

Degradation of DNA in High-Intensity Focused Ultrasonic Fields at 1 MHz

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Gassy and degassed aqueous solutions of DNA were treated ultrasonically while monitoring the field for discrete cavitation events. At irradiation intensities greater than about 500 W/cm², transient cavitation is shown to be responsible for the observed reduction in molecular weight. At intensities of 200 to 288 W/cm², degradation of DNA was observed which did not depend upon transient cavitation.

INTRODUCTION

Attempts to understand in detail the alterations induced in animal tissues by intense ultrasound have led investigators to examine such interactions at various levels of biological structure. As a result, biopolymers in solution, in particular the unique macromolecular structure deoxyribonucleic acid (DNA), have received considerable attention. While it is recognized that conformation of DNA *in vitro* bears little relation to that *in vivo*, such studies aid in assessing the mechanical stresses to which polymers *in vivo* are subjected during ultrasonic irradiation.

Much interest has arisen around the point of whether or not polymer degradation can occur in the absence of cavitation. The general approach has been to effect damage under certain conditions and then to take steps to suppress cavitation. Persisting degradation at a rate less than that existing before these steps have been taken has been used as an argument for the presence of a mechanism other than cavitation. There is general agreement that the main action of ultrasound on polymers is mechanical, since experiments show that degradation proceeds to a limiting molecular weight if irradiation is continued for long periods.¹ However, when working with cavitation in aqueous solutions where free radicals are produced, Alexander and Fox² assessed that 30% of their breakage could be due to chemical effects.

On ultrasonic irradiation of synthetic polymers at intensities from 5 to 700 W/cm² over the frequency range from 210 kHz to 2 MHz and simultaneously suppressing cavitation, several investigators reported no degradation.³⁻⁷ Some investigators have reported observing continued degradation, though at a reduced rate, with suppression of cavitation by irradiating samples under reduced ambient pressure.^{8,9} This latter method of suppressing cavitation has not been widely used and no quantitative measurements are available on the efficacy of this technique.

The DNA molecule is of a polymer of two strands coiled in a double helix about a common axis. The backbone of the individual strands consists of pentose residues connected together by phosphate bridges. The pentose residues, in turn, are linked to purine or pyrimidine bases. In the double helical form, the sugars and phosphates are on the outside and the bases on the inside of the helix. The main forces holding the two strands together are the base stacking forces which are the hydrophobic interactions between the heterocyclic bases as they stack in parallel arrays at right angles to the main helix axis.¹⁰

Electron micrographs of DNA fragments produced by cavitation at 7 kHz have shown that breakage of the molecule occurs by double backbone scission, i.e., the ends of the fragments were double rather than single stranded.^{11,12} An investigation of the viscosity of sonic fragments after irradiation with cavitating

ultrasound at 800 kHz and 15 W/cm² supports the view that double backbone scission, rather than an accumulation of single strand breaks, is the cause of DNA degradation.¹³ Chemical studies suggest that the main bond involved in the backbone cleavage is the C-O bond (90%) with 10% P-O rupture and no appreciable C-C damage.¹⁴ In an attempt to detect a mechanism for DNA breakdown which does not involve the effects of cavitation,¹⁵ degassed samples of DNA were irradiated at 981 kHz and 25–31 W/cm². The sample holder and field geometry were such that the distribution of the acoustic energy over the sample chamber did not vary by more than 10%. A sedimentation coefficient change from 32 to 16, corresponding¹⁶ to a molecular weight change from 2.2×10^7 to 4×10^6 , occurred in 15 sec. Irradiation for two minutes produced little additional degradation, showing that a limiting molecular weight had been reached. Several methods were employed simultaneously to detect the presence of cavitation and no evidence was observed suggesting its presence. It was considered then that cavitation was not present and that the degradation resulted from viscous stresses set up due to relative motion resulting from the density difference between the DNA molecule and the solvent molecules.

The degradation of DNA of molecular weight 10^7 exposed to 1-MHz ultrasound of 10 W/cm² and 30-msec repeated pulses has been observed to occur when the spherical sample holder was rotated during the irradiation period.¹⁷ No effect was observed in degassed solutions on irradiation without rotation of the sample holder. The degradation correlated with the sonochemical release of iodine from potassium iodide and the detection of the first subharmonic of the driving frequency. It was concluded that microstreaming around stable oscillating bubbles was responsible for the effect.

In order to study further the mechanism responsible for degradation, calf thymus DNA in solution was irradiated in a focused sound field at 1 MHz at intensities greater than those of previous studies.

I. METHODS AND MATERIALS

The 1-MHz (48-mm radius of curvature) PZT4 ceramic transducer, the irradiation tank, the preparation of degassed water, sound intensity measurement procedure, transient cavitation, and first-order subharmonic detecting systems are described elsewhere.¹⁹ All are established procedures except the cavitation detection scheme. Briefly, when the rf voltage applied to the transmitting transducer is rectified and filtered, transient signals may be detected when isolated transient cavitation occurs at the focal region of the field.

Commercially available highly polymerized calf thymus DNA (British Drug Houses Ltd.) was dissolved over chloroform in sterile B.P.E.S. buffer (0.006M Na₂HPO₄, 0.0002M NaH₂PO₄, 0.001M EDTA, 0.179N

NaCl, pH 6.8). After one week, the solution was filtered through Whatman G.P. filter paper to remove a gelatinous mass of denatured protein. The protein concentration of the filtrate was determined with the Folin-Ciocalteu reagent¹⁹ and was typically 6% of the DNA concentration. DNA concentrations were estimated at 260 nm ($D=1.0=48 \mu\text{g/ml}$). The stock solution of DNA was stored over chloroform, which was removed by degassing prior to ultrasonic irradiation to avoid any effect of the chloroform on the onset of cavitation, and 0.01% weight/volume of the antibiotic tris(hydroxymethyl)nitromethane was added. The flask was sealed with a cotton wool plug and allowed to stand for days to saturate the solution with air. The solution was not refrigerated, in order to avoid air bubbles on reheating to room temperature before treatment. When it was desired to suppress cavitation, DNA solution (10 ml) in a 20-ml test tube was degassed at approximately 3 cm Hg, during which time the glass was tapped to encourage bubble emission. Degassing was stopped when no further bubble release was produced in a 1-min interval. Dissolved oxygen was estimated with an oxygen electrode^{20,21} and the output of the polarizing and measuring circuit was applied to a strip chart recorder.

The DNA was irradiated in two types of containers, viz., a thin-walled aluminum cylinder of 13-mm i.d., 5-mm length, having a volume of 0.65 ml, and a lucite cylinder of 18-mm i.d., 12-mm length, with volume of 3.3 ml. Rubber O rings were used to attach 0.0005-in.-thick "Saran" (Dow Corning) foil windows to the cylinder. The DNA was loaded into the container with a wide-mouth pipet, the container being sealed without trapping air bubbles. The sample container was placed in the focal region of the acoustic field for the irradiation procedure.

The DNA molecular weight was estimated from empirical relations linking molecular weight and intrinsic viscosity.¹⁶ The intrinsic viscosity was determined from relative viscosity measurements in an electromagnetic modification²² of the Crowthers and Zimm low-shear-rate viscometer at a DNA concentration of 50 $\mu\text{g/ml}$. Since the main interest of this work was in relative degradations, measurements were made at only one concentration. It was considered that the concentration dependence of the intrinsic viscosity below 50 $\mu\text{g/ml}$ would not alter degradation estimates significantly.

II. RESULTS

Gassy and degassed samples of calf thymus DNA molecular weight 2.2×10^7 (100 $\mu\text{g/ml}$) irradiated in the aluminum container for 10 sec were diluted with equal volumes of B.P.E.S. buffer and their viscosities measured. The oxygen level in the degassed stock solutions varied from 5% to 12% before loading the DNA into the container. No degradation of degassed solu-

tions was observed below 400 W/cm^2 . In the intensity region from 1500 to 2000 W/cm^2 , degradation in the gassy samples varied from no change in molecular weight to a decrease in molecular weight to 4×10^6 . The results for the degassed samples ranged from no observable degradation to a lowest value of molecular weight of 13×10^6 . From the most degraded of the three gassy samples, a sharp cracking audible noise was emitted. Bubbles were visible in fewer than four of the eight degassed samples exhibiting degradation. Therefore degradation could occur in degassed samples without obvious signs of cavitation.

The degradation of undegassed calf thymus DNA in the lucite cylinder was later studied with an electroacoustic system¹⁸ capable of detecting single transient cavitation events (Table I). The sound was pulsed with 2 min between pulses (column 2, Table I). All transients greater than 90 mV were counted electronically. The maximum amplitudes of the cavitation events at the different intensities were comparable. The table suggests a correlation between the number of cavitation events and the reduction in molecular weight.

The transducer could be excited continuously below 550 W/cm^2 without overheating. The molecular weight of undegassed DNA ($56 \mu\text{g/ml}$) irradiated at 515 W/cm^2 in the lucite cylinder and the total number of transient cavitation events plotted against time both show a high rate of change in the first few minutes levelling off as the time of irradiation increased (Fig. 1). It will be seen that there is a strong correlation between the decrease in molecular weight and the number of cavitation events.

For the above and for 12 further samples of undegassed DNA from two stock solutions ($M_c = 15.53 \times 10^6$ and 20.0×10^6) irradiated at 515 W/cm^2 , the decrease in molecular weight, ΔM , is expressed as a fraction of the mean control molecular weight, $\Delta M/M_c$, to facilitate the comparison of the degradation from both stocks (Table II). The best-fit straight line when $\Delta M/M_c$ for the 16 samples is plotted against the number of cavitation events N (Fig. 2), independent of irradiation time, is $\Delta M/M_c = 4.67 \times 10^{-4}N + 0.038$. The 95% confidence limits for the intercept of the line on the ordinate are 0.038 ± 0.056 which, since zero is in-

TABLE I. Ultrasonic degradation of undegassed calf thymus DNA ($50 \mu\text{g/ml}$) and number of transient cavitation events greater than 90 mV.

I (W/cm^2)	Time regime (sec)	Total irradiation time (sec)	Number of transients	Maximum amplitude (V)	Molecular wt $\times 10^{-6}$
0	0	0	0	0	18.4 and 17.4
1470	10+8+6+5+6	35	163	6	13.2
1900	5+5+5+7+6	28	142	12	13.7
2000	5+5+5+6+6	27	293		7.7
2000	6+5+5+5+5	26	283		10.1
2050	8	8	360	7	10.1

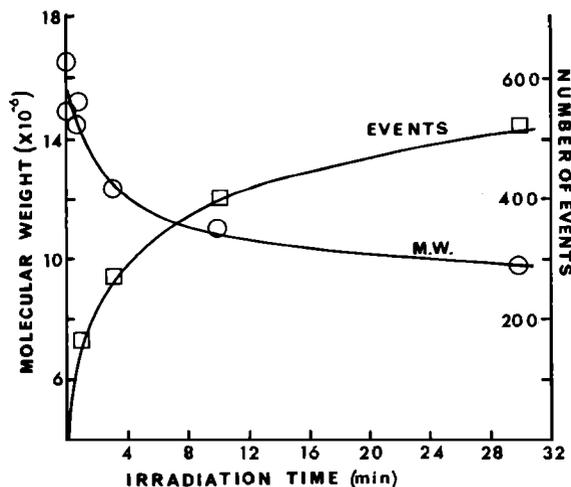


FIG. 1. Time dependence of molecular weight reduction of DNA under ultrasonic irradiation at 515 W/cm^2 and number of transient cavitation events greater than 85 mV.

cluded in the range, shows that transient cavitation alone could explain the degradation observed. The correlation coefficient, r^2 , for the 16 points is 0.68. The best-fit straight line for the 10 points for 30-min irradiation is $\Delta M/M_c = 4.59 \times 10^{-4}N + 0.023$ and r^2 is 0.69. A complete relationship ($r^2 = 1.0$) would not be expected because of the contribution to the variance by visco-

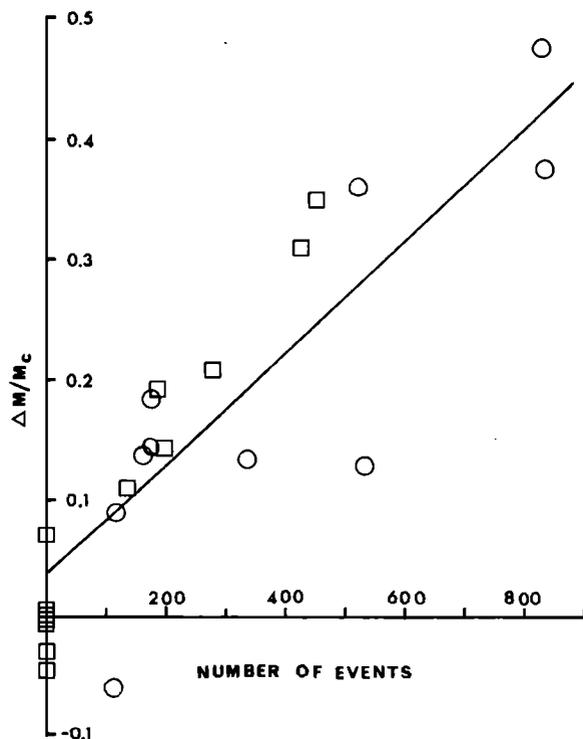


FIG. 2. Relative change in molecular weight $\Delta M/M_c$ as a function of the number of cavitation events for samples irradiated at 515 W/cm^2 from 0.75 to 30 min. The line is $\Delta M/M_c = 4.67 \times 10^{-4}N + 0.038$. (○: 30-min irradiation points only).

TABLE II. Degradation of undegassed calf thymus DNA treated at 515 W/cm² for various times. The control molecular weight estimate on the day of sonication, M_{c1} , and the mean molecular weight estimate, M_c from many measurements of the stock solution are shown. The decrease in M , ΔM , was calculated as $M_c - M$ and expressed as $\Delta M/M_c$. The concentration of the stock of $M_{c1} = 15.53 \times 10^6$ was 56 $\mu\text{g/ml}$ and that of the other stock was 46 $\mu\text{g/ml}$.

Irradiation time (min)	Control molecular wt ($M_{c1} \times 10^{-6}$)	Mean control molecular wt ($M_c \times 10^{-6}$)	$\Delta M \times 10^{-6}$	$\Delta M/M_c$	Number of transient events	Maximum amplitude of transients greater than 45 mV (V)
0.75	15.7	15.53	1.43	0.092	184	
3.0	15.7	15.53	3.23	0.208	276	
10.0	15.7	15.53	4.83	0.310	424	
30.0	15.7	15.53	5.63	0.362	523	4 V, max
1.0	14.6	15.53	1.73	0.111	134	
3.0	14.6	15.53	2.23	0.144	197	
10.0	14.6	15.53	5.03	0.324	450	4 V, max
30.0	14.6	15.53	5.83	0.375	835	2 V, max
30.0	20.0	20.0	2.6	0.130	535	3×3 V, max
30.0	20.1	20.0	9.5	0.475	839	21×5 V, 13×3 V
30.0	19.9	20.0	2.7	0.135	336	3×5 V, 8×3 V
30.0	20.1	20.0	2.9	0.145	173	1×6 V, 3×2 V
30.0	20.0	20.0	3.7	0.185	175	3×5 V, 2×2 V
30.0	19.9	20.0	1.8	0.09	116	1×5 V
30.0	17.4	17.4	2.4	0.138	164	3×6 V
30.0	17.4	17.4	-1.0	-0.058	106	1×0.8 V

metric estimates of M as evidenced by the cluster of the control points about the origin, and because the calculated correlation is with N alone and does not take account of the variations in transient amplitude with each total number of events. The amplitude distribution was not monitored during this work, but recently irradiation of tap water has shown it to be very positively skew.

Figure 3 shows that the average number of cavitation events in each minute of irradiation, of all 16 samples of Table II, and the average maximum amplitude of the cavitation events, calculated from maximum amplitudes recorded during each two-minute interval both fell by factors greater than 10. Since $\Delta M/M_c = 0.3$ for $N = 570$ (Fig. 2), it is assumed that approximately 0.3 of the volume of the container has been affected at least once by a cavitation event. This leads to an "average effective radius" of 0.77 mm, at 515 W/cm², of a transient event, considering the region of influence to be spherical. It has been shown that treating the transient event as a disturbance moving through a stationary cylinder of liquid does not change the estimate of this radius appreciably.²³

Two samples of undegassed DNA were irradiated at 1100 W/cm² (rows 3 and 4, Table III). When com-

pared with data from Table I (rows 1 and 2, Table III) and Table II (row 5, Table III), it can be seen that the degree of damage observed over a wide range of intensity and time of irradiation correlates well with a number of cavitation events, allowing for the fact that the transient amplitudes are larger at the higher acoustic intensities. This also supports the view that

TABLE III. Comparison of DNA degradation in samples irradiated over a wide intensity and time range.

I (W/cm ²)	Irradiation time (sec)	Number of transient events	Maximum amplitude of transients (V)	Relative change in molecular wt $\Delta M/M_c$
2050	8	360	7	0.436
1470	35	163	6	0.262
1100	9×10	139	2.5	0.133
1100	9×10	157	2.5	0.177
515	1800	835	2	0.375

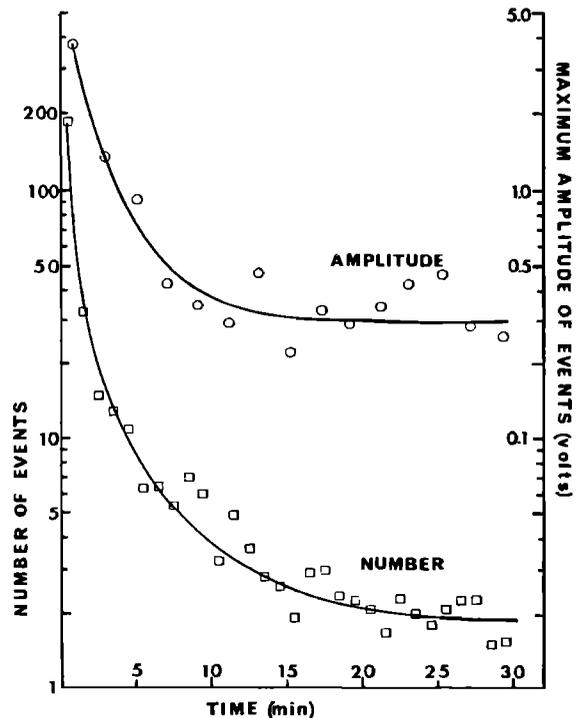


FIG. 3. The number of cavitation events per minute of irradiation (lower curve) and the maximum transient cavitation event amplitude in each 2-min interval, both averaged over the 16 samples of Table II.

TABLE IV. Intensity dependence of degradation of undegassed DNA (50 $\mu\text{g}/\text{ml}$) at 1 MHz.

I (W/cm^2)	Irradiation time (min)	$\Delta M/M_c$	Standard error of mean degradation
72	30	0.030	± 0.031
200	35-39	0.352	± 0.030
288	30	0.207	± 0.031
515	30	0.192	± 0.049

transient cavitation events are responsible for the damage at intensities greater than or equal to 515 W/cm^2 .

The degradation of DNA irradiated at 288 W/cm^2 for 30 min, plotted against number transient cavitation events (Fig. 4), shows one point at $N=266$ isolated from the other nine. If there is no source of degradation other than transient cavitation events, then (0,0) is included as a data point. The curve of Fig. 4 is the best-fit line to those nine points and passes through the origin. The value of the correlation index²⁴ R for the nine points about the line is 0.10, showing little correlation between damage and transient cavitation. This result was expected since a comparison of Fig. 2 and Fig. 4 shows that the damage at 288 W/cm^2 was greater by a factor of approximately 2 for the same number of transient events. The amplitudes of the transients were also less by a factor of 2 at 288 W/cm^2 than at 515 W/cm^2 . Thus a mechanism other than transient cavitation is required to explain the degradation at 288 W/cm^2 .

A series of results at 200 W/cm^2 , after many preliminary experiments to isolate and reduce the sources of experimental variation in the viscosity measure-

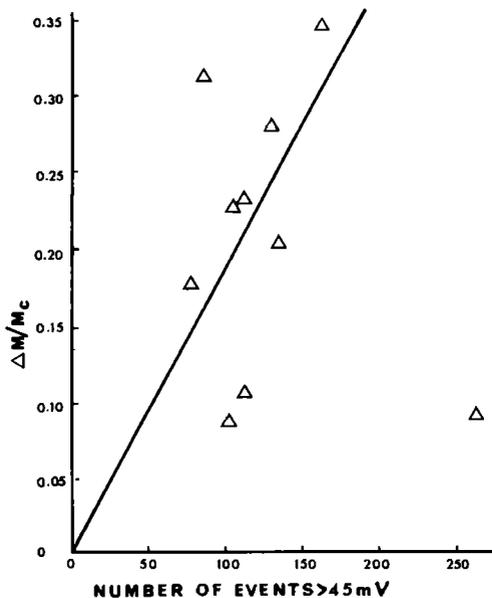


FIG. 4. Molecular degradation of DNA irradiated at 288 W/cm^2 for 30 min as a function of the number of transient events greater than 45 mV.

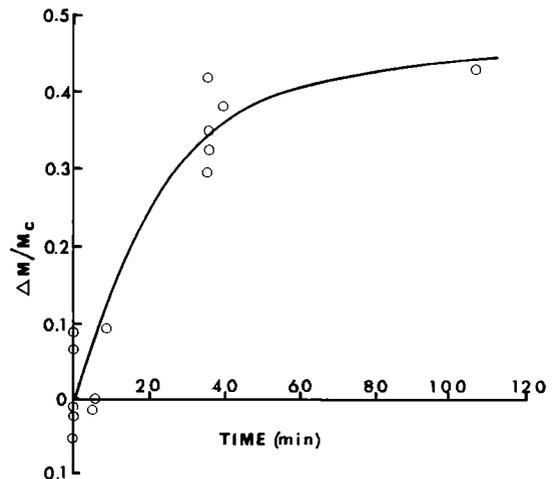


FIG. 5. $\Delta M/M_c$ for calf thymus DNA irradiated at 200 W/cm^2 vs exposure time.

ments, supports the suggestion of a mechanism which does not depend on transient cavitation events, as there was negligible correlation of degradation with transient cavitation numbers at 200 W/cm^2 . However, a plot of degradation against time (Fig. 5) supports the concept of a time-dependent mechanism, as the three points irradiated for up to 8 min show little degradation while there is marked degradation after 35 min.

A series of seven irradiations at 72 W/cm^2 for 30 min showed no detectable damage.

To summarize these results, Table IV shows that the degradation which is not strongly associated with transient cavitation events rises to a maximum and then decreases until at 515 W/cm^2 the breakage may be accounted for by the transient events alone.

A previous study of DNA degradation, in a system designed to stabilize subharmonic-emitting cavitation bubbles at 1 MHz, had an intensity threshold in the range 1-1.5 W/cm^2 , a maximum at a peak intensity in the range 7-10 W/cm^2 , and then decreased on further increase of intensity.¹⁷ The maximum degradation of DNA coincided with a maximum in the detected 500-kHz subharmonic signal. Even though the intensities of the above work were much lower than those presented in this paper, the occurrence of a maximum in the DNA degradation offered a possible explanation of the results of Table IV. This was particularly so since the subharmonic maximum¹⁸ in tap water treated in the open irradiation tank was at 200 W/cm^2 and the level of DNA degradations was also high at that point (Table IV). However, the 500-kHz subharmonic detected from an undegassed DNA sample when a bowl hydrophone was positioned vertically over the sample container was not very different from the signal from the degassed tank water in the absence of the container (Table V). This was surprising since the subharmonic signal detected from tap water was, at most intensities,

TABLE V. The mean of the zero-to-peak 500-kHz signal, measured every 15 sec in a 2-min period, from an undegassed DNA sample and from the tank in the absence of the sample container, as a function of intensity.

I (W/cm ²)	Mean DNA subharmonic (mV)	Mean tank subharmonic (mV)
8	0.3	0.3
32	0.6	0.59
72	2.0	0.95
128	2.75	2.15
200	3.25	3.6
328	3.95	3.8
515	5.45	5.50

an order of magnitude greater than that from degassed water.¹⁸

The 500-kHz signal from the DNA samples irradiated at 72, 288, and 515 W/cm² was measured at the end of each minute of the 30-min irradiation periods and the results averaged for each sample. The bowl hydrophone monitoring the subharmonic was in a fixed position for the early samples but later a technique was adopted for adjusting the bowl position to that of a maximum activity. The level of subharmonic activity did not show a marked decrease irradiation time—the greatest was one of 16% at 515 W/cm² for 30 min. It was not possible to detect a dependence of degradation on subharmonic activity at 72, 288, or 515 W/cm² when considering only those results for which the bowl hydrophone was adjusted as above (Table VI). The number of results at each intensity is small but the absence of any trend in these, and in the other experiments in which the bowl position was not adjusted, indicated that monitoring subharmonic emission in focused fields is not as useful in determining mechanisms of breakage as it has been shown to be in plane fields.¹⁷ The difficulties of interpreting the subharmonic signal were compounded because it was not steady during the irradiations, possibly because the amplitude at any instant was a function both of the number of emitting bubbles present and of their amplitude of vibration.

Since the undegassed DNA samples were separated from the degassed tank water by a Saran membrane, there was a gas concentration gradient across the membrane, and gas diffusion was accentuated during irradiation by bulk streaming inside and outside the container. The oxygen content in a stock DNA solution (50 μg/ml), measured with an oxygen electrode, was 95%. On treatment of a sample for 5 min (515 W/cm²), the oxygen level had fallen to 86%, and it fell to 42% in a sample treated for 30 min. Seventy-six percent of the transient events at 515 W/cm² occur in the first 5 min of irradiation (Fig. 3); therefore, the decrease in the rate of transient cavitation is much more rapid than the fall in gas content in the solutions, so it is unlikely that the two phenomena are related. The experiment did show, however, that significant gas

depletion occurs in samples treated for periods of the order of 30 min.

III. DISCUSSION

Transient cavitation has been shown to be the cause of DNA breakage at intensities of 515 W/cm² and higher. The number and amplitude of transient cavitation events recorded at 288 and 200 W/cm² were too small to account for the breakage observed. Since it was not possible to link 500-kHz subharmonic activity with degradation, as previously reported,¹⁷ the possibility of a noncavitation-linked mechanism of degradation cannot be completely discounted.

If a noncavitating mechanism depending upon a time-averaged force existed, the strain S experienced by a molecule in the sound field would be proportional to $I^{\frac{1}{2}}$ and also would depend upon the relaxation time T required for the molecule to respond to the stress. The streaming velocity of the molecule along the sound-beam axis is proportional to I . The value of the relaxation time required of the DNA molecule so that the strain at 200 W/cm² (S_{200}) may be greater than that at 400 W/cm² (S_{400}) may be calculated since

$$S \sim I^{\frac{1}{2}}(1 - e^{-0.693t/T}),$$

where t is the time the molecule spends streaming along the beam axis. This condition is fulfilled if

$$T > 0.82t.$$

The estimated speed of a cavitation event through the focal region at 515 W/cm² is 1 mm/msec.²³ This decreases to about 0.8 mm/msec at 400 W/cm². If it is assumed that the DNA molecule moves at the same speed as the event, then the molecule traverses the 13-mm length of the container in 16 msec. Thus T must be greater than (0.82×16) or 13 msec. The DNA molecule extended and oriented in hydrodynamic flow relaxes on stopping the flow suddenly with a spectrum

TABLE VI. First-order subharmonic emission and molecular degradation in undegassed DNA irradiated for 30 min at different intensities.

I (W/cm ²)	Mean sample subharmonic (mV)	Mean tank subharmonic (mV)	$\Delta M/M_e$
72	3.0	4.9	0.13
72	4.6	3.2	0.080
288	5.1	5.2	0.205
288	8.1		0.230
288	7.8	3.2	0.090
288	13.0	8.0	0.280
515	14.1	18.5	0.475
515	9.3	5.2	0.145
515	8.5	7.9	0.185
515	12.0	8.0	0.090

of relaxation times,²⁵ the longest of which is given as

$$T = 5.0 \times 10^{-14} M^{1.6}.$$

The value of T relevant to the present study is 20 msec, which fulfills the condition that T be greater than 13 msec. A large fraction of the DNA relaxes with the longest relaxation time.^{25,26} The fact the DNA relaxation time meets the requirements of a mechanism which would be noncavitational and yet explain the strange intensity dependence of degradation (Table IV) may be fortuitous but further experimentation involving irradiation of degassed DNA will be required before deciding between it and cavitational microstreaming.

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