Preparation of Adenosine Nucleotide Derivatives Suitable for Affinity Chromatography

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Methods of synthesizing a series of chemically-defined AMP, ADP, ATP, adenylyl imidodiphosphate and pyrophosphate derivatives suitable for affinity chromatography are extensively described. Each derivative has a single primary amino group at the end of a hexamethylene 'spacer' chain for attachment to CNBr-activated agarose. The synthesis of the derivative where the 'spacer' arm is attached directly to the 8 position of the adenine ring to produce 8-(6-aminohexyl)amino-AMP involves the direct bromination of AMP in the 8 position followed by displacement of the halogen by 1,6-diaminohexane. This monophosphate derivative can then be converted into the corresponding di- or triphosphate forms by direct phosphate condensation with carbonyl di-imidazole. A second series of adenosine phosphate derivatives with the phosphate moieties unsubstituted has been similarly prepared from *N*⁶-(6-aminohexyl)-AMP (Guilford *et al.*, 1972). A third type of ligand has been synthesized by condensing the phosphoryl imidazolide of AMP with 6-aminohex-1-yl phosphate. This compound, *P*¹-(6-aminohex-1-yl) *P*²-(5'-adenosyl) pyrophosphate, has an unsubstituted adenine ring. The synthesis of a fourth type of ligand, 6-aminohex-1-yl pyrophosphate, was done by heating 6-aminohexan-1-ol with crystalline pyrophosphoric acid under reduced pressure. The structures of the synthesized compounds were confirmed by chemical, electrophoretic and chromatographic methods and by u.v. spectrometry. The general applicability of the synthetic methods used is discussed in relation to the preparation of other affinity adsorbents. Examples are given where these derivatives have been successful in reversibly binding dehydrogenases, kinases and myosin and its proteolytic subfragments. The partial purification of rat liver glucokinase on an ADP derivative is shown.

The idea of basing protein-separation methods on the molecular affinities found in biological systems is not new (Starkenstein, 1910; Campbell *et al.*, 1951), but the introduction of agarose polymers as a support matrix together with the CNBr activation procedure (Axen *et al.*, 1967) has led to great exploitation of this technique in recent years (see reviews by Cuatrecasas & Anfinsen, 1971*a,b*). Most of the work in this field has concerned itself solely with enzyme purification although the wider implications of the technique for the investigation of more complex biological interactions are increasingly being realized. Initially the preparation of biospecific adsorbents for protein purification attempted to utilize immobilized substrates or substrate analogues primarily for the adsorption and elution of a single enzyme. More recently the concept of 'general ligand' affinity chromatography, where a ligand capable of interacting with a wide variety of enzymes is insolubilized, has received much attention, e.g. for the separation of nicotinamide nucleotide-dependent dehydrogenases on columns containing covalently-bound AMP or NAD⁺ derivatives (Mosbach *et al.*, 1972; Lowe & Dean, 1971).

Much of the work in this field has been done on affinity columns prepared by the methods outlined by Cuatrecasas (1970). These procedures involve the initial preparation of an agarose-'spacer-arm' conjugate to which the ligand of choice is then attached in some way. Unless the coupling is essentially complete and chemically unambiguous, the resulting biopolymer may still possess potentially reactive groups or the desired ligand may be covalently bound to the 'spacer' molecule in a number of ways, each of which could interact with enzyme(s) in a differing manner. This may be satisfactory if enzyme purification is the prime objective. It could, however, help explain some of the anomalies noticed during affinity chromatography (O'Carra *et al.*, 1973) and perhaps even give rise to a concurrent hydrophobic chromatography (Er-el *et al.*, 1972; Shalteil & Er-el, 1973). Some of those problems can be alleviated by preparing and purifying derivatives of the required ligand containing a primary amino group before coupling to an agarose matrix by the CNBr activation procedure. Much higher purifications on columns containing chemically defined ligands (Shaper *et al.*, 1973; Barker *et al.*, 1972) may be possible, thus justifying

the additional preliminary work. Certainly it is necessary to have more exact information about the chemical nature of the immobilized ligand if affinity chromatography is to be used for investigating more complex biological interactions, e.g. an investigation into enzyme mechanistic processes (Barker *et al.*, 1972; Trayer & Olsen, 1972), separation of isoenzymes (Brodelius & Mosbach, 1973), and the study of complex formation between enzymes and other ligands (Akanuma *et al.*, 1971).

This study describes the synthesis of a series of chemically-defined AMP, ADP and ATP derivatives that contain primary amino groups suitable for coupling to CNBr-activated agarose. The synthetic methods employed are all in fairly common use, enable high yields of the final product to be obtained and are capable of being applied, in some instances, to the immobilization of other nucleoside phosphates. Procedures are given for attachment of the adenosine phosphate through the 6-amino moiety and the 8-position of the adenine nucleus and by phosphodiester linkage through the terminal phosphate. The synthesis of a simple pyrophosphate ligand is also presented. The affinity columns so obtained clearly have numerous applications in enzyme chemistry and a few examples where they have been successfully employed in reversibly binding dehydrogenases, kinases, and myosin and its proteolytic subfragments are described.

Materials and Methods

Chemicals

Organic solvents (Analar grade) were dried for at least 48 h over molecular sieves, type 4A (BDH Chemicals Ltd., Poole, Dorset, U.K.) and were used without further purification. Other reagents, which were not further purified unless otherwise stated, were obtained from the sources indicated. 1,6-Diaminohexane, crystalline phosphoric acid, Celite analytic filter aid, phosphoryl chloride, triethyl phosphate and tributylamine were obtained from BDH Chemicals Ltd. Nucleotides and nucleosides, Dowex ion-exchange resins and β -NAD (type III) were purchased from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K. 6-Aminohexan-1-ol was bought from Aldrich Chemical Co., Milwaukee, Wis., U.S.A., crystalline pyrophosphoric acid was from Fluka A.G. Chemische Fabrik, Buchs, Switzerland, ethyl trifluorothiol acetate was from Pierce Chemical Co., Rockford, Ill., U.S.A., and CNBr was from either Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K., or R. Emanuel Ltd., Wembley, Middx., U.K. DEAE-cellulose (DE-52) was obtained from Whatman Biochemicals, Maidstone, Kent, U.K., and Sepharose-4B from Pharmacia (GB) Ltd., London W.5,

U.K. Carbonyl di-imidazole was purchased either from Sigma Chemical Co. Ltd. or Fluka A.G. Chemische Fabrik. Care must be taken before using this chemical (m.p. 113–115°C) and it is often supplied already decomposed to imidazole (m.p. 88–90°C). It should be kept under strictly anhydrous conditions and can, in our experience, be used satisfactorily if the m.p. is greater than 105°C.

Proteins

Yeast hexokinase (ATP-D-hexose 6-phosphotransferase, EC 2.7.1.1; type III) (14 units/mg) and lactate dehydrogenase (L-lactate-NAD oxidoreductase, EC 1.1.1.27; rabbit muscle, crude, type I) (80 units/mg) containing pyruvate kinase (ATP-pyruvate phosphotransferase, EC 2.7.1.40) activity (110 units/mg) were purchased from Sigma Chemical Co. Ltd. Human serum albumin was obtained from Pentex Biochemicals Ltd., Kankakee, Ill., U.S.A.

Enzyme preparations and assays

Myosin was extracted and purified from the longissimus dorsi of New Zealand White rabbits by the method of Trayer & Perry (1966). Heavy meromyosin was prepared from this by tryptic digestion (Szent-Gyorgyi, 1953) and subfragment 1 was prepared by digestion with Sepharose-papain (Bottomley & Trayer, 1973). Samples of rat liver supernatant containing hexokinase and glucokinase activities were prepared as described by Cheshier *et al.* (1973) except that the EDTA was omitted from the homogenizing buffer.

Measurements of the Ca^{2+} -stimulated ATPase (adenosine triphosphatase) activities of myosin and its subfragments were carried out either by a pH-stat method (Moos & Eisenberg, 1967) or by a direct determination of P_i (Fiske & Subbarow, 1925) after incubation as described by Trayer & Perry (1966). Glucokinase and hexokinase were assayed spectrophotometrically as described by Parry & Walker (1966). Lactate dehydrogenase activity was determined, by measuring the decrease in E_{340} as NADH is oxidized, with a procedure slightly modified from the method of Mosbach *et al.* (1972). The incubation medium contained NADH (0.66 μmol), sodium pyruvate (2.25 μmol), test solution (up to 0.15 ml) and 0.1 M-sodium phosphate buffer, pH 7.0, in a final volume of 3.0 ml. Pyruvate kinase activity was also determined from the decrease in E_{340} in a coupled procedure slightly modified from that described by Mosbach *et al.* (1972). The incubation medium consisted of NADH (0.66 μmol), ADP (5.0 μmol), phosphoenolpyruvic acid (2.0 μmol), lactate dehydrogenase (0.1 mg), test solution (0.1–0.2 ml) and 0.05 M-imidazole-HCl buffer, pH 7.6, (containing 0.12 M-KCl and 0.06 M- MgSO_4) in a final volume of 3.0 ml.

Preparation of tributylammonium salts of phosphate esters

The procedure given below was used for the preparation of tributylammonium phosphate. An identical sequence of operations was used to prepare the tributylammonium salt of pyrophosphate, imidodiphosphate and AMP. An aqueous solution of phosphoric acid (5mmol) in 5ml of water was passed over a column (6cm×0.6cm) of Dowex 50 (X8; pyridinium cycle; 20–50 mesh) resin to remove any inorganic cations. To the solution of pyridinium phosphate so obtained was added tributylamine (1.2ml, 5mmol) and after mixing, the solution was concentrated. The syrupy residue was dried down from pyridine (5×10ml) and then from dimethylformamide (3×10ml) and finally dissolved in dimethylformamide. When drying down from pyridine and dimethylformamide the rotary evaporator was connected to a mechanical vacuum pump through a solid-CO₂ trap. For most other purposes a water pump was sufficient.

Preparation of sodium imidodiphosphate

This was prepared by the hydrolysis of diphenylimidodiphosphoric acid as described by Yount *et al.* (1971). Diphenylimidodiphosphoric acid was synthesized from diphenylphosphoramidate and PCl₅ as outlined by Kirsanov & Zhmurova (1958). Phosphate analysis gave a formula weight corresponding to sodium imidodiphosphate, undecahydrate. The crystals were stored at –20°C.

Analytical methods

Elementary analyses were performed by the Department of Inorganic Chemistry, University of Birmingham, Birmingham, U.K. Acid-labile phosphate was determined on the nucleotide phosphate derivatives after heating for 30min at 100°C in 1M-HCl. P_i produced was measured as described by Bartlett (1959). Total phosphorus was determined as P_i by the same procedure after oxidation of the organic phosphate (Bartlett, 1959).

Unless otherwise stated molar extinction coefficients were determined on a weight basis on the free acids of the adenosine nucleotide derivatives after removal of cations by Dowex 50 (H⁺) and drying the sample overnight in a heated (40°C) vacuum desiccator. Phosphate analyses were generally based on this coefficient.

Quantitative ribose determinations were done by the procedure of Dische & Borenfreund (1957) on acid hydrolysates (3M-HCl at 100°C for 1h) of the sample by using AMP treated in an analogous manner as the standard.

Spectral data were collected on a Unicam SP. 1800 u.v. recording spectrophotometer (Pye Unicam Ltd., Cambridge, U.K.).

Chromatographic procedures

DEAE cellulose (HCO₃[–] cycle) was prepared by making a slurry of Whatman DE-52 in 1 litre of 0.4M-triethylammonium bicarbonate, pH7.5, filtering under suction and then washing the cake with water (about 3 litres). Dowex-50 (pyridinium cycle) was prepared by treating the acid form with aq. 2.5M-pyridine and washing with water until the washings were neutral. Dowex-1 was converted into the chloride form by standard procedures and the other forms were prepared from this. The bicarbonate form was obtained by treating the chloride form with 0.5M-NaOH, and then by washing with water until neutral, 0.4M-triethylammonium bicarbonate (1l) and finally water (2 litres). The acetate form was obtained by washing successively with 3M-sodium acetate until no more Cl[–] ions were detected in the filtrate, water (2l), 1M-sodium acetate containing 6M-acetic acid (1l), acetic acid (500ml) and finally water until neutral. The formate form was prepared in an identical manner by using sodium formate/formic acid washings.

Triethylammonium bicarbonate buffers were prepared by bubbling CO₂ through a sintered-glass diffuser into triethylamine solutions (either in an ice bath or at room temperature, depending on whether the column was to be operated at room temperature or at 4°C) until the pH fell to 7.5. After chromatography in this buffer, the triethylammonium bicarbonate could be removed from the appropriate fractions by repeated addition of and evaporation from ethanol at 40°C.

All columns in the first section of the Results section (under 'Synthesis of ligands') were operated at room temperature unless stated otherwise, whereas the affinity chromatography was performed at 4°C.

The progress of the various gradient elutions used in this study were monitored on a conductivity meter, type CDM (Radiometer, Copenhagen, Denmark).

Electrophoresis and chromatography

High-voltage paper electrophoresis was performed under varsol on Whatman no. 1 paper at a potential gradient of 50V/cm in all buffer systems. Pyridine-acetate buffers were used at pH6.5 (pyridine-acetic acid-water, 2.5:1:225, by vol.) and pH3.5 (pyridine-acetic acid-water, 1:10:90, by vol.) and formic acid-acetic acid at pH2.0 (90% formic acid-acetic acid-water, 1:4:45, by vol.).

Ascending paper chromatography for the organic compounds was carried out on Whatman no. 1 paper with butan-1-ol-acetic acid-water (5:2:3, by

vol.) as solvent 1; iso-butyric acid–conc. aq. NH_3 –water (66:1:33, by vol.) as solvent 2; and propan-2-ol–aq. 0.25M- NH_4HCO_3 (65:35, v/v) as solvent 3.

Chromatograms and electrophoretograms were developed with the ninhydrin–cadmium reagent for amines (Barrolier, 1957), molybdate spray for phosphates (Bandurski & Axelrod, 1951) and the periodate spray for sugar vicinal hydroxyl groups (Metzenberg & Mitchell, 1954). Adenine-containing compounds were also examined under u.v.

Preparation of Sepharose adsorbents

The ligands were coupled to CNBr-activated Sepharose 4B essentially as described by Axen *et al.* (1967). About 150–200mg of CNBr/g of packed wet gel was used during the activation procedure. Coupling was carried out in 0.1M- Na_2CO_3 , pH9.5, overnight at 4°C. This procedure generally gave a coupling efficiency of about 70–95% resulting in adsorbents with 3–5 μmol of ligand/g of packed wet gel. The extent of coupling was determined on the exhaustively washed gel by phosphate analyses after oxidation of the Sepharose–ligand conjugate by the method of Bartlett (1959).

Results and Discussion

The synthesis of the compounds* shown in Fig. 1 is given below. Compounds of type A and B contain a 1,6-diaminohexyl group at the 8 position (type A) or at the 6 position (type B) (Guilford *et al.*, 1972) of the purine ring and provide a nucleotide with a free phosphate substituent. Conversely, the synthesis of a phosphodiester bond between the terminal phosphate of ADP and 6-aminoheptan-1-ol as in compound C leaves a free adenosine substituent. Compound D is a simple pyrophosphate ester of 6-aminoheptan-1-ol and has proved particularly useful in work with myosin (H. R. Trayer, R. C. Bottomley & I. P. Trayer, unpublished work).

Synthesis of ligands

(a) *Adenosine phosphate ligands substituted in the 8 position of the adenine ring (type A compounds).* The synthetic sequence shown in Scheme 1 was used to prepare the AMP, ADP, ATP and AMP·PNP derivatives.

(i) 8-Bromo-AMP (II). The synthesis of this compound was based on the method of Ikehara & Uesugi (1969). Disodium AMP (I) (0.9g, 2mmol) was dissolved in 1M-sodium acetate buffer, pH4.0,

* Abbreviations: C8-AMP, C8-ADP, C8-ATP, C8-AMP·PNP, for compounds of the type A series; N⁶-AMP, N⁶-ADP, N⁶-ATP, N⁶-AMP·PNP, for compounds of the type B series; P-ADP, P¹-(6-aminohept-1-yl) P²-(5'-adenosyl) pyrophosphate; HAPP, 6-aminoheptan-1-ol pyrophosphate.

(80ml) and bromine–water (0.15ml of bromine, 3mmol, dissolved in 15ml of water with vigorous shaking) was added. After a thorough mixing of the reactants in a stoppered flask, the solution was stored in the dark at room temperature for 18h. The pale-yellow colour was discharged from the solution by addition of sodium metabisulphite (0.1g). The solvent was removed by rotary evaporation and the residue concentrated several times from ethanol to give a white solid. This was dissolved in water (100ml) and applied to a column (20cm×1.5cm) of Dowex 1 (X8; formate cycle; 200–400 mesh) which was washed with water (300ml) and then developed with 0.1M-formic acid. Two major u.v.-absorbing peaks were eluted. The first, which contained some 8–9% of the applied 260nm-absorbing material, had a u.v.-absorption spectrum identical with that of AMP. The second peak ($\lambda_{\text{max}}^{\text{H}^+}$ 262.5nm) was eluted between 1 and 2 litres and usually contained between 80–90% of the material applied as judged by extinction (260nm) measurements. Activated charcoal (1g) was added to the pooled fractions from the second peak and stirred for several minutes. The charcoal was separated by filtration and the filtrate monitored for u.v.-absorbing material. The process was continued until no appreciable material absorbing at 260nm was found in the filtrate; 4g of charcoal was used. The product was eluted from the charcoal by washing several times with aq. 50% (v/v) ethanol (250ml) containing 2% (v/v) conc. aq. NH_3 . The combined washings (1.5–2 litres), containing more than 90% of the 260nm-absorbing material eluted from the column were concentrated to dryness. The product, which is the diammonium salt of II, was obtained in about 75% overall yield based on u.v. absorption. The product migrated as a single spot on high-voltage paper electrophoresis at pH6.5 and pH3.5, and on paper chromatography (Table 1). Bromide ions could also be detected after a sample was subjected to the sodium-fusion technique. U.v.-absorption properties:

$$\begin{aligned}\lambda_{\text{max}}^{\text{H}^+} &= 262.5\text{nm} (\epsilon_{262.5}^{\text{litre}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}} = 16400); & \lambda_{\text{min}}^{\text{H}^+} &= 233\text{nm} \\ \lambda_{\text{max}}^{\text{H}^+} &= 264.5\text{nm} (\epsilon_{264.5}^{\text{litre}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}} = 15100); & \lambda_{\text{min}}^{\text{H}^+} &= 232.5\text{nm}\end{aligned}$$

(ii) 8-(6-Aminoheptylamino-AMP (III). The diammonium salt of compound II (0.48g, 1mmol) was dissolved in water (50ml), and 1,6-diaminohexane (3.48g, 30mmol) was added. (The large excess of the diamine was necessary to prevent the formation of compounds where two adenine rings are joined by one molecule of diamine.) The resulting solution was distributed between six thick-wall Pyrex test tubes and sealed into these. After heating these tubes at 140°C for 2h in an oil bath, the cooled fractions were pooled, diluted to 150–200ml with water and adjusted

Fig. 1. Adenosine phosphate and pyrophosphate ligands

A = 8-(6-Aminoethyl)amino-AMP (R = H) (C8-AMP). B = N⁶-(6-Aminoethyl)-AMP (R=H) (N⁶-AMP). The synthesis of the corresponding ADP, ATP and adenylyl imidodiphosphate analogues of compounds A and B, where

respectively, is also described. C = P¹-(6-aminohex-1-yl) P²-(5'-adenosyl) pyrophosphate (P-ADP). D = 6-Aminohexan-1-ol pyrophosphate (HAPP).

to pH11.5. This solution was applied to a column (20cm×1.5cm) of Dowex 1 (X8; acetate cycle; 200–400 mesh) and developed with water (200ml) to remove unchanged diamine. The product was eluted by applying a linear gradient formed from 1 litre each of water and 1 M-acetic acid. The major 260nm-absorbing peak, which was eluted in the first 15–20% of the gradient and which contained some 85% of the total 260nm-absorbing material applied to the column, was collected, concentrated to a small volume and freeze-dried. The product obtained was shown to be homogeneous by high-voltage paper electrophoresis at pH6.5 (neutral) and pH2.0 (cationic), and by paper chromatography (Table 1). The structure (III) given in Scheme I was assigned to

the product on the basis of this and the following information. The u.v.-absorption spectrum was characteristic of 8-aminoalkyl derivatives of the adenine nucleus (Muneyama *et al.*, 1971):

$$\begin{aligned} \lambda_{\text{max}}^{\text{pH } 1} &= 277 \text{ nm } (\epsilon_{277}^{\text{litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}} = 17700); & \lambda_{\text{min}}^{\text{pH } 1} &= 240 \text{ nm} \\ \lambda_{\text{max}}^{\text{pH } 11} &= 278 \text{ nm } (\epsilon_{278}^{\text{litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}} = 20400); & \lambda_{\text{min}}^{\text{pH } 11} &= 239 \text{ nm} \end{aligned}$$

Phosphate analysis (Bartlett, 1959), based on the u.v.-absorption data, revealed 1.0mol of phosphorus/mol of purine base; (elementary analyses; found: C, 41.4; H, 6.4; N, 21.2; C₁₆H₂₈N₇O₇P requires C, 41.6; H, 6.1, N; 21.3%).

Table 1. Paper chromatography data on compounds used in the synthetic schemes outlined

The key to the solvent systems employed and details of the staining techniques used are given in the Materials and Methods section. The staining patterns observed for each compound on chromatography were also found when the same compound was subjected to high-voltage paper electrophoresis.

Compound	<i>R_f</i> values in solvent systems			Detected by			
	1	2	3	Ninhydrin	U.v. light	Molybdate	Periodate
AMP	0.06	0.41	0.16	—	+	+	+
ADP		0.22	0.06	—	+	+	+
II	0.31			—	+	+	+
III	0.18	0.66	0.16	+	+	+	+
1,6-Diaminohexane		0.75		+	—	—	
IV	0.64	0.72	0.51	—	+	+	+
V	0.68	0.69	0.75	—	+	+	+
VIb		0.46		—	+	+	+
VIIa		0.59	0.31	+	+	+	+
<i>N</i> ⁶ -AMP		0.64	0.11	+	+	+	+
<i>N</i> ⁶ -(6-trifluoroacetamidohexyl)-AMP		0.52		—	+	+	+
Inorganic phosphate		0.25		—		+	
Inorganic pyrophosphate		0.13		—		+	
Inorganic imidodiphosphate		0.16		—		+	
6-Aminohexan-1-ol		0.77		+		—	
6-Aminohexan-1-ol phosphate		0.62		+		+	
6-Aminohexan-1-ol pyrophosphate		0.36		+		+	
<i>N</i> -Trifluoroacetyl 6-amino-1-ol phosphate		0.75	0.55	—		+	
<i>N</i> -Trifluoroacetyl 6-amino-1-ol phosphoryl imidazolidine		0.84	0.80	—		+	
<i>P</i> -ADP		0.56	0.34	+	+	+	+
Trifluoroacetyl derivative of <i>P</i> -ADP		0.68	0.61	—	+	+	+

This material can be attached directly to CNBr-activated Sepharose to provide an AMP affinity column or can be used for the preparation of the di- and tri-phosphate derivatives. The preparation of the diphosphate is described in detail below.

(iii) 8-(6-Trifluoroacetamidohexyl)amino-AMP. The product, III (0.5g, 1mmol), was dissolved in water (25ml) in an ice-bath and 1 ml of ethyl trifluorothiol acetate was added. The reaction mixture was vigorously stirred to produce a fine dispersion and was adjusted periodically to pH9.5 with 5*M*-LiOH. Additional ethyl trifluorothiol acetate (2–4ml) was added over a further 2h period until a sample of the reaction mixture no longer gave a positive ninhydrin test. After adjusting this reaction mixture to pH5 with trifluoroacetic acid, excess of reagents were removed by concentrating to dryness from water several times. The final residue was dissolved in water (10ml), adjusted to pH1.5 with trifluoroacetic acid and applied to a column (6cm×0.6cm) of Dowex 50 (X8; H⁺ cycle; 20–50 mesh) to remove unchanged amine; occasionally it was necessary to add methanol to achieve solution at pH1.5 before chromatography. Elution with water was continued until no more material absorbing at 278nm appeared from the column. The combined eluents were then concentrated to dryness. The yield was essentially quantita-

tive as shown by u.v.-absorption data, and the product (IV) migrated as a single anionic spot (negative to the ninhydrin test, but positive to the molybdate spray for phosphate) on high-voltage paper electrophoresis at pH6.5. This product (IV) was used in the next stage of the reaction without further characterization.

(iv) 8-(6-Trifluoroacetamidohexyl)amino-ADP (VIa). The preparation of the di- and tri-phosphates from the monophosphate is based on the carbonyl di-imidazole procedure (Staab *et al.*, 1959) as developed by Hoard & Ott (1965). A solution of compound IV (0.2mmol in 10ml of water) was applied to a column (6cm×0.6cm) of Dowex 50 (X8; pyridinium cycle; 20–50 mesh) resin, and washed with 10ml of water. Tributylamine (1.5ml, 6.2mmol) was added to the combined eluent and washings and the solution was concentrated *in vacuo*. The syrupy residue was dried by repeated addition and evaporation of anhydrous pyridine (5×10ml) and then of anhydrous dimethylformamide (3×10ml). The resulting anhydrous tributylammonium salt of IV was dissolved in 10ml of dimethylformamide and 1,1'-carbonyl di-imidazole (0.16g, 1mmol) (see the Materials and Methods section) was added. The mixture was shaken vigorously in a tightly stoppered container for 15–20min and then left in a desiccator at

Fig. 2. Purification of 8-(6-trifluoroacetamido-hexyl)amino-ADP (VIa) on DEAE-cellulose

The reaction mixture from the condensation of the imidazolidine (V) of 8-(6-trifluoroacetamido-hexyl)amino-AMP (0.2 mmol) with phosphate (20 ml, $E_{278} = 155$) was applied to a column (11 cm $\times$ 3 cm) of DEAE-cellulose. It was operated at room temperature at a flow rate of 50 ml/h and fractions (10 ml) were collected. The column was developed with aq. 50% (v/v) ethanol (150 ml) and water (50 ml) and then, at point A, with a linear gradient formed from 1.8 litres each of water and 0.4 M-triethylammonium bicarbonate, pH 7.5. The arrow, B, indicates the elution position of the triphosphate formed when the imidazolidine (V) was condensed with either pyrophosphate or imidodiphosphate and applied to DEAE-cellulose under identical conditions. ●, E_{278} ; ----, gradient concentrations.

room temperature for 6–12 h. Excess of di-imidazole was destroyed by the addition of methanol (0.013 ml, 0.3 mmol), and after 30 min the solution was concentrated to dryness at 40–50°C *in vacuo*. The formation of the phosphoryl imidazolidine (V) was quantitative and could be monitored by paper chromatography in solvent 3 (Table 1). The imidazolidine (V) is stable for several weeks if kept in dimethylformamide under anhydrous conditions.

Anhydrous tributylammonium phosphate (1 mmol in 10 ml of dimethylformamide) (see the Materials and Methods section) was added to the imidazolidine (V) in 10 ml of dimethylformamide and after vigorous shaking for 30 min the reaction mixture was left in a desiccator at room temperature for 24 h. The precipitate of imidazolium phosphate that formed was removed by centrifugation and the precipitate was extracted with 3 $\times$ 1 ml portions of dimethylformamide. The combined supernatants were mixed with an equal volume of methanol and the resulting solution was concentrated to dryness at 40°C. The residue was dissolved in 20 ml of aq. 50% (v/v) ethanol and then applied to a column of DEAE-cellulose and developed as described in Fig. 2. Three major peaks absorbing at 278 nm were eluted from the

column after applying the gradient. The peak eluted between tubes 75 and 97 represented about 20% of the total u.v.-absorbing material applied to the column and was chromatographically and electrophoretically identical with 8-(6-trifluoroacetamido-hexyl)amino-AMP (IV). The solutions in tubes 109–140, which contained some 50% of the 278 nm-absorbing material applied, were pooled and evaporated to a syrup, and the triethylammonium bicarbonate was removed as described in the Materials and Methods section. This material migrated as a single component on high-voltage paper electrophoresis at pH 6.5 (mobility relative to AMP: AMP, 1.0; VIa, 1.22; IV, 0.56) and on paper chromatography. It was ninhydrin-negative, but positive to the molybdate spray for phosphate.

The u.v.-absorption characteristics of this compound were identical with those of compound III. It was found to contain 2.01 mol of total phosphorus and 1.02 mol of acid-labile phosphorus/mol of adenine base (as determined by absorption at 277 nm at pH 1). The structure, VIa, given in Scheme 1 was thus assigned to this compound.

The yield of compound VIa was 50% based on u.v.-absorption data. In our experience the yields varied from 40–90% at this stage of the reaction sequence although 50% was a more commonly obtained figure. This may be a consequence of moisture in the condensation reaction since vigorously anhydrous conditions were not stressed.

(v) 8-(6-Amino-hexyl)amino-ADP (VIIa). The product, VIa (about 0.1 mmol), was dissolved in water (10 ml) with the aid of a few drops of methanol if necessary, and adjusted to pH 11 with 1 M-NaOH. After 3–4 h at room temperature, the mixture was adjusted to pH 7 with 1 M-HCl. This solution was strongly ninhydrin positive and contained a single component as judged by high-voltage paper electrophoresis at pH 6.5 (mobility relative to AMP: AMP, 1.0; VIIa, 0.50). Paper chromatography (Table 1) also showed a single component. This product still contained 2.01 mol of total phosphorus/mol of adenine base and had the same $\lambda_{\max}$ and molar extinction coefficient as compounds III and VIa. It was desalted on a column (140 cm $\times$ 3 cm) of Sephadex G-10 in the early parts of this study before characterization as described above, but could be coupled directly to CNBr-activated Sepharose 4B without desalting.

(vi) 8-(6-Amino-hexyl)amino-ATP (VIIb) and 8-(6-amino-hexyl)amino-adenylyl imidodiphosphate (VIIc). These compounds can be prepared by condensing either tributylammonium pyrophosphate (VIIb) or imidodiphosphate (VIIc) with compound V in the above reaction sequence. All other reaction conditions and separation procedures were identical except that the DEAE-cellulose chromatography was carried out at 4°C instead of room temperature. In the case of

these triphosphates only the fractions eluting from the column with a molar ratio of total phosphorus/purine base (determined from the E_{278}) of 3.0 ± 0.1 were pooled. (The addition of a pyrophosphate moiety does not significantly change the u.v.-absorption spectrum from that of compound III.) After removal of the triethylammonium bicarbonate by repeated evaporation from ethanol (at 25–30°C for the triphosphate derivatives), these fractions were shown to contain a homogeneous product by high-voltage paper electrophoresis at pH 6.5 (mobility relative to AMP: AMP, 1.0; VIIb, 1.29; VIc, 1.22) and by paper chromatography (Table 1). Each compound contained 2.0 mol of acid-labile phosphorus and 2.9 mol of total phosphorus per mol of compound (based on u.v.-absorption data). The product from this reaction could be made available for attachment to CNBr-activated agarose after hydrolysing the trifluoroacetyl blocking group by incubating at pH 11 for 2–3 h at room temperature. This procedure did not appear to liberate any P_i from the triphosphates when subjected to paper chromatography (Table 1) and the final product was strongly ninhydrin positive. The final yield of product during the preparation of the phosphate esters was about 20–30% based on u.v.-absorption data.

A shorter preparation of the triphosphate (VIIb) was also attempted by omitting the trifluoroacetylation of compound III (and hence the necessary hydrolysis as the last step). The imidazolidine of compound III was prepared and condensed with tributylammonium pyrophosphate in the usual manner. Subsequent chromatography of this reaction mixture, however, revealed a number of unidentified peaks absorbing at 278 nm in addition to those found with the method described above, and the yield of the triphosphate (VIIb) was considerably decreased. Presumably the nucleophilic primary amine gave rise to a number of side reactions (cf. Staab *et al.*, 1959).

(b) *Adenosine phosphate ligands substituted on the 6-amino position of the adenine ring (type B compounds)*. The comparative inertness of the 6-amino group of the adenine nucleus is well documented (see for example, Bock, 1960; Pullman & Pullman, 1963; Kochetkov & Budovskii, 1971). This atypical behaviour seems to be caused by interaction of the amino moiety with the heterocyclic purine ring. Consequently direct acylation of this group requires more drastic conditions than are normally associated with acylation of an amino function (Falbriard *et al.*, 1967; Wilchek *et al.*, 1971). These authors prepared N^6 -monoacyl cyclic AMP by reaction of an acid anhydride with cyclic AMP over an 8 day period. Very little success was achieved, however, when this method was applied to the synthesis of N^6 -(6-amino-hexanoyl)-AMP. Yields were very low and several unidentified compounds were found in the reaction mixture.

A more indirect approach to the synthesis of an N^6 -linked AMP derivative was used by Guilford *et al.* (1972). This involves a similar procedure to that employed above for the C-8-linked AMP derivative, i.e. the nucleophilic attack of a primary amine on the mono-halo substituted purine ring, in this instance, 6-chloro-9- β -D-ribofuranosylpurine 5'-phosphate. Although this is potentially a more time-consuming preparation, a homogeneous product, N^6 -(6-amino-hexyl)-AMP, in reasonably high yield is readily obtainable.

(i) N^6 -(6-Aminohexyl)-AMP (Fig. 1B, R = H). Inosine was converted into 6-chloro-9- β -D-ribofuranosylpurine in three steps via 2',3',5'-*O*-triacyl-inosine and 6-chloro-(2',3',5'-*O*-triacyl-9- β -D-ribofuranosyl)purine (Bredereck & Martini, 1947; Brown & Weliky, 1953; Gerster *et al.*, 1963). From 15 g of inosine, 4.90 g of the 6-chloro derivative was obtained in 31% overall yield. The product was homogeneous by paper chromatography and the u.v.-absorption data ($\lambda_{\text{max}}^{\text{pH } 7.0} = 263.5 \text{ nm}$) and melting point (168–170°C with decomposition) were consistent with the data given for 6-chloro-9- β -D-ribofuranosylpurine synthesized by the above authors.

Direct and selective phosphorylation of the 5'-position of 6-chloro- β -D-ribofuranosylpurine was carried out by using phosphoryl chloride containing water (5–10%, v/v) (Guilford *et al.*, 1972). This compound was converted directly into the N^6 -(6-amino-hexyl)-AMP by reaction with 1,6-diaminohexane and the subsequent purification of the product on Dowex 1 (X8; acetate cycle; 200–400 mesh). From 4.90 g of 6-chloro- β -D-ribofuranosylpurine, 4.20 g of N^6 -(6-amino-hexyl)-AMP was obtained in 55% overall yield.

The final product was shown to migrate as a single component on high-voltage paper electrophoresis at pH 6.5 (neutral) and pH 2.0 (cationic) and by paper chromatography (Table 1). The u.v.-absorption spectrum is characteristic of 6-alkylaminoadenosines (Windmueller & Kaplan, 1961):

$$\begin{aligned}\lambda_{\text{max}}^{\text{pH } 1} &= 263.5 \text{ nm } (\epsilon_{263.5}^{\text{litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}} = 19300), \\ \lambda_{\text{min}}^{\text{pH } 1} &= 231 \text{ nm.} \\ \lambda_{\text{max}}^{\text{pH } 11} &= 268 \text{ nm } (\epsilon_{268}^{\text{litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}} = 16700), \\ \lambda_{\text{min}}^{\text{pH } 11} &= 232 \text{ nm.}\end{aligned}$$

Phosphate analysis and ribose determinations were compared with adenine composition (u.v. absorption) and gave a ratio of 1.0:1.1:1.0 (phosphate:ribose:adenine), (elementary analyses; found: C, 42.8; H, 6.2; N, 18.5; $\text{C}_{16}\text{H}_{27}\text{N}_6\text{O}_7\text{P}$ (free acid) requires C, 43.1; H, 6.1; N, 18.8%). These data compare very well with those given by Guilford *et al.* (1972) and is consistent with the structure shown in Fig. 1B.

This product can be attached directly to CNBr-activated Sepharose 4B or can be used as the starting

material for the preparation of the di- and tri-phosphate derivatives.

(ii) *N*⁶-(6-Aminohexyl)adenosine 5'-di- and tri-phosphate. The preparation of the di-, tri- and imido-di-phosphate derivatives from the mono-phosphate was done exactly as described for the C-8-linked derivatives above (i.e. through the steps giving rise to compounds IV, V, VI and VII in Scheme 1). Again it was necessary to trifluoroacetylate the free amino function on the 'spacer' arm to obtain the desired product in high yield and to prevent side reactions. Chromatography of the reaction mixture on DEAE-cellulose resulted in elution of the di- and tri-phosphates of the *N*⁶-substituted derivatives in the same positions along the gradient as were found for their C-8-substituted adenosine phosphate analogues (Fig. 2). Similar yields based on u.v. absorption were also obtained. The spectral data ($\lambda_{\max}$, and molar extinction coefficient) for the di- and tri-phosphates were not significantly altered from those obtained for the AMP derivative. The products were shown to migrate as a single component on both high-voltage paper electrophoresis at pH 6.5 (having the same mobilities relative to AMP as found for their C-8-substituted analogues) and paper chromatography. The phosphate content based on u.v.-absorption data, indicated 1.1 mol of acid-labile phosphorus/2.0 mol of total phosphorus per mol of diphosphate derivative and 2.1/2.9 mol of phosphorus (respectively) for the triphosphate derivative. After removal of the trifluoroacetyl blocking group by incubation (10–20 μ mol/ml) at pH 11 for 2–3 h at room temperature, the ligands were attached to CNBr-activated agarose.

(c) *ADP ligand substituted through the terminal phosphate group (type C compounds)*. The synthesis of a phosphodiester link between the terminal phosphate of ADP and aminohexanol was based on the method of Barker *et al.* (1972, 1974) for the synthesis of a UDP-aminohexanol ester. This essentially involves many of the same methods described above.

The following procedures describe the synthesis of the ADP-aminohexanol ester (Fig. 1C).

*P*¹-(6-Aminohex-1-yl) *P*²-(5'-adenosyl) pyrophosphate (Fig. 1C). The preparation of *N*-trifluoroacetyl 6-aminohexan-1-ol phosphate was exactly as described by Barker *et al.* (1972, 1974). After converting it into the phosphoryl imidazolidine as described by these authors (with dimethylformamide as a solvent), it was left to condense with the anhydrous tributylammonium salt of AMP for 24 h. Occasionally, the imidazolidine of AMP was prepared and condensed with a threefold molar excess of *N*-trifluoroacetyl 6-aminohexan-1-ol phosphate. The yield of the final product was the same in either case. After the condensation the reaction mixture containing the desired trifluoroacetylaminohexanol-ADP (from 1 mmol of AMP) was passed down a column (27 cm $\times$ 1 cm) of Dowex 1 (X8; chloride cycle; 200–400 mesh)

and developed with aq. 50% (v/v) methanol (250 ml) and then with a linear gradient of 500 ml of 0.01 M-HCl as the starting solvent and 500 ml of 0.01 M-HCl containing 0.5 M-LiCl as the limit solvent. The elution profile was identical with that obtained by Barker *et al.* (1972, 1974) for their UDP-aminohexanol derivative. The appropriate fractions, containing the trifluoroacetylaminohexanol-ADP and accounting for approx. 60% of the starting u.v. absorbance, were pooled and concentrated to dryness. This material was used as a routine for the preparation of the *P*-ADP-agarose adsorbent without further characterization after incubation (10–20 μ mol/ml) at pH 11 for 2–3 h at room temperature to hydrolyse the trifluoroacetyl derivative to the free amine.

*P*¹-(6-Aminohex-1-yl) *P*²-(5'-adenosyl) pyrophosphate (Fig. 1C) was characterized and shown to be pure after desalting on a column (140 cm $\times$ 3 cm) of Sepharose G-10 developed in water. It migrated as a single anionic component on high-voltage paper electrophoresis, pH 6.5. Paper chromatography (Table 1) also revealed a single component. Based on a molecular weight of 538 for the dilithium salt the u.v.-absorption spectrum was identical with that of ADP:

$$\lambda_{\max}^{\text{pH } 7.0} = 259 \text{ nm } (\epsilon_{259}^{\text{litre}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}} = 15400)$$

Phosphate analysis based on u.v.-absorption spectrum revealed 2.01 mol of phosphorus/mol of compound. On this basis the structure shown in Fig. 1C was ascribed to this product.

(d) *Preparation of a pyrophosphate ligand (type D compounds)*. These ligands can be simply prepared by heating an amino alcohol with crystalline pyrophosphoric acid under reduced pressure.

6-Aminohexan-1-ol pyrophosphate (Fig. 1D). 6-Aminohexan-1-ol (11.7 g, 0.1 mol) was mixed with crystalline pyrophosphoric acid (17.6 g, 0.1 mol) in a round-bottomed flask. The flask was connected by a side arm to a second flask which was immersed in a solid CO₂-ethanol bath. After evacuating the apparatus to less than 0.1 mmHg, the reaction flask was heated to 150 $\pm$ 5°C on an isomantle. The reaction was continued for 18 h and the resulting amber solid was cooled to room temperature and dissolved in 200 ml of water. The solution was adjusted to pH 10.5 with 5 M-LiOH and the precipitated lithium pyrophosphate was removed by filtration under suction through a Celite pad. The filtrate was adjusted to pH 3 with acetic acid and passed over a column containing about 30 ml of Dowex 50 (X8; pyridinium cycle; 20–50 mesh) to remove any unreacted 6-aminohexan-1-ol. The ninhydrin-positive eluate was concentrated to dryness and pyridinium acetate was removed by heating at 100°C *in vacuo* for 2–3 h. The residue was dissolved in warm water (100 ml) and ethanol (about 100 ml) was added until the solution became cloudy and then the suspension was left overnight

Fig. 3. Purification of 6-aminohexan-1-ol pyrophosphate on Dowex 1-X8

Partially purified 6-aminohexan-1-ol pyrophosphate (0.6 g) after crystallization was dissolved in water (80 ml) and applied to a column (12 cm $\times$ 3 cm) of Dowex 1 (X8; bicarbonate cycle; 200–400 mesh) and washed through with water (80 ml). The column was operated at room temperature at a flow rate of 40 ml/h and fractions (8 ml) were collected. At A, a linear gradient from water to 0.5 M triethylammonium bicarbonate, pH 7.5, over a total volume of 1 litre was applied. Samples (0.01 ml) were taken for phosphate analysis (Bartlett, 1959) and the E_{830} (●) obtained was plotted directly. At B, the column was washed with a further 300 ml of 0.6 M triethylammonium bicarbonate, pH 7.5. This fraction was found to contain principally inorganic pyrophosphate with trace amounts of the desired product. ———, Gradient concentration.

at 4°C. The crystals were collected and washed with cold aq. 50% (v/v) ethanol. The yield was 19.8 g (72%). Paper chromatography and high-voltage electrophoresis of this material revealed the presence of trace amounts of P_i and PP_i , aminohexanol and the monophosphate derivative. It could be purified to homogeneity on Dowex 1-X8 as described in Fig. 3. The material eluted in fractions 97–135 was pooled and the triethylammonium bicarbonate was removed as described in the Materials and Methods section. This material migrated as a single component on electrophoresis at pH 6.5 (anionic) and on paper chromatography (Table 1). Phosphate analysis (based on weight) showed 1.99 mol of phosphorous/mol of free acid (elementary analyses; found: C, 26.1; H, 6.1; N, 4.9; $C_6H_{17}N_1O_7P_2$ required C, 26.0; H, 6.2; N, 5.1%).

This material was attached directly to CNBr-activated Sepharose.

Affinity chromatography on agarose-adenosine phosphate derivatives

Several examples of the application of these adsorbents in binding adenosine phosphate-utilizing

Fig. 4. Chromatography of human serum albumin and a crude commercially available preparation of lactate dehydrogenase containing pyruvate kinase activity on (a) C8-AMP-Sepharose and (b) P-ADP-Sepharose columns

Both columns (10 cm $\times$ 0.8 cm) were equilibrated and eluted throughout with 0.05 M sodium phosphate, pH 7.5, at a flow rate of 13 ml/h and fractions (1.3 ml) were collected. After applying the same protein mixture [human serum albumin (1 ml, $E_{280} = 0.52$) and 0.2 ml of lactate dehydrogenase ($\Delta E_{340} \cdot \text{min}^{-1} = 1.16$) containing pyruvate kinase activity ($\Delta E_{340} \cdot \text{min}^{-1} = 1.60$)] to each column, they were eluted with the above buffer. At the points indicated the following additions were made to this buffer: A, 0.5 M-KCl; B, nothing; C, 1 mM NADH. The fractions were monitored for enzymic activity as described in the Materials and Methods section. Δ , Pyruvate kinase activity; $\blacktriangle$, lactate dehydrogenase activity; $\bullet$, E_{280} (human serum albumin).

enzymes are given below. One example is shown in Fig. 4 where a commercially available preparation of crude lactate dehydrogenase (containing an approximately equal amount of pyruvate kinase activity) and a non-specific protein, human serum albumin, are applied to either a C8-AMP-agarose derivative (Fig. 4a) or a P-ADP-agarose derivative (Fig. 4b). In both cases, pyruvate kinase activity (together with the human serum albumin) appear in the void volume. No lactate dehydrogenase activity was found in these fractions, nor in those subsequently eluted with 0.5 M-KCl. The lactate dehydrogenase activity was eluted, however, when 1 mM-NADH was included in the buffer. The recovery of both enzyme activities was essentially quantitative. A similar type of experiment has been reported for the N^6 -AMP-agarose derivative (Mosbach *et al.*, 1972) and has been entirely reproducible with our preparation of this derivative. These results serve to illustrate two points. First, the specific nature of the interaction is indicated

Fig. 5. Chromatography of rabbit skeletal muscle myosin on an N^6 -ADP-Sepharose conjugate

The column (5 cm $\times$ 0.6 cm) and the myosin solution were equilibrated to 0.25 M-potassium acetate containing 5 mM-Tris-HCl buffer, pH 7.6, and 2 mM CaCl_2 . After charging the column with myosin, 0.02 M-sodium pyrophosphate was included in the above buffer at A. At B, the column was eluted with 1.5 M-KCl. The flow rate was 20 ml/h and fractions (2 ml) were collected. ●, E_{280} ; ▲, ATPase activity.

by the fact that lactate dehydrogenase is not eluted from these columns by non-specifically raising the ionic strength of the developing buffer, but can be eluted quantitatively by low concentrations of a specific displacing ion, NADH. Salt has been used as a non-specific displacer for this enzyme quite effectively by some workers using chemically ill-defined affinity adsorbents (Lowe & Dean, 1971; Lowe *et al.*, 1973*a,b*; Lowe & Dean, 1973). This probably means that lactate dehydrogenase binds less tightly to their biopolymers. Very high salt concentrations (1.5–2.0 M-KCl) will elute lactate dehydrogenase from our chemically-defined ligands, but at these concentrations the effect on the enzyme may be quite drastic and this does not represent a specific elution. Secondly, lactate dehydrogenase is capable of binding to a variety of AMP derivatives in which either the adenine ring (P -ADP) or the 5'-phosphate moiety (C8-AMP, N^6 -AMP) are unsubstituted. These observations are fully consistent with the detailed molecular model of the binary lactate dehydrogenase-NAD⁺ complex that has emerged recently (Rossman *et al.*, 1972; Chandrasekhar *et al.*, 1973).

The ADP-agarose derivatives bearing unsubstituted pyrophosphate moieties have proved very effective in their ability to interact reversibly with a variety of ATP-utilizing enzymes. It can be seen from Figs. 5,

Fig. 6. Chromatography of a papain digest of myosin on a C8-ADP-Sepharose conjugate

A water-soluble fraction (1 ml, $E_{280} = 0.65$) of a Sepharose-papain digest of myosin was equilibrated to 5 mM-Tris-HCl, pH 7.6, containing 2 mM- MgCl_2 and 2 mM-2-mercaptoethanol, and applied to the column (5 cm $\times$ 0.6 cm) equilibrated with the same buffer. The column was operated at a flow rate of 15 ml/h and fractions (2.1 ml) were collected. The arrow indicates where 0.02 M-sodium pyrophosphate was included in the developing buffer. ●, E_{280} ; ▲, ATPase activity.

6 and 7 that myosin and its biologically active subfragments, prepared by proteolytic digestion of the parent molecule (i.e. heavy meromyosin and subfragment 1), can form stable complexes with these immobilized ADP and pyrophosphate ligands which can be subsequently dissociated in a specific manner. Myosin is capable of binding to an N^6 -ADP-agarose derivative in the presence of Ca^{2+} (Fig. 5) and can be eluted by including pyrophosphate in the developing buffer. ATPase activity was only found in the pyrophosphate-eluted fraction. When the water-soluble fraction of a Sepharose-papain digest of myosin was applied to a C8-ADP-agarose conjugate (Fig. 6) the material absorbing at 280 nm which passed unretarded through the column possessed no ATPase activity. Quantitative recovery of the ATPase activity of the applied material could, however, be obtained after flushing the column with pyrophosphate. This material was indistinguishable from conventional preparations of subfragment 1 (Lowey *et al.*, 1969). Heavy meromyosin, isolated from a tryptic digest of myosin by gel filtration on Sephadex G-200, bound strongly to a HADP-agarose conjugate (Fig. 7) and in this case the immobilized complex was dissociated by applying a linear gradient of ATP. Again, the material passing unretarded through the affinity column possessed no ATPase activity whereas that eluted by the ATP was active. It is worth stressing that

Fig. 7. Chromatography of heavy meromyosin on a HAPP-Sepharose conjugate

Heavy meromyosin, prepared by tryptic digestion of myosin as indicated in the Materials and Methods section, was equilibrated to 5 mM-Tris-HCl, pH 7.6, containing 2 mM-MgCl₂ and 2 mM-2-mercaptoethanol. This solution (2 ml, $E_{280} = 1.3$) was applied to the column (10 cm × 0.8 cm) equilibrated to the same buffer. The flow rate was 20 ml/h and fractions (2 ml) were collected. At the point indicated, a linear gradient from 0 to 0.01 M-ATP in the above buffer system was applied over a total of 40 ml. The protein was detected in the course of the gradient by measuring light scattering at 360 nm (○) after thoroughly mixing a portion of the fraction (1.5 ml) with 15% (w/v) trichloroacetic acid (0.5 ml). ●, E_{280} ; ▲, ATPase activity; ----, gradient concentration.

Fig. 8. Partial purification of rat hepatic glucokinase on a C8-ADP-Sepharose conjugate

The supernatant (0.5 ml) from a 33% (w/v) rat liver homogenate in 0.25 M-sucrose, 0.01 M-KCl, 0.025 M-Tris-HCl, pH 7.2, 7.5 mM-MgCl₂, and 1 mM-dithiothreitol was applied to the column (5 cm × 0.8 cm) equilibrated in this buffer (minus sucrose). The column flow rate was 20 ml/h and fractions (2 ml) were collected. At the point indicated, 0.01 M-ATP was included in the column buffer (and the MgCl₂ concentration was also raised to 0.01 M). The fractions were monitored for enzymic activity as described in the Material and Methods Section. ●, E_{280} ; ○, total glucose-phosphorylating activity, i.e. glucokinase and hexokinase, measured at a glucose concentration of 0.1 M; ▲, hexokinase activity determined at a glucose concentration of 0.5 mM.

in each case, the affinity adsorbents were capable of distinguishing between those components in the preparation which possessed ATPase activity and those which did not. The eluted enzyme possessed a higher ATPase activity (after removal of inhibiting ions by dialysis) than did that originally applied to the columns. These experiments extend the observations reported earlier by Lamed *et al.* (1973) for the interaction of heavy meromyosin with their immobilized ATP derivatives. The affinity adsorbent described by Lamed *et al.* (1973) was not capable either of distinguishing between enzymically active and inactive heavy meromyosin or of binding myosin at the high ionic strength necessary to keep the parent molecule in solution. A possible explanation for this apparent discrepancy between their observations and the behaviour normally associated with a truly biospecific affinity column may be the chemical nature of their immobilized ATP derivative. The chemical modification of the ATP (periodate oxidation of the ribose moiety followed by reaction with a hydrazide), required for its coupling to agarose, could destroy the three-dimensional integrity of the ATP molecule in such a way that totally specific interactions with myosin are not possible.

The C8-ADP-agarose and N⁶-ADP-agarose derivatives (and the ATP derivatives) are also capable of interacting with a variety of kinase enzymes. An example of this is shown in Fig. 8, where the partial purification of glucokinase from a crude rat liver supernatant has been effected on a C8-ADP-agarose

conjugate. The main protein peak passing unretarded through the column does contain some hexokinase activity, but no glucokinase activity. Glucokinase, containing virtually no hexokinase activity, could be recovered from the column after the addition of ATP to the developing buffer. This particular ADP derivative seems to be capable of distinguishing between the two kinases despite the fact that free ADP will inhibit both enzymes. This again illustrates a difference in the substrate binding sites of the high- K_m and low- K_m (for glucose) glucose phosphorylating kinases which was also noted from their behaviour on a chemically-defined glucosamine-agarose conjugate (Chesher *et al.*, 1973). Rat liver hexokinase and glucokinase and yeast hexokinase will, however, bind to an N^6 -ADP-agarose conjugate (M. J. Holroyde, H. R. Trayer & I. P. Trayer, unpublished work). These results show how, by using chemically-defined ligands, some information about the active-site conformation of enzymes can be obtained.

The experiments reported in this section are only intended to illustrate some of the ways in which these ligands can be employed. Further investigations of the applicability of these affinity adsorbents will be reported upon later.

General discussion

The mono-, di- and tri-phosphates of nucleosides and many of their derivatives, such as the sugar nucleotides and the vitamin containing coenzymes, are substrates, inhibitors, effectors or cofactors for a large number of proteins in biological systems. Thus the attachment of nucleotide phosphates, particularly adenosine phosphates, to agarose should provide affinity adsorbents for the purification of a wide variety of enzymes. This report has concentrated on the preparation of adenosine phosphate derivatives containing a single primary amino group which enable them to be attached to an agarose support in a chemically unambiguous manner. The general method of preparing ligands of type A or type B (Fig. 1), monosubstituted in the purine ring, by nucleophilic attack of an amine nitrogen on a carbon bearing a halogen, can, in principle, be applied to the synthesis of other nucleoside or nucleotide derivatives. The synthesis of mono-bromo or mono-chloro derivatives of nucleosides other than adenosine is well documented (Frisch & Visser, 1959; Michelson *et al.*, 1962; Holmes & Robins, 1964; Wataya *et al.*, 1973) and these could be treated in a manner analogous to that described in Scheme 1 to provide a range of mono-substituted nucleoside or nucleotide derivatives. The same general methods could also be used to convert any monophosphate into the di- or tri-phosphate derivatives or to convert AMP into more complicated structures such as NAD⁺, FAD or CoA.

The general applicability of the methods involved in the synthesis of the type C (Fig. 1) ligands has been commented on elsewhere (Barker *et al.*, 1972, 1974). The preparation of the P -ADP ligand (Fig. 1C) reported here is similar to the synthesis of ATP-Sepharose described by Berglund & Eckstein (1972).

A few examples have been included in this report where these affinity adsorbents have been successfully employed in reversibly binding a variety of adenosine phosphate-recognizing enzymes. Methods for increasing the selectivity of 'general ligand' affinity chromatography and hence its versatility have been devised (Mosbach *et al.*, 1972; Ohlsson *et al.*, 1972; Lowe *et al.*, 1973b; I. P. Trayer, unpublished work). This increase in the selectivity of elution from a general ligand often involves the formation of a ternary complex between the enzyme, the immobilized ligand and some substrate analogue (Ohlsson *et al.*, 1972). Interpretation of this sort of data is made simpler and more meaningful by using chemically defined affinity adsorbents. This is especially true if information about the kinetic processes and the conformation of active sites of enzymes is to be extracted from affinity chromatographic data. The observation that rat liver hexokinase will bind much more strongly to an N^6 -ADP ligand than to a C8-ADP ligand shows how important it is to present the ligand of choice in a defined manner to the enzyme. Thus, by comparisons of this sort, some insight into the conformation of the enzyme-substrate complex can be obtained. Although the synthesis of chemically defined ligands may be time-consuming, it is often necessary in order to improve both the quality and quantity of the information that can be obtained by affinity chromatography studies.

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