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Role of Apurinic Sites in the Resistance of Methylated Oligodeoxyribonucleotides to Degradation by Spleen Exonuclease

By GEOFFREY P. MARGISON* and PETER J. O'CONNOR
Paterson Laboratories, Christie Hospital and Holt Radium Institute, Manchester M20 9BX, U.K.

and ATHEL CORNISH-BOWDEN
Department of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT, U.K.

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The effect of introducing methyl groups into DNA substrates was studied by using the spleen exonuclease (EC 3.1.4.1), an enzyme which hydrolyses oligonucleotides in a sequential manner by splitting off 3'-phosphomononucleotides starting from the 5'-hydroxyl terminus. Analyses of oligodeoxyribonucleotide 3'-phosphate substrates after reaction *in vitro* with dimethyl sulphate demonstrated that the resultant methylation pattern differed from that previously found for native DNA, particularly with respect to the relative amounts of 1- and 3-methyladenine produced. Although after treatment with increasing amounts of dimethyl sulphate the substrate became progressively resistant to degradation by the exonuclease, the methylation products themselves were only partially responsible for the observed inhibition of enzyme activity. The incomplete degradation encountered was apparently due to the presence of apurinic sites, which arose as secondary lesions after the spontaneous release of the labile alkyl purines from the methylated substrate. Inhibition of enzyme activity appeared to be competitive, being characterized by constant values for apparent $V_{max.}$ and increased values for apparent K_m. The interpretation of this, however, is complicated by the complex nature of the substrate, and these aspects are considered in some detail.

Considerable attention has been given to studies of effects of chemical methylating agents on cellular nucleic acids and in particular to their reaction with DNA, but so far relatively little is known about the effects of these DNA reactions on the activity of enzymes concerned with the degradative metabolism of DNA. During studies on the mechanism of action of carcinogenic methylating agents, we have investigated the ability of various deoxyribonucleases to degrade chemically methylated DNA. In contrast with certain nucleases with alkaline pH optima, two nucleases, namely deoxyribonuclease II (EC 3.1.4.6) and spleen exonuclease (EC 3.1.4.1), both of which exhibit acid pH optima, were restricted in their capacity to degrade methylated DNA substrates. A preliminary account of this work was given by Margison & O'Connor (1972). In the present paper we demonstrate that the methylation products themselves are only partially responsible for the inhibition observed with the spleen exonuclease. The incomplete degradation of methylated oligodeoxyribonucleotides by this enzyme apparently results from the presence of apurinic sites which arise as secondary lesions following the spontaneous release of labile alkyl purines from the methylated substrate.

* Present address: Unit of Chemical Carcinogenesis, International Agency for Research on Cancer, 150 Cours Albert Thomas, 69008 Lyon, France.

Experimental

Materials

Spleen exonuclease was purified by the method of Bernardi & Bernardi (1968) from pig spleens, which were a gift from T. Wall and Son, Godley, Hyde, Cheshire, U.K. Deoxyribonuclease II (2200 Kunitz units/mg) from pig spleen was purchased from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey KT2 7BH, U.K. Di[^{14}C]methyl sulphate (sp. radioactivity 140mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K.; the unlabelled compound for dilution of the radioactive material was purchased from Hopkin and Williams, Chadwell Heath, Essex, U.K., and distilled before use. Calf thymus DNA (highly polymerized) was purchased from Worthington Biochemical Corp., Freehold, N.J., U.S.A.; Dowex resins AG 50W X4 (–400 mesh) and AG X1 (200–400 mesh) were from Bio-Rad Laboratories, Richmond, Calif., U.S.A., and Sephadex G-25 (superfine)

was from Pharmacia (G.B.) Ltd., London W5 5SS, U.K.

Methods

Assay of spleen exonuclease activity. Bernardi & Bernardi (1968) showed that the single-stranded oligodeoxyribonucleotide 3'-phosphates produced by deoxyribonuclease II degradation of DNA were the preferred substrates for spleen exonuclease. These were prepared by incubating calf thymus DNA (0.8mg/ml) at 37°C for 3h with commercial deoxyribonuclease II (0.2mg/ml) in 0.1M-sodium acetate–0.07M-EDTA, pH5.6; the incubation was continued for a further 3h after the addition of the same quantity of enzyme. The samples were deproteinized by repeated extraction with chloroform–3-methylbutan-1-ol (4:1, v/v), then dialysed against 0.01M-sodium acetate to remove traces of organic solvent. For assay of exonuclease activity the oligodeoxyribonucleotide substrate was used at a concentration of 200μg/ml; the buffer used was sodium acetate (pH5.6), otherwise assay conditions were those of Bernardi & Bernardi (1968), except where indicated.

Methylation of oligodeoxyribonucleotides. Oligodeoxyribonucleotides were methylated to different extents by using various amounts of unlabelled or ^{14}C-labelled dimethyl sulphate in the presence of 0.5M-NaCl in 0.25M-sodium cacodylate buffer (pH7.0), by the method of Uhlenhopp & Krasna (1971). Excess of dimethyl sulphate and the hydrolysis products thereof were removed by exhaustive dialysis against 0.01M-sodium acetate. The extents of methylation of the oligodeoxyribonucleotides (in terms of the proportion of guanine residues converted into 7-methylguanine) were found as a routine by chromatography, on a column (1cm×25cm) of Dowex 50 (H^+ form), of 1.0M-HCl hydrolysates (100°C, 60min) of 2–3mg samples of the material. An example of this type of analysis is given in Fig. 1 and the procedures were as described elsewhere (O'Connor *et al.*, 1972). After measurement of the E_{260}, 2.0ml of each fraction (6.0ml) was assayed for radioactivity. This procedure and also Sephadex G-25 chromatography of the original di[^{14}C]methyl sulphate-treated oligomers showed them to be free of residual dimethyl sulphate, since radioactivity due to the unchanged alkylating agent was readily detected by these methods. The molar proportions of the various methylation products found after dimethyl sulphate treatment are given in Table 1. In some cases DNA was methylated before digestion with deoxyribonuclease II; use of these methylated oligodeoxyribonucleotides is indicated in the text.

Depurination of oligonucleotides. The minimum time required to remove all the heat-labile methylpurines was found by heating [^{14}C]methylated oligonucleotides at 100°C in 0.1×SSC (0.015M-NaCl–0.0015M-sodium citrate, pH7.1). They were then subjected to Sephadex G-25 chromatography to separate the free bases from the oligonucleotides. The extinction of each fraction was measured at 260nm and a small sample was assayed for radioactivity. It was shown that 10min was sufficient to release the heat-labile methylpurines under these conditions (Table 2). Normal oligodeoxyribonucleotides were made completely apurinic by using the method of Peterson & Burton (1964);

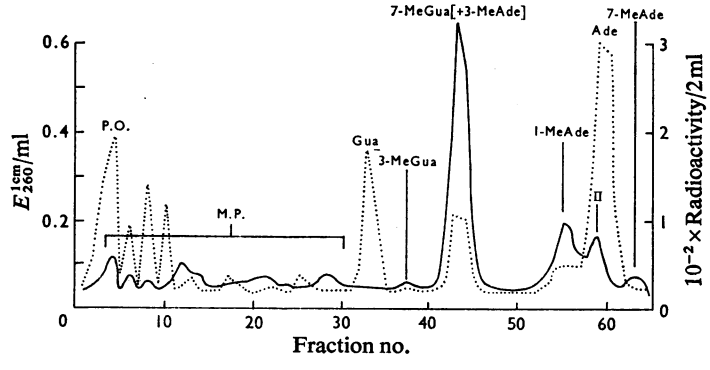

Fig. 1. *Dowex 50 (H^+ form) chromatography of a 1M-HCl hydrolysate of di[^{14}C]methyl sulphate-treated oligodeoxyribonucleotides*

P.O., Incompletely hydrolysed pyrimidine oligomers and mononucleotides; M.P., minor unidentified radioactive products; Gua, guanine; 3-MeGua, 3-methylguanine; 7-MeGua+3-MeAde, 7-methylguanine and 3-methyladenine; 1-MeAde, 1-methyladenine, and II, its HCl breakdown product 5-amino-4-(*N*-methyl)carboxamidoimidazole; 7-MeAde, 7-methyladenine; ——, radioactivity; ----, E_{260}. The relative amounts of the various products are given in Table 1.

Table 1. *Products of methylation of oligodeoxyribonucleotides by di[^{14}C]methyl sulphate*

The molar proportions of the products are expressed as a percentage of total radioactivity in DNA. For comparison, values for the methylation of native calf thymus DNA by dimethyl sulphate *in vitro* are given in parentheses (from Margison & O'Connor, 1973). Details of these analytical procedures are given in the text.

Products	Oligodeoxyribonucleotides		
3-Methylguanine	0.7		(0.9)
7-Methylguanine*	50.0	53.9	(74.7)
3-Methyladenine*	3.9		(19.7)
1-Methyladenine	15.7		(2.2)
5-Amino-4-(*N*-methyl)carboxamidoimidazole†	10.8	26.5	
7-Methyladenine	1.1		(1.5)
Minor products+ 3-methylcytosine	17.9		(1.1)

* 7-Methylguanine and 3-methyladenine co-chromatograph on Dowex 50 (H^+ form); separation was achieved by rechromatography on Dowex 50 (NH^+ form) (see Margison & O'Connor, 1973).

† Acid-degradation product of 1-methyladenine.

Table 2. *Release of heat-labile methylpurines from di[^{14}C]-methyl sulphate-treated oligodeoxyribonucleotides during heating at 100°C in 0.1×SSC*

The depurinated bases were separated from the oligomers by chromatography on Sephadex G-25.

Time of heating (min)	Total radioactivity remaining bound to oligodeoxyribonucleotides (%)
0	100
7.5	44
30	45
60	42
90	46

they were freeze-dried and stored at a concentration of 1–2mg/ml.

Analysis of products of degradation by spleen exonuclease by Dowex 1 (formate form) chromatography. After degradation by spleen exonuclease, the reaction products from digests of normal or apurinic oligonucleotides were deproteinized, adjusted to pH6–7 and diluted to an ionic strength of less than 0.02mol/l. Deoxyribonucleotide 3′-phosphates were separated on columns (1cm×20cm) of Dowex-1 (formate form), which were eluted with an exponential gradient produced by passing 0.8M-ammonium formate (pH5.0) into 500ml of water in an airtight mixing vessel. After measurement of the E_{260} a sample of each 6ml fraction was taken for the determination of deoxyribose by the Burton (1956) procedure.

Paper chromatography. Products from spleen exonuclease digests were spotted on Whatman no. 1

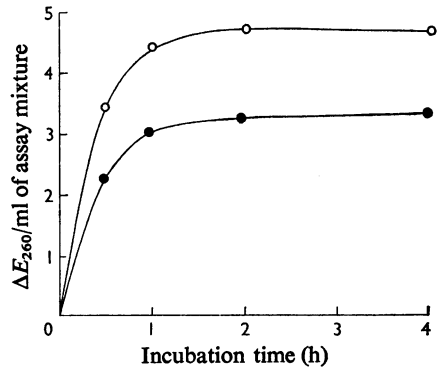

Fig. 2. *Degradation of oligodeoxyribonucleotides by spleen exonuclease*

Substrate (50μg) was incubated at 37°C with 20μl of the enzyme in 0.01M-EDTA–0.15M-sodium acetate, pH5.6, in a total volume of 0.25ml. The reaction was stopped by the addition of an equal volume of cold 2.5% $HClO_4$–0.25% uranyl acetate and the extinction at 260nm of a 3000*g* supernatant was recorded. Controls consisted of similarly incubated samples to which the enzyme was added after the reaction was stopped. ○, Normal oligomers; ●, methylated oligomers (44% 7-methylguanine).

paper and developed for 20h in a descending direction by using propan-2-ol – aq. NH_3 (sp.gr. 0.880)–water (7:1:2, by vol.).

Assay of radioactivity. Samples (2.0ml) of fractions obtained by chromatography on Dowex 50 and on Sephadex gels were counted for radioactivity in a scintillation 'cocktail' (10ml), consisting of Triton X-100 (1 litre) and toluene (1 litre), containing 16g of 2,5-diphenyloxazole and 0.4g of 1,4-bis-(5-phenyloxazol-2-yl)benzene. Appropriate internal standards were used to assess the efficiency of counting, which was 60–75%.

Results

Inhibition of exonuclease activity

Whereas spleen exonuclease can degrade normal oligodeoxyribonucleotides quantitatively to mononucleotide 3′-phosphates, part of the substrate became completely resistant to digestion after methylation by dimethyl sulphate (Fig. 2). Samples from spleen exonuclease digests of control or methylated oligodeoxyribonucleotides were tested by paper chromatography, and it was found that mononucleotides were readily generated from the control substrate, but only to a limited extent from the methylated substrate, even after overnight digests.

Comparison of several samples of methylated oligodeoxyribonucleotides demonstrated that the proportion of the substrate which was resistant

was linearly related to the extent of methylation (Fig. 3).

In the inhibition experiments the apparent value of $V_{max.}$ was constant, but the apparent value of K_m increased with the extent of methylation, as shown in Table 3. In other words the inhibition appeared to be competitive, but the detailed interpretation is complicated by the heterogeneous nature of the substrate and is discussed below. In some of the earlier experiments oligonucleotides were produced by digestion of methylated DNA (see the Experimental section). In these cases it was expected that this would lead to the production of oligonucleotides which differed considerably in length, owing to the inhibition of deoxyribonuclease II activity caused by methylation of the DNA (Margison & O'Connor, 1972). Attempts to demonstrate these differences were complicated by the effects of positive charges arising from the introduction of methyl groups into the polymer. However, data obtained for methylated oligonucleotides prepared in this way showed the same competitive-inhibition kinetics as described above. For both of these procedures values obtained for K_m varied to some extent with each preparative batch of oligonucleotides, but when the results were normalized (i.e. K_m for control oligonucleotides = 1) a linear relationship could be demonstrated between the increasing value for K_m and the increasing extent of methylation; data from both sources followed this relationship.

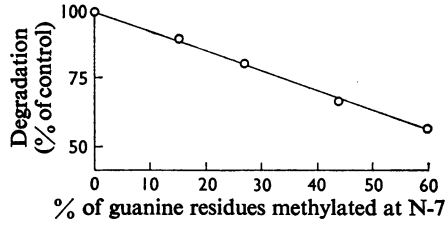

Fig. 3. *Relationship between the extent of methylation of oligodeoxyribonucleotides and their degradation by spleen exonuclease*

The assay conditions were as in Fig. 2. Incubation was for 2h.

Table 3. *Variation of K_m and $V_{max.}$ with increasing amounts of methylation in the oligodeoxyribonucleotide substrate*

For details see the text.

% of guanine residues methylated at N-7	K_m (μg)	$V_{max.}$ (E_{260}/h)
0	285	11.40
12.9	322	11.28
23.8	400	11.10

Nature of the inhibition of exonuclease activity

In an attempt to establish the nature of this resistance to exonuclease attack, a preparation of [^{14}C]-methylated oligodeoxyribonucleotides was exposed to the enzyme until degradation was maximal. The released 3'-mononucleotides and the resistant substrate were separated by Sephadex G-25 chromatography, pooled and the extent of methylation was determined. The small quantity of alkylpurines liberated by chemical hydrolysis (during the enzymic incubation) was also separated by this procedure and therefore does not spuriously raise the specific radioactivity of the degraded fraction. On comparison with the original substrate, it was seen that none of the methylation products was found exclusively in the resistant part (Table 4). However, compared with the original substrate, the relative amounts of 7-methylguanine and of 3-methyladenine were increased in the resistant material and slightly lower in the degraded material. These results indicate that methylated mononucleotides are liberated by the enzyme, but with lower efficiency compared with normal nucleotides. Although the lower specific radioactivity of the degraded fraction could be partially explained by the more rapid release of the 7- and 3-substituted alkyl purines (see the Discussion section), this cannot account for the fact that 1-methyladenine, which is a stable product in DNA, behaved in a similar

Table 4. *Distribution of methylated purines in the oligodeoxyribonucleotide substrate and in the products of its degradation by spleen exonuclease*

For details see the text.

Oligodeoxyribonucleotide material	Guanine (μmol) / Adenine (μmol)	% of guanine residues methylated at N-7	Total radioactivity (c.p.m./μmol of purine)	
			7-Methylguanine (+3-methyladenine)*	1-Methyladenine
Original	0.57	38	4970	1420
Resistant	0.56	53	10250	2080
Degraded	0.57	28	3130	1270

* The amount of 3-methyladenine was small (see Table 1).

manner. Further, the release of alkylpurines cannot explain the increased specific radioactivity of the resistant fraction compared with the original substrate.

Thus there appeared to be no direct methylation product which was entirely responsible for the resistance of methylated oligonucleotides to spleen exonuclease degradation. However, it is known that the major alkylation product, 7-methylguanine, is released spontaneously from DNA and that 3-methyladenine is even more susceptible to this process (Lawley & Brookes, 1963; Margison *et al.*, 1973; Margison & O'Connor, 1973). It was considered therefore that the resistance might be attributed to the resulting apurinic sites. These sites would be generated quite rapidly during incubation with the enzyme at pH5.6; approx. 4% of the 3-methyladenine residues and 0.6% of the 7-methylguanine residues would be released per h from DNA under these conditions (Margison *et al.*, 1973; Margison & O'Connor, 1973) and a higher rate would be expected for DNA oligomers (see the Discussion section). Since the depurination reaction approximates to a first-order process, the number of sites produced would be proportional to the initial amount of alkylation.

To test this hypothesis, methylated oligonucleotides were heated for the time required to release all of the heat-labile methylpurines and were then subjected to spleen exonuclease attack. Control oligonucleotides were treated similarly and the results (Fig. 4) indicated that heat treatment of the methylated oligonucleotides further increased the proportion resistant to the exonuclease, but had no effect on the degradation of the normal substrate.

In this experiment, the heat-labile bases were present during the enzyme incubation, and there was the possibility that these free bases were competing with oligonucleotides for active binding sites on the enzyme. To eliminate this, the free alkylpurines were removed from heat-treated methylated oligodeoxyribonucleotides by Sephadex G-25 chromatography. Oligonucleotides treated in this way were resistant to complete degradation by the exonuclease, as previously demonstrated (Figs. 5*a* and 5*b*). When apurinic sites were generated more slowly in a methylated substrate by heating at 80°C for various periods, as expected, resistance increased with the gradual release of alkylpurines (Fig. 6). The initial linear relationship obtained in these experiments provided a further indication of the role of these secondary lesions in the inhibition observed when the methylated substrate was heated.

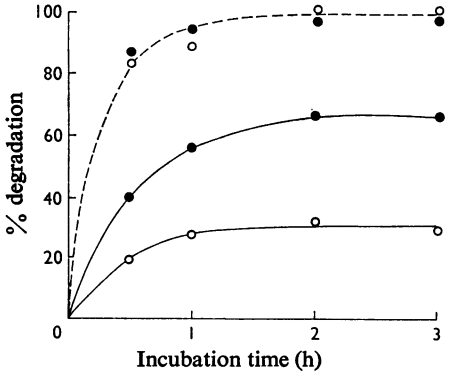

Fig. 4. *Degradation of heat-treated and control oligodeoxyribonucleotides by spleen exonuclease*

Samples were treated for 10min at 100°C in 0.1×SSC and assay conditions were as described under Fig. 2. Normal substrates, ●----●, unheated, and ○----○, heated; methylated substrates (44% 7-methylguanine), ●——●, unheated, and ○——○, heated.

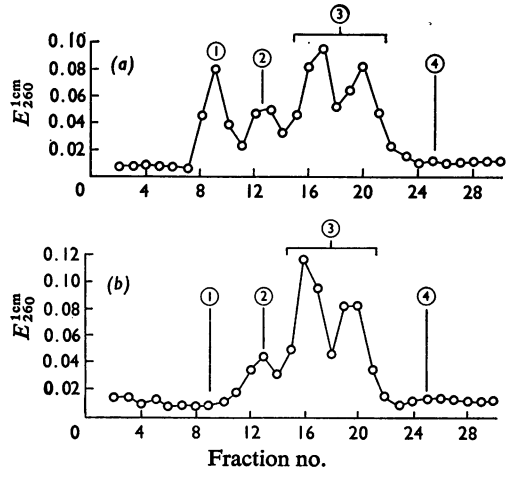

Fig. 5. *Sephadex G-25 column chromatography of heat-treated methylated and control oligodeoxyribonucleotides after digestion with spleen exonuclease*

The methylated substrate was heated for 20min at 100°C in 0.1×SSC and then subjected to gel filtration to separate the free alkylpurines from the oligonucleotides before exposure to the enzyme; the control substrate was treated similarly. Enzyme hydrolysates were then applied to columns (2.5cm×10cm) and eluted with 0.1×SSC; 2ml fractions were collected. The elution positions are identified as follows: 1, oligodeoxyribonucleotides; 2, incubation buffer salts; 3, 3′-mononucleotides; 4, the free alkyl purines. (*a*) Methylated oligonucleotides (63.7% 7-methylguanine) after incubation with spleen exonuclease until degradation was maximal; (*b*) control oligonucleotides treated as for (*a*).

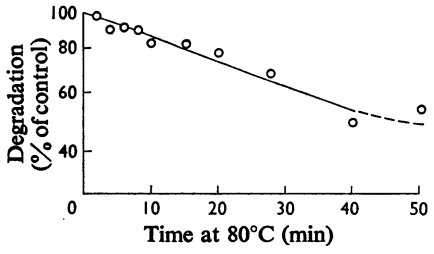

Fig. 6. *Degradation of methylated oligodeoxyribonucleotides* (63.7% *7-methylguanine*) *by spleen exonuclease after heating the substrate for various times at* 80°C *in* 0.1 × *SSC*

Samples were cooled in ice and then subjected to spleen exonuclease degradation for 2h under the incubation conditions given in Fig. 2.

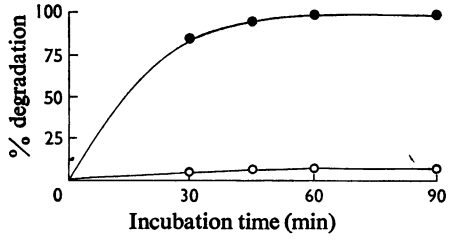

Fig. 7. *Degradation of normal and apurinic oligodeoxyribonucleotides by spleen exonuclease*

The incubation conditions were as described under Fig. 2, except for the concentrations of substrate and enzyme, which were 1.5mg and 0.6ml respectively per ml of the mixture. Samples were withdrawn for assay at the times shown above. ●, Normal substrate; ○, apurinic substrate.

The inability of this exonuclease to degrade beyond apurinic sites in oligodeoxyribonucleotides was demonstrated by using a completely depurinated substrate. Under the usual acid-soluble nucleotide assay conditions, apurinic oligodeoxyribonucleotides were almost completely resistant to degradation (Fig. 7). Chromatography on Dowex 1 (formate form) of a large quantity of exonuclease-degraded apurinic oligonucleotides taken after 90min incubation (see Fig. 7) showed that 2′-deoxyribose 3′-phosphate was below the level of detection (i.e. 0.001 μmol/ml) by using the Burton (1956) procedure (Fig. 8*a*). On the other hand the peak fractions for 3′-dCMP and 3′-dTMP contained 0.29 and 0.25 μmol/ml respectively, showing that the enzyme was able to degrade suitable termini. When an exonuclease digest of a small quantity of normal oligonucleotides was similarly fractionated (Fig. 8*b*) the Burton (1956) method was shown to operate

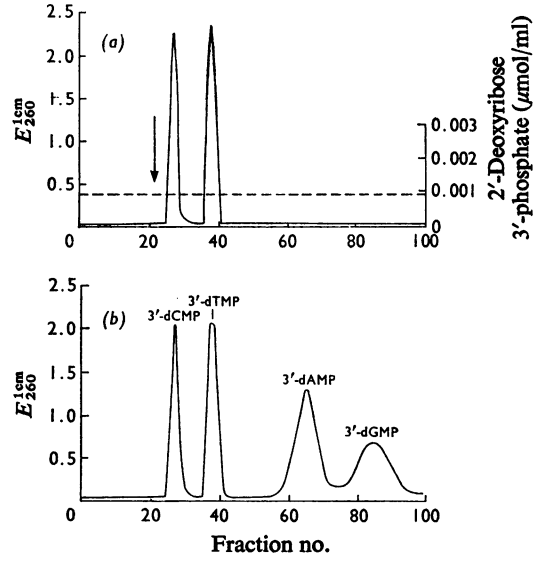

Fig. 8. *Dowex* 1 (*formate form*) *chromatography of products of spleen exonuclease degradation of oligodeoxyribonucleotides*

Samples for analysis were from a 90min digest (see Fig. 7) of (*a*) apurinic oligodeoxyribonucleotides and (*b*) normal oligodeoxyribonucleotides. The arrow indicates the expected position of 2′-deoxyribose 3′-phosphate, which was not detected by the Burton (1956) procedure (sensitive to 0.001 μmol/ml).

under these conditions; the amounts of the purine nucleotides determined spectrophotometrically corresponded very closely to those determined by the diphenylamine procedure.

Discussion

In this report we have attempted to assess some of the effects due to the introduction of methyl groups in substrate DNA which is subsequently used in degradative enzyme reactions. Initially, dimethyl sulphate was used for this purpose to limit, as far as possible, the complexity of reactions produced in DNA. This avoided the production of significant amounts of *O*-methylation products, as found with S_N1-type reagents (Loveless, 1969; Bannon & Verly, 1972).

Inhibition of spleen exonuclease activity

Although the inhibition of spleen exonuclease observed with methylated substrates appears to be competitive, the situation is complicated by the fact that the inhibitor and substrate form part of the same molecule, and by the fact that methylated oligonucleotides behave as poor substrates whereas

oligonucleotides with apurinic terminal or near-terminal sites are not hydrolysed at all. So there are three types of molecule that must be considered even in a first approximation, namely (i) unmodified substrate, represented as S, (ii) methylated material capable of reacting, represented as S′, and (iii) modified material with apurinic terminal or near-terminal sites capable of binding to the enzyme but incapable of reacting, represented as I. The situation is similar to the case of competing substrates considered by Foster & Niemann (1951), and the measured velocity is the sum of the hydrolysis rates of S and S′, given by

$$v = \frac{\frac{V_{max.}[S]}{K_m} + \frac{V_{max.}[S']}{K'_m}}{1 + \frac{[S]}{K_m} + \frac{[S']}{K'_m} + \frac{[I]}{K_i}}$$

where $V_{max.}$ and K_m are the maximum velocity and Michaelis constant respectively for S, $V'_{max.}$ and K'_m are the maximum velocity and Michaelis constant respectively for S′, and K_i is the inhibition constant for I. But the three concentrations are not varied independently, as in a conventional inhibition experiment, but in unison. The practical consequence of this is that it is impossible to overcome any competitive inhibition by saturating the enzyme with substrate. But in fact the observed maximum velocity is independent of the degree of methylation, which indicates that S and S′ must react with the same maximum velocity, i.e. $V_{max.} = V'_{max.}$, and that inhibition by I is insignificant, i.e. K_i is large. So the equation can be simplified to

$$v = \frac{V_{max.}\left(\frac{[S]}{K_m} + \frac{[S']}{K'_m}\right)}{1 + \frac{[S]}{K_m} + \frac{[S']}{K'_m}}$$

The observation that the apparent value of K_m increases with the degree of methylation implies that K'_m is greater than K_m, i.e. that S′ binds less tightly than S, which is chemically reasonable.

These conclusions may be summarized as follows: material with a methylated terminal appears to bind less tightly to the enzyme than the native substrate, but once bound it reacts with the same maximum velocity; material with a terminal or near-terminal apurinic site does not bind significantly to the enzyme, and so has no detectable reactivity either as a substrate or as an inhibitor.

Reactions with DNA

With regard to the methylation and depurination reactions two points are worthy of note. First, the extent of methylation at the N-3 position of adenine is very much less in the oligonucleotide than in calf thymus DNA (see Table 1), whereas the higher proportion of 1-methyladenine found in these oligonucleotides compared with native DNA would have been predicted from the expected degree of hydrogen-bonding, as shown by earlier studies with native and denatured DNA (Lawley & Brookes, 1963) and with polynucleotides (Ludlum, 1965; Pochon & Michelson, 1967). Secondly, the rate of release of methylpurines during heat treatment is much higher than that observed for their release from DNA heated in neutral solution (O'Connor *et al.*, 1975; Bannon & Verly, 1972) or similarly for the ethylpurines (Bannon & Verly, 1972). In this context it has been shown that methylated purines are released at a higher rate from nucleosides than from nucleotides (Lawley & Brookes, 1963), dinucleotides are next (Hendler *et al.*, 1970) and DNA exhibits the lowest rate of depurination (Lawley & Brookes, 1963; Margison *et al.*, 1973). Chemical depurination *in vivo* would therefore be expected to proceed at a higher rate in oligodeoxyribonucleotides than in DNA.

General considerations

In normal DNA apurinic sites are relatively rare (Lindahl & Nyberg, 1972), but after treatment of rats with low doses of methyl methanesulphonate about 10^7 out of a total of 10^{11} bases per liver cell will be in the form of methylpurines (Margison & O'Connor, 1973). Chemical depurination of the methylpurines therefore is likely to give rise to many apurinic sites *in vivo*. Recently it has been suggested that enzymic release of O^6-methylguanine and of 3-methyladenine as free bases may be the initial step in the repair of these lesions in bacteria (Kirtikar & Goldthwait, 1974). A combination of these processes could lead to a dramatic increase in the numbers of apurinic sites present in the DNA of animals treated with monofunctional alkylating agents. Also, apurinic sites are produced in DNA by a variety of other agents, e.g. bleomycin (Muller *et al.*, 1972), HNO_2 (Burnotte & Verly, 1971) and heat, which *in vivo* would be expected to produce these lesions at a low rate (Lindahl & Nyberg, 1972). Endonucleases which act specifically at apurinic sites in DNA may constitute part of a major repair mechanism, since nucleases of this type have now been found in bacteria, plant and mammalian tissues (Hadi & Goldthwait, 1971; Paquette *et al.*, 1972; Ljungquist & Lindahl, 1974; Verly & Paquette, 1973). The biological and chemical effects of apurinic sites have been discussed by Lawley (1974) and Verly (1974).

The biological role of exonucleases of the type characterized by the spleen enzyme is at present unknown, and any suggestions with regard to the

findings presented here are therefore purely speculative. However, deoxyribonuclease II, the enzyme which liberates the preferred substrate for this exonuclease (Bernardi & Bernardi, 1968) is more abundant in rapidly dividing tissues (Cordonnier & Bernardi, 1968), and is present in nuclei (Slor & Lev, 1971), particularly in the nuclei of very young as opposed to adult mice (Lesca, 1968). The likelihood, therefore, of a role for deoxyribonuclease II, and possibly for this exonuclease, which is associated with periods of nuclear activity and distinct from processes for example leading to the dissolution of redundant DNA or cell lysis and death, should be considered. Alternatively, a direct attack by the exonuclease on DNA might be envisaged. Bernardi & Bernardi (1968) found that this enzyme could attack denatured DNA, and, although native DNA was degraded to a much lesser extent, in both cases the initial reaction was quite rapid.

From the data presented here it might be expected that the action of this exonuclease in DNA metabolism would be inhibited by the presence of a 5'-terminal or near-terminal apurinic site in the substrate molecule unless, in the former instance, the adjacent nucleotide was exposed through the intervention of an enzyme which permitted the excision of a 2'-deoxyribose 3'-phosphate from apurinic-terminated DNA molecules. These data show that the spleen exonuclease is unsuitable for use in the quantitative base analysis of DNA containing a significant number of apurinic sites (e.g. methylated DNA), but that it may prove useful in the detection of apurinic sites in oligonucleotides which are too small to band on density gradients.

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