THE ASSAY OF TINCTURE OF DIGITALIS AND THE DETERMINATION OF THE CONSTITUENTS OF DIGITALIS SPECIES

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Introduction

In our previously published work[1,2] we dealt with the 3 : 5-dinitrobenzoic acid (Kedde[3]) assay, the chromatographic behaviour and the frog assay of the pure constituents of *Digitalis purpurea* and of tinctures of digitalis. Guinea-pig assays have now been made and the results are here reported.

In the earlier chromatographic work[1] we dealt with the pure constituents of *D. purpurea* and used paper partition methods in which the stationary phase was water and the mobile phase was an organic solvent saturated with water. It was found impossible to separate the secondary glycosides from their respective aglycones, and for this reason we attempted to evolve a chemical assay which would allow us to measure with reasonable accuracy the concentrations of a secondary glycoside and its aglycone in a mixture of both like that obtained in chromatographic eluates.

Several assay processes were investigated and it was found that 33 per cent. hydrochloric acid[1] could be used to estimate a secondary glycoside in the presence of its aglycone. Assays of such mixtures have now been made. Subsequently, chromatographic separations employing formamide, in particular those described by Jensen[4–7], have been introduced in which a good separation of the aglycone from its secondary glycoside can be obtained—the 3 : 5-dinitrobenzoic acid reagent cannot be used to estimate the concentration of each constituent in the eluates from such separations. For this reason there is much to be said for the use of the earlier chromatographic systems when the separated chromatographic eluates are to be assayed.

It was felt that the formamide—chloroform system would be more suitable for investigating qualitatively the constituents of digitalis preparations and that the use of Whatman 3 MM paper would allow relatively large volumes of an extract, for example a tincture, to be chromatographed so that any constituents present in low concentration might be identified. For this purpose, the Standard Preparation of Digitalis was used and a practicable chromatographic method has now been attempted.

If a reliable chemical assay process for digitalis preparations is at all feasible it is almost certain that the separation of the constituents before assay and an interpretation of the concentration of each constituent in terms of biological activity will be necessary. Furthermore, the biological behaviour of a mixture of pure constituents must be investigated since the effect of the combination may be additive, so that in a mixture, one drug can be substituted at a constant ratio for any proportion of another without altering the toxicity of the combination. On the other hand, there may

be potentiation characterised by a toxicity greater than that predicted from experiments with the individual constituents, or there may be antagonism, characterised by a toxicity less than that expected if the ingredients acted independently[8,9].

EXPERIMENTAL

Small quantities of about 50 mg. of the constituents investigated were very kindly supplied by Professor Arthur Stoll. Standard solutions containing 10 mg. to 100 mg. of the constituents in 100 ml. were prepared in absolute ethanol. Similar solutions of commercial samples of digitoxin and gitoxin were also prepared. The solutions were stored at room temperature and were protected from light.

Hydrochloric Acid Assays of Pure Constituents

The purpurea glycoside A, purpurea glycoside B, digitoxin and gitoxin were assayed by the hydrochloric acid method[1] and calibration curves constructed (Figure 1). The reaction obeys the Beer-Lambert law within the range 0·1 mg. to 0·6 mg./10ml. of the reaction mixture.

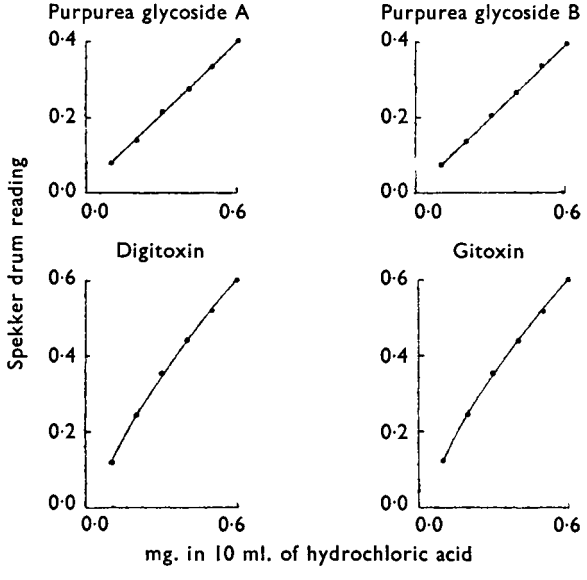

FIG. 1. Colour density/concentration calibration curves for the hydrochloric acid assay of digitalis constituents supplied by Professor Stoll.

Since each of the primary and secondary glycoside molecules contains 3 molecules of digitoxose, one would expect the molecular extinction coefficient for each glycoside to be constant and to be equal to 3 times that for digitoxose. Table I shows that it is approximately so in the case of the secondary glycosides but not in the case of the primary glycosides. This anomalous behaviour of the primary glycosides may be due to a non-quantitative reaction; the third digitoxose molecule in the primary

glycosides is combined with glucose and this linkage is not broken even by strong acid treatment[10].

TABLE I

MOLECULAR EXTINCTION COEFFICIENTS OF DIGITALIS CONSTITUENTS (STOLL). 0·5 MG. ASSAYED BY THE HYDROCHLORIC ACID METHOD

Glycoside or sugar	Molecular weight	Molecular extinction coefficient
Purpurea glycoside A	926	6190
Purpurea glycoside B	942	6250
Digitoxin	764	7950
Gitoxin	780	8060
Digitoxose (3 molecules)	444	8000

Assays of Mixtures containing Secondary Glycosides and the Respective Aglycones

Samples of commercial digitoxin and gitoxin were assayed by means of hydrochloric acid and the calibration curves (Fig. 2) constructed. These commercial samples of secondary glycosides were used in the assays of mixtures of secondary glycosides and their aglycones described below in order to conserve the purer samples obtained from Professor Stoll,

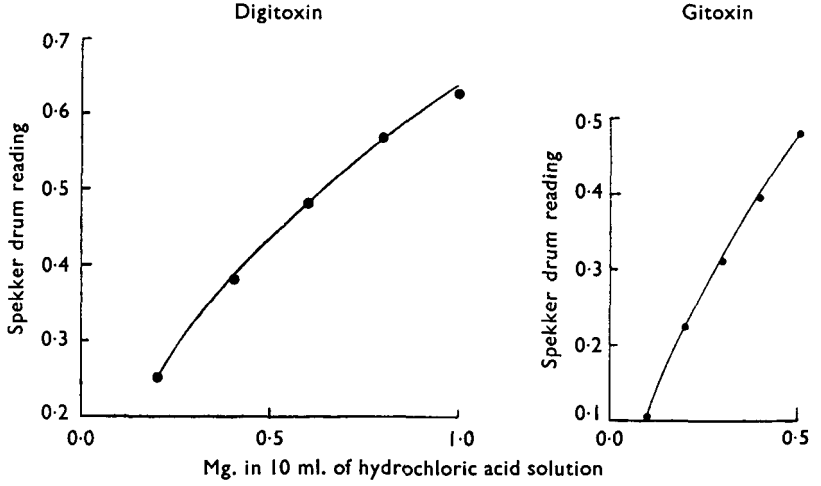

FIG. 2. Colour density/concentration calibration curves for the hydrochloric acid assay of commercial digitoxin and gitoxin.

since in assays of this type the results are not influenced by the purity of the sample. Furthermore, for the purpose of these assays, it is necessary to construct calibration curves for the secondary glycosides and for the aglycones when assayed by the 3:5-dinitrobenzoic acid reagent. Calibration curves of this type for the digitoxin and digitoxigenin used are reproduced in Figure 3 to illustrate the worked example shown below.

Solutions of a secondary glycoside and its respective aglycone were assayed by the alkaline 3:5 dinitrobenzoic acid and by the hydrochloric

acid methods. The former reagent estimates the glycoside and its aglycone and the latter reagent estimates the glycoside only. The reading obtained in the 3 : 5 dinitrobenzoic acid assay for the total aglycone content is expressed as mg. of the secondary glycoside and from this figure is

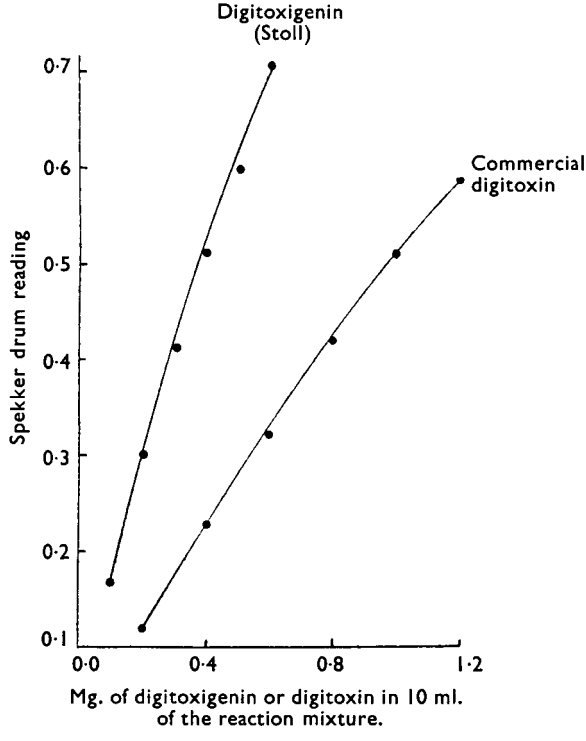

FIG. 3. Colour density/concentration calibration curves for the 3 : 5-dinitrobenzoic acid assay for digitoxigenin (Stoll) and commercial digitoxin.

subtracted the weight in mg. of the secondary glycoside estimated by the hydrochloric acid assay for secondary glycoside only. The difference is interpreted as weight of aglycone by reference to the standard curves for the glycoside and the aglycone. The example below illustrates the method.

A mixture containing 0·2 mg. of commercial digitoxin and 0·2 mg. of digitoxigenin was assayed. The Spekker drum reading 0·397 obtained by the aglycone assay (Fig. 3) is equivalent to 0·74 mg. of the digitoxin used, but by the hydrochloric acid assay (Spekker drum reading 0·242) only 0·2 mg. of secondary glycoside was found to be present (Fig. 2). Hence the residual absorption would be equivalent to 0·54 mg. of the digitoxin. Reference to the graph shows that 0·2 mg. of digitoxigenin gives the same colour density reading with the Kedde reagent as 0·54 mg. of digitoxin. Thus the calculated quantity of the aglycone agrees with the quantity actually taken.

Similarly a mixture containing 0·4 mg. of commercial digitoxin and 0·1 mg. of digitoxigenin was assayed as follows: Aglycone assay 0·370 ≡ 0·69 mg. of digitoxin, Glycoside assay 0·378 ≡ 0·40 mg. of digitoxin. The aglycone assay has estimated 0·40 mg. of digitoxin plus an amount of digitoxigenin equivalent to 0·29 mg. of digitoxin. Reference to the appropriate graph shows that 0·11 mg. of digitoxigenin produces the same colour density with the Kedde reagent as does 0·29 mg. of digitoxin.

The average results of triplicate assays are shown in Table II.

TABLE II

CHEMICAL ASSAYS OF MIXTURES OF SECONDARY GLYCOSIDES AND AGLYCONES

No.	Mixture assayed	Weight of constituents taken, mg.	Weight of constituents estimated, mg.
1	Digitoxin and digitoxigenin	0·10 0·30	0·11 0·31
2	,, ,, ,,	0·20 0·20	0·20 0·20
3	,, ,, ,,	0·40 0·10	0·40 0·11
4	Gitoxin and gitoxigenin	0·20 0·20	0·21 0·20
5	,, ,, ,,	0·40 0·20	0·42 0·19
6	Digoxin and digoxigenin	0·20 0·30	0·22 0·29
7	,, ,, ,,	0·40 0·20	0·40 0·19

BIOLOGICAL ASSAYS

Assays of some of the Constituents of D. Purpurea using Guinea-pigs

The lethal doses of purpurea glycoside A, purpurea glycoside B, digitoxin, gitoxin, digitoxigenin and gitoxigenin were experimentally determined by the procedure recommended by the British Pharmacopœia 1953 and Miles and Perry[11] for standardising tinctures of digitalis. Eight to 10 male guinea-pigs were used for each constituent and the solutions of the constituents were suitably diluted with sodium chloride solution so that the diluted solution caused the death of the animal 20 to 40 minutes after administration was commenced. 0·65 ml. per kg. weight, of the diluted solution was injected into the jugular vein every minute by means of a micro-burette. Digitoxigenin was found to be affecting the respiration of the animals and spasms were observed 8 to 10 minutes after the administration was started. The effect of digitoxigenin on respiration and its pharmacology are under investigation. Gitoxigenin was found to be of negligible potency which agrees with the results obtained with frogs[1]. The lethal doses of the constituents and their respective standard errors are given in Table III and for comparison the frog LD50 values obtained previously[1] are also tabulated.

Assays of Mixtures of Digitalis Constituents using Guinea-pigs

In order to study the effect of mixtures of these constituents, thereby simulating more nearly digitalis and its galenical preparations, 6 different mixtures were prepared, representing a mixture of the primary A and B

TABLE III

RESULTS OF THE BIOLOGICAL ASSAYS OF DIGITALIS CONSTITUENTS

Constituent or tincture	Frog assays		Guinea-pig assays		
	LD50 mg./kg.	Fiducial limits (P = 0·95)	Lethal dose mg./kg.	Fiducial limits (P = 0·95)	I.U. per mg.
Purpurea glycoside A	2·2	1·8–2·7	0·53	0·44–0·62	2·70
Purpurea glycoside B	5·7	4·8–6·7	1·44	1·25–1·63	0·99
Digitoxin	3·7	3·4–4·1	1·54	1·25–1·83	0·93
Gitoxin	16·3	12·6–21·2	13·34	10·97–15·71	0·11
Digitoxigenin	17·4	14·4–21·0	3·36	2·49–4·23	0·43
Gitoxigenin	>40	—	>80	—	—
Standard preparation of digitalis	—	—	109·0 (1·43) I.U.	79·0–139·0	0·01315

glycosides, a mixture of a primary glycoside and a secondary glycoside (purpurea glycoside A and digitoxin), a mixture of a primary glycoside and an aglycone (purpurea glycoside A and digitoxigenin), a mixture of a secondary glycoside and an aglycone (digitoxin and digitoxigenin), a mixture of an aglycone and a sugar (digitoxigenin and digitoxose) and a mixture of purpurea glycoside A, purpurea glycoside B, digitoxin, gitoxin and digitoxigenin. These mixtures contained equal volumes of those solutions of the respective constituents which caused death 20 to 40 minutes after administration.

The relative log potencies of the constituents (R_2, R_3 . . .) used in a particular mixture were calculated with reference to one of the constituents. The log potency of the mixture was calculated from the experimental data log ($\lambda_1 + \lambda_2 R_2 + \lambda_3 R_3$ . . .); its deviation (D) from the theoretical potency and its standard deviation (SD) were calculated. The values D/SD and the corresponding per cent. at which they are significant are shown in Table IV. The figure 5·24 (D/SD) in the second column is highly significant statistically. Other figures are not significant individually but all positive values taken together provide evidence of potentiation.

TABLE IV

RESULTS OF GUINEA-PIG ASSAYS WHEN DIGITALIS CONSTITUENTS WERE ADMINISTERED IN MIXTURES

Mixture	D/SD	Level at which significant, per cent.
1. Purpurea glycoside A and purpurea glycoside B	+0·60	55
2. Purpurea glycoside A and digitoxin	+0·90	37
3. Purpurea glycoside A and digitoxigenin	+5·24	0·0001
4. Digitoxin and digitoxigenin	+1·31	19
5. Purpurea glycoside A, purpurea glycoside B, digitoxin, gitoxin and digitoxigenin	+1·20	23
6. Digitoxigenin and digitoxose	No potentiation was observed	

Assays of Mixtures of Constituents using Frogs

In order to study the effect of the constituents in mixtures, 4 constituents (purpurea glycoside A, digitoxin, gitoxin and digitoxigenin i.e., a primary glycoside, 2 secondary glycosides and an aglycone) were mixed in suitable proportions.

Two dose values per kg. of frog which were expected to give 20 per cent. and 80 per cent. mortality were chosen for each of the constituents and also for the tincture prepared from the Standard Preparation of Digitalis, this information being obtained from their LD50 values. The lower and the higher doses were in each case in the ratio 2:3. The mixture contained a quarter of these quantities of each of the constituents. Thus 12 solutions were prepared for injection, 6 containing the lower dose (20 per cent. expected mortality) and 6 containing the higher dose (80 per cent. expected

TABLE V

COMPARISON OF THE ESTIMATED POTENCY TO FROGS OF A MIXTURE OF PURPUREA GLYCOSIDE A, DIGITOXIN, GITOXIN AND DIGITOXIGENIN WITH THE POTENCY OF THE MIXTURE PREDICTED ON THE HYPOTHESIS OF SIMILAR ACTION

Experiment	Potency ratio estimated: theoretical	Fiducial limits (P = 0·95)
First	1·30	1·09–1·57
Second	1·04	0·89–1·22
Pooled estimate	1·15	1·02–1·29

mortality) of the following—purpurea glycoside A, digitoxin, gitoxin, digitoxigenin, a mixture of the above 4 constituents in appropriate concentration and the tincture prepared from the Standard Preparation of Digitalis. The procedure adopted was the same as described for the 2 and 2, 18 hour frog assay method of the British Pharmacopœia 1953. Table V incorporates the results of two investigations made with 15 frogs for each dose of each constituent in the first experiment, and 20 frogs for each dose in the second. The individual slopes (b) and their respective variances (B) for the lines were calculated by the B.P. method. Since the figures for $X^2_{(4)}$, (3·12 and 7·0) were not significantly a line with a common slope ($\bar{b}$)[12] was fitted and used in the calculations.

Log dose/probit curves were drawn for each of the constituents and for the mixture, plotting the logarithms of 2 and 3 (ratio of doses) against the probits of mortality corresponding to these doses. A line with a common slope $\bar{b}$ was drawn on the same graph sheet. Another line parallel to this was drawn to intersect the original line in the centre, and the log dose ($\lambda 5$) corresponding to probit 5 was read from this new line. Regression equations for all these constituents were then calculated and the potencies (R_2, R_3, R_4) of the other 3 constituents of the mixture were calculated from the fourth (digitoxin) which was regarded as unity. From the values of R, the equation for the mixture was calculated on the hypothesis of similar action[12,13]. The potency ratio (estimated potency: theoretical potency i.e., the ratio of the experimentally determined potency to the predicted potency) and its fiducial limits were then calculated by the equations described by Finney[12]. These results suggest a 15 per cent. potentiation which is statistically significant when these 4 constituents are mixed in these proportions.

Biological assays of tinctures. Six commercial tinctures[2] (B, C, D, E, G and H) were assayed by the official guinea-pig method mentioned above[11].

The results along with the frog assay figures (experiments performed at about the same time) are given in Table VI. Thus an interpretation of the potency of each constituent and each tincture can be made in terms of International Units per mg. of constituent, or per ml. of the tincture (See Tables III and VI). Throughout the whole series of guinea-pig assays, the Standard Preparation of Digitalis was assayed at intervals as a control on the uniformity of the animals etc.

TABLE VI

POTENCY OF COMMERCIAL TINCTURES OF DIGITALIS EXPRESSED IN I.U./ML.

Tincture	Potency expressed as I.U./ml.			
	Guinea-pig assay January-March 1955	Frog assay March 1955	Frog assay March 1954	Per cent. deterioration
A	—	—	0·54	—
B	0·51	0·46	0·48	4·2
C	0·42	—	0·39	—
D	0·57	0·55	0·62	11·2
E	0·55	—	0·51	—
F	—	—	0·47	—
G	0·51	0·55	0·58	5·1
H	0·49	0·45	0·51	11·7
*S	1·315	1·315	1·315	—

* Tincture prepared from the Standard preparation of digitalis.

CHROMATOGRAPHIC EXAMINATION

The R_F values of a number of *D. lanata* constituents obtained from Professor Stoll were studied using the formamide system. The results are set out in Table VII. The Standard Preparation of Digitalis was qualitatively analysed. A 1 in 10 tincture was prepared by the B.P. method and stored in a refrigerator below 5° C. In order to detect constituents which might be present in smaller quantity, relatively larger volumes of the tincture had to be chromatographed. For this purpose, Whatman 3MM paper was found to be more satisfactory than Whatman No. 1 paper. The tincture was applied along the starting line which was about 20 cm. in length by means of a pipette drawn to a capillary. The solvent was allowed to evaporate and the sheets were then impregnated with the stationary phase (formamide) using a mixture of 3 volumes of formamide and 7 volumes of acetone. Chromatography was then commenced in glass chambers previously saturated with the chloroform vapour of the mobile phase. The solvent was allowed to run down the paper and development stopped when the solvent front had travelled about 35 cm. On such chromatograms the primary glycosides are not completely separated from each other. Whenever it was desired to separate the

TABLE VII

R_F VALUES OF SOME OF THE CONSTITUENTS OF *Digitalis lanata* USING FORMAMIDE AS STATIONARY PHASE AND CHLOROFORM AS THE MOBILE PHASE

Constituent	Approximate R_F
Lanatoside A	0·31
Lanatoside B	0·05
Lanatoside C	0·02
Digoxin	0·33
Digoxigenin	0·11
Purpurea glycoside A / Purpurea glycoside B	0·02
Digitoxin	0·95
Digitoxigenin	0·85
Gitoxin	0·60
Gitoxigenin	0·34

primary glycosides development of the chromatograms was allowed to continue for about 60 hours—the mobile phase dripping from the bottom edge of the paper. After about 60 hours development, purpurea glycoside. A was found to have travelled about two thirds and purpurea glycoside B about one third the length of the paper. The chromatograms after appropriate development were removed and the mobile phase allowed to evaporate. The heavy stationary phase was removed by heating the sheets in an oven at 60° C. to 70° C. for 3 to 4 hours. Vertical $\frac{1}{2}$ in. strips were cut from the developed chromatograms, parallel to the direction of flow, and sprayed with 2 or 3 of the commoner reagents to localise and identify the constituents. Horizontal strips were then cut from the remainder of the sheet and each strip was eluted with 70 per cent. ethanol. The eluate from each was evaporated to small volume under reduced pressure and rechromatographed with the same phase system on 3 in. wide paper strips. On each strip a solution of the pure constituent having the corresponding R_F value was also applied as a reference spot.

The reagents used for spraying the chromatograms were antimony trichloride in chloroform, trichloroacetic acid in chloroform, chloramine-trichloroacetic acid reagent and xanthydrol reagent[14]. None of these reagents is satisfactory alone but the use of 2 or 3 reagents on strips cut from the same chromatogram is helpful in recognising the substance as a member of the A or B series and as a glycoside or an aglycone.

The following constituents were found to be present in a tincture prepared from the Standard Preparation of Digitalis when 6 ml. of the tincture was applied along the starting line of a sheet 22 cm. × 50 cm.—purpurea glycoside A, purpurea glycoside B, digitoxin and gitoxin. In addition a substance was detected at about R_F 0·2 which fluoresced under ultra-violet light after treatment with the chloramine-trichloroacetic acid reagent. When this substance was extracted from the paper and tested with alkaline 3 : 5 dinitrobenzoic acid reagent, it gave a positive reaction indicating the presence of a ketonic group in the molecule.

Discussion

Of the methods which have been used for the chemical estimation of the constituents of digitalis, the hydrochloric acid method of assay for digitoxose containing glycosides seems to be accurate. It can estimate a secondary glycoside in the presence of its aglycone. This reagent which reacts with the sugar moiety, digitoxose, appears to be applicable to the assay of digitalis preparations, but unfortunately this reagent cannot be used for assaying tinctures, the pigments of which are always difficult to remove by any of the standard decolourisation processes. Nevertheless the method is of value in estimating the concentration of a secondary glycoside in a chromatographic eluate containing both the secondary glycoside and its aglycone in a reasonable state of purity.

After chromatographic separation, the constituents may be estimated by a chemical process and the biological potency of the mixture calculated provided that the constituents have an additive biological action.

Investigations have been made with a few mixtures of constituents and the results suggest potentiation.

It has been observed that the aglycone digitoxigenin affects the respiration of the guinea-pig and that this effect causes the death of the animal. The pharmacological action of digitoxigenin needs further investigation. A similar unusual effect has been noticed in the frog assays of digitoxigenin —namely spasms occuring soon after injection, rapidly followed by cessation of respiration and reflexes and apparent death of the animal although the frog's heart continues to beat and the animal often recovers.

The constituents present in the Standard Preparation of Digitalis seem to be limited to the primary and secondary glycosides and no aglycones were found. This does not exclude the possibility of the presence of very small amounts of aglycones. The extra band R_F 0·2, which was repeatedly found indicates the presence of an unknown substance which may possibly be identical with one of the new constituents reported by Jensen[15].

Summary

1. A chemical assay process for digitoxin, gitoxin and digoxin in the presence of their respective aglycones using hydrochloric acid has been described.

2. Six constituents of *D. purpurea* have been biologically assayed by the frog and by the guinea-pig methods.

3. The biological behaviour of mixtures of these constituents has also been studied using frogs and guinea-pigs.

4. Six samples of commercial tinctures were assayed by the frog and guinea-pig method.

5. The constituents present in the Standard Preparation of Digitalis have been identified by paper partition chromatography.

The authors appreciate the interest taken and help given by Professor A. D. Macdonald during the biological investigation. Our thanks are due to Mr. A. M. Walker of the Statistics Department of Manchester University for his advice in statistical methods.

During these investigations one of us, S. N. Sharma, was the holder of an Imperial Chemical Industries' Fellowship in Pharmacy and Pharmacology, at Manchester University.

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DISCUSSION

The paper was presented by DR. G. RIGBY.

DR. F. FISH (Glasgow) said he agreed that in principle a chemical assay was good, but did it give any indication of the therapeutic activity in man? The chemistry of digitalis was very complex, but even if they got a clear picture of the chemical constituents, were they any further forward? He thought not, because they did not know which pharmacological assay gave a true indication of clinical activity. The authors had shown that the relative activities of the primary glycosides, secondary glycosides and aglycones in either the A or B series differed in the frog and the guinea-pig; and figures were available in the literature to show that cat assays gave yet a third set of ratios. He wondered if the use of commercial digitoxin (Fig. 3) led to discrepancy between the practical and theoretical results obtained in the assays of mixtures of glycoside and genin. The authors stated that, from the graphs in Figure 3, 0·2 mg. of digitoxigenin gave the same colour density as 0·54 mg. of digitoxin, whereas theoretically 0·2 mg. digitoxigenin should be equivalent to 0·48 mg. digitoxin. Referring to Table VI, he asked how old the tinctures were to begin with, and he suggested that they had already reached the stable condition that Dr. Rowson had mentioned earlier. In Table V, he did not know whether it was possible to make sound deductions from only two estimations: one showing an excess of 30 per cent. and the other of 4 per cent.

DR. ROWSON (London) made a plea for consistency in the terminology of reagents, for example, the use of "3:5-dinitrobenzoic acid" instead of "Kedde" reagent. He wished the authors had been more precise in stating the reason why the molar extinctions of primary glycosides with hydrochloric acid were lower than for the secondary glycosides. This was surely due to the interaction of two of the three digitoxose molecules present only. The same phenomenon occurred when primary glycosides are estimated with other reagents, e.g., Keller-Kiliani and the phosphoric-sulphuric acid reagent of Tattje. He asked the authors to state the molar extinction values for different glycosides and their genins with dinitrobenzoic acid. The deviations in Figure 3 between the two graphs would be more clearly apparent from such molar extinction values. The high value quoted for digitoxigenin was probably due to the influence of acid used in the preparation of that genin producing "exaltation of colour." The wide deviation of results between the frog and the guinea-pig methods of assay was worrying, and it would appear that the more constant and reproduceable results from chemical methods were to be preferred. He asked for more details of the methods of chromatographic separation used to decolorise the tinctures before the hydrochloric acid assay was applied. Was the unknown glycoside, found chromatograhically in powdered digitalis leaf, digitalinum verum?

PROFESSOR BRINDLE said that he agreed with Dr. Fish that they ought to be able to decide which particular action of digitalis they wished to use in evaluating the drug. He did not, however, agree with his conclusion. In bioassays they were testing on animals, and up to the death of those

animals, which was quite different from the therapeutic use of the drug. They should try to analyse the constituents of digitalis and judge the potency by the amount of each individual active substance present.

DR. RIGBY, replying to points raised by Dr. Fish, said that Professor Brindle had answered him in some degree, and he had no wish to enter into a discussion on which bioassay should be preferred. His own opinion was that the cat and guinea-pig assays were the best. The literature of the past 20 years showed that there was not much hope of agreement as between chemical and bioassay. On the difference between primary and secondary glycosides, their method of approach was by chromatographic separation, and it was very easy to separate them in pure form. He had used commercial products for economy's sake. With regard to the tinctures, the labels from the bottles were available but Dr. Sharma, who had assayed the tinctures by the guinea-pig method, was unfortunately not available to report their age. It was true that there were only two estimations shown in Table V but they involved the use of 400 frogs, and the results had been checked statistically. Dr. Rowson had made a point about nomenclature of reagents which had always bothered them a little. The hydrochloric acid method was developed originally because at the time they could not separate clearly the secondary glycoside from the aglycone, so they had tried to get round it by estimating the two in a mixture. Personally, he had found results with the Bial reagent unreproducible. The molar extinction of the genins to dinitrobenzoic acid, they felt, might be due to the formation of anhydro compounds. On digitalinum verum, they had been thinking along the lines of Jensen's work, who had found a number of unidentified compounds. The hydrochloric acid assay for quantitative estimations after chromatography had not yet been used. In his view if one could rely on a good quality dried leaf—carefully collected and processed—one could probably find a high proportion of primary glycosides present. That could be checked chromatographically, and if one used a large volume one could test for smaller amounts of degradation products. Then the problem became simpler: the estimation of the potency of a dried leaf containing, roughly speaking, two active constituents.