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FAST CONSECUTIVE RADICAL PROCESSES WITHIN THE RIBONUCLEASE MOLECULE IN AQUEOUS SOLUTION
II. REACTION WITH OH RADICALS AND HYDRATED ELECTRONS

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SUMMARY

Transient absorption spectra generated by the action on aqueous solutions of ribonuclease of OH radicals in the pH range 3.5–7.3 and of hydrated electrons in the pH range 6.0–7.6 have been investigated by the technique of pulse radiolysis–kinetic spectroscopy. Using 100-ns pulses with both reagents a sequence of spectral changes occurs. Using sweep rates of 1 μs/cm or higher a series of spectra were obtained over a time range from about 10 μs after pulsing up to several seconds later. The first spectrum observed is that due to addition of the radical to ribonuclease. The subsequent transformations are first order and presumably correspond to intramolecular reactions in which radical sites migrate within the protein. Spectral data implicate aromatic and sulfur-containing amino acid residues as sites of transient radical intermediates. Attack of OH also appears to involve abstraction of hydrogen from saturated carbon atoms of the peptide link.

Both the occurrence of consecutive reactions and the sites of transient radicals resemble behavior observed when H atoms react with ribonuclease[1,2]. There are, however, significant differences characteristic of OH and e^-_{aq} attack.

Values of k[ribonuclease + OH] and the rates of the first order intramolecular changes are reported. The implications of intramolecular free radical migration in protein for enzyme chemistry are discussed.

INTRODUCTION

The first paper of this series[1] presented evidence obtained by the technique of pulse radiolysis–kinetic spectroscopy which indicated that the action of radiolytically generated H atoms initiates short intramolecular chain reactions in the ribonuclease molecule. Sulfur and aromatic radicals appear to be involved in the chains. This paper reports the application of the same methodology to the reaction of radiolytically generated OH radicals and hydrated electrons with ribonuclease

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and provides evidence of the generality of consecutive radical reactions within the protein molecule.

EXPERIMENTAL

Apparatus, methods and materials have been described previously. In the present work the various transient spectra were measured at times appropriate to their rates of appearance and decay. When the desired reactant was hydroxyl radical, solutions were swept for at least 1 h with Matheson N_2O which was first passed through alkaline aqueous pyrogallol to remove oxygen. When the desired reactant was the hydrated electron 0.1 or 0.2 M *tert*-butanol was used to remove hydroxyl radicals. Deaeration was accomplished by sweeping with argon for at least 1 h shortly before pulsing the solution.

Dose per pulse was monitored by determining the absorbance at 578 nm due to the hydrated electrons produced by pulsing 10^{-2} M aqueous propanol at pH 10.5 ($\varepsilon_{578} = 1.06 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$, $G(e^-_{aq}) = 2.7$, $G(OH) = 2.6$, $G(H) = 0.55$). The dose per 100-ns pulse during the period of these experiments fell in the range 306–512 rad. Transient spectra presented in this paper are normalized to a dose per pulse of 500 rad. A typical set of doses delivered by pulses of different duration and the corresponding instantaneous concentrations of OH radical produced in N_2O-saturated solution and of hydrated electrons produced in 0.1 M *tert*-butanol solution are:

50 ns: 330 rad, $1.8 \cdot 10^{-6}$ M OH, $0.92 \cdot 10^{-6}$ M e^-_{aq}
100 ns: 473 rad, $2.6 \cdot 10^{-6}$ M OH, $1.35 \cdot 10^{-6}$ M e^-_{aq}
200 ns: 703 rad, $3.9 \cdot 10^{-6}$ M OH, $2.00 \cdot 10^{-6}$ M e^-_{aq}
300 ns: 917 rad, $5.1 \cdot 10^{-6}$ M OH, $2.60 \cdot 10^{-6}$ M e^-_{aq}
500 ns: 1304 rad, $7.2 \cdot 10^{-6}$ M OH, $3.70 \cdot 10^{-6}$ M e^-_{aq}

Total dose to any solution was limited to about one incident radical per ribonuclease molecule although it was established that signals remained unchanged up to several times as large a dose.

RESULTS

Reaction of OH radicals

Pulses of OH radicals were obtained by employing solutions saturated with N_2O which converts hydrated electrons to OH radicals via the reaction:

$$e^-_{aq} + N_2O + H_2O \rightarrow OH + N_2 + OH^-$$

The rate constant, $k[e^-_{aq} + N_2O]$, is $5.6 \cdot 10^9$ $M^{-1} \cdot s^{-1}$ (see ref. 3) and the concentration in solutions saturated with N_2O at 1 atm pressure is $2.3 \cdot 10^{-2}$ M. The consequent pseudo first order rate constant for consumption of electrons, $1.3 \cdot 10^8$ s^{-1}, was more than one hundred times greater than the pseudo first order specific rate of consumption of electrons by the highest concentration of ribonuclease employed in this work. Under these conditions the ratio of OH radicals to H atoms is about 10:1.

Kinetic data were obtained from oscilloscope traces as described previously[1,2]. Values of k[ribonuclease + OH] were calculated at numerous wavelengths from signals produced by doses of about 300–1000 rad applied to $0.7 \cdot 10^{-5}$–$1.8 \cdot 10^{-5}$ M ribonuclease. No significant variation of k[ribonuclease + OH] with dose per pulse or concentration was observed and first order plots of the data of individual traces

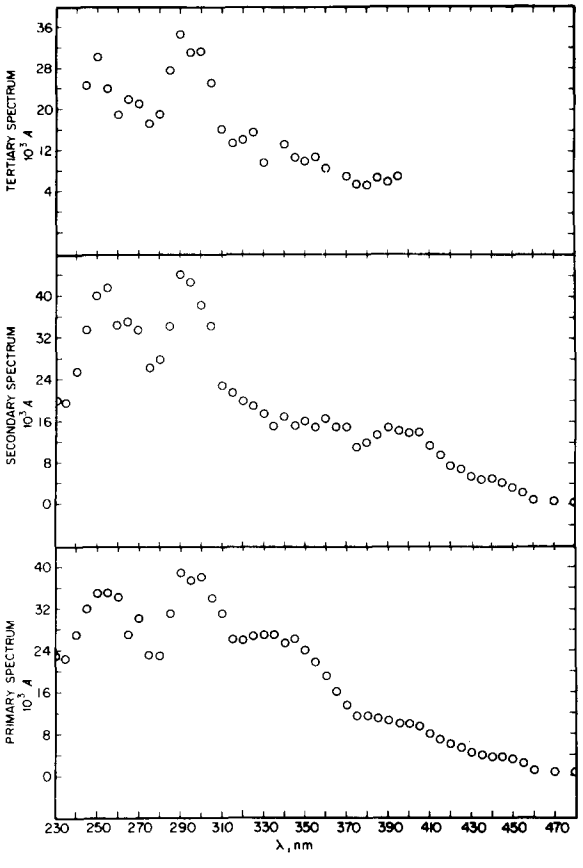

Fig. 1. Primary, secondary and tertiary transient spectra from reaction of $1.8 \cdot 10^{-5}$ M ribonuclease with OH at pH 3.5. 100-ns pulses [approx. 500 rad].

were linear. The resulting values of k[ribonuclease + OH] in units of 10^{10} $M^{-1} \cdot s^{-1}$ are: pH 3.5, 3.6 ± 0.5; pH 5.6, 1.9 ± 0.3; autogenous pH about 7, 2.4 ± 0.6. Thus, the specific rate of reaction of OH with ribonuclease is somewhat higher than that of H, $1.5 \cdot 10^{10}$ $M^{-1} \cdot s^{-1}$.

Sequential transformations of transient spectra could be resolved into four main stages. Corresponding primary (p), secondary (s), tertiary (t) and quaternary (q) transient spectra are displayed in Figs 1–3 while difference spectra, s–p, t–s and q–t are shown in Figs 4 to 6, respectively. Stable spectra observed under anaerobic conditions are displayed in Fig. 7. These were initially determined 5–10 min after irradiation and remained substantially unchanged for many hours thereafter. The dependence of first order rate constants determined directly from oscilloscope traces, *i.e.* k[p → s], k[s → t], and k[t → q], on wavelength and pH is summarized in Tables I–III while their dependence on dose per pulse and on concentration of ribonuclease is summarized in Tables IV–VI.

From Table I it is apparent that at each pH k[p → s] increases with wavelength, the largest variation occurring at autogenous pH. The p → s spectral trans-

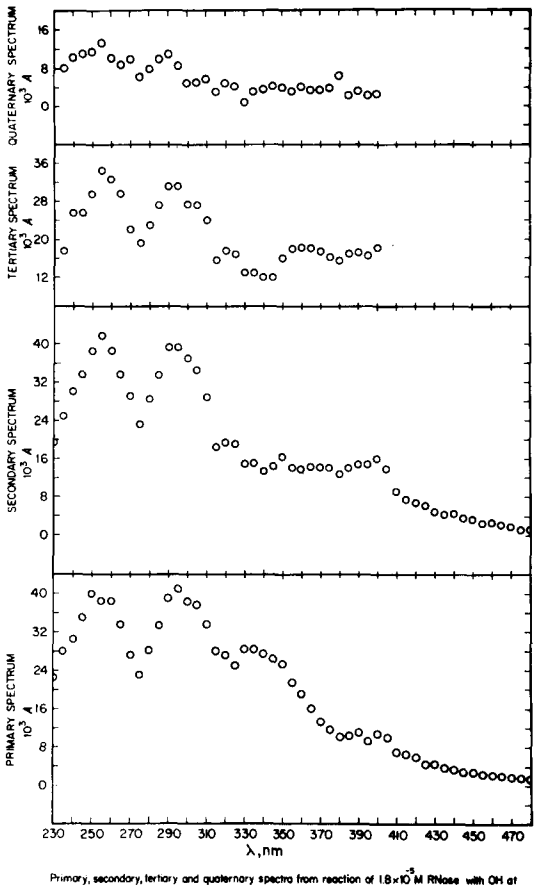

Fig. 2. Primary, secondary, tertiary, and quaternary transient spectra from reaction of $1.8 \cdot 10^{-5}$ M ribonuclease with OH at pH 5.5. 100-ns pulses [approx. 500 rad].

formation thus corresponds to at least two independent chemical transformations. Most noteworthy (see Figs 1 and 2) is the large increase in absorption around 400 nm at autogenous pH which, because of its greater rate, cannot be due to a chemical transformation consequent upon the reaction(s) leading to decreased absorption at shorter wavelength. The large pH dependence of $k[p \rightarrow s]$ around 400 nm may indicate that the spectral transformation observed at this wavelength at autogenous pH is due to a chemical transformation different from that occurring at lower pH.

The data of Table II show that $k[s \rightarrow t]$ is essentially independent of wavelength at both pH 3.5 and autogenous pH. These spectral transformations may therefore be due to single chemical reactions. It should be noted that the magnitude of $k[s \rightarrow t]$ at pH 3.5 is similar to that of $k[t \rightarrow q]$ at pH 5.5 and 7.3. Limited data (see Table V) indicate that the magnitude of $k[s \rightarrow t]$ at pH 5.5 is about one quarter of its value at autogenous pH. Spectral transformation at pH 3.5 comparable in rate to $k[s \rightarrow t]$ at pH 5.5 or 7.3 could not be detected.

The data of Table III show that $k[t \rightarrow q]$ is almost independent of wavelength

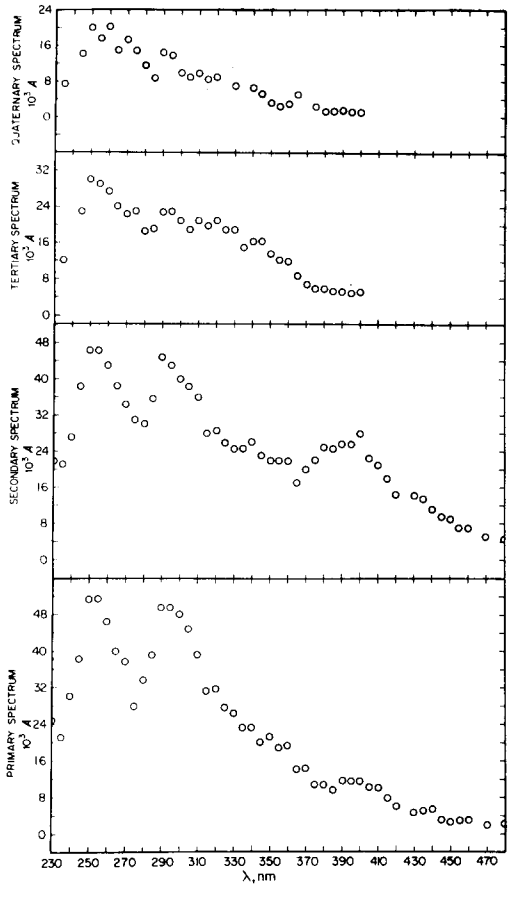

Fig. 3. Primary, secondary, tertiary and quaternary transient spectra from reaction of $1.8 \cdot 10^{-5}$ M ribonuclease with OH at pH 7.3. 100-ns pulses [approx. 500 rad].

over a range which, like the wavelength range of Table II, was limited by the emission properties of the deuterium lamp. The possibility exists here also that at each pH the spectral change is due to a single reaction. The magnitude of $k[t \rightarrow q]$ at autogenous pH is about three times greater than at pH 5.5, a difference similar to that for $k[s \rightarrow t]$.

The data of Table IV provide evidence as to the kinetic order of the spectral transformation $p \rightarrow s$. No significant dependence on either dose per pulse or concentration of ribonuclease is apparent at pH 3.5. Thus $p \rightarrow s$ appears to be truly first order at this pH. At pH 5.5 also, $k[p \rightarrow s]$ does not vary significantly with dose per pulse. The preponderance of data indicate that at autogenous pH $k[p \rightarrow s]$ is also independent of dose per pulse.

The data of Table V provide evidence of the kinetic order of the spectral transformation $s \rightarrow t$. At all values of pH there is no significant variation of $k[s \rightarrow t]$ with dose per pulse. Dependence of $k[s \rightarrow t]$ on concentration of ribonuclease was examined only at autogenous pH, where no significant variation is apparent. Thus,

TABLE I

DEPENDENCE OF k[p→s] FROM REACTION OF OH RADICALS UPON WAVELENGTH AND pH, 100-ns PULSES, $1.8 \cdot 10^{-5}$ M RIBONUCLEASE

Indicated uncertainties are mean deviations of replicate measurements.

λ (nm)	k[p→s] (10^3 s^{-1})		
	pH 3.5*	pH 5.5**	pH 7.3***
320	0.83	1.1±0.4	0.86
330	0.60	1.0±0.3	
335	0.80		3.5±0.9
340	0.66	1.0±0.2	
345	1.0±0.2	1.2±0.3	4.8
350	1.1	1.2	
355			9.3
360	1.1	1.3	
385	1.7		18
390	1.3	2.6±0.2	
395	1.5		21
400	1.4	3.2±0.6	23
405	1.8		20
415	1.9		27
420	3.2		
430	3.4		27

* $5 \cdot 10^{-4}$ M $HClO_4$.
** Buffered with 10^{-3} M KH_2PO_4.
*** Autogenous pH.

TABLE II

DEPENDENCE OF k[s→t] FROM REACTION OF OH RADICALS UPON WAVELENGTH AND pH, 100-ns PULSES, $1.8 \cdot 10^{-5}$ M RIBONUCLEASE

See Table V for data at pH 5.5.

λ (nm)	k[s→t] (s^{-1})	
	pH 3.5*	pH 7.3**
235		5.1
245	0.17	6.3
255	0.17	6.1
265	0.20	5.5
275	0.15	5.8
285	0.23	6.1
295	0.24	6.4
305	0.15	6.1
315		4.5
325		4.2
335		4.1
345	0.13	5.7
355		5.2
365		5.5
375		6.5
385	0.15	7.0
395		6.7

* $5 \cdot 10^{-4}$ M $HClO_4$.
** Autogenous pH.

TABLE III

DEPENDENCE OF $k[t\rightarrow q]$ FROM REACTION OF OH RADICALS UPON WAVELENGTH AND pH, 100-ns PULSES, $1.8 \cdot 10^{-5}$ M RIBONUCLEASE

λ (nm)	$k[t\rightarrow q]$ (s^{-1})	
	pH 5.5*	pH 7.3**
235	0.15	
245	0.18	0.42
255	0.23	
265	0.15	0.46
275	0.17	0.65
285	0.19	0.36
295	0.18	0.51
305	0.16	0.38
315		0.39
325	0.12	0.40
335		0.31
345	0.18	0.32
355	0.14	0.44
375	0.15	
385	0.17	0.60
395	0.16	0.49

* Buffered with 10^{-3} M KH_2PO_4.
** Autogenous pH.

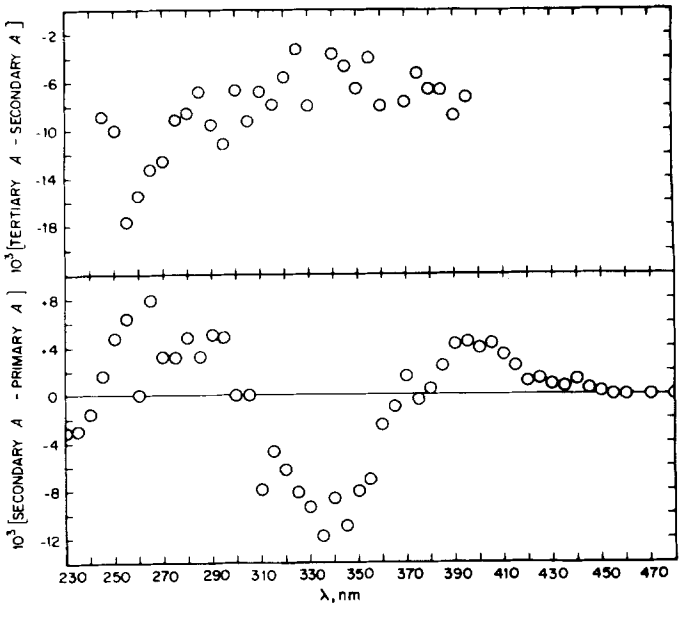

Fig. 4. Transient difference spectra from reaction of $1.8 \cdot 10^{-5}$ M ribonuclease with OH at pH 3.5 100-ns pulses [approx. 500 rad].

TABLE IV

DEPENDENCE OF k[p→s] FROM REACTION OF OH RADICALS UPON DOSE PER PULSE AND CONCENTRATION OF RIBONUCLEASE

Ratio of dose per pulse for 50-, 100- and 300-ns pulses about 1:1.7:3.5. Indicated uncertainties are mean deviations of replicate measurements.

λ (nm)	k[p→s] (10^3 s^{-1})		
	Pulse length:		
	50 ns	*100 ns*	*300 ns*
pH 3.5, 0.76 · 10⁻⁵ M ribonuclease*			
320	1.2		1.4
345	1.3		1.2±0.1
pH 3.5, 1.8 · 10⁻⁵ M ribonuclease*			
295	0.9	1.2±0.2	
320	1.3	0.8	1.4±0.05
345	1.0	1.0±0.2	1.2
390	1.7	1.3	1.0
*pH 5.5**, 1.8 · 10⁻⁵ M ribonuclease*			
295	2.4		2.7
325	1.4±0.3	1.4	1.2
345	1.1	1.2±0.3	1.2
390		2.6±0.2	4.3
*pH 6.3***, 0.37 · 10⁻⁵ M ribonuclease*			
325	1.7±0.3		1.9
400	82		99
*pH 6.7***, 0.73 · 10⁻⁵ M ribonuclease*			
325	3.3±1.4		4.0
400	90 ±27	95 ±4	118
*pH 7.3***, 1.8 · 10⁻⁵ M ribonuclease*			
325		1.6±0.3	3.3
400	64		58

* $4 \cdot 10^{-4}$ or $5 \cdot 10^{-4}$ M $HClO_4$.
** 10^{-3} M KH_2PO_4.
*** Autogenous pH.

at autogenous pH and possibly at pH 3.5 and 5.5 as well, the s → t spectral changes appear to be truly first order.

The limited data of Table VI show that k[t → q] does not vary significantly with dose per pulse or, at autogenous pH, with concentration of ribonuclease. Thus, at autogenous pH and possibly at pH 5.5 as well, the t → q spectral transformation appears to be truly first order.

Reaction of hydrated electrons

Hydroxyl radicals were scavenged by large excesses of *tert*-butanol (see ref. 4 for an example of the use of *tert*-butanol to scavenge OH radicals and for references to the background of this method). KH_2PO_4 buffer was used at a concentration low enough to make conversion of hydrated electrons to H atoms negligible. Under these conditions the ratio of electrons to H atoms is about 5:1.

The value of k[ribonuclease + e^-_{aq}] at autogenous pH, 7.1 ± 0.5, calculated

TABLE V

DEPENDENCE OF k[s→t] FROM REACTION OF OH RADICALS UPON DOSE PER PULSE AND CONCENTRATION OF RIBONUCLEASE

Ratio of dose per pulse for 50-, 100- and 300-ns pulses about 1:1.7:3.5. Indicated uncertainties are mean deviations of replicate measurements.

λ (nm)	$k[s \rightarrow t]$ (s^{-1})		
	pulse length:		
	50 ns	*100 ns*	*300 ns*
pH 3.5, $1.8 \cdot 10^{-5}$ M ribonuclease*			
315	0.23		0.16
390	0.15		0.30
*pH 5.5**, $1.8 \cdot 10^{-5}$ M ribonuclease*			
250	1.2		1.6
295	1.4		1.8
*pH 6.3***, $0.37 \cdot 10^{-5}$ M ribonuclease*			
295	6.0		6.5
355	3.7		3.7
400	4.9		5.5
*pH 7.3***, $1.8 \cdot 10^{-5}$ M ribonuclease*			
295	6.8	6.4	6.3 ± 0.1
355	4.9 ± 0.3	5.2	5.7
400	6.5	6.9	6.9

* $5 \cdot 10^{-4}$ M $HClO_4$.
** Buffered with 10^{-3} M KH_2PO_4.
*** Autogenous pH.

TABLE VI

DEPENDENCE OF k[t→q] FROM REACTION OF OH RADICALS UPON DOSE PER PULSE AND CONCENTRATION OF RIBONUCLEASE

Ratio of dose per pulse for 50-, 100- and 300-ns pulses about 1:1.7:3.5.

λ (nm)	$k[t \rightarrow q]$ (s^{-1})		
	Dose per pulse:		
	50 ns	*100 ns*	*300 ns*
pH 5.5, $1.8 \cdot 10^{-5}$ M ribonuclease*			
250	0.23		0.15
295	0.19 ± 0.3**	0.18	0.20
*pH 6.3***, $0.37 \cdot 10^{-5}$ M ribonuclease*			
295			0.41
325		0.53	0.54
355	0.28		0.27
400			0.41
*pH 7.3***, $1.8 \cdot 10^{-5}$ M ribonuclease*			
295	0.62	0.51	0.42
355		0.44	0.39
400		0.49	0.53

* Buffered with 10^{-3} M KH_2PO_4.
** Mean deviation.
*** Autogenous pH.

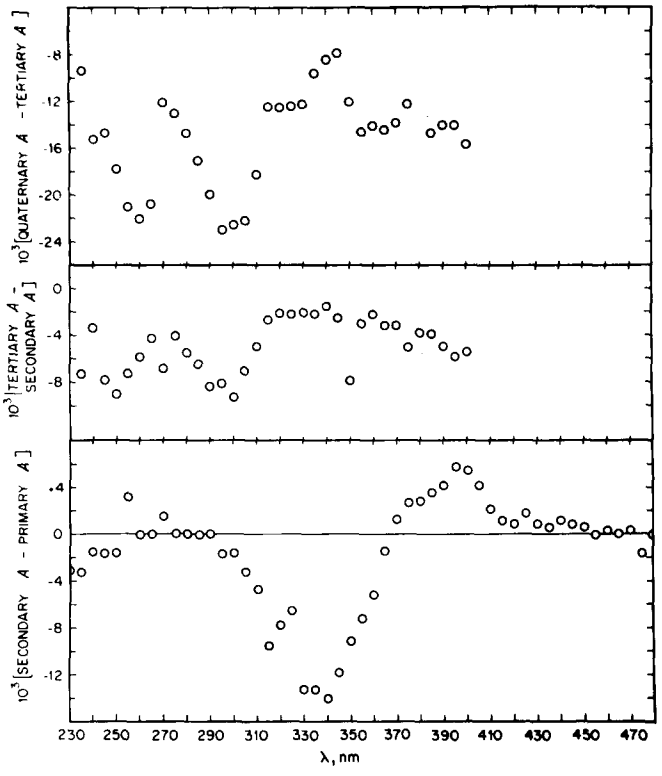

Fig. 5. Transient difference spectra from reaction of $1.8 \cdot 10^{-5}$ M ribonuclease with OH at pH 5.5. 100-ns pulses [approx. 500 rad].

from measurements at numerous wavelengths from signals produced by 100- or 200-ns pulses applied in the presence of *tert*-butanol to $1.5 \cdot 10^{-5}$–$3.5 \cdot 10^{-5}$ M ribonuclease was found to be $1.0 \cdot 10^{10} \pm 0.15 \cdot 10^{10}$ $M^{-1} \cdot s^{-1}$. Experiments involving application of 100-ns pulses to a $2.95 \cdot 10^{-5}$ M solution at autogenous pH, 7.6, containing $1.5 \cdot 10^{-5}$ M O_2 provided a pseudo first order rate of consumption of hydrated electrons, $6.9 \cdot 10^5 \pm 0.1 \cdot 10^5$ s^{-1}, from which, taking $k[O_2 + e^-_{aq}] = 1.88 \cdot 10^{10}$ $M^{-1} \cdot s^{-1}$ (ref. 3), k[ribonuclease + e^-_{aq}] was calculated to be $1.0 \cdot 10^{10}$ $M^{-1} \cdot s^{-1}$ in good agreement with the preceding results. The value of k[ribonuclease + e^-_{aq}] found in this work is in good agreement with that reported by Braams[5] for pH 6.8, $1.3 \cdot 10^{10}$ $M^{-1} \cdot s^{-1}$. Experiments involving application of 100-ns pulses to $1.5 \cdot 10^{-5}$ M ribonuclease at autogenous pH, 7.4, in the absence of *tert*-butanol so that OH radicals were not scavenged gave k[ribonuclease + e^-_{aq}] $= 0.9 \cdot 10^{10} \pm 0.12 \cdot 10^{10}$ $M^{-1} \cdot s^{-1}$. At pH 5.9 in the presence of 10^{-3} M KH_2PO_4 k[ribonuclease + e^-_{aq}] was found to be $1.7 \cdot 10^{10} \pm 0.2 \cdot 10^{10}$ $M^{-1} \cdot s^{-1}$. This can be compared to $2.9 \cdot 10^{10}$ $M^{-1} \cdot s^{-1}$ obtained by Braams at pH 5.5.

As was the case with transient spectra produced by reaction of OH, four sequential stages were resolved. The primary, secondary, tertiary and quaternary spectra observed at autogenous pH and in the presence of 10^{-3} M KH_2PO_4 are presented in Figs 8 and 9, respectively, and the difference spectra derived from them in Figs 10 and 11. Fig. 12 displays stable spectra of irradiated solutions observed under anaerobic conditions.

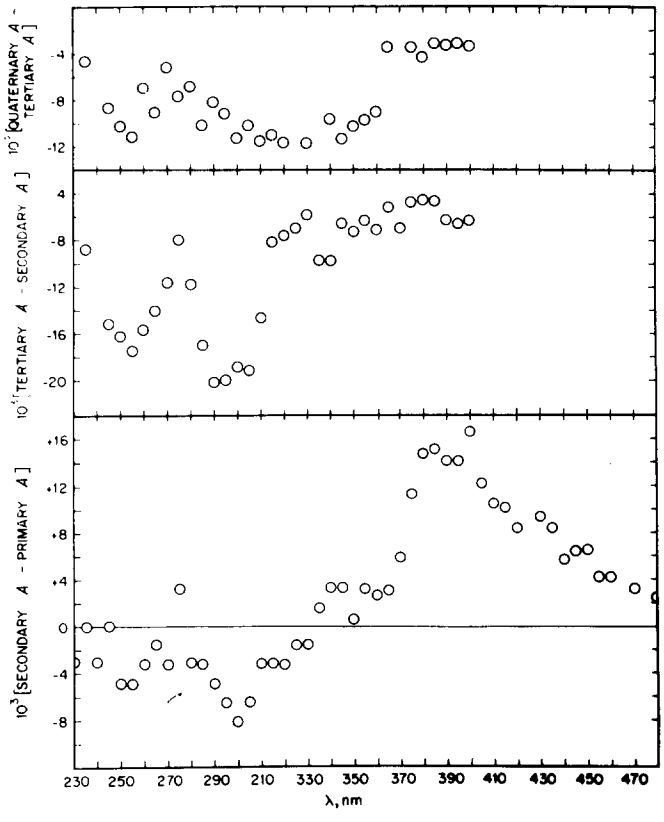

Fig. 6. Transient difference spectra from reaction of $1.8 \cdot 10^{-5}$ M ribonuclease with OH at pH 7.3. 100-ns pulses [approx. 500 rad].

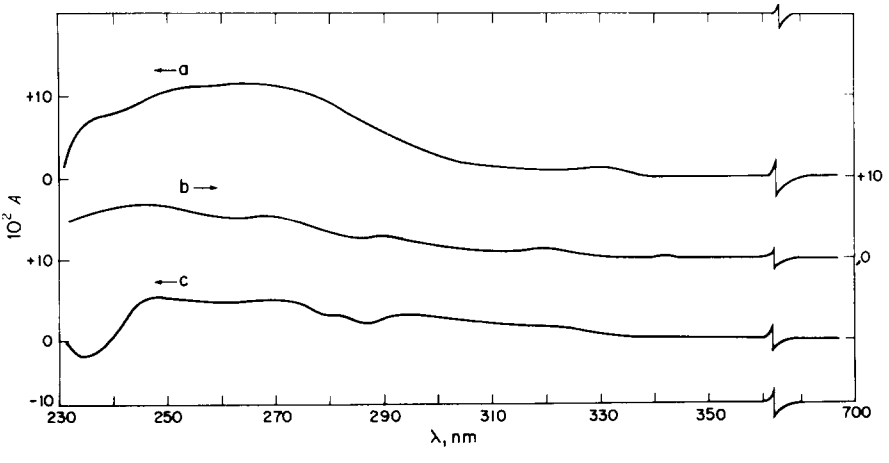

Fig. 7. Stable spectra from reaction of $7.5 \cdot 10^{-6}$ M ribonuclease with OH, eighteen 100-ns pulses [approx. 10 krad], *vs* unirradiated ribonuclease. a, pH 7.3; b, pH 5.3; c, pH 3.5.

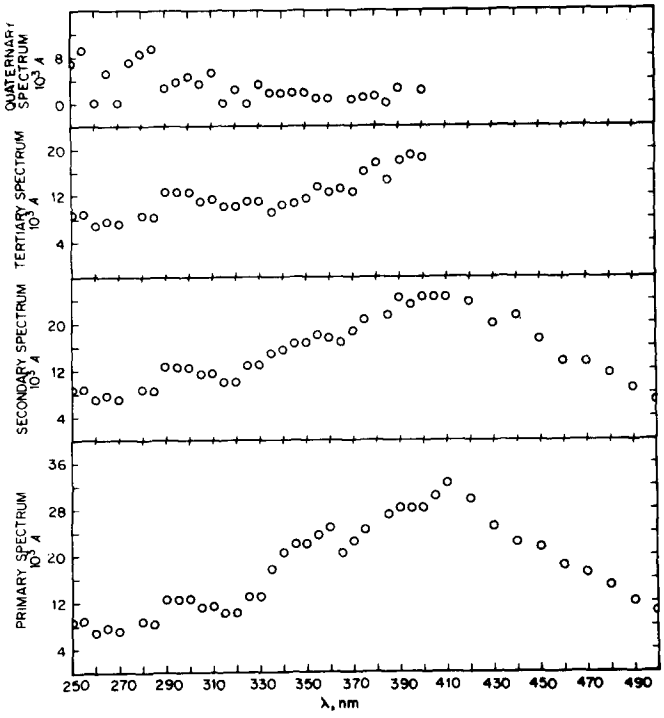

Fig. 8. Primary, secondary, tertiary and quaternary transient spectra from reaction of $3.0 \cdot 10^{-5}$ M ribonuclease with e^-_{aq} at pH 7.6. 100-ns pulses [approx. 500 rad].

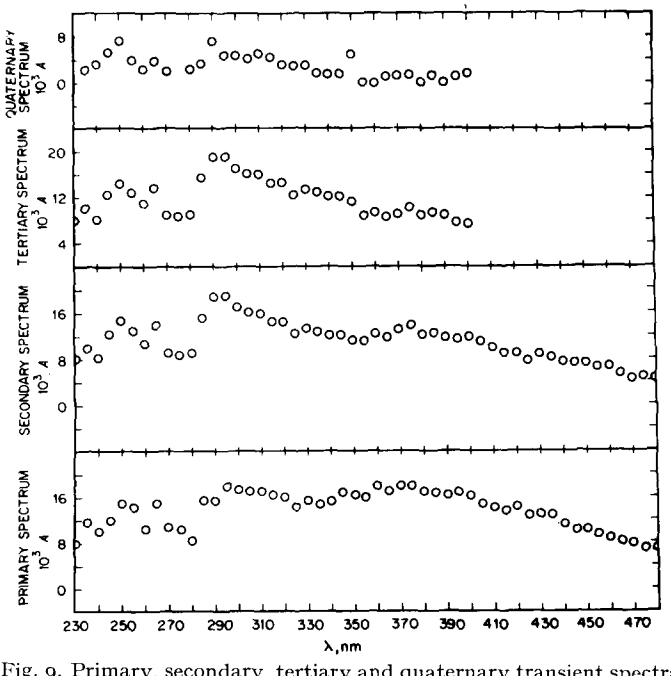

Fig. 9. Primary, secondary, tertiary and quaternary transient spectra from reaction of $1.8 \cdot 10^{-5}$ M ribonuclease with e^-_{aq} at pH 6.0. 100-ns pulses [approx. 500 rad].

From data resulting from application of 100-ns pulses to $3.0 \cdot 10^{-5}$ M ribonuclease at pH 7.6 $k[p \rightarrow s]$ was found to be $1.3 \cdot 10^2 \pm 0.1 \cdot 10^2$ s^{-1} at 350–360 nm. In the presence of $1.5 \cdot 10^{-5}$ M O_2 the intensity of the primary spectrum in the 330–400 nm region produced from the above solution by 100-ns pulses was diminished to less than one half that observed in the absence of O_2, as expected from the competition of O_2 with ribonuclease for electrons, and the rate of decay of the primary spectrum was increased about 10-fold around 350 nm, presumably because of scavenging by O_2 of radicals formed by attachment of electrons to the enzyme. Data obtained from application of 100-ns pulses to $1.8 \cdot 10^{-5}$ M ribonuclease buffered to pH 6.0 by 10^{-3} M KH_2PO_4 showed no significant variation of $k[p \rightarrow s]$ over the wavelength range 335–470 nm (12 different wavelengths) and gave an average value of $3.0 \cdot 10^2 \pm 0.5 \cdot 10^2$ s^{-1}. The value of $k[s \rightarrow t]$ at autogenous pH was found to be 2.5 ± 0.3 s at 295 nm and 3.5 ± 0.3 s^{-1} at 380 nm, both independent of dose per pulse (50- and 300-ns

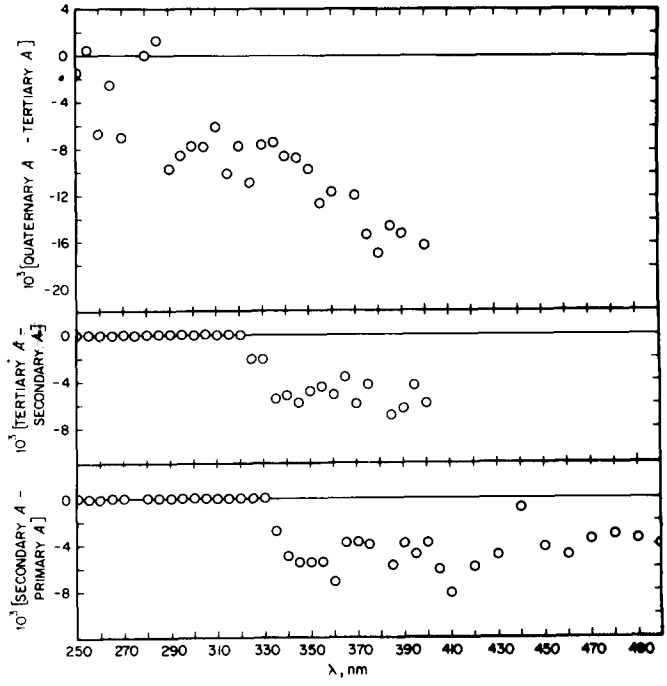

Fig. 10. Transient difference spectra from reaction of $3.0 \cdot 10^{-5}$ M ribonuclease with e^-_{aq} at pH 7.6. 100-ns pulses [approx. 500 rad].

pulses) and concentration of ribonuclease ($0.76 \cdot 10^{-5}$ and $1.8 \cdot 10^{-5}$ M ribonuclease). Experiments involving the same variation of dose per pulse and concentration of enzyme at autogenous pH gave $k[t \rightarrow q]$ equal to 0.38 ± 0.06 s^{-1} at 295 nm and 0.39 ± 0.06 s^{-1} at 380 nm. Rate constants were not evaluated for the spectral transformation $s \rightarrow t$ in phosphate buffer. As can be seen from Fig. 11 the $s \rightarrow t$ transformation was observed over a part of the accessible spectral region. From data obtained by applying 100-ns pulses to $1.8 \cdot 10^{-5}$ M ribonuclease, the value of $k[t \rightarrow q]$

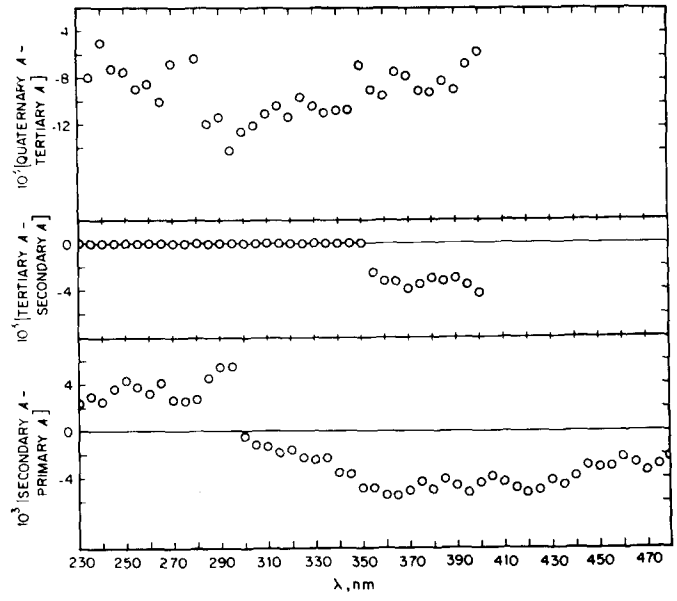

Fig. 11. Transient difference spectra from reaction of $3.0 \cdot 10^{-5}$ M ribonuclease with e^-_{aq} at pH 6.0, 100-ns pulses [approx. 500 rad].

in 10^{-3} M KH_2PO_4 at pH 6.0 was found to be 0.48 ± 0.05 s^{-1} independent of wavelength from 235 to 395 nm (15 wavelengths).

Reaction of sum of aqueous radicals

Fig. 13 represents the transient spectra obtained by pulsing $1.8 \cdot 10^{-5}$ M aqueous ribonuclease at autogenous pH in solutions which were deaerated but which contained neither N_2O nor *tert*-butanol. The resulting spectra are therefore superpositions of those resulting from the action of H atoms, hydroxyl radicals and hydrated electrons in proportion to their radiolytic yields, *i.e.* 0.55:2.6:2.7, assuming that

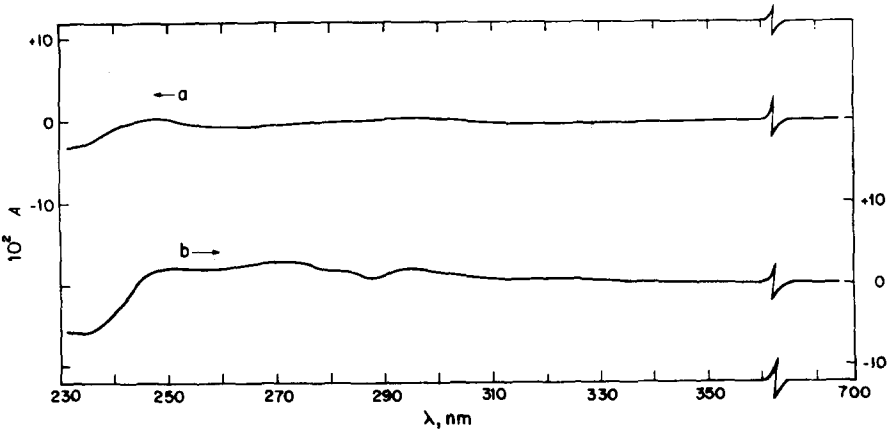

Fig. 12. Stable spectra from reaction of $7.5 \cdot 10^{-6}$ M ribonuclease with e^-_{aq}, thirty 100-ns pulses [approx. 15 krad], *vs* unirradiated ribonuclease. a, pH 6.9; b, pH 5.8.

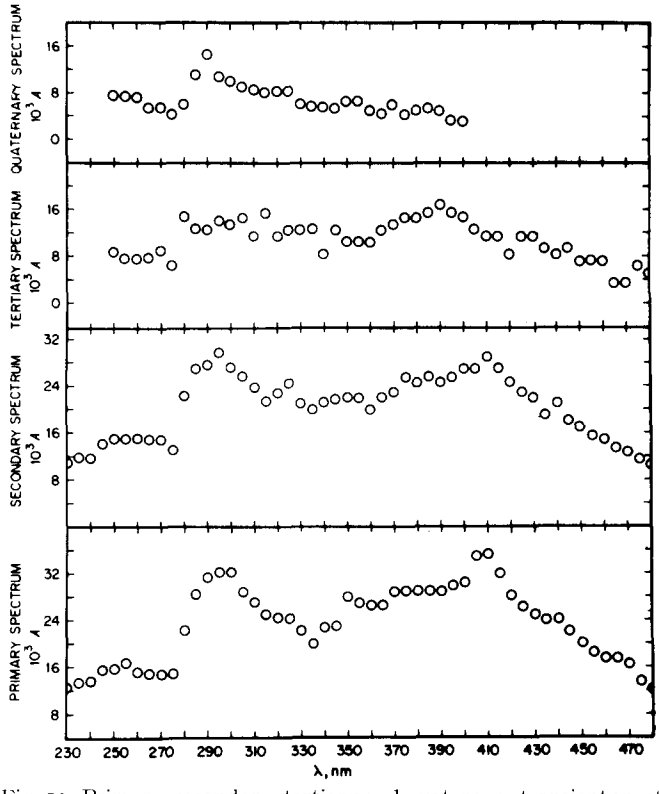

Fig. 13. Primary, secondary, tertiary and quaternary transient spectra from reaction of $1.8 \cdot 10^{-5}$ M ribonuclease with OH + e^-_{aq} + H at pH 7.1. 100-ns pulses [approx. 500 rad].

the p → s, s → t and t → q transitions are intramolecular. This follows from the fact that under the conditions of the experiment only one aqueous radiolytic radical adds to a given enzyme molecule.

DISCUSSION

Kinetics

Reaction of OH radicals. We assume here that all the observed spectral transformations which are first order in the sense that their first order specific rates are independent of initial concentration of OH radicals and concentration of ribonuclease are due to intramolecular transformations. This assumption is supported by the demonstration of such behavior for a large number of the transformations. However, the kinetic data do not exclude the possibility that any of the chemical transformations associated with the spectral transformations may involve water or buffer components. The observed first order behavior requires that any such reaction with species responsible for the primary and secondary spectra does not produce reactive fragments discrete from the protein which react at later stages.

There is no reasonable alternative to concluding that the chemical transformations associated with the p → s spectral transformations are reactions of radicals

produced by the initial action of OH. The present data do not determine whether the s → t and t → q spectral transformations are due to second and third consecutive steps or to parallel reactions with different rate constants. Chemical evidence[6,7] shows that the action of OH radicals, like that of H atoms, results in significant modification of only tyrosine, cystine and methionine units, but with an efficiency smaller than that of H atoms. This fact is suggestive of the following sequence: radical sites are produced initially almost at random on accessible surface sites of the protein by OH radicals, which are more reactive and less selective than H atoms[3]. Some of the radicals are eventually transmitted to the loci leading to the specific series of events damaging sulfur and aromatic amino acids. This transfer is less likely for the OH radical adducts than for H. Conversely OH radicals lead to greater damage at surface sites. This is supported by product studies which have shown[6,7] that inactivation of ribonuclease by OH radicals is associated with extensive dimerization of the protein. Dimerization is practically absent upon H atom attack. Formation of dimers must be a process second order in radicals. It is too slow to be observable on the time scales available to us.

It is interesting to compare the kinetics of interconversion of spectra produced by initial reaction with OH with corresponding data for H atom reaction. Over the pH range, k[p → s] is generally greater with OH than with H, the difference being largest around neutrality where it amounts to a factor of 10–100 depending on wavelength. The more random nature of the initial reaction of OH is reflected in the much greater variation of k[p → s] with wavelength. Two sequential spectral transformations were observed in the H atom reaction, a sequence of three after reaction with OH, except at pH 3.5 where only two were observed. At pH about 7 k[s → t] from reaction with OH is 2–10 times faster than k[s → t] from reaction with H. In this case, the specific rate of the transformation originating in reaction with OH is independent of wavelength, while k[s → t] from the H atom reaction varies sharply with wavelength. The transformation t → q, which was not observed in the H atom reaction, is about one tenth as fast at pH 5.5 and 7.3 as the change s → t. As has already been noted, the magnitude of k[s → t] from the reaction of OH at pH 3.5 is similar to those of k[t → q] at pH 5.5 and 7.3 and is about one fourth as large as k[s → t]from H atoms at pH 3.5.

Chemical interpretations of some of these changes are suggested in the discussion of the spectra.

Reaction of hydrated electrons. All of the relatively limited number of kinetic measurements were consistent with first order transformations of spectra. It seems probable that these transformations are also due to intramolecular chemical changes.

The magnitude of k[p → s] from the electron reaction at pH 6.0 differs from k[p → s] from the H atom reaction near this pH by a factor of less than two and, like it, varies little with wavelength, suggesting some similarity in chemical transformations. The values of k[t → q] from the electron reaction at pH 6.0 and 7.6 are very similar to k[s → t] from the H atom reaction at pH 3.5 but do not display the wavelength dependence which characterizes the latter at pH 6.6. The data seem to suggest that at some sites proton transfers can interconvert products of addition of H atoms and hydrated electrons. It is interesting in this connection that k[s → t] from the reaction of hydrated electrons at pH 7.6 is essentially identical with the (high) long

wavelength value of k[s → t] from the reaction of H atoms at pH 6.6, a relationship which is consistent with spectral similarities.

Spectra

Reaction of OH radicals. Primary spectra, recorded about 10 μs after pulsing, are similar at pH 3.5, 5.5 and 7.3. The principal features are well defined intense maxima at approx. 250 and 300 nm, broader somewhat less intense absorption centering around 330 nm, the suggestion of a weaker maximum around 400 nm with a tail gradually diminishing towards longer wavelength. The observed primary spectrum is the summation of spectra due to a number of different radical sites (each located in a different molecule of ribonuclease). Assignments of absorption bands is largely speculative.

An acceptable assignment for the peak at about 250 nm is to radicals of the type –CONHĊ(R)CO–. It has recently been shown[8,9] that radicals of the type RCONHĊR'R'' formed by attack of OH on *N*-alkyl amides have spectra characterized by maxima around 245 nm, ε_{max} about 7000 $M^{-1} \cdot cm^{-1}$ and at 350–400 nm, ε_{max} about 2000 $M^{-1} \cdot cm^{-1}$. The spectra of radicals of the type RR'ĊCONR''R''' are characterized by single maxima at 350–500 nm with ε_{max} approx. 1000. Radicals of the type –CONHĊ(R)CO– are related to both the latter types. Hydrogen abstraction by OH from the saturated carbon atoms of the peptide links is to be expected in view of their large number and of their fairly high individual reactivity. A specific rate of the order of several times 10^8 $M^{-1} \cdot s^{-1}$ is to be expected[8,9] for one such structural grouping. If the 250-nm peak is due to –CONHĊ(R)CO– radicals and ε_{max} of these radicals is similar to ε_{max} of RCONHĊR'R'' radicals then abstraction of H atoms from the saturated carbon of the peptide link accounts for about 20% of the total action of OH radicals. (Radicals produced by the action of OH on simple peptides in acidic solution have similar spectra. It has been suggested that the absorbing species in these cases is –CONHĊRCO$_2^-$H. See ref. 10.)

The maximum at 300 nm is at the same wavelength as the prominent feature of the primary spectrum produced by the action of H atoms on ribonuclease which is, in the latter case, more intense in the secondary spectrum. In spite of the identity of λ_{max} in the two cases, less than 10% of the primary absorption at 300 nm recorded in Figs 1–3 can be due to action of H atom. The position of the maximum is consistent with its being due to radicals formed by attachment of OH to aromatic or heterocyclic rings. Two possibilities are phenylalanine and histidine units[11]. If an extinction coefficient of the order of 5000 $M^{-1} \cdot cm^{-1}$ is assumed for such radicals then it would appear that about 25% of incident OH adds to such sites.

The similarity of the absorption band centering around 330 nm to absorption produced by the action of OH on tyrosine has been pointed out[11]. It is to be expected that –CONHĊ(R)CO– radicals would contribute to absorption in this region as well as to absorption at higher wavelengths.

It has already been noted (see Table I) that k[p → s] is strongly pH-dependent. The rate differences are paralleled by spectral differences. At pH 3.5 and 5.5 (see Figs 4 and 5) the s – p difference spectrum is characterized by a well defined negative peak at 340 nm and a smaller positive peak around 400 nm. Both these difference peaks, as well as the corresponding magnitude of k[p → s], *i.e.* about 10^3 s^{-1}, are similar to spectral changes which have been ascribed[12] to the conversion of the

adduct of OH radical to tyrosine into a phenoxy radical. In contrast, the most prominent feature of the p — s difference spectrum at pH 7.3 is an intense positive peak with λ_{max} about 400 nm. The negative difference peak around 340 nm is not observed at this pH but the shape of the 400-nm positive peak suggests that this may be due to the overlap of these difference peaks of opposite sign. A possible interpretation of the intense positive difference peak at 400 nm is that it is due to transfer of reducing equivalents from initially formed radicals to S–S links to give intensely absorbing –Ṡ–S̄– (ref. 13). The absence of a strong positive difference peak around 400 nm at acid pH may be due to the weaker absorption at this wavelength of the –S–Ṡ(H)– radical. Alternatively, it may be due to decay of the disulfide radical which, due to acid catalysis[14], is so rapid that it was not observed under the conditions of these experiments.

The t — s and q — t difference spectra are more diffuse. Negative difference peaks in the vicinity of 250 and 300 nm are their most prominent features. It is noteworthy that at pH 7.3 the relatively large magnitude of k[s → t] (see Table II) is associated with much larger negative difference peaks than are observed at acidic pH. Possibly deprotonation is associated with the more rapid decay of the radicals responsible for these peaks.

The principal feature of the stable spectra presented in Fig. 7 is pH-dependent diffuse positive absorption extending from 230 to 370 nm. The contrast with the stable negative absorption produced by the action of H atoms[1,2] is striking. A possible source of some of the permanent absorption originating in attack by OH radicals is the resultant formation of quinonoid structures[15].

Reaction of electrons. The primary spectrum produced by electron addition at pH 7.6 resembles that which has been reported by Adams *et al.*[11] at wavelengths above about 330 nm but lacks the relatively intense absorption around 300 nm reported by these workers. The most prominent features of this spectrum can reasonably be assigned to –Ṡ–S̄– radicals[13]. The weaker, more diffuse spectrum obtained at pH 6.0 is similar in both intensity and form to the primary spectrum produced by the action of H atoms on ribonuclease at pH 6.6. Possibly the difference between primary electron adduct spectra at pH 6.0 and 7.6 reflects a difference in the state of protonation of the electron adduct to disulfide and/or the chemical decay of this adduct[14]. Only decay of absorption is apparent in the difference spectra obtained at pH 6.0 and 7.6. A large part of this decay occurs at the t → q stage and the pH dependence of the q — t difference spectra is consistent with the dependence of the primary spectra on pH.

The stable spectrum obtained at pH 6.9 is very weak. Its main feature is weak negative absorption at the short wavelength end which resembles a feature of the stable spectrum obtained by addition of H atoms. This negative absorption is more pronounced at pH 5.8. It was suggested[1,2] in connection with the H adduct stable spectrum that this negative absorption may be associated with modification of sulfur containing moieties.

CONCLUSIONS

The kinetic data support the occurrence of consecutive intramolecular processes consequent upon addition of both OH radicals and hydrated electrons. The previous finding[1,2] of such intramolecular reactions originating in the action of H atoms on ribonuclease is thus generalized. Spectral data indicate the involvement of aromatic and sulfur-containing amino acid residues in the reactions of OH radicals with ribonuclease—a result similar to that obtained with H atoms and supported by an investigation[6,7] of the products of this reaction. The similarities of the reaction of OH radicals and H atoms are considerable and are due at least in part to the ability of some of the radicals formed by the initial action of OH to serve as intramolecular reducing agents. Electron adduct spectra indicate action on disulfide links.

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REFERENCES

1 N. N. Lichtin, J. Ogdan and G. Stein, *Biochim. Biophys. Acta*, 263 (1972) 14.
2 N. N. Lichtin, J. Ogdan and G. Stein, *Radiat. Res.*, 47 (1971) 349.
3 M. Anbar and P. Neta, *Int. J. Appl. Radiat. Isot.*, 18 (1967) 493.
4 M. Simic and M. Z. Hoffman, *J. Am. Chem. Soc.*, 92 (1970) 6096.
5 R. Braams, in M. Ebert, J. P. Keene, A. J. Swallow and J. H. Baxendale, *Pulse Radiolysis*, Academic Press, New York, 1965, p. 171.
6 L. K. Mee, S. J. Adelstein and G. Stein, *Radiat. Res.*, (1972) in the press.
7 L. K. Mee, S. J. Adelstein and G. Stein, *Radiat. Res.*, 47 (1971) 349.
8 E. Hayon, T. Ibata, N. N. Lichtin and M. Simic, *J. Am. Chem. Soc.*, 92 (1970) 3898.
9 E. Hayon, T. Ibata, N. N. Lichtin and M. Simic, *J. Am. Chem. Soc.*, 93 (1971) 5388.
10 M. Simic, P. Neta and E. Hayon, *J. Am. Chem. Soc.*, 92 (1970) 4763.
11 G. E. Adams, R. I. Willson, R. H. Bisby and R. B. Cundall, *Int. J. Radiat. Biol.*, 20 (1971) 405.
12 E. J. Land and M. Ebert, *Trans. Faraday Soc.*, 63 (1967) 1181.
13 G. E. Adams, C. S. McNaughton and B. D. Michael, in G. R. A. Johnson and G. Scholes, *The Chemistry of Ionization and Excitation*, Taylor and Francis, Ltd, 1967, p. 281.
14 A. Shafferman, *Israel J. Chem.*, (1972) in the press.
15 R. Shapira and G. Stein, *Science*, 162 (1968) 1489.