The physiological standardization of digitalis / by Charles Wallis Edmunds and Worth Hale.

TREASURY DEPARTMENT
Public Health and Marine-Hospital Service of the United States

HYGIENIC LABORATORY.—BULLETIN No. 48
DECEMBER, 1908

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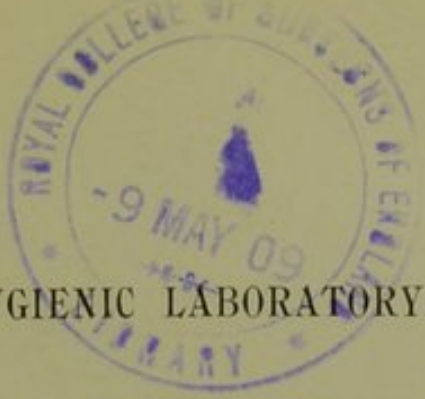

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THE PHYSIOLOGICAL STANDARDIZATION OF DIGITALIS.[a]

By CHARLES WALLIS EDMUNDS,

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and

WORTH HALE,

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One of the directions along which scientific medicine has advanced during the past few years is in the greater accuracy with which medicines are administered. A very essential factor in this tendency is that the drugs themselves shall be of a uniform strength, and to insure this the Pharmacopœia of the United States, in its last revision, provided a number of standards to which official preparations must conform. Especially is this true in the case of a number of drugs, such as opium, belladonna, nux vomica, etc., in which the active constituents are capable of isolation in a pure form. With a considerable number of others, however, this was not found to be possible for the reason that the active principles are either not known or are not capable of being isolated in pure state by any known chemical method. In this class occur such drugs in common use as digitalis and the other members of that series, ergot and cannabis indica.

By far the most important member of this group is digitalis itself, on account of its widespread use in cases of cardiac disease. At the present time it is impossible to secure a standardized preparation of the drug by any known chemical means[b] on account of the fact that the activity of the drug depends upon no single active principle, but upon several whose chemistry is not completely known and for the isolation of which there does not, at the present time, exist any satisfactory chemical method.

Our knowledge of the chemistry of the digitalis leaves we owe mainly to Schmiedeberg and Kiliani. The most important constituent is digitoxin, a glucoside, which is insoluble in water but soluble

[a] Submitted for publication December 4, 1908.

[b] Some firms put out preparations standardized so as to contain a certain percentage of digitoxin, but, as will be pointed out, this need not indicate a uniform physiological activity.

in alcohol and chloroform. In watery solutions of the drug it is probably made soluble by the presence of other constituents of the plant, most likely the saponin-like body, digitonin. The digitoxin is readily broken up into digitoxigenin and the sugar, digitoxose. Schmiedeberg obtained toxiresin as a decomposition product, a body which is related in action to the picrotoxin group.

A second glucoside is digitalinum verum or digitalin, which is soluble in alcohol but insoluble in chloroform or water. This breaks up into an inactive body digitaligenin and two sugars. The digitalinum verum is found mainly in the seeds.

Digitalein[a] resembles digitalin very closely, differing from it, however, in being soluble in water.

Digitophyllin, another glucoside, which has been isolated, resembles digitoxin.

While digitoxin is the most important constituent of the preparations of digitalis it does not necessarily follow that the activity of the drug runs parallel with the digitoxin content. At least this is true as far as we can judge by the method which is available for the isolation of this body, viz, the Keller method or the Keller method as modified by Fromme. Keller's method[b] for the isolation of digitoxin consists in the extraction of a certain amount of carefully dried and powdered leaves with dilute alcohol. The percolate is evaporated to drive off the alcohol and the residue is taken up with water and lead acetate added. A voluminous precipitate results, but only the first portion of the filtrate obtained is saved to avoid the uncertainty of attempting to wash the precipitate free from digitoxin. Sodium sulphate is added to the filtrate to precipitate any excess of lead and after standing four to five hours the clear liquid is decanted, made alkaline with ammonia, and shaken out with chloroform. The chloroformic solution is evaporated and a yellow resinous mass, the digitoxin, is left behind. This may be purified by taking it up with a small amount of chloroform and reprecipitating with a mixture of sulphuric and petroleum ether.

Several workers have tried to find a relationship between the digitoxin content and the activity of the preparations, as determined by biological methods, but the results, in almost every case, have proved a failure, as will be seen by a study of the following tables, which we have brought together for the sake of comparison.

Ziegenbein[c] conducted such a series of comparative experiments, estimating the toxic dose upon frogs, employing leaves obtained from various localities.

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[a] According to Kiliani, München. med. Wchnschr., 1907, LIV, 886, the widely advertised proprietary preparation digalen consists of a solution of impure digitalein.

[b] Keller, Ber. d. deutsch. pharm. Geselssch., Berl., 1897, VII, 125.

[c] Ziegenbein, Arch. d. Pharm., 1902, CCXL, 454.

No.	Specimen.	Digitoxin content.	Toxic dose for 100 gram frog.
		Per cent.	*Gram.*
1	Leaves from North Harz, 1901	0.33	0.03
2	Leaves from North Harz, 1901 (selected)	.163	.03
3	Leaves from South Harz, 1901	.14	.04
4	Leaves from South Harz, 1901 (selected)	.185	.03
5	Leaves from North Harz, 1901	.125	.03
7	Leaves from Thuringia	.115	.05
10	Leaves, old, 1900	.226	.06
11	Leaves, Harz (finely powdered)	.235	0.04–.05
12	Leaves, Harz (finely powdered)	.18	.05

Famulener and Lyons[a] carried out a similar research with the following results:

Specimen fluid extract of digitalis.	Purified digitoxin content.	Toxic dose for 40 gram frog.
	Per cent.	*Gram.*
A	0.196	0.026
B	.258	.029
C	.182	.038

Barger and Shaw[b] found that the ratio of toxicity of the tincture of digitalis to the toxicity of the digitoxin contained in the specimen varied in the series of nine tinctures from 3.5 : 1 to 6.9 : 1, or, in other words, tinctures from 3.5 to 6.9 times as toxic as the digitoxin which was obtained by chemical assay. Focke[c] and Fromme carried out a similar set of comparative experiments and concluded that the digitoxin content and the physiological activity as determined by experiments on the frog did not run parallel. Reed[d] and Vanderkleed, using guinea pigs, claimed to have found a certain parallelism, but a study of their results shows that the parallelism is by no means without exceptions:

No.	Preparation.	Chemical assay. Gram digitoxin in 100 cubic centimeters.	Physiological assay. M. L. D.[e] for guinea pig of 240 grams weight.
		Grams.	*c. c.*
1	United States Pharmacopœia tincture	0.0377	0.6
2	.....do	.023	1.0–1.25
3	.....do	.0277	.75
4	.....do	.0254	1.0
5	Fat free tincture	.027	1.0–1.25
6	Fluid extract	.264	0.1
7	.....do	.2405	.09
8	.....do		.1
9	Powdered extract	.234	.08
		[f]1.061	[g].019–0.025

[a] Famulener and Lyons, Proc. Am. Pharm. Ass., Phila., 1902, L, 415.

[b] Barger and Shaw, Chem. and Physiol. Assay of Digitalis Tinctures, Handbook of Pharmacy, 1904, 541; Pharm. J. and Tr., 1904, LXXIII, 249.

[c] Focke, Deutsch aerzt. Zeit., 1904, VI, 292.

[d] Reed and Vanderkleed, Am. J. Pharm., Phila., 1908, LXXX, 110.

[e] M. L. D.=minimum lethal dose.

[f] Per cent.

[g] Gram.

These figures certainly show a closer relationship than has been obtained by the previous workers.

Since we have then no satisfactory chemical method of estimating the activity of digitalis preparations, attention has been directed toward the development of assay by physiological methods. Several have been employed in the past ten years and the results obtained by their aid have served to emphasize the necessity of some way of standardizing these drugs. In both this country and in Europe many writers have demonstrated by these physiological tests the great variability of strength which exists not only in the galenical preparations of the drug but also in the so-called pure preparations themselves.

Among these investigators may be mentioned Bennefeld, who in 1881 showed that for rabbits the lethal doses of eight digitalis tinctures varied about fourfold.

Bührer (1900) demonstrated on frogs that some of the fluid extracts of digitalis were four times as strong as others.

In 1902 Fränkel showed that six infusions of digitalis varied from 100 to 275 per cent, and six tinctures from 100 to 400 per cent.

Siebert (1903) found in fifty experiments on digitalis leaves that the fatal doses per 100 gram frogs varied from 0.03 to 0.075 gram.

Edmunds in 1907 showed that seventeen tinctures of digitalis purchased in the open market varied in strength as 1 to 4, while Gottlieb (1908) found the same ratio to exist in Heidelberg. Reed and Vanderkleed (1908) found the toxic dose for guinea pigs of 240 grams weight varied in four tinctures from 0.6 to 1.25 c. c.

These variations in strength depend upon several factors, among which may be mentioned the source of the plant; for example, Ott[a] has pointed out that Bohemian leaves are more toxic than others. At the present time the English leaves are considered the best. The plants growing wild are preferred to the cultivated variety, and those growing in sunny places to those in the shade, and those grown in dry seasons to those gathered in rainy. However, the most important factor in determining the activity of the leaves is the manner of drying and the mode of preservation, the influence of which has been especially studied by Focke (p. 24).

The experiments of Bennefeld and others to determine the relative activities of different members of the group were carried out along essentially the same lines and embody the same principles as are employed to-day in the biological standardization of these drugs. It was but a step from one to the other; the first writers determined the relative activity, while the later writers merely corrected these variations by such means as concentration or dilution of the preparations. To get then any comprehensive idea of the development of modern physiological standardization it is necessary to go further back than

[a] Ott, Verhandl. d. Cong. f. innere Med., Wiesb., 1901, 89.

the first paper strictly devoted to the standardization problem and examine the earlier methods in vogue. We have therefore given short abstracts from earlier writings of such articles as bear upon the subjects before considering it in the light of more recent works, not only digitalis being discussed, but some other drugs belonging to the same group, such as strophanthus, as the same methods apply to all the members of the series.

In 1865 Fagge and Stevenson, in an address before the Royal Society, claimed that physiological tests would be of great medico-legal importance.[a] They had studied digitalin especially and said for this purpose frogs were better adapted than the higher animals, as the various organs could be examined, the animals exhibited no fear, and the drugs acted quickly upon them.

In their paper published in 1866 they made a report upon the relative activity of several members of the digitalis group, among them being "antiar," helleborus viridis, squills, digitalin, and the infusion of digitalis. The method employed was to weigh the frog, attach it to a piece of cork, and expose the heart, avoiding hemorrhage as far as possible. It was then injected beneath the skin of the thighs and flanks and the time noted at which systolic standstill of the ventricle occurred. The length of time between the injection of the drug and the stoppage of the heart determined the relative toxicity of the different drugs. Fagge and Stevenson considered that the season of the year did not alter the reaction of the frogs to the drugs.

Ten years later Koppe[b] published the results of his investigations upon the relative toxicity of the active constituents of digitalis, digitoxin, digitalin, and digitalein. His experiments were carried out upon frogs, cats, dogs, and rabbits. In his work on frogs he employed both Rana temporaria and R. esculenta and showed how much more susceptible the former were than the latter. His method was to expose the heart in the usual manner and inject the drugs, noting the time when systolic standstill of the heart appeared.

The dogs were either injected subcutaneously or the drug was administered by the mouth, changes in the rate of the heart and the strength of the beat being noticed, as well as the time when nausea and vomiting occurred. To cats the drug was administered both subcutaneously and by the mouth and the amount necessary to produce vomiting noted. Large doses produced alterations in the pulse rate. The drugs were given subcutaneously to rabbits and produced in them as the most prominent symptom a more or less complete paralysis associated with irregularity and slowing of the heart rate.

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[a] C. Hilton Fagge and Thomas Stevenson, Application of physiological tests for certain organic poisons, and especially digitaline. Reprinted from Guy's Hosp. Reports, 1866.

[b] Koppe, Arch. f. exper. Path. u. Pharmakol., Leipz., 1875, III, 274.

Fr. Bennefeld[a] in 1881 undertook an investigation of the relative activity of tinctures of digitalis obtained from different parts of Germany. He first tried to compare them by means of their specific gravities and the amount of the dried residue, but his results, confirming those of Schneider,[b] showed that such examinations were worthless. He then adopted the physiological method, employing rabbits as experimental animals.

The tinctures were prepared for examination by evaporating 25 c. c. and drying the resulting residue to constant weight over sulphuric acid. The residue was digested on a water bath for fifteen minutes at a moderate temperature with 15 c. c. of distilled water, was then filtered as quickly as possible and brought to a volume of 20 c. c. Before injection it was heated to body temperature.

The rabbits used were of the same age, weight, and resistance as far as possible. After inserting a cannula into a jugular vein, the modified tincture was injected at regular intervals in 0.5 c. c. doses until the animal died or the 20 c. c. was exhausted.

His results showed that the minimum lethal dose for the eight tinctures varied from 3.36 to 15.97 c. c. of the residue solution per kilogram, body weight, while the time of death varied from eleven to fifty-four minutes.

Frankel[c] adopted still another method to determine the comparative activity of different preparations of digitalis. Dogs weighing between 10 and 18 kilograms were curarized and the drug injected subcutaneously in doses not exceeding 10 c. c. His standard for comparison consisted in the amount which was necessary to produce the digitalis effect, viz, a decrease in the rate of the heart with an increase in the volume of the single beats. He also registered the blood pressure.

He found marked differences between the various preparations in the amount necessary to bring about the desired effects, which he thought might be partly due to individual variations in the dogs and also possibly to the curara. The tincture was found to be very weak, while the acetum was the stronger preparation, but the active amounts of both acetum and infusion were quite variable.

Laborde and Duquesnel[d] examined two samples of digitalien (Nativelle) as to their chemical purity and physiological activity, using for the latter purpose frogs and guinea pigs.

The hearts of the frogs were exposed and the drugs injected in 5-milligram doses, the effects being noted. With one preparation

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[a] Fr. Bennefeld, Über Digitalis-Tincturen, Comparativ-experimentelle Untersuchungen, Inaug. Dissert., Göttingen, 1881.

[b] von Schneider, Arch. d. Pharm., 1879, CCXV, 412.

[c] Frankel, Charité-Ann., Berl., 1881, VI, 207.

[d] Laborde and Duquesnel, Comp. rend. hebd. des Sèances et Mém. de la Soc. de Biol., 1884, XXXVI, 93.

the heart stopped in systole in two minutes, while with the second sample it continued to beat for hours. The difference in physiological activity was also shown by means of graphic records.

The results obtained on the frogs were confirmed by the injection of 5 milligrams of each sample into two guinea pigs, the length of time till death occurred serving as a means of comparison.

Similar methods of comparison were also employed by Laborde[a] in his study of the purity and activity of the digitalines in use at the hospitals of Paris.

Gley[b] compared the toxicity of ouabaine and strophanthin by means of experiments upon several varieties of animals. Upon frogs he showed that with equal doses of the two drugs ouabaine would stop the heart in half the time that strophanthin would. He then compared the toxic dose of the two drugs upon guinea pigs, dogs, and rabbits; in the latter case injecting the drugs into the vein of the ear, in the other cases using subcutaneous injection.

Reusing[c] carried out a series of experiments comparing the actions of strophanthin and digitalis upon the frog's heart, employing two methods in his work. In the first he studied the effect directly upon the exposed heart, the pericardium not being opened. In the second method the Ludwig-Coat's heart apparatus was employed, the perfusion fluid to which the drugs were added consisting of two parts of 0.65 per cent NaCl solution with 1 part of defibrinated hog blood.

Bardet[d] undertook in the same year an examination of the relative activity of the various active principles of digitalis and employed the simplest method which is in use to-day to standardize these drugs, viz, that of determining the lethal dose for animals, using frogs and rabbits.

An examination of a number of the so-called digitalines of commerce was made by Fouquet,[e] who ascertained the toxic dose of the different samples when they were injected hypodermically into frogs, rabbits, and dogs. In this way he studied the relative activity of crystallized and amorphous digitalin, digitoxin, and digitalein.

In 1893 Prevost[f] examined a number of the preparations which had been included in the Swiss Pharmacopœia III, among them being some members of the digitalis group. His experiments were carried out upon frogs (mainly R. temporaria), upon which he sought the minimal dose of the drug which would produce definite stoppage of

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[a] Laborde, Comp. rend. hebd. d. Sèan. et Mém. de la Soc. de Biol., 1884, XXXVI, 599.

[b] Gley, Comp. rend. des Sèances de l'Acad. de Sci., 1888, CVII, 348.

[c] Reusing, Über die Einwirkung des Strophanthin auf das Froschherz, Inaug. Dissert, Würzburg, 1889.

[d] Bardet, Bull. et Mém. Soc. de thérap., Par., 1889, 2d ser., XVI, 253.

[e] Fouquet, Bull. gén. de thérap., 1892, CXXII, 71.

[f] Prevost, Rev. Méd. de la Suisse Rom., Genève., 1893, XIII, 505.

the heart and death of the animal. He considered the frog as the most suitable animal upon which to test the relative activity of the members of this group as mammals vary considerably in their susceptibility to them. In his earlier experiments he did not weigh the animals, but later adopted this precaution, regarding the weight as being a factor of some importance, but at the same time not considering that the action of the drug upon different frogs is in any absolute relation to their weights. The frogs he employed were an average size, being between 25 and 30 grams. He confirmed some of the results obtained on frogs by experiments upon guinea pigs, the comparative results being as follows:

Animal used.	Toxic dose.		
	Fluid extract convallaria.	Fluid extract digitalis.	
		No. 26.	No. 6.
	c. c.	c. c.	c. c.
R. temporaria, average size	0.003	0.02	0.04
Guinea pig, per 100 grams	.006	.01	.02

It will be seen that the two digitalis preparations show exactly the same ratio on both animals.

In 1895 Prevost [a] repeated his experiments upon these preparations to see if they had deteriorated in the intervening time. In this case he used R. esculenta, realizing, however, that they did not offer the same susceptibility as R. temporaria.

Mlle. M. Piotrowska [b] studied the comparative toxicity of a number of the members of this group and the effect upon their toxicity of the mode of administration, whether subcutaneously or by the mouth. For this purpose she used frogs (R. temporaria) almost exclusively, determining the toxic dose and comparing them on a basis of 100 grams body weight. Some of her results she confirmed by determining the toxic dose for cats and rabbits, comparing them on the basis of kilograms of body weight.

The examination of the plant dialysates was made the subject of investigations by several workers, one of the earliest being Jacquet.[c] These dialysates were prepared by Golaz of Vevey, Switzerland, from fresh plants and made of such strength that 1 part of dialysate corresponded to 1 part of leaves. By using great care in the collection of the leaves, gathering them in the same region during sunny weather, using them fresh, and making the product up to a uniform volume, it was believed that a definite dosage of digitalis was attained, as

[a] Prevost, Rev. méd. de la Suisse Rom., Genève., 1895, XV, 453.

[b] M. Piotrowska, Inaug. Dissert., Geneva, 1896.

[c] A. Jacquet, Cor.-Bl. f. schweizer Aerzte, 1897, XXVII, 326.

so many of the disturbing factors had been eliminated. It was naturally impossible to make allowances for the yearly variation which was known to take place, and therefore the argument for a uniform product based upon these facts alone falls to the ground. Jacquet, who pointed out in his paper these advantages of the dialysates, made a number of physiological tests on the dialysates of digitalis and Adonis vernalis. He employed frogs (R. esculeuta and temporaria) with exposed hearts, injecting the drug into the lymph sacs and noting the length of time before death. He also studied their action upon the blood pressure and heart rate in rabbits and noted the toxic dose.

By a comparison of the results obtained in this way with those he obtained under similar conditions using digitoxin and digitalium verum he estimated the dose of the dialysates which it would be proper to employ in man, it being possible to do this as the therapeutic dose of the active principles for man was already known.

It appears that Jacquet approached more nearly to physiological standardization than any of his predecessors, as he points out the advantage of the dialysates in affording an exact dosage, but it was not until his second paper,[a] which appeared in December, 1898, that he seems really to have adopted physiological standardization. As stated above, he believed that a uniform preparation had been obtained by eliminating so many factors which go to make the great variations which are found in the ordinary galenical preparations; but as he stated then, he was unable to avoid the yearly variation in plants. This was evidently quite a large factor, as, examining the 1896 specimen, he was able to isolate 0.16 per cent of the active constituent, while in the 1897 preparation there was only 0.096 per cent. This variation he ascribed to the fact that the weather was unsettled at the time of gathering the leaves, and on that account the plants contained an unusually large amount of water. He therefore compared the two dialysates by physiological methods and found the same relative differences, for while ten drops of the 1896 specimen produced systolic standstill in frogs, it took twenty drops of the 1897 preparation. Likewise, it took double the amount of the 1897 preparation to kill rabbits.

Recognizing at this time the yearly variation, which is apparently of a good deal of importance, he suggested that either a yearly change of dose would be necessary or an adjustment of the solution by evaporation or dilution as might be necessary, so as to give a uniform product. The latter course he thought preferable. This paper, in which he really adopted the physiological method of standardization, was, however, preceded by one by Houghton which appeared in America in October, 1898.

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[a] A. Jacquet, Cor.-Bl. f. schweizer Aerzte, 1898, XXVIII, 745.

No reference is directly made in Jacquet's paper as to the effect of the weight of the frogs upon the results, except, in the protocols, the size is noted as "large" or "medium."

In his experiments upon rabbits he used two methods. Upon most he measured the effect upon the blood pressure and heart rate when the drug was injected into the jugular vein and calculated also the toxic dose per kilogram body weight. In other cases he injected the drug subcutaneously.

It appears strange that, although so much work had been done on the comparison of the relative strengths of the different members of the digitalis group, no practical application was made of the methods developed until more than thirty years after Fagge and Stevenson published their results. As pointed out, it was not until 1898 that the application of these physiological tests was advised for the production of uniform pharmaceutical preparations. In that year Houghton[a] published his method for the physiological assay of strophanthus, basing it upon the fact that the killing power of the cardiac drugs for frogs of definite size and species, kept under proper conditions, is constant per gram of body weight. He had tried rabbits, guinea pigs, and rats, as well as frogs, but came back to the latter as being most satisfactory as he found too great variation in the blood pressure of dogs and rabbits under the action of digitalis to allow of this method being of use. Any species of frogs may be employed, provided both the standard solution and the unknown preparation are tested upon the same species, for, while animals of different species vary in their reaction, those belonging to the same are much alike. Also frogs of any weight may be used, providing the proper allowance is made for the variation, but in testing a preparation, it is best to use frogs of nearly uniform size, varying from one another not more than 3 grams. The frogs to be employed should be freshly caught and carefully handled, being kept in wet moss until they can be placed in suitable pools at the laboratory.

The dose of the drug to be tested is calculated according to the weights of the frogs to be employed and made up with salt solution so that it will measure about one-half cubic centimeter, and is then introduced into the abdominal lymph sac by means of a pipette. The frogs are placed in separate jars and allowed to remain for twelve hours, at the expiration of which time they are examined and note made as to the dead and living animals. By a preliminary test in which the limits of dosage are quite wide some idea is formed as to the strength of the preparation and a second series of frogs is injected, still further narrowing the dose. When by this second group the probable toxic dose is obtained, a new series of five frogs is injected, and if the dose is correct at least three frogs out of the five should be

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[a] Houghton, J. Am. M. Ass., Chicago, 1898, XXXI, 959.

killed. By a simple calculation the relative strengths of the unknown and the standard preparation can then be obtained.

An important contribution to the subject of biological assay was furnished by Carl Bührer,[a] who investigated the activity of some of the toxic fluid extracts, among them being digitalis and convallaria. He selected frogs and rabbits as experimental subjects. The frogs, either R. esculenta or temporaria, were chosen with great care and obtained fresh every two weeks, but he used only one species for one preparation and for digitalis only R. temporaria. He seems to be the first to note any effect of sex upon the results and says he finally employed females exclusively. The effect of season upon the reaction of the frogs was corrected by repeating his experiments at various times of the year. Bührer seems not to have considered the size of the animal as being important, for while he weighed the frogs before the drug was injected, the weight was not used in calculating the dose or judging the results. The method he used was as follows: The frog was stretched out on its back and then, with as little hemorrhage and other injury as possible, its heart was exposed. The rate was counted at once and again after five minutes when the drug was injected into the thigh lymph sac and the animal put into a moist chamber and observed at intervals. The dose of the drug to be tested was prepared by diluting the fluid extract with different amounts of salt solution and injecting one-half cubic centimeter. The end reaction or standard consisted in finding the smallest amount of drug which would cause systolic stoppage of the heart within twenty-four hours. The comparative strengths of the different specimens were then reckoned as so many milligrams, according to the dilution of the drug, without paying any attention to the weight of the animal. By this method he found the specimens of 1897 varied in the ratio of 1 to 4, while the 1898 fluid extracts were weaker, but more uniform in strength. He though it was possible that this apparent weakness was due to a difference in the reaction of the frogs, as the 1898 specimens were examined one and one-half months after those of 1897, and he had found that even in from two to four weeks the animals might alter very considerably in their reaction to one and the same drug. This variation he thought was due to their winter fast, but in this case his supposition was proved incorrect by subsequent examination made in December, 1899.

Bührer further continued his experiments, employing rabbits which were arranged for blood pressure estimations. The diluted drug was injected intravenously at intervals of from five to ten minutes, until the lethal dose was reached. By carrying out several experiments

[a] Bührer, Untersuchungen über die Wirksamkeit einiger toxischer Fluid-Extrackte, Inaug. Dissert., Basel, 1900.

an average lethal dose was obtained, which was based upon the toxic amount per kilogram body weight.

The variations obtained on rabbits did not appear as great as with the frogs, being only 200 instead of 400 per cent. He tabulates his results as follows:

Fluid extract digitalis.	1897.		1898.	
	Frogs.	Rabbits.	Frogs.	Rabbits.
	Mg.	*Mg.*	*Mg.*	*Mg.*
A	17	400	33	590
C	25	630	25	780
D	33	770	25	740
B	67	890	.....	.....

The relation between the different preparations is not the same with the frogs as it is with the rabbits, and Bührer had expected this discrepancy, as he said the two animals are so entirely different. However, fluid extract A is shown to be the strongest by both methods

Fränkel[a] assayed Merck's digitoxin and later took up different infusions of digitalis which he obtained in and around Heidelberg. His method was to ascertain the amount of drug which would produce systolic standstill of the frog's heart in about an hour's time. If the heart stopped in a shorter time than fifty minutes the dose was lessened, while if it had not ceased to beat in one hour and ten minutes the dose was increased. The frogs (species not mentioned) were injected in a lymph sac, the amount of fluid being usually 1 c. c. except in some cases 2 or even 3 c. c. were employed. The animals were weighed and the toxic dose per 100 grams body weight calculated by a simple ratio.

His results showed that six infusions varied in toxic dose from 2.5 to 6.9 c. c., a difference of 100 to 275 per cent. Six tinctures examined varied from 0.6 to 2.5 c. c., 100 to 400 per cent, and seven strophanthus tinctures varied from 0.015 to 1.0 c. c. Fränkel thinks we are justified in transferring these results to the higher animals as Bührer was able to show a parallelism between the action on rabbits and on frogs, while he himself with four digitalis preparations showed the same ratio to exist on cats as on frogs. (Bührer's table, as he himself pointed out, shows a certain parallelism, but by no means an exact one.)

In 1902 Famulener and Lyons[b] undertook a study of the relative strength of various preparations of digitalis and related drugs. They employed frogs (species not mentioned), choosing those of about 40 grams weight and injecting the preparations to be tested into the ventral lymph sac by means of finely pointed pipettes. At the

[a] Fränkel, Therap. d. Gegenw., Berlin u. Wien, 1902, XLIII, 106.

[b] Famulener and Lyons, Proc. Am. Pharm. Ass., Phila., 1902, L, 415.

end of an hour the heart of the animal was exposed and examined. If the heart was found completely paralyzed the dose was too large; if pulsation still continued the amount was too small. The correct "end reaction" adopted by the writers was "paralysis" of the apex of the ventricle, but at the base of the ventricle there should occasionally be a faint wave of contraction while the auricle, though much distended, would continue to pulsate regularly. When the correct dose was found the amount necessary to kill a frog of standard weight (40 grams) was calculated by a simple proportion.

Famulener and Lyons found the reaction of the frogs very uniform, for not more than one or two out of a dozen healthy frogs showed a variation in susceptibility of as much as 10 per cent. To avoid this source of error, when they had determined the minimum dose in the manner indicated, they took a further series of three frogs, injecting into one the minimum dose, into a second a dose 10 per cent above the minimum, and into the third a dose 10 per cent below it. If any of this latter series showed irregularities further corrections were made. More uniform results were obtained by this method than could be obtained in the assay of such drugs as opium and nux vomica by chemical means. The authors give a table showing the results of their examination into the activity of the pure principles of digitalis and related drugs as well as of the galenical preparations. A very important contribution in their paper is a comparative study of the digitoxin content of three preparations of digitalis fluid extract with their activity as determined by physiological methods. This was referred to earlier in our article (p. 9).

Wolff[a] made a short report upon the biological dosage of digitalis preparations commenting upon the findings of Fränkel and quoting Professors Kobert and Gottlieb upon the necessity for a government station for the standardization of digitalis preparations. He then describes the method adopted by Brunnengräber, of Rostock, to bring into the market uniform preparations of these drugs. The leaves are collected on sunny days from wild plants before they begin to flower, carefully sorted to free them from any foreign leaves, and are then dried quickly in a vacuum at a definite temperature. In this way the fermentation which the leaves usually undergo during slow drying is avoided. Tinctures prepared from leaves, dried in this way, were examined by Professor Kobert, who reported them free from harmful split products and high in content of active principles. Strophanthus tinctures were also prepared from the fresh green seeds of S. Kombé which had not been freed from fat and these too were very active. An infusion made from 5 milligrams of digitalis leaves produced systolic standstill of the frog's heart as did also 0.02 c. c. of the tincture of strophanthus.

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[a] Wolff, Therap. d. Gegenw., Berlin u. Wien, 1902, XLIII, 423.

Ziegenbein[a] during 1901 and 1902 examined a number of specimens of digitalis leaves obtained from different localities estimating the digitoxin content and conducting comparative experiments by physiological methods. His results as given in the table on page 9 show that no definite parallelism between the two can be found. The toxicity of the digitoxin, which he was able to isolate by the Keller or Keller-Fromme method, only accounted for about one-third of the activity of the leaves from which it was obtained. He examined the toxicity of the extract which was left after shaking it out with chloroform to remove the digitoxin, and found that some principles which were insoluble in chloroform still remained and accounted for another one-third of the activity of the leaves, but the remaining one-third could not be accounted for. He carried out some experiments to determine the effect of the fineness of the drug upon the toxicity of the infusion which when powdered gave a higher value than did infusions made from leaves which were simply cut up. The toxic dose for the former was found to be 0.048 gram, while the latter required 0.064 gram to produce the same effect, and when finely powdered the leaves were most potent requiring only 0.03 gram, the difference probably being due to the greater surface of the drug exposed to the solvent. The method he employed for the physiological estimations was that originated by Hans and Arthur Meyer. In this method, freshly caught male frogs (R. temporaria) were secured upon a board and the heart exposed in the usual manner. Definite amounts of the drug to be tested were then injected into the thigh lymph sac and the smallest amount was sought which would produce systolic standstill within two hours. The minimum lethal doses thus found were calculated upon the basis of 100 grams frog weight in order to allow of comparisons. Frogs weighing about 25 grams were chosen for the experiments, but Ziegenbein did not think that a few grams difference in weight influences the results to any appreciable extent. He was also of the opinion that the season of the year makes very little difference in the susceptibility of frogs.

In the following year Moschkowitsch[b] published a critical study of the method of standardizing digitalis preparations by means of their action on frogs. His experiments were carried out on R. temporaria, which were fastened to a board and the sternum removed to permit of free observation of the heart, the pericardium being left intact to prevent drying. The drug to be tested was introduced into a lymph sac and its activity was measured by the time when it first produced an effect on the heart and by the time necessary to cause systolic stoppage.

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[a] Ziegenbein, Arch. d. Pharmacie, 1902, CCXL, 454. Ber. d. Deut. Pharm. Gesells., 1902, XII, 335.

[b] H. F. Moschkowitsch, Arch. d. Pharm., 1903, CCXLI, 358.

The author studied pure principles and also galenical preparations, and the most important fact which he brought out and especially emphasized in his work was the great variability in the reaction of frogs. The time of the first appearance of the action varied between two and six minutes, but a still greater variability was shown in the time of the appearance of systolic standstill. He said he was greatly discouraged by the great capriciousness in the reaction of the frogs, and the hope of an exact estimation by physiological methods received a mighty blow.

While in the estimation of his dosage he did not usually take into consideration the weight of the animal, yet in discussing his results he had several opportunities to compare the action of the drug upon animals of the same size. In some of his tables this factor seems to be important, while in others it would appear that it could be ignored; the hearts of frogs of the same size given the same dose stopping in widely different times, while those of different sizes stopped after like intervals when given the same sized dose. His conclusion upon this phase of the question was that it was best to take into consideration the weights of the frogs in making an assay. Upon the important question as to the effect of the season upon the susceptibility of frogs he found that summer (July) frogs possessed far greater resistance than winter frogs.

He points out that his results do not confirm those of the earlier workers, but he thinks the individuality of the animal is an important factor. According to this author, unless animals of about the same weight and of the same species can be obtained from a certain section of the country at the same time of year greater reliance can be placed upon chemical analysis. At any rate, he concluded that it was only in a very general way that we could tell from physiological experiments as to whether a preparation was good or bad, and while the method was not necessarily useless it was defective.

In the same year Brondgrest[a] also conducted a series of experiments to compare the action of the dialysate of digitalis grandiflora with digitalinum purum and the infusion of digitalis purpura. He employed for this purpose the frog's heart in situ, using the suspension method combined with the Williams perfusion apparatus. He concluded that the dialysate of digitalis grandiflora acts the same as D. purpuræ and as digitalinum.

Among the numerous writers upon the subject of standardization no one has contributed more than Focke,[b] who published several articles between 1902 and 1906. His method has undergone one or

[a] Brondgrest, Zentrbl. f. innere Mid., Leipz., 1903, No. 24, 906.

[b] Focke, Therap. d. Gegenw., Berlin u. Wien, 1902, XLV, 44; Arch. f. Pharm., 1903, CCXLI, 128, 669; Deutsche Aerzte Ztg., Berlin, 1904, VI, 272, 292; Therap. d. Gegenw., Berlin u. Wien, 1904, XLV, 250; Berlin klin. Wchnschr., 1906, XLIII, 642.

two important modifications since it was originally published, but otherwise it is essentially the same. He employs frogs (Rana temporaria) which are collected not earlier than the end of June. They are kept in a cellar in a box the bottom of which is covered with water, the latter being changed at least daily. The frogs should not be used before the third day of captivity and should be brought to the examining room six hours before they are needed, being placed in jars at a temperature not over 17° C. The examinations should be carried out in July, August, and September, during which months the susceptibility of the frogs does not differ greatly, and also during this time no distinction need be made with reference to sex. Animals weighing between 25 and 30 grams are selected and fastened to a board in the usual manner, and the heart is exposed without allowing bleeding or other injury (pithing is not permitted at this time). After opening the pericardium a slight pressure upon the abdomen will cause the heart to protrude from the opening, where it will remain in plain view. A measured quantity of the infusion to be tested is now injected into the two leg lymph sacs, about 0.3 c. c. into each or 0.6 c. c. in all. Both lymph sacs are used so that the distended skin will not force the drug out of the opening by pressing upon a large bulk of fluid. Systolic standstill of the heart is now watched for and it should appear in ten or fifteen minutes. When this is observed and the time noted, the frog is pithed and weighed.

Two more frogs are now selected of about the same weight, and the dose to be injected is determined by the effect of the primary dose. If the heart, in the first frog, stopped in less than ten minutes, a relatively smaller dose is used, while if it did not stop in twenty minutes a larger dose should be employed. A sufficient number of frogs should be used so that four are found in which the hearts have stopped between seven and twenty minutes. The toxic value (V) of the drug is then determined in relation to the size of the frog (P) divided by the dose of the drug given (d) multiplied by the time it takes for the heart to come to a systolic standstill (t): $(V) = \frac{P}{d \times t}$. The "Vs," having been determined for each trial, are averaged and the amount found is considered as representing the value of the preparation of digitalis which has been tested. Focke considers that a good specimen of leaves should give a standard value of 5. He originally accepted all readings between ten and twenty-five minutes, but the method was modified later to time limits of seven to twenty minutes, as he found the results were much more uniform. If the heart stops in a shorter time than seven minutes, the differences in the values found are greater, or if over twenty minutes, he found the readings were much more variable, and the "Vs" obtained were very small. For this reason he dislikes Ziegenbein's and Frankel's methods, the former

taking the least amount which will stop the heart which will usually occur anywhere between thirty-five and one hundred minutes (Ziegenbein says within two hours), while the latter takes as his end reaction the stoppage in from fifty to seventy minutes. Focke, therefore, prefers his method because it is more convenient, taking less time and because it is more exact, showing according to him only 8 per cent of error.

After Focke developed this method he considered a number of factors which influence the results of standardization and the variability of digitalis preparations. In order to determine the effect of season upon the frog's susceptibility, he examined leaves which had been very carefully dried and which would retain the same value at the various times of the year. He found that in the spring they gave an average value of 2.66; from the end of June to October, 4.36; and in October, 5. At this time he considered these variations due to differences in the nutrition of the frogs, but later researches[a] seemed to indicate that the temperature factor might also play some part in changing the V obtained.

Thus frogs operated on at a temperature of 17.5° to 18.5° in December showed a V of 5.5. At the same temperature the same drug had a V of only 4.7 the last of February, but at 19° to 21° gave nearly the original value. In June, to obtain this value, it was necessary to increase the temperature to from 22° to 23°, at 18.5°, the original December temperature, the value being only 3.8. At the same temperatures there seemed to be no difference in the reaction of frogs which had been captured recently and those kept in captivity for several months.

Physiological standardization may be carried out according to Focke, therefore, at any time during the year, but it is first necessary to standardize the animal against digitalis leaves of known value by regulating the temperature and then by cooling or heating the operating room to carry out the assay at the temperature found to show the standard value as arbitrarily adopted.

It has been recognized that wild plants are more toxic than cultivated, and in confirmation of this view Focke found the former to give a V of from 5 to 6, while the latter gave as Vs 2.6 and 3. The yearly variation in plants from the same district he found to be quite marked, the weakest giving a value of 4.3, while the strongest had a V of 8.5. It has been universally recognized also that the second-year plants just before flowering are more active than the first year. In agreement with this view Focke found that the second-year leaves collected in June have a toxic value 15 to 20 per cent higher than first-year leaves collected from plants grown in the same locality. Plants flowering in July seed in August, and at this

[a] Focke, Arch. d. Pharm., 1907, CCXLV, 646.

time the leaves show the effect by a lessened toxicity, the relation being reversed, as at this time the first-year leaves are stronger. The effect of daylight was found to be very slight, even if the leaves were exposed to its influence for a year. As to the effect of the aging of the leaves he found that if they were collected and dried in the air in the ordinary way and preserved in nonair tight flasks they will lose a large part of their activity.

The greatest factor in this deterioration Focke considers to be the moisture content, and the changes which the leaves undergo during the first few months after their collection are largely due to this, as in the presence of moisture certain ferments present in the leaves are able to destroy some of the active constituents. To avoid this, he heats the leaves rapidly and strongly at a temperature not over 100° C., and when they are dried so as to contain only about 1½ per cent moisture they are preserved in lots of 50 grams in dark, air-tight jars. He found that if dried and preserved in this manner they lost only 5 per cent in V in a year. The last 1 per cent of moisture left in the leaves seemed to be bound in some manner, so that it was not active.[a] To show the importance of the moisture content factor, Focke believed that only about one-fifth of the differences in strength were due to all the other factors.

To avoid the variation due to the different localities, the leaves from all the different sources should be powdered after drying and mixed, so as to give a uniform product.

Siebert[b] reports the efforts of himself and Ziegenbein to determine a method by which digitalis and strophanthus preparations could be standardized biologically. The method adopted by them was the one developed by Arthur and Hans Meyer, which has been described earlier in the discussion of Ziegenbein's work.

In fifty experiments with digitalis leaves the most active preparation showed a toxicity of 0.03 gram for 100 grams frog weight, while the weakest required 0.075 gram to produce death. They, therefore, adopted as a standard strength for digitalis leaves 0.04 gram for 100 grams frogs.

The variation in strophanthus preparations was as follows: Weakest 0.06, strongest 0.008 c.c. for 100 grams frogs. The standard they adopted was 0.02 c.c.

An important chapter on the subject relating to the comparative study of the chemical and biological methods of assay of digitalis

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[a] In this connection it is interesting to note that in 1867 Tourdes (Gaz. med. de Strasb., 1867, XXVII, 191) pointed out that the reason the digitalis preparations obtained in Strassburg were superior to those used elsewhere was because the leaves of the second year's growth were employed after having been selected carefully and dried first in the shade, and then in an oven at a temperature not over 40° C. The leaves were then preserved in tin or glass vessels away from the light and moisture.

[b] Siebert, Berl. klin. Wchnschr., 1903, XL, 813.

was contributed by Barger and Shaw[a] in 1904. Their investigations were carried out upon tinctures of digitalis prepared according to the British Pharmacopœia and obtained from various commercial sources. For the estimation of the digitoxin content they used Keller's method with slight modifications. For the biological experiments male frogs (Rana temporaria), weighing between 20 and 35 grams, were used almost exclusively, but comparative experiments were limited to those of nearly the same weight. The experiments were carried out in the early summer.

The tinctures were modified by evaporating a known amount over a hot-water bath and suspending the residue in hot water. The digitoxin employed in some of these experiments was dissolved in alcohol and later diluted with water so as to give it an alcoholic strength of 10 per cent, the digitoxin being injected in suspension. In all cases the drugs were injected into the dorsal lymph sac, the volume for decisive experiments being 1 c.c. The animals after injection were kept in a moist atmosphere and the time noted when the heart stopped, which always occurred within three hours, if at all.

After an estimation by chemical means of the digitoxin content in nine tinctures, they estimated the toxic value of the same tincture by the biological method described. The results obtained by the latter method show less variation between the tinctures than is usually found, as the strongest was only one and one-half times as toxic as the weakest.

They show that, on account of the slight toxic strength of digitalin and digitalein, a supposition exists that the toxicity of a tincture runs parallel to the digitoxin content (by Keller's method), but this is not correct, as they found that the ratio of toxicity of tinctures to the toxicity of the digitoxin content in them was as 1 to 3.5, 5, 3.7, 5.5, 6.9, 4.1, 4.1, 5, and 4.2.

The writers then estimated physiologically the toxicity of the water soluble active constituents, and this, added to the toxicity of the digitoxin, was compared to the total toxicity of the tincture. The results show that about 50 per cent of the tincture toxicity was still unaccounted for even when both water-soluble and insoluble constituents were considered.

In order to find the cause of this discrepancy they prepared a tincture from chaff and hay and added to this 0.04 per cent crystallized digitoxin. By biological methods 0.0375 per cent was demonstrated, while by Keller's method only 0.01 per cent was found. The cause of the difficulty was shown by further experiments to depend upon the fact that much of the digitoxin adheres to the resin which separates out when the alcohol is evaporated off from the tincture, this being lost in the chemical method of estimation, but appearing in the physiological.

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[a] Barger and Shaw, Pharm. J. & Tr., London, 1904, XIX, 249.

According to their results it appeared that 1 gram of the tincture would kill a 59-gram frog, but the water soluble active constituents would only kill a 15-gram frog. The 44 grams difference in frog weight therefore ought to be killed by the insoluble portion or digitoxin. The toxic value for the digitoxin for only a 17-gram frog had been demonstrated by the Keller method, however, leaving 27 grams of the toxicity of the digitoxin of the original tincture still unaccounted for and which probably remains in the resinous portion when Keller's method of determination is used. In other words, only about one-third to one-half of the digitoxin present is isolated by this method, and the authors conclude, therefore, that the only reliable method for the assay of digitalis tinctures is the physiological one.

Freund[a] studied the action of abyssinin and compared it with drugs of the digitalis groups, notably, digitalin, digitalein; digalen, the dialysate of digitalis, and strophanthin.

For the purposes of this investigation he used frogs and rabbits. With the former the heart was laid bare and attached to a suspension writing lever, thus obtaining tracings showing the progressive action of the drug. In some animals a solution of the drug was dropped directly on the heart, while in others it was injected into a lymph sac. In the case of rabbits Freund used the blood pressure changes as a method for comparing the relative action of the drugs.

In his study of the different heart drugs, Kakowski[b] examined a number of digitalis preparations, among them being the infusion and the tincture. He employed the isolated hearts of frogs (R. temporaria), rabbits, cats, and dogs and showed that the infusion acted more strongly upon some of the animals than the tincture.

Still another modification of the use of a frog's heart in estimating the activity of members of the digitalis group was adopted by Santesson[c] in his examination of the strength of different varieties of strophanthus seeds. In this method the drug was injected into a lymph sac of Rana temporaria. The animal was not tied down, but was held on its back in such a way that the contractions of the heart could be observed through the skin. In case it was necessary, a glass rod was passed into the esophagus so as to press the heart forward to facilitate the determination of its rate. Finally, to allow of exact timing of the appearance of the systolic standstill, the chest was opened and the organ observed directly. The standard dose employed by Santesson was that amount of the drug which would produce systolic standstill of the heart in thirty minutes, the dose being reckoned upon the basis of a 50-gram frog. Santesson's custom was to give a large dose at first, one that would be sure to kill, and on

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[a] Freund, Zeitschr. Exp. Path. u. Therap., 1905, I, 557.

[b] Kakowski, Arch. Internat. de Pharmacod. Gand et Par., 1905, XV, 21.

[c] Santesson, Skandi. Arch. f. Phys., 1905, XVII, 389.

subsequent injections to diminish the amount until that dose was reached which would stop the heart in the required time. He points out that the biological standardization is not easy on account of the irregularity of the reaction of frogs. To avoid this as much as possible there should be taken into consideration the effect of season, species, body weight, temperature, condition of health, and, finally, a factor which can not be reckoned upon, viz, individual idiosyncrasy. The frogs employed by Santesson were captured in the autumn and kept in a cool place until they were used, in November and December. Males of about 50 grams weight were selected and kept in the examining room at least one hour before they were used. With these precautions Santesson showed that tinctures prepared from different varieties of strophanthus seeds varied in strength in the ratio of 1 to 4.

A second method of comparison was to perfuse the frog's heart, using William's apparatus, comparing the effect of the different drugs upon the pulse frequency, circulation rapidity (number of drops perfused per minute), and the pulse volume.

The results obtained by the two methods furnish a very interesting comparison.

Preparation.	Lethal dose for intact frog.	Lethal dose, isolated heart.
1	0.063	0.047
2	.10	.123
3	.22	.25
4	.10	.046
5	.064	.027
6	.051	.0925

Santesson calls attention to the fact that a certain parallelism exists in the results obtained by the two methods, but it is certainly very slight. He explains the lack of closer agreement as being due to the fact that the poison would not be so uniformly distributed in the fluids of the intact animal as in a fluid used to perfuse an isolated heart.

Dixon,[a] in a paper on the biochemical standardization of drugs, considers the frog as the most suitable animal for digitalis standardization. In employing them the time of year should be taken into consideration, as they are most active and vigorous in the summer and least active in the spring, but their sensitiveness to digitalis will not vary over 50 per cent during the year. Those selected should be males of about 25 grams weight and free from abnormal conditions. The tincture, diluted with an equal amount of water, is injected into the dorsal lymph sac and 6 minims of this diluted tincture should

[a] Dixon, Pharm. J. and Tr., London, 1905, p. 155; Manual of Pharmacology, 1906, p. 34.

produce systolic stoppage of the heart in one hour. Dixon proposes this as a standard "unit," and if a certain tincture does not correspond to this strength the pharmacist should make the necessary calculations, so as to give the patient a uniform product. The writer thinks the method proposed above may be accurately controlled by experiments upon rabbits.

In his Manual of Pharmacology (1906, p. 34), after discussing the frog method, Dixon says: "These drugs can be standardized more accurately by perfusing the isolated rabbit's heart with Ringer's solution and subsequently adding the drug." However, Sowton's results, referred to later, hardly confirm this statement.

Haynes [a] sought to determine the relative activity upon the heart of the three important members of the digitalis group—digitalis, strophanthus, and squills—using tinctures which he prepared very carefully according to the British Pharmacopœia. He standardized these by determining the smallest dose of each which, when injected into the dorsal lymph sac of a frog, would cause systolic stoppage of the heart. He also carried out comparative tests upon rabbits with these preparations, injecting the drugs invariably into the stomach and watching for changes in the blood pressure as well as for the toxic effects upon the heart. In addition he studied their relative strength upon the excised hearts of rabbits, employing a modification of the Langendorff method and compared especially the effect upon the rate and the quality of the heart beat.

In respect to the suggestion that, on account of the variation in the strengths of galenical preparations of this series, the active principles should be substituted for them in general practice, Haynes says that in their isolation much of their potency is lost and that they require standardization even more than the galenicals.

The Pharmacopœia of Norway (1870) directed that official preparations of digitalis should be made from the plant growing wild in Norway. Wang [b] undertook to determine whether the plants growing in that country offered any advantage as regards strength over those growing elsewhere.

He used frogs (Rana temporaria) and adopted Focke's method of assay described earlier. His experiments were carried out in Strassburg in October, 1905. The frogs were kept in a cool cellar, and the evening before they were needed they were placed in a cool room, being brought to ordinary room temperature two or three hours before they were used. The animals were injected with the 10 per cent infusion through the lateral lymph sacs into the two thigh lymph sacs. In this manner any escape of the drug was avoided when the chest was open. The heart was exposed as soon as the animal was

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[a] Haynes, Bio-Chem. Jour., 1906, I, 63.

[b] Wang, Festschr. f. Olof Hammarsten, 1906.

injected and the time noted when systolic standstill appeared. Wang, in common with some other workers, found it hard to decide at just what time this end reaction occurred, and he therefore used the stoppage of the circulation as an end reaction. His results, using Focke's formula to give the toxic worth of the leaves, are very variable. In some of the tables, especially Table I, in which, in sixteen estimations of one preparation, he obtained values between 3.6 and 8.8, with an average of 5.2. The other tables are not nearly so irregular in their results, since in these he calculated his dose according to the body weight of the animal employed. Table III, for instance, only shows slight variations from 4.7 to 5.7, with an average worth for the specimen of 5.3.

Löwy [a] carried out experiments with infusions of digitalis to determine the effect of hydrochloric acid and pepsin, in the per cents in which they occur in the stomach, upon their action. He used 20-gram frogs (Rena temporaria), exposing the heart and after injecting 1 c. c. of the solution to be tested, noted the time of the appearance of systolic standstill.

Kochman [b] showed that five infusions of digitalis made from leaves obtained from different sources possessed different actions upon the blood pressure of dogs, only two of them producing the true digitalis effect of increased blood pressure and lessened pulse rate.

In a research to determine the uniformity of the preparations of digitalis and strophanthus which were upon the market Edmunds [c] examined seventeen preparations of the tinctures of digitalis and six of strophanthus. Although many of these were said to be physiologically standardized they showed great variation, two preparations (standardized) from one manufacturer having toxicities in the ratio of 1 to 2. Four nonstandardized preparations from one manufacturer varied as 1 to 4.

The method employed to standardize the drugs was the same as has been used in the pharmacological laboratory of the University of Michigan for a number of years and is essentially the same as that employed by Famulener and Lyons, [d] the only difference being that as an end reaction complete systolic stoppage of the heart is used, not only of the ventricle, as in the Famulener and Lyons method, but also of the auricles.

In his comparison of various tinctures of strophanthus which were on the market Hatcher [e] adopted Frankel's method of examination; that is, the amount necessary to produce systolic standstill of the frog's heart in about one hour, comparing the doses when calculated

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[a] Löwy, Wien klin. Wchnschr., 1906, XIX, 1157.
[b] Kochman, Bull. Soc. de Med. de Gand., 1906, LXXIII, 95.
[c] Edmunds, J. A. M. A., 1907, XLVIII, 1744.
[d] Famulener and Lyons, Proc. Am. Pharm. Ass., 1902, L, 415.
[e] Hatcher, J. A. M. A., 1907, XLVIII, 1177.

on the basis of 100 grams frog's weight. He also compared their action upon cats and dogs, these reacting by vomiting. He suggested on account of the regularity of the appearance of this symptom, that it might be thought of as an index of the potency of the drug but because of the variability of the reaction of the vomiting center he "considered it better to determine the amount necessary to produce systolic standstill which is, of course, promptly followed by death."

In his article "Ueber die physiologische Wertbestimmung von Arzneimitteln" Gottlieb[a], after discussing the work of Frankel, Zeigenbein, and Bührer upon physiological standardization, mentions the results of his investigation upon the uniformity of digitalis leaves used in the clinics around Heidelberg, showing that they varied as much as fourfold. He calls attention to the necessity of a government institution where such preparations can be examined, a fact to which attention has been called by several earlier writers.

In order to obtain a convenient method of designating the strength of preparations he suggests the adoption of a standard "unit," using the term in the same way as it is used in connection with sera work. For such a unit he has adopted the following: "The smallest amount of the solution which will call forth systolic standstill of the heart of a R. temporaria of 30 grams weight in thirty minutes exactly." In an infusion freshly prepared from 1 gram of good digitalis leaf powder there must be 30 to 40 units; the strongest leaves may contain 120 units.

He further points out that such a comparison on the frog's heart is only possible when made with similar active constituents, such as we have in the leaves, infusion, and tincture of digitalis, but could not hold good with dissimilar constituents as, for instance, a comparison of strophanthus with digitalis. A further fact which he calls attention to is that, in the methods usually employed in which the heart is to be brought to a systolic standstill in a certain short time, a strong solution of a substance, absorbed with difficulty, would appear weaker than a weak solution of easily absorbable substances.

Sowton[b] undertook to examine the activity of twenty-six specimens of the tincture of digitalis by means of perfusing the hearts of rabbits. He chose animals weighing 3 or 4 pounds each, and, using the coronary vessels, perfused the right ventricle with a solution of digitalis of a strength of 1 to 200 made up in Locke's or Ringer's solution. From his results he grouped the preparations into two classes, "strong" and "weak," but later found that such a classification was impossible, as five of his specimens upon which seventeen experiments had been carried out were placed some in one group and some in the other, and yet they were probably alike, having been made in exactly the same

[a] Gottlieb, München. med. Wchnschr., 1908, LV, 1265.

[b] Sowton, Brit. M. J., London, 1908, No. 2458, 310.

manner from the same lot of leaves. The results indicated the practical worthlessness of the method, as rabbits' hearts evidently exhibit marked variations.

Lutzkaja[a] published a critical study of the Focke method of standardization which he thinks is preferable to the one-hour method which, according to his views, is too inexact. He carried out the experiments according to Focke's directions with the exception that he used September and October frogs.

In his first experiments, with 0.5 milligram doses of digitoxin injected into frogs of different weights, he obtained toxic values showing as great variations as from 1.9 to 8.3 with an average of 4.5. For his second experiments he estimated the doses so as to stop the heart in about ten minutes, and by this means the results were much more uniform, differing only 80 per cent. In a third experiment his results varied still less, only 65 per cent. His conclusion is that, in spite of some irritating individual variation, if a sufficient number of experiments are carried out useful results may be obtained. He urges as an objection to the "short-time methods" that they might not give sufficient time for all the poison to be absorbed and by this means a very active preparation, absorbed with difficulty, would appear weaker than a weak preparation containing easily absorbable constituents.

His results show that digitoxin and digitalinum verum do not give the same ratio on frogs as their relative doses appear to indicate that they possess in man; in some preparations of digitalis he suggests there might be a great deal of digitoxin, but in others not as much, and while they might show the same effect on frogs they would not on man. He therefore thinks such a condition would render the standardization of the leaves on frogs of little value.

Practically every worker upon this subject has employed the frog in some way or other as a means of standardizing the preparations of the drugs under consideration and the results obtained upon this animal have been confirmed in some cases by control experiments carried out, usually upon the blood pressure, upon the higher animals. However, in 1908 Reed and Vanderkleed[b] introduced a method, using guinea pigs, as they object to the frog because the reaction of this animal is very irregular, being affected by the season of the year, the species, etc. They also point out that the action upon the heart is a toxic effect, a determination of the lethal dose, and therefore they substitute for the toxic dose in frogs the toxic dose in guinea pigs. To make the assay they prepare their tinctures by evaporating off the alcohol and diluting the residue with water. Progressively increasing doses are then injected subcutaneously into guinea

[a] Lutzkaja, Arch. internat. de Pharmacod, Gand et Par., 1908, XVIII, 77.
[b] Reed and Vanderkleed, Am. J. of Pharm., 1908, LXX, 110.

pigs of 240 grams weight until the amount is found which will kill the animal in one and one-half to two hours. Their standard dose then, which they confirm by injecting like amounts into five or six animals, is that amount which would kill a guinea pig of 240 grams weight in one and one-half to two hours. They carried out a series of experiments to compare the digitoxin content of tinctures with the physiological activity. These results are tabulated on page 9.

EXPERIMENTAL WORK.

As will be seen from a study of the literature that has been given there are essentially three methods used for the biological assay of the members of the digitalis series, the others being merely modifications departing more or less widely from what we may consider the primary groups. These may be classified as follows: A toxic method, in which frogs, guinea pigs, or some of the higher animals are used; second, a method using the frog's heart, which is perfused in some cases while in others it is simply exposed and systolic standstill is watched for (this in Focke's method is reached in from seven to twenty minutes, while in others a longer interval of one or two hours is allowed); the third class includes those methods which aim at a comparison by means of the relative effects upon the blood pressures of some of the higher animals.

One of the objects of the present study has been to examine into the results of these various methods to find if possible if one possessed a marked advantage over the others, and, most important of all, to see whether the different methods would give anything approaching uniform results in a comparative study of a number of digitalis preparations, some of which have been made according to the pharmacopœial requirements, while others had been manufactured according to some special method devised by individual pharmaceutical houses. A study of these preparations constitutes an important part of the work, but was not the primary object of the research.

With these objects in view we have examined nine preparations of digitalis, namely: Fluid extracts prepared according to the U. S. Pharmacopœia, VIII, by Messrs. Parke, Davis & Co., Detroit; Sharpe & Dohme, Baltimore; Nelson, Baker & Co., Detroit; Hance Brothers & White, Philadelphia. In addition to these we examined several proprietary preparations as follows: Three specimens of Digitalone, prepared by Parke, Davis & Co.; a fat-free tincture of digitalis (Digitol), prepared by H. K. Mulford & Co., Philadelphia; Digitalis, Specific Medicine, manufactured by Lloyd Brothers, Cincinnati; a Concentrated Tincture of Digitalis (1 to 4), prepared by Burroughs, Wellcome & Co., London, and finally a purified "Normal Tincture," prepared by William S. Merrell & Co., Cincinnati.

Digitalone is said to be a "nonalcoholic permanent solution of digitalis" corresponding in strength to the U. S. Pharmacopœia tincture and physiologically standardized. The manufacturers state that

Digitol is a preparation of tincture strength made from digitalis leaves from which the fixed and volatile oils have been removed. It is "assayed, tested physiologically," and is standardized to contain 0.025 gram digitoxin in 100 c. c. Digitalis (Specific Medicine) is made from fresh digitalis leaves, and is of such a strength that 480 grains of the drug are contained in each fluid ounce of "80 per cent absolute alcohol." Concentrated Tincture of Digitalis (1 to 4) is said to be physiologically standardized and is of such a strength that 1 c. c. added to 4 c. c. dilute alcohol makes a preparation corresponding in strength to the U. S. Pharmacopœia tincture. Purified Normal Tincture is said to be a standardized neutral tincture of prime digitalis freed from irritating fats and oils.

It was desired for the sake of comparison that all experiments with these various preparations be carried out upon the basis of the official tincture strength. No difficulty was experienced in doing this except with Merrell's Normal Tincture, the name of which might suggest that it was a 10 per cent preparation, but for reasons to be considered later, the dose prescribed, etc., it was considered to correspond to a fluid-extract strength. Accordingly a measured quantity of each preparation (excepting Digitol and Digitalone) was taken and after evaporating off the alcohol over a water bath it was diluted to ten times the original volume with physiological salt solution. Digitol was evaporated to free it from the contained alcohol and then diluted to the original volume only. Digitalone, being nonalcoholic, was not evaporated excepting in some experiments when it was concentrated in order to avoid such a large bulk of fluid as the doses given required. In all cases resinous bodies, precipitated by the evaporation of the alcohol and the addition of the salt solution, were injected in suspension to insure the introduction of any active bodies which might be carried down by their precipitation.

For the sake of uniformity the following abbreviations have been adopted to designate the above preparations in all of the tables which follow:

Digitol, bottle No. 1 = Mulford No. 1.
Digitol, bottle No. 2 = Mulford No. 2.
Normal Tincture Digitalis = Merrell.
Concentrated Tincture Digitalis = B., W. and Co.
Specific Medicines, Digitalis = Lloyd Bros.
Nelson Baker and Co., Fluid Extract Digitalis = N., B. and Co.
Sharpe and Dohme, Fluid Extract Digitalis = S. and D.
Hance Bros. and White, Fluid Extract Digitalis = H. B. and W.
Parke, Davis and Co., Fluid Extract Digitalis = P., D. and Co.
Parke, Davis and Co., Digitalone bottle No. 1 = Digitalone No. 1.
Parke, Davis and Co., Digitalone bottle No. 2 = Digitalone No. 2.
Parke, Davis and Co., Digitalone bottle No. 3 = Digitalone No. 3.

MINIMUM LETHAL DOSE METHOD OF ASSAY.

The method adopted for examination and the animals employed by us as illustrating the toxic methods of assay were as follows:

Mice.—White mice, kept under the same conditions and varying in weight from 15 to 25 grams, were used. The drug was injected hypodermically beneath the skin of the back, the dose being calculated at so many milligrams per gram of body weight and so diluted that a dose of 1 c. c. was not exceeded in any case. The mice were then watched and the death or survival of the animal noted. The data thus obtained (as were the results in all subsequent series of experiments) were confirmed by injecting a second series of mice several days later. The results of these tests are given in Table I.

TABLE I.—*Determination of the minimum lethal dose per gram body weight for white mice; subcutaneous injection.*

[Survived = −; dead = +.]

MULFORD NO. 1.

Number of animals used.[a]	Dose.	Results.
	mgm.	
1	2	−
4	3	−
5	4	+
3	5	+
3	6	+

MERRELL.[b]

1	3	−
1	4	−
2	5	−
1	5	+
3	6	+
2	7	+

B. W. AND CO.

3	3	−
2	4	+
1	5	+
1	6	+
2	7	+

H. B. AND W.

3	6	−
1	7	−
3	7	+
2	8	+
1	9	+

DIGITALONE NO. 1.

1	10	−
1	19	−
1	20	−
1	22	+
1	25	+
1	28	+
1	30	+

LLOYD BROS.

Number of animals used.[a]	Dose.	Results.
	mgm.	
2	18	−
2	19	−
2	20	−
1	20	+
2	21	−
1	21	+
1	22	−
2	22	+
3	23	−
2	23	+
2	24	−
4	24	+

N. B. AND CO.

3	7	−
2	8	−
2	8	+
3	9	+
1	11	+

S. AND D.

2	6	−
2	7	−
4	8	−
1	8	+
2	9	−
3	9	+
1	10	−
3	10	+

P., D. AND CO.

2	3	−
3	4	+
3	4	−
2	5	+
3	5	−
2	6	+
3	6	−
2	7	+
3	7	−
4	8	+

[a] The heading "Number of animals used" refers to the number of animals which received the dose indicated in the second column.

[b] Dose calculated on the basis that preparation was of fluid extract strength.

TABLE I.—*Determination of the minimum lethal dose per gram body weight for white mice; subcutaneous injection*—Continued.

SUMMARY.

Minimum lethal dose for mice per gram body weight.

Preparation.	Milligrams.
Mulford (digitol)	4
B. W. and Co. (concentrated tincture)	4
Merrell (normal tincture)	6
H. B. and W. (fluid extract)	7
P., D. and Co. (fluid extract)	8
N., B. and Co. (fluid extract)	9
S. and D. (fluid extract)	9
Digitalone No. 1 (P., D. and Co.)	22
Lloyd Bros. (specific medicine)	24

Guinea pigs.—Guinea pigs of about the same weight were injected beneath the skin of the abdomen with the drugs to be tested. The dose was reckoned according to the weight of the animal and the preparations were so diluted as to make the bulk of the fluid injected between 1 and 2 c. c. The results of these experiments are recorded in Table II.

TABLE II.—*Determination of the minimum lethal dose for guinea pigs—subcutaneous injection.*

[Survived = −; dead = +.]

MULFORD NO. 1.

Number of animals used.[a]	Dose.	Result.
	mgm.	
1	0.56	−
1	.625	−
2	.65	−
1	.70	−
1	.70	+
1	.75	+
1	.80	+

MERRELL.

1	0.40	−
2	.50	−
4	.50	+
3	.60	−
5	.60	+
1	.65	+
2	.70	+

B. W. AND CO.

1	0.25	−
2	.30	−
1	.35	−
2	.35	+
1	.40	+
1	.55	+
1	.68	+

LLOYD BROS.

Number of animals used.[a]	Dose.	Result.
	mgm.	
2	0.70	−
2	.80	−
2	.80	+
2	.90	−
3	.90	+
1	1.00	−
2	1.00	+
1	1.50	+
1	2.00	+

DIGITALONE NO. 1.

1	2.00	−
1	4.00	−
1[b]	5.00	+

N., B. AND CO.

1	0.50	−
1	.70	−
2	.80	−
2	.80	+
3	.90	+
2	1.00	+
1	1.20	+

[a] This heading refers to the number of animals which received the dose indicated in the second column.
[b] Guinea pig, weight 250 grams, injected with 12.5 c. c., digitalone No. 1. No digitalis symptoms whatever appeared, the animal passing at once into a comatose condition. The respiration became weaker and finally ceased. Cause of death (?).

TABLE II.—*Determination of the minimum lethal dose for guinea pigs—subcutaneous injection*—Continued.

DIGITALONE NO. 3.

Number of animals used.	Dose.	Result.
	mgm.	
1	0.90	−
1	1.50	[a]−

H. B. AND W.

1	0.30	−
2	.40	−
2	.50	+
1	.70	+
1	1.00	+

P., D. AND CO.

Number of animals used.	Dose.	Result.
	mgm.	
1	0.50	−
1	.60	−
1	.70	−
2	.70	+
2	.80	+

S. AND D.

1	0.70	−
3	.80	−
1	.80	+
1	.90	−
4	.90	+

[a] No symptoms.

Digitalone No. 3 was used in injecting two guinea pigs, one receiving 0.9 milligram and the other 1.5 milligrams per gram weight. Neither of these animals showed any symptoms whatever excepting that they were somewhat less active than normal.

SUMMARY.

Preparation.	M. L. D.[a]
	mgm.
B. W. and Co	0.35
H. B. and W	.50
Merrell	.60
Mulford No. 1	.70
P., D. and Co	.70
N., B. and Co	.90
S. and D	.90
Lloyd Bros	.90
Digitalone No. 1	5.00(?)
Digitalone No. 3	[b]1.50

[a] M. L. D.—minimum lethal dose. [b] Lived.

Cats.—The toxic dose was determined for these animals in the course of the blood-pressure experiments. After the initial injection of 1 c. c. of the 10 per cent solution, further injections of 0.5 c. c. each were administered at intervals of not less than 5 minutes, artificial respiration being maintained so that the toxic action was due to an effect upon the heart rather than upon the medulla. This was thought desirable because in some of the earlier experiments the respiration was observed to have failed, although a good circulation was being maintained, while in others the animal would make apparently normal respiratory efforts after the heart had stopped and the blood pressure had fallen to zero. The toxic doses were compared on the basis of the number of milligrams of the drug used per kilogram of body weight of the animal. The results of this series of experiments and the average for each drug used are given in the last two columns of Table III.

TABLE III.—*Determination of blood pressure and minimum lethal dose for cats, intravenous injection. Fluid extracts diluted to 10 per cent strength.*

	Heart rate.	Blood pressure.	Percentage increase in blood pressure.	Lethal dose.	Lethal dose per kilogram, body weight.
N., B. AND CO.					
				c. c.	*mgm.*
August 4, 1908, cat, 3,800 grams:					
Normal	126	55			
After 1 c. c	150	122	122	7	184
September 9, 1908, cat, 3,780 grams:					
Normal	90	38			
After 1 c. c	90	60	58	6	158
September 9, 1908, cat, 2,930 grams:					
Normal	120	36			
After 1 c. c	120	78	116	7	235
Average			99		192
MULFORD NO. 2.					
August 6, 1908, cat, 1,380 grams:					
Normal	162	60			
After 1 c. c	186	140	133	2	145
August 6, 1908, cat, 1,870 grams:					
Normal	192	68			
After 1 c. c	186	86	27	4	214
September 4, 1908, cat, 3,250 grams:					
Normal	198	66			
After 1 c. c	174	84	27	7	215
September 11, 1908, cat, 2,870 grams:					
Normal	180	94			
After 1 c. c	180	158	68	6	208
Average			64		195
DIGITALONE NO. 2.					
August 5, 1908, cat, 1,800 grams:					
Normal	174	66			
After 1 c. c	168	100	52	9	500
September 9, 1908, cat, 2,500 grams:					
Normal	150	38			
After 1 c. c	160	60	58	15	600
Average			55		550
B. W. AND CO.					
August 5, 1908, cat, ——:					
Normal	198	80			
After 1 c. c	210	104	30		
August 7, 1908, cat, 1,150 grams:					
Normal	180	39			
After 1 c. c	186	74	90	2	174
September 4, 1908, cat, 3,500 grams:					
Normal	138	50			
After 1 c. c	138	66	32	5	143
Average			51		158
P., D. AND CO.					
August 5, 1908, cat, 1,600 grams:					
Normal	114	40			
After 1 c. c	126	55	38	3	187
September 5, 1908, cat, 3,220 grams:					
Normal	174	56			
After 1 c. c	162	76	36	5	155
Average			37		171
S. AND D.					
August 7, 1908, cat, 1,150 grams:					
Normal	216	94			
After 1 c. c	210	112	19	2	174
September 5, 1908, cat, 1,690 grams:					
Normal	144	40			
After 1 c. c	114	64	60	5	296

TABLE III.—*Determination of blood pressure and minimum lethal dose for cats, intravenous injection. Fluid extracts diluted to 10 per cent strength*—Continued.

	Heart rate.	Blood pressure.	Percentage increase in blood pressure.	Lethal dose.	Lethal dose per kilogram, body weight.
S. AND D.—continued.					
				c. c.	*mgm.*
September 8, 1908, cat, 2,880 grams:					
Normal	158	56			
After 1 c. c	162	74	32	6	208
September 8, 1908, cat, 3,800 grams:					
Normal	162	64			
After 1 c. c	156	84	31	11	289
Average			35		242
H. B. AND W.					
August 4, 1908, cat, 2,400 grams:					
Normal	162	58			
After 1 c. c	204	100	72	4	166
September 10, 1908, cat, 3,050 grams:					
Normal	220	68			
After 1 c. c	190	78	15	7	229
September 10, 1908, cat, 3,900 grams:					
Normal	120	50			
After 1 c. c	120	62	24	9	231
September 11, 1908, cat, 3,620 grams:					
Normal	130	70			
After 1 c. c	140	85	21	9	249
Average			33		219
MERRELL.					
August 4, 1908, cat, 3,600 grams:					
Normal	138	54			
After 1 c. c	132	70	30	7	194
September 12, 1908, cat, 2,540 grams:					
Normal	200	90			
After 1 c. c	210	104	16	7	275
September 16, 1908, cat, 2,800 grams:					
Normal	140	54			
After 1 c. c	140	72	33	8	286
Average			26		252
DIGITALONE NO. 3.					
August 6, 1908, cat, 1,600 grams:					
Normal	138	70			
After 1 c. c	120	84	20	4	250
August 6, 1908, cat, 1,930 grams:					
Normal	120	46			
After 1 c. c	108	60	31	6	310
Average			26		280
LLOYD BROS.[a]					
August 5, 1908, cat, 2,000 grams:					
Normal	168	66			
After 1 c. c	168	84	27	8	400
September 11, 1908, cat, 2,730 grams:					
Normal	130	52			
After 1 c. c	130	63	21	13	476
Average			24		438
DIGITALONE NO. 1.					
August 5, 1908, cat, 1,800 grams:					
Normal	180	68			
After 1 c. c	210	48	[b] 29		
September 5, 1908, cat, 3,220 grams:					
Normal	160	60			
After 1 c. c	190	50	[b] 17		
Average			[b] 23		

[a] Specific medicine. Evaporated at room temperature and diluted to 10 per cent strength with salt solution.
[b] Per cent fall.

TABLE III.—*Determination of blood pressure and minimum lethal dose for cats, intravenous injection. Fluid extracts diluted to 10 per cent strength*—Continued.

SUMMARY OF TOXIC DOSES FOR CATS (AVERAGES).

Preparation.	Milligrams.
B. W. and Co	158
P., D. and Co	171
N., B. and Co	192
Mulford No. 2	195
H. B. and W	219
S. and D	242
Merrell	252
Digitalone No. 3	280
Lloyd Bros	438
Digitalone No. 2	550

Frogs.—Finally the toxic dose on frogs was found according to Houghton's[a] method (see p. 16). The frogs (R. pipiens, Schreber, Syn. R. virescens and halecina) were unpacked as soon as they arrived and placed in a large cage through the end of which flowed fresh water. They were only brought to the experimental room when needed and injected late in the afternoon. They were then placed in separate jars and early the following morning the death or survival of each animal was noted. The results of experiments in which the lethal doses of four of the digitalis preparations were determined are given in Table IV.

TABLE IV.—*Determination of the minimum lethal dose for Rana pipiens, twelve-hour method.*

[Survived = −, dead = +. Dose per gram body weight.]

MULFORD NO. 2.

Series.	Dose.	Result.
	c. c.	
1	0.016	−
	.018	−
	.020	+
2	.019	+
	.020	+
	.020	−
3	.020	+
	.020	+
	.020	−

B. W. AND CO.

1	0.010	−
	.012	−
	.014	−
2	.014	+
	.016	+
	.018	+
3	.015	+
	.015	+
	.015	+

P., D. AND CO.

Series.	Dose.	Result.
	c. c.	
1	0.024	−
	.026	−
	.028	−
2	.030	+
	.032	−
	.034	+
3	.029	+
	.030	+
	.032	+
4	.029	+
	.029	+
	.029	+

S. AND D.

1	0.025	−
	.026	+
	.027	−
2	.026	−
	.027	+
	.028	+
3	.027	+
	.027	+
	.027	−

[a] Houghton, J. Am. Med. Ass., Chicago, 1898, XXXI, 959.

TABLE IV.—*Determination of the minimum lethal dose for Rana pipiens, twelve-hour method*—Continued.

SUMMARY.

Preparation.	M. L. D.[a]
	c. c. mgm.
B. W. and Co.	0.015=1.5
Mulford No. 2	.020=2.0
S. and D.	.027=2.7
P., D. and Co	.029=2.9

[a] M. L. D.=minimal lethal dose.

Factors modifying effect.—Our experiences with the various factors which are supposed to influence the reaction of the frogs may be briefly summarized here.

Weight.—In regard to the weight of the animal many workers have apparently very largely ignored this factor, while others have made only an approximate allowance for it. For instance, Focke says to use frogs weighing from 25 to 30 grams, while others merely say "large" frog or "medium" or "small," as the case may be. In our experience, which covers some years, we have always weighed the animals fairly accurately, within the limits of 1 gram, and then calculated the dose per gram body weight.[a] This seems not to be absolutely necessary, judging from the reports of several writers quoted, but we think that it can not help but insure greater uniformity in the results, especially when there are certain factors such as idiosyncrasy which can not possibly be allowed for.

Sex.—Our experiences agree with those of Focke that unless it is in the springtime there is no particular difference in the reaction obtained from male and female frogs.

Season.—The effect of the season of the year upon the conditions of the frogs seems to be pretty generally recognized. Both Moschkowitsch and Dixon point out that the frogs are most active in the spring. The question has been most fully considered by Focke, who says that on account of the seasonal variations only summer frogs should be employed, as they not only react differently at this time but there is very much less variation in their reaction. According to a later research,[b] if they are to be used at any other season, they should themselves be standardized by testing them against a digitalis preparation of known value and using the temperature factor which will give the preparation its standard "V" as previously determined. The introduction of this factor, however, we believe to be entirely unnecessary excepting that comparative experiments should be car-

[a] In our former work we used as a standard for comparison doses calculated on the basis of a 20-gram frog, but in this work we have compared the preparations upon the basis of 1-gram body weight as being simpler.

[b] Focke, Arch. d. Pharm., 1907, CCXLV, 646.

ried out at the same temperature. That a given preparation should show a worth of 5 at one time and only 3 at another seems of very little importance if its keeping qualities are known to be good. All that is necessary is to keep such a preparation in stock and, at any time when an assay is to be made, to redetermine its activity and then make the unknown conform to the value found. The only essential is that the known and the unknown digitalis preparation be tested at the same temperature.

Focke[a] carried out a series of experiments especially designed to show the effect of season. (The temperature factor probably played some part in the results.) He employed a digitalis powder which had been very thoroughly dried and carefully preserved. During April, May, and the early part of June the average of all experiments gave the powder a worth of 2.66. From the last of June to the end of September a V of 4.36; during October, a V of 5, and on November 20 a V of 3.3. These variations he ascribes to the nutrition of the frogs which is lowest in the spring and gradually improves during the summer, reaching its highest point just before the winter sets in, when it falls off sharply until it reaches the low point in the spring. He also says in a later paper that in the spring the frogs react weakly, and this is supported by the results of experiments in which he shows that the temperature must be increased in order to obtain the same values for the same preparation as at other seasons. His tables show that reacting weakly the frogs require a larger dose of the drug or an increase in the temperature of the operating room to produce the same effect and therefore give a lower V to the specimen of leaves examined.

The effect of a lowered nutrition in increasing the resistance to the drug is rather surprising, especially as our experiments, while not as complete as Focke's, show exactly the opposite effect, viz, that in the summer when the animals are most vigorous they require a larger dose to produce systolic standstill of the heart. We worked with a different variety of frogs from those Focke employed, but it would hardly be expected that this factor should make such a fundamental difference. Our experience may be summarized as follows:

The U. S. Pharmacopœia tincture examined by Edmunds[b] in 1907 required a dose of from 0.16 to 0.20 c. c. to produce systolic standstill in one hour. In experiments carried out in August, 1908, frogs belonging to the same species (R. pipiens) and of the same size required 0.40 to 0.50 c. c. to produce the same effect.

To be still more exact, in April, 1907, a Parke, Davis and Co. tincture required 0.15 c. c. to produce the end reaction described above; in 1908 one of their fluid extracts made to correspond to tincture

[a] Focke, Arch. d. Pharm., 1903, CCXLI, 678. Ther. d. Gegenw., 1904, XLV, 251.
[b] Edmunds, J. Am. Med. Ass., Chicago, 1907, XLVIII, 1744.

strength required 0.42 c. c. As these preparations are both physiologically assayed to a definite strength, the difference must be ascribed to the variability of the reaction of the frogs. As a means of demonstrating the uniformity of these two preparations mentioned, we might say that the 1907 tincture corresponded in strength to a tincture made in the laboratory at Ann Arbor from leaves obtained from Parke, Davis and Co., while their 1908 preparation is practically the same strength as U. S. Pharmacopœia fluid extracts made by other firms and which were examined at the same time, the results being given in the table on page 44.

In striking contrast to these views are the results of some of Houghton's[a] assays, which were carried out to ascertain if a standard tincture would deteriorate in a year if kept under proper conditions. His two tables, which we give below, show not only that the standard preparations do not deteriorate, but also, what is equally important and interesting, that frogs react exactly the same at all times of the year.

TABLE III A.—*Standard strophanthus.*

Date.	Toxic dose.
July 6, 1897	0.00015
October 19, 1897	.00015+
December 20, 1897	.00015
February 23, 1898	.00015−
April 25, 1898	.00015

TABLE III B.—*Concentrated strophanthus.*

Date.	Toxic dose.
July 28, 1897	0.0000037
November 10, 1897	.0000037

Ziegenbein also did not think that season made much difference in the susceptibility of frogs. However, it may be said that any errors due to differences in the reaction of the frogs dependable upon season can be avoided, in the first place, by standardizing the frogs with reference to the temperature of the operating room after the manner of Focke, and, second and more simply, these differences may be avoided by always assaying a standard solution of digitalis, which is kept under proper conditions at the same time that the unknown solution is examined.

FROG-HEART METHODS OF ASSAY.

According to our classification, the second group is concerned with an action upon the frog's heart as a means of measuring the comparative strengths of the different preparations. In these methods the

[a] Houghton, J. Am. Med. Ass., 1898, XXXI, 959.

end reaction is the stoppage of the frog's heart in systole, the chief differences between the various methods being dependant upon variations in the time factor.

The one-hour method.—In this method the frogs are secured and kept in the manner already described, weighed, and such a dose is injected that the heart will be found in complete systolic contraction at the end of exactly sixty minutes. The drug, properly diluted so as to make a volume of 0.5 to 1 c. c., is injected into the anterior lymph sac by means of a glass pipette. Shortly before the hour is up the frog is pithed, tied to a frog board, and the heart is exposed in the usual manner. If the heart is still beating, the dose has been too small and must be increased in subsequent trials. In the first series of frogs doses are chosen with wide limits, which in a second and third series of animals are narrowed down until the smallest amount of the drug which will produce systolic standstill in one hour is found. Usually three series of frogs are sufficient to assay one preparation, but in case of any irregularity in the reaction of any of the frogs a fourth or even a fifth series may be necessary. The results of our assay of the different preparations are given in Table V.

TABLE V.—*Determination of the systolic stoppage of the heart of Rana pipiens in one hour—Drug injected into abdominal lymph sac. The doses given are in cubic centimeters per gram body weight.*

MULFORD NO. 1.

Number of animals used.	Dose.	Result.
	c. c.	
1	0.009	−
1	.01	−
3	.011	−
1	.0115	−
3	.012	+
1	.014	+
1	.016	+

DIGITALONE NO. 3.

2	0.035	−
1	.037	−
1	.038	−
1	.039	+
1	.040	−
2	.040	+
1	.041	−
1	.042	+

S. AND D.

1	0.025	−
1	.026	−
1	.026	+
1	.027	−
3	.027	+
1	.028	−
3	.028	+
1	.029	+

B. W. AND CO.

Number of animals used.	Dose.	Result.
	c. c.	
1	0.018	−
1	.019	−
1	.020	[a] +
1	.020	+
1	.021	+
1	.022	+

LLOYD BROS.

1	0.027	−
3	.028	−
3	.029	−
4	.030	+

DIGITALONE NO. 2.

1	0.025	−
1	.032	−
1	.040	−
1	.090	−
1	.105	−
1	.120	[b]

MULFORD NO. 2.

1	0.014	−
2	.015	−
1	.016	−
4	.016	+
1	.017	+

[a] Diastole.

[b] Diastole, later beating.

TABLE V.—*Determination of the systolic stoppage of the heart of Rana pipiens in one hour—Drug injected into abdominal lymph sac. The doses given are in cubic centimeters per gram body weight*—Continued.

MERRELL.

Number of animals used.	Dose.	Result.
	c. c.	
2	0.022	−
2	.023	−
1	.023	+
2	.024	−
3	.024	+
5	.025	+
1	.026	+

H. B. AND W.

Number of animals used.	Dose.	Result.
	c. c.	
2	0.022	−
1	.023	−
2	.024	−
2	.025	+
1	.026	−
2	.026	+
1	.027	+

N., B. AND CO.

1	0.020	−
2	.021	−
1	.021	+
1	.022	+
1	.023	+
1	.025	+

P., D. AND CO.

1	0.018	−
1	.019	−
1	.020	−
2	.020	+
2	.021	−
3	.021	+
3	.022	+

SUMMARY.

Preparation.	Dose.
	c. c. mgm.
Mulford No. 1	0.012=1.2
Mulford No. 2	.016=1.6
B. W. and Co	.020=2.0
P., D. and Co	.021=2.1
N., B. and Co	.022=2.2
Merrell	.024=2.4
H. B. and W	.025=2.5
S. and D	.027=2.7
Lloyd Bros	.030=3.0
Digitalone No. 3	.040=4.0
Digitalone No. 2	

"Focke" method.—The second method upon the frog's heart which we employed was that of Focke, modified, however, in one or two important particulars. In the first place, we pithed all the frogs, while Focke does not do this until the experiment is over. It is absolutely essential that this should be done for purely humanitarian reasons, and if this operation interferes with the accuracy of his method then the method can not be used. As a matter of fact, this was done very carefully so as not to lose any blood, and the piece of sharpened wood was left in the brain cavity so as to prevent hemorrhage. The other modification was patterned after Wang, who found it was hard to determine when the ventricle may be said to have come to a permanent systolic standstill. We found exactly the same difficulty, and so adopted Wang's modification of watching for the stoppage of the circulation. Wang does not mention how he determines this, but the method we used was to watch with the microscope the stoppage of the circulation in the web of the foot. This itself is not always satisfactory, because in some animals, due to

some unknown condition, very little movement will be found in the blood in the web vessels. The most important factor in the condition of the peripheral circulation seemed to be whether any hemorrhage whatever had occurred during the operation. If even a drop of blood had been lost its loss appeared to be shown by the poor circulation in the web. An important precaution we adopted was, after selecting the field which showed the most favorable circulation, to note the direction of the flow in the main streams. If this was not done, after the heart stopped or had become very weak the blood would fre-frequently flow in the reverse direction and continue doing so for a long time. If it was not known that the blood was no longer going in the normal direction it would be supposed that the circulation had not ceased.

In most cases it was very hard to tell exactly when the circulation did actually stop, because when the heart became weak the blood would stop flowing for a short period, to be followed by a renewal of the flow just as soon as enough pressure was obtained in the supplying arteries to overcome peripheral resistance. Both end reactions, either stoppage of the heart or of the circulation, are very indefinite, in our opinion, but probably if a sufficient number of experiments are carried out a fairly definite idea of the relative strength of the preparations can be obtained.

It is very essential in this method if a comparison of different preparations is to be carried out not merely to choose any dose which will stop the heart at any time between seven and twenty minutes, because quite different values are obtained if a dose is given which will stop all the hearts in eight minutes or in say eighteen minutes. As we found, and as Focke shows in his tables, the shorter times give higher values, so that in comparing two preparations it would be necessary to have the hearts stop at some average time, ten or twelve minutes, or to so regulate the doses that two hearts will stop in about eight minutes, two in twelve or thirteen, and two in eighteen. We adopted this rule and in this way a fairly good average is attained. However, even with these precautions, we believe that this method allows of greater variations and inaccuracies than any other method we employed. The results of the determination of the toxic dose of the different digitalis preparations by this method are given in Table VI.

TABLE VI.—*Determination of toxic dose for Rana pipiens, Focke method—drug injected into leg lymph sacs.*

Time is noted when circulation in web of foot stops. Dose is calculated per gram body weight, animals weighing between 25 and 30 grams each

MULFORD NO. 1.

Dose.	Time.	Value (V).
c. c.	*Min.*	
0.020	18	2.8
.020	18	2.8
.020	14	3.6
.0225	9	4.9
.0225	12	3.7
.023	11	3.9
.025	9	4.4
.025	9	4.4
Average		V=3.8

MERRELL.[a]

0.035	20	1.4
.035	15	2.0
.036	16	1.8
.0375	13	2.0
.0375	13	2.0
.038	12	2.2
.040	12	2.8
.040	10	2.5
Average		V=2.1

P., D. AND CO.

0.0375	17	1.6
.0375	14	1.9
.040	13	1.9
.040	14	1.8
.040	14	1.8
.0425	11	2.1
.0425	10	2.4
.0425	11	2 1
.045	10	2.2
.045	11	2.0
.045	8	2.8
.045	9	2.5
Average		V=2.1

LLOYD BROS.

0.035	15	1.9
.0375	13	2.1
.040	12	2.1
.040	10	2.5
.0425	12	1.9
.045	8	2.9
.045	9	2.4
Average		V=2.3

N., B. AND CO.

Dose.	Time.	Value (V).
c. c.	*Min.*	
0.015	27	
.020	18	2.7
.020	17	2.9
.0225	10	4.4
.025	20	2.0
.026	12	3.2
.027	15	2.5
.030	13½	2.5
.035	10	2.9
Average		V=2.9

H. B. AND W.

0.0325	14	2.2
.0325	15	2.0
.033	13	2.3
.035	12	2.4
.035	11	2.6
.0375	8	3.3
.0375	8	3.3
.038	7	3.7
.040	11	2.3
.040	7	3.6
Average		V=2.7

B. W. AND CO.

0.03	13	2.6
.03	16	2.2
.03	12	2.7
.035	15?	1.9
.035	10	2.8
.035	9	3.2
.04	8	3.1
.04	7½	3.3
Average		V=2.7

S. AND D.

0.036	16	1.8
.038	10	2.6
.038	12	2.2
.040	11	2.3
.045	10	2.2
.045	9	2.5
.045	8	2.8
.050	7	2.8
Average		V=2.4

[a] Calculated as of fluid extract strength.

Digitalone No. 1. Frog, weight 41 grams, injected with 0.5 c. c. per gram body weight. Five hours later heart rate was 16, weak contraction but dilating well.

Digitalone No. 2. Frog, weight 40 grams, injected with 0.5 c. c. per gram body weight. Five hours later heart still beating, rate 17, contractions weak, but dilating fairly well.

Digitalone No. 3. Frog, weight 39 grams, injected with 0.5 c. c. per gram body weight. Ventricle stopped in fifty-seven minutes; auricles beat two hours longer.

TABLE VI.—*Determination of toxic dose for Rana pipiens, Focke method—drug injected into leg lymph sacs*—Continued.

SUMMARY.

Preparation.	V (value).
Mulford No. 1	3.8
N. B. and Co	2.9
B. W. and Co	2.7
H. B. and W	2.7
S. and D	2.4
Lloyd Bros	2.3
P., D. and Co	2.1
Merrell	2.1
Digitalone 1, 2, 3	.....

PERFUSION OF FROG'S HEART.

The perfusion experiments upon excised frogs' hearts were carried out by tying a cannula in the left trunk of the aorta and a second in the posterior vena cava near the heart. The isolated heart was perfused under constant pressure with Ringer's solution to which digitalis in tincture strength was added so as to make a 1 per cent solution. The time was noted until systolic stoppage of the heart appeared and the figures recorded indicate the number of minutes the heart continued beating after the introduction of the drug. The results obtained by this method are recorded in Table VII.

TABLE VII.—*Determination of the time required to stop the isolated heart of Rana pipiens with Ringer's solution to which digitalis had been added to make the solution of 1 per cent tincture strength.*

	Minutes.
Mulford No. 2	23
	26
	21
	21
	18
	22
	14
	15
	15
	16
	21
	18
Average	19
Merrell	31
	35
	17
	35
	37
	34
	21
	35
Average	31
B. W. and Co	19
	30
	29
	21
	26
	17
	18
Average	23
Lloyd Bros	66
	71
	54
	48
	46
Average	57
Digitalone No. 3	53
	47
	78
	65
	97
	72
Average	69

	Minutes.
N. B. and Co	17
	18
	28
	19
	20
	16
	21
Average	20
H. B. and W	17
	15
	21
	23
	16
	18
Average	18
P., D. and Co	20
	32
	12
	33
	44
	26
	12
	16
	19
	22
	26
Average	24
S. and D	29
	27
	25
	32
	26
	32
	29
	20
Average	28

SUMMARY.

Preparation.	Minutes.
H. B. and W	18
Mulford No. 2	19
N. B. and Co	20
B. W. and Co	23
P., D. and Co	24
S. and D	28
Merrell	31
Lloyd Bros	57
Digitalone No. 3	69

THE BLOOD PRESSURE METHOD OF ASSAY.

The blood-pressure method, included in a third group by our classification, was carried out in the ordinary manner, cats being used as experimental animals. They were anæsthetized with chloretone, 0.4 gram per kilogram body weight, dissolved in a small amount of alcohol (2 c. c. to 1 gram chloretone) diluted with a little water and given by the stomach after the method of Edmunds and Cushny.[a] The comparison of the various preparations of digitalis is made upon the percentage of rise in blood pressure which followed the injection of 1 c. c. of the various solutions when diluted with normal salt solution to tincture strength. Only one preparation could be used upon one animal, and from two to four animals were used for each preparation in order that the average of the results obtained might more accurately represent the value of the drug used. The results of these experiments, which are tabulated in Table III, page 37, are summarized in the following table:

TABLE VIII.—*Summary of results obtained on blood pressure.—Average per cent rise after 1 cubic centimeter doses intravenously.*

Preparation.	Per cent increase.
N. B. and Co.	99
Mulford No. 2.	64
Digitalone No. 2.	55
B. W. and Co.	51
P., D. and Co	37
S. and D.	35
H. B. and W.	33
Merrell.	26
Digitalone No. 3.	26
Lloyd Bros.	24
Digitalone No. 1.	[1]—23

[1] Fall.

To enable a comparison to be made of the relative strengths of the different digitalis preparations as obtained by the different methods of assay employed, we have grouped all the findings in Table IX.

TABLE IX.—*Summary of results obtained by the several methods for the standardization of digitalis preparations.*

LETHAL-DOSE METHODS.

Mice.		Guinea pigs.		Frogs (twelve-hour).		Cats.	
	mg.		*mg.*		*c. c.*		*mg.*
Mulford No. 1.	4	B. W. and Co.	0.35	B. W. and Co.	0.015	B. W. and Co.	158
B. W. and Co.	4	H. B. and W.	.5	Mulford No. 2.	.020	P., D. and Co.	171
Merrell.	6	Merrell.	.6	S. and D.	.027	N. B. and Co.	192
H. B. and W.	7	Mulford No. 1.	.7	P., D. and Co.	.029	Mulford No. 2.	195
P., D. and Co.	8	P., D. and Co.	.7			H. B. and W.	219
N. B. and Co.	9	N. B. and Co.	.9			S. and D.	242
S. and D.	9	S. and D.	.9			Merrell.	252
Digitalone No. 1.	22	Lloyd Bros.	.9			Digitalone No. 3.	280
Lloyd Bros.	24	Digitalone No. 1.	5.0			Lloyd Bros.	438
		Digitalone No. 3.	[b]1.5			Digitalone No. 2.	550

[a] Edmunds and Cushny, Laboratory Guide in Experimental Pharmacology, p. 12. [b] Lived.

TABLE IX.—*Summary of results obtained by the several methods for the standardization of digitalis preparations*—Continued.

FROG-HEART METHODS.

One hour.		"Focke."		Perfusion.	
	c. c		*V.*		*min.*
Mulford No. 1	0.012	Mulford No. 2	3.8	H. B. and W	18
Mulford No. 2	.016	N. B. and Co	2.9	Mulford No. 2	19
B. W. and Co	.020	B. W. and Co	2.7	N. B. and Co	20
P., D. and Co	.021	H. B. and W	2.7	B. W. and Co	23
N. B. and Co	.022	S. and D	2.4	P., D. and Co	24
Merrell	.024	Lloyd Bros	2.3	S. and D	28
H. B. and W	.025	P., D. and Co	2.1	Merrell	31
S. and D	.027	Merrell	2.1	Lloyd Bros	57
Lloyd Bros	.030			Digitalone No. 3	69
Digitalone No. 3	.040				

BLOOD-PRESSURE METHOD.

	Per cent.		*Per cent.*
N. B. and Co	99	H. B. and W	33
Mulford No. 2	64	Merrell	26
Digitalone No. 2	55	Digitalone No. 3	26
B. W. and Co	51	Lloyd Bros	24
P., D. and Co	37	Digitalone No. 1	[a]—23
S. and D	35		

[a] Fall.

Table X has been arranged from Table IX to show in a numerical way the relative position the preparations experimented with occupy. The figures represent the relative position each preparation holds with reference to its activity as determined by the various methods of assay. In case two preparations showed the same strength (as for instance the Concentrated tincture, B. W. and Co. and Digitol, Mulford on mice) they have been given equal ranking.

TABLE X.—*Rearrangement of Table IX to show the relative activity of the following preparations as determined by the various methods of assay.*

Preparations.	Lethal dose methods.				Frog heart methods.			Blood Pressure.
	Mice.	Guinea pigs.	Twelve hour.	Cats.	One hour.	"Focke."	Perfusion.	Per cent increase.
B. W. and Co	1	1	[a]	1	2	3	4	4
Mulford	1	4	.....	4	1	1	2	2
Merrell	3	3	.....	7	5	7	7	8
H. B. and W	4	2	.....	5	6	4	1	7
P., D. and Co	5	4	.....	2	3	7	5	5
N. B. and Co	6	6	.....	3	4	2	3	1
S. and D	6	6	.....	6	7	5	6	6
Digitalone No. 1	8	9	.....	.....	.....	.....	.....	.....
Lloyd Bros	9	8	.....	9	8	6	8	10
Digitalone No. 3	.....	[b]	.....	8	9	.....	9	8
Digitalone No. 2	.....	.....	.....	10	.....	.....	.....	5

[a] The results obtained by the twelve-hour method are not included because too few experiments were carried out according to it to give the preparations their proper rank.

[b] Lived.

DISCUSSION OF METHODS OF ASSAY.

A superficial examination of the tables giving the summary of the results obtained would seem to indicate the practical worthlessness of the biological assay of digitalis preparations by the methods now in vogue. This is especially emphasized by a comparison of Digitol and the Concentrated tincture made by Burroughs, Wellcome & Co. These give comparative values as follows:

	Mice, toxic dose.	Guinea pigs, toxic dose.	Frogs, one hour.
	mgm.	*mgm.*	*c. c.*
Digitol No. 1	4.0	0.70	0.012
Concentrated tincture	4.0	.35	.020

Here are two preparations which according to the first test are of the same strength; by a second test one is half the strength of the other, and by a third method exactly the reverse relation is found. No explanation can be suggested for the results obtained on mice and guinea pigs; there was no possibility of a mistake, as the results were confirmed on numerous animals as the tables show, and with different solutions made up on different days. A closer examination of the table shows that these results are certainly exceptional, the vast majority being more uniform, as, for example, the two preparations named are shown by most of the methods to be the strongest of any of the specimens examined. Also comparing the four U. S. Pharmacopœia fluid extracts made by Hance Brothers & White, Parke, Davis & Co., Nelson, Baker & Co., and Sharpe & Dohme, these were found by several of the methods to be of very nearly the same strength, differing not more than 25 per cent. Also the Digitalone preparations and Lloyd's Specific Medicine appear at the bottom of the list in almost every table showing by every method their comparative weakness.

There appears thus to be some slight uniformity in the results obtained by the different methods. This is indeed far from close, but this fact is not surprising when the factors concerned in the different methods of assay are considered. In the first place it could hardly be expected that animals differing so widely as cats and frogs, mice and guinea pigs would react exactly alike to the different preparations. The cause of death is not the same in the mice and guinea pigs as in the frogs and the cats under the conditions of our experiments. With frogs the action is always one upon the heart; also in the cats we maintained artificial respiration so that the figures obtained there indicate a cardiac action. On both mice and guinea pigs, however, the cause of death in probably every case is not due

to an action upon the heart but upon the medulla. In every animal we examined we found the heart beating after the respiration had stopped, and it continued to beat as long as artificial respiration was maintained. It would not then be at all surprising if two solutions which showed a certain relationship when the cardiac action was concerned should show an entirely different relation when the central nervous system was the point of attack. One solution might be very weak in its action upon the heart and yet contain decomposition products of digitalis whose typical action is upon the medulla and it would therefore appear unduly strong when judged by such a standard. For this reason *we think that methods which employ as a standard the minimum lethal dose obtained upon the higher animals are not applicable to the physiological assay of the digitalis series.*

It is possible that still further complications would arise in using such methods in that on certain animals of the same species some would die from the cardiac effect and others from the medullary action. In some of the cats, for instance, distinct respiratory movements were seen after the heart had stopped and the blood pressure had fallen to zero; in others the respiration stopped first. As stated earlier, to avoid complications of this sort, in our experiments on cats we always employed artificial respiration.

Comparing the toxic doses obtained upon cats and frogs (twelve-hour method) we have in both cases a cardiac action and three of the four preparations show relatively the same activity; the Parke, Davis & Co. Fluid Extract alone being an exception. But that an exception should occur is not to be wondered at when the differences between the heart of the frog and that of the cat are considered. Also in the blood pressure experiments on the cat we have to do with substances acting upon the vessel walls as well as upon the heart and this necessarily introduces another factor as some of the digitalis active principles act more strongly upon vessel walls than others, and in two preparations of digitalis the active substances may not be present in the same relative amounts.

The "Focke" and the "one-hour" methods upon frogs might be thought to give a closer agreement, but here (aside from the difficulties connected with Focke's method which have been discussed) we have to deal with the question of ease of absorption. As has been pointed out by some earlier writers, in those methods in which the time element is very limited, a weak preparation with easily absorbable constituents would appear stronger than a strong preparation with constituents which are absorbed with difficulty. It would seem that such an objection might be of importance in the "Focke" method, where from seven to twenty minutes only are allowed. As to whether it would play a part in the "one-hour" method is of course possible

but not very probable, when it is remembered with what rapidity the action appears when digitoxin in solution is injected.

The reason that the "frog's heart profusion" method does not agree with the other frog methods is possibly due partly to the more uniform character of the solution of the poison in which the heart is bathed and also to the resinous precipitate which forms and falls to the bottom of the perfusion vessel and which no doubt contains some of the active constituents.

From this short critical review of the different methods for standardization it can easily be seen why no two methods of assay could be expected to give exactly the same relative values to a series of digitalis preparations, especially when they are made according to radically different methods, such differences as exist, for instance, between a "Fat free" tincture and a "Specific medicine." The agreement is much closer when the preparations are made according to the same formula as we have in the case of the official fluid extracts.

It would appear then most desirable if manufacturing houses could agree upon a certain method to be used which would insure the different preparations upon the market under the same name having the same potency. As to the choice of such a method difficulties would arise, but certainly none which would appear insurmountable. The method chosen should be as simple as possible, and above all *an action upon the circulation should be taken as a standard of comparison rather than an action upon the nervous system.* Judged by these rules, some of the methods now in use are not suitable for the purpose.

First, we have the toxic action upon the higher animals, which should not be used for the reasons given earlier. The technical difficulties of the Focke method, together with the question of completeness of absorption, exclude it. The perfusion of the frog's heart is also excluded for reasons mentioned and also because it requires more skill to carry it out, and even then the variations in results are very great. The blood-pressure method upon cats and dogs commends itself on account of the close relation it sustains to the use of the drug in clinical practice. The objections consist in the difficulty of procuring these animals at times and also the necessity of carrying out repeated experiments to confirm the results, which a study of our tables show will vary very greatly. A dose of a certain preparation which in one animal may cause a rise of perhaps 70 or 80 per cent in blood pressure may in others not raise it more than one-quarter as much, but probably repeated tests will give reliable results.

The toxic dose can also be obtained at the same time by using artificial respiration, but, as our tables show, there need be no agreement between the two, and it therefore seems superfluous. This leaves, then, the two frog methods—the "twelve-hour" toxic method and the

"one-hour" method. As between them it is impossible to choose with any degree of accuracy. With the "one-hour" method we have the advantage of there being no delay, it being possible to assay a preparation in three or four hours, and it probably takes fewer frogs than the "twelve-hour" method. On the other hand, with the latter method, although taking four or five days to complete the assay, it need not interfere with other work, as the frogs can be injected late in the afternoon and examined the next morning. Between these two methods, then, as far as can be judged, it is largely a question of personal preference and convenience, at least in the light of our present knowledge. Our results do not show that they give the same relative values to the four specimens examined by them, and as to which is correct it is impossible to say. They do roughly agree in that Burroughs, Wellcome & Co.'s Concentrated Tincture and Digitol are both stronger than the two fluid extracts of Parke, Davis & Co. and Sharpe & Dohme. This relationship is confirmed by every other method which we employed, excepting that using the toxic dose as found on cats. It would not seem, therefore, to be unreasonable to suppose that such a relationship would probably exist in man.

As to the desirability of manufacturing houses standardizing their preparations belonging to the digitalis series there can be no question.

It is true that the official preparations examined herewith are approximately of the same strength, but the crude drug obtained in another year may represent altogether a different toxicity to that employed in the manufacture of these preparations, and the only way at present to avoid like variations in the tinctures and fluid extracts is to standardize them by physiological methods. It is not claimed that such methods are infallible. They certainly are not, as we have shown; but, as we have also shown, most of the apparent discrepancies are due to the fact that actions upon quite different physiological functions are taken as a measure of activity. Such discrepancies can be avoided by the adoption of one or the other of these frog methods, and if it is thought necessary the results obtained can be confirmed by blood-pressure experiments upon cats or, preferably, dogs.

We believe the chief point to be kept in mind is that, in spite of annoying individual discrepancies, there is a general agreement between the various methods. This agreement, too, is much more complete in the case of those methods in which the toxic effect is due to an action upon the heart. A preparation found weak by one method appears weak by all methods, and one showing marked activity by one shows the same result in all.

The second part of the research, that which is concerned with the different preparations, requires little to be added to that already given in the tables. We have arranged, however, the different preparations in the order of their relative strengths as determined

by the results obtained by all the methods of assay. Such a list is found in Table XI. This is prepared from Table X by adding together the numbers denoting the relative position of each preparation and dividing the sum by the number of assay methods used. Naturally the quotients thus found will stand in inverse ratio with the strength of the preparation.

In this table the results obtained by the "twelve-hour" method on frogs were not used, as only four preparations were assayed according to it, so that no true ranking in relation to the results obtained by the other methods could be found.

TABLE XI.

Preparation.	Sum of ratings.	Number of assays.	Final result.
Mulford	15	7	2.14
B., W. and Co	16	7	2.28
N., B. and Co	25	7	3.57
H. B. and W	29	7	4.14
P., D. and Co	31	7	4.43
Merrell	40	7	5.71
S. and D	42	7	6.00
Lloyd Bros	58	7	8.28
Digitalone No. 3	34	4	8.50
Digitalone Nos. 1 and 2	----------	----------	----------

Such a table as this we think, on the whole, represents very fairly the relative strengths of the different preparations examined. It represents the final conclusion drawn from a very large series of experiments, and on that account may be considered fairly accurate.

There are only four preparations which would appear to require special mention. These are the normal tincture of digitalis made by William S. Merrell Chemical Company, of Cincinnati; digitalone made by Parke, Davis & Co., of Detroit; the specific medicine, digitalis made by Lloyd Bros., of Cincinnati, and tincture digitol made by H. K. Mulford & Co., of Philadelphia. Two bottles of digitol were examined. By the one-hour method on frogs they did not show the same strength, one requiring 0.012 c. c. to produce systolic stoppage of the heart in one hour and the other 0.016 c. c. (Table IX.) This preparation, as mentioned earlier, is "assayed, tested physiologically" and standardized to contain 0.025 gram digitoxin in 100 c. c.

The purified normal tincture of Merrell is said to be "a standardized neutral tincture of prime digitalis leaves freed from the irritating fats and oils." In our tables the dose of this preparation was always calculated upon the basis of fluid extract strength, which is not correct, as normal tinctures are said to be made from fresh leaves which are put into alcohol. Later the alcohol is expressed and by evaporation 1 kilo of the solution is made equivalent to 1 kilo of leaves. It is thus considerably weaker than a fluid extract on account of the moisture content of the fresh plant. But even calculated in this

way it compares fairly well with the fluid extracts, especially by the "toxic" methods. The maximum dose recommended is about 3 minims, or about the same as for a fluid extract.

The "Specific Medicine" digitalis, made by Lloyd Bros., of Cincinnati, offered some difficulties on account of its high alcohol content (80 per cent absolute). If this was evaporated off, a gummy resinous mass was left which was very hard to mix with water in such a way as to give uniform dosage. In many cases to avoid this trouble the solution was merely exposed in any open vessel at room temperature until part of the alcohol had evaporated, and the residue was then mixed with the water to get the desired dilution. The solutions thus obtained were always thoroughly shaken to insure a uniform distribution of the precipitate. In one method, namely, the perfusion of the frog's heart, it is not possible to get the full action of the drug, as the precipitate which forms when the drug is added to the Ringer's solution settles to the bottom of the bottle containing the perfusion fluid and only the soluble portion reaches the heart. It is for this reason that the drug appears so much weaker by this method than by the other methods in which the animal receives the entire drug. The results of our experiments show that the preparation is uniformly weaker than the pharmacopœial fluid extracts. According to the label its strength is 480 grains to the fluid ounce.

In the advertising literature of this firm the effect upon the system of the "Specific Medicines" is said to be about double that of ordinary fluid extracts and the dose should not be more than one-half the usual dose of fluid extracts. The dose recommended on the bottle is from one-third minim to 1 minim *every hour*, which is certainly not less than that of a pharmacopœial fluid extract. In general, its strength would appear to be about the same as the fluid extracts; it certainly is not stronger.

Three bottles of digitalone were examined. This is said to be an aseptic nonalcoholic, permanent solution of digitalis of the same strength as the U. S. Pharmacopœia tincture. Bottles Nos. 1 and 2 were not essentially different in the character of the preparation contained. In both cases it possessed a peculiar, somewhat aromatic odor, not exactly unpleasant, but somewhat sour. The fluids were both a dark brown and contained a rather heavy precipitate. Bottle No. 3, the odor was not as unpleasant, and somewhat suggested chloretone. The preparation was slightly lighter in color than an orange yellow, and contained a fairly abundant, fine white, flaky precipitate.

Their relative physiological activity can be most clearly illustrated by three experiments upon frogs carried out under similar conditions, the hearts being exposed and the drug being injected in relatively the same size doses.

DIGITALONE NO. 1.

August 17, 1908. Frog, 41 grams:
11.25. Heart rate 27 in twenty seconds.
11.29. Heart rate 28 in twenty seconds.
11.30. Injected 2.05 c. c. digitalone No. 1; 0.05 c. c. per gram body weight.
11.42. Heart rate 18 in twenty seconds.
11.50. Heart rate 16 in twenty seconds.
12.01. Heart rate 16 in twenty seconds.
12.30. Heart rate 17 in twenty seconds.
2.15. Heart rate 16 in twenty seconds; heart weak.
4.30. Heart rate 16 in twenty seconds; dilates poorly and very weak contraction.

DIGITALONE NO. 2.

August 17, 1908. Frog, 40 grams:
11.00. Heart rate 30 in twenty seconds.
11.05. Heart rate 37 in twenty seconds.
11.05. Injected 2 c. c. digitalone No. 2.
11.10. Heart rate 15 (?) in thirty seconds; irregular peristaltic contractions.
11.18. Heart rate 16 in twenty seconds; normal contractions.
11.27. Heart rate 16 in twenty seconds.
11.41. Heart rate 16 in twenty seconds; systole weak.
11.50. Heart rate 16 in twenty seconds.
12.30. Heart rate 15 in twenty seconds.
2.15. Heart rate 17 in twenty seconds; weak.
4.15. Heart rate 17 in twenty seconds; weak, dilating fairly well.

DIGITALONE NO. 3.

August 17, 1908. Frog, 39 grams:
10.55. Heart rate 26 in twenty seconds.
11.01. Heart rate 26 in twenty seconds.
11.02. Injected 1.95 c. c. digitalone No. 3.
11.06. Heart rate 11 in twenty seconds; systole prolonged.
11.12. Heart rate 10 in twenty seconds.
11.16. Heart rate 16 in twenty seconds; dilates little.
11.40. Heart rate 7 in twenty seconds, little dilatation.
11.59. Ventricle in systole; auricles, rate 7 in twenty seconds.
2.30. Auricle stopped; ventricle in systole.

Specimens Nos. 1 and 2 showed no digitalis action whatever, while No. 3 was quite weak.

To kill mice Digitalone No. 1 required from two and one-half to five times the dose that any other preparation examined required excepting Lloyd's "Specific Medicine, digitalis." On guinea pigs 2 milligrams per kilogram body weight caused death, but as pointed out earlier it was a question whether this was due to the digitalis per se or to the effect of an injection under the skin of 12.5 c. c. of fluid containing chloretone and possibly digitalis decomposition products. The animal became comatose at once, showing no characteristic digitalis symptoms. Preparation No. 2, tested by the twelve-hour method on frogs, did not cause death in doses as high as 0.07 c. c. per gram body weight, while the preparations made according to the

other methods caused death in doses from one-quarter to one-half as large. In the one-hour method Digitalone No. 2, in doses four times as large as were given of the weakest and ten times as large as the strongest of the other (excepting Digitalone) preparations, failed to call forth systolic standstill of the heart. Digitalone No. 3 seemed to be fairly active. By the one-hour method on frogs it was from one-third to two-thirds as strong as the other preparations. Focke's method was not very successful on account of the extremely large dose which would have required so much concentration on the water bath to bring it to a volume small enough to inject into the lymph sacs. This specimen failed to kill guinea pigs when used in doses twice as large as the average preparation required. Injected into rabbits Digitalone No. 1, instead of raising the blood pressure, lowered it 23 per cent, the curve resembling that given by the nitrite series. Digitalone No. 3 raised the blood pressure 26 per cent.

An interesting fact in connection with the value of the blood-pressure methods of assay is that Digitalone No. 2, which was shown by the exposed frog's heart to be almost devoid of digitalis action, caused a rise of 55 per cent in the blood pressure of two cats. It might be that some other substances are present (perhaps decomposition products) which, possessing no cardiac effect, yet act on the vasomotor system, probably upon the center. In specimen No. 1 other changes in the solution must have occurred, as at each injection a fall in blood pressure followed. The important conclusion to be drawn from these experiments is that Digitalone is not invariably a permanent solution. Those preparations which have decomposed are not only devoid of any digitalis action, but are distinctly harmful. A preparation which evidently had not deteriorated completely seemed to be about half the strength of an official tincture.

LIST OF HYGIENIC LABORATORY BULLETINS OF THE PUBLIC HEALTH AND MARINE-HOSPITAL SERVICE.

The Hygienic Laboratory was established in New York, at the Marine Hospital on Staten Island, August, 1887. It was transferred to Washington, with quarters in the Butler Building, June 11, 1891, and a new laboratory building, located in Washington, was authorized by act of Congress, March 3, 1901.

The following *bulletins* [Bulls. Nos. 1–7, 1900 to 1902, Hyg. Lab., U. S. Mar.-Hosp. Serv., Wash.] have been issued:

*No. 1.—Preliminary note on the viability of the *Bacillus pestis*. By M. J. Rosenau.

No. 2.—Formalin disinfection of baggage without apparatus. By M. J. Rosenau.

*No. 3.—Sulphur dioxid as a germicidal agent. By H. D. Geddings.

*No. 4.—Viability of the *Bacillus pestis*. By M. J. Rosenau.

No. 5.—An investigation of a pathogenic microbe (*B. typhi murium* Danyz) applied to the destruction of rats. By M. J. Rosenau.

*No. 6.—Disinfection against mosquitoes with formaldehyd and sulphur dioxid. By M. J. Rosenau.

No. 7.—Laboratory technique: Ring test for indol, by S. B. Grubbs and Edward Francis; Collodium sacs, by S. B. Grubbs and Edward Francis; Microphotography with simple apparatus, by H. B. Parker.

By act of Congress approved July 1, 1902, the name of the "United States Marine-Hospital Service" was changed to the "Public Health and Marine-Hospital Service of the United States," and three new divisions were added to the Hygienic Laboratory.

Since the change of name of the Service the bulletins of the Hygienic Laboratory have been continued in the same numerical order, as follows:

*No. 8.—Laboratory course in pathology and bacteriology. By M. J. Rosenau. (Revised edition, March, 1904.)

*No. 9.—Presence of tetanus in commercial gelatin. By John F. Anderson.

No. 10.—Report upon the prevalence and geographic distribution of hookworm disease (uncinariasis or anchylostomiasis) in the United States. By Ch. Wardell Stiles.

*No. 11.—An experimental investigation of *Trypanosoma lewisi*. By Edward Francis.

*No. 12.—The bacteriological impurities of vaccine virus; an experimental study. By M. J. Rosenau.

*No. 13.—A statistical study of the intestinal parasites of 500 white male patients at the United States Government Hospital for the Insane; by Philip E. Garrison, Brayton H. Ransom, and Earle C. Stevenson. A parasitic roundworm (*Agamomermis culicis* n. g., n. sp.) in American mosquitoes (*Culex sollicitans*); by Ch. Wardell Stiles. The type species of the cestode genus *Hymenolepis;* by Ch. Wardell Stiles.

No. 14.—Spotted fever (tick fever) of the Rocky Mountains; a new disease. By John F. Anderson.

No. 15.—Inefficiency of ferrous sulphate as an antiseptic and germicide. By Allan J. McLaughlin.

*No. 16.—The antiseptic and germicidal properties of glycerin. By M. J. Rosenau.

*No. 17.—Illustrated key to the trematode parasites of man. By Ch. Wardell Stiles.

*No. 18.—An account of the tapeworms of the genus *Hymenolepis* parasitic in man, including reports of several new cases of the dwarf tapeworm (*H. nana*) in the United States. By Brayton H. Ransom.

*No. 19.—A method for inoculating animals with precise amounts. By M. J. Rosenau.

*No. 20.—A zoological investigation into the cause, transmission, and source of Rocky Mountain "spotted fever." By Ch. Wardell Stiles.

No. 21.—The immunity unit for standardizing diphtheria antitoxin (based on Ehrlich's normal serum). Official standard prepared under the act approved July 1, 1902. By M. J. Rosenau.

*No. 22.—Chloride of zinc as a deodorant, antiseptic, and germicide. By T. B. McClintic.

*No. 23.—Changes in the Pharmacopœia of the United States of America. Eighth Decennial Revision. By Reid Hunt and Murray Galt Motter.

No. 24.—The International Code of Zoological Nomenclature as applied to medicine. By Ch. Wardell Stiles.

No. 25.—Illustrated key to the cestode parasites of man. By Ch. Wardell Stiles.

No. 26.—On the stability of the oxidases and their conduct toward various reagents. The conduct of phenolphthalein in the animal organism. A test for saccharin, and a simple method of distinguishing between cumarin and vanillin. The toxicity of ozone and other oxidizing agents to lipase. The influence of chemical constitution on the lipolytic hydrolysis of ethereal salts. By J. H. Kastle.

No. 27.—The limitations of formaldehyde gas as a disinfectant with special reference to car sanitation. By Thomas B. McClintic.

*No. 28.—A statistical study of the prevalence of intestinal worms in man. By Ch. Wardell Stiles and Philip E. Garrison.

*No. 29.—A study of the cause of sudden death following the injection of horse serum. By M. J. Rosenau and John F. Anderson.

No. 30.—I. Maternal transmission of immunity to diphtheria toxine. II. Maternal transmission of immunity to diphtheria toxine and hypersusceptibility to horse serum in the same animal. By John F. Anderson.

No. 31.—Variations in the peroxidase activity of the blood in health and disease. By Joseph H. Kastle and Harold L. Amoss.

No. 32.—A stomach lesion in guinea pigs caused by diphtheria toxine and its bearing upon experimental gastric ulcer. By M. J. Rosenau and John F. Anderson.

No. 33.—Studies in experimental alcoholism. By Reid Hunt.

No. 34.—I. *Agamofilaria georgiana* n. sp., an apparently new roundworm parasite from the ankle of a negress. II. The zoological characters of the roundworm genus *Filaria* Mueller, 1787. III. Three new American cases of infection of man with horsehair worms (species Paragordius varius), with summary of all cases reported to date. By Ch. Wardell Stiles.

*No. 35.—Report on the origin and prevalence of typhoid fever in the District of Columbia. By M. J. Rosenau, L. L. Lumsden, and Joseph H. Kastle. (Including articles contributed by Ch. Wardell Stiles, Joseph Goldberger, and A. M. Stimson.)

No. 36.—Further studies upon hypersusceptibility and immunity. By M. J. Rosenau and John F. Anderson.

No. 37.—Index-catalogue of medical and veterinary zoology. Subjects: Trematoda and trematode diseases. By Ch. Wardell Stiles and Albert Hassall.

No. 38.—The influence of antitoxin upon post-diphtheritic paralysis. By. M. J. Rosenau and John F. Anderson.

No. 39.—The antiseptic and germicidal properties of solutions of formaldehyde and their action upon toxines. By John F. Anderson.

No. 40.—1. The occurrence of a proliferating cestode larva (*Sparganum proliferum*) in man in Florida, by Ch. Wardell Stiles. 2. A reexamination of the type specimen of *Filaria restiformis* Leidy, 1880=*Agamomermis restiformis*, by Ch. Wardell Stiles. 3. Observations on two new parasitic trematode worms: *Homalogaster philippinensis*

n. sp., *Agamodistomum nanus* n. sp., by Ch. Wardell Stiles and Joseph Goldberger. 4. A reexamination of the original specimen of *Tænia saginata abietina* (Weinland, 1858), by Ch. Wardell Stiles and Joseph Goldberger.

*No. 41.—Milk and its relation to the public health. By various authors.

No. 42.—The thermal death points of pathogenic micro-organisms in milk. By M. J. Rosenau.

No. 43.—The standardization of tetanus antitoxin (an American unit established under authority of the act of July 1, 1902). By M. J. Rosenau and John F. Anderson.

No. 44.—Report No. 2 on the origin and prevalence of typhoid fever in the District of Columbia, 1907. By M. J. Rosenau, L. L. Lumsden, and Joseph H. Kastle.

No. 45.—Further studies upon anaphylaxis. By M. J. Rosenau and John F. Anderson.

No. 46.—*Hepatozoon perniciosum* (n. g. n. sp.); a haemogregarine pathogenic for white rats; with a description of the sexual cycle in the intermediate host, a mite (*Lelaps echidninus*). By W. W. Miller.

No. 47.—Studies on Thyroid.—I. The Relation of Iodine to the Physiological Activity of Thyroid Preparations. By Reid Hunt and Atherton Seidell.

No. 48.—The Physiological Standardization of Digitalis. By Charles Wallis Edmunds and Worth Hale.

In citing these bulletins, beginning with No. 8, bibliographers and authors are requested to adopt the following abbreviations: Bull. No. ——, Hyg. Lab., U. S. Pub. Health & Mar.-Hosp. Serv., Wash., pp. ——.

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