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PROTAMINE–HEPARIN COMPLEX AS A SUBSTRATE FOR PLASMIN

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SUMMARY

A method for estimating the proteolytic activity of plasmin using a protamine–heparin complex as substrate has been developed. The optimum conditions of substrate concentration, pH, temperature, etc., are described. Digestion by plasminogen–streptokinase mixtures is shown to be bi-phasic and the significance of this is discussed briefly.

INTRODUCTION

Many substrates have been used for the proteolytic assay of plasmin. They include casein[1–4], denatured haemoglobin[5], gelatin[6,7], collagen (in the form of azocoll)[8], as well as fibrin, which is usually considered to be the natural substrate.

Serum was found by HAGEDORN[9] to possess the property of breaking down complexes of proteins (*e.g.* insulin) with protamine. This observation was confirmed and extended by BRUNFELDT AND POULSEN[10] who showed that the enzyme involved was 'fibrinolysin' (plasmin). KJELDGAARD AND PLOUG[11] utilised this observation to estimate the proteolytic activity of plasmin by measuring the decrease in absorbancy of a suspension of protamine heparin complex when incubated with plasmin or a plasmin-generating mixture. They described their method briefly. The method appealed to us as a potentially simple one for the proteolytic assay of plasmin. We found however that the conditions described by KJELDGAARD AND PLOUG were not optimal, so a study of the effect of pH, salt concentration, temperature, etc., was undertaken and its results are reported in this paper.

MATERIALS

Plasminogen was prepared by the procedure of KLINE[12] from lyophilised COHN human plasma Fraction III, omitting the final step of precipitation with phosphate buffer at pH 6.4. It was exhaustively dialysed against distilled water to which just sufficient HCl was added to make the pH 2.0. The human plasma Fraction III was obtained from Pentex, Inc. Streptokinase was obtained as 'Varidase' (Lederle); it was dissolved in 0.025 M Tris buffer (pH 8.5). Protamine sulphate (salmine sulphate,

Lights) and heparin (powder, Boots) were dissolved in water to make stock solutions containing 50 mg/ml.

METHOD

The principle of the method is that the protamine in a complex of heparin and protamine is attacked by proteolytic enzymes and heparin is set free. The optical clearing of the suspension of the complex which occurs, is measured. KJELDGAARD AND PLOUG[11] have shown, by measuring the heparin set free, that the decrease in turbidity is proportional to the degree of breakdown of the complex.

The substrate

This is prepared by mixing equivalent amounts of protamine sulphate and heparin. It has been found sufficient to assume that a given weight of protamine sulphate will neutralize an equal weight of heparin.

Equal volumes of protamine sulphate solution and heparin solution, both at a concentration of 2 mg/ml are mixed. A stable turbid solution ("stock suspension") results. If more concentrated solutions are mixed, a useless, coarse and sticky precipitate results. The "stock suspension" can be stored indefinitely without change, at 4°. Merthiolate, 1 : 1000, is added. It is further diluted 20 times with 0.025 M Tris buffer (pH 8.5) for use. This provides a substrate with an absorbancy between 0.600 and 0.700 when read at 450 mμ in a 1-cm cuvette and gives optimum digestion rates (see *Substrate concentration*).

The absence of an excess of protamine or heparin in the substrate is of importance, as the digestion rates are considerably modified by an excess of either[13]. The absence of free protamine or heparin in the substrate is checked by centrifugation of an aliquot of the "stock suspension" at 20 000 rev./min at 4° for 30 min. Additional heparin or protamine is added to aliquots of the clear supernatant and the absence of precipitation or turbidity formation checked spectrophotometrically.

Assay

In most of our studies we have, in common with many other workers, used plasminogen activated with streptokinase as "plasmin". The assay is performed in two stages, *viz.*, the activation of KLINE plasminogen with streptokinase ("activation mixture") and then an aliquot of the activated material is transferred to the substrate or the substrate is added to the "activation mixture". The substrate and enzyme is designated "digestion mixture".

The "standard activation mixture" consists of 0.2 ml of plasminogen, 0.2 ml of 0.025 M Tris buffer (pH 8.5) and 0.1 ml of streptokinase solution. After an activation period, 2.5 ml of substrate is added and the zero reading taken immediately. This, the "standard digestion mixture", is then incubated at 37° and further readings made at 5, 10, 15 and 30 min. The absorbancy was measured at 450 mμ in 1-cm cuvettes with a Unicam SP 600 spectrophotometer against a distilled-water blank.

A plasmin unit (P.U.), as defined here, is the amount of enzyme which gives rise to a decrease of $1 \cdot 10^3$ absorbancy units/min, measured at 450 mμ during the linear phase of the reaction with a substrate concentration of 0.2 mg complex/ml in 0.025 M Tris buffer (pH 8.5).

It was found in work to be reported elsewhere[13] that optimum conditions for activation of KLINE plasminogen to plasmin were: streptokinase concentration, 500 μg/ml; Tris buffer 0.025 M (pH 8.5), activation period, 5 min. These conditions for the activation of plasminogen apply to the studies reported here on the digestion of the substrate by plasminogen–streptokinase activation mixtures.

EXPERIMENTAL

Factors influencing digestion

1. Temperature. Optimum digestion occurs between 37° and 45°; digestion is slow at room temperature or below. At 56° there is evidently rapid initial digestion

TABLE I

EFFECT OF TEMPERATURE ON DIGESTION RATE

Standard digestion mixture (see text).

Temperature °C	Digestion rate (P.U.) for digestion period (min)		
	0–5	5–15	15–30
4	4.0	0	5.3
21.5	12.0	2.0	12.7
28.0	16.0	8.0	16.5
37.0	46.8	23.6	23.1
45.0	52.0	21.0	19.7
56.0	56.4	9.2	10.0

but also rapid destruction or inactivation of the enzyme, so that the rate falls rapidly (Table I). The rapid initial phase (0–5 min) of digestion (see later) is also retarded by low temperatures but not as much as the subsequent slower phase (*cf.* rates for period 0–5 min and 5–15 min in Table I).

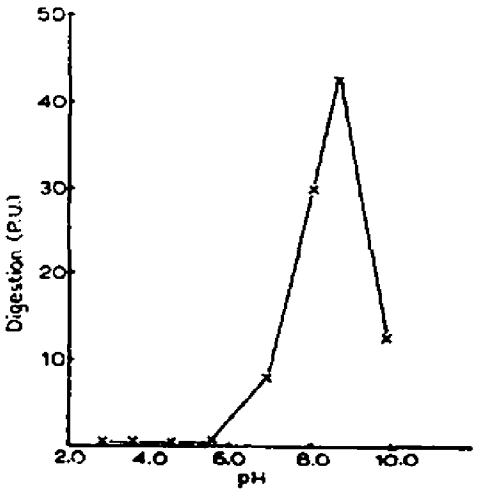


Fig. 1. The effect of pH of digestion mixture on digestion.

2. pH of digestion mixture. Aliquots of an "activation mixture" after 5 min activation, were transferred to aliquots of substrate adjusted to a pH ranging from 4 to 10 by addition of appropriate buffers (for pH 4–6, 0.05 M citrate buffer; for pH 7–9, 0.05 M Tris buffer and for pH 10, 0.05 M borax–NaOH buffer). The substrates were kept in an ice bath during addition of activation mixture; the pH of each was checked after its addition and adjusted if necessary. A zero reading was made and then the tubes incubated at 37°. Fig. 1 shows that the optimum pH lies

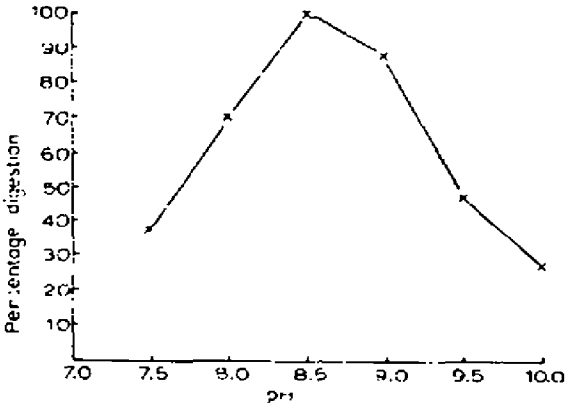


Fig. 2. The effect of pH of digestion mixture on digestion. The digestion at each pH is expressed as a percentage of that occurring at the optimum pH found, 8.5.

between 8 and 9; a more detailed study of the pH range 7 to 10 is shown in Fig. 2. Deterioration of plasmin is conspicuous at pH values above 9.0.

3. Ionic strength. We discovered early in our studies that the digestion rate is markedly affected by the concentration of salt in the digestion mixture. This effect is illustrated in Fig. 3. Very low concentrations (< 0.02 M) enhanced the rate of digestion but concentrations of 0.05 M and above are inhibitory to digestion, and

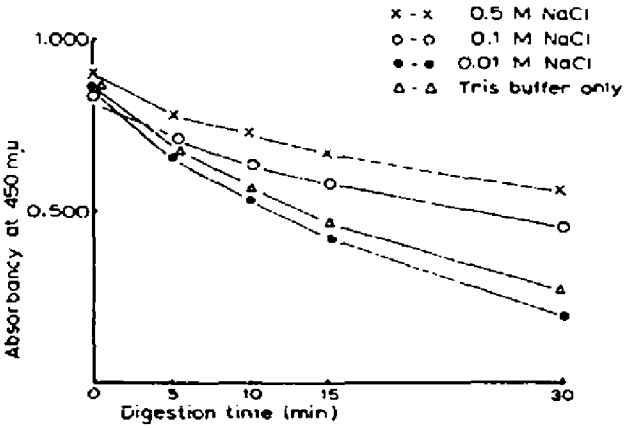


Fig. 3. The effect of salt on digestion.

hence the use of physiological saline (0.15 M) for diluting purposes, or of salt-containing buffers such as that of PALITZSCH[14] or OWREN[15], is precluded with this method.

Other cations were studied. Potassium chloride was identical in behaviour to sodium chloride. Magnesium chloride was inhibitory at 0.01 M and above, but was conspicuously activatory at 0.0001 M; calcium chloride behaved similarly (Table II).

TABLE II

EFFECTS OF SALTS ON DIGESTION

Digestion mixtures with substrate containing concentration of salt shown. The results are expressed as a percentage of the digestion occurring in standard digestion mixture (*i.e.* without salts). —, not tested.

Salt	*Percentage digestion*						
	Molarity						
	0.5	*0.25*	*0.10*	*0.05*	*0.01*	*0.001*	*0.0001*
NaCl	42	53	73	80	108	98	102
$MgCl_2$	28	22	71	—	87	125	146
$CaCl_2$	*	—	61		77	131	157

* The substrate was soluble in this concentration of calcium chloride solution.

Tris buffer was found to have a slight activating effect on digestion with increasing concentration up to 0.2 M in the substrate (Table III).

4. Substrate concentration. Well-marked substrate competition can be demonstrated with protamine–heparin complex. To demonstrate this, aliquots of an "activation mixture" were transferred to substrates containing from 4 mg of heparin–protamine complex per ml down to 0.1 mg/ml. At concentrations above 0.2 mg/ml, readings could not be made directly on the digestion mixtures, so aliquots were removed and diluted for reading. Fig. 4 shows a direct plot of the velocities against

TABLE III

THE EFFECT OF MOLARITY OF TRIS BUFFER (pH 8.5) ON DIGESTION

Standard digestion mixture.

Molarity of Tris buffer in substrate	*Digestion rate (P.U.)*
Nil*	8.0
0.01	35.3
0.025	38.7
0.05	42.1
0.1	46.5
0.2	44.8

* This tube contained distilled water-diluted substrate and was consequently unbuffered. Its pH was 7.2, *i.e.* well below the optimum for digestion.

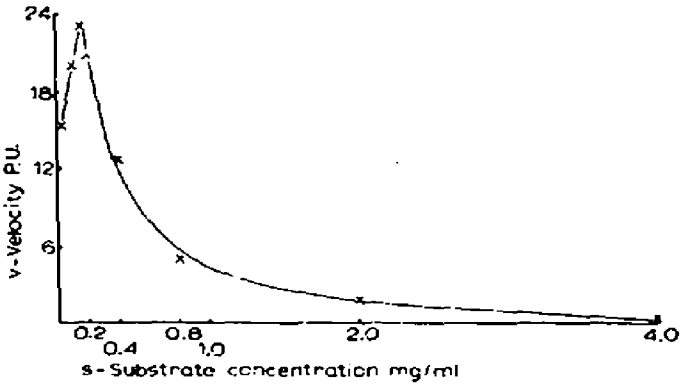


Fig. 4. Direct plot of velocity *vs.* substrate concentration. Standard digestion mixture. Velocity calculated from linear phase of digestion (5–15 min).

substrate concentration, and Fig. 5 a Lineweaver-Burk plot for the same data. K_m is 0.30 mg/ml for this plot.

It is a fortunate coincidence that the optimum substrate concentration is near to that providing the most suitable initial absorbancy for spectrophotometric measurement at 450 mμ.

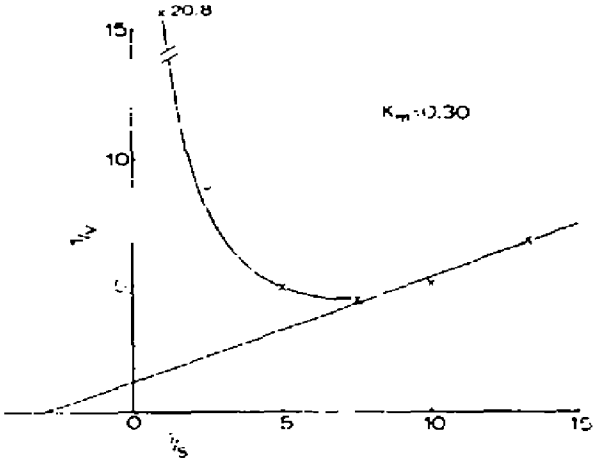


Fig. 5. Lineweaver Burk plot of data of Fig. 4.

Characteristics of the digestion of protamine heparin substrate by plasminogen-streptokinase mixtures

Fig. 6 shows a typical plot of the digestion of protamine–heparin substrate by an activation mixture of plasminogen and streptokinase. There is a more rapid phase of digestion during the first 5 min than during the succeeding three 5-min periods, when digestion is linear. This rapid initial phase tends to disappear when diluted KLINE plasminogen is used (see below). On account of the non-linearity of the initial

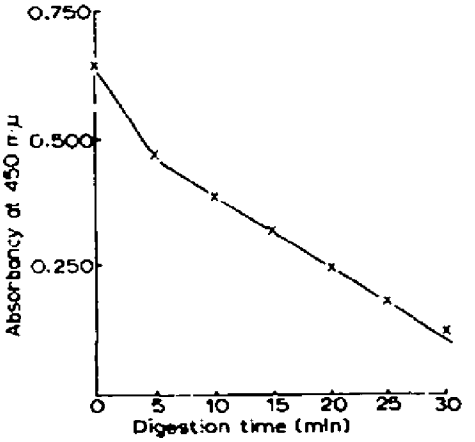


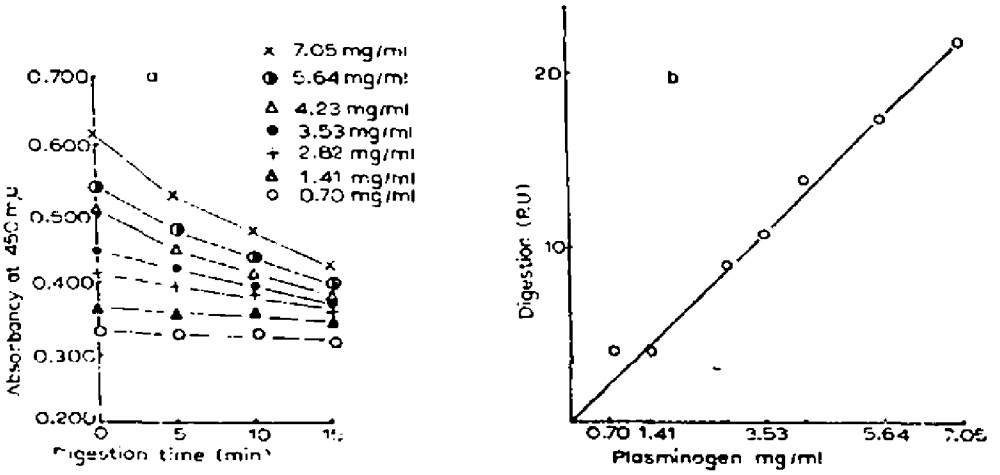
Fig. 6. Digestion of protamine–heparin complex by plasminogen–streptokinase mixture, showing initial rapid phase of digestion. Digestion for period 0–5 min = 88.0 P.U. Digestion for period 5–20 min = 30.0 P.U.

phase, we have used the digestion occurring during the linear phase (from the 5th to the 15th min) for our kinetic studies (*cf.* DERECHIN[3]).

Figs. 7 a and b show the relationship of plasminogen concentration to digestion, with constant substrate concentration. The disappearance of the initial rapid phase of digestion on dilution of the plasminogen is seen in Fig. 7a.

The digestion of protamine–heparin substrate by isolated plasmin

In a few experiments plasmin isolated from a plasminogen–streptokinase activation mixture by precipitation at pH 2.0 in the presence of 1 M sodium chloride[16]



Figs. 7a and b. Digestion of protamine–heparin substrate by a series of dilutions of plasminogen. Fig. 7b shows linear relationship of digestion to plasminogen concentration. Digestion was calculated from linear phase of digestion (5–15 min) in Fig. 7a for each plasminogen concentration.

has been used. In Table IV a comparison is made of the digestion by a plasminogen - streptokinase mixture and plasmin precipitated from it by 1 M NaCl at pH 2.0. There is a loss of activity, the isolated plasmin having 69% of the activity per unit protein possessed by the plasminogen–streptokinase mixture. This is in accord with the findings of others[17].

The effect of salt on the digestion of the substrate by plasmin is also shown in Table IV. There is the same pattern of inhibition of plasmin by salt as is found with plasminogen–streptokinase mixtures.

TABLE IV

COMPARISON OF ISOLATED PLASMIN AND PLASMINOGEN–STREPTOKINASE MIXTURE, AND EFFECT OF SALT CONCENTRATION ON PLASMIN DIGESTION

Digestion mixture	*Conc. of NaCl in substrate*	*Digestion (P.U./mg protein)*
Plasminogen + streptokinase	Nil	25.6
Plasmin	Nil	17.7
Plasmin	0.05 M	14.8
Plasmin	0.1 M	13.7
Plasmin	0.25 M	12.9
Plasmin	0.5 M	7.8

Comparison with other substrates

Dilutions of a single batch of KLINE plasminogen were tested against casein in parallel with protamine–heparin substrate. Casein and protamine–heparin substrates seem to be similar in sensitivity provided a digestion period of an hour is allowed for the higher dilutions with casein. With protamine-heparin substrate a digestion period of 15 min was satisfactory. The results of this experiment are shown in Table V. It seems likely from the ratios of caseinolytic to protamine–heparin digestion that the two substrates are measuring the same proteolytic enzyme.

TABLE V

COMPARISON OF CASEIN AND PROTAMINE–HEPARIN COMPLEX AS SUBSTRATES FOR PLASMINOGEN–STREPTOKINASE MIXTURES

Plasminogen conc. (mg/ml)	*Casein units (CU)/mg protein*	*Plasmin units** (PU)/mg protein*	*Ratio CU/PU*
2.84	4.6	28.2	1/6.1
1.92	5.7	31.2	1/5.4
1.15	5.5	34.0	1/6.0
0.77	5.2	29.9	1/5.7

* Casein unit = amount of enzyme which will release colour equivalent of 450 μg tyrosine in 60 min from 2% casein in borate–saline buffer (pH 7.6; I 0.2). Streptokinase 200 μg/ml plasminogen.

** Standard digestion mixture for protamine–heparin substrate. Plasmin units as defined in text.

DISCUSSION

The method described appears to be a simple and satisfactory procedure for assaying the proteolytic activity of plasmin. It is, of course, not specific, and the possibility exists of measuring a protaminase in a preparation instead of plasmin. However, the protaminase of human serum or plasma attacks the substrate very slowly indeed and a streptokinase-activated protaminase other than plasmin is not known. Human euglobulin preparations also attack the substrate very slowly under the experimental conditions described. On the other hand, if streptokinase is added to either native human serum or human euglobulin preparations, very rapid digestion occurs. Little is known about the protaminase[19] of human serum; it seems unlikely it would survive as a contaminant of KLINE plasminogen or of plasmin prepared by salt precipitation at pH 2.0. AMRIS[20] has recently confirmed BRUNFELDT AND POULSEN'S[19] demonstration that the "protaminase" activity in streptokinase-activated human serum or serum fractions is due to plasmin.

The substrate is simply prepared from readily obtainable materials and is stable for long periods of time. Incubation with isotonic media in the physiological pH range does not alter the turbidity to any significant extent. It is however soluble in NaCl solutions of 0.5 M and above at pH 8.5 on prolonged (> 2 h) incubation at 37°. GODAL[21] found the complex insoluble at neutral pH even at ionic strengths as high as 3.0, but does not mention temperature or time. He also showed that the heparin of the complex "protected" the protamine against plasma protaminase.

The assay procedure is exceedingly simple, reproducible, and requires no further apparatus than a spectrophotometer reading in the visual range. The assay is accomplished rapidly (*cf.* heated fibrin plate method[18]) and is particularly suitable for progressive studies.

The findings in respect of temperature are in line with previous observations using other substrates. The optimum pH for plasmin using casein has generally been recognised to be about 8.0 (ref. 22); the optimum with protamine–heparin substrate is near 8.5. It is to be noted that digestion is far from optimal below pH 8.0. (*cf.* the pH of the digestion mixture apparently used by KJELDGAARD AND PLOUG[11] *viz.* 7.2. This is inferred from their description of materials.)

The inhibitory effect of chloride (or increased ionic strength) in various fibrinolytic and plasmin proteolytic systems has been observed by various authors. PILLEMER *et al.*[23] observed that increasing ionic strength inhibited the fibrinolytic activity of serum activated by streptokinase and suggested that this effect is due to inhibition of activation of plasminogen by streptokinase. However, with protamine–heparin substrate, our results with isolated plasmin indicate that the inhibition described is a direct one on proteolysis.

It is of interest to note that if plasminogen is added to the substrate (standard concentration: 0.04 μg/ml) and streptokinase is added last, activation and/or digestion is very poor indeed[13]. Prior activation of plasminogen with streptokinase even for as short as 2 min is necessary. On the other hand, KLINE[24], KJELDGAARD AND PLOUG[11], and NORMAN[25] all recommend activation of plasminogen by streptokinase in the presence of the substrate (casein or other) in order to forestall the deterioration of the plasmin generated, which, it is claimed, is stabilised by the presence of sub-

strate. Our findings do not indicate such a rapid deterioration of plasmin in the activation or digestion mixture at 37° or below.

With casein as substrate, an initial lag period was found by DERECHIN[3] but not by MULLERTZ[22], or MARKUS AND AMBRUS[26]. Indeed, these latter workers, using sub-optimal streptokinase concentrations, found an initial rapid phase during the first minute or two, followed by a much slower, linearly rising activity. They attribute the two phases to two distinct enzyme forms which they refer to as α- and β-plasmin, and suggest that α-plasmin may be "activator".

Our demonstration of an inital rapid phase is, we think, confirmatory of the work of MARKUS AND AMBRUS. The temperature studies indicate that this rapid phase is enzymic and while it is not as depressed by lower temperatures as the slower phase is, it would appear that the activity is very labile. This early rapid phase is not as a rule seen with plasminogen precipitated from KLINE plasminogen at pH 7.9 with phosphate buffer[27]. As such phosphate-precipitated plasminogen attacks protamine–heparin substrate very slowly on streptokinase activation unless the supernatant of the precipitation procedure is also added[13], we also think that the early rapid phase is due to a distinct form of plasmin which has "activator" activity (and may be responsible for "streptokinase co-factor"[28] activity towards animal plasminogens) and that this form of plasmin is derived from a soluble (at neutral pH) form of plasminogen which we referred to in a previous publication[27] as a "pro-activator".

It could be argued that the rapid initial phase is true plasmin digestion and that the succeeding phase is due to plasmin destruction (*cf.* LASSEN[29]). However this is unlikely as the second slower phase is linear for about 15–20 min after the end of the initial rapid phase.

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