Digitalis assay / by W. Harrison Martindale.

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DIGITALIS ASSAY.

A comparison between Physiological and Chemical Results with an approximate simple Chemical Assay Method

BY

W. HARRISON MARTINDALE, *Ph.D. Marburg.*

For the purposes of this paper it will be well to briefly rehearse the knowledge of the Digitalis glucosides.

The glucosides chiefly concerned are Digitonin, Digitalin, Digitoxin and Digitalein.

It will be recalled that confusion arose at the outset by reason of the fact that Schmiedeberg* and Kiliani used as their raw material "Digitalinum Germanicum," which is mostly made from the seeds. They purified it by Tannin precipitation. In this way differences were found between the leaf and seed glucosides.

Digitalinum Germanicum consists of a mixture of Digitalin (Schmiedeberg), Amorphous Digitonin (50 per cent.) and Digitalein.

Digitonin Amorph. (Water soluble).—Schmiedeberg obtained this from Digitalinum Germanicum. Kraft suggests the name Digitsaponin for this body.

Digitonin Cryst. (Water soluble).—Kiliani prepared this also from the same material, and at first thought it to be a purified form of the last mentioned, but, on comparison with Schmiedeberg's original preparation, found that it was not; the

* For Schmiedeberg's original research see "Arch. Exp. Path. and Pharmakol." 3, 16; P.J., 3rd Ser., vol. v., 741, "Year Book of Pharmacy," 1875, 31.

name, however, was allowed to stand. It is obtained from seeds only.

Digitalin is an amorphous though definite (*seed*) glucoside. Kiliani called it Digitalinum Verum,* to distinguish from the various preparations of commerce. (It can be hydrolysed into Digitaligenin and Digitalose.)

Digitoxin is produced exclusively by the leaves (Kraft). Kiliani hydrolysed it into Digitoxigenin and Digitoxose.

Digitalein occurs in leaf and seed. Neither Schmiedeberg nor Kiliani could prepare it pure; the name stands for water soluble glucosides, which are certainly present. Kraft claims that it has been prepared in a pure condition as *Gitalin*.

Note that the above is by no means the order of importance. On the contrary Digitonin is relatively physiologically inactive, whilst the other bodies exercise well-known Digitalis action.

PREPARATION OF GITALIN.

Kraft's research (Arch. d. Pharm. 250, 1912, 118; see also Schweiz. Woch. Chem. Pharm., 1911, **49**, 161, 173) of the Harz leaves gave briefly the following:—

Cold water (1) was first used for extracting and then (2) *dilute* Alcohol.

(1) The *Aqueous Extractive* was precipitated with Neutral Lead Acetate and excess of lead in the filtrate removed by means of Sodium Phosphate. Concentrated Tannin solution (60 Gm. to the 2 kilos. of leaves taken) was used to precipitate the glucosides.

After precipitation he allowed the liquid to stand for 24 hours, collected, washed somewhat and pressed the resinous residue almost dry. He then proceeds as follows:—Rub the pressed cake with Zinc Oxide 30 Gm. and a little water to form a thick paste, dry with slight heat, powder and sift. Boil out three times with ½ kilo. of Methyl Alcohol (this decomposes the

* Kiliani, "Arch der Pharm.," 1895, 299; Y.B.P., 1896, 48. A method of preparation of Digitalinum Verum from Commercial Digitalin by Alcohol Extraction. Ether is added and precipitation effected with water.

Tannin Compounds and takes up the glucosides). Distil *in vacuo*—the syrupy residue contains all the water-soluble glucosides. The syrup (still warm) is shaken up with 100 Gm. of water, and after standing over night is filtered. A small amount of Anhydrogitalin remains as precipitate. Shake the Aqueous solution 20 times (five times is in reality sufficient) each with 40 Gm. Chloroform. Saponins remain behind whilst an active glucoside goes into solution. The Chloroform Extractive is dried by Sodium Sulphate and distilled *in vacuo* yielding crude *water-soluble active glucoside.*

The new active glucoside Gitalin can, it is stated, be prepared *direct* from the purified Aqueous extractive, as follows :—Shake out with Chloroform ; shake the chloroformic extractive with dry Soda and exsiccated Sodium Sulphate and then pour the Liquor into Petroleum Ether. Filter off the glucoside which is thrown out, dry it, purify by rapid crystallisation from cold dilute Alcohol, dissolve again in Chloroform, precipitate by adding to Petroleum Ether to obtain the easily soluble amorphous water-free modification—*Gitalin.*

The Saponins are fully described in the paper to which I refer—one of these appears identical with Schmiedeberg's Amorphous Digitonin, *Syn.* Digitsaponin Kraft, as already indicated.

PROPERTIES OF GITALIN.

Gitalin is described as an amorphous, white water-soluble glucoside (soluble 1 in 600 in cold water), insoluble in Petroleum Ether. A crystalline Hydrate can be prepared more soluble in *cold* than *hot* water, m.p. 150-155°. Gitalin rapidly decomposes in presence of almost all solvents except Chloroform and cold water. On heating a solution or keeping a solution in any solvent, except Chloroform, a precipitate forms which contains $1H_2O$ less, *i.e.*, Anhydrogitalin. This body is insoluble in most liquids. The yield of Gitalin is stated to be about 0·07%.

True Digitoxin is stated by Kraft to be insoluble in water. It could not be found in aqueous extractives of Digitalis leaves.

(2) *Alcohol Extraction.—Dilute* Alcohol must be used as otherwise Chlorophyll hinders the extraction without producing other or more glucosidal yield.

For 4·5 kilos. of the leaves previously extracted with water, 3 kilos. of Alcohol and 3 kilos. of water mixed were employed and the extraction repeated twice. For this quantity 0·5 kilo. of Lead Acetate in concentrated warm solution is used; the mixture is filtered and then 10 Gm. Calcium Carbonate added and the liquid evaporated to one-third volume. The precipitate which forms is collected, washed with 2% Soda solution, dried and boiled out with Chloroform 150 Gm. five times to yield Digitoxin contaminated with Gitalin and Anhydrogitalin. Directions for purifying are given by means of Ether, Benzol, &c.

Pure Digitoxin with the *Kiliani Test* gives a pure brown colour, and with the Keller-Kiliani test a brown ring at the juncture, and a blue to bluish green ring in the Acetic layer.

According to Kraft, Commercial Digitoxin on account of its admixture of Gitalin and Anhydrogitalin gives a violet tinge (? pink) to the typical *brown* of Digitoxin with the Kiliani Test.

(Care should be taken to distinguish in this paper the Keller-Kiliani Test from the Kiliani Reaction).

One may here mention *Nativelle's Digitalin*, which is generally considered as identical with Digitoxin. I have found the glucoside in the "Granules" of Commerce to yield a fine blue ring with the "K K" test and a pinkish brown with the "Fröhde mixed" reaction (to be described).

Flückiger, Neu Jahr. für Pharm., xxix, 129; Y.B.P., 1873, 226, provided a number of colour reactions for his specimen of Nativelle's Digitalin. The process for manufacture of Nativelle's Digitalin was communicated to the Moniteur Scientifique Questneville, February, 1867.

Nativelle (Jl. Pharm. Chem., 4th Ser. xvi, 430; Y.B.P., 1873, 223), writing on his glucoside, says, "It is *hardly* soluble in

water, 90% Alcohol dissolves it readily. Absolute Alcohol dissolves it less freely. Pure Ether only dissolves traces. Chloroform is the best solvent—dissolving it in all proportions."—See further Y.B.P., 1875, 25, for Improved method of Preparation.

The conclusions of Kraft's paper contain the following:—

Digitalein has been prepared pure as *Gitalin*. A *hydrate* $C_{28}H_{48}O_{10} + 4H_2O$ has been obtained crystalline. On heating and with reagents it changes easily to Anhydrogitalin; the latter can be hydrolysed into Anhydrogitaligenin and Digitoxose—hence for this reason it is allied to Digitoxin.

Digitalis leaves also contain the glucoside *Gitin*, which is inactive physiologically, closely allied to Digitonin cryst.

The leaves contain an amorphous saponin—a pentose derivative identical with Digitonin Amorph. Schmiedeberg from the seeds. For this the name *Digitsaponin* is suggested.

Digitoxin is a true glucoside. (Schmiedeberg states it is not.)

The so-called Digitoxin obtained in Keller's method of Assay consists principally of *Gitalin* with *little* Digitoxin.

Kobert, in a quite recent paper (Münch. Med. Woch., Aug. 20/12, p. 1865), in drawing attention to Withering's work (1785), points out that Withering's dosage was the equivalent of either five tablespoonsful of a 3·7 % *infusion* during a day, *i.e.*, 2·4 Gm. (approximately) of leaf or a *pill* containing at most 3 grains of powdered leaf* twice daily, *i.e.*, 0·3 Gm. in all—in other words, eight times as much *in infusion* form as in the form of powdered leaf.† The importance of this Kobert emphasises on the ground that the infusion is Digitoxin-free.

Kobert had shown the vasoconstrictor action of minute quantities of 10 different Digitalis glucosides fifteen years ago.

* Fothergill (B.M.J., No. 548, 553, Y.B.P., 1872) advocated powdered leaf in pill form in preference to the tincture. Administration by absorption through the skin by means of poultices and flannels soaked in the infusion applied to the abdomen and thighs where there is risk of disordering the stomach was spoken of at that time.

† Kobert's figures.

On this occasion he shows the constrictor effect of Gitalin (which he calls the only active substance in Digitalis Infusion) after previous dilatation with Yohimbine.

The constituents of Digitalis leaves and seeds may be divided according to Kobert. They both contain two groups of substances, viz., *Glucosides of the Digitalin group* and *Glucosides of the Saponin group*.

It has been shown that addition of a Saponin to a fine suspension of Digitoxin in water increases the action. Probably all the Digitalin group of bodies, and especially the absolutely insoluble ones in presence of Saponins, act more vigorously.

The majority of Saponins and several of the Digitalin group of bodies hæmolyse blood corpuscles, but Digitsaponin (soluble) and the insoluble Gitin do not possess this property.

Digitalis Infusion, Kobert repeats, contains only Gitalin and Digitsaponin, no Digitoxin. Infusion of the Seeds contains Gitalin and the water-soluble Digitonin of Schmiedeberg. An Infusion of the Seeds made with Physiological Salt Solution hæmolyses energetically, but the same strength or stronger leaf Infusion does not. If one extracts leaves or seeds even three times with boiling water, one does not render them inactive. The entire content of *Digitoxin*, *Digitophyllin* and *Gitin* remains, according to this authority, in the leaves. In the extracted Seeds a portion of the Digitalin and Digitonin of Kiliani remain, as all these substances are when pure only very slightly soluble in water.

As Leaf Infusion is therapeutically the best preparation, it follows that *Gitalin* is, in Kobert's opinion, an important substance in therapy. It is said by him to be present in "Digalen."

In therapy Kobert advises Digitoxin to be prescribed alternately with the Infusion to get the full effect of the leaf.

0·00025 Gm. of Digitoxin represents 0·1 Gm. of leaf = 0·25 %.

The following is a portion of a useful table on the latest aspect which is provided :—

Digitalis Purpurea.

Leaves contain				Seeds contain			
Heart Drugs.		Inactive Saponins.		Heart Drugs.		Active Saponins.	
Digitoxin. Toxiresin	*Gitalin.* Digitali-resin.	*Gitin.*	*Digit-saponin.*	*Digita-lin.* Digitali-resin.	*Gitalin* (?) Digitali-resin.	*Digitonin Schm.*	*Digito-nin Kil.*
Digitoxigenin	Digitoxose	Gitigenin	Digitsapogenin	Digitaligenin	Anhydrogitaligenin	Products not studied	Digitogenin
Digitoxose	Anhydrodigitaligenin	Galactose	Arabinose and glucose	Digitalose and glucose	Digitoxose	Decomposition	Galactose and glucose

ASSAY OF DIGITALIS.

Attempts to devise assay processes for Digitalis have been numerous during years past, and I do not propose to dwell more than briefly on these methods—they are readily available in pharmaceutical text-books, and comparatively seldom employed.

A few lines are, however, necessary to refer to the suggestions that have been put forward in the past.

Keller (Ber. d. Pharm., 1897, VII, 125; Y.B.P., 1898, 144) prescribed a *Digitoxin Assay process*, based on the ready solubility of Digitoxin in Chloroform and comparative insolubility of Digitonin and Digitalin therein. Basic Lead Acetate and Sodium Sulphate and Ammonia are used. Digitonin and Digitalin can also be assayed from the liquor after removal of the Digitoxin. The red colour obtained by treating Digitoxin with Sulphuric Acid alone was stated to be due to traces of "Digitalin" carried over in the Chloroform. He gives 0·26 to 0·62% as the content in the leaves, the amount in the seeds being considerably less.

Fromme (P.J. [4], **5**, 283; Y.B.P., 1898, 146), using Keller's process, assayed leaves of various kinds for Digitoxin from the Harz, Thuringia, Spessart, also English leaves collected in various months.

The content ranged from 0·1 to 0·3%, petioles contained frequently 0·2%, but no conclusion seems to be given as to the relative value of first and second year's leaves—the table covers a number of analyses.

Stoeder (Oest. Zeit. für Pharm., **39**, 836; Y.B.P., 1902, 71) directs a filtered aqueous extractive to be extracted with Chloroform and Ammonia. The Chloroformic solution is evaporated to a syrup—ether is added and then Petroleum Ether. The liquid is decanted and the precipitate collected and weighed. Aliquot parts are used throughout. Yield should be 0·25 to 0·35%. This is one of the simplest processes devised.—Note the method is in direct contradiction to the others quoted at commencement of this paper.

Gordon Sharp (P.J. [4], **14**, 236; Y.B.P., 1902, 228) stated that Digitalis Tincture is not so unstable as supposed. He suggested a Fermentation Test of the leaves, using Amygdalin. A portion of Digitalis leaves was to be placed in a bottle with water and a little Amygdalin. Another bottle was to be arranged containing the Amygdalin and the water, but no leaves. The one with the leaves should have a bitter almond odour after eight hours at a moderate temperature, and the other should be free from odour. A test with Fehling's Solution was also suggested. A sample of Tincture should not at once reduce this reagent showing that the glucosides are not decomposed.

Barger and *Shaw* (Y.B.P., 1904, 541) estimated Digitoxin on lines of Keller's process, and found it to be the chief active constituent of Digitalis Tincture. They evaporated the Tincture, using 146 Gm. of it, added water and precipitated with Basic Lead Acetate. Sodium Sulphate is subsequently added, and the liquor shaken out with Chloroform in presence of Ammonia, and this precipitated with Petroleum Ether and the Precipitate

treated further to bring it to a condition capable of being weighed. These workers found Digitoxin, Merck, to have a minimum lethal dose of 0·3 mgr. for a frog, as also had Nativelle's Digitalin cryst.—causing the heart to stop in systole, whilst the water soluble Digitalin and Digitalein caused it to stop in diastole. They found Keller's method to indicate only about half the Digitoxin really present in the tincture—much is lost in adding water and evaporating—the Digitoxin was found to be thrown out with the resin.

Barenstein (Pharm. Zeit. 1911, **56**, 128; Y.B.P. 1911, 118) estimated Digitoxin in radical leaves to be 0·53%, in flowers 0·58% (crude), in seeds 0·215%.

J. Burmann (Bull. Soc. Chim. 1912, **11**, 221; Y.B.P. 1912, 194) provides a detailed process for assay of leaves, in which there are some useful "tips"—one of these is to avoid rubber corks in the apparatus. The final Glucoside obtained by Petroleum Ether precipitation is weighed and reckoned to consist of about 77% pseudo-digitoxin and 23% Digitoxin.

The general conclusion seems to be that Digitoxin content ranges from about 0·1 to 0·3% in the leaves, whilst the seeds yield less. It is said (N.S.D.) to be most abundant in the leaves in August, hot, dry weather increasing the content.

These are a few abstracts of the work that has been done, not to mention the physiological methods, to which reference shall be made later.

It occurred to me that there would be considerable utility and value in a simple mode of assay, if such could be devised—a process, in fact, which would, if possible, render the pharmacist in future independent of the physiologist. This is the main theme of my paper. At first, however, a comparison between results obtained by a physiologist of repute with existing approximate tests seemed imperative.

It is well known that great diversity of strengths exists in

Digitalis leaves collected at different seasons in the same locality; also that the soil, the prevailing climatic conditions and so forth, may cause marked variation in activity; hence, a systematic study of diverse leaves was resolved upon. The information gained in this manner is divided into four portions :—

(1) Examination of infusions of various leaves, and

(2) Tinctures of the same by available approximate chemical tests.

(3) The physiological examination of the Tinctures.

(4) The devising of an approximate simple chemical assay method and the comparison with physiological results. Glycerin Extractives and Tables of Solubility and Colour Reactions.

The general opinion seems to have been that an Assay process should be based on Digitoxin content—the reason being apparently that the water-soluble glucosidal constituents are not so well defined. (It has been stated that there is a fairly constant ratio between chemical assays based on Digitoxin and physiological results on guinea pigs.) In view of the fact that the water-soluble bodies are by no means therapeutically impotent, and that they are obviously present when the powdered leaf or the infusion are given—all of which receive praise on various sides—it seems a pity that they cannot also be brought into a method of assay, however approximate.*

Preparations of leaves in the form of Infusion and Tincture from various parts of Great Britain, also from several European countries, were examined (see Table, Column 1). Glycerin Extracts and a Tincture of the Seeds were also included. I took occasion to write to various parts of the world for good-sized

* Note.—Many references are, of course, to be found in Digitalis literature, pointing to the fact that Digitoxin does not represent the entire physiological action of the plant. England (Am. Jl. Ph., 1899, 379; Y.B.P., 1900, 122), for example, quotes K. Hofmann to the effect that : "The effects of Digitoxin are not seen in less than six hours. Digitalis and some of its principles produce their effect in half an hour."

samples of leaves, thinking that their examination (see Table) might throw some useful light on the subject. The following, however, could not help me with supplies, for reasons given :—

Argentine.—None grown so far as could be ascertained. All imported from Germany.

Australia.—My friends were unaware that any is grown.

Greece.—Digitalis grows native only in small amount.

India.—The systematic cultivation of Digitalis is no longer continued, but a 2-lb. sample of leaves was kindly obtained from the Kumaun Government Gardens (see Table).

South Africa.—My friends had tried on several occasions to grow Digitalis, but could not meet with success.

U.S.A.—Unable to obtain supplies grown in U.S.A. Was informed that all the leaves handled there are imported from Europe.

I.—EXAMINATION OF INFUSIONS.

Initially the Keller-Kiliani test for Digitoxin was applied in the manner given in P.G.V.

Experience with this test shows that the coloration varies greatly with the manner of procedure (amount of mixing, also the Chlorophyll content). Applied, however, to a simple Acetic Solution of Commercial Digitoxin the colour was typical.

1 in 2,000 gives a "strong" reaction in about 10 to 15 seconds with blue changing to *green* colour above.

1 in 5,000—a "weak" reaction with *blue* colour above.

1 in 10,000—a very weak reaction with blue. The last two take somewhat longer to produce.

(These were shaken slightly so as to mix and create a zone of about 0·5 to 1 Cm. in a 1 × 5 Cm. test tube.)

To ascertain to what extent Digitoxin can be detected in an *Infusion* of the drug, Commercial Digitoxin to the extent of 0·001 Gm. was added to 20 Cc. of Infusion as prepared for the test (*i.e.*, 0·1% in the leaves) using the Infusion of the "C" leaves which gave a weak "KK" reaction from their Infusions

in the Table. (The mgr. was mixed with the powdered leaves before making the infusion). The result was as follows:—

Control Infusion (without addition) = olive-green above and brown-red below.

Infusion containing Digitoxin = same as above; there was no appreciable difference.

It is clear, therefore, that the Test is by no means delicate. At a subsequent stage the detection of Digitoxin in Infusions is referred to again. (At the time that much of this early experimental work was done the recent statements as to the insolubility already dwelt upon had not been seen.)

In Column II. of the table are given the data obtained with the various samples.

II.—CHEMICAL EXAMINATION OF TINCTURES.

The Keller-Kiliani Method of examination of the leaves, as given in P. Helv., seemed first worthy of trial.

10 Gm. of the Tincture with 10 Gm. of water are evaporated to 10 Gm. on the water-bath and filtered. After precipitation with Liquor Plumbi Subacetatis the filtrate is shaken out with 10 Cc. of Chloroform, the Chloroform evaporated, and the residue dissolved in 3 Cc. of Acetic Acid, to which a trace of Ferric Chloride is added and "layered" beneath with Sulphuric Acid. Coloration is produced. It is not satisfactory on account of the Chlorophyll admixture.

The *Hungarian Pharmacopœia* directions are practically the same, thus:—Dissolve the dry residue from 10 Gm. of the Tincture in 10 Cc. of hot water. Add whilst shaking or stirring 5 drops of Basic Lead Acetate Solution and filter into a separator. Add 10 Cc. of Chloroform and shake for two minutes thoroughly. Evaporate and add 3 Cc. of Conc. Acetic Acid, to which 1 drop of N/2 Ferric Chloride Solution is added and "layer" on to Sulphuric Acid. A Carmine Red Colour is produced at the juncture with bluish ring above.

In addition the following test (in P. Helv.) was conducted :—

Digitalis Tincture 2 Cc. are mixed with 10 Cc. of water and filtered.

A little Tannic Acid Solution added produces a milky opalescence.

In Column III. in the table are the results with the various leaves by the Hungarian Pharmacopœia test, and in Column IV. the Tannin Test Results.

LIMIT OF DELICACY OF THE KELLER-KILIANI TEST

The Test in examining Tinctures is a qualitative one of fair delicacy, as the following showed :—

(*a*) Application of the Test to a Tincture of a Drug containing no glucoside. Result—No blue or green coloration, but brown zone as anticipated.

(*b*) Addition of Commercial Digitoxin in certain proportions, e.g., 0·001 Gm. and upwards to 10 Cc. was clearly discernible.

We should expect this proportion (0·01%) *at least* in a B.P. Tincture. The leaves contain on an average 0·2%* and the Tincture being 1 in 8 should contain on an average 0·03%. It became imperative to determine whether the test could be clearly seen with a *weak* Tincture, e.g., 1/10 of the average ; in other words employing 0·0003 Gm. Digitoxin in 10 Cc.—this was *not* clearly visible. 0·01% (1 in 10,000) is about the limit of delicacy.

TANNIN TEST.

The Tannin Test (1 Cc. of 50% Tannin Solution was added to 12 Cc. of the mixture in the test) gave anomalous results relative to the Keller-Kiliani test—this may be due to the Tannin

* Note.—Kiliani, Arch. Pharm., 1899, 446, Y.B.P., 1900, 45, states : Digitalein occurs in Infusion and in the Tincture. The Infusion from 10 kilo. of leaves yielded 2 Gm. of a product 0·6 mgr. of which produced systole in the frog, while the Alcoholic Extract from 10 kilos. yielded 150 Gm. of a body, 1·5 mgr. of which produced systole—naturally these would not be in a state of purity.

indicating all the glucosides, whilst the Keller-Kiliani test indicates roughly Digitoxin only. The results with the test are given in Column IV., whilst in Column IVa results are shown with a hot water infusion 1 in 10 under the same conditions.

In Column V. are the extractives and the specific gravities of the Tinctures.

ATTEMPT TO FIND A BETTER TEST THAN THE KELLER-KILIANI TO INDICATE DIGITOXIN IN AN INFUSION OR TINCTURE.

Effect of Reagents on Digitoxin of Commerce both Solid Substance and in Solution.

Many attempts were made empirically to discover if possible some new coloration or reaction. These were taken as a matter of routine—not with the idea that any important conclusion could be drawn.

A Solution 1 in 5,000 was prepared by diluting a 1 in 2,000 Acetic Acid Solution with water, and was used where "Solution" is indicated in the following :—

Alkaloidal Reagents, e.g., Mayer's Solution, Gold Chloride, Dragendorff's, &c., gave nil.

Calcis Chlorinatæ Liquor gave nil with solution and solid.

Chromic Acid Solution—Nil.

Fröhde's Reagent (layered), i.e., Sulphuric Ammonium Molybdate, gave blue ring as the Keller-Kiliani Test on solution and reddish brown colour with solid substance changing to violet brown.

Hydrokinone and Acid gave a blue upper layer, but only about half as delicate as the Keller-Kiliani Test.

Mandelin's Reaction gave brown as with Sulphuric Acid alone.

Nitric Acid alone on solid substance gave reddish violet gradually changing to yellowish brown, but nil with solution.

Phenol-Disulphonic Acid (as used for estimating Nitrates) layered under 1 in 2,000 Solution in Acetic Acid gave blue green layer, but Sulphuric gives this alone.

Phloroglucin and Vanillin Solution evaporated with the Solution gave nil.

Phospho-Molybdic Acid (Sonnenschein's Reagent)—Nil.

Phospho-Tungstic Acid—Nil.

Potash Solution and Bromine Water—Nil.

Potassium Iodate on Solid Substance.—Nil.

Pyrogallol.—Nil.

Rimini's Aldehyde Test on Solution.—Nil.

Sodium Nitrite on Solution and Solid.—Nil.

Sodium Peroxide on Solid.—Nil.

Note.—It was determined that the Keller-Kiliani Test must be conducted with concentrated Sulphuric Acid.—Dilute Acid is useless.

Tests applied both to Commercial Digitalin and Commercial Digitoxin for comparison.

Brunner's Test (Sulphuric Acid and Bile) gives a better red colour with Digitalin without the bile added.

Buckingham's Test.—A Sulpho-Molybdic Acid Test similar to Fröhde, but very much stronger, namely 1 in 16, gives an intense blue upper layer with a 1 in 2,000 Solution of Digitoxin and with Digitalin, but to a less extent. It gives, however, a colour with other tinctures, e.g., that of Hyoscyamus treated when used as a layer test. The Test gives a blue colour of similar depth with 1 in 10,000 Digitoxin and 1 in 2,000 Digitalin.

Czumpelitz's Reagent (Zinc Chloride, Hydrochloric Acid and Water) gives yellow solution and green specks with Digitoxin, brown with Digitalin on solid substances.

Dragendorff's Chloral Test.—Nil with Digitoxin, not very characteristic with Digitalin.

Dragendorff's Bromine, Potassium Hydrate and Sulphuric Acid Test.—Red with Digitalin, brown with Digitoxin.

Erdmann's Nitro-Sulphuric Acid Test.—Red with Digitalin, brown with Digitoxin, not changing to any great extent.

Fröhde's Test (Ammon. Molybdate 1 per cent. in Sulphuric Acid) is the most distinctive test for Digitalin. On solid substance gives cherry to violet red with Digitalin, and dark orange to brown with

Digitoxin, according to concentration. See also Table of Colour Reactions.

Grandeau's Bromine and Sulphuric Acid Test.—On solid substances red with Digitalin, brown with Digitoxin.

Kiliani's Test (Ferric Sulphate 0.05 gm. dissolved in water 1 Cc. and Sulphuric Acid added to 100 Cc.) gives exactly the same colours as Fröhde's Reagent (*c.f.* Table).

Lafon's Test (Alcohol and Sulphuric Acid).—Digitalin yellow, Digitoxin violet brown.

Linke's Test (Aldehyde and Sulphuric Acid).—Not characteristic (yellowish brown with each).

Experiment to Determine the Cause of the Blue Colour by the Layered Fröhde Test (Blue Ring.)

Assuming that the colour is either a matter of oxidation or reduction, numerous substances were tried in Acetic Solution.

The following gave a blue ring similar to that with "Digitoxin":—

Glacial Acetic Acid alone—slight, after a short time.

Acetic Acid diluted—slight, after a short time.

Both the above are very much less than is produced with a 1 in 30,000 solution of Digitoxin.

Sodium Bisulphite.

Ferrous Sulphate (strong reaction).

Formaldehyde.

Phenol (strong reaction).

Vegetable extracts and similar substances often give a dark green colour.

Hydrogen Peroxide, also Nitric Acid, do *not* cause blue coloration.

The test, therefore, seems to be due to reducing action. The substance known as Blue Oxide of Molybdenum, formed by action of reducing agents on acid solutions of Molybdic Acid, is probably of formula Mo_3O_8.

In no case was the typical *pink* colour produced *on mixing*—hence of the two, the Fröhde mixing test is obviously more definite than the layered test.

Similar Experiments with the Keller-Kiliani Reaction.

On the above lines of all the substances tried in the experiments, both reducing and oxidising agents, *none* gave the blue ring.

The conclusion is that the Keller-Kiliani test will distinguish actual Digitoxin from other possible confusing substances better than the layered Fröhde test, but I claim that the glucosidal residues obtained by my finally adopted method are sufficiently pure to prevent confusion of this kind and that the mixing Fröhde test can be used to indicate water-soluble glucosides and will show Digitoxin also if present—it shows water-soluble by the pink colour and Digitoxin by the brown—the presence of the latter can be confirmed by the layering method.

It is in fact a most useful test capable of use in the two ways and more sensitive than the Keller-Kiliani Reaction.

Notes from Various Pharmacopœias.

The British, United States, Belgian and Danish Pharmacopœias give no remarks on the assay of leaf or tincture.

The Dutch Pharmacopœia employs the Keller-Kiliani test on the Tincture using Basic Lead Acetate as in the *Swiss Pharmacopœia*, *q.v.* page 14.

The Hungarian Pharmacopœia mentions the bitter taste regarding the leaves, and requires that an infusion 1+10 cooled should give a copious precipitate with Tannic Acid Solution which on adding a further quantity of Solution redissolves only with difficulty. For Tincture see page 14.

The Swedish Pharmacopœia specifies that the leaves using 0·1 Gm. are to give the Keller-Kiliani Test for Digitoxin.

The Swiss Pharmacopœia specifies Powdered leaf (F.I.) not

to be kept longer than one year. The tincture in this instance is tested for Digitoxin, *c.f.* page 14. A Tannin test is also given, *vide* page 15.

III.—PHYSIOLOGICAL ASSAYS.

In Column VI. in the Table the Physiological results on frogs are indicated in the customary manner, *i.e.*, the minimum lethal dose per 100 Gm. body weight. If the amount required to kill the frog is in the neighbourhood of 0·75 Cc. per 100 Gm., the Tincture is designated a "standard" one, by general consensus of opinion in this country. A minimum lethal dose of less than this quantity indicates a strong or "above standard" Tincture, and *per contra* if *more* is required for the M.L.D., a weak Tincture is under consideration.

My physiologist reports, "On the whole, therefore, these Tinctures are well up to standard.

"Tested on the excised frog's heart it was found that after removal of its Digitoxin, in which it was rich,* the Seed preparation 'L' proved to be weaker than an ordinary Tincture, though its M.L.D. (0·6 Cc. per 100 Gm. frog) was still low." The physiologist goes on to say that as it appears to be therapeutically weaker and yet more toxic than leaf Tincture it does not seem a desirable preparation. Digitoxin is generally considered to be irritant to the alimentary canal.

"One must not place too much reliance on comparison of 'L' with the others by simple M.L.D. tests, as seeds and leaves differ in their saponins as well as in their useful glucosides. It is desirable to manufacture one's Digitalis Tincture at least thrice a year."

A few references to past work will now be desirable.

The period for gathering Digitalis was being discussed as far back as 1870, and probably earlier—vide Bull. Soc. Pharm., 1870, 164; Y.B.P., 1870, p. 75; also Y.B.P., 1871, 97. The result of an investigation showed then that leaves are best

* Digitoxin is not usually viewed as a seed constituent to any extent.

collected at the end of August or beginning of September, with even the radical leaves of the plants, which are not to flower until the following year. With these an infusion was obtained, which gave the wished for (chemical) reactions immediately—Ferrocyanide and Tannin precipitates.

Bernbeck (Ph. Zeitung, 1879, 506; Y.B.P., 1880, 173) found "by chemical analysis" second year's leaves better than first year's.

Ziegenbein (Arch. Pharm. 240, 454; Y.B.P., 1903, 208), writing on the fallacy of valuing Digitalis on the *Digitoxin content*, says:—Physiological Tests are alone reliable. There is no relation whatever between Digitoxin content and physiological activity. Thus, leaves containing only 0·125% of this Glucoside are twice as toxic as those containing 0·226%. It was also shown that a solution of pure Digitoxin in the same proportion as found in the leaves is from two to six times less active than an equivalent quantity of the soluble extract of the same leaves.

Martin, Brit. Pharm. Conf., 1909, gave a very complete account of the method of procedure, selection and variation of frogs, &c. Digitalis Tincture, according to him, retains its activity for nine months certain.

Burman, Schweiz. Woch., 1910, 410, per Merck; Berichte for 1910, p. 159, states "physiological standardisation shows only toxicity, but not curative action. Chemical investigation more trustworthy."

Though many workers are in favour of physiological methods yet just as existing simple chemical means of assay of Digitalis have been seen to be unsatisfactory, physiological methods are, I think, not very much better.* We have, for example, the

* I have heard of a case in which a manufacturing chemist submitted a double strength Tincture for examination, the report being "M.L.D. = 0·9 Cc." Not satisfied with this result the Tincture was submitted to another physiologist, who gave "0·35 to 0·4 Cc." The former result was undoubtedly incorrect. It is only fair to say that in my own experience I have not come across such great variations as this in physiological reports.

existence of differences in standards adopted (though 0·75 Cc. per 100 Gm. frog is, I believe, generally accepted) and the variation in reports, frog variation, &c.

Physiologists frequently differ; for example one reads:— "Physiological tests have shown second year's leaves are stronger than the first (*Farr* and *Hynes*, P.J., i./07, 198, also Jl. Am. Ph., July, '08, p. 330), *i.e.*, in proportion of 10 to 8¼. This may be due to petiole excess in the first year's growth." Another physiologist (*Hale*), however, says first year's are 28 to 40% more active than the best second year's obtainable. *He disposes of the idea that the leaves of wild-growing plants are more potent than those of cultivated ones*; nevertheless, some of the former are more potent than the latter, but cultivation *per se* has nothing to do with the fact. The real cause of the variation in the potency of Digitalis leaves seems to be connected with the nature of the soil and the character of the season, &c.—P. J., i./11, 578.

With regard to the activity of Digitalis at various times, *Focke* states that second year's leaves have their highest value at the time of flowering and the first year's leaves attached to them the highest value in late summer.

Recently, *Gordon Sharp* and *Lancaster* found that active preparations can be made with the first year's leaves or with the leaves of second year's plants before flowering. Dried leaves kept for ten years gave active Tinctures. Tinctures retain activity for a year. Therapeutic trial is necessary in addition to animal experiments.—P.J., i./11, 102.

Alexander Goodall, B.M.J., i./12, 887; Y.B.P. 1912, 437, employing as Standard 15 m. per 100 Gm. frog, found of 23 samples of Tincture 12 to be average, 6 under and 5 over average. He also goes fully into the question of the keeping qualities of the Tincture.

Gordon Sharp, B.M.J., July 6, 1912, p. 47, says that contrary to Dr. Goodall's finding there is no great variation in potency in Tinctures made by Chemists of repute. He is sure

that variation in his own city does not exceed 10 %. (See also F. W. Price, B.M.J. i./12, 1514.) He enquires as to the 'end points' in Goodall's experiments on frogs. Must the frogs be quite limp—the peripheral circulation stopped and all three heart chambers still within four hours with the 3 m. dose for 20 Gm. frog? One may find a frog active or with the circulation in the web active and yet the ventricle is still in systole when the chest is opened.

Goodall subsequently (B.M.J., July 20, 1912, p. 149) said his Tinctures were laboratory samples, not those of trade. His end point is absolute death in four hours.

Goodall says further, if the Tincture given to a patient is weak the medical man will increase the dose—if too potent the practitioner's opportunity may cease entirely.

FOCKE'S PHYSIOLOGICAL ASSAY METHOD.

At this point I should like to break off to bear briefly on Dr. Focke's Physiological Assay Method, which has been used in Germany. (See "Archiv. der Pharmacie," 1910.) *Rana temporaria seu muta* is to be used. From May to October both male and female are suitable, but from November onwards only males can be employed. He has used frogs from weight of 18 to 40 Gm.

For the experiments he employs Infusions (1 : 10), but these are not filtered bright through paper—on the contrary they are muddy filtrates obtained by using a piece of an old linen handkerchief. It appears that variable amounts of the "colloidal substances" are kept back by using good filter paper —results with (clear) filtered solutions being only about one-third of those *not* filtered.

In making the infusion 24 Cc. of water are made alkaline by adding 8 drops of a 5% Sodium Carbonate Solution, and this is boiled and poured on to 2 Gm. of the powder and allowed to stand 30 minutes.

Preparations containing Alcohol, Chloretone, &c., have to be specially treated. Those containing Glycerin cannot be examined by the method as it is stated that (as in the case of Digalen which contains 25%) absorption is hindered to a high degree.

With co-operation of large growers in Germany a value, "V" = 4 or 4·5, seems to have been taken as a Normal.

Powdered leaves retain their full activity for a length of time, *e.g.*, a V = 4·3 in 1903 was the same after seven years.

Then follow directions for maintaining the temperature of the frog (18 to 20°, according to the month) and the details as to the Syringe, etc., used. In the technique of the method one reads: The frog is bound to the board with thick string in such a manner that to improve the circulation the limbs are stretched in a sharp angle. With forceps and scissors a strip is cut away from the breast, and the heart belt exposed. By slight pressure on the abdomen the ventricle is made to exude. Two frogs are prepared in this manner. They are then warmed for a quarter of an hour to bring up the temperature to that employed. The rhythm and beat of the heart is observed. An animal that shows too sluggish a movement is rejected and another one taken. After the injection of the Solution (about ½ to 1 Cc.) just above the knee and after arrest of the heart action the

$$\text{Valor} = \frac{p \text{ the weight of the frog}}{d \text{ (dose of the 10\% infusion)} \times t \text{ (time for arrest)}}$$ is

obtained, *e.g.*, $$V = \frac{20 \text{ (Gm.)}}{0{\cdot}5 \text{ (Cc.)} \times 10 \text{ minutes.}}$$

$$= 4.$$

Discussing the "humanity" of the method, the author brings as his cover that millions of animals, horses, oxen, sheep, pigs, &c., are castrated annually; further, that a frog, if wounded in the trunk or limbs, uses them as strongly as ever, unless there is great loss of blood or the wound is too severe. If there were pain this would, according to him, be impossible. Further it is made out that a dog allows itself to be tended for a wounded

foot with apparent gratitude by the veterinary surgeon because *rest* is necessary for the healing, but with frogs and other lower animals in which even a bad wound heals without resting, pain, which would stop them, would not only be useless but annihilating to their existence. It is conceded that the frog would notice something that was occurring to him, but that it feels no pain. In conclusion, it is claimed that in the 12-hour method advocated in America the heart in the non-narcotised animals must go through a peristalsis of one to three-quarters of an hour, whilst cyanosis and asphyxiation slowly set in. In Focke's method, on the other hand, from the commencement of peristalsis to heart-arrest occupies one to three minutes without asphyxiation setting in. In the examples given actual arrest takes place in times in the neighbourhood of ten minutes—this is deemed an average—in fact the mean time of 8½ to 10 minutes has to be arranged for by adequate dose, concentration, &c.

In the same paper Focke makes a comparison of the various Physiological methods for standardising in vogue :—

(1) The killing methods on mice, guinea-pigs, cats, frogs, *i.e.*, minimum lethal dose methods ;

(2) Frog heart-arrest methods (*a*) after one hour, (*b*) according to Focke's method, (*c*) on the isolated heart transfused with Ringer's Solution, with which the substance to be tested is mixed ;

(3) Blood pressure measurements on cats.

Houghton's "one hour arrest" method entailed knocking the frogs on the head first of all, but as this in some instances may have entailed bleeding and consequent irregular heart-action, the method is not favoured by Focke.

In Houghton's "kill in twelve hours" method a matter of at least twenty frogs are wasted for each experiment. Houghton's method of "Heart Tonic Units" is greatly condemned by Focke—action, according to him, of such various heart tonics is not parallel with the action on the human being.

Then follows a criticism of Martin's "quick-kill" (two hours) method, in which one does not observe the arrest of heart action, but lack of response to pinching, also by aid of the microscope one has to observe cessation of peripheral circulation.

The paper in question concludes with a reference to the work of Schmiedeberg, whose researches date from 1874. Schmiedeberg deems results from animals only "helping figures" to reduce laboratory work, but they will not help dosage at the bedside.

Focke in another paper (Archiv. d. Pharmacie, 1911) deals further with the question of the effect of use of filter-paper on Digitalis Infusion for Physiological Assay. In this communication he negatives his previous statement. It is stated as a conclusion that filter-paper of certain kinds does *not* affect the value of Inf. Digitalis.

Hence one must assume from this that the bulk of the glucosidal constituents required for the assay is in a soluble condition.

In view of the fact that ½ doses of a 20 % Infusion had the same action as whole doses of a 10 % preparation, it was thought that a 10 % Infusion was nothing approaching a *Saturated* Infusion. In this paper he set himself to determine the amount of loss in value with a high concentration.

Experiment showed that a fluid extract made 1 = 1 with about 35 % Spirit, diluted with water to the Infusion strength (1 : 10), had the same strength as the infusion, *showing that there was no loss even in a 1 in 1 preparation.* (Glycerin, as already explained, was unsuited to the test.)

Again, a simple Aqueous Infusion (using no Spirit) of strength 1 in 2 can retain the active principles, i.e., *there can be no question of saturation in a 10 % preparation.*

Further work showed that a *10* % Infusion contains in reality about 80 per cent. of the active principles. With special care and secondary washing 85 % can be included. A *10* % Infusion made by Focke's method contains about 74% of the active

principles. A second washing will bring the strength up to 85 %.

In spite of this, and for various reasons, he does not propose to alter his general method for determination of the Value V. The reason *why* a 10 % Infusion, although by no means saturated with the water-soluble active principles, leaves about 11 % of them in the leaves, Focke says, must be the subject of further research.

I may add that the leaves "R" in the Table had a "Value" according to Focke of 4·0. This evidently supports my figure "2" in the Table (Column VII.) for the Tincture of the leaves in question.

IV.—THE DEVISING OF A SIMPLE CHEMICAL ASSAY METHOD.

At the conclusion of these Physiological Tests, and as they were not altogether in accordance with the preliminary Keller-Kiliani results, it was thought desirable to work on a Digitoxin-free tincture, or, rather, a water extractive of the residues obtained on evaporation of the tinctures, the idea being, if possible, to obtain data indicating active water-soluble glucoside effect rather than Digitoxin effect.

It will be clear that in the physiological assay methods, there is no great attempt at separation of the effects of the various constituents of Digitalis—these methods give simply a conjoint killing power—the constituents might be present in very varying proportion; but a simple chemical separation process on these lines does not appear practicable.

Preliminary Tests.

Ten Cc. of each of the Tinctures were evaporated, and the residues taken up with 10 Cc. of water and insoluble matter filtered out in the cold; the filtrates were evaporated to 1 Cc. or less, and extracted with Chloroform; the Chloroform evaporated and the residues taken up with water. The solution in each

case was filtered and evaporated, and the residue dissolved in 2 Cc. Glacial Acetic Acid and 0·2 Cc. of this solution mixed with 1 Cc. of Fröhde's Reagent in a narrow test tube.

Second Preliminary Test.

Another Series of Experiments by this Preliminary Test was conducted, but by using only 2½ Cc. of water to take up the evaporated residue of the Tincture (this would reduce the Digitoxin content, assuming that it is soluble, and increase the water-soluble glucosides proportionally to the Digitoxin). The water extractive was evaporated to dryness and taken up with Alcohol (5 Cc.) instead of using Chloroform (the water soluble glucosides being more soluble in Alcohol than in Chloroform). The Alcohol extractive was evaporated and the residue dissolved in 2 Cc. of Glacial Acetic Acid and tested (0·05 Cc. of Solution) as by the Preliminary Test, *i.e.*, by mixing with 1 Cc. of Fröhde's Reagent.

Solubility of Digitoxin.

Digitoxin is only very slightly soluble in water, by experiment about 1 in 20,000. This solubility was corroborated by washing out Commercial Digitoxin repeatedly with water and applying the actual Keller-Kiliani and Layered Fröhde Tests; the response was typical in both cases. It is true there may be some Saponin body or impurity that assists its solution. Our physiologist states Digitoxin is sufficiently soluble to produce physiological effect, but whether this solubility is caused by concurrent impurity is uncertain. As much as 0·003 Gm. could theoretically be present in the 10 Cc. of Tincture; hence the 10 Cc. of water used in the first method was not sufficient to dissolve this theoretical quantity of Digitoxin. The idea of separating the assay in this manner was discarded in favour of a joint assay method. (See Inset opposite page 36.)

The colours produced with the Tinctures C.D.F.H.I.J.K.L.M. and O. in the Preliminary Tests agreed in proportion with the

physiological results, but the Acetic Solutions were not nearly sufficiently pure, especially those of the Alcohol extractives. (Note.—The colours had no relation to the percentages of extractives, they were undoubtedly due to the very impure glucosides.) It became necessary, therefore, to devise some more stringent mode of procedure.

Digitoxin and Digitalin Additions to Tinctures.

To determine whether Digitoxin was carried through and interfered with the results by the "Fröhde mixed" test, experiments were made by adding both Commercial Digitoxin and German Digitalin to a blank Tincture (Tinct. Agropyri was taken). German Digitalin gives reactions similar to Digitalein, Gitalin, and probably Digitsaponin of the leaf. The amounts added were 0·04 % (=1 in 2,500) of the Tincture (= 0·32 % of the leaves) in Digitalin, and 0·02 % (= 1 in 5,000) of the Tincture in Digitoxin = 0·16 % of the leaves.

The results with the first Preliminary Test were :—

No. 1, with addition of both Digitalin and Digitoxin = Strong (pink).

No. 2, with addition of Digitoxin only = Very weak (light pink).

No. 3, with addition of Digitalin only = Strong (light pinkish brown).

Control—(no addition) — Negligible colour.

The conclusion of this series was that although the test will indicate very clearly by the pink colour a proportion of 1 in 5,000 of Commercial Digitalin in Acetic Acid, when one adds even double this proportion (1 in 2,500) to a Tincture containing Chlorophyll there is no marked distinction between the colour produced by this and by a "Digitoxin addition" of the same strength. Nos. 1, 2, and 3 were repeated in order to eliminate the possibility of error by the method, but the same result was reached. It was found that whilst

a very characteristic pink is given by a simple Acetic Solution of "German Digitalin," and a brown is given with Digitoxin, when we conducted the above test by carrying the glucosides through a tincture "German Digitalin" produced a pinkish brown, and Digitoxin produced a light pink. There also is much loss. The Chlorophyll is difficult to get rid of; if it passes through to the final Acetic Acid Solution there is confusion of the colour—the typical pink "Digitalin" colour is hindered. There is also a possibility that Commercial Digitoxin contains some Digitalin-like body. Hence the question at issue—whether Digitoxin is carried through under the conditions of the test—is to be answered to the effect that Digitoxin probably passes through in proportion to the volume of liquids employed, but this "Addition" experiment was inconclusive for reasons stated. The matter is reverted to again later.

Additions of German Digitalin to Tinctures Giving Respectively a Weak and Strong "Fröhde" Reaction.

The next step was to add 4 milligrammes of Commercial Amorphous Digitalin to 10 Cc. of Tincture "D" (a weak one), and to 10 Cc. of Tincture "J" (a strong tincture), and to test by the first Preliminary Test, comparing with controls without these additions. In both cases a distinct increase in the colour reaction was seen.

Tests Devised on the Lines of Kraft's Methods (Archiv. der Pharm., 250, p. 118).

Kraft in following up Schmiedeberg's methods of isolation proceeds first with the water-extractive of the leaves from which he obtains his "Active Water-Soluble Glucosides," Gitalin, &c., and then with an alcoholic extractive for Digitoxin, &c, as already indicated.

Somewhat on these lines the following were conducted;—

(1) Took 10 Cc. of the tincture, added 10 Cc. water, precipitated with about 2—4 Cc. or *q.s.* of 10 % Neutral Lead

Acetate Solution, filtered. Removed excess of lead with Sodium Phosphate. Filtered, evaporated, extracted the residue by repeated treatment with Chloroform (5 to 10 times necessary) and tested with Fröhde's Reagent.

Result.—Typical pink colour, but absence of blue ring with the Keller-Kiliani test.

(2) Took 10 Cc. of the Tincture, added water, treated with lead, filtered (no Sodium Phosphate used in this instance), added a small quantity of Calcium Carbonate to the liquor, evaporated to small bulk, collected and washed the precipitate formed, dried and boiled out with Chloroform with reflux condenser, evaporated the Chloroform, dissolved residue in Acetic Acid and tested by mixing with Fröhde's Reagent.

Result.—Brown colour, but no blue ring with Keller-Kiliani test.

Conclusion of these experiments.—Residues not sufficiently pure, and in the case of (2) some loss thought to occur.

"Tannin Method."

The idea of this method was, if possible, to combine the tests into one process.

Took 10 Cc. of a strong Tincture ("C"), vide Table, and 10 Cc. of a weak one ("P").

Added Lead Acetate Solution 2·5 Cc. Removed Lead with Sodium Phosphate, added Saturated Tannic Acid Solution in excess to filtrate, collected precipitate, rubbed with Zinc Oxide (about 0·1 to 0·2 Gm.), dried and extracted with Methyl Alcohol thoroughly. Evaporated and extracted Methyl Alcohol residue with water, then tested the portion dissolved by water for glucoside, and the residue not dissolved. The results were :—

Tincture ("C") For Water Soluble { With Fröhde = Distinct typical pink. ,, K.K. = Slight (?).

	For Digitoxin	With Fröhde	Slightly more than "P."
		,, K.K.	=Nil.
Tincture ("P")	For Water Soluble	,, Fröhde	=Pink less than "C."
		,, K.K.	=Nil.
	For Digitoxin	,, Fröhde	=Slight.
		,, K.K.	=Nil.

Further work showed that the tannin precipitation is not satisfactory, and the extraction with Methyl Alcohol is lengthy. The following was tried :—

"No. 2" Method.

To 10 Cc. of Tincture "C" and 10 Cc. of Tincture "P" added excess of Lead Acetate Solution (3 Cc.) then Sodium Phosphate. *No Tannin.*—Evaporated to about 2 Cc. extracted with Chloroform five or six times in a separator. (Testing minute quantities of each successive liquor shows how the extraction is proceeding, using Fröhde as a layer test.) Evaporate Chloroform Solution to dryness on water bath; extract the residue with water (10 Cc.) and test with Fröhde = water soluble glucoside; then dissolve *residue* in Chloroform and evaporate this and test again = Digitoxin.

In the course of this series of investigations I found that a *substance giving typical Digitoxin effect by the Keller-Kiliani layer test was extracted by the water.*

The actual results were :—

Tinct. "C" ...	Water-Soluble.	With Fröhde	Decided pink (more than "P")
		,, K.K.	Distinct greenish blue.
	Final Chloroform Soluble	,, Fröhde	More than "P."
		,, K.K.	Nil.

Tinct. "*P*" ...	Water-Soluble	With Fröhde	Slight.
		,, K.K.	Nil.
	Final Chloroform Soluble	,, Fröhde	Slight.
		,, K.K.	Nil.

In other words, relying on the Keller-Kiliani test, the extraction with Chloroform *finally* (after the use of water) seems to be useless. Seeing that nothing giving a K.K. colour reaction is left behind after treatment with water, the only reasonable conclusion is that the Digitoxin (not necessarily *all* that is present) is sufficiently soluble in the amount of water used to produce the reaction, and that the K.K. colour from the water extractive is due to *Digitoxin*. Note: The blue colour is not produced by Gitalin, which, like the rest of water-soluble glucosides, gives a pink or red with Fröhde's mixing or layered test.

Experiments to Determine Whether the Addition of a Substance to Hinder Hydrolysis in Evaporation Greatly Affects the Result, and Further Notes as to the Solubility of Digitoxin in Water.

10 Cc. of the (weak) Tincture "I" were treated by the final process (see opp. p. 36) with and without Calcium Carbonate added, a final separation being made into Chloroform-Soluble Extractive and water-soluble, using Chloroform after the water as hitherto.

Plus $CaCO_3$.

Aqueous Extractive,	*Chloroform Extractive,*
slight Digitoxin effect by the Fröhde layered test.	gave no Digitoxin effect (by Fröhde layered).
Decided pink colour by Fröhde mixed = water-soluble glucosides.	Slight (?) impurity by Fröhde mixed—negligible.

Minus $CaCO_3$.

Aqueous Extractive,	*Chloroform Extractive,*
gave Digitoxin effect, slight, if anything *less* than the above.	gave no Digitoxin effect by the layered test.
Decided pink by the mixed Fröhde test. Equal to the above —slightly more, if anything.	Slight by Fröhde mixed (impurity as above).

The conclusion from the above was that Calcium Carbonate makes little difference. Further that Digitoxin, or some allied substance, is soluble in water—giving the typical blue Digitoxin ring. It will be seen that nothing giving the colour went into Chloroform after water treatment.

Neutral versus Basic Lead Acetate.

An experiment was conducted with the Tincture " P " using 10 Cc. of same according to my final process, but with respectively Neutral and Basic Lead Acetate Solutions.

The final depth of colour with Fröhde's Reagent, using the Neutral Solution, was very slightly greater than that with the Basic preparation. It could be argued that in the case of the Subacetate some of the Digitoxin or body producing the recognised K.K., reaction had been precipitated. The test, in fact, with the Neutral Acetate alongside showed that it probably had been, and it was decided that the *Neutral Acetate* is preferable. Dorvault warns against the Subacetate in the manufacture of Digitaline Crystallisée.

The question whether the Neutral Acetate Solution precipitates Digitoxin was answered by several careful tests practically in the negative, as follows :

The combined precipitates by using Neutral Lead Acetate Solution from five London Tinctures of commerce extracted with Chloroform before and after acidifying, gave a reaction with Fröhde layered, which, if reckoned in respect of one Tincture, could be disregarded. Also Digitoxin of commerce in 0·03%

solution in 60% Alcohol treated with Neutral Lead Acetate Solution and Sodium Phosphate Solution gave no great difference in brown coloration with the mixed Fröhde Reagent against a control.

Tinctura e Herbe Recente ("U" in the Table).

The Question of Ferment Decomposition.

Opinion has been expressed that a Tincture made from fresh leaves might have the advantage of non-enzyme-decomposition of the glucoside contained.

Accordingly (November, 1912) 300 Gm. of fresh leaves were collected and macerated 24 hours in 480 Cc. of 96 % alcohol. On pressing off 580 Cc. of Tincture were obtained. Reckoning about 80 % moisture in the leaf this Tincture should be 1 in 12, i.e., about $\frac{2}{3}$ strength of the B.P. Tincture.

By proceeding according to *Kobert* (Munch. Med. Woch, August, 1912) one may possibly overcome enzyme action, and Digitoxin and the other insoluble bodies may possibly, according to him, also be brought into solution.

Physiologically tested—

The result with this Tincture was a M.L.D. between 0·2 and 0·3 Cc., *i.e., a Tincture weaker than the B.P. strength in leaf gave a preparation physiologically stronger than the average B.P. preparations.*

Chemically tested, the result did not agree with the above.

Further work seems here to be necessary, using fresh leaves in all experiments. Only one experiment has been conducted, and that on November 20th last; repetition is necessary; the question at issue had to be left over owing to closing for press.

Kobert (Am. Jl. Ph., 1887; Y.B.P., 1888, 199) dwelt on fermentation. He stated that when the leaves are imperfectly dried a species of fermentation may occur, which may decompose the three essential components of the drug.

The ferment, according to *Briessemont*, Jl. de Ph. (6) VII, 481; Y.B.P., 1899, 139, combines oxidising with hydrolytic

properties, and must hence be classed with the oxydases. Its proportion is greatest in recently dried leaves, gradually becoming less on keeping. This may appear a little confusing. It does not follow, of course, that old leaves are best by any means.

Bosse (Cent. Bl. f. Inn. Med., July, 1899 ; Y.B.P., 1900, 190) advocated a dialysed preparation of fresh leaves 1=1. This relieved cardiac symptoms and diuretic effect said to have been good.

One has to add to these older references the following of *Gordon Sharp* and *F. W. Branson*, (B.P. Conf., 1912) ;—

The object of their work was to determine whether a tincture made with 90 % alcohol retained its activity for a longer time than the ordinary pharmacopœial preparation. It was thought that the glucosidal deterioration might be due to a ferment, and that a stronger alcoholic menstruum might destroy it. The physiological testing was done by noting the action of a 60 and 90 per cent. alcoholic tincture on frogs after the preparations had been made four months. They came up to standard, although on the whole the stronger alcoholic preparation was not quite as toxic as the other. After a further period of time a subsequent test showed that the stronger alcoholic tincture was much less toxic than the other. The authors believe that the stronger alcohol decomposes the glucosides of the plant.

The authors also believe that a potent preparation can be produced from either wild or half cultivated plants ; also, that leaves gathered in November are as active as those gathered in August ; that leaves from plants which had flowered and from plants which had not yet flowered were equally toxic.

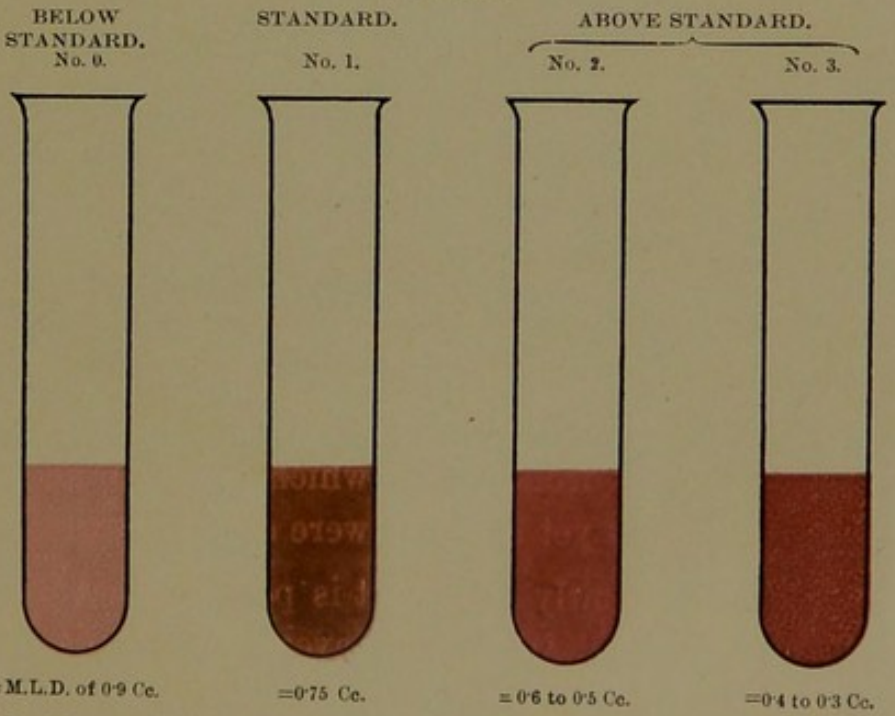
Kobert quite recently says :—It is possible that in collecting the drug, during rain, &c. (wild leaves are specified in various pharmacopœias), the only substances of any action in heart disease, viz., Gitalin, Digitoxin and Digitalin may become hydrolysed into useless or dangerous secondary glucosides.

FINAL METHOD ADOPTED FOR

ASSAY OF DIGITALIS TINCTURE (OFF.) IN TERMS OF PHYSIOLOGICAL EQUIVALENTS.

To determine whether a Tincture is up to Physiological Test requirements (usually taken at M.L.D. = 0·75 Cc. per 100 Gm. body weight of frog) mix 10 Cc. of the Tincture with 10 Cc. of water, precipitate with 10% Neutral Lead Acetate Solution (about 3 Cc.), adding a little Kieselguhr. Allow to stand for a ¼ hour, filter off on the pump, wash the precipitate slightly. Remove excess of lead from the filtrate with 10% Sodium Phosphate Solution (about 2 Cc. required) and filter. Add a little Calcium Carbonate (about 0·2 Gm.) to the filtrate, and evaporate to dryness on a water-bath. Add about 2 Gm. of dry washed sand to the residue and extract with Chloroform five times by thorough trituration, using about 10 Cc. on each occasion. Filter and evaporate the Chloroformic Solution and extract the residue with warm water on the water-bath, using 10 Cc. and 5 Cc. and again employing sand. Filter, evaporate to dryness in a porcelain basin, extract the residue again with cold Chloroform to purify it (about three or four quantities of 5 Cc. each, using dry sand and triturating thoroughly with a small pestle) and filter. Evaporate the combined Chloroformic Liquors and dissolve the residue in 4 Cc. of Glacial Acetic Acid. Mix 0·1 Cc. of this Acetic Solution with 1 Cc. of Sulphuric Ammonium Molybdate Reagent in a 5 × 1 Cm. test tube and compare the depth of colour after five minutes with the scale below—this coloration indicates the content of combined "active water soluble" Glucosides. Further, if 0·1 Cc. of the Acetic Solution be mixed with 0·5 Cc. of Glacial Acetic Acid. and this be layered upon 1 Cc. of the Sulphuric Ammonium Molybdate Reagent, the typical blue ring showing presence of Digitoxin should be formed.

SCALE.



It should not be lost sight of that a tincture becoming weak physiologically might still give the same colour test as originally—this can only be found out by keeping a tincture for a length of time.

In a series of experiments using this method it was possible to obtain depths of colour comparing most favourably with the physiological data, and it is claimed that the method can be made to yield results which may obviate physiological assay of Digitalis Tincture by means of frogs.*

It is true there *may* be a very slight loss of Digitoxin and other bodies in the resin, &c. (*c.f.* Barger and Shaw *antea*). The matter has already been dealt with. The process aims at comparison with a standard under certain conditions, and in any case we have proved by testing step by step that the loss is negligible.

From a series of Solutions tested by the method containing the following :—

(*a*) 0·4 mgr. of Commercial Digitalin in 0·2 Cc. Acetic Acid.

(*b*) { 0·32 mgr. "Digitalin" with 0·08 ,, Digitoxin } in ditto

(*c*) { 0·3 mgr. "Digitalin" with 0·1 ,, Digitoxin } in ditto

(*d*) { 0·24 mgr. "Digitalin" with 0·12 ,, Digitoxin } in ditto

and so on, decreasing in proportion of Digitalin and increasing the Digitoxin to 0·4 mgr. of Digitoxin *alone*, the colours increasing from a pure pink to dark brown were very characteristic, and it was possible to judge that in the "final method" adopted there is a *proportion* of Digitoxin with the generally

* It is very handy to use a slab of "Plasticine" pressed down firmly on to a white tile as the stand for the little test tubes. Observe colours in good light (day-light in preference) with a white background.

acknowledged water-soluble substances—it would be hazardous to state the proportion—it may be in the vicinity of 1 to 4.

It could also be adduced by comparison of the colour formed by (*c*) above, which corresponded with the colour produced by the Tincture "J," that from this Tincture we obtained 0·12% of Digitalin-like bodies, and 0·04% of Digitoxin—the colour in question being probably produced by about 0·3 mgr. of Digitalin-like substances with about 0·1 mgr. of Digitoxin in the 0·1 Cc. of Acetic Acid employed in the assay of this Tincture.

It must be clearly understood that this is only an approximation.

Some very interesting results were obtained by my final process in examining a series, including tinctures "J" as control against "K," "P" (a weak Tincture), "T" (the Indian Tincture), "Z" a Stock Tincture, and an Infusion of "J" leaves made 1 in 8 diluted with an equal quantity of Alcohol 60% instead of water as in the first steps.

"J" came out strongest (third corroboration of this result), with "K" and "P" in proportion.

"T" Tincture was = about M.L.D. of 0·5 to 0·6 Cc. by the colour scale, *i.e.* No. 2. Subsequent physiological examination gave M.L.D. 0·4 to 0·5., *i.e.*, between Nos. 2 and 3 on the scale.

"Z" was up to standard.

With regard to the Infusion the colour scale indicated that this contained glucosides almost equal to a standard tincture.

The colours taken at, as nearly as possible, 5 minutes gave :—

"J" Very strong.	= No. 3 on Scale.
"K" strong.	= No. 2.
"P" weak.	= below standard.
"T" very strong.	= No. 2 to No. 3.
"Z" moderate.	= No. 1.
Infusion, strong.	= No. 2.

TABLE OF COLOUR REACTIONS.

PRODUCED BY FRESH GLACIAL ACETIC ACID SOLUTIONS OF THE GLUCOSIDES 0·05% W/V STRENGTH (0·1 MGR. IN 0·2 CC.)

Glucoside.	Keller Kil. Test (Layer).	Fröhde Mixed.	Fröhde Layered.	Kiliani Test (Ferric Sulphate & H_2SO_4).	—
Digitoxin	Brown ring at juncture and typical blue ring above; in two or three minutes (shake slightly)	Pinkish *brown*. (1 Cc. of reagent mixed with 0·2 Cc. of Solution.)	Eactly similar to the K.K. reaction, but much more delicate	Pinkish *brown* like the "Fröhde Mixed" *q.v.* If quite pure, *brown*. (Kraft).	—
German Amorph. Digitalin of commerce	Carmine-red ring at juncture in one minute or less. *No blue* colour	Decided clear pink (using 1 Cc. reagent with 0·2 Cc. Solution)	Pink ring	Pink not so marked as the Fröhde effect	—
DigitaleinSchmiedeberg	Pink ring; no blue colour	Pink	Pink ring	Pink. Not so marked as the Fröhde	All these are identical with those of German Digitalin
Gitalin	—	—	—	The solid substance dissolves with red colour (Kraft)	—
Anhydrogitalin ...	—	—	—	As above (Kraft)	—
Glac. Acetic Acid alone	—	—	Produces minute blue colour, not developing further on standing	—	—

NOTE.—The colours do not alter greatly on standing in open test tubes for two or three hours.

Mark.	Description of Leaf.	Col. II.* "K K" Test with Infusion.
A. ...	English, own cultivation, 1911, *i.e.*, fully six months old at time of examination.	None available, all made into tincture.
B. ...	Second year's leaves. Own cultivation, collected May, 1912, after a period of luxuriant growth owing to heavy rain.	Slight reaction. Contained little chlorophyll in comp. with C. & F.
C. ...	From same locality as A., July, 1912. Collected May, 1912, from wild plants.	Very poor reaction
D. ...	From the Harz Mountains, July, 1912. Collected about June, 1912.	Not available
E. ...	Ditto. Collected 1911, *i.e.* one year old before examination.	Ditto
F. ...	From Hungary. Collected ("Grown") June, 1912.	Weak
G. ...	German leaves (cultivated). 1912.	Weak
H. ...	English leaves. Cultivated in a different county from B., J., I., K., &c. Collected August, 1912.	Weak
I. ...	English, own cultivation. From fine second year's plants cultivated in the shade. Collected July 20th, 1912, flowering being almost over and seed pods ripening.	Strong
J. ...	From second year's plants which had *not* flowered, cultivated in the sun close to the preceding. Collected July 20th, 1912.	Very strong
K. ...	Same as J., except from *flowering* plants. Collected July 20th, 1912.	Weak
L. ...	Seeds from plants grown in the sun at the same place. Collected July 20th, 1912.	Very weak
M. ...	From English wild plants, non-flowering, in the sun. Collected July 27th, 1912. Same locality as J., &c.	Very strong
N. ...	Collected in neighbourhood of Abruzzi, Italy, July, 1912. Received September, 1912.	Strong
O. ...	From first year's plants grown in a North London garden. Collected August 3rd, 1912.	Strong
P. ...	From second year's plants, otherwise as "O."	Weak
Q. ...	Sterilised (recent) leaves collected from wild plants in the Vosges Mountains, received September, 1912.	Weak
R. ...	German leaves, received September 23, 1912. Collected July, 1912, in the Harz Mountains.	Strong
S. ...	German leaves grown near Lerbach, Zellerfeld.	Weak
T. ...	Leaves grown in India. Collected September, 1912.	Strong
U ...	From *fresh* leaves: own growing. Collected November, 1912. Tincture = 1 of dried leaves in 12.	—

Col. III.* K K Test with Tinctures, by modif. P. Hung Method.	Col. IV.* Ac. Tannic Test as P. Helv.	Col. IVa* Ac. Tannic Test on Infusion.	Col. V. Extractive and Specific Gravity.		Col. VI.† Physiolog. Test (Sept. 25 to Nov. 20, 1912). Calculated Dose per 100 Gm. Frog. Failed to kill.	Killed (min. leth. dose)	Col. VII. Colour Nos. by W. H. M.'s Process.
Not available	Not available	Not available	Not available		— (Above standard)	0·5 cc.	Not available
Weak (light green)	S.	S.	5·4	0·9404	— (Above standard)	0·6 cc.	2
Strong (dark green)	„	Very S.	5·3	0·9390	0·4 (Above standard)	0·5 cc.	2 to 3
Weak	„	Not available	4·87	0·9280	0·5 (Above standard)	0·6 cc.	2
W.	„	Not available	4·88	0·9278	0·6 (Above standard)	0·7 cc.	1 to 2
S.	„	Very S.	4·88	0·9385	0·5 (Above standard)	0·6 cc.	2
Not available	„	S.	4·15	0·9315	Not conducted		2
W.	„	W.	4·00	0·9260	0·5 (Above standard)	0·6 cc.	2
S.	Mod. S.	S.	3·5	0 9227	0·4 (Above standard)	0·5	2 to 3
W.	Very W.	S.	4·5	0·9285	0·3 (Very active)	0·4	3
W.	W.	W.	4·25	0·9285	0·6 (Standard)	0·75	1
W.	Very W.	W.	1·55	0·9200	0·3 (Very active)	0·4	—
S.	W.	W.	2·8	0·9200	0·8 (Just below standard)	0·81§	2
S.	Very S.	Very S.	4·15	0·9278	Not conducted		—
S.	W.	W.	3·4	0·9232	0·57 (Above standard)	0·6	2
Very W.	S.	S.	3·05	0·9200	0·8 (Below standard)	0·9	Below standard
S.	S.	Not available	3·55	0·9232	0·36 (Very active)	0·4	3
Very S.	Strong	Very S.	4·65	0·9327	Not conducted		2
Very W.	W.	S.	4·75	0·9305	Not conducted		2
S.	S.	Very S.	3·7	0·9178	0·4 to	0·5†	2 to 3
—	—	—	4·0	0·9280	0·2 to	0·3	Anomalous

* No great reliance can be placed on any of these methods.
§ Corrected subsequently, 0·5 to 0·6 cc.
† The usual standard is M.L.D. = 0·75 cc. per 100 Gm.

TABLE OF SOLUBILITIES.

Glucoside.	Water.	Alc. Abs.	Alc. 90 per cent.	Chlorof.	Ether.	Petrol Ether.
Digitoxin, commercial ...	1 in 20,000 possibly	1 in 125	1 in 140	1 in 3	Slightly	Very slightly
German Digitalin of commerce	1 in 1½	1 in 100	1 in 4	Slightly	Slightly	Practically insoluble
Digitalinum Verum K. ...	1 in 1,000 (Kiliani)	More than 1 in 100 (Kiliani)	—	—	—	—
Digitonin cryst. (Merck)...	1 in 1½	1 in 300	1 in 3	Slightly	Slightly	—
Digitalein Schmiedeberg...	1 in 3 partially	1 in 120	1 in 10	Slightly	Very slightly	Practically insoluble
Gitalin, Kraft	1 in 600	Decomp.	Decomp.	Soluble	Decomp.	Insoluble
Anhydrogitalin Kraft ...	Insoluble	Almost insoluble in most liquids except dilute alcohol.				—

Comparison Between Physiological Results and the Colour Scale.

It will be seen that the following leaves gave *Standard* or *above Standard* Tinctures :—

J. English second year's non-flowering grown in the sun (own growing) = Scale Colour No. 3.

I. English leaves growing in the shade (own growing) = Scale Colour No. 2 to 3.

B. English second year's, collected after a period of heavy rain = Scale Colour No. 2.

K. Flowering plants of "J" = Scale Colour No. 1.

H. English Leaves = Scale Colour No. 2.

C. English Leaves from another county = Scale Colour No. 2 to 3.

D. and R. from Harz Mountains = Scale Colour No. 2.

E. Harz Mountains Leaves, old = Scale Colour No. 1 to 2.

F. Hungarian = Scale Colour No. 2.

O. North London Garden = Scale Colour No. 2 (1st year's plants).

G. and S. German Leaves = Scale Colour No. 2.

Q. Vosges Mountains Leaves (sterilised—recent samples) = Scale Colour No. 3.

T. Indian Leaves = Scale Colour No. 2 to 3.

M. Physiologically was at first said to be weak. I was inclined to think from the result of my colour test that this must have been due to frog variation. My surmise was justified, because when this tincture was sent for re-examination a few weeks afterwards under the designation "V" instead of "M" the report came back "M.L.D. 0·5 to 0·6" = Scale Colour No. 2.

The following leaves were *weak* :—

P. Second year's leaves from a North London Garden—Below Standard.

The Tincture "L" of Seeds is not included in the above—it will be seen to be a "strong" tincture physiologically, but weak by the Colour Scale—there is no marked preponderance of Water Soluble Glucosides.

I have found Tinctures of Commerce to vary very greatly.

GLYCETRACTUM DIGITALIS.

This preparation, it was thought, might prove physiologically active. I had in mind, however, that Focke in his report (*v. antea*) found that Glycerin hinders absorption in the physiological test. A sample of Glycetract was submitted to my physiologist, October, 1912, who reported M.L.D. to be 0·45 to 0·5 Cc. only (this preparation is 1=1 in strength), also that perfused through the frog's heart the effect was less than would be expected from the above.

The result was in a manner disappointing, but one must remember in addition to the absorption question mentioned that, although weak physiologically, this Glycetract gave fairly strong chemical reactions. 1¼ Cc. diluted to 10 Cc., and treated by the final process, gave an equivalent on the Colour Scale to a No. 1 Tincture, i.e., the Glycetract appeared to be eight times as strong as an average B.P. Tincture.

GLYCETRACT DIGITALIS. IMPROVED FORMULA.

On mentioning Focke's work to my physiologist friend he contended that the question of non-absorption only enters with the "short" method used by Focke. He suggested a modification in the formula as follows :—

(*a*) Wash leaf thoroughly with Petroleum Ether* in a Soxhlet and dry.

* Bearing on this the following is of interest:—Extractum Digitalis Liquidum. Macerate dried leaf 1,000 two hours with Glycerin 50 Gm. and Dilute Alcohol 450 Gm., then percolate with Dilute Alcohol 6,000 Gm. Distil until only 1,000 Gm. remain. Dilute with 2,000 of water, evaporate to 1,500, filter and again evaporate to 500, to which Alcohol 500 are now added to obtain 1,000 Gm. Dose: 0·1 to 0·5 Gm.—Fayn, Jour. de Pharm. d'Anvers, Aug., 1893, per Y.B.P., 1894, 205. England (Am. Jl. Ph., 1899, 332; Y.B.P., 1900, 190) recommended a fat-free Tincture (Petroleum Ether used). Said to have been more prompt in assimilation.

(*b*) Prepare an Extract 1 in 1, from this washed leaf with Glycerin 20 volumes, Alcohol (absolute) *q.s.* to 100 volumes.

The leaves were percolated with the mixture, the first 75 being reserved and the marc percolated to exhaustion with absolute alcohol, the extractive added to the reserve and made up to 100 by volume with Absolute Alcohol.

It would seem that in the old form Glycetract there is a shortage of Digitoxin, though it is well up to standard in the active water soluble bodies. The result with the improved Glycetract containing Alcohol is both physiologically and by colour scale remarkably strong.

Of the Glycetracts the new formula was superior to the old, the strength being practically equal to eight times that of the tincture "J" (the preparation is eight times as strong in leaves and is hence economical).

The examination of the new preparation, however, was in agreement with expectation of the leaf used, the Glycero-alcohol menstruum rendering the Glucosides capable of physiological action, and by the colour produced it was predicted that the physiologist's report of the M.L.D. would be in the neighbourhood of 0·05 Cc.—the actual report received was 0·05 to 0·06.

Note.—The Petroleum Ether extractive was evaporated to dryness and extracted with 90% Alcohol and made of strength 1=2 of the original leaves. The physiological test of this Alcoholic Extract of the Petroleum Ether Wash Liquor was "M.L.D. greater than 0·8 Cc. per 100 Gm."—*i.e.*, there was little loss by the Petroleum Ether Extraction. The chemical test of same gave slight blue with layered Fröhde.

I acknowledge with thanks the assistance of Mr. H. Johnson, Ph.C., who has conducted a large number of the experiments under my direction.

To sum up therefore—

(1) Digitalis preparations can be assayed by a simple colorimetric chemical method.

(2) The process which I have devised, though not claiming absolute accuracy or comparison with the physiological methods, will, at any rate, show whether a tincture is above or below standard, and it will with certainty show an excessively strong or a weak preparation. The method uses only a small amount of Tincture. The apparatus and reagents are perfectly simple and such as a pharmacist would have at hand. Weighing of residues of questionable purity by means of an accurate balance is not introduced. The process takes about three hours to carry out.

(3) There are strong indications that Digitoxin is not entirely insoluble in water.

(4) The routine use of animals in assays is not justifiable if a chemical method can be devised to produce equivalent results. In particular methods involving vivisection for routine assay are to be deplored. Their use in experiments in research work is an entirely different matter. Furthermore, the pharmacist should, if possible, be able to assay all the drugs he dispenses.

(5) Selected leaves recently dried, as a general rule, will produce tinctures up to standard, but there is obvious danger in the variation which may occur. At present a patient might easily obtain a preparation twice as strong at one pharmacy as at another. Considering the fact that, with Digitalis, prolonged administration in the treatment of heart affections is almost always necessary, and that the initial doses of Digitalis are invariably large, it is evident that standardisation of its preparations is of great importance.

(6) There is much to be learnt as to the ideal conditions for growth of Digitalis. Speaking generally, I think a dry season favours potency. The most potent leaves I have examined were second year's leaves from plants grown in this country in a sunny exposed situation. These leaves at time of collection were from plants showing no flower spikes.

(7) An active Glycero-Alcohol Extract can be produced of strength 1 = 1, in fact, exactly equal in strength to eight times that of a B.P. Tincture.

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