General Ligands in Affinity Chromatography

COFACTOR-SUBSTRATE ELUTION OF ENZYMES BOUND TO THE IMMOBILIZED NUCLEOTIDES ADENOSINE 5'-MONOPHOSPHATE AND NICOTINAMIDE-ADENINE DINUCLEOTIDE

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(Received 24 November 1971)

1. Two different gels have been prepared suitable for the separation of a number of enzymes, in particular NAD^+ -dependent dehydrogenases, by affinity chromatography. For both the matrix used was Sepharose 4B. For preparation (a), NAD^+ -Sepharose, 6-aminohexanoic acid has been coupled to the gel by the cyanogen bromide method and then NAD^+ was attached by using dicyclohexylcarbodi-imide; for preparation (b), AMP-Sepharose, N^6 -(6-aminohexyl)-AMP has been coupled directly to cyanogen bromide-activated gel. 2. Affinity columns of both gels retain only the two enzymes when a mixture of bovine serum albumin, lactate dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase is applied. Subsequent elution with the cofactor NAD^+ yields glyceraldehyde 3-phosphate dehydrogenase whereas lactate dehydrogenase is eluted by applying the same molarity of the reduced cofactor. 3. The binding of both glyceraldehyde 3-phosphate dehydrogenase and lactate dehydrogenase to the gel tested, AMP-Sepharose, is strong enough to resist elution by gradients of KCl of up to at least 0.5M. A 0.0-0.15M gradient of the competitive inhibitor salicylate, however, elutes both enzymes efficiently and separately. 4. The elution efficiency of lactate dehydrogenase from AMP-Sepharose has been examined by using a series of eluents under comparable conditions of concentration etc. The approximate relative efficiencies are: 0 (lactate); 0 (lactate+semicarbazide); 0 (0.5mM- NAD^+); 80 (lactate+ NAD^+); 95 (lactate+semicarbazide+ NAD^+); 100 (0.5mM-NADH). 5. All contaminating lactate dehydrogenase activity can be removed from commercially available crude pyruvate kinase in a single-step procedure by using AMP-Sepharose.

Affinity chromatography or, as it also is called, bio-specific adsorption, has in recent years received considerable attention, as reflected by the number of surveys (Cuatrecasas & Anfinsen, 1971a,b; Feinstein, 1971; Friedberg, 1971). Dramatic enzyme purifications have been reported by using this technique, yet the method of achieving the very specificity that has contributed to the extraordinary success of affinity chromatography somewhat hampers the wider application of the method. Thus, in most cases, for each individual enzyme a different specific ligand has to be chosen and bound to the matrix, often at a certain distance from the matrix to permit binding of the particular enzyme, each new problem requiring a different and often elaborate chemical synthesis. With these factors in mind we report further on our investigations of systems that incorporate 'general' ligands showing affinity for a broad 'enzyme spectrum' (Mosbach *et al.*, 1971). We chose to immobilize AMP and NAD^+ , the former reported to be a competitive inhibitor for a number of enzymes and the latter forming binary complexes with a variety of NAD^+ -dependent dehydrogenases. In both cases

a hexacarbon 'spacer' of about $7\text{\AA}$ (0.7nm) length was introduced between the Sepharose-gel matrix and the ligands to improve their steric availability for enzymes. The general idea was that the bound enzymes could subsequently be eluted by using solutions of an appropriate cofactor, which ought to show greater affinity than the bound analogue for any particular enzyme. In addition, since enzymes differ in their K_m for substrates and/or cofactor, e.g. NAD^+ , separations of enzymes ought to be feasible by elution with either cofactor or appropriate substrate, thus preserving the specificity associated with the more familiar pattern of affinity chromatography.

Experimental

Materials

Chemicals. 6-Aminohexanoic acid, sodium salicylate, semicarbazide hydrochloride and dicyclohexylcarbodi-imide were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. Sepharose 4B was purchased from Pharmacia, Uppsala, Sweden. Cyanogen

bromide was obtained from Fluka A.G., Buchs, Switzerland. L-(+)-Cysteine hydrochloride was purchased from Merck A.-G., Darmstadt, West Germany. *N*⁶-(6-Aminohexyl)-AMP was prepared according to the procedure described elsewhere (Guilford *et al.*, 1972). ADP (grade I), β -NAD⁺ (grade III), β -NADH (grade III), DL-glyceraldehyde 3-phosphate (free acid), DL-lactate (grade DL-V) (sodium salt), pyruvate (type II) (sodium salt), phosphoenolpyruvate (tricyclohexylammonium salt) and bovine serum albumin were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Commercially available chemicals were used without further purification.

Enzymes. Lactate dehydrogenase (L-lactate-NAD oxidoreductase, EC 1.1.1.27, ox heart, type II) (240 units/mg), D-glyceraldehyde 3-phosphate dehydrogenase (D-glyceraldehyde 3-phosphate-NAD oxidoreductase, EC 1.2.1.12, rabbit muscle) (25 units/mg) and pyruvate kinase (ATP-pyruvate phosphotransferase, EC 2.7.1.40; rabbit muscle, crude, type I) (23 units/mg), containing lactate dehydrogenase activity (33 units/mg), were purchased from Sigma Chemical Co. All activities were determined by using the assay procedures outlined below.

Methods

Enzyme assays. In earlier experiments, enzymes were dialysed at 4°C overnight against the appropriate buffer. Subsequently, however, we found that the binding of the dehydrogenases is unaffected by the presence of (NH₄)₂SO₄. The results reported herein can be taken to be independent of whether or not the enzymes were dialysed before being applied to the column. Glyceraldehyde 3-phosphate dehydrogenase was used without removal of the NAD⁺ (1.9 mol/mol of protein), which is bound to the commercially available enzyme (D'Alessio & Josse, 1971). All enzyme assays were carried out at 20°C in 10mm quartz cuvettes by using a Perkin-Elmer 124 double-beam spectrophotometer to measure changes in *E*₃₄₀.

Glyceraldehyde 3-phosphate dehydrogenase activity was determined from the increase in *E*₃₄₀ as NADH is formed in a modification of the assay described by Warburg & Christian (1939). The incubation mixture consisted of NAD⁺ (0.5 μ mol), DL-glyceraldehyde 3-phosphate (3.6 μ mol), cysteine (50 μ mol), test solution (0.2–0.5 ml) and 0.1 M-sodium phosphate buffer, pH 7.5, to make a total volume of 3.0 ml.

Lactate dehydrogenase activity was determined from the decrease in *E*₃₄₀ as NADH is oxidized in an assay procedure slightly modified from the method of Warburg (1948). The incubation mixture consisted of NADH (0.5 μ mol), sodium pyruvate (1.0 μ mol), test solution (0.1–0.2 ml) and 0.1 M-sodium phosphate buffer, pH 7.5, to make a total volume of 3.0 ml.

Pyruvate kinase activity was similarly determined

from the decrease in *E*₃₄₀ in a coupled assay procedure slightly modified from that described by Bücher & Pfeleiderer (1955). The incubation mixture consisted of phosphoenolpyruvate (1.5 μ mol), ADP (4.3 μ mol), NADH (0.65 μ mol), lactate dehydrogenase (0.15 mg), test solution (0.5 ml) and 0.05 M-triethanolamine buffer, pH 7.6 (0.2 M with respect to NaCl and 0.1 M with respect to MgCl₂), to make a total volume of 3.0 ml.

Preparation of immobilized NAD⁺ (NAD⁺-Seph-*arose*). A slight modification of the published procedure was used (Larsson & Mosbach, 1971). Related, independent investigations of the preparation and affinity-chromatographic properties of variously immobilized NAD⁺ have been reported (Lowe & Dean, 1971). Thus Sepharose 4B (200 ml, representing 8 g dry wt.) was activated by the cyanogen bromide method (Axén *et al.*, 1967). 6-Aminohexanoic acid (20 g), dissolved in 0.1 M-NaHCO₃ (200 ml), was added and the final pH adjusted to 8.5 with 4 M-NaOH. After having been stirred for 15 h at room temperature, the gel was extensively washed with 0.1 M-NaHCO₃, 0.01 M-HCl, 0.5 M-NaCl and water. The gel was then filtered off and 80 g of the wet gel, corresponding to 3.9 g dry wt., was washed on a filter with a large volume of aq. 80% (v/v) pyridine. After filtration the gel was transferred to a bottle; this was followed by successive addition of NAD⁺ (0.8 g), dissolved in water (24 ml), and dicyclohexylcarbodi-imide (40 g) dissolved in pyridine (96 ml). The suspension was gently agitated on a rotary shaker for 10 days at room temperature. To remove formed dicyclohexylurea, the gel was then filtered off and washed successively with water, ethanol, warm (40°C) butan-1-ol, ethanol, water and aq. 80% (v/v) pyridine. The gel was then resuspended in the first filtrate and additional carbodi-imide (10 g) was added. After one more week, the above washing procedure was repeated but with the aq. pyridine omitted. In addition the gel was washed for 30 min with 1 M-HCl and for, at most, 5 min with ice-cold 0.1 M-NaHCO₃, followed by extensive washings with 0.5 M-NaCl and water. All washings were carried out at 20°C. The preparations were stored as moist gels at 4°C. Blank gels were prepared as above except for the omission of NAD⁺.

Preparation of immobilized N⁶-(6-aminohexyl)-AMP (AMP-Seph-*arose*). N⁶-(6-Aminohexyl)-AMP is prepared in seven steps from inosine as described in detail elsewhere (Guilford *et al.*, 1972). The starting material is converted by standard procedures into 6-chloro-9-(2',3'-O-isopropylidene- β -D-ribofuranosyl)-purine. This nucleoside is then transformed to the required AMP derivative by phosphorylation, displacement of the 6-chloro group by 1,6-diaminohexane and hydrolysis of the protecting groups.

Seph-*arose* 4B (10 ml, 0.4 g dry wt.), suspended in water (5 ml), was activated with a solution of cyanogen bromide (540 mg) in water (5 ml) as described above.

An ice-cold solution of *N*⁶-(6-aminohexyl)-AMP (158mg) in 0.1M-NaHCO₃ (10ml), pH8.4, was added to the activated gel and the suspension was stirred gently at 4°C for 24h. After this period, the gel was filtered off, extensively washed at 4°C with 0.1M-NaHCO₃, water, 1mM-HCl, 0.5M-NaCl and water and refiltered at reduced pressure on a sintered-glass filter. This preparation can be stored for long periods at 4°C. The binding yield was about 55%.

Elution efficiency tests. The tests of the relative efficiency of elution of lactate dehydrogenase from AMP-Sepharose by using various eluents were run in batches. Thus the enzyme (8 units) was added to a suspension of AMP-Sepharose (1g wet wt., i.e. 82mg of dry gel) in 0.1M-sodium phosphate buffer, pH7.5, in a screw-cap vial, which was rotated slowly for 90min at 4°C and equilibrated for 30min at room temperature. The gel was filtered off and washed with the buffer until no enzymic activity could be detected in the washings (in practice, usually there was no activity in the filtrate). From this gel was taken the required number of 0.1g batches, which were placed in screw-cap vials with their respective eluents.

Elution was carried out at room temperature for 30min with gentle rotation. Each eluate was rapidly filtered and the filtrate was assayed for lactate dehydrogenase activity by using essentially the standard assay procedure outlined above. Each batch was eluted with 0.1M-sodium phosphate buffer, pH7.5 (0.5ml), containing respectively lactate (62.5μmol), lactate (62.5μmol) + semicarbazide (100μmol), NAD⁺ (0.25μmol), NAD⁺ (0.25μmol) + lactate (62.5μmol), NAD⁺ (0.25μmol) + lactate (62.5μmol) + semicarbazide (100μmol) or NADH (0.25μmol). Each eluate was transferred to a cuvette, to which was added buffer and, except for the batch with NADH, appropriate amounts of any missing component (i.e. whichever of NAD⁺, lactate and semicarbazide was not present in the eluate) to give a final volume of 2.85ml. After 30min 10mM-NADH (0.05ml) and 10mM-sodium pyruvate (0.1ml) were added and the enzymic activity was assayed as usual. A preincubation period of 30min was sufficient for the conversion of NAD⁺ into NADH to allow a direct comparison of the observed activities. Thus if semicarbazide were added to, for instance, the NAD⁺+lactate eluate and the assay was run immediately, the activity found was only about 60–70% of that observed after a 30min preincubation period. This effect may be due in part to inhibition of lactate dehydrogenase by NAD⁺ (Nygaard, 1956).

Results and Discussion

Immobilized NAD⁺ was prepared by modifying Sepharose by linkage of 6-aminohexanoic acid with the cyanogen bromide method and subsequently coupling the pyridine nucleotide to the carboxylate

function of this 'spacer' by using dicyclohexylcarbodiimide (Larsson & Mosbach, 1971). The amount of bound nucleotide material was in the range of 50μmol/g of dry polymer. The precise mode of binding of NAD⁺ to the matrix is not yet definitely established. The information already to hand, however, suggests that the involvement of an amide linkage between the terminal carboxyl group of the Sepharose-spacer and the 6-amino group of the adenine moiety is unlikely since no shift in light-absorption maximum from 260nm to a higher wavelength can be observed, in contrast with that reported for a variety of *N*⁶-acyl derivatives of adenosine 3':5'-cyclic phosphate (Falbriard *et al.*, 1967) and of adenine (Brink & Schein, 1963). Model studies suggest that esterification of ribose secondary hydroxyl groups of adenosine derivatives is favoured over 6-aminoacylation (Rammner & Khorana, 1963) and several groups have reported syntheses of amino-acyl adenylates involving anhydride linkages between the amino acid carboxyl group and the phosphoric acid moiety of, for instance, AMP (Berg, 1958; Moldave *et al.*, 1959). In the absence, however, of direct definitive evidence (for instance, our i.r. spectroscopic investigations have not been fruitful) we can only speculate on the involvement of ester or anhydride coupling modes. The direct coupling of NAD⁺ to cellulose by using the cyanogen bromide method has been described by Lowe & Dean (1971). In our preliminary experiments, however, we had been unable to attach NAD⁺ directly to a Sepharose gel in like manner. Lowe & Dean (1971) also suggested that an analogous coupling of NAD⁺ to cellulose modified with 6-aminohexanoic acid involves an amide bond between the 6-amino group of the purine and the terminal carboxyl group of the spacer. This we find unlikely in our case for the reasons expressed above.

The gel-bound NAD⁺ is remarkably stable, permitting repeated use of the preparation. Some deterioration, however, could be observed on storage in water at 4°C over a period of months. This may well be due to slow hydrolysis of our putative ester linkages, although it is also possible that it is the adenine-ribose bond that is being cleaved. Immobilized AMP was prepared by direct coupling of *N*⁶-(6-aminohexyl)-AMP to Sepharose, again by using the cyanogen bromide method. The amount of bound nucleotide was on the average 400μmol/g of dry polymer. The preparation obtained is stable over periods of months and its binding capacity is not significantly diminished even after repeated use. Binding of the AMP analogue through its terminal amino group provides a hexamethylene spacer between the gel and the AMP moiety to improve steric availability for the enzymes. Recently covalent binding of AMP directly to cellulose via phosphate esters has been reported (Gilham, 1971). This preparation,

however, does not seem likely to be suitable for the generalized affinity-chromatography studies we envisage.

The successful separation of a mixture of serum albumin, glyceraldehyde 3-phosphate dehydrogenase and lactate dehydrogenase on a column of NAD^+ -Sephrose gel is depicted in Fig. 1. Both enzymes remain bound to the column material, whereas the inactive protein appears with the void volume. On elution with a solution of 0.15 mM- NAD^+ , glyceraldehyde 3-phosphate dehydrogenase is desorbed with about 80–90% recovery. No other activity was detected in these fractions. Only after applying a 0.15 mM solution of the reduced cofactor, NADH, was lactate dehydrogenase desorbed, with about 70–80% recovery, and free from glyceraldehyde 3-phosphate dehydrogenase activity. Subsequent washing with 10 mM-NADH eluted no further activity. The observed elution pattern may be rationalized in terms of the data in the literature for the binding affinity of the free cofactors for the two enzymes (Winer & Schwert, 1958; Eby & Kirtley, 1971). Thus K_m values of NAD^+ for glyceraldehyde 3-phosphate dehydrogenase ($5 \times 10^{-5} \text{ M}$) and lactate dehydrogenase ($1 \times 10^{-4} \text{ M}$) are consistent with the preferential elution

of the former enzyme with 0.15 mM- NAD^+ . The differences between the K_m of NAD^+ for glyceraldehyde 3-phosphate dehydrogenase alone and the K_m for the enzyme as supplied with NAD^+ bound to it are small enough not to affect the arguments presented here (Furfine & Velick, 1965). Also, the K_m for lactate dehydrogenase of NADH ($2.4 \times 10^{-5} \text{ M}$) is lower than that of NAD^+ , which is compatible with the observation that 0.15 mM-NADH elutes this enzyme whereas the same molarity of NAD^+ does not. Controls were run by using columns packed with gels prepared in analogous fashion to NAD^+ -Sephrose, except that NAD^+ itself was omitted from the procedure. No retention of any of the three components was observed on this carbodi-imide-treated 6-aminohexanoate-Sephrose gel.

In analogy with the above experiment, both enzymes were also retained on the immobilized AMP analogue, N^6 -(6-aminohexyl)-AMP. Apparently the affinity of this derivative for the two dehydrogenases is sufficient for its use as a biospecific adsorption agent, as might be predicted from the inhibitor constants of the parent analogue, AMP, which are available in the literature (Eby & Kirtley, 1971; McPherson, 1970). (Glyceraldehyde 3-phosphate

Fig. 1. Chromatography of a mixture of two dehydrogenases and bovine serum albumin on an NAD^+ -Sephrose column

The NAD^+ -Sephrose column (10.3 cm $\times$ 1.0 cm) contained about $17 \mu\text{mol}$ of nucleotide material and was equilibrated with 0.1 M-sodium phosphate buffer, pH 7.0. A mixture containing glyceraldehyde 3-phosphate dehydrogenase (4.1 units), lactate dehydrogenase (37 units) and bovine serum albumin (0.9 mg) in the same buffer (0.2 ml) was applied and subsequent elution was carried out with 0.15 mM- NAD^+ , 0.15 mM-NADH and 10 mM-NADH in that order. Serum albumin content was determined at E_{280} . Enzymic activities were measured as described in the Experimental section. The flow-rate was 1 ml/8 min, and 2 ml fractions were collected. $\blacktriangle$, Bovine serum albumin; $\circ$, glyceraldehyde 3-phosphate activity; $\triangle$, lactate dehydrogenase activity.

Fig. 2. Chromatography of a mixture of two dehydrogenases on an AMP-Sepharose column

The AMP-Sepharose column (11 cm $\times$ 0.5 cm) was equilibrated with 0.05 M-sodium phosphate buffer, pH 7.5. A 1 ml sample of the above buffer containing glyceraldehyde 3-phosphate dehydrogenase (2.8 units) and lactate dehydrogenase (35 units) was added to the column and buffer (20 ml) was applied. Subsequently a linear gradient of 0.0–0.5 M-KCl in buffer (total vol. 50 ml) was applied; 2 ml fractions were collected at a flow-rate of 1 ml/10 min. The column was washed with buffer (20 ml) and a linear gradient of 0.0–0.15 M-sodium salicylate in the above buffer (total vol. 100 ml) was applied. Fractions (1 ml) were collected at the same flow-rate. Enzymic activities were determined as described in the Experimental section. o, Glyceraldehyde 3-phosphate dehydrogenase activity; Δ , lactate dehydrogenase activity; —, gradient concentration.

dehydrogenase: $K_{I(AMP)} = 2.5 \times 10^{-3}$ M; lactate dehydrogenase M₄, dogfish: $K_{I(AMP)} = 5.5 \times 10^{-3}$ M.) On applying the eluents NAD^+ and NADH as above, a similar elution pattern was obtained. Controls were run with Sepharose 4B gels that had been activated by using cyanogen bromide but to which no nucleotide had been coupled; both serum albumin and the dehydrogenases eluted together in the void volume.

Having achieved a satisfactory separation of inactive protein and two dehydrogenases by using the cofactor-elution technique, we tested the same systems by applying gradients of increasing ionic strength to assess the magnitude of the affinity of the gels for the enzymes used and to ascertain whether buffer solutions of inorganic salt could be used as alternative eluents. To this end, after binding of the two enzymes to an AMP-Sepharose column, a gradient of 0.0–0.5 M-KCl was applied. As shown in Fig. 2, no activity was found in the effluents. In a parallel experiment it was shown that the enzymes could subsequently be eluted by using the low cofactor concentrations given above. These results provide compelling evidence that we are not dealing with an ion-exchange phenomenon. Our search for alternative eluents led us to test salicylate, an in-

hibitor competing for the NAD^+ -binding site of a number of NAD^+ -dependent dehydrogenases (Hines & Smith, 1964). On application of a gradient of 0.0–0.15 M-sodium salicylate in the same buffer to the same column, we were able to elute and resolve the same two dehydrogenases as shown in Fig. 2.

In certain circumstances the use of 'partial-inhibitor elution' (e.g. with salicylate) may be advantageous, besides being inexpensive. In particular, if it were desirable to remove dehydrogenase impurities from an enzyme also having affinity for NAD^+ or AMP, and if there were no interest in the salicylate-dependent enzymes *per se*, then this could be the method of choice. Other inhibitors, such as oxalate and oxamate (Novoa & Schwert, 1961), are not so advantageous, since binding takes place only in the presence of cofactors. There is no need to remove either KCl or salicylate before assaying fractions from the salt-inhibitor gradient columns. Potassium chloride does not interfere at all and salicylate inhibits only partially and thus does not prevent the measurement of relative activities in consecutive fractions.

The relative efficiency of elution of lactate dehydrogenase from the AMP-Sepharose by using the different eluents is presented in Fig. 3. Solutions of

the substrate lactate and of lactate+semicarbazide and of the cofactor NAD^+ do not desorb the enzyme to any measurable extent. Cofactor+substrate together, however, elute it quite efficiently but not so well as either cofactor+substrate+semicarbazide or reduced cofactor. NAD^+ (1 mM) is as ineffective an eluent as 0.5 mM- NAD^+ , whereas doubling the concentration of NADH results in no greater degree of enzyme elution. (On applying the solution of NADH under the specified conditions, a recovery of enzyme of the order of 25% was obtained. To permit a simple comparison of elution efficiencies, this value is arbitrarily assigned the value of 100 and other values are recorded as a percentage of this.) The fact that lactate dehydrogenase is not eluted by the lactate+semicarbazide mixture is consistent with the gradient studies by using KCl and confirms that the enzyme is tightly bound to the immobilized ligand. The finding that the reduced cofactor is far superior to the oxidized form is also consistent with the results obtained for selective elution of the two dehydrogenases as shown in Fig. 1. The fact that lactate+semicarbazide+ NAD^+ proved almost as efficient as added NADH we ascribe to the enzymic formation of NADH under the specified conditions.

Fig. 3. Batchwise elution of lactate dehydrogenase from an AMP-Sephacryl gel

The eluents depicted are (A) 125 mM-lactate + 200 mM-semicarbazide, (B) 0.5 mM- NAD^+ , (C) 125 mM-lactate + 0.5 mM- NAD^+ , (D) 125 mM-lactate + 200 mM-semicarbazide + 0.5 mM- NAD^+ and (E) 0.5 mM-NADH (for details, see the Experimental section). The efficiency of each eluent is recorded as the mean value of enzyme recovery relative to that of 0.5 mM-NADH under the conditions specified in the text. Standard deviation from the mean of four elutions was ± 5 –10%.

On omission of semicarbazide the efficiency in elution decreased by only one-fifth. This rather small decrease in elution efficiency is surprising in view of the fact that here the equilibrium is far to the side of NAD^+ . We attribute this result to cofactor and substrate acting together to induce conformational changes leading to weakening of the binding and probably ternary complex-formation (Everse *et al.*, 1971). Similar elution effects have been observed with NAD^+ +pyruvate as eluent (R. Ohlsson, P. Brodelius & K. Mosbach, unpublished work).

To exemplify the range of this affinity-chromatographic system we undertook a purification of a commercially available crude pyruvate kinase preparation. This preparation is also listed under 'lactate dehydrogenase' in the catalogue and is said to contain about equal specific activities of the two enzymes. The kinase is known to have no affinity for AMP (Reynard *et al.*, 1961). Accordingly, on application of the enzyme mixture to an AMP-Sephacryl column, pyruvate kinase activity appeared in the void volume completely free of measurable lactate dehydrogenase activity (Fig. 4). Subsequent elution of the same column with 1 mM-NADH solution eluted the lactate dehydrogenase in turn completely free from pyruvate kinase activity. The recovery of both enzymes as assayed in the mixture was essentially quantitative. In principle, there seems no reason why these preparations should not find wide application in the removal of dehydrogenase impurities from other enzymes.

In conclusion, both NAD^+ and N^6 -(6-aminohexyl)-AMP immobilized on Sepharose have proved to be efficient binding agents for affinity chromatography. Both preparations show the desired properties of general ligands having affinity for a broad enzyme spectrum. The attractive aspect of selectivity inherent in affinity chromatography is preserved by utilizing an elution procedure depending on the different affinities of the individual enzymes for the cofactors or cofactor and substrate. In this respect, pulse or gradient elution with NAD^+ or NADH are potentially very useful. The choice of enzymes is not, however, restricted to NAD^+ -dependent dehydrogenases. In principle any enzyme showing sufficient affinity for, e.g., the AMP analogue to be retarded, could subsequently be eluted by using a specific cofactor, substrate or inhibitor (e.g. AMP). The selective elution of one particular NAD^+ -dependent dehydrogenase from a bio-absorbed mixture of several can in principle be achieved by concomitant application of cofactor and the appropriate substrate. We think that in N^6 -(6-aminohexyl)-AMP we have not only a useful affinity ligand *per se*, but also a template for assembly of analogues of ATP, CoA, FAD and NAD^+ , suitable for immobilization through unambiguous and relatively unrestricted covalent linkages, capable of acting both as bio-

Fig. 4. Chromatography of crude commercially available pyruvate kinase (23 units/mg) containing lactate dehydrogenase (30 units/mg) on an AMP-Sepharose column

The AMP-Sepharose column (11 cm $\times$ 0.5 cm) was equilibrated with 0.1 M-sodium phosphate buffer, pH 7.0. After application of the mixture of enzymes (30 μ l) in buffer (0.5 ml), the column was eluted with the above buffer (40 ml), 1.2 ml fractions being collected at a flow-rate of 1 ml/10 min. Then 1 mM-NADH was applied, 0.4 ml fractions being collected at the same flow-rate. The fractions were monitored for enzymic activity as described in the Experimental section. ●, Pyruvate kinase activity; Δ, lactate dehydrogenase activity.

specific adsorbents and with a higher degree of co-enzymic function than previously observed in studies of NAD⁺-Sepharose (Mosbach, 1971; Larsson & Mosbach, 1971).

This work has been supported in part by the Swedish National Science Foundation.

References

- Axén, R., Porath, J. & Ernback, S. (1967) *Nature (London)* **214**, 1302
- Berg, P. (1958) *J. Biol. Chem.* **233**, 608
- Brink, J. J. & Schein, A. H. (1963) *J. Med. Pharm. Chem.* **6**, 563
- Bücher, T. & Pfeleiderer, G. (1955) *Methods Enzymol.* **1**, 435
- Cuatrecasas, P. & Anfinsen, C. B. (1971a) *Annu. Rev. Biochem.* **40**, 259
- Cuatrecasas, P. & Anfinsen, C. B. (1971b) *Methods Enzymol.* **22**, 191
- D'Alessio, G. & Josse, J. (1971) *J. Biol. Chem.* **246**, 4326
- Eby, P. & Kirtley, E. M. (1971) *Biochemistry* **10**, 2677
- Everse, J., Barnett, R. E., Thorne, C. J. R. & Kaplan, N. O. (1971) *Arch. Biochem. Biophys.* **143**, 444
- Falbriard, J. G., Posternak, Th. & Sutherland, E. H. (1967) *Biochim. Biophys. Acta* **148**, 99
- Feinstein, G. (1971) *Naturwissenschaften* **58**, 389
- Friedberg, F. (1971) *Chromatogr. Rev.* **14**, 121
- Furfine, C. S. & Velick, S. F. (1965) *J. Biol. Chem.* **240**, 844
- Gilham, P. T. (1971) *Methods Enzymol.* **22**, 191
- Guilford, H., Larsson, P. O. & Mosbach, K. (1972) *Chem. Scr.* in the press
- Hines, W. J. W. & Smith, M. J. H. (1964) *Nature (London)* **201**, 192
- Larsson, P. O. & Mosbach, K. (1971) *Biotechnol. Bioeng.* **13**, 393
- Lowe, C. R. & Dean, P. D. G. (1971) *FEBS Lett.* **14**, 313
- McPherson, A., Jr. (1970) *J. Mol. Biol.* **51**, 39
- Moldave, K., Castelfranco, P. & Meister, A. (1959) *J. Biol. Chem.* **234**, 841
- Mosbach, K. (1971) *Sci. Amer.* **224**, 26
- Mosbach, K., Guilford, H., Larsson, P. O., Ohlsson, R. & Scott, M. (1971) *Biochem. J.* **125**, 20P
- Novoa, W. B. & Schwert, G. W. (1961) *J. Biol. Chem.* **236**, 2150
- Nygaard, A. P. (1956) *Acta Chem. Scand.* **10**, 408
- Rammner, D. H. & Khorana, H. G. (1963) *J. Amer. Chem. Soc.* **85**, 1997
- Reynard, A. M., Hass, L. F., Jacobsen, P. D. & Boyer, P. D. (1961) *J. Biol. Chem.* **236**, 2227
- Warburg, O. (1948) *Wasserstoffübertragende Fermente*, p. 40, Verlag Dr. W. Sanger, Berlin
- Warburg, O. & Christian, W. (1939) *Biochem. Z.* **303**, 40
- Winer, A. D. & Schwert, G. W. (1958) *J. Biol. Chem.* **231**, 1065