JCI The Journal of Clinical Investigation

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J Clin Invest. 1942;21(5):533-537. https://doi.org/10.1172/JCI101330.

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STUDIES ON BLOOD COAGULATION: THE NATURE AND PROPERTIES OF A PROTEOLYTIC ENZYME DERIVED FROM PLASMA [1,2,3]

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(Received for publication May 28, 1942)

In the preceding paper (1) the preparation, by the action of chloroform on calcium and cell free plasma and subsequent precipitation of the globulins, of a globulin fraction having marked fibrinogenolytic and fibrinolytic properties was described. The present communication describes the chemical nature of these preparations. The preceding paper explains the activity of the chloroform plasma preparations on the basis of the presence, in them, of a proteolytic enzyme. The present communication offers evidence substantiating this assumption.

METHODS

Blood from the aorta of freshly stunned steers was collected in 20 per cent potassium oxalate solution. The final concentration of oxalate was 0.2 per cent. The plasma was removed by centrifuging. The plasma was then treated with chloroform. Chloroform plasma and its globulin derivatives were then prepared, as described in the preceding paper (1).

Determinations of pH were made by a glass electrode, non-protein nitrogen estimations by micro Kjeldahl methods, and viscosity determinations by the Ostwald viscosimeter at 37.5° C.

As substrates, fibrinogen, gelatin, and casein were used. Plasma was, in general, used as a source of fibrinogen and the lysis of precipitated fibrin followed by determinations of non-protein nitrogen at daily intervals. When casein and gelatin were used as substrates, 2 per cent solutions of these proteins were employed.

In the non-protein nitrogen studies, an equal volume of the standard enzyme preparations was added to the substrate and the mixture adjusted to pH 7. Samples were removed at suitable intervals of time, and the progress of hydrolysis followed by the non-protein nitrogen produced.

For viscosimetric studies a 4 per cent gelatin solution was employed and the amounts of enzyme noted in the text were added. Both the gelatin and enzyme solutions were adjusted to the required pH, all particulate matter removed by centrifuging, and the solution brought to a temperature of 37.5° C. before mixing. The mixtures were transferred to the viscosimeters and the initial viscosity times recorded. No attempt was made to calculate absolute or relative viscosity, only the percentage change in viscosity time being of immediate interest. The viscosity was determined at 15 minute intervals.

Bacteriological cultures on all preparations showed that contamination by bacteria was absent in both plate and broth culture. One per cent chloroform was used as a bacteriostatic agent in the non-protein nitrogen experiments. As buffers, either phosphate or imidazole buffer (2) was used.

EXPERIMENTAL

The first observations of true proteolysis were made on plasma after the addition of chloroform. As has been stated before (1), the clot which initially forms undergoes lysis. The disappearance of this protein clot in 3 or 4 days was suggestive of proteolysis. When samples from the reaction vessels were removed and analyzed at daily intervals, an increase in non-protein nitrogen was found. No bacterial contamination was present. This, and many similar observations, indicate that during the preparation of the enzyme, hydrolysis of protein progressed. Similar proteolysis was observed when saline solutions of globulin substances were treated with chloroform (Figure 1).

Proteolysis of casein and gelatin. When the saline solution of the globulin fraction of steer plasma, obtained after the action of chloroform, was added to an equal volume of gelatin at pH 7, a marked increase in non-protein nitrogen occurred, indicating that digestion by

[1] Presented, in part, by M. H. Kaplan, as a thesis, to the Faculty of Arts and Sciences of Harvard College, in partial fulfilment of the requirements of the Degree of Bachelor of Arts in Honors.

[2] Presented, in part, before the Boston Society of Biologists, April 15, 1942.

[3] This study was aided, in part, by a grant "In recognition of Doctor Francis W. Peabody's services to the Foundation" by the Ella Sachs Plotz Foundation; and, in part, by a gift to Harvard University from the Smith, Kline and French Laboratories, Philadelphia, Pennsylvania.

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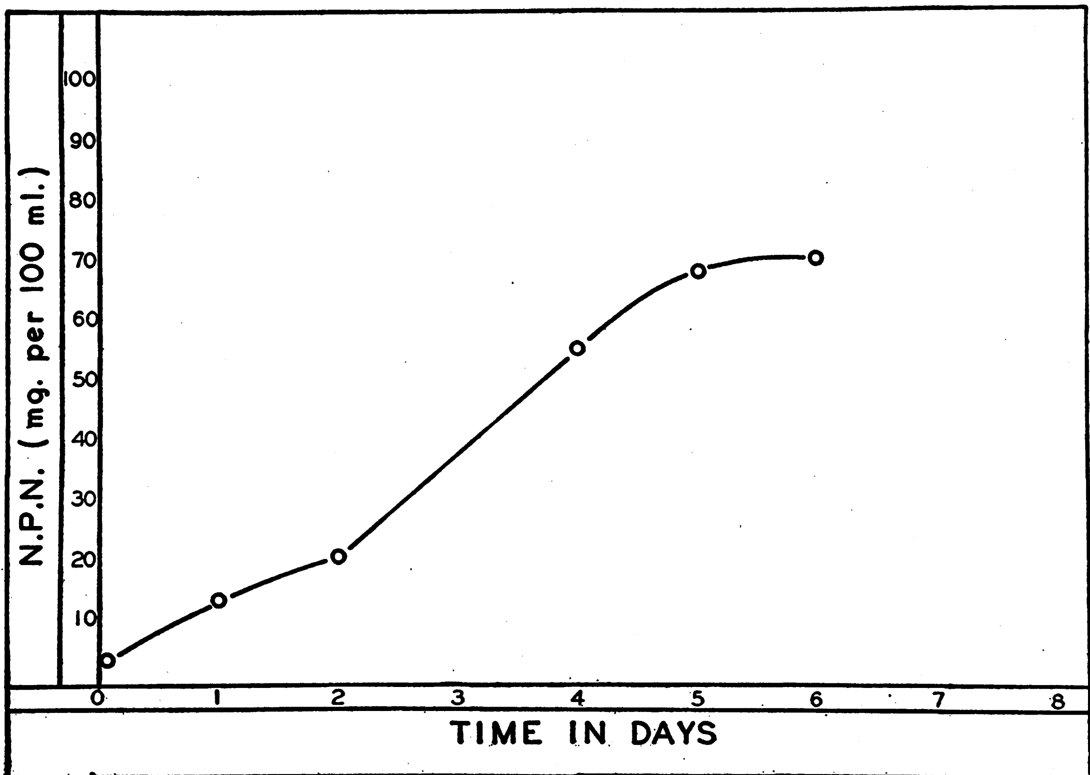

FIG. 1. INCREASE IN NON-PROTEIN NITROGEN FOLLOWING THE EXPOSURE OF PLASMA EUGLOBULIN TO THE ACTION OF CHLOROFORM

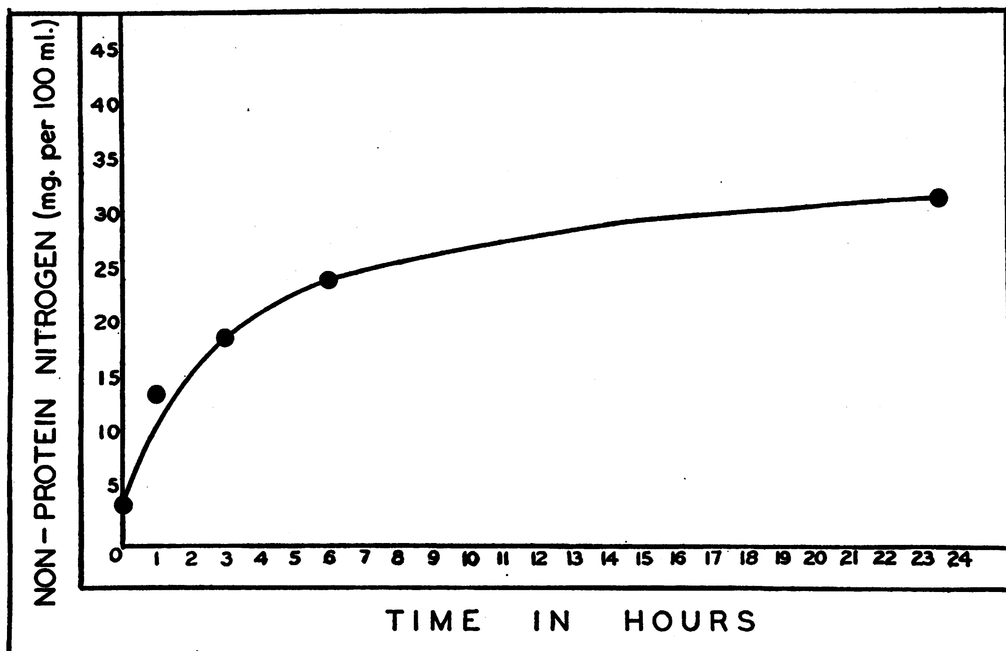

FIG. 2. HYDROLYSIS OF CASEIN BY THE PLASMA PROTEASE

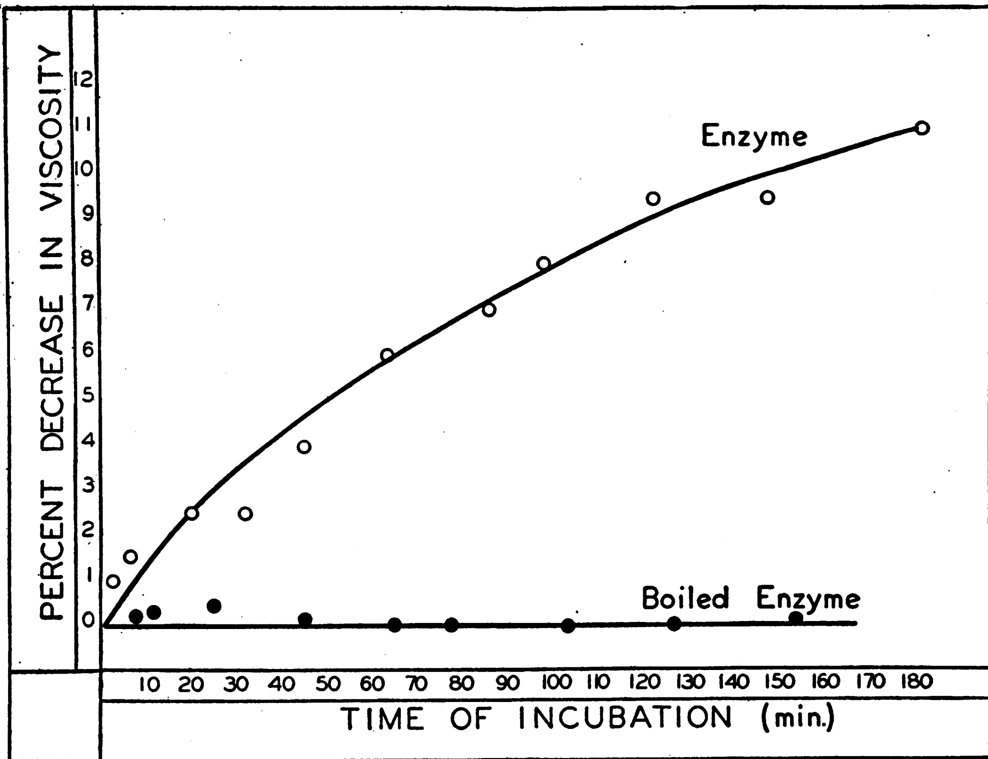

FIG. 3. THE EFFECT OF THE ACTION OF THE PLASMA ENZYME ON THE VISCOSITY OF A GELATIN SOLUTION

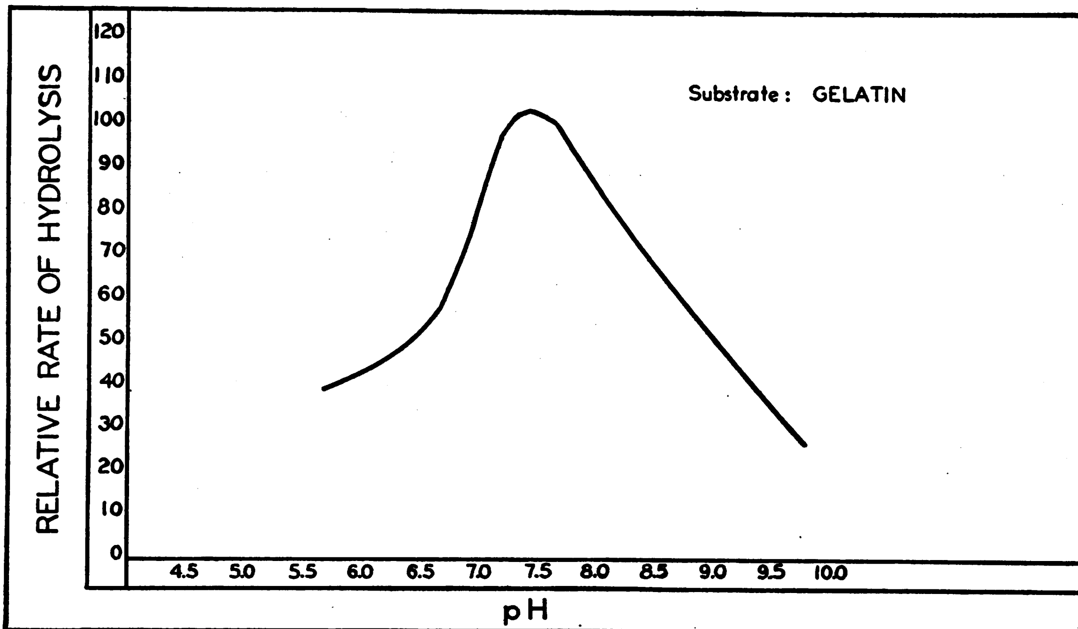

FIG. 4. THE EFFECT OF pH ON THE ACTIVITY OF THE PLASMA ENZYME

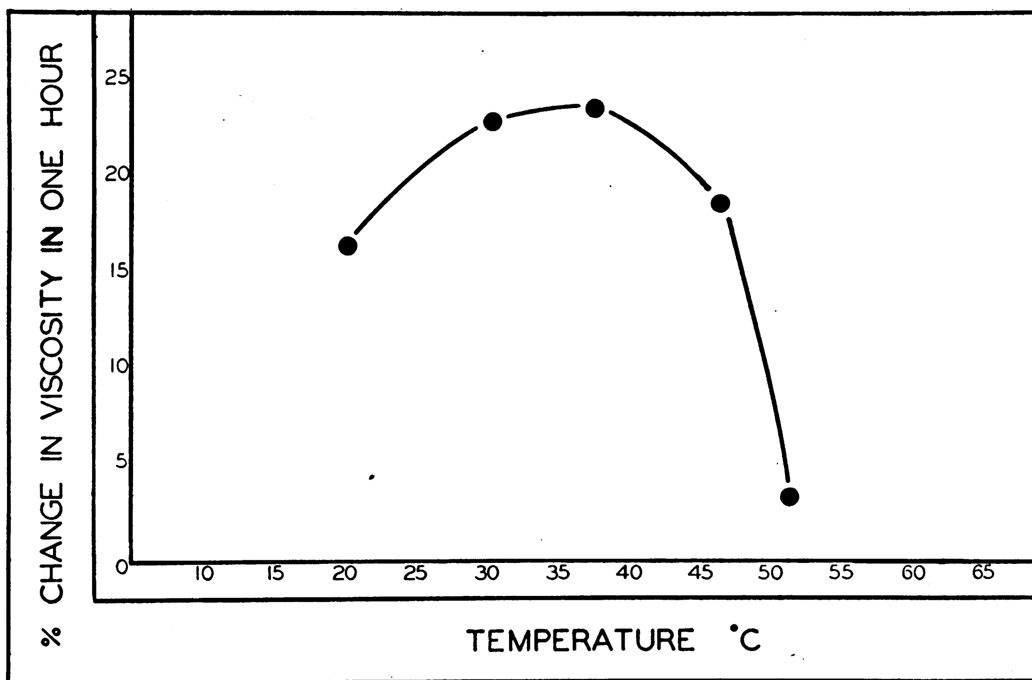

FIG. 5. THE EFFECT OF TEMPERATURE ON THE RATE OF HYDROLYSIS BY THE PLASMA PROTEASE

hydrolysis takes place (Figure 2). Entirely similar results were obtained when casein was used as a substrate.

Viscosimetric studies. To study the effect of pH and temperature on this enzyme, viscosimetric studies were employed. Figure 3 shows the effect of the active enzyme preparation on the rate of flow of a gelatin solution in a typical experiment. The lower curve is a control in which the enzyme solution was inactivated by boiling.

Since the enzyme preparations were obviously impure, there seemed no point in developing the reaction kinetics too far. The empirical equation of Schutz was applied to the reaction. Table I shows that the Schutz law holds remarkably well for the plasma enzyme.

The optimum pH of proteolysis by the plasma enzyme. Gelatin solutions, having an initial pH between 5.5 and 10, were made. The enzyme samples were adjusted to similar pHs and mixed. After each run, the pH was checked and the curve for change in viscosity time was drawn. The curve shown in Figure 4 was obtained by plotting the change in viscosity, 1 hour after the run was commenced, against the pH. It will be observed that the enzyme has an optimal pH at 7.4.

The effect of temperature on the plasma enzyme. As was shown in Figure 3, when a solution of the enzyme was boiled, its activity was destroyed. Figure 5 shows a composite of several enzyme curves giving the rates of viscosity change 1 hour after mixing at different temperatures from 2° to 50° C. The optimum temperature of the enzyme reaction was found to be approximately 36° C. There was no activity at temperatures above 50° C. The temperature coefficient (Q10) for the 10 degree interval between 20° and 30° C. was 1.5. The temperature coefficient was obtained by determining the ratio of the reaction velocities at 20° and 30° C. This coefficient was within the range found for most proteolytic enzymes. Trypsin alone has a higher temperature coefficient.

DISCUSSION

The foregoing evidence indicates that a true proteolytic enzyme can be prepared from oxalated or citrated steer plasma by the addition of chloroform. This proteolytic activity is associated with the globulin fraction of the plasma proteins, after treatment with chloroform.

As early as 1903, Delezenne and Pozerski (3) reported that normal serum treated with chloroform had a lytic effect on gelatin. Abderhalden (4), knowing that normal serum had no such proteolytic effect, dismissed this finding as being due to the action of chloroform on the leukocytes. Barker (5) believed that the fibrinolysis of clots, which he also observed, was due to entrapped mononuclear cells. In all this work, however, the manipulations required might well justify Abderhalden's criticism of an intracellular source of the enzyme. The first investigator to work with cell free plasma was Hedin (6) who in 1904, showed that it could digest both casein and gelatin but not coagulated egg albumin. Unfortunately,

Hedin's preparations were very weakly active, and precise data on the properties of the enzyme were not obtainable.

TABLE I

Application of the Schutz rule to the plasma protease

t Time of incubation in hours	y % change in viscosity	$K = \frac{y}{\sqrt{t}}$
2	9.0	6.4
4	15.3	7.7
6	20.1	8.3
8	24.3	8.6
10	28.0	8.8
12	30.9	8.9
14	33.0	8.8
16	34.5	8.6
18	35.4	8.3
20	36.0	8.0

The present data indicate that the active enzyme is a protein capable of lysing fibrin, fibrinogen, casein, and gelatin. Lysis is accompanied by an increase in split protein products and also by a diminution of the viscosity of gelatin. The enzyme is thermolabile, being destroyed by boiling, and having an optimal temperature *in vitro* of 36° C. Its optimal pH is between 7.4 and 7.9.

Schmitz (7), in 1936, was able to isolate from plasma an enzyme capable of producing slight increases in non-protein nitrogen when added to substrates of casein and gelatin. It is interesting that his data for optimum pH agree fairly well with our own, although the activity of his preparations was much less.

The plasma enzyme resembles trypsin although identification of the new enzyme with trypsin is not at present possible. It is probably not of pancreatic origin since derivatives of depancreatized dog plasma have been shown to be quite satisfactory for the formation of the enzyme (8).

The data of this communication deal with preparations of steer blood. Similar preparations from human and swine plasma were, by the methods described herein, equally active as proteolytic enzymes. However, horse plasma has been found deficient as a source of the enzyme.

CONCLUSIONS

(*1*) When chloroform acts on oxalated or citrated steer plasma and plasma globulins subsequently precipitated, these globulin fractions have the properties of an enzyme.

(*2*) This enzyme is capable of digesting fibrinogen, fibrin, gelatin, and casein as indicated by the progressive formation of non-protein nitrogen from the substrates. The non-protein nitrogen studies were confirmed in the case of gelatin by viscosimetric studies.

(*3*) The enzyme has an optimal pH of 7.4 at 37.5° C.

(*4*) The enzyme is destroyed by boiling and exhibits its optimal activity at a temperature of 36° C. at pH 7.

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