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Ion- and ligand-exchange chromatography of proteins using porous zirconium oxide supports in organic and inorganic Lewis base eluents

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ABSTRACT

The applicability of an eluotropic scale pertaining to the desorption of low molecular weight Lewis base solutes from zirconium oxide is examined for its ability to rationalize the retention of proteins on this substrate. The strongest Lewis base eluents (phosphate and fluoride) are able to bring about elution of nearly all proteins provided that their initial mobile phase concentration almost saturates the eluent's adsorption isotherm. In contrast, weaker Lewis bases such as borate, sulfate and bromide are able to elute only those proteins which are retained primarily by ionic interactions. In weak eluents, proteins that contain a large number of accessible Lewis base sites are not eluted from the support. The effect of ionic strength and a variety of Lewis base eluents were also examined.

INTRODUCTION

Early attempts in this laboratory to separate proteins on zirconium oxide based supports were unsuccessful [1]. Conventional protein ion-exchange elution strategies, consistent with the anticipated amphoteric ion-exchange nature of zirconia, failed to effectively elute many proteins. Elution of a few robust proteins was achieved by use of very high pH values. The resulting peaks were poorly shaped and the proteins were only marginally resolved despite large differences in their isoelectric points and sizes.

Subsequent work showed that proteins could be eluted with good selectivity and efficiency from "phosphated" zirconia supports [1–4]. Treatment of the native metal oxide with boiling phosphoric acid formed a surface coating of zirconium phosphate. This material was found to be very biocompatible and gave very well shaped chromatographic peaks for proteins, but it acted only as a cation exchanger for proteins that have rather high isoelectric points. Such proteins were well retained and could be eluted with ionic strength gradients. By and large, acidic proteins were unretained.

The bio-incompatibility of native zirconia supports is due to Lewis base interactions between protein and exposed Lewis acid (zirconium ion) sites on the particle surface. We have shown that these sites strongly bind those solutes which have accessible Lewis base moieties [5–8]. The "irreversible" binding of many proteins to bare zirconia is a consequence of these acid–base interactions [6,7].

We have shown that fluoride interacts very strongly with these Lewis acid sites, and at an appropriate concentration it can prevent "irreversible" protein adsorption [6,7]. When the fluoride

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concentration in the eluent suffices to nearly saturate the support's Lewis base adsorption isotherm, the residual ligand-exchange interactions between the surface and a protein results in well shaped peaks. Moreover, the balance of cation-exchange and ligand-exchange character of the support formed by this adsorptive surface modification provides unique chromatographic selectivity for the separation of proteins. In many ways, the retention mechanism of proteins on Lewis base modified zirconia surfaces is analogous to that of calcium hydroxyapatite. That is, on hydroxyapatite ligand exchange occurs on exposed calcium sites, and cation exchange takes place on ionized surface hydroxyl and phosphate groups [9–11].

For many proteins, fluoride competes much too strongly and retention is low. The main problem in achieving retention of acidic proteins is the ease of saturation of the fluoride adsorption isotherm [5,7]. Relatively low levels of fluoride block the ligand-exchange interactions and displace acidic proteins [6,7]. As a result, there is not much leeway for modulating the strength of the competitive ligand-exchange interaction through adjustments of the fluoride concentration. This results in a nearly an "on/off" retention process where the protein is either strongly retained or not retained at all. Obviously, adjusting selectivity and resolution is extremely difficult under these circumstances.

We now know that the addition of a very wide variety of inorganic and organic Lewis bases to the eluent can attenuate the interactions of low-molecular-weight solute with the surface [8,12,13]. Carboxylate groups on proteins are the main sites for ligand exchange with Lewis acid sites on zirconia.

In the above studies, a number of competing bases were evaluated in terms of their ability to cause elution of a series of low-molecular-weight carboxylic acids. This led to an eluotropic strength scale for some 30 different Lewis bases [13]. This ranking should be useful in predicting the effect of eluent Lewis bases on the retention of other types of Lewis base solutes in the absence of other strong interactions with the support. The applicability of this eluotropic series to the elution of proteins is the subject of this investigation.

EXPERIMENTAL

Chemicals

N-Tris (hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS), 2-(N-morpholino)ethanesulfonic acid (MES) and all proteins were obtained from Sigma (St. Louis, MO, USA). Sodium acetate was from Aldrich (Milwaukee, WI, USA). Hydrochloric acid, acetic acid and sodium fluoride were from Mallinckrodt (St. Louis, MO, USA). Sodium sulfate was obtained from EM Science (Gibbstown, NJ, USA) and 50% sodium hydroxide solution was obtained from CMS Scientific (Houston, TX, USA). Particle pretreatment and solution fluoride measurements were as described in detail earlier [1–7]. All chemicals were reagent grade or better.

Chromatographic supports

The porous zirconium oxide spherules were provided by the Ceramic Technology Center of the 3M Company and were described earlier [1–8, 12–14]. The particles used in this investigation had a nominal diameter of 5.3 μm ± 1.3 μm, an average pore diameter of 308 Å by mercury porosimetry and an average BET surface area of 30.5 m^2/μ. They were suspended in isopropanol and packed at 4500 p.s.i. into 50 × 4.6 mm I.D. columns by the upward slurry method. Titanium screens (2 μm) were used instead of frits to minimize protein losses [15] and to minimize contamination of the column by solubilized metal ions. Whenever the buffer was changed, the column was regenerated by flushing it with approximately 50 ml of 0.1 *M* sodium hydroxide solution followed by 50 ml of freshly boiled (carbon dioxide free) deionized water. This treatment is essential since it removes all irreversibly bound solutes from the zirconia and reproducibly prepares the surface for equilibration in the next buffer [5,7]. The zirconia particles are not damaged at all by this treatment. Some columns of zirconia have been subjected to over fifty cycles of such washes with alkali without any damage to the column.

Chromatographic systems

Chromatographic studies were carried out with a Hewlett-Packard (Avondale, PA, USA) Model 1090M liquid chromatograph equipped with a DR5 ternary solvent delivery system and a diode array detector. This system was outfitted with a 50 × 4.6

mm I.D. pre-column filled with 10–20-μm zirconia particles. This pre-column was placed before the injection valve to remove any contaminants in the buffer. The optional expanded pH range kit as well as ultrahigh-molecular-weight polyethylene piston seals (UPC-10) obtained from Bal Seal Engineering (Santa Ana, CA, USA) were installed. Data were processed using a Hewlett-Packard 9000/Series 300 computer with ChemStation software.

RESULTS AND DISCUSSION

Lewis base gradients at low ionic strength

A variety of proteins were chosen to cover a wide range in molecular weights and isoelectric points. A list of these proteins and their relevant properties is given in Table I [16,17]. These proteins were separated using Lewis base gradients. The results are shown in Table II. Phosphate is known to be the strongest Lewis base of the four shown in Table II [7,13]. It is, therefore, not surprising that most proteins are unretained in this medium. Because adsorption of phosphate is so strong and phosphate desorbs so slowly from zirconia that the surface is not fully equilibrated with the mobile phase before the start of the next gradient, many of the surface Lewis acid sites are occupied even when the eluent phosphate concentration is only 10 mM. Although these sorbed phosphate ions strongly inhibit the interaction of proteins with Lewis acid sites, they do provide additional sites for cation exchange by virtue of their ionized hydroxyl groups [2–4, 9–11].

The contribution of cation exchange to the retention of proteins on zirconium oxide in phosphate media is shown by the rather high retention of the cationic proteins (see Table II). Cytochrome *c*, α-chymotrypsin, ribonuclease and lysozyme have very high capacity factors (k') despite the high

TABLE I

PHYSICAL PARAMETERS FOR SELECTED PROTEINS

Values are taken from refs. 16 and 17.

Protein	Abbrev.	Type	pI	Molecular weight
Albumin	BSA	Bovine serum	4.7, 4.9	65 400
Albumin	HSA	Human serum	4.6–5.3	69 000
Apotransferrin	ATRH	Human	5.9, 6.0	80 000
Catalase	CAT	Bovine liver	5.8	247 000
Cellulase	CELL	*Asp. niger*	3.9, 4.2, 4.5	52 000
α-Chymotrypsin	αCHY	Bovine pancreas	8.8	21 600
Concanavalin A	CONA	*Can ensiformis*	4.5–5.5	71 000
Cytochrome *c*	CYTC	Horse heart	9.0, 9.4	12 360
Glucose oxidase	GLOX	*Asp. niger*	4.2	186 000
β-Glucuronidase	βGLU	Bovine liver	5.1, 5.9	290 000
Hemoglobin	HEMO	Human blood	6.9–7.4	64 500
Hexokinase	HEXO	Baker's yeast	4.9, 5.3	102 000
Holotransferrin	HTRH	Human		80 000
Laccase	LACC	*Pyr. oryzae*		
β-Lactoglobulin	βLAC	Bovine milk	5.2, 5.3	35 000
Lectin	LEC	Wheat germ		36 000
Lipase	LIP	Porcine pancreas	5.2, 5.5	60 000
Lysozyme	LYS	Chicken egg white	11.0	14 306
Mucin	MUC	Porcine stomach		40 000
Myoglobin	MYO	Equine muscle	6.8, 7.3	17 500
Ovalbumin	OVA	Egg white	4.7	43 500
Pepsin	PEP	Porcine mucosa	2.2, 2.8	33 000
Phosphatase, alk.	ALKP	Chicken intestine	5.7	140 000
Ribonuclease A	RNA	Bovine pancreas	9.3	13 683
Ribonuclease B	RNB	Bovine pancreas	9.3	14 700
Trypsin inhibitor	TINH	Soybean	4.5	20 091

TABLE II

CAPACITY FACTORS FOR VARIOUS PROTEINS WITH LEWIS BASE GRADIENTS AT LOW IONIC STRENGTH

Linear gradient from 2% to 100% B in 30 min then back to 2% B in 15 min followed by a 15 min-equilibration period. Injections were 10 μl of a 10 mg/ml protein in buffer A solution. Detection was at 280 and 410 nm. − = Elution not observed. Buffer A = 20 m*M* MES at pH 6.1 or 20 m*M* TAPS at pH 8.4.
Gradient I: B = 0.5 *M* sodium phosphate in 20 m*M* MES (pH 6.1) or 20 m*M* TAPS (pH 8.4).
Gradient II: B = 0.5 *M* sodium fluoride in 20 m*M* MES (pH 6.1) or 20 m*M* TAPS (pH 8.4).
Gradient III: B = 0.5 *M* sodium sulfate in 20 m*M* MES (pH 6.1) or 20 m*M* TAPS (pH 8.4).
Gradient IV: B = 0.5 *M* sodium borate in 20 m*M* MES (pH 6.1) or 20 m*M* TAPS (pH 8.4).

Protein	*k'*							
	Gradient I		Gradient II		Gradient III		Gradient IV	
	pH 6.1	pH 8.4	pH 6.1	pH 8.4	pH 6.1	pH 8.4	pH 6.1	pH 8.4
Pepsin	−0.3	−0.3	−	−0.3	−	−	−	−0.3
Cellulase	−0.2	−0.2	−	−0.3	−	−	−	−0.3
Glucose oxidase	−0.3	−0.3	−	−0.3	−	−	−	−0.3
Trypsin inhibitor	−0.3	−0.2	0.2	5.8	−	−	−	4.3
Bovine albumin	−0.3	−0.3	−	4.1	−	−	−	−0.3
Human albumin	−0.3	−0.3	−	3.5	−	−	−	−0.3
Concanavalin A	−	−0.3	−	−	−	−	−	8.3
Hexokinase	0.3	−0.3	0.6	3.9	2.9	−	4.1	3.7
β-Lactoglobulin	−0.3	−0.3	−	−0.3	−	−	−	4.4
Lipase	0.3	−0.2	0.6	2.2	2.9	1.9	3.5	3.7
β-Glucuronidase	−0.1	−0.3	0.8	3.4	3.1	−	4.1	4.1
Phosphatase, alk.	−0.3	−0.3	0.2	−0.3	3.0	0.2	4.1	0.1
Catalase	−0.3	−0.3	−	4.5	−	−	−	−0.3
Apotransferrin	8.3	−0.3	25.5	−	−	−	−	4.7
Myoglobin	9.3	0.0	24.1	5.9	−	−	−	5.7
Hemoglobin	25.1	−0.3	−	3.8	−	−	−	3.8
α-Chymotrypsin	22.0	11.0	22.8	23.2	−	−	−	3.8
Cytochrome	−	22.3	−	−	−	−	−	−
Ribonuclease A	20.9	8.6	23.7	21.0	−	20.6	−	−
Ribonuclease B	14.2	5.7	13.1	13.2	−	20.7	−	−
Lysozyme	15.9	7.9	11.9	14.9	−	17.8	−	−
Holotransferrin	8.5	−0.3	−	−	−	−	−	4.5
Laccase	0.1	−0.1	0.1	−	−	−	−	−
Lectin	−0.2	−0.2	−	−	−	−	−	−
Mucin	−0.2	−0.2	0.6	3.9	3.2	3.0	4.0	4.3

eluent ionic strength at the point of elution (approximately 0.35 *M* phosphate). Ligand-exchange interactions between the protein and the surface Lewis acid sites are still possible, but they are attenuated at this high phosphate concentration. This, however, does not preclude changes in selectivity due to minor contributions from ligand-exchange interactions. Fig. 1 shows that closely related proteins can be well resolved using strong phosphate eluents. In this case, commercial ribonuclease preparations are well resolved into the A and B sub-types which differ only in their degree of glycosylation.

The elution behavior of the acidic proteins in fluoride media at pH 6.1 (see Table II) is quite different from that observed in our previous studies [6,7]. This is because the initial fluoride concentrations were very different. In the earlier studies [6,7], the initial fluoride concentration was about 20 m*M*. This is sufficient to nearly saturate the fluoride adsorption isotherm and provides very strong competition for the incoming proteins. The net effect was to make protein retention quite weak, since Lewis base solutes are readily displaced by fluoride [5–7, 13].

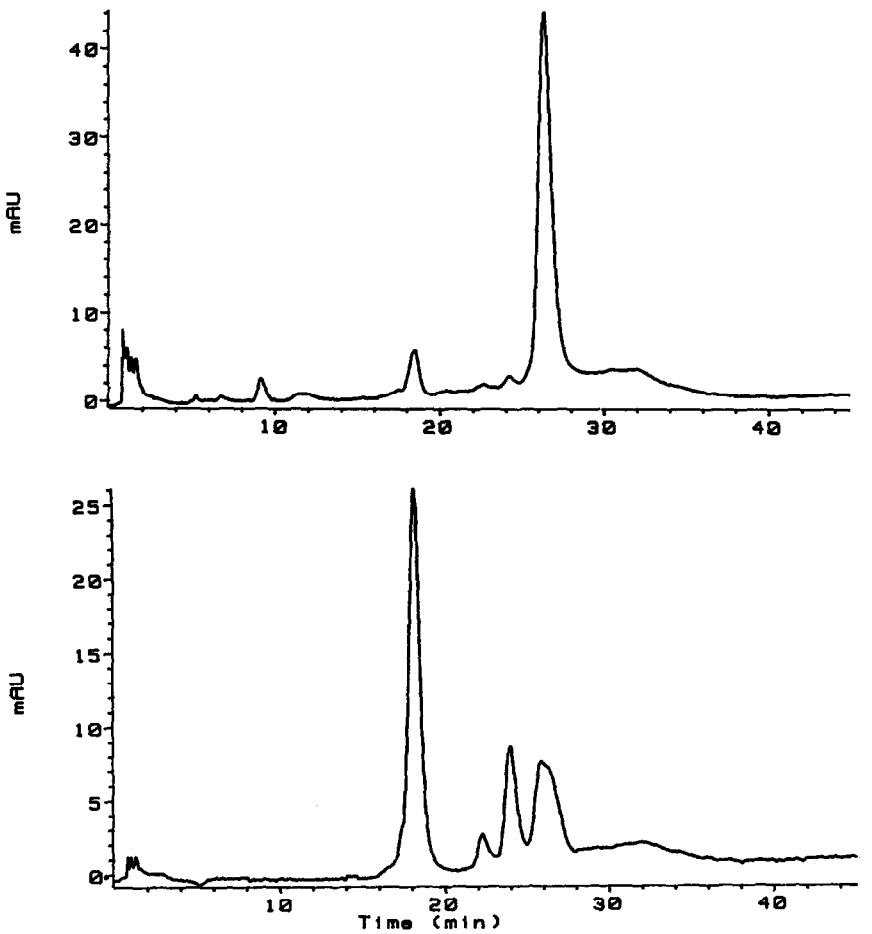

Fig. 1. Separation of commercial preparations of ribonuclease A (top) and ribonuclease B (bottom) from bovine pancreas. Linear gradient elution from 98% A (20 m*M* MES pH 6.1) to 100% B (0.5 *M* sodium phosphate, 20 m*M* MES pH 6.1) in 30 min, then back to 98% A in 15 min with a 15-min equilibration pertiod. Injections were 10-μl volumes of 10 mg/ml solutions in buffer A. Flow-rate was 0.5 ml/min at 35°C.

In the present experiment (Table II), the initial fluoride concentration was reduced to only 10 m*M*. This low concentration of fluoride relative to that used in prior work (20 m*M*) leaves a substantial number of Lewis acid sites available for interaction with Lewis base solutes. Thus proteins "stick" very strongly to the Lewis acid sites, and are not as readily displaced as when the an initially higher fluoride concentration is used. We believe that once one site on a polyvalent Lewis base is sorbed, additional Lewis sites on the same molecule then readily interact with unblocked Lewis acid sites on the support. One can view this as a local concentration effect or as an entropic effect related to the chelate effect. The result is that retention and elution are very strongly dependent on the number of available (unblocked) Lewis acid surface sites at the start of the gradient. Most likely, such multiply-coordinated solutes desorb rather slowly upon an increase in the fluoride concentration. This effect should be much more pronounced for acidic proteins and especially for those proteins containing proximal carboxylic acid groups capable of forming chelates with the surface.

We believe that the presence of proximal acidic amino acids in a protein plays an important role in determining the strength of the eluent needed to desorb a protein. Table III indicates the number and location of the acidic amino acids for some of

TABLE III

LOCATION OF ACIDIC AMINO ACID RESIDUES IN TEST PROTEINS

Taken from ref. 18.

Protein	Acidic residues
Cytochrome *c* (horse)	Asp(2), Glu(4), Glu(21), Asp(50), Glu(61), Glu(62), Glu(66), Glu(69), Glu(90), Glu(92), Asp(93), Glu(104)t[a]
Lysozyme (egg)	Glu(7), Asp(18), Glu(35), Asp(48), Asp(52), Asp(66), Asp(87), Asp(101), Asp(103), Asp(119)
Ribonuclease A (bovine)	Glu(2), Glu(9), Asp(14), Asp(38), Asp(53), Asp(83), Glu(86), Glu(111), Asp(121)

[a] t = Terminal residue

the proteins used in this study [18]. Many amino acids with ionized side chains are present on a protein's surface and may be available for interaction with a stationary phase. Lysozyme is retained on zirconium oxide in the presence of fluoride mainly by electrostatic interactions [6,7]. The amino acid content of lysozyme shows that 10 of its 129 amino acids are acidic [18]. However, most of these acidic residues are separated by other amino acids and offer little chance to form chelates on the zirconia surface. The aspartic acid residues at positions 101 and 103 may show some strengthening of the ligand exchange interaction due to their proximity, however, studies with small solutes have shown that the importance of chelation decreases rapidly as the ring size increases (*cf.* succunic acid *vs.* iminodiacetic acid [7,13]). As a result, lysozyme does not show any strong ligand exchange character.

Cytochrome *c* behaves quite differently. Table II shows that it is hard to elute cytochrome *c*, even when strong Lewis bases such as phosphate and fluoride are used. Its amino acid sequence suggests that this may be due to the presence of many (12 out of 104) proximal acidic amino acid residues. Ribonuclease A behaves similarly to lysozyme in that it shows little ligand-exchange character. This may be due to either a lower proportion of acidic amino acids (9/124) or the spatial separation of the acidic residues.

Weaker Lewis bases, such as sulfate and borate, are less effective in displacing proteins which are strongly retained by ligand exchange. This is especially true at eluent concentrations below the point of isotherm saturation. The result is "irreversible" binding of most proteins with no clear elution pattern (see solutes denoted as "−" in Table II).

When the same study was conducted at a higher pH, a few notable changes in the elution pattern were observed. These results are also shown in Table II. Most elution changes can be predicted based on the isoelectric points of the proteins. At higher pH values, the anion charge density on the stationary phase increases as the surface hydroxyl groups and bound phosphate groups become more ionized. This should increase retention for cationic proteins. However, this effect is offset by the increased competition by hydroxyl ions for the Lewis acid sites. The net result for a given protein is due to the relative contributions of cation and ligand-exchange to retention.

In phosphate media the retention of all proteins decreased upon increasing the pH. The same is true in sulfate media. However, in fluoride media the retention of hexokinase, lipase and mucin increased upon increasing the pH. In borate the retention of lipase and mucin increase, but only slightly, when the pH was raised. The decrease in retention is due to two factors. First, at higher pH the hydroxide concentration is greater. Hydroxide is the strongest known Lewis base towards zirconia and thus it is a very powerful displacing agent. Second, at higher pH the proteins are less positively charged and therefore are held less strongly by cation exchange. Clearly, pH is an important variable that can be used to elute proteins once the Lewis acid sites on the surface are moderated by use of a competing Lewis base in the eluent.

Lewis base gradients at high ionic strength

The study reported in Table II was repeated using a higher ionic strength to attenuate ion-exchange contributions to retention; the results are shown in Table IV. The ionic strength was increased by adding 0.5 *M* sodium chloride, since chloride is a very weak Lewis base towards zirconia [19]. The major trend observed in Table IV is that the retention of almost all proteins that were well retained (k' greater than 1) at low ionic strength (Table II) decreased

TABLE IV

CAPACITY FACTORS FOR VARIOUS PROTEINS WITH LEWIS BASE GRADIENTS AT HIGH IONIC STRENGTH

Conditions as in Table II except buffer A contained 0.5 *M* sodium chloride in addition to the MES or TAPS. – = Elution not observed.

Protein	*k'*							
	Gradient I		Gradient II		Gradient III		Gradient IV	
	pH 6.1	pH 8.4	pH 6.1	pH 8.4	pH 6.1	pH 8.4	pH 6.1	pH 8.4
Pepsin	0.0	−0.1	–	0.1	–	–	–	4.3
Cellulase	0.0	0.0	–	0.1	–	0.1	–	4.2
Glucose oxidase	0.0	0.0	–	–	–	–	–	–
Trypsin inhibitor	0.1	0.0	0.2	–	–	–	–	4.7
Bovine albumin	0.0	−0.1	–	–	–	–	–	4.5
Human albumin	0.0	−0.1	–	–	–	–	–	4.6
Concanavalin A	–	3.1	–	–	–	–	–	–
Hexokinase	0.1	0.0	0.2	2.5	2.4	0.1	1.9	4.1
β-Lactoglobulin	–	0.0	–	0.1	–	0.1	–	4.4
Lipase	0.1	0.1	0.2	1.3	2.5	0.7	1.7	4.0
β-Glucuronidase	0.1	0.0	–	0.1	2.6	0.1	3.1	4.5
Phosphatase, alk.	0.1	0.0	0.2	0.1	2.5	0.1	3.1	0.1
Catalase	0.1	0.0	–	0.1	–	0.1	–	4.9
Apotransferrin	0.8	0.0	–	22.2	–	–	–	4.7
Myoglobin	1.4	0.1	11.6	5.3	–	18.1	–	4.3
Hemoglobin	10.0	0.0	15.4	13.2	–	–	–	4.2
α-Chymotrypsin	3.2	1.3	12.4	6.9	25.6	18.0	–	6.4
Cytochrome *c*	35.1	11.5	13.9	–	–	–	–	–
Ribonuclease A	5.1	0.9	7.6	4.3	–	5.47	–	4.7
Ribonuclease B	1.4	0.4	0.9	4.6	–	5.2	–	4.5
Lysozyme	0.9	0.3	0.7	1.4	–	3.2	9.4	2.7
Holotransferrin	1.4	0.0	–	–	–	–	–	5.2
Laccase	0.0	0.0	0.1	–	–	–	–	–
Lectin	0.0	0.0	–	–	–	–	–	–
Mucin	0.0	0.0	60.2	2.5	3.0	0.1	3.5	4.9

upon increasing the ionic strength. This trend was followed by virtually all proteins at both pH values in phosphate media. There are a few significant exceptions most notably concanavalin A. For example, at pH 8.4 its *k'* is 3.1 at high ionic strength (Table IV) but drops to −0.3 at low ionic strength (Table II).

The changes in retention with ionic strength in fluoride buffer are similar to those in phosphate media except that almost all proteins are more retained in fluoride. Note that a few changes in the elution sequence do take place.

Not much was learned in sulfate and borate buffer because even upon increasing the ionic strength most of the proteins still did not elute. However, where proteins were eluted the changes in retention upon increasing the ionic strength were not as large as the effect in phosphate and fluoride media. Those proteins which do elute in these buffers show some decrease in retention suggesting that there are some ionic contributions to retention but that ligand exchange interactions are dominant.

Retention in borate media is clearly rather unusual. At low pH, only a few proteins elute at either low or high ionic strength. This indicates that the retention process is not ionic. "Irreversible" retention occurs for both acidic and basic proteins. Even more curious are the retentive properties at pH 8.4. Those proteins that are weakly retained at high pH and low ionic strength, such as pepsin through human albumin, actually show an increase in retention upon increasing ionic strength. This is very

strange behavior for an ion-exchange separation, but vaguely reminiscent of hydrophobic interaction chromatography. At low ionic strength, the borate system acts like a cation exchanger. This is consistent with studies which show that adsorption of borate reaches a maximum at a pH near borate's first pK_a (9.24) [20]. This results in a higher surface coverage by borate at pH 7.4 relative to pH 6.1. Borate is also more ionized at the higher pH, thereby generating a higher negative charge. Some acidic proteins are excluded, the others are retained by non-ligand-exchange interactions. Neutral proteins behave like the acidic proteins, but not all basic proteins are eluted under these conditions.

Upon decreasing the ionic interactions (Table IV), the residual retention of acidic, neutral and basic proteins appear to be similar. Most proteins are eluted at nearly the same borate buffer concentration. This effect may indicate non-specific adsorptive processes. Additional experiments in borate media are given below.

Retention of tightly bound proteins

A number of proteins are very well retained despite the use of very strong elution conditions (see Table V). In gradient I (see Table V for conditions) only a few proteins are retained. This may be due to any of three factors. First, the ionic strength is very high throughout the gradient (1.5 *M*). Second, the initial fluoride concentration (30 m*M*) virtually completely saturated the Lewis acid site adsorption isotherm, thereby strongly attenuating the ligand-exchange interactions. Finally, the starting conditions (0.5 *M* sulfate) also served to saturate the Lewis acid adsorption isotherm.

In gradient II, most proteins were retained. This

TABLE V

ELUTION OF TIGHTLY BOUND PROTEINS USING VARIOUS ELUTION STRATEGIES: CAPACITY FACTORS AT pH 6.1

Linear gradient elution at 0.5 ml/min and 35°C was used. Gradients were from 2% B to 100% B in 30 min then back to 2% B in 15 min followed by a 15-min equilibration period. Injections were 20 µl of 10 mg/ml solution of protein in 0.5 *M* K_2SO_4 20 m*M* MES pH 6.1. – = Elution not observed. Buffers were as follows:
Gradient I: A = 0.5 *M* K_2SO_4, 20 m*M* MES, pH 6.1; B = 1.5 *M* KF, 20 m*M* MES, pH 6.1.
Gradient II: A = 1.5 *M* KCl, 20 m*M* MES, pH 6.1; B = 1.5 *M* KF, 20 m*M* MES, pH 6.1.
Gradient III: A = 1.5 *M* KCl, 20 m*M* MES, pH 6.1; B = 0.5 *M* K_2SO_4, 20 m*M* MES, pH 6.1.
Gradient IV: A = 0.5 *M* K_2SO_4, 20 m*M* MES, pH 6.1; B = 50 m*M* KF, 0.5 *M* K_2SO_4, 20 m*M* MES, pH 6.1.

Protein	p*I*	Mol. wt.	*k'*			
			Gradient			
			I	II	III	IV
Albumins:						
ovalbumin	4.7	43 500	1.5	6.4	–	–
human serum	4.6–5.3	68 460	0.1	8.3	–	15.1
bovine serum	4.7, 4.9	65 400	0.1	6.4	–	16.2
bovine (maltosyl)			0.1	6.4	–	16.2
sheep serum			0.1	7.1	–	14.4
horse serum		68 600	0.1	8.0	–	15.7
pig serum			0.2	8.2	–	16.2
β-Lactoglobulin	5.1, 5.3	17 500	0.3	6.7	–	15.0
Hemoglobin	6.9–7.4	16 000	0.1	0.2	–	0.8
Alcohol dehydrogenase	8.7–9.3	41 000	0.2	6.1	–	7.4
Creatine phosphokinase	6.6–6.9	40 000	0.4	3.4	–	11.8
Transferrins:						
human, apo	5.9	80 000	0.0	1.8	–	6.9
human, holo			0.1	1.9	–	8.1
bovine, apo			0.1	6.3	–	14.1

clearly shows the effect of the higher Lewis basicity of sulfate compared to chloride since the same ionic strength was used in both gradients. Comparison of the retention data under gradients I and II demonstrates that, although the Lewis acid sites were nearly saturated by fluoride, the presence of sulfate obviously had a strong effect on retention. In gradient II most of the albumins had capacity factors of about 7, however, some selectivity between variants is evident. More dramatic differences in retention were observed with the apotransferrin variants. Human and bovine variants are well separated despite very minor differences in their amino acid compositions.

As shown by the data obtained in gradient III (which did not contain any fluoride) sulfate gradients cannot induce the tightly bound proteins to elute. The initial low concentration of sulfate (10 m*M*) does not saturate the adsorption isotherm and the lower binding constant of sulfate with zirconium ion, relative to fluoride [19], combine to preclude elution.

Gradient IV is a modification of the first gradient. These data demonstrate that only a small amount of fluoride is needed to bring about elution of the tightly bound proteins once ligand-exchange interactions have been attenuated by other species. The initial fluoride concentration was only 4 m*M*, which is far below the amount needed to saturate the adsorption isotherm. We conlude that sulfate must be responsible for some attenuation of the ligand-exchange interactions. Higher fluoride concentrations cover more Lewis acid sites. Most proteins elute when the concentration of fluoride is approximately 20 m*M* (isotherm saturation); since fluoride's formation constant with Zr(IV) is so much larger than the other competing bases it is by far the more important Lewis base. In gradient IV, as in gradient II, a fair degree of selectivity between variants was obtained for the albumins and the transferrins.

These results demonstrate some important operational aspects of controlling the ligand-exchange interactions between proteins and zirconia surfaces. As is also true with hydroxyapatite supports, adsorption and desorption do not correspond to the reversal of the same process [9–11]. Unless the strength of the Lewis acid sites are initially attenuated by addition of a competing ligand prior to loading the sample, these sites will cause very strong adsorption of proteins as described above. These observations are in good agreement with the hypothesis of Van Oss *et al.* [21] concerning protein binding processes. Adsorption, which is driven by long range interactions such as Coulombic and Van der Waals interactions, may be accentuated by other attractive interactions. Once long range interactions have drawn the attracting bodies into close proximity, short range interactions augment the attraction between these two bodies. In this work, the ligation of a single Lewis base to a Lewis acid site promotes further Lewis interactions. In addition, hydrogen bonding, which is normally masked in aqueous solutions, is facilitated by the close proximity of bonding groups. The result is that a very strong adsorptive interaction can take place once a single ligating bond is formed. Therefore, to overcome the deleterious effects of strong binding on chromatographic efficiency, the ligand-exchange interaction must be labilized as much as possible.

A final comment concerning the effect of the various displacing ligands on protein selectivity is in order. When only fluoride is used to desorb the tenaciously bound proteins, the elution pattern is different from when fluoride is used to displace proteins which are not tightly bound. In gradient II, bovine albumin was more retained than its maltosyl derivative. However, the change in retention mechanism in gradient IV allows the maltosyl derivative to be more retained than the underivatized albumin.

Retention of acidic proteins

A dozen acidic proteins were examined at pH 6.1 and 8.4 using linear Lewis base gradients under conditions of nearly constant ionic strength. Table VI shows the results using four Lewis base eluents ranging in strength from fluoride to acetate. Based on the acidic proteins tested here, fluoride is the strongest competitive ligand. All of the acidic proteins can be eluted at low pH, although some proteins are rather well retained. This high retention did not correlate with the protein's isoelectric point or molecular weight. Presumably, the high retention is due to the spatial arrangement of Lewis bases on the protein surface.

We also note that the pH effects are not simple. For some proteins, retention increased with pH

TABLE VI

ACIDIC PROTEIN RETENTION VERSUS LEWIS BASE STRENGTH: CAPACITY FACTORS

Linear gradient elution at 0.5 ml/min and 35°C from 2% to 100%B in 30 min followed by a return to 2% B in 15 min. A 15-min equilibration period followed each gradient run. Injections were 20 μl of 10 mg/ml solutions of protein in buffer A. Detection was at 280 and 410 nm. – = Elution not observed.
Gradient I: A: 1 *M* NaCl, 20 m*M* MES, pH 6.1; B: 1 *M* KF, 20 m*M* MES, pH 6.1.
Gradient II: A: 1 *M* NaCl, 20 m*M* MES, pH 6.1; B: 0.33 *M* Malic acid, 20 m*M* MES, pH 6.1.
Gradient III: A: 1 *M* NaCl, 20 m*M* MES, pH 6.1; B: 0.33 *M* Na_2SO_4, 20 m*M* MES, pH 6.1.
Gradient IV: A: 1 *M* NaCl, 20 m*M* MES, pH 6.1; B: 1 *M* Sodium acetate, 20 m*M* MES, pH 6.1.

Protein	*k'*							
	Gradient I		Gradient II		Gradient III		Gradient IV	
	pH 6.1	pH 8.4	pH 6.1	pH 8.4	pH 6.1	pH 8.4	pH 6.1	pH 8.4
Fetuin	4.0	–	–	–	–	–	–	–
Cellulase	0.2	–	0.3	0.1	1.8	3.7	2.4	3.5
Trypsin inhibitor	17.1	0.0	–	0.4	–	1.4	–	1.5
Ovalbumin	18.3	25.6	–	–	–	–	–	–
Bovine albumin	2.6	–	–	–	–	–	–	–
Human albumin	27.3	–	–	–	–	–	–	–
β-Lactoglobulin	24.7	–	0.1	–	–	–	–	–
Insulin	20.1	4.9	–	2.6	–	–	–	25.9
Peroxidase	0.5	2.5	0.2	0.3	2.0	1.9	2.8	8.4
Phosphatase, acid	0.2	0.3	0.3	0.4	1.7	4.3	2.2	2.1

whereas in other studies (Table II and IV) the general trend was for retention to decrease with an increase in pH. This suggests that acidic proteins can behave quite distinctly from neutral and basic proteins in these chromatographic systems. While this greatly complicates understanding the mechanism of retention it suggests that the separation chemistry exhibited by zirconia in Lewis base media offers great promise for unique selectivities.

Lewis bases that are weaker than fluoride leave many strongly retained proteins bound to the support. Some proteins which were weakly retained in fluoride buffers are eluted by the weaker bases. The capacity factors are, however, higher. This increase in capacity factor for these proteins closely follow the eluotropic strength of each of the Lewis bases as established in our study of the retention of benzoic acid derivatives. Deviations from this eluotropic sequence were not observed, except for horseradish peroxidase in malate buffer. This agreement occurs despite the many other types of interactions between proteins and the support.

Fig. 2 shows a correlation of the capacity factor for acidic proteins with eluotropic strength (denoted *E*). *E* is arbitrarily defined as the capacity factor for paracyanobenzoic acid in 20 m*M* Lewis base–20 m*M* MES at pH 6.1 [12,13]. The "levelling" of the

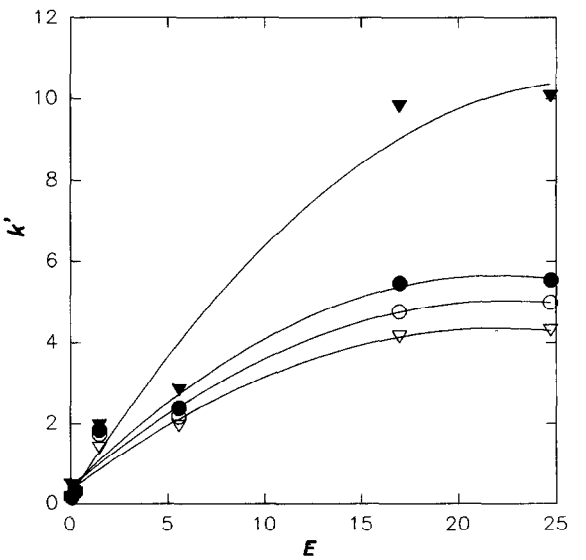

Fig. 2. Acidic protein retention *versus* Lewis base eluotropic strength. ▼ = Horseradish peroxidase; ● = cellulase; ○ = acid phosphatase; ▽ = hexokinase. Elution was a linear Lewis base gradient at 0.5 ml/min and 35°C. See Table II for gradient details.

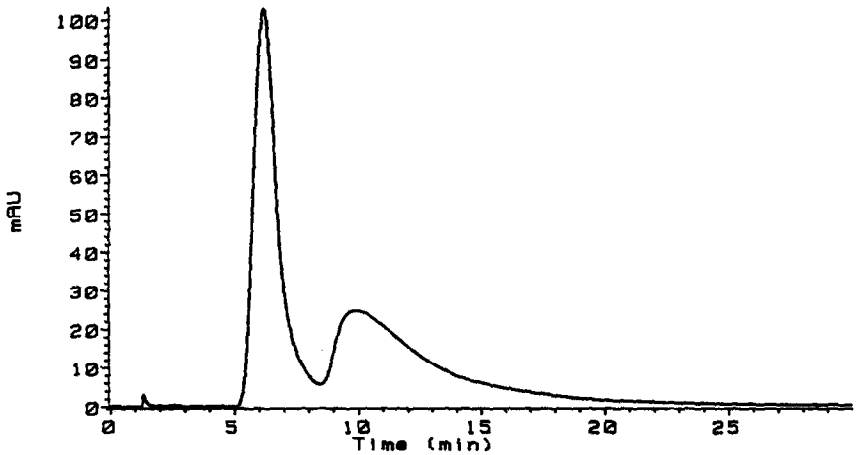

Fig. 3. Separation of hexokinase (baker's yeast) proteins PI and PII. Linear gradient elution from 98% A (1 *M* sodium chloride, 20 m*M* MES pH 6.1) to 100% B (1 *M* sodium bromide, 20 m*M* MES pH 6.1) in 30 min, then back to 98% A in 15 min with a 15-min equilibration period. Injections were 10-μl volumes of 10 mg/ml solutions in buffer A. Flow-rate was 0.5 ml/min at 35°C.

capacity factor at high *E* (low eluotropic strength) is due to the effect of the MES buffer. The sulfonic acid group on the MES molecule is a Lewis base which is stronger than either bromide or chloride, but weaker than the other bases. Therefore, the resulting eluotropic strength of the buffer is dominated by this buffer and not by the added ligand (bromide, chloride).

This does not mean that the weak displacing anion is of no importance. Fig. 3 shows that the hexokinase sub-types (PI and PII) are readily separated by a gradient from chloride to bromide. All other aspects of the weak and strong buffers were identical. Although the peak shapes are not satisfying, this separation shows that even weak Lewis base gradients can be useful. It should be noted that isocratic elution of the same sample in the chloride buffer failed to elute the second protein and when a bromide eluent was used the peaks were not resolved.

Ionic strength gradients

Based on the above, it is clear that gradient elution with changes in the concentration of the Lewis base are chemically and chromatographically very complex. In order to study the differential effect of a chemically wide variety of Lewis bases under the simplest possible conditions the experiment described in Table VII was implemented. In this study, the Lewis base concentration was held constant throughout the gradient and the ionic strength was increased by varying the concentration of sodium chloride.

The data in phosphate media show that many proteins are not affected by the changes in the gradient. The acidic proteins, which are not retained to any significant extent, remain relatively unretained. However, cationic proteins (*e.g.* β-lactoglobulin, apotransferrin, ribonuclease, etc.) all show significantly higher capacity factors relative to the conditions in Table II. This is not surprising since these proteins are primarily retained by cation exchange and the chloride gradient used in Table VII has a lower ionic strength than the phosphate gradient used in Table II. Additional retention probably results from ligand-exchange interactions. Since these proteins do show some ligand-exchange character, this retention process will be accentuated at a low, constant Lewis base concentration compared to conditions where the gradient rapidly swamps the ligand-exchange equilibria.

The results in the fluoride buffer are also interesting. A higher ionic strength was used here than in Table II. Given this, it is interesting to note that some proteins are more retained in this experiment than that described in Table II. Once again, the changes in retention do not correlate with protein isoelectric point or molecular weight. More likely, the differences are due to the complex balance of ligand- and ion-exchange contributions to retention in the systems reported here.

TABLE VII

PROTEIN RETENTION WITH IONIC STRENGTH GRADIENTS AND CONSTANT LEWIS BASE CONCENTRATIONS

Linear gradient elution from 2% B to 100% B in 30 min, then back to 2% B in 15 min with a 15-min equilibration period. Flow-rate was 0.5 ml/min at 35°C. Injections were 10 μl volumes of 10 mg/ml solutions of protein in 20 m*M* TAPS at pH 8.4. Detection was at 280 and 410 nm. Buffer A: 20 m*M* Lewis base in 20 m*M* TAPS at pH 8.4; buffer B: buffer A with 1 *M* sodium chloride. – = Elution not observed. Lewis bases: A = sodium phosphate; B = sodium fluoride; C = boric acid; D = citric acid; E = aspartic acid; F = tartaric acid; G = iminodiacetic acid; H = aminomethylphosphonic acid; I = ethylphosphonic acid; J = O-phospho-DL-serine.

Protein	*k'*									
	Gradient									
	A	B	C	D	E	F	G	H	I	J
PEP	−0.3	−0.3	−0.3	−0.3	−0.3	−0.3	−0.3	−0.3	−0.3	−0.3
GLOX	−0.3	–	−0.3	−0.3	1.2	−0.3	–	−0.3	−0.3	−0.3
TINH	−0.2	0.1	0.1	0.0	0.2	0.0	0.2	0.0	−0.2	−0.2
OVA	−0.2	–	–	−0.2	–	0.2	–	−0.2	−0.2	−0.2
HSA	−0.3	–	−0.3	−0.3	−0.3	−0.3	–	−0.3	−0.3	−0.3
HEXO	−0.2	1.6	1.9	0.5	1.3	1.3	3.0	0.1	−0.3	0.1
βLAC	–	–	–	–	–	–	–	−0.3	−0.3	−0.2
LIP	0.2	1.2	1.3	0.5	1.3	1.3	2.2	0.1	0.5	0.1
ATRH	12.0	–	–	0.1	–	0.0	–	−0.1	−0.2	−0.2
MYO	11.6	14.5	13.4	1.1	26.9	3.8	–	0.3	0.7	0.1
HEMO	19.4	20.0	14.4	−0.1	–	−0.1	–	−0.1	−0.2	−0.1
αCHY	13.8	–	–	12.5	22.5	14.5	–	6.3	14.2	8.8
CYTC	23.4	–	–	23.3	–	26.2	–	11.3	26.4	16.5
RNA	15.1	13.0	13.2	9.9	14.1	11.0	18.8	6.6	11.3	7.4
RNB	11.1	12.8	13.0	8.5	13.7	9.7	18.9	3.8	9.5	6.2
LYS	10.9	9.4	11.2	10.5	12.8	10.2	12.6	5.9	15.1	8.1

Retention in borate buffers is comparable to that in fluoride media. Proteins which are more retained in borate media rely more on ligand-exchange interactions than ionic interactions. However, slight differences in selectivity are apparent between fluoride and borate buffers, indicating that secondary interactions (hydrogen bonding, etc.) between the proteins and the bound Lewis bases may be important.

Lewis base buffers which contain more than one Lewis base functional group per molecule (*e.g.* citrate) stick much more tightly to the surface than do monovalent buffer Lewis bases (*e.g.* acetate). One consequence of this effect is that the adsorption isotherm for polyvalent Lewis bases will saturate at much lower buffer concentrations than those of monovalent bases. Therefore, lower concentrations of these species can be used to promote the elution of Lewis base solutes. This can also help promote elution by reducing the mean number of available Lewis acid sites.

To determine the effect that these polyvalent organic Lewis bases have on protein retention, a number of proteins were examined in such systems. The results of this study are shown in Table VII. According to our previously developed eluotropic scale, the displacing strength of the bases should be: citric acid > aspartic acid > tartaric acid > iminodiacetic acid. This trend was observed for many of the proteins studied here. Proteins such as hexokinase, lipase and ribonuclease A followed clear trends, while some proteins showed a different pattern of retention.

Acidic proteins, in general, were not well retained in polyvalent Lewis base buffers. The eluent base simply out-competed the protein for the Lewis acid sites on the surface. Basic proteins, on the other hand, may be retained by cation exchange in the absence of strong ligand-exchange contributions to retention.

Glucose oxidase was slightly retained in the aspartic acid buffer, but not in any other buffer, other than iminodiacetic acid. The elution strength of imi-

nodiacetic acid may not be sifficient to overcome ligand-exchange interactions. Aspartic acid is probably bound to the surface via its two carboxylate groups. The amino functional group is fully protonated at this pH and should be available for ionic interactions with solutes. This anion-exchange capacity would allow anionic proteins to be retained. Myoglobin, α-chymotrypsin, ribonuclease and lysozyme all show this interesting retention pattern in the aspartic acid buffer.

In contrast to the results obtained with monovalent bases, protein retention in polyvalent base eluents is not in good agreement with the eluotropic scale (*E*) described above. Based on the *E* scale fluoride should be a stronger competitor than any of the organic ligands. Many proteins did not elute in fluoride media but do elute in citrate, aspartate and tartrate eluents.

Secondary interactions are a second major source of differences in the behavior of polyvalent bases. As in the case of aspartic acid, the non-chelating portion of the buffer Lewis base is free to interact with the solutes. This can result in additional ionic and hydrophobic interactions with the solute.

It is often necessary to use very strong buffer Lewis bases to elute proteins from zirconium oxide. Phosphate and fluoride both suffice, but both produce more or less the same selectivity. An approach to inducing significant changes in selectivity while still maintaining high eluotropic strength is to use ligands with phosphate or phosphonic acid groups. This idea was tested by studying retention in eluents containing organophosphonates (see Table VII). Most of the acidic proteins were not well retained. The phosphate portion of the ligand competes too well for the available ligand-exchange sites and residual electrostatic interactions only serve to promote elution. Low retention is observed despite the presence of cationic amino groups on aminomethylphosphonic acid and O-phospho-DL-serine.

Basic proteins were well retained in these buffers. Secondary interactions, however, caused some significant changes in elution patterns among these proteins. All proteins which were retained had their greatest retention in the ethylphosphonic acid buffer. O-phospho-DL-serine is the next stronger Lewis base buffer, however, myoglobin was less retained in it than in aminomethylphosphonate. Aminomethylphosphonic acid is the strongest buffer and offers the greatest selectivity between ribonuclease A and B. This is no doubt due to the secondary interactions between the support and proteins.

Borate buffers

There were clear signs in the data of Tables II and IV that borate is an unusual eluent towards proteins. Blesa *et al.* [20] have shown that the driving force for adsorption of borate on zirconia is different from that of other Lewis bases. Most adsorbed borate is present in the form of esters of hydroxyl groups on the surface of zirconia. The remainder is present as ionized esters with tightly bound counterions. Such species can act to sterically block the Lewis acid sites, however, additional interactions between borate and the Lewis acid sites are possible.

Table VIII shows the retention of a variety of

TABLE VIII

PROTEIN RETENTION IN BORATE BUFFERS: CAPACITY FACTORS

Linear gradient at 1.00 ml/min and 35°C from 100% A to 100% B in 30 min followed by a return to 100% A in 15 min. Equilibration period was 15 min between runs. Injections were 10 μl of 1 mg/ml solutions of proteins in 20 m*M* MES pH 6. Detection was at 280 and 410 nm. – = Elution not observed.
Gradient I: A: 0.5 *M* H_3BO_3, pH 5.5; B: 0.5 *M* H_3BO_3, 1.5 *M* NaCl, pH 5.5.
Gradient II: A: 0.5 *M* H_3BO_3, pH 5.5; B: 0.5 *M* H_3BO_3, 0.5 *M* Na_2SO_4, pH 5.5.
Gradient III: A: 0.5 *M* H_3BO_3, pH 5.5; B: 0.5 *M* H_3BO_3, 0.5 *M* NaCl, pH 5.5.
Gradient IV: A: 0.5 *M* H_3BO_3, pH 7.0; B: 0.5 *M* H_3BO_3, 0.5 *M* NaCl, pH 7.0.

Protein	*k'*			
	Gradient			
	I	II	III	IV
Ovalbumin	–	–	–	–0.3
Bovine serum albumin	–	–	–	–0.3
Human serum albumin	–	–	–	–0.3
Transferrin	–	–	–	4.4
Myoglobin	–	–	–	13.8
Hemoglobin	–	–	–	27.3
Alcohol dehydrogenase	–	–	–	–0.3
α-Chymotrypsin	29.5	0.3	–	35.1
Cytochrome *c*	52.0	–	–	–
Ribonuclease A	20.9	25.9	48.7	30.7
Ribonuclease B	20.8	25.0	–	26.3
Lysozyme	19.7	28.0	52.6	30.0

proteins in highly concentrated borate buffers. At low pH, borate is scarcely ionized and the surface esters are likely uncharged. Acidic and neutral proteins are not eluted. Cationic proteins were well retained in both gradients I and II. Because both gradients employed were of equal ionic strength, differences in capacity factor cannot be attributed to differences in ionic interactions. The fact that sulfate (gradient II) proves to be a weaker eluent than chloride (gradient I) suggests that Lewis acid site interactions are not responsible for retention. The nonelution of the acidic proteins is inexplicable.

At higher pH values, the borate esters are more ionized. Anionic proteins are eluted at pH 7.0 (gradient IV), but not at lower pH values. Neutral proteins are reasonably well retained at pH 7.0. Cationic proteins are strongly retained. However, they are not as well retained as at the lower pH. The net effect of a change to higher pH is the conversion of this medium from an adsorbent to a cation exchanger. It should be noted that the surface coverage with borate must be quite high since very high ionic strengths are required to elute the proteins at pH 7.0

This unique elution behavior may be due to two effects. First, Hingston *et al.* [22] found that the adsorption capacity of Lewis bases on metal oxides was maximal at pH values near their pK_a. The first ionization constant of borate is 9.24, so at pH 7.0, a higher surface loading of borate is anticipated. This could help to displace the acidic proteins bound to Lewis acid sites. Secondly, the unionized borate surface might act as a non-specific adsorbent for proteins. When this surface becomes ionized at high pH, electrostatic interactions may dominate the adsorptive interactions and cause elution by ion exchange. This explanation is highly speculative, but it does account for the borate buffer results given in Tables II and IV.

CONCLUSIONS

A wide variety of acidic, neutral and basic proteins can be chromatographically separated on porous zirconium oxide particles, provided that an appropriate concentration of a strong hard Lewis base is present in the eluent. The proteins are retained by a complex balance of ion-exchange (on anionic sites) and ligand-exchange (on surface Lewis acid) sites. Elution can be initiated by increasing the concentration of the Lewis base or by increasing the ionic strength. The most critical parameter in achieving acceptable retention and peak shape is the initial concentration of the Lewis base in the eluent. The retention mechanism is analogous to that of calcium hydroxyapatite, but the selectivites are quite different and depend very strongly on the type of Lewis base used for elution.

Most proteins did not show a clear correlation between displacing ligand strength and protein capacity factor. This is not entirely surprising, since neutral and basic proteins have significant cation-exchange contributions to retention once the ligand-exchange mechanism has been attenuated. Acidic proteins, however, showed a fairly high degree of correlation between retention and Lewis base displacing strength since ligand exchange is the main retention mode for these proteins. Overall, the selectivity was not unlike calcium hydroxyapatite. However, this support is far superior to hydroxyapatite in chemical and physical stability.

The most important finding is that a fairly strong buffer Lewis base must be present, in sufficient concentration to nearly saturate the adsorption isotherm, for any of the proteins to be fully eluted from the phase. In practice, phosphate, fluoride, polyvalent organic ligands and organosphosphate ligands proved most suitable for the successful attenuation of the Lewis acid sites at low concentrations.

The complex Lewis bases proved most interesting as modulators of the ligand-exchange process. Not only were the slow desorption kinetics and strong binding properties advantageous in effectively blocking the Lewis acid sites, but the complex structures allowed secondary interactions between the protein and the Lewis base to occur. This resulted in different selectivities which were not predictable based on electrostatic or ligand-exchange interactions.

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