and whose short jacket is supplied with water from the tap. The function of the condenser or cooler is to promote circulation by increasing the specific gravity of the solution.

When properly set up the hot concentrated solution flows through the siphon with the condenser, from the flask to the cooling vessel where precipitation takes place through cooling. The precipitated material falls to the bottom of the cooling vessel where it collects until removed. It was found that the gentle current set up through the action of the two siphons would carry but very little of the solid material back to the flask. By means of the apparatus the solvent dissolves the crude material, or the desired substance from it, deposits it on being cooled and returns to the flask for a further quantity—the advantages being continuous operation, requiring no attention, and economy of solvent.

This apparatus has served well in the writer's laboratory in the purification of saponin from the dried crude plant extract by precipitation from hot alcohol by the Schrader method.

ASSAY OF DIGITALIS BY INTRAMUSCULAR INJECTION IN THE FROG.

BY M. S. DOOLEY AND C. D. HIGLEY.*

There is no satisfactory method for the standardization of the digitalis bodies in spite of the extensive efforts that have been made to find one. The present paper describes a method which presents advantages over the procedures now followed.

Observers have criticized the present official method mainly on account of lack of uniform absorption from the lymph sac. It is toward the obviation of this particular difficulty that our experiments have been directed. This lack of uniform absorption is not merely inconvenient, but it interferes with the reliability of the method. This is indicated by the fact that more than twenty different methods have been proposed since Famulener and Lyon published the one-hour method in 1902.[1] In the search for a better method investigators have covered a wide field—chemical as well as biological—but, with the exception of the cat method, none has attracted much more than passing notice.

In common with other observers we have long recognized great variability in the results obtained in routine assays by the lymph sac method, especially when the attempt was made to utilize it in research. This may be illustrated by an experience of one of us (D.) while working in Dr. Hatcher's laboratory in the summer of 1920 (the season is here referred to because the variabilities appear to be greater at this time). The problem under consideration was the question of the rate of elimination of digitalis substances—a problem in which it was desirable to know the potency of a given specimen in advance of attempting the study of its rate of elimination. Poor absorption from the lymph sac rendered the one-hour method useless for this purpose and the twenty-four-hour toxic method was finally substituted.

I. ORIGIN OF THE PROBLEM AND DESCRIPTION OF THE METHOD.

During the course of the above experiments upon the elimination of digitalis substances mention was made of the feasibility of making injections into the

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abdominal vein of the frog which led to an attempt to develop a method of assay of digitalis bodies by means of intravenous injections in the frog. The results of these intravenous injections proved disappointing but in the course of experiments the idea of an intramuscular method suggested itself. Only a few trials were required to demonstrate its apparent superiority over the official method.

The method of preparing the drugs for this study was, for the most part, as follows: In the case of alcoholic preparations, tinctures and fluidextracts, an amount equivalent to the percentage of alcohol contained was evaporated off, in most instances spontaneously, but in some cases by slight heat (not above 50° C.) over a water-bath. Enough alcohol was then added to the residue to make the final product contain 15%, when 0.7% sodium chloride solution was added sufficient to make it up to the original volume. The largest total amount of fluid injected into any animal in our whole series, with the exception of two experiments with digitalin in which doses of 0.5 and 0.51 cc were injected, was 0.47 cc and this was in the case of a very large frog. In general the total dose averaged much less than these figures. The fluid dose per gram of frog was, with a few exceptions, well below the allowable 0.015 cc specified by the Pharmacopoeia. In one series of six animals receiving digitalin the per gram dose was from 0.016 to 0.021 cc but in the cases tinctures and fluidextracts of digitalis no per gram dose was larger than 0.01 cc. In some instances preparations have shown such high potency as to require dilution for the sake of accuracy but none have required concentration. This was done with 0.7% sodium chloride solution and enough alcohol to make the final product contain 15%. In the case of ouabain a 1 to 10,000 solution in 0.7% sodium chloride was always used. Digitalin was made up in 0.7% sodium chloride containing 15% alcohol. Where precipitates occurred, as was always the case with tinctures and fluidextracts, these were dealt with as directed by the Pharmacopoeia—the sample was always thoroughly shaken before a dose was taken out for injection. These sediments were never very abundant or so coarse as to clog the fine needles (26 and 27 guage) used for the injections. A tuberculin syringe was used to measure the total dose and the injections made directly from it.

The procedure for intramuscular injections is simple and may be briefly described as follows: The frog is held with its back against the palm as in the official method, but with the head downward. The edge of the palm and the little finger fit in back of the fore legs and offer resistance to the pull on the hind legs being held, one between the thumb and fore finger, the other between the middle and ring fingers. In this way the hind legs are grasped below the knees and spread apart, care being taken not to stretch the skin over the thighs. The finest needle (27 guage) is used and half the total dose is injected slantingly into the thickest part of each thigh. A little experience taught us to avoid the large vessels as certain negative results could be attributed to hemorrhage.

In a preliminary report[2] the results of studies of ouabain and some preparations of digitalis have already been summarized in which it was shown that the required S. S. S. (systolic standstill) dose was always smaller, and the end-point sharper, than is true when equivalent doses are injected into the lymph sac. Additional data are now presented, much of which is even more conclusive than that originally published.

TABLE 1.

Frogs.	Drug No.	S. S. S. Dose in Mg./Gm. Lymph sac.	Intramuscular.	Percent.
		Crystalline Strophanthin (Ouabain).		
Lot 1	1	0.00045	0.00037	17.8
Lot 2	1	0.00051	0.00044	13.8
Lot 3	1	0.00032	0.00027	15.7
Lot 4	1	0.00039	0.00031	20.6
Lot 5	2	0.00059	0.00050	15.3
Lot 6	2	0.00057	0.00051	10.6
		Digitalis—Tinctures.		
Lot 1	1	0.70	0.57	18.6
Lot 2	2	0.59	0.49	17.0
Lot 3	3	0.72	0.55	23.6
Lot 3	4	1.03	0.72	30.0
		Digitalis—Fluidextracts.		
Lot 1	1	0.90 Neg.	0.70	22.3
Lot 2	2	0.60	0.50	16.7
Lot 3	3	0.58	0.49	15.6
Lot 3	4	0.92	0.60	34.7
Lot 3	5	0.64	0.56	12.5 (24 hr.)
		Digitalin—Merck. *Formerly known as "German."*		
Lot 1	1	0.025	0.022	12.0

Table 1 summarizes the results. It contains the condensed result of all experiments with ouabain, digitalin, tinctures and fluidextracts of digitalis. In all cases the intramuscular has been compared with the S. S. S. dose by lymph sac and has always been found smaller. The last column of the table shows the percent difference between the two in corresponding lots of frogs. The effective intramuscular dose of digitalin is seen to be 12% less (single series), while with tinctures and fluidextracts the differences average, respectively, 22 and 22.2%. With ouabain the difference lies intermediate—15% (approximately). It may be noted in passing that digitalin was one of the most difficult of our series to assay. It seems advisable, also, to call attention to the rather wide variations in the percent column for the digitalis preparations. The high values were derived from experiments during the summer season. Other observers have reported poorer absorption from the lymph sac during the summer and that has always been our experience. It is believed, therefore, as the other figures in the table show, that the differences will not average as great during the other seasons of the year.

DISCUSSION.

It is apparent from the above tabulations that the drugs under consideration develop a more intensive effect by from 10 to approximately 35% within the hour, injected intramuscularly, than when acting from the lymph sac. When one attempts to explain this discrepancy difficulties arise. One cause already cited—incomplete absorption from the lymph sac—is evident enough in some experiments, but by no means in all (see discussion of ouabain, next pages). The presence of fluid in the lymph sac at the end of an experiment is commonly set down as evidence of this but, of course, may or may not be correct, for, while it is usually associated

with a negative result, this is not a fixed rule. The fluid remaining may contain only a negligible fraction of active principle. In the case of intramuscular injections inspection of the regions of injections and the constancy of results indicate uniform absorption in nearly all cases, but other objective proof is desirable if it could be had. Intravenous injections would not serve as controls. The twelve- and twenty-four-hour toxic methods yield values far higher, hence, if completeness of absorption is to be judged solely by the size of the minimum dose required, this would indicate that absorption is far more complete by the intramuscular route. But, as complete absorption occurs in many of the animals in the twenty-four-hour experiments that have not died but which have received doses greatly in excess of those giving positive results in the one-hour intramuscular experiments, it is evident that there is at least one and, we believe, more than one other factor influencing the final result in a given case. These are the rates of absorption and elimination. As indicated below a distinction should be made between rate of and completeness of absorption, in relation to the time limits set for the experiments, although one of these factors is dependent upon the other. In going over our notes it is found that, in a number of instances in which animals have received equivalent dosage and in which complete absorption has been noted, the results have been partly negative, partly positive. How are such to be explained? The usual practice has been to dismiss the question by the stock expression that some of the animals are more susceptible than others. But this empiric explanation does not explain. Is it not more likely that variation in the rate of absorption has made the difference? Rather, it may be more correct to say that the final result will be conditioned upon the relative rates of absorption and elimination. If it is assumed that two animals are given the minimum effective dose, the one will absorb the total dose, let us say, in 35 minutes, whereas the other just completes the process by the end of the hour, is it not probable that systolic standstill will occur in one case and not in the other? It is evident that such difference in susceptibility is a matter of how promptly an effective concentration is built up in the circulation. It must be apparent that some such mechanism is back of the greater susceptibility from intramuscular injections. More especially is this the case when ouabain is used, for the protocols show that complete absorption occurred in almost every one of our numerous experiments in which it was used by both methods. It is believed, therefore, that the results warrant special emphasis upon this point, particularly as there is strong probability that most of the digitalis substances begin to be eliminated at variable rates as soon as they reach the circulation.

Hatcher has shown that, in some types of mammals, most of the digitalis substances begin to disappear from the circulation promptly on entering it but at different rates, compared one with another, and at different rates in different species of animals.[3] One of us (D.) has shown that the size of the minimum lethal dose for the cat, by vein, is governed materially by the rate of injection.[4] In the case of slow injections the minimum lethal dose was found to be much larger than where the dose was allowed to enter the vein at a fairly rapid rate. In the former case elimination was going on and had to be made good by further injection. As applied to the present problem any delay in absorption from the lymph sac or muscles would similarly influence the necessary dose. It is evident,

therefore, that the more adequately the rate of entrance into the circulation keeps pace with the rate of elimination the smaller will be the required dose for a given effect.

It is not a pure assumption that elimination goes on at a fairly rapid rate in the frog, for it has been observed repeatedly that if animals in which the ventricle is almost in systolic standstill at the end of the hour be kept under observation for a time, their hearts will resume rhythmical beating. We venture the suggestion that if any worker who assays by the official method would keep his animals under observation for a longer period than required by this method, some of the hearts recorded as positive would, in time, resume rhythmic activity. This is taken to mean that elimination has, meantime, lowered the concentration of the drug necessary to hold the heart in systole.

The following experiment may be cited as a more direct proof of the fact that most of the active digitalis substances are rapidly eliminated by the frog. By the device of injecting the dose in four or five equal parts into different regions of the muscles at half-hour intervals instead of injecting the whole amount at once and

TABLE 2.

Tabulation to Show Relative Constancy of Effects with Lymph Sac and Intramuscular Injections.

		Ouabain.			
Lymph Sac—Mg./Gm. Body Wt.		*Abs.*	*Intramuscular—Mg./Gm. Body Wt.*		*Abs.*
0.0005–0.00055	S. S. S. tend. beats	C	0.00047–0.00049	S. S. S. tend. s. b.[1]	C
0.00056	S. S. S. +	C	0.00049	S. S. S. +	C
0.00057 (2 expts.)	S. S. S. tend. s. b.[1]	C	0.0005 (3 expts.)	S. S. S. +	C
0.00058 (2 expts.)	S. S. S. +?	C	0.00051 (2 expts.)	S. S. S. tend. s. b.[1]	C
0.00058	S. S. S. +	C	0.00051	S. S. S. +	C
0.00059	S. S. S. tend. s. b.[1]	C	0.00052–0.0006	S. S. S. +	C
0.00059	S. S. S. tend irreg. b.	C			
0.00059	S. S. S. +	C			
0.0006	S. S. S. +	C			
0.0006	S. S. S. +?	C			
0.00065	S. S. S. +	C			

[1] s. b. = Slow beat.

then examining the animals at the end of two and a half or three hours it was found that a 25 to 30% larger dose was required. It is assumed that this 25 to 30% difference represents the amount the animal has eliminated during the longer interval of the experiment over that of the one-hour period with which it is compared.

Tables 2 and 3 show the contrast between the results afforded by the two methods that are discussed in this paper. They are inserted to illustrate the difference in sharpness of end-point. Table 2 is typical of the results obtained with ouabain by the two methods on the same lot of frogs. It will be noted that in the case of the intramuscular injections the results tend to be negative up to a certain intensity of dosage—in this experiment, 0.0005 mg. per Gm. of frog—and all dosages to be positive above that level, whereas, in the case of injections into the lymph sac, negative results are interspersed with positive over a wide range of dosage. With any of the preparations of digitalis the differences are usually more marked, as shown in Table 3. Tinctures and fluidextracts show the greatest difference,

TABLE 3.

Tabulation to Show Relative Constancy of Effects from Lymph Sac and Intramuscular Injections. Some of the Higher and Lower Dosages, Not Significant, Have Been Left Out of This Summary. It May Be Seen That If, under the Intramuscular Column, the Second 0.51 in Which a Hemorrhage Was Noted Is Excluded, There Is a Satisfactory Line of Demarcation between Positive and Negative Values. Under Lymph Sac Column the Not Unusual Ragged End-point Is in Evidence.

	Digitalis—Tincture.		
Lymph Sac—Mg./Gm. Body Wt.	*Abs.*	*Intramuscular—Mg./Gm. Body Wt.*	*Abs.*
0.49 S. S. S. tend. b. s.[1]	C	0.48 S. S. S. tend. b. s.[1]	C
0.50 S. S. S. +	C	0.49 S. S. S. +	C
0.50 S. S. S. tend. b. s.[1]	C	0.49 S. S. S. +	C
0.51 S. S. S. tend. b. s.[1]	C	0.50 S. S. S. tend. b. s.[1]	C
0.52 S. S. S. tend. b. s.[1]	C	0.50 S. S. S. +	C
0.53 S. S. S. +	C	0.50 S. S. S. +	C
0.54–0.58 S. S. S. nearly	Inc.	0.51 S. S. S. +	C
0.59 S. S. S. +	C	0.51 S. S. S. tend. hemorrhage	
0.60 S. S. S. +	C	0.52 S. S. S. +	C
0.60 S. S. S. ?	C	0.53 S. S. S. +	C
0.60 S. S. S. ?	C	0.53 S. S. S. +	C
0.61 S. S. S. tend.	C	0.53 S. S. S. + hemorrhage	C
0.61 S. S. S. nearly	Inc.	Absorption complete in this	
0.62 S. S. S. nearly	C	series so far as autopsy could show.	
0.63 S. S. S. +	C		
0.64 S. S. S. nearly	C		
0.65 S. S. S. tend. irreg.	C		
0.70 S. S. S. tend. b. s.[1]	C		
0.75 S. S. S. +	C		

[1] b. s. = Beats on stimulation.

just as they also show the greatest disparity in the S. S. S. dose, by the two methods. This combination of excessive dosage required by the official method and the uncertainty of end-point robs an assay of most of its value, and results furthermore in additional expense and much loss of time.

It has been suggested that frogs obtained from different localities may show differences in their capacities for absorption of the different digitalis bodies. This seemed to us to be worthy of investigation, for if such differences really exist the Pharmacopoeia should at least specify the region from which the frogs are to be taken.

As a matter of fact we have not observed any such differences dependent upon the source of supply. Frogs caught recently in the region about Syracuse behaved in this respect much like those obtained from Chicago. It appears to us, on the other hand, that the fundamental difficulty lies with the lymph sac as an absorbing mechanism for it may be here noted again that, even with a readily absorbable material like ouabain, an average of over 15% (see Table 1), or more than one-sixth of the total dose, is held back from absorption within the hour. It can be seen that even greater differences in absorption from the lymph sac will be observed when one uses different galenical preparations of digitalis made by different methods employed by different manufacturers. This can hardly fail to have a practical bearing on the problem of manufacturing standard preparations.

Most of the samples used in this research have it stated on the labels that they have been prepared by the official method, from assayed leaves. The inference

is drawn that assay has been by the official method but when an assay has been attempted by the same method utter failure has resulted in some cases and in others lack of a sharp end-point (see Table 3). We are prompted to ask how the manufacturer definitely accomplishes an assay when, in our experiments, most of the doses were found, in large part, in the lymph sac at the end of the hour.

That the rapidity of absorption plays an important rôle in the effects under consideration is still further evidenced by some recent experiments performed with strychnine. Exactly the same procedures have been carried out as with digitalis, except the adoption, as an end-point in this instance, of the interval required after the drug is injected for a convulsion to develop. Two main points have been developed. First, convulsions develop much sooner—our data are too meager to enable us to say how much sooner but in some cases, 100%—after intramuscular injections. Second, the minimum intramuscular convulsive dose will not induce convulsions acting from the lymph sac.

There are three factors that must be considered with reference to absorption from the muscle. In the first place it is well known that the muscles have an abundant blood supply. In the second place the animals are usually somewhat active following the injection, their movements serving to massage the area of injection, thus favoring the wider distribution of the drug and bringing it into contact with a larger surface, thereby promoting absorption. This could not happen in the case of the lymph sac. In the third place the injection of the dose in two parts in different areas serves this end also.

SUMMARY.

1. The need for a modification of the present Pharmacopoeial method of digitalis assay to insure more uniform absorption is pointed out.

2. A method involving the intramuscular injection in the frog is described. It is simpler and gives more nearly uniform results.

3. Data are presented to show that the dose is smaller and the end-point sharper than in corresponding injections into the lymph sac.

4. The more rapid and complete absorption plays a dominant rôle in developing greater toxicity from intramuscular injections.

5. The probability that the rate of elimination of drugs in the frog, as in mammals, plays an important rôle in the final result is discussed.

6. It is believed that the great variability of many presumably assayed commercial preparations is due to reliance having been placed in the method of injection into the lymph sac.

7. The better absorption from the muscles is due to division of the dose, better blood supply, and movements of the animals.

LITERATURE.

No attempt is made to review the literature on the development of methods for the assay of digitalis as an admirable summary down to 1916 is to be found in the following bulletins from the Hygienic Laboratory, Washington, D. C.:

(*a*) Edmunds and Hale, *Bulletin* 48, 1908.

(*b*) Hale, *Bulletin* 74, 1911.

(*c*) Roth, *Bulletin* 102, 1916.

One other contribution since that time may be noted:

(*a*) Knudson and Dresbach, *Proc. Soc. Exp. Biol. and Med.*, 19, No. 8, 1922.

(*b*) Knudson and Dresbach, *Jour. Phar. and Exp. Therap.*, Vol. 19, 1922.

1. Famulener and Lyon, PROC. AM. PHARM. ASSOCIATION, 50, 415, 1902.
2. Dooley and Higley, *Soc. Exp. Biol. and Med.*, 19, 250, 1922.
3. Hatcher, *Arch. Int. Med.*, 10, 169.
4. Dooley, *Jour. Phar. and Exp. Therap.*, 17, 277, 1921.

DETERIORATION OF THE TINCTURE OF DIGITALIS.*

BY CHAS. C. HASKELL, D. S. DANIEL, AND G. S. TERRY.

The keeping qualities of the tincture of digitalis has been the subject of many investigations. In one of the earliest papers reporting the results of such a study, Houghton and Hamilton[9] concluded that there was a loss in the strength of the tincture with age. Two years later, Sharp and Lancaster and Sharp and Branson[16,17] presented evidence indicating that there was a definite loss in the activity of certain tinctures, becoming manifest at the expiration of 15 months. Goodall,[3] from the examination of 23 samples, arrived at about the same conclusions as those reached by the authors just mentioned. According to his assays, there was no loss in strength up to 14 months; but, after this, some of the samples showed a decrease in potency; and, at the end of 3 years, one tincture possessed less than one-third of its original toxicity. He cites Hanes as saying that the tincture keeps for two years without material change in activity, while Moran makes the claim that there is no important amount of deterioration up to 3 years. From assays on a number of liquid preparations of digitalis, Hale[4] concluded that the rate of deterioration differed in different cases, but that some occurred with age in all of his samples. O'Brien and Snyder[11] observed a rapid loss in the activity of a tincture, originally of a very high degree of potency, this loss amounting to 55% in the course of $2^1/_2$ years. Even more remarkable rate of deterioration was reported by Schmidt and Heyl[15]—a tincture tested by them being found to have retained only 40% of its original strength at the end of 15 months. Indeed, all of the preparations examined by Schmidt and Heyl lost strength so rapidly that one is led to suspect that the conditions under which their experiments were carried out differed from the ordinary.

All of the investigators who have been cited employed one of the various frog methods in testing the specimens examined. While their conclusions as to the rate of deterioration are far from being in complete harmony, their results do agree in indicating that the tincture of digitalis loses strength in relatively short periods of time, when judged by the criterion of the frog test. From the meager reports of similar experiments carried out on guinea pigs, the same inference may be drawn. Employing the guinea-pig method, Pittenger and Mulford[14] reported a rate of deterioration surpassing even the unique observation of Schmidt and Heyl; for, after 7 months, a tincture tested by them retained little more than a quarter of its original toxicity. Only one of the specimens which Pittenger and Mulford examined failed to show deterioration; and they state ". . . that, in most cases, tincture of digitalis not only deteriorates, but deteriorates very rapidly." As Hamilton remarks,[5] the acceptance of such "revolutionary" conclusions as these is tantamount to holding that the tincture of digitalis is a useless preparation;

* Scientific Section, A. Ph. A., Cleveland meeting, 1922.