AN EXAMINATION OF

DIGITALIS PURPUREA AND ITS GLYCOSIDES

IN RELATION TO THE LOSS OF ACTIVITY ON STORAGE

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AN EXAMINATION OF DIGITALIS PURPUREA AND ITS GLYCOSIDES IN RELATION TO THE LOSS OF ACTIVITY ON STORAGE

A thesis presented by

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to the

UNIVERSITY OF GLASGOW

for the

DEGREE OF DOCTOR OF PHILOSOPHY

The School of Pharmacy,
Royal Technical College,
Glasgow

February, 1955

PREFACE

This research was undertaken in an attempt to explain the nature of the deteriorative changes which occur during the storage of tinctures of digitalis. These changes lead to a reduction of the potency of such tinctures to frog, but do not affect the potency to cat.

Results of work on tinctures of digitalis and ethanolic solutions of the digitalis glycoside, digitoxin, are recorded in this thesis and an explanation for the above anomaly between frog and cat results is presented.

Since, at present, there is no suitable chemical assay for all digitalis preparations, these are evaluated by determining their effect on animals. However, all existing biological assays are based on toxic reactions with a lethal end point and therefore an attempt was made to find a cardiotonic end point in the hope that a better indication of therapeutic efficacy would be obtained. Results of a preliminary study, involving electrocardiography of rabbit and frog, are disappointing but a brief description of the methods used is given in this thesis.

ACKNOWLEDGEMENTS

The author wishes to express his gratitude for guidance and encouragement given by Professor J.P. Todd, Ph.D.,F.P.S.,F.R.I.C., under whose supervision this work was conducted in The School of Pharmacy, The Royal Technical College, Glasgow.

Thanks are also due to Dr. E.S. Fairley and Mr. C.E. Hammond of the Department of Electrical Engineering, The Royal Technical College, Glasgow, who constructed the electrocardiograph and gave valuable advice on its use: to the Trustees of the McCallum Bequest who financed the purchase of certain equipment and materials: to Dr. G.E. Foster, Burroughs Wellcome and Co., London, who kindly supplied samples of digitoxin and digitoxose: to colleagues in the School of Pharmacy for their interest in this work; and to the following who supplied samples of commercial tinctures of digitalis:-

Mr. J. Chilton, M.Pharm., F.P.S. (Glasgow), Mr. H.B. Dunn, M.P.S. (Glasgow), Mr. F.C. Cumberland, F.P.S., (Blackburn), Miss P. Harrison, M.P.S., (Hull), Mr. E.W. Simpson, M.P.S., (Carshalton), Mr. P.B. Richardson, M.P.S., (Northampton), Mr. W. Robertson, M.P.S., (Southend-on-Sea), and Mr. M.F. Thatcher, M.P.S., (Shanklin, I.O.W.)

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INTRODUCTION

INTRODUCTION

The leaf of Digitalis purpurea (Linn.) is the best known member of a group of crude drugs which have a characteristic action on cardiac muscle. Many plant families are represented in the group, chiefly the Scrophulariaceae which includes the genus Digitalis, and the Apocynaceae which provides a large number of these drugs from the genera Acocanthera, Adenium, Apocynum, Cerbera, Nerium, Pachypodium, Strophanthus, and Tanghinia. Other genera yielding such drugs are Adonis, Antiaris, Caloptris, Cheiranthus, Convallaria, Coronilla, Erythrophleum, Helleborus, Rabelaisia, Scilla and Thevetia. Extracts of some of the plants have been used as arrow poisons and their classification into one pharmacological group has been rendered possible by systematic study of their action on animals.

It is interesting to note that similar substances are found in the Animal Kingdom, the secretions of the 'parotid' and other glands of the toad containing compounds which resemble, in their pharmacological action and to some extent their chemical structure, the glycosides of digitalis.

The first mention of the medicinal use of digitalis occurred in a treatise by the Welsh physicians of Myddvai in 1250[1]. A botanical description of the plant was first given in 1542 by Fuchs[2]: it was described by Book in a German herbal of 1546 and in medical textbooks by Monardes in 1565, Clausius in 1601 and Hurnung in 1625.

Some early English physicians used the drug both internally and externally for the treatment of many unconnected conditions such as epilepsy, ulcers and wounds. Gerarde, in 1597, and Parkinson, in 1640, mentioned it as an expectorant or emetic, Haller as a purgative.

The drug was introduced into the London Pharmacopoeia of 1650 and the Edinburgh Pharmacopoeia of 1783 but it had largely fallen into disuse until it was revived as a result of the treatise "An Account of Foxglove and some of its Medicinal Uses: with Practical Remarks on Dropsy and Other Diseases" by William Withering, published in 1785. Withering employed the drug as a diuretic but noted the cardiac action of the drug and even warned against over-digitalisation, saying that the drug should be stopped if any nausea was caused[3,4]. However he failed to relate the cardiac action to the diuretic effect. In 1789 Cullen speculated whether or not the diuretic effect arose from a general systematic action but it was Ferriar[5] who, in 1799, first emphasised the action of digitalis on the heart saying that the slowing of the pulse was "its true characteristic". Two years later Kinglake reported that the force of the pulse was increased by the drug thus indicating increased stroke-volume of digitalised hearts. Despite these accurate observations, a large amount of literature on the use of digitalis in phthisis accumulated during the early part of the 19th century.

The really effective use of digitalis began in the early part of the present century when Sir James Mackenzie[6] laid down definite rules for its use and established it as an indispensable therapeutic agent.

Despite the tremendous progress which has been made in the field of synthetic organic chemistry during the past two decades, this natural product still maintains its pre-eminent position in cardiac therapy.

Preparations of digitalis which have been used medicinally include capsules, extracts, infusions, injections, juices, pills, powders, tablets, tinctures, vinegars, mixtures of glycosides and single purified glycosides in the form of tablets or solutions for injection. Of these, tablets of the dried leaf and preparations of purified glycosides are being extensively used for the treatment of all forms of cardiac insufficiency such as congestive heart failure and auricular fibrillation, although some cardiologists still prefer to use tincture because of its more rapid and certain absorption. The action is partly vagal and partly direct on the heart muscle, the tone of which is increased. The heart rate is slowed and the ventricles caused to empty more completely and thus the cardiac output is increased.

Because of the complexity of the mixture of cardio-active principles and the great variations in pharmacological activity of different samples of dried leaf, preparations of digitalis are standardised by biological assays based on the toxicity of digitalis to certain animals. Many chemical methods of assay have been described but none gives an accurate measure of the therapeutic effect of digitalis on man.

CHEMICAL CONSTITUENTS OF DIGITALIS

CHEMICAL CONSTITUENTS OF DIGITALIS

Historical

The active constituents of digitalis leaf are a mixture of cardiac glycosides. Also present in the drug are saponins, oxidase and hydrolytic enzymes, and colouring matter.

Intensive research into the chemical nature of the digitalis principles has been pursued since the early part of the 19th century, but it was only within comparatively recent years that their constitution became clear and to date they have not been synthesised completely. The greatest barrier to progress was the similarity in physical and chemical properties not only between the various cardiac glycosides but also between them and the pharmacologically inert saponins whose presence retarded the crystallisation of the active fractions.

The earliest work proved so unfruitful that, in order to stimulate interest, the Societé de Pharmacie de Paris offered, in 1835, a prize of 500 francs for the isolation of "the active principles of digitalis". Five years later the sum was increased to 1000 francs and the award was made to Homolle and Quevenne[7] who isolated from the leaves of Digitalis purpurea a yellowish, partly crystalline cardio-active powder which they named "digitaline" and which probably contained digitoxin. Since then contributions to the chemistry of digitalis have been numerous.

Walz[8] isolated a series of fairly well defined substances which

he named digitalacrine, digitaline and digitalosine.

In 1869, Nativelle[9] succeeded in isolating a fairly pure, crystalline, highly potent substance which he called digitaline and which is now known to be identical with digitoxin. He described two other principles, the water-soluble digitalein and the chloroform-soluble digitine. Arnaud[10] prepared Nativelle's digitaline in a high state of purity and attributed to it the formula $C_{31}H_{50}O_{10}$.

In 1891, both Houdas[11] and Kiliani[12] stated that almost the whole of the water-soluble fraction consisted of a glycoside with the properties of a saponin. Houdas retained Nativelle's name of digitalein for this saponin but Kiliani called it digitonin, the name by which it is still known.

In this early work much confusion arose by failure to recognise that the cardiac glycosides and the saponins from the seeds of Digitalis purpurea differed from those of the leaf. Schmiedeberg[13] had, in 1875, isolated from the seeds four glycosides which he named digitonin, digitaleine, digitaline and digitoxin. Kiliani was of the opinion that Schmiedeberg's digitaleine was a mixture and that the three glycosides present were digitonin, digitalinum verum and digitoxin. In 1898, Cloetta[14] reported the isolation of digalen, of the same elementary composition as digitoxin but differing from the latter in being water-soluble. Krafft[15] realising the difference between the seed and leaf glycosides, isolated from the leaves digitsaponin and a glycoside, having the properties of Schmiedeberg's digitaleine, which he called gitalin.

Results of careful chemical and pharmacological investigations on digitoxin were published by Cloetta[16] in 1920. The work of Windhaus started in 1915, but published much later[17,18], gave the first real insight into the structural character of the glycosides and he assigned an empirical formula to digitoxin then showed that on acid hydrolysis it yielded one molecule of the aglycone digitoxigenin and three molecules of the sugar digitoxose.

$$\underset{\text{digitoxin}}{C_{41}H_{64}O_{13}} + 3H_2O = \underset{\text{digitoxigenin}}{C_{23}H_{34}O_4} + \underset{\text{digitoxose}}{3C_6H_{12}O_4}$$

Gitoxin was isolated in a pure state by Krafft[19] and later by Cloetta[20]. Windhaus[21] established its formula and its behaviour on hydrolysis.

$$\underset{\text{gitoxin}}{C_{41}H_{64}O_{14}} + 3H_2O = \underset{\text{gitoxigenin}}{C_{23}H_{34}O_5} + \underset{\text{digitoxose}}{3C_6H_{12}O_4}$$

Another glycoside isolated from the same material by Cloetta and investigated chemically by Windhaus is gitalin, which on hydrolysis yields only two molecules of digitoxose and one molecule of gitaligenin which is the hydrate of gitoxigenin, to which it reverts on dehydration.

$$\underset{\text{gitalin}}{C_{35}H_{56}O_{12}} + 2H_2O = \underset{\text{gitoxigenin-hydrate}}{C_{23}H_{36}O_6} + \underset{\text{digitoxose}}{2C_6H_{12}O_4}$$

Digitalinum verum obtained from the seeds of D. purpurea and first described by Schmiedeberg[15], was examined by Windhaus[22,23] who showed

its behaviour on hydrolysis:

$C_{36}H_{56}O_{14} + 2H_2O = C_{23}H_{34}O_5 + C_7H_{14}O_5 + C_6H_{12}O_6$

digitalinum verum — primary aglycone — digitalose — glucose

$C_{23}H_{34}O_5 = C_{23}H_{30}O_3 + 2H_2O$

digitaligenin (dianhydrogitoxigenin)

This glycoside has recently[24,25] been obtained from the leaves of D. purpurea, together with a new gitoxigenin-glycoside called gitorin.

In 1930, Smith[26] isolated from the leaves of Digitalis lanata a new glycoside named digoxin, from the hitherto unknown aglycone digoxigenin. Hydrolysis of the glycoside is represented as follows:

$C_{41}H_{64}O_{14} + 3H_2O = C_{23}H_{34}O_5 + 3C_6H_{12}O_4$

digoxin — digoxigenin — digitoxose

It was not realised until comparatively recently that the glycosides digitoxin, gitoxin and digoxin were themselves hydrolytic products of higher glycosides. Attention was first drawn to this possibility by Perrot and Goris[27] and it was proved experimentally by Stoll and Kreis[28] who isolated and described the primary glycosides.

The initial work was done on leaves of Digitalis lanata following the successful isolation of cardio-active glycosides from the bulbs of Scilla maritima (Linn.). The fresh leaves were ground at low temperature with ammonium sulphate to inactivate the enzymes which were present.

The mash was exhaustively extracted with ethyl acetate and from the solution chlorophyll etc. were removed by shaking with ether in which the glycosides, originally present as tannoids, are sparingly soluble. The tannins were subsequently removed by precipitation with lead hydroxide. On evaporation of the glycoside solution, solids separated and these were recrystallised from aqueous methanol. The substance, at first thought to be chemically pure and named digilanid, was found on hydrolysis to yield three aglycones namely, digitoxigenin, gitoxigenin and digoxigenin. After painstaking fractional crystallisations, using aqueous methanol and chloroform, the mixture was separated into the three primary lanata glycosides, the crystalline digilanids A, B, and C. These are the natural glycosides which exist in the living leaf and Stoll and Kreis have demonstrated the action of the enzyme digilanidase, also present in the leaf, on the digilanids as follows:

$C_{49}H_{76}O_{19}$	+	H_2O	=	$C_{43}H_{66}O_{14}$	+	$C_6H_{12}O_6$
digilanid A				acetyl-digitoxin		glucose
$C_{49}H_{76}O_{20}$	+	H_2O	=	$C_{43}H_{66}O_{15}$	+	$C_6H_{12}O_6$
digilanid B				acetyl-gitoxin		glucose
$C_{49}H_{76}O_{20}$	+	H_2O	=	$C_{43}H_{66}O_{15}$	+	$C_6H_{12}O_6$
digilanid C				acetyl-digoxin		glucose

The acetyl derivatives are largely decomposed during drying, so that in dried leaf, digitoxin, gitoxin and digoxin predominate.

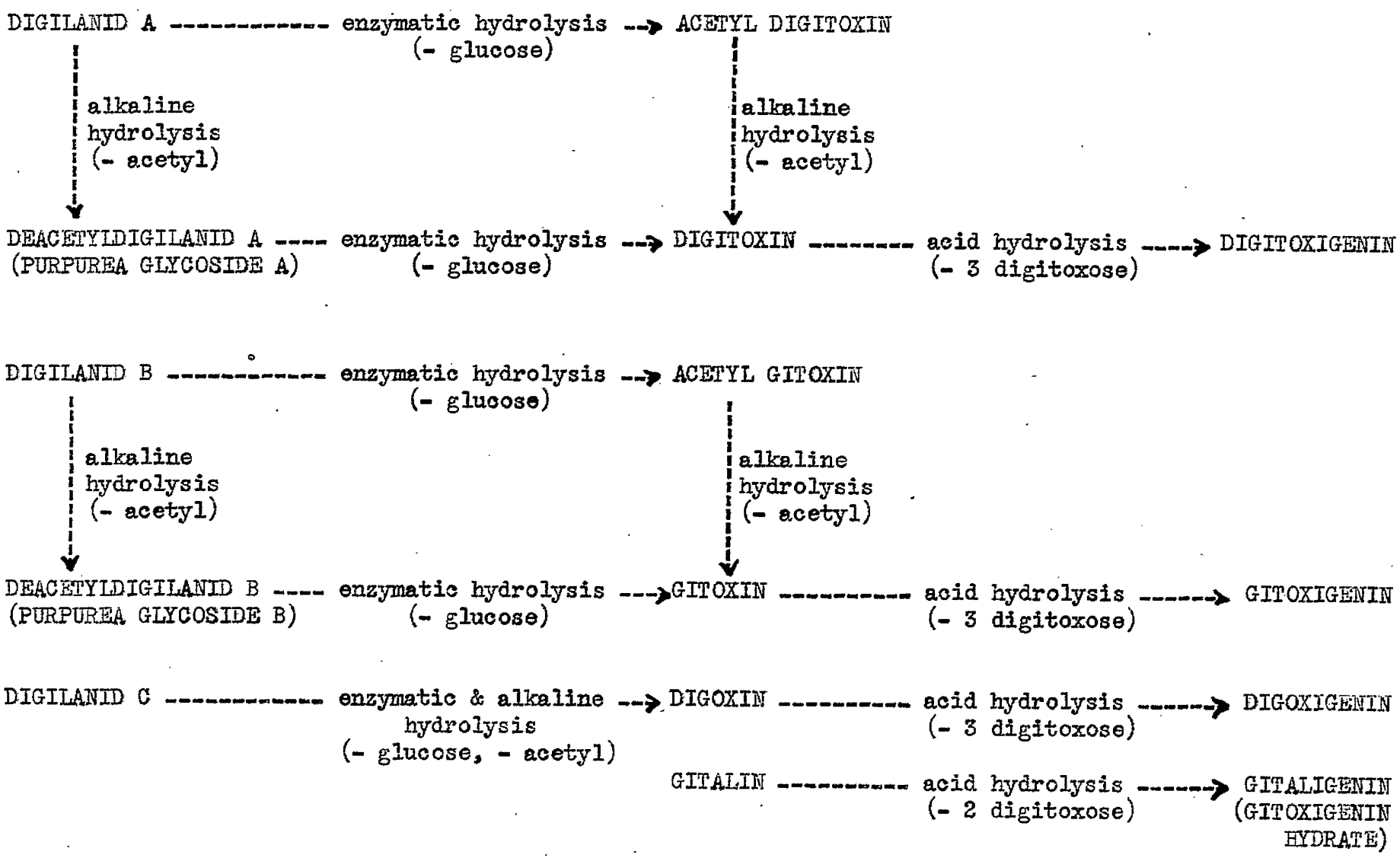
It was found possible to remove the acetyl group from the genuine glycosides by using calcium hydroxide under mild conditions, when deacetyl-digilanids A, B and C were formed[29].

Stoll and Kreis[30] next turned their attention to the natural glycosides of Digitalis purpurea, but this investigation proved more difficult because of the relatively lower glycosidal content and the higher rate of enzyme activity in these leaves. In addition, all attempts to crystallise the glycosides failed and it was extremely difficult to obtain a separation from large quantities of inactive impurities. Eventually, after a tedious process of fractionation between immiscible solvents (chloroform, methanol, water) involving considerable loss of material, two amorphous substances, purpurea glycosides A and B were obtained. From a study of their physical, chemical and pharmacological properties, these substances were shown to be identical with deacetyl-digilanids A and B respectively. Hydrolysis of these compounds, under the influence of the enzyme digipurpidase which is present in the leaves of D. purpurea, is similar to the hydrolysis of the lanata glycosides.

$C_{47}H_{74}O_{18}$	+	H_2O	=	$C_{41}H_{64}O_{13}$	+	$C_6H_{12}O_6$
purpurea glycoside A (deacetyl-digilanid A)				digitoxin		glucose
$C_{47}H_{74}O_{19}$	+	H_2O	=	$C_{41}H_{64}O_{14}$	+	$C_6H_{12}O_6$
purpurea glycoside B (deacetyl-digilanid B)				gitoxin		glucose

TABLE I

Inter-relationship of cardiac glycosides from the leaves of Digitalis lanata and Digitalis purpurea



A higher glycoside related to gitalin, or corresponding to digilanid C, could not be found in the leaves of D. purpurea.

Qualitatively the hydrolytic enzymes digilanidase and digipurpidase are mutually interchangeable, but quantitatively each shows a specificity for its own substrate.

The inter-relationship between the D. lanata and D. purpurea leaf glycosides is shown diagrammatically in Table I.

In 1948, a team of Japanese workers[31] claimed to have isolated a new glycoside, digicorin, from the leaves of specially cultivated plants of D. purpurea and D. lanata, but they have been unable to repeat this work and have therefore withdrawn the name digicorin from the literature[32].

Also in 1948, Ulrix[33], using adsorption chromatography on alumina, claimed to have found four new glycosidal substances in digitalis, while in 1953 Jensen[34] claimed to have separated five new glycosidal substances from stabilised leaf by means of paper partition chromatography. However, Jensen stated that two of the substances appeared to correspond to digicorin and its aglycone digicorigenin. Brindle et al.[35] could find no new glycoside-like substances in tinctures of digitalis examined by paper chromatography.

Digitalis Saponins

The presence of saponin glycosides along with the cardiac glycosides

in commercial preparations of digitalis was first recognised in 1875 by Schmiedeberg[13] who named the principle saponin, digitonin. Purer preparations of the substance were later described by Kiliani[12], but in 1913 Windhaus and Schneckenburger[36] discovered, in a supposedly pure sample of digitonin, a second glycoside which they named gitonin. On hydrolysis:

digitonin = digitogenin + 2 glucose + 2 galactose + xylose

gitonin = gitogenin + 3 galactose + a pentose

Windhaus, working on the saponins obtained from digitalis seeds, obtained degradation products other than the above and he suspected the presence of still other saponins. Although no other saponins have been isolated from this source, Jacobs and Fleck[37] isolated a new aglycone, tigogenin, from hydrolysed extracts of digitalis leaves. Tschesche[38] discovered the corresponding glycoside, tigonin, in extracts of Digitalis lanata leaves in which it appears to be the only saponin. On hydrolysis:

tigonin = tigogenin + 2 glucose + 2 galactose + rhamnose

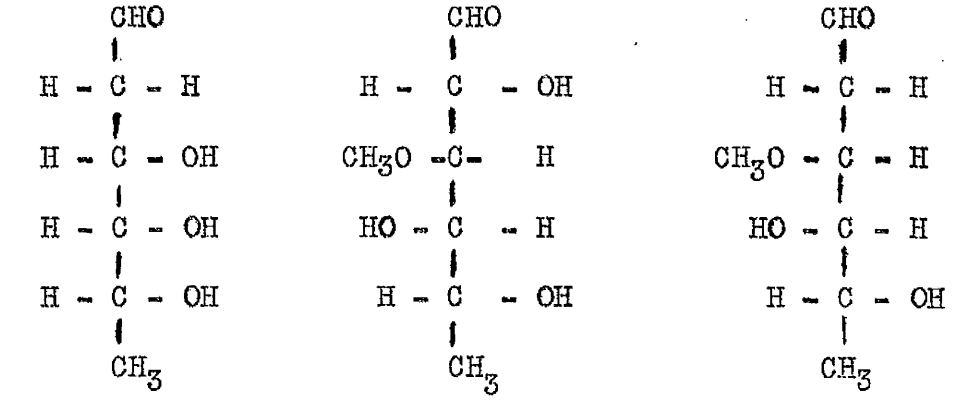
Structure of the Cardiac Glycosides

The digitalis glycosides consist of a parent skeletal structure, the aglycone or genin, to which three or four sugar molecules are attached. Digitoxin, gitoxin and gitalin contain the sugar digitoxose which has not been found elsewhere in nature. This rare sugar was described by Kiliani in 1922 as a 2:6 de-oxy hexose[39], its configuration was established in 1930[40] and its synthesis first described in 1944[41]. Jacobs[42] stated that the glycosides which contain the 2-deoxy sugars are readily hydrolysed under

mild conditions, contrasting with glycosides which contain 2-hydroxy sugars and are hydrolysed only under conditions so drastic as to cause changes in the aglycone. Digitalinum verum contains the 2-hydroxy sugar digitalose and on hydrolysis the aglycone loses two molecules of water with the formation of dianhydrogitoxigenin.

Also present in the leaves of D. purpurea is the pharmacologically inactive glycoside diginin which on hydrolysis yields digigenin and diginose[43].

Fig. 1.



The elucidation of the structure of the aglycones presented great difficulties. The digitalis aglycones, in common with those of other cardiac aglycones, contain 23 carbon atoms. It was assumed that all these aglycones, of closely related empirical formulae, would possess the same general skeletal structure. This proved to be the case and the transformation of several pairs of these into identical substances was accomplished.

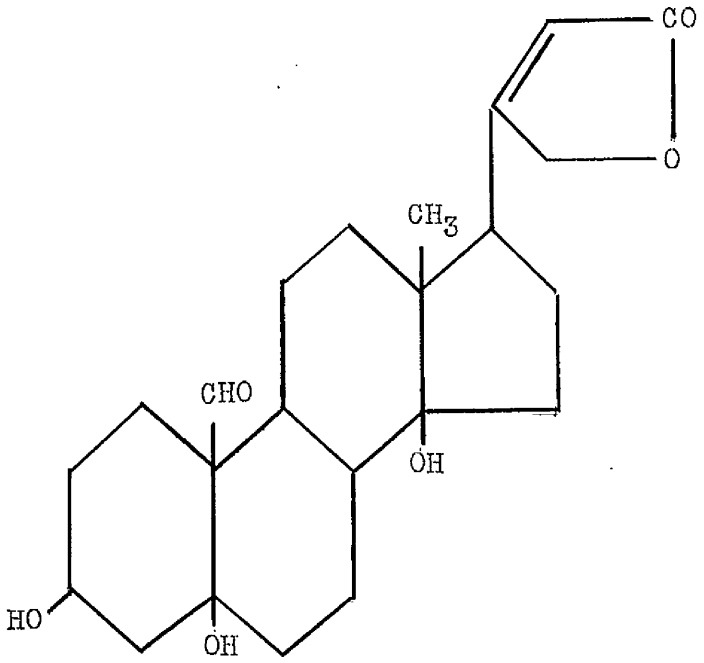
The field of investigation of the cardiac glycosides, previously restricted by lack of reference compounds , was opened up by Jacobs and Elderfield[44] in 1934 when they demonstrated the selenium dehydrogenation of the Strophanthus glycoside strophanthidin (Fig. 2a.) to Diel's hydrocarbon (γ-methyl-1-2-cyclopentenophenanthrene) (Fig. 2b.). This discovery linked the chemistry of the cardiac glycosides with the rapidly developing chemistry of the sterols and bile acids, from both of which Diel's hydrocarbon had been prepared. Further evidence was obtained when Jacobs[45] successfully degraded digitoxigenin (Fig. 2c.) to the bile acid, etiocholanic acid (Fig. 2d.). The attachment of the unsaturated lactone ring, already known to exist, was thus shown to be at C_{17}.

After studying the reactions of several of these related aglycones, Tschesche[46] and Jacobs[47] proposed formulae which were later supported by the theoretical deductions of Kon[48] and also by evaluations of X-ray photographs of suitable derivatives prepared by Bernal and Crawfoot[49].

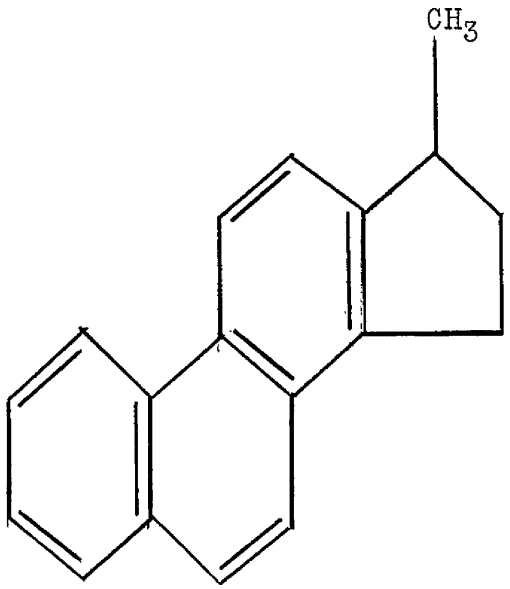
Gitoxigenin and digoxigenin each differs from digitoxigenin only by the presence of one additional hydroxy group in the molecule; in gitoxigenin at C_{16} and in digoxigenin at C_{12}.

Excellent reviews of the extensive literature on the chemistry of digitalis and other cardiac glycosides have been published by Stoll[50] and Turner[51].

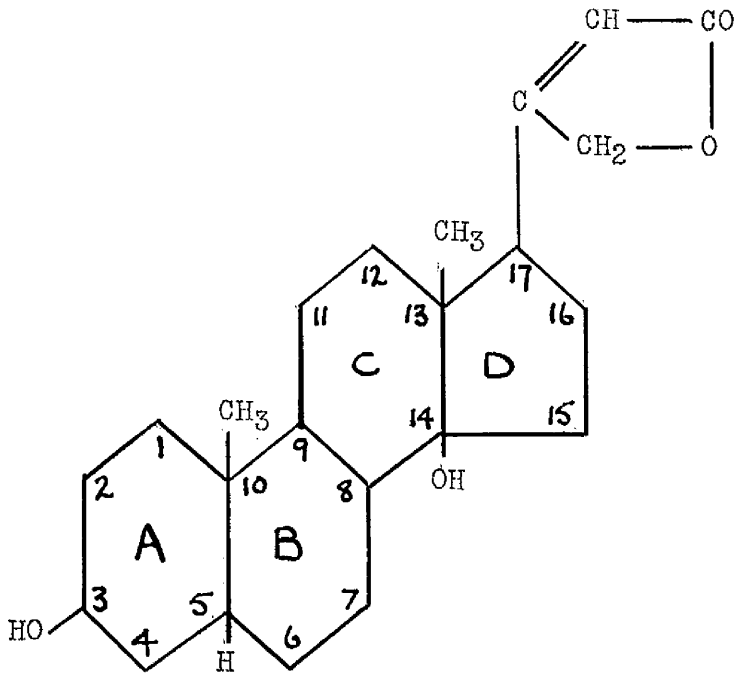
Fig. 2.



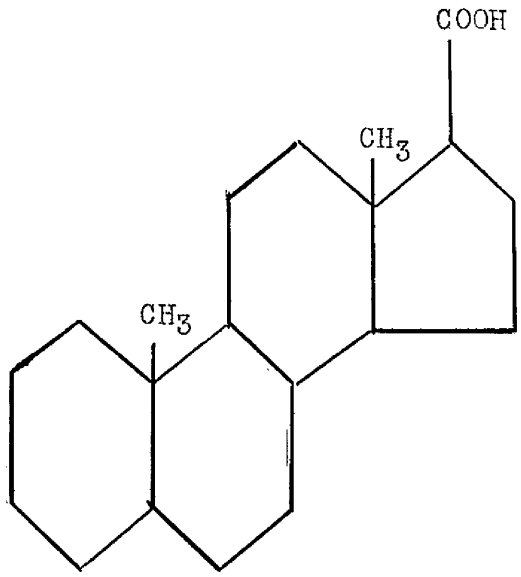
(a) Strophanthidin



(b) Diel's hydrocarbon



(c) Digitoxigenin



(d) Etiocholanic acid

Relationship between chemical structure and pharmacological activity

The sugar moieties of the cardiac glycosides, while themselves inert pharmacologically, greatly enhance the activity of the aglycones to which they are attached, being apparently responsible for water solubility, cell penetrability and persistence of cardiac action[52]. In all the cardiac glycosides so far examined, the sugar residue occupies the 3-position, where the aglycone is usually united with a deoxy sugar.

Relative potency does not seem to depend on the type of sugar present and consequent ease of hydrolytic fission of the sugar residue, but specific structure of the sugar seems to be important[53]. It has been stated that the potency of the glycosides is in an inverse ratio to the number of sugar molecules attached to the aglycone. Thus digitoxin, a trioside, is more potent (examined on cat) than the tetroside, purpurea glycoside A, derived from the same aglycone[54]. However, this does not appear to be true when the materials are tested on frog in which purpurea glycoside A is reported to be more potent than digitoxin[50,55].

The specific cardiotonic activity resides in the aglycone and it has been shown that for high activity the A and B rings must be cis fused. Uzarin, in which the A/B fusion is trans, has greatly reduced activity. The C and D rings are also cis fused and thus the cardiac genins are unique in this respect, differing from other naturally occurring steroids. The 14 hydroxyl group and the α,β unsaturated butenolide ring, both common to all cardiac glycosides, are β-orientated. That the lactone ring is

$\alpha\beta$ unsaturated, has been established by Elderfield et al[56] contrary to the earlier views of Jacobs, who considered the double bond to be in the $\beta\gamma$-position to the carbonyl group. This unsaturated lactone ring is intimately associated with cardio-activity. Jacobs[57], who with his co-workers, did much to solve the major problems of the structure of cardiac glycosides, has shown that if the ethylene linkage is saturated the toxicity is greatly reduced. If the lactone ring is opened without hydrogenation the activity falls to about one five-hundredth of the original. Fieser[58] regards the ring system merely as a support for the lactone ring and the solubilising sugars.

Is is worth noting that the saponins also contain the cyclopentenophenanthrene nucleus but are devoid of any cardiac action.

Attempted syntheses of cardiac glycosides

It was inevitable that once the main structure of the cardiac glycosides had been elucidated, attempts to synthesise these compounds would follow. One approach has been the addition of sugars to natural genins[59,60,61]. Elderfield prepared various glycosides by condensation of known genins with aceto-bromo-glucose and the compounds were reported[62] to be more potent than corresponding natural glycosides. Other workers have attempted to synthesise genins and methods for the construction of some of the characteristic groupings have been described[63,64,65,66,67,68,69,70] although the resulting compounds have had little or no cardiotonic activity.

STANDARDISATION OF DIGITALIS PREPARATIONS

BIOLOGICAL ASSAY METHODS

STANDARDISATION OF DIGITALIS PREPARATIONS

It has been shown that leaves of Digitalis purpurea may contain up to ten known cardio-active principles. In addition to variations in total glycoside content, there are differences in the proportions of the various active principles present and while these all show similar qualitative pharmacological effects, there are marked differences in quantitative activity. Further, in addition to D. purpurea there are numerous other species of Digitalis which contain cardiac glycosides and which show potencies differing from that of D. purpurea[71].

For these reasons, digitalis has been largely evaluated by measuring the effect on animals, but many chemical methods of assay have also been described.

BIOLOGICAL ASSAY METHODS

Review of methods used

The first biological method applied to digitalis was that described in 1898 by Houghton[72] who measured the smallest dose of an extract required to kill a fixed weight of frog in a given time, regardless of specific action. Since that time numerous assays have been described employing a wide range of test animals including Daphnia, Paramecium, Goldfish, Frog, Toad (heart), Turtle (heart), Tortoise (heart), embryonic Chick, embryonic Duck, Pigeon, Guinea Pig, Mouse, Rat, Rabbit, Cat, Dog and Man. A phytopharmacological method, using lupin seedlings, has been described but

in this case, as with goldfish, the observed effect was due to the saponins and not to the cardiac glycosides.

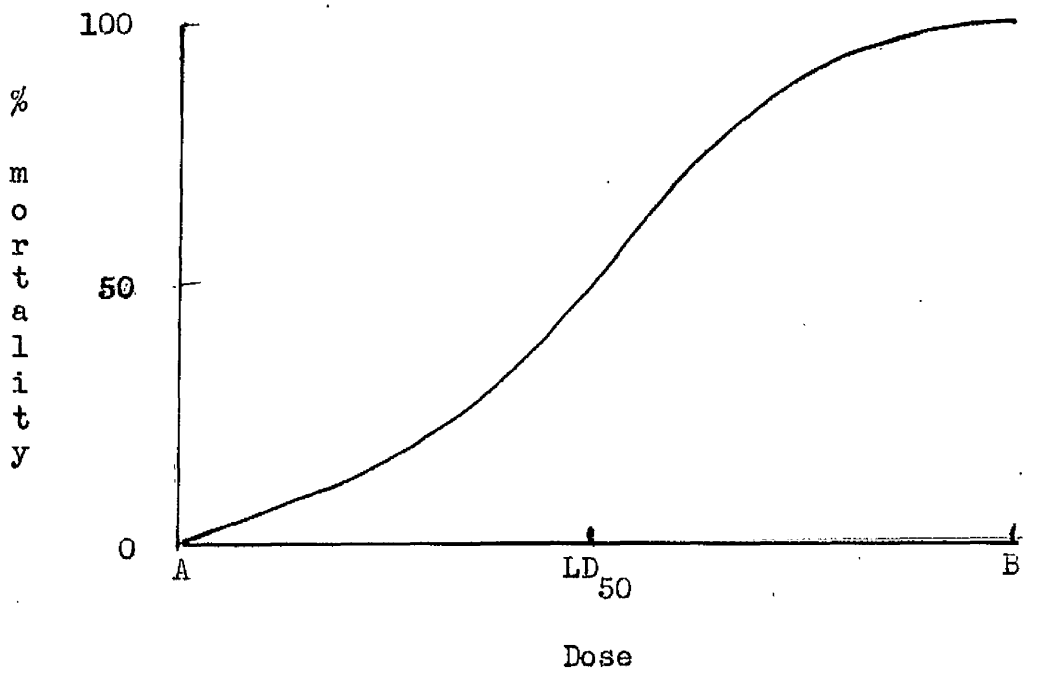
Before the introduction of comparative assays, the potency of preparations was expressed in animal units, one unit being the amount to cause death, or other effect, in the test animal. It was not appreciated that there were many variable factors which tended to make this unit a very inconstant quantity. An indication of this variation was shown by Burn[73] who quoted for the same sample of digitalis leaf, examined at different times of the year, values which ranged from 1310 to 3300 frog units per g. If, however, two preparations with the same specific effect were assayed simultaneously, under the same conditions, the ratio of their potencies was reasonably constant. The introduction of an International Standard Digitalis Powder in 1926, which was in 1928 defined as containing one unit of activity per g., made comparative assays possible, and all samples or preparations of digitalis are now determined by comparison with standard powder. Because the supply of original powder was almost exhausted, the Permanent Commission on Biological Standardisation decided, in 1935, to adopt the British Standard, to be known as the International Standard 1936 and which, possessing 125 per cent of the activity of the previous International Standard, contained one unit of activity in 0.08 g. In 1952, a third International Standard became necessary and this has been stated to contain one unit of activity in 0.076 g.

Of the numerous assay methods mentioned, many are of historical interest only and these are adequately dealt with by Munch[74] and Schwartz[75] who review all methods used prior to 1930. Methods which have been widely applied since that time will be described.

The 18-hour frog method

In 1927 Trevan[76] reported the results of toxicity tests on frogs (Rana temporaria) in which only the death or survival of the animal could be recorded. He injected groups of thirty frogs with graded doses of a digitalis preparation and plotted the percentage mortality in each group against the dose injected. The resultant graph was a sigmoid curve (Fig. 3.)

Fig. 3.



A dose represented by OA failed to kill any animal and was termed the LD_0. The dose represented by OB killed all the animals and was called the LD_{100}. Trevan found the ratio LD_{100}/LD_0 to be 4.8, which emphasised the big gap between the biggest dose failing to kill any animal and the smallest dose required to kill all the animals. He proposed that the lethal dose determined in tests of this kind, be that which causes a fifty per cent. mortality in the group, this dose being referred to as the LD_{50}.

In the British Pharmacopoeias of 1932 and 1948 an assay method based on Trevan's observations was described. A dose of the standard preparation calculated to kill fifty per cent. of the frogs injected, and suitably diluted with 0.6 per cent. sodium chloride solution, was injected into a group of not less than twelve healthy frogs, all of the same sex and preferably males, the dose being proportional to body weight. A dose of the preparation under test, similarly calculated and diluted, was injected into another group of not less than twelve frogs as near in weight and other respects to the first group as possible. The injections were made into the ventral lymph sac and the frogs left overnight. After twelve to eighteen hours the number of dead frogs in each group was counted. The percentage mortality observed in each group was referred to a table based on Trevan's curve and the potencies of the standard and test solutions obtained. The potency of the test preparation in terms of the standard preparation could then be calculated. The procedure was repeated on the second day, adjusting the doses, if necessary, to obtain fifty per cent. mortalities. The potency of the test preparation was taken as the average of the two estimations. This

This method involved the assumption that the standard dose/response curve was constant but it has been shown[77,78,79] that the slope may vary, not only in different laboratories but even from time totime in the same laboratory. Gaddum[77] observed that by plotting the logarithm of the dose against the deviation on the frequency distribution curve to which the percentage mortality corresponds (the normal equivalent deviation), the sigmoid dosage/response curve was converted to a straight line. This is the basis of the frog method of the 1953 British Pharmacopoeia and was described as an alternative method in the 1948 edition. The test solution is estimated in terms of the standard solution without reference to a pre-determined curve, the slope of the line being determined in each experiment by using two dose levels of both standard and test solutions and injecting into four groups each of not less than twenty frogs. The Pharmacopoeia recommends the use of doses estimated to produce twenty and eighty per cent. mortalities. It is more convenient to use a system of probits (probability units) obtained from a table by Bliss[80] in place of the normal equivalent deviation.

The 1-hour frog method

This method was at one time widely used and it was official in editions IX, X and XI of the United States Pharmacopoeia, being replaced in the following edition by the intravenous cat method.

Dilutions of the test and standard preparations were administered to groups of frogs, as in the 18-hour method, the injections being made into the ventral lymph sac. At the end of one hour the dead frogs in each

group were counted, each animal being dissected to check the end point which is stoppage of the heart in ventricular systole, the auricles being widely dilated. Results were calculated as stated for the previous method.

The guinea pig method

This method was first introduced for testing digitalis by Vanderkleed[81, 82] in 1908 and 1910, but the method was established in 1926 by Knaffl-Lenz[83] and it is his method which forms the basis of the method in the British Pharmacopoeias of 1932 and 1948.

Suitably diluted preparations of digitalis are injected intravenously, at a slow uniform rate, into anaesthetised guinea pigs in which respiration is maintained artificially. The injection is continued until the heart stops, the dose being determined in fourteen animals for the standard preparation and in six animals for the estimation of the preparation under test. The average lethal dose for the standard preparation is not required to be calculated at each examination of an unknown preparation. Jacobsen and Larsen[84] report variations in the sensitivity of the animals and recommend that standard and test preparations be tested simultaneously. This recommendation is adopted in the 1953 British Pharmacopoeia in which equal numbers of animals, not less than six, are used for the estimation of each preparation.

Animal weight[79] and rate of injection[85,86] both have an effect on the lethal dose, therefore weight range and duration of injection are both specified in the official assay.

Ether was the anaesthetic originally used, but it is said to give variable results and both urethane[87] and sodium phenobarbitone[85] have been recommended.

The disadvantage of the method is that respiration becomes irregular a considerable time before the heart finally stops beating and therefore the end point tends to be indistinct. However, because of the difficulty in obtaining suitable cats in sufficient numbers, the guinea pig method is widely used in this country.

The cat method.

This method was introduced by Hatcher and Brody[88] in 1910, modified in the laboratory of Magnus and described in detail by Lind van Wijngaarden[89].

The method as described in the British Pharmacopoeias of 1932 and 1948 was similar to the guinea pig method. Changes in the 1953 edition include the introduction of a weight factor since, on a body weight basis, heavier cats are relatively more sensitive to digitalis than lighter ones[79]. In place of the continuous injection, doses are given intravenously at five minute intervals to allow fixation of the slower acting glycosides[90], and the duration of the injection is specified. Again standard and test preparations are estimated concurrently on at least six cats for each extract and as far as possible the same number of cats should be given standard and test preparations during any one day. The test should be completed within fifteen days since Bliss[90] has shown that sensitivity of cats in any one laboratory will remain constant only for this period.

The pigeon intravenous method

In 1934 Haag and Woodley[91] used pigeons instead of cats in the Hatcher and Brody method, and comparing their results with those obtained on cats they found differences of up to twenty five per cent in the results. However, in 1948, Braun and Lusky[92] compared the cat and the pigeon intravenous methods and found the latter satisfactory and it was adopted as an official method of assay for digitalis preparations in the 14th. edition of the United States Pharmacopoeia and the 1953 British Pharmacopoeia. Details of the method are very similar to those in the cat assay except that ether is the one anaesthetic recommended, a number of injections are given into the alar vein and the assay is to be completed within thirty days.

This intravenous method is not to be confused with the pigeon emesis method of Hanzlik[93] who mistakenly regarded the amount of digitalis to cause vomiting in pigeons as a better therapeutic index than the lethal dose to animals.

Comparison of methods and relationship of results to clinical efficacy

Wasicky[94] has pointed out that different animal methods of assay give different indications of potency for the same digitalis preparations and it will be shown in the section dealing with deterioration of digitalis tinctures that there is marked difference in potency estimates of old tinctures by the intravenous cat method compared with the frog lymph sac method. Lack of agreement has also been reported between cat and

guinea pig results[85], although fair agreement has been reported by some workers[92,95] between pigeon and cat assays. The question arises as to which method gives the best indication of therapeutic efficacy in man. Gold et al.[96] stated that animal methods may give results which are misleading when applied to humans. This is especially true of frog results and the cat method is said to give better indications of strength although here again the results may be misleading when estimating pure substances[97]. Results of pigeon assays are also said to give a poor measure of oral potency to man[98].

Although the methods using cats, guinea pigs and pigeons are now more widely used than the frog lymph sac method, the effect on mammalian hearts being preferred to the effect on the amphibian heart, all intravenous techniques suffer a serious defect in not distinguishing between absorbable and non-absorbable materials since most digitalis preparations are administered orally to man. The same criticism applies to some extent to the 1-hour frog method in which some workers[99,100] have said that sufficient time is not allowed for the absorption of some of the less soluble glycosides.

Human bioassay

Because of the limitations of the animal assay processes, Gold and his co-workers[101] introduced a bioassay on humans based on the degree of T-wave and RT-T segment changes of the electrocardiogram after treatment with digitalis. They claimed to be able to distinguish 22 per cent. differences in dosage.

Pardee[102] had previously suggested the method while Dieuaide, Tung and Bien[103] had been able to detect a 25 per cent. difference in dosage by measuring the degree of shortening of the Q-T interval of the electrocardiogram.

Electrocardiographic methods on animals

The characteristic effect of digitalis on the electrocardiogram of the cat has been demonstrated by several workers[104,105,106,107,108,109,110]. Robinson and Wilson[104] showed that there was an inversion of the T-wave with about 25 per cent. of the lethal dose, definite prolongation of the P-R interval with about 50 per cent. and constant A-V dissociation with about 75 per cent. of the lethal dose. Krueger and Unna[105] reported agreement with previous workers in finding the onset of bradycardia with about 30 per cent. of the lethal dose and they noted that the dose of digitoxin which caused the appearance of the first ectopic beat had a definite relationship to the fatal dose. Rothlin[107,108,109,] could find no criteria for distinguishing between therapeutic and toxic symptoms.

Boyd[111] demonstrated the effect of microdoses of digitalis and other cardio-active drugs on the electrocardiogram of the frog, using a cathode ray oscilloscope as the recording instrument.

CHEMICAL ASSAY METHODS

CHEMICAL ASSAY METHODS

Gravimetric methods

Many of the earlier workers decolourised aqueous extracts with lead subacetate, removed excess lead, then extracted with chloroform. The glycosidal material was obtained by evaporation of the solvent, dried and weighed. Auenmüller pre-extracted digitalis leaves with ether to remove chlorophyll, fat etc. before chloroform extraction. Tschirch and Wolter weighed an acetone extract and determined its physiological activity on frogs. These methods are of historical interest only and are adequately reviewed by Schwartz[75].

Colorimetric methods

One of the first methods of this type to be used was described by Martindale[112] in 1912. He estimated the combined "active water-soluble" glycosides dissolved in glacial acetic acid by the density of the blue colour produced on the addition of sulphuric ammonium molybdate reagent, matched against a number of standard colours.

The current methods can be classified according to whether the reagent reacts with the butenolide side chain or with the sugar moiety of the glycosides, to give coloured products. These methods have been well reviewed in a number of recent articles[113,114,115,116,117], and therefore only a brief account of each follows.

(a) Methods dependent on the butenolide side chain

In the Legal reaction a red colour is formed when sodium nitroprusside is added to alkaline solution of a substance containing the butenolide group and it is specific for all the aglycones of the digitalis group. However the reagent has been shown to give a similar colour with related synthetic products[118]. The qualitative test has been used extensively by Jacobs[119,120], in research work on cardiac glycosides, but it has been criticised by Elderfield[56] who suggested replacing the sodium nitroprusside with potassium ferricyanide.

The test has been adapted by Kedde[121] for the quantitative estimation of cardiac glycosides by measuring the absorption photometrically in buffered solution(pH11) at wavelength 470mμ.

The Baljet reaction[122] was first used by Knudson-Dresbach[123] for the estimation of cardiac glycosides which give an orange-red colour with alkaline picrate solution. The method was used by Neumann[124] and has more recently been modified and extensively used by Bell and Krantz[125] and numerous other workers[115,126,127,128,129,130,131,132], for the spectrophotometric estimation of galenical preparations and pure glycosides of digitalis. Choice of wavelength in this, as in other spectrophotometric methods, is extremely important and much of the work of Bell and Krantz has been invalidated by the unfortunate choice of a wrong wavelength[116]. Rowson[115] was unable to confirm the claim of Bell and Krantz that better results were obtained using tetraethylammonium hydroxide as the alkali in place of sodium hydroxide.

Raymond[133] developed a quantitative method based on a reaction described by Marthoud[134] who reported that ethanolic solutions of cardiac glycosides with m-dinitrobenzene developed a blue colour on the addition of alkali. The method has been used for the estimation of digitoxin[113,135], and digitalis leaf[136]. Rowson[117] reports that this method, as described by Canback[113], is satisfactory but demands great precision in observation and is subject to inaccuracies due to the low colour developed. The absorption maximum is at wavelength 620 mμ and thus yellow colours from impurities have less effect than in the Knudson-Dresbach method where the maximum absorption for leaf extracts is at wavelength 480 mμ.

Hassall and Lippman[137] have used the red colour (max. 560 mμ) given when dilute solutions of cardiac glycosides or aglycones are mixed with m-dinitrobenzene in the presence of dilute sodium hydroxide solution or alkaline buffer at pH 11 - 13. The blue colour mentioned above is obtained with more concentrated alkali.

The Kedde method[138] utilises the purplish-brown colour obtained when digitoxin or clarified tinctures of digitalis are treated with ethanolic solution of 3:5 dinitrobenzoic acid and then made alkaline with sodium hydroxide. Pratt[139] proposed replacing this alkali with benzyl-trimethyl-ammonium hydroxide.

Rowson regards the 3:5 dinitrobenzoic acid method as the most efficient and accurate way of estimating cardiac glycosides on their aglycone content.

1:3:5: trinitrobenzene[140], 2:5 dinitrobenzoic acid[141], 1:3 dinitronaphthalene[142] and sodium naphthaquinone- 4-sulphonate[143] all give colours with cardiac genins and have been used as quantitative reagents.

(b) Methods based on the sugar moiety of the glycosides

Keller-Kiliani method.

The qualitative chemical test which is specific for digitoxose was first described in 1896[144] and consists of dissolving the sugar, or glycoside containing the sugar, in glacial acetic acid containing a trace of ferric chloride and superimposing this solution on a layer of concentrated sulphuric acid. The upper part of the sulphuric acid layer becomes brown while the lower part of the acetic acid layer changes to an indigo blue colour which is stable for some hours. If the layers are mixed a green colour results.

As early as 1906, Cloetta and Fischer[145] attempted to use this method for the estimation of cardiac glycosides. More recently, James et al.[146] described a photometric method based on this reaction for the estimation of digitoxin in tablets. Soos[147] has applied the method to the assay of digitalis leaf using the reagent recommended by Lindewald[148] and the method has been critically examined by Rowson[115] and Tattje[149].

The method, as described by Rowson, has been used in this present

work for the estimation of the chloroform-soluble glycosides in tinctures of digitalis.

The Pesez reaction.

This has been applied by Bellet[150] who described the yellow colour given by digitoxin with syrupy phosphoric acid. This colour has been used by Dequeker[151] for the photometric determination of leaf extracts but Brindle et al.[55] could not obtain reproducible results with this reagent applied to digitalis tinctures.

Bial's reaction.

This consists of the interaction of the sugar with orcine in the presence of hydrochloric acid. Langejan[152] has applied this reaction to the estimation of pure glycosides, but it does not give reproducible results with tinctures of digitalis[55].

33 per cent. hydrochloric acid.

This gives a stable yellow colour when heated with cardiac glycosides and it has been stated[55] that reproducible results can be obtained with this reagent.

Physical methods

Adsorption chromatography.

It is possible to separate various glycosidal constituents of

digitalis by chromatographing on columns of aluminium oxide[33,132], or silica gel[153]. Kaern separated digitoxin from digitoxigenin and determined each colorimetrically by the Knudson-Dresbach method.

Colouring matter which interferes with the spectrophotometric determinations of digitalis by the 3:5 dinitrobenzoic acid process can be removed on columns of alumina[35,154].

Partition chromatography.

An extensive literature is accumulating on the separation of cardiac glycosides and aglycones by means of paper chromatography[35, 55,155,156,157,158,159,160,161,162,163,164,165,166,167,168]. Both ascending and descending methods of development have been used and the solvents employed are: chloroform/methanol/water, chloroform/benzene/formamide, chloroform/formamide, chloroform/ethyl acetate/water, chloroform/ethyl acetate/benzene/water, ethyl acetate/benzene/water, toluene/propylene glycol.

The developed papers are dried and then sprayed with one of the following reagents: m-dinitrobenzene or m-dinitrobenzoic acid in alkaline ethanol, hydrochloric acid vapour, trichloracetic acid in chloroform or antimony trichloride in chloroform. Jensen[165] has also modified the Legal and Baljet reagents for use as chromatographic sprays. The trichloracetic acid spray, which gives well defined fluorescent spots with cardiac glycosides, is improved by the addition of chloramine[165] or hydrogen peroxide[167].

Silberman and Thorp[166] report a quantitative method of estimating cardiac glycosides after separation on paper. The chromatogram, sprayed with the trichloracetic acid reagent and heated for eight to ten minutes at 105-110°C., is photographed in ultra-violet light and a negative is prepared from the fluorescence photograph. Corresponding to the glycoside "spots" on the original chromatogram, a number of variable clear areas appear. Measurements of the size and density of these areas are made using a simple photoelectric photometer. Known quantities of pure glycosides are run on the same paper for comparative purposes.

Other physical methods.

In 1953 Jensen[169] described a quantitative determination of purpurea glycoside A, digitoxin and digitoxigenin based on the fluorescence obtained with the genin in a solution of hydrochloric acid and methanol containing ascorbic acid, on the addition of hydrogen peroxide. In 1954, Fruytier and van Pinxteren[170] described a fluorimetric method for the determination of gitoxin in a mixture of hydrochloric acid, glycerol and ethanol.

Ultra-violet spectroscopy[116,171] and polarography[172,173] have been applied to pure glycosides and genins.

Comparison of chemical methods

In the gravimetric methods, no account was taken of the water-soluble glycosides and the material extracted and weighed consisted of a variable mixture of glycosides and genins all with different pharmacological activities. This criticism also applies to the other chemical assay processes.

The colorimetric methods based on the sugar moiety suffer the defect that they measure the pharmacologically inactive part of the molecules. The method does not estimate free genins.

The methods based on the aglycone portion of the molecule are open to the criticism that no distinction is made between glycosides and the much less active genins. For this reason, the values obtained are often higher than the values obtained with biological assay methods. The aglycone methods are considered to give a better estimate of potency than the methods based on the sugar moiety.

Because of the presence in digitalis leaf of primary glycosides, secondary glycosides and possibly aglycones, all with different potencies, the ideal chemical method should give complete separation and estimation of the various components present. Some progress has been made in this direction, the Kedde[138] process being capable of distinguishing between glycosides and genins, while the method of

Tattje and van Os[174], employing a process of enzymatic degradation of the glycosides and utilising methods based on both the aglycone (picrate method) and sugar part of the molecule (Keller-Kiliani method) can estimate separately the primary glycosides, secondary glycosides and the aglycones.

A method of Tattje[175] based on the colour given with a reagent consisting of sulphuric acid, syrupy phosphoric acid and ferric chloride, and used to estimate gitoxigenin as distinct from digitoxigenin, has been used[176] to assay the relative proportions of glycosides related to these genins, in samples of digitalis leaf.

Use of chromatographic separation followed by estimation has definite possibilities, as shown by Silberman and Thorp[166,167] and Jensen[177].

Without more precise information on the relative pharmacological activities of the various constituents of digitalis leaf and, more especially, information on the relationship between pharmacological effects on animals and the therapeutic effects on man, detailed assessment of glycoside content by chemical assay does not provide the complete answer to this still outstanding problem of the evaluation of digitalis.

Comparison of chemical and biological assay methods

Many attempts have been made to correlate the results of

chemical assays with the results of biological assays but conflicting reports have appeared.

Of the earlier workers, Knudson-Dresbach[123], Ockaloen and Timmers[178] and Carratula[179] found agreement between a chemical assay based on the alkaline picrate (Baljet) reaction and biological assays, whereas Wasicky et al.[180] and Dyer[181] could find no such agreement. More recently Vos and Welsch[182] reiterated this lack of agreement. In 1946, Bell and Krantz[125] reporting results obtained by thirty collaborators in the comparison of the alkaline picrate and cat methods of assay on digitalis powders, tinctures, tablets and capsules stated that while some samples showed good agreement by both methods, excipients present in tablets and capsules caused wide errors. Further, they confirmed Hagemeier's[183] earlier observation that the inactive glycoside diginin reacted with the reagent and rendered the results of this chemical assay unreliable. Zöllner & Neuwald et al.[184], assaying leaf samples, found agreement between the modified Knudson-Dresbach method and both cat and guinea pig methods, but the bioassay results differed from the results of the Neuwald[185] method in which all the glycosides were hydrolysed to free genin before estimation with alkaline picrate. Brindle et al.[128] obtained agreement between the picrate method and the 18-hour frog method on tinctures prepared from one sample of digitalis leaf but results differed in the estimation of tinctures obtained from different sources. Eastland et al.[186] observed widely divergent results in the estimation of chloroform-soluble glycosides of digitalis tinctures by the picrate and guinea

pig methods. Braun and Lusky[187], comparing two commercial samples of digitoxin by the intravenous pigeon assay and the alkaline picrate assay, found the latter to give results 26 to 30 per cent. higher than the former, presumably due to the presence of the less pharmacologically active gitoxin in the samples.

The general consensus of opinion indicates non-agreement between the alkaline picrate and bioassay results.

Brindle et al. also stated that there was no agreement between results obtained by the Kedde 3:5 dinitrobenzoic acid process and the 18-hour frog method in the estimation of pure glycosides[55] or commercial tinctures[35]. However, Rowson and Dyer[188] using guinea pigs and Langejan and van Pinxteren[154] using cats, claimed that the bioassay and 3:5 dinitrobenzoic acid results agreed in the estimation of dried leaves, and Rowson[117] also found agreement in the estimation of the majority of commercial tinctures.

Chemical estimations based on the sugar part of the molecule using either Bial's reagent[154] or hydrochloric acid[55] yielded results which differed from bioassay results using the cat or frog.

In an extensive examination of several species of Digitalis, using several different chemical and biological assay methods, Fuchs et al.[114] found that while there was some agreement amongst the results of different genin methods, these did not agree with the results based on sugar estimations and that the 4-hour frog method gave different results than the guinea pig method. There was some

agreement between the results of Keller-Kiliani method and the biological methods. Rowson[117] points out that the findings of these workers can be criticised because of errors in the extraction process used.

Summarising it can be stated that although comparable results may be obtained in some cases by chemical and biological assay procedures, no one chemical method gives a true indication of the potency for all digitalis samples and that, at present, it is still necessary to standardise this crude drug and its preparations by biological assay.

STABILITY OF DIGITALIS PREPARATIONS

STABILITY OF DIGITALIS PREPARATIONS

Dried Digitalis Leaf

Gathercoal[189], Haskell and Miller[190], Rowe and Pfeifle[191], and Edmunds et al.[192], all employing a frog method of assay, could detect no loss of potency in samples of digitalis leaf which had been stored for periods between one and twenty-five years. Gold and DeGraff[193] and Haag and Hatcher[194], using cats, reported similarly on leaf which had been stored for five and fifteen years respectively. However, Schmidt and Heyl[195], examining only one sample of digitalis leaf on frogs, reported that it appeared to have lost about one-third of its activity after one year, while Christensen and Smith[196] stated that leaves containing 4.8 to 11.9 per cent. moisture deteriorated on standing.

The pharmacopoeial directions for drying the leaf as quickly as possible after collection at a temperature of 55° to 60°C. ensures that enzymatic breakdown of the cardiac glycosides is kept to a minimum during drying although the enzymes are not destroyed. During storage the moisture content must be kept low, preferably by use of a dessicating agent such as silica gel[197]. The limit of moisture in the British Pharmacopoeia is 8 per cent., in the United States Pharmacopoeia 6 per cent., and in the International Pharmacopoeia 5 per cent.

The general view is that digitalis leaf of low moisture content is stable on long storage.

Infusion of Digitalis

A very quick deterioration of aqueous extracts of digitalis is indicated by potency tests on frogs whereas these preparations appear to be relatively stable when tested on cats. Pomeroy[198] found a 33 per cent. potency loss to frogs after three weeks while Nuki et al.[199], by intravenous infusion into frogs, found no loss after two days, a 20 per cent. loss on the third day and a 40 per cent. loss in four weeks. Wokes and Elphick[200] found that infusion of Digitalis preserved with chloroform or thymol, to prevent growth of micro-organisms, showed no appreciable loss of activity to cat for at least three weeks. Hatcher and Egglestone[201] similarly showed that there was no change in potency to cats during three weeks or even after two and a half years, but there was a fifty per cent. loss of activity after eleven years. Haag and Hatcher[194] reported a loss of 75 per cent. potency in cat assays after eight years storage, but pointed out that the sample was still effective clinically, in appropriate doses.

Hintzelmann and Joachimoglu[202] stated that deterioration was more rapid in alkaline than in neutral or acid solution. Tainter[203] found a tendency for the infusions to increase in acidity during storage. It had been thought that deterioration was due to the action of micro-organisms but Tainter reported that loss of activity was not

prevented by the addition of preservatives, that the deterioration as tested by the one-hour frog method was as rapid in sterile as in contaminated infusions, and that deterioration seemed to be due to hydrolytic cleavage of the glycosides.

These observations on the infusion are interesting inasmuch as many of the comments apply equally to the tincture, in which the vehicle is seventy per cent. ethanol.

Tincture of Digitalis

In 1914 Symes [204] reported testing fifty-five tinctures which maintained their original potency for one month to one year then began to deteriorate, some of them losing as much as seventy per cent. of their potency in one year. Haskell et al.[205] examined four tinctures which had been stored for five years and found the potency to cats unchanged. Similarly Vanderhoof[206], examining an aged tincture which was giving poor clinical results, found the potency to cats unchanged but the frog-potency greatly reduced. This was the first of several papers to emphasise the discrepancy between the results obtained with the cat intravenous method and the frog lymph sac method in the estimation of aged tinctures of digitalis.

Wokes[207,208] found that during the first month of storage tinctures gave the same results when assayed by either the cat or the frog method but thereafter the potency to cats remained constant while the frog

potency decreased at an average rate of three per cent. of the initial potency per month for about nine months. The rate of deterioration then decreased until about one third to one half of the original potency was lost, at which point the potency remained constant for several years. Emig[209] also reported that after a period of deterioration, a state of equilibrium was reached. Gunn[210] found no loss of potency to frogs after one year, a ten to fifteen per cent. loss during two years and a twenty per cent. loss in eleven years. Stasiak[211], examining a three year old tincture, found a diminution in frog potency of 58.2 per cent. compared with a loss of only 10.3 per cent. in cat. Ichniowski and Thompson[212] detected no loss by the cat method but a definite loss by the one-hour frog method; on further storage, some of the potency lost to frogs appeared to be regained. Foster and van Dyke[213] found that a frog intravenous assay indicated a greater degree of deterioration than a cat intravenous assay.

Some of the earlier workers[206,214,215] found old tinctures to be weak clinically, which supported the frog assay results and they viewed cat assay results with suspicion since no account was taken of differences in absorption. Gold[96,216], however, stated that specimens found weak by the frog method were full strength when administered to man, the intensity of action corresponding to the number of cat units.

Hughes and Todd[217] advanced the theory that the difference between

the cat and frog assay results could be explained by assuming the degradation of the solubilising saponins, with consequent change in rate of glycoside absorption from the frog lymph sac. This theory has been shown to be untenable because Brindle and Rigby[128] showed that removal of saponin from fresh tinctures of digitalis caused no alteration of frog potency and the writer has shown[218] that addition of the digitalis saponin, digitonin, to aged tinctures does not restore lost potency when administered to frogs by the lymph sac route. It was also shown that a definite loss of potency was indicated by an intravenous frog assay in which the absorption factor is eliminated.

Haag[219] stated that deterioration probably rested on hydrolysis of digitoxin, gitoxin and gitalin to their respective genins. If this was true then loss of potency would certainly be more evident in frog where the glycoside/genin potency ratio is much higher than in cat[55,62,220]. A theory of hydrolysis is supported by the findings that tinctures prepared with absolute ethanol are more stable than those prepared with 70 per cent. ethanol[211,221] and both are much more stable than aqueous infusions.

While Foster and van Dyke[213] stated that digitoxin in absolute ethanol was unstable, the writer has found this glycoside to be stable in 70 per cent. ethanol, this confirming earlier reports on the stability of digitoxin[194,204].

On hydrolysis of the secondary glycosides, free digitoxose would be liberated and while its presence in tinctures of digitalis has been

reported[55] though not confirmed[35] by Brindle et al., the author has been unable to detect this sugar in aged tinctures.

Evidence is provided in this present work to show that deterioration of digitalis preparations is, to some extent at least, due to hydrolysis of the primary glycosides to the corresponding secondary glycosides without further breakdown to genins.

There have been conflicting reports on the effect of pH values on the stability of tinctures. Some workers[208,209] have stated that alkalis are more detrimental than acids; others[212,213] found no correlation between pH value and potency. Addition of acid[222] or of anhydrous sodium acetate[223] has been reported to increase stability but this view was contradicted by Carr and Krantz[224] who stated that there was no evidence to show that stability could be increased by the addition of acids or by buffering. In tinctures examined by the writer the pH values ranged from 5.58 to 5.98 and these showed considerable loss of potency to frog during storage.

Macht and Krantz[225], using their phytopharmacological method, found that polarized light had a deleterious effect on tinctures of digitalis, but Bond and Gray[226], using the cat method found the potency of digitalis preparations unaltered after exposure to ordinary or polarized light. However, it has been pointed out earlier in this work that neither method of assay is suitable for the estimation of deterioration in tinctures and in the author's experience the potency to frogs is reduced whether tinctures are stored in clear or dark coloured bottles. No comparisons were made to determine if storage in dark coloured containers reduced the rate of deterioration.

EXPERIMENTAL

MATERIALS

Collection, Stabilisation, Drying and Storage of Digitalis Leaf

The leaves used in the preparation of tinctures were collected in a number of localities in the West of Scotland, from wild plants of Digitalis purpurea (Linn.). Leaves were taken from first year rosettes and from second-year plants in flower, collection being made during dry weather following periods of sunshine, when the glycosidal content is regarded to be high[227].

Each batch, of six to eight kilogrammes, was thoroughly mixed before dividing into two equal portions. One half was dried immediately after collection in a drying cupboard maintained at 55 to 60°C. and the remainder was stabilised by treatment with alcohol vapour under pressure in order to inactivate the enzymes. The stabilisation method used was that of Hughes and Todd[211]. The leaves were placed in thin layers, on wire trays, inside a heated, water-jacketed vacuum oven fitted with a compound (vacuum-pressure) gauge. The air was withdrawn from the oven by means of a mechanical pump and then the alcohol vapour, generated in an autoclave which was connected to the vacuum oven by means of a flexible metal tube, was introduced into the oven to neutralise the negative pressure and then build up a positive pressure of five pounds per square inch. This pressure was maintained for five minutes and then released by withdrawing the alcohol vapour through a condenser. Condensation of alcohol vapour on the leaves was negligible and no leaching of the active principles occurred.

The stabilised leaves were quickly dried in a drying cupboard at a temperature of 55 to 60°C..

The dried leaves were reduced to a moderately fine powder in a disintegrator and then distributed into small glass jars with ground glass stoppers containing silica gel. The moisture content was about six per cent. and therefore no loss of activity during storage was expected to occur.

Preparation of tinctures of digitalis

Tinctures were prepared by macerating one part by weight of powdered leaf with ten parts by volume of 70 per cent. ethanol, shaking continuously for forty-eight hours[115, 228] in a mechanical shaker. This was more than adequate to ensure complete extraction of the active principles, since it has been stated recently[117] that this is achieved by shaking continuously for only one hour. Care was taken to reproduce the exact extraction conditions in the preparation of subsequent batches of tincture prepared from the same leaf. The tinctures were filtered through fast filter papers and then stored in securely stoppered bottles, some amber coloured, others of clear glass.

Some commercial tinctures were also used in this work and each was labelled with the date of manufacture and a statement that the preparation contained one unit of biological activity per ml..

Pure glycosides

In addition to tinctures of digitalis, ethanolic (70 per cent.) solutions of two samples of digitoxin were examined. Sample A was stated, by the suppliers, to contain 1369 units and Sample B about 900 units of activity per g..

Animals

The frogs (Rana temporaria) were kept in a specially designed tank with a sloping floor, constructed to allow continuous changing of the water. Any animals which were kept for several weeks were fed with live bait from time to time in order to keep them in a healthy condition. Subsequent to their arrival, a period of at least 48 hours was allowed to elapse before the frogs were used for experimental purposes and in any one experiment, frogs from the same batch were used.

Rabbits used in the electrocardiographic section of the work were of different breeds and no attempt was made to establish if any particular breed was more suitable than others for this type of work. Healthy animals of both sexes and weighing between 2 and 3 kg. were used.

BIOLOGICAL SECTION

Details of the 18-hour frog (lymph sac) method

Twenty-four frogs were selected at random and weighed to the nearest half g. after drying the skin and expressing the urine. Each animal was placed in a separate jar containing a little water and then left in an evenly illuminated part of the laboratory for two hours before injection. The animals were arranged in two groups so that as far as possible they were paired with regard to weight, an equal number of frogs of a given weight being present in each group.

The two solutions to be compared were suitably diluted with 0.6 per cent. saline solution, the dilutions being such as could be expected to produce fifty per cent. mortality in the group of frogs injected. For very weak tinctures, it was found that large doses were required and to avoid the injection of high concentrations of ethanol in these cases, the tinctures were evaporated to about half volume before dilution with saline solution.

The frogs in the first group received one of the dilutions at the rate of 0.02 ml. per g. of frog and those in the second group received the other dilution in the same dosage. Injection was made into the ventral lymph sac, the needle passing through a little of the thigh muscle to prevent leakage of the solution after withdrawing the needle. Care was taken to avoid penetration of the viscera.

After a period of eighteen hours the mortality in each group was observed, moribund animals being counted as dead.

The test was repeated using two further groups of twelve frogs and adjusting the strength of the dilutions, if necessary, in an effort to obtain fifty per cent. mortality in each group.

The comparative potencies of the two solutions were obtained from the percentage mortality figures by reference to a table in the 1948 British Pharmacopoeia, page 820. The final strengths were calculated by taking the average of the results obtained in the two experiments.

Assay of aged tinctures of digitalis by the 18-hour frog method

The potencies of three commercial tinctures (A. B. and C) were found by comparison with a tincture prepared by maceration from International Standard Digitalis Powder. The potencies of tinctures T.S.1 and D.1, prepared by the author, were found relative to the potencies of corresponding tinctures freshly prepared from the same raw materials.

Results.

Detailed results appear in Appendix I and the potency-losses during storage, together with the ages of the tinctures, are given in Table II.

Effect of added saponin on the potency of aged tinctures of digitalis as determined by the 18-hour frog assay

To a portion of each aged tincture previously shown to have lost potency during storage, the digitalis saponin digitonin was added in proportions ranging from 0.1 to 0.8 per cent., the strongest solutions being saturated with saponin. These tinctures with additional saponin were comparatively assayed against portions of the same original tinctures to which no saponin had been added.

Results.

Detailed results appear in Appendix II. The comparative potencies are shown in Table II from which it will be seen that the addition of saponin, even to the point of saturation, does not restore the potency of aged tinctures which have lost a high proportion of their initial activity during storage.

Effect of saponin on the rate of absorption from the frog lymph sac

It was noted that while the overnight mortality figures were not affected by the addition of digitonin, death appeared to occur more quickly in frogs receiving extra saponin.

A series of experiments with pure digitoxin and aged tinctures of digitalis was undertaken to compare the rates of absorption from the lymph sac of solutions with and without added saponin. The dilution and injection of solutions were done as previously described

and the number of deaths in each group of frogs was noted at intervals after injection.

Results.

From the results in Table III it can be seen that

(a) digitonin has no lethal effect when injected into the lymph sac of the frog.

(b) the presence of saponin facilitates the absorption of digitoxin but this relatively insoluble glycoside is completely absorbed, without added saponin, within twelve hours.

(c) there is sufficient saponin present in aged tinctures to ensure fairly complete absorption of the toxic principles within about three hours and certainly within twelve hours. Assays of very short duration, for example the one-hour frog method, may give misleading results due to incomplete absorption.

TABLE II

Effect of added saponin on the potency of aged tinctures of digitalis as determined by the 18-hour frog method

Tincture	Age of tincture	Potency lost during storage (per cent.)	Potency : average of assay results calculated from frog mortality figures
A	8 years	39.3	112.6
A plus 0.1 per cent. digitonin			111.7
B	6 years	16.2	118.0
B plus 0.1 per cent. digitonin			121.6
C	7 years	29.6	98.3
C plus 0.2 per cent. digitonin			107.4
T.S.1	7 months	46.2	95.8
T.S.1 plus 0.2 per cent. digitonin			106.3
D.1	1 year	59.9	91.9
D.1 plus 0.8 per cent. digitonin			89.1

TABLE III

Effect of saponin on the rate of absorption from the frog lymph sac

Expt. No.	Solution injected	No. of frogs used	Time after injection (hours) 1	1½	2	2½	3	12
			Mortality figures					
1	0.03 per cent. digitonin	23	0	0	0	0	0	0
	0.03 per cent. digitoxin	36	0	3	5	12	14	31
	0.03 per cent. each of digitoxin and digitonin	36	4	12	17	26	32	33
2	0.03 per cent. digitonin	6	-	-	-	-	-	0
	0.03 per cent. digitoxin	20	-	-	-	-	-	7
	0.03 per cent. each of digitoxin and digitonin	20	-	-	-	-	-	6
3	40 per cent. dilution of Tincture A	34	7	11	15	18	18	27
	40 per cent. dilution of Tincture A with 0.1 per cent. digitonin	34	9	14	19	21	21	27
4	40 per cent. dilution of Tincture B	12	4	-	6	6	-	9
	40 per cent. dilution of Tincture B with 0.1 per cent. digitonin	12	3	-	6	6	-	10

Assay of aged tinctures of digitalis and ethanolic (70 per cent.) solution of digitoxin by a frog intravenous method

In estimating the loss of potency to frogs (Rana pipiens) during the ageing of digitalis tinctures, Foster and van Dyke[213] used an intravenous method in which diluted tinctures, with most of the alcohol removed, were injected into groups of pithed frogs. The state of their hearts was examined after two hours and the percentage mortality recorded. A curve relating percentage mortality to relative dose was constructed, similar to the Trevan curve in the lymph sac method.

It was thought desirable, in this present work, to use a continuous infusion technique, as in the cat method, to measure the minimum lethal dose in anaesthetised frogs and to obtain figures for potency losses by both the intravenous and lymph sac methods on frog.

Details of the frog intravenous assay.

Anaesthetic.

Urethane is a suitable anaesthetic for amphibia[229] and may be used as a two per cent. aqueous solution in which the frog is immersed, or a ten per cent. solution which can be injected into the ventral lymph sac. The injection of one ml. of this solution per twenty-five g. of frog was found to give very satisfactory results, anaesthesia being established in from two to five minutes

and being maintained for at least one hour. Since this method was quicker and less variable than the immersion method, it was used throughout the series of experiments.

Route of Injection.

In previously recorded work necessitating intravenous injection into frogs, the musculo-cutaneous[230,231] and anterior abdominal veins[232] have been used. Both routes were tried and the left muscule-cutaneous vein was found to be the most accessible and convenient for the present purpose.

A median incision was made in the skin covering the ventral lymph sac and transverse incisions made in the skin covering the thorax and upper parts of the forelimbs. By reflexing the flaps of skin, the prominent musculo-cutaneous veins were seen and by careful dissection, the one to be used was cleared of surrounding tissue up to the point where it joined the brachial vein. A small portion of the anterior thoracic wall was removed to permit direct observation of the heart.

The injection was not started until ten minutes after the dissection was performed to allow for recovery from surgical shock and throughout the experiment the heart was kept moist by periodic application of frog Ringer solution.

Apparatus.

For the first experiment, the technique described by Trevan

and Boock[233] was employed, injecting a diluted tincture at the rate of 0.002 ml. every ten seconds by means of a hand-operated micrometer syringe. The injection was intermittent and tedious to perform, therefore an electrically operated continuous apparatus (Fig. 4.) was constructed by coupling the end of the micrometer to a synchronous motor. When in use the micrometer head was rotated once every minute and the plunger of the syringe pushed forward continuously to give a uniform injection at the rate of 0.01 ml. per minute.

The complete apparatus is shown in Fig. 5. The large insulated tank contained 0.6 per cent. saline solution, heated by an immersion heater and maintained at 27°C., by means of a toluene regulator, to avoid variations in the susceptibility of frogs to digitalis with changes in temperature[230,234,235]. Initially an electric stirrer was used to circulate the warm saline solution in the bath, but since the temperature at the surface remained constant $\pm$ 0.5°C. without this refinement, its use was discontinued. The injection apparatus, rigidly fixed to a wooden base board, was placed on a box so that the syringe could pass through a hole in the side of the tank, just above the surface of the saline solution. The dissected animal was supported on a cork frog-board fitted to a retort stand and so placed in the saline bath that the frog was partially submerged and in a convenient position to receive the needle into the exposed vein. Special very fine needles of 0.011 inch diameter were required because of the small size of the vein in some frogs.

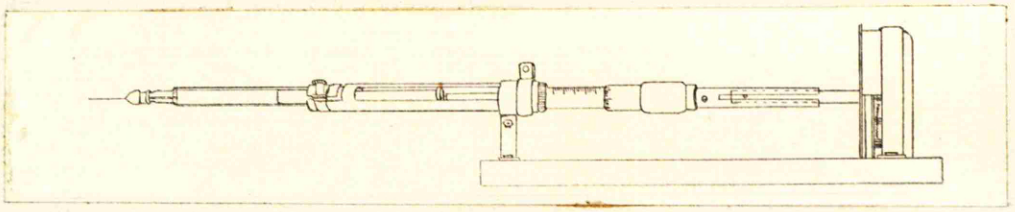


Fig. 4. Continuous injection apparatus.

Fig. 5. Complete apparatus for frog intravenous assay.

Method.

Ten ml. of the tincture to be assayed was evaporated on a boiling water-bath to a volume of five ml. to remove most of the ethanol, the presence of which leads to irregular results in intravenous assays[213]. Further evaporation led to precipitation of colouring matter etc., and an even suspension could not then be obtained on dilution. The concentrate was diluted with 0.6 per cent. saline solution to give a one in four, or in some cases a one in six, dilution of the original tincture. These dilutions were prepared freshly each day to obviate any errors due to rapid deterioration in aqueous solutions[198].

The amount of a diluted tincture required to stop the heart in ventricular systole was determined on each of several male frogs, the mean lethal dose of original tincture per 100 g. of frog being calculated.

By this method aged and fresh tinctures prepared from the same raw materials were compared, alternating the injection of the tinctures on successive frogs to avoid errors due to diurnal variation in the susceptibility of frogs, trying as far as possible to use animals of comparable weight in each group.

Eleven assays were conducted using one aged solution of digitoxin (Sample B) and four different aged tinctures, three prepared from leaf dried at 55°C. to 60°C. and one from stabilised leaf (T.S.1).

Results.

The classified results appear in Table IV and the detailed results, statistically treated for significance, appear in Appendix III.

Comparison of potency losses in aged tinctures of digitalis and ethanolic (70 per cent.) solutions of digitoxin as determined by the lymph sac and intravenous methods of assay on frogs

Some of the tinctures and two samples of digitoxin (A and B) in ethanolic solution examined by the intravenous method were also examined by the frog lymph sac (18-hour) method and the detailed results are given in Appendix I. Comparison of the potency losses indicated by the two assay processes is given in Table V.

It is evident from these results that on storage of digitalis tinctures there is a real loss of potency to the frog, a loss which is not due to failure of the absorption mechanism but which is nevertheless more pronounced in the lymph sac method of assay than in the intravenous method.

It appears that digitoxin in ethanolic (70 per cent.) solution is quite stable on long storage.

TABLE IV

INTRAVENOUS ASSAY OF A SERIES OF AGED DIGITALIS TINCTURES

Tincture	Age of tincture, weeks	Number of frogs used	Mean volume of tincture per 100 g. of frog	Standard error	Probability *t*-test, $p =$	Potency lost, per cent.
D.3	28	3	0·3301	0·006413	0·02	24·1
D.3 (*a*)	0	3	0·2504	0·020310		
D.3	36	12	0·1732	0·009473	0·05–0·02	19·2
D.3 (b)	0	12	0·1399	0·010810		
D.3	60	8	0·1624	0·006711	0·01–0·001	18·4
D.3 (*c*)	0	8	0·1326	0·005678		
D.1	38	12	0·1487	0·006290	0·01–0·001	21·3
D.1 (*a*)	0	12	0·1170	0·007698		
D.1	51	8	0·1564	0·012270	0·01–0·001	29·6
D.1 (*b*)	0	8	0·1101	0·003932		
T.D.1	18	8	0·1766	0·006301	0·001	21·5
T.D.1 (*a*)	0	8	0·1386	0·006231		
T.D.1	33	7	0·1515	0·011020	0·1–0·05	19·1
T.D.1 (*b*)	0	7	0·1225	0·009113		
T.S.1	21	8	0·1417	0·011730	0·7–0·6	4·7
T.S.1 (*a*)	0	8	0·1350	0·005123		
T.S.1	24	9	0·1897	0·015640	0·4–0·3	10·9
T.S.1 (*b*)	0	9	0·1690	0·013840		
T.S.1	36	8	0·1447	0·010730	0·05–0·02	21·4
T.S.1 (*c*)	0	8	0·1137	0·005825		
Digitoxin solution	145	8	0·1517	0·018970	0·7–0·6	5·9
" "	0	8	0·1427	0·008769		

TABLE V

COMPARISON OF FROG LYMPH SAC AND INTRAVENOUS ASSAY RESULTS ON A SERIES OF AGED DIGITALIS PREPARATIONS

Tincture	Age of tinctures, weeks	Loss of potency, per cent.	
		Lymph sac method	Intravenous method
D.1	51	59·9	29·6
T.D.1	33	54·0	19·1
T.S.1	36	46·2	21·4
Digitoxin A	25	+7·3	—
Digitoxin B	145	13·2	5·9

ANIMAL ELECTROCARDIOGRAPHY

ANIMAL ELECTROCARDIOGRAPHY

Apparatus.

The use of the cathode ray oscilloscope as a recording instrument in the electrocardiography of humans and frogs has been described by Boyd[111,236].

The recording apparatus used in this present work consisted of a double beam Cossor D.C. oscilloscope fitted with a geared motor-driven camera capable of operation at three different speeds. The output from the experimental animal was passed through a pre-amplifier before being led to one beam of the oscilloscope. The second beam was used as a time marker, the sinusoidal wave of the 50 cycle A.C. input being squared and differentiated and the peaks used to give a separate signal every 0.02 second.

Placed in parallel with the Cossor oscilloscope was a Furzehill oscilloscope with a long persistence screen on which the electro-cardiogram could be studied throughout the experiment, especially while photographic records were being taken on the other instrument.

To eliminate electrical "pick-up" from static charges and external A.C. interference, the test animals and the operator were inside a securely bonded, fine-mesh wire screening cage (Fig. 6.) constructed on lines suggested in a Post Office leaflet on radio interference[237]. All leads were made of screened cable.

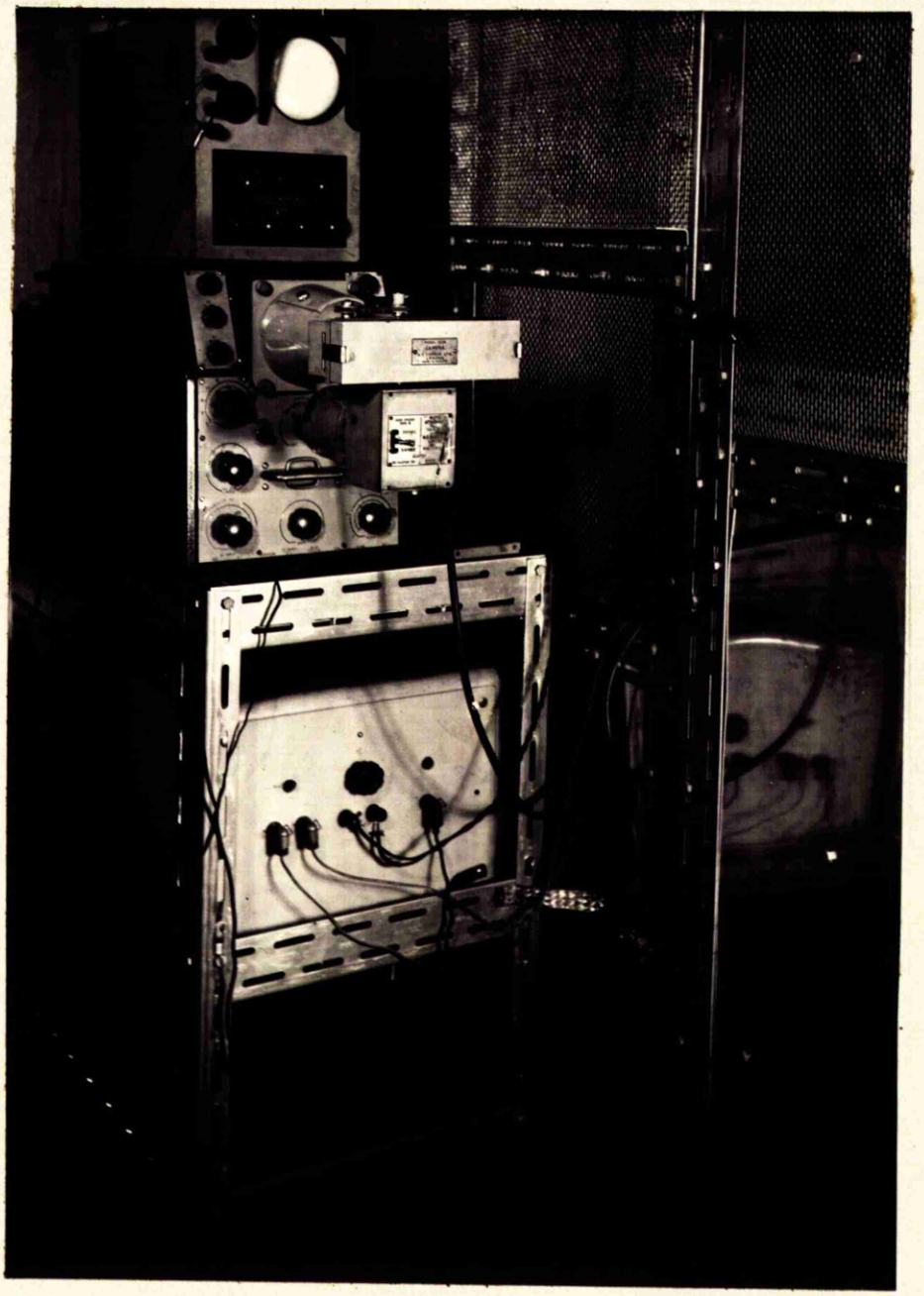


Fig. 6. Electrocardiograph

The electrodes used on rabbits were of the light-weight, spring-clip type and were made of phosphor-bronze wire with small brass plates attached[238]. Those used on frogs were pointed silver electrodes, ball and socket mounted so as to allow easy manipulation.

Electrocardiography of Rabbit

This study was undertaken to determine if it would be possible to select a group of animals whose responses to cardiac glycosides could be calibrated, for the purpose of standardising digitalis preparations.

Method.

Initially attempts were made to obtain tracings from unanaesthetised rabbits as described by Pizzi and Agosin[239], the animals being held in a specially constructed box[240], adapted to allow introduction of the electrodes. Distortion of the tracings due to animal tremors completely masked the wave form of the electrocardiogram. To remove this interference the animal was sedated by injecting pento-barbitone sodium intraperitoneally in a dose of 25-30 mg. per kg. of body weight[241]. This dosage calmed the animals for about two hours without producing deep anaesthesia.

To ensure good contact with the electrodes, the hair was removed from parts of the limbs by clipping. Attempts were made to shave the

areas but it was found that an erythema was produced and therefore after clipping, the remaining hair was completely removed by the application of a depilatory paste[242].

An electrode jelly recommended by Katz[243], or soft soap[244] was smeared on the areas in order to obtain good electrical contact between the skin and the electrodes. Equally good results were obtained with either contact medium.

The electrodes were fitted, according to the lead required, as follows :

Lead I - both forelimbs
Lead II - right forelimb / left hindlimb
Lead III - left forelimb / left hindlimb.

Normal heart tracings were recorded for each lead then a solution of ouabain in sterile 0.9 per cent. saline solution was injected into the ear vein in a dose within the range 0.01 to 0.03 mg. per kg. body weight.

Electrocardiograms were recorded at intervals up to two or three hours after the injection.

For each animal, a resting period of two weeks was allowed between experiments to allow complete elimination of the toxic principles.

The results of repeated experiments on nine rabbits were not

satisfactory since in several cases, a rabbit which showed no obvious electrocardiographic changes (Fig. 7.) with a certain dose of ouabain was killed with a very slightly increased dose on the next occasion. It became apparent that a group of rabbits whose responses to cardiac glycosides could be calibrated, would not be obtained and thus an assay process similar to that of Gold[101] who used humans, could not be adopted using rabbit as the experimental animal.

Electrocardiography of Frog

Although, as previously stated, Rothlin[107,108,109] could find no electrocardiographic criteria for distinguishing between therapeutic and toxic symptoms on the mammalian (cat) heart, no data has been published on similar work using the amphibian heart, therefore an attempt was made to correlate electrocardiographic changes in the frog with increasing doses of digitalis tinctures.

Method.

The animal was prepared for intravenous injection as described on page 55.

To prevent any deviations in the tracings due to ionisation potentials, the frog was not submerged in saline solution but the heart was kept moist by periodic application of frog Ringer solution, care being taken to allow this to drain off and not accumulate beneath the

heart. The experiments were conducted at room temperature. Particular care must be paid to earthing the animal and injection apparatus in order to avoid electrical interference.

One electrode tip was placed lightly on the base of the heart and the other on the liver just below the free apex of the heart and an electrocardiogram was recorded before the injection was started. During the continuous injection of diluted tinctures of digitalis or dilute solutions of pure glycosides, tracings were recorded at intervals until death occurred. In some experiments, 0.01 ml. of solution was injected at five minute intervals and tracings recorded after one minute and four minutes following each injection.

The injections caused the usual electrocardiographic changes associated with digitalis intoxication, namely decrease in frequency, depression or inversion of the T-wave, prolongation of the P-R interval and auriculo-ventricular dissociation. A typical recording is shown in Fig. 8. Because of the much slower normal heart rate of the frog as compared with the rabbit the camera speed was reduced from 1·2 to 0·4 inches per second and at this slower speed the time marker was ineffective because the signals were much too close together. However, since the film speed was known, the time intervals were obtained by direct measurement of the tracings.

In some experiments kymographic tracings (Fig. 9.) were made instead of electrocardiograms and it was seen that while the mechanical

recordings showed the slowing of the heart and eventual heart block caused by digitalis, finer assessment of cardiac action could be made from the electrocardiograms. However, no definite electrocardiographic change could be predicted with any particular dose of digitalis and therefore no suitable basis for quantitative estimation could be established.

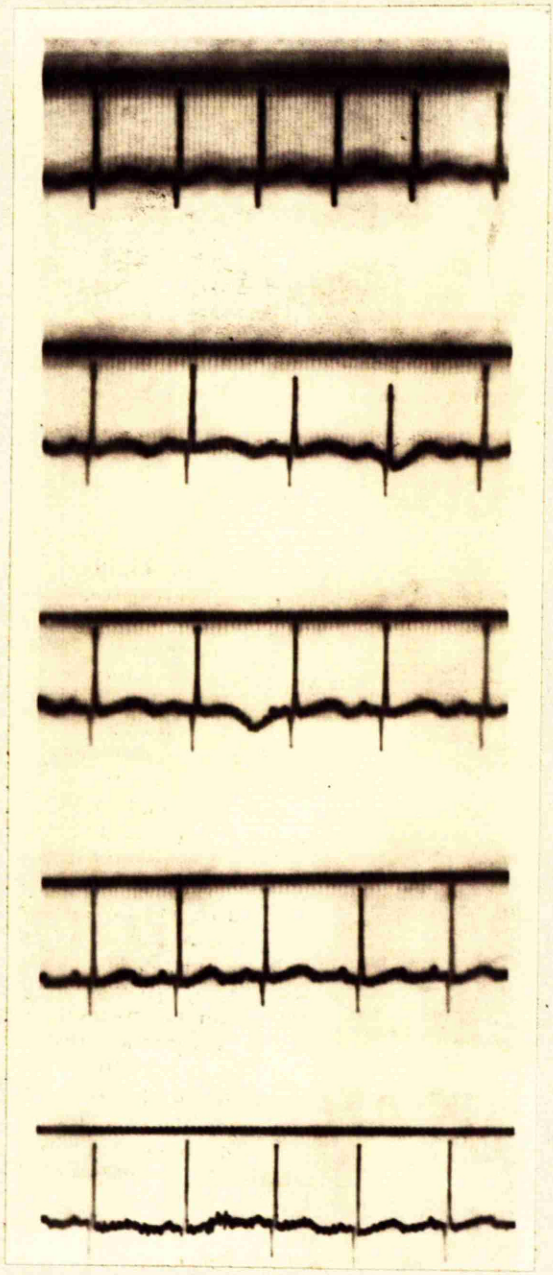


Fig. 7. Rabbit Electrocardiogram.

(Lead II. 2.5 kg. rabbit injected with 0.025 mg. ouabain)

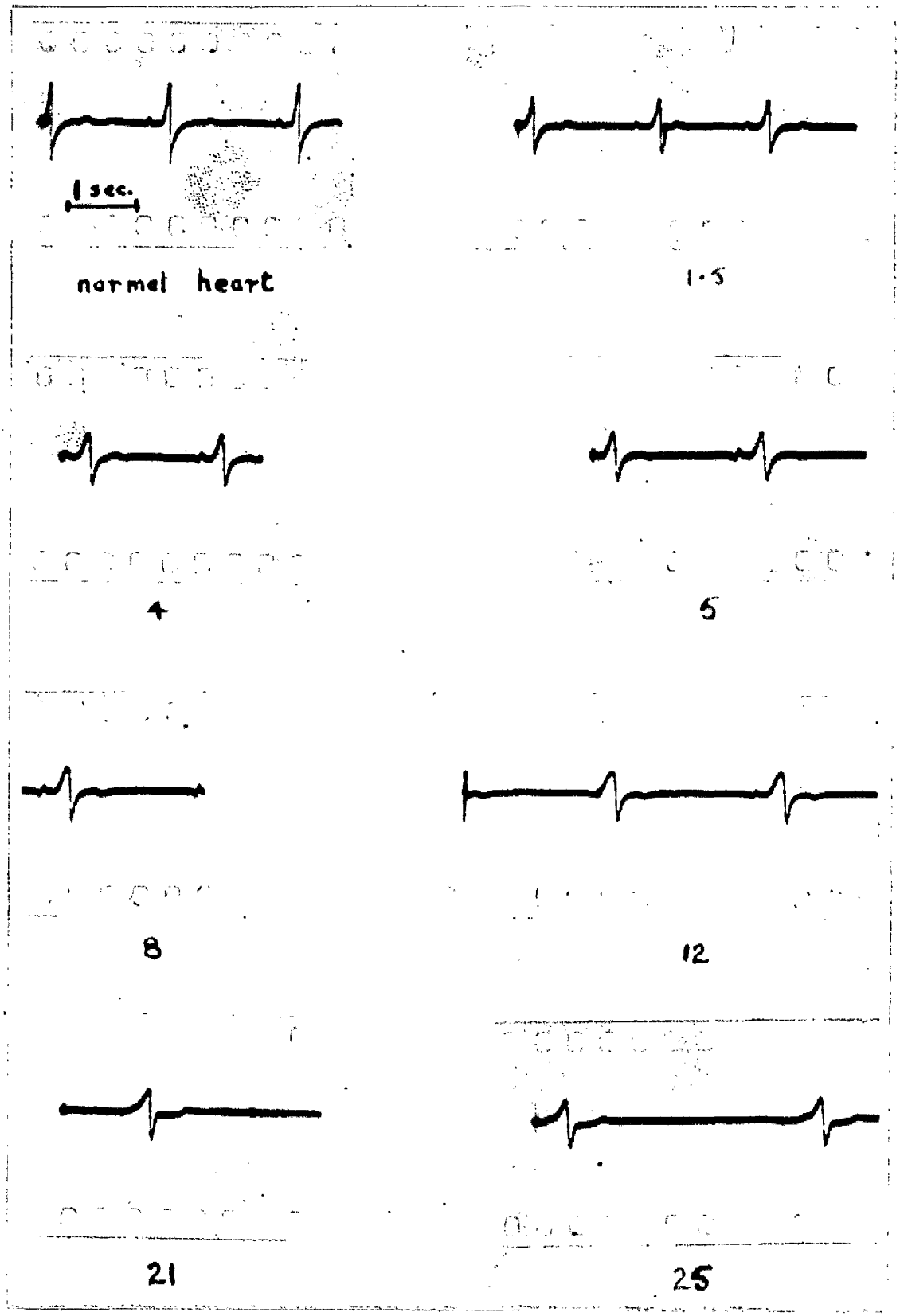


Fig. 8. Frog electrocardiogram showing effect of continuous injection of diluted tincture of digitalis.

(The number under each portion of the tracing indicates the time in minutes after the beginning of the injection)

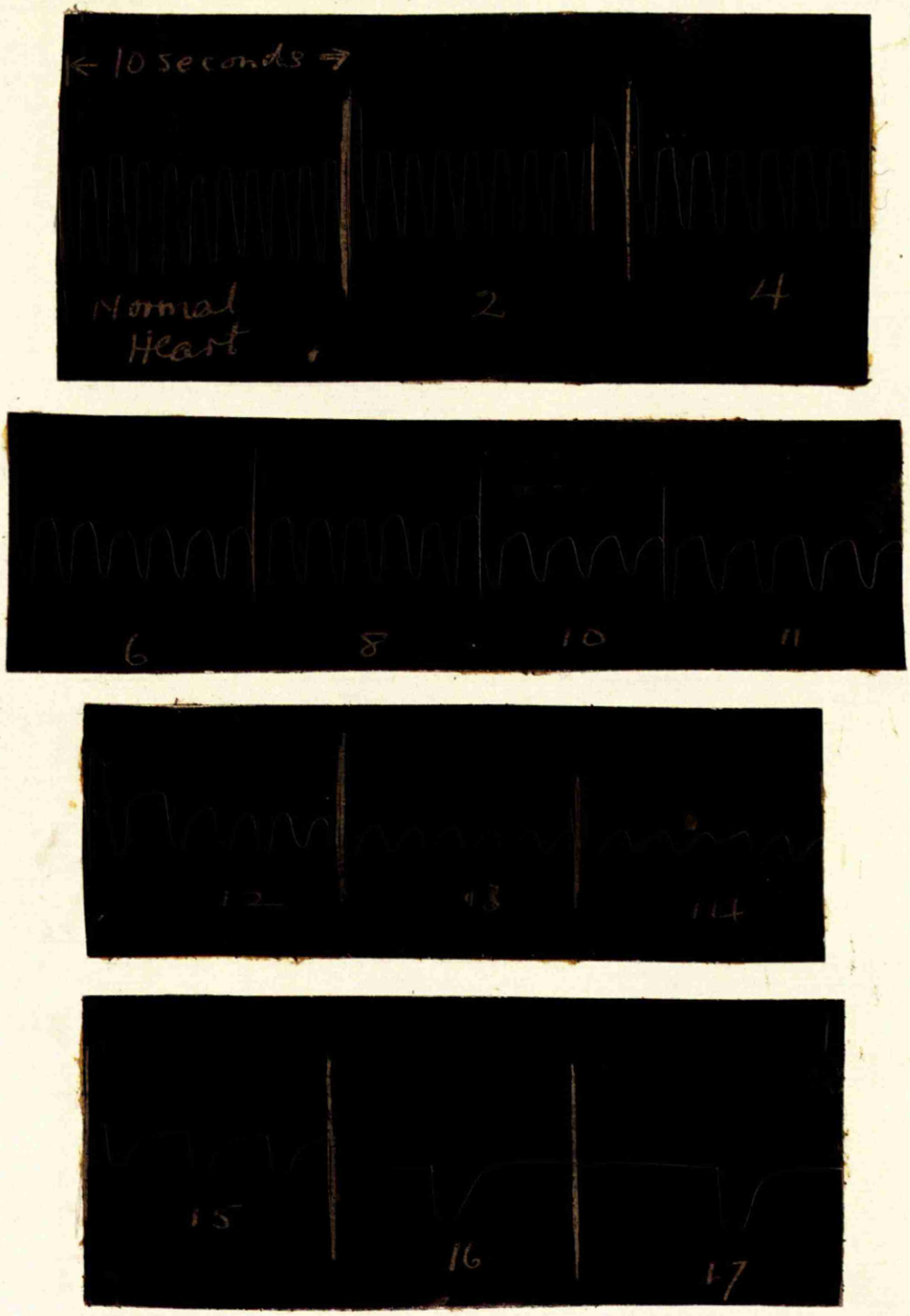


Fig. 9. Mechanical recording of frog heart beat showing effect of continuous injection of diluted tincture of digitalis.

(The number under each portion of the tracing indicates the time in minutes after the beginning of the injection)

CHEMICAL SECTION

CHEMICAL SECTION

The results of the biological assays on aged solutions of digitoxin indicated that this glycoside and presumably the other secondary glycosides, which are similar in structure, are stable when stored in ethanolic (70 per cent.) solution. It was felt however, that confirmation of their stability in tinctures was needed and this could be obtained by chemical investigation.

If the secondary glycosides suffered hydrolysis during storage, the unusual sugar digitoxose could be expected to be present in aged tinctures of digitalis. Paper partition chromatography has been widely used for the separation and identification of other sugars in vegetable materials and this method was used to examine for digitoxose in aged tinctures of digitalis.

Detection of digitoxose by paper chromatography

Apparatus.

It was found after preliminary experiments that separation of the sugars present in tinctures of digitalis could be achieved by either upward or downward development. Because of the economy in solvents using downward development, this method was used in the following work.

The glass chromatography tank used was 12 inches long, 8 inches wide and 15 inches deep; the lid was of ground glass and a tight seal

was ensured by spreading a layer of silicone grease on the part of the lid in contact with the tank. There were two small holes in the lid through which the solvent could be pipetted into the solvent-containers inside the tank, but the holes were securely closed by rubber stoppers during development of papers in order to maintain the all-important state of equilibrium of the vapours within the tank. Containers made from thick polythene tubing of 1 inch internal diameter and supported on an aluminium frame, held the developing solvent and each tube was slit longitudinally on its upper side to allow introduction of one end of the chromatographic paper. The paper was held firmly in position by means of a strip of glass also fitted into the slot in the polythene tubing and the free end of the paper passed over a clean glass rod alongside the solvent-container in order to prevent contact of the paper with the polythene tubing. Petri-dishes placed on the floor of the tank contained water or the aqueous fraction from the solvent mixture to provide a water saturated atmosphere inside the tank.

Whatman No. 1 paper for chromatography was used and two pieces 9 inches wide by 14 inches long could be accommodated in the tank at one time.

The spray reagents were applied in a fine even spray using a glass atomiser, the air pressure being applied from a rotary compressor and vacuum pump.

Solvents.

Of the two developing solvents used, namely 80 per cent. phenol

in water[245], and butanol 4 parts, ethanol 1 part and water 5 parts[246], the latter was preferred only because it was cleaner in use, although the sugars travelled further on the paper when phenol was used. In the butanol mixture, the components were shaken together thoroughly for several hours to ensure complete saturation of each phase with the other, then the aqueous fraction was separated and placed in the petri-dishes at the bottom of the tank, the organic fraction being retained for subsequent use to irrigate the papers.

Spray reagents.

Numerous reagents have been used for the detection of carbohydrate substances on paper chromatograms, each giving a characteristic colour reaction with some or all sugars. Many of the reagents are phenolic and react with the furfurals produced when the sugars on the paper chromatograms are heated.

In this work, 0.2 per cent. solutions of guaiacol, naphthoresorcinol, α-naphthol, orcinol, phloroglucinol, resorcinol and xanthydrol in absolute ethanol were prepared and nine ml. of the solution to be used was mixed with one ml. of syrupy ortho-phosphoric acid immediately before use, as suggested by Bryson and Mitchell.[247] After spraying with any of these reagents the papers were dried at 100° to 105°C. for 2 to 5 minutes. Other spray reagents used were :

(a) aniline hydrogen phthalate in water-saturated butanol[248] with which papers were heated at 105°C. for five minutes.

(b) 0.5 per cent. benzidine in a mixture of 20 parts glacial acetic

acid and 80 parts absolute ethanol[249]. The papers were heated at 90°C. for 10 minutes.

(c) the β-naphthylamine reagent of Novellie[250]. A higher temperature was required in this case, the papers being heated at 160° to 170°C. for 10 minutes.

To test the suitability of these reagents for the detection of digitoxose, a small drop of an aqueous solution of this sugar was applied to filter paper and dried. As a check on the efficiency of the reagent for the detection of sugars in general, several other sugars were applied to the same paper as separate spots. After drying, the paper was sprayed with one of the reagents and heated in a hot air oven for the specified time. The colours given with the various sugars and the colour of the paper were noted. All the reagents used gave coloured products with digitoxose, but the following gave the most satisfactory results because of the strong colours produced and the colourlessness or contrasting colour of the background.

β-naphthylamine: digitoxose gave a bright pink colour and other sugars gave pale brown colours against a yellow background.

aniline hydrogen phthalate: digitoxose gave a pale brown colour which changed to pink on standing for 24 hours. Other sugars gave brown to dark brown colours against a pale buff coloured background.

naphthoresorcinol: was an excellent reagent for sugars and gave with some, including digitoxose, a blue to green colour, and with others a deep red colour against a pink background.

guaiacol: the background remained colourless and digitoxose gave a pronounced grey colour. Some other sugars gave faint brown colours.
xanthydrol: gave a bright pink colour with digitoxose only, the paper becoming yellow.

Decolorisation and concentration of digitalis tinctures prior to chromatographic examination

To prevent streaking of the colouring matter of tinctures when these were applied to paper chromatograms, it was necessary to obtain an almost colourless solution for application, otherwise the colour reactions between the reagents and the sugars present would have been masked.

Since only very small amounts of free digitoxose could be expected in tinctures, it was also necessary to concentrate these. Samples of dried digitalis leaf contain about 1 per cent. of cardiac glycosides and the tincture represents a 1 in 10 dilution. On a weight basis, digitoxose comprises approximately one half of each glycoside molecule and therefore even assuming complete hydrolysis of the total glycosides, one could expect only about 50 mg. of digitoxose in every 100 ml. of tincture.

It was found that shaking with activated charcoal, or precipitating with lead subacetate, or passing the original tincture through a column of activated alumina did not remove all the colouring matter and on concentration a very dark brown solution was obtained.

By using the following method, a suitable clear concentrate was obtained. 50 ml. of tincture was evaporated to 10 ml. under reduced pressure to avoid breakdown of the thermolabile glycosides. The concentrate was shaken with 4 successive 10 ml. quantities of chloroform to remove green colouring matter, genins and unchanged glycosides. Sufficient kieselguhr was added to the separated dark brown aqueous liquid to form a stiff paste which was dried in a vacuum oven at 60°C. The product was powdered, sifted through a No. 20 sieve and extracted continuously for three hours with dry acetone in a Sohxlet apparatus. Since acetone has a low boiling point, there was no risk of charring the sugar with this solvent. The pale yellow extract obtained was concentrated by evaporation of the solvent, then added to a 10 g. column of activated alumina packed to a height of about 12 cms. The material was eluted with dry acetone and the eluate was collected in 10-ml. fractions. As shown by adding digitoxose to tinctures prior to treatment, any digitoxose present could be expected in the first two fractions of the eluate. These two fractions were mixed and concentrated by evaporation of the solvent, to a volume of about 1 ml.

Examination for digitoxose in aged tinctures of digitalis by means of paper chromatography

A total of 0.01 ml. of a concentrate obtained as described above was applied to a single spot near one end of the chromatographic paper and pure digitoxose in acetone solution was applied as a separate spot

on the same starting line to act as a marker or reference spot. With the size of paper used, several tinctures could be examined at the same time. When the applications had dried throughly, the paper was placed inside the tank, which was then sealed and the paper was left overnight to become saturated with water vapour. The developing solvent was pipetted into the solvent container and the paper was slowly irrigated for seven to eight hours after which it was removed from the tank and allowed to dry spontaneously. On removal from the tank the position of the solvent front was marked. The dried paper was sprayed evenly and completely with one of the spray reagents and heated at the temperature and for the time previously stated.

Using the butanol solvent, the Rf value for digitoxose (measured to the front of the spot) was found to be about 0.59 and other carbohydrate substances usually present in the tinctures were found to have Rf values of approximately 0.79, 0.51, 0.24 and 0.145. Slight variations in Rf values are encountered with changes in temperature but since digitoxose was used as a reference spot in all the experiments, these were conducted at laboratory temperature and conclusions were drawn by comparing the distances travelled by the sugars with the distance travelled by the digitoxose in each individual experiment.

Results.

In five aged tinctures examined repeatedly, no digitoxose was found

even when 1 litre of starting material was used. However when quantities of less than 2 mg. of pure digitoxose were added to 50 ml. of tincture and the above process carried out, the sugar could be detected readily.

In order to conserve the pure digitoxose, of which only a very small supply was available, a hydrolysate of digitoxin prepared as follows was added to large volumes of tincture when it was required to show that the method was capable of detecting digitoxose.

To 50 mg. of digitoxin, dissolved in 3 ml. of ethanol, 3 ml. of dilute sulphuric acid was added and the mixture allowed to stand at 37°C. for 2 hours. After dilution with 6 ml. of water the solution was allowed to stand at 37°C. for 18 hours then neutralised with strong solution of sodium hydroxide. The hydrolysate was shown to contain digitoxose by chromatographing against pure digitoxose.

After the completion of this work, Brindle et al.[54] reported the presence of digitoxose in two of six tinctures examined, therefore further work was conducted on ten aged tinctures using the quantities and decolorisation process used by these workers: this was the lead subacetate method previously rejected.

Using the 50 mg. level of lead per ml. of tincture, as recommended by Rowson[115], and removing the excess lead with sodium sulphate instead of hydrogen sulphide, better results were obtained than hitherto,

although the subsequent concentrate was still coloured. Nevertheless when digitoxose was added to tinctures in quantities of 100 µg. per 10 ml., either before or after clarification, its presence could be detected easily on paper chromatograms. No digitoxose could be found however in any of the ten aged tinctures unless it was previously added.

The absence of free digitoxose from aged tinctures was due either to the fact that no hydrolysis of the glycosides to genin plus sugar had occurred during storage, or, if sugar was split off, then some further change destroying the digitoxose had occurred.

In order to determine whether or not the glycosides had been hydrolysed, comparison was made of the total chloroform-soluble glycosides in aged and fresh tinctures of digitalis. It was necessary to use a chemical assay which differentiates between glycoside and genin, and therefore a method based on the sugar part of the molecule was used.

Spectrophotometric determination of the chloroform-soluble glycosides in aged and fresh tinctures of digitalis by a process based on the Keller-Kiliani reaction

Although this method has been criticised, partly because the extraction with chloroform is not quantitative, this objection was overcome by rigidly fixing the extraction conditions, and since the preparations

under comparison were identical in all respects other than age, a method similar to that described by Rowson[115] was deemed to be suitable.

Method.

The following procedure was carried out on pairs of tinctures, aged and fresh prepared from the same raw materials, and all the stages in the process were done concurrently on the two preparations.

5 ml. of tincture was mixed with 5 ml. of water and one ml. of strong solution of lead subacetate and the mixture centrifuged until the supernatant liquid was clear. This was separated and to exactly 5 ml. was added 2 ml. of a 6.3 per cent. solution of sodium sulphate ($Na_2SO_4.10H_2O$). The precipitate was removed by centrifugation and the clear supernatant liquid transferred to a separating funnel to be shaken with six separate 10 ml. quantities of chloroform. Initially, some difficulty was experienced due to extraction of the grease from the separating funnel taps but this was overcome by using "Quickfit" funnels in which the taps were so carefully ground that lubricant was not needed to prevent leakage of the chloroform. After each addition of chloroform, the mixture was shaken for thirty minutes in a mechanical shaker, the chloroformic layer allowed to separate and then run into a dry 100 ml. conical flask to be evaporated to complete dryness over a boiling water bath. To the total dried residue was added exactly 10 ml. of a reagent prepared as follows:

30 ml. glacial acetic acid (Analar)

0.33 ml. of a 15 per cent. solution of ferric chloride

2.5 ml. concentrated sulphuric acid (Analar)

The reagent was prepared freshly each day and allowed to cool to room temperature before use. Rowson stated that the colour density was reduced and the colour-development time increased with increase in the proportion of moisture present in the reagent, therefore an equivalent amount of 15 per cent. solution of ferric chloride was used in place of the 5 per cent. solution used by this worker.

Using the reagent as control, the colour densities produced by the residues from 5 ml. of fresh and aged tinctures were measured in the one-centimetre cells of a "Unicam S.P.600" spectrophotometer, at wavelengths over a range from 440 mμ to 630 mμ. It can be seen from Fig. 10. that the highest colour density is at wavelength 470 mμ with a second peak at 590 mμ. The absorption spectrum obtained with tinctures thus differed from the spectra obtained with pure digitoxin or digitoxose in the Keller-Kiliani reagent (Fig. 11) where similar peaks were obtained but the highest was at wavelength 590 mμ. The spectrum obtained with the pure glycoside was the same as that reported by other workers[115,116].

Several determinations of the colour development with time were made at wavelengths 470 mμ. and 590 mμ. and from Fig. 12. it can be seen that the colour develops more quickly, but is less stable at 470 mμ. The colour reaches maximum density at 590 mμ. within thirty minutes, is stable for a further thirty minutes, and then begins to fade very gradually.

In all subsequent determinations, measurements of colour density

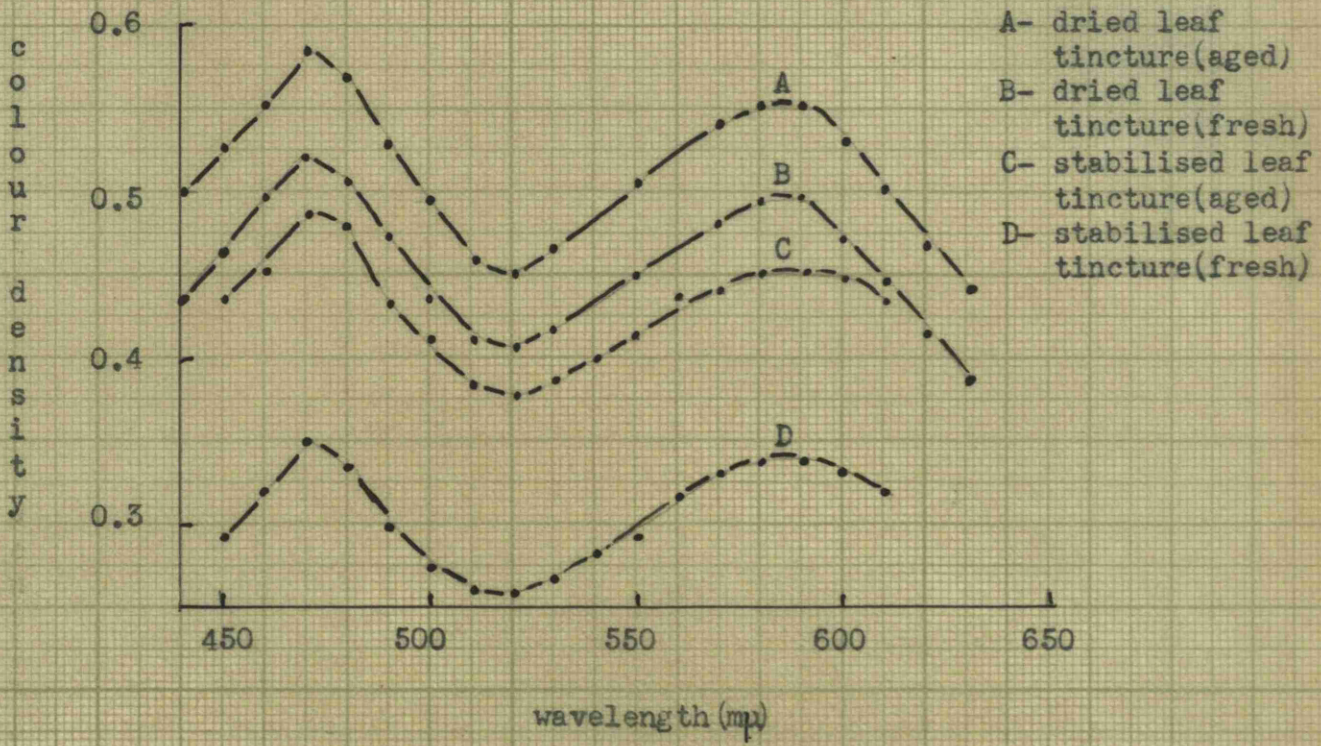


Fig. 10. Keller-Kiliani reagent and chloroform-soluble glycosides from tinctures of digitalis.

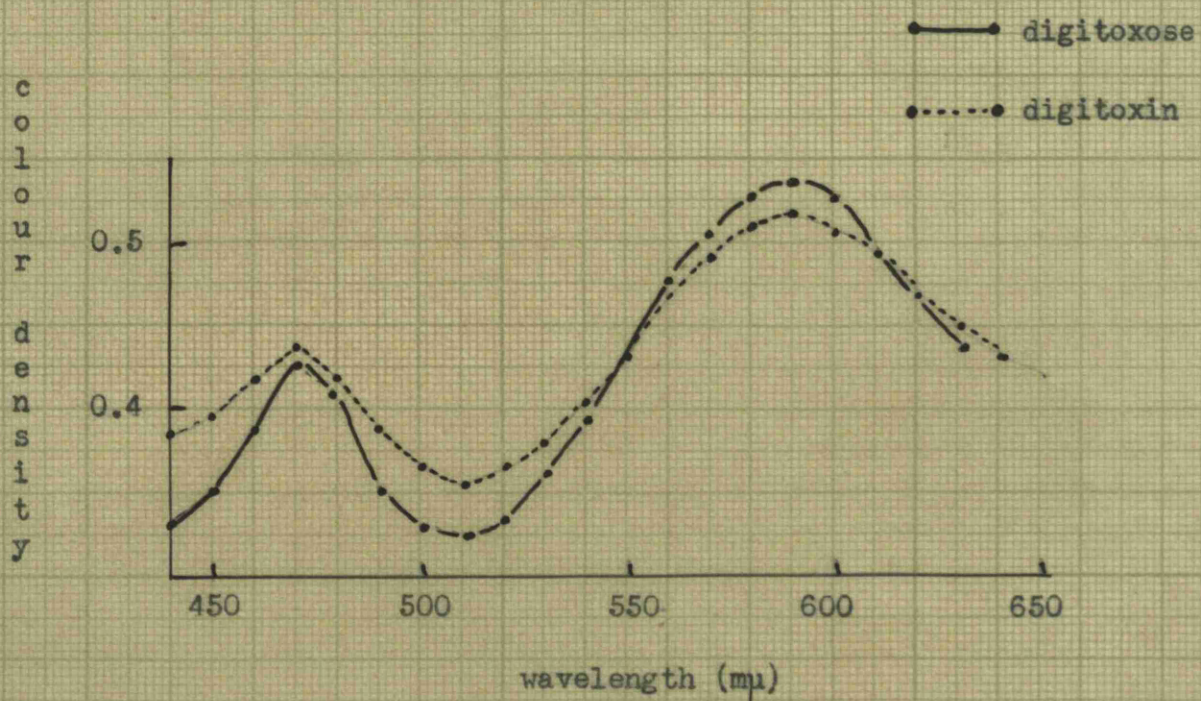


Fig. 11. Keller-Kiliani reagent with digitoxin and with digitoxose.

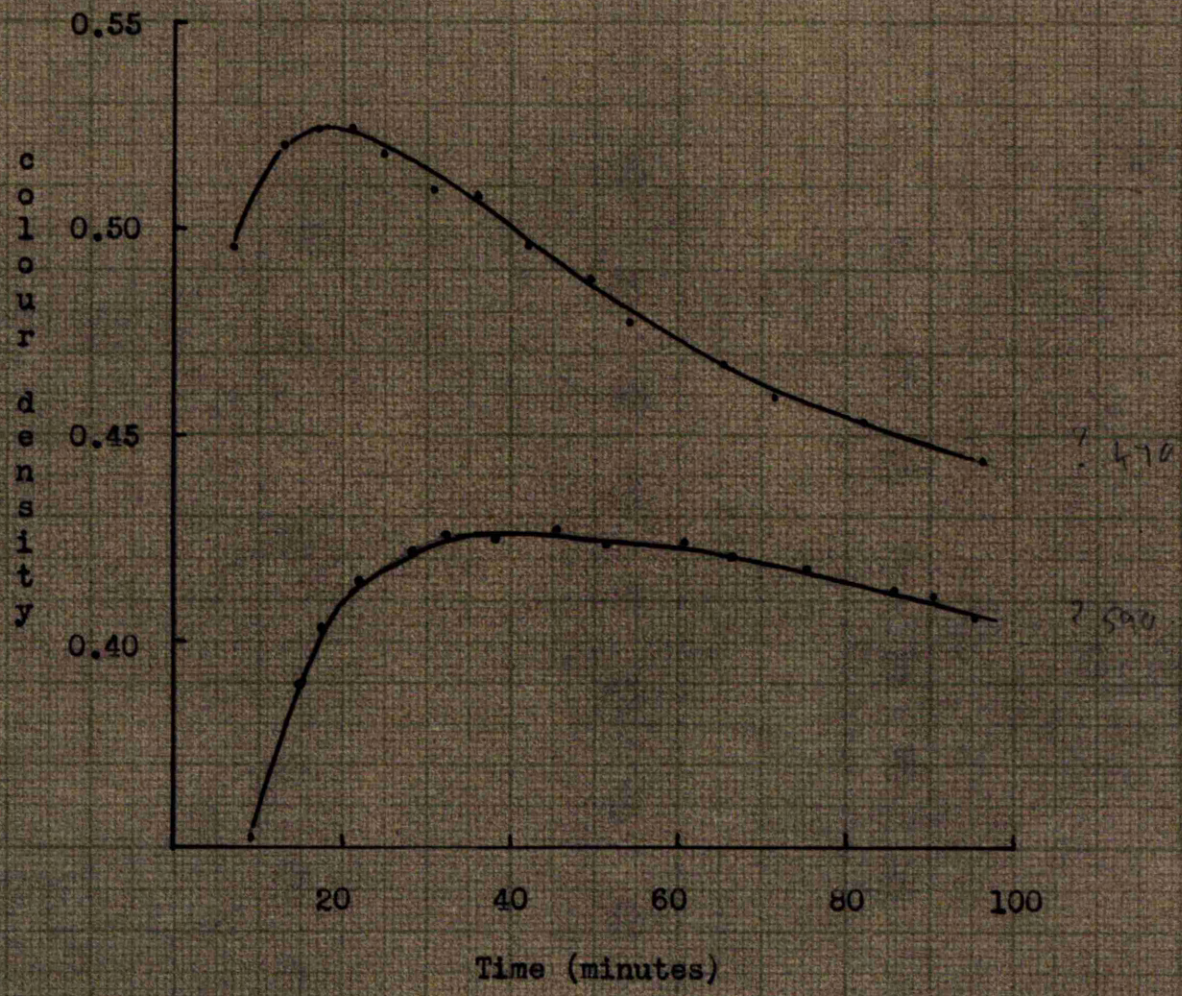


Fig. 12. Keller-Kiliani reagent and tincture of digitalis. Colour development with time at wavelengths 470 mμ and 590 mμ.

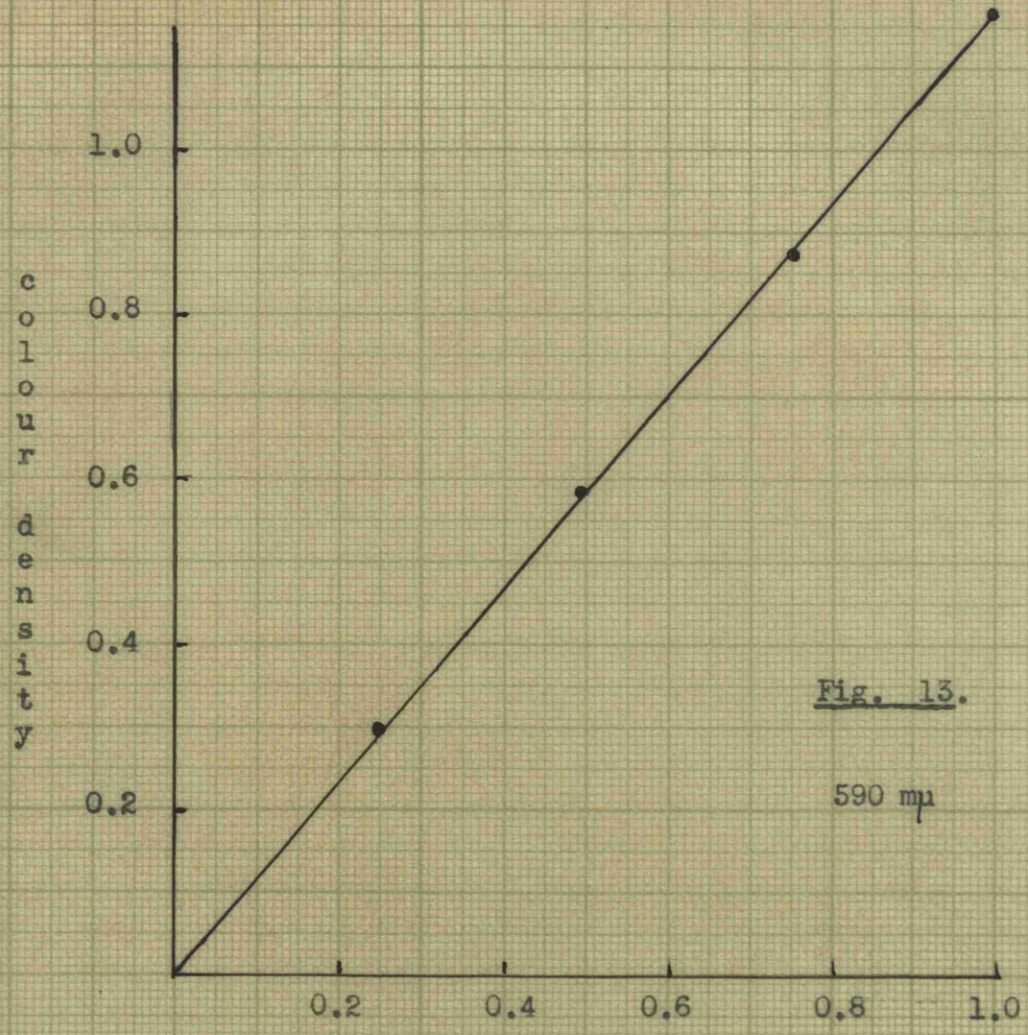


Fig. 13.

590 mμ

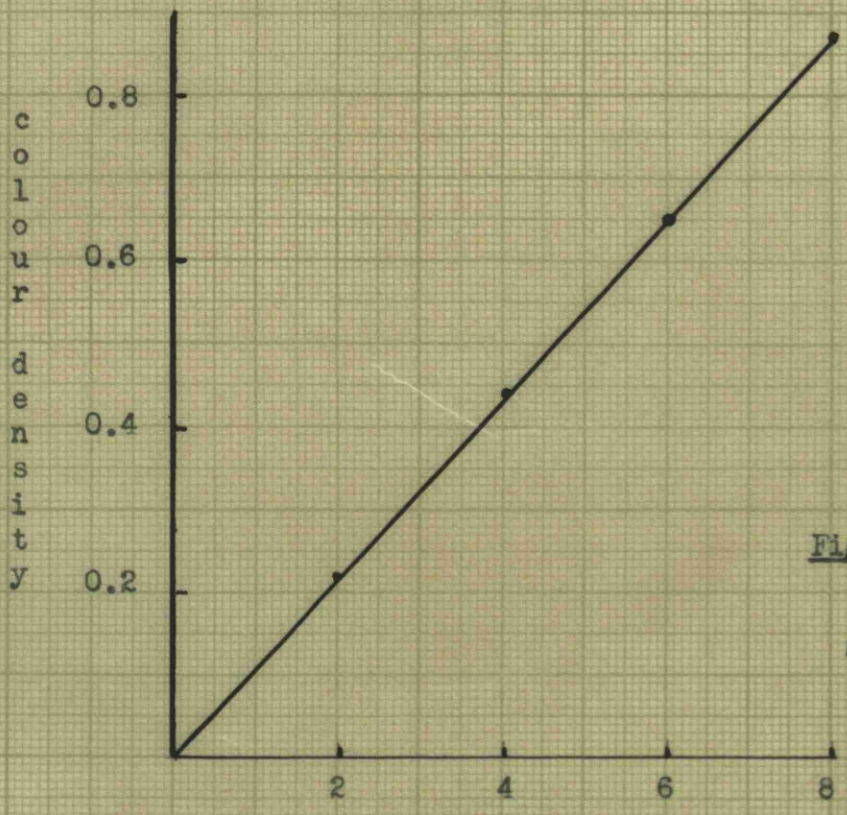


Fig. 14.

590 mμ

ml. tincture in 10 ml. Keller-Kiliani reagent

were made at wavelength 590 mμ, thirty minutes after mixing.

Results.

The results in Table VI show the comparison of colour densities obtained with aged and fresh tinctures. Each value is the average of duplicate or triplicate readings which did not differ from each other by more than 2 per cent.

The equivalent amounts of digitoxin shown in the table, were obtained by reference to a graph constructed from data obtained by measuring the maximum colour density at wavelength 590 mμ. obtained with pure digitoxin dissolved in the Keller-Kiliani reagent. From this graph (Fig. 13.) it can be seen that digitoxin in concentrations of 0.02 to 0.1 per cent. obey the Beer-Lambert law.

The colour densities given with the chloroform-soluble glycosides extracted from different volumes of the same tincture and dissolved in the reagent were also plotted graphically, and it can be seen from Fig.14 that the Beer-Lambert law is obeyed in the range shown. The values recorded in Table VI all lie within this range.

TABLE VI.

COLORIMETRIC ASSAY OF AGED AND FRESH TINCTURES OF DIGITALIS AND SOLUTION OF DIGITOXIN
CHLOROFORM EXTRACT FROM 5 ML. OF TINCTURE IN 10 ML. OF KELLER-KILIANI REAGENT

Tincture	Colour density (590 mμ)		Equivalent of digitoxin, mg.	
	Aged	Fresh	Aged	Fresh
G.D	0·562	0·507	0·477	0·430
M.D	0·650	0·571	0·552	0·485
T.D	0·414	0·388	0·351	0·329
D	0·485	0·470	0·412	0·399
G.S	0·459	0·450	0·390	0·382
M.S	0·600	0·572	0·509	0·485
T.S	0·531	0·480	0·451	0·407
Digitoxin B	0·429	0·432	0·365	0·365

The results shown above indicate that the proportion of chloroform-soluble glycosides, calculated as digitoxin, increases during the storage of digitalis tinctures. This increase could be accounted for only by assuming the breakdown of primary glycosides to secondary glycosides which are more soluble in chloroform and which, on a molar basis, give a greater colour density with the Keller-Kiliani reagent than the primary glycosides[116,251,252]. The aglycones do not react with this reagent and since any free sugar would be left in the aqueous fraction on shaking with chloroform, hydrolysis to genins would have been accompanied by a reduction in the spectrophotometric determinations. To further confirm this stability of secondary glycosides, an aged solution of digitoxin was assayed chemically.

Spectrophotometric determination of digitoxin in aged ethanolic (70 per cent.) solution.

A solution of digitoxin (Sample B) was stored at room temperature for nine months and a portion of the same solution was stored for eight months at room temperature and then one month at 55°C. The two portions of this solution were separately assayed by comparison with a freshly prepared solution of the same initial strength.

Method.

0.5 ml. of each solution, corresponding to 0.365 mg. of glycoside, was diluted with 4.5 ml. of distilled water and then shaken with six

separate 5 ml. quantities of chloroform. The mixed chloroform extracts were evaporated to complete dryness over a boiling water bath and the residue mixed with 10 ml. of freshly prepared Keller-Kiliani reagent. The colour density at wavelength 590 mμ. was measured thirty minutes after mixing.

Results.

The results for the two portions of aged solution were identical and the average value of duplicate readings taken for each portion is given in Table VI. Comparison with the colour density given with the freshly prepared solution shows that no change had occurred during storage.

This stability of digitoxin in solution and the absence of measurable quantities of free digitoxose from aged tinctures of digitalis indicate that the loss of potency during storage of tinctures is not due to hydrolysis of the secondary glycosides. non-sugar

Enzyme etc. present in Dr. not present in pure digitoxin. ∴ some may hydrolyse the digitoxins therein whilst the latter is stable

Comparison of chemical assay (Keller-Kiliani method) with biological assay (18-hour frog method) on samples of digitoxin

Two commercial samples of digitoxin were available for these determinations. Sample A was stated, by the suppliers, to contain 1369 units of biological activity per g., while Sample B, obtained from a different source, was reputed to contain only about 900 units per g.

Chemical assay.

For each sample of digitoxin, a volume of ethanolic solution equivalent to 0.4 mg. of glycoside was evaporated to dryness and 10 ml. of Keller-Kiliani reagent added to the residue. The colour density was measured at wavelength 590 mμ. thirty minutes after mixing.

TABLE VII

Comparative assay of two commercial samples of digitoxin by the Keller-Kiliani method

Colour density	
Digitoxin A	Digitoxin B
0.442	0.445

Results.

The results in Table VII showed that the two samples were equipotent as determined by this chemical assay.

Biological assay.

Ethanolic solutions of the two samples were assayed by the 18-hour frog method as described earlier (page 49).

TABLE VIII

Comparative assay of two commercial samples of digitoxin by the 18-hour frog method

Solution	Digitoxin A		Digitoxin B	
	1st. day	2nd. day	1st. day	2nd. day
Ml. solution per 10 ml. dilution	3.0	4.0	3.0	3.3
Overnight mortality	2/12	5/12	6/12	4/12
Mortality per cent.	16.66	41.66	50.0	33.3
Potency	76.66	94.7	100.0	89.0

Solution A contained 1 mg. of Digitoxin A in 1.113 ml.

Solution B contained 1 mg. of Digitoxin B in 1.369 ml.

Therefore potency of Digitoxin A in terms of Digitoxin B equals

(1) $\frac{76.66}{100.0} \times \frac{1.113}{1.369} \times \frac{3}{3} \times \frac{100}{1}$ = 62.26 per cent.

(2) $\frac{94.7}{89.0} \times \frac{1.113}{1.369} \times \frac{3.3}{4.0} \times \frac{100}{1}$ = 71.34 per cent.

Average = 66.8 per cent.

Since Digitoxin A contained 1369 units of activity per g.

Digitoxin B contained $\frac{66.8}{100.0} \times \frac{1369}{1}$ = 915 units of activity per g.

Results.

The results, given in Table VIII showed that the two samples were of quite different potency, the strengths being of the order stated previously.

The discrepancy between the results obtained by the two methods of assay on samples of digitoxin, serves to emphasise the inadequacy of the Keller-Kiliani method for the standardisation of digitalis preparations.

DISCUSSION

DISCUSSION

In this work it has been confirmed that tinctures of digitalis lose a considerable proportion of their potency to the frog during storage and it has been shown that tinctures prepared from stabilised leaf are not more stable than tinctures prepared from leaf which has been dried according to pharmacopoeial directions at 55°C.

The addition of saponin to aged tinctures of digitalis does not restore the lost potency and since aged tinctures, even without added saponin, are well absorbed within twelve hours the potency loss is not due to poor absorption from the frog lymph sac. This is further demonstrated in the results of frog intravenous assays which revealed a significant loss of potency in aged tinctures and which thus conflicted with published results of similar assays on cat.

The discrepancy between frog and cat results can be accounted for only by assuming that chemical changes occur in the tinctures during storage and that the breakdown products are less potent than their precursors to the frog but not to the cat. The most likely change appeared to be the hydrolysis of the glycosides.

It has been shown by both biological and chemical assays that digitoxin, which is typical of the secondary glycosides, is stable when stored in ethanolic (70 per cent.) solution at room temperature or even at 55°C. Further, from the results of

spectrophotometric determinations on aged tinctures which showed no reduction in the percentage of chloroform - soluble glycosides and from the absence from aged tinctures of measureable quantities of free digitoxose, which would result from hydrolysis of the secondary glycosides, it appears that no hydrolysis of these glycosides occurs during the storage of tinctures of digitalis.

Aged tinctures yield a chloroform extract which gives a greater colour density with the Keller-Kiliani reagent than an extract obtained from the same volume of a corresponding freshly prepared tincture. Since digitoxin gives a greater colour density with this reagent than an equimolar amount of purpurea glycoside A, the apparent increase in potency as shown by the spectrophotometric assay of aged tinctures could be explained by assuming the hydrolysis of the primary glycosides to their corresponding secondary glycosides. Such a change would result in loss of potency to frog, to which the secondary glycosides are less potent than their precursors, but no less would be shown by cat assays since the secondary glycosides are not less potent to cat than the primary glycosides. The splitting of the terminal glucose molecule from the sugar chain in primary glycosides of digitalis seems probable in such mild conditions as exist in tincture of digitalis and this theory of hydrolysis is offered as an explanation of the potency loss to frog during storage of the tinctures.

The decrease in potency of tinctures as shown by biological methods compared with the increase in potency of the same tinctures

as shown by a chemical method and the contradictory results obtained by these two types of assay on samples of digitoxin, emphasise the lack of suitable criteria for the true estimation of digitalis preparations. A biological method which could be correlated with therapeutic effects appears to offer the best means of assaying crude cardioactive materials and it is possible that a measurement of electrocardiographic effect would provide a solution to this difficult problem. However, preliminary work has shown that the rabbit is not a suitable experimental animal for this type of estimation and much still remains to be done to establish the method using the frog as experimental animal.

SUMMARY

SUMMARY

1. Tinctures prepared from either dried or stabilised digitalis leaf are unstable on storage, losing up to 60 per cent. of initial potency as determined by the 18-hour method of assay on frogs.

2. The loss of potency is not due to breakdown of solubilising agents with resultant loss of absorption from the frog lymph sac, since aged tinctures are well absorbed within twelve hours and the addition of saponin does not increase the potency of these tinctures.

3. Intravenous assays of aged tinctures of digitalis reveal a 20 to 30 per cent. loss of potency to frog during nine to twelve months storage.

4. The loss of potency to frog is not due to hydrolysis of the secondary glycosides because digitoxin, in ethanolic (70 per cent.) solution has been shown to be stable and no free digitoxose, which would result from hydrolysis of the secondary glycosides, could be found in aged tinctures.

5. Spectrophotometric assays based on the Keller-Kiliani reaction show a slight increase in strength of digitalis tinctures during storage. This indicates conversion of primary glycosides to secondary glycosides, a change which would account for the loss of

potency to frog, but would result in no potency loss to cat.

6. Samples of digitoxin found equipotent by chemical assay, using the Keller-Kiliani reagent, were of widely different biological potency measured on frog.

7. No satisfactory method exists for the accurate assay of all digitalis preparations and work is proceeding in an effort to establish a method based on cardiotonic effects as shown by changes in the electrocardiogram of frog.

APPENDIX I

RESULTS OF ASSAYS BY THE 18-HOUR FROG METHOD ON TINCTURES OF DIGITALIS AND ETHANOLIC (70 PER CENT.) SOLUTIONS OF DIGITOXIN

TABLE IX

Assay of aged commercial tincture A

Tincture	Standard		A	
	1st. day	2nd. day	1st. day	2nd. day
Ml. Tincture per 10 ml. dilution	3.0	2.5	3.0	3.5
Overnight mortality	10/12	7/12	2/12	4/12
Mortality per cent.	83.3	58.3	16.6	33.3
Potency	125.3	105.6	76.6	89.0

The standard tincture contained 1 unit per ml.

Therefore Tincture A contained:

(1) $\frac{76.6}{125.3} \times \frac{3.0}{3.0}$ = 0.6113 unit per ml.

(2) $\frac{89.0}{105.6} \times \frac{2.5}{3.5}$ = 0.6002 unit per ml.

Average = 0.6058 unit per ml.

Since Tincture A contained 1 unit per ml. when fresh 39.32 per cent. of the original potency had been lost during eight years storage.

TABLE X

Assay of aged commercial tincture B

Tincture	Standard		B	
	1st. day	2nd. day	1st. day	2nd. day
Ml. Tincture per 10 ml. dilution	3.0	2.5	3.0	3.0
Overnight mortality	10/12	7/12	7/12	7/12
Mortality per cent.	83.3	58.3	58.3	58.3
Potency	125.3	105.6	105.6	105.6

The standard tincture contained 1 unit per ml.

Therefore Tincture B contained

(1) $\frac{105.6}{125.3} \times \frac{3.0}{3.0}$ = 0.8428 units per ml.

(2) $\frac{105.6}{105.6} \times \frac{2.5}{3.0}$ = 0.8333 units per ml.

Average = 0.8380 units per ml.

Since Tincture B contained 1 unit per ml. when fresh 16.2 per cent. of the original potency had been lost during six years storage.

TABLE XI

Assay of aged commercial tincture C

Tincture	Standard		C	
	1st. day	2nd. day	1st. day	2nd. day
Ml. Tincture per 10 ml. dilution	2.5	2.5	3.0	3.0
Overnight mortality	7/12	9/12	4/12	6/12
Mortality per cent.	58.3	75.0	33.3	50.0
Potency	105.6	118.0	89.0	100.0

The standard tincture contained 1 unit per ml.

Therefore Tincture C contained :

(1) $\frac{89.0}{105.6} \times \frac{2.5}{3.0}$ = 0.7025 unit per ml.

(2) $\frac{100.0}{118.0} \times \frac{2.5}{3.0}$ = 0.7062 unit per ml.

Average = 0.7044 unit per ml.

Since tincture C contained 1 unit per ml. when fresh 29.56 per cent. of the original potency had been lost during seven years storage.

TABLE XII

Assay of aged tincture T.S.1

Tincture	T.S.1 (aged)		T.S.1(c) (fresh)	
	1st. day	2nd. day	1st. day	2nd. day
Ml. Tincture per 10 ml. dilution	5.5	5.0	3.5	3.3
Overnight mortality	8/12	2/12	10/12	6/12
Mortality per cent.	66.7	16.7	83.3.	50.0
Potency	111.3	76.7	125.3	100.0

Potency of the aged tincture compared with that of the fresh tincture :

(1) $\frac{111.3}{125.3} \times \frac{3.5}{5.5} \times \frac{100}{1}$ = 56.56 per cent.

(2) $\frac{76.7}{100.0} \times \frac{3.3}{5.0} \times \frac{100}{1}$ = 51.10 per cent.

Average = 53.82 per cent.

Therefore Tincture T.S.1 had lost 46.18 per cent. of its original potency during seven months storage.

TABLE XIII

Assay of aged tincture D.1

Tincture	D.1 (Aged)		D.1 (b) (Fresh)	
	1st. day	2nd. day	1st. day	2nd. day
Ml. Tincture per 10 ml. dilution	5.5	6.0	3.5	3.3
Overnight mortality	1/12	2/12	6/12	7/12
Mortality per cent.	8.33	16.7	50.0	58.3
Potency	63.3	76.7	100.0	105.6

Potency of aged tinctures compared with the potency of the fresh tincture:

(1) $\frac{63.3}{100.0} \times \frac{3.5}{5.5} \times \frac{100}{1}$ = 40.3 per cent.

(2) $\frac{76.7}{105.6} \times \frac{3.3}{6.0} \times \frac{100}{1}$ = 39.9 per cent.

Average = 40.1 per cent.

Tincture D.1 had therefore lost 59.9 per cent. of its original potency during one year storage.

TABLE XIV

Assay of aged tincture T.D.1

Tincture	T.D.1 (aged)		T.D.1(b) (fresh)	
	1st. day	2nd. day	1st. day	2nd. day
Ml. Tincture per 10 ml. dilution	5.0	5.5	3.0	3.0
Overnight mortality	3/12	2/12	8/12	4/12
Mortality per cent.	25.0	16.7	66.7	33.3
Potency	83.5	76.7	111.3	89.0

Potency of the aged tincture compared with the potency of the fresh tincture:

(1) $\frac{83.5}{111.3} \times \frac{3.0}{5.0} \times \frac{100}{1}$ = 45.01 per cent.

(2) $\frac{76.7}{89.0} \times \frac{3.0}{5.5} \times \frac{100}{1}$ = 46.98 per cent.

Average = 46.00 per cent.

Therefore Tincture T.D.1 had lost 54.0 per cent. of its original potency during 33 weeks storage.

TABLE XV

Assay of aged ethanolic (70 per cent.)

solution of digitoxin (Sample A)

Solution	Digitoxin A (aged solution)		Digitoxin A (fresh solution)	
	1st. day	2nd. day	1st. day	2nd. day
Ml. solution per 10 ml.dilution	4.0	3.0	3.3	3.0
Overnight mortality	11/12	6/12	7/12	5/12
Mortality per cent.	91.7	50.0	58.3	41.7
Potency	138.0	100.0	105.6	94.7

Potency of the aged solution compared with the potency of the fresh solution:

(1) $\frac{138.0}{105.6} \times \frac{3.3}{4.0} \times \frac{100}{1}$ = 108.9 per cent.

(2) $\frac{100}{94.7} \times \frac{3.0}{3.0} \times \frac{100}{1}$ = 105.6 per cent.

Average = 107.3 per cent.

This apparent increase of 7.3 per cent potency during 25 weeks storage was less than the experimental error in this type of assay, therefore the solutions were regarded to be of the same potency.

TABLE XVI

Assay of aged ethanolic (70 per cent.)

solution of digitoxin (Sample B)

Solution	Digitoxin B (aged solution)		Digitoxin B (fresh solution)	
	1st. day	2nd. day	1st. day	2nd. day
Ml. Solution per 10 ml. dilution	3.0	4.0	3.0	3.5
Overnight mortality	1/12	9/12	3/12	7/12
Mortality per cent.	8.33	75.0	25.0	58.3
Potency	63.3	118.0	83.5	105.6

Potency of the aged solution compared with the potency of the fresh solution:

(1) $\frac{63.3}{83.5} \times \frac{3.0}{3.0} \times \frac{100}{1}$ = 75.8 per cent.

(2) $\frac{118.0}{105.6} \times \frac{3.5}{4.0} \times \frac{100}{1}$ = 97.8 per cent.

Average = 86.8 per cent.

This apparent loss of 13.2 per cent. potency during 3 years storage was insignificant compared with loss of potency in tinctures and could be due to experimental error. The two solutions were therefore regarded as being of the same potency.

APPENDIX II

EFFECT OF ADDED SAPONIN ON THE POTENCY OF AGED TINCTURES OF DIGITALIS

RESULTS OBTAINED BY THE 18-HOUR FROG METHOD OF ASSAY

TABLE XVII

Assay of aged commercial tincture A

with added saponin

Tincture	A		A plus 0.1 per cent. digitonin	
	1st. day	2nd. day	1st. day	2nd. day
Ml. Tincture per 10 ml. dilution	3.5	4.0	3.5	4.0
Overnight mortality	9/18	20/24	7/18	21/24
Mortality per cent.	50.0	83.3	39.0	87.5
Potency	100.0	125.3	92.8	130.5
Average potency	112.6		111.7	

TABLE XVIII

Assay of aged commercial tincture B

with added saponin

Tincture	B		B plus 0.1 per cent. digitonin	
	1st. day	2nd. day	1st. day	2nd. day
Ml. Tincture per 10 ml. dilution	3.0	3.5	3.0	3.5
Overnight mortality	9/12	9/12	9/12	10/12
Mortality per cent.	75.0	75.0	75.0	83.3
Potency	118.0	118.0	118.0	125.3
Average potency	118.0		121.6	

TABLE IXX

Assay of aged commercial tincture C

with added saponin

Tincture	C		C plus 0.2 per cent. digitonin	
	1st. day	2nd. day	1st. day	2nd. day
Ml. Tincture per 10 ml. dilution	3.3	3.3	3.3	3.3
Overnight mortality	4/9	6/12	5/9	8/12
Mortality per cent.	44.4	50.0	55.6	66.7
Potency	96.6	100.0	103.5	111.4
Average potency	98.3		107.4	

TABLE XX

Assay of aged tincture T.S.1

with added saponin

Tincture	T.S.1		T.S.1 plus 0.2 per cent.digitonin	
	1st. day	2nd. day	1st. day	2nd. day
Mls. Tincture per 10 mls. dilution	5.0	6.0	5.0	5.0
Overnight mortality	2/12	11/12	5/12	9/12
Mortality per cent.	16.7	91.7	41.7	75.0
Potency	76.6	138.0	94.7	118.0
Average potency	95.8		106.3	

In the calculation of average potency for Tincture T.S.1 it was estimated that a 5 ml. dilution on the second day would have resulted in a potency estimate of 115.0

TABLE XXI

Assay of aged tincture D

with added saponin

Tincture	D		D plus 0.8 per cent. digitonin	
	1st. day	2nd. day	1st. day	2nd. day
Mls. Tincture per 10 mls. dilution	4.5	5.5	4.5	5.5
Overnight mortality	4/12	5/12	3/12	5/12
Mortality per cent.	33.3	41.7	25.0	41.7
Potency	89.0	94.7	83.5	94.7
Average potency	91.9		89.1	

APPENDIX III

RESULTS OF ASSAYS ON TINCTURES OF DIGITALIS AND ETHANOLIC (70 PER CENT.) SOLUTIONS OF DIGITOXIN USING THE FROG INTRAVENOUS METHOD

APPENDIX III

RESULTS OF ASSAYS ON TINCTURES OF DIGITALIS AND ETHANOLIC (70 PER CENT.) SOLUTIONS OF DIGITOXIN USING THE FROG INTRAVENOUS METHOD

The results in the following tables are stated in terms of undiluted tincture, the mean dose required to arrest the heart in ventricular systole being calculated per 100 g. of frog.

The standard error E for each set of results has been calculated from the formula:

$$E = \sqrt{\frac{\Sigma d^2}{n(n-1)}}$$

where d = the deviation of individual results from the mean M
and n = the number of animals injected.

Fisher's t-test was applied to determine if the two means in any one assay were significantly different, the value t being calculated from the formula:

$$t = \frac{M_1 - M_2}{\sqrt{E_1^2 + E_2^2}}$$

where M_1 = mean dose of aged tincture
M_2 = mean dose of fresh tincture
E_1 = standard error for aged tincture
and E_2 = standard error for fresh tincture

The probabilities p, corresponding to the values of t so calculated, were obtained by reference to Fisher's statistical tables[253]. The means were regarded as being significantly different when values $p = 0.05$, or less, were obtained.

TABLE XXII

Intravenous assay of aged tincture D.3.

Tincture	Frog weight g.	Volume ml. 1-4 dilution injected	Volume ml. tincture per 100g. frog	Mean volume ml. per 100g. frog (M)	Standard Error (E)
D.3 (aged 28 weeks)	18.0 18.4 20.3	0.244 0.234 0.270	0.3398 0.3180 0.3326	0.3301	0.006413
D.3(a) (freshly prepared)	16.5 22.5 23.8	0.192 0.208 0.218	0.2910 0.2312 0.2290	0.2504	0.020310

$$t = 3.744$$

corresponding to $p = 0.02$

$$\text{Loss of potency} = 100 - \left[\frac{0.2504}{0.3301} \times \frac{100}{1}\right]$$

$$= 24.1 \text{ per cent.}$$

Note: The above experiment was conducted using the hand-operated micrometer syringe. All subsequent experiments were performed with the continuous injection apparatus.

TABLE XXIII

Intravenous assay of aged tincture D.3.

Tincture	Frog weight g.	Volume ml. 1-4 dilution injected	Volume ml. tincture per 100g. frog	Mean volume ml. per 100g. frog (M)	Standard Error (E)
D.3. (aged 36 weeks)	21.6	0.1390	0.1609	0.1732	0.009473
	17.1	0.1568	0.2293		
	18.0	0.1500	0.2084		
	16.3	0.1096	0.1681		
	21.6	0.1418	0.1642		
	21.9	0.1562	0.1783		
	16.2	0.0798	0.1231		
	20.2	0.1156	0.1431		
	16.2	0.1272	0.1963		
	15.6	0.0812	0.1301		
	17.2	0.1442	0.2096		
	16.1	0.1074	0.1667		
D.3(b), (freshly prepared	17.0	0.0772	0.1135	0.1399	0.01081
	20.7	0.0780	0.0954		
	21.7	0.1218	0.1403		
	19.5	0.1560	0.2000		
	17.5	0.1482	0.2117		
	18.0	0.1070	0.1486		
	29.3	0.1276	0.1088		
	16.6	0.0694	0.1045		
	16.7	0.0958	0.1434		
	15.9	0.0980	0.1540		
	15.4	0.0642	0.1042		
	14.4	0.0890	0.1545		

$$t = 2.317$$

corresponding to $p = 0.05 - 0.02$

$$\text{Loss of potency} = 100 - \left[\frac{0.1399}{0.1732} \times \frac{100}{1}\right]$$

$$= 19.2 \text{ per cent.}$$

TABLE XXIV

Intravenous assay of aged tincture D.3.

Tincture	Frog weight g.	Volume ml. 1-4 dilution injected	Volume ml. tincture per 100g. frog	Mean volume ml. per 100g. frog (M)	Standard Error (E)
D.3. (aged 60 weeks)	23.8	0.1304	0.1370	0.1624	0.006711
	29.4	0.2146	0.1825		
	28.6	0.1872	0.1636		
	22.8	0.1302	0.1428		
	23.8	0.1682	0.1767		
	20.2	0.1170	0.1448		
	20.8	0.1548	0.1861		
	22.9	0.1520	0.1659		
D.3(c). (freshly prepared)	24.4	0.1188	0.1217	0.1326	0.005678
	25.3	0.1368	0.1352		
	23.9	0.1466	0.1498		
	24.1	0.1158	0.1200		
	20.4	0.1284	0.1573		
	28.4	0.1618	0.1421		
	23.5	0.1056	0.1123		
	18.0	0.0879	0.1220		

$t = 3.39$

corresponding to p = 0.01 - 0.001

Loss of potency $= 100 - \left[\frac{0.1326}{0.1624} \times \frac{100}{1}\right]$

$= 18.4$ per cent.

TABLE XXV

Intravenous assay of aged tincture D.1.

Tincture	Frog weight g.	Volume ml. 1-4 dilution injected	Volume ml. tincture per 100g. frog	Mean volume ml. per 100g. frog (M)	Standard Error (E)
D.1. (aged 38 weeks)	18.6	0.1060	0.1424	0.1487	0.00629
	18.1	0.0830	0.1147		
	14.5	0.0860	0.1483		
	20.6	0.1166	0.1416		
	17.7	0.0790	0.1154		
	15.6	0.0906	0.1452		
	21.9	0.1272	0.1452		
	16.7	0.1240	0.1857		
	14.7	0.1024	0.1742		
	23.5	0.1406	0.1496		
	16.6	0.0962	0.1449		
	14.4	0.1020	0.1771		
D.1(a) (freshly prepared)	17.0	0.0640	0.0941	0.1170	0.007698
	14.4	0.0718	0.1247		
	19.3	0.1038	0.1345		
	17.7	0.1160	0.1639		
	16.3	0.0626	0.0960		
	18.6	0.0976	0.1311		
	19.6	0.1106	0.1410		
	16.5	0.0756	0.1146		
	15.8	0.0906	0.1433		
	14.6	0.0474	0.0812		
	20.5	0.0704	0.0859		
	17.3	0.0644	0.0931		

$t = 3.189$

corresponding to $p = 0.01 - 0.001$

Loss of potency $= 100 - \left[\frac{0.1170}{0.1487} \times \frac{100}{1}\right]$

$= 21.3$ per cent

TABLE XXVI

Intravenous assay of aged tincture D.1.

Tincture	Frog weight g.	Volume ml. 1-6 dilution injected	Volume ml. tincture per 100g. frog	Mean volume ml. per 100g. frog (M)	Standard Error (E)
D.1. (aged 51 weeks)	21.6	0.2270	0.1752	0.1564	0.01227
	16.9	0.1640	0.1617		
	22.0	0.1914	0.1450		
	18.5	0.1904	0.1715		
	24.9	0.1594	0.1067		
	18.3	0.2436	0.2219		
	21.5	0.1716	0.1330		
	15.6	0.1272	0.1359		
D.1(b) (freshly prepared)	23.3	0.1652	0.1181	0.1101	0.003932
	16.0	0.0966	0.1007		
	22.7	0.1508	0.1108		
	17.6	0.1094	0.1036		
	25.1	0.1894	0.1258		
	15.3	0.0870	0.0948		
	20.1	0.1254	0.1040		
	18.3	0.1350	0.1229		

$t = 3.595$

corresponding to $p = 0.01 - 0.001$

Loss of potency $= 100 - \left[\frac{0.1101}{0.1564} \times \frac{100}{1}\right]$

$= 29.6$ per cent.

TABLE XXVII

Intravenous assay of aged tincture T.D.1.

Tincture	Frog weight g.	Volume ml. 1-4 dilution injected	Volume ml. tincture per 100g. frog	Mean volume ml. per 100g. frog (M)	Standard Error (E)
T.D.1. (aged 18 weeks)	20.0	0.1260	0.1575	0.1766	0.006301
	18.0	0.1136	0.1578		
	14.9	0.1112	0.1866		
	21.2	0.1434	0.1690		
	15.5	0.1186	0.1913		
	12.4	0.1036	0.2088		
	15.5	0.1080	0.1742		
	14.8	0.0990	0.1672		
T.D.1.(a) (freshly prepared)	19.7	0.1074	0.1363	0.1386	0.006231
	16.0	0.0970	0.1516		
	14.7	0.0866	0.1472		
	24.8	0.1128	0.1137		
	14.7	0.0984	0.1673		
	13.6	0.0738	0.1357		
	12.0	0.0674	0.1404		
	17.1	0.0800	0.1169		

$t = 4.289$

corresponding to $p = 0.001$

$$\text{Loss of potency} = 100 - \left[\frac{0.1386}{0.1766} \times \frac{100}{1}\right]$$

$= 21.5$ per cent.

TABLE XXVIII

Intravenous assay of aged tincture T.D.1

Tincture	Frog weight g.	Volume ml. 1-6 dilution injected	Volume ml. tincture per 100g. frog	Mean volume ml. per 100g. frog (M)	Standard Error (E)
T.D.1. (aged 33 weeks)	25.4	0.2212	0.1452	0.1515	0.01102
	20.8	0.1346	0.1078		
	19.7	0.1582	0.1339		
	30.3	0.3538	0.1946		
	17.8	0.1930	0.1807		
	15.3	0.1436	0.1564		
	23.4	0.1994	0.1420		
T.D.1 (b) (freshly prepared)	20.8	0.0925	0.0741	0.1225	0.009113
	20.8	0.1714	0.1374		
	25.5	0.1864	0.1218		
	22.4	0.1478	0.1100		
	17.9	0.1452	0.1352		
	17.0	0.1460	0.1432		
	26.3	0.2144	0.1358		

$t = 2.028$

corresponding to p = 0.01 - 0.05

Loss of potency $= 100 - \left[\frac{0.1225}{0.1515} \times \frac{100}{1}\right]$

$= 19.1$ per cent.

TABLE IXXX

Intravenous assay of aged tincture T.S.1.

Tincture	Frog weight g.	Volume ml. 1-4 dilution injected	Volume ml. tincture per 100g. frog	Mean volume ml. per 100g. frog (M)	Standard Error (E)
T.S.1. (aged 21 weeks)	18.5	0.0684	0.0925	0.1417	0.01173
	13.9	0.0956	0.1720		
	15.8	0.1120	0.1772		
	17.2	0.1040	0.1511		
	16.4	0.0844	0.1286		
	11.5	0.0830	0.1804		
	21.4	0.0986	0.1152		
	12.9	0.0600	0.1162		
T.S.1(a) (freshly prepared)	20.7	0.0856	0.1034	0.1350	0.005123
	16.2	0.0928	0.1432		
	15.5	0.0852	0.1374		
	19.0	0.1108	0.1457		
	20.5	0.1046	0.1276		
	21.4	0.1260	0.1472		
	19.5	0.1120	0.1435		
	18.6	0.0984	0.1322		

$$t = 0.5235$$

corresponding to p = 0.7 - 0.6

$$\text{Loss of potency} = 100 - \left[\frac{0.1350}{0.1417} \times \frac{100}{1}\right]$$

$$= 4.7 \text{ per cent.}$$

TABLE XXX

Intravenous assay of aged tincture T.S.1.

Tincture	Frog weight g.	Volume ml. 1-4 dilution injected	Volume ml. tincture per 100g. frog	Mean volume ml. per 100g. frog (M)	Standard Error (E)
T.S.1. (aged 24 weeks)	21.8	0.1362	0.1563	0.1897	0.01564
	14.8	0.0804	0.1358		
	17.3	0.1124	0.1625		
	22.1	0.1696	0.1919		
	23.8	0.2258	0.2372		
	17.4	0.1444	0.2075		
	16.9	0.1824	0.2699		
	16.4	0.1402	0.2137		
	31.0	0.1644	0.1326		
T.S.1(b) (freshly prepared)	20.9	0.1360	0.1627	0.1690	0.01384
	17.6	0.0968	0.1375		
	17.2	0.1078	0.1567		
	22.9	0.1470	0.1604		
	21.4	0.1556	0.1818		
	15.4	0.1530	0.2484		
	13.4	0.1172	0.2186		
	15.2	0.0832	0.1369		
	34.3	0.1600	0.1183		

$t = 0.9913$

corresponding to $p = 0.4 - 0.3$

Loss of potency $= 100 - \left[\frac{0.1690}{0.1897} \times \frac{100}{1}\right]$

$= 10.9$ per cent.

TABLE XXXI

Intravenous assay of aged tincture T.S.1

Tincture	Frog weight g.	Volume ml. 1-6 dilution injected	Volume ml. tincture per 100g.frog	Mean volume ml. per 100g. frog (M)	Standard Error (E)
T.S.1. (aged 36 weeks)	22.4	0.1806	0.1344	0.1447	0.01073
	27.8	0.3038	0.1821		
	22.4	0.1526	0.1135		
	26.9	0.2074	0.1285		
	29.1	0.1902	0.1089		
	23.0	0.2350	0.1775		
	25.4	0.2020	0.1326		
	21.8	0.2354	0.1800		
T.S.1(c) (freshly prepared)	16.0	0.1104	0.1150	0.1137	0.005825
	25.2	0.1836	0.1214		
	22.35	0.1454	0.1084		
	26.2	0.1858	0.1182		
	28.3	0.1492	0.0879		
	27.8	0.2376	0.1424		
	29.5	0.2120	0.1198		
	21.7	0.1256	0.0965		

$t = 2.54$

corresponding to $p = 0.05 - 0.02$

Loss of potency $= 100 - \left[\frac{0.1137}{0.1447} \times \frac{100}{1}\right]$

$= 21.4$ per cent.

TABLE XXXII

Intravenous assay of aged digitoxin solution

Solution	Frog weight g.	Volume ml. 1-8 dilution injected	Volume ml. solution per 100g. frog	Mean volume ml. per 100g. frog (m)	Standard Error (E)
Digitoxin Solution (aged 145 weeks)	23.0	0.3260	0.1772	0.1517	0.01897
	24.0	0.2340	0.1219		
	20.5	0.4393	0.2679		
	23.1	0.3194	0.1728		
	26.0	0.2630	0.1265		
	25.4	0.2248	0.1106		
	20.9	0.1864	0.1115		
	20.7	0.2070	0.1250		
Digitoxin Solution (freshly prepared)	21.9	0.2830	0.1615	0.1427	0.008769
	24.2	0.2528	0.1306		
	19.75	0.2834	0.1794		
	25.3	0.2432	0.1201		
	33.7	0.3366	0.1250		
	22.8	0.3042	0.1668		
	21.4	0.2528	0.1477		
	18.2	0.1610	0.1106		

$$t = 0.4306$$

corresponding to $p = 0.7 - 0.6$

$$\text{Apparent loss of potency} = 100 - \left[\frac{0.1427}{0.1517} \times \frac{100}{1}\right]$$

$$= 5.94 \text{ per cent.}$$

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