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ISOTACHOPHORETIC ELECTRODESORPTION OF PROTEINS FROM AN AFFINITY ADSORBENT ON A MICROSCALE

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SUMMARY

Cationic isotachophoresis was used for the desorption of mouse monoclonal antibody to transferrin strongly affinity-bonded to transferrin immobilized on polyethyleneglycol terephthalate powder. The electrodesorption under nondestructive conditions was effected in the capillary isotachophoresis apparatus of our own construction which was equipped with an adsorption element. The electrodesorption is on-line connected with quantitative isotachophoretic analysis of the antibody desorbed. Only a few tens of microliters of the affinity adsorbent and several nanomoles of the antibody are needed for the characterization of the capacity of the affinity adsorbent and the conditions of adsorption and desorption.

INTRODUCTION

One of the key problems in affinity chromatography is the release of the bio-affinity-adsorbed, soluble, complementary component or ligate [1] from the bond to the affinity ligand immobilized on a solid support. In some cases of very strong complexes, such as, for example, antigen—antibody complexes, the ligate cannot be set free from the bond to the affinity ligand by changes in the composition of the solution, pH, ionic strength, temperature or by the addition of chaotropic reagents or detergents without damage or even complete loss of the biological activity of the protein ligate. The nondestructive release of the ligate by an excess of the low molecular weight ligands, if available at all, leads to undesirable contamination and often requires an additional separation step. If the ligand—ligate complex is in equilibrium with its components [2] and if the ligate is of an ionogenic character, the ligate can be liberated by electrophoretic desorption according to Dean et al. [3].

The electrophoretic desorption (electrodesorption), also called electrophoretic elution by Grenot and Cuilleron [4], can be effected by all the three

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basic electromigration methods, i.e. by electrophoresis in a homogeneous electrolyte, by isotachophoresis in a discontinuous system of two electrolytes and, with amphoteric ligates, also by isoelectric focusing in a pH gradient of mixed ampholytic electrolytes. As yet the desorption of protein ligates, especially of antibodies, has been mostly carried out by electrodesorption in a homogeneous electrolyte [2—11] which represents a special mode of affinity electrophoresis (the affinity adsorbent occupies only a part of the separation chamber). A disadvantage of electrodesorption in a homogeneous electrolyte is not only a slow release of the antibodies and of other ligates in a dilute state but also the elaborate detection or isolation of the ligate released.

The focusing electromigration methods of electrodesorption of proteins were employed in the form of isoelectric focusing by Vesterberg and Hansén [12] and by Haff et al. [13]. In such a case the protein ligate is not only to be exposed to an unfavourable effect of the pH of the medium during the focusing but also to the risk of precipitation after the quasistationary state of the isoelectric point has been achieved.

The self-focusing and concentrating effect of isotachophoresis enables us to use for electrodesorption a properly selected isotachophoretic system of electrolytes where the pH of both the leading and the terminating electrolyte does not exceed values at which the protein desorbed is stable and at the same time shows an intermediary mobility with respect to both the leading and the terminating ion of the system. In the process of desorption the ligate released migrates between the leading and the terminating ion thus forming a zone of a relatively high steady-state concentration [14, 15] which can, moreover, be regulated by the concentration of the leading ion. The pH of this zone is always different from the isoelectric point of the protein desorbed and the ionic strength is higher than in the case of isoelectric focusing; the risk of precipitation of the protein desorbed is generally lower compared to isoelectric focusing. This study was undertaken in an effort to develop the conditions of the use of capillary analytic isotachophoresis for the release of the ligate and its on-line analysis and conditions of the characterization of the properties of the affinity adsorbent on a microscale; the affinity pair antibody—antigen served as a model.

MATERIALS AND METHODS

Chemicals

Acetic acid, A.G., hydrochloric acid, A.G. (Normanal), boric acid, A.G., potassium hydroxide, A.G., and sodium azide pure were purchased from Lachema, Brno, Czechoslovakia. Sodium borohydride (98%) was made in the Institute of Inorganic Chemistry, Czechoslovak Academy of Sciences, Rež, Czechoslovakia. β-Alanine (BALA) puriss., and ε-aminocaproic acid (EACA) puriss., were from Koch-Light, Colnbrook, Great Britain. 2-(N-Morpholino)-ethanesulfonic acid (MES) A-grade, was purchased from Calbiochem, San Diego, CA, U.S.A. Polyvinyl alcohol (PVA) Mowiol was a product of Hoechst, Frankfurt, G.F.R. The solution of mouse monoclonal antibody to porcine transferrin (MMAbT), IgG1 subclass [16], in phosphate-buffered saline (PBS, Serva PM 16, Heidelberg, G.F.R.) with the addition of 0.1% sodium azide, and

polyethyleneglycol terephthalate microgranular carrier (Sorsilen) from the Insolmer kit (a product of the Institute of Sera and Vaccines, Prague, Czechoslovakia), coated with porcine transferrin immobilized by glutaraldehyde (Insol-transferrin) were kindly supplied by Dr. F. Franěk, Institute of Molecular Genetics, Czechoslovak Academy of Sciences, Prague, Czechoslovakia. A part of the Insol-transferrin was treated with sodium borohydride according to the method of Royer et al. [17].

Instrumentation

Electrodesorption was carried out in the apparatus of our construction, made in the workshops of the Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague. The core of the instrument is the analytical capillary isotachophoresis apparatus. The separation takes place in a PTFE capillary of 0.45 mm I.D. and 0.7 mm O.D., 230 mm long. The capillary is accommodated in a thermostated bath filled up with the Savant EC 123 coolant and temperature-controlled over the range 7—25°C by Peltier thermocouples. The apparatus is provided with two universal potential gradient detectors connected in series and with a photometric detector of ultraviolet absorption at 254 nm. The adsorption element containing the affinity adsorbent is placed between the six-way application PTFE valve and the block of the terminating electrolyte; the element is connected to the apparatus by a PTFE capillary of 1 mm I.D. and 1.4 mm O.D. of variable length. The arrangement can be seen in Figs. 1 and 2.

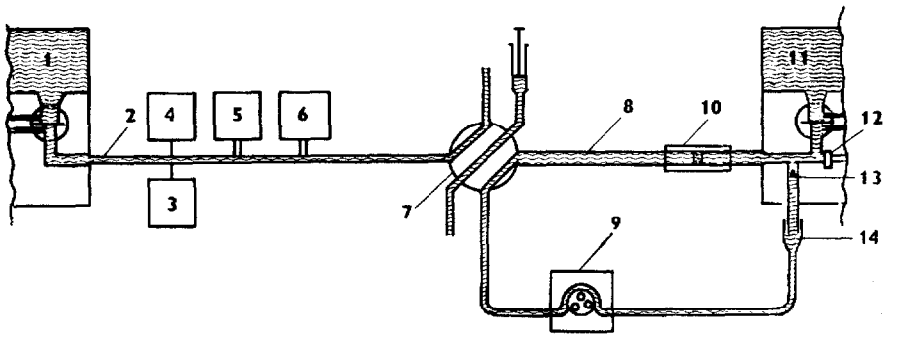

Fig. 1. Scheme of isotachophoretic apparatus for analytic desorption during sample application. 1 = Leading electrolyte compartment; 2 = PTFE capillary of 0.45 mm I.D.; 3 = UV source, 254 nm (low-pressure mercury lamp); 4 = photometric detector (photomultiplier); 5, 6 = potential gradient detectors; 7 = six-way valve for application of sample (the middle bore connected to a syringe containing the leading electrolyte); 8 = PTFE capillary of 1 mm I.D.; 9 = peristaltic micropump; 10 = adsorption element; 11 = terminating electrolyte compartment; 12 = PTFE-coated silicon rubber septum; 13 = outlet valve (open); 14 = solution reservoir.

The driving current is supplied by a high-voltage source of current stabilized over the range of 2—500 μA and 0.2—30 kV, manufactured in the Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague. The source is equipped with a low-voltage outlet and with a recorder of output voltage and current.

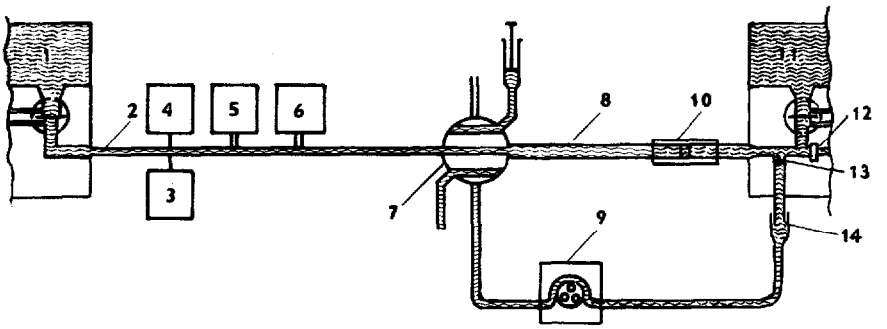

Fig. 2. Scheme of isotachophoretic apparatus for analytical desorption during separation; the designation of the functional elements is the same as in Fig. 1. The leading electrolyte compartment 1 represents the cathodic part of the system under conditions of cationic isotachophoretic separation; the peristaltic pump circuit 9 is disconnected and the outlet valve 13 is closed during isotachophoresis.

Adsorption elements

Four different models of adsorption elements shown in Fig. 3A—D were manufactured. The simplest element A represents a segment of a PTFE capillary of 1 mm I.D. and 1.4 mm O.D. with a porous polypropylene disc (Vyon, Porvair Ltd., Norfolk, Great Britain), 1 mm thick. Th suspension of the affinity adsorbent is placed on the disc, allowed to sediment, and subsequently the capillary is closed up also at the other side by another porous polypropylene disc. In the apparatus the extending terminal parts of the PTFE capillary are inserted into two PTFE connectors (not shown in the figure) and the position of the element is thus fixed.

Element B (Fig. 3B), more suitable for larger adsorbent volumes, is composed of a polyethylene tube of 3 mm I.D. and 5 mm O.D., provided with a porous polypropylene disc; the suspension of the adsorbent is placed on the latter, allowed to sediment and another disc is then used as a partition. The outside space beyond the disc is closed up by conically tapered tips limiting the effect of electric shading. If the particle size of the microgranular adsorbent is too small and the adsorbent particles are likely to penetrate through the porous discs, the adsorption element (model C, Fig. 3C) is covered with a microporous membrane of cellulose acetate (such as, for example, Membranfolien zur Elektrophorese, Membranfiltergesellschaft GmbH, Göttingen, G.F.R.), fixed in place by another polypropylene disc. The membrane will allow for the flow during the adsorption, but will not, however, let through the fine adsorbent of particle size around 3 μm. If it is necessary to change the volume of the affinity adsorbent, model D (cf. Fig. 3D) with removable PTFE tips is used to advantage. These tips serve as grooves for polypropylene porous discs. The disc diameter is in this case 1 mm smaller than the I.D. of the polyethylene tube. Table I shows the characteristics of the individual variants of the adsorption elements.

Procedure

The procedure of adsorption, electrodesorption, and isotachophoretic

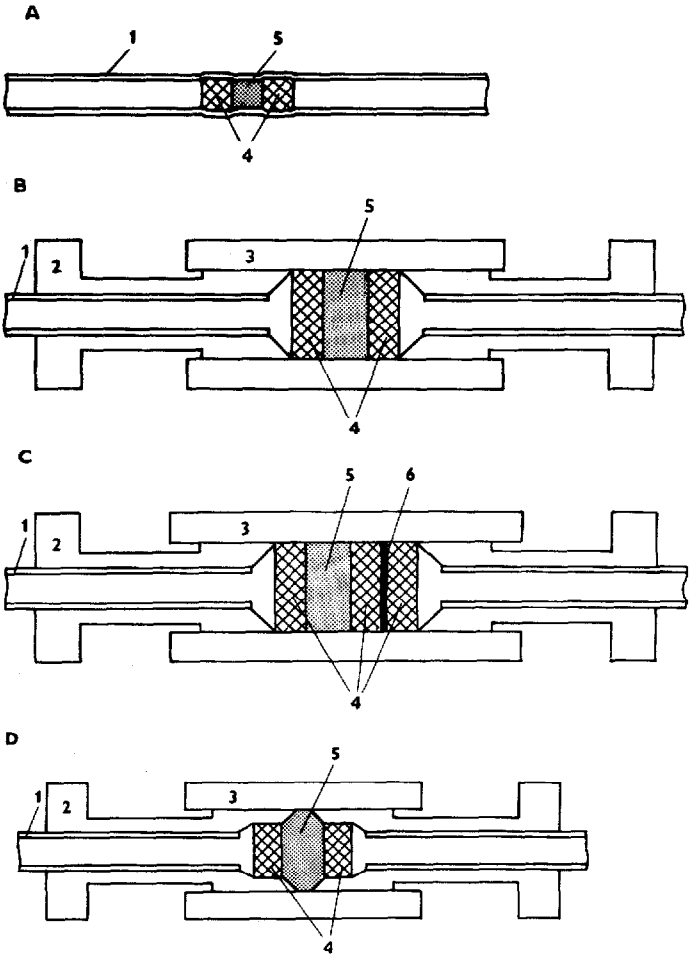

Fig. 3A—D. Scheme of adsorption element, models A—D: 1 = PTFE capillary of 1.0 mm I.D.; 2 = PTFE connector outlet; 3 = polyethylene tube of 3 mm I.D.; 4 = porous polypropylene disc; 5 = microgranular affinity adsorbent; 6 = cellulose acetate microporous membrane for electrophoresis (placed in the system closer to the outlet valve).

analysis of desorbed ligate consists of the following operations:

(1) Washing of affinity adsorbent in the adsorption element in the apparatus by the leading electrolyte (a volume of ca. 1 ml is used for model A whereas ca. 2 ml are necessary for models B, C, and D). Establishment of the boundary between the leading and the terminating electrolyte at the site of the outlet valve of the terminating electrolyte; closing of the valve.

(2) Blank electrodesorption—isotachophoretic analysis of the leading and terminating electrolyte with the adsorption element containing the void adsorbent to check on the possible leakage of the ligand from the adsorbent in the electric field.

(3) Filling. The application valve is turned to the filling position (cf. Fig. 1), the outlet valve is opened and the solution of the ligate is pumped by a peristaltic pump through the application valve to the adsorption element and back to the pump.

TABLE I

CHARACTERISTICS OF ADSORPTION ELEMENTS

The alphabetic designation corresponds to the models shown in Fig. 3A—D.

Type	Size of porous polypropylene discs (mm)		Volume of sorbent (mm^3)	Width of sorbent layer	Electrical screen
	Diameter	Distance			
A	1.0	1.0	0.8	Constant	None
		1.5	1.2		
		2.0	1.6		
B, C	3.0	1.0	7.1	Constant	Small
		1.5	10.6		
		2.0	14.1		
D	2.0	1.0	6.1	Variable	Medium
		1.5	9.6		
		2.0	13.2		

(4) The application stopcock is turned to "separation", the adsorption element is washed by the leading electrolyte and the boundary between the leading and the terminating electrolyte is established at the site of the outlet valve.

(5) Connection of the apparatus to the current source and electrodesorption

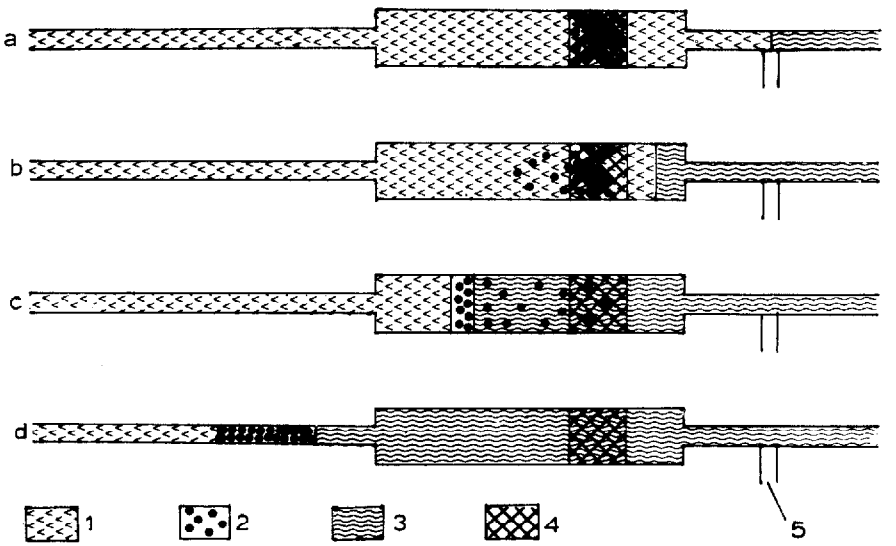

Fig. 4. Scheme of electrodesorption under conditions of isotachophoresis: a = before the start of isotachophoretic electrodesorption; b = initial stage — electrodesorption analogous to electrodesorption in a homogeneous electrolyte; c = formation of zone of desorbed ligate after passage of the terminating electrolyte boundary through the adsorption element; d = final stage — the ligate desorbed migrates in steady-state through a thin capillary to the detection system. 1 = Leading electrolyte; 2 = ligate; 3 = terminating electrolyte; 4 = adsorption element; 5 = outlet valve (closed).

of ligate under isotachophoretic conditions. The individual stages of the desorption are shown in Fig. 4. The course of the desorption was checked by recording the voltage of the stabilized current source (cf. Fig. 5).

(6) Isotachophoretic analysis of desorbed antibody; record of the passage of the individual zone boundaries through the detection system at decreased values of current passing through the apparatus.

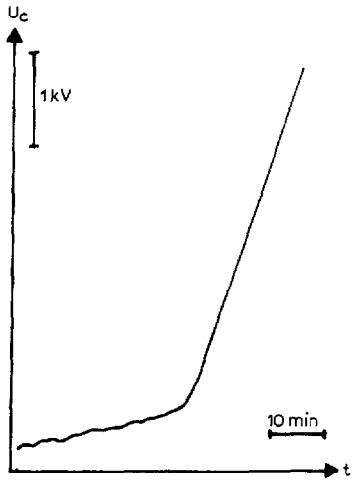

Fig. 5. Time profile of voltage of the high-voltage source of stabilized current. Inflection of the slope of the recorded curve indicates the passage of the boundary of terminating electrolyte or ligate from the 1-mm capillary to the application stopcock.

RESULTS AND DISCUSSION

The capillary isotachophoretic analysis of the MMAbT, carried out in electrolyte system I (see Table II), is documented by the record in Fig. 6 showing additional experimental data. Fig. 7 documents the result of isotachophoretic analysis of the desorbed MMAbT by electrodesorption in electrolyte system I. The quantity of the desorbed antibody, i.e. the ratio of MMAbT

TABLE II

COMPOSITION OF ELECTROLYTE SYSTEMS I AND II FOR ISOTACHOPHORETIC DESORPTION OF MMAbT FROM INSOL-TRANSFERRIN

BALA = β-alanine, EACA = ε-aminocaproic acid, MES = 2-(N-morpholino)ethanesulfonic acid, PVA = polyvinyl alcohol (Mowiol).

System number	Leading electrolyte				Terminating electrolyte		
	Leading ion (mol l^{-1})	Counterion	pH	Additive	Terminating constitutent (mol l^{-1})	Counterion	pH
I	K^+ 0.01	CH_3COO^-	5.2	PVA 0.02%	BALA 0.01	CH_3COO^-	5.0
II	K^+ 0.01	MES^-	5.9	—	EACA 0.01	Cl^-	5.2

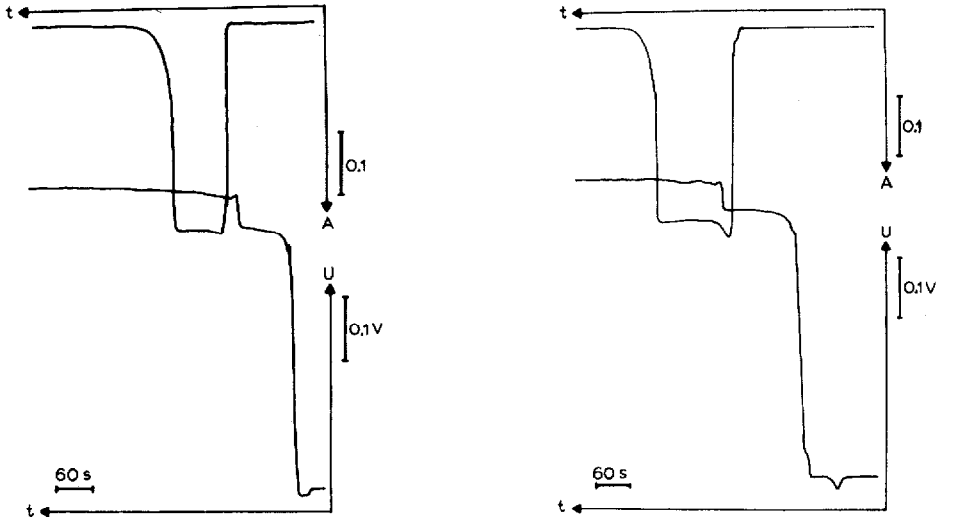

Fig. 6. Control analysis of ligate (isotachophoretic standard of MMAbT) in the absence of the adsorption element in electrolyte system I (see Table II). Sample, 5 μl of 1% solution of MMAbT in phosphate-buffered saline containing 0.1% sodium azide applied via a septum; current intensity 50 μA, during detection 20 μA; temperature 20°C.

Fig. 7. Isotachophoretic analysis of on-line desorbed fraction of MMAbT with binding affinity in electrolyte system I. Sample: 20 μl of 1% MMAbT in phosphate-buffered saline containing 0.1% sodium azide, made up to 1 ml with the leading electrolyte of system I, adsorbed at pump flow-rate of 2 ml h^{-1} for 2700 sec; adsorption element B; volume of affinity adsorbent 10 μl; length of 1-mm capillary 100 mm; time of electrodesorption 1 h at current intensity of 50 μA; detection at current intensity of 20 μA; capillary temperature in thermostated bath 20°C.

showing binding affinity for immobilized transferrin, was determined as follows. The length of the zone of a known quantity of antibody analyzed in the absence of the adsorption element (cf. Fig. 6) was compared with the length of the zone of the desorbed antibody (see Fig. 7), shorter by 10% because of decreased rate of zone migration due to changes in the electro-osmotic flow through the capillary in the presence of the adsorption element. The migration rate of the zone was determined as the ratio of the known distance between the potential gradient detector and the UV detector (15 mm in our apparatus) and the time during which the front boundary of the zone traveled over this distance at a current of 20 μA.

The quantity of the antibody desorbed was determined in this manner in two electrolyte systems (see Table III), in the B, C, and D adsorption elements (see Table IV), and also at various flow-rates during adsorption in element B (Table V).

The quantity of the unadsorbed fraction MMAbT in the sample solution after its passage through the adsorption element was determined colorimetrically by the method of Lowry et al. [18] and was a few per cent lower than the quantity of unadsorbed MMAbT determined as the difference between the quantity of MMAbT applied and desorbed. The low result can be accounted for both by the experimental error of the colorimetric determination of the un-

TABLE III

QUANTITY OF MMAbT WITH BINDING ACTIVITY IN ELECTROLYTE SYSTEMS I AND II

Conditions: pump flow-rate 2 ml h^{-1} during adsorption; time of circulation of 1-ml sample 2700 sec; for composition of sample see legend to Fig. 7; adsorption element B.

Electrolyte system	Applied amount of protein (μg MMAbT)	Desorbed amount of protein (μg MMAbT)
I	200	64—70
II	200	60—64

TABLE IV

QUANTITY OF DESORBED MMAbT IN VARIOUS MODELS OF ADSORPTION ELEMENTS IN ELECTROLYTE SYSTEM I

Conditions: flow-rate 2 ml h^{-1} during adsorption; adsorbent volume 10 ± 0.5 mm^3; adsorption time 2700 sec; for composition of sample see legend to Fig. 7.

Adsorption element type	Applied amount of protein (μg MMAbT)	Desorbed amount of protein (μg MMAbT)
B	200	64—70
C	200	66—68
D	200	62—64

TABLE V

QUANTITY OF DESORBED PROTEIN OF MMAbT (FRACTION WITH BINDING ACTIVITY) AS A FUNCTION OF FLOW-RATE UNDER CONDITIONS OF ADSORPTION IN ELECTROLYTE SYSTEM I

Adsorption element B; for composition of sample see legend to Fig. 7.

Peristaltic pump volumetric flow (ml h^{-1})	Adsorption element linear flow (μl mm^{-2})	Adsorption time (sec)	Applied amount of protein (μg MMAbT)	Desorbed amount of protein (μg MMAbT)
2	278	2700	200	70.0
8	1110	675	200	65.0
16	2220	338	200	27.6

adsorbed fraction of MMAbT and by irreversible adsorption which can never be eliminated completely.

The capacity of Insol-transferrin (reduced by $NaBH_4$) was determined as the quantity of antibody desorbed from a fully saturated adsorption element and represents 8.0 μg of MMAbT per μl of adsorbent.

The electrodesorption of MMAbT from Insol-transferrin in the isotachophoretic system of buffers, followed by isotachophoretic analysis of desorbed MMAbT, showed that the method of isotachophoretic desorption can be used for the desorption of ligates forming strong complexes with immobilized affinity ligands. Compared to the remaining electromigration methods which have been used for electrodesorption there are numerous advantages to the isotachophoretic procedure.

The experimentally determined steady-state concentration of electrophoretically desorbed MMAbT (3.2% in electrolyte system I and 4% w/v in electrolyte system II) is in good agreement with our earlier data [14] on the average steady-state concentration of polyclonal porcine immunoglobulin G and is, for example, ten times higher than the concentration of the sex hormone binding protein [10] obtained by electrodesorption in a homogeneous electrolyte. The concentration factor was 160 or 200 in our case if the original MMAbT concentration was 0.02%. Far more concentrated protein solutions can be obtained by isotachophoresis provided that there are no solubility problems; this factor is important from the viewpoint of future applications of the method. Another fact advantageous as regards these applications is that the antibody desorbed contains the counterion of the leading electrolyte only and there is no contamination with the carrier electrolyte or mixed ampholytes, a contamination which is observed when the electrodesorption is carried out by electrophoresis in a homogeneous electrolyte or by isoelectric focusing.

The conditions of isotachophoretic desorption (pH, ionic strength) must be chosen with respect to the stability of ligate and, if necessary, of ligand, and with respect to the solubility of the ligate. The deviation in pH of the terminating electrolyte zone in steady-state from the pH of the leading electrolyte can be calculated [19] and from these calculations the composition of the electrolyte system adjusted so that the values critical both from the viewpoint of ligate solubility and from the viewpoint of ligate and/or ligand stability may not be exceeded. The pH of the zone of desorbed ligate lies within the interval represented by the pH value of the leading electrolyte and the pH zone of the terminating electrolyte in steady-state. The calculated steady-state pH value of the leading electrolyte zone was 4.0 in system I and 5.0 in system II. System II [20] is more convenient as regards the stability of MMAbT whereas electrolyte system I is more advantageous from the viewpoint of the desorption rate. The electrodesorption process is most effective if the probability of the resorption of ligate, resulting from the decomposition of the complex, has been limited to a minimum. The probability of resorption can be decreased by decreasing the path of ligate ions in the adsorbent layer [6] and by increasing the migration rate of ligate ions in this layer [2]. The reduction of the path can be effected by a geometric arrangement of the adsorbent layer; a larger diameter and a thinner layer are more convenient. The increase of migration rate can be achieved by a proper choice of pH and ionic strength of the leading electrolyte and of the intensity of the electric field. As a rule, in common practice it is necessary to compromise and to choose the current density and the adsorbent cross-section to prevent thermal denaturation of ligate and/or ligand since the removal of Joule's heat is less efficient if the

cross-section is larger. Joule's heat was the critical factor when adsorption element A was used and the current density was 64 μA mm^{-2} : because of the small diameter of the adsorbent layer the thickness of the adsorbent had to be expanded to 10 mm in order that the properties of the individual adsorption element models could be compared. The isotachophoretic concentration of the antibody desorbed took place in the adsorbent layer. Hence, the specific conductivity in the adsorbent layer significantly decreased and thermal denaturation of the antibody occurred.

Our arrangement of the apparatus where the adsorption element of models B, C and D is connected in series to a capillary of 0.45 mm I.D. and 0.70 mm O.D. was used with relatively low current densities in the adsorbent layer, i.e. at 7 μA mm^{-2}. The maximal current densities in the adsorbent layer used by other authors are 100—250 μA mm^{-2} in adsorption elements without cooling and up to 1060 μA mm^{-2} in a cooled adsorption element [4]; the intensity of the electric field in this element did not exceed the value of 1.7 V mm^{-1}, whereas in our arrangement [21] these values go up to 5 V mm^{-1} similarly to the experiments of Haff [2] who showed that the intensity of the electric field is the decisive factor from the viewpoint of the efficiency of electrodesorption.

The time necessary for the desorption of MMAbT including its isotachophoretic analysis is 70—105 min. This period was controlled by the length of the capillary of 1 mm I.D. (see Figs. 1 and 2). The decrease in the desorption time results in a decrease in the quantity of desorbed ligate. More than 95% of MMAbT was liberated in 90 min.

The dependence of the quantity of MMAbT desorbed on the flow-rate of the sample solution during the adsorption (Table V) shows that the transferrin—MMAbT complex is kinetically very labile since a four-fold increase in the relatively high linear flow-rate resulted in a decrease of complex formation by only 7%.

If the quantity of the antibody is determined, it should be kept in mind that the size of the electroosmotic flow in the presence of the adsorption element is other than if this analysis is carried out in the absence of the adsorption element. The rate of zone migration is thus also different and the same real zone length may manifest itself in a different manner in the isotachopherogram. The rate of zone migration was in the individual cases 10—30% lower than the rate in the absence of the adsorption element. The zone length under the conditions of electrodesorption must therefore be corrected for zone length corresponding to the rate of zone migration in the absence of the adsorption element. If l_n is the uncorrected zone length, v_d the rate of zone migration under the conditions of electrodesorption, and v_a the rate of zone migration in the absence of the adsorption element, then the corrected zone length l_c can be expressed by the relation

$$l_c = \frac{v_d}{v_a} \cdot l_n$$

We used isotachophoretic desorption to check the quality of the Insol-transferrin adsorbent, i.e. its binding activity in the sense of its complex for-

mation with MMAbT. The determined capacity of this affinity adsorbent (8 $\mu g/\mu l$) is merely 2—3 times lower than the capacity of affinity adsorbent gels (mostly Sepharose derivatives) used by other authors for electrodesorption [2, 7]. The relatively high capacity of Insol-transferrin can be explained both by the high unit surface [22] of the polyethyleneglycol terephthalate carrier of the ligand [80—100 $m^2\ g^{-1}$) and also by the fact that the entire surface of the carrier particles is coated by the cross-linked antigen which, however, shows binding activity. The pellicular character of Insol-transferrin obviously contributes to the enhancement of the electrodesorption process since the electrodesorption rate is not limited by the ligate diffusion from the interior of adsorbent particles.

It appears that our on-line arrangement of qualitative isotachophoretic analysis may yield data on the specificity of sorbents of similar type and on the homogeneity of the ligate desorbed in the near future.

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