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PRELIMINARY NOTE ON A NEW PHARMACODYNAMIC ASSAY METHOD,

CARASSIUS AURATUS (GOLD FISH) AS TEST ANIMALS FOR THE DIGITALIS SERIES.

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"The purpose of the pharmacodynamic assay just as of the chemical assay is to secure a means of measuring therapeutic activity and to make it possible to furnish *uniform* preparations. A satisfactory method which meets these requirements may or may not involve the production of physiologic reactions similar to those for which the drug is intended to be the means of producing when used therapeutically. That the effect chosen as a means of standardization does not parallel the clinical effect sought is not sufficient to condemn the method. It is only necessary that the effect chosen as an earmark is always indicative of a good quality of the drug or preparation, and criticisms of methods on the ground that they are *toxic* methods or that the animal chosen is biologically much different than man are made only through a lack of conception of the real purpose of the pharmacodynamic test, namely, to secure *uniformity*. The determination of the real value of a drug in the treatment of disease in man is another matter entirely."[1]

[1] Quoted from "Modern Methods of Drug Standardization," by Stewart, Hitchins, Elgin, Vanderkleed and Pittenger, Monthly Cyclopedia and Medical Bulletin, January, February and March, 1913.

Since biologic standardization was first proposed in 1898 the methods of assay employed for the digitalis series have been for the most part, not new but merely quantitative applications of the methods which had been used to elicit the drugs' actions. Of the many methods proposed, with their various modifications, there are only three which have been extensively used for standardization purposes, i. e., Houghton's "12 hr." frog method, Famulener and Lyons' "1 hr." frog method and Reed and Vanderkleed's guinea pig method.

Although the above methods all give more or less satisfactory results and have been revolutionary in that they have enabled the manufacturer to supply the physician with preparations of known and definite activity, there are still some things to be desired, first, a reduction in the cost of the assay; second, a more sensitive method, and third, a method simple enough that any competent pharmacist or physician can carry it out equally as well as the expert.

Just three weeks ago, one of the authors[2] conceived the idea of using fish as test animals by placing them in varying dilutions of the drug and noting the minimum dilution which will cause the death of the animal in a given time.

In experiments on frogs and guinea-pigs we have always been of the opinion that after taking care of the weight and temperature factors, the most important cause of animals' dying or recovering "out of order" is the marked variation in rate of absorption. The great absorptive power of the gills of a fish, together with the fact that they contain a large number of blood vessels through which the blood circulates direct from the heart, made this animal present itself as a possible means of eliminating these variations due to absorption.

Accuracy being the prime requisite for any assay method, it was first necessary, therefore, to determine whether or not the fish would die in direct proportion to the strength of the dilution in which they were placed. Accordingly the following experiment was carried out on gold fish.

Preparation Employed, Fld. Ext. Digitalis No. 1—Experiment No. 1.

Dilution	Actual Amt. of F. E. Contained in 300 cc. Water	Temperature	Weight of Fish	Time Required to Cause Death
1— 750	0.4 cc.	25° C.	4.3 gm.	2 hours, 1 minute
1—1000	0.3 cc.	"	4.4 gm.	3 hours, 7 minutes
1—1250	0.24 cc.	"	2.5 gm.	4 hours, 59 minutes
1—1500	0.2 cc.	"	4.0 gm.	7 hours, 2 minutes
1—1700	0.192 cc.	21° C.	4.0 gm.	9 hours, 7 minutes
1—1800	0.166 cc.	"	3.4 gm.	21 hours, 3 minutes
1—1900	0.157 cc.	"	3.5 gm.	22 hours, 16 minutes
1—2000	0.15 cc.	"	3.2 gm.	24 hours, 58 minutes

The dilutions were made with "tap water," 300 cc. placed in an 800 cc. beaker and the fish added. No attention was paid to weight or temperature other than recording the same.

The experiment was repeated with tincture of digitalis, with the following result:—

[2] Pittenger.

Experiment No. 2.

Dilution	Actual Amt. of Tinct. Contained in 300 cc. Water	Temperature	Weight of Fish	Time Required to Cause Death
1— 75	4.0 cc.	27—28° C.	3.2 gm.	2 hours, 17 minutes
1—100	3.0 cc.	" "	4.1 gm.	11 hours, 45 minutes
1—125	2.4 cc.	" "	3.8 gm.	16 hours, 45 minutes
1—150	2.0 cc.	" "	2.9 gm.	20 hours, 15 minutes
1—170	1.92 cc.	" "	3.7 gm.	24 hours, 20 minutes
1—180	1.68 cc.	" "	4.0 gm.	Recovered after 30 hours

It will be noted from the above results that, although in some cases the strength of the dilutions varied only 5%, *every* fish died in order in accordance with the dilution of the solution in which it was placed.

Next the variation between the strength of the dilutions was still further diminished in order to determine the limit of sensitiveness of these animals.

Preparation Employed, Fld. Ext. Digitalis No. 1—*Experiment No.* 3.

Dilution	Actual Amt. of F. E. Contained in 300 cc. Water	Temperature	Weight of Fish	Time Required to Cause Death
1—1000	0.3 cc.	27° C.	4.4 gm.	2 hours, 23 minutes
1—1025	0.288 cc.	"	2.8 gm.	2 hours, 53 minutes
1—1050	0.285 cc.	"	3.3 gm.	3 hours, 40 minutes
1—1075	0.275 cc.	"	3.8 gm.	3 hours, 18 minutes

Preparation Employed, Fld. Ext. Digitalis No. 2—*Experiment No.* 4.

Dilution	Actual Amt. of F. E. Contained in 300 cc. Water	Temperature	Weight of Fish	Time Required to Cause Death
1—1000	0.3 cc.	26° C.	3.0 gm.	1 hour, 20 minutes
1—1025	0.288 cc.	"	3.8 gm.	1 hour, 25 minutes
1—1050	0.285 cc.	"	3.2 gm.	1 hour, 36 minutes
1—1075	0.275 cc.	"	2.1 gm.	1 hour, 35 minutes

Preparation Employed, Fld. Ext. Digitalis No. 2—*Experiment No.* 5.

Dilution	Actual Amt. of F. E. Contained in 300 cc. Water	Temperature	Weight of Fish	Time Required to Cause Death
1—1500	0.2 cc.	27.5° C.	2.8 gm.	1 hour, 15 minutes
1—1525	0.196 cc.	"	2.8 gm.	1 hour, 36 minutes
1—1550	0.194 cc.	"	3.0 gm.	1 hour, 10 minutes
1—1575	0.193 cc.	"	2.5 gm.	1 hour, 14 minutes
1—1600	0.187 cc.	"	3.4 gm.	1 hour, 38 minutes

Exp. No. 3 shows that with a variation as small as 2.5% in the strength of the dilutions, all but one fish died in order. Exp. No. 4 and 5, Fld. Ext. No. 2 (which was a preparation possessing greater activity than Fld. Ext. No. 1) show greater discrepancies. In this case, 3 of 9 fish died "out of order."

The facts that of Fld. Exts. No. 1 and 2, the weaker preparation gave the better results and that in Exp. No. 1 and 2 where the length of time required to cause

death was greater than in Exp. No. 4 and 5 (due to the use of a weaker preparation), not a single fish died "out of order," indicated that decreasing the strength of the dilutions would increase the sensitiveness of the test. The next experiments were, therefore, made with weaker solutions in order to prove this point.

Preparation Employed, Fld. Ext. Digitalis No. 2—*Experiment No.* 6.

Dilution	Actual Amt. of F. E. Contained in 300 cc. Water	Temperature	Weight of Fish	Time Required to Cause Death
1—1850	0.162 cc.	28° C.	2.4 gm.	1 hour, 10 minutes
1—1900	0.157 cc.	"	3.1 gm.	1 hour, 27 minutes
1—1950	0.154 cc.	"	3.4 gm.	1 hour, 49 minutes
1—2000	0.15 cc.	"	2.9 gm.	1 hour, 57 minutes.

Experiment No. 7.

Dilution	Actual Amt. of F. E. Contained in 300 cc. Water	Temperature	Weight of Fish	Time Required to Cause Death
1—3500	0.0857 cc.	29° C.	3.1 gm.	2 hours, 58 minutes
1—3550	0.0845 cc.	"	4.0 gm.	3 hours, 55 minutes
1—3600	0.0833 cc.	"	4.2 gm.	3 hours, 48 minutes
1—3700	0.0810 cc.	"	3.8 gm.	4 hours, 30 minutes
1—3800	0.0789 cc.	"	4.6 gm.	6 hours, 6 minutes

These results show very clearly that decreasing the strength of the dilution prolongs the time required to cause death and thereby greatly increases the sensitiveness of the test. It will be noted that a variation of less than 3% in the strength of the dilutions, or, in other words, a difference of only 0.0021 cc. in the actual amount of Fld. Ext. contained in the 300 cc. water in which the fish were placed, caused a difference of one hour and 36 minutes in the length of time required to produce death.

That the weight of the fish is of no importance is clearly demonstrated by the fact that in the foregoing experiments, although some of the fish weighed more than twice as much as others, practically all died in the order of the dilution of the solutions in which they were placed.

In order to determine whether or not *large* variations in the weight of the fish would influence the results, the following experiment was carried out:—The size of the large fish used made it necessary to place each animal of the series in 1000 cc. of the dilution instead of 300 cc., as was used in the preceding experiments.

Preparation Employed, Fld. Ext. Digitalis No. 2—*Experiment No.* 8.

Dilution	Temperature	Weight of Fish	Time Required to Cause Death
1—1000	27.5° C.	34.1 gm.	58 minutes
1—1000	"	27.9 gm.	52 minutes
1—1000	"	5.5 gm.	59 minutes
1—1000	"	5.0 gm.	47 minutes
1—1000	"	2.3 gm.	54 minutes
1—1000	"	3.1 gm.	55 minutes

It will be noted that, although the largest fish weighed *more than ten times as much as the smallest,* the length of time required to cause death, when immersed

in solutions of the same strength, varied but 12 minutes. These results would tend to prove conclusively that the weight of the animal can be disregarded when making tests by this method.

It was noticed that, although each series of dilutions gave concordant results within 2½%, there was a variation between the different series, in the length of time required to produce death by solutions of the same strength. This discrepancy suggested a variation due to differences in temperature. The following table shows the results of an experiment to determine the effect of temperature on the resistance of gold fish:—

Preparation Employed, Fld. Ext. Digitalis No. 2—*Experiment No.* 9.

Dilution	Temperature	Weight of Fish	Time Required to Cause Death
1—1000	38 to 39° C.	4.7 gm.	46 minutes
1—1000	26.5 to 27° C.	4.2 gm.	2 hours, 55 minutes
1—1000	12 to 17° C.	3.9 gm.	7 hours, 21 minutes

It is apparent from this single experiment that temperature is of the utmost importance and that in order to obtain concordant results it will be necessary to carry out all assays at exactly the same temperature. This can easily be accomplished, however, by immersing the beakers containing the dilutions in a constant temperature bath. The best temperature to employ still remains to be determined.

Next eight fish were placed in beakers, each containing exactly the same strength solution in order to determine the individual variation in susceptibility.

Preparation Employed, Fld. Ext. Digitalis No. 2—*Experiment No.* 10.

Dilution	Actual Amt. of F. E. Contained in 300 cc. Water	Temperature	Time Required to Cause Death
1—3500	0.0857 cc.	28.5° C.	2 hours, 47 minutes
1—3500	0.0857 cc.	"	3 hours, 12 minutes
1—3500	0.0857 cc.	"	3 hours, 18 minutes
1—3500	0.0857 cc.	"	2 hours, 59 minutes
1—3500	0.0857 cc.	"	2 hours, 43 minutes
1—3500	0.0857 cc.	"	3 hours, 8 minutes
1—3500	0.0857 cc.	"	3 hours, 4 minutes
1—3500	0.0857 cc.	"	2 hours, 51 minutes

The last fish died 35 minutes after the first one. For comparison 8 guinea pigs were injected with exactly the same dose (1½ times the usual m. l. d.) per 250 gm. body weight, and it was found that the sixth pig died 1 hr. and 28 minutes after the first. The remaining two pigs were still alive at the end of 12 hours. I was unable to obtain frogs in time to make a comparison with these animals, but it is generally admitted by most workers that the variation in frogs is more marked than in guinea pigs.

In experiment No. 2 with tincture digitalis, the presence of the alcohol which was not removed did not interfere with the results. It was possible, however, that the alcohol might have increased or decreased the length of time required to cause death for the whole series. Fish were, therefore, placed in four 1-1000 dilutions of Fld. Ext. Digitalis; two with 1.5 cc. alcohol added (the amount which would be

contained in 3.0 cc. Tinct Digitalis) and two without alcohol. The results follow:—

Experiment No. 11.

Dilution	Time Required to Cause Death
1—1000 without alcohol	2 hours, 35 minutes
1—1000 without alcohol	2 hours, 20 minutes
1—1000 1.5 cc. alcohol added	2 hours, 7 minutes
1—1000 1.5 cc. alcohol added	2 hours, 27 minutes

It would appear from the above results that alcohol to the extent of that contained in the U. S. P. tincture does not affect the results. More extensive experiments, however, are necessary before definite conclusions may be drawn in regard to this point.

The time being limited, it was impossible to carry out all the experiments which suggested themselves in time for this preliminary report. We shall, therefore, continue our investigations in an endeavor to determine the following:—

1. The best time limit and,
2. The most suitable dilution, leading to
3. Tentative standards.
4. Action of other members of the digitalis group.
5. Extent of seasonal variations.
6. Best temperature to employ.
7. More extensive experiments on the effect of alcohol.
8. The possibility of the application of this method to standardizing biologic products, such as antitoxins, sera, etc.

SUMMARY.

The results of the foregoing experiments, together with experience with other methods so far proposed, lead to the following conclusions:—

1. Gold fish are sensitive to variations of 2½% in the strength of the dilutions of digitalis in which they are placed.
2. Variations due to differences in the rate of absorption appear to be practically eliminated by the use of these animals.
3. Decreasing the strength of the dilution, increases the sensitiveness of the test.
4. The weight of the fish may be disregarded when making tests by this method.
5. Variations in the temperature markedly influence the resistance of gold fish to digitalis poisoning.
6. The individual variation in the susceptibility of gold fish is much less than that found in guinea pigs and frogs.
7. The gold-fish method is unquestionably the simplest so far proposed and can easily be carried out by those not especially skilled in the pharmacodynamic art.
8. The inexpensiveness of the assay is decidedly in its favor. Gold fish of the proper size can be purchased wholesale for from 45 to 60 cents per dozen.
9. A sufficient number of animals can be procured at all seasons of the year.

Pharmacodynamic Laboratory, H. K. Mulford Company, August 20, 1914.

DISCUSSION.

DR. EDWARD KREMERS:—Is the application of this method new in this country?

DR. PITTENGER:—New, so far as I know.

DR. KREMERS:—Gold fish have been used in other work?

DR. PITTENGER:—Yes, I think they have been used in other work, but so far as I am acquainted with the literature this is the first attempt that has been made to use these animals as a means of quantitatively testing the activity and thereby standardizing galenical preparation or antitoxins.

DR. KREMERS:—I think Dr. Stockberger could tell us something about that. Is he here in the room? Dr. R. H. True worked with gold fish along similar lines some years ago before he went to work in the Department of Agriculture.

DR. STOCKBERGER:—Yes, both Dr. Kahlenberg and Dr. True have conducted work with fish and tadpoles along somewhat similar lines, but the application, of course, was in an entirely different direction. The fish were used merely as a reagent, you might say, in testing the toxicity of various chemical solutions. I do not know that this method has been used in testing galenicals prior to Dr. Pittenger's work.

DR. KREMERS:—The principle was different.

DR. STOCKBERGER:—The principle was different, but the use practically was very similar.

MR. A. J. MEIER:—I would like to ask whether any experiments were carried out to determine whether or not the fish would live from twenty-two to twenty-four hours if left in ordinary water without changing at room temperature when such small amounts of water were employed?

DR. PITTENGER:—No. Such experiments were not necessary as Experiment No. 2 shows that one fish was still alive after being kept for thirty hours in only 300 cc. of a 1-180 dilution of tincture of Digitalis. Table No. 1 also shows that some fish lived for twenty-two to twenty-four hours in this amount of solution.

MR. MEIER:—Without any change of water, or getting oxygen in the water?

DR. PITTENGER:—Yes. It would not be advisable, however, to employ so small a quantity of solution if twenty-four hours should eventually prove to be the best time limit. As stated in the paper the best time limit has not yet been determined. The 300 cc. of solution were merely arbitrarily adopted at the beginning of the experiment when I had no idea as to the length of time which would be required for the fish to die when placed in the different strength solutions employed. It merely happened that some of these fish lived for twenty-four hours. If this should eventually be chosen as the time limit the quantity of the dilution would necessarily have to be increased. The fact that one fish recovered after twenty-four hours shows that such factors had no apparent effect in this time and that by using a shorter end point their effect would be practically *nil*.

DR. KREMERS:—Wasn't the fish which recovered seemingly dead? Is it hard to tell just when a fish dies?

DR. PITTENGER:—No it wasn't. Apparently it was just as much alive as when placed in the solution. It is very easy to note when a fish dies. It generally first turns over on its side and a little later becomes motionless.

DR. KREMERS:—Did you try to see if you could revive those which were seemingly dead by putting them in salt water?

DR. PITTENGER:—No, I did not as that would have interfered with our results. I know however, that gold fish breeders revive their sick fish by that method.

EXAMINATION OF CALYCANTHUS FLORIDUS FOR ALKALOIDS.

E. R. MILLER AND H. W. BROOKS.

One of the widely-known and popular plants indigenous to the Southeastern States, is *Calycanthus floridus,* a shrub from 2 to 8 feet high, growing from Virginia to Florida and Alabama.

Its chief characteristic, which has won for it almost first place among the wild flowers, is not the size and beauty of its blossoms, but rather their delightful fragrance. To this it owes the almost universal name of "Sweet Shrub."

As a medicinal plant it is not of much importance, but is said to be antiperiodic, stimulant and tonic. The bark has an agreeable, spicy, camphoraceous odor which no doubt suggested the names Carolina allspice, Florida allspice. etc.

Inasmuch as alkaloids have been discovered in the seeds of another species of