SCIENTIFIC SECTION

THE BIOASSAY OF DIGITALIS WITH OBSERVATIONS ON THE p_H FACTOR.*,[1]

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INTRODUCTION.

The literature is replete with reports showing that there is much disagreement as to the selection of the best method for the assay of Digitalis.

A problem concerning the assay methods for Digitalis in this laboratory centered itself about the cat or frog methods because most of the preparations of Digitalis which are placed at the disposal of the practicing physician are standardized by one or another of the several frog methods. A review of the literature likewise shows that the most important methods used in the standardization of Digitalis are based on procedures using the cat or the frog as the test object.

The following reports bear out the above statement: Hatcher and Brody (1), Haskell (2), Eckler (3), Eggleston (4), Rowntree and Macht (5), Colson (6), Rowe (7), O'Brien and Snyder (8), Haskell, Dowell and Terry (9), A. D. M. A. Proceedings (10), Haskell and Courtney (11), Haskell (12), Pittenger (13), Vanderhoff (14), Wible (15), Trevan, Boock, Burn and Gaddum (16), Rowe (17), Swanson and Hargreaves (18), Wokes (19), Edinburgh Conference of the League of Nations Health Committee (20), the Geneva Conference (21), Knaffl-Lenz (22) and the Frankfurt Conference (22).

It is significant that in the application of the cat method in the present work, ouabain was not used to terminate the tests, nor was the "Cat Unit" (based on ouabain) method of expressing potency peculiar to the Hatcher-Brody technique, employed in these studies. It is still more significant that the ouabain-complicating "Cat Unit" procedure of Hatcher and Brody apparently has been abandoned in Dr. Hatcher's own laboratory (23). The confusion is left remaining, however, because in spite of the fact that Digitalis alone is used in the procedure described, it is still referred to as the "Hatcher-Brody method" in the article (23).

The 1932 edition of the British Pharmacopœia requires that Digitalis and its preparations be standardized in terms of the International Standard Digitalis Powder and that in the actual assay, comparison of an unknown is made against the official standard preparation of powdered Digitalis. The frog method involving the use of a mortality curve, the procedures involving the use of the guinea pig or the cat slowly perfused with an infusion of the powder or a saline dilution of the tincture are listed by the B. P. as methods that are satisfactory.

The Heart Committee (23) of the N. Y. Tuberculosis and Health Association recommends that the Hatcher-Brody procedure be used in standardizing Digitalis,

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but as pointed out in the preceding paragraphs, the procedure used is not that of Hatcher and Brody (1) since ouabain is not involved in the test.

Edmunds (24), in replying to this recommendation of the Heart Committee, stated that the Revision Committees of the U. S. P. voted unanimously to keep the frog method in 1910, 1920 and 1930. Furthermore, he answers that pharmacists claim it would be practically impossible to carry out cat tests since the manufacturers could not obtain enough cats, concluding with a statement that not a single manufacturer voted to adopt the cat procedure.

The U. S. P., 10th revision, makes mandatory a bio-assay for Digitalis, Squill and Strophanthus and their preparations. The method of assay is the one-hour frog procedure with ouabain as the reference standard.

The eleventh revision of the U. S. P. which becomes official June 1, 1936, has as the official method for the assay of Digitalis and its preparations the one-hour frog technique with a Reference Standard Digitalis Powder for comparison. Furthermore, the U. S. P. XI potency requirements are expressed in units directly referable to the International Standard Digitalis Powder.

A consideration of the above list reveals that the official trend of thought, as far as standardization of Digitalis is concerned, favors and even makes mandatory the frog or cat or guinea-pig procedures. It was these methods, therefore, which received emphasis in the report which follows.

PROCEDURE, APPARATUS AND TECHNIQUE.

1. Preparation of the Dilutions.—The frog methods used in this study were: U. S. P. X and modifications (25), U. S. P. XI (26) and the Canadian "Over Night" technique described by Chapman and Morrell (27). The dilutions for injection into the frog were made according to the requirements of the particular method used. Whenever a given preparation was so weak that de-alcoholization was necessary, the required amount of the tincture that was necessary for the dilution was placed in a beaker and a current of air passed over the tincture, after which the calculated amount of water was added. This procedure was always carried out at room temperature.

The dilutions of the tinctures or of the extracts of Digitalis used for cat assays were made up with 0.9 per cent NaCl solution and in no instance did the dilution contain more than 10 per cent alcohol by volume.

2. Apparatus.—The equipment used for the assays was outfitted with a thermoregulating device that maintained a set temperature rigidly and accurately. A very rigid maintenance of temperature is necessary since the rates of absorption are intimately related to the temperature; *vide* Baker (28), Sollmann, Mendenhall and Stingel (29), Roth (30) and Smith and McClosky (31).

3. Procedure.—The frogs received in any shipment were placed in a storage tank and kept there for a period of at least a week before they were used in an assay. The injections and method of observation were followed as directed in the U. S. P. X and U. S. P. XI.

The modifications of the U. S. P. X procedure involved only the time period; that is, in the two-hour and four-hour methods the time only was increased as indicated by the name of the method which appears in the tables.

The cat method that was followed was in all essentials that of Hatcher (23). The procedure differed, however, from the Hatcher-Brody (1) method in two respects: *First*, a supplementary ouabain solution was not used to terminate the test—a Digitalis solution alone being used throughout the assay, and *second*, the duration of the assay was limited to a period of from thirty to forty-five minutes. Burn (32) recommends a thirty- to fifty-five-minute period.

Records of this department show that it matters little what period of time is selected for the duration of the assay (within 30 and 90 minutes) provided that the period of time selected is maintained throughout all the series of assays carried out in any particular study and further provided that a standard of comparison is used in an identical manner, *expressing the potency in*

terms of the reference standard rather than in "animal units." This is an extremely important departure from the usual practice of manufacturers and other workers.

Bio-assay Standards.—The standards used for the frog assays were: Ouabain, the official U. S. P. X standard, the Standard Digitalis Powder of the U. S. P. XI and the Canadian Standard No. 428 (85 mg. equivalent to 100 mg. International Standard) and a second official sample of Canadian Standard (88 mg. equivalent to 100 mg. of International Standard) that was distributed when the supply of Standard No. 428 had been depleted.

The ouabain was kept in a 1:1000 stock solution prepared by dissolving the ouabain in 70 per cent alcohol. Such a stock solution was kept in a refrigerator at a temperature of 1–2° C. except when making the necessary dilutions for the assay. Then the solution was taken from the refrigerator and brought to room temperature before withdrawing the necessary amount. These stock solutions, when prepared and kept as described above, were found to be stable for a year or more, as determined by frequent and careful checks against freshly prepared ouabain solutions.

The standard Digitalis powders, obtained sealed in ampuls of amber glass, were kept in a container in a refrigerator until used.

The simple determination of lethal doses of preparations by the cat method does not indicate reliably the potency of a given Digitalis product. The idea of expressing results in terms of "cat units" was abandoned and in its stead a comparison of unknown Digitalis preparations was made in terms of ouabain and, in the more recent studies of the report, in terms of Standard Digitalis Powders.

PART ONE—STUDY OF TINCTURE DIGITALIS BY THE ONE, TWO AND FOUR-HOUR FROG METHODS AND THE CAT METHOD.

The tinctures of this part of the study were obtained from Dr. J. C. Munch and are described by him (33). Studies and observations made on these samples date from February 1931, so that at the beginning of these studies these samples had been aged for two years. The tinctures were labeled with the following P.T. numbers: Blue A—P.T. 23, Flint A—P.T. 24, Amber A—P.T. 25, Blue B—P.T. 26, Flint B—P.T. 27 and Amber B—P.T. 28. These bottled samples were kept on a laboratory table exposed to diffused light and to the conditions of the room. The results on these preparations are given in Table I.

TABLE I.—COMPILATION OF THE FROG AND CAT ASSAY RESULTS OBTAINED WITH P.T. 23, 24, 25, 26, 27 AND 28.

	U. S. P. X One-Hour Frog.		2-Hour Frog.		4-Hour Frog.		Cat Method.	
Preparation.	Date of Assay.	Ouabain Equiv. of 1 Cc. Mg.	Date of Assay.	Ouabain Equiv. of 1 Cc. Mg.	Date of Assay.	Ouabain Equiv. of 1 Cc. Mg.	Date of Assay.	Ouabain Equiv. of 1 Cc. Mg.
P.T.	8-14-31	0.0550	2-24-31	0.0700	8-15-31	0.0500	3-6-31	0.1057
23	2-10-32	0.0354	8-17-31	0.0454	.....	....	5-27-31	0.0897
	3-15-32	0.0450	2-9-32	0.0347	3-14-32	0.0363	12-4-31	0.0883
	2-27-33	0.0857	3-10-32	0.0438	.....	....	12-20-32	0.1117
P.T.	2-25-31	0.0700	4-17-31	0.0625	4-17-31	0.0625	6-19-31	0.0889
24	4-18-31	0.0613	8-17-31	0.0500	8-15-31	0.0583	12-9-31	0.0906
	8-14-31	0.0523	2-9-32	0.0347	3-14-32	0.0375	12-23-32	0.0912
	2-10-32	0.0344	3-10-32	0.0500	.....	....	.....	....
	3-15-32	0.0430	.....	....	.....	....	.....	....
	2-27-33	0.0800	.....	....	.....	....	.....	....
P.T.	2-25-31	0.0786	4-21-31	0.0635	4-22-31	0.0435	5-1-31	0.1049
25	4-18-31	0.0625	8-18-31	0.0593	8-15-31	0.0573	12-10-31	0.0914
	8-14-31	0.0550	2-9-32	0.0385	3-14-32	0.0438	12-8-32	0.0966
	2-10-32	0.0370	3-10-32	0.0500	.....	....	5-17-34	0.0648
	3-15-32	0.0405	.....	....	.....	....	7-12-34	0.0699
	2-27-33	0.0857	.....	....	.....	....	6-20-35	0.0856
	3-29-34	0.0714	.....	....	.....	....	4-18-36	0.0891
	5-15-34	0.0875	.....	....	.....	....	.....	....
	7-12-35	0.0769	.....	....	.....	....	.....	....
	3-26-36	0.0769	.....	....	.....	....	.....	....

P.T.	3-19-31	0.050	10-24-31	0.0375	10-24-31	0.0375	4-10-31	0.0667
26	10-23-31	0.033	3-31-32	0.0167	4-2-32	0.0200	6-25-31	0.0755
	4-1-32	0.016	.....	....	.....	....	12-17-31	0.0671
							12-27-32	0.0691
P.T.	3-19-31	0.055	3-20-31	0.0450	10-24-31	0.0429	7-11-31	0.0698
27	10-23-31	0.040	10-27-31	0.0364	4-2-32	0.0180	12-18-31	0.0746
	4-1-32	0.019	3-31-32	0.0200	.....	....	12-29-32	0.0722
P.T.	4-18-31	0.0400	3-20-31	0.05	4-22-31	0.025	7-15-31	0.0623
28	10-23-31	0.0375	4-18-31	0.05	10-24-31	0.043	12-23-31	0.0535
	4-1-32	0.0200	10-27-31	0.04	4-2-32	0.020	12-16-32	0.0597
	3-27-36	0.0556	3-31-32	0.02	.....	....	4-28-36	0.0587

DISCUSSION OF TABLE I.

In 1931 and 1932 the preparations were assayed by the U. S. P. X one-hour and the two- and four-hour modifications of the U. S. P. X method and also by the cat method. The cat method as applied in 1931–1932 involved the use of a fixed anesthetic, chloretone. The chloretone in 50 per cent alcohol solution was injected intraperitoneally. During the induction stage the cats were anesthetized lightly with ether. The M. L. D. of ouabain determined on cats anesthetized as just described was 0.1112 mg. per K. of animal. The use of chloretone, or any fixed anesthetic, was abandoned after 1932 and in its stead ether alone was used, the cats being kept lightly anesthetized with this volatile anesthetic. This method of maintaining a rather regular and easily controlled depth of anesthesia was found to be much more practical and convenient than the procedure involving the use of a fixed anesthetic. Cats anesthetized lightly with ether were slightly more susceptible to ouabain and digitalis than when chloretone was used. Average M. L. D.'s determined over several periods of time were as follows: 0.097, 0.094 and 0.095 mg. per K. of animal, using from 5 to 20 cats per assay. Cats anesthetized with chloretone were less sensitive and gave M. L. D. values somewhat greater than 0.1 mg. per K. Bauer and Fromherz (34) in studying the influence of the narcotic on the M. L. D. of ouabain and digitaloids found the M. L. D. of ouabain to be 0.114 mg. and 0.096 mg. per K. of cat when the animals were anesthetized with a barbiturate and "light ether," respectively.

Reference to Table I reveals that by the cat method there was no demonstrable loss in potency in the tinctures. Furthermore, there was no definitely demonstrable difference in potency among the different samples of one series. The A tinctures, however, were definitely stronger than the B samples, whose potency, relative to A on the basis of the dilution made, was estimated quite accurately.

The frog methods revealed, on the other hand, at first a gradual drop in potency in terms of ouabain beginning with the 1931 assays and continuing through 1932. A survey of the results by the frog methods showed that these procedures gave good agreement between the relative potencies of the A and B tinctures but these potencies were very much lower than those shown by the cat assays.

Furthermore, a comparison of the frog methods, one against the other, disclosed that the four-hour technique yielded the more definite and clearer end-point. The absorption from the lymph sac, on the whole, was better than with the shorter

observation periods. The absorption in the frogs used in the shorter periods of assay was irregular and inconsistent; sometimes good and at other times poor.

Further studies on the potencies of these preparations were limited to observations on P.T. 25 because the relative potencies of these samples were definitely established and there was no purpose in repeating tests on similar preparations. The one-hour technique of the U. S. P. X was followed in these latter assays since it was the official procedure, and official opinion pointed to the retention of a one-hour technique in the U. S. P. XI. In the final set of assays, observations on P.T. 28 were included to see whether the 100:70 ratio of potency still held.

The estimations of potency carried out beginning with 1933 showed rather interesting results. The potency of P.T. 25 apparently had risen from a level of approximately 50 per cent U. S. P. X potency to a potency of slightly more than 100 per cent, in terms of ouabain. Assays in 1934 showed potencies of 86 per cent and 105 per cent of U. S. P. X requirements. Cat potencies for the period in 1934 were at a level of 79 per cent and 84 per cent in terms of ouabain, a rather good agreement between the official method and the cat method. In 1935, the frog method showed a potency of 93 per cent, while the cat technique gave a result of 103 per cent; again a good agreement. The 1936 assays disclosed the fact that P.T. 25, by the frog technique, was 93 per cent, and 107 per cent by the cat technique. P.T. 28 was 67 per cent by the frog method and 71 per cent by the cat assay.

TABLE II.

Preparation No.	Date.	M. L. D. of Prep. Cc. Tr.	Std. Deviation from the Mean; + or −.	Ouabain Equivalent of Prep. Mg.	Std. Deviation from the Mean; + or −.
23	3-6-31	1.05	0.04	0.1112	0.0162
	5-27-31	1.24	0.10	0.1112	0.0162
	12-4-31	1.20	0.06	0.106	0.0192
	12-20-32	1.02	0.06	0.114	0.0168
24	6-19-31	1.25	0.09	0.1112	0.0162
	12-9-31	1.17	0.07	0.106	0.0192
	12-23-32	1.25	0.06	0.114	0.0168
25	5-1-31	1.06	0.03	0.1112	0.0162
	12-10-31	1.16	0.10	0.106	0.0192
	12-8-32	1.18	0.09	0.114	0.0168
	5-17-34	1.50	0.16	0.09719	0.0223
	7-12-34	1.39	0.10	0.09719	0.0223
	6-20-35	1.10	0.07	0.0943	0.0052
	4-18-36	1.10	0.08	0.0981	0.0137
26	4-10-31	1.67	0.10	0.1112	0.0162
	6-25-31	1.47	0.09	0.1112	0.0162
	12-17-31	1.58	0.11	0.106	0.0192
	12-27-32	1.65	0.09	0.114	0.0168
27	7-11-31	1.59	0.12	0.1112	0.0162
	12-18-31	1.42	0.06	0.106	0.0192
	12-29-32	1.58	0.21	0.114	0.0168
28	7-15-31	1.78	0.14	0.1112	0.0162
	12-23-31	1.98	0.08	0.106	0.0192
	12-16-32	1.91	0.08	0.114	0.0168
	4-28-36	1.67	0.08	0.0981	0.0137

It is believed that such results offer substantial proof that the frog susceptibility to Digitalis and ouabain is not parallel from a quantitative standpoint,

thus making ouabain a wholly unsuitable reference standard for use in the bio-assay of Digitalis preparations by the frog method.

Table II is included as a supplement to Table I and it shows the standard deviation from the average for the cat assays shown in Table I. The calculations were made according to Burn (35).

PART TWO—THE HYDROGEN-ION CONCENTRATION STUDY OF TINCTURE DIGITALIS.

It was decided to undertake a p_H study of the Tincture of Digitalis discussed in Part One in order to observe whether there was any relationship between potency and p_H of these tinctures as they aged.

The procedure used in estimating the hydrogen-ion concentrations of the tinctures was in all essentials that suggested by Biilmann (36) for small quantities of material. The method involved the following system:

Pt	Quinhydrone KCl 0.09*M* + HCl 0.01*M*	Sat'd. KCl	Quinhydrone Unknown Solution	Pt

The capillary electrode vessels recommended and described by Cullen and Biilmann (37) were used as the containers for the solutions of the unknown half cells.

No correction was made for the error caused by the alcoholic content of the galenicals. Smith (38) believes, since it is inconvenient to report results in terms of e. m. f. (which is in reality what is determined), it is customary to calculate p_H using the ordinary constants for water. LaMotte workers stated (private communication) that in the case of such preparations as galenicals calculations of p_H were made without correcting for the error that the alcoholic content plays. The following tables, A and B, summarize the results of these tests.

TABLE A.

P. T. No.	Date.	Series No. 1 p_H.	Date.	Series No. 2 p_H.	Date.	Series No. 3 p_H.	Date.	Series No. 4 p_H.
23	8-21-31	5.39	1-30-32	5.62	4-29-32	5.66	2-9-33	5.18
24	8-21-31	5.66	1-30-32	5.75	4-29-32	5.84	2-9-33	5.75
25	8-21-31	5.69	1-30-32	5.63	4-29-32	5.71	2-9-33	5.55
26	8-22-31	5.22	1-30-32	5.50	4-29-32	5.54	2-9-33	5.03
27	8-22-31	5.65	1-30-32	5.58	4-29-32	5.77	2-9-33	5.73
28	8-22-31	5.74	1-30-32	5.59	4-29-32	5.71	2-9-33	5.59

TABLE B.

P. T. No.	Date.	p_H.	U. S. P. X % Potency.
302	2-10-33	5.51	70
317	2-10-33	5.48	106
303	2-10-33	5.45	110
306	2-10-33	5.02	120
*284	2-10-33	5.09	100
294	2-10-33	5.59	175
309	2-10-33	5.49	105
*110	2-10-33	5.12	140

* N. F. V.

DISCUSSION.

Results of series No. 3 showed on the whole a decrease in acidity when compared with series No. 2, while determinations of the No. 4 group showed an increase in acidity when compared with series No. 3. The differences in p_H among the vari-

TABLE III.—DETERIORATION DATA.

Fresh Preparations.

Identity of Prep.	U. S. P. X Method. Ouabain Equiv. of 1 Cc. Mg.	C.-M. Canadian Over Night. Can. Std. Powd. Equiv. of 1 Cc. Mg.	C.-M. Canadian Over Night. Ouabain Equiv. of 1 Cc. Mg.	Cat Method. Can. Std. Powd. Equiv. of 1 Cc. Mg.	Cat Method. Ouabain Equiv. of 1 Cc. Mg.
Tr. B 843	0.109	100.7	0.0481	103.9	0.1117
Tr. A 844	0.100	120.6	0.0576	92.6	0.0996
Tr. Conc. 845	0.444	537.6	0.2560	512.7	0.5514
Tr. F	* Not assayed	120.3	0.0870	96.7	0.1041

After 6 Months' Aging.

Identity of Prep.	U. S. P. X Method. Ouabain Equiv. of 1 Cc. Mg.	C.-M. Canadian Over Night. Can. Std. Powd. Equiv. of 1 Cc. Mg.	C.-M. Canadian Over Night. Ouabain Equiv. of 1 Cc. Mg.	Cat Method. Can. Std. Powd. Equiv. of 1 Cc. Mg.	Cat Method. Ouabain Equiv. of 1 Cc. Mg.
Tr. B 843	0.100	73.7	0.043	88.5	0.0990
Tr. A 842	0.100	85.5	0.0498	84.3	0.0942
Tr. Conc. 845	0.417	351.0	0.239	461.4	0.5165
Tr. F	0.1**	66.6	0.0454	86.8	0.0971

* Preparation 4.5 months old when first assayed

** 13.5 months old.

ous preparations were so small as to be of doubtful significance in relation to potency changes.

The observations reported in Table B are interesting, and further the contention that there is apparently no significant relationship between p_H and potency. All the preparations listed in this table were tinctures except P.T. 110 and P.T. 284 which were fluidextracts. P.T. 317 was P.T. 302 fortified by re-percolating more drug with P.T. 302.

In general, the findings reported herein are in agreement with those given by Haag and Jarrett (39), Wokes (40) and Foster and Van Dyke (41).

PART THREE—DETERIORATION DATA ON FOUR SAMPLES OF TINCTURE DIGITALIS.

In this observation four samples were assayed by three methods and their keeping qualities followed. Three samples, P.T. 843, P.T. 844 and P.T. 845, were samples prepared for commercial distribution while the Tincture F was not. The methods of preparation of the first three samples were not available. Tincture F was prepared by collecting 1000 cc. of percolate from 100 Gm. of a Canadian Digitalis Leaf. The U. S. P. X technique was used in preparing this latter tincture.

P.T. 845 was labeled four times tincture strength while the labels of P.T. 843 and P.T. 844 bore no special statements or explanations.

TABLE IV.

First Assays.

Preparation.	M. L. D. of Prep. Cc. per Kg.	Std. Deviation of the Mean; + or −.	Ouabain M. L. D. Mg. per Kg.	Std. Deviation of the Mean; + or −.	M. L. D. of Can. Std. Powd. Mg. per Kg.	Std. Deviation of the Mean; + or −.
Tr. B P.T. 843	0.84	0.063	0.09429	0.005	87.67	5.57
Tr. A P.T. 844	0.95	0.04	0.09429	0.005	87.67	5.57
Tr. Conc. P.T. 845	0.171	0.008	0.09429	0.005	87.67	5.57
Tr. F	0.91	0.04	0.09429	0.005	87.67	5.57

Preparation.	Second Assays. M. L. D. of Prep. Cc. per Kg.	Std. Deviation of the Mean; + or −.	Ouabain M. L. D. Mg. per Kg.	Std. Deviation of the Mean; + or −.	M. L. D. of Can. Std. Powd. Mg. per Kg.	Std. Deviation of the Mean; + or −.
Tr. B P.T. 843	0.99	0.08	0.09805	0.0137	87.67	5.57
Tr. A P.T. 844	1.04	0.09	0.09805	0.0137	87.67	5.57
Tr. Conc. P.T. 845	0.190	0.001	0.09805	0.0137	87.67	5.57
Tr. F	1.01	0.09	0.09805	0.0137	87.67	5.57

The samples were assayed by: (1) U. S. P. X, (2) Canadian Over Night and (3) cat methods. The Canadian reference standard powder used in this particular study had the following relation in the International Standard: 1.0 Gm. International Standard was equivalent to 0.88 Gm. Canadian Standard Powder. The results of the observations are reported in Table III. Table IV supplements the results in Table III in that it gives the standard deviation of the mean for the cat assays.

DISCUSSION.

The potency relationships existing among P.T. 843, P.T. 844 and P.T. 845 will be considered first. The concentrate was found to be four times the potency of A and B by the U. S. P. X procedure. The cat results for A and B are in agreement with the one-hour results but the cat value of the concentrate was 1.25 times greater than the U. S. P. X frog value and was 5.5 and 5 times the potency of A and B, respectively, when the cat values are referred to ouabain as the standard. The ratio of the potencies by the cat method in terms of the Canadian Standard were the same as when expressed in terms of the cat ouabain values. Therefore, in the case of the concentrate, the cat method revealed a definitely greater potency than the one-hour frog procedure. The Canadian over night technique showed that the concentrate was 5.3 times stronger than B and 4.4 times stronger than A when the results are referred to the Canadian Standard Powder. On the other hand, although the over night ouabain values indicated the potencies of the samples to be in the same ratio as those obtained with the Canadian Standard Powder, they did not agree with the U. S. P. X values. The over night ouabain potencies were from 50–60 per cent of the U. S. P. ouabain values.

These preparations, after standing six months on the laboratory table and under ordinary room conditions, did not show a loss of potency by the U. S. P. X procedure. The cat method showed a slight drop in potency but this loss is insignificant when one considers the margin of error accorded the procedure. By the Canadian technique, A and B deteriorated to 71 per cent and 73 per cent of their original respective potencies; the concentrate assayed 65 per cent of its original potency.

Tincture F could not be compared as fully as the above-discussed preparations since one-hour results on F were not obtained when first assayed because the available stock of frogs had been depleted in obtaining over night results. A comparison of the over night and cat methods, however, reveals again that deterioration by the cat method is insignificant while the over night method sets the potency of F at 55 per cent of its previous potency.

A rather significant point in the above deterioration study is the fact that the drop in physiologic activity for A, B and the concentrate ranged from 65 per cent

to 73 per cent by the Canadian technique, and the concentrate was deteriorating as rapidly as the ordinary tincture.

PART FOUR—THE RELATIVE MERITS OF PROCESSES OF EXTRACTION OF DIGITALIS POWDERS BY MACERATION, PERCOLATION AND SOXHLET EXTRACTION.

1. Residual Activity Studies.—In the course of an assay of a Digitalis preparation for Canadian distribution, it was found that this preparation yielded two entirely different results when assayed in terms of (*a*) the Canadian Standard extracted by Soxhlet for two hours with absolute alcohol, as required in the Canadian specifications (42), and (*b*) the Canadian Standard extracted as in (*a*) but with 95 per cent alcohol. The 95 per cent alcohol gave the more potent Standard extract. This difference in potency initiated an investigation of the Canadian procedure of extraction of Digitalis powders in comparison with other methods. Thus it became the object of this study to determine whether or not the Canadian technique, if rigidly followed, was fully efficient in extracting the total activity of a given Digitalis powder. This information would prove valuable to bio-assayists since manufacturers who distribute Digitalis in Canada standardize these products according to the Canadian procedure. Furthermore, if the Canadian technique does not yield a complete extraction, then the Digitalis preparations assayed and adjusted according to the results obtained by this method, would appear on the market actually weaker than the intended requirements.

The activity of the various extractives prepared was followed by using the regular Canadian Over Night Method of assay (27). At first, the only deviation, and that by necessity, from this technique was in the number of frogs used in estimating the potency of the majority of the residual extracts (q. v. Table V).

TABLE V.—DATA ON RESIDUAL EXTRACTIVES.

Identity of Preparation.	Ouabain Equiv. of Total Ext., after 1st Ext'n Mg.	Ouabain Equiv. of Total Residual Ext'n Mg.	Duration of Residual Ext'n in Hours.	Absolute Ouabain Equiv. of the Digitalis Powd. Mg.	% of Absolute Ouabain Equiv. Remaining after 1st Extraction.	Number of Frogs in the Residual Test.
1 2-hr. abs. ext. powd. P.T. 733 of 3-22-35	0.3320	0.11628	5.0	0.44828	25.9	10
2 2-hr. abs. ext. Can. Powd. of 3-22-35	0.36613	0.09030	5.0	0.45643	19.8	6
3 2-hr. abs. ext. Can. Powd. of 4-26-35	0.33330	0.09263	4.0	0.42593	21.7	10
4 2-hr. abs. ext. Can. Powd. of 5-23-35	0.42625	Negative	2.5	.....	..	10
5 2-hr. abs. ext. Can. Powd. of 6-27-36	0.40000	0.10137	6.75	0.50137	20.2	10
6 2-hr. abs. ext. Powd. No. 432 of 7-16-35	0.52035	0.13215	8.5	0.65250	20.3	5
7 2-hr. abs. ext. Can. Powd. of 7-16-35	0.38675	0.07469	8.5	0.46144	16.2	8
8 2-hr. abs. ext. Can. Powd. of 10-15-35	0.26917	0.04425	6.5	0.31342	14.1	6
9 5-hr. abs. ext. Can. Powd. of 1-7-36	0.49677	0.04092	12.0	0.53769	7.6	10

10	2-hr. abs. ext. Can. Powd. of 1-15-36	0.35900	0.07381	13.0	0.43281	17.5	5
11	2-hr. 95% alc. ext. Can. Powd. of 1-15-36	0.43038	0.04725	13.0	0.47763	9.9	2
12	2-hr. abs. ext. Can. Powd. of 4-15-36	2.06305	0.27380	5.0	2.33685	11.7	25
13	2-hr. 95% alc. ext. Can. Powd. of 4-15-36	2.381652	0.17020	5.0	2.551852	6.7	25
14	2-hr. abs. ext. U. S. P. XI Powd. of 4-15-36	2.885883	0.6355648	5.0	3.521531	18.1	25
15	2-hr. 95% alc. ext. U. S. P. XI Powd. of 4-15-36	3.380872	0.23840	5.0	3.619272	6.6	25

The reason for using less than twenty-five frogs was that the residual extract was obtained from the re-extraction of the small amounts of samples that are ordinarily used in the Canadian method. The samples extracted on 4-15-36 weighed approximately three Gm. or six times the usual weight of a sample used in ordinary routine assay. The purpose of using a larger quantity was: (1) to obtain a volume of residual extract that would provide a quantity sufficiently adequate for twenty-five frogs and (2) to reduce any possible error resulting in losses due to transfer of smaller volumes of extract. The menstruum which was used to extract the powder at first was also used for the re-extraction process.

The residual activity of any given sample was estimated by comparing the activity of the first extract and the re-extracted fraction with ouabain. In this way the total activity of any powder could be computed.

In another phase of this study, the activities of extracts obtained with menstrua other than absolute alcohol were compared. These results are reported in Table VI.

TABLE VI.—COMPARISONS OF EXTRACTION METHODS.

Identity of Preparation.	Date of Assay.	Equivalent of 100 Mg. Can. Std. Powder 2-Hour Abs. Ext. Mg.	% Equivalent of 2-Hour Abs. Ext. Can. Powder.
Can. Powd. 2-hr. abs. ext.	1-7-36	100.00	100.0
Can. Powd. 5-hr. abs. ext.	1-7-36	84.11	118.9
Can. Powd. 2-hr. 95% ext.	1-15-36	84.00	119.0
Can. Powd. 2-hr. abs. ext.	1-15-36	100.00	100.0
U. S. P. XI Std. Powd. 2-hr. abs. ext.	4-15-36	70.70	141.4
U. S. P. XI Std. Powd. 2-hr. 95% ext.	4-15-36	60.00	167.0
Can. Powd. 2-hr. abs. ext.	4-15-36	100.00	100.0
Can Powd. 2-hr. 95% ext.	4-15-36	83.10	120.3
U. S. P. XI Std. Powd. macerate	4-16-36	55.60	179.9
U. S. P. XI Std. Powd. percolate	4-16-36	58.10	172.1
Can Powd. macerate	4-16-36	89.40	111.9
Can. Powd. percolate	4-16-36	101.00	99.0

DISCUSSION.

In this study, the ouabain equivalents of both the first extract and the residual extract were always determined. The sum of the ouabain equivalents of the two total extracts indicates the total physiologic activity of any given sample. The following example is given to illustrate how the residual activity was calculated.

Example: 0.02 cc. per Gm. frog of an aqueous dilution of an extract of powder P.T. 733 caused 50 per cent mortality which corresponds to a dose number of 4.0 (cf. Chapman and Morrell (27) while 0.00035 mg. of ouabain caused 70 per cent mortality corresponding to a dose number of 4.22. Therefore 0.02 cc. is equivalent to:

$$\frac{4.0}{4.22} \times 0.00035 = 0.000332 \text{ mg. ouabain}$$

Since the total volume of this dilution was 20 cc. the ouabain equivalent of the total sample of this aqueous dilution, which represents the total amount of the sample of powder by the first extraction, becomes:

$$0.000332 \times \frac{20}{0.02} = 0.332 \text{ mg.}$$

The aqueous dilution of the residual extract or re-extracted portion in a dose of 0.025 cc. per Gm. of frog caused a mortaility of 40 per cent corresponding to a dose number of 3.9 while 0.00035 mg. of ouabain produced 70 per cent mortality. Thus 0.025 cc. of the aqueous diluted re-extractive is equivalent to:

$$\frac{3.9}{4.22} \times 0.00035 = 0.000323 \text{ mg. ouabain}$$

The ouabain equivalent of the total residual extract becomes:

$$0.000323 \times \frac{9}{0.025} = 0.11628 \text{ mg. ouabain,}$$

since the total volume representing the residual extract was 9 cc.

The true or absolute activity of this particular sample of powdered Digitalis becomes the equivalent of $0.332 + 0.11628 = 0.44828$ mg. of ouabain and the per cent residual activity is represented by:

$$\frac{0.11628}{0.44828} \times 100 = 25.9\%$$

A study of Table V reveals that: (1) 10–25% of the activity remains in the powders extracted with absolute alcohol for two hours, (2) a five-hour extraction with absolute alcohol and a two-hour extraction with 95 per cent alcohol yield extracts of the same potency and (3) the residual activity after a five-hour absolute, and two-hour 95 per cent alcohol extractions is less than 10 per cent of the total potency of the powder.

The period of time that was followed in the re-extraction processes varied as is indicated in Table V. The period of time selected at first for re-extraction was set at five hours since literature reports indicate that six to eight hours give complete extractions. The 1932 Edition of the British Pharmacopœia (43) specifies a six-hour extraction with absolute alcohol in a continuous extraction apparatus. Foster and Van Dyke (41) state eight hours' continuous extraction with absolute alcohol gives a complete extraction. However, they point out that four-hour extracts were not significantly less potent than the eight-hour extract.

Later it was deemed advisable to observe the influence of periods of time less and greater than the five-hour re-extraction period on the residual activity of the

Digitalis powders. The data compiled in Table V indicate that re-extraction of at least four hours is necessary in order to estimate the activity of these residues. Furthermore, the results show that the activity determined for the residual extracts of the powders extracted for five hours with absolute alcohol and those extracted for two hours with 95 per cent alcohol, is of such magnitude as to be insignificant from a standpoint of bio-assay standardization. This indicates that for practical purposes of routine standardization by the Canadian Over Night technique, a five-hour absolute alcohol extract and a two-hour 95 per cent alcohol extract may be considered as representing the full activity of the powder.

As a result of the studies reported in Table V, in which the optimum accuracy of the method used could not be realized because a sufficient number of frogs was not used in most of the instances, it was decided to compare the potencies of Digitalis extracts obtained by (1) the U. S. P. XI process for Tincture Digitalis (slow percolation), (2) maceration as prescribed for the U. S. P. XI Standard Digitalis with the single exception that 10 cc. of menstruum were used for each Gm. of powder taken and (3) Soxhlet extraction with 95 per cent and absolute alcohol. The potency determinations were made by following the Canadian Over Night Method.

After examination of Table VI, one comes to the conclusion that the two-hour period of extraction with absolute alcohol in a Soxhlet apparatus does not permit the realization of a complete extraction. On the other hand, two-hour extraction with 95 per cent alcohol, five-hour extraction with absolute alcohol in a Soxhlet apparatus, maceration and percolation yield approximately 20 per cent stronger extracts. An exception to this was noted in the case of the Canadian Standard Powder extracts obtained by percolation and maceration, in which case the percolate was equal in potency to the two-hour absolute alcohol extract while the macerate was 12 per cent stronger. These results indicate that if extraction in a Soxhlet apparatus is to be followed in preparing an extract for assay by the Canadian method, a five-hour extraction with absolute alcohol or a two-hour extraction with 95 per cent alcohol will give a more complete extraction than the present Canadian procedure. However, it must be pointed out that although the Canadian specifications do not permit a complete extraction, the technique does permit an assayist rigidly adhering to the prescribed steps of the method to obtain a fairly constantly uniform extract. By this last statement, it is meant that the relative potency of a given powder of Digitalis compared with the Canadian standard will be indicated quite accurately since the two powders will be extracted approximately to the same degree. This condition will not hold, on the other hand, when it is a question of standardizing a tincture of Digitalis prepared for commercial distribution, since the extraction factor in this case is eliminated. However, it is possible to obtain check assays on a tincture or fluidextract in terms of the Canadian Standard Powder, but the potency thus obtained will not indicate the true activity of that galenical. A tincture standardized by the present Canadian technique passes into the trade actually weaker than is indicated by the specified requirements. The true or absolute equivalent in mg. of Digitalis Standard of such a tincture may be expressed by the following formula:

$$T = A + \left(\frac{x}{y} \times A\right) \text{ in which}$$

T = The absolute value in mg. of standard

A = The apparent value of mg. of standard as found by the assay

$\frac{x}{y}$ = Fraction representing the residual activity.

2. Study of the Deterioration of the Aqueous Dilutions of the Soxhlet Extracts.—The Canadian technique requires that the extraction and dilution of the extract should be made within twenty-four hours of the beginning of injections. It was decided to obtain data on the potency of the aqueous dilutions as they aged. These aqueous dilutions, aged for periods of time ranging from one week to seven and one-half months, were assayed against freshly prepared extracts of the Canadian standard. The aging aqueous dilutions of the extracts were kept in glass containers well stoppered in a refrigerator at a temperature of 4° C. The results of this study are given in two tables, Table VII and Table VIII.

TABLE VII.—DETERIORATION DATA.

Identity of Preparation.	Age of Diluted Extract.	Equivalent of 100 Mg. of Fresh 2-Hr. Abs. Alc. Ext. of Can. Std. Powder Mg.	% Equivalent of Fresh 2-Hr. Abs. Ext.
Can. Powd. 2-hr. abs. ext.	Fresh	100.00	100.0
Can. Powd. 5-hr. abs. ext.	Fresh	84.11	118.9
Can. Powd. 2-hr. abs. ext.	7.5 months	113.63	88.0
Can. Powd. 2-hr. abs. ext.	6.5 months	129.63	77.0
* Powd. No. 432 2-hr. abs. ext.	5.75 months	179.37	47.7
Can. Powd. 2-hr. abs. ext.	5.75 months	138.88	72.0
Can. Powd. 2-hr. abs. ext.	4.5 months	140.85	71.0

* 85.6 mg. of Powder No. 432 were equivalent to 100 mg. of Canadian Standard Powder when freshly prepared.

TABLE VIII.—DETERIORATION DATA.

Identity of Preparation.	Age of Diluted Extract.	Equivalent of 100 Mg. of Fresh 2-Hr. Abs. Alc. Ext. of Can. Std. Powder Mg.	% Equivalent of Fresh 2-Hr. Abs. Ext.
Can. Powd. 2-hr. abs. ext.	Fresh	100.00	100.00
Can. Powd. 2-hr. 95% ext.	Fresh	84.00	119.0
Can. Powd. 2-hr. abs. ext.	1 week	111.40	89.8
Can. Powd. 5-hr. abs. ext.	1 week	98.43	102.0

DISCUSSION.

From a survey of Tables VII and VIII, it is evident that within a week the aqueous dilutions of the Digitalis extracts show a decrease in potency even when these dilutions are kept at the temperature of the refrigerator. Although actual biologic tests do not show a very appreciable deterioration in these week-old aqueous dilutions, the tests indicate that there is evidence of loss in potency. From the data on hand, it also appears that the deterioration rate reaches an equilibrium level at approximately 70 per cent of the original potency after aging four and a half months. Therefore, the requirements set forth by the Canadian method pertaining to the immediate use of the aqueous dilutions of the Digitalis extracts are perfectly rational.

3. Comparison of Extraction Techniques by the U. S. P. XI and Canadian Over Night Methods of Assay.—In this particular phase of the study of the relative potencies of Digitalis extracts obtained by maceration and percolation (as described in Part A) and Soxhlet extraction, it was decided to pay special attention to the physiologic activity of these extracts in terms of U. S. P. X and U. S. P. XI requirements. The standards for comparison in this series of observations were

ouabain and the macerate of the U. S. P. XI Standard Powder (1 cc. of macerate being equivalent to 100 mg. of this powder). Table IX gives the results of this investigation. Potency estimations by the Canadian Over Night Method are also included in this table for the purpose of comparing the one-hour method against the over night technique. Results by the Canadian technique are likewise referable directly to the macerate of the U. S. P. XI Digitalis Standard.

TABLE IX.—COMPARISON OF EXTRACTION METHODS.

Preparation.	U. S. P. X Method. Ouabain Equiv. of 1 Cc. Mg.	U. S. P. XI Method. U. S. P. XI Std. Powd. Equiv. of 1 Cc. Mg.	C.-M. Canadian Method. U. S. P. XI Std. Powd. Equiv. of 1 Cc. Mg.	C.-M. Canadian Method. Ouabain Equivalent of 1 Cc. Mg.	Cat Method. Ouabain Equivalent of 1 Cc. Mg.
Macerate of U. S. P. XI Powd.	0.1670	100.0	100.0	0.1232	....
Percolate of U. S. P. XI Powd.	0.1670	100.0	95.0	0.1169	....
Macerate of Can. Std. Powd.	0.1428	85.7	62.0	0.0761	....
Percolate of Can. Std. Powd.	0.1428	85.7	55.0	0.0673	....
2-hr. 95% Alc. Ext. U. S. P. XI Powd.	0.1670	100.0	92.0	0.1133	....
2-hr. abs. alc. ext. U. S. P. XI Powd.	0.1250	75.0	78.0	0.0961	....
2-hr. 95% alc. ext. Can. Std. Powd.	0.1250	75.0	67.0	0.0819	....
2-hr. abs. alc. Ext. Can. Std. Powder	0.100–0.125	60–75	55.0	0.0682	....
P.T. 843 Tr. B	0.1000	60.0	37.0	0.0430	0.0990
P.T. 844 Tr. A	0.1000	60.0	43.0	0.0498	0.0942
P.T. 845 Tr. Conc.	0.333–0.500	200–300	195.0	0.2385	0.5165
P.T. 25	0.0714	42.9	27.5	0.0321	0.0891
P.T. 28	0.0555	33.3	19.0	0.0221	0.0587
Tr. F	0.1000	60.0	33.3	0.0388	0.0971
Macerate of Powd. No. 432	0.1375	75.0	...	....	....
Percolate of Powd. No. 432	0.1375	75.0	...	....	....
Macerate of Powd. A	0.0917–0.1100	50–60	...	....	....
Percolate of Powd. A	0.1000	54.5	...	....	....
Macerate of Powd. P.T. 417	0.1053	63.2	...	....	....
Percolate of Powd. P.T. 417	0.1267	66.7	...	....	....
Macerate of Powd. P.T. 632	0.1250	75.0	...	....	....
Percolate of Powd. P.T. 632	0.1250	75.0	...	....	....
Macerate of Powd. P.T. 747	0.150–0.1670	75–100	...	....	....
Percolate of Powd. P.T. 747	0.1180	70.6	...	....	....
Macerate of Powd. Minn. Fol.	0.1670	100.0	...	....	....
Percolate of Powd. Minn. Fol.	0.1540	92.3	...	....	....

DISCUSSION.

Observation of these tests reveals that the maceration technique of the U. S. P. XI (26), two-hour extraction in a Soxhlet apparatus with 95 per cent alcohol and slow percolation yield extracts, in general, of the same potency. However, it must be pointed out that it appears that the macerate is a trifle stronger than the percolate when the potencies of these two types of extracts are considered in the light of both the one-hour and Canadian over night techniques.

In several instances the one-hour frog method did not yield clear-cut results since a range of potencies, rather than any one set potency, was the best that could be obtained. The Canadian technique eliminates the possibility of any personal interpretation, a factor which does arise and will persist as long as the so-called "minimum systolic standstill" is the end-point in the one-hour technique. In the case of comparatively strong and fresh preparations, the end-points as a rule are quite definite and easily discernible. On the other hand, if weak samples or aged samples of tinctures be assayed, then the end reaction is not so clear since stopped hearts (even after "massive" doses) are readily stimulated and will beat, then stop and beat again. Furthermore, with aged samples the absorption is erratic and this factor again complicates the picture. Is the poor absorption due to the inherent properties of an aged sample or of a weak sample which has to be concentrated before a suitable dilution can be made, or is the poor absorption caused by the failure of circulation after the frog has absorbed enough to cause complete heart stoppage?

A comparison of the results obtained by the Canadian technique and those obtained by the U. S. P. XI one-hour method shows that the results by the former method are on the whole lower than the potencies shown by the U. S. P. technique. Comparison of the ouabain equivalents by the over night and one-hour methods reveals that the frog is much more susceptible to ouabain by the over night method. As a consequence of this variation in susceptibility, a discussion of potencies in terms of ouabain equivalents is ruled out.

At this point it is worth while to include a brief summary of the conclusions and recommendations made by the Sub-Committee on Digitalis of the A. D. M. A. as reported in the proceedings (44) of this association. Among their recommendations are two that are quite pertinent: (1) the abandoning of ouabain as a reference standard for Digitalis and (2) the replacement of the M. S. D. one-hour frog technique by an M. L. D. technique of six hours or over night, so as to allow sufficient time for a complete absorption and physiologic effect. This particular A. D. M. A. report (44) is based on a collaborative study carried out by operators in twelve laboratories. Two samples of Tincture Digitalis were used in this study.

In 1935, this same Sub-Committee reported (45) the collaborative efforts of ten laboratories on two samples of Tincture Digitalis. One tincture, Sample B, was prepared by diluting a portion of the sample labeled "A" to two-thirds the potency of A. The tests were carried out by the one-hour M. S. D. and the over night M. L. D. frog methods with Canadian Standard Digitalis Powder No. 428 as a reference standard. The potencies of the tinctures assayed in this collaborative study were reported in terms of International Standard Powder. The results obtained in this study were much more uniform than in the previous year when ouabain was used as a reference standard. The average results of the ten laboratories

by the one-hour method were 94.8 per cent for A and 76.3 per cent for B and by the over night method 97.6 per cent for A and 69.8 per cent for B. Thus by the one-hour procedure B was 80.7 per cent of A and by the over night method B was 70.5 per cent of A. The one-hour results tend to show for B a greater potency than is theoretically possible, if the method of preparing B is kept in mind. This committee in analyzing the compiled data of the ten laboratories reveals that there were from four to six different conclusions that could be mathematically calculated and that it was impossible to say which of two or three of the more likely interpretations was actually correct. This committee cites as an example that the data obtained in testing Tincture A by the one-hour method gave interpretations ranging from 77 per cent to 100 per cent. The possibility of misinterpretation of results obtained by the Canadian technique is negligible if not absent. On the other hand, such a condition does not prevail if the one-hour technique as outlined by either U. S. P. X or U. S. P. XI is followed. The collaborative work carried out under A. D. M. A. auspices points to the adoption of an over night technique since such a procedure gives clear-cut results that are devoid of the difficulty of interpretation of an end-point reaction.

It is interesting from a comparative standpoint to see how the results of this laboratory on these two samples are related to the results reported by the Digitalis Sub-Committee:

Preparation.	A. D. M. A. Av. Result.	Result of the Pharmacology Lab. of the School of Pharmacy.
ONE-HOUR FROG METHOD.		
Tr. A	94.6%	100%
Tr. B	76.3%	86%
OVER NIGHT FROG METHOD.		
Tr. A	97.6%	109%
Tr. B	69.8%	75%

The results of this laboratory are in concordance with the general findings of the A. D. M. A. report. By the one-hour method B is 86 per cent of A and by the over night technique B is 68.8 per cent of A. Thus by the over night procedure this laboratory was able to estimate accurately the theoretical potency of B.

Some data obtained with cat assays are included in Table IX for the purpose of a review and limited comparison of the three widely used techniques for Digitalis Standardization. These cat assays were previously discussed in the deterioration studies and therefore no further comments on this particular group of assays will be made.

4. A Comparison of the Potency of Digitalis in Terms of the U. S. P. X and U. S. P. XI Requirements.—This study was undertaken with the view of determining the relative potencies when assayed (1) in terms of the U. S. P. X requirements and (2) in terms of U. S. P. XI requirements. The results of this investigation are reported in Table X.

The reference standards for comparison were ouabain (supplied by the Food and Drug Administration of the U. S. Department of Agriculture) and the U. S. P. XI Standard Digitalis Powder (supplied by the Board of Trustees of the U. S. P. Convention). This U. S. P. Reference Digitalis Powder is 1.34 times as strong as the "U. S. P. Digitalis Unit" which in turn is equivalent to 100 mg. of International Standard Digitalis since 74.5 mg. of the U. S. P. XI Reference Powder are equivalent to one U. S. P. Digitalis Unit.

TABLE X.—COMPARISON OF POTENCIES OF DIGITALIS EXTRACTS IN TERMS OF U. S. P. X AND U. S. P. XI REQUIREMENTS.

Preparation.	% U. S. P. X Potency.	% U. S. P. XI Potency.	Ratio of U. S. P. X Potency to U. S. P. XI Potency.	
Macerate U. S. P. XI Powd.	200	134	1.49	
Percolate U. S. P. XI Powd.	200	134	1.49	
Macerate Can. Std. Powd.	172	115	1.50	
Percolate Can. Std. Powd.	172	115	1.50	
2-hr. 95% ext. U. S. P. XI Powd.	200	134	1.49	
2-hr. abs. ext. U. S. P. XI Powd.	151	101	1.50	
2-hr. 95% ext. Can. Powd.	151	101	1.50	
2-hr. abs. ext. Can. Powd.	121–151	80–101	1.48	(average)
P.T. 843	121	80	1.51	
P.T. 844	121	80	1.51	
P.T. 845	401–602	268–402	1.50	(average)
P.T. 25	86	57	1.51	
P.T. 28	67	45	1.49	
Tr. F	121	80	1.51	
Macerate Powd. No. 432	166	101	1.64	
Percolate Powd. No. 432	166	101	1.64	
Macerate Powd. A	110–133	67–80	1.65	(average)
Percolate Powd. A	121	73	1.66	
Macerate Minn. Fol.	200	134	1.49	
Percolate Minn. Fol.	186	124	1.50	
Macerate P.T. 417	127	85	1.49	
Percolate P.T. 417	152	89	1.71	
Macerate P.T. 632	151	101	1.50	
Percolate P.T. 632	151	101	1.50	
Macerate P.T. 747	181–200	101–134	1.62	(average)
Percolate P.T. 747	142	95	1.49	

A survey of Table X shows that the U. S. P. XI potency requirements are definitely greater than those of the U. S. P. X. By actual assay the average of twenty-six tests revealed that the U. S. P. XI potency is 1.531 times as great as the U. S. P. X requirements.

The Canadian Standard Digitalis Powder used in this particular study had the following relationship: 88 mg. of Canadian Standard are equivalent to 100 mg. of International Standard. Thus this sample of Canadian Powder is 1.136 times as strong as the International Standard. The potency of this Canadian Standard referred to the International Powder through a comparison with the U. S. P. XI Reference Powder and as determined by the U. S. P. XI procedure, was found to be 115 per cent of International Powder potency.

Comparison of the U. S. P. XI Digitalis Standard Powder was made on four different occasions. The following table summarizes these tests:

Date.	M. S. D. of Ouabain Mg. per Gm. Frog.	M. S. D. of U. S. P. XI Std. Powder Mg. per Gm. Frog.
3-26-36	0.00050	0.300
3-31-36	0.00055	0.325
4-4-36	0.00050	0.300
4-7-36	0.00050	0.300

In three of the tests 100 mg. of the Digitalis Standard Powder were equivalent to 0.167 mg. of ouabain or 200 per cent U. S. P. X potency but only 134 per cent in terms of U. S. P. XI requirements. In the other test the ouabain equivalent of 100 mg. of this Digitalis was slightly greater—0.169 mg.

GENERAL DISCUSSION.

The over night frog method is to be preferred to the one-hour frog method because the over night procedure allows sufficient time for complete absorption, a factor of paramount importance in obtaining consistently good results, and thus full physiologic effect. At the same time this method eliminates the personal equation in the interpretation of the end-point effects as is often required in the one-hour technique.

The abandonment of ouabain as a reference standard for Digitalis standardization removes an objection to the U. S. P. X procedure for Digitalis standardization, since Digitalis and ouabain are not identical from a standpoint of absorption rate and frog susceptibility.

The most practical method, a method that should give more uniform results, for the preparation of a Digitalis extract of the reference standard is the maceration technique as described for the Reference Digitalis Powder of the U. S. P. XI (26). The maceration technique requires very little of the assayist's time. The variables incident to a percolation procedure are eliminated if a macerate be prepared for use in the assay. Berry and Davis (46), comparing the relative merits of maceration and percolation for the preparation of Tincture of Digitalis found that maceration was just as effective as percolation.

SUMMARY AND CONCLUSIONS.

1. The expression of potency of Digitalis assayed by the cat method in terms of "cat units" is a practice that should be abandoned.

The cat method should involve direct comparison of the unknown with the reference standard powder in order to eliminate differences in results among different workers who use different types and depths of anesthesia, different perfusion rates, etc. The application of identical technique to both unknown and standard and the expression of potency in terms of reference powder instead of "cat units" would eliminate a serious objection to the routine use of this method.

The cat method in the first portion of this study revealed very little loss in potency in tinctures of Digitalis (ouabain was used then as a reference standard) for a period of approximately five years.

2. The one-hour frog method of the U. S. P. X (with ouabain as the standard) yielded peculiar results in a deterioration study conducted over a period of approximately five years. A consistent decrease in potency of the tinctures followed by an increase in potency was observed.

3. When the deterioration of samples of Tincture of Digitalis was followed by the U. S. P. X, Canadian Over Night Frog and the Cat Methods, the Canadian technique was the only method to reveal a significant loss in potency of tinctures aged for approximately six months. The cat method showed a loss that was not mathematically significant.

4. The unsuitability of ouabain as a reference standard for Digitalis is confirmed.

5. No relationship between the potency and p_H of tinctures could be observed.

6. The Canadian extraction technique does not exhaust the activity of a given Digitalis powder. The activity remaining in the sample represents approximately 20 per cent of the total activity of the powder.

7. Maceration, percolation and a modified Canadian technique of Soxhlet extraction of five hours with absolute alcohol or two hours with 95 per cent alcohol yield quantitative extracts satisfactory for practical purposes.

8. The maceration technique as described in the U. S. P. XI for the extraction of the Digitalis Reference Powder was found to be the most satisfactory extraction process for use in the routine bio-assay of Digitalis.

9. The potency requirements for Tincture Digitalis U. S. P. XI are 1.53 times greater than those of U. S. P. X based on the relative susceptibilities of frogs to ouabain and Digitalis in the spring of 1936.

10. The over night technique of the Canadian method is the most satisfactory frog procedure for routine standardization of Digitalis products.

11. Although the U. S. P. XI and Canadian Tinctures of Digitalis are intended to be equivalent to 100 mg. of International Standard Digitalis Powder per cc., the U. S. P. XI tincture is considerably stronger than the Canadian tincture because of the fact that the Canadian extraction technique does not permit a complete extraction of the reference standard.

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STUDIES OF LECITHIN SOAP.*

I. BACTERICIDAL AND DETOXIFYING EFFECT ON INTESTINAL FLORA.

BY L. G. HADJOPOULOS AND SAUL CASPE.

The detergent properties of soaps have been known since their introduction into domestic use. A definite knowledge of their bactericidal value was gradually acquired at a much later period through the observations of such outstanding workers as Koch (1881), Noguchi (1907), Landsteiner and Erlich (1908), Lanar and Flexner (1911), Nichols (1920) and others.

During the last two decades, the bacteriostatic property of soaps has been intensively investigated and the results obtained can be summarized in the words of Eggerth (1–5):

> 1. That there exists within certain limitations, a direct relationship between the molecular weights of the fatty acids, and the corresponding germicidal properties of their respective soaps;
> 2. That the germicidal properties are more manifest in the higher p_H values of the soaps;
> 3. That the soaps of the various fatty acids exhibit selective germicidal values which can be increased or decreased by substituting the alpha hydrogen with hydroxyl, bromine or sulphydryl-groups.

R. R. Spencer (6) experimentally confirmed the selectivity of certain soaps for certain bacteria, and his interesting work on the visible effects of sodium ricinoleate in completely dissolving bacterium tularense, bacillus typhosus, bacillus pyocaneus, bacillus alkaligenes, can be used as a working hypothesis of the mode by which soaps act upon bacteria.

Larson (7) assumed that, in general, these intrinsic properties of soaps are dependent upon their surface tension reducing values. However, he was not able to account by this assumption for the effectiveness of soaps upon bacterial by-products

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