Formation of Alkaloid Artifacts in Plant Extracts by the Use of Ammonium Hydroxide and Acetone

By DWIGHT E. HOUSHOLDER and BENNIE J. CAMP

Treating plant extracts with ammonium hydroxide and acetone can give rise to the formation of artifacts, which are difficult to distinguish from the actual plant alkaloids. These artifacts give positive reactions with several of the standard qualitative alkaloid test reagents. Similar reactions are also obtained from mixtures of equal volumes of concentrated ammonium hydroxide and acetone.

DURING preliminary extraction procedures for the isolation of plant alkaloids, it was observed that the Dragendorff qualitative alkaloid tests became more intensely colored with time. This was observed only in extracts which had been treated with ammonium hydroxide and acetone. This observation initiated a study of the formation of compounds from these two reagents.

The reactions between carbonyl compounds and ammonia have been studied by several investigators. Patterson and McMillan (1) found that acetone saturated with ammonia, when allowed to stand 3 weeks, led to the formation of condensation products. Hack and Stuhlmann (2) isolated $Me_2C(OH)NH_2$ from a mixture of equimolar amounts of acetone and liquid ammonia. Matter (3, 4) reported the formation of acetonine when acetone was treated with gaseous ammonia under specified conditions. He reasoned the compound to be 2,2,4,6,6-pentamethyl-1,2,5,6-tetrahydropyrimidine. Bradbury *et al.* (5) presented detailed descriptions of reactions between acetone and ammonia. Hancox (6, 7) isolated 2,2,4,6-tetramethyl dehydropyridine from the reaction between acetone and ammonia.

Inghilleri (8–10) reported that a mixture of formaldehyde, concentrated ammonium hydroxide, and methyl alcohol, when sealed in a tube and exposed to direct sunlight for 7 months, caused the formation of several lower amines, hexamethylenetetramine, resinous products, and a crystalline compound with the empirical formula $C_6H_8ON_2$. This compound gave all the alkaloidal tests (8).

The work of these investigators indicate that ammonia or ammonium hydroxide can react with certain carbonyl compounds to form condensation products. This paper reports the formation of compounds from simple mixtures of ammonium hydroxide and acetone which give positive tests with several qualitative alkaloid reagents.

METHODS AND MATERIALS

Technique of Dragendorff's Test.—A small glass rod was used to apply small droplets of the sample to a small strip of 3-mm. filter paper. A forced air dryer was used to dry the droplets after they were applied. In this manner several drops could be applied in order to increase the concentration of the material being tested. Dragendorff's reagent (11) was then applied directly to the dried area with a dropping pipet. The sensitivity of the test could be greatly increased by adding 0.1 N H_2SO_4 to the spot after the addition of the Dragendorff's reagent (12).

Thin-Layer Chromatography (TLC).—TLC plates were prepared from a slurry of 30 Gm. of aluminum oxide G[1] shaken for 1.5 min. with 55 ml. of water. The slurry was allowed to stand 15 sec. in the applicator, then spread on 5 × 20 cm. glass plates. The plates were air dried for 15 min., then stored at 90° until used. Approximately 5 μl. of sample aliquots were applied to the plates, then developed one-dimensionally in benzene–chloroform–absolute ethanol (10:10:0.5).

Gas-Liquid Chromatography (GLC).—GLC studies were performed on a Warner-Chilcott Laboratory Instruments Division (formerly Research Specialties) model 600, with a β-ionization detector. A 3-ft. column of chromosorb W, 60–80 mesh, containing 20% diethyleneglycolsuccinate at 150° was used. Carrier gas was argon at 4 p.s.i.

Preparation of Reaction Mixtures and Chloroform Extracts.—Four reaction mixtures were prepared. Each mixture consisted of equal volumes of 15 M ammonium hydroxide and reagent grade acetone. They were aged 10 hr., 2 weeks, 7 weeks, and 15 months, respectively. The 10-hr. sample was aged while exposed to light and air. The other samples were exposed only to light.

A chloroform extract was made of each of the reaction mixtures by shaking a 10–15-ml. aliquot with 10 ml. of chloroform in a separator. The extract was concentrated to a volume of 0.5 ml. under a stream of nitrogen.

Color Test Reagents.—The following reagents were prepared by standard techniques: concentrated sulfuric acid with formaldehyde (13–15), saturated solution of chloranil in dioxane (13, 15), 5% benzidine in concentrated hydrochloric acid (13), and ceric nitrate (15). Concentrated sulfuric acid (13, 14) and concentrated nitric acid (13) were also used.

Qualitative Alkaloid Test Reagents.—Mayer's reagent (16), Valser's reagent (17), and Dragendorff's reagent (11) were prepared.

Spray Reagents.—The following reagents were used: Pauly's reagent (11) and 0.2% ninhydrin in ethanol (11).

RESULTS

Aliquots of each of the 4 reaction mixtures were used for color tests and qualitative alkaloid tests. All color tests, except with chloranil reagent, were negative. The Dragendorff's, Mayer's, and Valser's qualitative alkaloid tests were positive.

Received June 25, 1965, from the Department of Biochemistry and Nutrition, Texas A & M University, College Station.

Accepted for publication August 18, 1965.

Abstracted in part from a dissertation submitted by Dwight E. Housholder to the Graduate School, Texas A & M University, College Station, in partial fulfillment of Doctor of Philosophy degree requirements.

This study was supported in part by the Herman Frasch Foundation, New York, N. Y.

[1] Brinkmann Instruments, Inc., Westbury, Long Island, N. Y.

Thin-layer chromatography of the 15-month-old reaction mixture showed 15 areas after spraying with Dragendorff's reagent. A pink pigment was seen at R_f 0.8 before spraying.

The concentrated chloroform extracts of each reaction mixture were utilized for TLC, GLC, color tests, and qualitative alkaloid tests. All color tests were negative with the exception of the chloranil reagent, which gave an instantaneous reaction. All qualitative alkaloid tests were positive.

TLC separations of the chloroform extracts of the progressively older reaction mixtures revealed an increase in the number of areas present when sprayed with Dragendorff's reagent. The chloroform extract of the 10-hr. reaction mixture showed one large pink-orange area at the solvent front. Three areas were observed from extracts of the 2- and 7-week-old reaction mixtures. The extract of the 15-month-old mixture showed 11 areas almost evenly spaced from the origin to the solvent front.

GLC chromatograms of chloroform extracts of each reaction mixture were prepared. Three peaks were observed from extracts of the 10-hr. sample. Seven peaks were observed for the 2-week, 7-week, and 15-month reaction mixture extracts.

Exposure to light and air increased the rate of artifact formation. Under these conditions the chloroform extract of a freshly prepared reaction mixture gave a positive Dragendorff test strip within 10 hr. Another freshly prepared reaction mixture protected from light and air required 20–35 hr. before a positive Dragendorff test strip was obtained.

All concentrated chloroform extracts of the reaction mixtures, after standing a few days exposed to light and air, became dark red in color and developed an offensive odor.

DISCUSSION

In order to explain an unexpected intensified Dragendorff's reaction in procedures using ammonium hydroxide and acetone, mixtures of these reagents and chloroform extracts of these mixtures were subjected to various techniques. The results indicate the formation of condensation products from the reaction of these two compounds. Color and spray test reagents were employed to screen for primary, secondary, and aromatic amines, aromatic hydrocarbons, phenols, and hydroxy compounds. Chloranil reagent, used to detect secondary amines, gave the only positive color test on these reaction mixtures and their chloroform extracts.

To confirm the unusual Dragendorff's color change, two additional qualitative alkaloid reagents were employed. Both Valser's and Mayer's reagents gave positive results with the reaction mixtures and the chloroform extracts.

Both TLC and GLC studies showed that as these mixtures aged, a greater number of compounds were formed. The rate of formation of these compounds was affected by exposure to light and the atmosphere.

No further attempts were made to identify these compounds. The presence of compounds formed by the reaction of ammonium hydroxide and acetone explain the unusual Dragendorff's reaction originally noticed. Their presence differentiates this report from other references found in the literature on the "false-positive" Dragendorff's reactions of non-nitrogen-containing material (18, 19).

Although the reactions of ammonia and ammonium hydroxide with various carbonyl compounds have been studied, this report describes for the first time the reaction of ammonium hydroxide with acetone under the conditions described.

In view of these reactions, caution is advised in the use of these reagents in plant extraction procedures. The formation of artifacts can lead to false conclusions concerning the actual plant alkaloids.

REFERENCES

(1) Patterson, T. S., and McMillan, A., *J. Chem. Soc.* **119**, 269(1921).
(2) Hack, H., and Stuhlmann, H., *Ber.*, **61B**, 470(1928).
(3) Matter, E., *Helv. Chim. Acta*, **30**, 1114(1947).
(4) *Ibid.*, **31**, 612(1948).
(5) Bradbury, R. B., Hancox, N. C., and Hatti, H. H., *J. Chem. Soc.*, **1947**, 1394.
(6) Hancox, N. C., *Australian J. Sci. Res.*, **3A**, 450 (1950).
(7) Hancox, N. C., *Australian J. Chem.*, **6**, 143(1953).
(8) Inghilleri, G., *Z. Physiol. Chem.*, **80**, 64(1913).
(9) Inghilleri, G., *Zentr. Biochem. Biophys.*, **14**, 900(1912).
(10) *Ibid.*, **16**, 227(1913).
(11) Block, R. J., Durrum, E. L., and Zewig, G., "A Manual of Paper Chromatography and Paper Electrophoresis," 2nd ed., Academic Press Inc., New York, N. Y., 1958.
(12) Stahl, E., "Dunnschicht Chromatographie," Springer-Verlag, Berlin, Germany, 1962.
(13) Feigl, F., "Spot Tests," vol. 2, Elsevier Publishing Co., Amsterdam, The Netherlands, 1954.
(14) Stewart, C. P., and Stolman, A., eds., "Toxicology, Mechanisms and Analytical Methods," vol. II, Academic Press Inc., New York–London, 1961.
(15) Cheronis, N. D., and Entrikin, J. B., "Semimicro Qualitative Organic Analysis," 2nd ed., Interscience Publishers, Inc., New York, N. Y., 1957.
(16) Paech, K., and Tracey, M. V., eds., "Modern Methods of Plant Analysis," vol. IV, Springer-Verlag, Berlin, Germany, 1955, p. 373.
(17) Jenkins, G. L., Christian, J. E., and Hager, G. P., "Quantitative Pharmaceutical Chemistry," 4th ed., McGraw-Hill Book Co., Inc., New York, N. Y., 1953, p. 330.
(18) Furgiuele, A. R., Farnsworth, N. R., and Buckley, J. P., *J. Pharm. Sci.*, **51**, 1156(1962).
(19) Farnsworth, N. R., Pilewski, N. A., and Draus, F. J., *Lloydia*, **25**, 312(1962).