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# INSTABILITY OF (ARA-C)DNA UNDER ALKALINE CONDITIONS*

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**Abstract**—We employed both [5-$^3$H]ara-C and [2-$^{14}$C]ara-C labeled L1210 DNA for analysis following exposure to alkali under various conditions. The results demonstrated that the tritium label on $C_5$ of ara-C molecules incorporated in DNA was exchanged with water under alkaline conditions and, therefore, radioactivity was subsequently detectable in the acid-soluble fraction. The [$^{14}$C]ara-C labeled DNA, however, was not susceptible to loss of radioactivity by this mechanism, and the appearance of this isotope in the acid-soluble fraction required degradation of the DNA strand or pyrimidine ring. Our results indicated that the [$^{14}$C]ara-C labeled DNA was degraded by alkali, suggesting structural instability of this abnormal nucleic acid. These findings provide useful technical information on the purification of (ara-C)DNA labeled with different isotopes.

We have shown previously that 1-β-D-arabinofuranosylcytosine (ara-C) incorporates into leukemic cell DNA without significant amounts being detectable in RNA [1, 2]. These studies were performed under non-degrading conditions using cesium sulfate gradient analysis to distinguish between RNA and DNA. Zahn *et al.* employed cesium chloride sedimentation analysis under non-alkaline conditions and also demonstrated that ara-C was detectable only in DNA [3]. These findings are in contrast to those of others who have reported ara-C incorporation in both RNA and DNA when using alkaline hydrolysis to separate these nucleic acids [4, 5]. The varying conclusions reached in these studies suggested that the incorporation of ara-C in DNA might lead to the susceptibility of this nucleic acid to degradation under alkaline conditions.

In this report, we have investigated the stability of [5-$^3$H]ara-C and [2-$^{14}$C]ara-C labeled DNA under alkaline conditions. Our studies demonstrate that the tritium label of [5-$^3$H]ara-C is unstable and exchanges with water during exposure to alkali. We have circumvented this problem by using [2-$^{14}$C]ara-C and have further demonstrated that incorporated ara-C residues result in DNA strand scission under alkaline conditions.

## MATERIALS AND METHODS

*Cell culture.* L1210 cells were grown as a suspension culture in minimum essential medium (Eagle's S-MEM) with 10% fetal calf serum (FCS), 100 units of streptomycin/ml, 100 μg of penicillin/ml, 1% L-glutamine, and 0.05 mM 2-mercaptoethanol at 37° in a 5% $CO_2$ atmosphere.

*Labeling of L1210 nucleic acids.* L1210 cells in logarithmic growth phase were washed twice with phosphate-buffered saline (PBS) and were resuspended at $1 \times 10^6$ cells/ml in S-MEM medium. The cells were then incubated with one of the following: (a) 10 μCi/ml of [5-$^3$H]ara-C (26 Ci/mmole; New England Nuclear Corp., Boston, MA) for 6 hr; (b) 0.05 μCi/ml of [2-$^{14}$C]ara-C (55 mCi/mmole; Moravek Biochemicals, City of Industry, CA) for 24 hr; or (c) 7.5 μCi/ml of [5-$^3$H]dC (40 Ci/mmole; New England Nuclear Corp.) for 6 hr. The cells labeled for analysis by cesium sulfate gradient centrifugation were also labeled with 7.5 μCi/ml of $H_3{}^{32}PO_4$ (carrier-free, New England Nuclear Corp.) The purification of cellular nucleic acids has been described previously [1].

*Alkaline treatment of labeled L1210 DNA.* DNA labeled with either [$^3$H]ara-C or [$^3$H]dC was treated with various concentrations of NaOH for periods of 1.5, 8 and 16 hr at 37° or for periods of 0.5 hr at 100°. The [$^{14}$C]ara-C labeled DNA was treated with 0.4 N NaOH for 0.5 hr at 100°. The samples were then neutralized by the addition of 0.1 M Tris/HCl, (pH 7.8) and concentrated HCl. The acid-insoluble nucleic acids were then precipitated with 0.7 N perchloric acid for 30 min at 0°. Samples were spun at 15,000 *g* for 15 min in an Eppendorf Microfuge. The precipitate was then dried and redissolved in 0.1 M Tris/HCl (pH 7.8). The supernatant samples were neutralized with 4.0 M potassium bicarbonate with removal of the potassium perchlorate precipitate after incubation at 0° for 5 min. The acid-insoluble and acid-soluble fractions were then analyzed by high pressure liquid chromatography either directly or following enzymatic digestion.

*Enzymatic digestion of labeled L1210 DNA.* L1210 cellular nucleic acids were purified as described previously [1]. The purified DNA fraction was then digested to either 5′-nucleotides, 3′-nucleotides or nucleosides [2].

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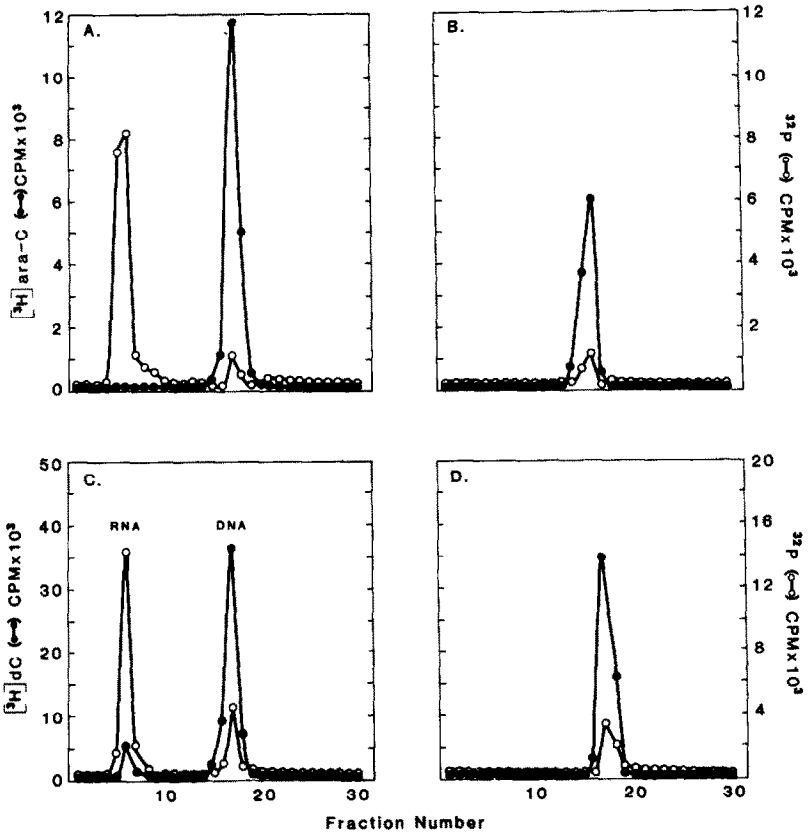


Fig. 1. Effect of alkali on incorporation of [$^3$H]ara-C and [$^3$H]dC into L1210 nucleic acids. L1210 cells in logarithmic growth phase at a concentration of $1 \times 10^6$ cells/ml were incubated with $10^{-7}$ M [$^3$H]ara-C or [$^3$H]dC and 10 $\mu$Ci/ml of $H_3{}^{32}PO_4$ for 6 hr as described in Materials and Methods. The total cellular nucleic acids were purified and analyzed by cesium sulfate density gradient centrifugation (A and C). Similar aliquots were treated with 0.4 N NaOH for 16 hr at 37°, neutralized, and then analyzed by cesium sulfate gradient analysis (B and D).

*High pressure liquid chromatography (HPLC) analysis.* The HPLC analyses were performed on a Varian 5020 high pressure liquid chromatograph (Varian Associates, Palo Alto, CA). Ion exchange chromatography was run on an AX-10 column (Varian Associates) at a flow rate of 2 ml/min. Buffer A was 80% acetonitrile/20% 0.01 M $KH_2PO_4$ (pH 2.85) and buffer B was 0.01 M $KH_2PO_4$ (pH 2.85). The elution program utilizing buffer B was as follows: (1) 0–40% for 5 min; (2) 40% for 10 min; (3) 40–100% for 5 min; and (4) 100% for 10 min. Analysis on a reverse phase MCH-10 column (Varian Associates) was performed using 0.1 M $KH_2PO_4$ buffer (pH 5.1) at a flow rate of 1 ml/min.

## RESULTS

L1210 nucleic acids labeled with either [$^3$H]ara-C or [$^3$H]dC and $^{32}$P were analyzed by cesium sulfate gradients (Fig. 1, A, and C). Similar nucleic acid preparations were treated with 0.4 N NaOH for 16 hr at 37° prior to density centrifugation (Fig. 1, B and D). The alkaline digestion resulted in the loss of $^{32}$P radioactivity banding in the RNA regions of the gradient. The alkaline treatment also resulted in a greater than 50% reduction in the [$^3$H]ara-C counts banding in the DNA region of the gradient, without a significant decrease in the $^{32}$P counts. In contrast, there was no loss of tritium radioactivity after treatment of DNA labeled with [$^3$H]dC with alkali.

The [$^3$H]ara-C labeled DNA was treated with alkali under various conditions to monitor the extent of tritium radioactivity released in the acid-soluble fraction (Fig. 2). Under these conditions, the appearance of tritium in the acid-soluble fraction was dependent upon concentration of NaOH, temperature, and time of exposure. The treatment of [$^3$H]ara-C labeled DNA with 0.4 N NaOH for 30 min at 100° resulted in over 80% of the tritium appearing in the acid-soluble fraction, while similar treatment of [$^3$H]dC labeled DNA resulted in all of the radioactivity remaining in the acid-insoluble fraction.

The acid-soluble fraction resulting from the alkaline treatment of [$^3$H]ara-C labeled DNA was analyzed directly on anion exchange HPLC (Fig. 3A). Under these conditions, the tritium radioactivity eluted near the void volume and co-migrated with ara-U and tritiated water. The digestion of the acid-soluble fraction with either DNase I and snake venom phosphodiesterase (Fig. 3B) or micrococcal nuclease and spleen phosphodiesterase (Fig. 3C) did not significantly alter the elution pattern. In contrast, no tritium radioactivity was detectable when the acid-insoluble fraction was analyzed without prior

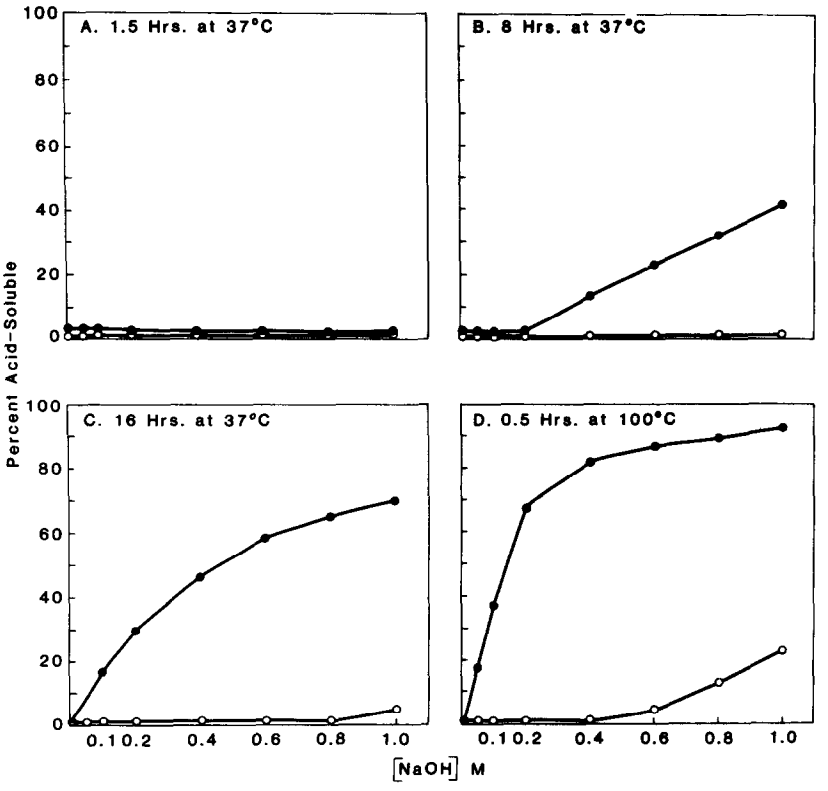


Fig. 2. Effect of alkaline treatment on [$^3$H]ara-C-DNA and [$^3$H]dC-DNA. L1210 cells were labeled with [$^3$H]ara-C (●) or [$^3$H]dC (○) as described in the legend to Fig. 1. The DNA was purified and treated with various concentrations of NaOH for 1.5, 8 and 16 hr at 37° or for 30 min at 100°. The samples were then neutralized and precipitated with 0.7 N perchloric acid and the counts were determined in both the acid-soluble and acid-insoluble fractions. The results are expressed as a percent of acid-soluble tritium radioactivity.

enzymatic digestion (Fig. 3D). However, digestion of this fraction to 5′-nucleotides resulted in co-migration of tritium radioactivity with 5′-ara-CMP and with 3′-ara-CMP after digestion to 3′-nucleotides. These findings demonstrate that ara-C remained in internucleotide linkage in the acid-precipitable material, while the tritium radioactivity in the acid-soluble fraction was no longer associated with a macromolecular species. In fact, lyophilization of the acid-soluble fraction resulted in loss of over 90% of the tritium counts, while similar treatment of the acid-insoluble fraction had no effect on recovery of radioactivity. These findings are consistent with exchange of the tritium to water from the [$^3$H]ara-C labeled DNA under alkaline conditions.

To circumvent the exchange of the tritium on [$^3$H]ara-C labeled DNA, L1210 cells were incubated with [$^{14}$C]ara-C, and the DNA fraction was purified. Digestion of the [$^{14}$C]ara-C labeled DNA with micrococcal nuclease and spleen phosphodiesterase confirmed the internucleotide linkage of over 95% of the residues as demonstrated by co-migration of the $^{14}$C radioactivity with 3′-ara-CMP (Fig. 4A). Under these conditions, less than 5% of the residues was present at the chain terminus as determined by co-migration of radioactivity with ara-C. The treatment of this DNA preparation with alkali (0.4 N NaOH, 0.5 hr at 100°) resulted in over 80% of the radioactivity co-migrating with ara-U near the void volume of the chromatogram and the remainder of the counts co-migrating with both ara-C and 3′-ara-CMP (Fig. 4B). It was of interest to determine whether the initial peak of radioactivity in this profile actually represented ara-U or a product that failed to bind to the column and had an early elution pattern. Analysis of the alkali-treated [$^{14}$C]ara-C labeled DNA on reverse phase HPLC is illustrated in Fig. 5. The majority of the $^{14}$C radioactivity also eluted on this column in the early fraction and did not co-migrate with ara-U. This radioactivity was not removed by lyophilization. This labeled material must, therefore, represent a degradation product of the [$^{14}$C]ara-C.

## DISCUSSION

We have demonstrated previously that ara-C incorporates into cellular DNA and not into RNA [1, 2]. Other investigators have reported that ara-C incorporates into both RNA and DNA when using alkaline conditions to distinguish these nucleic acids [4–6]. These findings suggested that labeled ara-C residues incorporated in DNA might appear in the alkaline hydrolysate and be interpreted as having been incorporated into RNA.

We have employed cesium sulfate gradient analy-

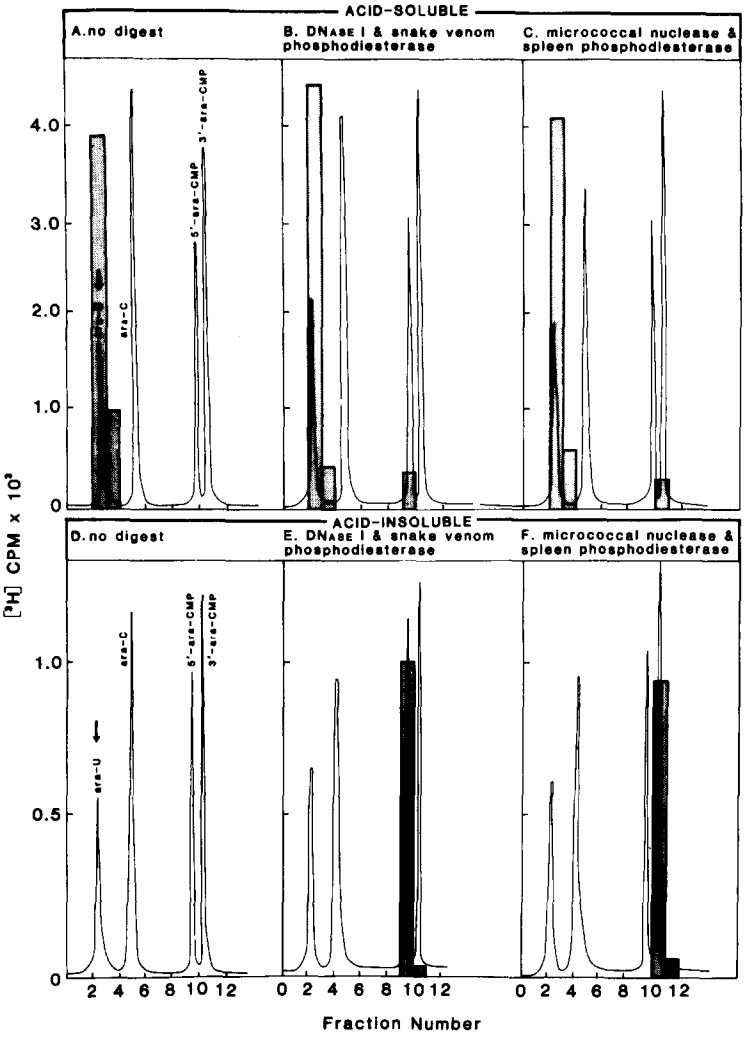


Fig. 3. Digestion of acid-soluble and acid-insoluble L1210 (ara-C)DNA after NaOH treatment. L1210 cells were labeled with [$^3$H]ara-C as described in the legend to Fig. 1. The DNA fraction was purified and treated with 0.4 N NaOH for 16 hr at 37°. The sample was neutralized and then precipitated with 0.7 N perchloric acid. The acid-soluble fraction was then neutralized and analyzed by anion exchange HPLC under the following conditions: (A) no enzymatic digestion; (B) after digestion with DNase I and snake venom phosphodiesterase; or (C) after digestion with micrococcal nuclease and spleen phosphodiesterase. The acid-insoluble fraction was analyzed under similar conditions (D–F). Reference standards were detected at 254 nm, 0.2 AU/MV. Shaded bars represent tritium radioactivity.

sis of L1210 nucleic acids labeled with both [$^3$H]ara-C and $^{32}$P to determine the effect of alkaline treatment on the DNA fraction. Under these conditions, there was loss of the radioactivity banding in the RNA region of the gradient and loss of significant amounts of tritium but not of $^{32}$P from the DNA region of the gradient. These findings were in contrast to those obtained when the DNA was labeled with $^{32}$P and [$^3$H]dC which was unaffected by alkaline treatment. Further, this release of radioactivity from the [$^3$H]ara-C labeled DNA was dependent on pH, temperature, and time of incubation.

The radioactive material released into the acid-soluble fraction following alkaline treatment was subjected to further analysis. When the acid-soluble fraction was analyzed directly or after enzymatic digestion on anion exchange HPLC, all of the radioactivity was recovered in the initial fractions where tritiated water and ara-U also migrate. Thus, the radioactivity did not co-migrate with 3′-nucleotides which are liberated following alkaline hydrolysis of RNA. Further, the absence of 3′- or 5′-nucleotides following micrococcal nuclease and spleen phosphodiesterase or DNase and snake venom phosphodiesterase enzymatic digestion of the acid-soluble fraction suggested that the radioactive material did not represent oligonucleotides. Subsequent analysis on reverse phase HPLC demonstrated that this radioactive product was not retained on the column and that it co-migrated with tritiated water. Finally, lyophilization demonstrated that this product was volatile and simply represented exchange of the tritium on the 5-position of the cytosine ring with water.

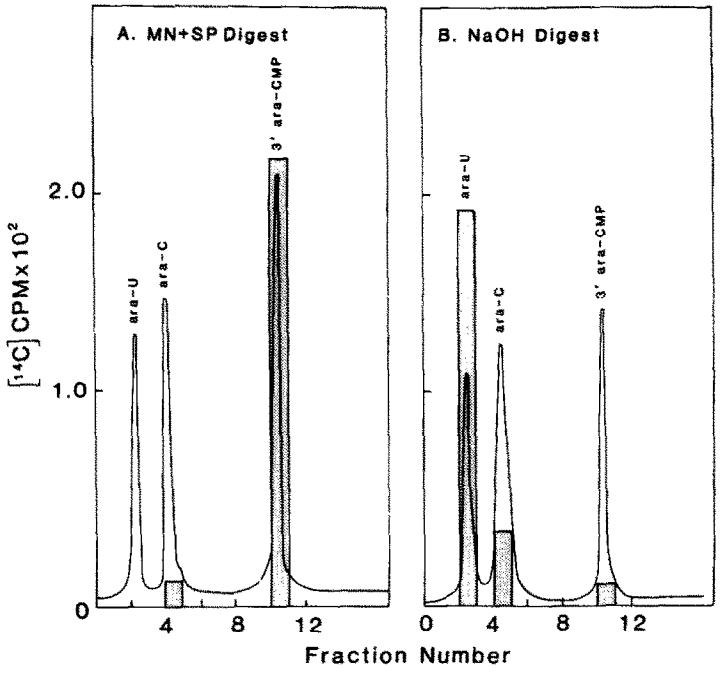


Fig. 4. Anion exchange HPLC analyses of acid-soluble [$^{14}$C]ara-C labeled DNA after (A) enzymic or (B) alkaline digestion. L1210 cells in logarithmic growth phase were incubated with $10^{-5}$ M [$^{14}$C]ara-C for 24 hr. The labeled DNA was purified and either digested with micrococcal nuclease and spleen phosphodiesterase (A) or treated with 0.4 N NaOH for 30 min at 100° (B). The alkali-treated aliquot was then neutralized and both samples were analyzed by anion exchange HPLC.

Further experiments were conducted with [$^{14}$C]ara-C labeled DNA to determine whether this DNA would be vulnerable to alkaline treatment. The $^{14}$C present at the 2-position of the cytosine ring would not be susceptible to exchange as found with the tritium-labeled (ara-C)DNA. However, alkaline treatment of the [$^{14}$C]ara-C labeled DNA also produced a radioactive material which was non-acid precipitable and which on both reverse phase and anion exchange chromatography was recovered in the early elution fractions. This labeled material was therefore, not a structure with an intact pyrimidine ring. The release of the $^{14}$C radioactivity into the acid-soluble fraction was also dependent upon pH, temperature, and time of exposure. This finding suggests that the cytosine ring incorporated into DNA as ara-C was labile to alkaline digestion and could represent an alkali-labile lesion that results in strand breaks.

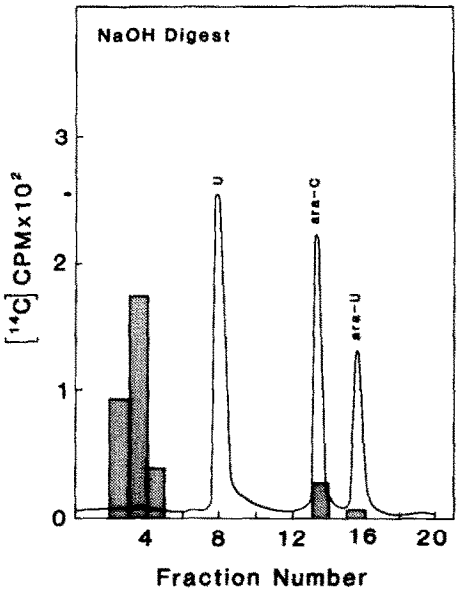


Fig. 5. Reverse phase HPLC analysis of [$^{14}$C]ara-C labeled DNA. The alkali-treated [$^{14}$C]ara-C labeled DNA, prepared as described in the legend to Fig. 4, was analyzed by reverse phase HPLC.

Previous studies have shown that 5-fluoro-1-$\beta$-D-arabinofuranosylcytosine will form a 6,2′-anhydro derivative under alkaline conditions [7]. It has been suggested that the 6,2′-anhydro compound could be formed with ara-C and could facilitate the deamination and exchange reaction at the C5-position of the pyrimidine ring [8–10]. Further, it has also been suggested that formation of the 6,2′-anhydro derivative could lead to cleavage of the pyrimidine ring [8]. The phosphodiester bonds would then be susceptible to cleavage under alkaline conditions and could result in strand breaks. We are presently attempting to answer this question using defined pieces of DNA [11] where ara-C residues have been incorporated.

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## REFERENCES

1. D. Kufe, P. Major, E. Egan and G. Beardsley, *J. biol. Chem.* **255**, 8997 (1980).
2. P. Major, E. Egan, G. Beardsley, M. Minden and D. Kufe, *Proc. natn. Acad. Sci. U.S.A.* **78**, 3235 (1981).
3. R. Zahn, W. Muller, W. Forster, A. Madhof and R. Beyer, *Eur. J. Cancer* **8**, 391 (1972).
4. F. Graham and G. Whitmore, *Cancer Res.* **30**, 2636 (1970).
5. M. Chu and G. Fischer, *Biochem. Pharmac.* **17**, 753 (1968).
6. S. Grant, C. Lehman and E. Cadman, *Cancer Res.* **40**, 1525 (1980).
7. J. Fox, N. Miller and R. Cushley, *Tetrahedron Lett.* 4927 (1966).
8. R. Notari, M. Chin and R. Wittebort, *J. pharm. Sci.* **61**, 1189 (1972).
9. W. Wechter, *Colln Czech. chem. Commun. Engl. Edn* **35**, 1991 (1970).
10. W. Wechter, *Colln Czech. chem. Commun. Engl. Edn* **35**, 2003 (1970).
11. L. Manuelidis, *Nucleic Acid Res.* **3**, 3063 (1976).