

*Mutation Research*, 34 (1976) 11–20

© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

## CAFFEINE ENHANCEMENT OF DIGESTION OF DNA BY NUCLEASE S<sub>1</sub>

C.J. CHETSANGA, K. RUSHLOW and V. BOYD

*Laboratory of Molecular Biology, Department of Natural Sciences, University of Michigan—Dearborn, Dearborn, Michigan 48128 (U.S.A.)*

(Received June 10th, 1975)

(Accepted August 17th, 1975)

### Summary

The activity of *Aspergillus oryzae* nuclease S<sub>1</sub> on DNA has been investigated under varying pH and metal ion conditions. Nuclease S<sub>1</sub> was found to preferentially digest denatured DNA. With native DNA as substrate the enzyme could only digest the DNA when caffeine was added to the reaction mixture. The enzyme was more active in sodium acetate buffer (pH 4.5), than in either standard saline citrate (pH 7.0) or sodium phosphate buffer (pH 6.8).

Caffeine was also found to affect the thermal stability of DNA, resulting in a melting profile characterized by two transitions. The first transition (poorly defined) was below the normal melting temperature of the DNA, while the next transition was at the normal melting temperature of the DNA. The susceptibility of caffeine-treated DNA to nuclease digestion seems to be a result of the local unwinding that caffeine causes in the regions of DNA that melt in the first transition. This selective destabilization presumably sensitizes the unwound regions to nuclease hydrolysis.

The hydrolysates of the DNA digested by nuclease S<sub>1</sub> were subjected first to ion exchange chromatography followed by paper chromatography. The results from this partial characterization of the digestion products showed that they contain mononucleotides as well as oligonucleotides of varying lengths. The base composition of the mononucleotide digests suggests that caffeine has greater preference for interacting with A–T base-pairs in DNA.

---

### Introduction

The treatment of X- and UV-irradiated *Escherichia coli* cells with caffeine has been reported to have a synergistic effect on nuclease hydrolysis of DNA, while mere treatment of *E. coli* cells by caffeine alone did not lead to the breakdown of their DNA [5]. In vitro, caffeine inhibits exonucleases of *E. coli* [11].

On the other hand when these cells were simultaneously treated with caffeine and phleomycin, there was an extensive breakdown of their DNA [4]. Recently caffeine has been shown to cause varying degrees of damage to the chromosomes of eucaryotic cells [3,6].

We have used nuclease  $S_1$  from *Aspergillus oryzae*, as a probe in examining the in vitro effect of caffeine on eucaryotic DNA. Nuclease  $S_1$  is specific for single-stranded nucleic acids [1]. We report here the destabilization of bihelical DNA by caffeine in a manner that enhances its degradation by nuclease  $S_1$ , even under reaction conditions that we had found to inhibit the activity of this enzyme. The digestion products, although almost completely acid-soluble, consist of both mononucleotides and oligonucleotides of different lengths. The treatment of native DNA with caffeine and nuclease  $S_1$  resulted in the releasing of some mononucleotides which were rich in A + T.

## Materials and methods

### Materials

Caffeine (1,3,7-trimethylxanthine) was obtained from Fisher Scientific. (methyl $^3\text{H}$ ) Thymidine was obtained from International Chemical and Nuclear Corporation, Irvine, Cal. *Aspergillus oryzae* crude  $\alpha$ -amylase and bovine serum albumin were obtained from Sigma. DEAE-Sephadex (A-50) and DEAE-Sephadex (A-25) were obtained from Pharmacia. dAMP, dCMP, dGMP and dTMP were obtained from Calbiochem. L1210 mouse leukemia cells were obtained from Associated Biomedic Systems, Buffalo, New York. Bleomycin was obtained from National Wholesale Drugs Co., Detroit, Michigan.

### Labeled DNA

Mouse L1210 cells were used as the source of labeled DNA. The cells routinely maintained in RPMI 1640 medium containing 5% horse serum were labeled with (methyl $^3\text{H}$ )thymidine (1  $\mu\text{Ci}$  per ml of culture medium) for 24 h at 37°C. DNA was prepared by the method of Marmur [8] from both L1210 cells and mouse liver.

### Nuclease $S_1$ assays

Nuclease  $S_1$  was prepared from *A. oryzae* crude  $\alpha$ -amylase by a slight modification of the method of Sutton [14]. The enzyme was eluted from a DEAE-Sephadex (A-50) column by using a step gradient of 0.1, 0.15, 0.25, 0.3 and 0.5 M NaCl buffered in 0.02 M potassium phosphate, pH 6.9. Nuclease  $S_1$  activity was measured in a 0.25 ml reaction mixture consisting of 0.1 M KCl, 0.1 mM  $\text{ZnSO}_4$ , and 0.025 M sodium acetate (KZS), pH 4.5. The bulk of high activity nuclease  $S_1$  was eluted with the 0.25 M NaCl, 0.02 M potassium phosphate, pH 6.9 solution. In some reactions  $\text{ZnSO}_4$  was replaced by other transition metal ions. The other reaction conditions used are described in the appropriate legends. Generally, the reaction mixtures also contained 5  $\mu\text{g}$  of native or heat denatured L1210 cell  $^3\text{H}$ DNA and 5  $\mu\text{g}$  of nuclease  $S_1$ , sometimes supplemented with varying concentrations of caffeine. Incubations were for 30 min at 37°C, unless stated otherwise. The reaction was stopped by chilling the reaction mixtures in an ice bath, adding 25  $\mu\text{g}$  of bovine serum albumin

to each tube, and precipitating the undigested material with 10% cold trichloroacetic acid. The acid-insoluble materials were collected on GF/C filters and the counts in them measured in a Beckman liquid scintillation spectrometer.

Some nuclease  $S_1$  assays were carried out with unlabeled DNA. In such cases, 50  $\mu\text{g}$  of unlabeled DNA was used as substrate and incubation terminated by adding 25  $\mu\text{g}$  of carrier calf thymus DNA to facilitate the precipitation of longer DNA fragments with 0.5 N perchloric acid. After chilling for 15 min the acid-insoluble precipitates were removed by centrifuging at 17,000  $g$  for 10 min, and the amount of digested material in the supernatant measured at A260. The enzyme had a specific activity of 220 units/mg. A unit of nuclease  $S_1$  activity is the amount of enzyme which causes the release of 1.0 A260 unit of acid-soluble material during 30 min of incubation at 37°C.

Chromatography of nuclease  $S_1$  digestion products. The digestion of DNA was carried out in 1.5 ml of KZS containing 200  $\mu\text{g}$  of denatured DNA and 20  $\mu\text{g}$  of nuclease  $S_1$ . In some cases 50  $\mu\text{g}$  caffeine was also added. After incubating for 20 h at 37°C, the samples were desalted by passing them through Bio-Gel P-2 (50-100 mesh), made 0.1 M in Tris-HCl, pH 8.3, and loaded on to a DEAE-Sephadex (A-25) column (1.5  $\times$  30 cm), pre-equilibrated with 0.1 M Tris-HCl, pH 8.3 [15]. A column volume of loading buffer was used to remove most of the nuclease  $S_1$  material from the column. The enzyme seems to poorly bind to the column matrix at pH 8.3. The nucleic acid hydrolysates were eluted with a linear gradient of 0.05–0.5 M NaCl made in loading buffer. Column effluents were monitored at 253, 259, 267 and 272 nm which are the wavelengths of maximum absorption of dGMP, dAMP, dTMP, and dCMP, respectively. Fractions in each peak were pooled, desalted, frozen in liquid  $N_2$ , and lyophilized.

The samples were each dissolved in 0.1 ml  $H_2O$  and 0.01 ml aliquots applied to Whatman No. 1 paper for descending chromatography using an isopropanol–conc. HCl–water (170 : 41 : 39) solvent system. Commercial deoxyribonucleoside monophosphates and caffeine were used as standards. Chromatography was allowed to take place for 16 h at 25°C. After drying, the sample spots were visualized by means of a UV light (257.3 nm), and marked in order to determine  $R_f$  values.

Bleomycin digestion of [ $^3H$ ]DNA. The conditions of treatment of the DNA with bleomycin were according to the procedure of Kuo et al. [7]. The final dialysis of the DNA was against a solution of 0.1 M KCl, 0.1 mM  $ZnSO_4$ , and 0.025 M sodium acetate, pH 4.5. This was followed by treatment of the 20  $\mu\text{g}$  of [ $^3H$ ]DNA with 10  $\mu\text{g}$  of nuclease  $S_1$  in a 0.5 ml volume reaction mixture. After incubation for 30 min at 37°C, the acid-insoluble material was collected on GF/C filters and counted.

### *Melting profiles*

For the melting temperature measurements, 0.5 A260 unit of calf thymus DNA, as well as that of caffeine–DNA complexes (1 : 1 mass ratio) in 0.015 M NaCl, 0.0015 M sodium citrate (0.1  $\times$  SSC) solution was used. The melting was measured in a Gilford 222 recording spectrophotometer equipped with a temperature programmer.

## Results

Fig. 1 shows the effect of the helix-coil configuration of DNA on the activity of nuclease  $S_1$ . The enzyme shows minimal activity with a native DNA substrate, but does almost completely digest denatured DNA. In the presence of caffeine, however, more than 35% of native DNA is digested by the enzyme. The kinetics of the caffeine-mediated digestion of native DNA somewhat resemble those of the digestion of denatured DNA in the absence of caffeine. The digestion in both cases is slow and approaches the plateau level after 40 min. As nuclease  $S_1$  is specific for single-stranded nucleic acids [1,14] caffeine probably enhances its digestion of native DNA by promoting regional unwinding of the double-helix. The loci of native DNA that undergo this special destabilization thus become subject to nuclease  $S_1$  digestion. Caffeine by itself did not render a detectable amount of native DNA acid soluble. Sleigh and Grigg [12] have reported a similar observation in their studies on the activity of *Neurospora crassa* conidial endonuclease in the presence of phleomycin and caffeine.

Nuclease  $S_1$  exhibits the highest level of activity in KZS. In this medium, 96% of denatured DNA was digested by the enzyme in 30 min in the presence of caffeine (Fig. 2). In either  $2 \times$  SSC, pH 7.0, or 0.12 M sodium phosphate buffer, pH 6.8, nuclease  $S_1$  digested only about 10% of denatured DNA in the absence of caffeine. It seems that the pH of the two media as well as partial renaturation of the DNA during incubation reduced the activity of the enzyme.

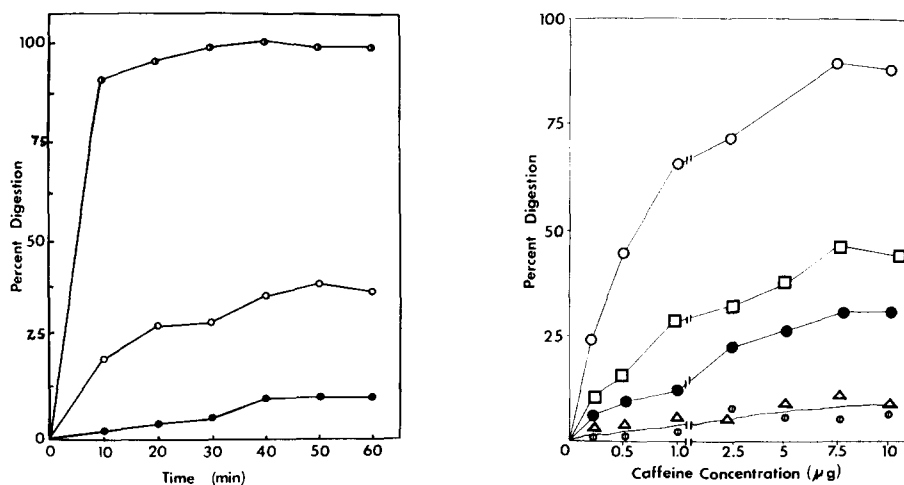


Fig. 1. Effect of caffeine on nuclease  $S_1$  activity. The 0.25 ml contained 10  $\mu$ g of native or denatured [ $^3$ H]DNA, 5  $\mu$ g nuclease  $S_1$ , and 10  $\mu$ g of caffeine. Incubation was at 37°C for the indicated time periods. Digestion of denatured DNA in absence of caffeine (○—○), native DNA (○—○) in the presence of caffeine, and of native DNA in absence of caffeine (●—●). Plotted values represent means with  $\pm 5.2\%$  variance.

Fig. 2. Effect of caffeine concentration and different media on digestion of DNA. The 0.25 ml of assay medium contained 10  $\mu$ g of native or denatured [ $^3$ H]DNA, 5  $\mu$ g nuclease  $S_1$ , and varying amounts of caffeine. After 30 min incubation at 37°C the reaction mixtures were chilled and acid-insoluble material collected for counting. Digestion of denatured DNA in KZS (○—○); in  $2 \times$  SSC with caffeine (□—□), and without caffeine (△—△); and in 0.12 M sodium phosphate buffer, pH 6.8 with caffeine (●—●), and without caffeine (x—x). Variance to the mean was  $\pm 4.4\%$ .

Fig. 2 also shows that the addition of caffeine to the reaction mixture increased the digestion of the DNA by nuclease  $S_1$  to about 45% in  $2 \times$  SSC, and 31% in 0.12 M sodium phosphate buffer, pH 6.8. This suggests that caffeine interferes with the renaturing of the DNA and thereby promotes the action of nuclease  $S_1$ .

As  $Zn^{2+}$  belongs to the transition metal ion group, we examined the effect of replacing the  $Zn^{2+}$  in KZS with other transition metal ions. We also examined the effect of supplementing some of these reaction mixtures with caffeine. Table I presents a summary of the results so obtained. In the sodium acetate buffer, pH 4.5, the reactions with  $Zn^{2+}$  showed the greatest nuclease  $S_1$  activity both in the presence and absence of caffeine when compared to the reactions with the other metal ions. In the same buffer, the activity of the enzyme in the presence of the other transition metal ions was remarkably enhanced by the presence of caffeine. In 0.01 M Tris-HCl, pH 7.4, the enhancement of nuclease  $S_1$  activity by caffeine was still lower than the almost complete digestion of the DNA observed in KZS. The results in these two buffer systems indicate that the enzyme exhibits greater activity at pH 4.5.

When DNA that had been treated with bleomycin (DNA : bleomycin ratio of 1 : 15) was subjected to nuclease  $S_1$  digestion, 99% of the DNA was rendered acid-soluble. This suggests that bleomycin destabilizes the DNA in a manner analogous to the action of caffeine. Kuo et al. [7] have reported that 20% of the DNA treated with bleomycin remains undegraded and is single-stranded. Our results clearly confirm their observations, as the almost complete digestion of the bleomycin treated DNA by nuclease  $S_1$  indicates that the fragments not degraded by bleomycin are single-stranded polydeoxyribonucleotides. Only 1% of the bleomycin treated DNA resists digestion by nuclease  $S_1$  probably because it had become renatured.

The absorption spectra of caffeine and of the caffeine-DNA complex in KZS are presented in Fig. 3. The profiles show that when caffeine is complexed with DNA, its absorption maximum at 205 nm is conserved, while that at 275 nm is shifted to 270 nm, presumably due to the influence of the DNA whose

TABLE I  
EFFECT OF TRANSITION METAL IONS AND BUFFER SYSTEM ON NUCLEASE  $S_1$  ACTIVITY.

Metal ion	Percentage of DNA digested			
	Sodium acetate, pH 4.5		0.01 M Tris-HCl, pH 7.4	
	-Caffeine	+Caffeine	-Caffeine	+Caffeine
ZnSO <sub>4</sub>	98	98	42	68
NiCl <sub>2</sub>	61	94	10	60
Cu(NO <sub>3</sub> ) <sub>2</sub>	16	77	10	66
CdSO <sub>4</sub>	45	96	13	59
Co(NO <sub>3</sub> ) <sub>2</sub>	74	95	10	68
FeCl <sub>3</sub>	18.4	92	30	65

Each 0.25 ml reaction mixture contained 0.1 M KCl, the appropriate metal ion and buffer, 10  $\mu$ g of denatured [<sup>3</sup>H]DNA and 5  $\mu$ g nuclease  $S_1$ . Some reaction mixtures were supplemented with 5  $\mu$ g caffeine. Incubation was at 37°C for 30 min.

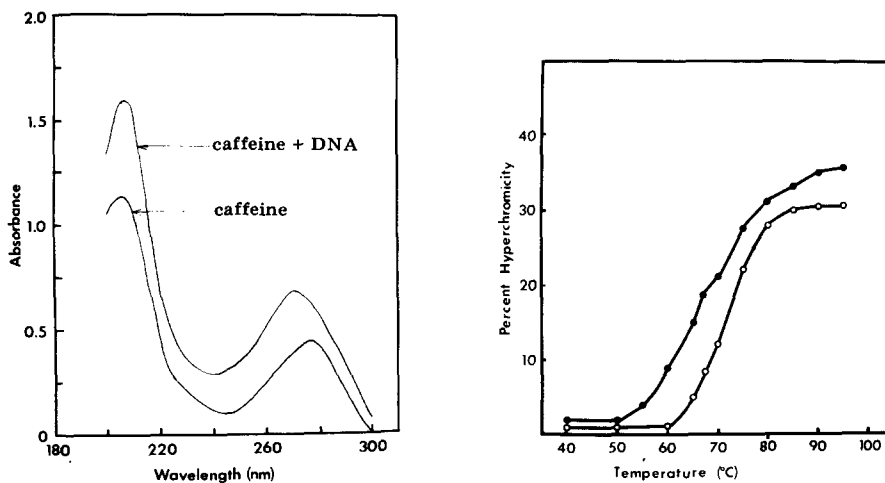


Fig. 3. Absorption spectrum of caffeine and caffeine—DNA complex in KZS. Caffeine alone ( $10 \mu\text{g}$ , lower curve) and caffeine—DNA complex ( $10 \mu\text{g}$  caffeine and  $20 \mu\text{g}$  DNA, upper curve).

Fig. 4. Melting profile of calf thymus DNA ( $\circ$ — $\circ$ ) and caffeine—DNA complexes ( $\bullet$ — $\bullet$ ) in  $0.1 \times$  SSC. About  $0.5 A_{260}$  unit samples were used in each measurement. Hyperchromic shifts were measured at  $260 \text{ nm}$  to facilitate comparison.

absorption maximum is at  $260 \text{ nm}$ . The absorption minimum of the complex becomes shifted from  $245$  to  $250 \text{ nm}$ . The absence of significant increase in absorbance at  $270 \text{ nm}$  upon the addition of  $20 \mu\text{g}$  of DNA to the caffeine solution whose absorbance ( $A_{270}$ ) is already  $0.5$ , suggests that some of the unwound DNA becomes involved in some type of a complex with caffeine leading to a reduction in the hyperchromic effect that would have been expected from the unwinding of the DNA.

Fig. 4 shows that the melting profile of the DNA—caffeine complex occurs over a broad two-phase transition ( $T_m$   $64^\circ\text{C}$ ,  $72^\circ\text{C}$ ). The  $T_m$  of DNA alone is  $72^\circ\text{C}$  [10,13]. The poorly defined transition at  $64^\circ\text{C}$  is reproducible. The nature of the profile is consistent with the notion that caffeine selectively destabilizes some regions ( $T_m$   $64^\circ\text{C}$ ) of the DNA while some regions retain the typical  $T_m$  ( $72^\circ\text{C}$ ) of calf thymus DNA. Presumably, it is the material melting at  $64^\circ\text{C}$  that becomes nuclease  $S_1$  sensitive after caffeine treatment. This notion is supported by the fact that the nucleotides released by nuclease  $S_1$  after caffeine treatment are high in A and T composition.

When the hydrolysates from heat denatured DNA digested with nuclease  $S_1$ , were subjected to chromatography on DEAE-Sephadex A-25, the material was separated into four peaks (Fig. 5). The absorbance of each fraction was measured at  $253$ ,  $259$ ,  $267$  and  $272 \text{ nm}$  to get a rough estimate of the relative amounts of dGMP, dAMP, dTMP and dCMP, respectively in each fraction. Absorbance measurements of the fractions in the first three peaks indicated that each fraction consisted of the four deoxyribonucleotides. The data do not show whether these were mononucleotides or polynucleotides. Fig. 5A shows that peak IV material contains dCMP ( $A_{272}$ ) and a minimal amount of dTMP ( $A_{267}$ ). Peak IV material also contains dGMP ( $A_{253}$ ) and dAMP ( $A_{259}$ ) (Fig.

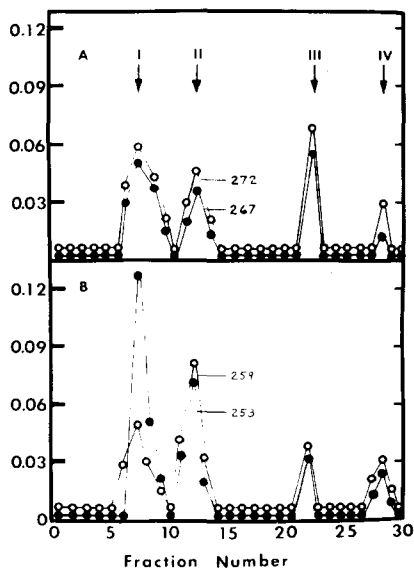


Fig. 5. Chromatographic separation of nuclease  $S_1$  hydrolysates of DNA. The hydrolysates of 200  $\mu\text{g}$  of denatured DNA digested by 20  $\mu\text{g}$  of nuclease  $S_1$  were fractionated on a DEAE-Sephadex A-25 column as described in the text. The absorbances of the fractions from the same column were measured at four wavelengths as follows: (A)  $\circ$ — $\circ$ , 272 nm for dCMP;  $\bullet$ — $\bullet$ , 267 nm for dTMP; (B)  $\circ$ — $\circ$ , 259 nm for dAMP;  $\bullet$ — $\bullet$ , 253 nm for dGMP.

5B). As the base ratio of the nucleotides in peak IV does not give evidence of base equivalence in the DNA, it can be inferred that this material consists of mononucleotides derived from one DNA strand, assuming that some of the nucleotides represented in this fraction are not constituents of homopolymers. There is a greater release of dGMP and dCMP from denatured DNA by nuclease  $S_1$ ; this suggests a specificity in the mode of action of this enzyme on single-stranded polydeoxyribonucleotides.

When attempts were made to resolve the material recovered in the four peaks from the DEAE-Sephadex column by paper chromatography, the separation was generally poor. The material from peak I did not migrate from the origin indicating that the material was composed of polynucleotides. The material from peak II consisted of low mobility oligonucleotides and a faster moving spot of material of  $R_f$  value corresponding to that of dAMP. Peak III consisted largely of dTMP, small amounts of dCMP and poorly resolved trailing material. Peak IV consisted of dCMP, small amounts of dGMP and dAMP and trace amounts of dTMP, thus confirming the  $\lambda_{\text{max}}$  measurements of the eluates from the DEAE-Sephadex column (Fig. 5).

DEAE Sephadex chromatography of the nuclease  $S_1$  digests of the caffeine treated DNA (not shown in Fig. 5) also resolved these hydrolysates into four peaks. Attempts were also made to resolve the nucleotide composition of the material in each of peaks I–IV. Caffeine, dAMP, dCMP, dGMP and dTMP were included on each chromatogram as markers. The caffeine standard migrated

TABLE II  
MOBILITIES OF NUCLEASE  $S_1$  DIGESTS OF CAFFEINE-DNA COMPLEXES.

Nucleotide recovered	$R_f$ value	$R_f$ value of nucleotide standard
dAMP	0.26	0.25
dGMP	0.17	0.184
dCMP	0.22	0.23
dTMP	0.36	0.362
Caffeine	0.33	

The digestion and paper chromatography conditions are given in Materials and Methods. The  $R_f$  values were determined from approximate mean distances migrated. Where trailing was extensive, no efforts were made to determine  $R_f$  values.

just behind dTMP standard as the  $R_f$  values in Table II show. The material in peaks I—III seemed to be made up of oligonucleotides of varying sizes as evidenced by the trailing nature of the mobility profile; peak I was larger than each of peaks II and III. Peak IV material consisted of mononucleotides whose A—T/G—C ratio was about 5; these estimates are based on quantitative measurements of the mononucleotides recovered from the paper chromatograms. The results suggest that caffeine sensitizes DNA to nuclease  $S_1$  by destabilizing the A—T rich segments of the double helix.

## Discussion

The finding that caffeine causes chromosome breakage [6] places it in the group of chemical mutagens. In this study we were interested in determining some of the mechanisms of the action of caffeine on DNA. The digestion of caffeine-treated DNA by nuclease  $S_1$  indicated that caffeine causes regional unwinding of the DNA. The base-stacking interaction of caffeine with DNA [9] and the increased affinity of caffeine for single-stranded DNA [17] seem to be responsible for promoting local unwinding.

The destabilization of DNA by caffeine was further demonstrated by the mode of the melting of DNA after exposure to caffeine. Such DNA showed a two-transition melting profile covering a broader range of temperature when compared to the melting of untreated DNA (Fig. 4). This broadening of the melting transition mediated by caffeine is comparable to that observed by Falaschi and Kornberg [2] in their work with DNA-phleomycin complexes. The first transition at 64°C is about 8°C below the normal  $T_m$  of calf thymus DNA (72°C). This value is comparable to the results obtained by Ts'o et al. [16].

The hydrolysates recovered when caffeine treated native DNA was treated with nuclease  $S_1$  consisted of oligonucleotides as well as mononucleotides. The mononucleotides were approximately five times richer in A + T than in G + C. We conclude that caffeine base-stacks largely with A—T rich regions of DNA, and that this interaction results in a local unwinding of the DNA, thus sensitizing double helix DNA to nuclease  $S_1$ .

In an effort to determine the nucleotide composition of the regions of DNA unwound by caffeine, we have tried to analyze the hydrolysates from the DNA



digested by nuclease  $S_1$ , both without caffeine treatment and after caffeine treatment. The  $\lambda_{\max}$  absorbance measurements of material in each peak was not really sensitive enough to tell us the base composition of the material. Such data alone made it difficult for us to make valid conclusions concerning the base composition of the regions of DNA that preferentially interact with caffeine. Consequently, the nuclease  $S_1$  digests of caffeine-treated DNA were next subjected to paper chromatography.

These results from paper chromatography indicate that nuclease  $S_1$  digests consist of a heterogeneous mixture of DNA fragments of different sizes. These fragments are apparently eluted from DEAE-Sephadex A-25 on the basis of size as well as binding affinity. The largest fragments were eluted in the first peak while subsequent peaks contained molecules of decreasing sizes; the last peak (IV) seemed to contain only mononucleotides. These results make us conclude that nuclease  $S_1$  attacks single-stranded DNA in such a way that it releases polynucleotides of different sizes as well as mononucleotides. In the case of nuclease digests from caffeine-treated DNA, the mononucleotides were greatly enriched in A-T composition.

### Acknowledgements

This work was supported in part by Institutional Research Grant No. IN-40N to The University of Michigan from the American Cancer Society, and Grants No. 121010, 360829 and 387003 from The University of Michigan.

### References

- 1 Ando, T., A nuclease specific for heat-denatured DNA is isolated from a product of *Aspergillus oryzae*, *Biochim. Biophys. Acta*, **114** (1966) 158-168.
- 2 Falaschi, A., and A. Kornberg, Phleomycin, an inhibitor of DNA polymerase, *Fed. Proc.*, **23** (1964) 940-945.
- 3 Fujiwara, Y., and T. Kondo, Caffeine-sensitive repair of ultraviolet light-damaged DNA of mouse L-cells, *Biochem. Biophys. Res. Commun.*, **47** (1972) 557-564.
- 4 Grigg, G.W., The effects of caffeine, ultraviolet and X-irradiation of DNA breakdown in *E. coli* B and some of its dark repair mutants, *Molec. Gen. Genet.*, **106** (1970) 228-238.
- 5 Grigg, G.W., Amplification of phleomycin induced death and DNA breakdown by caffeine in *Escherichia coli*, *Mol. Gen. Genet.*, **107** (1967) 162-172.
- 6 Kuhlmann, W., H.G. Fromme, E.M. Heege and W. Ostertag, The mutagenic action of caffeine in higher organisms, *Cancer Res.*, **28** (1968) 2375-2389.
- 7 Kuo, M.T., C.W. Haidle and L.D. Inners, Characterization of bleomycin-resistant DNA, *Biophys. J.*, **13** (1973) 1296-1306.
- 8 Marmur, J., A procedure for the isolation of deoxyribonucleic acid from microorganisms, *J. Mol. Biol.*, **3** (1961) 208-218.
- 9 Pitha, P.M., W.M. Huang and P.O.P. Ts'o, Physicochemical basis of the recognition process in nucleic acid interactions, IV. Costacking as the cause of mispairing and interaction in nucleic acid interactions, *Proc. Nat. Acad. Sci. U.S.A.*, **61** (1968) 332-339.
- 10 Rafi, A.A. and C.M. Wheeler, Radiation-induced hyperchromicity of DNA, *Int. J. Radiat. Biol.*, **24** (1973) 321-323.
- 11 Roulland-Dussoix, D., Degradation par la cellule hôte du DNA du bacteriophage lambda irradié par le rayonnement ultraviolet, *Mutat. Res.*, **4** (1967) 241-252.
- 12 Sleigh, M.J. and G.W. Grigg, Induction of local denaturation in DNA in vitro by phleomycin and caffeine, *FEBS Letters*, **39** (1974) 35-38.
- 13 Sober, H.A. (ed.), *Handbook of Biochemistry*, 2nd Ed., The Chemical Rubber Co., Cleveland (1970) J-175.
- 14 Sutton, W.D., Crude nuclease preparation suitable for use in DNA reassociation experiments, *Biochim. Biophys. Acta*, **240** (1971) 522-531.

- 15 Takanami, M., Analysis of the 5'-terminal sequences of ribonucleic acids, I. The 5'-terminal of *Escherichia coli* ribosomal RNA, *J. Mol. Biol.*, 23 (1967) 135-148.
- 16 Ts'o, P.O.P., G.K., Helmkamp and C. Sander, Interaction of nucleotides and related compounds with nucleic acid as indicated by the change of helix-coil transition temperature, *Proc. Nat. Acad. Sci. U.S.A.*, 48 (1962) 686-698.
- 17 Ts'o, P.O.P. and P. Lu, Interaction of nucleic acids. I. Physical binding of thymine, adenine, steroids and aromatic hydrocarbons to nucleic acids, *Proc. Nat. Acad. Sci. U.S.A.*, 51 (1964) 17-24.