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Publication Date
1965-08-23

UCRL-16358

University of California

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Berkeley, California

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Workshop Conf. on Space Radiation
Biology - Berkeley

UCRL-16358

UNIVERSITY OF CALIFORNIA
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Berkeley, California

AEC Contract No. W-7405-eng-48

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INTRODUCTION

It is well known that free radicals are produced and trapped in solid biological systems when exposed to ionizing radiation. Enzymes represent an important and suitable group of molecules for studying certain aspects of radiation damage, since the formation and fate of free radicals, as revealed by electron spin resonance (ESR) spectroscopy, can be correlated to a biological damage such as the loss of enzymatic activity.

Previous work has shown that the initial ESR centers formed by the radiation react rapidly at room temperature (1-2). These reactions lead both to the disappearance of ESR centers and to the formation of some more stable radicals, which here will be called the "secondary radicals." Most ESR work so far has been concentrated on the determination of the types and yields of the secondary enzyme radicals, and very little is known about the formation and types of the initial ESR centers and their subsequent reactions. Furthermore, both the fate of the secondary radicals and their role in the final steps of the inactivation mechanism are largely unknown. In this work the three enzymes ribonuclease, lysozyme, and trypsin have been irradiated with different types of ionizing radiations. Efforts have been made to study the effect of temperature on the early secondary reactions and on the formation of enzyme radicals.

In a previous study on glycine and trypsin irradiated with helium, carbon, and argon ions, it was found that the irradiation temperature had a pronounced effect on the yield of secondary radicals (3). The results indicated that the temperature effect was more pronounced for radiation with low LET. This work therefore represents an extension of these studies because the enzymes have been irradiated with various types of radiations, including 6.5-MeV electrons, in the temperature range 77° to 330°K. In an attempt to elucidate the significance of the secondary radicals for the inactivation mechanism, the radical data are correlated with the inactivation results previously reported for the same three enzymes by Brustad (4) and Fluke (5).

EXPERIMENTAL PROCEDURE

The ESR spectrometer and the experimental procedure used to obtain the radical concentration have been described elsewhere (6). Since the enzyme spectra are very sensitive to microwave saturation, these experiments have been carried out at a low microwave power level. Furthermore, in order to resolve details in the high-field part of the spectra, the modulation amplitude was kept small.

Ribonuclease and trypsin were obtained from Nutritional Biochemical Corp. and lysozyme from Worthington Biochemical Corp. The enzymes were irradiated in the polycrystalline form in the variable-temperature irradiation apparatus shown in Fig. 1. This irradiation equipment can be connected directly to the exit port of the heavy-ion linear accelerator (Hilac), and represents a part of the "main Faraday chamber." The same apparatus, which in these experiments was used also for the electron irradiation, had previously been used in the inactivation experiments on trypsin reported by Brustad (4).

The enzymes were irradiated in thin layers in brass holders mounted on a metal plate. The temperature of this plate was changed by letting different

liquids or gases circulate inside the plate; liquid nitrogen, nitrogen gas, and hot water were used. The temperature of the metal plate was measured by a copper-constantan thermocouple. The temperature variation during exposure was negligible. After each exposure the temperature was lowered in steps of approximately 20°. The irradiation chamber was evacuated during irradiation.

After exposure the samples were brought to liquid nitrogen temperature, transferred to glass sample tubes, evacuated, and sealed. When not otherwise stated the ESR measurements were carried out at room temperature after the samples had been kept at 295° K for 20 to 30 minutes (3).

The samples were irradiated with 6.5-MeV electrons and fast stripped helium, carbon, and argon ions from the Berkeley Hilac. The quality and dosimetry of the radiation have been described previously (6). The dose was of the order of 1 to 3 megarad given at a dose rate of 1 megarad per minute. The radical concentration is given in relative units.

RESULTS AND DISCUSSION

Types of Enzyme Radicals

Ribonuclease, lysozyme, and trypsin are all relatively small enzyme molecules with a molecular weight of less than 25 000. They all contain the sulfur amino acids cysteine or cystine (or both), and when irradiated at room temperature they exhibit a composite ESR spectrum consisting of a broad resonance and a doublet, as shown for lysozyme in Fig. 2 (upper spectrum) (3). As previously pointed out (7), the broad resonance in the protein spectra has been ascribed to a sulfur radical in which the unpaired electron is localized mainly on the sulfur atom in the cysteine residue, whereas the doublet has been ascribed to a radical in which the unpaired electron is localized in a

π orbital on an α-carbon atom in the protein backbone (either a $\cdot\rangle$ CH or a $\cdot\rangle$ CR fragment). The support for this interpretation can briefly be summarized as follows (see also Fig. 2 and Table I):

1. When thiols and disulfides of the cysteine-cysteamine group are irradiated, the sulfur radical $RCH_2S\cdot$ is usually formed (8-9). The unpaired electron in this radical can interact with one of the two protons in the CH_2 group adjacent to sulfur with the result that a doublet, with a splitting of approximately 9 gauss is observed for single crystals (8). For polycrystalline substances the doublet is smeared out at room temperature, but can be detected when the observation temperature is below about 160° K (9). A similar doublet was also observed for the enzymes (the bottom spectra in Figs. 2 and 3) when the measurements were carried out at 77° K.

2. The sulfur radical $RCH_2S\cdot$ has a large anisotropy, which in polycrystalline samples gives rise to a broad resonance. The principal g values can be roughly determined by using Kneubühl's method (10), and in Table I the results for the sulfur radicals formed in some thiols and disulfides are given together with the data obtained for the broad resonances in the enzyme spectra. The good agreement between these results strongly supports the interpretation that sulfur radicals are formed in all the three enzymes here studied.

3. It appears from Table I that there is a good correlation between the g value for the doublet (g_D) in the enzyme spectra and the average g value for the doublet induced in irradiated dipeptides. This observation, together with the fact that the doublet splitting is also the same for the two types of substances, is the main evidence for the assumption that a radical of the same type is formed. Single crystals were used in the dipeptide studies, and it was concluded that a radical of the type $\cdot\rangle$ CH was formed (7).

When the three enzymes are irradiated at low temperatures and measured before any heat treatments, completely different spectra are observed.

In Fig. 3 the results for ribonuclease are given (upper spectrum). The spectra observed for trypsin (7) and lysozyme (3) show that there is some spectral variation from one enzyme to the other, although the main resonance consists of a broad single line, like that shown for ribonuclease, with a g value of approximately 2.006 to 2.008. The ESR centers giving rise to these spectra have not been identified, but they are assumed to be the precursors for the secondary radicals. Thus, when the samples are annealed at room temperature the resonance spectra change rapidly and the sulfur radicals and the doublet-type radicals are formed as shown in Fig. 3. The bottom spectrum in this figure indicates that a third radical is also formed. Thus, the main doublet is poorly resolved and a new resonance line appears on the high-field side (see the dashed curve). In order to study this resonance in more detail the observation temperature was changed to 295° K. In Fig. 4 it is clearly shown that a resonance is superimposed on the main protein doublet. Although it was not possible to identify the resonance, a few characteristics should be noted.

a. The resonance, which results in the characteristic splitting of about 5 gauss in the center of the main doublet, is spread out over approximately 70 gauss. Thus, the weak line on the low-field side of the doublet (the line located 11.3 gauss from one of the sulfur peaks) is a part of the resonance and has nothing to do with the temperature-dependent sulfur doublet discussed above.

b. The resonance (or resonances) consists of three or more lines and seems to be centered at a g value approximately that of the free electron.

c. As demonstrated by the three spectra in Fig. 4, the resonance is relatively unstable and disappears more rapidly than for the two other secondary radicals at room temperature. This implies that when the enzymes are irradiated at room temperature, or at higher temperatures, the resonance

is much weaker and has almost disappeared before the first measurement can be made. This may partly explain the failure to observe this resonance in previous studies.

d. The same resonance was also found for lysozyme and trypsin, although it was much weaker for these enzymes. This observation suggests that the radicals responsible for this transient resonance may frequently be formed in irradiated proteins.

From the qualitative ESR studies it can be concluded that at least three different types of secondary radicals are formed when solid enzymes are exposed to ionizing particles. When the type of radiation was varied from 6.5-MeV electrons up to the densely ionizing argon ions no other types of secondary enzyme radicals were found. The relative importance of the radicals varies from one enzyme to another, and--for one particular enzyme--with the treatment of the sample before an actual measurement. For the three enzymes studied here, the backbone-type radical is the most important. However, the sulfur resonance gradually becomes more important upon heat treatment, or upon storage of the samples at room temperature. When the enzymes were irradiated with 6.5-MeV electrons in these experiments, it was found that sulfur radicals accounted for approximately 37% of the ribonuclease resonance, 30% of the lysozyme pattern, and about 16% of the trypsin spectrum. The results also seem to indicate that the relative percentage of the sulfur resonance decreases slightly with increasing stopping power of the radiation (6).

The Yield of Enzyme Radicals

The radical concentration was obtained from the integrated spectra by comparison with reference samples measured under the same conditions. The yields of secondary radicals depend upon the stopping power of the radiation.

Thus, it was found, for a number of different substances, that the yields were constant up to about 200 MeV g^{-1} cm^2, whereas above this level the yields decreased with increasing stopping power. The radical yield varied by a factor of from 2 to 5 for the different substances over the range of LET studied (6).

In Fig. 5 the yield of secondary radicals produced in trypsin irradiated with 6.5-MeV electrons and fast stripped argon ions is given as a function of the irradiation temperature. For both types of radiation the radical yield increases with the irradiation temperature above approximately 100° to 120° K. It appears that the effect of the irradiation temperature is larger for radiation with small stopping power. This observation has previously been made for glycine (3), and similar results were also obtained for lysozyme, as shown in Fig. 6. Thus, the relative increase in radical yield as a function of irradiation temperature over the yield observed at 77° K is higher for electron irradiation than for helium and carbon ions.

One possible explanation for the variation in the temperature effect with the stopping power may be suggested by assuming that the radicals formed along the track of the ionizing particle is the sum of those formed in the track core and those produced by the δ rays. It is reasonable to expect that the radical production by the δ rays would exhibit the same temperature dependence as that for electron irradiation. In the track core, however, the situation may be different. Norman and Spiegler (11) use the expression "thermal spike" to indicate that the "temperature" in the track core (if a concept such as temperature can be used for these small regions) initially is much higher than the ambient temperature. If we adopt this view it is reasonable to assume that some of the secondary reactions in the track core take place at a temperature, and a rate, higher than that characteristic for the

δ rays (see below). Consequently, for the heavy ions we probably have a mixed situation in which the radical production by the δ rays depends upon the ambient temperature more than does the radical production in the track core. This would imply that the sample temperature during irradiation would be less important the larger the stopping power of the radiation, i.e., less important for argon ions than electrons, etc.

In the above discussion it was assumed that the variation in radical yield with the irradiation temperature could be ascribed to secondary reactions that take place, to a greater or lesser extent, during irradiation. In an attempt to get some information about these processes the radical yield versus the irradiation temperature was plotted in so-called Arrhenius plots. In Fig. 7 the results for the two enzymes ribonuclease and lysozyme, irradiated with 6.5-MeV electrons, are presented. In this semilogarithmic plot the experimental data may be fitted by a curved line which can be described mathematically by a sum of exponential functions, i.e., straight lines in this plot. It appears that the lysozyme data can be adequately fitted by the sum of the three lines marked 1, 2, and 3, whereas for the ribonuclease only the two lines, 1 and 2, are necessary. The slopes of these lines can easily be determined. One possible interpretation, which emerges from the Arrhenius plots, is to assume that two (or three) processes are responsible for the radical production and that the activation energies for these processes are given by the slopes of the straight lines (see Table II). According to this interpretation the temperature-independent process (the lines marked 1 in Fig. 7) is the most important one up to temperatures of about 295° K. Because ESR measurements have been carried out only up to 330° K (at higher temperatures the decay of secondary radicals becomes too serious), most of the radical data can be adequately fitted by a sum of only two straight lines. This would indicate that the secondary radicals are mainly formed by two processes with

activation energies 0 and approximately 1 kcal/mole. As shown in Table II, the apparent activation energies for radical production are almost the same for all three enzymes here studied.

It was pointed out in a previous paper (3) that the above interpretation was questionable. Another explanation was proposed in which the temperature-independent parts of the curves in Fig. 7 (i.e., temperatures below 100° K, or above 10 in this figure) were neglected. The reason for this may be somewhat speculative, and involves the above-mentioned thermal spike model. Thus, absorption of radiation energy leads to a transient "localized heating" of small regions of the sample. For example; Ingalls et al. (12), using ^{60}Co γ rays, estimated the temperature in the "hot spots" to be several hundred degrees above the ambient temperature in a time interval less than 10^{-10} sec. This would imply that some of the secondary reactions take place, partly or completely, in the hot spots before they become thermalized. The "effective temperature" for the secondary reactions may be somewhat between the initial temperature in the hot spots and the average sample temperature. This is the reason for assuming that approximately 100° K is the lowest effective temperature attainable for the secondary reactions. If the results below 100° K are neglected the ribonuclease data can be adequately fitted by a sum of the two lines I and II (Fig. 7). Consequently, another interpretation of the radical data is to assume that they are produced by two processes with activation energies about 100 cal/mole and 2 kcal/mole (see Table II).

Brustad (4) and Fluke (5) have shown that the inactivation of the three enzymes here studied depends upon the irradiation temperature in a fashion similar to that demonstrated for the radical production. If the inactivation data are plotted in Arrhenius plots the same two types of interpretations can

be applied. In Table II the apparent activation energies observed from these plots are presented. Since in the inactivation experiments temperatures up to 440° K were used, a sum of three straight lines seems to give the best fit to the experimental data.

There appears to be a good correlation between the inactivation data and the radical data, independent of the way the temperature curves are analyzed. The only discrepancy is in the results obtained for lysozyme irradiated with electrons. Fluke's inactivation data for this enzyme resulted in much smaller activation energies than those found for ribonuclease and trypsin. The good correlation obtained makes it reasonable to suggest that the same processes are responsible for the formation of secondary radicals as well as for the loss of enzymatic activity. It may be that the secondary radicals somehow are connected to the final steps in the inactivation mechanism (3). However, nothing is known so far about which one of the three types of secondary radicals discussed above may be important for the loss of the enzymatic activity. Furthermore, it is not known to what extent the inactivation depends upon the free-radical formation. If the formation of a certain type of secondary radical subsequently leads to inactivation, it is also of interest to know how many radicals are necessary in order to inactivate one macromolecule.

The results presented here, as well as previous ESR experiments (1-3), have shown that the radicals we observe at room temperature (here called the secondary radicals) are the result of a sequence of events initiated by the energy absorption. The reactions in this sequence have relatively small activation energies in the range zero to about 7 kcal/mole. Unfortunately, little is known about the nature of these processes, because the ESR centers formed prior to the secondary radicals have not been identified. The formation of these ESR centers and their interactions in subsequent heat treatments are

currently being studied by irradiating polycrystalline samples and single crystals at liquid helium temperatures.

SUMMARY

The free radicals produced in the three enzymes ribonuclease, lysozyme, and trypsin, exposed to various types of ionizing radiations, have been studied by ESR spectroscopy. The enzymes were irradiated in the solid state, in vacuum, at different temperatures in the range 77° to 330° K.

For all three enzymes it was found that the resonance spectra at room temperature can be ascribed mainly to sulfur radicals and to a radical in which the unpaired electron is localized on an α-carbon atom in the protein backbone. However, for all three enzymes another unidentified resonance was found. The resonance is spread out over about 70 gauss, is centered at a g value approximately that of the free electron, and is more unstable than the two other secondary radicals.

The yield of secondary enzyme radicals increases with increasing irradiation temperature. The temperature effect depends upon the type of radiation, and is larger when the stopping power is the smaller. A plot of the radical yield versus the irradiation temperature can be described by a sum of exponential functions. One possible interpretation is that the secondary enzyme radicals are produced by several processes with activation energies from zero to approximately 7 kcal/mole. A good correlation was found between the production of secondary radicals and the inactivation of the three enzymes.

ACKNOWLEDGMENTS

I wish to express my gratitude to Professor C. A. Tobias and his group for the hospitality and working facilities offered me at Donner Laboratory.

It is a pleasure to acknowledge financial support from the U. S. Atomic Energy Commission and the National Aeronautics and Space Administration.

Table I. Observed g values for the enzyme spectra.[a]

Compound	g_1	g_2	g_3	g_D	Reference
Ribonuclease	-	2.024	2.056	2.0029	This work
Trypsin	-	2.022	2.055	2.0028	This work
Lysozyme	-	2.025	2.056	2.0030	This work
Cysteine	2.006	2.025	2.052	-	Henriksen (9)
Cysteamine	2.005	2.027	2.054	-	Henriksen (9)
Penicillamine	2.004	2.026	2.053	-	Henriksen (9)
Cystine, single crystal	2.003	2.025	2.053	-	Kurita and Gordy (8)
N -acetylglycine	-	-	-	2.0034	Miyagawa et al. (13)
Glycylglycine	-	-	-	2.0032	Katayama and Gordy (14)
Glycylglycine·HCl	-	-	-	2.0029	Box et al. (15)

a. The enzymes and the three thiols were studied in the polycrystalline state. According to Kneubühl (10), the principal g values correspond to maxima and zero point in the derivative curve. It was not possible to determine the g_1 value for the enzymes, since the high-field part of the sulfur resonance is masked by the doublet. The doublet, and the unidentified resonance, will also to a certain extent influence the determination of g_2. The g_D corresponds to the center of the doublet in the enzyme spectra. A small anisotropy was found for the dipeptides, which were studied as single crystals, and the values quoted in this table is the average of the principal g values.

Table II. Apparent activation energies for the processes leading to the formation of secondary enzyme radicals and the loss of enzymatic activity. [a]

Enzyme	Type of radiation	Type of measurement	E_1	E_2	E_3	E_I	E_{II}	Reference
			cal/mole					
Ribonuclease	Electrons	Radicals	0	1100	---	90	1900	This work
Lysozyme	Electrons	Radicals	0	1200	6600	100	2400	This work
Lysozyme	^{4}He ions	Radicals	0	1000	5000	90	2600	Henriksen (3)
Lysozyme	^{12}C ions	Radicals	0	1000	4000	100	2150	Henriksen (3)
Trypsin	Electrons	Radicals	0	1450	---	120	1900	This work
Trypsin	^{4}He ions	Radicals	0	1050	---	50	1650	This work
Trypsin	^{12}C ions	Radicals	0	1000	3900	70	2100	Henriksen (3)
Trypsin	^{40}Ar ions	Radicals	0	1300	---	120	1700	Henriksen (3)
Ribonuclease	Electrons	Inactivation	0	1060	6100	---	---	Fluke (5)
Lysozyme	Electrons	Inactivation	0	620	2540	---	---	Fluke (5)
Trypsin	^{2}D ions	Inactivation	0	1350	4700	110	2200	Brustad (4)
Trypsin	^{4}He ions	Inactivation	0	1350	5200	110	2200	Brustad (4)
Trypsin	^{11}B ions	Inactivation	0	1100	3400	70	2300	Brustad (4)
Trypsin	^{12}C ions	Inactivation	0	1150	4200	70	2300	Brustad (4)
Trypsin	^{20}Ne ions	Inactivation	0	1050	3800	70	2200	Brustad (4)
Trypsin	^{40}Ar ions	Inactivation	0	1300	3700	70	2300	Brustad (4)

a. The radical data refer to measurements after the samples have been kept at room temperature for 20 to 30 minutes. Because the enzyme radicals become unstable at temperatures above room temperature the radical experiments have been carried out only up to 330° K, whereas the inactivation experiments go up to 440° K. This implies that the radical data very often can be adequately described by a sum of only two exponential curves. In those experiments for which three lines seem to give a better fit, the third line and its activation energy E_3, are however, very uncertain. The results were interpreted in two different ways (see text). The three values E_1, E_2, and E_3 refer to the first interpretation, where the full curves were fitted by a sum of either two or three straight lines, and the two last figures, E_I and E_{II}, refer to the high-temperature parts of the curves (above 100° K).

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FIGURE LEGENDS

Fig. 1. The variable-temperature irradiation apparatus. The irradiation chamber can be mounted directly to the exit port of the Hilac.

Fig. 2. The ESR spectrum of lysozyme, irradiated at room temperature, and measured at 295° K (upper spectrum) and 77° K respectively. The spectra presented in this paper represent the first derivative of the actual absorption spectra. The microwave frequency was measured by a wavemeter and the magnetic field by a proton resonance field meter. The principal g values for the sulfur radicals correspond to maxima and zero point in the derivative curve. g_D is the g value for the center of the doublet. The dashed curves represent measurements at high gain. The temperature-dependent hyperfine splitting of the sulfur radical $RCH_2S\cdot$ is easily observed in the bottom spectrum.

Fig. 3. The ESR spectrum of ribonuclease irradiated with 6.5-MeV electrons at 77° K. All spectra in this figure are recorded at 77° K. After the first measurement (upper spectrum) the sample was annealed at room temperature for the period of time indicated and then measured again at 77° K. The relative spectrometer sensitivity is given by the columns to the left.

Fig. 4. The ESR spectrum of ribonuclease irradiated at 77° K and measured at room temperature. The same sample as that used in Fig. 3 has been followed after more heat treatments at 295° K. In contrast to Fig. 3, all spectra were recorded at 295° K. A high gain was used in order to study the weak and unstable resonance superimposed on the high-field part of the spectrum.

Fig. 5. The yield of secondary radicals in trypsin as a function of the irradiation temperature. The enzyme was irradiated in vacuum with 6.5-MeV electrons and fast stripped argon ions. The samples were kept at room temperature for 20 to 30 minutes before the ESR measurements were carried out. The dose was the same for all samples irradiated with one particular type of radiation. The results are given in relative units and the average value of the data below 100° K was set equal to 1.0.

Fig. 6. The yield of secondary radicals in lysozyme irradiated at different temperatures with electrons and fast stripped helium and carbon ions. Otherwise as in Fig. 5.

Fig. 7. The yield of secondary radicals in ribonuclease and lysozyme, irradiated with electrons, as a function of the reciprocal of the irradiation temperature. Otherwise as in Fig. 5. The curves were decomposed into a sum of straight lines (see text). For ribonuclease two different types of interpretations have been applied. In the first place, the total curve was analyzed into a sum of the two lines marked 1 and 2. According to the other interpretation only the high-temperature part of the curve was analyzed into the two lines I and II. The slopes for these lines are given in Table II.

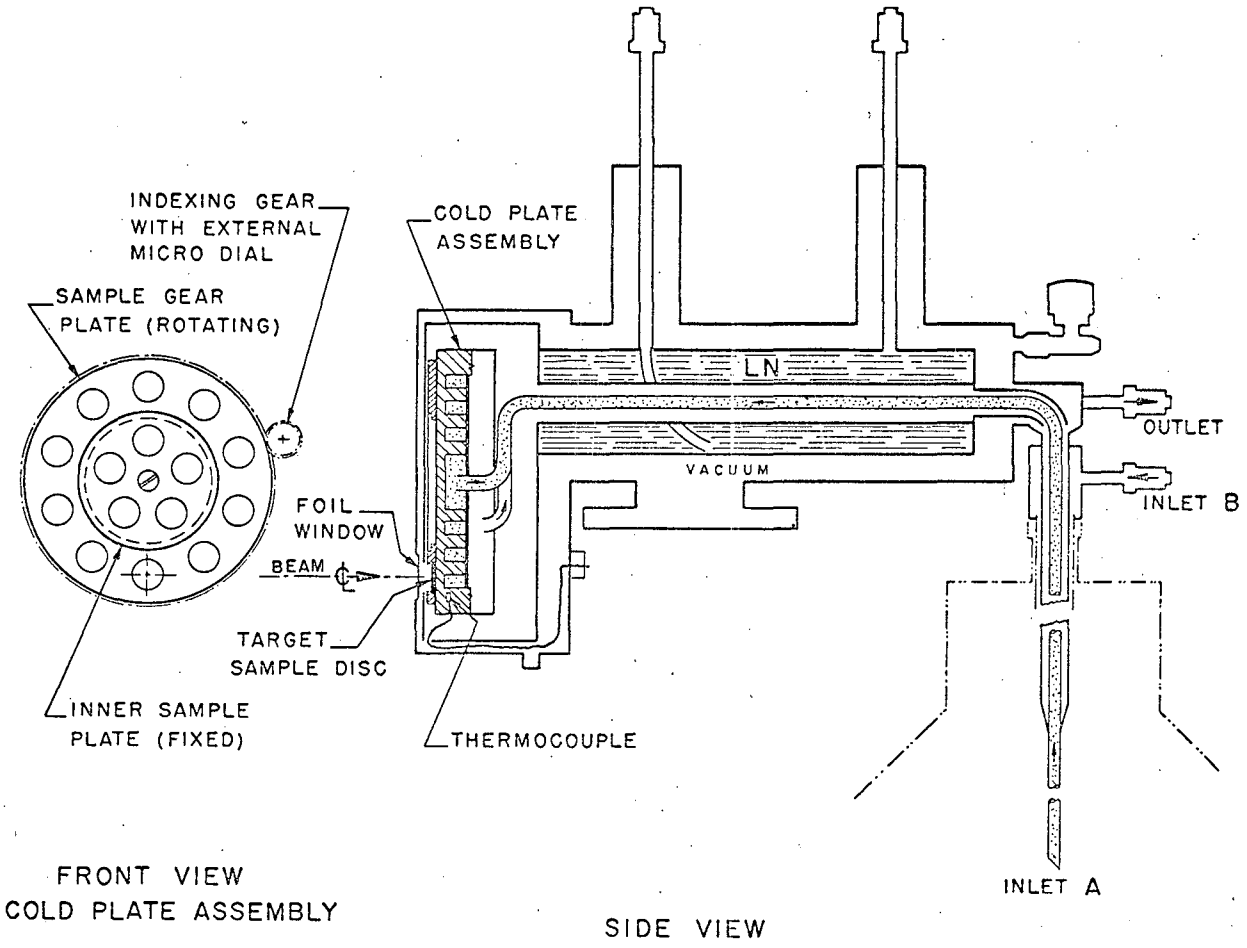

Fig. 1

MUB-7618

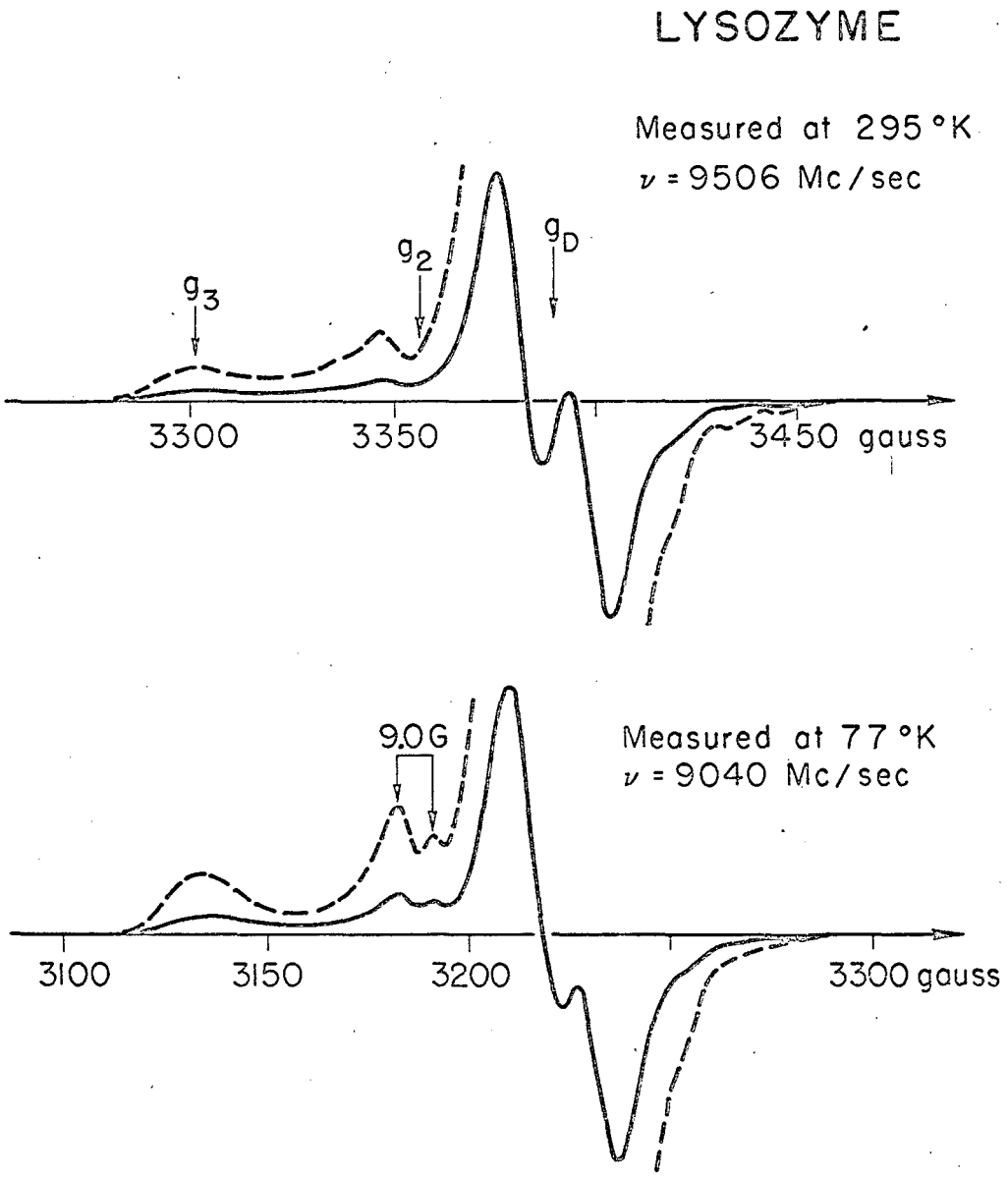

Fig. 2

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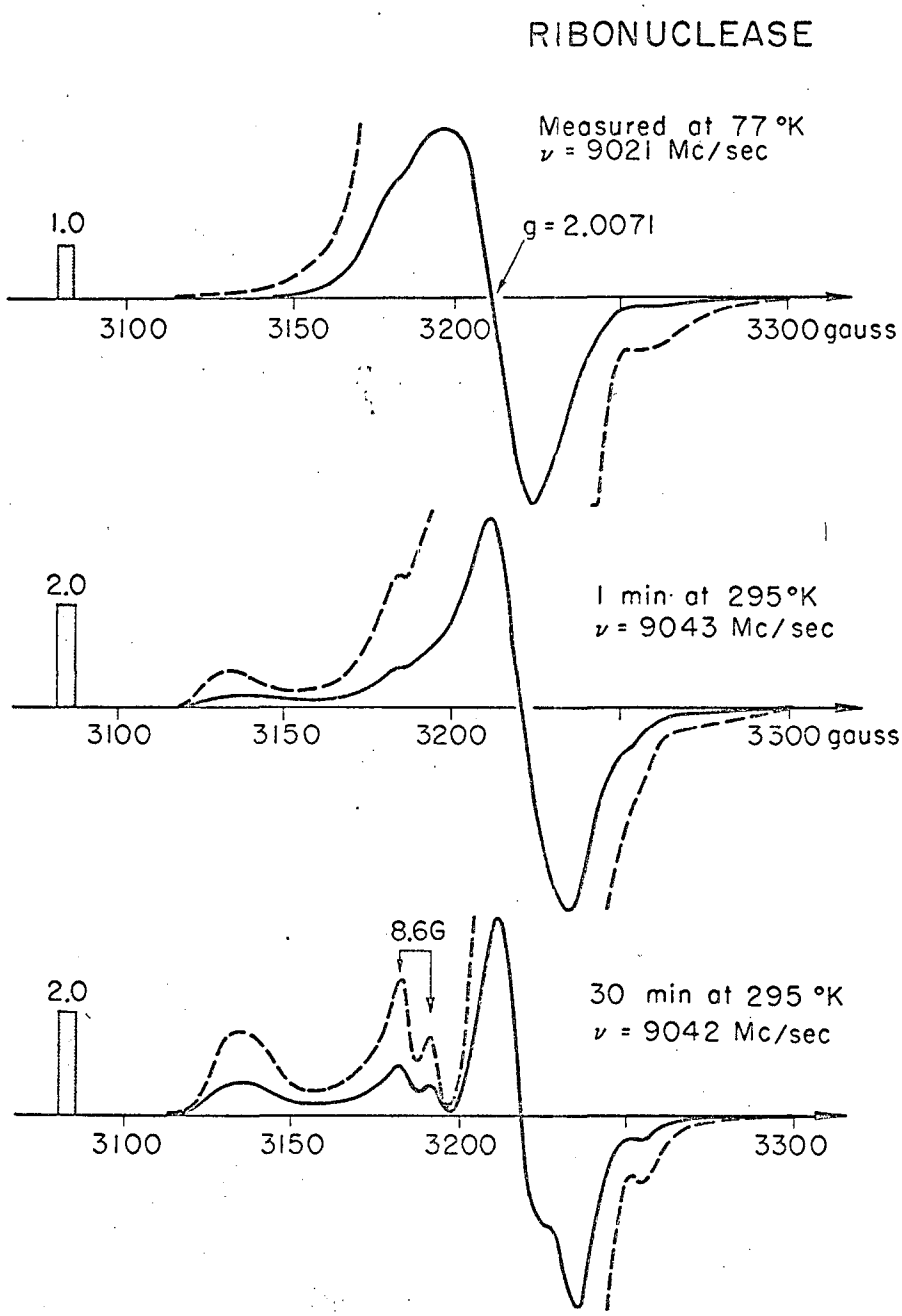

Fig. 3

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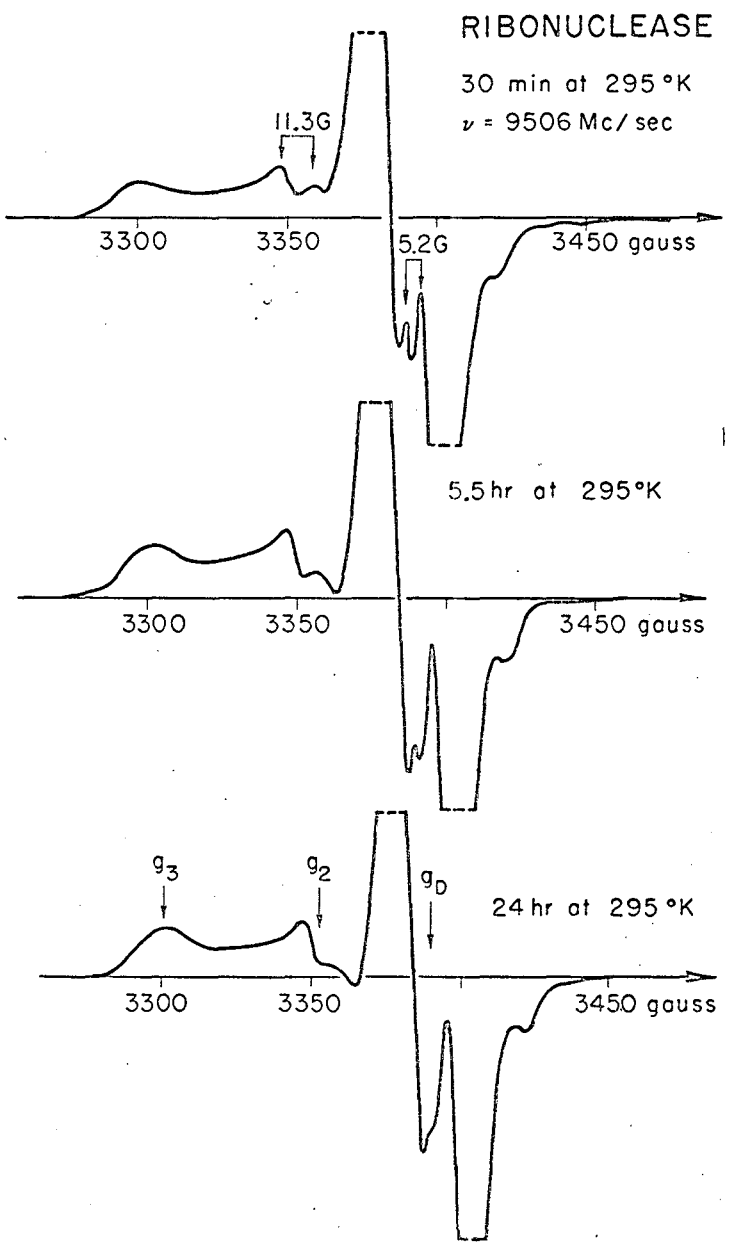
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3350
5.2G
3450 gauss
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3350
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g3
g2
gD
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3300
3350
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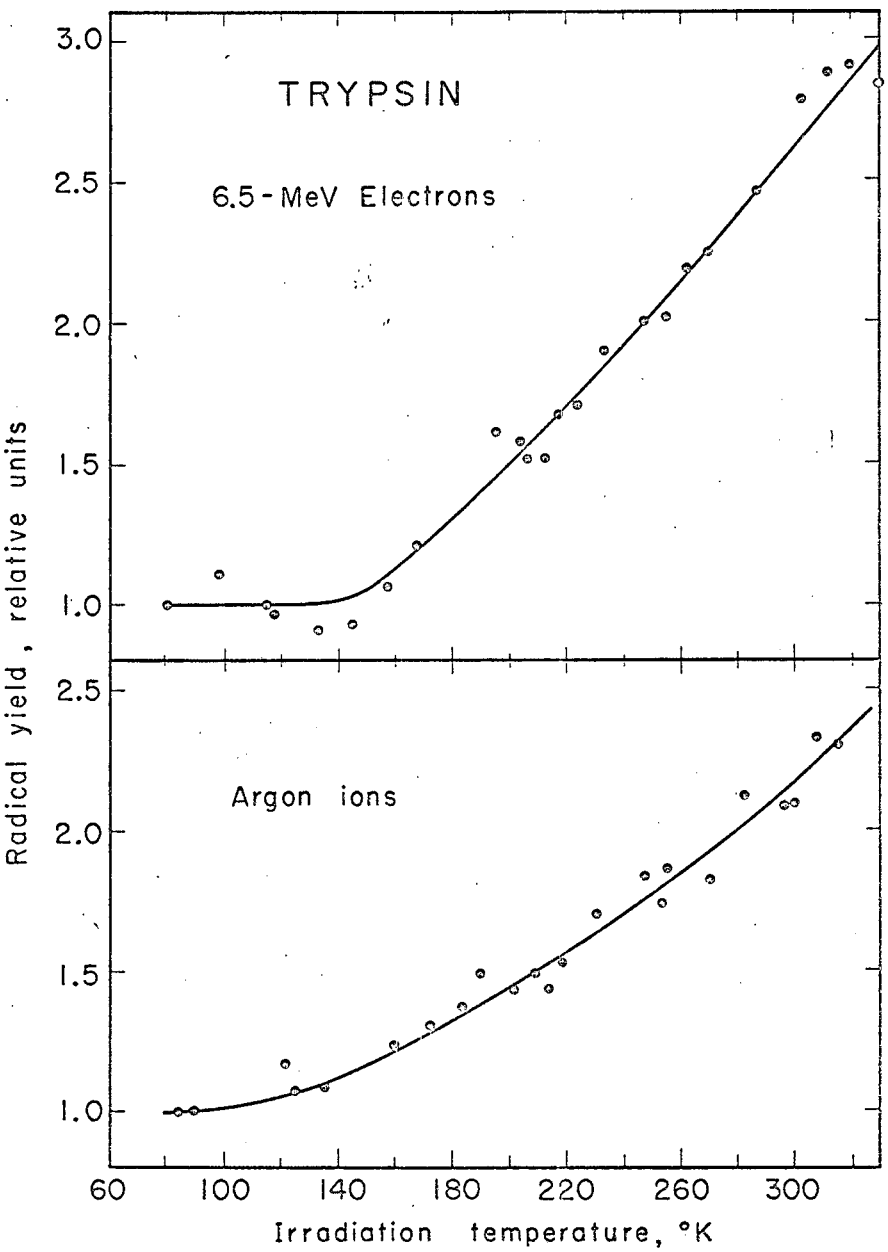
MUB-7677

Fig. 4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Argon ions
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2.5
2.0
1.5
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MUB-7678

Fig. 5

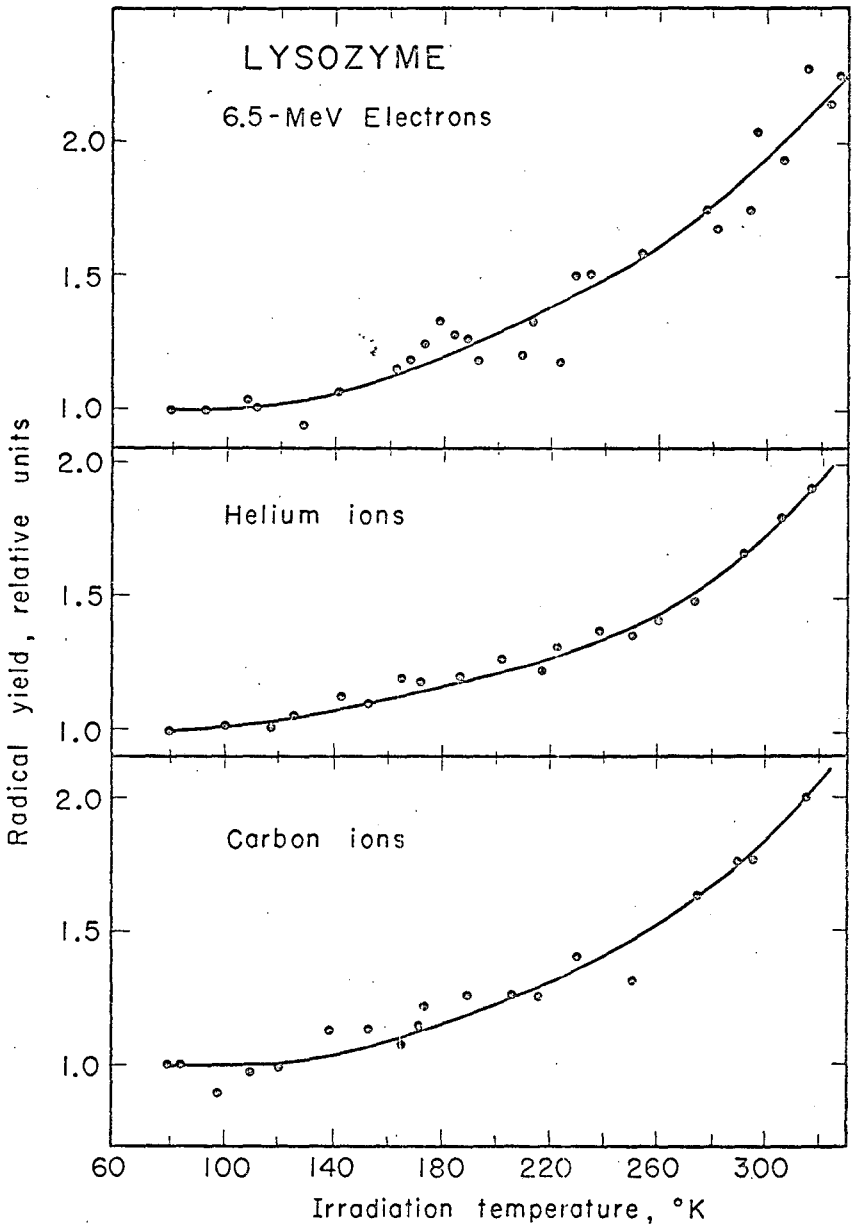
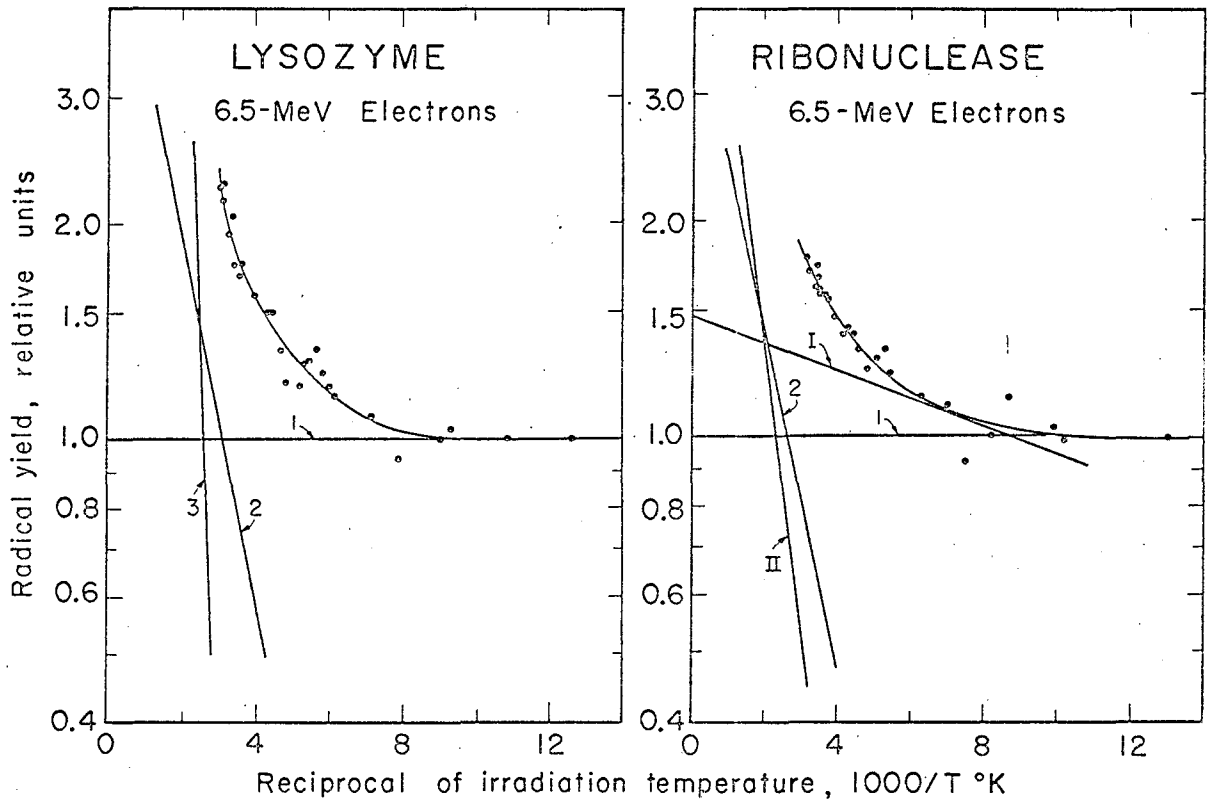


Fig. 6

MUB-7679



MUB-7680

Fig. 7