SINGLET OXYGEN AND RIBOSOMES: INACTIVATION AND SITES OF DAMAGE†

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Summary

The role of singlet oxygen ($^1O_2$) in the photosensitized inactivation of ribosomes by dyes, furocoumarins and ketones has been investigated. With many dyes, $^1O_2$ is a damaging species but not all of the inactivation seen with some dyes (*e.g.* acridine orange, pyronine Y etc.) can be attributed to it, indicating that type I processes are also involved. Furthermore, with some dyes (*e.g.* acridine orange and acriflavine) inactivation of ribosomes occurs even in the absence of oxygen. Inactivation by furocoumarins is mainly through $^1O_2$. However, $^1O_2$ plays a minor role in ketone-photosensitized systems where free-radical reactions seem to dominate.

Analyses of damage to the macromolecules within the ribosomes indicate no chain breaks in ribosomal ribonucleic acid on photosensitization with the dyes, furocoumarins or ketones. However, with some dyes, damage to ribosomal proteins is seen by gel electrophoresis.

1. Introduction

Singlet oxygen ($^1O_2$) is an important intermediate in photosensitized processes in aerated biological and biochemical systems. Several types of cells, enzymes, amino acids and guanosine are damaged by $^1O_2$ [1 - 7]. Additional mechanisms of sensitized photo-oxidations (photodynamic effect, type I) have also been suggested [2, 3, 8] and recent results on the photochemistry of dyes are consistent with these suggestions [2, 9 - 12]. Poppe and Grossweiner [13] showed that $^1O_2$ was involved in the furocoumarin-photosensitized inactivation of lysozyme. The major component of the photosensitized inactivation of *Escherichia coli* (*E. coli*) ribosomes by methylene blue (MB) and furocoumarins (8-methoxypsoralen (MOP) and 4,5',8-trimethylpsoralen (TMP)) was shown to be $^1O_2$ [14 - 16]. Ito [1] has reviewed the involvement of $^1O_2$ in cellular systems. There have also been suggestions

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that dye-sensitized photo-oxidation leads to deoxyribonucleic acid (DNA) chain breaks [17, 18]. However, with $^1O_2$, base damage of DNA is more probable than chain breaks [19].

The importance of $^1O_2$ in biological systems has been further enhanced by recent reports of its formation in enzymatic reactions. For example, Vidigal-Martinelli *et al.* [20] have shown that $^1O_2$ is formed in the horseradish peroxidase–malonaldehyde–oxygen system; Khan *et al.* [21] and Kanofsky [22] have shown the formation of $^1O_2$ in reactions of other peroxidases. Evidence for the generation of $^1O_2$ as a transient byproduct of the peroxidative decomposition of lipids in microsomes [23] and in intact organs [24] suggests that *in vivo* lipid peroxidation may also be a source of some $^1O_2$ in living systems.

Ribosomes are the central component of the *in vivo* protein synthetic machinery. Photoinactivation of 70S ribosomes by Rose Bengal (RB) [25] and changes in several proteins from the 30S ribosomal subunit [26] have been reported. We have been working with ribosomes to study the effects of various short-lived reactive species (free radicals, excited states) [14 - 16, 27 - 31]. Work on $^1O_2$ damage to ribosomes has been part of this study. Although methods have been developed by our colleagues to produce pure $^1O_2$ under high pressures of oxygen [32, 33], we have been able to use that technique only to establish qualitatively that $^1O_2$ inactivates ribosomes [34]. Ribosomes are delicate particles which do not lend themselves to investigation under pressurizing and depressurizing conditions required for such work. Therefore, we undertook to study ribosome inactivation, photosensitized by a series of dyes, furocoumarins and ketones. Some of the work has been published [14 - 16]. In this paper we describe further results on the involvement of $^1O_2$ as a damaging species in various photosensitized systems. The importance of $^1O_2$ in these systems varies from being a major damaging species in some dyes and furocoumarins to a minor species in some ketones.

2. Experimental details

2.1. Materials

The following dyes were obtained from Eastman Kodak Company: MB, RB, acridine orange (AcO), pyronine Y (Pyr-Y), safranine O (Saf-O) and ethidium bromide (EB). Bengal red B (BRB) and acriflavine (AcF) were obtained from Aldrich. The ketones used were acetophenone (AF) (BDH; spectral grade), benzophenone (BF) (James Hinton; zone refined) and camphorquinone (CQ) (Aldrich). The furocoumarins, MOP and TMP, were obtained from Elder Pharmaceuticals; the enzymes, proteinase-K and ribonuclease A and T_1 were obtained from Sigma.

2.2. Sample preparations

E. coli (MRE-600) ribosomes [29] and aminoacyl-transfer-ribonucleic acid synthetases [27] were prepared as previously described. The ribosome

samples for irradiation were prepared in 10^{-2} mol dm^{-3} Tris-HCl (2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride), pH 7.5 or pD 8.0, containing 10^{-2} mol dm^{-3} magnesium chloride and the appropriate photosensitizer. The D_2O solutions contained 3% H_2O or less, since stock solutions of ribosomes were always stored in H_2O buffer. The concentrations used for the dyes were as follows ($\times 10^{-5}$ mol dm^{-3}): MB, 3.1; RB, 2.43; AcO, 1.2; AcF, 1.2; Pyr-Y, 33; Saf-O, 0.76; EB, 7. The concentrations were 10^{-4} mol dm^{-3} and 7×10^{-6} mol dm^{-3} for furocoumarins MOP and TMP respectively and were 2.6×10^{-4} mol dm^{-3}, 4.3×10^{-5} mol dm^{-3} and 5.4×10^{-3} mol dm^{-3} for ketones AF, BF and CQ respectively. The stock solutions of AF, BF, MOP and TMP were prepared in ethanol and of CQ in *tert*-butanol. The total amount of ethanol or *tert*-butanol in the final solution was always kept below 1% and did not interfere with the biological activity assay of the ribosomes, as determined by control experiments.

2.3. Irradiations

The samples were irradiated by monochromatic light as described previously [14, 16, 17]. The wavelengths were selected on the basis of the λ_{max} of the photosensitizer and the intensity maxima of the lamp. These wavelengths and the corresponding incident light intensity were as follows: 590 nm and 3.6×10^{-3} W for MB, 559 nm and 1.8×10^{-3} W for RB and BRB, 556 nm and 3.7×10^{-3} W for Pyr-Y and 513 nm and 1.9×10^{-3} W for EB and Saf-O, in conjunction with a Corning filter 0-51 with a cut-off at 340 nm; 313 nm and 5.6×10^{-4} W for the ketones and furocoumarins in conjunction with a Corning filter 9-54 with a cut-off at 295 nm. The dose was monitored with a United Detector Technology optical power meter, 21A.

The biological activity of the ribosomes was assayed by Poly U-directed polyphenylalanine synthesis [29] using limiting concentrations of ribosomes. Gel electrophoresis of ribosomal ribonucleic acid (r-RNA) from proteinase-K-treated ribosomes was carried out on 3% acrylamide sodium dodecylsulphate (SDS) gels [35]. Unidimensional gel electrophoresis of proteins was carried out on ribonuclease A- and ribonuclease T_1-treated ribosomes by the method of Laemmli and Favre [36].

3. Results

3.1. Ribosome inactivation

3.1.1. Dyes

Different levels of ribosome inactivation were obtained with all the dyes used, on exposure to light. Controls with dyes in the dark showed no inactivation of ribosomes. In most cases, the presence of oxygen was necessary for ribosome inactivation; however, with some of the dyes inactivation was also observed in the absence of oxygen. The detailed results for some of the dyes are described below.

Photosensitization by RB at 559 nm is shown in Fig. 1. The inactivation (in air) is partially protected by the known $^1O_2$ quenchers, NaN_3 [37],

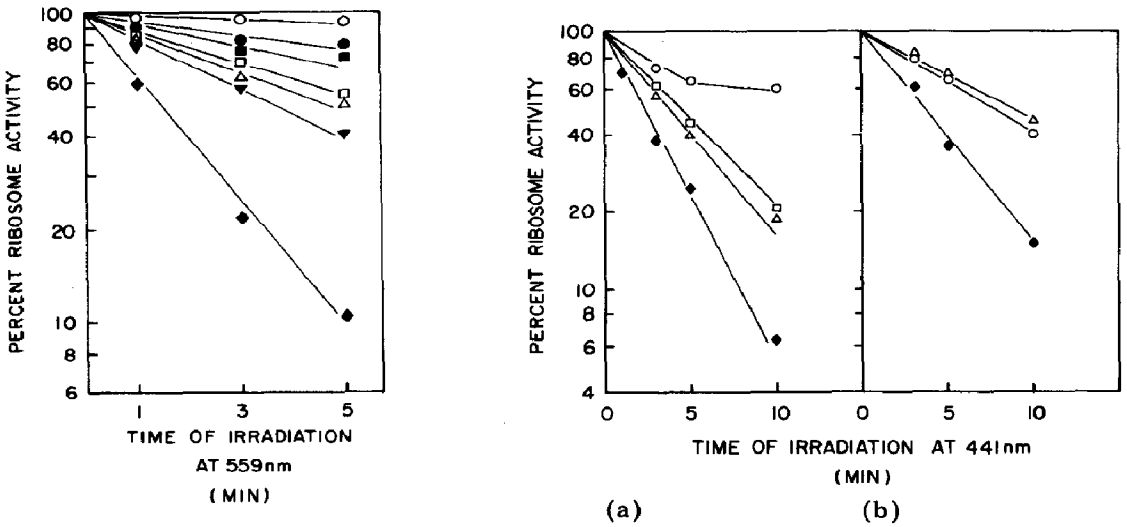


Fig. 1. Effect of additives on the RB-photosensitized inactivation of ribosomes in the presence and absence of oxygen: ♦, inactivation in air (no additives); ▼, 6×10^{-4} mol dm^{-3} AsA; △, 0.15 mol dm^{-3} NaN_3; □, 6×10^{-3} mol dm^{-3} ME; ■, 4.5×10^{-2} mol dm^{-3} ME; ●, 2.5×10^{-2} mol dm^{-3} Cys; ○, nitrogen-purged solution.

Fig. 2. Photosensitized inactivation of ribosomes by (a) AcO and (b) AcF in the presence and absence of oxygen: ♦, inactivation in air (no additives); △, 0.15 mol dm^{-3} NaN_3; □, 6×10^{-3} mol dm^{-3} ME; ○, nitrogen-purged solutions.

ascorbic acid (AsA) [37] and cysteine (Cys) [38], as well as by mercaptoethanol (ME), which has been found to protect $^1O_2$-mediated bleaching of 1,3-diphenylisobenzofuran [16]. The inactivation was negligible in the absence of oxygen (Fig. 1). Very similar results were obtained when BRB was used instead of RB.

Figure 2(a) shows the results of AcO-photosensitized inactivation of ribosomes. In this case, there is significant inactivation of ribosomes in the absence of oxygen. In the presence of air, the relative protection by both NaN_3 and ME is less than that for RB (Figs. 2(a) and 1). With AcF, the inactivation in the absence of oxygen is even greater (Fig. 2(b)). However, the protection of the inactivation in air by NaN_3 is similar to that observed in RB and greater than that in AcO.

3.1.1.1. The D_2O effect. Enhanced damage due to the longer lifetime of $^1O_2$ in D_2O is a good indicator of the intermediacy of $^1O_2$ [39]. The data for the D_2O effect on ribosome inactivation for various dyes and furocoumarins are shown in Figs. 3 - 5. On the basis of these data, the relative contribution of $^1O_2$ in the various systems is as follows: MB > RB = BRB > AcF > AcO > Pyr-Y > EB = 0. For EB, there appears to be no contribution by $^1O_2$ since the D_2O effect is zero. The furocoumarin MOP shows enhancement in ribosome inactivation (Fig. 5) similar to that seen with MB (Fig. 3), while that with TMP is slightly lower (Fig. 5).

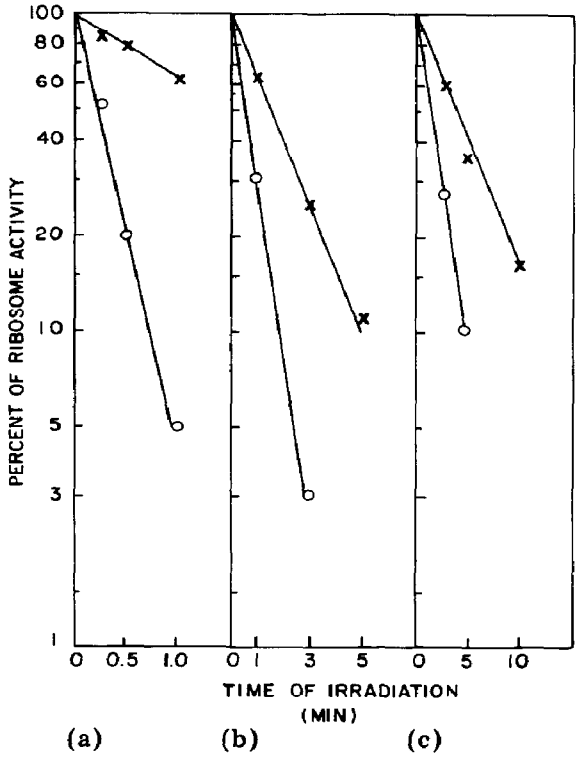


Fig. 3. Photosensitized inactivation of ribosomes in air by (a) MB ($E_T(35)$), (b) RB ($E_T(39)$) and (c) AcF ($E_T(51)$) in D_2O-buffered solutions (○) and H_2O-buffered solutions (×).

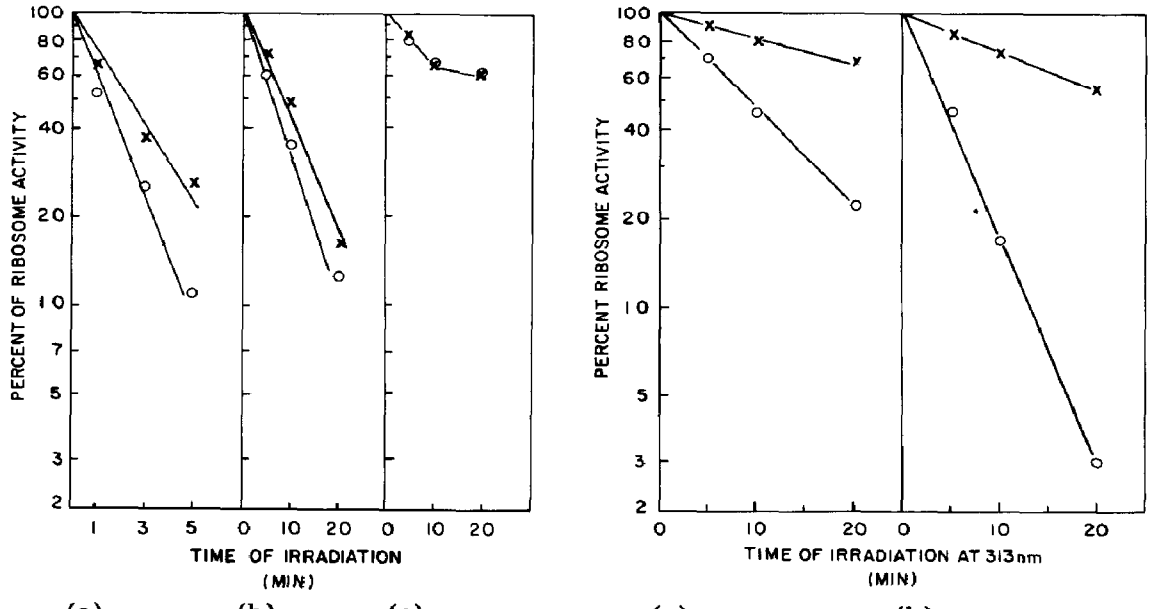


Fig. 4. Photosensitized inactivation of ribosomes in air by (a) AcO ($E_T(49)$), (b) Pyr-Y and (c) EB in D_2O-buffered solutions (○) and H_2O-buffered solutions (×).

Fig. 5. Furocoumarin-photosensitized inactivation of ribosomes in air in D_2O-buffered solutions (○) and H_2O-buffered solutions (×) of (a) TMP and (b) MOP.

On the basis of the protection provided by 0.15 mol dm^{-3} NaN_3, the order of $^1O_2$ contribution follows the same order as seen in the enhancement of inactivation by D_2O described above (Figs. 1 and 2 and unpublished data).

3.1.2. Ketones

We have extended our acetone work [27] to other ketones with lower triplet energies. These results with AF, BF and CQ are shown in Fig. 6. The D_2O effect is quite small in these cases also, indicating that the contribution of $^1O_2$ is small in these systems, with CQ showing the largest $^1O_2$ component. NaN_3 (0.15 mol dm^{-3}) provides very large protection against inactivation with AF and BF but somewhat lower with CQ. ME and AsA provide good protection with all three ketones. In the absence of air the ribosome inactivation is less than 10% for BF and CQ and about 30% for AF (Fig. 6).

Complex formation involving triplet ketones or ketyl radicals has been suggested [40 - 42]. However, our preliminary work using ^3H-labelled AF and BF shows that it is not important in our system since very little incorporation of ^3H-label on ribosomes was seen.

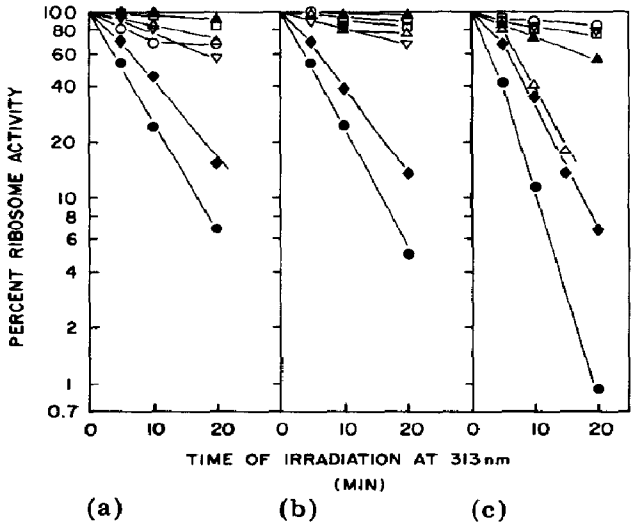


Fig. 6. Photosensitized inactivation of ribosomes by ketones (a) AF (E_T(76)), (b) BF (E_T(69)) and (c) CQ (E_T(50)) (the samples were made up in H_2O-based buffer and aerated unless otherwise stated): ♦, no additives; ▽, 5×10^{-4} mol dm^{-3} AsA; △, 5×10^{-2} mol dm^{-3} NaN_3; ▲, 0.15 mol dm^{-3} NaN_3; ■, 6×10^{-3} ME; ●, inactivation in air (D_2O-based buffer); ○, nitrogen-purged solutions (H_2O-based buffer).

3.2. Damage at macromolecular level

3.2.1. Chain breaks in ribosomal ribonucleic acid

In order to see whether the photosensitized inactivation of ribosomes leads to chain breaks in r-RNA within the ribosomes, SDS gel electrophoresis was carried out on proteinase-K-treated samples of both irradiated and control ribosomes. None of the r-RNA samples from ribosomes photo-

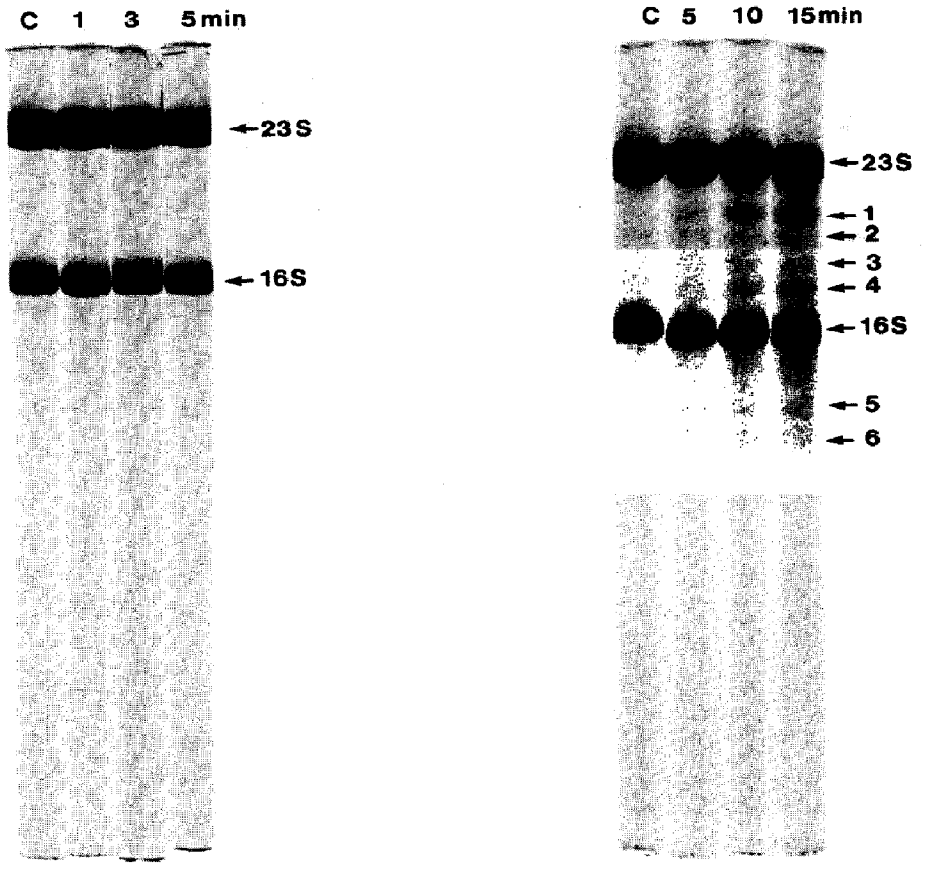


Fig. 7. Gel electrophoresis patterns of r-RNA from RB-photosensitized ribosomes. (a) r-RNA from ribosomes in the control sample C and in samples irradiated for 1, 3 and 5 min; only 23S and 16S r-RNA bands are visible. (b) For comparison, samples from γ-irradiated ribosomes, showing small fragments 1 - 6 from 23S and 16S r-RNA in samples irradiated for 5, 10 and 15 min but not in the control sample C.

sensitized by the dyes (MB, RB, AcO, AcF, Saf-O and EB), furocoumarins (MOP and TMP) and ketones (acetone, AF, BF and CQ) showed any chain breaks. Two examples of these results on r-RNA from RB-photosensitized (Fig. 7(a)) and MOP-photosensitized (Fig. 8) ribosomes are shown. No evidence of chain breaks was seen in the 23S and 16S r-RNA, which would normally show up as smaller fragments, *e.g.* fragments 1 - 6 (Fig. 7(b)), seen in r-RNA from ribosomes subjected to γ radiolysis, for example. Figure 8 shows results from MOP-photosensitized ribosomes in H_2O- and D_2O-based buffer. Ribosomes are inactivated to about 85% by 50 min of illumination in H_2O-based buffer, compared with more than 95% after 20 min of illumination in D_2O [16], and yet no fragmentation of r-RNA is seen. The chain breaks reported earlier with MB [15] were most probably due to *in vivo* ribonuclease contamination of the ribosomes prepared from *E. coli*

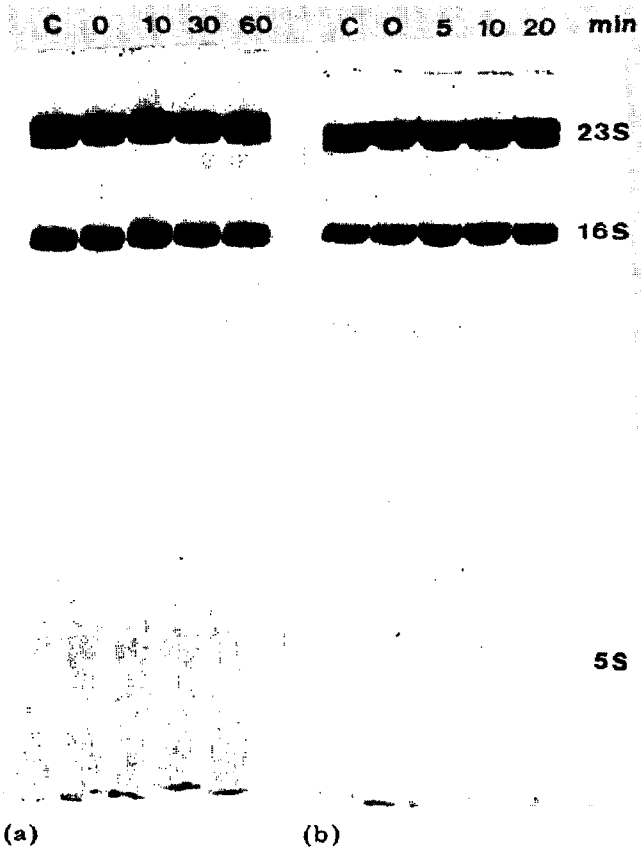
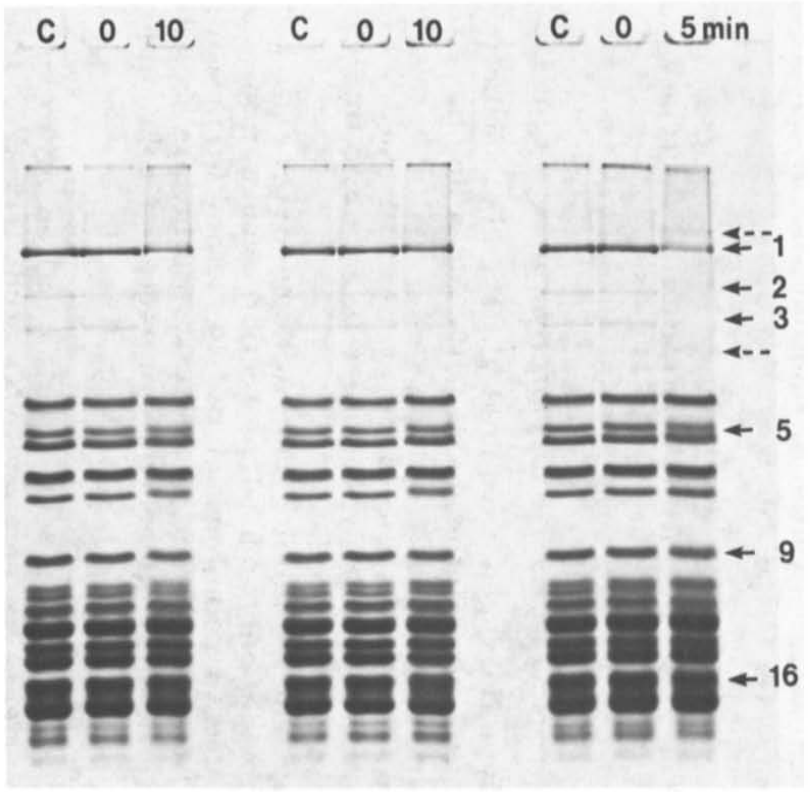


Fig. 8. Gel electrophoresis patterns of r-RNA from MOP-photosensitized ribosomes. r-RNA from ribosomes in (a) H_2O- and (b) D_2O-based buffers in control sample C without MOP and in samples irradiated for 0, 10, 30 and 60 min (in H_2O) and 0, 5, 10 and 20 min (in D_2O).

B cells. This contamination is not a problem with ribosomes from *E. coli* MRE-600 cells used in this study.

3.2.2. Damage to ribosomal proteins

Unidimensional gel electrophoresis patterns on ribosomal proteins from ribosomes photosensitized by AcF, AcO and RB are shown in Fig. 9. The bands labelled 1, 5, 9 and 16 show a decrease in intensity in all three cases, although more pronounced in RB-photosensitized ribosomes. Although bands 1, 5, 9 and 16 are the first to show decreases in intensity, almost all the proteins are affected to some extent, as can be seen in the absorbance scans of the stained gels (Fig. 10). Figure 10(a) shows the superimposed scans from control and AcF-photosensitized ribosomes. The shaded areas represent the decreases due to photo-oxidation in the proteins. Similar superimposed scans on protein gels from control and AcO-photosensitized ribosomes are shown in Fig. 10(b). The decreases in proteins in this case are



(a) (b) (c)

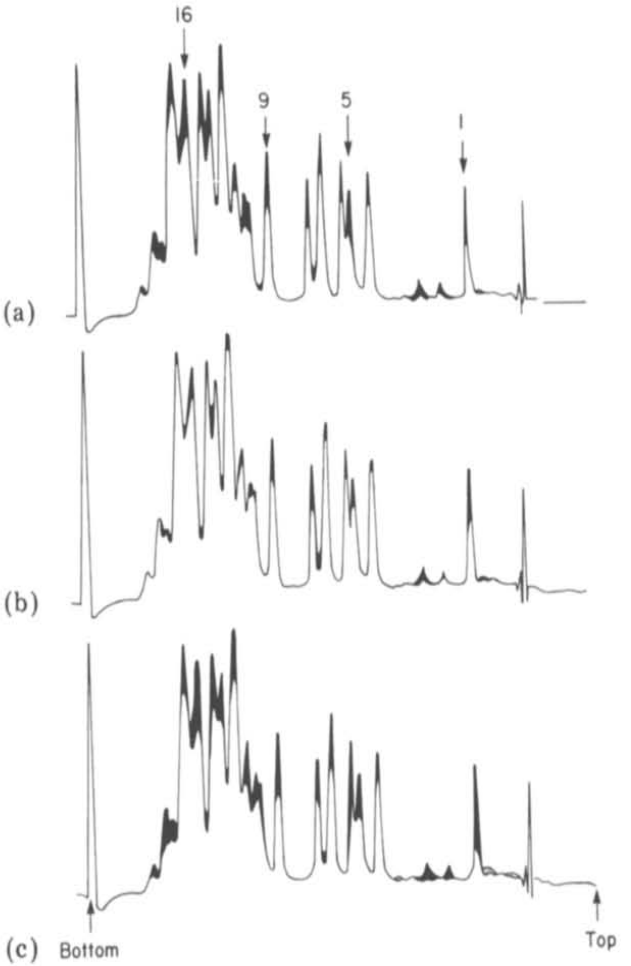


Fig. 9. Unidimensional gel electrophoresis patterns on ribosomal proteins from (a) AcF-, (b) AcO- and (c) RB-photosensitized ribosomes for samples irradiated for 5 or 10 *min*: C, control without dye; 0, control with dye in the dark.

Fig. 10. Absorbance scans on stained gels at 580 nm: (a) AcF; (b) AcO; (c) RB. The shaded areas represent decreases in the protein bands on irradiation. The conditions are similar to those for Fig. 9.

much smaller compared with those treated with AcF. These decreases are very much greater for RB-photosensitized ribosomes (Fig. 10(c)). For a similar degree of inactivation of ribosomes (about 90%) by the three dyes (AcF and AcO at 10 min and RB at 5 min), the relative decreases in proteins are in the order RB > AcF > AcO, in agreement with the relative order of decreasing $^1O_2$ production.

As indicated by the arrows in Figs. 9 and 10, bands 1, 5, 9 and 16 show greater loss than the rest of the proteins. Band 1 corresponds to S1 and bands 5, 9 and 16 to proteins S2, S6 and S15/16 respectively by analogy with the proteins identified in MB-photosensitized inactivation of ribosomes [15]. Our preliminary results with these dyes show that the protein damage is enhanced in D_2O compared with that in H_2O buffers, suggesting that $^1O_2$ is responsible for the damage. One very small peak in the AcF and AcO results and three small peaks in the RB results (shaded peaks in Figs. 10(a), 10(b) and 10(c) respectively) appear after irradiation. Although the identities of these peaks remain to be established, these could represent cross-linked proteins.

4. Discussion

4.1. Ribosome inactivation

4.1.1. Dyes

Photosensitized inactivation of ribosomes RiBH can occur via type I or type II processes [14, 16] as follows:

$$\text{dye} + \text{RiBH} + h\nu \xrightarrow[\text{free-radical formation}]{\text{electron transfer,}} \text{RiBH inactivation} \qquad \text{type I}$$

$$\text{dye} + O_2 + h\nu \longrightarrow {}^1O_2 \xrightarrow{\text{RiBH}} \text{RiBH inactivation} \qquad \text{type II}$$

While in the type II oxygen is required, the type I mechanism is applicable in the presence, as well as in the absence, of oxygen. In our system, $^1O_2$ formation is indicated by the D_2O effect as well as by the protection provided by NaN_3. The order of importance of $^1O_2$ varies with the dye as follows: MB > RB = BRB > AcF > AcO > Pyr-Y > EB = 0, as shown in Section 3. For EB, no $^1O_2$ seems to be produced.

In the systems containing RB, AcF and AcO (Figs. 1 and 2), the $^1O_2$ quenchers provide only partial protection against ribosome inactivation. This is attributed to a combination of two factors: (a) inactivation via type I processes and (b) $^1O_2$ formation by dyes absorbed on the ribosomes which would not be efficiently quenched by protectors in solution [14].

4.1.2. Ketones

Excited ketones would take part in (a) energy transfer to substrate, (b) energy transfer to oxygen to give $^1O_2$ (type II) and (c) free-radical forma-

tion (type I) [40, 43, 44]. The quantum yield of intersystem crossing is very high (about unity) and the lifetimes of the excited singlet states of ketones are quite short, less than 10^{-8} s [44]; $t_{1/2}$ for $^1CQ \lesssim 5 \times 10^{-9}$ s [45]. Therefore, only the reactions of triplet states of ketones need to be considered:

$$R_2CO \xrightarrow{h\nu} {}^1R_2CO^* \longrightarrow {}^3R_2CO^* \qquad (1)$$

4.1.2.1. Energy transfer to ribosomes. Ribosomes contain several components (*e.g.* pyrimidines and amino acids) whose triplet energies are lower than those of AF [46] but higher than those of BF and CQ. Thus, ribosome inactivation following energy transfer could occur only for AF:

$$^3AF^* + RiBH \longrightarrow AF + RiBH^* \longrightarrow RiBH \text{ inactivation} \qquad (2)$$

and would be reduced by oxygen due to quenching:

$$^3R_2CO^* + O_2 \longrightarrow {}^1O_2 \qquad (3)$$

4.1.2.2. $^1O_2$ formation. The D_2O effect (Fig. 6) with these ketones is small (20% or less), although it increases slowly from AF to CQ with decreasing triplet energy. Thus, the yields of $^1O_2$ from these excited ketone triplets are small. This is consistent with the results obtained with acetone [27] and those obtained by Wu and Trozzolo [47] with various ketones. It is reasonable to expect that the efficiency of the type I reaction (4) would decrease with decreasing triplet energy, thus increasing the proportion of triplets that react by reaction (3).

4.1.2.3. Free-radical reactions. Triplet ketones (reaction (1)) would lead to ribosome inactivation

$$^3R_2CO^* + RiBH \xrightarrow[\text{via free radicals}]{\text{type I}} RiBH \text{ inactivation} \qquad (4)$$

through free-radical reactions similar to those of acetone [27]. The protection seen with added ME and AsA (Fig. 6), for all the ketones used, is consistent with the type I process via free radicals. With BF and CQ, and to a lesser extent with AF, oxygen is required for inactivation (Fig. 6). This inactivation is attributed to fixation of damage through peroxy radical formation [30, 31]. We have found that the ketyl radical ($(CH_3)_2\dot{C}OH$), formed from isopropanol in radiolysis [30, 31] and in the photosensitized reaction of acetone [27], inactivates ribosomes. Similar inactivation by the ketyl radicals of the three ketones used in this work is quite probable. The inactivation efficiency of the ketyl radical decreases on reaction with oxygen, since the peroxy radical formed dissociates to give $O_2^{\cdot-}$ [48], which does not inactivate ribosomes [29]. Thus, in the presence of oxygen, the inactivation via ketyl radicals would decrease, but that due to free-radical formation on ribosomes would increase via ribosomal peroxy radical formation [31].

4.1.2.4. Role of NaN_3. The protection afforded by NaN_3 is much greater than the $^1O_2$ contribution as indicated by the D_2O effect (Fig. 6). This is attributed to quenching of the ketone triplets, as for acetone [27], where similar protection by NaN_3 was seen for acetone-photosensitized inactivation of ribosomes in the absence of oxygen. This is consistent with other reports of quenching of excited states by NaN_3 [49, 50].

4.1.3. Macromolecular analysis

4.1.3.1. Ribonucleic acid chain breaks. The work of von Sonntag and Schulte Frohlinde [51] has established that free-radical formation on sugar moieties, following $^{\bullet}OH$ attack, leads to phosphate release and concomitant chain breaks in DNA. A similar mechanism would be expected for chain breaks in r-RNA following $^{\bullet}OH$ attack, on radiolysis in air (Fig. 7(b) and ref. 35). However, $^1O_2$ is not expected to react with sugars, so a parallel mechanism is not possible. $^1O_2$ does react with bases, although the rate constants are very low (10^6 or less, compared with 10^8 or more for several amino acids). This would lead to opening of the guanine ring [1, 2, 19]. However, such ring opening is not likely to cleave the phosphodiester bond leading to chain breaks. Therefore, the lack of chain breaks seen with dyes and furocoumarins is consistent with the types of reactions expected from $^1O_2$. In view of our work, the mechanism of the reported chain breaks in DNA [17, 18] on photodynamic action needs to be further investigated. Our results also show that the type I component of damage by ketones and dyes does not lead to formation of free radicals on sugars that can, in turn, release phosphates and give chain breaks.

4.1.3.2. Protein damage. The observed damage to ribosomal proteins is consistent with the known reactivity of $^1O_2$ with amino acids and some proteins [37]. Some of the damage seen may also be due to the type I reactions.

Acknowledgments

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