THE ESTIMATION OF PEPSIN WITH HEMOGLOBIN

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A number of methods have been described for the estimation of peptic activity, many of which are accurate and convenient for comparative experiments but none of which give results which are accurately reproducible with different preparations of the protein used. This variation is due to the difficulty involved in obtaining reproducible protein preparations and keeping them unchanged.

Hemoglobin has been chosen as the protein substrate for the estimation of pepsin because it is easily prepared in large quantities, because it can be stored in solution for a long time without change, because it is rapidly digested, and because the rate at which it is digested by a given pepsin solution does not vary from one hemoglobin preparation to another. Not only is hemoglobin a reproducible protein which can be brought to reproducible conditions but the conditions of digestion have been so chosen that reasonable variations in these conditions have little effect on the amount of digestion.

In general the procedure used to estimate pepsin is this. A pepsin solution is added to acidified hemoglobin. After 5 minutes trichloracetic acid is added. The resulting precipitate which contains all the pigment and all the undigested hemoglobin is filtered off. The filtrate contains an amount of digested hemoglobin which is a measure of the amount of pepsin used. This digested hemoglobin is estimated by the blue color it gives with the phenol reagent, which reacts with tyrosine, tryptophane and cysteine groups, tyrosine being used as a standard. The intensity of this color is proportional to the amount of enzyme used and to the time of digestion.

Definition of Activity $[P.\ U.]^{Hb}$.—One unit of activity is defined as the formation per minute at 35.5°C. in 6 ml. of the standard digestion

mixture of an amount of digested hemoglobin not precipitable by trichloracetic acid which gives the same color as 1 milliequivalent of tyrosine, and the quantity of material possessing one unit of activity is that quantity which will cause this rate of change in the formation of digested hemoglobin when contained in 6.0 ml. of the standard digestion mixture (see Northrop, 1932, for a discussion of the definition of pepsin units).

In detail the procedure is this: 5 ml. of a 2 per cent solution of dialyzed ox carbon monoxide hemoglobin in 0.06 N HCl are pipetted into a 175 × 20 mm. test tube and brought to 35.5°C. or 25°C. 1 ml. of enzyme solution is added with an Ostwald pipette, which procedure takes about 4 seconds. The middle of the pipetting period is taken as the time of beginning. To mix the hemoglobin and enzyme solution the test-tube is whirled. After 5 minutes, 10 ml. of 4 per cent trichloracetic acid are poured into the hemoglobin solution from another test-tube, the suspension is poured back and forth from one test-tube to another, and filtered through fine paper. To 3 ml. of the filtrate in a 50 ml. Erlenmeyer flask are added 20 ml. of water, 1 ml. of 3.85 N NaOH, 1 ml. of the phenol reagent (Folin and Ciocalteau, 1927). Some of this NaOH is to neutralize trichloracetic acid. The phenol reagent and NaOH are added from automatic pipettes (A. H. Thomas Co.—No. 8210). The standard is made up from 3 ml. 0.1 N HCl containing 0.15 mg. tyrosine.[1] After 5 to 10 minutes, the blue colors are compared, the standard solution being set at 20, and the reading for the unknown solution being called X.

In addition to 0.15 mg. tyrosine, the standard solution contains from the reagents an amount of color-producing substance equivalent to 0.015 mg. tyrosine.[2] The color-producing substance in the 3 ml. of filtrate is therefore equivalent to $\frac{20}{X}(0.15 + 0.015)$ mg. tyrosine. Of this 0.015 mg. are due to the color-producing substance in the reagents and 0.01 mg. to the color-producing substance present in the trichloracetic acid filtrate even when no enzyme is added. The di-

[1] Copper sulfate can be used as a standard instead of the blue solution obtained from tyrosine with the phenol reagent. The colors match if a red color filter is used.

[2] The reagent blank is not measurable. It is deduced from the fact that colors given by tyrosine solutions in the relatively low range of concentrations used are not proportional to the tyrosine concentrations—doubling the tyrosine concentration decreases by some 5 per cent the color given per unit amount of tyrosine. Whether this deduction of a reagent blank equivalent to 0.015 mg. tyrosine is correct or not makes no significant difference to the validity of the calculations which merely provide a correction for the small deviation from proportionality.

gested hemoglobin in the 3 ml. of filtrate is therefore equivalent to $\frac{20}{X}$ (0.15 + 0.015) −0.015 −0.01 mg. tyrosine. This value must be multiplied by $\frac{16}{3}$ to obtain the digested hemoglobin in the whole 16 ml. of filtrate instead of in the 3 ml. taken for analysis; it must be divided by 5 to obtain the amount of non-precipitable digested hemoglobin produced in 1 minute instead of in 5 minutes; and it must be divided by 181, the molecular weight of tyrosine, to obtain the tyrosine equivalent as milliequivalents instead of as milligrams. The relation between the number of pepsin units in 1 ml. of enzyme solution and the colorimeter reading X is thus:

$$\text{P. U.} = \left[\frac{20}{X}(0.15 + 0.015) - 0.015 - 0.01\right]\frac{16}{3} \times \frac{1}{5} \times \frac{1}{181} = \frac{0.0195}{X} - 0.000147$$

If the digestion is carried out at 25°C. at which temperature it is 1.82 times slower than at 35.5°C. the right hand expression must be multiplied by 1.82 since the unit of activity is defined as the rate of reaction at 35.5°C. By the use of a curve in which P. U. is plotted against X, one can in practice avoid all calculations.

In the table are recorded actual estimations in terms of pepsin units of the activities of a series of solutions of five times recrystallized pepsin. The constancy of the specific activity, that is the activity per mg. protein nitrogen per ml., is an expression of the fact that the amount of non-precipitable color-forming substance liberated from hemoglobin is proportional to the pepsin concentration.

Protein N per ml. pepsin solution	Activity units $[\text{P. U.}]^{\text{Hb}}$		Specific activity $\left[\text{P. U.}\right]^{\text{Hb}}_{\text{mg. N}}$	
	35.5°C	25°C.	35.5°C.	25°C.
mg.				
0.0180		0.00326		0.181
0.0144		0.00260		0.180
0.0108	0.00194	0.00206	0.180	0.191
0.0072	0.00134	0.00136	0.185	0.189
0.0054	0.00100	0.00103	0.185	0.191
0.0036	0.00065	0.00064	0.180	0.178
0.0018	0.00034		0.189	
			Average.....0.184	

The Preparation of Hemoglobin.—Carbon monoxide is bubbled through whipped ox blood. The blood is centrifuged and the serum and white cells siphoned off. After the corpuscles are washed four times with cold 0.9 per cent sodium chloride, an equal volume of water and a sixth the total volume of toluol are added (Heidelberger, 1922). Carbon monoxide is bubbled through the solution which is then vigorously shaken and allowed to stand in the cold overnight. Next morning the hemoglobin layer is siphoned off, mixed gently with a tenth the volume of centrifuged alumina cream (Tracy and Welker, 1915), centrifuged, and then filtered through coarse paper. Finally the hemoglobin solution is dialyzed overnight in the cold in a shaking dialyzer (Kunitz and Simms, 1928) and stored in the cold under carbon monoxide with toluol as a preservative.

The concentration of hemoglobin is estimated by the Kjeldahl method, the nitrogen content being taken as 17.7 per cent. Hemoglobin is digested with greater difficulty than are the other common proteins. When copper is used as a catalyst, digestion is not complete. When mercury is used, digestion is complete but a preliminary evaporation is necessary to avoid serious foaming. With selenium oxychloride (Lauro, 1931) digestion is rapid and complete and no preliminary evaporation is needed. The concentration of the thrice recrystallized tyrosine is likewise determined by the Kjeldahl method—nitrogen content, 7.74 per cent.

To make up the acid solution of hemoglobin three volumes of 0.1 N HCl are added to two volumes of 5 per cent hemoglobin. This acidity (0.06 N) is sufficient to give the maximum rate of digestion and is safely on the acid side of the region in which the rate of digestion is sensitive to small changes in the hydrogen ion concentration. Changing the HCl concentration to 0.035 N or to 0.08 N does not change the rate of digestion more than 5 per cent. Hemoglobin is hydrolyzed very slowly in 0.06 N HCl. Acid solutions which have stood in the cold for 10 days have been used for the estimation of pepsin with results the same as those obtained from freshly acidified hemoglobin.

Increasing the hemoglobin concentration from 2 per cent to 3 per cent or decreasing it to 1.5 per cent has no significant effect on the rate of digestion.

Trichloracetic acid in the concentration chosen (2.5 per cent) gives a precipitate which filters rapidly. If the trichloracetic acid concentration is decreased 20 per cent, some 10 per cent more color-producing material is left unprecipitated. If the trichloracetic acid concentration is increased 20 per cent some few per cent less color-producing material is left unprecipitated. 10 per cent trichloracetic acid, as well as other so called protein precipitants, precipitates a great deal of the digested hemoglobin not precipitated by 2.5 per cent trichloracetic acid; so that the digested hemoglobin could probably be estimated nephelometrically as well as colorimetrically.

In general, then, the reproducibility of the amount of digestion obtained with a given pepsin solution from different hemoglobin preparations depends essentially on the reproducibility of hemoglobin as a protein rather than on accuracy in fixing

the concentrations of hemoglobin, hydrochloric acid, and trichloracetic acid, although in practice these concentrations are fixed accurately with little trouble.

The relations between the hemoglobin pepsin unit and other pepsin units are given by Northrop (1932).

A method will be described later for the estimation with hemoglobin of active, native trypsin in the presence of inactive, denatured trypsin, together with a discussion of the peculiar problems involved in the estimation of trypsin, in particular the prevention of the reversal of inactivation and denaturation. By measurements of tryptic digestion, furthermore, one can estimate denatured hemoglobin in the presence of native hemoglobin, for native hemoglobin is not digested by trypsin while denatured hemoglobin is digested at a rate which depends on its concentration.

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