Brit. J. Pharmacol. (1963), **21**, 491–499.

PRESENCE OF KALLIKREIN IN THE γ-GLOBULIN PERMEABILITY FACTOR OF GUINEA-PIG SERUM

BY

G. E. DAVIES AND J. S. LOWE

From Pharmaceuticals Division, Research Department, Imperial Chemical Industries Ltd., Alderley Park, Macclesfield, Cheshire

(*Received July* 18, 1963)

γ-Globulin fractions isolated from guinea-pig serum by chromatography on diethylaminoethyl cellulose exhibited all the properties of kallikrein. From a range of amino acid esters tested, only esters of arginine and lysine were hydrolysed; the esterase and capillary permeability-increasing activities of different samples were correlated. Both esterase and permeability-increasing activities could be increased by the addition of certain albumin-rich fractions or of glass beads previously treated with guinea-pig serum.

The 7 S γ-globulin fraction, isolated from guinea-pig serum by various methods, increases capillary permeability when injected intradermally into guinea-pigs (Davies & Lowe, 1960). Subsequent investigation showed that the permeability activity of the γ-globulins was markedly reduced by soya bean trypsin inhibitor, by dyflos and also by an albumin-rich fraction of guinea-pig serum isolated by zone electrophoresis on ethanolysed cellulose. The activity was not present in γ-globulins isolated from serum which had been heated at 60° C for 30 min, but similar heating did not reduce the activity of already isolated γ-globulins (Davies & Lowe, 1961). There appeared to be two possible explanations for these findings, either that the γ-globulins contained a substrate which was degraded by an enzyme in the skin to yield the ultimate mediator of increased permeability, or that the γ-globulins contained an enzyme which acted on a substrate in the skin.

The present study presents the evidence which led us to conclude that guinea-pig γ-globulins contain an esterase which has the properties of serum kallikrein. A preliminary account of some of this work has been published (Davies & Lowe, 1963).

METHODS

Isolation of γ-globulins. The 7 S γ-globulin fraction was isolated from fresh guinea-pig serum by chromatography on diethylaminoethyl cellulose as described previously (Davies & Lowe, 1961).

Fractionation of guinea-pig serum. Guinea-pig serum was fractionated by two methods: (a) zone electrophoresis on Munktell ethanolysed cellulose (A. Gallenkamp & Co.), using the apparatus described by Porath (1956) as modified by Lowe (unpublished); and (b) chromatography on diethylaminoethyl cellulose (Eastman Kodak) employing a cone-sphere gradient elution method with 0.01 M-phosphate buffer (*p*H 8.0, 1 l.) as the starting buffer and 0.3 M-sodium dihydrogen phosphate solution (*p*H 4.4, 500 ml.) as the limit buffer.

Esterase activity. The modification of the Hestrin method, described by Roberts (1958), was used to measure esterase activity. 1 ml. of a 0.06 M solution of substrate was used for all determinations. Synthetic amino acid esters were used as substrates: *p*-tosyl-L-arginine methyl ester, L-lysine methyl ester, *N*-acetyl-L-tryptophan ethyl ester, L-tyrosine methyl ester, L-phenylalanine ester, L-leucine ethyl ester, DL-alanine ethyl ester and *N*-acetyl-L-tyrosine methyl ester were purchased from Mann Research Laboratories, New York; the other esters were synthesized by our colleague Dr J. S. Morley.

Sedimentation velocity measurements. Sedimentation velocity runs were carried out by Dr W. E. F. Naismith (Fibres Division, I.C.I., Harrogate) using a Spinco Model E analytical ultracentrifuge. The γ-globulins (0.5%, w/v) were dissolved in phosphate buffer, $\mu=0.1$, *p*H 7, and centrifuged at 59,780 rev/min (250,000 *g*).

Starch gel electrophoresis. Starch gel electrophoresis was done by the method of Smithies (1955).

Proteolytic activity. Caseinolytic activity was determined by the method of Northrop, Kunitz & Herriott (1948) using light white, soluble casein (B.D.H.) as substrate. Fibrinolytic activity was determined by the method of Astrup & Müllertz (1952).

Immunoelectrophoresis. Immunoelectrophoresis was carried out on microscope slides using the LKB 6800A Immunoelectrophoresis Equipment and a pooled antiserum prepared by immunizing rabbits with fresh guinea-pig serum.

Detection of kinin activity. The release of kinin from dog globulins treated with acid was carried out by the method of Horton (1959).

Blood pressure was recorded by means of a mercury manometer from a cannula inserted into a common carotid artery of cats and guinea-pigs, anaesthetized with pentobarbitone sodium. Blood flow was recorded by means of an electromagnetic flowmeter attached to the femoral artery of dogs, anaesthetized with pentobarbitone sodium.

Determination of increased capillary permeability. Effects on capillary permeability were determined as described previously (Davies & Lowe, 1961), by intradermal injection into guinea-pigs previously "blued" by intravenous injection of Pontamine Sky Blue. In the present study the skins were not pinned on a cork mat but placed between two sheets of Perspex and the diameter of the blue area on the inner aspect of the skin measured immediately.

Activation of γ-globulins with glass. The method for activation of γ-globulins with glass is based on that described by Margolis (1958). Fresh guinea-pig serum was shaken for 2 min with glass ballotini (microspherules 0.1 mm in diameter), 1 g/ml. After removal of the serum, the ballotini were washed thoroughly with saline (usually four washings were sufficient to remove all permeability activity). A solution of γ-globulins was then added to the treated ballotini, shaken for 2 min and removed. As a control, ballotini were treated with saline, instead of serum, before the addition of γ-globulins.

RESULTS

Esterase activity of γ-globulins

In initial experiments, extracts of guinea-pig skin were prepared containing proteolytic enzymes capable of degrading γ-globulins, but there was no evidence that any of the products could cause an increase in capillary permeability. We therefore considered the alternative hypothesis that the γ-globulins themselves contained enzymes and, in particular, that they contained esterases because their effects were inhibited by dyflos. We were indeed able to show that they hydrolysed arginine esters such as *p*-tosyl-L-arginine methyl ester or benzoyl-L-arginine methyl ester. They were, however, devoid of proteolytic activity against casein and fibrin. These properties, together with the effects on permeability, suggested that the enzyme

might be kallikrein, which will hydrolyse arginine esters (Contzen, Holts & Raudonat, 1959; Habermann, 1959; Werle & Kaufmann-Boetsch, 1960; Webster & Pierce, 1961) and increase permeability by the formations of plasma kinins.

Kallikrein-like activity of γ-globulins

The γ-globulin fractions of four separate samples of guinea-pig serum were isolated and their esterase and capillary permeability activities determined. As can be seen from Table 1 these two activities appeared to be correlated. For the further

TABLE 1

ESTERASE AND PERMEABILITY ACTIVITIES OF FOUR SAMPLES OF GUINEA-PIG γ-GLOBULINS

Capillary permeability activity is expressed as μg which caused a lesion 8 mm in diameter. BAEe= benzoyl-L-arginine ethyl ester

No. of preparation	Esterase activity (μmoles of BAEe/hr/mg)	Capillary permeability activity (μg)
1	2·5	25
2	0·4	80
3	3·3	50
4	4·7	15

investigation of the properties of the γ-globulins, several batches of fresh guinea-pig serum were fractionated on diethylaminoethyl cellulose and the γ-globulin fractions dialysed and freeze-dried. The several fractions were then pooled, dissolved in a minimum volume of water and the solution was freeze-dried. This process provided 2.16 g of γ-globulins of 75% purity.

The esterase activity of this sample was tested against a variety of synthetic amino acid esters. The *p*-tosyl-L-arginine methyl ester, benzyl-L-arginine ester and L-lysine methyl ester were hydrolysed, the optimum *p*H for the hydrolysis of tosyl arginine methyl ester being 8.8, and for lysine methyl ester, 7.0. The following esters were not hydrolysed: *N*-acetyl-L-tryptophan ethyl ester, L-tyrosine methyl ester, L-phenylalanine ethyl ester, L-leucine ethyl ester, DL-alanine ethyl ester, *N*-acetyl-L-tyrosine methyl ester, *N*-α-carbobenzoxyaspartic acid methyl ester and *N*-α-carbobenzoxyglutamic acid methyl ester. The esterase and permeability activities of these samples were much higher than those of any of the batches shown in Table 1 (Table 2). This result raised the possibility that the large sample might have

TABLE 2

COMPARISON OF TWO TYPES OF GUINEA-PIG γ-GLOBULINS

Capillary permeability activity is expressed as μg which caused a lesion 8 mm in diameter. For description of "normal" and "more active" γ-globulins, see text. TAMe=*p*-tosyl-L-arginine methyl ester

Property	Type of γ-globulins	
	"Normal"	"More active"
Capillary permeability activity (μg)	25	1·0
Esterase activity (μmoles of TAMe/hr/mg)	0·4–5·0	72

contained traces of contaminating protein which had contributed to the activity. Although the preparation appeared homogeneous, with a sedimentation constant of 6.5 S when examined on the analytical ultracentrifuge, electrophoresis on starch gel revealed the presence of a contaminant in the albumin region, and at least three antigens, in addition to the main γ-globulins, were demonstrated by immunoelectrophoresis. After purification by further chromatography on diethylaminoethyl cellulose a sample was obtained which gave only one line on immunoelectrophoresis, but it still possessed the high esterase and permeability activities. Further properties of this purified sample are summarized in Table 3. When added to dog globulins

TABLE 3

KALLIKREIN-LIKE PROPERTIES OF "MORE ACTIVE" γ-GLOBULINS

TAMe=*p*-tosyl-L-arginine methyl ester; LMe=L-lysine methyl ester

Property or preparation used	Observation
Capillary permeability	1 μg caused a lesion of 8 mm diameter
Blood pressure	0·1 mg was hypotensive to cats and guinea-pigs
Femoral arterial blood flow	0·1 mg increased femoral arterial blood flow in dogs
Rat uterus and guinea-pig ileum	Contraction followed by tachyphylaxis
Dog globulins	Kinin produced
Esterase	70 μM-TAMe/hr/mg, *p*H 8·8, and 15 μM-LMe/hr/mg, *p*H 7·0, were broken down No action on tyrosine or tryptophan esters
Casein and fibrin	No proteolytic activity

treated with acid it produced a kinin which caused contraction of the rat isolated uterus and relaxation of the rat duodenum; intravenous injection into cats and guinea-pigs produced hypotension; intra-arterial injection into dogs increased femoral arterial blood flow. These properties, together with the effects on capillary permeability and its enzymic activities, suggested that the material contained kallikrein.

Isolation of γ-globulins from heated or acetone-treated serum

Werle & Maiser (1952) have shown that treatment of human serum with acetone activates the precursor kallikreinogen to give the active enzyme kallikrein. In an attempt to activate the enzyme in guinea-pig serum, a sample of serum was treated with acetone by the method of Webster & Pierce (1960). The γ-globulins isolated from this treated serum were, however, quite inactive both as esterases and as mediators of increased capillary permeability. Comparison of these inactive γ-globulins with normal active material by immunoelectrophoresis showed no difference. We have shown previously (Davies & Lowe, 1961) that similarly inactive γ-globulins could be isolated from heated guinea-pig serum. We have now shown that these γ-globulins also are without esterase activity but are still indistinguishable immunoelectrophoretically from γ-globulins prepared from normal guinea-pig serum. We were thus able to prepare a series of preparations of γ-globulins with esterase activities varying from 0 to 72 μmoles of *p*-tosyl-L-arginine methyl ester/hr/mg and, as Table 4 shows, with related permeability activities.

TABLE 4

ESTERASE AND CAPILLARY PERMEABILITY ACTIVITIES OF FIVE SAMPLES OF GUINEA-PIG γ-GLOBULINS

Capillary permeability activity is expressed as μg which caused a lesion 8 mm in diameter. TAMe= *p*-tosyl-L-arginine methyl ester

No. of batch	Esterase activity (μmoles of TAMe/hr/mg)	Capillary permeability activity (μg)
1	4·0	50
2	3·0	50
3	72·0	1
4	12·5	10
7	0	>100

Activation of guinea-pig γ-globulins by albumin-rich fractions

The above experiments suggested that guinea-pig γ-globulins either contained or were the enzyme kallikrein, and that the samples of different activities contained mixtures of kallikreinogen and kallikrein in varying proportions, the kallikreinogen having been activated to different degrees during the isolations. Treatment of guinea-pig serum with heat or acetone could destroy this activating system and thus lead to the isolation of γ-globulins containing only kallikreinogen and hence devoid of activity.

We have shown previously (Davies & Lowe, 1961) that the permeability activity of γ-globulins is inhibited by certain albumin-rich fractions. On one occasion an albumin-rich fraction actually increased the activity of γ-globulins (Table 5), which

TABLE 5

EFFECT OF ADDING AN AGED ALBUMIN-RICH FRACTION TO GUINEA-PIG γ-GLOBULINS

TAMe=*p*-tosyl-L-arginine methyl ester. *This value refers to the activity per μg of γ-globulins

Material	Esterase activity (μmoles of TAMe/hr/mg)
γ-Globulins	4·0
Albumin-rich fraction	0·7
γ-Globulins+albumin-rich fraction (1 : 10)	50·0*

suggests that it might have contained an activator. There were available eight batches of albumin-rich fractions prepared from guinea-pig serum either by diethylaminoethyl cellulose chromatography or by zone electrophoresis during the period 1959–1961. Each of these samples was injected intradermally into blued guinea-pigs both alone and mixed with a moderately active amount of γ-globulins. Four of these batches, those prepared by zone electrophoresis, contained an inhibitor, whereas the other four, prepared by chromatography, contained an activator. Examination of these activator-containing fractions by starch gel electrophoresis showed that they were very impure, but we have not yet made any attempt to separate the components. When the ratio of activator to γ-globulins was varied it became apparent from the esterase activities that the activator also contained some inhibitor

TABLE 6

EFFECT ON ESTERASE ACTIVITY OF VARIOUS RATIOS OF γ-GLOBULINS TO AGED ALBUMIN-RICH FRACTIONS

The albumin-rich fraction hydrolysed 0·7 μ moles of TAMe/hr/mg of protein. TAMe=*p*-tosyl-L-arginine methyl ester

Relative amounts (given together) of		Esterase activity (μmoles of TAMe/hr/mg of γ-globulins)
γ-Globulins (mg)	Albumin-rich fraction (mg)	
1	0	4·2
1	5	44·5
1	10	42·3
1	15	38·8
1	20	36·5

(Table 6), although this was not apparent from the permeability effects (Table 7). This difference could well be due to the greater sensitivity of the esterase method.

Attempts to confirm these results with freshly prepared albumin-rich fractions made by diethylaminoethyl cellulose chromatography met with only limited success. The activity of γ-globulins as permeability factors was increased but their esterase activity was unaltered. Some change had apparently taken place in these fractions during prolonged storage at 2° C. We argued that this change might involve a conversion of pro-activator to activator and that this conversion was also effected in guinea-pig skin when freshly prepared fractions were injected together with γ-globulins. Attempts to induce this conversion by the addition of various tissue extracts to freshly prepared fractions have so far been fruitless.

TABLE 7

CAPILLARY PERMEABILITY ACTIVITY OF VARIOUS RATIOS OF γ-GLOBULINS TO AGED ALBUMIN-RICH FRACTION

Amounts injected together of		Mean diameter of lesion (mm)
γ-Globulins (μg)	Albumin-rich fraction (μg)	
20	0	7·0
20	30	8·0
20	80	12·5
20	250	16·2
0	1,000	7·0

Activation of guinea-pig γ-globulins by ballotini treated with guinea-pig serum

An alternative approach to the problem was suggested by the work of Margolis (1958) who investigated the release of kinin from human plasma by 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 a release of kinin. In a later paper Margolis & Bishop (1962) suggested that component "A" might be kallikreinogen and that the activated component "A" might be kallikrein.

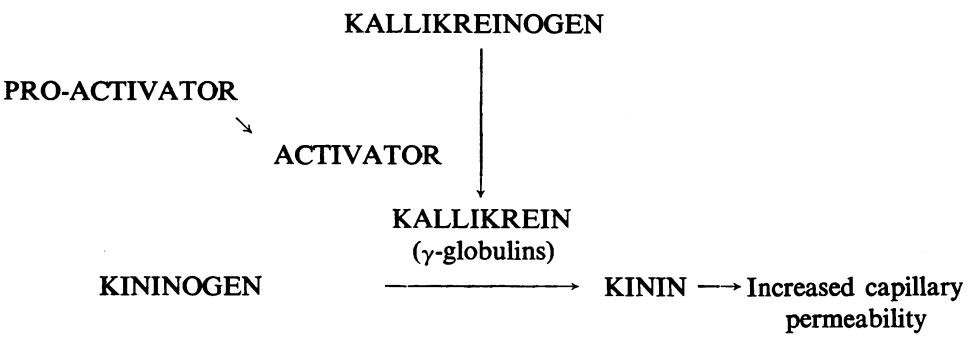
We therefore applied these findings to guinea-pig serum. Ballotini were treated with guinea-pig serum as described in Methods and washed until the washings were free from activity. A moderately active amount of γ-globulins was then added to

the treated, washed beads. The esterase activity of the γ-globulins was increased from 9 to 81 μmoles of *p*-tosyl-L-arginine methyl ester after treatment with the ballotini. There was a parallel increase in the capillary permeability-increasing activity, the amount of globulins required to produce an 8 mm diameter lesion decreasing from 10 to 0.8 μg.

DISCUSSION

The main objective in the work reported here was to explain the mode of action of guinea-pig γ-globulins as permeability factors. Our previous work presented evidence that the activity was not due to an artefact induced by the isolation procedures (Davies & Lowe, 1961), and also distinguished the γ-globulin permeability factor from the complement-dependent PF/P (Davies & Lowe, 1962).

The following scheme embodies the main findings of our present work.



According to this scheme, a kinin, analogous to bradykinin, is the ultimate mediator of increased capillary permeability. This kinin is derived from its precursor "kininogen" by the action of the enzyme kallikrein which, in turn, is derived from an inactive precursor kallikreinogen. We have shown that it is possible to obtain samples of guinea-pig γ-globulins containing various proportions of kallikreinogen and kallikrein, and that this conversion can be effected either by certain fractions from serum or by treatment with glass beads previously treated with serum. The nature of the activating system is unknown but it appears to be present in aged albumin-rich fractions prepared from guinea-pig serum by chromatography on diethylaminoethyl cellulose: it is not present, however, in purified guinea-pig serum albumin or in freshly prepared albumin-rich fractions, although these latter fractions do appear to contain a pro-activator. Mason & Miles (1962) have presented evidence which suggests that an α_2-globulin fraction might be concerned in this activation. Kagen, Laddy & Becker (1963) have described two permeability factors in human serum; one of these was associated with the γ-globulin fraction and the other with the β-globulins. They further suggested that the γ-globulin factor could be kallikrein.

There appears to be a direct relationship between the ability of a given sample of γ-globulins to hydrolyse arginine esters and its effect on capillary permeability.

Furthermore, both activities are increased by aged albumin-rich fractions or by ballotini treated with fresh guinea-pig serum.

Although we have shown that guinea-pig serum kallikrein activity is a property of the γ-globulin fraction, we are not yet able to conclude that the whole family of γ-globulin molecules possess this property nor that a complete molecule is necessary for activity. Indeed, we have not yet eliminated the possibility that kallikrein activity may be associated with protein fractions other than the γ-globulins. The role of kinins and kinin-liberating enzymes in the inflammatory process has been suggested by others (Hilton & Lewis, 1955 ; Edery & Lewis, 1962) and it is tempting to try to define this role in a little more detail. It is probable that the very first steps in an inflammatory process, at least in the guinea-pig, are mediated by released histamine. However, the effect of histamine is of short duration (Davies & Lowe, 1960) and it is necessary to postulate mediators which continue the process. Appearance of plasma proteins in extravascular sites, their presence having been mediated by histamine, would provide a source of kallikreinogen and the substance we have called " pro-activator." Surface effects, such as those provided by damaged cells or small fibrin clots, could then effect the change from pro-activator to activator and this in turn would lead to the appearance of kallikrein and hence to kinin. During later phases of inflammation the site would be infiltrated with lymphocytes which would synthesize γ-globulins and hence provide more kallikreinogen and the process would continue.

We should like to thank Miss J. M. Waite, Miss A. V. Worth and Mr R. A. Westhead for their expert technical assistance.

REFERENCES

ASTRUP, T. & MÜLLERTZ, S. (1952). The fibrin plate method for estimating fibrinolytic activity. *Arch. Biochem.*, **40**, 346–351.

CONTZEN, C., HOLTZ, P. & RAUDONAT, H. W. (1959). Über das bradykininbildende Prinzip des Bothrops-Giftes. *Naturwissenschaften*, **46**, 402.

DAVIES, G. E. & LOWE, J. S. (1960). A permeability factor released from guinea-pig serum by antigen-antibody precipitates. *Brit. J. exp. Path.*, **41**, 335–344.

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. (1962). Further studies on a permeability factor released from guinea-pig serum by antigen-antibody precipitates: relationship to serum complement. *Int. Arch. Allergy*, **20**, 235–250.

DAVIES, G. E. & LOWE, J. S. (1963). Guinea pig kallikrein system. *Nature* (*Lond.*), **198**, 94–95.

EDERY, H. & LEWIS, G. P. (1962). Plasma kinin-forming-enzyme activity in lymph after injury. *J. Physiol.* (*Lond.*), **163**, 48–49*P*.

HABERMANN, E. (1959). Über Zusammenhänge zwischen esterolytischen und pharmakologischen Wirkungen von Jararacigift, Kallikrein und Thrombin. *Naunyn-Schmiedeberg's Arch. exp. Path. Pharmkol.*, **236**, 492–502.

HILTON, S. M. & LEWIS, G. P. (1955). The mechanism of the functional hyperaemia in the submandibular salivary gland. *J. Physiol.* (*Lond.*), **129**, 253–271.

HORTON, E. W. (1959). The estimation of urinary kallikrein. *J. Physiol.* (*Lond.*), **148**, 267–282.

KAGEN, L. J., LEDDY, J. P. & BECKER, E. L. (1963). Isolation of two permeability globulins from human serum. *Nature* (*Lond.*), **197**, 693.

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

MASON, B. & MILES, A. A. (1962). Globulin permeability factors without kininogenase activity. *Nature* (*Lond.*), **195**, 587–588.

NORTHROP, J. H., KUNITZ, M. & HERRIOTT, R. M. (1948). *Crystalline Enzymes*, pp. 308–310. New York: Columbia University Press.

PORATH, J. (1956). Methodological studies of zone electrophoresis in vertical columns. 1. Fractionation in cellulose powder columns of substances of low molecular weight exemplified by amino acids and related compounds. *Biochim. biophys. Acta (Amst.)*, **22**, 151–175.

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

SMITHIES, O. (1955). Zone electrophoresis in starch gels: group variation in the serum proteins of normal human adults. *Biochem. J.*, **61**, 629–941.

WEBSTER, M. E. & PIERCE, J. V. (1960). Studies on plasma kallikrein and its relationship to plasmin. *J. Pharmacol. exp. Ther.*, **130**, 484–491.

WEBSTER, M. E. & PIERCE, J. V. (1961). Action of the kallikreins on synthetic ester substrates. *Proc. Soc. exp. Biol. (N.Y.)*, **107**, 186–191.

WERLE, E. & KAUFMANN-BOETSCH, B. (1960). Über esteratische Wirkungen von Kallikrein und Trypsin und ihre Hemmung durch Kallikrein—und Trypsininhibitoren. *Hoppe-Seylers Z. physiol. Chem.*, **319**, 32–63.

WERLE, E. & MAISER, L. (1952). Über die chemische und pharmakologische Unterscheidung von Kallikrein verschiedener Herkunft. *Biochem. Z.*, **323**, 279–283.