A Study of the Tryptic Activity of Pancreatin U. S. P.*

By CHARLES W. BAUER† and ARTHUR K. WHITE‡

The official assay for pancreatic trypsin is not an assay for a single enzyme but for a mixture of proteolytic enzymes. Many samples labeled Pancreatin U. S. P. possess three times the minimum U. S. P. trypsin requirements. Triple-strength pancreatin is frequently no stronger than ordinary U. S. P. Pancreatin.

PANCREATIN is described in the United States Pharmacopœia as "a substance containing enzymes, principally pancreatic amylase, trypsin, and pancreatic lipase, obtained from the fresh pancreas of the hog .....". Without mentioning trypsin directly, but only by inference, the monograph further states that "pancreatin converts not less than 25 times its weight of casein into proteoses."

"Trypsin" is the name coined by Kühne (1) in 1867 for a proteolytic enzyme found in pancreatic juice. It means "a substance that cleaves." Kühne also used the name "Pancreatin" for the trypsin-containing precipitate that he obtained when he treated the pancreatic extract with alcohol.

The proteolytic effect of pancreatic juice was first recognized by Purkinje and Pappenheim (2) as early as 1836. A more comprehensive study than theirs of the effect of pancreatic juice on egg albumin was made by Corvisart (3) in 1856. However, it remained for Kühne (1) and his co-workers to recognize the fact that the proteolytic enzyme or enzymes in pancreatic juice carried the decomposition of egg albumin beyond the peptone stage. They found that leucine and tyrosine were among the products of decomposition when peptones were treated with either pancreatic juice or minced pancreatic extract.

Northrop and Kunitz (4) obtained a crystalline proteolytic enzyme from the pancreas of cattle in 1932. They retained the name "Trypsin" to designate "the most important proteinase" found in the pancreas.

The trypsin found in Pancreatin U. S. P. is the proteolytic enzymes, that convert a special preparation of casein into decomposition products that are not precipitated by a specially prepared mixture of acetic acid and alcohol. The official assay is based upon the formation of proteoses by this process; but since trypsin, as Kühne (1) has shown, actually converts proteoses into simpler compounds, it appears advisable to base the assay, not upon proteoses, as is done in the Pharmacopœia, but upon the simpler compounds beyond the proteose stage, the amino acids. These can be assayed by Sørenson's Formol Titration Method (6).

The assay methods for trypsin found in pharmaceutical literature are varied and difficult to interpret. For example, the United States Pharmacopœia states that pancreatin will digest 25 times its own weight of casein into proteoses. *New and Nonofficial Remedies* (1941) states that one part of Trypsin-Armour "digests at least 100 parts of casein according to the Fuld–Gross method." Trypsin–Fairchild, according to the same authority, when treated by the Fuld-Gross method will convert "200 times its weight of casein to the standard end-point." From these statements one may be led to believe that the latter preparation is 8 times as potent as Pancreatin U. S. P. even though the assertion is not directly made. When these preparations are assayed by identical methods, as by the Formol Titration Method, a true comparison can be made, and it will be found that the potencies of these preparations do not vary in the proportion implied.

The assay method of trypsin given in the United States Pharmacopœia under pancreatin is based upon the digestion of casein by proteolytic enzymes for a definite period of time, to the stage where the digestion mixture fails to give a precipitate when treated with a specially prepared mixture of acetic acid and alcohol. The acetic acid mixture

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is made by mixing 1 cc. of glacial acetic acid with 9 cc. of distilled water and 10 cc. of alcohol. The main objection to this method is the difficulty of determining the official end point, that is, the absence of any precipitate after the addition of three drops of the acetic acid mixture at the end of the one-hour digestion period.

Willson (5) states that "the end point is far from easy to determine with certainty," and that "inconsistent results are obtained by different workers." This variation seems to be caused, for the most part, by the difficulty of making a clear casein solution by the official method.

EXPERIMENTAL

The digestion period as given in the Pharmacopœia appears to be too long. The data given in Table I show that the trypsin in Pancreatin U. S. P. is about three times as potent as the official definition indicates. The Pharmacopœia states that no precipitate is obtained in one hour. Experimental evidence shows that a faint precipitate is obtained in twenty minutes when Pancreatin U. S. P. is assayed by the U. S. P. method, and that no precipitate is obtained in any of the samples after thirty minutes. It appears that Pancreatin U. S. P. is actually from two to three times as potent as is required in the official monograph.

TABLE I.—LENGTH OF TIME OF THE DIGESTION PERIOD REQUIRED TO ASSAY THE POTENCY OF PANCREATIN FOR TRYPSIN BY THE U. S. P. METHOD

Test Tube No.	Casein Sol., Cc.	Pancreatin Sol., Cc.	Water, Cc.	Time in Minutes	Acetic Acid Mixture
1	5	2	3	20	Faint ppt.
2	5	2	3	25	Very faint ppt.
3	5	2	3	25	No ppt.
4	5	2	3	25	Very faint ppt.
5	5	2	3	30	No ppt.
6	5	2	3	30	No ppt.
7	5	2	3	35	No ppt.
8	5	2	3	40	No ppt.
9	5	Blank	5	30	Heavy ppt.

A Comparison of Pancreatin U. S. P. with That of a "Triple Strength" Pancreatin.—An experiment was made in an attempt to compare the strength of Pancreatin U. S. P. with that of a "Triple Strength" Pancreatin. The U. S. P. method was followed, and the results show that neither gave a precipitate at the end of the one-hour digestion period. Each sample was then used in solutions of one-half the strength of the U. S. P. solution. The result was the same. Finally, solutions of one-third the strength of the U. S. P. solution were used, and in each case only a very faint precipitate was formed at the end of an hour. No difference could be distinguished in the samples as may be seen by a comparison of data given in Table II.

TABLE II.—COMPARISON OF PANCREATIN U. S. P. WITH A "TRIPLE STRENGTH" PANCREATIN BOTH ASSAYED BY THE U. S. P. METHOD

Test Tube No.	Pancreatin U. S. P. Strength of Pancreatin Solution Used	Effect of the Acetic Acid Mixture
1	Full strength	No ppt.
2	One-half strength	No ppt.
3	One-third strength	Faint ppt.
	Pancreatin "Triple Strength"	
1	Full strength	No ppt.
2	One-half strength	No ppt
3	One-third strength	Faint ppt.

Willson's Assay Method for Pancreatic Trypsin

Because of the difficulty of making a perfectly clear solution of casein wherein it would be easy to see the end point by precipitation, an entirely different type of assay was tried, an assay based upon the formation and determination of amino acids. This assay, based upon a modification of Sørenson's Formol Titration Method (6), improved upon by E. R. Smith in 1912 (7), and again by F. E. Willson in 1930 (8), is performed as follows:

Solutions Required.—No. 1: A 37% formaldehyde solution to which 1 cc. of 0.5% phenolphthalein in 50% ethyl alcohol is added. This is brought to neutrality with normal sodium hydroxide.

No. 2: A 4% solution of casein neutralized by normal sodium hydroxide of an amount determined by titrating a small amount of the casein solution in the presence of phenolphthalein.

No. 3: A 1% unfiltered pancreatin suspension in distilled water.

Procedure.—Heat the casein solution and, at the same time, a supply of distilled water to 55°. Add 24 cc. of this water to 25 cc. of the casein solution and place the mixture in the water bath at 55°. Add 1 cc. of the pancreatin solution to the first solution and allow it to digest for twenty minutes. While the solution is digesting withdraw 20 cc. from the blank, add 10 cc. of solution No. 1, and titrate to a pink color with 0.1 *N* sodium hydroxide. Withdraw 20 cc. of the digested solution into a 50-cc. Nessler tube, add 10 cc. of solution No. 1, mix well, and titrate with 0.1 *N* sodium hydroxide to the pink color of the blank.

As may be seen, since Willson's blank does not contain boiled pancreatin, it does not give the true picture that a control should give; for it does not allow for the possibility that the pancreatin may contain substances—acid in reaction—that would be titrated along with the decomposition products of casein in the final titration with 0.1 *N* sodium hydroxide. Furthermore, the period of time allowed

by Willson does not afford sufficient time to carry out certain controls that appear to be necessary.

An experiment was performed using time intervals of 20, 40, 60, and 80 minutes. When these results are represented graphically, it is seen that more than half the digestion takes place in the first twenty minutes. After the first twenty minutes the relationship of digestion to time is fairly constant. Since about 75% of the digestion takes place in the first forty minutes, and since this time was found to be a satisfactory working period, forty minutes was used for the digestion period of this enzyme in the modification which was adopted. The data obtained from this portion of the investigation are shown in Fig. 1 and Table III.

TABLE III.—RELATIONSHIP OF DIGESTION TO TIME IN EACH CASE 20 CC. OF THE DIGESTION MIXTURE ADDED TO 10 CC. OF NEUTRAL FORMALDEHYDE SOLUTION

Pancreatin "Triple Strength"	Cc. of 0.1 *N* NaOH at Time Intervals of:			
Test Tube No.	20 Min.	40 Min.	60 Min.	80 Min.
1. Digestion Mixture	5.35	6.35	6.95	7.50
2. Control 1 Willson	1.90	1.90	1.90	1.90
3. Control 2 (Containing Pancreatin Previously Boiled)	2.95	3.05	3.20	3.30

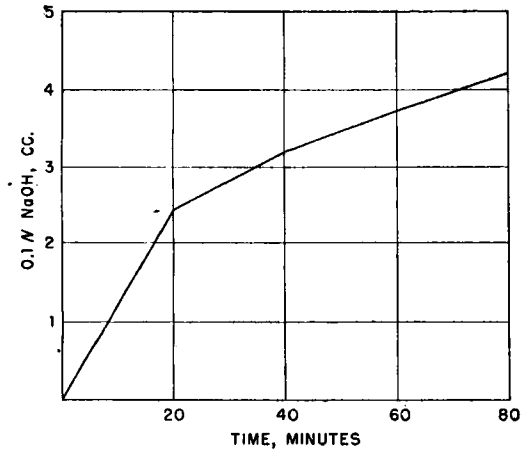

Fig. 1.—Graph showing relationship of digestion to time in assay of pancreatic trypsin.

Furthermore, it was found that when 5 cc. of the pancreatin suspension was used in place of the 1 cc., as used by Willson (8), there was more 0.1 *N* sodium hydroxide used, with the result that the larger figures cut down the percentage of error in the burette readings.

Another improvement in the method of assay was obtained by adding the digestion mixture to the formaldehyde solution already in the Nessler tube in order to stop the action of trypsin immediately at the end of the digestion period. This method reduces the possibility of error from the over-running of the time limit.

These data clearly indicate the value of using a control that contains the same amount of pancreatin, previously boiled, as is used in the digestion mixture.

Modification of Willson's Method.—Heat the casein solution and, at the same time, a supply of distilled water to 55°. Add 20 cc. of the water to 25 cc. of the casein solution and place in a water bath at 55°. Add 5 cc. of 1% pancreatin suspension and allow to digest for forty minutes. This is called the digestion mixture.

Control.—To 20 cc. of water add 25 cc. of the casein solution and place this mixture in the water bath at 55°. Then add 5 cc. of a 1% pancreatin suspension, which has been previously boiled for two minutes, and allow this mixture to stand for forty minutes.

Now add 10 cc. of the neutralized formaldehyde solution to each of two 50-cc. Nessler tubes. At the close of the digestion period withdraw 20 cc. from the digestion mixture, and from the control, and add to the formaldehyde solution in the Nessler tubes. Mix well and titrate each to a uniform pink color with 0.1 *N* sodium hydroxide.

The modifications of Willson's method may be briefly summarized as follows:

1. A control is used, which is identical to the digestion solution in every respect but one. The pancreatin solution has been boiled for two minutes before adding it to the mixture in order to destroy its enzymatic action.
2. The digestion period is forty minutes instead of twenty.
3. Five cubic centimeters of the 1% pancreatin suspension is used instead of 1 cc.
4. The formaldehyde solution is placed in a Nessler tube in advance and the digested mixture is added to it.

The Preparation of a Suspension of Casein More Nearly Homogeneous than Either Willson's or the Official Mixture.—Willson encountered some difficulty in dissolving the casein. In his directions for preparing a liter of solution he says: "40 Gm. of casein is added to 900 cc. of recently boiled distilled water to which approximately 25 cc. of 1 *N* NaOH has been added. The casein is brought into solution by stirring and mixing from time to time. If at the end of an hour or two all the casein is not dissolved a few more cc. of 1 *N* alkali are added to accomplish this."

The so-called solution obtained by this procedure was not homogeneous. Even upon adding more sodium hydroxide, and after allowing a longer period of time, the appearance did not improve. The casein seemed to swell; and after the suspension had been set aside, small undissolved particles settled to the bottom of the container.

In order to dissolve all the casein and to have a more nearly homogeneous suspension, the casein was

first powdered and sifted through a number 100 mesh sieve before it was treated with the solvent. When this powder was used a good suspension was formed, which was used with consistently satisfactory results. Pancreatin U. S. P. was assayed for casein digestive power with four different samples of casein by this modified method with nearly identical results. This indicates that the commercial samples of casein are similar in character to one another, and that they will give nearly homogeneous suspensions if the casein is first powdered and passed through a number 100 mesh sieve. All the experimental work, however, which followed, was done upon the same sample of casein.

Since it is impossible to put casein into solution by the U. S. P. method or by the Willson method, it appears advisable to modify the accepted procedure in order to bring about a more nearly homogeneous suspension of this substrate.

The Tryptic Activity of Various Samples of Commercial Pancreatin.—The discovery of pancreatic fibrosis and the renewed interest in pancreatic therapy for this condition, i.e., the inability to secrete trypsin, make it very important to know the tryptic activity of the available preparations of pancreatin or of the trypsin in order properly to estimate their medicinal value.

Commercial samples of pancreatin were assayed, in order to determine the exact amount of tryptic activity in them, by the modified Willson method instead of by the official assay for the minimum requirements indicated in the U. S. P. definition of pancreatin. If a sample is called "Triple Strength" Pancreatin, does it mean that it has three times the strength of the minimum requirements, or does it mean that it is three times as potent as ordinary Pancreatin U. S. P.? The U. S. P. test fails to distinguish between a sample which meets the minimum requirements and one which may be many times as potent.

TABLE IV.—COMMERICAL SAMPLES OF PANCREATIN ASSAYED BY THE MODIFIED WILLSON METHOD

Co.	Sample No.	Age in Mo.	Cc. of 0.1 *N* NaOH Used in Assay	Cc. of 0.1 *N* NaOH Used in Blank	Cc. of 0.1 *N* NaOH Actually Used
A	20	26	9.01	3.47	5.54
A	1A	24	9.55	2.97	6.58
A	11A	11	9.50	2.90	6.60
B	17	18	7.43	2.43	5.00
B	2A	67	7.03	2.28	4.75
B	3	67	7.18	2.57	4.61
B	3A	103	6.39	2.77	3.62
B	8A	67	6.98	2.72	4.26
C	10A	35	7.57	2.52	5.05
C	4A	53	7.47	2.47	5.00
C	4	89	7.77	2.57	5.20
C	12	23	7.60	2.68	4.92
C	3X[a]	Age unknown	10.36	3.28	7.08
D	7A	112	7.77	2.52	5.25
D	14	100	7.03	2.52	4.51
D	3Y[b]	Age unknown	10.58	3.37	7.21

[a] "Triple Strength" Pancreatin.
[b] "Triple Strength" Enteric Coated Capsule.

TABLE V.—SAMPLES OF TRYPSIN OBTAINED FROM VARIOUS SOURCES ASSAYED BY THE MODIFIED WILLSON METHOD

Sample No.	Cc. of 0.1 *N* NaOH Used in Assay	Cc. of 0.1 *N* NaOH Used in Blank	Cc. of 0.1 *N* NaOH Actually Used
1	9.06	3.14	5.02
1A	8.91	3.15	5.76
2	9.30	3.00	6.30
3[a]	10.25	3.46	6.79
3A[a]	10.30	3.59	6.71
4[b]	9.45	3.15	6.30

[a] "Triple Strength" Enteric Coated Tablets.
[b] Reference Standard Trypsin.

By assaying by the modified Willson method the samples of pancreatin obtained from different companies, it was found that most samples were two to three times as strong as the minimum requirements indicated on the label. Furthermore, in comparing a number of samples of Pancreatin U. S. P. (Company C) with a sample of "Triple Strength" Pancreatin from the same company, it was found that the U. S. P. samples were of approximately 70% the strength of the "Triple Strength" sample; whereas it would seem that they should be more nearly 33% as strong as the "Triple Strength" Pancreatin. However, a noticeable difference in the samples can be determined by the modified Willson method, whereas a previous experiment, when Pancreatin U. S. P. and "Triple Strength" Pancreatin were assayed by the U. S. P. method, merely showed that they both met the U. S. P. minimum requirements.

SUMMARY

1. The trypsin designated in the U. S. P. Pancreatin is a mixture of proteolytic enzymes.

2. The official assay is unsatisfactory, as no definite standard, with which different samples can be compared, is set up.

3. A modification of Willson's assay of pancreatin has been undertaken and has worked out successfully.

4. A number of commercial samples of pancreatin have been obtained and assayed by this method. A comparison of the strength of trypsin in various samples of pancreatin can be expressed in terms of the number of cc. of 0.1 *N* sodium hydroxide used to neutralize the amino acids produced

by digestion upon a specially prepared solution of casein.

5. A modification of both the U. S. P. and the Willson methods has been made in the preparation of the casein solution.

6. Samples of commercial trypsin were also assayed by the modified Willson method, and the results have been compared with U. S. Pharmacopœia Reference Trypsin.

7. It has been demonstrated by these assays that "Triple Strength" Pancreatin possesses three times the potency of the minimum requirements as stated in the official monograph; but, that it is misleading to imply that it posesses three times the potency of Pancreatin U. S. P.

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The Carboxypeptidase Activity of Pancreatin U. S. P.*

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Pancreatin U. S. P. contains the enzyme carboxypeptidase. It is a relatively stable enzyme. Pumpkin seed globulin serves as an excellent substrate for its analysis. Its activity may be expressed in terms of cc. of 0.02 *N* sodium hydroxide required to neutralize the amino acids produced from a definite amount of pumpkin seed *alpha*-globulin.

UNTIL a few years ago it was thought that proteins were first digested to peptones by the pepsin in the stomach, and were then completely digested into absorbable units by the trypsin of the pancreas. It is now known that the enzyme trypsin, in the strict meaning of the term, does not complete the digestion of proteins into absorbable units. It has been found that the enzymes aminopeptidase and carboxypeptidase accomplish this result. Since carboxypeptidase is secreted by the pancreas, it is of interest to determine whether this enzyme is present in the official preparation of pancreatin.

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The object of this investigation was to determine the presence and the relative amounts of carboxypeptidase in different samples of Pancreatin U. S. P.

In 1927 Waldschmidt-Leitz (1) and coworkers discovered the enzyme carboxypolypeptidase as the zymogen in the pancreas of certain of the higher animals. Two years later Waldschmidt-Leitz and Purr (2) shortened the name of carboxypolypeptidase to carboxypeptidase.

According to Bergmann (3) carboxypeptidase splits an end amino acid from its substrate, the enzyme attacking only those substrates containing a free carboxyl group next to the peptide linkage. The work of Bergmann and Fruton (4) indicates that the CO—NH—CH(R)—COOH groups are arranged in a counterclockwise order in which the alpha hydrogen atom of the CH(R) group is directed toward the enzyme. Anson (5) found carboxypeptidase to be the only known proteolytic enzyme that acts in the presence of formaldehyde.

One of the synthetic substrates employed for the measurement of carboxypeptidase activity is the expensive chloracetyl-*l*-tyrosine which was used by Waldschmidt-Leitz (2). Anson (7) found that a peptic digest