Catecholamine Oxidation and Ionization Properties Indicated from the H^+ Release, Tritium Exchange, and Spectral Changes Which Occur during Ferricyanide Oxidation*

W. H. Harrison, Walter W. Whisler, and B. J. Hill

ABSTRACT: Oxidations by ferricyanide ion of compounds with dihydroxyphenylethylamine structures to indole derivatives were studied by measurement of the H^+ release and spectral changes which occur in reactions maintained at constant pH by a pH-Stat technique. The results were verified in the case of adrenaline by measurement of the ^3H release accompanying oxidation of ^3H-ring-labeled adrenaline. The catecholamines and related compounds studied included adrenaline, noradrenaline, dopamine, α-methyladrenaline, α-methylnoradrenaline, dopa, epinine, adrenalone, noradrenalone, and isoproterenol. Their oxidation at pH 7 was found in each case to involve reduction of 4 equiv of ferricyanide (transfer of four electrons) and the release of 5 equiv of H^+, indicating formation of the aminochrome derivative. The stoichiometry indicated that release of 4 H^+ equiv resulted from the oxidation; the additional H^+ equivalent released reflects the protonation of the side-chain amino nitrogen atom which most probably is converted in the cyclization step into an imino nitrogen which has negligible affinity for H^+ at pH 7. Studies of oxidations of adrenaline, noradrenaline, and dopamine at pH <7 indicated that the oxidations proceed to the aminochrome stage but, however, are incomplete; in the presence of Zn^{2+}, the reactions proceed to a further extent, being complete down to pH 4 for noradrenaline and dopamine and to pH 3 for adrenaline. The method described should prove useful for the direct determination of the extent of protonation of other compounds of this type.

In the presence of a variety of electron acceptors, the physiologic hormones and drugs with dihydroxyphenylethylamine structures, such as adrenaline, noradrenaline, dopamine, and isoproterenol, are converted by oxidative cyclization into various indole derivatives *via* intermediate uncyclized *o*-quinones (Heacock, 1959, 1965; Harrison, 1963a,b; Harrison and Whisler, 1966; Harrison *et al.*, 1967). Also, similar reactions occur electrolytically at platinum or carbon paste anodes (Hawley *et al.*, 1967) or as a result of ultraviolet irradiation (Walaas, 1963). This reaction serves as the basis of a major chemical assay for catecholamines in biological sources (Udenfriend, 1962), and, although the major metabolic pathways have been established to be O methylation and oxidative deamination (Iverson, 1967), the additional participation of the catecholamines or their indolic oxidation products in biological electron transfer processes remains a definite possibility in view of the redox properties they exhibit in the presence of numerous isolated metalloproteins (Heacock, 1959).

The mechanisms of the oxidative cyclization step and the nature of the ensuing reactions of the indolic products are not well understood; the mechanisms of the direct electron transfer reactions involved in ferricyanide or iodine oxidations differ from the indirect electron transfer reactions occurring in the catalytic oxidations involving molecular oxygen as the ultimate electron acceptor. The chemical oxidative pathways of adrenaline which have been demonstrated together with those which have been postulated are described in Scheme I. In direct oxidations, such as with ferricyanide, adrenochrome (V) is believed to be the major product but it is not known whether it is formed by direct oxidative cyclization (pathway 2) or whether it is formed *via* leucoadrenochrome (III) by pathway 1. The indirect oxidations have been found to be considerably more complex, involving the formation of several intermediates and products, some identified as those indicated in Scheme I and others unidentified; typical examples are the catalytic oxidations involving transfer of electrons to molecular oxygen mediated by copper ion or copper ion–protein complexes (Hayaishi, 1962; Peisach *et al.*, 1966). In the latter case the catalyst functions by binding and activating the ultimate electron acceptor, O_2, and probably by binding catecholamine, the electron donor, as well. The products of the indirect oxidations are highly dependent upon reaction conditions such as pH, the type of buffer used, catalyst employed, and reactant concentrations and some are highly reactive, exhibiting

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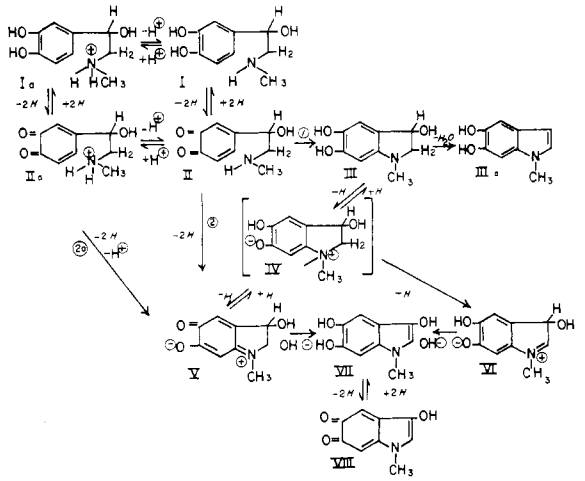

SCHEME 1: Possible oxidative pathways of adrenaline (I). The products shown are adrenaline-*o*-quinone (II), leucoadrenochrome (III), 5,6-dihydroxy-1-methylindole (IIIa), adrenochrome (V), 3,5,6-trihydroxy-1-methylindole (VII), and its *o*-quinone state (VIII), and structures (IV and VI) postulated by Harley-Mason (1950). Structures Ia and IIa are the protonated forms of I and II, respectively. Important details of this mechanism were originally postulated by Raper (1927).

a tendency toward polymerization to melanin-type (Bu'Lock, 1960) pigments. The indirect oxidations involving molecular oxygen occur with H transfer to the acceptor, oxygen, to form H_2O, in which case no net H^+ release accompanies oxidation. On the other hand, a direct oxidation such as with ferricyanide should occur with H^+ release concurrent with electron transfer and the initial objective of the present study was to verify this and to establish the stoichiometry of the H^+-release reaction for use in comparing the two reaction types. The reaction with ferricyanide was studied by measuring the H^+ release and the stoichiometry indicated was verified by measuring the spectral changes and ^3H release from ^3H-labeled compounds which occur during oxidation. It will be seen that this approach proved invaluable for verifying the H^+-release effect and for studying the mechanism as well as for determining various oxidation and ionization characteristics of several catecholamines.

Materials and Methods

The D-(−)-adrenaline (bitartrate), D-(−)-noradrenaline (bitartrate), 2-[3′,4′-dihydroxyphenyl]ethylamine (HCl) (dopamine), and L-(−)-3,4-dihydroxyphenylalanine (dopa) were supplied by Nutritional Biochemicals. The adrenalone (2-[3′,4′-dihydroxyphenyl]-2-oxo-*N*-methylethylamine) and noradrenalone (2-[3′,4′-dihydroxyphenyl]-2-oxoethylamine) were supplied by K & K Laboratories, Inc. The DL-α-methyladrenaline, DL-α-methylnoradrenaline, and 2-[3′,4′-dihydroxyphenyl]-*N*-methylethylamine (epinine) were kindly supplied by Dr. S. Archer of Sterling-Winthrop. The DL-isoproterenol (2-[3′,4′-dihydroxyphenyl]-2-hydroxy-*N*-isopropylethylamine) was supplied by Sigma Chemical Co. The DL-adrenaline-2,5,6-^3H was supplied by Nuclear-Chicago (200 mCi/mole). All other commercially supplied chemicals were reagent grade. Stock solutions (2×10^{-4} M) of the compounds studied were prepared in 0.01 M HCl to ensure stability.

The pH-Stat determinations were performed with a Radiometer titration assembly including a titrator 11, pH meter 27, and a titrigraph; a type G202 C glass electrode and type K 401 reference electrode were used and titration procedures recommended by Radiometer were followed. An Aminco-Bowman microtitrator was used used to dispense the potassium ferricyanide solution (2×10^{-2} M). A Radiometer ABU-1b autoburet was to dispense the CO_2-free standardized sodium hydroxide (*ca.* 0.01 N). A Beckman DU monochromator with Gilford attachments was used in the spectrophotometric studies. A Packard Tri-Carb no. 3003 liquid scintillation spectrometer was used in the ^3H tracer experiment.

In the experiments described in Figures 1 and 2, 25.0 ml of an adrenaline solution (8×10^{-5} M) was titrated with a $K_3Fe(CN)_6$ solution (2×10^{-2} M). In the experiments described in Figure 4, 25.0 ml of an adrenaline, noradrenaline, or dopamine solution (8×10^{-5} M) was titrated with a $K_3Fe(CN)_6$ solution (2×10^{-2} M) or with a mixture of $K_3Fe(CN)_6$ (2×10^{-2} M) and $ZnSO_4$ (2×10^{-2} M). The final concentration of NaCl in the above was approximately 4×10^{-3} M. The pH-Stat titrations were carried out at room temperature after ascertaining that the temperature control factor was not critical. Although most determinations were performed anaerobically, in the presence of nitrogen, it was determined that identical results were obtained in aerobic runs. Routinely, 0.10 ml of standardized HCl (*ca.* 0.01 M) was added to the reaction medium and titrated, this being done both before and after each run as a check of the constancy of sensitivity of the glass electrode and of the titer of the sodium hydroxide (*ca.* 0.01 M). The general procedure consisted of an automatic adjustment of the catecholamine solution to the desired pH, then a similar adjustment of a stock solution of the potassium ferricyanide or of a mixture of the zinc sulfate and potassium ferricyanide to the same pH, followed by stepwise addition of the latter to the catecholamine solution. The sodium hydroxide solution was introduced at a rate sufficient to maintain the pH within 0.1 pH unit during the oxidation and the stirring was set at a rate sufficient to prevent overtitration. The reaction was considered equilibrated if no change of pH occurred within 30 sec. In the spectrophotometric titration the runs were performed as described above with the exception that after addition of each equivalent of ferricyanide, a 3-ml aliquot was withdrawn, rapidly analyzed spectrally, and transferred back to the reaction mixture; quartz cuvets (Beckman) with 1-cm light path were used.

Details of the method employed in the ^3H tracer experiment with DL-adrenaline-2,5,6-^3H have been described in an earlier publication (Harrison and Whisler, 1966). The oxidations were carried out in 20-ml scintillation vials, each containing 20 mμmoles of adrenaline (including ^3H tracer) and 0, 20, and 40–180 mμmoles of potassium ferricyanide in 1 ml of 0.2 M phosphate buffer (pH 7). The oxidations were stopped after exactly 3.5

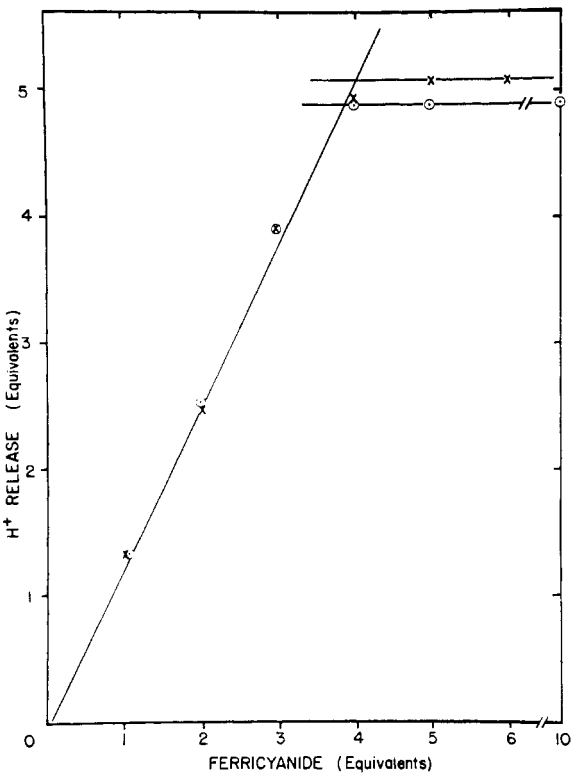

FIGURE 1: H^+ release in oxidation of adrenaline by $K_3Fe(CN)_6$ at pH 7. (×) H^+ release measured in this study. (⊙) H^+ release calculated from paper of Chaix *et al.* (1950).

min by addition of 0.1 ml of 1% ascorbic acid to each tube. Each solution was then evaporated to remove the liberated ^3H, as described previously (Harrison and Whisler, 1966); then the scintillation fluid was added and the sample counted for radioactivity. The total counts (zero time) in each tube were about 51,000 cpm.

Results

Oxidation at pH 7. H^+ release was measured during oxidation by ferricyanide ion at pH 7 of ten structurally related compounds. The titration of adrenaline, accomplished with the use of a pH-Stat, is described in Figure 1. Clearly, 4 equiv of ferricyanide ion marked the end point which corresponded to the release of 5 H^+ equiv. The same stoichiometry was demonstrated with noradrenaline, dopamine, α-methyladrenaline, α-methylnoradrenaline, dopa, epinine, adrenalone, noradrenalone, and isoproterenol. The same stoichiometry was found in reverse titration measurements in which the electron donor, the catecholamine, was added to a ferricyanide solution. The oxidations thus appeared to proceed quantitatively to the aminochrome stage with no side reactions. The latter was further verified in spectral studies of the oxidation of adrenaline, noradrenaline, and dopamine and it was found that an excess of ferricyanide was easily detected by noting whether a peak at 435 mμ appeared in addition to the major aminochrome peak (maximum 485 mμ); based on this, the ferricyanide titration was repeated with each amine by measuring its respective chrome derivative spectrophotometrically.

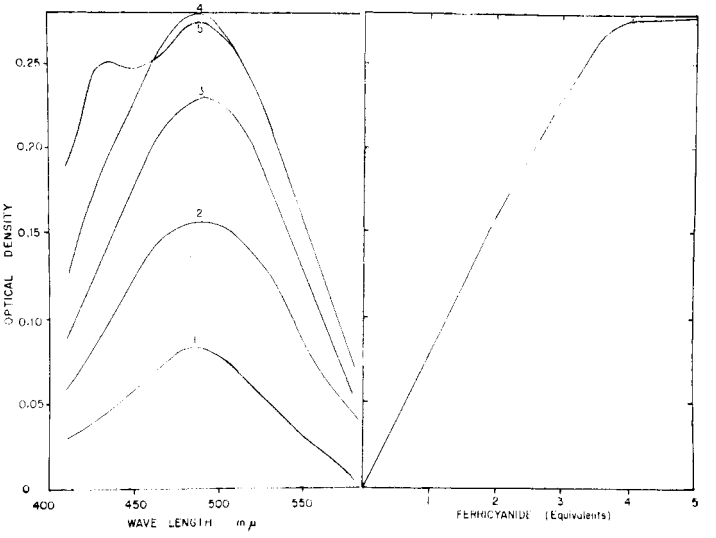

FIGURE 2: Adrenochrome formation in oxidation of adrenaline by $K_3Fe(CN)_6$. Numbers on spectral curves refer to the equivalents of $K_3Fe(CN)_6$ added. The points plotted in the right-hand graph refer to the peak optical densities.

The spectrophotometric titration of adrenaline is described in Figure 2, together with a plot of the spectral changes. This result served to validate the fact that 4 equiv of ferricyanide correspond to the end point and similar titrations with noradrenaline and dopamine gave identical results. The spectrophotometric titration at pH 7 could be performed by maintaining the pH with the aid of a pH-Stat or by performing the reaction in

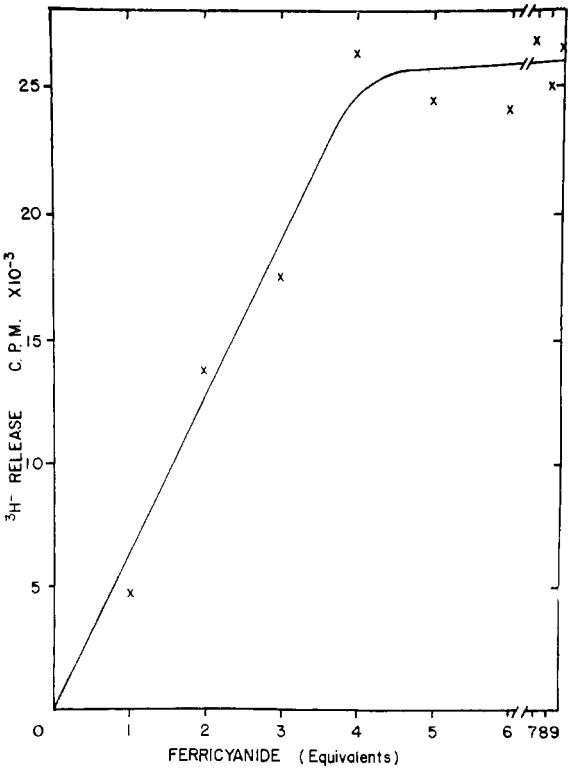

FIGURE 3: ^3H release in oxidation of DL-adrenaline-2,5,6-^3H by $K_3Fe(CN)_6$ in phosphate buffer (pH 7).

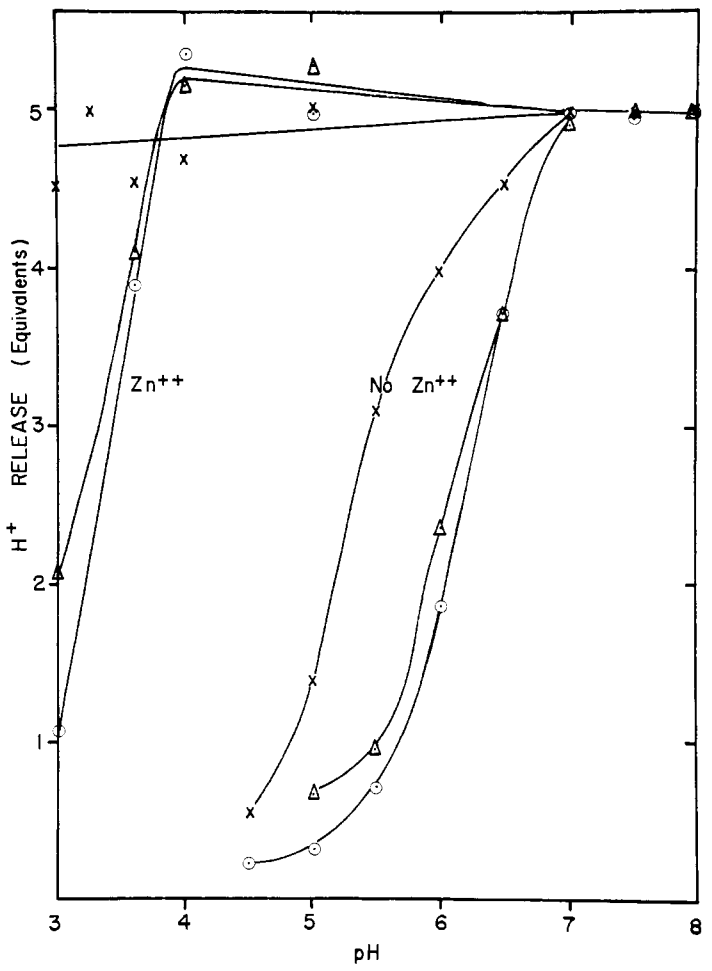

FIGURE 4: H^+ release as a function of pH in oxidation of adrenaline, noradranaline, or dopamine by $K_3Fe(CN)_6$ or by a mixture of $K_3Fe(CN)_6$ and $ZnSO_4$. (×) Adrenaline, (△) noradrenaline, and (⊙) dopamine.

phosphate buffer (pH 7, 0.1 M). The results with both methods were identical in respect to the end point but in the buffered runs the aminochromes were noted to slowly decompose as evidenced by a spectral shift from absorption in the visible range to an ultraviolet absorption peak (maximum 350 mμ); this is being examined further. As a further check, ^3H release from ^3H-ring-labeled adrenaline was measured; again 4 equiv of ferricyanide marked the end point (Figure 3). Following this, H^+ release from dopamine, noradrenaline, and adrenaline was studied as a function of pH from pH 3 to 8.

Effect of pH on Oxidation of Adrenaline, Noradrenaline, and Dopamine. In the absence of Zn^{2+}, commonly used to catalyze the reaction at low pH, adrenaline, noradrenaline, and dopamine were found to be incompletely oxidized below pH 7; no measurable oxidation occurred below pH 4.5 (Figure 4). In addition, the reactions were found to be sluggish in contrast to an almost instantaneous reaction at pH 7; the equilibration times decreased in the order: adrenaline > noradrenaline > dopamine. The linear relationship between H^+ release and ferricyanide added, demonstrated in the pH 7 titrations (Figures 1–3), prevailed only during addition of the first equivalent of ferricyanide. In the presence of Zn^{2+}, the reactions were found to proceed to a further extent at each pH (Figure 4), being complete for adrenaline, noradrenaline, and dopamine down to pH 4 and complete for adrenaline down to pH 3; however, in each case an excess of 1 equiv of ferricyanide ions was required and the linear relationship between H^+ release and ferricyanide added prevailed only during addition of the first equivalent of ferricyanide.

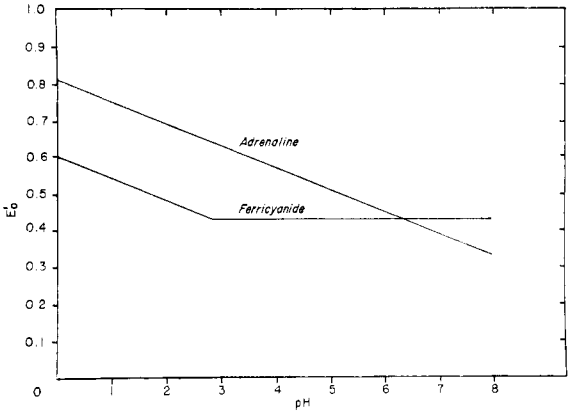

FIGURE 5: An approximate plot of potentials (volts at 50% reduction) *vs.* pH of adrenaline–adrenaline-*o*-quinone and ferricyanide–ferrocyanide redox systems (taken from data of Ball and Chen, 1933).

Discussion

Three lines of experimental evidence, *i.e.*, H^+ release, spectrophotometric, and ^3H release, indicated that the compounds studied convert into their respective aminochrome derivatives as a result of withdrawal of four electrons. The fact that the titrations of adrenalone and noradrenalone, both of which lack hydrogen atoms in the β positions of the side chain, were found to be identical with those of adrenaline and noradrenaline was indirect evidence for ruling out the possibility that any of the hydrogen ions released are derived from the β-carbon atoms of the side chains of the compounds studied; additional and more direct evidence for this comes from a study to be reported elsewhere in which adrenaline and noradrenaline labeled with ^3H in the β position of the side chain were found to undergo no loss of ^3H in a ferricyanide oxidation (W. W. Whisler and W. H. Harrison, unpublished data). From this, it can be concluded that three of the 5 H^+ equiv released represent, collectively, the extent of protonation of the three ionizable groups, which include the two phenolic groups and the one amino group, each of which has an affinity for protons at pH 7. When these groups are converted into their respective carbonyl and imino forms in the oxidation and cyclization process (Scheme I), the associated hydrogen ions are released since the groups formed would not be expected to exhibit proton affinity at pH 7. The remaining two proton equivalents are most probably

derived from carbon atom 6 of the ring and from the side-chain amino nitrogen since this is the site of the ring closure in the cyclization step. The aminochrome could form by either a two-step reaction involving an internal redox reaction of the Michael type to the leucochrome followed by its oxidation to the chrome derivative involving withdrawal of two electrons or by a one-step reaction, involving withdrawal of two electrons, in which a direct oxidative cyclization to the chrome takes place. Both mechanisms involve nucleophilic attack at carbon atom 6 by the side-chain nitrogen. In either mechanism, five protons would be released from one molecule of catecholamine oxidized simultaneously with the reduction of four molecules of potassium ferricyanide and this stoichiometry should prevail in acid media whether or not the reaction is completed.

The fact that the linear relationship found for H^+ release during addition of the 4 equiv of ferricyanide in the pH 7 oxidation (Figures 1–3) prevailed only during the addition of the first equivalent of ferricyanide in the oxidations below pH 7 reflects the effect of the H^+ concentration on the equilibrium state which is dependent upon the relative potentials of the pH-dependent redox systems, catecholamine–catecholamine-*o*-quinone and ferricyanide–ferrocyanide. To clearly show the pertinence of the latter factor to the present study the early data of Ball and Chen (1933) are presented in Figure 5. On consideration of Figure 5 and the Nernst equation, it is obvious why the reaction is incomplete below pH 7 and why it is essential that the ratio of ferricyanide to ferrocyanide be maintained high; it is also clear how a buildup of ferrocyanide ions would limit the extent to which the reaction can proceed.

A likely explanation of the effect of Zn^{2+} is that it promotes the reaction by complexing with the ferrocyanide ion, a property it is known to exhibit; in this way, Zn^{2+} could cause a shift in equilibrium in favor of catecholamine oxidation. Another important factor is that the cyclization reaction is irreversible; as Ball and Chen have pointed out, the reaction goes to completion in spite of an unfavorable equilibrium because the *o*-quinone, formed in exceedingly small quantities is rapidly removed by the irreversible cyclization step. The fact that adrenaline is oxidized completely at pH 3 in contrast to the incomplete reactions of noradrenaline and dopamine most probably is a result of the difference in the extent to which it is cyclized; in measurements of the cyclization rates of the catecholamines as a function of pH by an electrolytic method (Hawley *et al.*, 1967), it has been found that adrenaline is unique in this tendency toward cyclization at low pH. These low pH cyclizations serve as excellent examples of how nucleophilic reactions can occur even though the nucleophile, which is unprotonated nitrogen in this case, is present at very low concentrations which, for example, are infinitesimal at pH 3.

The fact that 5 H^+ equiv were released in the pH 7 oxidations of all compounds studied confirms the results of others who found compounds of the type studied to be fully protonated (structure Ia in Scheme I) at physiological pH, confirming previous titration studies (Lewis, 1954; Kappe and Armstrong, 1965) which differ from results of earlier studies (Tuckerman *et al.*, 1959). Also, it would seem that the results of this study are consistent with results of an earlier study (Chaix *et al.*, 1950) in which ferricyanide oxidation was followed by measuring CO_2 release resulting from H^+ release in oxidations performed in bicarbonate buffer. Although Chaix *et al.* found 5 H^+ equiv of CO_2 released they interpreted the results as indicating release of 4 H^+ equiv upon oxidation and 1 CO_2 equiv as a result of an unspecified decarboxylation; in light of the present results, it is clear that the fifth H^+ equivalent indicated the protonation of the amino group which is converted into the less basic imino group during the cyclization. The results of Chaix *et al.* are plotted in terms of H^+ equivalents in Figure 1 to show the remarkable close correspondence of the two methods.

Although numerous studies of others have indicated the relative acid stability of noradrenaline and dopamine in the presence of Zn^{2+} and ferricyanide ion in contrast to adrenaline which is readily oxidizable, the data of this study show conclusively that they nevertheless do undergo considerable oxidation. This was shown for noradrenaline in an earlier study in which a fluorescence spectrometric technique was employed (Harrison, 1963a,b). It can be concluded that all compounds studied are oxidized to varying extents and at varying rates to their cyclized chrome state in the range studied (pH 3–8). It can also be concluded that, in contrast to the catalyzed oxidation involving molecular oxygen, this reaction proceeds to the aminochrome stage with no side reactions; if this had not been the case, the results of the tracer, spectral, and H^+ measurements would not have been consistent.

The results indicate that measurements of the H^+ release on oxidation as a function of pH in regions of H^+ dissociation may be a valuable approach for the ascertainment of ionization constants of compounds of this type, which are unstable due to a tendency toward oxidation. The oxidation property, commonly a source of error in usual titration techniques, is utilized in this method. In the appropriate pH range the pK_a of each dissociable group would be reflected by an inflection point in plots of H^+ release *vs.* pH of oxidation. An investigation is underway to test this application by reassessing previous pK_a data by extending the study to the range, pH 8–12, the region of dissociation of the protonated groups. Noteworthy also are the kinetics of the systems involved; qualitative observations of the time required for equilibration of the different oxidations indicated that the amines are oxidized in the order: adrenaline > noradrenaline > dopamine (W. H. Harrison, unpublished data); this is in harmony with the order which Hawley *et al.* (1967) found in an electrochemical study for the rates of the rate-limiting cyclization step. It is striking that the relative rates of oxidation of dopamine and adrenaline by mushroom tyrosinase is the reverse of that observed for the chemical oxidation, clearly indicating the important role of the side chain of the substrate in respect to an interaction with a subsite on the active site of the enzyme as is emphasized in a recent publication (Harrison *et al.*, 1967), from this laboratory.

Acknowledgments

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