[CONTRIBUTION FROM THE COLLEGE OF PHARMACY, UNIVERSITY OF MICHIGAN.]

"SOME OF THE IMPORTANT CONSTITUENTS OF DIGITALIS."

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Much work has been done in an effort to determine the comparative values of various extracts of digitalis obtained by the use of different solvents, especially alcohol and hot and cold water, as a direct result of Kraft's careful and painstaking research in which he was able to demonstrate the presence of an exceedingly labile water-soluble substance in the leaves of digitalis.[1] Though this substance, which he called "Gitalin," was afterward shown by Kiliani[2] to be a mixture and not a pure substance, the name used by Kraft is still employed to designate the water-soluble, chloroform-soluble glucoside found in the leaves.

By purely pharmacological methods, Straub[3] has attempted to determine quantitatively the total amount of active substances present in the leaves, and the fraction of this obtained by extraction. He arrives at the conclusion that the leaves contain approximately 1% of active glucosides, a much higher proportion than it is possible to isolate by chemical processes.

No attempt has been made to determine whether these various extracts are of reasonably constant composition as far as the glucosides they contain are concerned. It is possible to reach a conclusion as to the identity of the glucosides by chemical methods only, but it is surely far-fetched to assume that one can determine by tests on cats, frogs, or other animals, just what glucosides may be present in any given fraction of the drug. It therefore, would appear of some value to determine whether it is possible to obtain fractions of uniform composition, using the same methods of procedure on different samples of the drug, and if so, just which constituents will be present in a given fraction.

Since, as stated previously, it is impossible to isolate all of the active material, the use of a pharmacological method of testing the relative values of the different fractions obtained is necessary. The M. L. D. method on frogs appears to be the only one now in use which can be adapted to such an investigation where it is desired to run a large number of tests in as short time as possible. The average chemical laboratory is a poor place in which to keep the number of frogs required for the tests, so that this presented quite a problem. After reviewing the results obtained on goldfish by Pittenger,[4] it was decided to try this method as it seemed to be simpler to obtain and keep these on hand.

[1] Kraft, *Arch. Pharm.*, **250**, 118 (1912).

[2] Kiliani, *ibid.*, **252**, 13 (1914).

[3] Straub, *Arch. exp. Path. Pharm.*, **80**, 52–71 (1916).

[4] Pittenger, *J. Am. Pharm. Assoc.*, April, 1915; *ibid.*, **8**, 893 (1919).

Some experiments were, therefore, carried out with goldfish, using the method given by Pittenger in his second paper and maintaining a constant temperature of 22°.

Tinc. used. Cc.	After 3 hours.
2.5	Alive
2.6	Alive
2.7	Alive
2.8	Alive
2.9	Alive
3.0	Dead
3.1	Dead
3.2	Alive
3.3	Dead
3.4	Dead[a]
3.5	Dead
3.6	Dead

[a] This fish appeared to be alive at the end of 3 hours but died a few minutes later.

The tincture tested 300% of the U. S. P. standard by the M. S. D. method on frogs, so that there is a wide discrepancy between these results and those reported by Pittenger, who sets the 3-hour M. L. D. on goldfish as 2.85 cc. of standard tincture. The fish used were fairly large, averaging 25 g. in weight, but Pittenger states in his earlier communication that the size of the fish does not affect the result. When adjusted to U. S. P. standard, the tincture used above gave the following results.

Tinc. used. Cc.	After 3 hours.
7.5	Alive
8.0	Alive
8.5	Dead
9.0	Dead
9.5	Dead

Since the method appeared to give fairly concordant results, and this was all that was desired, the question as to why there should be such a large discrepancy between the results by the 2 methods, was not investigated at this time.

The crude drug used in the investigation was all obtained from the Stearns Medicinal Plant Gardens, at the University of Michigan, and consisted of 3 lots, 2 of which were first-year plants, one 1918 crop, and the other 1919, the third being 1919 second-year plants. No special precautions were taken in handling and storing the leaves, the drying being carried out at ordinary temperature.

Before using, the leaves were ground to a coarse powder, and this was cleaned by sifting through an 80-mesh screen in a rotary sifting machine, all the material passing through being discarded. The siftings ran as high as 34% ash while the cleaned powder gave an ash content of 9 to 11%.

Cold Water Extract.

Two kg. of the coarsely powdered first-year 1919 leaves was treated with 10 liters of cold distilled water containing 5% of alcohol, and the mixture was agitated during 8 to 10 hours by means of an electric stirrer. Too violent agitation is to be avoided as it causes intense foaming. The watery extract was then pressed off, and the process repeated, the second extract being added to the first and the whole then evaporated to a third or a quarter of the original volume at a temperature not exceeding 30°. The results obtained by shaking out the total volume of aqueous extract with chloroform without the preliminary evaporation are much more satisfactory as regards the final yield of pure product, but it is almost impossible to handle such a large quantity of material in shaking out with the facilities of the ordinary laboratory. No matter which method is used, the addition of the small amount of alcohol used in the extraction seems advisable, in the first case to retard the development of molds during the evaporation, and in the second method, to prevent the formation of emulsions in the shaking-out process, something very difficult to avoid with a large volume of liquid containing a saponin. Experiments on a small scale indicate that the addition of such a small amount of alcohol has very little effect on the amount of active material extracted. The aqueous extract obtained by either method was then extracted with chloroform until the residue left upon evaporation of a small portion possessed only a faintly bitter taste.

The cold water extract prepared in this manner contained from 20 to 22% of extractive, calculated on the weight of crude drug used. The chloroform extract, however, contained only 1.4% of extractive. These results were checked by manipulating several 25-g. samples of the same crude drug as used above, using 250 cc. of H_2O.

Sample.	Extract. G.	$CHCl_3$ Ext.	%.
1	5.15	0.36	1.44
2	4.9	0.35	1.4
3	4.7	0.29	1.16

The residue from the chloroform extract after complete removal of the chloroform, re-dissolved in water, in which it is still completely soluble, possesses an activity 10% less than that of the chloroform-insoluble fraction, measured on goldfish, as has previously been shown by Straub.[1] Whether this chloroform soluble fraction loses part of its activity through the treatment with this solvent has not been conclusively determined although according to Kraft, "Gitalin," of which this fraction appears, to be composed, is not affected by chloroform.[2]

[1] Straub, *Arch. exp. Path. Pharm.*, **80**, 52–71 (1916).

[2] Kraft, *Arch. Pharm.*, **250**, 126 (1912).

The residue is a light brown in color and soluble in all the ordinary solvents. In order to purify it, it is dissolved in chloroform and precipitated as an amorphous powder by allowing the chloroform solution to run slowly into an excess of petroleum ether, the method followed by Kraft in the preparation of "Gitalin." A pure product can be obtained only by repeating this procedure several times. Treatment with animal charcoal seems to have little effect in removing the color, besides involving considerable loss due to absorption.

About 0.4 g. of a pure white amorphous susbstance was thus obtained, melting at 152°. Tested with Kiliani's modification of Keler's reaction, it gave a reddish-violet ring, and a blue color gradually developed in the acetic acid layer. A small portion dissolved in alcohol, on the addition of half its volume of water gave a precipitate of small crystalline aggregates, melting at 73–75°, the melting-point given by Kraft for his "Gitalin-Hydrat."

An attempt was made to separate digitoxin from the product, using a mixture of methyl alcohol and chloroform as a solvent and adding ether to precipitate any digitoxin present, but nothing was obtained. No conclusion could be drawn from this as the amount available for experiment was too small.

The water-soluble, chloroform-insoluble fraction, which contains approximately 55% of the activity of the water extract was evaporated to dryness at a low temperature, the final drying being done *in vacuo* over sulfuric acid. The dry substance was treated several times with an excess of acetone, which dissolves a large amount of yellow coloring matter, in addition to the active glucoside. The powder remaining can be purified further by solution in methyl alcohol and precipitation by means of ether. It proved impossible to obtain a crystalline substance from the purified product, and no attempt was made to separate it into α-, β- and γ-saponin, as done previously by Kraft.[1] In much higher concentration than usually used for the tests on the fish, it had no effect, except to cause them to come continuously to the surface for air. Two cc. of a 10% solution injected intravenously into 250 g. guinea-pigs had no effect whatever on the animals. Tests made, using one cc. of a 2.5% suspension of washed red blood cells from sheep with amounts of a 5% solution of the saponin ranging from 0.1 to 1 cc., the solution made up to 2 cc. with physiological salt solution, showed no hemolysis after an hour's incubation at 37°. The saponin substance of digitalis seeds (amorphous digitonin), possesses hemolytic properties.

It was attempted by various means to purify the acetone extraction of the original chloroform residue, but no successful method was worked out. In every case, the product obtained was a dark-brown amorphous

[1] Kraft, *Arch. Pharm.*, **250**, 121 (1916).

substance which resisted all attempts at further purification and upon continued treatment with solvents became more and more insoluble in water. A product was obtained, however, which was approximately 50% more toxic to the fish than the purified "Gitalin."

Alcohol Extraction.

The leaves, after extraction with cold water, were dried at a low temperature and treated with 3 liters of 50% alcohol after thoroughly moistening them with the same solvent. The mixture was allowed to stand overnight at room temperature, the liquid pressed off and the treatment repeated. After the second extraction, a test made on a portion of the extracted powder showed it to be inactive. The united alcoholic extracts were then precipitated with lead acetate, filtered, the excess lead removed with sodium phosphate and the final clear solution evaporated under reduced pressure on the steam-bath to a volume of about one liter. A copious precipitate was formed which was filtered off, dried at a low temperature, powdered finely and exhaustively extracted with 100 cc. of chloroform, by heating it on the steam-bath under a reflux condenser for 15 minutes. The chloroform solution was then evaporated, the greenish-brown residue taken up in hot alcohol, in which it is completely soluble, and digested with animal charcoal. After filtering, the solution was evaporated, the residue taken up in chloroform, the chloroform solution poured into an excess of ether and the mixture allowed to stand overnight. A peculiarity of the crude substance obtained from the chloroform extraction seemed to be that animal charcoal decolorized it much more efficiently in alcoholic solution than when chloroform was used as the solvent.

The precipitate obtained amounted to about 1.5 g. of an amorphous substance. Upon recrystallizing it from hot alcohol, long plates were formed, which melted at 244°, and showed a marked play of color between crossed nicols. They gave Keller's digitoxin reaction.

Subs., 0.1805, 0.1911: H_2O, 0.1387, 0.1471; CO_2, 0.4225, 0.4470.

Calc. for digitoxin, $C_{34}H_{54}O_{11}$: C, 63.90; H, 8.52. Found: C, 63.83, 63.79; H, 8.6, 8.58.

The filtrate obtained after the evaporation of the original alcoholic extract of the leaves had only a slightly bitter taste, and as it was without action on goldfish in the ordinary dilutions it was discarded.

According to Kraft,[1] after the exhaustive extraction of the precipitate obtained by evaporation of the alcoholic extract, there remains an inactive, chloroform-insoluble glucoside which he called gitin. However, after repeated extraction of the chloroform insoluble residue with hot alcohol and subsequent evaporation and purification of the united alco-

[1] Kraft, *Arch. Pharm.*, **250**, 138 (1912).

holic extracts, nothing was obtained except a trace of an amorphous substance and a few crystals resembling those of digitoxin.

Hot Water Extract.

The constituents of the infusion of the leaves appeared of special interest to us in view of some experiments performed by R. A. Hatcher.[1]

Two kg. of 1919 first-year leaf was treated with 8 liters of boiling water, the mixture allowed to stand on the steam-bath for an hour with occasional agitation, the watery extract pressed off and the process repeated. The united extracts were then strained through cotton and evaporated on the steam-bath in a current of air to a thick liquid. Evaporation takes place very rapidly in this manner, and the temperature of the liquid does not rise above 70°.

Instead of adding a large excess of alcohol at this point, which was the method followed by Hatcher, only enough was added to thin the syrupy liquid obtained from the evaporation. The precipitate was then removed and the aqueous-alcoholic mixture shaken out 5 or 6 times with 100 cc. of chloroform. The chloroform solution was evaporated, the brown resinous substance remaining dissolved in 90% alcohol, treated with animal charcoal, filtered, evaporated, dissolved in chloroform, and the chloroform solution diluted with twice its volume of petroleum ether. After standing for 48 hours, the amorphous precipitate was filtered off. Tests on a small portion showed it to be partially insoluble in a small amount of alcohol. Accordingly, the substance was dissolved in 5 parts of a mixture of equal volumes of methyl alcohol and chloroform, a solvent used by Kiliani in some of his later work on the digitalis glucosides, and an equal volume of ether was added. Upon standing overnight, a white incrustation appeared at the edge of the liquid. After this crust was filtered off and an equal volume of ether added to the clear solution, the latter became cloudy, and, after 24 hours later, a small amount of white incrustation had again appeared. The total amount of this substance obtained amounted to about 0.75 g. Upon recrystallizing it from alcohol, long plates, which melted at 240–245°, were obtained, exhibited a play of color under crossed nicols and gave Keller's digitoxin reaction.

Subs., 0.1556, 0.1485: CO_2, 0.3638, 0.3475; H_2O, 0.1196, 0.1140.
Calc. for $C_{34}H_{54}O_{11}$: C, 63.90; H, 8.52. Found: C, 63.77, 63.83; H, 8.61, 8.56.

The methyl alcohol-chloroform-ether solution was evaporated to dryness and the residue crystallized from alcohol. The product appeared to be a mixture of an amorphous substance with crystals of very irregular form when examined under the microscope. Careful recrystallization from alcohol in which the crystalline portion of the precipitate seemed to be insoluble gave an amorphous substance melting very definitely at

[1] A short summary appears in the *J. Am. Pharm. Assoc.*, 8, 913 (1919).

150–160°. Solution in a very small quantity of alcohol and the addition of an equal volume of water gave a precipitate of the smaller granular aggregates melting at 73–75° which had been obtained from the chloroform soluble portion of the cold water extract; further investigation of this fraction was not made because of the small amount of material available; we assumed it to be what Kraft designated as "Gitalin-Hydrat."

The crystalline portion of the precipitate from the methyl alcohol-chloroform-ether solution proved to be insoluble in practically all the ordinary solvents except dil. alcohol, and the crystals melted at 257°.

Subs., 0.1654, 0.1864: CO_2, 0.3864, 0.4352; H_2O, 0.1295, 0.1463.

Calc. for anhydrogitalin ($C_{28}H_{46}O_9$): C, 63.83; H, 8.8. Found: C, 63.70, 63.66; H, 8.76, 8.78.

The total yield of both amorphous and crystalline substance amounted to 1.1 g.

The activity of the chloroform-soluble portion of the infusion was approximately double that of the chloroform-insoluble portion. This latter, as in the case of the chloroform-insoluble fraction of the cold water extract, was evaporated, dried, and extracted with acetone, thus separating the saponins from the active glucoside. The same methods of purification were employed; but, as stated previously, no feasible means were found of getting the active substance in a pure condition.

The powder, after being extracted with hot water, was dried and extracted with 50% alcohol, the extraction and subsequent purification being carried out in identically the same manner as in the case of the cold-water extracted drug. The final purified product amounted to 0.9 g. of the long plates characteristic of digitoxin, melting at 244°.

All of these extractions were repeated with 2 other lots of drug, one consisting of 1919 second-year leaf, and the other 1918 first-year leaf, and closely tallying results were obtained. It should be borne in mind that little attention was paid to the yields obtained, the object being solely to get a pure product from the various fractions which could be positively identified. Care was taken to insure that the manipulation of each lot was the same as regards time, amount of solvent, etc.

Conclusions.

1. It is possible with careful manipulation and uniform methods of procedure to obtain crude fractions from the drug by means of various solvents which will have a reasonably constant chemical composition. In view of the difficulty of separating individual glucosides in a chemically pure state from these crude fractions, such an undertaking at present seems to have no commercial value.

2. The cold-water extract of the leaves contains either none or a very minute quantity of digitoxin, its activity being due entirely to a chloroform-soluble and to a chloroform-insoluble fraction. In view of the fact

that Kiliani reports that "Gitalin," made according to Kraft's process, is a mixture containing some digitoxin, this cold-water extract requires further investigation, especially as very little is known of the chloroform-insoluble portion.

3. The saponing substance of digitalis leaves, digitsaponin, is a totally inactive and non-hemolytic susbtance when purified. This disposes of one possible source of error in pharmacological tests on the leaf.

4. Hatcher's chloroform-soluble fraction of the infusion is a mixture of "Gitalin" and digitoxin. If the accepted theory that the cause of the deterioration of digitalis preparations is the water-soluble, chloroform-soluble substance or substances which we have designated as "Gitalin" is true, then such a fraction should also be unstable. Work is being continued on this point.

The writer is indebted to Dr. Henry Kraemer for his many suggestions, and for his assistance in obtaining the crude drug used in this investigation, which was obtained from plants grown by him at the Frederick Stearns & Co. Medicinal plant garden at the University of Michigan during 1918 and 1919.

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A FURTHER STUDY OF THE PROCESS OF PURIFYING PANCREATIC AMYLASE.

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In a recent paper from this laboratory[1] the proteolytic and amylolytic activities of some of the fractions obtained in the purification of pancreatic amylase were compared. The results showed that the attempts to purify pancreatic amylase by fractionation of pancreatin result in greatly concentrating both amylolytic and proteolytic activities in certain fractions, but also revealed large losses of both types of enzyme activity and indicated to us that the purification process should be studied in some detail in order that more light might be thrown upon the place and nature of the losses. Especially was it sought to determine in so far as practicable whether the loss of amylolytic activity which occurs, for example, when the partially purified amylase is dissolved and reprecipitated, is due to incompleteness of precipitation, to deterioration (inactivation) of the enzyme under such treatment, or conceivably to a change of amylase into protease or a shifting of amylolytic to proteolytic activity. Accordingly, the process of purification previously developed in this laboratory[2] was carried out with determinations of total solids and enzyme activity at

[1] Sherman and Neun, THIS JOURNAL, 41, 1855–62 (1919).

[2] Sherman and Schlesinger, *ibid.*, 33, 1195–1204 (1911).