Table II. Rates of Hydrolysis[a]

No.	pH 6 $t_{1/2}$, h	pH 6 K, h^{-1}	pH 8 $t_{1/2}$, h	pH 8 K, h^{-1}
1	60.1	0.0115	21.1	0.0329
2	14.8	0.0468	4.7	0.1480
3	75.8	0.0091	27.2	0.0255
4	26.6	0.0261	7.9	0.0879
5	10.0	0.0695	3.9	0.1790
6	10.1	0.0688	3.0	0.2310
7	63.7	0.0109	11.9	0.0583
8	182.0	0.0038	43.9	0.0158
9	80.1	0.0087	25.6	0.0271
10	111.0	0.0062	12.1	0.0575
11	4.7[a]	0.1470	1.4	0.5130
20	115.0	0.0060	61.1	0.0114

[a] The rates of hydrolysis studied conform to a pseudo-first-order reaction law, with the exception of 11 at pH 6. It appears that 11 reaches a state of equilibrium at about 10 h. Because of this exception the analysis was repeated and the same hydrolysis rate was obtained. At equilibrium there remained 24.3 mol % of ester.

Erylenmeyer flasks (reaction vessels). Aliquots of 5 mL were withdrawn from each reaction vessel and placed in 25-mL volumetric flasks (zero time). The reaction vessels were then immediately placed in a 37 °C H_2O bath and N_2 was bubbled through the solutions throughout the experiment. At selected time intervals, 5-mL aliquots were withdrawn, reacted with 2 mL of DTNB solution, and diluted with 25 mL of pH 7.5 buffer. The absorbance at 410 nm was recorded in 1-cm cells against a reagent blank (used to balance the spectrophotometer, a Cary Model 118).

Since the analytical method measured the growth of a reaction product rather than the decay of the ester under study, the concentration of intact ester at each sampling time was calculated. This concentration was proportional to the difference ΔA between the absorbance observed and that calculated for complete hydrolysis. First-order rate constants and half-lives were calculated by computer using a nonlinear least-squares fit to the first-order rate equation

$$C_t = C_0 \cdot \exp(-kt)$$

$$t_{1/2} = \ln 2/k$$

At pH 6, the rates of hydrolysis of all the esters studied, except 11, conformed to a pseudo-first-order reaction law (Table II). It appeared that 11 reached a state of equilibrium at about 10 h. Because of this exception the analysis was repeated and the same hydrolysis rate was obtained. At equilibrium there remained 24.3 mol % of ester. As expected, the hydrolysis rates for this series of compounds were more rapid at pH 8 than at pH 6.

Acknowledgment. We are indebted to Messrs. A. F. Zappala and A. Post and Dr. W. W. Holl of our Analytical and Physical Chemistry Section for the hydrolysis studies and calculations. We also thank Miss E. Reich and staff for elemental analyses.

References and Notes

(1) Presented in part at the 172nd National Meeting of the American Chemical Society, San Francisco, Calif., August 30–Sept 3, 1976.
(2) B. Blank, N. W. DiTullio, C. K. Miao, F. F. Owings, J. G. Gleason, S. T. Ross, C. E. Berkoff, H. L. Saunders, J. Delarge, and C. L. Lapière, *J. Med. Chem.*, 17, 1065 (1974).
(3) N. W. DiTullio, C. E. Berkoff, B. Blank, V. Kostos, E. J. Stack, and H. L. Saunders, *Biochem. J.* 138, 387 (1974).
(4) C. Hansch, S. Unger, and A. B. Forsythe, *J. Med. Chem.*, 16, 1217 (1973).
(5) G. L. Ellman, *Arch. Biochem. Biophys.*, 82, 70 (1959).

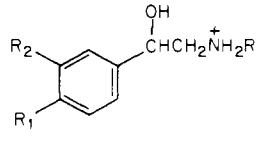
Relative Concentrations of Zwitterionic and Uncharged Species in Catecholamines and the Effect of N-Substituents

C. Robin Ganellin

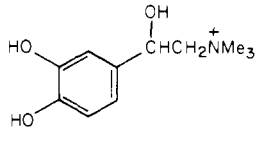
The Research Institute, Smith Kline & French Laboratories Limited, Welwyn Garden City, Hertfordshire, England.
Received July 12, 1976

The relative concentrations of zwitterionic and uncharged species for the series of N-substituted catecholamines (I, $R_1 = R_2 = OH$; R = H, Me, Et, *i*-Pr, *t*-Bu) are derived from the pK_a data published in 1962 by Sinistri and Villa. The concentration ratios, represented by the tautomeric equilibrium constant K_t, show a definite trend and are respectively 1.8, 4.3, 4.7, 4.7, and 7.1. These values suggest that any mechanism of action involving proton transfer, which might transform the zwitterion into the uncharged form, would be most favorable for norepinephrine and least favorable for the *t*-Bu derivative.

Knowledge of the chemistry of catecholamines is fundamental to understanding the molecular mechanisms of their biological actions.[1] Catecholamines exist as equilibrium mixtures of different ionic species and conformers and there have been various studies to determine proton dissociation constants[2] and conformational preference.[3] The effects of *N*-alkyl substitution are of particular interest; in the simple series classified by Ahlquist,[4] viz. norepinephrine, epinephrine, and isoproterenol (1, R_1 =



1



2

R_2 = OH; R = H, Me, *i*-Pr), isoproterenol is the least active at α-receptors but is the most active at β-receptors, and the chemical basis for selectivity imparted by the isopropyl substituent continues to intrigue medicinal chemists.[1b,e,f,5,6] Various molecular models for the receptor site interaction have been reviewed by Brittain et al.[5] There is, however, no proven mechanism of drug action and it is worthwhile to continue to examine features of catecholamine chemistry attributable to the *N*-alkyl groups.

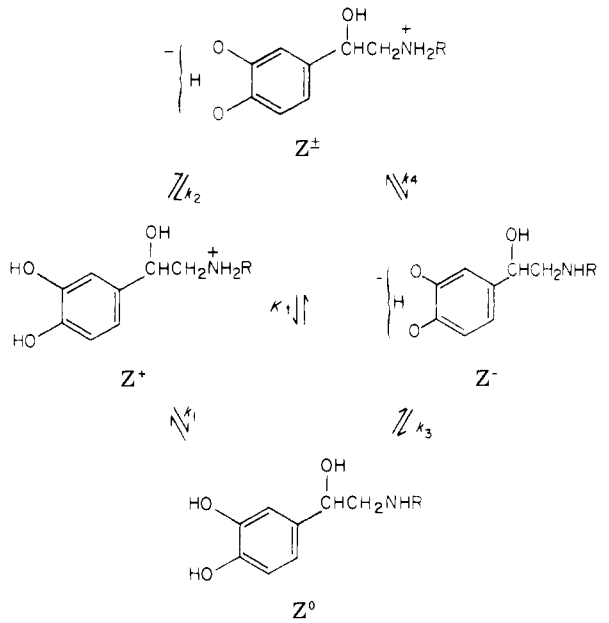
The author wishes to draw attention to a subtle effect of the alkyl group derived from proton dissociation. Determination of catecholamine proton acidities by potentiometric titration affords two stoichiometric pK_a values (e.g., for the epinephrine cation Lewis reported[2c] values of 8.7 and 9.9) but assignment of proton ionization to particular sites in the molecule is problematical. Making comparison with simpler molecules such as catechol (pK_a = 9.4) and phenylethanolamine (pK_a = 8.9) some authors attributed the lower pK_a to the $-NH_2{}^+R$ group[2a,d] and the higher pK_a to the phenolic OH.[2i,l] However, using a UV

Table I. Tautomeric Equilibrium Constants (K_t)[a] and Species Populations (Percentage of Total) of Catecholamines (1, $R_1 = R_2 = OH$) at 25 °C in Water at pH 7.4 and 8.4, Using Data from Sinistri and Villa[2g]

	R	K_t	pH 7.4				pH 8.4			
			Z^+	$Z^\pm$	Z^0	Z^-	Z^+	$Z^\pm$	Z^0	Z^-
Norepinephrine	H	1.8	95.1	2.9	1.6	0.4	67.8	18.1	10.0	4.1
Epinephrine	Me	4.3	95.9	3.2	0.7	0.2	71.1	21.8	5.1	2.0
N-Ethylnorepinephrine	Et	4.7	96.4	2.8	0.6	0.2	74.2	20.0	4.2	1.6
Isoproterenol	*i*-Pr	4.7	96.2	3.0	0.6	0.2	72.9	21.0	4.5	1.6
N-*tert*-Butylnorepinephrine	*t*-Bu	7.1	96.2	3.2	0.5	0.1	72.4	23.1	3.3	1.2

[a] K_t = antilog ($pk_1 - pk_2$). pk_1 is obtained by titration[2g] of the model compounds 1, $R_1 = R_2$ = OMe. pk_2 is obtained from the equation: $K_{a_1} = k_1 + k_2$. K_{a_1} is the macroscopic constant obtained by titration[2g] of the compounds 1, $R_1 = R_2$ = OH.

Scheme I. Ionic Species Equilibria of Catecholamines



method to determine the dissociation of the phenolic OH, such assignments were shown by Lewis[2c] and others[2b,j,m] to be in error and the assignment was reversed; i.e., the low pK_a was attributed to OH ionization. None of these authors considered that the measured pK_a's are composite values. As shown in Scheme I, proton dissociation of the cation (Z^+) occurs from NH^+ to give the uncharged species (Z^0), or from OH to give the zwitterion ($Z^\pm$), and dissociation of a second proton in either case affords the anion (Z^-) (further dissociation from Z^- gives the dianion Z^{2-} at high pH; e.g., norepinephrine has reported[2l] $pK_{a_3} = 11.1$). The equilibria between these species are represented by the four microscopic ionization constants k_1, k_2, k_3, and k_4. The stoichiometric ionization constants pK_{a_1} and pK_{a_2} determined by titration are thus mixed constants and are given by the equations[2k]

$$K_{a_1} = k_1 + k_2 \quad (1)$$

$$K_{a_2} = k_3^{-1} + k_4^{-1} \quad (2)$$

To assign pK_a's to the specific sites of proton ionization, it is necessary to determine the respective microscopic constants. An analysis was published in 1962 by Sinistri and Villa[2g] (see also ref 1b and 2k) using methyl-substituted analogues for a series of catecholamines. The dimethyl ethers (1, $R_1 = R_2$ = OMe) provided the dissociation constant of the $-NH_2^+R$ group in the absence of phenolic dissociation, which corresponds to pk_1 in Scheme I; in conjunction with the measured values for pK_{a_1}, pk_2 was calculated from eq 1. It is assumed that the *O*-methyl groups do not have a direct effect on ammonium pK_a; since the methyl groups are some distance from the respective ionization sites this seems likely to be valid, and it is supported by the excellent agreement between pk_2 and the value 8.90 determined for the quaternary ammonium analogue **2**. The values of pk_3 and pk_4 follow from these results and the measured values of pK_{a_2} in eq 2. The published[2g] microscopic ionization constants are very close together and it appears that altering the *N*-alkyl substituent affects pk_1 and pk_4 (i.e., NH^+ acidities) but has little effect on pk_2 and pk_3 (OH acidities).

From the published pK_a data we have calculated the relative populations of the species at pH 7.4 and 8.4 (see Table I). At pH 7.4 and 25 °C, the cation is the main species to an extent greater than 95%. There is a small proportion present as zwitterion and lesser amounts present as uncharged molecule and anion. At a higher pH of 8.4, the amount of cation falls to around 70%, and the zwitterion rises to around 20%. Altering the *N*-alkyl substituent has little effect on the populations of the two main species (cation and zwitterion) but it alters the populations of the minor forms. An interesting aspect is revealed by examining the relative concentrations of zwitterionic and uncharged forms. These two species are tautomerically related, differing only in the siting of a proton and the consequent charge distribution, and the ratio of concentrations is given by the tautomeric equilibrium constant

$$K_t = [Z^\pm]/[Z^0] = k_2/k_1 = \text{antilog}\,(pk_1 - pk_2)$$

We have derived K_t from the data in ref 2g (see also discussion in ref 2k) and discovered a trend in which the ratio of zwitterion to uncharged form increases, ascending the homologous series from norepinephrine, through epinephrine and isoproterenol, up to the value 7.1 for *tert*-butylnorepinephrine. Thus, altering drug structure alters the balance between the zwitterion and uncharged forms. From these findings it appears that *any mechanism of drug action involving proton transfer, during which time the zwitterion is transformed into the uncharged form, would be most favorable for noradrenaline and least favorable for the tert-butyl derivative.* A corollary, for structure–activity considerations of synthetic catecholamine analogues, is that introduction of strongly electron-withdrawing substituents into the catechol ring would lower pk_2 and substantially increase K_t.

The pK_a values refer to an aqueous system but, for compounds that act at receptors or on membranes, the medium may not be pure water, but structured and partly lipoid, and this may change the pK_a's;[7] furthermore, one does not know the pH that obtains at the active site; such factors alter the species populations. It is also problematical to know what importance to attach to minor species, although there is no reason a priori to exclude these from consideration, especially where transient

phenomena (e.g., intermediates or transition states) may be involved. An interesting question arises as to the structure of the zwitterion since there are two phenolic OH sites for proton dissociation and the possibility of internal hydrogen bonding.[2h] According to Sinistri and Villa[2h] there is little difference in the pK_a between respective *O*-monomethyl analogues, which suggests that ionization probably occurs about equally from the two sites.

References and Notes

(1) (a) P. Pratesi, E. Grana, L. Villa, A. La Manna, and L. Villa, *Farmaco, Ed. Sci.*, **18**, 920 (1963); (b) P. Pratesi and E. Grana, *Adv. Drug Res.*, **2**, 127 (1965); (c) P. Pratesi, E. Grana, and L. Villa, *Proc. Int. Pharmacol. Meet., 3rd, 1966*, **7**, 283 (1968); (d) P. Pratesi, E. Grana, and L. Villa, *Farmaco, Ed. Sci.*, **26**, 379 (1971); (e) B. Belleau, *Pharmacol. Rev.*, **18**, 131 (1966); (f) *Ann. N.Y. Acad. Sci.*, **139**, 580 (1967); (g) B. M. Bloom and I. M. Goldman, *Adv. Drug Res.*, **3**, 121 (1966); (h) E. J. Ariens, *Ann. N.Y. Acad. Sci.*, **139**, 606 (1967); (i) A. A. Larsen, *Nature (London)*, **224**, 25 (1969).

(2) (a) E. B. Leffler, H. M. Spencer, and A. Burger, *J. Am. Chem. Soc.*, **73**, 2611 (1951); (b) A. Albert, *Pharmacol. Rev.*, **4**, 136 (1952); (c) G. P. Lewis, *Br. J. Pharmacol. Chemother.*, **9**, 488 (1954); (d) M. M. Tuckerman, J. R. Mayer, and F. C. Nachod, *J. Am. Chem. Soc.*, **81**, 92 (1959); (e) H. Shapiro, *J. Theor. Biol.*, **1**, 289 (1961); (f) S. Riegelman, L. A. Strait, and E. Z. Fishcher, *J. Pharm. Sci.*, **51**, 129 (1962); (g) C. Sinistri and L. Villa, *Farmaco, Ed. Sci.*, **17**, 949 (1962); (h) *ibid.*, **17**, 967 (1962); (i) R. F. Jameson and W. F. S. Neillie, *J. Chem. Soc.*, 2391 (1965); (j) T. Kappe and M. D. Armstrong, *J. Med. Chem.*, **8**, 368 (1965); (k) R. B. Martin, *J. Phys. Chem.*, **75**, 2657 (1971); (l) K. S. Rajan, J. M. Davis, R. W. Colburn, and F. H. Jarke, *J. Neurochem.*, **19**, 1099 (1972); (m) P. J. Antikainen and U. Witikainen, *Acta Chem. Scand.*, **27**, 2075 (1973).

(3) For reviews with leading references, see G. C. K. Roberts in "Molecular and Quantum Pharmacology", E. D. Bergman and B. Pullman, Ed., Reidel Publishing Co., Dordrecht, Holland, 1974, p 77; J. P. Green, C. L. Johnson, and S. Kang, *Annu. Rev. Pharmacol.*, **14**, 319 (1974); P. S. Portoghese, *ibid.*, **10**, 51 (1970).

(4) R. P. Ahlquist, *Am. J. Physiol.*, **153**, 586 (1948).

(5) R. T. Brittain, D. Jack, and A. C. Ritchie, *Adv. Drug Res.*, **5**, 197 (1970).

(6) R. B. Barlow, "Introduction to Chemical Pharmacology", 2nd ed, Methuen, London, 1964, p 318.

(7) J. I. Brauman and L. K. Blair, *J. Am. Chem. Soc.*, **90**, 6561 (1968).

Isolation and Identification of an in Vivo Reaction Product of 6-Hydroxydopamine

Y.-O. Liang, P. M. Plotsky, and R. N. Adams*

Department of Chemistry, University of Kansas, Lawrence, Kansas 66045.

Received October 12, 1976

The product of oxidized 6-OHDA and GSH reacted in vitro has been identified by a variety of chemical and physical methods to be 2,4,5-trihydroxy-6-*S*-(glutathionyl)phenethylamine. Its chemical properties show it easily undergoes a variety of oxidative condensations and polymerization. Its oxidized form, the *p*-quinone, can be identified in small quantities in rat brain and mouse brain 1–3 h after 6-OHDA injection. This is believed to be the first report of a chemically identified species resulting from the in vivo interaction of 6-OHDA with CNS tissue.

The molecular mechanism whereby 6-hydroxydopamine (6-OHDA) exerts its powerful neurotoxicity toward catecholamine neurons is still in question. However, from the beginning it has been suggested that nucleophilic interaction (covalent bonding) of the 6-OHDA quinone with neuronal constituents might be crucial in this action.[1-4] This view has received recent support from several sources.[5,6] Our studies have shown that oxidized 6-OHDA (the *p*-quinone, 6-Q) reacts extremely rapidly with thiols. Using glutathione (GSH) as an example of a thiol known to be present in high concentration in the CNS milieu,[7,8] we characterized the rate and nature of this reaction, but the identification of the product remained somewhat uncertain.[9] We now have a complete chemical identification of the product from the in vitro reaction of oxidized 6-OHDA and GSH at pH 7.4. Further, we have been able to identify this same compound as resulting from the in vivo interaction when 6-OHDA is injected stereotaxically into rat brain.

Results and Discussion

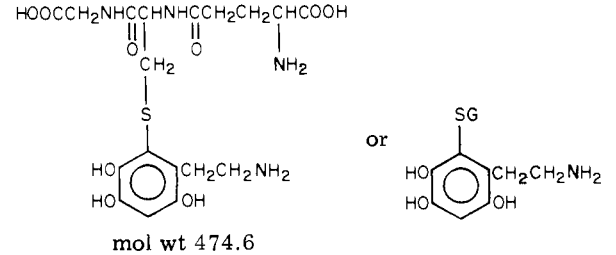
A. Characterization of the in Vitro Reaction Product of 6-OHDA and GSH. The reaction product (see Experimental Section) was hydroscopic and melted in air with decomposition at 195–198 °C. It gave a single peak by liquid chromatography with the same retention time as the new peak obtained when GSH is added to air-oxidized 6-OHDA solution. The UV spectrum in 0.1 M perchloric acid had a λ_{max} at 306 nm with $\epsilon = 3.2 \times 10^3$ M^{-1} cm^{-1}. The NMR spectrum in D_2O showed one aromatic proton at 6.6 ppm which we were able previously to show was the 5-position proton;[9] thus the GSH substitution occurs on the 2 position.

The mass spectrum of 6-OHDA showed *m/e* fragments at 152, 151, 150, 149, and 139. The mass spectrum of the product gave major peaks at *m/e* of 184, 183, 182, 181, and 171, each of which are 6-OHDA fragments with an attached sulfur (mass 32), consistent with the expected fragmentation pattern.

In deaerated citric–phosphate buffer (pH 7.4) the product can be oxidized very easily at the dropping mercury electrode with an $E^{0'} = -0.20$ V vs. SCE. This shows that the product is the reduced (hydroquinone) form.

The elemental analysis provided the following data. Anal. ($C_{18}H_{26}N_4O_9S$) C, H, N, S.

All of the above results confirm that 1 mol of oxidized 6-OHDA reacts with 1 mol of GSH by nucleophilic addition to form the RS-substituted 6-OHDA having the structure



mol wt 474.6