

**The standard for the biological assay of strophanthus / by J.H. Burn ;
together with a report on the assay by the frog method / by J.W. Trevan.**

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193

THE STANDARD FOR THE
BIOLOGICAL ASSAY OF
STROPHANTHUS

BY

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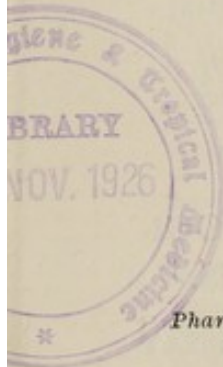
TOGETHER WITH A REPORT ON THE
ASSAY BY THE FROG METHOD

BY

J. W. TREVAN

Wellcome Physiological Research Laboratories.

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THE STANDARD FOR THE BIOLOGICAL ASSAY OF STROPHANTHUS.*

TOGETHER WITH A REPORT ON THE ASSAY BY THE FROG METHOD.

The Second International Conference on the Biological Standardisation of certain Remedies which met at Geneva in 1925 referred in its report¹ to the assay of *Strophanthus* in the following words: ". . . *strophanthus* tinctures can be assayed by corresponding methods, using as a standard for *strophanthus* tinctures *G. Strophanthin* (ouabain) . . ." The recommendation in this simple form suggests three points of inquiry:—

(1) To what strength, in terms of ouabain, should tinctures of *strophanthus* conform if they are to be considered satisfactory?

(2) Is the assay of a given tincture in terms of ouabain the same when the determination is made by (a) the cat method, (b) the frog method?

(3) Is the activity of all samples of ouabain the same? The work described attempts to give an answer to these three questions.

I.

In the United States Pharmacopœia, Xth Revision, it is laid down that "Tincture of *Strophanthus* injected into the ventral lymph sac of a frog has a minimum systolic dose of not less than 0.00,0055 C.c. and not more than 0.00,0065 C.c., equivalent to not less than 0.00,000,046 Gm., and not more than 0.00,000,054 Gm. of ouabain for each Gm. of body weight of frog." Thus the U.S.P. lays down not only the strength in terms of ouabain, but also the method by which tinctures of *strophanthus* are to be assayed.

The strength indicated may be expressed more simply, if no reference to the method is made, by saying that the U.S.P. requires that 1 C.c. of a tincture of *strophanthus* shall contain the activity of 8.33 milligrams of ouabain.

To confirm the expectation that this would be a reasonable standard to apply, six samples of tincture of *strophanthus* were obtained from different sources, and assayed by the cat method in terms of ouabain.

A tincture in accordance with the requirements of the British Pharmacopœia was prepared from *Kombé* seeds under the direction of Mr. Frank Browne, F.I.C., Ph.C., who reported that the seeds used gave a characteristic green colour with the sulphuric acid test, and, when dry, yielded to ether 31.9 per cent. of extractive (mostly oil). The other five

* Report to the Chairman of the International Conference on the Biological Assay of Certain Remedies.

tinctures were bought from different manufacturing houses in Great Britain.

Method of Assay.

The cat method was used according to the technique of de Lind van Wijngaarden,² with the exception that the blood pressure was recorded throughout each experiment, and the moment of the fall of the pressure to zero was taken as end point. The tincture was administered to the cat after being diluted 900 times.

Results of Assay.

In the following table, each figure represents the lethal dose for one cat in cubic centimetres of tincture (diluted 900 times) per Kgm. body weight of cat. The average given is of all the determinations made, and no cats have been excluded. It was seen from the blood pressure record, as well as from post-mortem examination of the heart, that the death in all cases was due to the action of the strophanthus on the heart.

TABLE I.

Tincture					
A	B	C	D	E	F
13.92	21.82	12.92	14.65	14.83	21.20
14.21	16.65	17.56	15.10	15.79	16.21
15.38	18.00	12.73	16.10	15.18	19.80
12.95	20.40	13.42	12.90	—	—
11.16	15.34	17.77	14.30	—	—
17.01	13.25	11.28	—	—	—
21.04	19.77	—	—	—	—
Average					
15.09	17.89	14.28	14.61	15.26	19.07

The lethal dose of a sample of ouabain kindly sent by Dr. E. W. Schwartz from the Bureau of Chemistry, U.S. Department of Agriculture, determined in the same way, was found to be 0.06115 Mgm. per Kgm. (The details of this estimation appear below.) Hence 15.09/900 C.c. of tincture A are equivalent to 0.06115 Mgm. ouabain, or 1 C.c. tincture A. = 3.64 Mgms. ouabain. In Table II. are the ouabain equivalents of the six tinctures.

TABLE II.

- 1 C.c. Tincture A. = 3.64 Mgms. ouabain.
- 1 C.c. Tincture B. = 3.07 Mgms. ouabain.
- 1 C.c. Tincture C. = 3.85 Mgms. ouabain.
- 1 C.c. Tincture D. = 3.76 Mgms. ouabain.
- 1 C.c. Tincture E. = 3.60 Mgms. ouabain.
- 1 C.c. Tincture F. = 2.88 Mgms. ouabain.

None of the figures in Table II. differ greatly; those for tinctures A, C, D, and E are so close together that these tinctures may be regarded as of the same strength. The value of tincture F is calculated from observations on only three cats, and the probable error of the estimation is accordingly greater. The standard deviation of the ouabain equivalent for A (3.64) is 0.271, and that for the ouabain equivalent for B (3.07) is 0.216. From this it may be calcu-

lated that the chance of tinctures A and B being actually different is 12.4 to 1. (I am indebted to Dr. Trevan for this calculation.) It is of some interest to point out that while the mean of the figures in Table II. is 3.46, the mean obtained by treating the individual cat values in Table I. as though they had been obtained with one tincture is also 3.46. On the other hand, the U.S.P. X. demands a minimum ouabain equivalent of 8.33 Mgm. per C.c. It will be evident that, on any consideration, the figures obtained represent an equivalent substantially less than one-half of this demand.

II.

The discrepancy between the values obtained for a series of representative tinctures and the U.S.P. figure, at once suggested that a different result was obtained by the cat method than would be obtained by the frog method demanded by the U.S.P. The two tinctures A and B were accordingly sent to Dr. J. W. Trevan, of the Wellcome Physiological Research Laboratories, who agreed to assay them by the frog method. No information was given to Dr. Trevan of the results by the cat method.

Dr. Trevan's Report.

Method.—All doses were injected into the dorsal lymph sac of frogs kept at room temperature in bright light. The doses were proportional to the body weight of the frogs injected, and were all made up to 0.5 C.c. with 0.6 per cent. saline before injection. Each group of frogs injected was accompanied by the group injected with the standard ouabain supplied by the United States Department of Agriculture. The twelve hours' mortality was noted in each group, and the relative potency indicated by the respective mortalities read off from the standard curve (see Fig. 1), which was

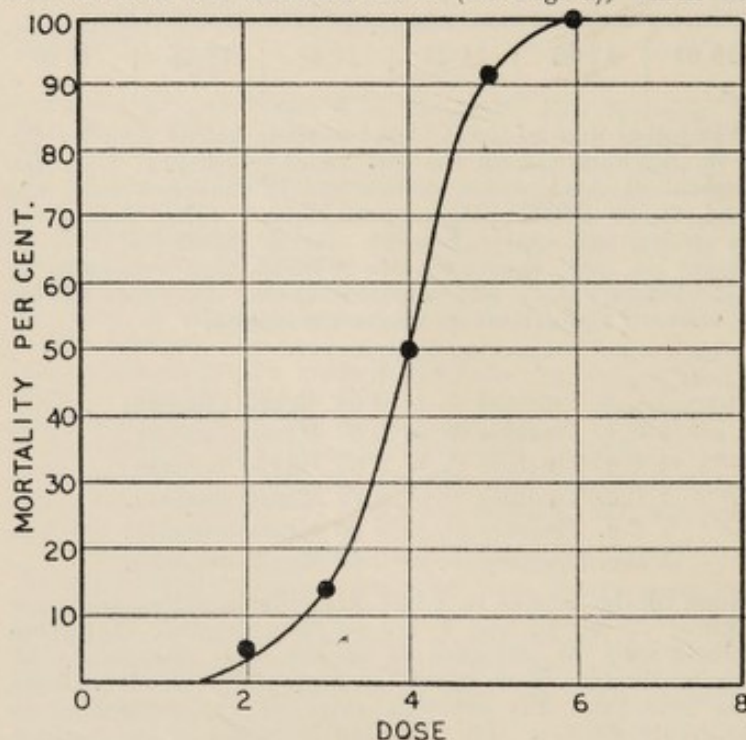


Fig. 1.—Standard curve showing relation between mortality and dose for frogs injected with strophanthus.

prepared some time ago by the injection of some hundreds of frogs with a strophanthus tincture. The shape of the curve is the same for digitalis and squill; and, like the insulin convulsion curve for mice, although the mortality for a given dose varies on different days, the variation is proportionately the same for all doses.

The results are given in Table III. The figures in the last column are obtained in the following manner. Tincture of strophanthus A gave a mortality of eleven out of twenty frogs (55 per cent.) with a dose of 0.014 C.c. on the first occasion on which it was tested. The ouabain at the same time gave a mortality of 3/20 (15 per cent.) for a dose of 0.000045 Gm. Reading from the curve, 55 per cent. mortality corresponds to a dose of 4.05 and 15 per cent. mortality to a dose of 3.0. 0.014 C.c. of A is therefore $4.05/3.0$ times as potent as 0.000045 Gm. of ouabain.

$$\therefore 1 \text{ C.c. A} = \frac{4.05 \times 0.000045}{3.0 \times 0.014} \text{ Gms. ouabain.}$$

The expected standard deviation is about 6 per cent., using 40 frogs in each group, and the average results are, in equivalents of ouabain per C.c.,

Tincture of Strophanthus A 0.00375 Gm.
Tincture of Strophanthus B 0.0029 Gm.

TABLE III.

Tincture.	Dose per 100 Gms. frog.	Mortality.	Standard Ouabain Dose per 100 Gms.	Mortality Ouabain.	Equivalent in Gms. of Ouabain for 1 C.c. of tincture.
Stroph A.	C.c.				
	0.014	11/20	0.000045	3/20	0.0043
	0.014	3/20	0.000055	8/20	0.0032
Stroph B.	0.018	7/20	0.000055	8/20	0.0029
	0.018	10/20	0.000055	12/20	0.0029

(End of Report.)

In Table IV. the results by the cat method and frog method appear side by side.

TABLE IV.

—	Cat.	Frog.
Tinc- ture A	1 C.c. = $3.64 \pm 0.271^*$ Mgms.	1 C.c. = $3.75 \pm 0.238^*$ Mgms.
Tinc- ture B	1 C.c. = $3.07 \pm 0.216^*$ Mgms.	1 C.c. = $2.90 \pm 0.184^*$ Mgms.

* Standard deviation.

It will be seen that the agreement between the two methods is extremely close, and is, in fact, more complete than can

be expected as a rule. Since the frog method gives a difference between tinctures A and B which is not only in the same direction but also of the same magnitude as that indicated by the cat method, the probability that tinctures A and B are actually different is considerably increased. As was mentioned above, judging from the cat figures only, the probability that A is stronger than B is only 12.4 to 1; Dr. Trevan points out, however, that since the frog method records the same difference, this probability is increased to 89 to 1. Even taking the one chance in 89 that the tinctures are identical, in which case the mean of the two cat figures and of the two frog figures may be taken, the assay by the cat method is 3.35 Mgms. per C.c., and by the frog method 3.325 Mgms. per C.c.

Hence the evidence shows beyond question that the cat method, when used for the assay of strophanthus in terms of ouabain, measures precisely those properties of the strophanthus and of the ouabain which are measured by the frog method.

III.

The sample of ouabain supplied by the United States Department of Agriculture differed in appearance from a sample of Merck's ouabain which Mr. T. Maben of Messrs. Parke Davis and Co. was good enough to give me. I determined the lethal dose of the two samples by the cat method. The ouabain was used in a solution containing 1 Mgm. in 200 C.c. The figures were as follows:—

<i>U.S.P. Ouabain.</i>	<i>Merck's Ouabain.</i>
0.0523 Mgm. per K.....	0.0985 Mgm. per K.
0.0991 " " "	0.0626 " " "
0.0476 " " "	0.0840 " " "
0.0651 " " "	0.0866 " " "
0.0668 " " "	0.1006 " " "
0.0576 " " "	0.0793 " " "
0.0493 " " "	0.0742 " " "
0.0458 " " "	0.0805 " " "
0.0669 " " "	0.0703 " " "
Average 0.06115 Mgm. per K.	0.0818 Mgm. per K.

The standard deviation of the mean 0.06115 is 0.00517, and of the mean 0.0818 is 0.00389, so that the difference between the two means is significant. The usual figure for the lethal dose for cats for ouabain Merck is 0.1 Mgm. per Kgm. The figure obtained here is lower, and the difference, I believe, is in part due to the fact that if the end point of each determination be taken at the moment of the fall of blood pressure to zero, a higher toxicity is recorded than if the end point is taken as suggested in the Report of the Geneva Conference. The absolute value of the toxicity is, of course, immaterial; it is of importance only in relation to the toxicity of the sample of U.S.P. ouabain.

In U.S.P. X. the specific rotation at 20° C. of anhydrous ouabain is given as -31.3° to -31.9°. The ouabain adopted as standard is stated to contain 12½ per cent. water. Consequently the specific rotation of the sample with which I was supplied by the U.S. Department of Agriculture would be -27.6°.

Dr. W. H. Linnell very kindly undertook to determine the specific rotation of the sample of ouabain Merck. He found this to be $[\alpha]_D^{20} = -22.6^\circ$. I am indebted to him for the following note: "The chemical characteristics of ouabain are such that when crystallised under varying conditions dif-

ferent amounts of water become associated with the molecule. For instance, on crystallisation at the ordinary temperature, $C_{30}H_{46}O_{12} \cdot 9H_2O$ is obtained, whereas if this operation be performed at 30° and $60^\circ C$, four and three molecules respectively of water become associated. Hence the specific rotation of any specimen of crystallised ouabain will vary inversely with the percentage of water present. The biological tests indicate that the Merck's sample is only 75 per cent. as active as that of the U.S.P. sample, and it is interesting to note that the specific rotations of the two samples are as 81.6 (Merck) is to 100 (U.S.P.). It is therefore logical to state that the different behaviour of the two specimens under test is due to varying amounts of water associated with the ouabain molecule."

Hence in prescribing ouabain as an international standard, it will be necessary at least to define the percentage of water in the sample adopted.

Discussion.

The surprising result that each of six tinctures of strophanthus obtained from different sources proved to have less than half the activity in terms of ouabain which is required by the United States Pharmacopœia, needs some consideration. In both countries the pharmacopœial tinctures are 10 per cent., but while the British Pharmacopœia restricts the seed to *Strophanthus Kombé*, the United States Pharmacopœia allows the use of *Hispidus* seeds in addition. Dr. Trevan states that in his experience the U.S.P. standard for strophanthus is impossibly high. He has tested tinctures from a good many sources, including some manufactured in the United States, and has never found one obtainable on the market which comes up to that standard, excepting, of course, tinctures that are marketed as concentrated. He states that a standard of 4.2 Mgms. of ouabain (for 1 C.c. strophanthus) is as much as can be expected with any regularity from good samples of strophanthus, and probably this is too high. Houghton³ also gives some evidence on the strength of strophanthus seed obtainable in America. In advocating the use of crystalline *Strophanthin Kombé* as a standard for tinctures of strophanthus, he stated in 1912 "0.000001 Gm. of this strophanthin is considered as the normal minimum fatal dose per Gm. body weight of frog, when properly injected into the abdominal lymph sac, and corresponds in physiological activity to 0.000075 C.c. of standard (U.S.P. 1900) tincture of strophanthus, which represents the average activity of first-class commercial strophanthus, *Kombé*, seeds as they appear on the American market." In the same paper he gives the minimum fatal dose per Gm. of frog of crystalline *Gratus Strophanthin* (Ouabain, Merck) as 0.00000048 Gm. From this it may be calculated that 1 C.c. tincture of strophanthus contains the activity of 6.4 Mgm. ouabain Merck. The strongest of the tinctures (C) described in this paper gives the value 1 C.c. = 5.15 Mgm. in terms of the sample of ouabain Merck which I used. The similarity between this figure and that calculated from Houghton's data suggests that the seed obtainable in Great Britain is not greatly inferior to that found in the United States, and the reason for the high potency demanded by U.S.P. X. remains unexplained.

The average activity of the six tinctures described in this paper is, in terms of the ouabain supplied by the United States Department of Agriculture, 1 C.c. = 3.46 Mgms. I propose to adopt as a standard for these laboratories, until

an official recommendation is made, the value that 1 C.c. = 3.5 Mgms. of U.S.P. ouabain, and to consider tinctures not differing from this value by more than 25 per cent. as satisfactory.

Summary.

(1) Six tinctures of *strophanthus* B.P., obtained from different sources, when assayed by the cat method in terms of ouabain supplied by the U.S. Department of Agriculture, gave the average value 1 C.c. = 3.46 Mgms.; the lowest was 2.88 Mgms., and the highest 3.85. All were less than half the potency demanded by U.S.P.X. (1 C.c. = 8.33 Mgms.).

(2) Two tinctures, independently assayed by the frog method, gave values almost identical with those obtained by the cat method.

(3) A sample of ouabain Merck was found to have only 75 per cent. of the toxicity of the ouabain supplied by the U.S. Department of Agriculture, and the specific rotation of the Merck sample was found to be correspondingly less than that given for U.S.P. ouabain.

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