NOTES ON PEPSIN PREPARATIONS.*

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This work on Pepsin solutions is largely a continuation of the investigations carried out in the same laboratory during the past two or three years.[1]

Some interesting observations recently have been made upon the rate of deterioration of various preparations containing pepsin. These observations have been extended, assays being repeated so that the curves show the deterioration of pepsin in the solutions over a period of three years. The results are fully shown on Graph Table No. 1. Preparations in which the pepsin strength had already fallen off so much that it was practically negligible were not assayed. In all cases the method of estimation was that of the U. S. P. IX with the slight modifications and use of controls of a known pepsin as described in a previous report.[1] The known pepsin used was one whose value had been determined by running about eighty assays upon it following closely the directions as given in the U. S. P. IX.

A number of preparations previously made up using different amounts of acid from those called for in the official methods of manufacture had shown some apparent relations between the acidity and rate of deterioration. These preparations were therefore assayed twice during the past year and their deterioration curves over the entire period are shown on Graph Table No. 2.

In view of the fact that these preparations of different acid strengths show deterioration which seems to be influenced by the amount of acid present, it was decided to study this possibility more closely. A preparation of pepsin was chosen which previous investigations had shown to deteriorate very rapidly.

Six samples of Liquor Pepsini N. F. IV were made up with varying amounts of acid according to the following formulæ:

LIQUOR PEPSINI.

		Deterioration after 5 months.
No. 1.		2575
Glycerite of pepsin	25 cc.	
Hydrochloric acid	5 cc.	
Glycerin	157.5 cc.	
Water q. s.	500 cc.	
No. 2		2475
As above except		
Hydrochloric acid	4 cc.	
No. 3		2420
As above except		
Hydrochloric acid	3 cc.	
No. 4		2125
As above except		
Hydrochloric acid	2 cc.	
No. 5		1940
As above except		
Hydrochloric acid	1 cc.	
No. 6		820
As above but with no hydrochloric acid added. This preparation contained only the acid present in the Glycerite used.		

The Glycerite of Pepsin used in the above was freshly prepared and assayed and adjusted to exact strength. The six preparations above were also assayed within two days after manufacture in order to be sure of their strengths at the start.

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[1] JOUR. A. PH. A., Vol. X, p. 595; Volume XI, p. 686.

After a period of five months these preparations were again assayed to ascertain deterioration of pepsin from the original strength of 1 to 3000.

It appears that the rate of deterioration increases with the amount of acid present. However, the total absence of acid does not seem to be advantageous as was previously shown in the case of Elixir of Pepsin (Table II). No doubt some acid is necessary in these preparations, but a very small amount seems to be the best and certainly much less than that required in the official formulas. The ultimate strengths of these may show no differences but the rate of falling off for the first few months seems to be dependent upon the amount of acid present.

PRESERVATIVES.

Previous reports of work done in this laboratory brought out the fact that samples of Essence of Pepsin containing small amounts of boric acid, chloroform, iodoform, and caffeine show no noticeable deterioration. Caffeine was used in varying amounts as small as 4.25 mg. per 100 cc. and all results were alike in showing little falling off of the pepsin strength.

A number of pepsin preparations containing various substances were therefore made up for the purpose of determining the preservative action of the added substances. These preparations were all assayed within three days after manufacture and adjusted to the correct 1:3000 pepsin strength. After six months all were again assayed with the following result:

	Assayed strength.		Assayed strength.
No. 61 Glycerite of Pepsin Caffeine, 0.01 Gm. per 100 cc.	2650	No. 67 Liquor Pepsin Sodium phosphate, 0.2 Gm. per 100 cc.	Inactive
No. 62 Elixir of Pepsin Caffeine, 0.01 Gm. per 100 cc.	1010	No. 68 Liquor Pepsin Sodium salicylate, 0.01 Gm. per 100 cc.	"
No. 63 Elixir of Pepsin Sodium salicylate, 0.02 Gm. per 100 cc.	1100	No. 69 Liquor Pepsini Methyl salicylate, 0.2 cc. per 100 cc.	"
No. 64 Elixir of Pepsin Methyl salicylate, 0.02 cc. per 100 cc.	200	No. 70 Elixir Pepsin and Bismuth Caffeine, 0.01 Gm. per 100 cc.	1350
No. 65 Liquor Pepsini No preservatives added	Inactive	No. 71 Elixir Pepsin and Bismuth Sodium salicylate, 0.02 Gm. per 100 cc.	2075
No. 66 Liquor Pepsini Caffeine, 0.01 Gm. per 100 cc.	"		

The first and last of these which showed the least deterioration were again assayed after a year and were still slowly losing strength.

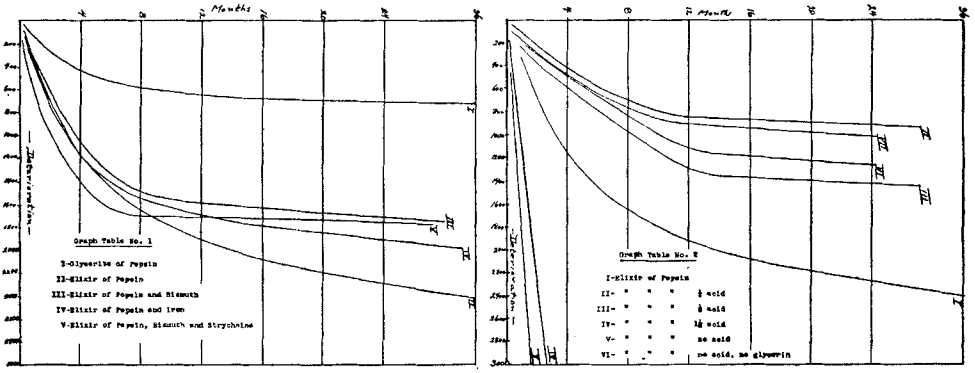
EFFECTS OF DIALYSIS OF PEPSIN.

This investigation seems to substantiate the belief that pepsin and rennin are two distinct ferments and that pepsin does have the dual property of digesting proteins and coagulating milk and that a measure of one property is a measure of the other.

Pepsin being a colloid and rennin being able to pass through a semi-permeable membrane offered a possibility of investigating these two properties of pepsin by a series of dialysis experiments. Accordingly a number of collodion sacs were

made and those of uniform thickness were used. Several samples of pepsin were used ranging from 1:2800 to 1:10,000 in strength as assayed.

It was found that the ordinary method of dialysis could not be employed because of the fact that molds began to appear in the solutions before the dialyses could be carried on any length of time. Rather than to attempt the use of any preservatives it was decided to use a method of continuous dialysis whereby the solution could be subjected to the action of a large amount of water in a comparatively short time. The method used consisted in having the pepsin solution in a beaker and immersing the collodion sac in it through which a continuous stream of water was passed. In all experiments the water used was acidified to the same strength as the pepsin preparation and a control solution of pepsin was also made up and set aside for comparison each time. After running the dialysis, both the control and the sample that had been subjected to the dialysis were made up to the same volume and acidity and portions of each were assayed by both the U. S. P. IX and "milk" methods. For a description of the milk method see previous report.[1] It was expected that if the dual property of pepsin were due to the presence of two different ferments, it would be indicated by a noticeable difference



in the relative activities in the two methods of assay. Without exception, a large number of solutions of pepsin so treated were found to have lost only a slight amount of proteolytic power and a corresponding amount of milk-curdling power. The small decrease in proteolytic power was undoubtedly due to the fact that a colloidal substance may pass through a semi-permeable membrane especially under the conditions in which this experiment was run.

These experiments, therefore, seem to point to the fact that pepsin is but one ferment, or at least that it cannot be separated into any components by dialysis, and that a measure of the milk-curdling power is a measure of the proteolytic strength.

CONCERNING THE MILK METHOD FOR THE ESTIMATION OF PEPSIN.

It has been shown that the coagulation of milk by rennin takes place in two steps. Rennin converts the caseinogen of milk into paracasein which is soluble. However, the calcium salts present in milk cause the precipitation of the paracasein as the curd, casein. The coagulation of milk by pepsin follows the same as in the coagulation by rennin.[1]

[1] Mellanby, *Jour. of Phys.*, 45, 345 (1922).

Considerable controversy has arisen at times relative to the identity of these two ferments but at present it seems well established that they are two distinctly different ferments. It has been found that both can be precipitated by magnesium salts but subsequently only the rennin precipitate is soluble. If a mixture of the two ferments be heated to 45° C., the rennin is more rapidly destroyed and eventually only the pepsin remains active. Furthermore, it has been observed that a larger amount of calcium salt is required to be present for the coagulation of milk by rennin than is needed when pepsin is used.

A method has been suggested for the estimation of trypsin by making use of its ability to curdle milk.[1] The hypothesis upon which this method is based is that the proteolytic ferments all coagulate milk provided the calcium content of the milk is suitable. Ferments which work in acid medium, such as pepsin, require only a small amount of calcium, while those that work in alkaline medium (trypsin) seem to require relatively larger amounts of calcium salts.

The method of estimating trypsin is as follows: Milk is diluted with an equal volume of $N/10$ calcium chloride solution. Two cc. of this and the solution to be tested are mixed and the volume made up to 3 cc. The time of coagulation at 38° C. is noted and from this time the quantity of ferment present is estimated. In order to make this estimation it is first necessary to construct a curve showing the time relation required for curdling to the amount of trypsin present. For the higher concentrations of trypsin the time seems to be inversely proportional to the amount of ferment present but in dilute solutions this does not seem to hold. Having once established such a curve by means of a series of actual determinations it is possible to determine the amount of trypsin present for any observed time of coagulation.

In the case of milk coagulation by pepsin it has been found that the acidity of the milk has a marked influence upon the time required for curdling, in fact, by using milk a little too old in which fermentation probably had started, it has been our experience to observe a shortening of the coagulation time to a marked degree in the course of an hour's time in the laboratory. For this reason no methods for the estimation of pepsin have been proposed other than ones comparing the time required for curdling with the time required for a standard. It has been shown that the time required for coagulation is inversely proportional to the amount of pepsin present or to the strength of the pepsin used, and that a measure of the milk-curdling power is also a measure of the proteolytic power of the pepsin as well. Traut and Vahlteich used a method in this laboratory[1] which has been found to be entirely satisfactory for the estimation of pepsin through its milk-curdling properties by comparison with a standard pepsin. The acidity of the milk should be such that coagulation is produced in about five minutes. Adjusting the acidity by the use of lactic and hydrochloric acids (2 to 1) gives a sharper end-point (coagulation) than the use of either alone.

Obviously, in assaying preparations of pepsin containing acid, there will be an error introduced if the acid is not first neutralized and then adjusted to the proper amount. This may be accomplished by the addition of a small amount of calcium carbonate until the preparation loses its acid reaction to litmus.

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[1] Mellanby and Wooley, *Jour. of Phys.*, 45, 372 (1912).

Another influencing factor in the time of coagulation is the calcium content. An addition of calcium salts was found to shorten the time required for coagulation but no definite relationship could be observed between the amount of calcium added and the change in time required for coagulation. The addition of oxalates to the milk also has been found to lengthen the time period due to its precipitation of calcium. With both the acidity and calcium content of milk influencing the time of coagulation, it seems that the use of milk for estimating pepsin will have to be one of comparison with a standard since acidity and calcium content are variable and the control of calcium content meets with many difficulties.

It has been shown that three essential factors influence the curdling of milk by pepsin. Casein, calcium salts, and acid seem to be essential. This offers an opportunity of preparing a standard reagent to be used for the assay of pepsin. Casein dissolved in lime water, for instance, and having some other calcium salts present in solution and sufficient acid strength can be coagulated by pepsin. However, the coagulation is not sharp and well defined as in the case of milk. The curd tends to come out in a few large slowly appearing pieces instead of the smaller particles that appear so well defined when milk is used. It is quite possible, however, that this method may be developed into a useful one. Some changes in acidity and calcium content as well as the concentration of casein are suggested as possible means of improving the end-point appearance.

For the preparation of casein there are a number of methods available. One using milk diluted with 3 to 5 times its volume of water and precipitated with acetic acid with two subsequent reprecipitations will give a very good grade of casein. After the final precipitation the casein should be put through a series of sieves starting with a No. 20 and ending with a No. 80, using absolute alcohol to remove water from the product and finally ether. The product resulting from such manipulation will be a very finely divided powder much superior to the usual coarse, hard material.

The proteolytic action of pepsin on casein does not seem to offer any possibilities for an assay method. It was our intention to use a casein of high grade as a uniform source of protein and to allow pepsin to act upon it for a given length of time after which the digested protein in solution or else the remaining casein would be determined by a nitrogen determination. The difficulty with this method is the slight action of pepsin upon casein, even on casein that has been dissolved and freshly precipitated for the digestion. Casein of a uniform degree of purity can be prepared easily and it seems possible that its use may offer a new source of protein for pepsin digestion assays.

YEAR BOOK OF THE AMERICAN PHARMACEUTICAL ASSOCIATION.

Volume 12 of the YEAR BOOK OF THE AMERICAN PHARMACEUTICAL ASSOCIATION has been mailed to members. General Secretary Day, by instruction, has sent a notice to all members, and a reply card, asking the member to state whether or not he desires to receive the next YEAR BOOK published, which will be during the year 1926. This request is made so that it may be determined whether the majority of the members make use of the YEAR BOOK and desire to receive it, as, if a considerable number of the members do not use it and do not care for it, the number printed can be cut down and result in a saving to the Association.