

THE EFFECT OF CONCENTRATION, RATE AND UNIFORMITY OF
INJECTION ON THE LETHAL DOSE OF CARDIOTONICS FOR PIGEONS

by

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Thesis submitted to the Faculty of the Graduate School
of the University of Maryland in partial
fulfillment of the requirements for the
degree of Doctor of Philosophy

1951

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ACKNOWLEDGEMENT

The author wishes to express his deepest gratitude to Dr. Clifford W. Chapman for his valuable guidance and criticism in the construction of this thesis; to Miss Georgianna Simmonds Gittinger for her help in the reading of the proof; to the American Foundation For Pharmaceutical Education whose generous financial assistance throughout his tenure of graduate study has made this work possible; and to the School of Pharmacy of the University of Maryland for the use of its facilities.

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PART I

INTRODUCTION

The biological assay of digitalis and related cardiotonics by the U.S.P.¹ intravenous pigeon method is very time consuming. Attempts to shorten the time of assay by increasing the rate of injection have been opposed because the value of the mean lethal dose apparently increases as the rate of injection is increased. The first part of this investigation was undertaken to determine whether a non-uniform rate of injection, which shortens appreciably the time of assay, would change the value for the mean lethal dose as determined by the U.S.P. assay. The relationship between the rate of injection and the mean lethal dose of cardiotonic for pigeons comprises the second part of the work. Different rates of injection are produced by varying the concentration of injection fluid; a uniform, periodic injection procedure is maintained in each case.

The methods for standardization of digitalis and allied cardiotonic drugs, after a half century, still remain in a state of flux. The application of statistical methods and the introduction of reference standards are improvements, but the universally accepted method has yet to be designed. The ideal method of standardization would be a chemical one which tests the full therapeutic activity of the drug. However such a

¹Pharmacopoeia of the United States

method is still wanting, and consequently one finds that only biological assay procedures have been used in official compendia with the exception of chemical assays for digitoxin and digoxin, which are now outlined in the U.S.P. XIV (86). The U.S.P. IX, X and XI made use of the one hour frog method (85), U.S.P. XII and XIII the intravenous cat method (86), and in the U.S.P. XIII Supplement and the U.S.P. XIV (87) the pigeon intravenous method became official. The B.P.¹ has used the overnight rather than the one hour frog method, the former being official in The United Kingdom. In addition to the above, many chemical, animal and plant assays have been proposed for the standardization of this group of drugs. In the following paragraphs these procedures will be briefly reviewed, and the reasons leading to the adoption of the intravenous pigeon assay as the official method in the United States Pharmacopoeia will be discussed.

Before a method for the standardization of a drug is devised, a need for such standardization is expressed. In the case of digitalis, where different samples of leaves often differ greatly in potency, numerous clinicians have appealed for a uniform product. There are of course a few, who even today, deny the need of a digitalis assay. A well known cardiologist, Thomas Lewis, has expressed the view that digitalis standardization is unnecessary (15). However the majority of clinicians believe as does Gold (16), that since pure principles of the drug are not available and since most of the medical practice is confined to the use of the crude material, a method

¹British Pharmacopoeia

of assay is necessary. Except for the chemical assay of digitalis and digoxin, biological assay procedures must be employed for the cardiotonics and their glycosides. The accurate determination of their potency is a necessity today, just as it was in 1906 when W. Dixon (20) said: "Many hundreds of patients die annually from digitalis and its allies not possessing the virtues which are required of them".

A method of assay for a drug is of little value unless a standard preparation of the drug, accurately defined, is used as a basis of comparison in determining the potency of other samples of the drug. The use of such terms as cat, frog and pigeon units should be discouraged as they have little absolute meaning, the unit varying with the individual animal used. International standard preparations of the cardiotonics were prepared under the auspices of The League of Nations and individual countries have prepared their own standards, several of them conforming to the international standards.

In 1916 ouabain was proposed by the U.S.P. as a reference standard for Tincture of Strophanthus (15). It was adopted by The League of Nations in 1923 and an international standard ouabain was prepared in 1928. Magnus in 1923 at the Edinburgh conference proposed an international standard for digitalis, which was adopted by the Geneva conference in 1925 and prepared by Magnus in 1926 (16). This International Standard Digitalis Powder, a sample of powdered digitalis leaves, was exhausted and a new one authorized by The League of Nations in 1936. It was decided at a conference in Frankfort (1928) that the inter-

national unit of activity should be that activity contained in 0.1 gram of the International Standard Digitalis Powder. As a standard of reference for unknown samples of digitoxin, the U.S.P. has adopted a Reference Standard Digitoxin Powder.

A great deal of effort has gone into the development of a chemical assay for the cardiotonics with primary emphasis upon the isolated glycosides, such as digitoxin. Gravimetric and colorimetric methods (75) have been employed for digitalis standardization, but an element of success has been achieved only in the case of a colorimetric procedure. Slow progress in the development of a chemical assay has finally culminated in the adoption by the U.S.P. XIV of a colorimetric procedure for the standardization of digitoxin (86). It should be noted however, that the U.S.P. retains a bioassay method for an identification test (87). Three pigeons used in the test, are injected by way of the alar vein with 0.5 mg. of the digitoxin sample. The sample is identified as digitoxin if each pigeon shows retching or emesis during the ensuing hour, and at least two out of three die from cardiac arrest within four hours.

The chemical assay for digitoxin had its beginning in 1918, when Baljet (2) developed the color reaction between picric acid and the digitalis glycosides. Later Knudson and Dresbach (51) (52) proposed the use of this procedure as a quantitative method for cardiotonic standardization. Finally Bell and Krantz (4) (5) developed a complete quantitative method for the estimation of digitoxin potency based on the work of Baljet and Knudson and Dresbach; and as a direct

result of their efforts the present official assay was adopted.

After Knudson and Dresbach introduced their method in 1922, many workers attempted to compare chemical with biological assays. Smith and McClosky (76) in 1925 found a lack of agreement between colorimetric results and results obtained by the intravenous cat method. Wible (97) in 1926 reported that colorimetric results did not parallel those obtained by the use of the one hour frog method. Allmark and Bachinski (1) as recently as 1948 stated that, though the chemical method is simple and practical, the presence of interfering substances gives results which differ greatly from those obtained by biological means, and occasional differences of as much as 50%. However Bell and Krantz (4) (5) have found fairly good agreement between their chemical and the intravenous cat method. Especially has this been true in the case of digitoxin. Therefore since most of the disagreement between chemical and biological methods has resulted when crude preparations of the cardiotonics were used, it seems logical to assume that the colorimetric assay of Bell and Krantz may serve a very useful purpose in the standardization of pure glycosides.

The proposed method of Macht and Krantz (56) (57) for the standardization of digitalis using the seedlings of Lupinus albus L. has been the only suggested plant assay cited in the literature. It was not well received and has been abandoned as a practical method for the determination of potency. This phytopharmacological procedure depends upon the retardation of growth by solutions of digitalis. "The growth of the seedlings

is inversely proportional to the concentration making possible a phytotoxic curve which can be calibrated in terms of cat units" (75). Munch (62) reported that the results he obtained with the seedling method did not coincide with the results obtained by bioassay procedures. In addition to the above the specificity of digitalis for the plant must be questioned. Is it not true that other toxic substances may retard the growth of the seedlings in the same manner as digitalis? The proposed method does not test any specific action of digitalis upon the heart.

There have been a multitude of procedures employing animals proposed for the standardization of the cardiotonics. These animals include a protozoan, an arthropod and several classes of chordates--fish, amphibians, birds and mammals. A discussion of these methods has been given by Munch (62) and also by Schwarz (75).

A method employing Paramecium caudatum L. was devised by Schneider in 1925 (74). Viehoefter (91) (92) (93) using Daphnia magna, a water flea, observed and measured the action of the drug upon the rate of the heart beat. Considerable investigation was carried out by Pittenger and Vanderkleed (67) (69) upon the Carrassius auratus L. (goldfish) and the authors maintained that constant and comparative results could be obtained. In regard to the above methods it can be said that as in the case of the plant seedling method of Macht and Krantz (56) (57), the tests are not specific for digitalis action with the possible single exception of the

daphnia method. Here, though the drug is tested upon the heart, it is unlikely that such action upon the heart of an insect runs parallel to the effect of the drug upon the heart of the cat, pigeon, human or even the frog. Lack of application of statistical methods to the above procedures is another reason why they have received little attention.

Numerous bioassays have been performed on amphibians and especially on the frog. The literature pertaining to such assays is voluminous, with particular emphasis on the one hour and overnight frog methods. A toad method has been described by Gunn and Epstein (30) employing the South African clawed toad (*Xenopus*). Zeigler (99) has reported a bioassay method using the turtle.

Since Houghton (49) introduced the frog method in 1898, many procedures, using frogs as the test animal have been devised. These include those performed on the intact animal as well as on the isolated frog heart. Famulener and Lyons (28) and Fraenkel (30) in 1902 described one hour frog methods. Both of the above assays in addition to the Focke (29) method and the 30-45 minute method of Gottlieb (35) used the stoppage of the heart in ventricular systole as the endpoint. In the United States it was the one hour method which was adopted as the official procedure for the standardization of the cardio-tonics.

The overnight frog method, also called 12-hour, 18-hour and M.L.D. method had many advocates after its introduction by Houghton (49). It was not until Trevan (81) (82) in 1926

and 1927 introduced statistical analysis for interpretation of results and constructed the characteristic S shaped curves that the method became generally accepted. Burn (14), who has also taken an especial interest in the statistical evaluation of results of bioassay procedures, inclines toward the M.L.D. frog method. Chapman and Morrell (18) in 1931 determined the characteristic curve for ouabain by the overnight method and obtained a curve similar to but even steeper than that of Trevan. It must be pointed out, however, that Chapman, while favoring the M.L.D. method, has reported close agreement between it and the one hour method (17).

Other methods using frogs include the intramuscular method of Dooley and Higley (21) (22) and the intravenous method of Uhlmann (83) (84). Injection is made into the thigh in the former and into the abdominal vein in the latter method. The isolated frog heart has also been used as a means of standardization. Munch (62) reports several attempts of investigators to develop a suitable assay by a heart perfusion procedure. In addition isolated strips of the frog heart have been used in the frog heart sinus method of Mansfeld and Horn (58).

It may be said that the one and eighteen hour frog assay methods have been improved to a high degree of accuracy by the use of a standard preparation and statistical analysis. The one hour method has been defended by Haskell (42), who stated that the procedure is both a good qualitative and quantitative test for the heart tonics. Trevan (81) found that two tinctures

of strophanthus gave almost identical results with both the 12-hour frog and cat methods. The M.L.D. frog assay has been further defended by Fittenger (68), who prefers it and the guinea pig method to the one hour frog or intravenous cat methods, and Rowe (72), who believes that the absorption factor, where the injection is made into the ventral lymph sac of the frog, increases the accuracy and value of the assay. Chapman (17) (18) has always expressed a preference for the M.L.D. frog method. Bhatia and Lal (8) have stated that the frog procedure of Trevan was superior to the cat method because it was more economical, simple to perform and capable of detecting deterioration.

The use of the frog for the assay of cardiotonics has been subject to considerable criticism. Nyiri and DuBois (63) believe that only warm blooded animals should be used, and that the best way of administering heart tonics in an assay is by intravenous injection. Wokes (98) and Miller, Bliss and Braun (60) report, that whereas deterioration of Tincture of Digitalis is noted by the overnight frog method, such deterioration is not observed in the cat method. Finally Eggleston (26) and Bliss (9) state that the results of frog assays are not comparable to results obtained by the use of the intravenous cat assay and therefore cannot be transferred to man.

On the strength of this criticism and also because of the preference expressed by Gold and others for the use of the cat in the assay of cardiotonics, the U.S.P. XII Revision Committee saw fit to abandon the one hour frog method as the official

assay for digitalis products in the United States. In its stead a modified Hatcher-Brody Cat Method was made official. Evidence supporting the cat in preference to the frog as the test animal will be presented (pages 11 and 12) in a discussion of the cat method.

The pigeon and chick embryo have also been used for cardio-tonic standardization. The pigeon emesis and fatal dose methods will be discussed in considerable detail below. A method for the assay of digitalis using the embryonic chick has been proposed by Hall (38), the endpoint being the arrest of the heart. Paff (64) made use of isolated hearts from 48-hour chick embryos in the determination of digitalis potency. The method has been subjected to little investigation, and statistical evaluation of it is noticeably absent from the literature.

Mice, rats, rabbits, guinea pigs, cats and dogs have all been used as test animals in attempts to devise a suitable assay for the heart tonics. Subcutaneous procedures using mice have been developed by Heinz (46) and Krogh (53). Wentz (96) and Beddow (3) used the rat in assaying tinctures of digitalis and strophanthus. An intravenous procedure was employed with injection being made into the saphenous vein. Methods with the rabbit as the test animal have been used by Hyiri and DuBois (65), Heinz (46) and Sowton (77). The latter employed a heart perfusion technique; whereas Hyiri and DuBois and Heinz used intravenous methods. The most important procedures using the guinea pig

include the subcutaneous method of Reed and Vanderkleed (70) and the intravenous method of Knaffl-Lenz (60). The B.P. recognizes the latter as acceptable for cardiotoxic standardization.

As a substitute for the frog methods, an intravenous type of assay is generally accepted in preference to subcutaneous or oral procedures. Although the guinea pig intravenous method was a fairly good one, the cat has been more often employed as the test animal.

Hatcher (44) suggested the use of a cat emetic assay in 1907 and the intravenous cat method of Hatcher and Brody (45) was described in 1910. Injection, into the femoral vein, was begun with the unknown drug and continued until toxic symptoms became manifest. Ouabain was then substituted and used until the death of the cat. Calculations were based upon "cat units" defined in terms of the amount of ouabain necessary to kill a kilogram of cat. This procedure consumed approximately 60-90 minutes. Modifications of the above method have included variations in type of anesthetic, changes in rate and method of injection, discontinuation of the use of ouabain to complete the assay, and the introduction of a reference standard in the calculation of relative potency.

The use of ouabain, in every cardiotoxic assay performed by the Hatcher-Brody cat method, increased the difficulty of the procedure. In order to simplify the method and because there appeared to be no real necessity for its use, the injection of ouabain to culminate an assay was abandoned and

the sample was administered until the death of the animal. This modified procedure was employed by Smith and McClosky (76), Rowntree and Macht (73) and van Wijngaarden (90). Many different rates of injection of the drug have been proposed and reported as suitable (43) (71) (73) (76) (90).

Eckler (83) suggested that the intravenous cat method was difficult, time consuming and not practical as a method of assay. Rowntree and Macht (73) stated that the cat method was more reliable than the frog method. Many other workers have argued as to the merits of different assay procedures. Arguments which have the most support are: (1) there exists a great similarity between the heart of the cat and the heart of man, (2) the best method of administration of a cardiotonic in an assay is intravenous and (3) an assay need not necessarily predict the clinical dosage for man.

The intravenous type of assay is preferred by many, among whom are Nyiri and DuBois (83). Eggleston (26) in 1913 expressed a preference for the cat because of the similarity of its heart to the heart of man. This view was restated by Gold et al. (32), who found that the results obtained with the cat assay could be transferred to humans. Eggleston (27) et al. (33) reported that reliable results as to the relative potency of cardiotonics could be obtained with the intravenous cat method and that the dosage for man could be based upon the determined potency. It is true that this procedure tests the toxic rather than the therapeutic action of the drug but therapeutic endpoints are difficult to obtain and toxic effects

are due to the same action (60).

Edmunds (34) believed that regardless of any animal standardization the physician must ascertain the dose for the individual patient. The results of an animal assay can be applied to man within certain limits, but a final evaluation of potency of a digitalis preparation must be based on determinations on man (54) (60). According to Gold et al. (32) the intravenous cat assay is satisfactory because the most that any method of bioassay can accomplish as regards the clinical problem of dosage is to supply suitable data concerning the relative potency of different specimens.

Berardi, Canan and McGuigan (7) devised a therapeutic method of assay employing the dog as the test animal. The therapeutic effect of digitalis upon the heart rate was noted after intravenous injection of the drug. Berardi (6) also made use of the Hatcher-Brody Cat Method, substituting the dog. There appears to be no reason to believe that the dog is more suitable for an intravenous cardiotoxic assay than the cat, especially since dogs would be more expensive and more difficult to obtain.

Hanzlik (39) in 1928 reported the pigeon emesis method for the estimation of digitalis potency. Emesis methods had been suggested for assay previously but this was the first instance in which the pigeon was used as the test animal. Hanzlik and Shoemaker (40) had observed the emetic reaction to digitalis in pigeons and they believed that it might serve as an index of therapeutic dosage for man. The method was

used for approximately a decade but never replaced the one hour frog or the intravenous cat assay as an official procedure. The introduction of the intravenous pigeon assay and its adoption by the U.S.P. XIV (88) makes the emesis method obsolete and should lead to its abandonment.

Hanzlik (39) said: "The principle of the pigeon method is directed towards the evaluation of the probable therapeutic dosage, by determination of the minimum (or average) emetic dose of digitalis". He devised the method believing that a fatal dose method of assay was unsatisfactory because it did not predict the therapeutic dosage for man, admitting at the same time that a pigeon fatal dose procedure could be used, but with the same qualification (39). Pigeons were preferred to cats because they were cheaper and easier to obtain. The emesis method was desirable because of its simplicity.

Hanzlik used pigeons, previously starved, weighing between 300 and 400 grams. The emetic dose of digitalis was injected into a wing vein with a number 23 hypodermic needle. After injection, the pigeon was placed in a cage and symptoms of vomiting observed. The vomiting had to occur in from three to ten minutes, depending on the dosage used, and was not a positive result if it were delayed longer. The minimum emetic dose was determined by injecting a series of pigeons and noting the minimum effective dose causing emesis in two out of three pigeons. The pigeons recovered after one or two weeks and could be used again if the veins were still in usable condition.

Differences in body weight only slightly affected the results. The M.Em.D. was higher in adult males than females, but close enough to permit the use of both sexes. The M.Em.D. was higher for immature and also for sick birds. Hanzlik (39) therefore stated that, if only mature, healthy pigeons in a given weight group were employed, a consistent value of the minimum emetic dose for a given drug could be obtained. This M.Em.D. of digitalis caused changes in the pigeon heart typical of digitalis action and was a measure of the therapeutic dosage in man.

Hanzlik and Stockton (41) tested tinctures of digitalis, assayed by the pigeon emesis method, on several patients with normal hearts. The doses administered, causing advanced digitalis action, agreed closely with the probable doses estimated from the pigeon M.Em.D. They observed in these patients nausea, emesis, slowing of the pulse, a fall of blood pressure and a reduced pulse pressure. Stockton (78) in a later work confirmed this agreement between clinically effective doses of digitalis and results by the pigeon emesis method. He cautions however, that because of the great variability of clinical dosage, close observation of the patient should be maintained, even though a standardized preparation is employed. This is of course in direct contradiction to the previous statements of Hanzlik and Stockton that the assay is a therapeutic one; that is, the M.Em.D. serves only to determine the relative potency and cannot accurately predict therapeutic dosage for man.

Burn (13) severely criticized the terminology and procedures used by Hanzlik and has suggested several improvements for the assay. Such terms as minimum emetic dose and pigeon units, he states, are not accurate expressions and have no place in bioassay procedures. He points out that several groups of three pigeons given the same dose of a sample will give varying results. In some cases none or all of the three pigeons will vomit and the number of groups in which two out of three respond will usually be in the minority. "The reaction of pigeons to digitalis by vomiting is another example of 'continuous variation' in a biological property" (13). If in each group three birds are used the error will be about 300% and only 30% if each group contains 25 birds. Burn believes that the method could be used if 25 animals are used in each group and a characteristic curve constructed relating percentage of emesis to the potency of the preparation. He states further that a standard preparation should be used making the assay comparative. In this way results might be obtained which resembled those obtained by the cat or frog methods.

Whereas Burn (13) attempted to improve the pigeon emesis method of Hanzlik, others denied that it could be used to standardize cardiotonics. Gold, Gelfand and Hitzig (34) stated that the pigeon emesis method of assay was inferior to the intravenous cat assay and did not predict the therapeutic dosage for man. They neither believed the method gave more consistent results than the cat assay, nor that the

emetic response paralleled the therapeutic response. They stated further that there were many inconsistencies and contradictions in Hanzlik's work and that much of it was based upon false assumptions. The use of a toxic, rather than a therapeutic method of assay, they said, did not account for the lack of correlation between the results of bioassay and the therapeutic potency of digitalis preparations in man. Indeed preparations found to be toxic for man were confirmed by the cat method.

During the course of his studies, Hanzlik (39) noted that the pigeon mean lethal dose for digitalis preparations compared favorably with that of the cat. This fact was to become of the utmost consequence for the future standardization of the cardiotonics. In 1934 Haag and Woodley (37), attempting to confirm Hanzlik's work by comparison of pigeon emesis and intravenous cat methods, proposed an intravenous pigeon fatal dose procedure. They observed the effect of rate of injection, state of health of pigeons and the administration of alcohol and saline solutions upon the M.L.D. They failed to suggest the use of a standard preparation but believed that a comparison of pigeon and cat units should be made.

Haag and Woodley used adult pigeons of either sex, starved for about 12 hours, and weighing between 300 and 425 grams. Ether was employed as the anesthetic and injection was made into the alar vein. The cannula used was a blunted hypodermic needle of gauge 20 to 23. Injection was made at

the rate of 0.5 cc. every ten minutes until death, regardless of the weight of the pigeon used. The M.L.D. however, was calculated by dividing the amount of digitalis in cc. or gm. by the weight of the pigeon in kg.

These authors were able to obtain some significant results. They found the M.L.D. for pigeons about 25% greater than for cats. That is pigeons were, on the basis of weight, more resistant to the drug. If they had been using a standard preparation, they would have been more able to appreciate this fact. Curiously enough pigeons appeared to be twice as tolerant to ouabain as cats. Increasing the rate of injection so that the pigeon died in less than 60 minutes, resulted in an increase in M.L.D. Sick pigeons gave high results, a fact previously noted by Hanzlik (39). Alcohol or normal saline solutions had little effect upon the M.L.D. Haag and Woodley observed that results were very consistent and as few as three pigeons would give an approximate value for the M.L.D.

The success of Haag and Woodley with the M.L.D. pigeon method and the difficulty of obtaining sufficient cats for assay purposes induced Braun and Lusky (11) to propose the substitution of pigeons for cats in the official procedure of the U.S.P. They patterned their procedure after that of the U.S.P. intravenous cat assay and compared results of intravenous cat and pigeon assays. They used adult pigeons of either sex weighing between 275 and 450 grams. Three breeds of pigeons were employed: White Kings, Homers and

Common Barn Pigeons. Injection was made into the alar vein using a blunted 22 gauge hypodermic needle. The injection solution was contained in a 10 cc. burette calibrated to 0.1 cc. Light ether anesthesia was employed during the assay. The sample was injected at the rate of 1 cc. per kg. of body weight every five minutes until the death of the pigeon.

The results Braun and Lusky obtained were very satisfactory. They were consistent results and there was lower coefficient of variation with pigeons than with cats. They also found that pigeons were more resistant to cardiotonics than cats. The average M.L.D. for U.S.P. Digitalis Reference Standard on pigeons was 96.22 mg. per kg. and on cats 84.83 mg. per kg. They also found more consistent results between assays, as well as within assays. Braun and Lusky (11) maintained that the pigeon paralleled the cat assay, was cheaper and that pigeons were more easily procured.

Lavallee and Allmark (55) confirmed the results of Braun and Lusky (11) and agreed that the method would be satisfactory. The cat could be replaced by the pigeon and less variable results could be obtained with the latter. An exception was noted in the case of digoxin, where differences of as much as 100% in potency were found between cat and pigeon results. The U.S.P. XIV Revision Committee (80) began a collaborative study of the intravenous pigeon assay with Dr. Haag as director. They found the results generally satisfactory but noted variation between laboratories. It was proposed to change the method of calculation of potency by the use of geometric

averages. A logarithmic calculation was employed and confidence limits of an assay determined. The U.S.P. XIV (87) official method of assay for the cardiotonics is essentially the procedure of Braun and Lusky (11) with the above change in calculation of potency.

Many clinicians have desired a human method of assay for the cardiotonics. Some believed that results obtained from animal bioassays were not applicable in human therapy. Others maintained that the potency obtained using animal procedures, even if a standard were used, was not correct. Is it not better to standardize digitalis orally on humans, than intravenously on cats? Of course one must always bear in mind the difficulty involved if humans are used as experimental subjects.

Human procedures have been proposed which involve the use of the electrocardiogram. Pardee (65) believed that the amount of drug necessary to produce a minimal change in the height of the T wave of an electrocardiogram could be used as a measure of potency of a digitalis preparation. Doses were given orally to humans after a control electrocardiogram had been taken. The depression of the T wave noted, represents one action of the drug and occurs simultaneously with the stimulation of the heart muscle. This method, said Pardee, is a practical one giving the minimum effective dose of a digitalis preparation. He did not use a reference standard preparation of digitalis, employing instead a "T wave unit" (66). Van Dyke and Li (89) made use of electrocardiographic

technique in another attempt at the clinical standardization of digitalis. They reported results which compared favorably with those obtained using mammalian procedures but which differed significantly from results obtained with the frog method.

Another therapeutic assay method employing humans has been proposed by Martin (59). The endpoint was the therapeutic effect, adjudged by the cardiac and respiratory improvement of the patient. The shortening of the auricular conduction time; i.e., the P-R interval was included among the criteria of improvement selected. Martin stated that the relative potency of dried digitalis leaves could be determined in this way. Gold et al. (51) used several changes in the electrocardiograms of subjects with normal hearts to determine digitalis potency. These changes included alterations in the T wave and RT or ST segments. The potency of an unknown sample was expressed in terms of a standard preparation. This human method of assay was being applied to the standardization of digitalis preparations of commerce, but the Food and Drug Administration objected to its use.

No methods have been proposed for shortening the duration of the intravenous pigeon assay. Haag and Woodley (37), observing the effect of rate of injection upon the M.L.D. for pigeons, concluded that there was an increase in M.L.D. at the faster rates, probably due to "overshooting". By maintaining an injection period of 60-90 minutes a lower value could be obtained; this they assumed to be the correct M.L.D.

Many different rates and types of injection have been used for the cat assay, and the findings of Haag and Woodley (37) are also applicable here. Hatcher and Brody (48), when devising the intravenous cat assay, injected 75% of the expected lethal dose of digitalis in fifteen minutes and the remainder in the following hour. Whether or not the assay was completed with ouabain, it consumed approximately ninety minutes. The duration of the assay; i.e., one to one and one-half hours was the same as that employed in present day cat methods, but the initial injection of 75% of the drug in fifteen minutes was unique.

Rowntree and Macht (73) injected 10 cc. in five minutes and then 1 cc. per minute until the death of the cat. Smith and McClosky (76) injected continuously. Haskell, Daniel and Terry (83) injected at the rate of 1 cc. every two and one-half minutes regardless of the weight of the cat. Rowe (71) believed uniform results could be obtained if the endpoint were reached in an average time of thirty minutes, the injections being made at a uniform rate. However, he thought that injecting rapidly at first and then giving 1 cc. every two minutes until the death of the animal was a better procedure. Wible (97) injected at a constant rate of 0.5 cc. per minute.

Many workers have attempted to shorten the intravenous cat assay using a uniform rate of injection. These include: Rowe (71), Edmunds, Moyer and Shaw (25), who administered the drug so as to kill the cat in forty minutes; Wasicky, Lasch and Schonovski (95), who calculated dosage necessary to produce

death in thirty to sixty minutes and van Wijngaarden (90), who used an assay period of thirty to fifty-five minutes. This reduction of the duration of the assay has been supported by Burn (15), who recommended shortening the time of the assay to thirty to sixty minutes.

Bliss (9), a proponent of the longer assay time adopted by the U.S.P. XII, believed that 1 cc. of the diluted material per kg. of cat should be injected. An injection is made within a few seconds and then repeated at five minute intervals with a dilution so prepared that the cat died within 13-19 doses; i.e., sixty to ninety minutes. He, like Haag and Woodley (37), observed that the M.L.D. was modified by the rate of injection. Nyiri and DuBois (65) stated that with fast as well as with slow administration, part of the drug was lost to the heart; therefore, it is best to set an arbitrary time as to drug administration as well as duration of the entire assay. They further stated that results of assays are only comparable when approximately the same amount of drug acts upon the heart within the same period of time.

The purpose of the work reported in this thesis is to determine the effect of rate of injection upon the mean lethal dose of cardiotonics administered intravenously to pigeons.

EXPERIMENTAL

Apparatus: The solution to be injected was contained in a straight, glass stoppered, Kimble (Exax) 10 cc. burette graduated in 0.05 cc. The tip was fitted with soft rubber tubing of 6 mm. outside diameter, a bore of 3 mm. and a length of 61-69 cm. As cannulae, Luer slip syringe needles 22-24 gauge, modified by shortening to approximately $1\frac{1}{2}$ cm. and blunting and grinding the hub to a cylindrical form to fit the rubber tubing, were used. Infusion or slip on hub needles and Luer slip needles fitted with adapters could be used. The burette with tubing and needle attached was mounted on a burette stand.

To measure five minute intervals a Hawkeye Measure Time (Fisher Scientific Co.) clock and interval timer with a spring mechanism was used. The pigeons were weighed on a Pelouze Dietetic Scale of 500 gram capacity. To anesthetize the pigeons raw absorbent cotton saturated with ether (diethyl-ether) was contained in the bottom of a desiccator having a diameter of 22 cm. and a depth of 20 cm.

The maceration, when necessary, was carried out in 125 cc. or 250 cc. Erlenmeyer flasks. The maceration was centrifuged at about 1800 RPM in a 110 volts AC or DC International Clinical Centrifuge fitted with 50 cc. tubes. Dilutions were prepared in 50 cc. and 100 cc. volumetric flasks, 1 cc., 5 cc. and 10 cc. Mohr pipettes being employed for measuring the

amount of sample used.

A Friden automatic calculator was used for calculating individual dosage on the basis of one cc. per kg. of body weight of pigeon, and for the calculation of all results.

The pigeons were securely fastened on pigeon boards (see figure 1), each wing being tied down with an ordinary 40 inch shoe lace and each leg with a 15 inch string tie. The vertical piece contained two nails at the lower end of each side to anchor the leg ties.

Animals: Adult pigeons (Homers or Common Barn Pigeons) of either sex weighing between 250 and 500 grams were used; one pigeon (table XXII) weighed 235 grams. The birds, neither sick nor emaciated, were starved 16-20 hours previous to use, except those in tables no. V, VI, VII, XVIII, XIX, and XX which were fed. The pigeons were obtained from Central Feed Company, Baltimore, Maryland. They were fed scratch feed containing cracked corn, whole oats and wheat and #4 pigeon feed, which consisted of whole corn, wheat and buckwheat, cracked rice, kafir corn, red millet and maple peas, both obtained from the Central Feed Company. Fresh water was supplied ad lib. A maximum of seven birds was kept in a cage 22 inches wide, 20 $\frac{1}{2}$ inches deep and 14 $\frac{1}{2}$ inches high.

Material: Samples used included digitalis tincture, tablets and leaves, digitoxin tablets and U.S.P. Reference Standard Digitalis, Digitoxin and Ouabain Powders. The U.S.P. digitalis and digitoxin menstruums were used to make macerations of the powders and tablets. U.S.P. Physiological Salt

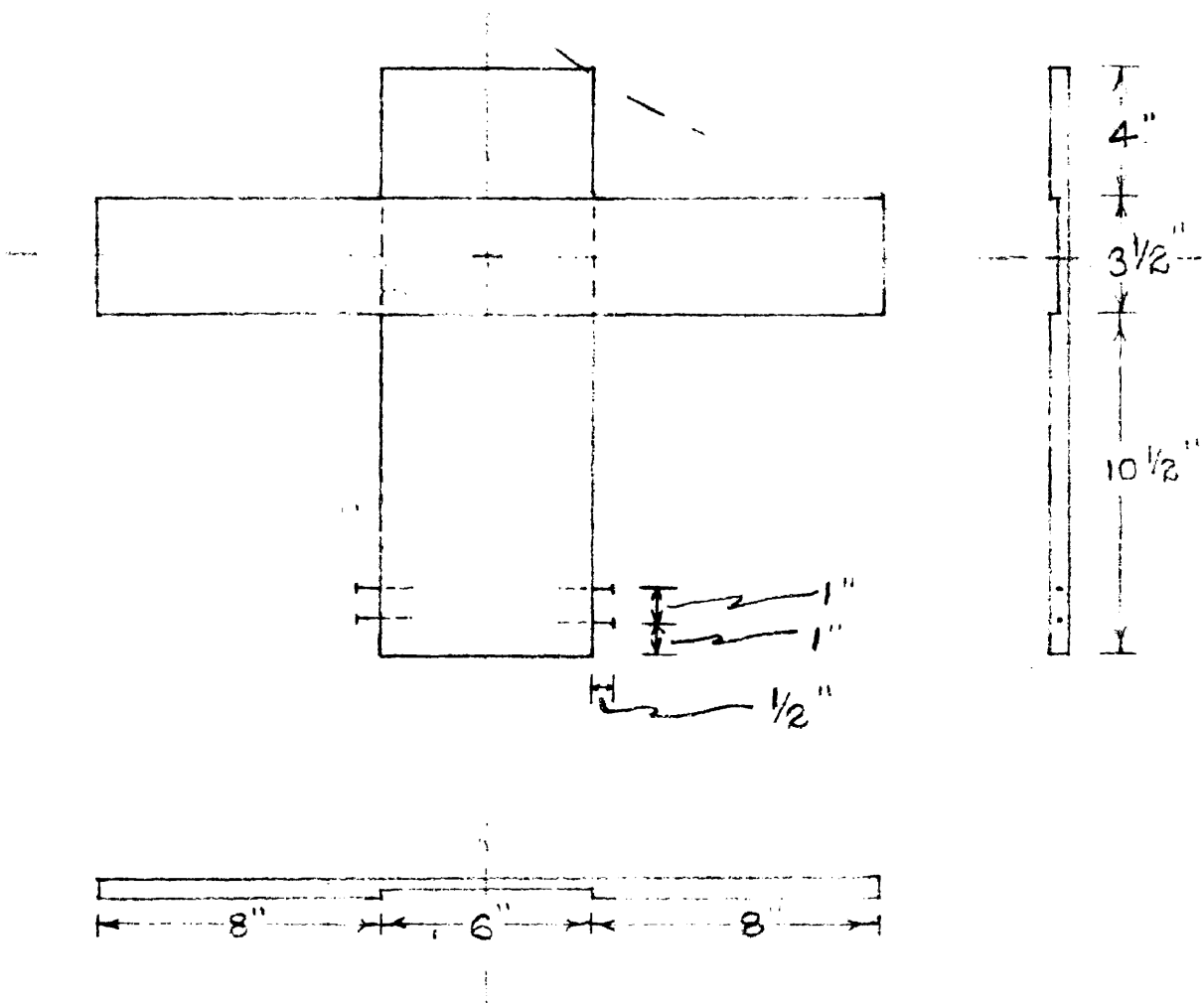


Fig. 1

TITLE: Assembled Pigeon Board
SCALE: 1" = 6"
REMARKS: Material - 1/2" Lumber, Four Common Nails, 1 1/2" Long Recess (Both Pieces) 1/4" Depth

Solution was employed in the preparation of dilutions. Ether (diethylether) was used as the anesthetic.

Procedure: On the day of the experiment a pigeon was accurately weighed to the nearest gram, anesthetized with ether in the desiccator and tied to the pigeon board. Some of the feathers covering the undersurface of one wing were removed to allow observation of the alar vein. This vein was then exposed and cannulated. The modified syringe needle was inserted into the vein after the distal end had been tied off. The cannula was then securely fastened in place with thread (Clark #40).

All macerations were prepared at least one day previous to the experiment. Liquid samples were used undiluted or were diluted on the day of the experiment. Samples macerated were centrifuged before use. The solution to be tested was diluted in such a way that the estimated fatal dose per kilogram of body weight was diluted to 15 cc. with normal saline solution. If 1 cc. per kg. of body weight were administered, the average lethal time was sixty to ninety minutes; i.e., 15-19 doses. An initial injection was given within a few seconds and additional injections every five minutes thereafter, intervals determined by interval timer, until death. If more than 1 cc. per kg. of body weight were administered initially, 5, 7, or 10 cc. per kg. were given at once and then additional injections at regular five minute intervals until the pigeon died. This shortened the time of injection by forty-five minutes if 10 cc. per kg. were given initially; the pigeons dying between

fifteen and forty-five minutes.

Death in the pigeon is due to failure of the circulation. The heart stops, the respiration is embarrassed and convulsions and death ensue. The endpoint is sharp with digitalis, much less so with digitoxin and ouabain. The endpoint used was the death of the animal, observations of toxic symptoms being made only to help determine the exact endpoint.

Sex: It is well known that it is impossible to determine the sex of pigeons because of the absence of external genitalia and any other external anatomical differences. In this work the sex, as recorded in the tables, was determined after death by dissection and observation of either a single ovary or testes. Since Haag and Woodley (37) had previously reported the absence of sex differences in lethal doses to cardiotonics, and since no differences were observed during the experiment no correlation of sex and lethal dose was made.

Body Weight: Haag and Woodley (37) found that the lethal dose increased with a decrease in body weight of pigeon. In the present experiments pigeons were selected on the basis of weight so that the mean body weight for groups tested by different methods on any individual sample was not significantly different. In this manner any effect upon the lethal dose due to differences in body weight was eliminated.

RESULTS

The results of the experiments are summarized in tables I-XXII. Two samples of Digitalis Reference Standard, three digitalis tinctures, one sample of digitalis tablets and one sample of digitalis leaves were tested by the U.S.P. and 10 initial dose methods. One of the digitalis tinctures (table VI) was also tested by a 5 initial dose method. Digitoxin Reference Standard was tested by the U.S.P. and 10 initial dose methods and digitoxin tablets by the U.S.P., 10 and 7 initial dose methods. Ouabain Reference Standard was tested by the U.S.P. and 10 initial dose methods.

In some instances, where small numbers of animals were involved, both procedures were performed on the same day. If a drug were tested by three methods or if a large number of animals were used, the period was often extended to a week and in a few cases to one to four months. Pigeons were selected on the basis of live body weight so that the arithmetic mean weights of the groups used for individual samples by different methods were approximately the same.

Dilutions of the drugs (column 5) were so prepared that the average lethal dose, if one cc./kg. were administered per dose, fell between 13 and 19 doses (mean of column 6). A few of the experiments contain pilot tests, performed to ascertain the dilution necessary to secure the desired time interval. In tables I-XXII, column 6 is headed no. of doses. In

the case of the U.S.P. method this number represents the actual number of doses of drug administered and is a direct indication of the time of the injection. In the initial dose methods, the number of doses was calculated by adding the number of initial doses to the remainder administered until the experiment was terminated; i.e., even if 10 cc./kg. were administered initially, this initial dose was indicated as ten. The number of doses in the latter instance is not a direct indication of injection time. This procedure was followed because the number of doses was proportional to the lethal dose and could serve as a comparison between the U.S.P. and modified methods.

The time in minutes is recorded in the last column. For assays performed by the official method it is equal to 5 (no. of doses - 1) because the initial dose was given immediately and the remainder at five minute intervals. The time in minutes for the modified methods is 5(no. of doses - initial no. of doses). Mean times for both methods were calculated and are given in table XXIII.

The lethal dose for each pigeon in terms of quantity of original sample has been included in tables I-XXII, column 7, as well as the mean lethal dose for each method. All of the mean lethal doses have been summarized in table XXIII for ready reference and comparison. The lethal dose was calculated by the short method; i.e., L.D. = no. of doses x % dilution because each dose was equivalent to one cc./kg.

All means included in the tables are arithmetic means

(averages). The standard deviation (σ) is equal to $\sqrt{S(x - \bar{x})^2 / (n - 1)}$; where \bar{x} = mean, x = an individual observation and n = number of animals. The standard error (ϵ) is equal to $\sqrt{S(x - \bar{x})^2 / n(n - 1)}$ or σ / \sqrt{n} . The value of $S(x - \bar{x})^2$ is determined by squaring each deviation from the mean and summing them or by employing the short cut method expressed by the equation, $S(x - \bar{x})^2 = Sx^2 - \bar{x}Sx$ (100). The statistic "standard error" is the standard error of the mean of samples and should not be confused with the standard deviation.

DISCUSSION

When comparing the U.S.P. and initial dose methods to determine if the mean lethal doses are the same, one must recognize that there are in fact two basic differences between the methods. The first is the difference in total time of injection and the second is the existence of a non-uniform rate of injection in the case of the initial dose method. If a quantity of drug is administered initially which is equivalent to ten doses of 1 cc./kg. each, the time of injection will be shortened by forty-five minutes provided that the lethal dose were the same in both instances. Similarly the time of injection will be thirty minutes less for a seven dose and twenty minutes less for a five dose initial method. The latter two methods have been included only to aid in the comparison of U.S.P. and 10 dose initial methods. A perusal of table XXIII will show that the difference of mean time of the two methods was not exactly forty-five minutes but varied in small measure either positively or negatively because of the difference of the value of the mean lethal dose. The mean difference of time for the ten comparisons is, however, 46.7 minutes. The use of the 10 initial dose method in place of the present U.S.P. method will reduce the injection time for an individual pigeon by at least 50%.

The non-uniform rate of injection is the result of the administration of a rather large sub-lethal dose initially

and subsequent small increments equal to 10% of this dose. This procedure has been used previously by Hatcher and Brody (45) who injected 75% of the expected lethal dose to a cat within fifteen minutes. The total time of their assays, however, was at least one hour and fifteen minutes. Rowntree and Macht (73) injected 10 cc. in five minutes and then 1 cc. per minute thereafter. The latter did not base their rate of injection upon the weight of the cat and the amount per kg. was thus considerably less. Therefore the mean injection time by the 10 dose initial method (table XXIII) was less than that of Rowntree and Macht, in most cases equal to less than half of the mean times of the assays performed by them.

An inspection of table XXIII reveals only small differences between the mean lethal doses as determined by each method. In many cases one could easily decide without statistical analysis that there exists no difference between them. However because the standard error of each mean is not considered in this comparison and also because of border line instances in which significance may exist, use of "Student's" t test has been made to facilitate comparison. There are of course other statistical procedures that could be used. The fact that the t test is well known and is essentially an analysis of variance, if only two means are considered; such analysis of variance being widely applied to biological data, has resulted in its use.

Table XXIV lists the calculated and theoretical (table) values of t. Degrees of freedom (d.f.) is an essential part of the table, especially where small numbers of animals were

used. The degrees of freedom are the number of independent comparisons which exist within a group and are usually equal to $n - 1$, where $n =$ no. of animals or independent observations. Where the means of two groups are being compared, d.f. = $(n_1 - 1) + (n_2 - 1)$ or $2n - 2$ if $n_1 = n_2$.

The equation for the calculation of t is $t = (\bar{x}_1 - \bar{x}_2) / \sqrt{\epsilon_1^2 + \epsilon_2^2}$, where \bar{x}_1 and \bar{x}_2 are two means and ϵ_1 and ϵ_2 are the respective standard errors of each. The theoretical value of t is the value for $P = 0.05$ (the probability of a deviation equal to or greater than t ; at this level of significance). If we assume this level of significance, we will be wrong not more than once in 20 trials in the long run.

The following example will help to explain the use of the terms and equations in a t test analysis. Let us consider a comparison of the mean lethal doses of digitoxin tablets, 9M-27C, obtained by the U.S.P. and 10 dose initial methods. First a "null" hypothesis that there is no difference between the two means is formulated. Then the t test is applied to prove or disprove this hypothesis. The respective means for the U.S.P. and 10 dose initial methods are 0.40 and 0.38 and the standard errors are both 0.02. The above data are found in tables XVIII and XIX. The value of t is $(\bar{x}_1 - \bar{x}_2) / \sqrt{\epsilon_1^2 + \epsilon_2^2}$ or $(0.40 - 0.38) / \sqrt{0.0004 + 0.0004}$ and is equal to 0.87. This calculated value of t is found in table XXIV together with its theoretical value. The latter can be found in a table of t (101) for $P = 0.05$ and degrees of freedom = 36, since (tables XVIII and XIX) $n_1 = 18$ and $n_2 = 20$. After 30 degrees of freedom most

tables give the value for d.f. = 40. The value 2.029 is thus an interpolated number. For n , the number of degrees of freedom, infinite, $t = 1.960$ for $P = 0.05$.

The hypothesis that there exists no difference between the two means must be accepted for a significance level of $P = 0.05$, since 0.67 is smaller than 2.029. That is, if it is said that the difference between the two means is not significant the statement will be wrong once out of 20 times. A level of significance equal to once in twenty is usually accepted. A word of caution must be interjected here lest a false conclusion be assumed that the two means are equal. The only conclusion that can be made is that under the conditions of the experiment no significant difference between the means is demonstrated. Table XXIV shows an absence of significant difference in all comparisons.

The belief (10) (37) that there is an increase in the value of the lethal dose with a decrease in time of injection is certainly not demonstrated by the results of these experiments. Such an increase in the lethal dose could coexist with a decrease due to the injection of a moribund dose resulting in an insignificant difference of the means. The injection of approximately 75% of the lethal dose within five minutes may conceivably cause a state of morbidity sufficient to decrease the lethal dose of the drug as determined by the U.S.P. method. The heart sounds of the pigeon were compared, using a stethoscope, after ten doses of 1 cc./kg. each and after a single dose of 10 cc./kg. No discernible difference in the con-

dition of the heart was noted. An electrocardiographic recording might be more revealing. The emetic reflex and struggling were usually absent after the initial 10 cc./kg. dose but also after ten single 1 cc./kg. doses.

CONCLUSIONS

1. A significant difference could not be demonstrated between the mean lethal doses of:
 - a. digitalis preparations, determined by the U.S.P. and 5 or 10 initial dose intravenous pigeon methods.
 - b. digitoxin preparations, determined by the U.S.P. and 7 or 10 initial dose intravenous pigeon methods.
 - c. a ouabain preparation, determined by the U.S.P. and 10 initial dose intravenous pigeon methods.
2. There was no observable increase in lethal dose with a decrease in injection time.
3. The initial multiple dose method can be used for the biological standardization of digitalis and its allies.
4. The 10 dose initial method, involving a non-uniform rate of injection, decreases the injection time for an individual pigeon by 45 minutes.

PART II

INTRODUCTION

This investigation was undertaken primarily to develop a procedure to shorten the time of the U.S.P. biological assay for cardiotonics. However it is important to note, even if this modified method (Part I) gives results not significantly different from results obtained by the official method, whether there is within a given range of concentrations an increase in the value of the mean lethal dose, with an increase in the amount of drug administered per dose. This increase in concentration of drug per dose shortens the injection time but the amount of dilution administered is uniform throughout; i.e., one cc./kg. of pigeon.

A certain amount of drug can produce a desired effect, such as death in an experimental animal, useful in the biological assay for the determination of the relative potency of the drug. However, as in the case of the intravenous pigeon assay for cardiotoxic preparations, it is impossible to ascertain the portion of drug which has contributed to the death of the animal (19). Thus one cannot state that the lethal dose is actually the amount of drug that has been administered, and that within certain finite limits the death of the pigeon is dependent upon the time of administration of this quantity of cardiotoxic; i.e., dependent upon the concentration per unit time. If the lethal dose were actually the

amount of drug administered, there would still exist an "overshooting" factor in any periodic injection method and it would, of course, be greater at the higher concentration.

Clark (19) has stated that a theoretical formula in which concentration is dependent upon time is impossible in biological phenomena. If this postulate were true, the lethal dose would be independent of time and therefore of concentration for all times and all concentrations. But as is stated by Clark (19) concentration \times time = constant ($CT = K$) implies that an infinite dilution produces an action in infinite time and that a sufficiently strong concentration will produce instantaneous action. These are both untruths because there is a minimum threshold active concentration and also a minimum time needed for the production of a response.

The problem is then, to determine whether in an intravenous type of injection in which all animals die, the mean lethal dose varies with the concentration of drug administered per unit time. This problem has been investigated in guinea pigs, cats, and in at least one instance in pigeons. Results have been inconclusive and contradictory.

The most contradictory results have been obtained with the Knaffl-Lenz method employing guinea pigs. Braun and Siegfried (12) state that Knaffl-Lenz showed that the quantity of digitalis leaf required to cause death was greater for a 0.5% solution than for a 1.0 to 2.5% solution; that Goldberg found an increase in lethal dose with an increase in the

quantity of drug injected per minute. Straub et al. (79) showed that toxicity decreased with dilution only up to a certain point. They used dilutions of gitalin, digilanide and k-strophanthin. Chapman, as quoted by Allmark (10), using guinea pigs found an increase in the lethal dose of tincture of digitalis with an increase in the concentration of the injection fluid.

Braun and Siegfried (12) were unable to observe an increase in the lethal dose of digitalis with an increase in concentration per injection, when the drug was administered intravenously to guinea pigs. They also state that Otterstrom, Levy and Otterstrom and Brun made the same conclusion. Muja and Holck (61) actually observed a decrease of lethal dose with increased concentration.

Hildebrandt (47) investigated the effect of an increase in concentration upon the lethal dose in cats. G-strophanthin showed maximum toxicity when injected at a rate of 0.03 mg./kg./hr. At higher or lower rates of injection the toxicity was appreciably less. For digitoxin, the lethal dose increased as the rate of injection was increased. Vos and Dawson (94) found that in cats at shorter injection times, produced by increasing the rates of injection, the mean lethal doses of digitalis, ouabain and digitoxin significantly increase. Holck et al. (48) observed a similar relationship between rate of injection and the lethal dose of tincture of digitalis.

Bliss (9) has stated that only part of the amount of cardiac glycosides injected into cats contributes to the death

of the animal. The lethal dose measured experimentally tends to increase as the concentration of digitalis in the injection fluid is increased because the contribution of the last injections is especially small. Thus Bliss was aware of the difficulty of measuring the lethal dose in a periodic injection assay and recognized the "overshooting" phenomenon, previously noted by Haag and Woodley (37). Bliss and Allmark (10) found a statistically significant increase in lethal dose with an increase in rate of injection in two out of three samples of digitalis powder.

Haag and Woodley (37) found that the lethal dose for pigeons varied with the rate of injection. They state that the very rapid injections gave a higher mean lethal dose than slower injections. This they believe is due to "overshooting", more apparent of course at the higher concentrations.

EXPERIMENTAL

Apparatus and Animals: The apparatus and pigeons used were the same as in Part I.

Material: Samples used included a digitalis leaf, and U.S.P. Reference Standard Digitoxin and Ouabain Powders. Menstruums, diluents, and anesthetic were the same as in Part I.

Procedure: The method of cannulation and the preparation of macerations and dilutions were performed as in Part I.

Injection was intermittent, one cc. of diluted sample per kg. of body weight of pigeon being administered at five minute intervals. Each of the six dilutions of the digitalis leaf sample were tested upon sixteen pigeons, twelve tests being performed in a single day upon two samples of each dilution. Experiments were carried out upon four dilutions of U.S.P. Reference Standard Digitoxin powder. Fifteen pigeons were used for each experiment and twelve daily tests included three of each dilution, except on each of two days (see page 43). The experiment on U.S.P. Reference Standard Ouabain was performed in the same manner as digitoxin, except that eighteen animals were used for each dilution. Thus 96 (digitalis), 60 (digitoxin) and 72 (ouabain) animals used respectively totaled 228, a sufficient number for statistical evaluation and analysis.

Sex and Body Weight: The sex was determined and the pigeons selected on the basis of body weight as in Part I (page 28).

RESULTS

Tables XXV to XXX contain the results of experiments on a digitalis leaf sample using six different concentrations of drug. Two tests were performed on each concentration on an individual day; the experiment was completed within 32 days. The tests performed with four concentrations of U.S.P. Digitoxin Reference Standard are summarized in tables XXXI to XXXIV. Three tests were performed on the 0.02 and 0.03 mg./cc. concentrations on each of five days (tables XXXI and XXXII). Three tests were performed on the 0.05 and 0.08 mg./cc. concentrations on each of three days and six tests on a fourth day (tables XXXIII and XXXIV); all of these were completed within 13 days. The results of the tests of four concentrations of U.S.P. Ouabain Reference Standard are contained in tables XXXV to XXXVIII. Three samples of each concentration were tested on a single day and the experiment completed within 43 days.

An increase in the concentration decreased the time of injection and vice versa. This variation in rate of injection resulted in a variable number of doses necessary to produce the lethal effect. The concentrations were so selected that the mean number of doses was equal to, less than or more than the number specified in the U.S.P. XIV (87). The mean no. of doses and mean time in minutes are summarized

in table XL. All tests were performed with a uniform rate of injection (1 cc./kg. every five minutes); thus the number of doses was a direct indication of injection time. The time in minutes was calculated, as in Part I; i.e., number of minutes = 5(no. of doses - 1).

The lethal doses were calculated as in Part I (page 30). Table XXXIX has been included for a rapid comparison of the mean lethal doses for the different concentrations. The values of the mean, standard deviation and standard error (tables XXV to XXXVIII) were calculated as in Part I (pages 30 and 31).

DISCUSSION

In order to determine whether there was an increase in the value of the mean lethal dose with an increased rate of injection; i.e., an increase in concentration of drug per injection, the results were subjected to an analysis of variance (100). The data for this analysis are contained in tables XLI to XLIII. The "null" hypothesis that there was no difference between the means was formulated. The analysis of variance necessary to prove or disprove this hypothesis is contained in table XLIV for the results of the digitalis experiments, in table XLV for the digitoxin experiments and in table XLVI for the ouabain experiments. The sources of variation considered were (a) the variation between groups, representing the variations between the mean lethal doses obtained with each concentration, and (b) the variation within groups, the errors of the individual assays. The following example will illustrate the calculation of the variance ratio in table XLIV and its use in determining whether the difference between the means is significant for $P = 0.05$.

The sum of squares between groups is calculated by subtracting the correction term, $(ST)^2/n$, from the sum of the squares of the individual group totals divided by the number of pigeons in each group, $S(T^2/n)$. The correction term for the data in table XLI is the summation of the totals squared divided by the total number of pigeons in the six groups or

$(5183.5)^2/96 = 279882.00$. $S(T^2/n) = 280839.70$, where $n = 16$, and the sum of squares between groups $= 280839.70 - 279882.00 = 957.70$. There are six groups of observation, five degrees of freedom, and the mean square (variance) is equal to $957.70/5 = 191.54$. The total sum of squares is calculated by subtracting the correction term from the summation of the squares of the ninety-six observations, Sx^2 . This is expressed algebraically by $Sx^2 - (ST)^2/n$, and is equal to $283711.75 - 279882.00 = 3829.75$.

The sum of the squares within groups (error) is equal to the summation of the sum of the squares of the deviations from the mean, $SS(x - \bar{x})^2$ and is equal to 2872.05. This is of course also equal to the total sum of squares minus the sum of squares between groups, since $T/n = \bar{x}$ and $S(x - \bar{x})^2 = Sx^2 - \bar{x}Sx$. The total number of degrees of freedom is 95, five of which have been used in the calculation of the mean square between groups; the remainder representing the degrees of freedom within groups. The mean square within groups is $2872.05/90 = 31.91$.

The variance ratio (F) is the quotient of the sum of squares between groups divided by the sum of squares within groups or $191.54/31.91 = 6.00$. The theoretical value of F (102) is obtained from a table of variance ratios. A significance level of $P = 0.05$ is used to determine whether a significant difference exists between the mean lethal doses obtained by testing the various concentrations of the digitalis sample. The table value of F for this 5% point is 2.33, and since

the calculated F is greater than this number it is concluded that there is a significant difference between the values of the means. This conclusion will be wrong once in twenty times because this is the probability of its occurring by chance.

The analysis of variance for the data of digitalis and digitoxin (table XLIV and XLV) show that there is a difference between the means. The analysis of variance for the data of ouabain (table XLVI) shows no such difference between the means. The value of F in the latter case is 0.77 and a value of F less than 1.00 cannot be assumed significant for any level of probability.

From the results of the above statistical analysis there appears to be a difference between digitalis and digitoxin on the one hand and ouabain on the other in regard to the effect of the rate of injection upon the mean lethal dose. Even in the case of digitalis and digitoxin, however, there appears to be no difference in the mean lethal doses within a range of 13.2 - 35.4 doses for the former and 16.8 - 23.9 for the latter (tables XXXIX and XL). The difference which exists may be due to the difference in the rate of absorption or fixation of the drug in the heart tissue or to the "overshooting" phenomenon, present in any periodic injection procedure, or both.

The rapid injection of the cardiotoxic may result in the loss of a portion of the drug because of its fixation in organs other than the heart or as seems more likely, there

may exist a difference in the time of fixation and action of the drug upon the heart itself; this period is greatest with digitoxin and least with ouabain. Further studies should be made to determine the time-action curves for the cardiotonics using isolated mammalian hearts. The action upon the vagus nerve in the intact animal should not, however, be overlooked.

The results obtained in Part I indicate that "overshooting" is the most significant factor responsible for the increase in lethal dose with an increased rate of injection. Even though the time of assay was considerably reduced using the 10 dose initial method, the value of the mean lethal dose remained unaltered. Here "overshooting" was of the same approximate magnitude as in the U.S.P. assay, since the last 25% of the lethal dose was administered with the same rate of injection. It is apparent, however, that an increase in lethal dose with an increased rate of injection may in small part be the result of the inability of the drug to be fixed and thus exert its action upon the heart.

The results of Part I and Part II appear to be contradictory. In the case of the former there was no increase of mean lethal dose with a decrease in injection time. In Part II there was a significant increase at the more rapid rates of injection for digitoxin and digitalis but not for ouabain. Thus even within the results of Part II there is a contradiction. This contradiction has been observed before with methods in which rate of injection is varied by the

concentration of drug injected per unit time (pages 39 - 41) and is probably caused by the poor design of the experiment. The experimental design in Part I and Part II both appreciably reduce the time of injection but in two very dissimilar ways. However, the results of the method in Part I, where a non-uniform rate of injection is used, are comparable to results obtained by the U.S.P. method; whereas the results of the method in Part II, where a uniform rate of injection was employed, do not.

An examination of table XXXIX reveals that there is a significant difference between the mean lethal dose as determined at the slowest and fastest rates of injection for digitalis and digitoxin. Table XL shows that at the largest concentration of digitalis (10.0 mg./cc.) the total injection time was 24.4 minutes and at the largest concentration of digitoxin (0.08 mg./cc.) the total injection time was 36.3 minutes. The largest concentration, of course, produced the most rapid rate of injection. Table XXIV reveals no significant differences between the results of the U.S.P. and the initial 10 dose methods. Table XXIII shows that the same digitalis sample (30-C), also tested by the procedure of Part II, was injected within 31.4 minutes by the 10 dose method and other digitalis samples in 25.8, 22.3, 22.5, 47.5 and 25.8 minutes. Table XXIII also shows that the same digitoxin sample (Digitoxin Reference Standard), tested by the procedure of Part II, was injected in 33.3 minutes. The time of injection was only 23.3 minutes for digitoxin tablets,

9M-27C.

Decreasing the injection time does not cause an increase in the mean lethal dose of cardiotoxic for pigeons. Even though the shorter methods of injection may be influenced by the ability of the pigeon heart to absorb and fix the drug, this influence does not cause an increase in mean lethal dose as measured by the U.S.P. method.

The method of determining the lethal dose in Part II is similar to the U.S.P. method in that a constant rate of injection (1 cc./kg.) is maintained throughout the experiment. The method employed in Part I is unlike the U.S.P. method because a non-uniform rate of injection was used. The reason for the contradictory results appears to lie elsewhere. Using a 10 dose initial method, little precision is lost, because the last injections are administered in the same manner as the official method and "overshooting" is of the same magnitude in both instances.

It is certainly apparent that the design of the experiments in Part I and Part II differ. This difference in design accounts for the discrepancy between the values of the mean lethal doses at the shorter injection times. A constant rate of injection is not the essential factor in comparing mean lethal doses obtained with different injection periods. In this case the rate of injection is varied by varying the concentration injected per unit time with a definite loss in precision because of the intermittent injection procedure.

That the design of the experiment in Part II is a poor

one is borne out in two ways. First the mean lethal doses obtained are not comparable because of the great variation in the amount of "overshooting" and secondly there are contradictions between experiments performed in the same manner. In regard to the latter a contradiction exists in Part II of this work, wherein results with digitalis and digitoxin differ from those of ouabain. Discrepancies found by other workers using this method are noted in pages 39 - 41. The first reason explains why the results of this method should not be compared with those of the official U.S.P. method; i.e., results which are not obtained with an equal loss in precision cannot be compared.

The use of the periodic injection method of the U.S.P. is certainly not without its failings. A continuous injection method to increase the accuracy of the experiment would certainly be an improvement. The purpose of this work is, however, to shorten the time of the official assay and the non-uniform rate of injection method does this without a loss in precision. This fact has been demonstrated by the values of the mean lethal doses obtained.

CONCLUSIONS

1. A significant difference was demonstrated between the mean lethal doses, determined by the intravenous pigeon method, of:
 - a. six concentrations of a sample of digitalis leaf.
 - b. four concentrations of a digitoxin sample.
2. A significant difference was not demonstrated between the mean lethal doses of four concentrations of a ouabain sample tested intravenously in pigeons.
3. There was an increase in mean lethal dose with an increase in the rate of injection of digitalis and digitoxin but not of ouabain.
4. There was no apparent increase in the mean lethal dose of digitalis within a range of 13.2 - 35.4 doses; i.e., 60.9 - 172.2 minutes.
5. The "overshooting" factor, present in a periodic injection procedure, is the chief cause of the increase of mean lethal dose with an increase in the rate of injection.

SUMMARY

A method has been described to shorten the time of the U.S.P. intravenous pigeon assay for cardiotonics. This method involves the use of a non-uniform rate of injection, in that an initial dose is administered equivalent to ten doses of one cc./kg. each. The remainder of the drug is given in increments of one cc. per kg. of pigeon. Comparisons have been made between this and the U.S.P. method with samples of digitalis, digitoxin and ouabain. Dilutions were so prepared that the mean lethal dose fell between 13 and 19 doses if tested by the U.S.P. method. No significant difference between the mean values obtained by the two procedures could be ascertained by a t test.

The relationship between lethal dose and rate of injection has been observed with a sample of digitalis leaf, Digitoxin Reference Standard and Ouabain Reference Standard. Six concentrations of digitalis and four concentrations of digitoxin and ouabain were used to vary the rate of injection. An analysis of variance showed a significant increase in mean lethal dose at the higher concentrations of digitalis and digitoxin. This increase was not demonstrated with ouabain. The lack of precision associated with a periodic injection procedure, resulting in considerable "overshooting", is proposed as the chief cause of the increase in mean lethal dose.

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TABLE I

Summary of Pigeon Data

Sample: U.S.P. DIGITALIS REFERENCE STANDARD, MACERATION NO. 2

Preparation of sample: One gm. in 10 cc. U.S.P. menstruum.

Injection: One cc./kg. every five minutes until death
(U.S.P. method).

Pigeon number	Date	Sex	Body wt. in kg.	Dilution cc./100 cc.	No. of doses	L.D. (mg./kg.)	Time in minutes
1.	2/23/50	F	0.347	6.0	13	72.0	60
2.	"	F	0.394	"	15	90.0	70
3.	"	M	0.430	"	15	90.0	70
4.	"	M	0.438	"	11	66.0	50
5.	"	M	0.398	"	13	78.0	60
6.	2/28/50	M	0.448	"	15	90.0	70
7.	3/11/50	M	0.370	"	15	90.0	70
8.	"	M	0.552	"	17	102.0	80
9.	"	F	0.350	"	12	72.0	55
10.	"	M	0.330	"	17	102.0	80
11.	"	F	0.312	"	16	96.0	75
12.	"	F	0.336	"	13	78.0	60
13.	3/18/50	F	0.316	"	13	78.0	60
14.	"	M	0.380	"	14	84.0	65
15.	"	M	0.400	"	13	78.0	60
<u>Mean</u>			0.373		14.1	84.8	65.7
<u>Standard Deviation</u> (σ)			0.044		1.8	10.6	
<u>Standard Error</u> (ϵ)			0.011		0.5	2.7	

TABLE II

Summary of Pigeon Data

Sample: U.S.P. DIGITALIS REFERENCE STANDARD, MACERATION NO. 2

Preparation of sample: One gm. in 10 cc. U.S.P. menstruum.

Injection: Ten cc./kg. at once; then 1 cc./kg. every five minutes thereafter until death (10 dose initial method).

Pigeon number	Date	Sex	Body wt. in kg.	Dilution cc./100 cc.	No. of doses	L.D. (mg./kg.)	Time in minutes
1.	2/25/50	M	0.495	6.0	14	84.0	20
2.	"	F	0.342	"	15	90.0	25
3.	"	F	0.438	"	13	78.0	15
4.	3/8/50	M	0.416	"	15	90.0	25
5.	"	F	0.400	"	15	90.0	25
6.	"	M	0.404	"	15	90.0	25
7.	"	M	0.468	"	14	84.0	20
8.	"	F	0.302	"	13	78.0	15
9.	"	M	0.356	"	12	72.0	10
10.	3/16/50	M	0.308	"	15	90.0	25
11.	"	F	0.364	"	17	102.0	35
12.	"	M	0.362	"	15	90.0	25
13.	"	F	0.358	"	15	90.0	25
14.	"	F	0.354	"	17	102.0	35
15.	"	F	0.400	"	15	90.0	25
Mean			0.383		14.7	88.0	23.3
Standard Deviation	(σ)		0.056		1.3	8.1	
Standard Error	(ϵ)		0.014		0.3	2.1	

TABLE III

Summary of Pigeon Data

Sample: U.S.P. DIGITALIS REFERENCE STANDARD, MACERATIONS NO. 3 & 4

Preparation of sample: One gm. in 10 cc. U.S.P. menstruum.

Injection: One cc./kg. every five minutes until death
(U.S.P. method).

Pigeon number	Date	Sex	Body wt. in kg.	Dilution cc/100 cc.	No. of doses	L.D. (mg./kg.)	Time in minutes
1.	4/11/50	F	0.260	6.0	16	96.0	75
2.	"	F	0.272	"	18	108.0	85
3.	"	F	0.438	"	15	90.0	70
4.	"	F	0.398	"	14	84.0	65
5.	"	F	0.340	"	16	96.0	75
6.	"	M	0.482	"	16	96.0	75
Mean			0.365		15.8	95.0	74.2
Standard Deviation	(σ)		0.090		1.3	8.0	
Standard Error	(E)		0.037		0.5	3.3	

TABLE IV

Summary of Pigeon Data

Sample: U.S.P. DIGITALIS REFERENCE STANDARD, MACERATIONS NO. 3 & 4.

Preparation of sample: One gm. in 10 cc. U.S.P. menstruum.

Injection: Ten cc./kg. at once; then 1 cc./kg. every five minutes thereafter until death (10 dose initial method).

Pigeon number	Date	Sex	Body wt. in kg.	Dilution cc./100 cc.	No. of doses	L.D. (mg./kg.)	Time in minutes
1.	4/12/50	F	0.294	6.0	17	102.0	35
2.	"	M	0.430	"	15	90.0	25
3.	"	M	0.418	"	14	84.0	20
4.	"	F	0.314	"	18	108.0	40
5.	"	F	0.350	"	14	84.0	20
6.	"	M	0.398	"	13	78.0	15
Mean			0.367		15.2	91.0	25.8
Standard Deviation (σ)			0.057		1.9	11.6	
Standard Error (ϵ)			0.023		0.8	4.7	

TABLE V

Summary of Pigeon Data

Sample: TINCTURE OF DIGITALIS, 9M-129.Preparation of sample: None.Injection: One cc./kg. every five minutes until death
(U.S.P. method).

Pigeon number	Date	Sex	Body wt. in kg.	Dilution cc./100 cc.	No. of doses	L.D. (cc./kg.)	Time in minutes
1.	9/3/49		0.413	5.0	13	0.65	60
2.	"		0.288	"	16	0.80	75
3.	"		0.378	"	13	0.65	60
4.	9/7/49		0.332	"	16	0.80	75
5.	"		0.410	"	13	0.65	60
6.	"		0.418	"	15	0.75	70
7.	"		0.370	"	12	0.60	55
8.	10/5/49	F	0.306	"	14	0.70	65
9.	"	M	0.438	"	16	0.80	75
10.	10/8/49		0.324	"	18	0.90	85
11.	"		0.368	"	15	0.75	70
12.	"		0.400	"	14	0.70	65
13.	10/12/49		0.404	"	14	0.70	65
14.	"		0.390	"	14	0.70	65
15.	"		0.440	"	12	0.60	55
<u>Mean</u>			0.379		14.3	0.72	66.7
<u>Standard Deviation (σ)</u>			0.047		1.7	0.08	
<u>Standard Error (ϵ)</u>			0.012		0.4	0.02	

TABLE VI

Summary of Pigeon Data

Sample: TINCTURE OF DIGITALIS, 9M-129.

Preparation of sample: None.

Injection: Five cc./kg. at once; then 1 cc./kg. every five minutes thereafter until death (5 dose initial method).

Pigeon number	Date	Sex	Body wt. in kg.	Dilution cc./100 cc.	No. of doses	L.D. (cc./kg.)	Time in minutes
1.	10/15/49		0.344	5.0	15	0.75	50
2.	"		0.310	"	16	0.80	55
3.	"		0.500	"	16	0.80	55
4.	"		0.380	"	17	0.85	60
5.	"		0.410	"	12	0.60	35
6.	"		0.412	"	14	0.70	45
7.	10/29/49	F	0.418	"	13	0.65	40
8.	"	F	0.307	"	13	0.65	40
9.	"	M	0.452	"	12	0.60	35
10.	"	F	0.383	"	13	0.65	40
11.	11/2/49	M	0.460	"	15	0.75	50
12.	"	M	0.444	"	14	0.70	45
13.	"	M	0.356	"	13	0.65	40
14.	"	F	0.473	"	15	0.75	50
15.	"	F	0.332	"	12	0.60	35
Mean			0.385		14.0	0.70	45.0
Standard Deviation	(σ)		0.058		1.6	0.08	
Standard Error	(ϵ)		0.015		0.4	0.02	

TABLE VII

Summary of Pigeon Data

Sample: TINCTURE OF DIGITALIS, 9M-129.

Preparation of sample: None.

Injection: Ten cc./kg. at once; then 1 cc./kg. every five minutes thereafter until death (10 dose initial method).

Pigeon number	Date	Sex	Body wt. in kg.	Dilution cc./100 cc.	No. of doses	L.D. (cc./kg.)	Time in minutes
1.	10/1/49		0.319	5.0	13	0.65	15
2.	"		0.426	"	15	0.75	25
3.	"		0.452	"	14	0.70	20
4.	"		0.446	"	14	0.70	20
5.	"		0.468	"	14	0.70	20
6.	"		0.362	"	14	0.70	20
7.	10/5/49		0.476	"	14	0.70	20
8.	"		0.315	"	15	0.75	25
9.	"		0.446	"	15	0.75	25
10.	10/8/49		0.408	"	14	0.70	20
11.	"		0.456	"	13	0.65	15
12.	"		0.370	"	15	0.75	25
13.	10/12/49		0.400	"	15	0.75	25
14.	"		0.422	"	17	0.85	35
15.	"		0.404	"	15	0.75	25
Mean			0.411		14.5	0.72	22.3
Standard Deviation (σ)			0.051		1.0	0.05	
Standard Error (ϵ)			0.013		0.3	0.01	

TABLE VIII

Summary of Pigeon Data

Sample: TINCTURE OF DIGITALIS, M-113.

Preparation of sample: None.

Injection: One cc./kg. every five minutes until death
(U.S.P. method).

Pigeon number	Date	Sex	Body wt. in kg.	Dilution cc./100 cc.	No. of doses	L.D. (cc./kg.)	Time in minutes
1.	9/8/50	M	0.394	5.0	15	0.75	70
2.	"	M	0.342	"	15	0.75	70
3.	"	F	0.250	"	16	0.80	75
4.	"	M	0.260	"	14	0.70	65
5.	"	M	0.420	"	15	0.75	70
6.	"	F	0.312	"	18	0.90	85
Mean			0.330		15.5	0.78	72.5
Standard Deviation (σ)			0.069		1.4	0.07	
Standard Error (ϵ)			0.028		0.6	0.03	

TABLE IX

Summary of Pigeon Data

Sample: TINCTURE OF DIGITALIS, M-113.

Preparation of sample: None.

Injection: Ten cc./kg. at once; then 1 cc./kg. every five minutes thereafter until death (10 dose initial method).

Pigeon number	Date	Sex	Body wt. in kg.	Dilution cc./100 cc.	No. of doses	L.D. (cc./kg.)	Time in minutes
1.	9/8/50	M	0.394	5.0	15	0.75	25
2.	"	F	0.310	"	14	0.70	20
3.	"	F	0.284	"	16	0.80	30
4.	"	F	0.250	"	14	0.70	20
5.	"	F	0.418	"	15	0.75	25
6.	"	F	0.306	"	13	0.65	15
Mean			0.327		14.5	0.73	22.5
Standard Deviation (σ)			0.066		1.0	0.05	
Standard Error (ϵ)			0.027		0.4	0.02	

TABLE X

Summary of Pigeon Data

Sample: TINCTURE OF DIGITALIS, 69-C.

Preparation of sample: None.

Injection: One cc./kg. every five minutes until death
(U.S.P. method).

Pigeon number	Date	Sex	Body wt. in kg.	Dilution cc./100 cc.	No. of doses	L.D. (cc./kg.)	Time in minutes
1.	9/22/50	F	0.447	4.5	17	0.77	80
2.	"	F	0.290	"	26	1.17	125
3.	"	F	0.410	"	18	0.81	85
4.	"	F	0.318	"	22	0.99	105
5.	"	M	0.412	"	19	0.86	90
6.	"	F	0.386	"	18	0.81	85
Mean			0.377		20.0	0.90	95.0
Standard Deviation	(σ)		0.061		3.4	0.15	
Standard Error	(ϵ)		0.025		1.4	0.06	

TABLE XI

Summary of Pigeon Data

Sample: TINCTURE OF DIGITALIS, 69-C

Preparation of sample: None.

Injection: Ten cc./kg. at once; then 1 cc./kg. every five minutes thereafter until death (10 dose initial method).

Pigeon number	Date	Sex	Body wt. in kg.	Dilution cc./100 cc.	No. of doses	L.D. (cc./kg.)	Time in minutes
1.	9/22/50	M	0.430	4.5	22	0.99	60
2.	"	F	0.352	"	16	0.72	30
3.	"	F	0.380	"	21	0.95	55
4.	"	F	0.297	"	20	0.90	50
5.	"	M	0.434	"	19	0.86	45
6.	"	M	0.400	"	19	0.86	45
Mean			0.382		19.5	0.88	47.5
Standard Deviation	(σ)		0.052		2.1	0.09	
Standard Error	(ϵ)		0.021		0.9	0.04	

TABLE XII

Summary of Pigeon Data

Sample: DIGITALIS TABLETS (0.1 Gm.), 2G-C.

Preparation of sample: Fifty tablets in 50 cc. U.S.P. menstruum
(1 tab./cc.).

Injection: One cc./kg. every five minutes until death
(U.S.P. method).

Pigeon number	Date	Sex	Body wt. in kg.	Dilution cc./100 cc.	No. of doses	L.D. (tab./kg.)	Time in minutes
1.	3/30/50	M	0.408	6.0	16	0.96	75
2.	"	M	0.455	"	15	0.90	70
3.	"	M	0.262	"	16	0.96	75
4.	"	M	0.446	"	15	0.90	70
5.	4/1/50	M	0.348	"	16	0.96	75
6.	"	F	0.370	"	16	0.96	75
<u>Mean</u>			0.382		15.7	0.94	73.3
<u>Standard Deviation</u> (σ)			0.072		0.5	0.03	
<u>Standard Error</u> (ϵ)			0.029		0.2	0.01	

TABLE XIII

Summary of Pigeon Data

Sample: DIGITALIS TABLETS (0.1 Gm.), 28-C.

Preparation of sample: Fifty tablets in 50 cc. U.S.P. menstruum
(1 tab./cc.).

Injection: Ten cc./kg. at once; then 1 cc./kg. every five
minutes thereafter until death (10 dose initial method).

Pigeon number	Date	Sex	Body wt. in kg.	Dilution cc./100 cc.	No. of doses (tab./kg.)	L.D.	Time in minutes
1.	4/6/50	F	0.350	6.0	16	0.96	30
2.	"	M	0.298	"	18	1.08	30
3.	"	M	0.360	"	13	0.78	15
4.	"	F	0.392	"	16	0.96	30
5.	"	F	0.446	"	14	0.84	20
6.	"	F	0.350	"	14	0.84	20
Mean			0.363		15.2	0.91	25.8
Standard Deviation	(σ)		0.051		1.8	0.11	
Standard Error	(ϵ)		0.021		0.7	0.04	

TABLE XIV

Summary of Pigeon Data

Sample: DIGITALIS LEAVES, 30-C.

Preparation of sample: Five grams of powdered leaves in 50 cc.
U.S.P. menstruum.

Injection: One cc./kg. every five minutes until death
(U.S.P. method).

Pigeon number	Date	Sex	Body wt. in kg.	Dilution cc./100 cc.	No. of doses	L.D. (mg./kg.)	Time in minutes
1.	3/18/50	M	0.405	4.5	10	45.0	45
2.	"	M	0.354	3.0	15	45.0	70
3.	3/21/50	F	0.302	"	19	57.0	90
4.	"	M	0.446	"	15	45.0	70
5.	"	M	0.344	"	15	45.0	70
6.	"	F	0.408	"	17	51.0	80
7.	3/25/50	F	0.368	"	15	45.0	70
Mean			0.375		16.0*	48.0	75.0*
Standard Deviation (σ)			0.048		1.7	4.7	
Standard Error (ϵ)			0.018		0.7	1.8	

* Mean of no. 2-7.

TABLE XV

Summary of Pigeon Data

Sample: DIGITALIS LEAVES, 30-C.

Preparation of sample: Five grams of powdered leaves in 50 cc.
U.S.P. menstruum.

Injection: Ten cc./kg. at once; then 1 cc./kg. every five
minutes thereafter until death (10 dose initial method).

Pigeon number	Date	Sex	Body wt. in kg.	Dilution cc./100 cc.	No. of doses	L.D. (mg./kg.)	Time in minutes
1.	4/13/50	F	0.394	3.0	19	57.0	45
2.	"	M	0.410	"	14	42.0	20
3.	"	M	0.360	"	16	48.0	30
4.	"	M	0.540	"	17	51.0	35
5.	"	M	0.404	"	15	46.0	25
6.	"	M	0.392	"	17	51.0	35
7.	"	M	0.446	"	16	48.0	30
Mean			0.392		16.3	49.0	31.4
Standard Deviation	(σ)		0.035		1.6	4.8	
Standard Error	(ϵ)		0.013		0.6	1.8	

TABLE XVI

Summary of Pigeon Data

Sample: U.S.P. DIGITOXIN REFERENCE STANDARD, SOLUTION NO. 1.
Preparation of sample: One-half mg. dissolved in 1 cc. U.S.P. menstruum (1-2,000 solution).
Injection: One cc./kg. every five minutes until death (U.S.P. method).

Pigeon number	Date	Sex	Body wt. in kg.	Dilution cc./100 cc.	No. of doses	L.D. (mg./kg.)	Time in minutes
1.	2/14/50	F	0.334	5.0	16	0.40	75
2.	"	F	0.292	"	19	0.48	90
3.	"	M	0.352	"	19	0.48	90
4.	"	M	0.374	"	21	0.53	100
5.	"	F	0.406	"	23	0.58	110
6.	"	M	0.372	"	19	0.48	90
7.	2/15/50	F	0.464	8.0	15	0.45	70
8.	"	F	0.292	"	19	0.57	90
9.	"	M	0.340	"	17	0.51	80
10.	"	M	0.330	"	17	0.51	80
11.	"	F	0.286	"	19	0.57	90
12.	"	M	0.496	"	16	0.48	75
13.	2/16/50	M	0.355	"	19	0.57	90
14.	"	M	0.322	"	20	0.60	95
15.	"	M	0.273	"	20	0.60	95
Mean			0.353		18.0*	0.52	85.0*
Standard Deviation (σ)			0.063		0.6	0.06	
Standard Error (ϵ)			0.016		0.2	0.02	

* Mean of no. 7-15.

TABLE XVII

Summary of Pigeon Data

Sample: U.S.P. DIGITOXIN REFERENCE STANDARD, SOLUTION NO. 1.
Preparation of sample: One-half mg. dissolved in 1 cc. U.S.P. menstruum (1-2,000 solution).
Injection: Ten cc./kg. at once; then 1 cc./kg. every five minutes thereafter until death (10 dose initial method).

Pigeon number	Date	Sex	Body wt. in kg.	Dilution cc./100 cc.	No. of doses	L.D. (mg./kg.)	Time in minutes
1.	2/16/50	F	0.283	6.0	18	0.54	40
2.	"	F	0.334	"	14	0.42	20
3.	"	M	0.430	"	15	0.45	25
4.	2/18/50	F	0.342	"	17	0.51	35
5.	"	F	0.300	"	16	0.48	30
6.	"	F	0.335	"	20	0.60	50
7.	"	F	0.292	"	17	0.51	35
8.	"	F	0.314	"	14	0.42	20
9.	"	F	0.262	"	18	0.54	40
10.	2/21/50	F	0.390	"	19	0.57	45
11.	"	M	0.398	"	15	0.45	25
12.	"	M	0.464	"	18	0.54	40
13.	"	M	0.380	"	18	0.54	40
14.	"	M	0.447	"	15	0.45	25
15.	"	F	0.370	"	16	0.48	30
Mean			0.356		16.7	0.50	33.3
Standard Deviation (σ)			0.062		1.8	0.05	
Standard Error (ϵ)			0.016		0.5	0.01	

TABLE XVIII

Summary of Pigeon Data

Sample: DIGITOXIN TABLETS (0.1 mg.), 9M-27C.

Preparation of sample: One tablet in 10 cc. U.S.P. menstruum.*

Injection: One cc./kg. every five minutes until death
(U.S.P. method).

Pigeon number	Date	Sex	Body wt. in kg.	Dilution cc./100 cc.	No. of doses	L.D. (tab./kg.)	Time in minutes
1.	9/3/49		0.360	10.0	11	0.22	50
2.	"		0.370	"	15	0.30	70
3.	11/5/49	M	0.462	20.0	16	0.32	75
4.	"	M	0.438	"	22	0.44	105
5.	"	F	0.380	"	24	0.48	115
6.	"	M	0.415	"	23	0.46	110
7.	11/12/49	F	0.445	26.0	18	0.47	85
8.	"	M	0.424	"	12	0.31	55
9.	"	M	0.376	"	16	0.42	75
10.	"	F	0.382	"	17	0.44	80
11.	"	M	0.390	"	17	0.44	80
12.	"	F	0.380	"	15	0.39	70
13.	11/23/49	F	0.392	"	16	0.42	75
14.	"	F	0.320	"	19	0.49	90
15.	"	M	0.490	"	15	0.39	70
16.	"	F	0.402	"	15	0.39	70
17.	"	F	0.362	"	16	0.42	75
18.	"	M	0.346	"	16	0.42	75
Mean			0.397		16.0**	0.40	75.0**
Standard Deviation (σ)			0.042		1.8	0.07	
Standard Error (ϵ)			0.010		0.5	0.02	

* Sample for no. 1 and 2 was prepared by macerating one tablet in 5 cc. U.S.P. menstruum (1-5,000 solution).

** Mean of no. 7-18.

TABLE XIX

Summary of Pigeon Data

Sample: DIGITOXIN TABLETS (0.1 mg.), 9M-27C.

Preparation of sample: One tablet in 10 cc. U.S.P. menstruum.

Injection: Ten cc./kg. at once; then 1 cc./kg. every five minutes thereafter until death (10 dose initial method).

Pigeon number	Date	Sex	Body wt. in kg.	Dilution cc./100 cc.	No. of doses	L.D. (tab./kg.)	Time in minutes
1.	11/26/49	M	0.394	26.0	17	0.44	35
2.	"	M	0.428	"	12	0.31	10
3.	"	M	0.432	"	12	0.31	10
4.	"	F	0.375	"	13	0.34	15
5.	"	M	0.414	"	11	0.29	5
6.	"	F	0.304	"	19	0.49	45
7.	12/15/49	M	0.347	"	18	0.47	40
8.	"	M	0.353	"	17	0.44	35
9.	"	M	0.383	"	14	0.36	20
10.	"	M	0.410	"	18	0.47	40
11.	"	M	0.410	"	10	0.26	0
12.	"	F	0.346	"	10	0.26	0
13.	"	M	0.344	"	17	0.44	35
14.	12/17/49	F	0.460	"	10	0.26	0
15.	"	M	0.316	"	19	0.49	45
16.	"	M	0.356	"	16	0.42	30
17.	"	F	0.348	"	17	0.44	35
18.	"	F	0.408	"	15	0.39	25
19.	"	F	0.390	"	16	0.42	30
20.	"	F	0.370	"	12	0.31	10
Mean			0.379		14.7	0.38	23.3
Standard Deviation (σ)			0.040		3.2	0.08	
Standard Error (ϵ)			0.009		0.7	0.02	

TABLE XX

Summary of Pigeon Data

Sample: DIGITOXIN TABLETS (0.1 mg.), 9M-27C.

Preparation of sample: One tablet in 10 cc. U.S.P. menstruum.

Injection: Seven cc./kg. at once; then 1 cc./kg. every five minutes thereafter until death (7 dose initial method).

Pigeon number	Date	Sex	Body wt. in kg.	Dilution cc./100 cc.	No. of doses	L.D. (tab./kg.)	Time in minutes
1.	12/23/49	F	0.376	26.0	16	0.42	45
2.	"	F	0.360	"	16	0.42	45
3.	"	F	0.303	"	16	0.42	45
4.	"	F	0.322	"	18	0.47	55
5.	"	F	0.440	"	17	0.44	50
6.	"	F	0.413	"	12	0.31	25
7.	1/7/50	F	0.390	"	15	0.39	40
8.	"	F	0.380	"	13	0.34	30
9.	"	F	0.321	"	17	0.44	50
10.	"	M	0.440	"	15	0.39	40
11.	"	F	0.458	"	16	0.42	45
12.	"	M	0.330	"	16	0.42	45
13.	1/11/50	F	0.340	"	18	0.47	55
14.	"	M	0.416	"	16	0.42	45
15.	"	F	0.400	"	14	0.36	35
16.	"	M	0.420	"	12	0.31	25
17.	"	M	0.464	"	13	0.34	30
18.	"	F	0.272	"	18	0.47	55
Mean			0.380		15.4	0.40	42.2
Standard Deviation (σ)			0.057		1.9	0.05	
Standard Error (E)			0.013		0.4	0.01	

TABLE XXI

Summary of Pigeon Data

Sample: U.S.P. QUABAIN REFERENCE STANDARD, SOLUTION NO. 1.

Preparation of sample: One mg. dissolved in 1 cc. U.S.P. menstruum (1-1,000 solution).

Injection: One cc./kg. every five minutes until death (U.S.P. method).

Pigeon number	Date	Sex	Body wt. in kg.	Dilution cc./100 cc.	No. of doses	L.D. (mg./kg.)	Time in minutes
1.	2/2/50	M	0.373	0.9	14	0.126	65
2.	"	M	0.330	"	15	0.135	70
3.	"	F	0.286	"	14	0.126	65
4.	"	F	0.310	"	17	0.153	80
5.	"	M	0.292	"	16	0.144	75
6.	"	M	0.359	"	18	0.162	85
7.	2/4/50	M	0.290	"	16	0.144	75
8.	"	M	0.384	"	16	0.144	75
9.	"	F	0.335	"	16	0.144	75
10.	"	M	0.340	"	15	0.135	70
11.	"	M	0.386	"	18	0.162	85
12.	"	F	0.314	"	17	0.153	80
13.	2/7/50	F	0.369	"	20	0.180	95
14.	"	F	0.400	"	13	0.117	60
15.	"	F	0.254	"	20	0.180	95
<u>Mean</u>			0.335		16.3	0.147	76.7
<u>Standard Deviation</u> (σ)			0.044		2.1	0.019	
<u>Standard Error</u> (ϵ)			0.011		0.5	0.005	

TABLE XXII

Summary of Pigeon Data

Sample: U.S.P. QUABAIN REFERENCE STANDARD, SOLUTION NO. 1.

Preparation of sample: One mg. dissolved in 1 cc. U.S.P. menstruum (1-1,000 solution).

Injection: Ten cc./kg. at once; then 1 cc./kg. every five minutes thereafter until death (10 dose initial method).

Pigeon number	Date	Sex	Body wt. in kg.	Dilution cc./100 cc.	No. of doses	L.D. (mg./kg.)	Time in minutes
1.	2/7/50	M	0.336	0.9	16	0.144	30
2.	"	M	0.336	"	17	0.153	35
3.	"	F	0.340	"	17	0.153	35
4.	2/8/50	F	0.300	"	17	0.153	35
5.	"	F	0.235	"	19	0.171	45
6.	"	F	0.254	"	22	0.198	60
7.	"	M	0.288	"	20	0.180	50
8.	"	M	0.270	"	19	0.171	45
9.	"	M	0.376	"	17	0.153	35
10.	"	M	0.276	"	16	0.144	30
11.	"	M	0.350	"	18	0.162	40
12.	2/11/50	F	0.384	"	14	0.126	20
13.	"	M	0.344	"	17	0.153	35
14.	"	M	0.306	"	15	0.135	25
15.	"	M	0.286	"	16	0.144	30
Mean			0.312		17.3	0.156	36.7
Standard Deviation	(σ)		0.045		2.0	0.018	
Standard Error	(ϵ)		0.012		0.5	0.005	

TABLE XXIII

A Comparison of the Mean Lethal Dose
and Mean Injection Time

Drug	Method	M.L.D.*	Mean time in minutes
Digitalis Ref. Std., Maceration No. 2	U.S.P.	84.8	65.7
" " " " " "	10 dose	88.0	23.3
" " " " " "			
No. 3 & 4	U.S.P.	88.0	74.2
" " " " " "			
No. 3 & 4	10 dose	91.0	25.8
Tct. Digitalis, 9M-129	U.S.P.	0.72	66.7
" " " " " "	5 dose	0.70	45.0
" " " " " "	10 dose	0.72	22.3
" " " M-113	U.S.P.	0.78	72.5
" " " " " "	10 dose	0.73	22.5
" " " 69-C	U.S.P.	0.90	95.0
" " " " " "	10 dose	0.88	47.5
Digitalis Tablets, 26-C	U.S.P.	0.94	73.3
" " " " " "	10 dose	0.91	25.8
Digitalis Leaves, 30-C	U.S.P.	48.0	75.0
" " " " " "	10 dose	49.0	31.4
Digitoxin Ref. Std., Solution No. 1	U.S.P.	0.52	85.0
" " " " " "	10 dose	0.50	33.3
Digitoxin Tablets, 9M-27C	U.S.P.	0.40	75.0
" " " " " "	10 dose	0.38	23.3
" " " " " "	7 dose	0.40	42.2
Ouabain Ref. Std., Solution No. 1	U.S.P.	0.147	76.7
" " " " " "	10 dose	0.156	36.7

*Mean lethal dose expressed as mg./kg. for powder or leaf,
cc./kg. for tincture and tablets per kg. for tablets.

TABLE XXIV

A Comparison of Means of U.S.P. and n Dose Initial
Methods by t Test ($F = 0.05$)

Drug	n*	d.f.**	t (calc.)	t (theoret.)
Digitalis Ref. Std., Maceration No. 2	10	28	0.94	2.048
Digitalis Ref. Std., Macerations No. 3 & 4	10	10	0.70	2.228
Tct. Digitalis, 9M-129	5	28	0.67	2.048
Tct. Digitalis, 9M-129	10	28	0.00	2.048
Tct. Digitalis, M-113	10	10	1.25	2.228
Tct. Digitalis, 69-C	10	10	0.29	2.228
Digitalis Tab., 26-C	10	10	0.75	2.228
Digitalis Leaves, 30-C	10	12	0.40	2.179
Digitoxin Ref. Std., Solution No. 1	10	28	1.00	2.048
Digitoxin Tab., 9M-27C	10	36	0.67	2.029
Digitoxin Tab., 9M-27C	7	34	0.00	2.034
Ouabain Ref. Std., Solution No. 1	10	28	1.29	2.048

* Initial number of doses in modified method.

**Degrees of freedom.

TABLE XXV

Summary of Pigeon Data

Sample: DIGITALIS LEAVES, 30-C.

Concentration of injection fluid: 1.5 mg./cc.

Pigeon number	Date	Sex	Body wt. in kg.	No. of doses	L.D. (mg./kg.)	Time in minutes
1.	4/26/50	M	0.352	39	51.5	190
2.	"	F	0.340	37	55.5	180
3.	4/29/50	M	0.312	37	55.5	180
4.	"	M	0.310	38	57.0	185
5.	5/3/50	M	0.376	38	57.0	185
6.	"	M	0.394	31	46.5	150
7.	5/10/50	M	0.435	37	55.5	180
8.	"	M	0.590	27	40.5	130
9.	5/13/50	F	0.354	35	52.5	170
10.	"	M	0.350	35	52.5	170
11.	5/17/50	F	0.370	32	48.0	155
12.	"	F	0.384	35	52.5	170
13.	5/20/50	F	0.318	35	52.5	170
14.	"	F	0.338	40	60.0	195
15.	5/27/50	F	0.390	32	48.0	155
16.	"	F	0.380	39	58.5	190
Mean			0.362	35.4	53.2	172.2
Standard Deviation (σ)			0.034	3.5	5.2	
Standard Error (ϵ)			0.009	0.9	1.3	

TABLE XXVI

Summary of Pigeon Data

Sample: DIGITALIS LEAVES, 30-C.

Concentration of injection fluid: 2.0 mg./cc.

Pigeon number	Date	Sex	Body wt. in kg.	No. of doses	L.D. (mg./kg.)	Time in minutes
1.	4/28/50	F	0.378	22	44.0	105
2.	"	F	0.334	23	46.0	110
3.	4/29/50	M	0.292	23	56.0	135
4.	"	M	0.272	25	50.0	120
5.	5/3/50	M	0.372	25	50.0	120
6.	"	M	0.410	27	54.0	130
7.	5/10/50	F	0.398	26	52.0	125
8.	"	F	0.390	25	50.0	120
9.	5/13/50	M	0.302	22	44.0	105
10.	"	M	0.318	25	50.0	120
11.	5/17/50	M	0.386	24	48.0	115
12.	"	M	0.366	25	50.0	120
13.	5/20/50	M	0.300	30	60.0	145
14.	"	M	0.302	34	68.0	165
15.	5/27/50	M	0.360	22	44.0	105
16.	"	M	0.380	26	52.0	125
Mean			0.348	25.6	51.1	122.8
Standard Deviation (σ)			0.044	3.1	6.3	
Standard Error (ϵ)			0.011	0.8	1.6	

TABLE XXVII

Summary of Pigeon Data

Sample: DIGITALIS LEAVES, 30-C.

Concentration of injection fluid: 3.0 mg./cc.

Pigeon number	Date	Sex	Body wt. in kg.	No. of doses	L.D. (mg./kg.)	Time in minutes
1.	4/26/50	M	0.370	15	45.0	70
2.	"	F	0.350	17	51.0	80
3.	4/29/50	M	0.316	17	51.0	80
4.	"	F	0.260	16	48.0	75
5.	5/3/50	F	0.384	16	48.0	75
6.	"	F	0.372	16	48.0	75
7.	5/10/50	M	0.405	14	42.0	65
8.	"	M	0.378	16	48.0	75
9.	5/13/50	F	0.292	18	54.0	85
10.	"	F	0.330	18	54.0	85
11.	5/17/50	F	0.350	20	60.0	95
12.	"	M	0.386	15	45.0	70
13.	5/20/50	M	0.312	19	57.0	90
14.	"	M	0.308	19	57.0	90
15.	5/27/50	M	0.378	17	51.0	80
16.	"	M	0.368	16	48.0	75
Mean			0.347	16.8	50.4	79.1
Standard Deviation (σ)			0.040	1.6	4.9	
Standard Error (ϵ)			0.010	0.4	1.2	

TABLE XXVIII

Summary of Pigeon Data

Sample: DIGITALIS LEAVES, 30-C.

Concentration of injection fluid: 4.0 mg./cc.

Pigeon number	Date	Sex	Body wt. in kg.	No. of doses	L.D. (mg./kg.)	Time in minutes
1.	4/26/50	M	0.334	14	56.0	65
2.	"	M	0.354	13	52.0	60
3.	4/29/50	F	0.240	15	60.0	70
4.	"	F	0.270	15	60.0	70
5.	5/3/50	M	0.380	12	48.0	55
6.	"	F	0.360	14	56.0	65
7.	5/10/50	M	0.420	11	44.0	50
8.	"	M	0.381	12	48.0	55
9.	5/13/50	F	0.290	14	56.0	65
10.	"	F	0.340	13	52.0	60
11.	5/17/50	M	0.400	12	48.0	55
12.	"	F	0.370	15	60.0	70
13.	5/20/50	M	0.328	11	44.0	50
14.	"	F	0.316	12	48.0	55
15.	5/27/50	M	0.408	13	52.0	60
16.	"	M	0.406	15	60.0	70
Mean			0.350	13.2	52.8	60.9
Standard Deviation (σ)			0.052	1.4	5.7	
Standard Error (E)			0.013	0.4	1.4	

TABLE XXIX

Summary of Pigeon Data

Sample: DIGITALIS LEAVES, 30-C.Concentration of injection fluid: 7.0 mg./cc.

Pigeon number	Date	Sex	Body wt. in kg.	No. of doses	L.D. (mg./kg.)	Time in minutes
1.	4/26/50	M	0.376	9	63.0	40
2.	"	M	0.340	8	56.0	35
3.	4/29/50	F	0.258	9	63.0	40
4.	"	F	0.330	8	56.0	35
5.	5/3/50	M	0.396	8	56.0	35
6.	"	F	0.358	8	56.0	35
7.	5/10/50	M	0.446	7	49.0	30
8.	"	F	0.432	8	56.0	35
9.	5/13/50	F	0.318	9	63.0	40
10.	"	M	0.354	8	56.0	35
11.	5/17/50	M	0.390	8	56.0	35
12.	"	F	0.370	8	56.0	35
13.	5/20/50	M	0.318	9	63.0	40
14.	"	F	0.324	9	63.0	40
15.	5/27/50	M	0.372	8	56.0	35
16.	"	F	0.368	8	56.0	35
Mean			0.359	8.3	57.8	36.3
Standard Deviation	(σ)		0.046	0.6	4.0	
Standard Error	(ϵ)		0.012	0.2	1.0	

TABLE XXX

Summary of Pigeon Data

Sample: DIGITALIS LEAVES, 30-C.Concentration of injection fluid: 10.0 mg./cc.

Pigeon number	Date	Sex	Body wt. in kg.	No. of doses	L.D. (mg./kg.)	Time in minutes
1.	4/26/50	F	0.346	6	60.0	25
2.	"	F	0.388	5	50.0	20
3.	4/29/50	M	0.264	6	60.0	25
4.	"	F	0.256	7	70.0	30
5.	5/3/50	F	0.410	5	50.0	20
6.	"	M	0.394	6	60.0	25
7.	5/10/50	M	0.428	6	60.0	25
8.	"	M	0.405	6	60.0	25
9.	5/13/50	M	0.362	5	50.0	20
10.	"	F	0.340	6	60.0	25
11.	5/17/50	M	0.372	5	50.0	20
12.	"	M	0.364	5	50.0	20
13.	5/20/50	F	0.314	6	60.0	25
14.	"	F	0.300	7	70.0	30
15.	5/27/50	M	0.390	7	70.0	30
16.	"	M	0.400	6	60.0	25
Mean			0.358	5.9	58.8	24.4
Standard Deviation (σ)			0.052	0.5	7.2	
Standard Error (ϵ)			0.013	0.1	1.8	

TABLE XXXI

Summary of Pigeon Data

Sample: U.S.P. DIGITOXIN REFERENCE STANDARD.

Concentration of injection fluid: 0.02 mg./cc.

Pigeon number	Date	Sex	Body wt. in kg.	No. of doses	L.D. (mg./kg.)	Time in minutes
1.	7/28/50	F	0.390	23	0.46	110
2.	"	M	0.316	25	0.50	120
3.	"	M	0.345	21	0.42	100
4.	8/1/50	F	0.370	27	0.54	130
5.	"	M	0.316	26	0.52	125
6.	"	M	0.338	23	0.46	110
7.	8/2/50	F	0.402	20	0.40	95
8.	"	M	0.475	25	0.50	120
9.	"	M	0.386	24	0.48	115
10.	8/4/50	F	0.320	23	0.46	110
11.	"	F	0.284	24	0.48	115
12.	"	M	0.260	25	0.50	120
13.	8/9/50	M	0.360	24	0.48	115
14.	"	F	0.365	23	0.46	110
15.	"	M	0.375	26	0.52	125
Mean			0.353	23.9	0.48	114.7
Standard Deviation	(σ)		0.052	1.9	0.04	
Standard Error	(ϵ)		0.013	0.5	0.01	

TABLE XXXII

Summary of Pigeon Data

Sample: U.S.P. DIGITOXIN REFERENCE STANDARD.
 Concentration of injection fluid: 0.03 mg./cc.

Pigeon number	Date	Sex	Body wt. in kg.	No. of doses	L.D. (mg./kg.)	Time in minutes
1.	7/28/50	M	0.372	18	0.54	85
2.	"	F	0.318	17	0.51	80
3.	"	F	0.550	17	0.51	80
4.	8/1/50	F	0.368	16	0.48	75
5.	"	F	0.377	15	0.45	70
6.	"	F	0.342	17	0.51	80
7.	8/2/50	M	0.400	15	0.45	70
8.	"	F	0.414	14	0.42	65
9.	"	M	0.414	18	0.54	85
10.	8/4/50	M	0.290	19	0.57	90
11.	"	M	0.270	20	0.60	95
12.	"	M	0.292	16	0.48	75
13.	8/9/50	F	0.350	17	0.51	80
14.	"	M	0.370	18	0.54	85
15.	"	M	0.376	15	0.45	70
Mean			0.354	16.8	0.50	79.0
Standard Deviation (σ)			0.044	1.7	0.05	
Standard Error (ϵ)			0.011	0.4	0.01	

TABLE XXXIII

Summary of Pigeon Data

Sample: U.S.P. DIGITOXIN REFERENCE STANDARD.
 Concentration of injection fluid: 0.05 mg./cc.

Pigeon number	Date	Sex	Body wt. in kg.	No. of doses	L.D. (mg./kg.)	Time in minutes
1.	7/28/50	M	0.370	12	0.60	55
2.	"	F	0.338	11	0.55	50
3.	"	M	0.336	11	0.55	50
4.	8/1/50	M	0.360	14	0.70	65
5.	"	M	0.356	14	0.70	65
6.	"	F	0.350	11	0.55	50
7.	"	F	0.350	11	0.55	50
8.	"	F	0.376	11	0.55	50
9.	"	M	0.342	13	0.65	60
10.	8/2/50	F	0.458	10	0.50	45
11.	"	M	0.420	10	0.50	45
12.	"	F	0.434	11	0.55	50
13.	8/9/50	F	0.300	11	0.55	50
14.	"	F	0.270	11	0.55	50
15.	"	M	0.352	11	0.55	50
Mean			0.361	11.5	0.57	52.3
Standard Deviation (σ)			0.048	1.2	0.06	
Standard Error (ϵ)			0.012	0.3	0.02	

TABLE XXXIV

Summary of Pigeon Data

Sample: U.S.P. DIGITOXIN REFERENCE STANDARD.

Concentration of injection fluid: 0.08 mg./cc.

Pigeon number	Date	Sex	Body wt. in kg.	No. of doses	L.D. (mg./kg.)	Time in minutes
1.	7/28/50	M	0.572	9	0.72	40
2.	"	F	0.324	9	0.72	40
3.	"	F	0.342	9	0.72	40
4.	8/2/50	M	0.415	8	0.64	35
5.	"	M	0.410	7	0.56	30
6.	"	M	0.404	9	0.72	40
7.	8/4/50	M	0.340	8	0.64	35
8.	"	M	0.298	10	0.80	45
9.	"	F	0.290	9	0.72	40
10.	"	F	0.292	8	0.64	35
11.	"	F	0.322	9	0.72	40
12.	"	M	0.304	6	0.48	25
13.	8/9/50	M	0.332	8	0.64	35
14.	"	F	0.394	8	0.64	35
15.	"	F	0.398	7	0.56	30
Mean			0.352	8.3	0.66	33.3
Standard Deviation (σ)			0.046	1.0	0.08	
Standard Error (ϵ)			0.012	0.3	0.02	

TABLE XXXV

Summary of Pigeon Data

Sample: U.S.P. OUABAIN REFERENCE STANDARD.

Concentration of injection fluid: 0.006 mg./cc.

Pigeon number	Date	Sex	Body wt. in kg.	No. of doses	L.D. (mg./kg.)	Time in minutes
1.	6/14/50	F	0.340	26	0.156	125
2.	"	M	0.330	27	0.162	130
3.	"	M	0.326	25	0.150	120
4.	6/17/50	M	0.406	25	0.150	120
5.	"	M	0.408	23	0.138	110
6.	"	M	0.396	24	0.144	115
7.	6/20/50	F	0.322	29	0.174	140
8.	"	F	0.298	25	0.150	120
9.	"	F	0.270	26	0.156	125
10.	6/23/50	M	0.324	24	0.144	115
11.	"	F	0.318	26	0.156	125
12.	"	M	0.332	25	0.150	120
13.	7/22/50	F	0.357	29	0.174	140
14.	"	F	0.408	29	0.174	140
15.	"	M	0.452	26	0.156	125
16.	7/26/50	M	0.406	23	0.138	110
17.	"	M	0.430	26	0.156	125
18.	"	M	0.388	20	0.120	95
Mean			0.362	25.4	0.153	122.2
Standard Deviation (σ)			0.051	2.3	0.014	
Standard Error (E)			0.012	0.5	0.003	

TABLE XXXVI

Summary of Pigeon Data

Sample: U.S.P. OUABAIN REFERENCE STANDARD.Concentration of injection fluid: 0.010 mg./cc.

Pigeon number	Date	Sex	Body wt. in kg.	No. of doses	L.D. (mg./kg.)	Time in minutes
1.	6/14/50	M	0.326	17	0.170	80
2.	"	F	0.334	18	0.180	85
3.	"	M	0.322	19	0.190	90
4.	6/17/50	M	0.390	14	0.140	65
5.	"	F	0.412	13	0.130	60
6.	"	M	0.380	16	0.160	75
7.	6/20/50	F	0.274	25	0.250	120
8.	"	M	0.276	17	0.170	80
9.	"	F	0.304	16	0.160	75
10.	6/23/50	M	0.402	13	0.130	60
11.	"	M	0.342	15	0.150	70
12.	"	M	0.318	13	0.130	60
13.	7/22/50	M	0.380	14	0.140	65
14.	"	M	0.386	17	0.170	80
15.	"	M	0.424	15	0.150	70
16.	7/26/50	F	0.392	16	0.160	75
17.	"	F	0.380	16	0.160	75
18.	"	M	0.390	14	0.140	65
Mean			0.357	16.0	0.160	75.0
Standard Deviation (σ)			0.046	3.0	0.029	
Standard Error (ϵ)			0.011	0.7	0.007	

TABLE XXXVII

Summary of Pigeon Data

Sample: U.S.P. OUABAIN REFERENCE STANDARD.

Concentration of injection fluid: 0.015 mg./cc.

Pigeon number	Date	Sex	Body wt. in kg.	No. of doses	L.D. (mg./kg.)	Time in minutes
1.	6/14/50	F	0.348	9	0.135	40
2.	"	M	0.328	9	0.135	40
3.	"	F	0.322	9	0.135	40
4.	6/17/50	M	0.380	11	0.165	50
5.	"	M	0.398	8	0.120	35
6.	"	M	0.398	11	0.165	50
7.	6/20/50	F	0.288	12	0.180	55
8.	"	M	0.278	14	0.210	65
9.	"	M	0.283	12	0.180	55
10.	6/23/50	M	0.344	15	0.225	70
11.	"	M	0.354	11	0.165	50
12.	"	M	0.316	16	0.240	75
13.	7/22/50	M	0.392	11	0.165	50
14.	"	M	0.430	8	0.120	35
15.	"	M	0.372	11	0.165	50
16.	7/26/50	M	0.382	9	0.135	40
17.	"	M	0.415	10	0.150	45
18.	"	M	0.380	11	0.165	50
Mean			0.356	10.9	0.164	49.7
Standard Deviation (σ)			0.046	2.3	0.034	
Standard Error (ϵ)			0.011	0.5	0.008	

TABLE XXXVIII

Summary of Pigeon Data

Sample: U.S.P. OUBAIN REFERENCE STANDARD.

Concentration of injection fluid: 0.025 mg./cc.

Pigeon number	Date	Sex	Body wt. in kg.	No. of doses	L.D. (mg./kg.)	Time in minutes
1.	6/14/50	M	0.342	8	0.200	35
2.	"	M	0.330	7	0.175	30
3.	"	F	0.352	5	0.125	20
4.	6/17/50	F	0.380	6	0.150	25
5.	"	F	0.392	7	0.175	30
6.	"	M	0.410	5	0.125	20
7.	6/20/50	M	0.314	7	0.175	30
8.	"	F	0.280	8	0.200	35
9.	"	F	0.288	6	0.150	25
10.	6/23/50	M	0.406	7	0.175	30
11.	"	F	0.372	6	0.150	25
12.	"	M	0.350	7	0.175	30
13.	7/22/50	M	0.402	5	0.125	20
14.	"	M	0.440	7	0.175	30
15.	"	M	0.350	6	0.150	25
16.	7/26/50	M	0.396	7	0.175	30
17.	"	F	0.386	7	0.175	30
18.	"	M	0.390	7	0.175	30
Mean			0.366	6.6	0.164	27.8
Standard Deviation (σ)			0.043	0.9	0.023	
Standard Error (ϵ)			0.010	0.2	0.005	

TABLE XXXIX

A Comparison of the Mean Lethal Doses
for Different Concentrations

Drug	Conc. of injection fluid (mg./cc.)	Mean lethal dose (mg./kg.)
Digitalis Leaves, 30-C	1.5	53.2
" " "	2.0	51.1
" " "	3.0	50.4
" " "	4.0	52.8
" " "	7.0	57.8
" " "	10.0	58.8
U.S.P. Digitoxin Ref. Std.	0.02	0.48
" " "	0.03	0.50
" " "	0.05	0.57
" " "	0.08	0.66
U.S.P. Ouabain Ref. Std.	0.006	0.153
" " "	0.010	0.160
" " "	0.015	0.164
" " "	0.025	0.164

TABLE XL.

A Comparison of the Mean Injection Times
for Different Concentrations

Drug	Conc. of injection fluid (mg./cc.)	Mean no. of doses	Mean time in minutes
Digitalis Leaves, 30-C	1.5	35.4	172.2
" " "	2.0	25.6	122.8
" " "	3.0	16.8	79.1
" " "	4.0	13.2	60.9
" " "	7.0	8.3	36.3
" " "	10.0	5.9	24.4
U.S.P. Digitoxin Ref. Std.	0.02	23.9	114.7
" " "	0.03	16.8	79.0
" " "	0.05	11.5	52.3
" " "	0.08	8.3	36.3
U.S.P. Ouabain Ref. Std.	0.006	25.4	122.2
" " "	0.010	16.0	75.0
" " "	0.015	10.9	49.7
" " "	0.025	6.6	27.8

TABLE XLI

Lethal Doses (mg./kg.) of Six Concentrations of Digitalis

	Concentration of Injection Fluid (mg./cc.)					
	1.5	2.0	3.0	4.0	7.0	10.0
1.	58.5	44.0	45.0	56.0	63.0	60.0
2.	55.5	46.0	51.0	52.0	56.0	50.0
3.	55.5	50.0	51.0	60.0	63.0	60.0
4.	57.0	50.0	48.0	60.0	56.0	70.0
5.	57.0	50.0	48.0	48.0	56.0	50.0
6.	46.5	54.0	48.0	56.0	56.0	60.0
7.	55.5	52.0	42.0	44.0	49.0	60.0
8.	40.5	50.0	48.0	48.0	56.0	60.0
9.	52.5	44.0	54.0	56.0	63.0	50.0
10.	52.5	50.0	54.0	52.0	56.0	60.0
11.	48.0	48.0	60.0	48.0	56.0	50.0
12.	52.5	50.0	45.0	60.0	56.0	50.0
13.	52.5	60.0	57.0	44.0	63.0	60.0
14.	60.0	68.0	57.0	48.0	63.0	70.0
15.	48.0	44.0	51.0	52.0	56.0	70.0
16.	58.5	52.0	48.0	60.0	56.0	60.0
Total (T)	850.5	818.0	807.0	844.0	924.0	940.0
Mean (\bar{x})	53.16	51.13	50.44	52.75	57.75	58.75

TABLE XLII

Lethal Doses (mg./kg.) of Four Concentrations of Digitoxin

	Concentration of Injection Fluid (mg./cc.)			
	0.02	0.03	0.05	0.08
1.	0.46	0.54	0.60	0.72
2.	0.50	0.51	0.55	0.72
3.	0.42	0.51	0.55	0.72
4.	0.54	0.48	0.70	0.64
5.	0.52	0.45	0.70	0.56
6.	0.46	0.51	0.55	0.72
7.	0.40	0.45	0.55	0.64
8.	0.50	0.42	0.55	0.80
9.	0.48	0.54	0.65	0.72
10.	0.46	0.57	0.50	0.64
11.	0.48	0.60	0.50	0.72
12.	0.50	0.48	0.55	0.48
13.	0.48	0.51	0.55	0.64
14.	0.46	0.54	0.55	0.64
15.	0.52	0.45	0.55	0.56
Total (T)	7.18	7.56	8.60	9.92
Mean (\bar{x})	0.479	0.504	0.573	0.661

TABLE XLIII

Lethal Doses (mg./kg.) of Four Concentrations of Ousabain

	Concentration of Injection Fluid (mg./cc.)			
	0.006	0.010	0.015	0.025
1.	0.156	0.170	0.135	0.200
2.	0.162	0.180	0.135	0.175
3.	0.150	0.190	0.135	0.125
4.	0.150	0.140	0.165	0.150
5.	0.138	0.130	0.120	0.175
6.	0.144	0.160	0.165	0.125
7.	0.174	0.250	0.180	0.175
8.	0.150	0.170	0.210	0.200
9.	0.156	0.160	0.180	0.150
10.	0.144	0.130	0.225	0.175
11.	0.156	0.150	0.165	0.150
12.	0.150	0.130	0.240	0.175
13.	0.174	0.140	0.165	0.125
14.	0.174	0.170	0.120	0.175
15.	0.156	0.150	0.165	0.150
16.	0.138	0.160	0.135	0.175
17.	0.156	0.160	0.150	0.175
18.	0.120	0.140	0.165	0.175
Total (T)	2.748	2.880	2.955	2.950
Mean (\bar{x})	0.1527	0.1600	0.1642	0.1639

TABLE XLIV

Analysis of Variance for the Data of TableXLI

Correction term, $(ST)^2/n = (5183.5)^2/96 = 279882.00$

Total sum of squares, $Sx^2 - (ST)^2/n = 283711.75 - 279882.00 = 3829.75$

Sum of squares between groups, $S(T^2/n) - (ST)^2/n = 280839.70 - 279882.00 = 957.70$

Sum of squares within groups, $SS(x - \bar{x})^2 = 2872.05$

Source of variation	d.f.*	Sum of squares	Mean square	F(calc.)**	F(theoret.)
Between groups	5	957.70	191.54	6.00	2.33
Within "	<u>90</u>	<u>2872.05</u>	31.91		
<u>Total</u>	95	3829.75			

* Degrees of freedom.

**Variance ratio.

TABLE XLV.

Analysis of Variance for the Data of Table XLIII

Correction term, $(\sum T)^2/n = (33.26)^2/60 = 18.4371$

Total sum of squares, $\sum x^2 - (\sum T)^2/n = 18.9422 - 18.4371 = 0.5051$

Sum of squares between groups, $S(T^2/n) - (\sum T)^2/n =$
 $18.7381 - 18.4371 = 0.3010$

Sum of squares within groups, $SS(x - \bar{x})^2 = 0.2041$

<u>Source of variation</u>	<u>d.f.*</u>	<u>Sum of squares</u>	<u>Mean square</u>	<u>F(calc.)**</u>	<u>F(theoret.)</u>
Between groups	3	0.3010	0.1003	27.86	2.78
Within "	<u>56</u>	<u>0.2041</u>	0.0036		
<u>Total</u>	59	0.5051			

* Degrees of freedom.

**Variance ratio.

TABLE XLVI

Analysis of Variance for the Data of Table XLIII

Correction term, $(ST)^2/n = 1.84736$ Total sum of squares, $Sx^2 - (ST)^2/n = 1.89449 - 1.84736 = 0.04713$ Sum of squares between groups, $S(T^2/n) - (ST)^2/n =$
 $1.84891 - 1.84736 = 0.00155$ Sum of squares within groups, $SS(x - \bar{x})^2 = 0.04558$

Source of variation	d.f.*	Sum of squares	Mean square	F(calc.)**	F(theoret.)*
Between groups	3	0.00155	0.00052	0.77	2.75
Within "	<u>68</u>	<u>0.04558</u>	0.00067		
<u>Total</u>	71	0.04713			

* Degrees of freedom.

**Variance ratio.