Free Radicals in Alkaloidal Color Identification Tests

By DAVID W. SCHIESER*

A number of color tests for alkaloids, some appearing in the U.S.P. and N.F., have been studied by electron paramagnetic resonance (e.p.r.) spectroscopy with the ultimate aim of ascertaining the chemical species responsible for the colors. In the case of the Marquis test for opium alkaloids, in which an intense purple is produced at once with morphine and quickly changes to deep blue-violet, it is shown that a different free-radical spectrum appears concurrently with each of these colors. Data are presented on the e.p.r. spectra of radicals thus formed from various alkaloids and several synthetic compounds. These conditions also produced free radicals from phenol and benzene; therefore, it is suggested that the site of radical formation in the alkaloids studied is the aromatic group. No proposal for a chemical mechanism is made, but it is suggested that a common mechanism is involved when aromatic compounds are treated with the Marquis reagent. Free radicals have also been observed in other color tests for alkaloids, such as the Mecke test for morphine. Like the Marquis test, the latter also uses concentrated sulfuric acid as solvent.

AN IDENTIFICATION test for morphine sulfate appearing in the U.S.P. XVI (1) involved treatment of morphine sulfate with a reagent consisting of a dilute solution of formaldehyde in concentrated sulfuric acid. An intense purple is produced at once which quickly changes to deep blue-violet. The reagent is sometimes referred to as the Marquis reagent for opium alkaloids (2–4). The test is described in other references (5) dealing with the identification of morphine. Conversely, the application of this color reaction with opium alkaloids is used as a very sensitive test for formaldehyde (6).

The test is not entirely specific for morphine or modified morphine type alkaloids. Colors are obtained with many aromatic compounds. For example, phenol and benzene give a qualitative red to brick-red color with this reagent. The test is referred to by Feigl (7) as the "Le Rosen Test for Aromatic Compounds," where the proposed reaction for the test involves the condensation of 1 mole of formaldehyde with 2 moles of the aromatic compound. Following the condensation step, oxidation by sulfuric acid occurs to produce unidentified colored quinoidal products.

Previous work in this laboratory has shown that free radicals can be stabilized in concentrated solutions of sulfuric acid (8). Quinoidal-type free radicals are known to occur in alkaline solution (9) and have also been reported to occur in acid solution (10). Hence, it was decided to examine several color tests by e.p.r. spectroscopy to determine if the colors were due to free radicals.

This work reports free radicals to be present in colored mixtures produced by the Marquis test as well as examples of free radicals occurring in colored mixtures produced by other tests in which sulfuric acid is the reaction solvent. In most cases the observed e.p.r. spectra exhibited a time dependency and indicated mixtures of free radicals.

EXPERIMENTAL

The e.p.r. spectrometer used in this study was previously described (11). This was modified by addition of a Varian 100-kc. field modulation and control unit, a Varian multipurpose cavity, and a flat sample cell (0.04 ml.). Good reviews of the technique and theory of e.p.r. spectroscopy are available (12).

The Marquis reagent was prepared by adding 1 ml. of 40% aqueous formaldehyde (Baker analyzed reagent) to 25 ml. of concentrated sulfuric acid (Baker and Adamson reagent). A dilute Marquis reagent was prepared using one-tenth of the above amount of formaldehyde in the same quantity of concentrated sulfuric acid. The Marquis reagents were freshly prepared every 2 or 3 days during the course of the e.p.r. measurements.

The Mecke reagent was prepared by adding 0.1 Gm. of selenous acid (Fischer analyzed reagent) to 20 ml. of concentrated sulfuric acid.

The Froehde reagent was prepared by adding 0.1 Gm. of sodium molybdate (Baker and Adamson reagent) to 100 ml. of concentrated sulfuric acid.

The Erdmann reagent was prepared by adding 10 drops of concentrated nitric acid (Baker and Adamson reagent) to 20 ml. of concentrated sulfuric acid. This reagent was prepared immediately before use.

The perhydrol reagent was prepared by adding 1 ml. of 30% hydrogen peroxide (Baker analyzed reagent) to 20 ml. of concentrated sulfuric acid. This reagent was prepared immediately before use.

Three general methods were used for the preparation of solutions for e.p.r. spectral determinations. (*a*) Approximately 1 ml. of dilute Marquis reagent or other color reagent was added to 0.01 mmole of

Received February 27, 1963, from the Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco.

Accepted for publication November 27, 1963.

Presented to Section NP, American Association for the Advancement of Science, Philadelphia meeting, December 1962.

The author gratefully acknowledges consultations with Dr. L. Dallas Tuck and Dr. J. F. Oneto in the preparation of this manuscript.

* Present address: State of California Department of Public Health, Bureau of Food and Drug Inspections, Berkeley.

TABLE I.—RESULTS FROM E.P.R. SPECTRA OF COLORED MIXTURES RESULTING FROM TREATMENT BY MARQUIS REAGENT OF ALKALOIDS AND SYNTHETIC COMPOUNDS

Parent Compd.	Final Concn., *M*	Time After Mixing and Color	Lines, No.	Splitting in Gauss	*g* Value
Morphine	0.01	10 min. red-violet	7	4.5	2.00305
Morphine	0.01	130 min. blue	4	9.0	2.00345
Morphine	0.005	Mixture of solns. violet to red-brown	37+	Varied	2.00345
Codeine	0.01	10 min. red-violet	7	4.6	2.00301
Codeine	0.01	48 hr. red-brown	3	...	2.0031
Codeine	0.005	Mixture of solns. blue-violet to reddish brown	3	6.2	2.00261
Pseudomorphine[a]	0.01	Brick-red or burgundy	Unresolved	...	2.0030
Apomorphine	0.01	5 min. green to blue	More than 4	...	...
Apomorphine	0.01	20 min. blue-green	4	9.9	2.0032
Apomorphine	0.005	Mixture of solns. blue-green	4	10.6	2.0030
Papaverine	0.02	Red-violet	Unresolved	...	2.0030
Meperidine	0.005	Mixture of solns. red or brick-red	Unresolved	...	...
Benzene	0.5	Brick-red	Unresolved	...	...
Salicylic acid	0.5	Red	Unresolved	...	...
Phenol	0.3	Brick-red	Unresolved	...	...
2 6-Dimethyl phenol	0.1	2 hr. deep brick-red	15 Doublets	1.92, 1.36, 0.3	2.00267
2,6-Dimethyl phenol	0.1	24 hr. deep brick-red	15	1.32	2.00266
2,6-Dichloro phenol	0.1	Red-violet	8 or 10	7.5	...

[a] This sample was of questionable purity.

solid alkaloid or synthetic compound. With respect to the dilute Marquis reagent, this represents a five-fold molar excess of formaldehyde. (*b*) Solutions of morphine sulfate U.S.P., codeine sulfate N.F., apomorphine hydrochloride, U.S.P., and 2,6-dimethylphenol (Eastman White Label) were prepared in concentrated sulfuric acid at 0.01 *M* concentrations. The solutions did not exhibit color formation even when allowed to stand for 1 week at room temperature. In an attempt to avoid complications due to the presence of a solid, the solutions were mixed with an equal volume of the dilute Marquis reagent or with a few drops of the previously mentioned color reagents immediately before the e.p.r. spectral determinations. (*c*) In a few instances radicals were not detectable at the above concentrations. In these cases the problem was overcome by either increasing the concentration of the solute or color reagent or both.

As soon as practical after the colored solutions were prepared e.p.r. spectra were obtained. Five to 10 minutes were usually required to measure the first spectrum. The spectral measurements were repeated at time intervals for several hours in those instances where a change in spectrum was apparent within 1 hour.

The *g* values[1] and hyperfine splittings of the spectra were obtained by simultaneously observing the spectrum of a 0.01 *M* solution of peroxylamine disulfonate (Aldrich Chemical Co.) in concentrated ammonia in a capillary fastened to the outside of the sample cell. The e.p.r. spectrum of a solution of peroxylamine disulfonate has been shown (13) to consist of three sharp peaks split by 13 gauss and with a *g* value of 2.00569.

[1] The *g* value is a property of a free radical relating the center of its e.p.r. spectrum to the magnetic field and the spectrometer frequency. See Blois, M. S., Jr., Brown, H. W., and Maling, J. E., *Arch. Sci. (Geneva)*, **13**, 243(1960); and "Free Radicals in Biological Systems," Proc. Symposium, Stanford, Calif., (1960), pp. 117–131. The true *g* value cannot be determined in cases where there are mixtures of free radicals present, but it is nevertheless a convenient notation for the location of the center of the spectrum and has value in comparing spectra in this report.

RESULTS AND DISCUSSION

The e.p.r. spectral data in Table I indicate that the action of the Marquis reagent produces free radicals with a variety of compounds. At room temperature no e.p.r. signals were observed in the Marquis reagent or in the sulfuric acid solutions of the compounds studied. The e.p.r. spectra of several of the colored solutions were observed to change with time (Table I). In each of several instances, a mixture of free radicals was indicated by the asymmetry of the e.p.r. spectrum. Normally, a single free radical shows an e.p.r. spectrum which is approximately symmetrical about a central point (12). In addition to the information given in Table I, measurements of concentration of free radicals were determined for a number of the spectra by double integration and comparison with a standard free radical spectrum (1,1-diphenylpicrylhydrazyl). The results from these calculations showed some variation, but in all cases it was within the range of 0.1 to 10% of the molar concentration of the parent compound.

Figure 1-A shows the e.p.r. spectrum of a solution prepared by adding dilute Marquis reagent to solid morphine sulfate. The same solution after a lapse of 2 hours yielded the spectrum in Fig. 1-B. When a solution of morphine sulfate prepared by method (*b*) was treated with the dilute Marquis reagent, a different spectrum was obtained, as shown in Fig. 2. The lack of symmetry of the spectrum probably indicates the presence of two or more radicals. While differing in appearance from Fig. 1-B, the spectrum in Fig. 2 has the same *g* value and could be a better resolved spectrum of the same free radicals. On the other hand, the dissimilarity in color of the two morphine solutions (Table I) would indicate the possibility of different free radicals.

The e.p.r. spectrum obtained 10 minutes after addition of dilute Marquis reagent to solid codeine sulfate is apparently identical with that obtained from morphine under the same conditions. After a lapse of time the two compounds no longer have

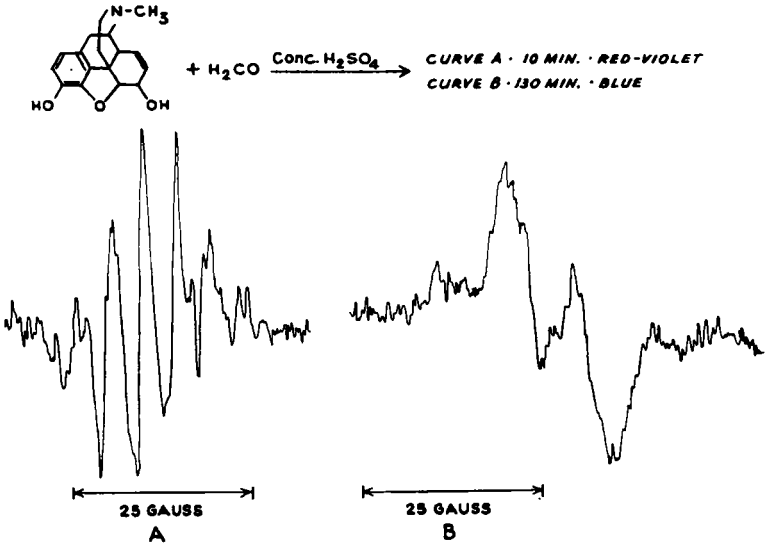

Fig. 1.—E.p.r. spectra of a solution prepared by the action of dilute Marquis reagent on *solid* morphine sulfate.

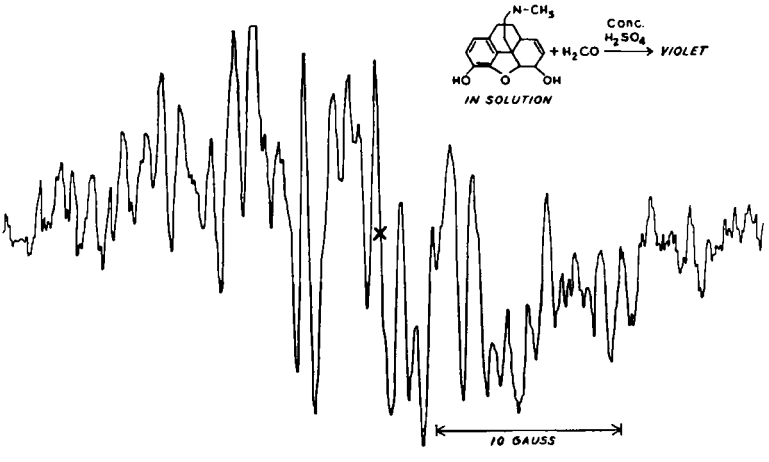

Fig. 2.—E.p.r. spectrum of a solution prepared by the action of dilute Marquis reagent on a sulfuric acid *solution* of morphine sulfate. The center of the spectrum is marked (×).

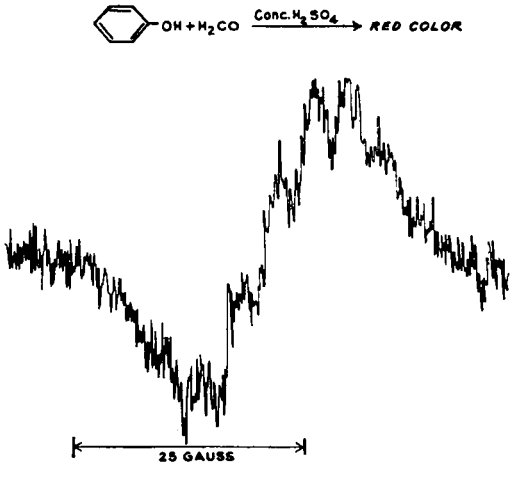

Fig. 3.—E.p.r. spectrum of a mixture prepared by the action of Marquis reagent on phenol. The sample contained a precipitate.

identical spectra, showing a difference in g values and in the number of hyperfine lines (Table I). The spectrum of the solution prepared from codeine by method (*b*) is similar to the secondary codeine spectrum from the solid.

The action of dilute Marquis reagent on apomorphine also produces a time-varying e.p.r. spectrum, but the initial spectrum was so transient that a complete graph was not obtained. The initial multilined spectrum changed rapidly to a broadened four-line spectrum. A solution of apomorphine in concentrated sulfuric acid treated with dilute Marquis reagent yields an e.p.r. spectrum which is comparable to the broad four-line spectrum having the same g value as that obtained from the solid.

The e.p.r. spectrum of the solution resulting from the treatment of papaverine with undiluted Marquis reagent consists of a single broad line which has the same g value as the initial spectra of solutions produced by the application of the same reagent to morphine, codeine, and pseudomorphine.

The action of the undiluted Marquis reagent on both benzene and phenol produced colored solutions with precipitation. Adjustments of concentrations

to produce highly colored solutions without precipitation and also a well-resolved e.p.r. spectrum were unsuccessful. Solutions which were sufficiently dilute to eliminate precipitation showed no detectable concentration of free radicals. Hence, the spectra reported in Table I and illustrated in Fig. 3 were obtained from solutions which contained precipitates. The lack of resolution in these spectra (Fig. 3) is probably due to the presence of mixtures of free radicals, some of which may be in the solid state.

The e.p.r. spectra obtained from solutions formed by the action of the undiluted Marquis reagent on meperidine and salicylic acid consist of broad single lines. Because of the unresolved nature of these spectra, the only possible inference is that free radicals are also formed in these two cases.

Since the only similarity in chemical structure of all the compounds shown above to produce free radicals by the action of the Marquis reagent is the aromatic ring, it is concluded that the ring is the site of the odd electron. The role of aromaticity in stabilizing free radicals is well known (12).

The reactions of the Marquis reagent on aromatic compounds, including alkaloids containing aromatic groups, may be similar to the acid-catalyzed reactions of the phenol-formaldehyde polymerization to resins as discussed by Walker (14). Since unsubstituted phenol produced a precipitate with the Marquis reagent, the 2,6-disubstituted compounds were tested in the hope of modifying the reactions by blocking the two reactive *ortho* positions.

2,6-Dimethylphenol, when treated with the Marquis reagent, exhibits a symmetrical e.p.r. spectrum (Fig. 4) consisting of at least 15 doublets. After standing 24 hours, the solution yielded a spectrum of the same g value and essentially the same basic 15 lines, but with the doublets unresolvable. The resulting solutions were deep brick-red and contained no precipitate, indicating decreased polymer formation. A higher concentration of free radicals was present than in the cases of benzene and phenol. The presence of the two methyl groups causes such a great number of hyperfine lines that the spectrum is difficult to analyze. The change in spectrum over a period of time also complicates the analysis of the hyperfine splitting.

2,6-Dichlorophenol was treated with the Marquis reagent in an attempt to simplify the e.p.r. spectrum while still blocking the two reactive *ortho* positions. This sample exhibited a spectrum of perhaps eight or ten lines, but so poorly resolved that conclusive analysis was not possible.

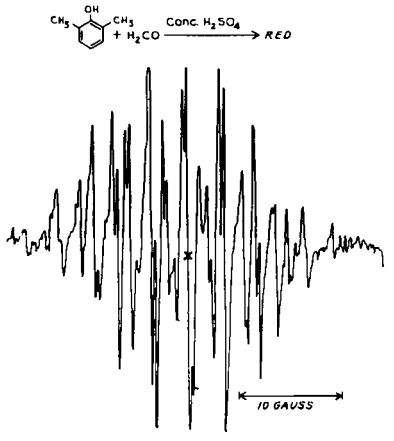

Fig. 4.—E.p.r. spectrum of a solution prepared by the action of dilute Marquis reagent on 2,6-dimethylphenol. The center of the spectrum is marked (×).

It is hoped that continued studies with other substituted phenols will lead to a better understanding of the hyperfine structure of the free radicals produced by reaction with the Marquis reagent and subsequently to the elucidation of the free radical structures. Once these spectral-structural relationships are established, the role of free radicals in the color tests will be clarified as will their role in the mechanism of acid-catalyzed phenol-formaldehyde polymerization.

Table II shows some e.p.r. data of compounds treated with several additional alkaloidal color reagents. The common feature of these reagents is the presence of an oxidizing agent in concentrated sulfuric acid. If only oxidation is involved, the action of any of these reagents on a given compound may result in the formation of identical radicals. There is sufficient similarity in the hyperfine structures and g values of morphine treated with the various reagents (Table II) to indicate that the principal free radical formed is the same in each case. Minor variations in the spectra may be due to (*a*) subtle differences in the relative concentration of minor radicals, (*b*) paramagnetic substances present as impurities in the color reagents, and (*c*) the presence of a paramagnetic transition element as a component of the reagent (Froehde reagent). In the latter case the observed spectrum consisted of the sum of the free radical spectrum and the paramagnetic spectrum of molybdenum.

TABLE II.-E.P.R. SPECTRAL DATA OF COLORED MIXTURES RESULTING FROM THE ACTION OF SEVERAL COLOR REAGENTS ON ALKALOIDS

Compd.	Reagent	Color	Lines, No.	Splitting in Gauss	g Value
Apomorphine	Mecke	Brown-green	Unresolved	...	...
Codeine	Mecke	Blue-green	12+	Uneven av. 0.28	2.00376
Morphine	Mecke	Blue-green	6	5.3 2.74	2.00363
Morphine	Froehde	Violet-green	6	4.06 2.56	2.00365
Morphine	Erdmann	Green-brown	6	4.77 2.65	2.00349
Morphine	Perhydrol	Green-brown	3	7.9	2.00355

The studies reported in this paper do not show conclusively that the colors are due to free radicals. In a few cases addition of water caused a marked decrease in the e.p.r. signal while the color became more intense; a striking example of this phenomenon is 2,6-dimethylphenol. It appears that the colors are due to a mixture of materials, some of which may be free radicals. This is supported by concentration measurements, which showed that the free radicals comprised less than 10% of the parent compound. The changes in color and e.p.r. spectrum with time provide evidence that several reactions are occurring simultaneously in the tests.

REFERENCES

(1) "United States Pharmacopeia," 16th rev., Mack Publishing Co., Easton, Pa., 1960, pp. 444, 303.
(2) Marquis, B. Z., *Anal. Chem.*, **38**, 467(1899); Marquis, B. Z., *Pharm. Zentralhalle*, **37**, 844(1896).
(3) Small, L. F., "Chemistry of the Opium Alkaloids," Supplement No. 103 to the Public Health Reports, 1932, p. 141.
(4) "The Merck Index," 5th ed., Merck and Co., Inc., Rahway, N. J., 1940, p. 827.
(5) Bentley, K. W., "The Chemistry of the Morphine Alkaloids," Oxford University Press, London, 1954.
(6) Fulton, C., *Ind. Eng. Chem.*, **3**, 200(1931).
(7) Feigl, F., "Spot Tests in Organic Analysis," Elsevier Publishing Co., Princeton, N. J., 1960, p. 135.
(8) Schieser, D. W., and Tuck, L. D., THIS JOURNAL, **51**, 694(1962).
(9) Adams, M., Blois, M. S., Jr., and Sands, R. H., *J. Chem. Phys.*, **28**, 774(1958).
(10) Bolton, J. R., and Carrington, A., *Proc. Chem. Soc.*, 1961, 385.
(11) Tuck, L. D., and Schieser, D. W., *J. Phys. Chem.*, **66**, 937(1962).
(12) Pake, G. E., "Paramagnetic Resonance," W. A. Benjamin, Inc., New York, N. Y., 1962; and Ingram, D. J. E., "Free Radicals as Studied by Electron Spin Resonance," Academic Press, Inc., New York, N. Y., 1958.
(13) Wertz, J. E., Vivo, J. L., and Dravnieks, F., "Free Radicals in Biological Systems," Blois, M. S., editor, Proc. Symposium, Stanford, Calif., 1960, p. 186.
(14) Walker, J. F., "Formaldehyde," A.C.S. Monograph Series, Reinhold Publishing Co., New York, N. Y., 1953, pp. 235 ff.

Powdered Particle Interactions: Suspension Flocculation and Caking IV

By ROBERT G. WILSON* and BERNARD ECANOW

The incompatibility of bismuth subnitrate with tragacanth mucilage has been reported in the literature. In this study, the phenomenon is discussed in terms of the chemical reaction that occurs between reactive bismuth sites on the bismuth subnitrate particle surface and the acidic groups of the D-galacturonic acid units of the complex acid polysaccharide present in the tragacanth gum.

AN AQUEOUS SUSPENSION containing bismuth subnitrate and one of a number of polysaccharide or carbohydrate derivatives such as tragacanth will exhibit a well known incompatibility (1, 2). The substances coagulate on mixing to form soft curd-like aggregates which settle to the bottom of the container. If the compounds are introduced at higher levels of concentration, the aggregates may set to hard, intractable masses.

The phenomenon has been explained (3, 4) as the interaction of positive bismuth or bismuth ions with the negatively charged tragacanth. It has been shown that the incompatibility does not appear in the presence of phosphate, citrate, or arsenate ions (3, 5). The negatively charged phosphate ion was thought to be strongly adsorbed on the tragacanth micelle and thus provided a protective action against the adverse effect of the positively charged bismuth. Nitrate ion was thought to be similarly adsorbed, but due to a weaker protective action could not prevent the occurrence of the incompatibility.

The chemistry of bismuth subnitrate in aqueous suspension has been explored in our recent work (6). The phosphate ion was shown to be a flocculating agent for an aqueous bismuth subnitrate suspension because strong interparticle bonds were formed by chemisorption of the phosphate ions at the surface of the bismuth subnitrate particles. An interaction of this type provides a basis for the discussion of the bismuth subnitrate-tragacanth or similar suspension systems.

EXPERIMENTAL

Materials.—The same lot of a commercial grade of bismuth subnitrate N.F. was used throughout the series of tests. It was obtained from Mallinckrodt Chemical Works, St. Louis, Mo. Two types of tragacanth U.S.P. were used in the work.[1] They will be more simply described as gum or ribbon

Received October 10, 1963, from the College of Pharmacy, University of Illinois at the Medical Center, Chicago.
Accepted for publication December 19, 1963.
Abstracted from a thesis submitted by Robert G. Wilson to the Graduate Faculty, University of Illinois at the Medical Center, Chicago, in partial fulfillment of Doctor of Philosophy degree requirements.
* Fellow of the National Science Foundation.

[1] Tragacanth Aleppo Ribbons No. 1 and Fine Powdered Gum Tragacanth Extra Grade No. 1, supplied by S. B. Penick and Co., New York, N. Y.