(10) M. Guggenheim and W. Löffler, *Biochem. Z.*, 72 (1916), 325.
(11) M. Guggenheim, *Die biogenin Amine*, Julius Springer, Berlin, 2nd Edition, 1924, 335.
(12) R. H. F. Manske, *Can. J. Research*, 5 (1931), 592.
(13) M. Greshoff, *Mededeeliigen uit Lands Plantantuin*, 7 (1890), 29 (cited by Romburgh and Barger).
(14) J. Marañon and J. K. Santos, *Philippine J. Sci.*, 48 (1932), 563.
(15) P. v. Romburgh and G. Barger, *J. Chem. Soc.*, 99 (1911), 2068.
(16) R. W. Jackson, *J. Biol. Chem.*, 84 (1929), 1.
(17) H. Wieland, G. Hesse and H. Mittasch, *Ber. deut. chem. Gesellsch.*, 64 (1931), 2099.
(18) K. K. Chen, H. Jensen and A. L. Chen, *J. Pharmacol. & Exper. Therap.*, 43 (1931), 13.
(19) G. Barger and H. H. Dale, *J. Physiol.*, 41 (1910-1911), 19.

## PHYTOCHEMICAL NOTES.*[1]

### No. 109. On the Non-Preëxistence of Azulene in Milfoil.

BY KATHERINE GRAHAM.[2]

The preëxistence of azulene in plants was first questioned by Tschirch and Hohenadel in 1895 (1), when they observed that sagapen yielded a yellow oil upon extraction with petroleum ether and that this oil became blue during fractionation. Not knowing whether the formation of the blue substance was due to the exposure of the volatile oil extracted with petroleum ether or to resin extracted at the same time, they prepared a resin-free volatile oil by steam distillation. This also was faintly yellow and only upon fractional distillation involving higher temperatures, *viz.*, abt. 200°, did they obtain a blue fraction. They, therefore, arrive at the conclusion that "without doubt, the blue oil is a pyrogenic decomposition product" (2). In 1917, however, Tschirch expresses himself as still in doubt as to whether the azulene is formed during the process of distillation (3).

Herzenberg and Ruhemann made a similar investigation in 1927 (4). They found that chamomile yielded only a small amount of a yellow oil to petroleum ether but that the extracted plant yielded a blue oil upon steam distillation. From this they concluded that azulene did not preëxist in the plant and that its formation was from sesquiterpenes by fermentative action especially since they had isolated a sesquiterpene which yielded a blue color by dehydrogenation. However, the experiment performed does not support this conclusion. If the azulene were formed from sesquiterpenes, it would not then be obtained from the extracted marc, from which the sesquiterpenes had been removed. Furthermore, fermentative action would not be expected during steam distillation, where the temperature is much above the thermal death point of enzymes. Nor is dehydrogenation likely to take place during steam distillation.

The existence of azulene in the plant may more logically be explained by the assumption of an acid addition product since it is known that azulene readily forms such a product which is not soluble in petroleum ether. The union of azulene with either phosphoric acid or an acid phosphate would presumably give

* From the laboratory of Edward Kremers.
[1] Scientific Section, A. PH. A., Miami meeting, 1931.
[2] Fritzsche Bros. Fellow.

a compound which could not be extracted with petroleum ether but which could be decomposed by the action of steam.

Another hypothesis which may explain the presence of combined azulene, *i. e.*, in a form which cannot be extracted with petroleum ether, is that it may exist in glucosidal combination. We usually consider glucosides to be ether-like products of the union of a sugar and an alcohol. It is barely possible that a hypothetical colorless alcohol, $C_{15}H_{19}OH$, when set free and at the higher temperatures involved in fractionation, loses a molecule of water thus yielding the blue hydrocarbon. This hypothesis is based on a possible analogy with the formation of a terpene, $C_{10}H_{16}$, observed by Kayser (5) in 1884 when he obtained it upon the hydrolysis of the glucoside, picrocrocin. The presumption in this case is that a terpene alcohol results upon hydrolysis of the glucoside and that under the conditions of the experiment the alcohol breaks up into the terpene and water.

The experiment of Herzenberg and Ruhemann made with chamomile has been repeated upon a larger scale with milfoil. The results found are in accordance with those of the earlier investigators.

The material used was collected in June 1930, from the garden of the Wisconsin Pharmaceutical Experiment Station and from land southwest of Madison, under the direction of Professor W. O. Richtmann. Having been air dried, the inflorescences were carefully removed and ground in a Grumbach mill.

*I. Extraction of the Flowers with Petroleum Ether and Subsequent Distillation.*—4988 Gm. of the ground milfoil flowers were extracted with petroleum ether in a Lloyd extractor. The flowers thus extracted were exposed so as to allow any adhering petroleum ether to evaporate. They were then transferred to a 60-liter Lentz copper still and subjected to steam distillation. The periods of distillation and the amount of aqueous distillate obtained in each are herewith tabulated:

| Period—Day. | Time—Hours. | Amount—Gallons. | Period—Day. | Time—Hours. | Amount—Gallons. |
|---|---|---|---|---|---|
| 1st | 2 | 7 | 6th | 0 | 0 |
| 2nd | 3 | 10 | 7th | 5 | 15 |
| 3rd | 2 | 12 | 8th | 1½ | 5 |
| 4th | 0 | 0 | 9th | 4½ | 20 |
| 5th | 0 | 0 | 10th | 1 | 5 |
| | | | Totals | 19 | 74 |

The distillation was discontinued when no more color could be extracted from the distillate with ether. The aqueous distillate, which gave no acid reaction with litmus paper, was shaken with ether, and the ethereal solution separated. The solvent was recovered by distillation and there remained 4.8 cc. (0.096 per cent) of a deep blue oil, which had a density of 0.9516 at 25°. This oil was treated with phosphoric acid, and the azulene-phosphoric acid compound hydrolyzed with water. The liberated azulene was extracted with ether and after the removal of the solvent 0.5 cc. of azulene remained (0.01 per cent).

The petroleum-ether extract resulting from the percolation of the flowers was distilled under reduced pressure to remove the solvent. The residue thus obtained weighed 186 Gm. When steam distilled, 12 cc. (0.24 per cent) of a light blue oil separated from the aqueous distillate. It had a density of 0.9105 at 25°. The oil was fractionated under atmospheric pressure with the following results:

| Temperature. | Amount. |
|---|---|
| −170° | 0.3 cc. |
| 170–180° | 2.0 cc. |
| 180–190° | 2.5 cc. |
| 190–200° | 2.1 cc. |
| 200°+ | 4.2 cc. |

*II. Extraction of the Azulene Compound with Chloroform. 1. Hot.*—100 Gm. of the ground flowers were placed in a continuous extractor and exhausted with chloroform. The heat of the vapors was sufficient to keep the small percolator warm during the extraction. The solvent was removed from the extract by distillation under reduced pressure and the extract washed with solvents, resulting in the following products:

(A) A petroleum-ether extract,

(B) An ether extract and

(C) A residue.

(A) The petroleum-ether extract was steam distilled and the distillate washed with ether. The ethereal solution was colored blue.

(B) The ether extract was steam distilled and the distillate washed with ether. The ethereal solution was also colored blue.

(C) The residue was steam distilled and the distillate washed with ether, yielding no blue color.

(D) The extracted flowers were distilled with steam and the aqueous distillate yielded no blue color when washed with ether.

*2. Cold.*—1000 Gm. of the dried flowers were extracted by maceration with chloroform at room temperature. From the chloroformic extract the solvent was removed by distillation under reduced pressure. This extract was treated as in the previous experiment and resulted in similar products:

(A) The petroleum ether was removed by distillation under reduced pressure. The extract was steam distilled and yielded 3.4 cc. (0.34 per cent) of a yellow oil.

(B) The ether extract was steam distilled after the removal of the ether. The distillate, when washed with ether, yielded a blue color.

(C) The residue was steam distilled and the distillate yielded a blue color.

(D) The extracted flowers, when steam distilled, yielded a faint blue color.

*3. Extraction by Percolation.*—Two samples of 1000 Gm. each of the ground flowers were packed in a percolator and extracted with chloroform. The chloroform was removed from the extract by distillation under reduced pressure. The extracts were treated as in the previous experiment:

(A) The petroleum ether was removed by distillation under reduced pressure. The extracts were steam distilled and 3 cc. and 3.1 cc., respectively, of a yellow oil resulted.

(B) The ether was removed by distillation under reduced pressure and the extracts steam distilled. The distillate from both extracts yielded a blue color.

(C) The residues were steam distilled and both yielded a blue color.

(D) The extracted flowers were distilled with steam and no blue color was obtained.

*4. Preparation of the Chloroform Extract on a Larger Scale.*—15,400 Gm. of the ground flowers were packed in percolators and extracted with chloroform. The chloroform was removed by distillation under reduced pressure and 1520 Gm. of extract resulted. The extract was washed with petroleum ether, resulting in two products:

(A) 377 Gm. of a petroleum-ether extract, and

(B) 790 Gm. of residue.

*III. The Azulene Compound.*—The 790 Gm. of chloroform extract deprived of its petroleum ether-soluble constituents and containing the azulene compound were used in the following experiments.

(*1*) *Determination of the Inorganic Constituents.*

(*a*) 0.8277 Gm. of the extract yielded no ash.

(*b*) 1.6176 Gm. of the extract yielded 0.0001 Gm. ash.

These results indicate the improbability of an acid salt addition product since azulene is not known to add to any organic acid except formic, with which it forms a liquid and easily hydrolyzed compound.

(*2*) *Acid Hydrolysis.*—If azulene is contained in the molecule in glucosidal combination, hydrolysis with acid should liberate it from the accompanying sugar molecule, which could be detected. The extract reduced Fehling's solution before hydrolysis. After hydrolyzing with dilute hydrochloric acid the amount of copper oxide was increased but constant values could not be obtained.

The extract was hydrolyzed for ten hours with 5 per cent sulphuric acid. After the reaction mixture was filtered it was divided and made neutral with sodium hydroxide. One-half of the neutralized solution was extracted with ethyl acetate. Both portions were then treated with Fehling's solution. The untreated portion did reduce Fehling's solution. The portion washed with ethyl acetate did not reduce the copper solution.

(*3*) *Ester Value.*—The ester value of the extract was determined in order to obtain an indication of the possibility of alkaline hydrolysis.

(*a*) 0.4204 Gm. of the extract required 0.379 cc. of normal potassium hydroxide for neutralization, corresponding to an acid value of 36. When heated for one-half hour, the sample reacted with 1.94 cc. of normal potassium hydroxide, corresponding to a saponification value of 184. Ester value = 148.

(*b*) 0.4046 Gm. of the extract required 0.4 cc. of normal potassium hydroxide for neutralization, corresponding to an acid value of 39. When heated, it reacted with 1.94 cc. of normal potassium hydroxide, corresponding to a saponification value of 191. Ester value = 152.

(*4*) *Alkaline Hydrolysis.*—The extract was treated for an hour with sodium hydroxide solution without heat. The mixture was filtered on a force filter and the residue (*a*) washed with water. The filtrate was made acid with dilute hydrochloric acid, added slowly and with continuous stirring. The resulting precipitate was separated on a force filter and washed with water (*b*). The filtrate was distilled to dryness and the residue extracted with alcohol, which was removed by distillation (*c*). The results of the hydrolysis are shown in the accompanying table:

| Reagent NaOH. | Extract. | Insoluble Substance (*a*). | Soluble substance (*b*). | Residue (*c*). |
|---|---|---|---|---|
| 5 p. c. | 4 Gm. | 0.05 Gm. | 0.7 Gm. | ..... |
| 5 p. c. | 10 Gm. | 0.3 Gm. | 4.5 Gm. | 3.0 Gm. |
| 5 p. c. | 10 Gm. | 0.1 Gm. | 2.0 Gm. | 7.2 Gm. |
| 5 p. c. | 10 Gm. | 0.1 Gm. | 4.3 Gm. | 3.5 Gm. |
| 2 p. c. | 10 Gm. | 2.7 Gm. | 1.2 Gm. | 5.0 Gm. |
| 2 p. c. | 10 Gm. | 2.7 Gm. | 1.9 Gm. | 4.5 Gm. |
| 2 p. c. | 10 Gm. | 3.7 Gm. | 1.0 Gm. | 4.5 Gm. |

The acid precipitated substances of the first four experiments were bulked and neutralized with sodium hydroxide. This sodium salt was used to prepare the barium and silver salts. 5 Gm. of the sodium salt yielded 2 Gm. of the barium salt, which was analyzed for barium content.

(*a*) 0.9198 Gm. of the barium salt yielded 0.0968 Gm. of barium carbonate corresponding to 7.32 per cent of barium.

(*b*) 1.0230 Gm. of the barium salt gave 0.1070 Gm. of barium carbonate, corresponding to 7.38 per cent of barium.

5 Gm. of the sodium salt were treated with silver nitrate and yielded 5.5 Gm. of silver salt, which upon analysis yielded the following results:

(*a*) 0.9760 Gm. of the silver salt gave 0.2395 Gm. of silver, corresponding to 24.5 per cent of silver.

(*b*) 1.0945 Gm. of silver salt gave 0.2690 Gm of silver, corresponding to 24.57 per cent of silver.

Attempts to form oximes or acetyl derivatives with other products of the hydrolysis yielded no results.

(*5*) *Carbonyl Oxygen.*—(*a*) Reaction with phenyl hydrazine. 0.5 Gm. of the extract was dissolved in alcohol. 2 cc. of acetic acid and 5 cc. of phenyl hydrazine were added. The mixture was warmed for 10 minutes. Upon cooling, small crystals of phenylhydrazine acetate, m. p. 127°, settled out and were filtered off. The mother liquor yielded an amorphous product with no characteristic melting point.

(*b*) Reaction with hydroxylamine. 2 Gm. of the extract were dissolved in alcohol and treated with 2 Gm. of hydroxylamine hydrochloride and sodium carbonate. Upon standing, a grayish green amorphous precipitate formed. This precipitate was filtered off and treated with hydrochloric acid. The resulting solution gave no reduction of Fehling's solution.

(*6*) *Hydroxyl Groups.*—(*a*) Acetylation. 5 Gm. of the extract were heated with acetic acid anhydride and sodium acetate for one hour. The product was washed with sodium car-

bonate solution, then with water and dried. The saponification value of this product could not be determined because of the color.

(*b*) Benzoylation. 13.5 Gm. of the extract were dissolved in 50 cc. of pyridine and cooled well. 15 cc. of benzoyl chloride were added in small portions, shaking after each addition. White crystals separated which were filtered off on a force filter and washed with pyridine. The platinum double salt of these crystals melted at 240° (indicating pyridine hydrochloride). The filtrate was diluted with ether. 2.2 Gm. of an insoluble residue remained, which when treated with sodium hydroxide gave an odor of pyridine. The ethereal solution was washed with dilute hydrochloric acid, with sodium carbonate solution and with water and the ether removed by distillation under reduced pressure. 14 Gm. of the ester were obtained.

(*a*) 1.0860 Gm. of the ester reacted with 4.88 cc. of normal sodium hydroxide, corresponding to a saponification value of 178.

(*b*) 0.9885 Gm. of the ester reacted with 4.39 cc. of normal sodium hydroxide, corresponding to a saponification value of 179.

7. *Oxidation with Nitric Acid.*—Since the action of nitric acid upon azulene is not known it would render the situation less complicated if the azulene could be removed from the molecule before oxidation. Steam distillation of the extract yielded the azulene very slowly, some blue color being obtained after 70 hours of distillation. Therefore, other means for the removal of the azulene were sought.

5 Gm. of the extract were mixed with 300 cc. of water and placed in a heavy-walled, tightly stoppered bottle and heated on an oil-bath at 150° for eight hours. The contents of the bottle were then steam distilled. The first distillate yielded a deep blue color and as distillation continued less and less of the color was obtained. However, after 60 hours of distillation some blue color was still obtained.

Acid hydrolysis, using 15 per cent hydrochloric acid in amyl alcohol solution (6), yielded no azulene when the contents of the flask were steam distilled. The same reaction was tried under pressure with no different results. Therefore, the attempts to remove azulene from the molecule were abandoned.

Oxidation of the extract was conducted in the following manner: 5 Gm. of the extract were mixed with 20 cc. of nitric acid, added slowly while the mixture was kept well cooled. The mixture was then allowed to warm slightly, almost to room temperature. As soon as brown fumes were evolved the mixture was cooled in an ice-bath so that the reaction proceeded slowly. When no more brown fumes were evolved from the mixture at room temperature, the reaction was regarded as complete. An insoluble substance rose to the surface of the liquid and was separated and washed with water. The reaction mixture was diluted with water and a flocculent precipitate resulted which was filtered off and washed with water. The bright yellow aqueous liquids were united and saved for further investigation.

The results of four such experiments are given in the following table.

| Extract. | Insoluble Substance. | Soluble Substance. | Total. |
|---|---|---|---|
| 5 Gm. | ..... | 1.19 Gm. | ..... |
| 5 Gm. | 3.89 Gm. | 0.45 Gm. | 4.34 Gm. |
| 5 Gm. | 1.6 Gm. | 1.6 Gm. | 3.2 Gm. |
| 5 Gm. | 1.6 Gm. | 1.22 Gm. | 2.82 Gm. |

The products were combined and washed with boiling alcohol. This procedure yielded 1.4 Gm. of a substance insoluble in alcohol, 1.2 Gm. of a substance which precipitated from the hot alcohol and 6 Gm. of an amorphous residue left upon evaporation of the mother liquid. This residue was acid to litmus, soluble in sodium hydroxide solution from which it was precipitated by acid. From the sodium salt a barium and a silver salt were prepared.

(1) 0.3330 Gm. of the barium salt yielded 0.0190 Gm. of barium carbonate corresponding to 4.1 per cent of barium.

(2) 0.7100 Gm. of the barium salt yielded 0.5000 Gm. of barium carbonate, corresponding to 4.7 per cent of barium.

(3) 0.8080 Gm. of the silver salt yielded 0.1762 Gm. of silver, corresponding to 21.8 per cent of silver.

(4) 0.7000 Gm. of the silver salt yielded 0.1470 Gm. of silver, corresponding to 20.0 per cent of silver.

The aqueous liquids from the oxidation mixture were distilled and resulted in a clear, acid distillate and a yellow, acid residue from which nothing solid separated. The distillates were combined and neutralized with barium carbonate. The mixture was filtered and the residues tested for organic matter. None was found present. The aqueous portion was concentrated and the crystals obtained did not char. This excludes the possibility of volatile acids resulting from the oxidation.

The residues were combined and neutralized with barium carbonate. The excess barium carbonate was filtered off and gave no test for organic matter. The aqueous portion when evaporated to dryness was found to contain organic material. A positive test for picric acid could not be obtained.

SUMMARY.

It has been shown that azulene does not preëxist in the flowers of milfoil but is formed during the process of distillation in the preparation of the volatile oil.

The azulene-yielding compound is contained in the chloroformic extract from which the petroleum ether-soluble constituents (bulk of the so-called volatile oil but minus the blue azulene) have been removed.

The nature of this compound has not yet been determined.

BIBLIOGRAPHY.

(1) *Arch. Pharm.*, 233 (1895), 259.
(2) *Ibid.*, 279.
(3) Tschirch, *Handbuch. d. Pharmakognosie*, Vol. II, page 964.
(4) *Ber.*, 60 (1927), 2464.
(5) *Ibid.*, 17 (1884), 2228.
(6) Power and Salway, *J. Chem. Soc.*, 103 (1913), 399.

---

## WASHINGTON BELLADONNA AND METHODS OF ASSAY.*

BY CLAIRE EVANS AND F. J. GOODRICH.

The somewhat unstable character of the active principles found in belladonna plants is well known and, in fact, the chemistry and structure of the important components have been thoroughly studied. The assay of the roots, as well as the leaves, of many belladonna plants for mydriatic alkaloids has been made, resulting in great variations with different samples. Some quantitative methods have been tried experimentally on prepared samples of the drug and many explanations offered as to the varying amounts of the alkaloids. Undoubtedly, numerous factors are responsible for the large differences in alkaloidal content of both roots and leaves.

It has been deemed of interest to investigate the alkaloidal content of belladonna roots collected over a period of years, using different, selected methods for making the determinations. Roots grown on the University of Washington campus were chosen because no assays, so far as could be learned, had been made on belladonna of western Washington. The purpose of the present study has been, therefore, to select a satisfactory method of assay and to determine the quantity of alkaloids from roots grown in the Pacific Northwest, collected in successive years and aged for varying periods.

---

* Scientific Section, A. PH. A., Toronto meeting, 1932.