Summary

1. New syntheses for 2,9-diaminofluorene and for 2-aminofluorenone have been developed. Neither the diamine nor 2-amino-9-hydroxyfluorene could be resolved.

2. Attempts to prepare 1-nitro-9-aminofluorene by nitration of 9-acetaminofluorene resulted in the formation of a compound which was probably 1,8-dinitro-9-acetaminofluorene, m. p. 236–238°.

3. The failure to resolve any 2,9-fluorene compound may be due to a lack of asymmetry or to the fact that the right conditions have not been found. In any case, the failure to resolve 2-amino-9-diazofluorene does not demonstrate that the carbon atom bearing the diazo group is not asymmetric.

4. *p*-Aminobenzophenone hydrazone, m. p. 139–140°, was prepared along with the ketazine, m. p. 225°. The former, on oxidation, yielded an unstable diazo derivative which could not be isolated.

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A METHOD FOR THE STUDY OF TOXICITY USING GOLDFISH[1]

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The use of derris root as a fish poison by the inhabitants of the localities to which this plant is native suggested the use of fishes as test animals in the study of the comparative toxicity of rotenone, an active constituent of that plant, and its derivatives. The goldfish, being a member of the carp family and so at home in sluggish waters, is more adapted to conditions in still water tanks and jars than most of the native fishes. The fact that fishes accustomed to active, cool waters are liable to suffocate when kept in still, comparatively warm water is a factor to be considered. The goldfish is also more easily obtained in large quantity and is inexpensive. The species sold for aquaria, *Carassius auratus*,[2] is the one used here.

In 1915 Pittenger and Vanderkleed[3] and in 1919 Pittenger[4] found that the goldfish was a suitable test animal in the assay of digitalis preparations. In 1917 Powers[5] reported an investigation testing the validity of this method and the use in general of the goldfish as the test animal in the

[1] Presented as a part of the Insecticide Symposium before the Division of Agricultural and Food Chemistry at the 77th Meeting of the American Chemical Society, Atlanta, Georgia, April 7 to 11, 1930.

[2] U. S. Dept. of Commerce, Bureau of Fisheries Econ. Circ. No. 68, p. 1 (1929).

[3] Paul S. Pittenger and Chas. E. Vanderkleed, *J. Am. Pharm. Assocn.*, **4**, 427–433 (1915).

[4] Paul S. Pittenger, *ibid.*, **8**, 893–900 (1919).

[5] Edwin B. Powers, Ill. Biol. Mono., **4**, No. 2 (1917).

study of toxicity. Powers' method was used as a starting point in the work reported in this paper; several changes, however, were deemed advisable.

Rotenone, prepared in this Laboratory from *Derris elliptica*,[6] was used as the toxic material. As this substance is but slightly soluble in water, some organic solvent non-injurious to the fishes had to be used in preparing stock solutions, from which aliquots could be taken to be added to the test solutions. Preliminary experiments were made with acetone, a good solvent for rotenone, to determine its action on the fishes. In a concentration of 2 parts of acetone to 1000 parts of water, fishes showed distress in half an hour. A concentration of 1 part of acetone to 1000 parts of water, however, was found to have no apparent effect on them. Therefore, stock solutions were made with acetone as the solvent of such concentrations that aliquots sufficiently large to be accurate could be taken without making the concentration of acetone in the test solutions greater than 1 part of acetone to 1000 parts of water.

It was found to be very important to use only goldfishes in good condition, those apparently but slightly out of condition always lowering the survival time appreciably. Fishes used in the tests were either taken from a stock tank which was heated to approximately the same temperature as that of the test solutions or allowed to warm up slowly to that temperature before the test was started. This eliminated any ill effect due to sudden change of temperature. This also is very important since a sudden change, either to a higher or lower temperature, may cause sickness and premature death of goldfishes. The difference between the temperature of the test solutions and that of unheated stock tanks with running water was often over 15° in winter.

Aside from any ill effect, sudden changes from lower to higher temperatures often result in marked temporary increase in activity of fishes.[7] In general the rate of metabolism increases with increase in temperature and diminishes with decrease. Wells[7] found that fishes react to so small a variation in the temperature of the surrounding water as 0.1°. It was found early in this experimental work that a varying temperature had considerable effect, thus necessitating the use of a constant temperature for the tests to achieve good comparative results. Small differences in temperature markedly influence the resistance of the fishes; an increase in temperature lowers the survival time, whereas a decrease in temperature raises it. Therefore, a constant temperature bath with a de Khotinsky regulator was used. Since the work was done in summer as well as in winter, it was difficult to maintain a temperature lower than 27°. This temperature therefore was selected as the temperature of the tests. Variation in temperature was usually less than half a degree.

To determine the size of the fishes used, they were at first weighed, but later were measured linearly from the mouth to the base of the tail, the latter procedure being the shorter. A number of fishes were both weighed and measured so that the weight of a fish could be estimated from the length when desired. Generally the large fish survived longer than the small ones; therefore fishes as nearly uniform in size as possible should be used.

In making the tests the ratio of one liter of solution to each fish was maintained. Some of the tests were carried out in three-liter wide-mouthed jars, known as "candy jars," which were found convenient when two liters of solution were used. The rest of the tests, in which six or eight liters of solution were taken, were carried out in three-gallon cylindrical jars.

In determining toxicity, the criterion used was the death point. Osterhout[8] has pointed out that "the relative toxicity of two substances may

[6] F. B. LaForge and L. E. Smith, THIS JOURNAL, **51**, 2574–2581 (1929).

[7] Morris M. Wells, *Trans. Ill. Acad. Science*, **7**, 48–59 (1914).

[8] W. J. V. Osterhout, *J. Biol. Chem.*, **23**, 67–70 (1915).

depend very largely on the stage of the reaction at which the measurement is made. . . . It is impossible to determine the precise moment of death," since the death curve "approaches the axis asymptotically." He objected to the death point as a perfectly satisfactory criterion of toxicity. The conclusion was soon reached in this work, however, that the death point is a more exact criterion for the goldfish than the loss of equilibrium, cessation of motion, or loss of irritability. No phenomena suitable for use as criteria, as suggested by Osterhout, have been found to appear at definite points along the course of the reaction with goldfishes. Powers came to the same conclusion and used the death point as the criterion. The death point, although not obtainable with precision from the observation of one or two fishes, was determined with fair accuracy when more (usually six to twelve) were used, and could, of course, be found with even greater accuracy by using a larger number

To find the death point of a fish repeated observations were made to follow the course of the action of the toxic material. The fish usually, but not always, after showing its first distress in swimming, suffered a loss of equilibrium, then lost its ability to swim and finally its irritability. From this point on careful observations were repeatedly made of any gill or mouth movements. These movements may be very faint and require close observation; they may, moreover, last for a long time, and the period is important in finding the actual survival time. It was found that when the fishes were taken out after close observation had failed to detect any gill movement whatsoever in one to one and one-fourth minutes and then were dipped into hydrochloric acid (about 1:3) about half were dead and half would still show faint signs of life.

Powers used only two goldfishes in each concentration in determining the survival time. In order to lessen the effect of individual variations in susceptibility, it was deemed advisable in this work to use a larger number of fishes, especially in the longer survival times. Naturally, variations from the mean survival time increased as the mean increased, that is, as the concentration decreased, but the percentage variation was found to be approximately the same.

Powers found that the survival time curve which is plotted by letting the ordinates represent the survival times of the goldfishes and the abscissas represent the concentrations of the test solutions is logarithmic in function. The middle portion, however, where the velocity of fatality (as measured by the reciprocal of the survival time) increases most rapidly with increase in concentration (usually when the survival time is between three-fourths and four hours) approaches an equilateral hyperbola. Therefore, the corresponding portion in the velocity of fatality curve which is drawn with the reciprocals of the survival times as the ordinates approaches a straight line. This straight line, designated the theoretical velocity of fatality curve,

when prolonged cuts the x-axis at a point which Powers designated as the theoretical threshold of toxicity. He suggested, for use in determining comparative toxicities, that if a represents this theoretical threshold of toxicity concentration and Θ the angle formed by the theoretical velocity of fatality curve with the x-axis, the toxicity of the substance may be expressed by the formula T (toxicity) $= \sqrt{\tan \Theta / a}$.

Table I shows the results of a typical group of tests with rotenone at one concentration, 0.075 mg. per liter, from which an idea of the reproducibility of the tests may be obtained.

TABLE I

TOXICITY OF ROTENONE TO GOLDFISH AT A CONCENTRATION OF 0.075 MG. PER LITER AND AT 27.0 ± 0.2°

Test no.	Length of fish in millimeters	Weight in grams[a]	Survival time of fish in minutes[b]	100 × the reciprocal of the survival time
1	42	2.3	93−	1.08
2	43	2.5	112−	0.89
3	42	2.3	117+	.85
4	39	1.8	108−	.93
5	39	1.8	108−	.93
6	45	2.7	123+	.81
7	46	2.8	128+	.78
8	48	3.4	133+	.75
9	39	1.8	113+	.88
10	47	3.3	119−	.84
Mean	43	2.5	115	.87

Probable error of a single observation.....8 min.
Percentage error of a single observation.....7%
Probable error of the mean.....2.4 min.
Percentage error of the mean.....2.1%

[a] Estimated from length. [b] Minus sign means fish was dead when taken out; plus sign means that fish was not quite dead, as shown by reaction to hydrochloric acid.

The condensed results of the complete series of tests is shown in Table II, with those of a statistical study of the method.

In this table the probable errors are calculated from the approximate equations $r = 0.8453 \frac{\Sigma v}{\sqrt{n(n-1)}}$ and $r_0 = 0.8453 \frac{\Sigma v}{n\sqrt{n-1}}$. In these equations r is probable error of a single observation; r_0 is probable error of the arithmetic mean; v is residual (difference between observed survival time and arithmetic mean); v is sum of individual residuals; n is number of observed survival times. These statistical data show that, although the percentage error of a single observation at points on the critical portion of the curve (that is, the portion approximating an equilateral hyperbola) may be as high as 20%, by using ten or twelve fishes the percentage error of the mean may be lowered to 7% or better. The same procedure used with compounds other than rotenone also gave a percentage error of the mean of 7%.

TABLE II

TOXICITY OF ROTENONE TO GOLDFISH AT 27.0±0.2°

Concn. mg. per liter	No. of fishes used	Mean length of fishes, mm.	Mean weight of fishes,[a] g.	Mean surv. time, min.	Mean $\frac{100}{\text{surv. time}}$	Probable error Of a single observ., min.	Probable error Of mean, min.	Percentage error Of a single observ.	Percentage error Of mean min.
4.0	4	40	2.0	46	2.19	...	...	..	...
3.0	3	37	1.6	46	2.16	...	...	..	...
2.0	4	37	1.6	48	2.08	...	...	..	...
1.0	4	39	1.8	49	2.06	...	...	..	...
0.90	7	38	1.7	52	1.91	...	...	..	...
.70	7	39	1.8	60	1.71	8	2.8	13	4.7
.50	8	39	1.8	57	1.82	8	2.5	14	4.5
.30	13	39	1.8	70	1.51	13	3.7	19	5.3
.20	16	38	1.7	65	1.55	5	1.2	7	1.8
.10	12	42	2.3	95	1.08	21	5.9	22	6.2
.075	10	43	2.4	115	0.87	8	2.4	7	2.1
.050	11	41	2.2	150	.70	27	8.2	19	5.7
.035	12	41	2.2	252	.42	51	14.8	20	5.9
.025	16	43	2.4	589	.23	244	61	41	10.3
.015	7	..	...	2400	.04	756	288	32	12.0

[a] Estimated from length.

The survival time curve and the velocity of fatality curve are plotted in Fig. 1. In the former the ordinates are survival times in minutes; in the latter the ordinates are the reciprocals of the survival times multiplied by

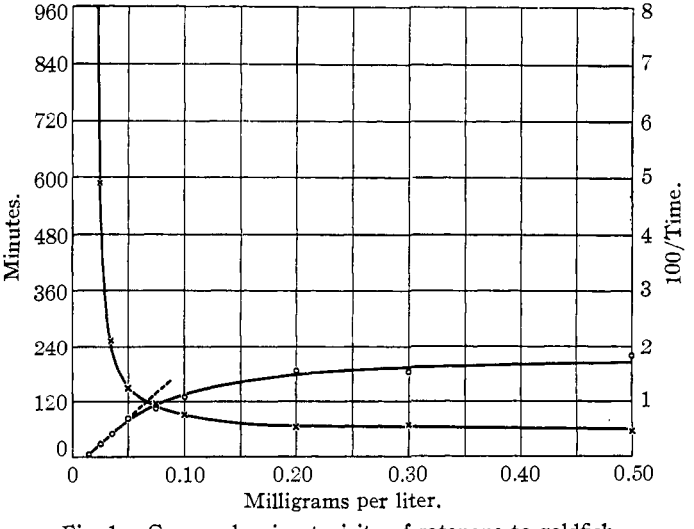

Fig. 1.—Curves showing toxicity of rotenone to goldfish.

100 to avoid decimals; in both curves the abscissas are concentrations. It is apparent that data falling outside of the critical portions of the curves are useless in a study of comparative toxicity. Along that portion of the

survival time curve which approaches the horizontal, great differences in concentrations correspond to comparatively small differences in survival times, whereas along that portion of the curve which approaches the perpendicular, very small differences in concentrations correspond to great differences in survival times. These curves are similar to those obtained by Powers except that in the case of rotenone the initial slow increase in the velocity of fatality with increase in concentration was not found. This may be due to the great toxicity of rotenone.

The value for rotenone obtained from the accompanying velocity of fatality curve by using the formula $\sqrt{\tan \theta/a}$ is 4 units of toxicity; those for phenol and potassium cyanide, calculated from the curves given by Powers, are 0.0008 and 0.16 unit, respectively. These values are not entirely comparable, since that for rotenone is obtained at 27° and those for potassium cyanide and phenol at 21.5°, but it is apparent that according to this formula rotenone is more toxic to goldfishes than is potassium cyanide, and the latter is 200 times as toxic as phenol.

Conclusions

The method described in this paper is suitable for the study of toxicity. The goldfishes should be as uniform in size as possible; they should not be even slightly out of condition. The tests should be carried out at a constant temperature. Ten or twelve fishes should be used at each concentration; the use of this number lowers the percentage error of the mean to 7% or less with the exception of long survival times (over five hours). The most suitable criterion is the death point. The death point is not so easily recognized with precision as is claimed by Pittenger,[4] but may be determined with fair accuracy as described in the foregoing method. A series of tests at various concentrations should be made in order that survival time curves and velocity of fatality curves may be plotted. Data falling outside that portion of the former curve which approximates an equilateral hyperbola are useless for a study of comparative toxicity. If substances are compared by the single survival time method, this survival time should fall on that critical portion of the curve.

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