CONCERNING A STANDARD FOR LIQUOR HYPOPHYSIS U. S. P. X.

BY ERWIN E. NELSON, PH.D.

I. INTRODUCTORY.

The question of a suitable standard for the biological assay of pituitary extracts has been a matter of considerable concern for a number of years. Although the first methods of assay were not published until 1912, Schäfer and Herring (1) in 1908 expressed the view that air-dried samples of perfectly fresh gland, when pulverized and kept in a dry stoppered bottle, were stable. "Thus prepared it appears to retain its active properties for months and even years.* * * * * We have also used saline extracts of the dried material which have been previously extracted with absolute alcohol or with ether, but obtained no diminution in activity after these reagents." Chloroform was always added to the fresh material to prevent putrefaction. This belief in the permanency of the preparations was, as pointed out by Dale and Laidlaw (2), only a impression, and not established by quantitative experiments. The latter authors state that "it may be that ultimately a freshly prepared decoction of such dried material will prove to be the best standard of reference," but they did not believe that such a condition yet existed. They used as a standard "the extract prepared by boiling the perfectly fresh and finely pounded infundibular material with a definite proportion of acidulated water, so as to produce a ten per cent. or twenty per cent. extract of the fresh moist substance. The extract is then sterilized by brief autoclaving in small phials. Some activity is lost in autoclaving but the preparation thereafter has great stability."

The same year (1912) Hamilton (3) published the details of the pressor method of assay. The description of the standard employed is as follows: "The preparation used for the standard is the dried, defatted, powdered gland, which is a stable product. Of this powder 0.001 gram corresponds to approximately 0.02 gram of the fresh gland.* The solution for injection is made by rubbing 0.1 gram of the powder in a mortar with successive portions of acidulated water until the yield is 100 cubic centimeters. This solution should be filtered from the sediment. One cubic centimeter of this solution contains the standard test dose." In a later publication by Hamilton and Rowe (4), it is stated that a completely water-soluble powder prepared by a method devised by Aldrich had been substituted for the simple dried and defatted powder. This preparation, which is patented, is made by extracting the fresh glands by the use of glycerine, and then, after filtration, precipitating the soluble material by the use of alcohol and acetone. The dried precipitate is completely soluble in water. The process differs from those used later apparently only in that glycerine is used for extraction.

The use of any preparation of the pituitary gland itself as a standard is theoretically unsound. This fact led Roth (5) in 1913 to suggest the use of histamine as a standard, and in 1914 (6) this suggestion was amplified, and directions given which were afterward incorporated into the U. S. P. IX. The experience of a number of workers showed that this was unsatisfactory, and in 1918 Spaeth (7), working in the same laboratory, suggested the use of potassium chloride as a standard. This in turn did not prove satisfactory, and has not been used practically.

* In view of later work, the fresh extracts used in evaluating this powder undoubtedly were quite weak.

During the period covered by this work, the procedure in various manufacturing laboratories differed, practically none of them complying with the U. S. P. requirements either as to strength of finished preparation, or standard for assay. During this time at least one commercial laboratory to our knowledge was using a dried, defatted, preparation of the posterior lobe, similar to that suggested by Schäfer and Herring and later used in the method of standardization devised by Hamilton.

In 1923 Burn and Dale (8) described a method for obtaining a solution of the active substances from the perfectly fresh gland by a relatively simple process. Their work was carried out in an exceedingly careful manner, and seemed to demonstrate that under laboratory conditions it was possible to prepare extracts of uniform strength. The Sub-Committee on Bio-Assays of the Revision Committee of the U. S. P. X then recommended tentatively the adoption of the standard solution made according to the method of Burn and Dale as the standard of reference for the assay of commercial preparations. Burn and Dale emphasized the necessity for care in the process and called attention to the possibility of loss of activity. Since few of the manufacturers obtain the posterior lobes immediately on removal from the animals, there would be some difficulty in the practical preparation of the standard according to the directions given. The Sub-Committee believed, therefore, that for actual standardization some stable dried preparation of the posterior lobes should be used, to be evaluated in terms of the Burn and Dale standard and distributed by some central laboratory.

Through the courtesy of Dr. Hamilton and of Parke, Davis & Company, the water-soluble powder developed by Hamilton (not the patented preparation of Aldrich) was offered to the Sub-Committee, and was conditionally accepted. This powder, according to directions furnished by Dr. Hamilton, is made by precipitating the concentrated watery extract of the fresh glands by acetone in the presence of a specified amount of milk sugar. A very fine powder results, which is dried and used in this form. It is completely soluble in water, and according to Dr. Hamilton is stable over long periods.

In 1923 after this action had been taken, Smith and McCloskey published the details of their modification of the preparation of the dried defatted powder suggested earlier, and at the time in use in some of the commercial laboratories. They stop all enzymatic action at once on removal of the gland by dropping it into acetone and also use this substance as a dehydrating and defatting agent.* The final solution for use is made by extracting the dried, defatted, and powdered gland with acidulated water. A surprising degree of uniformity of strength of such solutions is claimed. This, if substantiated, would make unnecessary the use of the solution according to Burn and Dale, since the powder can be evaluated once for all, and then used as the final standard, rather than as an intermediary as originally contemplated by the Sub-Committee.

Through the courtesy of Dr. Hamilton, Dr. Smith, and Dr. Fenger, it has been possible to examine these dried powders, and also to prepare fresh ones for

* We are informed that it has been the routine commercial practice of some manufacturing laboratories to preserve the various glands used in making biological products in acetone. This practice, however, has not been recorded in any scientific journal so far as we have been able to discover.

examination. The details of these examinations, together with some data from other laboratories, form the material for the remainder of this paper.

II. EXPERIMENTAL.*

Since this laboratory was without a direct access to a supply of fresh glands, Dr. Fenger was asked to supply certain preparations, the details of which follow. On October 21, 1922, a shipment of extracts in ampoules was received. The details of preparation of these extracts are quoted from a personal letter from Dr. Fenger.

"Each of the three lots made according to Dr. Dale's method represents forty glands, and the total amount of liquid in each case was 40 cc. The forty posterior lobes were carefully minced and mixed, and out of this mixture 4 grams were taken for the solutions.* * * * * * Lot No. 1 was made from strictly fresh glands. Immediately after removal from the sella turcica, the glands were placed in an enameled container surrounded by ice. After removal to the laboratory the posterior lobes were dissected out and minced by the aid of dissecting scissors. The time required for removal from the animals until the minced glands were covered with water for maceration was 35 minutes. The glands were extracted for one hour in distilled water at 18° C. After acidulation the liquid was heated according to Dale's directions, by immersing the flask in briskly boiling water for ten minutes. After chilling in ice water and filtration through paper the liquid was filled into ampoules. The ampoules were sterilized in boiling water for ten minutes for two consecutive days.

"Lot No. 2 was made as outlined above. The glands, however, were removed directly from the animal and placed at a temperature of 10° F. above zero for 18 hours before trimming and preparation. This lot should show the result of freezing, if any.

"Lot No. 3 was made from glands which were not chilled at all, but kept at room temperature (18° C.) for 18 hours before trimming. Here we should find a marked loss of activity if Dale's findings are correct.

"Lot No. 4 represents 10% pituitary liquid made from glands collected in the usual routine, macerated with acidulated water, and prepared by slow boiling as outlined in the present Pharmacopœia. The glands in this lot represent 3500 animals."

These preparations were assayed on the isolated uterus during the next few months. The results are of considerable interest, some of them being rather unexpected. The extract No. 4 was used as a standard. Placing its value at 100, the following comparative values were obtained:

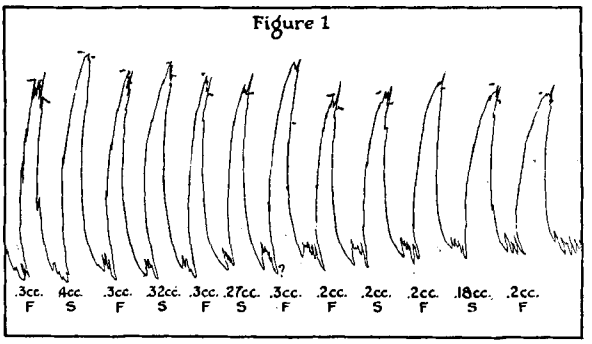
No. 1	180–200	No. 3	160–200
No. 2	70– 90	No. 4	100

It was of course to be expected that No. 1 should be the strongest, but the loss in activity of No. 2 which had been frozen, and the high strength of No. 3 which had been kept at room temperature for some time, are rather surprising. The explanation given by Dr. Fenger is as follows:

"The glands employed in preparing sample No. 2 were frozen in the whole form

* The author was assisted by Mr. Ferdinand R. Schemm in part of the experimental work.

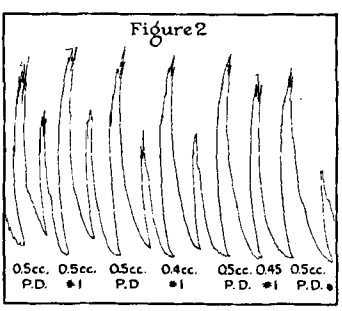
* * * * * The ice crystals formed in the glands tend to break down the cell walls, and render the active principle more available. When the glands are thawed out some of the active principle is dissolved in the melting ice. The pink serum which oozes out from the thawing glands contains the dissolved active principle. Since the liquid in this instance was derived from the entire gland we could not weigh it in as posterior lobe material. This explains the apparent loss in activity during the freezing process.* * * *



A comparison of the fresh extract according to Burn and Dale, with Smith's Extract D. F, the Burn and Dale solution made by Dr. Fenger, a 10% solution diluted 500 times. S, Smith's Extract D, a 5% extract, diluted 500 times. It can be seen that 0.2 cc. F = 0.2 cc. S, and while 0.32 cc. S is stronger than 0.3 cc. F, 0.27 cc. S is weaker than 0.3 cc. F.

"Sample No. 3 was made from fresh glands which had been kept at room temperature. * * * Weather conditions in the late fall (October 19) are not conducive to bacterial and putrefactive processes. Glands exposed to 18° for 18 hours will dry out considerably. The loss in weight and consequent increase in strength of the posterior lobes more than offset the deteriorating effect due to autolytic enzymes, possible bacterial activity, etc."

In connection with other assays to be noted later, this solution No. 1 from Dr. Fenger, a ten per cent. solution by the method of Burn and Deal, has been compared with a five per cent. solution made by Smith and McCloskey, which apparently differed in preparation only in that the fresh gland had been ground with sand instead of being cut up with scissors. The results of a number of comparisons showed that the five per cent. extract D had the same activity as ten per cent. extract by the Burn and Dale method (Fig. 1). The latter authors specifically state that their preparation does not possess the entire activity but only a constant fraction of it. Nevertheless the large discrepancy between the actual strength and the possible strength when somewhat better methods of extraction are used, would seem to make possible large errors. Smith and McCloskey in making up the Dale solution, found that successive preparations did not agree in strength. Most important of all, Dr. Dale himself, in a recent letter to the Chairman of Sub-Committee III, expressed the view that the standard is impractical, and in response to a request for permission to quote him, he makes the following statement in a letter to the author:



2/5-24. The original water-soluble powder received from Dr. Hamilton (PD), against the 10% solution according to Dale, from Fenger (No. 1). 1 cc. of each = 1:2,500,000 dry weight. The powder is practically the equivalent of dry gland. Or 1 mg. powder = 5 mg. fresh gland, as judged by this assay.

"* * * * our own experience gave such uniform results when we took pains to keep the conditions constant, that I hoped that others would adhere to our elaborately detailed description and obtain similar uniformity. Experience has proved that this hope was not justified."

These considerations of course make the use of the fresh extract as a standard problematical, either for comparison with commercial extracts directly or through some intermediary standard.

The Water-Soluble Powder.—The first specimen of the water-soluble powder supplied by Hamilton was received in January 1923. It was compared at once with the fresh extract No. 1 discussed above. Experimentally it was found that one cubic centimeter of a 1:2,500,000 dilution of the dry powder was equivalent to one cubic centimeter of a 1:500,000 dilution of the fresh 10% extract. If one assume that the glands are 80% water, then the final dilution of the gland substance in terms of dry weight is $1/50{,}000 \times 1/10 \times 1/5 = 1/2{,}500{,}000$. In other words, weight for weight the dry powder is equal to its own weight of dry gland. One year later the same powder was assayed against the same extract and the same value again found (Fig. 2). A sample of the powder was sent to Mr. E. E. Swanson, and using a Dale solution as a standard, he obtained the same values.

TABLE I—SUMMARY OF DATA ON THE STRENGTH OF THE ORIGINAL WATER-SOLUBLE POWDER, IN TERMS OF FRESH GLAND.

Determined by		Standard.	Value.
Hamilton		Fresh gland extract	2.5 times fresh gland
Smith	1923	Fresh extract D	1.8 times fresh gland
Nelson	1923	Fresh extract D	2.0 times fresh gland
Nelson	1923	Dale standard	5.0 times fresh gland
Nelson	1924	Dale standard	5.0 times fresh gland
Swanson	1924	Dale standard	5.0 times fresh gland
Anderson	1923*	Dale standard	2.5 times fresh gland

* Personal communication.

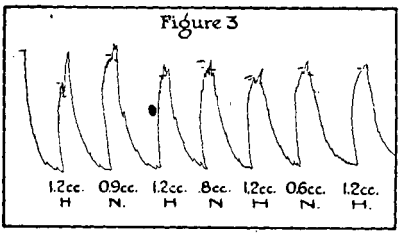
But this strength is twice that claimed by Hamilton for the powder, who states that 2% solution of the powder corresponds to a 5% solution of fresh gland. Smith examining the same sample found that one milligram of the powder was equal to 1.8 milligrams of fresh gland, and the author working in the Pharmacological Laboratory of the Bureau of Chemistry during the summer of 1923, using the Smith extract D as a standard, found essentially the same values as those reported by Smith, namely, that one milligram of the powder was equal to two milligrams of fresh gland. These various values are tabulated in Table I. It will be seen that there are two groups of values. When assayed against the Dale standard, the powder is twice as strong as when assayed against other extracts of fresh glands. The explanation for the discrepancy of course has already been given above, in that the extract according to Dale used here, and presumably also that of Swanson, was actually only half as strong as extracts made by other methods.

These findings of themselves offer no objection to the use of the water-soluble powder, but rather to the use of the Burn and Dale solution as a standard, either as the final standard or as a means of evaluating other powdered preparations. If it could be shown that the water-soluble powder was constant in strength in successive preparations, and was stable, it might still be used. Dr. Hamilton himself does not seem to hold to the view that such preparations are uniform in strength, since in the

first draft of the method of preparation sent to the Chairman of the Sub-Committee III, it is stated that "it is recommended that the powder be tested in comparison with the solution, since losses can always occur, and are not always avoidable.* * * * The resulting powder can be assayed against Dale's solution, and can be adjusted to any desired ratio of activity."

As a matter of fact, the preparation of this powder made in this laboratory, by the above-mentioned directions, is twice the activity of the most recent powder supplied by Dr Hamilton himself (Fig. 3). This recent powder from Hamilton in turn has been found in this laboratory to be weaker than the original specimen.

Figure 3

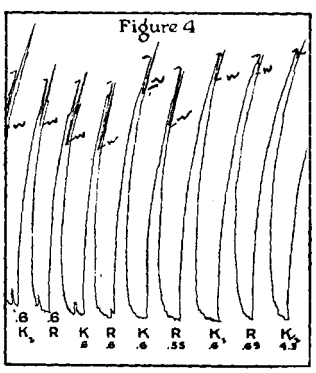


3/4–24. A comparison of the recent water-soluble powder received from Dr. Hamilton (H) with one prepared here (N). Both are in a 1:25,000 dilution. It is seen that 0.6 cc. N = 1.2 cc. H or that N is twice as strong as H.

The admitted lack of uniformity in the strength of the successive preparations of the water-soluble powder, and the impracticability of the use of the Burn and Dale standard solution of pituitary, has impelled the author to withdraw the earlier recommendation to the U. S. P. Sub-Committee III.

The Desiccated, Defatted, Powdered Posterior Lobe.—The pituitary powders made by acetone dehydration and defatting have been somewhat extensively examined. There have been available, through the courtesy of Dr. Smith, samples of his powders A_2, H_2, K_2. These powders were compared with each other in the Pharmacological Laboratory of the Bureau of Chemistry, and were found to be equal in activity. Two specimens of the powder have been prepared from material furnished by Dr. Fenger. These preparations, Fe and Re, have been compared with K_2, and found to have the same activity (Fig. 4). It was also found, working in the Washington Laboratory, that one milligram of A_2 was the equivalent of 6.4 milligrams of fresh gland, as represented in Smith's extract D. Recently Swanson (10) has published the results of examination of three such powders which he has prepared, his powders G, H, and I. The values which he gives are as follows:

Figure 4



1/4–24. A comparison of two preparations of the dried defatted posterior lobe. K_2, powder from Smith. 1 cc. = 1:100,000. Re, powder made by Nelson according to Smith's direction, 12/17–23. 1 cc. = 1:100,000. 0.6 cc. K_2 = 0.55 cc. Re., or K_2 is approximately equal to Re.

1 mg. G = 6.7 mg. fresh post. lobe
1 mg. H = 6.6 mg. fresh post. lobe
1 mg. I = 6.8 mg. fresh post. lobe

These figures are in close agreement with those given by Smith and McCloskey. All of the above data are summarized in Table II.

Because of the uniformity shown by the various preparations of the desiccated, defatted and powdered posterior lobe, and the resultant lack of necessity for standardization against fresh solutions, it is believed that the use of this preparation offers the best standard for the assay of commercial pituitary solutions.

TABLE II.—SUMMARY OF DATA ON THE COMPARISONS OF THE DESICCATED, DEFATTED, POWDERED POSTERIOR LOBE.

Determined by	Preparation.		Value.	Source of Preparation.
Nelson	A_2	=	H_2	Smith
	A_2	=	K_2	Smith
	A_2	=	6.4 mg. fresh gland (Extract D)	Smith
	Fe	=	K_2	Made by Nelson
	Re	=	K_2	Made by Nelson
Swanson	G		6.7 × fresh gland	Made by Swanson
	H	=	6.6 × fresh gland	Made by Swanson
	I	=	6.8 × fresh gland	Made by Swanson
Smith and McCloskey	A_2	=	7.2 × fresh gland	
	C_2	=	7.2 × fresh gland	
	E_2	=	6.7 × fresh gland	
	F_2	=	6.7 × fresh gland	
	G_2	=	7.5 × fresh gland	
	H_2	=	7.4 × fresh gland	

Absolute Value of the Final Standard.—The Pharmacopœial requirement as to strength of finished extract ought of course to conform to the average of accepted commercial practice. The author published in 1923 (11) the results of a number of comparisons of the various commercial extracts obtained on the open market, in terms of one of them. The results showed that there is a very large variation in commercial preparations, even among those which are said to be of the present U. S. P. strength. It is, therefore, very difficult to arrive at any figure for the average. However, it was recommended to the U. S. P. Sub-Committee III that there be adopted a standard representing a five per cent. extract in terms of the Burn and Dale solution.

In April 1923 Smith and McCloskey (9) reported a similar series of assays, finding about the same degree of variation as that found by the author. On the basis of their studies they recommended that the standard should be such that one cubic centimeter of the finished Liquor Hypophysis should correspond in activity to four milligrams of their powder, which in turn was the equivalent of a three per cent. fresh extract as they prepared it. The two recommendations apparently are not in accord. The discrepancy, however, is only apparent. As stated above, when the ten per cent. solution made according to the method of Burn and Dale was compared with the five per cent. solution made by Dr. Smith, the two were found to have the same activity. The earlier recommendation, that a five per cent. solution be used as a standard (which recommendation was made on the basis of experiments with this ten per cent. solution), therefore corresponds actually to a two and one-half per cent. solution by Smith and McCloskey's methods. The difference, then, in the early recommendation of the author to the Sub-Committee and that of Smith and McCloskey, is that between a two and one-half per cent. and a three per cent. solution, respectively. It is quite interesting that so nearly the same approximation of the average of commercial practice should be reached by two independent laboratories. In order to avoid the use of fractional terms, it has been recommended to the Sub-Committee on Bio-Assays that the strength of the finished solution should be such that one cubic centimeter of the commercial extract should have the same activity as that yielded by four milligrams of the dried, defatted, powdered gland.

III. SUMMARY.

The solution of fresh posterior lobe according to the method of Burn and Dale has not proved satisfactory in practical use as a standard for the assay of Liquor Hypophysis.

The water-soluble powdered preparation of pituitary glands made by precipitating the concentrated solution of the active principles with acetone is not uniform from preparation to preparation, and therefore requires standardization itself before being available for use as a final standard.

The dehydrated, defatted, powdered preparation made from fresh posterior lobes apparently is uniform from preparation to preparation. It is suitable for use as a standard. Its adoption has, therefore, been recommended.

It also has been recommended that the U. S. P. X require that the finished commercial preparation of Liquor Hypophysis be of such a strength that one cubic centimeter correspond in activity to that yielded by four milligrams of the dried, defatted, powdered gland.

The author wishes to express his appreciation to Dr. H. C. Hamilton, Dr. M. I. Smith, and especially to Dr. Frederic Fenger for the many courtesies extended to him.

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PRELIMINARY REPORT ON THE EFFECT OF FATTY ACIDS ON LINIMENTS AND EMULSIONS.*[1,2]

BY E. V. KYSER AND FRANK C. VILBRANDT.

In presenting this subject we have made no attempt to determine the critical points of emulsification. The two liniments which we have selected, lime and ammonia, are emulsions, or attempts at emulsions, depending upon the percentage of fatty acids in the oils from which they are made. For convenience we shall refer to them as emulsions. It is our purpose to point out, as they occur to us, certain phases of the effect of fatty acids on emulsions. The Pharmacopœia has no standard for the percentage of free fatty acids which U. S. P. fixed oils may contain and many operators have difficulty in preparing good emulsions by using U. S. P. methods. We believe that some standard for the percentage of free fatty acids of oils

* Scientific Section, A. Ph. A., Asheville meeting, 1923.

[1] Departments of Pharmacy and Chemistry, University of North Carolina.

[2] Samples of emulsions were made by J. E. Johnson, Ph.C., student at the University of North Carolina.