STUDIES IN BIOASSAYS: "THE STROPHANTHINS AND OUABAIN."*,[1]

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In the U. S. Pharmacopœia X Strophanthin is described as a glucoside or a mixture of glucosides obtained from *Strophanthus Kombe*. Descriptions follow as to its physical properties and tests are given for its identity and purity but no statement is made as to its physiological activity. This omission is of interest inasmuch as the next article described in the book is *Strophanthus Kombe* itself and for it a biological standard is prescribed, as indeed there is for all the other members of the digitalis group of drugs. The fact that strophanthin is an amorphous glucoside, and may even be a mixture of glucosides, adds to the difficulty of establishing exact chemical standards for its purity and uniformity and would seem to make a biological assay for the substance even more desirable. Of course, if it can be shown that, even with these difficulties, strophanthin as it appears on the market, prepared for medicinal use, is of uniform strength, then no biological standard of strength is necessary. It was the primary object of this research to answer this question.

Many secondary questions were also studied in addition to the primary one concerning the uniform activity of the strophanthins. Among these subsidiary questions may be mentioned a study of the relative activity of Ouabain (Crystalline Gratus-Strophanthin) and Kombe Strophanthin and indeed of the uniformity of different specimens of Ouabain itself. The activity of the powdered digitalis recommended as an international standard by the Second International Conference on Biological Standardization was also investigated and its strength relative to the U. S. P. Standard Ouabain was determined. The activity of powdered digitalis obtained upon the open market was also studied as well as the strength of different tinctures of strophanthus. A report upon the other phases of the work than strophanthin and ouabain will appear in a later communication. Finally, it may be said that the activity of these different glucosides, powders and tinctures was studied by various and sundry methods of assay which are used more or less extensively for the bioassay of the members of the digitalis group of drugs and by this comparative study not only was the activity of the drugs themselves investigated but also additional light was thrown upon the value of the methods employed.

The specimens of ouabain and strophanthin examined were as follows:

1. *Standard Ouabain U. S. P. X* obtained from Washington. It is stable at 130° C. and begins to soften at 180° C. The ordinary ouabain of commerce, crystallized from water, contains on an average about 20% of water of crystallization while the official standard preparation is crystallized from a mixture of alcohol and ether and contains 12.5% water. This fact is important in the present study, as it explains the slight variation in activity between this preparation and specimens of ouabain obtained from other sources.

2. *Ouabain, Merck No. 10182.* This specimen was furnished by Merck & Company in 1912 and the work done upon it at that time resulted in its adoption in U. S. P. IX as the standard for the digitalis series.

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3. *Ouabain, Merck No. 186450.* Obtained about ten years ago.
4. *Ouabain, Merck No. 21974.* Purchased in 1924.
5. *Strophanthin, Crystalline Kombe, Brauns-Closson* (1). Obtained in 1912.
6. *Strophanthin, Merck No. 10977.* Vial labeled "From Kombe Seeds." Obtained from a local druggist and probably an old specimen.
7. *"Strophanthin—puriss-cyst," Schuchardt.* Laboratory specimen over 20 years old.
8. *Strophanthin, Abbott Laboratories, No. 56304.* Labeled "From Kombe Seeds." 1927 specimen.
9. *Strophanthin, Merck No. 23427.* Labeled "From Kombe Seeds U. S. P. X" 1927 specimen.

The methods of assay employed in the study of the specimens enumerated above were as follows: *First*, the official one-hour frog method. *Second*, the intravenous frog method of Smith and McClosky (2). This method is practically the official method except that the drug to be tested is injected intravenously instead of being given into the lymph sac as directed in the official method. *Third*, the four-hour frog method. This method too is like the official frog method except that the period of observation is four hours instead of one hour. The suggestion that the time of observation be prolonged was made at the International Conference on Biological Standardization referred to above. *Fourth*, the frog minimal lethal dose method of Houghton. In this method the frogs are injected with the drug late in the day and the following morning the hearts are examined for systolic standstill. *Fifth*, the cat method (Hatcher-Magnus method). In this method cats of medium size were anæsthetized with urethane given by stomach and ether given as might be necessary during the operative procedures. Cannulæ having been inserted in the trachea and in a femoral vein and artificial respiration having been instituted, the warm dilute solution of the drug to be tested was injected slowly under constant pressure into the vein. The period of injection must be between 40 and 55 minutes, the end-point taken by us being final stoppage of the heart as determined by palpitation and stethoscope. *Sixth*, the final method employed was the colorimetric method of Knudson and Dresbach (3).

Before giving the results of the assays it may be well to make a few comments upon some precautions which we have found it necessary to take in order to get uniform results, not that by mentioning these anything especially new in bioassays will be revealed. In the first place, it was early realized that the mere assay of various samples of ouabain and strophanthin was by no means the whole story. Other facts of more general application might be uncovered which in turn might add to our knowledge of the question of absorption of the drugs and of the relative toxic doses to be obtained in the different methods of assay. For these and other obvious reasons great care has been taken in carrying out the work. An unusual number of frogs has been employed and in case the results in any assay were not satisfactory it was repeated—in some cases many times—until good results were obtained.

The work under the fellowship was started in the fall of 1927 by the Fellow in Pharmacology for that year (L), and in that academic year the fundamental figures were obtained. For purposes of verification this work was repeated and amplified by the second Fellow (B) in the academic year of 1927–1928. Thus, the two sets of assays served as a check upon each other. In case individual discrepancies in the results of the two years appeared, assays were repeated as often as necessary in order

to locate the difficulty. The figures given in the following table represent what is practically an average of all the results obtained and may, we believe, be accepted as giving a fairly accurate value to each preparation. Some variations in uniform results still appear in the table and we believe that even these would be smoothed out by further assays. The whole study has served to strengthen our confidence in the value of bioassay methods, but it has at the same time emphasized the absolute necessity of exercising the greatest care in attention to the details of the assay. In the first place, healthy frogs are absolutely essential. Those we obtained in 1928-1929 were clearly superior to those used in the previous year. A matter of prime importance is the maintenance of the proper temperature of the frogs during the course of the assay as well as in the twenty-four hours previous. Judging by previous assays reported, we imagine there is more looseness in this one detail than in any other. Coöperative assay reports too often give temperature readings with very wide limits of variation. Results obtained with frogs at 17° are very different from those obtained when the animals are at 21° or 22°.

In weighing frogs good results are doubtless usually obtained, but it is conducive to greater accuracy if the frogs are lightly wiped off and the urine expressed before they are weighed. Such precautions may easily change the weight by one gram and in frogs given doses near the border line an error of one Gm. may very well be responsible for a discordant result. It is unnecessary to call attention to common sources of error such as care in the dose measurement, etc., as these will naturally be avoided by any careful worker. It is in other directions that error is likely to creep in and cause serious discrepancies. For example, in the 4- and 12-hour methods it is absolutely necessary to expose the heart in order to come to a decision as to whether it is "plus" or "negative." Especially is this true in border line cases and neglect of this precaution is disastrous. Frogs may be limp and apparently dead and yet the heart be beating slowly. Unless the heart had been exposed it would have been considered "positive."

With these introductory remarks upon assay methods, the results obtained in the assay of the specimens enumerated are given in the following table:

TABLE I.

Specimen.		Frog methods. Official. Mg. per Gm.	Intravenous. Mg. per Gm.	4-Hour frog. Mg. per Gm.	M. L. D. Mg. per Gm.	Cat. Mg. per Kg.	Colorimetric.
1. Ouabain	U. S.	0.00045	0.00035	0.00035	0.00025	0.103	1
2. Ouabain	M. 10182	0.00045	0.00035	0.00035	0.00025	0.109	1.07
3. Ouabain	M. 186450	0.00050	0.00035	0.00035	0.00030	0.113	1.15
4. Ouabain	M. 21974	0.00050	.....	0.00035	0.00035	0.105	..
5. Strophanthin	B-C	0.00050	0.00035	0.00040	.....	0.111	1.15
6. Strophanthin	M. 10977	0.00045	0.00035	0.00035	0.00035	0.106	1.38
7. Strophanthin	Schuchardt	0.00100	0.00065	0.00060	0.00065	0.247	1.50
8. Strophanthin	Abbott	0.00100	0.00065	0.00070	0.00070	0.289	2.15
9. Strophanthin	M. 23427	0.00100	0.00065	0.00060	0.00065	0.255	2.16

Table I shows many interesting points which will have to be discussed separately. Considering first the results obtained by the official method, it will be seen (allowing for slight differences in results) that the glucosides fall into two groups in so far as strength is concerned—one group having a toxicity just twice that of the other. In other words, the "ouabain" group has a fatal dose of about 0.0005 mg. per

Gm. of frog weight, while in the "strophanthin" group the corresponding dose is 0.0010 mg. per Gm. There is no need to discuss individual differences from the average strengths of the ouabains mentioned except in the case of Specimens 1 and 2, which in some assays show a slightly higher toxicity than the others. This may be explained in the case of No. 1 by the low per cent of water of crystallization, as was mentioned earlier. This explanation, of course, will not hold good for No. 2. Taken altogether, however, the figures obtained on the nine specimens show a very satisfactory degree of uniformity and a relative toxicity of the two groups of 50 to 100. Two notable exceptions to the general rule are furnished by Specimens 5 and 6 which nominally are strophanthins, but which display an activity equal to the ouabains. These will be discussed more in detail a little later.

In the *intravenous frog* method it will be seen that here, too, there are the same two groups in so far as toxicity is concerned and also that the ratio of toxicity of the one group to the other is, as before, about 100:50. In the "ouabain" group (including there also strophanthins 5 and 6 as they display ouabain activity), the results of the individual assays agree very well as do those of the "strophanthin" group. In both groups the toxic dose required by the intravenous route is about 35% less than that required by the lymph sac route.

By the *four-hour frog* method the results obtained upon the ouabain group were remarkably uniform and the doses approach very closely those found by the intravenous method—in most instances being exactly the same. This is perhaps what would be expected, as ample time is available for absorption and all the glucoside is taken into the circulation just as when the drug is given intravenously. Within the strophanthin group the variation in dosage is slightly greater by this method than in the intravenous or in the official method. It may be said that in the case of these relatively weak strophanthins we have had more difficulty in establishing a fatal dose than in any other instance. Especially is this true when the longer periods of observation are concerned.

The M. L. D. method yielded rather irregular results which may be partly explained by the fact that they were obtained by only one of the workers (B), and thus there was no check by the second Fellow as in the case of the other assays. Discrepancies were therefore not eliminated by getting an average of two sets of figures as in the other instances. However, they show again the 100 to 50 ratio in activity between the two groups.

The four frog methods taken together, therefore, are absolutely conclusive in demonstrating the 100 to 50 ratio in activity existing between the two groups of glucosides designated for convenience the "ouabain" group and the "strophanthin" group.

When the results obtained by the *cat* method are studied, the same general relationship in activity which is shown by the frog methods is also found here. The actual figures obtained by us are in many cases slightly higher than those published by some other workers, but this is probably due to the fact that in every case we waited for the final standstill of the heart—proving it by palpitation and by the stethoscope. Some workers using this method and taking blood pressures at the same time have made their final reading when the pressure first fell to zero. This is, however, often not the final picture as usually with members of this series the heart will stop for a brief period and then start again and maintain a fairly satis-

factory circulation for a short time. By taking the reading when the heart finally stops the figures will naturally run a little higher when this method is used. Even so, the relationship is close as Hatcher's (4) figure for ouabain of 0.1 mg. per Kg. of cat weight is quite close to our average of four specimens of ouabain of 0.108 mg. per Kg. Here, too, strophanthins 5 and 6 fall into the ouabain group, leaving no doubt whatsoever as to where they belong in so far as toxicity is concerned. The remaining three strophanthins (7, 8 and 9) again display their relative weakness, which indeed appears to be much greater by this method than by the frog methods. Specimens 7 and 9 agree with each other, but in No. 8 there is a considerable discrepancy. It is true that only five cats were used in the assay of this specimen and it is quite possible that the use of a larger number might have brought the results into closer agreement with Nos. 7 and 9. The period of time during which the injection is made in this method is very important in determining the fatal dose, but in this case (No. 8) the three cats which died in approximately the same time gave rather widely divergent figures and the average of these three gave a fatal dose of 0.302 mg. It is irregular results such as these which are so discouraging in the use of the cat method. The results obtained by this method in the first six specimens are good but the last three do not stand in proper relation to the earlier groups as judged by all the frog assays, and then again No. 8 is a marked exception in its own group.

The *colorimetric* results are still more discouraging. The results on 1, 2, 3 and 5 are quite good; No. 6 shows marked deviation from its group and approaches No. 7, when by all the biological methods it is at least twice as strong as 7. Nos. 8 and 9 show good agreement but while No. 7 should agree with them, as a matter of fact there is a very great discrepancy. Figures such as these would not give any encouragement to the adoption of this method of assay. It should be said that all these observations were made by one person and in every instance readings were made up on duplicate specimens.

Considering now strophanthins Nos. 5 and 6, it was found that they had an activity corresponding exactly to the members of the ouabain group. No. 6 is labeled as being Strophanthin "made from Strophanthus Kombe," and yet it is twice as active by every test as is the specimen of strophanthin No. 9 made also from Strophanthus Kombe by the same firm. A similar interesting situation has evidently been encountered in other quarters, as a manufacturer of large quantities of strophanthin stated in a personal communication that his firm make their product from identified Kombe seeds and that by the official method it usually had a M. S. D. of 0.0010 mg. per Gm. of frog weight, equivalent to 0.0005 mg. of ouabain. (Our assays of the product confirm these figures.) But he said that "several times the finished product made from Kombe seeds according to the same process of manufacture has been double strength; that is, the M. S. D. has been the same as that of ouabain." He stated also that they have never had a product with the M. S. D. between these two figures. This peculiar fact is confirmed in our work by the figures given in Table I. The manufacturers further state that they have no explanation to offer unless it be that Kombe seeds as purchased upon the market vary in glucosidal content. However, it is not likely that the variation would be always in the ratio of 50 to 100 and never any ratio between these two figures. It would seem that the explanation would have to be sought elsewhere, and probably in the failure to identify properly the seeds.

Specimen No. 5, "Crystalline Kombe-Strophanthin," offers special difficulties. This strophanthin specimen is part of the original lot isolated by Brauns and Closson (1) in work reported in 1913. The same product has been studied recently from the chemical standpoint by Jacobs and Hoffmann (5). They found that it was in reality a mixture which could be separated into a chloroform-soluble and a water-soluble portion. They were able to show also that the chloroform-soluble portion was identical with cymarin of Canadian hemp. From the water-soluble portion they obtained K-Strophanthin-*B* and an amorphous glucoside or mixture of glucosides. Jacobs and Hoffmann say that it is obvious that extracts of Kombe seeds contain a mixture of strophanthidin glucosides and that it is not at all certain that previous workers have worked with a homogenous substance, even when working with identified seeds.

We have touched briefly upon the chemistry of this glucoside and the opinion of the workers mentioned above regarding the possible lack of uniformity of extracts made from Kombe seeds, as it may furnish a clue to the solution of some of the discrepancies we have encountered and discussed above. It does not, however, offer an explanation of our findings on Specimen No. 6. This, according to our figures, in so far as its toxicity is concerned, belongs in the "ouabain" group. By the frog methods, by the cat method and finally even by the colorimetric test it is of "ouabain" strength. This is surprising in view of the assay of the same glucoside by Brauns and Closson. For example, Table I in their paper gives for Merck's ouabain a M. L. D. for frogs (as determined by the Houghton method) of 0.00042 mg. and 0.0010 mg. for the Crystalline Kombe Strophanthin; a ratio of 4 to 10. By the official one-hour frog method, the M. S. D. was found to be 0.0013 mg. The results obtained in the various assays led them to conclude that their crystalline Kombe Strophanthin has about half the activity of crystalline Gratus Strophanthin (ouabain). There seems to be no explanation for this interesting discrepancy between the Brauns and Closson's results and our own. On the one hand, assays made sixteen years ago by them employing two methods show a toxicity ratio between ouabain and crystalline Kombe Strophanthin of about 2 to 1. Assays made to-day upon the same specimen by six different methods, all agreeing among themselves, show a toxicity ratio of 1 to 1. That these latter figures are correct is made still more certain by the fact that our results were reported to one of the most experienced workers in this country, and at our request he assayed the specimen and found that with a M. S. D. for U. S. P. ouabain of 0.00055 mg. the dose for Brauns and Closson strophanthin was 0.00060 mg. He thus confirmed our findings. As a matter of fact, it has been found impossible to duplicate the Brauns-Closson's figures.

Since the above work was completed we have, through the kindness of Dr. Jacobs, obtained a specimen of his crystalline K-Strophanthin, the equivalent of the Brauns-Closson's product. The specimen was very pure as it had been carefully purified by repeated recrystallization at the time when it was not suspected that it was a mixture. Biological assay of this product by the one-hour and by the four-hour frog method gave a toxicity per Gm. of frog as follows:

One-hour frog method	0.0005 mg.
Four-hour frog method	0.00045 mg.

These figures, as will be seen, agree almost exactly with those we obtained using the original Brauns-Closson's specimen.

By the M. L. D. method, Brauns and Closson found an average toxicity for their product of 0.00096 mg. per Gm. of frog as compared with our figure of 0.00045 mg. per Gm. for the Jacob's product, thus emphasizing again the discrepancy between our figures and those of Brauns and Closson.

CONCLUSIONS.

Strophanthin exists on the market to-day in at least two strengths differing greatly from each other in toxicity, one being practically twice as strong as the other. It seems justifiable, therefore, and indeed absolutely necessary to insert in the description of Strophanthin in the next revision of the Pharmacopœia a statement regarding its physiological activity.

The ouabains on the market appear to be of uniform activity and practically twice as strong as strophanthin.

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REPORT ON THE AVAILABILITY OF CERTAIN NEW BIOLOGICALS.

BY J. C. PEACOCK.*

Since the definite service of pharmacy is to provide and dispense remedial agents, it seems appropriate to report to this Section on the availability of new materia medica as the same comes into the market.

New biological products mean new medical progress, for the ability to combat and control disease has steadily gathered strength with the practical application of succeeding discoveries in this field; and pharmacists who are endeavoring to keep up with the onward march of the medical sciences are desirous of getting a better working knowledge of this class of remedies.

The mystery in which the packages of biological agents have been all too generally enveloped can be dispelled by a better comprehension of the nature of these preparations which are so intimately associated with the treatment of disease and the protection of health.

In a paper entitled "Biologicals Viewed as Pharmaceuticals" which this writer presented last year to the Section on Practical Pharmacy and Dispensing, the suggestion was made to look upon biologicals just as other pharmaceuticals are regarded; that is, to consider them with respect to their proximate composition, as preparations of the active factors or influences which they represent, in the same way that galenicals are thus thought of by the pharmacist, as he dispenses the latter.

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