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Effect of salt concentration gradient on separation of different types of specific immunoglobulins by ion-exchange chromatography on DEAE cellulose

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Abstract

A three-stage process, consisting of an ammonium sulfate precipitation step, dialysis desalination with microporous anion-exchange Neosepta membranes and anion-exchange chromatography on DEAE-cellulose DE-52 was used for the isolation of mouse monoclonal antibodies specific against different antigens. The ascites fluids contained monoclonal antibodies against human IgG, against horseradish peroxidase and against the heavy chain of human IgM. The effect of the salt concentration gradient in the elution buffer was examined with the aim of optimizing chromatographic conditions. The quality of separation of protein zones was determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis under non-reducing conditions. The immunoreactivity of purified monoclonal antibodies was determined by enzyme-linked immunosorbent assay using a solid-phase adsorbed antigens against which each monoclonal antibody type was directed.

Keywords: Salt concentration gradient; Immunoglobulins

1. Introduction

Monoclonal antibodies (mAbs) are increasingly used in biomedical studies [1]. Increasing the production and cloning of new types of mAbs stimulates the development of new purification methods and the improvement of the existing ones [2].

One of the traditional purification schemes [3] used by us consists of the following stages: clarification by centrifugation (micro-, ultrafiltration), precipitation of the protein fraction with ammonium sulfate, dialysis desalination and ion-exchange chromatography on DEAE-cellulose. DEAE-cellulose is one of the classical ion-exchange chromatographic supports which, due to the high hydrophilicity of the cellulose matrix, the absence of denaturing effect on IgG_1 and IgG_2 antibodies, and wide accessibility, is still in use in the isolation of mAbs from ascites and cell culture fluids [4–6] and in model laboratory studies, in spite of the availability of new supports possessing better chromatographic properties, such as affinity [7–9], ion-exchange [10,11], mixed-mode [12], hydrophobic [13] and hydroxyapatite [14] supports.

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Previously, we have shown [15] that replacing the dialysis membranes by the microporous ion-exchange Neosepta membranes allows optimization of the dialysis desalting step. Dialysis time decreased 20 times, 25–30% of the accompanying proteins such as mouse serum albumin (SA), transferrin (TF), proteases were partially separated. At the same time, the losses of mAbs did not exceed 1–5%.

Our preliminary study of the isolation of mAbs from the desalted solution of the protein fraction of mouse ascites fluids using ion-exchange chromatography on DEAE-cellulose showed that the mAbs and accompanying proteins cannot be separated without overlapping of their zones, if the desorption is carried out with a linear salt concentration gradient in the elution buffer [16].

In the case of sorption on DEAE-cellulose with a low volume concentration of the $-OCH_2CH_2N^+H(CH_2CH_3)_2$ groups (one group is present in a volume 13 700 $Å^3$ [17]), the protein molecules are bound to the carrier not only by electrostatic and hydrogen bonds; a contribution to the retention and transfer of protein molecules in this porous medium is made also by the dipole–dipole interactions arising between protein molecules. It is obvious that the migration of the zones of components in DEAE-cellulose is very sensitive to insignificant concentration changes and to the composition of the elution buffer and pH, which determines the charge of the ionogenic groups of the proteins undergoing separation.

The aim of this study was to investigate the effect of the salt concentration gradient in the elution buffer on the separation of the components of mouse ascites fluids containing different types of mAbs by ion-exchange chromatography on DEAE-cellulose and optimization of the chromatographic conditions.

2. Experimental

2.1. Ammonium sulfate precipitation of mouse ascites fluids

The ascites fluids containing immunoglobulins (Igs) specific against human IgG (heavy chain of human IgM or horseradish peroxidase) were prepared by injecting ~$5 \cdot 10^6$ antibody-producing mouse hybridoma cells into the peritoneal cavity of congenic mouse (Balb/C), pretreated with 0.5 ml pristane. After 7–14 days, ascites fluids containing specific Igs were collected from the intraperitoneal cavity and were pretreated with ammonium sulfate.

The protein fraction of an ascites fluid was precipitated by dropwise addition of an equal volume of saturated ammonium sulfate solution at room temperature. To complete precipitation, the solution was stirred for 2 h at room temperature and was refrigerated at 4°C overnight. The precipitate was centrifuged (4500 *g* for 30 min at 20°C) and the pellet was dispersed in the original volume of 50% ammonium sulfate solution. After a second centrifugation (4500 *g* for 30 min at 20°C), the pellet was dissolved in the original volume of deionized water. This solution of the protein fraction of mouse ascites fluid, after microfiltration through Whatman paper (Table 1), was desalted by dialysis through microporous anion-exchange Neosepta membranes prior to ion-exchange chromatography on DEAE cellulose DE-52.

2.2. Dialysis desalination of protein fraction of mouse ascites fluids through microporous anion-exchange Neosepta membranes

Dialysis desalination of a solution of the protein

Table 1
Characteristics of the protein fraction solution of mouse ascites fluids with different specificities

IgG_1 specificity	p*I* of IgG_1	Colloids diameter (nm)	pH	Ammonium sulfate (mg/ml) (*M*)	Organic substances (mg/ml)	Proteins (mg/ml)	IgG_1 (mg/ml)
Horseradish peroxidase	5.95–6.30	176±9	5.87	70.29 (0.53)	14.44	13.1	2.47
Heavy chain of human IgM	5.45–5.85	154±4	6.13	106.90 (0.81)	18.25	15.3	0.52
Human IgG	6.40–6.90	126±3	6.19	51.0 (0.39)	14.05	10.95	1.50

fraction of mouse ascites fluids was carried out in a flat three-compartment membrane cell [18] equipped with two membranes. The effective area of the membrane was 5.6 cm^2; the distance between the membranes 3 mm, the volume of diffusate (permeate) and dialysate (retentate) compartments of the membrane cell were 0.5 and 1.5 cm^3, respectively. The microporous anion-exchange Neosepta membranes used were kindly provided by Dr. Y. Mizutani and Dr. N. Ohmura (Tokuyama, Japan). They contained quaternary ammonium groups and differed in porosity, pore size distribution and concentration of ionogenic groups (Table 2).

The solution to be desalted (8 ml) and water in the chambers collecting the diffusate (10 ml) from the compartments of the membrane cell were thermostated at 25±0.1°C. Circulation of the solutions through the membrane compartments was maintained by a peristaltic pump. The flow-rate was 13 ml/min. After 30 min, the experiment was stopped and the electric conductivities of dialysate and diffusates were measured with digital conductivity meter (Philips, Cambridge, UK). Water in the diffusate chambers was replaced, and the process was continued. When the electric conductivity in the dialysate had reached its minimum of approximately 6 mS/cm, corresponding to the concentration of ammonium sulfate of 0.03–0.04 *M*, the dialysis was stopped. Before and after desalting, aliquots of the solution (5 ml) were lyophilized and the content of ammonium sulfate was calculated from the analysis of sulfates in the dry samples.

2.3. Ion-exchange chromatography

Desalted protein solutions were chromatographed on a column (13×1.2 cm I.D.) packed with DEAE cellulose DE-52 (Whatman, Maidstone, Kent, UK), equilibrated with 0.02 *M* Tris–HCl (Fluka, Buchs, Switzerland) buffer solution, pH 7.8 (buffer A). The anion-exchange chromatography was carried out at a flow-rate of 0.6 ml cm^{-2} min^{-1} in a linear (concave or stepwise) gradient of NaCl using the buffers obtained by mixing buffer A and buffer B (0.4 *M* NaCl in buffer A). The absorbances of the eluate fractions were measured with a diode array spectrophotometer (Hewlett-Packard, Vancouver, WA, USA) at wavelengths 260 and 280 nm and the concentration of proteins was calculated by the method of Warburg and Christian [19]. The concentration of Cl^- ions in the eluate fractions was determined by titration and pH was measured with a pH meter (WTW, Weilheim, Germany) with a pH microelectrode.

2.4. Detection of IgG_1 specificities

Mouse specific IgG_1s present in peaks eluted from the DEAE cellulose columns were detected by sandwich ELISA titration using standard antigens against which the definite type of specific Igs was directed [20].

2.5. SDS-PAGE electrophoresis

SDS-PAGE was carried out in a vertical Mini-Protean II apparatus (LKB, Uppsala, Sweden) by the discontinuous buffer method described by Laemmli [21]. The non-reducing sample buffer, the stacking gel (T 4%, C 2.5% filled to 1/10 of the total gel volume) and a separating gradient gel (T 6–15%, C 2.5%) were used with a spacer thickness of 1 mm. Gels were fixed and stained with a 50% methanol–7% acetic acid aqueous solution containing 0.1%

Table 2
Characteristics of microporous anion-exchange Neosepta membranes used

Membrane	Ion-exchange capacity (meq/ml)	Specific volume (ml/g)	Relative porosity (%)	Specific surface (m^2/g)
5A	0.037	2.15	62	12.1
6A	0.181	1.76	60	14.6
7A	0.151	1.85	59	10.0
8A	0.048	2.53	64	26.7
9A	0.044	2.53	65	59.1

Coomassie Blue R 250 (Aldrich, Steinheim, Germany). Destaining solution was 5% methanol–7% acetic acid aqueous solution. A standard Serva protein test mixture 4 was used as a high-molecular mass protein marker.

2.6. Isoelectric focusing (IEF)

A flat-bed separation with Servalyt Precote, pH 3–10 (Serva, Heidelberg, Germany) ultrathin prepacked polyacrylamide gels was performed with a

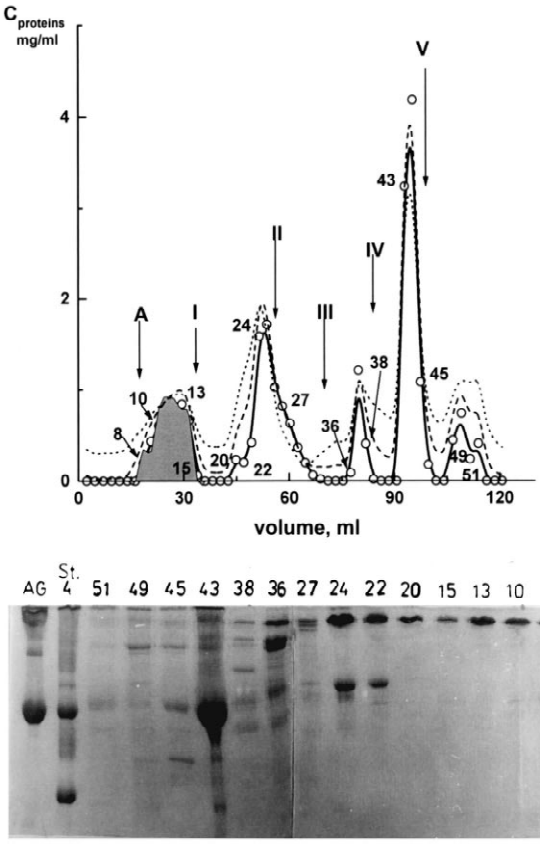

Fig. 1. (upper panel) Ion-exchange chromatography of the desalted solution of the protein fraction of mouse ascites fluid containing mAb vs. hIgG on DEAE-cellulose DE-52. Column (13×1.2 cm) was equilibrated with starting 20 m*M* Tris–HCl buffer (pH 7.8) (A). Volume of desalted protein solution 18.7 ml (pH 5.68). Elution under the stepwise concentration gradient of NaCl in the starting buffer A: I 0.025; II 0.05; III 0.1; IV 0.2; V 0.4 *M*. Flow-rate 0.4 ml min^{-1}. Dashed and dotted lines indicate the absorbance of eluate fractions at 260 and 280 nm, respectively. Shaded area of the peak indicates mAb vs. hIgG. The numbers indicate the eluate fractions analysed by SDS-PAGE. (lower panel) SDS-PAGE of eluate fractions: 8, 10, 13, 15: first peak; 20, 22, 24, 27: second peak; 36, 38: third peak; 43–45: fourth peak; 49, 51: fifth peak; AG: standard mixture of human serum albumin and γ-globulin; St.4: standard Serva protein test 4.

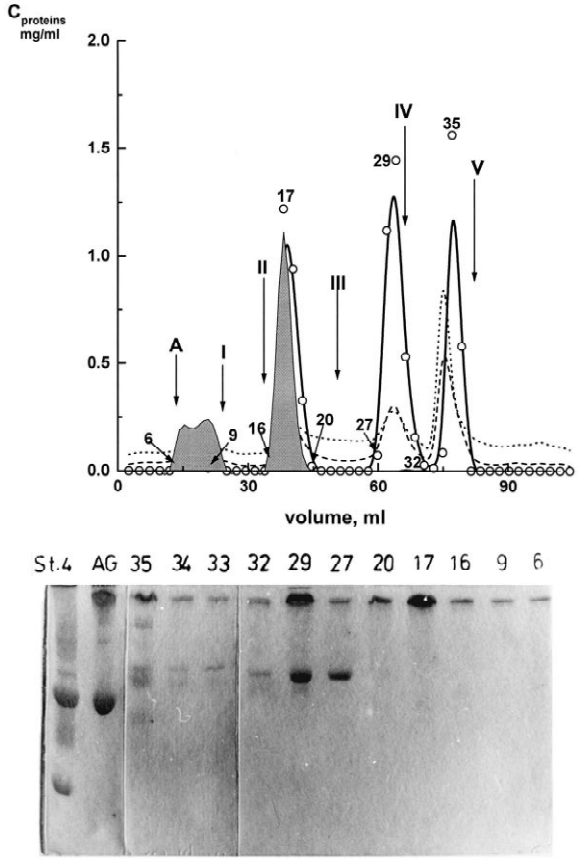

Fig. 2. (upper panel) Ion-exchange chromatography of the desalted solution of the protein fraction of mouse ascites fluid containing mAb vs. hIgG on DEAE-cellulose DE-52. Column (13×1.2 cm I.D.) was equilibrated with starting 20 m*M* Tris–HCl buffer (pH 7.8) (A). Volume of desalted protein solution 50 ml, (pH 7.8). Elution under the stepwise concentration gradient of NaCl in the starting buffer A: I 0.025; II 0.05; III 0.1; IV 0.2; V 0.3 *M*. Flow-rate 0.4 ml min^{-1}. Dashed and dotted lines indicate the absorbance of eluate fractions at 260 and 280 nm, respectively. Shaded area of the peak indicates mAb vs. hIgG. The numbers indicate the eluate fractions analysed by SDS-PAGE. (lower panel) SDS-PAGE of eluate fractions: 6, 9: first peak; 16, 17, 20: second peak; 27, 29, 32: third peak; 33–35: fourth peak; AG: standard mixture of human serum albumin and γ-globulin; St.4: standard Serva protein test 4.

layer thickness of 150 μm and size 125×125 mm. The following focusing conditions were used for the Multifor 2117 apparatus (LKB, Uppsala, Sweden): Servalyt was used for anode and cathode buffer solutions and samples were applied in the middle of the gel using an application strip; initial and maximum voltage were 100 V and 1500 V, respectively at 3000 Vh [22]. The pH gradient was determined with a Serva p*I*-marker mixture 9 [23]. Gels were stained and destained with the protein dye as described above.

3. Results and discussion

To optimize the separation conditions, isoelectric points of the basic proteins of the mixture were determined: TF, native mouse IgG, mAb vs. hIgG, mAb vs. HRP, mAb vs. hIgM and mouse SA. It was found using the isoelectrofocusing method that the proteins under separation have close isoelectric points, p*I* (Table 1): TF 5.7–5.7; mAb vs. hIgM 5.45–5.85; mAb vs. HRP 5.95–6.30; mAb vs. hIgG 6.40–6.90, native mouse IgG 4.0–5.9; and mouse SA 4.6–4.9. The p*I* values thus determined allow us to assume that the retention of the components of the protein mixture for each mAb type on DEAE-cellulose increases in the series:

1. mouse SA<TF=native mouse IgG<mAb vs. hIgG
2. mouse SA<TF=native mouse IgG=mAb vs. HRP
3. mouse SA<mAb vs. hIgM<native mouse IgG= TF

Consequently, with increasing salt concentration in the elution buffer, the order of displacement of the proteins from the carrier should be reversed.

As can be seen in the chromatogram (Fig. 1, upper panel), the components of the protein mixture 1 are separated into five non-overlapping zones. An analysis of fractions of eluate by SDS-PAGE (Fig. 1, lower panel) showed that mAbs elution starts virtually immediately after introducing the starting buffer A into the column and terminates on the addition of the buffer with 0.025 *M* salt concentration. Along with mAb vs. hIgG, the eluate fractions contained trace quantities of TF and of mAbs fragments. The recovery of mAb vs. hIgG reached 97%, purity was 95%. The yield of mAb vs. hIgG and its purification factor are presented in Table 3. With salt concentration in the elution buffer increasing up to 0.05 *M* native mouse IgG along with TF is desorbed in the second peak. The displacement of Fc fragments of mAb takes place in the third peak at 0.1–0.2 *M* salt concentration in the eluent. Mouse SA with an admixture of Fc fragments is displaced from the carrier at 0.3 *M* salt concentration in the eluent.

The results of elution are somewhat different if the protein mixture is rechromatographed after a preliminary separation of the peak of mouse SA. As can be seen in Fig. 2, mAb vs. hIgG are eluted in the first two peaks. The first peak, which again is eluted with buffer A, probably contained conformationally-altered or associated mAbs molecules. In the second peak, structurally unaltered mAbs molecules were eluted at a higher salt concentration in the eluent (0.025–0.05 *M*). The recovery mAb vs. hIgG amounted to 97%, purity was 95%. Native mouse IgG and TF (third peak) were eluted at a salt concentration in the elution buffer of 0.1–0.2 *M*. The fourth peak contained a small quantity of mouse SA

Table 3
Purification parameters of separation of mAb vs. hIgG

Purification step	Protein loaded (mg)	Protein yield (%)	mAb loaded (mg)	mAb recovery (%)	Purification factor
Ammonium sulfate precipitation	ND	ND	12.0	100	ND
Desalination	87.6	73.5	12.0	99.6	1.36
Anion-exchange chromatography	64.4	82.9	11.9	96.9	5.41

ND=not determined.

with native mouse IgG and was eluted from DEAE-cellulose also at higher (0.3 *M*) salt concentration in the eluent.

In the case of chromatography of the desalted solution of protein fraction of mouse ascites fluid containing mAb vs. HRP (Figs. 3 and 4), the separation of native mouse IgG, TF and mAb becomes a serious problem due to the closeness of their p*I* values. As shown in Fig. 3, native mouse IgG and TF begin to desorb at 0.025 *M* salt concentration in the eluent. The rear front of their zone overlaps with the leading front of the zone of mAb, which desorb at 0.05–0.1 *M* salt concentration in the eluent. The recovery of mAb reaches 85–90%, purity 75–80%. The yield of mAb vs. HRP and its purification factor are presented in Table 4. Fragments of mAb were displaced from DEAE-cellulose with a buffer solution of 0.2 *M* salt concentration. Mouse SA was desorbed with eluent having a salt concentration above 0.3 *M*. An attempt to apply the

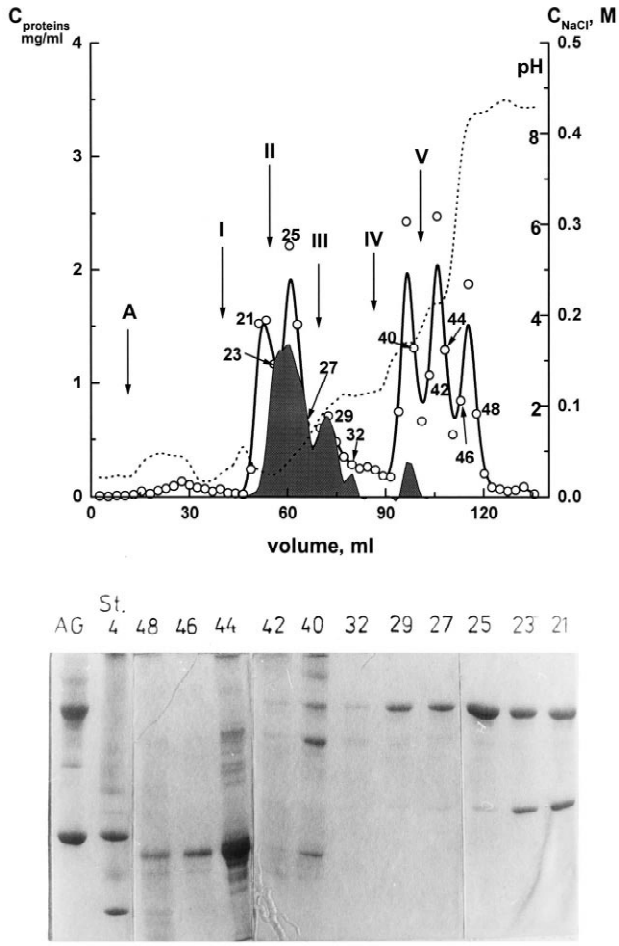

Fig. 3. (upper panel) Ion-exchange chromatography of desalted solution of protein fraction of mouse ascites fluid containing mAb vs. HRP on DEAE-cellulose DE-52. Column (13×1.2 cm I.D.) was equilibrated with starting 20 m*M* Tris–HCl buffer (pH 7.8) (A). Volume of desalted protein solution 15.2 ml (pH 5.87). Elution under the stepwise concentration gradient of NaCl in the starting buffer A: I 0.025; II 0.05; III 0.1; IV 0.2; V 0.4 *M*. Flow-rate 0.3 ml min^{-1}. Dotted line indicates the salt concentration in eluate fractions. Shaded area of the peak indicates mAb vs. HRP. The numbers indicate the eluate fractions analysed by SDS-PAGE. (lower panel) SDS-PAGE of eluate fractions: 21, 23: first peak; 25–27: second peak; 29, 32: third peak; 40, 42: fourth peak; 44, 46: fifth peak; 48: sixth peak; AG: standard mixture of human serum albumin and γ-globulin; St.4: standard Serva protein test 4.

Table 4
Purification parameters of separation of mAbs vs. HRP

Purification step	Protein loaded (mg)	Protein yield (%)	mAb loaded (mg)	mAb recovery (%)	Purification factor
Ammonium sulfate precipitation	ND	ND	19.76	100	ND
Desalination	104.8	91.0	19.76	99.2	1.09
Anion-exchange chromatography	95.4	79.8	19.6	89.6	4.0

concave salt concentration gradient in the elution buffer did not result in the expansion of zones of native mouse IgG, TF and mAb vs. HRP (Fig. 4, upper panel). In this case, all three components were eluted in a single narrow peak (Fig. 4, lower panel).

A similarly difficult task is the separation on

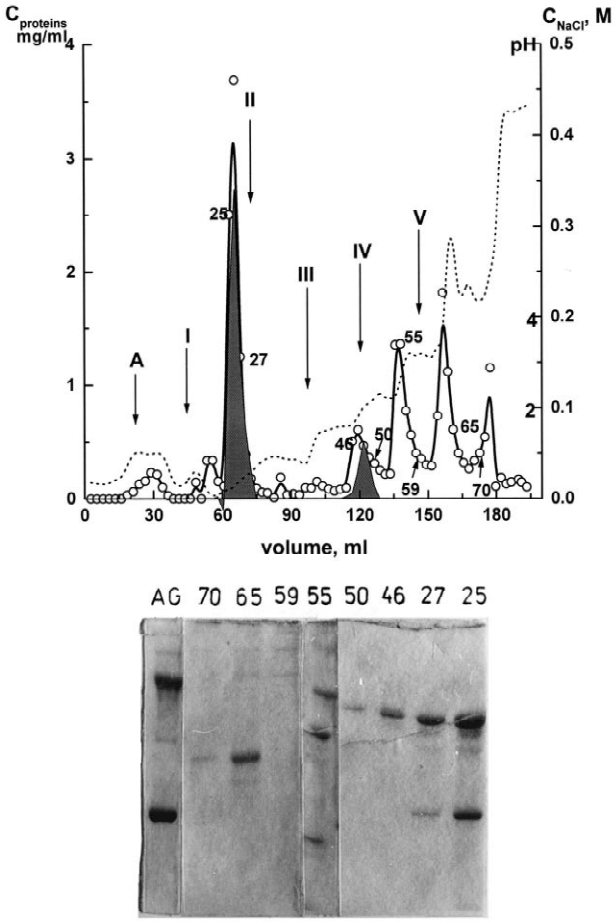

Fig. 4. (upper panel) Ion-exchange chromatography of desalted solution of protein fraction of mouse ascites fluid containing mAb vs. HRP on DEAE-cellulose DE-52. Column (13×1.2 cm) was equilibrated with starting 20 m*M* Tris–HCl buffer (pH 7.8) (A). Volume of desalted protein solution 18.4 ml (pH 5.92). Elution under the stepwise concentration gradient of NaCl in the starting buffer A: I 0.025; II 0.05; III 0.1; IV 0.2; V 0.4 *M*. Flow-rate 0.3 ml min^{-1}. Dotted line indicates the salt concentration in eluate fractions. Shaded area of the peak indicates mAb vs. HRP. The numbers indicate the eluate fractions analysed by SDS-PAGE. (lower panel) SDS-PAGE of eluate fractions: 25, 27: first peak; 46, 50: second peak; 55, 59: third peak; 65, 70: fourth peak; AG: standard mixture of human serum albumin and γ-globulin; St.4: standard Serva protein test 4.

DEAE-cellulose of components of the desalted solution of the protein fraction of mouse ascites fluid containing mAb vs. hIgM. Figs. 5 and 6 demonstrate the effect of the shape of the concave salt concentration gradient in the eluent on the separation of protein zones. A too-steep concave salt concentration gradient resulted in overlapping zones of native mouse IgG and TF eluted in the first peak with the zone of mAb; their fragments eluted in the second peak (Fig. 5). At a lower concave salt concentration gradient (Fig. 6), almost all native mouse IgG and

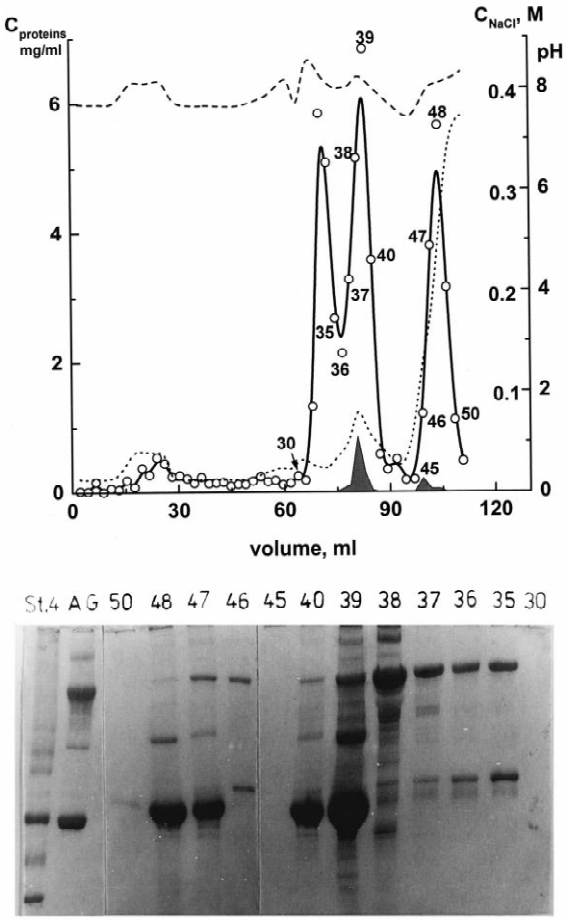

Fig. 5. (upper panel) Ion-exchange chromatography of desalted solution of protein fraction of mouse ascites fluid containing mAb vs. hIgM on DEAE-cellulose DE-52. Column (13×1.2 cm I.D.) was equilibrated with starting 20 m*M* Tris–HCl buffer (pH 7.8) (A). Volume of desalted protein solution 12.6 ml (pH 5.94). Elution under the concave concentration gradient of NaCl in the starting buffer A. Flow-rate 0.3 ml min^{-1}. Dashed and dotted lines indicate pH and salt concentration in eluate fractions, respectively. Shaded area of the peak indicates mAb vs. hIgM. The numbers indicate the eluate fractions analysed by SDS-PAGE. (lower panel) SDS-PAGE of eluate fractions: 30, 35, 36: first peak; 37–40: second peak; 46–50: third peak; AG: standard mixture of human serum albumin and γ-globulin; St.4: standard Serva protein test 4.

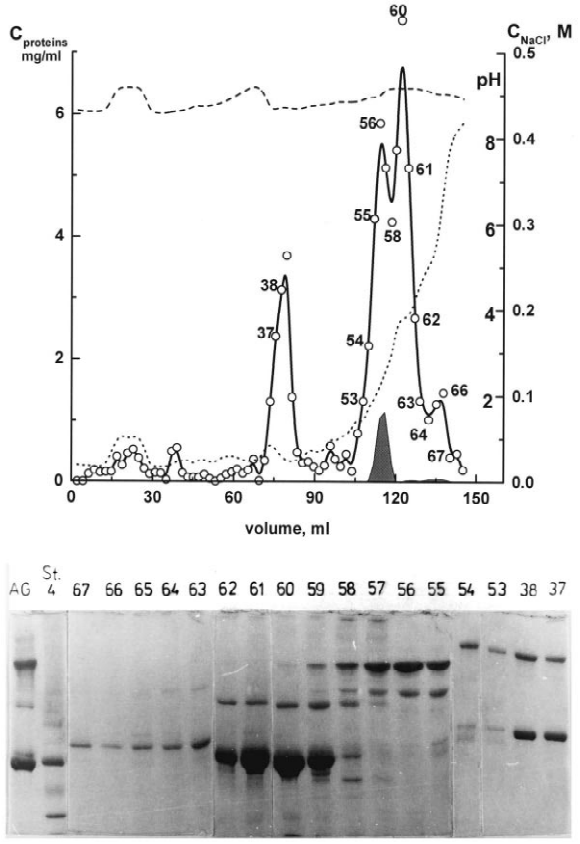

Fig. 6. (upper panel) Ion-exchange chromatography of desalted solution of protein fraction of mouse ascites fluid containing mAb vs. hIgM on DEAE-cellulose DE-52. Column (13×1.2 cm) was equilibrated with starting 20 m*M* Tris–HCl buffer (pH 7.8) (A). Volume of desalted protein solution 12.1 ml (pH 6.09). Elution under the concave concentration gradient of NaCl in the starting buffer A. Flow-rate 0.3 ml min^{-1}. Dashed and dotted lines indicate pH and salt concentration in eluate fractions, respectively. Shaded area of the peak indicates mAb vs. hIgM. The numbers indicate the eluate fractions analysed by SDS-PAGE. (lower panel) SDS-PAGE of eluate fractions: 37, 38: first peak; 53–58: second peak; 59–64: third peak; 65–67: fourth peak; St.4: standard Serva protein test 4; AG: standard mixture of human serum albumin and γ-globulin.

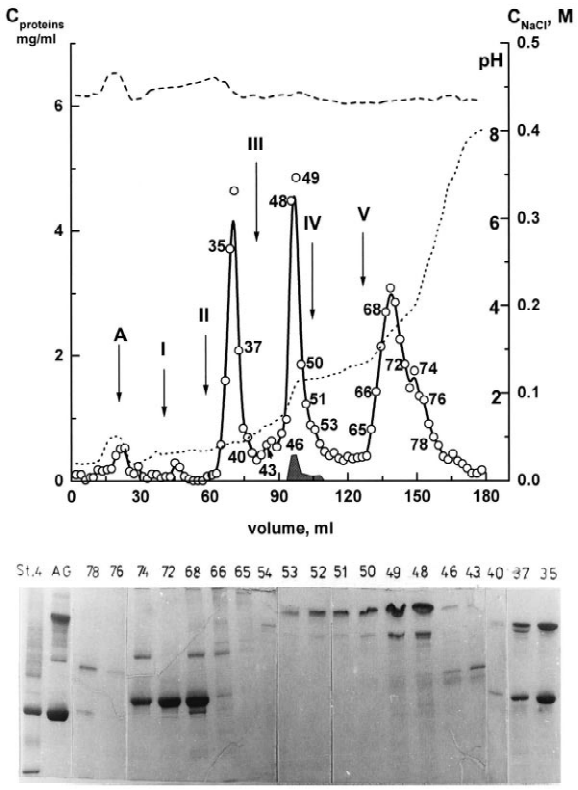

Fig. 7. (upper panel) Ion-exchange chromatography of desalted solution of protein fraction of mouse ascites fluid containing mAb vs. hIgM on DEAE-cellulose DE-52. Column (13×1.2 cm) was equilibrated with starting 20 m*M* Tris–HCl buffer (pH 7.8) (A). Volume of desalted protein solution 8.9 ml (pH 5.94). Elution under the stepwise concentration gradient of NaCl in the starting buffer A: I 0.025; II 0.05; III 0.1; IV 0.2; V 0.4 mol/l. Flow-rate 0.3 ml min^{-1}. Dashed and dotted lines indicate pH and salt concentration in eluate fractions, respectively. Shaded area of the peak indicates mAb vs. hIgM. The numbers indicate the eluate fractions analysed by SDS-PAGE. (lower panel) SDS-PAGE of eluate fractions: 35, 37, 40: first peak; 43, 46, 48–54: second peak; 65, 66, 68, 72, 74, 76, 78: third peak; AG: standard mixture of human serum albumin and γ-globulin; St.4: standard Serva protein test 4.

TF were replaced in the first peak, but the rear front of the zone of mAb overlapped with the leading front of the zone of mouse SA. Satisfactory separation of the protein zones was achieved using stepwise salt concentration gradient in the eluent (Fig. 7). In this case the first peak eluted at the salt concentration of 0.05 *M* contained native mouse IgG and TF, the second peak eluted at the salt concentration 0.1–0.2 *M* contained mAb with an admixture of their fragments, while in the third peak mouse SA with admixture of Fc fragments was eluted at the salt concentration 0.4 *M*. The yield of mAb vs. hIgM and its purification factor are presented in Table 5.

The experimental results obtained in this study confirm the assumed order of elution of the components of ascites fluids from DEAE-cellulose, based on the p*I* values of the proteins being separated. Sorption and elution of the protein proceeded using, as a background, 20 m*M* Tris–HCl buffer (pH 7.8). Under such conditions, according to the size of the negative charge and, consequently, strength of their binding on DEAE-cellulose, the proteins are arranged in the series:

Mouse SA>	mAb vs. hIgM>	Mouse IgG=TF> mAb vs. HRP>	mAb vs. hIgG
(0.4 *M*)	(0.1–0.2 *M*)	(0.05–0.1 *M*)	(0.025–0.05 *M*)

The salt concentration gradient in the elution buffer increased the purity of mAbs undergoing isolation. An analysis of purity of the eluate fractions investigated using SDS-PAGE and ELISA-titration showed that the largest expansion of zones of the components could be achieved with a stepwise and isocratic salt concentration gradient. At the same time, however, there was some overlapping of

Table 5
Purification parameters of separation of mAbs vs. hIgM

Purification step	Protein loaded (mg)	Protein yield (%)	mAb loaded (mg/ml)	mAb recovery (%)	Purification factor
Ammonium sulfate precipitation	ND	ND	4.16	100	ND
Desalination	122.4	99.0	4.16	99.5	1.01
Anion-exchange chromatography	121.2	68.7	4.14	70.0	2.5

protein zones with close p*I* values. Native mouse IgG and TF were eluted in a single peak independently of mAb specificity, being just a little ahead of the mAb vs. HRP zone. It seems clear that purification of the given mAb type requires highly-selective affinity supports. Contrary to this, 97% mAbs vs. hIgG with purity sufficient for diagnostic purposes can be isolated on DEAE-cellulose owing to the considerable difference between the p*I* values of the components of the protein mixture. In conclusion, the quality of separation of other mAbs and accompanying proteins will improve with increasing difference in their p*I* values.

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