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STUDY OF THE INTERACTION BETWEEN NADP-DEPENDENT DEHYDROGENASE AND IMMOBILIZED ADENOSINE 2′-MONOPHOSPHATE BY MEANS OF AFFINITY ELECTROPHORESIS

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SUMMARY

A water-soluble 8-substituted adenosine 2′-monophosphate-polyacrylamide (8-sub-2′-AMP-PA) was prepared as a new affinity ligand for the determination of the dissociation constants of the interactions between immobilized 8-sub-2′-AMP and NADP-dependent dehydrogenases (NADP-dependent DH), NAD-dependent dehydrogenases (NAD-dependent DH) and phosphorylase by means of affinity electrophoresis.

From the dissociation constants, it was found that NADP-dependent DH had a much stronger affinity to immobilized 8-sub-2′-AMP than did NAD-dependent DH and phosphorylase. On the other hand, NADP-dependent DH had a much weaker affinity to immobilized 8-sub-5′-AMP than did NAD-dependent DH. The effects of $NADP^+$ and NAD^+ on the interaction between immobilized 8-sub-2′-AMP and NADP-dependent DH were also investigated by means of affinity electrophoresis. $NADP^+$ inhibited the interaction specifically, but NAD^+ did not inhibit the interaction.

These results indicate that 8-sub-2′-AMP binds to the coenzyme binding site of NADP-dependent DH, while other compounds, such as 8-sub-5′-AMP and NAD^+, do not bind to NADP-dependent DH. Such a difference suggests that the phosphate group at position 2′ in 2′-AMP and $NADP^+$ is important for the binding at the coenzyme binding site of NADP-dependent DH.

INTRODUCTION

Adenine nucleotide derivatives have been utilized for studying the coenzyme binding to many NADP-dependent dehydrogenases and NAD-dependent dehydrogenases, including 6-phosphogluconate dehydrogenase[1–3], glucose 6-phosphate dehydrogenase[4,5], malic enzyme[6,7], lactate dehydrogenase[8–11] and malate dehydrogenase[12–14]. N6-substituted adenine nucleotides[15–18] and 8-substituted adenine nucleotides[19–21] immobilized on agarose have been utilized in affinity chromatography as general ligands.

Recently, we have reported the determination of the dissociation constants

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of lactate dehydrogenase isoenzymes for adenine nucleotides utilizing a water-soluble 8-substituted adenosine 5'-monophosphate-polyacrylamide (8-sub-5'-AMP-PA) by means of affinity electrophoresis[22].

Here, we report the synthesis of a water-soluble 8-substituted adenosine 2'-monophosphate-polyacrylamide (8-sub-2'-AMP-PA) as a new affinity ligand and the determination of the dissociation constants of NADP-dependent dehydrogenases, NAD-dependent dehydrogenases and phosphorylase for immobilized 8-sub-2'-AMP by means of affinity electrophoresis. The specificity of these enzyme–coenzyme analogue interactions and the effects of $NADP^+$ and NAD^+ on the interactions are discussed.

Abbreviations

2'-AMP = adenosine 2'-monophosphate; 5'-AMP = adenosine 5'-monophosphate; 8-sub-2'-AMP-PA = 8-substituted adenosine 2'-monophosphate-polyacrylamide; 8-sub-5'-AMP-PA = 8-substituted adenosine 5'-monophosphate-polyacrylamide; NAD^+ = nicotinamide adenine dinucleotide, oxidized form; $NADP^+$ = nicotinamide adenine dinucleotide phosphate, oxidized form; Tris = tris(hydroxymethyl)aminomethane; TNBS = 2,4,6-trinitrobenzene 1-sulphonic acid; TEMED = N,N,N',N'-tetramethylethylenediamine; EDTA = ethylenediaminetetraacetic acid; BPB = bromophenol blue; NMR = nuclear magnetic resonance.

Enzymes

NADP-dependent dehydrogenase: 6-phosphogluconate dehydrogenase (E.C. 1.1.1.44), malic enzyme (E.C. 1.1.1.40), glucose 6-phosphate dehydrogenase (E.C. 1.1.1.49) and isocitrate dehydrogenase (E.C. 1.1.1.42). NAD-dependent dehydrogenase: lactate dehydrogenase (E.C. 1.1.1.27) and malate dehydrogenase (E.C. 1.1.1.37). Phosphorylase (E.C. 2.4.1.1).

EXPERIMENTAL

Materials

β-$NADP^+$, β-NAD^+, 2'-AMP, 5'-AMP, nitroblue tetrazolium, phenazine methosulphate, tris(hydroxymethyl)aminomethane (Tris), 2,4,6-trinitrobenzene 1-sulphonic acid (TNBS), L-malic acid, lithium L-lactate, D-glucose 6-phosphate, 6-phospho-D-gluconic acid, *threo*-D_s(+)-isocitric acid 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'-tetramethylethylenediamine (TEMED), N,N'-methylenebisacrylamide, 2-mercaptoethanol, ethylenediaminetetraacetic acid (EDTA) and bromophenol blue (BPB) were obtained from Nakarai (Kyoto, Japan). Pre-coated silica gel F_{254} thin-layer chromatographic (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). 8-Substituted adenosine 5'-monophosphate-polyacrylamide was synthesized by a method described previously[22]. Crystalline L-malate dehydrogenase (E.C. 1.1.1.37) (pig heart mitochondrial and cytosol isoenzymes) were purchased from Sigma.

Preparation of crude extracts from rabbit tissues, carrot and potato

A male rabbit was deeply anaesthetized with 5 ml of 5% sodium pentobarbiturate solution by venous injection and the tissues (liver and brain) were removed immediately. Each tissue was chilled on ice and homogenized with an equal volume of 0.01 *M* phosphate buffer (pH 7.4). Each homogenate was centrifuged at 11,200 *g* for 1 h and the supernatant was stored at —20°C. Carrot was sliced and homogenized with an equal volume of 0.01 *M* phosphate buffer (pH 7.4). The homogenate was centrifuged at 7180 *g* for 1 h and the supernatant was stored at —20°C. Potato was sliced and homogenized with an equal volume of 10 m*M* EDTA and 100 m*M* 2-mercaptoethanol (pH 7.4). The homogenate was centrifuged at 7180 *g* for 1 h and the supernatant was stored at —20°C[23]. The protein contents of all crude extracts were determined by Warburg and Christian's method[24].

Analytical procedures

TLC data, UV spectra and molar absorptivities of 8-substituted 2′-AMP derivatives were obtained by the procedures described previously[22]. ^{1}H NMR spectra were obtained with a Jeol JNM-MH 100 NMR spectrometer operating at 100 Mz under the conditions detailed in the legend of Fig. 1. The phosphate contents of all of the 2′-AMP derivatives were determined by Allen's method[25].

Synthesis of 8-substituted 2′-AMP derivatives

8-Substituted 2′-AMP derivatives were synthesized by a method slightly modified from that described previously[22].

Synthesis of 8-bromo-2′-AMP

2′-AMP (free acid, 4.5 g, 10 mmole) was dissolved in 400 ml of 1 *M* sodium acetate at pH 4.0 and bromine–water (0.87 ml of bromine, 17 mmole, dissolved in 87 ml of water with vigorous shaking) was added. The solution was gently stirred and kept in the dark at 25°C for 24 h. The white precipitate that appeared in the reaction mixture was collected by filtration on a glass Büchner funnel and washed twice with 98% ethanol (100 ml). The resulting white solid was dissolved in a minimum volume of water (50 ml) and twice the volume of 98% ethanol was added to the solution. The white precipitate in the solution was collected and washed with 98% ethanol (100 ml) on a glass Büchner funnel. The resulting white solid (3.9 g, approximately 87% yield) was confirmed to be 8-bromo-2′-AMP from TLC, UV and ^{1}H NMR spectroscopic data.

Synthesis of 8-allyl-2′-AMP

8-Bromo-2′-AMP (1.0 g, 2.1 mmole) was dissolved in 10 ml of water to which allylamine (4.13 ml, 55 mmole) was added, and the pH was adjusted to 9.0 with 2.9 ml of concentrated hydrochloric acid. The solution was refluxed in a water-bath at 75°C for 38 h. After the coupling reaction was complete, the reaction mixture was diluted to 400 ml with water and then its pH was adjusted to 11.5 with 5 *M* sodium hydroxide solution. The solution was applied to a column (20 × 2.5 cm I.D.) of Dowex 1-X2 (CH_3COO^-, 200–400 mesh) equilibrated with water. After the column had been washed with water (800 ml) until unreacted allylamine was not detected in

the effluent by the TNBS colour test[26], the nucleotide was eluted with a linear gradient of acetic acid (0–1 *M*, total volume 500 ml). Fractions comprising a major peak of UV-absorbing materials, $\lambda_{max.}$ 279 nm, were pooled and lyophilized. The resulting white solid (590 mg, approximately 59% yield) was confirmed to be 8-allyl-2'-AMP from TLC, UV and ^{1}H NMR spectroscopic data.

Preparation of a water-soluble 8-substituted 2'-AMP-polyacrylamide (8-sub-2'-AMP-PA)

Acrylamide (100 mg), 8-allyl-2'-AMP (100 mg), TEMED (0.02 ml) and ammonium persulphate (5 mg) were dissolved in 5 ml of water in a small glass cylinder and 1 ml of water was overlayed on the solution. The solution was heated at 60°C for 3 h. After cooling to room temperature, the solution was dialysed against a large volume of water (2 l) several times until no UV-absorbing materials were detected in the outer dialysis solution. The content of 8-sub-2'-AMP incorporated in polyacrylamide was determined to be 420 μmole/g by UV absorption at 279 nm using the calibration graph obtained from 8-allyl-2'-AMP at 279 nm. The molecular weight of the 8-sub-2'-AMP-PA was presumed to be over $1 \cdot 10^5$ by gel filtration using Sephadex G-100 because it was eluted in the same fraction as Blue Dextran.

Electrophoresis

Polyacrylamide gel disc electrophoresis was carried out by the procedures described previously[22].

The synthesized 8-sub-2'-AMP-PA was added to the separating gel (5% polyacrylamide gel in Tris–hydrochloric acid buffer at pH 8.9 as described by Ornstein and Davis[27]) in an amount giving the desired concentration of immobilized 8-sub-2'-AMP. The protein sample (50 μl, containing 50–150 μg of protein of crude extracts or 2 μg of protein of crystalline enzyme solution) in 10% sucrose solution containing 30 m*M* sodium thioglycolate (pH 6.7) was applied to each gel tube. Electrophoresis in Tris–glycine buffer (pH 8.3) 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 migration distances of the enzymes were measured after each specific enzyme activity staining utilizing the nitroblue tetrazolium reagent[28–31] and iodide reagent[32].

The effects of $NADP^+$ and NAD^+ on the interactions between the enzymes and immobilized 8-sub-2'-AMP were investigated using the separating gel containing 8-sub-2'-AMP-PA together with $NADP^+$ or NAD^+. $NADP^+$ or NAD^+ was added to both the spacer gel and the running buffer solution at the same concentration as that of the separating gel. From the extent of the decrease in the retardation of the original enzymes mobilities due to $NADP^+$ or NAD^+, we calculated the strength of binding affinity of $NADP^+$ or NAD^+ to the enzymes.

Calculation of dissociation constants by affinity electrophoresis

The dissociation constants of the interactions between NADP-dependent dehydrogenases, NAD-dependent dehydrogenases and phosphorylase and immobilized 8-sub-2'-AMP were calculated from the extent of the retardation of mobility of the enzymes as a function of the concentration of immobilized 8-sub-2'-AMP in

the separating gel. We determined the dissociation constants from the following equation[33]:

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

where R_{mo} and R_{mi} are the relative migration distances of the enzyme to that of the tracking BPB band in the absence and the presence of immobilized 8-sub-2′-AMP in the separating gel, respectively, K is the dissociation constant of the interaction between the enzyme and immobilized 8-sub-2′-AMP and c is the concentration of immobilized 8-sub-2′-AMP in the separating gel. If $1/R_{mi}$ is plotted against c, a straight line will be obtained. The intercept of the line of the c axis gives $-K$.

RESULTS AND DISCUSSION

Chemical properties of 8-substituted 2′-AMP derivatives

2′-AMP and synthesized 8-sub-2′-AMP derivatives were detected as single spots on the TLC plates under UV light. As shown in Table I, they had different R_F values in each of the three solvent systems.

TABLE I

TLC AND UV ABSORPTION DATA ON 8-SUBSTITUTED ADENOSINE 2′-MONOPHOSPHATE DERIVATIVES AND 8-SUBSTITUTED ADENOSINE 5′-MONOPHOSPHATE DERIVATIVES

Solvent systems used in TLC: A, isobutyric acid–1 *M* ammonia solution (5:3) saturated with EDTA; B, ethanol–0.5 *M* ammonium acetate (9:4); C, 0.5 *M* lithium chloride solution. Molar absorptivities were determined in 0.1 *M* Tris–hydrochloric acid buffer at pH 7.5.

Compound	R_F *value*					*Molar absorptivity* ($l\ mol^{-1}\ cm^{-1}$)	$\lambda_{max.}$ *(nm)*
	Cellulose F		*Silica gel* F_{254}				
	A	*B*	*A*	*B*	*C*		
Adenosine	0.82	0.53	0.76	0.73	0.72	15100	260
2′-AMP	0.66	0.17	0.63	0.41	0.67	15400	259
8-Bromo-2′-AMP	0.72	0.27	0.70	0.63	0.70	17400	263
8-Allyl-2′-AMP	0.80	0.34	0.72	0.64	0.48	19500	279
5′-AMP*	0.57	0.08	0.58	0.39	0.68	15400	259
8-Bromo-5′-AMP*	0.62	0.21	0.65	0.60	0.73	17900	263
8-Allyl-5′-AMP*	0.77	0.32	0.68	0.65	0.57	20200	279

* Ref. 22.

The shift in the UV absorption maximum ($\lambda_{max.}$) of 8-sub-2′-AMP derivatives, $\lambda_{max.}$ at 259 nm for 2′-AMP, $\lambda_{max.}$ at 263 nm for 8-bromo-2′-AMP and $\lambda_{max.}$ at 279 nm for 8-allyl-2′-AMP, is characteristic of the substitution at the C8 position of the adenine nucleus[21,34]. No further shift in $\lambda_{max.}$ was observed after the reaction of the terminal allyl group with acrylamide to give a water-soluble 8-sub-2′-AMP-polyacrylamide. The molar absorptivities of these 2′-AMP derivatives were determined and are given in Table I. Phosphate group analysis of all derivatives demonstrated the presence of one phosphate group per molecule of the derivatives (0.94–0.97 mole per mole of derivative).

As shown in Fig. 1, the substitution of bromine or allylamine at the C8 position of the adenine nucleus was confirmed by the ^1H NMR spectra. In Fig. 1B, the signal of adenine-C8-H (δ:8.40) disappeared in ^1H NMR spectrum of 8-bromo-2'-AMP. In Fig. 1C, the signal of adenine-C8-H (δ:8.40) disappeared and the signals of allyl-C1-H_2 (δ:4.08), allyl-C2-H (δ:5.60–6.00), allyl-C3-H_2 (δ:5.08) and allyl-N1-H (δ:7.85–8.10) appeared in ^1H NMR spectrum of 8-allyl-2'-AMP.

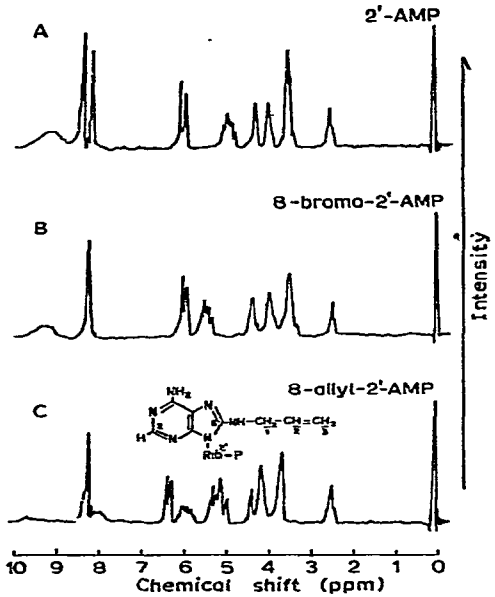

Fig. 1. ^1H NMR spectra of 8-substituted adenosine 2'-monophosphate derivatives. A, Adenosine 2'-monophosphate; B, 8-bromoadenosine 2'-monophosphate; C, 8-allyladenosine 2'-monophosphate. Conditions for ^1H NMR: solvent, dimethyl sulphoxide-D_6(ref. 21); internal reference, tetramethylsilane; room temperature.

Affinity electrophoresis

NADP-dependent dehydrogenase. Affinity electrophoretic patterns of 6-phosphogluconate dehydrogenase (rabbit liver) and malic enzyme (rabbit liver) are shown in Figs. 2 and 3, respectively. The electrophoretic mobility of 6-phosphogluconate dehydrogenase was markedly retarded in direct proportion to the concentration of immobilized 8-sub-2'-AMP in the separating gel (Fig. 2A), whereas the mobility of the enzyme was scarcely retarded in the presence of immobilized 8-sub-5'-AMP in the gel (Fig. 2B). With malic enzyme, similar results (Fig. 3A and B) to those with 6-phosphogluconate dehydrogenase were obtained. However, the extent of the retardation of the mobility of malic enzyme was smaller than that of 6-phosphogluconate dehydrogenase at the same concentration of immobilized 8-sub-2'-AMP in the gel.

NAD-dependent dehydrogenase. Affinity electrophoretic patterns of lactate dehydrogenase isoenzymes (rabbit brain) and malate dehydrogenase isoenzymes (rabbit brain) are shown in Figs. 4 and 5, respectively. The electrophoretic mobilities

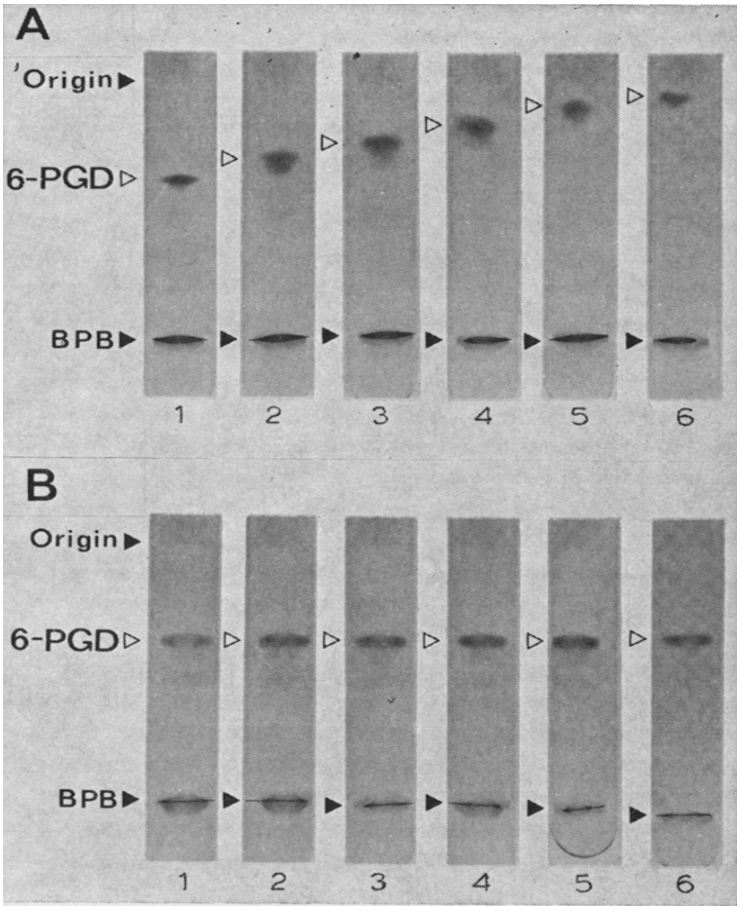

Fig. 2. Affinity electrophoresis of 6-phosphogluconate dehydrogenase (rabbit liver). A, Using gels containing immobilized 8-sub-2′-AMP; B, using gels containing immobilized 8-sub-5′-AMP. Concentration of immobilized 8-sub-2′-AMP and 8-sub-5′-AMP in the gels: 1, 0 m*M*; 2, 0.0125 m*M*; 3, 0.025 m*M*; 4, 0.05 m*M*; 5, 0.075 m*M*; 6, 0.1 m*M*. BPB▶, tracking BPB band; 6-PGD▷, activity band of 6-phosphogluconate dehydrogenase.

of lactate dehydrogenase isoenzymes were scarcely retarded in the presence of immobilized 8-sub-2′-AMP in the separating gel (Fig. 4A), whereas the mobilities of these isoenzymes were markedly retarded in direct proportion to the concentration of immobilized 8-sub-5′-AMP in the gel (Fig. 4B). With malate dehydrogenase isoenzymes, similar results (Fig. 5A and B) to those with lactate dehydrogenase isoenzymes were obtained.

In all instances, the mobilities of proteins other than these enzymes were not affected by the presence of either immobilized 8-sub-2′-AMP or immobilized 8-sub-5′-AMP in the gel. Therefore, the retardation of the mobilities of these enzymes is due neither to any change in the molecular sieving effect by addition of high-molecular-weight 8-sub-2′-AMP-PA or 8-sub-5′-AMP-PA to the separating gel nor to non-specific electrostatic interactions between these enzymes and 8-sub-2′-AMP-PA or 8-sub-5′-AMP-PA.

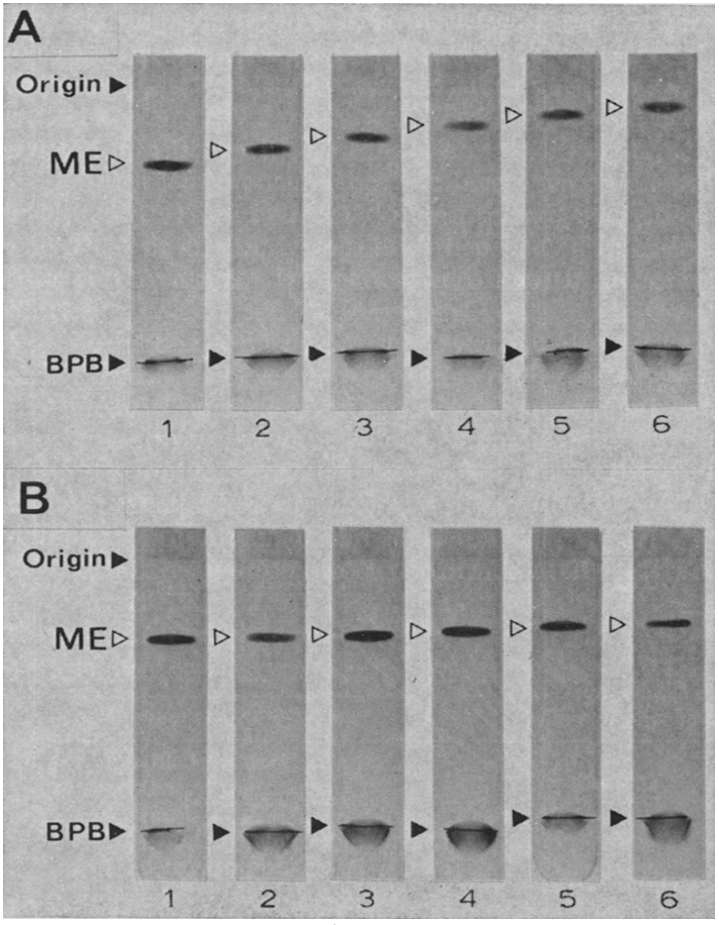

Fig. 3. Affinity electrophoresis of malic enzyme (rabbit liver). A, Using gels containing immobilized 8-sub-2′-AMP; B, using gels containing immobilized 8-sub-5′-AMP. Concentration of immobilized 8-sub-2′-AMP and 8-sub-5′-AMP in the gels: 1, 0 m*M*; 2, 0.125 m*M*; 3, 0.25 m*M*; 4, 0.5 m*M*; 5, 0.75 m*M*; 6, 1.0 m*M*. BPB▶, tracking BPB band; ME▷, activity band of malic enzyme.

Determination of dissociation constants

In Fig. 6, the reciprocal of the relative migration distances of 6-phosphogluconate dehydrogenase (rabbit liver) (Fig. 6A), malic enzyme (rabbit liver and carrot) (Fig. 6B), glucose 6-phosphate dehydrogenase (rabbit liver) (Fig. 6C), isocitrate dehydrogenase (rabbit liver) (Fig. 6D), lactate dehydrogenase isoenzymes (rabbit brain) (Fig. 6E), malate dehydrogenase isoenzymes (pig heart mitochondria and cytoplasm) (Fig. 6F) and phosphorylase (rabbit brain and potato) (Fig. 6G) are plotted against the concentration of immobilized 8-sub-2′-AMP or 8-sub-5′-AMP in the separating gel. From these plots, the dissociation constants (*K*) of the interactions between these enzymes and immobilized 8-sub-2′-AMP or 8-sub-5′-AMP were determined (Table II).

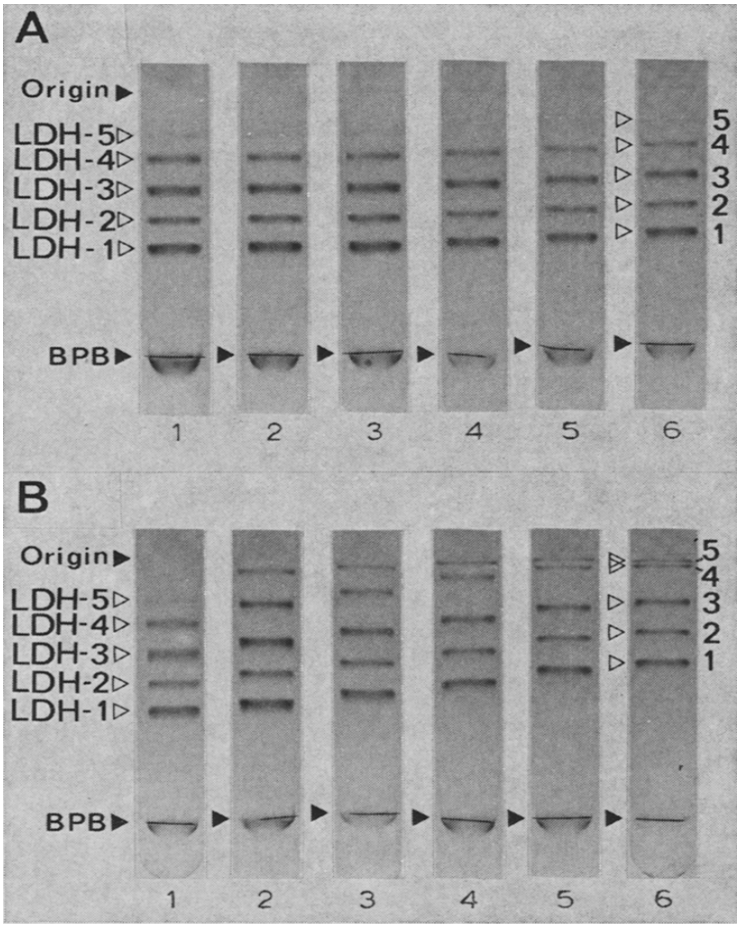

Fig. 4. Affinity electrophoresis of lactate dehydrogenase isoenzymes (rabbit brain). A, Using gels containing immobilized 8-sub-2′-AMP; B, using gels containing immobilized 8-sub-5′-AMP. Concentration of immobilized 8-sub-2′-AMP and 8-sub-5′-AMP in the gels: 1, 0 m*M*; 2, 0.125 m*M*; 3, 0.25 m*M*; 4, 0.5 m*M*; 5, 0.75 m*M*; 6, 1.0 m*M*. BPB▶, tracking BPB band; LDH-1, -2, -3, -4 and -5▷, activity band of lactate dehydrogenase isoenzyme-1, -2, -3, -4 and -5.

NADP-dependent dehydrogenase

The plot of the reciprocal of the relative migration distance of 6-phosphogluconate dehydrogenase against the concentration of immobilized 8-sub-2′-AMP was not a straight line (Fig. 6A). It appeared to be composed of two lines: a gently sloping line at low concentrations of immobilized 8-sub-2′-AMP and a steeply sloping line at high concentrations. The break in the line corresponds to $4.5 \cdot 10^{-5}$ *M* of immobilized 8-sub-2′-AMP. The dissociation constant was determined to be $8.5 \cdot 10^{-5}$ *M* from the initial gently sloping line. The inhibition constant of 2′-AMP for the enzyme has been reported as $4.4 \cdot 10^{-4} M$ (ref. 3) or $9.9 \cdot 10^{-4}$ *M* (ref. 2) by kinetic measurements. The dissociation constant determined by affinity electrophoresis is one fifth or one eleventh of the inhibition constant determined by kinetic measurements. At high concentrations of immobilized 8-sub-2′-AMP, the binding affinity of

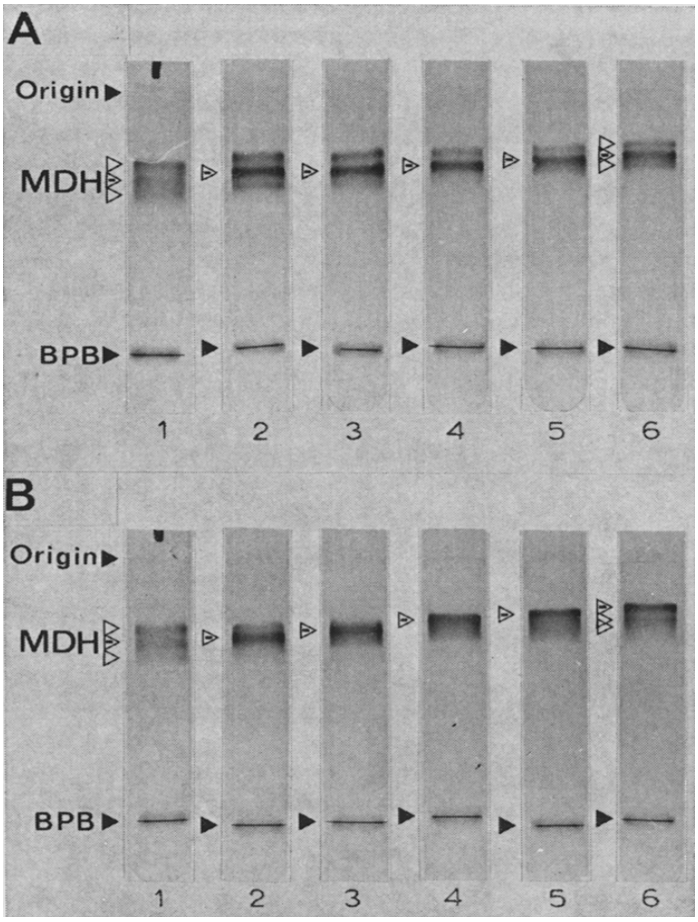

Fig. 5. Affinity electrophoresis of malate dehydrogenase isoenzymes (rabbit brain). A, Using gels containing immobilized 8-sub-2′-AMP; B, using gels containing immobilized 8-sub-5′-AMP. Concentration of immobilized 8-sub-2′-AMP and 8-sub-5′-AMP in the gels: 1, 0 m*M*; 2, 0.125 m*M*; 3, 0.25 m*M*; 4, 0.5 m*M*; 5, 0.75 m*M*; 6, 1.0 m*M*. BPB▶, tracking BPB band; MDH▷, activity band of malate dehydrogenase isoenzymes.

the enzyme to immobilized 8-sub-2′-AMP increased. Its dissociation constant was calculated to be $2.3 \cdot 10^{-5}$ *M* from the steeply sloping line. These results suggest that there would be cooperative enhancement of the binding affinity of the enzyme to immobilized 8-sub-2′-AMP. In contrast with immobilized 8-sub-2′-AMP, the enzyme showed only a weak affinity to immobilized 8-sub-5′-AMP. Its dissociation constant was over 100-fold greater than that of immobilized 8-sub-2′-AMP.

Malic enzyme from rabbit liver showed a much stronger affinity to immobilized 8-sub-2′-AMP than to immobilized 8-sub-5′-AMP. The dissociation constant of immobilized 8-sub-2′-AMP for the enzyme ($4.5 \cdot 10^{-4}$ *M*) was one fourteenth of that of immobilized 8-sub-5′-AMP. On the other hand, malic enzyme from carrot showed only a weak affinity to both immobilized 8-sub-2′-AMP and immobilized 8-sub-5′-AMP. The dissociation constant of immobilized 8-sub-2′-AMP for carrot

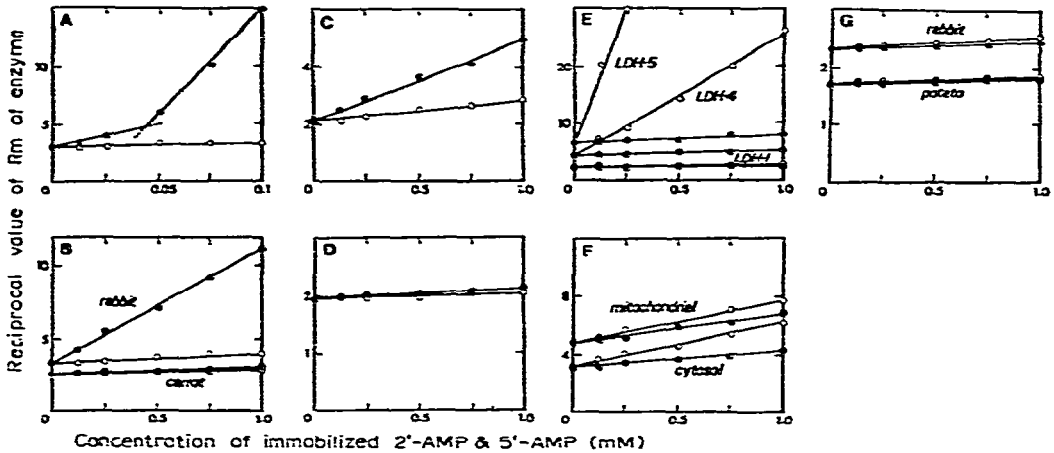

Fig. 6. Plots of the reciprocal of relative migration distances ($1/R_m$) of the enzymes against the concentration of immobilized 8-sub-2′-AMP and 8-sub-5′-AMP. A, 6-Phosphogluconate dehydrogenase (rabbit liver); B, malic enzyme (rabbit liver and carrot); C, glucose 6-phosphate dehydrogenase (rabbit liver); D, lactate dehydrogenase isoenzymes (rabbit brain); E, malate dehydrogenase isoenzymes (pig heart mitochondria and cytoplasm); G, phosphorylase (rabbit brain and potato). ●——●, With immobilized 8-sub-2′-AMP; ○——○, with immobilized 8-sub-5′-AMP.

TABLE II

DISSOCIATION CONSTANTS OF IMMOBILIZED 8-SUB-2′-AMP AND IMMOBILIZED 8-SUB-5′-AMP FOR NADP-DEPENDENT DEHYDROGENASES, NAD-DEPENDENT DEHYDROGENASES AND PHOSPHORYLASES

2′-AMP, immobilized 8-sub-2′-AMP; 5′-AMP, immobilized 8-sub-5′-AMP.

Enzyme	*Dissociation constant (M)*	
	2′-AMP	*5′-AMP*
NADP-dependent dehydrogenase		
6-Phosphogluconate dehydrogenase (rabbit liver)	$8.5 \cdot 10^{-5}$	$1.1 \cdot 10^{-2}$
Malic enzyme (rabbit liver)	$4.5 \cdot 10^{-4}$	$6.1 \cdot 10^{-3}$
Malic enzyme (carrot)	$7.3 \cdot 10^{-3}$	$1.0 \cdot 10^{-2}$
Glucose 6-phosphate dehydrogenase (rabbit liver)	$8.0 \cdot 10^{-4}$	$2.6 \cdot 10^{-3}$
Isocitrate dehydrogenase (rabbit liver)	$1.6 \cdot 10^{-2}$	$2.2 \cdot 10^{-2}$
NAD-dependent dehydrogenase		
Lactate dehydrogenase-1 (H_4, rabbit brain)	$1.6 \cdot 10^{-2}$	$3.2 \cdot 10^{-3}$*
Lactate dehydrogenase-2 (H_3M, rabbit brain)	$1.4 \cdot 10^{-2}$	$1.9 \cdot 10^{-3}$*
Lactate dehydrogenase-3 (H_2M_2, rabbit brain)	$9.4 \cdot 10^{-3}$	$1.2 \cdot 10^{-3}$*
Lactate dehydrogenase-4 (HM_3, rabbit brain)	$5.6 \cdot 10^{-3}$	$4.1 \cdot 10^{-4}$*
Lactate dehydrogenase-5 (M_4, rabbit brain)	$4.8 \cdot 10^{-3}$	$8.6 \cdot 10^{-5}$*
Malate dehydrogenase (rabbit brain supernatant)	$2.7 \cdot 10^{-3}$	$1.3 \cdot 10^{-3}$
Malate dehydrogenase (pig heart cytosol)	$2.8 \cdot 10^{-3}$	$9.6 \cdot 10^{-4}$
Malate dehydrogenase (pig heart mitochondrial)	$2.5 \cdot 10^{-3}$	$1.9 \cdot 10^{-3}$
Phosphorylase		
Phosphorylase (rabbit brain)	$1.4 \cdot 10^{-2}$	$1.0 \cdot 10^{-2}$
Phosphorylase (potato)	$4.3 \cdot 10^{-2}$	$1.7 \cdot 10^{-2}$

* Ref. 22.

malic enzyme ($7.3 \cdot 10^{-3}$ *M*) was over 10-fold greater than that for rabbit liver malic enzyme.

Glucose 6-phosphate dehydrogenase showed a greater affinity to immobilized 8-sub-2'-AMP than to immobilized 8-sub-5'-AMP. The dissociation constant of immobilized 8-sub-2'-AMP for the enzyme ($8.0 \cdot 10^{-4}$ *M*) was one third of that of immobilized 8-sub-5'-AMP.

Isocitrate dehydrogenase showed a very weak affinity to both immobilized 8-sub-2'-AMP and immobilized 8-sub-5'-AMP. The dissociation constants of both immobilized 8-sub-2'-AMP and immobilized 8-sub-5'-AMP for the enzyme were over $1 \cdot 10^{-2}$ *M*.

From these results, it was concluded that NADP-dependent dehydrogenases have different binding affinities to immobilized 8-sub-2'-AMP; however, the binding affinity to immobilized 8-sub-2'-AMP is always greater than that to immobilized 8-sub-5'-AMP. Thus, as the binding affinity to immobilized 8-sub-2'-AMP increased, the binding affinity to immobilized 8-sub-5'-AMP decreased.

NAD-dependent dehydrogenase

In contrast with NADP-dependent dehydrogenases, NAD-dependent dehydrogenases had a greater affinity to immobilized 8-sub-5'-AMP than to immobilized 8-sub-2'-AMP. Thus, the dissociation constant of immobilized 8-sub-2'-AMP for lactate dehydrogenase isoenzyme-5 (LDH-5, M_4-type isoenzyme) was determined to be $4.8 \cdot 10^{-3}$ *M* and was 55-fold greater than the dissociation constant of immobilized 8-sub-5'-AMP ($8.6 \cdot 10^{-5}$ *M*)[22]. Among LDH isoenzymes, LDH-1 (H_4-type isoenzyme) had the weakest affinity to both immobilized 8-sub-2'-AMP and immobilized 8-sub-5'-AMP, and hybrid isoenzymes, LDH-2 (H_3M), LDH-3 (H_2M_2) and LDH-4 (HM_3), composed of the more M-type subunit, have a greater affinity to both immobilized 8-sub-2'-AMP and immobilized 8-sub-5'-AMP.

Malate dehydrogenase isoenzymes showed a relatively strong affinity to both immobilized 8-sub-2'-AMP and immobilized 8-sub-5'-AMP and the affinity to immobilized 8-sub-5'-AMP was always greater than that to immobilized 8-sub-2'-AMP.

Phosphorylase

Phosphorylases from rabbit brain and potato showed a very weak affinity to both immobilized 8-sub-2'-AMP and immobilized 8-sub-5'-AMP. However, it has been reported that phosphorylase *a* had a strong affinity to N6-(aminohexyl)-5'-AMP-agarose[35] and 8-(aminohexyl)-5'-AMP-agarose[19] in affinity chromatography. One of the reasons for this discrepancy would be the difference in the spacer which was used for coupling of 5'-AMP to the matrix. We used allylamine as the spacer [–CH_2–NH–5'-AMP] which had a much shorter hydrocarbon chain than that of hexamethylenediamine [–NH–$(CH_2)_6$–NH–5'-AMP] used for coupling 5'-AMP to agarose. Therefore, the immobilized 8-sub-5'-AMP used in our experiment would not be able to attach to the 5'-AMP binding site of phosphorylase, which was localized at the subunit interface of the dimer of the enzyme and formed a sheltered pocket[36].

Effects of NADP⁺ and NAD⁺ on the binding of immobilized 8-sub-2′-AMP or 8-sub-5′-AMP to NADP-dependent or NAD-dependent dehydrogenases

As shown in Fig. 7A, when affinity electrophoresis of 6-phosphogluconate dehydrogenase (rabbit liver) was carried out using the separating gel containing 8-sub-2′-AMP-PA together with NADP⁺ (gel No. 1), the electrophoretic mobility of the enzyme returned to the native mobility, whereas such a return was not observed with NAD⁺ (gel No. 2). Similar results were obtained with malic enzyme (rabbit liver) and glucose 6-phosphate dehydrogenase (rabbit liver). On the other hand, as shown in Fig. 7B, when affinity electrophoresis of lactate dehydrogenase isoenzymes

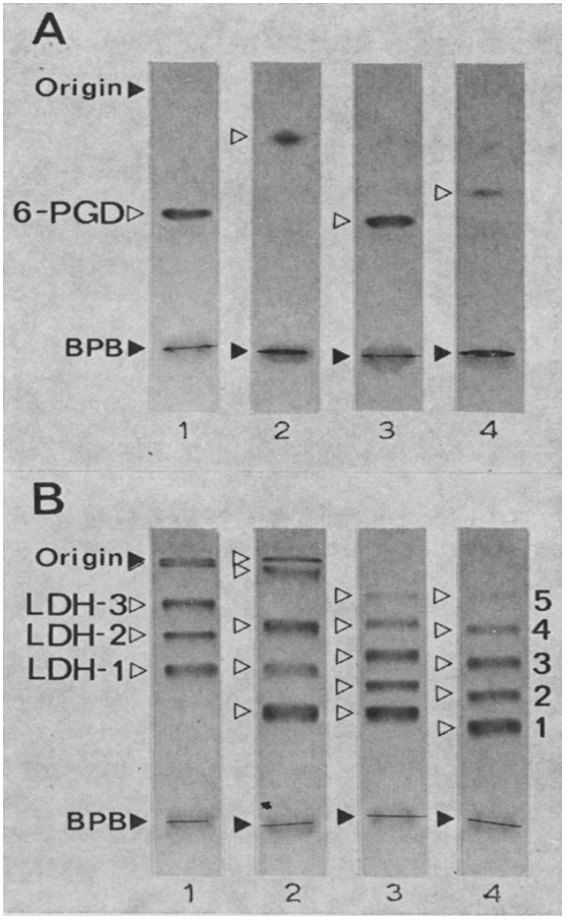

Fig. 7. Effects of NADP⁺ and NAD⁺ on the binding of immobilized 8-sub-2′-AMP or 8-sub-5′-AMP to NADP-dependent dehydrogenase or NAD-dependent dehydrogenase. A, 6-Phosphogluconate dehydrogenase (rabbit liver); B, lactate dehydrogenase isoenzymes (rabbit brain). Concentration of immobilized 8-sub-2′-AMP or 8-sub-5′-AMP together with NADP⁺ or NAD⁺ in the gels: A-1, 0.05 m*M* of 8-sub-2′-AMP together with 0.02 m*M* of NADP⁺; A-2, 0.05 m*M* of 8-sub-2′-AMP together with 0.02 m*M* of NAD⁺; A-3, 0.02 m*M* of NADP⁺; A-4, 0.02 m*M* of NAD⁺; B-1, 1.0 m*M* of 8-sub-5′-AMP together with 0.02 m*M* of NADP⁺; B-2, 1.0 m*M* of 8-sub-5′-AMP together with 0.02 m*M* of NAD⁺; B-3, 0.02 m*M* of NADP⁺; B-4, 0.02 m*M* of NAD⁺. BPB▶, tracking BPB band; 6-PGD▷ and LDH▷, activity band of 6-phosphogluconate dehydrogenase and lactate dehydrogenase isoenzymes, respectively.

(rabbit brain) was carried out using the separating gel containing 8-sub-5'-AMP-PA together with $NADP^+$ (gel No. 1), the retardation of the mobilities of the isoenzymes did not decrease, whereas the retardation of the mobilities of the isoenzymes was decreased with NAD^+ (gel No. 2). These results indicate that the immobilized 8-sub-2'-AMP binds to the $NADP^+$ binding site of NADP-dependent dehydrogenase specifically and the immobilized 8-sub-5'-AMP binds to the NAD^+ binding site of NAD-dependent dehydrogenase specifically. This binding specificity of immobilized 8-sub-2'-AMP and immobilized 8-sub-5'-AMP suggests that the phosphate group at the position 2' of the adenine ribose moiety of $NADP^+$ and 2'-AMP is important for the binding of these adenine nucleotides at the coenzyme binding site of NADP-dependent dehydrogenase.

Affinity electrophoresis is a simple and useful method for exploring biospecific interactions. Recently, studies of many biospecific interactions by this method have been reported[22,23,37–45]. In this paper, we have presented its application to the study of the interaction between NADP-dependent dehydrogenases and immobilized 8-sub-2'-AMP. By our method, the dissociation constants of enzyme–coenzyme analogue interactions can be determined with a very small amount of the enzyme (less than 0.1 mg) in a few hours. Further, there is no need to purify the enzyme.

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