# Contributed and Selected

## THE PHYSIOLOGICAL ASSAY OF ACONITE.

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The variability of aconite preparations when tested physiologically has shown that the chemical method of assay which is required by the U. S. P., VIII, for Aconitum Napellus, is not a measure of its activity. A preparation relatively rich in total alkaloids may have a low toxicity and vice versa. Other chemical methods than the official ones, which have been recommended have upon investigation been found to be equally unreliable. For this reason a search was made for a physiological test which might be used to determine the activity of aconite.

Before the consideration of any method of assay a brief review of the chemistry and pharmacology of aconite will be given to enable one to more properly value the various assay methods that will be mentioned and discussed in this paper.

For a long time the chemistry of aconite was in dispute on account of the fact that mixtures of alkaloids rather than pure principles were isolated from the plant.

Although an amorphous alkaloid had been isolated from the leaves of Aconitum Napellus by Geiger[1] in 1833, it was not until a half century later that its chemistry became better known, the especial credit for which may be given to Wright[2] and Dunstan.[3]

Dunstan[5] described three alkaloids in aconite,* namely, aconitine, benzaconine and aconine. These have all been found in the root in varying amounts. According to Dunstan their constitution is as follows:

Aconitine (acetylbenzaconine), $C_{24}H_{37}$ $(CH_3CO)$ $(C_6H_5CO)$ $NO_{10}$.
Benzaconine, $C_{24}H_{38}$ $(C_6H_5CO)$ $NO_{10}$.
Aconine, $C_{24}H_{39}NO_{10}$.

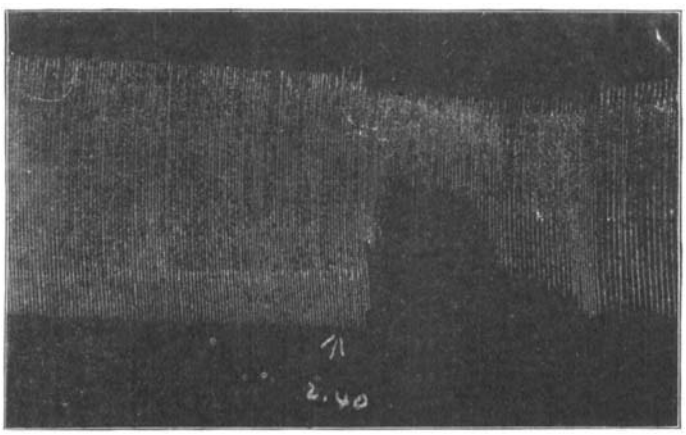
The activity of aconite depends mainly upon its most active alkaloid aconitine. This is a crystalline base which melts at about 188° C., is very sparingly soluble in water but readily soluble in alcohol. When heated to its melting point the alkaloid decomposes first into benzaconine and later into aconine. Benzaconine the product of the first stage of aconitine hydrolysis, is the chief constituent of the substances named napelline and picraconitine by the older workers. Dunstan first named it isaconitine. Benzaconine is an amorphous base, and like aconitine is very sparingly soluble in water but readily soluble in alcohol. Aconine is also an amorphous base, but is soluble in both water and alcohol. Although these alkaloids are present in the crude drug they may be present also in the

*By aconite is meant the official preparations made from the root of Aconitum Napellus.

galenicals as decomposition products. Dunstan and Umney[4] found aconite alkaloids remarkable in that they undergo decomposition easily, especially hydrolysis.

Besides these alkaloids there is considerable aconitic acid, a small amount of starch, some resin, fat, and sugar.

Aconitine, according to Cash,[5] is the most active of the alkaloids. Either to the frog (Rana temporaria) or the guinea pig, it is almost 200 times more toxic than benzaconine and about 1200 times as toxic as aconine. Upon the heart and circulation aconitine produces marked effects. In warm-blooded animals the heart is first slowed, due to a stimulation of the vagus center, and this slowing is accompanied by a great fall in blood pressure. The heart rate then increases and later becomes irregular with the development of a marked arrhythmia betweeen auricles and ventricles, the blood pressure fluctuating greatly.




Tracing of isolated frog's heart perfused with Aconitine hydrochloride.
At 2:35 p. m. Merck's Aconitine hydrochloride (1-400,000) was turned on. At 2:40 p. m. increased tonus appeared.

When applied to the tongue and mouth in dilute solutions it will produce the tingling sensation which is so characteristic of aconite.

Benzaconine in small doses causes a very slight rise in the blood pressure, and an acceleration of the heart rate, whereas with large doses a steady fall in the blood pressure occurs which is accompanied by a decrease in the heart rate. There is also developed a change in rhythm between the auricles and ventricles. No tingling is produced when applied to the tongue.

Aconine as compared with the former alkaloids is comparatively harmless. A slight rise in pressure usually occurs which is due to a more complete systole of the heart. In this respect it is antagonistic to aconitine and benzaconine. Like the latter, it does not produce tingling.

*Methods of Assay Investigated.* An investigation of seven methods was made: the lethal dose for frogs, one-hour frog method, the effect upon the blood pressure of the cat and the dog, the Squibb method, the reaction upon the perfused isolated frog's heart, and the lethal dose for guinea pigs.

Although frogs are so easy to obtain and handle they have never been extensively used in a method for the physiological assay of aconite. Some work has been done with this drug upon frogs by Mandelin,[6] Stevens,[7] and a few others, with unsatisfactory results. Buntzen and Mudsen[8] used them to determine the toxicity of various aconitines.

In the lethal dose method for frogs the amount of drug necessary to kill a frog of known weight is determined. This is essentially the method as used by Houghton[9] for the assay of digitalis. The aconite preparation is deprived of its alcohol by gentle heat, and then diluted with physiological salt solution so as to measure between 0.5 cc. to 1.0 cc., and then injected into the abdominal lymph sac. The frogs are injected in the latter part of the afternoon, placed under moist bell jars and allowed to remain until the next morning, when they are examined. It was usually found that when an animal was dead the body was rigid, but this was not necessarily true. The heart was examined and if stopped this was taken as an end reaction. The ventricle was usually found in systole and the auricles dilated.

This method of assay yielded very satisfactory results with some preparations, but with others very discordant results were obtained. Under these conditions it required from 0.0004 cc. to 0.004 cc. per gram of body weight of the official fluidextract to kill; 0.003 cc. to 0.004 cc. per gram of body weight, of the unofficial fluidextract of the leaves; and about 0.007 cc. per gram of body weight, of the official tincture. While in a general way this method shows the relative strengths of different preparations, it is very uncertain, inasmuch as there is a marked variation occurring in each series of animals. As an illustration, the assays of preparation "F", an official tincture, and preparation "C", an unofficial fluidextract of the leaves, will be given in full. Those marked (+) were found with the heart stopped, while the heart was beating, with those marked (—).

| PREPARATION "F". | |
|---|---|
| Dose (cc. per gram of body weight). | |
| 0.003 | — |
| 0.004 | — |
| 0.004 | — |
| 0.005 | — |
| 0.005 | — |
| 0.0055 | — |
| 0.006 | + |
| 0.006 | + |
| 0.006 | + |
| 0.0065 | + |
| 0.007 | — |
| 0.007 | — |
| 0.008 | — |
| 0.008 | — |
| 0.009 | + |
| 0.01 | + |

| PREPARATION "C". | |
|---|---|
| Dose (cc. per gram of body weight). | |
| 0.001 | — |
| 0.002 | — |
| 0.003 | — |
| 0.004 | — |
| 0.005 | + |
| 0.005 | — |
| 0.006 | — |
| 0.006 | — |
| 0.007 | + |
| 0.008 | + |
| 0.009 | — |

Preparation "C" shows a degree of uniformity in the results, but the fact that the heart was beating in the animal which received the largest dose and had stopped in the animals receiving smaller doses, makes the results more of a conjecture than a fact. The solutions injected were always quite dark and con-

tained considerable resinous material, traces of which could always be found in the lymph sac when examined at the end of the time.

In general, this method yielded more promising results than did the one-hour method, and although not strictly accurate for quantitative work, yet it shows gross differences in the activity of aconite preparations.

The one-hour frog method is a heart toxic method, and is also used in the assay of digitalis. The method in detail can be found in the work of Famulener and Lyons,[10] but a short description of it will be given. As in the lethal frog method, the diluted non-alcoholic drug is injected into the abdominal lymph sac and at the end of an hour the animal is pithed and the heart is exposed and examined. No definite end reaction except cardiac standstill was required, as the heart would be found in systole in many of the animals and in diastole in many others, the latter condition predominating in a given series. The stoppage of the ventricle alone could not be taken as an end reaction, as contractions supervened occasionally in the ventricle if the auricles were beating vigorously. The beat of the sinus, however, was disregarded. A large number of animals were used, but no constant results were obtained. The following series is typical for all the preparations used:

PREPARATION "X" (an official fluidextract.)

Dose (cc. per gm. of body weight.)

| (+) = heart stopped. | (—) = heart beats. |
|---|---|
| 0.02 — | 0.05 + |
| 0.02 — | 0.05 + |
| 0.02 — | 0.05 — |
| 0.025— | 0.05 — |
| 0.03 — | 0.05 — |
| 0.03 + | 0.06 + |
| 0.03 — | 0.06 + |
| 0.03 — | 0.06 — |
| 0.03 — | 0.06 — |
| 0.03 — | 0.07 — |
| 0.04 + | 0.07 + |
| 0.04 — | 0.07 + |
| 0.04 — | 0.09 + |

Further work on this preparation had to be discontinued because of the fact that the solution was too concentrated to be absorbed properly in the time limit. For example, a 30 gm. frog which received the largest dose was given 0.27 cc. of the modified fluidextract.

If we compare this method with the lethal frog method we find that it is more unsatisfactory in many ways. The absorption is more variable, there is a less definite increase in toxicity with the increase in the size of the dose, and larger doses are required than in the lethal frog method.

One of the effects of aconite is the production of a lowered blood pressure. This fact, therefore, was investigated, with the thought in mind of its being useful as a method of assay. After sufficient trial it was found that the effect upon the blood pressure could not be used as a measure of the activity of aconite, the chief objection to it being the relatively high toxicity of the drug. The experiments were performed on both dogs and cats. The animals were anaesthetized with morphine and chloretone and were given artificial respiration.

The drug was injected into the jugular vein, the rate of injection being uniform and slow and the amount of fluid injected being constant. As an illustration of the toxicity of the drug the following protocol will be cited. In a dog with intact vagi 0.25 cc. of tincture produced only a slight fall in the blood pressure. In a few minutes marked cardiac irregularities occurred, followed by a continued fall in pressure. In order to prevent the pressure from falling further the vagi were cut, which caused the heart irregularities to disappear. About fifteen minutes later a dose of 0.5 cc. was given, which caused a slight fall in pressure but no heart irregularities. This same amount was repeated after about ten minutes, producing a marked rise in pressure, followed by irregularities and a subsequent fall in pressure. After another interim of about ten minutes another 0.5 cc. dose was given, which caused the death of the animal, death being caused by a total of 1.75 cc. of the tincture given at intervals extending over a period of about forty-five minutes. Like results were obtained in the cat, a large animal being able to survive as large amounts of the drug as did the dog. With either intact or cut vagi no constancy in the fall or rise in pressure was observed, and the conclusion was reached that this method was altogether inadequate.

When the isolated heart of the frog (Rana pipiens) is perfused with aconitine in Ringer's solution, in certain dilutions, the organ responds first by going into what might be considered a state of incomplete tetanus with a marked increase in tonus (see Fig.) Relaxation of the heart gradually becomes lessened and the systole more complete or while the organ is in a state of marked tonus no regular beat occurs but a series of irregular contractions may pass from one side to the other. It was thought that solutions might be compared quantitatively by noting the dilution just necessary to produce this phenomenon, or perhaps a comparison might be made by noting the time required to cause this effect when using the same degree of dilution of unlike preparations.

The method is as follows: A large frog is selected and after exposing the heart an inflow cannula is inserted into the left anterior caval vein and tied firmly, and in a similar manner an outflow cannula is tied into the right branch of the truncus arteriosus. After tying all the remaining vessels and freeing the organ, the cannulae are then fixed securely with an iron clamp. A small spring clamp is then attached to the tip of the ventricle and tied to a Harvard heart lever, thus enabling one to obtain a tracing. The inflow cannula is then connected to a set of perfusion bottles which are placed at a height of about 80 mm.

The fluid passing through the outflow cannula is made to pass over the heart so that the organ is kept moist. The outflow cannula is about 30 mm. long and is held nearly upright, which gives the heart a constant amount of work. Inasmuch as each heart will permit of a single experiment, thus necessitating the use of a number of animals, it is quite essential that the technic be uniform. After the Ringer's solution* is turned on, the heart should beat at about the same rate as in the pithed animal.

In the following experiments Merck's aconitine hydrochloride was used.

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* Ringer's Solution: NaCl —0.6%
$CaCl_2$ —0.02%
KCl —0.0125%

Various dilutions were tested and after several trials for each dilution an average was made. The results are as tabulated below:

| Dilution | Number of experiments performed | Time elapsing before appearance of increased tonus. | Average |
|---|---|---|---|
| 1-200,000 | 4 | 6, 5, 4, 3 minutes | 4.5 minutes |
| 1-400,000 | 6 | 6, 10, 6, 5, 6, 5 minutes | 6.3 minutes |
| 1-600,000 | 6 | 9, 13, 23, 10, 14, 11 minutes | 13.3 minutes |
| 1-800,000 | 6 | 19, 7, 14, 18, 15, 16 minutes | 14.8 minutes |
| 1-900,000 | 2 | 23, 26 minutes | 24.5 minutes |

For an official tincture in 1 to 50 dilution an average of 11.6 minutes was required to produce increased tonus. With an official fluidextract in 1 to 500 dilution no typical reaction appeared, only a change in rhythm which occurred in 6 or 7 minutes. Further work with galenicals showed that not only the characteristic reaction did not appear with many preparations, but that the time for its appearance in case it did occur, showed greater variability than it did with aconitine. This may be due to the resins and other substances present in the galenicals as well as to the mixture of alkaloids.

The dilution necessary to produce this reaction was ascertained with Merck's aconitine hydrochloride and it was found to occur in as great a dilution as 1 in 2,000,000, during the months of October, November, and December, whereas it was obtained with considerable difficulty using a 1 to 800,000 dilution during the months of January, February and March. No sharp limit, as regards strength of solution, for the disappearance of this phenomenon could be determined.

This method might be useful to determine the activity of aconitines but is worthless for galenicals since the reaction is not constant when they are used.

Several decades ago Squibb[11] recommended a physiological test for the determination of the strength of aconite preparations, his endeavor being to secure some means whereby a good preparation might be known from a worthless one. At first he employed this merely as a qualitative test, but later he used it to determine the relative worth of various preparations.

This test takes for its end reaction the sensation produced on the tongue and mucous membranes of the mouth after allowing liquid preparations of the drug to remain in the mouth for a short period of time, and ascertains the dilution which is just necessary to give a distinct aconite sensation. The mouth is rinsed with distilled water, and four cubic centimeters of the required dilution, which has stood for an hour, is placed in the anterior part of the mouth and retained for one minute. After several minutes a distinct aconite impression is obtained, which, according to the test, should not amount to tingling, but should be very suggestive of it. This sensation should continue for about half an hour. As originally suggested, a fresh official fluidextract should produce the reaction in a 1 to 600 dilution.

This is perhaps the oldest physiological test for aconite and it has been investigated by a number of workers, many of whom have found it an admirable test for the quantitative estimation of the aconite preparations. In fact, it is used today by at least three drug manufacturers in conjunction with the chemical

method required by the Pharmacopoeia. What makes the method especially commendable is its simplicity, as it requires no expensive laboratory, and furthermore it can be carried out by almost any one. Lower dilutions were obtained than were supposed to be found. For example, an official fluidextract produced tingling in 1 to 375 dilution. This may have been due to the fact that distinct tingling was always required in my tests. The results of the tests will be given later.

The guinea pig test recommended by the Philadelphia Branch of the American Pharmaceutical Association[12] was given an extensive trial and yielded very satisfactory results. It is a lethal dose method and requires the determination of the minimum amount necessary to produce the death of a guinea pig within twelve hours, the dose injected being estimated per gram of body weight. This test was found to be especially delicate, the minimum lethal dose of an official fluidextract being about 0.00004 cc. per gram of body weight. It will be noticed that this amount is one-tenth of that designated in the report mentioned above.

The limits of toxicity are comparatively narrow, as will be seen from the assays below.

PREPARATION "B" (Tincture)

(+) = dead
(—) = alive

| Dose (cc. per gm. of body weight.) |
|---|
| 0.0003 — |
| 0.00035— |
| 0.00036— |
| 0.00037+ |
| 0.00039+ |
| 0.0004 + |
| 0.0005 + |

PREPARATION "C" (Fluidextract.)

(+) = dead
(—) = alive

| Dose (cc. per gm. of body weight.) |
|---|
| 0.00003— |
| 0.00004— |
| 0.00006— |
| 0.00008— |
| 0.0001 — |
| 0.00012— |
| 0.00014— |
| 0.00015— |
| 0.00016+ |
| 0.00018+ |
| 0.0002 + |
| 0.00025+ |

Before the further consideration of this method I shall give the results of the examination of eight samples by the three most satisfactory methods the lethal frog method, the lethal guinea pig method, and the Squibb method. The results appear in tabulated form below and are arranged according to the strength of the preparation examined, regardless of the actual ratio* which should exist between a tincture and a fluidextract.

| | Lethal Frog Method | | Squibb Method | | Lethal Guinea Pig Method | |
|---|---|---|---|---|---|---|
| Preparation | Cc. per Gm. | Ratio | Dilution | Ratio | Cc. per Gm. | Ratio |
| A—Fluidext. Leaves..... | 0.007 | 1 | 1-50 | 1 | 0.0005 | 1 |
| B—Tincture ..... | ? | ? | 1-60 | 1.2 | 0.00037 | 1.3 |
| C—Fluidextract ..... | 0.004 | 1.75 | 1-150 | 3 | 0.00016 | 3.1 |
| D—Fluidext. Leaves..... | ? | ? | 1-175 | 3.5 | 0.00009 | 5.5 |
| E—Fluidext. Leaves..... | 0.003 | 2.3 | 1-225 | 4.5 | 0.00009 | 5.5 |
| F—Fluidextract ..... | 0.0004 | 17.5 | 1-350 | 7 | 0.00004 | 12.5 |
| G—Fluidextract ..... | ? | ? | 1-375 | 7.5 | 0.000035 | 14.3 |
| | Gm. per gm. | | | | Gm. per gm. | |
| H—Merck's aconitine..... | 0.00000165 | 4242 | 1-225,000 | 4500 | 0.000000123 | 4065 |

* This could be found by dividing the ratio for the fluidextract by ten.

From this table the inadequacy of the lethal frog method is seen and a degree of parallelism is noticed between the Squibb method and the lethal guinea pig method, the latter showing a higher ratio except in the case of aconitine.

In summarizing this work, we must conclude that of the methods investigated the Squibb and the lethal guinea pig methods alone can be used with any degree of accuracy. The frog methods are undoubtedly worthless. Theoretically, a blood pressure method would be equally worthless, since we know that aconite contains alkaloids which have antagonistic effects on the circulation. Practically the inefficiency of such a method has been demonstrated in this investigation. The perfusion method is seen to be much more delicate than any other for aconitine, but can not be used with success with galenicals on account of the fact that they contain all three alkaloids which have dissimilar heart effects. The relative activity of aconitines, however, could be measured by the perfusion method, using a similar dilution of unlike preparations.

Many criticisms have been urged against the Squibb method, the subjective factor being regarded as detrimental. This we believe is an objection, since we found that our results were so much lower than those of other observers. However, if the individual is standardized against a good preparation, the test can be used, and we believe it is a measure of the activity of aconite, since the tingling is produced only by the aconitine and not by the other alkaloids in the drug. The guinea pig method is the most delicate toxic method investigated and showed little or no variability. The relative non-toxicity of the aconines in aconite and the parallelism noticed between the Squibb and the guinea pig method would indicate that the latter method is practically a measure of the aconitine content in aconite. The variability in the reaction of individuals to the Squibb test would lead us to conclude that the guinea pig method should be preferred as a method of biological assay.

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