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THE CHEMICAL AND PHYSIOLOGICAL ASSAY

OF

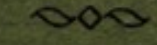
DIGITALIS TINCTURES

BY

GEORGE BARGER, M.A., D.Sc.

AND

W. VERNON SHAW, M.A., M.D.



WELLCOME PHYSIOLOGICAL RESEARCH LABORATORIES

W. DOWSON, M.A., M.D., *Director*

Brockwell Hall, Herne Hill

LONDON, S.E.

THE CHEMICAL AND PHYSIOLOGICAL ASSAY OF DIGITALIS TINCTURES.[1]

By George Barger, M.A., D.Sc.,

and

W. Vernon Shaw, M.A., M.D.

In many cases the active principle of a pharmaceutical preparation being well known and well characterised, chemical assay methods have been worked out which give perfectly reliable results. In others, such as that of diphtheria antitoxin, no such method being possible at present, we must rely entirely upon physiological methods. Between these extremes there are a number of cases in which physiological and chemical methods contend for superiority, and among these is that of Digitalis.

A chemical method for the quantitative estimation of the physiologically active glucosides in this plant has been proposed by Keller (*Ber. d. deutsch. pharm. Ges.*, 1897, **7,** 125). Of these glucosides, digitoxin is the chief contributor to the activity of the drug. This led Ziegenbein (*Archiv der Pharmacie*, 1902, **240,** 454) to compare the physiological activity of a number of samples of Digitalis leaves with the amount of digitoxin found in them by Keller's method. He finds that there is no relation between these two quantities, and concludes that the chemical assay method is unsatisfactory, but that the frog's heart satisfies all the demands which can reasonably be made.

In the course of an examination of a series of tinctures which had been obtained from various commercial sources, and had been prepared according to the directions of the British Pharmacopœia, third edition, we were led to a comparative study of

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the two methods, chemical and physiological. The chemical part was limited to the determination of the digitoxin contained in the various tinctures. The determination of digitalin and digitaleïn was not attempted, though Keller has given directions for accomplishing it. Both of the latter two principles are soluble in water, and it seemed doubtful whether they could be sharply separated from one another. The digitoxin is insoluble in water, but soluble in chloroform, and hence can be estimated with greater certainty. Moreover, as will be seen from our experiments, the digitoxin is the chief active constituent of Digitalis tincture.

For the estimation of digitoxin Keller's method was employed with the following slight modifications: Keller works with 20 Gm. of leaves, or with 200 Gm. of tincture. According to the Swiss Pharmacopœia, these quantities are equivalent, for 1 part by weight of leaves is extracted with 10 parts by weight of alcohol. The British Pharmacopœia directs that 125 Gm. of leaves should be percolated with 1000 c.c. of 60 per cent. alcohol. The density of this alcohol is 0·913, hence 1 Gm. of leaves is percolated with $\frac{0{\cdot}913 \times 1000}{125} = 7{\cdot}3$ Gm. of alcohol. In order to have quantities equivalent to Keller's 20 Gm. of leaves, we at first used 146 Gm. of tincture for an estimation; later we used 73 Gm., since the accuracy of the chemical estimations even then exceeded that of the physiological ones. The 146 Gm. of tincture were evaporated on a water-bath to 25 c.c. or less to remove the alcohol, and made up with water to 222 Gm. Here the first difficulty presents itself, for by the evaporation of the alcohol a resin separates out, which is mostly insoluble in water, and any digitoxin which may be contained in it will escape estimation. The water is best added in small quantities, and the dish containing the resin warmed on the water-bath while its contents are stirred vigorously, so as to have it suspended in as fine a condition as possible.

To the 222 Gm. of the turbid solution 25 Gm. of a saturated solution of basic lead acetate were added, and the precipitate was filtered off through an ordinary folded filter. In most cases the precipitation was complete, but in the case of two tinctures which contained an abnormally high amount of solids (4·7 and 4·9 per cent.) much more lead acetate was required, and so we did not use 146 Gm. of tincture, but two-thirds of that amount, keeping the other quantities the same. According to

Keller, the total solids in carefully prepared Digitalis tincture (Swiss Pharmacopœia) ought to be 3·5 to 3·7 per cent., though it generally falls below 3 per cent. Allowing for the difference in strength, good British tincture ought probably to contain $\frac{10}{7{\cdot}3}$ times as much, or 4·8 to 5·0 per cent.

Out of the nine tinctures examined, only two came up to this value, the average being 3·4 per cent.

The lead acetate produces in the turbid solution a voluminous yellow precipitate, which is filtered off through an ordinary folded filter. According to Keller, this precipitate from 20 Gm. of leaves weighs 7 Gm. We found for four different tinctures, after washing the precipitate very thoroughly and drying at 110°, 4·4, 5·4, 5·6, and 7·7 Gm. These tinctures contained respectively 2·7, 3·6, 3·7, and 4·9 per cent. of total solids. The average weight of our lead precipitates was therefore less than 6 Gm., but for the sake of simplicity we adhere to Keller's amount.

If to 222 Gm. of the turbid solution 25 Gm. of lead acetate be added, it makes a total of 247 Gm., and if the precipitate weighs 7 Gm., the solution—240 Gm.—is equivalent to 20 Gm. of leaves. Of these 240 Gm. of solution, 132 Gm. can readily be obtained by filtration, representing 11 Gm. of leaves. Keller now removes the excess of lead by adding 5 Gm. of sodium sulphate in 7 Gm. of water, filters, and takes 130 Gm. of the filtrate, corresponding to 10 Gm. of leaves. Clearly, there is a slight error, as the amount of the reagent added should be 11 Gm. (instead of $5+7=12$) to bring the 132 Gm. up to 143 Gm., 13 Gm. of solution being equivalent to 1 Gm. of leaves. Keller allowed the lead sulphate to settle down in an inclined flask, and then poured off the required 130 Gm. of liquid. We found some difficulty in completely precipitating the lead in this manner, and as lead sulphate is less soluble in dilute sulphuric acid than in water, we added 5 Gm. of sodium sulphate + 6 Gm. of 10 per cent. sulphuric acid. The decantation process proved troublesome, but the lead sulphate is easily and rapidly filtered off through a Schleicher and Schüll folded filter paper No. 584 (18½ Cm. diameter). Of the filtrate, 130 Gm. were made alkaline with 2 c.c. of 10 per cent. ammonia; the solution remained perfectly clear, and was shaken out four times with 30 c.c. of chloroform. The chloroform extract was filtered, evaporated to a small bulk, and then washed into a small wide weighing bottle with ground stopper, in which it was evaporated

to dryness, first on the water-bath, then in the steam oven, till of constant weight. The residue is "crude digitoxin." Keller purifies this by dissolving it again in chloroform, adding ether and petroleum ether, and collecting the precipitate formed on a small filter, from which it is dissolved again by hot absolute alcohol, after it has been washed with petroleum ether. Here again we departed slightly from Keller's method, as we found it troublesome to remove the precipitate (which only weighs a few milligrammes) quantitatively from the filter. We, therefore, placed the chloroform solution in a tall 50 c.c. or 100 c.c. stoppered measuring cylinder, in which the digitoxin was precipitated and allowed to settle overnight. The following day the clear liquid, containing impurities, was decanted, and the digitoxin washed by shaking it with a further quantity of petroleum ether. This was decanted, and finally the digitoxin, mixed with some petroleum ether, was dissolved in hot absolute alcohol. The solution was washed into a weighing bottle and evaporated; dry ether was added and evaporated off, and then the substance, which we will call "pure digitoxin Keller," was weighed. We must observe in this connexion that we have never seen the least trace of crystalline structure in this residue, as Keller claims to have found. As will be shown below, only two-thirds of Keller's "pure digitoxin" is really digitoxin. In the physiological estimation of the activity of the tinctures, and of the digitoxin obtained from them, frogs were used. The species was *Rana temporaria:* the weights were generally between 20 Gm. and 35 Gm., but comparative experiments were limited to frogs of nearly the same weight. Males were used almost exclusively, and the time of year was early summer.

The tinctures were tested by evaporating a known weight on the water-bath, and suspending the residue in a definite volume of hot water. The digitoxin "Keller" or digitoxin "Merck" was dissolved in the least possible quantity of absolute alcohol, and this was diluted with distilled water. The finely divided substance was injected before it could settle down, and always contained less than 10 per cent. of alcohol. The quantity injected, namely, 1 c.c. of 10 per cent. alcohol, was shown to be practically innocuous. Moreover, digitoxin kills by stopping the heart in systole, whereas with alcohol the heart continues beating long after reflexes are lost. The injections were made into the dorsal lymph sac, and for decisive experiments the volume of liquid was invariably 1 c.c. The animals were kept under

observation in a moist atmosphere, and the time was noted when the heart stopped. It was found that if death did not occur within about 3 hours, the animal survived.

The following table shows the density and percentage of total solids of the nine tinctures :—

Tincture.	I.	II.	III.	IV.	V.	VI.	VII.	VIII.	IX.
Density . .	0·927	0·934	0·928	0·933	0·932	0·930	0·930	0·936	0·926
Per cent. solids	2·6	4·7	4·1	3·2	3·7	2·7	3·1	4·9	3·6

The percentages of digitoxin found by Keller's method (slightly modified) are given below. Of the first three tinctures we made a larger number of estimations in order to see to what extent concordant results could be obtained.

—	Percentage of "Crude" Digitoxin.	Percentage of "Pure" Digitoxin.	Ratio of "Pure" to "Crude."
I. *a* . . .	0·081	0·032	0·40
b . . .	0·051	0·036	0·71
c . . .	0·076	0·034	0·73
d . . .	0·050	0·030	0·60
e . . .	0·043	0·030	0·69
f . . .	0·045	0·034	0·75
Mean . .	0·053	0·033	0·65
II. *a* . . .	0·058	0·042	0·73
b . . .	0·054	0·039	0·69
c . . .	0·053	0·033	0·62
d . . .	0·049	0·036	0·73
e . . .	0·060	0·039	0·66
f . . .	0·056	0·041	0·74
Mean . .	0·055	0·038	0·69
III. *a* . . .	0·038	—	—
b . . .	0·048	—	—
c . . .	0·054	—	—
d . . .	0·036	—	—
e . . .	0·040	—	—
f . . .	0·046	0·028	0·63
Mean . .	0·044		
IV. *a* . .	—	0·026	—
b . .	0·034	0·022	0·64
V. *a* . .	—	0·020	—
b . .	0·040	0·023	0·58

—	Percentage of "Crude" Digitoxin.	Percentage of "Pure" Digitoxin.	Ratio of "Pure" to "Crude."
VI. *a* . .	0·051	0·033	0·65
b . .	0·053	0·040	0·75
VII. *a* . .	—	0·029	—
b . .	0·045	0·027	0·60
VIII. *a* . .	—	0·039	—
b . .	0·060	0·038	0·63
IX. *a* . .	—	0·034	—
b . .	0·054	0·032	0·59
Mean for all tinctures	0·0485	0·031	0·64

It will be seen that in the values for pure digitoxin the maximum deviation from the mean is about 10 per cent. Many of the estimations were done on 73 Gm. of tincture, and some on two-thirds of this amount. The weight of the pure digitoxin is generally about two-thirds of the weight of the crude substance.

In the physiological part of the work the toxicity of all the tinctures was first determined. In the following tables M.L.D. signifies minimal lethal dose. The determinations of the M.L.D. for the first three tinctures are given in full, by way of illustration. In all the other cases only the results of the injection of doses immediately above and below the M.L.D. have been recorded.

Tincture Digitalis I.

Dose.	Weight of Frog in Gm.	Result.
0·5 c.c. . . .	25	died, 120 minutes.
0·5 c.c. . . .	24	died, 80 minutes.
0·4 c.c. . . .	23	died, 100 minutes.
0·4 c.c . . .	22	died, 80 minutes.
0·4 c.c. . . .	22	died, 80 minutes.
0·3 c.c. . . .	22	lived.
0·25 c.c. . . .	22	lived.
0·25 c.c. . . .	21	lived.

Hence M.L.D. = 0·4 c.c. Tincture I (for 22 Gm. frog).

Tincture Digitalis II.

Dose.	Weight of Frog in Gm.	Result.
0·5 c.c. . . .	34	lived.
0·5 c.c. . . .	31	died, 45 minutes.
0·5 c.c. . . .	36	died, 100 minutes.
0·5 c.c. . . .	20	died, 40 minutes.
0·4 c.c. . . .	31	died, 50 minutes.
0·4 c.c. . . .	32	died, 120 minutes.
0·4 c.c. . . .	35	died, 90 minutes.
0·35 c.c. . . .	32	died, 40 minutes.
0·35 c.c. . . .	32	died, 45 minutes.
0·35 c.c. . . .	30	lived.
0·35 c.c. . . .	35	lived.
0·31 c.c. . . .	31	lived.
0·31 c.c. . . .	30	lived.
0·31 c.c. . . .	34	lived.
0·31 c.c. . . .	36	lived.
0·31 c.c. . . .	31	lived.

Hence M.L.D. = 0·35 c.c. Tincture II (for 32 Gm. frog).

Tincture Digitalis III.

Dose.	Weight of Frog in Gm.	Result.
1·0 c.c. . . .	24	died, 55 minutes.
1·0 c.c. . . .	23	died, 80 minutes.
0·8 c.c. . . .	26	died, 80 minutes.
0·6 c.c. . . .	25	died, 85 minutes.
0·5 c.c. . . .	27	died, 120 minutes.
0·5 c.c. . . .	24	died, 110 minutes.
0·4 c.c. . . .	22	died, 100 minutes.
0·4 c.c. . . .	21	died, 90 minutes.
0·4 c.c. . . .	23	lived.
0·3 c.c. . . .	24	lived.
0·3 c.c. . . .	24	lived.

Hence M.L.D. = 0·45 c.c. Tincture III (for 23 Gm. frog).

Tincture.	Dose.	Weight of Frog in Gm.	Result.
IV. .	0·4 c.c.	24	died, 110 minutes.
		25	died, 100 minutes.
	0·3 c.c.	23	died, 180 minutes.
		22	lived.
	0·2 c.c.	22	lived.
		22	lived.
	M.L.D. = 0·35 c.c. for 23 Gm. frog.		
V. .	0·4 c.c.	36	died, 120 minutes.
		32	died, 100 minutes.
		29	died, 100 minutes.
		29	died, 100 minutes.
	0·3 c.c.	26	died, 120 minutes.
		26	died, 150 minutes.
		28	lived.
		30	lived.
	0·25 c.c.	29	lived.
		29	lived.
	M.L.D . = 0·35 c.c. for 27 Gm. frog.		
VI. .	0·4 c.c.	25	died, 130 minutes.
		24	died, 120 minutes.
	0·3 c.c.	25	lived.
		23	lived.
	M.L.D. = 0·4 c.c. for 24 Gm. frog.		
VII. .	0·6 c.c.	33	died, 140 minutes.
		32	died, 120 minutes.
	0·5 c.c.	25	died, 120 minutes.
		26	died, 140 minutes.
		32	lived.
		34	lived.
	M.L.D. = 0·5 c.c. for 29 Gm. frog.		
VIII. .	0·25 c.c.	21	died, 120 minutes.
		20	died, 90 minutes.
		20	died, 120 minutes.
		24	lived.
	M.L.D. = 0·25 c.c. for 22 Gm. frog.		
IX. .	0·4 c.c.	25	died, 100 minutes.
		23	died, 100 minutes.
	0·3 c.c.	20	died, 120 minutes.
		23	lived.
		20	lived.
	M.L.D. = 0·33 c.c. for 22 Gm. frog.		

The preceding tables are summarised below.

The M.L.D. for 100 Gm. of frogs has been calculated on the supposition (not verified) that the dose for frogs is proportional to their weight.

—	Average Weight of Frogs Receiving M.L.D.	Dose Given in Preceding Tables.	Calculated M.L.D. for 100 Gm. of Frog.
I.	22 Gm.	0·40 c.c.	1·7 Gm.
II.	32 Gm.	0·35 c.c.	1·0 Gm.
III.	23 Gm.	0·45 c.c.	1·8 Gm.
IV.	23 Gm.	0·35 c.c.	1·4 Gm.
V.	27 Gm.	0·35 c.c.	1·2 Gm.
VI.	24 Gm.	0·40 c.c.	1·6 Gm.
VII.	29 Gm.	0·50 c.c.	1·6 Gm.
VIII.	22 Gm.	0·25 c.c.	1·0 Gm.
IX.	22 Gm.	0·33 c.c.	1·4 Gm.

Approximately the toxicity of the strongest tinctures examined is about one and a half times that of the weakest. The toxicity of a number of samples of " pure digitoxin " obtained by Keller's method were next determined. The results are given in the following table :—

Tincture.	Digitoxin Estimation.	M.L.D.
I.	(*e*)	0·51 Milligrammes
II.	(*a*)	0·47 Milligrammes
II.	(*b*)	0·47 Milligrammes
II.	(*c*)	0·40 Milligrammes
IV.	(*b*)	0·41 Milligrammes
V.	(*b*)	0·425 Milligrammes
VI.	(*b*)	0·60 Milligrammes

Mean = 0·475 Milligrammes of purified digitoxin.

The M.L.D. for Merck's crystallised *Digitoxin* and for Nativelle's *Digitaline crystallisée* were next determined. Whether or not these two substances are identical remains at present doubtful ; Kiliani (*Archiv der Pharmacie*, 1897, **235,** 425) inclines to the view that the French digitaline is the same as his " digitophyllin." It was found that both products have the same M.L.D., viz., 0·3 Milligrammes. This was established by the following experiments :—

DIGITOXIN " MERCK."

Dose in Milligrammes.	Weight of Frog in Gm.	Result.
0·3	23	died, 90 minutes.
0·3	24	died, 100 minutes.
0·25	25	lived.
0·2	22	lived.
0·2	25	lived.

M.L.D. = 0·3 Milligrammes. The heart stops in systole.

NATIVELLE'S DIGITALINE CRYSTALLISÉE.

Dose in Milligrammes.	Weight of Frog in Gm.	Result.
0·35	32	died, 120 minutes.
0·35	29	died, 75 minutes.
0·35	27	died, 75 minutes.
0·30	35	lived.
0·30	30	died, 75 minutes.
0·30	26	died, 180 minutes.
0·30	25	lived.
0·30	22	lived.
0·25	31	lived.
0·25	22	died, 85 minutes.

M.L.D. = 0·3 Milligrammes. The heart stops in systole.

It will be seen that Merck's crystallised digitoxin (prepared according to Kiliani's method) is about one and a half times as active as Keller's purified digitoxin. Cloetta came to the same conclusion (*Chem. Centralblatt*, 1904, **1,** 1459). That Keller's purified digitoxin still contains 33 per cent. of impurities need cause no surprise, considering the roughness of his method of purification. We said above, page 6, that Keller's crude digitoxin generally weighs one and a half times as much as his purified product. This result we confirmed by physiological means; the toxicity of the crude digitoxin is two-thirds of that of the purified, as shown by the following table :—

CRUDE DIGITOXIN, OBTAINED BY KELLER'S METHOD.

Tincture.	Digitoxin Estimation.	M.L.D. in Milligrammes.
III.	(*b*)	0·70
III.	(*c*)	0·70
III.	(*e*)	0·74
		Mean = 0·71

Ratio of M.L.D. "crude" and "pure" = 0·71 : 0·475 = 1·5.

For the sake of completeness the M.L.D. of Merck's digitalin, Gehe's digitalin, and Merck's digitaleïn were also determined. The results were as follow for 25 Gm. frogs :—

Digitalin "Merck" . . . 1·25 milligrammes.
Digitalin "Gehe" . . . 2 milligrammes.
Digitaleïn "Merck" . . . 1·5 milligrammes.

With all these water-soluble preparations the heart stopped in diastole.

It will be seen that digitalin and digitaleïn are much less toxic than digitoxin. Hence it has been supposed that the amount of digitoxin present in a tincture gives an indication of

its toxicity. That this is not so—at least, if we take the digitoxin values as obtained by Keller's method—is seen by the following compilation, calculated from previous tables :—

Tincture.	Weight of Frog in Grammes Killed by 1 Gramme of Tincture.	Percentage of Digitoxin in Tincture.	Weight of Frog in Grammes Killed by Digitoxin in 1 Gramme of Tincture.	Ratio of Toxicity of Tincture to Toxicity of Digitoxin in it.
I. .	59	0·032	17	3·5
II. .	100	0·038	20	5·0
III. .	55	0·028	15	3·7
IV. .	71	0·024	13	5·5
V. .	83	0·022	12	6·9
VI .	77	0·036	19	4·1
VII. .	62	0·028	15	4·1
VIII. .	100	0·038	20	5·0
IX. .	71	0·033	17	4·2

The figures of the last column are very similar to those found for the same ratio by Ziegenbein (*loc. cit.*, p. 470) : he gives for six tinctures 5, 3·3, 6·6, 4, 2·6, and 4. As the digitoxin obtained in Keller's method only represents on the average one quarter of the total toxicity of the tincture, the toxicity of the aqueous solution containing digitalin and digitaleïn which remains after shaking out the digitoxin with chloroform was determined. This solution was concentrated on the water-bath, and at the same time the chloroform and ammonia contained in it were expelled. The results for six tinctures are given below :—

Number of Digitoxin Estimation.	Weight of Frogs Used in Grammes.	M.L.D. for One Frog in Gramme of Tincture.	M.L.D. for 100 Grammes of Frog in Grammes of Tincture.
I. (*d*) . .	25	1·75	7·0
I. (*e*) . .	30	1·8	6·0
I. (*f*) . .	30	2·25	7·5
II. (*b*) . .	25	0·8	3·2
III. (*d*) . .	25	1·65	6·6
V. (*b*) . .	25	1·5	6·0
VIII. (*b*) .	25	1·0	4·0
IX. (*b*) . .	25	1·8	7·2

From the above results we have calculated the weights of frog killed by the water-soluble substances in 1 Gm. of the tinctures. They are tabulated below, together with the weights of frog killed by 1 Gm. of the tinctures, and also by the digitoxin in 1 Gm. of the tinctures (as already given) :—

Number of Tincture.	Weight of Frog in Grammes Killed by 1 Gramme of Tincture.	Weight of Frog in Grammes Killed by Digitoxin in 1 Gramme of Tincture.	Weight of Frog in Grammes Killed by Water-soluble substances in 1 Gramme of Tincture.	Total Toxicity Accounted for (Grammes of Frog).	Percentage of Toxicity of Tincture Accounted for.
I. .	59	17	15	32	54 per cent.
II. .	100	20	30	50	50 per cent.
III. .	55	15	16	31	56 per cent.
V. .	83	12	17	29	35 per cent.
VIII. .	100	20	26	46	46 per cent.
IX. .	71	17	18	35	49 per cent.

As about half of the toxicity of the original tincture is unaccounted for, Keller's method would appear to be of little value. This conclusion is furthermore supported by the following experiment. To 94·7 Gm. of tincture No. II., which we had previously found to contain 0·038 per cent. of digitoxin "Keller," we added 0·0308 Gm. of digitoxin "Merck," equivalent to 0·032 per cent., dissolved in a few cubic centimetres of 60 per cent. alcohol. We then divided the mixture into halves, and estimated the digitoxin in each of these, finding :—

In the first half 0·050 per cent. of digitoxin.
In the second half 0·043 per cent. of digitoxin.

Subtracting in each case the 0·038 per cent. already known to be present, we find that of the 0·032 per cent. added, 0·012 per cent. was recovered in one case and 0·005 per cent. in the other, a very insignificant portion of the amount added.

We also made a similar experiment with amorphous digitalin, 30 Milligrammes of which were added to 97·4 Gm. of tincture II. We found the *crude* digitoxin to be : in the first half 0·053 per cent., in the second half 0·063 per cent., mean 0·058 per cent., which is practically identical with the mean for the tincture itself, i.e., 0·055 per cent. On the other hand, the water-soluble constituents of 1 Gm. of this artificially strengthened tincture now killed 34 Gm. of frog, as against 30 Gm. for the tincture itself. This increase corresponds to 20 Mgm. of digitalin instead of the 30 Mgm. actually added. That more was not found is due to the limitations of the physiological methods.

Our next step was to prepare a plant tincture similar to that of Digitalis, but free from toxic principles. For this purpose we percolated a mixture of chaff and finely cut hay with 60 per

cent. alcohol, and got a tincture the specific gravity of which was 0·932, and which left on evaporation 2·9 per cent. of solids, comparable therefore to the Digitalis tinctures investigated. The M.L.D. of this was determined by evaporating a known weight of the tincture, and making it up to a known volume with water, in the usual way. Three frogs lived, two frogs died with a dose corresponding to 13 Gm. of this tincture. It was therefore practically innocuous. We then added 0·04 per cent. of crystallised digitoxin, dissolved in a little alcohol, and tested this artificial tincture physiologically, with the following results :—

Dose.	Weight of Frog in Grammes.	Result.
1·0 Gm. . . .	23	died, 100 minutes.
1·0 Gm. . . .	19	died, 90 minutes.
1·0 Gm. . . .	19	died, 90 minutes.
0·8 Gm. . . .	23	died, 120 minutes.
0·8 Gm. . . .	23	died, 120 minutes.
0·8 Gm. . . .	21	died, 100 minutes.
0·7 Gm. . . .	29	lived.
0·7 Gm. . . .	26	lived.
0·6 Gm. . . .	25	lived.

M.L.D. = 0·8 Gm. of Tincture = 800 Milligrammes.

For the digitoxin added we had previously found M.L.D. = 0·3 Milligrammes, so that the physiological tests showed the presence of $\frac{0{\cdot}3 \times 100}{800} = {\cdot}0375$ per cent. digitoxin, whereas we had put in 0·4 per cent.

The digitoxin in this artificial tincture was next estimated by Keller's method, but we only found a mere trace (0·01 per cent.). This disappearance of the digitoxin is to be attributed to its being insoluble in water, and adhering to the resin which separates out when alcohol is evaporated off from the tincture. This resin is stirred up with water, but never properly enters into solution, and when the precipitate caused by lead acetate is filtered off, this resin with the digitoxin remains behind on the filter. We therefore evaporated the tincture, added water as usual, and then filtered off the resin. Resin and filtrate were both tested physiologically. For this purpose the resin was dissolved in dilute alcohol, the alcohol evaporated off, and the residue suspended in water. The results were as follow :—

Filtrate.

Solution concentrated to $\frac{1}{8{\cdot}3}$ of its original volume.

Dose in Grammes of Original Tincture.	Weight of Frogs in Grammes.	Result.
6·6	31	died, 120 minutes.
6·6	25	died, 100 minutes.
5·0	30	lived.
5·0	29	lived.

M.L.D. = 6·6 Gm. for 25 Gm. frog. 1 Gm. of filtrate kills 0·15 frog = 4 Gm. frog.

Resin.

Suspended in a quantity of water equal to $\frac{1}{2{\cdot}4}$ of the volume of tincture.

Dose in Grammes of Original Tincture.	Weight of Frog in Grammes	Result.
0·88	22	died, 120 minutes.
0·88	20	died, 100 minutes.
0·88	24	died, 140 minutes.
0·66	24	lived.
0·66	24	lived.

M.L.D. = 0·88 Gm. for a 22 Gm. frog. 1 Gm. kills 1·14 frog = 25 Gm. frog.

Adding together the toxicity of resin and filtrate, we get for the tincture :—

1 Gm. kills 0·15 + 1·14 = 1·29 frog or 4 + 25 = 29 Gm. of frog.

The M.L.D. for this artificial tincture had previously been found to be 0·8 Gm. for 22 Gm. frog, i.e., 1 Gm. kills 1·25 frog = 29·5 Gm. of frog.

As will be seen, we have proved (with an appearance of greater accuracy than is obtainable by our methods) that the toxicities of the parts are together equal to the toxicity of the whole. Of these two parts the resinous portion is by far the more active : it contains nearly all the digitoxin added to the chaff tincture, and hence this digitoxin escapes estimation by Keller's method. This result explains why Keller's method gave us a digitoxin

value for our artifical tincture which was much too low ; for the same reason too small values are obtained for the digitoxin in ordinary Digitalis tinctures. In order to confirm this result we evaporated down a quantity of No. 1 tincture (previously referred to), added water, filtered off the resin, and tested resin and filtrate as in the case of the chaff tincture.

Filtrate.

Seventy-three Gm. tincture = 10 c.c.

Dose of Tincture.	Weight of Frogs in Grammes.	Result.
0·73 Gm. . .	19	died, 120 minutes.
0·73 Gm. . .	15	died, 100 minutes.
0·73 Gm. . .	16	lived.
0·55 Gm. . .	18	lived.
0·55 Gm. . .	15	lived.

M.L.D. = 0·73 Gm. for 19 Gm. frog. Filtrate from 1 Gm. tincture kills 1·37 frog = 26 Gm.

Resin.

From 73 Gm. of tincture, made up to 20 c.c.

Dose of Tincture.	Weight of Frog in Grammes.	Result.
0·73 Gm. . .	21	died, 120 minutes.
0·73 Gm. . .	20	died, 100 minutes.
0·73 Gm. . .	18	died, 90 minutes.
0·55 Gm. . .	18	lived.
0·55 Gm. . .	15	died, 100 minutes.
0·55 Gm. . .	15	died, 100 minutes.
0·55 Gm. . .	23	lived

M.L.D. = 0·73 Gm. for 18 Gm. frog. Resin from 1 Gm. tincture kills 1·37 Gm. frog = 25 Gm. frog.

Previously it had been found that 0·4 c.c. of tincture I killed a 22 Gm. frog (page 6), i.e., 1 Gm. kills 2·7 frog = 59 Gm. of frog.

Total for resin + filtrate = 2·74 frogs = 51 Gm. of frog.

Previously, by working with Keller's method, it had been found that :—

Toxicity of digitoxin + toxicity of water-soluble part = 32 Gm. of frog.

It will be seen that, as with the chaff tincture, most of the digitoxin remains on the filter together with the lead precipitate. As the resin in the case of Digitalis tincture separates out with greater difficulty than in the case of the chaff tincture, the amount of digitoxin which passes through the filter is slightly larger, about one-third of the total amount. (Toxicity of 1 Gm. of tincture I = 59 Gm. of frog; water-soluble portion = 15 Gm. of frog, hence total digitoxin = 44 Gm. of frog. Of this an amount corresponding to 15 Gm. of frog was found, therefore approximating to one-third of the total amount present; see table, page 12).

Summary.

Our conclusions may be briefly summarised as follow :—

1. The amount of digitoxin found by Keller's method in the B.P. tincture of Digitalis is less than half the amount actually present.

2. As yet the only reliable method for the assay of Digitalis tincture is the physiological one.

THE WELLCOME PHYSIOLOGICAL RESEARCH LABORATORIES,
HERNE HILL, LONDON, S.E.