

ELECTRON TRANSFER FROM THE EXCITED STATE OF TYROSINE TO COMPOUNDS CONTAINING DISULFIDE LINKAGES

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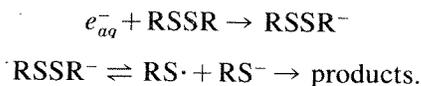
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Abstract—The flash photolysis of aqueous solutions of tyrosine has been studied in the presence of various concentrations of the cyclic disulfide sodium lipoate (thioctic acid, Na⁺ salt). In addition to the formation of phenoxyl radicals and hydrated electrons (and possibly H atoms) from the photoionization of tyrosine, the characteristic spectrum of the radical anion RSSR⁻ of lipoate was also observed in neutral as well as in alkaline solutions. From the dependence of these yields upon the concentration of lipoate, it was found that a long-lived triplet excited state of tyrosine, rather than the singlet excited state, is involved in these reactions. The negative radical ions RSSR⁻ are formed by two distinct pathways: (a) Na⁺-lipoate reacts with the solvated electrons which are ejected from the tyrosine triplets ³Tyr → RO· + e_{aq}⁻ + H⁺ followed by e_{aq}⁻ + RSSR → RSSR⁻, and (b) by direct interaction of lipoate with triplet excited tyrosine, resulting in the transfer of a negative charge from tyrosine to the disulfide linkage. At high lipoate concentrations, the singlet excited state of lipoate is quenched, k₄ = 1.6 × 10¹⁰ M⁻¹ sec⁻¹, but this reaction does not lead to the formation of RSSR⁻ radical ions.

INTRODUCTION

TYROSINE in its electronically excited state is a distinctly more reactive species than in its ground state. It has been found to become a stronger acid upon excitation (Feitelson, 1964) to the singlet excited state ¹Tyr, as well as to undergo photodissociation with the formation of phenoxyl radicals and solvated electrons (Jortner *et al.*, 1963; Land and Porter, 1963; Dobson and Grossweiner, 1965). The photoproducts, particularly the reactive solvated electron, can react further with other species present in the solution (Feitelson and Hayon, 1973). However, the optically excited tyrosine can also interact directly with other molecules in its environment and it is these direct reactions which are the subject of the present study.

The interaction in proteins of excited tyrosine with adjacent —S—S— linkages has recently attracted much attention (Cowgill, 1966; Shapira and Stein, 1968; Dose, 1968; Longworth, 1968; Arian *et al.*, 1970). For this reason a substance with an —S—S— group was chosen as a potential reaction partner for the excited tyrosine. Compounds containing disulfide bonds are known to react readily with hydrated electrons, e_{aq}⁻, (Hoffman and Hayon, 1972). Such reactions have been studied by pulse radiolysis (Hoffman and Hayon, 1972, Adams *et al.*, 1967, Karmann *et al.*, 1967, 1969), and were found to proceed by the general scheme:



At high pH and in the presence of compounds containing a sulfhydryl group, RSH, the equilibrium between RSSR⁻ and RS· + RS⁻ is displaced towards the left and consequently the lifetime of RSSR⁻ is increased. In neutral solutions (in presence or

absence of sulfhydryl compounds, provided the $\text{pH} \ll \text{p}K_a$ of RSH) the lifetime of the negative ion RSSR^- is relatively short for most disulfides (Hoffman and Hayon, 1972) and lies in the microsecond time range. It seems, however, that for compounds where the —S—S— bond forms part of an aliphatic ring the fragments $\text{RS}\cdot$ and RS^- cannot readily diffuse apart, and consequently the lifetime of RSSR^- is greatly enhanced. It was shown in the pulse radiolysis of thioctic acid (also known in the literature as lipoic acid), where the —S—S— bond forms part of a five-membered ring, that the lifetime of the corresponding negative ion reaches a few hundred microseconds (Willson, 1970; Hoffman and Hayon, 1972). Thioctic acid was therefore chosen as a reaction partner for the excited tyrosine in this study since the available time-resolution for the detection of short-lived intermediates produced by flash photolysis was $\geq 20\mu\text{sec}$.

EXPERIMENTAL

A flash photolysis apparatus of $\sim 2000\text{ J}$ energy and approximately $10\mu\text{sec}$ half-life was used in these experiments. The formation and decay of the transients as a function of wavelength were followed photometrically. The apparatus has been described in detail previously (Dogliotti and Hayon, 1967; Langmiur and Hayon, 1967). The solutions were contained in a 20 cm optical path quartz cell and could be deaerated in a reservoir connected to the cell by bubbling either N_2 or other gases, as required. The cells were fitted with an outer jacket which contained an appropriate solution filter. The pulse radiolysis set-up used has also been described elsewhere (Simic *et al.*, 1969; Keene *et al.*, 1969).

Fluorescence measurements were carried out on a Hitachi-Perkin-Elmer instrument.

Triply distilled water, which was radiolysed and subsequently ultraviolet-photolysed was used throughout. Tyrosine was obtained from Mann Research Laboratories. Thioctic acid was a Nutritional Biochemicals Company product and was checked by the Ellman method for sulfhydryl groups (Ellman, 1959). It was found to contain less than 0.3% RSH compounds. The pH was adjusted by boric acid-borax buffer of molarity less than 10^{-3} M or by addition of a small volume of 0.5 M KOH to the deaerated solution. *Tert*-butyl alcohol was supplied by Mallinckrodt.

RESULTS

The absorption spectra of tyrosine and of Na-lipoate are shown in Fig. 1. In order to optically excite tyrosine only, the exciting light was cut off at 280 nm by placing a 0.01 N sodium benzoate (pH 10.1) filter solution in the outer jacket of the optical cell. The absorption of light at wavelength between 280 and 300 nm was almost entirely due to tyrosine at all the concentrations of Na-lipoate employed. Blank experiments with Na-lipoate only in an identical experimental set-up did not produce any observable transients. Experiments were carried out in nitrogen and in N_2O -saturated solutions. The latter is known to be a good reactant for electrons



with $k = 6.5 \times 10^9\text{ M}^{-1}\text{ sec}^{-1}$ (Anbar and Neta, 1967). *Tert*-butyl alcohol was added to scavenge the OH radicals. The *t*-butyl alcohol radical produced (Simic *et al.*, 1969) is fairly inert and does not absorb above 280 nm. No indications were found for the reaction of this radical with the various species present in our solutions, and it was hence assumed not to interfere with our experiments.

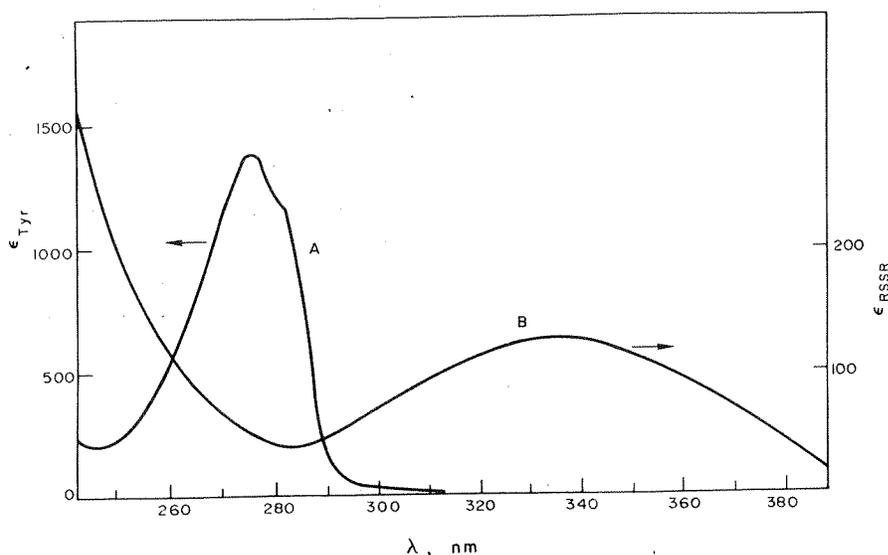


Figure 1. Absorption spectra of aqueous solutions of tyrosine (A) and of sodium lipoate (B) at pH 5-6.

About $8 \times 10^{-4} M$ aqueous tyrosine solutions were flash illuminated in the presence and absence of various concentrations ($5 \times 10^{-5} M$ to $5 \times 10^{-3} M$) of Na-lipoate. The solutions contained $1.0 M$ *t*-butyl alcohol and the pH was adjusted to 7.7 ± 0.1 , unless stated otherwise. The flash photolysis results obtained are presented in Fig. 2. In the absence of Na-lipoate, the characteristic spectrum of the phenoxyl radical with a sharp peak at ~ 410 nm and a broader band at ~ 390 nm was observed. The broad absorption band with $\lambda_{\max} \sim 710$ nm of the hydrated electron was also seen in more dilute solutions of tyrosine, in agreement with Dobson and Grossweiner's (1965) results. Another broad absorption band near 350 nm was observed, Fig. 2(A), and is attributed to the intermediate produced from the reaction of e_{aq}^- with tyrosine (Feitelson and Hayon, 1973). This band is not observed on flash photolysis of tyrosine in presence of N_2O (1 atm). Curves (B)-(D), Fig. 2, show the transient spectra obtained in the presence of Na-lipoate at two representative concentrations. The following features of these curves should be noted: At low lipoate concentration a striking change in the transient spectrum can be seen in N_2 -satd solutions, Fig. 2(B). The sharp peak at 410 nm still persists but superimposed is a broad spectral band which extends from 350 to 480 nm. In solutions saturated with N_2O the intensity of the transient is lower, Fig. 2(C), but its general spectral features are preserved. At somewhat higher lipoate concentrations the peak attributed to the tyrosine radical gradually disappears, and the results in N_2 approach those in N_2O solutions. At lipoate concentrations above $1.5 \times 10^{-3} M$ the spectra in N_2 and N_2O solutions coincide, Fig. 2(D).

The spectrum in Fig. 2(D) closely resembles the spectrum of the $RSSR^-$ radical anion of lipoate, Fig. 3, obtained by pulse radiolysis on reaction with e_{aq}^- . The molar absorption coefficient of $RSSR^-$ at 410 nm is $\epsilon = 9.2 \times 10^3 M^{-1} cm^{-1}$, and its decay constant obtained by pulse radiolysis was found to resemble that observed in the flash experiments, $k = 2.0 \pm 1.0 \times 10^9 M^{-1} sec^{-1}$. The rate constant $k(e_{aq}^- + \text{lipoate}) = 1.5 \pm 0.2 \times 10^{10} M^{-1} sec^{-1}$ (Hoffman and Hayon, 1972) was determined by pulse radiolysis.

The reaction of H atoms with lipoate is expected (Neta and Schuler, 1971) to have a rate constant of $\sim 1 \times 10^{10} M^{-1} \text{sec}^{-1}$. The production and the decay of the phenoxyl radical from tyrosine are not influenced by the presence of N_2O and/or *t*-BuOH in solution.

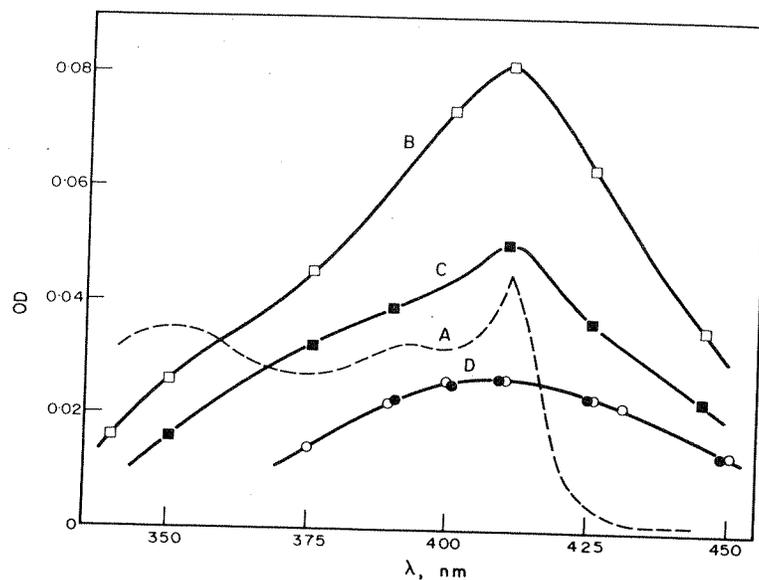


Figure 2. Spectra of transient species formed on optical excitation of $8 \times 10^{-4} M$ tyrosine at $\text{pH} = 7.7$ in presence of Na-lipoate: (A) no lipoate present; (B) $1.5 \times 10^{-4} M$ lipoate, N_2O -satd; (C) $1.5 \times 10^{-4} M$ lipoate, N_2O -satd; (D) $3 \times 10^{-3} M$ lipoate N_2O -satd (○) and N_2O -satd (●).

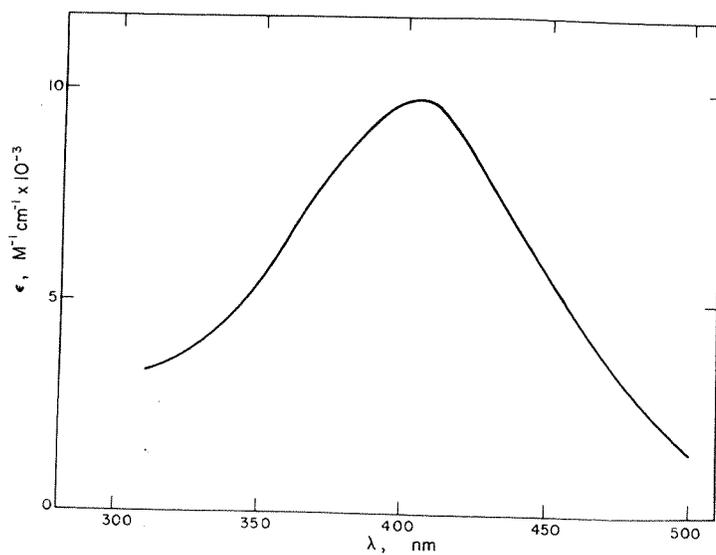


Figure 3. Optical absorption spectrum of the RSSR^- radical anion of lipoate obtained by pulse radiolysis (see text).

The absorbance at $\lambda = 410$ nm at low lipoate concentrations contains contribution from both RSSR^- and tyrosine radicals, while the absorbance at 450 nm is due to the disulfide RSSR^- ions only. These absorbances as a function of lipoate concentration are shown in Fig. 4 for results in presence and in absence of N_2O . The effect of N_2O in the flash photolysis experiments is to scavenge the hydrated electrons produced in the photoionization of tyrosine, since $> 90\%$ of them react with N_2O in the presence of up to 3×10^{-3} M lipoate. The difference in OD in the absence and in the presence of N_2O , $[\text{OD}(\text{N}_2) - \text{OD}(\text{N}_2\text{O})]_{410}$, at $\lambda = 410$ nm is therefore a measure of the lipoate RSSR^- ions produced by reaction with photo-ejected hydrated electrons. The same results are obtained from measurements at $\lambda = 450$ nm, Fig. 4, where the absorbances are smaller but no contribution from the tyrosine radical is expected at this wavelength. The ratio of extinction coefficients of the RSSR^- radical $\epsilon_{410}/\epsilon_{450} = 1.85$ (from Fig. 3). It can be seen from Fig. 4, under experimental conditions where all the electrons produced from the photoionization of tyrosine are scavenged by N_2O , that RSSR^- radicals are still produced.

Flash photolysis of tyrosine at pH 11.8 also yields phenoxy radicals. Similar to the experiments at neutral pH, RSSR^- radicals are also formed in the presence of Na-lipoate in N_2O saturated solutions. However, relative to the phenoxy radical yield more RSSR^- radicals are formed at pH 11.8 than at pH 7.7. In presence of 1.5×10^{-3} M lipoate, the ratio of the absorbances $\text{OD}_{\text{RSSR}^-}/\text{OD}_{\text{RO}} = 0.8$ at pH 7.7 and 2.2 at pH 11.8.

Fluorescence measurements of 5×10^{-5} M aqueous solutions in presence of varying concentrations of lipoate at pH ~ 7.6 gave a linear Stern-Volmer plot with a quenching constant of $k_q = 80$ liters mole $^{-1}$. Taking the lifetime of the lowest singlet excited state of tyrosine $\tau = 5$ nsec, a bimolecular rate constant for the quenching of singlet tyrosine by lipoate of $k_4 = 1.6 \times 10^{10}$ M $^{-1}$ sec $^{-1}$ was determined.

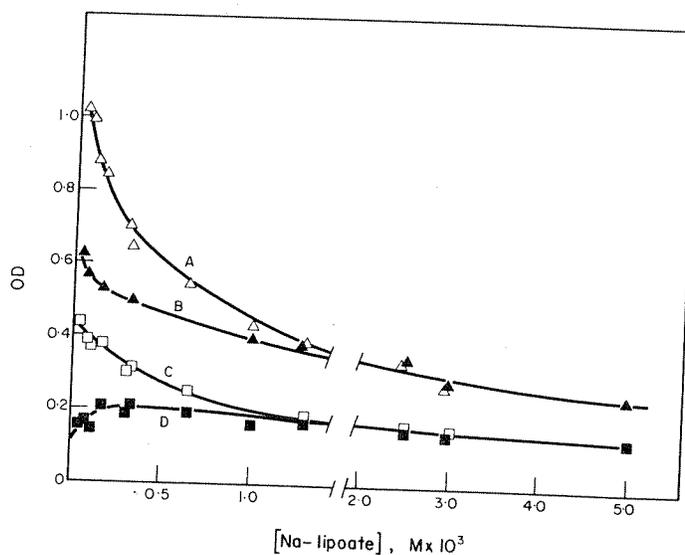
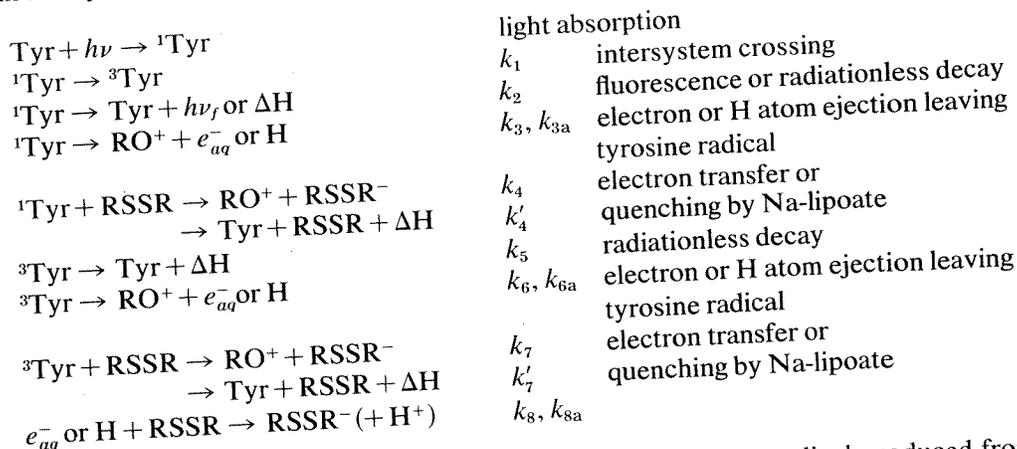


Figure 4. Absorbance of transients formed on flash photolysis of 8×10^{-4} M tyrosine, pH = 7.8, as a function of Na-lipoate concentration: (A) 410 nm, N_2 -satd; (B) 410 nm, N_2O -satd; (C) 450 nm, N_2 -satd; (D) 450 nm, N_2O -satd.

DISCUSSION

Since it has been found that solvated electrons are produced in the flash photolysis of tyrosine, the difference between the spectra obtained in the presence and in the absence of N_2O (Fig. 4) must be due to a product of the rapid reaction between these solvated electrons and other species present in the solution. The rate constant for the reaction of electrons with tyrosine at pH ~ 7.7 is $k = 2.8 \times 10^8 M^{-1} \text{sec}^{-1}$ (Feitelson and Hayon, 1973), while with lipoate it is $k = 1.5 \times 10^{10} M^{-1} \text{sec}^{-1}$ (Hoffman and Hayon, 1972). It follows, therefore, that in the presence of more than $2 \times 10^{-4} M$ lipoate the sole reaction product of electrons should be the negative ion $RSSR^-$ of Na^+ -lipoate. The above difference between the spectra in presence and absence of N_2O yields a broad band centered at about 400 nm. From the similarity between this band, the spectrum of the species obtained from the excited tyrosine at comparatively high lipoate concentration ($> 1.5 \times 10^{-3} M$), Fig. 2(D), and the spectrum obtained in the pulse radiolysis of lipoate (Fig. 3) we conclude that in all three cases one is observing the $RSSR^-$ radical anion of lipoate.

The following general scheme describes the various reactions that could take place in this system and lead to the production of $RSSR^-$ ions.



Here $RSSR$ denotes the Na^+ salt of lipoic acid, $RSSR^-$ is the radical produced from lipoate by attachment of an electron to the disulfide bond and RO^+ is the tyrosine radical ion. Reactions 3, 6 and 8 refer to electrons and reactions 3a, 6a and 8a to hydrogen atoms.

Since the experimental results described above indicates the formation of $RSSR^-$ radicals by a mechanism other than reaction (8), the question arises as to whether direct electron transfer processes between excited tyrosine (singlet or triplet) and lipoate can take place (reactions 4 or 7). We shall first discuss reaction 4 and reaction 3 followed by reaction 8, i.e. the processes directly involving the singlet 1Tyr state. From the fluorescence measurements carried out, Na-lipoate at a concentration below $10^{-3} M$ cannot quench the tyrosine fluorescence to any measurable extent. Since the quenching rate was found to be diffusion-controlled, $k_2 = 1.6 \times 10^{10} M^{-1} \text{sec}^{-1}$, this means that at these low concentrations the lipoate cannot interact with singlet tyrosine. It follows therefore that other reactions such as a direct electron transfer between the singlet state of tyrosine and Na-lipoate (i.e. reaction 4) can be excluded.

The fluorescence of tyrosine is, however, quenched at higher Na-lipoate concentrations and therefore some interaction between the singlet ^1Tyr and the disulfide molecule does probably take place. Curve (D) of Fig. 4 shows the RSSR^- ions produced in N_2O -saturated solutions. It can be seen that the reaction which leads to the quenching of the tyrosine fluorescence in the range of $1-5 \times 10^{-3} M$ Na-lipoate concentrations does not result in the production of RSSR^- ions. In fact, a decrease in absorbance of RSSR^- at 450 nm with increasing lipoate concentration is observed, Fig. 4(D).

From the difference between curves (A) and (B) or between curves (C) and (D) of Fig. 4, the absorbance of RSSR^- ions formed by electron scavenging, $[\text{OD}(\text{N}_2) - \text{OD}(\text{N}_2\text{O})]$, decreases with increasing Na-lipoate concentration until at $\sim 1.5 \times 10^{-3} M$ it disappears altogether. This can only mean that at this concentration no solvated electrons are formed any more because their precursor is wholly quenched by Na-lipoate. Again, because of its short lifetime, the tyrosine singlet cannot be this precursor and one must look for some longer-lived excited state. It is proposed that this state is the triplet excited state of tyrosine. The tyrosine radicals and solvated electrons are formed via reaction 6, (Feitelson and Hayon, 1973).

From Figs. 2 and 4 one can see that RSSR^- ions are also formed under conditions where no solvated electrons are produced. Figure 4, curve (D), shows the 450 nm absorbance of RSSR^- at very low ($10^{-4} M$) Na-lipoate concentration in N_2O -saturated solutions, and Fig. 2(D) the absorption spectrum of the RSSR^- ion formed at high concentrations ($\sim 3 \times 10^{-3} M$) of lipoate in presence of N_2O . Since N_2O does not react readily with H atoms, these RSSR^- ions could conceivably be produced via reactions 3a or 6a followed by reaction 8a. It has been shown by Feitelson and Hayon (1973) that the photodissociation of tyrosine at neutral pH results in the formation of solvated electrons and of H atoms in a ratio $[e_{aq}^-]/[\text{H}] \geq 4$. Reaction 6a is not likely to occur since, as shown above, the precursor of the H atoms, the triplet ^3Tyr , is almost completely quenched at lipoate concentrations above $10^{-3} M$ (see Fig. 4). Furthermore, the quantum yield of reaction 6a is expected to be much lower in alkaline solutions, whereas an increased yield of RSSR^- is observed at pH 11.8.

It follows then that the above mentioned quenching of tyrosine triplets by Na-lipoate results, at least in part, in a direct transfer yielding the RSSR^- ions, reaction 7. In this case, the decrease in the amount of solvated electrons formed in the presence of Na-lipoate should follow simple quenching kinetics of the triplet state. Now, from the rate constants of the reactions $e_{aq}^- + \text{lipoate}$ ($k_{e_{aq}^-} = 1.5 \times 10^{10} M^{-1} \text{sec}^{-1}$) and $e_{aq}^- + \text{tyrosine}$ ($k_{e_{aq}^-} = 2.8 \times 10^8 M^{-1} \text{sec}^{-1}$), at lipoate concentrations above $10^{-4} M$ practically all electrons are converted into RSSR^- ions. The amount of these ions, denoted $\text{RSSR}^- (\text{el})$, is a convenient measure of the solvated electrons formed. Their absorbance, as mentioned earlier, is given by the difference $[\text{OD}(\text{N}_2\text{O}) - \text{OD}(\text{N}_2)]$ between curves (A) and (B) or between curves (C) and (D) of Fig. 4. The OD value of $\text{RSSR}^- (\text{el})$ extrapolated to zero lipoate concentration, $\text{OD}_{\text{RSSR}^- (\text{el})}^0$, yields the absorbance of the $\text{RSSR}^- (\text{el})$ ions under the hypothetical conditions where all electrons are converted to RSSR^- ions and the precursor of these electrons, the triplet state, is not influenced by the presence of Na-lipoate.

From steady state kinetics one obtains

$$\frac{\phi_{\text{RSSR}^- (\text{el})}^0}{\phi_{\text{RSSR}^- (\text{el})}} = \frac{\text{OD}_{\text{RSSR}^- (\text{el})}^0}{\text{OD}_{\text{RSSR}^- (\text{el})}} = \frac{k_5 + k_6 + k_7[\text{RSSR}^-]}{k_5 + k_6} = 1 + k_7\tau[\text{RSSR}^-]$$

where ϕ denotes the quantum yield and superscript 0 indicates the absence of Na-lipoate. A graph of $OD_{RSSR^-(el)}^0 / OD_{RSSR^-(el)}$ vs. Na-lipoate concentration is shown in Fig. 5 to yield a quenching constant of $k_7\tau = 10,000$. If the rate constant for the reaction between tyrosine triplet and Na^+ lipoate, k_7 , is diffusion controlled, i.e. has a value of about $10^{10} M^{-1} sec^{-1}$, this would yield a lower limit for the triplet lifetime τ of approximately $1 \mu sec$.

Considering all the tyrosine radicals are derived from the triplet state then the concentration of these radicals formed in the absence of lipoate yields a lower limit for the initial triplet concentration. The absorbance of $RSSR^-$ ions produced in the presence of $1.5 \times 10^{-3} M$ Na-lipoate yields then the upper limit for the quantum yield of $RSSR^-$ produced through triplet quenching by lipoate. By using the extinction coefficients $\epsilon \approx 2500 M^{-1} cm^{-1}$ for the tyrosine radical (Feitelson and Hayon, 1973) and the value of $\epsilon = 9200 M^{-1} cm^{-1}$ for $RSSR^-$ ions at $\lambda = 410 nm$, one can conclude that at most 25 per cent of the triplets quenched produced $RSSR^-$ ions. Therefore the deactivation of the triplet must proceed both by pathways 7 and 7'.

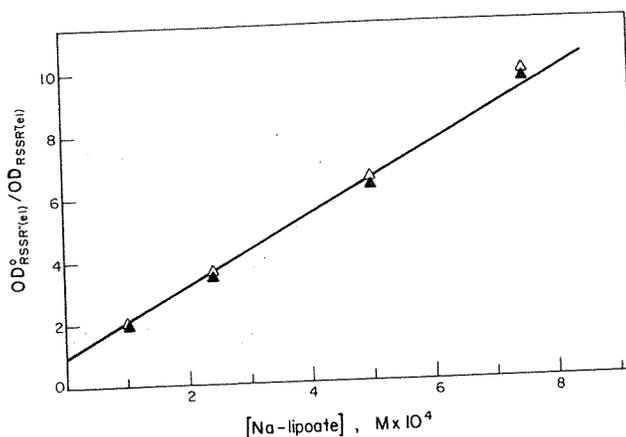


Figure 5. Reciprocal plot of $RSSR^-(el)$ as a function of low Na-lipoate concentrations obtained on excitation of $8 \times 10^{-4} M$ tyrosine, $pH = 7.8$, N_2O -satd. $OD_{RSSR^-(el)}$ values were obtained from Fig. 4 curves (A) and (B) (Δ), and from curves (C) and (D) (\bullet).

It was found that in the range of Na-lipoate concentrations of $1-5 \times 10^{-3} M$ the amount of $RSSR^-$ ions decreases slightly. In terms of our model this simply reflects the fact that the quenching of the *singlet* state by lipoate competes with the intersystem crossing and thereby causes a decrease in the amount of available tyrosine triplets. This assumption can be tested by a Stern-Volmer-like equation for the quantum yields of intersystem crossing as measured by the formation of $RSSR^-$ ions at lipoate concentrations between 1 and $5 \times 10^{-3} M$. $\phi^0/\phi = (k_1 + k_2 + k_3 + k_4[lipoate]) / (k_1 + k_2 + k_3)$ which should yield a quenching constant similar to that obtained from fluorescence measurements. Here ϕ is the yield of the disulfide radical ion, while ϕ^0 denotes the quantum yield of $RSSR^-$ ions under the hypothetical conditions where no singlet quenching takes place and all the triplets interact with Na-lipoate. The value of ϕ^0 is proportional to OD^0 and is obtained by extrapolating curve (D) of Fig. 4 between 1 and $5 \times 10^{-3} M$ to zero lipoate concentration. ϕ^0/ϕ equals OD^0/OD at 450 nm [curve (D), Fig. 4]. A plot

of the latter quantity against Na-lipoate concentration, Fig. 6, yields a value of $k_4/(k_1+k_2+k_3) = k_4\tau = 88$ liters mole⁻¹ which compares very well with a quenching constant of 80 obtained from fluorescence quenching data. This therefore indicates that both processes, i.e. decrease in fluorescence yield as well as decrease in the yield of RSSR⁻ ions, are due to a depletion of tyrosine singlets.

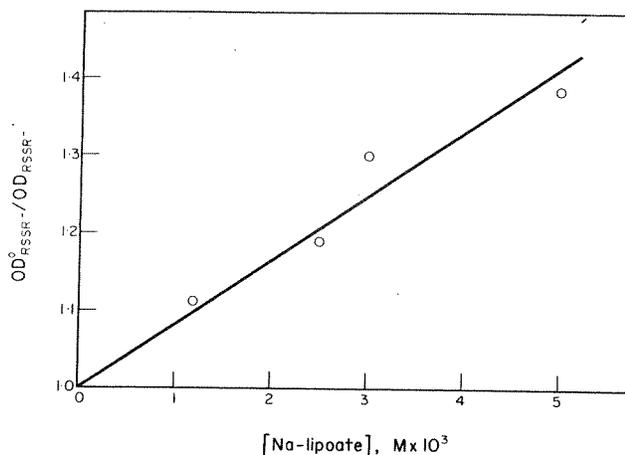


Figure 6. Reciprocal plot of [RSSR⁻] formed in 8×10^{-4} M tyrosine, N₂O-satd, pH ~ 7.8, in the presence of relatively high Na-lipoate concentrations.

Finally, the lifetime of the excited singlet of tyrosine is extremely short in alkaline solutions as seen from the absence of any measurable fluorescence. At high pH therefore only the longer-lived triplet excited state could interact with Na-lipoate. The transient produced in this interaction also has the absorption spectrum of the RSSR⁻ ion but its yield, relative to that of the phenoxyl radical formed in the absence of lipoate, is larger by a factor of 2.5 at pH 11.8 than in neutral solutions. This seems reasonable since it means that the direct electron transfer from a negatively charged triplet excited state ³RO⁻ to lipoate is more efficient than from a neutral excited molecule, ³ROH.

In conclusion, it is suggested that the disulfide negative ion of Na-lipoate is produced by two separate pathways: Na-lipoate reacts both with solvated electrons (and perhaps a small amount of H atoms), which are ejected from the triplet state of tyrosine, (reactions 6, 6a and 8, 8a) yielding RSSR⁻ ions, and also directly with the triplet excited tyrosine resulting in a charge transfer to the disulfide bond (reaction 7).

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