Preliminary Identification of the Antibacterial Principle "Madronin" from the Leaves of *Arbutus menziesii*

By BALACHANDRA KABADI and E. ROY HAMMARLUND

The antibacterially active compound "madronin" from the leaves of Pacific Madrona tree has been tentatively identified as a mixture of gallotannin and catechol tannin in addition to varying amounts of their degradation products, gallic acid, m-digallic acid, possibly trigallic acid, catechol, phloroglucinol, and glucose. Experimental evidence includes results of qualitative physical and chemical analyses, light absorption, paper chromatography, and microbiological fermentation tests. A simple modified laboratory extraction and purification procedure for the active principle is given. The previous observations made on madronin are explained according to its newly determined composition. Since tannin has been shown to be a rather potent antibacterial substance for many micro-organisms, its possible influence upon the various surveys for antibacterial agents in higher plants should be considered.

In 1952 HAMMARLUND, Pennington, and Rising (1) reported that the leaves of the Pacific Madrona tree (*Arbutus menziesii* Pursh., family *Ericaceae*) contained one or more antibacterial substances which were active *in vitro* mainly against Gram-positive and acid-fast bacteria. Their studies indicated the presence of several substances having similar chemical and antibacterial activities and the name "madronin" was given to the crude mixture of the active material (1, 2).

This current investigation was undertaken to characterize further and identify chemically the antibacterial principle by employing various improvements in the extraction and purification procedures.

Since light absorption studies made in this current investigation and most of the earlier qualitative tests indicated that madronin was probably a tannin although a few chemical tests reported earlier gave doubtful reactions for the presence of phenolic groups (1), numerous comparison tests were conducted with such substances as tannic acid N.F. (from *Quercus infectoria*), gallotannin (from *Rhus senialita*), and polyhydroxy phenolic compounds.

The active antibacterial substance prepared by using the present modified extraction and purification procedure, which is similar to that routinely used (3) for the preparation of tannins, is referred to in this presentation as "purified" madronin.

Received March 6, 1963, from the College of Pharmacy, University of Washington, Seattle.

Accepted for publication May 8, 1963.

This investigation was supported in part by funds provided for biological and medical research by the State of Washington Initiative Measure No. 171 and in part by Research Grant A1-04687-01 from the National Institute of Allergy and Infectious Diseases, U. S. Public Health Service, Bethesda, Md.

Abstracted from a thesis submitted by Balachandra Kabadi to the Graduate School, University of Washington, Seattle, in partial fulfillment of Master of Science degree requirements.

EXPERIMENTAL METHODS AND RESULTS

Modified Extraction Procedure.—Fresh leaves and stems were dried in a circulating hot air oven at 45° for about 48 hours. Six-hundred grams of the dried leaves were powdered and were repeatedly extracted in a Waring Blendor with 5 L. of distilled water. The aqueous extract was centrifuged, filtered, and dried under reduced pressure. The dried aqueous extract, weighing about 44 Gm., was repeatedly extracted in a large centrifuge tube by stirring with acetone in which sugar and other nonactive constituents were insoluble. The clear acetone solution was decanted following centrifugation and evaporated to dryness under reduced pressure. This yielded approximately 6.5 Gm. of material.

The residue from the acetone extract was dissolved in a minimal amount of water and filtered. This aqueous solution (approximately pH 3.7) was repeatedly extracted with ethyl ether to remove the coloring matter and gallic acid. To the aqueous solution 0.33 *M* phosphate buffer was added to raise the pH to 6.8, and the solution was extracted in several portions with 1 L. of ethyl acetate. The combined ethyl acetate solutions were air-dried and yielded about 4 Gm. of dried, antibacterially active residue.

Finally, the dried residue was dissolved in a minimal amount of water (approximately 15 ml.) and added to a 1 in. by 2-ft. chromatographic adsorption column packed with standard cellulose powder[1] prepared from a slurry in *n*-butanol (4). The chromatographic column was developed by the further addition of *n*-butanol, whereupon two colored bands formed. The lower band was eluted with *n*-butanol; the effluent was evaporated under reduced pressure and gave about 3.5 Gm. of yellow residue. The top purple band, which did not move with the butanol solvent, was eluted with water and rejected because it was antibacterially inactive.

The dried residue from the butanol effluent was dissolved in a minimal amount of acetone and poured slowly into 500 ml. of ethyl ether. A resultant white precipitate was filtered off and then immediately dried under reduced pressure. This was designated as "purified" madronin.

[1] Whatman B quality, standard grade, W. & H. Balton, Ltd., England.

TABLE I.—ANTIBACTERIAL ACTION OF MADRONIN AND RELATED SUBSTANCES

Test Compd.	Minimal Inhibition Concn., mcg. per ml. of the Medium
Madronin	4.5 to 5.0
"Purified" madronin	3.3 to 4.3
Tannic acid N.F.	2.5
Gallotannin[a]	4.9
Gallic acid[b]	11.6

[a] Gallotannin extracted from Chinese galls using 85% ethanol and dried under reduced pressure. [b] Mallinckrodt Chemical Works, New York, N. Y.

Antibacterial Study.—Tests made for the determination of the antibacterial activity of "purified" madronin and related substances were similar to those of Hammarlund, *et al.* (1). These tests were a quantitative serial dilution method for determining the minimal inhibitory concentration and a qualitative filter paper disk diffusion agar plate method for the qualitative detection of the antibacterial substances. The test organism was *Staphylococcus albus* C_{87},[2] and the media used for the serial dilution tests and agar plates was identical with that previously reported (1). The antibacterial activity of madronin, "purified" madronin, and related substances is recorded in Table I.

Qualitative Analyses of Antibacterial Principle.—The following chemical reactions which are generally characteristic of tannins were given by both the aqueous madronin and "purified" madronin solutions. Madronin formed a precipitate with gelatin, albumin, or starch (5). Madronin and tannic acid N.F. exhibited identical tanning properties with the Goldbeater's skin test using fresh untanned cowhide (3). Madronin gave a bluish-green color with ferric chloride and ferric alum solutions. Madronin was precipitated by salts of the heavy metals of copper, mercury, silver, and lead. Madronin gave a positive Sanio-bichromate test. Madronin precipitated with the alkaloids or alkaloidal salts of atropine, berberine, brucine, caffeine, cinchonidine, cinchonine, hydrastine, and strychnine (3). It formed a precipitate with the organic bases pyridine and phenazone (6).

The following tests indicated the presence of a gallotannin and a catechol tannin. The formaldehyde test (7) gave the characteristic pink fluffy precipitate of a catechol tannin and upon the addition of solutions of ferrous hydroxide and sodium acetate the filtrate became violet, which further indicated the presence of gallotannin. When madronin was heated above 210° for 30 minutes, a gas evolved and a sublimate formed on the sides of the vessel. The gas gave a white precipitate when passed through lime water and the sublimate turned red with ferric chloride solution and blue with ferrous sulfate solution. These tests on the sublimate as well as a paper chromatogram indicated the presence of pyrogallol, resulting from the gallic acid of gallotannin. A precipitate formed upon the addition of ammonium sulfide and ammonium molybdate, which are positive tests for gallotannin. Likewise, madronin precipitated with bromine water, which is positive for catechol tannin. The precipitate which formed upon the addition of lead acetate solution was soluble in acetic acid; this is characteristic of a catechol tannin. Madronin gave a positive phlobaphene test (8), indicating the presence of catechol tannin. The pine wood test (lignin reaction) gave the characteristic pink (7), indicating the presence of phloroglucinol from catechol tannin.

Ultraviolet Absorption.—Although madronin was thought to be a mixture of several substances instead of a pure substance, its absorption spectra were examined for characteristic features and were compared to those of tannic acid N.F. The ultraviolet absorption spectrum of an aqueous solution containing 16 mcg./ml. madronin or tannic acid N.F. was determined from 210 mμ to 350 mμ at various pH from 4–11 using a Cary recording spectrophotometer. Acetate, phosphate, and boric acid–sodium hydroxide–potassium chloride mixtures were used as the corresponding buffer solutions to maintain the desired pH. The same conditions were followed as previously employed by Hammarlund, Pennington, and Rising (1). The positions of the peaks in the spectra obtained for tannic acid N.F. (Fig. 1) were nearly identical at each pH with those previously reported for madronin; however, their magnitudes were greater, as reported by Sohn (9). The only essential difference was the presence of a more distinct absorption peak at 230 mμ for tannic acid N.F. at pH 10. For simplicity in Fig. 1 only the spectra for tannic acid at pH 6, 8, and 10 are given.

Infrared Absorption.—The infrared spectrum of 2 mg. of madronin and "purified" madronin in a 400-mg. KBr pellet was measured with a Beckman infrared recording spectrophotometer (model IR-5A) from 2 μ to 16 μ. The position of the absorption peaks was practically identical to that of an equal quantity of tannic acid N.F. with only insignificant shifts in the peaks as seen in Fig. 2. The infrared absorption spectra for the two madronin samples were identical.

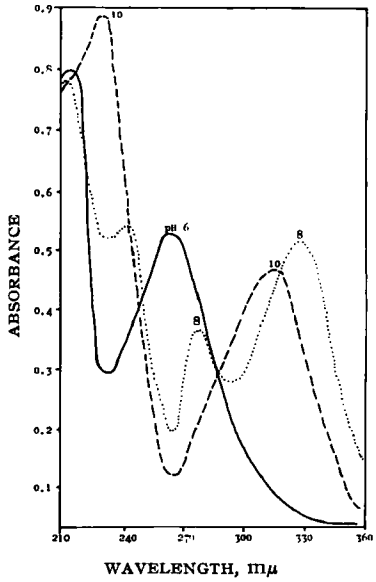


Fig. 1.—Ultraviolet absorption spectrum of tannic acid N.F. at various pH.

[2] Microbiology Department, University of Washington Medical School, Seattle.

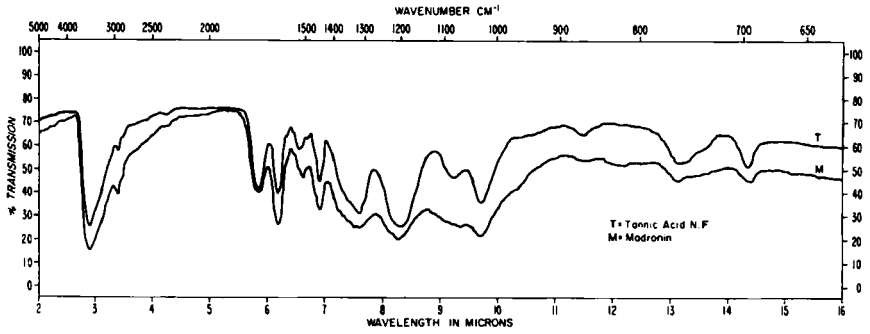


Fig. 2.—Infrared spectra of tannic acid N.F., madronin, and "purified" madronin. (The two madronin spectra are similar.)

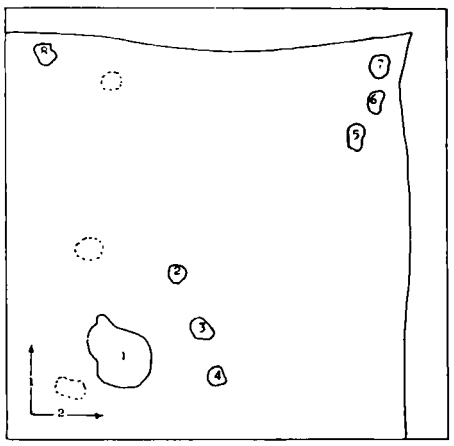


Fig. 3.—Two-dimensional chromatogram of madronin. Solvent system: first direction, 6% acetic acid; second direction, *n*-butanol:acetic acid:water (4:1:5). Reagent: 0.5% ammoniacal silver nitrate in methanol. Identified spots are (solid line): *1*, gallotannin; *2*, gallic acid; *3*, *m*-digallic acid; *4*, trigallic acid; *5*, catechin; *6*, phloroglucinol; *7*, catechol; *8*, glucose.

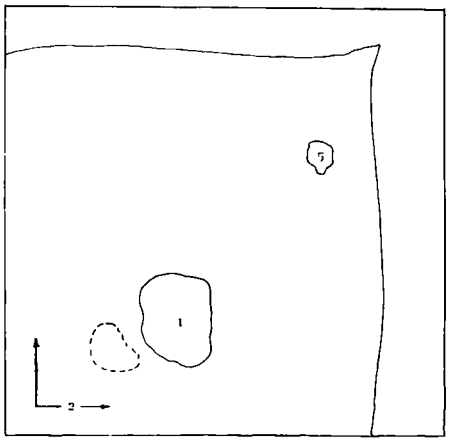


Fig. 4.—Two-dimensional chromatogram of "purified" madronin. Solvent system: first direction, 6% acetic acid; second direction, *n*-butanol:acetic acid:water (4:1:5). Reagent: 0.5% ammoniacal silver nitrate in methanol. Identified spots are: *1*, gallotannin; *5*, catechin.

Paper Chromatography.—The ascending filter paper partition chromatography of madronin using a water-saturated butanol solvent system on Whatman No. 1 paper previously reported by Hammarlund and associates (1) was repeated on tannic acid N.F. under identical conditions. Tannic acid also separated similarly into two antibacterially active spots.

The detailed chromatographic study of madronin and "purified" madronin was conducted on two-dimensional Whatman No. 1 chromatographic sheets, 14 in. square, by using the ascending method (4) and employing 6% acetic acid as the developing solvent in the first direction and *n*-butanol:acetic acid:water (4:1:5) in the second direction. The reagent employed for detecting the position of the spots was 0.5% ammoniacal silver nitrate in methanol (7, 10). The chromatogram of madronin is shown in Fig. 3, and the color of the spots of the various phenolic substances varied from brown to green-brown to blue-black. The following substances in madronin were identified by comparisons of the position and color of their spots with those given by known reagents used singularly or in combination: glucose, gallic acid, phloroglucinol, catechol, catechin, and tannic acid. In the case of gallotannin, *m*-digallic acid, and trigallic acid for which the pure reagents were not readily available, the relative position of the spots in each case were compared with similar chromatograms of tannins reported in the literature (4). This method likewise was used in determining the presence of protocatechuic acid and pyrogallol in the hydrolytic products. The spots encircled by dotted lines remain unidentified. Using the same method a chromatogram of "purified" madronin contained fewer spots (Fig. 4), showing only the presence of gallotannin and possibly catechin.

Hydrolysis of Madronin and Identification of the Hydrolytic Products.—One gram of madronin was acid-hydrolyzed by refluxing in 6% sulfuric acid for 20 hours. A two-dimensional paper chromatogram was made of the filtrate employing the same method described previously. The chromatogram is shown in Fig. 5 and the following spots were identified: gallic acid, catechin, phloroglucinol, catechol, glucose, and protocatechuic acid. A reasonably drastic acid treatment was made to obtain complete hydrolysis.

A 200-mg. sample of madronin was similarly alkali-hydrolyzed by dissolving it in an aqueous 5% sodium hydroxide solution which was shaken at room temperature for a few minutes. The resulting solution was chromatographed as previously described and the following spots were identified as shown in Fig. 6: gallotannin, gallic acid, *m*-digallic acid, trigallic acid, catechin, phloro-

glucinol, catechol, glucose, and protocatechuic acid. Since phloroglucinol and pyrogallol have the same R_f values for the two-dimensional solvents used, it was difficult to prove conclusively whether pyrogallol was there when phloroglucinol was present (10).

One-half gram of "purified" madronin was dissolved in 2% hydrochloric acid and kept at 95° for 1 hour. The resulting solution was then spotted on paper and two-dimensional chromatography was carried out (Fig. 7). By comparison with the chromatogram of "purified" madronin before hydrolysis (Fig. 4) which contained essentially

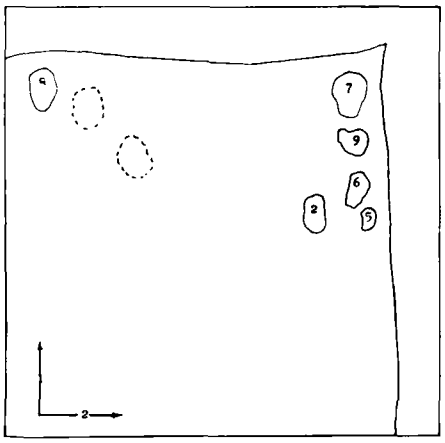


Fig. 5.—Two-dimensional chromatogram of acid-hydrolyzed madronin. Solvent system: first direction, 6% acetic acid; second direction, *n*-butanol:acetic acid:water (4:1:5). Reagent 0.5% ammoniacal silver nitrate in methanol. Identified spots are (solid line): *2*, gallic acid; *5*, catechin; *6*, phloroglucinol; *7*, catechol; *8*, glucose; *9*, protocatechuic acid. Length of hydrolysis: 20 hours with 6% sulfuric acid.

gallotannin and possibly catechin, the following additional hydrolytic products were obtained: gallic acid, *m*-digallic acid, trigallic acid, catechol, protocatechuic acid, and glucose. The hydrolysis was still incomplete because some gallotannin remained. Only a relatively mild acid treatment was required to show that "purified" madronin was hydrolyzable.

DISCUSSION

In the original study by Hammarlund, Pennington, and Rising (1) madronin was reported as being a mixture of unknown antibacterially active compounds. The current study revealed that the active principles were essentially gallotannin and catechol tannin in addition to some of the decomposition products of the tannins and that all of the results and observations made by the previous workers could be interpreted now on the basis of the newly determined composition of madronin.

The antibacterial activity of tannins has been extensively studied (11–16). In this present study madronin possessed slightly less antibacterial activity than tannic acid N.F. This might be due to the presence in madronin of the decomposition products of tannic acid, such as catechol, phloroglucinol, catechin, gallic acid, and glucose in addition to nonhydrolyzed gallotannin and catechol tannin. "Purified" madronin which did not contain the decomposition products showed greater antibacterial activity than did the original madronin but still not quite as much as did tannic acid N.F.

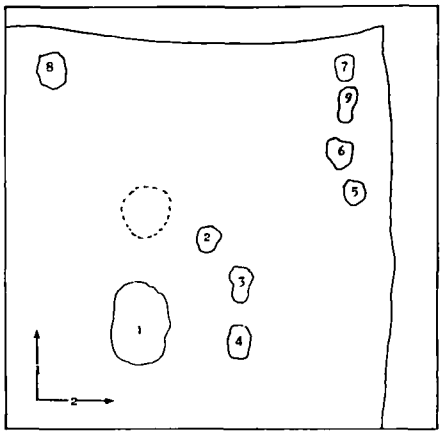


Fig. 6.—Two-dimensional chromatogram of alkali-hydrolyzed madronin. Solvent system: first direction, 6% acetic acid; second direction, *n*-butanol:acetic acid:water (4:1:5). Reagent: 0.5% ammoniacal silver nitrate in methanol. Identified spots are (solid line): *1*, gallotannin; *2*, gallic acid; *3*, *m*-digallic acid; *4*, trigallic acid; *5*, catechin; *6*, phloroglucinol; *7*, catechol; *8*, glucose; *9*, protocatechuic acid. Length of hydrolysis: 2 minutes with 5% sodium hydroxide solution.

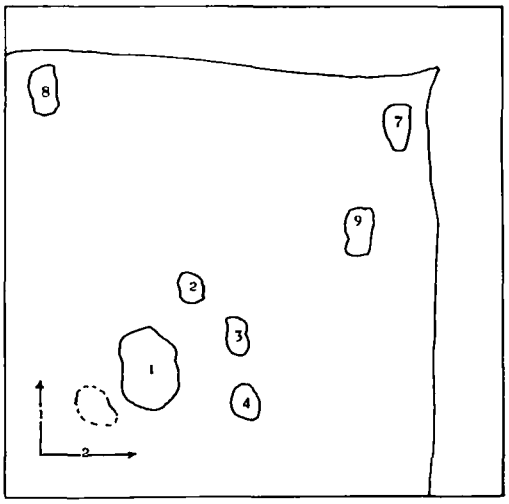


Fig. 7.—Two-dimensional chromatogram of acid-hydrolyzed "purified" madronin. Solvent system: first direction, 6% acetic acid, second direction, *n*-butanol:acetic acid:water (4:1:5). Reagent: 0.5% ammoniacal silver nitrate in methanol. Identified spots are (solid line): *1*, gallotanin; *2*, gallic acid; *3*, *m*-digallic acid; *4*, trigallic acid; *7*, catechol; *8*, glucose; *9*, protocatechuic acid. Length of hydrolysis: 1 hour with 2% hydrochloric acid.

The physical properties of madronin (yellow color, amorphous structure, and decomposition at 200°) are closely parallel to those of tannins. The levorotation of madronin is possibly due to the presence of catechol tannin which masked the usual dextrorotation of gallotannin. The loss of anti-

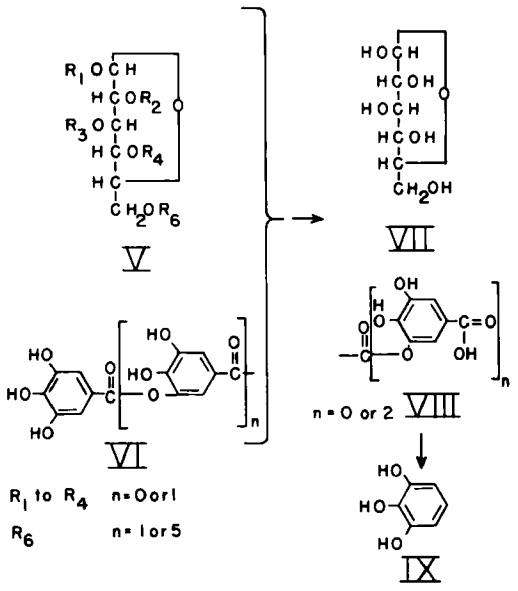


Fig 8—Degradation of gallotannin.

bacterial activity of madronin above pH 7 is due to the fact that tannins are less stable in alkaline solutions (3).

It was largely the fact that the qualitative chemical tests of madronin were similar to the reactions given by the various polyhydroxy phenolic constituents of tannic acid which led to the recognition that madronin was probably a tannin, particularly since no other antibacterial component was found present. Although various polyhydroxy compounds may give some of the individual chemical reactions, all the tests are given only by tannins (5).

The evidence that one of the tannins was gallotannin (V–VI, Fig. 8) was substantiated by the presence of gallic acid (VIII, n = 0) and glucose (VII). Tests for the presence of glucose in madronin, such as Molisch or yeast fermentation, were negative; however, glucose was identified from the hydrolytic products following purification (3) by both chemical and biological tests (positive osazones, Fehling's, Tollen's, yeast fermentation) and by paper chromatographic methods (Figs. 5, 6, and 7). Gallic acid and its decomposition product, pyrogallol (IX), were identified by their characteristic chemical tests and by paper chromatography.

The evidence that a catechol tannin (I, Fig. 9) was also present in madronin was obtained through the following series of reactions—the formation of a bluish-green color with iron compounds, the precipitation with bromine solution and formaldehyde solution, and formation of the red phlobaphene and a positive pine wood test which indicated the presence of phloroglucinol (II, Fig. 9). Finally, the identification by paper chromatography of protocatechuic acid (IV, Fig. 9) among the hydrolytic products of madronin is further evidence for the presence of catechol tannin.

Additional decomposition products of tannins such as catechol (III, Fig. 9), m-digallic acid (VIII, Fig. 8, n = 1), and trigallic acid possibly (VIII, Fig. 8, n = 2) were also identified by paper chromatography (Figs. 5, 6, and 7).

Because all evidence showed madronin to be a mixture of gallotannin and catechol tannin which hydrolyzed readily, the proportion of the various phenolic constituents and other hydrolytic products may vary greatly during extraction and purification. In addition, the normal seasonal variation could influence the concentration of the active tannin principle. Therefore, it is reasonable to assume that the yield of madronin and the antibacterial assays will show large variations depending upon the history of the sample tested.

One of the general observations which has resulted from this study is the reiteration of the importance of tannins as antibacterial substances in higher plants. Because of their natural wide occurrence in plants it is highly probable that in some cases the activity found in the higher plants during the course of the many surveys for antibacterial agents as reviewed recently by Nickell (17) might be due partly or entirely to the presence of tannins. Therefore, if antibacterial activity is found in extracts of higher plants, the extracts should be carefully tested for the presence of tannins.

SUMMARY AND CONCLUSIONS

A simple modified laboratory extraction and purification procedure was developed for the extraction of more antibacterially active madronin than was obtained previously.

Experimental evidence involving studies of light absorption, paper chromatography, qualitative chemical tests, and microbiological fermentation experiments shows that madronin is a mixture of gallotannin and catechol tannin and their various decomposition products of gallic acid, m-digallic

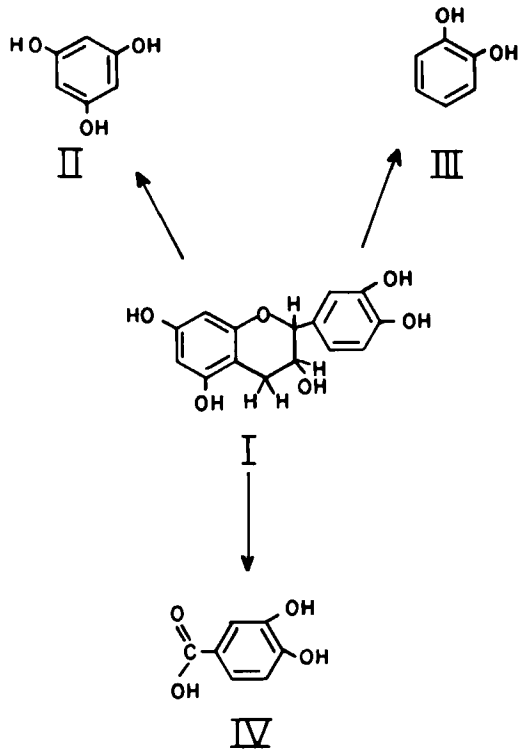


Fig. 9.—Degradation of catechol tannin (catechin).

acid, trigallic acid, catechol, phloroglucinol, and glucose.

Tannin has been shown to be a rather potent antibacterial substance; its possible influence upon the various surveys for the presence of antibacterial agents in higher plants should be investigated during the surveys.

REFERENCES

(1) Hammarlund, E. R., Pennington, D. E., and Rising, W. L., THIS JOURNAL, 41, 561(1952).
(2) Hammarlund, E. R., and Rising, L. W., *ibid.*, 42, 431(1953).
(3) Nierenstein, M., "The Natural Organic Tannins," The Sherwood Press, Cleveland, Ohio, 1928, pp. 1–120.
(4) Haworth, R. D., *Proc. Chem. Soc.*, 1961, 401.
(5) Nierenstein, M., "Allen's Commercial Organic Analysis," Vol. 5, 5th ed., P. Blakiston's Son and Co., Philadelphia, Pa., 1927, pp. 1–150.
(6) Shellard, E. J., "Practical Plant Chemistry for Pharmacy Students," Pitman Publishing Corp., New York, N. Y., 1957, p. 521.
(7) Paech, K., and Tracy, M. V., "Modern Method of Plant Analysis," Vol. III, Spring-Verlog, Berlin, Germany, 1955, pp. 521–525.
(8) Haas, P., and Hill, T. G., "Chemistry of Plant Products," Longmans Green and Co., London, England, 1928, pp. 266–300.
(9) Sohn, A. W., *Das Leder*, 2, 4(1951).
(10) Linkens, H. F., "Paper Chromatographic in der Botanik," Spring-Verlog, Berlin, Germany, 1959, p. 333.
(11) Kliewe, H., and Hillenbrand, H. I., *Deut. Apotheker Ztg.*, 54, 2211(1939).
(12) Floch, H., *Compt. Rend. Soc. Biol.*, 143, 450(1949).
(13) Suter, C. M., *Chem. Rev.*, 28, 269(1941).
(14) Plakhova, N. B., *Zhur. Khim. Biol. Khim.*, 1955, 15986; through *Chem. Abstr.*, 51, 11465(1957).
(15) Martin, J. D., Jr., and Fowler, C. D., *Ann. Surg.*, 99, 993(1934).
(16) Fuller, J. E., Muellar, W. S., and Swanson, R. W., *J. Dairy Sci.*, 25, 883(1942).
(17) Nickell, L. G., *Econ. Botany*, 13, 281(1959).

Colorimetric Method for Determination of Uracil Mustard and Related Alkylating Agents

By H. G. PETERING and G. J. VAN GIESSEN

A colorimetric method for uracil mustard has been developed which is equally useful for other aromatic and aliphatic nitrogen mustard antitumor drugs. This method has been used to determine these drugs in plasma pools and for the study of their kinetics of hydrolysis and reactivity with nucleophilic reagents.

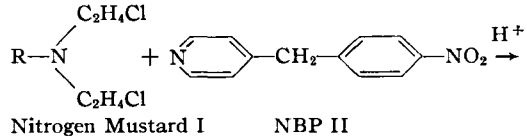
SEVERAL COLORIMETRIC methods for the determination of specific nitrogen mustards have been described by Klatt, Griffin, and Stahlin (1), Friedman and Boger (2) and Ausman, Crevar, Hagedorn, Bardos, and Ambrus (3). These methods all are based on the method for alkylating agents first reported by Epstein, Rosenthal, and Ess (4) which involves reaction of these agents with 4-(*p*-nitrobenzyl) pyridine. A photofluorometric method for ethylenimino and chloroethylamino groups has also been devised by Mellett and Woods (6). This latter method has the advantage of extreme sensitivity and thus may be useful for blood-level studies; it has the disadvantage of possible large losses because of involved and laborious manipulations.

The colorimetric methods are simpler than the fluorometric method and are useful in kinetic studies and in following the levels of these drugs when they are used in surgical perfusion studies. However, when we tried them with uracil mustard, 5-bis(2-chloroethyl) aminouracil, a number of difficulties were encountered. Since the method of Mellett and Woods was not found suitable for our purposes, we decided to attempt a modification of the colorimetric method of Klatt, Griffin, and Stahlin (1) so that a variety of nitrogen mustards could be determined.

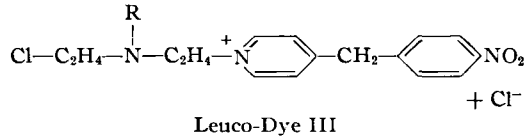
This method which is equally useful with aliphatic, aromatic, and heterocyclic nitrogen mustards and which can be conveniently used to determine these antitumor agents in perfusion blood pools, and on tissue extracts, is presented here.

EXPERIMENTAL

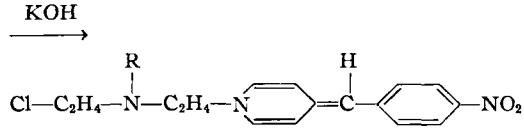
The reaction of alkylating agents with 4-(*p*-nitrobenzyl) pyridine (NBP) (II), which produces the chromophore, is carried out in our method under conditions similar to those originally described by Epstein, *et al.* (4). In the reaction formulated in Eq. 1, the solubility of the reaction product III is dependent on whether R is alkyl, aryl, or heterocyclic. Similarly the color formed from the chromophore IV when alkali is added will depend on the nature of R. In addition, Epstein, *et al.* (4), showed that the type of alkali used is of importance in the intensity of color formation. All of these matters were carefully considered in formulating the method outlined below.



Nitrogen Mustard I NBP II



Leuco-Dye III



Chromophore IV

The method worked out for uracil mustard (*Ia*) was found to give good results for dopan (*Ib*), chlorambucil (*Ic*), tris-chloroethylamine hydrochloride (*Id*), and HN-2 (Merck Mustargen) (*Ie*). The structures of these compounds, all of which are used as antitumor agents, are given in Table I.

Received February 25, 1963, from the Department of Pathology, The Upjohn Co., Kalamazoo, Mich.
Accepted for publication May 4, 1963.