Vol. 91, No. 3, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
December 14, 1979 Pages 1196-1200

PULSE RADIOLYTIC INVESTIGATIONS OF THE REDOX SYSTEM ALLOXAN-DIALURIC ACID : EVIDENCE FOR A RADICAL INTERMEDIATE

C. Houée-Levin, M. Gardès-Albert, C. Ferradini and J. Pucheault

Laboratoire de Chimie-Physique, Université René Descartes, 45, rue des Sts-Pères
75270 Paris Cedex 06, France

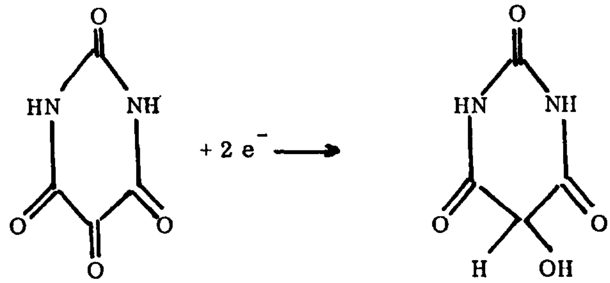
Received October 6, 1979

SUMMARY

The reduction of alloxan (A) into dialuric acid (AH_2) by $^{\bullet}COO^-$ radical has been investigated by pulse-radiolysis. A radical intermediate $^{\bullet}AH$ is shown to be formed with a rate constant k ($^{\bullet}COO^-$ + A) = $3.2 \times 10^7\ M^{-1}\ s^{-1}$. $^{\bullet}AH$ absorption spectrum is given. This radical disappears by disproportionation leading to dialuric acid, with a rate constant $2\ k = 1.6 \times 10^8\ M^{-1}s^{-1}$.

1. INTRODUCTION

The redox couple alloxan-dialuric acid has been known for a long time as a diabetogenic agent (1, 2, 3). Therefore a study of the redox properties of this couple in aqueous solution appears useful. First of all, we attempted to put into evidence the existence of a radical intermediate, $^{\bullet}AH$.



Alloxan (A) Dialuric acid (AH_2)

The reduction of alloxan was followed by fast spectrophotometry, and the chosen reducing agent was $^{\bullet}COO^-$ radical. It is known that, in the presence of formic acid and under an atmosphere of nitrous oxide, all free radicals produced by water radiolysis e^-_{aq}, $^{\bullet}H$, and $^{\bullet}OH$, are rapidly transformed into radicals $^{\bullet}COO^-$.

$$HCOO^- + {}^{\bullet}OH \longrightarrow {}^{\bullet}COO^- + H_2O$$

$$HCOO^- + {}^{\bullet}H \longrightarrow {}^{\bullet}COO^- + H_2$$

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

So after less than a microsecond, the solution contains only $^{\bullet}COO^-$ species.

0006-291X/79/231196-05$01.00/0

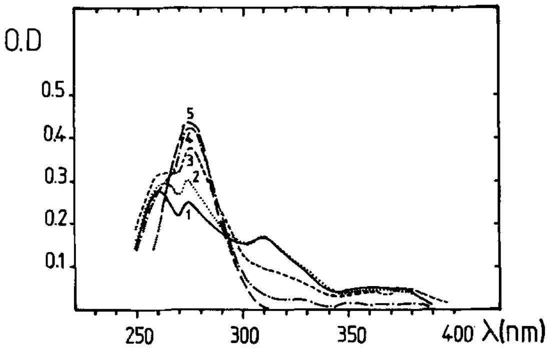


Fig 1 : Evolution of the differential absorption spectrum with delay after the pulse [Alloxan : 2.5 x 10^{-3}M; $HCOO^-$; 0.1 M; pH = 4.8; under atmosphere of N_2O : $p(N_2O)$ = 1 atm].

1 —— 22 μs after the pulse
2 --- 47 μs " "
3 —— 80 μs " "
4 —·— 130μs " "
5 —— 4180μs " "

2. EXPERIMENTAL PROCEDURE

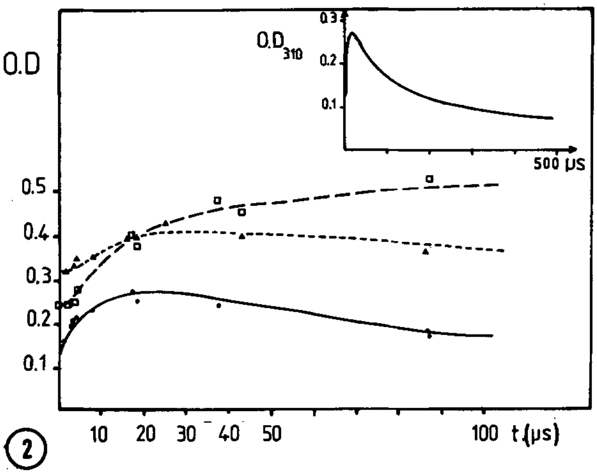
Irradiation were performed using a Febetron 708 which generates electron pulses (800 keV - 4 ns half-height). The doses were controlled by microcalorimetry and were chosen in order to get a $^{\bullet}COO^-$ concentration approximately equal to 1.5 x 10^{-4}M (measured by $^{\bullet}COO^-$ absorbance at 260 nm, with ε = 2200 $M^{-1}cm^{-1}$ (4)). A rapid spectrophotometric design allowed a measurement of absorbance evolution in the UV and visible wavelengths range, for times lying between 10^{-8}s and a few minutes.

Alloxan is a Sigma product. Sodium formate and sulfuric acid are Merck Pro Analysi products. Water is triply distilled so that its conductivity should not exceed $10^{-6}\Omega^{-1}$ cm^{-1}. N_2O is an "Air Liquide" gas. Its purity is superior to 99.99 %.

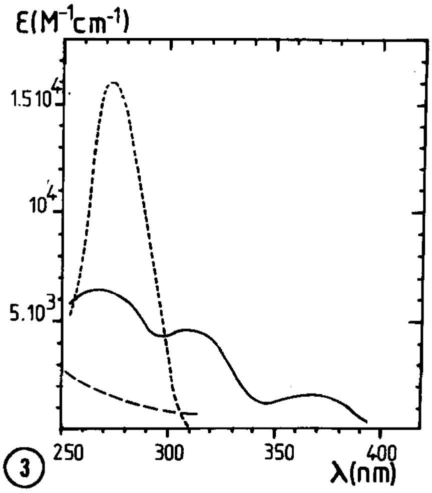
3. RESULTS

Two alloxan concentrations were used (1.25 x 10^{-3}M and 2.5 x 10^{-3}M). Figure 1 shows the differential absorption spectra of irradiated solutions versus the initial one, for several delays after the pulse. These spectra reveal two transient bands at 260 and 310 nm and an increasing peak at 275 nm. The final spectrum, that is mainly this latter peak, corresponds to dialuric acid. Identification was made by comparison with AH_2 produced by reduction of alloxan with sodium dithionite. Both other bands (at 260 and 310 nm) belong to a transient spectrum, which can be attributed to the radical intermediate $^{\bullet}AH$.

Therefore the variation of absorbance at these three wavelengths were peculiarly investigated (fig 2). One can see that optical density at 260 and 310 nm increase rapidly, and then decreases gently, whereas O.D. at 275 nm becomes stationary (insert of fig 2). It must be noted that in the first transient spectrum, a contribution due to $^{\bullet}COO^-$ ab-



②



③

Fig 2 : Variation of O. D. with time at 3 wavelengths : Curves are theoretical and compared to experimental points :
——Δ : 260 nm
--- □ : 275 nm
—— ● : 310 nm

in insert : the decrease of absorbance at 310 nm.

Fig 3 : Absorption spectra of the three species :
——— $^{\bullet}COO^-$
---- dialuric acid
—— transient radical $^{\bullet}AH$.

sorption is included, mainly at 260 and 275 nm. $^{\bullet}COO^-$ spectrum, which is shown on fig 3, has been determined by us and normalized using ε_{260} = 2200 cm^{-1} (4).

4. INTERPRETATION

In the whole range of wavelengths investigated, absorbance due to alloxan itself can be neglected because its extinction coefficients are very low.

The molar extinction coefficient of dialuric acid at 275 nm is known : ε_{275} = 16000 M^{-1} cm^{-1}(5).

So it can be seen that its formation is not stoechiometric with respect to the initial amount of $^{\bullet}COO^-$ (1.5×10^{-4}M) :

$[A]_0$	$[AH_2]_\infty$
1.25×10^{-3} M	2.33×10^{-5} M
2.5×10^{-2} M	3.08×10^{-5} M

This non-stoechiometry can be attributed to a partial disproportionation of $^{\bullet}COO^-$, which competes with its scavenging by A. So the reaction scheme may be written :

① $2\,^{\bullet}COO^- \xrightarrow{2\,k_1} C_2O_4^{=}$

② $H^+ + {}^{\bullet}COO^- + A \xrightarrow{k_2} {}^{\bullet}AH + CO_2$

③ $2\,^{\bullet}AH \xrightarrow{2\,k_3} A + AH_2$

In order to verify the validity of this mechanism, rate constants and molar extinction coefficients are to be determined :

a) Determination of $2\,k_1$: $2\,k_1$ was calculated with use of the 2nd-order decay of absorbance measured in the same condition but without alloxan :

$2\,k_1 = 1.2 \times 10^9\ M^{-1}s^{-1}$.

b) Determination of $2\,k_3$ and $\mathcal{E}^{310}_{AH}$: From the preceding results (see a), it appears that 47 μs after the irradiation, there is no more $^{\bullet}COO^-$ in solutions. So O.D. decay at 310 nm (insert of fig 2) must be due to the disappearance of $^{\bullet}AH$, for the absorbance of AH_2 is negligible at this wavelength. We verified it was a 2nd-order decay by drawing the curve 1/O.D. versus time, whichs is found to be a straight line. From its slope, $\dfrac{2\,k_3}{\mathcal{E}^{310}_{AH}}$ was calculated :

$$\frac{2\,k_3}{\mathcal{E}^{310}_{AH}} = 3.3 \times 10^4\ s^{-1}\ cm^{-1}.$$

The initial optical density at 310 nm, deduced by extrapolation of the straight line, is 0.30. The final concentration of AH_2 obtained by $^{\bullet}AH$ disproportionation, is 3.08×10^{-5}M (for an initial concentration : $[A] = 2.5 \times 10^{-3}$M). So the initial radical concentration must have been the double : 6.16×10^{-5}M. Hence $\mathcal{E}^{310}_{AH}$ is deduced :

$\mathcal{E}^{310}_{AH} = 4900\ M^{-1}cm^{-1}$

which gives : $2\,k_3 = 1.6 \times 10^8 M^{-1}s^{-1}$.

An analogous computation made from results obtained with the initial alloxan concentration of 1.5×10^{-3}M, leads to values of $\mathcal{E}^{310}_{AH}$ and $2\,k_3$, of the same order of magnitude.

c) Determination of k_2 : Immediately after irradiation $^{\bullet}AH$ and AH_2 are present in negligible quantities, so the only reactions to be considered are reactions ①and ②. As $2\,k_1$, $\mathcal{E}^{310}_{CO_2^-}$ and $\mathcal{E}^{310}_{AH}$ are known, the initial slope of O.D. at 310 nm evolution leads to an estimation of k_2 : $k_2 = 3.2 \times 10^7 M^{-1}s^{-1}$.

d) $^{\bullet}$AH spectrum : From the evolution of O.D. at 310 nm (where ε_{AH_2} and ε_A are quite negligible) the knowledge of ε_{AH} allows a calculation of $^{\bullet}$AH concentration at any time, and consequently the one of AH_2 at the same time :

$$[AH_2]_t = [AH_2]_\infty - \frac{1}{2}[AH]_t$$

So, when $^{\bullet}COO^-$ has disappeared (for example 47 μs after the pulse, when AH has not yet decreased too much) the absorbance at wavelength is equal to :

$$O.D. = \varepsilon^{\lambda}_{AH_2}\left([AH_2]_\infty - \frac{1}{2}[AH]\right) + \varepsilon^{\lambda}_{AH}[AH]$$

$\varepsilon^{\lambda}_{AH}$ can be deduced, because all other terms are known. The obtained spectrum is drawn on figure 3. The two bands at 310 nm and 370 nm show some analogies with a semiquinone radical's spectrum in this wavelength range (6).

In order to verify these values, a computation by iteration of the kinetic evolution at these three chosen wavelengths (260, 275 and 310 nm) was performed on a Wang 500 computer. The contribution of $^{\bullet}COO^-$ was taken into account. Theoretical curves are drawn on fig 2, and one can see a good fitting to experimental points.

Because of the existence of this radical intermediate which has been demonstrated here, the problem of dialuric acid cytotoxicity will perhaps need a reexamination.

REFERENCES

1. Brückmann, G. and Wertheimer, E. (1947) J. Biol. Chem., 168, 241-256.
2. Heikkila, R.E., Winston, B. and Cohen, G. (1976) Biochem. Pharmacology, 25, 1085-1092.
3. Weaver, D.C., McDaniel, M.L. and Lacy, P.E. (1978) Diabetes, 27(2), 71-77.
4. Neta, P., Simic, M. and Hayon, E. (1969) J. Phys. Chem., 73, 4207-4213.
5. Van Hemmen, J.J. and Meuling, W.J.A. (1977) Arch. Biochem. Biophys., 182, 743-748.
6. Rao, P.S. and Hayon, E. (1973) Biochim. Biophys. Acta, 292, 516-533.