A PHYTOPHARMACOLOGICAL STUDY OF DIGITALIS ASSAY.*

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INTRODUCTORY.

It is well known that up to the present time the only way of estimating the therapeutic efficiency of Digitalis preparations has been through biological assay. Such methods have been discussed in the United States by Edmunds (1), Hale (2) and a host of later writers, and in Europe by Ziegenbein (3), Führer (4), Storm Van Leeuwen (5), Heinz (6), Kobert (7), Straub (8), Heffter (9), Wiechowski (10), Modrakowski and Sikorski (11), Leyko (12) and others. It is equally well known that all physiological or biological assay methods as yet described are quite unsatisfactory. Every one and all of them have certain advantages on the one hand, and a great many disadvantages or disqualifications on the other hand. Thus the frog method as first introduced by Houghton (13), and later elaborated by Hamilton (14), Focke (15), Gottlieb (16), Lyons and Famulener (17) and others, while quite simple in its self, is subject to all kinds of variations; the species of frogs, the season of the year, the temperature at which the animals are kept, and the different susceptibility of different individual frogs, all play a rôle. The most important method at present in use, in the opinion of probably the majority of pharmacologists, is the cat method of Hatcher and Brody first described in 1910 (18) and later slightly modified by various other investigators. This method being an intravenous one and using warm-blooded animals gives, in the opinion of clinical pharmacologists, a better idea of the therapeutic efficiency of the Digitalis preparations tested. Its importance and value in pharmacological and clinical work has been emphasized by Rowntree and Macht (19), Eggleston (20), Haskell and Courtney (21), Storm Van Leeuwen, den Besten (22), Kuroda (23) and many others. Nevertheless even in this method marked variations are obtained which have not yet been satisfactorily explained, as pointed out recently by de Lind Van Wijngaarden (24) and others. Besides the above two principal methods of Digitalis assay which have been recognized by the U. S. P., other curious and interesting methods have been described. Thus Pittenger and Vanderkleed published a gold fish method (25), Wentz (26) suggests the rat (white rat) as a suitable test subject; and Reed and Vanderkleed (27) and recently Knaffl-Lenz (28) have recommended the guinea-pig as a suitable animal for testing Digitalis preparations. Schmiedeberg (29) and Straub recommended the use of excised frogs' hearts. A colorimetric method has also been described by Knudson and Dresbach (30) but has not met with much success in other hands (Wible (31)). The percentage of variation in the cat method has been shown to decrease, if vagotomy is first performed (Macht and Colson (32) and de Lind Van Wijngaarden).

In connection with studies in Phytopharmacology and Phytotoxicology carried on by the senior author, with various collaborators (33), (34), a few experi-

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ments were incidentally made on the effect of Digitalis solutions on the growth of some seedlings. A one per cent solution of Tincture of Digitalis was made in Shive solution and the growth of seedlings of *Lupinus albus* immersed in this, was compared with the growth of similar seedlings in a pure Shive or nutrient solution for plants. It was found that the digitalis solutions produced a distinct inhibition in the growth of the seedlings which was not accounted for by their alcohol content, inasmuch as control experiments with exactly equivalent amounts of alcohol alone without digitalis extract were found not to be as toxic. This observation stimulated the authors to further work and experiments were made with various concentrations of digitalis in Shive solution maintaining the alcohol content in every case the same. It was found that the inhibition of growth in the seedlings used was proportional to the concentration of the digitalis. This suggested the possibility of developing a new method for the assay of digitalis solutions by the use of living plants or seedlings. Accordingly a large series of experiments were begun with this end in view and the results obtained were so remarkable and constant that such a phytopharmacological method of testing was found to be very valuable in comparative studies of digitalis preparation along other lines, such as their deterioration by light as conducted by the authors. It is proposed to describe this plant or phytopharmacological method in the present paper.

METHOD.

The toxicity of digitalis preparations was studied by the authors on growth of the seedlings of *Lupinus albus* by measuring carefully the elongation of the well-defined single, straight rootlets. In order to eliminate all variations due to changes in hydrogen-ion concentration, bacterial growth, fungi, etc., tinctures of digitalis alone were employed exclusively in this work. The *Lupinus albus* is especially adapted to experiments in plant physiology and pharmacology because it can be easily germinated and the growth of the roots accurately measured. The procedure employed in the present experiments was as follows:

The dry seeds were soaked over night in tap water at the ordinary room temperature. On the following day the swollen seeds were planted with the hilum downward in finely ground moist sphagnum moss. The seeds were then placed in the dark and kept at a constant temperature of about 20° C. On the third day after planting the seeds, the seedlings are of convenient size, the length of the roots being generally from 20 to 30 millimeters. The length of these roots can be accurately measured because of a definite line of demarkation indicating the border line between root and stem. After recording the exact length of a root, the seedling is placed in an upright test-tube of hard glass containing a nutrient solution for plants, the seed resting on the upper edge of the tube. The solution employed was a so-called Shive solution (35), which contains calcium nitrate, magnesium sulphate and mono-potassium acid phosphate. Such a solution is prepared by mixing 10.4 cc. of a 0.5 molar solution of calcium nitrate, 30 cc. of a 0.5 molar solution of magnesium sulphate and 36 cc. of a 0.5 mono-potassium phosphate solution, with distilled water sufficient to make one liter. The normal growth of the Lupine rootlets was studied by immersing the seedlings in a mixture of normal Shive solution with an equal part of distilled water. The

effect of unknown substances, as studied by Macht and co-workers in other connections, was to add a definite percentage of the unknown to the distilled water and then mixing with an equal part of Shive.

In the present work the normal growth of the seedlings was first determined by using equal parts of distilled water and the Shive solution. Then a one per cent solution of tincture digitalis was made up by taking 1 cc. of tincture digitalis and adding to it sufficient water to make 50 cc. This was mixed with 50 cc. of Shive solution and the growth of seedlings in such a one per cent solution of digitalis tincture was noted. Inasmuch as tincture digitalis contains a definite amount of ethyl alcohol the growth of the seedling in the digitalis was compared not only with the growth in normal Shive but also with a Shive solution containing one per cent of alcohol of exactly the same strength as the original tincture. In this way it was found that a digitalis solution definitely and markedly inhibits the growth of the plants on the one hand, and that such an inhibition of growth was not due to the alcoholic content of the same. Such preliminary experiments with a one per cent solution of digitalis were followed by more elaborate experiments with various concentrations, the procedure followed being very much the same as the foregoing. In these extensive experiments the average growth of at least ten seedlings was determined for each solution used. First a series of ten or more hard glass test-tubes were filled with equal parts of Shive solution and distilled water and healthy seedlings were carefully measured and immersed in each. Then a series of an equal number of hard glass test-tubes was filled with a one per cent solution of alcohol, the alcohol concentration being exactly the same as that of the tincture to be examined, and another series of seedlings was carefully measured and immersed in this alcohol Shive solution. Next various percentages of tincture of digitalis were prepared, always maintaining the same alcohol contents while varying the amount of digitalis used. In this way a series of solutions containing tincture of digitalis of various strengths was prepared and their effect on the growth of the plants studied. To make this procedure clearer the following illustration may be given. A one per cent solution of a tincture digitalis is prepared by mixing 1 cc. of the tincture with 49 cc. of distilled water and 50 cc. of Shive solution. A 0.9 per cent solution of tincture of digitalis is prepared by taking 0.9 of the tincture and 0.1 cc. of a 71 per cent of alcohol solution and 49 cc. of water plus 50 cc. of Shive solution. A one-half per cent solution of tincture digitalis is prepared by taking 0.5 cc. of tincture plus 0.5 cc. of alcohol 71 per cent, and 49 cc. of H_2O and 50 cc. of Shive solution. In a similar way any desired percentage of concentration of digitalis for phytopharmacological study was prepared, always taking care to maintain the alcohol contents of all of the solutions exactly the same, in order to determine the effect of the digitalis and not of the alcohol.

After all the solutions have been prepared and all the seedlings have been measured and immersed in them, the plants are placed in the dark, preferably in a thermostat or closed space and left over night at a constant temperature. The temperature throughout the experiment was followed by means of a thermograph, although variations or fluctuations in the temperature were of no significant importance, inasmuch as all the plants or seedlings including the controls were always kept under the same conditions. After a period of twenty-four hours and some-

times twenty hours the seedlings are examined again. The roots are measured and the growth in various solutions is compared with the average growth in normal Shive and also with the average growth in a Shive plus normal alcohol. The results obtained with tincture digitalis are expressed in percentages as compared with the growth in the alcohol Shive control, and may be termed the coefficient or index of growth. In this way numerous experiments were performed with different tinctures, and the results analyzed and correlated.

Figure 1 illustrates four seedlings of *Lupinus albus*, two of which are immersed in normal Shive solution and two others in a solution to be tested.

Figure 2 illustrates how a large number of experiments can be performed at one time. The board in the figure can hold a large series of test-tubes so that each row of seedlings can be used for testing different solutions under exactly the same conditions of temperature, light, etc.

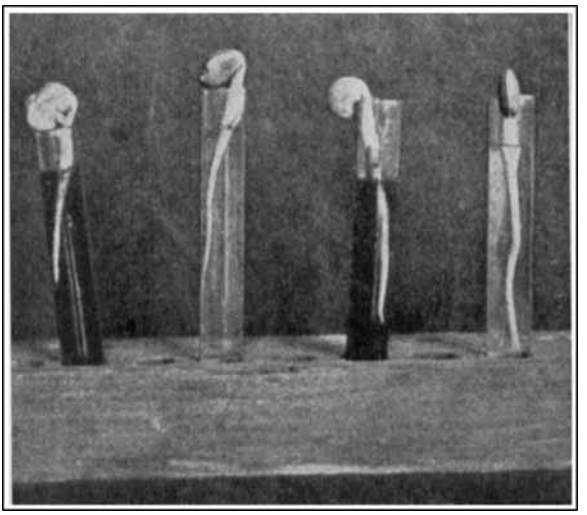


Fig. 1.—Lupinus albus seedlings in normal and test solutions.

RESULTS.

The U. S. P. Tincture of Digitalis was used throughout the present research. Ten different samples of tinctures obtained from various sources were studied

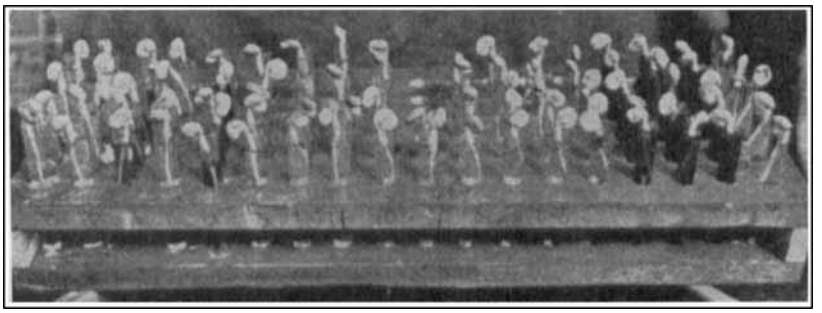
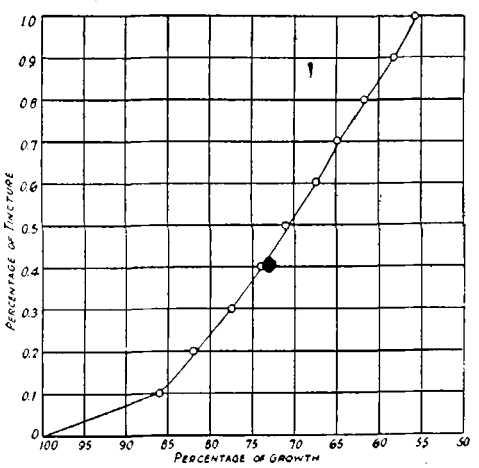


Fig. 2.—A series of experiments on Lupinus seedlings performed at the same time.

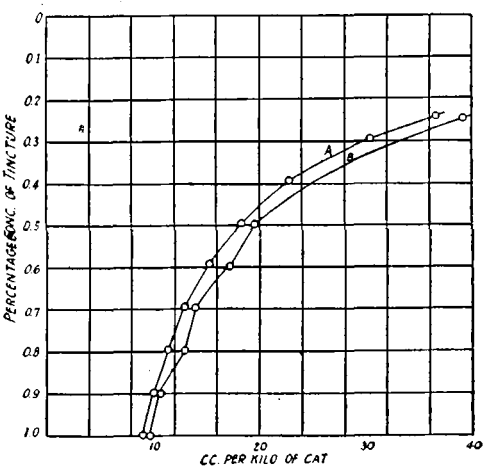
and, as might have been expected, great variations in the potency of the different preparations were noted.

The investigation was begun with an extensive and intensive study of Tincture No. 1. This tincture was prepared according to the specifications of the United States Pharmacopœia and contained 71% of ethyl alcohol. Solutions varying from one-tenth per cent to one per cent of this tincture were prepared

in Shive solution as heretofore described, always maintaining the amount of the alcohol the same, namely, one per cent. Thus when a one per cent solution of the tincture was desired, 1 cc. of the tincture was added to 49 cc. of distilled water and 50 cc. of Shive solution. When fractional percentages of the tincture were required the amount of alcohol was made up to one per cent by adding a sufficient amount of 71% ethyl alcohol to the fraction of cc. of the tincture employed. In all experiments at least ten seedlings were measured and their growth studied in each and every solution examined, so that the figures obtained were an average of at least ten individual biological test objects, that is seedlings. The results obtained with the various concentrations of the tincture were expressed in terms of per cent regarding growth in the one per cent alcohol Shive solution as the norm. In this way it was found that the growth of seedlings in various concentrations of digitalis solutions was, physiologically speaking, in direct ratio to the strength of the solution employed. A marked inhibition was obtained with the one per cent digitalis solution, and the amount of inhibition decreased with a decrease in the



Curve No. 1.—Concentrations of digitalis and growth of seedlings of lupinus albus.



Curve No. 2.—Concentrations of tincture of digitalis and potency on cat's heart.

percentage of the digitalis concentration, so that a curve could be easily plotted expressing the growth of the roots in terms of the concentrations of digitalis.

A large number of experiments were thus performed, using Tincture No. 1 and a composite curve was drawn based on the data thus obtained. The subjoined illustration gives the curve obtained in this way. (Curve No. 1.)

After having studied thoroughly the Tincture No. 1, other tinctures were examined in the same way and on comparing the results obtained with those obtained with the first or what we may call Standard Tincture, it was found that the effect on growth varied with different preparations used. In every case, however, the curve of relation between the toxicity for plants and percentage of digitalis solution used (0.1 to 1.0%) was practically the same as that obtained with Tincture No. 1, so that knowing the toxicity of a one per cent solution of any given tincture A, the toxicity of any other percentage of the same tincture between 0.1% and 1.0% could be calculated by simple proportion from the curve of Tincture No. 1. The variations obtained by this plant method with various

tincture samples agreed very well with the figures obtained concerning their potency by using the cat method. Table I exhibits concisely the results of all of the experiments with the various tinctures. In one column there will be noted the coefficients of growth obtained for the various tinctures by the phyto-pharmacological method. In the adjoining column are expressed the average potencies of the various tinctures obtained by the cat method. It may be stated at once in this place that the cat units obtained with the various tinctures as here expressed are an *average* of at least three and often five animal experiments, because great variations were obtained by using the cat method. The method of assay followed throughout this investigation was the Hatcher-Brody Method without the concluding Ouabain injection. It has been described by Rowntree and Macht elsewhere and consists briefly in diluting the tincture with ten times the amount of physiological saline solution, and injecting intravenously 10 cc. of the solution for the first five minutes and 1 cc. per minute after that until a stand-still of the heart was produced. In every case a satisfactory cat experiment required a stoppage of the heart before the failure of respiration.

It will be noted from Table No. I, that the results obtained by the plant method compare very favorably with the results obtained by the cat method. Indeed, having determined the potency of the first tincture for both plants and cats, the potency of any other unknown sample of digitalis tincture could be obtained in cat units by simply determing its phytotoxic index and comparing the same with the phytotoxic index of the first tincture.

TABLE I.—ASSAY OF TEN DIFFERENT TINCTURES BY THE PLANT AND CAT METHODS.

No. of tincture.	Plants' index of growth, in one-half per cent solution.	Cats' lethal dose per kilo wt., diluted with saline 1:10.
Tincture 1	72%	9.2 cc.
Tincture 2	76%	10.4 cc.
Tincture 3	70%	See Table No. II
Tincture 4	93%	13.7 cc.
Tincture 5	77%	11.0 cc.
Tincture 6	74%	9.3 cc.
Tincture 7	86%	12.0 cc.
Tincture 8	75%	9.8 cc.
Tincture 9	69%	9.1 cc.
Tincture 10	73%	9.4 cc.

TABLE II.—ASSAY OF TINCTURE NO. 3—BY PLANT AND CAT METHODS SHOWING REMARKABLE VARIATIONS BY THE CAT TESTS.

	Plants' index of growth.	Cats' lethal dose per kilo wt.
Experiment 1	70%	7.3 cc. (Tincture Diluted 1:10)
Experiment 2	70%	9.2 cc. (Tincture Diluted 1:10)
Experiment 3	72%	11.7 cc. (Tincture Diluted 1:10)
Experiment 4	71%	13.1 cc. (Tincture Diluted 1:10)
Experiment 5	73%	10.1 cc. (Tincture Diluted 1:10)

On examining Table No. I, a close agreement is noted between the plant and the cat methods. As a matter of fact the data obtained by the animal method are much more liable to wide variations than those obtained with an average of ten seedlings or more. The authors have found, as well as all other workers with the cat method, that sometimes quite uniform results may be obtained, but at other times and with other tinctures no uniformity in the cat units could be obtained at all. Thus for instance, in case of one of the tinctures studied (No. 3)

no definite information at all could be obtained by the animal method, because of the very different results obtained in each cat experiment as will be seen in Table No. II. It will be noted that the figures obtained from five experiments with plants were practically identical, while the toxicity given by five cat experiments was very different in each case. What the explanation for such great variations may be is still problematical. In Curve No. 2 are plotted the toxicity units obtained with various percentages of the same Tincture No. VIII. On comparing this curve with Curve No. 1 expressing a similar relationship between toxicity and concentration of digitalis as determined by the plant method it will be noted again that the cat curve is not as uniform as the plant curve.

COMMENT.

Although such curves as have been described may be useful in determining the potency of digitalis tincture for animals in terms of their toxicity for plants, the greatest usefulness of the phytopharmacological method of study is in research work, for the comparison of a large number of tincture samples. Thus for instance this phytopharmacological method enables one to compare easily the effect produced on the keeping qualities of digitalis preparations by various deleterious agents such as heat, light and other radiations; and indeed the authors have employed this method extensively in their study of the deterioration of digitalis published elsewhere. It has been not at all unusual in such work for the authors to test twelve or fifteen samples of digitalis tincture on ten seedlings each at one and the same time. It is evident that to make such a comparative examination of a large number of digitalis samples by means of cats using at least two or three animals for each preparation would involve an enormous expenditure of energy, quite a considerable cost, and a very much longer period of time. Furthermore, the plant experiments require a much smaller amount of any given preparation than the cat experiments. One cc. of a tincture and even less are quite sufficient for making a test on ten or more seedlings, whereas at least five cc. and usually more of the tincture are necessary for a single cat experiment.

Another and very interesting feature of the phytopharmacological examination of digitalis preparations must be mentioned in this place. Important work has in the past few years been published by various investigators concerning the influence of electrolytes on cardiac response. Thus it has been shown that the "pacemaker" may be shifted at will by changes in the Ca- and K-ion ratio (Sakai (36), Daly and Clark (37), Kolm and Pick (38)). The ratio of potassium and calcium ions in the blood is of great importance also in relation to the action of digitalis bodies. This has been emphasized especially by Loewe (39), Pick (40), and Horst-Meyer (41) and others. Briefly it may be stated that an increase in calcium tends to potentiate the toxicity of digitalis substances for the mammalian heart, causing a systolic stand-still; while an increase in potassium, as is well known, tends to paralyze the heart muscle and bring a stand-still in diastole. Indeed, it is possible to render a given preparation of digitalis much more toxic by adding a certain amount of calcium to it. Thus, for instance, the authors by adding small quantities of calcium chloride to Tincture No. VIII increased the toxicity from 9.8 cc. per kilo to 8.2 cc. per kilo weight of cat. Such an adulteration of digitalis preparations can be easily detected by the plant method.

The authors have studied the effects of increasing and decreasing potassium and calcium ions in Shive solution on the growth of *Lupinus albus* seedlings, and found that increase in the calcium produces an improvement in the growth of the plants, and in the same way slight increases in potassium ions in Shive solution also improve the growth of the seedlings. Additions of small quantities of calcium or potassium chloride, respectively, to tincture of digitalis were also found not to increase their toxicity for plants and, indeed, if anything to lessen it. This reaction of the plants is diametrically the opposite from the reaction of cats to such preparations of digitalis.

SUMMARY.

1. The effect of digitalis solution was studied on the growth of seedlings of *Lupinus albus* and found to inhibit it.

2. The inhibition on the growth of the seedlings is proportional to the concentration of the digitalis solution used, so that a simple curve of relationship between growth and concentration can be constructed.

3. Such a phytotoxic curve has been calibrated in terms of cat units and has been found useful in gauging the potency of digitalis tinctures.

4. The phytotoxic test is especially useful in the comparison of different samples of digitalis tinctures in order to detect differences in potency produced by various physical and other agencies.

5. By means of the phytopharmacological examination a large number of digitalis samples can be simultaneously tested, in a short time, at a smaller cost, and with less expenditure of labor than the cat method; and is superior to the latter in showing less variation and in certain other respects, notably in regard to the relationship of digitalis principals to potassium and calcium ions.

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THE DETERMINATION OF SMALL QUANTITIES OF BISMUTH IN TISSUE, EXCRETA, BLOOD AND BONE.

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There are a number of methods for the determination of bismuth in small quantities. These methods are mostly based on the determinations in colloidal solutions by using an alkaloid with the sulphide, the oxide or some other form of bismuth. As the accuracy of methods based upon colloidal suspensions is questionable, they are not to be recommended.

In 1887, Stone[1] found that when small quantities of bismuth in solution were treated with an excess of potassium iodide in the presence of sulphuric acid and sulphurous acid, a characteristic greenish yellow color was obtained. This was due to the formation of a complex bismuth potassium iodide having the formula $BiI_3.2KI.4H_2O$. Rowell[2] made use of this compound in analyzing ores and alloys containing copper, lead, etc., for small quantities of bismuth. He found that alkalis in excess, ammonium acetate, hydrochloric acid and chlorides bleached the color. The heavy metals such as arsenic, lead and copper form color and must be removed. Iron in the absence of sulfurous acid liberates free iodine.

C. A. Hill[3] recently in analyzing urine digested the organic matter by a method of wet ignition using nitric acid (Sp. gr. 1.42). He developed the color for comparison, however, by the aid of an alkaloid. Caille and Viel[4] in determining small quantities of antimony and bismuth ignited their samples in a muffle furnace.

Soon after the method to be described in this paper had been perfected, C. S. Leonard[5] published a method in which samples were digested by heating in a

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