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BIOLOGICAL ASSAY OF CARDIOTONIC DRUGS
BY A CHICK EMBRYO METHOD

By

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ASSAY OF CARDIOTONIC DRUGS
BY A CHICK EMBRYO METHOD

INTRODUCTION

Digitalis-like substances are a group of naturally occurring chemical compounds having a cardiac action. They may be divided into four main classes, two from the plant kingdom, glycosides and the Erythrophloeum alkaloids, and two from the animal kingdom, Bufagin and Bufotin. To date, investigations have been conducted on some sixty-two compounds from the four groups. The following discussion will be limited to the official digitalis and to the glycosides derived from it. The term glycoside may be defined as a conjugation product of sugar and aglycone (a non-sugar part). The various digitalis glycosides are qualitatively alike but quantitatively different in their pharmacological action.

Digitalis (foxtail, digitalis folium P.I., folk's glove, ladies' glove, dead men's bell, purple foxglove) as defined by the United States Pharmacopoeia is "the dried leaf of digitalis purpurea Linne (family Scrophulariaceae)." The plant itself is a "biennial pubescent herb whose first years aerial growth consists of a rosette of ovate oblong leaves. During the second year a hoary stem shoots up which rises to the height of 1 to 1.5 meters, bearing alternate, ovate lanceolate to ovate oblong pubescent leaves and terminal racemes of purple, rarely white, tubular campanulate flowers that are spotted within. The corolla is 4 to 5 cm. in length."

The plant is naturalized in North America and grows extensively, both wild and cultivated, in the United States.

First or second year leaves are gathered just before the expansion
of the flowers and are carefully dried since they are not legally per-
mittted to contain more than 6% moisture. Storage or transportation of
digitalis in any form must be in water-proof and air-tight containers
protected from light.

An interesting side light concerning the fresh green leaves is the
Baljet reaction, in which cells containing the glycosides are identified
by treating a mounted section with sodium picrate reagent (a mixture of
one drop of one percent picric acid with one drop of ten percent sodium
hydroxide). The glycoside containing cells are colored orange within
one to two minutes. This same reaction forms the basis for a proposed
colorimetric assay for the potency of digitalis.

The United States Pharmacopoea requires that "the potency of digitalis
shall be such that, when assayed as directed, 0.1 gm shall be equivalent to
not less than 1.0 U.S.P. unit. One U.S.P. digitalis unit represents the
potency of 0.1 gm of the U.S.P. Reference Standard digitalis."

This standard digitalis is prepared under the supervision of the
Committee of Revision and distributed through the Office of the Chairman
by the authority of the Board of Trustees of the United States Pharma-
copoeal Convention. The permanent Commission on Biological Standardization
has adopted a standard expressing potency in terms of units, and the U.S.P.
Reference Standard is analogous to that defined by the League of Nations
Commission. Reference Standard digitalis obtained from the Committee of
Revision is powdered digitalis in sealed, hard, amber colored glass ampuls.
It is prepared as needed by U.S.P. direction:

Weigh the contents of one ampul of digitalis
Reference Standard to the nearest milligram,
either in the original ampul or in a weighing
bottle, and transfer to a dry, hard glass,
glass-stoppered container of at least 50 cc. capacity. Complete the weighing within 5 minutes after opening the ampul. Add sufficient menstruum consisting of 4 parts of alcohol, by volume, and 1 part distilled water, by volume, so that the total volume of menstruum added corresponds to 1 cc. for each Gm. of powder. Insert the stopper, the upper third of which is greased lightly with petrolatum. Shake the mixture for 24 ± 2 hours at 25°C., ± 5°C. by mechanical means which continuously brings the solid material into fresh contact with the liquid phase. Immediately thereafter, centrifuge the mixture and decant into a dry, hard glass bottle having a tight closure, and preserve under refrigeration until used. Do not use for assay after a period of more than 30 days.

Powdered digitalis as defined by the U.S.P. is "digitalis dried at a temperature not exceeding 60°C. and reduced to a fine powder. The potency of powdered digitalis shall be such that when assayed as directed 0.1 gm. shall be equivalent to 1 U.S.P. digitalis unit."

Tincture of digitalis is prepared by U.S.P. direction:

**Digitalis fine powder 100 gms.**

To make about 1000 cc.

The drug is mixed with a sufficient quantity of a menstruum consisting of a mixture of 4 volumes of alcohol and 1 volume of water to render it evenly and distinctly damp. Allow it to stand for 15 minutes, transfer it to a suitable percolator and pack the drug firmly. Pour on enough of the prescribed menstruum to saturate the drug, cover the top of the percolator and when the liquid is about to drip from the percolator, close the lower orifice and allow the percolation to proceed slowly, (1 cc. of percolate per minute), gradually adding sufficient menstruum to produce 1000 cc. of tincture and mix thoroughly. The U.S.P. further directs that the potency of tincture of digitalis be such that, when assayed as directed, 1 cc. of the tincture shall be equivalent to 1 U.S.P. digitalis unit.

Another official preparation of digitalis which merits consideration

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is infusion of digitalis since it is preferred to the tincture or powdered leaf in various parts of the world. It is prepared according to The National Formulary Eighth Edition 1946:

Powdered digitalis 15 gms.
Alcohol 100 cc.
Cinnamon Spirit 5 cc.
Distilled water, a sufficient quantity to make 1000 cc.

Pour 900 cc. of boiling distilled water upon the powdered digitalis in a suitable vessel, cover tightly, and infuse 1 hour in a warm place. Then add the alcohol in which the Spirit of Cinnamon has been dissolved; filter and pass enough distilled water through the residue on the filter to make the product measure 1000 cc.

CAUTION: Only the U.S.P. XIII powdered digitalis is to be used in this preparation, and the infusion must not be dispensed unless freshly prepared.

The description of digitalis, and its preparation, was quoted directly from the U.S.P. XIII and National Formulary Eighth Edition because these books constitute a legal standard and the preparations are those commonly used in medical practice.

Although digitalis was used and misused for hundreds of years for a remarkable assortment of internal and external ailments, it was not until 1785 that a comprehensive record of its botanical identity and therapeutic value in the treatment of dropsy was made. A treatise, published by William Withering, a physician and botanist of Birmingham, England, removed digitalis from the list of secret nostrums. In spite of, or because of Withering's acute and logical recommendations, it was again used and misused for another hundred years, but this time by a more competent group of practitioners.
As a result of investigations during the early part of the twentieth century by Cusny and others, the major action of digitalis was properly understood to be upon the myocardium. At that time it was considered a specific in the treatment of auricular fibrillation. Twenty more years were required to establish its unique importance in the therapy of congestive heart failure. Patients with this pathology are usually forced to depend upon the action of digitalis for the remainder of their lives.

It may be given orally or rectally in any of the forms already described. There is only a partial absorption of digitalis through the gastro-intestinal tract, which varies between individuals. It may produce a gastric irritation due to a local irritant action. On the average, a therapeutic dose will be absorbed within two hours, and full cardiac effects may be manifest within six hours. Rapid digitalization may be accomplished by the intravenous injection of an isolated and purified glycoside obtained from digitalis. This technique is usually reserved for emergencies where time is a vital factor, but therapeutic results are obtained in five to fifteen minutes.

Digitalis is distributed rather uniformly in the body after its absorption. Bulky organs, obviously take up the major share of a given dose. There does not appear to be any unusual concentration in the heart.

The fate of the drug in the body is unknown with the exception of a small fraction that is eliminated unchanged by the bile and urine. Destruction or excretion is slow, and repeated therapeutic dosage results in a cumulative action. Avoidance of this condition requires a competent determination of the proper maintenance dose, based on the patient's
response. The rate of destruction or excretion is apparently dependent
upon the total amount of the drug present in the body at any given time.
Small amounts are destroyed or excreted slowly, while larger amounts
themselves accelerate the speed of their destruction. Beyond the maximum
rate, greater amounts of the drug extend the therapeutic effects to toxic
proportions. A single therapeutic dose of digitalis may exert its
effect on the heart in from two days to two weeks.

The pharmacological action of digitalis resides primarily in its
myocardial effect. Other actions may be considered secondary or
relatively unimportant. Digitalis acts directly on the cardiac muscle to
increase the force of systolic contraction provided that the functional
capacity of the heart is still present. Evidence for the specificity of
digitalis is well established with reports by Cattell and Gold, Cohn and
Stewart, among the more recent investigators. Alteration in cardiac
activity follows the initial change in logical sequence. Increased
systolic contraction, while occupying less time in the cycle, empties the
ventricles more completely, increases the absolute refractory period of
the heart muscle and allows a more complete venous return. Heart size is
decreased both in systole and diastole, but the cardiac output is increased.
Neurogenic control of the heart becomes less important and for practical
purposes sympathetic effects cease to be demonstrated. In a normal heart,
the sino-aortic node, and consequently its rate of discharge, may become
depressed with a full therapeutic dose of digitalis, but slowing of con-
duction time between the auricle and ventricle, due to a depression of the
bundle of His, always occurs. Marked slowing of conduction time renders
the individual cardiac fibres less responsive to neurogenic stimulation and,
if carried to extreme, kills them.

What influence the vagus has upon heart rate after digitalis administration is a matter for controversy. The drug may stimulate the medullary nuclei directly, or it may stimulate the carotid body and decrease the heart rate by reflex action. Animal experimentation shows that slowing of the digitalized heart occurs neither after bilateral vagotomy nor after the administration of atropine. However, in man atropine has no effect on the rate after digitalization, and many authorities agree that vagal factors are of minor importance in determining heart rate.

Blood pressure in man is not affected by digitalis although it has a direct constrictor action on the smooth muscle of blood vessels, in addition to a slight central vasomotor stimulation. Laboratory animals, in contrast, exhibit an elevation. Digitalis causes both constriction and dilation in different capillary beds with the net effect, an increase in blood pressure.

The pharmacological action of digitalis is utilized in the treatment of congestive heart failure. This pathological condition, in brief, is a lessening of the systolic power of the heart. The velocity of blood circulation slows, venous stasis takes place, and all the tissues of the body including the heart suffer from anoxia, lack of food, and an accumulation of metabolic waste products. The failure of muscular power may affect any single cavity or the whole heart. The reserve power of the myocardium is rapidly or slowly diminished. This reserve may be estimated by determining the amount of exertion required to produce symptoms of cardiac insufficiency, or by the relief of symptoms after digitalis.
therapy. A failing heart is the key factor in a vicious circle. It attempts to compensate for the deficiency by increasing its rate, but this results in decreased output, increased venous pressure and venous stasis.

Digitalis increases the force of contraction of failing hearts. The ventricles empty more completely and an increased venous return can be accommodated with a lowering of venous pressure. The compensatory tachycardia may be relieved although clinical improvement can occur without any alteration in rate. Impulses, in spite of slower conduction time from the sino-auricular node, still get through to the ventricle, and a normal rate is reestablished.

Secondary to the improvement in cardiac and circulatory functions, accompanying oedema is relieved by increased diuresis (no diuresis occurs if oedema is not present).

An extension of the action of digitalis gives toxic and lethal results. The mechanism by which this is accomplished is the same as that producing beneficial effects. Any digitalis preparation that fails to produce toxic symptoms is likewise devoid of therapeutic action. This is the basis for all biological assays to determine the potency of the drug.

The first symptoms of overdosage—anorexia, nausea and vomiting—ordinarily occur within a day or two after reaching a toxic concentration during therapy. Vomiting is mediated through a direct stimulation of the medullary center, or by reflex impulses from the heart carried by the phrenic nerve. This sequence of events does not always occur, however, and may be partly or entirely lacking in some patients. Extrasystoles are the most frequent danger signal. The source is in the auricle or ventricle because of an increased irritability of the myocardium. In turn, an acceleration of heart rate occurs, followed by a progressive decrease.
Lengthened conduction time and depression of the sino-auricular node results in dropped beats, then in complete auriculoventricular disassociation. After the complete block occurs, the auricles and ventricles may continue pulsations until the advent of fibrillation. Ventricular fibrillation is the most common cause of death due to digitalis.

Treatment in any suspected or proved case of digitalis poisoning is chiefly by mechanical removal of any unabsorbed drug and by the proper maintenance of diuresis. Nothing can be done to prevent or alleviate the progressive heart action. Although digitalis can be washed out of an excised heart with a resumption of normal activity, it is apparently not feasible to give the enormous amounts of fluid necessary to accomplish this in the intact body.
CHEMISTRY

The chemistry of the digitalis glycosides, as well as those of related drugs, proved to be a major stumbling block until recently. Claims and counterclaims of the isolation of various pure glycosides confused the issue even further. Complete synthesis of the cardiotonic principles is still impossible but the isolation and separation of the various components of digitalis has been accomplished. A single glycoside with all the pharmacological effects of digitalis is now commercially available.

An alcoholic extract of the whole leaf contains about one percent of the glycosides of digitalis in nearly equal proportions. Such an extract consists of the menstruum, the glycosides Gitalin, Digitoxin, Digitalin, Gitoxin, the saponins Digitonin, Digitsaponin, and Gitin, a volatile oil containing Digitalosmin, tannin, an irritant resin (Digitalis acid), a yellow coloring substance termed Luteolin and various enzymes. The chemical structures of all the glycosides of digitalis are similar. It has been shown that glycosides vary quantitatively in pharmacological action, but since Digitoxin is the most powerful and, consequently, the only one now commercially available, this discussion will be limited to that of Digitoxin.

The basic molecule consists of a methyl cyclopentanoperhydrophenanthrene nucleus with an attached lactone ring. Methyl groups are present at C-10 and C-13 (see Figure 1). There is a hydroxy group at C-14 with oxygen at C-3 to which is attached a side chain composed of three carbohydrate groups of 2,6 desoxyhexose. Enzyme action or acid
hydrolysis causes cleavage at this point, yielding three carbohydrate
groups (Digitoxose) and the aglycone Digitoxigenin.

Investigations to determine what portions of the entire molecule are necessary have yielded interesting results. Both the lactone ring and the double bond in the ring are indispensable for cardiac action. Either hydrogen saturation or saponification of this portion of the molecule results in a complete loss of activity. The hydroxyl group on C-14 and a spatial configuration in which the cis form of rings I and II exist are also vital. The sugar portion of the molecule, while having no cardiac action in itself, is apparently capable of influencing the potency of the glycoside. It is believed that this is accomplished by control of water solubility, cell penetrability, and persistence of cardiac action. The sugar groups are not essential.

Some success has been noted in the preparation of synthetic compounds by substitution on C-3 of various monosaccharides, singly or in combination. In the case of digitoxin, the substitution of a monoside yields a product (β-d glycoside) which is nearly twice as active as Digitoxin. Since Digitoxin possesses one thousand times the activity of digitalis and since it is administered in milligram doses, it is unlikely that further reduction in therapeutic doses would be desirable.
BIOLOGICAL ASSAY

Extreme variation of potency in samples of digitalis grown in various localities, as well as its extensive use in a serious condition, necessitated the establishment of a standard by which all preparations of digitalis could be measured and adjusted to a uniform value. In 1902 Focke, a German physician, introduced in a resumé a frog method suitable for the biological assay of digitalis which resulted in countless investigations. The major efforts of early workers were directed upon the amphibian heart, with attention directed toward methods of introducing the drug to the heart, toward length of time for results, and toward end points. By sheer force of accumulated data, even though conflicting, the United States Pharmacopoeal Revision Committee accepted, in 1916, a one hour frog method based on work by Cushny. This method was finally abandoned in favor of a cat assay in 1942.

Many workers have attempted to establish assays, utilizing as test objects, primates, amphibians, bony fish, seedlings of lupus albus, and even the lowly paramecium. The chief difficulty has been in transferring results obtained by any of the methods into a true measure of therapeutic potency for man.

A direct approach to the problem by Cattell and fellow workers produced the most accurate assay to date. Calibrations are made of the responses of suitable sensitive cardiac patients in which a twenty-five percent difference in the dosage of Reference Standard Digitalis can be distinguished by changes in the T wave of an electrocardiogram. In an assay, a tracing is taken and a dose of Reference Standard Digitalis is given; then a second tracing is taken twenty-four hours later to determine the effect on the T wave. A month is allowed to elapse for complete elimination of the digitalis,
and then the performance is repeated with a twenty-five percent larger dose of the Reference Standard. The unknown is then tested in the same manner on the same patient with doses that produce comparable effects. Blind comparison of the electrocardiograms from six or more patients so treated determines the potency of the unknown. However accurate, the method is self-limited to institutions where patients can be selected. It is very slow and expensive.

The introduction of purified digitoxin by industry, and its acceptance by the U. S. P. XIII is assuredly a step ahead. It is completely absorbed by the gastro-intestinal tract. Although the potency is checked by biological assay and by colorimetric methods, it is prescribed by a definite weight. Several methods of chemical assay have been proposed. The Baljet reaction, with modifications, is used for a colorimetric comparison of standard and unknown preparations, but no method has been accurate enough to warrant its use except in the potency determination of the purified glycoside.

The one hour frog method depended upon the fact that digitalis in toxic amounts produces a systolic standstill in the ventricle of amphibians. This was chosen as a suitable end point since it occurs within a reasonable time and is unmistakable. The Reference Standard is prepared, as previously described, then evaporated to one-half the volume on a steam bath. It is then restored to the original volume by the addition of distilled water. This reduces the alcoholic content of the preparation to twenty-five percent which is suitable for injection. Healthy animals of the species Rana pipiens Schreber between fifteen and thirty-five grams in weight are stored for
sixteen to twenty-four hours prior to the assay at a temperature of 20°C. ± 1°C. An hour before the assay, urine is expressed from each frog, and its weight is determined within 0.5 gm. At least twenty-five frogs are used in each of two series, one for the Reference Standard and one for the unknown digitalis preparation. The unknown preparation is also treated so that it contains no more than twenty-five percent alcohol. Digitalis, in identical amounts to the gram weight of the frog, is injected into the ventral lymph sac of the first frog in a series. Injection is done by means of a syringe calibrated to 0.01 cc. The first animal is then replaced in the constant temperature bath, and the succeeding frogs are injected at two minute intervals. Exactly 58 minutes later, the first animal of a series is pithed, the skin of the abdomen slit to expose the heart and to determine the presence of any unabsorbed digitalis (in which case the animal is rejected), and the reading is made. A record is made of each frog, indicating "beat" or "systolic standstill". The two minute interval between animals allows time for the operative and reading procedure so that each animal is observed exactly 60 minutes after injection.

In a given dose range, all or none of the frogs may show systolic standstill. In either event, the assay is repeated with dose modifications until not less than twenty-five and not more than seventy-five percent of the frogs in a series receiving the same dose show systolic standstill. Usually, completion of an assay results in several series of the Reference Standard and the unknown. The frogs in the smallest dose demonstrate systolic standstill. Gradations exist between the two extremes. Adjustment of the dose of the unknown is made until it corresponds as closely as possible to results obtained from the Reference Standard. Calculation is made by the
following formulas:

\[
\text{Dose of Standard} \times \frac{\text{Percent strength of}}{\text{Dose of Sample}} \times 100 = \text{Percent strength of the Reference Standard}
\]

\[
\text{Percent strength of sample in relation to Reference Standard} \times \frac{\text{Total amount of batch}}{100} = \text{Final Volume of the Batch}
\]

A standard error of ± 20% is allowed in the assay.

Therapeutic results in humans could not be predicted by the assay. The remarkable record of therapy by digitalis, in which very few fatalities from overdosage have been reported, is due to the fact that each physician, in effect, ran his own biological assay on each patient, using the units expressed by the frog method as merely a crude indication of potency.

Since the cat method, originated by Hatcher and coworkers, is the present U.S.P. method of biological assay of digitalis potency, it will be quoted directly. The Reference Standard is prepared as previously described.

The cats. Select domestic cats free from gross evidence of disease and weighing between 2.0 and 4.0 kg. Do not use cats which upon gross examination are either obese, emaciated, lactating, or pregnant. Withhold food for from 16 to 24 hours prior to use. Assign all cats at random with the restriction that the two groups, the one for the standard preparation and the one for the specimen to be assayed, shall not differ by more than 50% in the average of their weights. Lightly anesthetize the cat with ether and immobilize, preparatory to the injection. Insert a cannula into a femoral vein and arrange to inject the appropriate test solution from a burette calibrated to .1 cc. after insuring the absence of air bubbles from the injection apparatus. Maintain the anesthesia throughout the injection in such a state that pain is absent, the pupillary and corneal reflexes are present, the voluntary musculature is not relaxed and the cat occasionally moves its tail or makes some other voluntary movements.
Preparation of the Test Dilutions. Dilute the Standard preparation of Digitalis and the preparation to be assayed in such a way that the estimated fatal dose of each preparation per kg. of cat will be diluted to 15 cc. with isotonic solution of sodium chloride. Make test dilutions the day they are to be used.

Injection of the Dilutions. Inject 1 cc. of the diluted material for each kg. of the body weight of the cat within a few seconds. Repeat this dose at five minute intervals until the cat dies from cessation of the heart beat. Use a total of not less than 6 cats for the preparation to be assayed. If the average number of doses for any given dilution required to produce death is less than 13 or greater than 19, regard these data as preliminary. Use them as a guide, and repeat with fresh, higher or lower dilution. Complete the assay within a period of 15 days.

Calculation of Results. Express the lethal dose for each cat in terms of cc. of Tincture per kg. of live body weight. Find the average lethal dose for the Standard Preparation and for the preparation to be assayed, and compute the standard error for each of the two preparations. If the standard error for either of the two results exceeds 5.7% of the average, repeat the assay of the Standard Preparation or of the preparation to be assayed as the case may be, or use additional cats until the standard error falls within this limit. Express the potency of the preparation to be assayed in U.S.P. Digitalis units per cc. by dividing the average for the Standard Preparation by the average for the preparation to be assayed. To compute the standard error of the average, take the difference between the average and the value found for each cat. Square these differences, take their sum, divide this sum by the number of cats, and divide this quotient by the number of cats diminished by 1. The square root of the last quotient is the standard error of the average. The formula for the standard error (S.E.) is

\[ \sqrt{\frac{\text{Sum} \ (e-\bar{e})^2}{N(N-1)}} \]

where:
- \(e\) = fatal dose for cat.
- \(\bar{e}\) = average fatal dose for the group of cats.
- \(N\) = number of cats in the group.
Statistically speaking, the method represents a considerable advance over the one hour frog assay; however, as a basis for predicting potency in humans, it is as undependable as results from the frog method.

The human method of assay proves conclusively that digitalis can be biologically assayed, but there is no suitable method for doing so.

All biological assays must meet certain requirements, other than trustworthiness of information. They must be inexpensive, must utilize easily procurable test objects, and, to reduce errors to a minimum, must be fairly simple to perform.

Since there is, at present, no biological method of digitalis standardization which meets all the requirements, a valid reason exists for further investigation of the problem.
STATEMENT OF THE PROBLEM

A review of literature reveals that the possibilities of digitalis assay using chick embryos has been explored. Early researchers were in general agreement that the isolated and intact embryonic heart demonstrated digitalis effects in the same manner as mammalian hearts. It was also felt that the embryonic hearts did not demonstrate a sufficient sensitivity to digitalis to warrant further work.

An extensive investigation was undertaken in 1938 by Paff. He proposed a microscopic inspection of the excised, digitalized forty-eight hour embryonic heart for the appearance of an A-V block. Time for the appearance of the block was recorded on a number of experiments at different digitalis dilutions. By comparison of unknown dilutions with an established curve, he was able to identify the unknown dilutions with an average standard error of ±5% for all assays. The statistical results made the method comparable to the cat or frog assay. His next step was a preliminary correlation of results from his method to those obtained from a human assay, using the same Reference Standard and unknown. Results were no better than those obtained from the cat method.

It seemed reasonable to assume, on the basis of Paff's results that with elimination of two variables and with a simplification of the method, a chick embryo assay might be established which would be a better measure of potency.

One glaring omission in both the cat method and Paff's embryo method is the problem of absorption. Any attempt to measure digitalis potency must recognize the fact that variations in absorption and, consequently, in pharmacological action do occur in the oral administration of digitalis.
Furthermore, a traumatic effect on the excised embryonic heart is difficult to minimize though Paff has done so.

The purpose of this investigation is to standardize a technique, using chick embryos in situ, and to determine by means of a sufficient number of data the accuracy that can be expected from a master curve based on Reference Standard Digitalis.
**EXPERIMENTAL**

The method for the establishment of a curve, to be described in detail, consists of utilizing a chick embryo of approximately 40 somites. It is exposed to view by chipping a window in the egg shell. The egg is then placed, embryo up, on a suitable stage for temperature control. An accurately measured amount of de-alcoholized Reference Standard Digitalis is then run onto the surface of the embryo. Time necessary for digitalis to cause complete stoppage of the auricles and ventricles is measured.

The experimental work was begun in September, 1946, and continued through February, 1947. Eggs were purchased as needed in lots of four to ten dozens from farmers in the vicinity of Missoula, Montana. There is no commercial hatchery in the immediate neighborhood to insure delivery of viable eggs laid within a three day period. Difficulty was experienced in obtaining a steady supply of eggs, and as a result, embryos were used from three different breeds of chickens (Plymouth Rock, Buff Orpington, and White Leghorns). After the restriction of purchases to one source, seventy-five to eighty percent yield of embryos were obtained. Losses due to abnormal development, overdevelopment, and underdevelopment ranged from three to ten percent. At least 65 embryos could be expected from incubation of nine dozen eggs laid within a three day period.

Preliminary trials narrowed the optimum developmental stage of the embryo (about five days old) from thirty-five to forty somites, measuring 6.8 mm. to 7.8 mm. At 40 somites the heart is well formed. Though a double circulation has not been established at this time and valvular control between the four chambers is incomplete, a frontal view of the embryo shows a clearly exposed, and well defined heart. In addition,
elements of the vagus and sympathetic nerves have not migrated into the heart; therefore the neurogenic factor is absent.

Embryos were considered overdeveloped when the limb buds blocked a clear observation of the heart and its retreat into the thorax had begun. Underdevelopment was arbitrarily assumed if the tail was not directed forward, or if the retinal layer was not well marked.

The eggs were incubated at 37°C. in a 110 volt, 5.5 ampere, 60 cycle thermostatically controlled oven of the convection type commonly used in laboratories. Though not a precise humidity control, a 250 cc. beaker, continuously filled with distilled water, was placed in the bottom of the oven. Eggs in groups of twenty-four were placed on three racks, one above the other.

Unfortunately, the eggs could not be incubated for a prescribed period of time and produced a yield of embryos all in the same stage of development. A preliminary candling at the beginning of the fourth day and thereafter at intervals of eight hours was found necessary. Selection of the embryos was based upon the first observation of the retinal layer in the optic cup when the egg was viewed in a darkened room through a 3 cm. circular hole admitting light from a one hundred watt globe. It was found that one hundred eggs yielded about twenty percent of the viable embryos suitable for the experiment by the evening of the fourth day of incubation. Sixty percent of the embryos were ready the following morning, and the remainder by the evening of the fifth day. These times depend upon the time the eggs were first placed in the incubator. It is obvious that, by planning, any given batch can be available when the operator wants them.

Although the embryo was used in situ, it was advisable to eliminate the
temperature factor. A box was devised both to hold the eggs, and to give
some degree of control higher than room temperature. The box, 12" x 12" x
12", was fitted with a thirty watt incandescent lamp in the bottom. Four
oval openings were cut in the top to facilitate the placing of different
shaped eggs over the openings parallel to the long axis of the egg. At
least one-third of the egg was below the surface. The tip of a Centigrade
thermometer was placed just below the top surface of the box through a
small opening drilled for that purpose. The thermometer was suspended by
means of a clamp and ring stand. Two goose necked lamps, with one hundred
watt bulbs, were focused on the top surface of the box, both as an aid to
visual observation and as a source of heat. According to the temperature
recorded by the thermometer, any or all of the lights were turned on or off.
Although crude, the system permitted a temperature of 37°C. ±2° to be main-
tained during an experiment.

A Reference Standard Digitalis was prepared at thirty day intervals in
fifty cc. quantities. Just prior to use, an amount, based on the dose to
be used and the number of embryos available, was measured. The alcoholic
solution was then evaporated on a steam bath to one-sixth the original
volume. This volume was restored by the addition of distilled water during
frequent agitation. This procedure was followed because embryonic hearts are
quite sensitive to alcohol which must be removed. Fresh solutions were
prepared in this manner for each daily run.

The Reference Standard Digitalis, treated as described, was allowed to
run over the surface of the embryo, lying in the cup-like depression that
results when the embryo is exposed to view. Delivery of the measured quantity
of digitalis was accomplished by means of a one cc. pipette, calibrated to
.01 cc. The pipette was suspended three inches from the top of the table
and next to the temperature control box. Each egg was taken from the
incubator, the embryo exposed and the egg placed under the pipette. A
capillary nozzle, attached to the 1 cc. pipette by a short length of rub­
ber tubing, allowed the operator to place the digitalis on the exact
position of the embryo. A pinch clamp and screw clamp was connected to the
rubber tubing and gave excellent control over the rate of flow. The time
was recorded, the egg marked and placed in the depression on the top of the
temperature control box with the exposed embryo uppermost. Succeeding
eggs were prepared in exactly the same manner. As each embryo reached the
end point, the complete cessation of movement of auricles and ventricles,
it was discarded and another test embryo put in its place. By interspacing
the operation, it was easy to watch for the end points of four embryos. As
in other methods, this eliminates errors due to individual interpretation,
since a heart has ceased to function or it has not. One hundred embryos
were run in a three hour period. Anoxia was observed by the changing of
color of the blood from a bright red to a darker hue. While anoxia hastens
cardiac arrest, it also does so in the intact mammal and therefore this is
not an essential factor for consideration.

The A-V block was easily observed, and closer attention was directed
to the particular embryo so affected. The auricles generally continued
to pulsate after the ventricles had stopped, but no definite rule can be
made about the impending arrival of the end point. Some hearts stopped
abruptly while others continued spasmodic beating for some time. Occasion­
ally a heart stopped for ten to forty seconds, then resumed a fairly normal
function. To circumvent this, the embryo was left on the stand for at least
a minute after a recorded stoppage. If the heart resumed beating, the
recorded time was cancelled, and the heart was left in place until it stopped permanently. At least one embryo from each batch of eggs was used as a control. The hearts of all controls functioned well from one to four hours after the shell was broken. One source of irritation from this method was the inadvertent tearing of the blood vessels while exposing the embryo to view. Perhaps one egg in six had to be discarded because of hemorrhage. Future refinements may eliminate this factor.

All the embryonic hearts observed exhibited varying degrees of susceptibility to the action of digitalis. This is best shown by the twin embryos that were encountered. In all cases, they were lying in opposite ends of the egg, and a window for each was chipped in the shell. Identical amounts of digitalis were applied, and each twin demonstrated a different cardiac arrest time. An occasional embryo did not cease cardiac action after a given amount of digitalis, but continued beating for hours. It was felt that any embryo that failed to show complete stoppage by thirty minutes also failed to demonstrate normal digitalis effects. As a result, every record over that arbitrary time limit was rejected. From one to three embryos in each series were in this category; therefore, it indicates that cardiac resistance was the decisive factor.

To establish the curve, eight series of one hundred eggs each were run. All embryos in a series were given the same amount of digitalis and treated in the same manner. Amounts, increasing by .025 cc. steps, ranged from .025 cc. to .2 cc. These arbitrary limits depended upon the total volume of fluid which the egg can accommodate without loss, and upon the least volume which can be measured and placed upon the embryo with ease.

In all cases, after delivery of digitalis, the pinch clamp was closed
and the tip of the nozzle touched to the embryo. This insured complete
delivery of the digitalis.

The results were treated mathematically after the entire investigation
was complete in order to avoid errors. Appendix I consists of the formulas
used in the statistical calculations. Following immediately are tables of
statistical results, in turn followed by a discussion of the tables.
Table 1.

Statistical treatment of experimental data.

<table>
<thead>
<tr>
<th>U. S. P. Reference Standard (cc.)</th>
<th>.200</th>
<th>.175</th>
<th>.150</th>
<th>.125</th>
<th>.100</th>
<th>.075</th>
<th>.050</th>
<th>.025</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Determinations</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>98</td>
<td>97</td>
<td>100</td>
</tr>
<tr>
<td>Mean (minutes)</td>
<td>4.87</td>
<td>5.16</td>
<td>5.72</td>
<td>6.05</td>
<td>6.64</td>
<td>7.12</td>
<td>7.63</td>
<td>8.20</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>2.48</td>
<td>3.20</td>
<td>2.56</td>
<td>2.42</td>
<td>3.98</td>
<td>2.98</td>
<td>3.47</td>
<td>5.25</td>
</tr>
<tr>
<td>Standard Error</td>
<td>.25</td>
<td>.32</td>
<td>.26</td>
<td>.24</td>
<td>.40</td>
<td>.30</td>
<td>.35</td>
<td>.52</td>
</tr>
<tr>
<td>Coefficient of Variation (%)</td>
<td>59.2</td>
<td>62.2</td>
<td>64.6</td>
<td>40.0</td>
<td>59.9</td>
<td>41.9</td>
<td>45.5</td>
<td>64.0</td>
</tr>
</tbody>
</table>

Table 2.

Time Range.

<table>
<thead>
<tr>
<th>Reference Standard (cc.)</th>
<th>Minimum Time (Minutes)</th>
<th>Maximum Time (Minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>.200</td>
<td>1.3</td>
<td>16.9</td>
</tr>
<tr>
<td>.175</td>
<td>1.1</td>
<td>17.0</td>
</tr>
<tr>
<td>.150</td>
<td>1.8</td>
<td>14.1</td>
</tr>
<tr>
<td>.125</td>
<td>1.3</td>
<td>15.7</td>
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<tr>
<td>.100</td>
<td>1.5</td>
<td>22.3</td>
</tr>
<tr>
<td>.075</td>
<td>1.5</td>
<td>18.8</td>
</tr>
<tr>
<td>.050</td>
<td>3.0</td>
<td>17.5</td>
</tr>
<tr>
<td>.025</td>
<td>2.0</td>
<td>28.0</td>
</tr>
</tbody>
</table>

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DISCUSSION

The statistical data, based upon experimental results indicate that the proposed method shows a reasonable correlation between the length of the time for the appearance of the end-point and the potency of the digitalis.

The Standard Error is greatest for .100 cc. and .025 cc. It may also be seen by Table 2 that the maximum time for the appearance of an end-point was also greatest for these two series.

In all series, a radical departure from the Mean time can be attributed only to an inherent susceptibility of the embryonic heart to the action of digitalis. This phenomenon occurs in humans, and is to be expected.

The Mean time, as plotted in Table 3, shows a relatively smooth, progressive curve with one exception: the .150 cc. series resulted in a Mean time that is .1 minute above that which is expected. The error lies within the Standard Error for that series.

A few observations in each series of the time of appearance of an A-V block indicates that the same type of group deviations from the Mean would have occurred if an A-V block had been chosen as an end point; however, the data are not included since it is impossible to determine the time accurately. Compared to results obtained by Paff, the Standard Error is greater in all cases. However, Paff's writings indicate that he did not encounter the cases of extreme variations from the Mean which increase the Standard Error and Standard Deviation.

Since it is an accepted procedure in analytical chemical assays to reject results that vary greatly from the Mean, the question arises as to whether or not this practice can be utilized in calculating the Standard
Error and Standard Deviation of this series.

Accordingly, those results that differed markedly from the Mean were eliminated, and the entire series were again evaluated. Reference to Table 5 and Table 2 shows which data were eliminated.
Table 4.

Statistical treatment of experimental results.

<table>
<thead>
<tr>
<th>U. S. A. Reference Standard (cc.)</th>
<th>.300</th>
<th>.175</th>
<th>.150</th>
<th>.125</th>
<th>.100</th>
<th>.075</th>
<th>.050</th>
<th>.025</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Determinations</td>
<td>27</td>
<td>22</td>
<td>97</td>
<td>93</td>
<td>93</td>
<td>92</td>
<td>92</td>
<td>92</td>
</tr>
<tr>
<td>Mean (minutes)</td>
<td>4.60</td>
<td>4.78</td>
<td>5.38</td>
<td>5.23</td>
<td>5.77</td>
<td>6.63</td>
<td>7.11</td>
<td>7.73</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>1.93</td>
<td>2.17</td>
<td>2.19</td>
<td>2.12</td>
<td>2.79</td>
<td>2.40</td>
<td>2.90</td>
<td>3.34</td>
</tr>
<tr>
<td>Standard Error</td>
<td>.20</td>
<td>.23</td>
<td>.22</td>
<td>.21</td>
<td>.29</td>
<td>.25</td>
<td>.30</td>
<td>.35</td>
</tr>
<tr>
<td>Coefficient of Variation (percent)</td>
<td>42.0</td>
<td>45.5</td>
<td>46.4</td>
<td>36.6</td>
<td>16.3</td>
<td>35.9</td>
<td>40.7</td>
<td>43.2</td>
</tr>
</tbody>
</table>

Table 5.

Time Range.

<table>
<thead>
<tr>
<th>Reference Standard (cc.)</th>
<th>Minimum Time (minutes)</th>
<th>Maximum Time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>.200</td>
<td>1.3</td>
<td>10.3</td>
</tr>
<tr>
<td>.175</td>
<td>1.0</td>
<td>10.4</td>
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<tr>
<td>.150</td>
<td>1.5</td>
<td>10.9</td>
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<tr>
<td>.125</td>
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<td>.100</td>
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<td>.075</td>
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<tr>
<td>.050</td>
<td>3.0</td>
<td>14.9</td>
</tr>
<tr>
<td>.025</td>
<td>2.0</td>
<td>15.4</td>
</tr>
</tbody>
</table>

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DISCUSSION

While greatly reducing the Standard Deviation and Standard Error, the exclusion of some results destroyed the progressive continuity of the Mean curve.

It is interesting to note that the arbitrary rejection of embryos in which an end point was not observed after thirty minutes did not complicate the final results. Since each series demonstrated at least one resistant embryo, it is valid to state that the extended appearance of an end point does not demonstrate an average reaction to digitalis.

In comparison to all other methods for the biological assay of digitalis that have been discussed, this chick embryo method is the simplest to perform, with the added advantage of the operator being able to observe the intact heart as digitalis exerts its effect upon it.

In addition to the simplicity, the method allows for the absorption of digitalis before it can affect the heart. As previously pointed out, any other method for the assay of digitalis does not take this important factor into account, and it appears that this is a major reason for the marked discrepancy between potency expressed in terms of biological assay and the results obtained therapeutically.

A definite correlation between time for the appearance of an end point, and the amount of a reference standard digitalis applied to an exposed, intact embryonic chick heart has been demonstrated by eight series of approximately one hundred datum each. Each series varied by .025 cc. of reference standard digitalis, and ranged from .025 cc. to .200 cc. The Mean has been determined in all cases and the entire series graphed. Statistical data have been computed.
It remains for future investigation to determine how accurately the potency of an unknown sample of digitalis, assayed by this method, can be determined when compared to therapeutic results obtained in humans.

CONCLUSIONS

The chick embryo method appears to offer several advantages over the present United State Pharmacopoeial cat method. The end-point is a measure of glycosidal activity rather than toxicity, since the glycosides are absorbed through tissue by the embryo method. Another advantage is the simplicity and ease of carrying on the analysis.

Adequate control of the quality of the chick embryos may be easily maintained as opposed to the random selection of cats used in the official method.
Appendix I

Statistical Formulas

Statistical calculations were made according to the following formulas:

\[
\text{Mean} = \bar{X} = \frac{\sum X}{N}
\]

\[
N = \text{Number of cases}
\]

\[
\bar{X} = \text{Guessed Mean}
\]

\[
f^d = \text{Deviation of midpoint of each group from Guessed Mean}
\]

\[
\Sigma = \text{Sum of}
\]

\[
\text{Standard Deviation} = \sigma = \sqrt{\frac{\sum f^d^2}{N} - \left(\frac{\sum f^d}{N}\right)^2}
\]

\[
N = \text{Number of cases}
\]

\[
\sigma = \text{Standard Deviation}
\]

\[
f^d = \text{Deviation of midpoint of each group from Guessed Mean}
\]

\[
\Sigma = \text{Sum of}
\]

\[
\text{Standard Error of Mean} = \sigma_m = \frac{\sigma}{\sqrt{N}}
\]

\[
N = \text{Number of cases}
\]

\[
\sigma = \text{Standard Deviation}
\]

\[
\sigma_m = \text{Standard Error of Mean}
\]

\[
\text{Coefficient of Variation} = V = \frac{100 \sigma}{\bar{X}}
\]

\[
\bar{X} = \text{Mean}
\]

\[
V = \text{Coefficient of Variation}
\]

\[
\sigma = \text{Standard Deviation}
\]

*Calculations were made by Mrs. E. E. Webb of the Department of Mathematics, University of Montana.*
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35. Dille, James M., Principles and Practice of Biological Assay, 3rd Revision, Seattle, Washington, Department of Pharmacology, University of Washington, 1940, p. 54.


