251. Chemical Alterations in Native Histone Octamer Complexes Induced by the Attack of ·OH and $\cdot N_3$ Radicals

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Dedicated to Prof. *Tino Gäumann* on the occasion of his 60th birthday

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·OH Radicals generated by short electron-beam pulses were allowed to attack histone octamer complexes (extracted from calf-thymus chromatin) in N_2O-saturated dilute solution (0.5–1.3 g/l, [$NaClO_4$] = 1 – 2M, pH 9). They induced a volume contraction due to intra-complex cross-linking. In this process, essentially non-tyrosine moieties of the proteins were involved. Phenol coupling *via* tyrosyl radicals occurred mainly as an intramolecular reaction, *i.e.*, it was restricted to single histone molecules. Furthermore, it turned out that only about 55% of the tyrosine moieties were accessible to attacking ·OH and/or $\cdot N_3$ radicals. When $\cdot N_3$ radicals were generated *via* continuous irradiation of N_2O-saturated octamer solutions containing NaN_3 with ^{60}Co-γ-rays, dimers, trimers, and tetramers were detected by SDS gel electrophoresis, in contrast to pulse radiolysis where only dimers were found. These results were explained in terms of denaturation being induced by small chemical changes and causing partial or complete dissociation of the complexes thus permitting, in the course of the γ-irradiation, the attack and conversion of amino-acid moieties non-accessible in the native octamer complexes. Removal of steric restrictions for the combination of tyrosyl radicals may also play a role. By time-resolved absorption measurements, it was shown that, upon the attack of intact octamer complexes by ·OH radicals, tyrosyl radicals were formed which were converted to dityrosine groups according to two modes with half-lives of several 100 µs and 1–2 ms, respectively. Cross-linking of histone molecules occurred with a definitely lower rate (1st half-life: 50–100 ms). This process was detectable both by optical absorption measurements at λ = 300–400 nm and by light-scattering measurements.

1. Introduction. – In the course of our studies concerning the characterization of radiation damage in biopolymers, we recently started to investigate histone octamer complexes in the native state [1]. The complexes which consist of two sets of the histones H2A, H2B, H3, and H4 are major constituents of the nucleosomes in which double-stranded DNA, tied up by histone H1, is wrapped around the octamer complexes [2a] [3] [4]. This is illustrated in *Fig. 1*. The histones are proteins containing relatively large portions of arginine and lysine moieties (25–30 mol-%) which gives them a basic character and enables them to form strong associations with DNA at physiological pH ranges.

In preliminary studies, native histone octamer complexes, extracted from calf thymus chromatin, were irradiated in the isolated form in dilute, N_2O-saturated aqueous solutions with 16-MeV electron-beam pulses or ^{60}Co-γ-rays [1]. From the results of these experiments, it was inferred that the octamers underwent a volume contraction due to intra-complex cross-linking induced by ·OH radicals. Fluorescence measurements on irradiated octamer solutions yielded the characteristic emission of dityrosine (λ_{max} = 410 nm) indicating that crosslinking proceeded at least to a certain extent *via* 2,2′-biphenol coupling. Here we report extensively on these studies which include, apart from product analyses, time-resolved measurements of changes in the optical absorption and emission

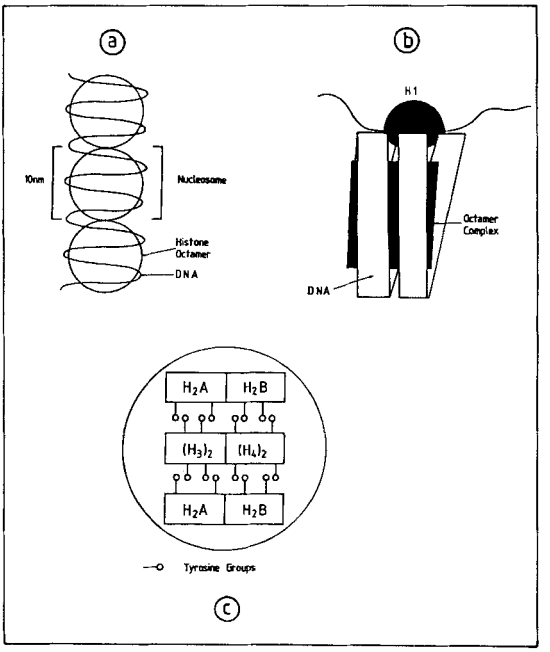


Fig. 1. *Structure of chromatin constituents.* DNA (146 base pairs per bead) is wrapped around globular histone beads (*a*, after *Kornberg* [2a]; *b*, after *Bradbury* [2b]) containing two sets of the histones H2A, H2B, H3, and H4 (MW: 1.1–1.5 × 10^4). The histone H1 (MW: 2.4 × 10^4) is located at the region where DNA enters and exits the core particle. The central globular domain of H1 is able to close two full turns of DNA around the histone octamer. In *c* the schematic arrangement of the histone molecules within the octamer complex is depicted. Only two tyrosine moieties per histone molecule are shown. The actual numbers are: 3 (H2A), 5 (H2B), 3 (H3), and 4 (H4).

as well as in the light scattering intensity (LSI). In addition to reactions induced by ·OH radicals also reactions of azide radicals, $\cdot N_3$, were studied. Azide radicals were readily generated by irradiating N_2O-saturated aqueous solutions of NaN_3 in which the hydrated electrons were converted to ·OH radicals and the latter reacted with the N_3^- ions [5–7]:

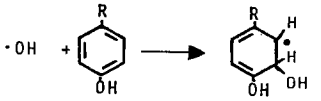
$$\cdot OH + N_3^- \rightarrow OH^- + \cdot N_3 \qquad (1)$$

Reactions of $\cdot N_3$ radicals with several proteins and peptides were recently investigated [8–11] and it was found that $\cdot N_3$ radicals possess a pronounced ability to selectively attack certain amino-acid moieties, *i.e.* tryptophan, tyrosine, and cysteine. Because calf-thymus histones do not contain tryptophan and only very little cysteine[1]) [12], reactions of $\cdot N_3$ radicals with tyrosine moieties are easily detectable. *Prütz et al.* [11], for example, observed the formation of tyrosyl radicals and their combination to 2,2′-biphenol groups subsequent to the reaction of $\cdot N_3$ radicals with several peptides and proteins including a commercial histone product consisting of a mixture of H1, H2A, H2B, H3, and H4.

Reactions of tyrosyl radicals, especially the formation of dityrosine groups, are considered, at present, to play an important role in the high-energy-radiation-induced inactivation of proteins.

[1]) Only histone H3 contains cysteine (2 cysteine moieties per molecule).

2. Reactions of ·OH and $\cdot N_3$ Radicals with Aromatic Amino Acid Moieties. – From the results reported below, it was inferred that phenylalanine and tyrosine moieties are involved in the product formation upon the reaction of ·OH and $\cdot N_3$ radicals with histone octamer complexes extracted from calf-thymus chromatin. Therefore, it seems helpful for the interpretation of the results to recapitulate what is known about these reactions. The addition of ·OH radicals to the benzene rings yields cyclohexadienyl type radicals:



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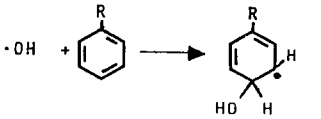
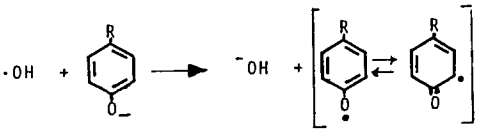


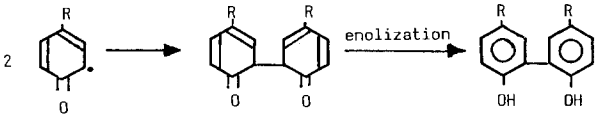
(3)

These radicals possess an absorption maximum around 330 nm [13]. Dihydroxycyclohexadienyl radicals formed according to *Reaction 2* can be converted to tyrosyl radicals by H_2O elimination [13]. In the case of tyrosine, an electron-transfer reaction can also occur in alkaline solution:



(4)

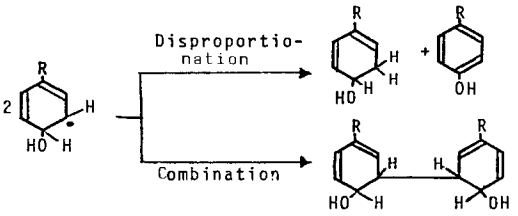
Tyrosyl radicals formed in this way absorb strongly at about 300 and 410 nm [7] [8]. They can combine to dityrosine:



(5)

Dityrosine strongly absorbes between 300 nm and 330 nm ($\varepsilon_{316\ nm} = 5.79 \times 10^3\ M^{-1}cm^{-1}$, in basic media) and fluoresces characteristically around 410 nm [11].

Cyclohexadienyl-type radicals, generated from phenylalanine, can combine or disproportionate. The latter reaction yields tyrosine which, at pH > 7, has an absorption maximum around 280 nm.



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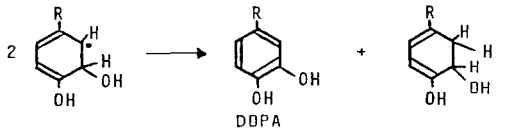
Upon disproportionation of OH-adduct radicals generated from tyrosine according to *Reaction 2*, 3,4-dihydroxyphenylalanine (DOPA) is formed:

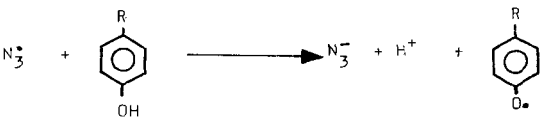


(8)

The absorption spectrum of DOPA ($\lambda_{max} \approx 280$ nm) is similar to that of tyrosine. The extinction coefficients, however, differ remarkably: $\varepsilon_{285\ nm} = 4918 M^{-1}cm^{-1}$ (DOPA) and $\varepsilon_{273\ nm} = 1721 M^{-1}cm^{-1}$ (tyrosine). $\cdot N_3$ radicals can react with ionized tyrosine moieties in alkaline media

(9)

The reaction with non-ionized tyrosine moieties is also feasible:



(10)

Rate constants of the reaction of $\cdot N_3$ radicals with (low molecular weight) tyrosine below and above the pK value of 10.1 were reported by *Prütz* and *Land* [8]: at pH 6.5, $k = 1.0 \times 10^8\ M^{-1}s^{-1}$ and at pH 11.8, $k = 3.6 \times 10^9\ M^{-1}s^{-1}$.

3. Experimental. – 3.1. *Materials. Native histone octamer complexes* were isolated from chromatin which was obtained from fresh calf thymus. The procedure was based on methods described in [14–19]. The thymus gland was homogenized in 0.14M NaCl soln. containing the disodium salt of ethylene diaminetetraacetic acid (2Na-EDTA) at a concentration of 10 mM, pH 8.0. The cell nuclei, so obtained, were washed several times with the same saline solution by renewed homogenization followed by centrifugation to recover the insoluble chromatin. Non-histone proteins and histone H1 were separated by extraction with 0.35M and 0.65M NaCl containing 10 mM 2Na-EDTA, pH 8, resp. The H1-depleted chromatin was dissolved in 1.98M NaCl, 6 mM $Na_2B_4O_7$, pH 9. After stirring for 24 h, the DNA was pelleted by ultracentrifugation for 16 h in a *Beckman 60Ti* rotor at 55,000 rpm. All procedures were carried out at 4°. Octanol was present during homogenization to prevent surface denaturation and 1 mM phenylmethanesulphenylfluoride (PMSF) (added as a 50 mM soln. in i-PrOH) was present in all solns. to prevent protease activity. PMSF, octanol, and impurities were removed by diafiltration against 1.98M NaCl, 6.85 mM $Na_2B_4O_7$, pH 9, using an ultrafiltration cell (*Amicon*, type *8400*) fitted with a *DIAFLO* ultrafiltration membrane (*Amicon*, type *YM 10*). Using the same set-up, the chloride ions were then exchanged for perchlorate ions. If necessary, the octamer concentration was increased using the ultrafiltration cell operating in the concentration mode. The octamer concentration was determined with the aid of the method of *Lowry et al.* [21]. Irradiation experiments were carried out at octamer concentrations of 0.5–1.3 g/l. The octamer samples were analyzed for DNA using the 'diphenylamine method' of *Burton* [20]. In all preparations, the DNA concentration was below the sensitivity limit of the method, *i.e.* below 2 μg/ml.

It was shown previously [22] [23] that the stability of histone octamer complexes depends on the ionic strength, the histone concentration and the pH of the soln. There is a pronounced tendency for dissociation of the complexes to $(H3–H4)_2$ tetramers and H2A–H2B dimers at relatively low ionic strength and at low and very high pH. The complexes were reported to be stable at pH 9, ionic strength of 1–2M and histone concentrations of 0.5–2.0 g/l. These conditions were chosen for the experiments carried out in this work.

3.2. *Irradiation of Histones.* N_2O-saturated solns. of histones were irradiated at pH 9 with 100 ns pulses of 16 MeV electrons. In addition, continuous irradiations with ^{60}Co-γ-rays were carried out. In the pulse radiolysis experiments, formation and decay of transients were monitored with the aid of optical absorption, light emission, and light scattering measurements. The absorbed dose was determined with the aid of the *Fricke* dosimeter using O_2-saturated solns.

3.3. *Product Analysis by Optical Absorption and Fluorescence Measurements.* The optical absorption and emission spectra of irradiated solns. were recorded with the aid of a UV/VIS spectrophotometer (*Perkin-Elmer*, model *550 S*) and a spectrofluorimeter (*Hitachi-Perkin-Elmer*, model *MPF 4*).

3.4. *Product Analysis by Gel Electrophoresis.* Prior to the determinations, octamer complexes were denatured by heating the irradiated solns. for 2 min at 100° after the addition of sodium dodecyl sulfate (SDS, 50 g/l), 2-mercaptoethanol (0.72M), trishydroxymethylaminomethane (TRIS; 2.5×10^{-3}M), and sucrose (140 g/l). The runs were carried out as described in [24] with an apparatus of *Pharmacia*, model *151*, using gel rods of 0.5 × 10.0 cm. The aqueous gel consisted of acrylamide (150 g/l), *N,N'*-methylenebisacrylamide (1 g/l), SDS (1 g/l), and TRIS (0.9M). Both, the lower and the upper electrode were surrounded by buffer solution of pH 8.3 (0.05M TRIS, 0.38M glycine, and 1 g/l SDS). The electrophoreses proceeded for 17 h towards the anode at 38 V. Subsequently, the gels were developed with the aid of amido black. For removal of non-adsorbed dye, the gel rods were destained at 60° with an aq. soln. containing 10% (v/v) glacial acetic acid and 30% (v/v) EtOH. After destaining, the gel rods were scanned at $\lambda = 600$ nm using a gel scanner in combination with a *Perkin-Elmer* UV/VIS spectrophotometer (model *550 S*). The slit width used for scanning the gels at a speed of 10 mm/min was 0.05 mm.

4. Results and Discussion. – 4.1. *Product Analysis after Electron-Beam (EB) Irradiation.* N_2O-saturated solutions of histone octamer complexes (pH 9, $[NaClO_4] = 2$M) were irradiated with single-electron beam pulses. $NaClO_4$ is inert with respect to its reactivity towards ·H and ·OH radicals, and the rate constant, $k(e_{aq}^- + ClO_4^-) = (2.8 \pm 0.4)\ 10^5$ $M^{-1}s^{-1}$ for the reaction of the hydrated electron with the perchlorate ion was determined in this work, which implies that practically all hydrated electrons were scavenged by N_2O, even at $[NaClO_4] = 2$M. Fluorescence measurements ($\lambda_{max} = 405$ nm, $\lambda_{exc} = 325$ nm) revealed the formation of dityrosine groups. As is shown in *Fig. 2a*, the conversion of

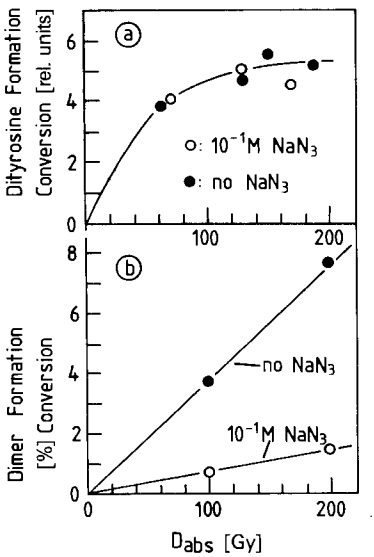


Fig. 2. *Irradiation of octamer complexes with 16-MeV electrons in N_2O-saturated aqueous solution* (pH 9) *containing $NaClO_4$* (2M) *and $Na_2B_4O_7$* (6.85 mM). [Histone] = 1.3 g/l. *a*) Dose dependence of the conversion of tyrosine to dityrosine groups in the absence and presence (10^{-1} M) of NaN_3 as determined by fluorescence measurements ($\lambda_{exc} = 325$ nm). *b*) Dose dependence of the conversion of histone molecules to chemically linked histone dimers in the absence and presence (10^{-1} M) of NaN_3 as determined by gel electrophoresis.

tyrosine into dityrosine groups increased with increasing absorbed dose and approached a limiting value. It is noteworthy that both in the absence and presence of NaN_3 the same results were obtained. Therefore, it seems that only a limited fraction of tyrosine moieties are prone to attack by ·OH or $\cdot N_3$ radicals and/or to combine to dityrosine groups.

The question now arises as to whether this behavior is due to the limited accessibility of tyrosine moieties to the attack of ·OH or $\cdot N_3$ radicals or to the fact that a fraction of

tyrosyl radicals formed by the attack of these radicals is prevented from interacting with each other because of the fixed conformation of the histone molecules in the octamer complex. It seems that both effects become operative. Actually, there is evidence for the non-accessibility of a significant portion of tyrosine groups: time-resolved spectroscopy studies revealed that only about 55% of the tyrosine moieties are capable of forming tyrosyl radicals *(vide infra)*. In this connection, it is interesting to draw the attention to the results obtained from gel electrophoresis experiments. It can be seen from *Fig. 2b* that intermolecular links were formed as indicated by the formation of dimers. The conversion of histones to dimers increased with increasing absorbed dose. NaN_3 strongly impeded dimer formation. From these results and the data shown in *Fig. 2a*, it can be concluded that upon attack by ·OH radicals non-tyrosine moieties play an important role in the formation of linkages between different histone molecules in octamer complexes and moreover, that phenol coupling involving tyrosyl radicals contributes to cross-linking but only to a limited extent.

Another interesting result, obtained during these studies, is the fact that multimers other than dimers were not detected. This is in contrast to the findings of the continuous γ-irradiations of octamer complexes, where higher multimers were detected *(vide infra)*. Light-scattering measurements with the native octamer complexes carried out before and after irradiation with EB pulses yielded, in the absence of N_3^- ions, a clearly detectable increase in the LSI, the extent of which was independent of the absorbed dose per pulse. The latter fact was taken as evidence that the observed effect is due to intra-complex cross-linking [1]. In the case of inter-complex cross-linking, the extent of LSI increase is expected to increase with the absorbed dose, because an increasing number of complexes should be aggregated with increasing absorbed dose. In accordance with the relatively low yield of dimers, the extent of the LSI increase was very low, actually just surpassing the detection limit, when NaN_3 was present in the solution.

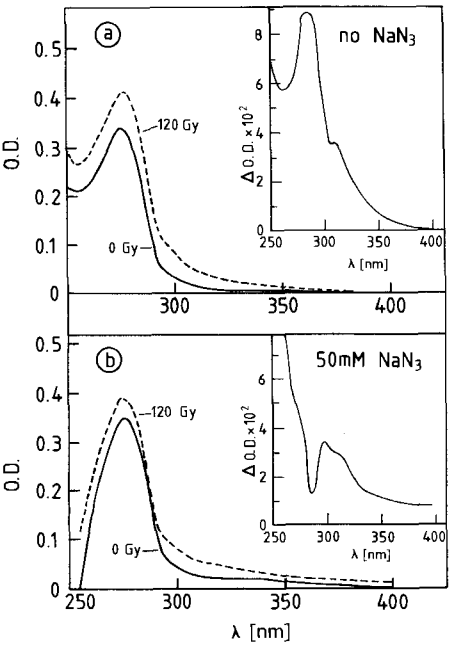


Fig. 3. *Irradiation of octamer complexes with 16-MeV electrons in N_2O-saturated aqueous solution* (pH 9) *containing $NaClO_4$*(1M) *and $Na_4B_2O_7$* (6.85 mM). [Histone] = 0.75 g/l. Absorbed dose 120 Gy. Optical absorption spectra recorded before (——) and after (– – –) irradiation in the absence (*a*) and in the presence (*b*) of NaN_3 (50 mM). Insets: Difference absorption spectra.

Difference optical-absorption spectra obtained from spectra taken before and after irradiation are shown in *Fig. 3a* and *b*. They revealed the formation of dityrosine groups in the presence of NaN_3 as indicated by the band at about 320 nm. In the absence of NaN_3, at least one additional product, possibly dihydroxyphenylalanine formed according to *Reaction 8*, was evidenced by the strong absorption band between 250 nm and 300 nm.

4.2. *Product Analysis after γ-Irradiation.* The characteristic fluorescence of dityrosine groups was also detected after γ-irradiation of octamer complexes under the same

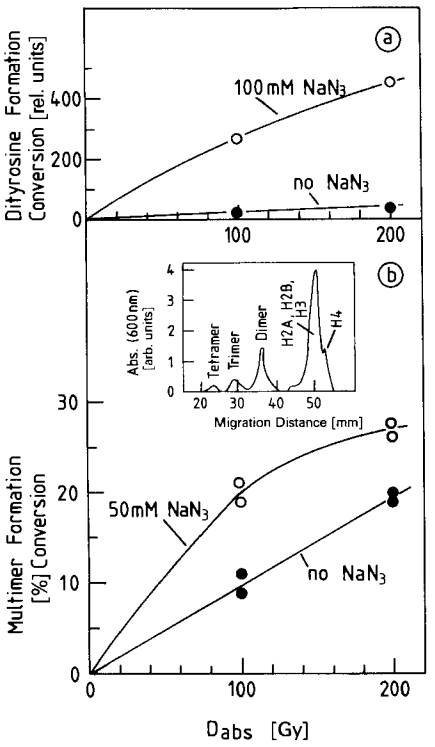


Fig. 4. *^{60}Co-γ-irradiation of octamer complexes in N_2O-saturated aqueous solution* (pH 9) *containing $NaClO_4$* (2M) *and $Na_2B_4O_7$* (6.85 mM). [Histone] = 1.3 g/l (*a*), 0.75 g/l (*b*). Absorbed dose rate: 100 Gy/h (*a*), 300 Gy/h (*b*). *a*) Dose dependence of the conversion of tyrosine to dityrosine groups in the absence and presence (10^{-1} M) of NaN_3 as determined by fluorescence measurements (λ_{exc} = 325 nm). *b*) Dose dependence of the conversion of histone molecules to chemically linked histone multimers in the absence and presence (10^{-1} M) of NaN_3 as determined by gel electrophoresis. Inset: Typical gel trace obtained after irradiation at 100 Gy in the presence of NaN_3 (50 mM).

conditions as in the case of EB irradiation. As can be seen from *Fig. 4a*, the conversion of tyrosine to dityrosine groups increased with increasing absorbed dose. In contrast to the results obtained with EB irradiation, dityrosine groups were formed with a much higher yield in the presence than in the absence of NaN_3. This result indicates that in the case of γ-irradiation, tyrosine moieties are rather easily converted to dityrosine groups which might be due both to an unhindered accessibility of tyrosine moieties to $\cdot N_3$ and to the removal of steric restrictions to radical combination in the course of the irradiation. A plausible explanation is that octamer complexes are denatured with time after being chemically altered initially, *i.e.* in the initial stages of the irradiation. Denaturation leads to a rather open structure, probably to a dissociation of complexes. The gel-electrophoresis results shown in *Fig. 4b* led to the same conclusion: In addition to dimers also trimers and tetramers were detected. These results also are understandable in terms of octamer complexes being disintegrated at low absorbed doses, so that cross-linking between

different histone molecules, including molecules from different octamer complexes by secondary processes, is quite feasible. In accordance with this explanation is the fact that multimerization was favored in NaN_3-containing solutions as can also be seen from *Fig. 4b*. In the absence of NaN_3 the yield of dimers (higher multimers were not detectable in this case) was much less pronounced than in its presence.

Moreover, gel electrophoresis together with fluorescence results demonstrate the importance of biphenol linkages in intermolecular cross-linking processes in octamer complexes induced by continuous γ-irradiation.

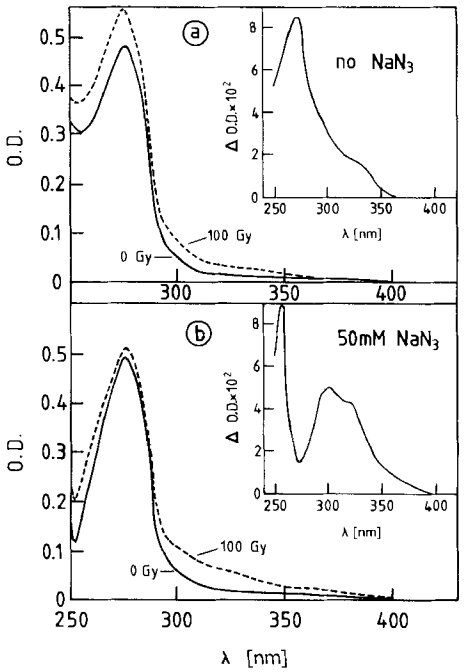


Fig. 5. *^{60}Co-γ-irradiation of octamer complexes in N_2O-saturated aqueous solution* (pH 9) *containing $NaClO_4$* (2M) *and $Na_2B_4O_7$* (6.85 mM). [Histone] = 0.9 g/l; absorbed dose = 100 Gy at a dose rate of 300 Gy/h. Optical absorption spectra recorded before (——) and after (- - -) irradiation in the absence (*a*) and presence (*b*) of NaN_3 (50 mM). Insets: Difference absorption spectra.

As can be seen from *Fig. 5*, absorption measurements of γ-irradiated octamer solutions led to similar conclusions as in the case of EB irradiations: in the presence of NaN_3, dityrosine groups were formed as indicated by the absorption band at $\lambda = 320$ nm, whereas, in the absence of NaN_3 in addition to these, other products were detectable by their absorption bands at $\lambda < 300$ nm.

With respect to end products which are formed as a consequence of ·OH addition to tyrosine or phenylalanine, it has to be considered that cyclohexadienyl-type radicals can disproportionate or combine according to *Reaction 6* and *7*. The products of both reactions absorb between 250 nm and 300 nm.

3,4-Dihydroxyphenylalanine is produced by disproportionation of OH-adduct radicals of tyrosine according to *Reaction 8*. As has been pointed out *(vide ante, Chap. 2)*, DOPA possesses like tyrosine an absorption maximum around 280 nm. However, its extinction coefficient is much greater than that of tyrosine. Consequently, the formation of DOPA is expected to lead to an increase in the permanent absorption between 250 nm and 300 nm, even when tyrosine is consumed.

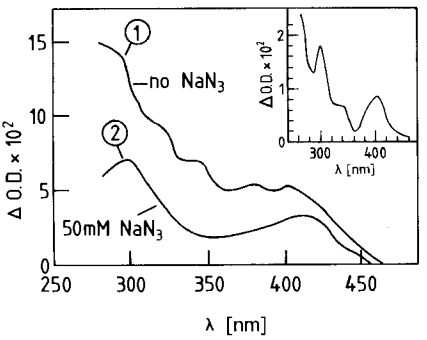


Fig. 6. *Irradiation of octamer complexes with 16-MeV electrons in N_2O-saturated aqueous solution containing $NaClO_4$* (1M) *and $Na_2B_4O_7$* (6.85 mM). [Histone] = 0.6 g/l. D_{abs} = 115 Gy. Transient absorption spectra recorded in the absence (*1*) and presence (*2*) of NaN_3 (50 mM). Inset: Difference absorption spectrum: $(O.D.)_{t=0} - (O.D.)_{t=900\mu s}$ recorded in the absence of NaN_3.

4.3. *Detection of Transient Species by Pulse Radiolysis. Fig. 6* shows transient absorption spectra that were recorded about 25 μs after irradiating N_2O-saturated octamer solutions with 100-ns single pulses of 16 MeV electrons. The spectra were formed with half-lives (about 5 μs) reflecting the reaction of ·OH or $\cdot N_3$ radicals with histone moieties. The spectrum obtained in the presence of NaN_3 *(Fig. 6 (2))* possesses maxima around 400 nm and 300 nm characteristic for tyrosyl radicals [7] [8] which were formed *via* the reaction of $\cdot N_3$ radicals with tyrosine moieties according to *Reaction 9*.

Because the experiments were carried out at pH 9, *i.e.*, below the *pK* value of tyrosine (10.1), the reaction of azide radicals with non-ionized tyrosine according to *Reaction 10* is also feasible. The spectrum obtained in the absence of azide (*1* in *Fig. 6*) is less characteristic than the spectrum obtained in its presence (*2* in *Fig. 6*). However, substraction of the spectrum recorded 900 μs after the pulse from the initial one yields a spectrum (see inset in *Fig. 6*) with maxima at 300 nm and 400 nm and a shoulder at 330 nm indicating the existence of tyrosyl radicals (300 and 400 nm) and of cyclohexadienyl-type radicals (330 nm), respectively. The latter were generated upon the addition of ·OH radicals to tyrosine or phenylalanine moieties, respectively, according to *Reactions 2* and *3*. Tyrosyl radicals were produced by electron-transfer reactions according to *Reaction 4*.

Evidence for the non-accessibility of a fraction of tyrosine moieties to the attack of $\cdot N_3$ radicals was obtained from the data shown in *Fig. 7*, where the calculated and the

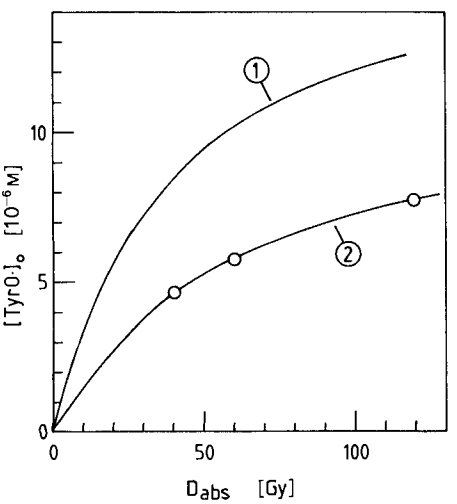


Fig. 7. *The initial concentration of tyrosyl radicals* vs. *the absorbed dose. Curve 1:* calculated according to *Eqn. 13. Curve 2:* measured at λ = 405 nm (ε = 3200 M^{-1} cm^{-1}, d = 1 cm). For reaction conditions refer to caption of *Fig. 6*.

measured initial concentration of tyrosyl radicals is plotted *vs.* the absorbed dose. The experimental values were obtained from the O.D. formed after the pulse at $\lambda = 405$ nm ($\varepsilon = 3200\ M^{-1}\ cm^{-1}$). It is obvious that the experimental values are about 45% lower than the calculated ones. Therefore, it is concluded that a significant portion of the tyrosine moieties is actually hidden, *i.e.*, not accessible to $\cdot N_3$ radicals. The calculation was based on the reaction mechanism and the rate constants shown in the *Table*. Moreover, it was assumed that the tyrosine moieties are homogeneously distributed in the solution. The radiation chemical yield of $\cdot N_3$ radicals was taken as being equal to the yield of hydroxyl radicals, *i.e.*, $G(\cdot N_3) = G(\cdot OH) = 5.6$. This implies that, at $[N_3^-] = 50$ mM, practically all $\cdot OH$ radicals are converted to $\cdot N_3$ radicals. That this assumption is justifiable results from the following consideration: with the rate constants given in the *Table*, the probability, α_b, for the occurrence of *Reaction b* in the *Table*, is obtained according to *Eqn. 11*:

$$\alpha_b = \frac{k_b [N_3^-]}{k_b [N_3^-] + k_a [\cdot OH]_o} \tag{11}$$

with $[N_3^-] = 50$ mM, α_b is almost unity, as shown in *Eqn. 12* for $D_{abs} = 115$ Gy:

$$\alpha_b = \frac{6.5 \times 10^9 \times 5 \times 10^{-2}}{6.5 \times 10^9 \times 5 \times 10^{-2} + 5 \times 10^9 \times 6.7 \times 10^{-5}} = 0.999 \tag{12}$$

Curve 1 in *Fig. 7* was calculated with the aid of *Eqn. 13*:

$$[TyrO\cdot]_o = \alpha_d [\cdot N_3]_o \tag{13}$$

with

$$\alpha_d = \frac{k_d [Tyr]}{k_d [Tyr] + [H] \Sigma k_{e,i} n_i + k_c [\cdot N_3]_o} \tag{14}$$

where [H] = concentration of histone octamer complexes
and n_i = number of moieties of amino acid i per octamer complex.

Table. *Formation and Reactions of $\cdot N_3$ Radicals.* AmA = amino acid moieties, other than tyrosine.

	Reaction	Rate Constant [$M^{-1}\ s^{-1}$]	Ref.
(a)	$\cdot OH + \cdot OH \xrightarrow{k_a} H_2O_2$	$k_a = 5 \times 10^9$	[27]
(b)	$\cdot OH + N_3^- \xrightarrow{k_b} OH^- + \cdot N_3$	$k_b = 6.5 \times 10^9$	[27]
(c)	$\cdot N_3 + \cdot N_3 \xrightarrow{k_c} N_6\ (3\ N_2)$	$k_c = 9 \times 10^9$	[7]
(d)	$\cdot N_3 + Tyr \xrightarrow{k_d}$ Products	$k_d = 0.9 \times 10^9$ (pH 9)	[8]
(e)	$\cdot N_3 + AmA \xrightarrow{k_e}$ Products	$k_e = 1 \times 10^6 - 2 \times 10^9$ $k_{N_3 + His} = 1 \times 10^6$ $k_{N_3 + Phe} = 1 \times 10^6$ $k_{N_3 + Cys} = 2 \times 10^9$ $k_{N_3 + Met} = 5 \times 10^6$	[8]

The kinetic analysis of the decay of the transient spectra revealed the following: tyrosyl radicals formed in NaN_3-containing solutions decayed according to two modes: a rapid first-order process with a half-life of about 300 μs and a slow second-order process with a 1st half-life of about 2 ms (at 115 Gy). This can be seen from *Fig. 8*, where an oscilloscope trace demonstrating the decay of the absorption at $\lambda = 405$ nm and the

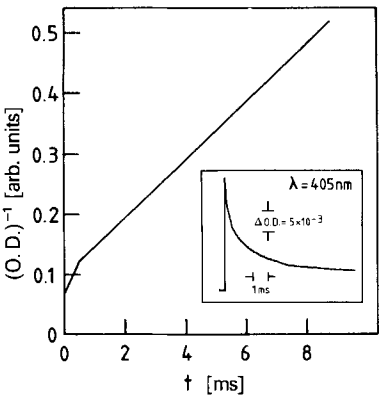


Fig. 8. *Pulse radiolysis of octamer complexes in N_2O-saturated aqueous solution* (pH 9) *containing NaN_3* (0.1M), *$NaClO_4$* (1M) *and $Na_2B_4O_7$* (6.85 mM). [Histone] = 0.75 g/l. D_{abs} = 120 Gy. Second-order plot of the optical density at 405 nm *vs.* the time after the pulse. Inset: Kinetic trace depicting formation and decay of the optical density.

corresponding second-order plot are shown. It is assumed that both modes reflect self-reactions of tyrosyl radicals. The rapid mode, which corresponds to about 50% of the tyrosyl radicals initially present, is ascribed to the reaction of tyrosyl radicals located in close proximity.

The existence of a slow mode, on the other hand, indicates that there are tyrosyl groups which are relatively mobile and can, therefore, approach each other without great difficulty within the octamer complex.

In the absence of azide, various radicals – including tyrosyl radicals – are formed by the attack of ·OH radicals. Oscilloscope traces demonstrating the decay of the transient absorption at 300 nm and at 400 nm are shown in *Fig. 9*.

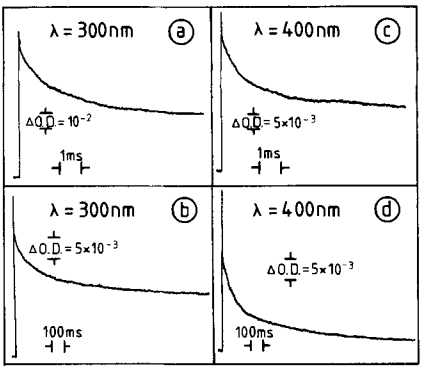


Fig. 9. *Pulse radiolysis of octamer complexes in N_2O-saturated solution* (pH 9) *containing $NaClO_4$* (1M) *and $Na_2B_4O_7$* (6.85 mM). [Histone] = 0.6 g/l. D_{abs} = 120 Gy. Kinetic trace illustrating formation and decay of the optical absorption at 300 nm (*a* and *b*) and at 400 nm (*c* and *d*).

At least three modes of decay could be discriminated: two relatively rapid ones with half-lives of several hundred μs and 1–2 ms, respectively (see *Fig. 9a* and *c*) and a rather slow one with a 1st half-life of 50–100 ms (see *Fig. 9b* and *d*). Comparing these results with those obtained in the presence of NaN_3, it seems that the rapid modes are essentially attributable to reactions of tyrosyl radicals, whereas the slow mode corresponds to the reaction of other radicals. Because the slow mode is detectable around λ = 400 nm, it can be possibly attributed to histidyl radicals which reportedly [25] absorb in this wavelength region. It is interesting to note that the light scattering intensity (LSI) increased with

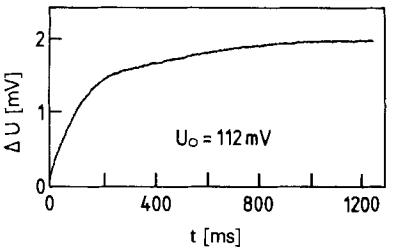


Fig. 10. *Pulse radiolysis of octamer complexes in N_2O-saturated solution* (pH 9) *containing $NaClO_4$* (1M) *and $Na_2B_4O_7$* (6.85 mM). [Histone] = 0.5 g/l. D_{abs} = 90 Gy. Kinetic trace illustrating the increase of the light scattering intensity.

about the rate at which the transient absorption decayed in the slow mode as can be seen from *Fig. 10*. The increase in the LSI was attributed to the contraction of the complexes as a consequence of intra-complex cross-linking [1]. It is, therefore, concluded that the slow mode of decay of the transient absorption reflects the rate of reaction of radicals within the complex leading (to some extent) to chemical linkages between different histone molecules.

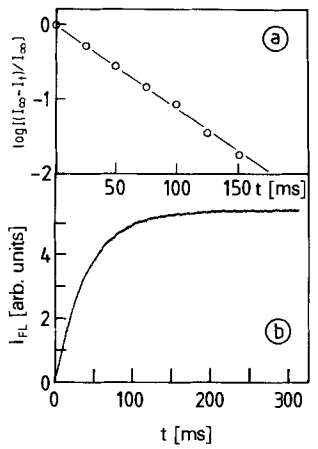


Fig. 11. *Pulse radiolysis of octamer complexes in N_2O-saturated solution* (pH 9) *containing $NaClO_4$* (2M), $Na_2B_4O_7$ (6.85 mM), and NaN_3 (50 mM). [Histone] = 1.18 g/l. First-order plot (*a*) and kinetic trace (*b*) illustrating the growth of the fluorescence after the pulse (100 ns) at λ = 410 nm (λ_{exc} = 325 nm).

Attempts to gain additional information concerning the nature of the transients from time-resolved fluorescence measurements failed. As can be seen from *Fig. 11*, the fluorescence (λ_{obs} = 410 nm) due to dityrosine groups increased according to a first-order law with a half-life of about 30 ms under conditions (50 mM NaN_3), where tyrosyl radicals coupled in a second-order process with a 1st half-life of about 2 ms as inferred from transient absorption measurements *(vide ante)*. Apparently, the rate of fluorescence growth is not connected to the coupling rate but to the consecutive enolization as depicted in *Reaction 5*. This finding corroborates the observation of *Hashimoto et al.* [26] who reported a first-order growth of the dityrosine fluorescence in the pH range 6.8–10.2 and a second-order growth at pH 10.7–12.5 in the dimerization of lysozyme. This result was interpreted in terms of enolization of the keto form of the dimer being relatively slow and, therefore, rate-determining at the lower pH range and radical coupling being rate-determining at the higher pH values because of the relatively fast enolization. In the present work, we were restricted to work at pH 9 in order to guarantee stability of the octamer complexes.

4.4. *Conclusions.* The results obtained in this work showed that native histone octamer complexes undergo a volume contraction due to intra-complex cross-linking upon the reaction with ·OH radicals. To assess the importance of phenol coupling *via* tyrosyl radicals in the cross-linking process, the reaction of octamers with $\cdot N_3$ radicals, which specifically attack tyrosine moieties, was investigated. It turned out that tyrosyl radicals contribute only to a limited extent to intra-complex cross-linking which is accomplished essentially *via* the formation of linkages between non-tyrosyl radicals. This process is strictly limited to single histone complexes as indicated by the fact that only dimers and no higher multimers were detectable by SDS gel electrophoresis. It must be emphasized that these processes pertain to native octamer complexes. Small chemical alterations cause denaturation of the complexes and make them capable of undergoing additional and different chemical reactions. This behavior was clearly demonstrated by comparing pulse radiolysis with continuous irradiation experiments. In the former case, the attacking radicals ·OH and $\cdot N_3$ were generated during the pulse (duration 100 ns), and only about 55% of the tyrosine moieties were found accessible to $\cdot N_3$ radicals. When, on the other hand, the latter were generated by continuous irradiation, higher multimers, *i.e.*, trimers and tetramers were evidenced by SDS electrophoresis. These findings indicated that octamer complexes were easily denatured as a consequence of chemical changes. The denaturation probably involves partial or complete dissociation of the complexes.

Time-resolved absorption measurements yielded evidence for the formation of cyclohexadienyl-type and tyrosyl radicals as well as of other radicals which could not be identified. With intact octamer complexes, all radicals were formed within about 20 μs after the pulse. Tyrosyl radicals generated upon attack of octamers by $\cdot N_3$ radicals were converted to dityrosine groups according to two modes with half-lives of several hundred μs and 1–2 ms. Histone molecules within octamer complexes cross-linked with a definitely lower rate, *i.e.*, 50–100 ms. This process was detectable both by absorption measurements at $\lambda = 300$–400 nm and by light-scattering measurements. Time-resolved fluorescence measurements following the formation of dityrosine groups yielded a rate much lower than that of the coupling of tyrosyl radicals. This was explained in terms of enolization of dimers in keto form *(cf. Reaction 5)* being the rate-determining step at pH 9.

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