KININOGEN MOLECULAR HETEROGENEITY BY AFFINITY CHROMATOGRAPHY ON CONCANAVALIN A AND *RICINUS COMMUNIS* LECTINS

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1. Introduction

Kininogen is a plasma glycoprotein exhibiting various types of heterogeneity. First, the well characterized molecular forms high relative molecular mass (HMW) and low relative molecular mass (LMW) kininogens with different M_r-values of 120 000 and ~50 000–78 000, respectively [1–3]. Another type of heterogeneity refers to the content of active bradykinin segment covalently bound to the HMW and LMW kininogens and specifically split off by kallikrein (EC 3.4.21.8). The LMW form, present in plasma in a higher concentration (~85% of total), is a potential source of the pharmacologically highly active bradykinin peptide, a possible mediator in inflammation [4,5]. The HMW kininogen functions as a cofactor in the contact activation of F XIIa and F XIa thus initiating the cascade reactions of intrinsic blood coagulation [6] independently of the kinin segment but depending on a histidine-rich peptide fragment covalently bound to the L-chain [2,3]. In the native HMW kininogen this histidine-fragment is connected with the H-chain through the bradykinin segment. We have shown that intrinsic in vitro activation of kallikrein through glass contact produces 3–4 HMW molecular forms which can be determined by radioimmunoassay (unpublished). It is likely that all forms of kininogen can be determined immunochemically through the antigenic determinants in the H-chain [7,8].

In [9,10] we investigated the charge heterogeneity of immunologically pure LMW kininogen prepared by using antibody specific affinity chromatography with monospecific anti-kininogen serum. The possibility that isoelectric heterogeneity primarily depends on the carbohydrate structure of kininogens was suggested. It seems that structural variations in the carbohydrate chains, other than the presence or absence of sialic acid residues alone, must play an important part in determining microheterogeneity. The relationship between the interactions of kininogens with lectins and isoelectric heterogeneity has not yet been described. This report deals with an investigation of kininogen heterogeneity utilizing the concanavalin A and *Ricinus* lectins.

Abbreviations: LMW, low relative molecular mass kininogen; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; Con A, concanavalin A–Sepharose 4B; RCA_1, *Ricinus communis* agglutinin of 120 000 M_r according to the nomenclature in [13]; IgG, immunoglobulin G

2. Materials and methods

2.1. *Kininogen preparations*

A crude LMW kininogen was prepared by Sephadex G-200 gel filtration of 500 ml human blood bank plasma (anticoagulant citrate–dextrose–phosphate, CPD) obtained from the Finnish Red Cross Blood Transfusion Service (by courtesy of Dr G. Myllylä). The K_{av} 0.34 fraction pool was collected and submitted to chromatography on DEAE-cellulose, DEAE-Sephadex and gel filtration as in [9,10]. A highly purified kininogen (LMW) was isolated from 1000 ml ammonium sulphate precipitated plasma according to [10].

A kininogen (H-chain) was isolated from Cohn's plasma fraction IV as in [7,11]. It contained about equal amounts of pI 4.2 and 4.4 components and lacked the main isoelectric component of native plasma kininogen. This kininogen has the characteristics of the H-chain by molecular size, amino acid composition, end-group analyses and antigenicity.

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Native plasma kininogen was a single donor human blood sample freshly prepared for focusing using 1 ml anticoagulant (citric acid, dextrose, trisodium citrate) to 9 ml blood. The plasma was separated by centrifugation 20 min at 1000 × *g*, 4°C (ACD plasma).

2.2. *Affinity chromatography on lectins*

Ricinus communis agglutinin (RCA_1) was isolated from 100 g castor beans (Deutsche Rizinus-Oelfabrik Boley Co.) essentially according to [12,13] by Sepharose affinity chromatography and subsequent Sephadex G-100 gel filtration. The RCA_1 agglutinin (195 mg) was dialyzed 20 h against 0.1 M $NaHCO_3$, 0.5 M NaCl (pH 8.5). It was mixed with 20 ml CNBr-activated Sepharose 4B (Pharmacia) as in [14]. CNBr (Eastman Kodak Co.) was 20 mg/ml in 25 ml. After coupling the gel was left standing for 30 min in 1 M ethanolamine (pH 8.0) and washed with 0.1 M $NaHCO_3$, 1 M NaCl (pH 8.5) and 0.05 M Na-phosphate, 0.2 M NaCl (pH 6.8) to remove unbound agglutinin and equilibrated with the latter buffer.

The binding capacity assays for RCA_1–Sepharose were performed in 2.5 ml 0.05 M phosphate buffer, 0.2 M NaCl (pH 6.8) and 0.5 ml RCA_1–Sepharose and started by addition of different amounts of lactose (0.36–18 mg). The tubes were shaken for 1 h at 4°C, centrifuged (8000 × *g*, 20 min) and the amount of unbound lactose determined as in [15].

Kininogen samples were applied on the RCA_1 columns, washed with 20 mM phosphate buffer (pH 7.2) containing 0.15 M NaCl at 20°C. The bound *Ricinus*-reactive material was eluted with 0.1 M D-galactose (Merck) in the same buffer. Fractions of 1.7–2.0 ml were collected.

Columns of Con A–Sepharose (Pharmacia) were equilibrated with 0.01 M Tris–HCl, 0.1 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.02% NaN_3, pH 7.5 at 20°C. After sample application the column was washed with 30 ml sugar-free buffer and the glycoproteins were eluted using 25 ml 0.1 M α-methyl-D-mannoside (grade III, Sigma) in the same buffer. Fraction volume was 2 ml. The binding capacity of Con A–Sepharose was determined in tubes in total volume of 3.4 ml essentially as in [16] using α-methyl-D-mannoside (25–500 μg) and 0.4 ml Con A Sepharose.

2.3. *Neuraminidase treatment*

Crude kininogen 1 mg (A_{280}) and 0.2 units of neuraminidase (*Clostridium perfringens*, Worthington, 0.63 U/mg) were incubated at 37°C for 1 h in 0.5 ml 0.1 M acetate buffer (pH 5.0). Immunological activity was measured without terminating the reaction.

2.4. *Radiolabelling*

Iodination was performed using the chloramine T method according to [17] with 10 μg immunoreactive LMW and 1 mCi $Na^{125}I$ (New England Nuclear, carrier free). Radiolabelled LMW was separated from free ^{125}I by gel filtration on Sephadex G-75. Radioactivity was measured in a Wallac GTL 300 500 gamma sample counter. The specific activity was ~5–10 μCi/μg immunoreactive kininogen.

2.5. *Analytical procedures*

Determination of kininogen and plasma proteins was by Ouchterlony double immunodiffusion analysis [18] and Maneini single radial immunodiffusion [19].

Radioimmunoassay was performed by incubating 0.2 ml samples or pure kininogen standards, 0.1 ml radiolabelled LMW (5000–10 000 cpm) with 0.1 ml diluted antiserum calibrated to bind 50% of the maximal radioactivity at 4°C for 18 h. Normal rabbit serum 5 μl was added as carrier. Antibody-bound ^{125}I-labelled LMW was precipitated by adding 0.25 ml 1:10 diluted sheep anti-rabbit-IgG and incubated for 2 h at 22°C with shaking. The precipitate was collected by centrifugation (5000 × *g*, 4°C) and counted for radioactivity.

Isoelectric focusing was performed in a LKB 8101 column (110 ml) with 1% Ampholine (LKB) pH 3.5–5 and 0.1% Ampholine pH 5–8 in a linear sucrose gradient. Fractions (~1 ml) were collected in ice using LKB peristaltic pump Zero-Max at 30 ml/h. The pH was measured within 2 h at 4°C with a Radiometer PHM 62 standard pH meter.

Sialic acid was determined by the thiobarbituric acid method [20] after hydrolysis with 0.1 N sulfuric acid for 1 h at 80°C. Neutral sugar analysis was performed by the phenol/sulfuric acid procedure [15] employing mannose as standard. Total hexosamine was determined by the Elson-Morgan procedure [21] using glucosamine as standard.

Homogeneity test on Ultrogel AcA (LKB) was done in 0.15 M NH_4HCO_3. All columns were calibrated with Blue Dextran 2000 (Pharmacia).

SDS–PAGE was performed using 8% or 12% polyacrylamide slab gels according to Laemmli [22].

Protein was determined by absorbancy at 280 nm using $A^{1\%}_{280} = 10$.

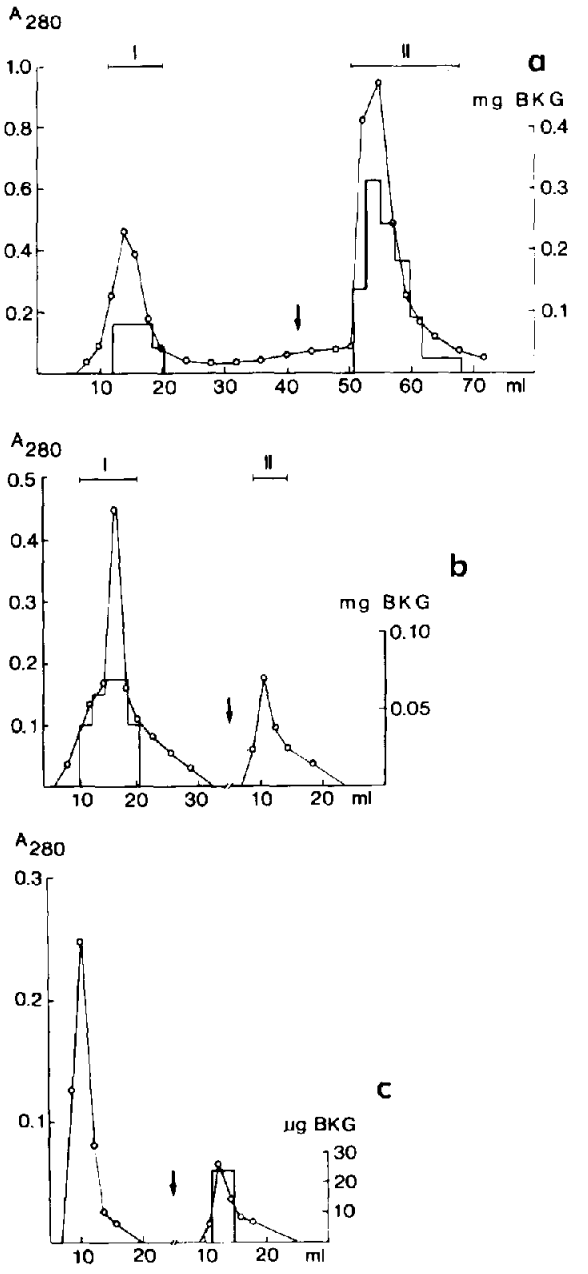

Fig.1. (a) Affinity chromatography of 17.5 mg crude kininogen on Con A–Sepharose (0.8 cm × 22 cm, flow rate 12 ml/h). Elution (at arrow) of bound protein with 0.1 M α-methyl-D-mannoside, fractions pooled as indicated, pool II (dialyzed, lyophilized) was chromatographed further (b) on *Ricinus*-lectin RCA_1 (0.7 cm × 16 cm, 12 ml/h) elution (at arrow) with 0.1 M galactose. Fraction pool I (dialyzed, lyophilized) was treated with neuraminidase and chromatographed on (c) RCA_1-lectin (0.7 cm × 16 cm, 12 ml/h). Protein by absorbancy (o-o) and mg kininogen (BKG) in fractions measured by single radial immunodiffusion (open area).

3. Results

The result obtained by affinity chromatography with Con A of crude kininogen is demonstrated in fig.1a. Kininogen separated in a non-reactive (25%) and a reactive fraction (75%). The crude kininogen contained 9% immunoreactive kininogen, α_2HS-glycoprotein and α_1-acid glycoprotein as main impurities and traces of albumin, ceruloplasmin and Gc-globulin. The binding capacity (K_a) of Con A was 12 mM^{-1} with α-methyl-D-mannoside. The Con A-reactive fraction (pool II) was further submitted to chromatography on RCA_1-lectin with the binding capacity of 0.5 µmol lactose/mg lectin. As demonstrated in fig.1b the reactive fraction from Con A did not bind to RCA_1. After neuraminidase treatment of the dialyzed and lyophilized protein fraction binding to RCA_1 occurred as seen in fig.1c.

The binding to Con A–Sepharose was repeated with purified, homogeneous (fig.2) LMW kininogen in comparison to the H-chain. The results are shown in fig.3. The distribution between the reactive (pool 2) and non-reactive (pool 1) LMW kininogen was 78% and 22% respectively, in agreement with the earlier result (fig.3a).

As demonstrated in fig.3b, 47% of the kininogen H-chain passed through and 53% was bound to Con A. The molecular size of the reactive and the non-reactive material was the same, 58 000, determined by SDS–PAGE (fig.3b inset). The inset in fig.3a shows SDS–PAGE analysis of purified LMW kininogen.

The antigenicity of the reactive and non-reactive

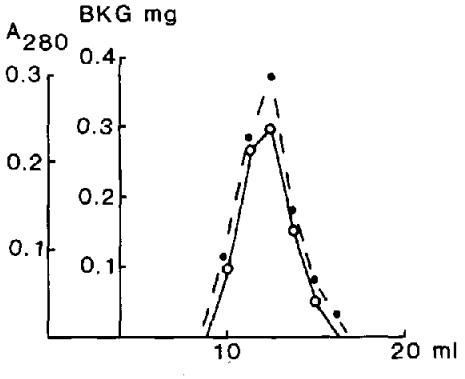

Fig.2. Homogeneity test on Ultrogel AcA 54 (0.9 cm × 30 cm, 8 ml/h, V_O = 11 ml, 0.15 M NH_4HCO_3) of 1 mg highly purified LMW kininogen (recovery 98%). Protein profile (o-o), kininogen (BKG) (· - - ·) by single radial immunodiffusion.

fractions of kininogen H-chain (fig.3b) was investigated by radioimmunoassay using anti-kininogen–H-chain serum. As demonstrated in fig.4 the Con A reactive fraction produced a radioimmunoassay inhibition curve parallel to the standard curve. The slightly different slope obtained with the non-reactive fraction still needs verification.

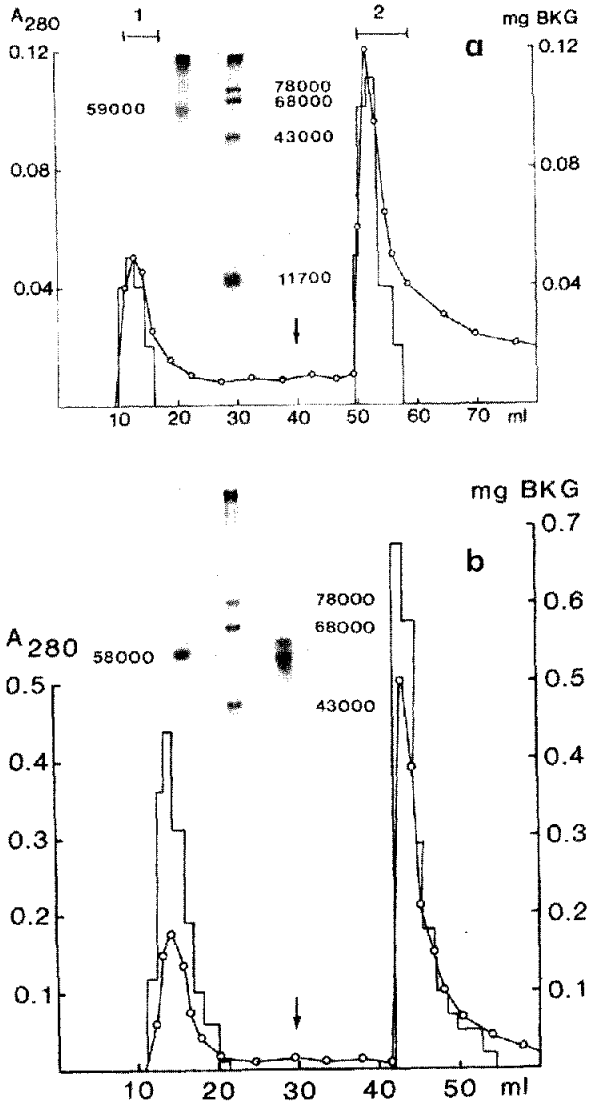

Fig.3. Affinity chromatography on Con A–Sepharose (0.8 cm × 22 cm, flow rate 10 ml/h) of (a) 1 mg homogenous highly purified LMW kininogen and (b) 4.8 mg H-chain preparation. Buffer, elution of lectin-bound fractions and symbols as in fig.1a. Insets: SDS–PAGE analysis (a) in 12% gel of 8 μg highly purified LMW and (b) in 8% gel of 6 μg Con A non-reactive (left) and 5 μg Con A-reactive kininogen. M_r markers 78 000, 68 000, 43 000 and 11 700.

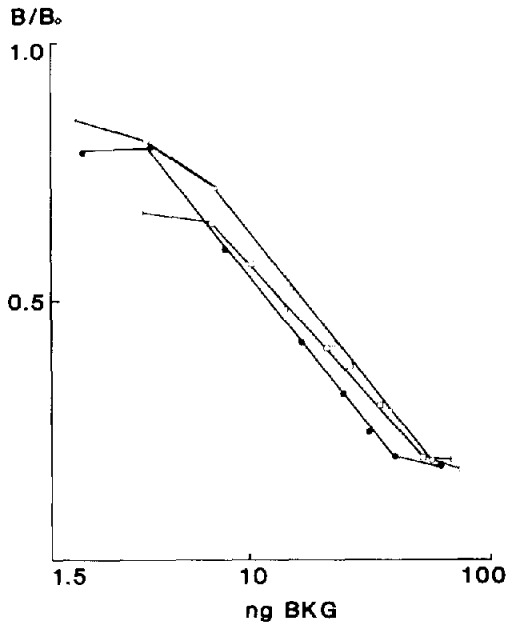

Fig.4. Radioimmunoassay inhibition curves obtained with the Con A-reactive (○ —○) and non-reactive (□——□) fractions of kininogen H-chain in comparison with a standard of LMW (●——●) kininogen. B/B_0 is the ratio of radioactivity bound in the presence and absence of inhibitor.

The non-reactive LMW fraction (fig.3a, pool 1) was dialyzed against distilled water, lyophilized, dissolved in 50 μl distilled water and iodinated. This material (total 3.7×10^6 cpm) was submitted to isoelectric focusing. Fig.5a presents the percentage distribution of radioactivity in relation to pI: 19% at pI 4.2, 12% pI 4.3, 21% pI 4.4, 41% pI 4.5–4.6 and 7% pI 4.7. When the Con A reactive fraction (fig.3a, pool 2) was focused (fig.5c) the corresponding distribution was: 13% at pI 4.2, 28% pI 4.3, 28% pI 4.4 and 31% pI 4.5–4.6. Fig.5b shows the focusing pattern of native plasma kininogen obtained with 2 ml fresh human ACD plasma. In all the cases the pI 4.5 component constitutes the main isoelectric fraction which in native plasma was 47%.

The carbohydrate compositions of LMW kininogen and H-chain are shown in table 1. The mol/mol content was calculated from the respective molecular masses obtained by SDS–PAGE (fig.3a,b insets). The total carbohydrate content of H-chain was somewhat lower (14.4% compared to 18.6%).

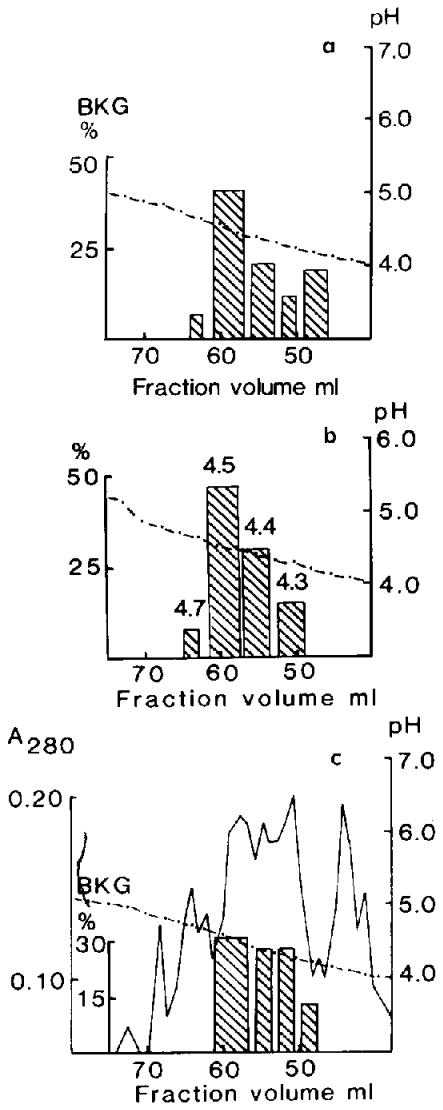

Fig.5. (a) Percent distribution of radioactivity after isoelectric focusing of 12 μg ^{125}I-labelled Con A non-reactive LMW. (b) Percent distribution of kininogen after focusing of 2 ml normal human single donor plasma determined by single radial immunodiffusion. (c) Percent distribution of 330 μg Con A reactive LMW after focusing. The focusing was performed between pH 3.5–5.0 (· · ·), 68–70 h, 4°C, 500–1000 V, fraction volume 1–1.2 ml; protein profile (——).

4. Discussion

The separation of kininogen into two components on Con A–Sepharose (fig.1a,3a) demonstrates a heterogeneity depending on carbohydrate structure. This adds a third type of heterogeneity common among plasma glycoproteins [23] to the earlier known heterogeneities depending on molecular size or charge [9,10]. The structural basis for the ability of Con A to bind kininogen has so far not been studied. The results show that the galactose residues are masked demonstrated by the binding to RCA_1 lectin after removal of all sialic acid. The binding differences when comparing Con A and RCA_1 suggest that glucosamine and mannose residues are available for binding despite the masked galactose residues. This is in agreement with recent findings comparing Con A and RCA lectin binding with vitamin B_{12}-binding protein and α-fetoprotein [24,25]. As seen in table 1 the only slightly higher sialic acid content in the LMW kininogen indicates that the sialic acid is primarily bound to the H-chain. This is in agreement with the low isoelectric point found with the isolated, pure H-chain [11] differing completely from the normal distribution of heterogeneous kininogen [8,10]. Present results sustain an earlier suggestion [11] based on fractionation of the H-chain by polyacrylamide gel filtration that variations in the location of carbohydrate units determines this type of charge heterogeneity manifested by low isoelectric points.

Table 1
Carbohydrate composition of purified kininogens

	% w/w		mol/mol	
	LMW	H-chain	LMW	H-chain
Neutral hexoses (as mannose)	6.0	4.8	19	16
Hexosamine (as glucosamine)	7.6	5.3	25	18
Sialic acid (as *N*-acetylneuraminic acid)	5.0	4.3	9.4	8.4
Total	18.6%	14.4%		

With LMW kininogen (fig.5) the isoelectric pattern did not appreciably differ comparing the Con A reactive and non-reactive fractions from the distribution of pI components in native plasma. The main fractions focused normally at pI 4.5–4.6 in agreement with earlier findings [10]. The role played by differences in carbohydrate and/or polypeptide portions of the kininogen molecule in the charge heterogeneity remains unclear. An earlier result [26] would suggest that the

desialylated kininogen focuses uniformly at pI 5.4–5.5.

The participation of carbohydrates in the antigenic determinants of glycoproteins was discussed in [28]. We have indicated [7] that the H-chain plays a dominant part in the antigenicity of kininogen. Comparison of the carbohydrate content with that of the LMW kininogen (table 1) shows that the main part of sialic acid and other carbohydrates are bound to the H-chain. As shown in [11] the H-chain kininogen preparation produces no detectable L-chain after reduction on SDS–PAGE. The result in fig.4 suggests that mannose and *N*-acetylglucosamine do not influence the antigenicity of the Con A reactive fraction. The slight difference in slope obtained with the non-reactive fraction would sustain the masking of these carbohydrates although both fractions had similar content of sialic acid.

Con A chromatography was applied for the purification of kininogen in [27]. We calculated their distribution to be 22% and 78% between the Con A non-reactive and reactive kininogen, respectively, which is in agreement with these findings. As seen by the SDS–PAGE patterns in fig.3b affinity chromatography on Con A–Sepharose can apparently be used to remove the main impurities ceruloplasmin and α_2HS-glycoprotein from the H-chain preparation [11]. Most of these proteins bound to Con A under these experimental conditions. On the other hand neither with LMW nor the H-chain preparations the Con A non-reactive fraction was totally free from contaminating plasma proteins.

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