THE BIOASSAY OF PICROTOXIN AND COCCULUS INDICUS PREPARATIONS.*

BY JAMES C. MUNCH AND AMELIA M. PONCE.[1]

Cocculus Indicus and its active principle, picrotoxin, have been used for poisoning fish, for increasing the bitter taste of beer, in "knockout drops" and externally against pediculi (17, 19, 24, 25, 27, 28). Pharmacologically, picrotoxin stimulates the medullary centers producing characteristic convulsions, slowing of the pulse (vagal), and an increase in blood pressure, which is followed by paralysis after large doses (1, 24, 25).

Because of the pharmacological and toxicological interest in picrotoxin, this investigation was undertaken with a view of developing a suitable bioassay.

Chemical studies have shown that Cocculus indicus contains between 1.5 and 5.0 per cent of a non-alkaloidal neutral principle, picrotoxin (1, 3, 27). Picrotoxin has been reported to have the formula $C_{30}H_{34}O_{13}$. On dissolving in water it is supposed to split into equal parts of picrotin and picrotoxinin, which are closely related chemically and have similar actions. Picrotoxinin is believed to be somewhat more potent, and some experiments suggest that picrotin, $C_{15}H_{11}O_7$ is inert on cold- and warm-blooded animals (4, 7, 14, 15). The available literature has not given any definite information regarding the relative toxicity of these three products. The data regarding toxicity have been compiled in Table I (1, 2, 3, 4, 5, 6, 8, 9, 12, 13, 14, 18, 20, 23, 26, 27). It is believed that when administered by mouth picrotin is eliminated partly unchanged in the urine, whereas picrotoxinin is decomposed (4). Picrotoxin is soluble in about 8 parts of alcohol or 240 parts of water (19, 25). In our experience some difficulty has been encountered in obtaining this strong a solution and our findings are in agreement with the statement that picrotoxin is slightly soluble in cold water and more soluble in hot water (19).

Chemical and toxicological studies on picrotoxin and its preparations are under way, and will be reported subsequently. This communication deals only with a method of physiological assay which has been developed.

BIOLOGICAL ASSAYS.

(*1*) *Fish.*—The use of picrotoxin as a fish poison suggests the possibility of developing a method for the bioassay of these preparations on fish. However, the data reported in the literature showed so wide a range of effectiveness, and the symptoms reported did not appear to be characteristic, so no quantitative methods have been developed on fish (1, 5, 6, 9, 11, 24).

(*2*) *Crabs.*—*Carcinas maenas*, a European sea crab is reported to be very susceptible, showing convulsions after a dose of 10 *gamma* of picrotoxin. Other crustaceæ are also believed to be susceptible. None of these animals were readily available and have not been studied in this connection. The symptoms reported did not appear to be characteristic of picrotoxin (20, 24, 27, 29).

(*3*) *Frogs.*—Qualitative tests may be conducted with picrotoxin. The injection of 0.5 to 1.0 mg. of picrotoxin to a 30-Gm. *Rana pipiens* produced a characteristic convulsion. Unfortunately cicutoxin produces a similar effect, although somewhat larger doses are required. The front legs are often folded across the breast, the hind legs extended with the web of the foot tightly stretched. A peculiar convulsant shriek is often heard. Death does not ensue for several hours.

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The susceptibility of frogs has been found to depend on the temperature, season and species. Frogs may be suitable for qualitative testing but did not appear useful for quantitative bioassays of picrotoxin and its preparations (1, 9, 10, 16, 24, 27).

(*4*) *Mice.*—In connection with studies on the potency of soporifics, Wieland and Pulewka found that the injection of 2.4 mg. of picrotoxin subcutaneously to male white mice was withstood, but that larger doses caused convulsions. They injected 4 mg. of picrotoxin per Kg. twenty minutes after the injection of the soporific or hypnotic studied (30). Rassers (22) found that picrotoxin subcutaneously injected into mice produced an S-curve response of the tail similar to that produced by morphine. Our studies on the toxicity of picrotoxin subcutaneously injected into the mice were in reasonable agreement among themselves, but the absolute lethal dose varied from day to day. We have, therefore, studied various factors believed to affect these results.

Although much of this work has been done with freshly prepared solutions, which were usually made to contain 1 mg. of picrotoxin per cc. in recently distilled water, solutions several months old have not been found to show any different toxicity. It would appear, therefore, that aqueous solutions are stable for several months.

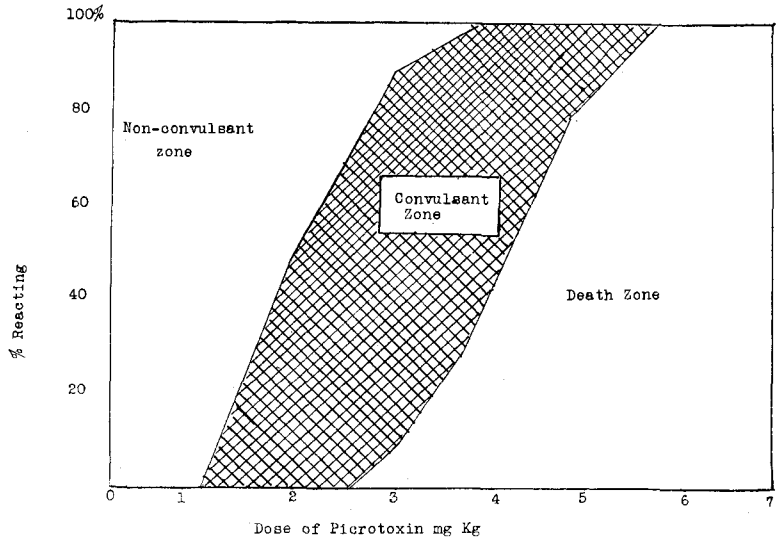

Fig. 1.—Effect of subcutaneous injections of Picrotoxin on mice.

To avoid individual differences in mice, several hundred animals were purchased at a time from different breeders. Mice were weighed to 0.5 Gm. and a measured volume of solution injected under the skin of the abdomen. Care was taken to prevent any loss of solution. The animals were placed in cages and kept under observation for several hours to note the appearance of symptoms. Very soon after injection the animals become quiet. Following this, convulsions develop which are very similar to, but readily differentiated from, those produced by strychnine. No consistent relationship was found between the interval until the development of convulsions, the period until death, and the dose administered. Many mice convulse, but recover. The tail may show various types of curvature, but does not give the characteristic S-curve of morphine.

In a preliminary assay, doses of 4, 5, 6 and 7 mg. of picrotoxin per kilo, and doses of 8 to 14 cc. per kilo of 1:20 dilution of fluidextract (or similar dilutions of other galenicals) are injected subcutaneously into groups of five mice each. The number of convulsions and of deaths are determined over a period of several hours. Under certain conditions, convulsions and deaths have developed within one hour: under other conditions three or four hours may be required. It is difficult to specify a specific time interval, although it appears that one hour would be advisable. Because of the peculiar variations in test animals, both the convulsant and killing doses are considered in reaching a preliminary conclusion. A desirable end-point is the production of convulsions or deaths in sixty to eighty per cent of the injected animals (Fig. 1).

Based upon the results of this preliminary assay, subsequent trials are made injecting groups of five mice each, in an effort to determine the dose of picrotoxin and of the unknown product which produce death in approximately eighty per cent of the injected mice within one hour ($_5LD_{80\%}$). The activity of the unknown product is then estimated in terms of picrotoxin.

The results obtained in testing several samples of tincture and fluidextract of fishberries are given in Tables II and III.

TABLE I.—TOXICITY OF PICROTOXIN AND PICROTOXININ.

Animal.	Subcutaneous. Picrotoxin.	Subcutaneous. Picrotoxinin.	Intramuscular. Picrotoxin.	Intramuscular. Picrotoxinin.	Oral. Picrotoxin.	Oral. Picrotoxinin.
Rana esculenta	...	1.1–2	2–10	...	...	...
Doves	...	...	1.4	1.6	...	...
Mice	2.5–6	1.6–2.0	...	...	...	...
Guinea pigs	0.3–0.8	...	...	...	...	...
Rabbits	1.3–2.8	1.35–1.6	...	...	2.5	...
Cats	2.0	...	...	...	3.5	...
Dogs	1.5–2.2	1.1	1.0	...	...	...
Man	...	...	...	...	1.0–1.5	...

TABLE II.—CONVULSANT ACTION OF PICROTOXIN ON MICE—SUBCUTANEOUS INJECTIONS.

Date.	Dose, Mg./Kg. 1.0 to 1.5.	2.0.	2.5.	3.0.	3.5 to 10.0.
12/27/32	0/3	1/1		1/1	5/5
1/9/33					2/2
1/16/33					1/1
1/17/33				2/2	4/4
1/18/33					1/1
1/20/33		1/1		1/1	1/1
1/24/33		0/2		1/2	4/4
1/27/33		0/2		1/2	8/8
1/30/33		2/2		2/2	2/2
1/31/33		1/1		2/2	1/1
2/1/33		0/1		1/1	2/2
2/5/33		0/1		1/1	1/1
2/6/33		0/2		2/2	3/3
		0/1		1/1	2/2
2/14/33		1/1		1/1	2/2
2/15/33		0/1		1/1	2/2
2/17/33		1/1		1/1	2/2
2/20/33		1/1		1/1	1/1
2/21/33		1/1		1/1	1/1
2/23/33		1/1		1/1	2/2
2/24/33					1/1
2/27/33		1/1		1/1	2/2
3/1/33		1/1		1/1	7/7
3/3/33	0/5		8/10	23/23	76/76
Total	0/8	12/22	8/10	46/48	133/133

This method appears promising for quantitative bioassays of picrotoxin and fishberries preparations.

(*5*) *Rats.*—A similar procedure was used in giving subcutaneous injections of picrotoxin and fishberries solution to white rats weighing between fifty and three hundred and fifty Gm., although most of the test animals weighed about one hundred and fifty Gm. The convulsant and lethal responses were much more erratic than those obtained in using mice. Results obtained with picrotoxin injected into rats are shown in Table IV.

Because of the variability of response, rats did not appear suitable for the bioassay of these products.

TABLE III.—THE TOXICITY OF PICROTOXIN TO MICE—SUBCUTANEOUS INJECTIONS.

Date.	Dose, Mg./Kg. 1 to 2.5.	3.0.	3.5.	4.0.	4.5.	5.0.	5.5.	6.0 to 10.
12/27/32	0/6	0/1		0/2		1/2		
1/9/33						1/2		
1/16/33						1/1		
1/17/33		0/2		0/1		2/3		
1/18/33						1/1		
1/20/33	0/1	0/1				1/1		
1/24/33		0/2				1/1		1/1
1/27/33	0/2	0/2		0/2		0/2		2/2
1/30/33	0/2	0/2				1/1		1/1
1/31/33	0/1	0/2				0/1		
2/1/33	0/1	0/1				1/1		1/1
2/5/33	0/1	0/1				1/1		
2/6/33	0/2	0/2				1/2		1/1
2/7/33	0/1	0/1				0/1		1/1
2/10/33	0/1	0/1				1/1		1/1
2/14/33	0/1	0/1				1/1		1/1
2/15/33	0/1	0/1				1/1		1/1
2/20/33	0/1	0/1				1/1		
2/21/33	0/1	0/1				1/1		
2/23/33	0/1	0/1				1/1		1/1
2/24/33						1/1		
2/27/33	0/1	0/1				1/1		1/1
3/3/33	0/1	0/1				1/1		1/1
3/6/33	0/15	16/21	5/23	6/15	7/30	4/4	6/6	5/6
8/7/33				4/10		5/10		
8/10/33				0/5		3/5		
Total	0/40	16/46	5/23	10/35	7/30	33/48	6/6	18/19

(6) *Man.*—It is stated that picrotoxin has been added to beer on account of its bitter taste, and the threshold concentration reported to be bitter is 1:80,000, corresponding to 12.5 mg. per liter (1, 21). The results obtained in testing various concentrations are given in Table V.

TABLE VI.—THRESHOLD LIMEN FOR PICROTOXIN TASTE TESTS.

Concentration, Mg./L.	Bitterness. Subject. 1.	2.	3.	4.	5.	Remarks.
5	0	0	0	0	0	
8	0	0	−	+	−	
10	0	0	0	++	0	Slightly astringent
12	0	0	−	−	−	Astringent
15	0	0	−	−	−	
20	0	0	−	−	−	
40	±	±	−	−	−	
50	+	+	−	−	−	
100	++	++	−	−	−	

In our tests on five subjects, one believed the product was definitely "bitter," whereas the other four obtained a taste which was characterized as "astringent." A peculiar numbing of the tongue develops, with various aberrations of sweet and salt tastes. The taste does not appear sufficiently characteristic to justify the use of this method for bioassay.

DISCUSSION.

As a result of our literature studies and of our laboratory tests, we have developed the following bioassay on mice:

TABLE IV.—TOXICITY OF FISHBERRY PREPARATIONS TO MICE.

Sample No.	Product.	Test.	Dose Injected as 10% Tincture Cc./Kg. 2.0.	2.4.	3.0.	4.0.	5.0.	6.0.	7.0.	8.0.	Picrotoxin Mg./Kg. 4.0.	4.5.	5.0.	6.0.	Conclusion.	Mg. Picrotoxin per Cc.
1	F.E.	1			0/2	0/3	0/2	1/4	1/2	4/4		5/10		10/10	0.8 cc. = 6 mg.	7.5
2	F.E.	1			1/5	3/5	7/10	4/5	4/5	4/5	1/2		1/2	1/1		
	F.E.	2				2/5	3/5				2/5		3/5		0.5 cc. = 5 mg.	10.0
3	F.E.	1			0/2	0/2	1/2	2/2			0/2		0/2	1/2		
	F.E.	2				1/5	5/10	8/10			4/10		6/10	5/5		
	F.E.	3				3/5	4/5				2/5		4/5		0.5 cc. = 5 mg.	10.0
4	33% Tr.	1														
	33% Tr.	2	0/5		5/5						2/5		4/5			
	33% Tr.	3	0/5	0/5							2/5		3/5			
											0/5		3/5		1 cc. more than 5 mg.	5.5
															0.8 cc. less than 5 mg.	
5	10% Tr.	1	0/5		0/5	2/5	3/5				0/5		3/5		5 cc. Tr. = 5 mg.	1.0

TABLE V.—THE TOXICITY OF PICROTOXIN TO RATS—SUBCUTANEOUS INJECTION.

Date.	Dose Mg./Kg. 1–2.	3.	4.	5.	6.	7–10.
12/27/32	0/4	1/2	2/2	2/2		4/4
4/17/33		0/5	0/5	2/5	2/3	
4/17/33			2/4	5/5		
		2/5	3/5			
Total	0/4	3/12	7/16	9/12	2/3	4/4

A large number of mice are stored under identical conditions for a sufficient time to insure uniformity. Groups of five mice each are weighed to 0.5 Gm. and injected subcutaneously under the skin of the abdomen with solutions of picrotoxin and of the unknown fishberry product. Typical picrotoxin convulsions and/or death should develop within one hour.

In a preliminary trial, doses of 4, 5, 6 and 7 mg. of picrotoxin per kilo may be administered, and quantities of the fishberry product estimated to be equivalent in picrotoxin content. Subsequent assays should be made, based on the results of this preliminary trial, to determine the amounts of picrotoxin and of the unknown product simultaneously injected which produce death in about eighty per cent of the injected mice. As a suggested standard, 5 mg. of picrotoxin is recommended.

CONCLUSIONS.

1. Great variations in the response of individual animals have been observed following the administration of picrotoxin and of its galenical preparations.

2. By the simultaneous injection of standard picrotoxin and of a galenical subcutaneously to mice, a feasible bioassay has been developed.

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NATIONAL STANDARDS FOR TINCTURE DIGITALIS* WITH SPECIAL REFERENCE TO U. S. P. X AND B. P. 1932 STANDARDS.[1]

BY L. W. ROWE.

A few years ago (1) experimental data were submitted showing the relation among five of the better known methods of bioassay of digitalis and the various standards proposed for these methods. At that time the powdered digitalis leaf standard of the Geneva Conference had not been available long enough to permit of more than a very few comparative tests and it was only then being seriously considered by various foreign countries as a national standard for digitalis assay. Since then Canada, in the Food and Drugs Act Regulations of 1928, and England, in the 1932 revision of the British Pharmacopœia, have definitely made the international powdered digitalis leaves their standard for the bioassay of digitalis. Meanwhile Ouabain has continued as the U. S. Pharmacopœia standard for digitalis and in spite of its being illogical from the standpoint of relative degree of absorption in the short time period (one hour), as previously pointed out, the majority opinion favors its retention in the U. S. P. XI. Very recently Defandorf (2) has advocated revision of the present U. S. P. "One-Hour Frog Method" to permit of a longer time period for more complete absorption and action of the diluted digitalis preparation which would be a valuable step forward. He failed, however, to observe that a difference in degree of absorption between Ouabain and digitalis in any given time and particularly in different frogs may also influence the accuracy of the assay method.

The assay methods which are official in the present U. S. P. and B. P. are sufficiently different to make a comparison of the official tinctures quite difficult and added to this, the marked difference between the standards, Ouabain and International Standard Digitalis Leaves, makes a series of experimental tests the only basis of comparison. This short paper presents such data accumulated since 1930 and permits a conclusion based on averages which should be approximately correct. It should be stated before the assay results are tabulated that both the official B. P. 1932 tincture of digitalis and the standard extract of the international powdered digitalis leaves are made without defatting the drug while the U. S. P. X tincture is made from defatted drug. For some unknown reason the assay results

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