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Potential Oxidative Pathways of Brain Catecholamines†

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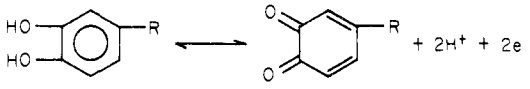
The possibility that catecholamines can be oxidized via aberrant pathways in vivo is open to question, but in vitro oxidation via aerobic manipulations is established. Assuming oxidation does occur, we have examined quantitatively the fast chemical reactions of the initial oxidation products, the *o*-quinones. The nature and rates of these reactions were studied under conditions simulating closely those which presumably exist in mammalian brain. The results are in close accord with existing literature and especially support oxidation pathways recently reported in [^{3}H]-norepinephrine binding to particulate cell fractions.

The possibility of aberrant oxidations of catecholamines (CA) has remained an active issue in the development of biochemical theories of mental illness, particularly schizophrenia. The original adrenochrome hypothesis seems relegated to history. However, the possibility of CA oxidations continues to surface in various reports of disturbances in oxidative metabolism, circulating rheomelanins,[1] and formation of the powerful neurotoxin, 6-hydroxydopamine.[2,3] Very recently Maguire et al.[4] have postulated that oxidative reactions of norepinephrine (NE) are involved in binding of [^{3}H]-NE with particulate fractions. They suggest that these findings may invalidate previous binding studies of adrenergic receptors.

Whether or not such aberrant oxidations can occur in vivo remains an unanswered question. That problem is not the object of the present study. Instead, we assume such reactions might occur under certain conditions in CNS. If so, the oxidized CA's produced even at picogram levels or less could have serious functional significance. The studies herein provide quantitative data about what would happen to the oxidized intermediates under conditions which exist in vivo in CNS. In addition, the results are highly pertinent to the particulate binding studies mentioned above.

The CA oxidations referred to are, of course, not the usual monoamine oxidase degradations, but conversion of the catechol moiety to the corresponding *o*-quinone as

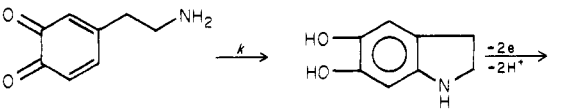


catechol *o*-quinone

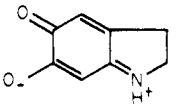
where R = $CH_2CH_2NH_2$ = dopamine (DA)
= $CH(OH)CH_2NH_2$ = norepinephrine (NE)
= CH_3 = 4-methylcatechol model analog

Other CA metabolites where the side chain is an alcohol or an acid function may be considered similarly. Dopamine is of primary interest in the present study and most of the reactions employed it or 4-methylcatechol as a model compound.

The major distinguishing feature of the primary oxidation product (the *o*-quinone) is that it is an electron-deficient species and therefore highly reactive with respect to nucleophiles. The most readily available nucleophile is the side-chain amine group of the *o*-quinone itself; hence, an intracyclization can take place near neutral pH as illustrated for dopamine-*o*-quinone (DOQ).



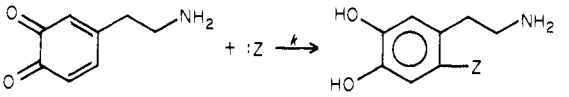
DOQ leucochrome



"dopamine-chrome"

The initial cyclized product, the leucochrome, is, of course, much more easily oxidized than the parent catechol; hence, oxidation proceeds further to the well-known aminochrome, in this case, dopamine-chrome. The complete electrochemical elucidation of this reaction has been reported previously[5] and Harrison et al.[6] have shown that chemical oxidations proceed similarly.

This intramolecular cyclization is just a special case of general nucleophilic reactions of the *o*-quinone. Despite the direct availability of the ethylamine side chain, the present studies show that more "aggressive" external nucleophiles can easily compete with the intracyclization as



where :Z represents a typical nucleophile species. Depending on the nature of Z, these addition products can also easily undergo further oxidation.

Hence, three reaction pathways of the original *o*-quinone must be considered as potentially important: (1) intracyclization yielding aminochromes, (2) addition of external nucleophiles known to be present in high concentration in brain, and (3) rereduction to the original catechol by endogenous reductants before either reaction 1 or 2 has time to occur.

The relative rates of these chemical reactions (all of which are possible in the CNS milieu) dictate the fate of any oxidized CA. From the rates one can predict what

† This paper is dedicated to the memory of Professor Edward E. Smissman, a dear friend and colleague whose enthusiastic support and interest in our studies is sorely missed.

final products to look for and, hence, possible ways to discover the consequences of suspected oxidative reactions in vivo or covalent bonding products in vitro. Quantitative data on these reaction pathways have been lacking because they are very rapid and complex under physiological conditions. Modern electroanalytical techniques are ideally suited to these studies and have provided the new information contained herein.

Experimental Section

The electrochemical techniques used are quite conventional and highly reliable. In general, the *o*-quinone intermediate was generated by electrolytic oxidation of the CA—in both the absence and presence of reactive nucleophiles, reducing agents, etc. For qualitative information about any chemical reactions which follow the initial oxidation, a rapid voltage-scanning technique (cyclic voltammetry) allows one to observe the formation of new oxidation–reduction couples produced. The rates of these reactions can be measured very simply by electrolyzing the CA at constant potential and following the current–time curve. In the absence of nucleophilic reaction with the *o*-quinone, the current–time curve decays inversely with $t_{1/2}$. If nucleophilic reactions of the type studied herein occur, the new product is also electrooxidizable at the given potential and "extra" current is observed during the current–time decay curve. Routine calculations allow one to relate the amount of "extra" current to the rate constant of the follow-up chemical reaction. A thorough discussion of these techniques applied to molecules of neuropharmacological interest, with examples similar to the present work, has been given in the literature.[7] Further details are mentioned here only when pertinent to an understanding of the particular experiment. The total time required to observe additions of nucleophiles to CA quinones varied greatly, from 5 msec to 15 sec. A Hewlett-Packard minicomputer system was utilized for experimental control, data acquisition, and analysis of the fast reactions. The computer operation is only a refinement of the basic experiment and involves no new concepts.

In general, the nucleophile was present in concentrations five times that of the *o*-quinone to provide approximate pseudo-first-order rates for comparison purposes. The actual rate evaluations were made following the procedures of Hawley et al.[5] In addition to the above techniques, controlled potential electrolysis with product separation and identification via high performance liquid chromatography was utilized. Since solutions of o-quinones slowly dimerize, electrolyses were carried out with 0.1 m*M* catechol solutions. Dimerization is then too slow to cause interference. All cyclic voltammetry and current–time experiments were run on carbon paste electrodes which have been adequately described.[7] All potentials referred to herein are vs. SCE as reference electrode.

Compounds were reagent grade and were used without further purification. Solutions were made in MacIlvaine pH 7.4 buffer which was thoroughly degassed to prevent premature air oxidation.

Results and Discussion

The individual rate measurements of the three possible reaction pathways of DOQ are discussed below. The intracyclization and external nucleophile additions data are examined together to better illustrate the competitive nature of the two processes.

A. External Nucleophiles vs. Intracyclization Rates. The rate studies in Table I include compounds representative of several classes of nucleophiles. Slow external nucleophile additions to DOQ are obscured by the faster intracyclization. For these slower reactions, the 4-methylcatecholquinone (which cannot intracyclize) was used as a model compound. The similarity of fast addition rates measured with *either* DA or 4-methylcatechol (bottom three entries in Table I) proves the latter is a good model for nucleophilic additions to DOQ.

The results show that most amino acids (except those with a sulfhydryl function) add relatively slowly. Thus, cystine, lysine, taurine, hydroxyproline, and the model compound, *p*-hydroxybenzoic acid, all add slower than the corresponding side-chain amino group (intracyclization), which is shown in the last column of Table I as a relative rate of unity. However, the rates of addition of the sulfhydrylamino acids, glutathione, and cysteine are *ca. three or four orders of magnitude greater* than any of the other naturally occurring amino acids tested and, indeed, *three orders of magnitude greater than the intracyclization reaction.* (The sulfhydryl additions are even 100–200 times faster than the physiologically unimportant but fast model compound, aniline.)

Table I. Nucleophilic Addition Rate Constants[c]

Nucleophile	Full time of expt	Addn rate[a] to DOQ	Addn rate[a] to 4-methylcatechol-quinone	Rate rel to cyclization
Cystine	15 sec		0.0033	0.22
p-Hydroxybenzoic acid	15 sec		0.0051	0.35
Lysine	12.5 sec		0.0065	0.47
Taurine	10 sec		0.0075	0.51
Hydroxyproline	5 sec		0.081	0.55
None (cyclization)	5 sec	0.147		1
Aniline	50 msec	14.5	13.7	9.9
Glutathione	25 msec	208[b]	122	1400
Cysteine	5 msec	315[b]	361	2100

[a] Pseudo-first-order rate constants in reciprocal seconds. [b] Due to unavoidable errors in the electrochemical measurement of these fast rates, the values for glutathione and cysteine could be too low by as much as 50%. Since the true rates are even faster than those given in the table, the data enhance rather than detract from any of the conclusions in the text. [c] pH 7.4 MacIlvaine buffer, 25°C.

B. Rereduction of *o*-Quinones to Catechols. Although these reactions with sulfhydryl compounds are extremely rapid (the approximate half-life of the reactive *o*-quinone is ca. 3 msec), an important alternative to this reaction is rapid, competitive rereduction of the *o*-quinone back to the catechol by physiologically important reducing agents. Ascorbic acid is a strong reducing agent and is present in the brain in high concentration, particularly in adrenergic neurons. Its ability to reduce quinone species, and thus prevent nucleophilic reactions, was examined as follows.

We first investigated whether ascorbic acid (AA) reduction could prevent the intracyclization reaction. A solution of DOQ was prepared by electrolytic oxidation of dopamine in pH 2.2 citrate buffer. At this pH, cyclization of DOQ is too slow to occur during the electrolysis. The concentration of DOQ was 10^{-4}, low enough to prevent dimerization of the quinone. A freshly prepared solution of AA in pH 8.7 Tris buffer was added rapidly to the DOQ solution. The concentrations were such that a final pH of 7.3–7.4 was quickly established and the AA and DOQ were equimolar. If cyclization occurred, the cyclized dopamine could be detected by the appearance of a redox couple at –0.3 V on the cyclic voltammogram. Figure 1 shows the results of two such experiments. In A, the Tris buffer contained no AA, so cyclization occurred normally. The couple at –0.3 V corresponds to the oxidation and reduction of 5,6-dihydroxyindoline, the cyclized DA product. In B, an equimolar concentration of AA completely prevents the appearance of the 5,6-dihydroxyindoline couple, indicating that AA reduced the quinone back to the original DA before cyclization could occur.

AA + DOQ ⇌ DA + dehydroascorbate

Thus, *AA reduction is faster than cyclization and will prevent aminochrome formation.* (In a similar experiment, AA prevented the even faster addition of aniline to electrochemically generated 4-methylcatecholquinone.)

The next problem was to see whether AA rereduction

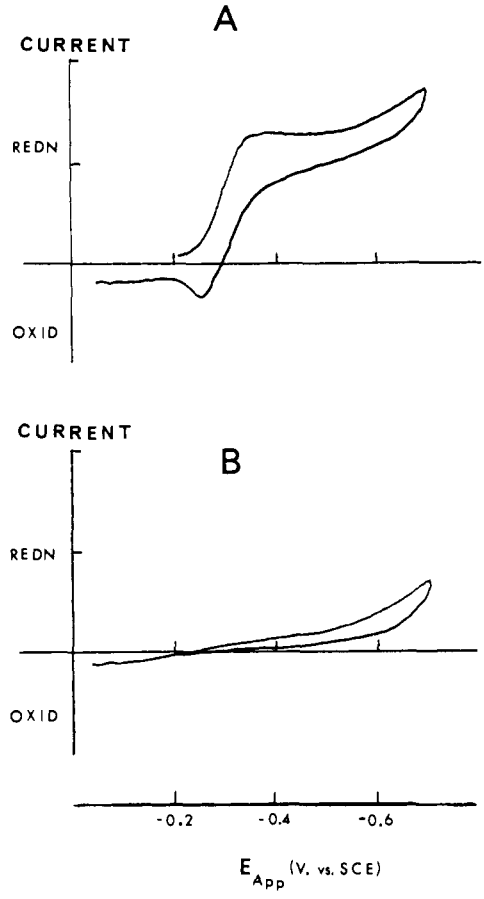


Figure 1. Prevention of dopamine cyclization by ascorbic acid: (A) cyclic voltammogram of dopamine alone at pH 7.4; (B) cyclic voltammogram of dopamine with equimolar ascorbic acid at pH 7.4.

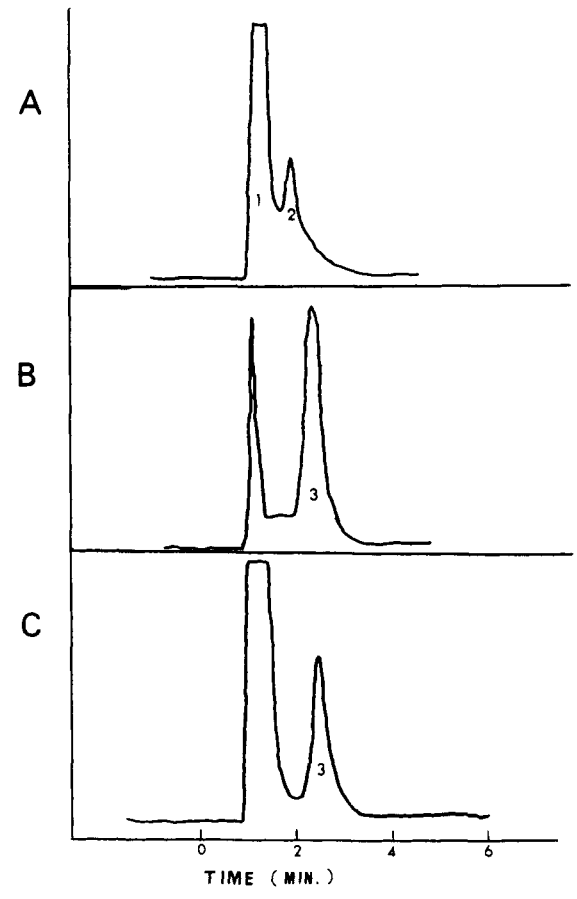


Figure 2. Addition of glutathione to oxidized 4-methylcatechol competition with ascorbic acid: (A) liquid chromatogram of partially oxidized 4-methylcatechol alone; (B) liquid chromatogram of partially oxidized 4-methylcatechol plus glutathione; (C) same as B but with equimolar glutathione and ascorbic acid.

could eliminate the very fast glutathione addition, and a slightly different experiment was devised. Neither the GSH–DA adduct nor the GSH–4-methylcatechol adduct differs enough electrochemically from their parent compounds to be distinguished by voltammetry. A high-pressure liquid chromatography system was utilized to separate and detect GSH–quinone adducts. To prevent possible interferences by cyclization of DOQ, the model compound 4-methylcatechol was used.

A solution of 4-methylcatecholquinone was prepared electrochemically under conditions where dimerization would not occur. The solution was mixed with a solution of GSH and AA and the mixture was chromatographed.

Figure 2, A, is a typical chromatogram of partially oxidized 4-methylcatechol (electrochemically oxidized at 0.7 V). Peak 1 is remaining unoxidized catechol and peak 2 corresponds to the *o*-quinone. This represents *o*-quinone in the absence of any nucleophilic additions at pH 7.4.

Figure 2, B, is a chromatogram of a similar mixture of *o*-quinone with an equimolar amount of GSH again at pH 7.4. Note peak 2 of the *o*-quinone is absent and is replaced by a peak, 3, due to the GSH nucleophilic addition product.

The AA competition experiment is seen in Figure 2, C. Here the same amount of 4-methylcatechol-*o*-quinone was prepared and to it was added a solution containing both GSH and AA. The final GSH concentration was identical with that of Figure 2, B, and, in addition, the ratio *o*-quinone–AA–GSH was 1:1:1. Clearly from the height of peak 3, there is a decrease in the yield of GSH–catechol adduct when AA is present. In another experiment when the concentration of AA was increased to five times that of GSH, the peak 3 adduct yield decreased another 50%. While AA rereduction competes seriously with the GSH addition, it cannot completely eliminate it. The GSH addition is sufficiently fast that some 60% of the reaction still occurs when AA and GSH are present at equimolar concentrations. This concentration ratio corresponds approximately to known whole brain levels in small animals.

Discussion and Conclusions

The rate measurements given above allow several useful conclusions to be drawn about the fate of oxidized dopamine if it were generated in small amounts in CNS tissue. First, the rereduction of the DOQ by endogenous AA is sufficiently rapid to prevent the formation of aminochromes, nonsulfhydrylamino acid adducts, or 6-hydroxylated products. This is historically consonant with the literature on possible in vivo formation of aminochromes, where all attempts to identify such compounds (e.g., adrenochrome) have been unsuccessful. The nucleophilic addition of OH^- to DOQ is ca. 10^{-4} sec^{-1} and the nonenzymatic formation of any 6-hydroxylated product such as 6-hydroxydopamine is highly unlikely.

Secondly, the possibility of any of the above reactions is further negated by the fact that sulfhydryl compounds, such as GSH, will react several orders of magnitude faster with DOQ. Even AA rereduction of DOQ cannot completely eliminate the fast sulfhydryl reactions. Hence, addition of GSH or perhaps protein or membrane sulfhydryl groups should be the overwhelmingly predominant reaction of any DOQ found in CNS. If the sulfhydryl function were part of a nerve terminal membrane struc-

ture, such interactions could result in substantial neuronal damage. This aspect of DOQ activity has been suggested previously.[3]

Nucleophilic interactions of the *o*-quinone of [^{3}H]-NE, with macromolecules as proposed by Maguire et al. (see Figure 14, ref 4), are completely consonant with the present findings. Furthermore, the quantitative data herein suggest that interaction of the *o*-quinone with particulate bound sulfhydryl functions constitutes the principal reaction.

All of the competitive reactions discussed herein are concentration dependent. The relative concentrations of reducing agents and nucleophiles have wide variations in brain regions and are not accurately known, especially at the microenvironmental level (i.e., nerve endings, etc.). Nevertheless, the comparative rates measured here provide useful guidelines for predicting the fate of any DOQ produced in vivo. It is pertinent in this respect to note that rat whole brain levels for ascorbic acid and glutathione are approximately equal when expressed on a molar basis (AA, 2.6 μmol/g;[8] GSH, 3.3 μmol/g[9]).

Finally, it should be noted that enzymatically promoted hydroxylations or oxidative condensations to melanin-like products in certain discrete CNS regions are not precluded by the arguments presented here.

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8-Chloro-(*S*)- and -(*R*)-10-[(*S*)- and -(*R*)-3'-methylethylaminopyrrolidino]-10,11-dihydrodibenzo[*b*,*f*]thiepins. Synthesis and Pharmacological Studies[1]

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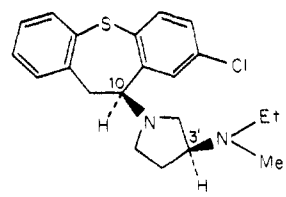
The synthesis for 8-chloro-(*S*)- and -(*R*)-10-[(*S*)- and -(*R*)-3'-methylethylaminopyrrolidino]-10,11-dihydrodibenzo[*b*,*f*]thiepins is presented. The absolute configuration at position 3' of the aminopyrrolidino side chain is known from synthesis and corresponds to the asymmetric carbon atom in (*S*)- or (*R*)-aspartic acid. The absolute configuration at C-10 of the dihydrodibenzo[*b*,*f*]thiepin ring system was deduced from ORD–CD analysis coupled with degradation of partially resolved (+)-8-chloro-10-amino-10,11-dihydrodibenzo[*b*,*f*]thiepin to (+)-(*S*)-1,2-diphenylethylamine. The four isomers were studied in mice for their ability to block conditioned avoidance responding, antagonize oxotremorine, and act as analgetics and anticonvulsants. These compounds were found to be nonselective antagonists of histamine, acetylcholine, and $BaCl_2$ in vitro. The compounds exerted effects similar to those of chlorpromazine. Stereoselective differences in activity between diastereoisomers, rather than between enantiomorphs, were generally observed.

Previous reports from this laboratory have considered the stereoselective biological properties of a number of enantiomorphic compounds synthesized from amino acids of known absolute configuration.[2-4] Among various D-(*R*)- and L-(*S*)-3-ethylaminopyrrolidino-substituted dihydrodibenzo[*b*,*f*]- and -[*b*,*e*]thiepins, xanthenes, and diphenylmethanes, the dihydrodibenzo[*b*,*f*]thiepin system seemed to warrant additional study for its potential antipsychotic activity.[4] To further explore stereostructure–activity relationships in a variety of biological systems we synthesized the four chiral isomers [3'*S*,10*S* (**1**); 3'*R*,10*R* (**2**); 3'*S*,10*R* (**3**); 3'*R*,10*S* (**4**)] of 8-chloro-10-(3'-methylethylaminopyrrolidino)-10,11-dihydrodibenzo[*b*,*f*]thiepin. The absolute configuration at position 3' of the aminopyrrolidino function corresponds to the asymmetric carbon atom in (*S*)- or (*R*)-aspartic acid; i.e., (*S*)-aspartic acid served as a precursor for **1** and **3** while (*R*)-aspartic acid served as a precursor for **2** and **4**. Studies leading to the assignment of absolute configuration at position 10 in each of the four isomers (**1**–**4**) are described in this article. The evaluation of these isomers as potential drugs for the treatment of psychotic disorders, depression, Parkinson's disease, convulsive disorders, and pain was carried out. It was of particular interest to us to determine whether diastereoisomeric and/or enantiomorphic differences in activity existed.



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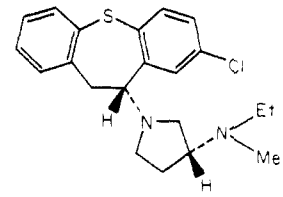

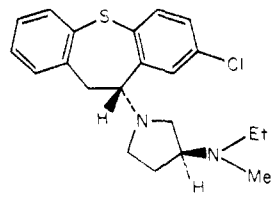

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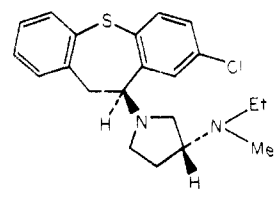

4

Synthesis. Tricyclic ketone **5**[5] served as starting

† This paper is dedicated to my major professor, Dr. Edward Smissman. Professor Smissman was an inspiring teacher and a very close friend; he gave to me considerably more than a scientific education.