

THE BIOASSAY OF DIGITALIS WITH OBSERVATIONS ON THE pH FACTOR

By
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of the University of Maryland in partial
fulfillment of the requirements for
the degree of Doctor of Philosophy

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Introduction

For a number of years literature reports have shown that various bioassay workers have disagreed as regards the best method for the assay of Digitalis.

A problem concerning the assay methods for Digitalis and evolving from the laboratories of the Department of Pharmacology of the School of Pharmacy centered itself about the cat or frog methods because most of the preparations of Digitalis which are put at the disposal of the practicing physician are standardized by either the cat or the frog methods. A review of the literature 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.

Hatcher and Brody (1) using the cat method found that the results would be 20% too high if only the Digitalis preparation were used for the perfusion throughout the assay. In order to eliminate this error, they modified the procedure in such a way that the assay was terminated by injecting an ouabain solution.

Haskell (2) concluded from his studies of the Digitalis assay procedures that the one-hour frog method is satisfactory and that ouabain standardization nullifies variations in the frogs.

Eckler (3) believes that the cat method is the most complicated and difficult technique when it is a question of the cat, frog and guinea pig methods.

Eggleston (4) on the other hand, states that of the three methods - cat, frog and guinea pig - the cat method is the best.

Rowntree and Macht (5) claimed that of the two commonly used procedures, cat and frog techniques, the cat method is the better. These workers used only a Digitalis preparation for the perfusion.

Colson (6) believes that the cat method is best and, on the whole, gives results which are lower than those obtained by the frog method.

Rowe (7) prefers the M.L.D. frog method to the cat and dog methods; and the cat, to the dog. However, he believes he did not work with enough cats.

O'Brien and Snyder (8) assayed Tinctures and Fluidextracts of Digitalis by the Hatcher cat method, one-hour and the twenty-four hour M.L.D. frog methods and the guinea pig method. These workers found the cat assays to show the least deterioration and the frog assays the most, in preparations which had aged for two and a half years.

Haskell, Dowell and Terry (9) concluded from their study on the deterioration of Tincture Digitalis that the frog and guinea pig methods show a rapid deterioration while the Hatcher cat method shows no apparent loss in potency even over a period as long as five years.

A report of the A.D.M.A. (10) states that four methods survived the test of time; (a) guinea pig, (b) one-hour frog, (c) M.L.D. frog and (d) cat methods. This report also states that the greatest variation

was observed in the comparison of the results obtained by the cat and frog techniques. Furthermore, the report reveals that some collaborators obtained results which were in good agreement by these methods.

Haskell and Courtney (11) believe that the Hatcher cat method excels the other methods of assay for Digitalis.

Haskell (12) studied the effect of age on the potency of Digitalis by the oral cat and the frog methods. His results, by these two methods checked. The Hatcher method, on the other hand, showed higher results in aged tinctures.

Pittenger (13) reporting at a meeting of the American Drug Manufacturers' Association, stated that A.D.M.A. members, members of the Committee on Biological Assaying of the A. Ph. A. and other experts found the guinea pig and M.L.D. frog procedures to yield results that were more accurate than those obtained by the one-hour frog or the cat technique. Furthermore, he stated that an objection to the guinea pig method was the cost of animals.

Vanderhoff (14) working with fresh and old tinctures found that the fresh tinctures were absorbed more rapidly than the old ones. These tests were carried out on healthy medical students, cats and dogs. The intravenous cat method showed no deterioration while the frog lymph sac, oral cat and the human being tests showed loss in absorbability - thus a loss in potency.

Wible (15) assayed nine tinctures by the frog and cat methods. He found discrepancies between these two methods but these variations were not consistent.

Trevan, Boock, Burn and Gaddum (16) who carried out a comparative study of bioassay methods on two samples of Digitalis powder reported that there is an agreement by frog, rabbit auricle, guinea pig and cat methods provided the sample is strong. However, if the sample be weak, then there is a serious disagreement among the results although all methods show the sample to be weak.

Rowe (17) states that the frog (.U.S.P.X and M.L.D.) methods and the cat method are inapplicable while the guinea pig method could be used but the animals are too expensive.

Swanson and Hargreaves (18) using frog, cat and guinea pig procedures found variations which were not consistent.

Wokes (19) gives results which show that with an increase in the age of the preparations the frog assays show a much lower potency when compared with the cat assay results. Likewise, he points out that the frog potency diminishes rapidly during the first few months of storage until the potency reaches a level of one-half to two-thirds of its original value at which point the potency becomes constant for several years. The cat potency, on the other hand, decreases much more slowly.

In the frog methods, the procedure attempts to account for the

absorption while the cat method does not since the solution is injected intravenously and thus one does away with the absorption factor.

The 1923 Edinburgh Conference (20) of the League of Nations Health Committee held the opinion that the activity of Digitalis and its allies could be determined with reasonable accuracy either by the lethal dose method on frogs or lethal dose method on the cat with slow intravenous infusion and further, the Conference recommended the investigation of the use of a sample of Digitalis leaves as a standard for Digitalis.

The succeeding Conference held at Geneva in 1925 (21) recommended the four-hour frog and the M.L.D. frog methods as well as the Magnus modification of the Hatcher cat method as sufficiently accurate procedures. Further the conference recommended that Digitalis and its preparations be assayed in terms of a standard Digitalis powder.

The results of an international study of three Digitalis powders are reported in the memoranda (22) prepared for the Permanent Commission on Standardization of Biological Products by Knaffl-Lenz. The report states that concordant results could be obtained in different countries with both the cat technique and the frog method of assay. The relative values obtained however were not absolutely identical.

Finally the Frankfurt Conference (22) held in 1928 decided to adopt a Digitalis powder as a reference standard. This powder became the "International Digitalis Powder." The methods of assay accepted as reliable and dependable were: the frog method as recommended at the

Geneva Conference or this frog method in its other modifications and the methods of slow intravenous infusion into a mammal; Hatcher-Magnus cat, Knaffl-Lenz guinea pig and Tiffeneau dog techniques.

The 1932 edition of the British Pharmacopoeia 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 techniques that are satisfactory.

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

C. W. Edmunds (24) in replying to this recommendation of the Heart Committee, stated that the Revision Committees of the U.S.P. voted un-animously 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 firms could not obtain enough cats and then he concludes 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 standard for comparison.

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 standard reference Digitalis powder for comparison. Furthermore, the U.S.P.XI potencies are expressed in units directly referable to International Standard Digitalis Powder potency.

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.

The problem and study, hereinafter described, were undertaken and surveyed from a standpoint of practical application of the frog and cat methods to the routine standardization and evaluation of Digitalis and its preparations.

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 "OverNight" 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, then the following procedure for removing the alcohol was carried out: 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. This procedure was always carried out at room temperature.

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

2. Apparatus. The equipment used for the assays was so constructed as to accommodate 200 frogs in individual compartments at one time. Also, the assay tank was outfitted with a thermo-regulating device that maintained a set temperature rigidly and accurately. A very rigid maintenance of temperature is necessary since the rates of absorption vary directly with the temperature; vide Baker (28), Sollmann, Mendenhall and Stingel (29), Roth (30) and Smith and McClosky (31). The frog apparatus used in this study constitutes a permanent part of the laboratory equipment.

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. This routine was followed in order to eliminate the less resistant ones and thus use the frogs which remained and were more uniformly resistant.

On the day before the assay, the temperature of the assay and storage tanks was regulated to 20° C, so as to have the frogs in 20° C. water for about twenty-four hours as is required. Then on the following day the actual assay was carried out according to the procedure used. A Luer tuberculin 1 cc. syringe, graduated in 0.01 cc. and fitted with a blunted needle point so as to prevent perforation of the skin was used to make the injections.

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

The cat method that was followed was in all essentials that of Hatcher and Brody (1). The procedure differed, however, from the original method in two respects; first, an ouabain solution was not used to kill the cat - a Digitalis Solution alone was 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 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".

Bioassay Standards.

The standards used for the frog assays were: ouabain, the official U.S.P.X standard, the Standard Digitalis Powder of U.S.P. XI and the Canadian Standard #428 (85 mgm. equivalent to 100 mgm. International Standard) and a second official sample of Canadian Standard (88 mgm. equivalent to 100 mgm. of International Standard) that was released when the supply of Standard #428 had been depleted.

The ouabain was kept in a 1:1000 stock solution prepared by dissolving the ouabain in 70% 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 was observed 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 against ouabain and, in the more recent studies of the report, against Standard Digitalis Powders.

In general, the study includes a survey of stability data with observations on the pH factor, a comparison of the cat method against the frog methods of long and short periods of observation and a comparison of the more widely used methods of extraction of Digitalis Powders.

The results obtained in this investigation are given in tabular form, each table being followed by comments and discussion.

PART ONE

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

HISTORY OF PREPARATIONS STUDIED

The tinctures in this study were obtained from Dr. J. C. Munch, Chairman of the Committee on Pharmacology and Bioassays of the A.Ph.A. Dr. Munch in his report (33) describes the preparation of these tinctures as follows: "Approximately seven pounds of dried Digitalis leaves were obtained from the Food and Drug Administration, Eli Lilly Company, H.K. Mulford Company, Norwich Pharmacal Company, Parke, Davis Company

and Sharp and Dohme. These ground crude Digitalis leaves were mixed to obtain a total of 18 kilos of crude drug. This was defatted with petroleum ether on February 3-4-5, 1929 then exposed on trays and dried. A total of 14.5 kilos of defatted material was obtained. This was converted into Tincture Digitalis strictly following the method outlined in U.S.P.X, except that a total of 145 liters was obtained and no adjustments for potency were made. This material was labelled "Tincture A".

"To a measured volume of 7000 cc. of Tincture A sufficient 70% alcohol was added on February 10, 1929 to make a total volume of 10 liters. This material which is 70% of Tincture A was labelled "Tincture B". Tinctures A and B were packaged in one ounce and four ounce flint, amber and blue glass bottles using the commercial procedure. All bottles were thoroughly washed and dried before use."

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.

These samples of the two tinctures were labelled 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
Amber B	P.T.	28

These bottles were kept on a laboratory table exposed to diffused light and to the conditions of the room.

The results on these samples are given in Table 1.

TABLE 1. Compilation of the Frog and Cat Assay Results Obtained with P.T. 23, 24, 25, 26, 27 and 28.

Preparation	U.S.P.X One-hour Frog		2 Hour-Frog		4 Hour-Frog		Cat Method	
	Date of Assay	Ouabain Equiv. of 1 cc. MGM	Date of Assay	Ouabain Equiv. of 1 cc. MGM	Date of Assay	Ouabain Equiv. of 1 cc. MGM	Date of Assay	Ouabain Equiv. of 1 cc. MGM
P.T. 23	8/14/31	0.0550	2/24/31	0.07	8/15/31	0.05	3/6/31	0.1057
	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. 24	2/25/31	0.07	4/17/31	0.0625	4/17/31	0.0625	6/19/31	0.0889
	4/18/31	0.0613	8/17/31	0.05	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	0.05				
	3/15/32	0.043						
	2/27/33	0.08						
P.T. 25	2/25/31	0.0786	4/21/31	0.0635	4/22/31	0.0435	5/1/31	0.1049
	4/18/31	0.0625	8/18/31	0.0593	8/15/31	0.0573	12/10/31	0.0914
	8/14/31	0.055	2/9/32	0.0385	3/14/32	0.0438	12/8/32	0.0966
	2/10/32	0.037	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. 26	3/19/31	0.050	10/24/31	0.0375	10/24/31	0.0375	4/10/31	0.0667
	10/23/31	0.033	3/31/32	0.0167	4/2/32	0.02	6/25/31	0.0755
	4/1/32	0.016					12/17/31	0.0671
P.T. 27	3/19/31	0.055	3/20/31	0.045	10/24/31	0.0429	12/27/32	0.0691
	10/23/31	0.04	10/27/31	0.0364	4/2/32	0.018	7/11/31	0.0698
	4/1/32	0.019	3/31/32	0.02			12/18/31	0.0746
P.T. 28	4/18/31	0.04	3/20/31	0.05	4/22/31	0.025	12/29/32	0.0722
	10/23/31	0.0375	4/18/31	0.05	10/24/31	0.043	7/15/31	0.0623
	4/1/32	0.02	10/27/31	0.04	4/2/32	0.02	12/23/31	0.0535
	3/27/36	0.0556	3/31/32	0.02			12/16/32	0.0597
						4/28/36	0.0587	

Discussion of Table 1.

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 1931-32 involved the use of a fixed anesthetic, chloretone. The chloretone in 50% alcohol solution was injected intraperitoneally to induce the anesthesia. During induction stage the cats were anesthetized lightly with ether. The m.l.d. of ouabain determined on cats anesthetized, as just described, had a value of approximately 0.1 mgm. per kilo of animal. The use of chloretone or any fixed anesthetic, was abandoned after 1932 and in its stead ether was used as the anesthetic. The cats were kept lightly anesthetized with this volatile anesthetic. This method of maintaining a rather regular 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 more susceptible to ouabain. Average m.l.d.'s. determined over several periods of time were as follows: 0.097, 0.094 and 0.095 mgm. per kilo of animal, using from five to twenty cats per assay. Cats anesthetized with a fixed anesthetic, chloretone or some barbiturate, were less sensitive and gave m.l.d. values somewhat definitely greater than 0.1 mgm. per kilo. 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 mgm., and 0.096 mgm. per kilo of cat when the animals were anesthetized with a barbiturate

and "light ether", respectively.

A close scrutiny of Table 1 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 the fact that by the four-hour technique a much better and clearer end point could be observed and also the absorption from the lymph sac, on the whole, was good. The absorption was much better than could be observed in carrying out the one and two hour-frog methods. The absorption in the frogs used in the shorter periods of assay was irregular and inconsistent; sometimes good and at other times poor. The end point, in frogs, of the one and two-hour techniques was, on the whole, less clear and definite than in the case of the four hour procedure.

Further studies on the potency 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 the 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 remarkable and interesting results. The potency of P.T. 25 had risen from a level of approximately 50% U.S.P.X potency to a potency of slightly more than 100%. Assays in 1934 showed potencies of 86% and 105% of U.S.P.X requirements. Cat potencies for the period in 1934 were at a level of 79% and 84% 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%, while the cat technique gave a result of 103%; again a good agreement. The 1936 assays disclosed the fact that P.T. 25, by the frog technique, was 93% and by the cat technique 107%, P.T. 28 was 67% by the frog method and 71% by the cat assay.

This rather peculiar behavior, that is, a gradual fall in potency and then an increase in physiologic activity was not the finding of this laboratory alone. Dr. Munch, commenting on the bioassay for Digi-

talis before the Pharmacology Section at the joint convention of the American Medical and Canadian Medical Associations held in Atlantic City in the spring of 1935 stated that the samples submitted for collaborative study by the A. Ph. A. were showing a remarkable and peculiar activity - the samples which in previous years had been deteriorating were now gaining potency.

An explanation offered by this laboratory for such a behavior is the fact that the frog susceptibility to Digitalis and ouabain is far from parallel from a quantitative standpoint. In 1931 and 1932 the m.s.d. for ouabain ranged from 0.00025 mgm. to 0.00005 mgm. per gm. of frog whereas in 1933, 1934, 1935 and 1936 the values were 0.0006 mgm., 0.0007 mgm., 0.0005 to 0.0006 mgm. and 0.0005 mgm. per gm. frog, respectively. Thus, in the years 1931-32 the frogs were very sensitive to ouabain requiring approximately from 2 - 13 times less ouabain per gm. of frog than in the subsequent years 1933-36. The m.s.d. of P.T. 25, traced in an analogous manner, ranged from 0.0045 cc. to 0.0015 cc. per gm. of animal in 1931-32 to 0.007 cc., 0.008 cc., 0.0065 cc., and 0.0065 cc. per gm. of frog for 1933, 1934, 1935 and 1936 respectively. From the above discussion it will be seen, in a general way, that the frog changes in sensitivity to ouabain were much greater than to Digitalis. Thus over a period of five years the range of m.s.d. for ouabain was much greater than for any given tincture.

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).

TABLE II

Preparation No.	Date	M.L.D. of Prep. cc. Tr.	Std. Deviation from the Mean	Ouabain equivalent of Prep. MGM	Std. Deviation from the Mean
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

PART TWO

THE HYDROGEN ION CONCENTRATION STUDY OF TINCTURE DIGITALIS

It was decided, although not originally planned, to undertake a pH study of the Tinctures of Digitalis discussed in Part One. The purpose of such an undertaking was to observe whether or not there was any relationship between the potency and the pH of these tinctures as they aged.

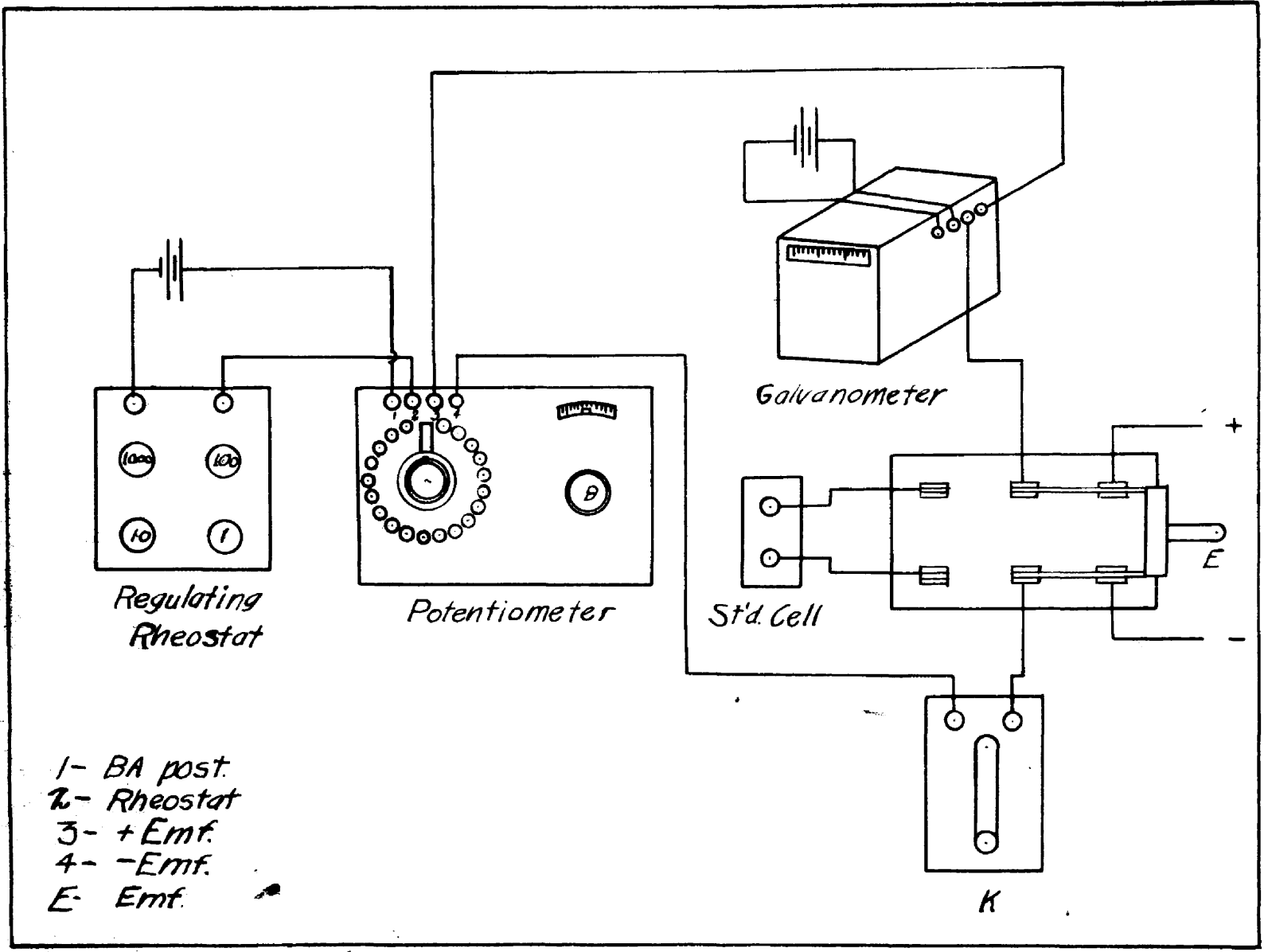
The regular procedures that are followed in the determination of pH values of solutions require approximately 15 cc. of the material for a single experiment. Since check results are usually carried out, it would necessary to use 30 cc. of the material at one time. Inasmuch as the work on these tinctures was to be carried out over a fairly long period of time and would require that the pH determinations be carried out every three or four months, it was found that 30 cc. would be too large a volume to use at one time. As a consequence an accurate micro-method had to be found. Such a method was first suggested by Billmann (36).

This method involved the following system:

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

If capillary tubes are used instead of the ordinary electrode vessels,

DIAGRAM #1



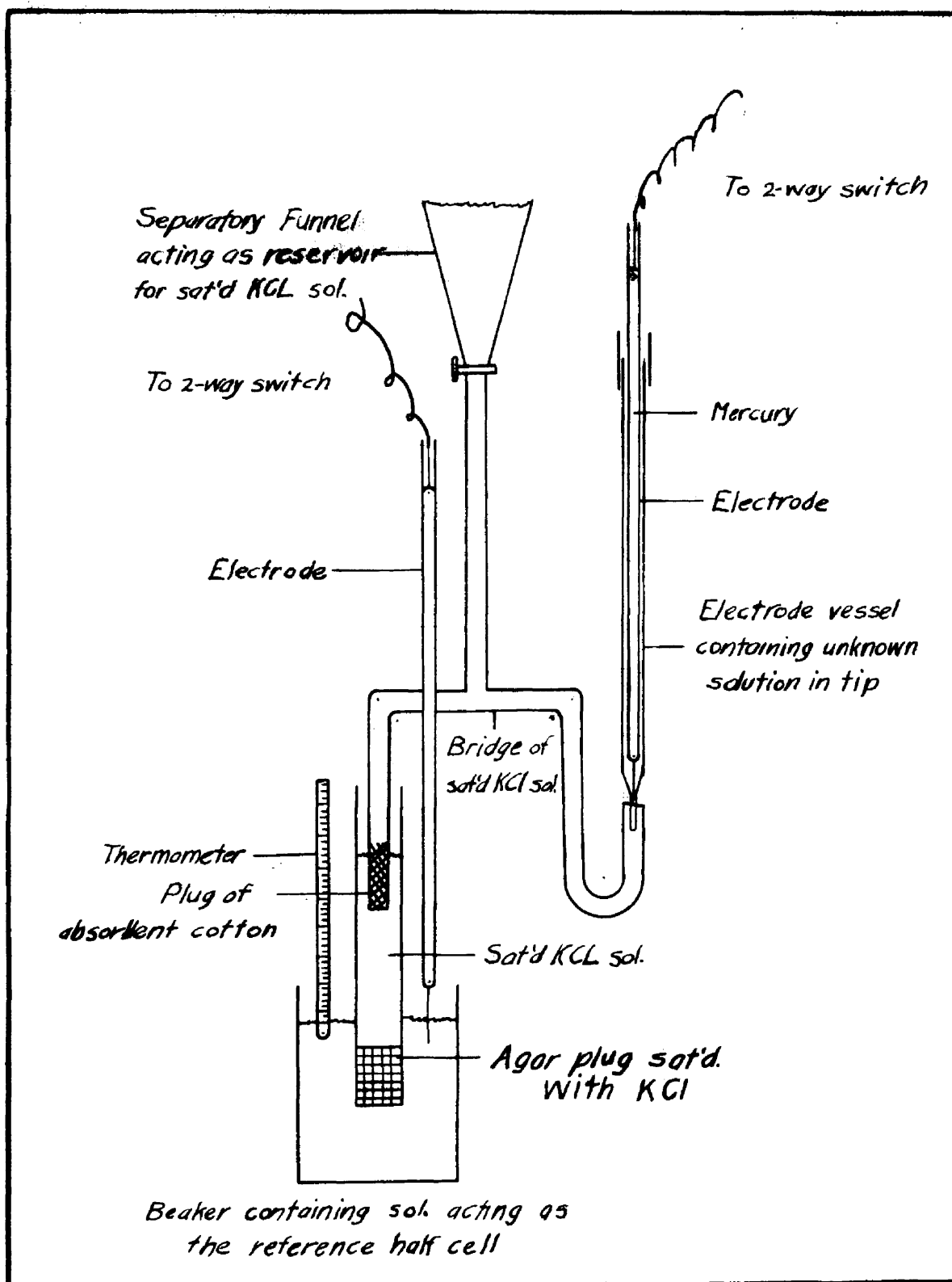


DIAGRAM #2

it is possible to determine the pH of solutions using only a few drops of the material in question. Therefore such a method would be economical when one takes into consideration the material being tested.

In the first series of determinations, a plain salt bridge without a reservoir for the saturated KCl solution was used (see diagram #2). The end dipping into the solution of the reference half-cell was plugged in with absorbent cotton previously soaked in saturated KCl solution. The "agar safety-tube" was not used at that time.

In the second and subsequent series of determinations, the arrangement (an improvement over that just described above) of the two half-cells, as shown in diagram #2, was used.

Material, Equipment and Procedure.

1. Standard Weston Cell (Eppley Labs.).
2. Student Potentiometer (Leeds and Northrup).
3. Lamp Galvanometer (Leeds and Northrup).
4. Two-way switch.
5. Three dry cells to supply current to the potentiometer.
6. Two dry cells for current to galvanometer.
7. A buffer solution 0.01 M.HCl
0.09 M.KCl
as the solution for the reference half-cell.
8. Agar gel saturated with KCl.

The electrode vessel which is to hold the unknown solution is the

one recommended by Cullen and Billmann (37) for small quantities of fluid.

Bright strips of platinum wire #21 gauge (B-S scale) sealed into ordinary fine glass tubing were used as the electrodes. These electrodes are contacted by means of mercury placed into these glass tubes. The electrode to be dipped into the unknown solution is first moistened with some of the unknown material and then dipped into some solid quinhydrone so as to make a few crystals adhere. The electrode, thus prepared, is placed into the capillary electrode vessel containing some of the unknown solution which has been drawn into the capillary.

A great many inconveniences resulted in using the salt bridge for the first series of pH determinations. First, the diffusion of the KCl solution from the bridge through the cotton plug into the solution of the reference half-cell made it imperative to change the buffer solution quite frequently. Secondly, there was no practical means for cleaning that portion of the bridge into which some of the unknown solution had diffused from the capillary. Thirdly, the maximum number of determinations that could be carried out with any degree of accuracy was limited to two or three. So that, all in all, the procedure used in the first series of determinations was very inconvenient and consumed too much time. Because of these disadvantages, the arrangement shown in diagram #2 was devised. Any solution that may diffuse from the bridge is caught in the "agar safety-tube". The rate of diffusion through the agar plug is negative

or practically negligible when observations were made on a diffusion test of from four to fifteen hours. After each determination the curved portion of the salt bridge is easily flushed by turning on the stop-cock of the separatory funnel.

The salt bridge shown in diagram #2 was constructed of ordinary soft glass tubing (0.5 cm. external diameter). The bridge was made 5.5 cm. in height and 5.4 cm. long. The curved portion measured 2.7 cm. in height.

After each determination the electrode and electrode vessel were removed and cleaned as follows: the electrode was dipped into a ($\text{H}_2\text{SO}_4 + \text{CrO}_3$) mixture, removed and washed with distilled water, alcohol and ether and then dried in a current of air. The same procedure was followed with the electrode vessel with this additional step: after drying the capillary tube, the capillary was rinsed with a portion of the unknown solution, then some of the unknown material was drawn up into the capillary; then the electrode, prepared with unknown solution and quinhydrone, as described previously, was placed into the electrode vessel.

The following was the method employed for preparing the agar gel and the agar safety-tubes: 2 Gms. of powdered agar (J. T. Baker's U. S. P. X brand) were rubbed to a thin paste in a glass mortar with 25 - 30 cc. of the saturated KCl solution. In the meanwhile, 75-70 cc.

of saturated KCl solution, in a beaker, were being heated to a boil. At this point the thin paste was poured into the beaker with constant stirring. This mixture was then heated to an incipient boil (distilled water in small amounts should be added to replace the water which has been driven off) and then the flame was removed. In order to prepare the "agar safety-tubes" a small portion of the hot agar mixture was poured into a small beaker until a layer 1 cm. deep was obtained. While the agar mixture was still hot, the desired number of tubes (thoroughly cleaned and dried) was inserted into the beaker and allowed to stand until the agar mixture gelled completely.

The ordinary potentiometric set-up (see diagram #1) was used in these determinations, the only difference being that the capillary tube was used as the container for the solution of the half-cell containing the unknown material. The solution of the reference half-cell was made by dissolving a small clump of quinhydrone in the HCl-KCl solution.

For the calculation of pH values the following formula (36) was used:

$$\text{pH} = 2.029 + \frac{\overline{\pi}}{0.0001984T}$$

in which $\overline{\pi}$ is the observed potential and
 T is the absolute temperature.

$\overline{\pi}$ is positive if the pH $>$ 2.029 and negative if $<$ 2.029.

No correction was made for the error caused by the alcoholic con-

tent (i. e. in the case of the galenicals) of the preparation. Ralph B. 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 pH using the ordinary constants for water. La Motte workers stated (private communication) that in the case of such preparations as galenicals, calculations of pH were made not correcting for the error that the alcoholic content plays.

With the arrangement used, equilibrium, in the system, is reached instantaneously and the potential is constant for about three minutes with aqueous solutions (buffers) and even longer than this period of time with preparations such as Tincture Digitalis. After this, there is a drift in potential due to diffusion changes that start to arise. Billmann (36) gives results which show that the use of the capillary vessel as the electrode vessel compares very favorably with the use of the ordinary electrode vessels employed. In order to check Billmann's view, the set-up used was tested against a series of buffers of known pH values.

For rinsing and preserving the electrodes, Billmann (36) states that it suffices ordinarily to wash with HCl and then with distilled water, and finally to place them in distilled water or dilute HCl or in the dry state preserved against dust. Even then the platinum electrode (he states) will undergo changes so that it becomes necessary to use a warm mixture of H_2SO_4 (conc.) + CrO_3 . Then the electrode is washed with distilled water and heated to redness in an alcohol flame. In heating

the electrode, one must be careful to avoid fissuring the glass tube since mercury may leak out and cover part of the platinum wire and thus give rise to false potentials. As stated above, the electrodes used in these experiments were cleaned in $\text{H}_2\text{SO}_4 + \text{CrO}_3$ mixture; the heating in an alcohol flame was omitted. When the electrodes were not used they were kept immersed in cold "cleaning solution".

The electrodes when prepared should be checked against a solution of known pH. If the measurements check the pH of the solution then one may proceed to use the electrodes. If the measurements obtained show the results to be inaccurate, then a new set of electrodes must be prepared.

Some results using electrode vessels whose capillaries were one-half the size recommended by Cullen and Billmann, were obtained in the course of the experiments used to check the potentiometric set-up with standard buffer solutions.

The accuracy of the set-up, from day to day, was controlled by the use of a freshly prepared 0.05 M. potassium acid phthalate solution before carrying out the pH determinations of the tinctures on any given day. In the case of the first series of values obtained, however, an HCl solution (pH 1.3) was used to check the system; thereafter, the phthalate solution was used.

Experimental

TABLE A

<u>Buffer Solutions</u> pH	pH Results Using	
	<u>10 mm. capillary</u>	<u>20 mm. capillary</u>
5.8 (phthalate)	5.59	5.82
	5.59	5.81
	5.66	
6.0 (phthalate)	5.76	6.01
	5.80	6.02
	5.83	
6.2 (phthalate)	5.94	---
	5.93	---
	5.98	---
5.8 (phosphate)	5.58	5.82
	5.64	5.81
6.2 (phosphate)	5.95	---
	6.04	---
2.8 (phthalate)	2.63	2.82
	2.66	2.80
3.8 (phthalate)	3.67	3.78
	3.70	3.79
5.8 (LaMotte)	---	5.80
	---	5.82
4.63 (Standard acetate)	---	4.62
		4.63

TABLE B

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

TABLE C

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

Discussion of the Hydrogen-Ion Concentration Study

The buffers used were prepared as directed in Clark's "The Determination of Hydrogen Ions" 2nd. Edition p. 99-109. The buffers prepared were checked (colorimetrically) against a freshly prepared 5.8 buffer obtained from La Motte Chemical Products Company. The same phthalate solution (0.2 M.) was used to make all of the phthalate buffers and the same phosphate solution (0.2 M.) was used to prepare all of the phosphate buffers. The phthalate and phosphate buffers of pH 5.8, pH 6.0 and pH 6.2 were checked with bromcresol purple standards (La Motte) and the 6.0 and 6.2 buffers of both sets were checked with bromthymol blue standards (La Motte) in order to check the consistency of the buffers. In all instances the results agreed. From the above statements it may be said that values assigned to the buffers were quite correct.

The first series of determinations on the buffers was carried out 1/15/32 - 1/16/32. Electrode vessels with the short capillary tip were used in the first series of determinations. The electrode vessels recommended by Cullen and Billmann (37) were used in the second series of results obtained in 1/29/32. From an inspection of the results in Table A, one may conclude that the vessel, as recommended by the two above mentioned workers must be used.

A great deal of emphasis can not be placed on the first series of determinations on tinctures (P.T. 23 - 28 inclusive) since these determinations were carried out while using the first type of salt bridge.

However, the results were quite accurate, since it was possible to check the pH of an HCl solution (pH 1.3) with that set-up.

In the subsequent series of results, the determinations were accurate to within the experimental error of the quinhydrone electrode. Results of series #3 showed, on the whole, a decrease in acidity when compared with series #2, while determinations of the #4 group showed an increase in acidity when compared with series #3. The differences in pH, among the various preparations were so small while the fluctuations in potency were so great and not consistent with the pH changes, that it was concluded that there was no relationship between potency and pH, that is, where the point under consideration is the pH of a tincture prepared by the ordinary procedures and without the addition of acid or alkali to either the extracting menstruum or the finished product.

The observations reported in Table C are interesting and further the contention that there is no relationship between pH and potency. All of the preparations in this table were tinctures except P.T. 110 and P.T. 284; these latter two were fluidextracts of Digitalis. P.T. 317 was P.T. 302 fortified by re-percolating more drug with P.T. 302. The pH values of these preparations and their potencies show no relationship.

Haag and Jarett (39) and Wokes (40) believe there is no relationship between potency and pH. Wokes made no attempt to regulate the pH of his tinctures; Haag and Jarett obtained results on samples of Tincture Digitalis which were obtained from pharmacies throughout Virginia. Foster

and Van Dyke (41) state that deterioration of tinctures could not be correlated with an increase in pH.

As a result of the study discussed above it was decided that further investigation of the pH factor of Tincture of Digitalis would be impractical and unnecessary.

The 1934 report of the A.D.M.A. (42) gives some very interesting information pertaining to the hydrogen-ion factor. Four chemists reporting pH values for two samples of Tincture Digitalis give the following values for one tincture: (a) 5.585 and 5.5, (b) 5.60, (c) 5.3 and (d) 5.3. Values for the second tincture are: (a) 4.48, (b) 4.3, (c) 4.5 and (d) unsatisfactory readings ranging from 4.609 to 4.915. These investigators used the quinhydrone electrode. One worker who checked his apparatus with a 0.05 M. potassium acid phthalate solution obtained good results for this buffer. Two others, of these workers, checked their sets with buffers; one merely reports that he checked his set and the other gives results of 6.6 and 6.6 for a buffer whose composition was not expressed. In the case of the first tincture the difference between the high and the low values for that preparation, was 0.3 of a pH unit while in the case of the second tincture the difference was 0.2 of a pH unit, when only the satisfactory results are considered. Thus these individual results prove that chemists determining the pH of the same tinctures are not able to obtain the consistency that is usually

accorded the quinhydrone method for aqueous solutions. It may be said, therefore, that differences of several tenths of a pH unit, when obtained with preparations such as Tincture Digitalis, are not very significant when attempting to correlate the potency and pH of a given preparation.

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 gms. of Canadian Digitalis Leaf. The U. S. P. X technique was used in preparing this latter tincture.

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

The methods followed in assaying these samples were: (1) U.S.P.X, (2) Canadian Overnight and (3) the cat techniques. The Canadian powder used in this particular study had the following relation to 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 3. Table 4 supplements the results in Table 3 in that it gives the standard deviation of the mean for the cat assays.

Table 3. Deterioration Data

Identify of Prep.	Fresh Preparations					After 6 Months Aging				
	U.S.P.X Method	C.-M. Canadian Overnight	Canadian	Cat Method		U.S.P.X Method	C.-M. Canadian Overnight	Canadian	Cat Method	
	Ouabain Equiv. of 1 cc. MGMS.	Can.Std. Powd.Equiv. of 1 cc. MGMS.	Ouabain Equiv. of 1 cc. MGMS.	Can.Std. Powd.Equiv. of 1 cc. MGMS.	Ouabain Equiv. of 1 cc. MGMS.	Ouabain Equiv. of 1 cc. MGMS.	Can.Std. Powd.Equiv. of 1 cc. MGMS.	Ouabain Equiv. of 1 cc. MGMS.	Can.Std. Powd.Equiv. of 1 cc. MGMS.	Ouabain Equiv. of 1 cc. MGMS.
Tr.B 843	.109	100.7	.0481	103.9	.1117	.100	73.7	.043	88.5	.0990
Tr.A 844	.100	120.6	.0576	92.6	.0996	.100	85.5	.0498	84.3	.0942
Tr. Conc. 845	.444	537.6	.256	512.7	.5514	.417	351	.239	461.4	.5165
Tr.* Not as- F sayed		120.3	.0870	96.7	.1041	.1 **	66.6	.0454	86.8	.0971

* Preparation 4.5 months old when first assayed.

** 13.5 months old.

Table 4
F I R S T A S S A Y S

Preparation	M.L.D. of Prep. cc. per Kg.	Std. Deviation of the Mean	Ouabain M.L.D. MGMS. per Kg.	Std. Deviation of the Mean	M.L.D. of Can. Std. Powd. MGMS. per Kg.	Std. Deviation of the Mean
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

S E C O N D A S S A Y S

Preparation	M.L.D. of Prep. cc. per Kg.	Std. Deviation of the Mean	Ouabain M.L.D. MGMS. per Kg.	Std. Deviation of the Mean	M.L.D. of Can. Std. Powd. MGMS. per Kg.	Std. Deviation of the Mean
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

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 potencies but the cat potency of the concentrate was 1.25 times greater than the U.S.P.X frog potency and was 5.5 and 5 times the potency of A and B respectively when the cat potencies 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 with 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 overnight 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 overnight ouabain values indicated the potencies of the samples to be in the same ratio as that obtained with the Canadian Standard Powder, they did not agree with the U. S. P. X values. The overnight ouabain potencies were from 50 - 60% 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 technique 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% and 73% of their original respective potencies; the concentrate assayed 65% of its original potency.

Tincture F could not be compared as fully as the just previously 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 overnight results. A comparison of the overnight and cat methods, however, reveals again that deterioration by the cat method is insignificant while the overnight method sets the potency of F at 55% 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% - 73% by the Canadian technique and furthermore the concentrate was deteriorating as rapidly as the ordinary tincture.

PART 4

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

A. RESIDUAL ACTIVITY STUDIES

In the course of an assay of a Digitalis preparation for Canadian distribution, it was found that this preparation was equivalent to a greater amount of Canadian Standard Digitalis when this commercial sample was compared with a two-hour absolute alcohol extract of the Canadian Standard prepared according to the regular two-hour Canadian technique (43) while this same sample had a lower potency in terms of a two-hour 95% alcohol extract. This observed difference in potency initiated an investigation of the Canadian procedure of extraction of Digitalis powders. 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 to be valuable to bio-assayists since manufacturers who distribute Digitalis in Canada standardize these products according to the Canadian procedure. Furthermore, if the Canadian technique did not yield a complete extraction, then the actual potency of galenicals, and possibly also the crude drug would be weaker in terms of the absolute potency of the standard.

The activity of the various extractives prepared was followed by using the regular Canadian Overnight method of assay (27). The only deviation,

and that by necessity, from this technique was the number of frogs used in estimating the potency of the majority of the residual extracts. The number of frogs used per dose of preparation is indicated in Table 5. Table 5 is a compilation of the "residual activity" data.

The reason for using less than twenty-five frogs was that the residual extract was obtained from the re-extraction of amounts of samples that are ordinarily used in the Canadian method. In these cases the amount of the Canadian Standard or of another sample of Digitalis is approximately one-half of one gram. Thus the volume containing the residual activity in detectable amounts was approximately 5-10 cc. Therefore a large number of frogs could not be injected when one considers that orienting assays had to be carried out. The samples extracted on 4-15-36 weighed approximately three grams 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 great enough to take care of twenty-five frogs and (2) to reduce any possible error resulting in losses due to transfer of smaller volumes of extract. The same menstruum that 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.

Ouabain lends itself admirably for such a comparison since it is a definite stable chemical entity.

In another phase of this study, the activity of extracts obtained with menstrua other than absolute alcohol was compared. These results are reported in Table 6 and will be discussed at length later.

Discussion

In this study, the ouabain equivalents of the first extract and also of 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% mortality which corresponds to a dose number of 4.0 (cf. Chapman and Morrell (27)) while 0.00035 mgm. of ouabain caused 70% mortality corresponding to a dose number of 4.22. Therefore 0.02 cc. is equivalent to:

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

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:

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

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

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

The ouabain equivalent of the total residual extract becomes:

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

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 mgm. of ouabain and the % residual activity is represented by:

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

The potency of the residual extract of the Canadian powder of 1-15-36 extracted with 95% alcohol is reported in Table 5 as less than 9.9% and also that only two frogs had been used in this estimation of potency. The orienting tests that were carried out to locate the final dose for injection had to be repeated several times and with completely negative results. At this point in the last test (on two frogs) all of the residual extract had been consumed and still the orienting assay

showed negative results. The potency, therefore, was calculated on the basis of the assumption that had the last dose of the aqueous dilution of the residual extract produced deaths then the residual activity would have been approximately 9.9%.

Again in the case of Powder #432 dated 7/16/35 the residual potency is recorded as being greater than 20.3%. In this instance, the orienting assay indicated a certain dose per gm. of frog as the L.D. 50. This dose upon injection produced a mortality of 100%. The test could not be repeated since all of the extract had been consumed. This maximal reaction indicated a residual potency of at least 20.3% of the total; therefore the recorded potency for this sample was given as greater than 20.3%.

A careful study of Table 5 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% alcohol yield extracts of the same potency and (3) the residual activity after a five-hour absolute and two-hour 95% alcohol extractions is less than 10% 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 5. 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 Pharmacopoeia (44) specifies a six-hour extraction with ab-

solute 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 5 indicate that re-extraction of a 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% alcohol, is of such a magnitude as to be insignificant from a standpoint of bio-assay standardization and thus this indicates that for practical purposes of routine standardization by the Canadian Overnight technique, a five-hour absolute alcohol extract and a two-hour 95% alcohol extract may be considered as representing the full activity of the powder.

TABLE 5. Data on Residual Extractives

Identity of Preparation	Ouabain Equiv. of Total Ext., after 1st. Ext'n. MGM.	Ouabain Equiv. of Total Residual Ext. MGM.	Duration of Residual Ext'n. in hours	Absolute Ouabain Equiv. of the Digitalis Powd. MGM.	% of Absolute Ouabain Equiv. Remaining after 1st. Extraction	Number of Frogs used in the Residual test
1 2 hr. abs. ext. powd. P.T. 733 of 3-22-35	0.3320	0.11628	5	0.44828	25.9	10
2 2 hr. abs. ext. Can. Powd. of 3-22-35	0.36613	0.09030	5	0.45643	19.8	6
3 2 hr. abs. ext. Can. Powd. of 4-26-35	0.33330	0.09263	4	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 6-27-35	0.40000	0.10137	6.75	0.50137	20.2	10
6 2 hr. abs. ext. Powd. # 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	0.53769	7.6	10
10 2 hr. abs. ext. Can. Powd. of 1-15-36	0.35900	0.07381	13	0.43281	17.5	5
11 2 hr. 95% alc. ext. Can. Powd. of 1-15-36	0.43038	0.04725	13	0.47763	9.9	2
12 2 hr. abs. ext. Can. Powd. of 4-15-36	2.06305	0.27380	5	2.33685	11.7	25
13 2 hr. 95% alc. ext. Can. Powd. of 4-15-36	2.381652	0.17020	5	2.551852	6.7	25
14 2 hr. abs. ext. U.S.P. XI Powd. of 4-15-36	2.885883	0.6355648	5	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	3.619272	6.6	25

As a result of the studies reported in Table 5, 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% and absolute alcohol for the periods of time indicated in Table 6 which contains the information and data on these various extracts. The Soxhlet extraction process was carried out following very closely the Canadian technique with special attention being given to the careful control of the rate of boiling. The potency determinations were made by following the Canadian Overnight method and using 25 frogs for the sample and 25 for the standard in each final assay.

After a careful examination of Table 6, 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% alcohol, five-hour extraction with absolute alcohol in a Soxhlet apparatus, maceration and percolation yield approximately 20% 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% 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% 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 assayer rigidly adhering to the prescribed steps of the method to obtain a 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 accurately since the two powders will be extracted 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 instance, 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 found for a tincture will be greater than the actual potency of that galenical. A tincture standardized by the present Canadian technique passes into the trade actually weaker than is indicated by the assay. The true or absolute equivalent in mgms. 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 mgms. of standard

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

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

TABLE 6. Comparisons of Extraction Methods

Identity of Preparation	Date of Assay	Equivalent of 100 mgms. Can. Std. Powder. 2 Hour Abs. Ext. MGMS.	% Equivalent of 2 Hour Abs. Ext. Can. Powder
Can. Powd. 2 Hr. Abs. Ext.	1-7-36	100	100
Can. Powd. 5 Hr. Abs. Ext.	1-7-36	84.11	118.9
Can. Powd. 2 Hr. 95% Ext.	1-15-36	84	119
Can. Powd. 2 Hr. Abs. Ext.	1-15-36	100	100
U. S. P. XI Std. Powd. 2 Hr. Abs. Ext.	4-15-36	70.7	141.4
U. S. P. XI Std. Powd. 2 Hr. 95% Ext.	4-15-36	60	167
Can. Powd. 2 Hr. Abs. Ext.	4-15-36	100	100
Can. Powd. 2 Hr. 95% Ext.	4-15-36	83.1	120.3
U. S. P. XI Std. Powd. Macerate	4-16-36	55.6	179.9
U. S. P. XI Std. Powd. Percolate	4-16-36	58.1	172.1
Can. Powd. Macerate	4-16-36	89.4	111.9
Can. Powd. Percolate	4-16-36	101	99

B. 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 7 and Table 8.

TABLE 7. Deterioration Data

Identity of Preparation	Age of Diluted Extract	Equivalent of 100 MGM. of Fresh 2 hr. Abs. Alc. Ext. of Can. Std. Powd. MGM.	% Equivalent of Fresh 2 hr. Abs. Ext.
Can. Powd. 2 hr. Abs. Ext.	Fresh	100	100
Can. Powd. 5 hr. Abs. Ext.	Fresh	84.11	118.9
Can. Powd. 2 hr. Abs. Ext.	7.5 Months	113.63	88
Can. Powd. 2 hr. Abs. Ext.	6.5 Months	129.63	77
* Powd. #432 2 hr. Abs. Ext.	5.75 Months	179.37	47.7
Can. Powd. 2 hr. Abs. Ext.	5.75 Months	138.88	72
Can. Powd. 2 hr. Abs. Ext.	4.5 Months	140.85	71

* 85.6 mgn. of Powder #432 were equivalent to 100 mgn. of Canadian Standard Powder when freshly prepared.

TABLE 8. Deterioration Data

Identity of Preparation	Age of Diluted Extract	Equivalent of 100 MGM. of Fresh 2 hr. Abs. Alc. Ext. of Can. Std. Powder MGMS.	% Equivalent of Fresh 2 hr. Abs. Ext.
Can. Powd. 2 hr. Abs. Ext.	Fresh	100	100
Can. Powd. 2 hr. 95% Ext.	Fresh	84	119
Can. Powd. 2 hr. Abs. Ext.	1 Week	111.4	89.8
Can. Powd. 5 hr. Abs. Ext.	1 Week	98.43	102

Discussion

From a survey of Tables 7 and 8, 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% 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.

C. COMPARISON OF EXTRACTION TECHNIQUES BY THE U. S. P. XI AND CANADIAN OVERNIGHT 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 mgm. of this powder). Table 9 gives the results of this investigation. Potency estimations by the Canadian Overnight Method are also included in this table for the sake of comparing the one-hour method against the overnight technique. Results by the Canadian technique are likewise referable directly to the macerate of the U. S. P. XI Digitalis Standard.

TABLE 9. Comparison of Extraction Methods

Preparation	U.S.P.X	U.S.P.XI	C.-M. Canadian Method		Cat Method
	Method Ouabain Equiv. of 1 cc. MGM.	Method U.S.P.XI Std. Powd. Equiv. of 1 cc. MGM	U.S.P.XI Std. Powd. Equiv. of 1 cc. MGM.	Ouabain Equivalent of 1 cc. MGM.	Ouabain Equivalent of 1 cc. MGM.
Macerate of U.S.P.XI Powd.	0.167	100	100	0.1232	
Percolate of U.S.P.XI Powd.	0.167	100	95	0.1169	
Macerate of Can. Std. Powd.	0.1428	85.7	62	0.0761	
Percolate of Can. Std. Powd.	0.1428	85.7	55	0.0673	
2 hr. 95% Alc. Ext. U.S.P.XI Powd.	0.167	100	92	0.1133	
2 hr. Abs. Alc. Ext. U.S.P.XI Powd.	0.125	75	78	0.0961	
2 hr. 95% Alc. Ext. Can. Std. Powd.	0.125	75	67	0.0819	
2 hr. Abs. Alc. Ext. Can. Std. Powd.	0.100-0.125	60-75	55	0.0682	
P.T. 843 Tr. B	0.100	60	37	0.0430	0.099
P.T. 844 Tr. A	0.100	60	43	0.0498	0.0942
P.T. 845 Tr. Conc.	0.333-0.500	200-300	195	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	0.0221	0.0587
Tr. F	0.100	60	33.3	0.0388	0.0971
Macerate of Powd. #432	0.1375	75			
Percolate of Powd. #432	0.1375	75			
Macerate of Powd. A	0.0917 - 0.110	50-60			
Percolate of Powd. A	0.100	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	0.125	75			
Percolate of Powd. P.T. 632	0.125	75			
Macerate of Powd. P.T. 747	0.150- 0.167	75-100			
Percolate of Powd. P.T. 747	0.118	70.6			
Macerate of Powd. Minn. Fol.	0.167	100			
Percolate of Powd. Minn. Fol.	0.154	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% 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 overnight techniques.

In several instances the one-hour technique 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 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 overnight and one-hour methods reveals that the frog is much more susceptible to ouabain by the overnight method and 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 worthwhile to include a brief summary of the conclusions and recommendations made by the Subcommittee on Digitalis of the A. D. M. A. as reported in the proceedings (45) 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 overnight, so as to allow sufficient time for a complete absorption and physiologic effect. This particular A.D.M.A. report (45) 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 Subcommittee reported (46) 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 labelled "A" to two-thirds the potency of A. The tests were carried out by the one-hour M.S.D. and the overnight M.L.D. frog methods with Canadian Standard Digitalis Powder #428 as a reference standard. The potencies of the tinctures assayed in this collaborative study were reported in terms of International Standard Powder potency. 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 was 94.6% for A and 76.3% for B and by the overnight method 97.6% for A and 69.8% for B. Thus, by the one-hour procedure B was 80.7% of A and by the overnight method B was 70.5% 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% to 100%. The possibility of interpretation of results obtained by the Canadian technique is negligible if not negative; 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 overnight 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 Subcommittee:

ONE HOUR FROG METHOD

Preparation	A.D.M.A. Ave. Result	Result of the Pharmacology Lab. of the School of Pharmacy
Tr. A	94.6%	100%
Tr. B	76.3%	86%

OVERNIGHT 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% of A and by the overnight technique B is 68.8% of A. Thus by the overnight

procedure this laboratory was able to estimate accurately the theoretical potency of B.

Some data obtained with cat assays are included in Table 9 for the sake 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 comments on this particular group of assays will be made.

D. 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 10.

The reference standard 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 mgms. of International Standard Digitalis since 74.5 mgms. of the U. S. P. XI Reference Powder are equivalent to one U. S. P. Digitalis Unit.

TABLE.10. 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. #432	166	101	1.64
Percolate Powd. #432	166	101	1.64
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 10 points to the fact 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 1.532 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 mgms. of Canadian Standard are equivalent to 100 mgms. 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% 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 MGMS. per Gm. Frog	M.S.D. of U.S.P. XI Std. Powder Mems. 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 mgms. of the Digitalis Standard Powder were equivalent to 0.167 mgms. of ouabain or 200% U. S. P. X potency but only 134% in terms of U. S. P. XI requirements. In the other test

the ouabain equivalent of 100 mgms. of this Digitalis was slightly greater - 0.169 mgms.

General Discussion

As a result of the experiences obtained in carrying out the frog and cat tests mentioned in this study, a certain routine procedure for the bioassay control of Digitalis was developed. At first, a preliminary assay is carried out on two or three cats giving approximately similar results in order to locate the approximate potency of any given Digitalis sample. For a complete assay, i. e. a cat assay, at least five cats are used. The cat m.l.d. results are always calculated and expressed in terms of a suitable reference standard. Then the assay is carried out on frogs. The purpose of such a preliminary cat assay is two-fold: (1) this procedure enables an assayist to select doses for injection into frogs without the preliminary orientation tests with frogs and (2) the cat results serve to check or control the frog assay since cat results obtained with the technique as applied in this laboratory are in rather good agreement with frog results. The cat technique giving results quite comparable to frog results is that method which involves anesthetizing the cat lightly with ether.

An outline of this cat technique may be described as follows: the femoral vein of the cat is exposed and cannulized. The strength of the

perfusing fluid is adjusted from a preliminary assay such that the m.l.d. per kilo is contained in ten cc. The digitalis dilution is injected into the animal at the rate of one cc. per minute until strong symptoms of Digitalis bradycardia are manifest. This rate of perfusion maintained throughout the bradycardiac stage of symptoms is slowed to a rate of three-quarters of a cc. with the first appearance of a tachycardia. As the tachycardiac symptoms develop and increase to a pronounced rapid rate, the rate of flow is checked still more until the flow is now one cc. every two to three minutes near the termination of the experiment. With the first pronounced signs of cardiac flutter the perfusion is stopped completely. The heart will then flutter to a stop within a short time of the first fibrillation.

The overnight frog technique is to be preferred to the one-hour frog method because the overnight 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 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 (47) 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 cat method as applied to the assay of Tincture of Digitalis showed very little loss in potency over a period of approximately five years when the method involves expression of the potency in terms of a reference standard.
2. The one-hour frog method of the U. S. P. X with ouabain as the standard revealed 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 Overnight frog and the cat methods, the Canadian technique was the only method to reveal an appreciable loss in potency of tinctures aged for approximately six months. The cat method showed a loss that was not very significant.
4. The unsuitability of ouabain as a reference standard for Digitalis is confirmed.
5. No relationship between the potency and pH of tinctures could be observed.
6. An accurate method for the determination of pH of small amounts of fluid is described.

7. The Canadian extraction technique does not exhaust the activity of a given Digitalis powder. The activity remaining in the sample represents approximately 20% of the total activity of the powder.
8. Maceration, percolation and a modified Canadian technique of Soxhlet extraction of five hours with absolute alcohol or two hours with 95% alcohol yield extracts of approximately equal potency.
9. The maceration technique as described in the U. S. P. XI for the extraction of the Digitalis Reference Powder and involving frequent agitation was found to be the most satisfactory extraction process for use in routine bioassay standardization tests of Digitalis.
10. The potency requirements for Tincture Digitalis U. S. P. XI are 1.53 times greater than the U. S. P. X requisites, based on the relative susceptibilities of frogs to ouabain and Digitalis in the spring of 1936.
11. The overnight technique of the Canadian method is the best frog procedure for routine standardization of Digitalis products.
12. A routine method for the laboratory control of Digitalis preparations is outlined.
13. Although the U. S. P. XI and Canadian official Tinctures of Digitalis are theoretically equivalent to 100 mgms. of International Standard Digitalis Powder, the U. S. P. XI tincture is approximately 20% stronger than

the Canadian tincture when the U. S. P. tincture is evaluated and compared by both the Canadian method of assay and the prescribed U. S. P. XI technique.

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