Electrochemical Studies of the Oxidation Pathways of Catecholamines

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Abstract: Modern, fast-sweep electrochemical techniques have been applied to the study of the oxidation pathways of catecholamines *in vitro*. These techniques allow positive identifications of the transient intermediates, *i.e.*, the open-chain *o*-quinones, and precise determinations of the rate of intramolecular cyclization to the substituted indole and its subsequent oxidation to the aminochrome. Significant differences in the rates of cyclization of adrenaline and noradrenaline are consistent with recent findings on the reactions of these catecholamines.

Although the hormonal actions and other functions of the catecholamines have been studied extensively, detailed information regarding their reactions at the molecular level is still uncertain. It is well established that the major metabolic pathway of adrenaline and noradrenaline is *via* O methylation.[2,3] However, due to the ease of oxidation of the catechol moiety at physiological pH, oxidative pathways of action cannot be dismissed entirely. Indeed, recent studies *in vitro* have shown that oxidative reactions of catecholamines are involved in various enzymatic and nonenzymatic processes.[4-8] The present study was undertaken to learn more about the chemical reactions which are coupled with oxidative electron transfer in the catecholamines.

Experimental Section

Materials. l-Adrenaline and adrenochrome were obtained from Sigma Chemical Co., dopamine came from Calbiochem. *l*-Noradrenaline *d*-bitartrate monohydrate and *dl*-α-methylnoradrenaline hydrochloride were kindly supplied by Dr. S. Archer of Sterling-Winthrop. *dl*-Isoproterenol sulfate dihydrate was that of the Aldrich Chemical Co. All these compounds were used as received. 5,6-Dihydroxy-N-methylindole was prepared by the method of Heacock[9] and recrystallized from benzene, mp 134–135° (lit.[9] 133–134°). All inorganic chemicals were reagent grade. Britton–Robinson buffers were used below pH 6 but could not be used above pH 6 because of interaction between borate and the *o*-dihydroxy moiety of the catecholamines. McIlvaine buffers (citrate–phosphate) were used for pH values above 6. Triply distilled water was used for all solutions.

Cyclic voltammetry was carried out using a triangular wave generator and potentiostat similar to that previously described.[10] The current–potential curves were recorded on a Moseley X–Y recorder, Models 7030A or 3S. For the potentiostatic experiments, a fixed potential was fed into the same potentiostat as used for the cyclic voltammetry. The current–time curves were recorded on a Leeds and Northrup Speedomax Model G recorder using an 8-in./min chart speed. The chronopotentiometry was conventional with the potential–time curves followed by a Leeds and Northrup Speedomax Model G recorder with an 8-in./min chart speed. A planar carbon paste electrode[11,12] was used for all experiments although a platinum button could also be used. The epr experiments were carried out by *in situ* electrolysis in the cavity of a Varian 4500 spectrometer equipped with a 6-in. magnet.

The kinetics of the cyclization reactions were followed using a potentiostatic technique similar to that used by Alberts and Shain.[13] The procedure used was as follows. Accurately weighed samples of catecholamine were diluted with deaerated buffer, and current–time curves were run at a potential approximately 100 to 200 mv anodic of the peak potential as determined by cyclic voltammetry. (For all catecholamines at pH 2.3–2.5, $it^{1/2}/C$ was a constant as expected in the absence of a following chemical reaction.) Current–time curves were then taken at various pH values and from the values of n_{app}/n, or in earlier experiments $it^{1/2}/it_{\infty}^{1/2}$ (see Results section) as a function of time values of kt could be obtained. The pseudo-first-order rate constants, k_{obsd}, were then calculated from the slope of plots of kt *vs.* t. All kinetic runs were at 25.0 ± 1°.

Results

The cyclic voltammetry of the various catecholamines was studied as a function of pH. The cyclic polarogram of adrenaline in 1.00 *M* H_2SO_4 is shown in Figure 1. On the first anodic scan a peak (1) is observed near +0.7 v *vs.* sce which corresponds to the oxidation of adrenaline to the open-chained quinone. Upon reversal of the potential scan, the rereduction of the *o*-quinone (2) to adrenaline is seen. On subsequent cycles, only this one quasireversible system is observed.

A pH 3.0, however, the cyclic polarogram is very much different as seen in Figure 2. On the first anodic scan, a peak (1) occurs at +0.6 v which corresponds again to the oxidation of adrenaline to the open-chain quinone. On potential reversal the rereduction of this quinone (2) is again observed, but the peak has diminished in intensity and another cathodic peak as well as two new anodic peaks appear on this and subsequent scans. The cyclic polarogram can be easily interpreted according to the mechanism of Harley–Mason[14,15] and is an example of an intramolecular 1,4 (Michael) addition reaction (eq 1–4). At low pH, the open-chained adrenalinequinone is protonated to a great extent (pK_a = 8.88), and hence the cyclization reaction is precluded. At pH 3, however, sufficient unprotonated quinone is available to allow the cyclization reaction to take place. The cyclic polarograms can now be interpreted. Peak

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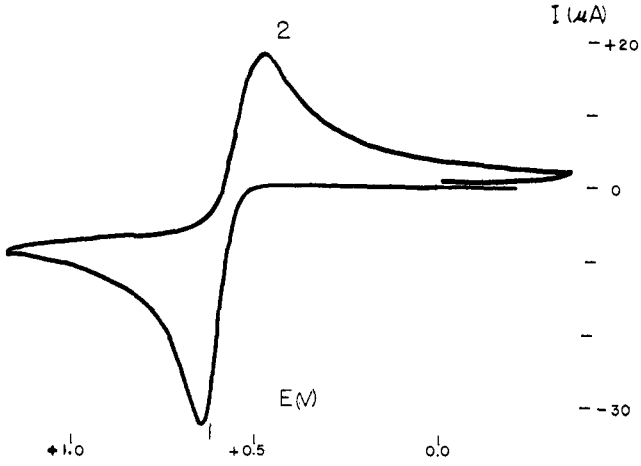


Figure 1. Cyclic voltammetry of 1.3×10^{-3} M adrenaline in 1.00 M H_2SO_4. Scan rate is 2.40 v/min.

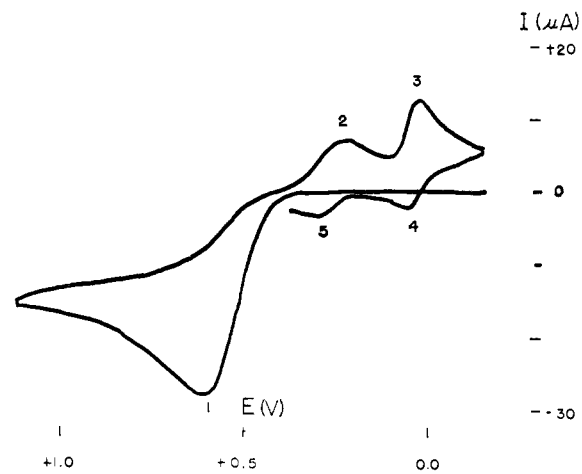
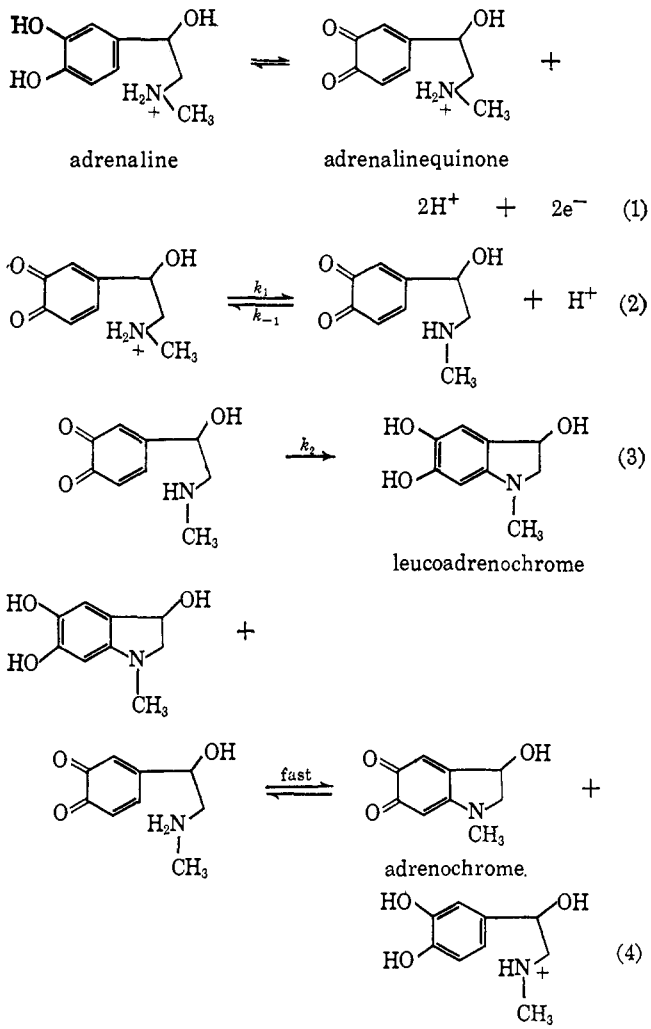


Figure 2. Cyclic voltammetry of 1.7×10^{-3} M adrenaline at pH 3.0. Scan rate is 2.40 v/min.

3 corresponds to the reduction of the cyclized product, adrenochrome, to leucoadrenochrome, while peak 4 is that of the reoxidation of leucoadrenochrome to adrenochrome. The adrenochrome–leucoadrenochrome couple was identified by comparison with the



cyclic voltammetry of an authentic sample of adrenochrome and by the observation of the red color of adrenochrome formed at the electrode surface upon oxidation of adrenaline. Electron paramagnetic resonance identification of the intermediates was precluded owing to the instability of the semiquinones of the species involved, in agreement with the observation of Borg.[8] Peak 5 was identified as the oxidation of 5,6-dihydroxy-N-methylindole by comparison with an authentic sample. This is formed by the dehydration of leucoadrenochrome.[15]

Because the initial cyclized product (leucoadrenochrome) is more easily oxidized than adrenaline itself, it can be oxidized by the adrenalinequinone (eq 4). Hence, the over-all mechanism is an ECC (electron transfer–chemical reaction–chemical reaction). The difference between this model and the more widely used ECE model has been discussed elsewhere;[16–18] thus it will suffice to say that the two models give the same results qualitatively, but not quantitatively. In this study the most consistent rate constants were obtained using the ECC mechanism. It can be seen from the mechanism written that as the chemical reaction (eq 3 and 4) occurs, the adrenaline is regenerated and hence can be reoxidized at the electrode surface. Thus as the chemical reaction takes place, the apparent number of electrons transferred, n_{app}, increases from the limits of $n = 2$ to $n = 4$. The following experiments have shown this to be the case. For an uncomplicated charge-transfer reaction, the chronopotentiometric constant $i_0\tau^{1/2}/C$ is a constant and proportional to n. Chronopotentiograms were run on *l*-noradrenaline at various current densities in pH 3 buffer, and $i_0\tau^{1/2}/C$ was a constant. At pH 6.5, however, the results were quite different. As τ increased from 6.2 to 18.9 sec n_{app}/n increased from 1.20 to 1.39 because the chemical reaction regenerating noradrenaline had more time to take place as the time of the experiment increased. Similar results were obtained from $i_p/V^{1/2}C$ *vs.* V in single sweep voltammetry and $it^{1/2}/C$ as a function of time using the potentiostatic technique.

Chronoamperometry (potentiostatic) was used for the quantitative study of the rate of cyclization since

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Table I. Rate of Cyclization of Open-Chain Quinone of Adrenaline at pH 3.5[a]

t, sec	$it^{1/2}/C$[b]	$it^{1/2}/it_\infty^{1/2}$	kt	k, sec^{-1}
2.33	66.7	0.526	0.056	0.024
3.11	67.7	0.535	0.074	0.024
3.89	68.8	0.544	0.094	0.024
5.44	71.0	0.561	0.131	0.024
7.77	74.1	0.585	0.191	0.025
11.66	78.9	0.623	0.302	0.026
15.54	83.4	0.658	0.400	0.026

[a] The open-chain quinone was prepared by electrochemical oxidation of a 4.58×10^{-3} M solution of adrenaline in pH 3.5 Britton and Robinson buffer at 25.0°. The area of the carbon paste electrode was 0.238 cm^2. The value of it_∞/C was 126.8×10^3 $\mu a\ sec^{1/2}$ l. $mole^{-1}$. [b] Units are 10^3 $\mu a\ sec^{1/2}$ l./$mole^{-1}$.

$$k_{obsd} = \frac{k_1 k_2}{k_{-1}(H^+) + k_2} \quad (5)$$

where k_1, k_2, and k_{-1} are those given in eq 1–4. This rate law can be obtained also by application of the steady-state approximation to eq 1–4. It can be seen that at low pH this rate law reduces to the form $k_{obsd} = k_2 K_a/(H^+)$ and k_2 can be estimated from a plot of k_{obsd} *vs.* $K_a/(H^+)$ assuming that the acidity constant of the side chain of the *o*-quinone is the same as that of the catechol. The values of k_2 listed in Table II were obtained in this way using only those points where no bending was observed in the pH profile. This includes all values for oxidized adrena-

Table II. Cyclization Rate Constants for Oxidized Catecholamines[a]

pH	Adrenaline	Noradrenaline	α-Methylnor-adrenaline	Dopamine	Isoproterenol
3.5	0.025				
4.0	0.098				0.020
4.5	0.27				0.055
5.0	0.99		0.019		0.15
5.5			0.043		
6.0		0.066	0.12		
6.5		0.15	0.23		
7.0		0.36	0.50	0.038	
pK_a	8.88	8.90	8.85	8.92	8.87
k_2[a,b]	7.4×10^3	5×10^1	8.1×10^1	...	1.2×10^3

[a] Units of rate constants are sec^{-1}. [b] k_2 calculated as discussed in text (Discussion section), assuming K_a of oxidized catecholamine is the same as the reduced form.

this technique is the only one for which the ECC case has been solved.[16,17]

Rate data can be obtained by following n_{app}/n or $it^{1/2}/it_\infty^{1/2}$ (where $it_\infty^{1/2}$ is the value of $it^{1/2}$ expected if the rate constant is infinity, *i.e.*, when $n = 4$) as a function of time, using a working curve which gives these ratios as a function of kt. An example of a typical experiment is given in Table I.

Table II shows k_{obsd} for the various catecholamine-quinones studied as a function of pH. The pH range covered is fairly small owing to the fact that the k_{obsd} measurable by this technique is 0.01 to 1 sec^{-1}. Also included in Table II are the pK_a values of the side chains of the various catecholamines, their structures, and, where applicable, k_2 (see Discussion).

Discussion

Figure 3 is a plot of log k_{obsd} for the cyclization of adrenalinequinone as a function of pH. Also included on the graph are some rate constants calculated from the data of Ball and Chen[19] who studied the potential of the adrenaline system as a function of pH using a flow technique. However, Ball and Chen used a value of $n = 2$ for their calculations, and hence their rate constants are higher than those obtained in this study. It can be seen that above pH 6, k_{obsd} becomes nearly independent of pH (see also the behavior of α-methylnoradrenaline). This behavior corresponds to a rate law of the form

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line and isoproterenol, pH 5–6 for α-methylnor-adrenaline but only pH 6 for noradrenaline. In-

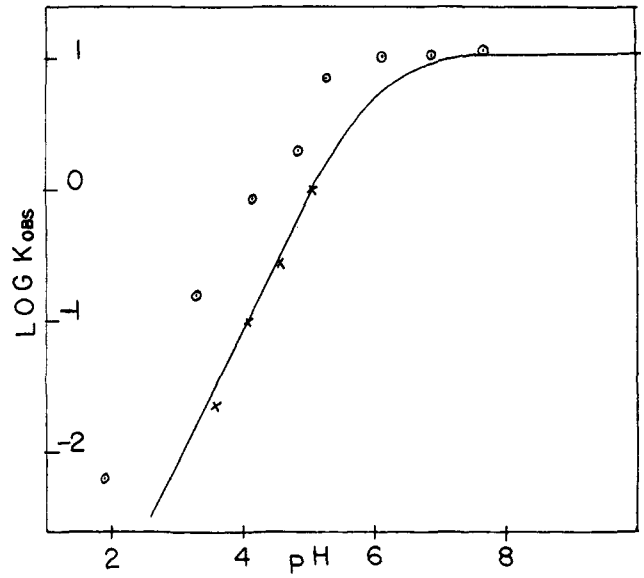

Figure 3. Cyclization rate of adrenalinequinone as a function of pH: ⊙, data of Ball and Chen[19] at 30°; ×, electrochemical data of the present study at 25°. The line is that calculated from eq 5 (see Discussion).

cluded in Figure 3 is a calculated pH profile using eq 5 for $k_2 = 10^4$ sec^{-1} (similar to adrenaline), $k_1/k_{-1} =$

$K_a = 10^{-9}$, and $k_{-1} = 10^{10}\ M^{-1}\ \text{sec}^{-1}$ (approximately diffusion controlled). From this plot it can be seen that the behavior is as predicted from the mechanism proposed by Harley-Mason.[14] At high pH eq 5 reduces to $k_{obsd} = k_1$, corresponding to a rate-limiting deprotonation reaction and a pH-independent rate constant. This behavior has recently been confirmed by use of a rotating disk electrode.[20]

One point of considerable interest is clear from the data of Table II. The cyclization rate (k_2) of adrenaline is 140 times faster than that of noradrenaline. These results are very interesting in the light of the recent studies of Walaas and Walaas.[4] In this work oxidation of reduced phosphopyridine nucleotides (DPNH and TPNH) was accomplished in a medium containing ceruloplasm and various catecholamines as substrates. Noradrenaline gave a considerably higher rate of oxidation than did adrenaline. It was suggested that adrenaline showed a greater tendency toward indolization and adrenochrome formation and hence lesser oxidative activity in the enzymatic oxidation of the DPNH. In a further study of the chemical transformation of catecholamines by ultraviolet irradiation, Walaas[5] again found much greater tendency toward ring closure with adrenaline. The importance of the uncyclized oxidation product of noradrenaline has been discussed by Walaas.[5] The present results on the relative cyclization rates are in perfect accord with the finding of Walaas. In this connection, the slow cyclization rate found for dopamine is of interest. The applications of the electrochemical techniques to melanization reactions would appear to be quite useful. The relative rate of adrenaline *vs.* isoproterenol is that expected from steric effects outweighing electronic effects.

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Oligonucleotide Syntheses on Insoluble Polymer Supports. I. Stepwise Synthesis of Trithymidine Diphosphate

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Abstract: An *insoluble* cross-linked polystyrene with pendant monomethoxytrityl chloride groups was prepared and condensed with thymidine to obtain a polymer containing approximately 500 μmoles of bound thymidine per gram of polymer. Condensation of the thymidine derivative with 3′-O-acetylthymidine 5′-phosphate (pTOAc) in the presence of dicyclohexylcarbodiimide (DCC) resulted in 54% conversion of bound thymidine to the dinucleoside phosphate. Deacetylation of the latter polymer and subsequent condensation with pTOAc and DCC gave the trinucleoside diphosphate derivative from which thymidylyl-(3′→5′)-thymidylyl-(3′→5′)-thymidine was isolated in 38% conversion based on polymer-bound dinucleoside phosphate.

The success achieved by Merrifield in stepwise synthesis of polypeptides on insoluble polymer supports[1] and the procedural advantages which the method affords prompted us to investigate the application of similar procedures to oligonucleotide synthesis. Reports of related studies in other laboratories have recently appeared.[2-4]

The support polymer under study in this laboratory consists of an *insoluble* styrene–divinylbenzene bead copolymer containing trityl chloride or 4-methoxytrityl chloride functional groups to which nucleosides are subsequently attached by trityl ether formation. Suitably protected nucleotides are then condensed with the polymer-bound nucleoside. The insolubility and form of this type of support confer physical and chemical characteristics which differentiate it from the soluble supports reported by Hayatsu and Khorana[3] and by Cramer, *et al.*[4] On the other hand, Letsinger and Mahadevan[2] utilized an insoluble popcorn polymer to which they attached amino-containing nucleosides through amide formation with polymer-borne carbonyl chloride groups.

Methoxytrityl Chloride Polymer Synthesis (Chart I). The synthesis of the supporting polymer was similar to procedures reported by Braun and Seelig[5] and is summarized in Chart I. Thus, a mixture of styrene and *p*-iodostyrene (mole ratio 4:1) containing 1% by weight of divinylbenzene (DVB) was polymerized in aqueous polyvinyl alcohol with benzoyl peroxide (Bz_2O_2) initiator to obtain cross-linked iodo copolymer (**1**) in the form of beads approximately 75 to 150 μ in diameter. The iodine content of the polymer corre-

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