Biochemical Pharmacology, Vol. 23, pp 2291–2303. Pergamon Press, 1974. Printed in Great Britain.

ISOLATION OF TWO FUNCTIONALLY DIFFERENT KININOGENS FROM HUMAN PLASMA—SEPARATION FROM PROTEINASE INHIBITORS AND INTERACTION WITH PLASMA KALLIKREIN*

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(*Received* 22 *October* 1973; *accepted* 14 *January* 1974)

Abstract—A high (HMW) and a low (LMW) molecular weight kininogen were isolated in highly purified form from human plasma, using QAE-Sephadex chromatography, followed by ammonium sulfate precipitation, gel filtration through Sephadex G-200, re-precipitation with ammonium sulfate, CM-Sephadex and SP-Sephadex chromatography. The initial preparative step was done at room temperature and the remaining procedures at 4°. In aqueous media, the apparent molecular size of the HMW-kininogen was about four times the size of the LMW-kininogen (200,000 vs 50,000). During the process of purification, proteinase inhibitors were separated from the two kininogens: α_1-antitrypsin and α_2-macroglobulin from the LMW-kininogen preparations; C1-inactivator and inter-α-trypsin inhibitor from the HMW-kininogen preparations. There was a well defined functional difference between the two kininogens with respect to kinin generation by plasma kallikrein. This enzyme released kinin at a much faster rate from the HMW-kininogen than from the LMW-kininogen. When equipotent preparations of kininogens were incubated for 10 min with kallikrein, 60 times more enzyme was required to release the same amount of kinin from the LMW-kininogen as from the HMW-kininogen.

THE GROUP of vasoactive peptides referred to as "kinins" are present in plasma in precursor form known as kininogens. Release of these peptides is brought about by interaction of the kininogens with kallikrein and other proteolytic enzymes. The peptides cause smooth muscle contraction,[1,2] induce pain,[3] hypotension[1,2] and enhance vascular permeability.[4] The literature on the kinin system is discussed at length in a comprehensive publication.[5]

For many years there has been some controversy about the number of kininogens in human plasma. Several investigators have isolated only one kininogen,[6–9] whereas others demonstrated the existence of two kininogens.[10–12] Some investigators isolated one, others two kininogens from animal plasmas.[13] Some of the low molecular weight kininogens have been isolated in highly purified form.[13]

In a previous communication, we described the isolation of two kininogens from human plasma in partially purified form.[14] This paper deals with further purification

* Supported by grants from the Atkinson Charitable Foundation, the Ontario Heart Foundation and the Medical Research Council of Canada (MT-1251).

† Holder of an MRC-Studentship. By presenting this paper at the SAMA-UTMB at Galveston, Texas, Flavio M. Habal received the first prize of the James McLoughlin Award and the second prize of the Mead Johnson Award for excellence in research.

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of these kininogens and with functional differences when the two substrates interact with plasma kallikrein. Subsequent publications will describe the physico-chemical differences of the two proteins in the native state, in the presence of dissociating agents and after reduction and alkylation.[15]

MATERIALS AND METHODS

Plasma. Non-glass contacted ACD (acid citrate dextrose) plasma was obtained from the Canadian Red Cross.

Isolation of kininogens. The plasma (which had not been cooled after collection of the blood) was dialysed against 0·1 M Tris–HCl, pH 8·0, containing 0·003 M EDTA (ethylenediaminetetra-acetate) at room temperature until it reached the conductivity of the buffer (approximately 4·8 mmho/cm). It was then applied to a siliconized glass column or polymethyl pentene column packed with QAE-Sephadex A-50, equilibrated with the dialysing buffer. Chromatography on QAE-Sephadex was done at room temperature and all other chromatographic procedures at 4°. The columns packed with QAE-Sephadex varied in size, depending on the amount of plasma. In earlier experiments, 5 × 45- and 10 × 50-cm columns were used to fractionate approximately 400–1800 ml plasma. In a recent experiment, 4 l. plasma was applied to a 37 × 15-cm column (one section of a KS/370 Pharmacia column). After applying 4 liters plasma, only the upper half of the QAE-Sephadex became discolored and there was no overload, since the effluent contained only IgG, prekallikrein and factor XI. Fractions containing kininogen (assayed as described below) were pooled, concentrated as described in a later section and rechromatographed, in several runs, through Sephadex G-200. A portion of the sample (25–30 ml) was passed through two interconnected columns (5 × 80 and 5 × 90 cm) equilibrated with 0·1 M Tris–HCl, pH 8·0, containing 0·003 M EDTA and 0·5 M NaCl. The partially purified kininogens were subsequently concentrated, dialysed and equilibrated against 0·03 M Na acetate–acetic acid, pH 5·8, containing 0·05 M NaCl and chromatographed on CM-Sephadex C-50, equilibrated with the same buffer. Column size was usually 2·5 × 20–25 cm. After passing 400–500 ml equilibrating buffer through the column, the adsorbed protein was eluted with a linear gradient of 500 ml equilibrating buffer and 500 ml 0·03 M acetate buffer, pH 5·8, containing 0·6 M NaCl. It had been ascertained earlier that the low molecular weight (LMW) kininogen was stable below its isoelectric point. This was done as follows. The kininogen preparation was dialysed against 0·03 M Na acetate, containing 0·05 M NaCl. The pH was then rapidly adjusted to pH 3·5 with acetic acid, followed by dialysis for 72 hr against 0·03 M Na acetate–acetic acid, containing 0·05 M NaCl. After neutralization, the kininogen was tested for kinin-forming activity and "spontaneous" kinin formation. No free kinin was detected, and the ability of kininogen to convert to kinin was unchanged after 3 days of dialysis at pH 3·5, whether tested with trypsin or with plasma kallikrein. Based on these findings the LMW-kininogen was chromatographed (after dialysis) on SP-Sephadex C-50. The cation exchanger was equilibrated with 0·05 M NaCl in 0·03 M Na phosphate–citric acid, pH 3·5, and packed into a 2·5 × 30-cm column. After washing the column with 500 ml equilibrating buffer, a linear gradient was applied, consisting of 500 ml starting buffer and 500 ml phosphate buffer containing 0·5 M NaCl.

Ammonium sulfate precipitation. The major protein contaminant present in the LMW-kininogen was albumin, and from a functional point of view, natural proteinase inhibitors were interfering factors. Furthermore, as described below, concentrating the chromatographic fractions presented a major problem. Ammonium sulfate precipitation was found to be a convenient method of overcoming these difficulties. Therefore, in more recent work, this procedure was introduced after several chromatographic steps. It was found that both kininogens precipitated at 40–45 per cent saturation, leaving the major contaminants in the supernatant as described under Results. Chromatographic fractions were dialysed against 0·1 M Tris–HCl (pH 8·0) and then brought to 40–50 per cent saturation by adding either solid or saturated ammonium sulfate. The precipitates were redissolved in a small volume of physiological saline and dialysed against the equilibrating buffer of the next chromatographic step.

Isolation of prekallikrein and kallikrein. The excluded peak obtained by fractionating plasma on QAE-Sephadex contained the prekallikrein, with IgG the major contaminant. The prekallikrein was further purified on two interconnected columns of Sephadex G-200 super fine (10 × 80 and 10 × 84 cm), equilibrated with Tris–HCl, as above. By applying the protein mixture to this column, the prekallikrein was rendered essentially free of other proteins, as described before with separation of kallikrein.[16,17] This partially purified prekallikrein (after full activation to kallikrein with prekallikrein activator)[16,17] was used in some experiments. In others, more highly purified preparations were used. Traces of IgG in the preparation obtained by gel filtration were removed by affinity chromatography. The columns were prepared as described before, with lysine–Sepharose.[18] When arginine was attached to Sepharose 4B, the prekallikrein became adsorbed and was subsequently desorbed with 0·04 M Tris–HCl, pH 8·5, containing 0·14 M NaCl and 0·003 M EDTA. Alternately rabbit anti-human IgG was linked to the Sepharose and the contaminating IgG was removed by adsorbing to the column. The human IgG used for immunizing the rabbits was rendered free of prekallikrein, by passing through SP-Sephadex.[19]

Concentration of chromatographic fractions. The use of positive pressure ultrafiltration, with continuous stirring, resulted in considerable loss of kininogen and "spontaneous" formation of kinin, and after several attempts it was abandoned. Two procedures were found feasible. Pooled chromatographic eluates were first placed into dialysing bags (visking membranes) and the bags covered with sucrose. When the volume in the bag fell to about one-third, the excess sugar was removed by dialysis. This process was repeated until the desired concentration was achieved. Subsequently, the partially concentrated solution was wither lyophilized or precipitated with ammonium sulfate, as described above.

Protein concentration. The protein concentration of chromatographic eluates was estimated as absorbance at 280 nm. The protein concentration of the purified preparations was determined by Lowry's modification of the Folin–Ciocalteux technique,[20] using bovine serum albumin as standards.

Detection of kininogen. Aliquots of chromatographic fractions (0·8 ml) were dialysed against 0·02 M phosphate-buffered saline (pH 7·4), containing 10^{-4} M 1,10-phenanthroline monohydrate (Sigma, St. Louis, Mo.). The fractions were incubated with 0·1 ml trypsin (1·0 mg/ml, Worthington, TPCK, 200 U/mg, Freehold, N.J.) for 10 min and the trypsin was neutralized with 0·1 ml lima bean trypsin inhibitor

(1·2 mg/ml, Sigma). The tubes were immersed for 10 min in boiling water, cooled and assayed on the estrous rat uterus, as described before.[16] The de Jalon solution contained 0·1 μg/ml of atropine sulfate. The volume of the bath was 5 ml. To eliminate serotonin as a possible spasmogen, 0·1 μg/ml of methysergide bimaleate (Sansert, Sandoz Pharmacenticals, Montreal, Que.) was added to some of the de Jalon solution and to some samples. In these experiments, both synthetic bradykinin (Sandoz) as well as serotonin (serotonin creatinine sulfate hydrate, Upjohn, Kalamazoo, Mich.) were used as standards. The kinin-containing samples were tested also on the guinea pig ileum, in the presence and absence of the antihistamine mepyramine maleate (0·1 μg/ml of Neo-Antergan, Poulenc, Montreal, Que.), histamine (Fisher Scientific, Toronto, Ont.) being used as standards. Another oxytoxic substance that had to be eliminated as a possible contaminant was anaphylatoxin. Cleavage products of both C3 and C5 contract the rat uterus.[21,22] However, both C3a and C5a induce tachyphylaxis. Therefore, our samples were checked for such activity. Furthermore, the kininogen preparations were tested for the presence of C3 and C5 by immunodiffusion. The anti-C3 was a commercial preparation (Behringwerke, Hoechst Pharmaceuticals, Toronto, Ont.) and the anti-C5 was a gift from Dr. E. L. Becker (University of Connecticut).

Tests for proteinase inhibitors. As indicated above, proteinase inhibitors of plasma[23] were present in both LMW- and HMW-kininogens. The former contained α_1-antitrypsin, α_2-antithrombin and α_2-macroglobulin and the latter $C\bar{1}$-inactivator and inter-α-trypsin inhibitor. Concentrated effluents were tested for these substances by double diffusion in agar, using commercial antisera (Behringwerke). Alpha$_1$-anti-trypsin, α_2-macroglobulin and inter-α-trypsin inhibitor were detected also by their ability to inhibit the arginine esterase (BAEe hydrolysis) activity of trypsin (10 μg/ml) and $C\bar{1}$-inactivator by its capacity to inhibit the arginine esterase activity of kallikrein.

Polyacrylamide gel electrophoresis. Disc electrophoresis was done by the method of Davis,[24] as described before.[17] For preparative work, the gels were cut at $\frac{1}{4}$-cm intervals, mixed with 1·0 M NaCl, squeezed repeatedly through a syringe and placed on a shaker for 16 hr; the supernatant was separated by centrifugation.

RESULTS

Isolation of kininogens. When plasma was chromatographed on QAE-Sephadex, prekallikrein eluted in the excluded peak. It hydrolysed BAEe and generated kinin from the kininogens. A mono-specific antibody, produced against highly purified kallikrein (see above) inhibited about 75 per cent of the esterolytic and about 90 per cent of the kinin-forming activity of the partially purified kallikrein. Kininogen eluted when the NaCl concentration was raised to 0·12 M and 0·35 M, respectively, as shown in Fig. 1. The earlier eluting kininogen fractions contained α_1-antitrypsin and $\alpha_2$macroglobulin, but their inhibitory activity was overcome by using excess trypsin for the release of kinin. The major protein constituent of these fractions was serum albumin. $C\bar{1}$-inactivator and inter-α-trypsin inhibitor were present in the kininogen fractions adsorbing firmly to the anion exchanger. In earlier experiments, the kininogen-containing pools were rechromatographed on QAE-Sephadex, using linear gradients, primarily in an attempt to eliminate the albumin. However, this step was found unnecessary and was subsequently abandoned.

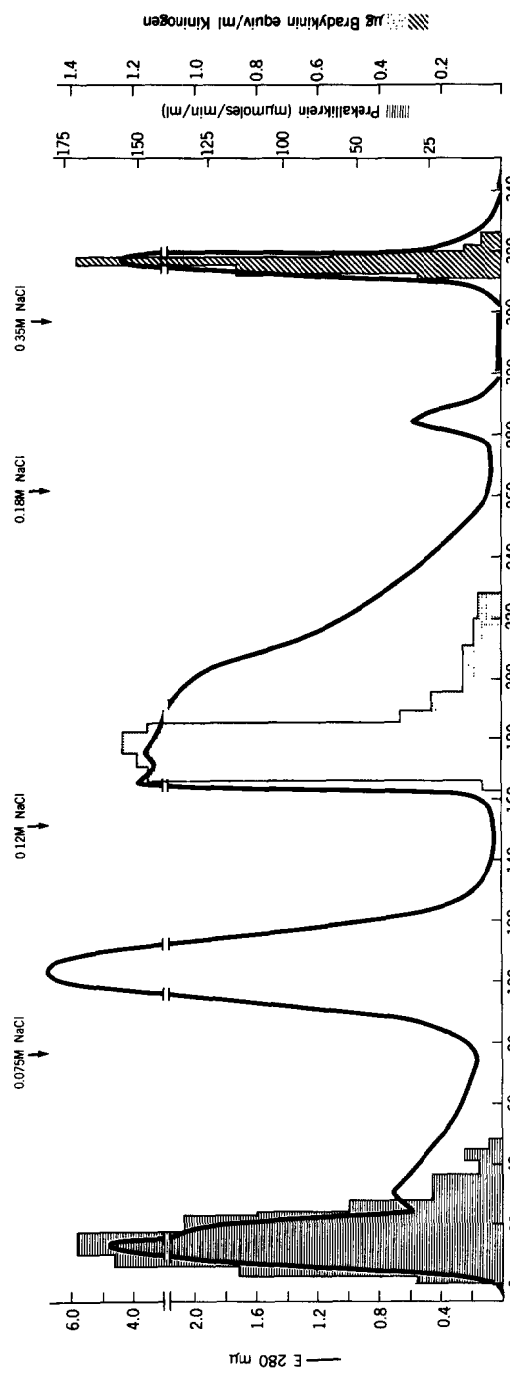

FIG. 1. Chromatography of 400 ml of non-contacted human plasma (total O.D. at 280 nm: 20,000) at room temperature on a 5 × 45-cm column of QAE-Sephadex, equilibrated with 0·1 M Tris–HCl (pH 8·0) at a flow rate of 60 ml/hr. The NaCl concentration was raised stepwise as indicated. Prekallikrein was recovered in the excluded peak and is expressed as nmoles BAEe hydrolysis/min/ml. The fractions showing esterase activity also released kinin from kininogens, and the esterase and kinin-forming activity were inhibited by monospecific antibody. Kininogen eluted when the NaCl was raised to 0·12 and 0·35 M respectively. It is expressed as μg/ml of bradykinin equivalents.

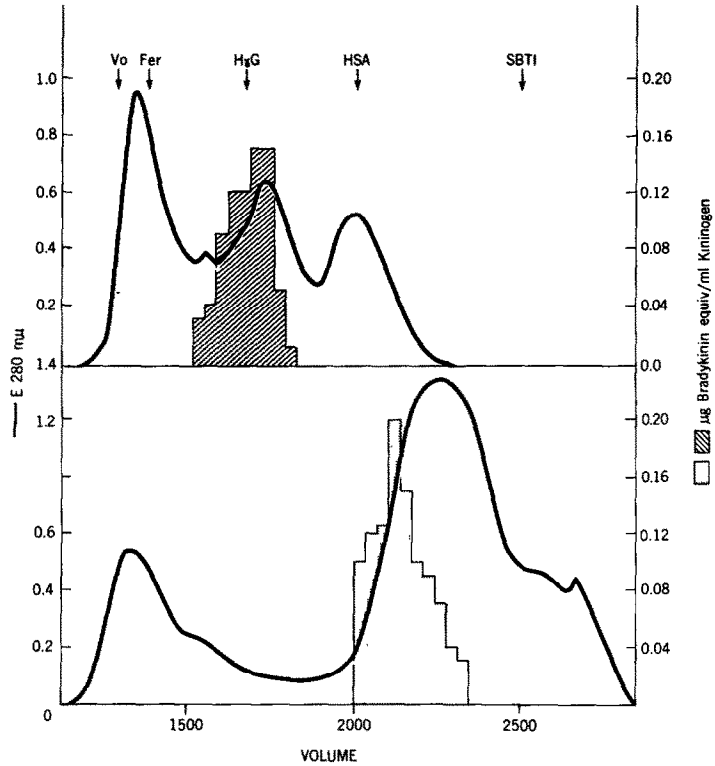

FIG. 2. Chromatography of 30 ml ($O.D._{280\,nm}$: 317) of HMW-kininogen (upper half of illustration) and 30 ml ($O.D._{280\,nm}$: 510) of LMW-kininogen (lower half) on two interconnected columns of Sephadex G-200 fine (5 × 80 and 5 × 90 cm) at a flow rate of 25 ml/hr. The columns were calibrated with apoferritin (Fer), human γ-globulin (HγG), human serum albumin (HSA) and soy bean trypsin inhibitor (SBTI).

The two pools of kininogen were further purified by gel filtration through Sephadex G-200, as shown in Fig. 2. The more anionic peak of kininogen eluted with an elution volume slightly smaller than human γ-globulin (IgG), whereas the less anionic peak had an elution volume slightly larger than human serum albumin. This pattern was obtained in all runs. This behaviour during gel filtration led to the use of the terms high molecular weight (HMW) and low molecular weight (LMW) kininogens,[13] a terminology which we propose to follow throughout this communication. During this preparative step, one of the proteinase inhibitors, α_2-macroglobulin, was eliminated from the LMW-kininogen, eluting with the first protein peak (Fig. 2). The two inhibitors, $C\bar{1}$-inactivator and inter-α-trypsin inhibitor, partially overlapped, with the HMW-kininogen, being slightly retarded.

Tubes in which kinin could be generated with trypsin were pooled, concentrated and applied to CM-Sephadex. Although the LMW-kininogen eluted with the equilibrating buffer, it was slightly retarded, and therefore, separated from most of the albumin and α_1-antitrypsin (Fig. 3). As shown in the lower half of Fig. 3, when HMW-kininogen was chromatographed under identical conditions, it became firmly adsorbed to the gel, eluting toward the end of the NaCl gradient. Figure 3 also shows that $C\bar{1}$-inactivator and inter-α-trypsin inhibitor became separated from the

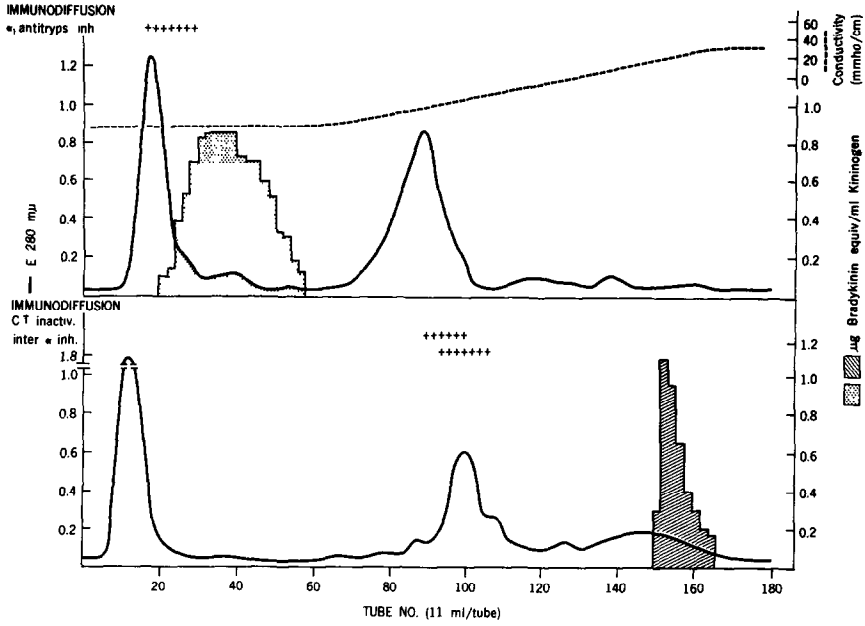

FIG. 3. Cation exchange chromatography on CM-Sephadex C-50 equilibrated with 0·03 M Na acetate–acetic acid and 0·05 M NaCl (pH 5·8). Column size 2·5 × 25 cm, flow rate 35 ml/hr. Linear gradient with same buffer containing 0·6 M NaCl as limiting buffer. Upper panel shows elution pattern of LMW-kininogen ($O.D._{280\,nm}$: 131) and lower panel of the HMW-kininogen ($O.D._{280\,nm}$: 92). The elution of α_1-antitrypsin, $C\bar{1}$-inactivator and inter-α-trypsin inhibitor is likewise shown, as determined by immunodiffusion.

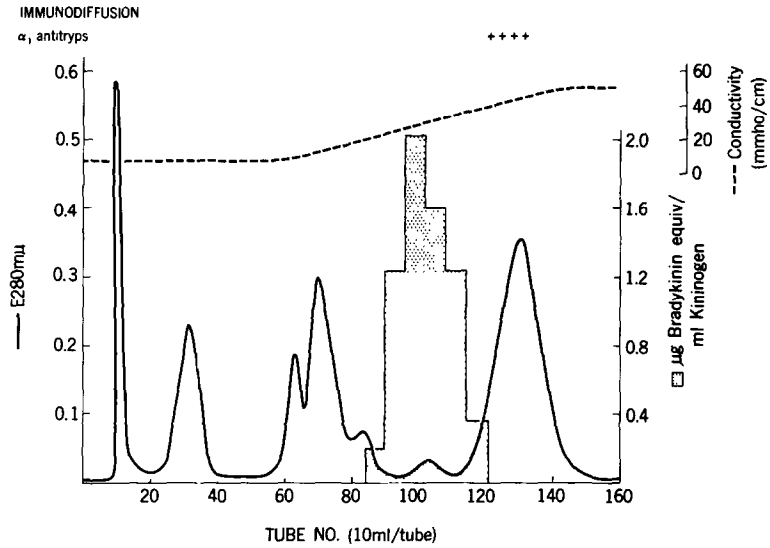

FIG. 4. Chromatography of LMW-kininogen ($O.D._{280\,nm}$: 82) on a 2·5 × 30-cm column packed with SP-Sephadex C-50, equilibrated with 0·03 M Na phosphate–critric acid (pH 3·5), containing 0·05 M NaCl. The limiting buffer contained 0·5 M NaCl. The flow rate was 35 ml/hr. The kininogen was concentrated 10-fold before testing.

kininogen, eluting in the first half of the salt gradient. This illustration shows only the results obtained by immunodiffusion. However, fractions in which the inhibitors were demonstrated by double diffusion inhibited also kallikrein and trypsin. Where the $C\bar{1}$-inactivator and inter-α-trypsin inhibitor did not overlap, only kallikrein or trypsin inhibition was detectable, $C\bar{1}$-inactivator inhibiting only kallikrein and inter-α-trypsin inhibitor only trypsin.

Next, attempts were made to further purify the kininogens, by cation exchange chromatography below their isoelectric point, which in earlier studies was found to be about 5·0.[14] As described under Materials and Methods, before chromatography, attempts were made to ascertain the stability of the two kininogens at low pH. Of the two, only the LMW-kininogen was found to be stable. It was, therefore, passed through an SP-Sephadex column at pH 3·5. It eluted with the NaCl gradient, becoming separated from the residual albumin and α_1-antitrypsin (Fig. 4).

Having established that functionally pure kininogens could be isolated by chromatographic procedures, a more rapid procedure was developed in which concentration and further purification was achieved by precipitation with 40–45% ammonium sulfate, as shown in Table 1. During this procedure, the kininogens were recovered from the precipitates and most of the inhibitors remained in the supernatants. Because both kininogens are present in plasma, the specific activity and yield could not be referred back to plasma, since their relative concentration in the starting plasma was not known. Rather the specific activity and yield are referred to QAE-Sephadex, the first step in which the separation of the two kininogens takes place. The degree of purification would have been considerably higher had it been possible to refer back to plasma.

With the procedure outlined, a high degree of purification was achieved with the LMW-kininogen which had a single band by acrylamide gel electrophoresis, whereas the HMW-kininogen had two close parallel bands. When the gels were sliced, kininogen was demonstrable in the fractional cuts corresponding to the appropriate bands (Fig. 5).

TABLE 1. ISOLATION OF LMW- AND OF HMW-KININOGENS, SPECIFIC ACTIVITY AND PER CENT YIELD*

Preparative step	Protein ($E_{280\,nm}$)	Bradykinin equivalent (μg)	Sp. act.	Yield (%)
LMW-kininogen				
Plasma (800 ml)	44,800	6400†	0·143	
QAE-Sephadex	20,192	1680	0·083	100·0
Sephadex G-200	8076	1504	0·186	89·9
CM-Sephadex	1935	1299	0·668	77·3
SP-Sephadex	96	768	8·0	45·7
HMW-kininogen				
Plasma (800 ml)	44,800	6400†	0·143	
QAE-Sephadex	9568	736	0·077	100·0
Sephadex G-200	4066	662	0·163	90·0
CM-Sephadex	51	397	7·821	53·4

* After each preparative step the protein was precipitated with ammonium sulfate as described in the text. All assays were done as described in Material and Methods, using trypsin to liberate kinin.

† One-tenth ml plasma was heated to 61° for 1 hr and then incubated with trypsin in the presence of 10^{-4} M *o*-phenanthroline.

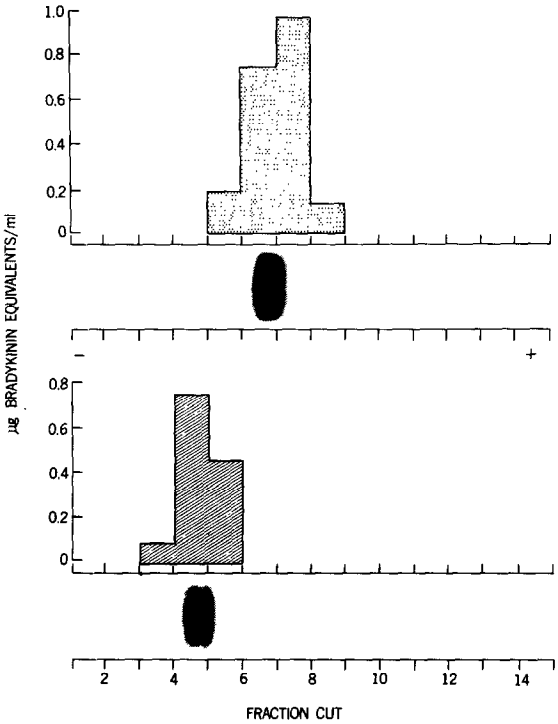

FIG. 5. Preparative polyacrylamide gel electrophoresis of LMW-kininogen (upper panel) and HMW-kininogen (low panel). One gel was stained and the remainder cut at 0·25-cm intervals.

TABLE 2. RELEASE OF KININ FROM HMW- AND FROM LMW-KININOGENS BY INCREASING CONCENTRATIONS OF KALLIKREIN*

Kallikrein in reaction mixture (μg)	Bradykinin equiv. (μg released from 1·0 mg kininogen) HMW-kininogen	LMW-kininogen
0·0862	0	
0·1725	0·14	
0·345	0·4	
0·7	0·68	0
1·4	1·0	0
2·8	1·15	0
5·6	1·15	0·05
11·2	1·15	0·325
22·4		0·575
44·8		0·825
89·6		1·0

* The two kininogens were adjusted to release the same amount of kinin when incubated with excess trypsin 100 μg/ml of kininogen). To a constant amount of kininogen (0·1 ml; HMW-kininogen 1·16 mg/ml; LMW-kininogen 1·83 mg/ml) increasing concentrations of kallikrein were added, the reaction mixture was adjusted to 2·0 ml with phosphate-buffered saline (pH 7·4) and incubated for 10 min. The reaction was stopped by immersing the tubes for 10 min in boiling water. After cooling and appropriate dilution with de Jalon solution, the reaction mixtures were assayed on the estrous rat uterus.

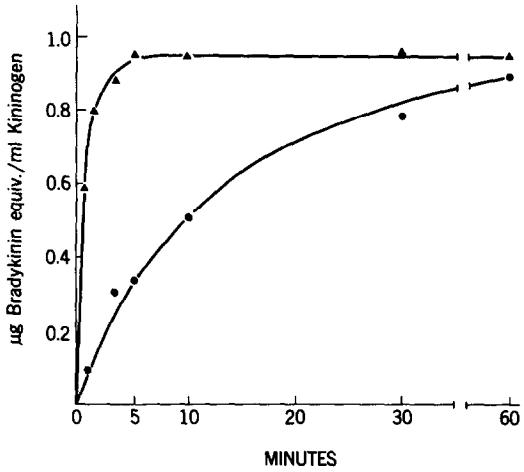

FIG. 6. Time course of the release of kinin from LMW- (closed circles) and HMW- (triangles) kininogens. The two kininogens were adjusted to the same potency by incubation with trypsin. They were then incubated with plasma kallikrein, as described in Materials and Methods, and aliquots were removed at various times and tested on the estrous rat uterus.

Interaction between the kininogens and kallikrein. Highly purified plasma kallikrein was incubated for 10 min with the two kininogens. As shown in Table 2, considerably more kallikrein was required to release the same amount of kinin from LMW-kininogen than from HMW-kininogen. When incubated for as long as 1 hr, the amount of kinin released from the two kininogens had about the same magnitude. However, the release of kinin from the HMW-kininogen was very rapid, reaching a peak within 5 min (Fig. 6). The rate of kinin release depended on kallikrein con-

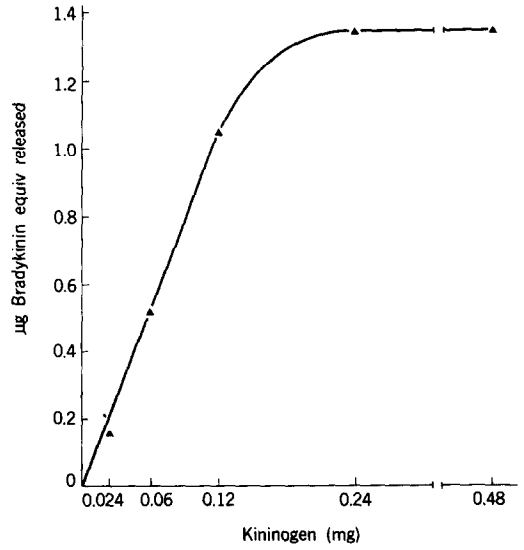

FIG. 7. Kinin release from HMW-kininogen, using a constant amount of plasma kallikrein (protein concn 1·0 μg) and increasing concentrations of kininogen (protein concn 1·2 mg/ml). Incubation time, 10 min

centration, whereas the magnitude of the released kinin was a function of kininogen concentration (Fig. 7).

That the product of kininogen–kallikrein interaction was kinin was ascertained by indirect means. Although the estrous rat uterus reponded with contractions to the application of serotonin, the addition of 0·1 μg/ml of methysergide to the de Jalon solution completely inhibited the contractions induced by the amine, without having any effect on the contractions induced by kinin. Anaphylatoxin induces tachyphylaxis after repeated applications, but no such effect was observed with kinin released from the purified kininogens. Furthermore, the kininogens were free of C3 and C5 as shown by immunodiffusion. Finally, when incubated with pancreatic carboxypeptidase B (Sigma, St. Louis, Mo.), the contractions of the rat uterus were abolished (Fig. 8).

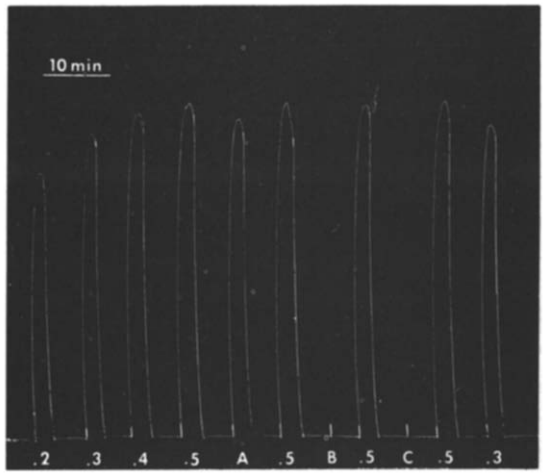

FIG. 8. Inactivation of the kinin released from HMW-kininogen by carboxypeptidase B. The numerals 0·2 to 0·5 represent contractions induced by synthetic bradykinin (ng/ml). At "A", kinin liberated from kininogen by kallikrein was applied to the bath. At "B", the substance applied at "A" was incubated for 5 min with 16 μg carboxypeptidase B (Sigma, 125 units/mg of protein) before application to the bath. At "C", synthetic bradykinin (2·5 μg in 5 ml) was incubated with 16 μg carboxypeptidase B and then applied to the bath.

DISCUSSION

Although studies currently in progress in our laboratory[15] indicate a close interrelationship between the two kininogens described in this paper, there is a clear-cut difference between the two kininogens. One designated high molecular weight kininogen by several investigators[13] seems to have about four times the molecular size of the low molecular weight kininogen, based on gel filtration in aqueous media. Furthermore, it seems to have a higher net over-all negative charge. More significantly, from a functional point of view, kallikrein can cleave kinin from the HMW-kininogen at a considerably faster rate. When equipotent kininogen preparations are used and incubated with kallikrein for 10 min, only about $\frac{1}{60}$ of the enzyme is needed to release the same amount of peptide from the HMW-kininogen as from the LMW-kininogen. These findings may explain, at least in part, some of the varied observations reported in the literature.[25,26]

Our findings with human plasma are in agreement with earlier publications claiming the existence of two kininogens and contradict the findings of others who could detect only one kininogen, as discussed in the introduction.[6–13] The kininogens in the plasma of various species are discussed in detail by Pierce.[13] In recent years Yano *et al.*[27,28] have studied in detail the kininogens in bovine plasma. Our findings are in good agreement with those of Yano and colleagues.

As shown in Figs. 1–4, the two kininogens contain natural porteinase inhibitors. The LMW-kininogen contains α_1-antitrypsin, α_2-antithrombin and α_2-macroglobulin and the HMW-kininogen $C\bar{1}$-inactivator and inter-α-trypsin inhibitor. It is very likely that these inhibitors interfered in earlier attempts to isolate and purify the kininogens. During the various preparative steps, the inhibitors are gradually eliminated. Relatively pure, inhibitor-free preparations are obtained after the second salting out, following Sephadex G-200.

In an earlier publication,[14] we indicated that the purification had to be carried out at room temperature. In the meantime, it was found that after the initial QAE-Sephadex chromatography and ammonium sulfate precipitation, the subsequent preparative steps could be carried out at 4°. The reason for this is not yet clear. In a carefully controlled experiment, the same amount of plasma (10·0 ml) was fractionated on QAE-Sephadex at room temperature and at 4° as described in this paper. The LMW-kininogen recovered from both columns was the same, the total releasable kinin with excess trypsin being 21·0 μg. However, only 1·8 μg kinin could be released from the HMW-kininogen fractionated at 4°, whereas from the HMW-kininogen obtained at room temperature 9·2 μg bradykinin equivalents could be released. Similar observations had been made over 2 years ago, when we first attempted to fractionate plasma at room temperature and at 4° on Sephadex G-200, as described originally by Jacobsen.[11]

Our highly purified preparations of kininogen were stable. However, the partially purified preparations were not stable. The data referring to "bradykinin equivalents" obtained at various purification steps refer to the kinin released from kininogen, after subtraction of "spontaneous kinin" present in the kininogen preparation. With the exception of Brocklehurst and Mawr,[6] in most papers published on this subject this aspect is not mentioned. In our experince, partially purified kininogen preparations contain from 0–20 per cent "spontaneously" developed kinin, depending on how much time has elapsed between termination of a preparative procedure and the kinin assay. At this time it would be premature to say exactly why such "spontaneous" conversion to kinin occurs, and this aspect is presently being investigated. However, it would appear reasonable to say that the conversion from kininogen to kinin is not "spontaneous", since the process can be stopped by small amounts of soy bean inhibitor and accelerated when the partially purified kininogens are kept at 37°.

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