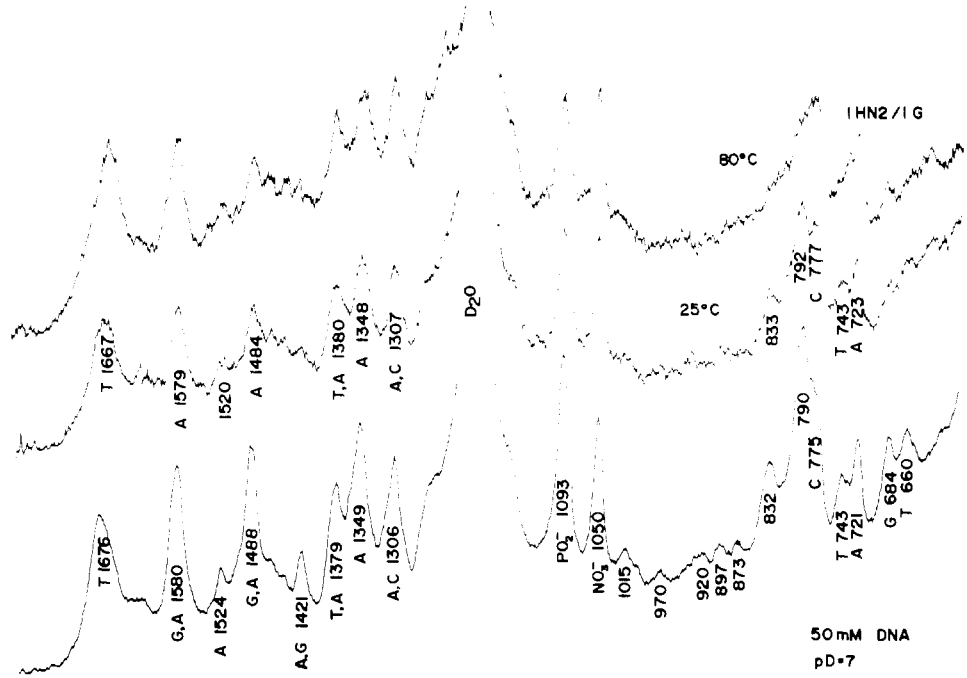


FIGURE 6: The Raman spectra of DNA alkylated with 1 mol of HN2 per mol of guanine base in aqueous solution (20 mg/ml), pD 7.0 at 25 and 80 °C.

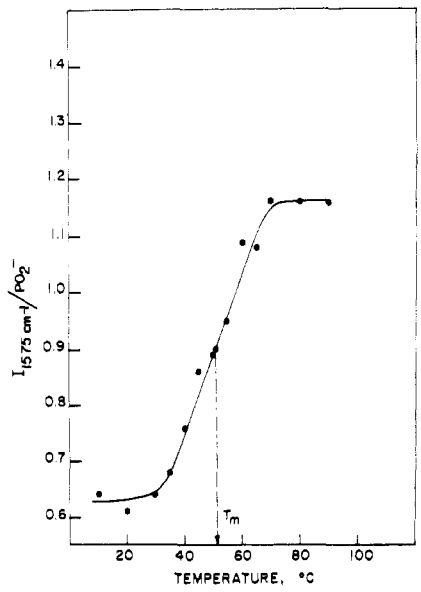


FIGURE 7: Melting of DNA fully alkylated with HN2 using the 1575-cm^{-1} purine band (predominantly adenine).

the band at 1490 cm^{-1} leads us to the conclusion that virtually all of the guanine residues are alkylated so that the remainder of this band at 1490 cm^{-1} is probably due to the adenine residues. This conclusion may be semiquantitatively verified by a comparison of Figure 5 with the Raman spectrum of a mixture of deoxymononucleotides at pH 7.0 in the same concentration ratio as that found in calf thymus DNA but missing completely the guanine residues (Erfurth and Peticolas, 1975). The absence of the 683-cm^{-1} guanine band also indicates the complete alkylation of the guanine residues. The presence of a band at 1535 cm^{-1} shows the existence of the alkylated guanine in the keto form. The presence of a band at 1516 cm^{-1} in DNA and a band of the same strength in the alkylated DNA also indicates that the alkylated guanine residues are not in the zwitterion form. Thus we see that Raman spectra allow us to determine which of the guanine structures are present in the alkylated DNA.

The significant changes in the Raman spectrum of DNA upon alkylation are given in Table II. Similar changes are observed in the Raman spectrum of alkylated DNA in D_2O. These changes are given in Table III. In particular, the 1484-cm^{-1} band becomes very weak, the 677-cm^{-1} band goes away completely, and the 1579-cm^{-1} band decreases in intensity.

Figure 6 shows the Raman spectra of fully alkylated DNA below and above its melting point of 52 °C in D_2O at pD 7.0. Two bands which increase in intensity upon melting are the 1575-cm^{-1} band of the purines (predominantly due to adenine) and the 1673-cm^{-1} band of thymidine. Plots of an actual melting curve using the 1575-cm^{-1} band are shown in Figure 7. The melting of DNA under identical conditions is about 85–87 °C (Erfurth and Peticolas, 1975).

Figure 8 shows the spectra of DNA fully alkylated with HN2 in aqueous solution at pH 7.0 at 25 and 80 °C, well above the mp of 52 °C. Here we see the large increase upon melting in the thymidine band at 1242 cm^{-1} which has previously been observed in ordinary DNA (Erfurth and Peticolas, 1975). However, unlike the earlier observations, this change in the cross-linked alkylated DNA is completely reversible.

Finally, we wish to discuss the possibility that the alkylating agent HN2 attacks adenine in the N-3 position. Careful examination of the Raman spectra of DNA fully alkylated with HN2 both in H_2O (Figure 8) and D_2O (Figure 6) shows no measureable change in the Raman spectrum of the adenine bands. This would not appear to be in accordance with the work of Lawley and Brookes (1963) and Kriek and Emmelot (1964) who have reported that adenine is alkylated in the N-3 position with about one-fourth the rate as the N-7 position of guanine. However, these authors used dimethylsulfonate and diazomethane, respectively, for alkylating agents and perhaps these alkylating agents behave differently toward DNA than does HN2. Perhaps the Raman is not sensitive to alkylation at the

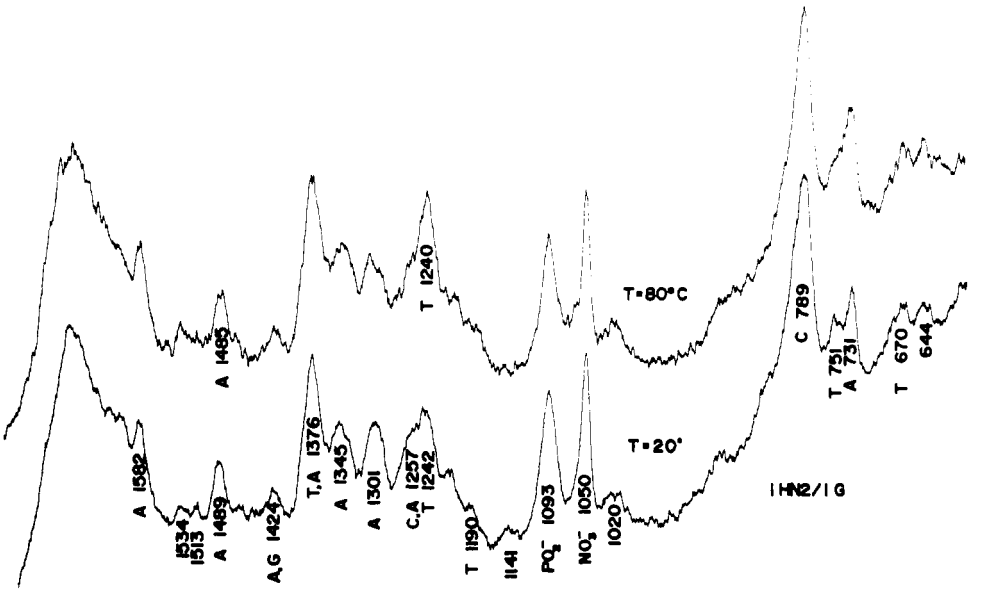


FIGURE 8: The Raman spectra of DNA alkylated with 1 mol of HN2/mol of guanine base in aqueous solution (20 mg/ml) at pH 7.0 at 25 and 80 °C.

N-3 position of adenine. In order to see if we could force an alkylation of adenine in a Crick–Watson double helix, we reacted the double helix poly(dA)·poly(dT) with HN2 and observed the effect on the adenine ring frequencies. Most of the adenine ring frequencies were found to decrease in intensity. In particular the adenine ring frequency at 1345 cm^{-1} was much less strong than the neighboring thymine ring frequency. We cannot at this point describe the reaction between HN2 and adenine that leads to these intensity changes.

In conclusion we may say that, in principle, Raman spectroscopy is useful for determining the structure of the alkylated bases in ordered and disordered double helical structures when the concentration of alkylated bases is sufficiently high. Unfortunately the ordinary Raman effect does not allow the in situ determination of minor alkylation products. Perhaps the resonant Raman effect with its greater sensitivity for dilute species will provide a better probe of minor alkylation products of DNA in situ.

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