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Diethyl pyrocarbonate can detect a modified DNA structure induced by the binding of quinoxaline antibiotics

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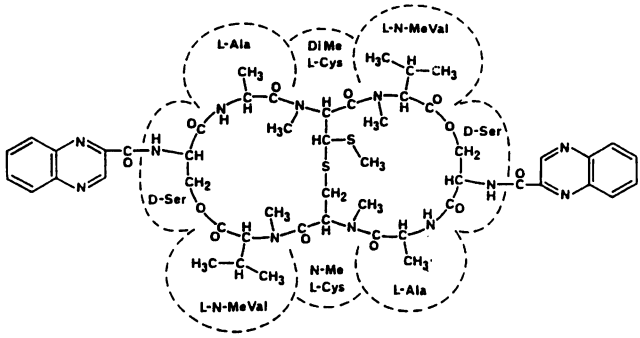
ABSTRACT

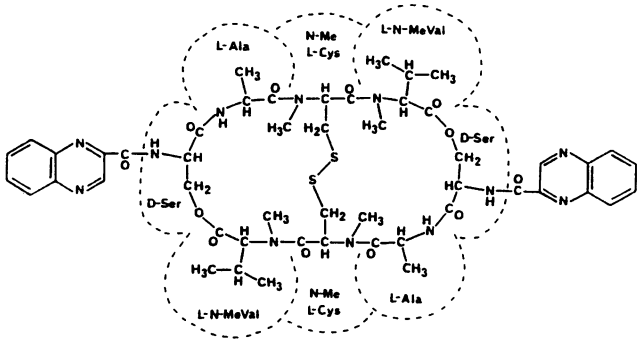
The reactivity of the 160 bp tyrT DNA fragment towards diethyl pyrocarbonate (DEPC) has been investigated in the presence of bis-intercalating quinoxaline antibiotics and the synthetic depsipeptide TANDEM. At moderate concentrations of each ligand, specific purine residues (mainly adenosines) exhibit enhanced reactivity towards the probe, and several sites of enhancement appear to be related to the sequence selectivity of drug binding. Further experiments were performed with echinomycin at pH 5.5 and 4.6 to facilitate the protonation of cytosine required for formation of Hoogsteen GC base pairs. No significant increase in reactivity was observed under these conditions. Additionally, no protection of deoxyguanosine residues from methylation by dimethyl sulphate was observed in the presence of echinomycin. We conclude that the structural anomaly giving rise to drug-dependent enhanced DEPC reaction is not simply the formation of Hoogsteen base pairs adjacent to antibiotic binding sites. Nor is it due to a general unwinding of the double helix, since we show that conditions which are supposed to unwind the helix lead to a uniform increase in purine reactivity, regardless of the surrounding nucleotide sequence.

INTRODUCTION

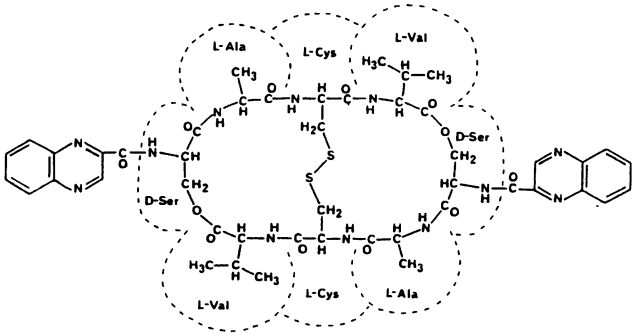
It has long been known that the binding of small ligands to DNA can affect its local structure [1-3]. Intercalators unwind and extend the DNA helix while other molecules may affect the width of the grooves or induce the formation of kinks [1,4,5]. Recent footprinting studies have demonstrated that sequence-selective drug binding can alter DNA conformation in regions at least one helical turn away from the actual drug binding site [6,7]. The determination of crystal structures for the bifunctional intercalators echinomycin and triostin A bound to short DNA fragments raised the possibility of more radical changes in DNA structure [8-10]. In these complexes the base pairs adjacent to the antibiotic binding site are not in the normal Watson-Crick configuration, but consist of Hoogsteen pairs [11] in which the purine nucleotides adopt a syn (rather than anti) conformation about the glycosidic bond. Hoogsteen pairing has been noted for both GC and AT pairs surrounding the central CpG sequence of the binding site. This raises the question as to



ECHINOMYCIN



TRIOSTIN A



TANDEM

Figure 1. Chemical structures of quinoxaline depsipeptides.

whether such radical changes can occur in solution, and within longer DNA fragments.

In a previous study, Mendel and Dervan [12] demonstrated that echinomycin produces enhanced sensitivity to diethyl pyrocarbonate (DEPC) at purines both proximal and distal to the antibiotic binding site. This result seemed consistent with the formation of Hoogsteen pairs at these positions, although the authors were careful to point out that the experiments did not prove their existence. Diethyl pyrocarbonate has previously been used as a probe for both Z-DNA [13,14] and cruciform structures [15,16]. In both instances it is presumed to interact with the purine N-7 atoms which are much more exposed than in B-DNA. In Hoogsteen pairs the N-7 atoms are directly involved in base pairing to the pyrimidine N3 and so are less likely to be reactive towards DEPC. It was therefore proposed that DEPC could react with purine N1 in Hoogsteen pairs [12], although experiments with the free nucleotide revealed that this position is not significantly reactive [17].

In this paper we use DEPC to investigate changes in DNA structure induced by binding of the quinoxaline antibiotics and discuss critically whether this chemical agent can discriminate between Watson-Crick and Hoogsteen base pairs.

MATERIALS AND METHODS

Antibiotic solutions

Echinomycin was a gift from Drs H. Bickel and K. Scheibli of Ciba-Geigy Ltd., Basel, Switzerland. Triostin A was a gift from Drs H. Otsuka and T. Yoshida of Shionogi & Co., Osaka, Japan. The des-N-tetramethyl analogue of triostin A (TANDEM) was synthesised by Dr R.K. Olsen, Department of Chemistry, Utah State University, USA. Actinomycin D is a product of Merck, Sharp and Dohme. Stock solutions (1mM) were prepared in absolute methanol and freshly diluted to the desired final concentration using 10mM Tris-HCl, pH 7.4 containing 50mM NaCl. In experiments performed to check the effect of methanol, a 5μM aqueous solution of echinomycin was prepared by shaking a sample of solid antibiotic with buffer for a few minutes and then filtering off the excess solid. The concentration in solution was estimated from the absorbance at 325nm (ε_{325}=11,500$M^{-1}cm^{-1}$). In the experiments performed at pH 5.5 or 4.6 echinomycin was made up to the desired concentration by diluting the stock solution with 20mM Na acetate (pH 5.5 or 4.6 as the case may be) containing 50mM NaCl.

DNA fragment

The 160 base-pair *tyrT* DNA fragment from *E.coli* containing the tyrosine

tRNA promoter was isolated and labelled as previously described [18,19]. The Watson (upper) strand can be labelled at the AvaI site on its 3'-end with [α-^{32}P]dCTP, and the Crick (lower) strand can be labelled at the EcoRI site on its 3'-end with [α-^{32}P]dATP.

Diethyl pyrocarbonate footprinting

The reaction with diethyl pyrocarbonate (DEPC) was performed by incubating 1µl of labelled tyrT DNA (3-4 pmoles in base pairs) with 19µl of the requisite antibiotic solution (final methanol concentration 10% v/v) for 15 min at 37°C. A control sample, containing 10% methanol, was subjected to identical treatment. The mixture was kept at 0°C for a few minutes and then 1µl of diethylpyrocarbonate (Sigma) was added. The reaction tubes were incubated for 15 min at room temperature with occasional mixing because DEPC is relatively insoluble in water. The reaction was terminated by addition of sodium acetate to 0.3M final concentration and sonicated calf thymus DNA (400µM final concentration) was added as carrier, followed by ethanol precipitation. In some preliminary experiments 400µM sonicated calf thymus DNA was added during the first incubation; this caused no differences in the final cleavage pattern. The pellet resulting from the ethanol precipitation was washed twice with 70% ethanol followed by vacuum-drying. The pellet was resuspended in 30µl of 1M piperidine, heated for 10 min at 90°C, lyophilized and resuspended in 2.5µl of 80% formamide containing 0.1% bromophenol blue and 10µM EDTA. Samples were heated at 100°C for 2 min prior to electrophoresis.

Methylation by dimethylsulphate

Methylation of tyrT DNA by dimethylsulphate in the presence and absence of the different antibiotics was performed as described by Maxam and Gilbert [20] but in the presence of 10% (v/v) methanol.

Gel electrophoresis and analysis of the results

The products of the DEPC and dimethylsulphate reactions were analysed on 0.3mm, 8% polyacrylamide gels containing 8M urea and tris-borate-EDTA buffer (pH 8.3). After 2h electrophoresis at 1500V, the gels were soaked in 10% acetic acid for 10 minutes, transferred to Whatman 3MM paper, dried under vacuum at 80°C and subjected to autoradiography at -70°C with an intensifying screen.

The intensity of modification at each purine residue after antibiotic treatment was compared with that in the control lane (lacking antibiotic). In general, differences that are not obvious to the unaided eye were ignored [14]. However, we have found it useful to scan the different gel lanes using

a Joyce-Loebl microdensitometer, which permits a comparison of the relative intensities of bands within each gel lane and corrects for any effects due to uneven loading of the gels. The results of such an analysis are presented as histograms where the heights are proportional to the relative intensity of the bands and thus to the extent of DEPC modification observed at each nucleotide.

RESULTS

Purines hyperreactive to DEPC in the presence of echinomycin

Patterns of diethyl pyrocarbonate (DEPC)-mediated DNA cleavage in the presence of varying concentrations of echinomycin are presented in Figure 2. We have used the tyrT DNA fragment since the echinomycin binding sites have already been located on it by DNAase I footprinting [18]. All the sites have been shown to centre around the dinucleotide step CpG, and every CpG dinucleotide in the tyrT fragment provides a site for echinomycin binding. Variations in gel band intensity are readily apparent and the patterns of hyperreactivity are presented as a histogram in Figure 3 where the CpG-containing antibiotic binding sites are boxed. It can be seen that nearly all the adenine residues show some degree of enhanced reactivity, and that certain guanines are also hyperreactive. However, it should be noted that the intensity of the bands in each gel lane is far from constant, which must reflect preferential reaction at some but not all purine residues.

Let us consider the relationship between these hyperreactive sites and the known echinomycin binding sites. Inspection of Figures 2 and 3 reveals that adenines with enhanced reactivity can frequently be found at either the 3'- or 5'-sides of antibiotic binding sites (see for example position 58 on the Watson strand). This is consistent with the previous study which showed DEPC hyperreactivity both distal and proximal to echinomycin binding sites [12]. However, it should also be noted that some enhancements are remote from known echinomycin binding sites. For example the adenine at position 44 (Crick strand) is strongly enhanced by antibiotic binding, yet is remote from the nearest CpG steps at positions 35 and 59. In addition, not all purines adjacent to binding sites show this enhancement. For example, neither the adenine at position 33 (Watson strand) nor the guanine at position 36 (Crick strand) exhibit enhanced reaction with DEPC, even at the highest echinomycin concentration used (100μM).

It is worth noting that the pattern of DEPC reactivity remains effectively constant above an antibiotic concentration of 6μM, consistent with the DNAase I footprinting studies which showed that the antibiotic-induced

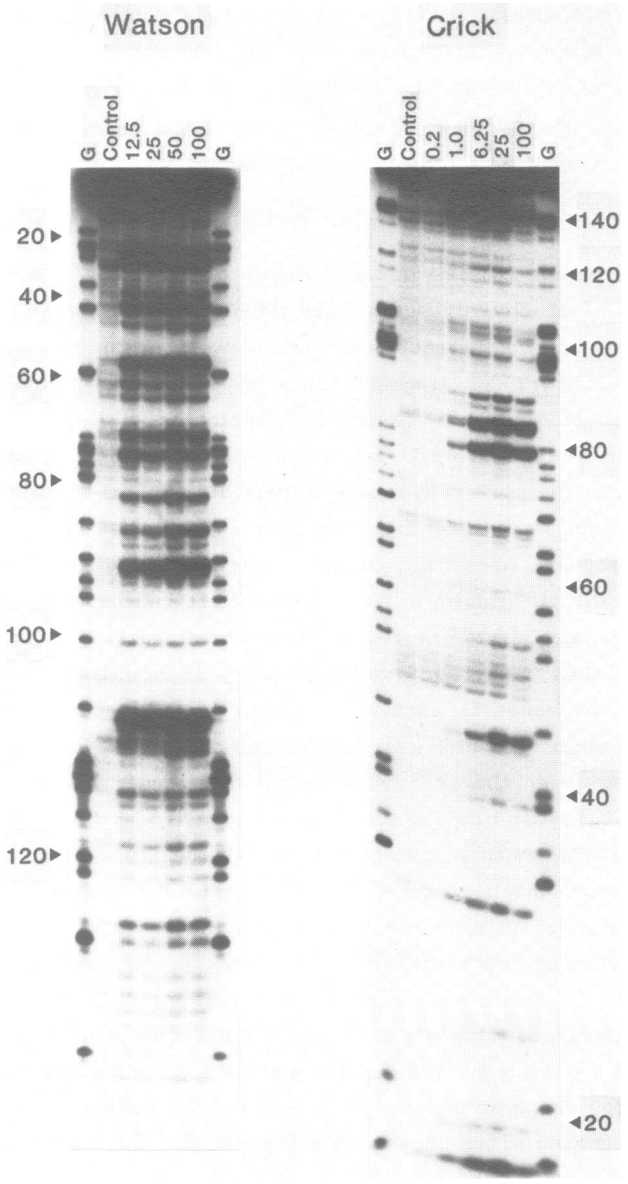


Figure 2. Diethyl pyrocarbonate reaction with the tyrT DNA fragment whose sequence is shown in Fig 3 in the presence of different concentrations of echinomycin (Watson = top strand and Crick = bottom strand, indicating which strand bears the 3' end label). All samples contained 10% methanol (v/v). The tracks labelled control contained no antibiotic. Tracks labelled "G" represent dimethylsulphate-piperidine markers specific for guanine.

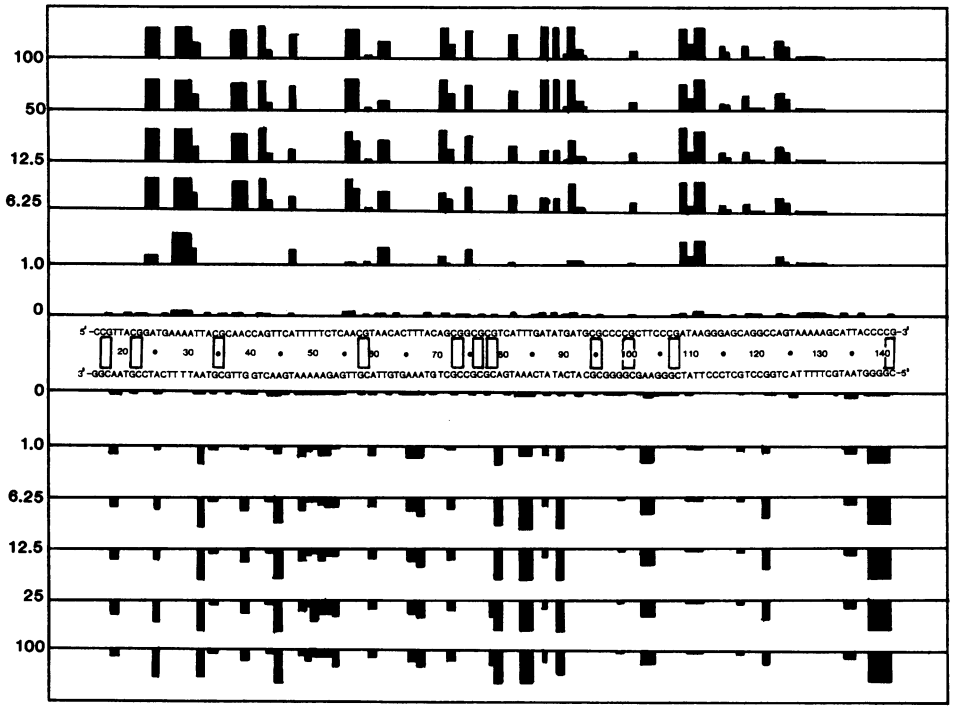


Figure 3. Summary map showing diethyl pyrocarbonate-reactive sites on tyrT DNA in the presence of different echinomycin concentrations as indicated on the ordinate. Reactive sites are represented in the form of bars with the height proportional to the enhancement of reaction deduced from the autoradiography film. The map was compiled from both densitometric tracings and visual inspection of several gels similar to those shown in Figure 2. Echinomycin binding sites (as deduced from DNAase I footprinting studies) are located around each of the CpG steps (boxed).

protection patterns tended to display all-or-none characteristics [18] in that no intermediate stages were observed between there being no effect of the antibiotic and the full effects found at the concentrations used in reference 18. The concentrations which produce a noticeable difference in the DEPC pattern are sightly lower than those which produced a threshold effect in the earlier work [18], although this may reflect the difference between positive footprinting (as in this case) and negative footprinting (ie the suppression of bands as in DNAase I footprinting).

The formation of a GC Hoogsteen base pair requires that the cytosine be protonated [11]. The pK_a of cytosine N3 is around 4.5 [21] so that under neutral conditions the formation of a Hoogsteen pair is unlikely. However,

the crystal structure of triostin A bound to the octanucleotide d(GCGTACGC) revealed that the terminal GC base pairs can adopt a Hoogsteen form even at pH 6.5 [10]. We have therefore investigated the reactivity to DEPC at low pH to see whether this would change the cleavage pattern by promoting the formation of GC Hoogsteen pairs. We chose pH 5.5 and 4.6 as a compromise so as to allow sufficient protonation of cytosine while preserving the integrity of the DNA double helix.

The results of such experiments are presented in Figure 4. They reveal that the drug-induced changes in the cleavage pattern are almost identical to those observed at pH 7.4 (see Figs 2 and 3). In particular, the notable reactivity of the guanine residue at position 75 on the Watson strand, which is tightly sandwiched between two strong echinomycin-binding sites, is totally unaffected by the acidity of the buffer. A decrease in pH does not therefore appear to affect the reactivity of guanine residues towards DEPC.

Because of the very low aqueous solubility of echinomycin (5μM) all the previous experiments were performed in the presence of 10% methanol. This concentration of organic solvent does not interfere significantly with nuclease digestion experiments [18] so we may assume that DNA structure is not grossly affected. Moreover, the effect of DMSO on the DEPC reaction (see below) demonstrates that this organic solvent does not have any significant effect at such a low percentage either. However, we have examined the effect of 4μM echinomycin in purely aqueous solution, prepared as described in Materials and Methods, on the DEPC modification reaction. The results (not shown) were similar to those seen in the presence of 10% methanol.

Purines hyperreactive to DEPC in the presence of triostin A

Patterns of DEPC modification of the tyrT DNA fragment in the presence of various concentrations of triostin A are presented in Figure 5. The lanes from antibiotic-treated samples reveal a pattern markedly different from the control and are very similar to those induced by echinomycin (Figure 2), though weaker in intensity. The equivalence of effect of these two antibiotics is consistent with their similar structures and sequence binding preferences [18,22]. Both echinomycin and triostin A form comparable complexes with oligonucleotides [8-10].

Purines hyperreactive to DEPC in the presence of TANDEM

Whereas triostin A and echinomycin both bind to DNA sites containing the dinucleotide step CpG the synthetic derivative des-N-tetramethyltriostin A (TANDEM) interacts preferentially with AT-rich DNAs [23] and seems to recognise the sequence TpA [22,24]. Typical patterns of DEPC modification of

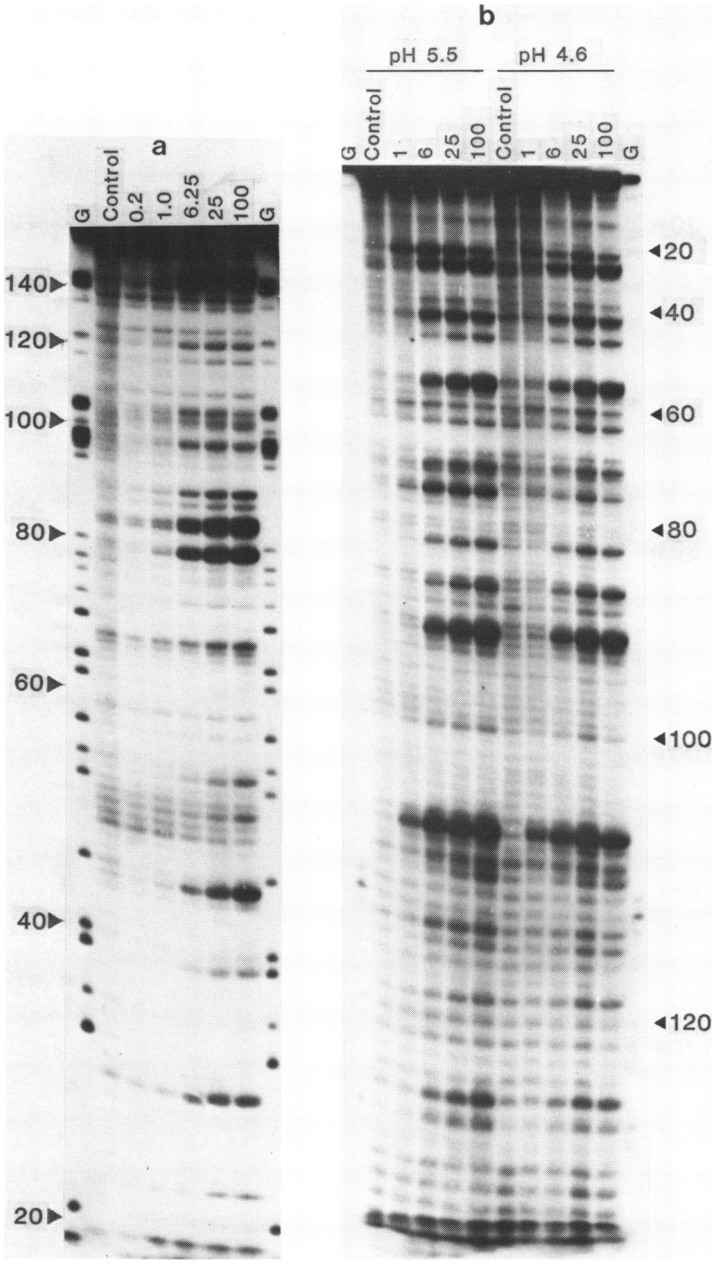


Figure 4. Diethyl pyrocarbonate reaction with tyrT DNA in the presence of echinomycin at acid pH. (a) Crick (bottom) strand at pH 5.5. (b) Watson (top) strand at pH 5.5 and 4.6. Details as described in Figure 2.

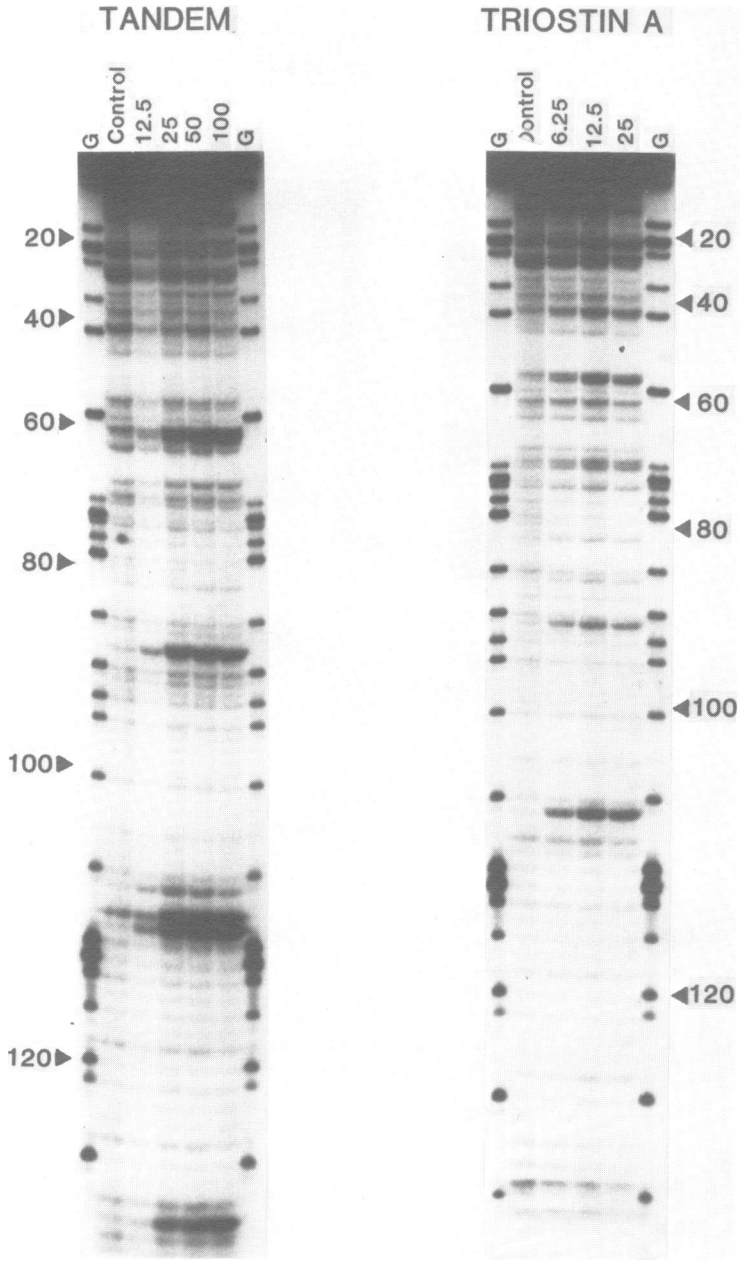


Figure 5. Diethyl pyrocarbonate reaction with the tyrT DNA fragment in the presence of different concentrations of TANDEM and triostin A. For both antibiotics only one of the DNA strands is shown. Other details as described in the legend to Figure 2.

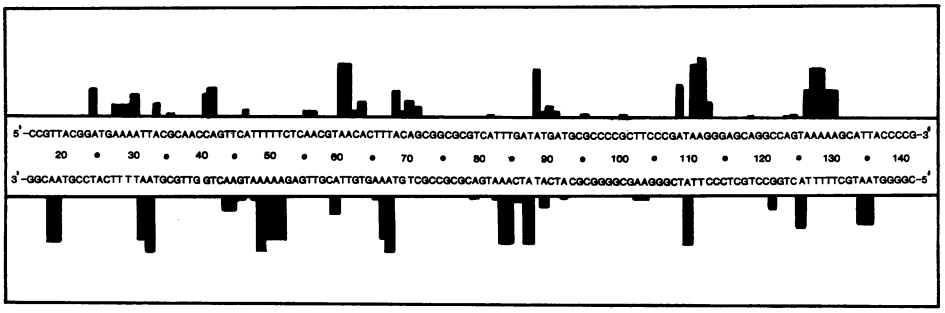


Figure 6. A summary map of diethyl pyrocarbonate reactive sites on tyrT DNA in the presence of TANDEM (25uM). Other details as described in Figure 3.

tyrT DNA in the presence of TANDEM are presented in Figure 5 and are represented as a histogram in Figure 6. Once again the pattern in the presence of the ligand is very different from that of the control, untreated DNA. In this case the hyperreactive bases are almost exclusively adenine residues. DNAase I footprinting has shown two clear binding sites for TANDEM on this DNA fragment at positions 88 and 110 [22]. Weaker binding around positions 60 and 32 has also been detected by micrococcal nuclease footprinting [25]. Adenines adjacent to, and within, these binding sites become hyperreactive to DEPC, although other adenines also display considerable reactivity, such as the long run of A residues in the vicinity of position 128 which is located well away from any known TANDEM binding sites. Indeed almost every adenine displays some enhanced reactivity to DEPC in the presence of TANDEM. The changes in susceptibility are only observed with TANDEM concentrations 2-5 times higher than those needed with echinomycin, probably due to the lower affinity of TANDEM for DNA, and again appear largely all-or-none in character with little additional change in the pattern at ligand concentrations above 20μM.

Effect of dimethylsulphoxide on the reactivity of purines to DEPC

Echinomycin, triostin A and TANDEM are all bifunctional intercalators which unwind the DNA helix about twice as much as known monofunctional intercalators such as actinomycin D and ethidium [26,27]. We have therefore considered the possibility that changes in reactivity to DEPC could arise from unwinding per se as opposed to more specific structural perturbations. To investigate this possibility we have used dimethylsulphoxide (DMSO), an organic solvent known to cause significant unwinding of DNA [19,28]. Figure 7(a) shows the effect of DMSO on patterns of DEPC reaction, where it can be

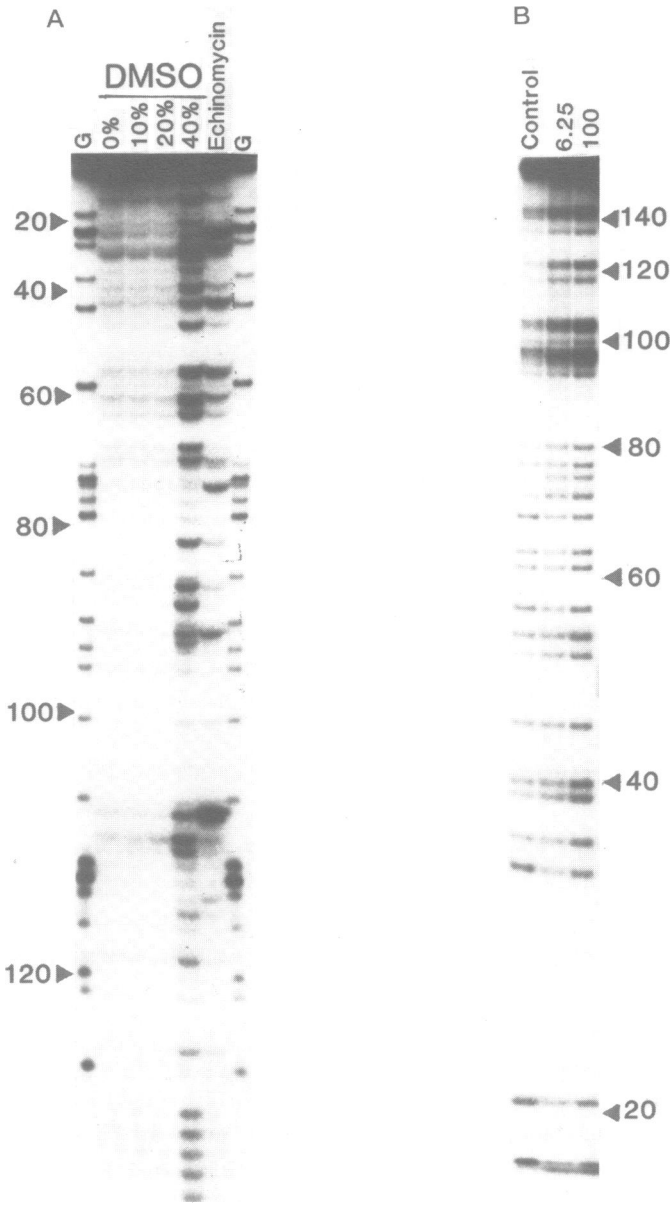


Figure 7. (A) Diethyl pyrocarbonate reaction with tyrT DNA in the presence of different concentrations (% v/v) of dimethyl sulphoxide (DMSO) together with a track containing 50uM echinomycin by way of comparison.
(B) Dimethylsulphate-piperidine reaction in the absence and in the presence of different concentrations of echinomycin.

seen that at solvent concentrations above about 30% there is indeed a marked increase in adenine reactivity. However, in contrast with the antibiotic-induced changes, the intensity of modification at each adenine residue is the same, regardless of its position within the sequence. In Figure 7(a) it is possible to compare the effects of 40% DMSO and 50μM echinomycin. It is quite clear that the organic solvent produces a general reactivity at adenine residues which cannot explain the distinctive pattern induced by binding of the antibiotics. Due to the poor solubility of DEPC in water, the reaction is normally performed in a two phase system, and it is possible that at least part of the increased reaction in 40% DMSO can be accounted for by the increased solubility of DEPC in the presence of an organic solvent. To investigate this possibility we surveyed the effects of a number of solvents, including methanol (frequently used to dissolve antibiotics of limited solubility) at concentrations up to 50% (v/v) in an effort to promote the solubility of the probe. No significant effect was observed (data not shown). Thus the enhanced reaction at adenosine residues in the presence of 40% DMSO is not merely due to solubilisation of DEPC, although we cannot rule out entirely some contribution from this effect.

Methylation by dimethylsulphate

Dimethylsulphate methylates guanine residues at the N7 position, in the major groove of the B-form helix, and adenines at N3 in the minor groove. Subsequent cleavage with piperidine yields the typical G track, whereas cleavage by acid produces a G>A reaction [20]. If the interaction between quinoxaline antibiotics and DNA caused the neighbouring DNA base pairs to undergo a transition to Hoogsteen pairing then the reactivity of these groups should be altered. The guanine N7 atom would be involved in base-pairing and would be redirected towards the minor groove so as to be less reactive to dimethylsulphate. Figure 7(b) shows the results of dimethyl sulphate modification in the presence and absence of echinomycin. Every band persists even in the highest concentration of echinomycin, suggesting that the accessibility of the N7 position of guanine is unaffected. These results argue against the formation of Hoogsteen GC pairs adjacent to echinomycin binding sites.

DISCUSSION

Diethyl pyrocarbonate has previously been used to detect Z-DNA [13,14] and hairpin loops (cruciforms) [15,16]. With Z-DNA the probe is presumed to interact with the purine N-7 (and the exocyclic N6 of adenine) which is exposed on the outer face of the helix. In Hoogsteen pairs N7 forms the

central, relatively shielded, portion of the base pair so that reaction with DEPC is more likely at N1 or N3 [12]. The results presented in this paper provide evidence that the binding of quinoxaline antibiotics to DNA causes a marked increase in reactivity towards DEPC, especially at adenine residues. The outstanding question concerns which of the four adenine nitrogens constitute the site(s) for this increased carboxyethylation and whether the reaction is consistent with the formation of Hoogsteen base pairs.

The failure of echinomycin to affect dimethylsulphate modification is important in that it suggests that access of a small probe to guanine N7 is unaffected by the binding of the antibiotic. This would argue against the formation of GC Hoogsteen pairs in which N7 is oriented quite differently and is involved in base pairing itself. Further evidence against Hoogsteen GC pairs is that the increased reactivity towards DEPC is not affected by lowering the pH to 5.5 or 4.6. The lower pH should facilitate protonation of cytosine and favour the formation of Hoogsteen pairs.

The situation with AT base pairs is less certain and we cannot dismiss the possibility that part of the increase in reactivity is due to the formation of Hoogsteen base pairs. Nevertheless, several observations are worth noting. Firstly, unwinding by DMSO at high concentrations also increases the reactivity of adenines to DEPC and the quinoxaline antibiotics are known to produce large unwinding angles [26,27]. However, general unwinding alone cannot explain the results since the drug-induced pattern is very specific, whereas the solvent-induced changes are uniform across every adenine residue. Local unwinding, caused by the specific binding of antibiotic to adjacent regions, could account for some of the increases in reactivity. But on the other hand, such monofunctional intercalators such as actinomycin D and ethidium do not affect the DEPC-mediated cleavage pattern (results not shown). In this context it is worth remembering that the quinoxalines differ from other intercalators as regards their structural effects, in that they are able to affect the positioning of DNA on nucleosome core particles [29,30].

Secondly, many of the adenines hyperreactive to DEPC are located at positions remote from known echinomycin binding sites. While it is possible to envisage small structural perturbations being transmitted along the DNA helix, such as changes in groove width, it is less easy to explain the formation of a Hoogsteen pair at a remote site. In the crystallographic work the rationale behind the formation of Hoogsteen pairs is that this allows closer contact between the peptide backbone of the antibiotic and the DNA

helix [8-10]. Clearly this cannot explain the formation of Hoogsteen pairs at a site displaced some distance from the antibiotic, especially if the intervening base pairs retain the normal Watson-Crick structure.

The results with TANDEM differ in that hyperreactivity is confined to adenosine residues. This ligand, which binds best to AT-rich DNAs, displays a binding constant for natural DNAs which is at least ten times weaker than that of the natural quinoxaline antibiotics [23], and as we have seen echinomycin and triostin A induce changes in the guanosine residues as well. It is conceivable that, whatever the molecular basis for the structural changes may be, reactivity towards DEPC is in some way related to the magnitude of the binding constants of the ligands and/or the rate constants for their dissociation from DNA. TANDEM may well be a less likely candidate for inducing Hoogsteen base pairs since a recent theoretical study suggests that it will bind best to a Watson-Crick structure in contrast to triostin A which will bind with equal affinity to the Watson-Crick helix and one containing Hoogsteen base pairs [31].

It is worth noting that the above discussion has focussed only on the preferred binding sites for these antibiotics, although it is known that they can interact with other secondary sites as well [25,27]. While antibiotic molecules associated with weaker sites may not yield enzymic footprints it is possible that they may remain bound for long enough to generate a DEPC-sensitive structural change. This could, in theory, explain some of the enhancements observed in regions remote from the preferred ligand binding sites.

In conclusion, we feel that the weight of evidence does not support the antibiotic-induced formation of Hoogsteen base pairs. It may be that some of the observed hyperreactive bases are in a Hoogsteen-like conformation, but this seems unlikely as a general explanation for the observed changes. We remain uncertain as to the real nature of structural transitions which result in enhanced reactivity to DEPC. The perturbed structure seen here is peculiar to the quinoxaline antibiotics and has not been observed with any other intercalating antibiotics. It is clearly not a standard B-type DNA and we can discount an A-type helix since double-stranded RNA is less reactive to DEPC than is DNA [32].

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