With hypophosphorous acid three experiments showed an average loss of 40%, and two of the three showed an average loss of 60%. The differences are not marked enough to be conclusive, but some other factors may be eliminated in further experiments to help in a decision.

One factor which does not appear in these experiments but which other experience has developed is the effect of heat and aeration in making the fluidextract. We have good evidence that heat and exposure sometimes injure this preparation quite seriously, a loss of 50% being known in one instance due to concentration by distillation under reduced pressure.

In the series of seven fluidextracts designated in the preceding paragraphs which were made individually, the conditions of distillation were not as uniform as they should be and some were exposed to more heat and aeration than others. Some of the erratic results may, perhaps, be due to such exposures. At any rate we feel that the next series should be based on fluidextracts made without heat, *i. e.*, by the fractional percolation method. We also hope to have some coöperative assistance in this work and an invitation is hereby extended to any who may be willing to join in an effort to secure a more stable preparation of ergot.

We desire to acknowledge with thanks the assistance of Mr. J. A Sultzaberger who made the p_H determinations on the preparations above quoted.

RESEARCH LABORATORIES OF PARKE, DAVIS & CO.,
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THE ASSAY OF PEPSIN.*, **

BY H. L. GREENBERG.[1]

INTRODUCTION.

The official assay for the proteolytic power of pepsin is a modified and improved procedure based upon the original test for the activity of Saccharated Pepsin which was made official in the pharmacopœial revision of 1880. The inability of experienced analysts, unfamiliar with the official method of assay, to secure concordant and reliable results using the official assay procedure led to the following critical study in an attempt to develop a more satisfactory method of measuring the proteolytic activity of pepsin.

DEVELOPMENT OF ASSAY METHODS.

According to Waldschmidt (1) the oldest method of quantitatively estimating peptic strength is that of Bidder and Schmidt (2) which dates back to 1852. Since that time there have been many methods worked out. Some of the more important of these may be grouped as follows:

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I. The enzyme is caused to act upon an insoluble protein and the rate at which the latter is digested into soluble products is observed. To this group belong the methods of Bidder and Schmidt (2), Brücke (3), Grünhagen (4), Grützner (5), Mett (6) and that of Witte (7).

II. The enzyme is made to act upon a solution or suspension of protein and the time required to carry the digestion to a definite state, or the amount of protein remaining undigested at the end of a definite time, is determined. The methods of Einhorn (8), Fuld and Levison (9), Gross (10), Jacoby-Selms (11), Rose (12), and that of Thomas and Weber (13), belong to this group.

III. The enzyme acts on the protein and the cleavage products are determined by chemical or physical methods. Abderhalden and Koelker (14), Allen (15), Foreman (16), Schütz (17), Sörenson (18), Van Slyke (19), Volhard (20), and Willstätter and Waldschmidt-Leitz (21), employed modifications of this procedure.

IV. The enzyme is allowed to act on a protein solution, and the progress of digestion is measured by change of electrical conductivity or change in turbidity or viscosity of the solution. To this group belong the methods of Hata (22), Liebermann (23), Northrop (24), and Spriggs (25).

EXPERIMENTAL.

The U. S. P. X Method.—A sample of commercial pepsin labeled "Pepsin U. S. P." was assayed repeatedly by the method of the U. S. P. X with results as follows:

Assay number.	Age of eggs in days.	Cc. of pepsin solution used.	Proteolytic strength.	Deposit of undissolved egg-albumen. (1)	(2)
1	Fresh	5		About 1.5 cc.	About 0.8 cc.
2	Fresh	6.5	1:2308 = 76.9%	Exactly 1 cc.	
3*	10	6.5		Exactly 1 cc.	About 1.3 cc.
4	10	6.5		About 0.9 cc.	About 0.8 cc.
5**	10	6.5		About 1.2 cc.	About 1.5 cc.
6	8	6.5		About 0.5 cc.	About 0.7 cc.
7***	8	6.0		About 0.8 cc.	About 0.6 cc.
8****	8	6.0		About 1.2 cc.	About 1.2 cc.
9	7	6.0		About 0.5 cc.	About 0.5 cc.
10*	10	5.8		About 1.2 cc.	About 1.2 cc.
11	10	5.8		About 0.9 cc.	About 0.7 cc.
12**	10	5.8		About 1.2 cc.	About 1.2 cc.
13****	5	5.7		About 1.3 cc.	About 0.9 cc.
14	5	5.7	1:2631 = 87.7%	About 1.1 cc.	About 0.9 cc.
15***	7	5.6		About 0.5 cc.	About 1.2 cc.
16	7	5.6		About 0.8 cc.	About 0.8 cc.
17	6	5.6		About 0.8 cc.	About 0.8 cc.
18	8	5.6		About 0.9 cc.	About 0.8 cc.
19	10	5.6		About 1.1 cc.	About 1.2 cc.
20	11	5.7		About 0.5 cc.	About 1.5 cc.
21	12	5.7		About 0.5 cc.	About 0.9 cc.
22***	5	5.5		About 0.9 cc.	About 0.9 cc.
23	6	5.5		Exactly 1 cc.	About 0.9 cc.
24	7	5.4	1:2778 = 92%	Exactly 1 cc.	Exactly 1 cc.
25	8	5.4		About 1.1 cc.	Exactly 1 cc.
26	7	5.3		About 1.3 cc.	About 1.2 cc.

27**	8	5.3	About 1.4 cc.	About 1.2 cc.
28	6	5.2	About 1.2 cc.	About 1.4 cc.
29	7	5.2	About 1.2 cc.	About 1.2 cc.
30	8	5.0	About 1.5 cc.	About 1.5 cc.
31	9	5.0	About 1.1 cc.	About 1.5 cc.

* One minute stirring. ** Unclean albumen. *** Too frequent agitation. **** Too infrequent or irregular agitation.

In the table, the standard pharmacopœial pepsin (1:3000) is taken as 100% pepsin. These results obtained with the same sample of pepsin assayed on different days indicate the effect of the age of the eggs and show the difference in proteolytic power obtained as the analyst became more experienced in the use of the method.

An extended study of the U. S. P. method of assay for pepsin revealed the following causes of inaccuracy

1.—Variations in the age of the eggs used as a source of albumen. Graber (26) reported that albumen from eggs 5 to 7 days old was more easily digested than that of eggs of other ages.

2.—Variations in the moisture content of coagulated egg albumen. Vahlteich and Glover (27) have shown that egg albumen is not a suitable substrate since its moisture content is irregular.

3.—Variations in the particle size of the coagulated albumen, in the amount and vigor of stirring during the assay, and in the cleanliness of the albumen, all are conditions which may lead to erroneous results.

All of these variable factors combined with the necessity for the accurate maintenance of a constant temperature for $2^1/_2$ hours with agitation at 10-minute intervals make the present official assay procedure inaccurate and tedious.

Modified Sörensen Titration Method.—This method is based on the so-called formol titration procedure of Sörensen (18). It involves the proteolytic splitting of protein, blocking of the free amino groups with formaldehyde and titration of the liberated peptid carboxyl groups with standard alkali solution. The method is similar to that proposed by Willson (28) for the assay of pancreatin in that the proteolytic activity of the enzyme is given directly in terms of cc. of standard alkali.

The materials required are: (1) a 0.1% solution of pepsin freshly prepared by dissolving 0.1 Gm. of pepsin in sufficient distilled water to make 100 cc. immediately before using. (2) An approximately 0.1 per cent solution of casein (Hammersten's Casein as supplied by Merck) prepared as follows:

Place 1 Gm. of casein in a 1000-cc. volumetric flask, add 54 cc. of normal HCl and agitate the flask to distribute the casein and prevent lumping. Add 600 cc. of recently boiled distilled water and warm the mixture on a water-bath until the casein dissolves. After the casein has completely dissolved, add 300 cc. of recently boiled distilled water and adjust the solution to a p_H of 1.4. Add 3 cc. of chloroform as a preservative. The solution of casein is not suitable for use when a sediment has formed, usually after 2 or 3 weeks. Adjustment of the p_H may be performed easily and with sufficient accuracy by colormetric methods. (3) 100 cc. of a freshly prepared solution containing about 40 per cent of formaldehyde to which 1 cc. of a 1 per cent solution of phenolphthalein in 95 per cent alcohol has been added, and the whole rendered neutral by the addition of sodium hydroxide solution.

The assay is performed as follows:

Pipette 25 cc. of the casein solution into a 350-cc. Erlenmeyer flask. Stopper the flask and warm to 40° C. in a constant temperature bath. Add 25 cc. of the pepsin solution, stopper,

invert the flask once, and allow to digest at 40° C. in the constant temperature bath for 15 minutes. Remove the flask from the bath, add 25 cc. of the formaldehyde solution and rotate the contents of the flask for 2 minutes. Add 100 cc. of 0.02 *N* sodium hydroxide solution, mix well and titrate the excess alkali with 0.02 *N* hydrochloric acid. Conduct a blank test in the same manner. Subtract the number of cc. of 0.02 *N* HCl consumed in the actual test from the number of cc. used in the blank. The difference represents the number of cc. of 0.02 *N* alkali required to neutralize the acidity developed during proteolytic digestion. Divide this result by 3 and multiply by 100 to obtain the percentage strength of the pepsin; the figure 3 is the equivalent in terms of 0.02 *N* alkali of pepsin of 100 per cent activity. Since each 0.1 cc. of difference in the amount of alkali found in the blank test corresponds to 3.33 per cent of proteolytic activity, all volumetric measurements must be made with considerable accuracy.

Commercial samples of pepsin purchased from retail and wholesale drug houses and labeled "Pepsin U. S. P." were assayed in duplicate by the U. S. P. method and by the titration method with average results as follows:

Sample.	Proteolytic activity by U. S. P. X method.	Proteolytic activity by the titration method.
1.	150%	155%
2.	110%	116%
3.	115%	116%
4.	90%	92%
5.	150%	145%
6.	100%	102%

While the activity obtained by the U. S. P. method can hardly be measured except in terms of round numbers, the results indicate that the titration method is as accurate, if not more so, than that now official.

In order to determine the time of digestion necessary to develop maximum acidity by the titration method, samples of casein were digested with pepsin in the same manner as in the assay process for 15, 30, 60, 90 and 180 minutes, respectively. The results showed that there is no increase in acidity after fifteen minutes. Further tests showed that doubling of the amount of the substrate, casein, did not affect the results.

The effect produced by varying the amount of pepsin in the assay process was studied with results as follows:

Concentration of pepsin.	Acidity developed in 0.02 *N* alkali.
0.1 Gm. in 150 cc.	1.90
0.15 Gm. in 150 cc.	2.70
0.30 Gm. in 150 cc.	5.95
0.60 Gm. in 150 cc.	11.40

When the concentration of the pepsin solution is plotted against the acidity developed during the peptic digestion of casein, a straight line is formed showing that the acidity developed is directly proportional to the amount of pepsin used. It indicates also that the acidity developed is directly proportional to the proteolytic activity of the pepsin.

The Gross Method.—The Gross (10) Method is based upon digestion of casein with pepsin and determination of the total disappearance of the substrate by the addition of acetic acid or sodium acetate. Modifications of the method in which the amount of undigested casein are determined by turbidimetric methods have also been used. The method has been studied by Bümming (29), Adowa and Smorodinzew (30), Brandrup (31), and others with widely varying results.

In our study of this method, the end-point was found to be uncertain and difficult to determine. The results from a given assay could not be duplicated using the same materials. The method was, therefore, discarded as unreliable.

CONCLUSIONS.

1.—The assay process for pepsin official in the U. S. P. X is too long and tedious; the accuracy of the assay is influenced by numerous factors; and it requires that the analyst be experienced in order to secure concordant results.

2.—A titration method has been studied and found to be simpler, more rapid, and it is believed, more accurate than the U. S. P. method.

3.—The Gross Method has been found to be unreliable.

4.—It is recommended that the titration method be made official in the next revision of the U. S. P.

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"Pharmacy has never deviated from the ancient tradition of its purpose—to prepare and compound the medicaments which prevent, control, and cure disease and relieve suffering."—Dr. Howard W. Haggard.