Pulse Radiolysis of Sulphur Compounds

Part 2.—Free Radical " Repair " by Hydrogen Transfer from Sulphydryl Compounds

By G. E. Adams,* G. S. McNaughton,† and B. D. Michael *

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The intense transient absorption spectrum of the radical-anion $RSSR^-$ produced in the pulse radiolysis of cysteamine solutions $NH_2CH_2CH_2SH$, (RSH) by the reaction $RS+RS^- \rightleftharpoons RSSR^-$ has been used to observe and measure the kinetics of hydrogen transfer reactions from the sulphydryl group to a number of simple organic radicals. This radical repair process is first order in cysteamine concentration and depends upon the dissociation constants of the SH group. In oxygenated solutions containing methanol, oxygen competes with cysteamine for the radical $\cdot CH_2OH$. Rate data are presented and the mechanisms discussed.

A mechanism involving hydrogen transfer from the SH group to polymer radicals has been proposed for the protection of polymers irradiated in the presence of sulphydryl compounds.[1] This radical repair model was later suggested in explanation of the protective effect of cysteamine, $NH_2CH_2CH_2SH$, on irradiated bacteriophage,[2] DNA[3] and also trypsin.[3] E.s.r. techniques have been used to provide some evidence of this type of process in irradiated proteins and other molecules (for a review see ref. (4)). Hydrogen transfer from SH compounds to simple organic free radicals has also been observed directly by pulse radiolysis [5-7] and the repair [5] by cysteamine, of alcohol radicals produced by reaction of OH radicals, was reported. Karmann and Henglein [7] have also observed independently, similar processes in aqueous H_2S solutions.

Pulse radiolysis of aqueous, N_2O-saturated solutions of cysteamine,[8, 9] cysteine,[9] mercaptoethanol [8] and simple mercaptans including the special case of H_2S [10] leads to the formation of strongly absorbing transient species with maxima around 4100 Å (3800 Å for H_2S). In all cases the absorptions have been assigned to radical-anion complexes of the species RS·, the general formula being $RSSR^-$.

The latter species can also be produced directly by electron attachment to disulphides.[9]

$$RSH \text{ (or } RS^-) \xrightarrow{OH} RS\cdot. \quad (1)$$

$$RS\cdot + RS^- \rightleftharpoons RSSR^- \leftarrow RSSR + e^-_{aq}. \quad (2)$$

In part 1 [9] the pulse radiolysis of cysteamine, and the corresponding disulphide cystamine, $(NH_2CH_2CH_2S)_2$, was investigated as a function of pH, solute concentration and other experimental parameters. Partly because of the interplay of the radical equilibrium (1) and the solute equilibrium (3)

$$RSH + OH^- \rightleftharpoons RS^- + H_2O \quad (3)$$

both the optical density of the absorption due to $RSSR^-$ and also, the lifetime of the species, are dependent upon the concentration of RSH (and RSSR when

* British Empire Cancer Campaign for Research, Research Unit in Radiobiology, Mount Vernon Hospital, Northwood, Middlesex, England.

† Institute of Nuclear Science, Private Bag, Lower Hutt, New Zealand.

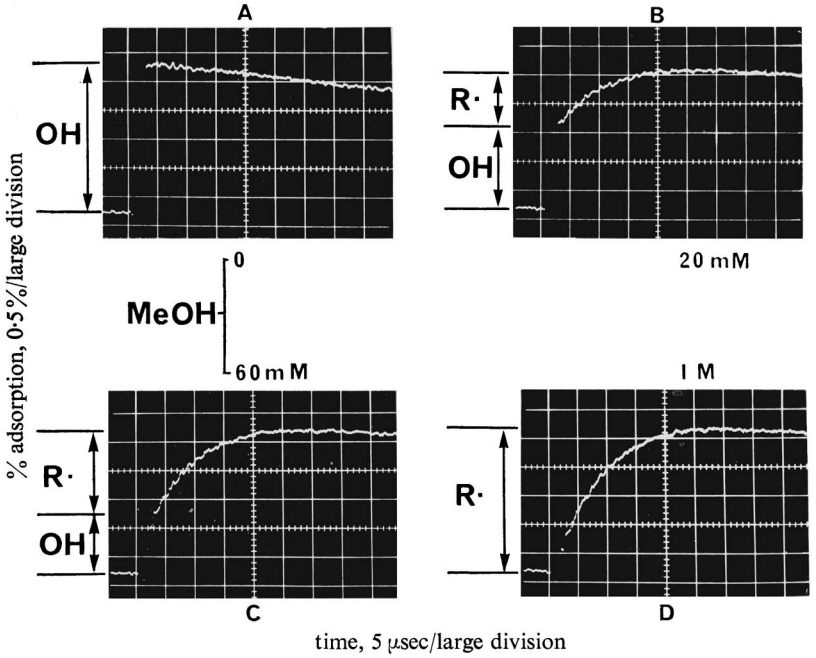


FIG. 1.—Oscillograms demonstrating the hydrogen transfer process. Absorption of $RSSR^-$ produced by direct reaction of OH and cysteamine (rapid rise) and by hydrogen transfer from cysteamine to the radical $\cdot CH_2OH$ (slower rise). 1·13 mM cysteamine; N_2O saturated; pH 7·6; [methanol] as indicated; dose 170 rads/pulse.

To face page 903.]

present in mixtures) and also upon the pH of the solution. Similar phenomena have been observed in solutions of H_2S and alkyl mercaptans.[10]

In the work discussed in this communication the transient absorption due to $RSSR^-$ is used as a marker for the RS radical in a more detailed investigation of some hydrogen-transfer repair reactions for a number of simple organic free radicals produced initially, by reactions of the hydroxyl radical.

EXPERIMENTAL

PULSE RADIOLYSIS

The general technique of pulse radiolysis have been discussed [11-15] and details of the present experimental arrangement have been published.[16] Relative dosimetry was reproducible to $\pm 2\%$. In view of their ease of oxidation, all solutions of cysteamine were prepared in de-aerated water and used as soon as possible thereafter. The pH of the solutions was controlled by the use of 5 mM phosphate buffer and, where appropriate, potassium hydroxide and sulphuric acid. Triply distilled water was used throughout.

RESULTS AND DISCUSSION

Direct observation of free radical repair by cysteamine arose in an experiment designed to measure the rate constant for the reaction between the hydroxyl radical (OH) and this sulphydryl compound (RSH)

$$OH\cdot + RSH \rightarrow RS\cdot + H_2O. \qquad (4)$$

The rate constant k_4 had been determined previously by observation of the build-up of the absorption due to the sulphydryl radical (measured as the complex, $RSSR^-$) at low concentrations of RSH. A value of $k_4 = 4{\cdot}9 \times 10^9\ M^{-1}\ sec^{-1}$ was obtained.[9] Attempts were then made to confirm this result by an independent competition experiment using methanol.

This solute reacts with OH but the product, the radical $\dot{C}H_2OH$, does not absorb at 4100 Å, the maximum of the $RSSR^-$ absorption. Therefore, in solutions of cysteamine containing increasing amounts of methanol, it was expected that the absorption due to $RSSR^-$ observed immediately after the pulse, would be decreased progressively according to the competition between the two solutes for the OH radical. Since the rate constant for the methanol reaction was known, the rate constant for OH with cysteamine could be verified directly. All solutions were deaerated and saturated with N_2O in order to convert hydrated electrons (e^-_{aq}) into OH radicals.

$$N_2O + e^-_{aq} \rightarrow N_2 + OH + OH^-. \qquad (5)$$

Fig. 1 shows some of the oscillograms demonstrating directly the repair process. In these traces (170 rads/pulse), the vertical (upwards) displacement of the light spot represents decreasing light transmission of the solution measured at 4100 Å (0·5%/large division) and the horizontal axis represents time (5μ sec/large division). The electron pulse (0·2 μsec) occurs at the right hand end of the base line trace on the left-hand side of each oscillogram.

Fig. 1*a* (no methanol present) shows the time scale for the formation of the $RSSR^-$ absorption in an N_2O-saturated solution containing 1·13 mM cysteamine buffered at pH 7·6. The maximum optical density (OD) is attained within 2 μsec of the end of the pulse. This is in keeping with the known rate constant for OH oxidation of cysteamine. The slow decline in the OD is due to the subsequent reactions of $RSSR^-$.[1]

Fig. 1*b* shows the development of the absorption when 20 mM methanol is added to the solution before irradiation. About 60% appeared in the first two μsec whereas the remainder is formed over a period of about 25 μsec. In fig. 1*c*, where the methanol concentration is 60 mM, the initial fraction of the absorption is decreased to about 41% and finally, when $[CH_3OH] = 1$ M, very little absorption appears during the first 2 μsec. The spectrum of the transient is unaffected by the presence of methanol and furthermore the subsequent decay kinetics are also unchanged.

We conclude therefore that in methanol solutions two independent processes occur, each of which leads to the formation of $RSSR^-$. Initially, OH radicals react competitively with both solutes (reactions (4), (6)):

$$OH + CH_3OH \rightarrow \dot{C}H_2OH + H_2O. \quad (6)$$

The fraction α of the total yield of OH radicals reacting with the alcohol is governed

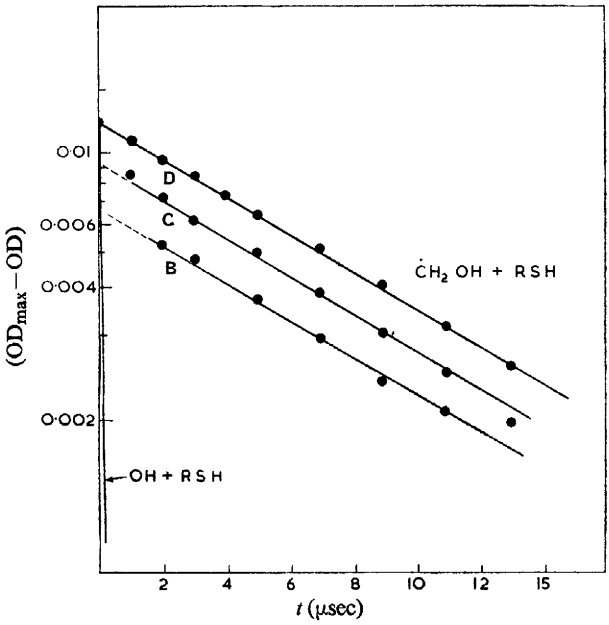


FIG. 2.—First-order kinetics of the repair reaction 1·13 mM cysteamine; N_2O saturated; pH 7·6.

line	methanol
B	20 mM
C	60 mM
D	1 M

by the relative concentrations of the two solutes and also, by the rate constant ratio, k_6/k_4. It can be shown that

$$\alpha = 1 + (k_6[CH_3OH]/k_4[RSH]). \quad (7)$$

The CH_2OH radical is then " repaired " by the hydrogen transfer reaction

$$\dot{C}H_2OH + RSH \rightarrow CH_3OH + RS\cdot, \quad (8)$$

the RS radical being observed as $RSSR^-$.

Therefore, the total observed transient absorption should be constant irrespective of the value of α, provided the kinetics of the build-up are unaffected by the slow second-order decay of the absorption. However, under the low dose conditions of this experiments, this complication was avoided. The rapid process, indicated in fig. 2, by OH corresponds to reaction (4) and the slower process indicated by R represents the repair reaction.

KINETICS OF REPAIR PROCESS

Reaction (8) is bimolecular and the rate of the reaction is given by

$$-\mathrm{d}[CH_2OH]/\mathrm{d}t = k_8[CH_2OH][RSH]. \quad (9)$$

Since the cysteamine concentration is in great excess of the radical concentration produced by the pulse (about 10^{-6} M), eqn. (9) becomes

$$-\mathrm{d}[CH_2OH]/\mathrm{d}t = k'[CH_2OH], \quad (10)$$

where

$$k' = k_8[RSH]. \quad (11)$$

The solution of eqn. 10 is

$$[\cdot CH_2OH] = (\cdot CH_2OH]_0 \exp(-k't), \quad (12)$$

where $[\cdot CH_2OH]_0$ is the initial concentration of $\cdot CH_2OH$ from reaction (6). From (12) and the stoichiometry of (4) and (6) it follows that

$$\begin{aligned}[RSSR^-] &= [\cdot OH](1-\alpha)+[\cdot OH]\alpha[1-\exp(-k't)] \\ &= [\cdot OH][1-\alpha\exp(-k't)],\end{aligned} \quad (13)$$

where [·OH] is the initial ·OH radical concentration. Since

$$\begin{aligned}[RSSR^-_{max}] &= [\cdot OH], \\ [RSSR^-_{max}]-[RSSR^-] &= \alpha\exp(-k't).\end{aligned} \quad (14)$$

Whence

$$\log_{10}(OD_{max}-OD) = \log_{10}(\alpha\varepsilon l)-k't/2{\cdot}303, \quad (15)$$

where ε is the decadic extinction coefficient (cm^{-1}) of the absorbing species and l is the path length (cm).

Fig. 2 shows that eqn. (15) is obeyed. The steep line represents the reaction of OH with RSH (4) and line D represents the repair process (reaction (8)). Lines B and C for 20 and 60 mM methanol respectively, show that the rate of repair is independent of methanol concentration. The parallel lines are displaced since the *proportion* of the total absorption formed by the repair reaction depends upon the relative solute concentrations.

Although the repair process is exponential under these conditions, the first-order rate constant, k' in eqn. (15), should be proportional to cysteamine concentration. To verify this requirement, the repair rate for $\cdot CH_2OH$ was measured over a range of RSH concentrations. Fig. 3 shows a linear relationship between k' and RSH concentration for several alcohols including methanol. The slope of the methanol line is equal to k_8. We find $k_8 = 6{\cdot}8\times10^7\ M^{-1}\ sec^{-1}$.

The conditions of the experiments were such that the kinetic complications which would be introduced by the radical-radical reaction

$$\cdot CH_2OH+\cdot CH_2OH\rightarrow(CH_2OH)_2 \quad (16)$$

were avoided. The dose per pulse was kept low (about 170 rads/pulse) and therefore the maximum concentration of $\cdot CH_2OH$ radicals did not exceed about 10^{-6} M. Even assuming a maximum diffusion-controlled rate constant of about $10^{10}\ M^{-1}\ sec^{-1}$ for reaction (16), the repair reaction will complete overwhelmingly for CH_2OH.

Rate constants were measured for a number of simple organic compounds. The results for methanol, ethanol, 1,4-butanol and t-butanol are shown in fig. 3. In all cases, the oscillographic traces were exponential and the half-lives were inversely

proportional to RSH concentration. The first-order behaviour indicates that if initial attack by OH· on the solute occurs at more than one site, then any differences in

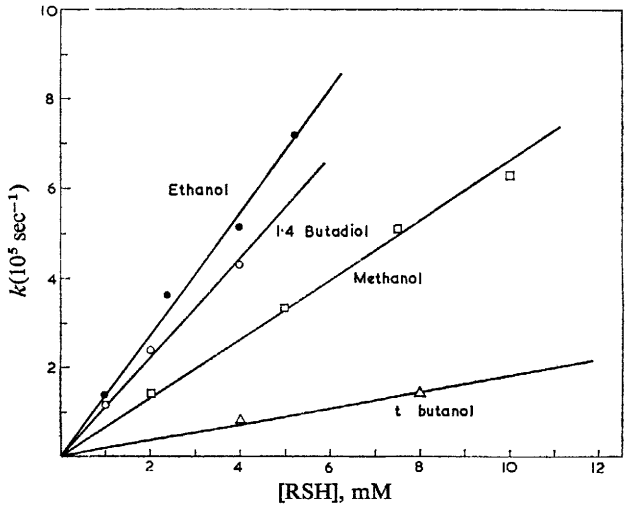


FIG. 3.—Dependence of radical repair rate on cysteamine concentration; N_2O saturated solutions.

the rate constants for repair of the radical products are too small to be revealed by any deviations from first-order build-up. From the slopes, the bimolecular rate constants were calculated and are given, together with the data for other solutes, in table 1.

TABLE 1.—BIMOLECULAR RATE CONSTANTS FOR VARIOUS RADICAL REACTIONS WITH CYSTEAMINE (SH UNIONIZED); ALL SOLUTIONS N_2O-SATURATED

solute	rate constant $10^7\ M^{-1}\ sec^{-1}$
isopropanol	42
acetone	~40
ethanol	14
isobutanol	14
1,4-butadiol	11
n-butanol	8·2
methanol	6·8
glucose	3·2
t-butanol	1·8
allyl alcohol	<1
thymidine	<1
uracil	<1

EFFECT OF pH ON FREE-RADICAL REPAIR

The —SH group in cysteamine ionizes, and therefore the fraction of the solute present in the unionized form depends upon the pH and the equilibrium constant of reaction (3). Since the mechanism of repair involves hydrogen transfer from the SH group, we investigated the kinetics of repair in a pH region where the SH group is unionized. The pK of the SH group in cysteamine is in the range 8·2-8·5.[17, 18] (The value used in part 1 [9] for the unsuccessful attempt to derive a unifying equation for the effect of solute concentration and pH on the life-time of $RSSR^-$, was too low.)

The rate constant for methanol radical repair was determined over the pH range from about 6 to 12. In all cases the reaction was exponential. The bimolecular rate constants calculated from the data, are plotted as a function of pH in fig. 4. The dotted line represents a theoretical dissociation curve of pK = 8·6. The experimental points do not lie on this pK curve although qualitatively, the rate constant does decrease with increasing pH. This effect is in accordance with the hypothesis that hydrogen transfer from a solvated RS^- ion either does not occur or is a very slow reaction.

Although the complex shape of the pH curve is surprising, the data can be reconciled with the known dissociation curves of a similar aminothiol, cysteine (Benesch and

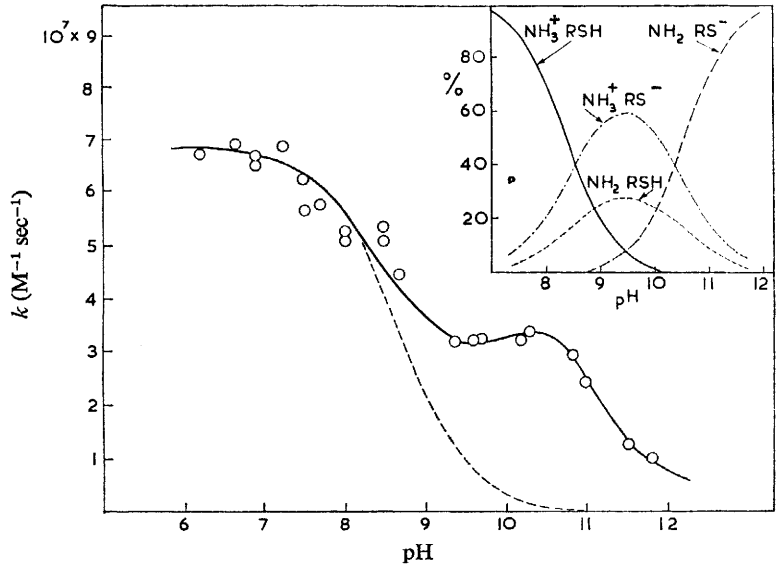


FIG. 4.—Effect of pH on the bimolecular rate constant for the reaction of CH_2OH with cysteamine. *Inset*—Dissociation curves for ionic forms of cysteine (data from ref. (19)).

Benesch [19]). Cysteine exhibits four microscopic forms in the pH range 7-12, viz., NH_3^+RSH, $NH_3^+RS^-$, NH_2RSH and NH_2RS^-. (The carboxyl group is ionized in all these forms.) The relative amounts present over a wide pH range were calculated by these authors and their data are reproduced in the insert to fig. 4. The % solute in which the —SH group is unionized is therefore the sum of the two curves for NH_3^+RSH and NH_2RSH. The compound dissociation curve for the SH group will be less steep than a simple theoretical pK curve and will be of a form not very different from the experimental curve for the rate data in fig. 4. The extent of the distortion will depend upon the relative amounts of two structures in which the SH group is unionized. This proportion may be somewhat different in cysteamine relative to cysteine. However, qualitatively, the data of Benesch and Benesch provide a realistic explanation of the form of the experimental curve in fig. 4. We conclude therefore that in these radical repair reactions, it is the unionized sulphydryl group which is involved in the transfer process.

EFFECT OF OXYGEN

Although the inter-relationship between oxygen and the protective effect of SH compounds in biological material is often experimentally complex, the effect of oxygen on the protective effect of cysteamine on irradiated bacteriophage was interpreted in

terms of competition between oxygen and cysteamine for free radical lesions produced in the bacteriophage by the radiation.[2] It was postulated that the extent of the —SH induced repair of the damaged molecule was decreased by the reaction of oxygen with the radical:

$$\sim\!\dot{\underset{|}{\text{C}}}\!\sim + O_2 \rightarrow \sim\!\underset{|}{\text{C}}\!\sim \quad (17)$$
$$\qquad\qquad\qquad\quad O_2^{\cdot}$$

The resultant peroxy radical was then presumed to be unreactive with, or irreparable by, cysteamine.

Using the simple methanol+cysteamine model, we have investigated the effect of oxygen on the observed repair reaction. Solutions containing 3 mM cysteamine, 1 M methanol and buffered at pH 7·5, were irradiated in the presence of small concentrations of oxygen. In all experiments, a high concentration of N_2O was maintained (~20 mM) in order to eliminate reaction between the hydrated electron and oxygen. Although the rate of repair of the $\cdot CH_2OH$ radical was unaffected by oxygen, the amount of absorption due to $RSSR^-$, indicating the *extent* of repair, was reduced

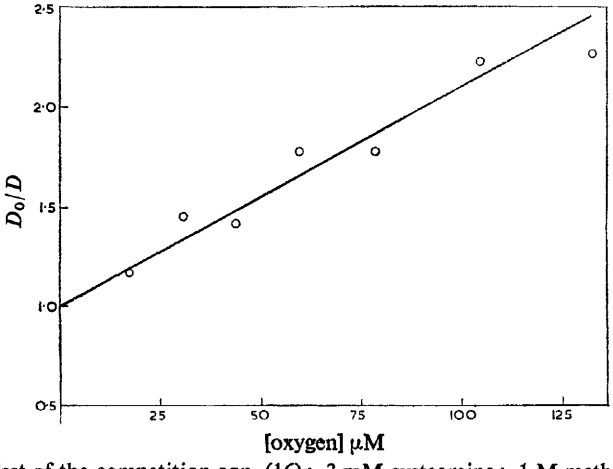


FIG. 5.—Test of the competition eqn. (16); 3 mM cysteamine; 1 M methanol; pH 7·6.

progressively by increasing concentrations of oxygen. Preliminary results were in accord with the hypothesis that reactions (18) and (8) were in competition:

$$\cdot CH_2OH + O_2 \rightarrow \underset{|}{O_2}\!\underset{|}{} \quad (18)$$
$$\qquad\qquad\qquad\quad CH_2OH$$

The usual form of the competition equation can be applied to these reactions. If D_0 is the optical density due to $RSSR^-$ produced by repair in the absence of oxygen, and D is the OD observed when reactions (18) and (8) are in competition, then

$$D_0/D = 1+(k_{18}[O_2]/k_8[RSH]). \quad (19)$$

The data obtained from the competition experiments are plotted according to eqn. (19) in fig. 5. The quantity D_0/D is linear with oxygen concentration as required and the line extrapolates at zero oxygen concentration to $D_0/D = 1{\cdot}0$. It would appear therefore, that simple competition occurs in this system, and from the slope of the time in fig. 5, we find k_{18}/k_8 to be 33, whence k_{18} is $2{\cdot}3\times10^9$ M^{-1} sec^{-1}. There are, however, uncertainties in this indirect estimate. The entity $RSSR^-$ is formed in a two-component step, and therefore, under some conditions, the intermediate $RS\cdot$ radical

may react to some extent with oxygen and at high oxygen concentrations, this reaction can occur during the period of the observed build-up, thus introducing kinetic complications. Because of these and other considerations, although the data clearly indicate that oxygen is very efficient in inhibiting radical repair, in the absence of more information on the mechanism, the experimental value for k_{18} should be taken as an order of magnitude estimate only.

GENERAL APPLICABILITY OF REPAIR MODEL

We believe that the simple methanol system provides a clear demonstration of the repair model. The main question to be resolved, however, is its general applicability to other free radical systems including complex polymeric and biologically important molecules. We have measured hydrogen transfer rate constants for a number of simple free radicals. These are listed in table 1.

The various alcohols all react with OH radicals by a hydrogen abstraction process:

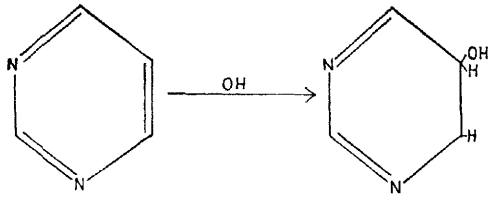
$$RCH_2OH + OH \rightarrow RCHOH + H_2O. \quad (20)$$

In all cases hydrogen transfer from cysteamine occurred, although some variation was observed in the magnitude of the rate constants.

In similar studies with H_2S, Karmann and Henglein [7] have shown that the transfer reaction from H_2S to alcohol radicals proceeds via an intermediate radical-H_2S complex. Under certain conditions, the first-order rate constant was not proportional to H_2S concentration. In the cysteamine experiments, however, there is a proportionality between cysteamine concentration and the rate of H transfer, and therefore, the rate-determining step in this case, is not the dissociation of a similar cysteamine + radical complex. It would follow from the data that the lifetime of the complex would be much less than ~ 1 μsec in cysteamine solutions.

The trend in the radical repair constants is due, probably, to a combination of steric hindrance and inductive effects on the strength of the α carbon-hydrogen bond. However, the overall spread is little more than an order of magnitude and therefore, since the reactions between oxygen and the various radicals will be probably diffusion-controlled, the relative efficiencies of peroxy radical formation to radical repair should be, in general, in the range 20-200. The extrapolation from the alcohol systems to bacteriophage may not then be as great as at first sight appears. Recent results show that repair can be observed directly in solutions of a simple polymer (polyethylene oxide) [20]; the H atom transfer rate was unaffected by large changes in molecular weight.

In table 1, there are some examples of solutes for which no repair was observed. These include an aromatic compound and some unsubstituted pyrimidines. It is known that OH radicals add to aromatic rings structures and also to the 5 position on the pyrimidine ring [21]



Hydrogen transfer to such a radical would not lead to the restitution of the original structure. The failure to observe any repair for radicals produced by OH attack on this type of molecule is in accord with the mechanism. However, this series of

experiments illustrates hydrogen transfer for radicals produced by OH oxidation only. Radicals produced in other processes, (direct absorption or reductive free radical attack) may be restorable by H transfer from SH compounds.

In pulse radiolysis experiments, which may be relevant to repair phenomena in radiobiological systems, Shalek, Ebert and Davies,[22] have observed transient absorption spectra in aqueous solutions of lysozyme. A transient, produced by reaction of OH radicals with the enzyme, appears to react with cysteine, although the formation of any cysteine radical-complex is masked by the absorption of the other lysozyme transient in this spectral region.

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