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STUDY OF THE INTERACTION BETWEEN PHOSPHORYLASE AND HYDROPHOBIC GROUPS BY MEANS OF AFFINITY ELECTROPHORESIS

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SUMMARY

A homologous series of water-soluble alkyl-dextrans varying in the length of their hydrocarbon side-chain [$-NH-(CH_2)_n-CH_3$; $n = 1-5$] were synthesized. When alkyl-dextrans were entrapped in polyacrylamide gel, the electrophoretic mobility of phosphorylase was retarded by hydrophobic interaction between phosphorylase and the immobilized alkyl groups. The dissociation constants of rabbit brain phosphorylase, rabbit skeletal muscle phosphorylase *a* and *b* and potato glycogen and starch phosphorylases were calculated from the extent of the retardation of mobility as a function of the concentration of the alkyl groups.

As the length of the hydrocarbon side-chains of alkyl groups increased, the affinity of the phosphorylases for the alkyl groups increased. The introduction of a hydroxyl or an amino group at the terminal position of the hydrocarbon side-chain diminished the affinity.

INTRODUCTION

Affinity electrophoresis is a type of electrophoresis that separates proteins on the basis of their affinity ligands being immobilized in the electrophoresis carriers[1–3]. In polyacrylamide gel disc electrophoresis, phosphorylase mobility was retarded when glycogen or starch was entrapped in the separating gel. The dissociation constant of the phosphorylase–glycogen interaction was calculated from the extent of retardation of mobility as a function of glycogen concentration[3].

In the same way, the dissociation constants of the reactions of α-amylase with starch[4], concanavalin A with various sugars[2] and dextran-specific myeloma protein with various dextrans[5] were calculated.

Hydrocarbon-coated agarose has been widely used in hydrophobic chromatography[6–10]. Shaltiel and Er-El[7] used this technique to purify glycogen phosphorylase and discussed the features of the interaction between phosphorylase and hydrophobic groups on agarose. We synthesized a series of water-soluble alkyl-dextrans varying in the length of their hydrocarbon side-chain [$-NH-(CH_2)_n-CH_3$; $n = 1-5$] and used them to determine the dissociation constants of phosphorylase–alkyl group interactions. This is the first report concerning the determination of dissociation constants of protein–hydrophobic ligand interactions by means of electrophoresis.

EXPERIMENTAL

Dextran T2000 was purchased from Pharmacia (Uppsala, Sweden). Ethylamine, *n*-propylamine, *n*-butylamine, *n*-pentylamine, *n*-hexylamine, 3-amino-1-propanol, 1,3-propanediamine, mercaptoethanol, EDTA and cyanogen bromide (CNBr) were purchased from Wako (Osaka, Japan). Tris(hydroxymethyl)aminomethane (Tris), 2,4,6-trinitrobenzene-1-sulphonic acid (TNBS) and adenosine 5'-monophosphoric acid (5'-AMP) were purchased from Sigma (St. Louis, Mo., U.S.A.). Acrylamide, N,N,N',N'-tetramethylethylenediamine (TEMED), N,N'-methylenebisacrylamide and bromophenol blue (BPB) were purchased from Nakarai Chemical Co. (Kyoto, Japan). Crystalline phosphorylase *b* was purchased from Boehringer (Mannheim, G.F.R.).

Preparation of potato and rabbit tissue crude extracts

Potato was sliced and homogenized with an equal volume of 10 m*M* EDTA and 100 m*M* mercaptoethanol (pH 7.4). The homogenate was centrifuged at 7180 *g* for 1 h and the supernatant was stored at −20°.

A male rabbit was deeply anaesthetized with 5 ml of 5% sodium pentobarbiturate solution by venous injection and the brain and skeletal muscle were removed immediately. These tissues were minced on ice and homogenized with an equal volume of 10 m*M* EDTA (pH 7.4). The tissue homogenates were centrifuged at 11,200 *g* for 1 h and the supernatants were stored at −20°.

The protein content was determined spectrophotometrically according to Warburg and Christian's method[11].

Synthesis of water-soluble alkyl-dextrans

A series of water-soluble alkyl-dextrans were prepared by using aliquots from the same dextrans activated with cyanogen bromide[7,12,13,19] as follows: 1.25 g (12 mmole) of cyanogen bromide was dissolved in 50 ml of water, then added with constant stirring to a solution of 5 g of Dextran T2000 (30 mmole of glycosyl residues) dissolved in 500 ml of water. The pH of the solution was adjusted to 11.0 by automatic addition of 1 *M* sodium hydroxide solution with a pH stat (Autotitrator or RAT 11, Hiranuma, Tokyo, Japan). After 15 min, the consumption of 1 *M* sodium hydroxide solution (about 13 ml) had ceased and the pH became constant. The pH of the solution was then decreased to 9.0 by the addition of 1 *M* hydrochloric acid and the whole solution (580 ml) was divided into five equal parts. These aliquots were added to 10 mmole of ethylamine, *n*-propylamine, *n*-butylamine, *n*-pentylamine and *n*-hexylamine dissolved in 10 ml of water, respectively. The pH of each solution was maintained at 9.0 by automatic addition of 1 *M* hydrochloric acid and they were allowed to stand for 24 h at 4° with gentle stirring.

Aminopropyl-dextran and hydroxypropyl-dextran were prepared with 1,3-propanediamine and 3-amino-1-propanol by the same procedure as described here.

Each reaction mixture was successively dialysed against a large volume of distilled water overnight with frequent changes of water, then twice with 2 l of 0.1 *M* sodium hydrogen carbonate buffer (pH 9.0) for 6 h, twice with 2 l of 0.1 *M* sodium acetate buffer (pH 6.0) for 6 h and, finally, a large volume of distilled water overnight with constant stirring. The dialysates (about 180 ml) were obtained and no un-

reacted alkylamines were detected in the outer dialysis solution by the TNBS colour test[14]. Then, each alkyl-dextran was concentrated to 100 ml by evaporation *in vacuo* and stored at 4°.

The contents of the alkyl groups conjugated on the alkyl-dextrans were determined by the Kjeldahl method. With aminopropyl-dextran, the terminal amino groups of the hydrocarbon side-chains were determined by the TNBS method[15]. A 1-ml volume of the sample solution was added to 1 ml of 4% sodium hydrogen carbonate solution, 1 ml of 0.1% TNBS and 1 ml of water, then stored in the dark at 40° for 2 h. A 1-ml volume of 1 *M* hydrochloric acid was added to the orange-coloured solution and the absorbance of the solution was measured spectrophotometrically at 340 nm. One millilitre of distilled water, instead of the sample solution, was used as a blank[15].

From 1 ml of the aminopropyl-dextran solution, 1.01 mmole equivalent nitrogen atoms were determined by the Kjeldahl method and 0.92 mmole equivalent free amino groups by the TNBS method. This result indicated that 92% of the total nitrogen atoms in the aminopropyl-dextran were derived from 1,3-propanediamine. Table I gives the contents of the alkyl groups conjugated on the dextrans. All alkyl groups conjugated at nearly equal rates.

TABLE I

STRUCTURES OF A SERIES OF WATER-SOLUBLE ALKYL-DEXTRANS USED IN THIS WORK AND RESULTS OF THE DETERMINATION OF THE CONTENTS OF CONJUGATED ALKYL GROUPS

Abbreviation	*Structure*	*Content of alkyl group (mmole/g)*
Dext-C_2	}–NH–CH_2–CH_3	1.6
Dext-C_3	}–NH–CH_2–CH_2–CH_3	1.5
Dext-C_4	}–NH–CH_2–CH_2–CH_2–CH_3	1.4
Dext-C_5	}–NH–CH_2–CH_2–CH_2–CH_2–CH_3	1.4
Dext-C_6	}–NH–CH_2–CH_2–CH_2–CH_2–CH_2–CH_3	1.3
Dext-C_3-OH	}–NH–CH_2–CH_2–CH_2–OH	1.4
Dext-C_3-NH_2	}–NH–CH_2–CH_2–CH_2–NH_2	1.4

Electrophoresis

Polyacrylamide gel disc electrophoresis was carried out by a slightly modified version of Takeo and Nakamura's method[3]. The separating gel (5 cm in height) was prepared as a 7.5% acrylamide gel (pH 6.7) in Tris–hydrochloric acid, using the buffer system for the spacer gel described by Ornstein and Davis[16]. The spacer gel (1 cm in height) was prepared as a 2.5% acrylamide gel (pH 6.7) in Tris–hydrochloric acid, using the same buffer system. Electrophoresis was run in a Tris–glycine buffer at pH 8.3. The synthesized alkyl-dextrans were mixed with the preparation mixture of the separating gel. To ensure a uniform concentration of alkyl-dextran throughout the separating gel, it was prepared by overlaying an alkyl-dextran solution of the same concentration as that of the separating gel, instead of water. The sample protein solution (50 μg of protein for tissue extracts and 1 μg of protein for crystalline phosphorylase *b*) were applied in a 10% sucrose solution to each gel tube[3].

Electrophoresis was carried out at 2 mA per gel tube for about 2 h until the BPB band had migrated 4.5 cm from origin in the separating gel. After electropho-

resis, a fine wire was inserted at the position of the BPB band. The phosphorylase band was revealed by the activity staining method[17,18] and the relative migration distance (*Rm*) was determined as the ratio of the distance travelled by the phosphorylase band to that of the tracking BPB band.

Theory of affinity electrophoresis

Calculation of dissociation constants for the interactions between phosphorylase and immobilized alkyl groups was based on the theory of affinity electrophoresis, developed for the phosphorylase–glycogen interaction[3].

The theory is based on the finding that the mobility of the phosphorylase–glycogen complex is zero and that the concentration of glycogen is very large relative to that of the enzyme, so that the total concentration of glycogen is almost equal to that of free glycogen. These assumptions are valid for the present experiment.

In the interaction between phosphorylase and an alkyl group, the dissociation constant (K) can be determined from the following equations:

$$P + R \rightleftharpoons PR \tag{1}$$

$$K = \frac{[P]\,[R]}{[PR]} \tag{2}$$

where [P], [R] and [PR] are the concentrations of free phosphorylase, free alkyl group conjugated on alkyl-dextran and phosphorylase–alkyl-dextran complex, respectively. As the average molecular weight of Dextran T2000 is reported to be $2 \cdot 10^6$, the mobility of the phosphorylase–alkyl-dextran complex should be zero in a 7.5% polyacrylamide gel. In the present experiment, the total concentration of alkyl groups (10^{-3}–10^{-4} M) was far higher than that of phosphorylase [P] (about 10^{-6} M), but the total concentration of alkyl groups (c) was almost equal to that of free alkyl groups conjugated on alkyl-dextran, [R]. Thus:

$$\frac{Rm_i}{Rm_0} = \frac{\text{concentration of free phosphorylase}}{\text{concentration of total phosphorylase}} = \frac{[P]}{[P] + [PR]} \tag{3}$$

where Rm_0 and Rm_i are the relative migration distances of phosphorylase in the absence and in the presence of the alkyl-dextran, respectively, in the separating gel. From eqns. 2 and 3:

$$K = \frac{c \cdot Rm_i}{Rm_0 - Rm_i} \tag{4}$$

Eqn. 4 can be transformed into

$$\frac{1}{Rm_i} = \frac{1}{Rm_0}\left(1 + \frac{c}{K}\right) \tag{5}$$

Therefore, if the reciprocal of Rm_i is plotted against c, a straight line will be obtained, the intercept on the c-axis giving $-K$.

RESULTS AND DISCUSSION

Fig. 1 shows the phosphorylase activity staining patterns of rabbit brain extract after disc electrophoresis in the presence of various concentrations of Dext-C_2 (A), Dext-C_5 (B), Dext-C_3-OH (C) and Dext-C_3-NH_2 (D) in the separating gel. In the absence of the alkyl-dextrans (gel No. 1 in each series), phosphorylase migrated about half the distance travelled by the tracking BPB band. When the gel contained alkyl-dextran, the phosphorylase mobility was retarded. As the concentration of alkyl-dextran was increased, the mobility of phosphorylase decreased progressively in proportion to the concentration of alkyl-dextran.

As can be seen in Fig. 1, the phosphorylase mobility approached zero in the separating gel containing 1 m*M* Dext-C_2 or Dext-C_5. In contrast, the mobility of other major protein fractions, except phosphorylase in brain extract, did not change in the separating gel containing 1 m*M* alkyl-dextrans.

This result indicates that the retardation of phosphorylase mobility is not due to any change in the molecular sieve effect by the addition of the high-molecular-

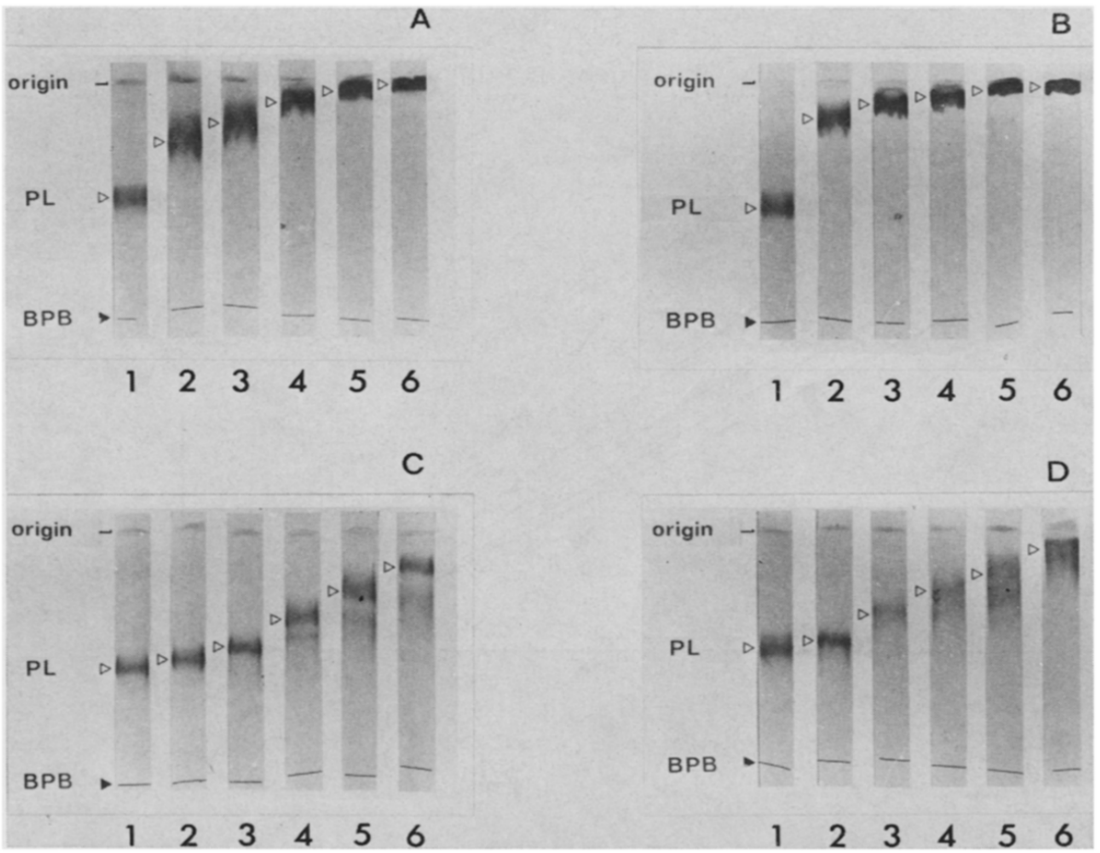

Fig. 1. Polyacrylamide gel disc electrophoresis of rabbit brain phosphorylase in the presence of varying amounts of a series of water-soluble alkyl-dextrans. (A) Dext-C_2; (B) Dext-C_5; (C) Dext-C_3-OH; (D) Dext-C_3-NH_2. Concentration of conjugated alkyl groups on dextran entrapped in polyacrylamide gel: 1, 0; 2, 0.3; 3, 0.6; 4, 1.2; 5, 2.4; 6, 4.8 m*M*.

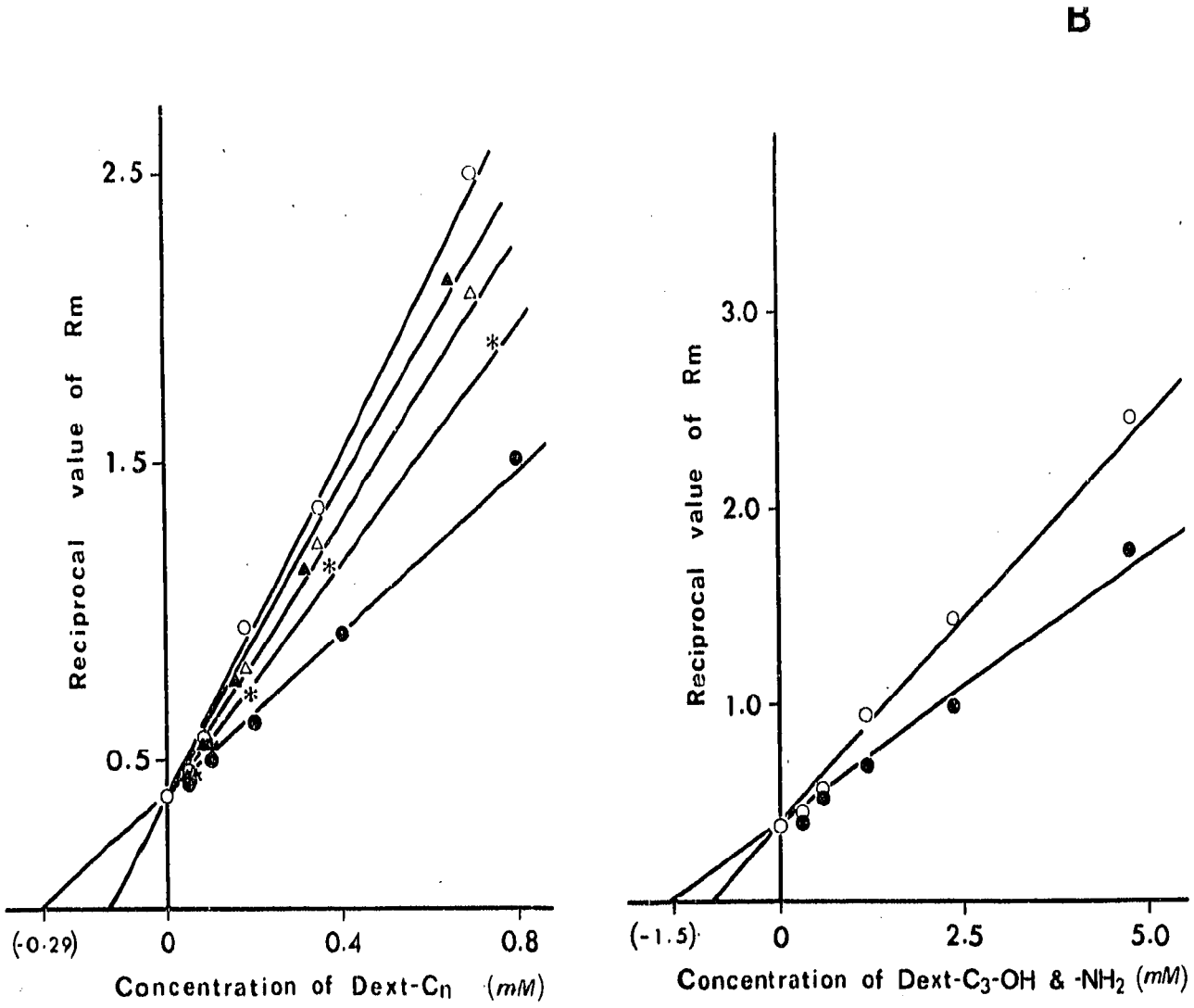

Fig. 2. Plots of reciprocal of relative migration distance ($1/Rm$) of rabbit brain phosphorylase against the concentration of conjugated alkyl groups on each alkyl-dextran entrapped in polyacrylamide gel. (A) Plot against Dext-C_2 (●), Dext-C_3 (✳), Dext-C_4 (△), Dext-C_5 (○) and Dext-C_6 (▲). (B) Plot against Dext-C_3-OH (●), and Dext-C_3-NH_2 (○).

weight alkyl-dextran to the separating gel, or to the positive charges introduced as a result of activation with cyanogen bromide and subsequent coupling with alkylamines. The retardation should be due to a specific interaction between phosphorylase and alkyl groups conjugated on dextran.

In Fig. 2, the reciprocal of *Rm* is plotted against the concentration of each alkyl-dextran in the separating gel. Each plot is a straight line, as predicted by the theory. From these plots, the dissociation constants of rabbit brain phosphorylase were calculated, as shown in Table II. Affinity is expressed as the reciprocal of the dissociation constant.

TABLE II

DETERMINATION OF DISSOCIATION CONSTANTS OF RABBIT BRAIN PHOSPHORYLASE-ALKYL-DEXTRAN COMPLEXES

Alkyl-dextran	*Dissociation constant (mM)*
Dext-C_2	0.29
Dext-C_3	0.19
Dext-C_4	0.16
Dext-C_5	0.13
Dext-C_6	0.15
Dext-C_3-OH	1.50
Dext-C_3-NH_2	1.00

As the length of the hydrocarbon side-chain on the dextran increased from Dext-C_2 to Dext-C_5, the dissociation constant decreased from $2.9 \cdot 10^{-4}$ to $1.3 \cdot 10^{-4}$ *M*, and hence the affinity of phosphorylase for the hydrophobic group increased more than 2-fold. However, further elongation of the hydrocarbon side-chain did not affect the affinity of the phosphorylase.

For alkyl groups with a hydroxyl or amino group in the terminal position, the dissociation constants of the phosphorylase were estimated to be $1.5 \cdot 10^{-3}$ and $1.0 \cdot 10^{-3}$ *M*, respectively. The introduction of a hydroxyl or amino group decreased the affinity of phosphorylase for alkyl groups to about one fifth or one seventh, respectively.

In the same way, the dissociation constants of various phosphorylase were calculated. In Fig. 3, phosphorylase activity staining patterns of rabbit skeletal muscle extract (A), crystalline phosphorylase *b* (B) and potato extract (C) are shown.

Two distinct phosphorylase bands were identified in the rabbit skeletal muscle extract. As can be seen in Fig. 3A, comparison of the skeletal muscle phosphorylases and crystalline phosphorylase *b* suggests that the faster band corresponds to phosphorylase *b*, while the slower band corresponds to phosphorylase *a*. In Fig. 4, the reciprocal of *Rm* is plotted against the concentration of Dext-C_5 in the separating gel. From those plots, the dissociation constants of the faster band and the slower band for Dext-C_5 were calculated to be $2.6 \cdot 10^{-4}$ and $1.0 \cdot 10^{-4}$ *M*, respectively. The dissociation constant of crystalline phosphorylase *b* was $2.6 \cdot 10^{-4}$ *M*. From these values, it is concluded that the faster band corresponds to phosphorylase *b* and the slower band to phosphorylase *a*.

Potato extract contains two phosphorylase isoenzymes (Fig. 3C); one was a

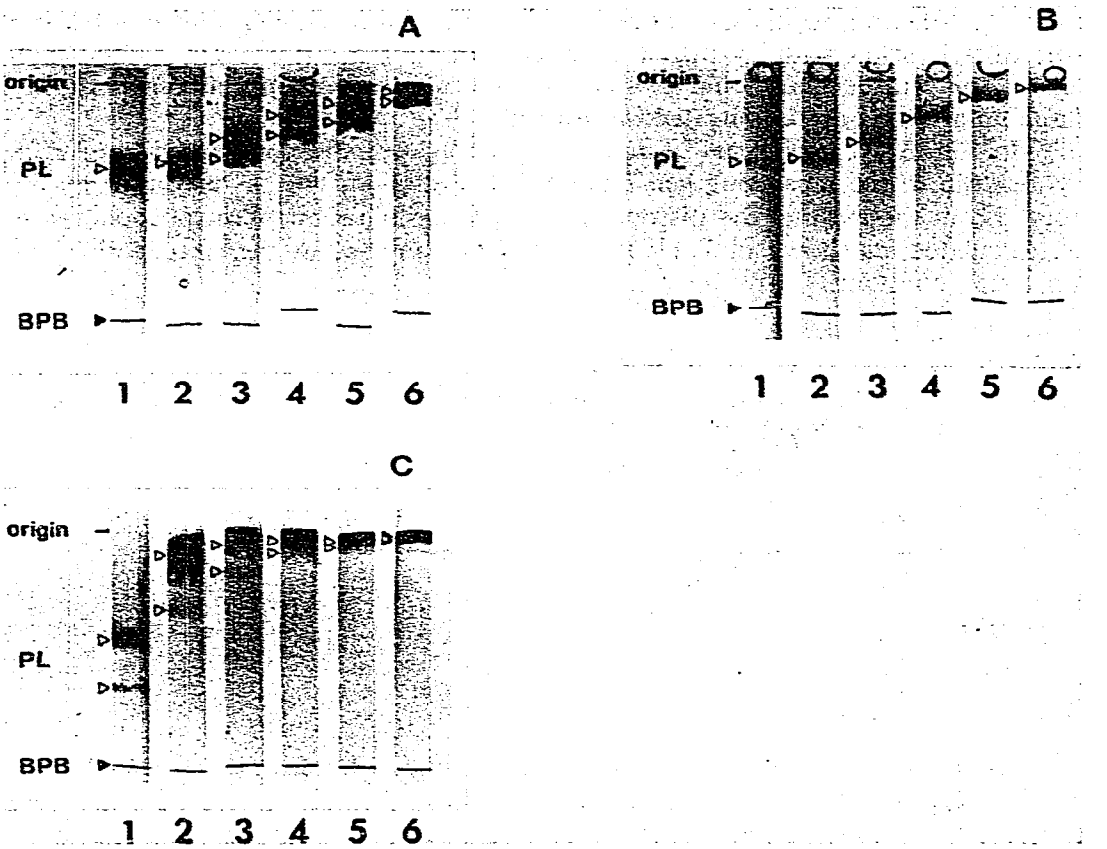

Fig. 3. Polyacrylamide gel disc electrophoresis of phosphorylase isoenzymes in the presence of varying amounts of Dext-C_5. (A) Rabbit skeletal muscle phosphorylase isoenzymes; (B) crystalline phosphorylase *b*; (C) potato phosphorylase isoenzymes. Concentration of conjugated alkyl group on Dext-C_5 entrapped in polyacrylamide gel: 1, 0; 2, 0.07; 3, 0.14; 4, 0.28; 5, 0.56; 6, 1.12 m*M*.

slower migrating main band which displayed almost no affinity for starch or glycogen upon affinity electrophoresis[4], whereas the other displayed a strong affinity for starch and glycogen and corresponded to potato glycogen phosphorylase. On the other hand, both potato phosphorylases have a strong affinity for alkyl groups. As can be seen in Fig. 3C, the mobility of both phosphorylases was strongly retarded. They approached zero mobility in the separating gel containing 0.28 m*M* Dext-C_5. The dissociation constants of the faster and the slower bands were $0.6 \cdot 10^{-4}$ and $0.4 \cdot 10^{-4}$ *M*, respectively. There were no significant differences between the two potato phosphorylases in their affinity for alkyl groups. Their affinities were greater than that of the other rabbit phosphorylase, as shown in Table III.

According to Shaltiel and Er-El[7,8], as columns of hydrocarbon-coated agarose (Seph-C_n) increased in the length of their hydrocarbon side-chain, their affinity for phosphorylase *b* increased progressively. One-carbon-atom alkyl-agarose (Seph-C_1) excluded phosphorylase *b*, while three-carbon-atom alkyl-agarose (Seph-C_3) retarded the enzyme and higher alkyl-agaroses adsorbed it. Thus, while phosphorylase *b* was eluted from four-carbon-atom alkyl-agarose (Seph-C_4) using a deforming buffer, the

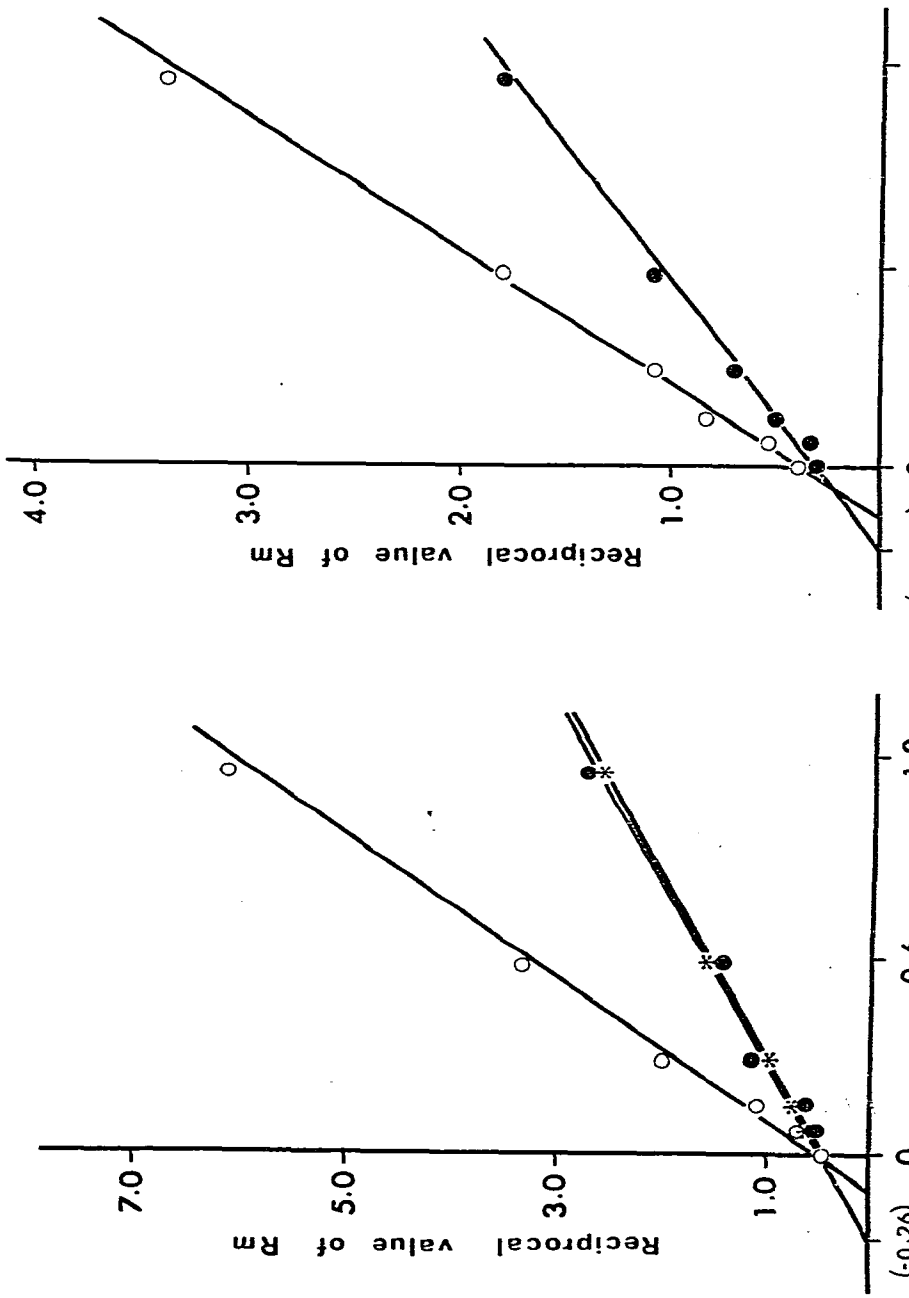

Fig. 4. Plots of reciprocal of relative migration distance ($1/Rm$) of phosphorylase isoenzymes against the concentration of conjugated alkyl groups on Dext-C_5 entrapped in polyacrylamide gel. (A) Plot for rabbit skeletal muscle phosphorylase isoenzymes: slower fraction (○); faster fraction (●); and crystalline phosphorylase *b* (✳). (B) Plot for potato phosphorylase isoenzymes: slower fraction (○); and faster fraction (●).

TABLE III

DETERMINATION OF DISSOCIATION CONSTANTS OF PHOSPHORYLASE ISOENZYME–DEXT-C_5 COMPLEXES

Phosphorylase isoenzyme	*Dissociation constant (mM)*
Potato phosphorylase:	
slower fraction	0.04
faster fraction	0.06
Rabbit skeletal muscle phosphorylase:	
slower fraction	0.10
faster fraction	0.26
crystalline phosphorylase *b*	0.26
Rabbit brain phosphorylase	0.13

recovery of the enzyme from six-carbon-atom alkyl-agarose (Seph-C_6) was possible only in an inactive form by washing the column with 0.2 *M* acetic acid[8].

On the other hand, the same progressive increase in affinity that phosphorylase had for the alkyl-agarose series was not observed with the alkyl-dextrans (Dext-C_n), as shown in Table II. The affinity of rabbit brain phosphorylase for six-carbon-atom alkyl-dextran (Dext-C_6) ($K = 1.5 \cdot 10^{-4}$ *M*) was only about twice as great as that for two-carbon-atom alkyl-dextran (Dext-C_2) ($K = 2.9 \cdot 10^{-4}$ *M*).

From the present results, the discrepancy between the affinity of phosphorylase for hydrocarbon-coated agarose and that for water-soluble alkyl-dextran could not be explained. However, one reason might have been a difference in the experimental conditions. In our study, affinity was calculated by affinity electrophoresis using polyacrylamide gel containing water-soluble alkyl-dextran with less than 1 m*M* of hydrophobic groups, whereas in hydrophobic chromatography, hydrocarbon-coated insoluble agarose beads with a much higher concentration of hydrophobic groups was used. Assuming the concentration of the hydrophobic groups coated on agarose is nearly equal to that of the cyanogen bromide activated groups on agarose, the concentration of hydrophobic groups in the affinity column would be approximately 1 *M*.

The affinity electrophoresis technique is a useful, simple method for exploring the interactions between enzyme and substrate or inhibitor, lectin and carbohydrate or glycoprotein, or antibody and antigen or hapten. Here, we have presented its application to a hydrophobic interaction. By this method, the dissociation constants of biospecific interactions can be determined with very small amounts of sample proteins (under 1 mg) in a few hours. Further, it is not necessary to purify the sample proteins if a specific staining method is available.

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