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Measurement of Trypsin Activity Using a Radioactive Substrate.* (26298)

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Recently the authors reported their use of RIHSA (radioactive iodinated human serum albumin—Abbott Laboratories) as a substrate for measuring pepsin activity(1). Because of its simplicity and reliability for assaying the activity of pepsin, its value for measuring trypsin activity was also tested. The procedure was similar to that reported earlier for pepsin, except for necessary changes in pH for optimum activity of trypsin, in enzyme concentration and in method of precipitation after incubation of enzyme and substrate.

This is a report of various factors affecting trypsin activity as measured by the RIHSA, Anson(2) and Kunitz(3) methods. Spectrophotometric measurements were also made of the supernatant fluid derived from analyses with hemoglobin as substrate. This latter technic which combines features from both the Anson and Kunitz methods is referred to in the text as the "spectrophotometric" method.

Materials and methods. Lyophilized crystalline trypsin—Armour Laboratories (Tryptar®) contained 2640 units of trypsin activity per milligram. The enzyme was dissolved in one of various aqueous solutions used in these studies. Control solutions for which the solvent was deionized water or McIlvaine's Buffer, were always included.

Egg albumin (Mallinckrodt Laboratories) dissolved in distilled water at a concentration of 4 mg/ml served as the stock substrate for the RIHSA studies. Immediately before its use, this stock solution was diluted 1:1 with 0.2 M Sorenson's phosphate buffer and one microcurie of RIHSA was added for each 10 ml of solution. This mixture was then passed through an anion exchange resin column to remove radioactive iodine not bonded to albumin(4). Stable albumin was added to the substrate to promote precipitation of the undigested protein at termination of the

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TABLE I. Trypsin Stability as a Function of Solvent pH.

pH	Trypsin activity* (with stand. errors)	pH	Trypsin activity* (with stand. errors)
2.5	80 ± 5.5	5.5	79 ± 6.2
3.0	86 3.1	6.0	63 6.3
3.5	94 4.7	6.5	46 3.2
4.0	100 5.8	7.0	37 1.6
4.5	100 5.7	7.5	34 1.6
5.0	90 6.4	8.0	34 1.6

* Arbitrary units.

Time between solvation and assay of trypsin—3 hr.

Incubation pH—7.5.

incubation period. Hemoglobin and casein substrates were prepared in accordance with published methods(2,3).

A pre-determined amount of one of these substrates was added to each tube containing trypsin after which all tubes were incubated in a water bath at 37°C for one hour. After incubation instantaneous inactivation of the enzyme and precipitation of both enzyme and substrate were achieved by addition of acid. Five % tricholoracetic acid was used as specified in the Anson and Kunitz methods. For the RIHSA method, 5% sulfosalicylic acid was found to be more effective in promoting precipitation than was 5% trichloroacetic acid or other concentrations of sulfosalicylic acid.

After centrifugation, aliquots were taken for the various analyses. For the RIHSA method the radioactive iodine freed from the digested albumin molecules remained in the supernatant fluid. The gamma radiation emitted by the iodine in an aliquot of the supernatant was assayed in a well scintillation counter as a measure of enzyme activity. Analyses of the supernatant from the hemoglobin and casein substrates were made according to the methods outlined by Anson and Kunitz.

Results. Preliminary investigations using RIHSA method. Trypsin stability as a function of solvent pH was investigated by dissolving trypsin in an aqueous solution containing varying amounts of 0.005 N citric acid and 0.005 N sodium hydroxide to give a pH range from 2.5 to 8.0. These solutions were permitted to stand for 3 hours prior to incubation. In this study it was necessary to increase the final concentration of the substrate buffer to 0.2 M to maintain the incubation pH at 7.5 ± 0.2. Results are shown in Table I.

A range of pH from 5.0 to 8.5 during incubation was used to study trypsin activity as a function of its incubation pH. This was accomplished by using varying concentrations of the components of 0.2 M phosphate buffer in making up the substrate. Results indicate that the pH for optimum tryptic activity is between pH 6.5 and 7.0 (Table II).

Trypsin activity as measured with RIHSA, hemoglobin or casein substrates. The activity of trypsin *vs.* its concentration was studied by incubating samples of trypsin varying in concentration from 50 to 8000 trypsin units per ml. A comparison of the results from the RIHSA, Anson and Kunitz methods is plotted in Fig. 1.

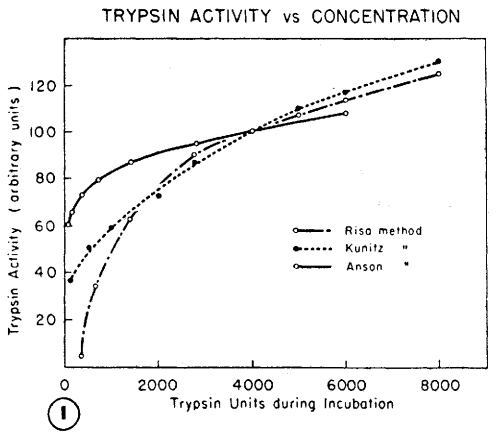
Results using the RIHSA, Anson, Kunitz and spectrophotometric methods for measuring tryptic activity at various incubation times are shown in Fig. 2. Trypsin concentration was maintained at 5280 T.U./ml for the RIHSA method and at 129 T.U./ml for the other methods.

Discussion. Our studies indicate that trypsin is most stable in solution at pH 4.5 which is near the pH for optimum stability of pepsin(1). The decrease in trypsin stability as the solvent pH approaches that for optimum activity of this enzyme is thought to be attributable to autolysis. The decrease in stability using solvents with pH values below 4.5 is possibly due to the effect of the citric acid used to control the pH. This pH for optimum trypsin stability is higher than the value of 2.0 reported by Kunitz and Northrop(5) and differs considerably from the

TABLE II. Trypsin Activity when Incubated with Substrate at Various pH Values.

pH	Trypsin activity* (with stand. errors)	pH	Trypsin activity* (with stand. errors)
5.0	50 ± 5.2	7.0	100 ± 2.7
5.5	67 3.8	7.5	94 4.3
6.0	86 2.8	8.0	80 4.2
6.5	100 1.9	8.5	61 5.6

* Arbitrary units.



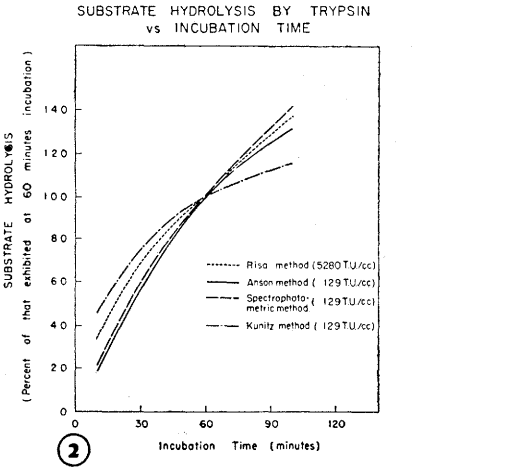


FIG. 1. Trypsin activity as a function of its concentration as measured by three methods.

FIG. 2. Substrate hydrolysis as a function of incubation time as measured by four methods.

work of Crewther(6) who found peaks of trypsin stability at pH 2.9, 6.6 and 10.7. It is possible that differences in substrates used for assay of trypsin activity may account for the greater part of these apparent discrepancies. To facilitate an evaluation of relative merits of these various methods, we chose to standardize the incubation pH at 7.6 for all studies in which these comparisons were being made.

In both the Anson and Kunitz methods the pH of the substrate used for assay of trypsin activity is maintained at 7.6.

Using 5% casein as the substrate Northrop and Kunitz(7) found a flat broad curve of optimum activity extending from pH 7.5 to 9.5. Bergmann and associates(8) show a sharp domed curve with a peak at pH 7.75 employing benzoyl-l-arginineamide as a substrate. These values are both above the optimum incubation pH as determined by the RIHSA method (pH 6.5-7.0).

Fig. 1 shows that for low concentration of trypsin, the RIHSA method is not as sensitive as either the Anson or Kunitz methods. Of these methods, that of Anson appears to be the most useful for measuring very small amounts of trypsin. With concentration of trypsin above about 2000 T.U./ml small differences in enzymatic activity are most readily detectable by the Kunitz or RIHSA methods.

As the concentration of trypsin was increased above about 2000 T.U./ml the results obtained by the Kunitz and RIHSA methods show good agreement. However, because of its simplicity, the RIHSA method is likely to be preferred for measuring the activity of relatively large concentrations of trypsin. These results of trypsin activity *vs.* its concentration agree favorably with those reported by Anson(2) and Kunitz(3).

There is a gradual decrease in trypsin activity with increasing incubation time for all the methods used. This is evidenced by the decreasing slope of the hydrolysis curve (Fig. 2). This is most apparent for long incubation times using Kunitz' method in which concentration of the substrate is likely the actual limiting factor for the reaction. Good agreement is found for all methods when incubation time is restricted to one hour or less.

Summary. Comparison has been made of measurements of trypsin activity by a substrate containing RIHSA (radioactive iodinated human serum albumin—Abbott Laboratories) and the commonly used methods of Anson and Kunitz. In this method, trypsin was incubated with an albumin substrate containing RIHSA, followed by precipitation of the undigested substrate with sulfosalicylic acid and measurement of radioactive digestion products in the supernatant fluid. Trypsin was found to be most stable in solution at a pH near 4.5 using the RIHSA method. Its activity was maximal at pH 6.5-7.0. The RIHSA method did not provide as sensitive

a test for low concentrations of trypsin as did the methods of Anson and Kunitz. However, because of its simplicity for assay of tryptic activity, the RIHSA method is considered to be of definite value when enzyme concentrations to be assayed are in excess of 2000 T.U. per ml.

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Endotoxin Shock and the Coagulation Mechanism: Modification of Shock with Epsilon-Aminocaproic Acid. (26299)

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Injection of a lethal dose of endotoxin into the dog is followed by a characteristic pattern of hemodynamic changes(1,2). Within a minute there is a marked decline in systemic blood pressure and rise in portal vein pressure. Fifteen to 30 minutes later the blood pressure tends to approach pre-injection level, then a gradual decline occurs, accompanied by oliguria or anuria, acidosis, and hemoconcentration. Death takes place within 24 hours.

We have reviewed elsewhere our investigations on the mechanism of vascular alterations induced by endotoxin in the dog(2,3.4). Briefly, the initial vasoconstriction caused by endotoxin is probably due to histamine or a histamine-like substance, release of which is mediated through a component or components of blood. Contraction of an isolated canine vein occurred when the vessel was exposed to a bath containing endotoxin and fresh whole blood. Enzymatic participation in the reaction was suggested by the latent period that occurred before contraction took place; and by an activating serum factor that was dialyzable and heat-labile. Involvement of proteolytic activity was considered, possibly associated with the coagulation mechanism. Such a concept is suggested by other investigations. Unger and Mist(5) postulated that specific antigen-antibody reactions, peptones or bacterial polysaccharides caused liberation of histamine through increased proteolytic activity of the serum. Others have concluded(6,7) that the Shwartzman reaction in rabbits is a result of coagulation alterations caused by endotoxin. Deutsch and Elsner(8) have stated that administration of endotoxin results in activation of the plasminogen-plasmin system in which an initial state of hypercoagulability is followed by accelerated fibrinolysis. Gans and Krivit(9, 10) reporting on some significant comparative studies on endotoxin in rabbits and dogs as to effects on the coagulation mechanism, concluded that endotoxin did not activate the plasminogen-plasmin system in rabbits. Therefore, in this species endotoxin causes extensive thrombosis, which is a feature of the Shwartzman reaction. Endotoxin does not produce the Shwartzman reaction in the dog because plasminogen is activated and thrombolytic activity is prominent. Pertinent to the present study they found that endotoxin in the dog caused a marked increase in activity of plasminogen-activator, and a decline in plasminogen concentration.

Epsilon-aminocaproic acid (hereafter referred to as EACA) is a potent inhibitor of plasminogen activation(11). Sherry and his associates(12,13) have studied EACA intensively, both *in vitro* and in human subjects,