PRELIMINARY NOTE ON A NEW PHARMACODYNAMIC ASSAY METHOD.*

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

(Continuation of a Previously Reported Paper.)[1]

BY PAUL S. PITTENGER.

In a previous publication the author states that

"Although the methods employed at the present time for biologically standardizing digitalis and its allies 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."

"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."

The result of several weeks' experimental work is then given as a basis for the following conclusions:

1. Gold fish are sensitive to variations of $2^1/_2$ percent 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.

---

*Read before Scientific Section, A. Ph. A., New York meeting, 1919.

[1] J. A. PH. A., April 1915.

The experiments which led to the above conclusions were preliminary in the extreme, as they covered a period of only three weeks before the annual meeting of the A. Ph. A. at which time the paper was read. It was apparent, therefore, that in order to prove the value of the above conclusions, more extensive and detailed experiments would be necessary.

The time being limited, it was impossible to carry out all the experiments which suggested themselves, and the author stated that the investigations would be continued 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.

Owing to the exigencies of abnormal conditions existing during the past three years the enormous increase in regular routine work made it impossible to attempt any research which was not absolutely essential.

During the last 8 months, however, detailed experiments involving the use of several thousand fish have been carried out in order to devise and perfect a satisfactory gold fish method and to arrive at definite conclusions as to its value for standardization purposes.

As the previous experiments indicated that temperature was an important factor, the first series of experiments was carried out in order to determine the influence of variations in temperature upon the resistance of gold fish to digitalis poisoning.

TEMPERATURE.

The results of these experiments verified our former conclusions that "*variations in the temperature markedly influence the resistance of gold fish to digitalis poisoning.*"

We found that the greater the temperature the lower the resistance of the fish and *vice versa.*

Of the many experiments conducted the following two tables are typical examples of the results obtained:

TABLE I.

Temperature.	Amount of tincture in 500 mils.	Time required to kill.
22° C.	2.1	4 hrs. 20 min.
22° C.	2.2	3 hrs. 44 min.
22° C.	2.3	2 hrs. 30 min.
22° C.	2.4	3 hrs. 41 min.
22° C.	2.5	3 hrs. 35 min.
22° C.	2.6	3 hrs. 26 min.
22° C.	2.7	4 hrs. 20 min.
22° C.	2.8	2 hrs. 57 min.
22° C.	2.9	2 hrs. 21 min.

TABLE II.

Temperature.	Amount of tincture in 500 mils.	Time required to kill.
29° C.	2.1	2 hrs. 15 min.
29° C.	2.2	2 hrs. 41 min.
29° C.	2.3	3 hrs. 5 min.
29° C.	2.4	2 hrs. 50 min.
29° C.	2.5	1 hr. 57 min.
29° C.	2.6	2 hrs. 6 min.
29° C.	2.7	2 hrs. 30 min.
29° C.	2.8	1 hr. 35 min.
29° C.	2.9	1 hr. 47 min.

It will be noted from the above tables that at a temperature of 29° C. the fish all die in from 1 hour and 47 minutes to 3 hours and 5 minutes, whereas at a temperature of 22° C. the same amounts of tincture of digitalis required from 2 hours and 21 minutes to 4 hours and 20 minutes to produce death, thus proving the fact that the lower the temperature the greater the resistance.

After proving that temperature exerts a marked influence upon the resistance of the fish, our next problem was to determine the most satisfactory temperature at which to carry out our tests, or in other words, at what temperature we would obtain the most concordant results. The results of our experiments along this line showed that equally satisfactory results could be obtained by any temperature from 18° C. to 25° C., so long as the same temperature was maintained throughout the test.

However, we arbitrarily chose 22° C. as standard temperature at which to carry out the test for the following reasons:

1. Approximately ordinary room temperature.
2. Approximate temperature at which fish are usually kept.
3. Easily maintained.
4. Approximate temperature of running "tap-water" in summer (Phila.).
5. Satisfactory results obtained and ease in maintaining this temperature for frog work.

All of the earlier experiments were conducted by placing the fish directly into beakers containing the solution of the drug made from tap water, regardless of the temperature of the tap water. The beakers were then immediately placed in the constant temperature bath.

Our experience with frogs suggested that we determine whether our results would be effected by bringing the temperature of both the drug solution and the water in which the fish were stored to the standard temperature of 22° C. for at least one hour before placing the fish in the solution.

Accordingly, several experiments were made and the following tables are typical examples of the results obtained.

Table III shows that three out of six fish died out of order, whereas in Table IV only one fish died out of order, showing that more concordant results can be obtained by keeping the fish at a constant temperature of 22° C. for at least one hour before placing them in the drugged solution. Again, it will be observed that on an average it required less time to kill in Table IV than in Table III.

TABLE III.

Fish taken from aquarium, temperature 12° C. and placed in drug solution temperature 12° C. and immediately placed in constant temperature bath at 22° C.

Amount of tincture in 500 mils.	Time required to kill.	Remarks.
2.0	4 hrs. 42 min.	Died out of order
2.1	4 hrs. 53 min.	
2.2	4 hrs. 20 min.	Died out of order
2.3	3 hrs. 45 min.	
2.4	2 hrs. 56 min.	Died out of order
2.5	3 hrs. 41 min.	

TABLE IV.

Fish kept at 22° C. for one hour before being placed in drug solution which has also been kept in constant temperature bath at 22° C.

Amount of tincture in 500 mils.	Time required to kill.	Remarks.
2.0	4 hrs. 55 min.	
2.1	4 hrs. 40 min.	
2.2	4 hrs. 8 min.	
2.3	3 hrs. 25 min.	
2.4	3 hrs. 15 min.	Died out of order
2.5	3 hrs. 28 min.	

END-POINT.

As our former experiments showed that dilutions of the drug of sufficient strength to cause the death of the fish in about 3 hours gave more concordant results than dilutions which caused death in 1 hour, our next problem was to determine the best end-point.

We therefore made a comparison between the results obtained with dilutions of the drug of sufficient strength to kill within 1 to 3 hours, 3 to 10 hours and 18 to 24 hours with the object of determining at which time limit the test is the most sensitive.

Exhaustive experiments involving the use of over 1,000 fish showed that the test was the most sensitive when using dilutions of the drug which produced death in approximately 3 hours.

The following three tables are characteristic of the results obtained:

TABLE V.

18 to 24 hours.

Amt. of tincture in 500 mils.	Time required to kill.	Order in which fish should have died.	Order in which fish died.	Remarks.
1.0	21 hrs. 20 min.	1	4	Died out of order
0.9	18 hrs. 25 min.	2	2	
0.8	36 hrs. 15 min.	3	6	Died out of order
0.7	19 hrs. 55 min.	4	3	Died out of order
0.6	23 hrs.	5	5	Died out of order
0.5	17 hrs. 47 min.	6	1	Died out of order
0.4	41 hrs.	7	7	

Temperature 22° C.

TABLE VI.

3 to 10 hours.

Amt. of tincture in 500 mils.	Time required to kill.	Order in which fish should have died.	Order in which fish died.	Remarks.
1.3	9 hrs. 17 min.	7	7	
1.5	5 hrs. 55 min.	6	2	Died out of order
1.7	6 hrs. 15 min.	5	4	Died out of order
1.9	6 hrs. 25 min.	4	5	Died out of order
2.0	6 hrs. 10 min.	3	3	
2.1	7 hrs. 10 min.	2	6	Died out of order
2.3	3 hrs. 40 min.	1	1	

TABLE VII.

1 to 3 hours.

2.3	3 hrs. 50 min.	15	15	
2.4	3 hrs. 42 min.	14	14	
2.5	3 hrs. 38 min.	13	13	
2.6	3 hrs.	12	11	Died out of order
2.7	3 hrs. 30 min.	11	12	
2.8	2 hrs. 55 min.	10	10	
2.9	2 hrs. 45 min.	9	9	
3.0	2 hrs. 34 min.	8	7	Died out of order
3.1	2 hrs. 39 min.	7	8	
3.2	2 hrs. 35 min.	6	6	
3.3	1 hr. 31 min.	5	2	Died out of order
3.4	2 hrs. 28 min.	4	5	
3.5	2 hrs. 10 min.	3	4	
3.6	1 hr. 59 min.	2	3	
3.7	1 hr. 52 min.	1	1	

Temperature 22° C.

It will be noted by Table V that five out of seven fish died "out of order" and that in Table VI four out of seven fish died "out of order," whereas in Table VII only three out of fifteen fish "died out of order." It is apparent, therefore, that the best results are obtained by dilutions of the drug of such strength that they will produce death within 3 hours. We accordingly adopted *3 hours as our time limit.*

TENTATIVE STANDARD.

Having decided as to the most satisfactory temperature and end-point, we are in a position to determine the amount of tincture digitalis of U. S. P. standard strength required to kill within the time limit, thus procuring the necessary data upon which to base a tentative standard.

Accordingly we adjusted a tincture of digitalis to standard U. S. P. strength by the "one hour frog method," after which we tested it upon gold fish, with the following results:

TABLE VIII.

Amt. tinct. digitalis in 500 mils.	Temperature.	Results at end of 3 hours.
2.65	22° C.	Alive
2.7	22° C.	Alive
2.75	22° C.	Alive
2.8	22° C.	Alive
2.85	22° C.	Dead
2.9	22° C.	Dead
2.95	22° C.	Dead
3.0	22° C.	Dead
3.1	22° C.	Dead

TABLE IX.

Amt. tinct. digitalis in 500 mils.	Temperature.	Results atend of 3 hours
2.7 mils	22° C.	Alive
2.7 mils	22° C.	Alive
2.7 mils	22° C.	Alive
2.7 mils	22° C.	Died
2.7 mils	22° C.	Alive
2.8 mils	22° C.	Died
2.8 mils	22° C.	Recovered
2.8 mils	22° C.	Died
2.8 mils	22° C.	Died
2.8 mils	22° C.	Recovered
2.8 mils	22° C.	Recovered
2.8 mils	22° C.	Died
2.85 mils	22° C.	Died
2.85 mils	22° C.	Died
2.85 mils	22° C.	Died
2.85 mils	22° C.	Died
2.85 mils	22° C.	Died
2.9 mils	22° C.	Died
2.9 mils	22° C.	Died

As a result of these experiments we adopted *2.85 mils as our tentative standard* as it will be noted that it is the smallest amount which will in practically every case cause the death of the fish within 3 hours.

ADOPTED METHOD.

The foregoing experiments led us to adopt a method which consists in determining the minimum amount of tincture of digitalis which in 500 mils of "tap-water" will prove fatal to gold fish within 3 hours, the fish being immersed in the solution which is kept at a constant temperature of 22° C.

The details of the method are as follows:

APPARATUS NECESSARY FOR EXPERIMENT.

Constant temperature bath,[1] 800 mil beakers, 500 mil volumetric flask, 10 mil pipette graduated in tenths and a 2 mil pipette graduated in hundredths.

Animals.—Common gold fish about $2^1/_2$ to 3 inches in length, in good healthy condition.

Preparation of Experiment.—Adjust constant temperature bath so that it maintains a temperature of 22° C. Wash and thoroughly dry six 800 mil beakers and label 1 to 6, respectively; accurately pipette to the hundredth of a mil, $^7/_{10}$ of the standard dose into the 500 mil volumetric flask and fill to the *mark* with "tap-water;" shake thoroughly and empty into beaker No. 1; five other solutions are similarly prepared containing $^8/_{10}$, $^9/_{10}$, $^{10}/_{10}$, $^{11}/_{12}$ and $^{12}/_{12}$, respectively, of the standard, and placed in the beakers 2 to 6, respectively; all six beakers are then placed in the constant temperature bath, together with another larger beaker containing 6 gold fish in plain "tap-water."

Actual Standardization.—After one hour the fish are removed from the large beaker and one is placed in each of the six beakers containing the various dilutions

[1] Pittenger, J. A. PH. A., Nov. 1916. p. 1261.

of the drug. In removing the fish from the "tap-water" to the drugged solution, care should be exercised that no water be transferred with them. Note the time that the fish are placed in the drugged solutions. Maintain constant temperature of 22° C. After 3 hours note should be made of those living and those which are dead.

The results of this preliminary test, in which the range of dosage is quite wide, enables the investigator to form some idea as to the strength of the preparation. Basing the dosage upon these results, other series of dilutions are made by progressively increasing or decreasing the strength of the dilutions, as the case may be, still further diminishing the variation between doses, until the smallest amount of tincture in 500 mils of water is found which will prove fatal within 3 hours.

The probable M. L. D. (minimum lethal dose) of the preparation, unless it deviates considerably from that of the standard, is generally obtained by one or two series of dilutions.

In order to determine whether or not this is the true M. L. D. this result is checked by carefully preparing a new series of four dilutions; two with the smallest amount of tincture which was found to kill and two with the largest amount of tincture which did not kill. If, however, any of this last series show irregularities, further correction must be made.

After thus determining the M. L. D. of the preparation its relative strength can be calculated by comparing the M. L. D. of the unknown with the standard M. L. D. of 2.85 by simple proportion.

It will be observed from Tables VIII and IX that this method is sensitive to variations of less than 2 percent in the strength of the dilutions of digitalis in which the fish are placed.

In our former contribution we stated:

> "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."

In order to arrive at a definite conclusion in regard to the effect of alcohol on the results we divided a tincture of digitalis into two portions. We then evaporated the one portion on a steam bath until it was freed from practically all of its alcohol, after which it was restored to its original volume with tap-water.

The two portions were then tested simultaneously with the following results:

TABLE X.

TINCTURE DIGITALIS.

70% alcohol.

Amt. of tincture in 500 mils.	Temperature.	Results after 3 hours.
2.5	22° C.	Recovered
2.6	22° C.	Recovered
2.7	22° C.	Recovered
2.8	22° C.	Recovered
2.85	22° C.	Died
2.9	22° C.	Died

M. L. D. = 2.85.

TABLE XI.

TINCTURE DIGITALIS.

Alcohol Removed.

Amt. of tincture in 500 mils.	Temperature.	Results after 3 hours.
2.5	22° C.	Recovered
2.6	22° C.	Recovered
2.7	22° C.	Recovered
2.8	22° C.	Recovered
2.85	22° C.	Died
2.9	22° C.	Died

M. L. D. = 2.85.

It will be noted that the above results confirm our former conclusions that *alcohol to the extent of that contained in the U. S. P. tincture does not affect the results.*

As a result of our experimental work to date we have arrived at the following conclusions:

1. Variations of less than 2 percent in the strength of tincture of digitalis can be accurately determined by the method outlined.

2. Variations due to difference in the rate of absorption appear to be practically eliminated by the use of these animals.

3. The weight of the fish may be disregraded when making tests by this method.

4. Variations in temperature markedly influence the resistance of gold fish to digitalis poisoning.

5. The individual variations in susceptibility of gold fish is much less than that in guinea pigs and frogs.

6. 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.

7. 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.

8. A sufficient number of animals can be procured at all seasons of the year.

9. Alcohol to the extent of that contained in the U. S. P. tincture does not affect the results.

10. A tincture of digitalis to be of standard strength should have a M. L. D. of 2.85 when assayed by this method.

Finally the author wishes to acknowledge his indebtedness to Mr. LeRoy Goinez for most of the laboratory work in connection with this paper.

PHARMACODYNAMIC LABORATORY,
H. K. MULFORD COMPANY,
JULY, 1919.

DIGITALIS STANDARDIZATION: A CONSIDERATION OF CERTAIN METHODS OF BIOLOGICAL ASSAY.*

BY L. W. ROWE.

The physiological standardization of the drugs comprising the digitalis series of heart tonics has received much consideration since Houghton[1] proposed the first method for the assay of Strophanthus preparations in 1898.

One of the more recent of the methods proposed for standardizing digitalis preparations and one which constantly appears to be receiving consideration is

*Read before the Scientific Section, A. Ph. A., New York Meeting, 1919.