

Transient free radicals from salicylate

(Received 5 October 1964; accepted 18 November 1964)

A ROLE for free radical intermediates in the action of phenothiazine drugs¹ and imipramine² has been proposed. Recently an electron paramagnetic resonance (EPR) spectrum of *o*-hydroxybenzoic acid was cited in a report of the chemistry of transient aryloxy radicals produced by ceric oxidation of substituted monohydric phenols in acid.³ Nevertheless, the possible significance of free radical mechanisms in the pharmacological chemistry of salicylates remains conjectural. In the absence of direct evidence of free radicals *in vivo*, further study of salicylate radicals *in vitro* was undertaken.

Salicylate oxidation was ascertained at redox potentials below those of the O_2/H_2O couple⁴ and thus within the range of biological interest: aqueous sodium salicylate or salicylaldehyde admixed with the univalent oxidant, ceric sulfate (in 0.2 N H_2SO_4), instantaneously reacted to give olive or tan products respectively. Interactions with $KMnO_4$ or $(NH_4)_2IrCl_6$ in neutral phosphate buffer produced gradual changes of color over several minutes. However, in 0.1 N NaOH, sodium salicylate immediately formed a green product with stoichiometric quantities of $KMnO_4$. This implied a homolytic oxidation of salicylate with concomitant univalent reduction of purple permanganate to green manganate, which is transiently stable in alkaline solution.

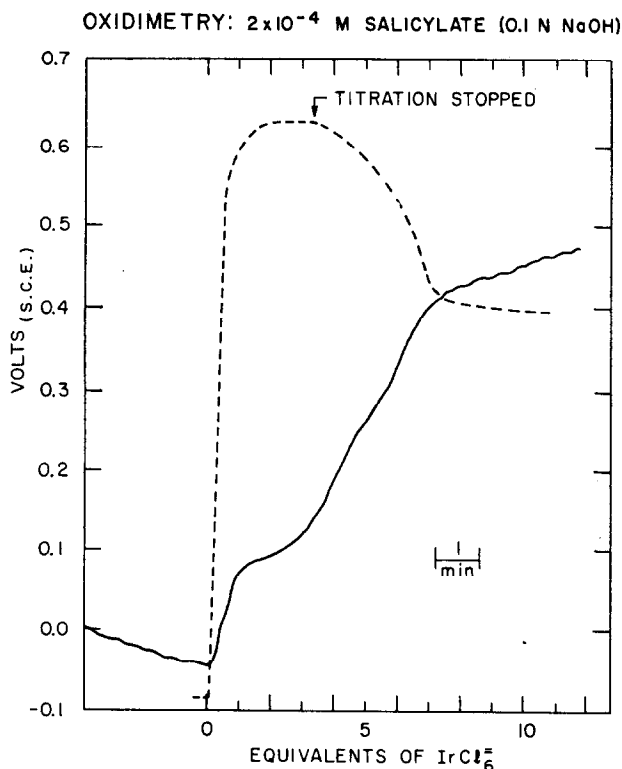


FIG. 1. Oxidimetric titration of salicylate in alkali by $IrCl_6^{3-}$. Potentials are vs. a saturated calomel reference electrode. Solid line: 4 ml of 0.2 mM sodium salicylate in 0.1 N NaOH were titrated continuously with ~ 7 mM $IrCl_6^{3-}$ (in 0.1 mM HCl). Dashed line: reference curve, with salicylate omitted, showing the effect of slow alkaline hydrolysis of Ir^{4+} .

Polarography (Sargent model XV polarograph) failed to confirm univalent oxidation of salicylate. No clear decomposition current was obtained with a rotating platinum electrode from pH 1 to 0.1 N NaOH, although in alkali the wave of apparent oxygen evolution occurred at a potential about 0.1 V lower than in the reference solution. In 0.2 N H_2SO_4 there was an equivocal suggestion of a very low anodic polarographic wave from approximately 0.2 V (vs. saturated calomel) to the voltage of oxygen formation. Presumably, polarographic analysis was obfuscated by the formation of irreversible final products and/or by contamination of the electrodes with adsorbed intermediates.

Continuous or intermittent potentiometric titrations of salicylate were carried out with equipment and procedures described previously.² Oxidimetry of salicylate titrated with alkaline KMnO_4 was incomplete, owing to insufficient separation of the redox potentials. With $(\text{NH}_4)_2\text{IrCl}_6$ in alkaline solutions, complications arose from gradual hydrolysis of the oxidant.⁵ Nonetheless, a rapid titration of salicylate in 0.1 N NaOH suggested one oxidation wave centered at about 0.1 V with slight indication of another wave at about 0.26 V; each wave represented roughly two or three oxidizing equivalents (Fig. 1).

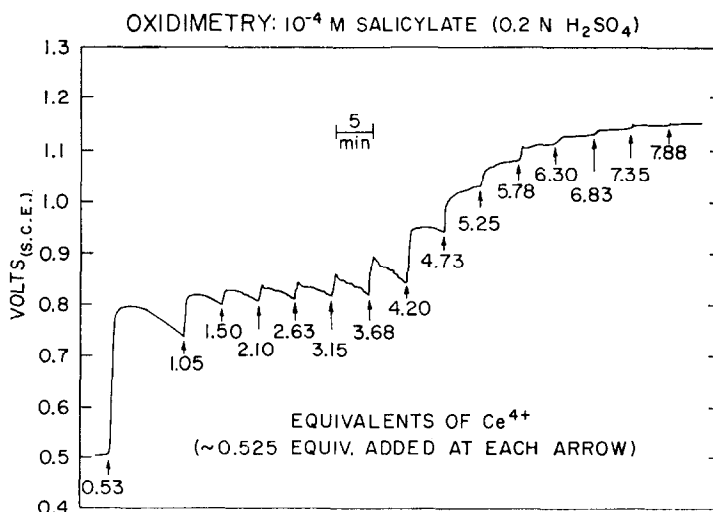


FIG. 2. Oxidimetric titration of salicylate in acid by Ce^{4+} ; 4 ml of 0.1 mM sodium salicylate in 0.2 N H_2SO_4 was titrated intermittently (see Ref. 2) with 10mM $\text{Ce}(\text{SO}_4)_2$ (in 0.2 N H_2SO_4). Each increment of Ce^{4+} was added in about 20 sec, and the drift of the solution potential between increments is seen to terminate after titration with approximately four equivalents of oxidant.

In neutral phosphate buffer salicylate reacted with iridium too slowly to yield a potentiometric titration curve. Despite this fact, clear evidence of a reaction was given by gradual drops in the potentials of salicylate-chloriridate mixtures under circumstances wherein control solutions of chloriridate were stable.

However, in 0.2 N H_2SO_4 a successful potentiometric titration of 0.1 mM sodium salicylate with 20 mM $\text{Ce}(\text{SO}_4)_2$ was achieved (Fig. 2). The reaction was slowly irreversible, with some downward drift of the solution potential following each increment of cerium. Oxidation of salicylate commenced at about 0.7–0.8 V, with a clear end point at approximately four electron equivalents (Fig. 2).

Free radical forms of salicylate would be expected to have very short lifetimes in solution, comparable to those observed for tyrosine or other substituted monohydric phenols.^{3, 6} Therefore the rapid reactions of salicylate or salicylaldehyde with ceric ion and of the former with alkaline permanganate were examined by EPR spectroscopy, by means of an improved continuous-flow apparatus described elsewhere.⁶ A Varian V-4502 spectrometer (100 kc/sec field modulation and 9-inch electromagnet controlled by Fieldial) was used to obtain EPR spectra of free radical reaction intermediates (Figs. 3–5).

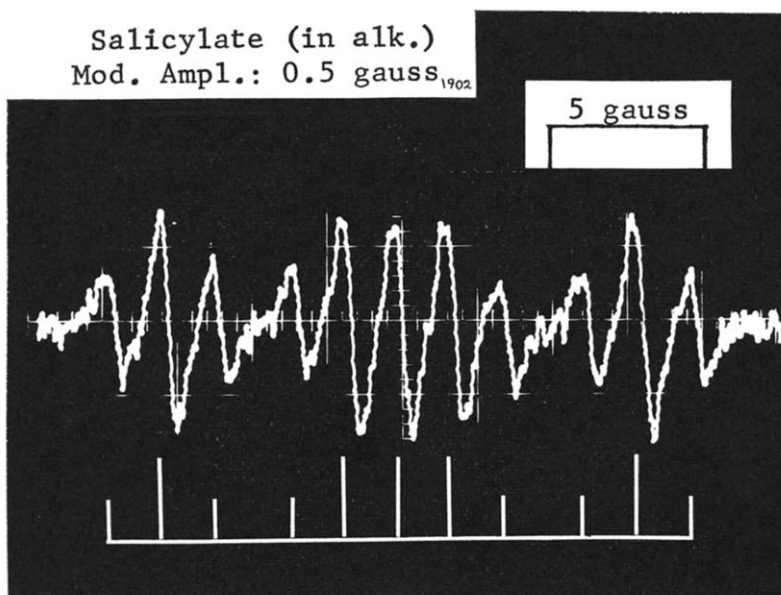


FIG. 3. EPR spectrum. Reactants: 10 mM Na-salicylate in H_2O and 10 mM KMnO_4 in 0.1 N NaOH, mixing in 1 : 1 ratio at room temperature. Spectrum scanned in ~ 15 sec with flow rate of 2.9 ml/sec. Magnetic field increases from left to right, with center of X-axis = g of 2.005 (at 9,503 mc/sec). Microwave power ~ 290 mW.

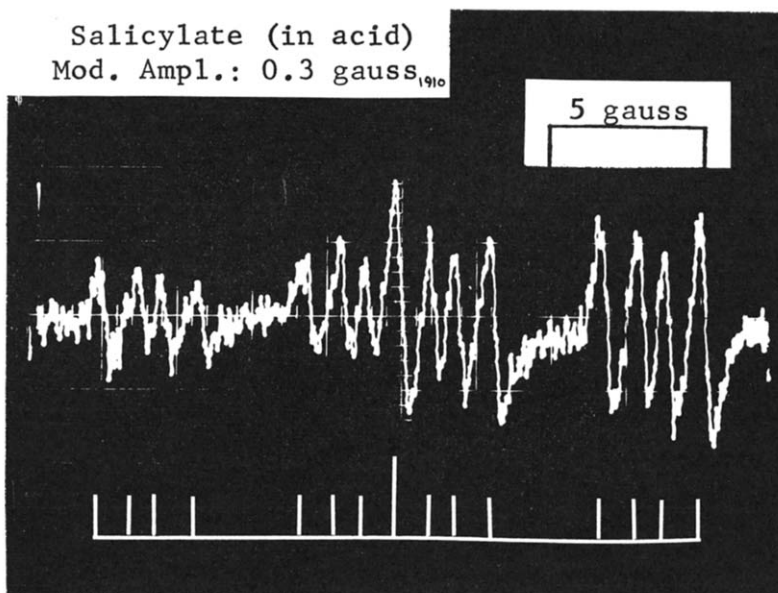


FIG. 4. EPR spectrum. Reactants: 20 mM Na-salicylate in H_2O and 20 mM $\text{Ce}(\text{SO}_4)_2$ in 0.33 N H_2SO_4 . Flow rate = 14.2 ml/sec. Microwave power ~ 480 mW. Other conditions as for FIG. 3.

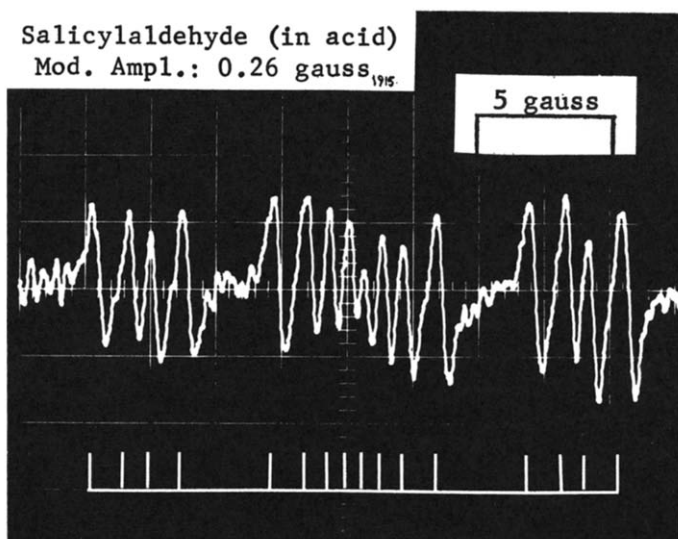
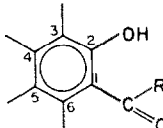


FIG. 5. EPR spectrum. Reactants: 20 mM salicylaldehyde in H_2O and 20 mM $\text{Ce}(\text{SO}_4)_2$. Spectrum scanned in ~ 6 sec with flow rate of 5.6 ml/sec. Microwave power ~ 480 mW. Other conditions as for Fig. 3.

As anticipated, the radicals were ephemeral: with unchanged instrumental amplification, a signal amplitude 60% greater than that of Fig. 3 was recorded by increasing the flow rate from 2.9 to 14.0 ml/sec. In the same manner, tripling the flow rate increased by one half the signal amplitude obtained from salicylate and cerium (Fig. 4). Stop-flow analysis of the same reaction revealed decay of EPR more rapid than the response time of the stop-flow system. Furthermore, the slow reaction of salicylate with KMnO_4 at neutral pH and equally slow interactions of acetylsalicylic acid with acidic $\text{Ce}(\text{SO}_4)_2$ or alkaline KMnO_4 failed to give EPR, even with high-velocity regenerative flow.⁶ This was not surprising because, even if labile free radical intermediates had been formed, the slow kinetics of the generating reactions would yield undetectable concentrations.

TABLE I. COUPLING CONSTANTS*

	$a_{\text{H}'}$	$a_{\text{H}''}$	$a_{\text{H}'''}$	$a_{\text{H}''''}$
Salicylate in alkali (Fig. 3)	10.2	6.5	1.9	1.9
Salicylate in acid (Fig. 4)	10.7	7.2	2.2	1.3
Salicylaldehyde in acid (Fig. 5)	10.3	7.3	2.3	1.4

* Absolute values, in gauss.

Despite some monotonic increase⁷ in the envelope of signal amplitude (Figs. 4, 5), coupling constants with hydrogen atoms of the aromatic nucleus (Table I) could be determined from the hyperfine line spacings of the EPR spectra (Figs. 3–5). The semiempirical spectra computed from the values of Table I are illustrated on the respective figures, where the correspondence with the experimental first-derivative spectra is seen to be good.

Although no molecular orbital calculations have been made, a presumptive assignment of the coupling constants of Table 1 may be made on the basis of the directive influence of substituent groups⁸ and on the work of Stone and Waters with substituted phenols.³ This leads to the tentative identification of $H' = H5$, $H'' = H3$, $H''' = H6$ (or $H4$), and $H'''' = H4$ (or $H6$).

By themselves the present data do not support a biological role for the free radical forms of salicylates. Nevertheless, it is germane that many other substituted phenols can be oxidized in a similar way.^{3, 6} Hence one may conclude that pharmacological behaviour cannot be determined *solely* by the presence of free radical intermediates, in any case. Substances that can be oxidized to form free radicals at similar redox potentials could have their biological effects modified by other molecular properties, especially those affecting penetration and distribution within cells.

Medical Research Center,
Brookhaven National Laboratory,
Upton, N. Y., U.S.A.

DONALD C. BORG

REFERENCES

1. D. C. BORG and G. C. COTZIAS, *Proc. nat. Acad. Sci. (Wash.)* **48**, 643 (1962).
2. D. C. BORG, *Biochem. Pharmacol.* **14**, 115 (1965).
3. T. J. STONE and W. A. WATERS, *J. chem. Soc.* 213, (1964).
4. P. GEORGE and J. S. GRIFFITH, in *The Enzymes*, P. D. BOYER, H. LARDY and K. MYRBÄCK, Eds., 2nd ed., vol 1, p. 380, Academic Press, New York (1959).
5. R. R. FERGUSON, *J. Amer. chem. Soc.* **78**, 741 (1956).
6. D. C. BORG, *Nature (Lond.)* **201**, 1087 (1964).
7. H. M. MCCONNELL, *J. chem. Phys.* **25**, 709 (1956).
8. L. F. FIESER and M. FIESER, *Organic Chemistry*, p. 556. Heath, Boston (1944).

Biochemical Pharmacology, 1965, Vol. 14, pp. 631-633. Pergamon Press Ltd., Printed in Great Britain.

The effect of various psychotropic drugs on the activity of tyrosinase

(Received 18 August 1964; accepted 20 November 1964)

ONE of the unusual side effects associated with the therapeutic use of the phenothiazine tranquilizers is the production of a Parkinsonian syndrome.¹⁻⁵ Little, however, is understood concerning the biochemical mechanism by which the phenothiazines produce the disturbance. One of the most striking biochemical lesions seen with the natural Parkinson's disease is the depigmentation of the substantia nigra. Marsden⁶ has demonstrated that the pigment of the substantia nigra probably arises from the action of a polyphenolase, most likely tyrosinase. It thus appeared that one might gain insight into a possible mechanism whereby the phenothiazines, such as chlorpromazine, produce this syndrome by studying the effects of these drugs on tyrosinase activity. Since the ability to produce the neurological disturbance is related to the psychotropic activity or potency of the compounds,^{7, 8} it appeared of interest to examine, also, the effects of other psychotropic drugs, such as the monoamine oxidase (MAO) inhibitors.

METHOD

Tyrosinase activity was measured spectrophotometrically by a Beckman DK-2 spectrophotometer. The reaction was followed by the change in optical density at 310 m μ . Mushroom tyrosinase (Sigma pfs grade) served as the enzyme. The substrates, enzyme, and all additions were put into solution with 0.1 M Na₂HPO₄-NaH₂PO₄ buffer, pH 6.8. The working solution of tyrosinase contained 0.1 mg of the enzyme/ml and 0.1 ml was used in the assay. The final concentration of the substrates ranged from 0.25×10^{-3} M to 1.0×10^{-3} M. The Michaelis constants (K_m) for 3,4-dihydroxyphenylalanine (dopa), 3,4-dihydroxyphenethylamine (dopamine), norepinephrine, and epinephrine were determined from Lineweaver-Burk double reciprocal plots.