brated glassware, by which results in close agreement with the theoretical values may be obtained.

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THE PHARMACOLOGY OF PYRETHRUM FLOWERS.*[1]

BY HARRY ROSEN AND MARVIN R. THOMPSON.[2]

The literature pertaining to the chemistry and pharmacology of pyrethrum flowers is reviewed in the original thesis and by other recent writers (1) (2), and hence will not be repeated here. This report is intended to embrace only the experimental studies described in the thesis.

In the course of the experimental work it was noted that pyrethrum powder or solutions exposed to air deteriorated rapidly. It was also observed that roaches and flies apparently protected from contact with the powder but not from possible vapors, readily exhibited symptoms of pyrethrum action. The possibility of an active volatile fraction was therefore investigated.

EXPERIMENTAL.

Six hundred grams of insect flowers were steam distilled and 4 liters of distillate collected in 250-cc. fractions. Some of the early fractions on injection into the ventral lymph sac of frogs showed toxicity. These combined distillates were saturated with sodium chloride, extracted with petroleum ether, and the solvent allowed to evaporate at room temperature. The residue was taken up in 25 cc. alcohol and precipitated by the addition of saturated sodium chloride solution, a light brown semi-solid mass resulting. This also showed some toxicity to frogs but was not investigated further because of a very meager yield. Four more samples of insect flowers were likewise steam distilled, but the distillates showed no toxicity to frogs and were found to exert no action on isolated rabbit intestine. The frog and intestine test methods are described in detail elsewhere in this paper.

The remainder of each of these steam distillates was saturated with sodium chloride and divided into two portions. One portion was extracted with petroleum ether and assayed chemically and the other was extracted with purified kerosene and assayed by the Peet-Grady fly

TABLE I.—RESULTS OF STEAM DISTILLATE ASSAYS.

Sample	Assay of Drug before Distillation. Fly Method		Chemical Method. Total Pyrethrins.	Pyrethrin I.	Pyrethrin II.	Fly Method.		Assay of Steam Distillate. Chemical Method. Total Pyrethrins.	Pyrethrin I.	Pyrethrin II.
A	68%	Kill	0.81%	0.361%	0.449%	3%	Kill	0.0015%	0.001%	0.0005%
D	83%	Kill	0.80%	0.344%	0.456%	4%	Kill	0.0035%	0.0025%	0.0010%
F	88%	Kill	0.83%	0.365%	0.465%	5%	Kill	0.0036%	0.002%	0.0016%
J	84%	Kill	0.835%	0.365%	0.470%	6%	Kill	0.0031%	0.0017%	0.0014%

* Scientific Section, A. PH. A., Dallas meeting, 1936.

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[2] Abstracted from a thesis submitted in May 1936 by Harry Rosen to the Faculty of the Graduate School of the University of Maryland in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

method. The following table shows the steam distillates to be inactive, since the actual results appearing in the table for the distillates are negative in the range of significant figures.

Control determinations by the fly method, using only purified kerosene, resulted in a fly mortality of 4–7%.

Distillation of various alcoholic percolates and macerates was also resorted to, but the results failed to prove the existence of an active volatile fraction in the drug.

THE SITE OF ACTION OF PYRETHRUM FLOWERS.

Pyrethrum flowers were found to be toxic to both warm- and cold-blooded animals, depending upon the dosage and the route of administration employed.

A hydro-alcoholic suspension of an extract of pyrethrum flowers, when injected into the ventral lymph sac of a frog produced, in a few minutes a very marked increase in reflex excitability resembling that resulting from the administration of strychnine. The increased reflex excitability soon began to pass into a state of decreased reflex excitability and in a short time the animal was completely paralyzed, with death rapidly ensuing.

If the spinal cord of the frog was destroyed before administration of the drug, the first symptoms of increased reflex excitability were not manifested. However, if the brain was destroyed, leaving the spinal cord intact, these symptoms were observed as in the normal frog. This evidence indicated that the drug exerted its action on the spinal cord.

An olive oil suspension of an active extract injected subcutaneously into white rats produced effects analagous to those observed in the frog. A typical protocol follows:

3:55—Olive oil suspension representing 12 mg. pyrethrum flowers per Gm. rat administered subcutaneously
4:00—Symptoms of distress
4:05—Increased reflex excitability
4:15—Animal in definite clonic convulsive state
4:40—Clonic convulsions ceased and paralysis developed
4:45—Respiration ceased
4:47—Cardiac arrest.

Cats after having been fed insect flowers mixed with food to the extent of 10%, over long periods of time, showed no toxic symptoms and appeared normal in all respects. However, relatively large doses of solutions of pyrethrum extracts administered to cats, intravenously or intraperitoneally, quickly produced death, especially by the intravenous route.

The following is a typical protocol showing symptoms produced by intraperitoneal injection to cats:

10:43—Olive oil suspension of active pyrethrum extract representing 4 Gm. of the flowers per Kg. cat administered intraperitoneally
10:48—Visible signs of discomfort
12:00—Abnormal movement of hind legs in locomotion and hyperactive response to mechanical stimuli in these limbs
1:00—Hind legs becoming hypoactive and forelegs hyperactive
2:30—Both fore and hind legs non-responsive to mechanical stimuli, fast and labored respiration, no readily demonstrable signs of cerebral depression, corneal reflex apparently still normal
3:00—Signs of cerebral depression—corneal reflex depressed
4:12—Respiratory failure
4:15—Cessation of heart beat.

The symptoms produced in the foregoing experiments indicated that insect flowers produce an ascending paralysis of the spinal cord, preceded by a transitory stimulation.

Experimentation with a view of determining action on the autonomic nervous system was conducted, but significant effects were not apparent from reasonable doses. Large intravenous doses of pyrethrum flowers produced no change in blood pressure of the cat. Due to this fact it appears that the drug has no action on the autonomic nervous system supplying circulation, since any significant effect at all would have shown an alteration in blood pressure. Three such experiments were performed.

PROOF OF SITE OF ACTION OF PYRETHRUM FLOWERS.

The first experiments to prove the site of action of insect flowers were concerned with its effects on the gastrocnemius muscle and sciatic nerve of the frog.

APPARATUS.

The apparatus consisted of a Harvard inductorium, platinum electrodes to stimulate the muscle, non-polarizable boot-electrodes for stimulation of the nerve, a moist chamber and a dry cell of 1.23 volts and 0.08 amperes as a source of current.

PROCEDURE.

One leg of a spinal frog, excepting the sciatic nerve, was ligated as high up as possible and amputated just below the ligature. A muscle nerve preparation was made and the minimal stimulus which elicited response of the muscle was determined by stimulation of the nerve with the boot electrodes. Likewise, by use of the platinum electrodes, the minimal stimulus causing response in the muscle was determined by direct stimulation. The results were recorded as the number of centimeters the secondary was removed from the primary coil, and also the number of degrees the secondary was rotated from the iso-axial plane of the primary coil.

A sample of pyrethrum, in a hydro-alcoholic macerate containing 10% alcohol, of which 0.0002 Gm. per Gm. of frog was pre-determined to produce paralysis in 25 to 35 minutes, was injected in the same dosage into the ventral lymph sac of the frog. After 45 minutes the remaining leg was amputated and the tests for minimal stimuli were conducted as for the first leg.

Another group of frogs was used as controls. The same recordings were made as for the experimental frogs.

TABLE II.—RESULTS OF DRUG ACTION ON NERVE AND MUSCLE.

				Before Drug.		After Drug.		
No.	Gm. Wt.	Sex.	Leg.	Nerve.	Muscle.	Leg.	Nerve.	Muscle.
1	19	M.	Left	10 cm. 45°	8.0 cm. 40°	Right	10 cm. 55°	10.0 cm. 0°
2	16	F.	Left	11 cm. 40°	7.8 cm. 0°	Right	11 cm. 40°	7.6 cm. 0°
3	13	F.	Left	9 cm. 20°	7.0 cm. 0°	Right	9 cm. 30°	7.0 cm. 0°
4	19	M.	Right	10 cm. 0°	8.0 cm. 25°	Left	9 cm. 20°	8.0 cm. 25°
5	15	M.	Right	11 cm. 35°	7.2 cm. 0°	Left	11 cm. 35°	7.1 cm. 0°
6	18	M.	Right	10 cm. 20°	7.6 cm. 0°	Left	12 cm. 55°	7.8 cm. 0°
				Controls with 10% Alcohol.				
7	18	F.	Left	12 cm. 60°	7.5 cm. 0°	Right	12 cm. 30°	7.0 cm. 0°
8	17	M.	Left	12 cm. 40°	8.0 cm. 10°	Right	12 cm. 40°	10.0 cm. 10°
9	20	M.	Right	13 cm. 35°	8.2 cm. 20°	Left	12 cm. 35°	7.9 cm. 0°
10	17	M.	Right	11 cm. 55°	7.5 cm. 0°	Left	11 cm. 55°	8.4 cm. 0°
11	17	F.	Right	10 cm. 55°	7.7 cm. 0°	Left	12 cm. 30°	8.4 cm. 0°

On examination of Table II, it is seen that small differences in intensity of stimulus necessary to elicit response in the nerve and muscle before and after the drug are sometimes present. However, comparable differences are also found in the controls, and thus it is readily seen that the drug exerts no action of any consequence on the muscle or the motor nerve.

For further studies, spinal frogs were again used, but here, the spinal cord and sciatic nerve were observed for effects produced by the drug.

APPARATUS.

The same inductorium and source of current were employed as above, but fine copper wire electrodes were used to stimulate the spinal cord and platinum electrodes were used to stimulate the sciatic nerve.

PROCEDURE.

The spinal cord, above the junction of the sciatic nerve (5th lumbar vertebra), and the sciatic nerve of one leg were exposed. The threshold stimulus was determined for each and the drug administered as in the previous experiments. Then at various intervals, recordings of the threshold stimuli were made. Here, also, control frogs injected with 10% alcohol were tested and the results compared to those obtained from the frogs exposed to the drug.

TABLE III.—RESULTS OF DRUG ACTION ON CORD AND NERVE.

No.	Gm. Wt.	Sex.	Before Drug. Cord.	Nerve.	Time.	After Drug. Cord.	Nerve.
1	21	M.	8.4 cm. 15°	10.3 cm. 50°	31 Min.	6.2 cm. 0°	10.3 cm. 55°
2	15	M.	8.4 cm. 10°	11 cm. 55°	28 "	7.1 cm. 0°	11.0 cm. 40°
3	21	F.	9.0 cm. 0°	9 cm. 30°	20 "	7.0 cm. 0°	9.0 cm. 35°
					45 "	2.0 cm. 0°	9.0 cm. 30°
4	23	F.	9.0 cm. 0°	9 cm. 40°	28 "	6.3 cm. 0°	9.0 cm. 30°
					54 "	5.5 cm. 0°	9.0 cm. 50°
5	24	M.	9.0 cm. 20°	9 cm. 15°	40 "	6.8 cm. 0°	9.0 cm. 40°
6	25	M.	5.8 cm. 0°	8.5 cm. 25°	30 "	4.6 cm. 0°	8.5 cm. 30°
					62 "	2.2 cm. 0°	8.5 cm. 35°
7	23	M.	4.1 cm. 0°	9 cm. 10°	46 "	2.3 cm. 0°	9.0 cm. 60°
8	22	F.	8.5 cm. 15°	9 cm. 45°	41 "	6.3 cm. 0°	9.0 cm. 55°
9	19	F.	7.0 cm. 0°	8.4 cm. 30°	60 "	5.3 cm. 0°	8.4 cm. 40°
10	22	F.	9.4 cm. 0°	10 cm. 35°	45 "	8.2 cm. 0°	10.0 cm. 40°
					65 "	6.4 cm. 0°	10.0 cm. 40°
			Controls with 10% Alcohol.				
1	20	M.	7.3 cm. 60°	10.8 cm. 25°	30 Min.	7.2 cm. 0°	10.9 cm. 30°
2	26	M.	7.0 cm. 0°	8.2 cm. 0°	30 "	7.0 cm. 0°	8.2 cm. 0°
					90 "	7.2 cm. 0°	8.3 cm. 45°
3	27	F.	7.3 cm. 0°	9.0 cm. 20°	37 "	7.2 cm. 0°	9 cm. 35°
					87 "	6.8 cm. 0°	9 cm. 45°
4	25	F.	8.5 cm. 15°	10.0 cm. 50°	38 "	8.5 cm. 50°	10 cm. 55°
					56 "	8.5 cm. 50°	10 cm. 40°
5	22	M.	9 cm. 30°	10.0 cm. 20°	40 "	9 cm. 0°	10 cm. 25°
					75 "	9 cm. 15°	10 cm. 40°
6	22	F.	7.8 cm. 0°	10.0 cm. 20°	34 "	7.7 cm. 0°	10 cm. 40°
					69 "	7.7 cm. 0°	10 cm. 45°

Table III shows again that the motor nerve is not affected, but the spinal cord is definitely depressed, because the secondary coil had to be brought closer to the primary coil of the inductorium (greater stimulus) in order to carry the impulse down the cord and over the sciatic nerve to elicit a response of the muscle. This depression is an ascending one, since it was observed in intact animals that the hind limbs and then the fore limbs were paralyzed. The paralysis, therefore, involves the anter r horn of the cord, since direct stimulation of the cord showed the paralysis.

Proof of the presence or absence of action on the posterior portion of the cord was next undertaken.

APPARATUS.

The same inductorium as above was used with two dry cells of 3.0 volts and 51 amperes, platinum electrodes being used for purposes of stimulation.

PROCEDURE.

Two frogs were used for each experiment one being a spinal frog and the other a completely pithed frog, both sciatic nerves of the spinal frog and the right sciatic nerve of the completely pithed frog being exposed. The left sciatic nerve of the spinal frog and the right sciatic nerve of the completely pithed frog were contacted by means of a wire bridge and the stimulus was applied to the right sciatic nerve of the spinal frog. The threshold stimulus necessary to pass over the posterior portion of the cord of the spinal frog, down the left sciatic nerve, into the right sciatic nerve of the completely pithed frog, and producing a response in the leg, was ascertained. Then the drug solution was injected into the ventral lymph sac of the spinal frog and at various intervals the threshold was again determined.

Control frogs were also used here and the results compared to those obtained from the frogs injected with pyrethrum flowers. The results were recorded the same as those in Tables II and III.

TABLE IV.—RESULTS OF DRUG ACTION ON POSTERIOR HORN OF SPINAL CORD.

No.	Gm. Wt.	Sex.	Before Drug.	Time.	After Drug.
1	25	M.	5.0 cm. 0°	128 Min.	2.5 cm. 0°
2	29	F.	7.4 cm. 0°	82 "	4.7 cm. 0°
3	25	F.	4.9 cm. 0°	80 "	3.7 cm. 0°
4	25	F.	3.8 cm. 0°	115 "	*0.2 cm. 0°
5	24	M.	5.7 cm. 0°	69 "	4.1 cm. 0°
6	24	M.	4.2 cm. 0°	90 "	*0.2 cm. 0°
7	25	M.	5.3 cm. 0°	130 "	2.0 cm. 0°
8	19	M.	7.0 cm. 0°	59 "	5.6 cm. 0°
9	24	M.	6.7 cm. 0°	96 "	2.8 cm. 0°
10	25	F.	6.3 cm. 0°	127 "	4.8 cm. 0°
			Control Frogs.		
1	20	M.	6.5 cm. 0°	130 Min.	6.2 cm. 0°
2	25	F.	5.5 cm. 0°	114 "	5.8 cm. 0°
3	24	F.	6.3 cm. 0°	72 "	6.2 cm. 0°
4	25	M.	7.1 cm. 0°	104 "	6.8 cm. 0°
5	26	M.	8.3 cm. 0°	140 "	7.9 cm. 0°

* No response here. Secondary coil could not be moved closer to primary coil.

Analysis of Table IV shows that depression of the posterior portion of the spinal cord caused by drug action is far in excess of the slight changes occurring in the control frogs over a period of time, proving that the sensory portion of the spinal cord is depressed by the drug.

A review of all results thus far obtained shows that pyrethrum flowers exert no changes on the threshold for either striated muscle or motor nerves. However, the drug does depress both the anterior and posterior portions of the cord after a transitory period of stimulation.

This does not entirely dismiss the possibility of changes in the phases of an isotonic contraction of striated muscle. The threshold stimulus may not be changed, but nevertheless the phases through which a muscle passes in an isotonic contraction may be altered.

In determining the effects of the drug on phases of contraction of striated muscle, both cold- and warm-blooded animals were used.

PROCEDURE.

For the cold-blooded animal experiments, a muscle nerve preparation of a frog's gastrocnemius muscle was made and stimulated directly with platinum electrodes, recording the phases by means of a kymograph. The muscle was irrigated with pyrethrum in frog Ringer's solution and again the phases of contraction were recorded and compared to the original phases before drug action.

In the experiments with warm-blooded animals, anesthetized cats were used. The proximal portion of the gastrocnemius muscle was left intact, but the distal tendons were detached and connected to a muscle lever. The phases of contraction produced by direct stimulation with platinum electrodes were recorded and the muscle was then irrigated with pyrethrum solution. After considerable irrigation the phases were again recorded, the results being compared to the original.

Analyses of the phases of contraction of the frog and cat muscles show no significant changes after exposure of the muscles to the action of pyrethrum flowers.

After the effects of the drug on striated muscle were ascertained, the action of pyrethrum flowers on intestinal muscle was next determined.

PROCEDURE.

Segments of rabbit intestine from the pyloric portion each about one centimeter long, were bathed in 50 cc. of Tyrode's solution maintained at 37.5° C. After a suitable control recording had been obtained, pyrethrum solution was added and the results noted.

It was found that pyrethrum produced a decrease in amplitude of contraction and a decrease in tonus of isolated rabbit intestine.

For further localization of this action, the response of the muscle to a definite dose of 1% barium chloride solution was observed, the barium solution was replaced by new Tyrode's solution and pyrethrum added. After an elapse of ten minutes the original dose of barium chloride was added and, at two-minute intervals, two larger doses of barium chloride were administered.

This procedure showed that the action of barium which is directly on the muscle is inhibited by pyrethrum, thus indicating that pyrethrum acts directly on intestinal musculature.

THE BIOLOGICAL ASSAY OF PYRETHRUM FLOWERS.

The assay of pyrethrum flowers has been the object of much research in recent years, numerous chemical and biological methods having been proposed and used by various test laboratories. The best chemical methods and the fly method which appears to be the peer of the biological assay procedures, require either elaborate apparatus or consume very much time. In addition, many investigators are unable to agree on the superior suitability of any one method.

In view of these facts, Chevalier (3), and Chevalier and Ripert (4) have suggested the use of the frog as a test object, and Perrot and Gaudin (5) and Rigal (6) have used isolated rabbit intestine as a means of evaluating pyrethrum flowers.

In this work, both of these suggestions were further investigated and a method has been developed from each.

THE OVER NIGHT FROG METHOD OF ASSAY.

Various symptoms produced by pyrethrum flowers on the frog were considered as possible end-points in determining the potency of unknown samples of drug and it was finally concluded that over night mortality was the best end-point. This necessitated the preparation of a mortality curve in order to compare the relative strengths of an unknown pyrethrum and the one that would be used as a standard.

PREPARATION OF MORTALITY CURVE—APPARATUS.

The apparatus consisted of a storage tank for the frogs with constant running water at a temperature below 15° C. in order to reduce the metabolic rate of the frogs, so that feeding would

not be necessary. A large tank, the temperature of which could be maintained at 20° C. ± 0.5° C., equipped with individual compartments, was used for keeping the frogs over night after administration of the drug. The individual compartments had no bottom and were placed on wire screens, immersed in the water to a depth of about one centimeter.

ANIMALS.

The frogs used in the preparation of the mortality curve weighed from 15 to 35 Gm. and were stored in the tank, with running water below 15° C., for one week before use.

PROCEDURE.

Seven series of frogs, each series of one sex weighing within a range of 5 Gm., were divided into eight groups, each group of equal number ranging from 10 to 25 frogs depending on the number available. On different days, 24 hours before use, each series of frogs was removed from the storage tank and placed in the individual compartments at 20° C. ± 0.5° C. Before administration of the drug, each frog was dried with a towel, the urine expressed and the weight recorded within one-half gram. The drug was used as a 10% macerate in alcohol, diluted with distilled water so that the solution contained less than 25% alcohol and so that no frog received more than 0.02 cc. per Gm. The same 10% alcoholic macerate was used in all the seven series of frogs but the dilutions were prepared as needed for each group of frogs. Eight doses, one for each group of each series, were selected so that the lowest dose produced no mortality and the highest dose produced 100% mortality. The frogs of each group were injected in the ventral lymph sac with the assigned doses and each frog was placed in its separate compartment. The following day, the frogs were examined and the percentage mortality was recorded for each dose. A curve was plotted for each series of frogs, and all of the curves were then superimposed on a 50% point. Finally, the various percentage mortalities were averaged and a composite curve of all the individual results was prepared.

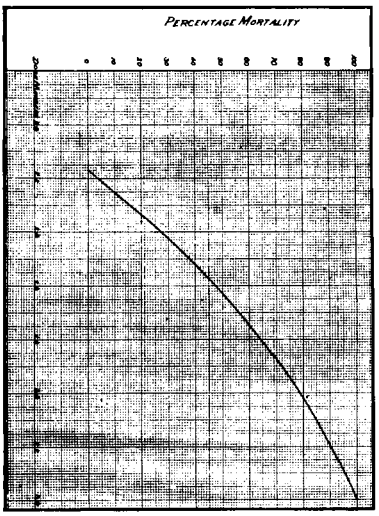

Fig. 1.—Composite of all frog mortality curves.

TABLE V.—DOSE NUMBERS CORRESPONDING TO PERCENTAGE MORTALITY.

Per Cent Mortality.	Dose Number.	Per Cent Mortality.	Dose Number.
0	2.17	52	2.65
4	2.20	56	2.70
8	2.23	60	2.75
12	2.27	64	2.80
16	2.30	68	2.85
20	2.33	72	2.91
24	2.37	76	2.97
28	2.41	80	3.03
32	2.44	84	3.10
36	2.48	88	3.17
40	2.52	92	3.24
44	2.56	96	3.32
48	2.60	100	3.40

From the composite curve, dose numbers for all possible percentage mortalities, based on the use of 25 frogs, were calculated and recorded for use in obtaining the potency of an unknown pyrethrum in terms of a standard pyrethrum.

ASSAY PROCEDURE—APPARATUS AND ANIMALS.

The apparatus and animal requirements are the same as for the preparation of the curves.

SOLUTIONS.

The drug, if not already ground, is reduced to a No. 40, or finer, powder. Five grams of both the standard and unknown powders are accurately weighed and placed in separate 25-cc. volumetric flasks. Sufficient alcohol is added to each to make 25 cc. and the solutions are placed in the dark at 1–2° C. for 24 hours, with occasional agitation, after which they are ready for use. The solution is brought to room temperature before use and the supernatant fluid is decanted without shaking.

ASSAY PROCEDURE.

Frogs weighing between 15 and 35 Gm. and of one sex are used in the assay. For any one determination, the frogs must all weigh within a range of five Gm. The diluted solutions for injection must not have an alcohol content of more than 25% and if necessary, any excess alcohol is removed with a current of air. Not less than 0.01 cc. nor more than 0.02 cc. per Gm. frog may be injected, no frog receiving a dose of less than 0.25 cc. The dose of both standard and unknown producing a mortality of approximately 50% is determined by injecting varying doses into groups of three frogs to each dose. The frogs are kept below 15° C. until 24 hours before the assay and then removed to a 20° C. ± 0.5° C. temperature. They are dried with a towel, urine expressed and weighed to within 0.5 Gm. just prior to their use. After orientation of the 50% mortality dose for both standard and unknown, 50 frogs of the above specifications are selected, and 25 injected with each, the standard and unknown. The following day, the mortality percentages are determined and by means of the curve-dose numbers the potency of the unknown is determined in terms of the standard. The percentage mortalities may be from 20% to 80% but never below or above these figures. The frogs which recover may be used again in preliminary orientation of dosage, but never in final determinations.

DISCUSSION OF A TYPICAL FROG ASSAY.

From experience, it has been found that pyrethrum flowers do not have as wide a range of potency in terms of a good commercial sample, which may be chosen for a standard, as do many other drugs. The potency may vary from 0% to 150% but it has been most unusual to obtain a specimen that assayed above this figure. For this reason, in orienting the over night M. L. D., not so wide a range of doses is necessary and one preliminary determination of three frogs to each dose often suffices, especially if the assayist has carried out recent determinations and knows the approximate M. L. D. of the standard. If such is not the case, two preliminary tests may be necessary. The object is to find the dose which will result in a mortality as near 50% as possible.

For the sake of explanation of the preliminary assay, let it be assumed from previous results, that 0.0004 Gm. per Gm. frog, of the standard, resulted in approximately 50% mortality. This dose can be used for the standard in the final assay, since this figure does not vary greatly in monthly periods. For the unknown, doses are selected which are based on 0.0004 Gm. per Gm. frog as the 50% M. L. D. of a 100% drug or the equivalent of the standard. The doses of the unknown are calculated on the basis of possibilities of 25, 50, 75, 100, 125 and 150 per cent of the standard. The solution is properly diluted and injected into the ventral lymph sacs of three frogs for each dose. The following day the results were found to be, for example, as follows:

Suspected Potency.	Grams per Gram Frog.	Results of 3 Frogs.
150%	0.000266	− − −
125%	0.000320	− + −
100%	0.000400	+ − +
75%	0.000533	+ + −
50%	0.000800	+ + +
25%	0.001600	+ + +

These results show that the 50% M. L. D. of the unknown lies between 0.000320 and 0.00040 Gm. drug per Gm. frog.

For the final assay, the average, 0.00036 Gm. per Gm. frog, is used as the dose and 25 frogs are injected. Likewise, 25 frogs are injected with 0.00040 Gm. standard per Gm. frog.

The following day it is found, for example, that the standard produced 52% mortality and the unknown produced 36% mortality.

Consulting Table V, it is seen that
0.004 Gm. (S) = 52% mortality = 2.65 dose number
0.00036 Gm. (X) = 36% mortality = 2.48 dose number

$$\text{Thus } 0.00036 \text{ Gm. (X) multiplied by } \frac{2.65}{2.48} = 0.004 \text{ Gm. (S)}$$

$$0.000384 \text{ Gm. (X)} = 0.004 \text{ Gm. (S)}$$
$$1.0 \text{ Gm. (X)} = 1.042 \text{ Gm. (S)}$$
$$\text{(X)} = 104\% \text{ of (S)}$$

THE BARIUM INHIBITION OR ISOLATED RABBIT INTESTINE METHOD OF ASSAY.

It has been previously shown that pyrethrum flowers decrease amplitude and tonus of rabbit intestine, and that this action is exerted directly on the muscle, Fig. 1, since the effect of barium on the intestine is inhibited by pyrethrum. On further investigation, it was found that this inhibition occurs quantitatively and for this reason, the reaction has been utilized as the basis of a method of biological assay.

APPARATUS.

The apparatus for this assay consisted of an isolated tissue bath equipped with two 50-cc. tissue chambers and a constant temperature control. The tissue chambers were so equipped that they could be emptied and refilled with the tissue bathing fluid without undue exposure of the tissue to air. The chambers were also equipped with an oxygen supply which could be regulated as necessary for various tissues.

TISSUE.

The tissue was obtained from the pyloric portion of the rabbit's intestine, the rabbit being preferably a mature one.

SOLUTIONS.

The pyrethrum solutions for this assay were prepared as for the frog method. The barium solution consisted of a suitable strength (usually 1%) solution of barium chloride in distilled water and Tyrode's solution was used as the bathing fluid for the tissues.

ASSAY PROCEDURE.

The rabbit is killed by a blow on the head and approximately 50 cm. of the pyloric portion of the intestine are removed. The extirpated tissue is placed in a beaker containing Tyrode's solution and may be used immediately. The remainder of the tissue, after the first strips are taken for use, is kept at 1–2° C. Two 1.5–2-cm. portions of the intestine, both of equal length, are cut and suspended in each of the tissue chambers and the upper ends are attached to suitable

magnifying levers in order to record the results. The oxygen is adjusted as necessary for the two strips of tissue, care being taken that both receive exactly the same supply. The temperature is maintained between 37.5° C. and 38° C. Tension is applied, the same to both tissues, as necessary and the tissue is allowed to relax. Then similar doses of barium are added to each chamber, usually 0.2 to 0.4 cc. being sufficient, and a significant response of 2 to 4 minutes' duration is recorded. A 1% solution of barium chloride usually suffices, but occasionally it is necessary to use a 2% or 3% solution. After recording the first response the bathing fluid is removed, new fluid is introduced and the tissues are allowed to relax. Finally equal doses, smaller than the first, are added to each chamber and the responses are recorded. These latter doses enable the assayist to determine whether or not the tissues are reacting consistently, and also insure against a maximal response, which is shown by the second response being less than the first. If the reactions are consistent and a submaximal response has been obtained, these responses are accepted as controls.

The tissues are again freed of the Tyrode's solution containing the barium, and new Tyrode's solution is introduced. Next, the unknown pyrethrum solution is added to one chamber immediately after washing; and one minute later the standard pyrethrum solution is added to the other chamber. Usually, 0.25 cc. to 0.5 cc. of a 1 to 3 dilution of the stock solution of pyrethrum (Tyrode's solution being the diluent) is satisfactory to inhibit the quantities of barium suggested above. Ten minutes later, the original submaximal dose of barium is added to each chamber containing, respectively, the above-mentioned pyrethrum solutions. After the tissues return to the control line, two subsequent doses of barium are added to each chamber. The second and the third doses are usually twice and three times the strength of the first dose, respectively. From the degree of inhibition produced by both the standard and unknown pyrethrum solutions, the potency of the unknown is determined in terms of the standard. New strips are required for each determination, since the tissues do not recover within a reasonable period of time.

A TYPICAL ASSAY BY THE BARIUM INHIBITION METHOD.

Although the intestine removed from the rabbit for use in this procedure is always satisfactory for one day's use, it was possible, with patience, to successfully use strips from the same intestine for two or even three days. However, on the second or third day, the tissue responds very slowly and for the saving of time it is recommended that new tissue be used each day.

New strips are used for each determination, although evidence is at hand which shows that the drug effects can be washed out and the tissues used again. The washing and subsequent recovery are very prolonged, and since an abundance of tissue is available for one day's work, the loss of time in waiting for recovery is not justifiable.

For the purpose of conveying some idea as to the procedure for obtaining the potency of an unknown pyrethrum powder by this method, figures of an actual assay are presented.

A dilution of I, representing 0.6666 Gm. pyrethrum flowers per cc., was assayed in terms of a dilution of A, representing 0.7123 Gm. pyrethrum flowers per cc.

In the first determination, 0.3 cc. of each was used and it was found that I produced greater inhibition of response of the intestine to barium, than did A, thus being more than 107% of the potency of A.

Then, the dose of A was increased to 0.4 cc. while the original dose of I was retained, and as a result this dose of A was more potent than 0.3 cc. of I, showing I to be less than 142% of A.

In the next determination, a dose of 0.25 cc. of I was compared to 0.3 cc. of A and was found to be more potent than the dose of A, being therefore more than 128% of A.

From these results it is seen that the potency of I is between 128% and 142% of A. For commercial purposes, any further attempts to orient the potency are not

necessary and an average of the two figures, which is 135%, may be assigned to I. Further increase in the number of determinations permit of somewhat greater precision.

The dilutions of the stock macerate of the drug must be prepared each day as they lose potency rapidly after dilution.

The percentage inhibition produced by the pyrethrum in this assay is of great importance. If too much inhibition of barium is obtained, the relative potencies of the two preparations cannot be easily deduced. The pyrethrum dosage must be so selected as to provide a definite barium response at the end of the ten-minute period.

The use of more than one dose of barium following the ten-minute elapse of time for the production of pyrethrum paralysis, is advisable in all assays. This helps clarify results and removes much of the possibility of misinterpretation of potency.

This method of assay is accurate, without difficulty, within plus or minus 10%. On various occasions the writers obtained an accuracy within plus or minus 5%, when assaying different dilutions of one preparation prepared by other members of the laboratory, the writers being totally without knowledge of their relative potencies.

APPLICATION OF THE NEW BIOASSAY METHODS.

Ten commercial samples of pyrethrum flowers were obtained and identified as A to J, inclusive. Sample A, which was considered to be a good commercial sample, was used as the standard. It was preserved in ampuls, in the dark, at 1–2° C. Each ampul contained approximately 5 Gm. which is the quantity indicated for use in the assay procedures.

The remaining nine samples were assayed in terms of A by the two new methods and in addition, by the Seil chemical method (7) and the Peet-Grady fly method (8).

TABLE VI.—RESULTS OF ASSAYS.

Sample.	Total Pyrethrin Content.	Pyrethrin I Content.	Pyrethrin II Content.	Fly Kill in 24 Hours.	Potency in Terms of Total Pyrethrins.	Potency in Terms of Pyrethrin I Content.	Potency in Terms of Pyrethrin II Content.	Potency by Frog Method.	Potency by Intestine Method.
A	0.81 %	0.361%	0.449%	68%	...	...	...	..	...
B	0.32 %	0.133%	0.187%	48%	40%	37%	42%	41%	25%
C	0.121%	0.062%	0.059%	16%	15%	17%	14%	25%	25%
D	0.80 %	0.344%	0.456%	83%	99%	95%	102%	86%	99%
E	0.87 %	0.371%	0.499%	89%	107%	103%	111%	62%	133%
F	0.83 %	0.365%	0.465%	88%	102%	101%	104%	98%	91%
G	0.87 %	0.375%	0.495%	80%	107%	104%	110%	89%	109%
H	0.76 %	0.350%	0.410%	69%	94%	97%	91%	86%	107%
I	0.77 %	0.364%	0.406%	77%	95%	101%	94%	64%	135%
J	0.835%	0.365%	0.470%	84%	103%	101%	105%	60%	125%

DISCUSSION OF RESULTS OF ASSAYS.

Table VI shows that the results obtained by the frog and isolated rabbit intestine methods of assay are not always in good agreement with those resulting from the chemical assay.

The frog assays, while showing good agreement with the chemical assay, involving six of the samples, show low results in the remaining three samples.

The isolated intestine determinations show good agreement with the chemical assays in six of the comparisons and show no great disagreement in the remaining three tests.

It is significant to note that the disagreements of both of the biological methods with the chemical method occur in the same samples, the frog assay showing a lower potency and the isolated intestine assay showing a higher potency than the chemical assay.

In some instances, the percentage fly kill shows some agreement with the assay results of the other methods, but the absence of a standard of comparison deprives the fly results of any great significance, showing only the relative positive or negative fly killing powers of a sample of pyrethrum flowers.

SUMMARY AND CONCLUSIONS.

1. Pyrethrum, by the methods so far employed, was found to contain no volatile active constituent.

2. Pyrethrum flowers are toxic to both warm- and cold-blooded animals, depending upon the dosage and route of administration.

3. Cold-blooded animals (frogs and insects) are much more susceptible to the action of pyrethrum than warm-blooded animals (rats and cats).

4. Skeletal muscle and the motor nerves supplying this type of muscle are not affected by pyrethrum flowers.

5. Following toxic doses, pyrethrum flowers produce a transitory stimulation of both the anterior and posterior horns of the spinal cord, followed by an intense depression.

6. The principal site of action of pyrethrum is the spinal cord. The character of the action may be described as a transitory stimulation followed by depression and paralysis of a distinctly ascending type, ultimately reaching the medullary centers.

7. The autonomic nervous system appears not to be directly affected by pyrethrum flowers. Any alterations in function of autonomically controlled organs are slight, and are induced reflexly.

8. Rabbit intestine is depressed by pyrethrum flowers, the drug exerting its action directly on the musculature.

9. Two new methods of assay, the Over Night Frog and Isolated Rabbit Intestine methods have been developed and used for assay purposes.

10. Nine samples of pyrethrum flowers have been assayed in terms of a reference standard, by the Seil chemical method, Peet-Grady fly method and the two new biological assay methods.

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