The ash contained a notable quantity of manganese which was suspected because of the green color. The identification was confirmed by the usual qualitative procedure.

By distillation of the bark with steam, there was obtained a white, aromatic solid which was not acid and was soluble in ether. The odor was somewhat like borneol but no oxime or semicarbazone could be obtained from the oxidized product. The original substance melted under 60° C. but this was very indefinite. It was apparently a paraffin hydrocarbon mixed with some odorous substance. The amount obtained was too small to admit of a more careful examination.

SUMMARY.

A partial analysis was made of the bark of American larch and the results were tabulated. It contained starch, pentosans, tannins of the catechol type, saponins and 10 per cent of hard, brittle resin, partly acid, but no alkaloid. By steam distillation there was obtained a small amount of white solid which may be paraffin.

THE UNITED STATES PHARMACOPŒIAL STANDARD DIGITALIS POWDER.*

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One of the points of difficulty which has been encountered in connection with the biological standardization of drugs has been the necessity of supplying some standard preparation of uniform potency against which the strength of the unknown might be measured and in terms of which its value could be expressed. While this is true for all the drugs which are subjected to this form of assay, it is of special importance in connection with the members of the digitalis series on account of their wide-spread use in circulatory disorders and the well-recognized variability of the crude drugs. At the same time digitalis itself offers special difficulties, as it contains no active principle which is suitable for use as a standard. Accordingly, in the U. S. P. IX, which was issued in 1916, and the first pharmacopœia in which biological standardization was recognized officially, crystalline g-strophanthin (ouabain) was adopted as the standard for members of the digitalis group of drugs. This glucoside was selected for the reason that pharmacologically it belongs to the digitalis series and chemically it is easily identified by its physical characteristics such as solubilities, melting point, etc. For the next twenty years, therefore, ouabain served as the standard in the United States for digitalis and its allies and, on the whole, it proved quite satisfactory. However, at the Edinburgh conference held in 1924 under the auspices of the Health Committee of the League of Nations, it was pointed out that, while ouabain was satisfactory as a standard for preparations of strophanthus, it was not suitable as a standard for digitalis for international usage. This was due to the different species of frogs which are in use in European countries and to the different methods of assay which are employed, factors which make it practically impossible to get comparable relationships between ouabain and digitalis in assays carried on in the different countries.

Accordingly, at this conference it was decided to investigate the possibility of

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using a powder of digitalis as a standard for that drug, and Professor Magnus of Utrecht undertook its preparation, using the Hatcher-Brody cat method of assay as it had been modified at the University of Utrecht. The Edinburgh experimental powder, when injected in the form of an infusion, proved fatal to cats in a dose of 91.1 mg. per Kg. of body weight of cat. Since a cat unit had been defined as the amount of drug which will kill one kilo of cat, this powder contained 10.9 cat units in each gram of powder. As a result of this work, the Geneva conference held the next year, adopted as an International standard a powder of digitalis which should be prepared by Professor Magnus and be within ten per cent of the potency of the Edinburgh powder. Such a powder was secured by mixing ten different lots of powder obtained from various sources and when these were finally blended and assayed on twenty-three cats, the fatal dose was 89.7 mg. per Kg. of cat. Each gram therefore contained 11.1 cat units. This powder sealed in ampuls, containing about three Gm. each, serves to-day as an International standard. An International Digitalis unit is defined as being the equivalent in potency of 0.1 Gm. of the International standard powder and the U. S. P. Digitalis unit is identical in potency with the International unit. The International standard powder is stored in London and is furnished to the different countries to be used as a standard, and in turn each country prepares its own standard powder which is assayed and its potency determined in terms of the International Powder.

One of the first countries to adopt this standard and to prepare its own powder was Great Britain. The standard powder prepared for that country in 1928 and adopted by the British Pharmacopœia of 1932 was made by the careful drying and thorough mixing of nine different samples of dried leaves. It was assayed in terms of the International Powder both by the frog lethal dose method and by the cat method. In comparison with the International standard it possesses a potency of 138 per cent when assayed on frogs and judged by this method it contains 13.8 digitalis units per gram. On the other hand when assayed on cats it has a potency of 116 per cent, each Gm. therefore containing 11.6 digitalis units. According to these figures one International digitalis unit is contained in 0.0725 Gm. of the British powder when the assay is carried out by the frog lethal dose method and in 0.0862 Gm. when it is assayed on cats. When other methods of assay are used it is suggested that the intermediate figure of 0.079 Gm. be taken as representing one "unit."

The Canadian standard powder (No. 428) of 1928 also possesses a potency greater than the International in that 0.85 Gm. of the former is equivalent to 1 Gm. of the latter. The lethal dose (twelve to eighteen hours) method using frogs (*R. pipiens*) was employed in assaying this powder.

In the United States the new Pharmacopœia (XI), which became official on June 1, 1936, adopted a powder of digitalis as a standard for digitalis in place of ouabain, it being understood that its potency was to be determined in terms of the International standard. Such a powder was secured by blending three separate powders all of which had been carefully selected and dried. Two-thirds of the amount was from an American source, while one-sixth was secured from England and one-sixth from Germany. In the study of this composite specimen it was decided not only to determine its potency in terms of the International Powder but also in relation to the British and the Canadian standards. Such a study would not only serve as a check upon the accuracy of the methods employed but in addition might furnish valuable information upon the relative strengths of the different standards.

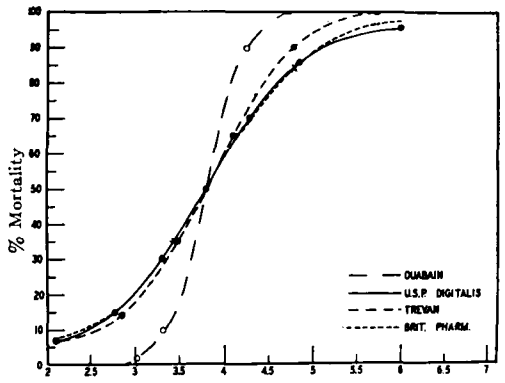

Fig. 1.

Fig. 1.—Characteristic mortality curves for frogs injected with digitalis or strophanthus—injection being made into the lymph sac. For the U. S. P. digitalis curve *Rana pipiens* was employed and the one-hour period of observation. For the ouabain curve (Chapman and Morrell) *R. pipiens* and the lethal dose method were used and for the Trevan digitalis curve *R. temporaria* and the lethal dose method. The two crosses on the U. S. P. curve indicate the mortality obtained when *R. pipiens* were used with the lethal dose method.

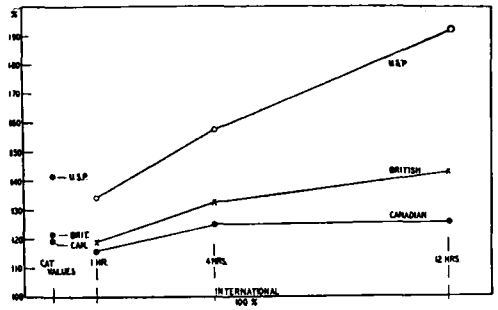

Fig. 2.

Fig. 2.—Potency of the three national standard powders of digitalis in relation to the International standard powder as determined on frogs using the one-, four- and twelve-hour period of observation. The values obtained by the cat method are indicated at the left of the curves and show how closely the cat and one-hour frog results agree.

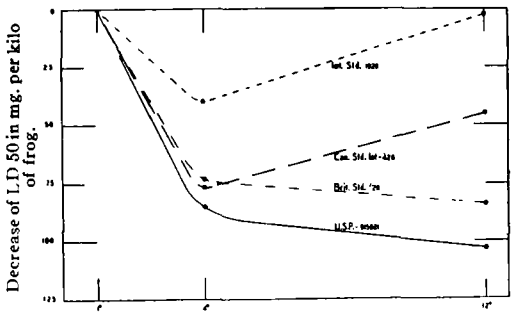

Fig. 3.

Fig. 3.—Curves of the four standard digitalis powders drawn on basis of decrease in M. L. D. 50, the one-hour value being taken as zero. The curves show the great decrease in dosage for the four-hour period observation with two powders still showing decreased dosage in the twelve-hour period while the Canadian and International Powders require large doses. Each of the two pairs of curves take a parallel course in the 4–12-hour period but in opposite directions.

	L. D. per kilo of frog.			
	U. S. P.	Brit.	Can.	Int.
One hour	391	446	470	524
Four hours	306	372	394	484
Twelve hours	288	362	425	522

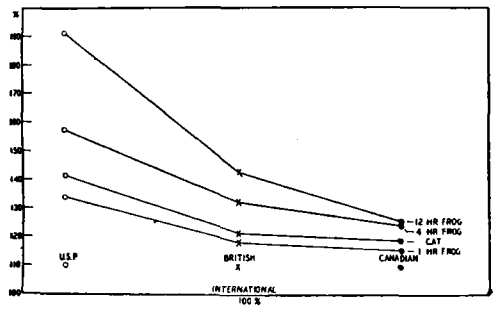

Fig. 4.

Fig. 4.—Curves showing the potency of the three National Standard digitalis powders in relation to the International Powder as determined by the cat and frog methods of assay. A considerable degree of parallelism is shown and a close approximation of results especially between the cat and one-hour frog results.

THE ASSAYS.

The assays on frogs were carried out by the official U. S. P. one-hour method; by the four-hour method as recommended by the Health Committee of the League of Nations; and by the twelve-hour period of observation (lethal dose method). The latter method was studied as it had been employed in the assay of the British and the Canadian standards and had also been used in this country by certain workers since the earliest days of biological standardization. In addition to the various modifications of the frog methods which were used, the cat method was also employed as will be described later.

For the assays on frogs the various powders were injected as tinctures. These were prepared by adding 10 cc. of 80 per cent alcohol to each gram of drug in a glass-stoppered flask and allowing the same to stand with frequent shaking at room temperature for twenty-four hours as directed in the Pharmacopœia. Before injection the alcohol content was reduced to 20 per cent by evaporation over a water-bath at a temperature not exceeding 50° C., the initial volume being restored by adding distilled water. The undiluted tinctures, when not in use, were kept in a refrigerator and no two tinctures were compared which differed in age from each other by more than a week.

In carrying out the assays upon frogs the statistical method of Trevan was employed. For this purpose it was necessary to construct a standard curve, inasmuch as the curve used by Trevan is based upon the lethal dose method using *Rana temporaria*. The curve employed by the Canadian workers, Chapman and Morrell, is also based upon a similar period of observation but uses ouabain as a standard and employs *Rana pipiens*. Accordingly, a curve was constructed in this laboratory using *Rana pipiens* and the official one-hour U. S. P. method and the new U. S. P. standard powder. The curve was constructed in a manner similar to that employed by Chapman and Morrell using 570 frogs for the purpose. The range of variation in doses of digitalis between those killing some frogs (1–5%) and those killing 100 per cent was shown by this curve to be in the ratio of about 1 to 5.00. Trevan found by the lethal dose method on *Rana temporaria* a ratio of 1 to 4.85, practically the same ratio. On the other hand, the ratio of variation in killing dosage of ouabain was found by Chapman and Morrell to be as 1 is to 1.60. In Fig. 1 (p. 292) the Trevan curve is shown superimposed upon the U. S. P. curve and it is seen that the two curves are practically identical. The Chapman and Morrell curve, also shown in Fig. 1, is much steeper than the other curves so that in its middle portion for each increasing increment in dosage the mortality percentage rises very rapidly. In order to make a further comparison possible between the U. S. P. curve and the Trevan curve, with the idea of checking the effect of using different varieties of frogs, we located the principal points for another curve using the lethal dose method with *Rana pipiens*. These points, also shown in Fig. 1, were gained by using 180 frogs, and it is seen that they coincide almost exactly with those found by the one-hour method. It would seem, therefore, that regardless of the period of observation (one-hour or lethal dose) or variety of frogs (*R. pipiens* or *temporaria*) the characteristic curve for digitalis has the same slope and that the two species of frogs display the same degree of variation to toxic doses of digitalis. (This statement holds good, at present, only for the lymph sac method of injection.) These findings would therefore seem to justify the use of the statistical method when either species of frogs is used or either period of observation (one or twelve hours) without first establishing an integrated curve in the individual laboratory. In other words, the Trevan-U. S. P. curve apparently can be used in any laboratory for the assay of digitalis when a frog lymph sac method is used. On the other hand the curve of Chapman and Morrell obtained with ouabain is much steeper than the digitalis curve and therefore it should not be used in the assay of digitalis. Chapman and Morrell have pointed out that errors due to the use of the ouabain curve for digitalis may be minimized by using doses which would bring the mortality between the 25 and 75 per cent points. It is true that this procedure would lessen the errors as the differences between the two curves are less within these limits—the deflection being much greater above and below these percentages.

In Table I are given the results of the assays of the different powders when the official U. S. P. method (one hour) is used, the tinctures employed being fresh and prepared under identical conditions and injected into the anterior lymph sacs of the frogs.

TABLE I.—ONE-HOUR FROG METHOD. USING 10 PER CENT TINCTURES OF THE DIFFERENT POWDERS PREPARED AS DESCRIBED IN THE TEXT. TWENTY FROGS WERE USED FOR EACH ASSAY.

Date, 1935.	Preparation.	Dose per Gram of Frog.	Number of Frogs Discarded.	Number of Frogs Dead.	Per Cent Mortality.	U. S. P. Curve.	Potency of Powder in Per Cent of Int. Std., 1926.
Oct. 28	Int. Std., 1926	0.006	2	12	66.6	4.18	145.1
	Int. Std., 1926	0.005	3	10	58.8	4.00	126.5
	U. S. P. 915921	0.005	1	17	89.4	5.06	...
Oct. 31	Int. Std., 1926	0.005	2	9	50.0	3.80	...
	U. S. P. 915921	0.004	1	11	57.9	3.98	130.9
Nov. 5	Int. Std., 1926	0.005	4	8	50.0	3.80	...
	U. S. P. 915921	0.004	1	11	57.9	3.98	130.9
	Brit. Std. 1928	0.0045	3	9	52.9	3.86	112.8
Nov. 6	Int. Std., 1926	0.005	2	7	38.8	3.54	...
	U. S. P. 915921	0.004	0	11	55.0	3.90	137.7
	Brit. Std., 1928	0.005	2	11	61.1	4.04	114.1
	Can. Std., 1928	0.005	0	12	60.0	4.02	114.0
Nov. 7	Int. Std., 1926	0.006	3	11	64.7	4.14	...
	Brit. Std., 1928	0.004	0	8	40.0	3.58	129.7
	Can. Std., 1928	0.004	0	6	30.0	3.32	120.3
	Can. Std., 1928	0.005	0	11	55.0	3.90	113.0

The average of these results shows that in terms of the International standard powder the three powders assayed possess potencies as follows:

International standard powder considered as	100.00%
U. S. P. powder No. 915921	134.22%
British 1928 standard	118.87%
Canadian 1928 standard	115.77%

In Table II are to be found the results obtained by assaying the same four powders by the frog method using a four-hour period of observation.

TABLE II.—FOUR-HOUR FROG METHOD. TEN PER CENT TINCTURES USED AS GIVEN IN TABLE I AND TWENTY FROGS EMPLOYED IN EACH ASSAY.

Date.	Preparation.	Dose per Gram of Frog, Cc.	Number Dead.	Per Cent Mortality.	U. S. P. Curve.	Potency of Powder in Per Cent of Int. Std., 1926.
Nov. 20	Int. Std., 1926	0.0055	15	75	4.42	...
	U. S. P. 915921	0.004	18	90	5.10	154.9
Nov. 20	Int. Std., 1926	0.005	11	55	3.90	...
	U. S. P. 915921	0.0035	13	65	4.15	152.0
Nov. 20	Int. Std., 1926.	0.0045	7	35	3.45	...
	U. S. P. 915921	0.003	10	50	3.80	165.2
Nov. 30	Int. Std., 1926	0.0045	9	45	3.68	...
	Brit. Std., 1928	0.004	13	65	4.14	135.0
	Can. Std., 1928	0.004	10	50	3.8	124.5
Jan. 26	Int. Std., 1926	0.005	11	55	3.9	...
	Brit. Std., 1928	0.004	12	60	4.04	130.0
	Can. Std., 1928	0.004	11	55	3.9	125.0

From these results it appears that in terms of the International Powder the potencies of the three national standards are as follows:

International standard powder as	100.00%
U. S. P. powder No. 915921	157.37%
British 1928 standard	132.5 %
Canadian 1928 standard	124.7 %

In Table III the same powders have been assayed by the twelve-hour or lethal dose method, that being the method which was first used to assay digitalis in the latter part of last century.

TABLE III.—TWELVE-HOUR FROG (LIMITLESS) METHOD. TEN PER CENT FRESH TINCTURES USED AND TWENTY FROGS USED IN EACH ASSAY EXCEPT AS NOTED.

Date.	Preparation.	Dose per Gram of Frog, Cc.	Number Dead.	Per Cent Mortality.	U. S. P. Curve.	Potency of Powder in Per Cent of Int. Std.
Nov. 16	Int. Std., 1926	0.0045	6	30	3.32	...
	U. S. P. 915921	0.003	11	55	3.90	176.2
	U. S. P. 915921	0.0025	7	35	3.44	185.5
	Thirty Frogs Used in Each Assay.					
Nov. 19	Int. Std., 1926 (X)	0.0048	7	23.3	3.20	187.5AX 190.6AY 202.7BX
	Int. Std., 1926 (Y)	0.0053	10	33.3	3.48	205.8BY
	U. S. P. 915921 (A)	0.0028	11	36.6	3.50	...
	U. S. P. 915921 (B)	0.0033	13	76.6	4.46	...
Dec. 2	Int. Std., 1926	0.0048	6	30	3.32	...
	Brit. Std., 1928	0.004	12	60	4.04	145.2
Dec. 2	Int. Std., 1926	0.0048	6	30	3.32	...
	Can. Std., 1928	0.004	7	35	3.44	124.3
Jan. 27	Int. Std., 1926	0.0055	12	60	4.04	...
	Brit. Std., 1928	0.004	13	65	4.14	140.8
Jan. 30	Int. Std., 1926	0.0055	15	75	4.43	...
	Can. Std., 1928	0.0045	16	80	4.59	126.6

NOTE: The frogs were followed for an additional twelve hours after the designated period of twelve hours with no change in mortality statistics; hence these figures may also be considered as being derived by the so-called limitless time method.

Table III shows that by this method the following relative potency results were obtained:

International standard powder as	100.00%
U. S. P. powder No. 915921	191.4 %
British 1928 standard	143.0 %
Canadian 1928 standard	125.4 %

The figures in Tables I, II and III, indicating the relative potencies of these powders as obtained by different frog methods, are collected in Table IV and with these are given the amounts of the individual powders which would correspond in potency to 1 Gm. of the International Powder as determined by the different methods.

TABLE IV.

	U. S. P.		British.		Canadian.	
International	100.00%	1.0000	100.00%	1.0000	100.00%	1.0000
One-hour frog	134.22	0.7441	118.87	0.8419	115.77	0.8637
Four-hour frog	157.37	0.6341	132.50	0.7407	124.7	0.8019
Twelve-hour frog	191.40	0.5224	143.00	0.6993	125.4	0.7974

A comparison of these results with those obtained upon the same powders by other workers is important not only as indicating the approximate accuracy of these results but also as demon-

strating the feasibility of securing comparable results in different laboratories and indeed even when using different species of frogs. At our request Dr. Chen of Indianapolis very kindly assayed the U. S. P. powder by the U. S. P. method and reported a potency of 135% in relation with the International Powder as compared with our findings of 134.2%. Trevan and Gaddum, using *Rana temporaria* found the British powder to be 138% of the International while we, using *Rana pipiens* found it to be 143%. In each case the twelve-hour method was used. Our results differed only 5% from the British figures even though a different species of frog was used.

The Canadian powder as assayed in the Laboratory of Hygiene at Ottawa had a potency of 117.5% of the International powder, while our results gave it a potency of 122.5% when using the "O" curve used in the Canadian laboratory (or 125.4% using the U. S. P. curve), a difference again of only 5%. Also it must not be forgotten that seven years have intervened between the time the British and the Canadian workers assayed their powders and when they were assayed in this laboratory. Such comparative assays also furnish evidence as to the keeping qualities of digitalis powder when it is stored under proper conditions.

It is apparent from the results given above that the U. S. P. powder is much more potent than any of the others. This is due to the fact that it is a blend of three carefully selected specimens while the International Powder, for example, was intended to represent a good average quality product, being derived from ten different sources. It is a question whether, when the present supply of U. S. P. powder is exhausted, it would not be better to select a powder of only average strength than one which is so potent as such powders are more likely to give better comparative results than those which differ widely in potency. It is interesting to note that the new Canadian standard which has just been issued is very close to the International standard in potency.

A study of the percentage figures given in Table IV emphasizes again what others have pointed out before, *viz.*, that with different periods of observation the potencies of different powders in relation to a standard vary considerably—in general showing an indicated ascending degree of potency as the period of observation is lengthened. An important point, however, is that the lines of indicated increase for the different powders do not run parallel. For example, in this series the strongest powders show the greatest degrees of divergence from the others in the longer periods of observation (Fig. 2 (p. 292)). This is true not only for the U. S. P. powder but also for the British, while the deflection of the Canadian standard from the International is much less. Also with the Canadian powder it is seen that the greatest deviation from the International takes place in the interval between the one- and four-hour periods of observation. This lack of parallelism between the results obtained by the different methods (1-, 4-, 12-hour) disposes of the thought that possibly a definite ratio might be found in the results so that figures obtained by one method might be used as a basis for estimating the probable results to be obtained by another. For instance, if such a ratio existed a potency figure obtained by the lethal dose method might be used to calculate the potency that would be obtained by the one-hour method. These findings show that this is impossible. For example, if an unknown powder should happen to be identical with the U. S. P. powder, a twelve-hour lethal dose percentage of 191 per cent of the International would signify a one-hour figure of 134 per cent, in other words, a ratio between the two values of about three to two. On the other hand, with a weaker powder, intermediate between the U. S. P. and the International (such for example as either of the two powders herein studied) even though the twelve-hour value was known it would be impossible to calculate the one-hour value from it. There would no

longer be a ratio of three to two but with a powder reacting like the British a ratio of seven to six and with the Canadian a ratio of about twelve to eleven. It will be seen therefore that the possibilities for trouble would be considerable.

The reason for such varying relationships among these powders is not at all clear. So far as these four powders are concerned they fall into two groups—a strong group and a weaker—which in this instance is represented by the International Powder, the strength of which was based upon a mixture of ten powders obtained from different sources. The strong group is represented by the U. S. P. powder—a blend of three very carefully selected samples. It would seem reasonable to suppose that these strong powders contain a larger percentage of those glucosides which are absorbed with difficulty or at least require a longer period of time to unfold their full strength.

In order to get further light upon this question the lethal doses for 50 per cent of the frogs injected (LD50) were determined and expressed in mg. per kilo of frog, using the different periods of observation and basing the curves so obtained upon the one-hour period, considering it as zero. The other points were obtained by calculating the decrease in mg. which represent the LD50 for the different powders and observation periods. The results are shown in Fig. 3 (p. 292). The LD50 in each instance is the mean of three groups of twenty frogs each and the integrated frequency curve for digitalis was used in the correcting of the mean dosage to LD50. All determinations were made in January 1936, and for a single method they were carried out upon all the powders in a single day so as to avoid possible variations due to diurnal or seasonal influences.

The LD50 dosages for all the preparations are less for the four-hour and for the twelve-hour interval than for the one-hour period. This decrease in dosage has been noted by many workers and it holds good for digitalis, strophanthus and ouabain. In the present instance the percentage decreases for the different powders for the different observation periods are as follows:

	Four-Hour.	Twelve-Hour.
International standard 1926	7.6%	0.4%
Canadian standard No. 428	16.1	9.6
British standard, 1928	16.1	18.8
U. S. P. standard No. 915921	21.7	26.3

The curves (Fig. 3 (p. 292)) show very clearly that the greatest deviation from the one-hour figures occurs in the "one–four" hour interval and certainly lends weight to the argument which has been made that a longer period than one hour should be allowed for complete absorption. This possible source of error might not be of so much importance in the practical use of the method as it would appear at first sight, if it could be assumed that approximately equal amounts of glucosides would be absorbed in the one-hour period, which, of course, would be assuming entirely too much. However, in spite of this theoretical objection to the method it has on the whole proved satisfactory in the thirty-five years that it has been used in this country. Some further light upon this question appears later in this paper. It is certainly true, that, as shown by the findings of Dr. Chen and ourselves in the present study, accurate comparative results can be obtained by the one-hour method if attention be paid to follow carefully the essential details of the technique.

In some comparative assays which have been made, much valuable time and material have been wasted by failure to observe the fundamental directions for

carrying out the assay. For example, it is impossible to draw conclusions of any value as to the comparative potency of different products from figures gained in assays which have been carried out with no regard to temperature control. It is essential in biological assays, as in chemical assays, that in order to secure trustworthy results, care must be taken to control each factor entering into the procedure. This should be simple with the new Pharmacopœia, as it provides a powder of digitalis for the standard and the potency of the unknown is to be compared with the standard, each powder being prepared for use and injected under like conditions.

Figure 3 (p. 292) shows further that in the "four–twelve" hour period again the curves do not run parallel, in that for the U. S. P. and the British powders a further decrease in dosage is shown, while for the Canadian and International Powders the "twelve-hour" fatal dose is greater than for the four-hour period. In the case of the International Powder the LD for the twelve-hour period is practically the same as for the one-hour; 522 and 524 mg. Thus the fatal doses of two of the powders increase in the four–twelve-hour interval while the doses for the other two decrease, displaying a sharp difference of reaction between the two groups. This phenomenon accounts for the increased relative potency of the U. S. P. and British standards in comparison with the International.

Another factor which must play a part in the assays is the destruction or excretion of the glucosides during the period of observation. Such features would doubtless appear most prominently in the twelve-hour period and be seen best with the weaker powders. With such powders it is not uncommon to observe, when borderline doses have been given, that hearts which have been seen to be at a standstill at an earlier hour may again be beating at the twelfth hour. It is evident that recovery from a mild state of poisoning has taken place in the interval.

The directional change in the curve (Fig. 3 (p. 292)) for the International standard which was noted in this laboratory has also been described by Rowe who found the M. S. D. of this powder was 600 mg. per kilo by the one-hour method; M. L. D., 375 by the four-hour method and 498 mg. by the twelve-hour. Only in this powder does he have a complete series of figures for digitalis but in several instances he has comparisons of the one- and twelve-hour doses in which the twelve-hour dose was higher than the one-hour, a condition which we have not found in our studies. Finally these curves show that the main differences in the ratio among these powders in relation to the International occur in the first four hours; this difference continues to grow greater with the U. S. P. and British powders but in the case of the Canadian it runs parallel to the International—the only deviation being in the four-hour period.

To recheck the potency findings obtained, four fresh tinctures were prepared using amounts of the U. S. P. powder which our results had shown corresponded in potency to one Gm. of the International standard when tested by the different periods of observation. The amounts of powder taken, to which in each instance 10 cc. of 80% alcohol were added, were as follows:

For the standard tincture of the International Powder	1.000 Gm.
U. S. P. powder. One-hour frog method	0.7441 Gm.
U. S. P. powder. Four-hour frog method	0.6341 Gm.
U. S. P. powder. Twelve-hour frog method	0.5653 Gm.*

* The original adjusted tincture for the twelve-hour frog method was made on the basis of the early assays which had given findings which were too high. Thus 0.5653 Gm. was used instead of 0.5224 Gm. which the completed assays showed to be correct. Accordingly the final percentage found was recalculated upon the basis of the corrected figure.

Each of the three adjusted tinctures was then assayed in comparison with the International by two of us (M. and S.) working independently, comparative assays being made on the same day. In each assay twenty frogs were used and the percentage potency calculated from the mortality, using the integrated frequency curve for digitalis.

TABLE V.—ASSAY OF ADJUSTED TINCTURES—CALCULATED TO POTENCY OF 1 GRAM OF THE INTERNATIONAL POWDER AND COMPARED WITH ORIGINAL VALUES (TABLE IV).

Date.	Preparation.	Dose in Cc. per Gram Frog.	Per Cent Mortality.	Curve Number.	U. S. P. Powder in Per Cent of Internat. on Bases of 1 Gm. of Powder. Per Cent of Int. Std., 1926.	Adjusted Tincture.	Unadjusted Tincture, Table IV.
			One-Hour Frog Method.				
Dec. 10	Int. Std., 1926	0.005	40	3.57	....	....	....
Dec. 10	U. S. P.	0.005	55	3.9	109.27	146.64	....
Dec. 11	Int. Std., 1926	0.005	50	3.8	.. .	....	....
Dec. 11	U. S. P.	0.005	55	3.9	102.62	137.77	....
				Mean	105.94	142.21	134.22
			Four-Hour Frog Method.				
Dec. 11	Int. Std., 1926	0.005	65	4.12	...	....	....
Dec. 11	U. S. P.	0.005	60	4.02	97.1	153.12	157.37
			Twelve-Hour Frog Method.				
Mar. 20	Int. Std., 1926	0.0055	20	3.18	...	....	....
Mar. 20	U. S. P.	0.0055	40	3.54	112.2	....	....
Mar. 21	Int. Std., 1926	0.0055	40	3.57	...	....	....
Mar. 21	U. S. P.	0.0055	50	3.8	106.4	....	....
		Mean on basis of 0.5653 Gm.			109.3		
		Recalculated on basis of 0.5224 Gm. and on basis 1 Gm. powder			101	193.3	191.4

The potencies of these adjusted tinctures should theoretically have been found to be of 100 per cent strength, barring errors inevitable in any bioassay; and a survey of the findings in Column six shows that they all do in fact fall within six per cent of the theoretical 100 per cent. The findings on the adjusted tinctures were then recalculated on the basis of the use of 1 Gm. of powder in each case and these figures given in Column seven may be compared with the original findings (Table IV) as recorded in Column eight. These results obtained with tinctures using quite different amounts of powdered drug and carried out with a different lot of frogs at different times of the year show a maximum variation of 8 per cent and a minimum difference of 2 per cent, all well within the limits of error allowable in the biological method of assay.

A study of Table V giving the results obtained with the adjusted tinctures confirms the earlier findings as to quantitative differences in the potency relationship obtained by the different methods with the four powders. Age of the powder apparently does not play any conspicuous part, as the British and International are almost the same age and yet they act quite differently, the British 1928 acting like the U. S. P. of 1935 and not like the Canadian which was adopted in the same year as the British (1928).

Finally it may be said that these studies have been carried out with a considerable degree of completeness, as it is with such methods of assay that the manufacturer is concerned when he uses the U. S. P. powder in testing his products.

ASSAY OF STANDARD DIGITALIS POWDERS ON CATS.

In a continuation of this study all the digitalis powders were assayed by the cat method using the Magnus modification of the original procedure in which the drug is injected in the form of a dilute infusion. These infusions were made in one-half per cent strength keeping them upon a water-bath for fifteen minutes according to the directions in the Dutch Pharmacopœia. They were always used fresh, in no case more than six hours after they had been prepared. During the course of the work certain changes in technique were made leading to greater accuracy in results. The most important of these was the adoption of a certain definite period of time during which the drug was injected resulting in death of the animal. The time period chosen was forty minutes being intermediate between the thirty- and fifty-five-minute interval which is in common use. The variation in length of the interval was rarely more than a minute or two.

The infusions were injected at a uniform rate by means of an electrically driven pump, the speed of injection being varied to conform to the weight of the cat and the strength of the powder by changing the length of the stroke of the piston. The rate of injection was determined from the results obtained upon the first three cats. In the first cat, 5 mg. of the drug (1.0 cc. of 0.5% infusion) were administered per minute. From these findings the lethal dose for the cat was calculated and to the next two cats this amount was administered at such a rate that it should kill in forty minutes. From the results thus obtained on the three cats the average fatal dose per kilo was computed and injected into the other animals in the required time of forty minutes, the amount to be actually injected being adjusted according to weight of the individual cat. All the cats were anæsthetized with urethane, one Gm. per kilo given intraperitoneally, in the form of a freshly prepared fifty per cent solution. Artificial respiration was used throughout and blood pressures were taken, the final reading of the amount of infusion needed to kill being taken when the pressure fell to zero and no sign of heart activity was present.

All series of tests were conducted on a sufficiently large number of cats so that the average of the deviations of individual lethal doses from the mean, expressed in per cent of the mean, was less than $6.67\sqrt{n-1}$. The results of the assays upon cats of the four powders are given in Table VI.

TABLE VI.—ASSAY ON CATS OF THE THREE NATIONAL STANDARD DIGITALIS POWDERS IN TERMS OF POTENCY OF THE INTERNATIONAL POWDER.

Preparation.	Anæsthetic.	Number of Cats.	Mean Weight of Cats.	Calculated Duration of Assay.	Actual Mean Injection Time.	Mean Fatal Dose; Mg. per Kilo.	Per Cent of Intrnl. Std., 1926.	S. D. of Mean (Mg.).	$6.67\sqrt{n-1}$ Deviation Allowed.	$6.67\sqrt{n-1}$ Actual Deviation.
Intnl. Std., 1926	Urethane	17	2.4	40′	38′	110.35	....	2.3	27.43%	5.9 %
U. S. P. 915921	Urethane	24	2.42	40′	37′	77.90	141.65	1.53	32.66%	7.67%
Intnl. Std., 1926	Ether	6	2.13	40′	38.1′	93.0	....	2.93	16.3%	5.15%
U. S. P. 915921	Ether	7	2.28	40′	37.4′	64.96	143.0	2.47	16.3%	8.20%
Brit. Std., 1928	Urethane	7	2.42	40′	41′	90.75	121.60	3.25	16.3%	6.92%
Can. Std., 1928 No. 428	Urethane	7	2.35	40′	40′	92.53	119.26	3.275	16.3%	7.05%
Intnl. Std., 1926	Urethane	5	2.06	55′	55′	93.4	....	5.9	13.34%	11.45%
U. S. P. 915921	Urethane	7	2.31	55′	56.3′	66.1	141.3	3.4	16.3%	11.7 %

In the assay of the International standard powder when a sufficient number (17) of cats had been used so as to give a fair idea of its potency it was found that the average lethal dose was 110.35 mg. per kilo of cat weight. This was very different from the original value of 89.7 mg. found in Utrecht in 1926. This marked discrepancy of over 20 mg. per kilo would certainly indicate deterioration of the powder unless some other explanation could be found for it. Inasmuch as we were using urethane as an anæsthetic, whereas the original assays were made using ether, a number of additional assays (6) were made in which ether was substituted for the urethane. Under these conditions an average dose of 93 mg. per kilo was found. According to this the etherized animals required only 84 per cent as much drug as did the urethanized animals. But, as the table shows, the potency of the U. S. P. powder was found to be 141.65 per cent of the International when urethane was used and 143.3 per cent when ether was employed, the results being practically identical. These facts being established, the remaining powders were all assayed using urethane on account of the ease of its administration and the uniformity of the degree of anæsthesia.

The results obtained in the present study when using ether, therefore, were practically the same (93 mg.) as those found at Utrecht in 1926 (89.7 mg.) and are important in that they indicate the excellent keeping qualities of powdered digitalis when stored under proper conditions. They also demonstrate the possibility of getting comparable results when comparable methods are employed, and above all, the absolute necessity of securing such identical or comparable conditions. If these factors are controlled it is possible, probably, to vary the conditions somewhat if the same deviation in method is followed with both powders to be assayed and yet to obtain comparable results.

A very important point in connection with these studies is brought out by a comparison of the results obtained with cats and by the various frog methods. One argument which has been made for the cat method is that the cat biologically is much closer to man than is the frog and therefore is much more suitable as a test animal for the assay of drugs which are to be administered to man. Such an argument is, of course, hard to contradict. Another advantage which is urged in favor of the cat method is, that here, the drug being injected intravenously, there is no question as to its absorption as there is in the case of the lymph sac injection into frogs. For these reasons a comparison of the results obtained on frogs with those gained on cats is especially interesting. Such comparative figures are given in Table VII based on the International Powder considered as 100 per cent. The comparative results are also shown in Fig. 2 (p. 292).

TABLE VII.—PERCENTAGE POTENCY OF STANDARD POWDERS IN TERMS OF INTERNATIONAL STANDARD (1926) AS 100%.

Method.	U. S. P. Powder.	British.	Canadian.
Cat	141.65%	121.60%	119.26%
One-hour frog	134.22	118.87	115.77
Four-hour frog	157.37	132.50	124.70
Twelve-hour frog	191.40	143.00	125.40

A study of these results shows that the cat method and the one-hour frog method yield results which are remarkably close, differing in the case of the U. S. P. Powder by 7%; the British 3% and the Canadian 3.5%. On the other hand the four- and the twelve-hour results differ much more widely from the cat results indicating that in spite of the short period of observation and other theoretical objections the results by the one-hour method agree well with those obtained with cats.

It is worthy of note that the British workers found for their standard (by the

etherized cat method) a potency, relative to the International standard, of 116% while we found 121.6%; and by the twelve-hour frog (*R. temporaria*) the British report a potency of 138% as compared with our findings (*R. pipiens*) of 143%. Such checking and cross-checking demonstrate anew the accuracy attainable by biological methods. The figures giving the relative potency of the Canadian powder again demonstrate that it reacts in a manner very similar to the International Powder which it approaches in potency more closely than do the other powders and the assay results obtained upon it by all the four methods do not differ more than about 10 per cent.

In order to check this relationship still further a tincture was prepared from the U. S. P. powder, using the amount indicated by its potency in relation to the International standard, as determined by the cat method, *viz.*, 0.7060 Gm. in 10 cc. of 80% alcohol. This tincture was assayed by the various frog methods in order to see which frog value would approach most closely the theoretical potency indicated by the cat assay.

TABLE VIII.—ASSAY ON FROGS, USING THE 1-, 4- AND 12-HOUR PERIODS OF OBSERVATION, OF A TINCTURE OF THE U. S. P. POWDER OF A STRENGTH AS DETERMINED BY ITS ASSAY ON CATS.

Date.	Preparation.	Amount of Powder.	Dose in Cc. per Gram of Frog.	Mortality.	Per Cent Mortality.	Curve.	Per Cent of Int. Std., 1926.	Per Cent of Int. Std. on Bases of 1 Gm. of Powder.	Per Cent of Int. Std. on Basis of Unadjusted Tincture, Table IV.
				One-Hour Frog Method.					
12/10	Int. Std.	1 Gm.	0.005	8	40	3.57	....	....	....
12/10	U. S. P.	0.7060	0.005	7	35	3.45	96.64	136.79	134.24
				Four-Hour Frog Method.					
11/29	Int. Std.	1 Gm.	0.005	14	70	4.28	....	....	....
11/29	U. S. P.	0.7060	0.005	16	80	4.6	107.47	....	....
11/30	Int. Std.	1 Gm.	0.0048	9	45	3.69	....	....	....
11/30	U. S. P.	0.7060	0.0048	11	55	3.9	105.70	....	....
						Mean	106.59	150.98	157.7
				Twelve-Hour Frog Method.					
12/2	Int. Std.	1 Gm.	0.0048	6	30	3.32	....	....	....
12/2	U. S. P.	0.7060	0.0048	13	65	4.14	124.7	176.6	191.4

Here again the close relationship between the potency findings as determined by the cat and the one-hour frog methods is clearly shown, a difference of only 2.5% being present, against a 7% difference by the four-hour method and 15% by the lethal dose method.

Rate of Injection.—An important point in the assay of digitalis by the cat method, and one to which reference was made earlier, is the effect of varying the rate of injection upon the potency figures. To study this feature further we compared the results of injecting infusions of two of the powders at a slower rate than we had used in the earlier assays, taking 55 minutes in place of our average of 38 minutes and using five cats for the International powder and seven for the U. S. P.

The following table gives the results obtained by the two rates of injection.

TABLE IX.—EFFECT OF VARYING THE RATE OF INJECTION OF THE DIGITALIS INFUSION UPON THE ABSOLUTE AND RELATIVE TOXICITY OF THE DRUG IN THE CAT.

	Period of Injection—55 Min. Mean Mg. per Kilo.	Per Cent of International.	Period of Injection—38 Min. Mean Mg. per Kilo.	Per Cent of International.
International	93.4	...	110.35	....
U. S. P. Powder	66.1	143.3	77.90	141.65

Here again, as in the case of the figures obtained using different anæsthetics, the individual absolute results for the two rates of injection are entirely different but when comparative assays are made, under like conditions, the percentage potency ratios at the two rates are again practically identical. From the theoretical standpoint it might have been supposed that with the longer period of injection larger amounts of the drug would be tolerated than when it was administered more rapidly, but, as a matter of fact, in each instance it took 18 per cent more drug to kill in 38 minutes than it did in 55 minutes indicating that with the longer period the drug has a better opportunity to unfold its full activity and therefore the smaller dose is toxic. From this it might be thought that the longer period is to be preferred, but when the final calculations are made the comparative percentage results are identical. These findings explain the favorable results obtained by the one-hour frog method in comparison with the longer periods of observation, in that, while the longer period gives greater opportunity for absorption, apparently comparable degrees of absorption occur in the shorter period. An objection which may very properly be made to the use of the prolonged period of injection, at least, in so far as our experience goes, is that the individual variation of the experimental animals increases as the period is lengthened. This is shown by the relatively high average of the deviations of the L. D. from the mean which is expressed in per cent of the mean L. D.

In view of the fact that a prolongation of fifteen minutes in the time of injection makes such a difference in the toxic dose of digitalis, certainly a more exact time limit for the injection should be adopted than one varying anywhere between thirty and fifty-five minutes or even ninety minutes as some advocate. It also demonstrates how indefinite is the term "cat unit." This term is employed to denote the amount of digitalis which will prove fatal to the cat calculated as per kilo of body weight when the drug is injected under standard conditions. But as we have shown, the absolute amount of drug necessary to kill a certain weight of cat means little, as the fatal dose will vary depending upon the rate of injection of the drug and upon the anæsthetic employed. Where ether is used the fatal dose, and therefore the "cat unit," is much smaller than where urethane is employed. Our results have also been confirmed and the general subject reviewed very recently by Haskell.[1] In his experiments he found that with cats anæsthetized with ether digitalis proved fatal in doses much smaller than when the animals were given dial-urethane.

It would seem necessary to emphasize again the effect of the type of anæsthetic employed upon the toxic dose of digitalis for cats and therefore upon the quantity of drug in a "cat unit." In recent published directions for the carrying out of the assay it is said that "light ether may be used or local anæsthesia—one per cent procaine or ten per cent phenol rubbed into the skin." Since ether, urethane, dial and a variety of other anæsthetics discussed by Haskell affect the size of the toxic dose of digitalis, it may well be questioned what effect procaine or phenol would have. With very little doubt the toxic dose when ether is used would hardly be comparable to that when procaine or phenol is employed.

Since there is this deviation in indicated potency depending upon anæsthetic and rate of injection, there will always be discrepancies in results as obtained by

[1] Haskell, *J. Pharm. and Exp. Therap.*, 58, 111 (1936).

different assayists when potency is indicated in cubic centimeters or milligrams per kilo of cat weight. On the other hand, comparisons of the unknown powder with a standard preparation, both assays being carried out under standard conditions, will remove the difficulty of expressing and comparing results. The new Pharmacopœia provides for such a comparison by directing that "Powdered Digitalis" shall be of such strength that 0.1 Gm. shall possess a potency equivalent to 1 U. S. P. unit which in turn is equal in potency to one International unit, that being the potency contained in 0.1 Gm. of the International Powder.

Basing the calculations upon the figures which have been given in Table VI the number of units in the U. S. P. powder, as determined by the different variations in the cat method, is as follows:

TABLE X.

Anæsthetic.	Injection Time.	L. D. in Mg. per Kilo. Internatl.	L. D. in Mg. per Kilo. U. S. P.	L. D. of U. S. P. Powder Calculated to Potency of 100 Mg. Internatl.	Digitalis Units in 1 Gm. U. S. P. Powder.
Urethane	35 min.	110.35	77.9	70.59	14.16
Ether	35 min.	93.0	64.96	69.85	14.31
Urethane	55 min.	93.4	66.1	70.77	14.13
					Mean 14.2

Thus by the cat method the U. S. P. powder contains 14.2 digitalis units in each Gm. and by the one-hour frog method 13.42 digitalis units.

Having established the relative potencies of the four powders both by the cat and by the three frog methods, it became a matter of interest to see whether any definite ratio or parallelism could be demonstrated between the results obtained on the two species of animals. Various efforts have been made to establish such a relationship; however, in general they have not been very successful. In spite of this failure, some still hold that some close correlation should exist between the various sets of toxicity figures. Such a comparison can be made in the present instance by comparing the results we have obtained with those reported by the English workers when their own standard was being prepared. These figures are given in the following table.

TABLE XI.—COMPARISON OF POTENCY OF BRITISH STANDARD POWDER IN TERMS OF INTERNATIONAL STANDARD AS DETERMINED IN ENGLAND IN 1928 AND IN THIS COUNTRY IN 1936.

	British Findings.	U. S. A. Findings.
12-hour frog	138%	143.0%
Cat method	116%	121.6%
Cat figures in per cent of frog figures	84%	85.0%

The results are seen to be practically identical, showing that compared with the International Powder the British and the U. S. P. powders display by the cat method a potency of 85 per cent of that shown by the frog method. We realize that these figures are too few to state that such a relationship would always exist. Our own figures do indeed demonstrate a considerable degree of parallelism between the potency findings on the three powders assayed by the different methods. As the curves given in Fig. 4, p. 292 show, the most complete agreement is again to be found between the cat and the one-hour frog methods. The greatest discrepancy is in the twelve-hour result with the U. S. P. powder. This discrepancy lessens rapidly through the weaker British powder to approach most nearly the findings obtained by the other methods in the case of the one-hour result for the Canadian powder.

In an earlier study (1929) carried out upon strophanthus six tinctures were assayed by different methods and a similar parallelism was shown to exist. However, in that study the cat values were about 30 per cent lower than the one-hour frog values—exactly the reverse of the findings with digitalis in the present work.

The British Pharmacopœia permits, in case some other method of assay than the twelve-hour frog or cat method is used, that an amount of powder be employed which is an average between the values obtained by the two methods mentioned. Thus by the frog method their powder has a potency of 13.8 units per Gm., and by the cat method 11.6 units per Gm. However, if other methods are employed, an average of these two figures or 12.7 units is considered to be close enough for practical purposes. It is of interest to see how such a rule would work if it were followed in the case of these powders for which we have definite figures of potency.

TABLE XII.

	U. S. P.		British.		Canadian.	
	Potency.	Units per Gram.	Potency.	Units per Gram.	Potency.	Units per Gram.
Average of cat and 12-hour frog figures allowed by British Pharmacopœia	166%	16.6	133%	13.3	122%	12.2
Our findings by 1-hour frog method	134%	13.4	119%	11.9	116%	11.6
Our findings by 4-hour frog method	158%	15.8	135%	13.5	125%	12.5

These figures, showing values which would be permitted under the rule adopted in the British Pharmacopœia and the actual results we obtained in the assay of the three powders show what a large discrepancy would occur if the British rule were used in the case of a strong powder such as the U. S. P. specimen. An error of almost 20 per cent would be at once introduced in adopting an "average" value and using the one-hour frog method. The error would be over 10 per cent in the case of the British powder, while in the case of the Canadian powder it is perhaps not so serious. In bioassays a certain degree of error is perhaps unavoidable but, if to this unavoidable error an initial error of 20 per cent is added, the discrepancy may be very considerable. Such an initial error would be especially important in the case of the one-hour frog method, as this method yields results which approach more closely those given by the cat method than do the four- or twelve-hour methods.

SUMMARY.

An integrated mortality curve for the assay of digitalis by the one-hour frog method was found to agree substantially with the curve obtained by Trevan using the lethal dose method. By means of the statistical method the potency of the U. S. P., British and Canadian standard powders was studied in relation to the International standard. The results showed that these powders stood in relative potency to the International as follows, in order of decreasing potency: U. S. P., British, Canadian. The twelve-hour frog method gave the highest relative potency and the one-hour the lowest, the four-hour being intermediate. Tinctures made up of various strengths, based upon the values obtained in the above assays, were assayed by the different methods and yielded figures which were strictly comparable within the limits of error.

The same powders were assayed by the Magnus modification of the cat method. The relative values obtained by this method coincided quite closely with those obtained by the one-hour frog method.

The effects of anæsthetic and rate of injection of the drug were studied and were found to have a marked influence upon the amount of drug required to kill and therefore upon the amount of drug in a "cat unit." Such discrepancies can only be avoided by the adoption of some uniform method of technique or by the use of a standard powder of digitalis with which to compare the unknown. Such a standard, based upon the potency of the International standard, is provided by the U. S. Pharmacopœia of 1936.