NORTHROP'S PHENOMENON

The Digestion of Proteins by Enzymes in the Presence of Gelatin

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Northrop (6, 8) observed that the digestion of casein and gelatin by crude and crystalline trypsin proceeded at the same rate whether present in separate solutions or in the same solution. Northrop (10) pointed out that this fact could not be explained if it is supposed that an intermediate enzyme-substrate compound is formed according to addition compound theory, unless it be assumed either that there are two enzymes or two different active groups, which amounts to practically the same thing.

As a first point under consideration, the question arises whether this phenomenon is specific for trypsin-casein or whether it is general for all proteases and for other substrates different from casein. In this work, the behavior of crude and crystalline trypsin, papain, and pepsin with respect to casein and hemoglobin either alone or in the presence of gelatin was studied in an attempt to give, partially at least, an answer to this question.

EXPERIMENTAL

Enzymes.

Trypsin, twice crystallized salt-free, and crude pancreatic protease, Worthington. Papain, twice crystallized, lyophilized, and lyophilized fraction 3A, Worthington. Pepsin, Worthington twice crystallized, and Difco crude pepsin.

Solutions.

1, 2, and 5 per cent solutions of Merck casein according to Hammarsten in phosphate buffer following Kunitz' (4) procedure.

1, 2, and 5 per cent solutions of hemoglobin denatured by urea prepared by Anson's method (1).

1, 2, and 5 per cent pH 1.9 hemoglobin substrates prepared according to Anson (1).

1, 2, and 5 per cent Difco gelatin in 1/15 M Na_2HPO_4–KH_2PO_4 pH 7.6 buffer.

1, 2, and 5 per cent Difco gelatin in urea solution like the hemoglobin solutions.

1, 2, and 5 per cent Difco gelatin dissolved in 0.06 M HCl, pH adjusted to 1.9.

1/15 M pH 7.6 Na_2HPO_4–KH_2PO_4 buffer.

Urea solution: 40 gm. urea, 8 ml. NaOH 1 M, and 10 ml. KH_2PO_4 1 M made up to 100 ml. with distilled water.

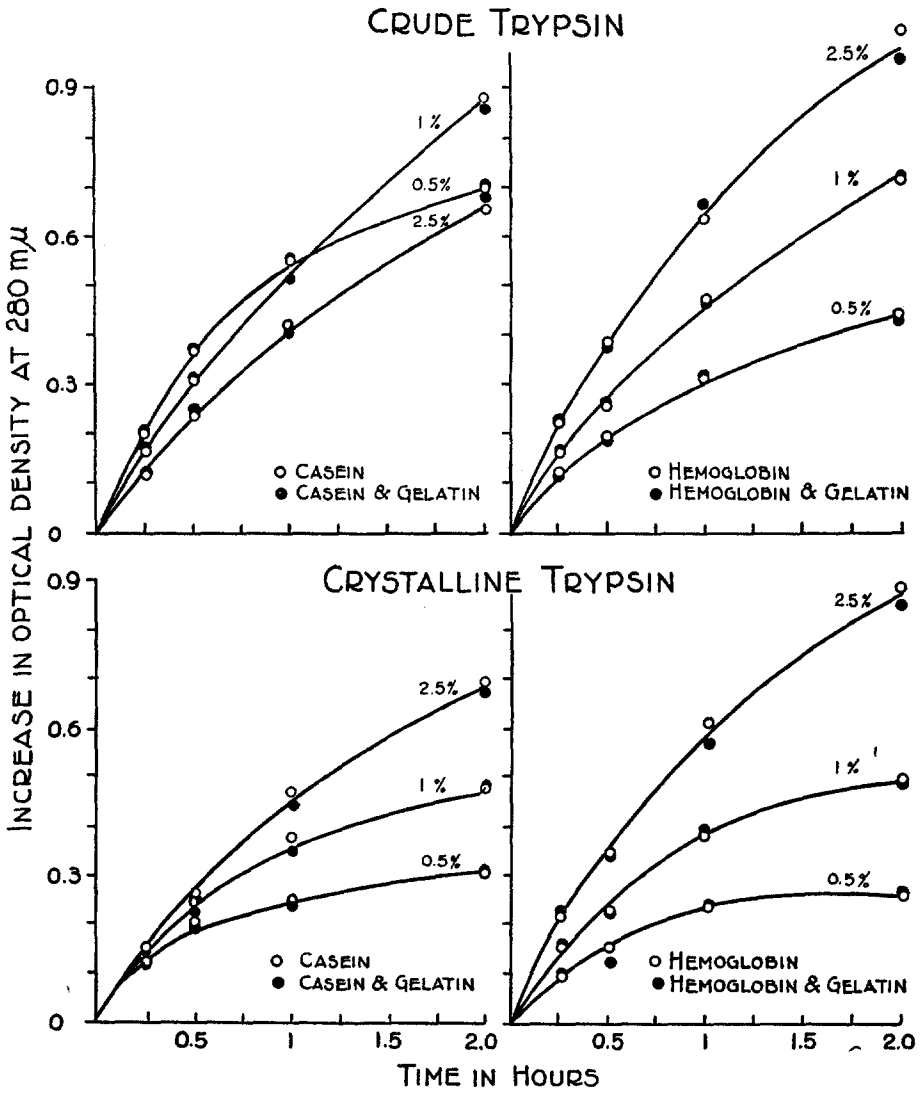

FIG. 1. Comparative digestion in function of time of casein, hemoglobin, and mixtures of gelatin with casein or hemoglobin by crude and crystalline trypsin.

HCl 0.06 M.

NaCN 2 M.

Determination of Proteolytic Activity.—All proteases were dissolved in distilled water. Papain was activated with NaCN following Anson's instructions (1). Of each enzymatic solution 0.5 ml. was measured and added to 1 ml. of substrate prepared in the following way:

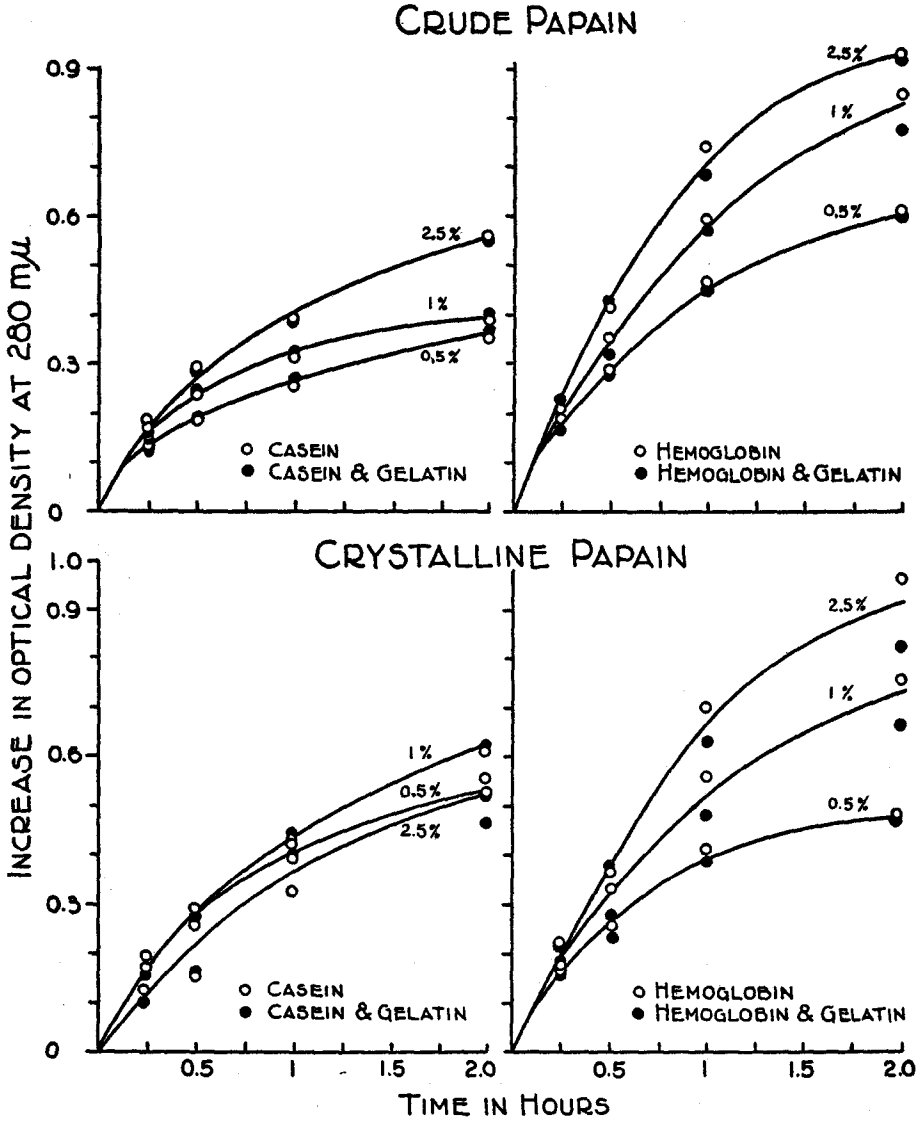

FIG. 2. Comparative digestion in function of time of casein, hemoglobin, and mixtures of gelatin with casein or hemoglobin by crude and crystalline papain.

Casein, 0.5, 1, and 2.5 per cent; 0.5 ml. of 1, 2, and 5 per cent casein are pipetted into a series of test tubes and 0.5 ml. of phosphate buffer added to each. Casein-gelatin, 0.5, 1, and 2.5 per cent; 0.5 ml. of 1, 2, and 5 per cent solutions of casein are mixed with 0.5 ml. of gelatin in phosphate buffer solution at the same concentration.

Hemoglobin and hemoglobin with gelatin, 0.5, 1, and 2.5 per cent; diluting 0.5 ml.

of each dilution of hemoglobin with 0.5 ml. of either urea solution or the corresponding concentration of gelatin-urea. pH 1.9 hemoglobin or hemoglobin-gelatin; 0.5 ml. of each solution of acid hemoglobin plus 0.5 ml. of 0.06 M HCl or the same concentration of pH 1.9 gelatin respectively.

The digestion mixtures were incubated at 37°C. in a water bath during 15, 30, 60, and 120 minutes or 18 hours. Then the hydrolysis was stopped by precipitation with 3 ml. of 10 per cent trichloracetic acid for casein substrates or 5 per cent trichloracetic acid for digestion mixtures containing hemoglobin. In spite of not being precipitable by trichloracetic acid, most of the gelatin is carried down with the heavy coarse pre-

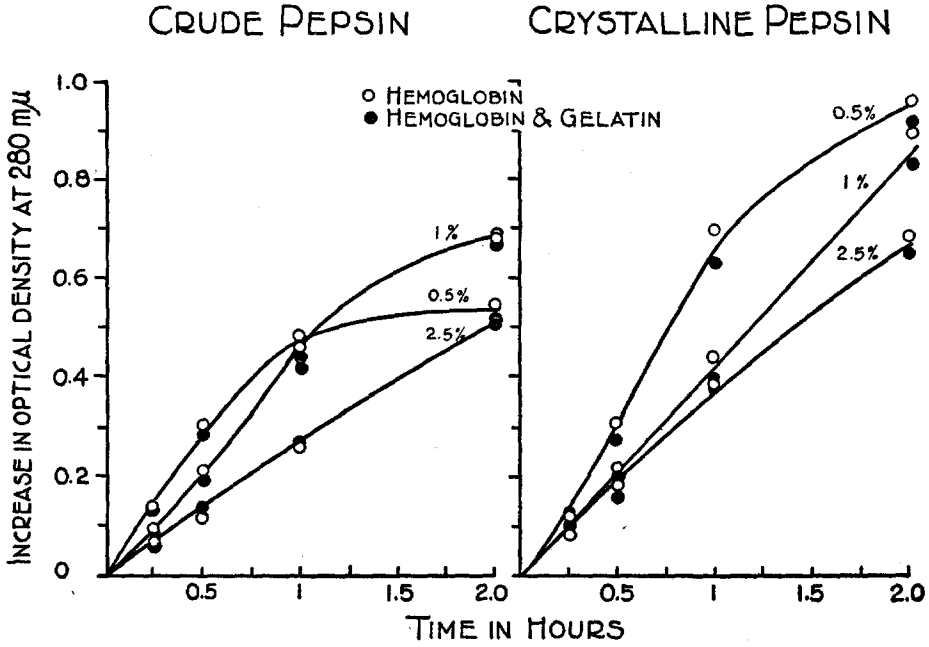

FIG. 3. Comparative digestion in function of time of acid hemoglobin and of mixtures of hemoglobin with gelatin by crude and crystalline pepsin.

cipitate of hemoglobin or casein in the course of 24 hours. The error due to the remaining gelatin that goes into the clear supernatants can be estimated by running blanks made up of substrate and enzyme which had been precipitated immediately after mixing. The increase in optical density in comparison with blanks of the supernatants separated after centrifugation was read at 280 mμ in a Beckmann quartz spectrophotometer. In hydrolysates containing dilute gelatin the proteolysis rate could also be measured colorimetrically by means of the Folin-Ciocalteu phenol reagent.

RESULTS AND DISCUSSION

In the present work only the digestion of casein or hemoglobin was measured, even though they were mixed with gelatin, since the optical density

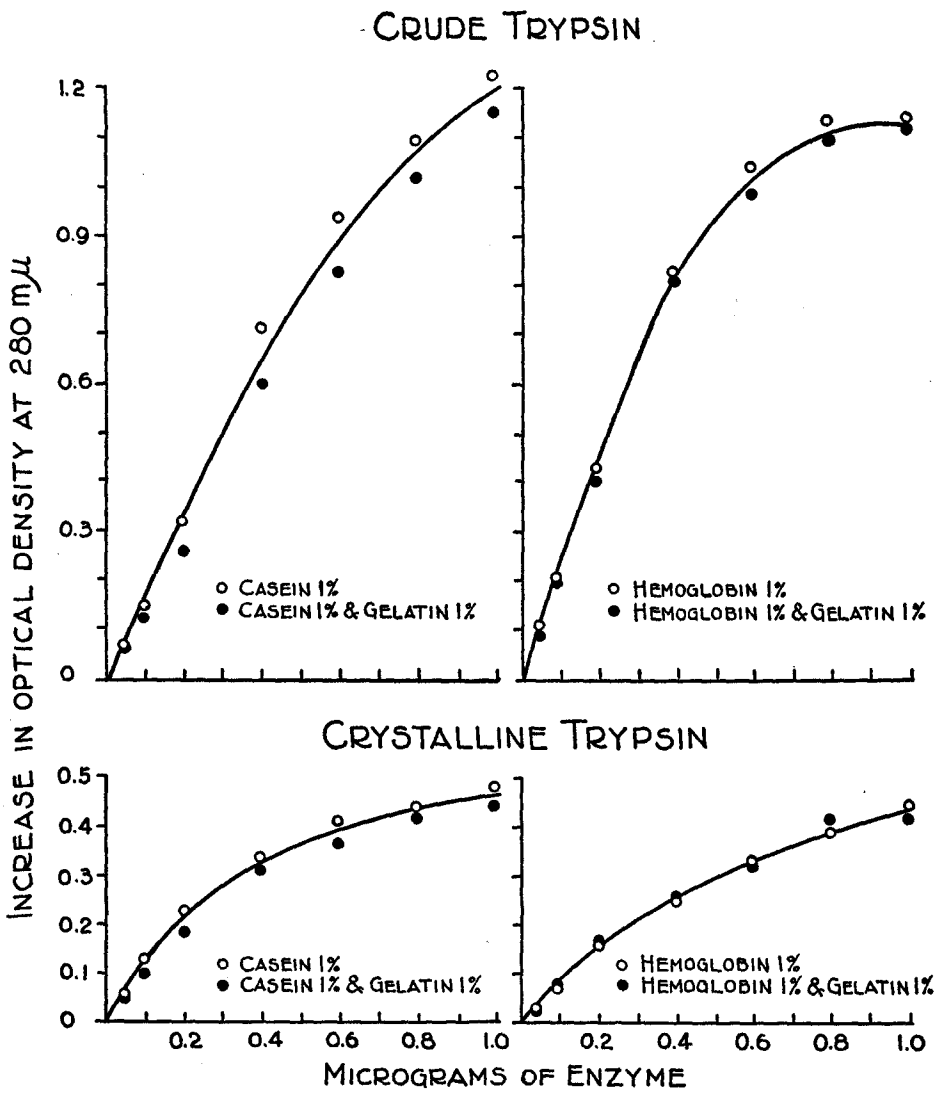

FIG. 4. Proteolytic activity of crude and crystalline trypsin during a digestion time of 18 hours on casein, hemoglobin, and mixtures of gelatin with casein or hemoglobin as a function of micrograms of enzyme.

readings were made at a wave length of 280 mμ, which corresponds to the maximum of the absorption spectrum of tyrosine and tryptophane (10), amino acids which are lacking or present in negligible proportions in gelatin (2, 3). However, it is known that gelatin is hydrolyzed by any of the proteases studied here, and Northrop (6, 8), following methods which permitted the

determination of gelatin digestion (increase in non-proteic nitrogen, formol titration, decrease of viscosity index), found that the rate of hydrolysis of a mixture of casein and gelatin by trypsin was equal to the sum of the two individual rates estimated separately. The procedure for determining proteolytic activity used throughout this work, based upon Anson's (1) and Kunitz' (4) methods, permits the estimation of the proteolytic activity of any protease upon casein or hemoglobin in the presence of gelatin without interference caused by the hydrolysis of the latter protein.

The data presented in Figs. 1 and 2 show that the proteolytic activities (measured by the increase in optical density at 280 mμ) in function of time

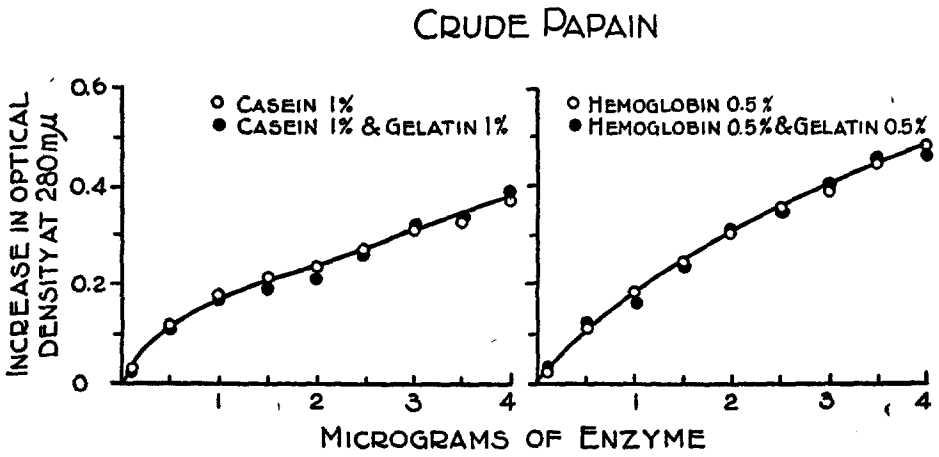

FIG. 5. Proteolytic activity of crude papain during a digestion time of 18 hours on casein, hemoglobin, and mixtures of gelatin with casein or hemoglobin as a function of micrograms of enzyme.

of crude and crystalline trypsin and papain were approximately the same when each of these enzymes acted on a single protein, *i. e.* casein or hemoglobin, or when the substrates acted upon were casein or hemoglobin associated with gelatin. No difference was apparent in the behavior of the above mentioned proteases, regarding the action of gelatin, on the three distinct substrate concentrations used. It is observed on Fig. 1 that the rate of hydrolysis of casein by crude trypsin up to 1 hour is inversely proportional to the substrate concentration and from 1 to 2 hours the digestion rate on 0.5 per cent substrate decreased more quickly than on 1 per cent substrate. This fact makes possible the suggestion that after a more prolonged time of action the rate of digestion would be proportional to substrate concentration as happens in most cases. Similar data have been already reported (5, 7–9). The behavior of crystallized papain on casein (Fig. 2) resembles that of crude trypsin except during the

first half hour, in which time 0.5 and 1 per cent substrates were hydrolyzed at the same rate. The action of crude and crystalline trypsin and papain on hemoglobin did not demonstrate any anomaly in relation to substrate concentration.

The hydrolysis of acid hemoglobin by crude and crystalline pepsin followed nearly the same curves as the digestion of hemoglobin plus gelatin (Fig. 3). Here, can be observed again, in both crude and crystalline pepsin, especially the latter, the inverse proportionality between digestion rate and substrate concentration, which has been previously described.

During 18 hours curves of activity of variable small quantities of proteases indicate that the addition of gelatin to casein or hemoglobin modified very little or not at all the rate of hydrolysis of each isolated protein (Figs. 4 and 5) in spite of the very long time that the enzyme remained with the substrates.

SUMMARY

1. Evidence has been found that Northrop's phenomenon (so called by us) is produced in the digestion of casein or hemoglobin brought about by trypsin, papain, and pepsin either crude or crystalline in the presence of gelatin.

2. Anson's and Kunitz' methods permit the measure of proteolytic activity of any protease on casein or hemoglobin substrate in the presence of gelatin, even in very small quantities and with prolonged digestion time.

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