

THE ESTIMATION OF TRYPSIN WITH HEMOGLOBIN

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The method for the estimation of trypsin described in this paper is essentially the same as our method for the estimation of pepsin (Anson and Mirsky, 1932). Trypsin is allowed to digest denatured hemoglobin in a slightly alkaline phosphate solution. Precipitation of the denatured hemoglobin by the phosphate or by salt added with the enzyme is prevented by urea. The undigested hemoglobin is precipitated with trichloroacetic acid. The amount of digested hemoglobin not precipitated, which is a measure of the amount of trypsin used, is estimated by the blue color which the tyrosine and the tryptophane in the digested hemoglobin give with the phenol reagent. Cysteine and heme (even heme whose iron is in the ferric state) also can reduce the phenol reagent. But there is very little cysteine in hemoglobin and all the heme is precipitated with trichloroacetic acid.

The procedure has several advantages. Many estimations can be made in a short time; the results are entirely reproducible; the hemoglobin solution keeps for at least a month without change; and the rate of digestion is not sensitive to considerable amounts of acid, alkali, urea or glycerol added with the enzyme.

The procedure has two disadvantages. In the first place, the hemoglobin solution cannot be used for the estimation of the active native trypsin in a mixture of active native and inactive denatured trypsins because inactive denatured trypsin changes into active native trypsin in the hemoglobin solution just as it does in all the protein solutions which have hitherto been used for the estimation of trypsin. This change can be prevented by making the hemoglobin solution more alkaline as is described in a following paper. In the second place,

the activity of a crude pancreatic extract is higher when measured by the digestion of hemoglobin than when measured by the change in the viscosity of gelatin. The reasons for this are being studied. In the experiments with purified trypsin so far carried out the two methods yield the same results.

Commercial dried proteins can be used instead of hemoglobin prepared in the laboratory. They are of dubious reproducibility and they contain considerable and variable amounts of color-producing substances not precipitated by trichloroacetic acid.

When gelatin, casein or any other non-reproducible protein substrate is used for the estimation of trypsin by any method, the procedure can be calibrated by means of a solution of purified trypsin whose activity has been measured by the hemoglobin method which yields reproducible results. The calibration curve states the extents to which a particular sample of protein is digested under given conditions by different known amounts of trypsin. A sufficiently purified trypsin can be prepared from commercial trypsin in a few minutes by a modification of the Northrop-Kunitz procedure (1932) which avoids several filtrations. A solution of this partially purified trypsin which digests hemoglobin at the same rate as a solution of crystalline trypsin also has the same effect on the viscosity of gelatin as does the crystalline trypsin.

The Procedure.—1 ml. of enzyme solution is added to 5 ml. of the hemoglobin solution to be described later. The 175 × 20 mm. test-tube containing the 6 ml. of digestion mixture is whirled and placed in a water bath at 25°C. After 5 minutes 10 ml. of 5 per cent trichloroacetic acid are poured in from another test-tube, the suspension is poured back and forth, 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 of Folin and Ciocalteu (1927) diluted three times (*cf.* Wu, 1922, and Greenberg, 1929). The reagent is added drop by drop with stirring and is always added 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.

If the trichloroacetic acid suspension is filtered immediately instead of after 5 minutes the first half of the filtrate contains some undigested hemoglobin in fine suspension and this first portion must accordingly be rejected or refiltered. Centrifugation can be used instead of filtration without any difference in results.

Preparation of Tyrosine Standard.—The tyrosine is thrice crystallized and its concentration is estimated by Kjeldahl (100 mg. tyrosine = 7.74 mg. nitrogen).

It is stored at room temperature in 0.2 N hydrochloric acid containing 0.5 per cent formaldehyde. Some preservative is needed to prevent the destruction of tyrosine by mould even in the cold. Formaldehyde does not affect the color value of tyrosine.

Copper sulfate solution or a blue glass inserted in the plunger of the colorimeter can be used as a standard instead of the blue solution obtained from tyrosine. Although these standards do not match the tyrosine blue in white light they do match it in the fairly monochromatic red light transmitted by the Corning Glass Filter No. 241.

Rubber, even after being boiled with alkali, contains reducing substances which can be extracted by the reagents so all contacts with rubber should be avoided.

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.¹ 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.

To denature the hemoglobin and to remove substances not precipitated with trichloroacetic acid which give a color with the phenol reagent, one proceeds as follows. A mixture of 220 ml. 10.5 per cent hemoglobin and 11 ml. 1 N sodium hydroxide is brought to 50–60°C. and is added to 1300 ml. of water previously brought to 100°C. There is then added with mechanical stirring 26 ml. of a solution 5 M in respect to sodium chloride and 0.5 M in respect to KH_2PO_4 . The resulting suspension is filtered on a folded paper, the precipitate is washed with water, transferred to a beaker, weighed and enough water added to make the weight 400 gm. 400 gm. of urea are then stirred up with the precipitate and 160 ml. of 1 N sodium hydroxide are added. After solution of the protein and the urea 200 ml. of 1 M KH_2PO_4 plus 240 ml. water are added. The solution is stored in the cold with toluol as a preservative. The solution is the same as would be obtained by adding 40 gm. of urea to 100 gm. of a solution which contains 2.2 gm. denatured hemoglobin (about 5 per cent of the protein is lost) and the equivalents of 100 ml. of 0.2 M KH_2PO_4 and 80 ml. of 0.2 M sodium hydroxide.

Preparation of Solutions of Commercial Dried Proteins.—25 gm. of edestin (La Roche) hemoglobin (Eimer and Amend) or casein (after Hammarsten) are mixed with 400 gm. urea. This mixing facilitates the solution of the protein. In the case of casein and edestin, the protein and urea are simply put together in a

¹ These washed corpuscles can be stored frozen and after being dialyzed and acidified can be used for the estimation of pepsin instead of the purified hemoglobin solution already described (Anson and Mirsky, 1932) which is more difficult to prepare. The one acid hemoglobin solution which we have prepared from frozen corpuscles was digested at the same rate as the purified hemoglobin.

flask which is whirled. In the case of hemoglobin the protein and urea are ground together in a mortar. 240 ml. water and 160 ml. 1 N sodium hydroxide are added to the urea-protein mixture and the solution is brought to room temperature. After the protein is dissolved (and denatured) 200 ml. 1 M KH_2PO_4 and 375 ml. water are added. Eimer and Amend's hemoglobin dissolves more readily than other commercial hemoglobins we have tried. Although it is labelled pure it is contaminated with other proteins. Of the three proteins, edestin is the most rapidly digested. A sample of the Hoffman-La Roche edestin, however, was not digested at the same rate as crystalline edestin prepared in the laboratory.

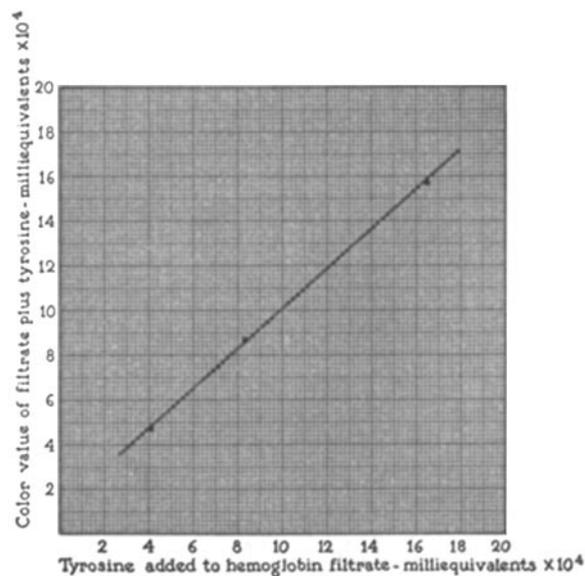


FIG. 1. Color values of various amounts of tyrosine dissolved in hemoglobin filtrates.

Calculations

What is measured is the color value of 5 ml. of the trichloroacetic acid filtrate from digested hemoglobin in terms of the amount of tyrosine which would give the same color under the same conditions. For the purposes of calibration it must, therefore, first be determined how much color would be given by various known amounts of tyrosine in the trichloroacetic acid filtrate from undigested hemoglobin which contains in addition to trichloroacetic acid, phosphate and urea a small amount of color-producing substance not precipitated by trichloroacetic acid.

10 parts 5 per cent trichloroacetic acid are added to a mixture of 5 parts hemoglobin solution and 1 part water. To 5 ml. portions of the filtrate are added 1 ml. portions of 0.1 N hydrochloric acid containing various amounts of tyrosine. The colors developed with sodium hydroxide and the phenol reagent are read against the color developed from 0.15 mg. or 0.00083 milliequivalents, tyrosine dissolved in 5 ml. 0.2 N hydrochloric acid plus 1 ml. water. Fig. 1 shows how many

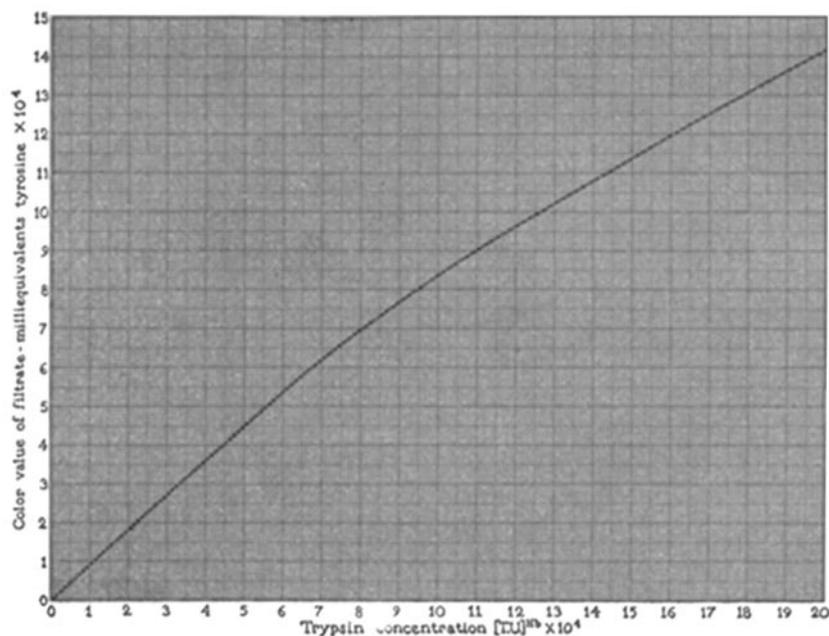


FIG. 2. Relation of trypsin concentration to color value of digestion products, 5 ml. filtrate, 5 minutes digestion at 25°C.

milliequivalents of tyrosine in hydrochloric acid are needed to give the same color as any given amount of tyrosine dissolved in the trichloroacetic acid filtrate.

Digestion is now carried out with various amounts of enzyme. Fig. 2 gives the color values of the filtrates in terms of the amounts of tyrosine in the filtrate which would give the same colors. In practice since the properties of the hemoglobin solution are constant one avoids calculations by using a curve in which the amounts of trypsin

are plotted directly against the colorimetric readings when the standard is set at 20. One does not have to use different calibration curves if different periods of digestion are used because increasing the digestion time n times is always equivalent to increasing the enzyme concentration n times.

For the purpose of using the hemoglobin-urea procedure the trypsin units may be considered as arbitrary numbers which are proportional to the amounts of trypsin which give the amounts of color-producing substances expressed by the curve. In order, however, to make the hemoglobin trypsin unit comparable with other units of proteolytic activity (Northrop, 1932; Anson and Mirsky, 1932) the following definition has been adopted. One unit of trypsin produces in 1 minute at 35.5°C. in 6 ml. of the digestion mixture an amount of color-producing substance not precipitable with trichloroacetic acid which gives the same color as 1 milliequivalent of tyrosine. This definition assumes that the extent of digestion is proportional to the concentration of enzyme and to the time of digestion. These assumptions are correct only when the amount of digestion is small since as digestion proceeds the trypsin is inhibited by the products of digestion. The slope of the curve of Fig. 2 for small amounts of digestion is $1/5 \times 16/5 \times 1.75$ or 1.12 times less steep than it would be if the determination were carried out as described in the definition because the digestion is carried out for 5 minutes instead of 1, only 5 ml. of filtrate are used in the colorimetric estimation instead of the total 16 and the digestion is carried out at 25°C. instead of at 35.5°C. at which it is 1.75 times faster.

Effect of Variations in the Composition of the Digestion Mixture on the Extent of Digestion

Hemoglobins from the bloods of different individual animals are digested at the same rate. Doubling the hemoglobin concentration or reducing it 10 per cent has no detectable effect. The amount of urea can be increased or decreased 5 per cent, or the equivalent of 1 ml. of 0.1 N hydrochloric acid, 0.1 N sodium hydroxide or 10 per cent glycerol can be added to the digestion mixture without changing the extent of digestion 3 per cent.

Preparation of Purified Trypsin for the Standardization of Non-

Reproducible Proteins.—1 gm. of Fairchild's trypsin is suspended in 25 ml. 0.1 N hydrochloric acid, heated for 1 minute at 80°C. and cooled rapidly to room temperature with ice water. After 10 minutes, 6 gm. of ammonium sulfate are added and the suspension filtered. To each 10 ml. of the filtrate are added 2 gm. ammonium sulfate. The resulting precipitate is centrifuged and dissolved in enough 0.005 N hydrochloric acid to make the final volume 25 ml. This final solution has about 0.01 activity unit per ml.; *i.e.*, it has to be diluted about 10 times for estimation.

SUMMARY

The formation from hemoglobin of split products not precipitable by trichloroacetic acid is taken as a measure of tryptic activity. The split products are estimated colorimetrically.

Many measurements of tryptic activity can be made in a short time and different samples of hemoglobin yield the same results.

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