autofluorescent cells was as in the differentially involuted thymi of normal rats of the same age. This indicated that the development of these cells is not dependent on a process starting with thymic involution. Secondly, rats aged 3 weeks were placed on a fat-free diet until they reached 10 weeks. Their considerably involuted thymi contained as many autofluorescent cells as thymi of normal rats of the same age. This revealed the endogenous origin of the lipids of their granules as well as the fact that the formation of these cells is independent of thymic lymphocytopoiesis and lymphopoiesis, both phenomena having been considerably reduced by the inanition. Finally, rats aged 2 months were cortisone-treated or the thymus was irradiated. Thymic autofluorescent cells remained at the cortico-medullary junction while, in the cortex, macrophages were actively engulfing degenerating lymphocytes. This demonstrated that the thymic autofluorescent cells are not mobilizable macrophages.

The author thanks Mrs. M. Koekebakker for her devoted technical help.

1. Sainte-Marie, G., Anat. Rec., 1965, v151, 133.
2. Metcalf, D., Ishidate, M., Austr. J. Exp. Biol. Med. Sci., 1962, v40, 57.
3. Loewenthal, L. A., Smith, C., Anat. Rec., 1952, v112, 1.
4. Siegler, R., The Thymus in Immunobiology, R. A. Good, A. E. Galbrielsen, eds., Hoeber, New York, 1964.
5. Sainte-Marie, G., Anat. Rec., 1965, v153, 71.
6. Andreasen, E., Acta Path. Microbiol. Scand., 1943, Suppl. 49, 1.
7. Ishidate, M., Metcalf, D., Austr. J. Exp. Biol. Med. Sci., 1963, v41, 637.
8. Ito, T., Hoshino, T., Z. f. Zellforsch., 1962, v56, 445.
9. Regaud, C., Crémieu, R., Compt. Rend. Soc. de Biol., 1912, v1, 253.

Received April 29, 1966. P.S.E.B.M., 1966, v122.

The Separate Determination of Human Pepsin and Gastricsin.* (31231)

LUCIANO CHIANG,† LETICIA SANCHEZ-CHIANG,‡ STEWART WOLF AND JORDAN TANG
Psychosomatic and Neuromuscular Section, Oklahoma Medical Research Foundation, and Departments of Biochemistry and Medicine, University of Oklahoma Medical Center, Oklahoma City

The isolation and the study of 2 proteolytic enzymes, pepsin and gastricsin, from human gastric juice have been previously reported from our laboratory(1,2). Separate determination of the 2 enzymes from a mixture has been difficult because although the pH optima of the two enzymes are different(2), their respective proteolytic activities remain high throughout the acidic pH range. Thus quantitative analysis of pepsin and gastricsin separately required a fractionating technic. Such a method using a small ion-exchange column was developed(3). It allowed a quantitative measurement of the 2 enzymes in a solution with only about 5% error. The present communication describes a much less tedious method based on the discovery that the synthetic dipeptide substrate, N-acetyl-L-phenylalanyl-L-diiodotyrosine (APDT) is hydrolyzed by human pepsin but not by gastricsin. Baker previously reported that porcine pepsin also hydrolyzed this substrate(4). In the light of this finding, a method has been devised which allows a simple measurement of the 2 enzymes in a mixture. The principle of the method is to determine the amount of pepsin present using APDT as the substrate

* This investigation was supported by Research Grant AM-01107 from Nat. Inst. of Arthritis and Metab. Dis. and Institutional Cancer Grant 14-63 from Am. Cancer Soc.

† On leave of absence from Dept. of Physiology, University of Concepcion, Concepcion, Chile. Recipient of a Fulbright Travel Grant.

‡ On leave of absence from Dept. of Biochemistry, University of Concepcion, Concepcion, Chile.

and then to measure the total proteolytic activity on the conventional bovine hemoglobin substrate. The quantity of the gastricsin can then be simply calculated by the difference between these activities.

Materials and methods. Human pepsin and gastricsin were purified from human gastric juice as previously described(1,2). APDT was purchased from Cyclo Chemical Corp. (lot S 1022 B). 3,5Diiodo-L-tyrosine dihydrate was obtained from Mann Research Laboratories (lot 1040), and bovine hemoglobin was obtained from Pentex Inc. (lot 28).

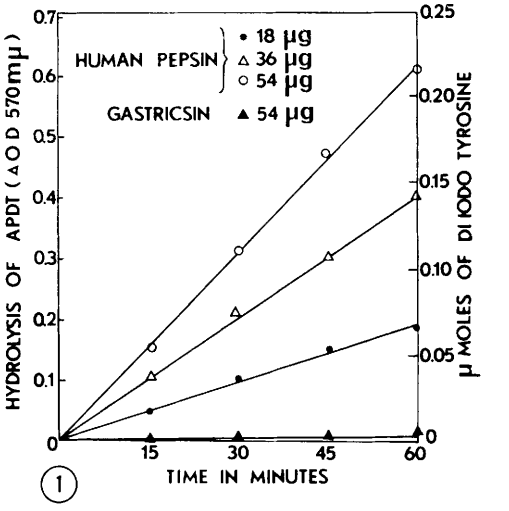
Stock solutions of human pepsin and gastricsin containing 0.5 mg enzyme per ml were prepared by dissolving the purified enzymes in 0.01 N HCl. More exact concentrations of the enzyme solutions were calculated from the absorbance at 278 mμ. The molar extinction coefficients used were 52,104 and 47,952 for human pepsin and gastricsin, respectively. These values are based on molecular weights of 33,400 for pepsin and 31,000 for gastricsin(5). Samples of gastric juice were obtained from patients of the University Hospital, University of Oklahoma School of Medicine, Oklahoma City. The gastric juice samples which were collected before and after histamine stimulation, were filtered and then dialyzed against 0.01 N HCl in order to eliminate dialyzable ninhydrin positive substances.

Determination of the hydrolysis of APDT by pepsin. Solutions of known concentrations of the enzymes were prepared by diluting the stock enzyme solutions with 0.01 N HCl to give solutions containing from 12 to 72 μg of enzyme per ml. Gastric juice samples were prepared by diluting 1 ml of gastric juice to 7.5 ml with 0.01 N HCl. The reaction mixture was composed of 0.75 ml of the enzyme solution or diluted gastric juice and 0.25 ml of 0.002 M APDT in 0.005 M NaOH. The mixture was then incubated in a water bath at 37°C for one hour (or as otherwise stated). Thereafter, 0.5 ml of acetate-cyanide buffer was added followed by addition of 0.5 ml of ninhydrin. The color developed according to the method of Rosen(6) using 5 ml of 50% isopropanol diluent and the absorbancy was read at 570 mμ against water. All samples were run in duplicate. Duplicate blanks were run by adding the substrate after the enzymes were inactivated by addition of the acetate-cyanide buffer.

The amount of substrate hydrolyzed was calculated from the ninhydrin molar extinction coefficient of diiodotyrosine (which was found to be 20.3) and corrected for ninhydrin positive substances detected in the blank.

Determination of total proteolytic activity with hemoglobin. This determination is a modification of that described by Anson and Mirsky(7). The acidified hemoglobin substrate was freshly prepared by addition of 7 ml of 0.3 N HCl to 20 ml of a 10% hemoglobin solution. This solution was diluted with water to 100 ml (final pH 3.1). Standard solutions of the enzymes were prepared by diluting the stock enzyme solutions with water so that the final concentration of gastricsin was 4 to 30 μg per ml and that of pepsin was 10 to 60 μg per ml. Samples of gastric juice were prepared by diluting 0.5 ml of gastric juice with water to a volume of 5 ml. All determinations were run in duplicate. To 0.5 ml of the enzyme solution or diluted gastric juice was added 0.5 ml of 0.1 M sodium citrate buffer (pH 3.1) and 5 ml of acidified hemoglobin solution (pH 3.1). The reaction mixture was then incubated for 10 minutes at 37°. (The hemoglobin solution and enzyme solutions were warmed to 37° prior to their mixing.) The reaction was stopped by addition of 10 ml of a 5% trichloroacetic acid (TCA) solution. After filtering through Whatman No. 50 filter paper, the optical density was measured at 280 mμ in an Hitachi Perkin-Elmer Model 139 spectrophotometer. A blank was run in which the TCA was added prior to the enzyme. The value of Δ O. D. 280 mμ represents the difference of the absorbancy of the sample tube and that of the blank.

Results. Hydrolysis of APDT by human pepsin. Incubation of APDT with human pepsin resulted in a linear increase of ninhydrin color with time, indicating hydrolysis of APDT by human pepsin. The product of the enzymic hydrolysis was analyzed on a Spinco Model 120 B Amino Acid Analyzer using a 15-cm column which was eluted with 0.2 M



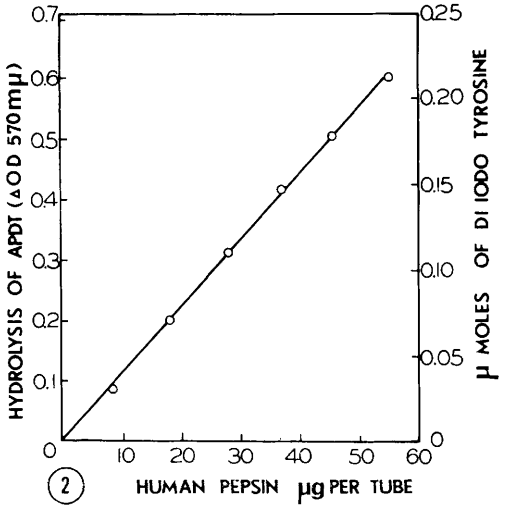


FIG. 1. Hydrolysis of 0.5 µmole of APDT by human pepsin and gastricsin at 37°C.

FIG. 2. Effect of human pepsin concentration on hydrolysis of APDT during one hour of incubation at 37°.

sodium citrate, pH 5.28. Only one ninhydrin positive product was formed and it was eluted from the column at the same position as authentic diiodotyrosine. These results indicated that the direct ninhydrin color determination measures the peptic hydrolysis of APDT to form N-acetyl-L-phenylalanine and diiodotyrosine and that other reactions, such as transpeptidation, do not occur.

The rate of hydrolysis of APDT by several concentrations of human pepsin under the experimental conditions described above was linear for 60 minutes (Fig. 1). Gastricsin, under the same conditions, did not hydrolyze APDT (Fig. 1).

The rate of hydrolysis of APDT of up to 0.21 µmoles per hour was found to be directly proportional to the amount of human pepsin present (Fig. 2).

An optical density of 0.6 at 570 mµ corresponds to the formation of 0.21 µmoles of diiodotyrosine. Thus the amount of pepsin can be determined directly from the standard curve shown in Fig. 2.

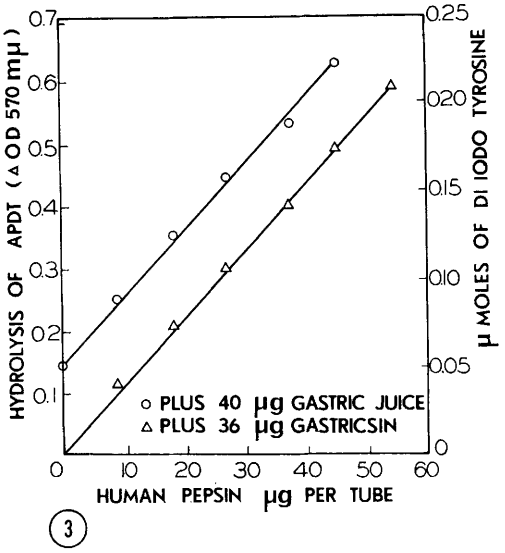
Effect of presence of gastricsin and other gastric contents on hydrolysis of APDT by human pepsin. In order to use APDT as the substrate for determination of pepsin, the effect of the presence of other gastric juice components was investigated. It was found that the hydrolysis of APDT by pepsin was not affected by the presence of either gastricsin or human gastric juice. Fig. 3 shows that when 36 µg of gastricsin were added to varying amounts of human pepsin ranging from 0 to 54 µg, the rate of hydrolysis of APDT was essentially the same as that of human pepsin alone. When 40 mg of lyophilized human gastric juice were added, the slope of the line remained the same. The elevation of the line in this case was clearly due to the human pepsin in lyophilized gastric juice.

Determination of human pepsin and gastricsin in a mixture. The total proteolytic activity, pepsin plus gastricsin, can be determined by the hemoglobin method. The quantity of human pepsin in a mixture can be determined using APDT as the substrate since it is not hydrolyzed by gastricsin. Thus the difference between the total proteolytic activity using hemoglobin substrate and that using the APDT substrate yields the activity of gastricsin. Similarly one can construct standard curves to determine these enzymes in a mixture.

Shown in Fig. 4, the rate of hydrolysis of hemoglobin (Δ O. D. 280 mµ) is linearly related to the amount of enzyme up to 15 µg of gastricsin or 30 µg of pepsin. When both enzymes were present in the sample the proteolytic activity with hemoglobin as substrate was found to be additive. The comparative proteolytic activity of pepsin against hemo-

globin and APDT is shown graphically in Fig. 5.

After the rate of hydrolysis of APDT (O. D. 570 mμ) and the rate of hydrolysis of hemoglobin (Δ O. D. 280 mμ) had been determined for a given enzyme mixture, according to the procedures described under *Methods*, the quantities of pepsin and gastricsin in the



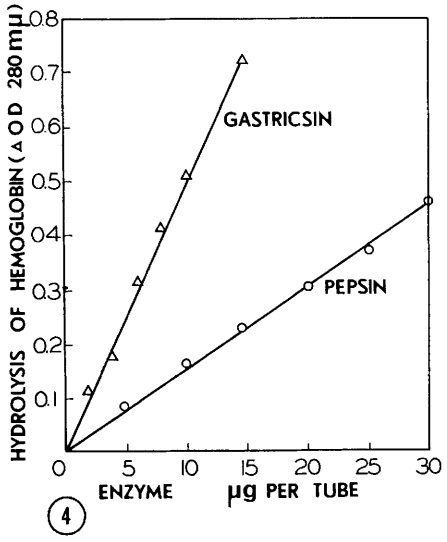


FIG. 3. Hydrolysis of APDT by human pepsin (0 to 54 μg per tube) in presence of 40 μg gastric juice or 36 μg gastricsin.

FIG. 4. Effect of human pepsin or gastricsin concentration on hydrolysis of hemoglobin during 10 min incubation at 37°.

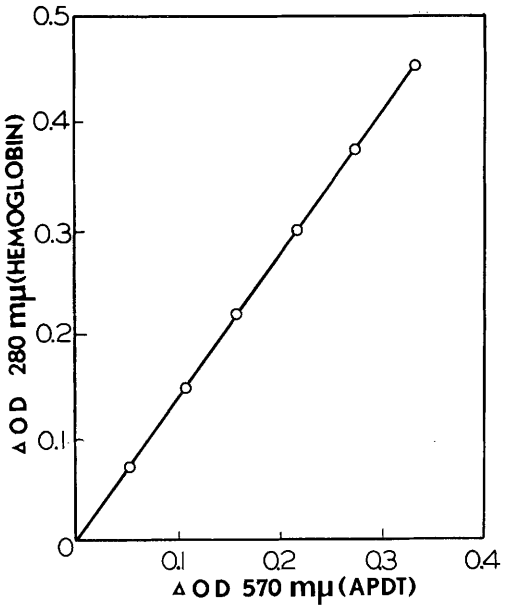


FIG. 5. Plot of hydrolysis of hemoglobin (280 mμ) and APDT (570 mμ) by 6 different concentrations of human pepsin.

sample were then calculated according to the following equations:

$$\mu g \text{ Pepsin/ml gastric juice} = \frac{\Delta \text{O.D. } 570/\text{hr} \times 10^*}{\text{Sp } 570} = \frac{\Delta \text{O.D. } 570/\text{hr} \times 10^*}{0.0111}$$

$$\mu g \text{ Gastricsin/ml gastric juice} = \frac{\text{Total } \Delta \text{ O.D. } 280/10 \text{ min} \times 20^* - (\text{Sp } 280 \times \mu g \text{ Pepsin})}{S_G\ 280}$$

$$= \frac{\text{Total } \Delta \text{ O.D. } 280/10 \text{ min} \times 20^* - (0.015 \times \mu g \text{ Pepsin})}{0.050}$$

Where: Sp 570 = Δ O.D. 570/hr/μg Pepsin = 0.0111
Sp 280 = Δ O.D. 280/10 min/μg Pepsin = 0.015
S_G 280 = Δ O.D. 280/10 min/μg Gastricsin = 0.050

§ Dilution Factor.

These specific activity values are valid only under the exact time, concentration, and volume conditions described before. Small variations of less than 10% in the specific activity value (Sp 570) were obtained with different batches of APDT.

An alternative is the graphical determination as outlined in the following:

TABLE I. Analysis of Pepsin and Gastricsin in the Mixture of Reconstituted Enzymes.

Theoretical		Found	
Gastricsin	Pepsin	Gastricsin	Pepsin
μg			
0	50	0	48
10	40	10.5	41
20	30	21.0	29
30	20	31.3	21
40	10	38.8	10
50	0	47.5	0

(i) Convert O. D. 570 mμ to μg of pepsin using Fig. 2.

(ii) Convert O. D. 570 mμ to Δ O. D. 280 contributed by pepsin using Fig. 5.

(iii) Subtract the result in (ii) from Δ O. D. 280 mμ determined from the hemoglobin assay.

(iv) Convert the result in (iii) to μg of gastricsin using Fig. 4, line for gastricsin.

Test of method on reconstituted mixture of human pepsin and gastricsin. In order to test this analytical method, determinations were carried out on samples of known amounts of pepsin and gastricsin, reconstituted from purified enzymes. The results are shown in Table I, which indicates that the values found are very near the theoretical values. In all cases, the error was less than 5%.

Analysis of pepsin and gastricsin in human gastric juice. The contents of pepsin and gastricsin in the gastric juice from 9 individuals, both before and after histamine stimulation, were analyzed and are shown in Table II.

Summary. The synthetic substrate, N-acetyl-L-phenylalanyl-L-diiodotyrosine was found to be hydrolyzed by pepsin but not at all by gastricsin. This difference has made possible the separate determination of pepsin and gastricsin in a mixture. The proteolytic activity of the mixture against APDT yielded the amount of pepsin present. The proteolytic activity of the mixture against bovine hemoglobin yielded the total amount of pepsin and gastricsin present. The amount of gastricsin was then simply calculated from the differences in these activities. The accuracy of the method was demonstrated using a reconstituted mixture of human pepsin and gastricsin. The content of pepsin and gastricsin in the native gastric juice was measured in 9 individuals both before and after histamine stimulation. Histamine stimulated the secretion of both pepsin and gastricsin.

TABLE II. The Contents of Pepsin and Gastricsin in the Gastric Juice of Individuals Before and After Histamine Stimulation.

	Before stimulation			After stimulation		
		Gastricsin	Pepsin		Gastricsin	Pepsin
Individuals	pH*	μg/ml gastric juice		pH*	μg/ml gastric juice	
1	8.5	0	0	1.3	150	144
2	2.2	100	304	1.5	144	460
3	1.7	175	455	1.0	175	525
4	1.7	112	156	1.2	136	330
5	8.5	0	0	1.5	165	275
6	1.6	155	205	1.3	200	575
7	1.5	240	500	1.5	320	720
8	1.7	260	360	1.2	520	540
9	1.5	380	820	1.2	410	870

* The pH of the samples of gastric juice were taken immediately after collection, using a pH meter.

The authors are grateful to Dr. John Mills, Mr. Gene May and Mr. Lee Poyer for helpful suggestions in preparing this manuscript.

1. Richmond, V., Tang, J., Wolf, S., Trucco, R. E., Caputto, R., Biochem. Biophys. Acta, 1958, v29, 453.

2. Tang, J., Wolf, S., Caputto, R., Trucco, R., J. Biol. Chem., 1959, v234, 1174.

3. Tang, J., Tang, K. I., ibid., 1963, v238, 606.

4. Baker, L. E., ibid., 1951, v193, 809.

5. Mills, J., Ph.D. Dissertation, University of Oklahoma, 1965.

6. Rosen, H., Arch. Biochem. Biophys., 1957, v67, 10.

7. Anson, M. L., Mirsky, A. E., J. Gen. Physiol., 1932, v16, 59.

Received February 17, 1966. P.S.E.B.M., 1966, v122.