

The Pharmacology of Pyrethrum Flowers

By

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Doctor of Philosophy**

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THE PHARMACOLOGY OF PYRETHRUM FLOWERS

Part I. The History and Uses of Pyrethrum Flowers.

The early history of pyrethrum flowers or insect powder is very much confused but it is known that the people of eastern Europe were aware of the value of pyrethrum as an insecticide more than a century ago and that insect flowers were used still earlier in Dalmatia under the name of "Polvere de Pulisi" (1).

The use of insect powder was exposed to western Europe by Sumttoff, an Armenian merchant, who while traveling through the Caucasian territory became cognizant of the fact that insect powder was made from the flowers of *Pyrethrum roseum* and *Pyrethrum carneum* (2). Sumttoff's son became the first manufacturer of the powder on a commercial basis. Another report states that Tcherkess prisoners related the formula of insect powder to Russian military authorities.

About 1840, *Pyrethrum cinerariaefolium* was produced in Dalmatia and soon began to replace the Persian species in Europe. The origin of the rise of this species as an insecticide is very interesting. A German woman living in Ragusa, Dalmatia, picked a bunch of flowers for decorative purposes, later throwing them in a corner after they had become withered. After a short time she noticed dead insects near the flowers and believing that the insects died because of some action of the flowers, she undertook the manufacture of insect powder, which was continued after her

death by a pharmacist of Ragusa (3).

Pyrethrum powder was introduced into the United States about 1860 (4). Until 1914 practically all pyrethrum used in this country was imported from Dalmatia but the World War closed this market and Japan was quick to seize this opportunity to supply insect powder. Japan was able to do this owing to the fact that pyrethrum flowers had been introduced into that country from England in 1881, from Dalmatia in 1884 and 1885, and from Austria in 1886.

In recent years, enormous quantities of insect powder have been imported into the United States from many countries, but the quantities from Japan are far in excess of the combined quantities from all other countries.

The imported powder must be obtained from *Chrysanthemum* (*Pyrethrum*) *cinerariaefolium* (Trev.) Bocc., *Chrysanthemum* (*Pyrethrum*) *roseum* Web. and Mohr., or *Chrysanthemum* *Marshallii* Aschers (synonym, *Pyrethrum* *carneum* M.B.), all of these being recognized as sources of insect flowers by the Insecticide and Fungicide Board of the United States Department of Agriculture (5). Of the three, *Pyrethrum cinerariaefolium* is the most important commercially, since very small quantities of *Pyrethrum roseum* and *Pyrethrum carneum* are imported into the United States.

Table I supplied by the United States Department of Commerce shows the extent to which Japan monopolizes the pyrethrum importations into this country.

TABLE I. Imports into United States of Pyrethrum Flowers, Crude.

	<u>1923</u>		<u>1924</u>		<u>1925</u>	
	Pounds	Value	Pounds	Value	Pounds	Value
Japan	2,514,251	\$1,218,545	3,473,534	\$1,404,207	4,957,232	\$ 985,944
Yugoslavia	67,296	34,857	1,187	491	224,075	35,582
Italy	389,056	142,880	322,068	138,673	1,177,514	198,303
Austria	3,250	1,628	103,939	44,807	32,881	4,603
United Kingdom	---	---	11,975	4,386	700	410
Hongkong	---	---	22,400	11,200	4,928	998
Canada	---	---	---	---	33,600	9,132
Netherlands	---	---	---	---	4,475	1,720
Total	2,973,863	\$1,397,910	3,945,101	\$1,603,764	6,435,405	\$1,236,892

	<u>1926</u>		<u>1927</u>		<u>1928</u>	
	Pounds	Value	Pounds	Value	Pounds	Value
Japan	8,061,209	\$1,024,472	7,787,098	\$1,063,755	12,024,148	\$3,299,099
Yugoslavia	221,919	26,380	562,072	79,315	1,032,370	249,721
Italy	1,512,711	160,779	536,236	63,429	503,844	109,717
Austria	---	---	---	---	112,000	30,568
Belgium	---	---	---	---	2,202	623
France	---	---	---	---	10,691	1,400
United Kingdom	786	200	1,330	322	2,414	629
Mexico	---	---	---	---	1,340	369
Canada	225	80	23,718	3,233	---	---
China	56,000	7,454	---	---	---	---
Germany	---	---	5,477	856	---	---
Netherlands	---	---	32,826	3,653	---	---
Russia	---	---	44,352	5,544	---	---
Total	9,859,850	\$1,219,365	8,993,109	\$1,220,107	13,689,009	\$3,692,126

	<u>1929</u>		<u>1930</u>		<u>1931</u>	
	Pounds	Value	Pounds	Value	Pounds	Value
Japan	8,814,931	\$2,013,020	8,223,939	\$1,281,199	3,871,842	\$ 452,833
Yugoslavia	88,646	22,439	44,545	6,682	1,561	167
Italy	73,000	20,153	267,942	45,135	365,364	40,656
France	16,500	2,272	---	---	92,058	10,549
United Kingdom	---	---	---	---	14,784	1,370
China	20,000	3,000	---	---	---	---
Russia	---	---	---	---	61,938	4,572
Greece	---	---	---	---	44,506	4,339
Netherlands	---	---	---	---	65,803	7,239
Canada	---	---	---	---	3,287	378
Total	9,013,077	\$2,060,884	8,536,426	\$1,333,016	4,521,143	\$ 522,103

	<u>1932</u>		<u>1933</u>		<u>1934</u>	
	Pounds	Value	Pounds	Value	Pounds	Value
Japan	11,061,949	\$1,206,692	9,066,040	\$1,373,450	10,093,612	\$1,985,603
Yugoslavia	612,965	59,910	838,256	94,372	298,684	47,596
Italy	412,658	41,865	446,197	52,072	113,533	17,152
United Kingdom	---	---	---	---	42,369	6,340
Russia	14,164	625	3,855	3,855	38,093	1,714
Brazil	---	---	---	---	4,480	1,086
Total	12,110,736	\$1,309,092	10,434,559	\$1,523,749	10,590,771	\$2,059,492

In addition to the foregoing imports of crude pyrethrum, special provision has been made in the import statistical records for "Pyrethrum, advanced in value or condition."* Entries under this description have been as follows:

<u>Year</u>	<u>Pounds</u>	<u>Value</u>	<u>Year</u>	<u>Pounds</u>	<u>Value</u>
1923	185,603	\$81,270	1929	23,240	\$ 6,524
1924	44,777	19,829	1930	31,200	6,817
1925	31,908	9,895	1931	15,770	3,056
1926	13,638	3,015	1932	25,010	3,066
1927	30,786	6,237	1933	60,747	12,147
1928	58,721	14,364	1934	150,370**	36,502

At one time, pyrethrum was grown commercially in central California, but the hand labor required for picking the flowers made costs of crop production too high to compete with imported supplies.

In addition to insecticidal properties, it is claimed that pyrethrum flowers also have some virtue as an anthelmintic. The earliest reports

* "Pyrethrum advanced in class," is probably pyrethrum ground to a very fine powder.

** Japan 149,250 pounds (\$36,148) and Italy 1,120 pounds (\$354).

of such use were made by Schipulinsky (6) in 1854, by Frontali (7) in 1858, and by Noodt (8) in 1858. The first suggestion in this country of vermicial properties was made by a physician in 1898 (9) who through an accident to a child discovered this property of insect flowers. In more recent years, Chevalier (10, 11), Gaudin and Carron (12), Anglade, Gaudin, and Arcony (13), and Perrot (14) have reported such widespread successes with pyrethrum as an anthelmintic that several preparations are now marketed in France and are extensively used for this type of medication. Gnadinger (15) in the United States reports that his successes in this direction have not been nearly as spectacular as those of the other investigators.

Insect flowers have also been described as useful in the treatment of scabies, by Lemaire and Gaudin (16) in France and by Sweitzer and Tedder (17) in the United States. An ointment containing 0.75 per cent pyrethrins, representing 83 grams pyrethrum per 100 grams of product has recently been made available to physicians in the United States for use in the treatment of this disease.

Part II. The Active Principles of Pyrethrum Flowers.

The middle of the nineteenth century marked the beginning of many attempts to isolate the active principle or principles of insect flowers. Many conclusions appeared, such as the presence of volatile oils, santonin, resins, acids, oleoresins, and esters (18), all of which at some time were considered responsible for the insecticidal powers of insect flowers. However, for various reasons, none of these conclusions have withstood the test of time.

In 1924, Standinger and Ruzicka (19) published a series of papers in which were described the isolation, chemical investigation, and attempts to synthesize two active constituents designated as pyrethrin I and pyrethrin II. These two substances were reported as representing the total activity of insect flowers.

It is unfortunate that the name pyrethrin was used to designate the active constituents of insect flowers because this name had already been used to identify the active principle of *Anaelyclus pyrethrum* (20) also known as pellitory. *Anaelyclus pyrethrum* was official in U.S.P. IX and is used as an irritant, apparently having absolutely no insecticidal properties.

During the course of the work to be described, it was noted that pyrethrum powder or solutions exposed to air very quickly deteriorated. It was also observed that roaches and flies apparently protected from

contact with the powder but not from vapors possibly emitted, readily showed symptoms of pyrethrum action. This led to the belief that some samples of pyrethrum may contain some proportion of volatile activity.

In following this suggestion, 600 grams of insect flowers were steam distilled and 4 liters of distillate collected in 250 c.c. 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 c.c. 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 will be described in detail in Part IV of this paper.

Distillation of various alcoholic percolates and macerates was also resorted to but in no case was any evidence obtained to substantiate the activity present in the first steam distillate.

Part III. 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 solution of an extract of pyrethrum flowers, when injected into the ventral lymph sac of a frog, produced in a very few minutes, a very marked hyperirritability, resembling that resulting from the administration of strychnine. The hyperirritability soon began to pass into a state of hypoirritability 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 hyperirritability 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 solution 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 mgm. pyrethrum flowers per gram 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 stoppage.

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

A chronological order of symptoms produced by intraperitoneal injection is as follows:

10:43 - olive oil suspension of active pyrethrum extract representing 4 grams of the flowers per kgm. 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 decreased.

4:12 - respiratory failure.

4:15 - cessation of heart beat.

The symptoms produced in the foregoing experiments strongly 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. In Fig. 1 it can be seen that large intravenous doses of pyrethrum flowers produced no change in blood pressure and 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.



Fig. 1 - Effect of Pyrethrum Flowers on Carotid Blood Pressure.

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 hydro-alcoholic solution containing 10% alcohol, of which 0.0002 grams per gram of frog was predetermined 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

No.	GM. WT.	Sex	Leg	Before Drug		Leg	After Drug	
				Nerve	Muscle		Nerve	Muscle
1	19	M.	Left	10 cm. 45°	8 cm. 40°	Right	10 cm. 55°	10 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 cm. 0°	Right	9 cm. 30°	7 cm. 0°
4	19	M.	Right	10 cm. 0°	8 cm. 25°	Left	9 cm. 20°	8 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°
C O N T R O L S W I T H 10% A L C O H O L								
7	18	F.	Left	12 cm. 60°	7.5 cm. 0°	Right	12 cm. 30°	7 cm. 0°
8	17	M.	Left	12 cm. 40°	8 cm. 10°	Right	12 cm. 40°	10 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 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		Time	After Drug	
			Cord	Nerve		Cord	Nerve
1	21	M.	8.4 cm. 15 ⁰	10.3 cm. 50 ⁰	31 min.	6.2 cm. 0 ⁰	10.3 cm. 55 ⁰
2	15	M.	8.4 cm. 10 ⁰	11 cm. 55 ⁰	28 "	7.1 cm. 0 ⁰	11 cm. 40 ⁰
3	21	F.	9 cm. 0 ⁰	9 cm. 30 ⁰	20 "	7.0 cm. 0 ⁰	9 cm. 35 ⁰
					45 "	2.0 cm. 0 ⁰	9 cm. 30 ⁰
4	23	F.	9 cm. 0 ⁰	9 cm. 40 ⁰	28 "	6.3 cm. 0 ⁰	9 cm. 30 ⁰
					54 "	5.5 cm. 0 ⁰	9 cm. 50 ⁰
5	24	M.	9 cm. 20 ⁰	9 cm. 15 ⁰	40 "	6.8 cm. 0 ⁰	9 cm. 40 ⁰
6	25	M.	5.8 cm. 0 ⁰	8.5 cm. 25 ⁰	30 "	4.6 cm. 0 ⁰	8.5 cm. 30 ⁰
					62 "	2.2 cm. 0 ⁰	8.5 cm. 35 ⁰
7	23	M.	4.1 cm. 0 ⁰	9 cm. 10 ⁰	46 "	2.3 cm. 0 ⁰	9 cm. 60 ⁰
8	22	F.	8.5 cm. 15 ⁰	9 cm. 45 ⁰	41 "	6.3 cm. 0 ⁰	9 cm. 55 ⁰
9	19	F.	7.0 cm. 0 ⁰	8.4 cm. 30 ⁰	60 "	5.3 cm. 0 ⁰	8.4 cm. 40 ⁰
10	22	F.	9.4 cm. 0 ⁰	10 cm. 35 ⁰	45 "	8.2 cm. 0 ⁰	10 cm. 40 ⁰
					65 "	6.4 cm. 0 ⁰	10 cm. 40 ⁰
CONTROLS WITH 10% ALCOHOL							
1	20	M.	7.3 cm. 60 ⁰	10.8 cm. 25 ⁰	30 min.	7.2 cm. 0 ⁰	10.9 cm. 30 ⁰
2	26	M.	7.0 cm. 0 ⁰	8.2 cm. 0 ⁰	30 "	7.0 cm. 0 ⁰	8.2 cm. 0 ⁰
					90 "	7.2 cm. 0 ⁰	8.3 cm. 45 ⁰
3	27	F.	7.3 cm. 0 ⁰	9 cm. 20 ⁰	37 "	7.2 cm. 0 ⁰	9 cm. 35 ⁰
					87 "	6.8 cm. 0 ⁰	9 cm. 45 ⁰
4	25	F.	8.5 cm. 15 ⁰	10 cm. 50 ⁰	38 "	8.5 cm. 50 ⁰	10 cm. 55 ⁰
					56 "	8.5 cm. 50 ⁰	10 cm. 40 ⁰
5	22	M.	9 cm. 30 ⁰	10 cm. 20 ⁰	40 "	9 cm. 0 ⁰	10 cm. 25 ⁰
					75 "	9 cm. 15 ⁰	10 cm. 40 ⁰
6	22	F.	7.8 cm. 0 ⁰	10 cm. 20 ⁰	34 "	7.7 cm. 0 ⁰	10 cm. 40 ⁰
					69 "	7.7 cm. 0 ⁰	10 cm. 45 ⁰

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 anterior 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 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°

C O N T R O L F R O G S

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°

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

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

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 muscle in Figs. 2 and 3 and the cat muscle in Figs. 4 and 5 show no significant changes after exposure of the muscle 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 c.c. of Tyrode's solution maintained at 37.5° C. After a suitable control recording had been obtained, pyrethrum solution

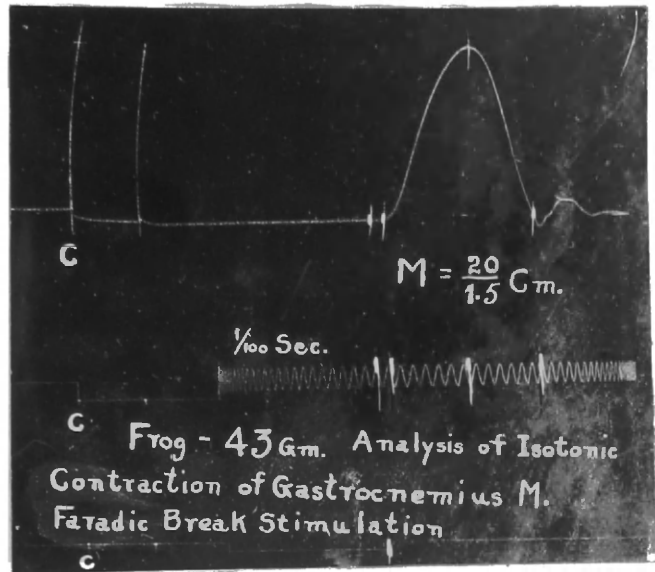


Fig. 2 - Frog gastrocnemius muscle before drug action.

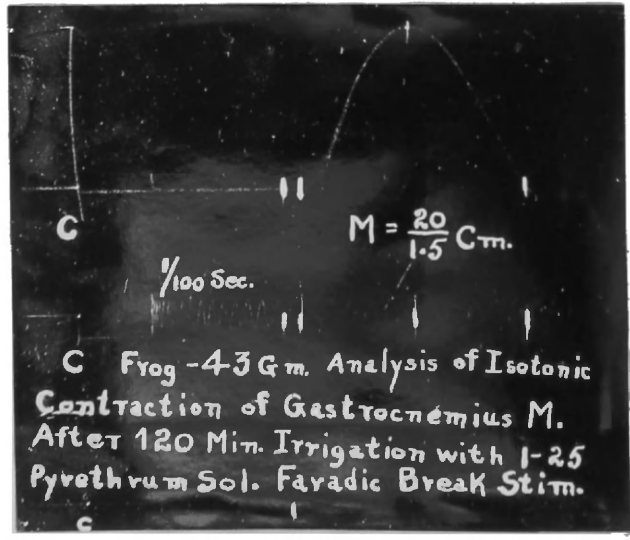


Fig. 3 - Frog gastrocnemius muscle after drug action.

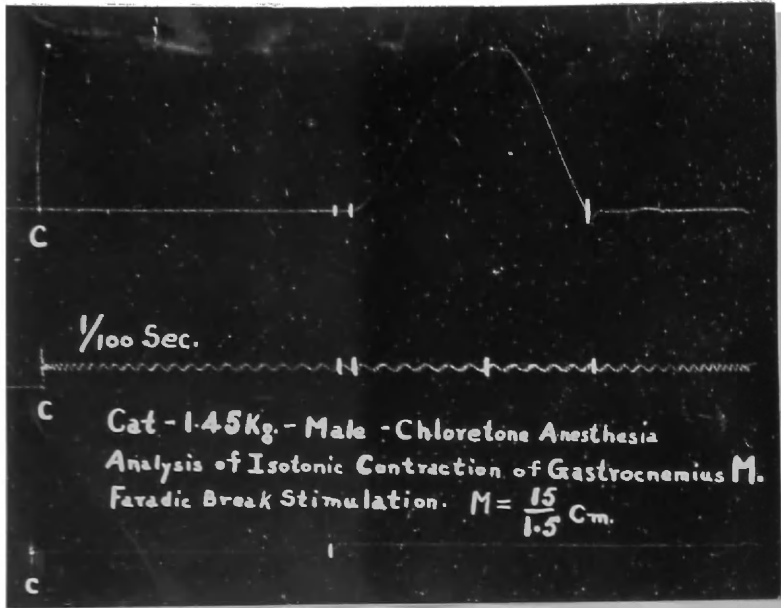


Fig. 4 - Cat gastrocnemius muscle before drug action.

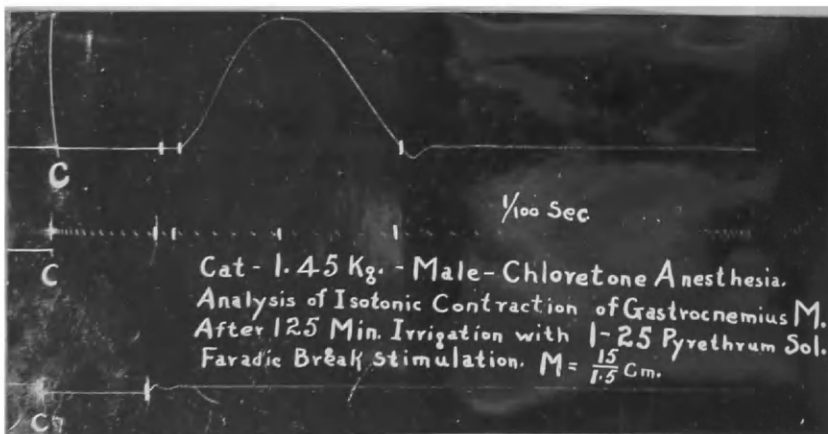


Fig. 5 - Cat gastrocnemius muscle after drug action.

was added and the results noted.

Figure 6 shows that pyrethrum produces 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.

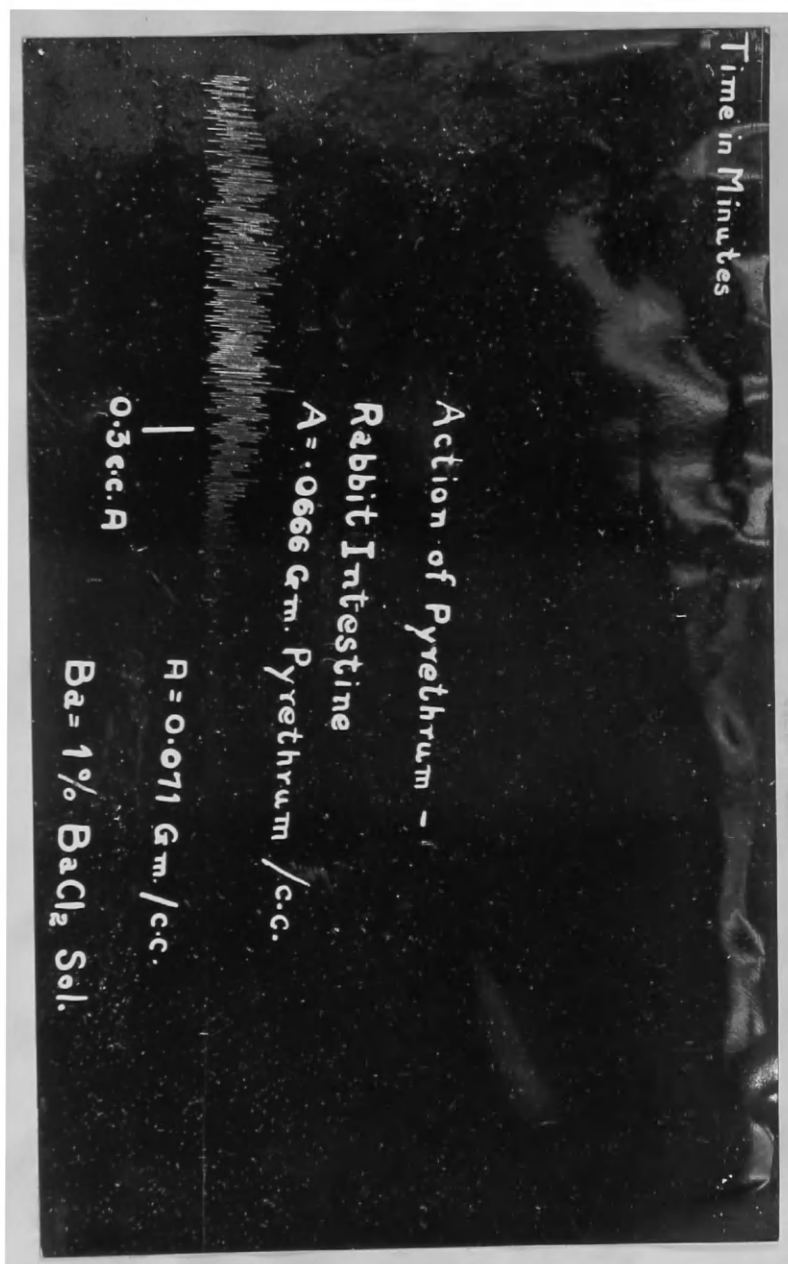


Fig. 6 - Action of Pyrethrum on Rabbit Intestine.

Figure 7 shows that the action of barium which is directly on the muscle is inhibited by pyrethrum, thus indicating that pyrethrum acts directly on intestinal musculature.



Fig. 7 - Pyrethrum Inhibition of Barium on Rabbit Intestine.

Part IV. 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 (21), and Chevalier and Ripert (22) have suggested the use of frogs as a test object, and Perrot and Gaudin (23) and Rigal (24) 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 OVERNIGHT 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 overnight 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 $20^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$, equipped with individual compartments, was used for keeping the frogs overnight 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 grams 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 grams, 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 $20^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$. 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 gram. 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 into 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, Fig. 8,

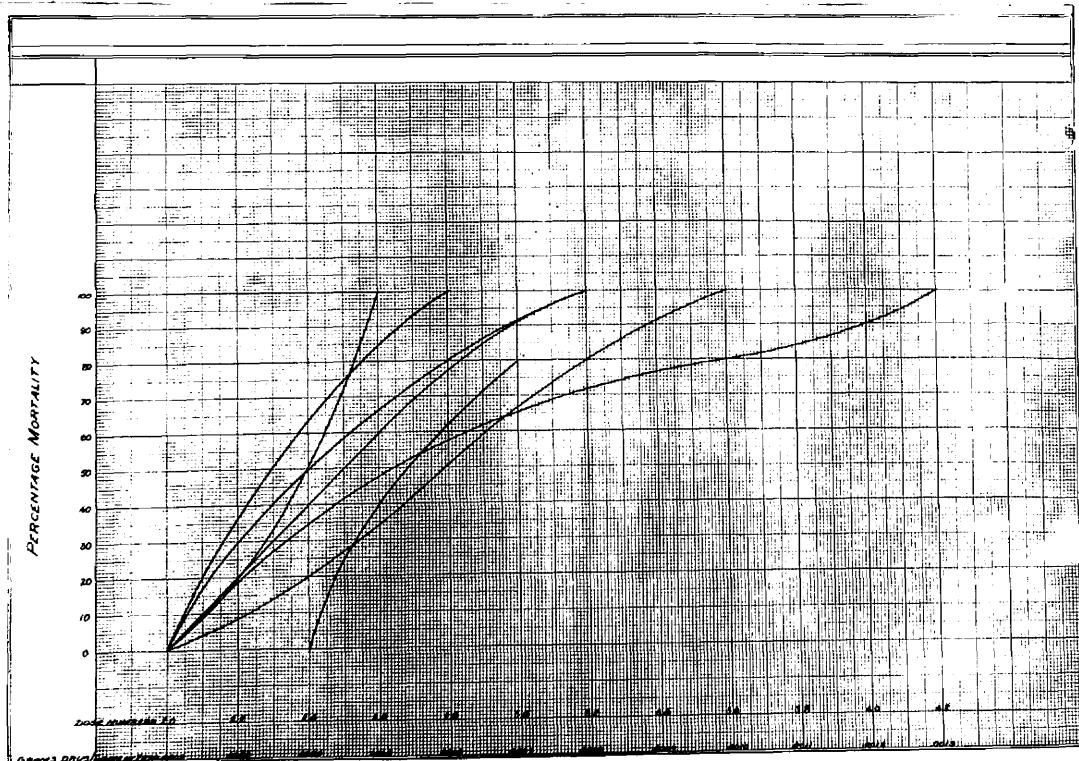


Fig. 8 - Frog mortality curves.

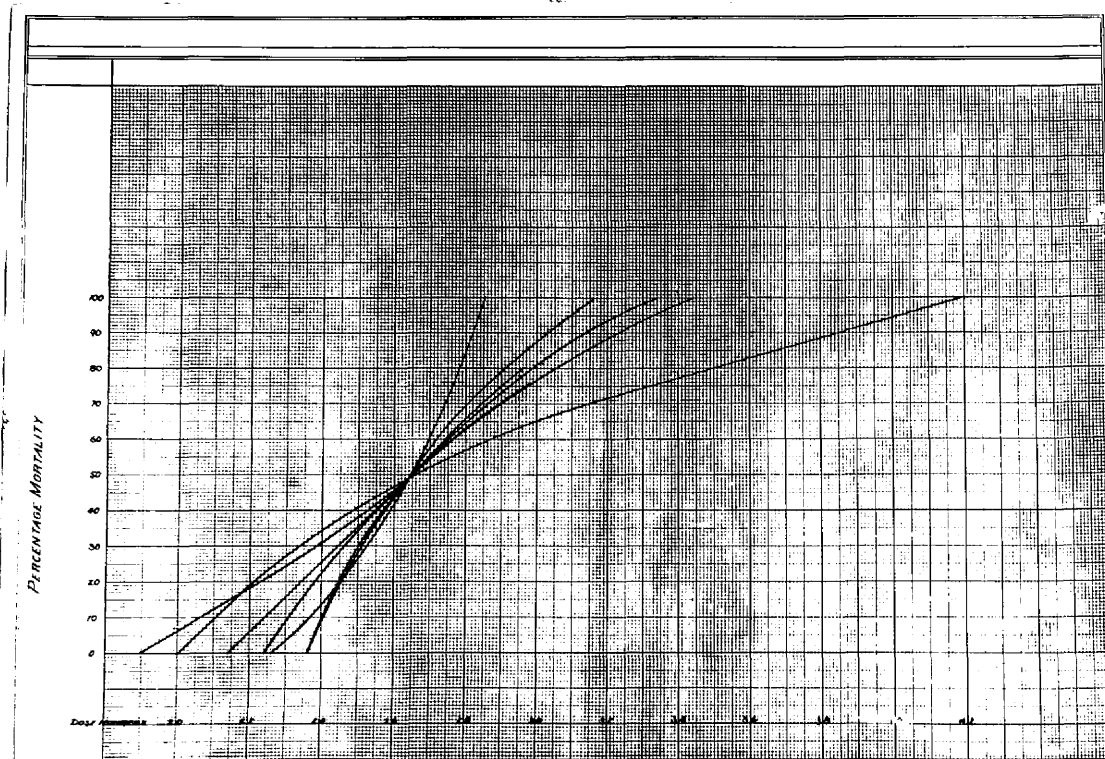


Fig. 9 - Frog mortality curves superimposed on a 50% point.

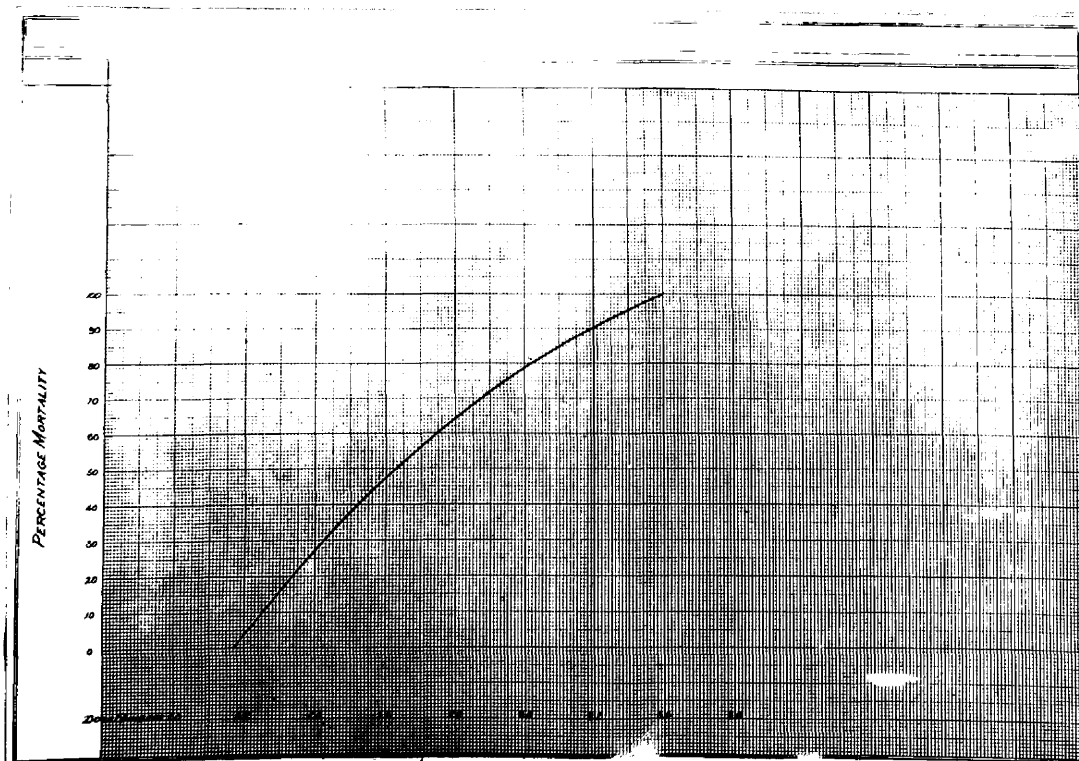


Fig. 10 - Composite of all results.

and all of the curves were then superimposed on a 50% point, Fig. 9. Finally, the various percentage mortalities were averaged and a composite curve of all the individual results was prepared, Fig. 10.

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.

TABLE V. Dose Numbers Corresponding to Percentage Mortality.

Percent Mortality	Dose Number	Percent 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

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 c.c. volumetric flasks. Sufficient alcohol is added to each to make 25 c.c. 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 grams and of one sex are used in the assay. For any one determination, the frogs must all weigh within a range of five grams. 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 c.c. nor more than 0.02 c.c. per gram frog may be injected, no frog receiving a dose of less than 0.25 c.c. 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 $20^{\circ} \text{C} \pm 0.5^{\circ} \text{C}$ temperature. They are dried with a towel, urine expressed, and weighed to within 0.5 grams 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 never has the worker obtained a sample that assayed above this figure. For this reason, in orienting the overnight 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 assayer 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 in view 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, it is assumed that from previous results, it is known that 0.0004 grams per gram 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 gram 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 are 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 grams drug per gram frog.

For the final assay, the average, 0.00036 grams per gram frog, is used as the dose and 25 frogs are injected. Likewise, 25 frogs are in-

jected with 0.00040 grams standard per gram 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

0.0004 grams (S) = 52% mortality = 2.65 dose number

0.00036 gram (X) = 36% mortality = 2.48 dose number

Thus 0.00036 grams (X) multiplied by $\frac{2.65}{2.48}$ = 0.0004 gram (S).

0.000384 gram (X) = 0.0004 gram (S)

1.0 gram (X) = 1.042 gram (S)

(X) = 104% 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, Fig. 6, and that this action is exerted directly on the muscle, Fig. 7, 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 c.c. 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 centimeters 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 centimeter 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 c.c. 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 insures 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 c.c. to 0.5 c.c. 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 solution. After the tissues return to the base 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.

In the interpretation of the results which are obtained, it is readily seen in Fig. 11, the first determination of a typical assay, in which like

doses of A and I were used, that I produced greater inhibition of barium response than A, thus being more than 107% of the potency of A.

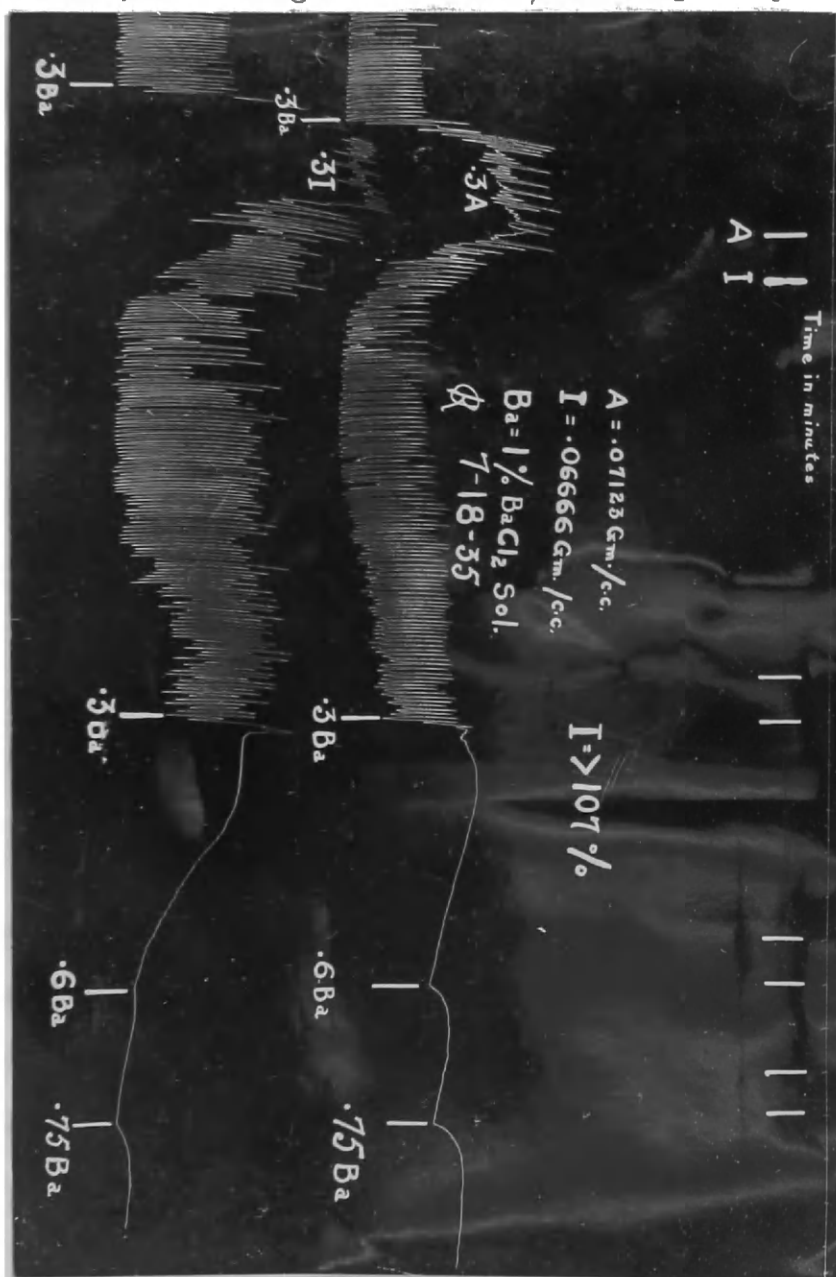


Fig. 11. Assay of I in terms of A.

In Fig. 12, the dose of A was increased to 0.4 c.c. while the original dose of I was retained, and as a result it is apparent that this dose of A is more potent than the dose of I; therefore I is less than 142% of A.

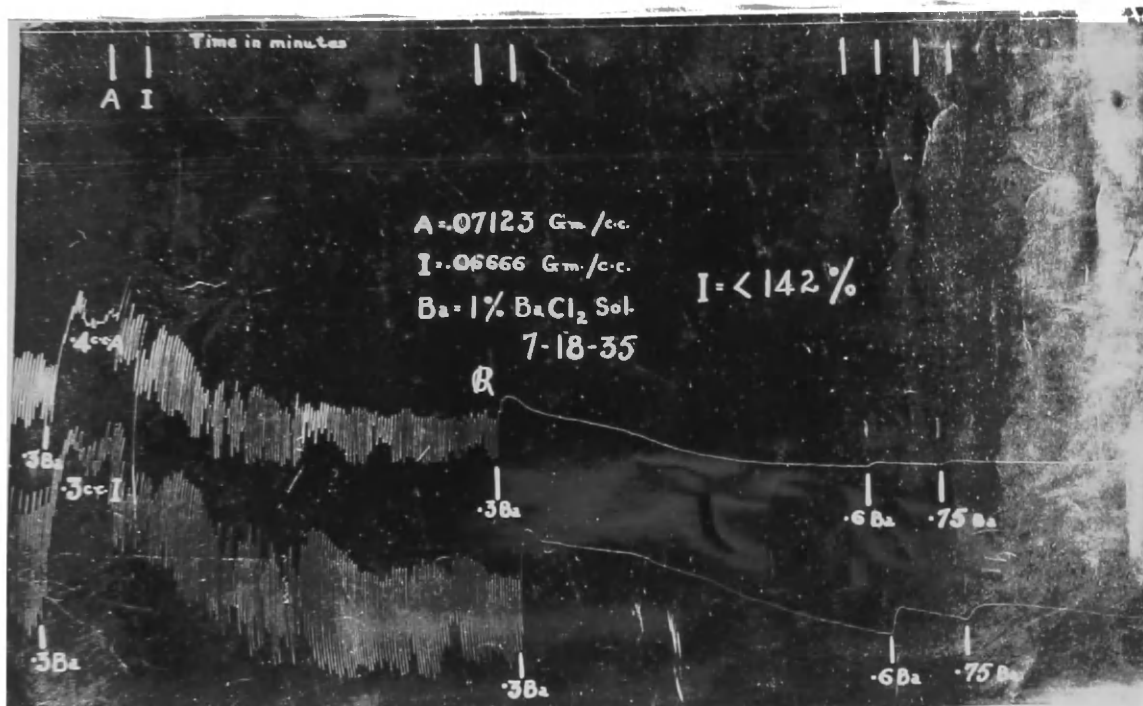


Fig. 12. Assay of I in terms of A.

Fig. 13 shows 0.25 c.c. of I to be more potent than 0.3 c.c. of A. Therefore I is greater 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.

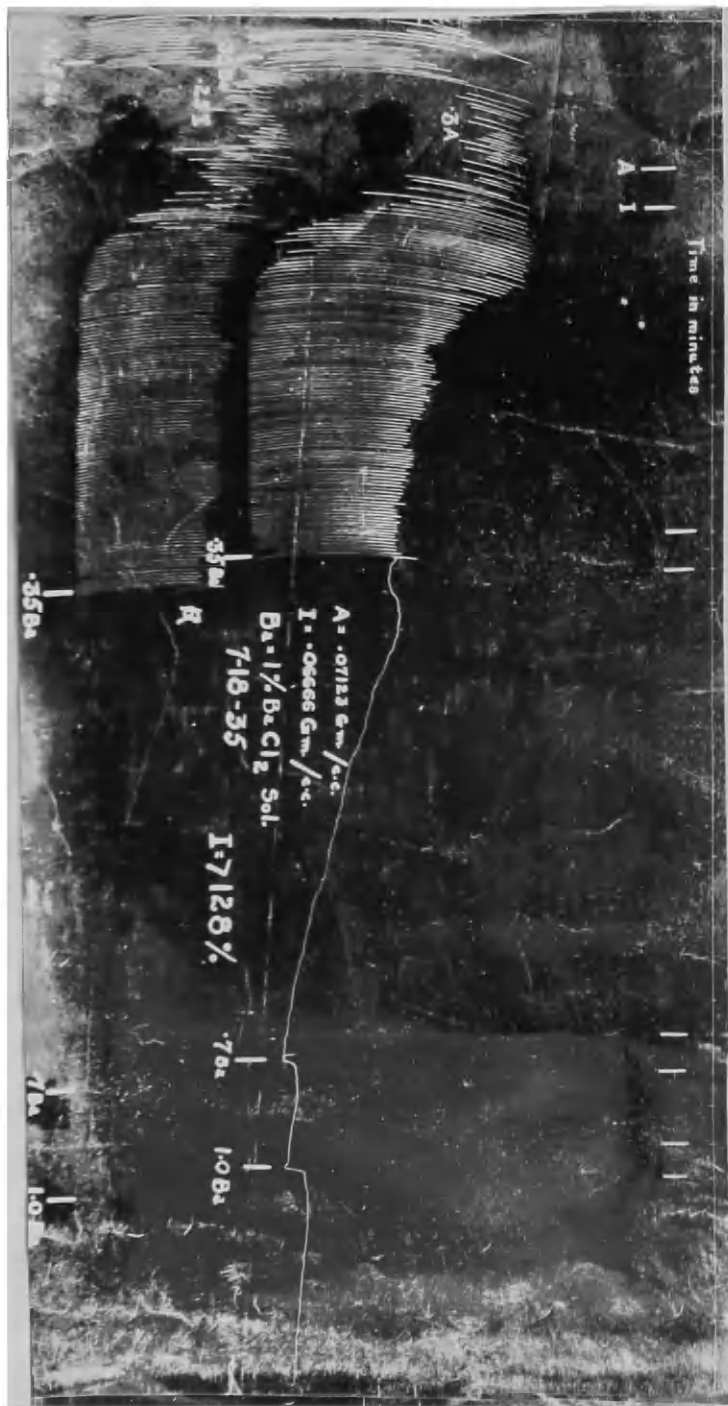


Fig. 13. Assay of I in terms of A.

The dilutions of the stock macerate of the drug must be prepared each day as they lose potency rapidly after dilution. Fig. 14 in which the dilution of A was 24 hours old, shows I to be greater than 160% of A, while Fig. 15, in which a new dilution of A was used, shows I to be much less than 160% as compared to A. Figs. 11, 12 and 13 bear out the fact that I is much less than 160% of A.

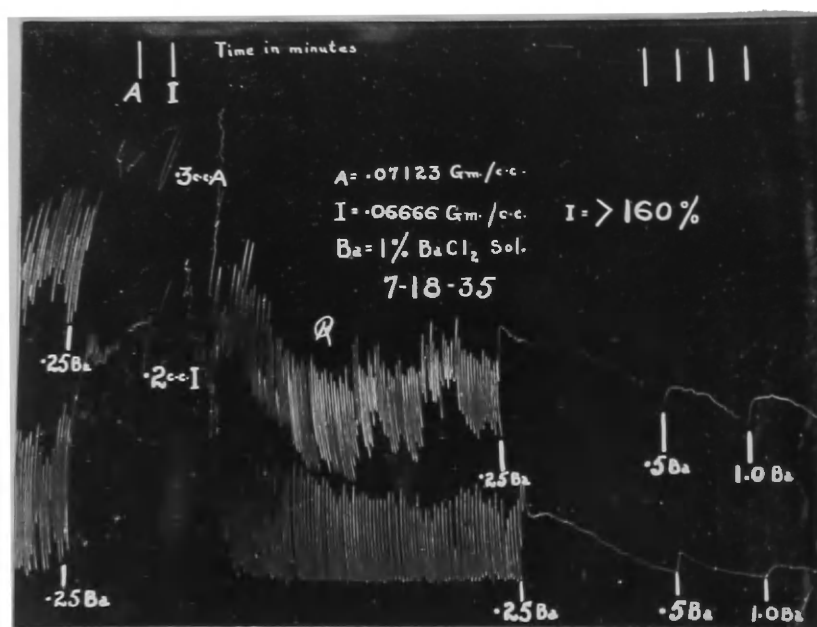


Fig. 14. Assay of I in terms of A using 24 hour old dilution of A.

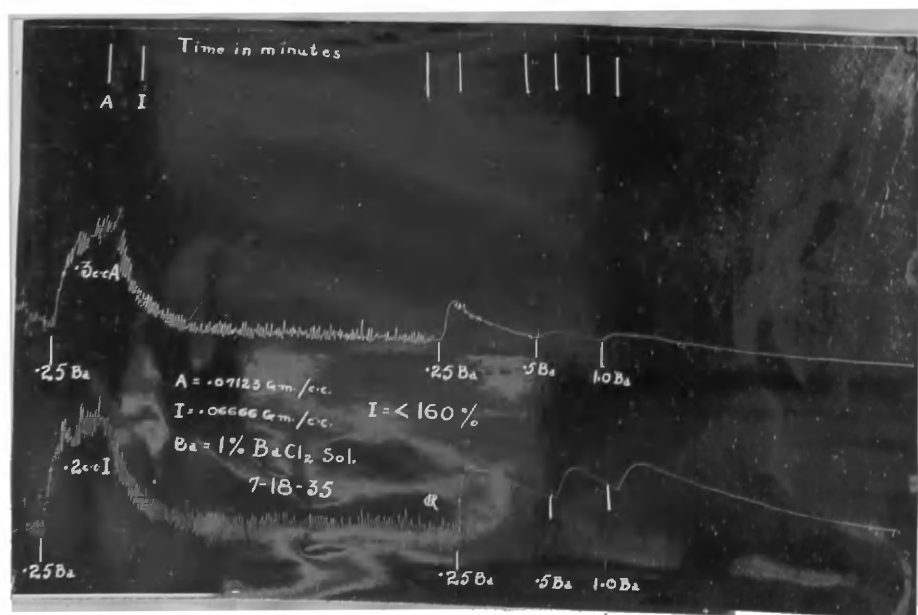


Fig. 15. Assay of I in terms of A using new dilution of A.

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. Fig. 16 shows clearly that, with the great degree of inhibition obtained, it is not possible to assign a potency for F in terms of A. The pyrethrum dosage must be so selected as to provide a definite barium response at the end of the ten-minute period.

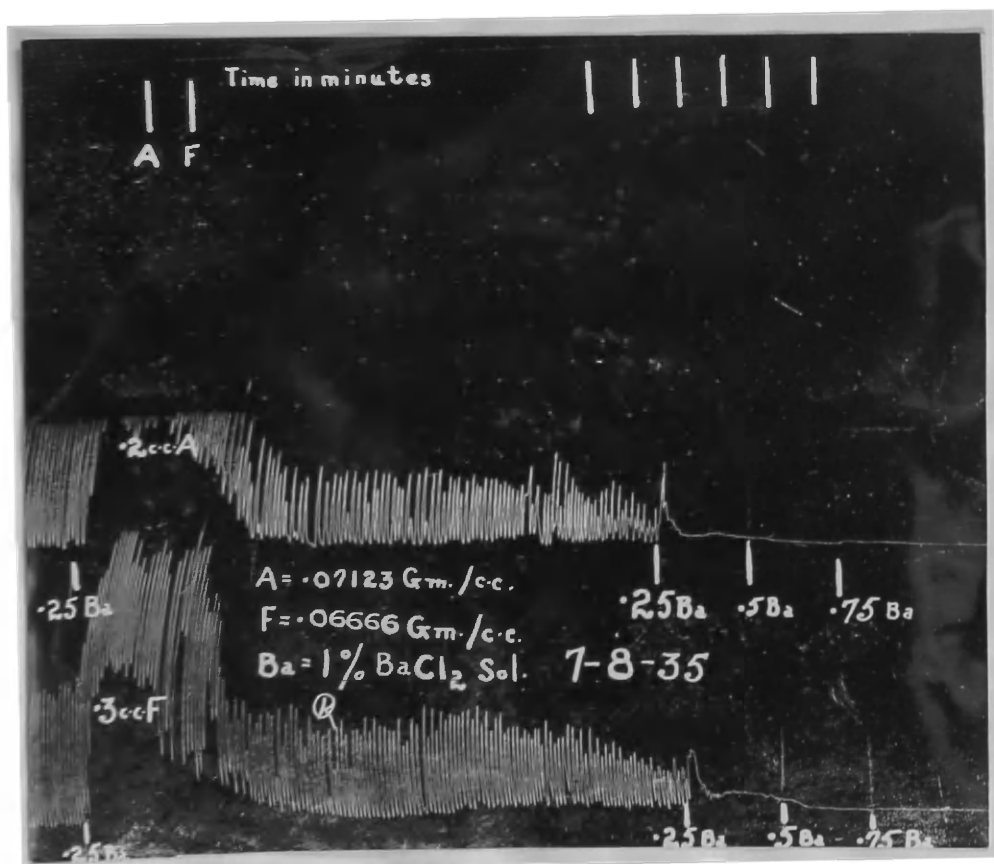


Fig. 16. Assay of F in terms of A.

The advisability of using more than one dose of barium, following the ten minute elapse of time for the production of pyrethrum paralysis, is demonstrated in Fig. 17. The first addition of barium shows 0.35 cc. D to be approximately as strong or possibly slightly weaker than 0.25 cc. A, but the two subsequent doses remove any trace of doubt, showing D to be definitely less potent than A.

This method of assay is accurate, without difficulty, within plus or minus 10%. On various occasions the write obtained an accuracy within plus or minus



Fig. 17. Assay of D in terms of A.

5%, when assaying different dilutions of one preparation, prepared by other members of the laboratory, the writer 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 five grams, 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 (25) and the Peet-Grady fly method (26).

TABLE VI. Results of Assays

Sam- ple	Total Pyre- thrin Content	Pyre- thrin I Content	Pyre- thrin II Content	Fly Kill in 24 hours	Potency in terms of			Potency by	
					Total Pyre- thrins	Pyre- thrin I Content	Pyre- thrin II Content	Frog Meth- od	Intes- tine 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	0.78%	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.

The results in Table VI show 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 (frog, earthworm, and insects) are much more susceptible to the action of pyrethrum than warm blooded animals (rat, cat, and guinea pig).

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

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

6. The principle 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 alteration 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 Overnight 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, and Peet-Grady fly

method, and the two new biological assay methods.

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