

Kinetic Investigations of the Autoxidation of Adrenalin

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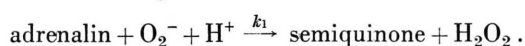
Adrenalin, Hydroxylamine, Superoxide Dismutase, Superoxide Anions, Rate Constants

Generation rates of superoxide anions (O_2^-) by autoxidizing adrenalin at pH 9.5 were determined in solutions containing either superoxide dismutase ($0.85\text{ M}^{-1}\text{ s}^{-1}$) or hydroxylamine ($0.0185\text{ M}^{-1}\text{ s}^{-1}$) as competitive scavengers. The rate constants of O_2^- with adrenalin and hydroxylamine were calculated for neutral and alkaline solutions. The respective values were for adrenalin: $5.6 \times 10^4\text{ M}^{-1}\text{ s}^{-1}$, pH 7.8; $7.0 \times 10^3\text{ M}^{-1}\text{ s}^{-1}$, pH 9.5 — and for hydroxylamine $5.9 \times 10^4\text{ M}^{-1}\text{ s}^{-1}$, pH 7.8; $3.4 \times 10^4\text{ M}^{-1}\text{ s}^{-1}$, pH 9.5. The effects of various competitors and O_2^- -sources on the rate constants were compared.

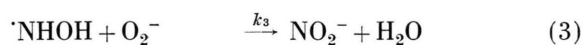
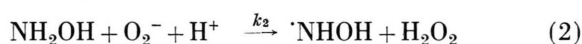
Introduction

In the course of our investigations on the autoxidation of adrenalin [1], we found that the dissociated catecholamine is capable of directly transferring an electron to oxygen. This generation of superoxide anions (O_2^-) was demonstrated by the superoxide dismutase-inhibitable formation of nitrite from hydroxylamine [2]. In the present paper we report the quantitation of the reaction of adrenalin with oxygen, using competition kinetics and steady-state treatment of the data.

As further evaluation of the autoxidation of adrenalin we re-determined its reaction rate with O_2^- . Following an approach of Asada and Kanematsu [3], the calculations were based on the assumption that the initial reaction of the four-electron oxidation of adrenalin to adrenochrome [4, 5] is rate-limiting:



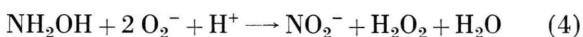
Whereas the oxidation of hydroxylamine to nitrite by O_2^- proceeds by two discrete steps (unpublished results),



Abbreviation: SOD, superoxide dismutase (EC 1.15.1.1).

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and therefore does not involve a ter-molecular reaction according to the stoichiometry [2],



the rate-limiting step again is the initial attack of O_2^- (R. 2). We were thus able to determine this rate constant, which we could not obtain by pulse-radiolytic methods [6].

Materials and Methods

Adrenalin, hydroxylammonium chloride and sodium formate were from Merck (Darmstadt); xanthine and xanthine oxidase (EC 1.2.3.2) from Boehringer (Mannheim); SOD (erythrocyte; EC 1.15.1.1) was a gift of Dr. Weser (University of Tübingen). Triply distilled and pyrolyzed water was used for all solutions and — except for the xanthine oxidase-mediated co-oxidation of adrenalin at pH 7.8 — was free of any buffers to prevent artefactual chelating or scavenging effects. The pH was adjusted with NaOH or HCl and remained constant during the autoxidation reaction within 0.2 pH-units.

Adrenochrome formation was monitored both at 310 and 485 nm, taking the rate of absorption increase in the linear region. The higher molar absorptivity of adrenochrome at 310 nm ($2.28 \times 10^4\text{ M}^{-1}\text{ cm}^{-1}$ vs. $3.8 \times 10^3\text{ M}^{-1}\text{ cm}^{-1}$ at pH 9.5) could be utilized only in the presence of SOD [1]. The total yield of nitrite over the respective reaction times was determined by the formation of the diazo com-



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pound with sulfanilic acid and α -naphthylamine, for which we obtained a molar absorptivity of $1.04 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ at 530 nm. Autoxidation was initiated by switching from nitrogen to oxygen bubbling in the cuvette. Saturation with oxygen was very rapid in the small volume, as determined with an oxygen electrode, giving a concentration of $1.23 \times 10^{-3} \text{ M/l}$ at 25 °C. Spectra were taken on an Unicam SP 800 spectrophotometer.

γ - or X-radiolysis of oxygenated solutions containing 0.01 M sodium formate was considered to be the most reliable steady-state source of O_2^- . However, it allowed only the determination of the rate constant of hydroxylamine with O_2^- , as adrenochrome was too unstable under these conditions [7] to be analyzed separately. A compromise had to be found between the dose rate of the radiation source and the yield of nitrite as excessive long irradiation times could result in secondary reactions of the radiation products. We used a X-ray source (Siemens Dermopan) with a dose rate of 8 krd/min. The exposure time was 3, respectively 5 min with continuous oxygenation of the solution.

Results

The assumption that O_2^- is generated by dissociated adrenalin is based primarily on the fact, that the SOD-inhibitable formation of NO_2^- from NH_2OH is observed only above pH 8.5, corresponding with the pK_a -value of adrenalin [8]. The saturation behaviour of nitrite formation by autoxidizing adrenalin (Fig. 1 a) allowed a linear plot of the data in reciprocal coordinates (Fig. 1 b). Only at very low concentrations of adrenalin ($< 10^{-4} \text{ M}$) we observed a deviation from the extrapolated curve (Insert in Fig. 1 a). The plots depicted in Fig. 1 a, b were obtained at pH 10.2, where the generation rate was higher than at pH 9.5 (1.85×10^{-2} vs. $8.8 \times 10^{-2} \text{ M}^{-1} \text{ s}^{-1}$). From these plots and the kinetic derivation, as presented in the discussion, the generation rate of O_2^- by autoxidizing adrenalin was calculated. The values are compiled in Table I.

For the case of adrenochrome formation in the presence of SOD Fig. 2 shows a linear plot of the inverse generation rate of adrenochrome taken at 310 and 485 nm *vs.* the concentration of SOD,

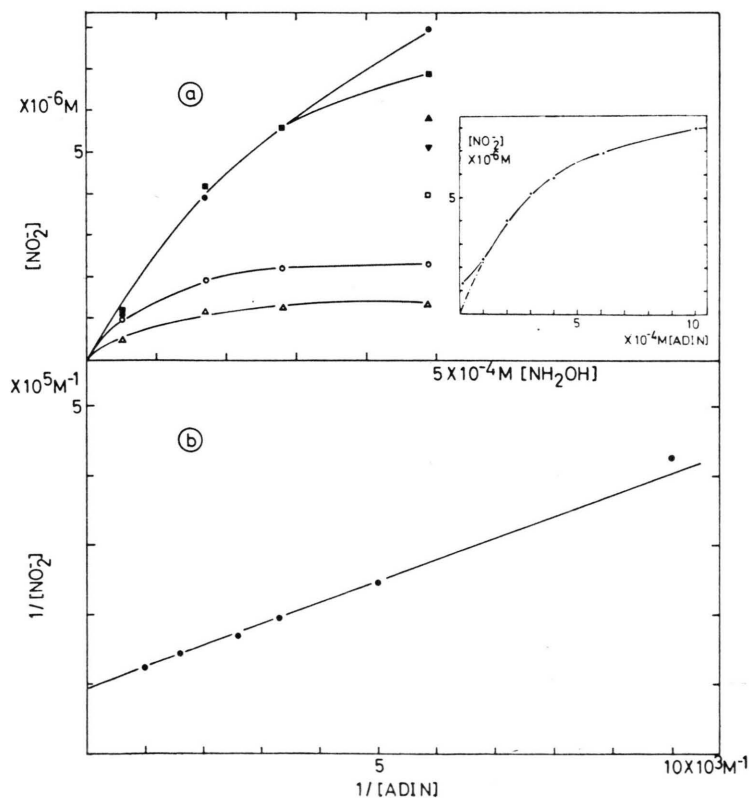


Fig. 1. Generation of nitrite by autoxidizing adrenalin. a) NO_2^- -yield for various concentrations of hydroxylamine and adrenalin (insert). b) Linear relationship of the reciprocal nitrite- and adrenalin concentrations. Unbuffered solutions at pH 10.2; determination of NO_2^- after 5 min autoxidation as diazo compound at 530 nm. The NO_2^- -yields at the highest NH_2OH concentration ($4.85 \times 10^{-4} \text{ M}$) were taken for the plots in the insert to Fig. 1 a and in Fig. 1 b. The insert also depicts the respective concentrations of adrenalin. Least-square analysis of the linear plot in Fig. 1 b gave an ordinate intercept of $9.37 \times 10^4 \text{ M}^{-1}$ and a slope of 30.84. (The respective values at pH 9.5 were: 20 min oxidation of $9 \times 10^{-4} \text{ M}$ NH_2OH , intercept $14.45 \times 10^4 \text{ M}^{-1}$, slope 34.56).

Table I. Generation rates of superoxide anions by autoxidizing adrenaline.

pH	Product observed	Competitor	Generation rate [M ⁻¹ s ⁻¹]	Method of determination
9.5	NO ₂ ⁻ (530 nm)	NH ₂ OH	1.85 × 10 ⁻²	slope
10.2	NO ₂ ⁻ (530 nm)	NH ₂ OH	8.8 × 10 ⁻²	slope
9.5	ADOM (310 nm)	SOD	0.99	ordinate
9.5	ADOM (485 nm)	SOD	0.64	ordinate

based on equation (8) (see discussion). The mean value for the generation rate of O₂⁻ in the presence of Cu/Zn-SOD of $0.85 \pm 0.11 \text{ M}^{-1} \text{ s}^{-1}$ at pH 9.5 (average of all determinations) is higher by a factor of 46 as compared to the presence of hydroxylamine. Steady-state concentrations for O₂⁻ were calculated as $4.3 \times 10^{-10} \text{ M}$ for the presence of hydroxylamine, respectively $4.5 \times 10^{-8} \text{ M}$ with added SOD, both at pH 9.5.

The reaction rates of adrenalin and hydroxylamine with O₂⁻ were determined from a least-square fit of the standard competition plot [9] given in Fig. 3 a, b. The values for several O₂⁻-sources, respectively competitors, are compiled in Table II. The reference rate constant of SOD with O₂⁻ (previously taken as $2.37 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$; ref. [10]) was corrected for the influence of the ionic strength according to McAdam [11] and amounts to $3.7 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ in oxygenated formate solutions. As can be seen, the rate constants are generally lower in alkaline solutions and differ for autoxidizing adre-

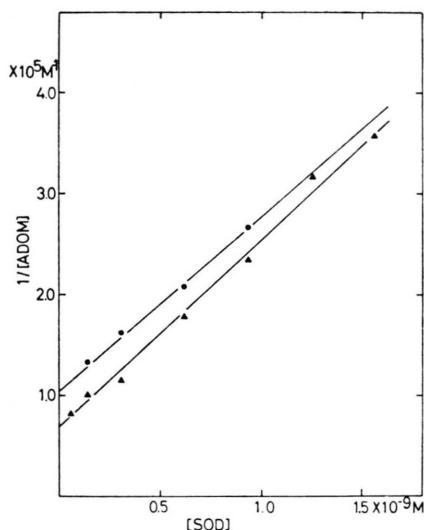


Fig. 2. Linear relationship of the reciprocal adrenochrome concentration *vs* the concentration of SOD. Autoxidizing adrenalin ($2 \times 10^{-4} \text{ M}$) at pH 9.5 (unbuffered solution). Rate of autoxidation determined as linear absorbance increase per minute at 310 (▲) and at 485 nm (●). Regression analysis gave at 310 nm: $1.862 \times 10^{14} \text{ M}^{-2}$ (slope) and $6.85 \times 10^4 \text{ M}^{-1}$ (ordinate intercept) and at 485 nm: $1.711 \times 10^{14} \text{ M}^{-2}$ and $1.060 \times 10^5 \text{ M}^{-1}$, respectively.

naline, xanthine/xanthine oxidase and X-radiolysis as respective O₂⁻-sources. The values which are considered to be most accurate are emphasized (see discussion).

Discussion

For the determination of generation rates a steady-state method was used, which took into account the production of O₂⁻ by adrenalin. The

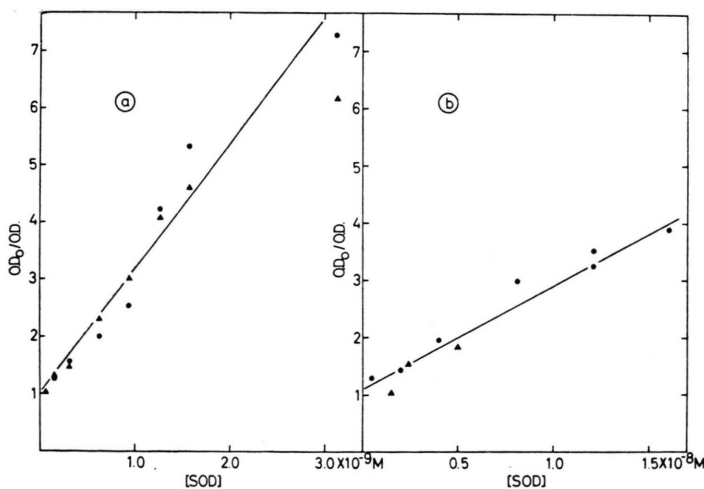


Fig. 3. Competition plots for reaction rates of superoxide anions. a) Autoxidizing adrenalin as O₂⁻-source, SOD as competitor: concentration of adrenalin $2 \times 10^{-4} \text{ M}$, unbuffered solutions at pH 9.5; rate of autoxidation per minute by the formation of adrenochrome at 485 nm (●) and at 310 nm (▲) in the linear region of absorbance increase. Regression analysis (least-square fit) resulted in ordinate intercept of 0.981 and slope of 2.215×10^9 . b) Steady-state radiolysis of oxygenated formate solutions, SOD as competitor: concentration of NH₂OH $4.85 \times 10^{-4} \text{ M}$, formate 10^{-2} M , pH 9.5. (●) X-radiolysis: irradiation time 3 min, 23 krd average dose; (▲) γ-radiolysis: irradiation time 60 min, 5.8 krd average dose. NO₂⁻ was determined as diazo compound at 530 nm. An ordinate intercept of 1.104 and slope of 1.814×10^8 were obtained.

Rate determined	O_2^- -source	Product observed	Rate constant $[M^{-1} s^{-1}]$		$\frac{k_{neutr.}}{k_{alkal.}}$
			neutral (pH 7.8)	alkaline (pH 9.5)	
ADIN + O_2^- (k_1)	xanthine/XOD	ADOM (485 nm)	5.6×10^4 ^b	2.5×10^4	2.2
	autoxidation of ADIN	ADOM (310 + 485)	—	7.0×10^3	—
$NH_2OH + O_2^-$ (k_2)	xanthine/XOD	NO_2^- (530 nm)	2.7×10^4 ^c	—	—
	xanthine/XOD	NO_2^- (530 nm)	1.0×10^4	2.1×10^3	4.9
	X-radiolysis	NO_2^- (530 nm)	5.9×10^4	3.4×10^4	1.8

Table II. Compilations of rate constants (obtained from competition experiments with superoxide dismutase ^a).

^a Rate constant of SOD with O_2^- (k_d) from ref. [10] corrected for influence of ionic strength according to ref. [11].

^b Value of Asada and Kanematsu (ref. [3]) corrected according to footnote ^a; we obtained identical result.

^c Value obtained by calculation of data in ref. [2].

linear equation for the reciprocal plots of Fig. 1 b and Fig. 2 were derived under the assumption, that the rate-limiting reactions of O_2^- are exclusively with adrenalin (ADIN), hydroxylamine or superoxide dismutase in the employed systems. The depletion of O_2^- is then given by:

$$-\frac{d[O_2^-]}{dt} = k_1[ADIN][O_2^-] + k_2[NH_2OH][O_2^-] - k_0[ADIN][O_2] \quad (1)$$

respectively:

$$-\frac{d[O_2^-]}{dt} = k_1[ADIN][O_2^-] + k_d[SOD][O_2^-] - k_0[ADIN][O_2]. \quad (2)$$

For steady-state conditions Eqn (1) is transformed to:

$$k_0[ADIN][O_2] = k_1[ADIN][O_2^-] + k_2[NH_2OH][O_2^-]. \quad (3)$$

The unknown concentration of O_2^- at steady-state levels can be expressed as a function of either NO_2^- or adrenochrome (ADOM) being formed. At saturating amounts of NH_2OH the yield of O_2^- corresponds to the yield of NO_2^- with negligible accumulation of ADOM. Assuming constant concentrations of O_2^- — which is implicit for the steady-state system — and of NH_2OH , we can integrate the kinetic expression

$$\frac{d[NO_2^-]}{dt} = k_2[NH_2OH][O_2^-] \quad (4)$$

leading to

$$[NO_2^-] = k_2[NH_2OH][O_2^-]t. \quad (5)$$

Substituting this in Eqn (3) and solving for k_0 , one obtains:

$$k_0 = \frac{[NO_2^-](k_1[ADIN] + k_2[NH_2OH])}{k_2 t [NH_2OH][ADIN][O_2]} \quad (6)$$

which after rearrangement finally gives:

$$\frac{1}{[NO_2^-]} = \frac{k_1}{k_0 k_2 t [NH_2OH][O_2]} + \frac{1}{k_0 t [O_2]} \frac{1}{[ADIN]} \quad (7)$$

where $1/[NO_2^-]$ is a linear function of $1/[ADIN]$ and k_0 can be calculated from the slope.

Similar steady-state assumptions for Eqn (2), integration of the yield of adrenochrome and transformation into a linear equation, resulted in:

$$\frac{1}{[ADOM]} = \frac{1}{k_0 t [ADIN][O_2]} + \frac{k_d}{k_0 k_1 t [ADIN]^2 [O_2]} [SOD] \quad (8)$$

on which the plot of Fig. 3 is based. In this case k_0 can be calculated from the ordinate intercept. We thus have both in Eqn (7) and (8) kinetic terms for k_0 , which are independent of other rate constants.

In the formulation of Eqn (1), a possible contribution of R. (3) was excluded. As shown in the insert in Fig. 1 a, the involvement of R. (3) can be

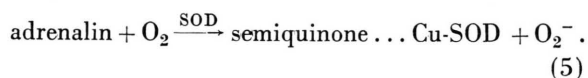
neglected above a concentration of adrenalin of $1 \times 10^{-4} \text{ M}$ — where the kinetic term $k_3 [\text{'NHOH}] \cdot [\text{O}_2^-]$ is much smaller than $k_1 [\text{ADIN}][\text{O}_2^-]$ or $k_2 [\text{NH}_2\text{OH}][\text{O}_2^-]$. Since the presence of 'OH radicals would increase the yield of 'NHOH[6], thus enhancing the influence of R. (3), the absence of 'OH radicals during the autoxidation of adrenalin had to be ascertained. Previous pulse-radiolytic investigations of adrenalin [12] and hydroxylamine [13] have shown that both compounds are much more liable to attack by 'OH radicals as compared to O_2^- . Hydroxyl radicals could be excluded both by the failure of excess *t*-butanol to inhibit the reaction and by the absence of any bleaching of *p*-nitrosodimethylaniline, an efficient [14] and specific [15] 'OH radical scavenger (manuscript submitted).

The determination of the rate constants of both adrenalin and hydroxylamine with O_2^- was also subject to the presence of 'OH radicals. While the competition method derived for pulse-radiolytic evaluation of rate constants is independent of the generation respectively concentration of the mutual reaction partner O_2^- [16], the adventitious presence of 'OH radicals in chemical generation systems leads to the observed discrepancies of the k_1 - and k_2 -values. This is particularly the case for the system xanthine/xanthine oxidase, where the formation of 'OH radicals has repeatedly been postulated [17, 18]. We therefore consider the rate constants obtained in X-irradiated oxygenated solutions containing formate as an exclusive O_2^- -source to be more correct.

The higher value of k_1 in the xanthine/xanthine oxidase system as compared to autoxidizing adrenalin, furthermore, may be caused by the generation of O_2^- by the catecholamine itself. Both the generation of O_2^- by the reaction of dissociated adrenalin with oxygen and the reaction of adrenalin with O_2^- are slow, thus a lower concentration of SOD is required to inhibit the overall reaction. Acceleration by xanthine oxidase as an additional O_2^- -source (and potential 'OH-source) may thus partially reflect more efficient subsequent reactions of O_2^- in the oxidation

sequence. The fact of a genuine competition with SOD for autoxidizing adrenalin is supported by the good linearity and the ordinate intercept close to unity of the experimental results in Fig. 3 a.

Both the generation rate and the steady-state concentration of O_2^- at pH 9.5 were significantly higher in the presence of Cu/Zn-SOD as compared to hydroxylamine. This quantitative expression reflects the previously reported stabilization of the adrenalin semiquinone in the presence of SOD [19], which has also been observed by circular dichroism spectroscopy (W. Schubotz, personal communication):



The reaction parallels the complex formation of *o*-benzosemiquinone with Cu^{2+} , which also results in stabilization of the radical [20]. Conversely Cu^{2+} has been found to accelerate the oxidation of adrenalin [21]. One might therefore argue that SOD stimulates an O_2^- -generating process, yet which on balance is masked by the more efficient inhibition of an O_2^- -dependent reaction. [1].

A directly comparable bimolecular generation rate of O_2^- to our knowledge has been reported only once: in the case of O_2^- -formation from the electron transfer from ubi-semiquinone to oxygen during the mitochondrial generation of hydrogen peroxide ($40 \text{ M}^{-1} \text{ s}^{-1}$; ref. [22]). Generally biochemical generation rates of O_2^- are expressed in nM/min/mg protein, which leaves the molarity of the generating protein to be defined. On the other hand, rate constants for the generation of O_2^- derived from pulse-radiolytic investigations [23] are less relevant as they involve only electron transfer equilibria. In view of the low k_0 -values obtained for adrenalin and the fact that another catecholamine, 6-hydroxydopamine [24], generates O_2^- much more readily, a quantitative kinetic evaluation of the autoxidation of the latter compound would be worthwhile.

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