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DNA STRAND BREAKAGE BY ^{125}I DECAY: PLASMID DNA IN DILUTE AQUEOUS SOLUTION

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ABSTRACT

The question of the extent of damage to DNA, in particular double strand breaks, that can be caused by the decay of the Auger emitter ^{125}I when it is covalently incorporated into the DNA requires resolution. In particular, experiments with plasmid DNA reported by Linz and Stöcklin (1) would seem to indicate that the range of effect extends over the whole length of the molecule (a few 1000's base pairs (bp)) in contrast to the generally accepted view that the decay is very localized (*i.e.* effect over a few 10's bp). This raises the question of whether a long range energy migration process is operative in DNA or whether the fragmentation can be accounted for by free radical diffusion. We report here experiments to help resolve this issue by investigating and trying to eliminate the possible effects of free radicals. Our results which are broadly consistent with the findings of Linz and Stöcklin (1) indicate that long-range damage in labeled DNA in aqueous solution is due to effects of free radicals. We confirm the high-LET nature of the ^{125}I decay.

INTRODUCTION

The role of Auger emitters in biology is of both practical and fundamental interest. The high energy densities produced in the local regions surrounding the nuclide upon radioactive decay possibly lead to effects similar to those produced by high-LET particles (2). However, the relevance of this damage may depend on the spatial relationship of the nuclide to critical biological targets which in the case of high-LET particles would be a stochastic relationship.

One critical biological target is DNA and it is of fundamental interest to know the extent of damage resulting from Auger decays that take place within or close to the DNA duplex. Iodine-125 has traditionally been chosen for such studies because of the ease with which it can be covalently bonded to DNA. ^{125}I introduced into cells in the form of a labeled base that becomes incorporated into the cell nucleus is very much more cytotoxic than ^{125}I introduced in a form that excludes it from the nucleus (3). If it is assumed that each decay results in at least a double strand break in the nuclear DNA at the site of decay (not unreasonable considering the combined effects of electron release, charge build-up and transmutation involved in the decay), then for many normally sensitive cell lines some 60 such decays would appear, on average, to be lethal to a mammalian cell.

Early studies with DNA species specifically labeled with ^{125}I indicated that multiple single strand breaks could be formed up to the order of 20 base pairs (~ 10 nm) from the site of decay (4). Calculations, based upon track structures for the electron emission upon Auger decay, confirm these conclusions (5). It may then be concluded that ^{125}I decay within a DNA molecule is a highly localized event with damage confined to within a few DNA diameters of the site of the decay, but with the likelihood of considerable damage within that region leading to the loss of genetic information.

Subsequent experiments (1) seemed to indicate that the above picture was incomplete. Plasmid DNA, linear pBR322 labeled at both ends with ^{125}I, was stored either in phosphate buffer or sodium chloride solution to allow radioactive decay and then analyzed for double strand breakage using electron microscopic imaging. These studies revealed that double strand breaks could occur at positions remote (several hundreds of bp) from the sites of

radioactive decay. The conditions under which these studies were made did not prevent the possibility that free radicals resulting from the radioactive decay might cause damage after migration through the aqueous medium, although experiments in which ^{125}I was present in the solution, but not incorporated into the DNA, did not suggest that radical migration would be effective in causing damage. Alternatively it has been suggested that a long range energy migration process might be operative in DNA stimulated by the 'far from equilibrium' condition created by the Auger decay (6).

The purpose of the experiments described in this paper is to resolve the question of whether the fragmentation observed by Linz and Stöcklin (1) is due to free radical effects or energy migration. It is stressed that breaks formed in close proximity to the site of the label can not be measured by the techniques employed here, and so these experiments shed no light on the mechanism by which the local effects of ^{125}I decay occur.

MATERIALS AND METHODS

^{125}IdCTP was purchased from Dupont New England Nuclear at a specific activity of 81.4 TBq/mmole and a radioactive concentration of 37 MBq/ml. The radiolabeled base was supplied in an ethanol:water mixture of 3:1, the majority of which was removed under vacuum immediately prior to use to give a radioactive concentration of approximately 370 MBq/ml. Enzymes were purchased from Life Science Technologies (Gibco BRL), the Klenow enzyme at a concentration of 2 units per pmol. pSVL plasmid DNA in pure vector form, Sephacryl S-1000 and S-400 gel filtration media, and cold dNTPs were obtained from LKB Pharmacia. Agarose and Sephadex gel filtration media were obtained from Sigma, and all other general laboratory chemicals either from Sigma or BDH.

Isolation of pSVL Plasmid DNA

The vector form of pSVL plasmid was multiplied in *Eshericia coli* HB101 from in house supplies, isolated and purified by base/acid extraction and Sephacryl S-1000 gel filtration following the protocol of Bywater *et al.* (7). This method routinely produced DNA preparations with a high percentage of supercoiled molecules.

Preparation of Plasmid for 3'- Endlabeling

The plasmid was initially linearized by digestion at 37°C with the restriction endonuclease EcoR V, which produces blunt ends, at a concentration of 3 units of enzyme per microgram of plasmid. A second digestion at 37°C with the enzyme Xba 1 at a concentration of 5 units per microgram of plasmid produced a sample containing a mixture of 4.2 kb and 700 base pair plasmid, each possessing one blunt and one cohesive end. After each digestion the reaction was terminated with EDTA to a final concentration of 20 mM, heated to 65°C for 5 min, and then immediately incubated on ice to dissociate any annealed cohesive ends.

Separation of Restriction Fragments

Restriction fragments were separated using a Sephacryl S-1000 gel filtration column, the dimensions of which were 2.5 X 50 cm. The gel was initially equilibrated in 0.5 M NTEB (0.5 M NaCl, 10 mM Tris pH 7.5 and 1 mM EDTA) and a sample of 200 µg in a volume of < 500 µl was loaded onto the column and eluted at a flow rate of 25 ml per hour.

3'-Endlabeling

The Klenow fragment of DNA polymerase 1 was employed for this purpose in a reaction mixture containing 260 nM of DNA cohesive ends, 260 nM Klenow enzyme and 4 µM ^{125}I dCTP in 12.5 mM NaCl, 5 mM magnesium chloride and 50 mM Tris-HCl pH 7.4 polymerase buffer adapted from Kuchta *et al.* (8). Initially the plasmid and ^{125}I dCTP in the polymerase buffer were incubated at 20°C for 5 min independently of the enzyme which was then added to initiate the reaction. The reaction was terminated with EDTA to a final concentration of 20 mM after an incubation time of 40 min.

Spun column chromatography as described by Maniatis *et al.* (9) was used to remove the excess labeled deoxynucleotides. All gel filtration media employed during this stage had first been equilibrated in 10 mM NaCl except for the sample that was to be stored in 10 mM phosphate buffer pH 7.0 which was treated independently in columns prepared under phosphate conditions. The first column was packed with Sephacryl S-400 to simultaneously remove excess nucleotides and any contaminating protein. This was followed by at least one more elution down a Sephadex G-50 spun column to remove the

last traces of labeled nucleotides. Using the Klenow enzyme we have found that there is inevitably some extraneous incorporation. Indeed, we have noticed that the enzyme is capable of blunt end labeling, the extent of which is dependent upon the recognition site. This is, however, to a much lesser degree than with cohesive ends. Therefore, to determine to what extent this had occurred, an aliquot of the plasmid was further digested with Sal 1 under conditions of 5 units of enzyme per microgram of DNA at 37°C. The digest was analyzed by agarose gel electrophoresis and revealed a 3% extraneous incorporation probably at the blunt ends.

Sample Preparation, Storage, and Analysis

All samples were prepared for storage immediately after labeling. The DNA was already present both in the 10 mM NaCl pH 7.0 and independently in the 10 mM phosphate buffer pH 7.0. The volume of both these samples were thus adjusted to give a final DNA concentration of 20 μg/ml. A portion of the DNA sample in 10 mM NaCl was taken before dilution and glucose added to a final concentration of both 50 and 500 mM. This also applies to the 10 mM and 100 mM Tris pH 7.5. Control samples were prepared under identical conditions using cold dCTP and the effect of unincorporated ^{125}I studied by the addition of free labeled base to samples of cold labeled plasmid. All solution experiments were stored in heat sterile screw cap vials at 4°C in a moist environment. In the labeled samples the molar ratio of ^{125}I:DNA was 0.5, while for the unlabeled samples the ratio was 2. Samples were imaged using electron microscopy and analyzed by methods described in Baverstock *et al.* (10).

Estimation of Strand Break Yields

Due to measurement errors the means of the distributions of DNA fragments vary from one sample to another. This complicates the estimation of break yields on the basis of the fractions of DNA remaining unbroken. An alternative method, and arguably a better one when fragment length information is available, is to estimate the mean length of the unbroken fragments. This is essentially a simple task when break yields are low. By dividing the total length in the distribution by this length estimate, the number of fragments of unbroken DNA that would constitute the sample are obtained.

If n DNA molecules each have p breaks per molecule (on average), then there will be $(n+pn)$ fragments in the sample. Thus, for a sample exposed to radiation which was found to have f fragments,

$$p = (f - n) / n$$

Breaks caused in the handling and preparation of the DNA, or spontaneous breaks formed during storage of the samples, can be estimated from yields observed in the control samples.

RESULTS

Figure 1 shows double strand breakage in DNA labeled at one end with ^{125}I and stored in phosphate buffer solution. Samples were analyzed at intervals to observe the dependence of breakage on the fraction of ^{125}I decayed. Also shown in the figure are the break yields for solutions in which the ^{125}I was not incorporated in the DNA but present in solution as the labeled base. In both cases the breakage observed in DNA subjected to the same procedures, but without exposure to radioactive iodine, has been subtracted. Fifty percent of the molecules in the sample are labeled with iodine so the molar ratio of ^{125}I to DNA was 1:2. In the non-incorporated sample it was 2:1. Fig. 2 shows a similar plot for DNA stored in sodium chloride (pH 7).

In both these cases the extent of breakage observed was very small but somewhat greater in the experiments where the ^{125}I was incorporated in spite of the higher ^{125}I ratio. Indeed the breakage observed was markedly less than that observed by Linz and Stöcklin (1), but in common with them we observed an indication of slightly higher yields in the sodium chloride solution. As indicated by the fitted lines (see DISCUSSION) the results from the labeled samples are consistent with breakage being related to the square of the dose, as would be expected if the effect were due to free radical attack on the DNA. However, because of the low levels of breakage, a linear fit would be equally acceptable.

Figures 3 and 4 show the effect of added glucose at 50 and 500 mM. Glucose acts to scavenge free radicals generated from the radiolysis of water. The reduced yields of breaks observed in these experiments (virtually undetectable even when 90% of the activity had decayed) and absence of any

detectable trend of breakage with fractional decay would suggest that such breakage as is observed in the salt and buffered solutions is generated by free radical attack. This conclusion is confirmed by the absence of breakage in similarly labeled DNA stored in Tris buffer at 10 and 100 M as shown in Figs. 5 and 6.

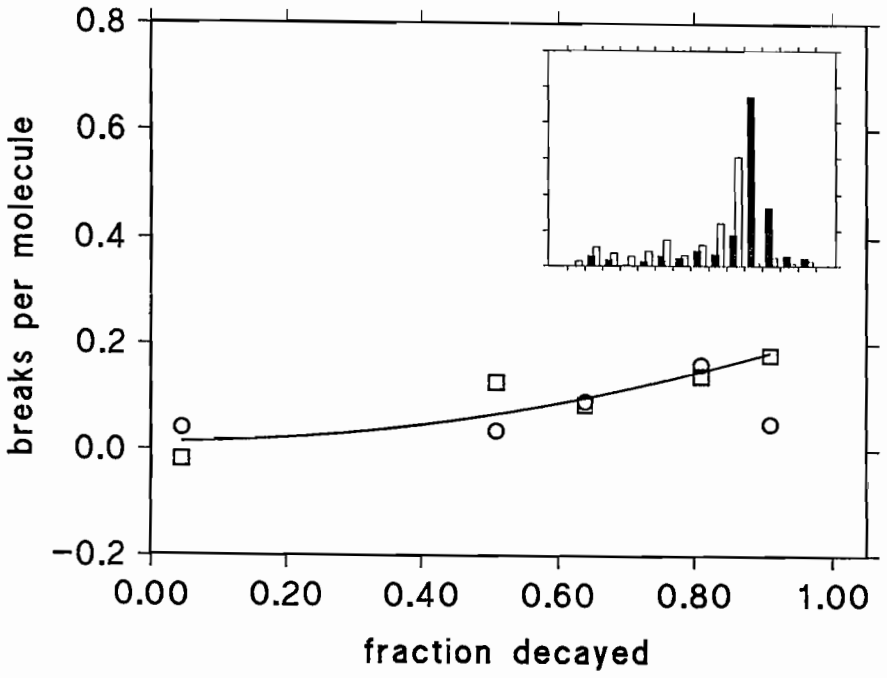

FIG. 1. Measured numbers of double strand breaks in linearized pSVL plasmid DNA molecules either labeled with ^{125}I (open squares) or with labeled base in solution (open circles) stored in 10mM phosphate buffer (pH 7) at 4°C. Break yields were determined as described in the text. Typically between 100 and 200 μm of DNA fragments (initially 1.05 μm in length) were measured per sample. Labeling efficiency in the labeled experiments was about 50%. In the unincorporated samples four times as much ^{125}I was present in solution. The fitted line is to the points for the labeled samples only and is of the form $y = m\,x^2 + b$. The inset diagram shows the fragmentation pattern (number of fragments of DNA on the y axis vs length (x axis)) for the sample at 91% decay (fifth point) (open squares) compared to unlabeled DNA stored under the same conditions. Each bin (x axis) corresponds to 0.1 μm length of DNA. The solid bars refer to the unlabeled sample and the open bars to the labeled sample.

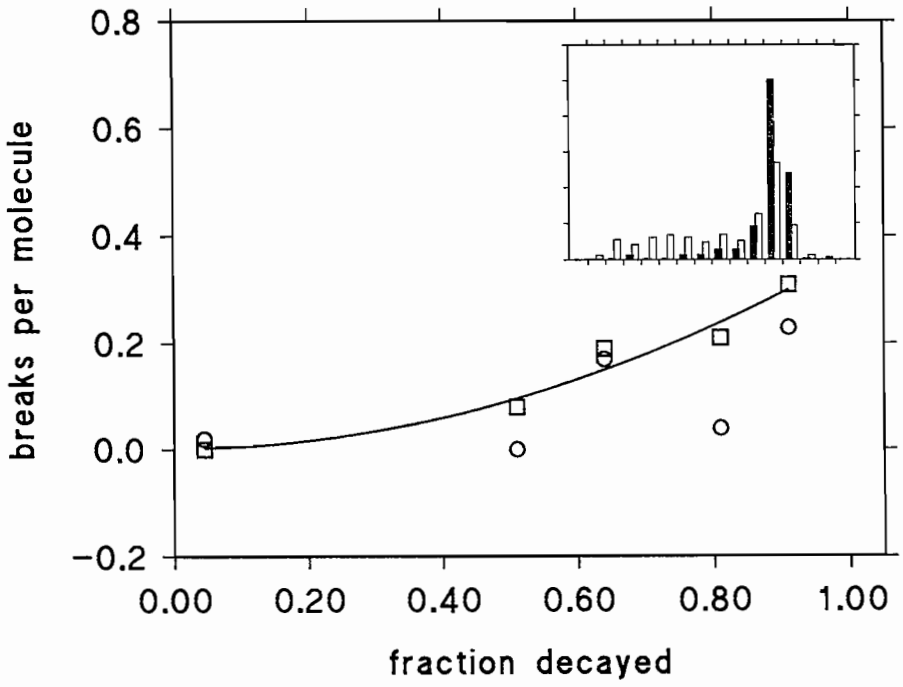

FIG. 2. As for Fig. 1 but samples stored in 10 mM sodium chloride.

To assess the relationship between strand breakage and radiation dose under the experimental conditions (*i.e.* DNA at 20 μg/ml in either sodium chloride solution or phosphate buffer) similar solutions were irradiated with gamma rays. Full decay of the ^{125}I in the incorporated samples would be equivalent to a dose of 6 Gy. At such a dose ~ 0.05 double strand breaks per molecule are observed. In solutions containing glucose no damage is observed at such doses. In the experiments with non-incorporated ^{125}I, where the ratio of iodine to DNA is four times greater, the expected number of double strand breaks per molecule is about 4.5.

DISCUSSION

The experiments reported above were designed to determine whether the extensive fragmentation of the labeled DNA observed by Linz and

Stöcklin (1) was due to a long range energy migration process in the DNA or to free radical migration following ionization of the aqueous medium. In addition to this fragmentation, localized effects in the DNA within 10-20 bp of the site of the label are also expected, but the techniques employed here can not detect damage at this resolution.

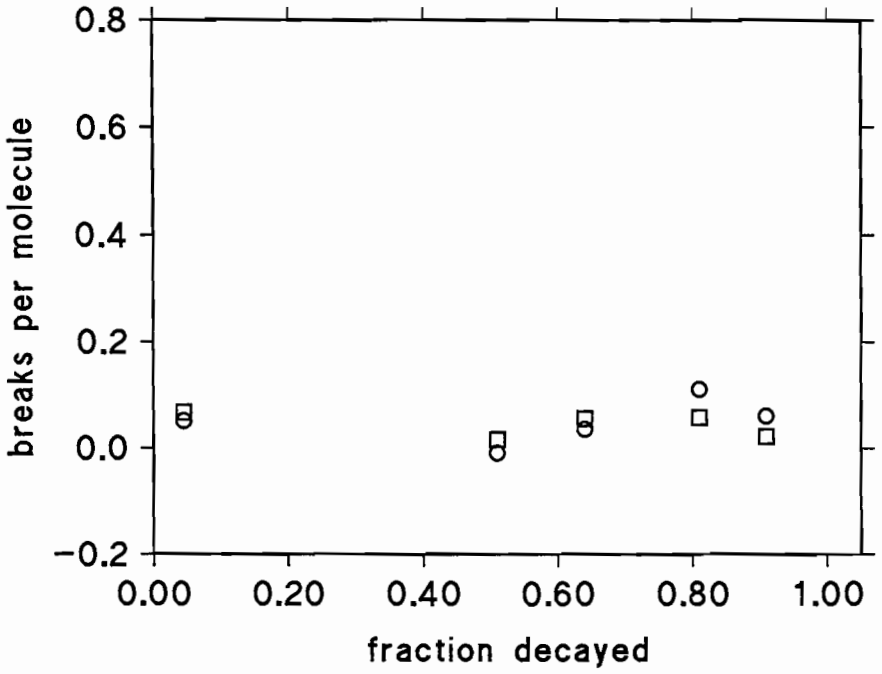

FIG. 3. As for Fig. 2 but with 50 mM glucose added.

Linz and Stöcklin (1) used a plasmid linearized by cutting with the enzyme Sal 1 and labeled with ^{125}I at each end. The labeled plasmid was stored in sodium chloride solution (10 mM) or phosphate buffer (10 mM) with and without the presence of oxygen for up to 6 months. Upon imaging with electron microscopy the DNA was seen to be fragmented, especially in the samples stored in sodium chloride. Two possible explanations were offered, namely, that free radicals generated from water radiolysis had caused single strand breaks to be formed, the combined effects of which was to fragment the DNA, or that the π-electron structure of DNA had enabled energy migration to points on the DNA molecule remote from the site of decay. In support of

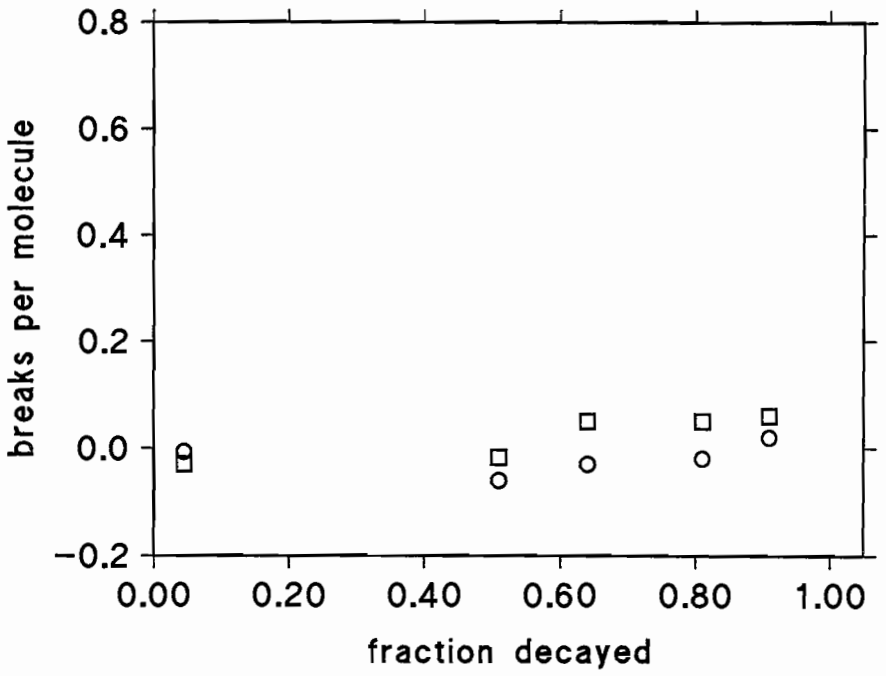

FIG. 4. As for Fig. 2 but with 500 mM glucose added.

this latter contention the authors noted that the size of fragments generated in sodium chloride solutions corresponded to the intervals between regions of the plasmid DNA rich in the base thymine which might act as a trap for migrating energy. In experiments in which the ^{125}I was not incorporated into the DNA, but free in solution, no significant breakage was observed. When the doubly labeled plasmid was recircularized through ligation of the ends linearization of the circle was observed on decay but fragmentation was much less severe than in the case of the molecules originally stored in the linear state.

Our experiments differed from those of Linz and Stöcklin (1) in the following ways:

a) only a single ^{125}I labeled base was incorporated;
b) only half the DNA molecules contained a label;

c) labeling was carried out with the Klenow fragment of DNA polymerase I, which lacks the 5 to 3' exonuclease activity of DNA polymerase I used by Linz and Stöcklin (1);
d) samples were taken at intervals during the decay of the radionuclide to obtain the relationship between decay of the label and breakage in the DNA.

These departures from the Linz and Stöcklin (1) protocol were not deemed to change the essential nature of the experiment, but rather to simplify the interpretation of the results. For example, in molecules labeled at both ends, the state of the DNA when the second decay occurred would not be known. By leaving half the molecules unlabeled we would expect, if the energy migration was intramolecular, to see half the molecules intact at 100% decay but not if the mechanism was intermolecular. In none of the experiments, including those with no radical scavenger present, was fragmentation on the scale reported by Linz and Stöcklin (1) observed.

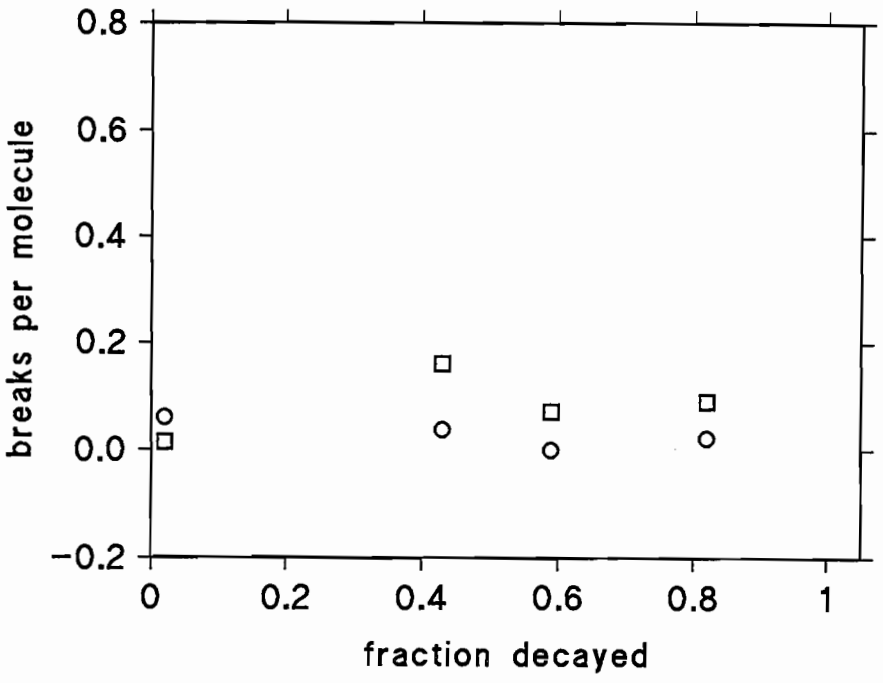

FIG. 5. As for Fig. 1 but stored in 50 mM NaCl with 10 mM Tris buffer at pH 7 .

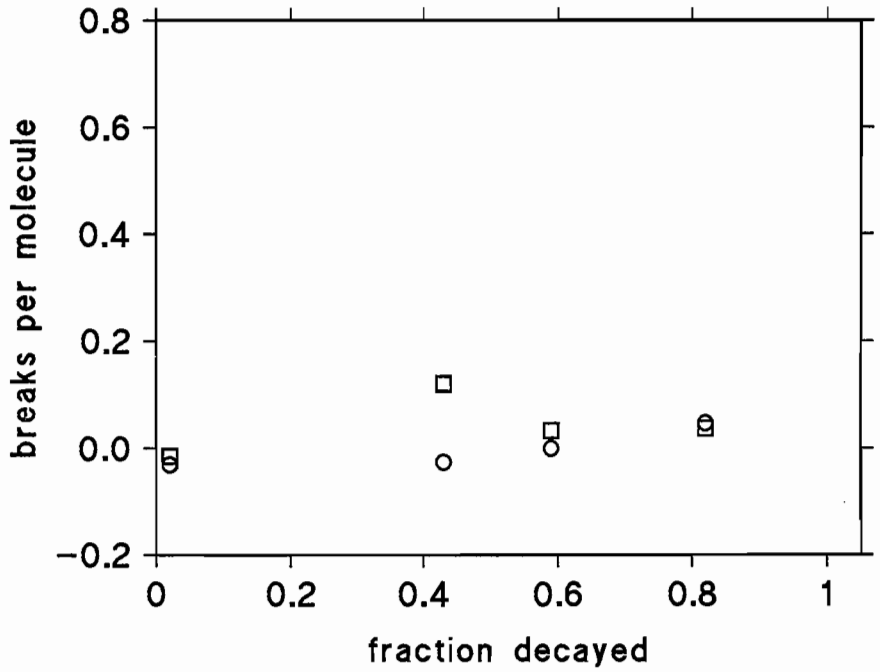

FIG. 6. As for Fig. 5 but with 100 mM Tris buffer.

We observed some degree of breakage of DNA in sodium chloride solutions, about half this amount in phosphate buffer, but apparently no significant breakage in the presence of 50 mM and 500 mM glucose or with 10 and 100 mM Tris buffer. Both the glucose and the Tris buffer would be expected to scavenge free radicals, thus suggesting that any breakage we did observe might be due to radical damage. However, in common with Linz and Stöcklin (1), we did not observe significant damage when the ^{125}I was not incorporated into the DNA even though in our experiments the concentration of ^{125}I was four times greater than for the incorporated samples.

On the basis that radical damage was indicated by those findings we fitted the results for DNA with incorporated ^{125}I in sodium chloride and phosphate buffer to the following relationship:

$$p = mD^2 + b,$$

where p is the number of breaks per molecule and D is the dose in terms of fraction of decay of ^{125}I. This is based on the assumption that double strand breaks in DNA subject to radical attack result from two single strand breaks formed on opposite strands of the DNA, but in close proximity. Assuming this relationship and a random distribution of single strand breaks, we can extrapolate the conditions of our experiment (*i.e.* one ^{125}I per molecule, 50% of the molecules labeled) to the assumed conditions of Linz and Stöcklin's experiment (*i.e.* two ^{125}I atoms per molecule, all molecules labeled). When this is done we estimate that Linz and Stöcklin (1) would observe about 5 breaks per molecule in sodium chloride solution and about 2 breaks per molecule in phosphate buffer. These estimates are in reasonable agreement with the results of Linz and Stöcklin (1), given that the extrapolation is quite substantial and therefore bound to be somewhat uncertain.

We also compared the effects, on similar DNA solutions, of external gamma irradiation. For a dose equivalent to 100% decay of the ^{125}I in the incorporated samples (~ 6 Gy) we found about 0.05 double strand breaks per molecule in both sodium chloride solutions, and phosphate buffer; significantly less than expected on the basis of the experiments with incorporated DNA.

We rationalize these results in the following way. The very localized nature of the energy deposition following the decay of ^{125}I leads to a high concentration of free radicals around the site of decay. If there are no reactive solute molecules (in this case DNA) in the immediate vicinity, the dominant reaction is recombination of radicals to form non-reactive molecular products, H_2O_2, H_2 and of course H_2O. However, if a reactive solute molecule is close by, there will be a competition between the reaction of free radicals with that molecule and recombination. These are precisely the circumstances one would expect in the case of a high-LET radiation and would lead to the results we observe. Namely, damage to DNA where the decay of the ^{125}I occurs close to it, but not where there is no correlation between the sites of decay and the DNA molecules, as is the case in the experiments where the ^{125}I is not incorporated. Thus, we conclude that the fragmentation observed by Linz and Stöcklin (1) was due to free radical effects and not energy migration within the molecule and that, in spite of the lower yields of fragmentation we observed, our results are broadly compatible with theirs.

The first decay in a doubly labeled molecule leads to little double strand breakage, but provides latent damage in the form of single strand breaks. The

second decay adds to the single strand damage causing a dramatic increase in double strand breaks and thus extensive fragmentation.

This interpretation is not apparently fully consonant with the observation of Martin and Haseltine (4) which has subsequently been confirmed (see paper by Martin; this volume) and supported by theoretical calculation (5) in so far as single strand breaks seem to be confined to the bases within 15 bp of the site of decay. Martin and Haseltine (4) stored their samples in 50 mM Tris-borate buffer and so molecules capable of scavenging free radicals were present. However, this level of scavenger would not be expected to remove all free radicals but a small background yield of single strand breaks distributed evenly along the DNA molecule might be difficult to detect. In our experiments 10 mM Tris buffer was sufficiently effective to prevent double strand breaks from forming, and we suggest that the much longer DNA (4300 bp compared to between 50 and 75 bp used by Martin *et al.* (4)) molecule is capable of forming a random coil in the solution so creating a reactive 'cage' environment around the iodine giving a locally very high effective scavenging cross section that can compete with recombination at early times (10^{-8} - 10^{-6} s) after decay of the ^{125}I. The much shorter molecules used by Martin and Haseltine (4) would not be capable of this.

The calculation by Charlton (5) only considered direct excitation of the DNA and it is probable that the breaks close to the site of decay in our experiments were caused by this mechanism rather than radical attack.

The biological implications of this result are that under circumstances where DNA is 'packed' such as in chromatin, some degree of indirect effect might be expected, but that the damage caused is unlikely to lead to double strand breakage by this mechanism unless more than one decay occurs in the same local region.

As for other observations by Linz and Stöcklin (1), namely that when the doubly labeled molecule is recircularized fragmentation was reduced; we suggest this can be explained if it is assumed that the effect of the Auger decay is to excise bases in the immediate vicinity of the decay. Upon recircularization the two labels are located within six base pairs apart. When the first iodine decays, there would seem to be a high probability of excising the remaining label and thus separating the largely intact DNA molecule for the remaining ^{125}I. This would seem to confirm that the decay of ^{125}I in an isolated molecule of DNA does not form a clean double strand break but

rather that there is a high probability of loss of genetic information even if the lesions can be repaired in a mechanical sense (see paper by Yasui; this volume).

That intramolecular energy migration has not been observed in this instance, does not rule out the possibility in other circumstances - for example solid state DNA.

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DISCUSSION

Halpern, A. 1) Which radical scavengers did you use? 2) It is highly questionable whether the results reported by Linz and Stöcklin can be interpreted in terms of the usual concepts of radiation chemistry. The most

plausible explanation of their results to be suggested is long-range energy transfer in DNA.

Baverstock, K. F. 1) Tris buffer (pH 7) and glucose. 2) I think the presence of scavengers which seem to protect against DNA fragmentation must strongly suggest that it is not an intramolecular energy migration process that is responsible. We seem then to be left with a radical mediated effect. This explains most, but not all, of the observations of Linz and Stöcklin. There is probably room for further experiments designed to resolve these issues.

Adelstein, S. J. The role of hydrated (solvated) electrons could be classified by using H_2O in the absence of oxygen.

Baverstock, K. F. I think the main damaging agent for DNA is overwhelmingly the OH radical. Solvated electrons are comparatively unreactive. I do, however, think that some basic radiation chemistry of the ^{125}I decay would be beneficial and they of course should include the influence of H_2O and other selective scavengers. This would help to characterize the competition between radical combination and reaction with solutes such as DNA.