Physiologic Fitness of Enterokinase* (34126)

J. Haskell Milstone, Melvin V. Goldblat,[1] and Vivian K. Milstone
Department of Pathology, Yale University School of Medicine, New Haven, Connecticut 06510

The recent finding that plasmin activates trypsinogen (1) called attention to the need for a simplified method for the assay of trypsinogen activator. It would be desirable for the method to be applicable to the assay of different activators, so that their relative potency might be compared. Such a method is herein described, and used to compare the two activators present physiologically.

When the pancreatic secretion reaches the intestine, its trypsinogen is activated by the enterokinase of the intestine (2). The resulting trypsin then converts various pancreatic zymogens to their active forms (3). Trypsin also activates its own precursor. But the present results indicate that enterokinase is better adapted to activate trypsinogen physiologically.

The principle of the method is that the rate of activation of trypsinogen is proportional to the concentration of activator used, provided that the effect of complications is not too great. In his masterly analysis, Kunitz (2) took into account the complications due to the action of trypsin: autocatalytic activation, formation of inert protein, and autolysis. All of these complications were reduced by using dilute trypsinogen, 0.065 mg/ml. In addition, Kunitz further limited autolysis by activating at 5°, and formation of inert protein by activating below pH 6.0. He then defined his enterokinase unit in terms of these conditions and described a standard method which involved trypsin assays on a series of samples taken during several hours of activation. At the same time Kunitz described a simplified procedure in which a sample was assayed for trypsin after a 30-min activation at pH 7.5–7.6 and 25°. The concentration of enterokinase in standard units was read from an empirically constructed chart. In the present method dilute trypsinogen is activated at pH 7.7 and 23°, and samples are assayed for trypsin at 1 and 61 min.

In their detailed study of the autocatalytic reaction, McDonald and Kunitz (4) found that formation of inert protein was suppressed by concentrations of calcium ion greater than 0.02 *M*, and that the rate of activation by trypsin increased with increasing concentrations of calcium chloride through 0.5 *M*, the highest concentration reported. Comparable results were obtained in this laboratory with activation by trypsin; whereas the optimal concentration of calcium chloride was 0.02 *M* when the activator was plasmin (1). It is now reported that a third, and different, optimum was found when enterokinase was the activator.

Materials and Methods. Tris: Tris (hydroxymethyl)aminomethane. Sigma Chemical Co. Enterokinase (ex hog duodenal fluid): Batch 21567. Pierce Chemical Co. A solution containing 3 mg/ml was dialyzed against 0.05 *M* Tris buffer, pH 7.7, diluted with the same buffer to contain 1 mg protein per ml and stored at —23°. With a sensitive test (5), this material was found to contain chymotrypsinogen-activator, most, but not all of which was separated from enterokinase on a column of DEAE-cellulose. However, all tests reported below, including tests for other substrates of enterokinase, were done on the unfractionated material. The name "enteropeptidase," has been recommended for this enzyme (6); but a current textbook (7) has retained the name, "enterokinase," which has considerable historical interest. Trypsin

* This study was supported by USPHS Grant HE-03906 from the National Heart Institute.

[1] Recipient of a USPHS 2T5-GM-37-05 Summer Fellowship, 1966. The results were developed from those used to satisfy the thesis requirement for graduation from the Yale University School of Medicine.

(bovine pancreas): Twice crystallized, salt-free, lyophilized. Worthington Biochemical Corp. A stock solution, containing 1 mg/ml 0.001 *M* HCl, was diluted with 0.001 *M* HCl immediately before the tests. Trypsinogen (bovine pancreas): Crystallized by method of Balls (8). Worthington Biochemical Corp. Stock solutions contained 0.5 mg/ml 0.001 *M* HCl. Inhibitor-free trypsinogen: Prepared from trypsinogen by method of Kunitz and Northrop (2). This procedure, which involves precipitation by 2.5% trichloroacetic acid, would be expected to remove not only the inhibitor described by Kunitz and Northrop (3), but also the inhibitor described by Kazal, Spicer, and Brahinsky (9). The latter inhibitor was soluble in 2.5% trichloroacetic acid and was only crystallized from it at 5° after the pH was adjusted to 3.25 with NaOH. TAMe : *p*-Tosyl-L-Arginine methylester hydrochloride. Mann Research Laboratories. LMe : L-Lysine methylester dihydrochloride. Mann Research Laboratories.

Estimation of protein. Method of Lowry *et al.* (10), with crystallized bovine albumin as standard.

Assay of esterase. 5.0 ml 0.1 *M* TAMe in 0.15 *M* KCl, 0.8 ml 0.5 *M* $CaCl_2$ and 3.0 ml 0.15 M KCl were added to a 50-ml vessel of the Radiometer pH-stat. The solution was kept under an atmosphere of nitrogen at 23 ± 1° and it was stirred by motor as 0.200 ml of 0.05 *M* NaOH in 0.15 *M* KCl was added from the 2.5-ml buret of the pH-stat. One milliliter of the sample to be tested was then added and the solution was maintained at pH 8.00 by the addition of NaOH in KCl by the pH-stat. The rate of hydrolysis of TAMe was determined from the average amount of base added per minute during the third and fourth minutes after starting the reaction. Activities were expressed as micromoles per minute per milliliter of initial hydrolysis mixture. In tests for hydrolysis of LMe, the mixture contained 5.0 ml 0.02 *M* LMe in 0.15 *M* KCl, 0.8 ml 0.5 *M* $CaCl_2$, 1.6 ml 0.15 *M* KCl, 1.6 ml 0.05 *M* NaOH in 0.15 *M* KCl and 1.0-ml sample.

Activation of trypsinogen. 0.60 ml of a $CaCl_2$ solution at five times the desired final concentration was added to 1.5 ml 0.05 *M* Tris buffer, pH 7.7 in 0.3 *M* KCl and 0.3 ml Tris buffer, pH 7.7, at 23 ± 1°. One minute after the addition of 0.3 ml trypsinogen (0.5 mg/ml), 0.3 ml of the material to be tested as activator was added. Samples were assayed for TAMe esterase activity at 1 and 61 min. The increase in 60 min was a measure of activator. For this system, one unit of trypsinogen activator per milliliter of activation mixture was defined as the amount that produced enough trypsin to hydrolyze TAMe at the rate of 1 μmole/min/ml activation mixture or 0.1 μmole/min/ml of hydrolysis mixture.

Results. In a previous study in this laboratory (1), trypsinogen was used at 0.2 mg/ml in order to make the test more sensitive to the activating effect of plasmin; but there was some indication of autocatalysis. More trypsin was produced in the second hour of activation, (when more trypsin was present), than in the first. This observation was now repeated in a series of activations by 0.005 mg enterokinase per milliliter, in the presence of 0.005 *M* calcium chloride. More trypsin was produced in the second hour of activation than the first, when trypsinogen was initially 0.2 mg/ml. But when the initial concentration of trypsinogen was 0.1 mg, 0.05 mg, or 0.025 mg/ml, there was no obvious indication of autocatalysis. With initial concentration of trypsinogen 0.05 mg/ml and calcium chloride 0.0002 *M*, there was still a tendency for the yield of trypsin to be greater near pH 6 than near pH 8, when incubation was continued for 24–48 hr at 24°. Higher yields were still obtainable after long incubation at 4° than at 24°. So, complications were not altogether eliminated. However, they did not cause obvious interference when tests were limited to the amount of trypsin produced in 1 hr at 23 ± 1°. For this determination there was a broad optimal range extending from pH 7.0 to pH 8.0. This is reminiscent of the broad plateau shown by Kunitz for the velocity constant of activation by enterokinase (2). However, present conditions were different; and there was no superimposed peak near pH 6.2, as observed by Kunitz. Activations were then performed routinely for 61 min at pH 7.7.

Figure 1 shows the results obtained with this system in the presence of 0, 0.0000064, 0.000032, 0.00016, 0.0008, 0.004, 0.02, 0.1, and 0.5 *M* added calcium chloride. When the activator was trypsin, most activation occurred in the presence of 0.5 *M* calcium chloride, as reported long ago by McDonald and Kunitz (4). When the activator was enterokinase, most trypsin was produced in the presence of 0.00016 *M* calcium chloride. Enterokinase in the presence of 0.0002 *M* calcium chloride proved to be more effective than trypsin with 0.5 *M* calcium chloride. This superiority was still manifest when the activations were performed at 37° or when inhibitor-free trypsinogen was used as the zymogen. Results were essentially the same when the trypsinogen had been dialyzed against 0.001 *M* hydrochloric acid.

When trypsinogen was activated by 0.01 mg enterokinase per milliliter in the presence of varying concentrations of magnesium chloride, 0.00016 *M* was optimal, although the curve rose much less than with calcium chloride. Nevertheless, it was more than twice as high as the best results with 0.01 mg

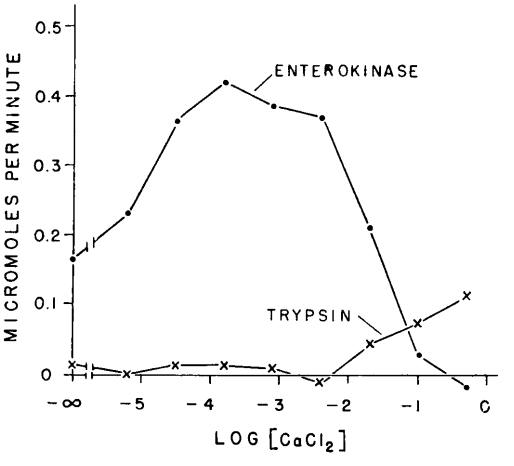

FIG. 1. Comparison of enterokinase and trypsin as activators of trypsinogen in the presence of various concentrations of calcium chloride. Trypsinogen, 0.05 mg/ml. Enterokinase or trypsin, 0.01 mg/ml. Degree of activation expressed as trypsin activity produced in 1 hr, and plotted as μmoles TAMe hydrolyzed per minute. Points below zero imply that there was less activity at 61 min than at 1 min. These small differences are within the limits of experimental error.

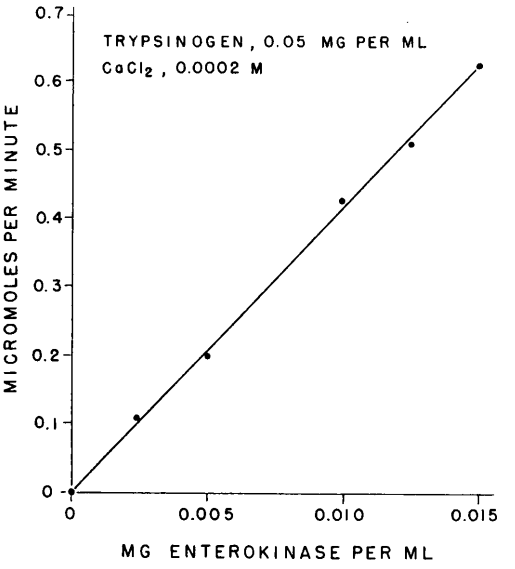

FIG. 2. Relation of trypsin activity produced in 1 hr to concentration of enterokinase used to activate trypsinogen. Trypsin activity plotted as μmoles TAMe hydrolyzed per minute.

trypsin per milliliter as activator, which were obtained with 0.5 *M* magnesium chloride, the highest concentration tested. Finally, enterokinase was a better activator than trypsin in the presence of 0.001 *M* calcium chloride plus 0.0005 *M* magnesium chloride. The inclusion of the magnesium salt along with the calcium did not offer an obvious chemical advantage.

In the presence of 0.0002 *M* calcium chloride, there was a linear relation between the concentration of enterokinase and the trypsin produced in 1 hr, as shown in Fig. 2. If unit concentration of trypsinogen activator in this system was taken as that which produced enough trypsin to hydrolyze TAMe at the rate of 0.1 μmole/min/ml hydrolysis mixture, there was an average value of 414 units/mg enterokinase for the activations in Fig. 2. In contrast, the average value for trypsin was 115 units/mg when it functioned in the presence of 0.5 *M* calcium chloride. Although enterokinase was a stronger activator of trypsinogen, it had a much weaker hydrolytic action on LMe and TAMe, as shown in Table I.

Discussion. The range of calcium concentrations listed for the pancreatic, duodenal,

TABLE I. Activities of Enterokinase Compared with Those of Trypsin.[a]

	Trypsinogen activation (units/mg)	LMe hydrolysis (μmoles/min/mg)	TAMe hydrolysis (μmoles/min/mg)
Enterokinase	414	<0.1	1
Trypsin	115	2.8	390

[a] Trypsinogen activations were performed in the presence of 0.0002 *M* calcium chloride for enterokinase, and 0.5 *M* calcium chloride for trypsin. Tests for hydrolysis involved action of enterokinase and trypsin, respectively, on 0.01 *M* LMe and on 0.05 *M* TAMe.

and jejunal secretions of man and other mammals corresponds to 0.0002–0.0032 *M* calcium chloride (11). Throughout this range, enterokinase was much more effective than trypsin as an activator of trypsinogen. In contrast, the enterokinase preparation had very weak hydrolytic effect on LMe and TAMe. According to Yamashina (12), even the low activity on synthetic substrates shown by his highly purified enterokinase might be due to a contamination by trypsin.

All this may signify that enterokinase is narrowly adapted to its function. It remains to be asked whether enterokinase functions merely as the starter for the autocatalytic reaction, or whether it activates most of the trypsinogen, physiologically. That the latter possibility deserves consideration is suggested by the fact that Kunitz found an amount of enterokinase in duodenal fluid (13) that was 13 times the concentration he used to produce the fastest activation of dilute trypsinogen at pH 7.6 (2). To the extent that autocatalysis might occur physiologically, when trypsinogen is more concentrated, it would be less efficient, due to the parallel formation of inert protein in the presence of physiologic concentrations of calcium. It would also be susceptible to competition from other substrates for trypsin, including other zymogens. In the final analysis, the answer will depend on observations made under conditions more closely approximating the physiologic.

Summary. For the activation of trypsinogen by enterokinase the optimal concentration of calcium chloride was near 0.0002 *M*, which is at the lower end of the range of calcium concentrations prevailing in the upper small intestine. Magnesium chloride was not so effective. For an hour's activation at pH 7.7 and 23°, in the presence of 0.0002 *M* calcium chloride, the rate of trypsin production was directly proportional to the concentration of enterokinase. This system was used for a simplified assay of trypsinogen-activator; and calcium was varied according to whether the activator was enterokinase or trypsin. Although partially purified enterokinase had much more trypsinogen-activating effect than an equal weight of crystallized trypsin, it had much less hydrolytic effect on lysine methylester and tosylarginine methylester. In comparison with trypsin, enterokinase is more precisely adapted to the activation of trypsinogen.

1. Goldblat, M. V., Kline, D. L., and Milstone, J. H., Proc. Soc. Exptl. Biol. Med. **125**, 763 (1967).

2. Kunitz, M., J. Genl. Physiol. **22**, 429 (1939).

3. Northrop, J. H., Kunitz, M., and Herriott, R. M., "Crystalline Enzymes," 2nd Ed., Columbia Univ. Press, New York (1948).

4. McDonald, M. R. and Kunitz, M., J. Genl. Physiol. **25**, 53 (1941).

5. Milstone, J. H. and Milstone, V. K., Thromb. Diath. Haemorrhag **17**, 388 (1967).

6. International Union of Biochemistry, "Enzyme Nomenclature," Elsevier Amsterdam (1965).

7. White, A., Handler, P., and Smith, E. L., "Principles of Biochemistry, " 4th Ed., McGraw-Hill, New York (1968).

8. Balls, A. K., Proc. Natl. Acad. Sci. U. S. **53**, 392 (1965).

9. Kazal, L. A., Spicer, D. S., and Brahinsky, R. A., J. Am. Chem. Soc. **70**, 3034 (1948).

10. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem **193**, 265 (1951).

11. Altman, P. L. and Dittmer, D. S., "Blood and Other Body Fluids," Fed. Am. Soc. Exptl. Biol. Washington, D.C., 1961.

12. Yamashina, I., Acta Chem. Scand. **10**, 739 (1956).

13. Kunitz, M., J. Genl. Physiol. **22**, 447 (1939).

Received April 7, 1969. P.S.E.B.M., 1969, Vol. 131.