More than 2,000 years ago Xenophon noted the toxicity of honey made from the flowers of certain species of Rhododendron. During the past 50 years investigations carried on, both in Europe and in Japan, have resulted in the isolation of a toxic principle or principles from various Rhododendrons, while no toxicity was observed in others. No investigation appears to have been made on R. californicum, although it has been reported as poisonous to sheep. The present investigation was made in an attempt to isolate the poisonous andromedotoxin from the leaves of this plant.

Of the several methods of isolation used, only two produced results. These were modifications of Hardikar's method of isolation from a water extract, which had been prepared after killing with boiling water any enzyme present in the leaves. The two modifications were in the method of purification of the aqueous extract. The first consisted in treatment with neutral lead acetate solution and hydrogen sulfide gas. The second (Britt's method) effected isolation by fixing with magnesium oxide the tannins present in the water extract, treating it with 95% alcohol, and concentrating to produce fine crystalline needles. The crude products, when given subcutaneously to experimental animals in amounts twice as great as the minimum lethal dose of andromedotoxin, were usually found to be inactive. Further animal experimentation is, however, necessary using larger doses since the crude product may contain large amounts of impurities and consequently small amounts of the poison. Color reactions for the substance obtained from Britt's method were similar to that of andromedotoxin, but its melting point was considerably below that reported for this compound. It is doubtful, therefore, whether andromedotoxin has been isolated from the leaves of R. californicum, but the final conclusion can not be drawn at present. Further investigation must be carried on to purify and identify the crystals which were isolated, and to learn more concerning their physiological action.
ANDROMEDOTOXIN IN RHODODENDRON CALIFORNICUM

by

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# TABLE OF CONTENTS

| Part   | Page |
|--------|------|
| I      | INTRODUCTION .......................... | 1 |
| II     | BOTANICAL ............................ | 3 |
| III    | CHEMICAL ............................ | 6 |
| IV     | PHARMACOLOGICAL ....................... | 10 |
| V      | EXPERIMENTAL ......................... | 14 |
|       | 1. Moisture and Ash content determination | 14 |
|       | 2. Preparation of Extracts ............ | 15 |
|       | 3. Isolations .......................... | 16 |
|       | 4. Tests on Crystals isolated .......... | 23 |
| VI     | CONCLUSION ............................ | 26 |
| VII    | SUMMARY .............................. | 28 |
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INTRODUCTION

Andromedotoxin, a poisonous principle found in a number of plants belonging to the Ericaceae family, is very toxic to the higher animals. It was first prepared by Eykman (6) in 1882 from *Andromeda japonica* and called by him, after the Japanese name of the plant, "Asebotoxin." Plugge (16) discovered it about the same time, first in the above plant and later in *Andromeda polifolia* (18), *A. calyculata* (19), *A. catesboei*, and in several other Ericaceae not belonging to the genus *Andromeda*, e.g., *Azalea indica*, and *Rhododendron ponticum* (20). Plugge gave it the name "Andromedotoxin."

A systematic investigation of the substance, chemical and pharmacological, was made by H. De Zaayer (5) in 1886, a summary of which was published (in Dutch) (5) by Plugge (17) in 1887. An extensive pharmacological investigation was made by S. W. Hardikar (7) in 1922, however very little is known about the chemistry of andromedotoxin.

Plugge (16) has investigated a number of plants which belong to the Ericaceae family and determined the presence or absence of andromedotoxin in them, but his list is, of course, far from complete. It has been reported from the Pacific coast that the leaves of *Rhododendron californicum* Hook., which belongs to the Ericaceae family,
are poisonous to sheep. The present investigation was undertaken to determine whether or not these leaves are highly toxic, and if so, to ascertain whether this toxicity is due to andromedotoxin.
Rhododendron californicum Hooker, belongs to the genus Rhododendron of the Ericaceae family. It is commonly known as California rhododendron or California Rose Bay. The plant is described by Jepson (90) as follows:

"R. californicum Hooker, is a rather closely erect shrub which attains a height from four to eight feet; or in the northern red woods it is a small tree up to twenty-six feet high. The shrub is evergreen. The leaves are coriaceous, oblong or elliptic, sharply acute, green above, rusty or lighter beneath, and two and a half to five and a half inches long. Calyx is five-lobed, low, obtuse, and a half line long. Corolla are turbinate-companulate, rose-purple and one and one-fourth inches long; each lobe is broad, undulate and the upper lobe is greenish-dotted within. There are ten stamens which are not exserted. Ovary is densely red-silky."

"The shrub spreads along the Pacific coast, Maplaso Creek; Waddell Creek; Pescodero; Mt. Tamalpais; abundant on the Mendocino and Humboldt coasts; W. Siskiyou Co.; N. to Washington."

Plugge (21) in 1889 examined a large number of Ericaceae plants, with a view to determine the presence or absence of andromedotoxin.
Plants containing the poison are as follows:

1. **Andromeda japonica** Thunb.  Leaves and woods
2. **A. polifolia** L.  Leaves and young twigs
3. **A. catesboei** Walt.  Leaves and flowers
4. **A. calyculata** L.  Leaves and young twigs
5. **A. polifolia angustifolia**  
6. **Rhododendron ponticum** L.  
7. **R. chrysanthum** L.  
8. **R. hybridum**  
9. **R. maximum** L.  
10. **Azalea indica** L.  Leaves and flowers
11. **Kalmia latifolia** L.  
12. **Kalmia angustifolia** L.  
13. **Monotropa uniflora** L.  
14. **Pieris formosa** Don  
15. **P. ovalifolia** Don  
16. **Rhododendron Falkoneri** Hook.  
17. **R. grande** Wight  
18. **R. barbatum** Wallich  
19. **R. flugens** Hook.  
20. **R. cinnabar** Roxb.  
21. **R. punicum** Smith  

(12-21 were investigated in 1892 (22)).

Those in which the poison was not found are as follows:

1. **Rhododendron hirsutum** L.  
2. **Ledum palustre** L.  
3. **Clethra arborea**
4. Celthra alnifolia
5. Arctostaphylos officinalis (Uva ursi) Wimm.
6. Chimaphila umbellata Nuttall
7. Oxydendron arboreum
8. Gaultheria procumbens L.
9. Arbutus Andrachne L.
10. A. canariensis Lem.
11. A. intergrifolia L.
12. A. Unedo L.
13. Arctostaphylos alpina Spr.
14. A. glauca Lindl.
15. Erica arborea L.
16. Pyrola maculata L.
17. P. rotundifolia Lin.
18. Ledum latifolium Lam.
19. Rhododendron ferrugineum L.

(9-19 were investigated in 1891 (23)).

Alfred J. M. Lasche (10) confirmed the presence of andromedotoxin in Kalmia angustifolia L., K. latifolia L., (the entire herb), and Monotropa uniflora L. (the entire herb) in 1890. In addition to those mentioned above the following plants have been found to contain the same poison:

1. Rhododendron occidentale Gray (12)
2. R. Hunnewellianum (30)
3. R. hymenanthes (11)
Chemically andromedotoxin has not been well investigated and needs further study. According to Eykman (6) asebotoxin, which is said to be identical with andromedotoxin is a toxic glucoside; while Peterson (15) states that andromedotoxin is an indifferent non-nitrogenous organic compound and not a glucoside. The latter view has been supported by many other investigators since andromedotoxin after hydrolysis does not reduce Fehling's solution.

Eykman (6) describes andromedotoxin or asebotoxin as a colorless substance, soft while moist, but brittle and transparent when dried at gentle heat. When covered with water it cakes together, becomes soft below 100° C., but does not melt until the temperature 120° C. is reached, and then forms a transparent light-brown mass. Mrs. Stedman (26), who made a chemical investigation of this compound recently under Professor Barger, states that the pure andromedotoxin crystallizes in fine, white needles containing no water of crystallization, and melts at 258° C. The melting point of this compound given in Beilstein (2) is, however, 228-229° C. and that by Chu and How (3) is 273° C.

There is some disagreement among investigators concerning the molecular weight and chemical formula for this
compound. Plugge (22) gives the formula, \( C_{31}H_{51}O_{10} \), which is also given in Beilstein (2); while Hardikar (7), and Chu and How (3), who found andromedotoxin in \( R. \) \textit{Hymenollium} \textit{newellianum}, give the formulas, \( C_{19}H_{30}O_{6} \) and \( C_{19}H_{31}O_{6} \) respectively. Makino (11) investigated \( R. \) \textit{hymenanthes} and found a principle which he called "Rhodotoxin", giving a formula, \( C_{31}H_{51}O_{10} \), which is identical with the formula for andromedotoxin given by Plugge and in Beilstein.

Solubilities of this compound in various solvents as given in Beilstein are:

| Solvent          | Solubility  | Temperature |
|------------------|-------------|-------------|
| Water            | 2.81%       | at 25°C     |
|                  | 0.61%       | at boiling point |
| Alcohol (94%)    | 11.10%      | at 25°C     |
| Amyl alcohol     | 1.14%       | "           |
| Chloroform       | 0.26%       | "           |
| Ether            | 0.07%       | "           |
| Benzene          | 0.004%      | "           |
| Ligroin          | insoluble   | "           |
| Carbon disulfide | "           | "           |

Eykman (6) states, however, that the compound is only slightly soluble in cold, more readily in hot water. He also adds that it is readily soluble in ether mixed with alcohol, but only sparingly soluble in pure ether; it is somewhat soluble in ammonia, less so in solution of soda. Plugge (16) states that the poison is freely soluble in
chloroform, glacial acetic acid, and slightly soluble in glycerin and oil of turpentine.

Solutions of the compound in water, in alcohol and in amyl alcohol are levo rotatory, while a solution of it in chloroform is dextro rotatory. Figures taken from Beilstein (2) are as follows:

A 2.8% solution in water, alcohol or amyl alcohol (α) \(12^\circ/D = -9.7^\circ\)

A .41% solution in Chloroform (α) \(12^\circ/D = +10.1^\circ\)

Chu and How (3) give an optical activity of - 52.2° for an alcoholic solution of this substance.

The solutions of andromedotoxin are neutral in reaction. The solution is not precipitated by any of alkaloidal reagents and heavy metals such as ferric chloride, copper sulfate, mercuric chloride, silver nitrate, gold chloride, platinum chloride, and does not reduce Fehling's solution, even after hydrolysis. Andromedotoxin does not decompose with dilute acetic acid. Several color reactions of the compound are mentioned by Plugge (22) and in Beilstein (2).

1. Concentrated sulfuric acid. Concentrated sulfuric acid gives a dark reddish-brown, which becomes deeper red on warming and turns light mulberry-red on dilution with water. The addition of alkali removes the color, which reappears on acidifying.
2. **Dilute sulfuric acid.** Evaporation with dilute (1:5) sulfuric acid gives a beautiful rose-red color. The pure material gives off no odor during this evaporation, but if not completely purified, a strong and very characteristic odor of ericinol is evolved.

3. **Phosphoric acid.** Evaporation with phosphoric acid gives a mulberry-red residue, clearly perceptible with very minute quantity, as in the case of the other acids.

4. **Hydrochloric acid.** According to Mrs. Stedman (26), hydrochloric acid also gives red color on heating. Eykman (6) mentions another color reaction of the compound with strong hydrochloric acid. When strong hydrochloric acid is added to its alcoholic solution, a magnificent blue color develops, accompanied by a peculiar odor resembling that of Spiraea ulmaria. This blue color, under various condition, changes to violet-red to reddish-gray.
Several epidemic poisonings of sheep from eating rhododendron have been reported by Parkinson (13) and by Slipper (25). The chief symptoms are stated to be salivation, vomiting, weakness, staggering and, in a few cases, death.

The lethal dose of andromedotoxin by subcutaneous administration varies in different animals. In the case of the frog it appears to lie between 2.5 and 3 mgm. per kilo. of body weight, but the rabbit requires only 0.3 mgm. 1.4 mgm. andromedotoxin per kilo. hypodermically caused death in 15 minutes; while .28 mgm. per kilo. hypodermically caused death in 12-24 hours in rabbit. De Zaayer (5) likewise has shown that andromedotoxin is less toxic to lower forms of life than to the higher animals. There is no influence upon unicellular organisms like Paramocium Coli and Oplina ranarum even in a saturated solution. Lumbricus terrestris remained quite normal in a solution of 1:8000. In a strength of 1:400, the worms at the commencement made active movements, became quiet shortly afterwards, but death did not occur until after the lapse of 24 hours. In frogs De Zaayer (5) observed vomiting, arrest of respiration, and a paralysis resembling that of curare. In mammals he describes the occurrence of emesis and purging, severe respiratory disturbances and dyspnoea, convulsions, and
death through arrest of respiration.

There is some difference in opinion as to the cause of the various symptoms which occurred upon administering andromedotoxin to the experimental animals. De Zaayer (5), and Chu and How (4) agree as to the cause of respiratory depression. They think it is due to a direct action upon the respiratory center because it appears in the frog before any symptoms of general paralysis, and in rabbits the nerve ends are shown to be intact by the convulsions which attend the failure of respiration and which may be prevented by artificial inflation. The respiration in the rabbit is retarded without any previous acceleration; the changes observed could not be due to an action on the vagus endings in the lungs, for reaction of the vagus does not produce similar changes. This view is supported by Hayashi and Muto (6), Archangelsky (1) and Payne (14). Hardikar, however, attributes this depression to an action of the poison on the vagus, for the poison at first stimulates and then paralyzes the terminations of the vagus. According to Chu and How (4) increase of blood pressure by andromedotoxin is due to a direct action on the heart, but Hardikar (7) states that it is due to an effect upon the vagus. Although Hardikar was unable to determine the seat of emetic action, De Zaayer (5) states that it may be caused by a direct action of the substance upon
the vomiting center, since vomiting occurred in a dog three minutes after a subcutaneous injection.

All the previous investigators, however, agree to the cause of death. In small doses, death occurs from the arrest of respiration from paralysis of phrenics. With a very large dose, according to Hardikar (7), death is due to a direct action upon the heart, the ventricles being arrested in diastole or partial systole.

In addition to those mentioned above, the following pharmacological actions of andromedotoxin have been observed by Hardikar (7):

1. Dyspnœa of the asthmatic type due partly to stimulation of afferent fibers and partly to spasm of the bronchial muscle from stimulation of its motor nerve, the vagus.

2. Increase of bronchial secretion.

3. Slowing of the heart and fall of blood pressure followed by acceleration and rise of pressure.

4. Repeated evacuation of the bowels.

(He attributes these four symptoms to stimulation followed by paralysis of terminations of the vagus).

5. Paralysis of the motor nerve ends in striped muscle. While the paralysis is developing, the muscle and nerve are more easily fatigued, but regain their excitability after a period of rest. In a stronger concentration, the poison also affects the muscle substance
itself which thus permanently loses its excitability. The manifestation of this fatigue is seen best and earliest in the nerves and muscles which have to be constantly in action, viz, the phrenics and diaphragm. The same action has been observed by Hayashi and Muto (8).

6. A narcotic action upon the higher center in the brain, the spinal cord being not affected.

7. A condition of arrhythmia in the heart dependent either upon a direct depressant action upon the conductivity tissue between the auricle and ventricle leading to heart-block, or upon the excitability of the ventricle itself. The period required for diastolic relaxation of the ventricle is increased and the diastole is incomplete.

8. The perfused frog heart was arrested with the ventricle as well as auricle in diastole; while in frogs injected with a fatal dose, the ventricle was arrested in total or partial systole with the auricle distended. The perfused mammalian heart was arrested in systole of the ventricle, but in the death of an animal injected with the poison, the right side was distended and left empty or in partial systole.

9. Involuntary muscle which is not supplied by the vagus was not affected.

10. At least a third of the poison injected hypodermically leaves the body unchanged in the urine.
Preparation of Materials

Two samples of leaves were collected—one in June, 1935 in the woods near the coast, at a point about ten miles south of Newport, Oregon, and the other in December 1936 near Eugene, Oregon. Both samples were air-dried at about 25°C by spreading on the floor of a room in the pharmacy building, in Corvallis. A portion of the first sample "A" was ground on April 17, 1936, separating the leaves from the stems and grinding each separately. The rest of the same sample "B" was ground on June 6, 1936 in the same manner. Sample "C", after drying was used without grinding or separating leaves from stems, of which the sample contained very few. The dry weight of these samples were as follows:

|       | leaves (Gm.) | stems (Gm.) | leaves (Gm.) |
|-------|--------------|-------------|--------------|
| Sample A | 1589.        | 252.        | ---          |
| Sample B | 2163.        | 303.        | ---          |
| Sample C | 8074.        | ---         | 19190.       |

Thus it is seen that in sample "C", the weight of the air-dried leaves represented 42% of the weight of the fresh leaves.

Moisture and Ash content

For the determination of the moisture and ash con-
tent of the air-dried materials, the sample "A" was used. In determining the moisture content, the leaves and stems were dried at 110° C. in an oven; and in determining ash content the air-dried sample was incinerated in a crucible until a constant weight was reached. The following results were obtained:

|         | Determined on air-dried Sample | Calculated on fresh Leaves |
|---------|-------------------------------|----------------------------|
|         | Moisture Content | Ash Content | Moisture Content | Ash Content |
| Leaves  | 16.43%            | 2.93%        | 64.90%           | 1.23%       |
| Stems   | 17.03%            | 3.30%        | ---              | ---         |

**Preparation of Extracts**

Since the poison is very soluble in water and alcohol, several alcoholic extracts were made. Two hundred grams of leaves of the sample "A" were used in the first two cases, and the extracts were made by percolation. In the last case 80 grams of the same sample were extracted in a Soxhlet extraction apparatus, using 95% alcohol. The extract was weighed after evaporating to apparent dryness on a water bath.

| Extract obtained | per cent yield |
|------------------|---------------|
| D 15% alcohol extract | 74.3 | 37.15 |
| E 25% alcohol extract | 89.0 | 44.5  |
| F 95% alcohol extract | 28.0 | 35.0  |
Isolation

Since the most extensive work on this subject was done by Hardikar (7), his methods of isolation were first employed.

Method 1 - Alcoholic extract

The extract "F" was taken up in a small quantity of water, precipitated with neutral lead acetate solution (10%), filtered, made free from the excess lead by treating with hydrogen sulfide gas and filtering. The aqueous filtrate thus obtained was evaporated to dryness on a water-bath. The residue was dissolved in a small quantity of 95% alcohol and precipitated with an equal volume of ether. This was repeated several times till the alcohol-ether treatment extracted nothing more.

At first a white milky solution was obtained, but on standing the solution became clear, leaving a heavy sticky brown precipitate at the bottom of the container. Hardikar (7) obtained from this alcohol-ether mixture a crystalline substance which was found to be physiologically inert. There not being enough precipitate to collect, the ether was driven off and the clear alcoholic solution, "G", was kept for further experiment.

Method 2 - Water extract (Hardikar)

One hundred grams of leaves of the sample "A", were
moistened with boiling water in a large beaker, which was then covered tightly with paper, and allowed to stand for 15 minutes. The moistened drug was boiled for twelve hours, replacing water lost by evaporation. The water extract thus obtained was evaporated to a syrupy consistency, mixed with saw-dust which had previously been purified with chloroform, and then extracted with chloroform in a Soxhlet extraction apparatus. The extraction was discontinued after being run for eighty hours although evaporation a small portion of the fresh extract still left some residue on a watch glass. The chloroform extract was evaporated to dryness, the residue left was purified by treating it several times with ether, which was decanted off each time. This purified residue was dissolved in n-butyl alcohol to crystallize since Mrs. Stedman (26) suggests that n-butyl alcohol is a good crystallizing solvent for andromedotoxin.

Several crystallizations from n-butyl alcohol produced a yellow powder, "H", instead of the crystals which Hardikar was able to obtain. The total weight of the yellow powder was 0.0842 gram, corresponding to a yield of 0.0842% based on air-dried drug.

This product was tested for toxicity on a 900-Gm. guinea pig, using a dose of 2 mgm. in a half cc of physiological salt solution and was found to be inert.
The product did not reduce Fehling's solution either before or after hydrolysis.

Method 3

If andromedotoxin is a glucoside as stated by Eykman (6), an enzyme will probably accompany it in the plant, and the enzyme will hydrolyze the glucoside when leaves are treated as in the previous experiments. Based on this assumption, it would be necessary, before attempting to isolate the glucoside, to kill the enzyme, which might have caused the unsuccessful results obtained in the previous experiments.

For this experiment, 280 grams of the dried sample, "C", was used. Into a 12-liter balloon flask was put six liters of water, which was brought to boiling and leaves were introduced while the water was boiling. This treatment kills any enzyme which might be present in the leaves. Boiling was continued for one hour, whereupon the water was poured off and replaced with fresh water. In this manner, several batches of extract were made. The combined extract was evaporated to a small volume on a water-bath. Half of this concentrated extract was mixed with saw-dust (previously purified with chloroform) and extracted with chloroform for thirty hours. The chloroform extract was evaporated to a small volume and treated several times with ether,
which was decanted off. The purified residue thus obtained was dissolved in n-butyl alcohol to crystallize.

A brown sticky mass, "I", with a sweetish odor was obtained. An attempt was made to remove the brown color by recrystallizing it from n-butyl alcohol, but it did not change the appearance of the substance. The total amount obtained was 0.669 gram with a per cent yield of 0.477. It did not reduce Fehling's solution either before or after hydrolysis.

A frog injected with 2.8 mgm. of this substance dissolved in a half c.c. of physiological salt solution showed in a minute after injection a convulsion of front legs, extending backwards, and five minutes after the injection a severe paralysis of the entire body, both front legs and back legs being stretched out. The heart was beating normally but the frog died in one hour.

To check the above result, a guinea pig was used since there is a difference in tolerance towards drugs between the cold blooded animals and higher animals. A guinea pig weighing 677 grams was injected with a solution of 1.695 mgm. of the substance dissolved in one c.c. of physiological salt solution. Contrary to the above result the substance did not show any toxic effect in the animal.
Method 4

A method given in the Peterson's toxicology book (15) was tried, using 89 grams of the extract "D". The extract was dissolved in a small amount of water, and extracted with a large quantity of ether to remove ether soluble impurities, especially chlorophyll, "J". The aqueous portion was then extracted with chloroform in a separatory funnel and evaporated to dryness in air.

The result was very poor—nothing but a trace of chlorophyll was left in the evaporating dish. Ether extract, "J", and the aqueous solution, "K", were kept for further investigation.

Method 5

According to Peterson (15) andromedotoxin is present only 0.005% in plants. It was, therefore, necessary to do the isolation on a larger scale than that had been done in the previous experiments. In Beilstein (2) it is mentioned that the poison is much more soluble in cold than hot water. In this experiment, therefore, the water extract was strained off after being cooled, rather than straining it right after boiling, which had previously been done.

Water placed in a large kettle was brought to boiling and 1000 grams of the sample of leaves, "C", was intro-
duced all at once. Boiling was continued for thirty minutes then the mixture was left to cool. The water extract was decanted off and evaporated to a syrupy consistency on a water-bath. As the second batch of the water extract was destroyed by fire, the third batch was prepared and combined with the first.

The total extract, "L", obtained was 519.0 grams with a percentage yield of 51.9%, based on air-dried leaves.

**Method 5-A**

The above water extract contained large amounts of tannins, which had to be removed before proceeding. The following method for their removal was suggested by Mr. Britt, an assistant professor of pharmaceutical analysis in School of Pharmacy at Oregon State College.

Of the extract "L" above, 295 grams were mixed with 300 grams of heavy magnesium oxide and made into a paste, allowing a reaction to take place between the tannic acids and magnesium oxide to form an alcohol-insoluble tannate. The paste was dried and pulverized yielding a powder which weighed 624 grams. The powder was then warmed with two liters of 95% alcohol in a large Erlenmeyer flask with constant stirring for two and a half hours at a constant temperature below 60° C. After
standing over night, the supernatant liquid was decanted off into a large graduated cylinder. An additional 750 c.c. of 95% alcohol was introduced into the flask and treated in the same manner as before for the same length of time. Both extracts were combined and evaporated to dryness.

Amber-colored sticky needles, "M", weighing 8.8 grams were deposited in the evaporating dish. This represents a yield of 2.98% of the water extract "L", or 1.55% of the air-dried leaves, but only 0.65% based on fresh leaves. When the crystals were recrystallized from n-butyl alcohol and examined under a microscope, fine colorless needles clustered in fern-like aggregations were observed.

Method 5-B

Using 78 grams of the extract "L", Hardikar's method (Method No. 1) was tried.

In the thick greenish liquid a large quantity of crystals was formed. They were collected on a filter paper and washed with water. Greenish yellow crystals, "N", were obtained. Further evaporation of the thick green liquid produced no crystals but only a mass of granules, which could not be filtered off or separated from the liquid. The weight of the first crop obtained
was 1.827 grams, corresponding a yield of 2.34% based upon the weight of the water extract, "L", or 1.22% of the dried leaves, or 0.51% of green leaves. No attempt was made to obtain more crystals from the granular mass.

Tests on the Crystals, "M" and "N"

1. Solubilities

|       | M             | N             | Andromedotoxin (Plugge) |
|-------|---------------|---------------|-------------------------|
| Water | soluble       | soluble       | soluble                 |
| Alcohol (95%) | soluble       | readily soluble | very soluble           |
| n-Butyl alcohol slowly soluble | soluble       |               |                        |
| Benzene | insoluble     | insoluble     | insoluble               |
| Ether  | insoluble     | insoluble     | insoluble               |
| Chloroform | insoluble     | insoluble     | insoluble               |

2. Color reactions (Refer to Page 8)

|       | M                                      | N                                      | Andromedotoxin                  |
|-------|----------------------------------------|----------------------------------------|---------------------------------|
| Concentrated Sulfuric acid | 1. Deep red on warming | 1. Orange red to deep red on warming | 1. Dark red-dish brown to deep red on warming |
|       | 2. Reddish purple on dilution with water | 2. Pinkish red on diluting with water | 2. Mulberry red on dilution with water |
|       | 3. The color disappeared in alkaline solution, but reappeared in acid solution | 3. Similar | 3. Similar |
|                | M                  | N                  | Andromedotoxin         |
|----------------|--------------------|--------------------|------------------------|
| Dilute Sulfuric acid | A fine red         | Dirty purple when warmed | Rose red               |
| Phosphoric acid   | Reddish-pinkish-red | Pinkish-red        | Mulberry-red           |
| Concentrated Hydrochloric acid to the alcoholic solution | Violet | Colorless | Blue color changes to violet-red to reddish-gray |
| Hydrochloric acid  | Pink               | Colorless          | Red                    |
| Fehling's solution | a. Before hydrolysis | No reduction | No reduction | --------------- |
|                | b. After hydrolysis | Reduction | Slight reduction | --------------- |

3. Melting point determination

For the melting point determination the crystals, "M", were recrystallized from water and dried in a desiccator (M'). An attempt to recrystallize this substance from n-butyl alcohol was not successful since the sticky crystals did not go into solution. The crystals, "N", were recrystallized from water, (N'), and from n-butyl alcohol (N''), and dried in a desiccator. The results compared with that of andromedotoxin are as follows:
184.6°C. 187.7°C. 185.4°C.  273°C.  228-229°C.
(Corrected melting point).

4. Toxicity of the crystals, "M" and "N"

Into a guinea pig, weighing 672 grams, were injected 0.373 mgm. of the crystals "M" dissolved in a half c.c. physiological salt solution. The injection was followed by no observable physiological effects.

Into another guinea pig, weighing 806 grams were injected 4.5 mgm. of the crystals "N" dissolved in a half c.c. physiological salt solution. This injection also showed no physiological action.
CONCLUSION

For Hardikar's method for the isolation of andromedotoxin (chloroform extraction of a concentrated aqueous extract) produced only a yellow powder which did not show any physiological action in the experimental animals. However, when this method was modified so as to kill any enzyme which might be present in leaves of *Rhododendron californicum* while making the water extract, a sticky brown mass was obtained. According to Eykman (6) andromedotoxin is converted into a transparent light-brown mass when heated above 120° C. Assuming that the brown mass obtained might be the same as that obtained by Eykman, its physiological action was tested on a frog and guinea pig. The frog showed marked reaction to the injection into the ventral lymph sac. Convulsions began within one minute and death resulted in one hour. On the contrary, the guinea pig did not respond to this substance at all, even with ten times the minimum lethal dose of the suspected andromedotoxin. Although more animal tests on this substance are necessary to confirm the above results, it may be doubted whether this substance is really andromedotoxin, since the latter is reported to exhibit greater physiological activity on the higher animals.

Hardikar's second method consisted in killing any
enzyme in the leaves by boiling with water. When cool, aqueous solution was separated, concentrated, and purified by treatment with neutral lead acetate and hydrogen sulfide gas. This process produced light greenish-yellow crystals, the color reactions and melting point of which did not correspond with those of andromedotoxin. Furthermore, this substance did not show any physiological effect upon a guinea pig even when injected subcutaneously in amounts corresponding to twice the minimum lethal dose of andromedotoxin.

The Britt method, (isolation by removing the tannins with magnesium oxide and extracting with 95% alcohol), produced amber-colored needles. These crystals showed color reactions identical with those of andromedotoxin, but the melting point was considerably lower than those reported for andromedotoxin. It was found that the substance was inactive physiologically when injected subcutaneously into a guinea pig in amounts corresponding to twice the minimum lethal dose of andromedotoxin.

While it is probable that andromedotoxin has not been isolated from the leaves of Rhododendron californicum, it is not at all certain that these leaves do not contain this toxic substance. Further purification is necessary and more animal experimentation must be carried out before a definite conclusion may be reached.
SUMMARY

1. Moisture and ash contents of leaves of *Rhododendron californicum* were determined.

2. The methods of isolation of andromedotoxin described by Hardikar and by Peterson, Webster and Haines did not prove successful.

3. When the Hardikar's chloroform extraction method was repeated, using water extract prepared by killing any enzyme which might be present along with the poison, a sticky brown mass resulted, which was found to be physiologically inert in higher animals but to cause convulsions in frogs.

4. When a portion of a water extract, prepared from 1000 grams of leaves after first killing any enzyme, was treated with magnesium oxide and extracted with alcohol (95%), a crystalline substance was obtained. Its color reactions were identical with that of andromedotoxin, but its melting point was much lower, and it was found to be physiologically inert.

5. When a portion of the water extract prepared in the same manner as before was treated with lead acetate and hydrogen sulfide gas as mentioned by Hardikar, there was obtained crystalline substance which was also found physiologically inert.

6. Thus andromedotoxin has probably not been isolated
from leaves of *Rhododendron californicum*. A tremendous amount of time was required to try each method of isolation, and with the limited time available it was not possible to cover the entire problem. Much has been left untouched at present but the investigation will be continued to determine if possible what really is the toxic constituent of the leaves of *Rhododendron californicum*. If this be andromédotoxin, then an explanation must be found for the anomalies in the melting point and in the action observed on guinea pigs.
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