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

of edestin was a satisfactory substrate for his investigations of carboxypeptidase.

Waldschmidt-Leitz (1) asserts that the zymogen found in the pancreas is activated by the enterokinase of the intestines. It has been suggested by Sumner and Somers (6) that the activation of the zymogen may be due to the action of trypsin.

MATERIALS AND METHODS

Substitute Substrate for Edestin.—Since chloracetyl-*l*-tyrosine was too expensive to be used in this investigation, the peptic digest of edestin was first employed as the substrate. The formalized solution of edestin was prepared according to Anson (7), using edestin (Eimer and Amend), decolorizing charcoal (Merck), granulated pepsin (Wilson Laboratories), and Filter-Cel (Johns-Manville). After several preliminary experiments with the edestin solution, the supply of edestin ran out, and no more could be obtained anywhere. The experiments performed with edestin clearly indicated the presence of carboxypeptidase in Pancreatin U. S. P. In order to continue this investigation it became necessary to find a satisfactory substrate to take the place of edestin.

In 1940 Vickery, Smith, and Nolan (8) reported the use of pumpkin seed globulin as a substitute for the globulin edestin obtained from hemp seed. These workers found the yield of globulin prepared from pumpkin seed by their method to be about 10%, by weight of the seed, a percentage somewhat higher than that of hemp seed. An added advantage of pumpkin seed globulin is that it is cheaper than edestin. Hence, pumpkin seed globulin was used as the substrate for the major portion of the experimental work reported in this paper.

Theory of Formol Titration and Modification.—In the determination of proteolytic enzymes other than carboxypeptidase by formol titration, the usual procedure for obtaining a standard consists of adding formaldehyde to the substrate solution to stop the enzymatic digestion, then of adding phenolphthalein and a sufficient amount of sodium hydroxide to bring the solution to a standard color. This preparation constitutes the standard or control. The increase in amount of alkali used over that of the control is a measure of the number of amide linkages split. This procedure is inadequate, according to Anson (7), and has to be slightly modified for the determination of carboxypeptidase, since the carboxypeptidase activity, unlike other proteolytic activities, is not stopped by the addition of formaldehyde. The blank is run on a substrate solution in the absence of the enzymatic preparation but in the presence of formaldehyde, phenolphthalein, and sodium hydroxide.

In the experimental investigation which follows, the blank was further modified by adding a boiled enzyme preparation which contained some amino acids not obtained from carboxypeptidase digestion. This modification eliminates the danger of including the amino acids, which are normally present in the crude pancreatin, from being considered to be the result of enzymatic action upon the substrate.

Preparation of Pancreatin Suspension.—A 5% pancreatin suspension was found by trial to be very satisfactory for this investigation. The suspension of pancreatin should be as homogeneous as possible in order that equal measurements may contain about equal amounts of pancreatin. All suspensions were made in distilled water about one half-hour before starting the assays.

Since heating might possibly have hydrolyzed some additional acids in the pancreatin, it was necessary to determine the acidity of both the boiled and the unboiled pancreatin suspensions. The effect of heat on the hydrolysis of pancreatic proteins had to be determined in order to have a correct control or blank. Phenolphthalein was used as the indicator, and the boiled suspension was first cooled to room temperature before assaying. The experimental results indicate that boiling has no effect upon the acidity of the preparation, as is shown by the fact that the amount of alkali was practically the same in each instance.

While the acidity of different samples of pancreatin may vary, the difference between the boiled and unboiled pancreatin in any one sample is about the same.

Preparation of Standard Formaldehyde Solution.—To 50 cc. of water add 15 cc. of formaldehyde 37%, 2 cc. of 0.5% phenolphthalein indicator solution, enough 5 *N* sodium hydroxide to make the solution just detectably pink, and enough distilled water to make the total volume measure 100 cc. This solution contains the same amount of formaldehyde as the formalized substrate solution.

Preparation of Pumpkin Seed Globulin.—To 150 Gm. of freshly ground pumpkin seed, in a previously warmed dry mortar, add sufficient 10% sodium chloride solution, warmed to 60–65°, to form a very soft paste. Triturate; add about one liter of sodium chloride solution and triturate for an additional five minutes. Strain through a number 20 sieve into a large evaporating dish and return the residue to the mortar for a second treatment. Use the remainder of the sodium chloride solution, two liters of which are prepared for the preparation, for the second extraction. Triturate again; then strain the contents into the large evaporating dish. Heat for one hour at 60–65° with constant stirring. Transfer the contents to a two-liter flask and tie a double fold of cheesecloth over the neck of the flask in the form of a small sack.

Invert the flask and contents into a stepfolded filter paper that has been washed with warm 10% solution of sodium chloride. It is desirable to use a water-jacketed funnel, maintained at 60–65°, for the filtration process, for, if the funnel and paper become cold, the globulin will precipitate and will clog the pores of the filter paper.

Dilute the filtrate with distilled water to a 2% concentration of sodium chloride and set it aside at room temperature for four to five hours; then place it in the refrigerator at 5° for twelve hours. Then siphon off the supernatant liquid. Filter the precipitate through a large Jena filter or a glass sintered filter, and wash the precipitate twice with a small amount of distilled water. Dry the precipitate over a steam-heated radiator or in a warm place, powder it finely, and pass it through a bolting cloth. The extracted globulin is light yellow-brown in color and behaves very similarly to edestin, as a control experiment showed. The yield by the foregoing method is about 6–7%.

The pumpkin seed globulin served as a satisfactory substrate for the determination of carboxypeptidase activity in Pancreatin U. S. P. Anson's (7) procedure was used to prepare the formalized substrate solution, but pumpkin seed globulin was substituted for the edestin.

Preparation of Digestion and Control Flasks and the Method of Assay

The Preparation of the Standard Color for the Blank.—To flask 1S, the standard color flask, add 10 cc. of 5% boiled pancreatin suspension previously cooled to room temperature, one drop of phenolphthalein indicator, and titrate to a pink color with 0.02 *N* sodium hydroxide in order to neutralize the amino acids present in the pancreatin suspension. Then add 5 cc. of neutralized standard formaldehyde solution. The pink color disappears on the addition of the formaldehyde solution because of the release of the carboxyl groups of the amino acids present as zwitter ions in the neutralized pancreatin. Make the suspension pink again with 0.02 *N* sodium hydroxide. Stopper the flask, and let this preparation represent the color standard for flasks 2a and 2b.

Blank Determination of Flasks 2a and 2b.—Add 10 cc. of 5% cooled, boiled pancreatin suspension, and one drop of phenolphthalein indicator to flasks 2a and 2b, which are to be employed as blanks for the digestion flasks 4a and 4b.. Titrate the mixtures with 0.02 *N* sodium hydroxide to the same pink color as that of the standard color flask, 1S; then add 5 cc. of formalized substrate solution. The pink color disappears after this addition, but returns on the addition of more alkali. After the desired pink color is obtained, stopper the flasks and place them in a constant temperature bath for thirty minutes at 37°. The standard color flask, 1S is also placed in the constant temperature bath at the same time. The amounts of 0.02 *N* sodium hydroxide used need not be recorded, for the alkali is used only to neutralize the acids in the pancreatin suspension as well as any amino acids that may be liberated when the formaldehyde solution is added. None of these acids is a product of the digestion with the enzyme preparation.

At the end of thirty minutes—the period allowed for digestion in flasks 4a and 4b—remove the flasks from the temperature bath. Titrate the contents of the flasks 2a and 2b with 0.02 *N* sodium hydroxide until the color of each matches that of the standard flask. Record the number of cc. of 0.02 *N* sodium hydroxide required to produce the desired color in flasks 2a and 2b. This is the amount, in terms of cc. of 0.02 *N* sodium hydroxide, necessary to neutralize the acids that may be produced during the thirty-minute digestion period. Theoretically there should be no amino acids produced, since the pancreatin suspension was boiled for seventy-five seconds to destroy all enzymatic activity. Flasks 2a and 2b are employed as blanks for the digestion flasks 4a and 4b, which contain the unboiled, enzymatically active, pancreatin suspension.

The Preparation of the Standard Color for the Digestion Flasks.—To flask 3S, which is to be the standard color flask for flasks 4a and 4b, in which digestion occurs, add 10 cc. of unboiled 5 per cent pancreatin suspension, one drop of phenolphthalein indicator, and titrate to a pink color with 0.02 *N* sodium hydroxide. Add 5 cc. of neutral standard formaldehyde solution and again add 0.02 *N* sodium hydroxide until the standard color is obtained. Stopper the flask, and use it as the standard color flask for 4a and 4b.

The Preparation of the Flasks 4a and 4b.—Add 10 cc. of unboiled 5% pancreatin suspension to flasks 4a and 4b; then add one drop of phenolphthalein indicator and titrate to the standard color. Then add 5 cc. of neutral formalized substrate solution and immediately titrate to the standard color; stopper, and place the three flasks in the constant temperature bath at 37° for thirty minutes. The amount of sodium hydroxide is not recorded. However, after the removal of the flasks from the temperature bath, the number of cc. of 0.02 *N* sodium hydroxide required is an indication of the degree of carboxypeptidase activity in the pancreatin suspension.

At the end of thirty minutes remove the flasks from the thermostatic bath. If the color of the standard color flask, 3S, be markedly faded or be of such a weak shade that it is difficult to compare or reproduce, introduce a known amount of 0.02 *N* sodium hydroxide until the desired color is obtained. Titrate the other flasks, 4a and 4b, with 0.02 *N* sodium hydroxide until their color matches that of the standard (3S). Subtract the number of cc. of 0.02 *N* sodium hydroxide used to produce the desired color in the standard color flasks, 3S, from the total number of cc. used in each of the two digestion flasks 4a and 4b.

Interpretation of the Assay Described.—Boiling the pancreatin suspension for seventy-five seconds destroys the enzyme present in the blank flasks 2a and 2b. Therefore, if the average number of cc. of 0.02 *N* sodium hydroxide used in flasks 2a and 2b equals the average number used in 4a and 4b, which contain the unboiled pancreatin suspension, the activity shown would not be caused by the enzyme, but by something else. However, if the average amount of 0.02 *N* sodium hydroxide used in 2a

and 2b be less than the average amount used in 4a and 4b, it may be assumed that part or all of the activity was caused by the carboxypeptidase, because carboxypeptidase is the only known proteolytic enzyme that will act in the presence of formaldehyde.

Experimental Data.—A sample of Pancreatin U. S. P. was obtained from one of the larger pharmaceutical houses. The determination of carboxypeptidase was determined by the previously described method of assay. The average requirement of the control flasks was 0.7 cc. of 0.02 *N* sodium hydroxide while the average requirement of the digestion flasks was 5.9 cc. The control average, 0.7 cc., subtracted from the digestion flask average, 5.9 cc., gives 5.2 cc. of 0.02 *N* sodium hydroxide used. This is the actual carboxypeptidase activity measured in terms of cc. of 0.02 *N* sodium hydroxide.

TABLE I.—THE AMOUNT OF CARBOXYPEPTIDASE IN SAMPLES OF PANCREATIN OBTAINED FROM COMPANIES A, B, C, AND D EXPRESSED IN TERMS OF CC. OF 0.02 *N* SODIUM HYDROXIDE REQUIRED TO NEUTRALIZE THE ACIDS LIBERATED BY THE ENZYME

Co.	Sample No.	Age in Mo.	Av. No. of Cc. of 0.02 *N* NaOH for Blanks	Av. No. of Cc. of 0.02 *N* NaOH for Digestion Flasks	Av. Carboxypeptidase Activity in Terms of Cc. of 0.02 *N* NaOH
A	1A	24	0.99	5.275	4.285
A	11A	11	1.15	5.190	4.040
B	4A	53	0.775	5.300	4.525
B	10A	35	0.675	5.275	4.600
B	12	23	0.500	5.400	4.900
B	12A	21	0.600	5.120	4.520
C	7	66	0.685	5.335	4.650
C	7A	112	0.900	5.350	4.450
C	14	100	0.400	5.700	5.300
C	15	46	1.115	6.100	4.985
D	3	67	0.700	5.650	4.950
D	2A	67	0.700	5.900	5.200
D	3A	103	0.635	6.100	5.465
D	17	18	0.800	6.150	5.350
D	5	73	0.410	5.950	5.540
D	8A	67	0.475	6.010	5.535
D	6	163	0.690	5.650	4.960
D	6A	85	1.060	5.500	4.440

The most recently manufactured sample of pancreatin assayed was eleven months old, the oldest sample was 163 months old, while the average age of the samples was about 53 months. The average carboxypeptidase activity of 10 cc. of a 5% suspension of pancreatin on 5 cc. of a pumpkin seed globulin substrate made according to the Anson method, expressed in terms of 0.02 *N* sodium hydroxide solution is 4.71 cc.

CONCLUSION

The experimental data definitely indicate the presence of carboxypeptidase in Pancreatin U. S. P. Since Pancreatin U. S. P. contains trypsin, a carboxypeptidase activator, it was impossible to determine the amount of enzyme in the form of its zymogen. The assay is based entirely upon active carboxypeptidase. Since all experimental work was carried out on an impure enzymatic preparation, it was impossible to express the amount of enzyme in Anson units (7), which are based on a crystalline carboxypeptidase. However, the amount of carboxypeptidase may be expressed in terms of the cc. of 0.02 *N* sodium hydroxide required to neutralize the amino acids liberated by carboxypeptidase from a definite amount of substrate. The amount of carboxypeptidase in samples of Pancreatin U. S. P. seems not to be affected to any significant extent by the age of the samples.

SUMMARY

1. Pancreatin U. S. P. contains carboxypeptidase.

2. Pancreatin suspensions may be boiled to destroy the enzyme without affecting the degree of acidity.

3. Pumpkin seed globulin is a satisfactory substrate for the determination of carboxypeptidase.

4. Boiled Pancreatin must be used in the control in order to avoid a misinterpretation of the carboxypeptidase activity of the unboiled pancreatin on the pumpkin seed globulin.

5. The activity of carboxypeptidase may be expressed in terms of cc. of 0.02 *N* NaOH required to neutralize the amino acids produced from a definite amount of substrate.

6. The amount of carboxypeptidase in samples of Pancreatin U. S. P. was practically the same for all samples, which were obtained from different sources, and which were stored for different periods of time. This uniformity seems to indicate that carboxypeptidase is a fairly stable enzyme.

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