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HIGH-PERFORMANCE ADSORPTION CHROMATOGRAPHY OF PROTEINS ON DEFORMED NON-POROUS AGAROSE BEADS COATED WITH INSOLUBLE METAL COMPOUNDS

II. COATING: ALUMINIUM AND ZIRCONIUM (HYDR)OXIDE WITH STOICHIOMETRICALLY BOUND PHOSPHATE

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SUMMARY

Two novel supports for adsorption chromatography of biomolecules, based on deformed non-porous cross-linked agarose beads coated with aluminium or zirconium (hydr)oxide, were prepared. Some fundamental chromatographic properties of these bed materials are discussed, including the adsorption mechanism and high-resolution separations of mixtures of proteins, nucleotides and coenzymes. Both aluminium and zirconium (hydr)oxide agarose irreversibly and stoichiometrically bind phosphate ions, thus some of the chromatographic properties resemble those of calcium phosphate and hydroxyapatite. Fractionation of a β-glucosidase preparation into two components possessing enzymatic activity was achieved on the aluminium (hydr) oxide phosphate agarose, whereas chromatography on zirconium (hydr)oxide phosphate–agarose removed several inactive contaminants but gave only one peak associated with enzyme activity. This finding indicates that the adsorption mechanisms of the two adsorbents are not identical, although other experiments show that there are several analogies. For instance, model proteins chromatographed on these two supports were eluted in the same order, although the relative retention times differed, *i.e.*, the chromatograms did not have the same appearance. Compressed beds of cross-linked non-porous agarose beads, including those coated with aluminium and zirconium (hydr)oxide, have the unique feature that the resolution is roughly independent of flow-rate and that there is no need for small beads of narrow size distribution.

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INTRODUCTION

Transition metals, such as titanium, zirconium, iron, tin and vanadium have been used to activate the surfaces of various matrices, for instance cellulose, nylon, glass and chitin[1,2] to give derivatives to which proteins, cells and antibiotics can be attached[1–5]. Such activated matrices have been shown to be useful tools in biotechnology, one application being the preparation of enzyme reactors[6].

Zirconium phosphate has been employed earlier as a chromatographic support[7].

The use of aluminium oxide (alumina) for chromatographic purposes was reported long ago. Its high capacity for the adsorption of different biological substances makes it an attractive alternative to the bed materials based on silica or polymers. Adsorption on aluminium oxide has been described for chromatographic separation of biological macromolecules[8,9], for purification of cyclic nucleoside monophosphates[10], steroid hormones[11] and polypeptides[12]. Aluminium oxide for high-performance liquid chromatography (HPLC) can be obtained commercially (5 μm Spherisorb A5Y from Chrompack). In this form it is macroporous and permits separation of proteins by size exclusion.

Amorphous (hydr)oxides of aluminium and zirconium are known to adsorb proteins[4,13], but unfortunately there is no possibility to use these materials directly for chromatographic experiments because of their poor flow properties. To overcome this problem we coated non-porous cross-linked agarose beads with aluminium and zirconium (hydr)oxides, respectively. This approach combines the adsorption characteristics of these (hydr)oxides with the good flow properties of the agarose support. The simplicity and relatively low cost of preparation also make these supports attractive. The subtitles used in this paper are analogous to those employed in the preceding paper (Part I[14]) on ferric oxyhydroxide phosphate agarose in order to facilitate comparison of the chromatographic properties of the adsorbents.

CHEMICALS AND EQUIPMENT

Agarose was a kind gift from Dr. R. Armisén (Hispanagar, Burgos, Spain), human transferrin (p*I* 5.5) from Dr. L.-O. Andersson, (KabiVitrum, Stockholm, Sweden) and γ-glycidoxypropyltrimethoxysilane from Mr. H. Gustafsson (Sikema, Stockholm, Sweden). Ovalbumin (p*I* 4.7), carbonic anhydrase (p*I* 5.9), conalbumin (p*I* 5.9), myoglobin from horse muscle (p*I* 7.3), α-chymotrypsinogen A (p*I* 8.8), cytochrome *C* (p*I* 9.2), lysozyme (p*I* 10), AMP, ADP, ATP, oxidized nicotinamide-adenine dinucleotide (NAD) and oxidized nicotinamide-adenine dinucleotide phosphate (NADP) were obtained from Sigma (St. Louis, MO, U.S.A.). β-Glucosidase from sweet almonds and 3-nitrophenyl-β-glucopyranoside were from Serva (Heidelberg, F.R.G.). All salts were of analytical grade from Merck (Darmstadt, F.R.G.).

Before use all buffer solutions were filtered through a 0.22-μm Millipore filter and degassed.

The chromatographic equipment was as described in Part I[14]. The charge of the metal (hydr)oxides was studied by free zone electrophoresis in an horizontal quartz tube rotating around its long axis (40 rpm) to eliminate disturbing convection[14,15].

EXPERIMENTAL AND RESULTS

Preparation of non-porous agarose beads

The procedure was the same as that described in Part I[14]. γ-Glycidoxypropyltrimethoxysilane was used as a cross-linker.

Precipitation of aluminium and zirconium (hydr)oxide onto non-porous agarose beads

We followed the method employed for the preparation of FeO(OH)–agarose (see Part I[14]) with the exception that the ferric chloride solution was replaced by 10 ml of an aqueous solution of 1 *M* $AlCl_3$ or 0.65 *M* $ZrCl_4$. The columns were packed in distilled water and the beds were compressed as outlined in Part I[14]. The mechanical properties of both adsorbents (including the relationship between flow-rate and back pressure) were similar to those of FeO(OH) agarose (Part I[14]).

Elemental analysis of aluminium and zirconium (hydr)oxide-coated agarose beads

The samples for elemental analysis were taken from columns equilibrated with potassium phosphate and then washed with water as described for $FeO(OH)(PO_4)$–agarose in Part I[14]. The metal-to-phosphate molar ratio in this case was also close to 2:1. Since phosphate ions are thus irreversibly bound it may be appropriate to introduce the terms aluminium (hydr)oxide phosphate and zirconium (hydr)oxide phosphate in analogy with the notation ferric oxyhydroxide phosphate used in Part I[14]. In this paper we also use the expressions $Al(OH)(PO_4)$ and $Zr(OH)(PO_4)$, in analogy with $FeO(OH)(PO_4)$ in Part I[14].

Chromatographic characterization of $Al(OH)(PO_4)$–agarose

A separation of a model protein mixture of ovalbumin, human transferrin, myoglobin, conalbumin, lysozyme and cytochrome *c* was performed on a column of $Al(OH)(PO_4)$–agarose under the different equilibration and elution conditions described below. The following buffers were used for equilibration: 0.01 *M* sodium acetate, pH 5.5; 0.01 *M* sodium cacodylate, pH 6.0 and 7.0; 0.01 *M* Tris–HCl, pH 7.5; 0.01 *M* potassium phosphate, pH 7.0 and 0.01 *M* sodium borate, pH 9.1. After equilibration of the column the sample was applied (75 μg of each of the above proteins dissolved in 80 μl of the equilibration buffer). In an attempt to elute the proteins we chose a linear 30-min salt gradient (0–0.2 *M*) in the equilibration buffer. The gradient was created by potassium chloride and sodium salts of chloride, sulphate, EDTA and cacodylate. These salts appeared to have little or no eluting power. However, all proteins were desorbed easily by a concentration gradient of potassium phosphate, pH 7.0, from 0.002 to 0.2 *M* in the absence of other salts (Fig. 1a). It should be added that equilibration of the column at high pH (0.01 *M* borate buffer, pH 9.1) gave a very weak adsorption of acidic proteins, whereas equilibration at low pH (0.01 *M* acetate buffer, pH 5.5) caused very strong adsorption of all proteins tested except ovalbumin.

We also equilibrated the column with 0.01 *M* sodium cacodylate buffer, pH 7.0, and eluted with the same buffer containing a linear gradient in potassium phosphate from 0 to 0.2 *M*, pH 7.0 (Fig. 1b). A comparison of Fig. 1a and b shows that this second elution procedure including sodium cacodylate gave sharper peaks in the chromatogram and thus a better separation, which is in analogy with the experiments on $FeO(OH)PO_4$, presented in Part I[14].

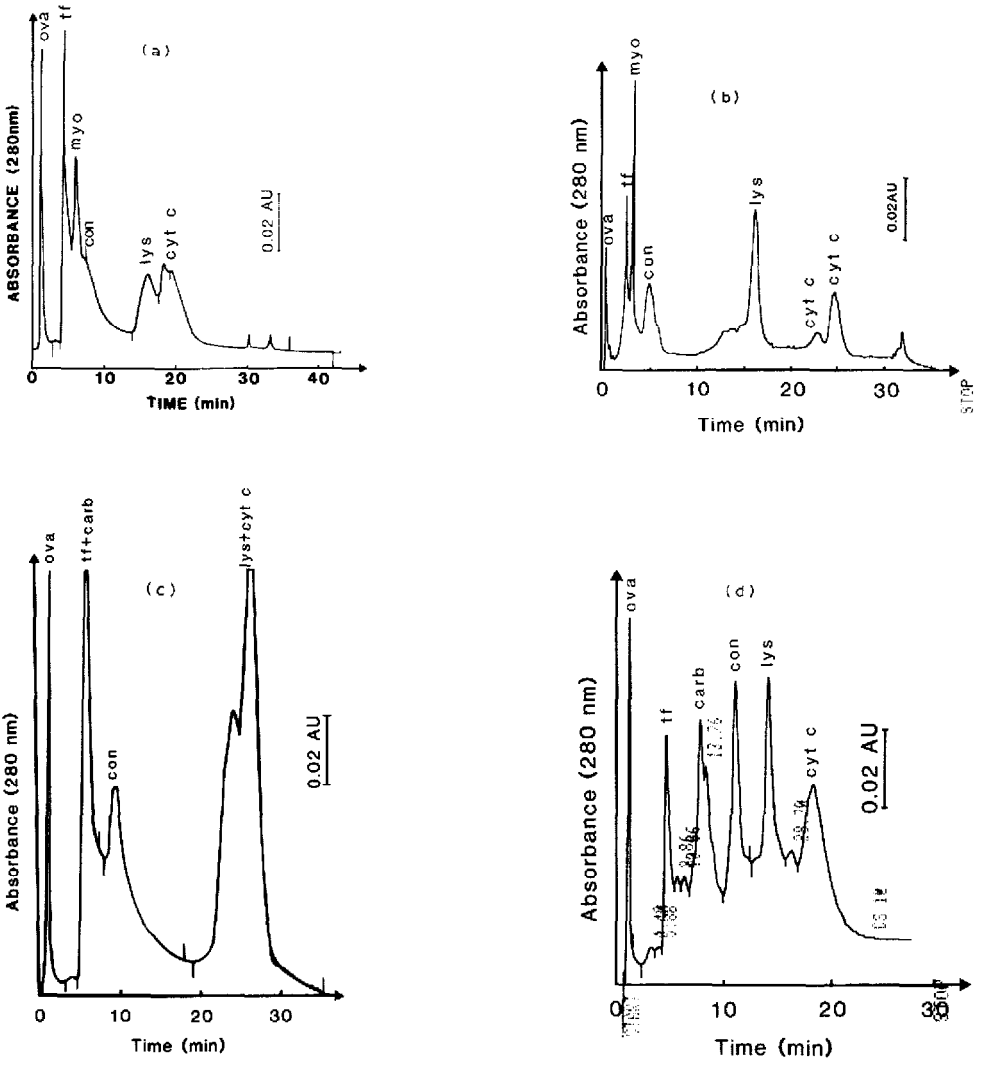

Fig. 1. Chromatography of model proteins on aluminium and zirconium (hydr)oxide phosphate agarose. Column dimensions: 30 mm × 6 mm I.D. Flow-rate: 1.0 ml/min. (a) $Al(OH)(PO_4)$–agarose. Sample: 75 μg each of the following proteins in a total volume of 80 μl: ovalbumin (ova); transferrin (tf); myoglobin (myo); conalbumin (con); lysozyme (lys); cytochrome *c* (cyt c). Elution: a linear gradient of potassium phosphate from 0.002 to 0.2 *M* over 30 min (pH 7.0). (b) $Al(OH)(PO_4)$–agarose. Sample as in (a). Elution: a linear gradient of potassium phosphate from 0 to 0.2 *M* over 30 min in 0.01 *M* sodium cacodylate, pH 7.0. (c) $Zr(OH)(PO_4)$–agarose. Sample as in (a) with the difference that myoglobin was exchanged for carbonic anhydrase (carb). Elution: a linear gradient of potassium phosphate, pH 6.0, from 0.002 to 0.2 *M* over 30 min. (d) $Zr(OH)(PO_4)$–agarose. Sample as in (c). Elution: a linear gradient of potassium phosphate from 0 to 0.2 *M* over 30 min in the presence of 0.01 *M* sodium cacodylate, pH 6.0. A comparison of (a) with (b) and (c) with (d) shows the favourable effect of cacodylate ions on the resolution.

Chromatographic characterization of $Zr(OH)(PO_4)$–agarose

The experiment was performed as discussed above for aluminium (hydr)oxide phosphate–agarose with the exception that myoglobin in the model protein mixture was exchanged for carbonic anhydrase (transferrin and carbonic anhydrase coeluted in all experiments except that shown in Fig. 1d). In Fig. 1c a chromatogram is presented for a column of zirconium (hydr)oxide phosphate–agarose following equilibration with 0.002 *M* potassium phosphate, pH 6.0, and elution with a linear 30-min gradient in potassium phosphate (pH 6.0) from 0.002 to 0.2 *M*. An higher resolution was obtained, however, by equilibration of the same column with 0.01 *M* sodium cacodylate buffer, pH 6.0, and elution with a 30-min gradient in potassium phosphate (pH 6.0) from 0 to 0.2 *M* in the equilibration buffer (Fig. 1d).

The pH dependence of the protein adsorption was similar to that described above for $Al(OH)(PO_4)$–agarose. Other similarities were: (1) among the eluting agents tested only phosphate ions can be used for desorption; (2) there was no obvious correlation between the p*I*-values of the proteins and their order of elution and (3) model proteins were eluted in the same order from aluminium and zirconium (hydr)oxide phosphate–agarose (Fig. 1).

In all subsequent experiments on columns of aluminium and zirconium (hydr)-oxide phosphate–agarose, *i.e.*, metal (hydr)oxide agarose equilibrated with phosphate we used a cacodylate buffer for equilibration and a phosphate buffer for elution.

Resolution on aluminium and zirconium (hydr)oxide phosphate–agarose as a function of different chromatographic parameters

The aluminium (hydr)oxide phosphate–agarose column was equilibrated with 0.01 *M* sodium cacodylate buffer (pH 7.0) and eluted with a concentration gradient of potassium phosphate (pH 7.0) from 0 to 0.2 *M* in the presence of the equilibration buffer. The same buffers were employed for the zirconium (hydr)oxide phosphate–agarose column, but the pH was 6.0 instead of 7.0. Eqn. 1 in Part I[14] was used for calculation of the resolution between the same model proteins (chymotrypsinogen A and carbonic anhydrase) as were used for the $FeO(OH)(PO_4)$–agarose bed[14].

Resolution versus gradient time. About 50 μg of each of the above two model proteins were dissolved in 100 μl of the equilibration buffer. Using a constant flow-rate of 1 ml/min, the resolution was determined at different gradient times for both the aluminium (Fig. 2a) and the zirconium (hydr)oxide phosphate–agarose column (curve 1 in Fig. 2b). Since the latter column exhibited a time-dependent adsorption, we performed two additional sets of experiments in which the column with the proteins adsorbed was washed with the equilibration buffer for 20 and 50 min, respectively, before the start of the gradient elution. The results are presented in Fig. 2b as curves 2 and 3, respectively. A comparison between these curves and curve 1 in the same figure indicates that the resolution as a function of gradient time is dependent on the residence time of the proteins.

Resolution versus flow-rate. The total gradient volume was constant at 7 ml. The gradient times were inversely proportional to the flow-rates. For other details, see Part I[14]. Curves (a) and (b) in Fig. 3 show that the resolution varies only slightly with the flow-rate.

Resolution versus protein load. The samples consisted of 25–1500 μg of each of the model proteins dissolved in 100 μl of the equilibration buffer. The flow-rate was 1

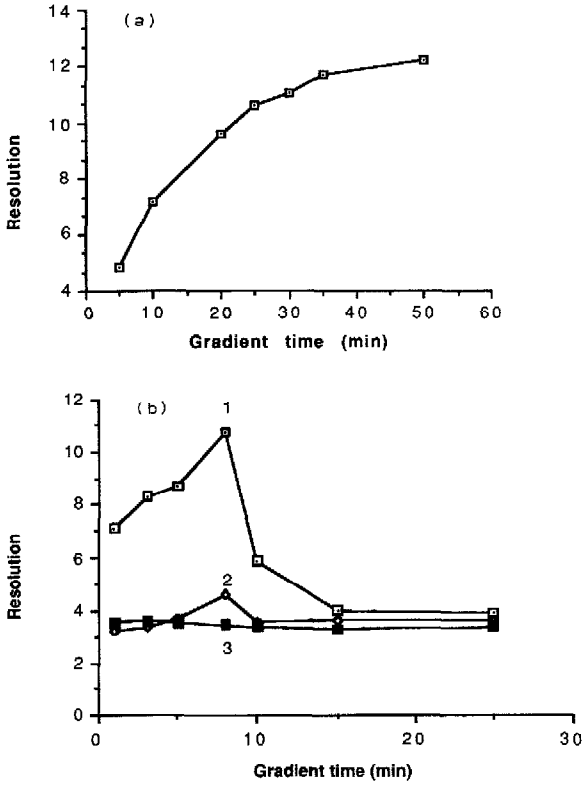

Fig. 2. Resolution (R_s) of chymotrypsinogen A and carbonic anhydrase *versus* gradient time on aluminium and zirconium (hydr)oxide phosphate–agarose. For details see Experimental and Results. (a) Al(OH)-(PO_4)–agarose; (b) Zr(OH)(PO_4)–agarose. Residence times: 0 (1), 20 (2), 50 min (3).

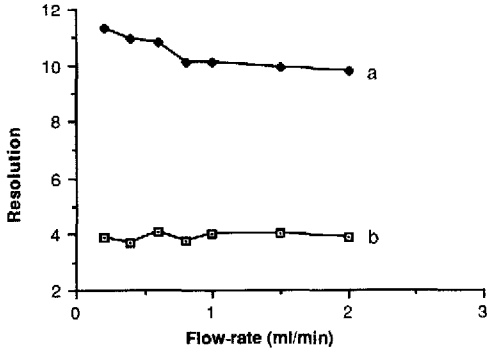

Fig. 3. Resolution of chymotrypsinogen A and carbonic anhydrase *versus* flow-rate on aluminium and zirconium (hydr)oxide phosphate–agarose. For details see Experimental and Results. (a) Al(OH)(PO_4)–agarose; (b) Zr(OH)(PO_4)–agarose.

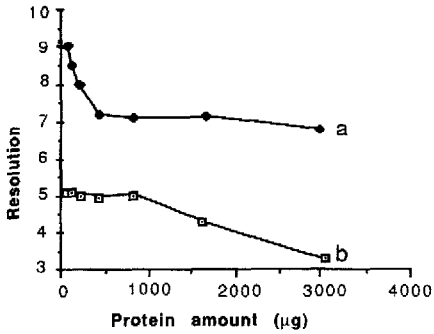

Fig. 4. Resolution of chymotrypsinogen A and carbonic anhydrase *versus* amount of protein in the sample on aluminium and zirconium (hydr)oxide phosphate–agarose. For details see Experimental and Results. (a) Al(OH)(PO_4)–agarose; (b) Zr(OH)(PO_4)–agarose.

ml/min and the gradient time 10 min. From Fig. 4 one can conclude that the resolution was relatively little impaired upon an increase in the total protein load up to as much as 6 mg.

Chromatography of a commercial preparation of β-glucosidase on aluminium and zirconium (hydr)oxide phosphate–agarose columns

The experimental conditions were similar to those used for the purification of the same preparation of β-glucosidase on FeO(OH)(PO_4)–agarose (Part I[14]) with the

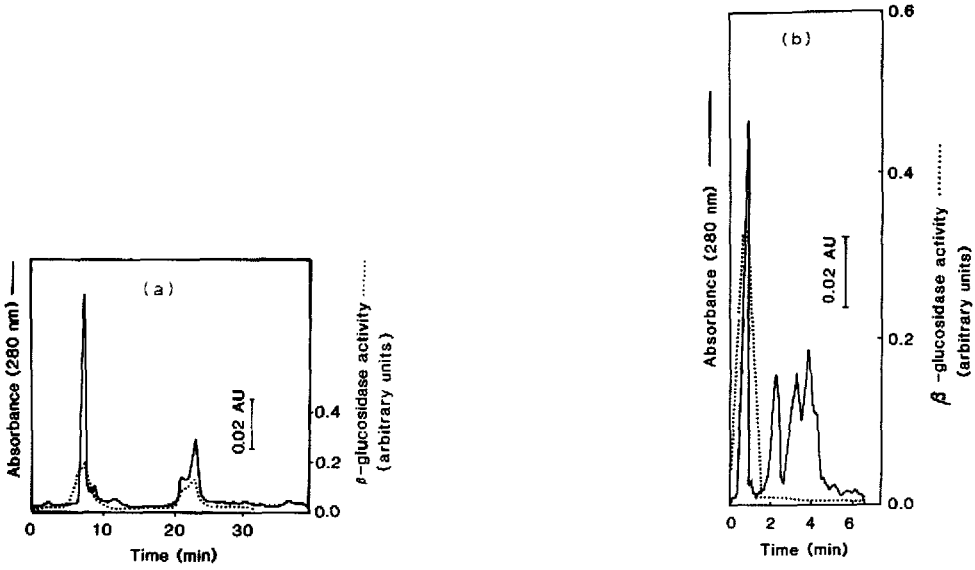

Fig. 5. Chromatography of a commercial preparation of β-glucosidase on aluminium and zirconium (hydr)oxide phosphate–agarose. Column dimensions: 30 mm × 6 mm I.D. Flow-rate: 0.5 ml/min. Sample: β-glucosidase, 50 μg dissolved in 50 μl of equilibration buffer. (a) Al(OH)(PO_4)–agarose: elution with a linear gradient of potassium phosphate from 0 to 0.35 *M* over 20 min in 0.01 *M* sodium cacodylate, pH 7.0. (b) Zr(OH)(PO_4)–agarose: elution with a linear gradient of potassium phosphate from 0 to 0.2 *M* over 30 min in 0.01 *M* sodium cacodylate, pH 6.0.

exception that the buffer concentrations were somewhat different (see the legend to Fig. 5). The gradient times for the $Al(OH)(PO_4)$–agarose and $Zr(OH)(PO_4)$–agarose were 20 and 30 min, respectively, at a flow-rate of 1 ml/min. The UV pattern for the former adsorbent showed two main peaks, both with enzyme activity (Fig. 5a), whereas the latter adsorbent showed several peaks of which only that corresponding to the void volume showed enzyme activity (Fig. 5b). The recovery of the enzyme activity was 98% on the $Al(OH)(PO_4)$–agarose column and 99% on the $Zr(OH)(PO_4)$–agarose column.

Separation of nucleotides and coenzymes on $Al(OH)(PO_4)$–agarose

An $Al(OH)(PO_4)$–agarose column was equilibrated with 0.01 *M* sodium cacodylate buffer, pH 7.0. A sample was applied containing 50 μg each of adenosine monophosphate (AMP), diphosphate (ADP) and triphosphate (ATP) dissolved in 150 μl of the equilibration buffer. The column was washed for 10 min with this buffer and then eluted in the presence of the same buffer with a linear gradient of potassium phosphate from 0 to 0.4 *M* over 30 min. Fig. 6a shows three well resolved peaks of the nucleotides. AMP was not adsorbed to the column and ADP and ATP were eluted at potassium phosphate concentrations of 0.16 and 0.21 *M*, respectively.

In another experiment the sample consisted of 50 μg each of NAD and NADP dissolved in 100 μl of the equilibration buffer. The elution conditions were the same as mentioned above, with the difference that the gradient time was 20 instead of 30 min. The chromatogram is presented in Fig. 6b. NAD was not adsorbed to the column, whereas NADP required a potassium phosphate concentration of 0.29 *M* for desorption.

Fig. 6. Chromatography of nucleotides and coenzymes on aluminium (hydr)oxide phosphate–agarose. Column dimensions: 30 mm × 6 mm I.D. Flow-rate: 1 ml/min. (a) Sample: 50 μg of each nucleotide (AMP, ADP and ATP) in a total volume of 150 μl. Elution: a linear gradient of potassium phosphate from 0 to 0.4 *M* over 30 min in 0.01 *M* sodium cacodylate, pH 7.0. (b) Sample: 50 μg of each coenzyme (NAD and NADP) in a total volume of 100 μl. Elution: a linear gradient of potassium phosphate from 0 to 0.4 *M* over 20 min in 0.01 *M* sodium cacodylate, pH 7.0.

DISCUSSION

Aluminium (hydr)oxide phosphate–agarose

In acidic solutions aluminium ions coordinate water. Some of these water molecules may be displaced by hydroxyl groups on and in the agarose bead. As the solution becomes less acidic a successive deprotonation takes place to yield $Al(OH)^{2+}$ and $Al(OH)_2^{+}$; of course, free hydroxide ions are also created by deprotonation of bulk water molecules and coordinate to the aluminium ion. This process terminates in the formation predominantly of $Al(OH)_3$ when the pH approaches neutrality[16].

At this stage $Al(OH)_3$ gives colloidal (amorphous) complexes of indefinitely large size. When aluminium trichloride is used in the preparation of aluminium hydroxide some of the chloride ions may be present in the $Al(OH)_3$ complex formed. Whether this complex changes upon ageing as proposed for the formation of $FeO(OH)(PO_4)$–agarose is not known.

Upon free zone electrophoresis in the presence of cacodylate and phosphate buffers (pH 7.0), aluminium hydroxide did not migrate, which confirms the existence of uncharged hydroxide in neutral solutions. The amphoteric nature of aluminium hydroxide makes possible an interaction with anions and cations by exchange[17]. It is also known that compounds containing phosphate groups can be specifically adsorbed to aqueous aluminium oxide under conditions where non-phosphorylated compounds show no interaction. Therefore, it is not surprising that specific elution can be obtained by an aqueous solution of inorganic phosphates[17]. Indeed, elemental analysis shows that some phosphate is irreversibly bound at a metal-to-phosphate ratio of 2:1.

Most of the proteins adsorbed on the column of $Al(OH)(PO_4)$–agarose could not be eluted by increasing the ionic strength of the elution buffer, except with regard to phosphate. This adsorbent is thus not a true cation exchanger, although proteins were eluted from the column roughly according to their p*I* values. Unsuccessful separations due to strong adsorption of the proteins to $Al(OH)(PO_4)$–agarose equilibrated at low pH (acetate buffer pH 5.5) and weak adsorption when the column was equilibrated at high pH (borate buffer pH 9.1) suggests that $Al(OH)(PO_4)$–agarose has ion-exchange properties. However, they must be complemented by other mechanisms of adsorption, since, as mentioned, only phosphate ions can effect desorption.

This effectiveness of phosphate ions as an eluting agent indicates that phosphate groups in biomolecules may play an important rôle in the interaction with $Al(OH)(PO_4)$–agarose. This conclusion is supported by our finding that AMP, ADP and ATP are eluted from an aluminium (hydr)oxide phosphate–agarose column in the order of increasing number of phosphate groups (Fig. 6a) and that NADP, but not NAD, is adsorbed (Fig. 6b).

To summarize, our experiments provide support for the idea that several of the above-mentioned mechanisms of adsorption to aluminium (hydr)oxide phosphate agarose may function in combination.

Zirconium (hydr)oxide phosphate agarose

$Zr(OH)(PO_4)$–agarose was prepared in a similar way to $Al(OH)(PO_4)$–agarose and the earlier reported $FeO(OH)(PO_4)$–agarose[14]. When zirconium tetrachloride is dissolved in water, the zirconium ion should coordinate eight water molecules. How-

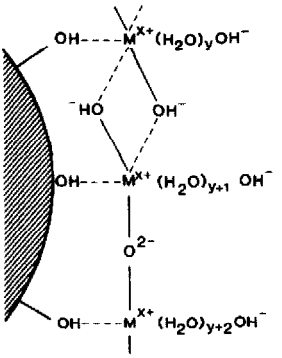

Fig. 7. Proposed structure of the metal (hydr)oxide coating on agarose beads. The two types of connections between metal ions (M^{x+}) thought to be involved in the structure are both drawn, *i.e.*, oxy bridges and double hydroxy bridges. The number of water molecules bound by the metal depends on the coordination number of the metal of interest. The proportion of deprotonated water molecules depends on the pH of the surrounding liquid and the charge of the metal ion. These hydroxide ions can in turn be shared with other metal ions, thus increasing the metal (hydr)oxide layer in all directions. When the metal (hydr)oxide agarose is equilibrated with phosphate buffer, some of the phosphate ions become irreversibly bound with a molar ratio between metal and phoshate close to 2.0. How these phosphate ions are incorporated into the structure is not known, but probably they replace some of the water molecules or hydroxide ions.

ever, even in acidic solution, zirconium salts hydrolyze to form a polymeric aggregate, which has been shown in ultracentrifuge studies to have a molecular weight of about 30 000[18].

These polymeric forms adsorb strongly to dextran, presumably by forming several complexes with vicinal hydroxyl pairs on the dextran surface[19]. Both in solution and adsorbed onto dextran, these polymers readily form outer-sphere complexes with especially divalent and polyvalent anions such as chromate and phosphate[20]. We assume that agarose behaves like dextran in these respects. A proposed structure of the metal (hydr)oxide coating on agarose beads valid for aluminium, zirconium and ferric ions is presented in Fig. 7. Upon equilibrating the column with phosphate buffer, a layer of irreversibly bound phosphate is formed similar to that observed with Al(OH)(PO_4)–agarose and with FeO(OH)(PO_4)–agarose in Part I[14]. This phosphate is bound in a metal-to-phosphate ratio of 2:1.

Electrostatic interaction between the negatively charged phosphate and hydroxide ions on the surface of the metal (hydr)oxide and positively charged groups of the protein might occur, thereby giving the zirconium (hydr)oxide phosphate agarose cation-exchange properties like those of the well known zirconium phosphate cation exchangers[7]. Such a mode of protein adsorption is indicated by the fact that proteins were adsorbed strongly to the column of Zr(OH)(PO_4)–agarose at low pH and hardly at all at high pH; upon free zone electrophoresis in phosphate and cacodylate buffers (pH 6.0), zirconium (hydr)oxide migrated towards the positive electrode, supporting the hypothesis that the surface of the (hydr)oxide is covered with negatively charged hydroxide groups. Adsorbed proteins may also displace coordinated water molecules, *cf.*, the analogous discussion in Part I[14] on protein interaction with FeO(OH)(PO_4)–agarose. The elution of the adsorbed proteins (not in the order of their p*I* values) together with the ineffectiveness of high salt concentrations (with the exception of

potassium phosphate) for elution of proteins from the zirconium (hydr)oxide phosphate–agarose suggest the combined action of the mechanisms described above. Unlike the other two metal (hydr)oxide phosphate-coated agarose supports investigated, *i.e.*, those based on $FeO(OH)(PO_4)$[14] and $Al(OH)(PO_4)$, zirconium (hydr)oxide phosphate–agarose exhibited time-dependent adsorption of proteins (Fig. 2b).

General

The high recovery of the enzymatic activity of β-glucosidase upon chromatography on ferric, aluminium and zirconium (hydr)oxide phosphate–agarose indicates that adsorption to the agarose coated with metal (hydr)oxide phosphate does not change the biological activity of the enzyme.

The use of non-porous agarose beads prevents zone-broadening caused by diffusion of proteins into and out of the bead. Upon compression of the beds the resolution becomes almost independent of the flow-rate[21,22] which permits high flow-rates without lowering the resolution. Some decrease in resolution is observed, however, for aluminium (hydr)oxide phosphate–agarose, which may be explained by the assumption that aluminium (hydr)oxide forms a thicker layer than zirconium (hydr)-oxide and that this layer is permeable to proteins. This hypothesis receives some support from our finding that the former adsorbent has an higher protein capacity (Fig. 4). An alternative explanation may be that the adsorption/desorption proceeds more slowly on $AlOH(PO_4)$–agarose.

The same columns of ferric, aluminium and zirconium (hydr)oxide phosphate–agarose were used over a period of 3 months. More than 200 analyses were performed on each using different buffers over a wide range of pH values and concentrations. No changes in performance or "leakage" of ferric, aluminium and zirconium (hydr)oxide phosphate from the columns was noticed during this time, as judged from the binding of different proteins and capacity studies. This testifies to the high stability of the gel material, which may be explained in terms of binding to vicinal hydroxyl groups and anchorage of the coating in the same way as proposed for $FeO(OH)(PO_4)$–agarose[14].

One of the main technical advantages of agarose beads coated with insoluble metal compounds is that large amounts of gel containing (hydr)oxides and salts of different metals can be prepared from a stock of shrunken and cross-linked agarose beads in a comparatively short time. The procedure does not require any expensive and time-consuming chemical modification of the agarose matrix.

Although the binding mechanisms of the three adsorbents which are based on deformed cross-linked non-porous agarose beads coated with (hydr)oxides of different metals (Fe, Al, Zr) and which have been described in this series of articles are not known in detail, there should be some analogies, since the adsorbents have many similar chromatographic properties. For instance, phosphate from the buffer was irreversibly bound at a metal-to-phosphate molar ratio of 2:1 in all three cases. Furthermore, proteins adsorbed to the columns were eluted only by a concentration gradient of phosphate and not by several other salts tested. From these points of view the adsorbents resemble brushite, $CaHPO_4 \cdot 2H_2O$[23], and hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$[24]. (Hydroxyapatite has a metal-to-phosphate molar ratio of 1.67:1.) One should also observe that the elution order of AMP, ADP and ATP is the same on hydroxyapatite[25] and aluminium (hydr)oxide phosphate–agarose (Fig. 6). This may indicate that the number of available phosphate groups in the solute plays

some rôle in the adsorption to aluminium (hydr)oxide phosphate–agarose, as has been proposed for hydroxyapatite[25]. It should be mentioned that AMP, ADP and ATP show little or no adsorption to ferric and zirconium (hydr)oxide phosphate–agarose. In addition, the following proteins are eluted in the same order on hydroxyapatite[24], ferric[14], aluminium (Fig. 1b) and zirconium (hydr)oxide phosphate–agarose (Fig. 1d): ovalbumin, transferrin, lysozyme and cytochrome *C*.

The resolution of the proteins eluted can be improved by the addition of sodium cacodylate (pK_a 6.3) to the buffers, but the reason for this is not yet clear. Its better buffering capacity at pH 6.0 may be important in the case of zirconium (hydr)oxide phosphate–agarose, but this was not valid for aluminium (hydr)oxide phosphate–agarose, where the pH of the buffers is 7.0, *cf.*, columns of hydroxyapatite which give higher resolution of tRNA and proteins when the phosphate buffer is supplemented with sodium chloride[26].

The obvious differences observed are the chromatograms obtained in the purification of β-glucosidase (Fig. 6 in ref. 14, and Fig. 5 herein), the time-dependent adsorption of proteins to zirconium (hydr)oxide phosphate–agarose (Fig. 2b) and the somewhat higher capacity of the aluminium (hydr)oxide phosphate–agarose (Fig. 4 herein and Fig. 5 in ref. 14). An effective purification of proteins should be obtainable upon combining two or three of the adsorbents studied. For example, if the β-glucosidase preparation is first purified on zirconium (Fig. 5b) or ferric (hydr)oxide phosphate–agarose (Fig. 6 in Part I[14]) a considerable fraction of the inactive material can be removed. The peak which is enzymatically active can then be subjected to chromatography on aluminium (hydr)oxide phosphate–agarose for further purification and particularly for fractionation of β-glucosidase into two enzymatically active components.

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