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SUBSTRATE- AND PRODUCT-AFFINITY RESINS FOR ADENOSINE DEAMINASE OBTAINED BY IMMOBILISATION OF ADENOSINE AND INOSINE VIA 2′,3′-CYCLIC ACETAL DERIVATIVES

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ABSTRACT

Immobilised inosine (**6a**) and adenosine (**6c**) and their 5′-phosphates have been synthesized. Reaction of the nucleosides with ethyl levulinate, followed by saponification or phosphorylation and then saponification, gave the 2′,3′-*O*-[1-(2-carboxyethyl)ethylidene] derivatives **3** and **4** and the corresponding 5′-phosphates **2b** and **2d**. 6-Aminohexylagarose (**5**) was severally coupled to **2b**, **2d**, **3**, and **4** through the carboxyl groups to give the polymers **6a–d**. Adenosine deaminase converts **3** into **4**, and **6c** into **6a**. The polymers can be used as affinity resins for adenosine deaminase, which is bound more strongly to **6c** than to **6a**. The operational capacity of **6a** for adenosine deaminase is constant at 15–25°, but decreases by ~16% from 25° to 35°. The resin **6a** has been used to separate adenosine deaminase from mixtures containing other enzymes, for example, guanase or alcohol dehydrogenase.

INTRODUCTION

Immobilised nucleosides[1] have been used for affinity chromatography[2,3] and to study the interaction between an enzyme and its polymer-linked substrate[4]. The immobilisation of a nucleoside can be accomplished through covalent coupling to a polymer involving the nucleobase[5,6], the ribose moiety[7], or the phosphate group[8]. Each type of immobilisation offers particular advantages.

For the synthesis of a resin specific for adenosine deaminase (mol. wt. 35,000)[9], it is important to consider the binding properties of modified substrates. The interaction between the enzyme and adenosine derivatives depends mainly on the structure of the nucleobase. The deamination step involves[10] the transition state **7**. Structural alteration of HO-2′ and HO-3′ of the ribose moiety only slightly influences the deamination reaction. The reaction of the enzyme with 2′,3′-*O*-isopropylideneadenosine has a V_{max} ~50% of that for adenosine, whereas, for N^6-ethyladenosine, only

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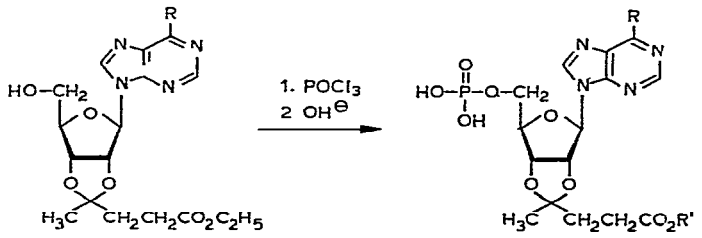
0.15% of the reaction velocity is reached[11]. Adenosine deaminase also shows specificity for the 5′-substituents, and adenosine-5′-phosphate can be deaminated only by a specific adenosine-5′-phosphate deaminase. Therefore, an affinity resin specific for a nucleoside deaminase should not link the base through N-6, but through O-2′ and O-3′

We have developed[12] a new method for immobilising ribonucleosides and ribonucleotides, in which binding to the polymer is effected through HO-2′,3′, and we now report on the preparation of agarose-bound inosine derivatives and their interaction with adenosine and guanine deaminases.

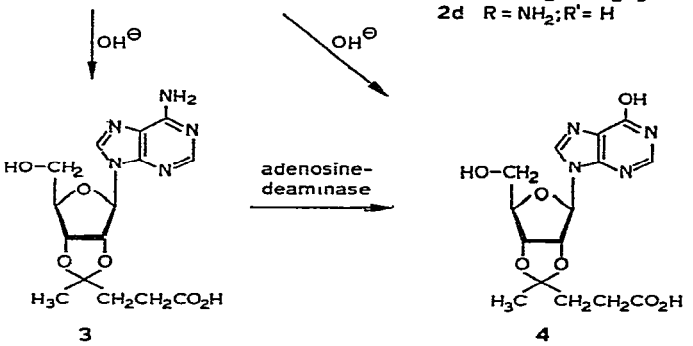
RESULTS AND DISCUSSION

Treatment[13] of adenosine with ethyl levulinate in the presence of hydrogen chloride and ethyl orthoformate gave the 2′,3′-*O*-[1-(2-ethoxycarbonylethyl)ethylidene] derivative **1b** in high yield. Under similar conditions, inosine gave the corresponding acetal **1a** (57%). Although the acetal carbon in **1a** and **1b** is a chiral centre, only one diastereoisomer was almost exclusively formed in each case, as indicated by ^{13}C-n.m.r. spectroscopy. Thus, 2′,3′-*O*-isopropylideneinosine, for example, gave ^{13}C signals for the acetal methyl groups at 25.1264 and 27.0044 p.p.m., whereas the acetals **1a** and **1b** showed only one such ^{13}C signal at 23.443 and 23.505 p.p.m., respectively, indicating that only one isomer was formed.

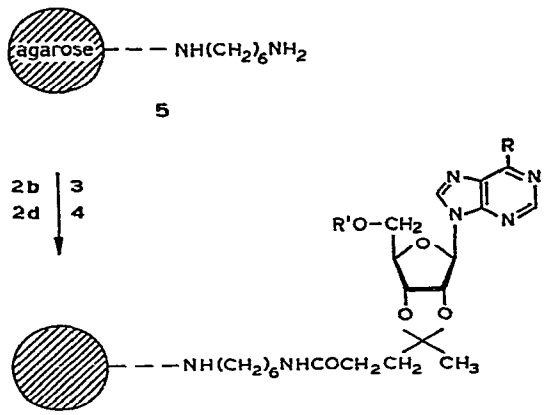


1a R = OH
1b R = NH_2

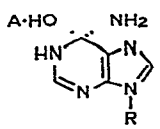
2a R = OH, R′ = C_2H_5
2b R = OH, R′ = H
2c R = NH_2; R′ = C_2H_5
2d R = NH_2; R′ = H



3 **4**



6a R = OH; R' = H
6b R = OH; R' = PO_3H_2
6c R = NH_2; R' = H
6d R = NH_2, R' = PO_3H_2



7 A = Adenosine deaminase
R = β-D-Ribofuranosyl

Saponification of **1a**, as already found for **1b**, affords the 2',3'-*O*-[1-(2-carboxyethyl)ethylidene]derivative **4**. Treatment of **1a** with phosphorus oxychloride in triethyl phosphate yielded the 5'-phosphate **2a**, which was not isolated but converted into the acid **2b** and isolated by ion-exchange chromatography on DEAE cellulose. The structure of **2b** was confirmed by phosphate determination and by its conversion into **4** by alkaline phosphatase.

Because of the good water-solubility of the inosine derivative **4**, coupling with the moist 6-aminohexylagarose derivative **5** to give the polymer **6a** was mediated with *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride in aqueous solution. Likewise, the 5'-phosphate **2b** was coupled to give the polymer **6b**. Reaction of the adenosine acetal **3** to give the polymer **6c** required aqueous *p*-dioxane.

Guanidino derivatives can be formed[14] by reaction of the nucleoside with the condensation reagent, but for the above reactions it was shown by t.l.c. (silica gel, solvent *B*) that no such products were formed.

The content of bound ligand (190 and 215 μmol/g, respectively) in the dry derivatives **6a** and **6c** was determined on the basis of the u.v. absorbance after hydrolysis, and for **6b** and **6d** (186 and 223 μmol/g, respectively) on the basis of

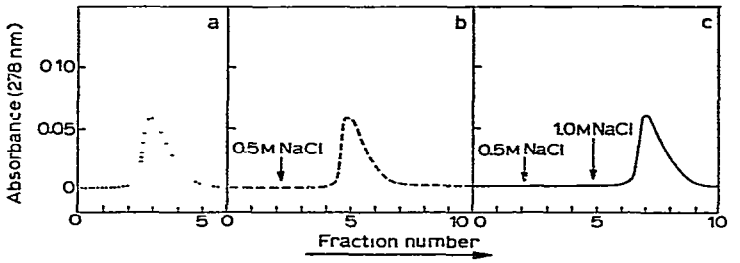


Fig. 1. Affinity chromatography of adenosine deaminase on the resins **5** (*a*), **6a** (*b*), and **6c** (*c*); 0.2 A_{278} unit of enzyme was applied to columns (20 × 0.9 cm) equilibrated with 0.15M phosphate buffer (pH 7.6) and eluted with sodium chloride.

the phosphate contents determined, after oxidation of the polymers, from the absorbance of the molybdate complex at 820 nm. For moist gels containing 96% of water, the values (μmol/g) are **6a** 7.4, **6b** 7.4, **6c** 8.6, and **6d** 8.9.

Since adenosine deaminase effects nucleophilic displacement of the amino group of the purine moiety by hydroxyl, a similar reaction was expected for the conversion of the acetals **3** → **4**. A quantitative reaction occurs within 2 h on incubation with adenosine deaminase at 37° in 0.1M Tris–HCl buffer at pH 7.6, as shown by t.l.c. Thus, the ligands **3** and **4** are bound specifically into the active site of the enzyme.

Because of the difference in the binding constants[10] of adenosine (K_M = 3.1 × 10^{-5}) and inosine (K_i = 16 × 10^{-5}) for adenosine deaminase, it was expected that the polymer **6c** would bind the enzyme more strongly than the polymer **6a**. The chromatographic behaviour of adenosine deaminase on the polymers confirmed this expectation (Fig. 1b,c). Whereas the enzyme was eluted from agarose within the void volume (Fig. 1a), 0.5M sodium chloride was required to elute the enzyme from **6a**, and M sodium chloride from **6c**. These results indicate a specific binding process.

In addition, the conversion **6c** → **6a** was observed on incubation of **6c** with an excess of adenosine deaminase in 0.15M phosphate buffer (pH 7.6) at 37° for 3 days. The structure of the product **6a** was confirmed by hydrolysis with 0.5M hydrochloric acid at 100° followed by thin-layer electrophoresis (solvents *C* and *D*) which revealed adenosine, inosine, and hypoxanthine. The last compound was not found in hydrolysates of **6c**. The enzymic conversion **6c** → **6a** demonstrates the specificity of the polymers to the enzyme.

The operational capacity of **6a** was 10.8 nmol of adenosine deaminase/g of moist polymer. This capacity was relatively independent of temperature at 15–25°, but decreased by ~16% from 25–35°.

By dividing the concentration of the ligand (7.2 μmol/g of moist gel) by the operational capacity, the molecular ratio of inosine ligands to bound adenosine deaminase is shown to be 650:1.

Affinity chromatography of the enzyme by elution with a linear gradient [5M sodium chloride (25 ml)/water (25 ml)] at 20 ml/h yielded an elution pattern of the Poisson type; at 80 ml/h, the curve was approximately of the Gauss type. The high

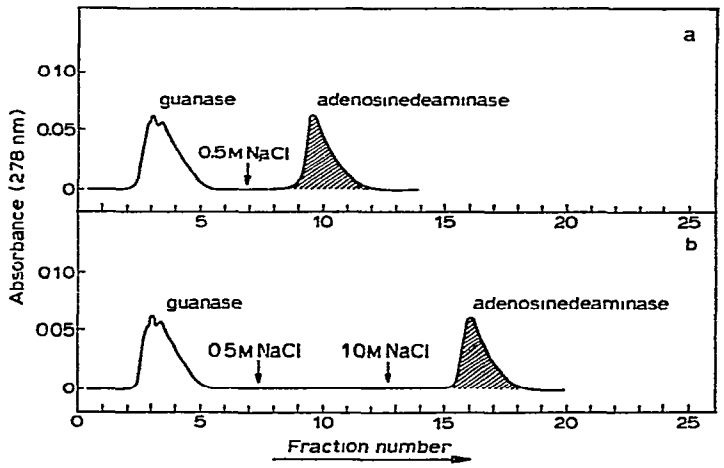


Fig. 2. Affinity chromatography of adenosine deaminase in the presence of guanase on the resins **6a** (*a*) and **6c** (*b*). A mixture containing 0.2 A_{278} unit each of adenosine deaminase and guanase was applied to columns (20 × 0.9 cm) equilibrated with 0.15M phosphate buffer (pH 7.6). Guanase was eluted with the void volume of buffer, whereas adenosine deaminase was eluted with sodium chloride.

flow rate counteracts the back reaction of the enzyme with the ligand shown in the step k_5/k_6 in the following scheme.

$$[\mathrm{ADA}] + [\mathbf{6c}] \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [\mathrm{ADA} \cdot \mathbf{6c}] \underset{k_4}{\overset{k_3}{\rightleftharpoons}} [\mathrm{ADA} \cdot \mathbf{6a}] \underset{k_6}{\overset{k_5}{\rightleftharpoons}} [\mathrm{ADA}] + [\mathbf{6a}]$$

The width of the elution peak therefore decreases, with an advantageous effect on the number of theoretical plates of the column. The step k_1/k_2 involves the adsorption (k_1) and the elution (k_2) of the enzyme for the substrate gel **6c**, k_3 refers to the conversion **6c** → **6a**, and k_5/k_6 relates to the adsorption (k_6) and elution (k_5) of adenosine deaminase for the product gel **6a**.

When mixtures of adenosine deaminase and guanase or alcohol dehydrogenase were eluted from columns **6a** and **6c** (Fig. 2), the latter enzyme was eluted with the void volume of buffer (Sørensen, pH 7.6), whereas adenosine deaminase required aqueous sodium chloride. Adenosine deaminase activity was assayed according to the method of Kalckar[15].

EXPERIMENTAL

General. — Melting points were determined on a Büchi SMP 20 apparatus and are not corrected. The ^{1}H- and ^{13}C-n.m.r. spectra were measured on solutions in Me_2SO-d_6 (internal Me_4Si) with Bruker HX-60 or WP-270 spectrometers, and chemical shifts are reported in δ values (p.p.m.). U.v. spectra were measured with Zeiss PMQ 3 or Shimadzu UV-200 spectrometers. Microanalyses were performed by Mikroanalytisches Labor Beller, Göttingen.

Column chromatography was performed on DEAE cellulose (DE-52, Whatman) using an UltroRac fraction collector with a Uvicord III (LKB Instruments, Sweden) as detection unit. T.l.c. was performed on silica gel F-254 (Woelm, Eschwege, Germany) with *A*, methanol–water (1:4); and *B*, 0.25M LiCl. Thin-layer electrophoresis (t.l.e.) was performed on silica gel in a TLE Double Chamber (Desaga, Heidelberg, Germany) with *C*, 0.1M sodium formate (pH 3.5); and *D*, 0.1M sodium borate (pH 10.0).

The triethylammonium hydrogen carbonate buffer was prepared by bubbling carbon dioxide through a solution of triethylamine (720 ml) in water (4280 ml) until pH 7.0 was obtained.

Affinity chromatography was performed on jacketed columns (20 × 0.9 cm) of the appropriate gel and equilibrated with twice the void volume of 0.15M phosphate buffer (pH 7.6). The columns were attached to a Uvicord 3 photometer (LKB Instruments, Sweden), on which the absorbance at 280 nm was read. Fractions (2 ml) were collected with an UltroRac or RediRac fraction collector using a Vario Perpex pump II (LKB Instruments, Sweden).

6-Aminohexylagarose (AH-Sepharose 4B, **5**) was purchased from Pharmacia (Uppsala, Sweden), nucleosides from Pharma Waldhof (Düsseldorf, Germany), and adenosine deaminase (EC 3.5.4.4), guanase (EC 3.5.4.3), and alcohol dehydrogenase (EC 1.1.1.1) from Boehringer (Mannheim, Germany).

Phosphate determination. — An aliquot (0.7 ml) of a freshly prepared 1:6 mixture of 10% aqueous L-ascorbic acid and 0.42% ammonium molybdate tetrahydrate in 0.5M H_2SO_4 was added to a solution (0.3 ml) of the unknown phosphate. The mixture was kept at 37° for 1 h and the absorbance was read at 820 nm.

Cleavage of 5′-phosphates with alkaline phosphatase. — A suspension (10 µl) of *E. coli* alkaline phosphatase (EC 3.1.3.1.; Boehringer, Mannheim, Germany; 1 mg/ml) was diluted with 0.25M Tris–HCl buffer (90 µl, pH 8.0). A portion (10 µl) of this solution was added to the substrate (10 µl) and incubated at 37° for 2 h.

2′,3′-O-[1-(2-Ethoxycarbonylethyl)ethylidene]inosine (**1a**). — To a suspension of inosine (10.73 g, 40 mmol) in dry *N,N*-dimethylformamide (150 ml), ethyl levulinate (11.52 g, 80 mmol) and triethyl orthoformate (10 ml, 60 mmol) were added, and insoluble material was dissolved by adding a solution (20 ml) of 7M hydrogen chloride in dry *p*-dioxane. The mixture was kept for 24 h at room temperature and then poured into ether (1.5 l). The upper layer was decanted, and the oily residue was washed twice with ether and then dissolved in chloroform by the addition of 2% aqueous sodium hydrogen carbonate. The organic layer was washed with water, dried, and concentrated, and the residue was crystallised and recrystallised from methanol–water to give **1a** (8.92 g, 57.2%), m.p. 207–209°, R_F 0.88 (solvent *A*), λ_{max}^{MeOH} 245 nm (ε 10,500). ^{1}H-N.m.r. data: δ 8.3 (s, H-2), 8.1 (s, H-8), 6.15 (d, H-1′), 5.35 (q, H-2′), 4.95 (q, H-3′), 4.2 (m, H-4′), ~4.06 (q, ester CH_2), 3.5 (d, H-5′,5′), 2.3 (m, aliphatic CH_2), 1.35 (s, acetal Me), and 1.2 (s, ester Me). ^{13}C-N.m.r. data: δ 172.475 (C=O), 156.465 (C-6), 113.761 (acetal C), 89.591 (C-1′), 86.756 (C-4′), 83.851 (C-2′), 81.154 (C-3′), 61.341 (C-5′), 59.83 (O-CH_2), 33.333 (CH_2-C=O), 28.077 (acetal CH_2), 23.443 (acetal Me), and 13.969 (ester Me).

Anal. Calc. for $C_{17}H_{22}N_4O_7$: C, 51.77; H, 5.62; N, 14.21. Found: C, 51.70; H, 5.73; N, 14.23.

2',3'-O-[1-(2-Ethoxycarbonylethyl)ethylideneadenosine[12] (**1b**). — ^{13}C-N.m.r. data: δ 172.548 (C=O), 156.104 (C-6), 152.606 (C-2), 148.814 (C-4), 139.693 (C-8), 119.064 (C-5), 113.735 (acetal C), 89.347 (C-1'), 86.535 (C-4'), 83.331 (C-2'), 81.305 (C-3'), 61.428 (C-5'), 59.859 (O-CH_2), 33.411 (CH_2-C=O), 28.115 (acetal CH_2), 23.505 (acetal Me), and 13.992 (ester Me).

2',3'-O-[1-(2-Carboxyethyl)ethylidene]inosine (**4**) *and its 5'-phosphate* (**2b**). — A solution of **1a** (2 g, 5.07 mmol) in a 1:1 mixture (120 ml) of ethanol and M sodium hydroxide was kept for 30 min at room temperature, and then neutralised (glass electrode) with Amberlite IR-120 (H^+) resin, filtered, and concentrated. The residue was crystallised from water–acetic acid to give **4** (1.76 g, 89%) as colourless crystals. Recrystallisation from the same solvent mixture gave material with m.p. 188–190°, R_F 0.56 (solvent *B*), and λ_{max}^{MeOH} 245 nm (ε 9,900). ^{1}H-N.m.r. data: δ 8.3 (s, H-4), 8.1 (s, H-8), 7.6 (m, 3 OH), 6.15 (d, H-1'), 5.26 (q, H-2'), 4.96 (q, H-3'), 4.25 (m, H-4'), 3.55 (d, H-5',5'), 2.2 (m, aliphatic CH_2), and 1.3 (s, acetal Me). ^{13}C-N.m.r. data: δ 175.71 (C=O), 156.946 (C-6), 147.807 (C-4), 146.575 (C-2), 138.85 (C-8), 124.432 (C-5), 114.476 (acetal C), 89.57 (C-1'), 86.673 (C-4'), 83.676 (C-2'), 81.112 (C-3'), 61.4 (C-5'), 35.062 (CH_2-C=O), 30.5 (acetal CH_2), and 23.441 (acetal Me).

Anal. Calc. for $C_{15}H_{18}N_4O_7$: C, 48.18; H, 4.95; N, 15.29. Found: C, 48.24; H, 5.04; N, 15.26.

To a solution of **1a** (394 mg, 1 mmol) in triethyl phosphate (5 ml) was added $POCl_3$ (180 μl, 2 μmol) at 4°. After storing for 12 h at 4°, the solution was neutralised with M sodium hydroxide and then concentrated *in vacuo*. A suspension of the residue in 0.5M sodium hydroxide (20 ml) was stirred for 30 min and the precipitate was dissolved with M hydrochloric acid. The solution was diluted with water (300 ml) and eluted from a column (25 × 3.5 cm) of DEAE cellulose DE-52 with a linear gradient derived from 0.5M triethylammonium hydrogen carbonate buffer (1 litre) and water (1 litre). Compound **4** was eluted first followed by the phosphate **2b** at ~0.3M buffer. The phosphate-containing fractions were combined, and concentrated *in vacuo*, and excess of buffer was removed by repeated evaporation of aqueous ethanol from the residue to yield **2b** (4410 A_{245} units, 42%, using ε of **1a**) as a colourless, amorphous solid, R_F 0.61 (solvent *B*); **4** had R_F 0.54.

Cleavage of **2b** with alkaline phosphatase gave **1a** as the only reaction product. 0.44 A_{245} unit of **2b** (0.042 μmol) gave 0.96 A_{820} unit of molybdate complex, corresponding to 0.040 μmol of phosphate.

Coupling reactions of 6-aminohexylagarose (**5**). — (*a*) To a solution of **4** (50 mg, 0.14 mmol) in water (10 ml), agarose gel (**5**, 5 ml) was added at pH 6. The suspension was shaken for 10 min, and the ligand was coupled by the addition of *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (100 mg, 0.52 mmol). Shaking was continued overnight, and the agarose derivative was collected, washed with 0.1M sodium hydrogen carbonate (pH 8.0, 250 ml), 0.5M sodium chloride (250 ml), and water to give the coupled product **6a** (5 g).

A portion (250 mg) of moist **6a** was hydrolysed in 0.5M hydrochloric acid (5 ml) for 30 min at 100°, and the cooled solution was diluted to 50 ml. Standard samples of pure **4** (1.25 μmol) and **5** (250 mg) were treated in the same manner. The absorbances at 245 nm (1-cm path-length) were: **6a**, 0.680; **5**, 0.346; and **4**, 0.058. Thus, the hydrolysed sample contained 0.680 − 0.346 = 0.334 absorbance unit. Since 1.25 μmol of **4** gave an absorbance of 0.058, 7.2 μmol of ligand/g of gel were covalently linked in **6a**.

Portions (500 mg) of moist **6a** and **5** were dried for 72 h at 95° in high vacuum to constant weight. The dry powders (9.3 mg, each) and **4** (10.021 μmol) were hydrolysed and diluted as described above. The absorbances at 245 nm (1-cm path-length) were: **6a**, 0.717; **5**, 0.422; and **4**, 1.67. Thus, the hydrolysed samples contained 0.717 − 0.422 = 0.295 absorbance unit ≙ 1.77 μmol/9.3 mg of powder ≙ 190.2 μmol of ligand/g of powder.

(*b*) The 5′-phosphate **2b** (0.2 mmol, 2100 A_{245} units) was coupled to moist **5** (5 g), as described in (*a*), to give **6b**. A portion (1 g) of the packed gel was dried for 72 h at 95° in high vacuum to constant weight; there was ~96% loss in weight. The dry powder (3.8 mg) was mixed with water (0.5 ml) and 10% $Mg(NO_3)_2$ in ethanol–water (1.5 ml, 1:1), swollen for 15 min, and then incinerated. The residue was hydrolysed with M hydrochloric acid (7.5 ml) for 30 min at 100°. An aliquot (300 μl) of the solution was mixed with phosphate-determination reagent (solution, 700 μl) and stored at 37° for 1 h. The absorbance at 820 nm (1-cm path-length) was then 0.68, and since 1 μmol of phosphate = 24 A_{820} units, this corresponds to 184.4 μmol of ligand/g of dry gel, and 7.4 μmol of ligand/g of moist gel.

(*c*) The coupling of the adenosine derivative **4** with **5** was carried out according to the method of Seela and Waldeck[12] to give **6c**. Dry **6c** (5.8 mg), dry **5** (5.8 mg), and 1.25 μmol of **3** (467 μg) were treated as described in (*a*). The resulting absorbances were as follows: **5**, 0.020; **6c**, 0.370; and **3**, 0.345. Thus, the hydrolysed solution of **6c** contained 0.37 − 0.02 = 0.35 absorbance unit, corresponding to 215 μmol of ligand/g of dry **6c**, and 8.6 μmol/g of moist **6c**.

(*d*) The 5′-phosphate **2d** was coupled to **5**, as described by Seela and Waldeck[12], to give **6d**. The determination of bound ligand was carried out as described in (*b*), and gave a value of 223 μmol of phosphate/g of dry **6d**.

Affinity chromatography of adenosine deaminase on the polymers **6a** *and* **6c**. — The gels **6a** and **6c** were thoroughly washed with water and packed in jacketed columns (20 × 0.9 cm). The flow rates (10, 20, and 80 ml/h) were adjusted with a Vario Perpex pump 2. The columns were first equilibrated by washing with 2–3 void volumes of Sørensen buffer (pH 7.6). For each run, 0.4 A_{278} unit of adenosine deaminase, guanine deaminase, or alcohol dehydrogenase was applied to each column. Elution was monitored on the basis of absorbance at 278 nm and was quantitative.

For determination of the operational capacities, 0.8 A_{278} unit was applied to a column of **6a** at 25°; 0.1 A_{278} unit was recovered in the effluent. By using 0.5M sodium chloride, 0.705 A_{278} unit of bound enzyme was eluted. Therefore, 1 ml of

6a can bind 337 μg of enzyme (*i.e.*, 10.9 nmol). At 15° and 35°, operational capacities of 10.7 and 9.1 nmol/ml of **6a**, respectively, were determined.

When mixtures of 0.4 A_{278} unit of adenosine deaminase with either the same amount of alcohol dehydrogenase or guanase were applied to columns of **5**, **6a**, and **6c**, the latter enzymes were eluted with the void volume and contained no adenosine deaminase activity, whereas the adenosine deaminase required 0.5M sodium chloride for elution from **6a**, and M sodium chloride for elution from **6c**. In the 10-ml effluents of all peaks, samples (100 μl) were diluted with adenosine solution (900 μl, 10 μg/ml), and the absorbances at 265 and 245 nm were read. Since a large excess of enzyme was used, complete deamination took place within 1 min, and ΔA_{265} and ΔA_{245} values (−0.254 and +0.083, respectively) were determined in the case of retarded material.

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