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RADIATION CHEMISTRY OF CHROMOSOMES

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By

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ABSTRACT

In this study, chromosomes and chromosome constituents have been exposed to electrons and hydrogen atoms, which are intermediates expected to be produced by ionizing radiation. Early work was concerned with elucidating the reactions of hydrogen atoms with purines and pyrimidines and it was shown that characteristic ESR spectra are obtained for each base. With this background it was possible to study the reactions of hydrogen atoms with DNA. It was found that hydrogen atoms react non-specifically by addition to the bases in DNA. The presence of histones was found to inhibit hydrogen atom attack on the DNA and result in preferential formation of radicals in the histone component. A tritium labeling technique was used to identify the radical sites on various amino acids of the histone samples. A high degree of specificity was found in that 67% of the radicals formed were on lysine residues.

The study of electron reactions with chromosome constituents was then initiated. Electrons were generated photolytically in an alkaline glass at low temperatures. It was found that electrons reacted readily with thymine, cytosine, adenine and uracil to form anions; but only the thymine anion reacted further by protonation. Interpretation of the ESR spectrum showed that protonation occurs at the 6-position of thymine. Thus the same radical is formed by electrons as is observed in the radiolysis of DNA. The reaction of electrons with amino acids was shown to be dependent on the nature of the substituent side groups. For glycine and amino acids with alkyl side groups the anion formed preferentially deaminates to form a carbon radical. For amino acids containing disulfide linkages electron attachment to the -S-S- group occurs. For methionine which contains a R-S-CH₃ group, electron attachment to the sulfur results in cleavage of a S-C bond and CH₃ radicals are formed. Many of the radicals observed in this study have also been observed

in ESR studies of irradiated amino acids and the role of electrons in radiolysis has thereby been clarified. Current work with dipeptides suggests that electrons preferentially deaminate terminal amino groups.

In a chromosome there are many sites of electron attachment but charge migration within DNA and histone molecules may occur. If this is the case then on the basis of our results we would postulate that electrons would remove terminal α -amino groups on histones and form free radicals on thymine base groups in DNA.

I. INTRODUCTION

The most important effects of radiation on mammalian tissue are the genetic effects, that is the changes induced in the chromosomes. An understanding of such effects in terms of molecular alterations in the chromosomes is being sought in this study. Effects of radiation on bacteria, such as cell death and mutation, have been linked to molecular changes such as chain breaks, crosslinking and base alteration in DNA. Mammalian chromosomes, which consist of histones and RNA as well as DNA, may be affected by these changes as well as changes involving other parts of the chromosome. Crosslinking of histone to DNA may be visualized to occur for example; the biological consequence of such an alteration is unknown. The focus of this study is to understand molecular alterations to chromosomes occurring during radiolysis in terms of elementary reactions of intermediates generated by the radiation. It is hoped that the information gained in this study will eventually be useful in devising treatment for radiation exposure.

The overall approach taken in this study is to expose chromosomes and chromosome constituents to intermediates expected to be produced by ionizing radiation. Direct interaction of radiation with chromosomes in cells is expected a priori to produce electrons, positive ions and excited molecules. Subsequently these species may form secondary free radicals which will react further. In addition chromosomes are subject to indirect attack by the hydrogen atoms, solvated electrons and hydroxyl radicals which are formed in the aqueous media of cells during radiolysis. This project has been concerned primarily with the reactions of hydrogen atoms and electrons with DNA, histones and their constituents. The reactions of these intermediates are followed by detecting radicals formed in lyophilized samples by ESR spectroscopy. Interpretation of the spectra is aided by the use of computer analysis.

The studies of hydrogen atom reactions are illustrative of the general approach. Initially the work was concerned primarily with elucidating the reactions of hydrogen atoms with purines and pyrimidines, the code molecules of DNA. Then the reactions of hydrogen atoms with DNA and nucleotides was investigated. It was shown that hydrogen atoms react preferentially with the nucleic acid bases and very little with the deoxyribose. Each base is attacked in proportion to its concentration in DNA. This detailed understanding was based upon the information obtained from the earlier studies of purines, pyrimidines and nucleotides. When the hydrogen atom work was extended to chromosome samples, the reaction was shown to be with the histone constituent. This stimulated a more detailed investigation in which a tritium labeling technique was used to identify radical sites in proteins.

The electron reaction study began with an investigation of reactions of nucleic acid bases. It was found that the anions of thymine-containing compounds rapidly protonate whereas other base anions do not. Following this, detailed information about the electronic structure and reactivity of purine and pyrimidine anions was obtained by observing the ESR spectra of these anions in solution. Also the reactions of electrons with amino acids was investigated. It was possible to identify radicals formed from amino acid anions and to evaluate the role of electron reactions in the radiolysis of amino acids. An extension of this study to dipeptides is now being made and promises to elucidate the mechanism of localization of damage in irradiated proteins.

II. REACTIONS OF HYDROGEN ATOMS

A. Reactions of H-atoms with Purines and Pyrimidines (Ingalls, Holmes & Myers)

The initial phase of this study was devoted to an investigation of the reaction of hydrogen atoms with purines and pyrimidines and substituted analogues. In these studies hydrogen atoms are produced by a microwave-powered discharge in H_2 at 1 torr pressure. The hydrogen atoms impinge on powdered samples of the bases to be investigated. The powdered samples are supported on quartz wool in the ESR cavity and radical formation in the samples is followed during bombardment.

These studies showed that hydrogen atoms add to both purines and pyrimidines and that the radicals formed have characteristic ESR spectra. From the spectra it was concluded that the locus of addition in pyrimidines is the 5,6 double bond. In purines the results strongly indicated the site of addition to be the C_8 atom on the five-membered ring. The results of this work are published in two papers which are included in this report as Appendices A and B.

11.-B. The Reaction of Hydrogen Atoms with DNA (Holroyd and Glass)

Introduction

This study of the reactions of hydrogen atoms with DNA was done for the purpose of evaluating the role of hydrogen atoms in the radiolysis of DNA. In vivo, DNA molecules in chromosomes of cells are subject to attack during radiolysis by radicals formed both from water molecules and from other parts of the chromosomes. There is a small but significant yield of hydrogen atoms from the radiolysis of water ($G \sim 0.6$).¹ In addition, hydrogen atoms may result from direct interaction of the radiation with the chromosomes. Hydrogen atoms have been detected by ESR techniques in samples of gamma irradiated DNA.^{2,3}

Previous ESR investigations of the reactions of hydrogen atoms with DNA have not identified the site or sites of attack. An ESR study of the reaction of hydrogen atoms with calf-thymus DNA yielded a spectrum devoid of hyperfine structure, in particular, there was no evidence of formation of a radical on thymine.⁴ In another study, ESR spectra were obtained which showed about five, wide hyperfine lines spread over 76 gauss for salmon sperm DNA and a broad singlet for E-coil DNA.⁵ The reaction of hydrogen atoms with the closely related acid RNA has been studied at 77°K.⁶ An ESR spectrum was obtained which was interpreted as indicating that hydrogen atoms add to purine and pyrimidine rings. Investigations of DNA nucleotides have shown that hydrogen atoms add primarily to the purine and pyrimidine rings^{7,8} but some reaction with deoxyribose constituent has been suggested.

In this study, dry samples of DNA were bombarded with hydrogen atoms at 300°K. Stable radicals were thereby formed and the ESR spectra showed considerable hyperfine structure. These spectra were interpreted in terms of the various radicals formed in DNA. This required first investigating the reactions of hydrogen atoms with the four DNA nucleotides (TMP, DGMP, DAMP, AND DCMP) and deoxyribose. The radical sites

formed in each DNA nucleotide were characterized as well as possible on the basis of the ESR spectra. The composite spectrum obtained with DNA was assumed to be the envelope of the spectra obtained for the various nucleotides. By means of a computer spectra simulation technique,⁹ it was possible to determine the relative amount of each type of radical present in the bombarded DNA sample.

Experimental

Samples of DNA, DNA nucleotides and deoxyribose were obtained commercially from Calbiochem and Worthington. Analysis of the samples by the Folin phenol method¹⁰ showed that all samples of salmon sperm DNA contained less than .5% protein whereas the calf thymus samples contained about 2% protein.

For the reaction with hydrogen atoms, the biological samples were placed in short pieces of quartz tubing (OD = 4 mm) and quartz wool was used to hold the samples in place. These tubes were then placed in a larger quartz tube passing through the ESR cavity. This tube was evacuated and then filled with flowing hydrogen gas. Hydrogen atoms were produced in the flowing hydrogen stream by a microwave discharge. The pressure in the discharge region was ~ 0.1 torr. Ions were screened out magnetically and direct photolysis of the samples by the light from the discharge did not occur.

The ESR spectrometer (Varian Model V 4500-10A) used to detect radicals was equipped with 100 kc/sec field modulation unit. A TE_{102} mode cavity was employed which permitted placing the samples in the cavity.

To facilitate the interpretation of ESR spectra, and to verify assignments of radical species, a computer spectra simulation technique was employed.⁹ Frequently, an observed spectrum consisted of a superposition of those of several radical species. To identify all the radicals and to determine the percentage of each radical present, the spectra of the individual radicals were first calculated based on assumed hyperfine splittings and a particular line shape. Then the spectra of the radicals were combined in various proportions. A "best fit" to the experimental spectrum

was selected and differences between calculated and experimental curves were used to improve on the initial combination. Continued repetition of this procedure often led to a determination of the number and nature of all radical species present.

Results and Discussion

Deoxyribose. Deoxyribose was investigated since the backbone of the DNA polymer is made up of alternating deoxyribose and phosphate groups. Further, it had been suggested in a recent study⁸ that hydrogen atoms attack the deoxyribose group in DNA nucleotides as well as the purine and pyrimidine rings. A more detailed study of this reaction was made in hopes that the ESR spectra would provide some information about the nature of the radicals formed.

The reaction of hydrogen atoms with deoxyribose was found to give a five-lined ESR spectrum (figure 1). The same spectrum was obtained when deuterium atoms were used in place of hydrogen atoms, which suggests that the radical is formed by hydrogen atom abstraction. A similar spectrum has been observed in the gamma-radiolysis of deoxyribose.¹¹ From changes in the intensities of various lines upon standing after bombardment, it was concluded that the spectrum was due to at least two radicals. One of the radicals (Type I) gave a 21 gauss triplet and the other (Type IIa) was responsible for the two lines separated by 19 gauss. A computer generated spectrum, consisting 60% of Type I and 40% of Type IIa was (dotted curve in figure 1) consistent with the experimental spectrum.

Since deoxyribose in the crystalline state is in the pyranose (six-membered ring) structure,¹² the reaction of hydrogen atoms with deoxyribose-5-phosphate which has the furanose (five-membered ring) structure was also investigated. This would be expected to be more representative of DNA since the deoxyribose in nucleotides is also a five-membered ring. The ESR spectrum (figure 2) which resulted in this case showed very little fine structure and positive identification of the radicals was not possible. However, as in the case of deoxyribose, the

H + DEOXYRIBOSE

Figure 1. Spectrum obtained on reaction of hydrogen atoms with deoxy-ribose. Solid line is experimental spectrum, recorded with a modulation of 1.5 gauss. Dotted curve is computed spectrum consisting 60% of radical I and 40% of a 19 gauss doublet; lines are assumed to be Lorentzian and 8 gauss wide.

H + DEOXYRIBOSE PHOSPHATE

Figure 2. Spectrum obtained on reaction of hydrogen atoms with deoxy-ribose-5-phosphate. Upper curve is experimental recorded with a modulation of 1.0 gauss. Lower curve is simulated spectrum consisting 15% of Type I, 70% of Type IIb, and 15% of a central line displaced 1 gauss upfield; lines for the three types are assumed to be 9, 10, and 9 gauss wide, respectively.

spectrum is not very wide and no large splittings are present (see following paragraph). It was found that the spectrum could be simulated (figure 2) by assuming that it consisted of 15% of a 21 gauss triplet (Type I), 70% of a 14 gauss doublet (Type IIb), and 15% of a singlet in the center of the spectrum. Thus, most of the radicals formed in this case are similar to those formed in deoxyribose but there is much less of the Type I radicals which lead to the 21 gauss triplet spectrum.

The spectra observed for deoxyribose and deoxyribose-5-phosphate are attributed to radicals resulting from ring rupture. The basis of this conclusion is as follows: Abstraction of a hydrogen atom from any position would lead to a radical with β -hydrogens. It is known that β -hydrogens in five and six-membered rings can give large splittings for example 35 and 41 gauss in cyclopentyl¹³ and 39 gauss in glucose.¹⁴ Such large splittings are not observed here and it is suggested that ring opening occurs following H-atom abstraction. Such carbon-carbon bond cleavage has been suggested to occur in straight-chain sugars following radical attack.¹⁴ The triplet spectra observed for deoxyribose may be due to a terminal radical of structure CH_2OR . Such a radical could be formed, for example, by cleavage of the $\text{C}_4\text{-C}_5$ bond following abstraction of a hydrogen atom from the C_4 position. Plausible structures of the radicals which result in the doublet ESR spectra are not obvious but ring opening may be involved here too.

DNA Nucleotides. The reaction of hydrogen atoms with thymidine-5'-phosphate (TMP) at 25°C leads to an eight-lined spectrum of 131 gauss total width (figure 3). The major lines in this spectrum are attributed to a substituted thymyl radical (Type III) formed by H-atom addition to C_6 .⁷ In addition to this radical, other radicals must be present since there are additional lines in the center of the spectrum.^{7,8} It has been suggested that these central lines are due to deoxyribose radicals.⁸

H + TMP

Figure 3. Spectrum obtained on reaction of hydrogen atoms with TMP at 25°. Upper curve is experimental recorded with a modulation of 3 gauss. Lower curve is computer simulated spectrum for 22% of sugar radical I, 22% of sugar radical II, and 56% of the thymine radical III. Lines are Lorentzian and 9, 10, and 8 gauss wide, respectively.

To analyze the spectrum shown in figure 3, it was assumed that thymyl radicals (Type III) as well as deoxyribose radicals (Types I and IIb) were present. In the thymyl radical, the unpaired spin interacts with the hydrogens in the methyl group at C₅ and with the two protons at C₆. The best match to the positions of the lines was obtained for splittings of $a_{\text{CH}_3}^{\text{H}} = 20.3$ gauss and $a_6^{\text{H}} = 32$ and 38 gauss. A previous study⁷ indicated that $a_{\text{CH}_3}^{\text{H}} = 20.5$ and $a_6^{\text{H}} = 38$ for this radical at 77°K. The spectrum of this thymyl radical (Type III) was mixed with varying proportions of the spectra of the deoxyribose radicals (Types I and IIb) by the computer simulation program until a match of the experimental spectrum for TMP was obtained. The best fit (figure 3) contained 56% of the Type III thymyl radical, 22% of Type I, and 22% of Type IIb deoxyribose radicals. The large percentage of deoxyribose radicals is somewhat surprising since in a one-to-one mixture of thymine and deoxyribose, only thymine radicals were formed. Further, the rate constant for hydrogen atom addition to thymine should be comparable to, or greater than, the rate constant for addition to uracil and the latter is reported to be 136 times greater than the rate constant for reaction with deoxyribose.¹⁵ Apparently the 5-membered deoxyribose ring in TMP is more reactive than deoxyribose itself.

The ESR spectrum obtained when hydrogen atoms reacted with deoxyguanosine-5'-phosphate (DGMP) at 25° is shown in figure 4. This spectrum has been attributed to a radical (Type IV) formed by hydrogen atom addition to the C₈ position in the guanine ring^{7,8} and the free spin is located mainly at N₇. The main lines in the spectrum constitute a 1:2:1 triplet due to the two protons at the C₈ position for which $a_8^{\text{H}} = 36.5$ gauss. The shoulders which are most apparent on the central peak are attributed to nitrogen splittings. The nitrogen splitting is anisotropic and varies from 1.5 to 21 gauss depending on the angle between the magnetic field and the molecular plane.¹⁶ In a powdered sample, all orientations are possible

H + dGMP

Figure 4. Spectrum obtained on reaction of H-atoms with DGMP. Upper curve is experimental recorded at 3 gauss modulation. Lower curve is a computer simulated spectrum consisting 36% of a 36 gauss triplet, 3.7% of a sextet corresponding to the shoulders on the main lines and 80% of a singlet at $g = 2.004$.

and the resultant spectra is an overlap of many spectra. Intensity is found to be strongest at magnetic field positions near $A_{||}$ and $A_{\perp}$ and lines ± 21.5 and ± 1.5 gauss from the center would be expected. Because of the line width only the larger splitting is observed and accounts for the shoulders on each side of the main triplet lines in the observed spectrum (figure 4). The asymmetry in the spectrum is due to a difference in $g_{\perp}$ and $g_{||}$ which amounts to 2×10^{-3} .

No effort was made to estimate the extent of attack on deoxyribose in this nucleotide because the sugar radicals have lines in the central portion of the spectrum and would thus be masked by the central lines of the spectrum of the guanine radical. The computer program GLAD was used to "curve fit" the experimental spectrum. The resulting computed spectrum (figure 4) simulated the experimental one reasonably well and therefore provided an analytical description of the DGMP spectrum which could be used in the computer program to interpret the DNA results.

The reaction of hydrogen atoms with deoxyadenosine-5'-phosphate (DAMP) at room temperature led to an ESR spectrum similar to that shown in figure 4 for DGMP. The three lines of the triplet were present as well as shoulders, which could be attributed to the nitrogen splittings. Since hydrogen atoms add to the five-membered ring of the purine base in both nucleotides⁸ and since the structure of this ring is identical in both, similar spectra would be expected. In the analysis of the DNA spectra (see below), the DAMP and DGMP spectra are taken to be identical.

The reaction of hydrogen atoms with deoxycytidine-5'-phosphate (DCMP) resulted in an ESR spectrum consisting of six broad lines (figure 5). After termination of the hydrogen atom reaction, the spectrum changed; the outer four lines decayed relative to the central component (doublet). Thus, two radicals are formed in the reaction. The principal radical (Type V) is one in which hydrogen atoms add to the 5,6 double bond in the cytosine ring.^{7,8} In this, radical splittings are to be expected from the hydrogens at the C_5 and C_6 positions. Values of $a_{CH}^H = 21$ and

H + dCMP

Figure 5. Spectrum obtained for reaction of hydrogen atoms with DCMP, Upper curve is experimental recorded at 3.7 gauss modulation. Lower curve is a simulated spectrum in which radicals present are assumed to be 65% Type V and 35% of the radical giving the 15 gauss doublet; lines are 14 gauss wide.

$a_{\text{CH}_2}^{\text{H}} = 12.5$ and 50 gauss have been reported.⁷ In this work, it was found that values of $a_{\text{CH}}^{\text{H}} = 19$ and $a_{\text{CH}_2}^{\text{H}} = 19$ and 41 gauss gave a satisfactory fit to the line positions in the spectra. The nature of the radical responsible for the doublet spectrum is not known. Such doublets have been observed in hydrogen atom bombardment⁷ as well as in the radiolysis^{17,18} of cytosine containing compounds. It was found possible to simulate the experimental spectrum by combining 65% of the spectrum of the Type V radicals with 35% of the 15 gauss doublet spectrum (figure 5).

DNA. The reaction of hydrogen atoms with salmon sperm DNA resulted in an ESR spectrum consisting of 11 hyperfine lines spread over 133 gauss (figure 6). During a 30-minute reaction, the number of radicals increased but there was no change in the ESR spectra. The same spectrum was obtained for samples of salmon sperm DNA obtained from various commercial sources and for samples which had been subjected to prior vacuum drying at 60°C. Samples of calf spleen and calf marrow DNA, when reacted with hydrogen atoms, gave ESR spectra essentially identical to that shown in figure 6. This is to be expected since the chemical composition and nucleic acid base ratio of calf DNA is comparable to that of salmon sperm. Samples of calf thymus DNA, on the other hand, gave an ESR spectrum in which the hyperfine lines were discernible only as shoulders on a broad signal. The poor resolution in this case was attributed in part to the presence of a small amount of protein (2%) (see below) as well as to the physical nature of the sample.²

-
2. It was generally found that the samples which reacted rapidly yielded better resolved spectra. Presumably the more reactive samples were of higher surface area which allowed more of the sample to be exposed to the hydrogen atom flux. In samples of lower surface area, the radicals are more likely to be formed closer together and line broadening can occur by spin-spin interaction.
-

H + DNA

Figure 6. Spectrum obtained by reaction of hydrogen atoms with salmon sperm DNA at 25°. Upper curve is experimental recorded at 3.7 gauss modulation. Lower curve is a computed spectrum for which radicals are 9% Type I, 9% Type IIb, 23% Type III, and 49% Type IV, 10% Type V.

The effect of histone impurities on the reaction of hydrogen atoms with DNA was investigated. Histones are intimately associated with DNA in vivo and thus are likely to be present as impurities in isolated DNA samples. For nucleohistone samples, which contain considerable histone ($\sim 60\%$), the ESR spectrum was similar to that obtained with pure histone indicating no attack on the DNA. The spectrum obtained on reaction of hydrogen atoms with histone is a 17 gauss doublet¹⁹ very much like that obtained in the radiolysis of proteins.^{20,21} To elucidate the effect of histones synthetic mixtures containing varying amounts of histone and DNA were prepared. These were prepared by lyophilization of solutions containing the appropriate amounts of salmon sperm DNA and nucleohistone. In the reaction of hydrogen atoms with a sample containing 30% protein, the characteristic hyperfine lines of the DNA spectrum were observed during the first few minutes of the reaction but continued bombardment resulted in a broad "singlet" with weak shoulders at the positions of the hyperfine lines in the DNA spectrum. The ESR spectrum obtained for a synthetic sample containing 3% histone showed the hyperfine lines in the DNA spectrum. The ESR spectrum obtained for a synthetic sample containing 3% histone showed the hyperfine lines of the DNA spectrum but the lines were not as well resolved as in figure 6. This effect of protein impurities in obscuring the resultant ESR spectrum is attributed to preferential reaction of hydrogen atoms with the protein component. It is suggested, on the basis of these results, that the histone which coats the DNA in cells in vivo will protect the DNA from hydrogen atom attack during radiolysis.

The percentage of radicals on each base and sugar group in the DNA sample after hydrogen atom bombardment was estimated from the ESR spectrum in figure 6 as follows. Radicals located on the individual nucleotides in DNA were assumed to

give rise to the ESR spectra obtained for the nucleotides (figures 3, 4 and 5). These spectra were then added together in various proportions by means of the GLAD computer program. A key to the analysis of the DNA spectrum is the intensity of the outer lines relative to the total spectrum. Since these outer lines are due to a radical on a thymine ring, the contribution of this radical (Type III) to the total can be determined quite accurately. Another characteristic feature of the spectrum is the presence of the guanine triplet due to radicals of Type IV (lines marked G in figure 6). The percentage of radicals on DGMP was adjusted to match the intensity of these lines. A trial synthetic spectrum generated by adding 46% of the spectrum obtained with TMP to 54% of the spectrum obtained for DGMP gave a satisfactory fit to the experimental spectrum. However, it is reasonable to expect some reaction to occur with DCMP as well. Therefore, varying amounts of the spectrum obtained for DCMP (figure 5) were added to the trial fit. The addition of 10% of this spectrum to the trial spectrum did not change it a great deal perhaps because of the nearly featureless spectrum observed for DCMP. Addition of more than 15% of the DCMP spectrum, however, began to markedly diminish the intensities of the outer lines of the TMP spectrum and those of DGMP. It is, therefore, estimated that no more than 10% of the radicals are cytosine radicals.

The final "best fit" synthetic spectrum (lower line, figure 6) consisted 41% of the spectrum obtained for TMP, 49% of the spectrum obtained for DGMP, and 10% of the spectrum obtained for DCMP. This simulation reproduces reasonably well the intensities of the TMP and DGMP lines, indicating that the percentages of radicals on thymine and guanine are correct. Particularly significant is the fact that the overall asymmetry of the spectrum is reproduced. This is due to a difference in g-values for the radicals involved. For the carbon radical in TMP, $g = 2.0032$,²² and for the nitrogen radical in DGMP, $g = 2.0040$.¹⁶ This difference amounts to a 1.3 gauss difference in centers of the two radicals and accounts for the asymmetry.

The final analysis indicated that 82% of the radicals formed are on base groups and 18% are on deoxyribose groups. Of the base radicals, 23% are on thymine and 49% are on purines. (Since the spectra obtained with DGMP and DAMP were not distinguishable, it was not possible to assess the relative amounts on each purine base.) Approximately 10% of the radicals were formed on cytosine bases. The attack on the purine and pyrimidine bases is nearly in proportion to their abundances in DNA, since in salmon and calf DNA the bases are 29% thymine, 21% cytosine, and 50% purines. Thus, although hydrogen atoms are selective in that reaction with the nucleic acid bases is more important than backbone attack, they add indiscriminately to the various bases.

The use of deuterium atoms in place of hydrogen atoms verified the fact that most of the radicals were formed by addition. The ESR spectrum obtained on reaction of D atoms with DNA was not as wide as the spectrum obtained with hydrogen atoms. This is expected if radicals are formed by addition as is the case for bases since a large hydrogen atom splitting is replaced by a smaller deuterium atom splitting. For deoxyribose, similar spectra are expected for deuterium and hydrogen atom reactions. A simulated spectrum obtained by assuming that the same percentages of radicals were present as determined for hydrogen atoms but with the appropriate proton splittings replaced by D-atom splittings was in reasonable agreement with the experimental curve. This provided further support to the validity of the analysis.

Conclusion. The importance of hydrogen atoms in the radiolysis of DNA can be evaluated by comparing the present results to ESR studies of the direct radiolysis of DNA. It has been shown by several workers that a principal radical formed in the radiolysis of DNA is the thymyl radical^{11,23,24,25,26} specifically no evidence of purine or cytosine radicals has been obtained. By contrast, this work shows that hydrogen atoms are unselective to the extent that they add nearly randomly to all the nucleic acid bases in DNA and only 23% of them alter thymine bases.

The unique formation of the thymine radical observed in radiolysis must be attributed to some other reaction. A plausible mechanism of thymine radical formation in the radiolysis of DNA is suggested²⁷ to be localization of the electron on thymine bases followed by protonation of the thymine anion.

This paper has been accepted for publication in Radiation Research.

II.- C. Reaction of Chromosomes with Hydrogen Atoms (Holroyd and Glass)

The sources of chromosomes used in this study were ascites mouse tumors and human lymphocytes. Metaphase chromosomes were isolated by Prof. Amos Norman of UCLA using the method of Cantor and Hearst.²⁸ To prepare a sample for irradiation, concentrated suspensions of chromosomes were placed in quartz tubes (1/4" OD) containing quartz wool and freeze-dried to remove the solvent. Such samples typically contained a few milligrams of chromosomes which adhere to the quartz wool in the tube. Cell nuclei samples were prepared in a similar fashion. These were studied as examples of chromosomes in interphase.

The reactions of hydrogen atoms with metaphase chromosomes and cell nuclei were studied at 25° and -90°C. The low temperature was used to increase the sensitivity for detection of radicals and to stabilize the radicals formed. Reaction to form radicals was observed in all cases. At 25° the ESR spectrum is a wide (35 gauss) unresolved singlet for both metaphase chromosomes and nuclei. Similar wide singlets were obtained on reaction of hydrogen atoms with protein samples.

At -90° the reaction of hydrogen atoms with metaphase chromosomes resulted in the ESR spectrum shown in Figure 7. A similar result was obtained for cell nuclei. The spectrum is a triplet, splitting approximately 43 gauss. These spectra are unlike those obtained with DNA samples (see part II-B).

They are quite similar to the ESR spectra that were obtained when hydrogen atoms were reacted with tyrosine and phenylalanine. The spectrum obtained with tyrosine is shown in the figure for comparison. Triplet spectra of this type are characteristic of cyclohexadienyl radicals²⁹ formed by addition of hydrogen atoms to the phenyl rings in the protein component.

H + CHROMOSOMES (-90°C)

Fig. 7. Solid line is ESR spectrum of radicals formed by reaction of H-atoms with metaphase chromosomes at -90°C, 6 gauss modulation. Dotted line is spectrum of radicals induced by reaction of H with tyrosine.

Thus at both temperature hydrogen atoms appear to react with the protein component of chromosomes and do not attack the genes. The two reactions tentatively concluded to occur in chromosomes are abstraction of the α -hydrogens from proteins (see Part II-D) and addition to phenyl rings in proteins. The lack of resolution of the ESR spectra obtained with chromosome samples prevented making any more definitive conclusions about the nature of the damage sites.

II.- D. Reaction of Hydrogen Atoms with Histone (Holroyd, Glass and Riesz)

As a continuation of the study of the reactions of hydrogen atoms with chromosome constituents, the reactions of hydrogen atoms with histone were investigated; histone is a major protein constituent of chromosomes. Hydrogen atoms may be important secondary radicals generated by reactions within the protein molecule, as has been suggested by Wheeler.³⁰ In a recent series of papers, Stein, et al (see review³¹) presented evidence that hydrogen atoms act at very specific sites and effectively deactivate enzymes. Although the reactions of hydrogen atoms with amino acids have been investigated,³² it is of interest to determine if there is any change in reactivity of amino acid residues towards H-atoms when they are present in proteins.

This study was undertaken to provide more detailed information about the reaction of hydrogen atoms with histones. The reaction with nagarse, a cystine-free enzyme, was also investigated.

Two approaches were used. In one the ESR spectra of the radicals formed by H-atom attack were analyzed to determine the structure of the radicals formed. In the second approach the protein samples were exposed to $^3\text{H}_2\text{S}$ following H-atom bombardment. This treatment labeled the radical sites with tritium.²⁰ Subsequently the sample was analyzed and the activity in each amino acid determined.

The ESR spectra of radicals formed in a histone sample on exposure to hydrogen atoms is shown in Figure 8. There are two side lines separated by approximately 19 gauss and weaker lines on either side which are separated by about 100 gauss. Very similar spectra have been obtained for other samples such as nucleohistones and chromosomes which contain a large component of histone. Nagarse, a sulfur free enzyme, also leads to a similar doublet.

H + HISTONE

Fig. 8. Solid line is ESR spectrum of radicals from H + histone (CT), 3 gauss modulation. Dotted line is spectrum of radicals from H + GlyGly, modulation 3 gauss.

The two strong central lines are believed to be due to a radical formed by abstraction of the hydrogen at the α -position forming the α -carbon radical

in amino acids. The α -carbon radical formed in amino acids containing an adjacent CH_2 group (17 out of the 20 common amino acids fit in this category) would give a doublet spectrum. This is because one of the hydrogens on the CH_2 group would be near the node of the p-orbital of the free electron.³³ The hydrogen is constrained in such a position by the bulky

side chain which is forced out of the plane by steric hindrance. Thus if one hydrogen is 15° out of the plane the other one would be at an angle at 45° from the p-orbital and splittings of 2.5 and 18 gauss would be expected for these hydrogens. The observation of the doublet spectrum leaves unanswered the question on which amino acids radicals are formed and raises a further question regarding why hydrogen atoms preferentially abstract α -hydrogens from amino acid residues in proteins when H atoms react quite differently but very specifically with certain amino acids.³² The results described below and in Part II-E provide partial answers to these questions.

To ascertain the distribution of radical sites among the various amino acids of histone and nagarse a tracer technique was employed.²⁰ Radical sites formed in these proteins were tritium labeled by reacting $^3\text{H}_2\text{S}$ with the sample subsequent to exposure to H-atoms or gamma radiolysis. Bombardment of ~10 mg samples of these proteins resulted in the formation of about 3×10^{16} radicals per sample. The $^3\text{H}_2\text{S}$ was found

to react readily with the radicals; 95% of the radicals were titrated in 15 mins. Subsequently the protein sample, after dissolution in water and lyophilization,

contained several microcuries of tritium. The activity in each amino acid was determined (with the cooperation of P. Riesz of NIH) with a Beckman-Spinco amino acid analyzer as has been described.²⁰ The results are shown in Table I and compared with the results obtained in tritium labeling of radical sites in gamma irradiated proteins.

The results show that a very similar distribution of activity is obtained in both H-atom bombardment and gamma radiolysis. Since in radiolysis it had been concluded that the radicals actually titrated by $^3\text{H}_2\text{S}$ are secondary radicals it may also be concluded that hydrogen atoms react like primary radicals to produce the same final radical distribution or that the radicals titrated in the H-atom case are also secondary radicals.

Perhaps one of the most significant results of this work is the high percentage of damage to lysine residues in histone. Lysine has the highest specific activity of 36%. Since histone is 13% lysine, this indicates that approximately 67% of the radicals are on lysine. In nagarse the specific activity of lysine is much lower and a higher percentage of the radicals are found on methionine. The reasons for such specificity are unclear at present. It has been suggested that the differences among different proteins are related to the secondary and tertiary structure.²⁰ Clearly the relative reactivity of the amino acids is not decisive in determining radical sites. Instead features such as conformation and proximity to end groups (see Section III-C) is more important in the final distribution of radicals in peptides. Since lysine is a basic amino acid, it presumably is involved in neutralizing phosphate groups of DNA in the complex mammalian chromosome. Selective alteration of lysine residues which are presumably adjacent to

TABLE I
TRITIUM DISTRIBUTIONS IN NAGARSE & HISTONE

Amino Acid	Nagarse		Histone	
	<u>Gamma Radiolysis</u>	<u>H-Atom Exposure</u>	<u>Gamma Radiolysis</u>	<u>H-Atom Exposure</u>
ASP	0.87	0.99	0.52	1.23
THR	6.20	7.07	4.12	4.81
SER	6.17	5.60	3.07	3.74
GLU	1.54	1.99	4.94	0.52
PRO	13.09	11.49	8.45	5.26
GLY	5.12	5.34	2.19	6.82
ALA	6.41	1.73	1.26	.83
CYS/2*	-	-	6.72	9.45
VAL	1.42	1.81	.44	.32
MET	26.98	27.0	13.9	5.86
ILE	1.35	2.33	.35	.04
LEU	5.71	6.25	1.53	1.09
TRY	5.88	7.57	2.84	2.41
PHE	3.91	9.45	1.23	1.19
LYS	2.61	3.74	26.8	36.5
HIS	4.51	5.24	24.4	18.5
ARG	8.23	2.28	1.66	1.16

*Histones generally contain no cystine, the sample employed contained .2% CYS 2

the DNA in chromosomes may conceivably result in damage to the DNA. A report describing this work is being prepared and will be submitted for publication.

II.- E. Reactions of Hydrogen Atoms with Polyalanine and Polyleucine (Holroyd, Glass and Sevilla)

In order to elucidate the sites of hydrogen atom attack in proteins several polypeptides were investigated which contained only one type of amino acid. The ESR spectra were analyzed to determine the nature of the radicals formed.

Poly -L-leucine was chosen because reaction of hydrogen atoms with leucine results exclusively in abstraction of the tertiary hydrogen and the radical formed has a very characteristic 16-line ESR spectrum (see Fig. 9). With polyleucine the ESR spectrum is mainly a large central doublet but the outer lines of the 16-lined spectrum of the tertiary leucyl radical are also evident. A computer integration of the spectrum indicates that 80% of the radicals result from abstraction of the α -hydrogen and only 20% from abstraction of the tertiary hydrogen. Similar results were obtained for D,L-leucyl-D,L-leucine. Thus, if the doublet spectrum is due to the α -carbon radical the α -hydrogen in polyleucine is much more labile than the α -hydrogen in leucine where it does not react at all.

Polyalanine was investigated to determine the relative importance of the formation of radicals on the α -carbon. Abstraction of the α -hydrogen in this polypeptide would be expected to produce an ESR spectrum with four lines of relative intensities 1:3:3:1. The reaction of hydrogen atoms with poly-D,L-alanine does in fact lead to a four-lined spectrum in which the average line separation is 18 gauss but the intensities are in the ratio: 1:5:5:1 (Fig. 10). A computer integration indicated most of the radicals were α -carbon radicals (Type I) as expected; although another species is present which produces lines in the central portion of the spectrum.

H + LEUCINE

-33-

Fig. 9 ESR spectrum of radicals formed by H-atom reaction with leucine

The reaction of hydrogen atoms with poly-L-alanine leads to an entirely different ESR spectrum than obtained with poly-D,L-alanine (compare Figs. 10 and 11). With poly-L-alanine the ESR spectrum is consistent with the formation of radicals of type II; that is C-N bond cleavage occurs. A similar reaction has been shown to occur on reaction of hydrogen atoms with alanine.³² Computer analysis of the ESR spectrum indicates 80% of the radicals are of type II and that a small amount (20%) of type I is also formed.

It is concluded that hydrogen atoms react in a variety of ways with proteins. Both abstraction and addition reactions occur. In proteins abstraction is largely from the α position. Addition can occur on the amino group which results in main chain cleavage, as has been suggested by Garrison.³⁴ The relative importance of these various processes apparently depends on the conformation of the protein. The solid state structures of poly-D,L- and poly-L-alanine are different in that poly-L-alanine contains a higher percentage of α -helix structure.³⁵ The radicals observed by ESR to be formed by H-atom abstraction may depend on the structure. We postulate that initially hydrogen atoms preferably add to amino groups to break the C-N bond and form radical II and that this radical then abstracts to form α -carbon radicals of structure I. In poly-L-alanine the radicals of type II are stabilized by the rigid structure of the α -helix and this secondary process cannot occur. Apparently abstraction can more readily occur in poly-D,L-alanine. The tritium labeling

Fig. 10

ESR spectrum of radicals formed
by H-atom reaction with poly-
D,L-alanine. Integration
indicated the spectrum was due
mainly to α -carbon radicals.
Upper curve - experimental
lower curve - computed

Fig. 11 ESR spectrum of radicals formed by H-atom reaction with poly-L-alanine. Integration of the spectrum indicated 80% of the radicals were $\cdot\text{CH}(\text{CH}_2)\text{CO}-$, and 20% were α -carbon radicals.
Upper curve - experimental
Lower curve - computed

experiments described below confirm that the free radicals detected in H-atom bombarded enzymes are secondary radicals. The fact that the radical II is not observed in the radiolysis of poly-L-alanine is attributed to the formation of these radicals on end groups where they are not as constrained by the helix and can abstract to form α -carbon radicals. This work is complete and a note is being prepared for publication.

III. ELECTRON STUDIES

A. Reactions of electrons with pyrimidines (Holroyd and Glass)

The study of the reactions of electrons with chromosome constituents was initiated with an investigation of pyrimidines. The electrons were produced photolytically in alkaline glasses at 77°K and allowed to react with pyrimidine solutes. It was found that all pyrimidines attach electrons to form anions, but anions of thymidine and thymidine-5'-phosphate readily undergo proton transfer from surrounding water molecules to the C₆ position of the thymine ring. Anions of uracil and cytosine do not undergo corresponding proton transfer reaction in the aqueous matrix at temperatures below 200°K. It is shown that electron attachment to thymine followed by protonation of the anion can account for the formation of "thymyl" radicals which are observed in the radiolysis of thymine containing compounds including DNA. A paper describing this work has been published²⁷ and a reprint is included here as appendix C.

III-B. Reactions of Electrons with Amino Acids (Sevilla)

Reactions of the electron have often been suggested to be important in the radiolysis of amino acids.^{34,36a-e} For example, in electron spin resonance (ESR) studies of irradiated crystalline L-alanine and L-glycine at 77°K it has been suggested that the first radical species formed is the anion which on warming undergoes reductive deamination to form a carbon radical.^{36a,b} This mechanism has also been shown to be consistent with the products formed in the radiolysis of L-glycine and L-alanine.³⁷ Since in radiolysis radical formation may result from reactions of other species such as positive ions and excited molecules it is interesting to clarify the role of the electron in the radiolysis of amino acids in general. We have therefore studied the reactions of photolytically-produced electrons with amino acids.

The source of electrons was the photolysis of $\text{Fe}(\text{CN})_6^{-4}$ ion in an alkaline glass at 77°K.^{27,38} This glass is prepared from a solution of 5 mM $\text{K}_4\text{Fe}(\text{CN})_6$, 8 N NaOD (92% D_2O) and 1-50 mM amino acid. The concentration of amino acid that can be used depends upon its absorbance at 2.57 Å. Amino acids such as glycine and those with alkyl side groups were not found to absorb appreciably at this wavelength and therefore the higher concentrations could be used. The solutions are deaerated by purging with argon prior to freezing. Photolysis of the glass at 77°K produced electrons, reaction 1.

At this temperature the electrons are stable and readily observable both visually, by the blue color of the glass, as well as by a strong ESR absorption at $g = 2.00$. The electrons become mobile on illumination of the glass with red light at 77°K and react with the amino acid.

Figure 12. Electron spin resonance (ESR) spectra of radical species produced by reaction of electrons with glycine in a deuterated alkaline glass. A. First species dianion at 90°K. B. Second species $\cdot\text{CH}_2\text{CO}_2^-$ after warming to 180°K. C. Third species $\cdot\text{CH}(\text{ND}_2)\text{CO}_2^-$ at 190°K.

Figure 13. ESR spectra of radical species produced by reaction of electrons with L-alanine in a deuterated alkaline glass. A. First species dianion at 90°K. B. Second species $\cdot\text{CH}(\text{CH}_3)\text{CO}_2^-$ at 180°K. C. Third species $\text{ND}_2\dot{\text{C}}(\text{CH}_3)\text{CO}_2^-$ at 195°K. D. Third species $\text{NH}_2\dot{\text{C}}(\text{CH}_3)\text{CO}_2^-$ at 190°K in alkaline H_2O . Note loss of resolution due to the proton splittings on nitrogen.

Figure 14. ESR spectra of radical species produced by reaction of electrons with L-leucine. A. First species dianion at 90°K. B. Second species $\cdot\text{CHRCO}_2^-$ at 100°K. Another radical species increases the intensity of the central component. C. Third species $\text{HD}_2\text{CRCO}_2^-$ at 195°K.

Figure 1. ESR spectra of radical species produced by reaction of electrons with L-isoleucine. A. Second species $\cdot\text{HRCO}_2^-$ at 10°K . The central component arises from another radical species. B. Third species $\text{HD}_2\dot{\text{C}}\text{RCO}_2^-$ at 185°K . The diion radical spectrum consisted of a 10G wide singlet.

Figure 16. ESR spectra of radical species produced by reaction of electron with L-Valine. A. Second species, $\cdot\text{SPRCO}_2^-$ at 180°K. Central component again arises from another radical species (perhaps the third species). B. Third species, $\cdot\text{DRCO}_2^-$ at 19°K. (9). Anion radical spectrum consisted of 11' wide $2g = 1.9$.

Table II

HYPERFINE SPLITTINGS OF RADICALS FORMED BY ELECTRON ATTACHMENT TO AMINO ACIDS

Alkyl Amino Acids	Dianion	Second Radical	Third Radical
	$\begin{array}{c} \text{ND}_2 \\ \\ \text{R}-\text{CH}-\text{CO}_2^- \end{array}$	$\begin{array}{c} \cdot \\ \\ \text{R}-\text{CH}-\text{CO}_2^- \end{array}$	$\begin{array}{c} \text{ND}_2 \\ \\ \text{R}-\text{C}-\text{CO}_2^- \end{array}$
Glycine (R = H)	$a_{\beta}^{\text{H}} = 23\text{G}$ (doublet)	$a_{\alpha}^{\text{H}} \cong 21.5\text{G}$ (anisotropic splittings resolved)	$a_{\alpha}^{\text{H}} = 13\text{G}$ $A_{\parallel}^{\text{N}} \cong 14\text{G}, A_{\perp}^{\text{N}} < 7\text{G}, g_{\parallel} \cong g_{\perp}$
Alanine (R = CH_3)	12G wide "singlet"	$a_{\beta}^{\text{CH}_3} \cong 24.5\text{G}$ $a_{\alpha}^{\text{H}} \cong 22\text{G}$ (anisotropic splittings resolved)	$a_{\beta}^{\text{H}} = 13\text{G}$ $A_{\parallel}^{\text{N}} \cong 12\text{G}, A_{\perp}^{\text{N}} < 4.5\text{G}, g_{\perp} - g_{\parallel} = 1.0 \times 10^{-3}$
Leucine (R = $\text{CH}_2 - \text{CH} \begin{array}{l} \nearrow \text{CH}_3 \\ \searrow \text{CH}_3 \end{array}$)	12G wide singlet	$a_{\beta_1}^{\text{H}} \cong 48\text{G}$ $a_{\beta_2}^{\text{H}} \cong 18\text{G}$ $a_{\alpha}^{\text{H}} = 18\text{G}$	$a_{\beta_1}^{\text{H}} = 12\text{G}, a_{\beta_2}^{\text{H}} < 7\text{G}$ $A_{\parallel}^{\text{N}} \cong 13\text{G}, A_{\perp}^{\text{N}} < 7, g_{\parallel} \cong g_{\perp}$
Isoleucine (R = $\text{CH} \begin{array}{l} \nearrow \text{CH}_3 \\ \searrow \text{CH}_2 - \text{CH}_3 \end{array}$)	Ca. 10G wide singlet	$a_{\beta}^{\text{H}} < 9\text{G}$ $a_{\alpha}^{\text{H}} \cong 22\text{G}$	$a_{\beta}^{\text{H}} < 6\text{G}$ $A_{\parallel}^{\text{N}} \cong 12\text{G}, A_{\perp}^{\text{N}} < 6\text{G}, g_{\perp} - g_{\parallel} = 1.2 \times 10^{-3}$
Valine (R = $-\text{CH} \begin{array}{l} \nearrow \text{CH}_3 \\ \searrow \text{CH}_3 \end{array}$)	Ca. 11G wide singlet	a_{β}^{H} Ca. 10G (inferred) a_{α}^{H} Ca. 22G (inferred)	$a_{\beta}^{\text{H}} < 7\text{G}$ $A_{\parallel}^{\text{N}} \cong 12\text{G}, A_{\perp}^{\text{N}} < 7, g_{\perp} - g_{\parallel} = 1.2 \times 10^{-3}$

1. Glycine and L-Amino Acids with Alkyl Side Groups

The results found for the reaction of electrons with glycine and amino acids with alkyl side chains, conform to the following general mechanism. Reaction of the electron at 77°K produces a radical attributable to the dianion radical (Reaction 2)

This species reacts upon warming to temperatures $\leq 170^\circ\text{K}$ to form a second radical. The ESR spectra show that this radical is formed by deamination, (Reaction 3)

Upon further warming to 190°K the alkaline glass softens and a new radical species is formed. Interpretation of the ESR spectra of this species shows it to have the structure $\begin{array}{c} \text{ND}_2 \\ | \\ \text{R}-\dot{\text{C}}-\text{CO}_2^- \end{array}$ which suggests the second radical is abstracting via reaction 4 from the parent compound, which is always in excess.

The ESR spectra for the radicals formed by electron attachment to glycine alanine, leucine isoleucine and valine are shown in Figures 12-16. These spectra have been interpreted and reduced to hyperfine splittings. The results are presented in summary in Table II and are discussed below in greater detail.

The ESR spectra of the first radical species formed through electron attachment at 77°K, which are attributed to the dianions, shows resolution of a hyperfine splitting only for glycine. The glycine spectrum (Fig. 12) consists of a 25 g doublet. The splitting must arise from one of the protons on

the carbon adjacent to the carboxyl carbon. The carboxyl carbon is the site of maximum spin density; about 0.7 of the unpaired spin is on the carbon, with the remainder divided between the oxygen atoms, see Section II-B-4. (Henceforth such alkyl protons adjacent to positions of high π -spin density will be referred to as β -protons). The splitting found in glycine dianion can be used to determine the spatial configuration of the β -protons. The relation between the β -proton splitting, A_{β}^H , and spatial configuration is given by

$$A_{\beta}^H = B_0 \rho^{\pi} + B_2 \rho^{\pi} \cos^2 \theta \quad (I)$$

where θ is the dihedral angle between the β -proton carbon-hydrogen axis and the Z axis of the P_z orbital on the adjacent carbon, B_0 and B_2 are empirical constants and ρ^{π} is the spin density.³⁹ Our work for the acetate dianion which has a similar electronic structure to glycine has given values of $B_0 \rho^{\pi} = 3.2$ and $B_2 \rho^{\pi} = -30$. (see Section III-D). Using these values we find that $\theta \cong +20^\circ$ for the β -proton of 25 G splitting. The lack of resolution of the second proton indicates that $\theta \cong -100^\circ$ for this proton.

The dianions of L-alanine, L-leucine, L-isoleucine and L-valine have one β -proton but their spectra do not show resolution of a splitting. This suggests that the β -proton is in an orientation (θ) which produces a small coupling ($50 \leq \theta \leq 90^\circ$).

The second radical species formed by warming the dianions to temperatures $\leq 170^\circ\text{K}$ yield ESR spectra which can only be interpreted on the basis that the dianions have undergone reductive deamination. Thus for glycine an anisotropic spectrum (Fig. 12b) due to two α -protons with a 21G isotropic component is observed in agreement with that expected for the $\cdot\text{CH}_2\text{-CO}_2^-$ radical.^{36b,d} The identity of this species has also been confirmed by reaction of electrons with α -chloroacetic acid (see below). For L-alanine a

spectrum arising from a 24.5G splitting due to 3 equivalent β -protons and an anisotropic α -proton with an approximate 22G isotropic component (Fig. 13b) is observed in agreement with the splittings expected for the $\cdot\text{CH}(\text{CH}_2)\text{CO}_2^-$ radical.^{56a,b,6} Reaction of the electron with α -chloropropionic acid has been used to confirm the identity of this species (see discussion of chlorinated acids below).

The second species for L-leucine (Fig. 14b) can also be interpreted in terms of a deaminated radical. A large 48G splitting due to a single proton and a 18G splitting due to two approximately equivalent protons are found. Owing to its magnitude the large 48G splitting must arise from a β -proton. Thus the α -proton and the remaining β -proton in the leucine α -carbon radical structure have a splitting of approximately 18G. The large linewidth (12G) found in this radical indicates that these two protons may have significantly different splittings, i.e., values of 15G and 21G would not be resolved and would average to 18G. The spatial configuration of the β -protons necessary to produce the observed splittings can be estimated by use of relation I. For radicals of this type $B_\beta \rho^\pi$ and $B_\alpha \rho^\pi$ are typically 0 to 4G and 45G respectively. The observed splittings would then arise for $\theta_1 \sim 0^\circ$ and $\theta_2 \sim 120^\circ$. Results found for the reaction of α -chlorobutyric acid with the electron tend to confirm this interpretation.

The ESR spectrum of the second radical of L-isoleucine (Fig. 15a) shows only a 22G doublet presumably due to the α -proton and no further splitting. Since the radical species expected has a single β -proton as well as an α -proton, this indicates that the β -proton is not coupling significantly and is in a configuration so that θ is at 75° to 90° . This configuration is reasonable from considerations of the steric interactions of the alkyl substituent in the radical.

The valine second species should have a structure similar to that of isoleucine, i.e., one β -proton and one α -proton. The observed spectrum (Fig. 16a) shows a 32G total spread. A second species interferes in the central portion of the spectrum, thus a complete analysis is not possible. The 32G spread is 10G wider than expected for only an α -proton. This infers a 10G β -proton splitting for the only β -proton, which would correspond to an angle θ of about 65° . Thus the results found for leucine, isoleucine and valine are in good agreement with those expected for a deaminated radical species. This suggests that deamination is a general mechanism for all dianions of amino acids with alkyl side groups.

Warming the alkaline glass to Ca. 190°K where it softens results in a nearly complete conversion of the second radical species to the third. Interpretation of the ESR spectra of the third species for each amino acid provides strong evidence for the abstraction reaction (reaction 4).

The ESR spectrum observed for species III in glycine consists of a 13G doublet with small line components 13G on either side of the main doublet (Fig. 12c). This spectrum can be interpreted on the basis of a radical of the structure $\text{ND}_2-\dot{\text{C}}-\text{C}\begin{smallmatrix} \text{O}^- \\ \diagup \end{smallmatrix}$. The 13G doublet must then arise from the α -proton and the smaller line components from an anisotropic nitrogen with $A_{||} \cong 14\text{H}$, $A_{\perp} < 7\text{G}$ and $g_{\perp} \sim g_{||}$.

By analogy to glycine the spectrum observed for the third species of alanine (Fig. 13c) would be expected to arise from the radical $\text{ND}_2-\dot{\text{C}}(\text{CH}_3)-\text{CO}_2^-$. For this radical a quartet splitting due to the methyl group would be expected which should be approximately equal to the α -proton splitting in the third radical species of glycine. In addition, an anisotropic nitrogen splitting with parameters similar to those for the glycine radical should be found. Analysis of the spectrum yields a 13G splitting due to 3 equivalent protons and an anisotropic nitrogen splitting with the parameters

$A_{||} \cong 10G$, $A_{\perp} < 4.5$ (linewidth) and $g_{\perp} - g_{||} = 0.0010$. The results therefore provide convincing evidence for the identity of the radical species and consequently for the abstraction reaction.

The third spectra found for leucine (Fig. 14c), isoleucine (Fig. 15b) and valine (Fig. 16b) are also interpretable on the basis of a radical of the structure $R-\overset{\text{ND}_2}{\underset{\cdot}{C}}-\text{CO}_2^-$. Leucine shows a 12G doublet and the anisotropic splittings of the nitrogen ($A_{||} \cong 13G$, $A_{\perp} < 7$, $g_{||} \sim g_{\perp}$). The doublet must arise from one of the β -protons, the other must be less than the linewidth in magnitude. Values of θ near 45° and 75° would produce such β -proton splittings. Isoleucine and valine show nearly identical third spectra. No β -proton splittings are observable; only the anisotropic nitrogen splittings are observed with $A_{||} \cong 12G$, $A_{\perp} < 6G$, $g_{\perp} - g_{||} = 1.2 \times 10^{-3}$ for isoleucine and $A_{||} \cong 12G$, $A_{\perp} < 7G$, $g_{\perp} - g_{||} = 1.2 \times 10^{-3}$ for valine. The lack of observation of a β -proton splitting suggests a spatial configuration (θ) for the β -proton near 90° . This orientation is in good agreement with that expected from the steric interactions of the alkyl group in the radical.

To further confirm steps 2 and 3 of the reaction sequence we reacted the electron with α -chloroacetic and α -chloropropionic acid. These compounds react with the electron by cleavage of the carbon-chlorine bond³⁸ and thus should produce the same radicals as found for the second species of glycine and alanine but different third species. Figures 17 and 18 show that this is indeed the case. Spectra for the second species are identical but the third species differ since a chlorine atom has replaced the amino group.

The reaction of the electron with α -chlorobutyric acid was also investigated since its molecular structure and steric interactions are such that the radical species formed should have similar hyperfine splittings to

Figure 17. ESR spectra of radical species produced by reaction of electrons with α -chloroacetic acid. A. First species $\cdot\text{CH}_2\text{CO}_2^-$ at 160°K. This spectrum is identical to that found for the second radical species of glycine. B. Second species $\text{Cl}-\dot{\text{C}}\text{HCO}_2^-$ at 190°K. As expected, this spectrum differs from that found for the third species of glycine. Dianion radicals are not observed for the α -chloro acids.

Figure 13. ESR spectra of radical species produced by reaction of electrons with α -chloropropionic acid. A. First species $\cdot\text{CH}(\text{CH}_3)\text{CO}_2^-$ at 180°K. This spectrum is identical to that found for the second species of alanine. B. Second species $\text{Cl}\dot{\text{C}}(\text{CH}_3)\text{CO}_2^-$ at 190°K.

Figure 19. ESR spectra of radical species produced by reaction of electrons with α -chlorobutyric acid. A. First species $\cdot\text{CH}_2\text{CHCO}_2^-$ at 160°K. B. Second species $\text{CH}_2\text{CHCO}_2^-$ after warming to 190°K.

the second and third species found for leucine. Figure 19a shows that this is the case for the first species formed by α -carbon-chlorine bond cleavage. The spectrum can be interpreted in terms of a 14G splitting due to a single proton and two protons with splittings of 0.1, 21.3. These values are very close to those found for leucine. Warming to 100° produces a 14G doublet with no further splitting, as would be expected for a radical of the form

$$\begin{array}{c} \text{Cl}-\dot{\text{C}}-\text{CO}_2^- \\ | \\ \text{CH}_2-\text{CH}_2 \end{array}$$

The agreement of the results found here for electron attachment reactions and those found in the radiolysis of glycine is striking. In irradiated single crystals of glycine the species $\text{NH}_2-\text{CH}_2\text{CO}_2\text{H}$ (77°K), $\cdot\text{CH}_2-\text{CO}_2^-$ (180°K), and $\text{NH}_2^+-\dot{\text{C}}\text{H}-\text{CO}_2^-$ (275°K) have been identified by electron spin resonance studies.^{27b,c,d,41} Except for the fact that the first and third species are protonated these species are identical to those identified here for glycine after electron attachment in an alkaline glass. For alanine the same first and second species are found but the third is not found on warming the single crystal containing the second species to room temperature.^{27a,b}

Radiolysis studies in which careful control of the temperature is maintained have as yet not been attempted for single crystals of leucine and isoleucine. A study of irradiated single crystals of valine at 77°K has shown that an ionic species deaminates to produce the same radical species as found here.⁴¹ The third species however is found to be a product of abstraction of a hydrogen from the tertiary carbon on the valine alkyl group. Also, some results for powdered samples of leucine, isoleucine and valine are available which show that the stable species at room temperature is formed by hydrogen abstraction from the tertiary carbon of the alkyl group not the α -carbon as is found here.^{36a}

2. Other Amino Acids

The amino acids discussed below were not found to conform to a general mechanism. In most cases they reacted in a unique manner depending on their side group. Thus they are discussed individually.

L-Asparagine - Reaction of the electron with asparagine at 77°K produces a radical whose ESR spectrum shows a well-resolved 24G doublet (Fig. 20a). Upon warming to 180°K the doublet splitting increases slightly to 26G. Upon further warming to 190°K the doublet converts directly to a spectrum (Fig. 20b), nearly identical to that found for leucine, showing a 13.5G doublet splitting further split by an anisotropic nitrogen with $A_{||}^N \cong 14$, $A_{\perp}^N < 7G$, and $g_{\perp} \cong g_{||}$.

Since doublet splittings were not found for carboxylic dianions with alkyl side groups such as alanine, leucine, isoleucine, and valine, and since the amide group of asparagine was found to be quite reactive to the electron (see section III-4) the first radical species is very likely the anion of the amide group of asparagine. The magnitude of the doublet splitting is in good agreement with that expected for a β -proton adjacent to the amide carbon at an angle of approximately 25° (this calculation assumes that the values of B_{ρ}^{π} and B_z^{π} for acetamide and the asparagine amide group are the same). The remaining β -proton which is unresolved must then be at an angle (θ) near -90°.

The final spectrum observed upon warming to 190°K is obviously that associated with abstraction of the α -proton. In the other amino acids studied an intermediate radical produced by deamination is observed. In this case none was found. The results could be explained by reaction 5

Further work is necessary to verify this mechanism.

Figure 17. ESR spectra of radical species produced by reaction of electrons with L-asparagine. A. First species, the anion, at 175°C. B. Second species $D_2CRCO_2^-$ at 190°C. The second species resulted directly from the first. A radical species of the form $CRCO_2^-$ was not observed.

Proline - Reaction of the electron with proline at 77°K produces a dianion whose ESR spectrum shows three lines (Fig. 21a). Upon warming to 170°K a second radical species is formed. Since Proline has two possibilities for carbon-nitrogen bond breakage to form radical A or B,

the observed spectrum (Fig. 21b) could arise from either species. If the species were radical A, β -proton hyperfine splittings of 48 and 21G and a α -proton splitting of 21G would reproduce the line positions but the intensity of the central component would not be in agreement (a case similar to that found in leucine). If the species were radical B four protons at Ca. 22G would reproduce the line intensities but the line positions would be in slight disagreement. Since there is some evidence for an interfering signal in the central line component, the assignment to radical A is favored although not unequivocally proven.

A third radical species is formed upon warming to 190°K. The ESR spectrum (Fig. 21c) shows five hyperfine components. The central component does not appear at the center of the remaining four lines suggesting it is due to another species. If this is so the remaining four line spectrum can be interpreted in terms of a radical with the α -hydrogen abstracted. Two β -protons of splitting 34G and 11G would then reproduce the spectrum. The results suggest that proline behaves similarly to the amino acids with alkyl side groups.

β -Alanine - Reaction of the electron with β -alanine at 77°K produces a dianion whose ESR spectrum (Fig. 22a) consists of a 24G doublet with a further splitting on the up-field line. Upon warming to 190°K the spectrum changes slightly to a more nearly symmetric doublet (Fig. 22b) possibly due to a reorientation of the β -protons, but deamination does not occur.

Figure 1. ESR spectra of radical species produced by reaction of electrons with propylene. A. First species, dianion, at 90°K. Second species at 160°K. C. Third species at 165°K.

Figure 1. ESR spectra of methyl radicals produced by reaction of laser light with benzene. A. First species, diamond, at 10°K. B. Second species after warming to 10°K. No further reaction was observed at this temperature. The change in spectrum is attributed to a change in the configuration of the radical.

Further warming to 190°K results in a loss of signal without observance of a second or third radical species. A comparison of these results to those for α -alanine, which readily deaminates, shows that deamination from the β -position is more difficult than from the α -position.

L-Lysine - The reaction of electrons with lysine produces the dianion. Upon warming to 160°K the dianion apparently deaminates from the α -carbon position to produce a second species similar to that found for L-leucine but weak in intensity. A large singlet spectrum interferes in the central portion of the spectrum. Warming to 190°K results in a third spectrum characteristic of hydrogen abstraction from the α -carbon of a parent lysine molecule. Analysis of the spectrum for the third species yields a 10G doublet splitting due to a β -proton and an anisotropic nitrogen splitting with $A_{\parallel}^N \cong 13G$, $A_{\perp}^N < 7G$ and $g_{\perp} \cong g_{\parallel}$.

L-Methionine - Reaction of the electron with methionine at 77°K results in the spectrum in Figure 23a. The sharp 1:3:3:1 quartet of 22.5G separation is undoubtedly due to the methyl radical. A second singlet species ($g = 2.003$) which is probably the carboxylic dianion is also present in the spectrum.

The production of methyl radicals must occur by carbon-sulfur bond cleavage:

To insure that the methyl radical did not arise from photolysis, samples were photolyzed in the absence of $\text{K}_4\text{Fe}(\text{CN})_6$. The results showed no signal due to the methyl radical. Thus it is certain that the methyl radicals are produced by reaction of the electron with methionine.

Figure 25. ESR spectra of radical species produced by reaction of electrons with L-methionine. A. First species formed at 77°K. The sharp four-line spectrum is due to the methyl radical. The broad signal in the central portion of the spectrum is most likely the carboxylic dianion. B. Second radical species formed at 180°K. The outer lines are background lines and do not arise from methionine. The other line components may arise from several radical species which have not been identified.

Upon warming the sample the methyl radical and the carboxylic dianion signals disappear and a new spectrum appears (Fig. 23b). This spectrum could and probably does arise from several species formed by reaction of the methyl radicals and the dianion and no assignment is attempted.

L-Cystine - Cystine proved to be very reactive towards the electron. Photobleaching a solution of cystine (1 mM) and the electron at 77°K produced a bright yellow color associated with the disulfide anion.⁴³ The ESR spectrum shows a single broad (10G wide) resonance at $g = 2.012$. The high g value is indicative of a sulfur anion. During warming the signal decays without the growth of a secondary species.

L-Cysteine - Cysteine was not found to be as reactive towards the electron as cystine. Photolysis of cysteine in the absence of $K_4Fe(CN)_6$ was found to produce a sulfur radical ($g = 2.01$). Therefore low concentrations of cysteine (0.5 mM) were used. Reaction of the electron with cysteine does not produce a sulfur radical but a radical at $g = 2.002$ indicative of a carboxylic dianion. Warming to 190°K resulted in a change in g value to 2.004 but no further resolution. Further warming resulted in a loss of signal. The lack of production of a sulfur radical is quite reasonable. In 8N NaOD cysteine is in the RS^- form (not RSH).⁴⁴ The electron would not be expected to react with RS^- but rather to form a carboxylic dianion.

Contrary to the results found in this work for cysteine, studies of irradiated single crystals of cysteine hydrochloride at 77°K show that a neutral sulfur radical is formed.⁴⁵ The electron could be a precursor of this radical since in single crystals cysteine is in the RSH form and would be expected to produce RS^- and $\cdot H$. The hydrogen atoms could then abstract from other RSH molecules to form the neutral sulfur radical, $RS\cdot$.

In two studies of irradiated single crystals of cystine hydrochloride at 77°K the radical product formed has been suggested to be the negative⁴⁶ as well as the positive ion.⁴⁷ In both studies the g-value of the radical was reported to be 2.0125 which is essentially that found in this work. Our results therefore tend to confirm the assignment of the species observed in radiolysis to the anion, but the possibility that the cation has the same g-value cannot be ignored.

In previous radiolysis studies of L-methionine single crystals at 77°K methyl radicals have not been identified.⁴⁸ Since we have shown that electrons react with methionine to produce methyl radicals, the methyl radicals must not be stabilized in crystalline methionine and must combine or abstract to form secondary radicals. This mechanism may account for the radicals observed in crystalline methionine.

ESR studies of γ -irradiated L-lysine single crystals have shown that the first species formed at 77°K, the dianion, loses the α -amino group upon warming to 90°K.⁴⁹ Only a very small amount of radical characteristic of deamination of the ϵ -amino group is found. These species proved to be stable in crystalline lysine and upon further warming did not react further. The results found in the alkaline glass differ in that we find that the second radical species abstracts from the α -carbon position of a parent molecule.

Detailed radiolysis studies of asparagine and proline have not been performed as yet. However, some recent work on β -alanine suggests that the anion which is first formed subsequently deaminates upon warming.^{36c} Deamination was not found to occur in our work in an alkaline glass.

The work on the reactions of electrons with amino acids is complete and a manuscript is being prepared for publication.

III-C. Reactions of Electrons with Dipeptides (Sevilla)

Reactions of the electron with dipeptides are being studied as the next step towards the understanding of electron reactions with the protein constituents of chromosomes. The dipeptides, L-alanylglycine, glycyl-L-alanine, and glycyl-L-leucine have thus far been investigated. On reaction with the electron at 77°K each of the dipeptides reacts to form a dianion. Upon warming to 180°K the dianion is found to deaminate to form a radical on the N-terminal amino group. Further warming to 190°K produces a third species characteristic of abstraction from the " α -carbon" position. For glycyl-L-leucine another radical formed by abstraction from the tertiary carbon of the alkyl group on leucine is observed as well. This radical species is not found to be as stable as the " α -carbon" radical and subsequently decays in intensity. These results may have great significance in the understanding of the radiolysis of large peptides. It may be conjectured that reductive deamination of the end amino group is the first step in free radical formation, followed by abstraction from an " α -carbon". Future work will test this hypothesis.

Thus far radiolysis studies of dipeptides have only dealt with species stable at room temperature.⁵⁰⁻⁵³ For example, the stable species found in the radiolysis of single crystals of glycylglycine is the acid terminal " α -carbon" radical.⁵¹⁻⁵³ The results found here for the reaction of electrons with dipeptides suggest that this species is formed only after the anion formation, deamination, and abstraction steps have occurred.

This work is continuing.

III-D. Reaction of Electrons with Acetate and Acetamide (Sevilla)

Reactions of the electron with amino acids and dipeptides are under investigation in this laboratory through use of electron spin resonance (ESR) spectroscopy.⁵⁴ To elucidate the structure of the anion radicals formed it was desirable to investigate model compounds for these systems. Acetate ion and acetamide were chosen because the acetate dianion should have a π -electronic structure similar to α -anions of amino acids⁵⁵ and the acetamide anion should closely approximate the electronic structure of an anion of the peptide bond. These species were prepared through electron attachment in an alkaline glass and are found to exhibit interesting properties dealing with restricted rotation of their methyl groups which are described in this note.

Experimental

The experimental procedure was essentially that of Ayscough, Collins and Dainton⁵⁶ as modified by Holroyd and Glass.²⁷ In this method a deoxygenated 8N NaOD (94% D₂O) solution containing 5×10^{-3} M K₄Fe(CN)₆ and 1 to 10 mM solute is cooled to 77°K. The glass formed is photolyzed with 2537 Å UV light at 77°K for approximately 1 minute. The photolysis produces a dark blue solid solution of the electron. At this point, an ESR spectrum is taken of the sample to insure that photolysis of the organic solute is minimal. The sample is then photobleached at 77°K with light from an infrared lamp for approximately 4 minutes. The electrons become mobile, attach to the solute, and the ESR spectrum of the anion can be then observed.

A Varian V4510 ESR spectrometer equipped with a dual cavity and Fieldial magnetic field regulator was employed in this work. Measurements of hyperfine splittings were made vs potassium peroxyamine disulfonate ($A = 13.0$ Gauss).

Results

Photolysis of a deuterated alkaline glass containing sodium acetate (10 mM) produces mainly the electron. Photobleaching results in a radical whose ESR spectrum shows a large doublet splitting (Ca. 27G) (Figure 24a). A small amount of methyl radical is formed in the photolysis. Photobleaching results in no increase in the methyl radical concentration; thus this species is not produced by a reaction of the electron. Upon warming the sample, the doublet spectrum changes gradually (Figure 24b) until at 180°K it appears as a quartet whose lines have approximately the same intensity (Figure 24c). Cooling the sample after initially warming to 180°K does not reproduce the line intensities in the intermediate temperature region (Figure 24d); however, at 85°K a similar doublet splitting, 26.5G, to that initially formed is found (Figure 24e). After this first annealing of the sample repeated cycling of the temperature between 85°K and 180°K results in reproducible spectra at each temperature identical to those found in Figure 24c,d, and e. Increasing the temperature to 190°K results in a loss of the ESR signal.

The results for acetamide are shown in Figure 25. To prevent hydrolysis, the alkaline solution was frozen quickly after the acetamide was dissolved in the deoxygenated alkaline solution. The results are similar to those found for "annealed" samples of sodium acetate. Photolysis again produces electrons and only a small amount of methyl radical. Photobleaching at 77°K results in a radical whose ESR spectrum at 85°K consists of a large 30.8G doublet splitting. Warming the sample to +180°K produces a 13.5G quartet in the intensity ratio 1:3:3:1. The conversion from doublet to quartet is reversible and can be repeated indefinitely without loss of signal. At intermediate temperatures ($85^{\circ}\text{K} < T < 180^{\circ}\text{K}$) a gradual change from doublet to quartet is observed (Figures 25b,c) which is also reversible. Again at

Figure 24. First derivative ESR spectra of the acetate dianion in a deuterated alkaline glass at various temperatures. A. Spectrum taken immediately after photobleaching, $T = 8^\circ\text{K}$. The very weak outer lines are due to the methyl radical. B. Spectrum after warming to 15°K . C. Spectrum at 180°K . D. Spectrum after cooling to 150°K . E. Spectrum at 35°K . After first warming to 180°K the spectra at any given temperature are reproducible.

Figure 25. First derivative ESR spectra of the acetamide anion in a deuterated alkaline glass at various temperatures. A. 5°K. The weak methyl radical signal has been removed by warming and recooling. B. 153°K. C. 146°K. D. 100°K.

temperatures near 190°K, the ESR signal is lost.

Preparation of samples of acetate and acetamide in alkaline H₂O solutions results in spectra with larger linewidths. At lower temperatures both species show resolved doublets with similar splittings to those found in D₂O solutions. At 180°K the spectrum of acetamide anion appears as a singlet, $\Delta H_{ms} \sim 25G$, while that of acetate dianion is only partially resolved.

Discussion

The results show that reaction with the electron at 77°K produces the acetate dianion and acetamide anion. Further, the spectra at 85°K show that the methyl groups of these anions are not free to rotate rapidly and can be considered locked in a spatial configuration corresponding to a potential minimum so as to produce one large splitting. The spectra at 180° indicate that the methyl groups are rotating more rapidly. The results are complicated by the change in line intensities noted in the spectra of acetate dianion before and after annealing. This is considered to be either a result of rehybridization so that the acetate molecular framework converts to a more stable configuration,* or a change in the nature of the alkaline glass immediately about the dianion to accommodate the extra negative charge. The fact that this effect was not found for acetamide anion suggests the former. Further discussion of the results found for the acetate dianion after annealing and acetamide anion is given below.

The orientation of the methyl group protons in the locked configuration found at 85°K can be determined by recourse to relation (1) which describes the β -proton splitting, A_{β}^H , as a function of angle:³⁹

$$A_{\beta}^H(\theta) = B_0 \pi + B_2 \pi \cos^2 \theta \quad (1)$$

* Rehybridization has also been invoked to explain results found in crystalline alanine (see Ref. 55).

where B_0 and B_2 are empirical constants with $B_2 \gg B_0$, ρ^π is the spin density in the Pz orbital of the π -carbon atom, and θ represents the dihedral angle between the z axis of the Pz orbital and the alkyl C-H bond axis. The doublet spectrum observed would result only if the three methyl protons were at angles near 0° , 120° and 240° . This would produce a large splitting for the proton at $\theta = 0^\circ$ and two small (apparently unresolved) splittings for the protons at $\theta = 120^\circ$, 240° .

As the temperature is increased above 85°K , the methyl group undergoes increasingly rapid hindered rotation (interconversion between potential minimum) until in the case of the acetamide anion (180°K) "free" rotation occurs (rapid interconversion in comparison to the hyperfine splittings in Mc/sec.). In the case of acetate dianion, the methyl group does not interconvert rapidly even at 180°K . Consequently, line intensities are observed which are similar to those for acetamide anion at 146°K . By comparison to the acetamide anion spectral behavior the splittings for a freely-rotating methyl in the acetate dianion is estimated to be 11.8G.*

The lack of resolution of the two protons at 120° and 240° in the low temperature spectrum of both anions can be understood by first solving for values of $B_0\rho^\pi$ and $B_2\rho^\pi$ using equation (1) and the experimental splittings for the "locked" and freely-rotating cases. The assumption must be made that there is no significant temperature dependence in $B_0\rho^\pi$ and $B_2\rho^\pi$. It is found that $B_0\rho^\pi = -3.2$ and $B_2\rho^\pi = 29.4$ for acetate dianion, and $B_0\rho^\pi = -3.8$ and $B_2\rho^\pi = 24.6$ for the acetamide anion. The calculated splittings of the protons at 120° and 240° in the low temperature case are then + 3.9G (acetate)

* The spectra in the intermediate temperature range between the locked and the freely-rotating cases can be understood in terms of the effect of hindered rotation on linewidths and splittings of methyl groups. See J. H. Freed, J. Chem. Phys. 43, 1710 (1965) and references therein for quantum mechanical and classical treatments of this phenomenon.

and + 4.8G (acetamide), values which are well within the observed linewidth of 10 and 13G.

Since theoretical spin densities are essential to the computation of theoretical values of the methyl proton splittings for comparison to experiment, McLachlan SCF-MO calculations were performed for the acetate and acetamide radical ions (Table III).⁵⁷ The effect of the methyl group is simulated by the use of typical hyperconjugation parameters and a small inductive effect. The results of this calculation are given in Table I. The coulomb (h) and resonance (k) parameters employed are: $k_{\text{C}\ddot{\text{O}}} = (k_{\text{C-O}} + k_{\text{C=O}})/2 = 1.2$, $k_{\text{C=O}} = 1.6$, $h_{\text{O}} = 1.5$, $k_{\text{N}} = 0.8$, and $h_{\text{N}} = 1.5$.⁵⁸ Moderate changes in these parameters are not found to affect the spin density distribution significantly. For example, a value of $h_{\text{O}} = 2.5$ raised ρ_{O}^{π} by only 0.09 for acetate dianion. Two methods are available to obtain theoretical methyl splittings for comparison with experiment. First the spin density on the carboxyl carbon, ρ_{C}^{π} , can be used to estimate the methyl group hyperfine splittings for free rotation assuming a relation of the form $A_{\text{CH}_3}^{\text{H}} = Q_{\text{CH}_3} \rho_{\text{C}}^{\pi}$ where $Q_{\text{CH}_3} \cong 20\text{G}$.^{58b} Second, the methyl proton hyperfine splitting can also be calculated from the spin density in the pseudo π orbital composed of a linear combination of methyl hydrogen 1s orbitals, by use of the relation $A_{\text{CH}_3}^{\text{H}} = 217 \rho_{\text{H}}^{\pi}$.^{59a,b} For acetate dianion it is found that $A_{\text{CH}_3}^{\text{H}} = 13.9$ and 14G for the first and second calculation respectively. The results for acetamide anion are slightly lower, 13.7 and 13.8G. The two types of calculations are in excellent agreement with one another and in reasonable agreement with experiments. Thus the calculated and "experimental" spin density distributions should also be in reasonable agreement.

The spectra of acetamide anion show no resolution of nitrogen hyperfine splittings whether isotropic or anisotropic. This is explained by the spin

density calculations which predict only a small spin density and consequently a small splitting for nitrogen. The deuteriums bonded to nitrogen of course should give rise to negligible hyperfine splittings.

The results found in this work suggest that anions can be made of small and perhaps even unstable molecules quite easily. Further they show that by a suitable choice of the molecular species and use of a deuterated alkaline glass ESR spectra can be obtained which are well resolved and easily interpretable.

Finally, these results should aid in the analysis of the spectra found in irradiated acetamide and sodium acetate where these anions may be produced and stabilized at very low temperature. Presently neither of these species has been observed in radiolysis studies at 77°K.^{60a *} It should be noted, though, that the acetate dianion has been reported as a product of the radiolysis of sodium acetate;⁶¹ however, the actual species observed has been shown to be the methyl radical through ¹³C substitution studies.^{60a,b} Our work supports this assignment. Since anions have been shown to be important species in radiolysis,^{62,63} it should be possible to stabilize them in these compounds as well. This may require cooling to even lower temperatures than have been previously employed.

This work is complete and has aided the analysis of ESR spectra obtained from the reaction of electrons with amino acids. The acetamide anion aided in the analysis of the spectrum of asparagine and should aid in the future peptide work. The acetate dianion aided in the analysis of the amino acid dianion spectra. This paper has been submitted for publication.

* However, a protonated acetate dianion has been reported to be formed in irradiated acetic acid at 77°K (I. Miyagawa and W. Gordy, J. Am. Chem. Soc. 83, 1036 (1961)). The results found for this species are in good agreement with those found here but suggests an appreciable effect of protonation on the hyperfine splittings.

Table III
McLACHLAN SPIN DENSITY CALCULATIONS^a

<u>Position</u>	<u>Acetate Dianion</u>	<u>Acetamide Anion</u>
1	0.0646	0.0636
2	0.032	-0.033
3	0.693	0.685
4	0.137	0.228
5	0.137	0.057

- a. Hyperconjugation parameters used were: $h_1 = 0.5$, $h_2 = -0.10$, $h_3 = -0.10$,
 $k_{12} = 2.5$, $k_{23} = 0.9$.

III-E. ESR Study of Purine and Pyrimidine Anions (Sevilla)

It is well known that electrons are formed during gamma radiolysis and since purines and pyrimidines react readily with the electron, anions of these molecules (which are constituents of DNA) are expected to be formed during radiolysis. A knowledge of the electronic structure of these anions is useful in understanding their subsequent reactions. Electron Spin Resonance (ESR) spectroscopy in combination with molecular orbital calculations provide a powerful means for obtaining information about electronic structure. In this work we have prepared the anions of purine, 6-cyanopurine, pyrimidine and 5-methylpyrimidine (Figure 26) and have analyzed their ESR spectra in terms of hyperfine splittings.

Experimental. Anions studied in this work were produced by electrolysis. The electrochemical cell used is similar to that used by Henning.⁶⁴ A tungsten electrode sealed in a 3 mm capillary acted as the cathode. A thin platinum plate acted as the anode. A fritted glass disc separated the cathode and anode. Intra muros electrolysis was performed by inserting the cathode in the lower third of the resonant cavity (TE_{102} mode) where it did not significantly affect the signal level. The cell was filled with a 0.1M solution of the supporting electrolyte, n-tetrabutylammonium iodide, in dimethylformamide. The concentration of the substance to be reduced was varied for maximum signal intensity while maintaining high resolution. Typically the concentrations were from 1×10^{-3} to 5×10^{-3} M. To purge oxygen from the cell the solution was "bubbled" with argon for 10 minutes, degassed by evacuation and the cell was filled with inert gas. The temperature at which the electrolysis took place was varied in order to maximize the signal.

A

B

Figure 26. The structure and numbering scheme for the molecules in this study. A. Pyrimidine (5-methylpyrimidine). B. Purine (6-cyanopurine) shown as the 9H tautomer. The numbering scheme for 5,5'-dimethyl-2,2'-bipyrimidine is based on that of 5-methylpyrimidine.

Results and Discussion

Pyrimidine Anion: Electrolysis of a $3 \times 10^{-3}M$ solution of pyrimidine in dimethylformamide at $-55^{\circ}C$ yielded a well-resolved spectrum of the pyrimidine anion (Figure 27). This spectra differs slightly from that previously reported by Talcott and Myers.⁶⁵ They obtained the anion by electrolysis in liquid ammonia and found that the largest hydrogen splitting was a multiple of the nitrogen splitting. In dimethylformamide this condition was not found. We therefore have been able to make an unequivocal determination of these splittings which verified their conclusions. The results of the spectral analysis are reported in Table IV along with those previously reported. The differences between the two results are small but beyond the limits of error and must therefore be attributable to a solvent effect.

Since the 9.54G splitting arises from two equivalent protons and the 3.34G splitting arises from two equivalent nitrogens they may be unequivocally assigned to the 4, 6 and 1, 3 positions, respectively. The smaller splittings have been assigned by analysis of the spectrum of 5-methylpyrimidine.

5-Methylpyrimidine Anion: The anion of 5-methylpyrimidine was prepared to aid in the assignment of splittings to positions in the pyrimidine anion. The spectrum of 5-methylpyrimidine anion prepared by electrolysis of a $2 \times 10^{-3}M$ solution at -55° is depicted in Figure 28. A straightforward analysis of the spectrum gave the hyperfine splittings reported in Table IV.

From the results for the 5-methylpyrimidine anion it can be seen that the methyl group has replaced the proton of 1.24G splitting in pyrimidine. The possibility that the proton of 0.72G splitting has been replaced and that the 1.24G splitting has been reduced to 0.67G is considered unlikely owing to the small effect of the methyl group on the larger splittings and

Table IV
EXPERIMENTAL HYPERFINE SPLITTINGS FOR THE
PYRIMIDINE AND PURINE ANIONS

Anion	Position	Splitting (Gauss)	Temperature (°C)
Pyrimidine	1 (N)	3.34 ± 0.01 (3.26) ^a	-55°
	2 (H)	0.72 ± 0.01 (0.72) ^a	
	4 (H)	9.54 ± 0.03 (9.78) ^a	
	5 (H)	1.24 ± 0.01 (1.31) ^a	
5-Methylpyrimidine	1 (N)	3.34 ± 0.02	-58°
	2 (H)	0.67 ± 0.02	
	4 (H)	9.26 ± 0.03	
	5 (CH ₃)	1.56 ± 0.02	
5,5'dimethyl-2,2'- bipyrimidine	1 (N)	1.92 ± 0.01	-24°
	4 (H)	<0.14	
	5 (CH ₃)	3.19 ± 0.01	
Purine	3 (N)	4.4 ± 0.3	-55°
	6 (H)	10.4 ± 0.2	
	8 (H)	4.4 ± 0.3	
	(H)	(1.85) ^b	
	(N)	(1.25) ^b	
6-Cyanopurine	1 (N)	2.43 ± 0.03	+25°
	2(9) (H)	0.18 ± 0.02	
	3 (H)	3.80 ± 0.05	
	7(9) (N)	0.74 ± 0.02	
	8 (H)	4.12 ± 0.05	
	9(7) (N)	0.99 ± 0.02	
	11 (N)	1.55 ± 0.02	

a. Liquid Ammonia Solvent Ref. 65.

b. Line components from these splittings are not completely resolved.
The splittings were inferred from a spectrum simulation.

2G

Figure 27. Approximately one-half the first derivative ESR spectrum of pyrimidine anion in dimethylformamide at -55°C . (Dot marks center.)

Figure 1. Approximately one-half the first derivative ESR spectrum of β -methyltryptophan anion in dimethylformamide at -50°C .

the correspondence of the magnitude of the smaller splittings in 5-methylpyrimidine. The smaller splittings in pyrimidine have therefore been assigned accordingly. The assignment of splittings to positions for 5-methylpyrimidine is of course unequivocal.

Upon prolonged electrolysis of $10^{-2}M$ solutions of 5-methylpyrimidine at temperatures greater than $-30^{\circ}C$ a secondary spectrum appeared (Figure 29). The spectrum extends over 30G and the intensity is not sufficient to resolve the end components. This always leads to some uncertainty in the interpretation, but several factors indicate that the spectrum is due to a "dimer" anion. First the spacing, regularity, and intensity ratios indicate a large number of equivalent protons such as would be found for two equivalent methyl groups. Second the fact that this spectra appeared at higher concentrations of 5-methylpyrimidine and only after prolonged electrolysis, is consistent with dimer formation. Of the several possible dimers of 5-methylpyrimidine the only dimer that could yield such a regular spectrum is 5,5-dimethyl-2,2'-bipyrimidine. Analysis of the spectrum on the basis of this structure yielded the splittings reported in Table IV. The splitting due to the four meta protons is not resolved and is therefore less than the line width (Ca. 0.15G). A spectrum computed on the basis of this analysis is shown in Figure 29. The excellent fit between theory and experiment is considered strong evidence for a 2,2'-dimer.

Purine: Purine anion was formed by electrolysis of 0.001M solutions of purine at temperatures between -34 and $-55^{\circ}C$. The ESR spectrum observed for this radical is only moderately resolved, $\Delta H_{m.s.} \sim 0.9G$ (Figure 30). The large line width is most likely a result of unresolved hyperfine splittings. There is also evidence for a second radical species, present in lower concentration, in the spectrum. It is possible that the second radical is associated with one of the two possible tautomeric forms of purine, i.e.,

Figure 29

UPPER CURVE: Approximately one-half the first derivative ESR spectrum of 5,5'-dimethyl-2,2'-bipyrimidine at -24°C .

LOWER CURVE: Calculated spectrum using a 3.19G splitting arising from 6 hydrogens and a 1.92G splitting arising from 4 nitrogens. The linewidth for the computed spectrum is slightly larger than the experimental.

Figure 30

UPPER CURVE: One-half the first derivative ESR spectrum of purine anion at -55°C .

LOWER CURVE: Calculated spectrum using 10.4 and 4.4G splittings arising from one hydrogen each and a 4.4G splitting arising from one nitrogen ($\Delta H_{\text{m.s.}} = 0.9\text{G}$).

the hydrogen can be at the 7 or 9 position. Analysis of the spectrum in Figure 30 yields 10.4 and 4.4G splittings due to one proton each and a 4.4G nitrogen splitting. Owing to the poor resolution the two 4.4G splittings could differ by as much as 0.6G and still not be resolved. Two smaller splittings of approximately 1.8G (proton) and 1.2G (nitrogen) are inferred from analysis of the end portion of the spectrum. These splittings account for only 5 of the 9 possible. The remaining three splittings must be less in magnitude than the linewidth, 0.9G. A computer reconstruction using the above splittings is shown in Figure 30. The excellent agreement between the experimental and theoretical spectra suggests that the analysis is correct. Assignment of the splittings to positions on the purine molecule is not possible from the experimental results, since all positions on the purine molecule are inequivalent. The splittings have therefore been assigned where possible by recourse to McLachlan SCF MO theory (discussed below) as shown in Table IV.

6-Cyanopurine: The anion of 6-cyanopurine was prepared to aid in the analysis of the purine anion spectrum and to confirm the assignment by theory of the 10.4 gauss splitting to the 6 position. 6-cyanopurine was chosen since the cyano group has been shown to have a relatively small electron withdrawing effect,^{66,67} i.e., in electron withdrawing power $CN < COOCH_3 < COCH_3 < CHO < NO_2$. It is therefore reasonable to assume for the case of 6-cyanopurine anion that the cyano group will have only a modest effect on the spin density distribution.

The spectrum of 6-cyanopurine was observed from -50° to +25°C. Best resolution occurred at 25°C (Figure 31). Analysis of this spectrum is complicated due to the fact that all positions on the molecule are inequivalent. There are both nitrogen and hydrogen splittings, and there are a large number

Figure 31

UPPER CURVE: One-half of the experimental ESR spectrum of 6-cyanopurine anion at 25°C.

LOWER CURVE: Computer simulated spectrum using hyperfine parameters discussed in text.

of theoretical hyperfine lines. Interpretation of the spectrum proceeded by examination of the end portion of the spectrum. This yielded the splittings 0.18, 0.74, 0.99, 1.55 and 2.43G. The spectrum shows that the 0.18G splitting is obviously a proton splitting. The other splittings were all shown to be due to nitrogens by spectrum simulation. The remaining larger splittings were determined to be 4.12G (proton) and 3.80G (nitrogen) by an inspection of the central portion of the spectrum and many computer simulations. The reconstruction of the 6-cyanopurine spectrum with the above splittings is shown in Figure 6 and is in excellent agreement with experiment. The hyperfine splittings clearly show that the proton of 10.4G splitting in purine has been replaced by the cyano group in 6-cyanopurine. The remaining proton splitting unaccounted for in the analysis must be less in magnitude than the linewidth of 0.13G.

As in the case of purine 6-cyanopurine also has two possible tautomeric forms 9H or 7H. The clearly resolved spectrum for the anion shows that one of the species predominates. Molecular orbital calculations performed for both of these possible species (see below) are used to assign the hyperfine splittings to positions on the 6-cyanopurine structure where possible (see Table IV).

Spin Density Calculations

McLachlan SCF-MO calculations⁵⁸ were performed for the radical anions in this study to aid in the assignment of experimental splittings to molecular positions, to compare experimental and theoretically calculated splittings, and to possibly determine which of the tautomeric forms of the purine anions predominate. The heteroatom coulomb and resonance parameters used in these calculations are given in Table V. The parameters for the aromatic nitrogen are considered to be the most critical to the calculation.

Table V

M.O. PARAMETERS USED IN CALCULATIONS

<u>Group</u>	<u>Parameters</u>	<u>Ref.</u>
$\begin{array}{c} \diagup \text{N} \diagdown \\ \diagdown \end{array}$	$h_N = 0.8$ $k_{CN} = 1.08$	65
$\begin{array}{c} \text{H} \\ \\ \diagdown \text{N} \diagdown \\ \diagup \end{array}$	$h_N = 1.5$ $k_{CN} = 1.0$	59
$\begin{array}{c} \diagup \text{C}' \diagdown \\ \diagdown \end{array} - \text{C} \equiv \text{N}$	$h_N = 1.0$ $k_{C \equiv N} = 2.0$ $k_{C' - C} = 0.9$	69
$\begin{array}{c} \diagup \text{C} \diagdown \\ \diagdown \end{array} - \text{C}' \equiv \text{H}_3$	$h_{\text{H}_3} = 0.5$ $h_{C'} = -0.1$ $h_{C \equiv \text{H}_3} = 2.5$ $h_{C - C'} = 0.93$	72

The value of h_n was therefore varied between 0.4 and 1.0. The results are found to be relatively insensitive to the different values of h_n and no improvement in the overall agreement with experiment is found. The spin density calculations are reported in Table VI. The comparison of theory with experiment is best accomplished by calculation of theoretical values of the hyperfine splittings. This required relationships which relate spin density and isotopic hyperfine splitting. The relationships $A_H = -24.5\rho_C^\pi$, $A_N = 27.3\rho_N - 1.7(\rho_C + \rho'_C)$, $A_{CH_3}^H = 217 \rho_H^\pi$ and $A_N = 23.1\rho_N - 6.6\rho'_C$ for >C-H , $\text{C}\equiv\text{N}$, $\text{>C}-CH_3$, and $\text{>C}'-C\equiv N$ have been employed with good results in similar molecules and are used in the calculation of hyperfine splittings.^{65,65,68,69} These results are listed in Table VI.

The results found for pyrimidine anion are in reasonable agreement with experiment and have been discussed elsewhere.⁶⁵

The calculated splittings for 5-methylpyrimidine are nearly identical to those calculated for pyrimidine discounting the methyl proton splitting. In agreement with theory the experimental results for these two anions show only a small effect of the methyl group. Methyl groups do not usually result in large shifts in spin density but such a small change is unusual.⁷⁰ This can be understood from the form of the Hückel molecular orbital (HMO) occupied by the unpaired electron in pyrimidine. This MO has a node at position 5 where the methyl group is substituted and consequently the methyl group has no effect on the coefficients of this MO, but several of the MO's associated with other energy levels do not have a node at position 5 and thus are affected by the methyl group. Although the HMO spin densities are unaffected by the methyl group the McLachlan spin densities are altered slightly since they are calculated using atom-atom polarizabilities which depend on the coefficients of all the molecular orbitals.

Table VI

THEORETICAL SPIN DENSITIES AND HYPERFINE SPLITTINGS

Anion	Position	McLachlan Spin Density		Theoretical Hyperfine Splittings (gauss)		Experimental Hyperfine Splittings (gauss)
Pyrimidine	1	0.177		4.23		3.34
	2	-0.028		0.69		0.72
	4	0.375		9.20		9.54
	5	-0.078		1.91		1.24
5-Methyl- Pyrimidine	1	0.177		4.23		3.34
	2	-0.027		0.66		0.67
	4	0.377		9.24		9.26
	5	-0.073		-		-
	6	-0.000		-		-
	7	-0.0082		1.78		1.56
5,5'-dimethyl ^a - 2,2'-bipyrimidine	1	0.082		1.91		1.92
	2	0.210		-		-
	4	-0.013		0.32		< 0.10
	5	0.137		-		-
	6	-0.003		-		-
	7	0.0180		3.90		3.19
Purine		9H	7H	9H	7H	
	1	0.048	0.082	0.49	1.63	
	2	0.048	0.013	1.18	0.32	
	3	0.162	0.156	4.22	4.06	4.4
	4	0.070	0.112	-	-	-
	5	0.007	-0.034	-	-	-
	6	0.436	0.391	10.68	9.59	10.4
	7	0.017	0.022	0.07	0.19(N) 0.55(H)	-
	8	0.220	0.274	5.38	6.72	4.4
	9	-0.009	-0.016	0.64(N) 0.22(H)	1.09	
6-Cyanopurine		9H	7H	9H	7H	
	1	0.090	0.107	1.84	2.36	2.43
	2	0.011	-0.003	0.27	0.07	0.18 (< 0.15)
	3	0.152	0.153	3.99	4.00	3.80
	4	0.081	0.112	-	-	-
	5	0.030	-0.005	-	-	-
	6	0.353	0.329	-	-	-
	7	0.002	0.007	0.28	0.17(N) 0.18(H)	0.74 (0.99)(N) ^a
	8	0.172	0.217	4.22	5.31	4.12
	9	-0.010	-0.018	0.72(N) 0.25(H)	1.05	0.99 (0.74)(N) ^a
	10	0.032	0.027	-	-	-
	11	0.0872	0.0794	1.79	1.65	1.55

^a. The 0.18G splitting could also arise from this position.

The steric interaction of the pyrimidine groups of 5,5'-dimethyl - 2,2'-bipyrimidine is such that a nonplanar structure would be expected. For biphenyl a molecule with a similar molecular structure but perhaps more steric interaction from its ortho hydrogens, the dihedral angle between the phenyl groups has been estimated to be approximately 40° .⁷¹ For this reason theoretical splittings for this 5-methylpyrimidine dimer have been calculated as a function of the angle between the two aromatic planes. The angle of twist has been simulated in the calculation by a reduction in the resonance integral between the carbon atoms joining the two rings. The relation $k^* = k \cos \theta$ describes the reduction in the resonance parameter (k) with the angle of twist (θ) and has been both experimentally and theoretically justified.³⁹ Results of the calculations showed that the methyl proton splitting is nearly invariant with angle while the nitrogen splitting is more sensitive. Best results are found at $k = 0.8$, $\theta = 37^\circ$. The results of this calculation are given in Table VI. The uncertainty in the value of θ must be considered large (Ca. 15°) due to the only moderate change in splitting with angle and the uncertainty in the calculation of the nitrogen hyperfine splitting constant. The results clearly suggest that the anion is nonplanar. In addition the good agreement between the calculated and experimental splittings is further evidence for the structure of this molecule and consequently the dimerization reaction.

The molecular orbital calculations for purine and 6-cyanopurine listed in Table VI are especially important since they must be used to assign hyperfine splittings to molecular positions. Further they may aid in the assignment of the anions to specific tautomers.

For purine the calculated hyperfine splittings for both the 9H and 7H tautomers given in Table VI show that positions 3, 6 and 8 are the positions of large splitting in agreement with experiment. The calculation with the

hydrogen at position 9 is in much better agreement with the individual magnitudes of the larger splittings. This suggests that the 9H tautomer predominates in the spectrum of purine anion. A second indication of which tautomer may be the more stable is the total HMO π -electron energy of the anions. For this case the energies are virtually identical suggesting no one species greatly predominates over the other.

The calculated hyperfine splittings for the 6-cyanopurine 7H and 9H tautomers are quite similar. The agreement between the calculated results and experiment for the two larger splittings is better for the 9H tautomer, but from the overall agreement it isn't possible to differentiate between the two tautomers. The total π -electron energy indicates that the 9H tautomer is favored by 0.5 Kcal ($\beta = 16$ Kcal). The splittings have been assigned to positions on the 6-cyanopurine structure based on the agreement of both calculations where possible.

The results for the purine and 6-cyanopurine anions suggest that the 9H tautomers are favored in both anions. However, for purine anion, the experimental spectra as well as the calculation of the total π energy suggests that there may be a significant concentration of the 7H tautomer.

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V. PUBLICATIONS AND PRESENTATIONS

A. The following papers have been published

1. D. E. Holmes, R. B. Ingalls and L. S. Myers, Jr., "An ESR Study of Free Radicals Formed by Reaction of Nucleotides and Their Constituents with Hydrogen Atoms," International J. Rad. Biol., 12, 415 (1967).
2. D. E. Holmes, R. B. Ingalls and L. S. Myers, Jr., "ESR Spectra of Free Radicals Induced in Pyrimidines, Purines and Related Analogs by Exposure to Hydrogen Atoms," International J. Rad. Biol., 13, 225 (1968).
3. R. A. Holroyd and J. W. Glass, "Radicals Formed by Electron Transfer to Pyrimidine Derivatives," International J. Rad. Biol., 14, 445 (1968).

B. The following paper has been accepted for publication

1. R. A. Holroyd and J. W. Glass, "The Reaction of Hydrogen Atoms with DNA," Radiation Research.

C. The following paper has been submitted for publication

1. M. D. Sevilla, "An Electron Spin Resonance Study of the Acetate Dianion and Acetamide Anion."

D. The following presentations were made

1. R. B. Ingalls, L. S. Myers, Jr., D. E. Holmes and J. W. Glass, "An ESR Study of Reactions of 1,3-dideutero-uracil and 1,3-dideutero-5-bromouracil with Hydrogen Atoms," at 15th Annual Meeting of Radiation Research Society, San Juan, Puerto Rico.

2. R. A. Holroyd and J. W. Glass, "Reaction of Hydrogen Atoms with DNA and Nucleoproteins," at the 16th Annual Meeting of Radiation Research Society, Houston, Texas, April (1968).
3. R. A. Holroyd, "Radiation Chemistry of Chromosomes," at Univ. of Calif. at Los Angeles, Feb. 25, 1969.
4. R. A. Holroyd, "Radiation Chemistry of Chromosomes," at University of Oklahoma, April 11, 1969.
5. M. D. Sevilla, "The Reactions of Electrons with Amino Acids and Di-peptides," at 158th Meeting of ACS, Sept., 1969.

An E.S.R. study of free radicals formed by reaction of nucleotides and their constituents with hydrogen atoms†

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Cytosine, pentoses, pentose phosphates, nucleosides, and nucleotides were suspended on quartz wool by freeze-drying and exposed to hydrogen atoms at room temperature inside an E.S.R. cavity. Stable free radicals were observed in every case. Spectra of sugar radicals do not change shape during the course of the reaction. Those of cytosine radicals show a loss of resolution as the exposure progresses, suggesting that side reactions involving the amino group occur. The spectra of nucleoside and nucleotide radicals change in shape as the exposure progresses. The initial spectra resemble those of the constituent bases, whereas late spectra contain a large component of a sugar radical spectrum. Comparison of spectra induced by hydrogen atoms with those induced by deuterium atoms suggests that atoms add to the bases, abstract from the sugars, and bring about both types of reactions with the nucleosides and nucleotides. The nucleosides and nucleotides appear to form hydrogen atom addition products on the base moiety initially; and on further exposure to give sugar radicals by a process which depends in part on the presence of base radicals. Thus hydrogen atom attack on bases is in effect transferred to the sugar. This process may be important in bringing about damage to the polymer backbone when nucleic acids are exposed to ionizing radiation.

1. Introduction

Thymidine exposed to gamma-rays yields an electron spin resonance (E.S.R.) spectrum similar to that of irradiated thymine (Shields and Gordy 1959). This spectrum is believed to result in large part from a radical in which a hydrogen atom is added to the thymine part of the molecule (Pershan, Shulman, Wyluda and Eisenger 1964, Pruden, Snipes and Gordy 1965). Evidence tending to substantiate this conclusion has been obtained in studies of the reactions of hydrogen atoms with thymine (Herak and Gordy 1965, Heller and Cole 1965, Holmes, Ingalls and Myers 1966, Holmes, Myers and Ingalls 1966) and thymidine (Holmes, Ingalls and Myers 1966, Holmes, Myers and Ingalls 1966). In both cases spectra similar to that of irradiated thymine have been obtained. Irradiated uridine and

† Supported by the Division of Biology and Medicine of the U.S.A.E.C.

cytidine, on the other hand, yield E.S.R. spectra which differ from those of the corresponding irradiated bases (Shields and Gordy 1959, Sheng, Blumenfeld, Kalamanson, and Pasynskii 1959). These latter spectra have not been fully explained, but there is reason to believe that they may be due to radicals formed by addition or abstraction of hydrogen atoms. Investigation of the reactions of hydrogen atoms may therefore contribute directly to an understanding of radiation effects on nucleic acids. We have therefore undertaken a general study of the reactions of hydrogen atoms with nucleic acids and their constituents, which complements previous analogous studies of the reactions of $\text{OH}\cdot$ free radical in solution (Myers, Ward, Tsukamoto and Holmes 1965).

In this paper we report on work dealing with nucleosides and nucleotides. Brief preliminary reports have been published (Holmes, Myers and Ingalls 1965 a, b, c).

While this paper was in preparation a paper was published by Herak and Gordy (1966 b) on a similar subject. Our experimental procedures were different from theirs, and this has led to two important differences in results. With our procedures:

(1) All the usual bases, nucleosides, and nucleotides are observed to form free radicals on exposure to atoms of either hydrogen or deuterium at room temperature, and

(2) Spectral changes can be observed as the reactions with the nucleosides and nucleotides proceed, and these changes demonstrate that more than one radical is formed in each of these reactions and that the proportion of the various radicals changes with the extent of reaction.

2. Experimental

The experiments have been described previously (Holmes, Myers and Ingalls 1966). Commercially-available materials were used without further purification. Samples from different manufacturers gave identical results in several instances. Samples were suspended on quartz wool by freeze-drying a rapidly frozen aqueous solution in a tube packed with the wool and were placed in an E.S.R. cavity, where they were exposed at room temperature to hydrogen atoms produced by a microwave discharge in hydrogen gas. Spectra were recorded at intervals during the exposure, beginning with times as short as one-tenth of a minute. The power level to the cavity was usually 0.35 mW.

3. Results

Some important generalizations can be made about the rate of accumulation of radicals and the steady-state signal levels in various substrates during exposure to H or D atoms. All the reactions studied appear to occur in three fairly distinct stages. At first there is a very rapid accumulation of radicals, which we call 'the explosion', characteristically only a few seconds long. The explosion is followed by a period of almost linear growth which begins to level off into a steady state within a few minutes. The time spent in each of the stages appears to be a function of the structure of the substrate. Thus the pentoses and pentose phosphates react slowly and never yield high radical concentrations. Both the rates of reaction and maximum signal intensity show considerable variation from run to run, suggesting some complicating factor such as water adsorption. The bases, on the other hand,

yield high radical concentrations quickly and reproducibly. Nucleosides and nucleotides at the beginning of the exposure yield radicals with a spectrum similar to the base radicals, but at a rate somewhat slower than shown by pure bases. On continued exposure to the atoms, spectra similar to those shown by the pentoses dominate. These 'sugar' spectra are stronger than those observed in the reactions of the pure sugars.

On termination of the hydrogen atom flow, the E.S.R. signal usually undergoes a slight decrease in intensity, but then remains constant for days or weeks if the sample is not exposed to air.

3.1. Bases

Figure 1 shows early (scanning time one-fourth of a minute) and late spectra of the cytosine hydrogen atom reaction product formed at room temperature. The

Figure 1. Early (A) and late (B) E.S.R. spectra of cytosine during exposure to hydrogen atoms.

early spectrum consists of six lines with splitting of $\text{CH} = 18$ and $\text{CH}_2 = 32$ gauss. On continued exposure the lines appear to broaden with consequent loss in resolution and overlap of the two central lines. Exposure to deuterium atoms gives a different spectrum with fewer lines. Corresponding spectra of thymine radicals obtained by our technique have been published (Holmes, Myers and Ingalls 1966); spectra of other base radicals will be discussed elsewhere. However, none of the other bases investigated, except 5-methyl cytosine and dimethyluracil, show changes in spectral shape on continued exposure.

3.2. *Pentoses and ribose phosphate*

Spectra of ribose, deoxyribose, and ribose phosphate after exposure to hydrogen atoms are given in figures 2 and 3. They show obvious differences from one another, but each contains a pair of lines separated by approximately 50 gauss. Exposure to deuterium atoms results in radicals with the same spectra as those formed by hydrogen atoms. The signal intensities are much lower than those obtained with the bases, and consequently early spectra were difficult to obtain and the spectra in the figures are steady state spectra. These appear to be the same as early spectra so far as we could determine.

Figure 2. Steady state E.S.R. spectra of deoxyribose after exposure to (A) hydrogen atoms, (B) deuterium atoms.

3.3. *Nucleosides*

Figure 4 shows the early spectrum of deoxycytidine after exposure to hydrogen atoms at room temperature. It is similar to the cytosine spectrum, but shows less resolution in the central region. On further exposure the central lines gradually merge to give a spectrum which appears to contain a central singlet plus a residual contribution from the original highly resolved spectrum.

Figure 5 shows the spectrum of uridine at several times after beginning hydrogen atom exposure, and after admitting air. The first observable spectrum is almost identical with the spectrum of uracil (to be published in a later paper). It has six lines with coupling constants $CH = 19$ and $CH_2 = 33$, in excellent agreement with the values for cytosine and the values of Herak and Gordy (1965) for uracil

Figure 3. Steady state E.S.R. spectra of (A) ribose, and (B) ribose phosphate after exposure to hydrogen atoms.

Figure 4. E.S.R. spectrum of deoxycytidine during exposure to hydrogen atoms and before the centre lines completely merge into a single line.

Figure 5. E.S.R. spectra of uridine during exposure to hydrogen atoms, (A) early spectrum, (B) 2 min after the early spectrum, (C) late spectrum, (D) spectrum of the sample after atom exposure and air has been admitted.

powders exposed at 300°K. Within 2 min the spectrum loses much of its structure, and ultimately it takes on the appearance of a broad absorption peak with several unresolved shoulders. The general shape is similar to that of ribose phosphate except for a small amount of additional hyperfine structure. Thymidine (Holmes, Myers and Ingalls 1966) and deoxycytidine behave in an analogous way, i.e. the initial spectrum observed during reaction with hydrogen atoms is well resolved and distinct from the initial deuterium atom induced spectrum, but continued exposure to either hydrogen or deuterium atoms yields spectra with a single broad unresolved peak.

The purine nucleosides, deoxyadenosine (figure 6) and deoxyguanosine, behave in a manner similar to the pyrimidine nucleosides, but do not yield as distinctive spectra because they have less resolved hyperfine structure.

In these reactions, the steady state level of the well resolved portions of the spectra are much lower than in the spectra of the free bases, which they resemble. The rate of growth of the broad unresolved peak is greater than in the pentoses or pentose phosphate. After a steady state is reached, the intensity of the broad peak appears to be considerably greater than that of the well resolved portions. There is considerable variation from experiment to experiment in the overall signal intensity of the nucleoside radicals, possibly because of variable amounts of adsorbed water in the samples.

3.4. Nucleotides

The nucleotides under hydrogen atom bombardment give spectra and spectral changes with bombardment time which are almost identical to those given by the corresponding nucleosides, but the steady state signal level is lower. Thymidylic acid (thymidine-5' phosphoric acid), for example, gives spectra which closely resemble those of thymidine except that there is less contribution from the central peak. This peak saturates at a higher power level than the rest of the spectrum, resembling the saturation behaviour of the deoxyribose rather than the pyrimidine radical. Uridine-3'-(2')-phosphoric acid and uridine-5'-phosphoric acid likewise resemble uridine, except that with the latter the central peak again contributes

Figure 6. E.S.R. spectra of deoxyadenosine (A) and purine (B) after hydrogen atom exposure.

slightly less to the steady state spectrum than with the nucleoside. Cytidine-5'-phosphoric acid and deoxycytidine-5'-phosphoric acid on bombardment with hydrogen atoms give spectra which show no differences from each other and which resemble those obtained in the exposure of cytidine. Adenosine-5'-monophosphate, adenosine-5'-diphosphate (ADP), and adenosine-5'-triphosphate (ATP) give almost the same spectra with hydrogen atoms as the purine nucleosides, but there is slightly less resolution of the outer peaks and a smaller steady state radical signal level. The second and third phosphate have essentially no effect. The polynucleotides likewise give spectra which resemble those obtained from the corresponding nucleosides and nucleotides, but the contribution of the central peak to the steady state signal is much greater.

4. Discussion

4.1. Bases

With our experimental techniques a spectrum was readily obtained at room temperature upon exposure of cytosine to hydrogen atoms. This is in contrast to the results of Herak and Gordy (1965) who were unable to obtain spectra with cytosine powders at room temperature. The difference is probably due to differences in sample configuration. The freeze-dried samples which we used have a large specific surface area and an open structure and thus make a relatively

large fraction of the sample material available for reaction. This is an important factor in determining the sensitivity of the method since hydrogen atoms diffuse only a short distance from the surface before reacting (Wall and Ingalls 1964).

The fact that the reaction is carried out within the E.S.R. cavity enabled us to observe spectral changes as the reaction proceeds. Cytosine spectra change more than those of any of the other bases, changing within a minute or two from a well resolved six-line spectrum to a spectrum very similar to that reported by Herak and Gordy (1966 a) for exposure at 77°K. Dimethyluracil and 5-methyl cytosine spectra undergo similar changes. These changes may be due to side reactions with the amino or N-methyl groups.

Exposure of cytosine to deuterium atoms gives spectra which are different from those given by hydrogen atoms, indicating that the spectra are due to addition of atoms to the base. This result is in agreement with previously reported findings with other purine and pyrimidine bases.

4.2. *Pentoses*

In contrast to the bases the spectra observed during hydrogen atom and deuterium atom reactions with pentoses are almost identical, suggesting that abstraction is the dominant reaction. These abstraction reactions appear to be slower than additions to the bases since the initial rate of radical formation is smaller and the steady state concentration is lower. This is consistent with the higher activation energies for abstraction reactions than for addition reactions which are expected on theoretical grounds (Semenov 1958). The kinetics of these reactions will be considered further in a later section.

4.3. *Nucleosides and nucleotides*

Initially, the reactions of hydrogen atoms with nucleosides and nucleotides produce radicals with E.S.R. spectra which differ from the spectra observed during reaction of these compounds with deuterium atoms. These 'first observed' spectra are almost identical with the spectra of the corresponding bases. Thus the radicals formed during the early stages of exposure in every case are clearly the base-hydrogen atom addition product. These radicals are stable on standing in vacuum, and resemble those observed after low temperature hydrogen atom bombardment of powders (Herak and Gordy 1966 b) except for differences due to temperature effects. On continued exposure of nucleosides and nucleotides at room temperature the spectrum undergoes a qualitative change. A signal from a radical with a single broad absorption grows into the spectrum rapidly while the signal from the base radical increases slowly or remains constant in intensity. The following evidence suggests that the single broad absorption is due to a pentose radical:

(1) The general shapes of the late spectra are similar to that of a pentose spectrum with an additional small amount of hyperfine structure from a base radical. Also, the additional lines observed in the late deoxyadenosine spectrum (figure 6) which are not present in the purine spectrum may be attributable to the pentose moiety.

(2) No significant changes in spectral shape were observed on exposure of bases alone over periods much longer than those required for the change in nucleoside or nucleotide spectra. Cytosine (figure 1) did show changes, but these are

not sufficient to account for the changes in nucleoside spectra, and they are completed early in the exposure, while changes in the sugar containing compounds proceed over a greater time period.

(3) The saturation characteristics of the central region of the late spectrum of the thymidylic acid radical resemble those of the deoxypentose radical (power saturation of the other nucleoside radical spectra was not investigated).

(4) The late spectrum, once formed, remains constant in shape and intensity for many minutes of exposure, a behaviour consistent with the growth of a specific radical to a steady state.

(5) Deuterium atom exposure of nucleosides results in spectra which lack most of the hyperfine structure, while the central region is the same as with hydrogen atom exposure, i.e. as the deuterium atom induced pentose spectrum.

These observations are strong support for the hypothesis that continued exposure of nucleosides and nucleotides to hydrogen or deuterium atoms results in the build-up of a large steady state concentration of pentose radicals.

4.4. Reaction mechanisms and kinetics

It has already been established with some certainty that the atoms add to bases and abstract from the sugars. It is also apparent that the atoms react with both base and sugar moieties of the nucleotides and nucleosides. However, it is not clear why the sugar spectrum is dominant after the prolonged exposure of nucleosides and nucleotides to the atoms. Consider the following:

Assuming these to be the only important reactions, it is easy to show that there is a tendency for the concentration of any radical in the system to approach asymptotically a value of $(k_1/k_2)M$ where (M) is the concentration of reactant (base or sugar moiety in this case) and k_1 and k_2 are rate constants for the production and destruction of free radicals by reaction with hydrogen atoms (Wall and Ingalls 1964). If $k_{1\text{B}} > k_{1\text{S}}$ then the base radical will appear first; but if $k_{2\text{B}} \gg k_{2\text{S}}$ then at long reaction times the sugar radical will dominate. However, one would expect $k_{2\text{B}} \approx k_{2\text{S}}$ since both reactions are atom-radical reactions and are probably diffusion limited. $k_{1\text{B}}$ is also high (Scholes and Simic 1964), probably of the order $10^9 \text{ M}^{-1} \text{ sec}^{-1}$, but abstraction of a hydrogen atom from the sugar is probably comparatively slow, of the order of $k_{1\text{S}} \approx 10^7 \text{ M}^{-1} \text{ sec}^{-1}$. Therefore, it is not likely that the appearance of the base radical, followed by the dominance of the sugar radical can be explained by these four equations. It is also important to point out that all these reactions are probably diffusion controlled in the sense that the half depth of penetration of the atoms into the solid samples (away from the exposed surface) is small (Ingalls and Hardy 1960), certainly less than 10^{-5} cm for

the highly reactive bases, nucleosides, nucleotides, and but a little more for the more slowly reacting sugars. This is because on the average the atoms react before they can diffuse very far into the solids. Where both base and sugar are present the ratio of products formed should reflect the reactivity of the reactants in the system because diffusion into the sample merely limits the number of hydrogen atoms available for reaction at a given depth, not the ratio of probabilities of reaction with one or the other of the two components. This point of view has been found to be applicable to a chemically analogous hydrocarbon system: an equal molar mixture of polymethylmethacrylate and polystyrene, (Tiedeman, C. T., and Ingalls, R. B., unpublished results). Upon exposure of this model system to hydrogen atoms one observes exclusively the radical formed by addition of hydrogen atoms to polystyrene rather than the radical formed by abstraction from polymethylmethacrylate. It is therefore difficult to see why the sugar radical should dominate when the base is present in comparable concentration unless some reaction other than those of (1) to (4) occurs.

This additional reaction must be one which is proportional to the base radical concentration since it does not occur in the early stages of the exposure, and which results in conversion of a base radical to a pentose radical. A further requirement is that the reaction must not proceed at an observable rate in the absence of atom bombardment, since the spectra do not change significantly on standing at room temperature.

It is possible that the heat of reaction warms the sample enough to promote abstraction from the sugar moiety by the base radical. This reaction would probably occur with about 10 kcal activation energy (or less) (Semenov 1958) and would therefore occur long before any 'charring'. (We have never observed 'charring' in any of our experiments in which the sample was supported on quartz wool, although it can be induced in unsupported polymer fluffs by high atom fluxes.)

It does not seem likely that hydrogenation of the base is the explanation of these observations since this would not lead directly to the enhancement of the sugar radical.

Another possible type of explanation of the appearance of the sugar radical during the reactions of nucleosides and nucleotides is some type of energy migration. Such a process has been suggested by Singh and Charlesby (1964) to explain their results with adenine compounds irradiated with ^{60}Co γ -rays at 77 K: the E.S.R. spectrum changes from an adenine radical at 77 K to a sugar radical when the temperature is raised to 295 K. Additional experimental evidence that some kind of radical or excitation energy migration may take place in nucleosides and nucleotides under certain conditions may be found in a quantitative E.S.R. study of irradiated compounds carried out by Müller (1964). Paradoxically, transfer in the opposite direction, from the pentose to the base, was required to explain high yields of base radical in irradiated nucleotides.

Whatever their explanation, the phenomena which we have observed during bombardment of the nucleosides and nucleotides with hydrogen atoms, almost certainly occur during radiolysis. It is difficult, of course, to determine their relative importance as compared with other radiation induced processes. It is not impossible, however, that the biologically significant damage to nucleic acids occurs in part by $\text{H}\cdot$ atom addition to the base followed by transfer of this chemical activation to the sugar moiety with resulting damage to the nucleic acid backbone.

5. Summary

All the usual purine and pyrimidine bases, nucleosides, and nucleotides, if present as rapidly frozen freeze-dried materials suspended on silica wool, will form stable free radicals when exposed to hydrogen atoms. The free bases react by addition of a hydrogen atom. The nucleosides and nucleotides react first to form hydrogen atom addition products on the base moiety; on further exposure the signal from the base radical becomes constant, and a signal believed to be a pentose radical formed by hydrogen atom abstraction becomes predominant. The results suggest that base radicals in nucleosides or nucleotides are converted to pentose radicals during the exposure period.

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Cytosine, pentoses, phosphates de pentose, nucléosides, et nucleotides ont été suspendus sur de la laine de quartz par lyophilisation et exposés à des atomes d'hydrogène à température ambiante tandis qu'ils étaient à l'intérieur de la cavité d'un E.S.R. Des radicaux libres stables ont été observés dans chaque cas. Les spectres de radicaux glucidiques ne changent pas de forme pendant la réaction. Ceux des radicaux de cytosine montrent une perte de résolution au cours de l'exposition, indiquant que des réactions secondaires se produisent au niveau du groupement amine. Les spectres des radicaux de nucléosides et de nucléotides changent de forme au cours de l'exposition, allant de certains ressemblant à ceux des bases constituantes, à d'autres qui semblent contenir un composant important d'un spectre glucidique. La comparaison de spectres induits par des atomes d'hydrogène à ceux de deutérium, indique que des atomes s'ajoutent aux bases, sont soustraits des glucides, et causent ces deux types de réaction avec les nucléosides et les nucleotides. Les nucléosides et les nucleotides semblent former initialement des produits d'addition hydrogénés sur les composants basiques, et avec plus d'exposition paraissent donner des radicaux glucidiques par un processus qui dépend en partie de la présence de radicaux basiques. Ainsi l'attaque des atomes d'hydrogène sur les bases est un effet transféré au glucide. Ce processus est peut-être une cause importante du dommage infligé à la structure polymère quand les acides nucléiques sont exposés aux radiations ionisantes.

Cytosin, Pentosen, Pentosephosphate, Nucleoside and Nucleotide wurden mittels Gefrier-trocknung auf Glaswolle aufgetragen und in einer E.S.R.-Kammer bei Raumtemperatur Wasserstoffatomen ausgesetzt. In jedem der Versuche wurden stabile freie Radikale beobachtet. Während Spektren von Zuckerradikalen ihre Gestalt im Verlauf der Reaktion nicht ändern, zeigen solche von Cytosinradikalen eine Abschwächung der optischen Auflösung während fortschreitender Behandlung mit Wasserstoff, was möglicherweise Nebenreaktionen der Aminogruppe andeutet. Die Spektren von Nucleosid- und Nucleotidradikalen verändern während der Behandlung ihre Form von jenen der Basenanteile zu solchen, welche einen erheblichen Bestandteil eines Zuckerradikals zu enthalten scheinen. Vergleiche von mit Wasserstoff- und Deuteriumatomen induzierten Spektren deuten an, daß die Atome sich an die Basen anlagern und sich von den Zuckern ablosen und mit Nucleosiden und Nucleotiden beide dieser Reaktionen eingehen. Die Nucleoside und Nucleotide scheinen zunächst Additionsprodukte der Wasserstoffatome mit den Basenanteilen zu bilden, während fortgesetzte Behandlung Zuckerradikale schafft, ein Vorgang, der zum Teil von der Gegenwart von Basenradikalen abhängt. Dadurch wird effektiv der Angriff der Wasserstoffatome von der Base auf den Zucker übertragen. Wegen der dadurch verursachten Schädigung des Polymeren-Rückgrates konnte dieser Vorgang dort von Wichtigkeit sein, wo Nucleinsäuren ionisierender Bestrahlung ausgesetzt werden.

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E.S.R. spectra of free radicals induced in nucleic acid purine and pyrimidine bases and selected analogues by exposure to hydrogen atoms†

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Purine and pyrimidine bases, selected to permit determination of effects of structure, were exposed to hydrogen or deuterium atoms within an E.S.R. cavity. Spectra were recorded at intervals during the exposure. Conclusions about pyrimidines are: atoms add to all pyrimidines tested, addition is to the 5-6 double bond of 1,3-dideuterouracil; methyl groups on carbons 5 or 6 exert a dominant directive effect on addition; dihydropyrimidinyl radicals appear to have a near planar conformation at room temperature, but 1,3-dimethyldihydropyrimidinyl radicals are non-planar, steric blocking of H atom addition appears to be unimportant, and oxo and amino groups on carbon 4 have slightly different effects on the electronic structure of the radicals. Reaction of H atoms with 5,6-dihydropyrimidines gives the same radicals by abstraction as are formed by addition to the corresponding pyrimidine. All purines tested give radicals with nearly identical electronic structures.

1. Introduction

Pyrimidine and purine bases and their derivatives are useful compounds for the investigation of some of the hydrogen atom chemistry involved in the irradiation of biological systems. They are a known site of attack by both ionizing radiation (Salovey, Shulman and Walsh 1963, Ehrenberg, Ehrenberg and Lofroth 1963) and hydrogen atoms (Herak and Gordy 1966) on nucleic acids, and several investigators have proposed, on the basis of electron spin resonance (E.S.R.) studies, that radicals with a hydrogen atom added to the 5-6 double bond of the pyrimidine ring (Pershan, Shulman, Wyluda and Eisinger 1964, Pruden, Snipes and Gordy 1965, Heller and Cole 1965) are formed during irradiation.

In previous papers (Holmes, Myers and Ingalls 1966, Holmes, Ingalls and Myers 1967), we have shown that E.S.R. spectra similar to those observed in irradiation studies are obtained by exposing freeze-dried thymine and cytosine to

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thermal hydrogen atoms within the E.S.R. cavity. We have also reported on the reactions of hydrogen atoms with several nucleosides and nucleotides and have shown that the radicals formed in the first stage of the reactions are the radicals formed by addition of hydrogen atoms to the bases, and that on continued exposure the radicals change to give spectra resembling the spectrum obtained by exposure of the respective pentose atoms.

The E.S.R. spectra of pyrimidine and purine powders which had been exposed to hydrogen atoms prior to examination have been reported by other authors (Heller and Cole 1965, Herak and Gordy 1965, 1966).

In this paper we report a study of the reactions of hydrogen atoms with the nucleic acid bases and selected analogues, with emphasis on the effects of chemical structure on the mode of reaction.

2. Experimental

The techniques and equipment have been described in detail elsewhere (Holmes *et al.* 1966). Commercially available compounds were freeze-dried on quartz wool for support and exposed to hydrogen atoms within an E.S.R. cavity. This technique allows observation of the formation of free radicals during the reaction. Thus the rate of free-radical accumulation, as well as structural changes which may occur during the reaction, may be determined.

1,3-dideuterouracil was prepared by exchange in D_2O solution for 16 hours at $60^\circ C$.

3. Results

Freeze-dried purine and pyrimidine bases were found to produce free radicals immediately on exposure to hydrogen atoms. These radicals are observable within the time required to obtain an E.S.R. spectrum ($\frac{1}{4}$ min), but continue to accumulate until a steady state is reached in about 5 min. The shapes of the spectra do not change significantly during continued exposure under our experimental conditions, except for relatively minor changes in cytosine, 5-methyl-cytosine, and 1,3-dimethyluracil. The changes in the cytosine spectra have been discussed (Holmes *et al.* 1967) and may be due to side reactions with the amino or N-methyl groups. Once formed, the radicals are stable for several days in vacuum or in hydrogen gas. On admission of dry air or oxygen, the signal intensity decreases, and the spectrum changes shape; in moist air, the signal decay is enhanced.

For all the bases the first spectra observed during deuterium atom exposure are different from the first observed hydrogen atom spectra. The deuterium spectra show less hyperfine structure and less overall width. All purines investigated yield spectra similar to that of purine itself, regardless of the substituents on carbons 2 and 4.

Splittings and the number of lines of the radical spectra are listed in the table. Previously unreported spectra are presented in figures 1-6.

4. Discussion

The family of compounds investigated was selected so as to permit determination of structural effects on the reactions of uracil and some of its derivatives with hydrogen atoms. Among the subjects considered are: (1) the site of hydrogen atom attack on uracil; (2) the type of reaction, i.e. whether addition or abstraction; (3) the influence of substituents on the type of site of attack on uracil derivatives;

(4) the conformation of some of the radicals; and (5) the relative stability of radicals formed.

Herak and Gordy (1965) assigned the spectrum of the hydrogen atom-uracil reaction product to the free radical formed by addition of the hydrogen atom to the 5-6 double bond of uracil. We have confirmed this experimentally by examining 1,3-dideuterouracil. This compound gives a spectrum identical to that given by uracil, proving that the observed hyperfine splittings are not due to the exchangeable hydrogen atoms, and therefore that the addition must occur at the 5-6 double bond. Differences observed in hydrogen and deuterium atom induced spectra confirm that addition occurs, rather than abstraction.

Figure 1. E.S.R. spectrum of radicals formed during exposure of 6-methyluracil to hydrogen atoms.

Pyrimidines with methyl substituents on either the 5 or 6 positions produce somewhat more complicated spectra on reaction with hydrogen atoms. Freeze-dried 5-methyluracil (thymine) reacts with hydrogen atoms to produce free radicals with an eight-line E.S.R. spectrum similar to the spectrum observed by Herak and Gordy (1965) after hydrogen atom bombardment of thymine powder. This spectrum was assigned by Herak and Gordy to the 5-methyl-5,6-dihydrouracil-5-yl radical (I). (Previous papers have given common names to several of the free

radicals under discussion. In this paper we have attempted to follow a systematic nomenclature (Christiansen 1960) using, however, the accepted trivial names for the parent compounds.) We observed a similar eight-line spectrum with 6-methyluracil (figure 1). This suggests interaction of the unpaired electron with

the same number of hydrogen atoms in approximately the same relative conformation as in the radical formed from thymine, and leads to the conclusion that the radical is the 6-methyl-5,6-dihydrouracil-6-yl radical (II). The shift in the position at which a H atom adds with a change in the position of the methyl group indicates that the methyl group exerts a directive effect which exceeds other directive effects in the molecule. The slight differences in splittings shown by the 5- and 6-methyldihydrouracil radicals are probably due to differences in the electronegativity between the N-H and C=O groups.

Figure 2. E.S.R. spectrum of radicals formed very early during exposure of dimethyluracil to hydrogen atoms.

Substitution of methyl groups on the nitrogen atoms of the ring leads to formation of a radical which gives a five-line spectrum (figure 2). This spectrum can be constructed from an overlapping doublet of triplets with an intensity distribution of 1:2:2:2:1, and is presumably due to the 1,3-dimethyl-5,6-dihydrouracil radical ((III) or (IV)). The splittings (table) are grossly different from those obtained for the analogous 5,6-dihydrouracil radical or for any of the other compounds studied at room temperature, which have unsubstituted ring nitrogen atoms. They are similar, however, to those observed by Herak and Gordy (1966) for the dihydrouracil radical at 77°K (doublet splitting 46 gauss, triplet 19.5).

If we accept the proposed primary structures of the radicals, it appears likely that the different splittings arise from different conformations of the otherwise nearly identical radicals.

The uracil molecule in crystals at room temperature is planar (Parry 1954) and in the lactam (keto) form (Mason 1957, Short and Thompson 1952). The

planarity can be considered to arise from resonance structures which have double bonds in the rings. These resonance structures are analogous to the structures which account for the planarity of amides. The uracil molecules are connected into sheets by hydrogen bonds between the N and O atoms of adjacent molecules. X-ray crystal studies have apparently not been made on dimethyluracil, but its crystal structure must differ from that of uracil in one important respect: hydrogen bonding between molecules is impossible because of the methyl substituents on the ring N atoms. Consequently forces between dimethyluracil molecules are much weaker than between uracil molecules, as evidenced, for example, by the greater solubility and lower melting point of dimethyluracil.

Compound	Number of lines observed	Doublet splittings (gauss)	Triplet splittings (between lines)	Quartet splittings
Uracil	6	19	33	—
1,3-dideuterouracil	6	19	33	—
5-methyluracil (thymine)	8	—	38	20
6-methyluracil	8	—	35	18
1,3-dimethyluracil	5	45	20	—
5,6-dihydrouracil	6	20	33	—
5-methyl-5,6-dihydrouracil	8	—	38	19
6-methyl-5,6-dihydrouracil	8	—	35	18
6-carboxyuracil (orotic acid)	4	18, 34	—	—
Cytosine	6	18	32	—
5-methylcytosine	8	—	37	18
Purine	3	—	39	—
Adenine	3	—	38	—
Guanine	3	—	38	—
Xanthine	3	—	39	—

Characteristics of hydrogen atom adduct radical spectra.

Addition of a hydrogen atom to either the 5 or 6 position of uracil or 1,3-dimethyluracil has several effects which would tend to change the conformation. The 5,6-carbon-carbon 'double' bond would become more nearly a single bond and would tend to become longer, the saturated carbon atom would tend to become tetrahedral, and the radical centre would be relatively flexible. Pruden *et al.* (1965) have presented evidence that the triplet splitting in the dihydrothymidyl radical, and by analogy, in the dihydrouracil radical, is due to the nearly equal interaction of the unpaired electron with the two hydrogen atoms on the saturated carbon atom. This suggests that in uracil crystals at room temperature, the intermolecular forces and geometry prevent any appreciable change in conformation when the $\text{H}\cdot$ adduct radical is formed, i.e. the room temperature dihydrouracil radical, like uracil and the dihydrothymidyl radical, is planar, or nearly so. The intermolecular forces in 1,3-dimethyluracil crystal are much weaker, and evidently permit changes in conformation, so that one of the hydrogen atoms on the saturated carbon interacts strongly with the unpaired electron. Several possibilities for bringing this about suggest themselves, among them, relative rotation of a planar $\text{C}(6)\text{-N}(1)\text{-C}(2)\text{-N}(3)$ group and a planar $\text{C}(2)\text{-N}(3)\text{-C}(4)\text{-C}(5)$ group about an axis coincident with the $\text{C}(2)\text{-N}(3)$ bond so as to lengthen the $\text{C}(5)\text{-C}(6)$ bond

distance and decrease the N(2)-C(6)-C(5) bond angle (addition of H $\cdot$ to C(6) assumed), or less likely, a change in conformation of N(3) from planar to pyramidal.

The change in conformation of uracil which occurs as the temperature is lowered may be caused by a solid-phase transition.

Fessenden and Schuler (1964) have investigated the importance of conformation on the E.S.R. spectrum of cyclohexyl radical. A conformational difference is consistent with the suggestion of a different physical structure advanced by Herak and Gordy to explain the effect of temperature on the uracil-hydrogen atom adduct radical spectra. A comparison of the room temperature splittings with those obtained on addition of a hydrogen atom to the aromatic ring of polystyrene (Wall and Ingalls 1964) (triplet with splittings of 45 gauss between lines) shows that the room temperature dihydrouracilyl radical is by no means completely aromatic.

Figure 3. E.S.R. spectrum of radicals formed during exposure of orotic acid to hydrogen atoms.

During the exposure of orotic acid to hydrogen atoms a quartet is obtained, which can be accounted for by a doublet of doublets with 18 and 34 gauss splittings (figure 3). Addition at carbon 5 to form a planar radical with two equivalent hydrogen atoms (V) would be expected to give a 19 gauss 1:2:1 triplet by analogy with the room temperature dihydrouracilyl radical spectrum. If the radical is non-planar with non-equivalent hydrogen atoms, this structure would be expected to give a spectrum which contains a large splitting (~ 45 gauss) by analogy with the 1,3-dimethyldihydrouracilyl radical spectrum. Thus the addition is probably not at carbon 5. Addition at carbon 6 to form a planar radical (VI) is consistent with the observation that the magnitude of the two splittings are very similar to the magnitude of room temperature dihydrouracilyl radical splittings. Therefore

the radicals probably have similar conformations, and the orotic acid-hydrogen atom adduct radical probably has hydrogen atoms on both the 5 and 6 carbon atoms and is planar. It follows that steric blocking of addition to a carbon bonded to the carboxyl group is not important, and the electronic directive effect of the carboxyl group dominates sufficiently to produce the secondary 6-carboxy-5,6-dihydrouracil-5-yl radical.

Figure 4. E.S.R. spectrum of radicals formed very early during exposure of 5-methylcytosine to hydrogen atoms.

The spectrum induced in cytosine is similar to that observed in uracil under similar conditions. However, the line-widths are slightly greater, and the splittings are slightly less than for the dihydrouracil radical. Thymine and 5-methylcytosine (figure 4) give spectra which differ in line-widths and splittings in a similar way. These differences reflect the effect of the amino group on the spin density distribution. The increase in line-width may be the result of unresolved hyperfine splitting due to the amino nitrogen. These observations are consistent with addition to carbon 6 of cytosine leaving the larger spin density on carbon 5 (VII).

The spectra observed during the reaction of 5,6-dihydrouracil, 5,6-dihydrothymine, and 6-methyl-5,6-dihydrouracil (figures 5, 6, and 7) with hydrogen atoms are almost identical to the spectra obtained from corresponding unsaturated molecules under similar conditions. The reaction of deuterium atoms with these saturated compounds yield generally similar spectra. These results suggest that the dominant reaction is abstraction to give the same free radical as was obtained by hydrogen atom addition to the 5-6 double bond in the corresponding pyrimidines. The dihydropyrimidine-5-or-6-yl radical appears, therefore, to have greater thermodynamic stability than other radical products.

Figure 5. E.S.R. spectrum of radicals formed during exposure of 6-methyl-5,6-dihydrouracil to hydrogen atoms.

Figure 6. E.S.R. spectrum of radicals formed during exposure of 5,6-dihydrothymine to hydrogen atoms.

Figure 7. E.S.R. spectrum of radicals formed during exposure of 5,6-dihydrouracil to hydrogen atoms.

Heller, Schlick and Cole (1967) have reported spectra which differ slightly from the spectrum in figure 6 for radicals produced by exposure of 5,6-dihydrothymine powder to hydrogen atoms. This difference may be due to differences in experimental techniques. Figure 6 is a spectrum obtained at low conversion, and we believe it to be the spectrum of essentially pure dihydrothymine-5-yl radical.

All purines investigated yield spectra similar to that previously reported for purine itself (Holmes *et al.* 1967) regardless of the substituents attached to carbons 2 and 4. Proton couplings are given in the table. Deuterium atoms produce spectra different from those produced by hydrogen atoms, which indicates that atom addition occurs. The addition probably is to carbon 8 in the imidazole ring portion of the molecule. However, the central peak of the purine triplet is relatively larger than would be expected from interaction with two equivalent protons. Since late spectra show even larger central components relative to the two outer lines, it may be that part of the central line is due to other purine radical structures.

5. Summary

The study of the reactions of nascent thermal hydrogen atoms with purine and pyrimidine bases dispersed by freeze-drying has led to several interesting conclusions:

- (1) Experiments with 1,3-dideuterouracil have demonstrated that addition of H atoms to uracil takes place at the 5,6 double bond.
- (2) Methyl groups attached to carbons 5 or 6 of the pyrimidine ring exert a dominant directive effect on addition of H atoms to give tertiary radicals.
- (3) The conformation of the dihydropyrimidinyl radicals has been inferred by comparisons of the 1,3-dimethyl-5,6-dihydrouracil radical spectrum with the spectra of radicals with unsubstituted ring nitrogen atoms.
- (4) Steric blocking of hydrogen atom addition appears to be relatively unimportant, as demonstrated by results with orotic acid.
- (5) Replacement of an oxo with an amino group on carbon number 4 has a small but significant effect on the electronic structure of the radical.
- (6) Reaction of H atoms with saturated 5,6-dihydropyrimidine compounds leads to formation of the same radicals as are formed from the corresponding unsaturated compounds, illustrating the high thermodynamic stability of dihydrouracil radicals as compared with possible alternative products.
- (7) All purine compounds investigated appear to give radicals with nearly identical electronic structure irrespective of substituents.

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Des bases puriques et pyrimidiques ont été exposées à des atomes d'hydrogène et de deutérium dans une cavité de R.S.E. Les spectres ont été déterminés au cours de l'exposition. Les conclusions concernant les composés pyrimidiques sont les suivantes: les atomes s'ajoutent à tous les composés examinés; l'addition se produit à la double liaison en 5-6 du 1,3-didéutérouracile; les groupements méthylés des carbones 5 ou 6 exercent un effet dominant et directeur sur l'addition; les radicaux dihydropyrimidinyles semblent avoir une conformation presque plane à température ambiante, tandis que les radicaux diméthyl-dihydropyrimidinyles n'ont pas une conformation plane; le blocage stérique de

L'atome d'hydrogène paraît sans importance, et les groupements oxo- et amino- sur le carbone 4 ont des effets légers sur la structure électronique du radical. Les réactions de l'atome d'hydrogène avec les 5,6 dihydropyrimidines conduisent à la formation par soustraction des mêmes radicaux que ceux formés par addition sur les composés non saturés correspondants. Toutes les purines examinées donnent des radicaux dont les structures électroniques sont presque identiques.

Purin- und Pyrimidinbasen wurden der Einwirkung von Hydrogen und Deuterium Atomen in der FSR-Kammer unterworfen und die resultierenden Spektren bestimmt. Pyrimidin-Verbindungen bilden Additionsprodukte mit diesen Atomen. Mit 1,3-Dideuterouracil findet die Addition an der 5,6-Doppelbindung statt. Methylgruppen an den Kohlenstoffatomen 5 oder 6 der Pyrimidinverbindungen üben eine entscheidende Richtungswirkung auf die Addition des H-Atoms aus. In Raumtemperatur haben Dihydropyrimidinyl Radikale fast ebene Konformationen aber die der 1,3-Dimethyldihydropyrimidinyl Radikale sind nicht eben. Stereochemische Hinderung der Addition des H-Atoms scheint unwichtig zu sein. Oxo- und Aminogruppen am Kohlenstoff 4 der Pyrimidinverbindungen haben geringe jedoch signifikante verschiedene Wirkungen auf die elektronische Struktur der Radikale. Reaktion des H-Atoms mit 5,6-Dihydropyrimidine bildet dieselben Radikale durch Abstraktion wie diejenigen die durch Addition zu den entsprechenden Pyrimidinen gebildet werden. Purine ergeben Radikale mit nahezu gleichen elektronischen Strukturen.

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Radicals formed by electron transfer to pyrimidine derivatives†

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The reactions of electrons with some pyrimidine derivatives were investigated. The electrons, produced photolytically in alkaline glasses, were reacted with solutes to form anions. It was found that anions of thymidine and thymidine-5'-phosphate easily undergo proton transfer from surrounding water molecules to the C₆ position of the thymine ring. Anions of uracil and deoxycytidine do not undergo corresponding proton transfer reactions in the aqueous matrix at low temperatures. The role of electrons in the radiolysis of thymine-containing compounds including DNA is discussed. It is shown that electron reactions can account for the formation of the eight-lined E.S.R. spectrum formed in the radiolysis of these compounds.

1. Introduction

A knowledge of the reactions of electrons is essential to understand the effects of ionizing radiation. In the case of DNA, it is known that the purine and pyrimidine base groups react readily with electrons at rates approaching the diffusion controlled rate (Hart, Gordon and Thomas 1964). Furthermore, chemical modification of these base groups may occur as a result of such reactions. It has been suggested that free radicals are formed on thymine (Ormerod 1965) and cytosine (Kamal and Garrison 1965) following electron attachment. The purpose of this work was to investigate these suggestions by studying the reactions of some pyrimidine derivatives with electrons produced by a non-radiolytic technique.

The source of electrons was the photolysis of $\text{Fe}(\text{CN})_6^{-4}$ ion in an alkaline ice glass at 77°K (Ayscough, Collins and Dainton 1965), reaction (1):

At this temperature the electrons are stable and readily observable both visually, by the blue colour of the glass, and by a strong E.S.R. absorption at $g \approx 2$. The electrons become mobile on illumination of the glass with red light or on warming the matrix to temperatures $> 173^\circ\text{K}$ and will react with certain solutes present at concentrations as low as 10^{-4} M. For example, if the solute is an alkyl halide, dissociative electron attachment occurs to form alkyl radicals (Ayscough *et al.* 1965). In the work reported here various pyrimidine derivatives were used as solutes and the radicals formed by electron attachment to these solutes were detected by E.S.R.

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2. Experimental

The alkaline glasses were prepared from H_2O which had been de-ionized and triply distilled from an all-quartz apparatus. The D_2O used was 99.75 per cent D (USAEC). The NaOD (Isotopes, Inc.) was obtained as a 40 per cent solution and filtered before use. The organic solutes were obtained from Schwarz and Calbiochem and used as received.

The E.S.R. spectrometer used to detect radicals was a Varian Model V 4500-10A X-band instrument equipped with a 100 kc/s field modulation unit. A TE_{012} mode cavity was employed so that samples could be placed in a thermostated quartz dewar located in the cavity.

Figure 1. (a) E.S.R. derivative spectrum of the electron in H_2O at 77°K ; glass contains 8 N NaOH and 5 mM $\text{K}_4\text{Fe}(\text{CN})_6$; modulation, 3 gauss. (b) E.S.R. derivative spectrum of the electron in D_2O at 77°K ; glass contains 8 N NaOH and 5 mM $\text{K}_4\text{Fe}(\text{CN})_6$; modulation, 2.4 gauss.

The solutions to be photolysed contained 5 mM $\text{K}_4\text{Fe}(\text{CN})_6$, 8 N sodium hydroxide, and 0.5 mM of an organic solute. The concentrations were such that more than 90 per cent of the $2537 \text{ \AA}$ light was absorbed by the ferrocyanide to minimize radical formation by direct photolysis of the organic solute (Helene, Santus and Douzou 1966). Solutions were de-aerated by bubbling with argon for 30 min. To prepare a frozen sample a piece of Suprasil tubing 20 cm long and 5 mm OD diameter was immersed in the de-aerated solution, capped at the top, withdrawn, quickly dried on the outside and immediately plunged into the liquid N_2 . This produced a frozen glassy sample approximately 35 mm long.

The samples were photolysed for 90 sec while frozen at 77°K with $2537 \text{ \AA}$ light from a low pressure Hg arc. After the glasses were photolysed they appeared dark blue and the E.S.R. spectra were singlets at $g \approx 2$. In H_2O the E.S.R. line width is 15 gauss and in D_2O it is 7.0 gauss (figure 1). This is in agreement with other observations of the width of the absorption line in this matrix (Ayscough *et al.* 1965, Moorthy and Weiss 1965). Usually no

radicals other than the electron were present in the glass after photolysis. The number of spins in the sample was of the order of 6×10^{16} , which is equivalent to an electron concentration of ~ 0.3 mM.

After photolysis the frozen samples with the electrons were either bleached with red light at 77°K or thermally annealed in a thermostated quartz dewar (Varian temperature control unit No. V 4540). Either of these techniques caused the electron signal to disappear. A different radical signal appeared in its place if a reactive organic solute was present; in the absence of a solute no E.S.R. spectrum was observed. E.S.R. spectra were usually recorded at 80°K .

Theoretical E.S.R. spectra were generated by an IBM 360-50 computer for comparison with experiment. The programme GLACES (Young 1968, private communication) was used to calculate line positions and intensities; and the programme GLAD (Medeiros and Young 1963) was used to generate the theoretical spectra.

3. Results

With thymidine (TdR) as solute, the E.S.R. spectrum shown in figure 2 (a) grew in as the electron signal decayed at 180°K ; no other intermediate radical species were observed. This radical was stable up to 193°K but decayed rapidly above that temperature. Similar results were obtained by red light bleaching at 77°K , except that the spectrum was slightly less resolved in the centre in this case. Since in the absence of thymidine no radical build-up accompanied the electron decay, the observed E.S.R. spectrum is concluded to be that of a radical formed on thymidine.

The conversion of electrons to radicals is very efficient in these glasses. Double integration of the spectra indicated that more than 50 per cent of the electrons were converted to solute radicals at a solute concentration of 0.5 mM. Concentrations of thymidine as low as 0.1 mM were sufficient to trap enough electrons to provide a detectable number of solute radicals.

The spectrum of the thymidine radical shown in figure 2 consists of eight prominent lines spread over 137 gauss. The outer lines are separated by approximately 20 gauss; this splitting is identified with the protons on the methyl group (Pershan, Shulman, Wylunda, and Eisinger 1964) of thymine. Integration of the spectrum showed that the relative intensities of the lines are 0.5 : 2.9 : 4.6 : 7.0 : 6.8 : 3.8 : 3.0 : 0.6. To account for the total width of the spectrum and the intensities of the lines, it is necessary to conclude that two protons with an average splitting of 38.5 gauss must also be interacting with the unpaired spin. Computer simulation showed that a poor fit to the experimental curve is obtained if the doublet splittings are equal. The best match was obtained with doublet splittings of 34 and 43 gauss; the computed spectrum based on these splittings is shown in figure 2 (b). The relative intensities of the eight lines in the theoretical spectrum are 1 : 3 : 5 : 7 : 7 : 5 : 3 : 1, very close to the observed values. This interpretation of the spectrum is very similar to that given for the 5-methyl-5, 6-dihydrouracil-5-yl radical, which is obtained when hydrogen atoms add to thymine (Heller and Cole 1965, Holmes, Ingalls and Myers 1968, Herak and Gordy 1965). The non-equivalence of the beta protons can be attributed to a puckering of the ring at the C_6 position.

Very similar results were obtained with thymidine-5'-phosphate (TMP) and thymine. The E.S.R. spectra obtained with these solutes were essentially identical to that shown in figure 2 (a). The overall widths were 137 gauss for TMP and 141 gauss for thymine. The increase in width for thymine is associated with an increase in the doublet splittings. Thymine was anomalous in its reactivity during red light bleaching. Whereas bleaching at 77°K of glasses

Figure 2. (a) E.S.R. derivative spectrum of the radical formed on reaction of the electron with thymidine (0.5 mM) at 180°K; spectrum recorded at 77°K at 3 gauss modulation and 20 μ W power. The line at the extreme left appears slightly larger than it should because of a background line at this position. (b) Computer simulated derivative spectrum of the radical formed from thymidine. The quartet splitting is 20 gauss and the doublet splittings are 34 and 43 gauss; lines are gaussian and 9 gauss wide.

containing either thymidine or TMP resulted in the eight-line spectrum, this did not occur when the solute was thymine. Even after the blue colour had gone, the spectrum consisted of a large line near $g = 2$ with smaller satellite lines. However, when the bleached sample was warmed to 180°K, the eight-line spectrum appeared and remained when recooled to 80°K. Although definite identification could not be made, it is suggested that this unresolved singlet observed with thymine after bleaching is due to a thymine anion (see discussion of uracil below).

With uracil as solute, the E.S.R. spectrum of the solute radical formed when the electron reacted at 190°K was a 16 gauss doublet, which became a 24 gauss wide singlet when the sample was cooled to 80°K. With deoxycytidine as solute, the spectrum of the solute radical is a 24 gauss wide unresolved singlet at 200°K.

To verify that the hydrogen adduct radical is formed in the thymine compounds and to establish the source of the hydrogen, the photolyses were performed in D₂O matrices. Transfer of a D atom would lead to a radical whose E.S.R. spectrum would be predictably different from that of the radical formed in an H₂O matrix.

Reaction of the electron with thymidine at 183°K in a D₂O glass yielded the spectrum shown in figure 3 (a). Further warming to 200°K resulted in increased resolution of the central lines and disappearance of the outermost pair of lines (figure 3 (b)). There are eighteen observable lines spread over 112 gauss in the

Figure 3. (a) E.S.R. derivative spectrum of the radical formed on reaction of the electron with thymidine in D₂O; annealed at 183°K; spectrum recorded at 77°K and 3 gauss modulation. (b) Same sample and conditions as in (a) except that sample has been annealed at 193°K; the line at the far left was present in the background spectrum. (c) Computer-generated spectrum of the radical formed by D-atom transfer to thymidine; quartet splitting is 20 gauss, the CH doublet is 40 gauss and the D-atom splitting is 5.7 gauss. Lines are gaussian and 4.8 gauss wide.

final spectrum. It is quite different from that obtained in H_2O glasses and supports the suggestion that a proton transfers from the water to the radical. The spectrum can be accounted for if the 20 gauss methyl splitting remains unchanged and one of the doublet splittings is replaced by the equivalent D-atom triplet splitting. A computer-simulated spectrum based on a 20 gauss quartet, a 40 gauss doublet and a 5.7 gauss D-atom triplet (equivalent to 37 gauss proton splitting) is shown in figure 3 (c). The line positions are in good agreement with the experimental spectrum.

A comparison of the E.S.R. spectrum in figure 3 (a) with figures 2 (a) and 3 (b) indicates that both H and D-adduct radicals are formed at 183°K in the D_2O matrix. A computer analysis indicated that approximately 30 per cent of the radicals are H-adduct radicals which arose because of the presence of 10 per cent NaOH in the matrix. The preference for H transfer in this matrix corresponds to an isotope effect of 4. In an NaOD- D_2O matrix only the D-adduct radical was observed. The improvement in resolution with temperature shown in figure 3 results from a faster decay of H-adduct radicals which permits the D-atom radical to be more clearly resolved at the higher temperature. Apparently the two radicals do not decay at the same rate.

Spectra very similar to that shown in figure 3 were obtained with thymine as solute D_2O glasses. The similarity in behaviour of thymine and thymidine in this respect indicates that water is the source of the protons which transfer. Little, if any, transfer of a proton from the deoxyribose group of thymidine occurs.

4. Discussion

All of the compounds investigated were found to react with the electron to form detectable radicals. In the case of thymine, thymidine, and TMP, the E.S.R. spectra show that the radical formed is the same as that formed when hydrogen atoms add to the C_6 position of the thymine ring. It is therefore concluded that electron capture by these compounds, reaction (2), is followed by proton transfer to the anion radical, reaction (3). This sequence is illustrated here for thymidine; the S in the structures represents the deoxyribose group:

The results in D_2O matrices confirm proton transfer and also show that water is the source of the proton. Anions of thymidine and TMP are the most reactive and protonate even at 77°K in the alkaline glass. Thymine anion is slightly less reactive, as step 3 does not occur until the sample is annealed. The results indicate that the same radical is formed in thymine, thymidine and TMP. In all three cases the unpaired spin is on the thymine ring.

The ESR spectra obtained by reaction of the electron with uracil and deoxycytidine are very unlike those produced by hydrogen atom addition to these compounds (Holmes *et al.* 1968, Herak and Gordy 1965, 1966). It is postulated that the observed spectra are due to anions and that proton transfer does not occur under our conditions for these anions. This suggestion is reasonable, since in the pyrimidine (Falcott and Myers 1967) and benzene (Fessenden and Ogawa 1964) anions, the nitrogen and hydrogen hyperfine splittings are found to be small. These splittings would also be expected to be small for uracil and deoxycytidine anions. This would explain the fact that no splittings were resolved and makes the assignment of these singlet spectra to anions reasonable.

Another possible explanation of these results is that the electrons first react with water resulting in hydrogen atoms, reaction 4, and that the hydrogen atoms add to the solute

This alternative is considered unlikely, since the rate of this reaction at the temperatures employed (Faub and Eiben 1968, Shubin, Zhigunov, Zolotarevsky and Dolin 1966) is less than the rate of reaction with the solute, e.g. reaction 2. Furthermore, with uracil and deoxycytidine the radical formed is an anion rather than a hydrogen atom adduct.

These results are significant to the gamma radiolysis of compounds containing pyrimidines. The hydrogen adduct radical shown to be formed here by electron attachment to thymine is a major radical species formed in the radiolysis of thymine (Ormerod 1965, Heller and Cole 1965), and thymidine (Ormerod 1965, Pruden, Snipes and Gordy 1965). ESR studies of the radiolysis of thymine compounds resemble the results reported here, in that irradiation of solid samples at 77 K leads to a singlet-like ESR spectrum, and the eight-lined spectrum, similar to that shown in figure 2, is observed when the samples are warmed above 200 K. In the radiolysis of thymine-containing compounds, electrons will readily attach to the thymine ring to form an anion (Hart *et al.* 1964). The work reported here indicates that this anion undergoes proton transfer in an aqueous medium to form the 5-methyl-5,6-dihydrouracil-5-yl or 'thymyl' radical. It is suggested that the formation of 'thymyl' radicals in the radiolysis of thymine, thymidine and TMP (see references) is a result of transfer of a proton from a surrounding molecule to the thymine anion formed by electron capture.

More complex molecules, such as DNA, contain numerous reactive sites where electrons may react, yet the 'thymyl' radical is also a principal radical observed in ESR studies of irradiated DNA (Peirshian *et al.* 1964, Ormerod 1965, Muller 1967, Ehrenberg, Rupprecht and Strom 1967, Elliott 1968). We suggest that in DNA the 'thymyl' radical may be formed by proton transfer to the thymine anion. If the anions of the other bases are less reactive and if electrons can migrate in DNA, protonation of the thymine-base anion would be expected to occur preferentially in DNA. More data will be required on the rates of proton transfer, particularly at other pH's and temperatures to test this hypothesis further.

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On étudie les réactions d'électrons avec des dérivés de pyrimidine. Les électrons produits par photolyse dans des verres alcalins réagissent sur les solutés pour former des anions. On constate que les anions de thymidine et de thymidine-5'-phosphate subissent facilement le transfert de protons à la position C₆ de l'anneau thyminique des molécules d'eau environnantes. Les anions d'uracile et de desoxycytidine ne subissent aucune réaction correspondante de transfert de protons dans la matrice aqueuse à basse température. On discute le rôle des électrons dans la radiolyse des composés contenant de la thymine, y compris du DNA. On voit donc que des réactions d'électrons peuvent expliquer la formation du spectre RPE de 8 lignes que l'on observe dans la radiolyse de ces composés.

Die Reaktionen der Elektronen mit einigen Pyrimidinderivaten werden untersucht. Die in Alkaliglasern photolytisch erzeugten Reaktionen wurden mit den aufgelösten Stoffen reagiert, um Anionen herzustellen. Man findet, daß die Anionen des Thymidins und des Thymidin-5'-Phosphats leicht den Protonenübertrag aus den umgebenden Wassermolekülen in die C₆-Position des Thyminringes erfahren. Die Anionen des Uracils und des Desoxycytidins erfahren die entsprechenden Protonenübertragsreaktionen nicht in der Wassermatrix bei niedrigen Temperaturen. Die Rolle der Elektronen wird durch Radiolyse von thyminhaltigen Verbindungen, einschließlich DNS, diskutiert. Es zeigt sich, daß die Elektronenreaktionen die Bildung des ESR-Spektrums von 8 Linien, die man durch Radiolyse dieser Verbindungen beobachtet, erklären können.

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