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STUDY OF THE INTERACTION BETWEEN L-LACTATE DEHYDROGENASE ISOENZYMES AND IMMOBILIZED 8-SUBSTITUTED ADENOSINE 5'-MONOPHOSPHATE BY MEANS OF AFFINITY ELECTROPHORESIS

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SUMMARY

A water-soluble 8-substituted adenosine 5'-monophosphate–polyacrylamide (8-sub-5'-AMP–PA) was prepared as an affinity ligand for the affinity electrophoresis of L-lactate dehydrogenase (LDH).

According to the principles of affinity electrophoresis, the dissociation constants of the interaction between immobilized 8-sub-5'-AMP and rabbit LDH isoenzymes [LDH-1(H_4), LDH-2(H_3M), LDH-3(H_2M_2), LDH-4(HM_3) and LDH-5(M_4)], beef LDH-1(H_4) and LDH-5(M_4) and pig LDH-1(H_4) were calculated. Both rabbit and beef LDH-5 (muscle-type isoenzymes) had approximately a 35-fold stronger affinity to immobilized 8-sub-5'-AMP than has their LDH-1 (heart-type isoenzymes).

The effects of free nucleotides on the interaction between immobilized 8-sub-5'-AMP and LDH isoenzymes were also investigated by affinity electrophoresis. NAD^+ had a stronger affinity to LDH-5(M_4) than had 5'-AMP and 5'-IMP, while NMN, adenosine and fructose 6-phosphate had no affinity.

INTRODUCTION

Affinity electrophoresis separates proteins according to their biospecific interactions with their affinity ligands being immobilized in the electrophoretic carriers. In polyacrylamide gel disc electrophoresis, the electrophoretic mobility of a protein is retarded by an affinity ligand being immobilized in the separating gel. The dissociation constant of the interaction between the protein and the affinity ligand can be determined from the extent of the retardation of the mobility of the protein as a function of the concentration of the affinity ligand immobilized in the separating gel. According to this principle, we studied the interactions between phosphorylase and glycogen, starch[1] or oligosaccharides[2], α-amylase and starch[3,4], concanavalin A and various sugars[5], dextran-specific myeloma proteins and various dextrans[6], and phosphorylase and synthetic hydrophobic ligands[7]. According to the same principle, studies of the interactions between lectins and various sugar–acrylamide copolymers[8–12] or lectin-binding glycoproteins[13–17] have been reported.

5′-AMP or NAD^+ immobilized on agarose has been widely used in affinity chromatography. Lowe and co-workers[18–20], Mosbach and co-workers[21–23] and Trayer *et al.*[24] used this technique to purify NAD^+-dependent enzymes or phosphorylase and discussed the features of the interactions between those enzymes and 5′-AMP or NAD^+ immobilized on agarose.

We have synthesized a water-soluble 8-sub-5′-AMP–polyacrylamide and used it to determine the dissociation constants of the interactions between LDH isoenzymes and immobilized 8-sub-5′-AMP. We have also investigated the specificity of certain nucleotides in binding with LDH isoenzymes. This is the first report concerning the determination of dissociation constants of enzyme–coenzyme analogue interactions by means of electrophoresis.

EXPERIMENTAL

Materials

5′-AMP, 5′-IMP, NMN, β-NAD^+, adenosine, fructose 6-phosphate, nitroblue tetrazolium, phenazine methosulphate, tris(hydroxymethyl)aminomethane (Tris), 2,4,6-trinitrobenzene-1-sulphonic acid (TNBS), and Dowex 1-X2 (Cl^-, 200–400 mesh) were purchased from Sigma (St. Louis, Mo., U.S.A.). Allylamine, bromine, acrylamide, N,N,N′,N′-tetramethylenediamine (TEMED), N,N′-methylenebisacrylamide, lithium L-lactate and bromphenol blue (BPB) were obtained from Nakarai (Kyoto, Japan). Pre-coated silica gel F_{254} TLC plates and pre-coated cellulose F TLC plates were purchased from Merck (Darmstadt, G.F.R.). Sephadex G-100 was obtained from Pharmacia (Uppsala, Sweden). Crystalline rabbit muscle LDH-5(M_4), beef muscle LDH-5(M_4), beef heart LDH-1(H_4) and pig heart LDH-1(H_4) were purchased from Sigma.

Preparation of rabbit brain crude extract

A male rabbit was deeply anaesthetized with 5 ml of 5% sodium pentobarbiturate solution by venous injection and the brain was removed immediately. The brain was chilled on ice and homogenized with an equal volume of 0.01 *M* phosphate buffer (pH 7.4). The homogenate was centrifuged at 11,200 *g* for 1 h and the supernatant was stored at −20°. The protein content of the crude extract was determined by Warburg and Christian's method[25].

Analytical procedures

Thin-layer chromatography was performed in three different solvent systems (see Table I). 5′-AMP derivatives on the developed plates were detected under ultraviolet (UV) light.

UV spectra were obtained with a Hitachi Model 200-20 spectrophotometer. ^1H Nuclear magnetic resonance (NMR) spectra were obtained with a Jeol JNM-MH 100 NMR spectrometer operating at 100 MHz.

The phosphate contents of 5′-AMP derivatives were determined by Allen's method[26].

Synthesis of 8-substituted 5′-AMP derivatives

Synthesis of 8-bromo-5′-AMP (8-Br-5′-AMP). 8-Bromo-5′-AMP was synthesized according to the methods of Lowe[19]. The product, obtained in approximately

85% overall yield, was confirmed to be 8-bromo-5'-AMP from thin-layer chromatographic, UV and ^{1}H NMR spectroscopic data.

Synthesis of 8-allyl-5'-AMP (8-allyl-5'-AMP). Allylamine (4.13 ml, 55 mmol) was dissolved in 5 ml of water and the pH of the solution was adjusted to 9.0 with 2.9 ml of concentrated hydrochloric acid. 8-Br-5'-AMP (1.0 g, 2.1 mmol) was added to the solution, which was refluxed in a water-bath at 75° for 38 h. The mixture was cooled, allowed to stand overnight at room temperature, diluted to 500 ml with water and the pH was adjusted to 11.0 with 5 *M* sodium hydroxide solution. The solution was applied to a column (20 × 2.5 cm) of Dowex 1-X2 (CH_3COO^-, 200–400 mesh) equilibrated with water. Unreacted allylamine was washed out with water (800 ml) until the TNBS colour test[27] on the effluent showed no positive results. Then, the nucleotide was eluted with a linear gradient of acetic acid (0–1 *M*, 500 ml total volume). Fractions comprising a major peak of UV-absorbing material, max. λ 279 nm, were pooled and lyophilized. The product (566 mg), obtained in approximately 56% overall yield, was confirmed to be 8-allyl-5'-AMP from thin-layer chromatographic, UV and ^{1}H NMR spectroscopic data.

Preparation of a water-soluble 8-substituted 5'-AMP–polyacrylamide (8-sub-5'-AMP–PA). Acrylamide (100 mg), 8-allyl-5'-AMP (100 mg), TEMED (20 μl) and ammonium persulphate (5 mg) were dissolved in 5 ml of water (in a glass cylinder) and 1 ml of water was overlayed on the solution to a depth of 1 mm. The solution was then heated at 60° for 3 h. After cooling to room temperature, the solution was diluted to 10 ml with water and dialysed against a large volume of water several times until no UV-absorbing material was detected in the outer dialysis solution. The content of 8-sub-5'-AMP incorporated in the polyacrylamide was determined to be 400 μmol/g by UV absorption at 279 nm using the calibration graph obtained from 8-allyl-5'-AMP at 279 nm. The molecular weight of the 8-sub-5'-AMP–PA was presumed to be over $1 \cdot 10^5$ by gel filtfation using Sephadex G-100 because it was eluted in the same fraction as blue dextran.

Affinity electrophoresis

Polyacrylamide gel disc electrophoresis was carried out by a slightly modified form of Takeo and Nakamura's method[1]. The separating gel (5 cm in height) was 5% polyacrylamide gel in Tris–HCl buffer (pH 8.9) as described by Ornstein and Davis[28], containing the synthesized 8-sub-5'-AMP–PA at various concentrations. To ensure a uniform concentration of 8-sub-5'-AMP–PA throughout the separating gel, it was prepared by overlaying a solution containing 8-sub-5'-AMP–PA at the same concentration as that in the separating gel, instead of water.

The spacer gel (1 cm in height) was 2.5% polyacrylamide gel in Tris–HCl buffer (pH 6.7). The running buffer was Tris–glycine buffer (pH 8.3). A sample solution was prepared by mixing a thioglycolate (67 m*M*, pH 6.7) and 40% sucrose solution. The sample solution (50 μl, containing 20 μg of protein of rabbit brain extract or 0.1 μg of protein of crystalline LDH isoenzymes) was applied to each gel tube. Electrophoresis was carried out at 2 mA per gel tube for 2 h until the tracking BPB band had migrated 4.5 cm from the origin in the separating gel. After electrophoresis, a fine metal wire was inserted at the position of the BPB band. The gels were stained for LDH activity utilizing the nitroblue tetrazolium reagent[29], and for protein using Coomassie brilliant blue (0.02% Coomassie brilliant blue dissolved in 7% acetic acid).

The mobilities of LDH isoenzymes were expressed as R_{m0} or R_{mi}, the migration distances of LDH isoenzymes relative to that of the tracking BPB band in the absence or presence of 8-sub-5′-AMP–PA in the separating gel, respectively.

The effects of free nucleotides on the interaction between LDH isoenzymes and immobilized 8-sub-5′-AMP were investigated using gel containing 8-sub-5′-AMP–PA together with one of the nucleotides. The same procedure was used in the analysis of the effects of oligosaccharides on the interaction between phosphorylase and polyglucan[2]. The separating gel was 5% polyacrylamide gel in Tris–HCl buffer (pH 8.9) containing 0.2 m*M* of 8-sub-5′-AMP–PA together with 25 μM of the nucleotide (β-NAD$^+$, NMN, 5′-AMP or 5′-IMP). The same concentration of the nucleotide was added to both the spacer gel and the running buffer solution. The extent of the decrease in the retardation of the original LDH isoenzyme mobility due to the nucleotide was expressed as $(R_{mj}-R_{mi}/R_{m0}-R_{mi})\cdot 100(\%)$, where R_{mj} is the migration distance of LDH isoenzyme relative to that of the tracking BPB band in the presence of 8-sub-5′-AMP–PA together with the given concentration of the nucleotide in the separating gel, R_{mi} is the migration distance of the LDH isoenzyme relative to that of the tracking BPB band in the presence of 8-sub-5′-AMP–PA without the nucleotide in the separating gel and R_{m0} is the migration distance of the LDH isoenzyme relative to that of the tracking BPB band in the absence of 8-sub-5′-AMP–PA in the separating gel.

Calculation of dissociation constant by affinity electrophoresis

The dissociation constants of the interaction between LDH isoenzymes and immobilized 8-sub-5′-AMP were calculated according to the theory of affinity electrophoresis[1]. From the equation

$$\frac{1}{R_{mi}} = \frac{1}{R_{m0}}\left(1 + \frac{c}{K}\right)$$

where R_{m0} and R_{mi} are the relative migration distances of LDH isoenzyme in the absence and the presence of 8-sub-5′-AMP–PA, respectively, in the separating gel, K is the dissociation constant of the interaction between LDH isoenzyme and immobilized 8-sub-5′-AMP and c is the concentration of immobilized 8-sub-5′-AMP in the separating gel, we can determine the dissociation constant. If the reciprocal of R_{mi} is plotted against c, a straight line will be obtained. The c-intercept of the line gives $-K$.

RESULTS AND DISCUSSION

Water-soluble polymer ligands are essential for affinity electrophoresis to determine the dissociation constants of biospecific interactions. In those materials, natural products such as soluble starch[1,3,4], glycogen[1,2,5] and dextrans[6] or synthetic ligands such as hydrophobic groups immobilized on dextran[7], blue dextran[30] and water-soluble O-glycosyl–polyacrylamide copolymers[8–12] have been used. We synthesized a water-soluble 8-substituted 5′-AMP–polyacrylamide to determine the dissociation constants of the interaction between LDH isoenzymes and immobilized 8-substituted 5′-AMP by means of affinity electrophoresis.

Synthesis of 8-substituted 5'-AMP derivatives and their structures and chemical properties

The synthetic pathways of 8-substituted 5'-AMP derivatives are shown in Fig. 1. 5'-AMP is brominated at the C-8 position of the adenine nucleus, then there is a nucleophilic displacement with allylamine. Subsequent copolymerization with acrylamide gives a water-soluble 8-sub-5'-AMP–polyacrylamide.

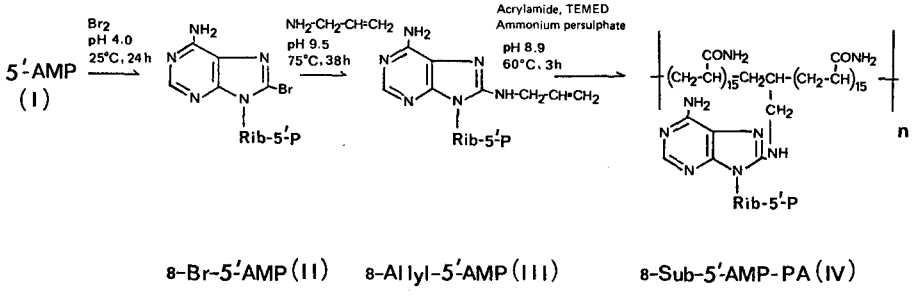

Fig. 1. Synthetic pathways of adenosine 5'-monophosphate derivatives substituted at the C-8 position of the adenine nucleus and a water-soluble 8-substituted 5'-AMP–polyacrylamide. Rib, ribose; P, monophosphate; NH_2–CH_2–$CH=CH_2$, allylamine.

8-Bromo-5'-AMP has $\lambda_{max.}$ at 263 nm and 8-allyl-5'-AMP at 279 nm. This shift of $\lambda_{max.}$ is characteristic of 8-sub-5'-AMP derivatives[19,24]. No further shift of $\lambda_{max.}$ was observed after the reaction of the terminal allyl group with acrylamide to give a water-soluble 8-sub-5'-AMP–polyacrylamide. The molar absorptivities of these 5'-AMP derivatives were determined (Table I).

TABLE I

THIN-LAYER CHROMATOGRAPHIC AND UV ABSORPTION DATA FOR ADENOSINE 5'-MONOPHOSPHATE DERIVATIVES SUBSTITUTED AT THE C-8 POSITION OF THE ADENINE NUCLEUS

Solvent systems: A = isobutyric acid–1 *M* aqueous ammonia (5:3) saturated with EDTA; B = ethanol–0.5 *M* ammonium acetate (9:4); C = 0.5 *M* LiCl. Molar absorptivities were determined in 0.1 *M* Tris–HCl buffer (pH 7.5).

Compound	*R_F value*					*Molar absorptivity (mol^{-1} cm^{-1})*	*λ_{max} (nm)*
	Cellulose F		*Silica gel*				
	A	*B*	*A*	*B*	*C*		
5'-AMP	0.56	0.07	0.57	0.37	0.68	15,400	259
8-Bromo-5'-AMP	0.63	0.12	0.61	0.56	0.73	17,900	263
8-Allyl-5'-AMP	0.79	0.20	0.64	0.62	0.56	20,200	279

Phosphate group analysis of all derivatives demonstrated the presence of one phosphate group per molecule of the derivatives (0.95±0.1 mol per 1.0 mol of derivative). 5'-AMP and synthesized 8-sub-5'-AMP derivatives were detected as single spots on the thin-layer chromatographic plates. As shown in Table I, they had different R_F values in each of the three solvent systems.

As shown in Fig. 2, the substitution of allylamine at the C-8 position of the

adenine nucleus was confirmed by the ^1H NMR spectra. In Fig. 2B, the signal of adenine-C8H (δ 9.34) disappeared and the signals of allyl-C3H$_2$ (δ 5.52), allyl-C2H (δ 5.78–6.16), allyl-C1H$_2$ (δ 4.2–4.48) and allyl-N1H (δ 7.9–8.12) appeared in ^1H NMR spectrum of 8-allyl-5′-AMP.

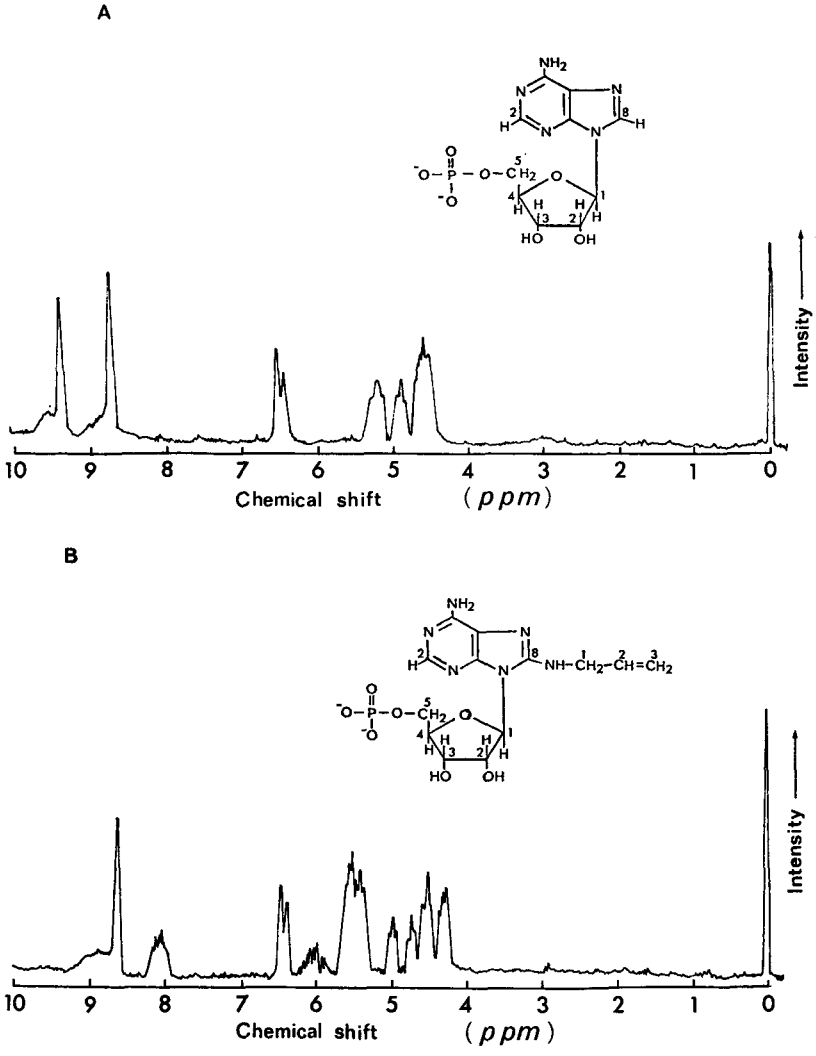

Fig. 2. ^1H NMR spectra of adenosine 5′-monophosphate derivatives substituted at the C-8 position of the adenine nucleus. (A) Adenosine 5′-monophosphate; (B) 8-allyladenosine 5′-monophosphate. Conditions for ^1H NMR: solvent, trifluoroacetic acid; internal reference, tetramethylsilane[31]; room temperature.

Determination of the dissociation constants of the interaction between LDH isoenzymes and immobilized 8-substituted 5′-AMP

In Fig. 3, LDH isoenzyme affinity electrophoretic patterns of rabbit brain extract (A), crystalline rabbit muscle LDH-5(M$_4$) (B), beef muscle LDH-5(M$_4$) (C),

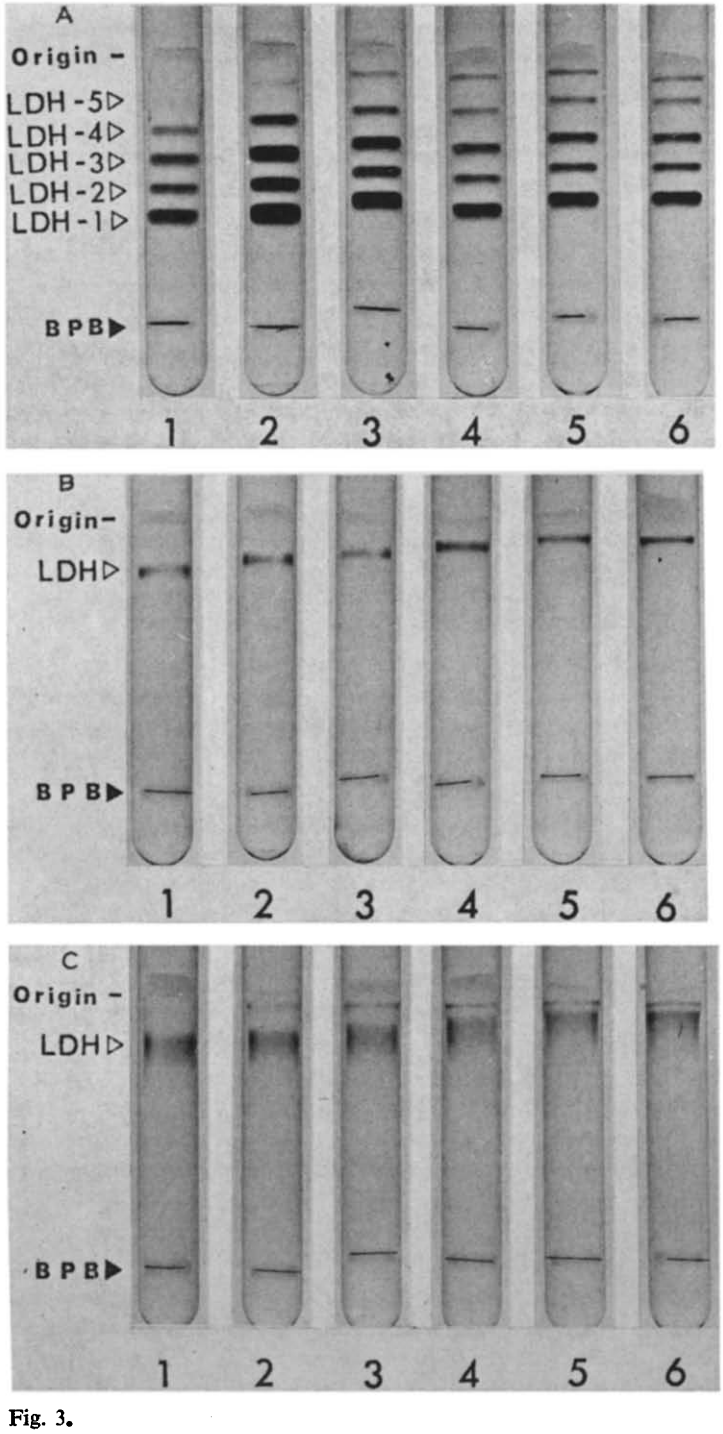

Fig. 3.

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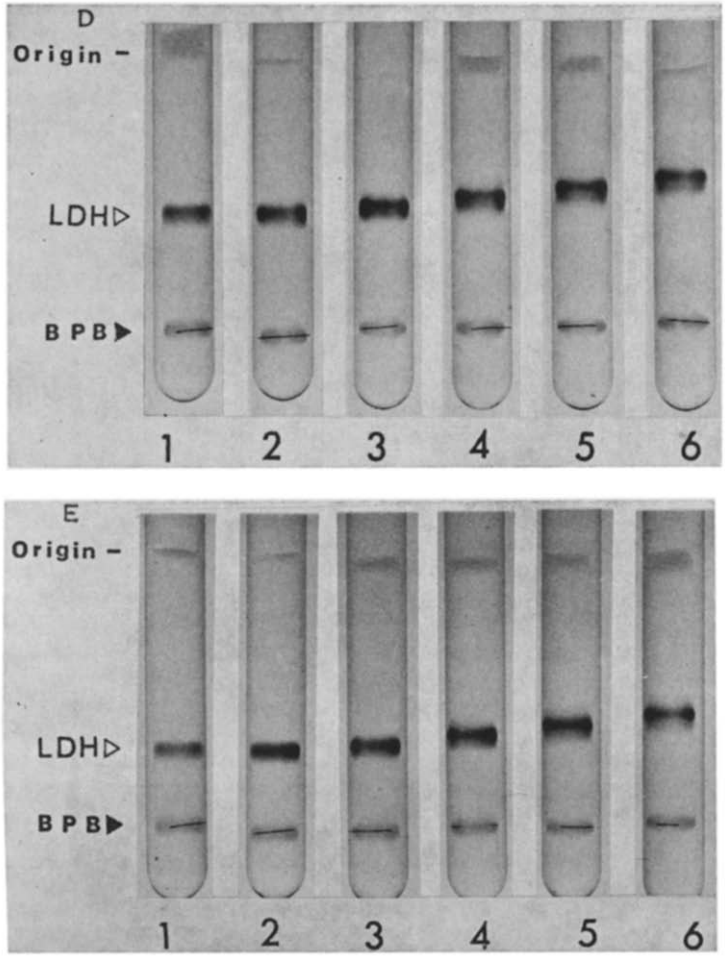

Fig. 3. Affinity electrophoresis of LDH isoenzymes using gels containing various concentrations of immobilized 8-substituted 5′-AMP. (A) Rabbit brain extract; (B) crystalline rabbit muscle LDH-5 (M_4); (C) crystalline beef muscle LDH-5 (M_4); (D) crystalline beef heart LDH-1 (H_4); (E) crystalline pig heart LDH-1 (H_4). Concentrations of immobilized 8-substituted 5′-AMP in the separating gel: (A) 1, 0 m*M*; 2, 0.062 m*M*; 3, 0.125 m*M*; 4, 0.25 m*M*; 5, 0.375 m*M*; 6, 0.5 m*M*; (B and C) 1, 0 m*M*; 2, 0.025 m*M*; 3, 0.05 m*M*; 4, 0.1 m*M*; 5, 0.15 m*M*; 6, 0.2 m*M*; (D and E) 1, 0 m*M*; 2, 0.125 m*M*; 3, 0.25 m*M*; 4, 0.5 m*M*; 5, 0.75 m*M*; 6, 1.0 m*M*.

beef heart LDH-1(H_4) (D) and pig heart LDH-1(H_4) (E) are shown. In rabbit brain extract, five LDH isoenzyme activity bands were observed: LDH-1(H_4), LDH-2(H_3M), LDH-3(H_2M_2), LDH-4(HM_3) and LDH-5(M_4) in order from the bottom of the separating gel. The mobilities of LDH isoenzymes were decreased to different extents when immobilized 8-sub-5′-AMP was present in the gel. When the concentration of immobilized 8-sub-5′-AMP was increased, the mobilities of LDH isoenzymes decreased in direct proportion to the concentration of immobilized 8-sub-5′-AMP. The mobilities of proteins other than NAD^+-dependent enzymes were not affected by the presence of 1 m*M* of immobilized 8-sub-5′-AMP. This result indicates

that the retardation of the mobilities of LDH isoenzymes is due to the specific interaction between LDH isoenzymes and immobilized 8-sub-5′-AMP, and not to any change in the molecular-sieving effect by the addition of high-molecular-weight 8-sub-5′-AMP-PA to the separating gel or to its negative charges.

In Fig. 4, reciprocal relative migration distances of rabbit brain LDH isoenzymes (A), crystalline rabbit muscle LDH-5(M_4) and beef muscle LDH-5(M_4) (B) and crystalline pig heart LDH-1(H_4) and beef heart LDH-1(H_4) (C) are plotted against the concentration of immobilized 8-sub-5′-AMP in the separating gel. From these plots, the dissociation constants were determined (Table II). The dissociation constant of rabbit brain LDH-5(M_4) was $8.60 \cdot 10^{-5}$ *M*, approximately one fortieth that of LDH-1(H_4) ($3.37 \cdot 10^{-3}$ *M*). With crystalline rabbit muscle LDH-5(M_4), almost the same dissociation constant ($7.60 \cdot 10^{-5}$ *M*) as that of rabbit brain LDH-5(M_4) was obtained. The dissociation constants of the hybrid LDH isoenzymes were related as follows: LDH-1(H_4)>LDH-2(H_3M)>LDH-3(H_2M_2)>LDH-4(HM_3)>LDH-5(M_4). Similarly, the dissociation constant of crystalline beef muscle LDH-5(M_4) was $9.60 \cdot 10^{-5}$ *M*, approximately one thirty-fifth that of beef heart LDH-1(H_4) ($3.23 \cdot 10^{-3}$ *M*). These results indicate that the binding affinity of the muscle-type LDH subunit to immobilized 8-sub-5′-AMP is much stronger than that of the heart-type LDH subunit. This result agrees with those obtained by Lowe[19] from affinity chromatography using 8-substituted 5′-AMP immobilized on Sepharose.

Lowe *et al.*[20] reported that the binding affinity of the muscle-type LDH subunit to immobilized N6-substituted 5′-AMP was almost the same as that of the heart-type LDH subunit by means of affinity chromatography using N6-substituted 5′-AMP immobilized on Sepharose. This discrepancy between our results and those of Lowe *et al.* may be due to differing positions of immobilized 5′-AMP derivatives on the matrix. In our experiment, there was a substitution of the spacer at the C-8 position of the adenine nucleus and in their experiment the substitution occurred at the N-6 position of the adenine nucleus.

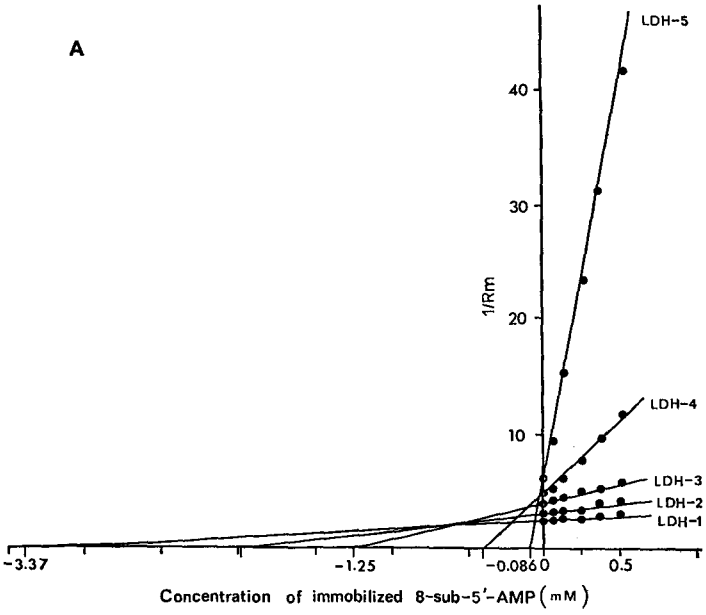

Fig. 4.

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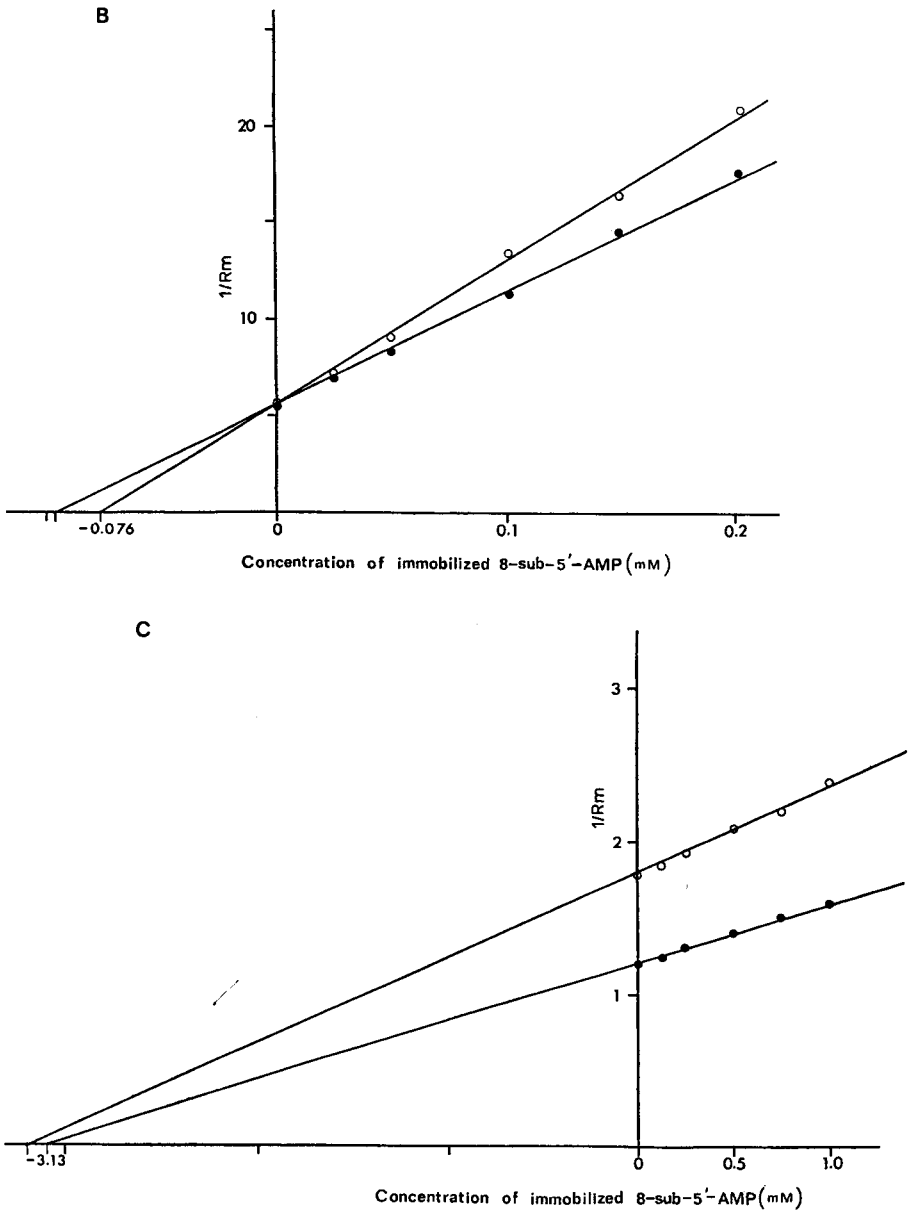

Fig. 4. Plots of the reciprocal of the relative migration distance ($1/R_m$) of LDH isoenzymes against the concentration of immobilized 8-substituted 5'-AMP in the separating gel. (A) Rabbit brain LDH-1 (H_4), LDH-2 (H_3M), LDH-3 (H_2M_2), LDH-4 (HM_3) and LDH-5 (M_4); (B) crystalline rabbit muscle LDH-5 (M_4) (○) and crystalline beef muscle LDH-5 (M_4) (●); (C) crystalline beef heart LDH-1 (H_4) (○) and crystalline pig heart LDH-1 (H_4) (●).

Effects of some nucleotides on the interaction between LDH and immobilized 8-substituted 5'-AMP

As shown in Fig. 5, when affinity electrophoresis of rabbit muscle LDH-5(M_4) was carried out with a separating gel containing immobilized 8-sub-5'-AMP (0.2 m*M*)

TABLE II

DISSOCIATION CONSTANTS OF THE INTERACTION BETWEEN LDH ISOENZYMES AND IMMOBILIZED 8-SUBSTITUTED 5′-AMP

LDH isoenzyme	*Dissociation constant (M)*
Rabbit brain LDH-1 (H_4)	$3.37 \cdot 10^{-3}$
LDH-2 (H_3M)	$1.94 \cdot 10^{-3}$
LDH-3 (H_2M_2)	$1.25 \cdot 10^{-3}$
LDH-4 (HM_3)	$4.07 \cdot 10^{-4}$
LDH-5 (M_4)	$8.60 \cdot 10^{-5}$
Crystalline beef heart LDH-1 (H_4)	$3.23 \cdot 10^{-3}$
pig heart LDH-1 (H_4)	$3.13 \cdot 10^{-3}$
beef muscle LDH-5 (M_4)	$9.60 \cdot 10^{-5}$
rabbit muscle LDH-5 (M_4)	$7.60 \cdot 10^{-5}$

together with NAD^+(25 μM), the retardation of the mobility of LDH decreased (gel No. 3). Similarly, the retardation of the mobility of LDH decreased with 5′-AMP (gel No. 4) and 5′-IMP (gel No. 5). On the other hand, no decrease in the retardation of the mobility of LDH was observed with NMN, adenosine and fructose 6-phosphate (gel No. 6). These results indicate that the decrease in the retardation may be due to a specific effect of the adenine nucleotide on the interaction between LDH and immobilized 8-sub-5′-AMP.

The extents of the decrease in the retardation of the mobility of LDH-5(M_4) with NAD^+, 5′-AMP and 5′-IMP were calculated as 43%, 26% and 20%, respectively. Because the exact concentration of these nucleotides in the separating gel could not be determined, the dissociation constants of the interaction between LDH-5(M_4) and these nucleotides were not calculated. However, from the extents of the decrease in the retardation of the mobility of LDH-5(M_4), it can be assumed that NAD^+ has a higher affinity to LDH-5(M_4) than 5′-AMP and 5′-IMP. This relationship is supported by other kinetic data[32–34].

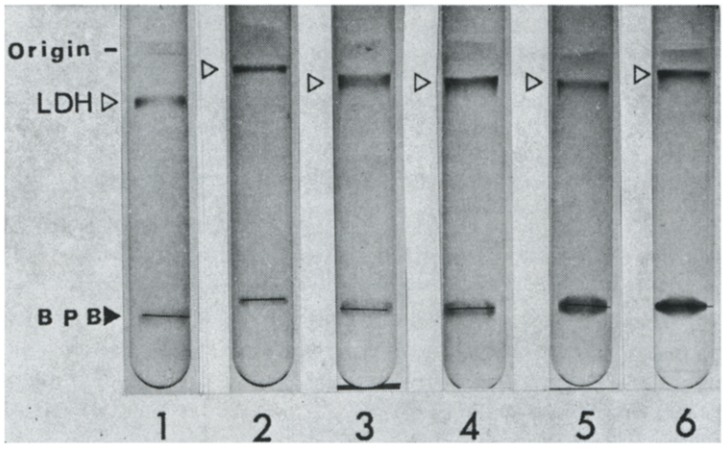

Fig. 5. Affinity electrophoresis of crystalline rabbit muscle LDH-5 (M_4) using gels containing immobilized 8-substituted 5′-AMP together with some nucleotides. Concentrations of immobilized 8-substituted 5′-AMP and the nucleotides in the separating gel: 1, 0 mM of immobilized 8-sub-5′-AMP without the nucleotides; 2, 0.2 mM of immobilized 8-sub-5′-AMP without the nucleotides; 3, 0.2 mM of immobilized 8-sub-5′-AMP with 25 μM of NAD^+; 4, 0.2 mM of immobilized 8-sub-5′-AMP with 25 μM of 5′-AMP; 5, 0.2 mM of immobilized 8-sub-5′-AMP with 25 μM of 5′-IMP; 6, 0.2 mM of immobilized 8-sub-5′-AMP with 25 μM of NNH, adenosine or fructose 6-phosphate.

Affinity electrophoresis is a simple and useful method for exploring biospecific interactions. Here, we have presented its application to the study of the interaction between LDH isoenzymes and immobilized 8-substituted 5'-AMP. Recently, Tichá *et al.*[30] have reported the affinity electrophoresis of proteins interacting with blue dextran. By these methods, the dissociation constants of enzyme–coenzyme interactions can be determined using a very small amount of sample proteins (less than 1 mg) within a few hours. Further, the dissociation constants of several isoenzymes can be determined simultaneously without purification.

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