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ROLE OF HAGEMAN FACTOR IN THE ACTIVATION OF GUINEA-PIG PRE-KALLIKREIN

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Davies and Lowe (1963) showed that the 7 S γ-globulin fraction of guinea-pig serum contained the enzyme kallikrein. The abilities of the γ-globulin to increase vascular permeability and to hydrolyse esters of arginine and lysine appeared to be related properties, and both could be increased by shaking solutions of γ-globulin with glass beads which had been treated with guinea-pig serum or by the addition of certain albumin-rich fractions obtained from guinea-pig serum. This activation led to the conclusion that the γ-globulin fraction contained both active kallikrein and its precursor. This precursor has been referred to in the literature by a variety of names—for example, kallikreinogen, prokininogenase, pro-kallikrein. We propose to adhere to the *Recommendations of the International Union of Biochemistry on Enzyme Nomenclature* (1965) and refer to the precursor of kallikrein (E.C.3.4.4.21) as pre-kallikrein.

The work summarized above supported the findings of Margolis (1958) that kinin was released when human plasma came into contact with glass. He postulated that glass adsorbs and activates Hageman factor and this in turn activates a component "A" which, together with a component "B," causes release of kinin. In a later paper, Margolis & Bishop (1962) suggested that component "A" might be pre-kallikrein. Ratnoff & Miles (1962, 1964) have also implicated Hageman factor in the activation of the kallikrein system; they found that vascular permeability increasing activity was gradually evolved when diluted human plasma was incubated in silicone-coated tubes with isolated activated human Hageman factor.

In this paper we present more evidence to show that Hageman factor is involved in the conversion of guinea-pig pre-kallikrein to kallikrein.

METHODS

Isolation of γ-globulins

The 7 S γ-globulin fraction was isolated from fresh guinea-pig serum by chromatography on diethylaminoethyl cellulose (Whatman Powder, D E 50) as described previously (Davies & Lowe, 1961).

Esterase activity

This was determined by the colorimetric procedure of Roberts (1958) using the conditions previously described (Davies & Lowe, 1963). N^{α}-tosyl-L-arginine methyl ester hydrochloride (TAMe) (Mann Research Laboratories) was the substrate.

Estimation of increased vascular permeability

Effects on vascular permeability were measured as described previously (Davies & Lowe, 1961) by intradermal injection into guinea-pigs previously "blued" by intravenous injection of Pontamine Sky Blue. The maximum and minimum diameters of the blue areas were measured immediately after the skins had been pinned out and not on the following day as in the previous study. The standard errors of the measurement with groups of four animals was ± 0.4 mm.

Activation of pre-kallikrein with glass

In a modification of the method described by Margolis (1958) fresh guinea-pig serum, in various dilutions, was shaken for varying times with different quantities of glass ballotini (microspherules 0.1 mm in diameter). After removal of the serum, the ballotini were washed thoroughly with saline (usually three or four washings were sufficient to render the supernatant liquid free from permeability activity). A solution of γ-globulin of known concentration was then added to the treated ballotini, shaken for a given time and then removed. Control ballotini were treated with saline instead of serum before the addition of γ-globulins. Dilutions of serum were made with physiological saline and γ-globulin preparations were dissolved in buffer of the following composition: sodium chloride, 85 g; sodium barbitone, 3.75 g; barbituric acid, 5.75 g; water to 2 l. Before use the buffer was diluted 1:5 with distilled water.

Preparation of Celite eluate

Our original preparations of Celite eluate were made by the method of Nossell (1964). For most of the work described in this paper the eluate was prepared as follows: Celite 545 (Johns Manville, London), (15 g) was mixed into a slurry with physiological saline and then poured into a glass chromatography column to make a column of Celite 17×1.7 cm. The Celite was thoroughly washed with saline before use. Fresh guinea-pig serum (30 ml.), the ability of which to increase both esterase and permeability activities of γ-globulin had been checked by the ballotini method, was put on the column and washed through with saline. The diluted effluent serum was passed through the same column repeatedly until it no longer rendered ballotini capable of activating pre-kallikrein. Usually eight such passages were sufficient but occasionally 9 or 10 were required. All activator was then assumed to have been removed from the serum. The column was washed with one column volume of physiological saline and the active material eluted with 10% $^{w}/_{v}$ sodium chloride, the eluate being collected in 10 ml. fractions. Each fraction was tested for its ability to activate pre-kallikrein by the method described below. The active fractions were bulked, dialysed against saline and stored at 2° C when not in use.

Assay of activator activity in Celite eluates

For this assay the pH Stat (Radiometer, Copenhagen) was used. One ml. of substrate (TAMe 0.06 M) was incubated with 0.005 M phosphate buffer pH 7.6 (1 ml.) and γ-globulin (1 mg dissolved in 1 ml. saline and pH adjusted to 7.6). The amount of alkali (N/40 NaOH) required to keep the pH constant at 7.6 was then measured for 10 min. Celite eluate (0.1 ml.) was added and the amount of alkali used in the ensuing 10-min period was recorded. Blanks of TAMe (1 ml.) buffer (1 ml.) and Celite eluate (0.2 ml.) were also determined. An increase in the amount of alkali used after the addition of the Celite eluate to the γ-globulin was taken as a measure of the ability of the eluate to convert pre-kallikrein to kallikrein.

Incubation of γ-globulin with Celite eluate

A solution of γ-globulin (5 ml. of 1 mg/ml.) was incubated at 37° C for 10 min with the appropriate volume of Celite eluate (usually 0.1 to 0.5 ml.). Portions of this mixture were used for determinations of esterase and vascular permeability activities. Blanks were measured on the Celite eluate with saline (5 ml.) in place of the solution of γ-globulin, and also on the solution of γ-globulin plus saline (0.1 to 0.5 ml.).

Use of collagen for adsorption and activation of Hageman factor

The appropriate weight of collagen (see text) was suspended by shaking in various dilutions of guinea-pig serum (2 ml.) contained in polypropylene centrifuge tubes. The suspensions were then

shaken in a metabolic incubator (Gallenkamp) at 37° C for varying times. After shaking, the tubes were centrifuged for 1 min at 1,000 *g*. The supernatant was discarded and the collagen re-suspended in saline (4 ml.) and again centrifuged. The washing was repeated four times.

Activation of pre-kallikrein with serum-treated collagen

A solution of γ-globulin was added to the collagen previously treated with guinea-pig serum and washed as described above. The suspension was agitated to disperse the collagen and then shaken at 37° C in the metabolic incubator for the times indicated in the text. After the shaking the suspension was centrifuged and the supernatant decanted and used for studies on its esterase and vascular permeability activities.

Preparation of collagen

Tendon collagen (Sigma Chemical Company) was cut into small pieces with scissors. No attempt was made to achieve pieces of uniform size.

Human plasma

Hageman-trait human plasma was a gift from Dr. Margaret E. Mackay of the Lister Institute of Preventive Medicine, London. Normal human plasma was obtained from volunteers.

RESULTS

Conditions for activation with ballotini

Initial experiments showed that the esterase and vascular permeability activities of γ-globulin remained unchanged when the protein solution was shaken with untreated ballotini but an increase in the activity of the γ-globulin occurred when the ballotini had been treated with guinea-pig serum. With undiluted serum, there was less activation with 0.25 g ballotini/ml. γ-globulin solution than with 0.5 or 1.0 g/ml. (Table 1).

When the concentration of serum added to the ballotini was varied, satisfactory activation was achieved with a dilution of 1/100 but not with 1/1,000 (Table 2).

TABLE 1

ACTIVATION OF GUINEA-PIG PRE-KALLIKREIN WITH SERUM-TREATED BALLOTINI: VARIATION IN QUANTITY OF BALLOTINI

Varying weights of ballotini were shaken for 2 min with undiluted serum (2·5 ml.) The ballotini were then washed three times with saline (20 ml.) and shaken with a solution of γ-globulin (2·5 ml. at 0·2 mg/ml.)

Weight of ballotini (g/ml. γ-globulin)	Vascular permeability Lesion diameter (mm)	Esterase activity (μ-mole TAMe/hr/mg)
1 in serum	11·9	23
0·5 in serum	12·0	23
0·25 in serum	10·0	7·0
1 in saline	6·9	
0·5 in saline	7·1	
0·25 in saline	7·6	

TABLE 2

EFFECT OF VARYING THE DILUTION OF SERUM USED TO SHAKE WITH A FIXED WEIGHT OF BALLOTINI AND THEN TO ACTIVATE A CONSTANT AMOUNT OF γ-GLOBULIN

A fixed weight of ballotini (0·2 g/ml.) was shaken with serum of different dilutions, the ballotini were then washed three times with saline and used to activate a fixed amount of γ-globulin solution

Serum dilution	Vascular permeability Lesion diameter (mm)	Esterase activity (μ-mole TAMe/hr/mg)
Undiluted	14·1	22·8
1:10	15·0	19·6
1:100	13·4	18·0
1:1000	9·0	8·9
γ-globulin alone	8·0	

Variations were made in the shaking times both of the ballotini with serum and of the treated, washed ballotini with the solution of γ-globulin. Maximum activation was achieved when both periods were 2 min. When the shaking time of γ-globulin with treated beads was extended to 60 min, there was less apparent activation, suggesting that some active material had been destroyed (Table 3).

TABLE 3

EFFECT OF VARYING (1) THE TIME OF SHAKING BALLOTINI WITH SERUM, (2) THE TIME OF SHAKING SERUM-TREATED BALLOTINI WITH γ-GLOBULIN ON THEIR ABILITY TO ACTIVATE PRE-KALLIKREIN

The ballotini (0·1 g/ml.) were shaken with undiluted serum or saline for varying times, washed with saline five times and then shaken with γ-globulin solution (0·2 mg/ml.) for varying times. C.B. = beads shaken in saline only

Time of shaking with serum (min)	Time of shaking with γ-globulin (min)	Vascular permeability Lesion diameter (mm)
2	2	15·6
60	2	15·4
2	60	13·1
60	60	10·0
C.B. 2	60	4·0
C.B. 2	2	9·1
γ alone—	—	5·5
Last saline wash	—	9·7

When the serum was heated at 60° C for 1 hr no loss occurred in its ability to activate a γ-globulin so that the factor responsible for the activation must be heat-stable under these conditions.

Activation with ballotini shaken with Hageman-trait plasma

The work of Margolis (1959), Margolis & Bishop (1962), and our own previous results (Davies & Lowe, 1963), together with those reported above, suggested that Hageman factor might be responsible for the conversion of pre-kallikrein to kallikrein. An opportunity to test this suggestion arose when we were given a small sample of Hageman-trait human plasma by Dr M. E. Mackay. At the same time, we had to establish that normal human plasma could take the place of guinea-pig serum in the activation process with ballotini. The results presented in Table 4 clearly show that normal human plasma can activate γ-globulin while the Hageman deficient plasma does not. The results again support the notion that Hageman factor is directly concerned in the conversion of pre-kallikrein to kallikrein.

TABLE 4

ACTIVATION OF γ-GLOBULIN WITH NORMAL AND HAGEMAN-TRAIT HUMAN PLASMA

Ballotini (0·5 g) were shaken with the plasma (1 ml. of 1:25) for 2 min, washed five times, added to the γ-globulin solution (5 ml. of 0·5 mg/ml.) and again shaken for 2 min. The supernatant was tested for activity as an esterase and vascular permeability factor.

Treatment of ballotini	Vascular permeability Lesion diameter (mm)	Esterase activity (μ-mole TAMe/hr/mg)
Saline	11·4	5·0
Normal human plasma Sample 1	15·1	50·0
Normal human plasma Sample 2	14·9	44·8
Hageman-trait plasma	12·0	4·7

Activation with Celite eluate

It is now well established that treatment of fresh human plasma with Celite removes the contact factors—for example, Hageman factor and plasma thromboplastin antecedent (Nossell, 1964)—and that these factors can then be eluted from the Celite. The work of Schoenmakers, Matze, Haanen & Zilliken (1965) has established that Hageman factor is an esterase with specificity similar to that of kallikrein. Hence, before we could use Celite eluate in an attempt to activate pre-kallikrein we had to ensure that the concentration of Celite eluate employed had a sufficiently low esterase and vascular permeability activity. Table 5 shows that this particular eluate was satisfactory at a dilution of 1:625 (1.1 mg/ml.) while Table 6 presents evidence which demonstrates that Celite eluate produces the same activation as treatment of γ-globulin with serum-treated ballotini.

TABLE 5

THE ESTERASE AND VASCULAR PERMEABILITY INCREASING ACTIVITY OF CELITE ELUATE

The esterase activity of the Celite eluate was determined by the method given in the text. Dilutions of the eluate were made with buffer and injected intradermally into blued guinea-pigs to measure their ability to increase vascular permeability

Amount of Celite eluate injected (mg/ml.)	Vascular permeability Lesion diameter (mm)
720	16·8
140	15·0
28	13·5
5·6	12·0
1·1	4·5
Buffer alone	2·5

Esterase activity of this eluate was 7·2 μ-mole TAMe/hr/mg.

TABLE 6

ACTIVATION OF γ-GLOBULIN WITH CELITE ELUATE

Solution of γ-globulin diluted to 0·5 mg/ml. and shaken for 2 min with serum-treated ballotini or a solution of γ-globulin at 1 mg/ml. mixed with an equal volume of Celite eluate (1:1000)

Treatment of γ-globulin	Vascular permeability Lesion diameter (mm)	Esterase activity (μ-mole TAMe/hr/mg)
No treatment	6·0	1·0
Celite eluate	13·5	27·7
Celite eluate with no γ-globulin	0·8	0
Serum-treated ballotini	13·7	28·4
Saline-treated ballotini	7·3	1·0
Buffer with no γ-globulin	0·4	0

Activation with serum-treated collagen

Niewiarowski, Bańkowski & Fiedoruk (1964) and Niewiarowski, Bańkowski & Rogowicka (1965) have recently found that Hageman factor is almost selectively adsorbed from human plasma by collagen and that the factor is activated following adsorption. Because of these findings they suggested that collagen may act as an intrinsic trigger of blood coagulation *in vivo*. Since our previous work had implicated Hageman factor in the activation of pre-kallikrein, it appeared reasonable to see whether treatment of guinea-pig serum with collagen might lead to adsorption and activation of Hageman factor. In our initial experiments the collagen was incubated with either serum or

plasma in glass centrifuge tubes. However, we subsequently established that some Hageman factor remained adsorbed to the glass and this contributed to the effect of the Hageman factor adsorbed on the collagen surface in converting pre-kallikrein to kallikrein. All experiments reported here were carried out in polypropylene tubes. Serum, undiluted or diluted 1:50 (2 ml.) was added to the collagen (50 mg) and shaken with an amplitude of 1.5 in at a rate of 150 oscillations/min in a metabolic incubator at 37° C. The shaking was carried out for either 10 or 20 min, after which the collagen was washed five times with saline (5 ml. on each occasion). Pre-kallikrein solution was then added to the collagen (3 ml. of 1 mg/ml.) and shaking was again carried out under the same conditions but for either 15 or 30 min. The collagen was separated off and esterase values determined on the supernatants. Results are recorded in Table 7.

TABLE 7

ACTIVATION OF GUINEA-PIG PRE-KALLIKREIN WITH SERUM-TREATED COLLAGEN

Collagen (50 mg) was shaken at 37° with serum (2 ml.) either undiluted or diluted 1:50 for 10 or 20 min. The collagen was then washed five times with saline (5 ml. each wash) and then shaken with γ-globulin (3 ml. at 1 mg/ml.) for either 15 or 30 min at 37°

Time of shaking collagen with serum (min)	Dilution of serum	Time of shaking serum-treated collagen with pre-kallikrein (min)	Esterase activity (μ-mole TAMe/hr/mg)
10	Undiluted	15	26·1
10	Undiluted	30	49·5
10	1:50	15	22·5
10	1:50	30	48·5
20	Undiluted	15	49·2
20	Undiluted	30	48·0

Esterase activity of pre-kallikrein shaken with saline treated collagen was 6·7 μ-mole TAMe/hr/mg.

It can be seen that the treated collagen effected a marked conversion of pre-kallikrein to kallikrein and that serum diluted 1:50 was about as effective in achieving this conversion as was undiluted serum.

DISCUSSION

Previous work from these laboratories has established that guinea-pig 7 S γ-globulin contains a mixture of pre-kallikrein and kallikrein. It is probable that in normal circumstances the circulating γ-globulin contains the enzyme entirely in the precursor form and that the variable activities of isolated material arise from some activation during the process of isolation. On rare occasions, γ-globulin fractions are obtained which have no activity before activation (Davies & Lowe, unpublished observations) and these fractions presumably contain pre-kallikrein together with " inert " γ-globulin. The various procedures used *in vitro* for the conversion of pre-kallikrein to kallikrein all depend on the presence in guinea-pig serum (or plasma) of a substance analogous to activated Hageman factor. Thus treatment of guinea-pig serum with glass, Celite or collagen, procedures which are known to adsorb and activate Hageman factor from human plasma, all lead to the activation of pre-kallikrein. Moreover, the albumin-rich fractions obtained from guinea-pig serum and used by Davies & Lowe (1963) to convert pre-kallikrein to kallikrein have been shown by Dr J. G. G. Schoenmakers to contain

" low but definite and specific Hageman factor activity " (personal communication). The present work clearly shows that a sample of human plasma deficient in Hageman factor failed to serve as a source of activator while normal human plasma was fully satisfactory.

One interpretation of these results is that activated Hageman factor acts directly on pre-kallikrein. However, the use of Celite and glass implies that contact factor might be implicated in the sequence. Formation of contact factor is dependent on the presence of plasma thromboplastin antecedent (PTA) and Margolis (1959) has shown that the formation of plasma kinin is normal in PTA-deficient patients. More recently Ratnoff & Miles (1964) have shown that ellagic acid, a substance known to activate Hageman factor, produces the same amount of permeability factor when added to normal or PTA-deficient human plasma but none when added to Hageman-deficient plasma. The evidence available therefore does not support any role for PTA in the pre-kallikrein—kallikrein system.

Ratnoff & Miles (1964) have shown that partially purified Hageman factor will convert pro-PF to PF/Dil and that this conversion had the characteristics of an enzymic reaction. They suggest that PF/Dil in turn is responsible for the conversion of pre-kallikrein to kallikrein. If this suggestion is correct then it implies that the Celite eluate used in our studies must also contain PF/Dil and that the serum-treated ballotini must contain PF/Dil on their surface, since there was no evidence that our γ-globulin contained any PF/Dil. Moreover, if PF/Dil is adsorbed on the ballotini it is not eluted with physiological saline since the fourth saline washing had no vascular permeability activity. Our work establishes beyond doubt that Hageman factor is implicated in the activation of pre-kallikrein and we are currently attempting to resolve the role of pro-PF and other factors in this system.

SUMMARY

1. Pre-kallikrein, contained in the γ-globulins isolated from guinea-pig serum can be converted to active kallikrein by a brief shaking with glass beads or collagen previously treated with guinea-pig or human serum or plasma and then washed.

2. Plasma taken from a patient with Hageman-trait did not render glass beads capable of activating pre-kallikrein.

3. An activator of pre-kallikrein can be adsorbed on Celite and subsequently eluted.

4. The experiments support the view that Hageman factor is implicated in the activation of pre-kallikrein.

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REFERENCES

DAVIES, G. E. & LOWE, J. S. (1961). The effects of guinea-pig gamma globulins on capillary permeability and serum complement. *Immunology*, **4**, 289–300.

DAVIES, G. E. & LOWE, J. S. (1963). Presence of kallikrein in the γ-globulin permeability factor of guinea-pig serum. *Br. J. Pharmac. Chemother.*, **21**, 491–499.

INTERNATIONAL UNION OF BIOCHEMISTRY (1965). *Enzyme Nomenclature.* p. 38. Elsevier, Amsterdam.

MARGOLIS, J. (1958). Activation of plasma by contact with glass. Evidence for a common reaction which releases plasma kinin and initiates coagulation. *J. Physiol. Lond.*, **144**, 1–22.

MARGOLIS, J. (1959). The role of Hageman factor in plasma/surface reactions. In *Hemophilia and Other Hemorrhagic States*. Ed. by K. M. Brinkhous. Chapel Hill. University of North Carolina Press, pp. 208–228.

MARGOLIS, J. & BISHOP, E. A. (1962). Interrelations between different mechanisms of release of biologically active peptides from blood plasma. *Nature, Lond.*, **194**, 749–751.

NIEWIAROWSKI, ST., BAŃKOWSKI, E. & FIEDORUK, T. (1964). Adsorption of Hageman factor (Factor XII) on collagen. *Experientia*, **20**, 367–368.

NIEWIAROWSKI, ST., BAŃKOWSKI, E. & ROGOWICKA, I. (1965). Studies on the adsorption and activation of the Hageman factor (Factor XII) by collagen and elastin. *Thromb. Diath. haemorrh.*, **14**, 387–400.

NOSSELL, H. L. (1964). *The Contact Phase of Blood Coagulation*. p. 124. Oxford: Blackwell.

RATNOFF, O. D. & MILES, A. A. (1962). Activation of a vascular permeability-increasing factor in human plasma incubated with purified activated Hageman factor. *J. Lab. clin. Med.*, **60**, 1009.

RATNOFF, O. D. & MILES, A. A. (1964). The induction of permeability-increasing activity in human plasma by activated Hageman factor. *Br. J. exp. Path.*, **45**, 328–345.

ROBERTS, P. S. (1958). Measurement of the rate of plasmin action on synthetic substrates. *J. biol. Chem.*, **232**, 285–291.

SCHOENMAKERS, J. G. G., MATZE, R., HAANEN, C. & ZILLIKEN, F. (1965). Hageman factor, a novel sialoglycoprotein with esterase activity. *Biochim. biophys. Acta.*, **101**, 166–176.