False-Positive Alkaloid Reactions Obtained with Extracts of *Piper methysticum*

By A. R. FURGIUELE, N. R. FARNSWORTH, and J. P. BUCKLEY

Piper methysticum Forst. (Piperaceae) has been reported by several investigators to contain alkaloids. Several screening methods utilized in this study substantiated these findings; however, after comparing a variety of test procedures, these "positive" alkaloid tests were shown to be due to certain non-nitrogenous α-pyrone compounds. The α-pyrones were also demonstrated to react with a modified Dragendorff spray reagent, after thin-layer chromatography on silica gel G plates. The orange color produced after spraying these α-pyrones was typical of the usual alkaloid positive result, but the color persisted for less than 24 hours. The use of heat to dissolve extracted residues in 1% hydrochloric acid for testing was shown to intensify the alkaloid-like reactions. The results are discussed relative to the screening methods employed.

A NUMBER of reference sources have indicated that the common alkaloid precipitating reagents (Mayer's, Wagner's, Dragendorff's, Sonnenschein's, Hager's, *etc.*) are capable of precipitating certain nonalkaloid materials from solution. Certain proteins, amino acids, glycosides, tannins, betaines, choline, carbohydrates, and ammonium salts have most frequently been implicated as substances present in plants that will give such false-positive reactions (1–11).

Piper methysticum Forst. is a plant indigenous to many islands of the South Pacific. The roots of this plant have been used in the form of a beverage, known as Kava, Kawa, or Awa, by natives of many of these islands to allay anxiety and reduce fatigue (12). Other uses claimed for Kava have been as an aphrodisiac, antiseptic, tonic, anodyne, sudorific, diuretic, stimulant, and intoxicant (13). Local application of the drug is reported to produce a burning pain followed by loss of sensitivity (14). Perhaps the most significant biological effects produced by extracts of this plant are the induction of intoxication of a silent and drowsy nature, accompanied by incoherent dreams when large doses are ingested (13).

Six α-pyrone compounds have been isolated from Kava. They are kawain (*K*), dihydrokawain (*DHK*), methysticin (*M*), dihydromethysticin (*DHM*), yangonin (*Y*), and desmethoxyyangonin (*DMY*), (15–20). See Fig. 1. Methysticin and dihydromethysticin have been demonstrated to be the most effective sedative compounds of the six pyrones isolated to date (20). Recently, Hansel, *et al.* (21), have isolated two new compounds, flavokawin *A* and flavokawin *B* from Kava, but these were shown to be devoid of sedative properties. In addition, gum, starch, sugar, resins, glucosides (22), and alkaloids (22–24) have been reported to be present in Kava.

Since the alkaloids reported present in this plant had not been thoroughly investigated, our initial studies were directed to the isolation, separation, and identification of these compounds. The present report is concerned with evidence pointing to a class of non-nitrogenous chemical substances present in *P. methysticum* that render alkaloid-positive reactions with a number of common alkaloid-detecting reagents.

EXPERIMENTAL

Materials.—The plant material used in this investigation was collected in Hawaii during the fall of 1959 and consisted of the roots of *Piper methysticum* Forst. (Piperaceae).[1] The *Hamamelis virginiana* L. and *Prunus serotina* Ehrh. used as alkaloid negative controls were obtained commercially as powdered leaves and bark, respectively.[1] Thin-layer chromatography experiments were conducted using the DeSaga apparatus,[2] designed to prepare a 200 μ matrix on 200 x 200 mm. glass plates. The adsorbent matrix was prepared from silica gel G[2] with all prepared plates activated at 105° for 30 minutes, followed by cooling to room temperature in a soda-lime desiccator. All solvents and chemicals used in this investigation were of reagent grade quality unless otherwise specified.

Comparison of Screening Methods.—All attempts made to isolate crude alkaloids from Kava by the usual methods employed in phytochemical studies were unsuccessful. Since the possibility existed that alkaloids were not present, small samples were screened for alkaloids according to the methods of Wall, *et al.* (25, 26), Wall method; Kiang and Douglas (27), Kiang method; and Swanholm, *et al.* (24), Swanholm method. Using the reagents to detect alkaloids that were recommended for each method, it was found that pre-

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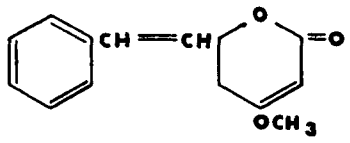
[1] Supplied through the S. B. Penick and Company, New York 8, N. Y.
[2] Distributed by Brinkmann Instruments Inc., Great Neck, L. I., N. Y.



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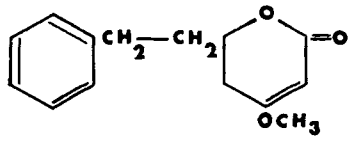
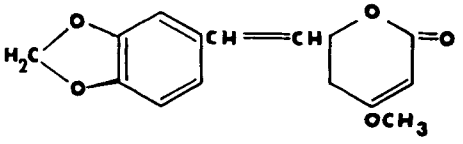
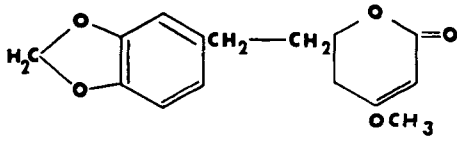


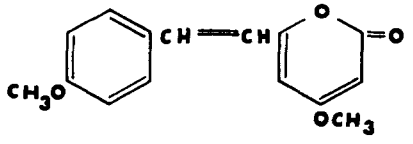
DHK



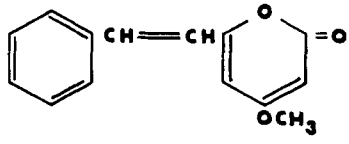
M



DHM



Y



DMY

Fig. 1.—Structures of *P. methysticum* α-pyrones: kawain, (*K*); dihydrokawain, (*DHK*); methysticin, (*M*) dihydromethysticin, (*DHM*); yangonin, (*Y*); desmethoxyyangonin, (*DMY*).

liminary tests for the alkaloids using the Wall method were positive, while confirmatory tests were negative. The Kiang method indicated that alkaloids were present corresponding to *ca.* 0.01% (as brucine hydrochloride). This is in general agreement with the reported occurrence of alkaloids in Kava of 0.022% by Winzheimer (22) and 0.012% by Scheuer and Horigan (23). The methods utilized for alkaloid estimation were not indicated by these investigators. Both Swanholm methods (direct hydrochloric acid extraction and Prollius extraction) gave results indicative of the presence of alkaloids in this plant.

Since the confirmative test by the Wall method was negative, and the methods of Kiang and Swanholm were indicative of the presence of alkaloids, a series of experiments were designed to clarify these conflicting results. Preliminary experiments had led us to believe that the material possibly responsible for the positive tests was chloroform-soluble. Therefore, it was decided to simultaneously compare the results obtained by screening *P. methysticum* and two plants known to be devoid of alkaloids, or alkaloid-like material, but to which chloroform extracts of *P. methysticum* had been added.

The Swanholm method was utilized and the leaves of *H. virginiana* and the bark of *P. serotina* were included as controls. It was necessary to include controls in this study since extraneous plant materials (resins, pigments, *etc.*) present in the extracts could alter the separation and/or resolution of the α-pyrones present in the *P. methysticum* chloroform extractives. Four 5-Gm. samples of finely powdered *P. methysticum* roots were continuously extracted with chloroform for 3 hours in suitable Soxhlet

TABLE I.—ALKALOID SCREENING RESULTS OF *P. methysticum* AND *P. methysticum* CHLOROFORM-SOLUBLE TREATED CONTROLS

Reagent	*P. methysticum* Method A[a]		*H. virginiana* + *P. methysticum* Method A[a]	B[b]	*H. virginiana* Method A[a]	B[b]	*P. serotina* + *P. methysticum* Method A[a]	B[a]	*P. serotina* Method A[a]	B[b]
Mayer's	tr	tr	tr	tr	—	—	tr	tr	—	—
Wagner's	+	+	+	+	—	—	+	+	—	—
Picric acid	tr	tr	tr	tr	—	—	tr	tr	—	—
Dragendorff's	+	+	+	+	—	—	+	+	—	—
Sonnenschein's	++	++	++	++	—	—	++	++	—	—
Silicotungstic acid	tr	tr	tr	tr	—	—	tr	tr	—	—

[a] A, acid extract; [b] B, Prollius extract. Key: ++, moderate precipitate; +, light precipitate; tr; trace (turbidity); —, no turbidity or precipitate.

extractors. Two of these chloroform extracts were mixed with separate 5-Gm. samples of finely powdered *H. virginiana* leaves and two were mixed with separate 5-Gm. samples of powdered *P. serotina* bark. These four samples were then heated to dryness on a steam bath.

One set of samples consisting of 5 Gm. each of *P. methysticum*, *H. virginiana* treated with *P. methysticum*, *H. virginiana*, *P. serotina* treated with *P. methysticum*, and *P. serotina* was screened by the Swanholm method utilizing direct 1% hydrochloric acid extraction. The samples were mixed with sufficient 1% hydrochloric acid to form a slurry, macerated at 80° for 4–6 hours, cooled to room temperature and filtered. Each filtrate was adjusted to 2 ml. by passing 1% hydrochloric acid through the marc on the filter paper.

A duplicate group of 5-Gm. samples was extracted with *ca.* 30 ml. of Prollius fluid (ether/chloroform/ethanol/ammonia) (25:8:2.8:1)(v/v) for 56 hours at room temperature in tightly stoppered flasks. After the maceration period, each sample was filtered and the residue on each filter paper was washed with an additional 20 ml. of Prollius fluid. The filtrates were then evaporated to dryness on a steam bath. Each residue was then mixed with *ca.* 4 ml. of 1% hydrochloric acid and allowed to macerate at 80° for 1 hour with occasional stirring. After cooling to room temperature, the extracts were filtered and sufficient 1% hydrochloric acid added to each sample to make 2 ml.

The samples were each tested for alkaloids by the addition of 0.1 ml. of reagent to 0.2 ml. of extract (equivalent to 0.5 Gm. of dried plant material). Mayer's, Wagner's, picric acid, Dragendorff's, Sonnenschein's, and silicotungstic acid reagents were used. The results are presented in Table I and clearly indicate the presence of material in the chloroform-soluble *P. methysticum* extracts that produce precipitates with the reagents indicated.

Since most alkaloids are known to exist in plants as chloroform-insoluble salts, the presence of chloroform-soluble, nonalkaloid material as being responsible for the alkaloid-like reactions was further suspected.

For comparative purposes, duplicate samples of *P. methysticum* were screened by the Prollius extraction method with one sample treated as previously described, using heat to aid in dissolving the Prollius residue in 1% hydrochloric acid. The second sample was treated in a similar manner; however, no heat was applied. This evaluation was considered necessary since the original Webb method (11) was found to give positive alkaloid tests with *P. methysticum* by direct acid extraction (heat applied at 60° for 1/2 to 1 hour), but negative tests by the Prollius method in which heat was not applied. When *P. methysticum* was screened by the Arthur modification (28) of the Webb method, negative results were obtained with the direct acid extraction (no heat) as well as with the Prollius extraction (no heat). The results of this study are indicated in Table II and point out the effect of heat on increasing the false-positive alkaloid reactions with these reagents.

TABLE II.—THE EFFECT OF HEAT ON *P. methysticum* PROLLIUS EXTRACTION ALKALOID SCREENING RESULTS

Reagent	*Piper methysticum*[a] Heat	No Heat
Mayer's	tr	—
Wagner's	+	—
Picric acid	tr	—
Dragendorff's	+	—
Sonnenschein's	++	+
Silicotungstic acid	tr	—

[a] ++, moderate precipitate; +, light precipitate; tr, trace (turbidity); —, no turbidity or precipitate.

Thin-Layer Chromatography Studies.—It is common practice in many laboratories to detect alkaloids in plant material by means of chromatographic methods using a modified Dragendorff spray reagent. After chromatograms are treated with the Dragendorff spray, the appearance of typical yellow-orange to orange spots is generally accepted as good evidence of the presence of alkaloids in the material chromatographed. Since the standard Dragendorff reagent produced a visible alkaloid-like reaction with extracts of *P. methysticum* in test tube experiments, it was decided to determine the effect of the Munier and Macheboeuf modified Dragendorff spray reagent (29) on chromatograms prepared from the previously described Prollius extracts, in addition to certain controls.

An additional aid in allowing us to verify that chloroform-soluble *P. methysticum* extracts contained material responsible for false-positive alkaloid reactions was found in treating chromatograms of these extracts with sulfuric acid. Chloroform extracts of *P. methysticum*, in addition to the known α-pyrones isolated from this plant (chloroform-soluble), were found to react with sulfuric acid to produce red or yellow colors.

Silica gel G plates were spotted with separate solutions containing 1γ/1λ of each of the known α-pyrones (kawain, dihydrokawain, methysticin, dihydromethysticin, desmethoxyyangonin, yangonin) in chloroform. In addition, a solution containing a mixture of 1γ/1λ of each was prepared. Ten lambda of each solution was applied and a mixture of Skelly B/ethyl acetate (1:1) was used as the eluent. After proper development, the plates were removed from the chambers, air-dried and sprayed with sulfuric acid. The areas of color formation were quickly traced and the colors noted. All compounds reacting with sulfuric acid to produce a yellow color quickly faded, while those components producing red colors were more permanent. The result of this chromatographic separation is illustrated in Fig. 2. A suitable solvent system for the complete resolution of the six α-pyrones could not be devised, but in mixtures of appropriate concentration, differences in migration, combined with the sulfuric acid color reaction, were sufficient to allow detection of desmethoxyyangonin (yellow) and yangonin (yellow) from mixtures of kawain (yellow)-dihydrokawain (yellow) and methysticin (red)-dihydromethysticin (red).

Several dilutions of the known α-pyrones were prepared, applied to duplicate silica gel G plates, and chromatographed as previously described. One set of plates was sprayed with sulfuric acid and the other set with the Munier and Macheboeuf-Dragendorff spray in order to ascertain the minimal quantities detectable with each reagent. The results are indicated in Table III.

The initial reaction of the α-pyrones to the Dragendorff spray was quite similar to that produced by known alkaloids; however, it should be pointed out that the reaction was not of long duration. The initial orange color faded to yellow within 1 hour and, dependent on concentration, was usually rendered colorless after standing for 24 hours. This is in sharp contrast to most alkaloids that we have treated in a similar manner, in which the orange color has been observed to persist for several weeks.

Duplicate silica gel G plates were prepared for thin-layer chromatography, by the application of 100λ each of the 1% hydrochloric acid solutions of the Prollius residues, prepared as previously described. These solutions included *P. methysticum*, *H. virginiana* treated with *P. methysticum*, *H. virginiana*, *P. serotina* treated with *P. methysticum*, *P. serotina*, *P. methysticum* chloroform-soluble extract in 1% hydrochloric acid (unheated), and *P. methysticum* chloroform-soluble extract in 1% hydrochloric acid (heated). In addition, 20λ of a chloroform extract of *P. methysticum* containing the equivalent of 1 Gm. of drug in 50 ml., and 20λ of the α-pyrone mixture (1γ/1λ) in chloroform were applied. Both plates were eluted with Skelly B/ethyl acetate (1:1) as previously described and were air-dried. One plate was sprayed with sulfuric acid and the duplicate plate with the Munier and Macheboeuf-Dragendorff spray. The results are illustrated in Figs. 3 and 4 and point out that the known α-pyrones, present in chloroform extracts of *P. methysticum*, react with Dragendorff's spray reagent. Sufficient quantities of these compounds are soluble in heated 1% hydrochloric acid, as indicated by the chromatographic results, to account for the previously described alkaloid-like reactions

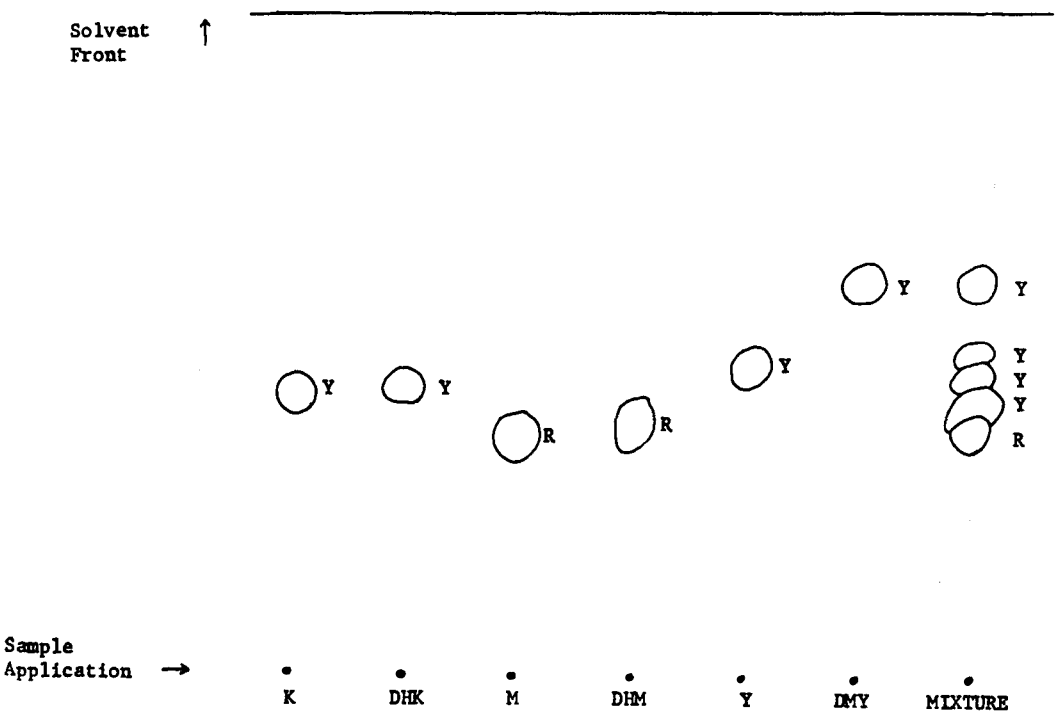


Fig. 2.—Thin-layer chromatogram of the six known *P. methysticum* α-pyrones in chloroform. Key (after sulfuric acid spray): Y = yellow, R = red.

TABLE III.—THE SENSITIVITY OF THE KNOWN *P. methysticum* α-PYRONES TO SULFURIC ACID AND DRAGENDORFF SPRAY REAGENTS[a]

α-Pyrone	Sulfuric Acid Spray: Minimal Detectable Quantity (γ) *ca.*	Sulfuric Acid Spray: Color	Dragendorff Spray: Minimal Detectable Quantity (γ) *ca.*	Dragendorff Spray: Color
Kawain	1	Yellow	10	Orange, fades to yellow
Dihydrokawain	4	Yellow	15	Orange, fades to yellow
Methysticin	1	Pink to red	10	Orange, fades to yellow
Dihydromethysticin	1	Pink to red	10	Orange, fades to yellow
Yangonin	1	Yellow	10	Orange, fades to yellow
Desmethoxyyangonin	4	Yellow	15	Orange, fades to yellow

[a] After thin-layer chromatography on silica gel G plates.

using Prollius extracts prepared with heat. Extracts prepared without the use of heat were only slightly soluble in 1% hydrochloric acid as evidenced by these results and undoubtedly the concentrations present in these Prollius extracts were too low to be detected by the precipitating agents employed in this study.

DISCUSSION

The results of this investigation clearly indicate that certain α-pyrone derivatives in *P. methysticum* can induce false-positive alkaloid reactions with several of the common alkaloid precipitating reagents. In addition, these compounds can react to produce similar results on chromatograms treated with the modified Dragendorff spray reagent. A critical analysis of the methods used to detect alkaloids in plant material can aid in the prevention, as well as in the interpretation of these false-positive reactions. If the Swanholm, Webb, or Kiang methods are to be utilized, heat should be avoided, or an additional purification step, as included in the Wall method, should be considered. The appearance of orange spots on chromatograms after treatment with the modified Dragendorff spray reagent should be

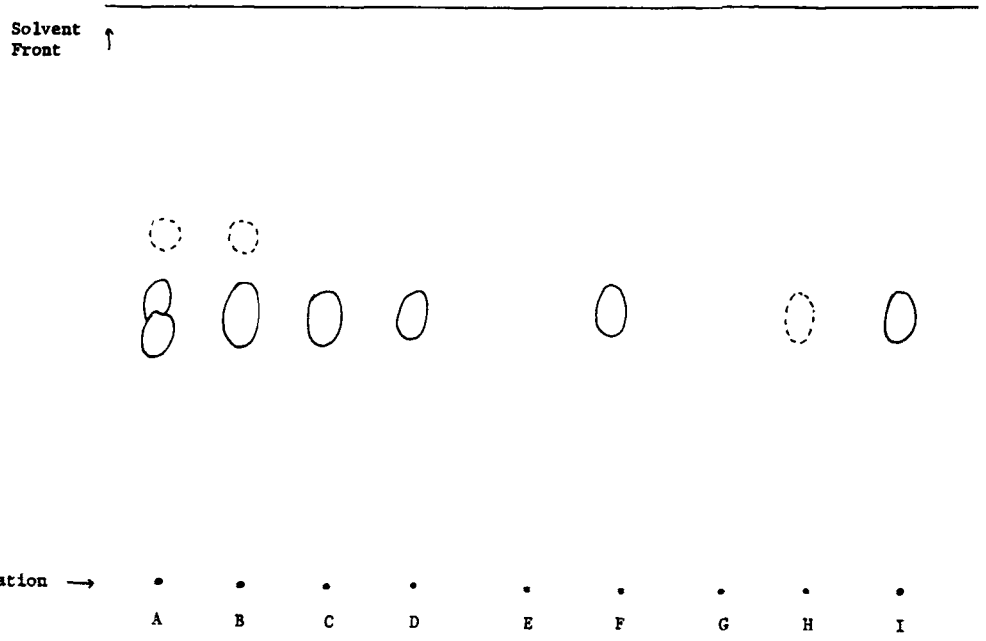


Fig. 3.—Thin-layer chromatogram of certain *P. methysticum* extracts, *P. methysticum*-treated control extracts, and control extracts. Key (after sulfuric acid spray): O = orange, P = pink, R = red, Y = yellow. Broken lines indicate trace concentrations. A, six known α-pyrones in chloroform; B, chloroform extract of *P. methysticum;* C, P. *methysticum* Prollius extract; D, *P. methysticum* Prollius extract + *H. virginiana;* E, *H. virginiana* Prollius extract; F, *P. methysticum* extract + *P. serotina;* G, *P. serotina* Prollius extract; H, *P. methysticum* extract + 1% HCl (unheated); I, *P. methysticum* extract + 1% HCl (heated).

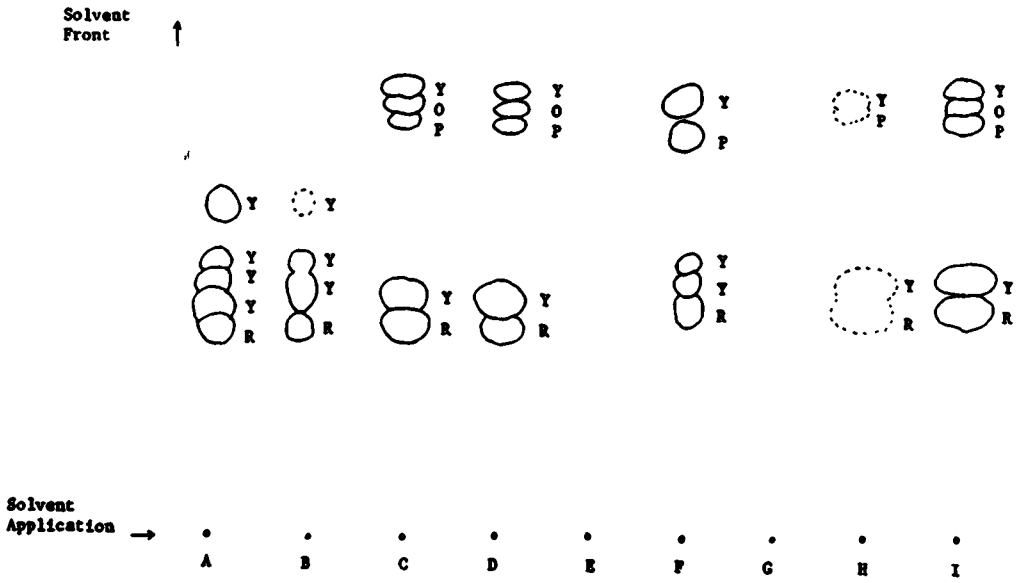


Fig. 4.—Thin-layer chromatogram of certain *P. methysticum* extracts, *P. methysticum* treated extracts, and control extracts following treatment with Dragendorff spray reagent. Circled areas indicate orange spots. Broken lines are indicative of trace concentrations. A, six known α-pyrones in chloroform; B, chloroform extract of *P. methysticum;* C, *P. methysticum* Prollius extract; D, *P. methysticum* Prollius extract + *H. virginiana;* E, *H. virginiana* Prollius extract; F, *P. methysticum* extract + *P. serotina;* G, *P. serotina* Prollius extract; H, *P. methysticum* extract + 1% HCl (unheated); I, *P. methysticum* extract + 1% HCl (heated).

considered indicative of the presence of alkaloids only when the suspected alkaloid spot persists for several hours, since compounds of the α-pyrone type, and perhaps others, will not retain the typical alkaloid-positive orange color with this reagent.

From the data presented, it is reasonable to assume that the occurrence of alkaloids in *P. methysticum*, as reported by several investigators, has been brought about through observations of the reactions due to the nonalkaloid α-pyrone constituents in this plant. These observations have prompted us to investigate other structurally related non-nitrogenous compounds, synthetic as well as naturally occurring, in order to gain additional information concerning the limitations of alkaloid detecting reagents as used in phytochemical studies. These results will be published at a later date.

SUMMARY

1. The nonalkaloid α-pyrone compounds present in *P. methysticum* have been shown to exhibit alkaloid-like reactions with several common alkaloid precipitating reagents.

2. The use of heat in alkaloid testing procedures increases the solubility of the α-pyrone compounds. This increased solubility is sufficient to allow detection of them with standard alkaloid reagents.

3. The α-pyrones present in *P. methysticum* will produce alkaloid-like color reactions on chromatograms treated with the Munier and Macheboeuf modified Dragendorff spray reagent. This effect is discussed and the sensitivity of the reaction on thin-layer chromatograms of the α-pyrones is reported.

4. The color reactions and sensitivity of kawain, dihydrokawain, methysticin, dihydromethysticin, yangonin, and desmethoxyyangonin after thin-layer chromatography and subsequent sulfuric acid spray, is reported.

5. Alkaloids have been shown to be absent from *P. methysticum*. Previous reports concerning the presence of alkaloids in this plant have been evaluated and discussed in view of the experimental results from this investigation.

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Complex Formation Influence on Reaction Rate I

Effect of Caffeine on Riboflavin Base-Catalyzed Degradation Rate

By DAVID E. GUTTMAN

The rate of the base-catalyzed decomposition of riboflavin was investigated in aqueous solution in the absence and presence of caffeine at a number of different temperatures. It was found that at all temperatures the apparent velocity of the reaction was decreased by the presence of caffeine and that the magnitude of this effect was dependent on the concentration of caffeine in the system. A mathematical relationship, expressing the relative degree of stabilization as a function of the caffeine concentration and the dissociation constant for the complex, was derived by assuming that a 1:1 complex formed and that the complexed riboflavin did not undergo reaction with the base. Dissociation constants which were calculated for the complex from the kinetic data were found to be in close agreement with values previously obtained by nonkinetic techniques. Free energy, enthalpy, and entropy changes which characterize the association were calculated to be −2,000 cal., −5,750 cal., and −12 e.u., respectively. The energy of activation for the degradative reaction was found to be the same in the presence of caffeine as in its absence.

A NUMBER of investigations have demonstrated that molecular complex formation can influence the rate at which participating species undergo chemical reaction. For example, the rate of the coupling reaction between β-naphthol and *p*-diazobenzenesulfonic acid was found by Overbeek, Vink, and Deenstra (1) to be lowered by the addition of caffeine to the reaction medium. The lowering of the reaction velocity was explained on the basis of lowered reactivities of the reactants in the complexed forms since both phenol and the sulfonic acid were shown to form 1:1 complexes with caffeine. Similarly, Higuchi, Lachman, Ravin, and Guttman (2,3,4) showed that the rates of hydrolysis of local anesthetic esters were substantially reduced by the addition of complexing agents to their aqueous solutions. Their investigations indicated that the esters, in the complexed forms, did not undergo hydrolytic cleavage at the ester linkage.

Pharmaceutical interest in this particular manifestation of complex formation is quite naturally derived from the possibility of utilizing such behavior to stabilize labile medicinal agents. More comprehensive studies of this phenomenon would, therefore, be of value in amplifying and extending this interest and could, in addition, be of more fundamental importance in providing further insights into the nature of the bonding forces responsible for molecular associations. The extent to which the reactivity of a compound is affected by complexation might serve as an indication of the type of interactive mechanism that is primarily operant. Correlation and comparison of such studies with results obtained by nonkinetic methods might also serve to pinpoint functional groups as sites of contact between components in the complex. Alternatively, it is possible that a kinetic approach to the determination of the energetics of complex formation might be more direct and preferred in some cases to many of the classical approaches which are commonly used.

This communication summarizes the results of a preliminary investigation of the effect of mo-

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