Stability of Pyrimidine Oligodeoxyribonucleotides Released during Degradation of Deoxyribonucleic Acid with Formic Acid–Diphenylamine Reagent

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(Received 25 November 1974)

A small release of P_i from a diphenylamine–formic acid digest of DNA was detected after elimination of interpurine phosphodiester bonds was complete. Minor components in the DNA digest were identified as pyrimidine oligonucleotides which had lost one terminal phosphate. Isolated pyrimidine tracts released P_i on redigestion with the formic acid–diphenylamine reagent in amounts that increased with the number of nucleotides in the oligonucleotide taken. The oligonucleotides were also partially degraded by the formic acid–diphenylamine reagent and the degradation (2–3% of phosphodiester bonds between consecutive nucleotides) was almost independent of chain length. The cleavage was random with no preference for a phosphodiester bond flanked by particular nucleosides. This minor lack of specificity in the formic acid–diphenylamine-catalysed degradation of DNA can, however, account for the low recoveries of long pyrimidine tracts previously reported. Any analysis of pyrimidine tracts in a DNA molecule should make some correction for this small degree of degradation if exact assignments of the numbers of pyrimidine tracts are to be made.

Burton & Petersen (1957, 1960) demonstrated that DNA could be degraded by formic acid (66%, v/v) containing diphenylamine (2%, w/v) and that this reaction was potentially valuable for the study of sequences in DNA. The more recent detailed studies of DNA molecules such as each of the separated strands of bacteriophage T7 DNA and bacteriophage λ DNA (Mushynski & Spencer, 1970), the viral and complementary strands of bacteriophages ϕ X174 and S13 DNA (Hall & Sinsheimer, 1963; Darby *et al.*, 1970; Cerný *et al.*, 1969) and guinea-pig satellite DNA (Southern, 1970) have indeed demonstrated the usefulness of this reagent.

The degradation of DNA by diphenylamine in acid solution yields pyrimidine nucleoside diphosphates of general formula, $dPyd_nP_{n+1}$,† free purine bases and inorganic orthophosphate, and the reaction appears to go to completion without the occurrence of side or exchange reactions (Burton & Petersen, 1960). No degradation of a short pyrimidine tract ($dCyd_4P_5$) was observed on reincubation of the isolated oligo-

nucleotide with the formic acid–diphenylamine reagent although minor decomposition would not have been detected (Petersen & Burton, 1964). H_2SO_4 (0.1 M) at 100°C also degrades DNA, releasing the same products as the formic acid–diphenylamine reagent (Shapiro & Chargaff, 1957; Spencer & Chargaff, 1963), but the two methods do not give the same frequencies of pyrimidine tracts from an identical sample of DNA. It has been demonstrated that not only is the release of interpurine phosphate incomplete, but also additional reaction products are obtained after acid digestion of DNA at 100°C (Petersen & Burton, 1964; Spencer *et al.*, 1969). For this reason all recent studies of the pyrimidine content of DNA molecules have used the formic acid–diphenylamine degradation procedure.

It was observed during an investigation of the pyrimidine tracts in bacteriophage f1 DNA (Petersen & Reeves, 1969; Tate & Petersen, 1974a,b) that the longest tract of length 19 or 20 nucleotides was recovered in an amount representing about 0.3–0.4 sequence per DNA molecule. A low recovery of the pyrimidine tract of the same chain length in bacteriophage fd DNA, and of the longest pyrimidine tracts in the separated strands of bacteriophage T7 DNA, was also obtained (Ling, 1972; Mushynski & Spencer, 1970).

In an attempt to account for the poor recoveries of the long pyrimidine tracts, the release of P_i from a ^{32}P -labelled sample of bacteriophage f1 DNA has

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† Abbreviations: dPyd, pyrimidine deoxyribonucleoside; dCyd, deoxycytidine; dThd, thymidine; P , phosphate esterified with the nucleoside; formic acid–diphenylamine reagent, 2% (w/v) diphenylamine in 66% (v/v) formic acid.

been carefully examined and, in addition, the stability of pyrimidine deoxyribonucleotides in the acid-diphenylamine reagent has been further investigated. This report demonstrates that a small but detectable decomposition of the pyrimidine tract occurs when isolated pyrimidine deoxyribonucleotides are incubated with formic acid containing diphenylamine.

Experimental

Materials

Carrier-free [^{32}P]P_i was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Polyethylene glycol 20000 (Fisher Scientific Co., Fairlawn, N.J., U.S.A.) was used as a stock solution in 0.5M-NaCl. Diphenylamine (BDH, Poole, Dorset, U.K.), AnalaR-grade, was decolorized with charcoal and recrystallized twice from ethanol before use. AnalaR 98–100% formic acid (BDH) was used without further purification. Urea (May and Baker, Dagenham, Essex, U.K.), as a saturated solution, was passed through a column (20cm×5cm diam.) containing a mixture of Darco G60 activated charcoal (Darco Corp., New York, N.Y., U.S.A.) and Celite 545 (Koch-Light Laboratories, Colnbrook, Bucks., U.K.) in the ratio of 1:5 (w/w) covered with a layer of DEAE-Sephadex G-25 (coarse). The microgranular DEAE-cellulose anion-exchange resin, DE-32 (Whatman Biochemicals, Maidstone, Kent, U.K.), was used for all studies. Calf thymus or sheep thymus DNA was used as non-radioactive carrier DNA.

Bacteria and bacteriophage. *Escherichia coli* K13 and *E. coli* K12 Hfr 3300 used in this study were kindly supplied by Dr. N. Zinder, Rockefeller University, New York, N.Y., U.S.A., and Dr. H. Schaller, Max-Planck-Institut für Virusforschung, Tübingen Germany, respectively. Bacteriophage f1 was kindly supplied by Dr. N. Zinder.

Methods

Phage growth and purification. Bacteriophage f1 was grown with *E. coli* K13 as host bacterium in 1 litre of medium used by Marvin & Schaller (1966). Alternatively, virus was prepared in 30ml cultures of *E. coli* K12 Hfr 3300 in T.C.G. medium (Abelson & Thomas, 1966) as described by Schaller (1969). [^{32}P]P_i (2mCi) was added to the cultures (2×10^6 cells/ml) 10min after infection with bacteriophage f1 (multiplicity 1–10). After the bacteria had been removed the phage were precipitated at pH3.8 and purified by selective precipitation with polyethylene glycol (Sutton & Petersen, 1971; Yamamoto *et al.*, 1970). Such preparations were shown to be free of contaminating bacterial fragments when examined by electron microscopy.

Isolation and degradation of the DNA. The bacteriophage DNA was isolated by a modification of the procedure of Marvin & Schaller (1966) as previously described (Tate & Petersen, 1974a). Bacteriophage f1 DNA prepared in this manner showed little evidence of fragmentation when analysed by electrophoresis in an agarose gel (Hayward & Smith, 1972). The DNA was degraded with 66% (v/v) formic acid containing 2% (w/v) diphenylamine at 30°C for 20h (Burton & Petersen, 1960). The diphenylamine and formic acid were extracted as previously described (Tate & Petersen, 1974a).

Fractionation of pyrimidine oligonucleotides on DEAE-cellulose. Detailed conditions for the complete fractionation of pyrimidine oligonucleotides derived from bacteriophage f1 DNA on the basis of chain length were previously described (Tate & Petersen, 1974a). Columns (50cm×1cm diam.) of the microcrystalline DEAE-cellulose strain, DE-32 (Whatman), were used and the pyrimidine tracts were eluted with 0.1M-sodium acetate, pH5.4, 7M-urea, 1mM-sodium diethyldithiocarbamate and a four-step gradient of NaCl concentration (0–0.25M in a total volume of 3.5 litres). The pooled fractions containing a pyrimidine oligonucleotide of a particular chain length were desalted and the salt-free oligonucleotides were separated according to base composition when appropriate (Tate & Petersen, 1974a).

[^{32}P]P_i determination. The [^{32}P]P_i released from DNA on incubation with the formic acid-diphenylamine reagent was examined by the following method. Samples (0.1ml) were removed at various times from a reaction mixture of ^{32}P -labelled bacteriophage f1 DNA and 2% (w/v) diphenylamine in 66% (v/v) formic acid, diluted to 10ml with deionized water, and loaded on to columns (10cm×1cm diam.) of DEAE-cellulose DE-32. The [^{32}P]P_i was eluted at the beginning of a NaCl concentration gradient (approx. 0.01M) in 0.1M-sodium acetate, pH5.4, and was well separated from the pyrimidine nucleoside [^{32}P]diphosphates.

Incubation of pyrimidine tracts with diphenylamine in acid solution. ^{32}P -labelled pyrimidine oligonucleotides of a particular chain length, isolated from bacteriophage f1 DNA as described (Tate & Petersen, 1974a), were mixed with 4–5mg of calf thymus and further incubated with the formic acid-diphenylamine reagent for 20h at 30°C. After removal of the reagent by ether extraction the oligonucleotides were separated on the basis of chain length by ion-exchange chromatography. Control samples for each ^{32}P -labelled oligonucleotide or group of oligonucleotides used in the above studies were mixed with carrier non-radioactive oligonucleotides of chain lengths 1–15 and also chromatographed on ion-exchange columns. In some studies, oligonucleotides of particular base composition were isolated as described previously, mixed with 4–5mg

of calf thymus DNA, and incubated with the formic acid–diphenylamine reagent for 20h at 30°C. The tracts were then separated on the basis of chain length or base composition.

Separation of oligonucleotides by high-voltage electrophoresis. Samples were applied as a narrow band on strips of Whatman no. 1 paper moistened with the electrophoresis buffer, 0.1 M-sodium formate, pH2.7. The paper (24cm×5cm) was placed between two plastic sheets and electrophoresis of the samples was carried out in a Shandon high-voltage electrophoresis apparatus. For the separation of pyrimidine nucleoside monophosphates (dPyd_nP_n) according to base composition, 0.1 M-sodium formate, pH2.7, was used.

Radioactivity determinations. The Cerenkov radiation of ³²P-labelled samples was determined by counting 10ml in polyethylene vials in a Packard liquid-scintillation spectrometer model 2002. The optimal efficiency of counting was 32% in polyethylene vials. The radioactivity of urea solutions (7M) was counted with an efficiency about 5–10% greater than that of aqueous salt solutions. For each experiment all samples were counted in the same solvent and radioactivity data were corrected for isotope decay and background.

Results

Release of orthophosphate from DNA

A careful study of the release of [³²P]P_i from ³²P-labelled bacteriophage f1 DNA on degradation with the formic acid–diphenylamine reagent showed that although the release was essentially complete after 24h there was a small increase in the concentration of [³²P]P_i in samples removed after this time (Fig. 1). This suggests that either the release of pyrimidine tracts is, in fact, not complete after 24h digestion, or a small proportion of pyrimidine tracts lose either one or both of their terminal groups during the reaction, or that the pyrimidine oligonucleotides undergo some further degradation which results in the release of phosphate from internal positions.

Minor components in formic acid–diphenylamine digests of DNA

No evidence of incomplete digestion of the DNA was obtained in analyses of the catalogue of pyrimidine oligodeoxyribonucleotides in the bacteriophage f1, fd and M13 DNA molecules (Tate & Petersen, 1974a,b). Minor products, however, were obtained from formic acid–diphenylamine digests of DNA. As shown in Fig. 2, two types of minor peaks were obtained when the products of a ³²P-labelled bacteriophage f1 DNA digest were separated on the basis of chain length. An initial

Fig. 1. Release of [³²P]P_i from DNA on digestion with 2% diphenylamine in 66% formic acid

Samples (0.1ml) were removed at the indicated times from a digestion mixture of ³²P-labelled bacteriophage f1 DNA (6×10⁷ d.p.m.) and 2% diphenylamine in 66% formic acid, diluted to 10ml with deionized water, and chromatographed on DEAE-cellulose DE-32 columns (10cm×1cm diam.). The [³²P]P_i was eluted with 0.01 M-NaCl in 0.1 M-sodium acetate, pH 5.4. The P_i peak was well separated from the radioactive pyrimidine oligonucleotides. The radioactivity in the P_i peak was expressed as a percentage of total radioactivity applied to the column.

Fig. 2. Minor components eluted from DEAE-cellulose DE-32 columns on chromatography of the products of formic acid–diphenylamine digest of DNA

The oligonucleotide components of a formic acid–diphenylamine digest of ³²P-labelled bacteriophage f1 DNA (6×10⁷ d.p.m.) were separated on the basis of chain length by ion-exchange chromatography as previously described (Tate & Petersen, 1974a). An initial minor peak (A) and a number of minor peaks (B) between the major peaks were obtained.

peak of ^{32}P -labelled material (about 0.1% of the total digest) did not absorb to the ion-exchange resin (type A, Fig. 2) and this was shown to contain mainly $[\text{P}]_1$ and a small amount of pyrimidine oligonucleotides of chain lengths 1–3. Small peaks of material both radioactively labelled and u.v.-absorbing were eluted before the first major peak of pyrimidine deoxyribonucleoside diphosphates (dPyd_2P_2) and between the subsequent major peaks of general formula $\text{dPyd}_n\text{P}_{n+1}$ (type B, Fig. 2). The minor species labelled (B) in Fig. 2, eluted after the pyrimidine mononucleotide diphosphate (dPyd_2P_2) and immediately before the corresponding dinucleotide component (dPyd_2P_3), was resolved into three components by high-voltage electrophoresis. These components were identified as the three dinucleotides, dCyd_2P_2 , $\text{dCydThd}_2\text{P}_2$ and dThd_2P_2 from the ratios E_{280}/E_{260} , their elution position on electrophoresis and the elution position of the minor peak containing the three species as shown in Fig. 2. This suggested that these small peaks, eluted from the ion-exchange columns between the major peaks of pyrimidine deoxyribonucleotide diphosphates, represent oligonucleotides which have one less terminal phosphate than the major peaks of general formula $\text{dPyd}_n\text{P}_{n+1}$ and that the large peak following each minor component has the same number of nucleotides.

Minor degradation of pyrimidine tracts by formic acid–diphenylamine

The low and variable recoveries of the long pyrimidine tracts (Tate & Petersen, 1974b) and the appearance of the small partially dephosphorylated peaks (dPyd_nP_n) as described above prompted an investigation into the stability of the pyrimidine oligonucleotides when incubated with the formic acid–diphenylamine reagent. A mixture of long tracts of chain lengths 9–19, octanucleotides, tetranucleotides and mononucleoside diphosphates from ^{32}P -labelled bacteriophage f1 DNA were each incubated with diphenylamine in formic acid as described under 'Methods'. The elution profiles of the control and redigested tetranucleotide sample are shown in Fig. 3. Small radioactive peaks of orthophosphate and pyrimidine oligonucleotides of each chain length from 1 to 3 were obtained from the sample of tetranucleotide incubated with the formic acid–diphenylamine reagent but not from the control sample. This demonstrates that some degradation of the pyrimidine tracts released from the DNA by formic acid–diphenylamine can occur at this stage. The control samples for the octanucleotides and the mixture of oligonucleotides of chain lengths 9–19 also showed significant amounts of degradation although less than the corresponding redigested samples, and the significance of this is

Fig. 3. *Stability of pyrimidine tracts of chain length 4*
One portion of ^{32}P -labelled pyrimidine tracts of chain length 4 (3×10^5 d.p.m.) was mixed with 4–5 mg of calf thymus DNA and incubated with the formic acid–diphenylamine reagent while the other was kept at -20°C as a control. After removal of the digesting reagent, both samples were chromatographed on columns of DEAE-cellulose DE-32 to separate the components on the basis of chain length. Non-radioactive oligonucleotides of chain lengths 1–3 were added to the control sample before chromatography. (a) Treated sample; (b) control sample; $\circ$, radioactivity; —, u.v. absorption at 271 nm.

described elsewhere (Tate & Petersen, 1975). The results of the above studies were corrected for the amount of degradation that had occurred in the control sample in each case and expressed to compare the stability of pyrimidine tracts of each chain length and also to compare the cleavage per phosphodiester bond in each of the oligonucleotides redigested (dPyd_2 , dPyd_4P_5 , dPyd_8P_9 and $\text{dPyd}_{9-19}\text{P}_{10-20}$) (Fig. 4). The sample containing a mixture of chain lengths 9–19 has been plotted as if it had an average chain length of 11 in Fig. 4. There is an increase in the degradation of pyrimidine oligonucleotides which corresponds to the increase in chain length of the tract. The susceptibility to cleavage of each phosphodiester bond (about 2–3%) within the pyrimidine oligonucleotide is not affected, however, by the chain length of the oligonucleotide (Fig. 4). It can be concluded therefore that the increased degradation of long pyrimidine tracts by the formic acid–diphenylamine reagent is a result of the larger number of phosphodiester bonds within these tracts.

The release of P_i during redigestion of the oligonucleotides discussed above is plotted as a

Fig. 4. Stability of pyrimidine tracts of different chain lengths

One portion of each of the ^{32}P -labelled pyrimidine tracts of chain lengths 1, 4, 8, 9–19 (plotted as 11 in the Figure) was mixed with 4–5 mg of calf thymus DNA and incubated with the formic acid–diphenylamine reagent while the other sample in each case was kept at -20°C as control. After removal of the digesting reagent the treated and control sample for each chain length were chromatographed on columns of DEAE-cellulose DE-32 to separate components on the basis of chain length. Non-radioactive carrier oligonucleotides were added to control samples. The percentage degradation of each treated oligonucleotide (corrected for the degradation in the control samples) is plotted against its chain length (○). The corrected percentage degradation is expressed to give the percentage degradation of each link between consecutive pyrimidine nucleotides in the tracts incubated with formic acid–diphenylamine reagent (●).

percentage of the total phosphate in the oligonucleotide sample and also as a percentage of the terminal phosphate in that sample (Fig. 5). The P_i released increases as the chain length of the oligonucleotide increases. There is also a small increase in the loss of terminal phosphate residues as the chain length of the oligonucleotide increases, but interpretation of this is difficult as new termini are created as a result of the simultaneous degradation of the oligonucleotide.

The formic acid–diphenylamine degradation of DNA would be of limited value for quantitative analyses of pyrimidine tracts in DNA molecules if the degradation of the pyrimidine tracts observed was more specific for a link between particular consecutive pyrimidines (e.g. dCydPdCyd or dThdPdThd or dCydPdThd). The degradation of sequences of particular base composition was investigated therefore to determine whether the diphenylamine–formic acid cleavage of pyrimidine oligonucleotides was, in fact, specific for a link between pyrimidine nucleotides containing particular bases. Two of the nona-nucleotides, dCyd₄dThd₅P₁₀ and dCyd₂dThd₇P₁₀, and the two undecanucleotides, dCyd₄dThd₇P₁₂ and dCyd₂dThd₉P₁₂, found in bacteriophage f1 DNA

Fig. 5. Release of inorganic phosphate from pyrimidine oligonucleotides incubated with formic acid–diphenylamine

The radioactivity released as P_i from each of the pyrimidine oligonucleotides incubated with formic acid–diphenylamine (Fig. 4) is expressed as a percentage of the total radioactivity in each sample (●). The radioactivity released as P_i from each of the pyrimidine oligonucleotides incubated with formic acid–diphenylamine is expressed as a percentage of the total radioactivity in the terminal phosphates of the oligonucleotide (○). In both cases the P_i liberated has been corrected for release in control samples.

Table 1. Stability of tracts of particular base composition on incubation with the formic acid–diphenylamine reagent

^{32}P -labelled oligonucleotides (1.5×10^4 d.p.m.) of a particular base composition were incubated with formic acid–diphenylamine as described under 'Methods', and the products separated according to base composition. The radioactivity in each sequence is expressed as a percentage of the total radioactivity recovered from the column.

Chain length	Sequence	% radioactivity recovered in sequence
9	dCyd ₄ dThd ₅ P ₁₀	78
9	dCyd ₂ dThd ₇ P ₁₀	77
11	dCyd ₄ dThd ₇ P ₁₂	75
11	dCyd ₂ dThd ₉ P ₁₂	75

were isolated and each was redigested with the formic acid–diphenylamine reagent. The stability of each sequence was determined by reisolating each tract according to its base composition (Tate & Petersen, 1974a) and determining the recovery of the appropriate sequence. There was no preferential stability of one of the two sequences in each of the pairs studied (Table 1). The stability of the link between consecutive cytosine nucleotides and consecutive thymine nucleotides was also determined. The pyrimidine oligonucleotides dCyd₄P₉ and

Table 2. *Stability of the link between two consecutive pyrimidine nucleotides containing two cytosine or two thymidine bases*

^{32}P -labelled oligonucleotides dCyd₄P₅ (2×10^4 d.p.m.) and dThd₄P₅ (5×10^4 d.p.m.) were incubated with the formic acid-diphenylamine reagent and the products were separated on the basis of chain length by ion-exchange chromatography, as described under 'Methods'. The radioactivity in each peak was expressed as a percentage of the total c.p.m. recovered from the column.

Peak	% of total radioactivity recovered	
	dCyd ₄ P ₅	dThd ₄ P ₅
^{32}P	2.86	3.03
dPydP	0.20	0.50
dPydP ₂	0.43	1.02
dPyd ₂ P ₂	0.20	0.20
dPyd ₂ P ₃	0.87	1.36
dPyd ₃ P ₃	7.80	0.90
(or dPyd ₄ P ₃)		
dPyd ₃ P ₄	1.90	1.31
dPyd ₄ P ₄	0.86	1.08
dPyd ₄ P ₅	84.88	90.60

dThd₄P₅ were each redigested with the formic acid-diphenylamine reagent and the products were separated on the basis of chain length. The degree of degradation of each sequence is shown in Table 2. The results demonstrated that there was a small difference only between the stabilities of the bonds between the two types of consecutive nucleotides dCydPdCyd and dThdPdThd.

Discussion

The careful investigation of the action of the formic acid-diphenylamine reagent on DNA made by Burton & Petersen (1960) did not detect any lack of specificity in the degradation. Examination of the release of P_i from DNA digested with the formic acid-diphenylamine reagent in the present study revealed that there is, in fact, a small but detectable continuous loss of P_i from the pyrimidine tract mixture after 18 h by which time Burton & Petersen (1960) had concluded that the release of pyrimidine tracts was complete. No evidence for incomplete digestion of DNA was obtained in detailed studies of the pyrimidine tracts in the DNA molecules of bacteriophages f1, fd and M13 (Tate & Petersen, 1974*a,b*) and the P_i released at long incubation times could therefore arise through two possible alternative mechanisms: (1) a proportion of the pyrimidine tracts lose either one or both of their terminal phosphate groups during the reaction or (2) the pyrimidine oligonucleotides undergo some further degradation with the release of phosphate from internal positions.

Minor components in a diphenylamine digest of DNA were identified as partially dephosphorylated oligonucleotides of general formula dPyd_nP_n. These components could arise from cleavage of the link between consecutive pyrimidines in a pyrimidine tract, i.e.:

or by dephosphorylation of a pyrimidine oligonucleotide

The amounts of each minor component of type dPyd_nP_n and the relative frequencies of occurrence of those of different chain lengths (data not shown) suggested that these components were formed after the loss of one of the terminal phosphates from a pyrimidine oligonucleotide of the same chain length. It seemed clear therefore that at least part of the release of inorganic phosphate observed when the liberation of pyrimidine tracts was complete resulted from this type of dephosphorylation.

Isolated pyrimidine tracts of various chain lengths released P_i and were partially degraded when redigested with the formic acid-diphenylamine reagent. The results obtained in these experiments showed that if this phosphate is assumed to arise entirely from the terminal positions of the oligonucleotide, the susceptibility to dephosphorylation increases with chain length of the oligonucleotide. This may, of course, result from the further dephosphorylation of the products of degradation of the longer tracts. Although no direct evidence was obtained for release of P_i from an internal position in the pyrimidine oligonucleotides, a proportion of the phosphate liberated may arise from this source. The main degradation products formed on redigestion of pyrimidine tracts with the formic acid-diphenylamine reagent co-chromatographed with carrier oligonucleotides of general formula dPyd_nP_{n+1} (Fig. 3). This observation suggests that the cleavage of pyrimidine tracts by the formic acid-diphenylamine reagent involves cleavage of the pyrimidine base-sugar bond followed by elimination of the phosphodiester bonds to give two shortened pyrimidine oligonucleotides in a manner analogous to the action of the formic acid-diphenylamine reagent on DNA.

The long pyrimidine oligonucleotides are more susceptible to degradation by the formic acid-diphenylamine reagent than the shorter tracts. This is mainly due, however, to their greater number of phosphodiester bonds, as the probability of cleavage of any phosphodiester link between consecutive pyrimidine nucleotides in oligonucleotides of any chain length is almost the same (about 2–3% of the available dPyd-P-dPyd links). It is perhaps not surprising that

this small percentage cleavage was not detected in the original work of Burton & Petersen (1960) since the degradation products are numerous and present in very small amounts.

The investigation into the specificity of cleavage of pyrimidine tracts during digestion with formic acid–diphenylamine reagent revealed no apparent bias towards links between particular pyrimidine nucleotides. Each of the nonanucleotides, dCyd₄dThd₅P₁₀ and dCyd₂dThd₇P₁₀ and the undecanucleotides, dCyd₄dThd₇P₁₂ and dCyd₂dThd₉P₁₂, were recovered in almost identical amounts. After redigestion with formic acid–diphenylamine the recovery of dCyd₄P₅ was slightly lower than that of dThd₄P₅ (6%), but the small difference probably implies a minor contamination in the dCyd₄P₅ sample. The P₁ release was the same for both samples.

Although the cleavage of pyrimidine tracts during digestion with the formic acid–diphenylamine reagent is minor and random, it contributes significantly to the low recoveries of the long tracts. Although such a low percentage has little effect on recoveries of tracts up to a chain length of about 5, it can account for loss of about one-half of the longest pyrimidine oligonucleotide (chain length 19 or 20) found in bacteriophage f1 DNA.

Any analysis of pyrimidine tracts in a DNA molecule should make some correction for this small degree of degradation if exact assignments of the numbers of pyrimidine tracts are to be made.

This work was supported by grants to G. B. P. by the Medical Research Council of New Zealand, the Medical Distribution Committee of the Golden Kiwi Lottery and the New Zealand University Grants Committee and the Bank of New Zealand Medical Research Fund.

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