SUMMARY AND CONCLUSIONS.

1. A proximate analysis of Ma Huang has been made.

2. A study of the assay methods of Ma Huang shows that there is much variation in the results obtained by using the different methods.

3. A new method of assay using barium hydroxide for liberating the alkaloids from the plant tissue has been developed and is recommended for the determination and isolation of the alkaloids.

4. Good species of Ma Huang should yield close to 2% total alkaloids.

5. A catechol tannin has been found to be present as one of the constituents of Ma Huang. This tannin may be catechol, protocatechuic acid or both.

6. A crystalline substance possessing glucosidal properties has been isolated.

7. A saponin has been isolated and determined as one of the constituents of Ma Huang.

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THE ASSAY OF PREPARATIONS CONTAINING PEPSIN OFFICIAL IN THE NATIONAL FORMULARY.*,[1]

BY GLENN L. JENKINS AND EDWARD M. HOSHALL.

INTRODUCTION.

The medicinal value of pharmaceutical preparations containing pepsin is commonly considered to be dependent upon the activity of this enzyme in the digestion of proteins. The present official method of assay of pepsin (1) based on the digestion of egg albumen has been shown to yield erroneous results due to numerous variable factors (2), (3). Methods have not been developed for the assay of preparations containing pepsin, consequently the only criterion of the quality of a

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preparation containing pepsin is that based on the activity of the pepsin employed in the compounding.

The results of a prescription survey by Gathercoal (4) indicate that the National Formulary preparations containing pepsin are extensively prescribed in modern pharmacy. It is considered highly desirable, therefore, to develop methods for the assay of these preparations with the intent that a satisfactory method might be made official in the next edition of the National Formulary. A positive result in this study would make possible the preparation of standardized preparations containing pepsin, and it would also make possible studies of methods for the stabilization of these preparations.

METHODS OF ASSAY.

Numerous methods have been proposed for the estimation of the proteolytic activity of pepsin. These methods may be grouped into three main classes, namely:

1. Methods based on determination of unaltered substrate.
2. Physical methods based on determination of cleavage products.
3. Chemical methods based on determination of cleavage products.

To the first group belong the methods of Ebstein and Grutzner (5), Mett (6), Volhard (7), Gross (8), Rona and Keinmann (9), Fuld and Levison (10), Rose (11), Jacoby-Selms (12) and Waldschmidt (13). This class contains the methods of historical significance; and while they permit relative measurements, they are not of sufficient accuracy to consider for this work.

The second group comprises the work of Fermi (14), Palitzsch and Walbum (15), Henri and Bancels (16), Abderhalden and Koelker (17), and Allen (18), Northrop (19) and Spriggs (20). With the exception of the method of Abderhalden and Koelker which is only applicable for use with optically active peptides, the others are too restricted in their applicability and not suited for a general method.

The remaining group contains the methods of Van Slyke (21), Sörensen (22), Willstätter and Waldschmidt-Leitz (23), Volhard (24), Foreman (25) and Jenkins and Greenberg (26). The methods were all reviewed but since that of Greenberg is by far the simplest and appears to be the most generally applicable, it was selected for a study of the determination of pepsin in National Formulary preparations.

EXPERIMENTAL PART.

In order to obtain a working knowledge of the method, four samples of commercial pepsin were obtained. Pepsin A had been repeatedly assayed by the U. S. P. X method and its proteolytic activity was determined to be 92 per cent. Pepsin B was assayed by the A. O. A. C. method (27), and using Pepsin A as standard, a proteolytic activity of 115 per cent was found for Pepsin B. Pepsin C and Pepsin D were unassayed, samples each from a different manufacturer.

The following pepsin containing preparations of the National Formulary 5th Edition (1926) were compounded: **Elixir Pepsini, Elixir Pepsini Bismuthi Et Strychninæ, Elixir Pepsini Compositum, Elixir Pepsini Et Bismuthi, Elixir Pepsini Et Rennini Compositum, Liquor Pepsini, Liquor Pepsini Antisepticus, Liquor Pepsini Aromaticus, Glyceritum Pepsini, Pepsinium Saccharatum.**

The methods used in their preparation were essentially the same as specified in the National Formulary, except that all weighings were made on the analytical balance, volumes were made at 20° C. in volumetric apparatus, and little or no agitation was used to effect solution of the pepsin. The solutions were carefully filtered (covered to prevent evaporation) into four-

ounce, narrow-mouth, amber bottles, stoppered and sealed with wax, and kept at 15° C. except when a sample was withdrawn. In all cases, the maximum amount of pepsin specified by the National Formulary was used. The pepsin used in all the preparations was Pepsin A.

The method of Greenberg was followed with one modification, namely, 20 cc. of 0.1*N* NaOH was added instead of 100 cc. of 0.02*N* NaOH; this reduced the total volume and made the titration more accurate. The method as modified was:

"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 20 cc. of 0.1*N* NaOH, mix well and titrate the excess of alkali with 0.02*N* HCl. 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 the result by 3 and multiply by 100 to obtain the percentage strength of the pepsin; the figure 3 is 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."

Using the modified method, six duplicate determinations were made on Pepsin A and Pepsin B, with the following results:

TABLE I.—PROTEOLYTIC ACTIVITY.

Pepsin A.			Pepsin B.	
1.	2.		1.	2.
97.3	100.0		96.7	98.3
100.3	108.0		106.3	109.3
100.0	101.7		103.3	108.3
105.0	...		108.5	109.7
98.3	103.3			
102		Average	105	

The checks on duplicate determinations are within experimental error, but a 12 per cent maximum deviation on determinations leaves much to be desired. The proteolytic activity of the pepsin samples as determined by the U. S. P. X method check the proteolytic activity as here determined by 10 per cent.

The method of assay was applied to the following pepsin preparations as tabulated below. In each case the preparations were taken from storage at 15° C. and when at 20° C. a volume was removed, such that when diluted, 25 cc. of the solution contained 0.025 Gm. of Pepsin A. The proteolytic activity was calculated the same as in the original method, namely, that the number of cc. difference of 0.02*N* HCl between the sample and the blank determination, was multiplied by 33.33, which expressed the proteolytic activity in per cent. The following results were obtained:

TABLE II.

Preparation.	Proteolytic Activity in Per Cent. 1.	2.	3.	4.	Ave.
Glyceritum Pepsini	47.6	56.6	50.3	54.3	52.2
Pepsinum Saccharatum	105.3	104.7	101.0	102.7	103.4
Elixir Pepsini	67.3	66.3	...	...	66.8
Liquor Pepsini Aromaticus	68.0	69.0	...	...	68.5
Liquor Pepsini Antisepticus	67.7	67.0	...	...	67.4
Liquor Pepsini	100.3	103.0	...	...	101.7
Liquor Pepsini Et Bismuthi	142.7	...	...	...	142.7
Liquor Pepsini Et Strychninæ	139.7	147.7	...	...	143.7

The result of this series of determinations shows that except in the case of **Pepsinum Saccharatum** and **Liquor Pepsini** the method is not applicable to the assay of these preparations.

Thus only in two cases out of eight does the amount of pepsin added compare favorable with that found. The other six samples vary from 40 per cent to 152 per cent. The causes for these variations were considered to be as follows:

1. With one exception (**Pepsinum Saccharatum**), diluted hydrochloric acid is used in making up the preparations, and the acidity of the sample is not accounted for in the assay. For example, it can be calculated that for Elixir Pepsini this error would be equivalent to a proteolytic activity of 16 per cent. In other preparations the error would be still greater.
2. Essential oils and other flavoring materials are employed to make the preparations. If there were a reaction of these materials with any reagents used, due to the fact that no blank is run on the sample, this would be unaccounted for.
3. There may be a reaction between the formaldehyde and the pepsin.
4. The p_H of the digestion mixture may be a factor. Although the p_H of the substrate, casein, is prepared at p_H 1.4, the added pepsin containing solutions of various acidities will change this p_H.
5. The difficultly determined end-point.

It was then decided to temporarily discontinue work on National Formulary preparations and using samples of commercial pepsins, to evolve a method where variations due to the "reagent blank" might be accounted for, and to subject the method to further study in the light of the following factors:

1. The acidity of the aqueous solution of pepsin,
2. The reaction between pepsin and formaldehyde,
3. The effect of p_H on degree of proteolytic activity, and
4. The difficultly determined end-point.

1. Acidity of the Aqueous Solution of Pepsin.—The four commercial samples of "U. S. P. Quality" Pepsin as previously described, were used as representative. Solutions of 0.25 per cent were prepared in pure distilled water and a 10-cc. aliquot titrated with 0.02*N* NaOH, using two drops of 1 per cent phenolphthalein in neutral alcohol as indicator, to a faint pink.

TABLE III.

Pepsin.	Concentration.	No. Cc.	Cc. 0.02*N* NaOH.	
A	0.25	10.0	1.20	1.25
B	0.25	10.0	0.47	0.46
C	0.25	10.0	2.75	2.80
D	0.25	10.0	0.90	0.85

On the basis of the factor used in the Greenberg assay, namely, 3.00 cc. of 0.02*N* acid equivalent to 100 per cent proteolytic activity, the results here indicate that due to the acidity of the sample an apparent proteolytic activity ranging from 15 per cent to 93 per cent could be obtained. No account of this factor was taken in the original assay, obviously it must be accounted for, either by another titration or by its inclusion in the "reagent blank."

2. The Reaction between Pepsin and Formaldehyde.—Three commercial samples of pepsin (Pepsin A, C and D) were used. A 0.25 per cent solution, in pure distilled water of each sample, was prepared and a 10-cc. aliquot was titrated with 0.02*N* NaOH, using phenolphthalein (1 per cent in neutral alcohol), to a faint pink color. To this was added 10 cc. of a 40 per cent solution of "Reagent Quality" formaldehyde, which had been previously rendered neutral with sodium hydroxide, using phenolphthalein as indicator. Upon the addition of the formaldehyde the solution became acid: the acidity then being determined by titration with 0.02*N* NaOH, using phenolphthalein, to a faint pink color.

TABLE IV.

Pepsin.	Concentration Per Cent.	No. Cc. Pepsin (Neutral).	No. Cc. Formaldehyde (Neutral).	No. Cc. 0.02*N* NaOH (to Neutralize).	
A	0.25	10.0	10.0	1.81	1.80
C	0.25	10.0	10.0	2.65	2.60
D	0.25	10.0	10.0	2.20	2.24

Table IV shows that there is a definite reaction between the pepsin in aqueous solution and the formaldehyde.

To determine if this reaction was proportional to the amount of pepsin present, varying amounts of pepsin were treated with neutral formaldehyde.

TABLE V.

Pepsin.	Concentration Per Cent.	No. Cc. Pepsin (Neutral).	No. Cc. Formaldehyde (Neutral).	No. Cc. 0.02*N* NaOH (to Neutralize).
A	0.25	10.0	10.0	1.80
A	0.25	20.0	10.0	3.55
A	0.25	30.00	10.00	5.67
A	0.25	40.00	10.0	7.32

Table V indicates that the reaction is directly proportional to the amount of pepsin present.

The amount of pepsin was then held constant and the amount of formaldehyde varied, with the following results:

TABLE VI.

Pepsin.	Concentration Per Cent.	No. Cc. Pepsin (Neutral).	No. Cc. Formaldehyde (Neutral).	No. Cc. 0.02*N* NaOH (to Neutralize).
C	0.25	10.0	1.0	2.15
C	0.25	10.0	2.0	2.35
C	0.25	10.0	5.0	2.55
C	0.25	10.0	10.0	2.65
C	0.25	10.0	20.0	2.64

It appears from Table VI, that, within limits, the amount of formaldehyde is not a factor in this reaction.

A definite reaction, therefore, proportional to the amount of pepsin present, takes place when formaldehyde is added to aqueous solutions of pepsin. The blank accordingly, of the Greenberg Assay, should take care of this reaction or else a separate titration and correction should be applied to the existent method. In view of the lack of positive knowledge concerning the structure of pepsin, Hammarsten (28), the cause of this reaction can only be conjectured, Sherman (29) and Johannessohn (30) indicate that formaldehyde in concentrations from 5 to 10 per cent does not inhibit the proteolytic activity of pepsin.

3. The Effect of Hydrogen-Ion Concentration on the Proteolytic Activity of Pepsin.—The lack of a satisfactory quantitative method precludes experimental work, but a survey of the literature relative to the proteolytic action of the enzyme pepsin on casein, notably the work of Northrop (31) and Grant (32), indicates that the optimum proteolytic activity is approximately at p_H 1.8 to p_H 2.0. In the following experimental work this acidity is maintained within those limits, rather than at p_H 1.4 as was the case in the Greenberg Assay.

4. The Difficultly Determined End-Point.—The determination of the end-point is effected by titration of the digestion mixture, the formaldehyde and the excess alkali (a total volume of some 200 cc.) with 0.02*N* acid. A check to within 0.30 cc. (5 drops) is difficult, save only to the analyst who has performed the titration many times. Jodidi (33) comments upon the unsatisfactory end-point in a like titration. It was found by experiment that slight modifications in the procedure gave a better end-point, namely: 1. It is possible to use 20 cc. of 0.1*N* NaOH in place of 100 cc. of 0.02 NaOH, with a decrease of volume and an increase of accuracy. 2. The pepsin solution may be made more concentrated, 10 cc. of a 0.25 per cent solution being used instead of 25 cc. of a 0.10 per cent solution. 3. The amount of formaldehyde was reduced from 25 cc. to 15 cc. as the latter amount was found to be sufficient. 4. By the use of a blue light to titrate, the red tinge due to the phenolphthalein could be more closely followed, and more accurate results obtained.

DISCUSSION OF RESULTS.

The application of the Sörenson formal titration method as modified by Greenberg, to the assay of pepsin containing preparations official in the National Formulary, was unsuccessful. The method was considered unsuited for the following reasons:

1. In an effort to find the source of difficulty, commercial samples of pure pepsin were assayed by the Greenberg Method. The results for two samples were in fair agreement with those obtained by Mr. Greenberg, but when other samples were analyzed by the same method, serious discrepancies were observed, and it was indicated that the results obtained were not in proportion to the proteolytic activity.

2. Several samples of pepsin tested showed definite and different acidities in aqueous solution. Because of the fact that a blank was not run on the pepsin solution, this initial acidity was entirely unaccounted for. Thus a pepsin with a high acidity would assay a high proteolytic activity.

3. For all samples of pepsin examined, there was a definite reaction with formaldehyde and different amounts of acid were liberated, which affected the results of the assay.

4. The consensus of recent work with the enzymic action of pepsin on casein indicate that p_H 2.0 is the optimum hydrogen-ion concentration rather than p_H 1.4 as used.

5. The determination of the end-point in the method was difficult, and the modifications suggested and used, although rendering the titration more accurate still leave much to be desired.

6. The formaldehyde was a source of apparent error, commercial samples being difficult to neutralize, turning brown and quickly developing acidity on exposure to air.

7. Another point is suggested when we find the ratio of pepsin to casein as 1:1, while pepsin will digest 3000 times its weight of egg albumen.

FOREMAN'S METHOD.

Foreman (25) proposed a method for the assay of pepsin based on the proteolytic splitting of protein and titration of the liberated carboxy groups in 80 to 90 per cent ethyl alcohol solution, where the free amino groups form no compounds with the phenolphthalein used as indicator, and consequently the acid alone can be titrated with standard alkali in alcoholic solution. The improvements on the orignal method by Willstätter and Waldschmidt-Leitz (23) indicate a method that is generally applicable for most proteolytic degradation products. The method was slightly modified, and the following procedure was adopted in order to determine the proteolytic activity of samples of pepsin previously assayed by the U. S. P. X method.

Reagents:

1. A 0.25 per cent solution of pepsin, freshly prepared, in pure distilled water.

2. Casein solution, prepared as follows: to 45 cc. of 0.5N HCl in a 250-cc. volumetric flask, add 5 Gm. of Hammarsten's Casein (Merck), and 150 cc. of distilled water. Heat on a water-bath until a clear solution is obtained. Remove, cool and fill to mark. Add 5 cc. of xylene as preservative.

3. Phenolphthalein indicator solution. A 1 per cent solution of phenolphthalein in neutral alcohol.

4. Alcoholic 0.02N HCl.

5. Alcoholic 0.1N NaOH.

Procedure:

Measure 10 cc. of the casein solution into a 250-cc. Erlenmeyer flask, warm to 40° C. then add 10 cc. of the pepsin solution, shake with a swirling motion and allow to remain in the bath 20 minutes. Remove, cool to room temperature, add 100 cc. of ethyl alcohol, 10 cc. of 0.1N NaOH and 2 cc. of indicator solution. Titrate to the disappearance of the pink color.

The blank is determined as follows: To 10 cc. of the casein solution add 100 cc. of alcohol, 10 cc. of 0.1 alcoholic NaOH, 10 cc. of pepsin solution and 2 cc. of indicator. Titrate to the same end-point as above.

The difference in the number of cc. of 0.02N alcoholic HCl required between the blank and the sample, indicate the apparent proteolytic activity.

Several determinations were carried out by this method. The temperature was maintained at 40° ± 2° C. The casein solution as prepared was of p_H 1.8 and when the sample was added the p_H was 1.85 to 1.90. The time of digestion was 20 minutes. For the same sample of pepsin (Pepsin A), the following results were obtained, expressed in cc. of 0.02N alcoholic HCl difference between the blank titration and the titration of the digested protein.

TABLE VII.

No. of Run.	Cc. 0.02*N* HCl (Difference).	Cc. 0.02*N* HCl (Difference).	Casein Solution.
1	4.06	4.18	A 2.0 Gm./100 cc.
2	0.32	0.40	B 2.0 Gm./100 cc.
3	4.26	3.80	C 2.0 Gm./100 cc.
4	3.60	3.96	C 2.0 Gm./100 cc.
4	3.95	3.78	C 2.0 Gm./100 cc.
Average	3.95		

With the exception of Run No. 2, the results were fairly reproducible. The checks on duplicate determinations are poor and the maximum deviation from the average is about 9 per cent.

To test the applicability of the method to National Formulary pepsin containing preparations, samples of Elixir Pepsini, Pepsinium Saccharatum and Glycertium Pepsini (see page No. 3), were removed from storage (they were four months old), and a quantity diluted so that 10 cc. contained the equivalent of 0.025 Gm. of Pepsin A, the same amount of pepsin as used in the above runs. The results are as follows:

TABLE VIII.

Preparation.	Cc. 0.02*N* HCl (Difference).	Cc. 0.02*N* HCl (Difference).	Casein Solution.
Pepsinium Saccharatum	3.68	3.78	D 2.0 Gm./100 cc.
Elixir Pepsinium	3.46	..	D 2.0 Gm./100 cc.
Glyceritum Pepsinium	3.42	3.33	D 2.0 Gm./100 cc.

The acidity developed for the above preparations compared with like amounts of Pepsin A is lower, as would be expected due to decrease of proteolytic activity resulting from agitation in preparation and storage. The question then arises as to whether the acidity developed is directly proportional to the amount of pepsin present. Varying amounts of pepsin were then used to digest the casein, following the same method as before.

TABLE IX.

Sample.		Cc. 0.02*N* HCl (Difference).	Cc. 0.02*N* HCl (Difference).	Casein.
10 cc. (heated to 100)		0.21	..	2.0 Gm./100 cc.
5 cc.	0.0125 Gm.	2.75	2.45	(New lot which
10 cc.	0.0250 Gm.	3.10	3.06	dissolved with
15 cc.	0.0375 Gm.	4.39		difficulty.)
20 cc.	0.0500 Gm.	4.78		

By inspection of the above results it is seen that when the concentration of the pepsin is varied the acidity developed is not directly proportional to the amount of enzyme present. A partial explanation is that the substrate, casein, must be increased if proportional results are to be obtained. Furthermore, according to Volhard and Löhlein (34) casein combines with a definite amount of HCl, and if the casein is digested by the enzyme, the combined acid is liberated. Thus in any case of casein digestion by pepsin, there will be two sources of acidity developed. 1. Acidity due to the proteolytic splitting of the protein which is titratable if the amino groups are "blocked off" with either formaldehyde, acetone or alcohol. 2. Acidity developed by digestion of the casein combined with HCl, with the subsequent liberation of the HCl.

The method was considered unsuitable, for the following reasons:

1. A 2 per cent solution of casein is difficult to prepare and to preserve. Different lots of Hammarsten's Casein (Merck) were found to have different solubilities. Regarding the preservation, Treyer (35) and Price (36) show that chloroform injures all enzymes to a varying extent, and they further find that ether and alcohol have very little effect while toluol has even less effect on enzymes. Toluol was first used, then xylene was tried, and found to be appreciably better as a preservative.

2. Titration with 0.02*N* alcoholic HCl in a volume of 125 cc. using phenolphthalein, is difficult, and checks within 0.30 cc. are unusual, and obtained only with practice.

3. The acidity developed is not in direct proportion to the amount of pepsin present.

4. Results with identical solutions of casein were not reproducible within 10 per cent, and even greater variations were found with the use of different batches of casein.

5. The titrated acidity in any of the methods whereby the amino group is blocked off, is not due to the presence of the remaining acid group alone, but also another factor: the breakdown of an addition product of casein with HCl, which liberates HCl that is titrated along with the proteolytically developed acidity.

6. This method was discarded.

ACETONE TITRATION METHOD.

Although it has been indicated that in any method where casein is digested, and the amino groups "blocked off," the results are not directly proportional to the amount of pepsin present, and are of doubtful value, it was decided to investigate the method recommended by Waldschmidt (37). According to K. Linderström-Lang (38) the proteolytic mixture is titrated with 0.1*N* HCl in alcoholic solution, after adding acetone from 85–95 per cent concentration. This method will effectively block off most amino groups. With some slight modifications the method was essentially the same as that of Forman. The procedure is as follows:

Add 10 cc. of the casein solution to a 250-cc. Erlenmeyer flask, heat to 40° C. and then add 10 cc. of pepsin solution shaking with a swirling motion; allow to remain on bath 20 minutes, remove, cool to room temperature and add 100 cc. of acetone, 10 cc. of 0.1*N* alcoholic NaOH and 2 cc. of indicator solution. Titrate with 0.02*N* alcoholic HCl to a faint pink.

The blank is determined as follows: To 10 cc. of the casein solution add 100 cc. of acetone, 10 cc. of 0.01*N* alcoholic NaOH, 10 cc. of the pepsin solution and 2 cc. of the indicator solution. Titrate to the same end-point as above.

The difference in the number of cc. of 0.02*N* alcoholic HCl required, is a measure of the apparent proteolytic activity.

Three determinations were made by the above method, and then the concentration of the pepsin was varied. The results are given:

TABLE X.

Sample (Pepsin A).	Cc. 0.02*N* HCl (Difference).	Casein.
10 cc.	3.41	2.0 Gm./100 cc.
10 cc.	3.90	(casein was
10 cc.	3.60	difficultly
5 cc.	2.76	soluble)
10 cc.	3.60	
15 cc.	4.39	
20 cc.	4.78	

The results show that close checks on identical samples were not obtained, and that the developed acidity was not directly proportional to the pepsin present. The end-point of the titration was much more accurate than when the alcohol was used to "block-off" the amino groups. The casein itself is a source of trouble, since it is difficultly soluble, does not form a clear solution and ultimately settles out. To offset this, the use of solid casein instead of the solution was next investigated. The method was the same except that 0.5 Gm. of Hammarsten's Casein (Merck) was used, and 10 cc. of 0.1*N* HCl added, giving a p_H of 1.9.

TABLE XI.

Sample.		Cc. 0.02*N* HCl (Difference).	Casein.
10 cc. (heated to 100)		0.10	0.5 Gm.,
2 cc.	0.010 Gm.	10.11	(Hammarsten's)
5 cc.	0.025 Gm.	13.54	
10 cc.	0.050 Gm.	13.71	
20 cc.	0.100 Gm.	14.80	

Under the new conditions, increasing both the concentration of pepsin and casein, it was found that increased acidity was the result. As before, the acidity is not satisfactorily proportional to the amount of pepsin. The increase in the developed acidity may be explained by increased enzyme concentration and also increased casein, the latter contributing acidity from the breakdown of its HCl addition product, as previously explained. The following conclusions are given for this method:

1. Acetone is more satisfactory as a "blocking-off" medium than alcohol, since the former allows of a more accurately determined end-point.
2. The acidity developed is not in direct proportion to the amount of pepsin present.
3. The use of solid casein is recommened for digestion, and in greater concentrations than hitherto used.
4. The method has possibilities as an empirical method, but an accuracy within 10 per cent would be difficult to obtain.
5. This method was discarded.

VOLHARD'S METHOD.

Volhard and Löhlein (34) suggest a method based upon the fact that casein combined with a definite amount of HCl, and if the casein has been digested by the enzyme, the combined HCl is liberated. The increase in the amount of free HCl compared with that of the original solution, as determined by titration, serves as a measure of enzyme activity. The acidity of the pepsin solution is determined and added to that of the original solution. The method is as follows:

"A 5 per cent casein solution is prepared by suspending the casein in a small amount of water, dissolving in NaOH solution (8 cc. of N NaOH per 10 Gm. of casein), making up to volume with water and warming to 90° C. Then 150 cc. of $0.7N$ HCl and 100 cc. of the casein solution are mixed, the mixture is warmed to 40° C. and the proper amount of pepsin solution is added, and the mixture is diluted to 300 cc. with water warmed to 40° C. At the end of the incubation period (24 hours usually), 100 cc. of 20 per cent sodium sulphate is added, thus precipitating the undigested casein. The solution is filtered and 100 cc. of the filtrate is titrated with $0.1N$ NaOH using phenolphthalein as indicator. The difference between the titration value of the digest and that of the original solution, and of the pepsin solution indicates the increase in acidity of the digest. When the increase in acidity is denoted by v, and the time of digestion in hours by t, the quantity of enzyme used by f, and x the actual pepsin concentration, then according to the Schütz-Borrissov Equation (39),

$$x = (f \times t)^2/v^2$$

A consideration of the above method from a practical standpoint will show that it is too involved and lengthy to be readily applicable. Some points of criticism are as follows:

1. A third titration is necessary to account for the acidity of the pepsin solution. 2. The casein solution is difficult to prepare and to preserve. 3. The acidity of a solution prepared as above, and after the pepsin had been added was found to be p_H 2.4 which is well above the optimum p_H for casein digestion by pepsin. 4. The time, 24 hours, must be reduced, if a practical rapid method is desired. 5. The volumes are too large, making for bulky apparatus, and requiring a large working space.

A tentative method was devised as follows:

Reagents:

Casein "Technical Grade."

Pepsin Solution (freshly made 0.1% in distilled water), or a preparation adjusted by dilution to contain 0.010 Gm. of pepsin in 10 cc.

Sodium sulphate, 20% on distilled water.

Phenolphthalein Indicator, 1% in neutral alcohol.

$0.1N$ Hydrochloric acid.

$0.5N$ Hydrochloric acid.

$0.5N$ Sodium hydroxide.

Method:

Into a 250-cc. Erlenmeyer flask introduce 3 Gm. of powdered casein, 50 cc. of distilled water and 10 cc. of $0.5N$ hydrochloric acid. Heat the flask to 90° C.

on the water-bath and then cool to 40° C. Then add the sample of pepsin and sufficient water from a burette to make the total volume 80 cc. Maintain the mixture at 40° C. exactly thirty minutes, with occasional shaking. The flask should be lightly stoppered. Remove from the bath, add exactly 20 cc. of the sodium sulphate solution, shake by swirling and filter using a folded filter. To 50 cc. of the filtrate (one-half the original volume), add from a burette sufficient 0.5*N* sodium hydroxide to have about 2 cc. excess and back titrate the alkali with 0.1*N* hydrochloric acid using phenolphthalein indicator. Titrate rapidly to the first colorless end-point.

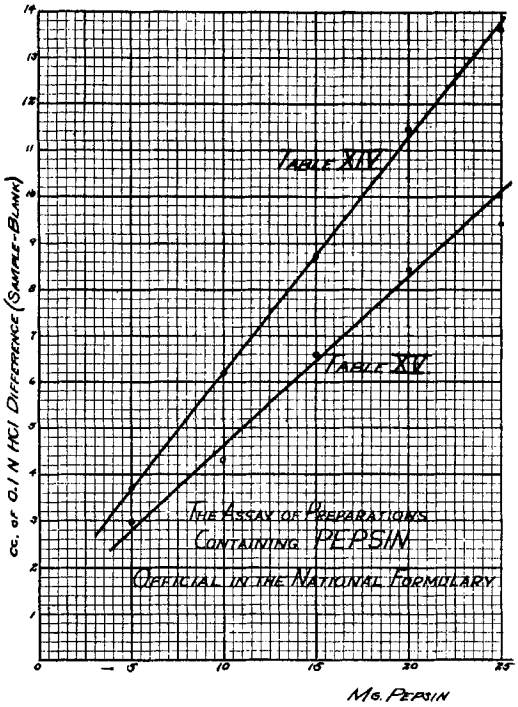

Fig. 1.—See Tables XIV and XV.

A blank is run simultaneously as follows: Into a 250-cc. Erlenmeyer flask introduce 3 Gm. of casein, 50 cc. of distilled water and 10 cc. of 0.5*N* hydrochloric acid. Stopper lightly, heat to 90° C. on a water-bath and cool to 40° C., then add sufficient water from a burette to make the total volume 80 cc. Maintain the mixture at a temperature of 40° C. for thirty minutes. Remove from the bath and add 20 cc. of sodium sulphate solution, shake by swirling and filter. To 50 cc. of the filtrate (one-half the original volume), add about 2 cc. excess of 0.5*N* sodium hydroxide, and one-half the volume of pepsin solution used in the determination. (This is necessary in order to correct for the acidity of the pepsin solution.) Back titrate with 0.1*N* hydrochloric acid to the same end-point as in the determination.

Calculation: The difference between the titration of the sample determination and the blank determination expressed in cc. of 0.1*N* hydrochloric acid may be taken as an expression of the relative proteolytic activity of the product being assayed.

The standard is calculated from a series of determinations using pepsin of known proteolytic activity (standardized by U. S. P. X Method), and plotting a graph showing the relationship between the amount of pepsin used and the cc. of 0.1*N* hydrochloric acid difference in titration between the blank and the sample.

Example: Standard will be Pepsin X, with a proteolytic activity of 100% (U. S. P. X). By the above method:

0.005		3.68
0.010	Gm. pepsin	6.20 cc. 0.1*N* HCl (difference in titration)
0.015		8.74

Sample made into solution of 0.01 Gm. of pepsin (based on declaration) per 10 cc. By above method 0.010 Gm. gave a difference in titration of 5.84 cc.

By interpolation we find that the sample will have a proteolytic activity of 93%.

$$\frac{6.20}{2.52} \quad \frac{5.84}{2.16} \quad \frac{3.68}{2.52} \times (0.010-0.005) = 0.00428$$

$0.005 + 0.00428 = 0.00928$ Gm. Standard $= 0.010$ Gm. $\frac{0.00928}{0.010} \times 100 = 92.8\%$

Using varying amounts of Pepsin A, determinations were made by the above method with the following results:

TABLE XII.

Sample of Pepsin.		Cc. 0.1*N* HCl (Difference).	Comments.
2 cc.	0.002 Gm.	3.40	Casein 5 Gm.
5 cc.	0.005 Gm.	4.08	p_H of mixture 1.80
10 cc.	0.010 Gm.	5.00	Time, 30 min.
15 cc.	0.015 Gm.	8.20	Pepsin A
20 cc.	0.020 Gm.	10.20	

The method appears to be promising. The end-point, however, is uncertain, due to the rapid return of the red color of Phenolphthalein, after the first colorless end-point is reached. This can be rectified by titrating rapidly. In spite of the inaccuracies of the end-points a straight line relationship is indicated within the limits (0.005 to 0.200 Gm. pepsin).

A second series of determinations by this method gave results as follows:

TABLE XIII.

Sample.		Cc. 0.1*N* HCl (Difference).	Comments.
10 cc. (inactive)*		0.14	Casein 5.0 Gm.
5 cc.	0.005 Gm.	2.30	p_H 1.80
10 cc.	0.010 Gm.	3.98	Time, 30 min.
15 cc.	0.015 Gm.	7.70	Pepsin A
20 cc.	0.020 Gm.	8.92	
25 cc.	0.025 Gm.	12.88	
50 cc.	0.050 Gm.	18.36	

* Sample of pepsin inactivated by heating to boiling.

The straight line relationship as found in the first series of determinations (Table XII) was confirmed, and with fair agreement, except for samples of pepsin containing less than 0.010 Gm. The end-points were still a source of considerable difficulty, and checks were difficult.

For a different sample of pepsin the straight line relationship was again determined between the limits of 0.005 and 0.025 Gm. of pepsin.

TABLE XIV.

Sample.		Cc. 0.1*N* HCl (Difference).	Comments.
10 cc. (inactive)*		0.03	Casein 3.0 Gm.
5 cc.	0.005 Gm.	3.68	p_H 1.85
10 cc.	0.010 Gm.	6.20	Time, 30 min.
15 cc.	0.015 Gm.	8.74	Pepsin B
20 cc.	0.020 Gm.	11.44	
25 cc.	0.025 Gm.	13.62	
50 cc.	0.050 Gm.	14.84	
100 cc.	0.100 Gm.	15.84	

* Sample of pepsin inactivated by boiling.

In these determinations the amount of casein used was decreased to three Gm., the result being that the end-points were more readily determined.

The next determination was carried out with the reduced amount of casein, and the results were as follows:

TABLE XV.

Sample.		Cc. 0.01*N* HCl (Difference).	Comments.
5 cc.	0.005 Gm.	2.96	Casein 3.0 Gm.
10 cc.	0.010 Gm.	4.32	p_H 1.85

15 cc.	0.015 Gm.	6.64	Time, 30 min.
20 cc.	0.020 Gm.	8.46	Pepsin A
25 cc.	0.025 Gm.	9.40	

Since Pepsin A is the reference standard, by means of the Schütz-Borrissov Rule, the proteolytic value of Pepsin B may be calculated. The values obtained in Tables XIV and XV are plotted and the straight lines drawn. Values obtained from the graph are given in the following table, and also by application of the equation the proteolytic value of Pepsin B was determined.

TABLE XVI.

Pepsin A.		Pepsin B.		
	Proteolytic Activity		Proteolytic Activity.	
Gm.	(Standard).	Gm.	From Curve.	By Equation.
0.005	92%	0.005	134	198
0.010	92%	0.010	128	177
0.015	92%	0.015	125	169
0.020	92%	0.020	123	165

The proteolytic activity of Pepsin B having been determined to be about 115 per cent, it would appear that the Schütz-Borrissov Equation is not applicable when the time of digestion is short in fact, when using the equation the time is always taken as 24 hours. The values obtained from a comparison of the straight line relationships agree within 10 per cent.

Following the same procedure as in the preceding determinations, several samples of Pepsin A and Pepsin B of 0.020 Gm. each were assayed with the following results.

TABLE XVII.

Pepsin A.		Pepsin B.	
Cc. 0.1*N* HCl (Difference).	Proteolytic Activity (Standard).	Cc. 0.1*N* HCl (Difference).	Proteolytic Activity (Comparison).
8.78	92%	10.36	
8.26	92%	10.96	$(10.88/8.51) \times 92 = 118\%$
8.44	92%	11.44	
8.24	92%	10.74	
8.40	92%		
8.92	92%	Ave. 10.88	
Ave. 8.51			

A series of determinations, all checking within 10 per cent, indicate a proteolytic activity of 118 per cent for Pepsin B compared with the 115 per cent experimentally determined.

National Formulary Preparations were next assayed by the same procedure. The preparations were removed from storage (at 15° C.) warmed to 20° C. and a portion taken, such that when diluted with pure distilled water, 20 cc. would contain 0.020 Gm. of Pepsin A.

TABLE XVIII.

	Cc. 0.1*N* HCl (Difference).		
Preparation.	I.	II.	Proteolytic Activity (Comparison).
Pepsinium Saccharatum	8.30	8.56	88
Elixir Pepsini	5.72	6.16	62
Liquor Pepsini	7.58	7.28	82
Liquor Pepsini Aromaticus	7.32	6.78	79
Glyceritum Pepsini	7.84	6.82	85
Elixir Pepsini et Bismuthi	8.46		91
Elixir Pepsini et Rennini Comp.	9.80		106
Elixir Pepsini Bismuthi et Strychninæ	7.56		82
Pepsin A (ave. of 8 determinations)	8.51		92 (by exp.)

Most of the preparations show a decreased proteolytic activity due to agitation and storage. (They were 6 months old.) Pepsinium Saccharatum gives an average of 91 per cent which

checks well with 92 per cent for the Pepsin A. Elixir Pepsini et Bismuthi and also Elixir Pepsini et Rennini Comp. were but a month old which would account for the higher values obtained.

This method as developed, appears to be applicable to pepsin and pepsin containing preparations of the National Formulary. It is as accurate as the egg-albumen digestion method, when applied to pepsin itself, and checks within 10 per cent are obtained with pepsin preparations. The method is simple, and a complete assay can be performed in about an hour. The greatest difficulty is an accurate determination of the end-point of the titration. It is believed that this can be solved by further study, which is contemplated.

CONCLUSIONS.

1. Pepsin containing preparations of The National Formulary are extensively prescribed in modern pharmacy.
2. The Assay of Pepsin as proposed by Greenberg is not practicable for the assay of pepsin containing preparations.
3. Assay methods based on proteolytic digestion of casein and subsequent blocking-off of the amino group, are inapplicable to pepsin or pepsin containing preparations.
4. The Schütz-Borrissov Rule for proteolytic digestion of casein is inaccurate when the time of digestion is short.
5. A modified Volhard's Method has been developed which will permit the measurement of proteolytic activity in pepsin or pepsin containing preparations, within 10 per cent or less.
6. The method developed is rapid and simple, except for the end-point of the titration.

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THE PHARMACOLOGICAL ACTION OF PEIMINE AND PEIMININE.*

BY K. K. CHEN, A. LING CHEN AND T. Q. CHOU.

The crude drug, Pei Mu, has been used in Chinese medicine as an antipyretic, cough sedative, expectorant and lactagogue (1). In combination with other ingredients, it has been advocated for the treatment of difficult labor, retention of placenta, blurring of vision and spider and snake bites.

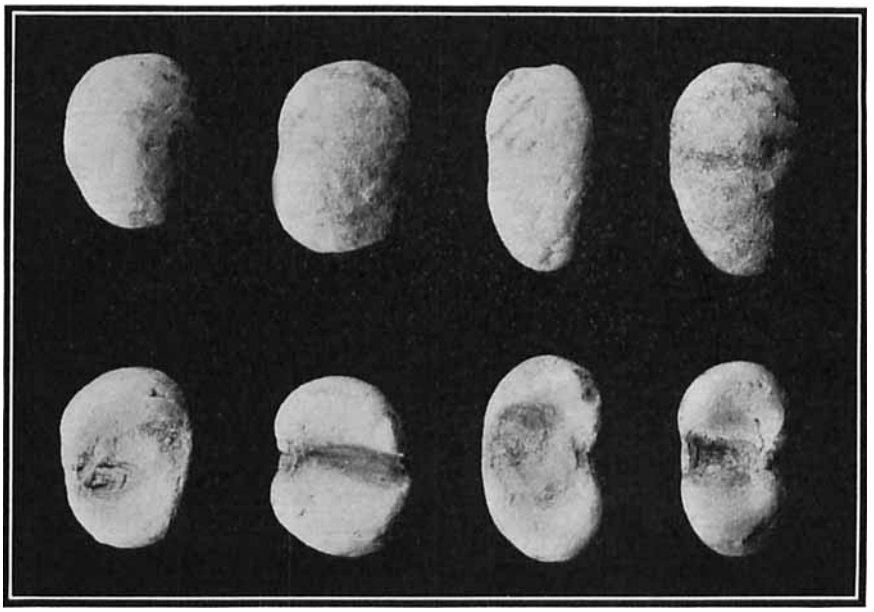

Fig. 1.—Pei Mu from Chekiang Province.

Pei Mu is made of the bulbs or corms of a liliaceous plant which is identified as *Fritillaria roylei* by Stuart (2), but as *F. verticillata*, Willd. *var. Thunbergii*, Bak. in Botanical Nomenclature (3). The corms produced in Chekiang Province are kidney-shaped, as shown in Fig. 1, each weighing on the average 3.5 Gm., and

* From the Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, and the Institute of Materia Medica, National Academy of Peiping, and the Sino-French University, Peiping, China.