

Radiation Effects in Organosulphur Compounds and Proteins: An E.S.R. Study

A Thesis submitted by

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of the

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*"I have described nothing but what I
saw myself, or learned from others of
whom I made the most careful and
particular enquiry."*

Thucydides

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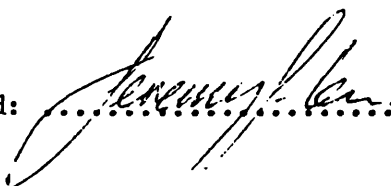
STATEMENT

The accompanying Thesis submitted for the Degree of Doctor of Philosophy entitled "Radiation Effects in Organosulphur Compounds and Proteins: An ESR Study" is based on work conducted by the author in the Department of Chemistry of the University of Leicester mainly during the period between October 1983 and October 1986.

All the work recorded in this Thesis is original unless otherwise acknowledged in the text or by references.

None of the work has been submitted for another degree in this or any other University.

Signed:

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Date: 10/2/87....

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CHAPTER 1

Introduction

1.1 INTRODUCTION

Since the advent and first use of the atomic bomb with all its terrible consequences, and the dawn of the nuclear age, the harmful effects of high energy radiation have become a major concern of the scientific community. Fields of study have extended from all aspects and applications of radiation involving workers from all disciplines.

Of greatest significance to this particular study has been the damage caused radiolytically to biological tissue which has assumed even greater importance with the use of radiotherapy in the treatment of cancer.

Much of the work in the fields of biochemistry and biophysics which concerns radiation is being carried out - specifically aimed with respect to the treatment of cancer. This aspect is discussed later in this section.

Many new fields have been opened by the use of radiation in its various forms. Radioactive isotopes have been particularly useful in the elucidation of chemical and biochemical pathways and mechanisms, for example the use of ^{32}P in DNA strand-break experiments. Pulse radiolysis experiments and the techniques of laser flash photolysis have revealed much in terms of "fast reaction" kinetics, which is expanding further with the increasing availability to research workers of Synchrotron radiation with its associated techniques.

The major technique employed in this study is electron spin resonance spectroscopy. The theory pertaining to this particular technique has been fully explained by several authors¹⁻³ but it may be helpful in terms of this particular study to list some of its applications and limitations. Electron spin resonance (e.s.r) concerns the study of paramagnetic molecules (or ions), that is a molecule containing one or more unpaired

electrons. These electrons will, under the influence of a magnetic field, become parallel or antiparallel to the direction of the field. This lifts the degeneracy of the $\pm\frac{1}{2}$ states of the unpaired electron. The spectroscopic act is then the flipping of the spin between these two levels [resonance occurs when the applied magnetic field induces a splitting equal to that of the applied microwave energy.]

The technique was developed at about the same time in England and Russia just after the Second World War,^{4,5} much of the initial work being on transition-metal and lanthanide complexes. Once it had been demonstrated that free radicals could be detected by this method,⁶ the field of study widened considerably. Various methods were used to generate the free radicals, amongst the most common being photolysis and radiolysis; photolysis often being used to generate free radicals later studied by u.v. spectroscopy (flash photolysis). The microwave cavity of the e.s.r. spectrometer is fitted with a grating designed to allow light into the sample cell, but not to perturb the microwaves. In this way high stationary concentrations of radicals can be accumulated (lifetime of the order of milliseconds per fluid solutions). Two of the main experiments arising from this technique are (1) the study of intermediates arising from the photolysis of the molecule of interest and (2) the use of a photoactive molecule as a sensitizer to promote reactions to be studied.

Thermolysis⁷ and redox processes⁸ can also be used to generate radicals. However, the method used primarily in this thesis is radiolysis. Photolysis in the near u.v. region of the spectrum leads to bond homolysis to form free radicals as in:



whereas radiolysis causes initially electron ejection as in:



usually followed by electron capture as in:



In low temperature studies as used in this work, very often the electron-gain and the electron-loss centres go on to give secondary radicals which can also be detected by e.s.r. (hence the mechanism of a particular radical reaction may be elucidated). Combining radiolysis with e.s.r. has yielded a large amount of information on all aspects of radical structure and radical mechanisms, one area of this field being of primary consideration here. In general, low-temperature solid-state studies as presented here do reveal primary or significant secondary radicals. How then is the technique of use in biochemical or biological studies?

When considering intrinsic radical signals from biological samples, it is worth remembering that large organic π -radicals, which are fairly stable, are probably responsible for acting as intermediates in electron-transfer systems. They are, however, present only in low concentrations and the signals they provide give little information (broad singlets in the free-spin region).

Of greatest significance to this particular study are the signals arising from irradiated biological samples. Studies employing photolysis as a method of irradiating samples have to a great extent been concerned with the photosynthetic systems.⁹ Until recently, studies involving γ - or X-rays as a source of radiation have been largely confined to small biochemically related compounds [see Chapters 2-5] as work on macromolecules and whole bio-systems did not provide easily interpretable data. However, the increased usage of model systems¹⁰ and of spin-label techniques¹¹ (much of the latter being concerned with lipid/protein

membrane-like systems¹²) has yielded a great deal more information.

This forms a very brief summary on both the techniques employed and the types of information to be gained. The latter part of this Chapter is concerned with its applications to the particular systems studied here.

The work presented in this thesis covers two main areas of research; (1) organosulphur compounds, (2) protein and DNA/protein related systems. There is, however, a common theme which runs throughout all the work, and is best described under the broad title "radiation protection".

To understand this, consider the rôle of organosulphur compounds. In particular, various RSH compounds are thought to be of major importance in the initial repair of damaged macromolecules. These compounds can be classified as [1] the naturally occurring non-protein thiols (npsh), e.g. glutathione, [2] the possible use of synthetic RSH compounds as radioprotectors, e.g. 2-pyridine methanethiol, [3] the sulphhydryl groups and disulphide linkages within the rigid framework of proteins, e.g. the immunoglobulins.

1.1.1 Rôle of Thiols

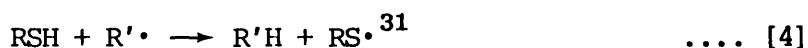
A great deal of work has been carried out with respect to the radiation protection properties of naturally occurring non-protein thiols [see Chapter 2] and much of the known radioprotective nature of the thiol group has been derived from a large amount of initial work on cysteine and cysteamine.¹³⁻²⁰ Glutathione itself has become the subject of several more recent studies. These include the study of radiolytic damage occurring in cells deprived of glutathione.²¹⁻²³ Also the binding of thiols to macromolecules such as DNA²⁴⁻³² is being studied in order to probe their mechanism of protection. There is great interest in duplicating and improving the action of the cellular non-protein thiols

by the use of synthetically produced thiol-containing compounds such as the aforementioned 2-pyridinemethanethiol³³ [see Chapter 3].

As to the radiation protection itself, the major way by which a cell protects or rather repairs itself from the effects of ionizing radiation is by directing the damage (in the form of free radicals) away from the target molecule and then "killing" the radicals produced by this process. As mentioned, the thiols form one group of compounds which perform this function.

Thiols may act as radical scavengers where the thiols locate around critical sites in macromolecules scavenging free radicals produced by radiation and hence protecting the macromolecule. It is through the protection of the DNA molecule itself (discussed later in this Chapter) that much of the organosulphur study was directed [see Chapter 8] and much of the work presented in Chapters 2-4 involves trying to understand what happens to the sulphur compound after it has acted as a radio-protector.

Sulphydryl (thiol) compounds act by hydrogen donation in the manner



where R' is the damaged macromolecule. It is the fate of the RS· radical which has become the centre of some controversy. Most sulphydryl compounds produce very similar e.s.r. spectra when subjected to γ-radiation. In particular, a spectrum with three major g-values of ca. 2.06, 2.025 and 2.002 (the "sulphur resonance") referred to in this thesis as "species X"⁷⁰ is often observed. A full discussion of this radical is presented in Chapter 6. In early studies, the radical responsible for these features was thought to be RS·.³⁴⁻³⁷ Symons³⁸ pointed out that the e.s.r. spectra for RS· should vary enormously with its environment, especially when there is hydrogen bonding to sulphur,

whereas the spectra for species "X" did not change with the change in environment. At about the same time, elegant work by Hadley and Gordy³⁹ showed that "species X" contained two inequivalent sulphur atoms. This led to the proposal of two alternative structures for "species X" (1) the perthiyl formulation $\text{RSS}\cdot$ put forward by Hadley and Gordy³⁹ and (2) the weak adduct formulation RSSR_2 put forward by Symons.³⁸ The work described in Chapters 2-6 attempts to resolve this controversy.

1.1.2 Rôle of Disulphides

Disulphide linkages can be vital as to the structure and functions of proteins. For example, the disulphide linkages in the immunoglobulins [Fig. 1.1] affect the interaction between the light and heavy chains of

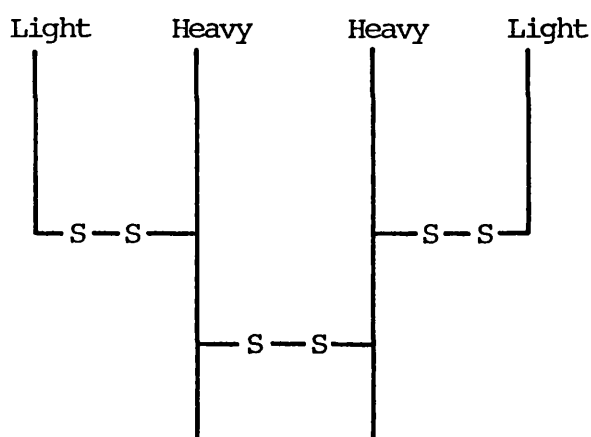


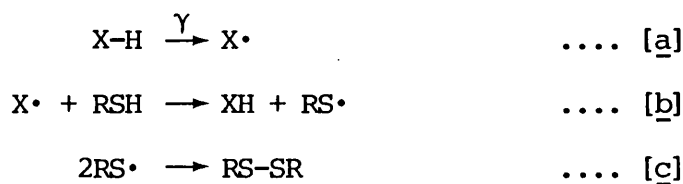
FIG. 1.1

Model for an immunoglobulin molecule showing two light and two heavy chains linked by disulphide bonds.

the molecule.⁴⁰ In this respect they are crucial for the molecules binding to the antigen under attack and hence have a very important part to play in the general immune response of the organism. Obviously damage to these particular sites occurring radiolytically could seriously impair the ability of the molecule to perform its function. The conver-

sion of a disulphide RSSR into two RSH units, breaking the sulphur-sulphur bridge between two peptide chains also occurs radiolytically, thus forming significant damage centres in protein molecules.

Disulphides are also linked to thiols in that they are the final products when thiol compounds carry out a repair (much in a reverse manner of the radiolytic damage to disulphides mentioned above). Thus in the manner shown:



Here, [a] is any radiolysis ^{resulting} in H-atom loss, and [c] is not meant to imply a precise mechanism.

1.1.3 Damage to DNA and Proteins

The latter part of this thesis deals not with the radioprotective agents but with the target molecules themselves. Chapter 7 deals with the possible site of electron-loss in polyamino acids and proteins. The final Chapter attempts to tie together the work not only on proteins but also that with thiols, to the target of primary importance within the cell - the DNA molecule itself.

The research group at Leicester University has been working on the damage caused to DNA by irradiation for some time⁴¹ but, more recently, this has been expanded into work of greater biological significance. This has involved work with DNA/histone complexes up to the study of the cell nucleus itself [see Chapter 8]. DNA is damaged continuously throughout the cells' lifetime. There are various sorts of damage and healthy cells have ways of repairing this damage. DNA is a very large and relatively easily damaged molecule. Despite this, bacterial genes can survive many millions of replication cycles without damage.⁴² The

integrity of the genetic information stored in the base pair sequence of DNA is protected to a great extent by enzymes capable of accurately repairing certain kinds of damage or defects in one strand of DNA as long as its complimentary strand remains intact.

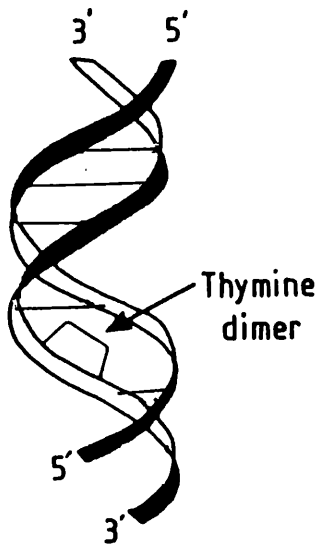
An example of this enzymatic process can be illustrated with the repair of one of the commonest known forms of damage which occurs in DNA, i.e. the thymine dimer produced by u.v. radiation⁴² [Fig. 1.2]. This form of enzymatic repair is brought about by the sequential action of four steps involving two enzymes. The thymine dimer is detected by the 5' → 3' nuclease activity of DNA polymerase I, which has a patrolling function and makes an incision at the 5' side of the defect via its endonuclease activity. The defective segment is then replaced, with the correct bases to complete the sequence, by the polymerase function of DNA polymerase I. The enzyme then uses its exonuclease activity to remove the defective segment. The new strand is then formed at its 3' end to the 5' end of the remaining intact strand, through the action of DNA ligase.

If a double strand break occurs, repair is more difficult, but still possible. Such double strand breaks have been shown [see Chapter 8] to occur quite frequently when DNA is irradiated,⁴¹ and hence may prove to be one of the more important types of damage in terms of permanent change to the molecule itself.

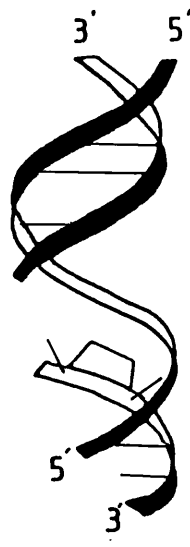
Clearly if RSH compounds can replace hydrogen rapidly even at only one damage centre of a pair, then a double strand break is avoided.

FIGURE 1.2

Repair of thymine dimer.



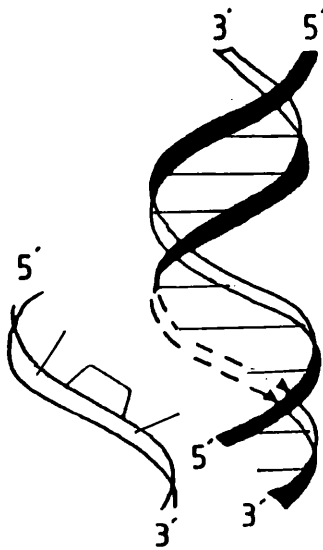
**Defect produced
by thymine dimer**



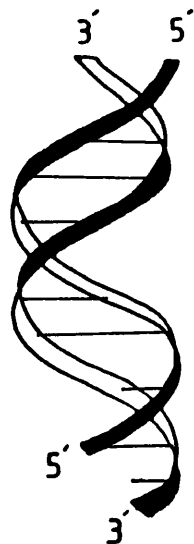
**Incision by an
endonuclease**



**Patching by DNA
polymerase I**



**Excision of defective
piece by 5' → 3' exonuclease**



Sealing by DNA ligase



CHAPTER 2

Dithiothreitol and Associated Thiols

2.1 INTRODUCTION

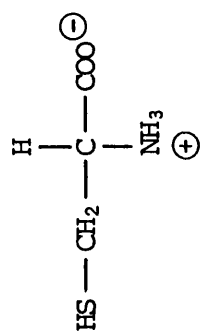
Although dithiothreitol is the major compound studied in this work, a variety of other thiols are briefly considered [Fig. 2.1]. As already mentioned [see Chapter 1] many of these compounds have been used in radioprotection studies for some time. In early studies, cysteine and cysteamine were favoured;¹³⁻²⁰ more recently, however, considerable attention has been given to mercaptoethanol,⁴³⁻⁴⁹ mercaptopropionylglycine (MPG),^{50,51} and especially dithiothreitol⁵²⁻⁵⁶ and glutathione.^{21-23,57,66,67} All these thiols prove to be good radioprotectors, particularly aminothiols [cysteamine is often used as a standard].

Previously work has ranged from the more straightforward observations on the effect on the radiolytic damage to macromolecules¹³⁻²⁰ when thiols are employed as additives to the more complex work on the influence of thiols on the behaviour of other additives in systems of this type.¹⁵ Such work is not limited to the use of thiols as additives in purely model systems, it extends to studies of endogenous thiols in cellular environments.

Much of the latter work has centred on glutathione. Glutathione (γ -glutamylcysteinyl glycine) is sometimes regarded as the cell's own radioprotector. In the light of recent publications in the literature, this would appear to be a fairly accurate statement. In 1984 Révész¹⁶ stated that the number of strand breaks induced in DNA by radiation in the presence of oxygen or misonidazole appears to be critically dependent on [glutathione]. This has been supported by several other workers.^{21,23} Held and coworkers^{55,56} postulated that in oxygenated conditions, thiol radioprotection is consistent with the scavenging of hydroxyl radicals, whilst in anoxic conditions there is additional protection due to

FIG. 2.1

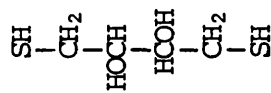
Structures of Thiol Compounds Studied



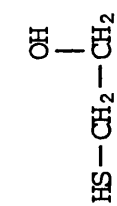
Cysteine



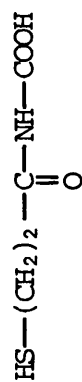
Cysteamine



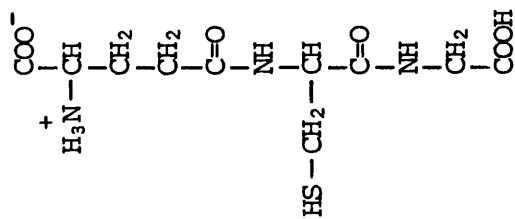
Dithiothreitol



Mercaptoethanol



Mercaptopropionylglycine (MPG)



Glutathione

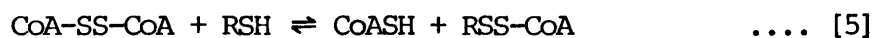
hydrogen atom donation from the thiol to radiation induced DNA lesions.

The concept of hydrogen atom donation is crucial to this particular study. As stated in Chapter 1, the reaction,



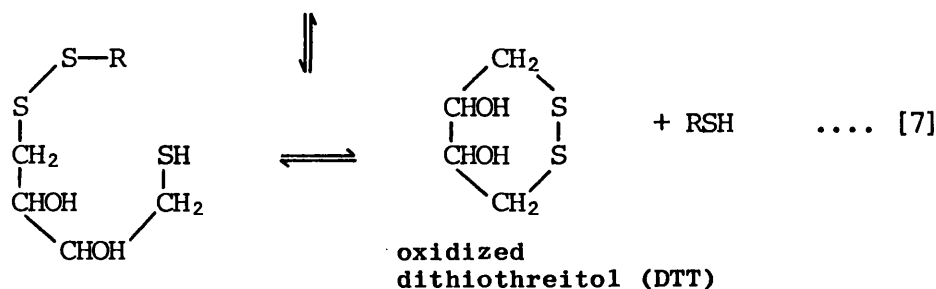
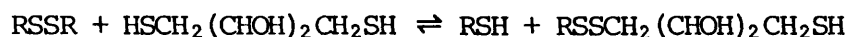
where $\text{R}\cdot'$ is a macromolecular radical that is repaired by the thiol compound, is closely involved with radioprotection studies. Reaction [4] is effective because $\text{RS}\cdot$ radicals are relatively inert. They are protected further by a facile reaction with the parent RSH molecules. E.s.r. has been very useful in attempts to study such reactions but there remain many ambiguities, and our aim has been to endeavour to probe the radical chemistry of these compounds a little more deeply in the hope of shedding more light on these complexities.

Initially, the usage of exogenous thiols as additives was not involved with radioprotection but more the preservation of endogenous thiol-groups in the reduced state and also reducing endogenous disulphides quantitatively. Thiol groups such as those of coenzyme A [CoA] and some other enzymes are readily oxidized in air to the disulphide. To maintain these groups in the reduced state, another thiol such as cysteine, glutathione or mercaptoethanol was often added so that interchange takes place according to reactions



However, the equilibria of these reactions is near to unity so an excess of the thiol additive had to be employed for the reactions to proceed. But if the reaction [6] was intramolecular and RSSR was a sterically favoured cyclic disulphide, there would be two products from one reactant so that the equilibrium should be displaced to the right, particularly in dilute solutions.

Cleland⁵² showed that, because of its low redox potential, dithiothreitol is capable of maintaining monothiols completely in the reduced state in the manner,



When this was monitored spectrophotometrically the oxidized form of dithiothreitol was found to predominate.

We felt that this property could have a particularly beneficial effect as far as radioprotection was concerned. It was mentioned in Chapter 1 that an important radical arising from these thiol compounds has two possible structures: [1] The perthiyl radical $\text{RSS}\cdot$, favoured by Hadley and Gordy,³⁹ [2] the $\text{RSS}(\text{H})\text{R}$ adduct, favoured by Symons.³⁸ Because of this structural ambiguity, the species having g -values close to 2.06, 2.025 and 2.00, is often referred to simply as species "X". It was hoped that a study on these thiols and dithiothreitol in particular would yield more evidence concerning this particular controversy.

2.2 EXPERIMENTAL

All compounds mentioned in this Chapter were obtained from Sigma chemicals and were of the highest grade available. They were purified by standard techniques and then checked by n.m.r. spectrometry. Both CD₃OD and D₂O were supplied by Aldrich Chemicals and, again, were of the highest purity. Typical preparations of both powder and glass samples are described here.

2.2.1 Powder Samples

The compounds were kept desiccated and, if necessary, below 5°C. A sample of the compound to be studied was taken and ground to a fine powder using a mortar and pestle. [This was to ensure that there were no crystallite effects when the spectra were taken. It was established that such grinding did not affect the reactions studied.] The sample was then placed in a sample bottle and irradiated at 77 K in a ⁶⁰Co Vickrad source at a dose rate of 0.4 Mrad/hr.

2.2.2 Glass Samples

A solution of the compound was made up in a CD₃OD/D₂O (70:30) mixture to a known concentration (usually 40 mM). Oxygen-free nitrogen gas was then bubbled through the solution (for up to 20 minutes) to ensure that there was no dissolved oxygen in the system. [Irradiation of oxygenated solutions produces the radical RO₂•,^{58,59} which would tend to swamp the radicals of interest in this study.] Glassy beads of the solution were made by adding an aliquot of the solution dropwise to liquid nitrogen. The resulting beads were then irradiated as with the powder samples.

Various doses, up to 1.2 Mrads, were used to ensure that dosage was not a factor in the radicals observed. E.s.r. spectra were measured on an X-band Varian E-109 spectrometer calibrated with a DPPH marker, or in

the case of Q-band studies, measurements were taken on a Bruker ER-200 spectrometer with an ER-053QRD microwave bridge. Annealing experiments were carried out by decanting liquid nitrogen and monitoring the spectrum. Liquid nitrogen was added whenever significant changes were observed, and the spectra were re-measured at 77 K. Temperature related studies were carried out by allowing the sample to warm to a predetermined temperature, allowing it to equilibrate at this temperature and then recooling to 77 K in order to observe the spectrum.

2.3 RESULTS AND DISCUSSION

Our results were remarkably similar throughout the range of these compounds containing one or more sulphhydryl (thiol) groups. Reasonably good spectra were obtained both with powder samples and dilute CD₃OD/D₂O (70:30) glass systems. In several of these samples, species "X", which is the main point of interest in this study, was not formed directly on irradiation at 77 K. When this was the case, the sample was annealed until "X" was observed, then re-cooled so that the radical could be observed at leisure.

Results for both powder and glass samples followed a similar pathway. Rather than present the results for each individual compound, general observations are presented here together with spectral examples from the whole range of compounds studied. The initial sulphur radical observed was found to vary in g_{\max} -value ($g_{\max} > 2.1$) and also in the structure of the signal recorded [Figs. 2.2a, b, c]. From previous studies by several workers,^{60-64,68} the signals were assigned to the radical RCH₂S• which would result via hydrogen atom loss (or electron loss followed by deprotonation) from the parent compound.

On annealing, these compounds give rise to a second sulphur radical which, like RCH₂S•, was easy to characterise, although its assignment is somewhat more ambiguous. This radical, found in all the compounds studied, has principal g -values at ca. 2.06, 2.025 and 2.002. This radical has been called a sulphenium radical⁶⁹ or as it will be referred to here, species "X"⁷⁰ [Figs. 2.3a, b, c].

Species "X" has featured in a large range of publications and has been the centre of some controversy in the literature.^{38,39,60,70-73} The whole question as to the nature of species "X" will be discussed in

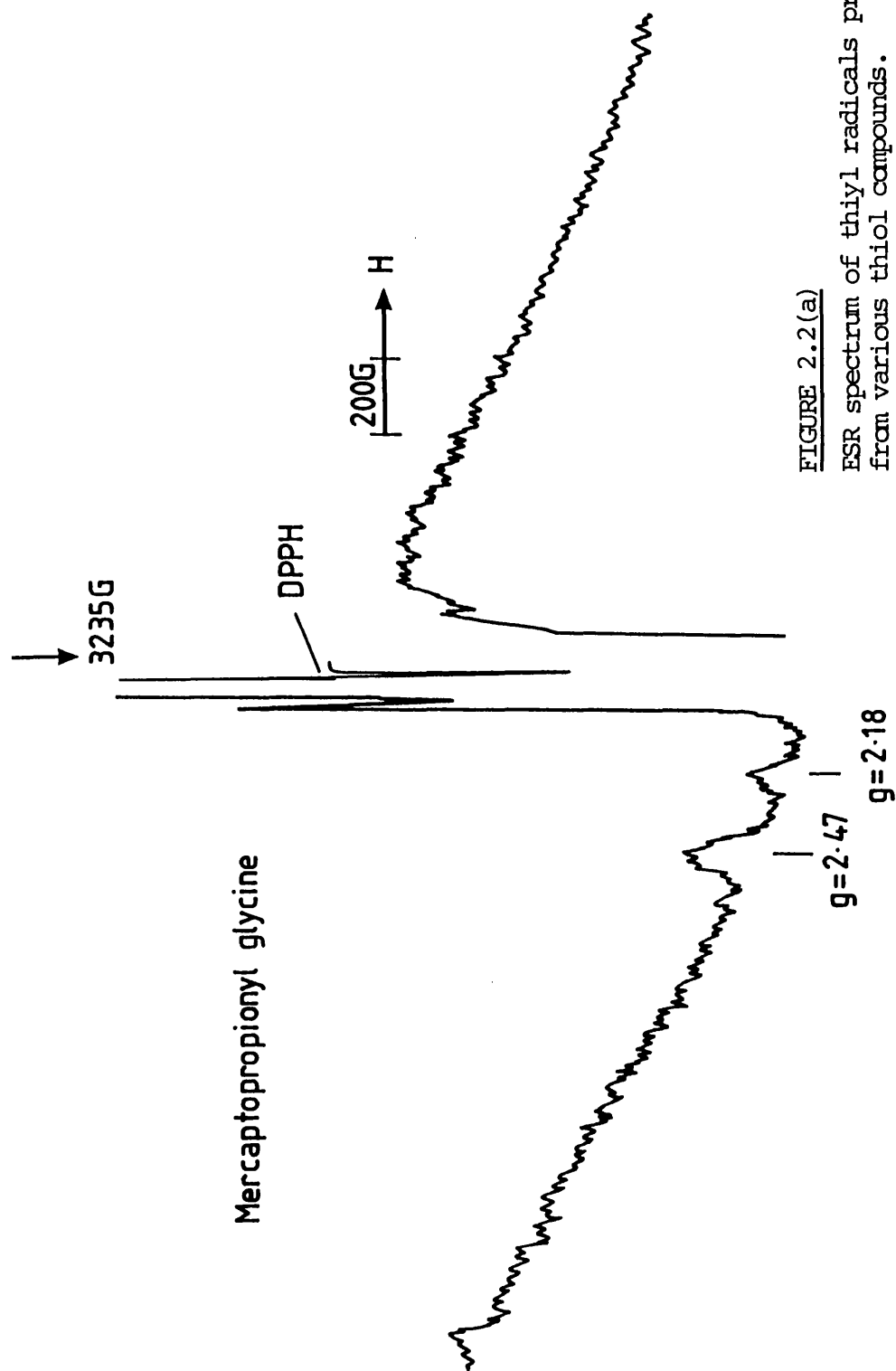


FIGURE 2.2(a)
 ESR spectrum of thiyl radicals produced
 from various thiol compounds.

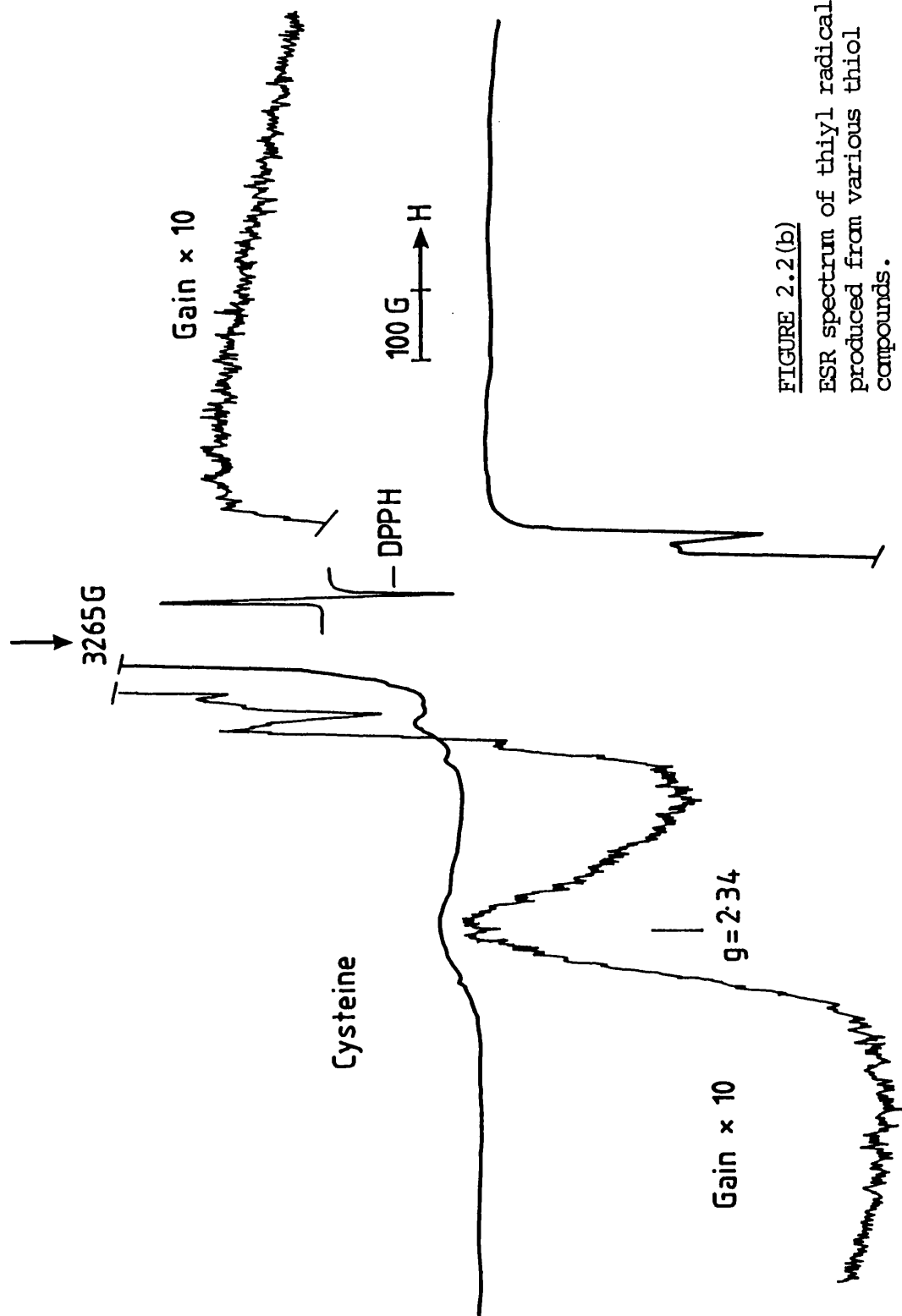


FIGURE 2.2 (b)
ESR spectrum of thiyl radicals
produced from various thiol
compounds.

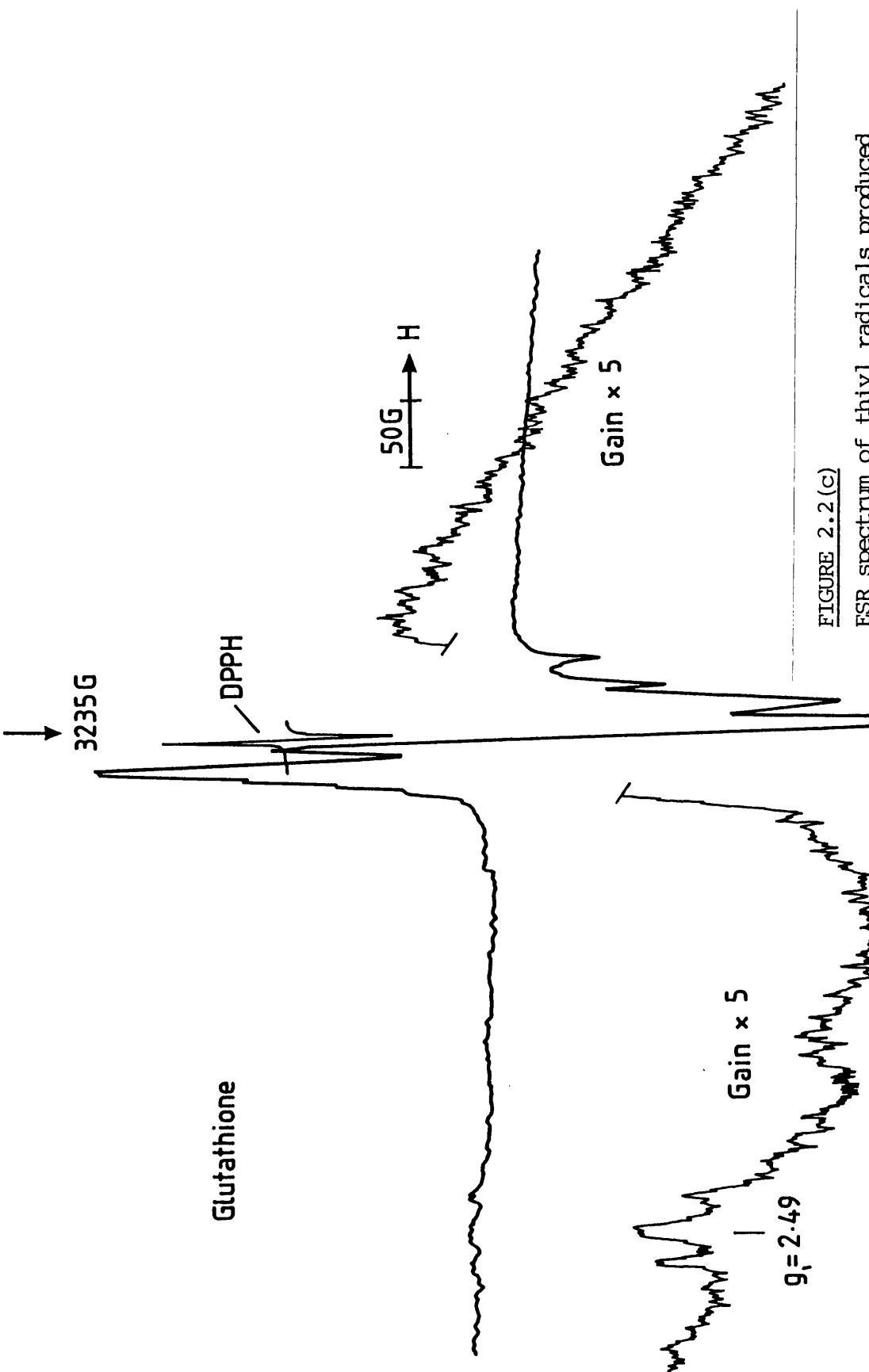


FIGURE 2.2(c)

ESR spectrum of thiol radicals produced from various thiol compounds. The g_1 feature at 2.49 shows hyperfine coupling to two equivalent protons ($\text{RCH}_2\dot{\text{S}}$) $A = 25.5$ G. [The doublet splitting seen in this spectrum and in many others assigned to species "X" is due to hyperfine coupling to one of the two methylene protons in the $\text{RCH}_2\text{S-S} \cdot$ unit.]

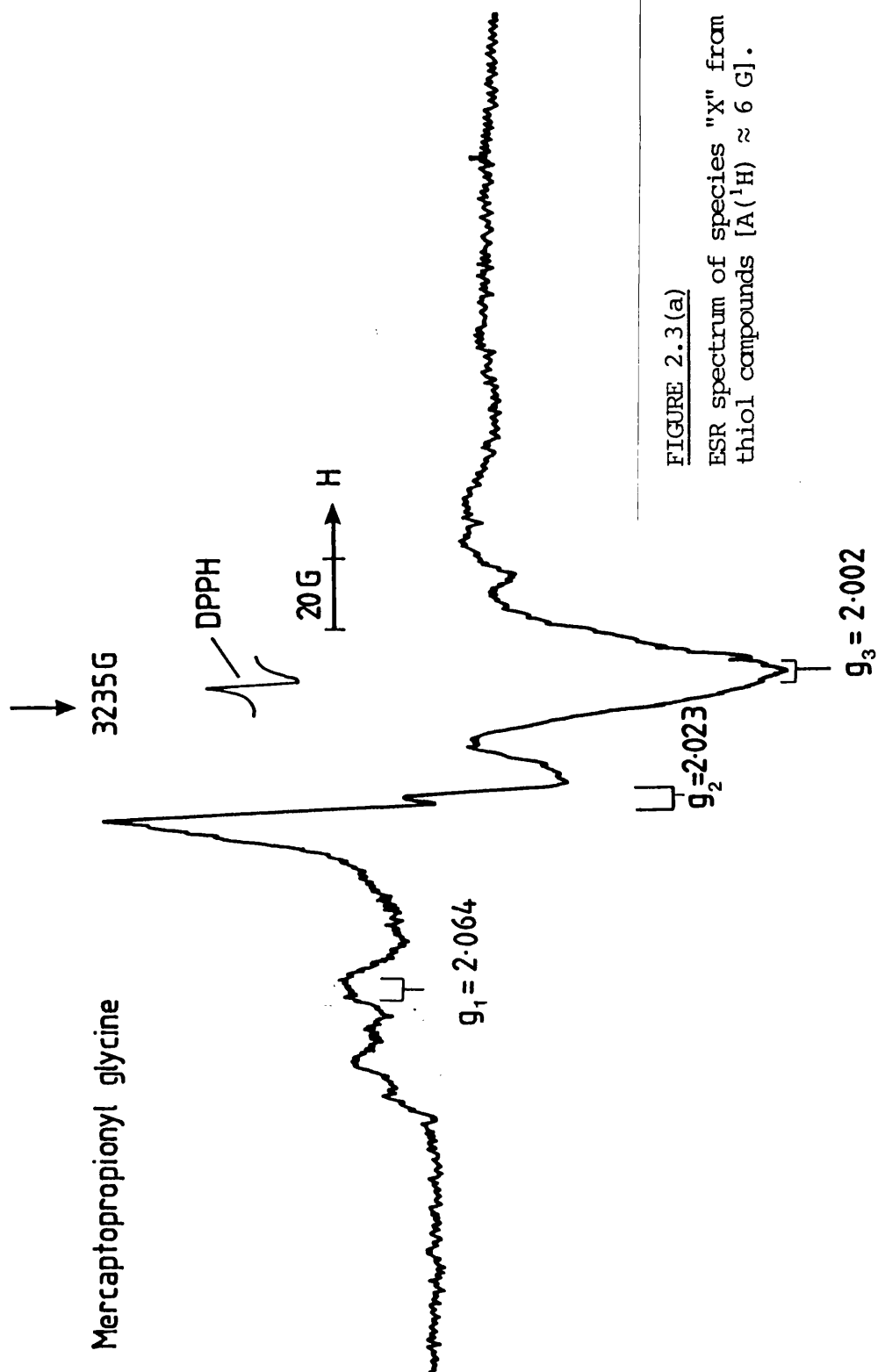


FIGURE 2.3 (a)
ESR spectrum of species "X" from
thiol compounds [$A(^1H) \approx 6$ G].

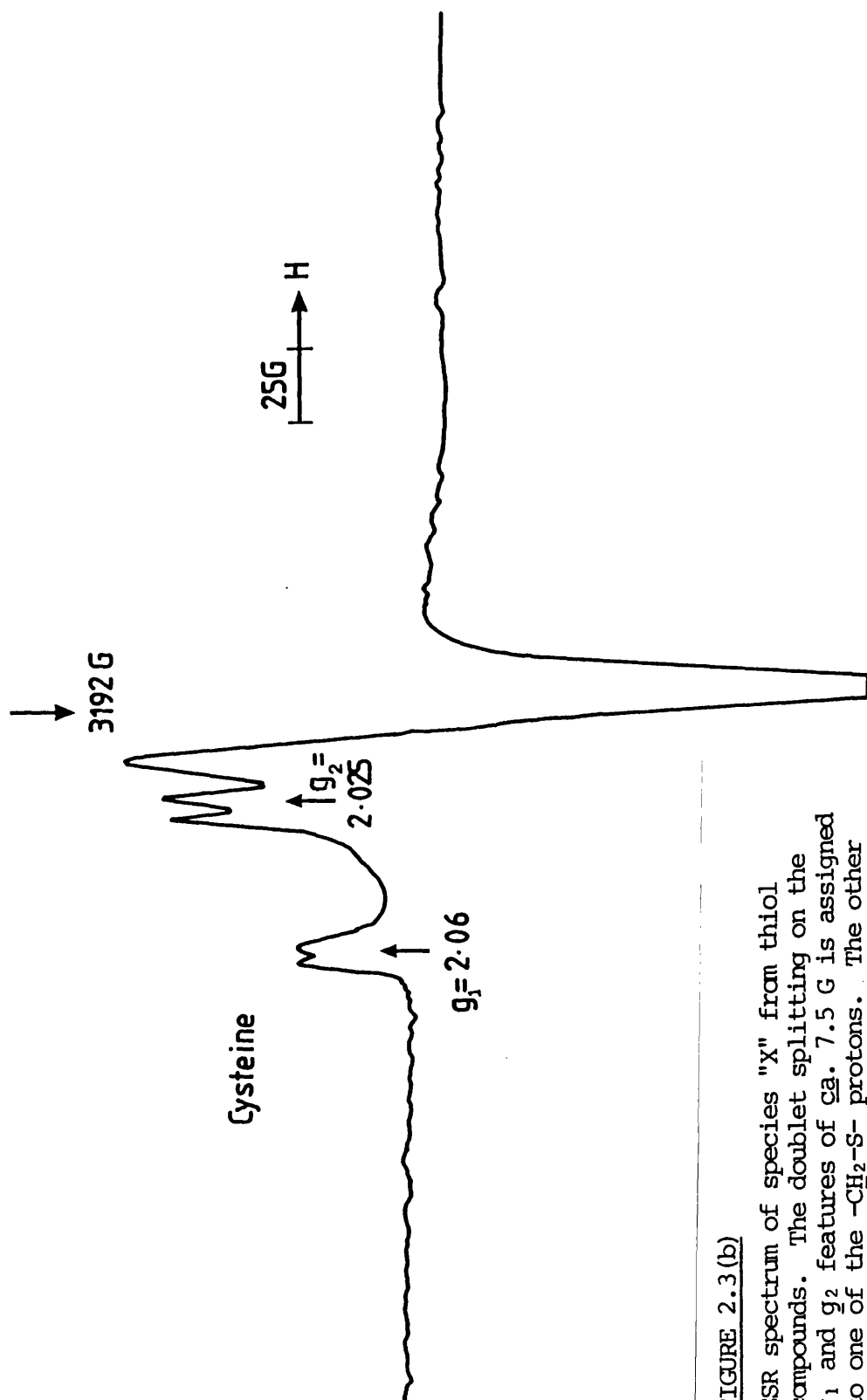


FIGURE 2.3 (b)

ESR spectrum of species "X" from thiol compounds. The doublet splitting on the g_1 and g_2 features of ca. 7.5 G is assigned to one of the $-\text{CH}_2-\text{S}-$ protons. The other has $A \approx 0$. This applies to many of the spectra for species "X" shown in the following pages.

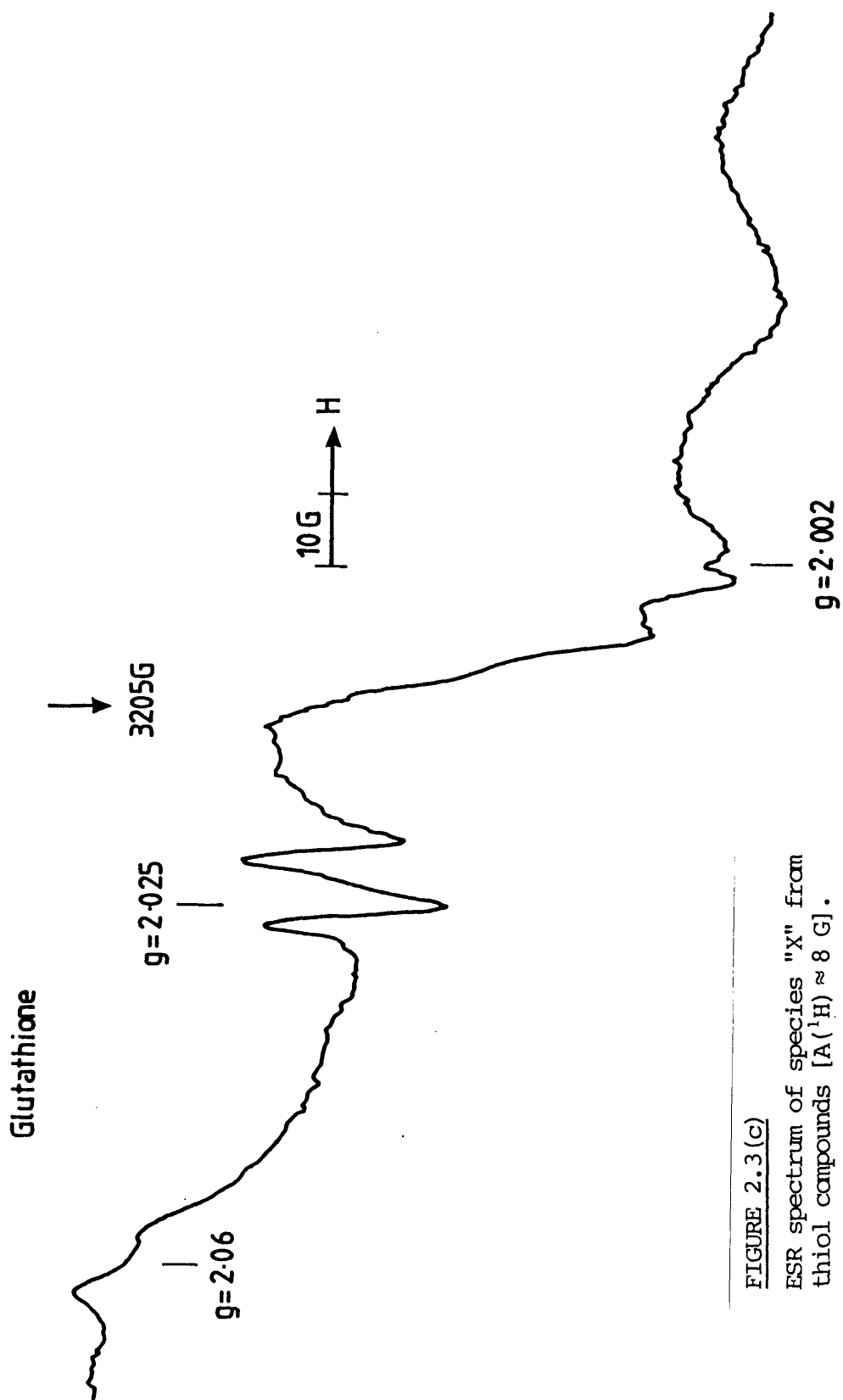


FIGURE 2.3(c)
ESR spectrum of species "X" from
thiol compounds [$A(^1\text{H}) \approx 8 \text{ G}$].

far greater depth in a later chapter [see Chapter 6]. The important results to be drawn from these compounds is the initial observation of RS^\bullet followed by apparent conversion of RS^\bullet into species "X" on annealing.

The set pattern observed with the thiol compounds seemed never to vary. This prompted studies of a more incisive nature and Cleland's reagent,⁵² dithiothreitol, was selected for this purpose.

2.3.1 D,L Dithiothreitol Powder Spectra

An immediate change was noticed on irradiation prior to any spectra being taken. This normally white powder turned yellow on irradiation, the significance of this is discussed later. The initial powder spectrum of dithiothreitol, taken at 77 K, appears quite complex. The interpretation of the spectrum is made somewhat easier if it is divided into separate species A, B and C, as illustrated [Fig. 2.4] and each is considered in turn.

As has been shown by Symons in previous studies^{38,60} and earlier in this Chapter, the g_{\parallel} feature attributable to the radical $RCH_2\dot{S}$ must vary in its position and hence g -value according to the environment of the radical. It is suggested here that species A is an example of this variation, with the parallel features of the radical shifted to a higher than average g -value of 2.47. We believe that it is this large shift to a relatively high g -value for the parallel features that, in this case, allows us to observe the perpendicular features for this radical at ca. $g = 1.90$. Usually the perpendicular features of RCH_2S^\bullet radicals are "lost" within the central signal in the free-spin region. [Note that for such radicals when g_{\parallel} deviates strongly from free-spin, g_{\perp} tends to shift to low g -values, both shifts being the result of orbital angular momentum.]

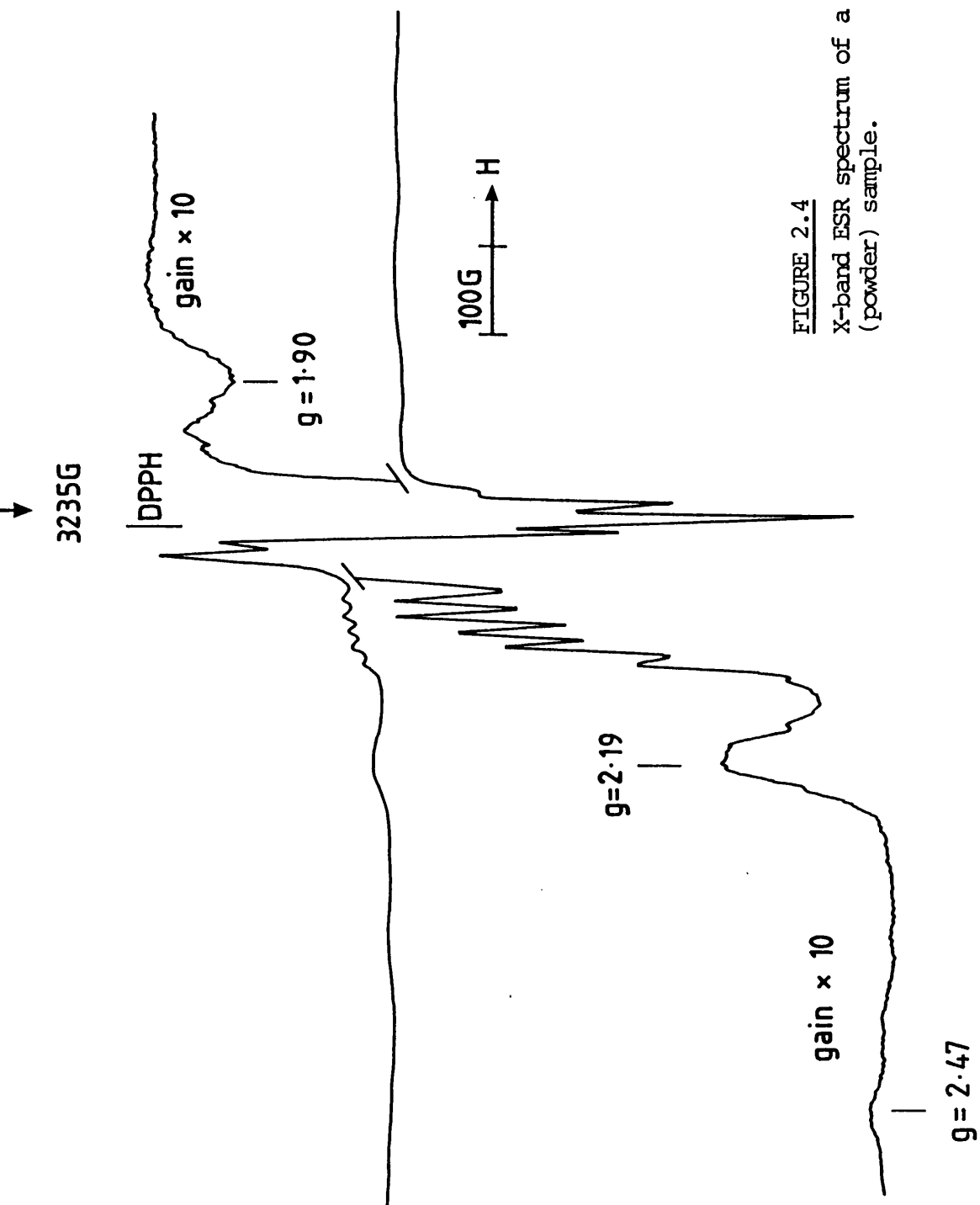


FIGURE 2.4
X-band ESR spectrum of a dithiothreitol
(powder) sample.

Species B is much easier to interpret, the parallel features observed at $g = 2.19$ and the "triplet-like" appearance are indicative of the more commonly observed form of the $\text{RCH}_2\text{S}\cdot$ radical.^{38,60-64} It can be noted, in this case, that the perpendicular features are not visible, being masked in the central region of the spectrum. This result was later confirmed by Q-band e.s.r. studies on this compound showing the same signal at $g = 2.19$. From this it is suggested that, like species A, species B is an $\text{RCH}_2\text{S}\cdot$ radical.

Unlike species A and B, the central signal arising from species C, whose parallel features appear to comprise of a quintet is not so straightforward to assign. However, when this signal was compared to results from earlier work by Akasaka *et al.*⁶⁴ on cystine dihydrochloride crystals and our own studies on lipoic acid [see Chapter 4], certain analogies could be noted. Treated as a quintet, species C has g -values of 2.067 (for the centre of the five-line parallel features), 2.01 and 2.000. These g -values greatly resemble those for a radical observed by Akasaka *et al.* (α_2 in their nomenclature) and also our own lipoic acid spectra.

They proposed that this signal was due to an "unrelaxed" form of the structure which we call species "X". Relaxation of $-\text{CH}_2$ groups within the radical giving rise to the more conventional values already observed. Q-band e.s.r. studies confirmed these g -values, and from this it is suggested that species C is an unrelaxed form of species "X" [see also Chapter 6].

Unlike the monothiols, dithiothreitol gives rise to species "X" (albeit in an unrelaxed form) directly on irradiation. When the dithiothreitol powder was annealed, the five-line species changed into one with data typical of the radical species "X" [Fig. 2.5]. The most noticeable

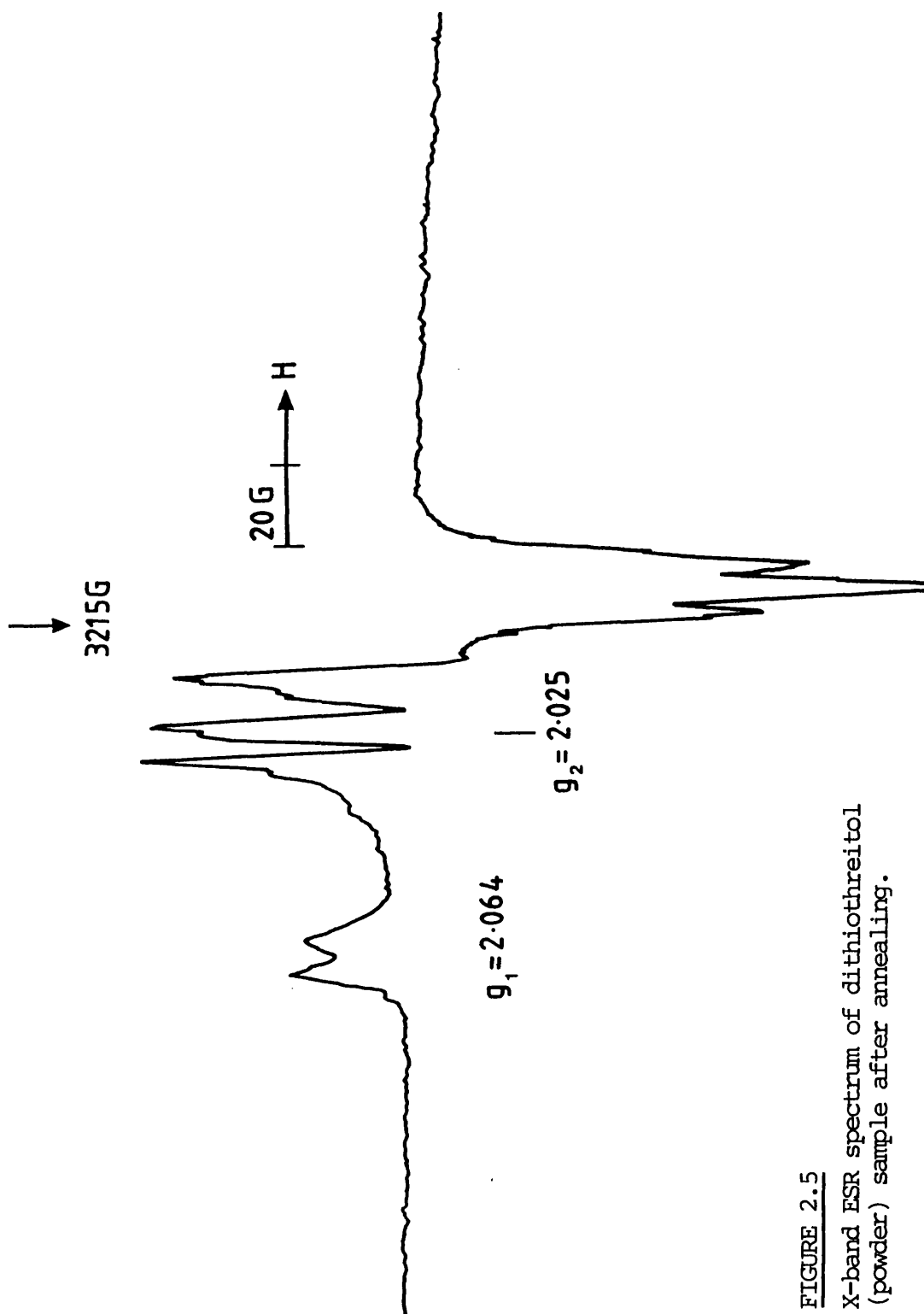


FIGURE 2.5
X-band ESR spectrum of dithiothreitol
(powder) sample after annealing.

result was that species C, thought to be the unrelaxed form of species "X", was lost completely and irreversibly. Concurrent with the appearance of the normal form of species "X", a reduction in the signal due to the $\text{RCH}_2\text{S}\cdot$ radical was noted.

In order to find out whether the $[\text{RCH}_2\text{S}\cdot]$ was indeed falling, the relative intensity of the signal was measured as a function of increasing temperature, as shown in Fig. 2.6. From this plot it can be seen that the intensity remained fairly constant until a temperature of ca. 180 K was reached and that it then decayed rapidly. The intensity of the signal from the "relaxed" form of species "X" was also measured and was found to increase in intensity above a temperature of ca. 175 K.

Hence, initially normal species "X" comes first from the loss of species C (possibly due to the relaxation of a constrained form of species "X") and later at a higher temperature more normal "X" is formed from the loss of $\text{RCH}_2\text{S}\cdot$ radicals.

2.3.2 D,L-Dithiothreitol Glass Spectra

The results gained with the powder samples of this compound prompted experiments to try and observe the behaviour of dithiothreitol in $\text{CD}_3\text{OD}/\text{D}_2\text{O}$ glass matrices irradiated at 77 K. Initially, a spectrum characteristic of the glass matrix only was observed [Fig. 2.7] together with the normal purple colouration characteristic of trapped electrons. As shown, features due to deuterium atoms and contaminating hydrogen atoms were observed, as well as an intense singlet centred around free-spin assigned to solvent $\dot{\text{C}}\text{D}_2\text{OD}$ radicals.⁷⁶

When a wider scan-range was employed [Fig. 2.8], a weak parallel feature was observed at $g \approx 2.18$. This is assigned to $\text{RCH}_2\text{S}\cdot$ radicals and is characteristic of $\text{RS}\cdot$ radicals in CD_3OD matrices.⁶⁰ It was observed to be much weaker than its powder counterpart, as expected,

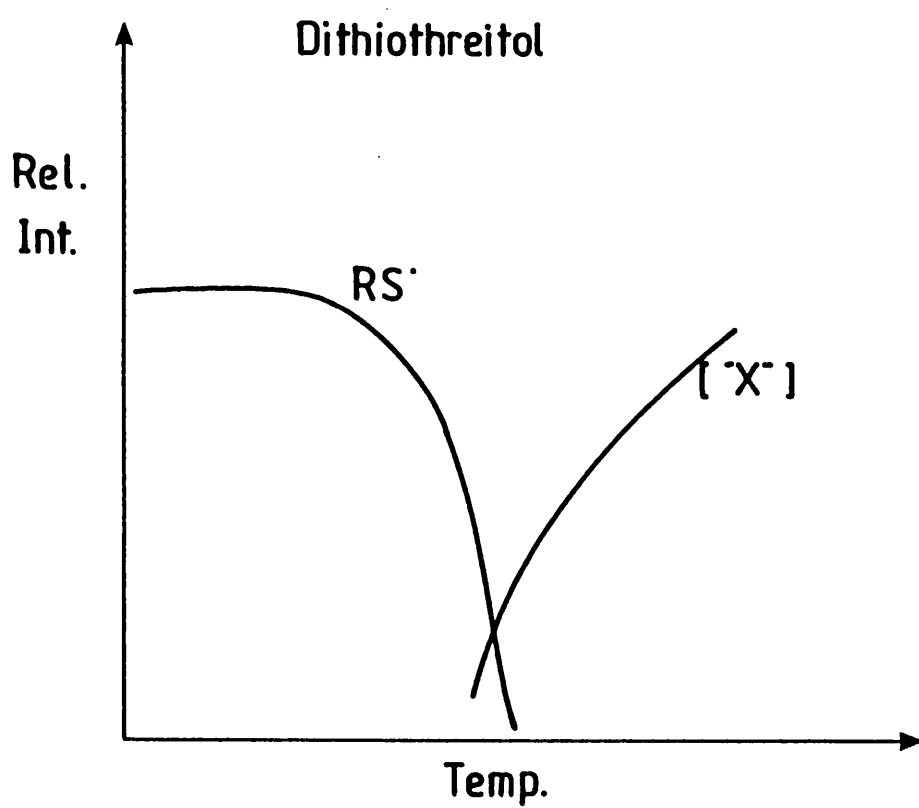


FIGURE 2.6

Plot of relative radical signal intensity of the thiyl radical of dithiothreitol against increasing temperature.

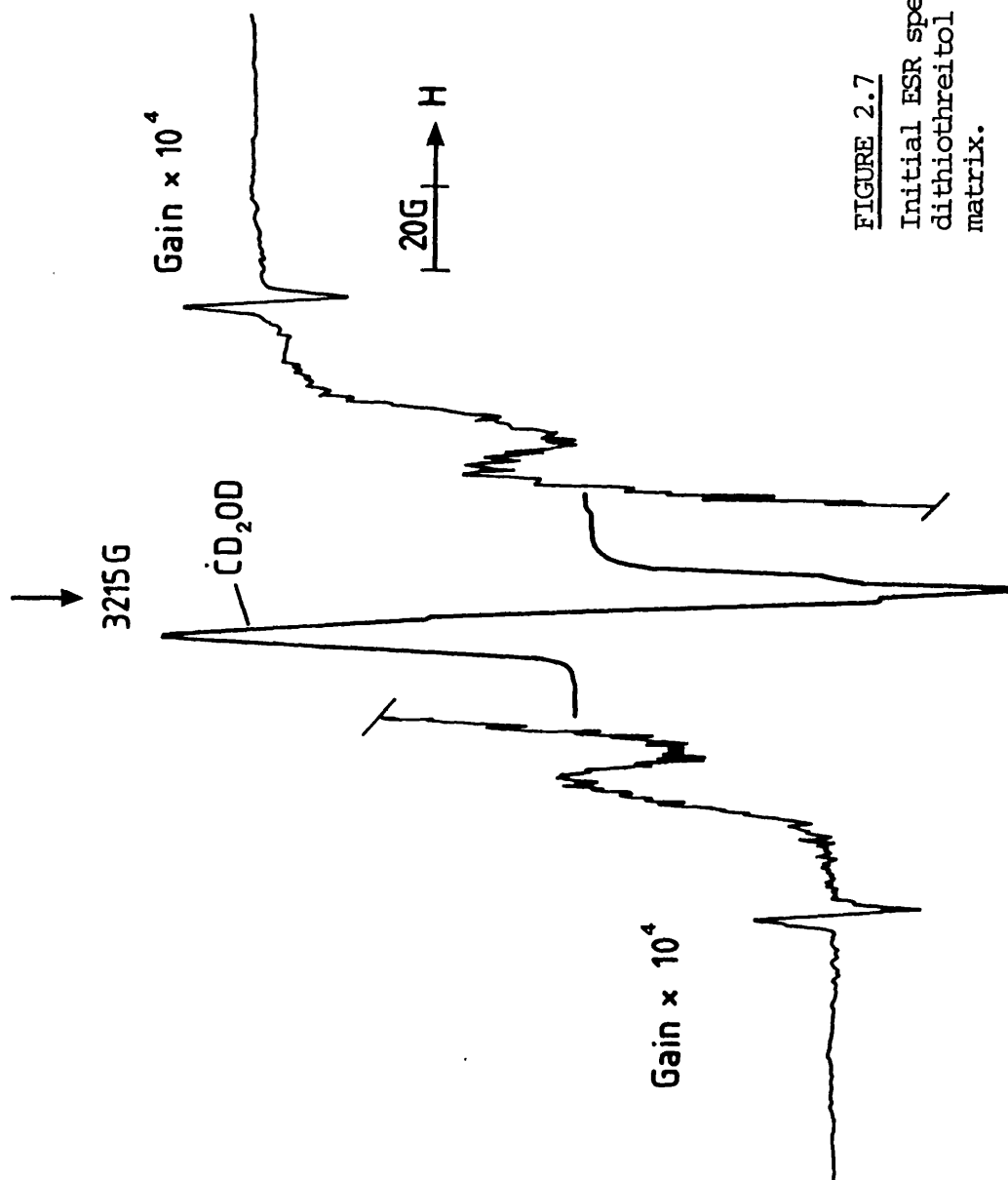


FIGURE 2.7

Initial ESR spectrum obtained from dithiothreitol in a CD₃OD/D₂O glassy matrix.

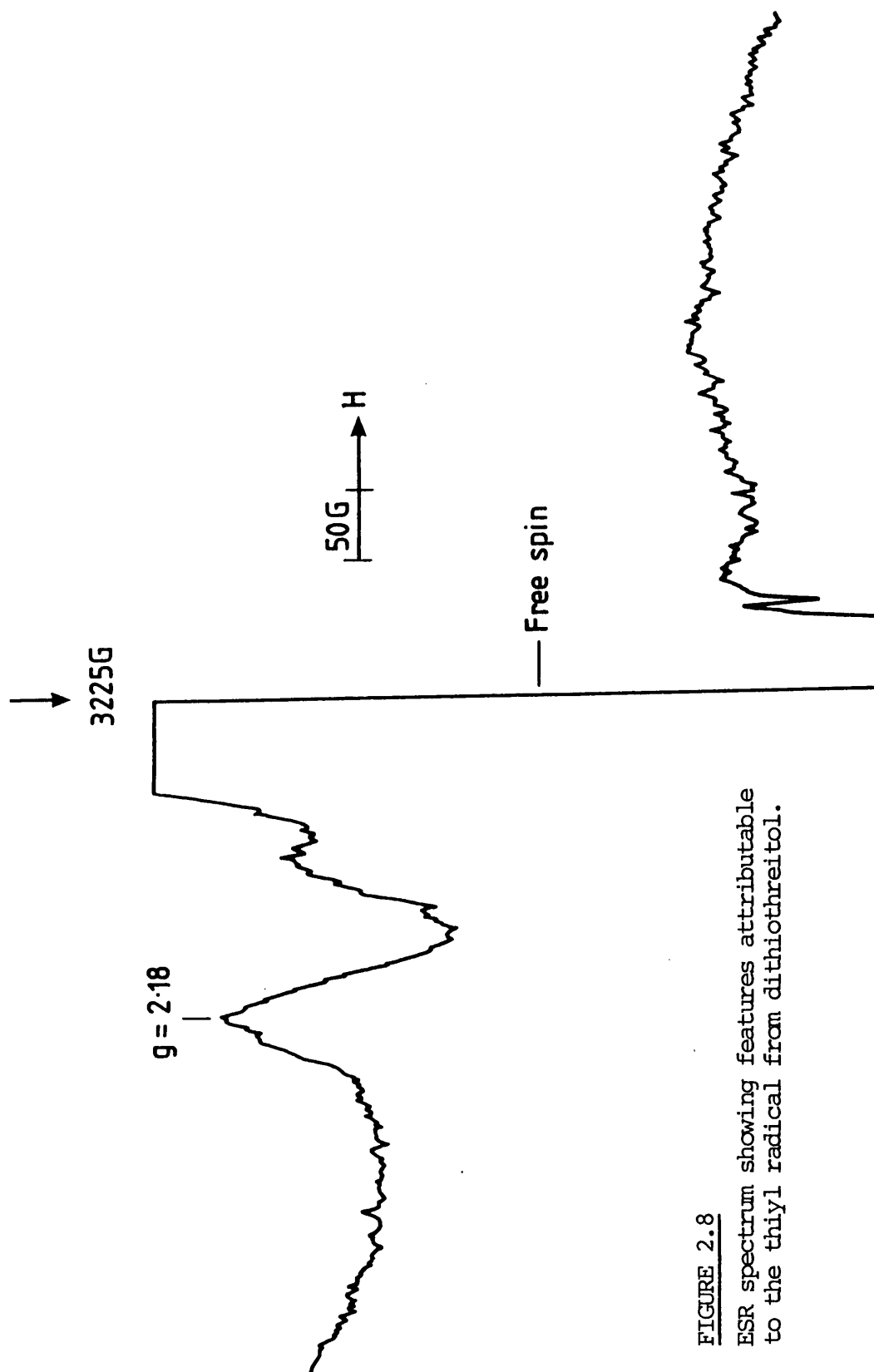
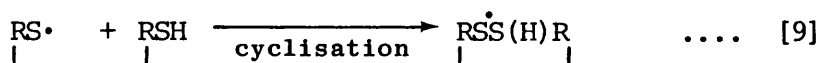
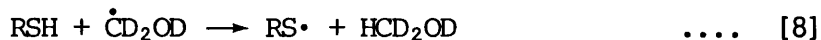


FIGURE 2.8
ESR spectrum showing features attributable to the thiyl radical from dithiothreitol.

since this is an electron-loss centre which are only rarely formed from solutes in this medium.

As before, the main point of interest arose when the sample was annealed [Fig. 2.9]. The solvent signal due to $\dot{\text{C}}\text{D}_2\text{OD}$ was lost, as was the purple colouration of the sample. In its place the normal species "X" was obtained, it was also noticed that the sample had turned yellow in colour. Previous pulse radiolysis studies⁶⁹ have indicated that a radical with an "X"-like structure according to Symons^{60,70} has an absorption band at ca. 380 nm. This ended our assignment, if this is considered along with the fact that when species "X" was observed directly on irradiation with the powder sample, this also had a yellow colour. After further annealing, as with the powder, a reduction in signal from the $\text{RCH}_2\text{S}\cdot$ radical was noticed.

It is therefore possible that the reactions involved are:



Therefore, under alkaline conditions, species "X" may lose a proton in the manner



This prompted a pH study of this particular system to investigate this possible equilibrium. Initial results seemed to support this theory [Fig. 2.10a,b]. High pH appearing to favour the formation of the radical anion, RSSR^- , shown by the spectrum consisting of a singlet with g-features at 2.02. This equilibrium is examined in greater depth in the study on lipoic acid [see Chapter 4].

In order to gain more information the L. isomer of dithiothreitol was

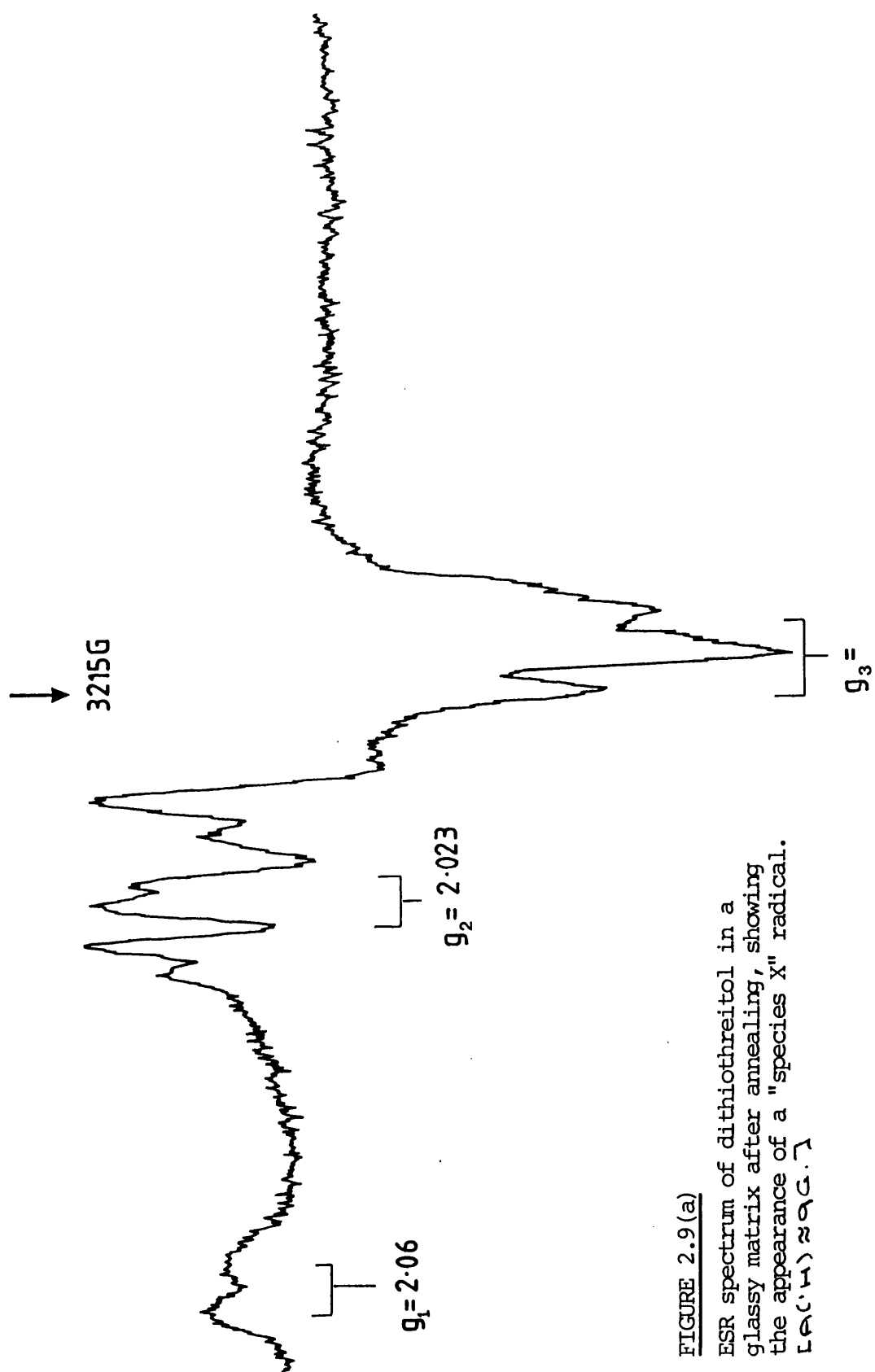


FIGURE 2.9(a)

ESR spectrum of dithiothreitol in a glassy matrix after annealing, showing the appearance of a "species X" radical.

[A(14) 396.7]

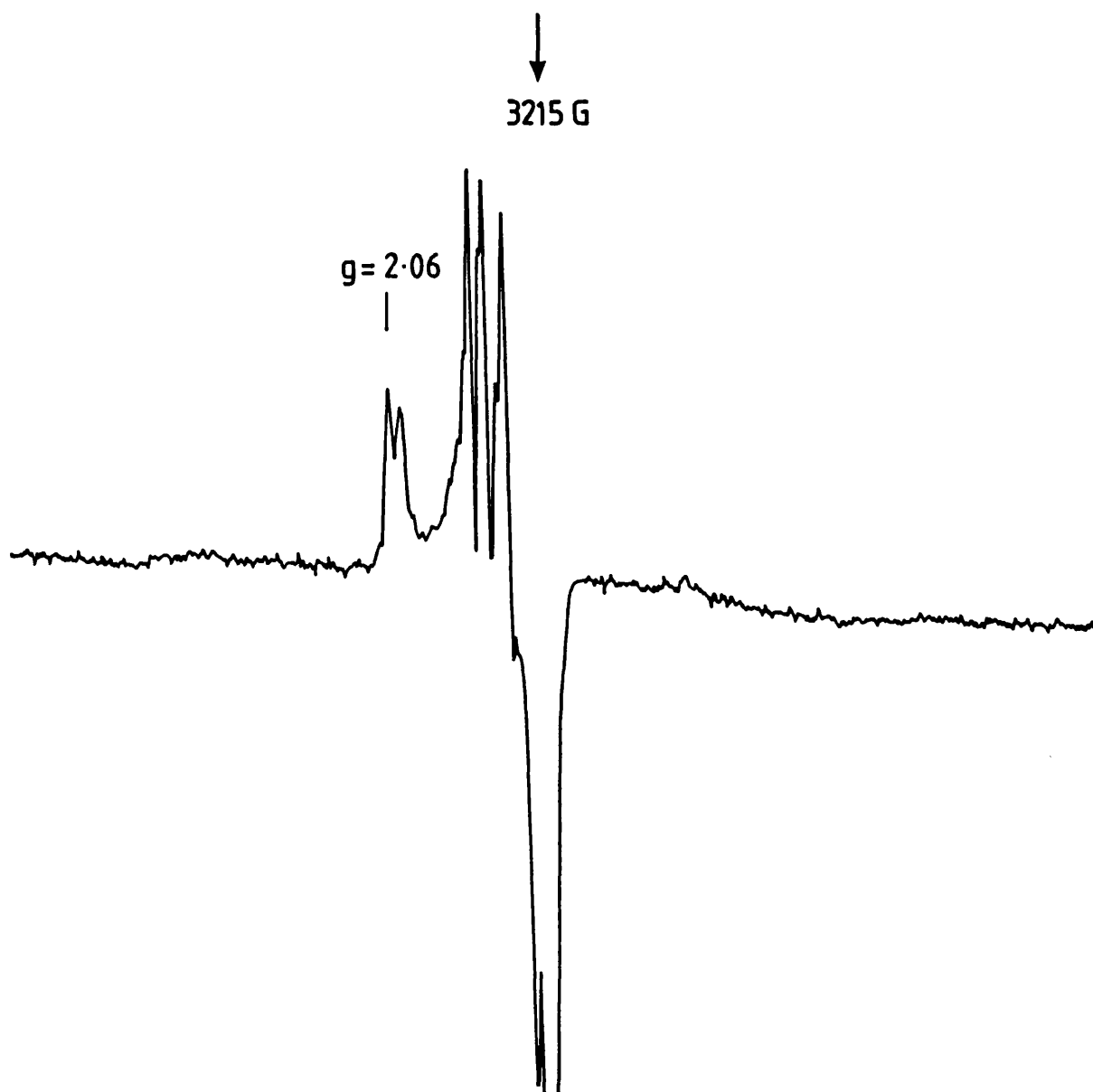


FIGURE 2.9(b)

Scan showing that the thiyl radical $RS\cdot$ was not detected. $[A(\cdot H) \approx 8G.]$

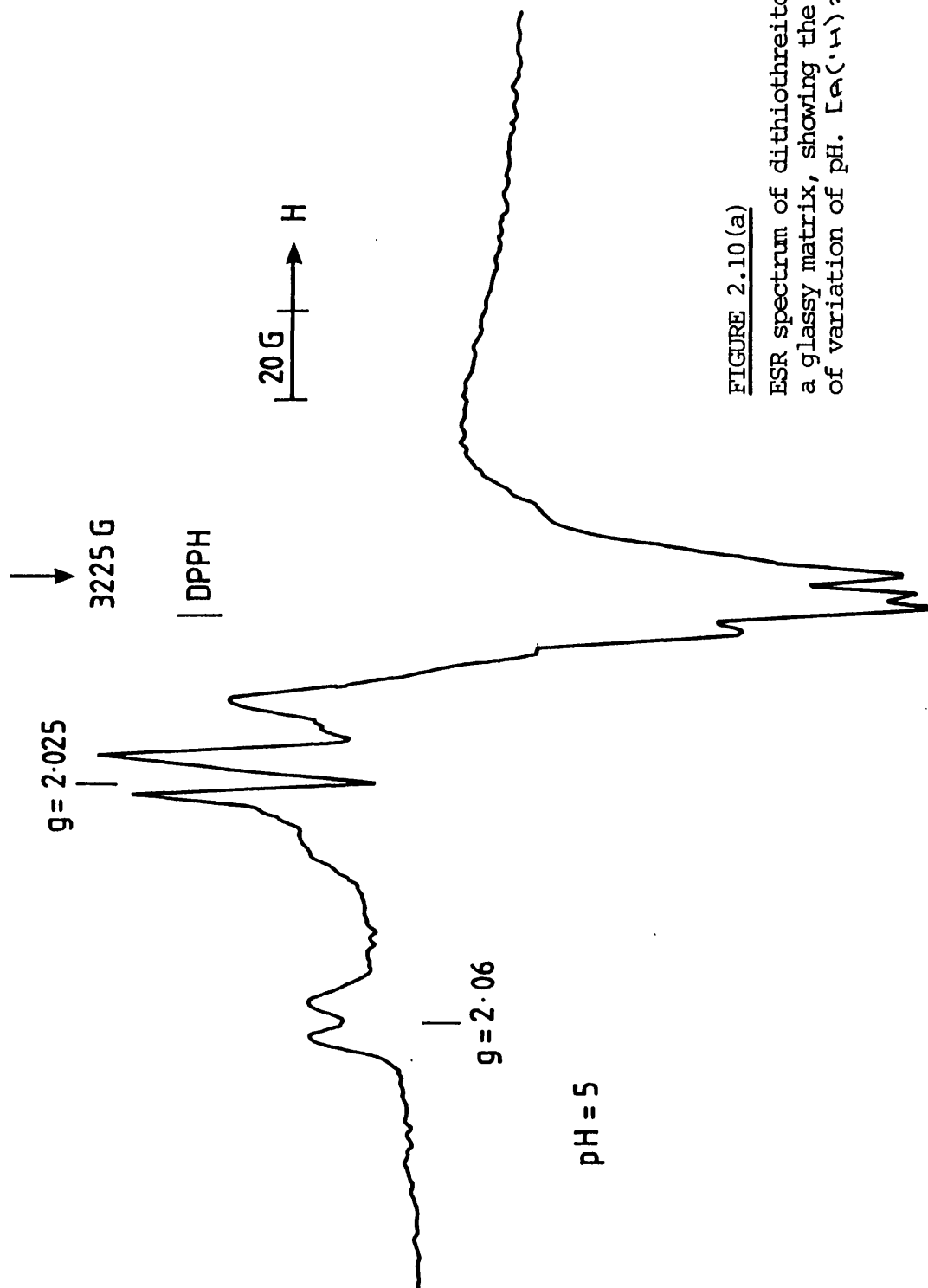


FIGURE 2.10 (a)

ESR spectrum of dithiothreitol in a glassy matrix, showing the effect of variation of pH. [A(·H) ≈ 8 G.]

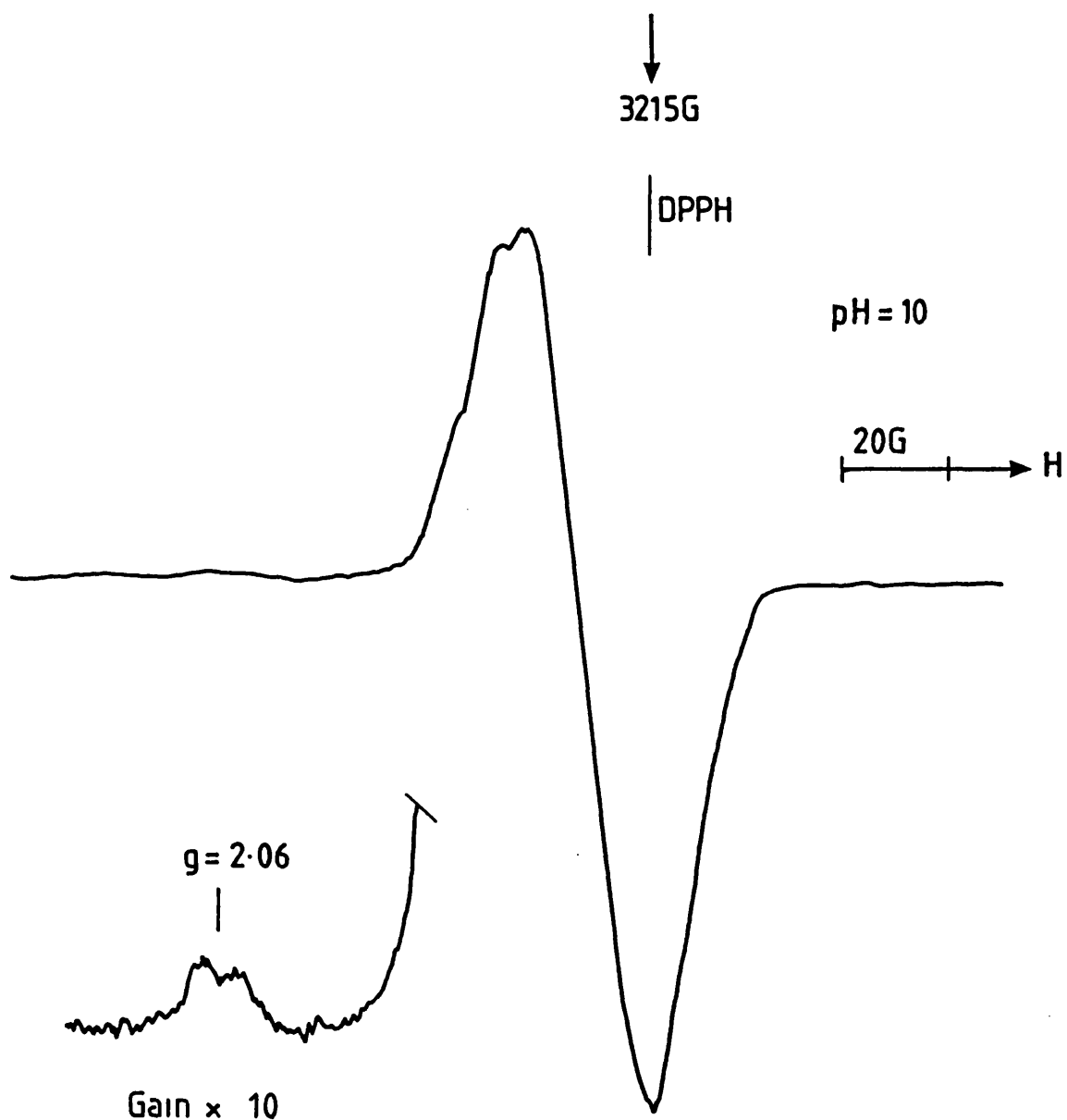


FIGURE 2.10 (b)

ESR spectrum of dithiothreitol in a glassy matrix, showing the effect of variation of pH. [$a(H) \approx 8 \text{ G.}$]

also studied both as powder and glass samples. These produced much the same final result as the D,L-isomer glass spectra with the normal form of species "X" being observed. There were, however, large differences in the initial spectra from the powder samples [Fig. 2.11a, b, c]. It can be seen that the parallel signals attributable to $\text{RCH}_2\text{S}^\bullet$ radicals in the L. isomer are quite different from those observed in the racemate. As the sample was very carefully annealed, the multiple signals in Fig. 2.11a collapse to the quadruplet in Fig. 2.11c as $\text{RCH}_2\text{S}^\bullet$ radicals formed initially in differing environments, relax to one favoured structure as the sample is warmed. This nicely illustrates the variable nature of the e.s.r. signal from $\text{RCH}_2\text{S}^\bullet$ radicals. These results were supported when the i.r. spectra of both the L. isomer and the racemate were studied. These spectra showed that hydrogen bonding to the S-H groups in the L. isomer is different from that found in the racemate. This would account for the differences in the e.s.r. signals, since g_{\parallel} is controlled by the degree of H-bonding.

In general, the L. isomer of dithiothreitol followed the pattern of events found for the racemate, namely, initial formation of $\text{RCH}_2\text{S}^\bullet$ radicals followed by the production of a signal due to species "X".

2.3.3 Possible Mechanism

This is a theme, which I return to over the next few chapters, concerning the mechanisms of reaction of thiol and disulphide compounds. For the moment if it is assumed that Symons' assignment of species "X" is correct, i.e. $[\text{RSS}^\bullet(\text{H})\text{R}]$, then a possible pathway for its production from dithiothreitol has already been postulated (see reaction [9]). As for the other compounds studied in this Chapter, the formation of species "X" would also be relatively simple [see Scheme 2A]. With dithiothreitol, at least in dilute solutions, cyclisation is expected to occur in favour

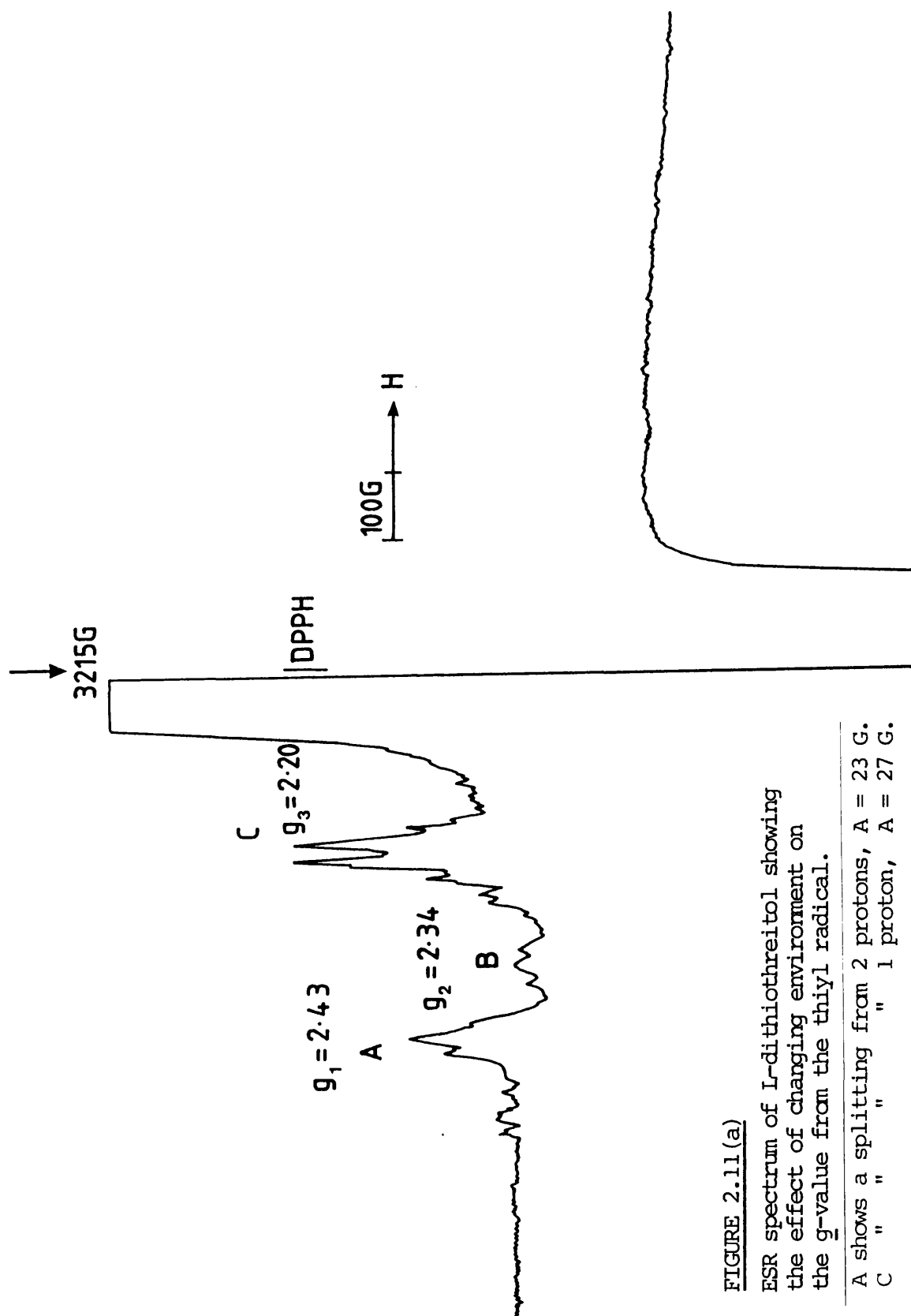


FIGURE 2.11(a)

ESR spectrum of L-dithiothreitol showing the effect of changing environment on the \bar{g} -value from the thiyl radical.

A	shows a splitting from 2 protons, $A = 23$ G.
C	" " 1 proton, $A = 27$ G.

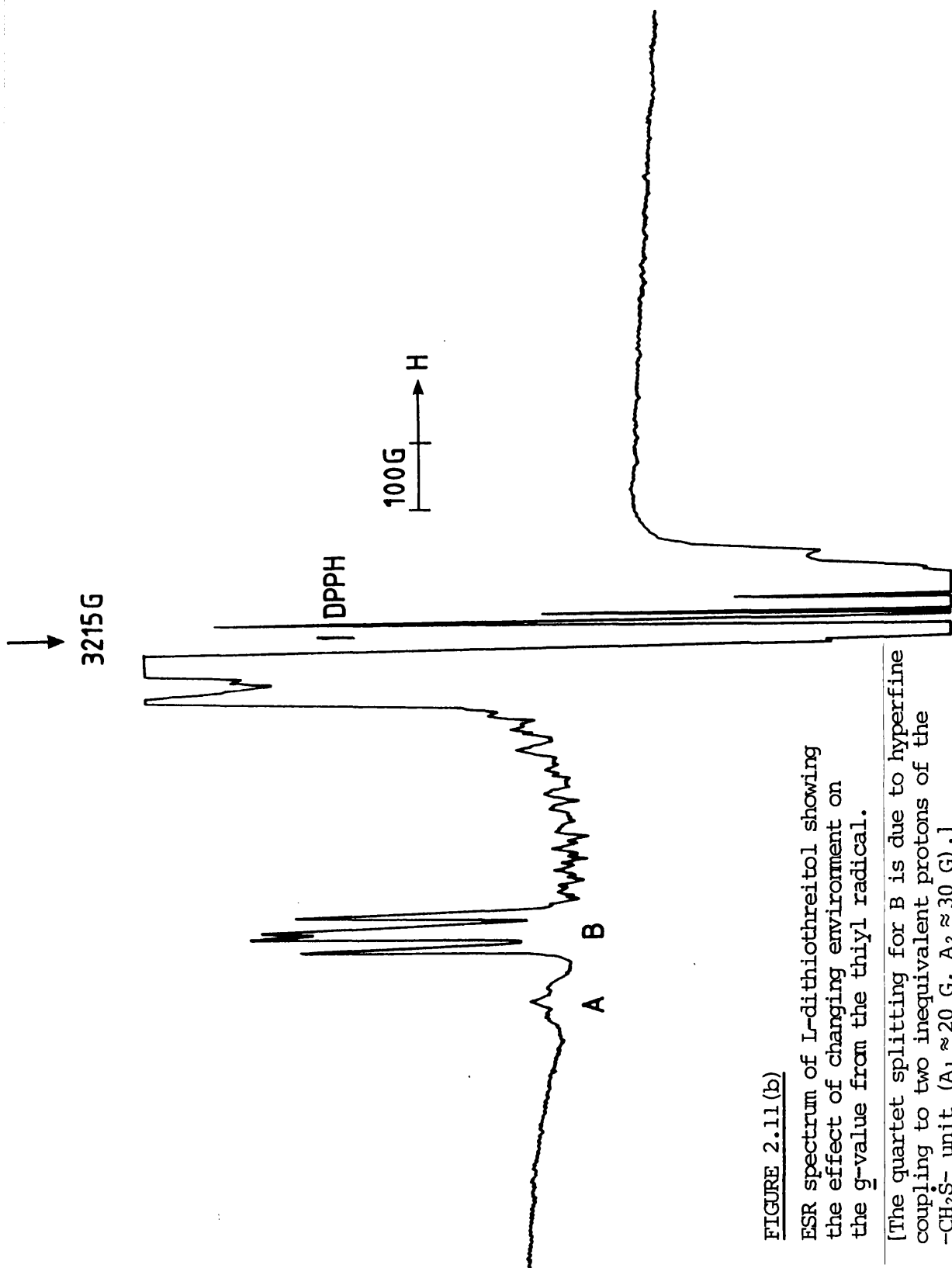


FIGURE 2.11 (b)

ESR spectrum of L-dithiothreitol showing the effect of changing environment on the g -value from the thiyl radical.

[The quartet splitting for B is due to hyperfine coupling to two inequivalent protons of the $-\text{CH}_2\dot{\text{S}}-$ unit ($A_1 \approx 20 \text{ G}$, $A_2 \approx 30 \text{ G}$).]

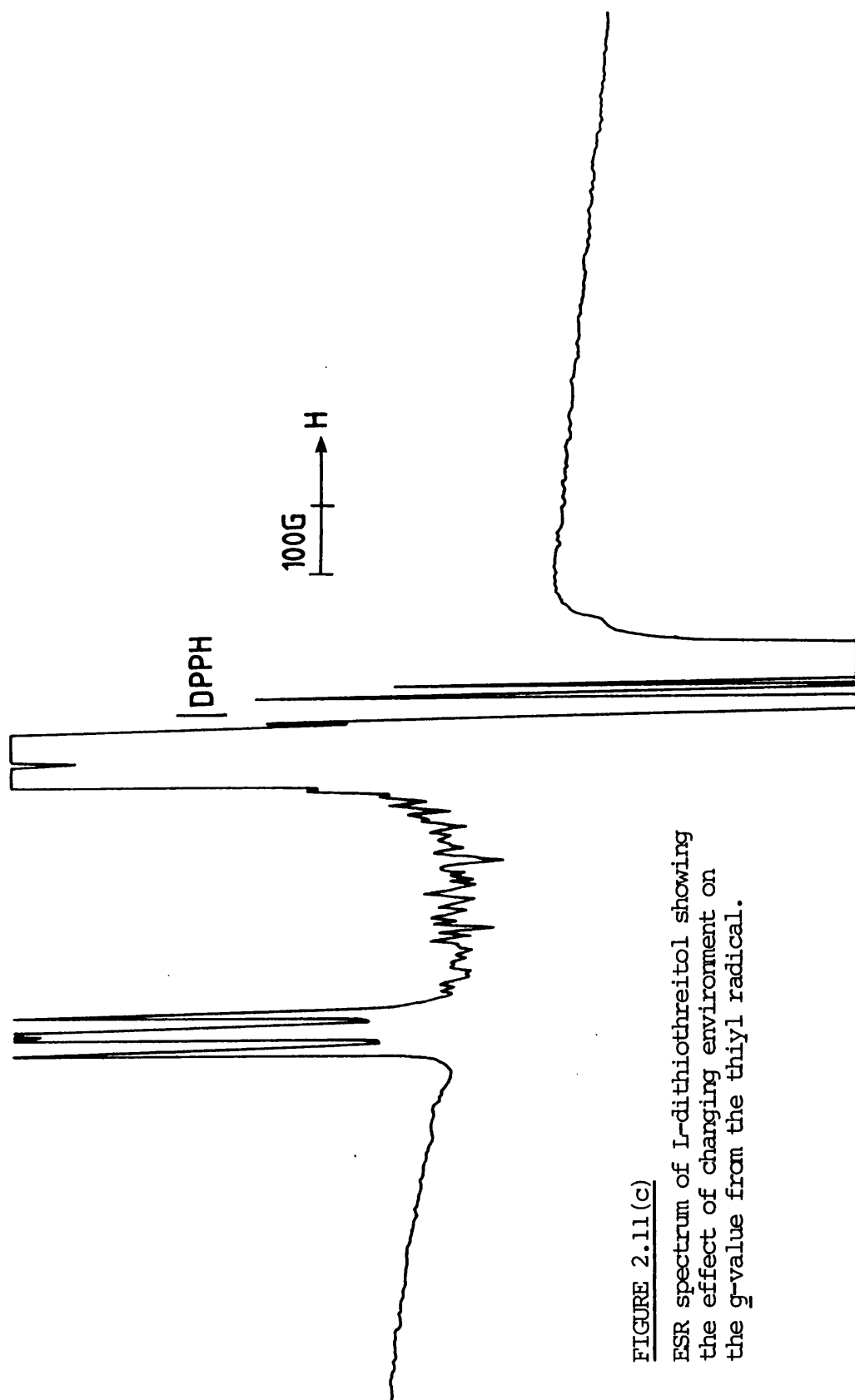
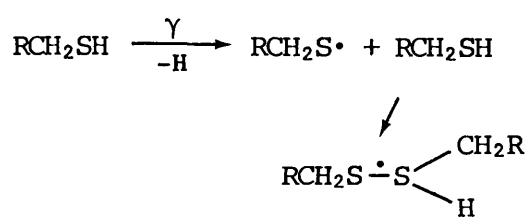
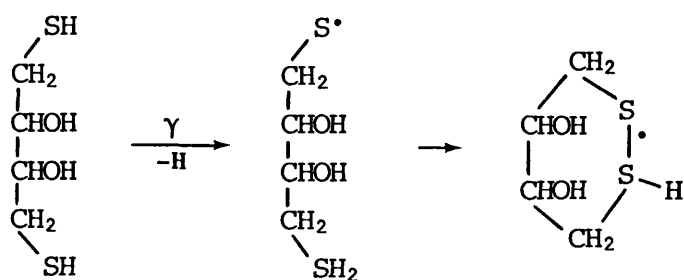


FIGURE 2.11(c)
ESR spectrum of L-dithiothreitol showing the effect of changing environment on the g -value from the thiyl radical.

SCHEME 2A



of normal "dimerisation" as occurring with the other thiol compounds. This is because the reagents are permanently held close together facilitating the cyclisation reaction. This reaction would cause the formation of a six-membered ring, part of which would be the disulphide radical. When considering the "dimers" formed by monothiols to produce species "X" radicals, the extra stability offered by the ring stricture of oxidized dithiothreitol would appear to make the cyclisation process more energetically favourable. This would appear to be supported by the pH studies on dithiothreitol, the radical anion RSSR^- being favoured in alkaline conditions with species "X" [possibly $\text{RSS}(\text{H})\text{R}$] being favoured in more acidic media. As shown previously, the pathway between the two is a simple protonation/deprotonation equilibrium.

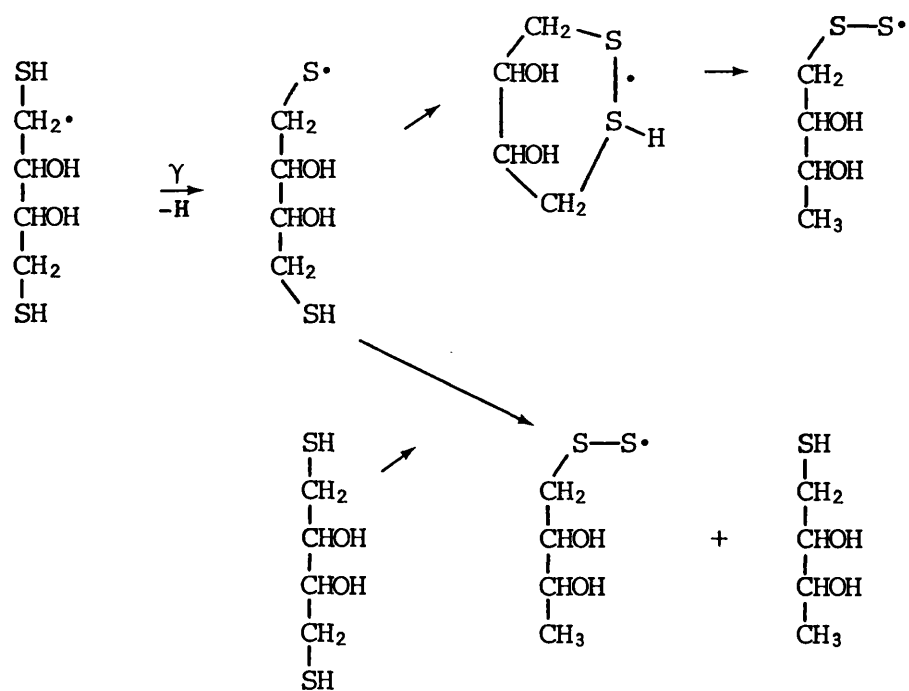
If the alternative perthiyl assignment of species "X" is considered, then the pathway to its formation is somewhat less straightforward, both for dithiothreitol [Scheme 2B] and for the other thiol compounds [Scheme 2C]. In the case of dithiothreitol the "stable" six-membered ring would not be formed, instead the possibly more reactive perthiyl radical would remain. Also, we are unable to formulate any pH dependent link between the formation of the radical anion and $\text{RSS}\cdot$ radicals such as was observed for dithiothreitol.

2.3.4 Concluding Remarks

The variability of the spectra for $\text{RCH}_2\text{S}\cdot$ radicals was apparent throughout this study, more particularly with the L. isomer and racemate samples of dithiothreitol, showing its dependence on its environment.

One of the major results to come from this study is that these compounds appear to be damaged initially to give $\text{RCH}_2\text{S}\cdot$ radicals, and that these go on to give species "X" via the mechanisms postulated. This was supported by the radical intensity experiments on dithiothreitol and

SCHEME 2B



SCHEME 2C



it is conceivable that, in future work, this should be extended to cover the other thiols.

Taken together with the other results obtained from dithiothreitol, i.e. cyclisation, the pH study and the production of a yellow colouration in the glass system as predicted,⁶⁹ the results of dithiothreitol would appear to favour Symons' assignment.



CHAPTER 3

Aromatic Thiols

3.1 INTRODUCTION

The monomer and dimer forms of the compound [Fig. 3.1] studied in this Chapter are important examples of radio-protective drugs currently under test. The general purpose of this study was two-fold. It was hoped that experimentation on the radiation chemistry of this compound would lead to greater understanding of its possible beneficial use in in vivo studies.

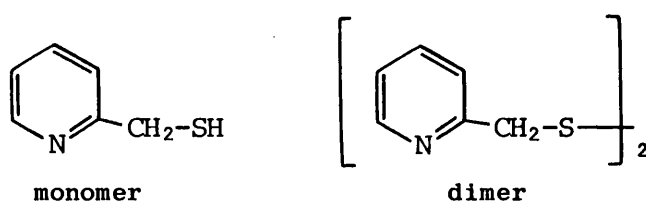
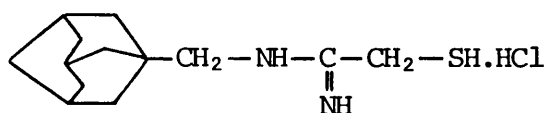


FIG. 3.1

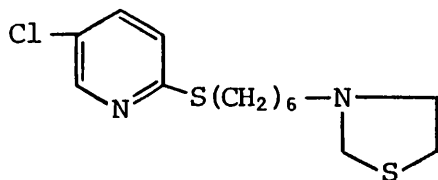
The Structure of 2-pyridinemethanethiol (monomer and dimer) forms.

That aromatic sulphur containing compounds might be of use in radio-protection has been supported by various workers.^{33,74} Vos and Roos-Verhey⁷⁴ studied three compounds designated WR109342, WR158490 and WR159243 [Fig. 3.2] and observed dose reduction factors (DRF) of 1.7 and 1.8 after oral administration to mice [the DRF in mice was calculated by dividing the L.D._{50/30} of protected mice by the L.D._{50/30} of non-protected mice]. However, blood taken from these mice offered virtually no protection to HeLa cells in vitro whereas blood from cysteamine-treated mice did provide protection. From these results Vos and Roos-Verhey postulated a different mechanism of action for the aromatic drugs compared with that for cysteamine.

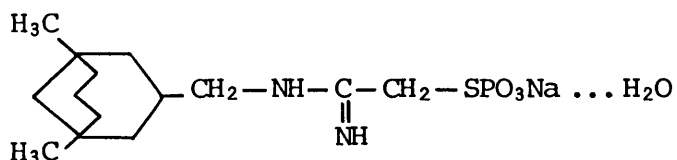
The relationship between these compounds⁷⁴ and those discussed in this Chapter is not marked. Work far more applicable to results presented in this Chapter was published by Barnes et al.³³ on N-hetero-



WR 109342 : N-(1-adamantanemethyl)-2-mercaptoacetamidinium chloride



WR 158490 : 5-Chloro-2(3-thiazolidinyl)heptyl thio pyridine



WR 159243 : Sodium hydrogen S-(3,5-dimethyl-1-adamantanemethyl amidino)methylphosphorothioate

FIG. 3.2

The Structures of compounds studied by Vos and Roos-Verhey [Ref. 74].

cyclic compounds as radio-protectors, the more important being that studied in detail in this Chapter, namely, 2-pyridinemethanethiol [Fig. 3.1].

As stated in Chapter 1, over the past few years numerous modifications of the chemical structure of 2-aminoethanethiol (MEA, the classical radioprotecting agent) have been made with the express purpose of obtaining agents having improved radioprotective properties. This has been done to obtain more information concerning the relationship between the structure and the biological properties of compounds within this category. These studies are summarised by Klayman and Copeland⁷⁵ and by Sweeney.⁷⁷

The work of Barnes et al.³³ was also concerned with these objectives. The difference in this case was the synthesis and screening in mice, of compounds having a different structural feature. This was the fact that the nitrogen atom and its adjacent carbon atom of the MEA moiety form part of the heterocyclic compound 2-pyridinemethanethiol. Therefore, 2-pyridinemethanethiol can be regarded as an aromatic analogue of MEA. However, unlike MEA, which exists as a highly polar zwitterion and is a water soluble solid,³³ the pyridine compound exists as an oil which is poorly water soluble. The aromatic compound was observed to provide good protection against γ -radiation, when administered intraperitoneally; furthermore, it was protective against a supralethal dose of radiation. The DRF being close to that for MEA when injected under similar conditions.

The corresponding disulphide derivative [Fig. 3.1] provided one hundred per cent protection against an L.D.₁₀₀ ($\frac{1}{2}$ L.D.₅₀) of radiation; this suggests that the disulphide like cysteamine may be cleaved in vivo to the thiol. When given by the oral route both 2-pyridinemethanethiol and its corresponding disulphide were significantly protective but less so when given intraperitoneally; MEA given by the oral route has no radioprotective action.

Hence it has been shown that these aromatic compounds do possess a degree of radioprotective efficiency. However, there would appear to be some doubt as to the mechanism of action of these particular compounds.

In Chapter 2 the opportunity to contain a radical with a $RS-\dot{S}R_2$ structure, within a more rigid framework was first observed. This concept is expanded here with the disulphide form of 2-pyridinemethanethiol, especially with respect to the prospect of the disulphide cleaving

in vivo to give the thiol.

The structural resemblance of 2-pyridinemethanethiol to cysteamine which had already been noted,³³ led to the question as to whether these aromatic compounds had similar behaviour to cysteamine in terms of their radiation chemistry in vitro. It would also reinforce the possibility of species "X" being a direct product from the radioprotective reactions of these sulphur containing compounds, making the structure of the species "X" radical of even greater importance.

In an attempt to clarify some of these points, the study of the effects of γ -radiation on 2-pyridinemethanethiol was undertaken.

3.2 EXPERIMENTAL

Both 2-pyridinemethanethiol hydrochloride and its associated dimer derivative were obtained from the Royal College of Military Science, Shrivenham, and were used without further purification. Powder and glassy solution samples were prepared and e.s.r. studies were carried out as per the Experimental section in Chapter 2.

3.3 RESULTS AND DISCUSSION

As has been stated in the Introduction to this Chapter, this study concentrates on the monomer and dimer forms of 2-pyridinemethanethiol hydrochloride [Fig. 3.1]. The results are based on the powder and deoxy CD₃OD/D₂O (70:30) glass spectra of both of these forms. All were irradiated and studied at 77 K.

3.3.1 Powder Spectra (dimer)

The compound is a yellow powder (stable at room temperature) which did not change colour on irradiation. When the spectrum is examined [Fig. 3.3] features are seen at ca. $g=2.058$ and 2.025 as well as a

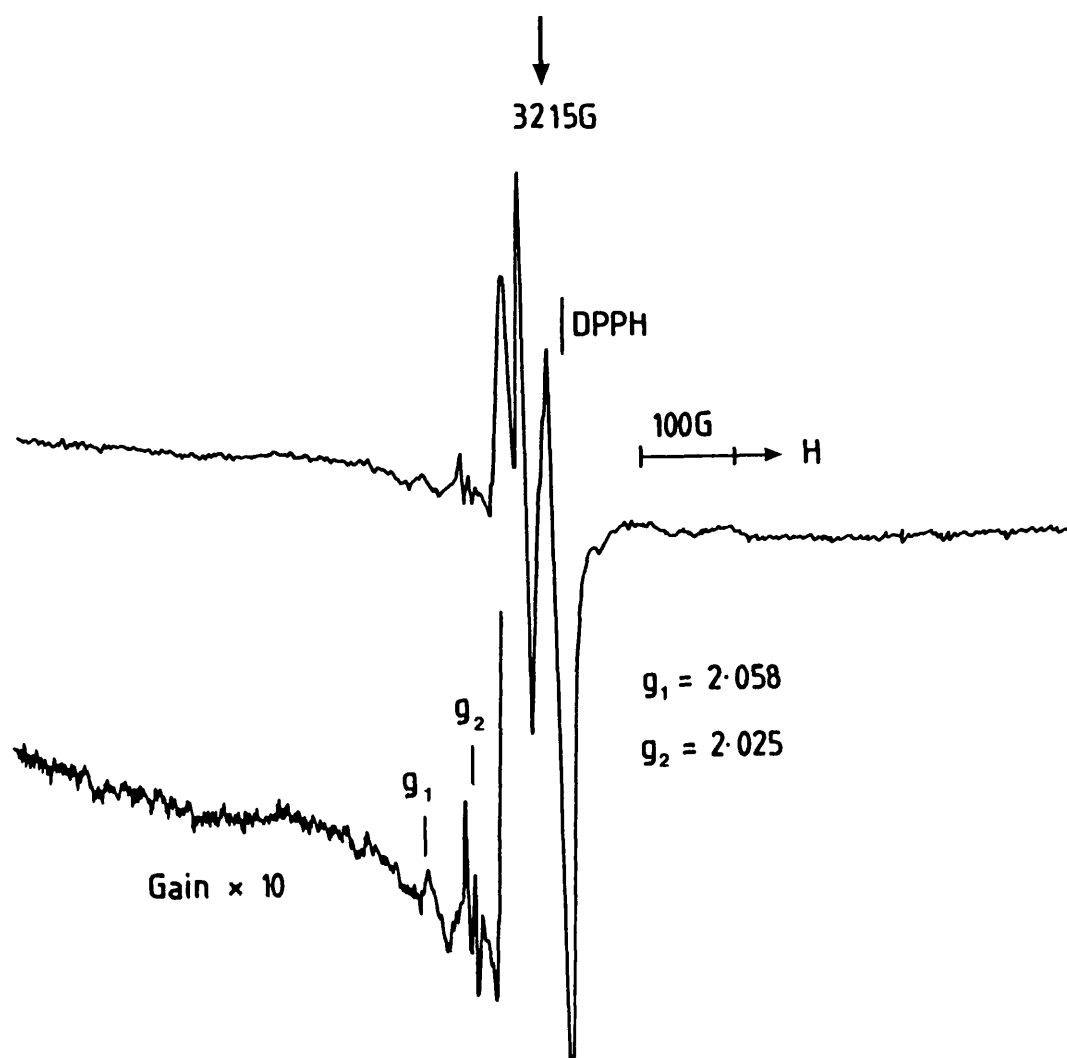


FIGURE 3.3

ESR spectrum of 2-pyridinemethanethiol (dimer) powder taken at 77 K after exposure to γ -radiation.

major central signal centred around free spin. These features are easy to identify, as they are fairly good fingerprints for the species "X" radical, the third feature for "X", at $g = 2.002$, being "lost" within the central signal in the spectrum.

The central signal itself is very large and broad in comparison with those features ascribed to species "X". It is suggested that this central signal is due not only to the radical anion $RS\dot{-}SR^-$ but to other unidentified radical centres.

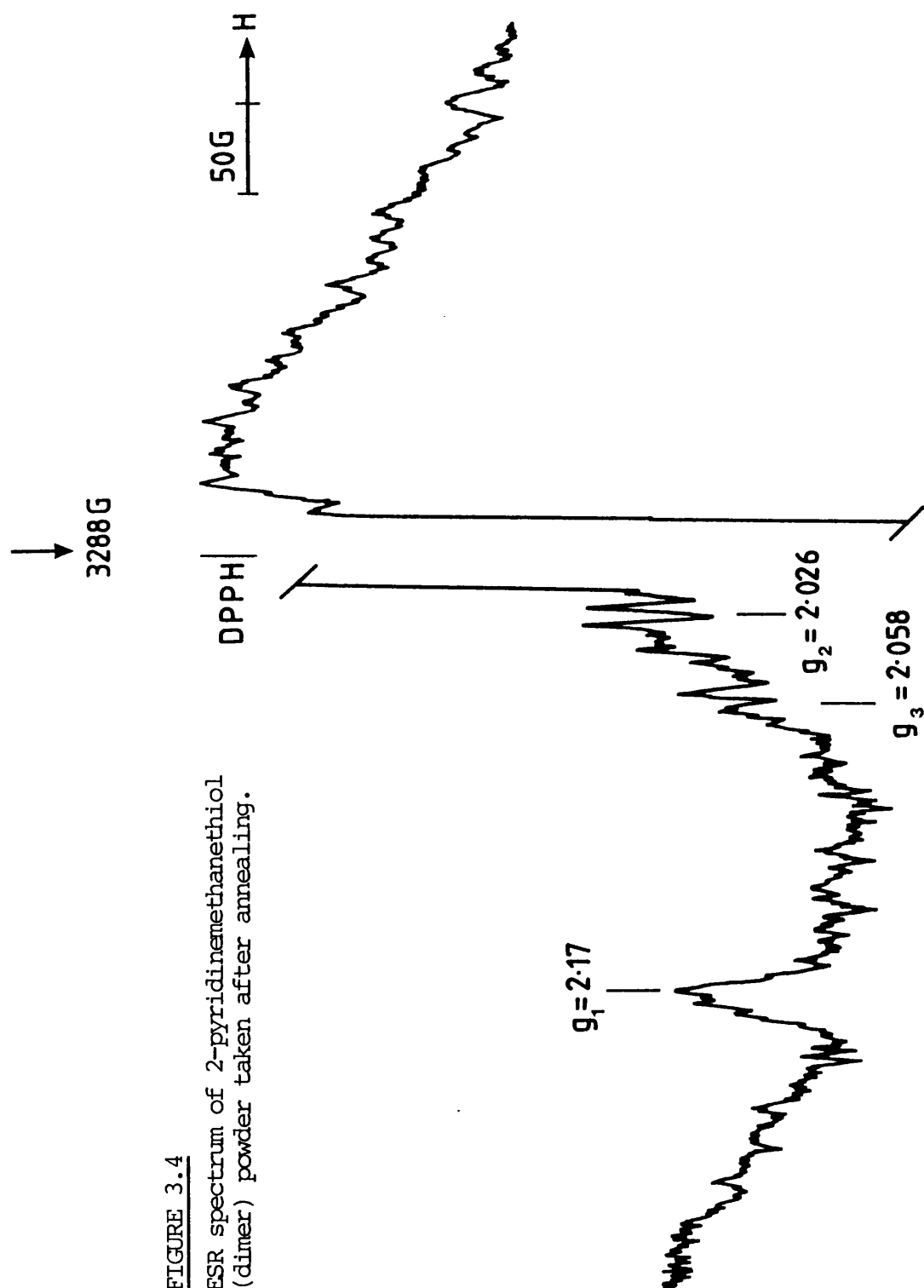
As with other compounds the main point of interest arose when the sample was annealed. The spectrum [Fig. 3.4] showed species "X" still present with its clear features at $g = 2.058$ and 2.026 . However, a new feature at $g = 2.17$ grew in, which displayed a triplet-like structure. From our own previous studies [see Chapter 2] this signal was assigned to an $RCH_2S\cdot$ type radical. The significance of this is discussed later in this section.

3.3.2 Deoxy CD_3OD/D_2O Glass Spectra (dimer)

To be able to compare these results with those of dithiothreitol, etc., samples of the dimer were also prepared in oxygen-free CD_3OD/D_2O glass matrices. Immediately after irradiation at 77 K the sample had a deep purple colour and its spectrum consisted of an intense singlet centred about free spin. The colour is assigned to "trapped" or solvated electrons within the system and the remaining signal to $CD_2\cdot OD$ radicals [Fig. 3.5]. No other signals were observed.

The sample was then carefully annealed. The purple colouration changed to yellow and signals due to solvent radicals were reduced. The yellow colour strongly suggests the presence of $RS\dot{-}SR^-$ radical anions,^{53,69} or possibly species "X". When the e.s.r. spectrum for this annealed sample was examined [Fig. 3.6], features attributable to species

FIGURE 3.4
ESR spectrum of 2-pyridinemethanethiol
(dimer) powder taken after annealing.



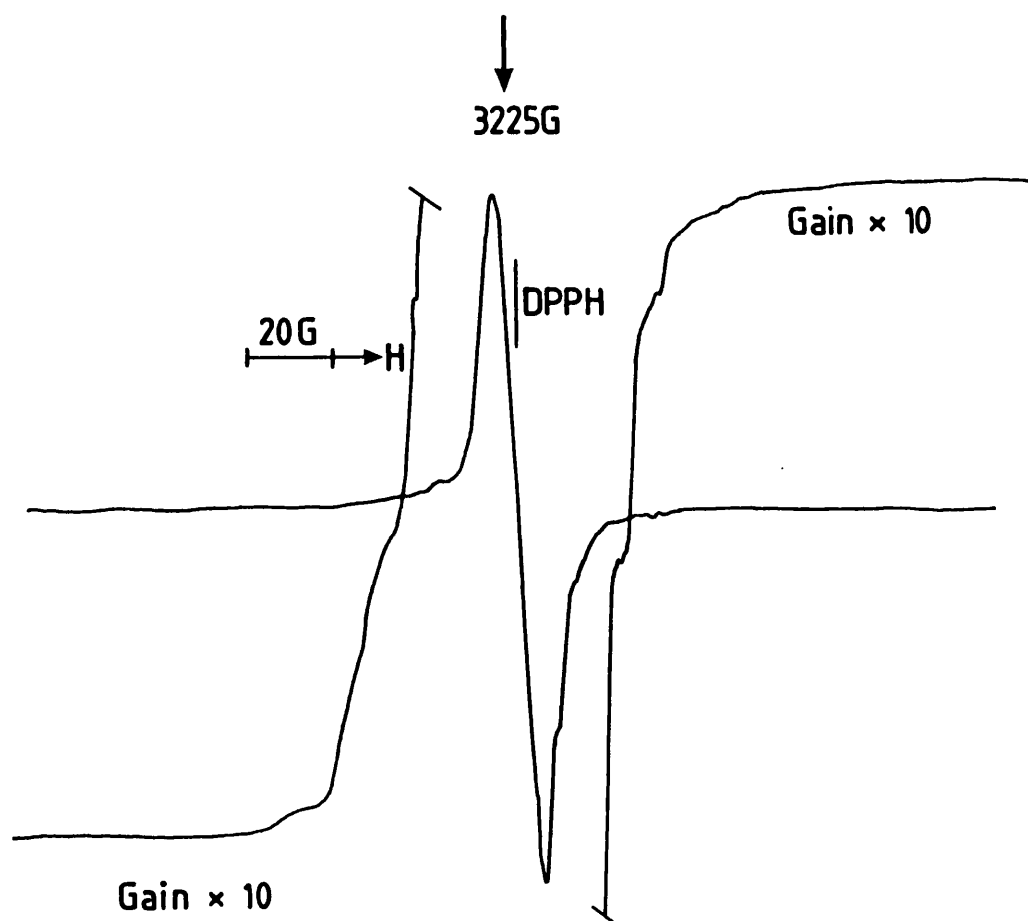


FIGURE 3.5

Initial ESR spectrum of 2-pyridinemethanethiol (dimer) in a $\text{CD}_3\text{OD}/\text{D}_2\text{O}$ glassy matrix showing solvent radicals observed at 77 K.

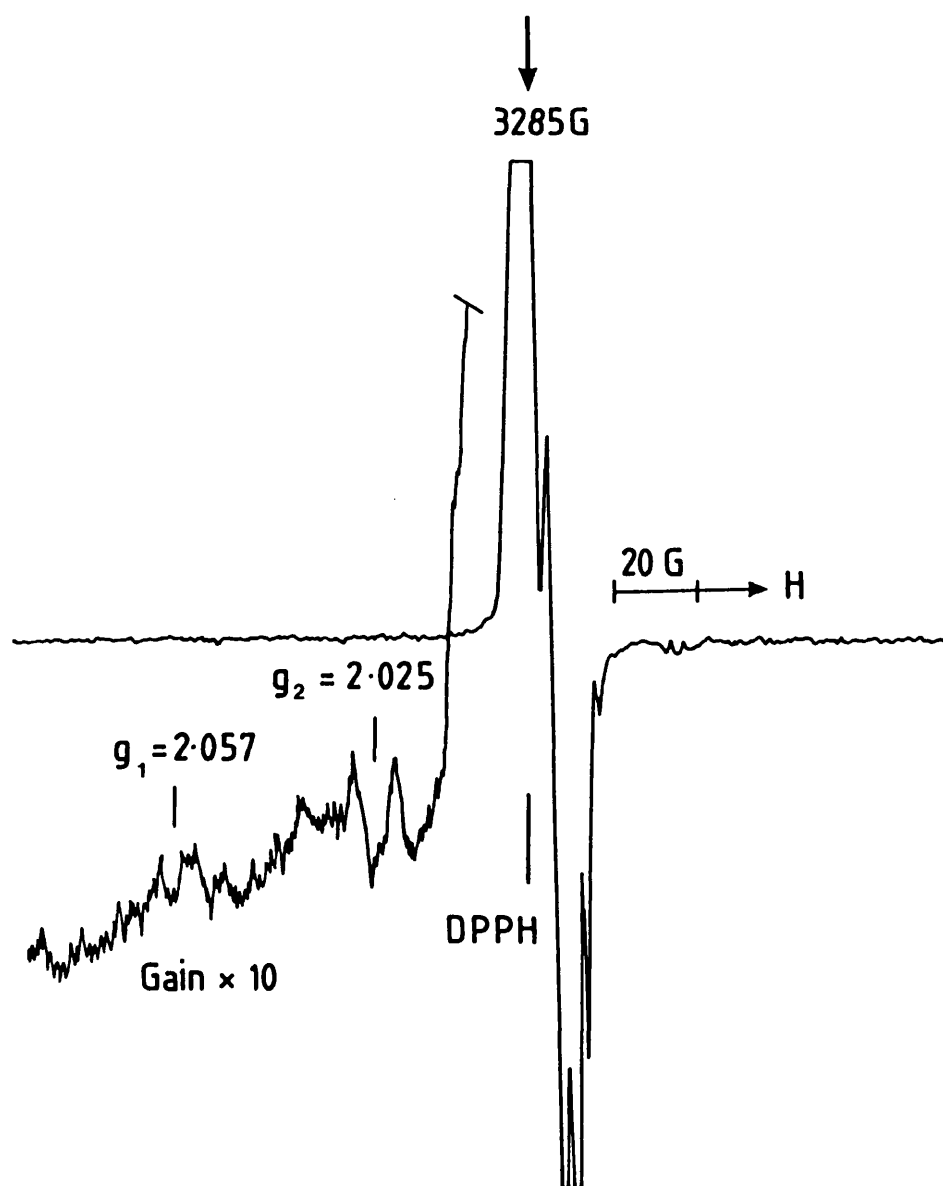


FIGURE 3.6

ESR spectrum of 2-pyridinemethanethiol (dimer) in a $\text{CD}_3\text{OD}/\text{D}_2\text{O}$ glassy matrix after annealing, showing production of a species "X" type radical.
 $[\Delta(\text{H}) \sim 8 \text{ G.}]$

"X" were detected at $g = 2.057$ and 2.025 . As with its powder counterpart, the third feature for this radical at 2.002 was masked within the central signal. It is suggested that this central signal is due to a single radical, in this case, the radical anion $RS^{\cdot-}SR^{\cdot-}$ ^{69,78}. This being the predicted product of samples irradiated in this matrix.³⁸ A wider scan showed clearly features at $g = 2.20$ and 2.35 [Fig. 3.7] which, as with other samples, were assigned to an RCH_2S^{\cdot} radical. Hence the dimer of 2-pyridinemethanethiol hydrochloride behaves similarly both as a powder sample or in a glassy system. Attention is now turned to the monomer.

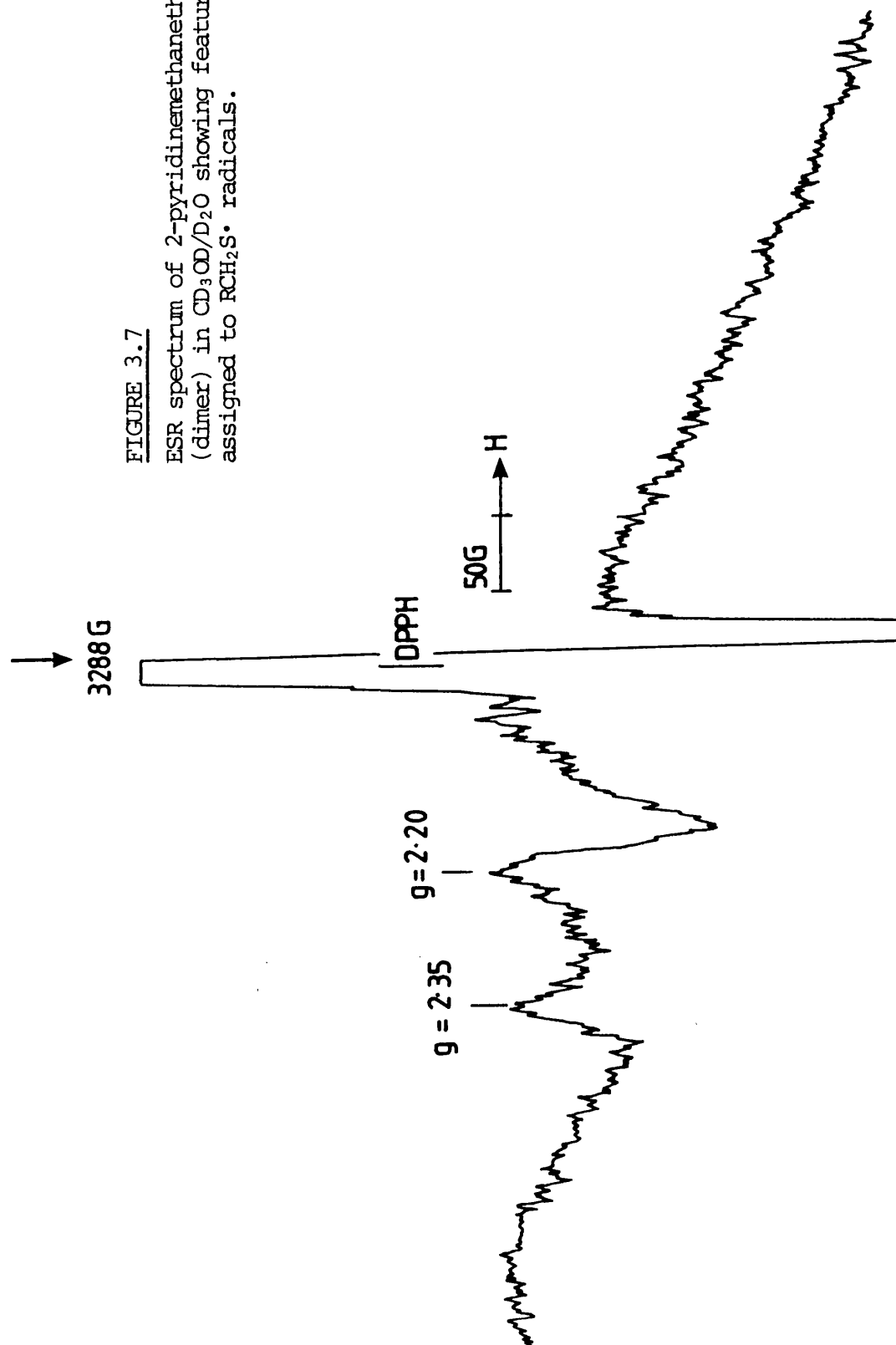
3.3.3 Powder Spectra of 2-pyridinemethanethiol (monomer)

As with dithiothreitol the monomer is a white powder which turned yellow on irradiation. The similarity with dithiothreitol continued when spectra of this sample were taken. In contrast with the dimer, at 77 K there were no features attributable to species "X" [Fig. 3.8]. There is a central signal due in all possibility to a combination of the radical anion and cation of the parent compound. There was, however, a signal due to RCH_2S^{\cdot} radicals at $g = 2.23$.

The first anneal again showed considerable change in the spectra observed [Fig. 3.9]. Immediately noticeable was the growth of a clear "X"-like species with features at $g = 2.055$ and 2.027 . The central signal could be attributed to the radical anion, with the third feature of species "X" "lost" within this larger signal. The parallel feature for RCH_2S^{\cdot} radicals at $g = 2.23$ was still present but with reduced intensity. This became even more pronounced after further annealing experiments when the colour of the sample became pale yellow with the g_1 feature of the "X"-like species present in the e.s.r. spectrum, whilst the signal due to RCH_2S^{\cdot} radicals were undetectable [Fig. 3.10a,b].

FIGURE 3.7

ESR spectrum of 2-pyridinemethanethiol (dimer) in $\text{CD}_3\text{OD}/\text{D}_2\text{O}$ showing features assigned to $\text{RCH}_2\text{S}^\bullet$ radicals.



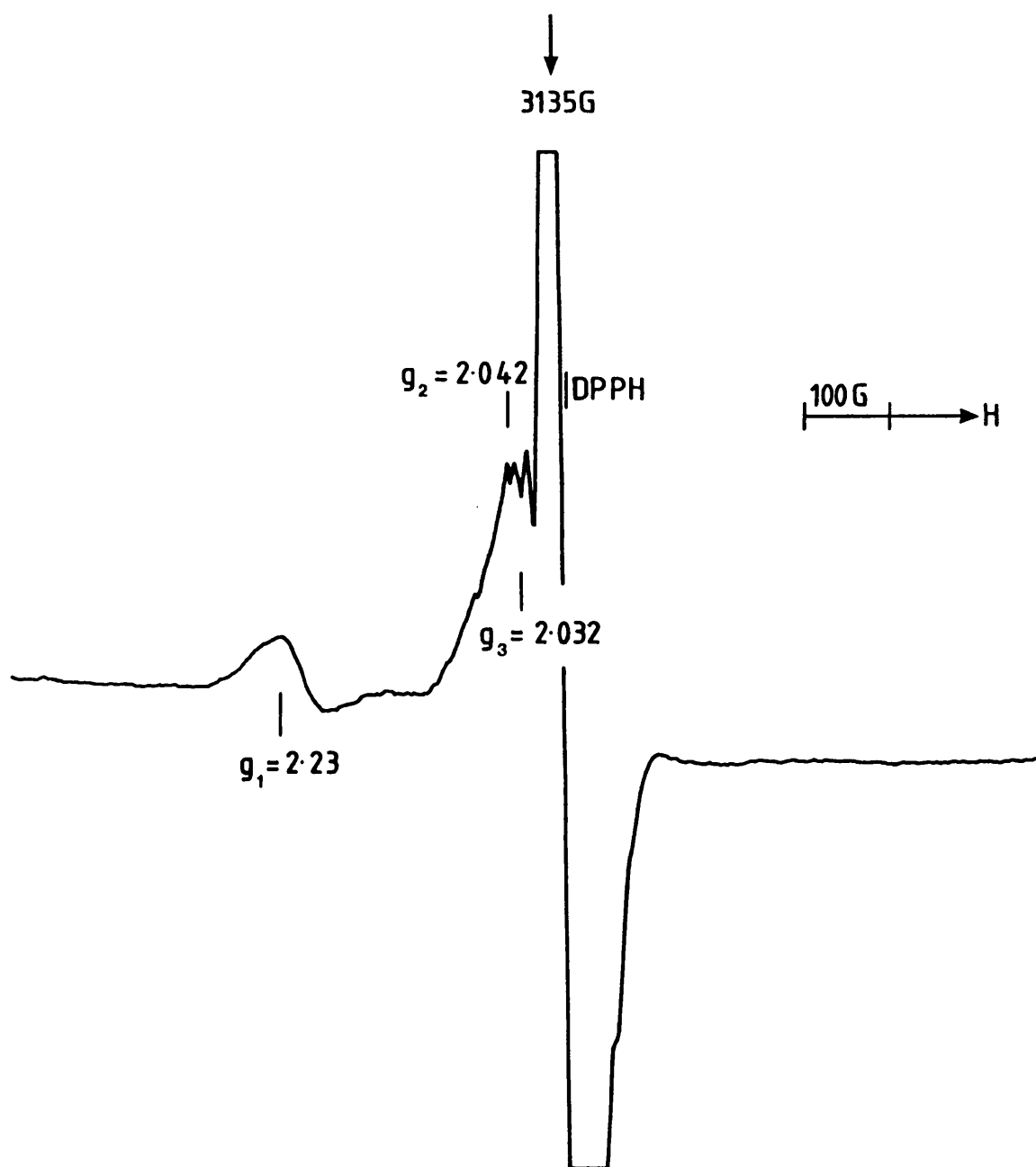
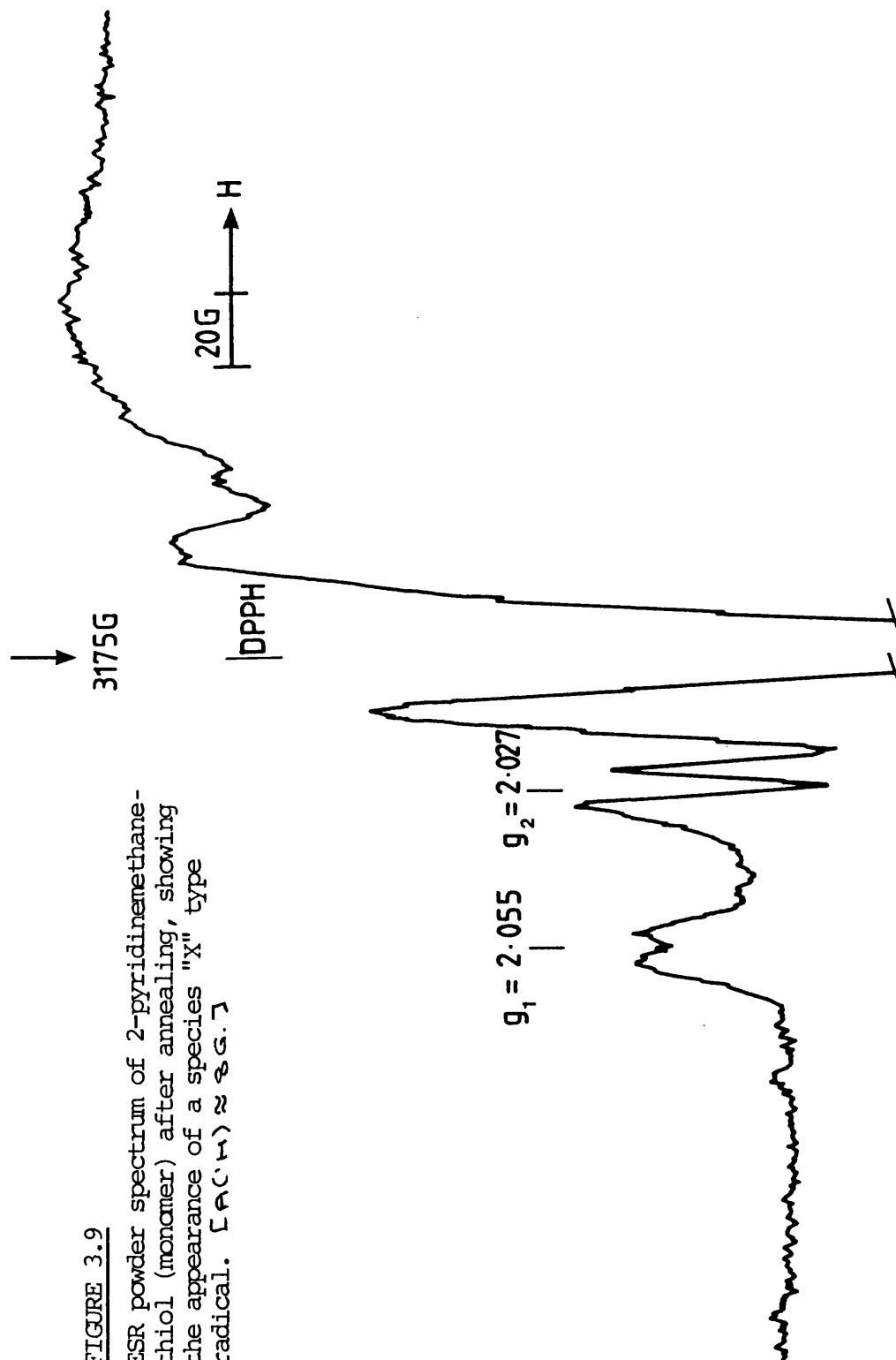


FIGURE 3.8

ESR powder spectrum of 2-pyridinemethanethiol (monomer) showing a feature assigned to an $\text{RCH}_2\text{S}^\bullet$ radical.

FIGURE 3.9

ESR powder spectrum of 2-pyridinemethanethiol (monomer) after annealing, showing the appearance of a species "X" type radical. $[A(\cdot H)] \approx 8 \text{ G.}$



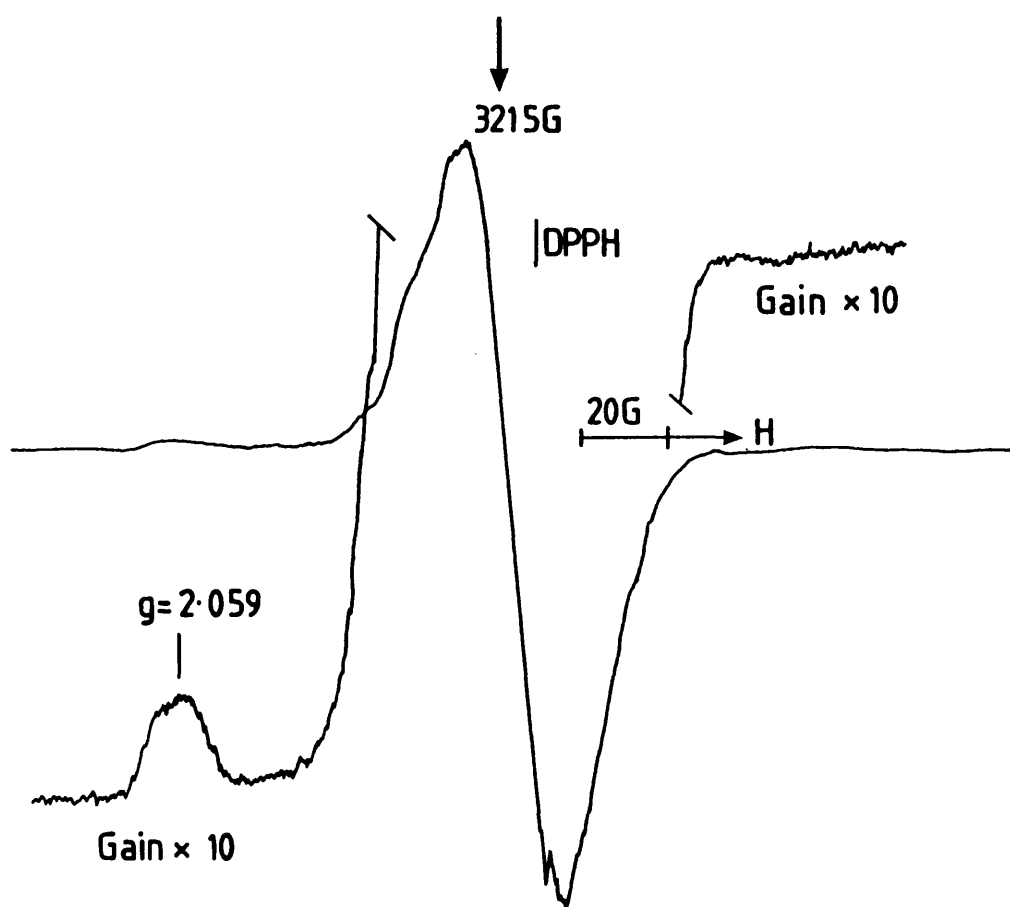


FIGURE 3.10(a)

ESR spectrum showing a species "X" radical.

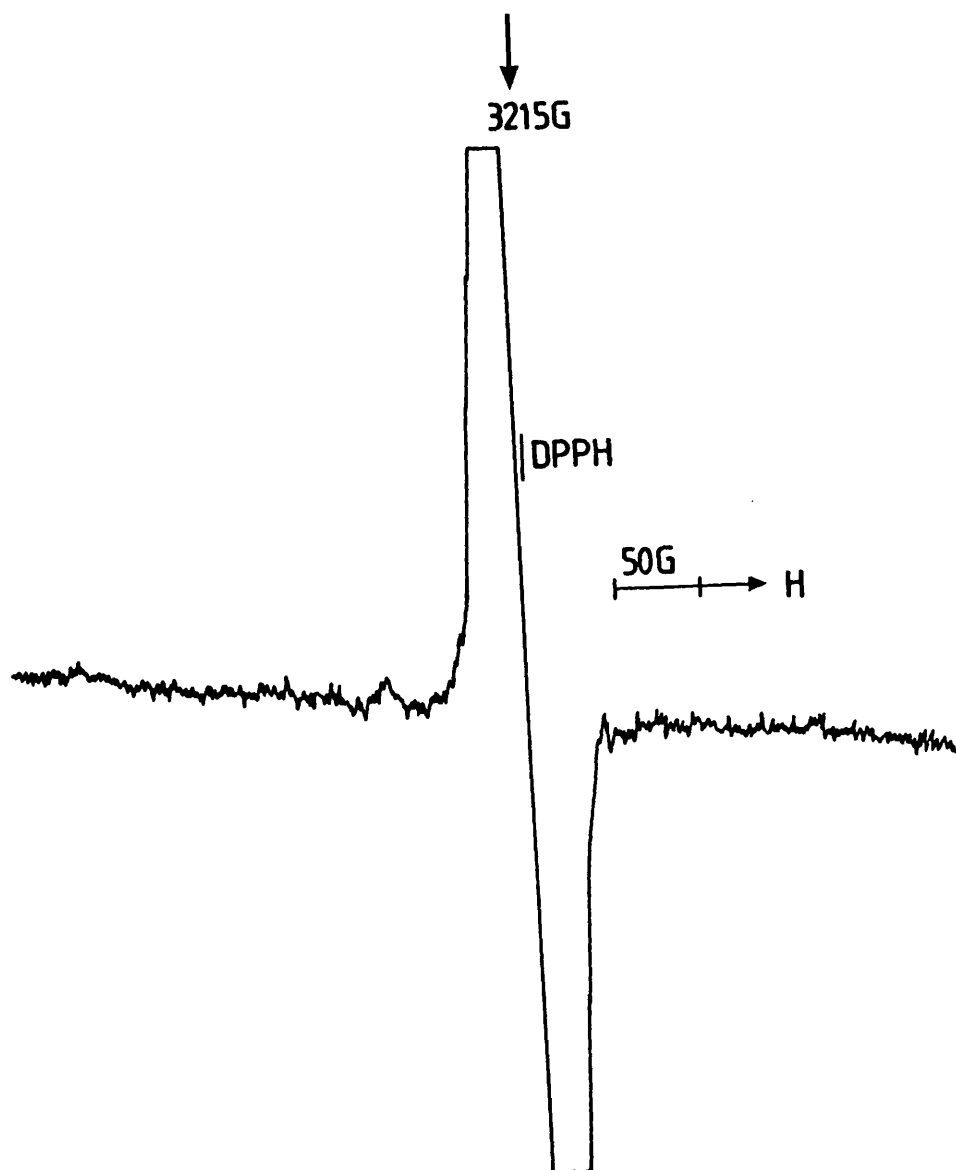


FIGURE 3.10(b)

ESR spectrum with no detectable $\text{RCH}_2\text{S}\cdot$ radicals.

3.3.4 Deoxy CD₃OD/D₂O Glass Spectra (monomer)

The pattern set by the powder experiments with the monomer was adhered to here with experiments in a CD₃OD/D₂O glass matrix. Again, a parallel feature at $g = 2.24$ is assigned to RCH₂S• radicals [Fig. 3.11]. After the sample was annealed, an "X" type radical was observed, together with a yellow colour of the sample [Fig. 3.12]. Further annealing experiments showed a relative strengthening of species "X" and a diminution of the RCH₂S• species [Fig. 3.13].

3.3.5 Concluding Remarks

Geoffroy and Reddy⁷⁹ suggested that sulphur containing aromatic compounds could be very important in future radioprotection studies and, as stated in the Introduction to this Chapter, this was partly the basis for this particular study. However, it was the structure of species "X" which was paramount in this section. It was hoped that by using the more rigid framework of the disulphide dimer more evidence could be brought to light. It is suggested here that this has, up to a point, been successful.

The results are considered in turn beginning with the dimer.

The experiments carried out both with powder and dilute glassy samples produced much the same results concerning the reactions of species "X". It was noted that, unlike previous compounds studied, the initial radical observed was species "X". Then after the sample was annealed not only species "X" but RCH₂S• radicals were observed. If it is assumed that RCH₂S• and species "X" are inter-related (as postulated in the previous Chapter) then the process for RCH₂S• radical formation is very simple [Scheme 3A] for Symons' assignment³⁸ of species "X", a breaking of the sulphur-sulphur bond being the only requirement to produce RCH₂S• radicals.

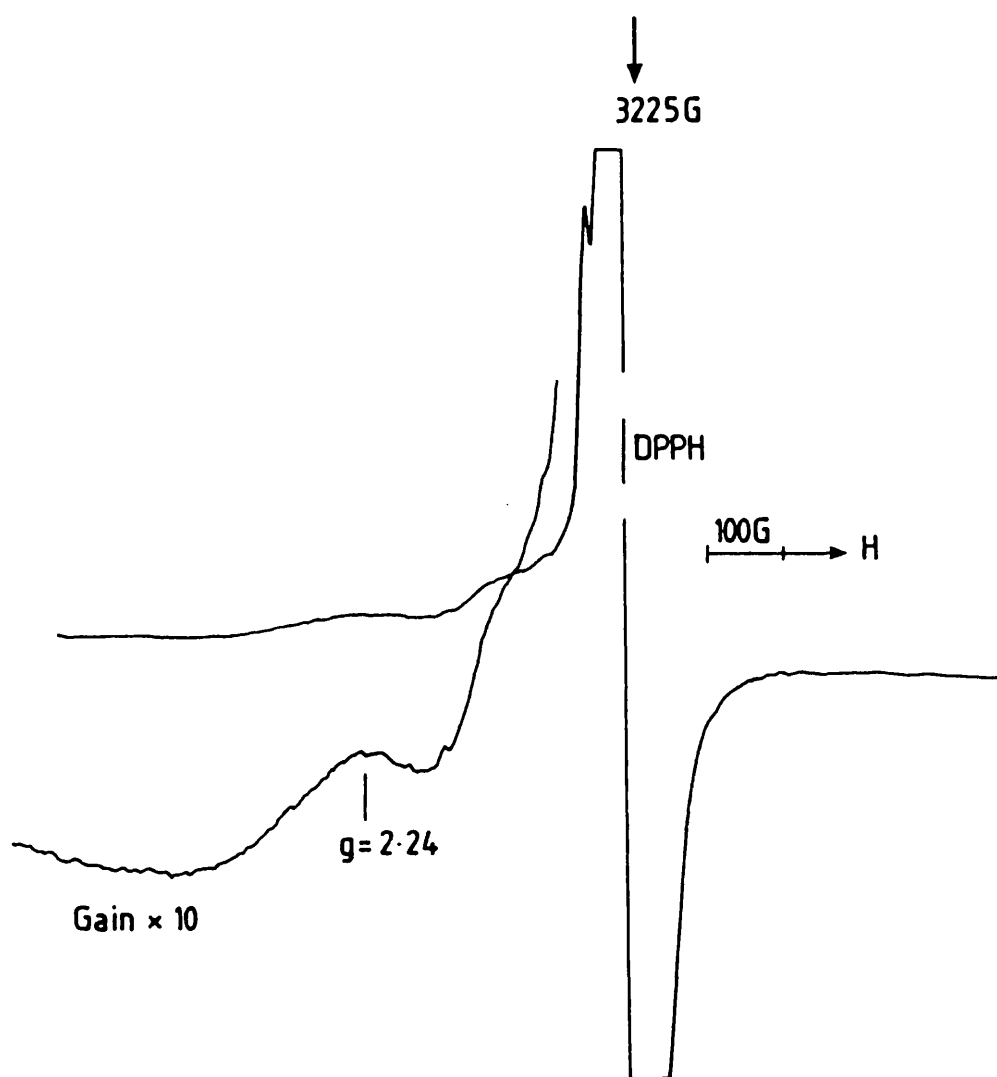


FIGURE 3.11

ESR spectrum of 2-pyridinemethanethiol (monomer) in $\text{CD}_3\text{OD}/\text{D}_2\text{O}$ showing feature assigned to $\text{RCH}_2\text{S}^\bullet$ radicals.

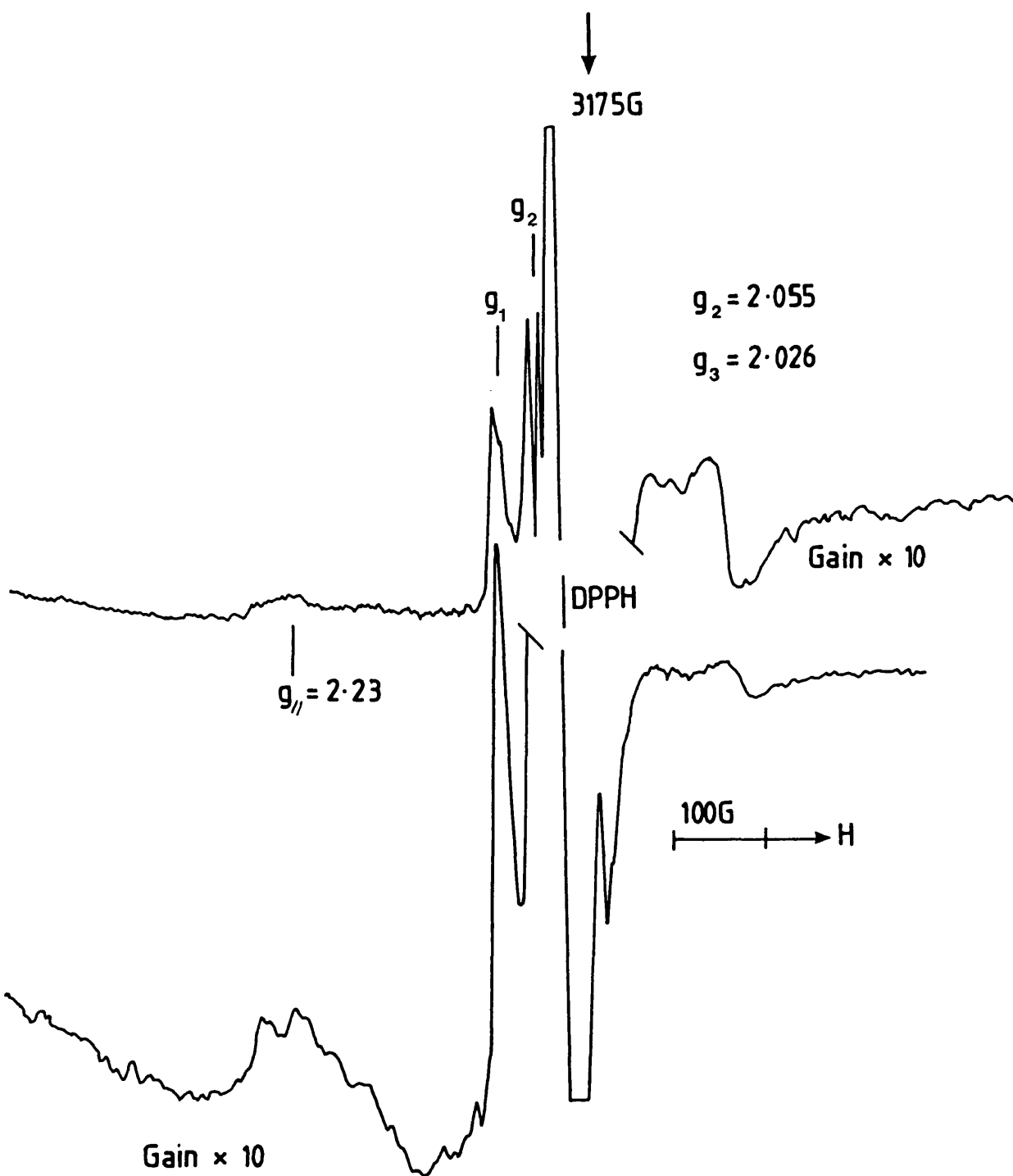


FIGURE 3.12

ESR spectrum of 2-pyridinemethanethiol (monomer) in $\text{CD}_3\text{OD}/\text{D}_2\text{O}$ showing presence of a species "X" radical with an $\text{RCH}_2\text{S}^\bullet$ radical.

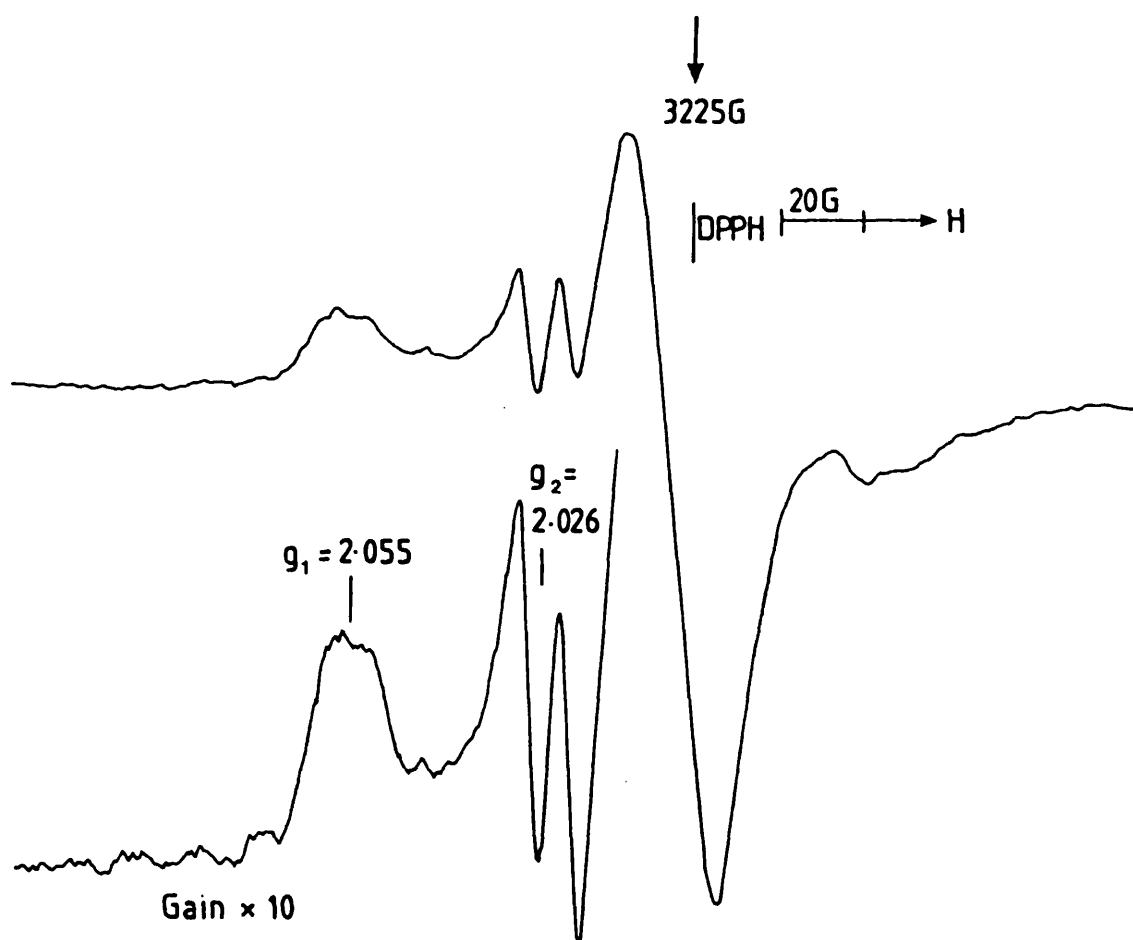
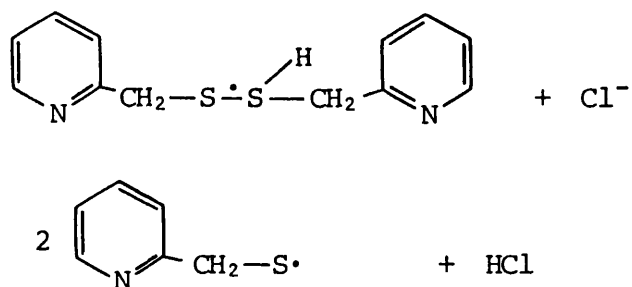


FIGURE 3.13

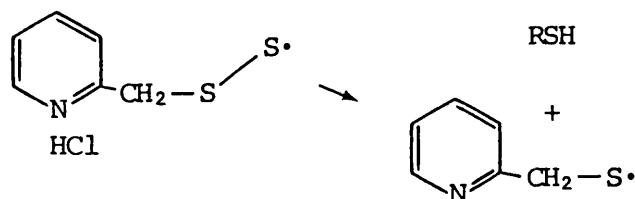
ESR spectrum of 2-pyridinemethanethiol (monomer) in $\text{CD}_3\text{OD}/\text{D}_2\text{O}$ showing growth of the species "X" radical. $[\text{A}(\text{H}) = 8 \text{ G.}]$

SCHEME 3A



If, however, the alternative perthiyl radical assignment⁷¹⁻⁷³ is considered, then the chemistry appears to be more complex in order to give $\text{RCH}_2\text{S}\cdot$ radicals as a product. For this to occur, a sulphur atom would have to be removed from the disulphide radical $\text{RSS}\cdot$, possibly to form a thiol group as shown [Scheme 3B]. This seems less likely than the simple dissociation of RSS(H)R radicals.

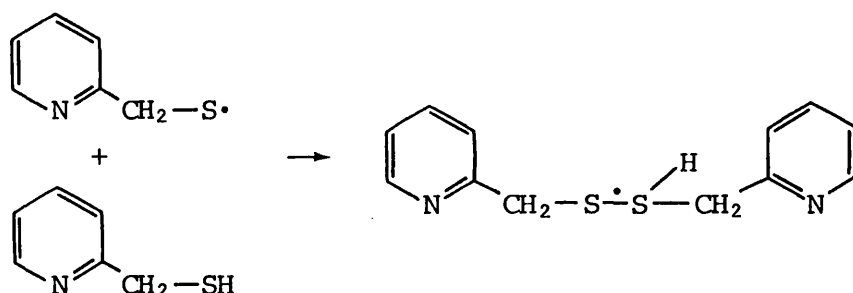
SCHEME 3B



These results are complemented and supported by those from 2-pyridine-methanethiol hydrochloride. The results give the reverse of those obtained for the dimer. The monomer behaves much in the same manner as dithiothreitol and the other thiol compounds studied in Chapter 1. As with the dimer, the results for powder and glass samples of the monomer are similar. Initially, $\text{RCH}_2\text{S}\cdot$ radicals were observed with no evidence of species "X". A signal ascribed to species "X" was only observed after the sample had been annealed, and then in conjunction with $\text{RCH}_2\text{S}\cdot$ radicals. Again in the glass sample a yellow colour was noticed as species "X" was formed.

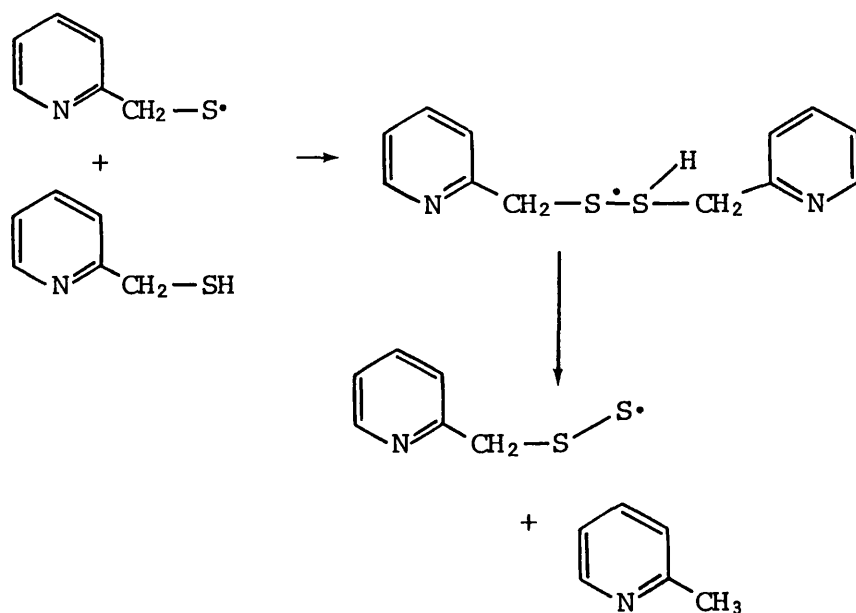
When the chemistry is considered, if species "X" is a radical of the form RSS(H)R then, in this case, its formation from $\text{RCH}_2\text{S}^\bullet$ radicals would be a dimerisation process with a thiol as illustrated [Scheme 3C].

SCHEME 3C



As before, the formation of the perthiyl radical from these aromatic $\text{RCH}_2\text{S}^\bullet$ radicals, is chemically more complex [Scheme 3D]. Initially, it

SCHEME 3D



is similar to the previous scheme, with a dimerisation taking place. However, another reaction step would be required, involving the breaking of a sulphur-carbon bond to give the perthiyl radical. It is suggested that this would be energetically less favourable than the single dimerisa-

tion process to form the RSS(H)R radical. In either case, one has to postulate the existence of the adduct formulation.

Considered overall, the study on 2-pyridinemethanethiol hydrochloride and its dimer, would appear to favour Symons' assignment of the features at $g = 2.06$ and 2.025 to $\text{RS}\dot{\text{S}}\text{SR}_2$ radicals rather than to $\text{RSS}\cdot$ radicals.



CHAPTER 4

Lipoic Acid

4.1 INTRODUCTION

α -(+)-Lipoic (6,8-thioctic) acid [Fig. 4.1] is an essential coenzyme for all systems of α -keto acid dehydrogenase complexes.⁷⁹ Its biochemical rôle is one of the reasons this compound was chosen for study. Taken in detail this rôle can be best described by the fact that lipoic acid acts as a carrier for an acetyl group in the intermediary oxidative processes in carbohydrate metabolism. Lipoic acid is involved primarily in the breakdown of pyruvic acid under aerobic conditions.

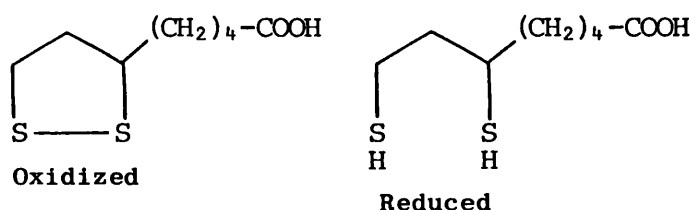
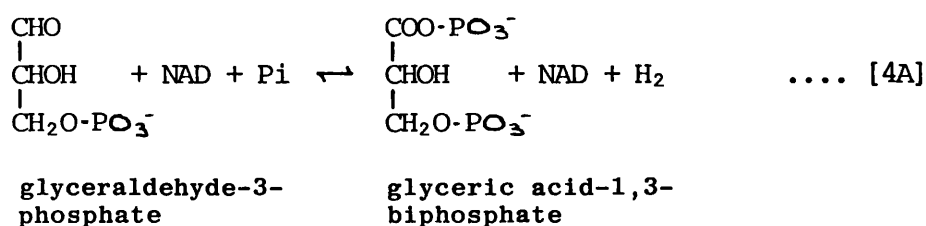


FIG. 4.1

The Structure of Lipoic Acid.

Pyruvic acid is the penultimate compound of the glycolytic pathway, and under anaerobic conditions the NADH_2 produced in the reaction,



occurring within this pathway is reoxidized to NAD by reducing pyruvic acid. Under aerobic conditions, on the other hand, NADH_2 can be reoxidized to NAD via the respiratory chain, so that, in this case, there is no need for pyruvic acid to act as an acceptor for hydrogen. Instead, pyruvic acid, most of which arises from degradation of carbohydrate, is broken down by oxidation.

The first stage in the breakdown is an oxidative decarboxylation.

This reaction proceeds in several steps, catalysed by the enzyme complex pyruvate dehydrogenase. In the first step, an acetyl group is removed from pyruvic acid, leaving carbon dioxide, and then this acetyl group is passed from one carrier to another until it reaches the carrier coenzyme A (CoASH).

The first of these molecules that carry the acetyl group is thiamine pyrophosphate (TPP) [Fig. 4.2]. The next carrier is lipoic acid. Thus

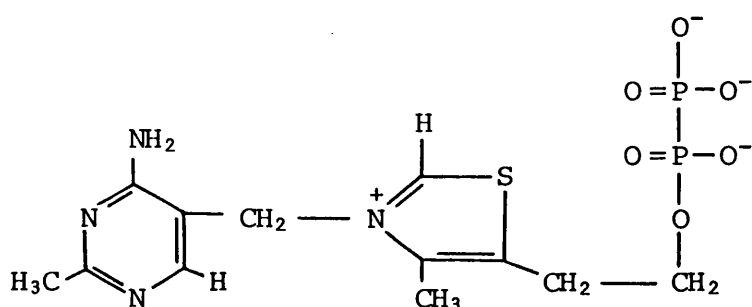
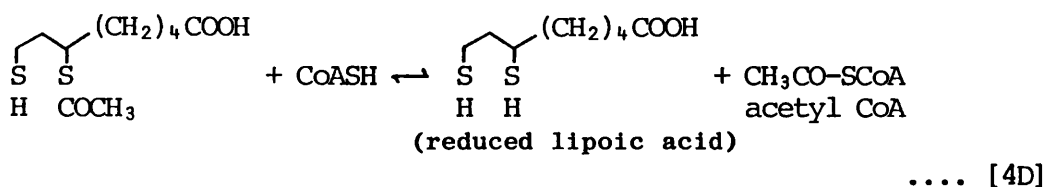
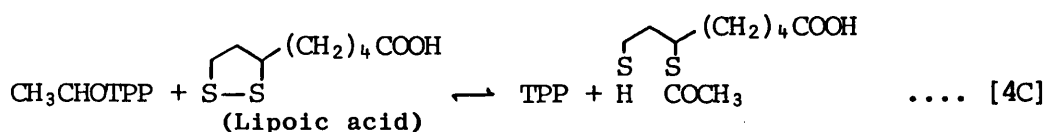
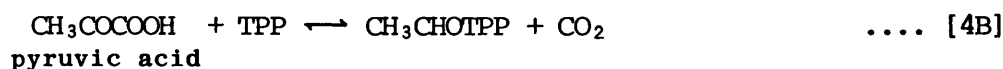


FIG. 4.2

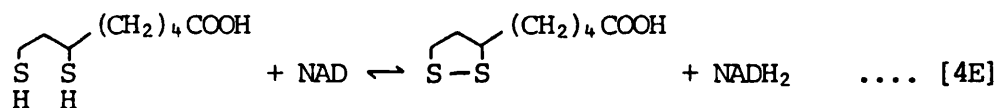
The Structure of Thiamine Pyrophosphate.

as far as the formation of acetyl CoA from pyruvic acid is concerned the reaction sequence can be written:



This is a similar situation to the accumulation of NADH_2 during glycolysis. One result of the oxidative decarboxylation of pyruvic acid is that lipoic acid becomes reduced. Since lipoic acid is a coenzyme and is present only in small quantities in the cell, it must be reoxidized

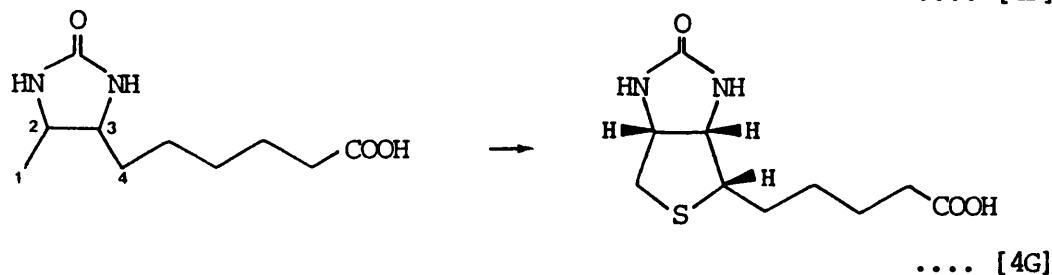
if it is to function in the breakdown of further molecules of pyruvic acid. In fact, reduced lipoic acid is reoxidized by NAD in a reaction catalysed by lipoate dehydrogenase.



Acetyl CoA which is the result of these reactions is one of the central compounds of intermediary metabolism, its functions including a) a breakdown product of fatty acids and some amino acids and b) the starting point for the synthesis of fatty acids. The acetyl group's fate to be oxidized via Kreb's cycle.

So it can be seen that lipoic acid in both oxidized and reduced form plays a vital rôle in the pathways of carbohydrate metabolism.

The biosynthesis of lipoic acid is intriguing in itself.⁸¹ In 1964 Reed et al.⁸² reported that octanoic acid serves as a specific precursor of lipoic acid in E. coli. This suggests that the dithiolane ring system of lipoic may be generated via the introduction of sulphur at two saturated carbon atoms. The biosynthesis of the vitamin (+)-biotin from (+)-dethiobiotin, a transformation subsequently confirmed, demonstrating that the mode of sulphur introduction in lipoate biosynthesis does indeed bear some resemblance to the mechanism of sulphur introduction in biotin biosynthesis.



This result has been confirmed by synthetic preparations of lipoic acid⁸² which have not only confirmed the structure of lipoic acid but also showing a) the $pK_a \approx 5.0$, b) polarographic half-wave potential and hydrogen ion reduction potentials more nearly correspond to the values for six-membered than five- or seven-membered disulphide rings showing its stability in the oxidized form, and c) that octanoic acid is the precursor of lipoate.⁸²

Hence, biologically lipoic acid is very important, but why then the need to understand its behaviour after exposure to ionizing radiation?

It was thought that by studying lipoic acid, a link would be formed between the work on dithiothreitol and its associated compounds and the synthetic aromatic compound 2-pyridinemethanethiol.

Lipoic acid does indeed share common properties with both groups of compounds. As with glutathione [see Chapter 2] lipoic acid is one of the cells non-protein thiols with its own specific functions and, like dithiothreitol, lipoic acid exists as a disulphide and as a dithiol, thus forming a necessary part of its biochemical function. The purpose of studying lipoic acid was to be able to compare its behaviour after irradiation to that of other cyclic compounds [see Chapter 3].

There have been several previous studies on the radiolysis of lipoic acid. Indeed, it has been studied previously by this group.⁷⁰ In this paper, there is a suggestion that lipoic acid gave rise to two species "X" radicals. This interesting result is discussed later in this Chapter. The electrochemistry of lipoic acid has also been studied.⁸³ The chemical reactivity of the lipoic acid disulphide group has been extensively studied. Much of the earlier work was limited to polarographic investigations in aqueous media.⁸⁴ However, the work of Howe *et al.*⁸³ concentrated on the electrochemistry of lipoic acid in both

aprotic and aqueous media. Briefly summarizing their findings, they stated that although reduction of lipoic acid by two successive one-electron transfers is thermodynamically possible, reduction actually occurs by an atom transfer mechanism. However, reduced lipoic acid is oxidized by one-electron transfer processes.

The behaviour of the disulphide group of lipoic acid is critical to the study in this particular Chapter.

4.2 EXPERIMENTAL

The powder and glass samples for e.s.r. spectroscopy were prepared as per the Experimental section in Chapter 2.

For the pH experiments in this Chapter, solutions of lipoic acid were made up in $\text{CD}_3\text{OD}/\text{D}_2\text{O}$ mixtures as normal (35 mg ml^{-1}). The pH of these solutions was measured and altered to the desired value with D_2SO_4 and NaOD . In equivalent experiments with $\text{CH}_3\text{OH}/\text{H}_2\text{O}$ solutions, pH was controlled with H_2SO_4 and NaOH . The concentration of the lipoic acid was monitored throughout the range of pH solutions and was kept constant at 35 mg ml^{-1} .

Relative signal intensities of the radical species were calculated using the ratio of signal of the sample to the signal of an iron acetyl acetate standard measured at the same receiver gain, which was placed in the cavity of the spectrometer at the same time as the sample.

4.3 RESULTS AND DISCUSSION

To maintain continuity between the studies of this compound and those of the other sulphur compounds, lipoic acid was also studied as both a powder and in a $\text{CD}_3\text{OD}/\text{D}_2\text{O}$ glass matrix. As with 2-pyridinemethanethiol only the disulphide (oxidized) form of lipoic acid can be studied

in the powder form, the thiol (reduced) compound existing as a yellow oily liquid at room temperature.

4.3.1 Lipoic Acid (oxidized) Powder Spectra

On irradiation the pure white powder of lipoic acid acquired a yellow colour. The significance of this is discussed later in this Chapter. When the e.s.r. spectrum of this sample was examined at 77 K, a radical with a species "X"-like signal was detected [Fig. 4.3]. However, when the g -values for g_1 , g_2 and g_3 were measured, it was noticed that the g_2 value was lower than that expected of the usual species "X" (2.016 as opposed to 2.025). When a wider scan was employed no $\text{RCH}_2\text{S}\cdot$ radicals were detected [Fig. 4.4].

In a series of annealing experiments, progressively raising the temperature of the sample (followed by recooling to 77 K when a spectral change was observed) the signal around g_2 became less well resolved. A power dependence study showed the possibility of the presence of two radicals displaying a species "X" signal [Fig. 4.5a,b]. Finally, on annealing to ca. 230 K, only one signal could be detected and this was due to a normal "X" radical with g -values of 2.06, 2.025 and 2.002 [Fig. 4.6a,b].

The possibility of there being two forms of the species "X" radical was further explored with the aid of Q-band e.s.r. spectrometry [Fig. 4.7]. This spectrum showed quite definitely that each of the three principal g -values was split into doublets. In addition to this, the spectrum also showed that there were two separate species "X" radicals, the g_1 and g_3 values being identical, but the g_2 value splitting to give signals at 2.016 and 2.025. The results from this experiment show that the "abnormal" X-species ($g_2 = 2.016$) changes irreversibly into the "normal" species ($g_2 = 2.025$) on annealing. In order to explain this

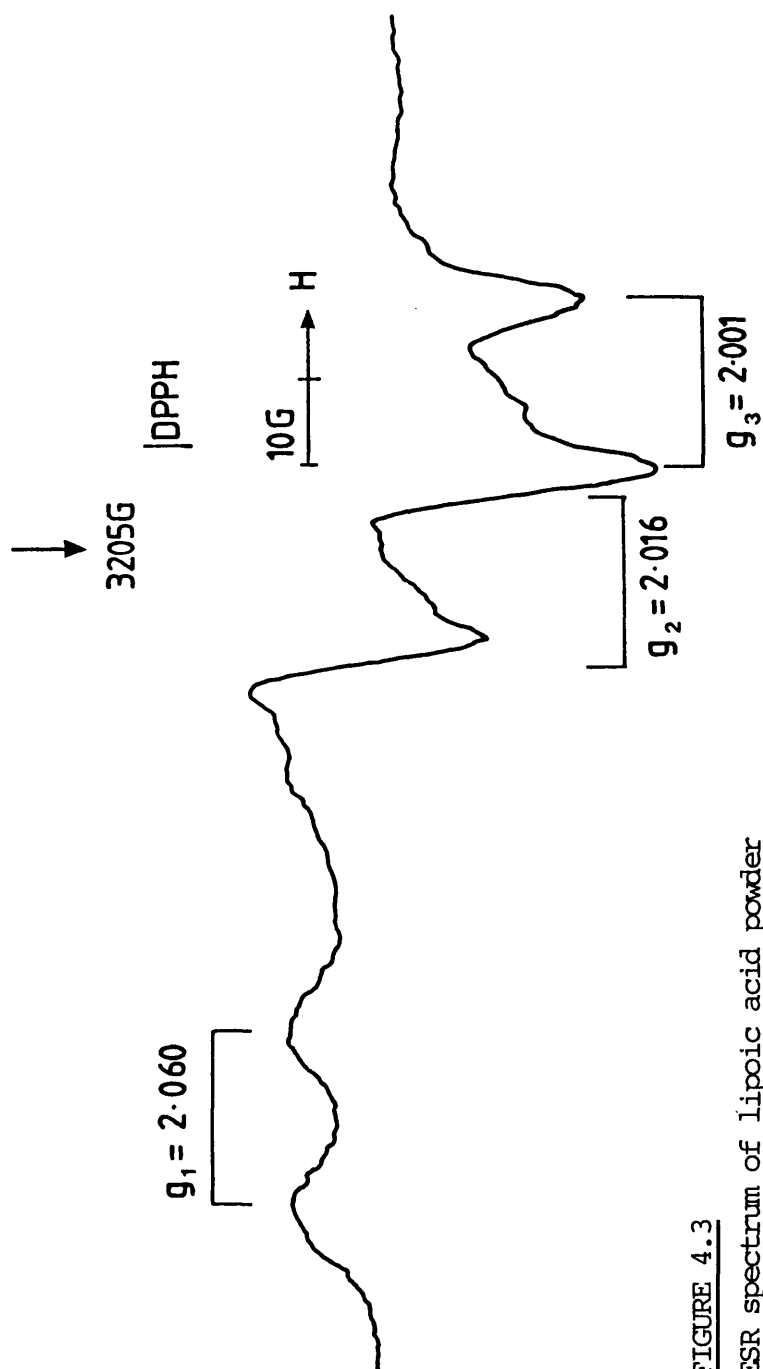


FIGURE 4.3
 ESR spectrum of lipoic acid powder
 after exposure to γ -radiation examined
 at 77 K. $[A(\cdot H)] = 2.06 \cdot 10^{-17}$

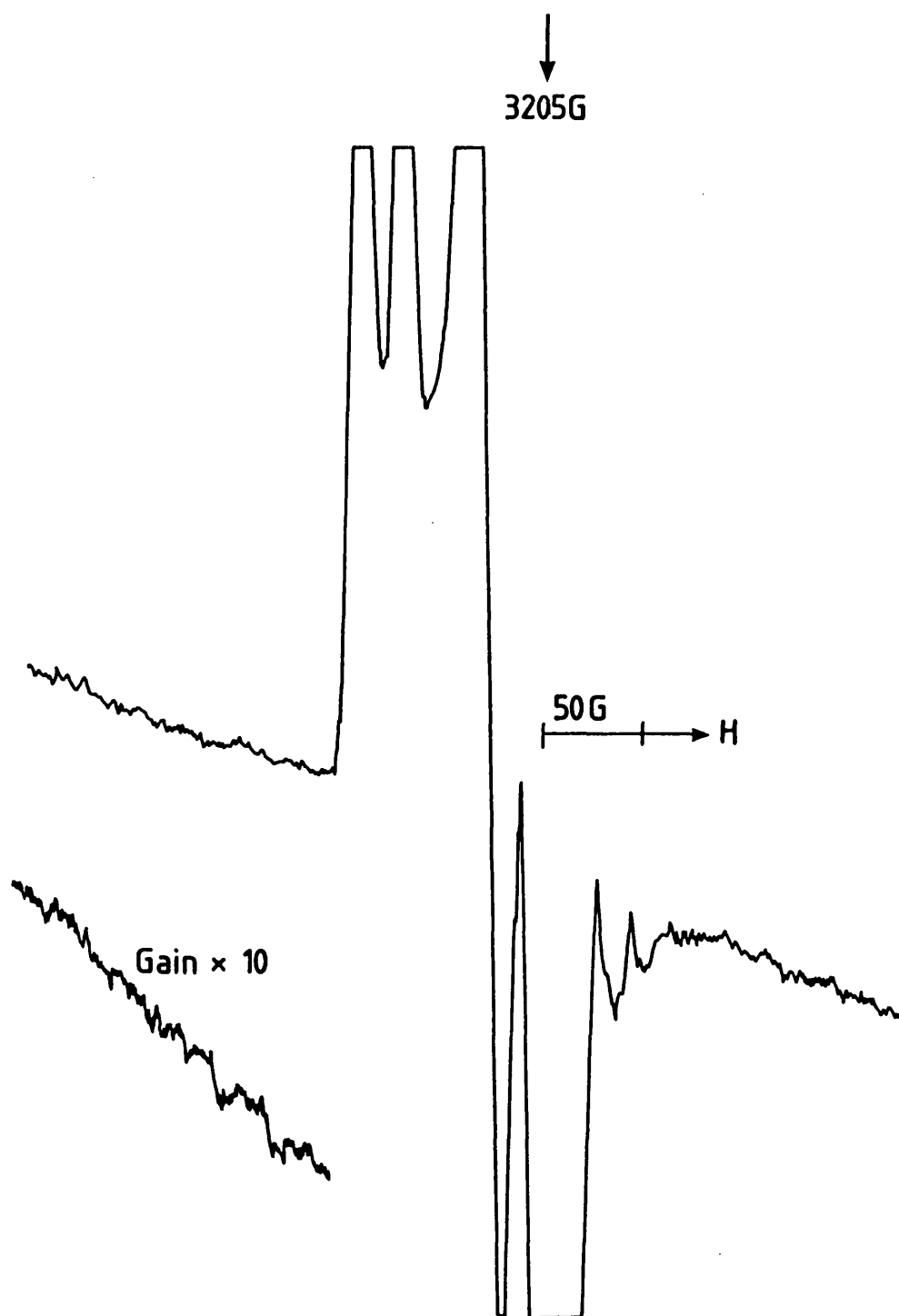


FIGURE 4.4

ESR spectrum of lipoic acid powder displaying no evidence for $\text{RCH}_2\text{S}^\bullet$ radicals.

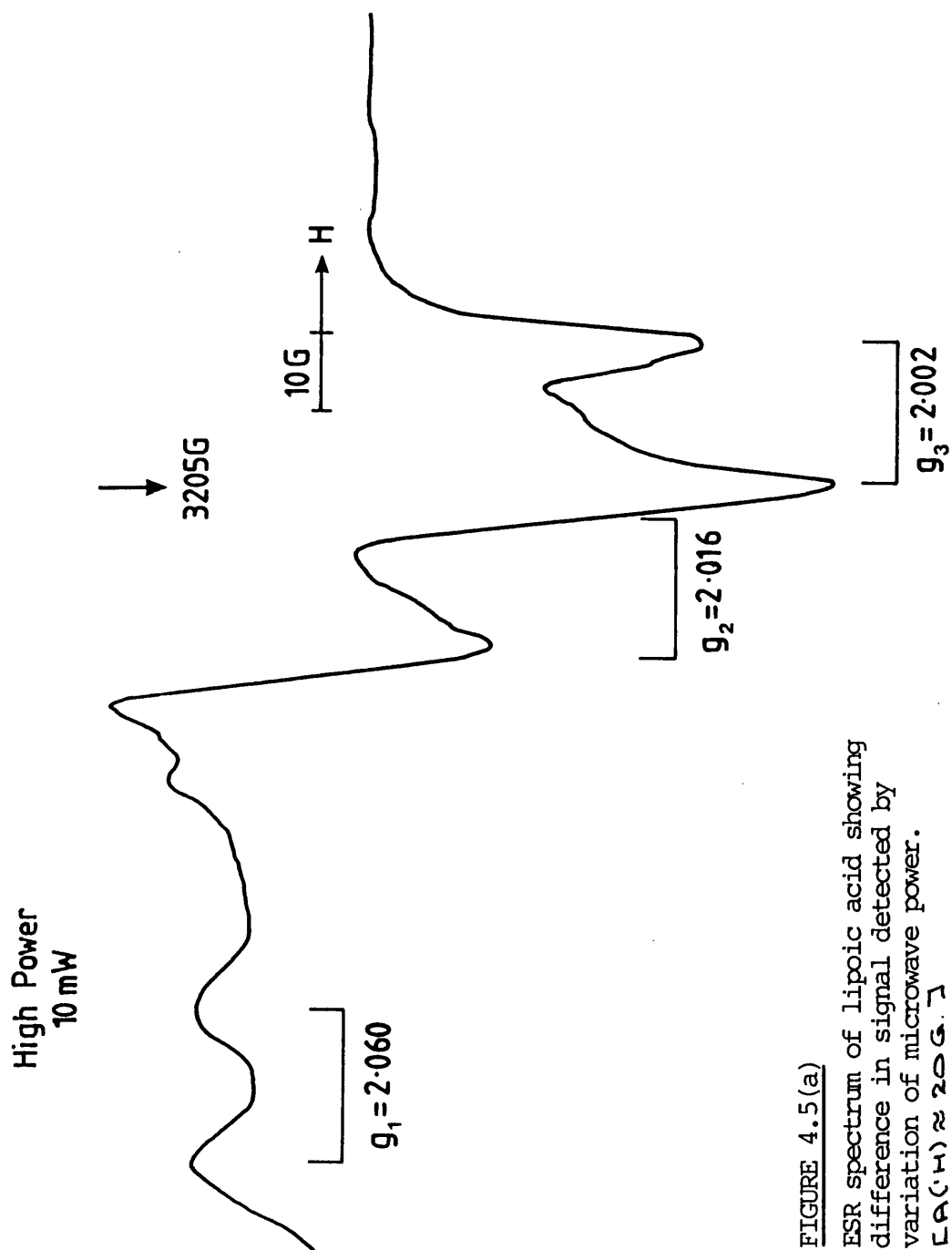


FIGURE 4.5(a)
ESR spectrum of lipoic acid showing
difference in signal detected by
variation of microwave power.
[A(14) \approx 20 G.]

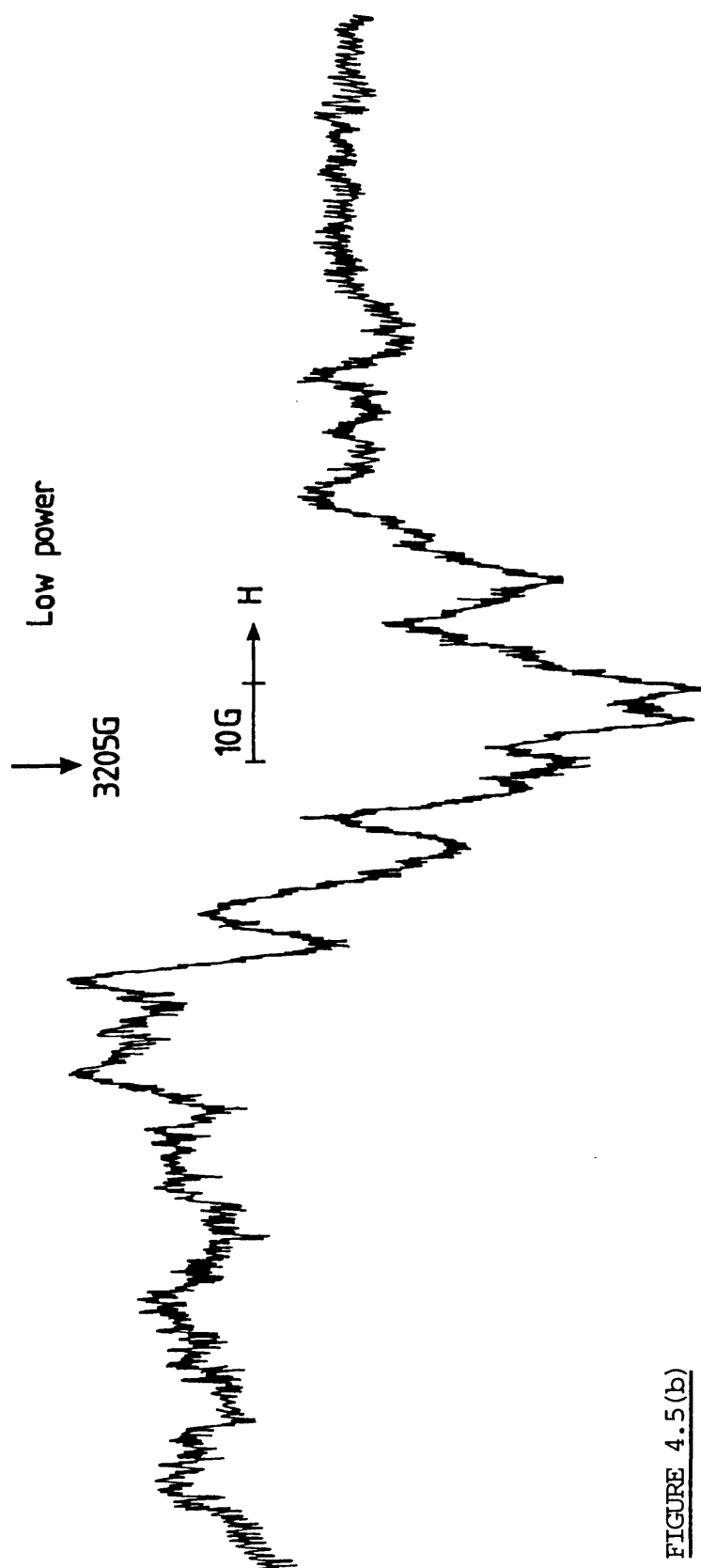


FIGURE 4.5(b)
ESR spectrum of lipoic acid showing difference
in signal detected by variation of microwave
power.

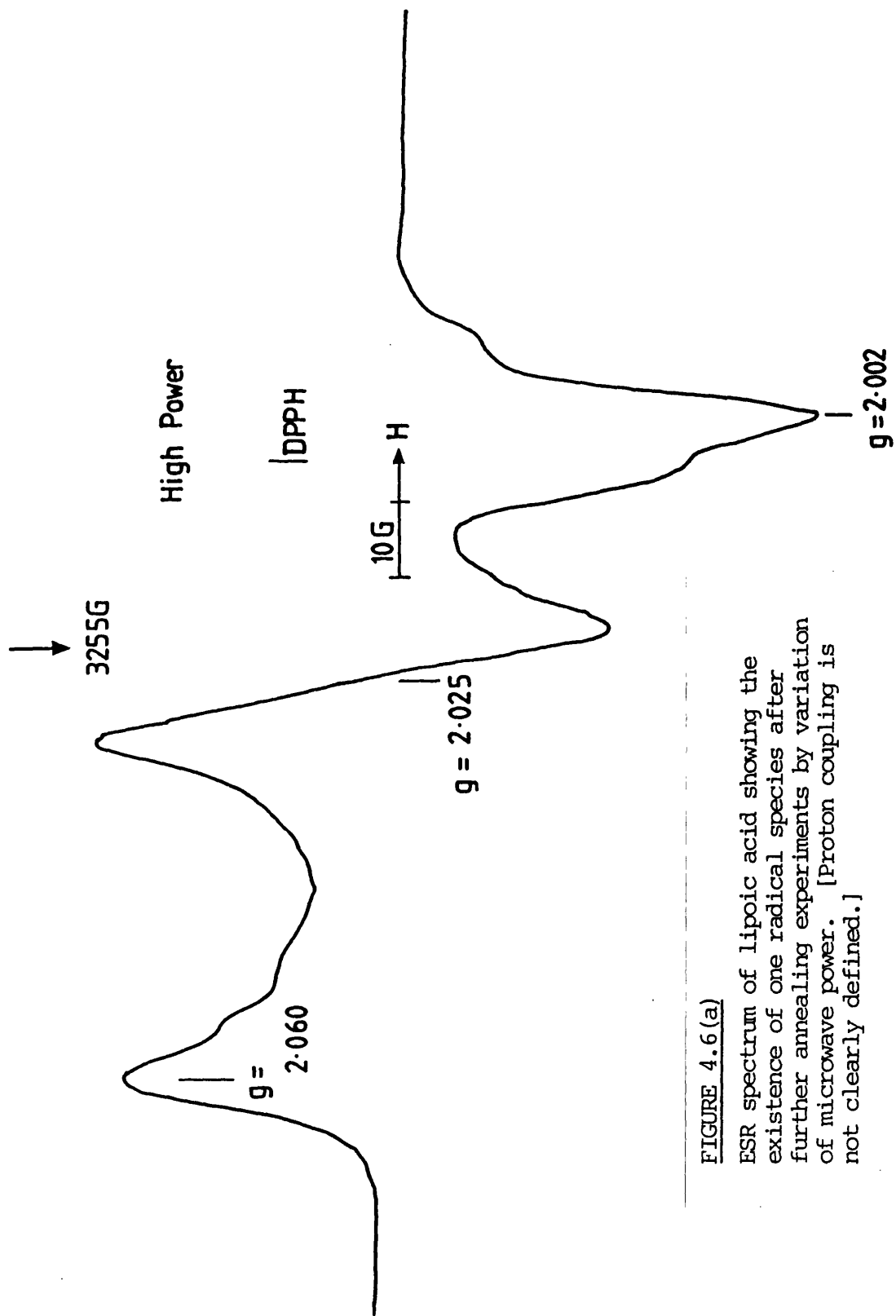


FIGURE 4.6 (a)

ESR spectrum of lipoic acid showing the existence of one radical species after further annealing experiments by variation of microwave power. [Proton coupling is not clearly defined.]

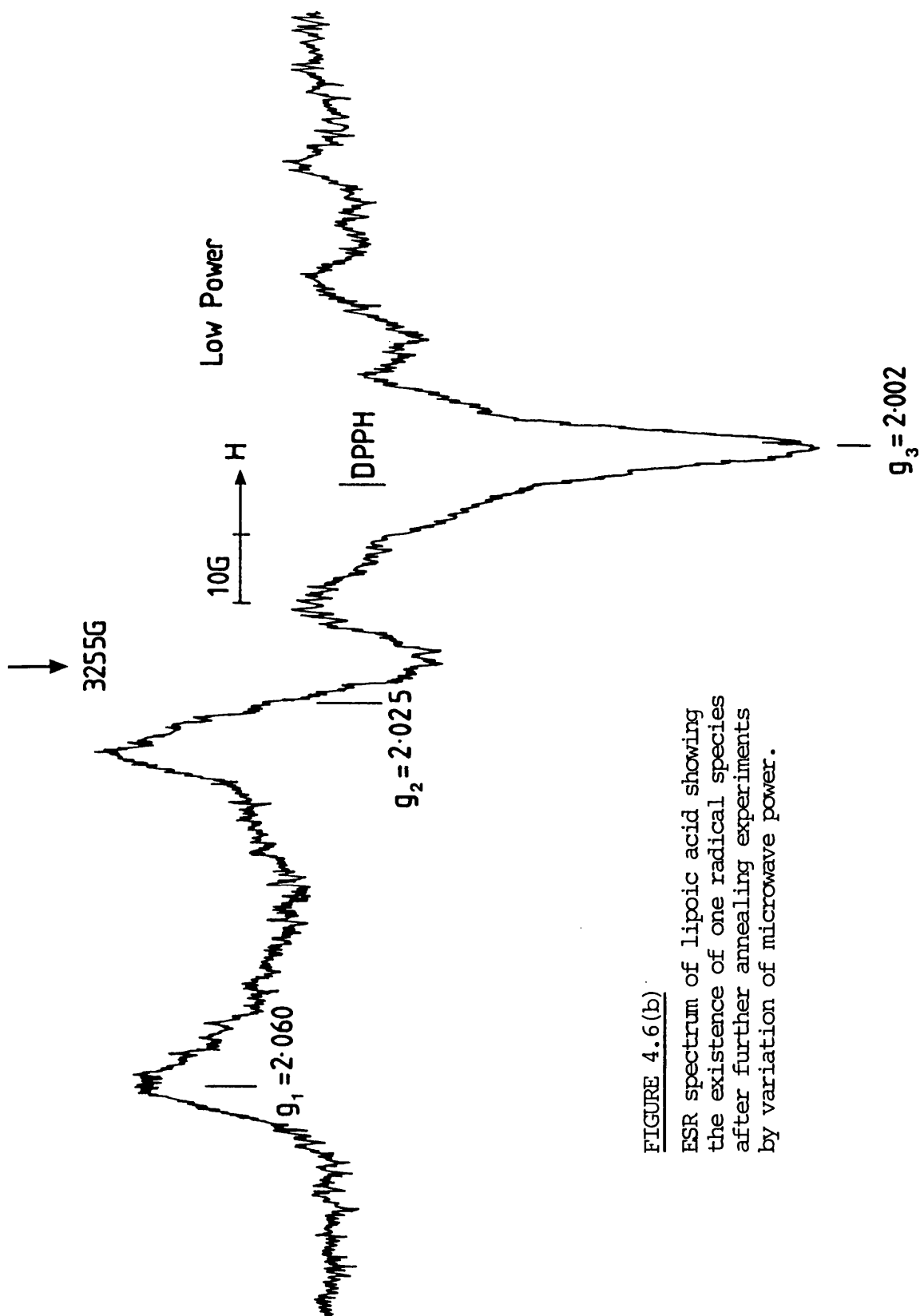


FIGURE 4.6(b)

ESR spectrum of lipoic acid showing the existence of one radical species after further annealing experiments by variation of microwave power.

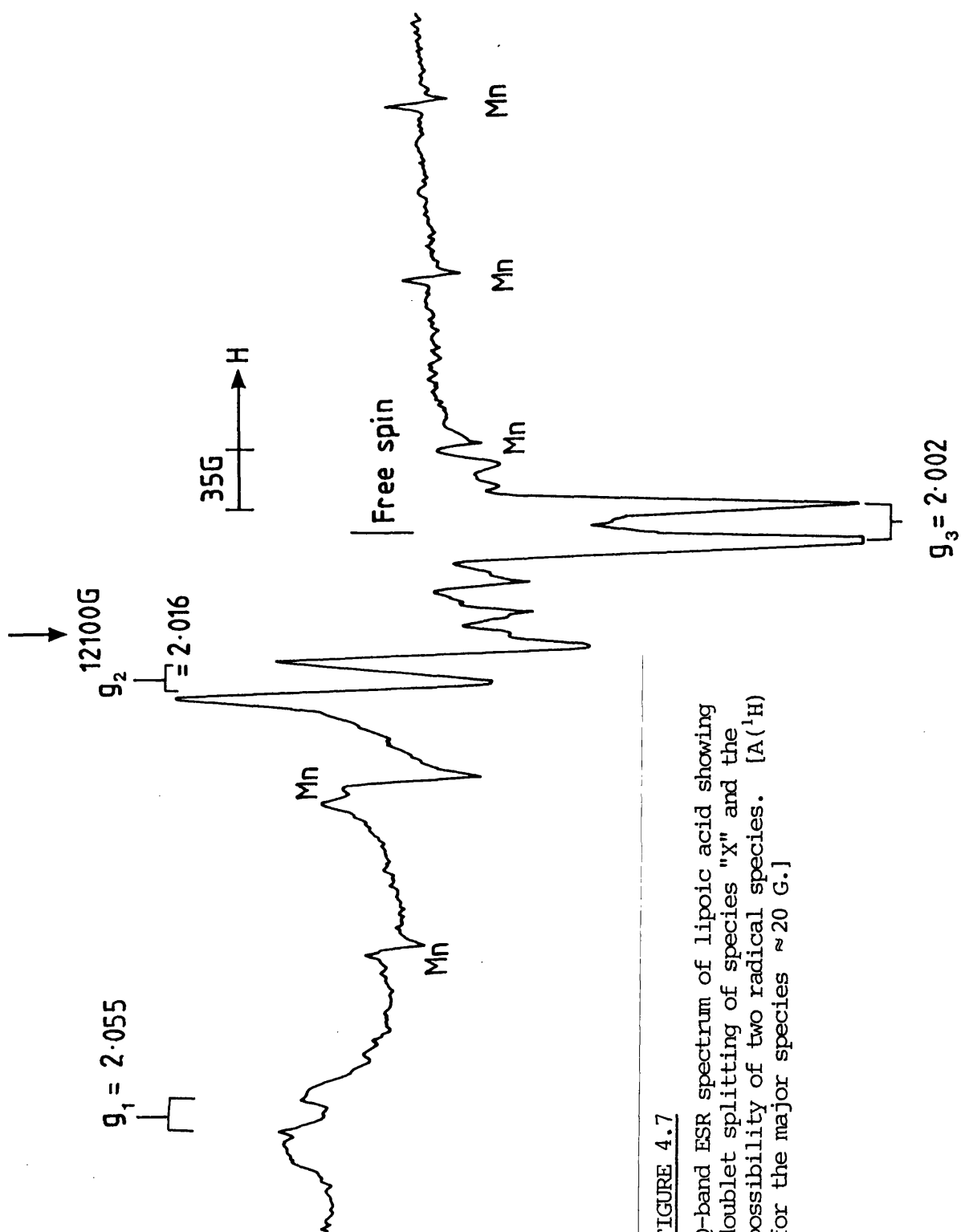


FIGURE 4.7

Q-band ESR spectrum of lipoic acid showing doublet splitting of species "X" and the possibility of two radical species. $[A(^1\text{H})$ for the major species $\approx 20\text{ G.}]$

unusual g_2 value, work by Akasaka et al.⁶⁴ on cysteinyl dihydrochloride has to be considered. They obtained a species, labelled α_2 , with similar g -values ($g_2 = 2.010$) which changed into a normal X-species on annealing. Earlier in this thesis [see Chapter 2], this work has been compared to values obtained for dithiothreitol powder signals where a similar low temperature radical was detected, which also changed into a normal "X"-radical on annealing.

Akasaka et al. proposed that both radicals were of similar structure and that the radical giving the g_2 value = 2.016 is a constrained or "unrelaxed" configuration of the radical giving the g_2 value = 2.025. Akasaka et al. also observed the apparent conversion from one signal to the other at higher temperatures and postulated that this was due to the constrained form relaxing (probably via rotation of CH_2 groups within the radical) to its more stable configuration.

Our results would appear to agree quite well with those of Akasaka et al. These observations, within the context of the structure of species "X", are discussed in greater detail later in this Chapter.

4.3.2 Lipoic Acid (oxidized) Glass Spectra

Samples of oxidized lipoic acid in a deoxy $\text{CD}_3\text{OD}/\text{D}_2\text{O}$ glass matrix were colourless prior to irradiation, but as with other samples in this matrix, post-irradiation the sample beads had become purple coloured due to the presence of "trapped" electrons within the system. The e.s.r. spectrum was also typical of these solvents comprising of an intense singlet due to solvent radicals ($\dot{\text{C}}\text{D}_2\text{OD}$), associated e_t^- centres, and also probably some contribution from the disulphide radical anion (RSSR^\cdot) [Fig. 4.8]. As has been described previously [see Chapters 2 and 3], in order to examine any signal arising from the solute, annealing experiments were carried out. Results analogous to those of the lipoic

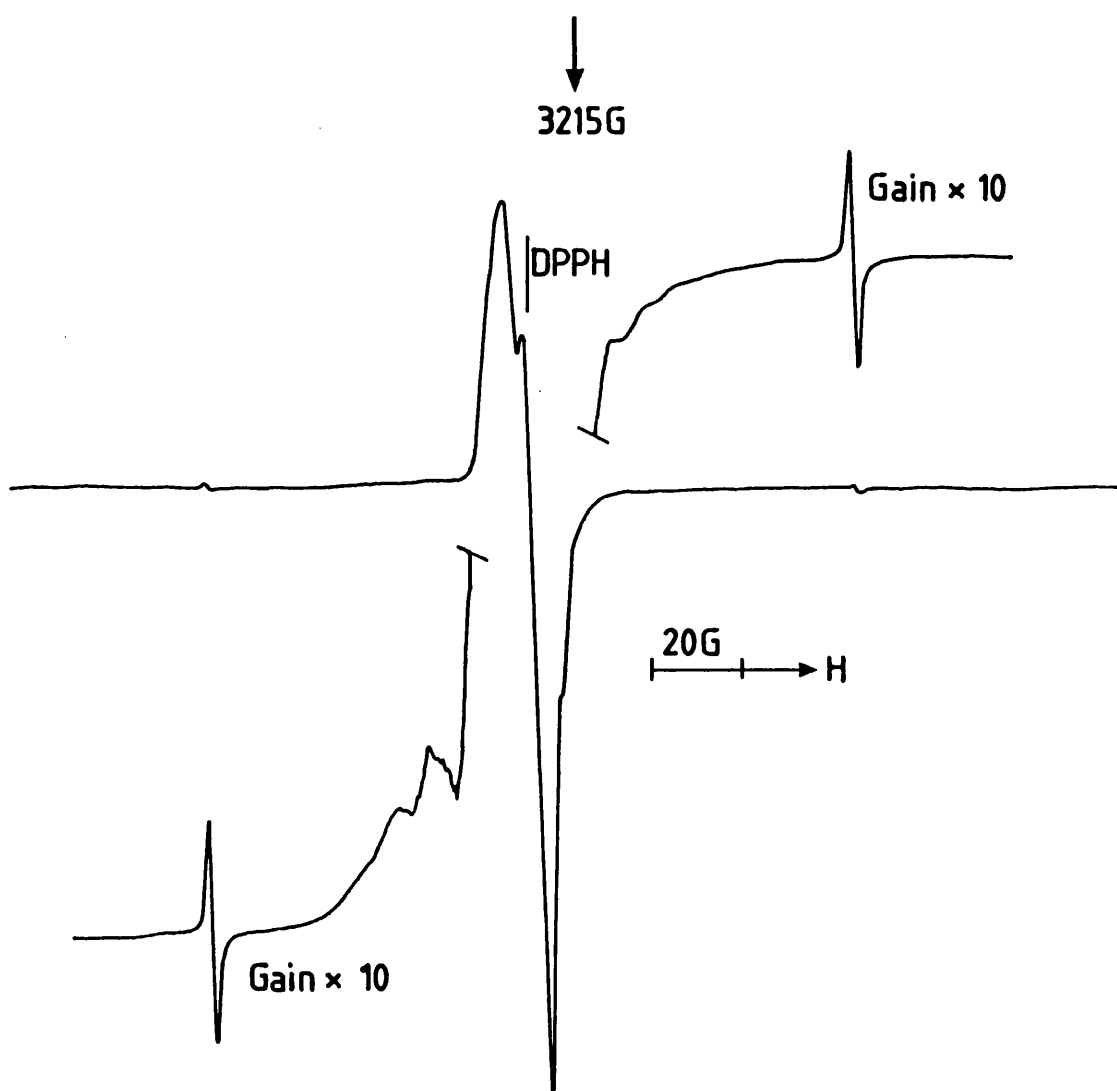


FIGURE 4.8

ESR spectrum of lipoic acid in CD_3OD taken at 77 K showing initial signal detected.

acid powder spectra were obtained. A normal "X"-like radical species was detected with g -values of 2.061, 2.025 and 2.002 respectively [Fig. 4.9]. The radical anion, $\text{RSSR}^{\bullet-}$ was found to be still present, this being the expected product in this matrix. This pattern was maintained throughout several increases in the temperature of the sample. Although these are general results two important observations were made. Unlike the powder samples, a constrained or unrelaxed form of species "X" was not observed, only the more common "relaxed" configuration of species "X" being detected. Secondly, at no time during the experiment were $\text{RCH}_2\text{S}^{\bullet}$ radicals detected. The importance of these observations is discussed later in this Chapter.

As in earlier experiments [see Chapter 2], species "X" was produced in an environment which would be expected to give the radical anion. The initial results from the lipoic acid glass spectra (as with the dithiothreitol) raised the question of a simple relationship between species "X" and the radical anion $\text{RSSR}^{\bullet-}$.

If species "X" is RSS(H)R then this relationship can be visualised as a straightforward protonation/deprotonation equilibrium;



However, if "X" is the perthiyl radical RSS^{\bullet} , then any interaction occurring with the disulphide radical anion would be more complex and certainly not reversible.



Therefore, if it were possible to establish a simple pH dependence, high values favouring $\text{RSSR}^{\bullet-}$ and low values favouring "X", at temperatures so low that reaction [4I] is unlikely to occur, this would constitute good evidence for the RSS(H)R structure. In order to study this depend-

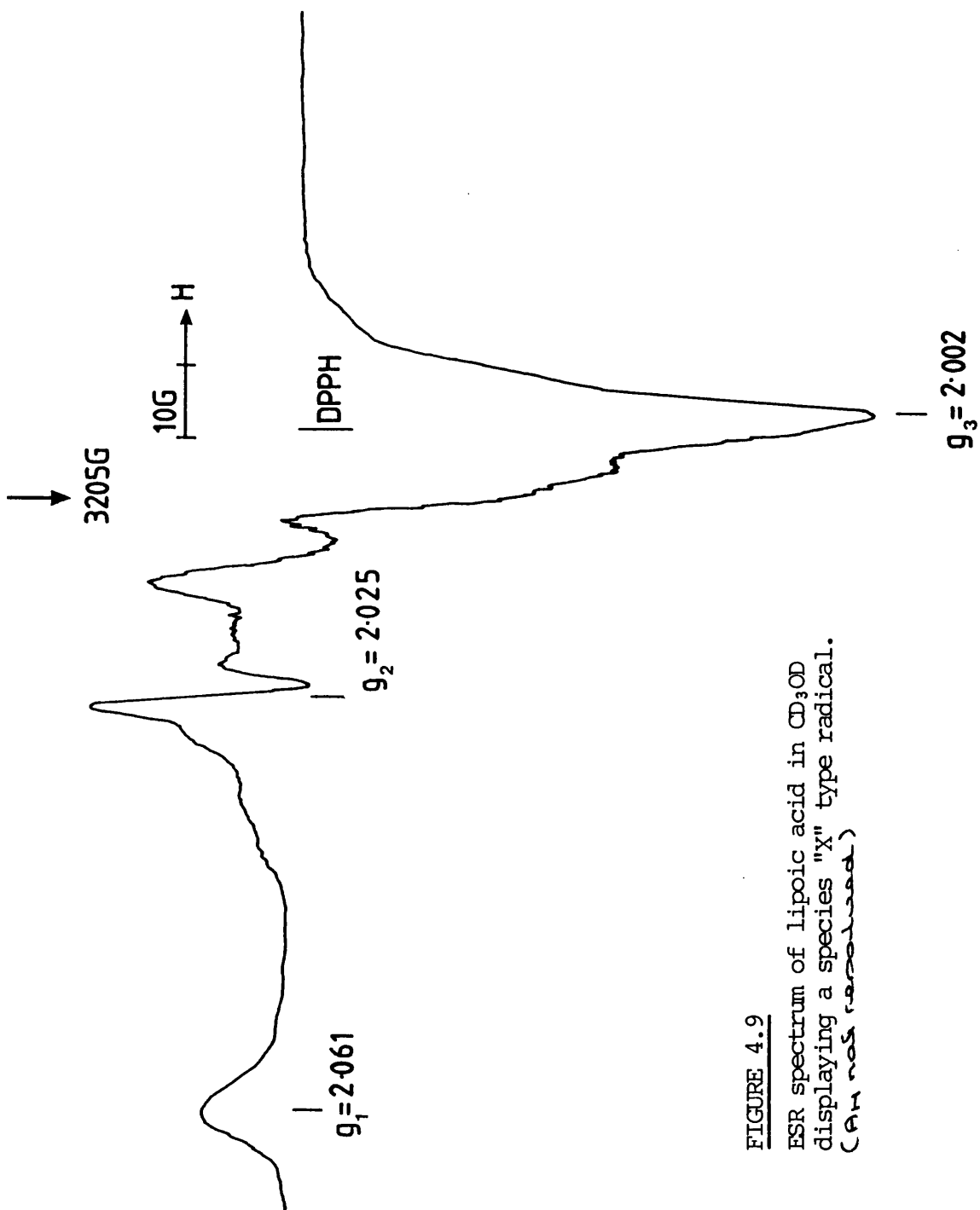


FIGURE 4.9
 ESR spectrum of lipoic acid in CD_3OD
 displaying a species "X" type radical.
 (All not resolved)

ence, a series of samples of oxidized lipoic acid in deoxy $\text{CD}_3\text{OD}/\text{D}_2\text{O}$ solutions at varying pH values was prepared, and the e.s.r. spectrum of each studied. Each sample was studied at the same temperature ($\approx 180\text{ K}$) and the relative intensity of the signals of both species "X" and the radical anion measured against an iron acetylacacetate standard and then plotted against pH [Fig. 4.10].

Clearly species "X" is favoured at low pH, and $\text{RSSR}^{\cdot-}$ at high pH. However, the signal intensity and hence ["X"] falls in intensity at $\text{pH} > (7.5)$ whilst $[\text{RSSR}^{\cdot-}]$ falls at $\text{pH} < (3.5)$. At pH (5.5) the signal intensity and hence concentration of both species is approximately equal. This is close to the pK_a of lipoic acid at pH (5.3).

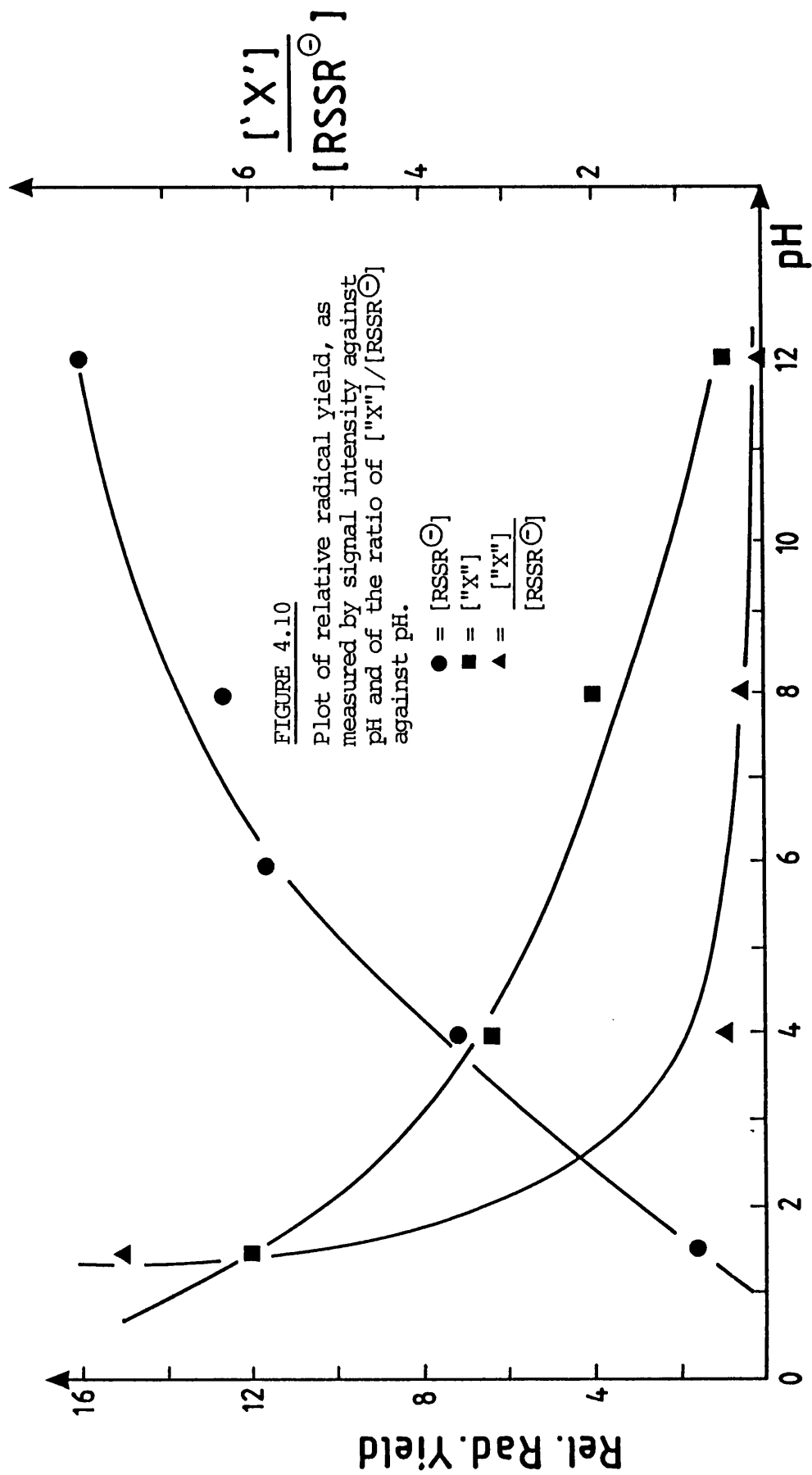
These results imply that a conversion between species "X" and the radical anion at the pK_a of the compound.

Bearing in mind that these are solid-state studies, the results do support the idea of a simple protonic equilibrium between species "X" and $\text{RSSR}^{\cdot-}$. This is not a new idea but previous studies have concentrated on liquid phase samples which also support the RSS(H)R structure observed in these solid-state systems.⁶⁹ This also corresponds to liquid phase UV/vis spectrophotometric studies showing that on formation of the radical anion ($\text{RSSR}^{\cdot-}$) a transitory band is observed at ca. 380 nm.⁶⁹ This conforms with the pale yellow colour of samples in which species "X" has been detected. An absorption of this kind is not expected of the perthiyl radical ($\text{RSS}\cdot$).

In an attempt to clarify these results further, the reduced form of lipoic acid [Fig. 4.1] was also studied.

4.3.3 Lipoic Acid (reduced) Spectra

Initial $\text{CD}_3\text{OD}/\text{D}_2\text{O}$ glass spectra examined were typical of these samples, comprising of features for solvent radicals and e_t^- centres.



In addition to this was a weak g_{\parallel} feature at $g = 2.18$ which is characteristic of $RS\cdot$ radicals in this medium [Fig. 4.11a,b]. This result was not surprising in the context of the results in previous chapters and in published work. On annealing, the $RCH_2S\cdot$ radical signal was lost and species "X" (2.053, 2.024, 2.002) was observed [Fig. 4.12] with an associated yellow colouration of the sample. In this respect the reduced form of lipoic acid behaved similarly to dithiothreitol [see Chapter 2]. This result prompted experiments as to the behaviour of reduced lipoic acid on variation of the pH of the solution. Under alkaline conditions, $RCH_2S\cdot$ radicals were initially detected at 77 K [Fig. 4.13] followed by the production of species "X" on minor annealing (with a concurrent yellow colouration of the sample), together with loss of $RCH_2S\cdot$ radicals [Fig. 4.14a,b]. However, when the temperature of the sample was increased further the signal attributed to species "X" was seen to decay and features characteristic of $RS\dot{S}R^-$ radicals grew in [Fig. 4.15].

Under acidic conditions, reduced lipoic acid again gave $RCH_2S\cdot$ radicals [Fig. 4.16] initially, followed by the production of species "X" [Fig. 4.17]. However, in this case, no conversion into $RS\dot{S}R^-$ radical anions could be detected. After these further annealing experiments, species "X" was still detected with only its g_1 feature resolved [Fig. 4.18]. Centred around free-spin was another signal which was attributed to the radical cation $RS\dot{S}R^+$.³⁸ These results would on first observation appear to support those of oxidized lipoic acid and are discussed in greater detail in the next section.

4.3.4 Concluding Remarks

There are several key results arising from the work in this Chapter. Firstly, an "X"-like species having an unusually low g_2 value (2.016)

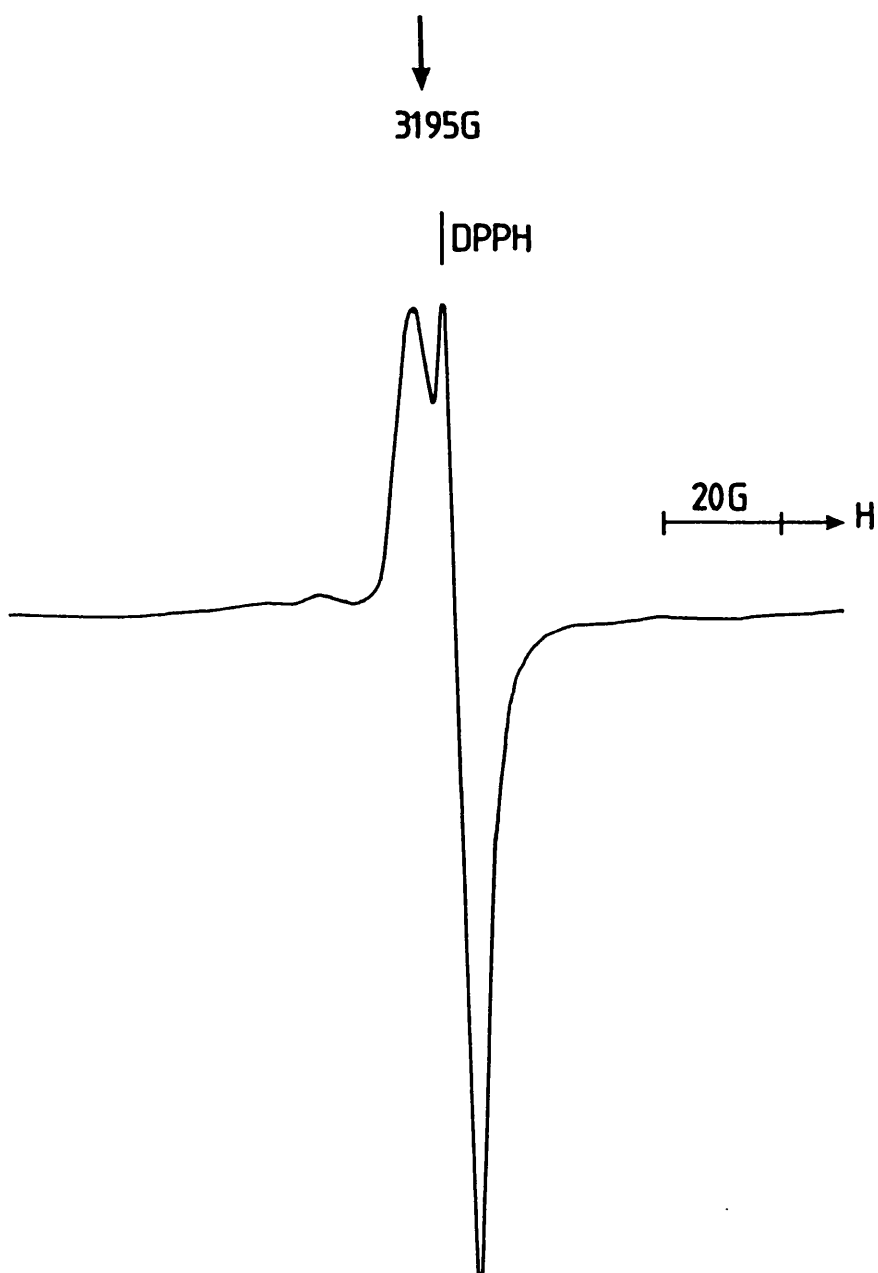


FIGURE 4.11(a)

ESR spectrum of reduced lipoic acid in CD_3OD displaying the solvent radicals.

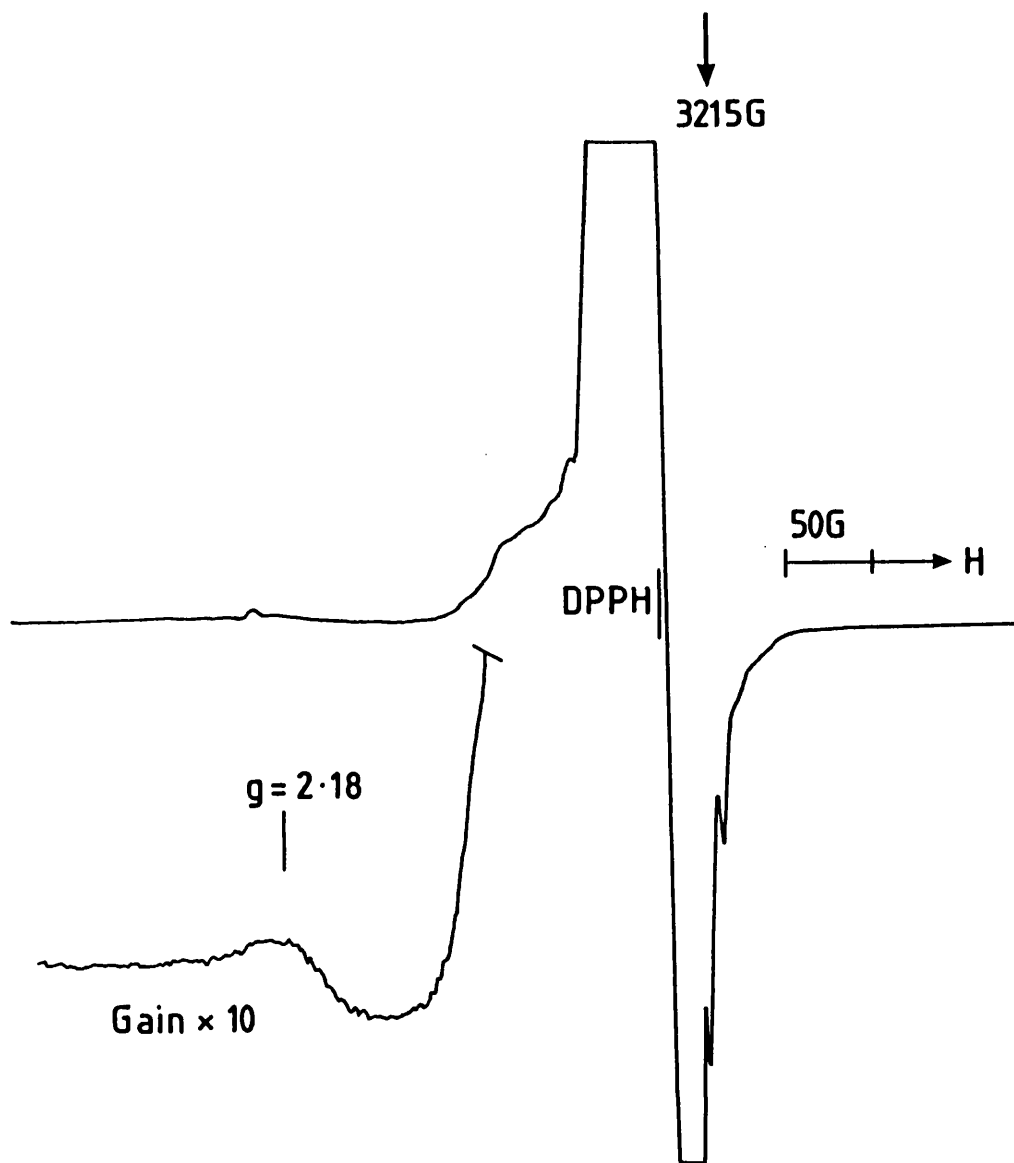


FIGURE 4.11(b)

ESR spectrum of reduced lipoic acid in CD_3OD displaying the $\text{RCH}_2\text{S}^\bullet$ radicals.

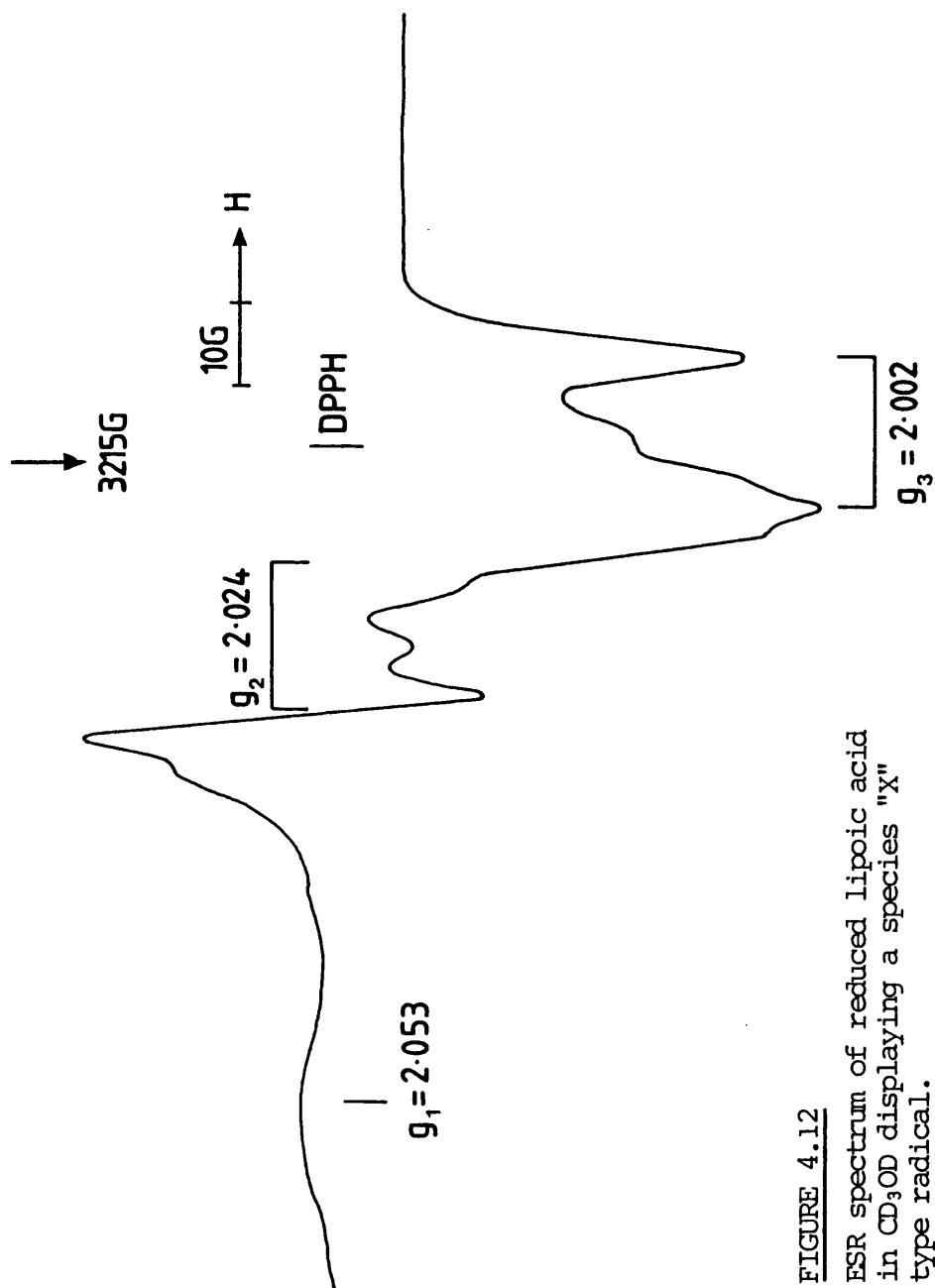


FIGURE 4.12
ESR spectrum of reduced lipoic acid in CD_3OD displaying a species "X" type radical.

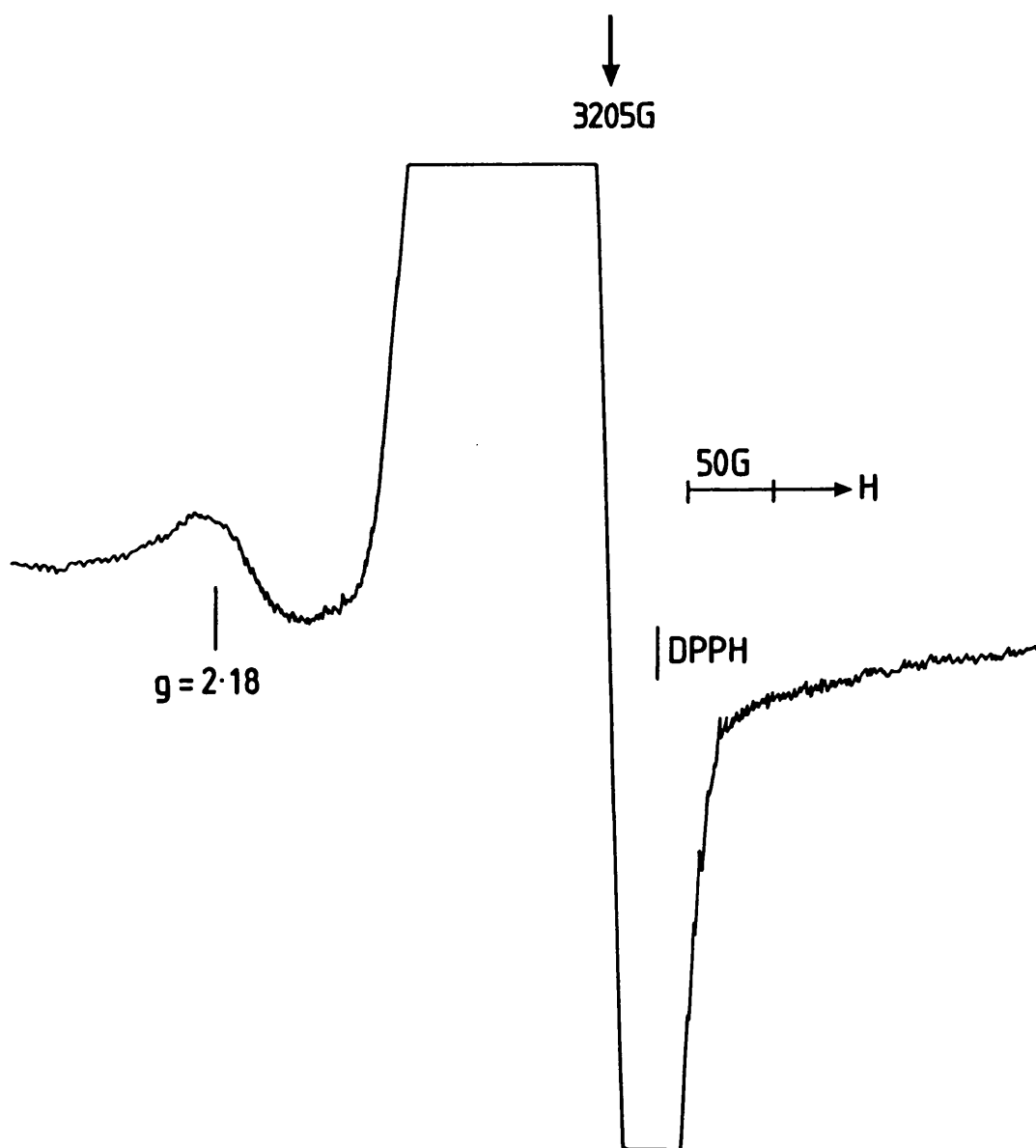


FIGURE 4.13

Initial ESR spectrum of reduced lipoic acid in CD_3OD under alkaline conditions showing existence of an $\text{RCH}_2\text{S}^\bullet$ radical.

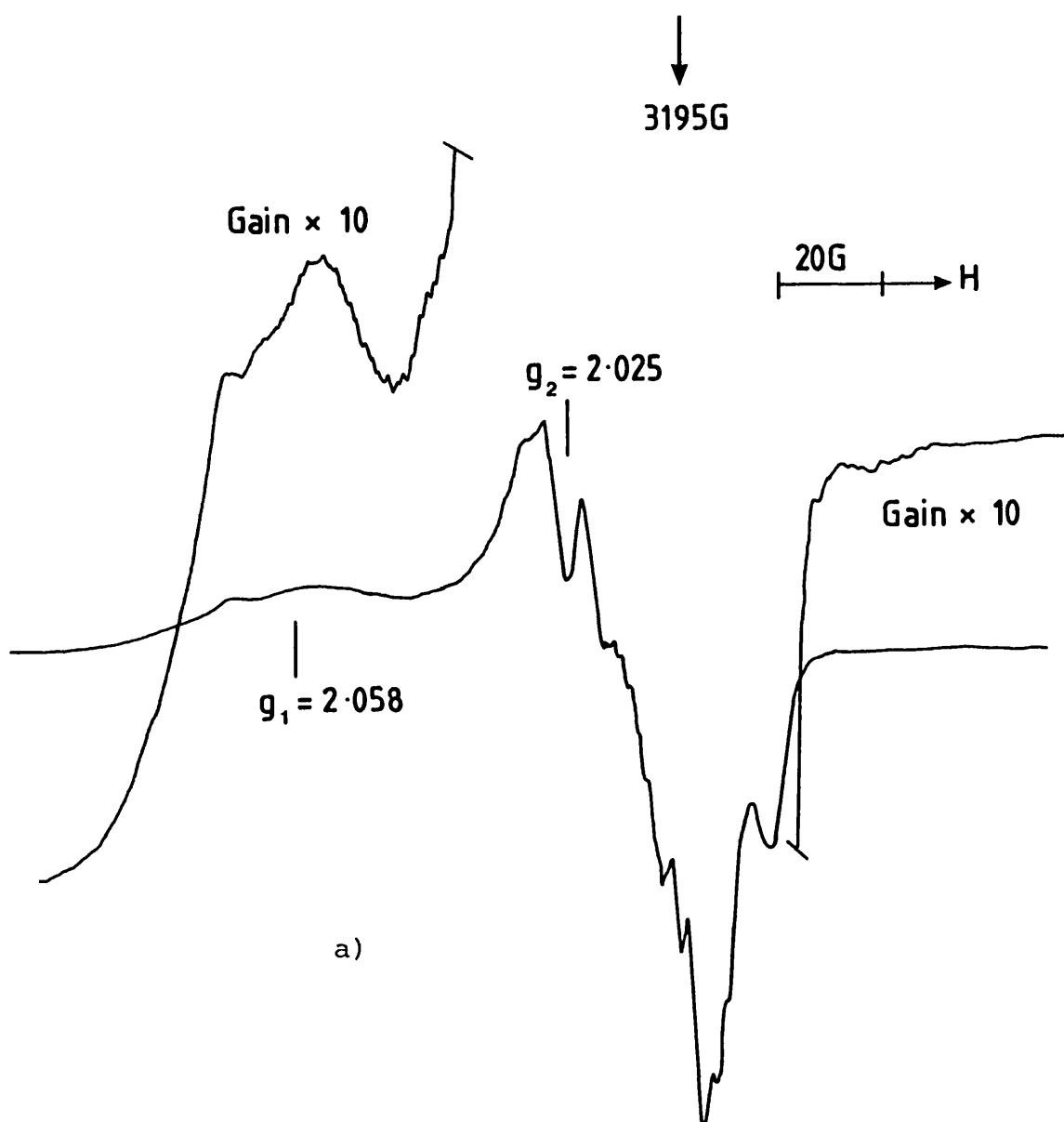


FIGURE 4.14

ESR spectra taken after annealing of reduced lipoic acid in CD_3OD under alkaline conditions showing detection of a species "X" radical (a) with the loss of $RCH_2S\cdot$ radicals (b).

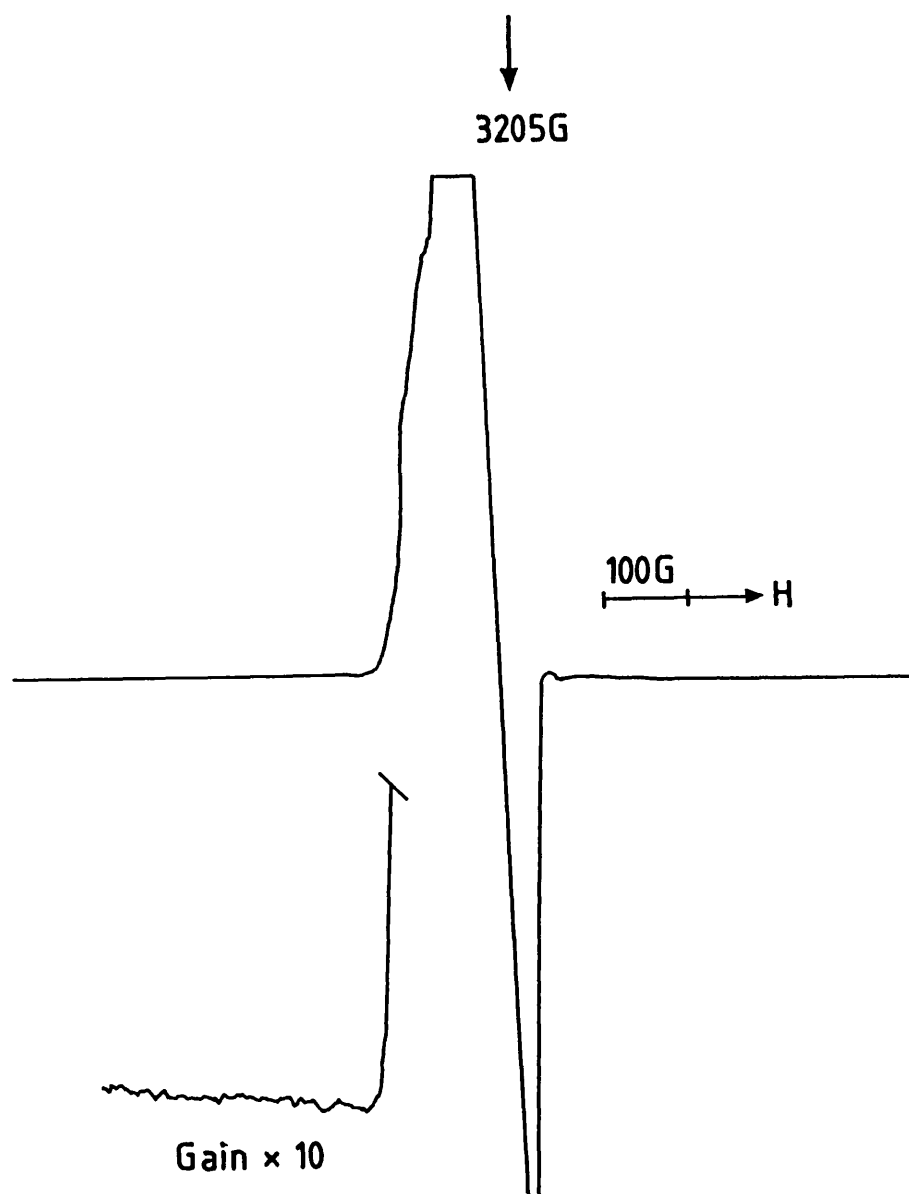


FIGURE 4.14(b)

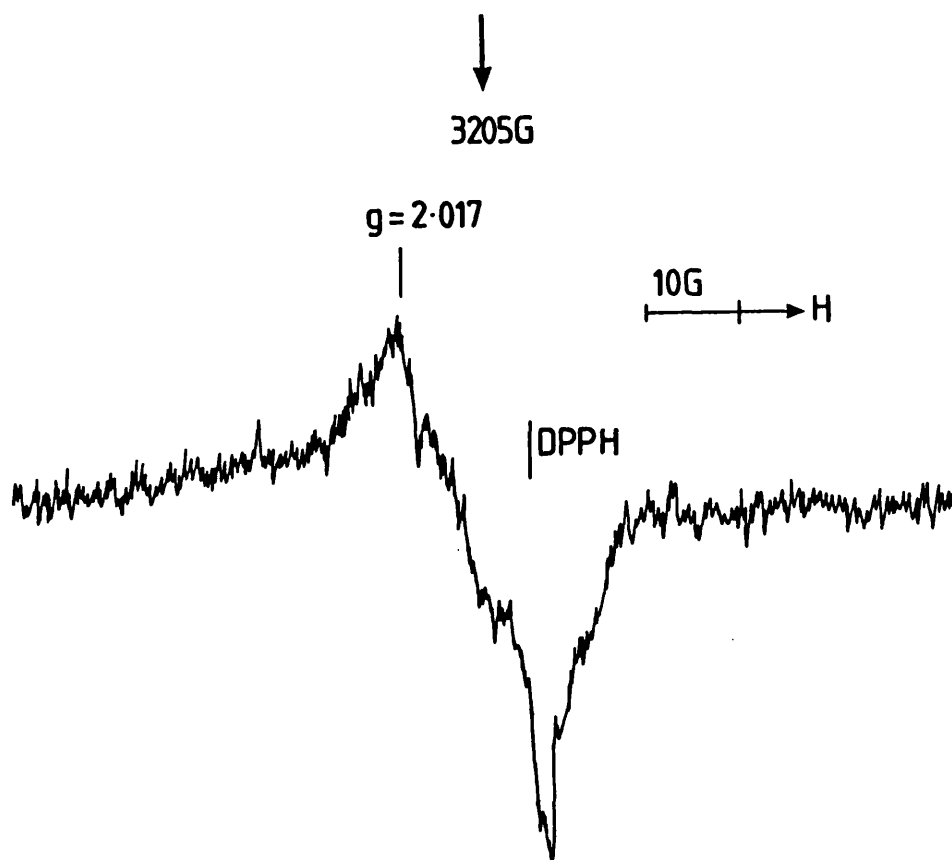


FIGURE 4.15

ESR spectrum of reduced lipoic acid in CD_3OD in alkaline conditions showing production of RSSR^\ominus radicals.

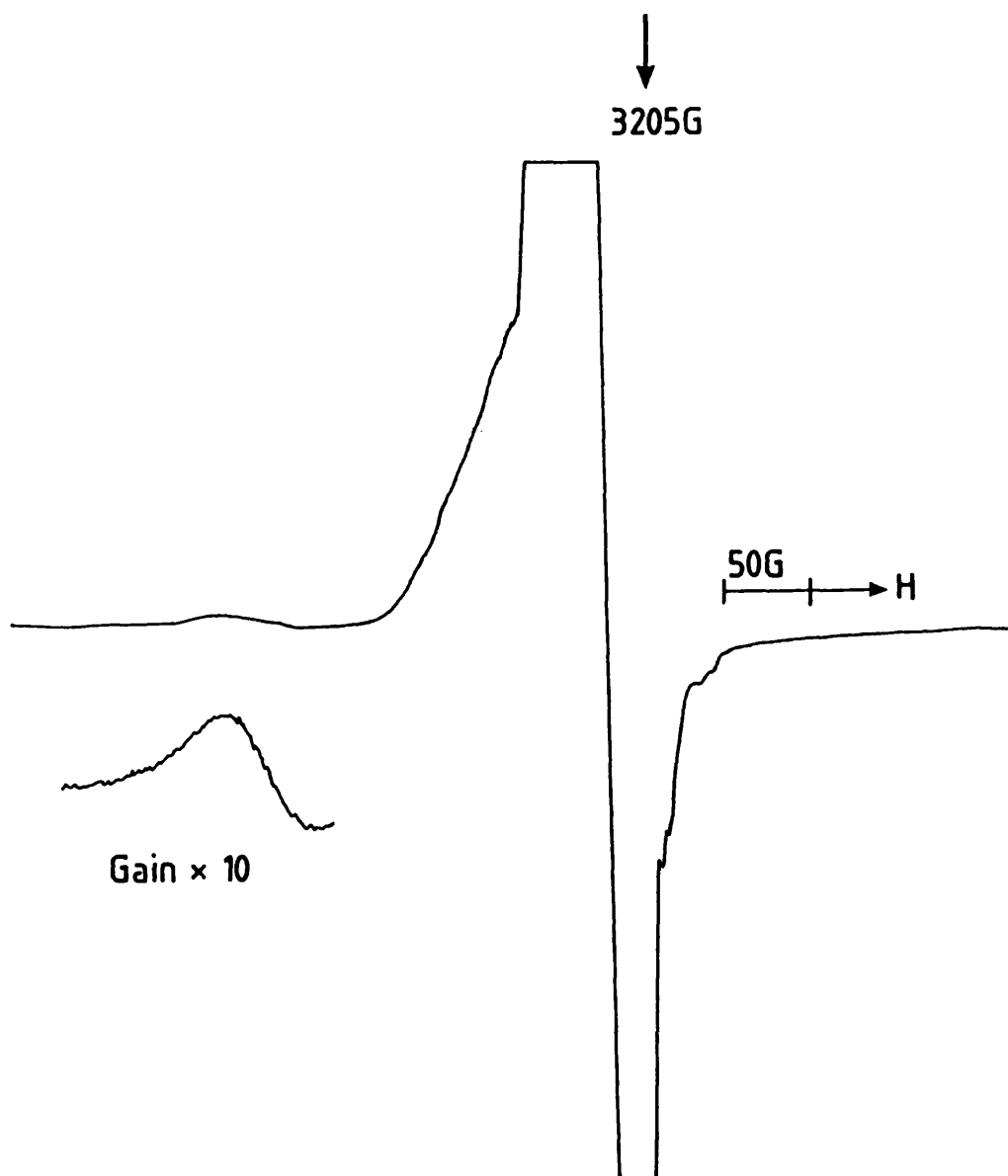


FIGURE 4.16

Initial ESR spectrum of reduced lipoic acid under acidic conditions showing production of $\text{RCH}_2\text{S}^\bullet$ radicals.

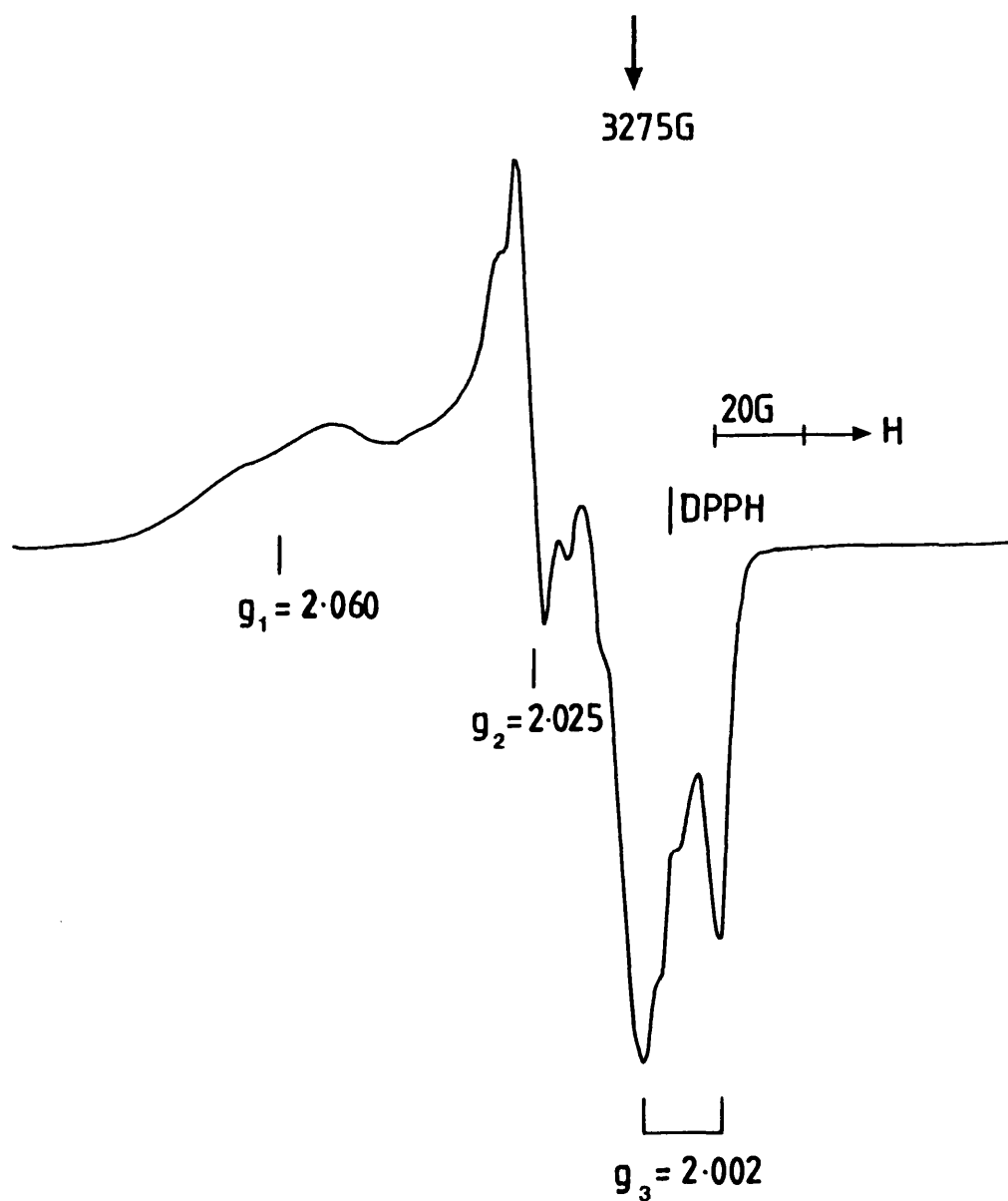


FIGURE 4.17

ESR spectrum of reduced lipoic acid in CD_3OD under acidic conditions showing production of a species "X" type radical.

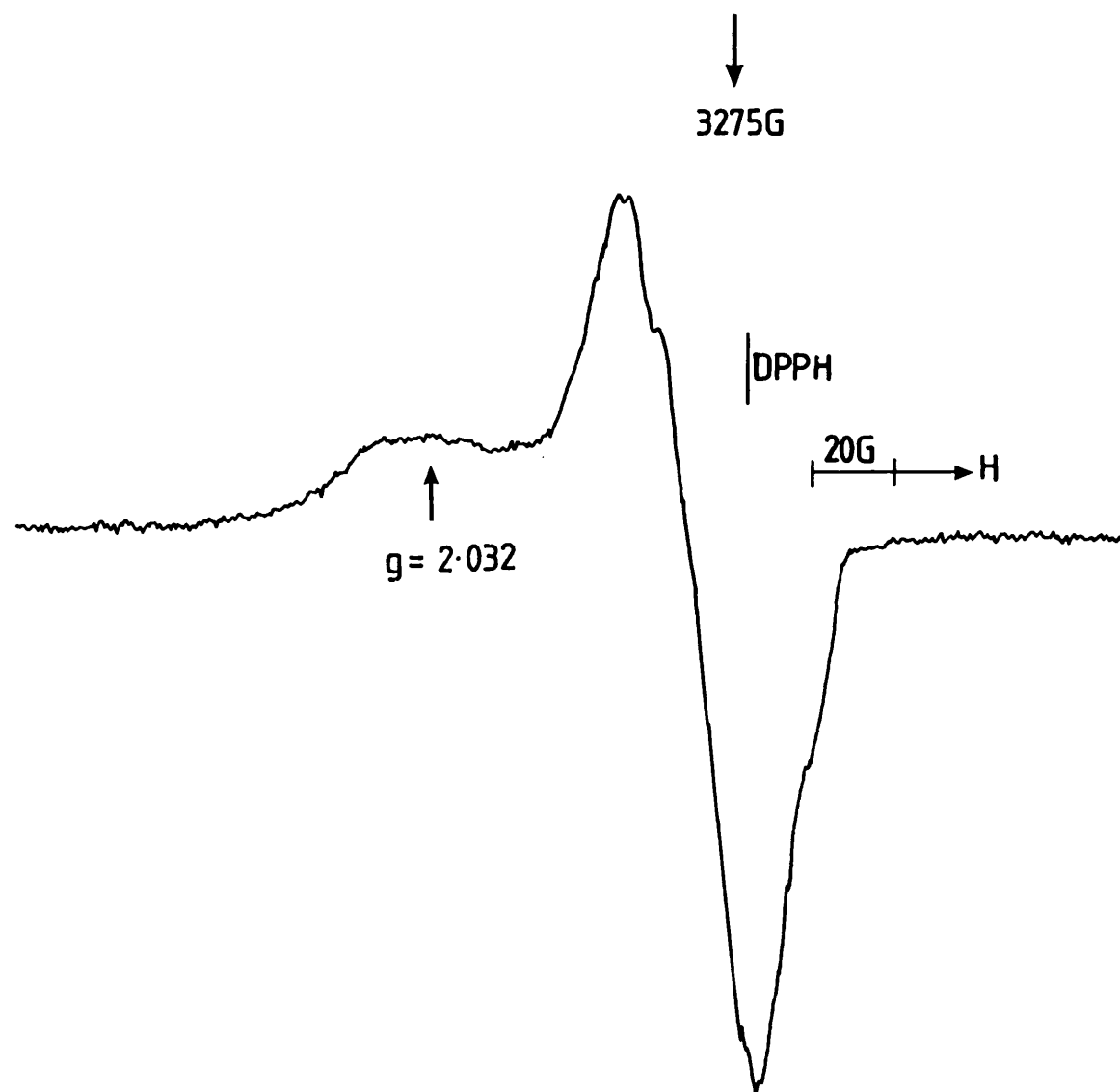


FIGURE 4.18

ESR spectrum of reduced lipoic acid in CD_3OD under acidic conditions showing production of the radical cation RSSR^\oplus .

was detected in the powder samples. This was found to change irreversibly into the normal form of species "X" ($g_2 = 2.025$) on annealing. Similar results were obtained for dithiothreitol [Chapter 2] and for cysteinyl dihydrochloride by Akasaka *et al.*⁶⁴ It is suggested that the "2.016" species is a constrained form of species "X", the constraint being lifted on annealing. A detailed analysis of these results is given in Chapter 6 but, briefly, these results would appear to support the $\text{RSS}(\dot{\text{H}})\text{R}$ structure due to the type of structural change involved.

Secondly, an apparent interconversion between the disulphide radical anion $\text{RSSR}^{\cdot-}$ and species "X" has been observed, both from RSSR and RSH parent compounds. It was found that the radical anion was favoured in alkaline media and species "X" in acidic media. This leads on to an interesting conclusion concerning the structure of species "X". When the oxidized form of lipoic acid (RSSR) is the substrate, both assignments for the structure of species "X" can be accommodated via reactions [4H] and [4I], though [4I] seems unlikely at these low temperatures. However, when the reduced dithiol form of lipoic acid was employed, species "X" was detected and the production of the disulphide radical anion was again found to be pH dependent. This follows naturally in terms of a simple acid/base reaction if species "X" is $\text{RSS}(\dot{\text{H}})\text{R}$ but seems quite impossible in terms of the perthiyl radical RSS^{\cdot} as this would involve the reverse of reaction [4I] alkylating the radical which, at these low temperatures, seems highly unlikely.

Finally, samples containing species "X", but no clear signals for the disulphide radical anion are always pale yellow. Although optical studies seem indicated, it is suggested here that there is an intense near UV band which is possibly the 380 nm band assigned to the protonated disulphide.⁶⁹ We know of no evidence for RSS^{\cdot} radicals

having an intense near UV band, nor is such an absorption probable on structural grounds.



CHAPTER 5

Ethylperthiyl Chloride

5.1 INTRODUCTION

In the previous chapters, the studies have concentrated on gathering evidence to attempt to solve the problem of assignment of the disulphide radical species "X", whether as (1) $\text{RSS}(\text{H})\text{R}^{38}$ or (2) $\text{RSS}\cdot^{39}$ or even both. Initial analysis of our own results seem to favour the former assignment, but there remains weighty evidence in favour of the RSS formation [see Chapter 6].

In this Chapter the approach to the problem is changed somewhat. By studying EtSSCl and RS_4R it was hoped that genuine examples of the perthiyl radical $\text{RSS}\cdot$ would be produced, thereby eliminating some of the confusion as to the assignment of its e.s.r. spectrum. That is, to play the rôle of devil's advocate to the preceeding chapters.

This approach is by no means new, for example, Geoffroy et al.⁷¹ have generated $\text{RSS}\cdot$ from an RSSH derivative. This is discussed in greater detail in the next chapter, [see Chapter 6]. Of more importance here are the studies of Bennett and Brunton, which form the basis of the work in this chapter. In 1979 they published an e.s.r. study of the t-butylperthiyl radical.⁷² Briefly summarising their work, they observed in solution, a singlet which they attributed to $\text{tBuSS}\cdot$ radicals. The radical, characterised by $\Delta H = 4 \text{ G}$ and $g_{\text{av}} = 2.025$, decayed via a second order process, with a rate constant ($2k_t$) of $2 \times 10^8 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$ at -86°C .

The g -value measured for the $\text{tBuSS}\cdot$ radical was very similar to that detected during the photolysis of di-n-butyl disulphide⁸⁵ (2.0262). On the basis of their results they suggested that this was likely to arise from the n-butylperthiyl radical $\text{nBuSS}\cdot$.

Unfortunately, frozen solutions of this species were not studied. However, solid-state photolysis (77 K) of tBuSSCl gave a dominant species

having g -values of 2.059, 2.026 and 2.001. The spectrum, which was not recorded, was described as containing at least two species, and the authors were unwilling to place much relevance on these features. Nevertheless, they suggested that their g_{av} value (2.025), was close enough to the average g -value (≈ 2.029) of species "X" in the solid state, to support the perthiyl assignment of this radical in solid state systems.

We accept that the liquid-phase species was $RSS\cdot$. However, the discrepancy between this g_{av} value of 2.025 and the average value for their solid-state species (2.029) left considerable room for doubt as to the identity of this species. This prompted these particular studies in an attempt to produce genuine $RSS\cdot$ radicals in a solid-state environment. This concentrated on two methods of producing the perthiyl radicals (1) radiolysis and (2) photolysis.

(1) Radiolysis

We used ethylperthiyl chloride instead of t-butylperthiyl chloride. This was made up in a dilute deoxy CD_3OD/D_2O solution, frozen into a glassy matrix, irradiated (γ -rays, ^{60}C source) and subsequently studied by e.s.r. at 77 K. Our firm expectation was that only e^- capture would occur, which we anticipated would give $EtSS\cdot + Cl^\ominus$.

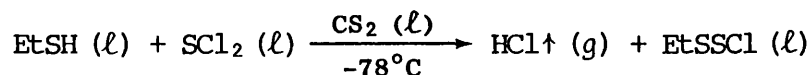
(2) Photolysis

These experiments were performed by Dr. R. L. Petersen on RS_4R compounds synthesized in this laboratory by the methods of Tsurugi and Nakabayashi.⁸⁶

5.2 EXPERIMENTAL

5.2.1 Synthesis of Ethylperthiyl Chloride

The compound was synthesized according to the methods outlined by Fehér and Kruse.⁹⁵ The synthesis follows the reaction;



Ethanethiol (2 cm³) and sulphur dichloride (30g) were carefully mixed at -78°C (using a dry ice/acetone slush bath) using carbon disulphide (30 cm³) as a solvent. As the sulphur dichloride was added dropwise, hydrogen chloride gas was given off. When all the sulphur dichloride had been added and the release of HCl had ceased, the mixture was allowed to warm to room temperature (cooling if necessary, to prevent an over-violent reaction).

The solvent was then distilled off at room temperature and the residue was distilled under vacuum ($\approx 0.1\text{mmHg}$), the products being condensed in a cold trap at -78°C. Purity was checked by standard procedures.

5.2.2 Synthesis of Dibenzyl tetrasulphide

This was prepared by Dr. R. L. Petersen using several standard procedures and the purity was carefully checked.⁸⁶

5.2.3 Preparation of samples for e.s.r.

Aliquots of ethylperthiyl chloride were made up in CD₃OD/D₂O solutions and prepared for e.s.r. analysis by the procedures outlined previously [see Chapter 2].

Samples of dibenzyl tetrasulphide were prepared for photolysis by adding the compound in the form of a yellow oily liquid dropwise into liquid nitrogen. The beads formed by this process were placed in a sample tube and photolysed at 77 K with a medium pressure Hg-lamp.

5.3 RESULTS AND DISCUSSION

5.3.1 Ethylperthiyl chloride

Ethylperthiyl chloride (EtSSCl) is a yellow oily liquid and, prior to irradiation, EtSSCl samples frozen in CD₃OD/D₂O glass matrices were also yellow in colour. Post-irradiation, however, the sample beads had changed colour to purple. This was thought to be due to "trapped" (or solvated) electrons within the system (as before with other samples in this matrix). This was supported by the initial spectrum taken from this sample. It comprised, as had been observed previously [see Chapter 2], of an intense signal centred around free-spin. This signal was assigned to solvent radicals in the matrix [Fig. 5.1].

After an increase in temperature (post-annealing) the spectrum observed had altered quite considerably. Contrary to previous observations of organosulphur compounds in this glassy matrix [see Chapters 2, 3 and 4] no evidence of RCH₂S• radicals was detected either in the pre- or post-anneal spectra [Fig. 5.2]. The major signal [Fig. 5.3] was indeed very "X"-like resembling qualitatively the spectra for dithiothreitol and lipoic acid. It was only when the g -values of the three major signals were measured that any difference was noted. The intermediate (g_2) and high-field (g_3) values did have "X"-like values at ca. 2.024 and 2.002 respectively. The difference occurred with the primary (g_1) value. This was calculated to be at $g = 2.042$, which is lower than the normal range of values of "X"-species detected (2.055 - 2.062).

[Features in the free-spin region of the spectrum were not easily distinguishable, signals from solvent radicals complicating the signal observed, making assignment all but impossible.] However, the main result is the abnormally low g -value (2.042) for the g_1 feature of the major product. This remained unchanged despite increasing temperature

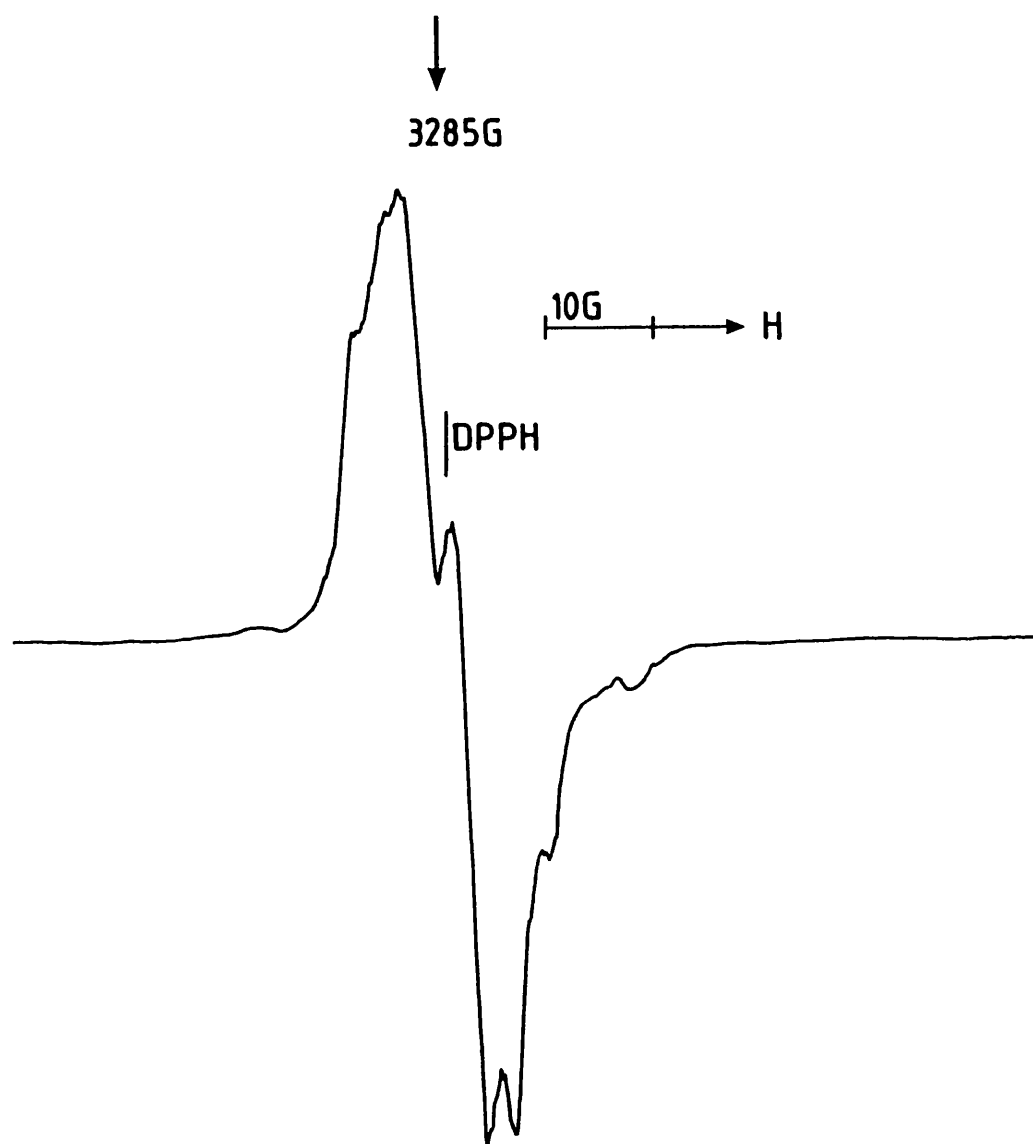


FIGURE 5.1

ESR spectrum of EtSSCl in CD₃OD taken at 77 K.

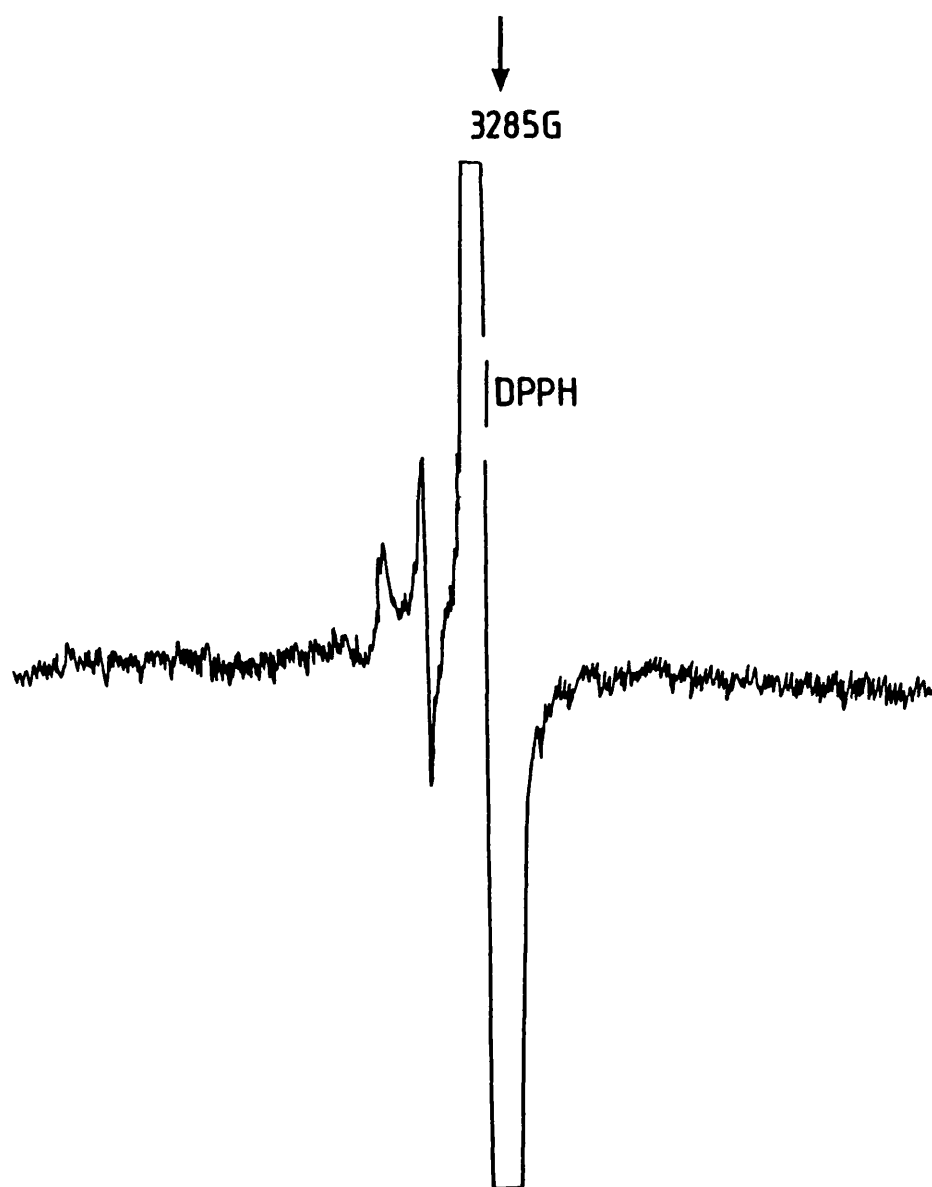
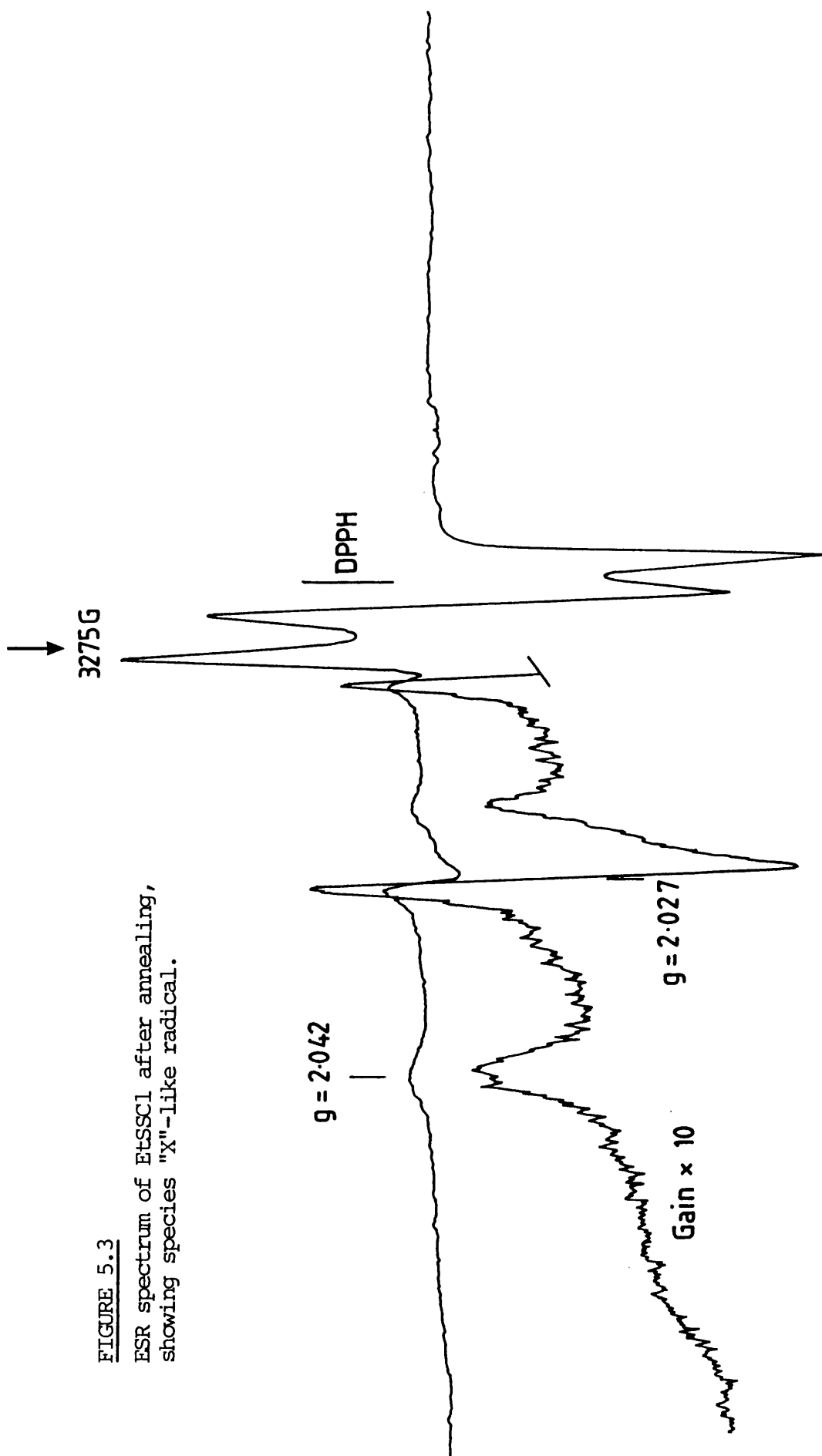


FIGURE 5.2

ESR spectrum of EtSSCl in CD_3OD after annealing, showing lack of any thiyl radicals.

FIGURE 5.3

ESR spectrum of EtSSCl after annealing,
showing species "X"-like radical.



[Fig. 5.4].

An additional factor was the colour of the sample. In the preceeding chapters great emphasis has been placed on the colour of these organo-sulphur samples in a glassy matrix, after an increase in temperature has removed the solvent radicals. At this stage, the predominant sulphur radicals are detected, concurrent with a change to a yellow colouration of the sample. With EtSSCl no such colour change was detected. Instead the sample turned white. The significance of this will be discussed later in this Chapter.

5.3.2 Dibenzyl tetrasulphide

When post-photolysis spectra of this compound were examined [Fig. 5.5] it was noted that these too were very "X"-like in appearance. These spectra do, however, have several features in common with those of EtSSCl. Again, there is the discrepancy in the g -value of the major g_1 feature (2.042) as opposed to the more normal "X" values (2.055 - 2.062) and also no $RCH_2S\cdot$ radicals were detected. Unlike EtSSCl there is no other radical present. No colour change was reported.

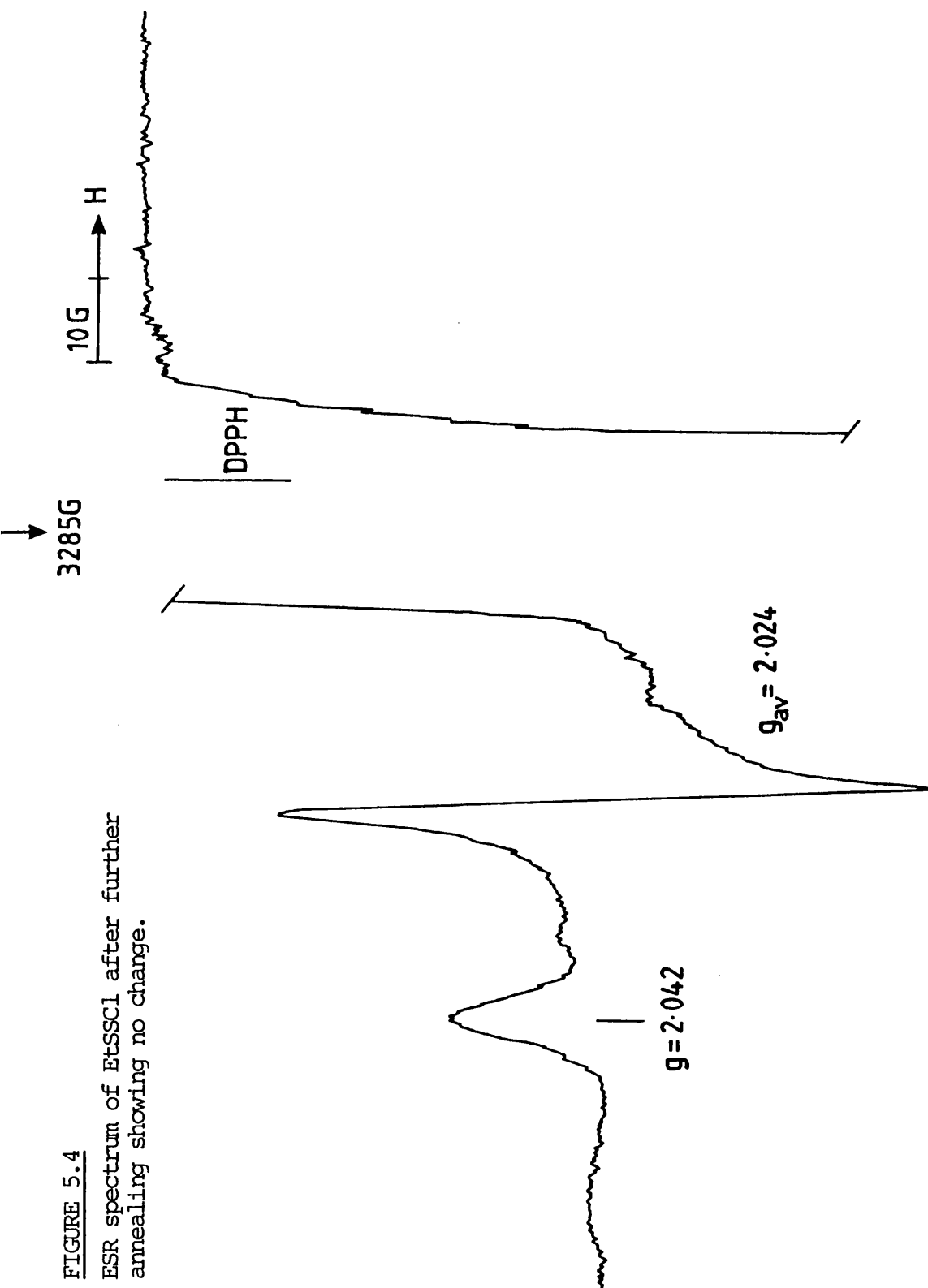
Comparison of spectra from ethylperthiyl chloride and dibenzyl tetrasulphide [Fig. 5.6] showed good alignment of the principal g -features, particularly at $g = 2.042$. It is suggested here that this is good evidence that the major radical observed is of similar structure, in both samples.

5.3.3 Concluding Remarks

The selection of ethylperthiyl chloride and dibenzyl tetrasulphide was not made at random. It was thought that these experiments would provide good examples of the perthiyl radical $RSS\cdot$. Taken in detail, gamma irradiation of EtSSCl in a CD_3OD/D_2O matrix should remove the chloride from the parent compound leaving the $EtSS\cdot$ radical [Scheme 5A]. The lack of evidence for $RCH_2S\cdot$ radicals in the e.s.r. spectra would

FIGURE 5.4

ESR spectrum of EtSSCl after further annealing showing no change.



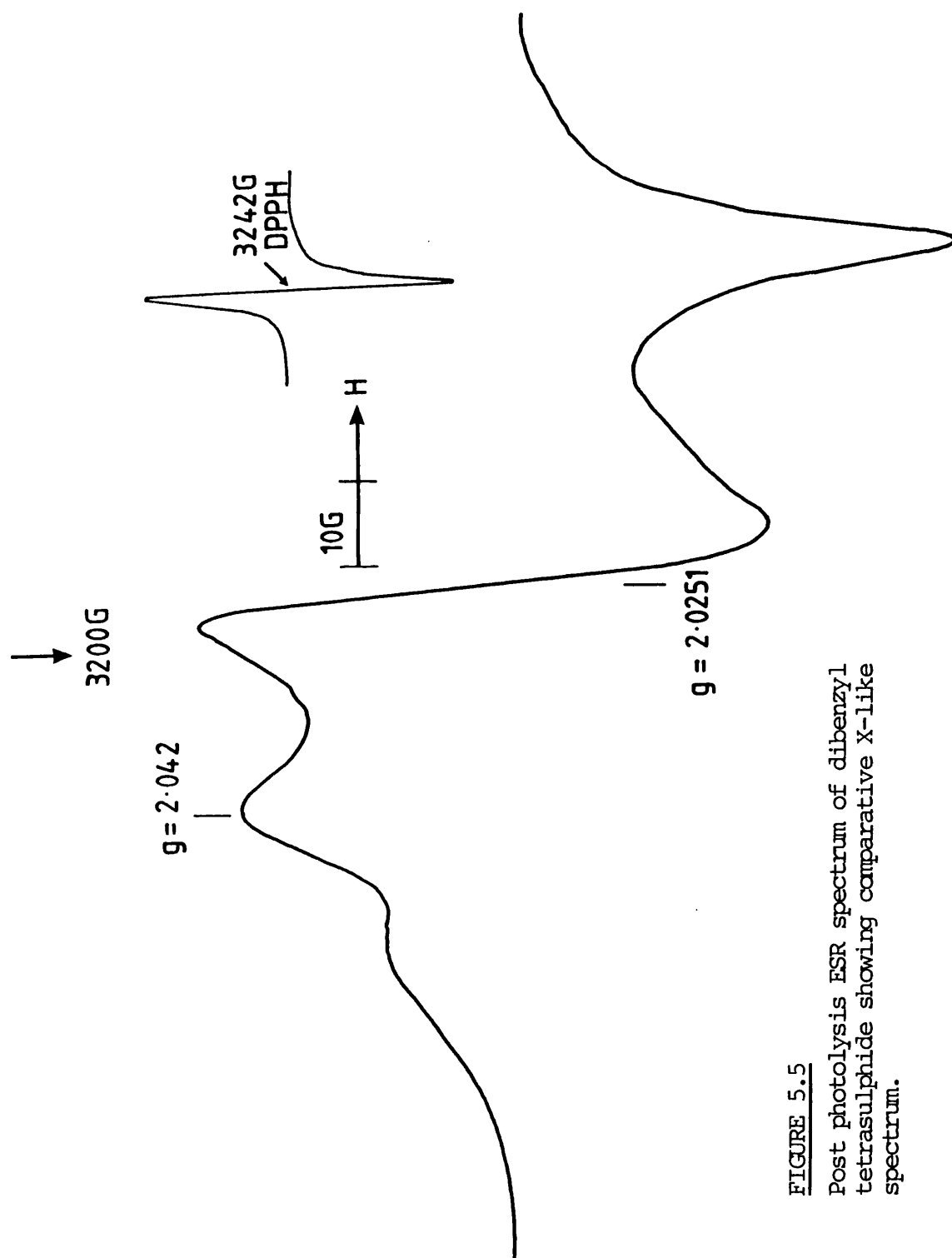
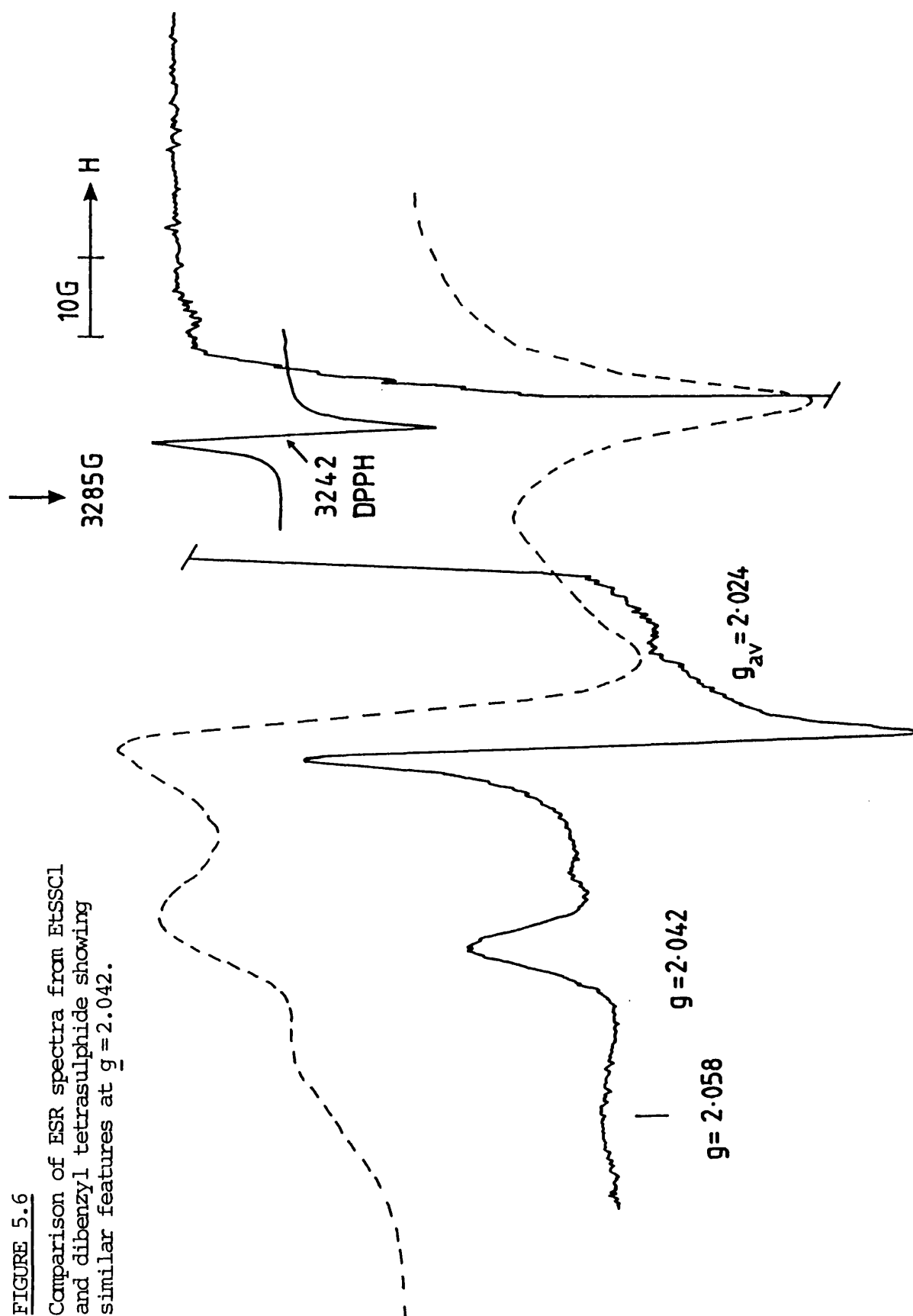


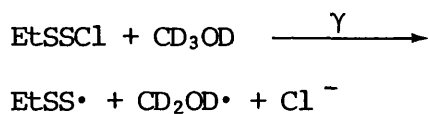
FIGURE 5.5
 Post photolysis ESR spectrum of dibenzyl
 tetrasulphide showing comparative X-like
 spectrum.

FIGURE 5.6

Comparison of ESR spectra from EtSSCl and dibenzyl tetrasulphide showing similar features at $g = 2.042$.



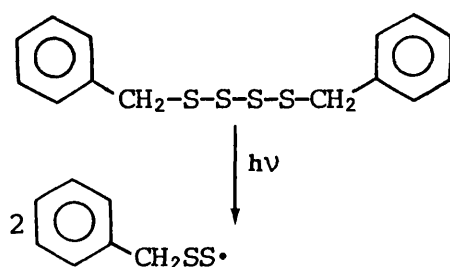
SCHEME 5A



appear to support this hypothesis. To return to the work of Bennett and Brunton,⁷² it is suggested here that breakage of the -S-Cl bond, in their sample (tBuSSCl) is by no means a certainty, when the compound is photolysed. Indeed, a referee of the paper suggested that the radical may be more of the type Bu^t(RS)S·S-Cl (where R = Bu^t or Cl) as previous studies⁸⁷ had indicated that radicals of this type may readily undergo α-scission. In other words, photolysis is more likely to break the S-S bond rather than the S-Cl bond.

The breakage of the centre S-S bond is even more likely in the case of the photolysis of dibenzyl tetrasulphide [Scheme 5B]. Absence of RCH₂S· radicals provides indirect evidence that this is the more likely reaction mechanism.

SCHEME 5B



Hence the spectra observed are thought to be due to perthiyl radicals of the type RSS·. Considering Geoffroy and coworkers' studies⁷¹ it is, however, surprising that RSS·-----RSS· pairs were not detected with the dibenzyl tetrasulphide.

It can also be noted [Fig. 5.6] from the spectra that in both EtSSCl and ØCH₂S₄CH₂Ø samples there is some indication of a signal at ca. g = 2.058. This signal would be more in keeping with a typical "X"-like

spectrum. However, due to the far more intense feature at $g=2.042$, which as stated is shifted to a value lower than any seen for species "X", it would seem possible that this signal (2.058) is due to a radical with an $RSS(R')R$ structure contaminating the sample. This radical with its own features at $g=2.025$ and 2.002 being swamped beneath the far more intense perthiyl radical signal. This would seem all the more likely, when the radical structure $Bu^t(RS)S\cdot SCl$, put forward by the referee to the paper by Bennett and Brunton,⁷² is considered.

Another factor aiding the assignment of this particular spectrum was the colour change observed for $EtSSCl$ samples in CD_3OD/D_2O matrices. After an increase in temperature, the other organosulphur compounds studied have exhibited a yellow colour concurrent with the appearance of a species "X" radical. In the previous chapters it has been stated that this absorption would be expected⁶⁹ of radicals with an $RSS(H)R$ type structure. This absorption was not predicted for radicals of the type $RSS\cdot$ and, at this stage, the $EtSSCl$ samples did not turn yellow but white instead. Again this would appear to support the assignment of the radicals from $EtSSCl$ and $\phi CH_2S_4CH_2\phi$ to be of the structure $RSS\cdot$.

This does, however, raise one very important question. If the signal observed at $g=2.058$ [Fig. 5.6] is due to radicals of the type $RSS(H)R$, why then is the yellow colour, observed for other radicals of this type, not observed here?

It is suggested here that there are two possible explanations of this. Since the signal at $g=2.058$ is far less intense than that at $g=2.042$, it is possible that the concentration of the perthiyl radical is so much greater than that of the contaminating species "X", that the yellow colour of the species "X" radical is simply not observed. An alternative to this explanation rests with the perthiyl radical itself. It was noted

with the studies on lipoic acid [see Chapter 4] that two forms of species "X" exist, a "relaxed" and an "unrelaxed" form. This agreeing with earlier work by Akasaka et al.⁶⁴ on cysteine dihydrochloride. When this is considered in terms of the results from EtSSCl and $\text{OCH}_2\text{S}_4\text{CH}_2\text{O}$, comparisons can be made.

With lipoic acid an increase in temperature results in the signal from the "unrelaxed" form of species "X" being lost. The radical adopts its "relaxed" form and the conventional g -values are obtained. Similarly, when samples of EtSSCl are annealed, the signal at $g = 2.058$ is lost [Fig. 5.6] with only the signal at $g = 2.042$ remaining. It is suggested here that the signal at $g = 2.058$ may not be due to a species "X" radical, but to an "unrelaxed" form of the perthiyl radical which, on annealing, relaxes to give the "conventional" perthiyl radical g_1 signal at $g = 2.042$. This would explain the lack of a yellow colour in the EtSSCl samples despite the signal at $g = 2.058$.

Finally, while the work of Bennett and Brunton⁷² does not claim to resolve the assignment of species "X" in the solid state, they favour the perthiyl assignment of species "X". This is on the basis that the g_{av} value obtained for the $\text{tBuSS}\cdot$ radical in solution (2.025) is close to the average g -value of species "X" in the solid state (2.029). However, when the average g -value (2.023) of the major radicals observed for both EtSSCl and $\text{OCH}_2\text{S}_4\text{CH}_2\text{O}$ in the solid state, is calculated, it agrees more closely with that of Bennett and Brunton rather than to species "X" values obtained both in this thesis and previously.³⁸

When all factors from this Chapter are considered, it is suggested that the radical in the solid state, known as species "X", giving g -values of 2.058, 2.025 and 2.002 is due to a radical of the structure $\text{RSS}(\text{H})\text{R}$ and not $\text{RSS}\cdot$. The results obtained for EtSSCl and $\text{OCH}_2\text{S}_4\text{CH}_2\text{O}$ suggest that

the perthiyl radical $\text{RSS}\cdot$ has a similar spectrum to RSS(H)R radicals, but with a vital g -shift, which distinguishes it as being a completely different radical. The whole question as to the structure of species "X" is concluded in the following chapter.

The firmness of this conclusion, however, must be tempered somewhat in view of the convincing results of Geoffroy et al.⁷¹



CHAPTER 6

A Case for Species "X"

6.1 INTRODUCTION

The importance of sulphur-centred radicals acting as intermediates in biological reactions has been discussed in Chapter 1. However, there remains a question of more chemical significance, that is the assignment and possible structure of the radical designated species "X".

This has been the cause of controversy in the literature, so I start with a summary of the history of this particular radical. E.s.r. spectroscopy was first used to study sulphur containing radicals in 1955 by Gordy and his coworkers⁶ and has been used extensively ever since, along with optical techniques^{88,89} (using matrices at low temperatures and pulse-radiolysis). The greatest difficulty encountered has always been that of assignment. Certainly in the case of workers employing e.s.r. techniques, this has been due mostly to the fact that the magnetic isotope of sulphur, ^{33}S (0.74% abundance and $I = 3/2$) has very low abundance. In practical terms, this means that if enrichment of a sample with ^{33}S is impossible, there is no clear finger-print. There is, however, a way around this problem. Spectroscopists working in this field have placed great emphasis on positive g -shifts (i.e. shifts to a high g -value) as being the major indication of sulphur-centred radical formation.^{6,34,35} In actuality, this has been found to be not always the case - it very much depends on the radical itself. If, within the radical, there are low-lying magnetically coupled excited states involving orbitals having significant spin-density on sulphur, then the large spin-orbit coupling constant for sulphur relative to that for oxygen will lead to a higher g -shift. This occurs in the case of $\text{RCH}_2\text{S}\cdot$ radicals but not for the $\cdot\text{SO}_3^-$ radical which has a g -tensor very close to the free-spin value (2.0023).

With assignment being a problem, obviously many incorrect ones have

been made. This is particularly true for species "X", where for quite some time it was thought to be an $\text{RCH}_2\text{S}\cdot$ radical. The same difficulties arose concerning the identification of $\text{RO}\cdot$ radicals until the work of Box and coworkers^{58,59} who identified $\text{RCH}_2\text{O}\cdot$ radicals in irradiated sugar crystals.

As to the identification of $\text{RS}\cdot$ radicals the orbitals must be considered. When the structure of these radicals is examined in detail, it is apparent that in the absence of any external perturbation the radical would be orbitally degenerate, so that the " π_x " and " π_y " orbitals on sulphur would have to be combined to give $|\pm 1\rangle$ orbitals. If this was the case, g -values would be in the range 0-4. However, with the exception of $\cdot\text{SH}$ radicals, small Jahn-Teller distortions would lift this degeneracy to a small extent, even if there were no environmental effects. In truth, these radicals have been detected in frozen (77 K) samples probably because the environmental effects far outweigh the Jahn-Teller distortions.

Indicative of these environmental effects are the results of Symons and coworkers concerning hydroxyl ($\cdot\text{OH}$) radicals in ice.⁹⁰ They suggested that it is hydrogen bonding which is responsible for the major quenching of the orbital angular momentum observed ($g_{\parallel} = 2.06$). For a clear e.s.r. spectrum to be obtained it would require that each radical would have to be similarly bonded. In a later paper Symons, Hüttermann and coworkers⁹¹ showed that when $\cdot\text{OH}$ radicals are trapped in imprecise glassy environments, the characteristic g_{\parallel} feature is greatly broadened, or even completely lost.

Hence, the g -tensor components for $\text{RS}\cdot$ radicals are not characteristic of these radicals, but are a measure of the strength of the asymmetric environmental interaction that must be present to give its spectrum

[Fig. 6.1].

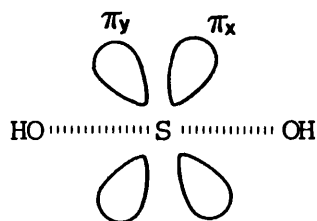


FIG. 6.1

Figure showing the orbitals around the sulphur atom showing the possibility of changing environment.

This was the major proof that radicals having g -values of ca. 2.06, 2.025 and 2.002 (species "X") could not be radicals of the type $RS\cdot$ which was their previous assignment. This is because these values are seen to be constant in a wide range of compounds and in different environments both in this thesis and by other workers.⁹² Further proof was provided by Hadley and Gordy³⁹ who showed, in elegant experiments using ^{33}S hyperfine features, that species "X" contained two inequivalent sulphur atoms and they suggested the perthiyl assignment $RSS\cdot$. At approximately the same time as Hadley and Gordy, Symons³⁸ suggested that since species "X" was formed from thiols, then it was a weak adduct formed between the primary $RS\cdot$ radicals and another RSH molecule and assigned species "X" the σ^* structure $RS-\dot{S}R_2$ (where R is H or alkyl). Both assignments could be expected to show coupling to two inequivalent ^{33}S nuclei.

This then has been the cause of controversy in the literature which this section of the thesis (the organosulphur compounds) hoped to resolve. Arguments and evidence for each assignment are extensive and each will be presented here.

6.2 THE PERTHIYL RADICAL

Much of the evidence for this structure is based on crystal studies employing e.s.r. spectroscopy and, in this respect, would be thought to be irrefutable. Primarily this concerns the work of Geoffroy and co-workers⁷¹ and Henriksen and coworkers.⁹³⁻⁹⁴ Considering Geoffroy's work initially. In 1984, Franzi, Geoffroy and Bernadinelli⁷¹ published the results of a study of the photolytic damage in triphenylmethyl-disulphane single crystals (Ph_3CSSH), the main points of the study being the crystal structure of Ph_3CSSH and the e.s.r. study of the $\text{Ph}_3\text{CSS}\cdot$ radicals, which are trapped in pairs. The e.s.r. eigenvectors were compared to the bond directions in the parent molecule determined from the crystal structure of Ph_3CSSH [space group $P2_12_12_1$, $a = 7.571(1)$, $b = 7.608(1)$, $c = 27.790(5)$, $z = 4$]. Both radical pairs of $\text{Ph}_3\text{CSS}\cdot$ were trapped along with isolated $\text{Ph}_3\text{CS}\cdot$ and $\text{Ph}_3\text{CSS}\cdot$ radicals.

From the crystal data it would seem that the -S-S and C-S bond lengths are not dissimilar to those already reported,⁹⁶ though the SSC angle would appear to be slightly larger for the disulphane (108°) than for the disulphide (103°).

Concerning the radicals observed there can be little doubt that the radical with g -values 2.112, 1.993 and 1.963 is indeed a radical of the type $\text{Ph}_3\text{CS}\cdot$. Data from this study agrees closely with requirements outlined for the formation of this radical by Symons⁶⁰ and also Box⁶² and Sagstuen and Alexander.⁹⁷

The main issue arising from this study is with the other two radicals observed, designated B and C. The eigenvalues of the g -tensors for species B and C are the same and it was suggested that the signals from B and C were from the same species with C corresponding to the isolated radical and B to the trapped radical pairs.

Geoffroy and his coworkers favoured the perthiyl assignment for their radical. Their argument is based on the fact that migration of Ph_3C to form the alternative structure, $\text{Ph}_3\text{CSS}(\text{H})\text{CPh}_3$, is highly improbable within the crystal lattice. The lack of any proton coupling, together with the agreement between calculated and experimental directions of g_{max} and g_{min} supports this assignment.

Thus they concluded that one of the major effects of u.v. radiation on Ph_3CSSH is to produce pairs of $\text{Ph}_3\text{CSS}\cdot$ radicals, via the scheme shown [Fig. 6.2]. It is claimed that this mechanism is facilitated by the crystal lattice, whose main feature is the existence of "channels" which are orientated along the b -axis and in which the SSH moieties are located.

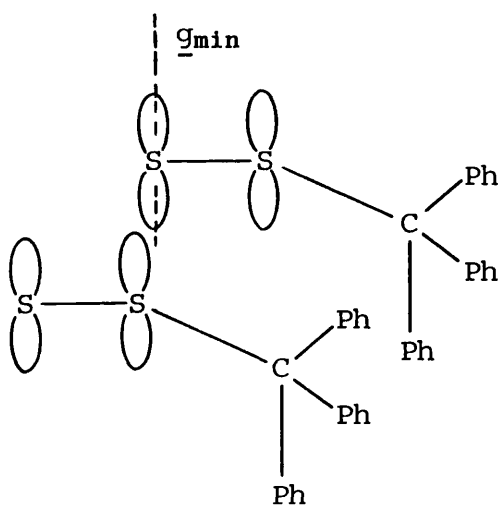


FIG. 6.2

Scheme for production of $\text{Ph}_3\text{CSS}\cdot$ radical pairs as shown by Geoffroy and coworkers [Ref. 71].

Further evidence for the perthiyl radical $\text{RSS}\cdot$ comes from the work of Sagstuen, Jørgensen and Henriksen.⁹³ They irradiated single crystals of 2-thiouracil [Fig. 6.3] and studied the e.s.r. spectrum at 77 K. In addition to the detection of an $\text{RS}\cdot$ radical, they also obtained a second

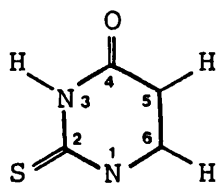


FIG. 6.3

Structure of 2-thiouracil.

sulphur-centred radical which exhibited g -values of ca. 2.060, 2.025 and 2.002. When considering the crystal and molecular structures, this group also favoured the perthiyl radical ($\text{RSS}\cdot$) assignment. An additional result was that they could observe no direct relationship between the thiyl radical and their own species "X", which they designated "the sulphur resonance".⁹³ This was, in their opinion, supported by the mechanism they proposed for the formation of the disulphide radical. Studies on aliphatic thiols have led to the suggestion that $\text{RS}\cdot$ radicals act as precursors for disulphide radicals via the reactions:



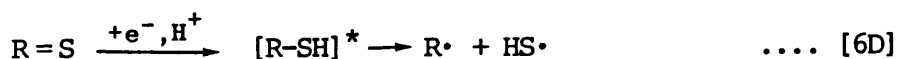
or



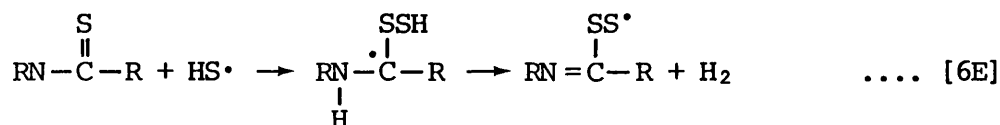
followed by



However, Henriksen and his coworkers obtained no evidence of thiyl radicals acting in this manner. They proposed that rotation of large molecular fragments such as the aromatic residues involved which would be required to bring two sulphur atoms into close contact is unlikely as this would necessitate a movement of $5\text{--}6 \text{ \AA}$.^o Therefore, they proposed an alternative mechanism;



where homolytic bond scission takes place from an excited state [*].



Aside from the work of Geoffroy and his coworkers and Henriksen and coworkers, there is more evidence for the perthiyl assignment. As discussed in Chapter 5, Bennett and Brunton⁷² proposed that a species formed by photolysis of Bu^tSSCl was the radical Bu^tSS•. They observed in solution a singlet signal which they assigned to a Bu^tSS• radical. The g -value measured for this radical was very similar to that obtained for the photolysis of di-*n*-butyl disulphide.⁸⁵ On the basis of their results, they suggested that this was likely to be due to the radical nBuSS•. Arguments for and against the identification of the radical from Bu^tSSCl are given in Chapter 5.

Giles and Roberts⁷³ prepared radicals of the structure F₃CS•SR₂, by addition of F₃C[•]S radicals to dialkyl sulphides in the liquid phase. This work clearly establishes that such adducts can be formed. However, they were found to have g_{av} of 2.0133. Since this value is well below that for species "X" ($g_{\text{av}} \approx 2.029$) they argued that species "X" cannot have this type of structure. This result would appear to disprove Symons' assignment completely.

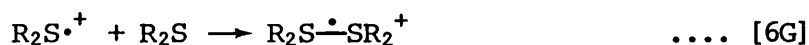
This summates the major evidence for the perthiyl radical assignment, RSS•.

6.3 THE RS•SR₂ STRUCTURE

It is the object of this section to discuss the literature evidence for the radical structure RS•S(H)R (or, in general, RS•SRR'). Evidence obtained in the present study is discussed later in this Chapter.

Both the disulphide "dimer" radical cations and anions (R₂SSR₂⁺ and

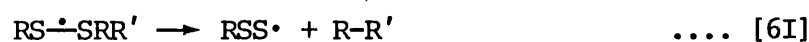
RSSR⁻) are formed readily from thiol derivatives:



Examining this it could be expected that the "intermediate" reaction should also occur.



However, to form the perthiyl radical RSS[•] from thiols, it is necessary to postulate [6H] in order for the reaction



to occur. Attempts to detect the product R-R' have been made but the results were inconclusive, even when species "X" has been formed in high abundance.^{61,97-100} We consider that further work on the species R-R' is required before any argument can be made for or against reaction [6I].

There is a difference between the two structures of species "X" in terms of their expected optical properties. Since both RSSR^{•-} and R₂SSR₂^{•+} have intense absorption spectra in the visible region, radicals with the structure RS[•]SR₂ should also have an intense low energy optical band.⁶⁹ However, absorption of this type is not expected for the perthiyl radical.⁹⁸ In fact, an intense band at ca. 380 nm observed in systems containing radicals of this type can be assigned to species "X".

Several other arguments would seem to support Symons' assignment.³⁸ Thus, from the literature there appears to be evidence for and against both radical structures. It is possible that their g-tensor components are very similar, so that both theories are correct. It is also possible that RSS[•] radicals are not formed from pure thiols but from RSSH impurities.^{101,102}

6.4 DISCUSSION

The whole issue of the structure of the radical species "X" would appear to be somewhat confused. However, it is the purpose of this section of this Chapter to weigh the evidence for each radical structure and, by including the results from this thesis, hopefully to resolve this controversy.

Considering initially the work of Geoffroy and coworkers,⁷¹ this is an exceedingly well-planned experiment, and were it not for results in this thesis, it would be completely convincing. Both mechanistic and crystallographic aspects appear to agree well with the perthiyl assignment $\text{RSS}\cdot$ for their species. However, as discussed in the previous section, Symons¹⁰³ postulated that $\text{RSS}\cdot$ radicals observed in samples of thiols were due to RSSH contamination. This agrees with Geoffroy's results, from the compound studied (Ph_3CSSH). Thus, their radical almost certainly is $\text{Ph}_3\text{CSS}\cdot$. But can one generalise from this to all other "X" species?

Much of the other major evidence for the perthiyl structure is less compelling. For example, the work of Sagstuen, Jørgensen and Henriksen,^{93,94} based on crystal studies using e.s.r. spectroscopy. The mechanism proposed for the production of $\text{RSS}\cdot$ refutes the action of $\text{RS}\cdot$ as a precursor of disulphide radicals. This may well be true of this particular compound but seems unlikely in terms of the wealth of evidence showing $\text{RS}\cdot$ as both the precursor of $\text{RSS}\cdot$ and $\text{RS}-\dot{\text{S}}(\text{H})\text{R}$ radicals.^{38,60,70} As to the disulphide radical they reported, with g -values 2.06, 2.025 and 2.002, it seems unlikely that this is a "normal" $\text{RS}-\dot{\text{S}}(\text{H})\text{R}$ type radical or an $\text{RSS}\cdot$ type since it has the wrong basic structure [Fig. 6.4]. The carbon adjacent to the sulphur atom is trigonally coordinated and hence it, and the aromatic moiety can surely

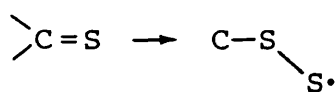


FIG. 6.4

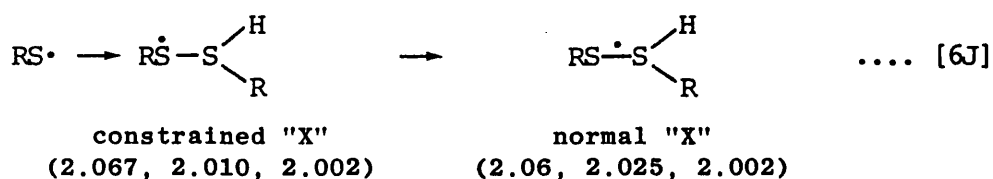
Configuration around carbon in 2-thiouracil according to Henriksen, Sagstuen and Jørgensen [Ref. 93].

be part of the π -SOMO. There would seem to be little conclusive evidence indicating sulphur atom attachment to form the perthiyl radical and, in view of the wealth of evidence suggesting disulphide radical formation from thiyl radicals, the mechanism suggested by Sagstuen, Jørgensen and Henriksen would seem a little improbable. Furthermore, it could be expected that the major radical produced would be formed from the radical cation or the radical anion and since it is not formed from the radical cation then the anion must be the precursor. It is possible that the major radical formed could be a carbon-centred radical anion of the type $\text{R}_2\dot{\text{C}}-\text{S}^-$ which would, indeed, have a large Δg , with g_{max} along the C-S bond.

As discussed earlier in this Chapter [see also Chapter 5], the results of Bennett and Brunton⁷² and Giles and Roberts⁷³ are inconclusive. As it appears in the literature much of the physical properties of species "X" would appear to favour the perthiyl assignment, whilst the chemical properties appear to favour the $\text{RS}-\dot{\text{S}}(\text{H})\text{R}$ structure. It is with this dicotomy that much of the work in this thesis is concerned. The work on dithiothreitol, 2-pyridinemethane thiol and lipoic acid would all appear to favour the $\text{RS}-\dot{\text{S}}(\text{H})\text{R}$ assignment.³⁸ On the other hand, when attempts to study genuine perthiyl radicals were made, several anomalies between these and species "X" were observed. Samples of EtSSCl in $\text{CD}_3\text{OD}/\text{D}_2\text{O}$ were devoid of colour after irradiation and partial annealing. This is to be expected of perthiyl radicals, whereas samples containing species "X" from other compounds have a yellow colour (ca. 380 nm band). Also

with these radicals, g_{\max} was observed at $g = 2.042$ which is far lower than expected for normal species "X". [As a counter to this, the work of Geoffroy and his coworkers gives g_{\max} at 2.06 for the $\text{Ph}_3\text{CSS}\cdot$ radical.]

I now return to a consideration of the species with g -values close to 2.067, 2.010 and 2.002. Up to now it has been discussed in terms of an unrelaxed form of species "X" [see Chapters 2 and 4] as proposed initially by Akasaka and coworkers.⁶⁴ It was, however, mentioned by Hadley and Gordy³⁹ who assigned it to a monosulphide radical of the type $\text{RS}\cdot$. However, an examination of the spectrum shows that, if the most of the spin-density is located on one of the sulphur atoms, then the parameters also fit the disulphide adduct structure. Hence, we can write,



Various mechanisms can be considered for this process, which would cause the constrained form of species "X" to resemble more closely the thiyl radical $\text{RS}\cdot$.

It was noticed that no monothiol compound (e.g. glutathione) exhibited this constrained configuration, only the "relaxed" form of species "X" being observed. The only samples which displayed this signal were disulphides, i.e. cysteinyl dihydrochloride or oxidized lipoic acid, or in the case of dithiothreitol, a compound where cyclisation producing a disulphide which was known to be favourable.⁵² Also the original study by Akasaka and coworkers^{64,65} was on single crystals of cysteinyl dihydrochloride and this "constrained" radical was only observed in

irradiated pure samples of lipoic acid and dithiothreitol. Solutions of these compounds [$\text{CD}_3\text{OD}/\text{D}_2\text{O}$ glassy matrix] producing only the "relaxed" radical centre. Therefore, requirements for this radical seem to be a disulphide at low temperature in a crystalline environment.

The observation that relaxation of some form, involving the CH_2 groups within the radical, causes the transition between the "unrelaxed" and the "relaxed" configuration of the radical, made by Akasaka and coworkers,⁶⁴ is thought to be correct. This may have a direct application to the problem of the structure of species "X".

The two possible structures of this radical "X" are considered, first of all in terms of the $\text{RSS}(\text{H})\text{R}$ assignment. When a compound displaying the "constrained" species "X" is annealed then it will adopt its most stable or most energetically favourable conformation. Hence on annealing the transition between the "constrained" species "X" signal and the signal attributable to the "relaxed" form of species "X" is observed.

In addition to this, dithiothreitol, lipoic acid and cysteinyl dihydrochloride share a facility for hydrogen bonding, be it intra- or inter-molecularly. Both the cysteinyl dihydrochloride and the lipoic acid contain carboxylic acid functional groups, and the dithiothreitol, two hydroxyl groups. Thus the crystal forces are expected to be strong. This will inhibit the movement required to form the relaxed form of the radical, at 77 K.

Similar arguments can be used for the $\text{RSS}\cdot$ formulation, except that one would not expect such strong resistance to the conformational change which stabilises the radical.

There is also a third possibility. This is that the species formed at 77 K is a radical of the structure $\text{RSS}(\text{H})\text{R}$ and that this gives $\text{RSS}\cdot$ on annealing via the reaction:



However, it has been mentioned earlier in this Chapter that there is no conclusive evidence for this particular reaction. It could, though, be a possible explanation of the e.s.r. changes.

In conclusion, consideration of all the evidence both in the literature and that gained in this thesis, it would appear that species "X" with \underline{g} -values 2.06, 2.025 and 2.002 is due to a radical with the structure $\text{RSS}(\text{R}')\text{R}$ where $\text{R}' = \text{H}$ or alkyl and that whilst the species observed by Geoffroy and coworkers⁷¹ is probably $\text{Ph}_3\text{CSS}\cdot$ it is thought to be coincidental that its \underline{g} -values resemble those of species "X", as our own studies on perthiyl radicals produce very different $\underline{g}_{\text{max}}$ values.

Further work is certainly indicated to resolve this controversy finally, possibly along the lines of further pH studies or optical studies on these particular radicals, perhaps employing specifically synthesized "models" as with Geoffroy and coworkers.⁷¹



CHAPTER 7

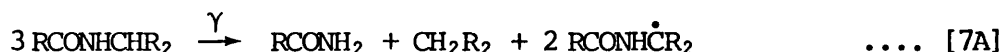
Polyamino Acids and Protein

7.1 INTRODUCTION

This Chapter marks a departure from the previous work on organo-sulphur compounds. Here the study is more concerned with the transfer of energy, in the form of ionizing radiation, through the peptide backbone of a protein or more particularly polyamino acids used as model systems for proteins. In this sense it is more related to the studies on bovine cell nuclei in the following chapter [see Chapter 8].

Much of the work within this field concerns the fate of electron transfer usually within metalloproteins,¹⁰⁴ usually residing with e.s.r. or electrochemical studies on the metal centre itself. This study, however, presents the alternative, i.e. the fate of the positive hole the electron leaves behind. Work in this area is not as extensive as that on metalloproteins but is still significant.

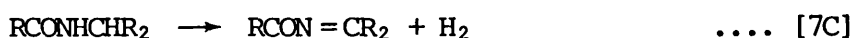
However, to continue the link into the cell nuclei study, it has been found that covalent DNA-protein cross-links are formed when whole cells are exposed to U.V. or γ -radiation.¹⁰⁵⁻¹⁰⁸ The implications of this being that radiation chemical damage to a single amino acid residue of histone could lead to a modification of a biological function of the DNA-histone complex. This is further explored later in this Chapter. To attempt to probe this particular possibility, this study concentrates on the products arising from the exposure of certain polyamino acids and protein to γ -radiation. Earlier work in this field being the subject of several publications and reviews.¹⁰⁹⁻¹¹¹ In 1967 Garrison et al.¹¹² postulated that main-chain cleavage with formation of amide and fatty acid function was a major reaction in the radiolysis of peptides in the solid state in the manner;



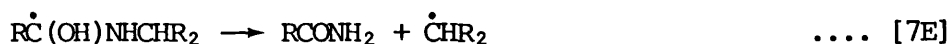
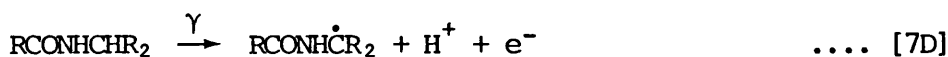
where $\text{RCONH}\dot{\text{C}}\text{R}_2$ represents the long-lived radical products observed at room temperature using e.s.r. spectroscopy.¹¹³ Further work by Garrison¹¹⁴ on chemical separations of products formed in the radiolysis of polyalanine and N-acetylalanine gave $G(\text{amide}) \approx 3$, $G(\text{propionic acid}) \approx 2$, $G(\text{pyruvic acid}) \approx 1$ and $G(\text{dimer}) \approx 2$. The dimer arises as a result of dissolution of the irradiated solid in deoxygenated water via;



The evidence from these separations being that the keto acid is produced through the dehydrogenation

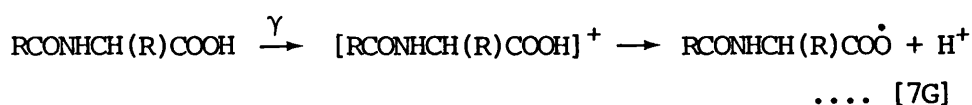


E.s.r. studies indicated that the stoichiometries observed for [7A] for polyamino acid derivatives of the simpler α -amino acids results from the intermediate ionic processes:^{115,116}

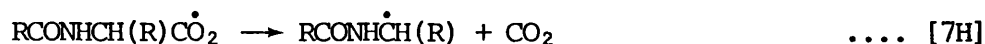


This would give the overall stoichiometry in [7A].

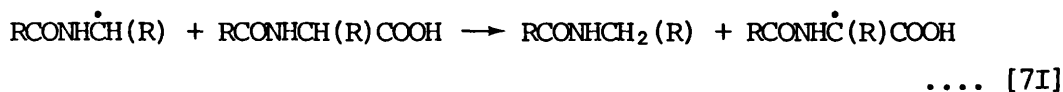
The e.s.r. work of Sevilla and coworkers^{116,117} and others¹¹⁰ claimed that the radiation chemistry of the lower-molecular weight peptides such as the N-acetyl amino acids and the di- and tripeptides is somewhat more complicated than that given in the reactions [7D-7F]. In these systems it would be the carboxyl group that would represent the major locus of positive hole formation, i.e.



This is then followed by decarboxylation:



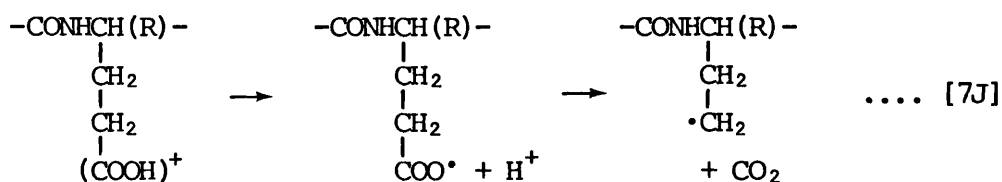
and then



to yield a long-lived α -carbon radical. Spin-trapping studies have yielded the decarboxylated radical, $\text{RCONH}\dot{\text{C}}\text{HR}$ and the α -carbon radical, $\text{RCONH}\dot{\text{C}}\text{(R)}\text{COOH}$, on dissolution of γ -irradiated N-acetylamino acids and dipeptides.¹¹⁸

Cleavage of a peptide main chain with the formation of amide and fatty acid functions is claimed to be the major process in solid state radiolysis of peptide derivatives of almost all aliphatic amino acids.¹¹¹ Sevilla *et al.*¹¹⁶ stated that the α -carbon radical, $-\text{CONH}\dot{\text{C}}\text{(R)}-$ is the long-lived radical species observed by e.s.r. spectroscopy of irradiated solids.

However, differences appear when considering other peptide derivatives. For example, with aspartic and glutamic acids, the evidence presented indicates that the positive hole is located preferentially at the side chain carboxyl group¹¹⁷ then loses H^+ and $\dot{\text{C}}\text{O}_2$ from the main chain to form α -aminobutyric acid.



Other studies^{117,119} indicate that the C=O linkage of the peptide bond appears to be the major trapping centre for e^- in the radiolysis of methionine and cysteine peptides in the solid state.

Garrison *et al.*¹²⁰ found that the yield of main chain cleavage as measured in terms of amide production is lower with aromatic amino acid peptide derivatives. The e.s.r. evidence is that the unsaturated side

chains of these residues competes with the peptide bond for e^- in solid state studies.¹¹⁷

As for proteins themselves, the yield of major degradation products formed by γ -radiolysis in the solid state (both fibrous and globular proteins) have been claimed to be¹²¹⁻¹²³ amide function ($G \sim 2.5$), keto acid plus aldehyde ($G \sim 1.0$), fatty acids ($G \sim 1.0$) and long-lived free radicals ($G \sim 5.0$). This would appear to substantiate the claim the stoichiometries presented [7A] are also of importance in the radiolysis of solid proteins.

Sevilla and coworkers¹¹⁶ have made extensive studies of model peptides using e.s.r. spectroscopy and has indicated that the major long-lived radical species observed by Gordy and Shields¹²⁴ and Riesz and White¹²⁵ are α -carbon radicals $P-\text{CONH}\dot{\text{C}}(\text{R})-P$.

However, by [7A] cleavage of the protein main chain must occur but this does not mean that the lower molecular weight fragments will be observed. It has been found that the average number molecular weight of polyamino acids and fibrous proteins does increase on irradiation in the solid state.¹²² However, globular proteins display this production of fragments to a lesser extent despite treatment to reduce intramolecular disulphide bonds.^{122,126}

The reason for this is likely to be that in globular proteins, the constraints imposed by the secondary and tertiary structures will make recombination competitive with dissolution of the fragments, whereas fibrous proteins and polyamino acids have far fewer constraints placed upon them, making dissolution more favourable. What then the purpose of this study? Firstly, the connection with the cell nuclei/DNA-histone work is clear. Whilst it is assumed that electron transfer will occur to the DNA molecule [see Chapter 8], what are the possible fates of the

positive hole it leaves behind? Also, there seems to be no definitive statement as to the behaviour of the positive hole within polyamino acid/protein systems after solid-state γ -radiolysis, only conclusions drawn from di- and tripeptide model systems.¹¹⁶ To this end, to try and confirm and/or resolve this situation, this study was undertaken, employing the polyamino acids, polyglycine and polyalanine and, to extend this study in proteins, bovine serum albumen.

7.2 EXPERIMENTAL

All samples [poly-L-alanine, polyglycine and bovine serum albumen] were obtained from the Sigma Chemical Company and were of the highest grade available. They were used without further purification.

Samples (35 mg ml⁻¹) were made up in D₂O [Aldrich] containing potassium ferricyanide (15 mM) [British Drug Houses]. The solution was then deoxygenated by bubbling oxygen-free nitrogen gas through the sample for up to 30 minutes. An aliquot was taken, frozen into beads, irradiated and examined in the manner previously described [see Chapter 2].

Powder samples of the pure compound were also prepared and studied as per the Experimental Section in Chapter 2.

7.3 RESULTS AND DISCUSSION

Powder samples of polyglycine were not found to be particularly useful as the signal produced [Fig. 7.1] was assigned to a combination of the radical cation and the radical anion, which provided no conclusive results as to the fate of the positive hole in a peptide chain. Hence it was decided to use an electron scavenger in an attempt to produce the

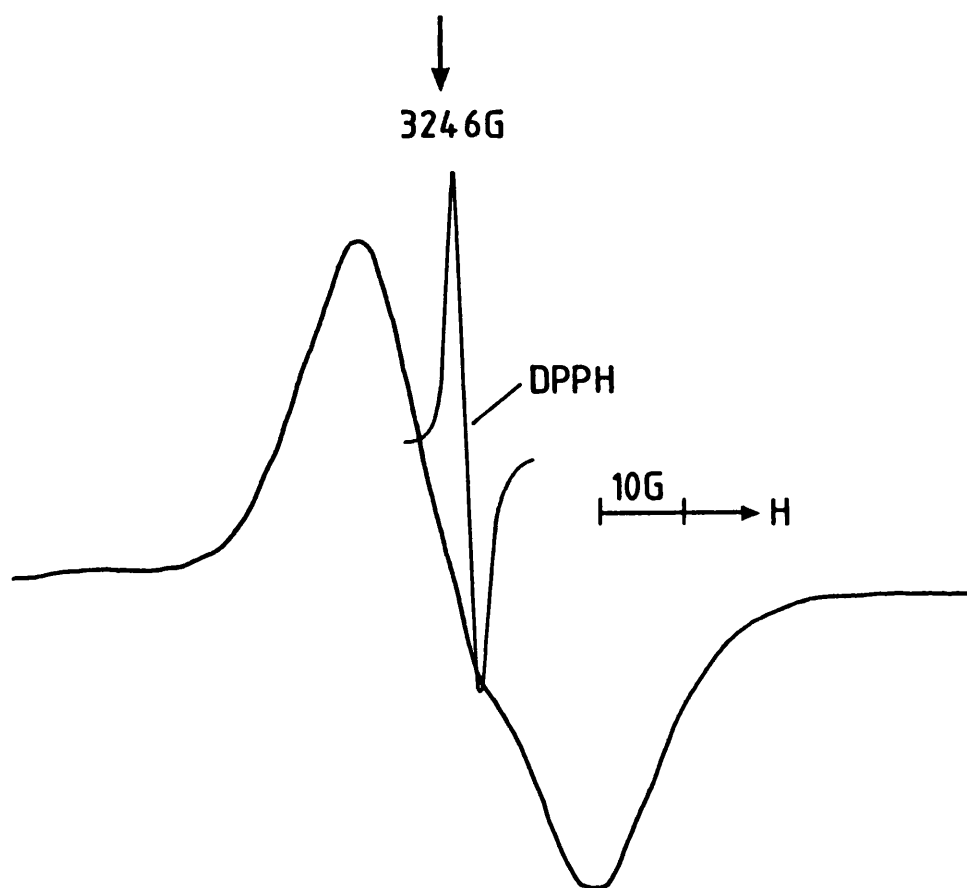


FIGURE 7.1

ESR spectrum of a powder sample of polyglycine at
 ≈ 120 K.

isolated radical cation, in this case, potassium ferricyanide was selected.

7.3.1 Polyamino acid, potassium ferricyanide, D₂O samples

Dealing with the simplest first, the initial studies were made on polyglycine [Fig. 7.2] where the more complex side groups of other amino

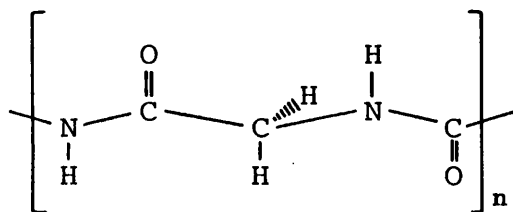


FIG. 7.2

The Structure of Polyglycine.

acids are replaced by protons. At 77 K the only signal observed after exposure to γ -radiation was that assigned to $\cdot\text{OD}$ radicals [Fig. 7.3]. As with other systems of this type, the signal attributable to the sample was only observed once the sample had been annealed. At ca. 140 K the $\cdot\text{OD}$ radical signal had diminished completely and the signal attributable to the polyglycine radical cation was observed [Fig. 7.4]. The major signal consisted of a doublet from one proton with a coupling of 25 G. Also, however, there was a minor two-line signal which was attributed to an $\pm 1\text{N}$ coupling of $\approx 45\text{G}$. When this sample was annealed further this minor signal was lost ($\approx 170\text{K}$) leaving only the doublet major signal [Fig. 7.5]. This remained unchanged until all signal was lost at ca. 230 K. To be able to compare this to the events in proteins, a slightly more complex polyamino acid was employed - poly-L-alanine. Before considering the results from this sample, its analogue poly-d,L-alanine was also studied to determine the effects of conformation. It was found that, in this particular case, conformation made no difference

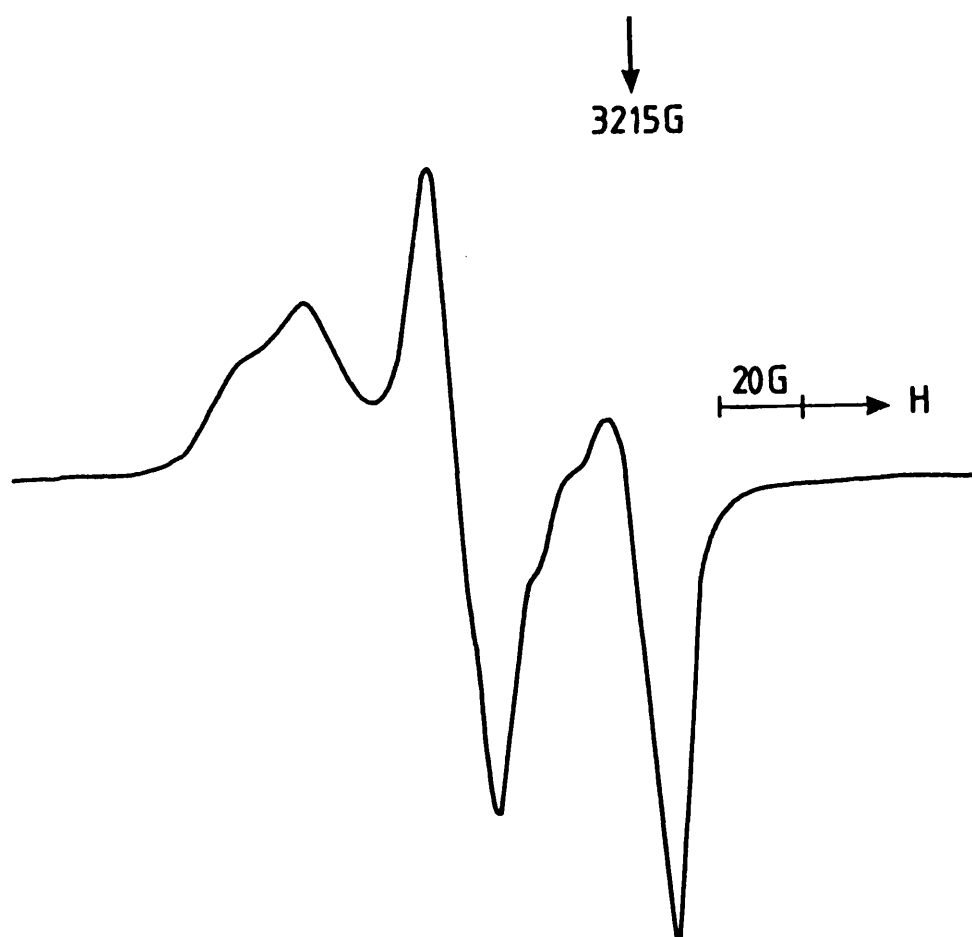


FIGURE 7.3

ESR spectrum of an ice sample of polyglycine/D₂O and potassium ferricyanide taken at 77 K.

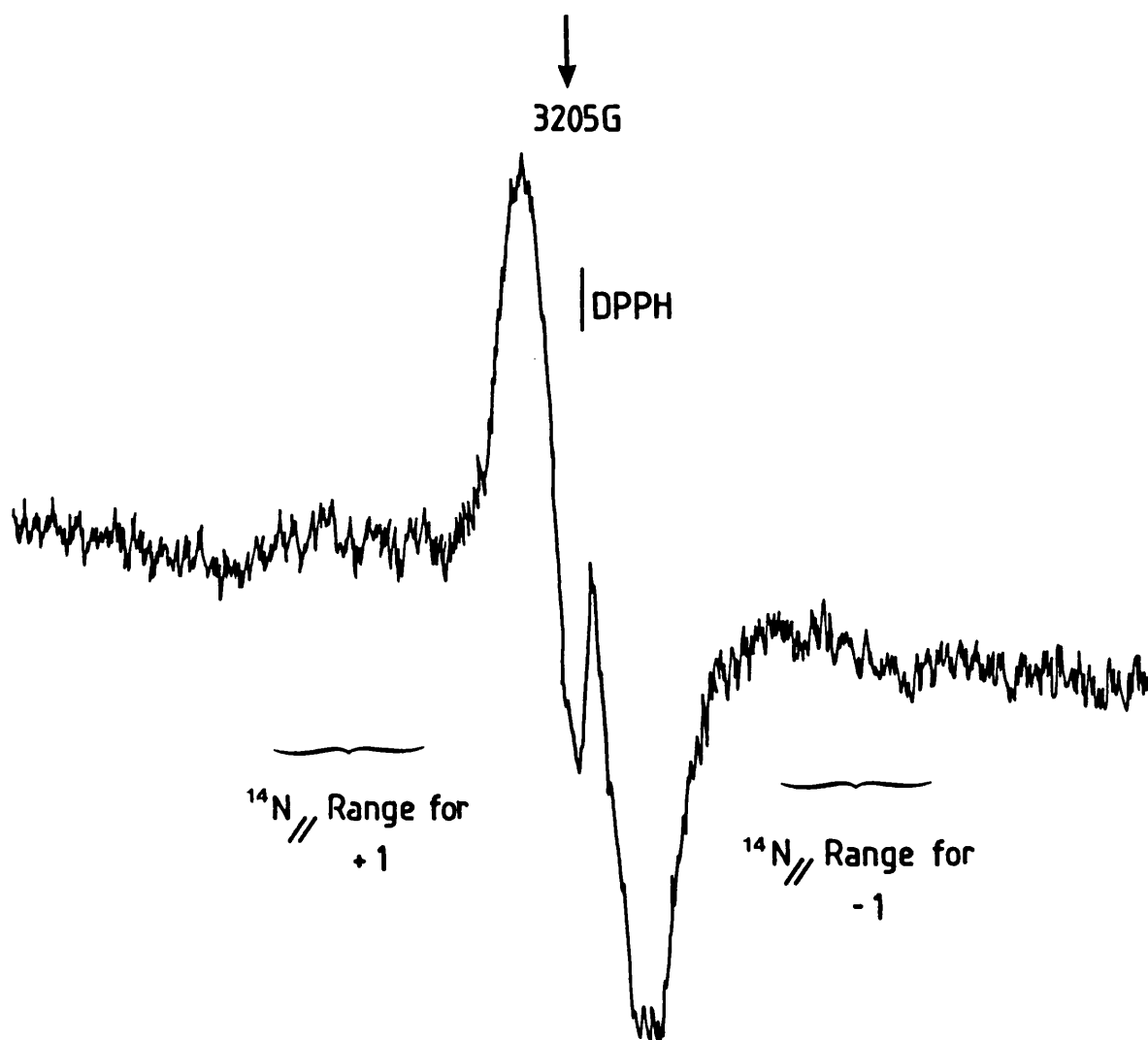


FIGURE 7.4

ESR spectrum of a polyglycine sample after annealing to ≈ 120 K.

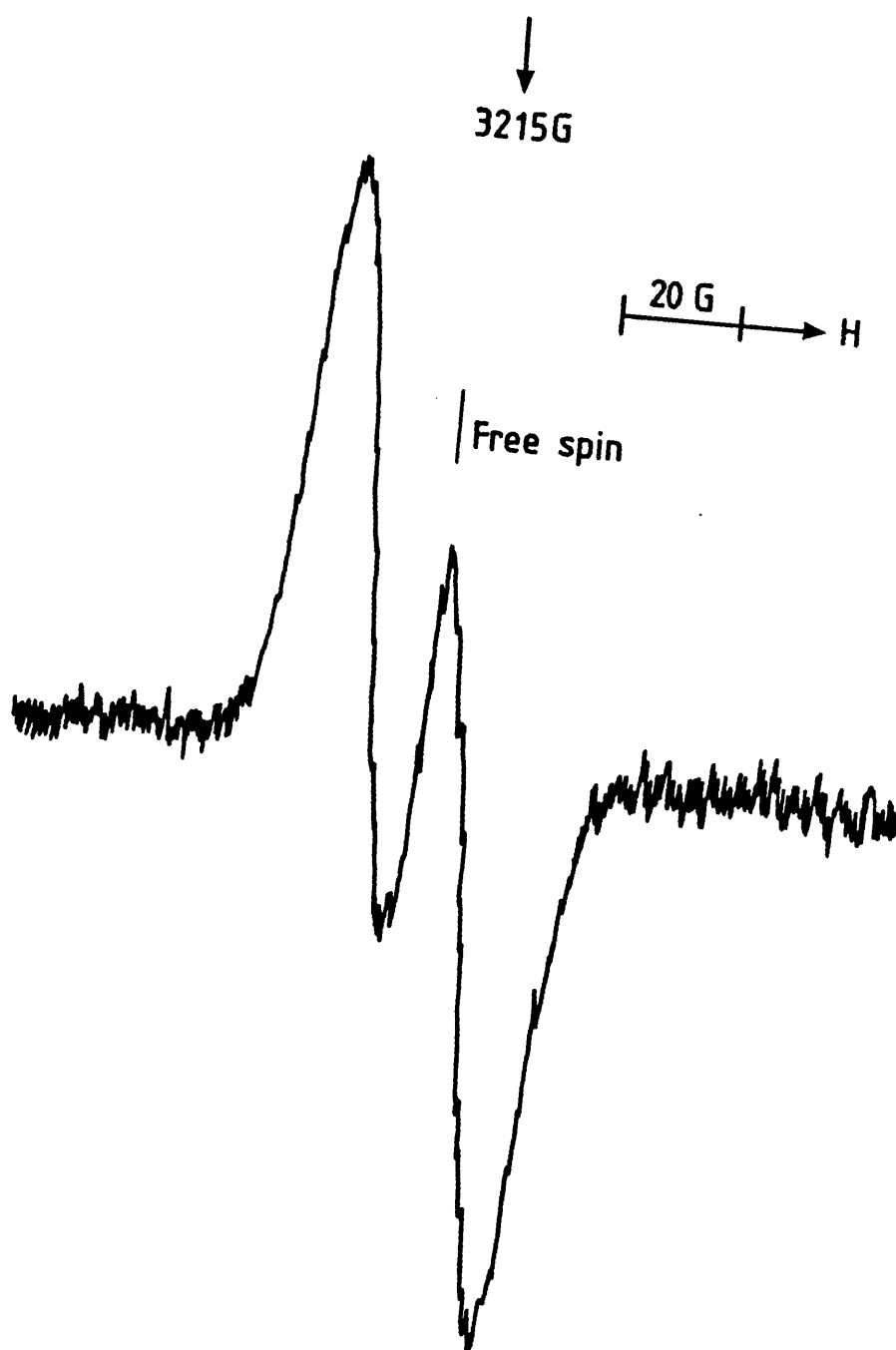


FIGURE 7.5
ESR spectrum of a polyglycine sample after
annealing to ≈ 150 K.

to the signals obtained. As with the polyglycine at 77 K, the major signal observed in poly-L-alanine [Fig. 7.6] samples was assigned to

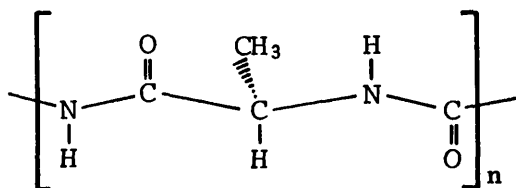


FIG. 7.6

The Structure of Poly-L-alanine.

•OD radicals [Fig. 7.7]. However, on closer examination at higher gain another signal was observed at higher field. In order to observe the signal from the polyamino acid radical cation the sample was annealed to ≈ 140 K. In this case, identification of the major signal was relatively easy [Fig. 7.8]. It consists of a 1:3:3:1 quadruplet centred around free-spin forming coupling from a methyl group adjacent to a carbon-centred radical, as per Sevilla and coworkers.¹¹⁶ However, it can be seen that on the minor lines of the quadruplet additional signals or shoulders are present which corresponds to an N_{\parallel} signal of ≈ 45 G ($A_H = 8$ G).

To determine whether these additional shoulders to the coupling from the methyl group were due to a separate radical, a power saturation study was conducted. It can be seen that from the power saturation studies [Fig. 7.9a, b, c] (Table 7.1) that there are two separate radical signals, one attributable to the carbon-centred radical, the other to a

TABLE 7.1

Power (mW)	Relative Signal Intensity	
	+1N $_{\parallel}$	-1N $_{\parallel}$
0.02	0.060	0.069
2.00	0.065	0.071
20.00	0.090	0.075

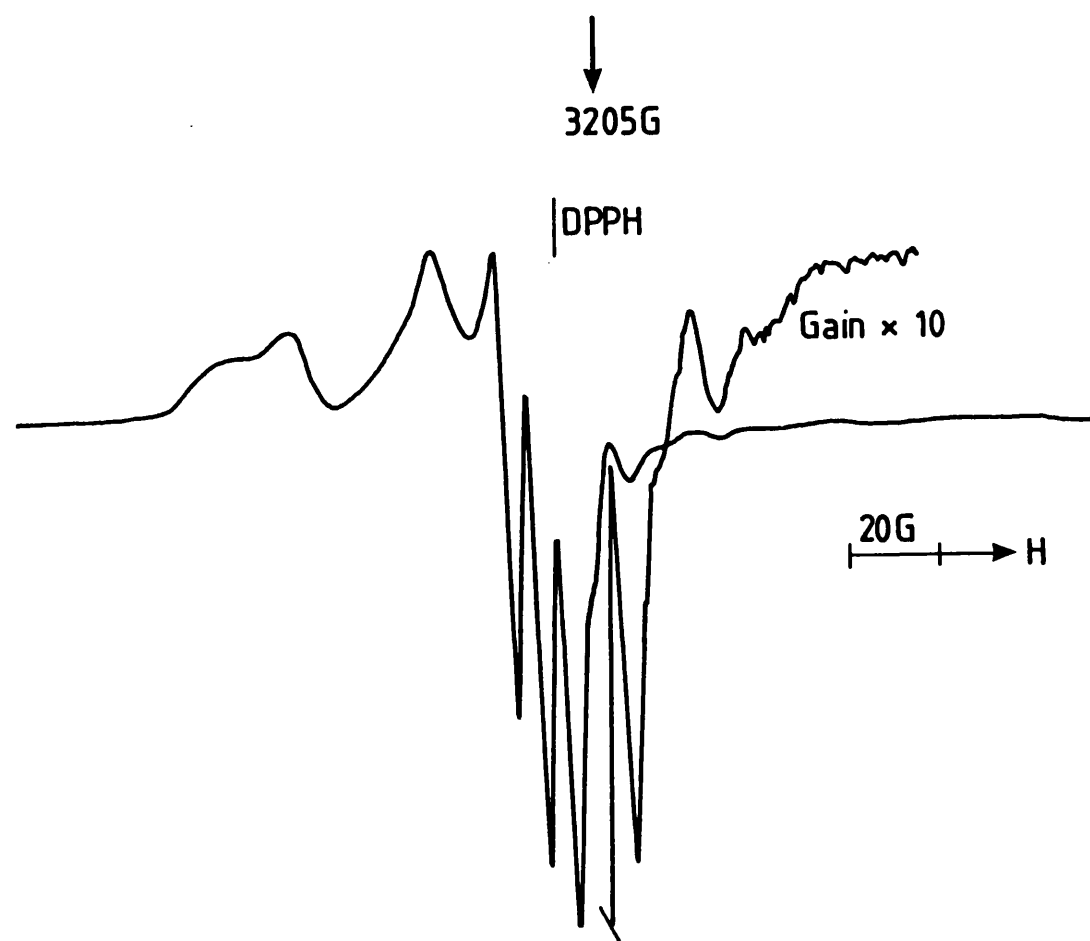


FIGURE 7.7

ESR spectrum of poly-L-alanine sample in D₂O plus potassium ferricyanide taken at 77 K.

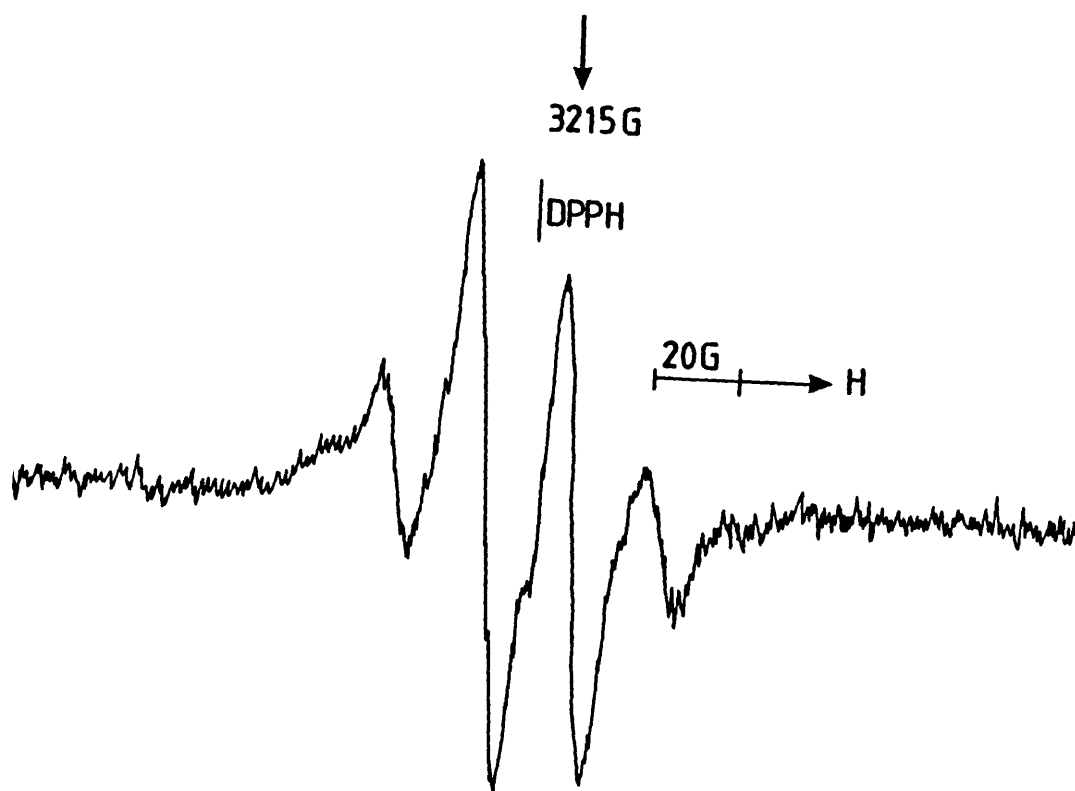


FIGURE 7.8

ESR spectrum of poly-L-alanine sample after annealing to ≈ 140 K.

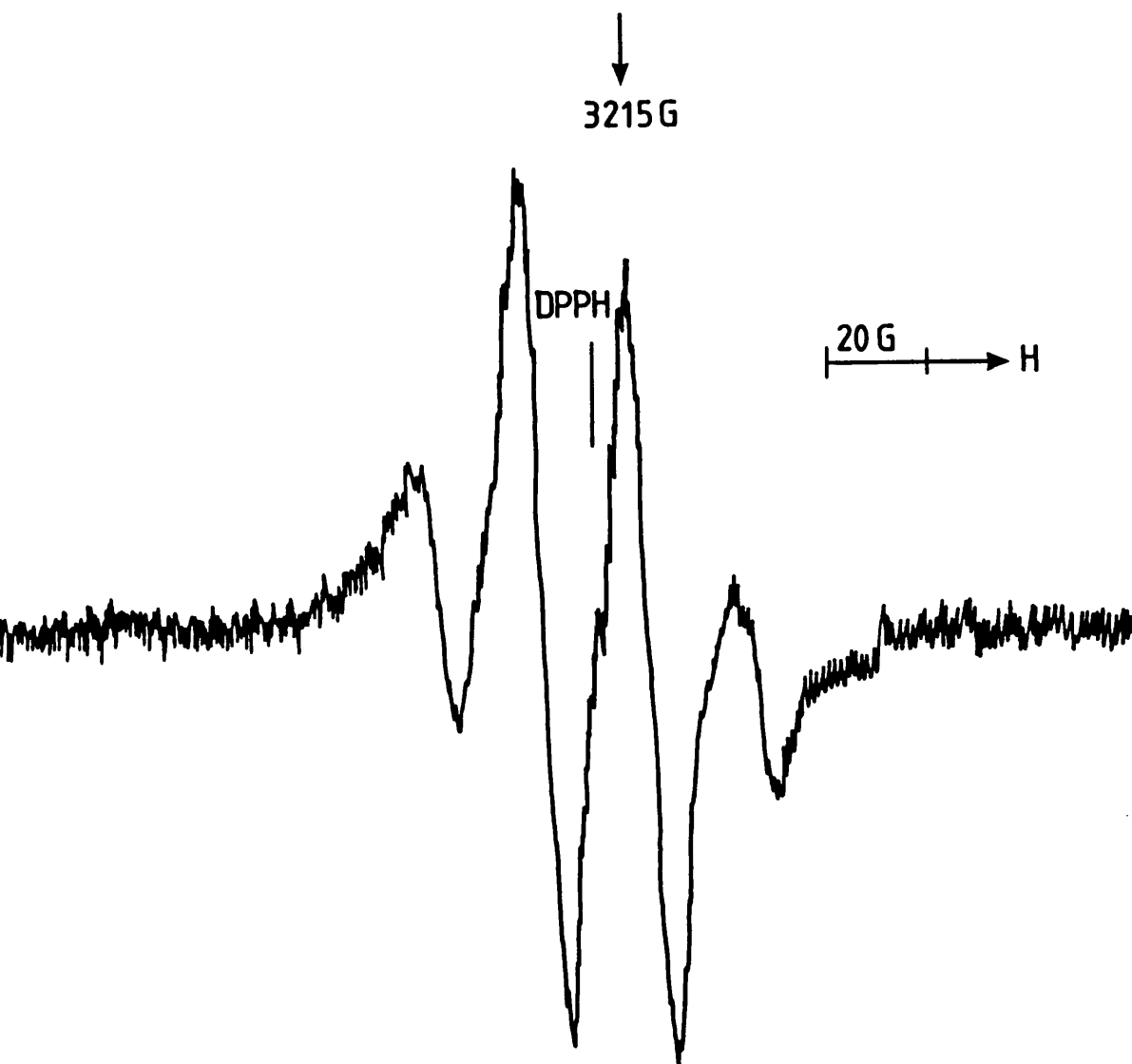


FIGURE 7.9(a)

ESR spectrum of poly-L-alanine sample at ≈ 140 K taken at 0.02 mW microwave power.

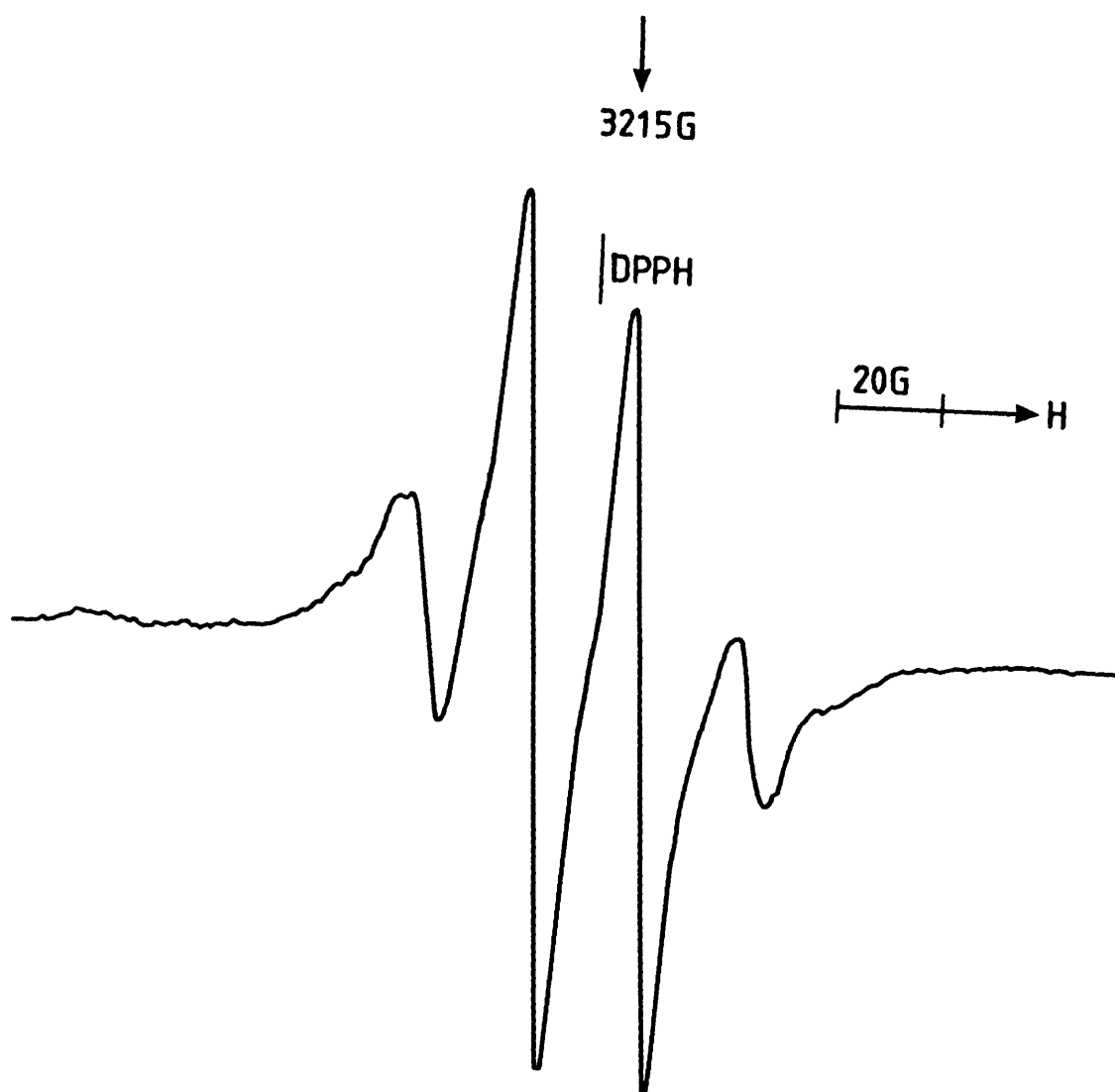


FIGURE 7.9(b)

ESR spectrum of poly-L-alanine sample at ≈ 140 K
taken at 2 mW microwave power.

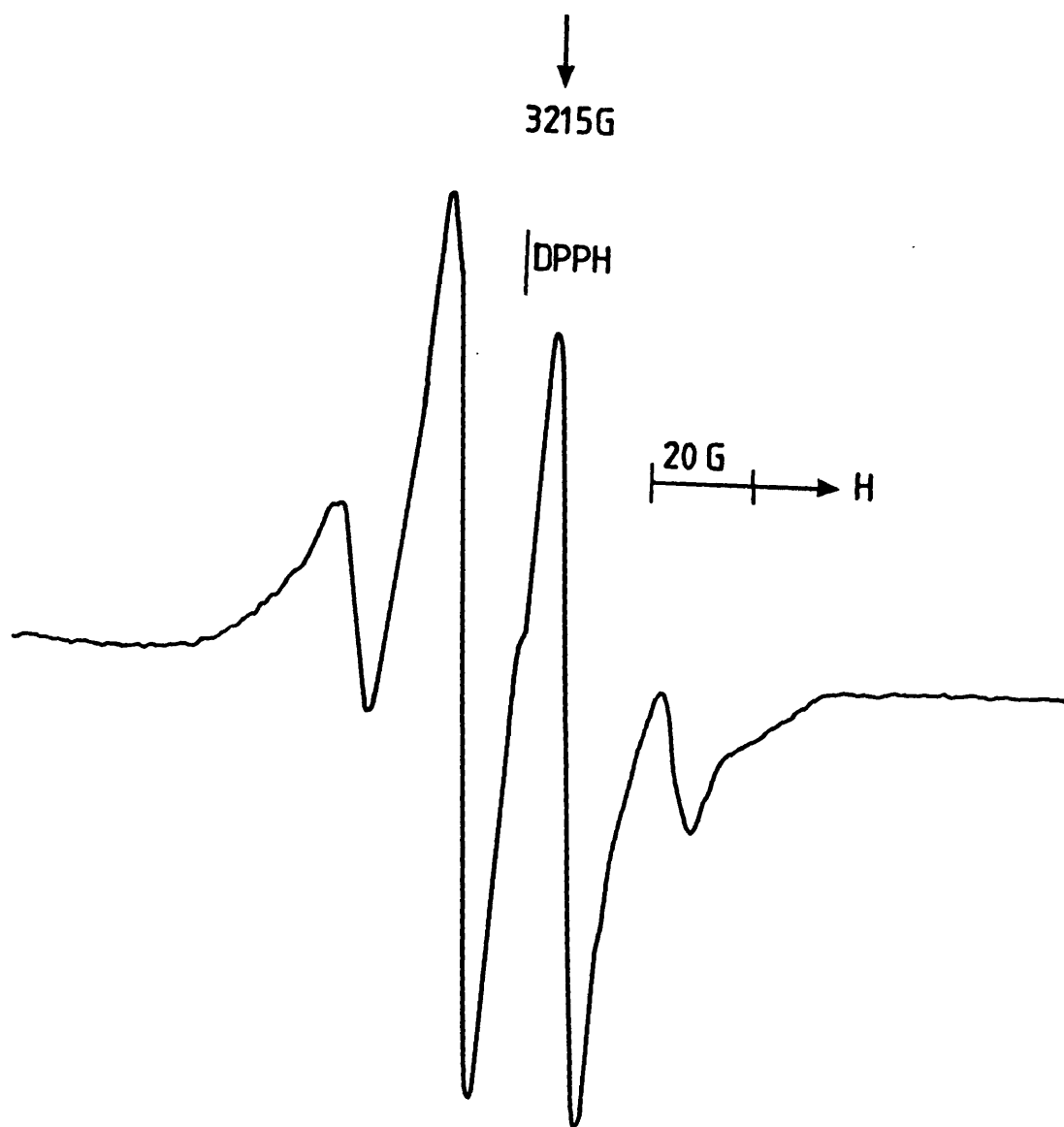


FIGURE 7.9(c)

ESR spectrum of poly-L-alanine sample at $\approx 140\text{ K}$
taken at 20 mW microwave power.

nitrogen-centred radical. [When the additional signal in the spectra taken at 77 K was compared to those at ≈ 140 K it was found that this corresponded to the outer high-field line of the quadruplet coupling from the methyl group.] These signals persisted until all signals were lost at ≈ 240 K.

7.3.2 BSA, potassium ferricyanide, D₂O samples

Serum albumens bind free fatty acids tightly and serve to transport these molecules between adipose tissue and other tissues or organs in vertebrates. They have an approximate molecular weight of 68,000 and, unlike the polyamino acids, have a varied peptide derivative chain and up to 17 -S-S- linkages but no metallo centre. Samples were prepared as before, with the potassium ferricyanide playing an even more important rôle preventing the production of RSSR^\bullet radicals which might conceal the radical cation signal. As before at 77 K, the only signal was from the $\bullet\text{OD}$ radicals. This time no additional radical signal could be observed [Fig. 7.10]. OD^\bullet radicals produced in this way are "phase-separated" from the protein, i.e. as the sample is frozen the D₂O freezes excluding everything else from it. Hence OD^\bullet radicals produced in this way cannot attack the protein sample. [They are confined to the ice and form D₂O₂ at ≈ 140 K.] At 140 K, however, the signal observed was assigned to the protein radical cation. Not surprisingly, the central signal [Fig. 7.11] was not so well resolved as with the polyamino acids, only a broad doublet was detected (resulting from the averaging of signals from the different peptide derivatives). However, additional signals are also visible. These outer signals greatly resemble the shoulders observed in polyalanine. Indeed, these shoulders would appear to be representative of $\pm 1\text{N}$ lines, with $A \approx 44$ G.

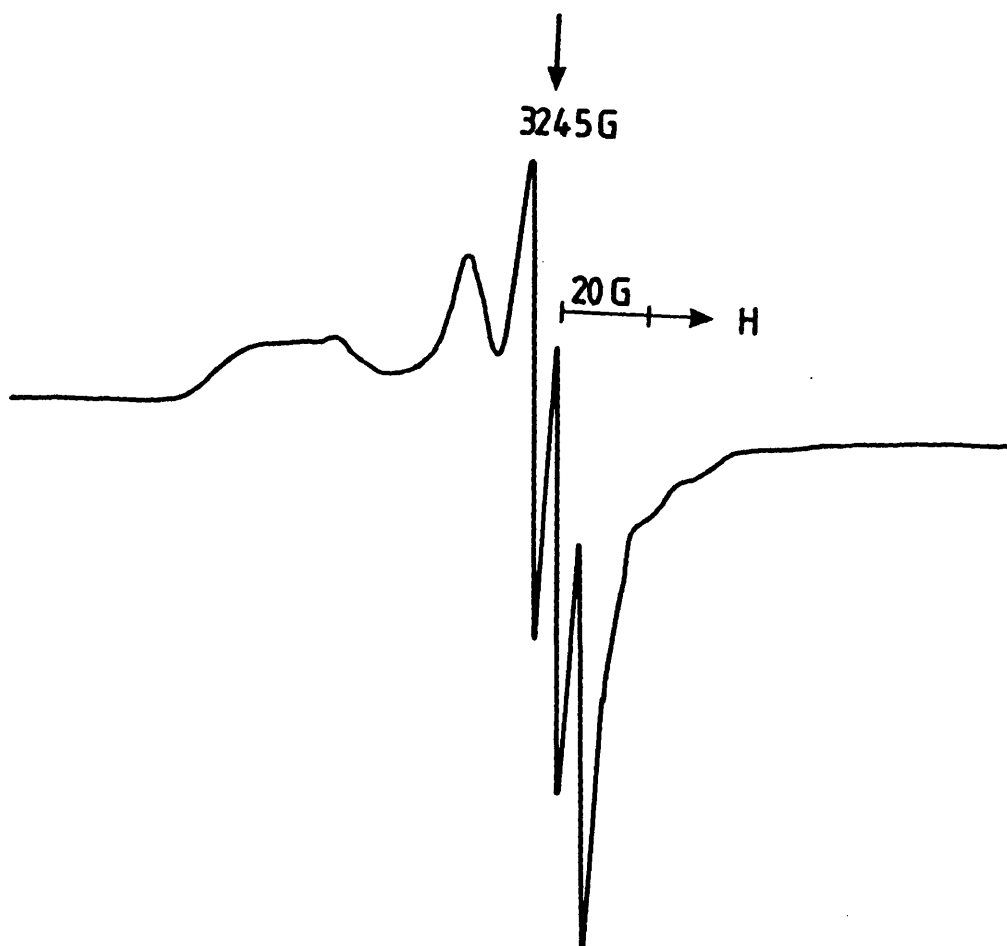


FIGURE 7.10
ESR spectrum of BSA sample in D_2O plus potassium
ferricyanide taken at 77 K.

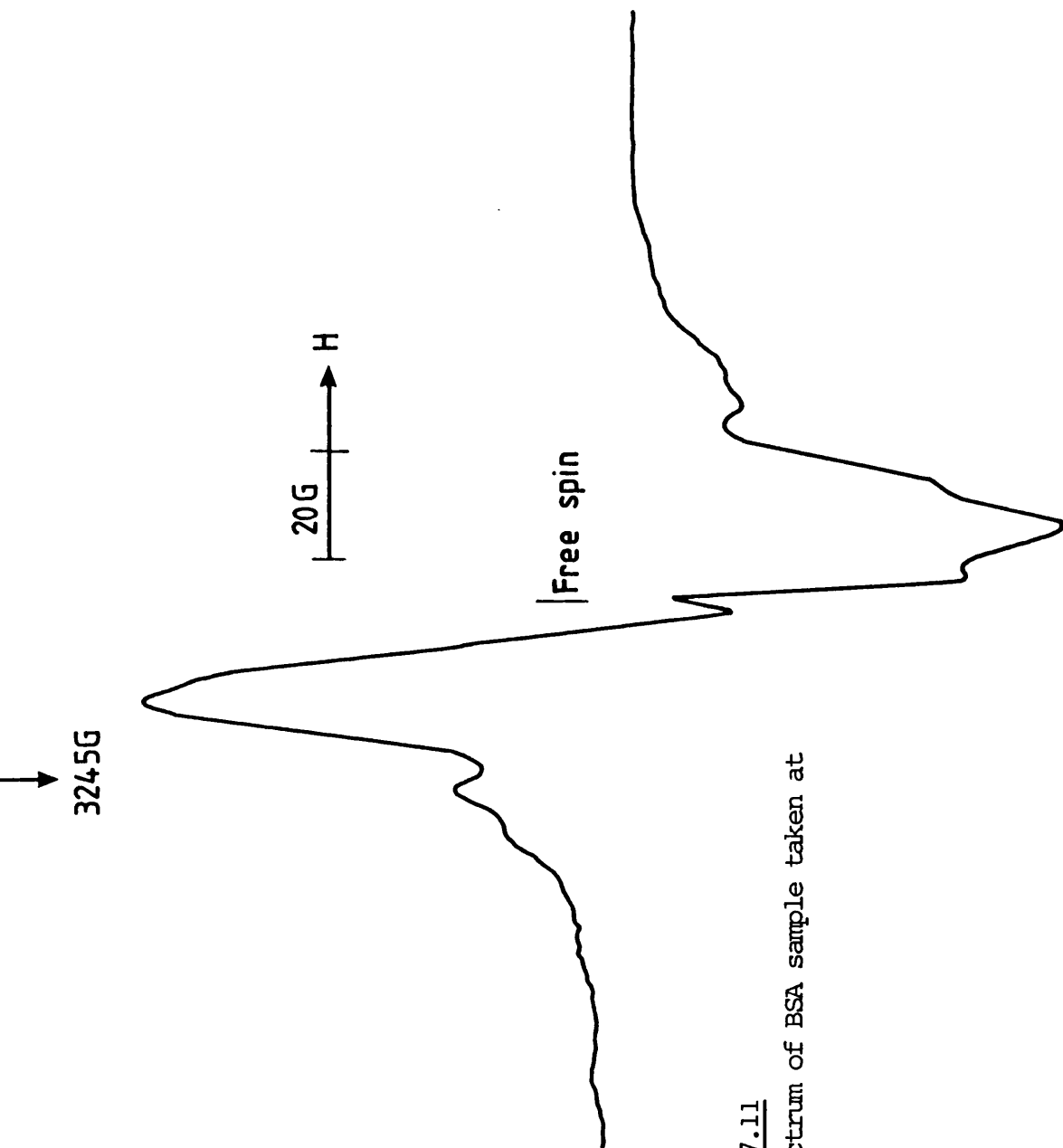
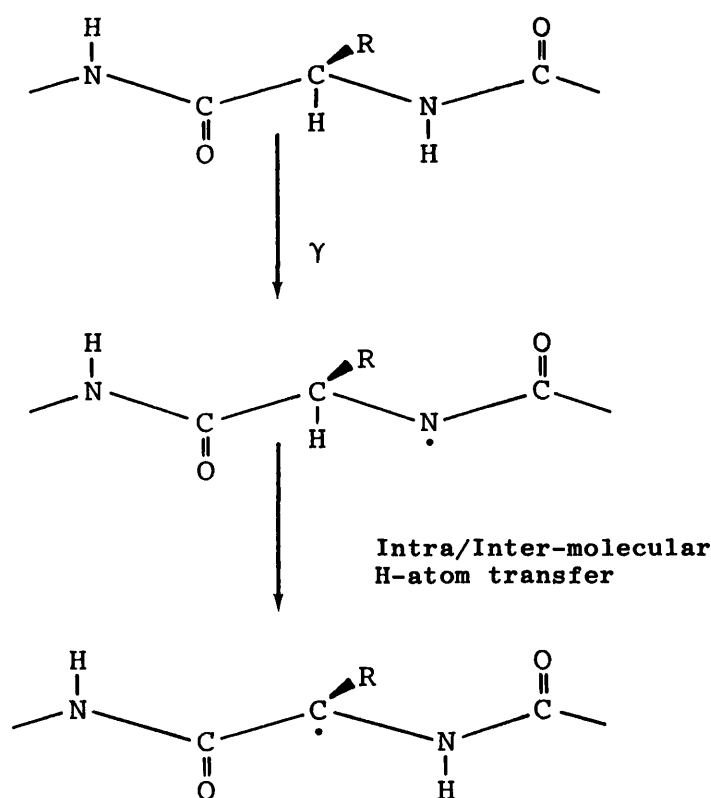


FIGURE 7.11
ESR spectrum of BSA sample taken at
 $\approx 135\text{ K}$.

7.3.3 Possible Mechanisms

The results obtained give the same basic pattern with only the underlying signal differing in each case. The final signal observed is attributed to the carbon-centred radical $R'CON\dot{H}C(R)COR''$, as stated by other workers^{116,117} but no mention is made by them of any possible role for the nitrogen within the peptide chain. From radical cation studies, it is predicted that initial electron loss would be from the nitrogen atom (that is, from the π -system of the amide units) which would then be followed by intramolecular/strand transfer of a hydrogen atom to form the carbon-centred radical [Scheme 7A] via H-atom transfer.

SCHEME 7A



From the results obtained there would appear to be fairly good evidence for this process. With polyglycine the signal attributable to the nitrogen-centred radical was lost on annealing to give the carbon-centred

radical alone. This is further supported by the work on polyalanine. The power dependence studies show that the signal attributable to an N// signal is, indeed, separate from the main signal from the carbon-centred radical. This is very important, substantiating the claim that nitrogen does, indeed, play a rôle in the radiation chemistry of peptides and proteins. This rôle of the nitrogen atom was not predicted by Sevilla and coworkers¹¹⁶ or others.¹¹⁵

This can now be extended to the studies of proteins with bovine serum albumen being used as a model for other non-metalloproteins. Here again, both the nitrogen-centred radical and the carbon-centred radical are shown to be present again possibly as the spin-transfer is occurring with intramolecular/strand hydrogen atom transfer to the nitrogen with resolution of the carbon-centred radical being impossible.

7.3.4 Concluding Remarks

It has been shown in this Chapter that the carbon-centred radical, proposed by Sevilla and coworkers¹¹⁶ and others,¹¹¹ is a site favoured for the positive hole in peptides and proteins. However, there is an alternative and, probably, a primary site for the electron-loss centre, that is the nitrogen within a peptide derivative at 77 K after γ -irradiation. Annealing brings about hydrogen atom transfer to give the carbon-centred radical. Also from the results from BSA it would appear that this mechanism can be extended to proteins themselves.

The applications of this are fairly wide, not only in terms of the radical chemistry of proteins or in the radio-sterilization of foods but also in the study of radiation damage in biochemical/biological environments, i.e. DNA, DNA-histone complexes. In the following chapter damage to these systems up to the cell nucleus itself is considered. It was first thought from this work that all damage in these systems

of direct damage was sited on DNA as the primary target. However, work on histones and chromatin systems¹⁰⁵ has shown that not all damage from electron-loss centres is sited on DNA. Some is found on the histone proteins with signals greatly resembling those for BSA.

Hence, non-metallo proteins can no longer be thought of as "highways" for radiation damage simply to pass through. The electron can, and indeed does, simply travel out of the protein to an alternative target (e.g. DNA/histone) or passes on to a metallo-centre if the protein has one. The positive hole, however, can be "trapped" within the protein possibly resulting in differing types of damage to the integrity of chromatin structure, ultimately leading to yet another pathway to mutagenesis, albeit not as strident as that for DNA but perhaps to be considered with some measure of severity.



CHAPTER 8

DNA and some Cell Nuclei Studies

8.1 INTRODUCTION

The purpose of this Chapter is to "link" the work with thiols and that of polyamino acids to the work carried out within the research group in general. It has already been mentioned that much of the radioprotective work with thiols has been carried out with DNA as the primary target. This will be dealt with in greater depth later in this Chapter. Also with the polyamino acids the purpose of this study arose from some of the work on cell nuclei. Hence each section of this thesis relates to another. Therefore, if the most important biochemical factor is considered initially.

8.1.1 The Cell Nucleus

The cell's complement of DNA is contained within the cell nucleus, which itself is contained within a double membrane called the nuclear envelope, which distinguishes eucaryotic cells from procaryotes. It is with eucaryotic nuclei that this study is concerned. The nuclear envelope separates the genetic processes of DNA replication and RNA synthesis from the cytoplasmic ribosomes, where the genetic message is translated into protein. This separation of RNA and protein synthesis appears to be the most important function of the nuclear envelope. Eucaryotic DNA is tightly complexed with specialized proteins - the histones, that help regulate DNA's activity. It is now known that DNA is an enormously long, unbranched linear polymer that can contain many millions of nucleotides arranged in an irregular but non-random sequence, the genetic information being contained in the exact order of the nucleotides. The linear four-letter genetic code is a straightforward solution to the problem of storing a large amount of genetic information in a small amount of space. In the DNA molecule, every

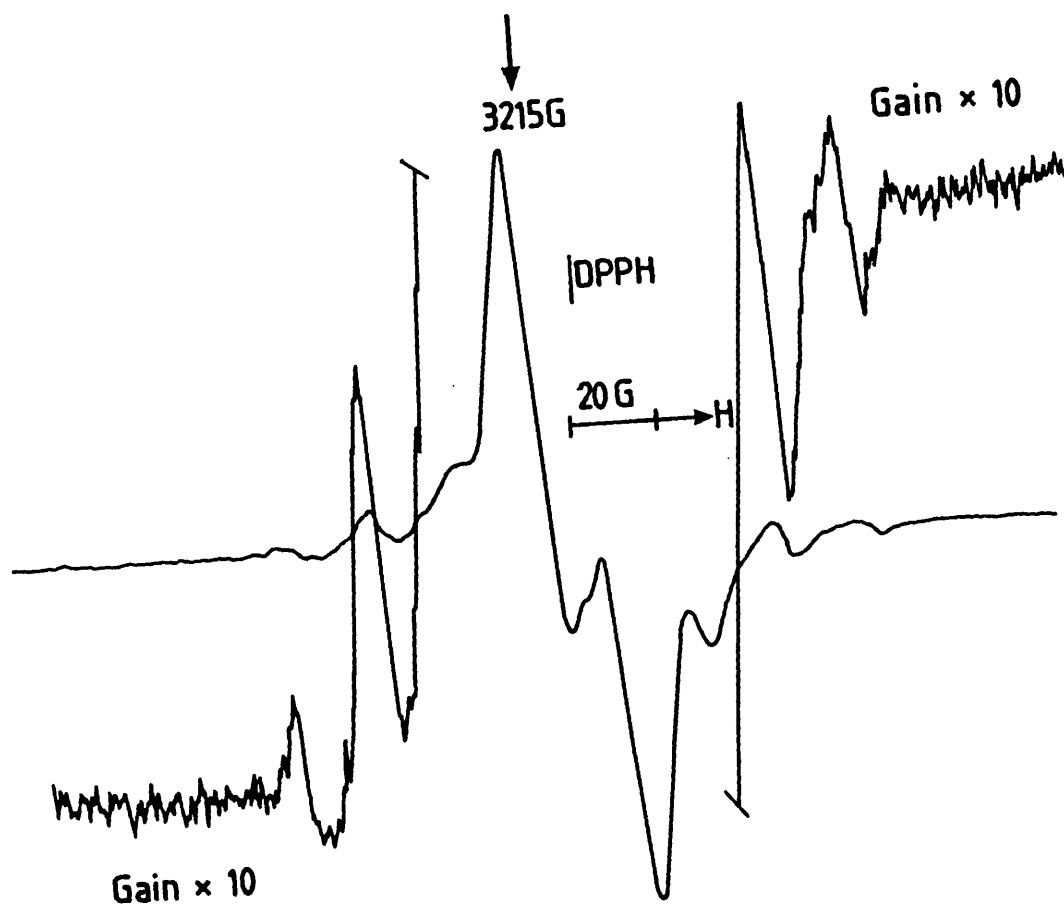


FIGURE 8.1
ESR spectrum showing the characteristic eight-line
spectrum attributable to the thiamyl radical TH[•]
from DNA.

million nucleotides take up a linear distance of only 3.4×10^5 nm (0.034 cm) and occupy a total volume of approximately 10^6 nm³ (10^{-15} cm³). Thus, for a typical human cell, 20 μ m in diameter with a haploid DNA sequence of 3×10^9 nucleotides - all of the genetic information could, in theory, be packed into a cube 1.5 μ m (1.5×10^{-4} cm) on each side.

Yet with all this space saving only a small proportion of the DNA actually codes for proteins or for essential non-translated RNA's such as transfer and ribosomal RNA's. It is customary to refer to these regions as coding DNA. The functions of the non-coding DNA sequences are, at present, not fully understood. It is possibly that information for biological information such as DNA folding is contained within these sequences.

DNA folding in eucaryotes is important in two ways. It is essential for packing DNA in an orderly way in the nucleus, also the exact manner in which a region of genome is folded can determine gene activity in that region. Specialized eucaryotic proteins (the histones) play a central part in the folding of eucaryotic DNA.

DNA in all organisms is closely associated with many different binding proteins. These proteins act in many ways as enzymes which mediate such processes as RNA synthesis or, for example, the histones which organise DNA in the nucleus. In fact, these proteins are divided into two classes in eucaryotes: 1) histones and 2) non-histone chromosomal proteins. The latter serves as a large umbrella for the many hundreds of different proteins with differing functions. Contrary to this, the histones exist as a well defined group of structural proteins. These far outweigh the other class of nuclear proteins with a total mass nearly equivalent to that for the DNA. DNA associated with histones

is called chromatin.

Histones are relatively small proteins with a very high proportion of positively charged amino acids (lysine and arginine). The positive charge helps the histones to bind tightly to DNA regardless of its nucleotide sequence. It is likely that histones remain bound to DNA at all times, and so are likely to play an important rôle in reactions involving the genome.

There are five types of histone within a eucaryotic cell and these fall into two main groups:

- 1) Nucleosomal histones, which are small and are responsible for folding the DNA into nucleosomes; these are designated H2A, H2B, H3 and H4. They are one of the most highly conserved group of proteins. Such conservation suggests that these proteins have crucial functions involving nearly all their amino acids.
- 2) The H1 histones - there are several different but closely related varieties in each cell. Except for a central core region, the sequence of H1 histones has been much less conserved than the other four.

If stretched out, the DNA double helix in each human chromosome would be on average about 5 cm long. The histones are responsible for packing this long DNA molecule into a nucleus only a few μm in diameter. A major advance in the understanding of chromatin structure came in 1974 with the discovery of the fundamental packing unit known as the nucleosome. It is this nucleosome which gives chromatin its "beads on a string" appearance in electron micrographs taken after initial unfolding. Each nucleosome bead contains a set of 8 histone molecules, 2 copies of each of the 4 highly conserved nucleosomal histones H2A, H2B, H3 and H4. These form a protein core around which the double-

stranded DNA is wound.

In undigested chromatin, the DNA extends as a continuous thread from nucleosome to nucleosome. Each nucleosome bead is separated from the next by a region of linker DNA, which varies in length but is approximately 60 base pairs long. This linker DNA plus the nucleosome bead constitutes the entire nucleosome. The human haploid genome of 3×10^9 DNA base pairs contains 1.5×10^7 nucleosomes.

In the living cell, chromatin must be kept in a highly compacted state. The nucleosomes are packed upon each other to generate regular arrays in which DNA is highly condensed. When nuclei are very gently lysed onto an electron microscope grid, most of the chromatin is seen as a fibre with a diameter of ≈ 30 nm.

Histone H1 molecules would appear to be responsible for packing nucleosomes into this 30 nm fibre. Each H1 molecule has a globular central region linked to extended amino-terminal and carboxyl-terminal "arms". The globular portion binds to a unique site on each nucleosome, and the arms are thought to extend to cover the linker DNA near the point where it joins the nucleosome bead as well as to contact the histone cores of adjacent nucleosomes. In this way, histone H1 proteins pull the nucleosomes together into a regular repeating array.

Further folding occurs to contain the DNA within the nucleus but this basically covers the rôle of the histone proteins.

Most of the non-histone chromosomal proteins are present in very small amounts. For example, there are only about 10,000 molecules of some known gene regulatory proteins per cell or about one copy per 3000 nucleosomes. Such rare proteins are very difficult to isolate in sufficient amounts for study. For this reason, only the most abundant non-histone proteins have thus far been investigated in detail. These

are present in quantities as great as 1 molecule for every 10 nucleosomes (over a million molecules per cell), so it is unlikely that most recognize specific DNA sequences. Instead they probably influence more general structural features of chromatin, such as those required for gene transcription.

The abundant non-histone proteins include the "high-mobility group" or H.M.G proteins, so called because they are relatively small and highly charged and they, therefore, move quickly during electrophoresis.

8.1.2 DNA and Thiols

The damage caused by ionizing radiation to the DNA molecule has been well documented,^{128,129} exposure of frozen, deoxygenated aqueous solutions of DNA to ^{60}Co γ -rays at 77 K resulting in the formation of guanine-centred radical cations (G^+) and thymine-centred radical anions (T^-). Both of these primary centres are thought to be capable of leading to DNA strand breaks. This is a very serious form of damage since it is apparent from the previous section that the informational content of the macromolecule is not easily restored. [This is an example of direct damage resulting from exposure to ionizing radiation. There is, however, an alternative, indirect method of damage resulting from interaction with the radiolysis products of water ($\cdot\text{OH}$, e^- , $\text{H}\cdot$). This Chapter will, however, only concern direct damage as we feel that this has a significant rôle in in vivo damage mechanisms.]

As to the repair of the DNA molecule itself, the cell has evolved a variety of ways of correcting the damage induced not only by ionizing radiation but also by other chemical effects. It is common to think in terms of radical scavengers, particularly when concerned with the hydroxyl radical, which prevent reactive radicals interacting with DNA and other macromolecules. This is more applicable to the indirect form

of damage, however, concerning direct damage it is thought that radicals formed in the DNA molecule may be repaired thus preventing further damage occurring. It is into this group that thiols fall. Finally, the base pair sequence of DNA is protected to a great extent by enzymes capable of accurately repairing certain kinds of damage or defects in the DNA strand.

As to the action of thiols, this has already been discussed in this thesis [see Chapter 1] and interest has grown in the rôle of glutathione (γ -glutamylcysteinyl glycine) in modifying the cell's response to ionizing radiation.¹³⁰ Repair of DNA being strongly implicated. This study was more involved with the chemistry involved when thiols react with DNA radicals.

In the past, many studies have concentrated on the rôle of thiols in repair processes after indirect damage, i.e. after initial hydroxyl radical attack on DNA. Also in the past, we have argued^{41,131,132} that the usage of dilute aqueous systems of DNA as in indirect damage studies does not provide a good representative model DNA within the nuclear envelope, as it seems unlikely that bulk water is present within the nucleus. Hence studies of frozen aqueous solutions of DNA plus additive allow for examination of the direct damage component as the macromolecule plus additive phase separates from the bulk water and then can be studied directly using e.s.r. spectroscopy. From this it was hoped that the effects of thiols on the direct damage pathway could be established both by e.s.r. and strand break analysis.

In an attempt to unify the studies of frozen aqueous DNA solutions with the more "in vivo" studies, bovine thymus gland nuclei were also studied.

8.2 EXPERIMENTAL

The strand-break analysis was carried out by Dr. M. Sweeney and the e.s.r. spectroscopy of DNA/thiol systems was carried out by Mr. G. D. D. Jones.¹²⁷

8.2.1 Strand-Break Analysis

Plasmid DNA (pBr 322) was isolated according to a modified procedure of Birnboim and Doly,¹³³ pBr 322 DNA preparations contained ca. 85% of the superhelical Form I DNA. Agarose-ME was obtained from the Miles Laboratory. Hydrogen peroxide was purchased from British Drug Houses (Poole). Tris(hydroxymethyl)methylamine (TRIS) buffer, ethylenedinitrilo-tetraacetic acid (EDTA), 3,5-diamino-S-ethyl-6-phenylphenanthridinium (ethidium bromide), calf thymus DNA (for e.s.r. studies) were obtained from the Sigma Chemical Company. The thiol and thiol derivatives employed, γ -L-glutamyl-L-cysteinyl glycine (glutathione), 2-mercaptoethylamine hydrochloride (cysteamine, MEA), L-cysteine, DL-2,3-dihydroxy-1,4-dithiobutane (dithiothreitol), 2,2'-dithio-bis[ethylamine]dihydrochloride (cysteamine), DL-cystine dihydrochloride and 5-methyl-L-cysteine were all purchased from Sigma Chemical Company and were of the highest quality.

The assay for DNA breaks has been described previously.^{41,131,132} Variations were introduced in order to optimise the separation of the linear Form III DNA from the relaxed Form II DNA. A solution of the thiol or thiol derivative (10 μ l) was added to the plasmid DNA solution (90 μ l; 80 μ g ml⁻¹) in Tris hydrochloride buffer (10 mM; pH 7.6) containing EDTA (1 mM) to give the final additive concentration. Samples with and without additive were γ -irradiated in a Vickrad ⁶⁰Co γ -ray source under the appropriate conditions. Oxygenated and deoxygenated samples were prepared by gas purging with oxygen or thoroughly

deoxygenated nitrogen for 60 minutes, then sealed and frozen prior to irradiation. Following irradiation and annealing to room temperature, a dye - EDTA (6 μ l; 56% glycerol v/v; 50 mM EDTA; 0.05% bromophenol blue w/v) was added and aliquots removed for analysis by agarose gel electrophoresis.

Samples of irradiated DNA (0.7 - 1 μ g) were layered onto agarose slab gels (1.3%) and subjected to electrophoresis in a horizontal gel apparatus at 40V for ca. 16 hours at room temperature using Tris buffer (90 mM, pH 8.3) containing boric acid (90 mM) and EDTA (2.5 mM). After electrophoresis the gels were stained with ethidium bromide (2.5 μ g ml^{-1}) in electrophoresis buffer for at least 15 minutes and the excess removed by washing. The stained gels were then excited with a trans-illuminator and photographed with a Polaroid MP-4 Land Camera using a red filter [Kodak Wratten Filter No. 9] and Polaroid type 55 film. The negative films of gels were used for densitometric scanning.

The negative films of the ethidium bromide-stained gels were scanned using a UV/visible spectrophotometer with gel-scanning attachment [Pye-Unicam, SP8-100]. The superhelical Form I of the plasmid takes up less ethidium bromide than the other two Forms. We have used a staining efficiency of 80% (as demonstrated for pBR 322¹³⁴) to normalize our data. No further uptake of ethidium bromide was observed on prolonged incubations. All samples were analysed in duplicate and average values are reported.

8.2.2 γ -Irradiation and ESR Measurements

Frozen samples were prepared by cooling in liquid nitrogen in a Pyrex tube containing an oxic or anoxic solution of DNA (50 mg ml^{-1} ; Type 1 sodium salt DNA, Sigma) with and without additive (0.1 - 140 mM). On extrusion from the tube, samples were irradiated as described above,

and the e.s.r. spectra recorded (X-band Varian E-109 spectrometer, 100 KHz field modulation) at 77 K. The samples were annealed to a particular temperature for 2 minutes through the use of a second spectrometer (a Varian E-3 X-band) equipped with a variable temperature accessory. The samples were then re-cooled (77 K) prior to the e.s.r. spectra being taken.

To judge the decay of the spectral features of the DNA ± additive samples with temperature, the spectra at $g = 4.3$ of a bead containing tris(acetylacetonato)di-iron (III) was recorded in tandem with the DNA samples under identical e.s.r. parameters and this was employed as an internal intensity standard [see Experimental section, Chapter 2]. In conjunction with the Fe(III) bead samples of DNA not containing additive were co-irradiated with the DNA additive samples, these were employed to normalize the DNA-additive spectra.

E.s.r. data at Q-band frequencies were recorded as before [see Experimental section, Chapter 2].

8.2.3 Isolation of Cell Nuclei

This was taken and adapted from the method outlined by Garrard and Hancock¹³⁵ and originally based on that of Blobel and Potter.¹³⁶

Fresh bovine thymus tissue was transported to the laboratory at 0°C in 200 ml of solution A (see below). The membrane and connective tissue were removed and 25g of tissue were minced in 50 ml of solution A.

Solution A: Homogenization medium: 0.3M sucrose - 0.05M Tris buffer - 0.025M potassium chloride, 5 mM magnesium chloride, pH 6.5 at 4°C.

Solution B: 2.15M sucrose - 0.05M Tris buffer - 0.025M potassium chloride - 5mM magnesium chloride, pH 6.5 at 4°C.

The 25g of fresh minced thymus in 200 ml of solution A was placed in a pre-cooled blender and homogenized for five minutes. The homogenate

was then filtered through two layers of cheesecloth. The filtrate was then subjected to centrifugation (800g for 10 minutes). The supernatant was discarded and the sediment re-suspended in 40 ml of solution A and filtered through cheesecloth to remove any lysed nuclei. To this filtrate was added 2.5 of solution B. This was then mixed and distributed to centrifuge tubes leaving room for a 20% (by volume) underlay with solution B; this underlay is added and the sample centrifuged (33000g for 3 hours). The supernatant was discarded and the tube wiped. The pellet was purified cell nuclei which were later re-suspended in Tris buffer (pH 6.5) [all steps were performed at 4°C].

8.2.4 Assay for [DNA]

A sample (100 mg) of frozen cell nuclei were suspended in ice-cold sodium chloride (0.14M, 4 ml) containing EDTA (10^{-3} M). This was stirred with a glass rod until the cells had thawed, the suspension was heated (to 37°C) and lysozyme (0.2 mg ml^{-1}) was added, this was then incubated with gentle shaking. After 10 minutes lysis of the cell nuclei occurs, the suspension clearing and becoming viscous. An equal volume of 90% phenol was added (shaken twice beforehand with 0.1M borate). This was shaken gently for twenty minutes to keep the phases well dispersed. The phases were separated by centrifugation (1000g, 5 minutes). The aqueous phase was removed without disturbing the denatured protein at the interface, this was then twice extracted with ether. The DNA was precipitated with an equal volume of 2-ethoxyethanol added slowly down the side of the beaker. The fibres of DNA were removed by winding on a glass rod. The fibres were washed with ethanol then pressed dry with filter paper and dissolved in 0.14M sodium chloride containing 10^{-3} M EDTA. It was left at 4°C for sixty minutes then assayed for DNA and protein. [Protein] was measured using a Lowry assay, DNA by its A_{260}

[1 mg ml⁻¹ DNA A₂₆₀ = 20].

8.3 RESULTS AND DISCUSSION

Before discussing the effect of thiols in these systems and the damage to cell nuclei caused by ionizing radiation, it is necessary to explain some of the previous work on direct damage to DNA.^{41,131,132,137}

It is now generally accepted that exposure of frozen aqueous solutions or dry thin films of DNA to γ -radiation at 77 K gives rise to the guanine radical cation (G \cdot^+) and the radical anion (T \cdot^-) in approximately equal yields. The thymine radical anion protonates to give the 5-thymyl radical TH \cdot , characterised by its well known eight-line spectrum [Fig. 8.1]. The fate of the radical cation is less clear. However, a recent study has suggested that the radical loses a proton from nitrogen to give the neutral radical GN \cdot (high spin density on the nitrogen) occurring at ca. 220 K for orientated DNA fibres.¹³⁸ Under oxic conditions both radical cation and radical anion give rise to RO₂ \cdot [Fig. 8.2] radicals, and it has been demonstrated previously that under both anoxic and oxic conditions strand breaks, double and single, occur.⁴¹

8.3.1 Effect of Thiol Compounds

With addition of moderate concentrations of a range of thiols (0.1–10 mM) to DNA samples, the yield of G \cdot^+ and T \cdot^- was not affected, following irradiation at 77 K. On raising the concentration of mercaptoethylamine (40–140 mM) there was a small initial decrease in the yield of T \cdot^- . This is likely to be due to contaminating disulphide (oxidized mercaptoethylamine) present in the system. This is a highly effective electron scavenger, as has been stressed in earlier chapters [see Chapters 2 and 4]. Once these thiol containing samples were annealed, the DNA radical signals were lost concurrent with the growth of sulphur-centred

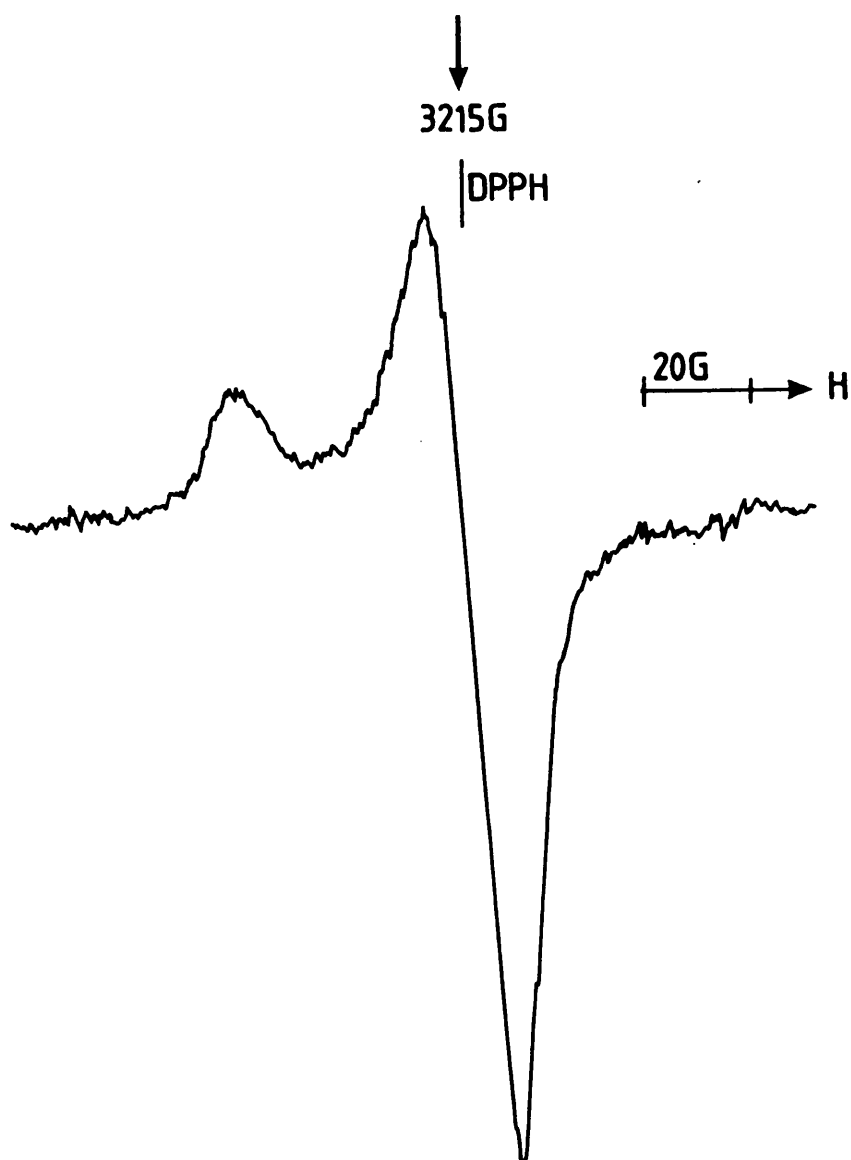


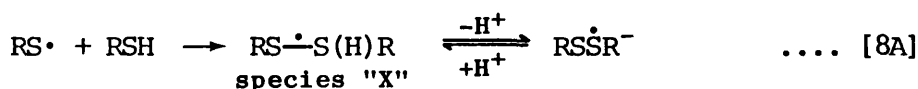
FIGURE 8.2

ESR spectrum showing features attributable to $\text{RO}_2\cdot$ radicals arising from the irradiation of DNA in the presence of oxygen.

radical signals due to the disulphide radical anion $\text{RSSR}^{\bullet-}$. These features were well characterized by Q-band studies [Fig. 8.3a,b]. These must be formed from transient thiyl radicals (RS^{\bullet}) as discussed previously [see Chapter 1]. This loss of DNA radical signals was at thiol concentrations as low as approximately 0.5 mM (1:150 base pairs) [Fig. 8.4].

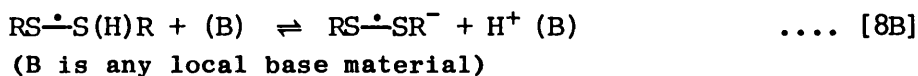
On the other hand, under oxic conditions, addition of thiols reduced the yield of RO_2^{\bullet} radicals. However, higher concentrations of thiol (≥ 10 mM) were required to produce this effect [Fig. 8.4].

It has been postulated previously [see Chapter 6] that the pathway to the radical anion from thiol is:



The RS^{\bullet} radicals are not detected by e.s.r. spectroscopy. However, since its spectrum is dependent on its environment (i.e. hydrogen bonding) and this itself is variable in these systems, this is not surprising.

An important result is that, in no case could species "X" be detected. Presumably proton loss is rapid even in these rigid systems. Our own studies¹²⁷ together with those of others⁶⁹ indicate that the pK of disulphide radical anions is ≈ 5.5 . Such a value would allow deprotonation to be favourable in our system.



In a recent comparative study the dimer form of 2-pyridinemethanethiol was employed as an additive in these systems¹³⁹ and, in this case, species "X" was detected (g-values of 2.055 and 2.026). It seems likely that addition of this compound alters the pH of the system or the species "X" produced is less pH dependent, allowing for its detection.

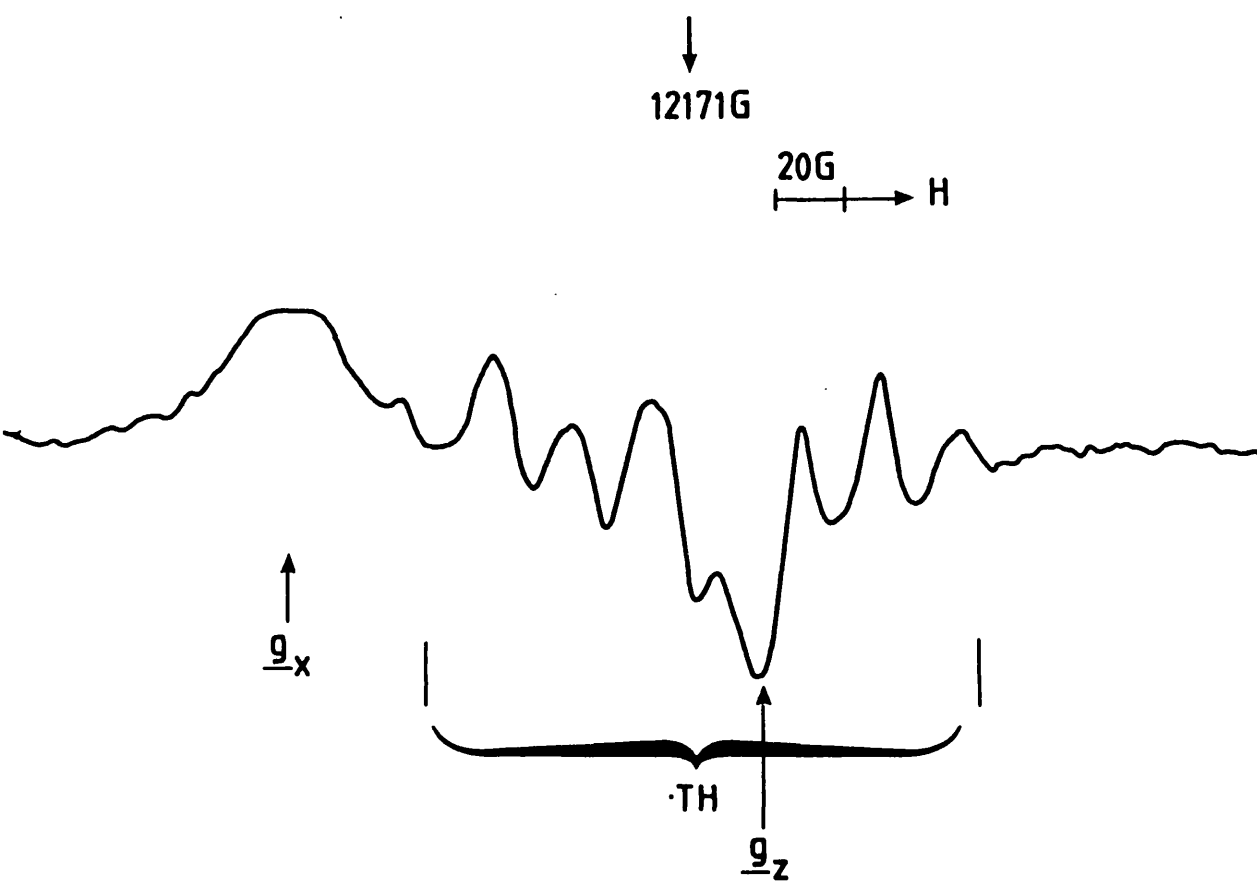


FIGURE 8.3(a)

Q-band ESR spectrum at $\approx 100\text{ K}$ of an anoxic solution of DNA ($\sim 50\text{ mg ml}^{-1}$) containing cysteamine (40 mM) after exposure to γ -radiation at 77 K and annealing to 195 K showing g_x and g_z features for RSSR^- radicals together with features for residual $\cdot\text{TH}$ radicals.

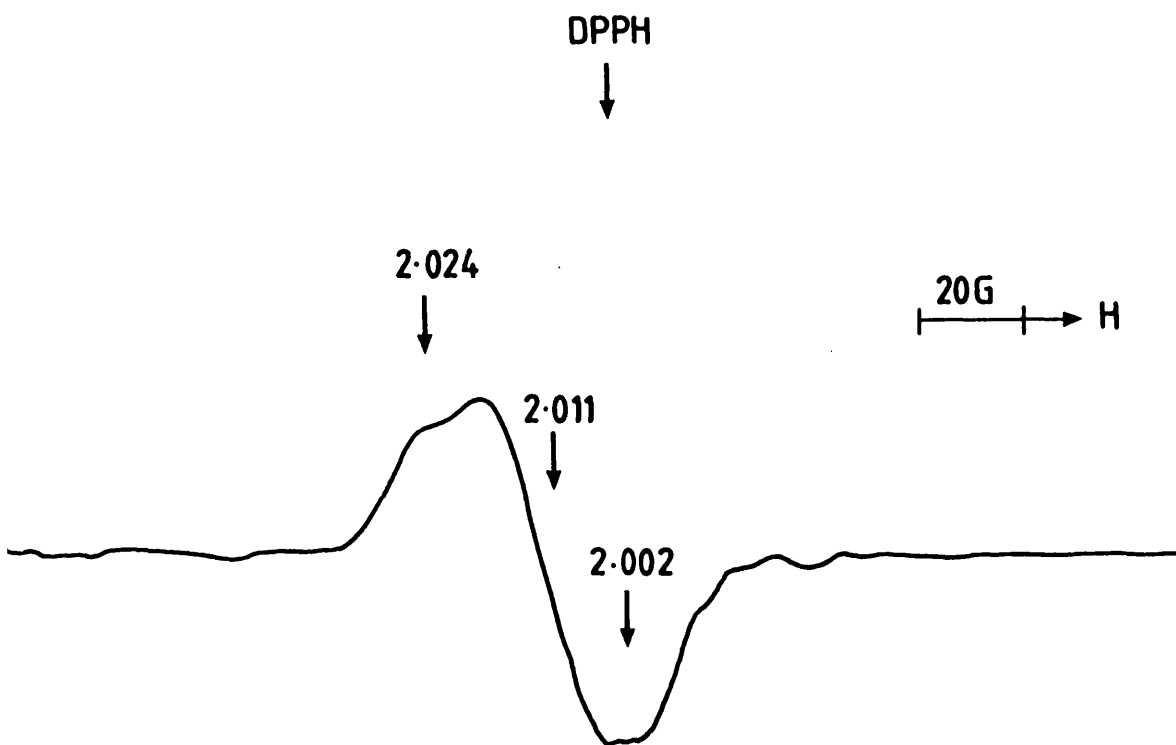
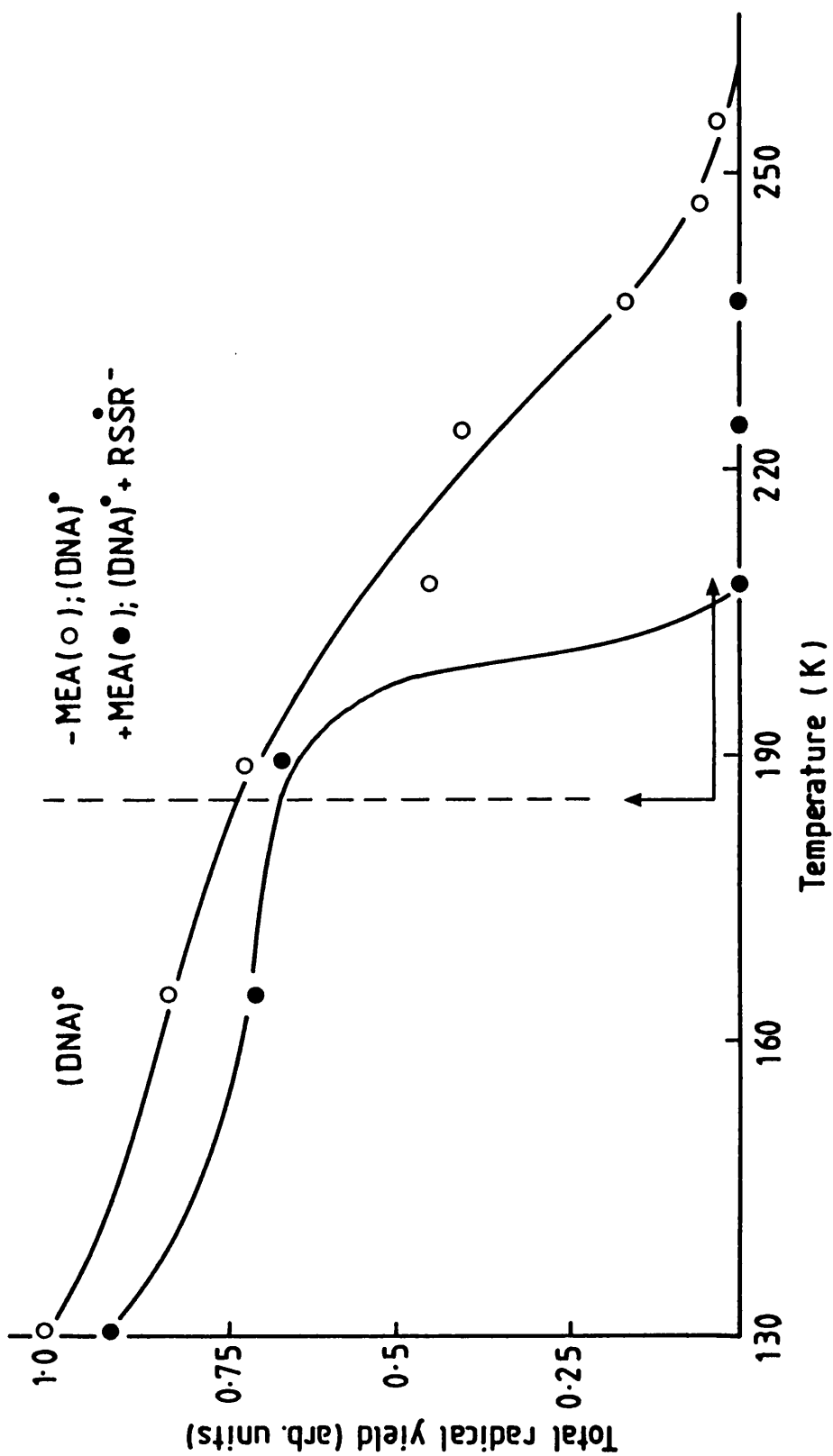


FIGURE 8.3(b)

The resultant X-band ESR spectrum at 77 K for an anoxic DNA solution (50 mg ml^{-1}) containing cysteamine (40 mM) after exposure to γ -radiation and annealing to 190 K, on computer subtraction of overlapping signals from $\cdot\text{TH}$; g -tensor components for RSSR^- can be seen.

FIGURE 8.4

Plot of relative radical yield against temperature showing the effect of cysteamine (40 mM) as an additive in solutions of DNA (50 mg ml⁻¹). Temperatures for the onset and duration of RSSR⁻ radicals existence are shown (↗↘).



8.3.2 Effects of Oxygen

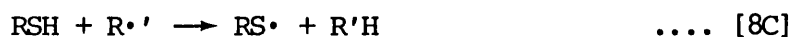
In addition to the normal $\text{RO}_2\cdot$ signals which grew in at 190 K but to a diminished extent, clear features at $g_x = 2.025$ and $g_y = 2.017$ were observed [Fig. 8.5]. These features agree with those observed by Copeland,¹⁴⁰ who assigned them to $\text{RSO}_2\cdot$ radicals. Our own work on the addition of oxygen to $\text{RS}\cdot$ radicals supports this argument.¹⁴¹ The signals were lost at ca. 208 K.

8.3.3 Strand Break Analysis

It was observed that plasmid DNA (pBR 322) in the presence of mercaptoethylamine (40 mM) was protected from both single and double strand breaks caused by exposure to γ -radiation at 77 K. Despite changes in the e.s.r. spectra this was found to be largely unaffected by the presence of oxygen. At lower concentrations of the thiol protection was reduced under anoxic conditions and further reduced under oxic conditions (suggesting opposing competitive reactions of thiols and oxygen).

8.3.4 Possible Mechanisms

It would appear from the results that the loss of DNA radicals at a temperature lower than normal shows that the thiols are acting as efficient hydrogen atom donators to DNA base radicals, in terms of the reaction;



However, the question arises, is this the only mechanism available?

Liquid phase results indicate that, whilst these reactions do occur in most cases for DNA (particularly with respect to $\text{G}\cdot^+$ formation), electron transfer dominates, in the manner;



with the thiolate anion as the active species. Whilst it would appear

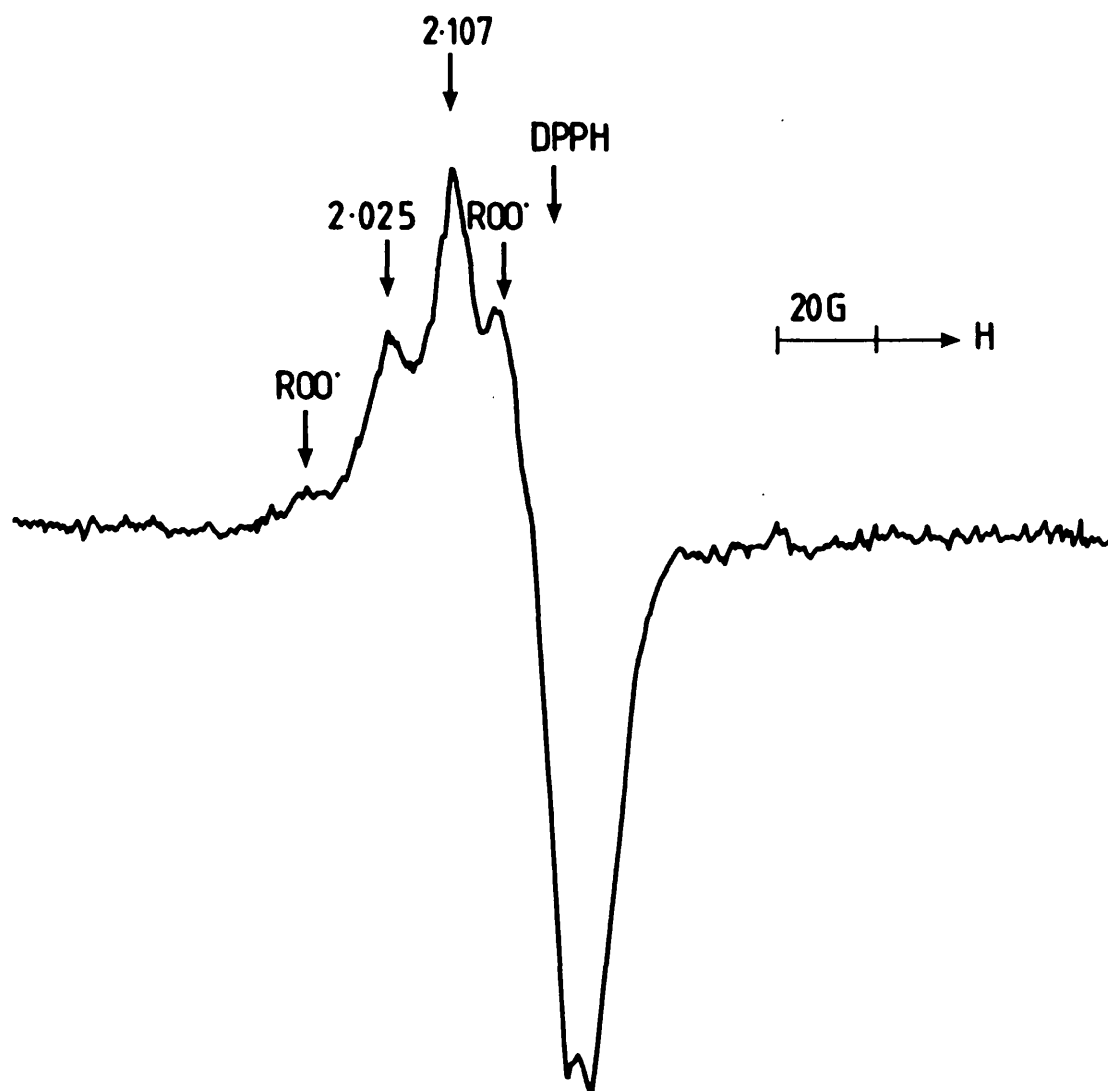


FIGURE 8.5

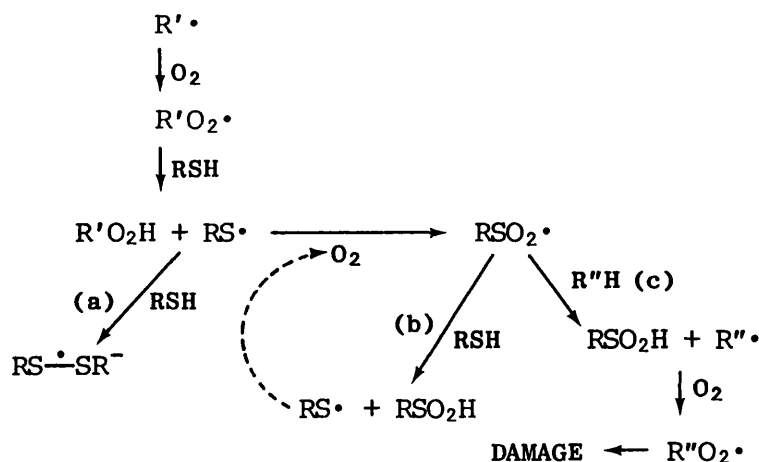
ESR spectrum at 77 K for an oxidic solution of DNA (50 mg ml^{-1}) containing cysteamine (40 mM) after γ -radiation and annealing to 190 K, showing g-tensor components of ROO^\bullet radicals and residual peroxy radicals.

unlikely that this reaction could occur directly with RSH molecules, a study was made on the effects of RSM⁺e compounds. These were found to have little effect on DNA damage and no RS⁺•Me radicals were detected.¹⁴²

Another study made was to raise the pH of the DNA/RSH samples to generate RS⁻ anions. This, however, modified the DNA structure and the nature of the damage. At low temperatures (well below those for thiol action) in the range 130-145 K a small growth in RSSR⁻• features was observed. At higher temperatures G⁺• converts to GN[•] and charge transfer reactions are no longer possible.

Since nothing of this type was observed in neutral systems and that at pH 6.5 the thiolate anion RS⁻ cannot comprise of more than $\approx 2.5\%$ of the total thiol present, then thiol mediated electron transfer cannot have a major rôle to play in the repair of direct damage in DNA frozen aqueous systems. Thus, for these systems RSH compounds must react by hydrogen atom donation.^{139,141} Clearly such 'repair' should lower the yield of SSB and DSB, as observed.

Under oxic conditions and at low [thiol] the principal sulphur containing radical detected is RSO₂• arising from the addition of O₂ to RS•. As the effect of thiol is competitive with that of oxygen (at these concentrations) this would be consistent with the Scheme shown [Scheme 8A]. Hence hydrogen atom donation by thiol to base peroxy radicals occurs to give thiyl radicals. These add oxygen to give RSO₂• rather than forming the disulphide radical anion. When the thiol concentration is increased, pathway (b) will dominate. Therefore, oxygen is consumed and DNA is protected. As a direct result of an increase in thiol concentration reaction (a) will also occur to give RSSR⁻• and, again, DNA will be protected.



SCHEME 8A

Alternatively at a low thiol concentration, the reactions (a) and (b) will be prevented. Pathway (c) will occur and this could well account for the attenuation by oxygen of protection at low thiol concentrations. Although 'repair' has occurred, if hydrogen donation is to, say, $\cdot\text{TH}$ it gives TH_2 , which is therefore a misrepair. TH_2 units are also formed as part of the postulated SSB mechanism, and have indeed been identified under conditions of direct irradiation.¹²⁷ There is rather less evidence concerning the fate of $\text{G}\cdot^+$, if however, it loses a proton to give $\text{G}(\dot{\text{N}})$ ¹³⁸ then hydrogen atom donation by thiol will restore the base.

8.3.5 Irradiation of Bovine Cell Nuclei

As mentioned in the Introduction to this Chapter, the cell nucleus contains the cell's complement of DNA. However, on examining the e.s.r. spectra after γ -irradiation at 77 K, only hydroxyl radicals were observed [Fig. 8.6].

After annealing to ≈ 130 K the ice $\cdot\text{OH}$ radicals were lost, as usual, and both $\text{G}\cdot^+$ and $\text{T}\cdot^-$ were observed [Fig. 8.7] (together with histone radical signals) under anoxic conditions. Further annealing to 180 K and the eight-line spectrum of $\text{TH}\cdot$ was observed [Fig. 8.8]. There was no further change in signal and the $\text{TH}\cdot$ signals decayed at ≈ 240 K.

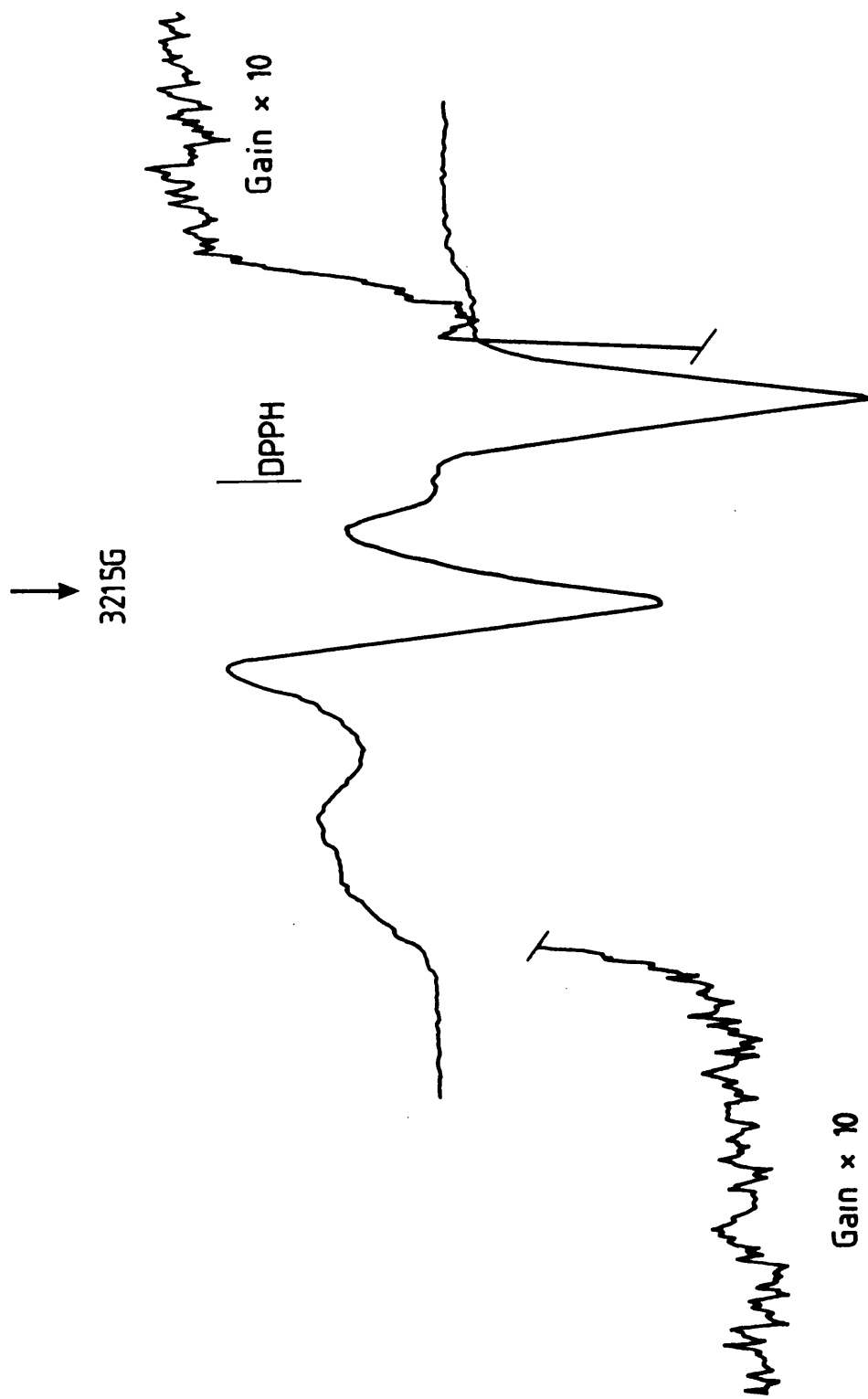


FIGURE 8.6

ESR spectrum of cell nuclei suspension in H_2O at 77 K after γ -radiation, showing the presence of hydroxyl radicals ($\cdot\text{OH}$).

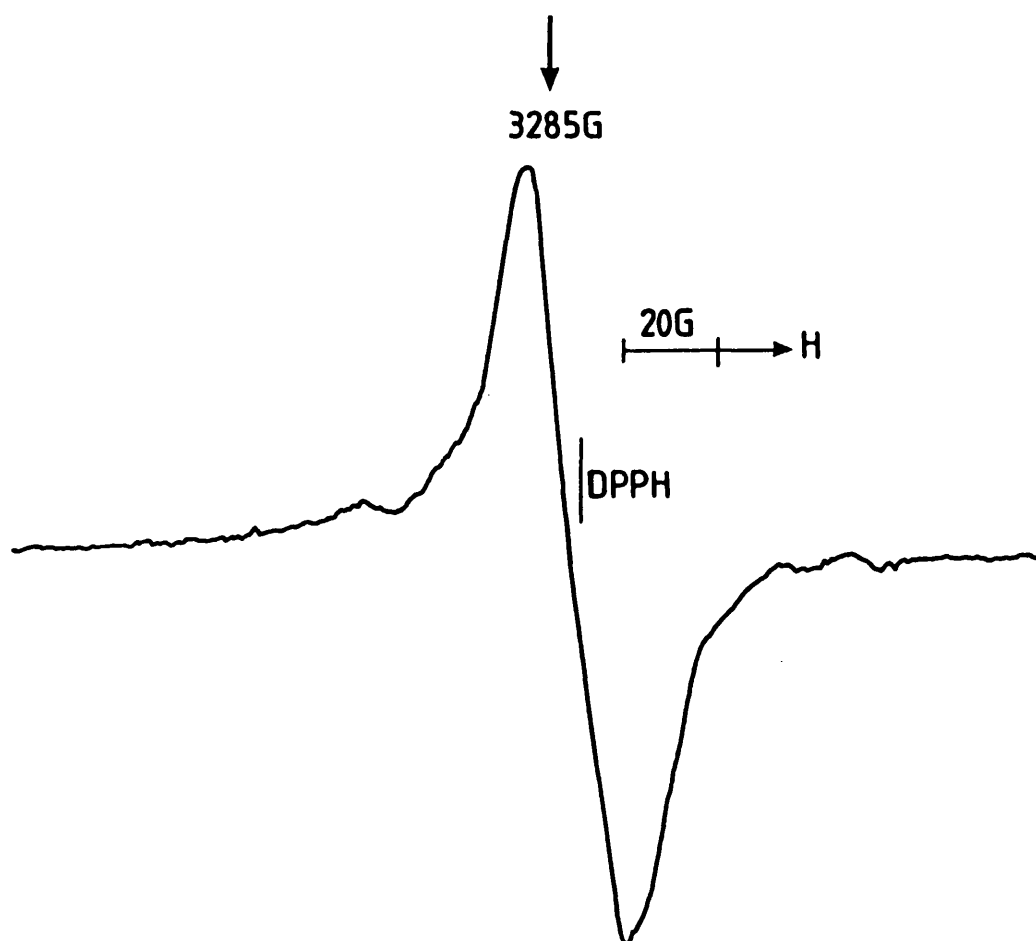


FIGURE 8.7

ESR spectrum of cell nuclei suspension in H_2O at 77 K after γ -radiation and annealing to $\approx 135\text{K}$, showing features assigned to DNA radicals.

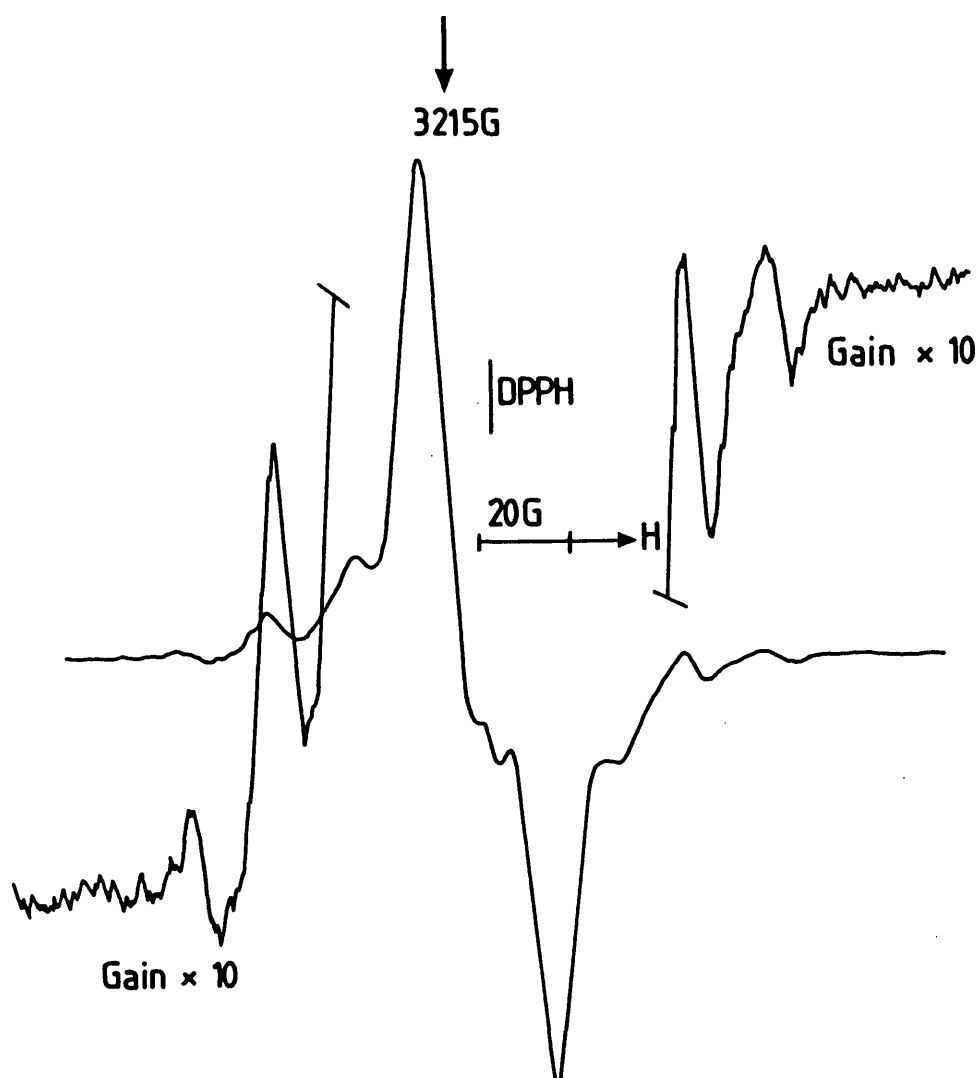


FIGURE 8.8

ESR spectrum of cell nuclei suspension in H_2O at 77 K after γ -radiation and annealing to ≈ 190 K, showing features assigned to $\bullet\text{TH}$ radicals.

Hence it would appear that DNA forms the principal target for radiation damage within the cell nucleus.

8.3.6 Concluding Remarks

From the results with DNA/thiol systems it would appear that under these conditions thiols are effective repair agents. Whilst this is not an original statement, it is still significant as in most studies dilute aqueous solutions of DNA are employed where indirect damage from $\cdot\text{OH}$ radical attack dominates. In our system efficient hydrogen atom transfer was observed under direct damage conditions leading to a reduction in biologically significant lesions.

This lends support to our claim that this is a viable method for observing the radical chemistry of DNA/additive systems. This would appear to be substantiated by the study of bovine cell nuclei where it would appear that DNA is the principal target.

The detection of species "X" and the disulphide radical anion confirm that thiyl radicals do not attack DNA but react with other thiol molecules to give the species "X" adduct which deprotonates to give $\text{RSSR}^{\cdot-}$.

The results under oxidic conditions are more complex but would appear to confirm Copeland's¹⁴⁰ results and show that $\text{RSO}_2\cdot$ radicals are formed, which can then lead to further damage.

As for the cell nuclei, initially it was thought that all damage was transferred completely to the DNA molecule. However, from the work in the previous Chapter, it would appear that the electron-loss centre can become "trapped" within a protein molecule and that only the electron-gain centre is transferred completely. [A later study showed that when yields of $\text{G}\cdot^+$ and $\text{T}\cdot^-$ were measured in irradiated nuclei, the yield of $\text{T}\cdot^-$ is greatly enhanced, whereas the yield of $\text{G}\cdot^+$ is slightly lower than

those for an equivalent DNA sample.^{41]}

Hence within the cell nucleus, DNA is the principal target making the rôle of thiol radioprotecting agents even more important.



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Appendix

The g-value

The g -value is the spectroscopic splitting factor and is the proportionality constant in the equation (for a system with $S = \frac{1}{2}$ and $I = 0$):

$$h\nu = g \mu_B B \quad \dots [E.1]$$

[where B is in G, ν is in MHz, $g = 0.71446 \times \nu/B$] ν is the fixed frequency of the microwave radiation and B is the magnitude of the static field at resonance. It measures the rate of divergence of the $M_S = \pm \frac{1}{2}$ levels in a magnetic field [Figure A.1].

If the electron spin is the only source of magnetism, then $g_e = 2.0023$ ($g \approx 2$ and not 1 is explained by the Dirac equation¹ and is related to the spin quantum number being $\frac{1}{2}$ and the deviation from 2 can be explained by the theory pertaining to quantum electrodynamics). It is true that for certain atoms and linear radicals, orbital angular momentum may be present, in which case the orbital and spin angular momenta will combine together to give a total J splitting; e.s.r. spectroscopy will monitor transitions between the J levels. Mostly, however, angular momentum is quenched as the covalent bonds lift the degeneracy of the orbitals involved.

If the unpaired electron is in a level far removed from other levels, g will be close to the free-spin value of 2.0023. An advantage of the technique is that g -values can be measured very accurately and, hence, small deviations from 2.0023 help to identify the species involved.

The g -value is characteristic of the molecule as a whole and is independent of any electron-nuclear hyperfine interactions [see next section]. Generally speaking, g is anisotropic, having three principal values along three orthogonal axes [Figure A.1]. Deviation from free-

spin arises due to the presence of orbital magnetism that adds to or subtracts from spin magnetism. This contribution is induced by the applied magnetic field (for most free radicals); the extent of this contribution depending on the magnitude of the spin-orbit coupling constants. Since these constants increase rapidly with atomic number, the shift in g -value may be large if there is a significant amount of spin-density on the p or d orbitals of heavier atoms within the molecule. [Hence the deviation from $g = 2.0023$ with sulphur-centred radicals - see Chapter 2.] Therefore, the form of the g -tensor is set by the contribution of spin-density from the various atoms within the molecule.

The magnetic moment of the electron arising from p -orbital angular momentum is 1. The g -value moves below 2 when the unpaired electron acquires orbital angular momentum (negative Δg). Conversely, if the coupling occurs via a filled rather than an empty level it is, in effect, a positive "hole" that moves and g -values shift to greater than 2 (positive Δg).

Frequently, the g -tensor is axial and values for g_{\parallel} and g_{\perp} are obtained from the spectra. However, in some cases, there are three principal g -values (g_x , g_y and g_z) [Figure A.1]. This occurs as different levels are coupled by the magnetic field along these different directions. Hence powder spectra (in the absence of hyperfine coupling - see Figure A.1) give g -values directly since spectral changes only occur at turning points. Therefore, the g -tensor can provide information about magnetically coupled excited states and the direction dependence of the coupling. This contrasts with the hyperfine interaction which is property of the ground state [see next section].

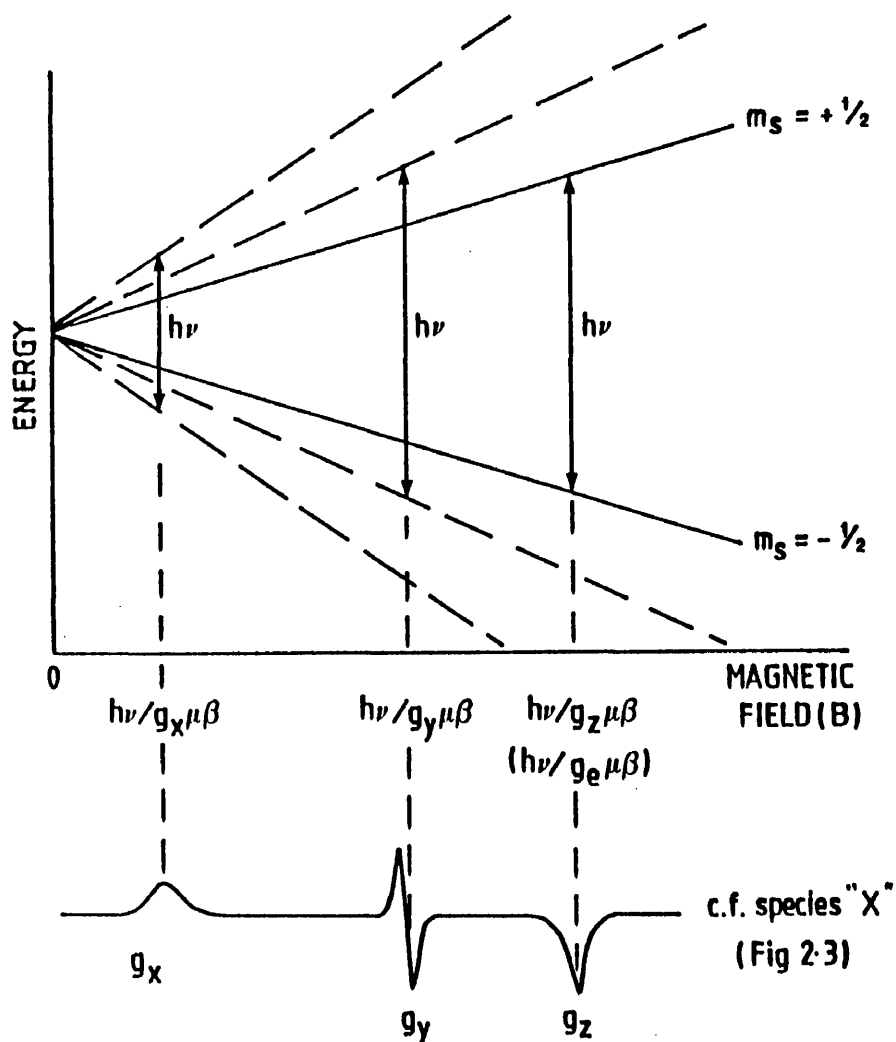


FIGURE A.1

The effect of an applied magnetic field (B) on the $m_s = \pm\frac{1}{2}$ levels of an unpaired electron. These are degenerate at zero field. The thick lines represent the spin-only behaviour; the transition ($h\nu$) occurring at a field corresponding to $g_e = 2.0023$. The dashed lines are for a radical having a neighbouring filled level, giving $g > 2.0023$. This figure illustrates the source of the g_x , g_y and g_z values of the species radical described as "X" in Chapters 2-4.

Hyperfine Coupling

Proton ($^1\text{H}_1$) hyperfine coupling

Hyperfine coupling is the term used to describe the magnetic coupling that can occur between the spin of the unpaired electron (S) and those of nearby magnetic nuclei in the molecule (I). In this respect it resembles spin-spin coupling in n.m.r. spectroscopy.

Employing a high-field approximation, the interaction can be thought of in the manner shown [Figure A.2] for a single nucleus with $I = \frac{1}{2}$. Clearly from the figure the levels $M_S = \pm \frac{1}{2}$, $M_I = +\frac{1}{2}$, $M_S = +\frac{1}{2}$, $M_I = -\frac{1}{2}$ differ in energy. So, if the nuclear spin does not become re-orientated during the electron-spin transition, which is generally the case, two lines will be detected in the e.s.r. spectrum.

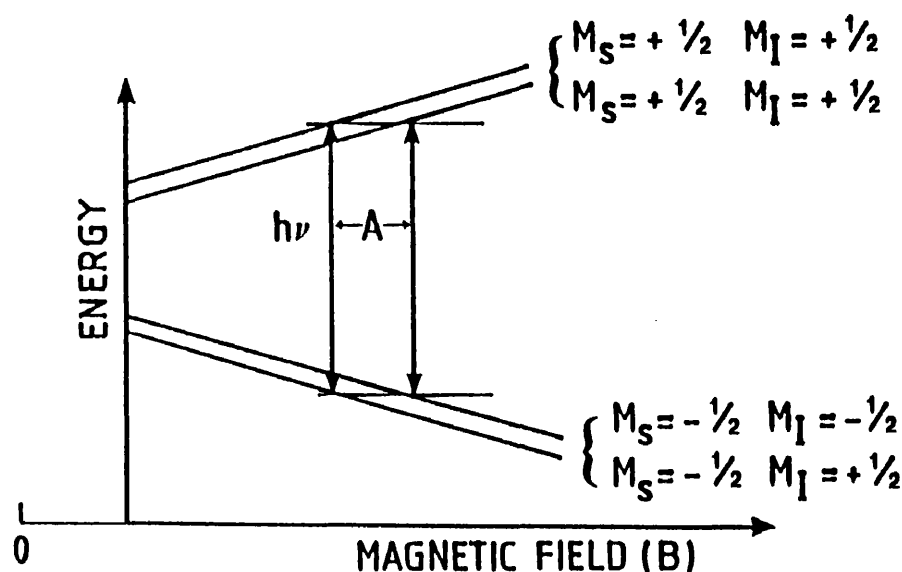


FIGURE A.2

Divergence with field of the $M_S = \pm \frac{1}{2}$ levels in the presence of a single nucleus having $I = \frac{1}{2}$ in the high-field approximation.

Prior to the application of an external field, the electron and nuclear spins are already coupled and are treated in terms of their combined spins. This can lead to apparent 'triplet' and 'singlet'

states (+1, 0, -1) and 0 for a single $I = \frac{1}{2}$ nucleus, which differ slightly in energy. When a magnetic field has been applied, the ± 1 states diverge, but the 0 states are initially unaffected. As the field increases the four states merge with the high-field states. This "zero-field splitting" has to be taken into account if the hyperfine interaction energy is greater than, or comparable with, the measuring frequency. When this is not the case, the high-field approximation is valid and the approximation [E.2] can be used.

$$h\nu = g \mu_B (B + M_I A) \quad \dots [E.2]$$

This defines A, the hyperfine coupling constant, in the manner in which it is measured - as the separation between component peaks of the spectrum.

Hence with reference to the sulphur-centred radicals, i.e. species "X" [Chapters 2-4], the signal from the radical is split into doublets via coupling to one proton in the $\text{RCH}_2\dot{\text{S}}\text{SR}$ unit, as in Figure A.3.

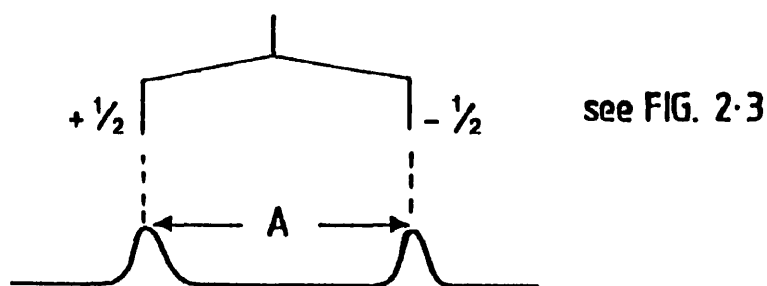
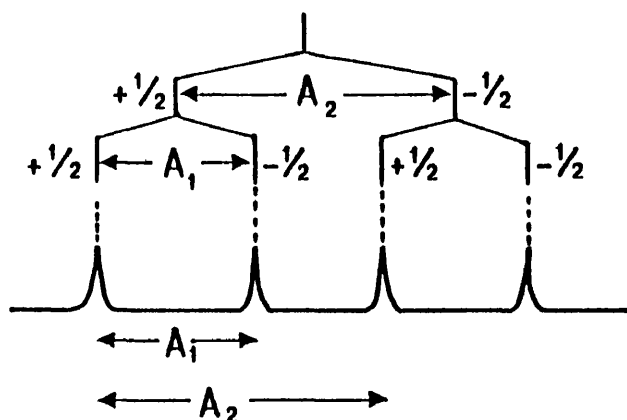


FIGURE A.3

But with thiyl radicals of the type $\text{RCH}_2\dot{\text{S}}\cdot$, which are much more environmentally dependent, coupling can be observed from two inequivalent β -protons as in Figure 2.11(b) [see Chapter 2], as in Figure A.4. If the two β -protons are equivalent, a 1:2:1 triplet results (Figure A.5).

To understand why the two β -protons can be magnetically different,



see FIG. 2.11b
i.e. a doublet of doublets is observed.

FIGURE A.4

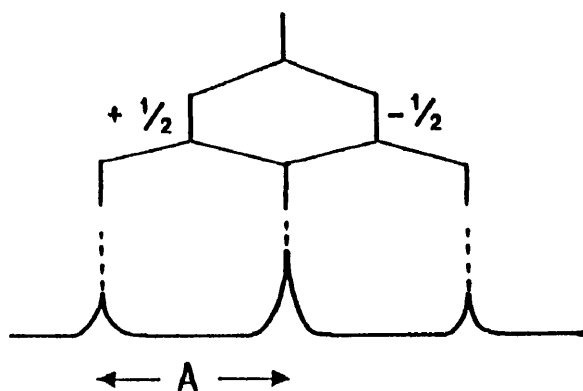


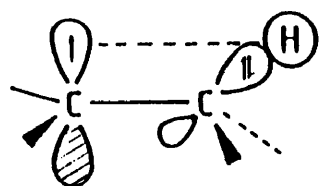
FIGURE A.5

the source of this hyperfine coupling needs to be considered.

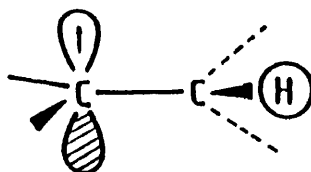
Delocalization of the unpaired electron onto the β -protons is thought to occur via a process of 'hyperconjugation' or σ - π delocalization. This, shown in Figure A.6, depends on θ , which controls the extent of overlap between the $2p$ orbital on carbon and the C-H σ -orbital. If this lies in the radical plane, i.e. in the nodal plane of the $2p$ orbital, the coupling is expected to be close to zero and to reach a maximum along the normal to this plane. This is expressed by

$$A(\beta H) = A_{\max} \cos^2 \theta + B \quad \dots [E.3]$$

where B is small.



Maximum σ - π overlap
for C-H bond $\theta = 0^\circ$



Zero σ - π overlap for
C-H bond $\theta = 90^\circ$

FIGURE A.6

Overlap (σ - π or hyperconjugative) for a C-H bond in a radical $R_2\dot{C}-CHR_2$ showing how this depends upon θ , the angle between this bond and the radical plane.

The experimental dependence of hyperfine coupling of β -protons on angle is well established.¹ Methyl groups generally rotate freely, giving a 1:3:3:1 quartet even at 77 K [see Chapter 7]. However, in the case of steric hindrance, this rotation may be quenched giving rise to inequivalent protons in a manner depending on matrix forces. Therefore for species "X" [see Chapters 2-4] examined in the solid state at 77 K, only one proton is observed from the $RCH_2\dot{S}SR$ unit as the other proton is at such an angle that gives rise to little or no coupling.

In this case, deuterium has $I=1$ (3 lines, +1, 0, -1, of equal intensity). It can be seen that there is a large difference in the coupling observed for deuterium compared to that for hydrogen. This is a difference which is frequently exploited in complex systems by specific deuteration, or by the use of deuterated solvents [see Chapters 2-4] to reduce the interference from solvent radicals. The difference in the couplings is expressed in the equation:-

$$6.5 A(^2H) = A(^1H) \quad \dots [E.4]$$

Other Centres (^{33}S)

^{33}S has low natural abundance [see Chapter 6], i.e. 0.74% and $I = 3/2$.

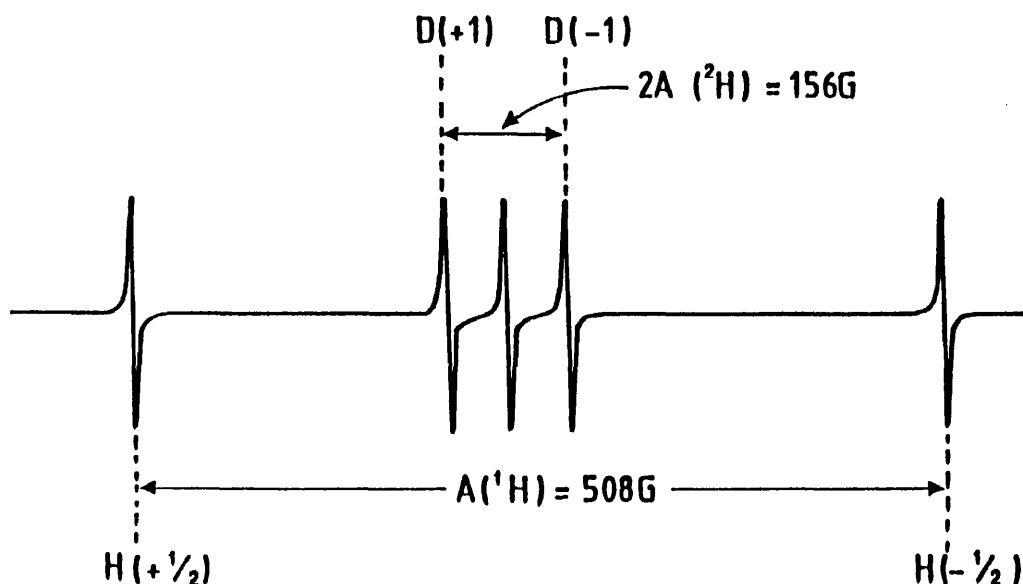


FIGURE A.7

First-derivative e.s.r. spectrum displaying features for trapped hydrogen and deuterium atoms.

Hence, there is no fingerprint readily available for sulphur-centred radicals (deviations in g -value being the primary method of identification). However, if enrichment with ^{33}S is possible, in the liquid phase this gives rise to four isotropic lines corresponding to $+3/2$, $+1/2$, $-1/2$ and $-3/2$ transitions which will lead to determination of A_{iso} .

However, there is need to consider the anisotropy for solid-state work. Usually, hyperfine coupling is axial giving rise to A_{\parallel} and A_{\perp} , i.e. $A_x \sim A_y$. A typical first-derivative powder spectrum for $I = 3/2$ where it is assumed that $A_{\parallel} > A_{\perp}$ and that g is isotropic, i.e. $g_1 = g_2 = g_3$, is shown in Figure A.8.

The alternative extreme, in which Δg is very large, is exemplified in Figure A.9.

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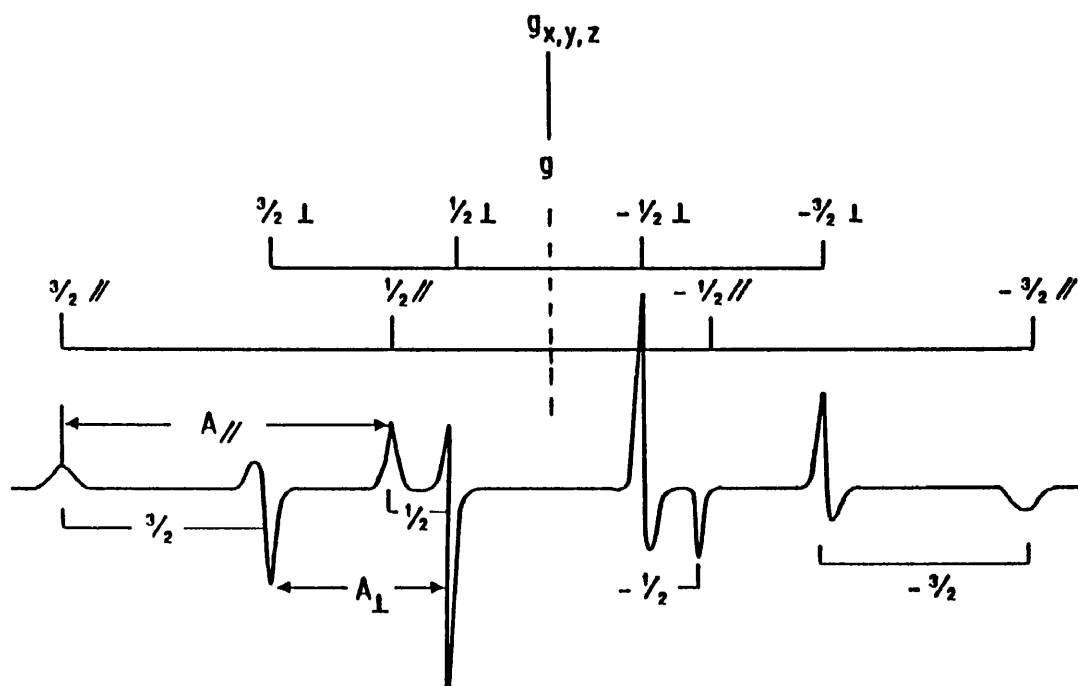


FIGURE A.8

Form of an e.s.r. spectrum for a radical 100% enriched in ^{33}S with one interacting nucleus. The g -value is taken as isotropic and $A_{||} > A_{\perp}$, as is usually observed.

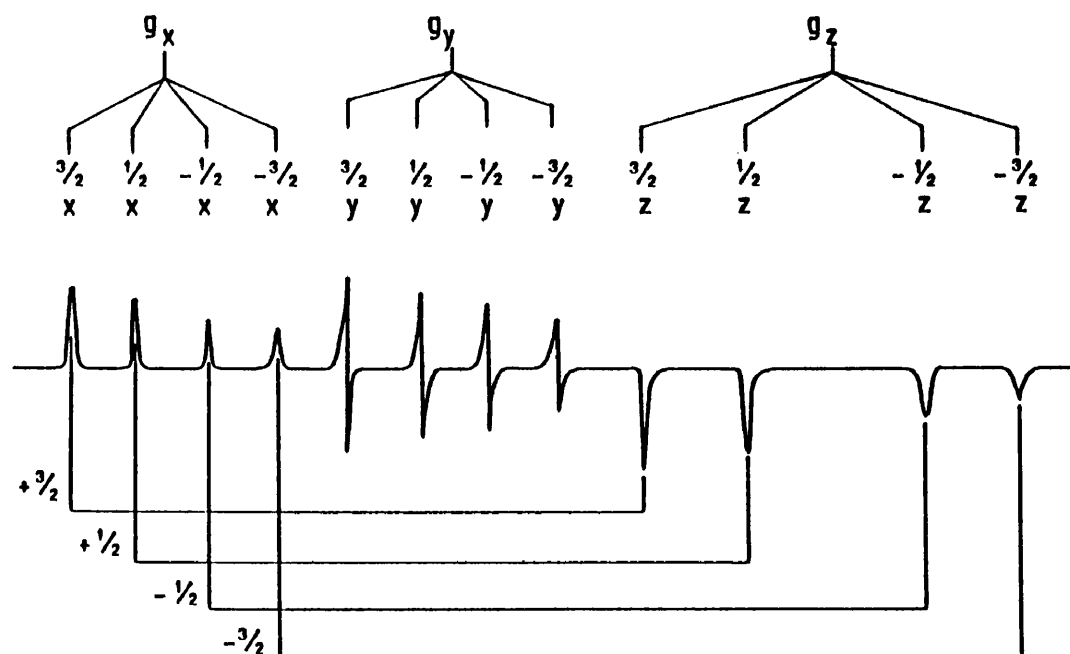


FIGURE A.9

Effect of a large Δg on the e.s.r. features [see previous figure] is to pull out each set of quartets in the manner shown.

Radiation Effects in Organosulphur Compounds and Proteins: An E.S.R. Study

Jeremy S. Lea

ABSTRACT

The technique of electron spin resonance spectroscopy was employed primarily to study the effects of ionizing radiation, in the form of γ -rays (^{60}Co source), on various organosulphur compounds, selected polyamino acids and proteins, and DNA. The work was carried out exclusively at low temperature ($<240\text{ K}$), powder, frozen aqueous or $\text{CD}_3\text{OD}/\text{D}_2\text{O}$ deoxy glass systems being studied.

The underlying principle of the work presented here was "radioprotection". To this end, several known thiol radioprotectors have been studied, along with thiol compounds where previous research has not been so extensive. The aim of this was to try to resolve a long-standing controversy in the literature which has both chemical and biochemical implications. The problem is the fate of radicals generated in the thiol "radioprotector" after it has completed its act of repair in a biochemical system. This is studied, evaluated and discussed in Chapters 2-6 and has direct bearing on the work presented in Chapter 8.

The reverse of this radioprotection is discussed in Chapters 7 and 8 that is radiation damage to bio-macromolecules. Chapter 7 is concerned primarily with damage occurring via an electron-loss centre in polyamino acids and proteins whereas Chapter 8 involves damage to the cells primary target - the DNA molecule itself.