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TRANSIENT FREE RADICAL FORMS OF HORMONES: EPR SPECTRA FROM CATECHOLAMINES AND ADRENOCHROME*

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The initial mechanisms of action remain unknown for all hormones, despite the considerable effort that has been devoted to their study. It is not unlikely, however, that the chemical properties manifested *in vitro* differ little from at least some of the properties that characterize the initial steps of their actions *in vivo*.

In fact, the effects of certain hormones on enzyme reactions *in vitro* have led others to infer that hormonal free radicals were formed reversibly therein. This conclusion is consistent with electron paramagnetic resonance (EPR) evidence that short-lived radicals can be formed chemically from some structurally similar non-hormonal molecules: for example, transient aryloxy radicals from a number of substituted phenols.¹⁻³ Nonetheless, with one possible exception (discussed below), no direct experimental confirmation of free radical forms of hormones has been reported heretofore.

We have studied the initial redox reactions *in vitro* of a number of hormones and their analogues. There was evidence of anodic oxidation of several hormones in solution, but no EPR was detected when electrolysis was performed within the microwave cavity. However, EPR confirmation of hormonal free radicals was obtained by using continuous flow techniques.³⁻⁵ From this work we wish to report in this initial communication only sufficient EPR data to establish firmly the generalization that free radical forms of many hormones exist in nature—at least *in vitro*—and that in most instances the radicals are very short-lived. Data will be presented in this article concerning catecholamines and adrenochrome, and a second paper⁶ will treat other indole hormones, thyronines, estrogens, and insulin.

Usually the oxidation-reduction potentials of the reactions that were studied fell within the range of oxidative metabolism, and presumably the free radical forms are relevant to some of the physiological chemistry of these same hormones *in vivo*. However, evidence supporting these arguments is indirect and will not be compiled here. By themselves the present demonstrations are moot regarding biological significance.

Experimental.—An improved flow system was used to provide higher stationary concentrations of short-lived radicals within the detection volume and to minimize the duration of flow required to record EPR spectra. The details of this system have been published.^{3, 4}

Most spectra were scanned in about 3 sec, but some required 5–6 sec. Volume flow rates varied from 6 to 15 ml/sec, with reactants kept equimolar at concentrations of $8\text{--}10 \times 10^{-3} M$.

In the figures magnetic field increases from left to right. The uncertainty in *g*-value is approximately ± 0.001 .

Results and Discussion.—*Catecholamines:* Phenoxy free radicals of epinephrine and norepinephrine have been proposed to explain the kinetics of their oxidation by ceruloplasmin in the presence of oxygen, although no optical or EPR spectroscopic confirmation was obtained for the existence of such hypothetical reaction intermediates.^{7, 8} A study of the kinetics of epinephrine oxidation by atmospheric oxygen at different pH's also led to the inference that free radical pathways might be involved.⁹ These hypotheses are plausible from the chemical point of view, because EPR evidence of phenoxy radicals of catechol and some of its derivatives stabilized in alkaline media has been available for several years.¹⁰⁻¹²

Figure 1 depicts the EPR spectrum of the transient semiquinone cation from epinephrine, following oxidation by ceric ion. As might be anticipated, the spectral hyperfine structure (Fig. 1) resembled that known for nonhormonal catechols,¹¹ although there were some differences.

FIG. 1.—EPR spectrum of the cationic phenoxy radical from epinephrine. Reactants: L-epinephrine·HCl (in water); $\text{Ce}(\text{SO}_4)_2$ (in $N/5 \text{ H}_2\text{SO}_4$). $g = 2.004$ for the catecholamine derivatives and analogues shown here (Figs. 1-7).

FIG. 2.—Norepinephrine·HCl (water); $\text{Ce}(\text{SO}_4)_2$ ($N/5 \text{ H}_2\text{SO}_4$).

Norepinephrine oxidized in the same way and recorded with identical instrumental settings gave rise to an EPR spectrum (Fig. 2) that was virtually congruent with that of epinephrine (Fig. 1). This is consistent with their sharing a common catechol moiety, which is also known to be essential for characteristic endocrinological action among catecholamine hormone analogues.¹³ Nonetheless, the correlations of spectra with molecular structure are complicated, because dihydroxyphenylalanine (dopa) and dopamine gave somewhat different EPR spectra. For example, Figure 3 depicts the EPR spectrum of the cationic radical from dopa following ceric oxidation.

In general, the free radicals were highly unstable in acid: for instance, reducing the dead time for flow from the locus of reaction to the midregion of the detection volume from about 12 to 5 msec increased the EPR signal amplitude of epinephrine oxidized by cerium by roughly 45 per cent.

If free radical forms of epinephrine are concerned with any of its primary actions upon effectors, its rapid-acting sympathomimetic analogue, phenylephrine, also would be expected to yield free radical products under comparable circumstances. Ceric oxidation did indeed yield a short-lived free radical ion from phenylephrine.

FIG. 3.—Dihydroxyphenylalanine (water); $\text{Ce}(\text{SO}_4)_2$ ($N/5 \text{ H}_2\text{SO}_4$). See ref. 14.

FIG. 4.—Phenylephrine·HCl (water); $\text{Ce}(\text{SO}_4)_2$ ($N/5 \text{ H}_2\text{SO}_4$).

FIG. 5.—L-epinephrine·HCl (water); $K_3Fe(CN)_6$ ($N/10$ NaOH).

FIG. 6.—Dihydroxyphenylalanine (water); $K_3Fe(CN)_6$ ($N/10$ NaOH). See ref. 18.

The EPR spectrum of the radical (Fig. 4) closely resembled tyrosine,³ a chemically related monohydric phenol.

Semiquinone free radicals formed by electron donation or dehydrogenation from a hydroxyl group usually are most stable at alkaline pH's,^{15, 16} and EPR has been recorded without fast reaction techniques from many semiquinone radicals that could be stabilized in alkaline media.^{10-12, 17} In our hands the radical anions of substituted dihydric phenol hormones formed in alkali persisted for less than a tenth of a second, even in $N/10$ NaOH. Nevertheless, they were appreciably more stable than the corresponding cationic forms in most instances.

Figure 5 presents an example of EPR from the epinephrine semiquinone anion in alkali, as oxidized by permanganate or ferricyanide. It is typical of the spectra of radical ions of catecholamine hormones and their analogues in the way in which it differs from the respective cationic radical in acid (Fig. 1); namely, the hyperfine pattern is appreciably altered, the greater stability of the radical is reflected by a marked increase in the recorded signal-to-noise ratio, and the over-all width of the resonance signal is less. A similar comparison may be made between the phenoxy radicals of dopa in acid (Fig. 3) and in alkali (Fig. 6).^{14, 18}

Semiquinones are notoriously less stable at neutrality than in acid or alkali.^{15, 16} Nevertheless, it was often possible to elicit at least poorly resolved EPR spectra

FIG. 7.—L-epinephrine·HCl (water); $KMnO_4$ ($M/10$ phosphate buffer). Final pH of product solution = 7.0.

FIG. 8.—Adrenochrome ($M/16$ HCl·KCl buffer); $TiCl_3$ ($M/16$ HCl·KCl buffer). $g = 2.003$ for adrenochrome (Figs. 8-11).

FIG. 9.—Adrenochrome ($M/16$ $\text{HCl}\cdot\text{KCl}$ buffer); TiCl_3 ($M/16$ $\text{HCl}\cdot\text{KCl}$ buffer). Final pH = 2.0. Stopped-flow curve.

FIG. 10.—Adrenochrome ($N/10$ NaOH); $(\text{NH}_4)_2\text{IrCl}_6$ ($M/10$ phosphate buffer). Final pH = 7.0.

from hormonal free radicals at pH ~ 7 . Figure 7 is an example of the formation of the epinephrine free radical at neutrality. The 1:2:1 hyperfine triplet of Figure 7 suggests a dominant unpaired electron density delocalized over two equivalent aromatic hydrogen nuclei.

Adrenochrome: Adrenochrome is an indolized oxidation product derived from epinephrine. Adrenochrome itself is hormonally active, and in some biological test systems it is more potent than epinephrine.¹⁹⁻²¹ Should epinephrine free radicals be involved in any of the actions mimicked by adrenochrome, then semiquinones of the latter also would be anticipated. In fact, Walaas and her colleagues hypothesized a semiquinone imine structure of adrenochrome as an intermediate in the over-all oxidation of epinephrine catalyzed by ceruloplasmin.⁸ In his correlation of data from catecholamine hormone oxidation *in vitro* in various enzymic and nonenzymic reactions, Harrison also concluded that semiquinones of adrenochrome could form by univalent oxidation or reduction steps.²²

In our experiments adrenochrome was made from L-epinephrine oxidized by Ag_2O , according to slight modifications of published syntheses,^{23, 24} and optical measurements on the product were checked with known values.²³ EPR study of the free radicals produced from adrenochrome by both oxidation and reduction at different pH's revealed a complex pattern, occasionally involving multiple free radical species in sequence. One such example is from the short-lived free radical produced by reduction in acid (Fig. 8). The transient nature of the radical was attested by stopped-flow analyses (Fig. 9).

Oxidation generated free radicals from adrenochrome. In one reaction carried out at neutral pH, weak EPR signals were recorded with high velocity flow in the regenerative flow apparatus, although only a poorly resolved spectrum without evident fine structure was obtained (Fig. 10).

FIG. 11.—Adrenochrome ($N/10$ NaCl); $\text{Na}_2\text{S}_2\text{O}_4$ ($N/10$ NaOH).

In alkaline media the reactions were extremely complex. Rapid reduction by dithionite produced a radical with a complicated EPR pattern (Fig. 11). However, despite its prompt formation, the radical persisted for several minutes under anaerobic conditions in *N*/10 NaOH. When deoxygenated solutions of adrenochrome in saline or KCl were mixed 1:1 with oxygen-free *N*/10 NaOH, a broad EPR singlet appeared. The signal amplitude increased as the reactant flow rates decreased. Upon cessation of flow, the spectrum converted over several seconds to a pattern very similar to Figure 11 and then persisted for a few minutes. Presumably a spontaneous disproportionation of adrenochrome had occurred in alkali, in conformity with Harrison's observation that the oxidation products of epinephrine were different in alkaline solutions.²²

The existence of a relatively stable free radical produced from epinephrine by air oxidation in alkaline media was reported by Blois some years ago.¹⁵ However, the EPR spectrum subsequently described¹² differs appreciably from the transient free radical formed upon alkaline oxidation of epinephrine in this work (Fig. 5). Rather, it resembles quite closely a spectrum like that observed from the spontaneous reduction of adrenochrome in alkali (Fig. 11). With the high velocity flow system, as used under regenerative conditions in these experiments, the primary free radical products are removed too rapidly for the occurrence of secondary reactions.^{2, 3} Therefore, Figure 5 should reflect the semiquinone anion from direct oxidation of epinephrine. Since autoxidation of epinephrine to adrenochrome is known to occur rapidly in oxygenated alkaline solutions and the present data indicate that at a high pH adrenochrome can disproportionate slowly to yield relatively long-lived radical products with complex EPR spectra (Fig. 11), it is likely that the signal attributed to an epinephrine semiquinone by Blois *et al.*^{12, 15} actually denoted a product of adrenochrome.

Summary.—A high-velocity continuous flow apparatus permitted EPR spectroscopy of the labile free radicals formed in solution by inorganic redox agents from a number of hormone molecules and their congeners. In this report the existence of hormonal free radicals was confirmed for catecholamine hormones (epinephrine, norepinephrine) and adrenochrome. A concluding part of this communication will present similar data regarding iodothyronines, indole hormones (serotonin, indole acetic, and butyric acids), estrogens (steroids and nonsteroidal synthetics), and one protein hormone (insulin).

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RNA CODEWORDS AND PROTEIN SYNTHESIS, V.
EFFECT OF STREPTOMYCIN ON THE FORMATION OF
RIBOSOME-sRNA COMPLEXES

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Flaks *et al.*,¹ and Speyer *et al.*² have shown that streptomycin (SM) inhibits poly U-directed C¹⁴-phenylalanine incorporation into protein in *E. coli* extracts. In accord with the hypothesis of Spotts and Stanier,³ SM-sensitive sites were found by Cox *et al.*⁴ and Davies⁵ to reside on 30 S ribosomes. Davies, Gilbert, and Gorini⁶ recently demonstrated that SM alters the specificity of polynucleotide-dependent amino acid incorporation into protein in extracts prepared from SM-sensitive *E. coli*. In addition, it seems probable that the binding of SM to resistant ribosomes also increases some error related to codeword recognition, for Gorini and Kataja⁷ have reported SM-activated suppression in SM-resistant *E. coli* growing in the presence of the antibiotic.

In this report, the effect of SM upon the recognition of RNA codons prior to peptide bond synthesis is studied by directing the binding of C¹⁴-AA-sRNA to ribosomes with poly- and trinucleotide templates. The results indicate that SM affects the recognition of poly- and trinucleotides.