

THE ESTIMATION OF ACTIVE NATIVE TRYPSIN IN THE PRESENCE OF INACTIVE DENATURED TRYPSIN

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Trypsin, which can catalyze the hydrolysis of proteins, is itself a protein (Northrop and Kunitz, 1932). The denaturation of proteins is reversible (Anson and Mirsky, 1931). When trypsin is denatured its proteolytic activity is completely lost. When the denaturation of trypsin is reversed the original proteolytic activity is completely restored (Northrop, 1932). The denaturation of trypsin and its reversal can therefore be followed by measurements of proteolytic activity. The present paper describes a technique for the measurement of the activity of native trypsin in the presence of inactive, denatured trypsin. Later papers will describe the application of this technique to the study of the effect of various denaturing agents such as heat, acid, alcohol and urea on the equilibria between native and denatured trypsin and on the rates of the denaturation of trypsin and its reversal.

To estimate trypsin the enzyme is added to a solution of protein and the rate of digestion is measured. In all the protein solutions which have hitherto been used reversal of the inactivation and denaturation of trypsin take place. If active, native trypsin is to be estimated in the presence of inactive, denatured trypsin, such change of inactive into active trypsin during the very estimation must obviously be avoided. This we have succeeded in doing by adding a suitable amount of the denaturing agent, urea, to the digestion mixture.

We have already described a method for the estimation of trypsin with hemoglobin (Anson and Mirsky, 1933) which can be used when the reversal of inactivation need not be considered. Digestion of

denatured hemoglobin by trypsin is allowed to take place in a slightly alkaline solution buffered with phosphate. Precipitation of the denatured hemoglobin is prevented by urea. The undigested hemoglobin is precipitated with trichloroacetic acid. The digested hemoglobin not precipitated by trichloroacetic acid, which is a measure of the amount of trypsin used, is estimated by the blue color it gives with the phenol reagent. Considerable amounts of acid, alkali or glycerol can be added with the enzyme without any effect on the rate of digestion.

The change of inactive into active trypsin which takes place in the standard hemoglobin solution used for the ordinary estimation of trypsin can be prevented by adding more urea or (as is done in the procedure to be described) more alkali. If too much urea or too much alkali is added not only is the change from inactive to active trypsin prevented but the active trypsin is inactivated so fast that no measurement of activity is possible. In a digestion mixture in which there is neither reactivation nor a too rapid inactivation the rate of digestion is slower than it is in the standard hemoglobin solution used for the ordinary estimation of trypsin and the rate of digestion is much more sensitive to small changes in pH and urea concentration.

The general method of preventing reactivation during an analytical procedure by having present an inactivating agent such as urea may prove to be of use not only in the study of the denaturation of pure proteins but in testing for the protein nature of biologically active substances which have not been isolated but which are present in extremely dilute solutions together with many impurities. One would expect a protein substance regardless of its concentration or of the presence of impurities to lose its activity if exposed to denaturation procedures or to the proteolytic activity of trypsin. Trypsin, itself, however, which is a protein, can be heated in acid to 100°C. without any permanent loss of activity, for the denaturation and inactivation which take place on heating are reversed on cooling. There are proteins like hemoglobin which in their native form are not attacked by trypsin although in their denatured form they are rapidly digested. If a substance does not lose its activity when exposed to a denaturation procedure or to trypsin it may mean, there-

fore, not that the substance is not a protein but that there has been, as in the case of trypsin, reversal of denaturation or that, as in the case of hemoglobin, the protein must be denatured to be digested by trypsin. To make sure that there has been no inactivation by denaturation the activity should be measured under conditions which prevent the reversal of denaturation. To make sure that the substance is not digestible by trypsin the substance should first be exposed to conditions known to cause denaturation and then brought to the slightly alkaline solution suitable for tryptic digestion in the presence of some substance such as urea which will keep the substance denatured.

The Procedure.—5 ml. of the hemoglobin solution to be described are poured from a 175 × 20 mm. test-tube into 1 ml. of the enzyme solution in a second tube, the solution is poured back and forth, and the two test-tubes are placed in a water bath at 25°C. Sometime during the digestion period the small amount of solution in the test-tube from which the digestion mixture was last poured is drained into the other test-tube. After 5 minutes 10 ml. 5 per cent trichloroacetic acid are added, the suspension is mixed with the few drops of digestion mixture still left in the third tube, allowed to stand 5 minutes and filtered. To 5 ml. of filtrate are added 10 ml. of 0.50 N sodium hydroxide and 3 ml. of the phenol reagent (Folin and Ciocalteu, 1927) diluted three times are added dropwise with stirring and always in the same way. After 1 to 10 minutes the blue color is read against the color developed from 0.00083 milliequivalents (0.15 mg.) of tyrosine dissolved in 5 ml. of 0.2 N hydrochloric acid containing 0.5 per cent formaldehyde. If the colorimetric comparison is made in the fairly monochromatic red light transmitted by the Corning glass filter No. 241 almost any blue glass or a blue solution can be used as a standard.

Preparation of the Hemoglobin Solution.—Defibrinated bovine blood is centrifuged, the serum and white corpuscles are siphoned off and the red corpuscles are washed once with an equal volume of 0.9 per cent sodium chloride solution. Water is added to give a solution containing in 100 ml. 10.5 gm. hemoglobin or 1.86 gm. nitrogen. This solution is stored frozen in paraffined paper ice-cream containers.

150 ml. of 10.5 per cent hemoglobin plus 7.5 ml. 1 N sodium hydroxide at 50–60°C. are added to 900 ml. of water at 100°C. There is then added with mechanical stirring 18 ml. of a solution 5 M in respect to sodium chloride and 0.5 M in respect to KH_2PO_4 . The precipitate is filtered, washed and made up to 390 gm. with water. 390 gm. of urea and then 20 cc. 1 N sodium hydroxide are added and the solution is brought to room temperature to insure the complete solution of the hemoglobin. Finally, 160 ml. of 0.5 M boric acid and 430 ml. water are added and the solution is stored at 5°C. with toluol as a preservative. The

solution is the same as would be obtained by adding 39 gm. of urea to 100 gm. of a solution containing 1.5 gm. denatured hemoglobin and the equivalents of 80 ml. 0.1 M boric acid and 20 ml. 0.1 M sodium hydroxide.

Calibration.—Digestion is carried out with various dilutions of a trypsin solution whose activity in terms of trypsin units has been measured by the standard

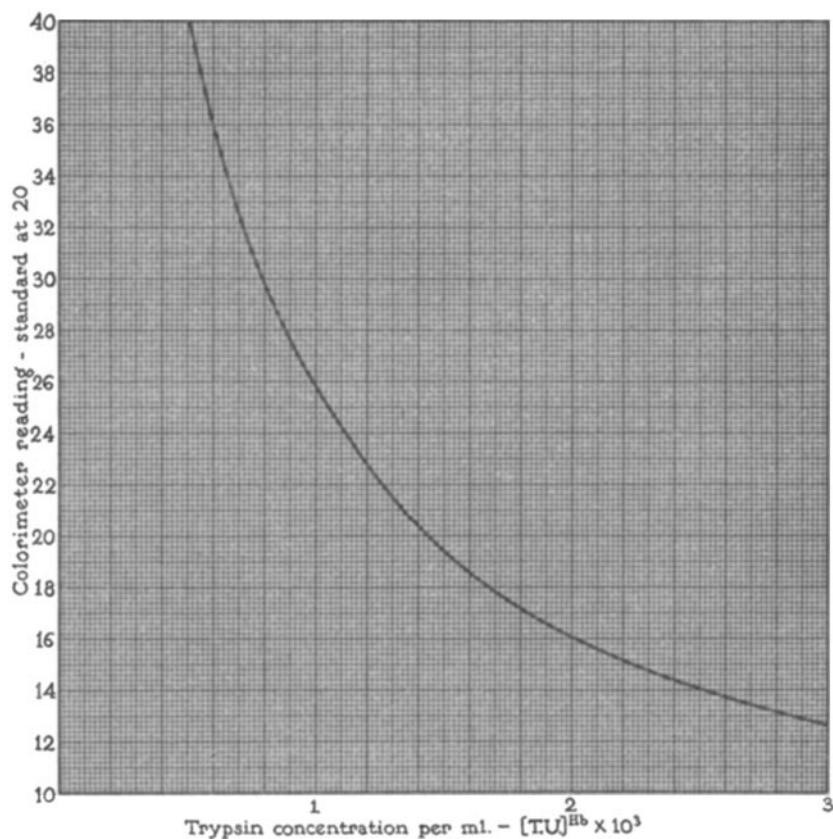


FIG. 1. Relation of trypsin concentration to color value of digestion products.

hemoglobin method. The colors developed from 5 ml. of trichloroacetic acid filtrate are read against the color developed from 5 ml. of the standard tyrosine solution. Fig. 1 gives the relation between the trypsin units and the colorimetric readings when the standard is set at 20. The hemoglobin solution may be kept at least 2 months without any change in the calibration curve and different preparations of the hemoglobin solution yield the same calibration curve.

The Effect of Variations in the Composition of the Digestion Mixture on the Rate of Digestion.—Increasing the urea concentration 5 per cent reduces the extent of digestion 2 per cent. Decreasing the urea concentration 5 per cent increases the extent of digestion 2 per cent. The addition to the digestion mixture of the equivalent of 1 ml. of 5 per cent glycerol causes an increase of 6 per cent in the digestion; 1 ml. of 0.06 N hydrochloric acid causes an increase of 4 per cent; and 1 ml. of 0.06 N sodium hydroxide causes a decrease of 6 per cent. If more than 0.01 N acid or alkali is added with the enzyme then 0.5 ml. instead of 1 ml. of enzyme solution is used and there is added to the 5 ml. of hemoglobin solution 0.5 ml. of a solution which exactly neutralizes the acid or alkali added with the enzyme.

Evidence of the Prevention of the Reversal of Denaturation.—When trypsin is heated to 60°C. in 0.01 hydrochloric acid for 2 minutes it is completely inactivated and denatured. On cooling the solution the original activity is restored. If 5 ml. of the hemoglobin solution plus 0.5 ml. of water are poured into 0.5 ml. of a hot 0.01 N hydrochloric acid solution of trypsin which before heating contained 24×10^{-3} units of active enzyme and digestion is carried out for 10 minutes only 0.65×10^{-3} units of activity are found.

When trypsin is heated or cooled to 45°C. in 0.01 N hydrochloric acid it is about half inactivated and denatured. The activity of such a mixture is the same whether or not the denatured half of the trypsin is first precipitated with salt and removed. The experiment is carried out as follows: Into 0.5 ml. of trypsin (1.72×10^{-3} [T.U.]^{ab}) in 0.01 hydrochloric acid heated to 45°C. for 5 minutes are poured 5 ml. hemoglobin solution plus 0.5 ml. water. 1 ml. of the mixture is immediately added to 5 ml. of a mixture of 5 parts hemoglobin solution and 1 part water. The digestion is carried out for 10 minutes after the hemoglobin solution was first poured into the enzyme solution. For the estimation with salt, 2 ml. of 5 M sodium chloride in 0.01 N hydrochloric acid are poured into 2 ml. of the heated trypsin solution. The resulting suspension is centrifuged. 1 ml. of the supernatant solution is added to 5 ml. hemoglobin solution; 1 ml. of this mixture is then immediately added to 5 ml. of a mixture of 5 parts hemoglobin solution to 1 part water and the digestion is carried

out as before. By both methods the heated solution is found to have 0.86×10^{-2} units of activity.

Effect of Variations in the Composition of the Digestion Mixture on the Extent of the Reversal of Inactivation.—The less urea in the digestion mixture the more reversal of inactivation. If the amount of urea is decreased 5 per cent the amount of reversal is still less than 5 per cent. If the amount of urea is decreased a third about 20 per cent reversal takes place. The results under these conditions are not very reproducible. Acid favors reversal, alkali the reverse, but the addition of 1 ml. of 0.04 N hydrochloric acid or sodium hydroxide (which is more than permissible if the rate of digestion by active trypsin is to be kept constant) has no significant effect on the extent of reversal. Glycerol favors reversal. The addition of the equivalent of 1 ml. of 5 per cent glycerol to the digestion mixture increases the amount of reversal 60 per cent.

SUMMARY

Inactive denatured trypsin changes into active native trypsin in the protein solutions which have been used to estimate tryptic activity. If the digestion mixture, however, is alkaline enough and contains enough urea this change does not take place. Such a digestion mixture can be used to estimate active native trypsin in the presence of inactive denatured trypsin.

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