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Department of Biophysics

Solvent Effects in the Radiation Degradation of DNA Solutions

A Thesis in

Biophysics

by

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ABSTRACT

Calf Thymus DNA was prepared in three solvents and irradiated at 77°K for a series of doses and concentrations with cobalt 60 gamma rays. Alkali and neutral band sedimentation was used to obtain initial molecular weight distributions and the molecular weight of both single and double strand samples at each dose. This information was then converted to the number of molecular scissions per molecule by an equation for random scissions.

The data were analyzed in terms of direct and indirect radiation effect and the results were compared with those of other workers. The damage to DNA irradiated at 77°K and warmed to room temperature for analysis was enhanced when phosphate was present; this enhancement was attributed, in part, to indirect radiation action. The rate of single strand break production, in terms of breaks per 10^6 daltons per magarad, was 0.9 in tris solvent, 1.1 in a modified SSCT solvent, and 5.2 in a phosphate solvent. Differences in these rates were attributed to solvent modifications of radiation action. The double strand break production could be interpreted as resulting from an accumulation of single strand breaks and from directly-produced double strand breaks which occurred when a sensitive core (approximately 3 Angstroms in diameter) is hit.

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INTRODUCTION

Radiobiology, the study of the interaction of radiation with biological systems, can be considered from two points of view. In one, biological responses are used to amplify the effects of radiation-induced chemical changes in biological molecules. As biological systems become better understood, this approach becomes increasingly useful. In the other general approach, biological responses to radiation are examined for clues to the affected structures and mechanisms. The importance of the information storage molecules, the nucleic acids, was confirmed in this way by radiation studies of DNA-containing viruses, by Pollard and co-workers (63), and of RNA-containing viruses, by Ginoza et al. (32, 33).

In this review, discussion will be limited to sparsely ionizing gamma- and X-radiation. The results of early work with virus inactivation were generally interpreted in terms of the size of the radiation-sensitive target, which was normally the nucleic acid molecule. This was one of the first methods used to estimate the size of biologically active DNA molecules. Early attempts at interpreting radiation inactivation of more complex systems in these terms were generally not successful, because other intra- and intercellular components and relationships, which were not well characterized, also had to be considered.

The most profitable studies of radiation inactivation from the point of view of understanding which molecules and mechanisms are affected, have involved simple biological systems. These systems can be easily examined both for biological effects and for changes at the molecular level. Bacteria, the simplest self-contained living systems, are particularly well-suited because their genetic and biological simplicity and short generation time greatly reduce the complexity and duration of experiments. The large numbers that can be studied enhance the observation of low probability events and improve the statistical significance of the results. A major disadvantage is the limited control over the immediate DNA environment. Viruses, although needing a host to express biological activity, have all the advantages of bacteria; in addition, because of the chemical and physical simplicity of the virus, which is composed of a nucleic acid molecule in a protein capsule, its irradiation environment can be more precisely controlled, and its nucleic acid can be easily extracted and examined.

A further reduction in the complexity of the DNA environment resulted from the finding that some appropriately treated bacteria can incorporate the purified DNA from viruses, in transfection, or from other bacteria, in transformation, and allow its biological expression. It therefore has become possible to investigate radiation damage to purified DNA and to assess its effect on biological expression.

Studies with these simplified biological systems have been generally classified as <u>in vivo</u>, when the irradiation environment

contained the complete living system, and as in vitro, when it did not. The latter classification would apply to the irradiation of the purified DNA preparations: the former, to irradiation of bacteria.

The effects of ionizing radiation have been separated into two general classes. Damage produced directly on the target material by its interaction with ionizing radiation, such as the production of free radicals on dry DNA, has been termed the direct effect. The indirect effect results from damage produced by radiation-induced species in the environment reacting with the target material. An example of this is the reaction in aqueous solution of DNA with OH radicals, which are produced by the direct interaction of radiation with water.

Simplification of the biological systems used in radiation studies was accompanied by investigation of the irradiation environment when it became apparent that the environment could modify radiation effects. Components such as oxygen (3, 6, 49, 50), copper (7, 46), iron and phosphate (32, 46, 64) enhanced biological inactivation by radiation. This enhancement was considered a result of an increased indirect effect. Measures have been taken to control this enhancement and, in fact, to reduce or eliminate the indirect effect. Nitrogen gas has been used to flush dissolved oxygen from the environment; catalase (31), to remove radiation-produced peroxides; freezing (21, 23, 35), to reduce and immobilize active agents so that they can decay harmlessly; drying (3), to remove water from the environment altogether; broth (32, 42), to scavenge

diffusible agents, and sulfur-containing compounds (32, 33, 42), to scavenge radicals.

At the same time that the effects of radiation damage on biological expression was being characterized, the chemical and physical alterations of the target materials were also being considered. Nucleic acid damage was detected by changes in molecular weight, indicating backbone scissions or crosslinks, by changes in ultraviolet absorption, indicating denaturation or base damage, and by the presence of free phosphate and DNA bases in solution, indicating backbone damage and disruption of the base-sugar linkages, respectively.

Since most biological systems contain a considerable amount of water, the effect of the water environment on the type and extent of molecular damage has been of great interest. Schwarz (73), in a review of the radiation chemistry of aqueous solutions, has summarized the major reactive species found in irradiated water. They are the aqueous electron, which is the predominant reducing radical, the hydrogen atom, another reducing radical, the hydroxyl radical, which is the predominant oxidizing radical, and the perhydroxyl radical, which can function as either an oxidizing or a reducing agent. The perhydroxyl radical can be formed from the reaction with oxygen of organic radicals or of hydrogen atoms and electrons. Baxendale (10) has discussed the effects of pH and oxygen on the reactions of these species. The chemical changes in DNA and related compounds, when irradiated in aqueous solutions, has been summarized (33). The principal agent, at neutral pH and high DNA concentration is the

hydroxyl radical (12, 13, 14, 22) which can attack the DNA bases or the sugar moieties (37, 71, 91).

The kinetics of radiation-induced DNA degradation have been studied by many physical and chemical methods; light scattering (55, 58), viscosity (55, 70), sedimentation (12, 14, 27, 30, 36, 81), ultraviolet absorption (17, 34, 45, 59, 65), and TCA precipitate (64) are some of the more classic techniques. The conclusion reached is that the principal lesions from sparsely ionizing radiation are base destruction and single strand breaks. Ward and Urist (90), in examining synthetic polynucleotides, found that in irradiated aqueous solutions the helical configuration protects the bases from damage, when compared to the coil configuration.

Another physical chemical approach to the investigation of radiation effects is the detection and localization of free radicals produced in irradiated materials. By ESR techniques, free radicals can be detected and, under favorable conditions, assigned to definite sites in the target materials. Because of the instability of free radicals in aqueous solutions and the sharper signal at low temperatures, samples are often irradiated and observed in the frozen or dry state at temperatures as low as 4.2°K. Important information has come from the investigation of irradiated DNA by this technique; however, it has proven difficult to assign definite sites for the localization of free electrons. Gordy and co-workers (57, 75), with samples at 4.2°K, irradiated and observed DNA and its components with varying amounts of water present. They concluded that the DNA spectrum could not be constructed from the spectrum of the individual

components, and that with water present fewer radicals resided on the DNA than predicted by calculations from dry sample data. For dry DNA at room temperature, Ehrenberg et al. (24) suggested that the DNA spectrum resembled the thymine spectrum, while Van der Vorst and co-workers (87) suggested that the radicals were localized on G-C pairs. In temperature studies, irradiating dry DNA at 77°K and observing at temperatures from 77°K to room temperature, Alexander and co-workers (4) concluded that the DNA spectrum at room temperature resembled that of thymine, while Singh and Charlesby (76) concluded that the spectrum resembled that of adenine at 77°K, and changed, upon warming to room temperature, to resemble that of a sugar moiety. The variety of conclusions expressed by these authors demonstrates the ambiguities encountered in analyzing multi-component samples.

ESR techniques have been particularly useful in exploring the phenomenon of radiation protection. Sulfur-containing compounds, such as glutathione, in both the oxidized and reduced forms have been shown to reduce biological inactivation when present during irradiation (32). Working with a mixture of DNA and sulfur-containing compounds, many workers (15, 38, 56, 62) have shown, upon warming an irradiated sample from 77°K, a decrease in the DNA-like spectrum and a corresponding increase in a spectrum associated with a sulfur radical. A likely explanation is that when the sample is warmed the unpaired electrons migrate to the sulfur atoms, resulting in a partial healing of the DNA. Braams (15) has suggested the following

mechanism, in which R_1 is the biologically active molecule and R-S-H is the reduced form of the sulfur-containing compound:

$$R\text{-}S\text{-}H + \dot{R} \rightarrow R\text{-}\dot{S} + R_1\text{-}H$$

Recent work with frozen aqueous solutions of model compounds has supported this view (62).

In spite of the advances made in understanding the interaction of radiation with matter and, in particular, with biological systems, the mechanisms suggested for radiation-produced biological inactivation are still equivocal (see the review by Ginoza (33)). Of course, damaged molecules may be subjected to different stresses, and various enzymatic repair systems may operate in an organism; thus, biological activity could be expressing such stresses or the presence or efficiency of such repair systems. This may explain the conflicting results of Freifelder (28, 29), using T7 and B3 viruses, and Summers and Szybalski (79, 80), using Ø29 virus, who found the principal inactivating lesions to be double strand scissions, and Taylor and Ginoza (83), using the replicating form of ØX174, who found the principal inactivating lesions to be single strand breaks and base or other damage. Support for this explanation is found in experiments with transforming DNA (80). Single strand breaks are implicated as the inactivating lesions and the authors suggested that different conditions must be met by the transforming DNA when compared with viral DNA, namely incorporation into the bacterial genome. If high stresses are experienced here, but not in viral infection, this difference may explain the conflicting results.

However, the possibility still remains that these differences might also be explained by assuming that the irradiation conditions affected the types and relative amounts of DNA damage. Although protecting environments were used in some of the studies--Freifelder used histidine, Taylor and Ginoza used freezing--the presence of phosphate, which has been demonstrated to enhance radiation action (32, 64, 84), in Freifelder's studies, the possibility of indirect energy transfer, shown by Sanner (69) for frozen aqueous solutions, in Taylor and Ginoza's study, and the presence of magnesium in Summers and Szybalski's study lend credibility to this suggested explanation.

Recently, studies by Hagen (36), using Calf Thymus DNA, and Blok (12, 13, 14, 22), using ØX174 DNA, have characterized the radiation degradation of purified DNA in liquid aqueous solutions. In these studies, possible modification of radiation damage by salts and buffers was either not considered (36), or was insignificant when compared to the influence of dissolved gases (14). This finding does not agree with the work of Pollard and Weller (64), who found considerable differences in the kinetics of radiation-induced DNA degradation between liquid solutions of phosphate, acetate, and tris buffers; no differences were found which were due to the presence of dissolved oxygen or nitrogen. In Blok's studies, it is possible that the damage from the indirect effect was caused by so many mechanisms, possibly in competition, that changes in the ionic components demonstrated little variation in the overall radiation damage. Nevertheless, these conflicting results point up the difficulties of interpreting radiation damage in terms of actual mechanisms and agents.

Since indirect radiation action is not fully understood, it should be rewarding to investigate the influence of buffers and salts on radiation damage under conditions where the indirect effect has been reduced in magnitude. This situation may exist in frozen solutions, where radiation damage has been considered to result almost equally from direct and indirect radiation action (35). This system is presently being investigated by workers using ESR techniques to follow the production and migration of free radicals (61, 69). The results of these studies will help characterize free radicals in frozen aqueous solutions. Physical and chemical alterations in DNA molecules must also be characterized before conclusions can be drawn concerning the mechanisms of radiation damage in frozen aqueous solutions.

The present study, which is the expansion of a previous study (82), was designed to explore the effects of various commonly used solvents on the kinetics of DNA strand scissions resulting from irradiation under conditions where the indirect effect is minimized by physical means, a temperature of 77°K. The production of DNA strand scissions--both single strand breaks and double strand breaks--will be calculated from radiation-induced changes in the average sedimentation coefficient of calf thymus DNA samples dissolved in three different solvents. Radiation-induced molecular scissions for each of these solvents will then be compared for variations in dose dependence and concentration dependence.

EXPERIMENTAL MATERIALS AND METHODS

DNA

High molecular weight, double strand calf thymus DNA (C. T. DNA)[1] from Worthington Biochemical Corporation was dissolved in SSCT (see below), and treated with boiled RNAase at a final concentration of 50 µg/ml at 37°C for 30 minutes, pronase at a final concentration of 50 µg/ml at 37°C for 30 minutes, and an equal volume of freshly distilled, water-saturated phenol at 50°C for 20 minutes, and precipitated with two volumes of 4°C isopropanol, according to established purification procedures (1, 40, 51, 53, 68). The DNA was redissolved in 0.01 M Tris, separated into three lots and extensively dialyzed against the three solvents used in this study:

1. SSCT

   0.015 M NaCl, 0.015 M NaCitrate, 0.005 M Tris (HCl) at pH 7.4

2. Tris

   0.01 M Tris (HCl) at pH 8.0

3. PO_4

   0.01 M PO_4 (sodium salt) at pH 7.4

This DNA preparation is denoted C. T. DNA #1. All C. T. samples should be assumed to be #1 unless otherwise designated.

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[1] See Appendix D for a glossary of terms and abbreviations.

Another sample of calf thymus DNA, prepared in the same way, was sheared in a Virtus homogenizer to reduce its molecular weight (11). This sample is denoted C. T. DNA #2.

The samples were passed through a 0.45 μ millipore filter and checked for purity by UV absorption at 230, 260, and 280 mμ; acceptable ratios were 260:230 = 2.0-2.2 and 260:280 = 1.75-1.85. Thermal melting transitions, at 260 mμ were also performed to verify that the sample was double strand DNA. The concentration of the DNA was determined by UV absorption at 260 mμ using $\varepsilon = 20.0$ cm^2/g, from Eigner and Doty (25).

Sample Storage

Samples of C. T. DNA were prepared at various concentrations, quick frozen in 0.2 ml volumes by liquid nitrogen, and stored at -60°C until used.

Irradiation

The frozen samples were warmed to 20°C, gently stirred by rotating the sample containers, and allowed to equilibrate with air. The samples were then submerged in liquid nitrogen and irradiated by cobalt 60 gamma rays at a dose rate that changed during the course of these experiments from 1.07 to 0.8 Mrads/hour. After irradiation, the samples were stored at 77°K until they were tested.

Sedimentation Analysis

Sedimentation experiments were performed in a Beckman Model E analytical centrifuge equipped with UV optics and a four cell mask and timer. UV absorption pictures were recorded on Kodak Commercial Film, which was processed with Kodak D-19 developer and rapid fixer.

Analytical band runs were done in 1.0 M NaCl, at pH 12.6 or pH 7.4, and boundary runs were done in SSCT, at pH 7.4. Both solvents were at 20°C. The viscosity and density of the solutions were measured; the partial specific volume of the sodium salt of DNA was assumed to be 0.556 cc/g, from Bruner and Vinograd (16). Appropriate controls were performed to ensure that the sedimentation coefficients were not affected by concentration (88) nor by the rotor-speed phenomenon (67).

The weight average molecular weight was calculated for each sedimentation coefficient by empirical equations: the single strand equation of Studier (78) was used for all samples sedimented at pH 12.6, and the double strand equation of Studier was used for all samples sedimented at pH 7.4.

Computer Analysis

The irradiation for single strand breaks were analyzed by an IBM 360/67 computer, using a least squares fit program for non-linear equations, Share Library Program NLIN.

The molecular weight distribution of each sample was calculated by the method of Young and Sinsheimer (92) also using the computer; the method was confirmed by agreement with a boundary run calculation.

Another computer program was used to correct the irradiation data for the molecular weight distributions. This entailed solving an implicit second order equation by iteration.

Crosslinks

The samples were examined for interstrand crosslinks by the method of thermal denaturation (44); no crosslinks were detected for gamma irradiated DNA. Controls, with UV irradiated DNA, showed that DNA with 1% of its strands crosslinked (50) could be detected by this method (11).

EXPERIMENTAL RESULTS

DNA samples from each solvent preparation were irradiated in liquid nitrogen at different doses, with concentration constant at 100 μg/ml, and at different concentrations, with dose constant for each solvent. The sedimentation coefficients for both single strand and double strand DNA were then determined for the control and irradiated samples as described in the section on "Methods." These sedimentation data, along with other variables to be described below, are shown in Tables 1 through 10.

Table 1. Single Strand Breaks versus Dose. Calf Thymus DNA #2 in SSCT. DNA was sheared.

Conc. = 100 μg/ml		β = 10.9	$M_n = 1.21 \times 10^6$		pH = 7.4
Dose in Megarads	Average $S_{w,20}$	Number of Points	$M_w \times 10^{-6}$	$M_w(0)/M_w(D)$	Z*
0.0	15.0	11	1.32	1.00	0.000
0.068	14.5	2	1.24	1.06	0.125
0.135	14.8	2	1.31	1.01	0.028
0.202	13.8	3	1.10	1.20	0.405
0.540	13.2	4	0.981	1.35	0.694
1.07	11.6	2	0.714	1.85	1.60
1.60	10.7	4	0.587	2.25	2.28
2.14	9.45	3	0.429	3.08	3.63
2.66	9.65	6	0.452	2.92	3.37
3.17	8.76	3	0.355	3.72	4.64
9.21	6.45	8	0.165	8.00	11.2
16.5	5.22	4	0.097	13.6	19.7

*Z represents the number of chain scissions per 10^6 daltons.

Table 2. Single Strand Breaks versus Dose. Calf Thymus DNA #1 in SSCT.

Conc. = 100 μg/ml		β = 2.12	$M_n = 4.87 \times 10^6$		pH = 7.4
Dose in Megarads	Average $S_{w,20}$	Number of Points	$M_w \times 10^{-6}$	$M_w(0)/M_w(D)$	Z*
0.0	29.2	8	7.16	1.00	0.00
0.92	12.7	2	0.897	7.98	2.01
1.84	10.8	2	0.605	11.8	3.09
3.23	8.97	2	0.376	19.0	5.10
4.23	8.42	2	0.321	22.3	6.02
5.00	8.35	2	0.315	22.8	6.15
6.00	7.81	2	0.266	26.9	7.30

*Z represents the number of chain scissions per 10^6 daltons.

Table 3. Single Strand Breaks versus Dose. Calf Thymus DNA in Tris.

Conc. = 100 µg/ml		β = 3.60	$M_n = 6.86 \times 10^6$		pH = 8.0
Dose in Megarads	Average $S_{w,20}$	Number of Points	$M_w \times 10^{-5}$	$M_w(0)/M_w(D)$	Z*
0.0	31.6	8	8.76	1.00	0.000
0.97	16.8	2	1.81	4.85	0.939
1.90	14.9	2	1.34	6.55	1.33
2.85	12.0	2	0.779	11.2	2.41
3.80**	10.8	2	0.581	15.1	3.30
3.91	10.6	2	0.560	15.6	3.42
4.75	10.0	2	0.494	17.8	3.90
5.70	9.03	6	0.383	22.9	5.08
7.00	8.17	2	0.298	29.4	6.56
8.40	7.70	2	0.257	34.1	7.64

*Z represents the number of chain scissions per 10^6 daltons.

**Irradiated in the single strand state.

Table 4. Single Strand Breaks versus Dose. Calf Thymus DNA in PO_4.

Conc. = 100 μg/ml β = 1.90 $M_n = 3.57 \times 10^6$ pH = 7.4

Dose in Megarads	Average $S_{w,20}$	Number of Points	$M_w \times 10^{-6}$	$M_w(0)/M_w(D)$	Z*
0.0	26.1	12	5.43	1.00	0.00
0.076	11.2	2	0.655	8.29	2.74
0.150	9.55	2	0.440	12.4	4.24
0.230	8.67	2	0.349	15.7	5.48
0.507	7.73	2	0.259	21.0	7.41
1.00	6.56	2	0.172	31.6	11.3
1.38	5.84	2	0.129	42.4	15.2
1.79	6.17	2	0.148	36.8	13.2
1.84	5.92	2	0.133	40.8	14.7
3.50	4.69	2	0.074	73.1	26.6
4.00	4.80	2	0.079	63.9	25.0

*Z represents the number of chain scissions per 10^6 daltons.

Table 5. Double Strand Breaks versus Dose. Calf Thymus DNA #1 in SSCT.

Conc. = 100 µg/ml		β = 6.42	M_n = 16.0x10^6		pH = 7.4
Dose in Megarads	Average $S_{w,20}$	Number of Points	M_wx10^{-6}	$M_w(0)/M_w(D)$	Z*
0.0	28.8	8	18.4	1.00	0.0000
0.92	26.3	2	14.2	1.30	0.0413
1.84	23.9	2	10.7	1.71	0.0945
3.23	21.9	2	8.35	2.21	0.154
4.23	19.6	2	6.06	3.04	0.251
5.00	18.2	2	4.89	3.77	0.334
6.00	17.8	2	4.58	4.02	0.362

*Z represents the number of chain scissions per 10^6 daltons.

Table 6. Double Strand Breaks versus Dose. Calf Thymus DNA in Tris.

Conc. = 100 µg/ml		β = 4.97	M_n = 18.0x10^6		pH = 8.0
Dose in Megarads	Average $S_{w,20}$	Number of Points	M_wx10^{-6}	$M_w(0)/M_w(D)$	Z*
0.0	30.5	6	21.7	1.00	0.0000
0.97	28.0	2	17.0	1.28	0.0320
1.90	26.3	2	14.2	1.53	0.0596
2.85	26.6	2	14.6	1.48	0.0543
4.75	22.3	2	8.80	2.47	0.155
5.70	19.2	10	5.67	3.83	0.286
7.00	19.8	2	6.19	3.51	0.256
8.40	17.9	2	4.66	4.66	0.365

*Z represents the number of chain scissions per 10^6 daltons.

Table 7. Double Strand Breaks versus Dose. Calf Thymus DNA in PO_4.

Conc. = 100 µg/ml		β = 3.89	M_n = 13.5x10^6		pH = 7.4
Dose in Megarads	Average $S_{w,20}$	Number of Points	$M_w x10^{-6}$	$M_w(0)/M_w(D)$	Z*
0.0	28.0	11	17.0	1.00	0.000
0.115	22.7	2	9.26	1.83	0.114
0.230	24.0	2	10.9	1.56	0.078
0.507	22.6	2	9.14	1.86	0.117
1.00	19.7	2	6.15	2.76	0.232
1.38	16.2	2	3.49	4.86	0.488
1.79	13.9	2	2.24	7.57	0.810
1.84	17.2	2	4.15	4.09	0.394
2.87	12.2	2	1.54	11.0	1.22
3.50	14.8	2	2.68	6.34	0.664
4.00	13.3	2	1.98	8.60	0.932
4.65	10.3	2	0.943	18.0	2.04

*Z represents the number of chain scissions per 10^6 dalton.

Table 8. Single Strand Breaks versus Concentration. Calf Thymus DNA #1 in SSCT.

Dose = 2.15 Mrad.	β = 2.12	$M_n = 4.87 \times 10^6$	pH = 7.4		
Conc. in µg/ml	Average $S_{w,20}$	Number of Points	$M_w \times 10^{-6}$	$M_w(0)/M_w(D)$	Z*
Control	29.2	8	7.16	1.00	0.00
55	10.1	2	0.502	14.3	3.78
210	10.3	2	0.528	13.6	3.57
370	10.4	2	0.548	13.1	3.44
650	10.7	2	0.585	12.3	3.21
1125	10.5	2	0.558	12.8	3.37

*Z represents the number of chain scissions per 10^6 daltons.

Table 9. Single Strand Breaks versus Concentration. Calf Thymus DNA in Tris.

Dose = 1.856 Mrad.	β = 3.60	$M_n = 6.86 \times 10^6$	pH = 8.0		
Conc. in µg/ml	Average $S_{w,20}$	Number of Points	$M_w \times 10^{-6}$	$M_w(0)/M_w(D)$	Z*
Control	31.6	8	8.76	1.00	0.00
70	12.8	2	0.916	9.60	2.03
330	13.3	2	1.00	8.74	1.84
460	14.8	2	1.30	6.72	1.37
640	13.8	2	1.12	7.86	1.64
1280	13.7	2	1.08	8.08	1.68

*Z represents the number of chain scissions per 10^6 daltons.

Table 10. Single Strand Breaks versus Concentration. Calf Thymus DNA in PO_4

Dose = 0.85 Mrad.		β = 1.90	$M_n = 3.57 \times 10^6$		pH = 7.4
Conc. in µg/ml	Average $S_{w,20}$	Number of Points	$M_w \times 10^{-6}$	$M_w(0)/M_w(D)$	Z*
Control	26.1	12	5.43	1.00	0.00
60	6.65	2	0.178	30.6	11.0
225	6.55	2	0.172	31.8	11.4
560	7.67	2	0.242	21.5	7.62
890	9.28	2	0.369	15.0	5.22
1500	11.7	2	0.728	7.47	2.44

*Z represents the number of chain scissions per 10^6 daltons.

ANALYSIS AND DISCUSSION

The molecular weight of each DNA sample was calculated from its sedimentation coefficient, using the widely accepted empirical relations established by Studier (78). Based on the work of Aten and Cohen (8) and Eigner and Doty (25), these molecular weight values were assumed to be the weight average values. Crothers and Zimm (18) have suggested a refinement in the empirical equation for double strand DNA that would include the effect of helix stiffness, important at low molecular weights. This equation was not used because the high molecular weight of the unirradiated samples made a correction unnecessary, and the irradiated samples, although of lower molecular weights, have many single strand breaks which would compensate for any tendency toward helix stiffening (26).

Radiation-induced crosslinking has been shown to occur in DNA irradiated at very high concentrations (2, 48, 49). Intramolecular crosslinking can be either intrastrand or interstrand. It was assumed that the intrastrand type would not significantly affect the sedimentation coefficients. Interstrand crosslinking, which would increase the sedimentation coefficients of alkali denatured DNA, was investigated by reversible thermal denaturation, a method used by Szybalski (44), Freifelder and Davison (26) and others. In this study, UV-induced interstrand crosslinks could be detected in 1% of

the DNA; this value is based on the production rate for these lesions cited by Marmur and Grossman (52). However, no interstrand crosslinks were detected by this method for cobalt irradiated DNA at 100 μg/ml after exposure to doses up to 18 megarads (11). No tests were conducted to detect intermolecular crosslinks; however, based on the work of other authors (48, 49), it was assumed that this class of crosslinks would not affect the data in this study until concentrations of over 1 mg/ml were irradiated. This assumption will be discussed further when the concentration dependence is examined.

The change in the molecular weight of each sample after irradiation can be related to the number of molecular scissions with the aid of an equation derived from the work of Inokuti (43):

$$\frac{M_w(0)}{M_w(D)} = \left(1 + \frac{1}{\beta}\right) \frac{x^2}{2} \left[x - 1 + \left(1 + \frac{x}{\beta}\right)^{-\beta}\right]^{-1}$$

where $M_w(0)$ is the unirradiated weight average molecular weight,

$M_w(D)$ is the weight average molecular weight after D dose,

β is a parameter describing the spread of a gamma distribution of molecular weights, and

x is the number of molecular scissions per number average molecule.

See Appendix A for derivation.

This equation describes the molecular weight changes as a function of molecular scissions, provided that the initial molecular weight distribution can be described by a generalized Poisson distribution and that the scissions are random; both assumptions are reasonable for these data. Theoretical curves were generated using this equation to determine how the distribution parameter, β (where $M_w = [1 + \frac{1}{\beta}] M_n$), affected the relationship between molecular weight changes and scissions. See Figure 1. This relationship appears to be linear for β values between 0.25 and 2.0. For β values larger than 2.0, a slight upward curvature appears in the early portion of the curve, that is, up to an average of 2 scissions per molecule. This plot demonstrates that the equation is well-behaved in the range of values used in this study. Thus, the equation will be used to convert molecular weight changes to numbers of scissions without further concern. The value of the slope, which will be used to calculate the radiation efficiencies, does change considerably for various β values, necessitating the calculation of β for each sample analyzed.

The experimental distribution parameter, β, was found for every condition--solvent, source of DNA, single and double strands--by using the method of Young and Sinsheimer (92) to calculate the initial molecular weight distribution. The x,y co-ordinates of each peak in a band run (50 to 150 points were used to describe each curve) were fed to a computer which was programmed to divide each peak into 10 equal areas, calculate the sedimentation coefficient for each area, convert these values to molecular weights, and

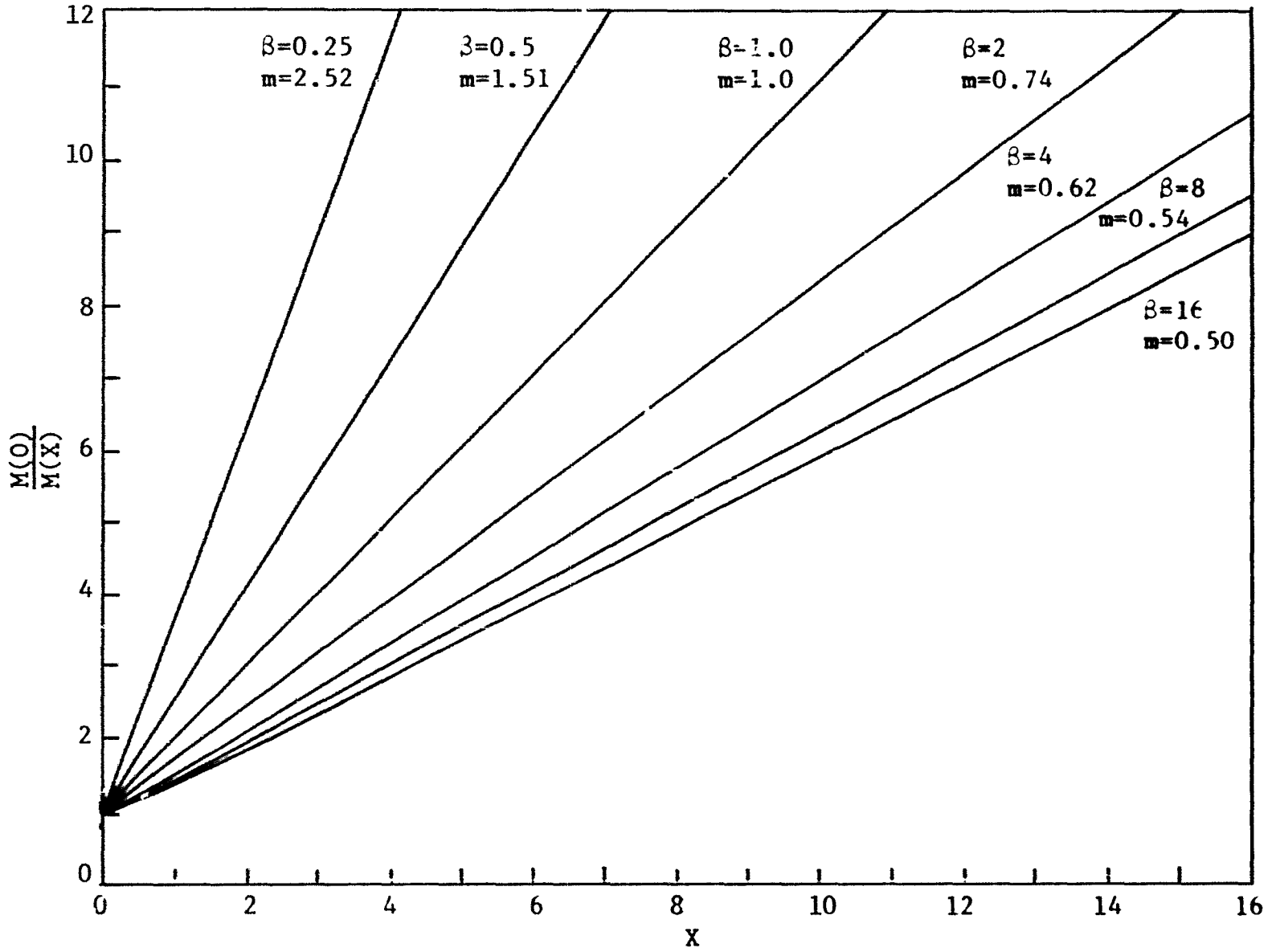

Figure 1. Relationship between Weight Average Molecular Weight and Molecular Scissions. The molecular weight distribution parameter, which is to be calculated from experimental sedimentation data, is represented by β, while the final slope, represented by m, is related to the rate of production of molecular scissions. The number of molecular scissions is represented by x

calculate the weight and number average molecular weights and β. Subdividing the peak into 20 areas did not change the results.

The molecular weight data were converted to number of molecular scissions per number average molecule by means of a computer program that solved the equation derived from Inokuti by the Mueller iterative method. In order to simplify a comparison of the data for the various cases, the scission data were normalized to Z values, which are the number of molecular scissions per 10^6 dalton number average molecule. These data, along with the corresponding sedimentation coefficients, molecular weights, and molecular weight ratios, are included in Tables 1 through 10 for the various cases investigated.

Single Strand Breaks

Let us first examine the single strand data, remembering that it was obtained from sedimentation runs done at pH 12.6, which denatures the double strand molecule. Figure 2 displays the number of ssb[2] as a function of radiation dose for calf thymus DNA in PO_4, in Tris, and in SSCT at both large and small molecular weights. It is apparent that the PO_4 and SSCT curves are nonlinear and could be the sum of two effects, while the Tris data appears to be best represented by a straight line; in addition, the rate of strand breakage is greatest in PO_4 and least in Tris. In spite of their different β values, it appears that both SSCT samples can be

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[2]See Appendix D for a glossary of terms and abbreviations.

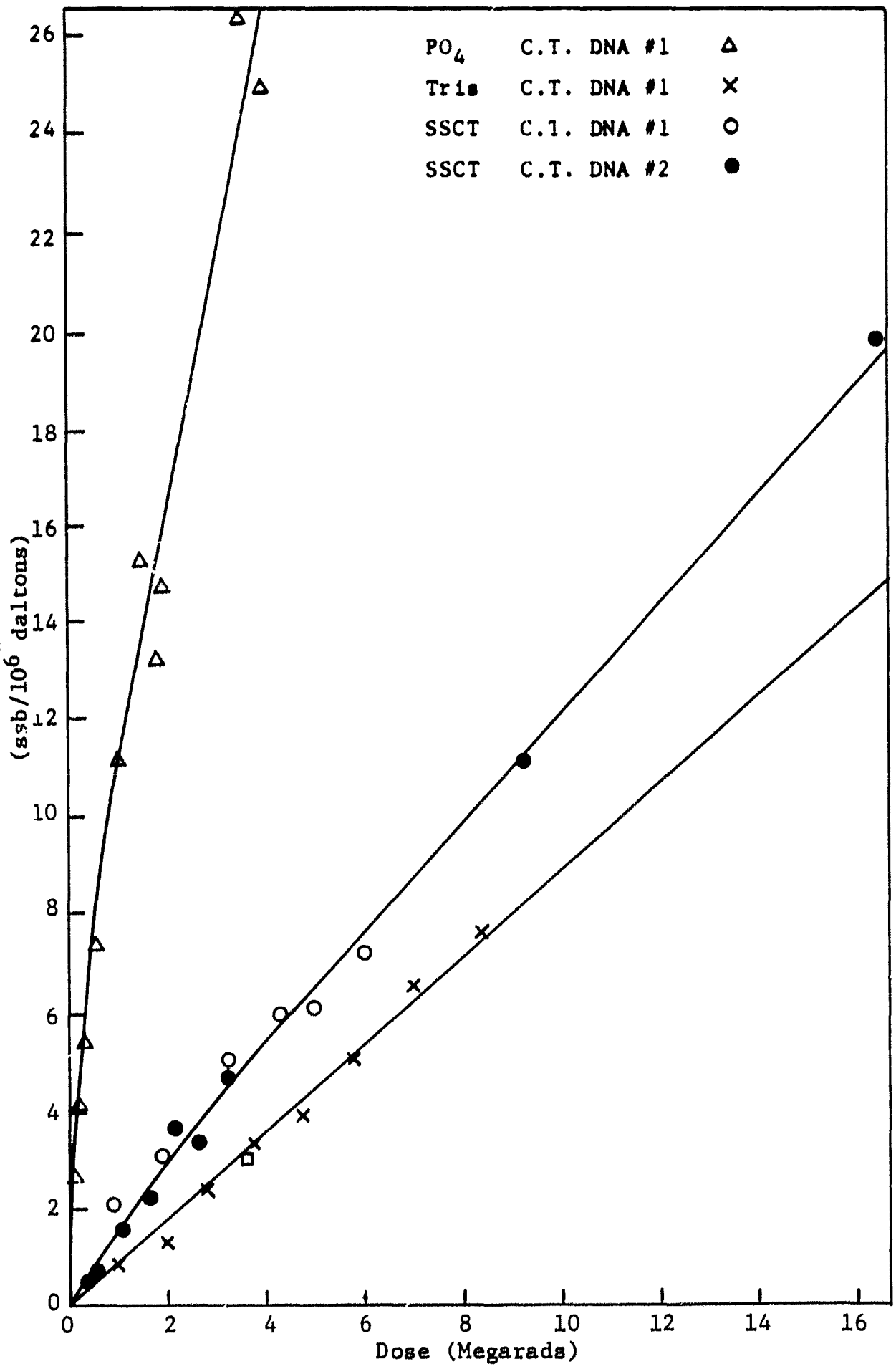

Figure 2. Single Strand Breaks versus Dose. Symbols are experimental points and curves are least squares fit of data to scheme 3. DNA concentration is 100 µg/ml. The square represents the irridation in Tris of single strand DNA.

described by the same line, which supports the method used to calculate β. See Figure 3 for an enlargement of the SSCT and Tris data. The final feature is the result of irradiating a single strand DNA sample in Tris. The number of ssb corresponds to the number expected for the irradiation of double strand DNA, which supports the assertation made above, that interstrand crosslinks do not affect these data.

The dependence of ssb on concentration of DNA is shown in Figure 4. The dose for each solvent was not the same, but was large enough to be on the second component portion of the dose dependence curve, wherever two components were evident. The dose for PO_4 was 0.85 megarads, for SSCT, 2.15 megarads, and for Tris, 1.86 megarads. If the data were plotted on an equivalent dose basis, the PO_4 curve would be shifted up, while the Tris and SSCT curves would be moved down and slightly closer together. The shapes of the curves would remain unchanged. A reduction in number of scissions with increasing concentration, as would be expected for an indirect effect, is quite evident in the PO_4 case, but only suggested in the other cases. An alternative explanation for the curvature in the concentration study, Figure 4, is the production of intermolecular crosslinks. Other authors (48, 49) have found no evidence for this type of crosslink at DNA concentrations below 1 mg/ml. Since the curvature demonstrated in Figure 4 starts at very low DNA concentrations, 50-100 μg/ml, it is highly unlikely that intermolecular crosslinks are the cause of this curvature. Before the experimental

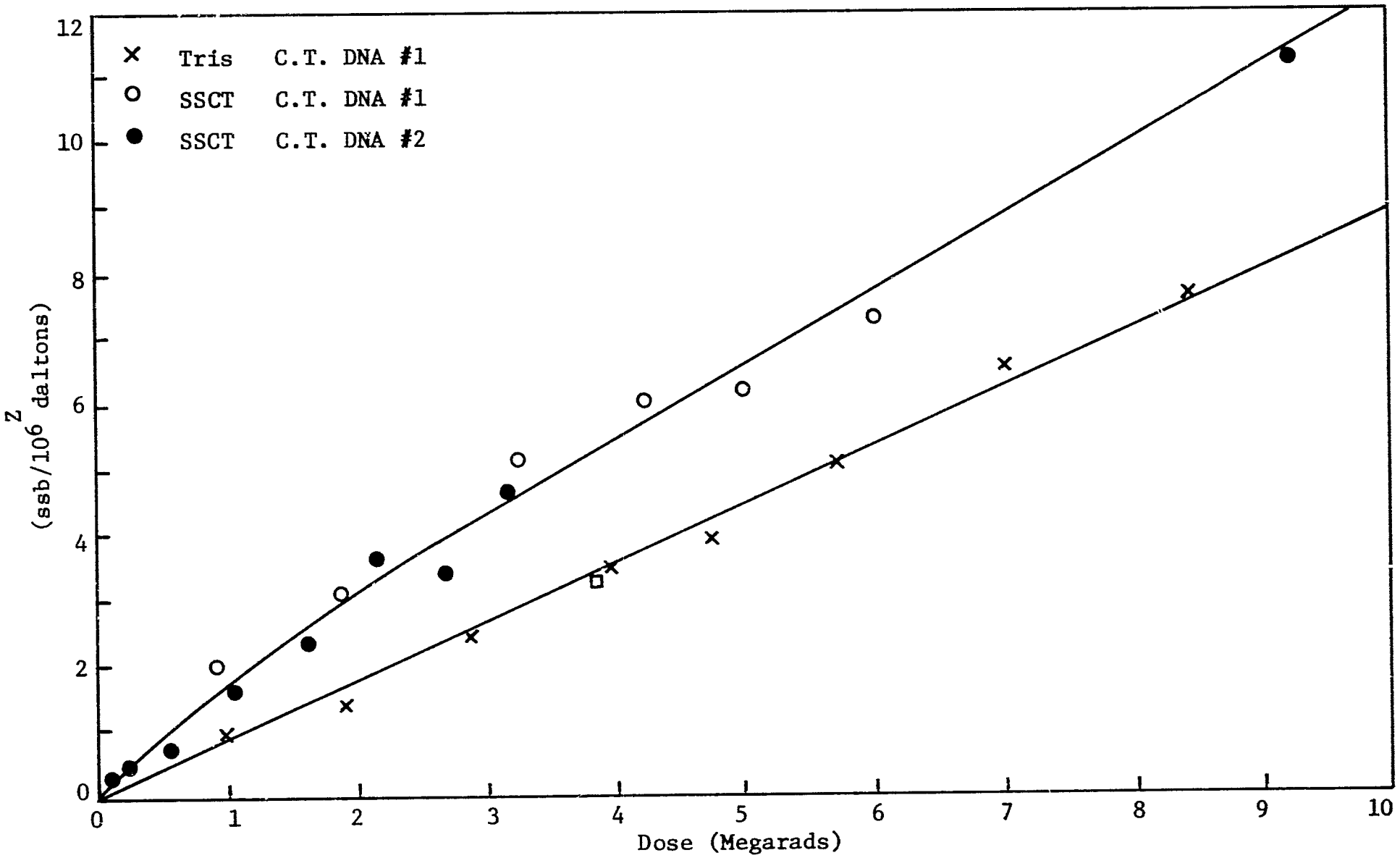

Figure 3. Single Strand Breaks versus Dose: An Enlargement. Symbols are experimental points and curves are least squares fit of data to scheme 3. DNA concentration is 100 µg/ml. Note fit of the two SSCT data sets to the same theoretical curve. The square represents the irradiation in Tris of single strand DNA.

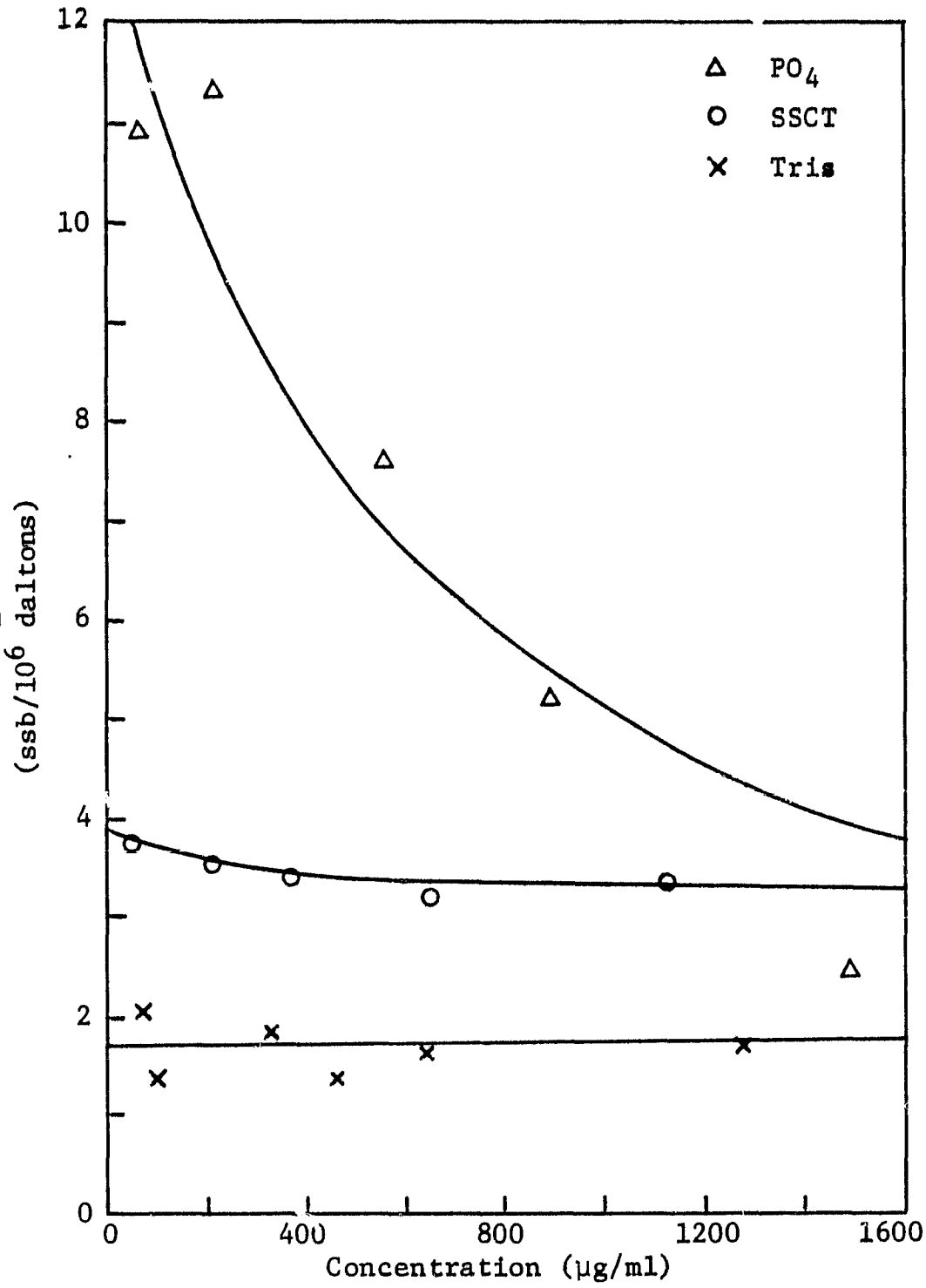

Figure 4. Single Strand Breaks versus DNA Concentration. Data is for Calf Thymus DNA #1. The radiation dose in megarads is 0.85 for PO_4, 2.15 for SSCT, and 1.86 for Tris.

results are analyzed further, the contributions to radiation damage expected from direct and indirect radiation action will be discussed.

Damage due to the direct effect is assumed to increase linearly with dose and to be independent of the concentration of the DNA molecules. The rate of radiation destruction is proportional to the size of the affected volume, which is normally considered to be the size of the affected molecule. It is possible, however, that in frozen aqueous solutions this volume includes a sensitive shell around the molecule, as suggested by Sanner (69). If present, this shell would increase the proportionality constant for the direct effect. The indirect effect is assumed to arise from the diffusion of solvent products or from energy migration either at the irradiation temperature or upon warming. For example, hydrogen atoms produced in irradiated water are trapped at 4°K but can migrate at 77°K, while OH radicals remain trapped at 77°K and become mobile at 120°K to 140°K. Damage due to the indirect effect, when expressed in terms of scissions per molecule, is expected to decrease as the DNA concentration increases. This response is the result of competing reactions involving the indirect agent and the dilution of the damaged molecules by the undamaged ones.

To compare the results quantitatively, reaction schemes must be devised that fit the experimental data and allow a calculation of the parameters that are associated with the direct and indirect processes. The results may be affected by a limited amount of oxygen which was present because the samples were in equilibrium with air at 20°C before they were frozen. It is unlikely that

oxygen would diffuse into or out of the samples once they were frozen, since oxygen molecules are probably immobile at 77°K. There also exists the possibility that a solvent species, responsible for some of the damage, could become saturated. The reaction schemes must be devised with these factors in mind.

There are many reaction schemes that could be considered; however, few can be expressed in terms of rate constants and the known variables, and still fewer can be solved explicitly for scissions as a function of the other variables and constants. The reaction schemes presented below were all derived with the assumptions that the direct and indirect effects are independent and that reactions of activated species occur upon warming. Appendix B contains the derivation of the final equation for each reaction scheme.

Scheme 1. Single strand breaks are produced by direct action and by indirect action. The latter involves the interaction of DNA with a species, in limited supply, that has been activated by radiation. The reaction scheme is:

$$A \xrightarrow{k_o D} A^*; \quad A^* \xrightarrow{k_2} Z; \quad A^* + DNA \xrightarrow{k_1} X$$

where A is a parent species,

Z is a product not resulting in a scission, and

X is a ssb.

The equation for the number of ssb per strand is:

$$X = k_D D + \frac{M_n}{\frac{k_2}{k_1} m_o + DNA} A_i \left[1 - \exp(- k_o D)\right]$$

where X is the number of ssb per initial single strand,

k_D is the probability of directly producing a ssb per strand per megarad,

D is the exposure dose in megarads,

M_n is the initial number average molecular weight of a strand in daltons,

m_o is the monomer molecular weight in daltons,

DNA is the DNA concentration in grams per liter,

A_i is the initial concentration of the parent species in moles per liter,

k_o is the probability of producing an activated species per unit dose, and

k_1 and k_2 are as shown above.

Scheme 2a. Single strand breaks are produced by direct action and by radiation-induced lesions that require the mediation of a solvent species, which is in limited supply. If this lesion can also react with some other solvent species, so as not to produce a scission, the scheme is:

$$\text{DNA} \xrightarrow{k_A D} \text{DNA}^*; \quad \text{DNA}^* + \text{A} \xrightarrow{k_1} \text{X}; \quad \text{DNA}^* \xrightarrow{k_2} \text{Z}$$

where * denotes a radiation activated species, which gives the equation:

$$X = k_D D + \frac{M_n}{DNA} A_i \left[1 - \exp\left(-\frac{k_1}{k_2} DNA \left[\frac{k_A}{m_o} D - \frac{X}{M_n}\right]\right)\right]$$

where the symbols have been described above. This expression can not be solved explicitly for X. However, this problem can be overcome by making a further assumption which is given in the next scheme.

Scheme 2b. The previous scheme can be solved for X with the assumption that all of A reacts with the DNA lesions before the lesions decay by first order or pseudo-first order kinetics to a non-scission product. The equation thus becomes:

$$X = k_D D + \frac{M_n}{DNA} A_i \left[1 - \exp\left(-\frac{k_1 k_A}{2} \frac{DNA}{m_o} D^2\right)\right]$$

where the symbols are as described above.

Scheme 3. Single strand breaks are produced by direct action and by the interaction of DNA with a radiation-activated solvent species which can be destroyed by radiation. The reaction scheme is:

$$B \xrightarrow{k_1' D} B^*; \quad B^* \xrightarrow{k_2' D} W; \quad B^* \xrightarrow{k_3} Y; \text{ and}$$

$$B^* + DNA \xrightarrow{k_1} X$$

where B is the solvent species,

W is a radiation-produced decay product of B*, and

Y is the non-radiation-produced decay product; neither product ultimately produces a strand scission. The reaction equation is:

$$X = k_D D + \frac{M_n}{\frac{k_3}{k_1} m_o + DNA} \frac{k_1'}{k_2'} [1 - \exp (- k_2' D)]$$

where the symbols have been explained above.

Scheme 4. Strand scissions are produced by three processes, each independent of the others: direct action, interaction of DNA with a radiation-activated solvent species, B*, and interaction of radiation-activated DNA with another solvent species, A, which is in limited supply. The reaction scheme is:

$$B \xrightarrow{k_4 D} B^*; \quad DNA \xrightarrow{k_6 D} DNA^*; \quad B^* \xrightarrow{k_3} Z;$$

$$B^* + DNA \xrightarrow{k_5} X; \quad DNA^* + A \xrightarrow{k_7} X; \quad DNA^* \xrightarrow{k_2} Y$$

and the equation is:

$$X = k_D D + \frac{M_n k_4' D}{\frac{k_3}{k_5} m_o + DNA} + \frac{M_n}{DNA} A_i [1 - \exp (- \frac{k_6 k_7}{2} \frac{DNA}{m_o} D^2)]$$

where k_4' is equal to $k_4 B$ and the other symbols are as described above. This equation is essentially the combination of Schemes 2b and 3, with the exception that B* is not destroyed by radiation. One form of the reaction scheme suggested by Szybalski (81) can be represented by this equation if A_i is assumed to be the initial oxygen concentration.

These schemes are quite simple and may not represent what is actually happening in this complex radiation environment. They all

do predict equations that seem to fit the experimental results; consequently, it would not be rewarding to develop more complex schemes in an attempt to determine the precise reactions without the benefit of data from some simpler, better detined systems. Two assumptions that appear, in some ways, quite restrictive seem to be valid. The assumption that activated species decay harmlessly by first order, or pseudo-first order kinetics is in accordance with the mode of decay for activated water (5) and for radiation-induced free radicals in polycrystalline organic samples (39). The important and critical assumption, that each reaction which results in scissions is independent, was made to simplify the solution of tne differential equations that occurred in the derivations of these final equations. Even if all the damaging reactions are not independent, the general characteristics of the resulting equations would be affected only if competitive reactions account for a significant proportion of the damage. Thus, because the reaction schemes include so many assumptions, the final equations should be regarded as empirical and the values found for the parameters should only be used to indicate differences between solvents, and not as absolute values for particular processes.

The curves drawn in Figures 2, 3, and 4 were determined by computer fitting of the data to the equation of scheme 3. The calculation was done by a program designed for least squares fitting of nonlinear equations, NLIN, from the IBM Share Library. The agreement between the theoretical lines and experimental data indicates that the general analytical approach is correct. However, the

similarity between the equations for all reaction schemes together with the experimental scatter make it impossible to distinguish between the schemes on the basis of their agreement with the data. Because of this ambiguity and the possibility that other schemes not considered here could better fit the data, the numerical values of the calculated parameters will not be stressed, although they are given in Appendix C. The experimental results will therefore be compared on a qualitative basis but before doing so, let us consider the possible causes of the indirect damage.

Damage due to the indirect effect in aqueous solutions has been attributed to diffusable agents, which include water species and solvent components, such as buffers, noteably phosphate (32, 64), salts (54), and oxygen (6, 45, 74, 86). The effect of radiation on DNA can be modified by other components in the solvent; broth (32, 33) and sulfur compounds (14, 41, 56, 62) are two examples. Most studies of these processes have been done in liquid solutions and the results may not be applicable to irradiation of frozen solutions where the indirect effect is greatly reduced (21, 23). Nevertheless, ESR techniques have shown that some indirect agents, specifically, the hydrogen atom and the OH radical, do exist and can migrate in frozen aqueous solutions, and that some sulfur-containing compounds can act as protecting agents at temperatures below freezing. It is also possible that other mechanisms such as energy transfer processes (9, 60) play important roles in the production of radiation damage at low temperatures. In this study

the samples were thawed for examination, so there is a possibility that damage associated with both conditions can be seen.

Damage, in ssb per 10^6 daltons, resulting from indirect action would be expected to decrease at a given dose with increasing DNA concentration. This is expressed by the DNA factor in the denominator of the equations derived above. The results of the concentration dependence study, Figure 4, show that, for the conditions and concentrations used in this study, ssb are considerably reduced by increasing the DNA concentration in PO_4, and only slightly reduced in SSCT. The results for Tris do not appear to vary over the DNA concentration range studied. Thus, it appears that indirect action is present in PO_4 and SSCT. Whether this action takes place upon warming, or at the irradiation temperature can not be determined here. One factor which could considerably affect these results is the dose at which the concentration dependence was determined. If the indirect effect is not saturated at that dose, then the dependence of the indirect effect on concentration would not necessarily result in downward curvature of the data for increasing concentration.

The dose dependence for each solvent, Figure 2, does show nonlinearity for both PO_4 and SSCT, but not for Tris. This nonlinearity can be interpreted as the saturation of an indirect effect agent; thus doses used for PO_4, 0.85 MR, and for SSCT, 2.15 MR, are at values that can be considered in the region where a concentration study would be valid. Alternative explanations for the nonlinear response, such as radiation destruction as well as creation of an active agent (scheme 3), also result in the same conclusion. The

Tris data can be interpreted as exhibiting no indirect effect, or an indirect effect that has not been saturated at the doses used in this study. Thus, it seems reasonable to conclude that indirect radiation action can damage the DNA in PO_4, in SSCT, and possibly in Tris, under the conditions used in this study.

The single strand dose and concentration data show that the presence of PO_4 considerably enhances the production of ssb and that this effect is principally an indirect one. The values of the final slopes in Figure 2 for dose dependence, which are the k_D values found by scheme 3, are 0.89 ssb/MR/10^6 daltons in Tris, 1.13 in SSCT, and 5.2 in PO_4. The difference between these values could be caused by a larger diffusion radius for particular solvent radicals or by a specific interaction between DNA and one or more solvent components or by different energy transfer processes. Another possibility is that in PO_4 alkali-labile bonds are produced which result in ssb at the pH used to measure the single strand sedimentation coefficients. The effect of this would be to increase the value of k_D. Freifelder has shown that such lesions occur in DNA-dye complexes exposed to UV (29), but suggests that they don't occur for x-irradiated DNA in PO_4 (28). However, Rhaese and Freese (66) have found that OH attack does produce lesions in DNA which result in ssb upon exposure to high pH. Without more data, further discussion of k_D is not warranted.

The greater rate of ssb production at low doses may be caused by the presence of a solvent component in limited concentration. Thus, oxygen, which is present, may account for part or all of this

rate increase. Although several authors have found no oxygen dependence for chain scissions (28, 64), it has been suggested (77) that oxygen could combine with $\dot{H}$ and e^-_{aq}, species which would normally combine with $\dot{O}H$, thus allowing a greater percentage of $\dot{O}H$ to react with the DNA. Then, upon depletion of the oxygen, these radicals would compete with the DNA for $\dot{O}H$. The principal objection to this scheme is that $\dot{H}$ and e^-_{aq} are also capable of damaging the DNA to an extent which varies with the DNA concentration (14). Whatever the explanation for the non-linear dose responses in PO_4 and SSCT, the linear response in Tris, irradiated under the same conditions, must also be considered. If Tris provides a protecting environment, as suggested by Pollard and Weller (64), then it might compete with oxygen for the $\dot{H}$ and e^-_{aq} species, allowing a linear response for dose and concentration dependence in the ranges studied. Since Tris is also a component of SSCT, at one half the concentration of the Tris solvent, the slight curvature in the SSCT data may be the result of oxygen depletion, which allows the $\dot{H}$ and e^-_{aq} a better chance to compete with DNA for the $\dot{O}H$. Other explanations can be devised for these data, but without further data from less complex systems, it would be just speculation to suggest roles for the various components. But in spite of these difficulties, this data can be compared with the results of other workers.

Taylor and Ginoza (83), who irradiated frozen aqueous Tris solutions of RF-DNA from Øx174, have calculated the production of ssb/MR/10^6 daltons by a method completely different from that used

here. Their value, 1.17, agrees reasonably well with 0.89, found in this study.

Freifelder (28), working at room temperatures with free DNA in PO_4, found a value of 330 ssb/MR/10^6 daltons, which is considerably higher than our value of 5.2 in PO_4. Although his dose response for single strand breaks was linear, he did not carry his experiments beyond approximately 0.3 ssb/10^6 daltons, which, based on the results of this study (Figure 2), would not have been enough degradation to demonstrate a nonlinear dose response similar to the one found here for PO_4. Our initial slope has a value of 31 ssb/MR/10^6 daltons, which is still far below Freifelder's value. It appears that this lack of agreement represents the influence of the different physical and thermal conditions used in the two studies. Freifelder finds no oxygen dependence for ssb, which suggests either that oxygen is not the species causing the nonlinearity in this study or that oxygen reacts differently in frozen solutions.

Hagen's study (36) of irradiated calf thymus DNA in NaCl adjusted to pH 7.0 has some confusing results. When his value for the probability of a ssb per link per rad is converted to the units used in this study, with the assumption that the number of links refers to a number average molecule, the value is 1340 ssb/MR/10^6 daltons. This is four times the rate cited by Freifelder, and may reflect differences in the solvents. However, this value was obtained by using Hagen's number average calculations. If instead his weight average values are analyzed by the methods used in this

study, with the assumption that the initial molecular weight distribution is as stated, a value of 446 is calculated, a value much closer to the 330 found by Freifelder. Another result also appears, nonlinear dose response. A closer examination of the relation between the weight average and number average molecular weights for irradiated samples reveals that the molecular weight distribution of the sample does not approach the normal distribution for random scissions, $\beta = 1$. Instead, it seems to approach a limit of 0.28 for both single and double strand DNA, which means that the distribution is becoming very broad. However, a look at Hagen's distribution of sedimentation coefficients shows that they are becoming narrower as the dose increases. This conflict can be resolved by assuming that nonrandom scissions are being produced or that the calculation of either the weight average or number average molecular weight is in error. The closer agreement with Freifelder for the rate of production of ssb when determined from the weight average data, and the appearance of a nonlinear dose response, similar to the one observed for SSC in this study, suggest that the last explanation may be best.

Double Strand Breaks

Let us now consider the double strand break data in Figure 5. A general description of the data is that dsb appear to increase with the square of the dose as shown by Thomas (85) for pancreatic DNAase treatment of double strand DNA. His equation can be written for small numbers of breaks as:

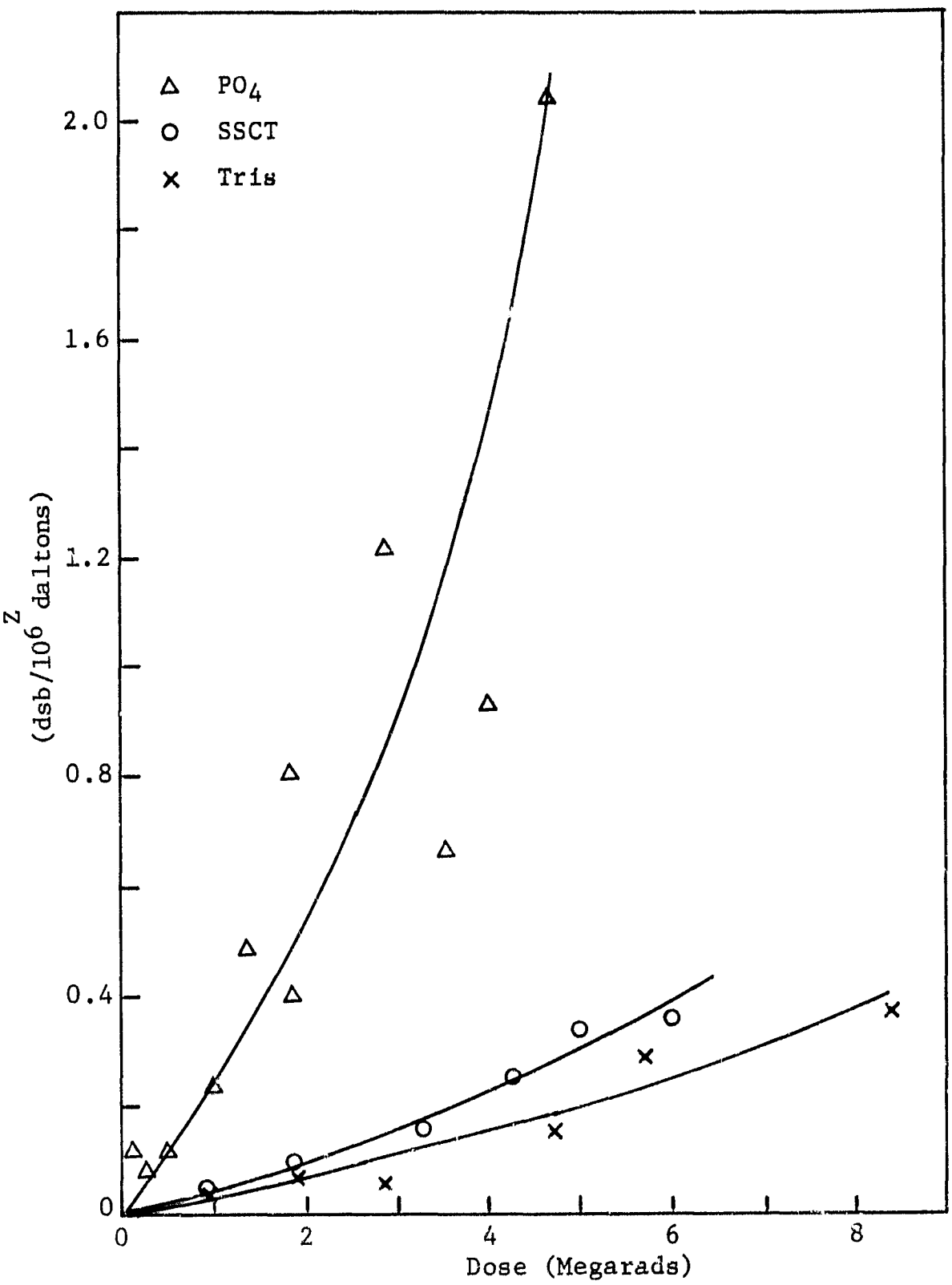

Figure 5. Double Strand Breaks versus Dose. Data is for Calf Thymus DNA #1, irradiated at 100 µg/ml.

$$\frac{2\ (dsb)}{L} = B\ \frac{k_1 D}{L}\ \ (\frac{k_1 D}{L} + \frac{A}{L})$$

where L is the number of links per 10^6 daltons,

A is the number of pre-existing ssb per 10^6 daltons,

dsb is the number of double strand breaks per 10^6 daltons,

k_1 is the number of links broken per 10^6 daltons per Mrad,

D is the irradiation dose in Mrads, and

B is the number of links between ssb on opposite strands that result in a dsb.

Before plotting dsb versus D^2, the term A/L will be considered. The average number of pre-existing ssb per double strand molecule is 0.32 for Tris, 0.64 for SSCT, and 0.89 for PO_4. These values were calculated using the equation:

$$\text{ssb per ds molecule} = (dsM_n)/[2(ssM_n)] - 1$$

where dsM_n is the double strand number average molecular weight, and

ssM_n is the single strand number average molecular weight.

The number of pre-existing ssb per 10^5 daltons is then 0.0175 for Tris, 0.0401 for SSCT, and 0.0661 for PO_4. When $k_1 D$ is 1.0, the contribution to dsb from $k_1 DA$ is only 2% for Tris, 4% for SSCT, and 6% for PO_4; thus, this term is negligible for doses greater than 1 Mrad.

The double strand break data is plotted versus dose squared for Tris and SSCT in Figure 6, and for PO_4 in Figure 7. The nonlinear response for all three solvents continues beyond 1 Mrad and resembles the ssb data; this behavior may be a result of the nonlinear response of ssb versus dose. In the equation cited at the beginning of this section, which described dsb as a function of ssb, k_1D was assumed to be equal to ssb. Since this has been demonstrated to be untrue, the dsb equation can be corrected for nonlinear ssb dose dependence by replacing k_1D with ssb read from the line fitted to the ssb data at the dose being considered. Figure 8 shows dsb for PO_4 plotted as a function of ssb squared, according to the equation:

$$dsb = \frac{B}{2L}\left[(ssb)^2 + (ssb)A\right]$$

where ssb is the number of ssb per 10^6 daltons and other symbols are as explained above. The best line through the data appears to be a straight line through the origin, although the data are quite scattered. The number of ssb at the lowest dose used in this study is 3.4, which makes (ssb)A equal to 2% of $(ssb)^2$. If B is the same for both terms, then the value of B is 12, only slightly larger than values cited by other workers; Thomas (85), using enzyme degradation, found B = 5, Hagen (36), using sedimentation data, found B = 7, and Peacocke and Preston (58, 59), using light scattering, found B = 9. This agreement of B with other published values suggests that the irradiated DNA was not exposed during handling to high shear forces that would enhance the number of dsb. When the data for Tris and SSCT are plotted as a function of ssb squared,

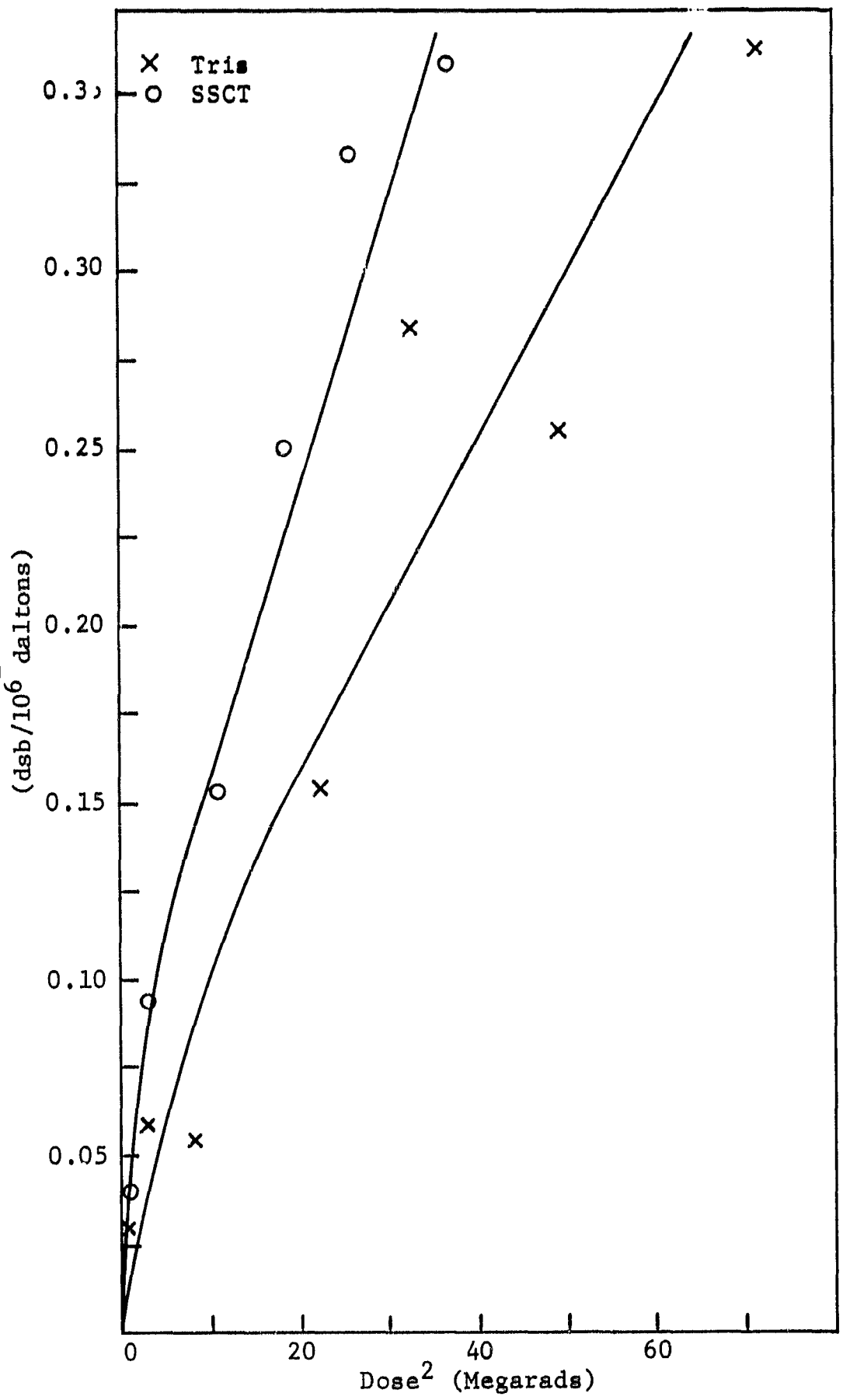

Figure 6. Double Strand Breaks versus Dose Squared. Tris and SSCT. C.T. DNA at a concentration of 100 µg/ml.

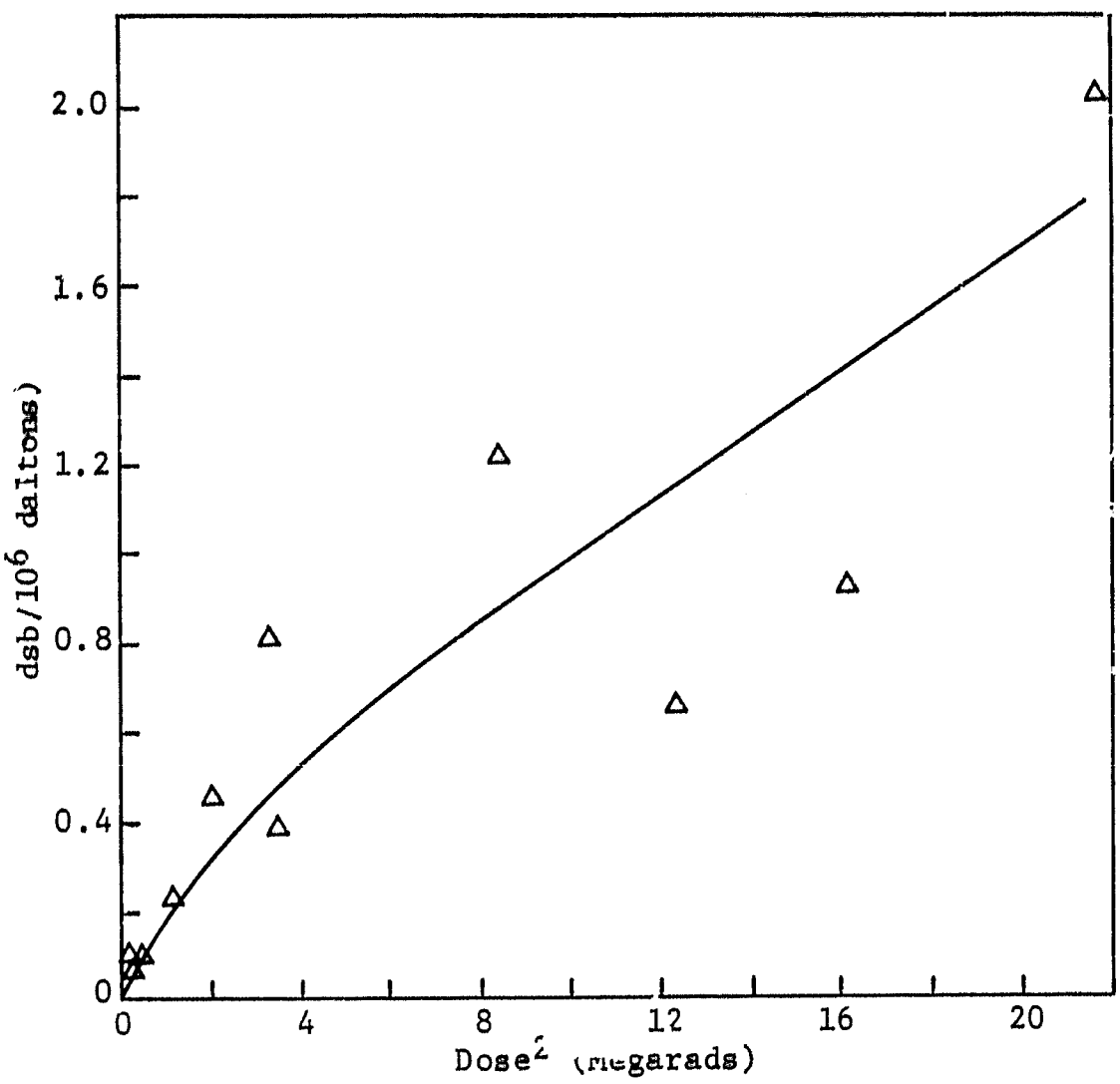

Figure 7. Double Strand Breaks versus Dose Squared. PO_4. C.T. DNA at a concentration of 100 µg/ml.

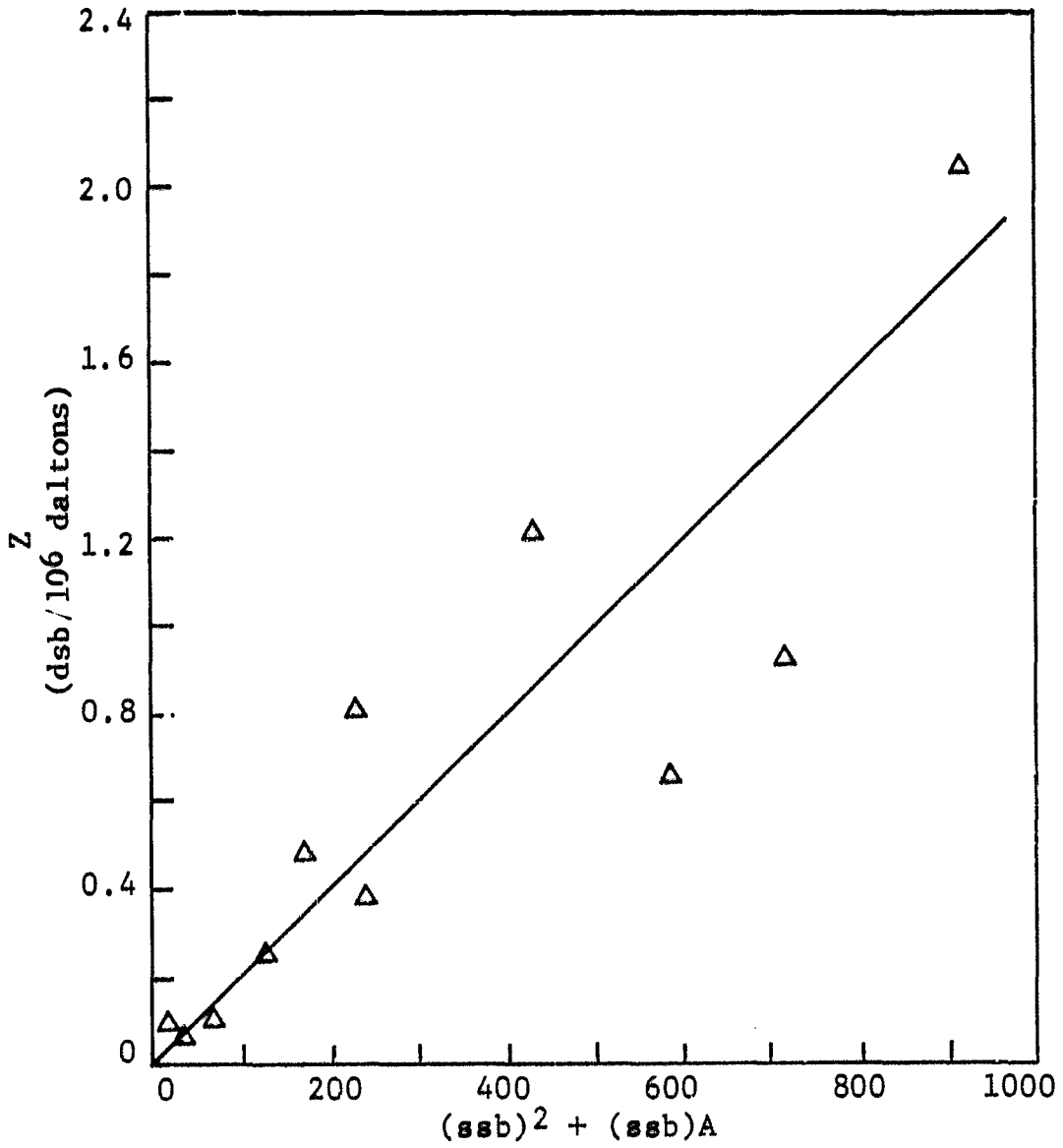

Figure 8. Double Strand Breaks versus a Function of Single Strand Breaks Squared. PO_4. C.T. DNA is at a concentration of 100 µg/ml and A represents the number of pre-existing single strand breaks per 10^6 daltons

Figure 9, straight lines can be drawn through the data points for both solvents, but they do not pass through the origin. Here again, the effect of the pre-existing single strand breaks is negligible. The slope of the linear portions of these curves gives B = 42 for Tris, and B = 38 for SSCT. If the lines are drawn through the origin, as would be expected, the B values increase at low doses. These values are approximately four times larger than those cited by other authors and might be explained by assuming that these samples had been exposed to higher shear forces or higher temperatures than the samples in PO_4. This is not the case, however, since all samples were handled at room temperature and in the same manner. The major stresses occurred when the samples were transferred to the centrifuge cell in a partially filled #18 needle. Based on previous work (20), it does not appear that this size molecule would experience sufficient stresses under these conditions to cause molecular scissions. The fact that B = 12 for PO_4, confirms this suggestion.

One possible explanation for the high B values for Tris and SSCT is that ssb are produced nonrandomly. Since tris is used as a buffer in SSCT, as well as being the only component in Tris, either tris or a contaminant in tris might cause sensitization of a section of DNA opposite a ssb. This suggestion is just conjecture; however, Douglas Vizard of this laboratory (89), using biologically active ØX174 DNA, has found that the kinetics of radiation-induced biological inactivation in frozen solutions are similar for Tris and sucrose (sucrose is known to be affected by radiation (72)) but considerably different than that observed for distilled water.

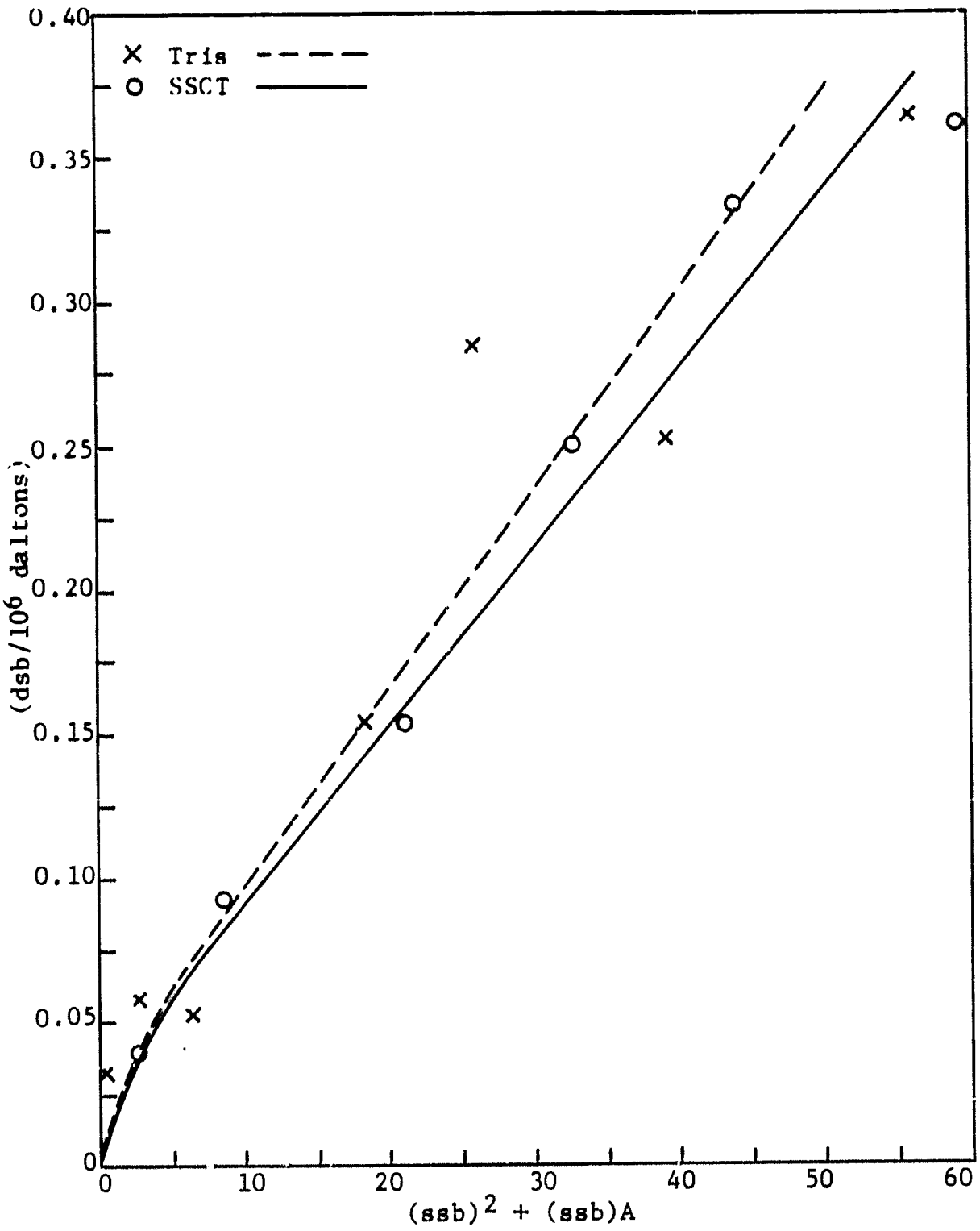

Figure 9. Double Strand Breaks versus a Function of Single Strand Breaks Squared. Tris and SSC. C.T. DNA is at a concentration of 100 µg/ml and A represents the number of pre-existing single strand breaks per 10^6 daltons.

This result suggests that tris does modify radiation damage in frozen solutions. Pollard and Weller (64) found that in liquid solutions tris protects from radiation damage compared to acetate buffer. Thus there is enough experimental evidence to suggest that tris is a radiation-active component in the DNA environment. The role oxygen may have in the initial rate at low doses can not be determined by the experimental results presented here.

It is also possible that the conditions used to examine the samples after irradiation can alter the initial radiation damage. If alkali-labile bonds are created by radiation, they could be broken when exposed to the pH 12.6 centrifuge solution, which is used to produce single strand molecules. The radiation-induced formation of labile phosphate groups was mentioned as early as 1956 (19), and used to interpret the viscosity "after effect" (70). As noted above, Freifelder found no evidence for alkali-labile damage in DNA exposed to x-rays (27), but there is evidence that OH radical attack can produce such a lesion (66). If alkali-labile bonds, which result in ssb at pH 12.6, were produced in this study, they would be experimentally indistinguishable from the ssb that are actually present at pH 7.4. Thus, the calculated value of B, the number of bonds between two ssb on opposite strands which result in a dsb, would be smaller than it, in fact, should be. Any correction for the effect of alkali-labile bonds would increase the value of B, and the problem of explaining such a large value for B would still remain.

One further possibility that will be duscussed here is that dsb can be produced directly, that is, linearly with radiation dose in addition to those produced by coincident singles. The double strand break equation would then be:

$$dsb = \frac{B}{2L}[(ssb)^2 + (ssb)A] + k_2 D$$

where k_2 is the number of dsb per 10^6 daltons per Mrad, and all other symbols are as described above. To examine this possibility, the data are plotted as dsb/D versus $[(ssb)^2 + (ssb)A]/D$, where the intercept gives k_2 and the slope gives B times a constant. Figure 10 displays the Tris and SSCT data according to this equation. The data are scattered but the slope and intercept, determined by the method of least squares, give B = 1.63±9.7 S.D. in Tris, and 15.8±4.4 S.D. in SSCT, and k_2 = (2.5±0.7 S.D.)$\times 10^{-12}$ in Tris, and (3.8±0.5 S.D.) $\times 10^{-2}$ in SSCT. The possibility that dsb occur linearly with dose has been suggested by Freifelder (27) to explain his B value of approximately 100, but he gives no estimate of a production rate. Taylor and Ginoza (83), who used the same irradiation conditions as were employed in this study, cite values of 4 ssb/molecule/Mrad and 0.076 dsb/molecule/Mrad in their work with the RF of ØX174 DNA. Using the above equation with B = 10, $k_2 = 2.0 \times 10^{-2}$ dsb/10^6 daltons/Mrad, which is consistent with the data of this study. An estimate of the target diameter for a cylindrical sensitive volume can be obtained by using Lea's graph of D_{37} versus spherical target diameter for gamma rays (47) and then performing the necessary algebraic conversion to a cylindrical volume. If Lea's relationship

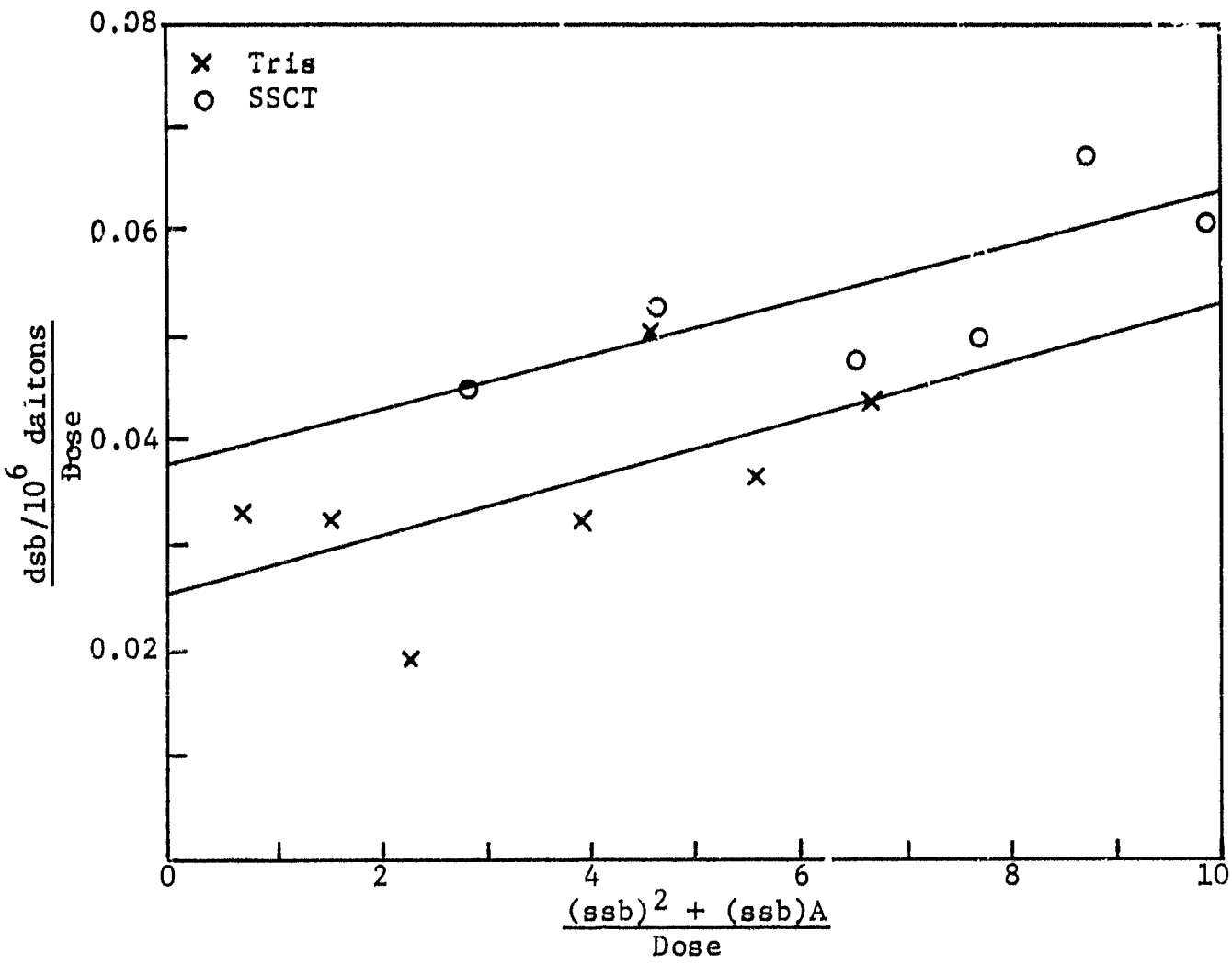

Figure 10. Function of Double Strand Breaks versus a Function of Single Strand Breaks Squared. Tris and SSCT. C.T. DNA is at a concentration of 100 μg/ml, dose is in megarads, and A represents the number of pre-existing single strand breaks per 10^6 daltons.

is correct for cylindrical targets, the diameter in Angstroms of the sensitive core of the native double strand DNA, in which a hit results in a double strand break, is, for this study, 2.7 in Tris and 3.3 in SSCT. For Taylor and Ginoza (83), the sensitive diameter in Tris is 2.5 Angstroms. These values should be considered as rough approximations because of the scatter in the data for this study and the small number of points cited for Taylor and Ginoza's work. The results do suggest that for cobalt 60 gamma rays a hit inside a small core (about 3 Å diameter) of double strand DNA can result in a dsb.

The data from PO_4, when plotted according to the above equation, are too scattered to allow an analysis. It is possible that the large number of indirectly produced ssb in PO_4 result in enough dsb to obscure the directly produced dsb, which would explain the straight line through the origin in Figure 8. However, more work must be done before this explanation can be accepted or rejected.

The last possibility that will be considered is that B for pre-existing single strand breaks is larger than B for radiation-induced ssb. The data would be expected to show a faster rise at low doses, corresponding to the larger B, and then curve over to a line describing the smaller B associated with solely radiation-induced ssb. Although the Tris and SSCT do display this type of behavior, the data do not show the effect that would be expected for the 100% more pre-existing ssb in SSCT than in Tris. In addition, the large B value for radiation-induced ssb is still not

explained, nor is the fact that PO_4 does not follow this pattern. Since no other evidence has been found to support this hypothesis, it will be assumed that B is the same for both types of ssb.

Of course, it is possible that some combination of the above explanations might best describe the experimental results. Unfortunately, the data are too scattered to allow any assignment of specific explanations, although the directly produced dsb hypothesis is most attractive from a theoretical point of view.

The following conclusions can be made from the results of this study:

1. Solvents do modify the effects of radiation on frozen aqueous DNA solutions. This modification may be a change in the energy transfer processes during or after irradiation at 77°K, or a change in the processes associated with the neutralization of activated species at 77°K or upon warming to room temperature.
2. In these experiments, the linear portion of the dose response curve gives a rate of 0.89 ssb/MR/10^6 daltons in Tris, 1.1 in SSCT, and 5.2 in PO_4. Thus some process in the phosphate buffer system appears to enhance the effects of radiation when compared to Tris or SSCT.
3. The magnitude of the ssb production rates in frozen solutions is approximately 100 times less than the rates cited for liquid solutions of DNA. This demonstrates that in frozen solutions the indirect effect is greatly reduced. The nonlinearity in the production

of ssb with dose in SSCT and PO_4, and the reduction in ssb per molecule at increased DNA concentrations, show that the indirect effect is present. This nonlinearity may be caused by the presence of dissolved oxygen, but if it is, then the Tris results suggest that the effect of oxygen can be modified.

4. The production of dsb in general goes as ssb squared. However, to fit the data this relationship requires a large value for the distance between ssb on opposite strands that would result in a dsb. This distance can be reduced by assuming either that ssb are not produced randomly or that dsb can also be produced directly, that is, linearly with dose. If double strand breaks do occur directly, the data predict a sensitive core approximately 3 Angstroms in diameter in the DNA molecule, in which a radiation hit would result in a double strand break.

SUMMARY

Calf Thymus DNA was prepared in three solvents and irradiated at 77°K for a series of doses and concentrations with cobalt 60 gamma rays. Alkali and neutral band sedimentation was used to obtain initial molecular weight distributions and the molecular weight of both single and double strand samples at each dose. This information was then converted to the number of molecular scissions per molecule by an equation for random scissions.

The data were analyzed in terms of direct and indirect radiation effects and the results were compared with those of other workers. The damage to DNA irradiated at 77°K and warmed to room temperature for analysis was enhanced when phosphate was present; this enhancement was attributed, in part, to indirect action. The rate of single strand break production was approximately 100 times less than the corresponding rate reported for irradiation of liquid solutions, which suggests that the indirect action is greatly reduced in frozen solutions. The rates, in terms of single strand breaks per 10^6 daltons per megarad, were 0.9 in a tris solvent, 1.1 in a modified SSC solvent, and 5.2 in a phosphate solvent. These differences were significant and demonstrated that solvent modification of radiation action did exist under the conditions used in this study.

Double strand breaks could be interpreted as resulting from two single strand breaks on opposite strands that are 16 or fewer base pairs apart, if the single strand breaks are produced nonrandomly, or if double strand breaks also occur directly, that is, linearly with dose. If double strand breaks do occur directly, the data predicts a sensitive core approximately 3 Angstroms in diameter in the DNA molecule, in which a radiation hit would result in a double strand break.

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APPENDIX A

Weight Average Molecular Weight Changes versus Single Strand Breaks

From Inokuti (43), we have the weight average degree of polymerization $P_w(\alpha)$ given by

$$P_w(\alpha) = \frac{2u}{x}\left\{1 - \frac{1}{x}\left[1 - (1 + \frac{x}{\beta})^{-\beta}\right]\right\} \quad ; \alpha = \frac{x}{u}$$

where x denotes the number of random scissions per number average degree of polymerization,

u denotes the initial number average degree of polymerization, and

β denotes a parameter expressing the broadness of the initial molecular size distribution, m(p,o), which has the following form:

$$m(p,o) = \frac{\beta^{\beta+1}}{u^2\Gamma(\beta+1)}\left(\frac{p}{u}\right)^{\beta-1} e^{-\frac{\beta p}{u}}$$

where p is the degree of polymerization.

This distribution is called a generalized poisson type.

For $\beta = 1$, the distribution is Poisson

$\beta > 1$, the distribution is narrow

$\beta < 1$, the distribution is broad

$\beta = \infty$, the distribution is uniform.

The weight average degree of polymerization is also given by

$$P_w = X_w = \sum_{x=1}^{\infty} x' W_x$$

where x' denotes the number of structural units in a given polymer, and

W_x denotes the weight fraction of x.

The weight average molecular weight is given by

$$M_w = m_o \sum_{x=1}^{\infty} x' W_x$$

where m_o denotes the monomer molecular weight.

Combining the two equations, we have

$$M_w = m_o P_w \qquad \text{or} \qquad M_w(\alpha) = m_o P_w(\alpha)$$

Now, considering the case of $\alpha = 0$, we have from Inokuti (43)

$$M_w(0) = m_o P_w(0) = m_o \left(1 + \frac{1}{\beta}\right) u$$

Solving for u,

$$u = \frac{M_w(0)}{m_o} \left(1 + \frac{1}{\beta}\right)^{-1}$$

Substituting this expression for u in the above equation for $M_w(\alpha)$, we get

$$M_w(x) = M_w(kx) = \frac{2M_w(0)}{[1 + (1/\beta)]x} \left\{1 - \frac{1}{x}\left[1 - \left(1 + \frac{x}{\beta}\right)^{-\beta}\right]\right\}$$

where $k = 1/u$, a constant.

The working equation is then

$$\frac{M_w(0)}{M_w(D)} = \left(1 + \frac{1}{\beta}\right) \frac{x^2}{2} \left[x - 1 + \left(1 + \frac{x}{\beta}\right)^{-\beta}\right]^{-1}$$

APPENDIX B

Derivation of Reaction Equations

Scheme #1

Single strand breaks are produced by direct action and, independently, by indirect action. The latter involves the interaction of DNA with a species, in limited supply, that has been activated by radiation. The reaction scheme is:

$$A \xrightarrow{k_o D} A^*;\ A^* + Y \xrightarrow{k_2'} Z;\ A^* + L \xrightarrow{k_1} X$$

where A is the parent species,

A* is the activated species,

Z is a product not resulting in a scission,

L is the number of links, and

X is the number of broken links. All these values are in moles/cc. The rate equations can now be written:

$$\frac{dz}{dt} = k_2' Y A^*;\ \frac{dx}{dt} = k_1 A^* L;\ \frac{dA^*}{dt} = - k_2' Y A^* - k_1 A^* L$$

Using the boundary condition, $L_o - X = L$, where L_o is the initial number of L, the chain rule, $\frac{dR}{dt} = \frac{dR}{dx} \cdot \frac{dx}{dt}$ and the assumption that dz/dt is a pseudo-first order reaction, where $k_2' Y = k_2$ the rate of change of A* with X can be written:

$$\frac{dA^*}{dx} = \frac{dA^*}{dt} \cdot \frac{dt}{dx} = \frac{- k_2 A^* - k_1 A^* (L_o - x)}{k_1 A^* (L_o - x)} = - 1 - \frac{k_2}{k_1} \frac{1}{(L_o - x)}$$

This differentiated equation is integrated to give:

$$A^* = \frac{k_2}{k_1} \ell n\ (L_o - x) - x + C$$

The two boundary conditions, $A^* = A_i^*$ at $x = 0$, and $A^* = 0$ at $x = x_f$, where f denotes the final value, give two different functions for C. Equating these two functions for C gives

$$A_i^* = X_f + \frac{k_2}{k_1} \ell n \left[\frac{1}{1 - \frac{X_f}{L_o}} \right]$$

The symbols are now changed to terms used for the observables in the experiments:

$$L_o = \frac{DNA}{m_o} \; ; \; X_f = X \frac{DNA}{M_n}$$

where DNA is the DNA concentration in gm/cc,

m_o is the nucleotide molecular weight in daltons,

M_n is the number average molecular weight in daltons,

and

X is the number of single strand breaks per molecule.

This equation is then:

$$A_i^* = X \frac{DNA}{M_n} + \frac{k_2}{k_1} \ell n \left[\frac{1}{1 - X \frac{m_o}{M_n}} \right]$$

Since $X \frac{m_o}{M_n}$ is always very much less than one in these experiments, $\left(1 - X \frac{m_o}{M_n}\right)^{-1}$ can be replaced by $\left(1 + X \frac{m_o}{M_n}\right)$ by selecting the first

two terms of the binominal expansion. Now, for $-1 < X \frac{m_o}{M_n} < 1$, a condition which is met in this study, $\ell n \left[1 + X \frac{m_o}{M_n}\right]$ can be replaced by $X \frac{m_o}{M_n}$, the first term in the expansion of the natural log. The equation then becomes:

$$A_i^* = X \frac{DNA}{M_n} + \frac{k_2}{k_1} X \frac{m_o}{M_n}$$

which can be solved for X and rewritten:

$$X = \frac{M_n}{\frac{k_2}{k_1} m_o + DNA} A_i^*$$

This equation describes the number of indirectly produced single strand breaks as a function of DNA concentration and of the indirect agent concentration.

The same relation can be obtained from Allen (5), by assuming that G_A is the number of A* produced by 100 ev, and all of A* disappear by reactions with either solute L or Y to give products P_L and P_Y. Then the probability that a radical will react with L is:

$$\frac{k_L [L]}{k_L [L] + k_y [Y]} = \frac{1}{1 + \frac{k_y [Y]}{k_L [L]}}$$

and the yield of P_L is

$$G(P_L) = \frac{G_A}{1 + \frac{k_y[Y]}{k_L[L]}}$$

This expression is in the form $x = \frac{A_i^*}{1 + \frac{k_2}{k_1} L_o}$ where, for $\frac{x}{L_o} << 1$, L equals L_o and k_2 is a pseudo-first order rate constant. When changed to terms of the observables, the equation becomes

$$X = \frac{M_n}{\frac{k_2}{k_1} m_o + DNA} - A_i^*$$

This equation must now be modified to satisfy the requirement that A_o, the concentration of the parent species, is limited. The amount of A_i^* is a function of dose, and can be described by the differential equation

$$\frac{d A_i^*}{dD} = k_o (A_o - A_i^*)$$

Solving this equation and converting the answer to the exponential form gives:

$$A_i^* = A_o [1 - \exp (- k_o D)]$$

The equation to describe single strand breaks per molecule as a function of dose and DNA concentration then is:

$$X = k_D D + \frac{M_n}{\frac{k_2}{k_1} n_o + DNA} \cdot A_o \left[1 - e^{-k_o D}\right]$$

Scheme #2a.

Single strand breaks are produced by direct action and, independently, by radiation-induced lesions that require the mediation of a solvent species which is in limited supply. The lesion can also react with some other solvent species not resulting in a scission. The reaction scheme is:

$$L \xrightarrow{k_o D} L^*;\ L^* \xrightarrow{k_2} Y;\ L^* + A \xrightarrow{k_1} X$$

where L is the number of links,

L* is the number of excited links,

Y is a product not involving a scission,

A is the concentration of the species in limited supply that mediates the scissions,

X is the number of single strand breaks, and

k_2 is a pseudo-first order rate constant; concentrations are in terms of moles/cc.

The rate equations can be written as

$$\frac{dY}{dD} = k_2 L^*;\ \frac{dx}{dD} = k_1 L^* A;\ \frac{dL^*}{dD} = \frac{dY}{dD} + \frac{dx}{dD}$$

It will be assumed that $L^* = k_o D(L - L^* - X)$ for the doses used in this study. It can be seen that

$$\frac{\frac{dL^*}{dD} - \frac{dx}{dD}}{\frac{dx}{dD}} = \frac{\frac{dY}{dD}}{\frac{dx}{dD}} = \frac{k_1 L^* A}{k_2 L^*} = \frac{k_1 A}{k_2}$$

Solving this expression for $\frac{dx}{dD}$ gives:

$$\frac{dx}{dD} = \frac{k_o DL}{\frac{k_2}{k_1 A} + 1}$$

Since A is in limited supply, it is replaced by the expression $(A_o - x)$ before integrating to give:

$$X = A_o \left\{ 1 - \exp \left[- \frac{k_1}{k_2} (L k_L D - X) \right] \right\}$$

This expression is then converted to the observables used in this study:

$$X = \frac{M_n}{DNA} A_o \left\{ 1 - \exp \left[- \frac{k_1}{k_2} DNA \left(\frac{k_L}{m_o} D - \frac{x}{M_n} \right) \right] \right\}$$

With the above conditions imposed on the derivation, the equation which results cannot be solved explicitly for x where x is the ssb produced by the indirect action of radiation. If these conditions are relaxed, the expression can be solved for x as presented in the following scheme.

Scheme #2b.

The scheme is the same as 2a except that the lesions react with the solvent species in limited supply until the species is

depleted before reacting with other species to produce a non-scission product. The rate equations are:

$$\frac{dx}{dD} = k_1 L^* A; \quad \frac{dL^*}{dD} = k_o(L_o - x) = k_o L_o$$

where $A = A_o - x$.

Integrating the expression for $\frac{dL^*}{dD}$ gives $L^* = k_o L_o D$ which then can be used to solve the expression for $\frac{dx}{dD}$. The solution is:

$$X = A_o \left[1 - \exp\left(-\frac{k_o k_1}{2} L_o D^2\right)\right]$$

which then can be converted to include the experimental observables:

$$X = \frac{M_n}{DNA} A_o \left[1 - \exp\left(-\frac{k_o k_1}{2} \frac{DNA}{m_o} D^2\right)\right]$$

Thus the final equation for ssb is:

$$X = k_D D + \frac{M_n}{DNA} A_o \left[1 - \exp\left(\frac{k_o k_1}{2} \frac{DNA}{m_o} D^2\right)\right]$$

Scheme #3

Single strand breaks are produced by direct action and, independently, by the interaction of DNA with a radiation-activated solvent species, which can be destroyed by radiation. The reaction scheme is:

$$B \xrightarrow{k_1' D} B^*; \quad B^* \xrightarrow{k_2' D} W; \quad B^* \xrightarrow{k_3} Y;$$

$$B^* + L \xrightarrow{k_1} X$$

where B is the solvent species,

W is the radiation-produced decay product of B*, and

Y is the non-radiation-produced decay product of B*;

neither product ultimately produces a strand scission.

The derivation of the basic equation is the same as that for Scheme #1. This gives for single strand breaks produced by indirect action:

$$X = \frac{M_n}{\frac{k_3}{k_1} m_o + DNA} B^*$$

The production and destruction of B* by radiation is described by:

$$B^* = \frac{k_1'}{k_2'} [1 - \exp (- k_2' D)]$$

The equation for single strand breaks then becomes:

$$X = k_D D + \frac{M_n}{\frac{k_3}{k_1} m_o + DNA} \frac{k_1'}{k_2'} [1 - \exp (- k_2' D]$$

Scheme #4.

Strand scissions are produced by three independent processes: direct action, interaction of DNA with a radiation activated solvent species, B*, and interaction of radiation-activated DNA with another solvent species, A, which is in limited supply. The reaction scheme is:

$$B \xrightarrow{k_4 D} B^*; \quad L \xrightarrow{k_6 D} L^*; \quad B^* \xrightarrow{k_3} Z;$$

$$B^* + L \xrightarrow{k_5} X; \quad L^* + A \xrightarrow{k_7} X; \quad L^* \xrightarrow{k_2} Y$$

Since these processes are independent, the equation of Scheme #2b can be used directly. The basic equation derived in Scheme #1, $X = \frac{M_n}{\frac{k_3}{k_5} m_o + DNA} B^*$, with B^* replaced by $k_4' D$, where $k_4' = k_4 B$, can also be used. The final equation is then:

$$X = k_D D + \frac{M_n}{\frac{k_3}{k_5} m_o + DNA} k_4' D + \frac{M_n}{DNA} A_o \left[1 - \exp\left(-\frac{k_o k_1}{2} \frac{DNA}{m_o} D^2\right)\right]$$

APPENDIX C

Parameter Values Calculated by Least Squares Fitting
of Data to Reaction Scheme #3

Solvent and Variable	$k_D \times 10^{-6}$	$\frac{k_3}{k_1} \times 10^{-6}$	$k_1' \times 10^{-16}$	$k_2' \times 10^{-6}$
Calf Thymus DNA				
Tris, Dose	0.9	---	---	---
SSCT, Dose	1.1	2.7	9.6	1.0
PO_4, Dose	5.2	2.0	56.0	5.8
Tris, Conc.	0.92	---	---	---
SSCT, Conc.	1.5	0.45	4.4	3.6
PO_4, Conc.	6.3	1.9	250	2.6

where the rate constants are as follows:

k_D is the rate of production of single strand breaks per 10^6 daltons per megarad, produced durectly by the interaction of DNA and radiation,

$\frac{k_3}{k_1}$ is the ratio of the rate of non-radiation-induced decay of an activated solvent species to the rate of reaction with DNA to produce a single strand break,

k_1' is the rate of radiation activation of a solvent species,

and k_2' is the rate of radiation induced decay of an activated solvent species.

APPENDIX D

Glossary

B	the number of links between ssb on opposite DNA strands that result in a dsb.
β	parameter describing a generalized Poisson distribution of molecular weights.
C.T. DNA	calf thymus DNA. In SSCT, #1 is unsheared DNA, #2 is sheared.
dsb	double strand break(s).
M_n	number average molecular weight.
M_w	weight average molecular weight.
PO_4	DNA solvent composed of 0.01 M PO_4 at pH 7.4.
ssb	single strand break(s).
SSCT	DNA solvent composed of 0.15 M NaCl, 0.015 M NaCitrate, and 0.005M Tris at pH 7.4.
$S_{w,20}$	sedimentation coefficient corrected to the value in water at 20°C.
Tris	DNA solvent composed of 0.01 M Tris, at pH 7.4 by HCl.
Z	number of chain breaks per 10^6 dalton molecule.

VITA

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