STUDIES IN THE GENUS *DIGITALIS*

PART II. A COMPARISON OF THE COLORIMETRIC AND BIOLOGICAL METHODS FOR THE EVALUATION OF *DIGITALIS PURPUREA*

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IN a previous paper[1] of this series a number of colorimetric methods for the estimation of digitalis glycosides have been investigated and conditions under which they may be applied to the examination of digitalis preparations were determined. This present paper reports the results obtained when 16 different powdered leaf samples of *Digitalis purpurea* were examined by these colorimetric processes and also by biological methods.

EXPERIMENTAL

Chemical. All leaf samples were extracted by mixing 1 part by weight of powdered leaf with 10 parts by volume of ethanol (70 per cent.) and allowing to macerate with gentle, continuous agitation for 48 hours. This process has been shown to extract the total glycosides present and has been employed regularly by one of the present authors (F.J.D.). An apparatus was designed for the agitation of a number of samples; 4 galleries each holding ten 60-ml. screw-capped bottles were built onto a wheel which was rotated by means of a low-speed electric motor provided with a variable resistance for control of speed of rotation. The efficiency of the newly designed apparatus for extraction was checked upon a commercial sample of Digitalis Pulverata containing 10 I.U./g. Tinctures were prepared by agitation for 48 hours and also for 72 hours; each was then estimated colorimetrically by the recommended process for estimation.[1] Colour densities for tinctures prepared by the two periods of maceration were the same.

It has been shown in a previous paper[1] that the behaviour of different volumes of one decolorised and diluted tincture of digitalis in the dinitrobenzoate process of estimation was in accordance with the Beer-Lambert law. A more detailed investigation of the application of this law to different extracts from the sample of Digitalis Pulverata was designed. The recommended process for estimation was modified by:—(*a*) tinctures containing between 0·5 and 1·75 I.U./ml. were prepared by using different weights of leaf sample and 10 ml. quantities were used for decolorisation; (*b*) a bulk tincture containing 1·75 I.U./ml. was prepared, aliquot volumes being used along with added ethanol (70 per cent.) for decolorisation to give the same range of concentrations as those prepared in (*a*) above; (*c*) a volume of 10 ml. of the bulk tincture containing 1·75 I.U./ml. was decolorised and different volumes of clear filtrate were employed for estimation. All solutions were estimated by means of the dinitrobenzoate process and concordant values were obtained at each concentration level

for each of the 3 types of leaf extract (*a*), (*b*) and (*c*) above. Over the range of concentrations of 5 to 15 I.U. per cent. for reaction mixture, the value of k (10 I.U. per cent.) was 0·274 to **0·286** to 0·301. At the level of 17·5 I.U. per cent. k (10 I.U. per cent.) = 0·261; thus decolorised tinctures when estimated by the dinitrobenzoate process obey the Beer-Lambert law if between 5 and 15 I.U. of activity are present in each 100 ml. of final reaction mixture. The picrate estimation at 3·5 per cent. ethanol level was also applied to each of the filtrates and similar concordant results were obtained over the range of 7·5 to 17·5 I.U. for tinctures, k (10 I.U. per cent.) = 1·07 to **1·11** to 1·15. Thus the recommended method of extraction of digitalis leaf powder may be conveniently applied to a range of samples differing widely in the amounts of glycosides present, and proportionate differences in colour densities will be obtained in their colorimetric estimations.

The conversion into equivalent digitoxin contents of figures obtained by the picrate estimations of tinctures of digitalis have been shown[1] to be of little value, hence it seemed desirable that all estimations of leaf samples should be expressed in terms of international units of activity. A sample of Standard Preparation of Prepared Digitalis supplied by the Medical Research Council and containing one I.U. in 76 mg. was examined. 4 tinctures of 1 in 10 concentration were prepared as described above and were examined by the recommended process of estimation, values obtained were k (10 I.U. per cent.) = 0·265, 0·275, 0·289, 0·294, average = 0·281. Decolorisations were also carried out at the higher lead level of 50 mg. of Pb. to each ml. of tincture and also all solutions were estimated by the picrate process at 3·5 per cent. ethanol level. These results are shown in Table I.

TABLE I

STANDARD PREPARATION OF PREPARED DIGITALIS

Colour densities k (10 I.U. per cent.)

	Lead level employed in decolorisation (mg. of Pb per ml. of tincture)	
	25	50
Dinitrobenzoate estimation ..	0·281	0·270
Picrate estimation in 3·5 per cent. ethanol	1·138	0·985

10 samples of the powdered leaf of *Digitalis purpurea* were estimated by these processes. The samples were harvested in September, 1951, from first year plants grown in the Museum Experimental Grounds from seeds of this species collected in different geographical areas. All leaf samples were rapidly dried at 55° C. immediately after collection, were reduced to powder, were further dried and securely bottled. Each wide-mouthed bottle for storage was fitted with an aluminium container, holding silica gel, rivetted onto an aluminium disc which acted as a washer to the screw-capped closure; the perforated lower half of the silica gel container was lined with a layer of filter paper thus preventing the contamination of the

leaf sample. Moisture contents of the 10 samples after 6 months' storage under these conditions, determined by drying at 105° C. for 5 hours, were 3·8 to 5·3 per cent. Tinctures were prepared in duplicate by maceration with agitation for 48 hours, they were decolorised by the recommended process described previously,[1] using lead subacetate both at the 25 mg and 50 mg. levels of Pb to each ml. of tincture. The decolorised filtrates were then estimated by both the picrate process in 3·5 per cent. ethanol and the dinitrobenzoate process. In each estimation 2 different quantities of decolorised filtrate were taken (e.g., 2 ml. and 3 ml. for dinitrobenzoate estimation) to ensure that in every reaction mixture the Beer-Lambert law was operating. Results were calculated in terms of I.U./g. of powdered leaf by using the values of Table I. Every preparation followed the Beer-Lambert law and concordant results between duplicate tinctures from each sample of leaf were obtained. Results are given in Table III. The total solids present in each tincture was estimated by the evaporation

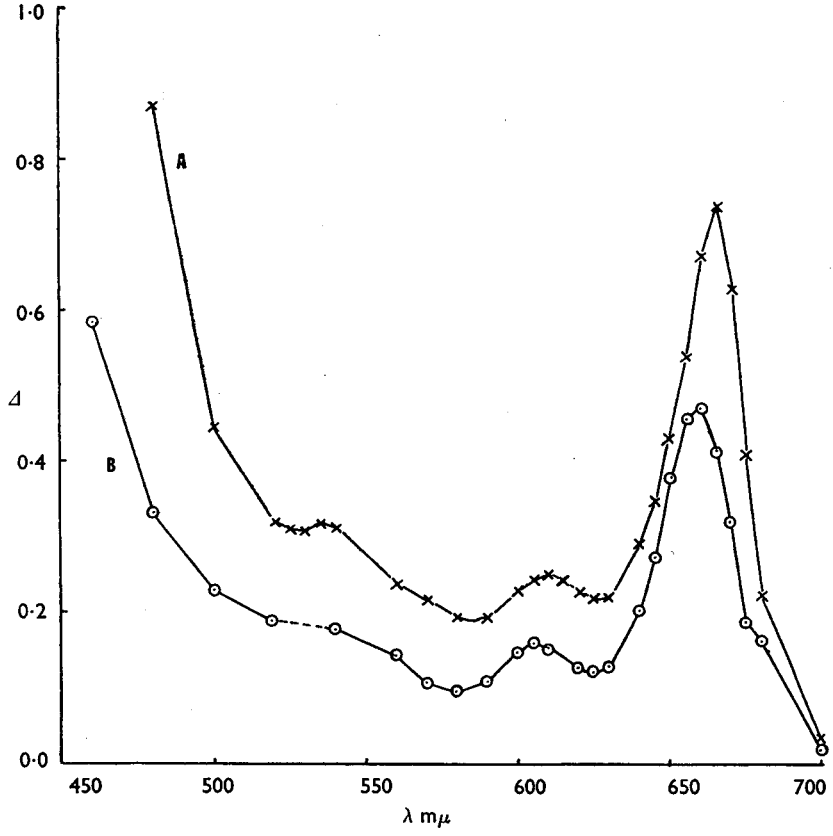

FIG. 1. Colour density of tincture of digitalis (diluted 1 in 10).
A. Dilution of tincture from leaf sample A.
B. Dilution of tincture of digitalis B.P.

of 5 ml. quantities and drying at 105° C. for 18 hours. Results for duplicate tinctures from each sample of leaf were in agreement and values for each sample are also given in Table III.

A subsidary investigation was made of the changes occurring in the decolorised filtrates upon storage at laboratory temperatures. The values reported in Table III were based upon estimations made within 4 hours of the decolorisation of the tinctures. These 40 filtrates were re-examined after 24, 48 and 72 hours' storage and it was found that results varied between +1·1 to **0·23** to 1·8 per cent. from those obtained initially, which is within the order of experimental error.

During the systematic biological estimation of many digitalis leaf samples one of the present authors (F.J.D.) formed an impression that a poor assay figure is often associated with an inferior colour of the tincture prepared for estimation, but as a result of this present work the impression has not been sustained. Preliminary experiments showed that a 10-fold dilution of tincture of digitalis with ethanol (70 per cent.) could be examined in the spectrophotometer. Colour densities at wavelengths between 400 and 700 mμ were determined for commercial tincture of digitalis and also for a 1 in 10 tincture prepared from sample A, using ethanol (70 per cent.) as a blank. Values are plotted in Figure 1, from which it is seen that good agreement between the 2 samples was obtained. 2 maximum colour density peaks were found at 610 mμ and, most pronouncedly, between 650 and 670 mμ. A subsidiary peak also exists at

TABLE II

MAXIMUM COLOUR DENSITIES AND WAVELENGTHS OF MEASUREMENTS FOR TINCTURES OF DIGITALIS DILUTED 10-FOLD

Sample	λ of observation mμ	Colour density
A to K	665	0·526–0·910 (See Table III)
Digitalis Pulverata (comme cial)	665	0·317
Tincture of digitalis B.P. No. 1 ..	660	0·472
Tincture of digitalis B.P. No. 2 ..	655 and 660	0·440

TABLE III

ESTIMATION OF DIGITALIS LEAF POWDERS

	Dinitrobenzoate estimation I.U./g.		Picrate estimation in ethanol (3·5 per cent.) I.U./g.		1 in 10 tincture	
Sample \ Decolorisation at lead level per ml. of tincture	25 mg.	50 mg.	25 mg.	50 mg.	Total solids per cent.	Colour density of 1 in 10 dilution at 665 mμ
A	13·1	12·6	12·8	14·3	3·87	0·728
B	12·9	13·6	14·0	15·3	3·51	0·526
C	11·7	12·8	13·1	14·3	3·80	0·799
D	14·4	13·8	13·8	14·4	3·92	0·752
E	12·5	13·3	12·8	13·9	3·88	0·777
F	12·0	13·5	14·1	13·9	3·65	0·572
G	15·0	14·8	14·7	14·9	3·89	0·910
H	11·9	11·5	13·9	15·1	3·79	0·767
J	13·7	13·7	15·3	14·7	3·80	0·728
K	15·6	15·6	14·8	16·4	3·74	0·893

535 mμ. The peak between 650 and 670 mμ was steep and this peak wavelength differed for the 2 tinctures but it was chosen for the examination of each of the 10 leaf samples. Colour densities were determined at wavelengths differing by 5 mμ intervals between 650 and 670 mμ and the value for maximum colour density was employed. These values are expressed in Tables II and III: figures for samples A to K are averages of readings obtained from duplicate tinctures and each pair showed good agreement.

The samples of which the estimations are given in Tables III and V were all grown, dried and stored under similar conditions and at the same time; also their activities show them to be average to good samples. It was thus desired to examine commercially produced samples which were average to poor in activities. These were decolorised at the 25 mg. lead level and estimated by the recommended process for estimation; picrate estimations were also carried out. The results are shown in Table IV.

TABLE IV

ESTIMATION OF COMMERCIAL LEAF SAMPLES (AVERAGE TO POOR)

Sample	Dinitrobenzoate estimation I.U./g.	Picrate estimation in ethanol (3·5 per cent.) I.U./g.
I	11·3	13·2
II	14·0	14·4
III	7·9	7·2
IV	13·5	13·7
V	11·7	16·6
VI	15·3	15·8

Biological. In a previous study upon the content of active principle in strains of digitalis (Mather and Dyer)[2] frogs were used to assess the potency. The British Pharmacopœia, 1948, permits the use of cats, guinea-pigs and frogs, and at the same time indicates that any other biological method fulfilling the basic principles of biological standardisation, viz., the use of standard and test preparations simultaneously, may be employed. In the present series of assays, guinea-pigs were used, the method followed being that originally described by Knaffl-Lenz,[3] as modified by Gage.[4] When this method was employed as a routine procedure in the Pharmacological Laboratory of the Pharmaceutical Society, and later in the School of Pharmacy of the University of London, the error of the test when 14 animals were used for the standard and not less than 6 for each test preparation was calculated by Emmens[5] to be ± 14 per cent. ($P = 95$). This calculation of accuracy was based on the procedure where the animal is allowed to respire unaided, i.e., without tracheal tube attached to a respiratory pump. In the present series some of the experiments have been done using this procedure, whilst in a few others tracheal tube respiration was used. Consequently, for some of the experiments, a laboratory standard figure of 1·28 I.U./kg. of body weight is used, and for the others (artificially respired) a figure of 2·25 I.U./kg. is employed.

It is, of course, understood that the potency of a "test" preparation is always determined against the International Standard, or a Laboratory sub-standard checked periodically against the International Standard. Although the chief purpose of this series of bioassays is to provide a "yard-stick" to measure the validity of the chemical methods described by Rowson,[1] there is also a subsidiary purpose. Some of the animals employed were purchased from breeders or dealers and may be described as mixed breed: others were obtained from a closed colony and the animals are, therefore, more genetically homozygous than the very heterozygous animals designated as mixed breed. From the results of assays based upon these 2 types of animals an indication was sought whether the closed colony cavies would give a greater degree of accuracy than the mixed breed animals. In this respect the present series can only be regarded as preliminary.

In brief the method of bioassay employed was as follows:—A freshly prepared tincture, representing 10 per cent. w/v of digitalis leaf in ethanol (70 per cent.), was diluted 10-fold with physiological saline solution and slowly infused intravenously into guinea-pigs previously anæsthetised by giving urethane 1·25 g./kg. by the intra-peritoneal route. The rate of infusion was regulated so that cessation of heart-beat occurred not sooner than 20 minutes nor later than 50 minutes, the animals being maintained at uniform temperature throughout the experiment. The lethal dose calculated in terms of ml. of the test tincture (or I.U. of Standard)/kg. of body weight of guinea-pigs, was recorded for at least 4 animals per "unknown" tincture, and for at least 10 animals on the standard tincture. The results of a series of assays made on 10 cultivated leaves and upon several commercial samples are shown in Tables V and VI.

Discussion of Results

Before referring to the intrinsic merits of the chemical sorting test for digitalis, a few observations on the bioassay may be appropriate. In the course of these assays there were used guinea-pigs from 2 main sources, the

TABLE V

Bioassays of digitalis leaf powders

Digitalis leaf	Number of guinea-pigs used	Potency values: I.U./g.	
		Range of results	Mean
International standard	15	11·0 to 19·1	[13·2]
A	8	11·7 „ 16·2	14·1
B	8	9·3 „ 15·3	12·4
C	4	10·8 „ 14·4	12·0
D	4	10·6 „ 14·9	12·7
E	4	10·8 „ 12·3	11·5
F	4	11·4 „ 14·8	13·1
G	8	11·7 „ 13·0	12·2
H	4	11·7 „ 13·6	12·7
J	4	10·9 „ 14·0	12·7
K	4	15·1 „ 16·7	15·9
International standard	10	11·8 „ 15·3	[13·2]

Note: Using 10 animals on Standard and 4 animals on a "Test" preparation, the error of the test may be expressed 100 ± 22 ($p = 0.95$).

TABLE VI

BIOASSAYS OF COMMERCIAL LEAF SAMPLES

Commercial digitalis leaf	Number of guinea-pigs used	Potency values: I.U./g.
International standard	10	(13·2)
I	6	7·9
II	6	5·2
III	5	4·9
IV	4	13·1
V	8	10·6
VI	8	12·0

TABLE VII

COMPARISON OF RESULTS

Cultivated samples			Commercial samples		
Sample	Recommended colorimetric estimation	Bioassay	Sample	Recommended colorimetric estimation	Bioassay
A	13·1	14·1	I	11·3	7·9
B	12·9	12·4	II	14·0	5·2
C	11·7	12·0	III	7·9	4·9
D	14·4	12·7	IV	13·5	13·1
E	12·5	11·5	V	11·7	10·6
F	12·0	13·1	VI	15·3	12·0
G	15·0	12·2			
H	11·9	12·7			
J	13·7	12·7			
K	15·6	15·9			

first being commercially bred, and the second closed-colony reared animals. It has emerged, during this investigation, that the lethal dose (I.U./kg.) for the International Standard varies from 1·28 to 1·66 according to their source of supply. Again, experience in 2 different laboratories, employing slightly varying technique, gives figures differing as widely as 1·28 and 2·25 I.U./kg. There is some evidence that, within limits, light-weight animals (190 to 260 g.) show a higher lethal value than others belonging to a heavier weight-group (350 to 520 g.). This merely emphasises the necessity for carrying out simultaneous determinations of T and S—or at least of frequently redetermining any "accepted" laboratory value of S—in every estimation of potency. Although the main object of this investigation is to compare 2 different types of sorting test (bearing in mind the limits of error of each), an ancilliary purpose was to collect data so as to decide whether closed-colony cavies give statistically more accurate results than mixed breed animals. Preliminary indications are in favour of the animals from the closed-colony.

Previous work has indicated[1] that the colorimetric estimation of digitalis preparations by means of dinitrobenzoic acid is more accurate and less susceptible to interference than when the alkaline picrate process is employed. The present investigation has confirmed these findings. In Table III both processes of estimation have been applied to preparations decolorised by 2 different amounts of lead subacetate; results for which are the same by the dinitrobenzoate method but are different by the picrate

process. Moreover, when results obtained by biological estimations are compared with those yielded by these 2 colorimetric processes it is seen that a closer approach is achieved by the dinitrobenzoate method. All these findings confirm the preferential choice of the dinitrobenzoate process for estimation, and although the absolute loss of glycoside in the decolorisation process has been found to be 15 to 20 per cent., this would appear to be a constant figure; hence it is cancelled out when the estimations of unknown samples are compared with those for the Standard Preparation of Prepared Digitalis.

A critical comparison of the results obtained by methods for the chemical estimation of 16 samples of digitalis leaf with those obtained by biological assay is obtained by examining Tables III to VII. A deviation in value of ± 10 per cent. may be considered reasonable in the values for chemical estimation, although a smaller error is probable. For two samples, II and VI, there is no good parallelism between either colorimetric estimation and those obtained biologically; for one sample, A, the picrate estimation is in closer agreement with the biological assay but for the remaining 13 samples the dinitrobenzoate estimations show a closer agreement with the biological assays than do the values by the picrate process. The results expressed in Table III for dinitrobenzoate estimations at the 25 mg. Pb level of decolorisation of tinctures prepared from samples A to F and H to K are in agreement, within the limits of experimental error, with those for biological estimations. Thus, if this colorimetric estimation were used as a sorting test, these 9 leaf samples would have been passed as of pharmacopœial quality with values ranging to only a limited extent from that of the Standard Preparation of Prepared Digitalis (13·2 I.U./g.). Sample K possesses the highest activity as shown by both estimations. There is a marked high assessment of sample G by colorimetric estimation as compared with the biological assay, although some difficulties have been experienced with the examination of this sample and it is still the subject of investigation.

The results of Table IV are a more critical test of the colorimetric method as at present proposed, for the samples are derived from different commercial sources and were deliberately chosen for the inferior quality of some of them. Sample I was known to be of continental origin. When the dinitrobenzoate assay is used as a sorting test, sample III would be rejected as poor, sample I might be suspect and samples IV to VI would be passed as satisfactory, thus agreeing with the biological assay although over-valuing samples I and VI. The results for sample II are, however, violently in conflict and the sample is being further examined.

It is apparent that the colorimetric process for the estimation of digitalis is of use, in association with biological methods. It is fully appreciated that, as used at present, this method estimates total glycosides plus aglycones and this may account for the occasional falsely high assessment obtained of a poor drug such as sample II. Such a faulty assessment is probably coupled with bad conditions of preparing the drug for the market by which hydrolysis of glycosides may occur. If the conditions of harvesting, drying and storage of digitalis leaf samples are carefully controlled,

as for those of Table III, then the colorimetric process, as at present proposed, may be used with some confidence as a sorting test.

Fuchs, Soos and Kabert[6] have compared the colorimetric and biological assessments of a number of *Digitalis* species. They found some agreement between biological values and those obtained by the dinitrobenzoate or other butenolide-estimating processes, but a closer parallel was obtained with the Keller-Kiliani process. All of these estimations both chemical and biological, are based upon aqueous extraction of the leaf, precipitation with lead subacetate and extraction of glycosides with three quantities of chloroform. Such extractions are subject to considerable losses of glycosides and so the value of the comparisons is somewhat weakened. That loss in glycosides had occurred in the Fuchs work is seen by the equivalent digitoxin yields calculated, which are of the order of 0·1 to 0·2 per cent. for leaf samples of *D. purpurea* and similar values are quoted by Langejan.[7] If values obtained for samples A to K in Table III are calculated on the basis of k (1 mg. per cent. of digitoxin) = 0·192 (see Rowson,[1] Figure 2) then digitoxin contents of the order 0·5 per cent. for these leaf samples of *D. purpurea* are obtained. It is thus concluded that the present work gives a more faithful comparison between the estimations of leaves of this species by biological methods and by certain colorimetric processes. These investigations, including the examination of deviations reported above, are being continued.

Summary and Conclusions

1. The choice of alkaline solution of 3:5-dinitrobenzoic acid for the colorimetric estimation of preparations of *Digitalis purpurea* is preferred to that of sodium picrate reagent.

2. Results of the bioassays, in which guinea-pigs from 2 distinct sources were used, provide evidence in favour of a slightly greater degree of accuracy in the closed colony animals.

3. Slight differences in weight of animals, or in details of technique, may seriously affect the lethal doses of digitalis per kg. of guinea-pig. Hence simultaneous determinations of the "laboratory standard value" is essential, whenever a bioassay of digitalis is made.

4. Estimations have been made of the potency of 10 samples of *D. purpurea* leaves grown and dried under good conditions. They show a very close correlation for 9 samples between results for bioassays and for colorimetric estimations using the dinitrobenzoate process. The tenth sample shows a deviation of 19 per cent. between the two processes.

5. 6 leaf samples, chosen because they were average to very poor, were examined by chemical and biological methods. A fair agreement between the 2 methods was found for 4 samples and marked disagreement for 1 sample. This was a very deliberate "weighting" of the test by selecting bad samples and it cannot be regarded as a random sampling of commercial specimens of *Digitalis purpurea*.

6. It is concluded that the recommended process for colorimetric estimation may be used as a sorting test for leaf samples which have been prepared for the market in accordance with pharmacopœial instructions.

We wish to thank Miss M. E. Cammiade and Mr. H. J. Fearn for technical assistance with bioassays. One author (J.M.R.) is also indebted to the Agricultural Research Council for a grant towards the cost of the drug drying plant employed in the Museum Experimental Grounds.

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DISCUSSION

The three papers on Digitalis were discussed together. The paper on Digitalis Glycosides was presented by Mr. C. J. Eastland, and the other two by Dr. J. M. Rowson.

The CHAIRMAN referred to Picot's work with *Rana esculenta* and *Rana temporaria*. Marked differences in effect were observed with similar doses in the two species. No species variation was observed with a preparation made with hot water. There appeared to be no explanation or contradiction of that work.

MR. C. J. EASTLAND commented on the high degree of colour reported by Dr. Rowson in the Baljet reaction, involving the use of alkaline picrate with alcohol. He confirmed the intense coloration when industrial alcohol was used, but with Analar ethanol the amount of colour produced was very small. Blank readings, however, had been carried out with the alcohol actually used. All results were obtained on samples of mixed chloroform-soluble glycosides, and it was possible that the discrepancy between the colorimetric and biological assays might be greater when dealing with a mixture of relatively pure glycosides than when much cruder extracts were being tested. The latter preparations contained, in addition to the chloroform-soluble glycosides, the water-soluble glycosides.

DR. J. M. ROWSON agreed that he used industrial spirit, but his work had been of an introductory nature and primarily concerned with proving his methods. Mr. Eastland had dealt with the very important point of colour produced by ethanol, but had he investigated the effect illustrated in Figure 4, which showed that with the leaf preparations the colour density was markedly affected by variation in the ethanol levels? A 1 per cent. change in ethanol level, for example from 25 to 24 per cent., brought about a 12 per cent. change in the glycosidal content as shown in the assay. He pointed out that Mr. Eastland was dealing with a different type of material and was separating it fractionally. Therefore different results were understandable. He did not like the picrate process very much. He noted that Mr. Eastland stressed that 20 minutes should be allowed for colour development and asked whether he had plotted

the colour development against time? In his experience with preparations of the leaf, the maximum depth of colour did not develop until 40 to 45 minutes had elapsed.

Mr. Eastland had criticised the "chemical assay" on the basis of the results he had obtained, his criticism should have been directed to the particular chemical assay procedure which he had used. He asked whether Mr. Eastland could say anything about the quality of the digitoxin used as a standard; it was stated in the paper to have a potency of 1000 I.U./g. which indicated that it was only about 50 per cent. pure. Pure digitoxin had a potency of 1750 I.U./g. but was not available in this country.

DR. G. E. FOSTER (Dartford) said that no attempt should be made to determine colour visually; a photo-electric instrument should always be used. It was a pity that pharmacologists did not record the actual dosage at which the guinea-pig heart began to slow and the dosage at which it stopped, because there was a difference between the cardiotoxic and cardiotonic effects and the ratio was not the same for different batches. In a paper which was to be published shortly it was shown that if less alkali were used in the dinitrobenzene reagent the colour obtained was red and the test was useful in the determination of individual glycosides. Some progress had been made in determining individual glycosides by using paper chromatography. If a glycosidal mixture were run on paper using a mixture of chloroform, methanol and water, then dried off and heated to 100° C. spots of different glycosides would be seen clearly.

DR. W. MITCHELL (London) said that Pratt, in a recent paper in *Analytical Chemistry*, had described a method which was similar to Dr. Rowson's procedure using 3:5-dinitrobenzoic acid in alcohol but, instead of sodium hydroxide, he used benzyltrimethylammonium hydroxide, claiming that hydrolysis was thereby diminished. Pratt did not allow time for the development of colour and read at 550 mμ, a bluish red colour.

MR. A. F. CALDWELL (Singapore) said that in a colorimetric assay of morphine tablets he had overcome a difficulty due to the effect of alcohol on the colour by distilling it over quicklime immediately before use. A method of detecting deterioration in digitalis galenicals quickly was needed in the tropics.

DR. J. G. DARE (Kippax) said that the problem of higher lethal doses for light-weight animals than for heavier animals had been investigated by two independent groups of workers, whose results were in agreement with those of Dr. Rowson. The observations of one of the two groups, an American team, were that if the number of mg. of powdered digitalis were divided by the number of hundreds of g. of guinea-pig body-weight to the power of 0·64, a linear relationship would be found between the weight and the toxic dose from which the adjustment could be made.

DR. N. EVERS (Hertford) said it would have been useful if Mr. Eastland had included limits of error for his biological results. If they had been the same as those of Dr. Dyer their results might have been the same.

Professor H. BRINDLE (Manchester) asked Mr. Eastland whether his samples 1 to 12 were prepared in the same way as samples 13 to 25,

which were described as digitoxin. Did not samples 1 to 12 give a Keller-Kiliani test or give it only very weakly? If prepared from reasonable leaf samples a good Keller-Kiliani test should be obtained. It would appear that Mr. Eastland used the Ulrix chromatographic process for separating the glycosides. He had tried that method, and had obtained only a partial qualitative separation. He agreed that for a complex mixture of primary and secondary glycosides and aglycones the colorimetric test would not give as good a measure of activity as the biological test. For example, he found that gitoxin, which gave a high colour, had very little effect upon frogs so that digitoxin containing gitoxin gave a good colour but gave a low result in the biological test.

The biological test was not the last word. The action of the glycosides on the normal heart was not the same as on the diseased heart and both these actions differed from that causing death in animals. Biological standardisation did not therefore necessarily give a correct idea of therapeutic value. Estimation of the separate glycosides should be attempted. He supported the suggestion that the trouble experienced by Dr. Rowson was due to impurities present in the industrial spirit used. The speaker had experienced no trouble when using pure ethanol distilled over caustic potash.

MR. R. L. STEPHENS (Brighton) asked Dr. Rowson whether he had considered drying the leaves by radio frequency dielectric heating by which drying could be completed in 1 to 1½ minutes.

DR. T. WALLIS (London) pointed out that two sets of specimens had been used by Dr. Rowson, one supplied commercially and one grown by himself. This might account for the variation in the results. At Dr. Rowson's request he had examined one sample giving a very divergent result and had found pollen grains present which were not from digitalis flowers. It was possible that the presence of weeds in the sample of digitalis affected the results.

MR. C. J. EASTLAND, in reply, said that as glycosides were used it was possible to have a constant volume of alcohol so that the question of alcohol level using the Baljet reaction did not arise. He had not studied the effect of varying the alcohol strength of the tincture. He confirmed that with comparatively pure glycosides the maximum colour was produced in about 10 to 12 minutes; in all cases it was obtained in 15 minutes, and a standard time of 20 minutes had been adopted. The weight yields of the chloroform-soluble glycosides varied enormously, from 0·38 g. to 1·2 g. per lb. of dry leaf. Digitoxin of only medium potency was adopted as standard because it was anticipated that samples of prepared glycosides with activity of between 800 and 1200 I.U./g. would be obtained. He agreed that it would be interesting to compare the results of the current methods of assay of digitalis preparations with therapeutic efficiency. In answer to Dr. Evers, the standard errors were not greater than 8 per cent. and were given in the paper. In regard to the difference between samples 1 to 12 and 13 to 26, samples 1 to 12 were all prepared in the same way using the same volume of chloroform for each extraction and some of the series 13 to 17 were also prepared

in that way, showing that the latter set of leaf samples did contain a much higher proportion of digitoxin. Ulrix's method using chloroform and methanol had been tried in chromatographic work. It gave some idea of the different glycosides present. The method had shown that many commercial samples of digitoxin of high activity contained as much as 5 per cent. of gitoxin in addition to a small percentage of primary glycosides.

DR. J. M. ROWSON, in reply, said that Dr. Dyer had certain reservations concerning artificially respired guinea-pigs, which seemed to die smoothly, whereas with ordinary conditions of respiration the cardiotonic effect was observed. In reply to Dr. Mitchell, Figure 2 showed that the wavelength effects were not pronounced, and the curve, which was flat topped, could be used over a wide range; thus, Pratt's use of 550 mμ seemed reasonable. Professor Brindle had referred to the bioassay using frogs. When the assay was carried out using guinea-pigs, gitoxin had the same order of activity as digitoxin. It was gratifying to learn that the American work mentioned by Mr. Dare was in agreement with that now reported by Dr. Dyer and himself. He had not yet tried drying by radio frequency heating.