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A PULSE RADIOLYSIS STUDY OF SOME FREE RADICAL REACTIONS WITH ERYTHROCYTE MEMBRANES

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SUMMARY

The reactions of the free radicals e_{aq}^-, OH and Br_2^- with haemoglobin-free erythrocyte ghost membranes have been studied by producing the radicals by pulse radiolysis and monitoring their reactions by optical spectroscopy. Hydrated electrons react rapidly with the membrane, but no attack at disulphide links was observed. Hydroxyl radical attack produced transient species absorbing weakly in the ultraviolet, which may arise from carbohydrate residues, such as *N*-acetyl neuraminic acid and *N*-acetyl glucosamine, on the membrane surface. No evidence was obtained for OH attack at ring-containing amino acid residues of the protein component. The Br_2^- radical, a more selective electrophile than OH, reacted only slowly with erythrocyte ghosts. Solubilization of the membranes with dodecylsulphate or digestion with alkali exposed protein containing tyrosine and tryptophan residues which reacted with Br_2^-. These results support other evidence for the absence of reactive protein at the membrane surface.

INTRODUCTION

In the few investigations of the effects of radiation on biological membrane systems which have been reported [1, 2], no attempt has been made to investigate the initial reactions between free radicals and a membrane. The technique of pulse radiolysis [3] permits direct observation of the reactions of radiolytically produced free radicals on a sub-microsecond timescale. We have now applied this method to study free radical attack on erythrocyte ghost membranes, producing the radicals by a short pulse of high energy electrons and monitoring the subsequent reactions by kinetic optical absorption spectroscopy.

Pulse radiolysis studies of the reactions of radiation-produced radicals such as the hydrated electron (e_{aq}^-) or the hydroxyl radical (OH) with proteins and enzymes have revealed certain amino acid residues which are particularly reactive with these

Abbreviations: GlcNAc, *N*-acetyl-D-glucosamine; AcNeu, *N*-acetyl neuraminic acid.

radicals [4–8]. Inorganic radical anions of the form X_2^-, obtained by reaction of OH radicals with halogen and thiocyanate ions, have been found to react much more selectively than OH with some amino acid residues, and these "probes" have been used to identify some essential amino acid residues of various enzymes [9].

We have investigated the reactions of the radicals e_{aq}^-, OH and Br_2^- with erythrocyte ghost membranes, and have observed changes in the reactivity of Br_2^- on solubilizing the membranes with detergent or alkali, which reflect the accessibility of reactive amino acid residues to this "probe".

METHODS

Haemoglobin-free erythrocyte ghosts were prepared in 5 mM phosphate buffer (pH 7.8) from sheep's blood by the method of Dodge et al. [10]. The cream-coloured "buttons" obtained on centrifugation were removed, since they have been reported to contain proteinases [11]. For irradiation the ghost suspension was diluted to a protein concentration of 75 μg/ml (determined using the Folin reagent [12]) in 5 mM phosphate buffer (pH 7.8).

Sodium dodecylsulphate (B.D.H. specially pure grade), ethanolamine (Hopkins and Williams GPR), choline chloride (Sigma), *N*-acetyl neuraminic acid (Sigma, synthetic, Type IV) and *N*-acetyl-D-glucosamine (Sigma) were used as received. Triply distilled water was used throughout.

Samples were irradiated in a silica spectrophotometric cell (20 mm light path) with single 0.2 μs electron pulses from a 1.8 MeV linear accelerator. Details of the equipment [13] and solution handling [14] have been published.

RESULTS AND DISCUSSION

Reactions with OH and e_{aq}^-

Irradiation of neutral, deaerated water produces the free radicals OH, e_{aq}^- and H with yields (G values) of about 2.9, 2.7 and 0.6 radicals per 100 eV ($1.6 \cdot 10^{-17}$ J) energy absorbed. Saturation with N_2O converts the e_{aq}^- into OH so that 90 % of the radicals are OH:

$$e_{aq}^- + N_2O \rightarrow N_2 + OH + OH^- \quad (1)$$

Saturation with air or oxygen leaves OH unchanged but converts e_{aq}^- and H into O_2^- at pH 7.8:

$$e_{aq}^- + O_2 \rightarrow O_2^- \quad (2)$$

$$H + O_2 \rightarrow HO_2 \quad (3)$$

$$HO_2 \rightleftharpoons H^+ + O_2^- \quad pK = 4.9 \quad [15] \quad (4)$$

Fig. 1 shows the absorption spectra observed 0.1 ms after pulse radiolysis of suspensions of erythrocyte membranes saturated with either N_2, N_2O or O_2. Weak absorptions were observed in all three solutions: spectra are presented as the product of the yield G (radicals/100 eV) and the decadic molar extinction coefficient $\varepsilon(M^{-1} \cdot cm^{-1})$. Thus for N_2O-saturated solutions, $G\varepsilon \cong 1400$ at 300 nm and since $G(OH) \cong 6$ then the species resulting from OH attack on the membrane has $\varepsilon \cong 230\ m^{-1} \cdot cm^{-1}$ at 300 nm.

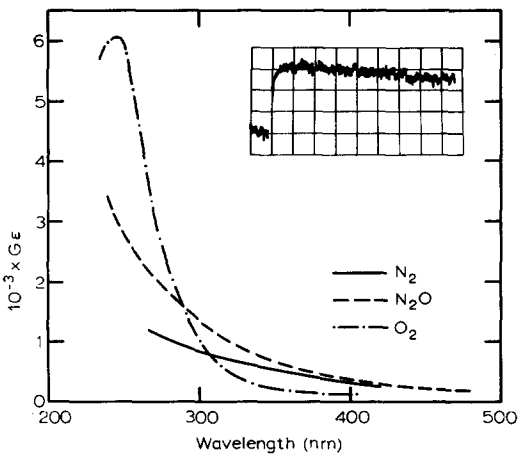


Fig. 1. Transient absorption spectra produced by pulse radiolysis of erythrocyte ghost suspensions saturated with N_2, N_2O or O_2. Spectra were measured 0.1 ms after a 1-krad, 0.2 μs pulse. Protein concentration 75 μg/ml, pH 7.8. Inset: Oscilloscope trace showing formation and decay of the transient absorption at 300 nm in an N_2O-saturated suspension. The initial horizontal trace lasting for 50 μs before the pulse (bottom left) represents the 100 % transmission baseline. Ordinate: transmission 0.2 %/division, abscissa: time 50 μs/division.

The nitrogen-saturated suspensions gave a very weak absorption in the ultraviolet after the strong visible absorption of e_{aq}^- had decayed (half-life $t_{\frac{1}{2}} \cong 0.6$ μs). When e_{aq}^- reacts with protein containing disulphide links, strong absorptions from $(RSSR)^-$ free radicals are observed, with $G\varepsilon \cong 1.5 \cdot 10^4$ at $\lambda_{max} = 410$ nm [4–7]. The absorption attributable to a product of e_{aq}^- reaction with erythrocyte ghosts was about two orders of magnitude smaller at this wavelength, suggesting that cystine-containing protein is not situated close to the membrane surface. It was confirmed by centrifugation that the hydrated electrons were reacting with the membranes and not with other dissolved material: the half-life of e_{aq}^- in the deaerated supernatant was an order of magnitude longer than that observed for the membrane suspension.

The decay of e_{aq}^- (first-order rate constant approx. $1.2 \cdot 10^6$ s^{-1} at 75 μg/ml membrane protein concentration) indicates a high reactivity for e_{aq}^- with the membrane. Proteins with molecular weight of about 10^4 such as ribonuclease and lysosyme have bimolecular rate constants of the order of $(1–3) \cdot 10^{10}$ $M^{-1} \cdot s^{-1}$ [5, 7, 8, 16] or about $1–2\,l \cdot \mu g^{-1} \cdot s^{-1}$. The apparent rate constant for reaction of e_{aq}^- with the membrane is even higher at approx. $16\,l \cdot (\mu g \text{ protein})^{-1} \cdot s^{-1}$. Although very high rate constants (up to $2 \cdot 10^{13}$ $M^{-1} \cdot s^{-1}$) have been measured and accounted for theoretically for high molecular weight DNA [16], it is probable that simple solution kinetics are not adequate to describe the rate of a reaction occurring in a suspension such as that of the erythrocyte membranes.

N-Acetyl neuraminic acid (AcNeu) and *N*-acetyl glucosamine (GlcNAc) are thought to be present at the erythrocyte membrane surface [17]. We found both sugars to be fairly unreactive to e_{aq}^- ($k \leqslant 10^7$ $M^{-1} \cdot s^{-1}$), as also were the hydrophilic group of the phospholipids thought to form an asymmetric bilayer [18]: choline ($k = 8 \cdot 10^7$ $M^{-1} \cdot s^{-1}$) and ethanolamine ($k = 2 \cdot 10^7$ $M^{-1} \cdot s^{-1}$), all measured at pH 7.8. Phosphatidylserine lipids [18] are also probably unreactive towards e_{aq}^-

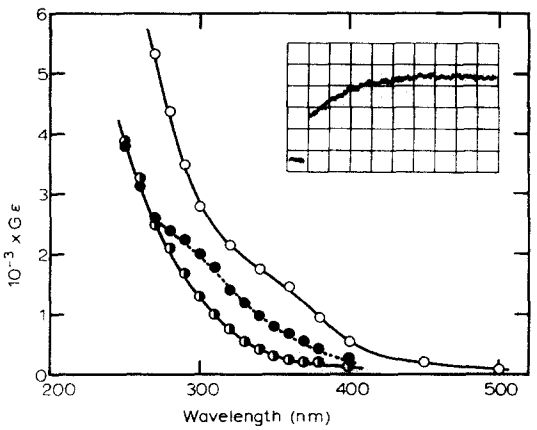


Fig. 2. Transient absorption spectra from pulse radiolysis of N_2O-saturated solutions of *N*-acetyl glucosamine (50 mM, pH 7.0, 1.0 krad/pulse) and *N*-acetyl neuraminic acid (0.5 mM, pH 7.5, 1.4 krad/pulse). ○, GlcNAc, 50 μs after the pulse; ◑, AcNeu, 10 μs after the pulse; ●, AcNeu, 0.4 ms after the pulse. Inset: transient absorption at 340 nm produced by pulse irradiation of AcNeu. Ordinate: transmission 0.1 %/division, abscissa: time 50 μs/division.

($k(e_{aq}^- + \text{serine}) < 3 \cdot 10^7\ M^{-1} \cdot s^{-1}$ [19]). The choline phospholipids are thought to occur in the external half of the lipid bilayer [18] but the difficulties of comparing rate data for attack on isolated constituents with attack on membranes are too great to permit the site of reaction of e_{aq}^- with the membrane to be deduced.

In membrane suspensions saturated with nitrous oxide, the radiation-produced transient absorption was increased relative to that in the nitrogen-saturated suspensions, indicating that much of the absorption at wavelengths less than 300 nm arose from the product(s) of OH attack on the membrane (N_2O approximately doubles $G(OH)$). This weak absorption is quite unlike the spectra observed from OH attack at the more reactive amino acid residues such as tryptophan, tyrosine, phenylalanine and histidine [4, 20, 21]. By comparison, the product of the reaction of OH with tryptophan gives a spectrum with maxima at approx. 300 and 520 nm and $G\varepsilon = 3 \cdot 10^4$ and $1 \cdot 10^4$, respectively [20].

We found that the spectrum produced from OH attack on the membrane was quantitatively similar to those observed after reacting OH with AcNeu or GlcNAc (see Fig. 2). With AcNeu a subsequent exponential grow-in ($t_{\frac{1}{2}} \cong 70$ μs) of absorption occurred at longer wavelengths, but the initial spectrum resembles that shown in Fig. 1. Unfortunately, such spectra are really too weak and featureless to prove that the sites of OH attack on membrane surfaces are indeed the carbohydrate residues, such as AcNeu and GlcNac, thought to be there [17].

Unfortunately, the rate of reaction of OH radicals with the membrane could not be measured from the rate of product formation, due to the low intensity of the transient absorption observed in N_2O-saturated solution.

In membrane suspensions containing oxygen, the pulse-induced spectrum is considerably more intense at wavelengths below 280 nm than in anaerobic suspensions. In Fig. 1 the absorption shown with $\lambda_{max} \cong 245$ nm is similar to that expected for O_2^-, which is unreactive. ($G\varepsilon \cong 6500$ at 245 nm expected for O_2^- in aerated pure

water at pH 7.8 [15].) At the concentration of membrane used, oxygen scavenges most of the hydrated electrons in aerated suspensions, since $G\varepsilon = 6000$ at 245 nm.

Reactions with Br_2^-

In solutions containing sufficient Br^- so that OH reacts predominantly with Br^- and not with other reactive solutes, the Br_2^- radical is produced with a yield $G(Br_2^-) \cong 3$ or 6 depending on whether the solution is aerated or saturated with nitrous oxide:

$$OH + Br^- \rightarrow OH^- + Br \quad (5)$$

$$Br + Br^- \rightleftharpoons Br_2^- \quad (6)$$

The Br_2^- has a strong absorption with $\lambda_{max} = 360$ nm [22] and decays relatively slowly by a second-order process:

$$2\ Br_2^- \rightarrow Br_3^- + Br^- \quad (7)$$

and half-life dependent on dose and ionic strength but typically hundreds of microseconds under our experimental conditions.

If the Br_2^- reacts with an oxidisable amino acid residue A to give a product P, e.g. by

$$Br_2^- + A \xrightarrow{k_a} 2\ Br^- + P + H^+ \quad (8)$$

then the reaction may be monitored either by the appearance of a new absorption of the free radical P or by the increase in decay rate of the Br_2^- absorption. If, as is usual, $[A] \gg [Br_2^-]$ then the decay of Br_2^- will now have a first-order component, with rate constant $k_a[A]s^{-1}$.

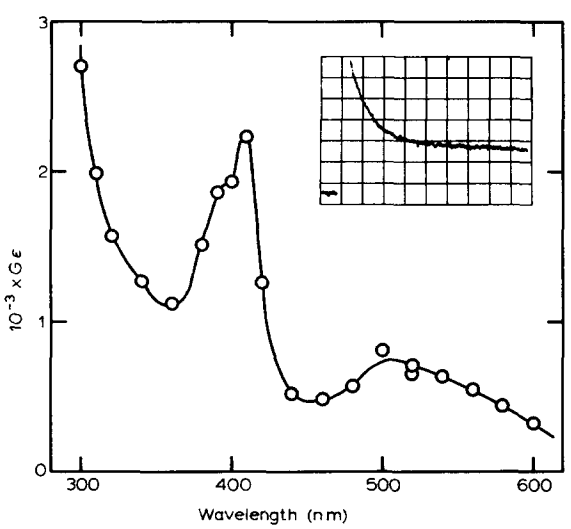
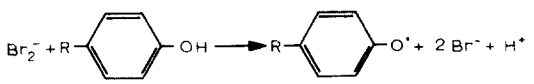


Fig. 3. Transient absorption spectrum produced by pulse radiolysis of an aerated suspension of erythrocyte membranes containing 50 mM KBr and digested with 5 mM dodecylsulphate. Spectrum measured 0.5 ms after a 400-rad pulse. Inset: oscilloscope trace showing rapid decay of Br_2^- at 410 nm, leaving a residual product absorption decaying much more slowly. Ordinate: transmission 0.2 %/division, abscissa; time 100 μs/division.

Aerated membrane suspensions containing 75 μg/ml erythrocyte ghosts and 50 mM NaBr were irradiated with a 200-rad pulse and the decay of the Br_2^- absorption at 360 nm measured. The first-half-life (about 170 μs) was similar to that observed from solutions of NaBr alone and no absorption in the 400–600 nm region expected from a product P was detectable.

Solubilizing the membranes [23] by digestion with dodecylsulphate for 1 h at room temperature had a marked effect on the reactivity of Br_2^- with the membranes. With 5 mM dodecylsulphate the first half-life of Br_2^- was decreased from 170 μs to 75 μs and as Br_2^- decayed a new absorption was produced, with maxima at 410 and 500 nm (Fig. 3).

We ascribe these absorptions at 410 and 500 nm to free-radical products of the oxidation by Br_2^- of tyrosine and tryptophan residues in the membrane. Adams et al. [24] have shown that of the common amino acids at pH 7.8, Br_2^- reacts only with tryptophan ($k = 8 \cdot 10^8\ M^{-1} \cdot s^{-1}$), tyrosine ($k = 2 \cdot 10^7\ M^{-1} \cdot s^{-1}$) and histidine ($k = 1.5 \cdot 10^7\ M^{-1} \cdot s^{-1}$). These reactions are thought to involve an electron transfer [24]. For instance the reaction of Br_2^- with tyrosine results in formation of the phenoxy radical [21, 24, 29]:



(9)

which is recognisable by its characteristic absorption spectrum. The products of reaction of Br_2^- with tyrosine or tryptophan have maxima at 440 and 510 nm, respectively [24], both with $\varepsilon \cong 2 \cdot 10^3\ cm^{-1}$ at pH 7.8. The predominance of the tyrosine radical absorption in Fig. 3 may reflect a large tyrosine:tryptophan ratio in the membrane protein, particularly in view of the approx. 40-fold greater reactivity of free tryptophan over free tyrosine at this pH [24]. However, the reactivity of a tyrosine residue in a protein may not be the same as the free amino acid [25].

In Fig. 4 we show the effect of dodecylsulphate concentration upon the first-order component of the Br_2^- decay, and on the magnitude of the tyrosine product absorption at 410 nm. Dodecylsulphate did not affect the Br_2^- decay itself. In both plots the solid lines we have drawn are those from Kirkpatrick et al. [23] for the solubilization of total membrane protein as a function of dodecylsulphate concentration. The good correlation between membrane solubilization and reactivity with Br_2^- strongly suggests that reactive protein sites (tryptophan, histidine and tyrosine residues) are not readily accessible on the membrane surface.

When the membranes were digested with 0.5 M NaOH for 2 h at 40 °C, and returned to pH 7.6, a further 4-fold increase in reactivity with Br_2^- was observed over that found after solubilization with sufficient dodecylsulphate to cause 100 % protein solubilization [23]. This may indicate that dodecylsulphate binds to the protein membrane and partially protects reactive protein against reaction with Br_2^-; alternatively the alkali digestion may expose more reactive sites than does dodecylsulphate.

A mixture of lactoperoxidase, H_2O_2 and $^{125}I^-$ has been used to label exposed membrane proteins with radioactive iodine [26, 27]. Under some conditions it appears that iodination occurs by a free radical mechanism [27]. We suggest that the free radical species is I_2^-, which reacts somewhat similarly to, although more slowly than, Br_2^- [24, 25]. The lactoperoxidase technique reveals that only one [26]

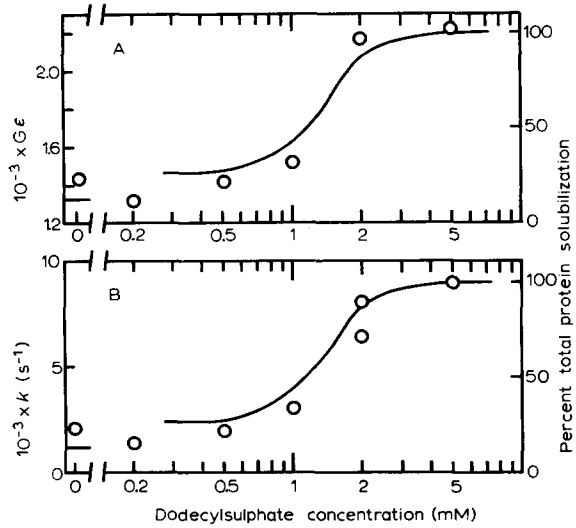


Fig. 4. Comparison of the effect of dodecylsulphate concentration on total membrane protein solubilization, and the reaction of Br_2^- with suspensions of erythrocyte membrane (75 μg protein · ml^{-1}) at pH 7.8. The solid lines are taken from Kirkpatrick et al. [23] for total protein solubilization. The data points in graph A are our observations for the formation of a 410-nm product absorption 0.1 ms after the pulse. In graph B the points represent the first-order rate constants for decay of the Br_2^- absorption.

or two [27] of the membrane proteins are available for reaction at the surface.

Amino acid analysis of membrane protein fragments from human erythrocyte membranes [30–33] shows some of them to possess unusually high proportions of the hydrophobic amino acids. The sequence of one such hydrophobic polypeptide from the major glycoprotein of human erythrocytes has been determined [33]. The major glycoprotein is thought to span the membrane with the hydrophobic region of the protein in association with the inner hydrophobic region of the lipid bilayer [18, 34]. Tyrosine and tryptophan are the two most hydrophobic amino acids [28] and would therefore be expected to be located in such a hydrophobic portion of the protein. In the native membrane we have found that tyrosine and tryptophan residues of the membrane protein are relatively inaccessible to a reagent (Br_2^-) which does react with these residues when the membrane is solubilized with dodecylsulphate or alkali. Our experiments therefore support the current model of the erythrocyte membrane.

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