electron on a sulphur atom. Recent work by Box and Freund[6] on a single crystal of cystine dihydrochloride irradiated at 77° K led them to suggest that the observed spectrum arose from the ion —S⁺—S—. Akasaka *et al.*[7] concluded from similar measurements that the observed species was a disulphide ion, probably —S⁻—S—. Truby[8] has investigated polycrystalline amyl disulphide irradiated at 77° K. He suggested that ionic species on the sulphur atom were observed, one of which, the negative one, could be bleached by light. In all cases the sulphur ions had a *g*-value higher than the free spin value. This evidence leads us to postulate that the unpaired electron on a sulphur atom observed in proteins at 77° K is an ion. In the case of a translucent sample of keratin from goose feather quill a brown coloration was observed at 77° K which faded on warming. It seemed probable that this colour was associated with the sulphur ion, but attempts to bleach the colour at 77° K with ultra-violet failed.

Besides the formation of a sulphur ion there was also a considerable increase in the yield of unpaired electrons in the presence of sulphur, and it appears that energy deposited by the radiation in the protein which would not normally give observable unpaired electrons, that is, radicals or ions, is utilized by the sulphur. At present we have no positive information about the mechanism by

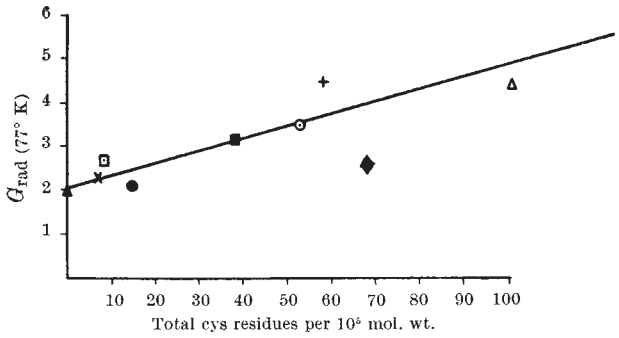

Fig. 2. The *g*-value for radicals in proteins irradiated at 77° K plotted against the number of cystine and cysteine residues in the protein. Taken from data by Henriksen (ref. 5). △, insulin; ○, BSA; ×, zein;, ●, ovalbumin; ▲, gelatin; +, ribonuclease; □, GAPDH; ◆, lysozyme; ■, papain

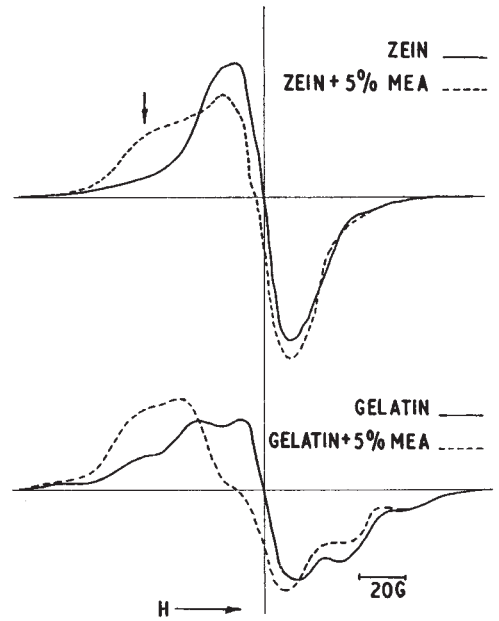

Fig. 3. Electron spin resonance spectra of zein and gelatin and their complexes with 5 per cent MEA recorded at 77° K after irradiation at that temperature

which this takes place. Probably, either excitation energy, which cannot ionize with the rest of the protein, migrates to and ionizes the sulphur atom, or the sulphur in the cystine and cysteine residues interferes with some of the ionic reactions leading to radical formation.

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RADIOLYTIC DEGRADATION OF AQUEOUS CYTOSINE: ENHANCEMENT BY A SECOND ORGANIC SOLUTE

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THE purine and pyrimidine moieties represent ma)or loci of chemical change in the radiation-induced degradation of nucleic acids in aqueous solution[1]. Investigations of the radiation chemistry of the individual base-water systems have provided considerable information on the nature of some of the important reactions[2–9]. Of particular interest has been the finding that the presence of oxygen during irradiation leads to a two- to three-fold increase in the amount of base destroyed[6–8]. The available results suggest that some type of reconstitution reaction or back reaction is involved in the radiolysis of the oxygen-free solutions[8,9]. In this article we offer a formulation of this reconstitution reaction and show that certain added organic solutes are as effective as molecular oxygen in increasing the degradation of cytosine in irradiated aqueous solution.

The actions of ionizing radiation on solutes in dilute aqueous solution are initiated by the radiation-induced step $H_2O \rightarrow H_2O^+ + e^-$ which is followed within 10^{-11} sec by the reactions $H_2O^+ + H_2O \rightarrow H_3O^+ + OH$, $e^- + H_2O \rightarrow e_{aq}^-$ where e_{aq}^- represents the hydrated electron[10]. Subsequent reactions of OH and e_{aq}^- with added solutes determine the radiation chemistry of the system. For γ-rays, the yields of those species per 100 eV absorbed energy is given by:

$$G_{e_{aq}^-} \simeq 2{\cdot}9, \; G_{OH} \simeq 2{\cdot}4 \text{ (ref. 11)}.$$

Scholes and Weiss[2] and Ekert and Monier[3] have shown that the OH radical reacts rapidly with the pyrimidine

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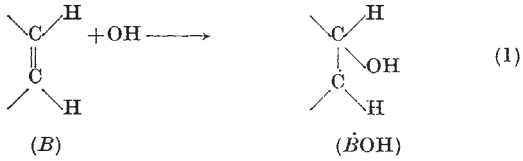
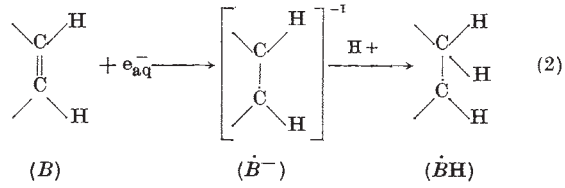
bases by addition at the 5,6 double bond to give the hydroxyhydropyrimidyl radical:



(1)

The production of glycols, hydroxyhydroperoxides and related derivatives of the pyrimidine bases in oxygen-flushed solutions[1,2] can be interpreted in terms of reaction (1) as the principal path for OH removal. The 100 eV yield for base destruction, $G(-B)$, in 10^{-3} M solutions of thymine, uracil and cytosine under γ-rays approximates the primary yield of OH radicals, namely, $G \simeq 2{\cdot}4$. The hydrated electron e_{aq}^- reacts preferentially with molecular oxygen to form O_2^- which is unreactive toward the pyrimidine bases.

In the absence of oxygen, the G value for destruction of the pyrimidine and purine bases has been found to be about one-third that observed in oxygenated, neutral solution. Latarjet *et al.*[8] report $G(-B) \simeq 0{\cdot}8$ for oxygen-free 10^{-3} M thymine solutions under γ-rays, and the datum of Ponnamperuma *et al.*[6] gives $G(-B) = 0{\cdot}95$ for aqueous cytosine under similar irradiation conditions. Now, as we have already noted, the evidence is that the OH radical is quantitatively scavenged via reaction (1) at relatively low concentrations of the pyrimidine bases. Hart *et al.*[10] have established by physical methods that the reactions of e_{aq}^- with the pyrimidines are among the fastest of known radical–molecule reactions. It would appear then that some type of reconstitution reaction is acting to reduce the G-value for base destruction in the oxygen-free system. If, as seems likely, the hydrated electron, e_{aq}^-, also adds to the labile 5,6 position:

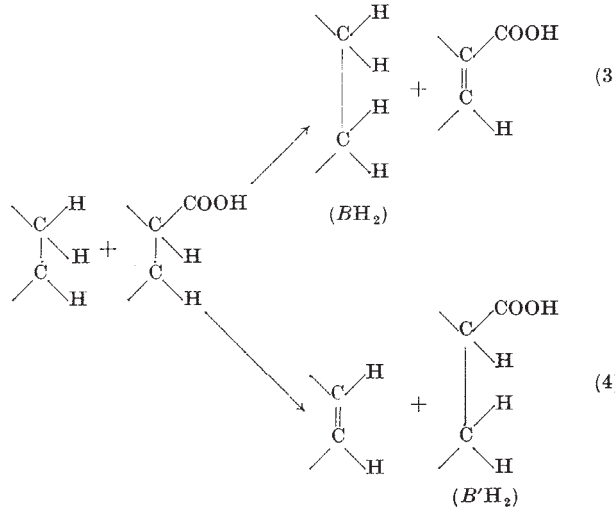


(2)

then the reconstitution reaction may be interpreted in terms of water regeneration via $\dot{B}H + \dot{B}OH \rightarrow 2B + H_2O$ or $\dot{B}H + \dot{B}OH \rightarrow B + \dot{B}H_2O$, followed by $\dot{B}H_2O \rightarrow B + H_2O$ or both. On this basis it follows that addition of a second organic solute, that is preferentially reactive towards OH via $RH + OH \rightarrow \dot{R} + H_2O$, would lead on replacement of OH by $\dot{R}$ in reaction 1 to an enhancement in $G(-B)$ since the possibility for self-protection through water elimination would be excluded. This increase in $G(-B)$ would correspond to an increase in the yield of products saturated at the 5,6 position.

Cytosine was chosen for investigation here because the dihydrocytosine derivatives obtained on saturation of the 5,6 double bond hydrolyse readily to give the corresponding 5,6 dihydrouracil derivatives and ammonia[12], a product conveniently followed analytically. Sodium formate and ethanol were used as second solutes; each of these compounds is relatively inert towards e_{aq}^- and at the same time is extremely reactive towards OH via $HCOO^- + OH \rightarrow \dot{C}OO^- + H_2O$ and $CH_3CH_2OH + OH \rightarrow CH_3\dot{C}HOH + H_2O$ (refs. 10 and 11). The effects of added formate and ethanol on ammonia yields in the γ-radiolysis of oxygen-free 0·06 M solutions of cytosine at pH ~ 7 are shown in Fig. 1. We see that $G(NH_3)$ increases abruptly with increasing solute concentration and reaches a limiting value of approximately 2·9 at the higher formate concentrations.

Now, if our interpretation of this enhancement is correct, the hydrated electron e_{aq}^- is removed via reaction (2) and the OH radical is converted in the presence of formate to the $\dot{C}OO^-$ radical which in turn is removed via the analogue of reaction (1). The radical products of these reactions then undergo disproportionation via reaction (3) or reaction (4) or both.



(3) (4)

where BH_2 and $B'H_2$ represent labile 5,6-dihydrocytosine derivatives that undergo rapid hydrolysis[12] to give ammonia and, respectively, hydrouracil (5,6-dihydrouracil) and a hydrouracil carboxylic acid. To confirm the formation of the hydrouracil nucleus, the irradiated cytosine-formate solutions were passed through 'Dowex 50' in the acid form to quantitatively remove cytosine[13]. A series of control runs with authentic materials showed that neither hydrouracil or hydrouracil-6-carboxylic acid are retained by 'Dowex 50'. The water effluent was freeze-dried, redissolved in water at pH ~ 7 and then read spectrophotometrically over the range 210–350 mμ against an unirradiated sample which had undergone the identical treatment. No characteristic absorption maxima were found under these conditions. However, addition of sodium hydroxide to a concentration of 0·1 N resulted

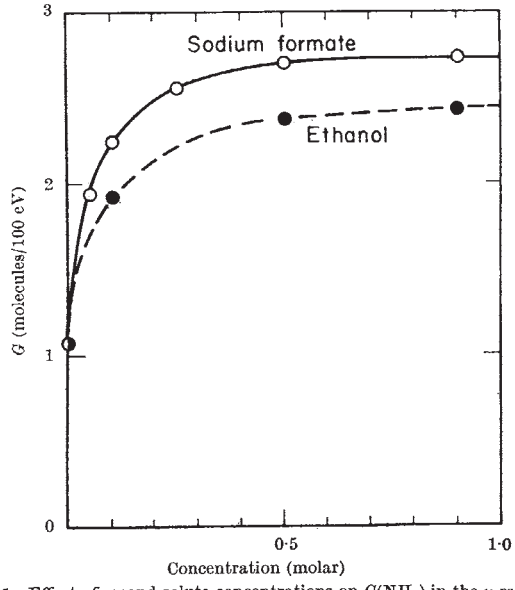


Fig. 1. Effect of second-solute concentrations on $G(NH_3)$ in the γ-radiolysis of 0·05 M cytosine, evacuated, pH 7 ± 0·3. Cytosine (Calbiochem., *A* grade, Lot 35887) was purified by fractional recrystallization from cold water. Solutions were irradiated in evacuated pyrex ampoules under cobalt-60 γ-rays at a dose rate of $1{\cdot}2 \times 10^{18}$ eV/g/min for a total dose of $6{\cdot}5 \times 10^{18}$ eV/g. Ammonia was separated by the Conway diffusion method (ref. 15) following addition of 1 ml. of sample to 9 ml. of 10 N potassium carbonate. The diffusates were assayed by means of the Nessler reaction

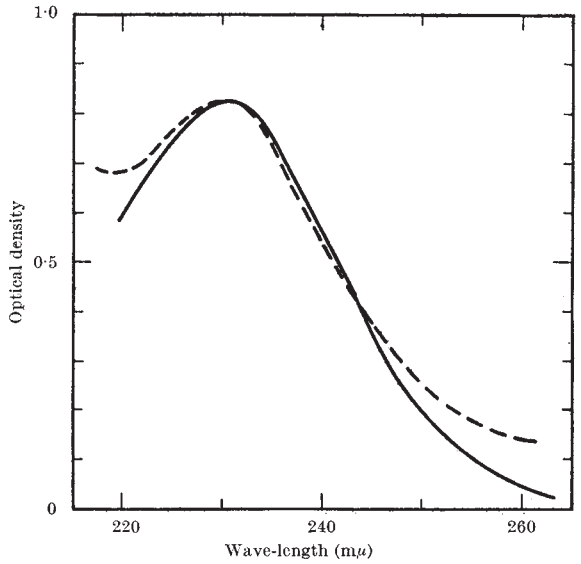


Fig. 2. Absorption spectrum in 0·1 N sodium hydroxide of the dihydrouracil derivatives formed in the γ-radiolysis of 0·05 M cytosine is given by dashed line. The solid curve represents the normalized spectra of authentic dihydrouracil and dihydrouracil-6-carboxylic acid which are practically indistinguishable with the Beckman *DB* spectrophotometer

in the immediate development of the characteristic absorption maximum at 230 mμ which is exhibited by hydrouracil and its derivatives in alkaline solution at *p*H values above 12–13 (ref. 14). The differential absorption curve of the irradiation products is compared in Fig. 2 with absorption spectra for authentic dihydrouracil and dihydrouracil-6-carboxylic acid. Since the spectra of these two substances are essentially identical, other methods had to be employed to distinguish their relative contribution to the absorption given by the product material. Advantage was taken of the fact that the effect of base on the optical absorption of hydrouracil and its derivatives is associated with the production of enol forms which are unstable and are rapidly hydrolysed with characteristic first-order rates to give the corresponding ureido acid. Batt *et al.*[14] have reported half-times of 5·0 min and 11·1 min, respectively, for the hydrolysis of hydrouracil and hydrouracil-6-carboxylic in 0·1 N sodium hydroxide. The irradiation products, the initial absorption spectra of which in 0·1 N sodium hydroxide are shown in Fig. 2, showed a decrease in optical density at λ_{max} = 230 mμ corresponding to a half-time of ~ 12·1 min over a minimum of three half-time periods. Reaction (4) seems to occur preferentially over reaction (3). Preliminary spectrophotometric determinations of $G(B'H_2)$ based on $\varepsilon_{max} \simeq 8 \times 10^3$ (ref. 14) gave values considerably below that anticipated on the basis of the indicated mechanism. Apparently $B'H_2$ undergoes a slow hydrolytic dark-reaction to form the corresponding ureido acid. However, the ureido acids revert to the original hydrouracil derivatives in the presence of dilute mineral acid[14]. Treatment of the irradiated cytosine solutions with 0·1 N hydrochloric acid for several hours prior to analysis gave $G(B'H_2) \simeq 2{\cdot}4$ in good agreement with the reaction mechanisms described here[16].

These findings would appear to cast some doubt on recent proposals regarding the nature and locus of the primary radiobiological lesion. Various authors have commented on the fact that the pronounced enhancement by oxygen of the radiation-induced degradation of the pyrimidines and purines in aqueous solution is closely paralleled by the two- to three-fold increase in radiobiological damage effected by oxygen. And it has been suggested[16] that this similarity in oxygen response is a strong argument in favour of a common radiation chemical process. However, we have found here that radicals of certain organic substances are as effective as oxygen in increasing the degradation of cytosine in irradiated aqueous solution. The possibility that organic radicals in anoxic biological systems under irradiation are equally effective in the enhancement of cytosine degradation cannot be dismissed. Hence, the interpretation of the oxygen-effects in radiobiology in terms of the radiation chemistry of pure base-water systems may prove to be misleading.

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NATURE OF THE UNREACTIVE SULPHYDRYL GROUPS IN HUMAN HAEMOGLOBIN

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ALL the mammalian haemoglobins so far examined contain sulphydryl (SH) groups[1]. These fall into two categories which have been termed 'reactive' and 'unreactive'. The reactive SH groups are those which show a chemical reactivity comparable with that of simple thiols. The unreactive SH groups show a very low reactivity in native haemoglobin but become indistinguishable from the reactive groups when the protein is denatured.

The existence of unreactive SH groups in haemoglobins, and other proteins, has been known for many years, but an adequate explanation for their behaviour has been lacking. The low reactivity observed would be consistent with some form of covalent bonding, such as a thiazoline ring[2]. However, the conditions required for denaturation[3,4], such as the addition of sodium dodecyl sulphate or lowering the *p*H to 3·5, are too mild to cause the rupture of covalent bonds.

Cecil and Snow[5] examined the effect of modifying the unreactive SH groups of haemoglobin on various properties of the protein, for example, absorption spectrum, dissociation and rate of urea denaturation, and concluded that these SH groups are involved in some form of non-covalent, probably intra-chain, bonding. Cecil[6] recently reviewed the evidence relating to the role of SH groups in proteins generally and concluded that there were comparatively few well-established instances of SH groups