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THE BIOLOGICAL AND CHEMICAL ASSAY OF TINCTURES OF DIGITALIS

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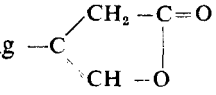
INTRODUCTION

THE biological assay of digitalis preparations has been attempted by a variety of methods, employing many of the more commonly used experimental animals. Satisfactory agreement has not been obtained between the results of these various biological assays, and this is probably due to the effects of a large number of factors such as the variation in concentration of the different digitalis glycosides in different digitalis preparations, the effects of saponins, the adsorption of the glycosides by heart and skeletal muscle and by serum albumins, and the great variations in absorption, accumulation and excretion of the glycosides which may result from the adsorption effect. The possible effects of these various factors, make it very unlikely that any single method of biological assay, can render a complete picture of the therapeutic value of every preparation of digitalis. Chemical assays in which the digitoxin content, or the mixed glycoside content of digitalis preparations was weighed have been described by Keller[1], Martindale[2] and Tschirch and Wolter[3].

A colorimetric estimation based upon the orange colour developed when suitable solutions of the active principles of digitalis preparations are mixed with an alkaline picrate solution was reported by Baljet[4] in 1918. This reagent, it is claimed, is specific for molecules containing the unsaturated lactone grouping  found in the aglycones of the digitalis glycosides. The Baljet reaction has been used by Knudson and Dresbach[5], and Bell and Krantz[6] as the basis of a colorimetric assay of digitalis preparations. Hagemeier[7] found that the greatest colour activity, using the Baljet reagent, was given by an inert glycoside diginin, whilst Knudson and Dresbach[5], Ockeloen and Timmers[8] and Carratala[9] found that an assay based on this reaction yielded results which agreed with one or other of the biological assays. Dyer[10], Wasicky and co-workers[11], and Vos and Welsh[12], however, found no agreement between such a colorimetric assay and one or other of the biological assays. Anderson and Chen[13] used a modified Raymond method, based on the blue colour which develops when alcoholic glycosidal solutions are mixed with an alkaline solution of *meta*dinitro-

benzene in alcohol, for the colorimetric assay of digitalis tinctures. Cänback[14] has criticised this assay method. Wasicky[15] has described colour reactions of the digitalis glycosides and aglycones with 3:5-dinitrobenzoic acid, and with sodium nitroprusside by which he could estimate the concentrations of glycoside and of aglycone in solutions where both are present. The results of a colorimetric assay of digitalis preparations would be expected to agree with those of a biological assay, only if the intensity of the colour produced by each glycoside were proportional to the degree of physiological change it produced. Colorimetric and biological assays might conceivably yield comparable results when one is dealing with pure glycosides, but the results of a colorimetric assay of digitalis preparations are not likely to agree consistently with those of a biological assay.

EXPERIMENTAL

I. INVESTIGATION OF THE BALJET REACTION

For this investigation, and for the comparative assays carried out as described in the following pages, a standard tincture of digitalis was prepared as follows:—

The Preparation of Tincture A. 100 g. of a coarsely powdered sample of digitalis leaf, containing 11·4 units of activity per g. were continuously extracted for 6 hours in a Soxhlet extractor, with 500 ml. of absolute alcohol. The resulting extract was cooled, adjusted to 500 ml. by the addition of absolute alcohol, allowed to stand for 48 hours and clarified by filtration through coarse filter paper. This tincture represents a 1 in 5 extract of the digitalis leaf.

The Method of Developing the Colour from Various Samples of Tincture A. The volume of tincture used was added to 15 ml. of distilled water and 2 ml. of freshly prepared 12·5 per cent. solution of lead acetate added. The volume was adjusted to 25 ml. with distilled water and filtered through paper. 12·5 ml. of the filtrate was placed in a 25-ml. volumetric flask, and 2 ml. of a 4·7 per cent. solution of disodium phosphate containing 7 molecules of water of crystallisation, was added. The contents of the flask were mixed and the whole diluted with distilled water to 25 ml., mixed, and filtered through paper. This decolorisation process has been described by Knudson and Dresbach[5]. The filtrate was again clarified by filtration through a sintered glass filter number D4, to remove traces of the phosphate precipitate which are not retained by the coarser Whatman No. 1 filter papers, used in the above operations. 12·5 ml. of this final filtrate was placed in a dry-stoppered bottle. Fresh samples of the Baljet reagent were prepared by mixing stock aqueous solutions of sodium hydroxide in 10 per cent. w/v concentration, and of picric acid in 1 per cent. w/v concentration, in the proportions of 5 volumes and 95 volumes respectively. The mixture was then filtered through a sintered glass filter, number D4. 12·5 ml. of the Baljet reagent was added to the 12.5 ml. of glycosidal filtrate in the bottle and mixed. 12·5 ml. of the Baljet reagent was mixed with 12·5 ml. of distilled water

for use as the "control" solution. The intensities of the coloured solutions obtained by this colorimetric method, were compared by means of the "Spekker" photoelectric absorptiometer H 454. The colour filters OB1, supplied with the instrument were used.

The Variation with Time of the Intensity of the Colour Developed in the Baljet Reagent. Bell and Krantz in their original work, measured the intensity of the coloured solutions 20 minutes after mixing the Baljet reagent with the glycoside filtrates. In their later papers they have extended this period from 20 to 40 minutes. The variation of colour intensity with time was therefore investigated. 10 ml. of each of the decolorised filtrates from (a) 1·0 ml., (b) 1·5 ml., (c) 2·0 ml. and (d) 2·5 ml. of tincture A was mixed with 10 ml. of the Baljet reagent. The filtrates and the Baljet reagent were all clarified by filtration through Whatman No. 50 filter papers, 10 ml. of the Baljet reagent was mixed with 10 ml. of distilled water to form the "control." The intensity of the colour developed was measured by comparison with this "control" at intervals of 5 minutes for 90 minutes, and then at intervals of 20 minutes for a

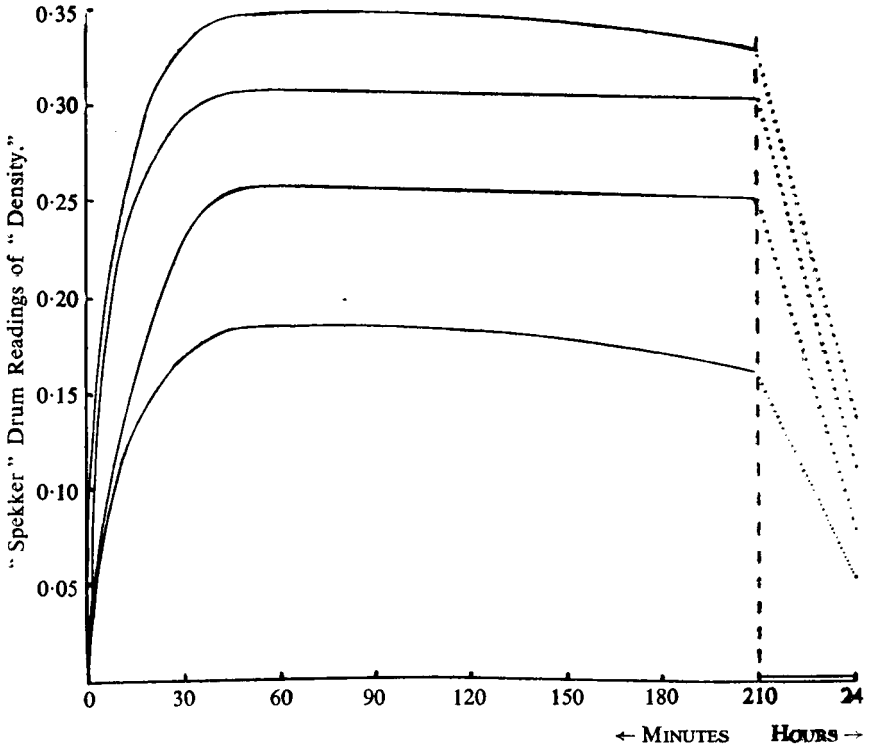


FIG. 1. Effect of time on the colour developed in the Baljet Reaction. Topmost graph, 2·5 ml. of tincture A; middle graphs, 2·0 ml. and 1·5 ml., and the lowest graph, 1·0 ml. of tincture A.

further 2 hours. A final reading was made after 24 hours. The results are shown in Figure 1.

The results presented in Figure 1 show that the difference in intensity of colour between the solutions tested and the reagent control takes 40 to 60 minutes to reach a maximum, after which the readings are fairly stable for about one hour, and fall very slightly over a period of about 3 hours. The more dilute glycosidal solutions differ slightly, as can be seen in the case of the readings for 1·0 ml. of tincture A where the readings fall from about 0·185 to 0·170 quite markedly, within about 3 hours.

After 24 hours, the readings have fallen very considerably. The question arises, whether this fall in the readings is due to a decrease in intensity of the colour of the glycosidal solutions with age, or to an increase in intensity of that of the Baljet reagent with age, or to both effects. In order to study the variation with time, of the intensity of the colour (a) developed from a glycosidal solution and (b) of the Baljet reagent itself, four glycosidal solutions were prepared from four different volumes of tincture A, clarified through a Whatman No. 50 filter paper, and 10 ml. of each mixed with 10 ml. of Baljet reagent. 10 ml. of the Baljet reagent was also mixed with 10 ml. of distilled water to form the Baljet reagent control solution. After 1 hour, readings were made as follows. The two blue filters O.B.1 were used. The orange filter O.Y.2 for the right hand side of the absorptiometer was placed in position. The water cell was placed on the left hand side of the instrument and the absorptiometer set at zero. The orange filter was removed and one of the five coloured solutions, in a 1 cm. path absorptiometer cup, was placed in position. The galvanometer was brought back to zero by closing the right hand light shutter, by turning the calibrated drum. The reading on the drum was taken. The readings were repeated 24 hours later. The results are shown in Table I.

TABLE I

EFFECT OF TIME ON THE COLOUR DEVELOPED IN THE BALJET REACTION

Volume of tincture A in ml.	Difference in " density " between the orange filter O.Y.2 and the solutions under test		Differences in " density " between the intensity of each coloured glycoside solution and the " control "	
	after 1 hour	after 24 hours	after 1 hour	after 24 hours
1·0	0·247	0·337	0·182	0·065
1·5	0·174	0·315	0·255	0·087
2·0	0·130	0·286	0·299	0·116
2·5	0·088	0·249	0·341	0·153
Nil :— " Control " ...	0·429	0·402		

The results given in Table I and Figure 1 show that the intensity of the colour developed from these glycosidal solutions when mixed with alkaline picrate solution, rises to a maximum in 40 to 60 minutes and

begins to decrease 2 or more hours after mixing. The intensity decreases very markedly during the 24 hours after mixing. The intensity of the colour of the alkaline picrate reagent is reasonably constant during the 24 hour-period following its preparation, the intensity of the colour increasing very slightly. It was decided to make all Spekker readings of "density" 1 hour after mixing the glycosidal filtrates with the Baljet reagent.

The Preparation of Calibration Curves for Tincture A. Method A. The results obtained using the method of colour development described on page 881 are shown in graphical form in Figure 2. In most cases each point plotted represents the average of 10 individual estimations of "density" for the volume of tincture A used.

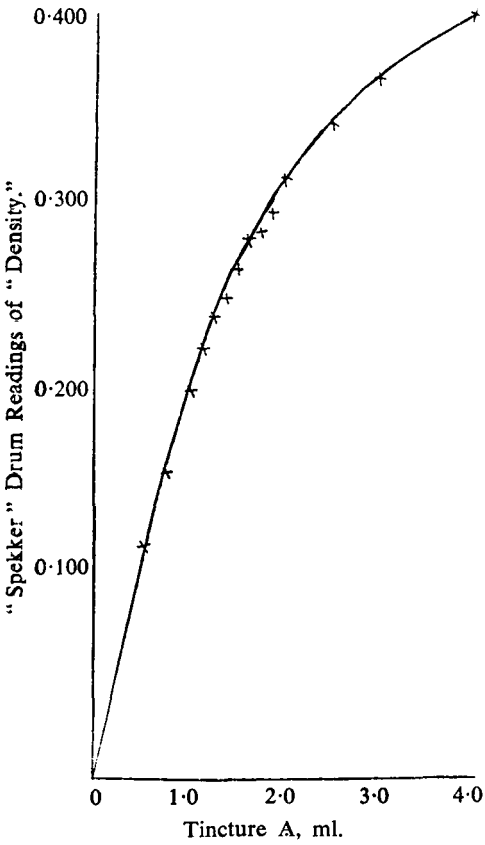


FIG. 2. Calibration curve for tincture A using method A.

Method B. Method A corresponds in essentials with the method described by Bell and Krantz. It can be seen that the increase in the intensity of the colour developed becomes less as the volume of tincture used increases, so that volumes of about 3·0 ml. or more of a normal pharmacopœial tincture cannot be estimated colorimetrically with a high degree of accuracy. For this reason a second calibration curve was prepared in which the final coloured solutions were diluted with an equal volume of distilled water before estimating the intensity of colour by means of the Spekker absorptiometer. The procedure was as follows:—

1. The final filtrates from the decolorisation process were filtered through acid-washed Whatman No. 50 filter papers.

2. 10 ml. of each filtrate was mixed with 10 ml. of the Baljet reagent, also filtered through a Whatman No. 50 filter paper.

3. 20 ml. of the "control" solution was prepared by mixing 10 ml. of the Baljet reagent and 10 ml. of distilled water.

4. The colours were allowed to develop for 1 hour.

5. Each of the coloured solutions including the "control" solution, was then diluted with 20 ml. of distilled water.

6. Immediately after dilution, the intensity of the colour of each of the glycosidal solutions was estimated by comparison with the diluted "control" solution.

The results are presented in graphical form in Figure 3. Each point represents the average of 20 individual estimations of "density" for the volume of tincture A used. The standard deviation of the results using these 2 methods was about the same, and varied between 2 per cent. and 8 per cent., so that there is free choice of method. The method B has been used by the authors, since, as can be seen from Figures 2 and 3, the curvature of the calibration curve is much less in the case of Figure 3 for the larger volumes of tincture A.

II. The comparison of the potencies of tinctures X and A using the colorimetric method

The Preparation of Tincture X. This tincture was prepared by macerating for 2 days with 500 ml. of alcohol (70 per cent.), 50 g. of the same sample of powdered digitalis leaf as was used to prepare tincture A. The liquid was then strained off, the marc pressed, and the expressed liquid mixed with the liquid strained from the marc—allowed to stand for 48 hours and clarified by filtration through coarse filter paper.

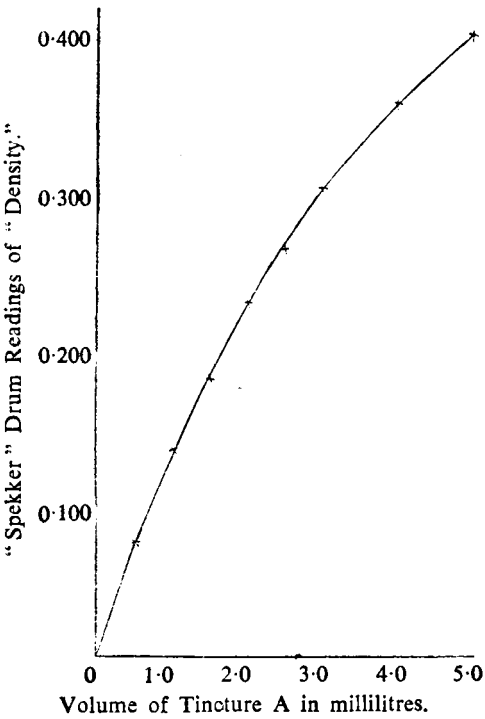


FIG. 3. Calibration curve for tincture A using method B.

The Comparison of the Colour Intensities developed by Tinctures X and A. Five comparisons were made using for each:—(1) Two volumes of tincture X, (2) Two volumes of tincture A, (3) One volume of tincture A. Each volume of tincture was decolorised by the method of Knudson and Dresbach (page 881) and the intensities of the coloured solutions which were produced when the decolorised filtrates were mixed with the Baljet reagent, were compared by the method B described above.

The Calculation of Relative Potency. The potency of tincture X

expressed as a percentage of tincture A was calculated from the formula:—

$$50 \times \left(\frac{R_X - R_{100}}{R_{100} - R_{50}} \right) + 100,$$

where R_X = the reading of "density" for the coloured solution delivered from two volumes of tincture X, R_{100} = the reading of "density" for the coloured solution developed from two volumes of tincture A, and R_{50} = reading of "density" for the coloured solution developed from one volume of tincture A. This formula was used by Bell and Krantz[6] in their colorimetric assay. The volumes of tinctures used by them were:—5·0 ml. of tincture of unknown strength, 5·0 ml. of the U.S.P. Reference Standard Powder Tincture, and 5·0 ml. of the U.S.P. Reference Standard Powder Tincture, diluted with an equal volume of alcohol (71 per cent.). The results of the five assays are shown in Table II.

TABLE II

COMPARISON BY THE COLORIMETRIC METHOD OF TINCTURES A AND X

Assay number	Volume of tincture X used, in ml.	Reading of "Density" (R_X)	Volume of tincture A used, in ml.	Reading of "density" (R_{100})	Volume of tincture A used, in ml.	Reading of "density" (R_{50})	The percentage potency of tincture X relative to the potency of tincture A as obtained from the Bell & Krantz formula
1	1·0	0·078	1·0	0·141	0·5	0·077	50
2	2·0	0·135	2·0	0·233	1·0	0·136	50
3	3·0	0·183	3·0	0·309	1·5	0·185	49
4	4·0	0·230	4·0	0·360	2·0	0·233	49
5	5·0	0·263	5·0	0·405	2·5	0·262	50

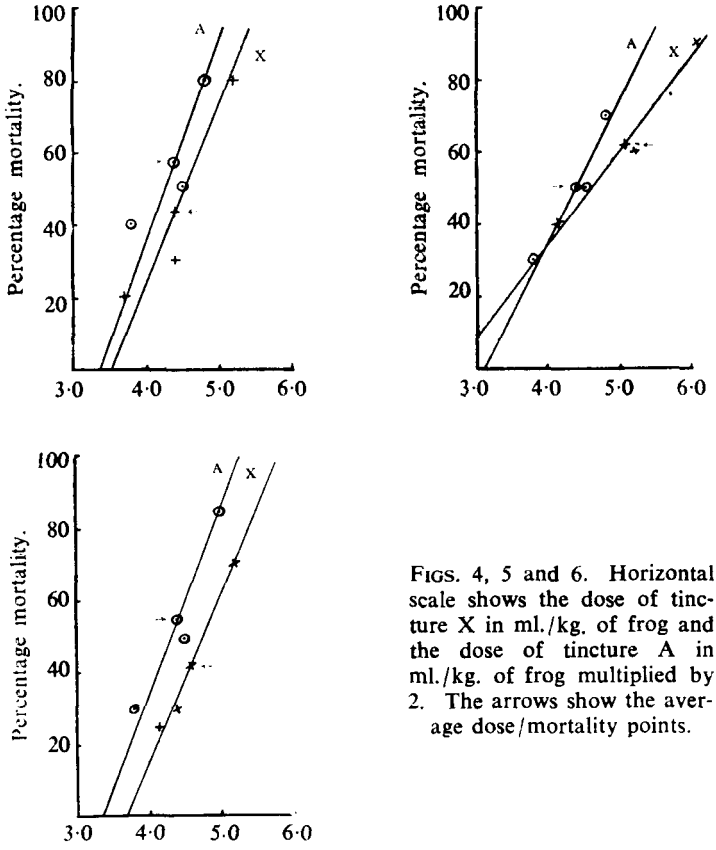
$$\text{The mean ratio} \frac{\text{potency of tincture X}}{\text{potency of tincture A}} = \frac{0{\cdot}5}{1{\cdot}0}$$

III. THE COMPARISON OF THE POTENCIES OF TINCTURES A AND X BY BIOLOGICAL METHODS USING FROGS

Three assays using the method described by Møller[16]. Six groups of frogs were chosen for each assay, and 3 dilutions in 0·6 per cent. saline solution of each of the 2 tinctures were made so that the expected mortalities for the 3 doses of each tincture were about 20 per cent., 50 per cent., and 70 per cent. Each of the dilutions was made so that the dose volume was 0·02 ml. per g. of frog. Injection was made into the ventral lymph sac, the needle being inserted in the thigh and directed upwards to the lymph sac so as to pierce a small portion of the thigh muscle. The frogs were inspected after 18 hours, and the mortality ratio for each dose value was recorded.

The average dose value, and the average mortality ratio for each tinc-

ture were calculated from the results obtained, and these figures, along with the 3 appropriate experimental results were used to plot a dose/mortality line for each tincture, which was drawn by eye to pass through the average dose/mortality point. The doses of the tinctures used, in ml. per kg. of frog, form the abscissæ and the percentage mortalities of the frogs form the ordinates. From these two lines, the dose of each tincture corresponding to 50 per cent. mortality, i.e., the LD50, was read off, and the ratio between the LD50's of the 2 tinctures was recorded as the ratio of their potencies. The results of these three assays are recorded in Tables III, IV and V and Figures 4, 5 and 6. In these figures, the volumes of tincture A used have been multiplied by 2 since tincture A is a 1 in 5 extract, and tincture X is a 1 in 10 extract of the sample of leaf.



FIGS. 4, 5 and 6. Horizontal scale shows the dose of tincture X in ml./kg. of frog and the dose of tincture A in ml./kg. of frog multiplied by 2. The arrows show the average dose/mortality points.

The Calculation of Regression Equations for Tinctures A and X. Owing to the poor and irregular supply of frogs during the past years, it was decided that any suitable frogs left over from the comparative assays, and too few for a complete assay, should be used to obtain additional dose/mortality readings. The combined results could then be used

TABLE III

BIOLOGICAL ASSAY NO. 1, CARRIED OUT ON SIX GROUPS OF TEN FROGS

Tincture used	Doses of tinctures in ml./kg. of frog	Mean dose in ml./kg. of frog.	Number of deaths	Mortality, per cent.	Mean mortality, per cent.
X	3·7	4·4	2/10	20	43
X	4·4		3/10	30	
X	5·2		8/10	80	
A	1·9	2·2	4/10	40	57
A	2·25		5/10	50	
A	2·4		8/10	80	

TABLE IV

BIOLOGICAL ASSAY NO. 2, CARRIED OUT ON SIX GROUPS OF TEN FROGS

Tincture used	Doses of tinctures in ml./kg. of frog	Mean dose in ml./kg. of frog	Number of deaths	Mortality, per cent.	Mean mortality, per cent.
X	4·15	5·1	4/10	40	63
X	5·2		6/10	60	
X	6·06		9/10	90	
A	1·9	2·2	3/10	30	50
A	2·25		5/10	50	
A	2·4		7/10	70	

TABLE V

BIOLOGICAL ASSAY NO. 3, CARRIED OUT ON SIX GROUPS OF TWENTY FROGS

Tincture used	Doses of tinctures in ml./kg. of frog	Mean dose in ml./kg. of frog	Number of deaths	Mortality per cent.	Mean mortality, per cent.
X	4·15	4·6	5/20	25	42
X	4·4		6/20	30	
X	5·2		14/20	70	
A	1·9	2·2	6/20	30	55
A	2·25		10/20	50	
A	2·5		17/20	85	

TABLE VI

COMBINED RESULTS OF THE THREE BIOLOGICAL ASSAYS

Assay number	LD50 of Tincture X in ml./kg. of frog	LD50 of Tincture A in ml./kg. of frog	Potency of Tincture X / Potency of Tincture A
1	4·55	2·15	0·47
2	4·6	2·2	0·48
3	4·8	2·15	0·45

$$\text{The average ratio } \frac{\text{potency of tincture X}}{\text{potency of tincture A}} = 0{\cdot}47$$

to calculate a regression line. The method described by Finney[17] was used.

(i) *The calculation of a regression equation for Tincture X.* The combined results obtained for tincture X are shown below (Table VII).

TABLE VII

BIOLOGICAL ASSAY OF TINCTURE X

Dose of tincture X in ml./kg. of frog	(X) Log of dose	(n) No. of animals injected	(r) No. of animals which died	Mortality, per cent.	Probit Y
3·7	0·5682	27	4	14·7	3·95
4·15	0·6180	30	9	30	4·47
4·4	0·6435	39	14	36	4·64
5·2	0·7160	47	31	66	5·41
6·06	0·7825	10	9	90	6·28

The final regression equation was:—$y = 4{\cdot}84 + 10{\cdot}22\ (x - 0{\cdot}685)$

$= 10{\cdot}22x - 1{\cdot}84.$

The log. LD50 $= m = 0{\cdot}67.$

$\chi^2_{(3)} = 0{\cdot}19 \quad V_{(m)} = 0{\cdot}000117$

$V_{(b)} = 3{\cdot}52 \quad \sigma_{(m)} = 0{\cdot}0108$

and $\sigma_{(b)} = 1{\cdot}88$

The fiducial limits for the LD50 of tincture X at the 95 per cent. probability level are 4·45 ml. and 4·91 ml./kg. of frog. The best estimate is 4·68 ml./kg. of frog.

(ii) *The calculation of a regression equation for tincture A.* The combined dose/percentage mortality results for tincture A are shown in Table VIII.

TABLE VIII

RELATION BETWEEN DOSAGE AND THE PERCENTAGE MORTALITY IN THE BIOLOGICAL ASSAY OF TINCTURE A

Dose of tincture A in ml./kg. of frog	(x) Log dose	(n)	(r)	Mortality, per cent.	Probit Y
1·90	0·2788	48	17	35·4	4·39
2·25	0·3522	60	34	56·6	5·12
2·41	0·3820	40	29	73	5·42
2·50	0·3979	34	27	79·4	5·58

The equation for the regression line is:—$y = 5{\cdot}25 + 9{\cdot}46\ (x - 0{\cdot}35)$

$= 9{\cdot}46x + 1{\cdot}95$

$\sigma_{(b)} = 2{\cdot}22 \quad \sigma_{(m)} = 0{\cdot}0119$

$V_{(b)} = 4{\cdot}92 \quad V_{(m)} = 0{\cdot}00014$

$\chi^2_{(2)} = 0{\cdot}676 \quad m = 0{\cdot}3229$

The fiducial limits for the LD50 of tincture A at the 95 per cent. probability level are:—
1·95 ml. and 2·20 ml./kg. of frog.
The best estimate for the LD50 is 2·10 ml. of tincture A per kg. of frog.

(iii) *The calculation of the relative potency of Tinctures A and X from their regression equations.* The common slope $b_{(AX)}$ of the regression lines for the two tinctures is $b_{(AX)} = 9{\cdot}9 \pm 1{\cdot}4$.

A χ^2 for parallelism was calculated to be 0·073. There is one degree of freedom, and this figure is not significant. There is no evidence of any conflict with the hypothesis that the two lines are parallel.

After inserting the figure $b_{(AX)}$ into their regression equations, new estimates for the log. LD50 (m) of tinctures A and X, were obtained:—

$$m_{(A)} = 0{\cdot}324$$
$$m_{(X)} = 0{\cdot}674.$$

From these values, the fiducial limits for the potency of tincture X with respect to tincture A—the standard tincture—were calculated to be 0·41 and 0·48, at the 95 per cent. probability level. The best estimate for the ratio $\frac{\text{potency of tincture X}}{\text{potency of tincture A}}$ is 0·45.

IV. The comparison of the results of the biological and chemical assays of tinctures A and X

The results for the ratio $\frac{\text{potency of tincture X}}{\text{potency of tincture A}}$ are (i) by the chemical assay, 0·50; (ii) by the bio-assay of Møller, 0·47; (iii) by the comparison of regression equations for the frog mortality experiments, 0·45. If the third result is taken to be the most accurate, since it is based on the reactions of 335 frogs, then the figure obtained by the chemical assay differs by about 11 per cent., and the figure obtained by Møller's assay differs by about 4 per cent. On the whole, there is good agreement between the results of the three assay methods. The use of the simple dose/mortality line, as employed by Møller, is open to some criticism. Gaddum[18] and many other workers have described the advantages of the use of the probit, and of the logarithm of the dose volumes. It was therefore decided to use the Møller assay only for a preliminary estimation of relative potency, and to employ the type of assay described by Gaddum[18] where the slope of the probit/log dose curve is determined in each experiment.

(a) *The Effect of Saponins on the Biological and Colorimetric Assays of Digitalis Preparations.* The discrepancy between the results of cat and frog assays, in the comparison of two tinctures of digitalis may be due to the presence of digitalis saponins in one of the tinctures, since saponins are reported to be much more toxic to cold-blooded animals than they are to warm-blooded animals. Saponins may also be responsible for the discrepancy between the results of the frog and the colorimetric assays, reported by many workers, since the digitalis saponins do not give a positive reaction with the Baljet reagent, as they do not contain an unsaturated lactone grouping in the sapogenin part of their molecules.

(i) *Test for the Presence of Saponin in the Sample of powdered Digitalis leaf, used in the Preparation of Tinctures A and X.* A 1 in 10 infusion

was prepared from 5 g. of the powdered digitalis leaf. To the filtered infusion, sufficient sodium chloride was added to produce a concentration of 0·9 per cent. To 2·0 ml. of this solution in a test tube 2·0 ml. of a 1 in 40 dilution in normal saline of washed horse red blood cells was added. The contents of the test tube were mixed, and re-mixed after 15 minutes—allowed to stand for 24 hours and inspected for hæmolysis. This test was repeated with 1·0 ml., 0·5 ml. and 0·25 ml. volumes of the saline-infusion solution. In all cases the volume was adjusted to 2·0 ml. with normal saline, before adding the blood dilution. Hæmolysis occurred in all cases.

(ii) *The Preparation of Tinctures I, J, O and P.* From the same sample of powdered digitalis leaf as was used to prepare tinctures A and X the following tinctures were prepared.

Tincture I. This was a 1 in 10 tincture prepared by the continuous extraction of 100 g. of the powdered leaf, with 1000 ml. of absolute alcohol in a Soxhlet extractor for six hours. The resulting extract was adjusted to 1000 ml. by the addition of absolute alcohol, and clarified after standing for 48 hours, by filtration through coarse filter paper.

Tincture J. 50 g. of the powdered leaf was macerated for 2 days with 500 ml. of alcohol (70 per cent.) according to the pharmacopœial method. The resulting tincture was clarified by filtration through coarse filter paper, after standing for 48 hours. Pénau and Hardy[19] and Mellanoff[20] have reported that digitonin forms an insoluble complex with various sterols, of which the cholesterol-digitonin complex is the least soluble. Pénau and Hardy describe the preparation of a digitonin-ergosterol complex, which can be used for the quantitative estimation of ergosterol. The solution in which the complex forms has an alcoholic strength of about 90 per cent. This process was used as a basis for the removal of saponin from a sample of tincture J as follows.

Tincture O. 100 ml. of tincture J was mixed, in a 500-ml. stoppered bottle, with 100 ml. of absolute alcohol, and 100 ml. of a 0·175 per cent. solution of ergosterol in absolute alcohol was added. This yields a mixture of approximately 90 per cent. alcoholic strength. The mixture was allowed to stand for 18 hours, at room temperature, and the bulky precipitate of saponin-ergosterol complex, and other precipitated matter, was filtered off through a sintered glass filter, and the bottle and filter washed 3 times with a total of 50 ml. of absolute alcohol. (The precipitate was later washed with 3 20-ml. quantities of alcohol (70 per cent.). These washings when evaporated yielded only a slight Keller-Kiliani reaction.) The filtered solution and washings were then reduced to about 70 ml. by distillation under reduced pressure at a temperature not exceeding 60°C. The contents of the distillation flask were removed and adjusted to 100 ml. with the washings of the distillation flask. Absolute alcohol was used for these washings. The final 100 ml. of treated tincture was filtered through coarse paper after being allowed to stand for 7 days.

Tincture P. 100 ml. of tincture J was mixed in a 500-ml. stoppered

bottle with 200 ml. of absolute alcohol and allowed to stand for 18 hours. The mixture was then filtered through a sintered glass filter, the bottle and filter washed with 50 ml. of absolute alcohol and the filtrate and washings evaporated, as in the case of tincture O, to yield a final volume of 100 ml. This was allowed to stand for 7 days and filtered through a coarse filter paper. Tincture P was prepared as a control for tincture O.

(iii) *Test for the Presence of Saponin in Tinctures of Digitalis.* Tinctures A, X, I, J, O and P were tested for saponin by the following method. 20 ml. of each tincture was evaporated to dryness in an evaporating dish, heated on a water-bath. The residue was thoroughly mixed with 40 ml. of normal saline solution and the mixture filtered. It was found that those tinctures which were later shown to contain saponin yielded a residue which very easily passed into solution when saline solution was added. Such solutions could be filtered clear only with difficulty. The saponin-free tinctures yielded a residue which mixed with saline only with difficulty and could be clarified very easily—filtration through Whatman No. 1 filter papers was efficient. In each case, test tubes were set up containing (1) 5·0 ml., (2) 1·0 ml., (3) 0·5 ml., (4) 0·25 ml., (5) 0·125 ml., (6) 0·05 ml., (7) 0·025 ml. and (8) 0·01 ml. of filtrate, the latter quantities being obtained by dilution of the primary filtrate with saline. The volume of the liquid in tubes 3 to 8 inclusive was adjusted to 1·0 ml. by the addition of normal saline. To each tube was added 1·0 ml. of a 1 in 40 dilution in normal saline of washed horse red blood cells. Each tube was mixed and re-mixed 15 minutes later, allowed to stand for 24 hours and inspected for hæmolysis by viewing from above against a white background. Where hæmolysis had occurred, the solution was clear and red—the deposit if any was fine, and white, or grey, not red—this is due to red cell stroma. Where no hæmolysis occurred the deposit was red, and on shaking the mixture became opaque. The results are shown in Table IX.

TABLE IX

RESULTS OF HÆMOLYSIS TEST FOR THE PRESENCE OF SAPONIN IN TINCTURES A, X, I, J, O AND P

Millilitres of filtrate	A	X	I	J	O	P
5·0	—	*	—	*	—	*
1·0	—	*	—	*	—	*
0·5	—	*	—	*	—	*
0·25	—	*	—	*	—	*
0·125... ...	—	*	—	*	—	*
0·05	—	*	—	*	—	*
0·025... ...	—	slight	—	*	—	slight
0·01 ...	—	?	—	?	—	—

* indicates hæmolysis.

Thus 0·025 ml. of filtrate from tinctures X, J and P just cause hæmolysis, and since the filtrates are 1 in 2 dilutions of the original tinctures, this means that 0·0125 ml. of the original tinctures will just cause hæmolysis under these conditions. The total final volume is 2·0 ml., the Hæmolytic Index of these three tinctures is therefore about $\frac{1 \times 2}{0{\cdot}0125} = 160$. In the case of tinctures A, I and O no hæmolysis occurred even when 5·0 ml. of filtrate were used, and the hæmolytic index must therefore be less than 2·4. This shows that there is a clearly defined difference in saponin content between tinctures A, O and I on the one hand, and tinctures X, J and P on the other. With regard to tincture O, and its control tincture P, these results show that the method employed for the removal of saponin is efficient. With regard to tinctures A, I and J, these results indicate that absolute alcohol will extract a negligible amount of saponin, whereas alcohol (70 per cent.) will extract appreciable amounts, from a leaf which contains saponin.

(b) *The Biological and Colorimetric Assays of Tinctures I, J, O and P.*

(i) *The biological comparison of tinctures I and J.* Six groups each of 10 frogs were injected with appropriate doses of the 3 tinctures following the method described by Møller. The ratio $\frac{\text{potency of Tincture J}}{\text{potency of tincture I}}$ was found to be 0·84.

Three further comparisons were made using the method described by Gaddum[18].

Assay No. 1. Four groups, each of 10 frogs, were used. The ratio $\frac{\text{potency of tincture J}}{\text{potency of tincture I}}$ was found to be 0·86. The limits within which this ratio lies, at the 95 per cent. probability level, corresponding to $\pm$ 1·96 standard deviations, were calculated to be 0·79 and 0·95. The weight of the estimate was calculated to be 2158.

Assay No. 2. Four groups each of 10 frogs were used. The ratio $\frac{\text{potency of tincture J}}{\text{potency of tincture I}}$ was found to be 0·81. The limits within which this ratio lies at the 95 per cent. probability level were calculated to be 0·75 and 1·09. The weight of the estimate was 853.

Assay No. 3. Four groups each of 25 frogs were used. The ratio $\frac{\text{potency of tincture J}}{\text{potency of tincture I}}$ was found to be 0·89. The limits within which this ratio lies at the 95 per cent. probability level were calculated to be 0·83 and 0·96. The weight of the estimate was 3,415.

The weighted mean of the 3 results obtained in these assays, for the ratio $\frac{\text{potency of tincture J}}{\text{potency of tincture I}}$ was calculated to be 0·87. The total weight of this estimate was 6426 and, at the 95 per cent. probability level, the

limits within which the weighted mean of the results of the three assays lay, were 0·85 and 0·90.

C. As before (page 887), the combined results from the above assays were used to calculate regression equations for tinctures I and J as shown below.

Tincture I. Altogether 186 frogs were used to prepare a regression line for tincture I. The regression equation was calculated to be $y = 11{\cdot}04x - 1{\cdot}51$. The best estimate of the LD50 was found to be 3·9 ml of tincture I per kg. of frog. The fiducial limits of the LD50 at the 95 per cent. probability level were 3·7 ml. and 4·1 ml. per kg. of frog.

Tincture J. Altogether 133 frogs were used to prepare the regression line. The regression equation was calculated to be $y = 11{\cdot}34x - 2{\cdot}51$. The best estimate of the LD50 was 4·6 ml. of tincture J per kg. of frog. The fiducial limits of the LD50 at the 95 per cent. probability level were 4·4 ml. and 4·9 per ml./kg. of frog. A calculation of the relative potency of these 2 tinctures from the above data is made below: page 895.

(ii) *The biological comparison of tinctures O and P.* Three comparisons were made using the method described by Gaddum[18].

Assay No. 1. Four groups, each of 10 frogs, were used. The ratio $\frac{\text{potency of tincture O}}{\text{potency of tincture P}}$ was found to be 1·03. The limits within which this ratio lies at the 95 per cent. probability level, were calculated to be 0·88 and 1·21. The weight of the estimate was 787.

Assay No. 2. Four groups, each of 10 frogs, were used. The ratio $\frac{\text{potency of tincture O}}{\text{potency of tincture P}}$ was found to be 0·99. The limits within which this ratio lies at the 95 per cent. probability level were calculated to be 0·85 and 1·16. The weight of the estimate was 886.

Assay No. 3. Four groups, each of 15 frogs, were used. The ratio $\frac{\text{potency of tincture O}}{\text{potency of tincture P}}$ was found to be 0·96. The limits within which this ratio lies at the 95 per cent. probability level were calculated to be 0·82 and 1·11. The weight of the estimate was 986.

The weighted mean of the three results for the ratio $\frac{\text{potency of tincture O}}{\text{potency of tincture P}}$ was calculated to be 1·00. The limits within which this ratio lies at the 95 per cent. probability level were calculated to be 0·92 and 1·09. The total weight of the estimate was 2659.

The combined results from the above assays were used to calculate a regression equation for tincture O.

Altogether 107 frogs were used to calculate the regression equation. The equation for the regression line was calculated to be $y = 9{\cdot}32x - 1{\cdot}37$. The best estimate of the LD50 of tincture O was calculated to be 4·8

ml./kg. of frog. The fiducial limits for the LD50 at the 95 per cent. probability level were 4·5 ml. and 5·4 ml. of tincture O per kg. of frog.

The combined results from the above assays were used to calculate a regression equation for tincture P. Altogether 123 frogs were used to calculate the regression line. The regression equation was calculated to be $y = 10{\cdot}59x - 1{\cdot}94$. The best estimate of the LD50 of tincture P was 4·5 ml. per kg. of frog. The fiducial limits for the LD50 of tincture P at the 95 per cent. probability level were 4·3 ml. and 4·8 ml./kg. of frog.

(iii) *The comparison of the potencies of tinctures I, J, O and P from their regression equations.* The common slope " b " of the regression lines for the four tinctures is

$$b = 10{\cdot}57 \pm 1{\cdot}39.$$

A χ^2 test for parallelism was calculated to be 0·26. There are three degrees of freedom, so that this figure is not significant, and there is no evidence of any conflict with the hypothesis that the 4 lines are parallel.

New estimates for the log. LD50 (m) were obtained from the four regression equations into which the figure for the common slope (10·57) had been inserted:—$^{m}(I) = 0{\cdot}590$, $^{m}(J) = 0{\cdot}660$, $^{m}(O) = 0{\cdot}680$, $^{m}(P) = 0{\cdot}656$.

From these values the following ratios were calculated:—

(a) The potency of tincture J in terms of tincture I.
(b) The potency of tincture O in terms of tincture I.
(c) The potency of tincture P in terms of tincture I.
(d) The potency of tincture O in terms of tincture P.

The results are shown in table X.

TABLE X

RELATIVE POTENCIES OF TINCTURES I, J, O AND P

	Potency of tincture in terms of tincture I or P	Fiducial limits at the 95 per cent. probability level	Best estimate
(a)	Tincture J...	79·3 to 90·2 per cent.	84·6 per cent.
(b)	Tincture O...	75·5 to 86·7 per cent.	81 per cent.
(c)	Tincture P...	77·8 to 94·7 per cent.	85·9 per cent.
(d)	Tincture O expressed as a per cent. of tincture P.	87·6 to 101·5 per cent.	94·3 per cent.

The figures for the relationship between tinctures O and P indicate that tincture O is some 5 per cent. weaker than tincture P. This difference in potency falls within the limits of experimental error.

The removal of saponin from this sample of Tincture of Digitalis has caused no significant difference in the potency of the saponin-free tincture as estimated by this method of biological assay. The combined results of the biological assays, for the ratios of the potencies of tinctures I, J, O and P, are shown in Table XI.

TABLE XI

RATIOS OF TINCTURES I, J, O AND P, OBTAINED BY THE DIFFERENT PROCEDURES

Method of calculation	Ratio of potencies of tinctures :—			
	$\frac{J}{I}$	$\frac{O}{I}$	$\frac{P}{I}$	$\frac{O}{P}$
Møller Assay	0·84	—	—	—
Gaddum Assay	0·87	—	—	1·00
Regression Equations	0·85	0·81	0·86	0·94

(iv) *The colorimetric comparison of tinctures I, J, O and P. The estimation of the relative potency of Tinctures I and J using the Bell and Krantz formula.* Three assays were carried out using for each: (a) 2 ml. of tincture J, (b) 2 ml. of tincture I, (c) 1 ml. of tincture I. Each volume of tincture was decolorised by the method of Knudson and Dresbach. Final clarification was effected by means of Whatman acid-washed No. 50 filter papers. The colours were developed, diluted and their intensities compared by the method B described on page 884. Three more assays were carried out in the same way using: (a) 3 ml. of tincture J, (b) 3 ml. of tincture I, (c) 1·5 ml. of tincture I.

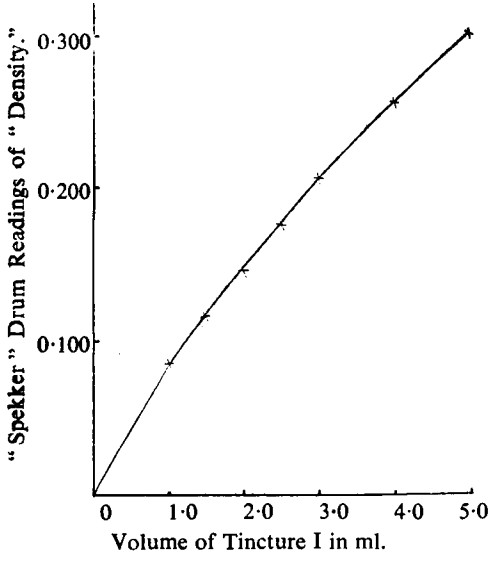


FIG. 7. Calibration curve for Tincture I.

For each of these 6 assays, the intensity of the colour developed from tincture J was compared with that developed from tincture I by means of the Bell and Krantz formula (page 886). The results are shown in Table XII, the potency of tincture being expressed, not as a percentage of that of tincture I, as in the Bell and Krantz formula, but as the corresponding ratio, in which the potency of tincture I is regarded as unity.

2. *The Estimation of the Relative Potency of Tinctures I and J by the comparison of Calibration Curves for the two Tinctures. The calibration curve for tincture I.* A "density" calibration curve was contructed for tincture I. In most cases 10 separate experiments were carried out for each volume of tincture, and the colours were developed, diluted, and estimated as described on page 884. The results are shown in graphical form in Figure 7.

The calibration curve for tincture J. The combined results for tincture J are shown in graphical form in Figure 8. A comparison of the two curves (Figures 7 and 8) at the 0·100, 0·200 and 0·150 " density " levels, indicates that the mean ratio $\frac{\text{potency of Tincture J}}{\text{potency of tincture I}} = 0{\cdot}87$.

TABLE XII

POTENCY OF TINCTURE J EXPRESSED IN TERMS OF TINCTURE I AS IN THE BELL AND KRANTZ FORMULA

Assay No.	Tincture J		Tincture I		Tincture I		Ratio :— Potency of tincture J / Potency of tincture I
	Volume in ml.	Reading of " density "	Volume in ml.	Reading of " density "	Volume in ml.	Reading of " density "	
1	2·0	0·133	2·0	0·150	1·0	0·088	0·86
2	2·0	0·129	2·0	0·153	1·0	0·083	0·86
3	2·0	0·125	2·0	0·145	1·0	0·084	0·84
4	3·0	0·186	3·0	0·207	1·5	0·119	0·88
5	3·0	0·182	3·0	0·205	1·5	0·115	0·87
6	3·0	0·188	3·0	0·212	1·5	0·120	0·87

Average ratio $\frac{\text{Potency of tincture J}}{\text{Potency of tincture I}} = 0{\cdot}86$

3. *The Comparison of Tinctures O and I Using the Bell and Krantz Formula.* Six assays were carried out by the method used to compare tinctures J and I, and described above. The mean ratio of the six assays was calculated to be $\frac{\text{potency of tincture O}}{\text{potency of tincture I}} = 0{\cdot}86$.

4. *The Comparison of Tinctures P and I Using the Bell and Krantz Formula.* Six assays were carried out as described above. The mean ratio of the 6 assays was calculated to be: $\frac{\text{potency of tincture P}}{\text{potency of tincture I}} = 0{\cdot}88$.

5. *The Results Obtained for the Colorimetric Comparison of the Potencies of Tincture I, J, O and P.* These are shown in Table XIII. As can be seen from the result in column 5, the removal of saponin from a saponin-containing tincture of digitalis has no effect on the chemical

TABLE XIII

RESULTS OF COLORIMETRIC ASSAY OF TINCTURES I, J, O AND P

(1) Method of calculation employed	Ratios of Potencies of tinctures :—			
	(2) $\frac{J}{I}$	(3) $\frac{O}{I}$	(4) $\frac{P}{I}$	(5) $\frac{O}{P}$ from the results shown in columns (3) and (4)
The Bell and Krantz formula ...	0·86	0·86	0·88	$\frac{0{\cdot}86}{0{\cdot}88} = 0{\cdot}98$
Comparison of calibration curves ...	0·87	—	—	—

assay of the tincture when a method employing the Baljet reagent is used.

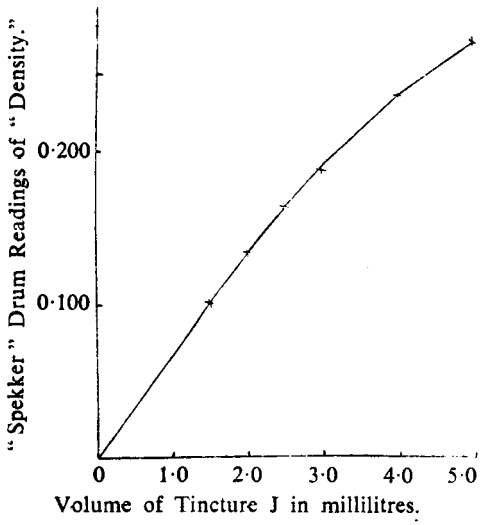


FIG. 8. Calibration curve for Tincture J

(v) *The Comparison of the Results of the Biological and Chemical Methods of Assay for Tinctures I, J. O and P.* A comparison of the results recorded in Tables XI and XIII shows that the chemical and biological comparisons agree closely in the cases of tinctures J and I and of tinctures P and I. In the comparisons of tinctures O and I there is a difference of about 6 per cent. between the result of the colorimetric assay using the Bell and Krantz formula and the result obtained by the comparison of regression equations for the frog mortality experiments. This difference is again apparent in the comparison of the biological and chemical assays of tinctures O and P, when the result of the "regression" method is compared with that of the chemical method. The result of the Gaddum assays, however, agrees closely with that of the chemical method. It can be said in conclusion that the removal of saponin from a saponin-containing tincture of digitalis has no appreciable effect on its potency as estimated by the method of assay using the frog described above, or as estimated by the colorimetric method using the Baljet reagent. The results of the "frog" assays and of the colorimetric assays agree quite well, but it should be remembered that all 6 of the tinctures compared—A, X, I, J, O and P—were prepared from the same sample of powdered Digitalis Leaf.

SUMMARY

1. The comparison of the potencies of tinctures of digitalis, by a chemical method employing the Baljet reaction, has been investigated. Several modifications have been employed, and a method in which the final coloured solutions were diluted with an equal volume of distilled water has been preferred. The period during which the colours are allowed to develop has been extended to 60 minutes, and a method of calculation using the Bell and Krantz formula has been used.

2. Biological assays using the methods described by Møller and by Gaddum have been employed, and regression equations have been calculated. The results of these biological assays agree amongst themselves, and also agree fairly well with the results of the chemical assays.

3. The effect of the removal of saponins from a saponin-containing tincture of digitalis has been investigated. Both biological and chemical methods of comparison indicate that the removal of saponin has no effect on the potency of a saponin-containing tincture of digitalis.

4. All the tinctures used in these comparisons were prepared from the same sample of powdered digitalis leaf. It is not claimed that the results of a chemical assay such as the one employed would agree with the results of a biological assay if the potencies of tinctures prepared from various samples of digitalis leaf were compared.

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DISCUSSION

This paper was read in abstract by Mr. G. Rigby.

MR. K. L. SMITH (Nottingham) remarked that the paper would have been more valuable if the authors had compared more than one sample of digitalis powder instead of examining all the tinctures prepared from one sample. He did not think that the paper proved that the chemical method gave results equivalent to the biological assay; it would be possible to think of other physical characteristics of the solution which, under the author's conditions, would have given the same potency ratio.

DR. G. E. FOSTER (Dartford) said that in his experience the Baljet reaction, unless used with a very sensitive absorptiometer, could be a snare and a delusion. The reagent, alkaline sodium picrate, was yellow and the colour formed was orange. Assays made with a visual colorimeter were unreliable owing to the inability of the eye to match colours of that nature. Digitalis was used therapeutically to slow down the heart but pharmacologically it exerted two effects: one was a cardiotonic effect which might be measured by the minimum dose required to slow the heart in a guinea-pig assay, and the other was the cardiotoxic effect which would stop the heart and kill the animal. Were these two pharmacological properties comparable? If the cardiotoxic effect was measured, was that a measure of the cardiotonic effect which was used clinically?

In some recent Japanese work, a glycoside of an acidic character had been isolated, and in that work it had been suggested that the cardiotonic and cardiotoxic principles were not the same. At this stage, it would be extremely dangerous for anybody to attempt to base the biological standardisation on the chemical assay. There was another difficulty with the biological assay of digitalis preparations. The international standard was a sample of dry powdered digitalis leaf. That was sometimes used in order to standardise such preparations as digitoxin. The substance chosen for the standard was not of the same nature as the substance under test. It would be better, if one were going to standardise a preparation of digitoxin, to use as standard a purified preparation of digitoxin as was done in the U.S. Pharmacopœia.

MR. H. S. GRAINGER (London) commented on the altered use of digitalis tablets in recent years. Clinicians had expressed the opinion that with the digitalis tablets now in use it was possible to achieve the desired clinical effect with a smaller dose, about half the previous requirement. This might be due to improved methods of preservation or of tableting and it would be interesting to know whether any alterations had been made in the criteria of the analysts in doing these assays.

MR. C. J. EASTLAND (London) asked whether the authors had carried out parallel assays on aged tinctures made from the same batch of leaf. He had examined a very large number of samples of the chloroform-soluble glycosides, which were a variable mixture of digitoxin, digitalin and some of the aglycones, and when a chemical assay was applied to such samples, there was a great variation in the ratio of these results to those obtained biologically. In a series of 14 samples, the ratio varied from 1 : 4 to 5. This was quite easy to understand if it was realised that the Baljet reaction depended upon the unsaturated lactone grouping in the glycoside molecule. If hydrolysis occurred to the extent of splitting up the sugar moiety, there would still be presumably the intact saturated lactone ring and that would still give the reaction. In a tincture there would be the aglycone present in the same proportion as in the original glycoside, but in isolated glycosides a good deal of the sugar fraction would have been eliminated and there would be a much higher proportion of the aglycone. If digitoxin were hydrolysed to give the aglycone and the aglycone were tested by the method in question, one would get a colorimetric assay roughly twice that given by the original glycoside. As ageing occurred, one would get an increasing quantity of aglycones, and he would like to ask whether, in the opinion of pharmacologists, such a tincture, though giving an assay figure corresponding to that of the freshly-made tincture, would have the same therapeutic effect.

MR. H. DEANE (Sudbury) said that there was not much evidence that the therapeutic activity of digitalis was closely proportional to the frog-killing power, and the different samples of digitalis did not produce parallel results when frogs, cats or guinea-pigs were used. He thought that everybody who had had to have physiological tests done would be

pleased if they could use a chemical method instead. The Pharmacopœia laid down limits of 7·6 and 12·4 and there were very few chemical tests which could not give closer results than that. They should be as close to the therapeutic effect as the biological method.

MR. G. RIGBY, in reply, said that the use of tinctures prepared from different samples of digitalis leaf was the next step in their programme. They must also attempt to separate the glycosides digitoxin and gitoxin in order that an assay of the content of each could be made in any particular tincture or sample of digitalis leaf. It was not claimed that the colorimetric assay would give satisfactory results if it were used for tinctures prepared from different samples of leaf.

For estimating the intensity of the orange colour produced in the Baljet reaction they had used the Spekker absorptiometer with the blue filters supplied with the instrument. The results were quite reasonable, with a standard deviation of 2 to 8 per cent. The fact that the biological assay depended for its end-point on the death of the animal and thus differed from the therapeutic object, was common to many such assays. However, it was the most suitable means available at the moment for assaying digitalis preparations. With regard to the cardiotonic and cardiotoxic effects he believed that it had been suggested that the adsorption of large amounts of digitoxin on heart or skeletal muscle and the various tissues of the body was irreversible while the adsorption of aglycones and of small amounts of digitoxin was reversible, or partially so, and that this irreversible adsorption led to accumulation of the drug which eventually might lead to the cardiotoxic effect. The international standard powder was of no use in making a comparison of a particular glycoside, but pure samples of digitoxin were available and one would, he imagined, compare an unknown sample of digitoxin with a pure one. As yet he had only assayed tinctures. He could think of no reason why the potency of digitalis tablets should be increased as reported by Mr. Grainger. A good deal of work had been done on the ageing of tinctures, and he thought that there was agreement that a tincture made with absolute alcohol was much more stable than one made with a weaker alcohol. This was possibly connected with the hydrolysis of the glycoside to the genin and aglycone. Such hydrolysis would not occur to any extent in a tincture prepared with absolute alcohol.

PROFESSOR H. BRINDLE, who also replied, said that the work described in the paper had been undertaken because in the American literature it was reported that the Baljet reaction gave a fairly accurate figure with regard to the activity of digitalis preparations when compared with biological methods. There was no satisfactory account from English workers and he felt it desirable that some work on the problem should be done in this country. Only one sample of digitalis leaf had been examined as it was thought better to examine the tinctures from this sample of digitalis thoroughly before proceeding with other studies. Over 1000 frogs had been used and the work had occupied about two years.