lar region or fed orally through a catheter. After a short period of rest and assimilation, blood for analysis was withdrawn from the ear lobe by severing one of the larger veinlets, which bulge on the outer surface, and allowing the blood to drop into a clean crucible containing a small amount of potassium oxalate to prevent coagulation. An exact amount of the oxalated blood was then pipetted from the dish and analyzed according to the method of Folin and Wu (7).

In the appended graph, the blood sugar reading is given at the left, in milligrams for each 100 cc. of blood, and the time in minutes from left to right. Dotted curve Number 2 represents the effect of feeding glucose hypodermically to starved rabbits. Solid line Number 3 shows the effect upon the same rabbits after administering Neomyrtillin hypodermically.

CONCLUSIONS.

1. The entire pharmacology of blueberry leaf has been studied.
2. The glucoside $C_{24}H_{36}O_{16}$ was isolated, purified, studied and the non-descriptive name Neomyrtillin given to it.
3. Neomyrtillin was found to possess hypoglycemic properties when administered to rabbits having induced alimentary hyperglycemia.

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A STUDY OF THE ASSAY OF ACONITE AND THE STABILITY OF ITS PREPARATIONS.*,[1]

BY GEO. BAKER[2] AND DEAN CHAS. B. JORDAN.

Aconite, though employed in medicine since the thirteenth century, has never had a completely satisfactory assay. Much confusion existed in the early investigation of the drug. It was not until about 1900 that the alkaloids existing in Aconite were definitely identified and their relative physiological action determined. The eighth revision of the United States Pharmacopœia (U. S. P. VIII) possessed a chemical assay for the drug and its preparations while the ninth revision carried an alternative physiological assay. The tenth revision of the U. S. P. dropped the chemical assay and made the bioassay official. The chemical assay method was unsatisfactory in that it determined total alkaloids. The alkaloids of *Aconitum Napellus* are aconitine, benzoylaconine, and aconine. Aconitine is the active agent and a practical assay must therefore determine the aconitine present in the drug and its preparations.

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* Scientific Section, A. Ph. A., Portland meeting, 1935.

[1] Based upon a thesis by Geo. L. Baker submitted to the Faculty of Purdue University in partial fulfilment of the requirements for the degree of Doctor of Philosophy, June 1935. (A fairly complete bibliography accompanies the thesis.)

[2] Eli Lilly & Co. Fellow, 1933–1935.

The purpose of this investigation was to work out a satisfactory chemical assay procedure for Aconite and its preparations and to further check the work of previous investigators on the stabilization of Aconite preparations. Such an assay has been worked out in this laboratory and is presented on the following pages. Work has been carried out on stabilization of the tincture and fluidextract and the determination of any change in p_H during storage of the tincture and fluidextract.

EXPERIMENTAL—PART I.

Stabilization and Changes in p_H of Aconite Preparations.—The studies undertaken are indicated in the title. Two phases were investigated regarding the stability of the official preparations of Aconite. The first of these involved the change in the p_H value of the tincture and fluidextract during storage and this phase will be discussed here.

For this determination tincture and fluidextract of Aconite were obtained from Eli Lilly and Company of Indianapolis, Indiana, and the p_H determined. Samples of 60 cc. each were then stored in the following manner: (*a*) two samples in colorless glass bottles were stored in diffused light, one remaining closed throughout storage and one being opened occasionally to simulate drug store conditions; (*b*) two samples in amber-colored bottles were stored and treated as in (*a*); four samples, two in colorless glass bottles and two in amber-colored bottles, were stored in a dark compartment and treated in the same manner as the samples in diffused light. The hydrogen-ion concentrations of these samples were determined after thirteen months' storage. They were assayed biologically at about the same time. Results are given in the table.

TABLE I.—TINCTURE OF ACONITE, U. S. P. (p_H 4.62 AT 25 DEGREES, 10/19/33).

Sample No.	Color of Bottle.	Conditions of Storage. Light Conditions.	Closed or Open.	p_H at Storage 10/19/33.	p_H after 13 Mos'. Storage 11/14/34.	Bioassay 1/12/35 M. L. D.
I	Amber	Diffused	Closed	4.62	3.53	0.000346 cc.
II	Amber	Diffused	Opened	4.62	3.53	......
III	Colorless	Diffused	Closed	4.62	3.45	0.00035
IV	Colorless	Diffused	Opened	4.62	3.35	......
V	Amber	Dark	Closed	4.62	3.35	0.000325
VI	Amber	Dark	Opened	4.62	3.41	......
VII	Colorless	Dark	Closed	4.62	3.28	0.000346
VIII	Colorless	Dark	Opened	4.62	3.29	......

In the following table are given the results obtained with the fluidextract.

TABLE II.—FLUIDEXTRACT OF ACONITE, N. F. (p_H 4.22 AT 25 DEGREES, 10/22/33).

Sample.	Color of Bottle.	Conditions of Storage. Light Conditions.	Closed or Open.	p_H at Storage 10/21/33.	p_H after 13 Mos'. Storage 11/14/34.	Bioassay 1/12/35 M. L. D.
B	Amber	Diffused	Closed	4.22	3.15	0.000035 cc.
A	Amber	Diffused	Opened	4.22	3.12	......
C	Colorless	Diffused	Closed	4.22	3.12	0.000035
D	Colorless	Diffused	Opened	4.22	2.99	......
E	Amber	Dark	Closed	4.22	3.15	0.000035
F	Amber	Dark	Opened	4.22	3.07	......
G	Colorless	Dark	Closed	4.22	3.20	0.0000375
H	Colorless	Dark	Opened	4.22	3.17	......

The obvious conclusion from the change in p_H value is that there was liberation of an acid or acids. This confirms the conclusions previously advanced that aconitine is hydrolyzed into benzoylaconine and acetic acid and that benzoylaconine is hydrolyzed into aconine and benzoic acid. Since these are official preparations and must therefore have conformed, when the study was begun, to the standards set forth in the U. S. P. and N. F., the minimum lethal dose of the fluidextract must not have been greater than 0.00004 cc. per Gm. body weight of guinea pig and that of the tincture not less than 0.00035 cc. and not more than 0.00045 cc. per Gm. body weight of guinea pig. Reference to the preceding tables will show that in the majority of the samples there has been an increase in activity, *i. e.*, a smaller amount was required to produce death than is required of the official preparations. We have no explanation to advance for this increase in biological activity. It is possible that the inaccuracy of bioassay methods may be the explanation.

The second phase involves stabilization by the addition of acid. For this experiment tincture and fluidextract of Aconite were prepared from Aconite root provided by Eli Lilly and Company. The potency of these preparations was determined biologically. The tincture had a minimum lethal dose of 0.00046 cc. per Gm. body weight of guinea pig and the fluidextract had 0.000039 cc. per Gm. body weight of guinea pig. Samples were brought to definite p_H values by the addition of varying amounts of hydrochloric acid. One-ounce samples of the tincture with p_H values of 1.88, 2.30, 2.50, 2.83, 3.05, 3.20, 3.40, 3.55, 3.83, 3.95, 4.19, 4.40, 4.68 and 5.11, respectively, were stored on December 23, 1933, and a selected few again assayed on April 22, 1935. The results are as follows:

Sample assayed 0.00046 cc./Gm. body weight before storage.
Sample with p_H of 1.88 assayed 0.00045 cc./Gm. body weight after storage.
Sample with p_H of 2.83 assayed 0.00050 cc./Gm. body weight after storage.
Sample with p_H of 3.05 assayed 0.00055 cc./Gm. body weight after storage.
Sample with p_H of 3.95 assayed 0.00065 cc./Gm. body weight after storage.
Sample with p_H of 5.11 assayed 0.00400 cc./Gm. body weight after storage.

One-ounce samples of the fluidextract having p_H values of 1.50, 2.20, 2.65, 2.80, 3.00, 3.26, 3.35, 3.56, 3.80, 4.05, 4.25, 4.40, 4.60 and 4.90, respectively, were stored on December 22, 1933, and a selected few again assayed on April 4, 1935. The results are as follows:

Sample assayed 0.000039 cc./Gm. body weight before storage.
Sample with p_H of 1.50 assayed 0.000035 cc./Gm. body weight after storage.
Sample with p_H of 2.65 assayed 0.000035 cc./Gm. body weight after storage.
Sample with p_H of 3.00 assayed 0.000035 cc./Gm. body weight after storage.
Sample with p_H of 4.05 assayed 0.000040 cc./Gm. body weight after storage.
Sample with p_H of 4.90 assayed 0.000055 cc./Gm. body weight after storage.

Careful inspection of the preceding tabulated data will show that in the case of the tincture decomposition began to take place to a marked degree when the p_H of the sample was 3.95 or above and in the fluidextract when the p_H was greater than 4.05. The increasing minimum lethal doses of the samples are indicative of this conclusion. Swanson (1) has shown that increased acidity tended toward stabilization and later (2) stated that above a p_H of 4.1 the preparations rapidly lost in potency. Wm. I. Baker (3) checked the work of Swanson and agreed with his conclusions. The experiments performed in this laboratory check those of Swanson and Wm. I. Baker and confirm their conclusions that a p_H of 2.5 to 3.0 would be the ideal range for stabilization of the preparations of Aconite.

EXPERIMENTAL—PART II.

Assay.—Before assay procedures could be developed, it was necessary to obtain the alkaloids of Aconite in a chemically pure condition. It was found that only one of the alkaloids, namely aconitine, could be purchased, and this only in an impure form from Merck & Company at Rahway, N. J. It therefore became necessary to purify it, and prepare the two hydrolytic products, benzoylaconine and aconine, from this alkaloid.

Aconitine as purchased from Merck is labeled "Aconitine, Potent, Merck, 10 to 15 times strength of amorphous." No other statement as to its purity is made. The melting point of the product, as received, was 179 degrees which does not check with the accepted value of 195 degrees for aconitine. Titration of accurately weighed samples of the product showed the sample to be only 96% pure. The product was then dissolved in 1% HCl and placed in a vacuum desiccator over phosphorus pentoxide. The crystals of aconitine hydrochloride formed upon concentration were washed with ether and then dried between filter papers. The base was then liberated from the hydrochloride, washed with water, and dried. The melting point was still not sharp at 180° C. The alkaloid was again dissolved in weak HCl acid, crystallized and dried as before and again liberated. This procedure was repeated several times until the hydrochloride melted at 151–153° C. which is well within the range of 149–153° C. given in the literature. The free base melted at 193° C. Titration of accurately weighed samples of the purified base gave values indicative of 99.4% aconitine. Further attempts at purification gave negative results, therefore this alkaloid was accepted for use in the following experiment.

Several methods of preparation of benzoylaconine and aconine were studied and a few tried out. Those giving the best results are given below in detail.

The hydrochloride of aconitine was prepared by dissolving pure aconitine in 1% hydrochloric acid and the solution concentrated in a vacuum desiccator over phosphorus pentoxide and then allowed to crystallize. The crystals were filtered off and dried. Three to four per cent aqueous solutions of the salt were prepared and sealed in thick-walled pyrex test-tubes. These solutions were heated at 120° to 130° C. in an oil bath from three to four hours. The solutions were then filtered and concentrated on a steam-bath. The crystals formed during concentration were filtered off and dissolved in distilled water. This solution was concentrated and the procedure repeated until the crystals of benzoylaconine hydrochloride were of a constant melting point. The melting point of this salt was 212° to 214° C. (uncorr.) which agrees well with that given in the literature.

Liberation of the benzoylaconine from this salt was carried out by carefully adding weak ammonia water and extracting with ether. After evaporating the ether and drying, the base melted at 125° C., which also agrees with other data. This method was employed throughout for the preparation of benzoylaconine and its hydrochloride.

Aconine was prepared by aqueous hydrolysis of aconitine. A mixture of aconitine and water (1 to 2 Gm. in 40 to 50 cc. of water) was sealed in a tube and heated at 120° to 130° C. for several hours. The aqueous solution was then extracted with ether and the ether rejected. After addition of sulphuric acid the solution was again extracted with ether to remove benzoic acid. The aqueous solution was then evaporated to a low volume to drive off the acetic acid. Barium hydroxide was carefully added until precipitation of the sulphate radical was complete. After filtration to remove the barium sulphate the solution was evaporated to dryness. The residue was washed with ether and then dried. The base thus obtained melted at 130–132° C. and its hydrochloride melted at 174–175° C. These values satisfy those given in the literature for aconine and its hydrochloride.

In order to work out an assay for this drug and its preparations, the most feasible method would be one in which the alkaloids could be separated. Attempts were made to do this based upon solubility and precipitants. Since aconine is insoluble in ether in the presence of water this alkaloid is immediately eliminated

by extraction of the other alkaloids with ether. Then the problem becomes one of separating aconitine and benzoylaconine.

Several alkaloidal precipitants were employed in this procedure using the pure alkaloids in the form of their hydrochlorides. In each case a small quantity of each alkaloidal salt was dissolved in a weakly acid aqueous solution and the precipitant added. In every case with the exception of Na_2CO_3 solution and NH_4OH solution saturated or partially saturated with NH_4Cl the two alkaloids, aconitine and benzoylaconine, are precipitated. With the carbonate and hydroxide solution aconitine is precipitated but benzoylaconine is not.

The results show that the two alkaloids cannot be separated by the ordinary alkaloidal precipitants since both are precipitated. There exists in the reactions with ammonium hydroxide and the carbonates some possibility. After repetition of these experiments for confirmatory purposes, a deeper investigation involving these agents was carried out. The results are given later.

Solubility determinations were made on aconitine and benzoylaconine as follows:

The pure alkaloids were first employed by placing a small quantity of each alkaloid on separate watch glasses and adding equal amounts of the solvent to each watch glass. Both alkaloids were found to be soluble in benzene, carbon bisulphide, fairly soluble in *n*-propyl and iso-propyl alcohols, and insoluble, or practically so, in *n*-butyl and amyl alcohols. Both are soluble to about the same extent in di-iso-propyl, dibutyl and di-iso-amyl ethers.

Determinations of the solubility at 25° C. of aconitine hydrochloride and benzoylaconine hydrochloride in water and ethyl alcohol (U. S. P.) were carried out. The procedure followed was to heat a watch glass at 100° C. to constant weight, cool and then place a small quantity of a saturated solution of the salt in question upon it. The two were then quickly weighed and placed in a desiccator over calcium chloride until dry. The glass and solid were then heated at 80° C. for one hour, cooled and weighed. Aconitine hydrochloride was found to be soluble to the extent of 0.1139 Gm. per Gm. of water and 0.7346 Gm. per Gm. of alcohol, while the corresponding values for benzoylaconine were 0.0803 Gm. per Gm. of water and 0.1499 Gm. per Gm. of alcohol. These experiments lead to the conclusion that the alkaloids could not easily be separated on solubility behavior.

The fact that von Planta (4) found aconitine to be partially soluble in sodium carbonate solution and that this aconitine was later found to be of dubious purity, led to the question of whether or not pure aconitine was soluble in sodium carbonate solution. To determine the possibilities appearing in von Planta's work, samples of the pure salts of these alkaloids, aconitine and benzoylaconine, were dissolved in water and treated with bases. The results of these qualitative tests have been mentioned. It will be noted that the surmise of impurity in the aconitine being responsible for the action he observed is somewhat borne out. The results show that the alkalies employed precipitate the aconitine but do not precipitate the benzoylaconine. Advantage was taken of this as a possible means of separation of the two alkaloids. Experiments were carried out in an empirical way on known quantities of the aconitine by dissolving the hydrochloride in a small volume of water and then adding the alkaline solution. Immediate filtration of the precipitate was prevented because the first precipitate of aconitine was very fine and passed through the paper and precipitation was complete only after a lapse of a

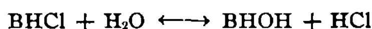
few minutes. Immediate filtration was also hindered since, in some cases, a slight precipitate of benzoylaconine was thrown down which returned to solution after a few minutes. The time required for these adjustments could not be cut below thirty minutes. The precipitated aconitine was dissolved in standard acid and titrated with standard base and showed a loss too great to permit this procedure to be of practical value. Apparently the loss was due to hydrolytic decomposition of aconitine because the loss of alkaloid increased as the time of cold digestion of the precipitate increased. The alkaloids could not be separated by ether because the precipitated aconitine was soluble in it and it also extracted the benzoylaconine from the aqueous solution.

The methods described above proved unsatisfactory and the methods tried by other workers also were not of practical use. It therefore seemed to us that the problem should be attacked from a new angle.

It will be recalled that many alkaloids of very similar composition, both empirically and structurally, often vary widely in certain chemical properties. The purine bodies, caffeine, theophylline and theobromine, are a group of such alkaloids. Though these three are quite similar in composition, they do vary widely in some respects. The variation of prime interest at this point is in their dissociation constants. Many other closely related alkaloids show variation in this respect. A similar situation might exist in the case of aconitine and benzoylaconine, and this led us to a study of their dissociation constants. Landolt-Bornstein gives the dissociation constant of aconitine as 3×10^{-8}. The corresponding value of benzoylaconine could not be found. It therefore became necessary to determine this value.

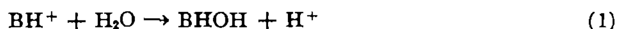
From the relationship existing between the hydrolysis constant of a salt and the hydrogen-ion concentration of the solution, the dissociation constant of the base is readily calculated.

In an aqueous solution of the salt the equilibrium is expressed by the equation:



$$BHCl + H_2O \longleftrightarrow BHOH + HCl$$

which in terms of the ionization theory becomes



$$BH^+ + H_2O \rightarrow BHOH + H^+ \quad (1)$$

Applying the law of mass action to this equation, we get

$$\frac{(BHOH)\,(H^+)}{(BH^+)} = K_h \quad (2)$$

where K_h is the hydrolytic constant of benzoylaconine hydrochloride.

If we multiply equation (2) by (OH^-), we obtain

$$\frac{(OH^-)\,(BHOH)\,(H^+)}{(BH^+)\,(OH^-)} = K_h = \frac{K_w}{K_b}, \text{ or,}$$

$$K_b = \frac{K_w}{K_h} \quad (3)$$

Therefore, we need but to determine K_h in order to find K_b.

Using conductivity water as the solvent, an $N/50$ solution of benzoylaconine hydrochloride was prepared and the p_H of the solution determined using the standard hydrogen electrode. The reading on the potentiometer was 0.649 volts. The p_H may then be calculated from

$$pH = \frac{E_h - E_o}{(0.0001983)T} = \frac{0.649 - 0.303}{(0.0001983)298} = 5.85$$

In a solution whose pH is 5.85 the C_{H}^{+} equals 1.41×10^{-6}
Employing equation (2), we obtain

$$K_h = \frac{(1.41 \times 10^{-6})^2}{0.02} = 10^{-10}$$

Using this value for K_h in (3)

$$K_b = \frac{10^{-14}}{10^{-10}} = 10^{-4}$$

the dissociation constant of benzoylaconine.

A solution whose hydroxyl-ion concentration falls between 3×10^{-8} and 1×10^{-4} should then extract the weaker base, aconitine, and leave the stronger base, benzoylaconine, in solution as the salt. Proceeding on such a basis a solution having a concentration of hydroxyl ion equal to 3×10^{-7} should be approximately correct.

Since, in a $1/100N$ solution of ammonium hydroxide, the ionization constant is approximately 1.8×10^{-5}, an amount of NH_4Cl can be added to depress the hydroxyl ion to the desired concentration. If we assume complete ionization of the NH_4Cl then

$$\frac{1.8 \times 10^{-5}}{3 \times 10^{-7}} \text{ equals } 0.6 \times 10^2 = 60,$$

or the desired solution must be $0.6N$ in NH_4Cl and $0.01N$ in NH_4OH.

Before employing such a solution, it is necessary to determine whether or not aconitine will suffer hydrolysis in it. To determine this, samples of aconitine hydrochloride were dissolved in a small quantity of water and then added to 50 cc. of a solution of $0.01N$ in NH_4OH and $0.3N$ in NH_4Cl. The alkaloid was then extracted with successive small portions of ether, standard acid added in excess, the ether evaporated, and the acid titrated. Results were as follows:

Aconitine Used.	Aconitine Reclaimed.
43.3 mg.	42.8
46.0	45.5
45.2	45.0

This shows that a solution more strongly basic than is required for separation of the alkaloids will not hydrolyze aconitine during the extraction procedure.

The strength of the solution in NH_4OH was varied from $0.01N$ to $0.016N$ and in NH_4Cl from $0.6N$ to $0.85N$ and combinations of these strengths used in attempts to separate the alkaloids. These solutions were employed in extracting the alkaloids separately to determine which solution would set free the smallest quantity of benzoylaconine and still set free all or nearly all the aconitine as it theoretically should. Of the solutions employed, a solution $0.0159N$ in NH_4OH and $0.75N$ in NH_4Cl gave the best results.

The procedure was as follows:

The hydrochloride of the alkaloid was dissolved in 5 cc. of water and this solution added to 50 cc. of the above buffer. The weakly basic aqueous solution was then extracted with ether. Four extractions of 20 cc. each were found to be sufficient to easily remove all the aconitine when each extraction portion was gently shaken for 10 minutes. Ten cc. of standard acid are then added to the combined ether extracts and the ether evaporated. The excess acid is then titrated with standard base. Results are as follows:

Aconitine Used.	Aconitine Reclaimed.	Loss.	Percentage Loss.
41.5 mg.	41.0	0.5	1.2
41.1	40.8	0.3	0.7
41.8	41.5	0.3	0.7
43.2	42.7	0.5	1.1
41.5	41.1	0.4	0.9
40.4	40.0	0.4	1.0
		Average per cent loss	0.93

The following results were obtained for benzoylaconine in the same procedure:

Benzoylaconine Used.	Benzoylaconine Reclaimed.	Per Cent Reclaimed.
44.4 mg.	4.0	9.00
42.4	4.6	10.84
45.2	4.4	9.73
44.5	4.5	10.00
42.6	4.2	9.72
41.3	4.1	9.87
	Average per cent reclaimed	9.86

Then, from such a solution, 99% of the aconitine may be isolated while only 9.86% of the benzoylaconine will be removed. Experiments were then carried out on mixed samples and calculations made in the following manner: From a sample of "x" mg. of aconitine 99% would be removed, or 0.99x mg. Then $\frac{0.99x}{6.45}$ = the cc. of 0.01*N* acid required to neutralize this base. Likewise, from a sample of "y" mg. of benzoylaconine 9.86% would be removed, or 0.0986y mg., and $\frac{0.0986y}{6.03}$ = cc. of 0.01*N* acid required to neutralize this base. The sum of these two quantities of acid should then be the same as the cc. of acid actually required in such a mixed sample. The results below show the comparison of the actual result and the theoretical.

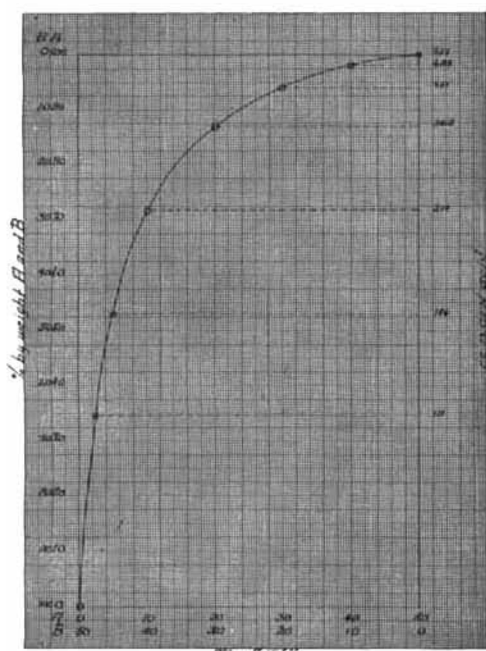
Aconitine used.	$\frac{0.99x}{6.45}$ = Cc.	Benzoylaconine Used.	$\frac{0.0986y}{6.03}$ = Cc.	Total Cc.	Actual Cc.	Difference.
26.6	4.08	18.2	0.26	4.34	3.88	0.46
25.3	3.88	19.1	0.32	4.20	4.26	0.06
26.7	4.09	17.3	0.29	4.38	4.44	0.06
23.8	3.66	16.9	0.29	3.95	4.08	0.07
27.2	4.17	18.8	0 32	4.49	4.44	0.05
25.1	3.85	16.4	0.27	4.12	4.23	0.11

Comparison of the theoretical number of cc. of acid with the actual number of cc. required as given in the above data shows close agreement with exception of the first sample. Some error, probably a burette reading, has apparently crept in since the other 5 samples are in good harmony. It does not appear amiss to assume from this data that the procedure holds on the mixed alkaloids.

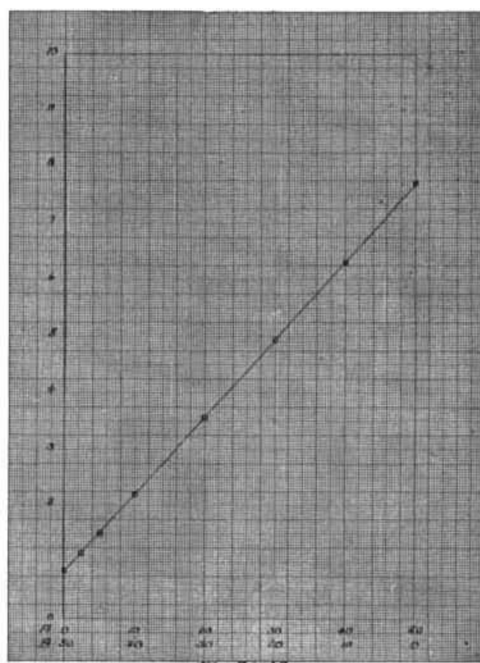
In any mixed sample of the two alkaloids treated in the preceding manner the extracted mixture of alkaloids will be composed of a certain amount of aconitine and the remainder will be benzoylaconine. If a graphical relationship is set up for these facts as explained below, certain other useful data may be derived. If we base the comparison on a mixture of the alkaloids weighing 50 mg. and then select various combinations which equal 50, it is found that the per cent of one

alkaloid in the extracted mixture, following the previous procedure, varies with the composition of the original mixture. These per cent values plotted against total weight give rise to the following curve. The points may be located as follows: In a mixture in which 40 mg. of aconitine and 10 mg. of benzoylaconine are present originally, 39.6 mg. of aconitine and 0.986 or 1 mg. of benzoylaconine will be extracted making a total of 40.6 mg. of extracted alkaloids. Then the amount of aconitine present in 40.6 mg. of total alkaloids is 97.54% and likewise there is present 2.46% benzoylaconine. This point then is plotted as indicated. Others are found in the same manner.

Using other total quantities of alkaloids in the original mixture, calculating and plotting as before gives rise to the same type of curve. If, at these loci, as in the preceding graph, the amount of acid required to neutralize such a mixture is calculated on the basis of aconitine, it is found that these points on the ordinate are



Graph 1.



Graph 2.

a logarithmic function. If now the acid ordinate is plotted in units the following graph is obtained and likewise with a total of 100 mg. a straight line is obtained which possesses the same slope but a different intercept.

With the proper constants introduced to control the intercept and slope, it should be possible to calculate the amount of aconitine present in a sample from the equation of this line.

In this graph, if h equals the total alkaloids calculated as aconitine and A equals the mg. of aconitine, then (h − A) equals the mg. of benzoylaconine.

Therefore, $\frac{0.99A + (0.0986)(h - A)}{6.45}$ equals the cc. of 0.01N acid required to titrate the combined alkaloids, and this is expressed on the y axis of the graph.

Then $y = 0.1382A + 0.01592h$ (4)

As a check on this equation, let A = 100;

$$\text{Then } y = 13.8 + 1.5 = 15.3$$

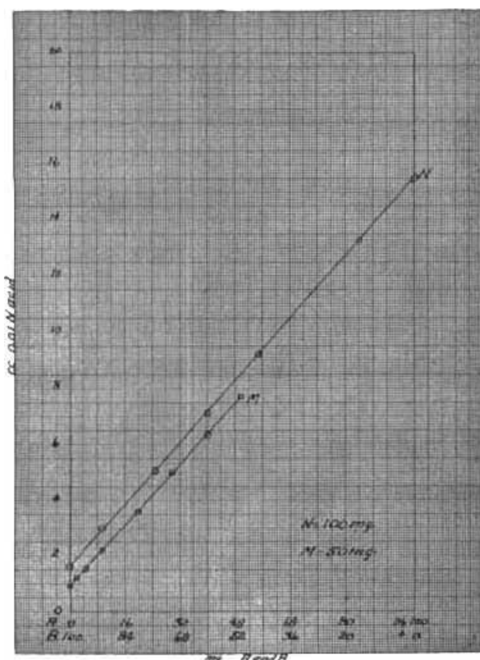
Reference to the immediately preceding graph shows this value to check. Proceeding with equation (4) and solving for A,

$$A = 7.236y - 0.1106h$$

This is the equation from which aconitine may be calculated in mg. where "y" is the cc. of 0.01 acid required to combine with the extracted alkaloids and "h" is the total mg. of alkaloids in the sample. To check this we need but refer to the preceding table of mixed samples. Selecting Sample 2 as an example and proceeding by equation

$$A = (7.236)(4.26) - (0.1106)(44.4)$$
$$A = 25.9$$

This is in good agreement with the original quantity of aconitine, 25.3 mg., used.



Graph 3.

To apply such procedure to the preparations of Aconite, the fluidextract prepared earlier in this work and used for determining the change in p_H on storage was selected as the test preparation. To 10 cc. of the fluidextract, 1 cc. of 10% sulphuric acid was added and the alcohol evaporated on a steam-bath. Twenty cubic centimeters of water were then added and the whole filtered and the filter paper and slight residue washed well with water. The filtrate was then shaken out with 20 cc. of ether to remove coloring matter. The aqueous portion was then carefully made alkaline with 10% ammonium hydroxide and shaken out with three 20-cc. portions of ether, shaking each portion 5 minutes. This was found to remove all the alkaloids. From 4 samples the following results were obtained:

Sample.	Mg. of Alkaloids.
1	41.3
2	42.1
3	41.1
4	42.1
Average	41.7

In order to separate the aconitine from the benzoylaconine, the neutralized solutions of alkaloids from above were each added to 50 cc. of the buffer, 0.0159N NH_4OH–0.75N NH_4Cl, and the solution extracted with four portions of ether of 20 cc. each. Standard acid was added to the ether extracts, the ether evaporated, and the excess acid titrated with standard alkali. Results were as follows:

Sample	Mg. Aconitine *
1	25.3
2	25.1
3	25.3
4	24.9
Average	25.2

* By equation.

The fact that Na_2SO_4 had been added to the buffer solution when the neutralized solution of alkaloids was added was checked both theoretically and practically at this point. Theoretically a mixture of 10 cc. of 0.01*N* H_2SO_4 and 10 cc. of 0.01*N* NaOH would not produce sufficient Na_2SO_4 to destroy the buffer. Practically, the result was tested by determining the p_H of the buffer and then determining the p_H of 50 cc. of the buffer plus 20 cc. of the Na_2SO_4 solution. The change in p_H was not noticeable by the potentiometric method.

To test this method of assay samples of the same fluidextract were used with the addition of known quantities of aconitine. After addition of the alkaloid the same procedure of separation was followed. Results are as follows:

Sample.	Cc. F. E.	Added Aconitine.	Total Alkaloids (b).	Cc. 0.01*N* Acid (y).	Total Aconitine.	Difference (See Below).
1	10	23.9	54.5	6.60	42.6	17.9
2	10	25.9	63.2	8.16	52.0	26.8
3	10	22.9	60.9	7.46	47.3	22.1
4	10	20.9	62.3	7.31	46.0	20.8
5	10	21.2	61.1	7.35	46.4	21.2
6	10	22.3	61.2	7.64	48.5	23.3

(According to previous assay each 10 cc. of fluidextract had 25.2 mg. aconitine. Therefore, the difference equals the total aconitine minus 25.2 and this should be compared with the added aconitine.)

This test, as applied, shows that the procedure of assay employed determines the amount of aconitine present within reasonable expectancy. With the exception of Sample 1 the difference between the total aconitine removed and the aconitine presumably present in the fluidextract as shown previously checks the known added amount of pure alkaloid within reasonable error. This method, then, may be employed to determine the amount of aconitine present.

Aconitine of the U. S. P., when administered subcutaneously, has a minimum lethal dose of not less than 0.00,000,005,5 Gm. and not more than 0.00,000,006,5 Gm. for each Gm. of body weight of guinea pig. The standard for the U. S. P. tincture, when administered subcutaneously, is a minimum lethal dose of not less than 0.00,035 cc. and not more than 0.00,045 cc. for each Gm. of body weight of guinea pig. The standard in the N. F. for the fluidextract, when administered subcutaneously, is a minimum lethal dose not greater than 0.00,004 cc. for each Gm. of body weight of guinea pig.

Assuming that aconitine is the only toxic agent, then by mathematical calculation, each 100 cc. of U. S. P. tincture should contain not less than 14.4 mg. and not more than 15.7 mg. of aconitine and each 10-cc. portion of fluidextract should contain not less than 14 mg. of aconitine. This, then, should give something of a check between the bioassay and chemical assay methods of Aconite preparations. However, we must not lose sight of the fact that in the bioassay the hydrolytic products of aconitine and other extractive from the drug may have a considerable effect on the toxic action of aconitine.

Four samples of preparations of Aconite made by reputable manufacturers were obtained for investigation. Two of these samples were tinctures and two were fluidextracts. These were subjected to the chemical assay process developed in this study. The results of the assay along with pertinent remarks on the label of

the preparation are tabulated below. Key letters are employed in order that reference to research data may be easily made if desired.

Sample A: Labeled—

"Tincture of Aconite. Physiologically standardized. This product differs from U. S. P. tincture in being slightly acidified to retard deterioration."

Chemical assay yields 18 mg. aconitine per 100 cc.

Sample B: Labeled—

"Tincture of Aconite, U. S. P. X. Physiologically standardized."

Chemical assay yields 12.9 mg. aconitine per 100 cc.

Sample C: Labeled—

"Fluidextract of Aconite. Physiologically standardized. This product differs from the N. F. fluidextract in being slightly acidified to retard deterioration."

Chemical assay yields 14.2 mg. aconitine per 10 cc.

Sample D: Labeled—

"Fluidextract of Aconite, N. F. Standard—Minimum lethal dose 0.00005 cc. per Gm. body weight."

Chemical assay yields 17.5 mg. aconitine per 10 cc.

From these results definite conclusions cannot be drawn. However, certain indications may be noted. In the case of the samples of Tincture of Aconite: Sample A was stabilized by addition of acid and, for this reason, cannot be labeled U. S. P., but it was physiologically standardized and, no doubt, to meet the U. S. P. requirements. This sample was high in aconitine according to chemical assay. Sample B, according to the label, is U. S. P. and cannot have had acid added for stabilization purposes. This sample yields but 12.9 mg. of aconitine when assayed chemically which is low. This would be expected due to deterioration.

It is more difficult to draw conclusions from the data on fluidextracts because no definite standard, or rather limit of standard is required by the N. F. However, to meet the requirements of the N. F. they must assay not less than 14 mg. of aconitine per 10 cc. as previously shown. Both of these samples come up to standard but there are indications that the producer of Sample D may have put out a product of excessive potency, with the idea that the ultimate consumer would have at his disposal a product which would meet the requirements of the standard. Sample C, being stabilized, needs no such protection and assays very nearly the standard amount of aconitine.

From these results it appears reasonable to suggest the following assay for Aconite preparations:

To 10 cc. of the fluidextract, or 100 cc. of the tincture, add 1 cc. of 10% H_2SO_4 and evaporate the alcohol on a steam-bath. Add about 20 cc. of water and filter off the insoluble residue, washing the paper and its contents several times with small portions of water and finally with one or two small portions of acidulated water, collecting the filtrate and washings in a separatory funnel. Shake out with 20 cc. of ether to remove coloring matter and discard ether. Carefully add 10% NH_4OH until the solution is faintly basic to litmus and then completely extract the alkaloids with successive portions of ether. Add 10 cc. of $0.01N$ acid to the combined ether extracts, evaporate the ether on a steam-bath, cool, add 3–4 drops of methyl red and titrate with $0.01N$ base. The number of cc. of $0.01N$ acid consumed multiplied by 6.45 gives the mg. of total alkaloids calculated as aconitine. Transfer the neutralized solution of total alkaloids to a separatory funnel containing 50 cc. of the buffer solution ($0.0159N$ NH_4OH–$0.75N$ NH_4Cl) and extract with four portions of ether, of 25 cc. each, shaking each extraction about 10 minutes. Again add 10 cc. of standard acid to the combined ether extracts and evaporate the ether on a steam-bath, cool, add

one drop of methyl red and titrate the excess acid with $0.01N$ base. The number of cc. of 0.01 acid consumed is the value substituted for "y" in the equation

$$A = 7.236y - 0.1106h$$

in which h = total alkaloids. A = the number of mg. of aconitine present in the sample.

SUMMARY.

Aconitine, considered to be the active toxic principle of Aconite and its preparations, is easily hydrolyzed and some of its hydrolytic products cannot be readily separated from it by the usual chemical methods such as precipitation, extraction, etc. It therefore becomes necessary to determine the aconitine in the presence of at least one of its hydrolytic products, benzoylaconine, which can be done by a method which is based on a study of the dissociation constants of the two alkaloids. By extracting the alkaloids with ether from their solution in which the hydrogen-ion concentration is controlled by a buffer solution a definite percentage of the total amount of each alkaloid present is removed, and a curve indicating this relationship can be constructed. An equation may be derived from this curve by means of which the amount of aconitine can be determined. On the basis of this relationship a satisfactory assay process has been developed.

CONCLUSIONS.

1. Preparations of Aconite change in p_H value on storage, becoming more acidic thus bearing out the supposition that an acid is liberated by the decomposition of aconitine.
2. Addition of an acid (preferably HCl) to produce a p_H of 2.5 to 3.0 stabilizes the preparations of Aconite. This agrees with the results of other investigations.
3. Aconitine and benzoylaconine possess dissociation constants of 3×10^{-8} and 1×10^{-4}, respectively.
4. On this basis the two alkaloids may be extracted with ether from a buffered solution in a ratio such that the equation,

$$A = 7.236y - 0.1106\,h,$$

in which A = mg. aconitine, y = cc. of $0.01N$ acid required in the final titration, and h = the total alkaloids present in the sample, yields the amount of aconitine.

REFERENCES.

(1) Swanson, E. E., and Walters, A. L., JOUR. A. PH. A., 12, 957 (1923), "The Standardization and Stabilization of Aconite Preparations, Paper I."

(2) Swanson, E. E., JOUR. A. PH. A., 13, 1108 (1924), "The Standardization and Stabilization of Aconite Preparations, Paper II."

(3) Baker, W. B., JOUR. A. PH. A., 23, 974 (1934), "Deterioration and Stabilization of Aconite Preparations, Part I."

(4) Von Planta, A., *Ann.*, 74, 257 (1850), "Unteruschung über die Zusammensetzung einiger natürlich organischer Salzbasen; III Aconitin."

Since the completion of the thesis, seven more preparations of Aconite have been assayed by this method with the following results:

1. "Fluidextract Aconite No. 3, 3,056,164. Standard-M. L. D. 0.00004 cc. per Gm. body weight." Assayed chemically—16.0 mg. per 10 cc.
2. "Fluidextract Aconite No. 3, 3,066,570. Standard-M. L. D. 0.00004 cc. per Gm. body weight." Assayed chemically—15.3 mg. per 10 cc.

3. "Fluidextract Aconite No. 2, 910,036A. Biologically standardized." Assayed chemically—14.3 mg. per 10 cc.
4. "Tincture Aconite No. 1, 3,071,282. Standard-M. L. D. 0.0004 cc. per Gm. body weight." Assayed chemically—13.6 mg. per 100 cc.
5. "Tincture Aconite No. 1, 909,454A. Biologically standardized." Assayed chemically —14.0 mg. per 100 cc.
6. "Tincture Aconite 'E.' " Assayed chemically—11.0 mg. per 100 cc.
7. "Tincture Aconite for Experimental Purposes." Assayed chemically—14.1 mg. per 100 cc.

Of the three samples of Fluidextract of Aconite, all of them met the requirements which we calculated to be equivalent to the N. F. requirements, that is, they contained at least 14 mg. of aconitine per 10 cc.

Of the four samples of Tincture of Aconite, one (Sample No. 6) should be eliminated because it was not indicated to be of U. S. P. strength, and one (Sample No. 4) fell below the strength we calculated to be equivalent to that of the U. S. P., that is, 14.4 to 15.7 mg. per 100 cc.

The other two tinctures met the requirement calculated to be equivalent to the U. S. P. requirement.

This is further evidence that the chemical assay process for Aconite and its preparations as recommended by us should be substituted for the present U. S. P. process.

VOLATILE OIL FROM WESTERN YARROW.*

BY R. L. MCMURRAY.[1]

Considerable discussion has occurred as to whether the yarrow of the western states is the same species as that which grows in the eastern states. In 1834 Thomas Nuttall (1) classified the western yarrow as *Achillea lanulosa* Nuttall. This work was made on material collected while with the N. B. Wyeth expedition in the region of the falls of the Columbia River east to the first navigable waters of the Missouri River. The Index Kewensis (2) lists this not as a new species, as did Nuttall, but the same plant as *Achillea Millefolium* Linné. C. V. Piper (3) made a third classification by combining the preceding and giving it the name *Achillea Millefolium lanulosa* (Nutt.) Piper. Since taxonomists disagree on this plant, or plants, it was considered of possible interest to determine the constants of the volatile oil and compare them with those reported for other volatile oils of *Achillea Millefolium* Linné.

The material was gathered from various local habitats in Whitman County, Washington, during the second week of July, 1935. The flower-heads were clipped off and collected into bags. The stems and leaves were rejected. All foreign materials were carefully excluded. Collecting was done during the entire day. The flower-heads amounted to about 76 pounds in the fresh condition. They were packed the same day into a small steam distillation apparatus (capacity about 20 pounds of material) and distilled with "high pressure" steam.

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* Scientific Section, A. PH. A., Portland meeting, 1935.

[1] Ohio State University, College of Pharmacy; operations performed at Washington State College, School of Pharmacy.