

## Isolation and characterization of rat complement factor B and its interaction with cell-bound human C3

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**Summary.** Factor B was isolated from fresh rat plasma by sequential chromatography on QAE-A50, Biorex-70, gel filtration on Sephadex G-200 superfine and rechromatography on QAE-A50. That B<sup>rat</sup> was isolated in its native form was indicated by its migration during immunoelectrophoresis and by its capacity to react with cobra venom factor (CoVF) in the presence of human D to form a C3 convertase capable of cleaving purified rat C3 and human C3. The recovery of B<sup>rat</sup> was between 8 and 15%; the final material was homogeneous according to SDS-PAGE analysis. Reduction of B<sup>rat</sup> with DTT in the presence of urea and SDS did not produce detectable peptides of lower molecular weight. Both reduced and unreduced B<sup>rat</sup> had an apparent molecular weight of 100,000. An antiserum against B<sup>rat</sup> induced in rabbits recognized only one protein in fresh rat plasma as indicated by immunodiffusion and immunoelectrophoretic analysis. Zymosan treatment of rat serum resulted in the

Abbreviations: VBS, isotonic Veronal-buffered saline; GVB, VBS containing 0.1% gelatin,  $5 \times 10^{-4}$  M magnesium, and  $1.5 \times 10^{-4}$  M calcium; DGVB<sup>2+</sup>, half isotonic GVB<sup>++</sup> containing 2.5% dextrose; EDTA, ethylenediaminetetraacetate; GVB-EDTA, GVB containing 0.04 M EDTA. SDS, sodium dodecylsulphate; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; CoVF, cobra venom factor (*Naja naja*); EAC<sup>4hu3hu</sup>, sheep erythrocytes (E) sensitized with rabbit antibody (A) and bearing the major cleavage fragments of human C4 and C3.

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cleavage of B<sup>rat</sup> into two fragments with  $\alpha$  and  $\gamma$  mobility. Native B<sup>rat</sup> has a  $\beta$  electrophoretic mobility. The plasma concentration of B<sup>rat</sup> in Wistar rats was  $215 \pm 38$   $\mu$ g/ml (mean  $\pm$  SD).

### INTRODUCTION

The complement (C) system consists of multiple serum proteins that interact sequentially to mediate a wide variety of inflammatory and immune reactions. Most of the interactions of the C system are now well understood, especially the reaction mechanism of the human complement system, and several reviews of the pathways of complement activation have been published recently (Götze & Müller-Eberhard, 1976; Fearon & Austen, 1976). Factor B appears to play a major role in the initiation of the alternative pathway (Fearon & Austen, 1977; Schreiber, Pangburn, Lesavre, Müller-Eberhard, 1978) and in amplification of C3 cleavage (Müller-Eberhard & Götze, 1972; Fearon, Austen & Ruddy, 1973). Although the human complement system has been studied extensively, reliable animal models for *in vivo* studies of autologous complement components and their degradation products are not available. Reports of the isolation and characterization of the complement components C3 (Daha, Stuffers-Heiman, Kijlstra & van Es, 1979) and C4 (Daha & van Es, 1979) in the rat were published recently; this paper describes the isolation and characterization of rat factor B. It is also shown that purified B<sup>rat</sup> is able to react with cell-bound human C3 and generate an

amplification convertase in the presence of human  $\bar{D}$  and  $\bar{P}$ .

## MATERIALS AND METHODS

Benzamidine, HCl (Sigma Chemical Co, St Louis, MO), agarose oligomers of lactalbumin (BDH, Amsterdam), sodium dodecyl sulphate (Merck, Amsterdam), Quaternary amino ethyl (QAE)-Sephadex-A50, Sephadex-G-200 superfine (Pharmacia Fine Chemicals, Inc., The Hague), and dithiothreitol (Biorad Labs, Pleuger, Amsterdam) were purchased as indicated.

Fresh blood from 4-month old Wistar rats was drawn and immediately mixed with EDTA and benzamidine to obtain concentrations of 0.002 M and 0.001 M, respectively. The plasma was separated from the cellular components by centrifugation at 5° and used immediately.

### Assays

Isotonic Veronal-buffered saline (VBS) (pH 7.5) containing 0.1% gelatin,  $5 \times 10^{-4}$  M magnesium ( $Mg^{2+}$ ), and  $1.5 \times 10^{-4}$  M calcium ( $Ca^{2+}$ ) (GVB<sup>2+</sup>); half-isotonic GVB<sup>2+</sup> (DGVB<sup>2+</sup>); and GVB containing 0.04 M EDTA (GVB-EDTA) were used as diluents for the haemolytic assay (Nelson, Jensen, Gigli & Tamura, 1966).

During the first two isolation procedures for B<sup>rat</sup> B activity was assayed by incubation of 25  $\mu$ l dilutions of rat plasma or column fractions in DGVB<sup>2+</sup> with 25  $\mu$ l portions of purified cobra venom factor (CoVF, 1 mg/ml) (Cochrane, Müller-Eberhard & Aikin, 1970), purified rat C3 (1 mg/ml), and purified human D (5  $\mu$ g/ml). After incubation for 30 min at 37° residual C3 haemolytic activity was determined with the cellular intermediate EAC1<sup>SP4</sup><sup>hu2SP</sup> (Ruddy & Austen, 1969) and convertase sites were developed with guinea-pig serum diluted 1:30 in GVB-EDTA for 60 min at 37°. Using the purified B<sup>rat</sup> from the first two runs it was found that a cell-bound amplification convertase could be formed with EAC4<sup>hu3</sup><sup>hu</sup> (Fearon *et al.*, 1973) bearing 4000 C3b molecules per intermediate in the presence of purified human  $\bar{P}$  and  $\bar{D}$ . This assay was carried out as described for human B (Daha, Fearon & Austen, 1976). Convertase sites were formed during a 30 min period at 30° and subsequently developed with rat serum (diluted 1:15) in GVB-EDTA for 60 min at 37°. The number of haemolytic sites generated was calculated using EAC<sup>hu3</sup><sup>hu</sup> incubated with buffer as a

reagent blank. A linear dose-response relationship was found between the input of purified B<sup>rat</sup> and the number of haemolytic sites generated in the assay system; 0.01  $\mu$ g B<sup>rat</sup>/ml yielded an average of one site (Z) per cell. Assessment of B with EAC4<sup>hu3</sup><sup>hu</sup> could not be used for whole rat serum because of inhibition of B activity, presumably by inhibitors present in the rat serum. Since the assay of B<sup>rat</sup> activity with the intermediate EAC<sup>hu3</sup><sup>hu</sup> was more rapid than the CoVF assay, EAC4<sup>hu3</sup><sup>hu</sup> intermediates were used to detect B activity in purified B preparations or in column fractions. Human  $\bar{P}$  and  $\bar{D}$  (Fearon & Austen, 1975), C3 (Tack & Prahl, 1976) and rat C3 (Daha *et al.*, 1979) were purified as indicated.

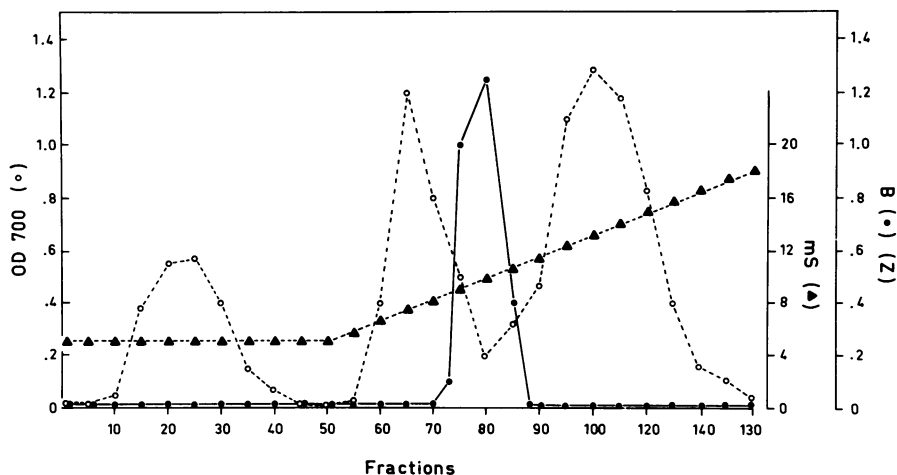
B<sup>rat</sup> in plasma was quantified immunochemically by radial-immunodiffusion in the presence of 0.002 M EDTA with purified B<sup>rat</sup> as a standard. Purified B<sup>rat</sup> was quantified by Folin analysis (Lowry, Rosebrough, Farr & Randall, 1951) using B<sup>hu</sup> as a standard. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 6% gels containing 1% SDS. Aliquots of 25  $\mu$ l containing 20–50  $\mu$ g protein were diluted with 25  $\mu$ l 1.5% SDS in a 10 M urea solution, incubated for 60 min at 37°, and applied to the gels. Reduction of protein samples was performed in the presence of 8 M urea, 1% SDS, and 0.02 M dithiothreitol (DTT) (Daha *et al.*, 1979). Electrophoresis was carried out at 8 mA/gel and 30° until the buffer front reached the end of the gel (Weber & Osborn, 1969). Oligomers of lactalbumin were used as mol. wt markers, subjected to electrophoresis in a parallel gel, and stained with Coomassie blue.

Immuno-electrophoresis was performed in 1.5% agarose, 0.025 M Veronal, and 0.002 M EDTA (pH 8.4) at 15 V/cm for 30 min, and precipitation areas were developed with an antiserum to whole rat serum and monospecific antiserum to B<sup>rat</sup>; the latter was obtained from rabbits previously immunized with homogeneous preparations of B<sup>rat</sup>.

## RESULTS

### Anion exchange chromatography

One hundred millilitres of fresh rat plasma containing 0.002 M EDTA and 0.001 M benzamidine were adjusted to a pH of 8.0 with 0.01 N NaOH, then diluted to a conductivity of 5.5 mS with ice-cold water containing EDTA and benzamidine, and subsequently applied to a 2.6  $\times$  60 cm QAE-A50 column, which had previously been equilibrated with 0.01 M Tris-HCl

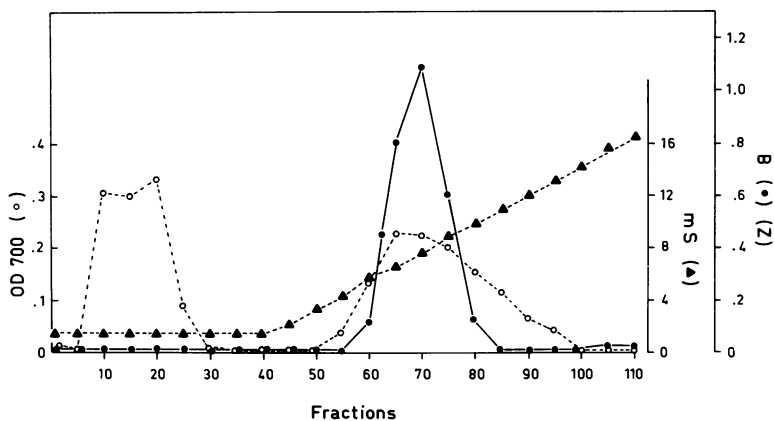


**Figure 1.** QAE-A50 chromatography of rat plasma. Protein content was measured by Folin analysis (○); the ionicity of the fractions is indicated (▲); B-haemolytic activity was assessed with EAC<sup>4hu3hu</sup> in the presence of excess D<sup>hu</sup> and P<sup>hu</sup> (●).

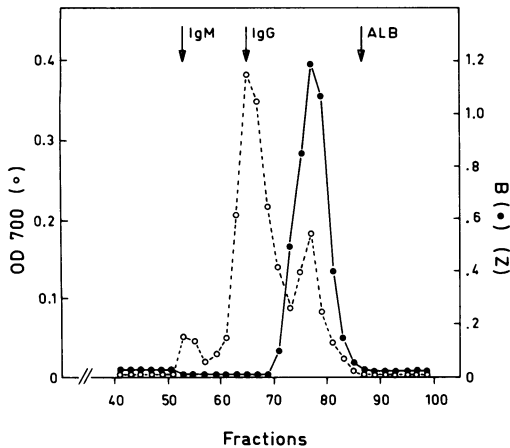
buffer (pH 8.0), 0.002 M EDTA, 0.001 M benzamidine, and enough NaCl to yield a conductivity of 5.5 mS. After collection of 40 fractions of 10 ml each, a linear NaCl gradient in 1000 ml starting buffer was applied to the column. For every fifth fraction, the protein content in a 25  $\mu$ l aliquot was measured by Folin analysis. B haemolytic activity eluted between 8 and 12 mS (Fig. 1). Fractions 75–86 were pooled and dialysed for 4 h against 3 l of 0.01 M sodium acetate buffer, pH 6.0.

#### Cation exchange chromatography

One hundred and twenty millilitres of the dialysed B pool from the QAE-A50 step were applied to a 1.5  $\times$  30 cm Biorex-70 column which had been equilibrated with 0.01 M sodium acetate buffer, 0.002 M EDTA, 0.001 M benzamidine (pH 6.0), and enough NaCl to yield a conductivity of 1.5 mS. After collection of 35 fractions of 5 ml each in one drop of 0.5 M Tris-base in order to neutralize the pH of the fractions, a linear NaCl gradient of 600 ml was applied to the column.



**Figure 2.** Cation-exchange chromatography of the QAE-pool of rat B on Biorex-70. Protein content (○), ionicity (▲), and B haemolytic activity of the fractions (●) are indicated.



**Figure 3.** Gel filtration of the concentrated Biorex pool of  $B^{\text{rat}}$ . protein content (○) and B haemolytic activity (●) are indicated. The filtration positions of IgM, IgG and albumin are indicated by arrows; these were determined previously.

The protein content was measured in 50  $\mu\text{l}$  aliquots of every fifth fraction; B haemolytic activity was assessed in 1:50 dilutions of the fractions and the conductivity was measured at 0° (Fig. 2). B haemolytic activity was eluted from the column between 5 and 9 mS. Fractions 62–72 were pooled and concentrated by ultrafiltration to 5.7 ml.

### Gel filtration

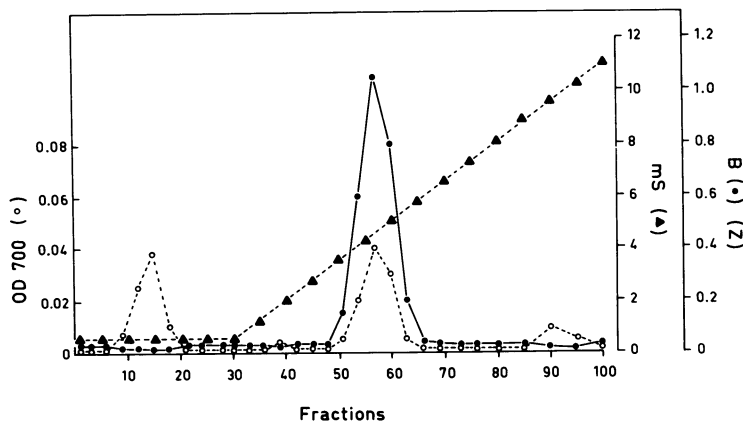
A volume of 5.5 ml of the concentrated Biorex-pool

containing B was applied on a 2.5 × 90 cm Sephadex G-200 superfine column, equilibrated with VBS, 0.002 M EDTA, and 0.15 M NaCl. The column was previously calibrated with albumin IgG and IgM as mol. wt markers. Fractions of 2.5 ml were collected, and the B haemolytic activity was measured in 1:60 dilutions of the fractions. B activity was found in fractions 72–85 with a calculated molecular weight of 105,000, coinciding with the third protein peak (Fig. 3). Fractions 75–81 were pooled and dialysed against 1 l of 0.01 M Tris-HCl (pH 8.0), 0.002 M EDTA, and enough NaCl to yield a conductivity of 1 mS.

### Repeat chromatography on QAE-A50

Nineteen millilitres of the dialysed G-200 pool were applied on a 1.5 × 10 cm QAE-A50 column which was equilibrated with dialysis buffer. After collection of 25 fractions of 2.5 ml each, a linear NaCl gradient in 200 ml Tris-HCl buffer was applied to the column. The protein content was measured in 100  $\mu\text{l}$  aliquots of the fractions by Folin analysis and B hemolytic activity was measured in 1:50 dilutions of the fractions. (Fig. 4). B haemolytic activity was associated with the second protein peak. Using a rabbit antiserum produced against purified  $B^{\text{rat}}$  it could be shown that the protein profile coincided with B as measured immunologically by radial immunodiffusion.

This post QAE-A50 B preparation contained 10% of the initial plasma B protein and 11% of the original plasma B activity as assessed with the CoVF assay (Table 1).



**Figure 4.** Rechromatography on QAE-A50 of the post G-200 pool of  $B^{\text{rat}}$ . Protein content (○), ionicity (▲) and B haemolytic activity (●) of the fractions are shown.

**Table 1.** Method for B purification

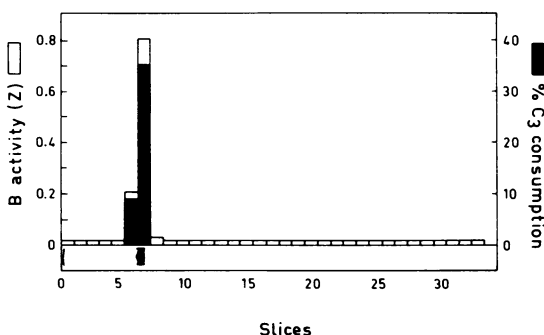
Step	Total B* ( $\mu\text{g}$ )	Yield (%)
1. Plasma	21.500	100
2. Post-QAE pool	12.470	58
3. Post-Biorex pool	6.450	30
4. Post-G-200 pool	4.300	20
5. Post-QAE pool	2.150	10

\*Determined by radial immunodiffusion against monospecific anti-rat B.

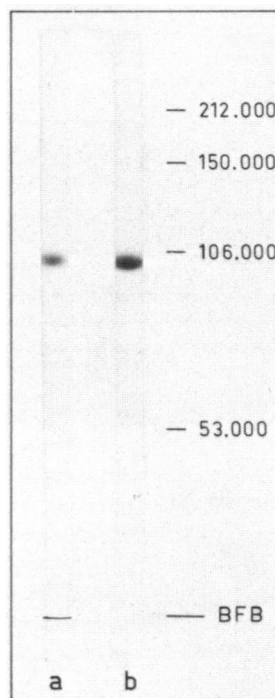
### Characterization of B

In order to determine whether the B<sup>rat</sup> activity as assessed with the CoVF assay coincided with the B<sup>rat</sup> activity measured with EAC4<sup>hu3hu</sup>, 50  $\mu\text{g}$  quantities of purified B<sup>rat</sup> were applied to two 6% polyacrylamide gels. After electrophoresis, one gel was stained with Coomassie blue and the other was sliced in sections. In each slice the B activity was measured with the CoVF-assay and with EAC4<sup>hu3hu</sup>. Both methods showed B activity only in slices 6 and 7, associated with the only detectable protein band (Fig. 5).

To determine the chain composition of B<sup>rat</sup>, 35  $\mu\text{g}$  B before reduction and 50  $\mu\text{g}$  B after reduction with DTT were subjected to 6% SDS-PAGE analysis. After electrophoresis the gels were stained and the mol. wt compared with markers run on a parallel gel (Fig. 6). The mol. wt of B before reduction was  $100,000 \pm 4200$  (mean  $\pm 1$  SD,  $n=6$ ) and after reduction



**Figure 5.** PAGE analysis of 50  $\mu\text{g}$  B<sup>rat</sup> from the last QAE-A50 step. B activity was determined in the eluate of a parallel gel and assessed either with CoVF, D<sup>hu</sup> and C3<sup>rat</sup> in a 1:3 dilution of the eluates (solid bars) or with EAC4<sup>hu3hu</sup> in the presence of D<sup>hu</sup> and P<sup>hu</sup> in a 1:50 dilution of the gel eluates (open bars).



**Figure 6.** SDS-PAGE analysis of 30  $\mu\text{g}$  B<sup>rat</sup> under non-reducing conditions (a) and 50  $\mu\text{g}$  B<sup>rat</sup> under reducing conditions (b). The position of the molecular weight markers is shown and was obtained from a parallel gel.

$99,000 \pm 4100$  ( $n=6$ ). No additional protein bands were detectable in the gels.

Antiserum to rat B was produced in two rabbits, each immunized with 50  $\mu\text{g}$  B from the last QAE-A50 step incorporated in Freund's complete adjuvant. Both rabbits produced antibodies that recognized one protein in whole rat plasma. This protein gave a line of identity with purified B<sup>rat</sup>.

The mobility of B<sup>rat</sup> in serum was comparable with that of human B, and treatment of rat serum with 2 mg/ml zymosan for 30 min at 37° resulted in cleavage of B into two fragments (Fig. 7), one with  $\gamma$ -mobility and one with  $\alpha$ -mobility. Using the monospecific antibody to rat B, the concentration of B was found to be  $215 \pm 38$   $\mu\text{g}/\text{ml}$  (mean  $\pm 1$  SD, 12 rats) in 4 month old Wistar rats.

### Interaction of B<sup>rat</sup> with cell-bound human C3b

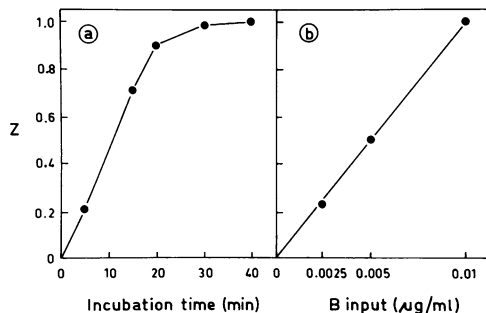
To determine whether B<sup>rat</sup> was capable of forming a cell-bound convertase on EAC4<sup>hu3hu</sup>, 0.01  $\mu\text{g}$  B<sup>rat</sup> was



**Figure 7.** Immunoelectrophoretic analysis of normal rat serum (upper well) and zymosan-treated rat serum (lower well). The troughs contain antiserum against whole rat serum (anti-RtS), rabbit anti-B<sup>rat</sup> (anti-B) and phosphate-buffered saline (PBS).

incubated with  $1 \times 10^8$  EAC4<sup>hu3hu</sup> in the presence of  $1.4 \mu\text{g } \bar{P}$  and  $15 \text{ ng } \bar{D}$  in 2 ml DGVB<sup>2+</sup> at 30°. At timed intervals 0.2 ml of the reaction mixture were removed and incubated with 0.3 ml rat serum diluted 1:15 in GVB-EDTA as a source of C3–C9. After incubation for an additional 60 min at 37°, 1.5 ml isotonic saline were added to each tube and the extent of haemolysis was determined. The average number of haemolytic sites (*Z*) was calculated with a reaction mixture lacking B as the reagent blank. The reagent blank never exceeded more than 2% lysis of the total input of intermediates. The results, as depicted in Fig. 8a, indicate that convertase formation was optimal during the 30–40 min interval at 30°. For further experiments a period of 30 min was chosen for convertase formation. No convertase formation was found in the presence of 0.002 M EDTA. Convertase activity was resistant to washing at 0° with 0.002 M GVB-EDTA.

In order to determine whether generation of the



**Figure 8.** (a) Time-dependent generation of cell-bound convertase sites (*Z*) by interaction of B<sup>rat</sup> (0.01 μg/ml) with EAC4<sup>hu3hu</sup> in the presence of  $\bar{D}^{\text{hu}}$  and  $\bar{P}^{\text{hu}}$ . (b) Dose-dependent formation of cell-bound convertase sites (*Z*) upon interaction of various concentrations of B<sup>rat</sup> with EAC4<sup>hu3hu</sup> in the presence of  $\bar{D}^{\text{hu}}$  and  $\bar{P}^{\text{hu}}$ .

number of convertase sites was dependent on the input of B<sup>rat</sup>, 0.01, 0.005 and 0.0025 μg B were assayed for their capacity to generate convertase sites on EAC4<sup>hu3hu</sup> in the presence of  $\bar{D}$  and  $\bar{P}$  for 30 min at 30°. As may be seen from Fig. 8b, a linear dose-response relationship for the number of convertase sites generated was obtained under the conditions described above. Under identical conditions purified human B was capable of generating 110 sites per cell at an input of 0.01 μg.

## DISCUSSION

Initial attempts to purify rat B from freshly frozen plasma yielded very poor results. The addition of benzamidine and EDTA, the immediate use of fresh plasma obtained from healthy rats and rapid chromatography during the first QAE-A50 step led to conservation of B activity. The presence of benzamidine in the first two columns is essential. Although freshly frozen plasma yields B preparations containing native B, better results may be obtained with fresh plasma. The final yield of B ranged between 8 and 15% on four occasions (one of which is summarized in Table 1).

The protein was homogeneous according to SDS-PAGE analysis (Fig. 6). The molecular weight of rat B was found to be  $100,000 \pm 4200$  and  $99,000 \pm 4100$  for the non-reduced and reduced forms, respectively. These molecular weights are in close agreement with those determined for human B (Boenisch & Alper, 1970; Götze & Müller-Eberhard, 1971), guinea-pig B (Brade, Cook, Shin & Mayer, 1972) and mouse B (Bitter-Sauermann, Burger, Brade & Hadding, 1976).

Since measurements of B<sup>rat</sup> activity by determining its capacity to form a cell-bound amplification convertase on the cellular intermediate EAC4<sup>hu3hu</sup> is more

rapid than measurement of B activity with the CoVF assay, assessment of B activity in column fractions was performed with the cellular intermediate EAC4<sup>hu3hu</sup>. B<sup>rat</sup> activity measured with both methods showed a close overlap in eluates of gel slices (Fig. 5) indicating that B<sup>rat</sup> was indeed measured with the cellular intermediate.

The formation of a cell-bound convertase of B<sup>rat</sup> with EAC4<sup>hu3hu</sup> in the presence of human D̄ and P̄ is Mg<sup>2+</sup>-dependent and is optimal between 30 and 40 min at 30°. These results are in agreement with the formation of the homologous convertase between human B and EAC4<sup>hu3hu</sup> (Fearon *et al.*, 1973). Although B<sup>rat</sup> is capable of reacting with EAC4<sup>hu3hu</sup> to form a convertase, on a weight basis it is approximately 100 times less efficient than human B. Whether these differences are attributable to a lesser affinity of B<sup>rat</sup> for human C3b or that human D̄ is less efficient in the cleavage of B<sup>rat</sup> once it is bound to C3b<sup>hu</sup> is not clear at the moment and requires further investigation. In addition human P̄ may also be less efficient in stabilizing the heterologous convertase composed of C3b<sup>hu</sup> and Bb<sup>rat</sup> as compared to the C3b<sup>hu</sup>Bb<sup>hu</sup> convertase.

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