Study on Isozymes of Isocitrate Dehydrogenase from a Psychrophilic Bacterium, *Vibrio* Sp. Strain ABE-1

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CONTENTS

PART I

SUMMARY

It was previously found in our laboratory that an obligately psychrophilic Vibrio sp. strain ABE-1 had two isozymes of $NADP^+$-specific isocitrate dehydrogenase [IDH; EC 1.1.1.42] which were markedly different in their temperature dependency and so on [Ochiai et al. (1979), J.Biochem., 86: 377-384]. In order to elucidate their physiological significance and their evolutionary relationship, it was absolutely necessary to purify these isozymes. However, these isozymes could not be purified so far. Then, firstly I attempted to develop the purification procedures of the enzymes, and subsequently biochemical and physical properties of the enzymes were investigated. Next, catalytic properties of the isozymes were revaluated. Finally, their structural relation was evaluated by comparison of the immunoreactivities, the amino acid sequences at the amino terminals, and the complete nucleotide sequences (Part II).

Application of the hydrophobic chromatography in an early step of the procedure resulted in complete separation of the two isozymes from each other followed by being successful in purification to electrophoretically homogeneous state. Apparent molecular weights of the isozymes estimated by gel filtration were 88,100 for isozyme-I (IDH-I) and 80,500 for isozyme-II (IDH-II), whereas 49,100 for IDH-I and 79,500 for IDH-II by gel

electrophoresis in the presence of sodium dodecyl sulfate, indicating that IDH-I was dimer and IDH-II was a single polypeptide. The isoelectric points of IDH-I and -II were found to be 4.9 and 5.2, respectively. The absorption maximum and shoulder in ultraviolet region of the isozymes were observed at 278 nm and 292 nm, and values of $\underline{E}^{1\%}_{1cm}$ at 280 nm were 6.15 for IDH-I and 4.01 for IDH-II. Although the two isozymes were similar in amino acid compositions with slight differences in the contents of nonpolar and hydroxyl amino acids, the NH_2-terminal amino acid sequences were quite different. Moreover, Ouchterlony double immunodiffusion and immunotitration of the each isozyme with the respective counter antibody revealed that the isozymes were different in immunochemical property from each other. There observed remarkable differences not only in thermostability but also in thermal inactivation mode between the isozymes, that is, IDH-I was inactivated linearly in the incubation above 45°C, whereas IDH-II was inactivated biphasicaly above 20°C. Some nucleotides and metabolites in glycolysis, tricarboxylic acid cycle and glyoxylate bypass acted as inhibitors to the IDH isozymes. Potassium ion was found to be the best monovalent cation for activities of the both isozymes, even though the range of optimum concentration of K^+ for IDH-II was broader than that for IDH-I. Additionally, the isozymes were different in kinetic and thermodynamic parameters. From these results, I conclude that IDH-II

is more adaptive than IDH-I under the condition which is low temperature and high salt concentration

INTRODUCTION

The life of bacteria is greatly influenced by temperature as similar as other most life forms, however, in general, the bacteria can survive in a much greater thermal range than any other [1].

Bacteria are classified into thrmophiles, mesophiles, and psychrophiles according to the ranges of their growth temperature [2]. Although the existence of psychrophiles has already been affirmed firstly at the latter half of the 19th century, many aspects of those are waiting for clarification [3]. Vibrio sp. strain ABE-1 (Vibrio ABE-1), which was isolated from sea water at Abashiri Bay, Hokkaido, is a psychrophilic bacterium with an optimum and a maximum growth temperature at 15°C and between 20 and 25°C, respectively [43]. As a line of the investigation on adaptation strategy or compensation mechanism of Vibrio ABE-1 and some psychrotrophic bacteria to low temperature, studies on protein synthesis [4-9], characterization of enzymes [10-12], energy metabolism [13-17], and changes in lipid compositions in membrane [18-22] have been carried out in our laboratory. The purpose of this study is to grope the adaptation strategy to low temperature of psychrophilic enzymes, on the basis of their biochemical properties.

$NADP^+$-specific isocitrate dehydrogenase [IDH: EC 1.1.1.42] is a member of enzymes in tricarboxylic acid cycle and catalyzes the oxidative decarboxylation of

isocitrate to yield α-ketoglutarate and carbon dioxide coupling with $NADP^+$ reduction [23]. IDH has been purified from numerous bacteria [10, 24-35, 44, 46, 59]. Of particular interest is that an inactivation and a reactivation of the enzyme from enteric bacteria is controlled by phosphorylation/dephosphorylation reaction which is catalyzed by IDH phosphorylase/kinase [36].

Previously, Ochiai et al. reported that Vibrio ABE-1 possessed two distinct forms of IDH markedly differing in thermostability, salt-dependency, kinetic characteristic [47], and molecular structure [60]. They designated the thermostable dimeric isozyme and the thermolabile monomeric one as IDH-I and -II, respectively. Such a coexistence is unusual since a bacterial strain has been reported to contain only one form of IDH except Acinetobacter calcoaceticus [28]. However, the impurity of the IDH isozyme preparations prevented further characterization.

To ressolve the problems with the IDH isozymes that remained unclear, I first attempted to develop the improved procedures for the purification of the isozymes in good yield. In "Part I" of this thesis, the results of the purification are presented. There also report the comparative studies between these isozymes in biochemical and immunochemical characteristics examined with the homogeneous preparations.

In respect to the relation between enzymatic kinetic parameters and temperature, many reports have alrea-

dy been published. Among them, it is noted that Hochachka suggested the basic strategies and mechanisms for enzyme adaptation to temperature [37]. Somero and Hochachka [38], Moon [39], Somero [40] and Horwitz and Hettinger [41] proposed that either of two or more isozymes, which are extremely different in their kinetic parameters at a given temperature, may contribute to the biochemical adaptation of ectothermic organisms to the temperatures. Similarly, Segel [42] described a general appreciation of a constitute in enzymes with variant forms and the same function by comparison of kinetic parameters. Revaluation in this study on catalytic properties of the IDH isozymes gave slightly different results from the previous report [47]. Finally, I discuss whether Vibrio ABE-1 IDH isozyme system is obeyed by the above proposal or not by consideration with kinetic properties together with other catalytic characteristics.

MATERIALS AND METHODS

Chemicals

DL-Isocitrate, 2-mercaptoethanol, acrylamide, N,N'-methylenebisacrylamide, N,N,N',N'-tetramethylethylenediamine, Agarose LE, and protamine sulfate were obtained from Nacalai Tesque Inc. Butyl-Toyopearl 650M, DEAE-Toyopearl 650M, and TSK gel DEAE-5PW were purchased from TOSOH Co. DEAE-Sephadex A-50, Procion Red Sepharose CL-6B, Sephadex G-200 (superfine), and pI marker proteins were obtained from Pharmacia. DEAE-cellulose was from Brown Co. Hydroxylapatite gel was prepared by the method of Tiselius et al [43]. Marker proteins for molecular weight were purchased from Boehringer-Mannheim. Ampholine was a product of LKB. Reagents and solvents used for determination of NH_2-terminal amino acid sequence were all of "sequencing grade" from Wako Pure Chemical Industries, Ltd. All other reagents used were of analytical grade.

Bacterial Strains and Growth

Some bacterial strains were kindly donated as follows: Azotobacter vinelandii strain IAM 1078 was from Institute of Applied Microbiology, the University of Tokyo, Salmonella typhimurium and Salmonella paratyphi B was from Prof. Toshikazu Makino, Department of Biology, Hokkaido University, Vibrio parahaemolyticus Y-4 was from Dr. Kazuyuki Kimura, Department of Microbiology,

School of Medicine, the Hokkaido University, respectively.

Vibrio ABE-1 [44] and V. parahaemolyticus were cultured at 15°C and 37°C, respectively, with vigorous shaking in nutrient medium containing 1 % peptone and meat extract with 0.5 M NaCl. Cells were harvested in the early stationary phase, washed three times with chilled 0.5 M NaCl, and frozen at -80°C until use. Escherichia coli K-12, S. typhimurium, S. paratyphi, Bacillus subtilis, Pseudomonas aeruginosa, Serratia marcescens, Micrococcus lysodeikticus were cultured in the above nutrient medium with 50 mM NaCl instead of 0.5 M NaCl at 37°C for the former five strains or at 25°C for the latter two strains. The culture and washing condition of A. vinelandii was followed by the method described in [45].

Standard Assay Conditions

$NADP^+$-specific isocitrate dehydrogenase activity was assayed spectrophotometrically at 40°C for IDH-I or at 20°C for IDH-II in a mixture (final volume 2 ml) containing 33 mM Tris-HCl (pH 8.4 at 20°C), 250 mM NaCl, 0.67 mM $MnCl_2$, 0.12 mM $NADP^+$, 2 mM DL-isocitrate, and an appropriate amount of enzyme. The reaction was started by the addition of $NADP^+$ or DL-isocitrate, and measured the initial rate of increase in absorbance at 340 nm. One unit of enzyme activity is defined as the amount of enzyme catalyzing the reduction of 1 μmol of $NADP^+$ per

min.

Protein concentrations were estimated by the Bio-Rad protein assay procedure which is based on the method of Bradford [46] with bovine plasma γ-globulin as a standard.

Electrophoresis

Disc gel electrophoresis of native enzyme was performed in 7.5 % polyacrylamide gel containing 4 mM isocitrate and 10 mM $MgCl_2$ at a constant current of 2 mA per gel at 4°C [47]. Sample solutions were dialyzed for 4 h against 20 mM potassium phosphate (pH 7.5) containing 4 mM isocitrate, 10 mM $MgCl_2$, 1 mM dithiothreitol to preserve the enzymatic activity before electrophoresis. Protein(s) on gels was detected by staining either for protein with Coomassie brilliant blue R-250 or for IDH activity as described in [47]. Gel electrophoresis in the presence of sodium dodecyl sulfate was carried out by the procedure of Weber and Osborn [48] with 7.5 % polyacrylamide disc gel or that of Laemmli [49] with 10 % slab gel. Analytical isoelectric focusing was performed according to the LKB manual, using 1 mm thick slab gel consisting of 5 % acrylamide, 2 % Ampholine of pH 3.5-10, and 4 mM isocitrate. Condition for dialysis of the purified isozymes before electrophoresis was the same as described above. The gel was run for 80 min at a constant power of 15 watts in electrolyte containing 4 mM isocitrate and 1 mM dithiothreitol.

The pH gradient was estimated from the migration distance of pI marker proteins. Focused proteins on the gel was fixed and stained by the method as described in the LKB manual.

Analytical gel filtration

For estimation of relative molecular weight of IDH isozymes, gel filtration was carried out using 1 X 36.5 cm column of Sephadex G-200 (superfine) equilibrated with 50 mM potassium phosphate (pH 7.5), 250 mM NaCl, 2 mM $MgCl_2$, and 10 mM 2-mercaptoethanol. Both isozymes were dialyzed against the same buffer before gel filtration. The molecular weight markers used were as follows: catalase (240 kDa), aldolase (158 kDa), albumin (68 kDa), ovalbumin (45 kDa), chymotrypsinogen A (25 kDa), cytochrome c (12.5 kDa).

Amino Acid Analysis

Lyophilyzed isozyme proteins after exhaustive dialysis against deionized water were hydrolyzed in 6 N HCl in evaculated sealed tubes at 110°C for 24, 48, and 72 h. The hydrolysates were subjected to a Hitachi model 835 amino acid analyzer. The values for threonine and serine were obtained by extrapolation to zero time [50], and those for valine and isoleucine were determined with 72 h hydrolysate [51]. Cystein and methionine were estimated as cysteinic acid and methionine sulfone after performic acid oxidation [52]. Tryptophan was deter-

mined by hydrolysis in methane sulfonic acid [53].

NH_2-Terminal Amino Acid Sequence Determination

Automated Edman degradation was carried out with Beckman liquid-phase sequencer, type 890-C, or Applied Biosystems 477A gas-phase protein sequencer. The PTH amino acids were identified by HPLC with a TSK gel ODS-80Tm reverse-phase column [54] for the former, or by an on-line PTH analyzer model 120A (Applied Biosystems) for the latter.

Immunological Studies

Homogeneous IDH-I (860 µg) or IDH-II (930 µg) emulsified with Freund's complete adjuvant was injected subcutaneously into a young New Zealand white rabbit. Two and three weeks later, booster injection was performed with a half dose of the first injection. Twenty days after the last injection, blood was collected from ear vein, allowed to clot at room temperature, and then left overnight in cold room. An antiserum was obtained by centrifugation at 3,000 X g for 10 min. The IgG fraction was prepared by precipitation with 50 % ammonium sulfate, followed by passage through a column of DEAE cellulose [55]. Double immunodiffusion in 1 % agarose supplemented with 0.1 % sodium azide was performed according to the method of Ouchterlony [56]. For the immunotitration, Vibrio ABE-1 and V. parahaemolyticus cells were sonically disrupted in 20 mM potas-

sium phosphate buffer (pH 7.5) containing 0.5 M NaCl, 2 mM $MgCl_2$, 1 mM EDTA and 10 mM 2-mercaptoethanol. Cell free extract obtained by centrifugation at 15,000 X g for 10 min was diluted with the above buffer to give IDH-I activity in a range from 1.0 to 3.0 units/ml or IDH-II activity 0.3 to 0.5 units/ml. Anti-IDH-I and -II antisera were also diluted from 4- to 16-fold and from 1.33- to 4-fold, respectively, with the above buffer containing 1 % bovine serum albumin. To 100-300 µl of the diluted cell free extract, the same volume of each antiserum was added and mixed thoroughly, and the mixture was incubated for 6 h at 15°C or 1 h at 37°C. Then the mixture was rapidly cooled in an ice bath, and centrifuged at 8,000 X g for 10 min. The remaining enzymatic activity in the supernatant was measured under standard condition. Pre-immune rabbit serum was used as a control. The preparation of cell-free extracts from other bacterial strains was also carried out by sonic disruption in 10 mM potassium phosphate (pH 6.5), 0.5 M KCl, and 2 mM $MgCl_2$ for E. coli, S. marcescens, P. aeruginosa, and M. lysodeikticus, in 50 mM potassium phosphate (pH 7.5) with 150 mM NaCl for B. subtilis, in 45 mM Tris-HCl (pH 7.7) for S.typhimurium and S. paratyphi, or in 20 mM potassium phosphate (pH 7.5) containing 1 mM glycerol for A. vinelandii. Each IDH activity in these bacterial cell free extracts was diluted with the same buffer as used in Vibrio ABE-1 to give enzymatic activity from 0.3 to 0.5 units/ml. For

B. subtilis, the cell free extract was diluted with 20 mM potssium phosphate (pH 6.5), 2 mM $MgCl_2$, 1 mM EDTA and 10 mM 2-mercaptoethanol. Dilution of anti-IDH-I and -II antisera were 2- and 4-fold, respectively. After incubation and centrifugation as above, the remaining activity except that of V. parahaemolyticus was determined in the standard assay mixture omitting NaCl.

Thermostability

IDH-I (52 μg/ml) and -II (42 μg/ml) in an appropriate buffer were placed in a water bath equipped with a thermal controller, and incubated at various temperatures. The pH of the sample solutions was adjusted to 8.0 at each temperature. After an appropriate interval of time period, aliquots of the samples were withdrawn for determination of the activity under the standard assay condition. Remaining activities were plotted as a per cent of the activity at 0 time of the incubatiuon.

Kinetic Study

The specific activities of the IDH isozymes used in this study were 65.2 to 67.1 units/mg for IDH-I and 55.8 to 57.6 units/mg for IDH-II. For determination of kinetic parameters as a function of temperature, the pH value of the reaction mixture was adjusted to 8.0 over the experimental temperature according to the correction factor of Tris buffer, dpH/dt as -0.028 pH units/deg [57]. To obtain unique values of kinetic parameters,

Vmax and Km values for DL-isocitrate or $NADP^+$ were determined by the matrix method [58]:reaction velocities were determined by using six different subsaturating concentrations of the substrate. For this purpose, the substrate concentration was increased in a manner of geometric series. Four independent experiments were carried out at each concentration of the substrate, and the data were averaged and analyzed by the following equation,

$$v = Vmax[A][B]/K_{ia}K_m^B + K_m^B[A] + K_m^A[B] + [A][B]$$

where v is the initial velocity, Vmax is the maximum velocity, [A] and [B] are the concentration of $NADP^+$ and DL-isocitrate, K_{ia} is the true equilibrium dissociation constant for isocitrate dehydrogenase-$NADP^+$ complex when isocitrate concentration approaches to zero. K_m^A and K_m^B are the Michaelis constants for $NADP^+$ and DL-isocitrate, respectively.

The value of activation energy (Ea) was computed from the slope of an Arrhenius plot determined by the method of least squares, and the Q_{10} value was calculated by following equation:

$$\log Q_{10} = 10\ Ea/2.303RT_1T_2$$

where T_1 and T_2 are absolute temperatures differing in 10 K, R is the gas constant.

The thermodynamic parameters for activation, $\underline{\Delta H}^{\neq}$ (enthalpy) and $\underline{\Delta S}^{\neq}$ (entropy), were caluculated by following equation:

$$\log \underline{V}max/\underline{T} = -\underline{\Delta H}^{\neq}/2.303\underline{R}\ 1/\underline{T} + (\log \underline{k_b}/\underline{h} + \underline{\Delta S}^{\neq}/2.303\underline{R})$$

where $\underline{k_b}$ is the Bolzman's constant and $\underline{h}$ is the Plank's constant. The data obtained from the plot $\underline{V}max/\underline{T}$ (K^{-1}) *versus* $1/\underline{T}$ (K^{-1}) were analyzed by the method of least squares. The free energy of activation ($\underline{\Delta G}^{\neq}$) was calculated from the below equation.

$$\underline{\Delta G}^{\neq} = \underline{\Delta H}^{\neq} - T\underline{\Delta S}^{\neq}$$

RESULTS

Effect of pH and Buffer on Stabilities of IDH Isozymes in Cell-Free Extract

Preceding the purifications, I examined the effects of pH and buffer on stabilities of *Vibrio* ABE-1 IDH isozymes in order to find the best condition for purification of the isozymes. A small amount of the cell-free extract was prepared as described in the paragraph of purification (step 1) of "RESULTS" and diluted appropriately with one of Tris-HCl, HEPES, and potassium phosphate buffers in accordance with various pHs. The diluted cell-free extract was allowed to leave at 4°C for 24 h, and the remaining activities of IDH isozymes were measured under the standard assay condition (Fig. 1). It can be seen that potassium phosphate buffer was preferable to other buffer systems at any pH tested. Optimum pH on stability was found to be 7.0 for IDH-I and 7.5-8.0 for IDH-II. In addition, the stability of the each isozyme was differently affected by the addition of some chemicals. Glycerol and citrate were found to stabilize for IDH-I, but not for IDH-II. On the other hand, reducing agents such as 2-mercaptoethanol and dithiothreitol, and the presence of an appropriate concentration of salt; *e.g.* NaCl or KCl, were indispensable for protection of IDH-II from the inactivation [47], while such a compound was ineffective for IDH-I.

Purification

In order to purify the each isozyme in the respective suitable conditions, I attemped to separate the isozymes from each other in an early step of the purification. After then, IDH-I and -II were purified separately by the method described previously [47] with some modifications. All operations were carried out at 4°C or below, unless otherwise indicated.

Step 1. Preparation of Cell-Free Extract

Frozen cells (usually 60 g wet weight) were thawed, suspended in about an equal volume of chilled 20 mM potassium phosphate (pH 7.5) containing 0.5 M NaCl, 2 mM $MgCl_2$, and 10 mM 2-mercaptoethanol (referred to as Buffer A) and subjected to sonic oscillation for four times of 1.5 min with each interval of 1 min under continuous cooling. Cell-free extract was obtained by centrifugation of the lysate at 12,000 X g for 30 min.

Step 2. Protamine Sulfate Treatment

2 % protamine sulfate in Buffer A was added dropwise to the cell-free extract with gently stirring to give a ratio of 1 mg of protamine sulfate per 5 mg of protein. The mixture was stirred further 20 min and centrifuged at 12,000 X g for 20 min. The precipitate was discarded.

Step 3. Ammonium Sulfate Fractionation

To the supernatant, powdered ammonium sulfate was added gradually to give 45 % saturation. The solution was stirred for 1 h, centrifuged at 12,000 X g for 20

min, and the precipitate was discarded. Ammonium sulfate was further added to the supernatant to give 75 % saturation, and the solution was stirred and centrifuged as described above. The precipitate was saved. During this step, the pH of the solution was adjusted to 7.5 by the addition of 1 M NH_4OH.

Step 4. Hydrophobic Chromatography

The resultant pellet was dissolved in a small volume of Buffer A to give a concentration of ammonium sulfate of about 1.5 M, and the solution was cleared by centrifugation. The obtained amber supernatant was applied to a column of Butyl-Toyopearl 650M (2.5 X 20 cm) equilibrated with Buffer A containing 1.5 M ammonium sulfate at the flow rate of 3 ml/min. The column was washed exhaustively with the same buffer until the absorbance at 280 nm of eluent was below 0.1, then a linear gradient from 1.5 to 0 M ammonium sulfate was developed in a total volume of 1 liter. IDH-I was eluted at near 1.0 M ammonium sulfate. After gradient was over, the column was washed with Buffer A to elute a large amount of IDH-II absorbed to the column. Two different IDH activities were able to be completely separated in this step (Fig. 2). Fractions containing the respective IDH activity were pooled separately.

IDH-I Step 5. DEAE Toyopearl 650M Chromatography

The IDH-I solution was concentrated by ultrafiltration with a UP-20 membrane (Toyo Roshi Co.), and dialyzed against Buffer B (20 mM potassium phosphate (pH

6.8) containing 50 mM KCl, 5 mM potassium citrate, 2 mM $MgCl_2$, and 10 % glycerol). The dialyzed enzyme was cleared by centrifugation and applied to a column of DEAE Toyopearl 650M (1.6 X 15 cm) equilibrated with Buffer B at the flow rate of 0.5 ml/min. After washing the column with four column volume of Buffer B, IDH-I was eluted with a linear gradient from 50 to 250 mM KCl in a total volume of 300 ml (Fig. 3A). Fractions containing high activity were collected, concentrated with polyethylene glycol #20,000, and dialyzed against Buffer C (20 mM potassium phosphate (pH 6.5) containing 2 mM $MgCl_2$, and 10 % glycerol).

IDH-I Step 6. Procion Red Sepharose CL-6B Chromatography

The dialyzed enzyme was applied to a column of Procion Red Sepharose CL-6B (1 X 13 cm) equilibrated with Buffer C at the flow rate of 0.25 ml/min. After washing the column with the same buffer until the absorbance at 280 nm of eluent was negligible, a linear gradient from 0 to 40 mM potassium citrate was developed in a total volume of 150 ml (Fig. 3B). Eluted fractions with IDH-I activity were collected, concentrated as above, and dialyzed against Buffer B omitted 50 mM KCl.

IDH-I Step 7. HPLC on TSK gel DEAE-5PW

IDH-I was finally purified by HPLC (Tosoh Co.). The dialyzed solution was applied to a column of TSK gel DEAE-5PW (0.7 X 7 cm). HPLC was carried out at room

temperature under the following conditions: flow rate, 0.5 ml/min; gradient, 100 % Buffer B omitted 50 mM KCl/13 min, 0-38 % Buffer B containing 0.5 M KCl/1 min, 38-40 % the same buffer/20 min, and 40-100 % the same buffer/1 min. IDH-I was eluted coincidentally with a main peak of absorbance at 280 nm as shown in Fig. 3C. The resulting preparation was dialyzed against Buffer B containing 40 % glycerol, and stored at -20°C until use.

IDH-II Step 5. Hydroxylapatite chromatography

The pooled IDH-II solution after the step 4 was concentrated with a UP-20 membrane, and dialyzed against Buffer D (20 mM potassium phosphate (pH 8.0), 250 mM NaCl, 2 mM $MgCl_2$, and 10 mM 2-mercaptoethanol). The dialyzed material was cleared by centrifugation, and applied to a column of hydroxylapatite (1.6 X 10 cm) equilibrated with Buffer D at the flow rate of 7.2 ml/h. After washing the column with three column volume of the same buffer, a linear gradient from 20 to 300 mM potassium phosphate was developed to elute IDH-II in a total volume of 250 ml (Fig 4A). Fractions having activity were collected, concentrated with polyethylene glycol # 20,000, and dialyzed against Buffer E (20 mM potassium phosphate (pH 8.0), 100 mM NaCl, 2 mM $MgCl_2$, and 10 mM 2-mercaptoethanol).

IDH-II Step 6. DEAE Sephadex A-50 Chromatography

The dialyzed enzyme was applied to a column of DEAE Sephadex A-50 (1.6 X 10 cm) equilibrated with Buffer E at the flow rate of 18 ml/h. The column was washed

with three column volume of the same buffer, then IDH-II was eluted with a linear gradient from 100 to 400 mM NaCl in a total volume of 300 ml (Fig. 4B). Fractions containing high activity were collected, concentrated with polyethylene glycol # 20,000, and dialyzed against Buffer E.

IDH-II Step 7. Elution by Substrate from DEAE Toyopearl 650M

The final step of IDH-II purification was specific elution of the enzyme by substrate from a DEAE Toyopearl column. The dialyzed material from the previous step was applied to a column of DEAE Toyopearl 650M (1.6 X 10 cm) equilibrated with Buffer E at the flow rate of 2 ml/min. After washing the column with the same buffer, IDH-II was eluted with 4 mM DL-isocitrate in Buffer E (Fig. 4C). Fractions near the peak of the activity were subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate to check their purities. The fractions containing only one band corresponding to IDH-II were pooled, concentrated by ultrafiltration, and dialyzed against Buffer A supplemented with 4 mM isocitrate. The purified IDH-II was stored at -20°C until use.

Supplement to Purification

Despite the examination of a variety of column chromatography, including ion exchanger, affinity matrices, and elution buffers, the complete separation of the

IDH isozymes was not accomplished unless the hydrophobic chromatography on Butyl Toyopearl 650M was introduced (Fig. 2). The elution profile of IDH-II from a column of Butyl Toyopearl was greatly influenced by the concentration of NaCl or KCl employed; e.g. 50 mM KCl could not elute IDH-II independently of IDH-I, while that of IDH-I was not affected by changing NaCl or KCl concentration.

After the separation, three sequential chromatographic techniques as described above led IDH-I to be homogeneous state. Borthwick et al. [59] reported that E. coli IDH, which is similar to IDH-I in molecular structure and thermal property (see below), was completely retained by a column of Procion Red Sepharose equilibrated with potassium phosphate buffer containing potassium citrate, followed by elution with a "pulse" of $NADP^+$. But this technique could not be applied to IDH-I, because IDH-I was neither absorbed to this matrix in the presence of 5 mM potassium citrate, nor eluted by $NADP^+$ even if the enzyme was absorbed in the absence of potassium citrate [data not shown].

The purification of IDH-II was hampered by the problems originated from salt dependency of the enzyme. Because the activity of IDH-II was lost rapidly in a condition without 0.1-0.4 M KCl or NaCl, only a few techniques with commercially available matrices could be applicable. Although a lot of proteins other than IDH-II were eliminated by hydroxylapatite (Fig. 4A) and

DEAE-Sephadex column chromatography (Fig. 4B), a small number of minor contaminants could not be removed even if these techniques were applied repeatedly.

Typical results of the purifications of the isozymes are summarized in Table I.

Purity, Molecular Weight and Isoelectric Point

The purified IDH isozymes gave only a single band of protein or enzyme activity on the gels of electrophoreses both under nondenaturing and denaturing conditions (Fig. 5A and 5B). When the gels were stained for IDH-II activity, tailing of the band was appeared with the time of the incubation [47]. From the migration distance of the each isozyme on the gels in the presence of sodium dodecylsulfate, molecular weights of IDH-I and -II were estimated as 49,100 and 79,500,respectively. On the other hand, apparent molecular weights determined by analytical gel filtration were 88,100 for IDH-I and 80,500 for IDH-II, respectively (Fig. 6). These observations confirm the previous results [60] that IDH-I is a dimeric form and IDH-II is a single polypeptide.

As shown in Fig. 7, the purity of the purified isozymes was confirmed again by an electro-focusing, and the isoelectric points of IDH-I and -II were found to be 5.2 and 4.9 ,respectively.

Absorption Spectra

The ultraviolet absorption spectra of native IDH isozymes in 20 mM potassium phosphate (pH 7.5) containing 250 mM NaCl, 2 mM $MgCl_2$, and 1 mM dithiothreitol, at 15°C, resulted in ordinary protein absorption spectra with a maximum at 278 nm and a shoulder at 292 nm. Values of $E^{1\%}_{1cm}$ at 280 nm of IDH-I and -II were obtained as 6.15 and 4.01, respectively, on the basis of the protein concentration determined by the method of Bradford [46].

Amino Acid Composition

The amino acid compositions of the isozymes are represented in Table II on the basis of Mr of 49,000 for IDH-I and of 80,000 for IDH-II. It seems that IDH-I contains more non-polar amino acid residues such as valine, methionine, isoleucine, and tryptophan, and IDH-II possesses significantly higher amounts of hydroxyl amino acids such as serine and threonine than the respective counterpart. The calculated values of the hydrophobicity indices (Hø) [61] of IDH-I and -II were 1.102 and 0.963 Kcal/residue, which are comparable to those of cytoplasmic proteins obtained from several non-halophilic and halophilic bacteria, respectively. Relatedness among six different IDH proteins purified from five different bacterial strains was estimated by using the parameter SΔQ which was expressed by Marchalonis and Weltman [63] as the sum of the square of the

difference in mole fraction of each amino acid residue (Table III). The fact that all $S\Delta Q$ values listed in the Table are less than 100 indicates that these bacterial IDHs are comparatively similar in their amino acid compositions. Especially, close relatedness is found between IDH-I and -II (15.5), and between IDHs of E. coli and B. stearothermophilus (12.2).

NH_2-Terminal Amino Acid Sequence

IDH-I and -II were subjected to 40 and 28 cycles, respectively, of automated Edman degradation. In analyses, both isozymes gave only one PTH-amino acid at each cycle, suggesting that IDH-I is composed of chemically identical or highly homologous subunits. The NH_2-terminal sequences of the isozymes are shown in Fig. 8.

Immunological Property

By Ouchterlony double diffusion [56], it was revealed that IDH-I and -II formed a precipitation line only with the antibody raised against the respective isozyme (Fig. 9). Non-immunocrossreactivity between the isozymes was confirmed again by immunotitration method (Fig. 10), that is, rabbit anti-IDH-I antiserum was unable to neutralize the activity of IDH-II at all. These results suggest the possibility that the each antiserum against the IDH isozymes may be useful to distinguish the other bacterial IDHs in immunochemical

and structural properties. Thus, the activities of some bacterial IDHs were immunotitrated with each one of the antisera (Table IV).

Thermostability

Thermostabilities of the IDH isozymes were examined under the conditions as described in "MATERIALS AND METHODS", and the results were essentially the same as described with impure preparations [47]. As shown in Fig. 11, IDH-I was stable up to 40°C for at least 20 min, but inactivated linearly in the course of time period on semi-logarythmic plots with increasing temperature incubated above 45°C. In contrast, IDH-II was extremely thermolabile, and inactivated above 20°C. The rate of inactivation of IDH-II was very rapid in the first 5 min, and became in the second phase.

Effect of Nucleotides and Other Compounds on Catalytic Activity

The effects of some compounds on enzymatic activities of both isozymes are summarized in Table V. As similar as the report by others [27, 30] that citrate inhibits the activity of some dimeric IDHs, citrate inhibited specifically the activity of IDH-I. On the other hand, glyoxylate inhibited only the activity of IDH-II, and the concerted inhibition by oxalaetate and glyoxylate was severer in IDH-II as reported previously [47]. The inhibitory effects of ATP and α-ketogluta-

rate on IDH-I were found to be higher about twofold than those on IDH-II. The inhibitory degree of the compounds on IDH-I activity was independent on assay temperature at 40 °C and 20°C. Neither acceleration of the enzymatic activities nor concerted inhibition by any combination other than glyoxylate and oxalacetate were observed so far.

Effect of Monovalent Cation on Activity

Figure 12 illustrates the effects of monovalent cations on the activities of the IDH isozymes as expressed relatively to 100 % activity of the value measured under the standard assay condition (in the presence of 250 mM NaCl). For both isozymes, it was found that K^+ was preferable to any other cations tested, and was broader the range of optimum concentration for activity of IDH-II than that of IDH-I.

Kinetic Property

The Km values of the two IDH isozymes were determined within the temperature range that each isozyme could not be inactivated during the assay. For both isozymes, the apparent Km values for DL-isocitrate increased linearly (Fig. 13A). Such a linearlity was also observed in the changes of the Km values for $NADP^+$ of IDH-II, whereas the relationship between temperature and the Km values for $NADP^+$ of IDH-I showed a synclinal curve with a minimum point at 20°C (Fig. 13B). Over

the temperature range from 5° to 20°C, the Km values for isocitrate and $NADP^+$ of IDH-II were considerably lower than those of IDH-I. These results indicate that IDH-II shows much higher affinity for substrate than IDH-I, especially at lower temperatures.

The values of the maximum velocity measured at various temperatures are listed in Table VI. As shown in Fig. 14, the Arrhenius plot of IDH-I was best fitted by a biphasic linear curve showing a break at 20°C, whereas that of IDH-II was monophasic. The Vmax/Km values were always higher in IDH-II than in IDH-I under the permissive temperature range for the growth of *Vibrio* ABE-1 (Table V). Since the value of Vmax/Km of IDH-II increased with decrease of temperature, the reaction catalyzed by IDH-II may proceed preferentially *in vivo* under low temperature.

Table VII shows the apparent thermodynamic activation parameters for the two IDH isozymes. The value of the activation energy was calculated from the slopes in Fig. 14 and the others were from the equations described in "MATERIALS AND METHODS". The Q_{10}, Ea, $\Delta H^{\neq}$, and $\Delta S^{\neq}$ values of IDH-I are significantly higher than those of IDH-II. Compared with the differences of the four pairs of parameters of the two isozymes described above, $\Delta G^{\neq}$ values differed a little from each other.

DISCUSSION

Two distinct forms of $NADP^+$-specific isocitrate dehydrogenase were purified to be homogeneous states from Vibrio ABE-1. Some improvements were found to lead to the success of the purifications. Primarily, choice of suitable buffers and the additives, which can minimize the inactivations of the isozymes, was essential for the purification (Fig. 1). This means that one should take care of buffer systems used in the purification of a protein from a certain source, that is, the buffer system, which has already been reported as suitable for the purification of a similar protein from other sources, should not be applied easily without critical examination. Secondly, the complete separation of the isozymes was accomplished by introduction of hydrophobic chromatography (Fig. 2). Thirdly, in each final step, HPLC for IDH-I (Fig. 3C) and specific elution by substrate from an ion-exchange chromatography for IDH-II (Fig. 4C), were effective to eliminate a few remaining contaminants. Finally, an adequate amount of bacteria (usually 60 g wet weight) was employed as a starting material to finish the all steps of purifications within a week.

Bacterial IDHs have been reported to show a molecular weight ranging 75-100 kDa, and they are classified according to their molecular structures into a dimeric type composed of identical subunits in size of 40-50 kDa

[24, 29, 32, 34-35] and a monomeric one with the molecular weight of 75-80 kDa [33, 45]. The molecular weights and structures of the two IDH isozymes in Vibrio ABE-1 also fall into the above classification. However, the coexistence of such different types of IDHs in a bacterium has not yet been observed in any other bacterial strains. So, the relationship between the isozymes is an attractive problem as well as the physiological role of them.

Generally, isozymes can be categorized into two groups; one group is the true isozymes encoded by distinct stretches of DNA, and the other is the secondary isozymes derived by post-translational or epigenetic modification of a single gene product [64]. In respect to the IDH isozymes of Vibrio ABE-1, there observed many differences in their properties such as amino acid composition (Table II), NH_2-terminal amino acid sequences (Fig. 9), immunological property (Fig. 10 and 11) and thermostabilities (Fig. 12). Apparently, these differences suggest that the IDH isozymes seem to have the respective structural genes. This conclusion is supported by the previous observation [70] that IDH-I was inducibly synthesized by acetate, whereas IDH-II was not. But the most conclusive evidence for the above question is an identification of the genes encoding the IDH isozymes, and it will be shown in "Part II" in this thesis.

It is of quite interest to note the results of the

immunotitration (Table IV) that each rabbit antiserum raised against IDH-I and -II could specifically inhibit the activity of either types of bacterial IDHs; e.g. anti-IDH-I-antibody inhibited the activities of IDHs of E. coli and S. typhimurium, and anti-IDH-II-antibody inhibited the activity of IDH of A. vinelandii. These results suggest that bacterial dimeric and monomeric IDHs were found to have type-specific antigenicities, or in other words, the two types of bacterial IDHs were immunologically distinguishable. In addition, S Δ Q values among the homologous type of IDH proteins were usually lower than those among the heterologous types (Table III). These observations may imply that two types of IDHs were originated from a different primitive gene or evolved independently from a common ancestor diverged in much long time ago. Although the data on the molecular structures of IDHs of the following bacterial species are not yet available, IDHs of S. paratyphi B, S. marcescens, B. subtilis are presumed to be a dimeric type because of their immunocrossreactivity with anti-IDH-I-antibody. As similarly, IDH of V. parahaemolyticus may be a monomeric type (This speculation has been proven recently by the study with purified enzyme of this bacterium [Imagawa S. in the thesis for the degree of Master of Science, Hokkaido University in 1988]).

Vibrio ABE-1 is a marine bacterium, and requires an appropriate concentration of salt for the growth [44].

Some cellular functions or components of Vibrio ABE-1, in practice, are stimulated or stabilized by the addition of salt such as NaCl or KCl [16]. Also in the case of the IDH isozymes, NaCl, KCl, or possibly other monovalent cations, were found to be essential for their stabilizations. Especially, salt dependency was striking in IDH-II. When 50 mM KCl was employed in place of 0.5 M NaCl in the buffer system, IDH-II was coeluted with IDH-I from a column of Butyl-Toyopearl in a partially inactive state (data not shown). And, it was reported that the apparent molecular weight of IDH-II became larger with the loss of enzymatic activity when the buffer containing 50 mM NaCl was used for gel filtration than when 0.5 M NaCl was used instead of 50 mM [60]. Furthermore, excessive tailing on the band stained specifically for IDH-II (Fig. 5A, lane 6) was observed. These results suggest that an appropriate concentration of salts was necessary to form the compact and highly active conformation of IDH-II. Such the conformation may be that much more hydrophobic residues are exposed to the molecular surface in the presence of salt, or negative charges of external hydrophilic residues are screened by monovalent cations, to become more hydrophobic nature. Some enzymes of tricarboxylic acid cycle were suggested to loosely bind to an inner membrane of mitochondria [65-66]. IDH-II may also bind reversibly to an inner membrane of the bacterium with hydrophobic interaction enhanced by salts. Ochiai et

al. reported that IDH-I and -II were slightly and greatly, respectively, activated by NaCl or KCl [47]. Compared with the above results, the study using the purified preparations of the isozymes showed that optimal concentrations of monovalent cations, and also, the extent of activation of IDH-II were rather low (Fig. 13) (16.5-fold at 300 mM NaCl and 20-fold at 1.2 M KCl in [47], while 1.7-fold at 200 mM NaCl and 400 mM KCl in this study). This discrepancy seems to be partly due to the difference in the purity of IDH-II. However, the most crucial factor may be derived from difference in the experimental conditions employed. Ochiai et al. [47] examined the salt-dependency of the isozymes dialyzed for 2 h against the buffer omitting salt. As described in [47], more prolonged dialysis than 2 h in such a condition induced the irreversible inactivation of these isozymes. Furthermore, IDH-II was found to be inactivated reversibly even within 2 h of the dialysis. Accordingly, I examined the salt-dependency with the enzyme dissolved in phosphate buffer containing 250 mM NaCl, although 2.5 mM NaCl was carring originally in the solution. Thus, previous results may reflect the effects of salts on the reactivation of IDH-II. The intracellular concentrations of NaCl and KCl of Vibrio ABE-1 were recently estimated to be in a range of 16-68 mM for NaCl and 377-675 mM for KCl [15]. The latter value is in agreement with an optimum concentration for IDH-II. IDH-II found to be more adaptive than IDH-I in

salinary profile.

Many reports have been published on the existence of various isozymes in prokaryotes differing from one another in their localizations [67], physiological role [68], or substrate specificity [69]. One of the unique features of the $NADP^+$-IDH isozymes of Vibrio ABE-1 is that the isozymes accomplish apparently identical function and coexist in cytoplasm. Under such a circumstance, the two IDHs would compete for a common substrate and cofactor. Thus, it is important to know exact values of their kinetic and thermodynamic parameters for understanding their physiological roles in a given environmental condition. The Km values of IDH-II for substrate and cofactor were lower than that of IDH-I at any temperature (Fig. 13), indicating that IDH-II has higher enzyme-substrate affinity than IDH-I does. The maximum velocities were always lower in IDH-I than IDH-II (Fig. 14), in addition, the Q_{10} value of IDH-I was increased below 20°C (Fig. 14, Table VII). As the results, the Vmax/Km values were higher in IDH-II than in IDH-I under the permissive temperature range for the growth of Vibrio ABE-1 (Table VII), and the fact that the lower temperature, the larger Vmax/Km value was observed, suggests that temperature compensation was predominant in IDH-II than in IDH-I, consequently IDH-II is concluded a favorable isozyme in low temperature. At low substrate concentration, the relative contributions of the isozymes will depend upon the Vmax/Km

ratios of the two isozymes [42]. Therefore, assuming the cellular contents for the two IDH isozymes are equal, the relative ratio of contribution of the IDH-II to an oxidative decarboxylation of isocitrate is three and fifteen times higher than that of IDH-I at temperature of 20° and 5°C, respectively. Moreover, the ratio could be expected to increase practically because much more level of IDH-II was observed in the cells [70]. Analysis of the thermodynamic activation parameters of the IDH isozymes were consistent with the above conclusions speculated from the kinetic parameters. Hochachka proposed that in the strategies of biochemical adaptation to low temperature of enzymes were included an acquisition of a direct Km-temperature relationship as shown in Fig. 13 and the reduction of $\Delta G^{\neq}$ value which include $\Delta H^{\neq}$ and $\Delta S^{\neq}$ in itself [37]. Judged from the above conclusion together with the results of the thermostabilities of the two IDH isozymes (Fig. 12), IDH-II is undoubtly a much more adapted to low temperature and serves as a principal catalyst in vivo. On the other hand, IDH-I seems to be a mesophilic enzyme and scarecely contribute to the oxidative decarboxylation of isocitrate.

Many investigators suggested that isozyme systems are used in compensating for the depressant effects of decreased thermal energy in ectothermic organisms [37-41]. Different mechanisms are contained in the compensation. For example, the "on-off" synthesis of unique

isozymic forms [39], relative changes in specific isozyme members of a complex isozyme system [40], and the dependence on altered kinetic characteristics without changes of amounts of variants [41]. In the case of Vibrio ABE-1 IDH isozymes, the level of IDH-I was temperature-independent, while that of IDH-II increased with elevating growth temperature [Ochiai T., unpublished data]. If one suppose from this result, the IDH isozymes was seems to belong the isozyme system of the second of the above three mechanisms. However, most data obtained in this study indicate that IDH-I is comparable with mesophilic counterpart, and I failed to find any superiority of IDH-I on kinetic and thermodynamic traits to IDH-II below and even above the maximum growth temperature of Vibrio ABE-1. IDH-I may be an "intruder" in the process of evolution of this bacterium. This idea is supported by the observation that the growth rate of a defective mutant in IDH-I, YF-83, was essentially the same as parental strain at any temperature and growth media [72]. Moreover, no examples like the IDH isozyme of Vibrio ABE-1 were found in any other isozyme systems so far as I know. I, therefore, conclude that Vibrio ABE-1 IDH isozymes are the instance to be quite novel.

PART II

SUMMARY

Synthetic oligonucleotide probes designed according to the NH_2-terminal amino acid sequences of the proteins were used to identify and isolate the genes encoding the two distinct forms of $NADP^+$-specific isocitrate dehydrogenase [IDH: EC 1.1.1.42] from an obligately psychrophilic marine bacterium, Vibrio sp. strain ABE-1. A comparison of the restriction maps of DNAs cloned from Vibrio ABE-1 genomic library revealed that the genes were tandemly located in order of icd-I gene (coding for a dimeric isozyme with mesophilic in nature, IDH-I) preceded by icd-II gene (coding for a monomeric isozyme with psychrophilic in nature, IDH-II). The open reading frames of icd-II and icd-I contained 2,226 and 1,245 base pairs, respectively. The molecular weights calculated from the deduced amino acid sequences were 45,013 for IDH-I and 80,493 for IDH-II, which were essentially the same as the values previously estimated with the IDH isozyme proteins. Amino acid compositions calculated from deduced primary structures of the two IDH isozymes were consistent with the respective amino acid compositions obtained by chemical analysis of proteins. No significant homology was found between the amino acid sequences of IDH-I and -II, in addition, no repeated sequence in IDH-II was detected by harplot analysis. These suggest that the isozymes originated from the different primitive ancestor, or evolved from a common

ancestor but diverged in extremely early stage from each other, and that IDH-II was not a product as the result of the gene duplication of a putative genetic unit. Homology search using database showed that IDH-II shared none of other proteins, on the other hand, IDH-I shared a 73.8 % homology with Escherichia coli IDH. In addition, there was a homologous region between IDH-I and isopropylmalate dehydrogenase from Thermus aquaticus, which also showed a faint homology with a part of IDH-II. The adenine plus thimine content of the coding region and that of the third letter of codons was 61.6 % and 74.7 % for IDH-I, and was 60.3 % and 74.8 % for IDH-II, respectively.

INTRODUCTION

Recent advances in molecular biological techniques have porovided the new concepts and methods for the study of the genetics, biochemistry and physiology of bacteria at molecular level [74]. In fact, not to speak of Escherichia coli and Salmonella typhimurium [75], many bacteria were investigated in such a line [76].

In "Part I" of this thesis, I reported the purifications and physicochemical and kinetical characterizations of $NADP^+$-specific isocitrate dehydrogenase [IDH; EC 1.1.1.42] isozymes (IDH-I and -II) from an obligately psychrophilic marine bacterium, Vibrio sp. strain ABE-1. However, many questions such as the phylogenetical relationship of protein species, the identification of the amino acid residues in catalytic sites, and why IDH-II is thermolabile and able to show high activity in low temperature range, etc., still remain unclear. In addition, the synthesis of these isozymes were shown to be differentially regulated when cells were grown on acetate or other carbon sources [69]. And it is expected that genetic approaches are available for the elucidation of all the above problems.

Icd gene coding for IDH was firstly isolated from Escherichia coli [76], and subsequently the structure of the IDH was studied by a X-ray crystallographic analysis [77, 93]. As the results, E. coli IDH was revealed to

have no conserved sequence which has been found in several other pyridine nucleotide-specific dehydrogenases. Thus, a new and interesting questions are raised concerning the study on the evolution of the bacterial IDHs. But at present time, there are no comparable data on primary structure of other bacterial IDHs.

In "Part II", I report the results of cloning and sequencing of Vibrio ABE-1 icd-I and icd-II genes, which encode IDH-I and -II, respectively. This is the first report on the cloning of genes from Vibrio ABE-1, and the isolation of bacterial monomeric IDH gene has not been yet reported until the present study.

MATERIALS AND METHODS

Chemicals

[γ-^{32}P]ATP (>4,000 Ci/mmol) and [α-^{32}P]dCTP (~3,000 Ci/mmol) were purchased from ICN Biomedicals, Inc.(USA). Restriction endonuclease *Ban*I, *Sac*II and T4 polynucleotide kinase were obtained from Toyobo (Osaka, Japan). Other restriction endonucleases, DNA polymerase (Klenow fragment), T4 DNA ligase, alkaline phosphatase, exonuclease III, mung bean nuclease, DNase I, RNase A, and M13 reverse primer were obtained from Takara Shuzo (Kyoto, Japan), or Nippon Gene Company (Toyama, Japan). Hybond-N was a product of Amersham International (England). All other reagents used were of analytical grade.

Bacteria and Growth

Vibrio sp. strain ABE-1 [44] was cultured as the same in "Part I". *Escherichia coli* XL1-Blue [78] which used as a host in transformation was cultured in 2 X TY medium (1.6 % Bactotryptone (Difco), 1 % yeast extract (Difco), 0.5 % NaCl) at 37 ^{o}C with vigorous shaking.

DNA

Vibrio ABE-1 chromosomal DNA was prepared by the method of Bedbrook *et al*. [79] with some modifications. Harvested and washed cells were suspended in 100 volume of 0.5 M sucrose with vigorous shaking for 1 h. Cells

were collected, and added by eight volume of 20 mM Tris-HCl (pH 7.5) containing 1 mM EDTA and 0.1 % lysozyme, followed by incubation at 37°C for 3 h. Then 20 % lauroyl sarcosyl sulfate and pectinase were added to give 1 % and 500 μg/ml, respectively, and incubated at 60°C for 10 min, then successively at 37°C overnight. The lysate was added ethidium bromide and CsCl, and ultracentrifuged at 35,000 rpm for 36 h in Hitach RP-50T rotor. Banding DNA was recovered, destained by 2-propanol saturated with CsCl and TE (10 mM Tris-HCl (pH 7.5) containing 1 mM EDTA), and dialyzed exhaustively against TE. Chromosomal DNA was stored at 4°C until use.

Vibrio ABE-1 genomic library was constructed in the λDASH vector (Stratagene) by partial digestion with Sau3A [80]. The syntheses of oligonucleotide were performed on a DNA synthesizer (7500; Milligen or 381A; Applied Biosystems) against the parts of NH_2-terminal amino acid sequences determined from proteins described in Fig. 15.

The preparations of plasmid and phage DNAs were carried out according to the methods in [82].

Southern and Plaque Hybridization

The nylon membrane upon which digested, followed by electrophoretically separated DNAs were blotted was soaked at 44°C for 5 h in prehybridization solution containing 6 X SSC (1 X SSC is composed of 0.15 M NaCl

and 0.015 M sodium citrate), 5 % Irish Cream Liqueur (Baileys, Ireland), 0.5 % sodium dodecylsulfate, and 1 mg/50 ml of sonicated calf thymus DNA. Then the membrane was placed onto hybridization solution composed of prehybridization solution plus synthetic oligonucleotide probes end-labeled with [γ-^{32}P] ATP, and was incubated overnight at 44^{o}C. Washing of the membrane was performed with vigorous shaking as follows:2 X SSC at room temperature for 30 min, 2 X SSC containing 0.1 % sodium dodecylsulfate at 44^{o}C for 2 h with a change of washing buffer.

Transfer of phage particles formed on NZY agar plate (1 % NZ Amine, 0.5 % yeast extract, 0.5 % NaCl, 0.2 % $MgSO_4$-H_2O, 1.5 % agar), and alkali-denaturation followed by neutralization were carried out acoording to the method in [81]. Prehybridization, hybridization and washing of nylon membrane were done as above.

Determination of Nucleotide Sequence

Double-stranded template DNA was prepared from the bacterial cultures incubated overnight according to the alkaline lysis method [81] followed by treatment of the DNA pellet with RNase A and precipitation with polyethylene glycol # 6000. The purified DNA was then denatured with 0.2 M NaOH [81] and used as a template. Nucleotide sequences of pBluescript vector (Stratagene) containing various length of inserts were determined for both strands of DNA by the dideoxy method of Sanger

et al. [83], using Sequenase kit (United States Biochemical). Overlapping deletions were generated by using the technique of Henikoff [84]. Computer analyses of nucleic acid and deduced amino acid sequence data were performed by using the programs GENETYX (Software Development Co., Tokyo) on a NEC PC9801 computer.

RESULTS AND DISCUSSION

Genomic Southern Hybridyzation

Vibrio ABE-1 chromosomal DNA was completely digested with *Eco*RI, *Eco*RV, *Hin*dIII and *Xba*I, and fragments were electrophoresed on 1 % agarose gel followed by blotting onto a membrane filter. Genomic southern hybridization was performed using 5'-labeled synthetic oligonucleotide probes for IDH-I and -II prepared as described in "MATERIALS AND METHODS" (Fig. 16).

Isolation of Recombinant DNA

Approximate 20,000 recombinants of a *Vibrio* ABE-1 genomic library were screened using the synthetic oligonucleotide probes. Of the numerous positive signals, two plaques for *icd-I* and one for *icd-II* were selected for further characterization. From analyses of restriction enzyme maps of these recombinant DNAs (Fig. 17), it was identified that these clones were overlapped and *icd-I* gene was tandemly located at downstream of *icd-II*. Each *Xba*I-*Xho*I fragment for *icd-II* or *Xho*I-*Xba*I one for *icd-I* was subcloned into pBluescript KS(+) or SK(+) (Stratagene), and sequenced for both strands.

Nucleotide Sequence and Deduced Amino Acid Sequence

The complete nucleotide sequences of *icd-I* and *icd-II*, and the flanking region are shown in Fig. 18. *icd-I* and *icd-II* genes were separated by 276 bases of spa-

cer. Icd-II spans 2226 base pairs long for coding region and has a putative ribosome binding site [85] at 29 bases upstream of a predicted initiation site for translation, while icd-I possesses 1245 bases for coding and a Shine-Dalgarno sequence at 9 bases upstream of a predicted initiation codon. The NH_2-terminal amino acid sequences determined from proteins (expressed as the underlined peptides in Fig. 18) were identical with the respective regions deduced from nucleotide sequences except for first methionine of both genes and for 26th glycine to aspartic acid of icd-I.

It is, at present, unknown whether the two genes constitute a polycistronic operon or are transcribed independently. However, putative stem-loop structure followed by a run of uridine which is in consensus as rho-independent termination signal in prokaryotes [86] was observed in the downstream region of the each gene (Fig. 19).

The molecular weights caluculated from 2nd to 742nd of the open reading frame for icd-II and from 2nd to 415th of that for icd-I were 80,493 and 45,013, respectively. These were comparable to the values estimated from proteins (80,500 by gel filtration or 79,500 by denaturing polyacrylamide gel electrophoresis for IDH-II, and 49,100 by denaturing polyacrylamide gel electrophoresis for IDH-I). As shown in Table VIII, the amino acid compositions derived from the sequences were consistent with those obtained by chemical analysis of the

proteins.

The homology score [87] between IDH-I and -II was less than 29, and the harplot analysis [88] of IDH-II failed to detect any repeated sequence. These results indicate that the two IDH isozymes were originated from a different ancestral genes, or evolved from a common ancestor but diverged in extremely early stage from each other. In addition, it can be concluded that no gene duplication had occurred in the formation of icd-II. The nucleotide sequence for the open reading frame of icd-I exhibited 67.0 % homology with that of E. coli icd gene, and 73.8 % of amino acids predicted from the sequence was identical with those of E. coli IDH protein, and 22.1 % conservative (Fig. 20) [76]. E. coli IDH has been shown to be phosphorylated at serine 113 by IDH kinase [76]. A serine residue was found at the same position in the predicted sequence of IDH-I. Amino acid sequences following this serine residue were also homologous between IDH-I and E. coli IDH, while the preceding two residues to the serine were different, -Ile-Arg- for E. coli IDH, and -Met-Ser- for IDH-I. It is of interest to examine whether or not IDH-I is phosphorylated by E. coli IDH kinase. Most recently, Hurley et al. [93] reported the three-dimentional structure of the enzyme-substrate complex of E. coli IDH and identified the amino acid residues of that which form hydrogen bond with isocitrate and Mg^{2+}. Surprisingly, all those amino acids were located at the same positions

in IDH-I. Rossman structure, which has been proposed as the typical nucleotide binding domain of pyridine-nucleotide specific dehydrogenases [89], was not detected in both sequences of IDH-I and -II, as reported with E. coli IDH [76]. This result suggests that the binding of cofactor to the bacterial IDH may be accomplished by alternative mechanism from that of other dehydrogenases [89]. Homology search with IDH-I using the FASTP algorithm with ktup = 1 [90] in SWISS-PLOT amino acid database resulted in the identification of homologous regions between IDH-I and 3-isopropylmalate dehydrogenases from Thermus aquaticus [91] and Bacillus coagulans [92], as reported in E. coli IDH [76]. These regions were shown in Fig. 21. On the other hand, any significant sequence homology with IDH-II was not detected in the same database. However, careful scanning revealed that homologous region between the dimeric IDHs and the 3-isopropylmalate dehydrogenases was also present in the sequence of IDH-II (Fig. 21).

Nucleic Acid Base Composition and Codon Usage

The G + C contents of open reading frames of icd-I and icd-II genes were 38.4 % and 39.7 %, respectively, which were a little higher than total G + C content (33 %) of this bacterium calculated previously [44].

As shown in Table IX and X, codon utilization for IDH-I and -II exhibited a preference for the third letter of codons with A or T, either A or T ratio of the

third letter of the codons for IDH-I and -II reached to 74.7 % and 74.8 %, respectively. Interestingly, high G + C content was reported for codon utilization in thermophilic bacteria [91].

The molecular genetical research of Vibrio ABE-1 has just started, and more detailed investigation must be driven. Recently, monomeric IDH was purified from Vibrio parahaemolyticus [Imagawa, S. in the thesis for degree of Master]. This IDH is very similar with Vibrio ABE-1 IDH-II in many respects, except for thermostability. Comparative study of these IDHs in molecular level may provide a clue for clarification of potential mechanism controlling thermolability of proteins.

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TABLE I. Purification of ICDH isozymes from V. ABE-1.[a]

Step		Total protein (mg)		Total activity (units)		Specific activity (units/mg protein)		Yield (%)	
		I	II	I	II	I	II	I	II
1.	Crude extract	6145		868.6	2045.7	0.14	0.33	100	100
2.	Protamine sulfate	4291		905.2	1972.1	0.21	0.46	104.2	96.4
3.	Ammonium sulfate	2051		553.3	1472.9	0.26	0.72	63.7	72.0
4.	Butyl Toyopearl 650 M	366.6	420.8	522.4	1392.1	1.43	3.31	60.1	68.0
5-I.	DEAE Toyopearl 650 M	18.40		455.9		24.78		52.5	
6-I.	Procion Red Sepharose CL-6B	5.59		313.2		56.07		36.1	
7-I.	HPLC TSK gel DEAE-5PW	2.66		177.6		66.77		20.4	
5-II.	Hydroxylapatite		56.05		1350.3		24.09		66.0
6-II.	DEAE Sephadex A-50		26.17		1326.1		50.68		64.8
7-II.	DEAE Toyopearl 650 M		17.53		1009.7		57.59		49.4

[a]About 60 g (wet weight) of the cells was used as a starting material.

TABLE II. Amino acid compositions of *Vibrio* ABE-1 IDH isozymes [a]

Amino acid	IDH-I		IDH-II	
	Integer	Mol %	Integer	Mol %
Asx	43	9.37	76	9.99
Thr	22	4.79	43	5.65
Ser	34	7.41	72	9.46
Glx	55	11.98	83	10.91
Pro	21	4.58	31	4.07
Gly	52	11.33	76	9.99
Ala	45	9.80	89	11.70
Cys	4	0.87	5	0.66
Val	32	6.97	46	6.04
Met	11	2.40	13	1.71
Ile	27	5.88	37	4.86
Leu	34	7.41	61	8.02
Tyr	13	2.83	19	2.50
Phe	13	2.83	23	3.02
Lys	27	5.88	45	5.91
His	7	1.53	15	1.97
Arg	15	3.27	23	3.02
Trp	4	0.87	4	0.53
Total	459		761	

[a] The results are based on molecular weights of 49,000 and 80,000 for IDH-I and -II, respectively.

TABLE III. $S\Delta Q$ values during bacterial IDHs

Bacterial strain	Type of IDH	IDH-I	*E. coli*	*B. stearothermophilus*	*R. spheroides*	*A. vinelandii*
Vibrio ABE-1; IDH-I	Dimer	-				
Escherichia coli	Dimer	24.0	-			
Bacillus stearothermophilus	Dimer	39.7	12.2	-		
Rhodopseudomonas spheroides	Dimer	22.4	25.4	34.4	-	
Azotobacter vinelandii	Monomer	45.9	39.2	46.7	24.2	-
Vibrio ABE-1; IDH-II	Monomer	15.5	55.6	71.5	30.4	28.7

TABLE IV. Immunotitration between bacterial IDHs and the antisera raised against *Vibrio* ABE-1 IDH isozymes.

Bacterial Strain	Type of IDH	Inhibition (%)	
		Antiserum against	
		IDH-I	IDH-II
Vibrio ABE-1 IDH-I	Dimer	100	0.8
Escherichia coli	Dimer	50.6	0.2
Salmonella typhimurium	Dimer	53.4	0.8
Vibrio ABE-1 IDH-II	Monomer	0	97.2
Azotobacter vinelandii	Monomer	2.0	23.4
Vibrio parahaemolyticus	Monomer	0	38.5
Bacillus subtilis	——	65.4	4.6
Salmonella parathyphi B	——	75.1	1.3
Pseudomonas aerginosa	——	75.2	4.3
Serratia marcescens	——	40.8	6.1
Micrococcus lysodeikticus	——	0	4.1

TABLE V. Inhibition of Vibrio ABE-1 IDH isozymes by intermediates in glycolysis, tricarboxylic acid and glyoxylate cycles, and adenine nucleotides. All assays were performed under standard condition at 40°C for IDH-I or 20°C for IDH-II.

Additive (1 mM [+1 mM])	Inhibition (%)	
	IDH-I	IDH-II
None	0	0
Phosphoenolpyruvate	0	3.3
Pyruvate	0	0
Citrate	30.2	0
α-Ketoglutarate	12.7	5.9
Succinate	0	0
Fumarate	0	0
Malate	0	0
Oxaloacetate	6.2	21.5
Glyoxylate	0	7.3
Oxaloacetate + Glyoxylate	71.8	100.0
ATP	37.6	18.1
ADP	11.5	8.9
5'-AMP	0	3.0

TABLE VI. Kinetic parameters for Vibrio ABE-1 IDH isozymes as a function of temperature

T(°C)	K_m^{Isoc} (uM)		$K_m^{NADP^+}$ (uM)		V_{max}(umol/min/mg)		V_{max}/K_m^{Isoc}		$V_{max}/K_m^{NADP^+}$	
	IDH-I	IDH-II	IDH-I	IDH-II	IDH-I	IDH-II	IDH-I	IDH-II	IDH-I	IDH-II
40	81.68		98.23		99.46		1.218		1.013	
35	62.07		89.95		82.94		1.336		0.922	
30	55.94		62.09		62.25		1.113		1.003	
25	47.68		55.43		47.72		1.001		0.861	
20	40.46	21.25	36.38	15.95	35.96	63.42	0.889	2.984	0.998	3.976
15	32.38	13.62	41.37	12.69	24.87	54.25	0.768	3.983	0.601	4.272
10	30.03	9.02	58.48	8.80	17.17	44.98	0.572	4.987	0.294	5.111
5	27.39	5.00	87.91	6.29	12.88	36.47	0.470	7.294	0.147	5.789

TABLE VII. Apparent thermodynamic activation parameters for *Vibrio* ABE-1 IDH isozymes

Parameter	IDH-I		IDH-II
	5-20°C	20-40°C	5-20°C
Q_{10}	1.97	1.66	1.45
Ea (Kcal/mol)	11.0	9.2	6.0
$\Delta H^{\neq}$ (Kcal/mol)	9.8	8.6	5.4
$\Delta S^{\neq}$ (cal/deg·mol)	-18.0	-21.3	-31.7
$\Delta G^{\neq}$ (Kcal/mol)	15.1[a]	15.3[b]	14.7[a]

[a]Value at 20°C, [b]Value at 40°C

TABLE VIII. Amino acid compositions of Vibrio ABE-1 IDH isozymes.

Amino acids	Isozyme	
	IDH-I	IDH-II
Asx	43 (43)[a]	79 (76)
Asp	24	47
Asn	19	32
Thr	22 (22)	48 (43)
Ser	22 (34)	47 (72)
Glx	38 (55)	79 (83)
Glu	27	51
Gln	11	28
Pro	20 (21)	31 (31)
Gly	39 (52)	46 (76)
Ala	35 (45)	90 (89)
Cys	5 (4)	4 (5)
Val	31 (32)	52 (46)
Met	14 (11)	20 (13)
Ile	36 (27)	43 (37)
Leu	33 (34)	65 (61)
Tyr	14 (13)	19 (19)
Phe	11 (13)	24 (23)
Lys	29 (27)	48 (45)
His	11 (15)	27 (23)
Arg	11 (15)	27 (23)
Trp	6 (4)	4 (5)
Total	414	741
Cal. mass	45,013	80,493

a) Data from amino acid analysis.

TABLE IX. Codon usage in *Vibrio* ABE-1 IDH-I.

First	Second				Third
	U	C	A	G	
U	Phe 11	Ser 4	Tyr 13	Cys 4	U
	Phe 0	Ser 0	Tyr 1	Cys 1	C
	Leu 14	Ser 8	Term 0	Term 0	A
	Leu 4	Ser 3	Term 1	Trp 6	G
C	Leu 10	Pro 10	His 4	Arg 2	U
	Leu 1	Pro 1	His 1	Arg 2	C
	Leu 1	Pro 6	Gln 11	Arg 3	A
	Leu 3	Pro 3	Gln 0	Arg 0	G
A	Ile 21	Thr 7	Asn 14	Ser 6	U
	Ile 7	Thr 8	Asn 5	Ser 1	C
	Ile 8	Thr 4	Lys 24	Arg 4	A
	Met 15	Thr 3	Lys 5	Arg 0	G
G	Val 16	Ala 9	Asp 19	Gly 24	U
	Val 0	Ala 4	Asp 5	Gly 10	C
	Val 11	Ala 16	Glu 24	Gly 3	A
	Val 4	Ala 6	Glu 3	Gly 2	G

Total number of codons = 415
Percentage of A or T for third letter = 74.7%

TABLE X . Codon usage in *Vibrio* ABE-1 IDH-II.

First	Second				Third
	U	C	A	G	
U	Phe 18	Ser 17	Tyr 12	Cys 4	U
	Phe 6	Ser 0	Tyr 7	Cys 0	C
	Leu 40	Ser 17	Term 1	Term 0	A
	Leu 5	Ser 2	Term 0	Trp 5	G
C	Leu 13	Pro 11	His 12	Arg 13	U
	Leu 0	Pro 3	His 2	Arg 3	C
	Leu 5	Pro 15	Gln 24	Arg 1	A
	Leu 2	Pro 2	Gln 4	Arg 0	G
A	Ile 31	Thr 20	Asn 19	Ser 7	U
	Ile 9	Thr 7	Asn 13	Ser 4	C
	Ile 3	Thr 13	Lys 36	Arg 5	A
	Met 21	Thr 8	Lys 12	Arg 0	G
G	Val 27	Ala 35	Asp 41	Gly 32	U
	Val 5	Ala 11	Asp 6	Gly 10	C
	Val 15	Ala 27	Glu 36	Gly 2	A
	Val 5	Ala 17	Glu 15	Gly 2	G

Total number of codons = 742
Percentage of A or T for third letter = 74.8%

LEGENDS TO FIGURES

Fig. 1. Effects of buffer and pH on the stabilities of Vibrio ABE-1 IDH isozymes.

Cell-free extract of Vibrio ABE-1 was diluted with 100 mM of three different buffer systems of various pHs for IDH-I (A), or with the same buffers containing 100 mM NaCl and 10 mM 2-mercaptoethanol except for potassium phosphate for IDH-II (B), and incubated at 4°C. Protein concentrations of IDH-I and -II were 20 µg/ml and 46.7 µg/ml, respectively. After incubation for 24 h, the remaining activities of the IDH isozymes were measured under standard condition. (○-○), potassium phosphate; (△-△), HEPES-NaOH; (□-□), Tris-HCl.

Fig. 2. Elution profile of butyl-Toyopearl 650M column of Vibrio ABE-1 IDH isozymes.

Experimental details were as described in the text. (○-○), IDH-I activity; (●-●), IDH-II activity; (----), absorbance at 280 nm; (-·-·-), $(NH_4)_2SO_4$ concentration.

Fig. 3. Elution profile from chromatography in the purification of Vibrio ABE-1 IDH-I.

After the complete separation of Vibrio ABE-1 IDH isozymes, IDH-I was purified through DEAE-Toyopearl 650M column (A), Procion Red Sepharose CL-6B column (B), and HPLC on TSK gel DEAE-5PW column (C). Details were as

in the text. (●-●), activity; (----), absorbance at 280 nm; (-·-·-), salt concentrations indicated in (A), (B), or (C).

Fig. 4. Elution profile from chromatography in the purification of Vibrio ABE-1 IDH-II.

After the complete separation of Vibrio ABE-1 IDH isozymes, IDH-II was purified through hydroxylapatite column (A), DEAE-Sephadex A-50 column (B), and DEAE-Toyopearl 650M column (C). Details were as in the text. (●-●), activity; (----), absorbance at 280 nm; (-·-·-), salt concentrations indicated in (A), (B), or (C). In (C), the column was washed with 4 mM DL-isocitrate at arrow (1), and with 150 mM NaCl at arrow (2).

Fig. 5. Polyacrylamide gel electrophoresis of the IDH isozymes.

Electrophoresis was performed under non-denaturing (A) or denaturing (B) condition. (A), 10 μg of purified IDH isozymes was loaded on each lane. Lane 1-3; IDH-I, lane 4-6; IDH-II. Lane 1 and 4 were stained for protein, and lane 2, 3, 5, and 6 were stained for enzymatic acrivity. Lane 2 and 5 were incubated for 1 h, and lane 3 and 6 were for 3 h in the staining solution. (B), Lane 1 and 5 were 1 μg of marker protein as follows: phosphorylase b (97.4 kDa), glutamate dehydrogenase (55.4 kDa), lactate dehydrogenase (36.5 kDa),

trypsin inhibitor (20.1 kDa). Lane 2; IDH-I (20 μg), lane 3; IDH-I (20 μg) plus IDH-II (20 μg), lane 4; IDH-II (20 μg).

Fig. 6. Molecular weight determination of Vibrio ABE-1 IDH isozymes by gel filtration.

The purified IDH isozymes were applied to a column of Sephadex G-200 (superfine) (1 x 36.5 cm) at a flow rate of 0.7 ml/h. The molecular weights of the IDH isozymes were determined from the relationship between the elution volumes and the molecular weights of standard proteins. Standard proteins used were as follows: aldolase (158 kDa), albumin (68 kDa), ovalbumin (45 kDa), chymotrypsinogen A (25 kDa), cytochrome c (12.5 kDa).

Fig. 7. Isoelectric focusing of Vibrio ABE-1 IDH isozymes.

Experimental details were as described in "MATERIALS AND METHODS". Lane 1, IDH-I (30 μg); lane 2, IDH-II (30 μg); lane 3, marker proteins having the pI values indicated on the right.

Fig. 8. NH_2-terminal amino acid sequences of Vibrio ABE-1 IDH isozymes.

Purified IDH-I (20 nmol) and IDH-II (1 nmol) were subjected to automated Edman degradation with Beckman liquid-phase sequencer, type 890-C, and Applied Biosys-

tems 477A gas-phase protein sequencer, respectively. Identification of the PTH amino acids were carried out by HPLC with a TSK gel ODS-80Tm reverse-phase column (the former), or by an on-line PTH analyzer model 120A (Applied Biosystems) (the latter).

Fig. 9. Ouchterlony double immunodiffusion test of Vibrio ABE-1 IDH isozymes.

Wells I and II contained purified IDH-I and -II, respectively. Wells labeled AbI and AbII contained anti-IDH-I-IgG and anti-IDH-II-IgG, respectively.

Fig. 10. Immunotitration of Vibrio ABE-1 IDH isozymes with an antiserum raised against IDH-I (A) or IDH-II (B).

A 100 µl aliquot of each purified isozyme (IDH-I, 1.0 unit/ml or IDH-II, 0.45 units/ml) in potassium phosphate buffer (Buffer A described in the text) containing 1 mM EDTA was mixed with an equal volume of antiserum diluted as indicated in the figure with Buffer A containing 25 % (v/v) preimmune rabbit serum. After incubation at 15°C for 6 h, the mixtures were centrifuged at 8,000 X g for 10 min, then the remaining enzyme activity in the supernatant was measured. (○ - ○), IDH-I; (● - ●), IDH-II.

Fig. 11. Thermostability of Vibrio ABE-1 IDH isozymes. IDH-I (52.2 µg/ml) and IDH-II (42.0 µg/ml) in potassium phosphate (pH 7.5) containing 250 mM NaCl, 2 mM $MgCl_2$, and 1 mM dithiothreitol were incubated at (○-○), 42.5°C; (△-△), 45°C; (□-□), 47.5°C; (▽-▽), 50°C; (●-●), 20°C; (▲-▲), 25°C; (■-■), 27.5°C; (▼-▼), 30°C. The remaining activities were assayed immediately after withdrawal (each 20 ul) at desired time. (A), IDH-I; (B), IDH-II.

Fig. 12. Effect of monovalent cation on activity of Vibrio ABE-1 IDH isozymes.

A 20 ul of each isozyme in potassium phosphate (pH 7.5) containing 250 mM NaCl, 2 mM $MgCl_2$, and 1 mM dithiothreitol was added to assay mixture (total 2 ml) containing indicated monovalent cations instead of 250 mM NaCl. Accordingly, 2.5 mM NaCl in a final concentration was carried over in the assay mixture. (A), IDH-I; (B), IDH-II. (○-○), LiCl; (●-●), NaCl; (△-△), KCl; (▲-▲), NH_4Cl.

Fig. 13. Effect of temperature on Km value of Vibrio ABE-1 IDH isozymes for DL-isocitrate (A) and $NADP^+$ (B).

The pH value of reaction mixture was adjusted to 8.0 at various temperatures. Km value was determined by the matrix method as described in "MATERIALS AND METHODS". Each Km value was expressed as average of quadruplicate assays. (○-○), IDH-I; (●-●), IDH-II.

Fig. 14. Arrhenius plot of Vmax values at various temperature of Vibrio ABE-1 IDH isozymes.

Vmax value was determined by the matrix method and expressed as the average of quadruplicate assays. (O - O), IDH-I; (● - ●), IDH-II.

Fig. 15. Sequence of the synthetic oligodeoxyribonucleotide used for probing the cloned DNAs for Vibrio ABE-1 IDH isozymes.

R, Y, H, D, and I represent purine, pyrimidine, not G, not C and inosine, respectively.

Fig. 16. Genomic southern hybridization of Vibrio ABE-1 IDH isozyme genes.

Vibrio ABE-1 chromosomal DNA was digested with restriction endonucleases as indicated, then separated by 1 % agarose gel electrophoresis (3.24 μg DNA per lane). After blotting onto a nylon membrane, hybridization was performed using 5'-labeled synthetic DNA probe for icd-I (A) and that for icd-II (B). Molecular weight markers are shown on the center (in kilobases) of (A) and (B).

Fig. 17. Restriction enzyme map of genomic clones of Vibrio IDH isozyme genes.

Two clones were perfectly overlapped at indicated region. Arrows represent open leading frame for respective isozyme.

Fig. 18. Nucleotide sequence of Vibrio ABE-1 icd genes and their flanking regions.

NH_2-terminal amino acid sequences determined by chemical analysis were boxed. Underlines indicate the putative ribosome binding sites. Two possible stem-loop structures involved in the downstream region of the transcription termination are shown by two arrows pointing toward each other.

Fig. 19. Putative transcription terminaton sequences.

The $\underline{G}_0$ values are -20.2 Kcal/mol for the left and -15.6 Kcal/mol for the right.

Fig. 20. Homology between Vibrio ABE-1 IDH-I and Escherichia coli IDH.

Asterisk indicates the same amino acid, and dot a conservative residue. Upper strand; Vibrio ABE-1 IDH-I, lower strand; Escherichia coli IDH.

Fig. 21. Alignment of amino acid sequences of Vibrio ABE-1 IDH isozymes, Escherichia coli IDH, and Thermus aquaticus isopropylmalate dehydrogenase (IPDH).

Homologous residues observed in more than three enzymes are shadowed.

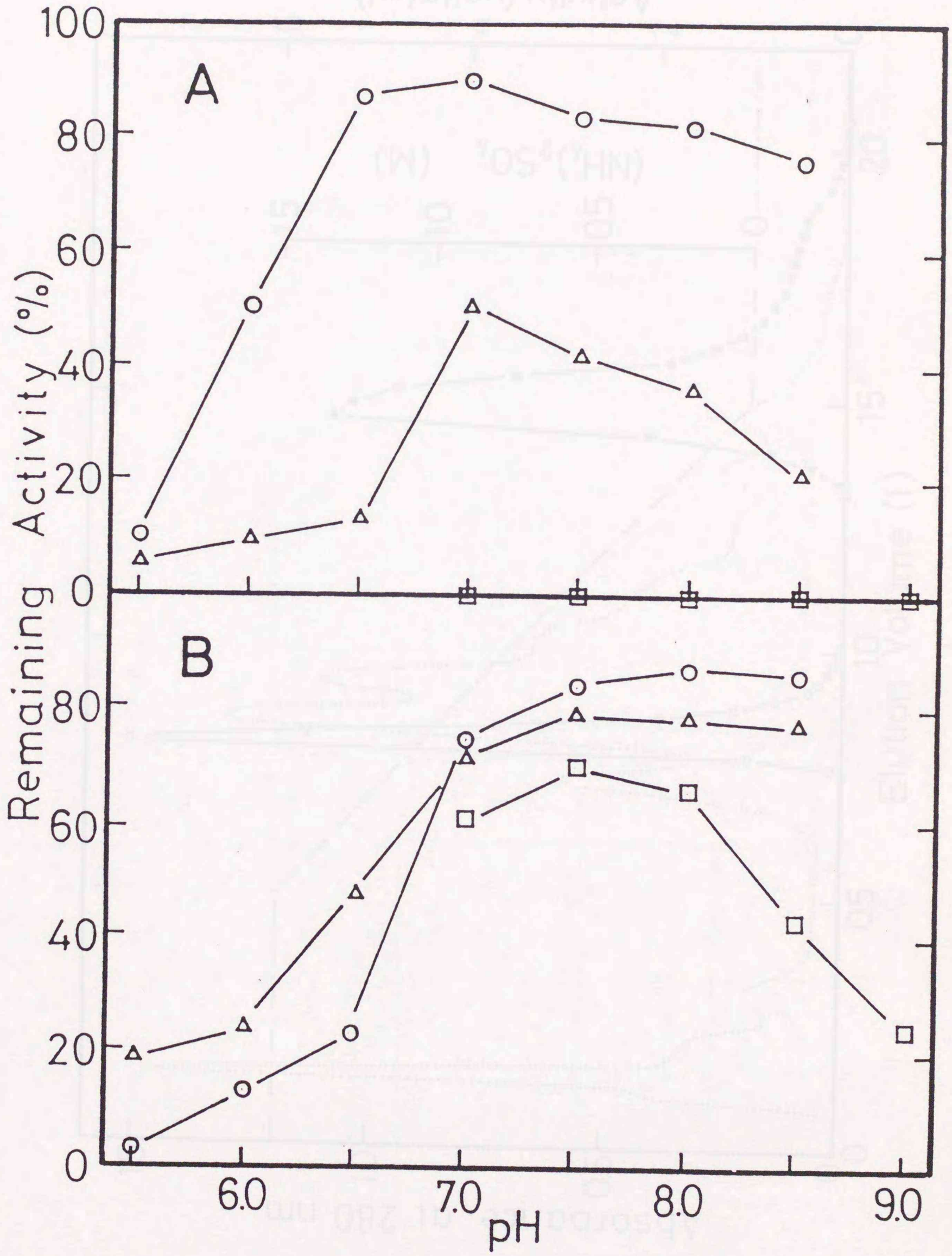
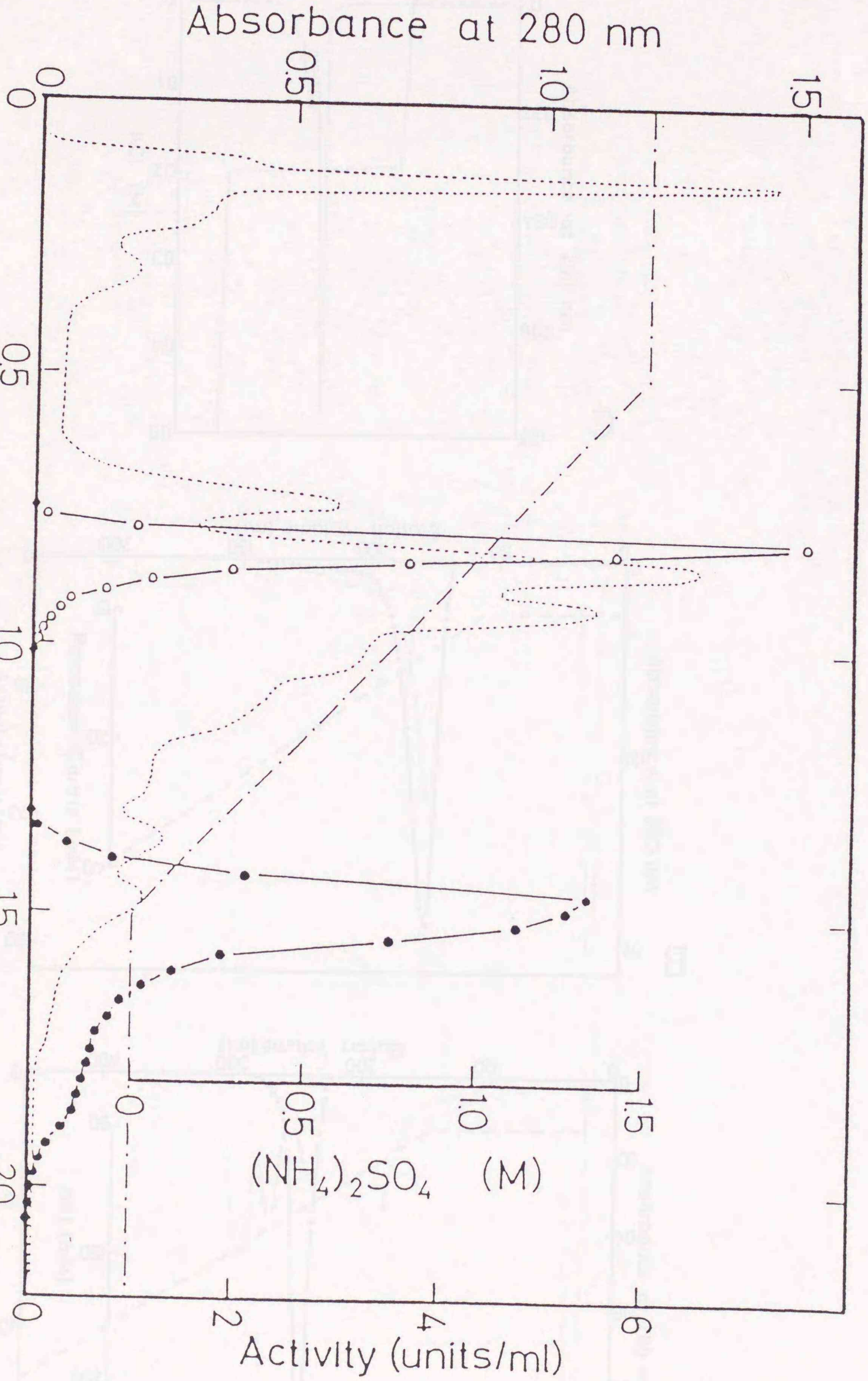


Figure 1

Figure 2


Absorbance at 280 nm
0
0.5
1.0
1.5
Elution Volume (l)
0
5
10
15
20
0
0.5
1.0
1.5
$(NH_4)_2SO_4$ (M)
0
2
4
6
Activity (units/ml)

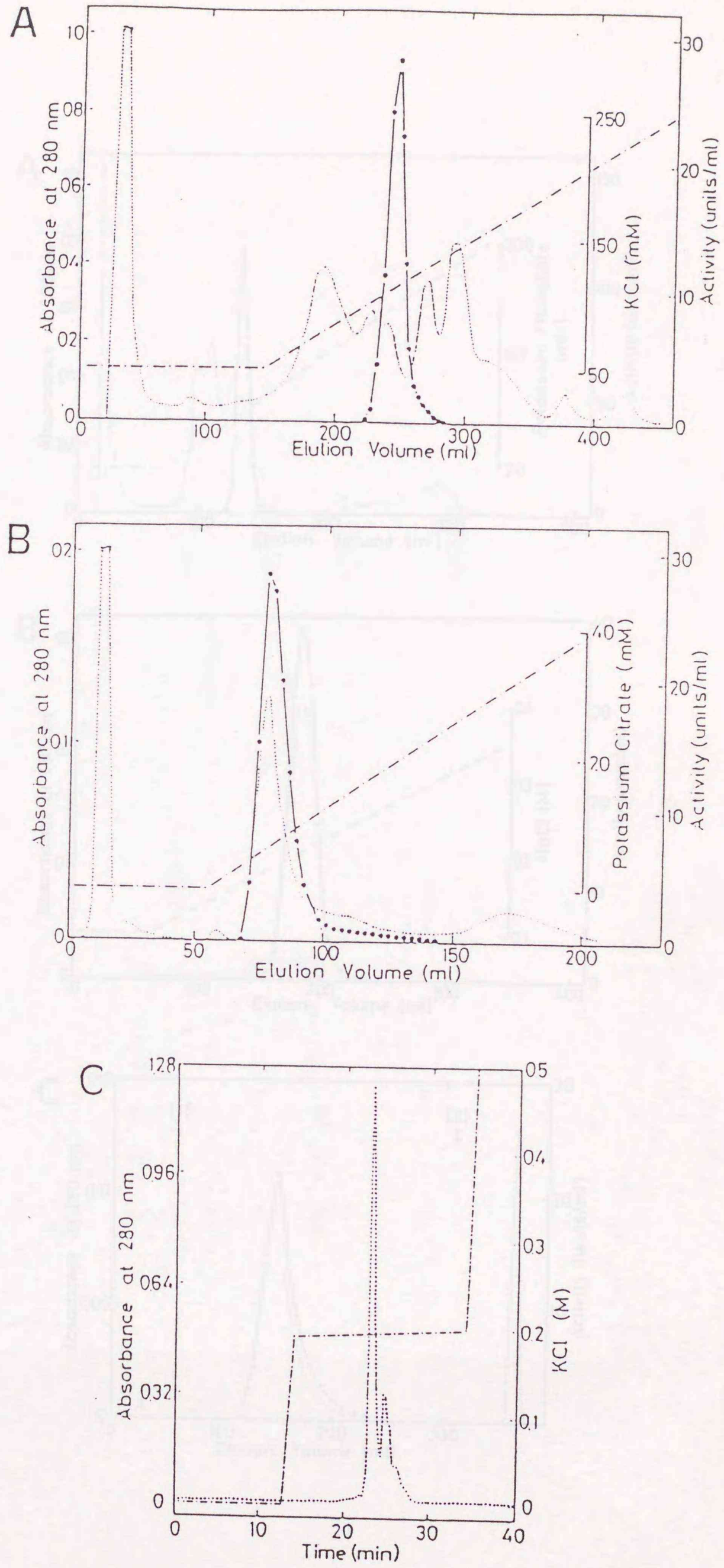

A
Absorbance at 280 nm
Activity (units/ml)
KCl (mM)
Elution Volume (ml)
B
Absorbance at 280 nm
Potassium Citrate (mM)
Activity (units/ml)
Elution Volume (ml)
C
Absorbance at 280 nm
KCl (M)
Time (min)

Figure 3

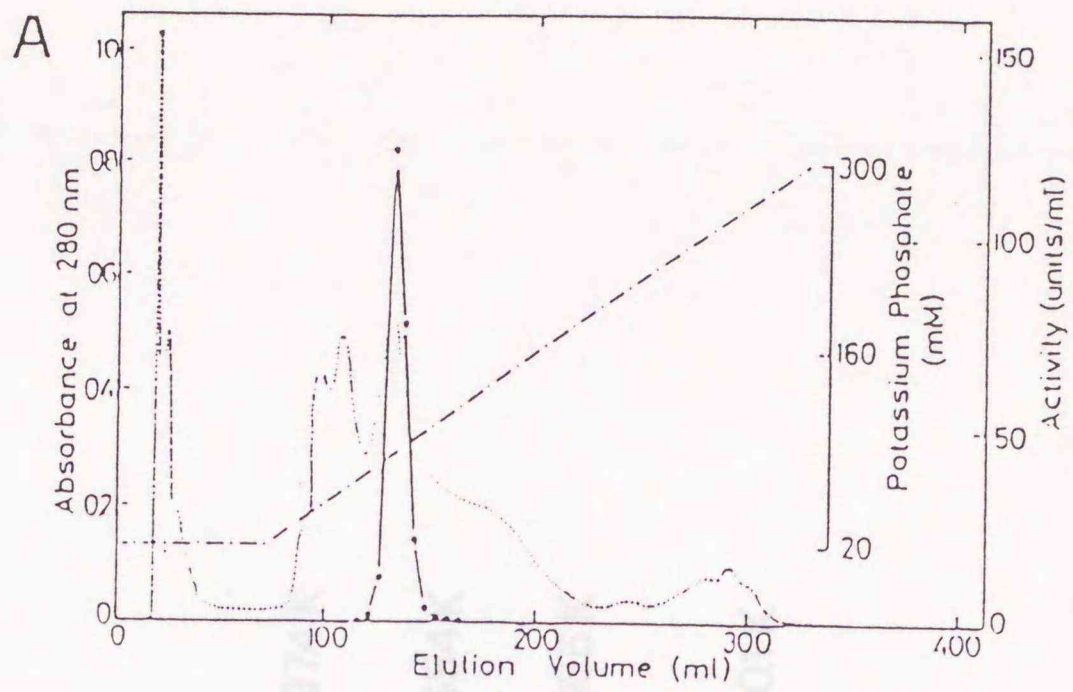

A
Absorbance at 280 nm
Potassium Phosphate (mM)
Activity (units/ml)
Elution Volume (ml)
B
Absorbance at 280 nm
NaCl (M)
Activity (units/ml)
Elution Volume (ml)
C
Absorbance at 280 nm
(1)
(2)
Activity (units/ml)
Elution Volume (ml)

Figure 4

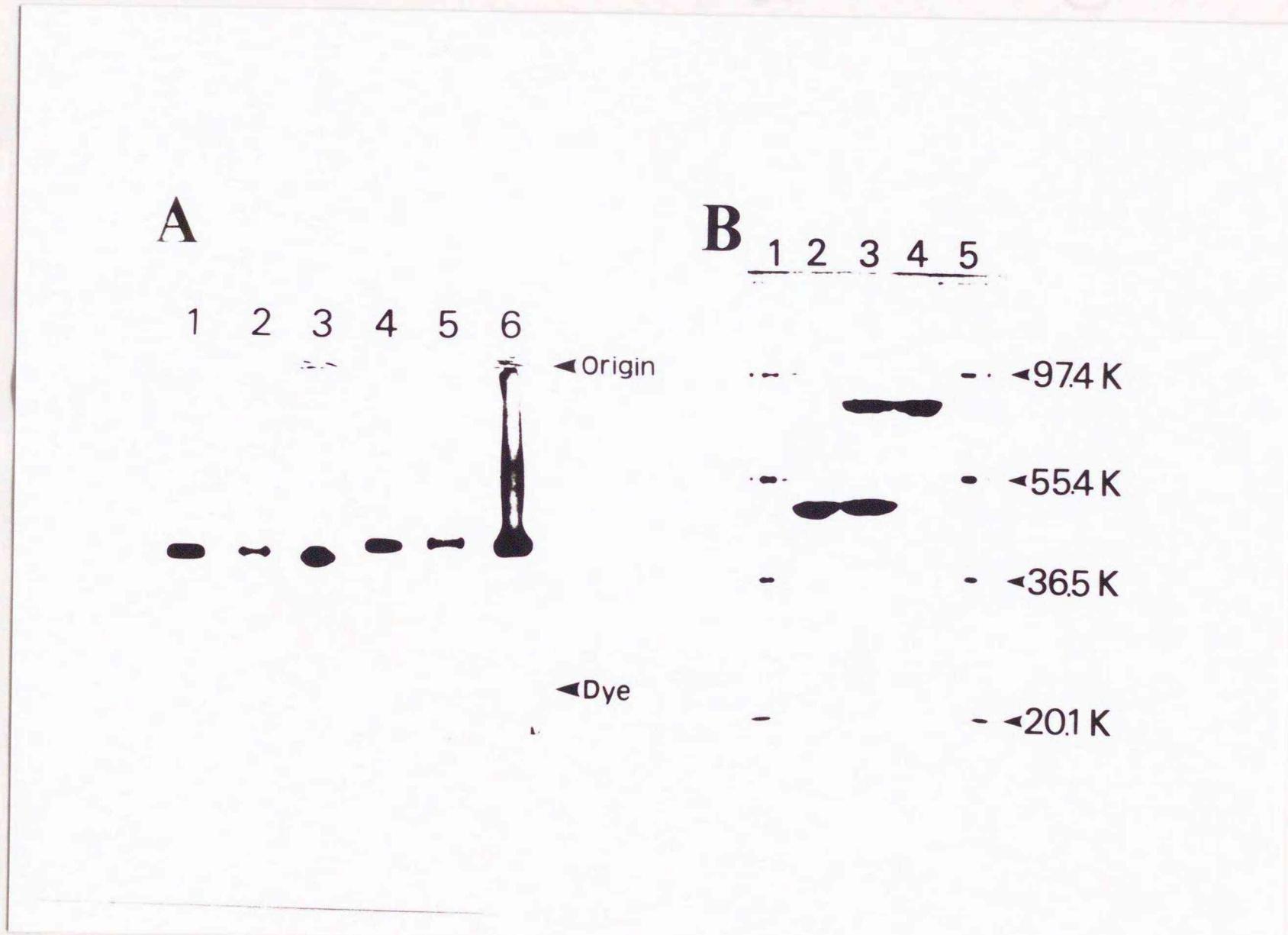


Figure 5

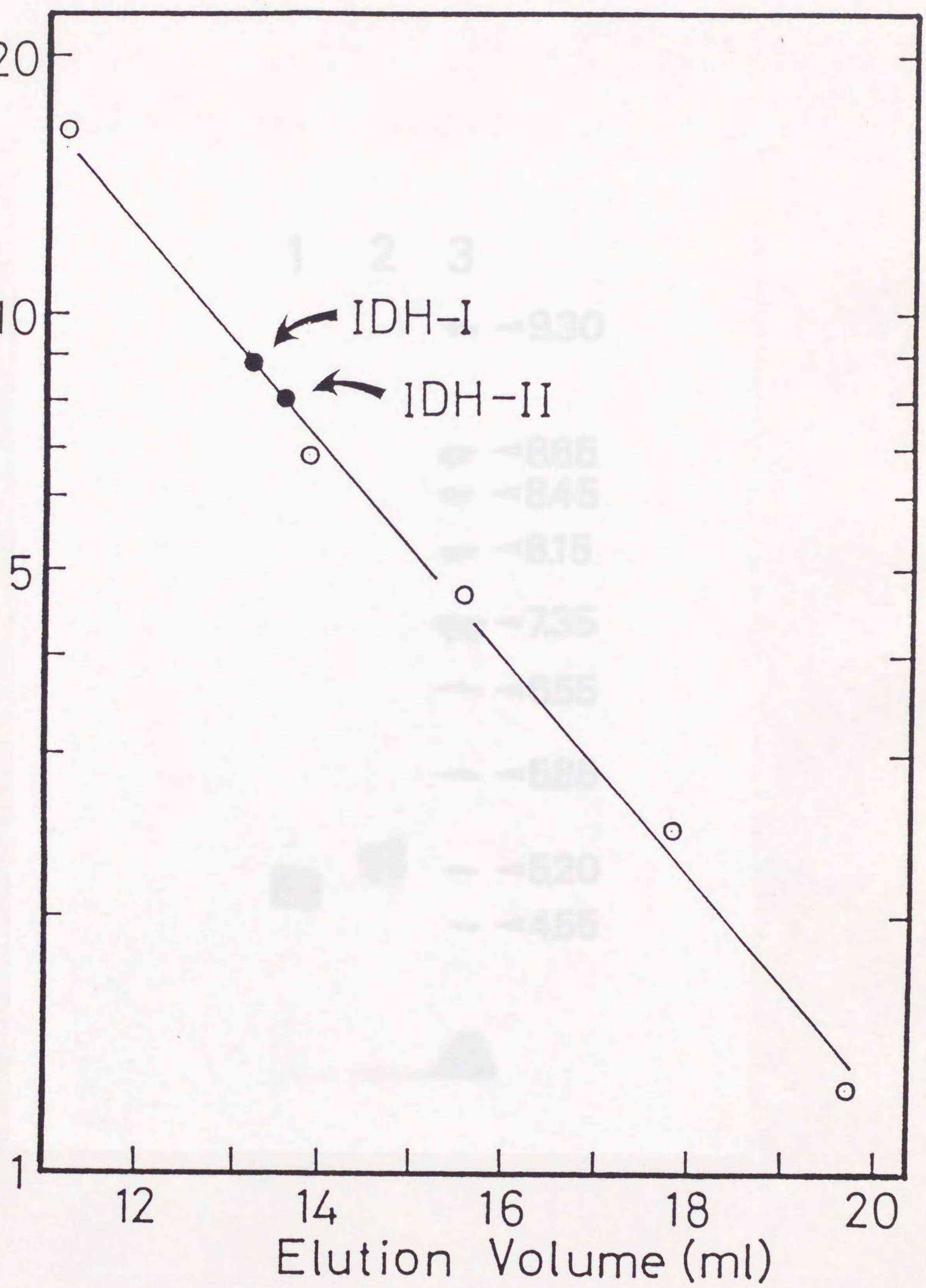

Molecular Weight (x10-4)
20
10
5
1
IDH-I
IDH-II
12
14
16
18
20
Elution Volume (ml)

Figure 6

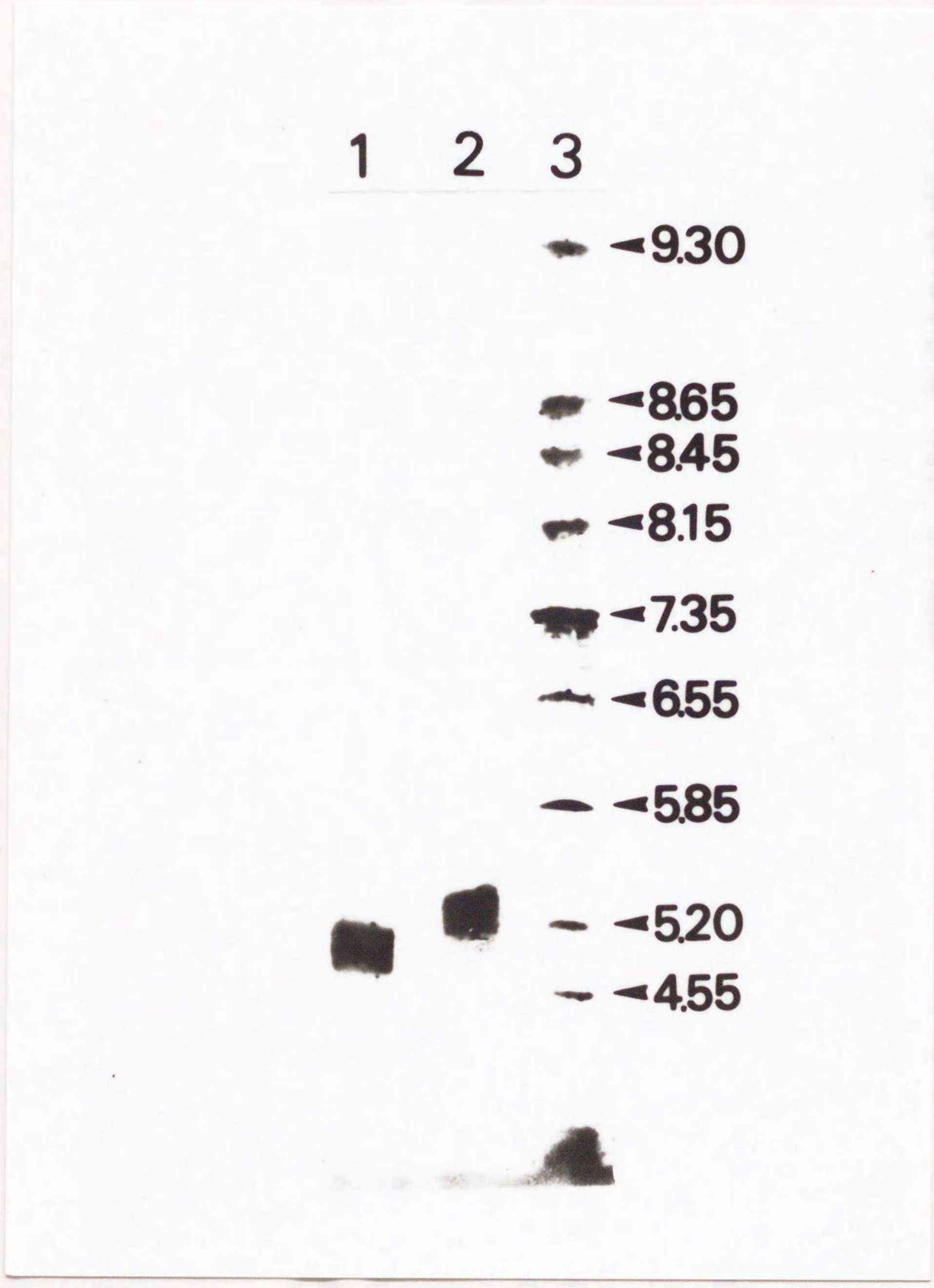


Figure 7

	1				5				10				15			
IDH-I	Thr	Asn	Lys	Ile	Ile	Ile	Pro	Thr	Thr	Gly	Asp	Lys	Ile	Thr	Phe	
IDH-II	Ser	Thr	Asp	Asn	Ser	Lys	Ile	Ile	Tyr	Thr	Ile	Thr	Asp	Glu	Ala	
					20				25				30			
IDH-I	Ile	Asp	Gly	Lys	Leu	Ser	Val	Pro	Asn	Gly	Pro	Ile	Ile	Pro	Tyr	
IDH-II	Pro	Ala	Leu	Ala	Thr	Tyr	Ser	Leu	Arg	Pro	Ile	Ile	Gln			
					35				40							
IDH-I	Ile	Glu	Gly	Asp	Gly	Ile	Gly	Val	Asp	Val						

Figure 8

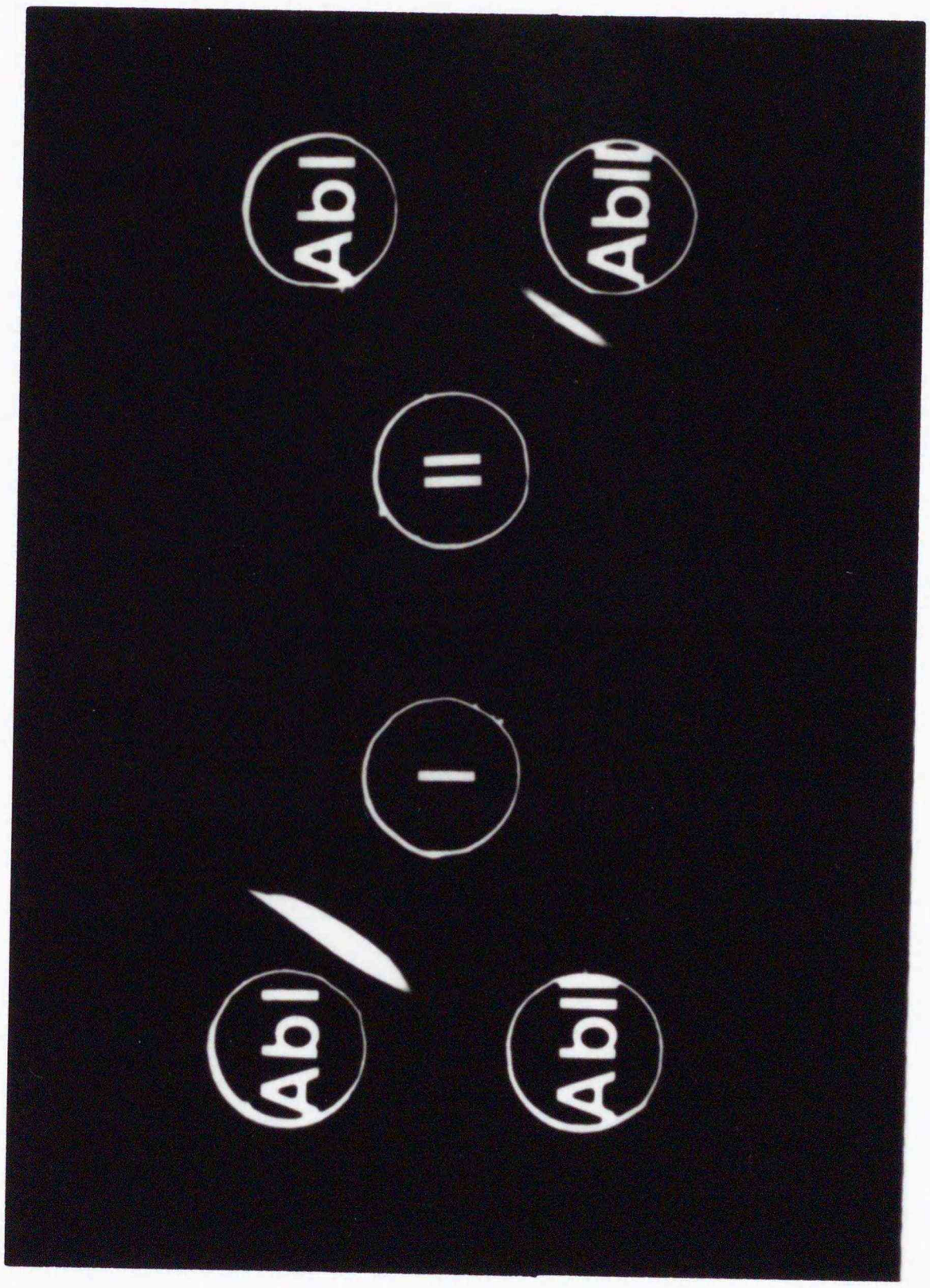


Figure 9

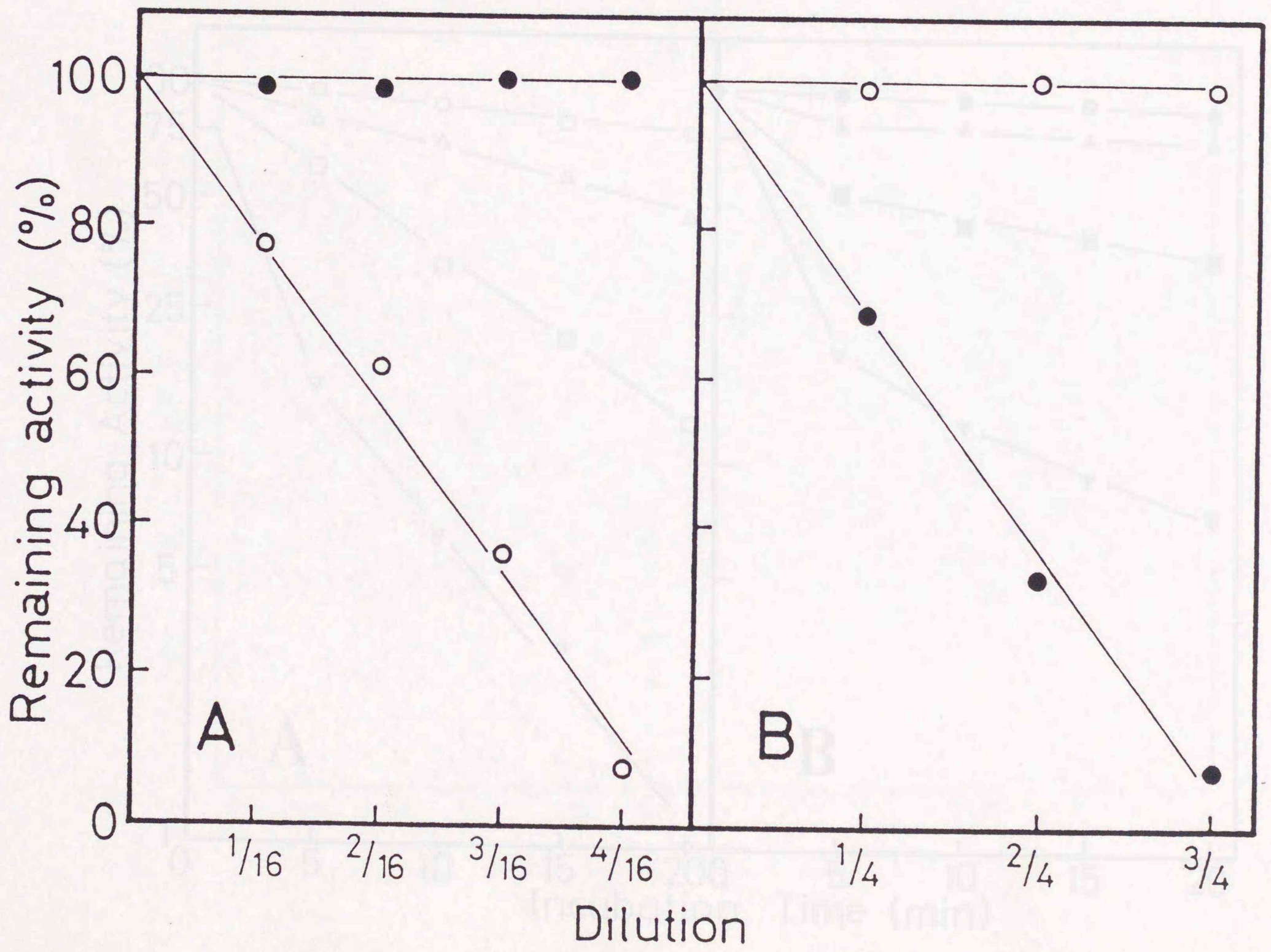

100
80
60
40
20
0
Remaining activity (%)
A
B
1/16
2/16
3/16
4/16
1/4
2/4
3/4
Dilution

Figure 10

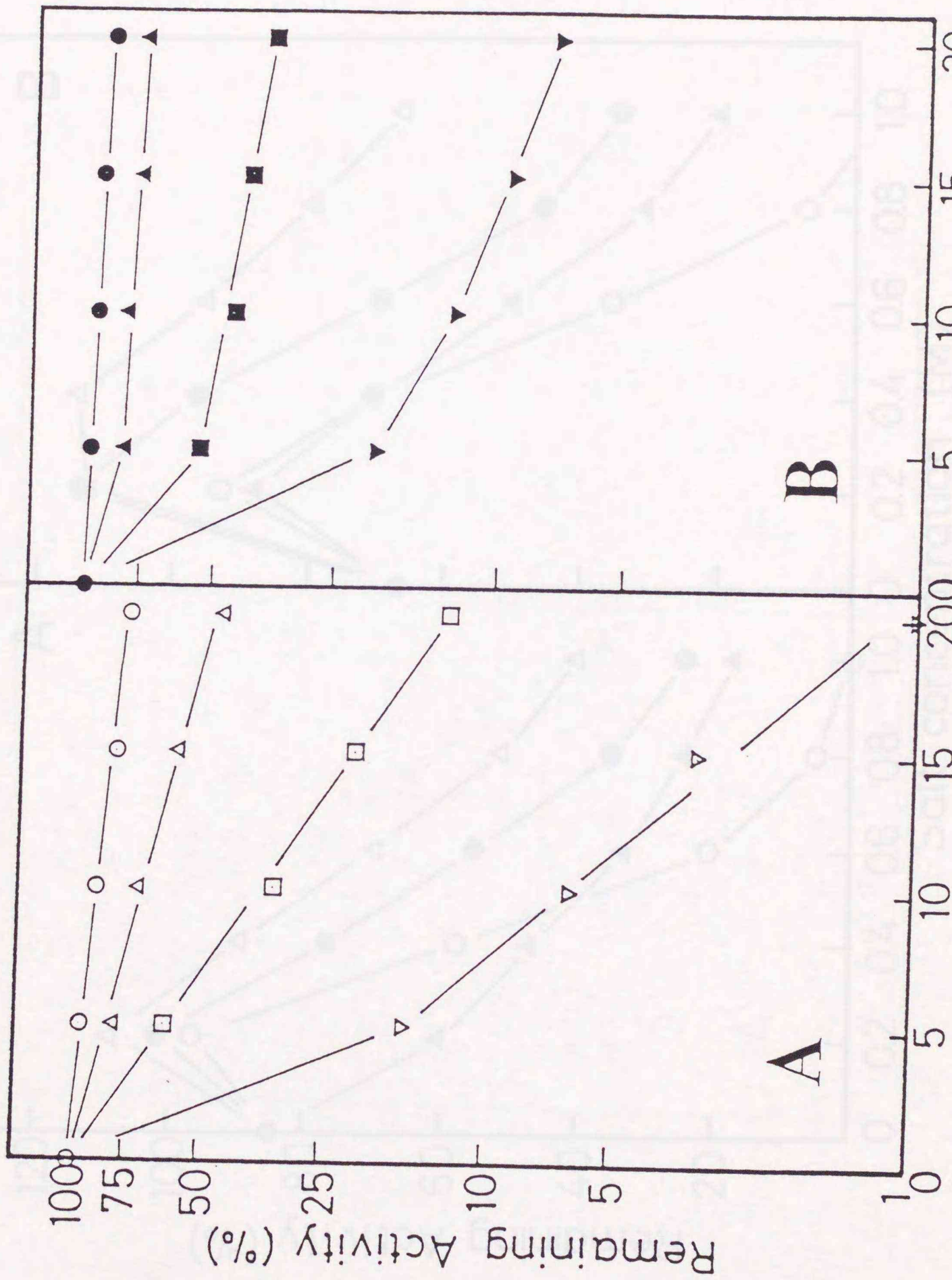

Remaining Activity (%)
100
75
50
25
10
5
1
A
B
5
10
15
20
Incubation Time (min)
5
10
15
200

Figure 11

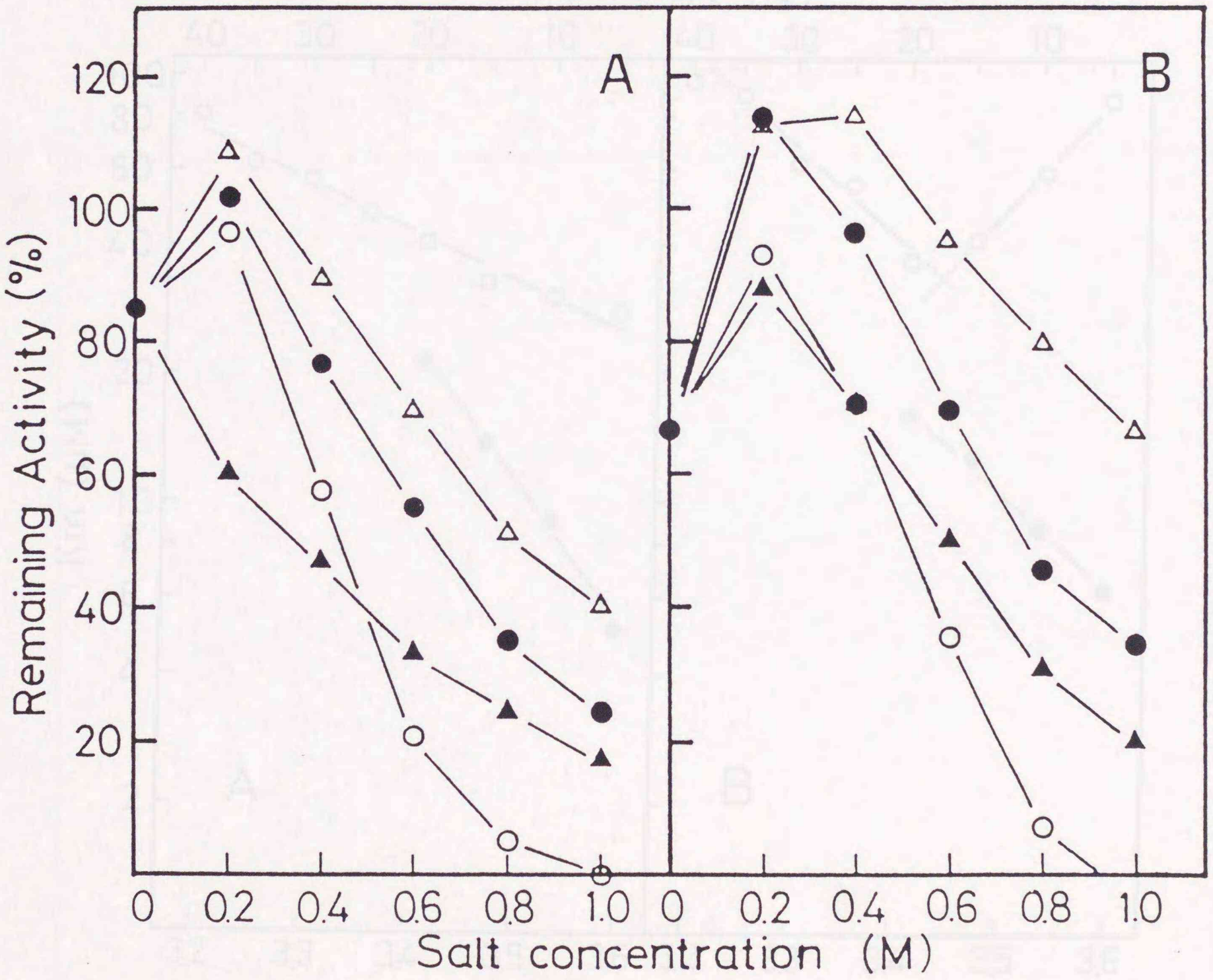

A
B
120
100
80
60
40
20
Remaining Activity (%)
0
0.2
0.4
0.6
0.8
1.0
Salt concentration (M)

Figure 12

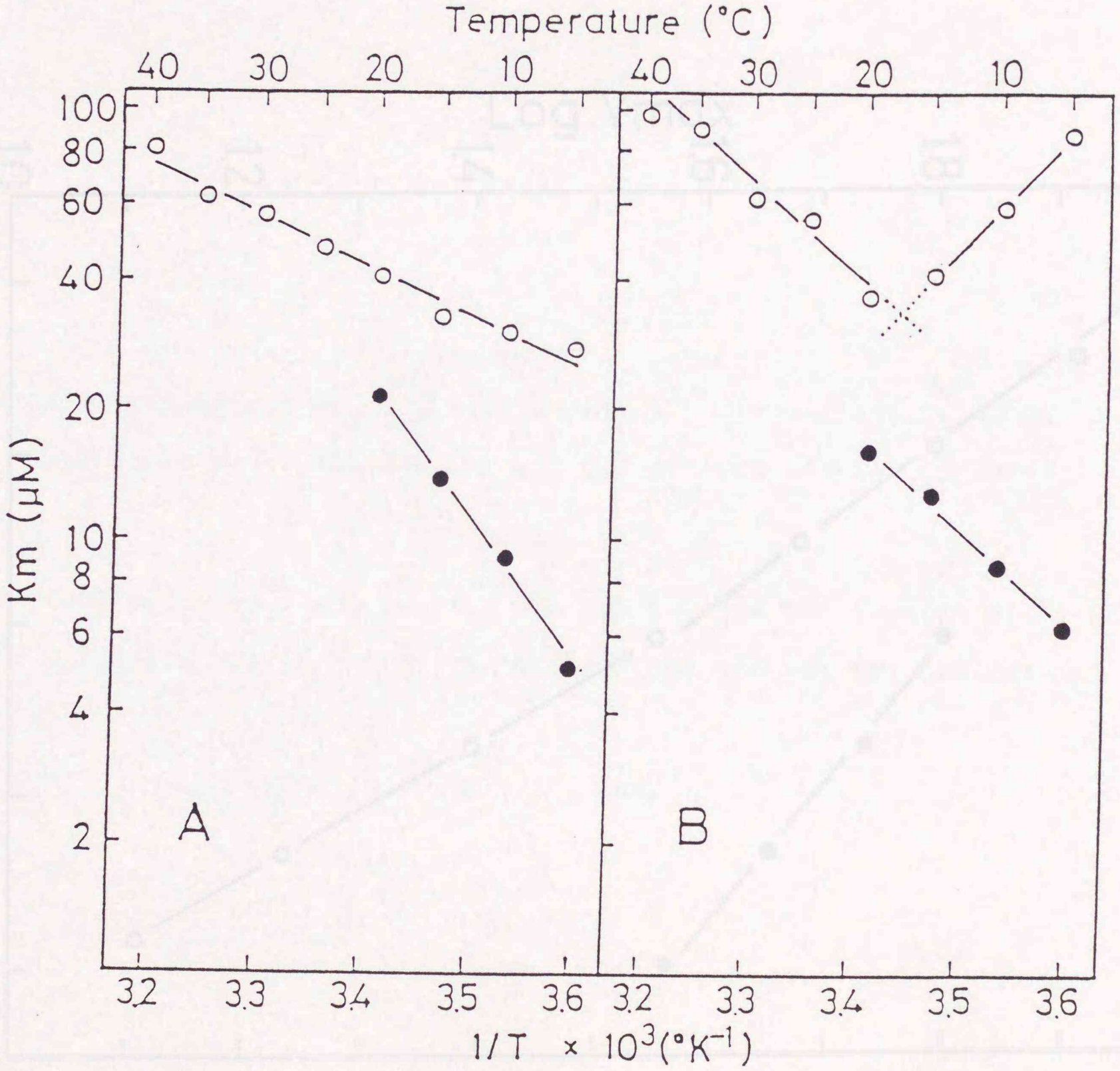

Temperature (°C)
40
30
20
10
40
30
20
10
100
80
60
40
20
10
8
6
4
2
Km (μM)
A
B
3.2
3.3
3.4
3.5
3.6
3.2
3.3
3.4
3.5
3.6
1/T × 10³(°K⁻¹)

Figure 13

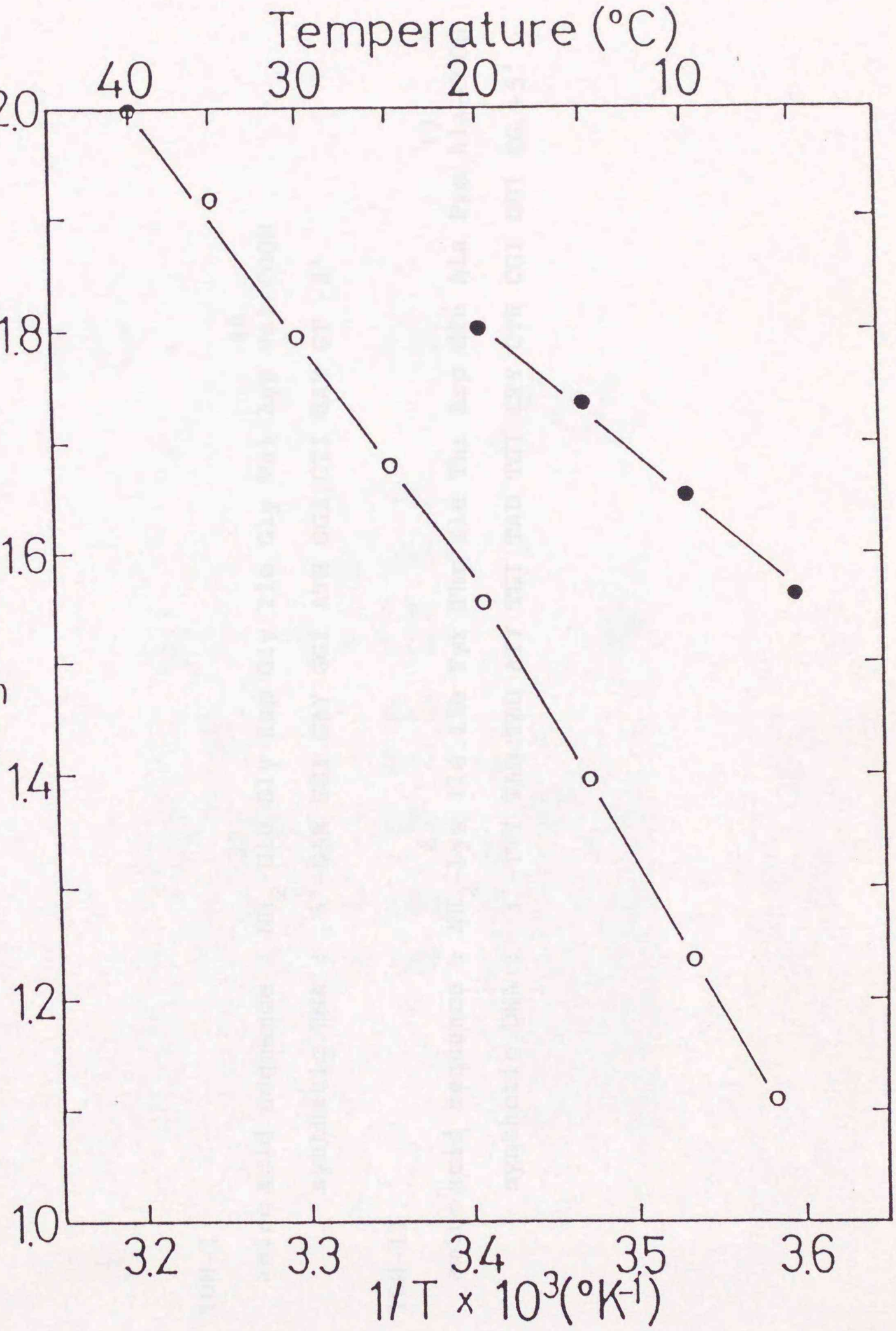

Temperature (°C)
40
30
20
10
2.0
1.8
1.6
1.4
1.2
1.0
Log Vmax
3.2
3.3
3.4
3.5
3.6
1/T x 10³(°K⁻¹)

Figure 14

IDH-I

amino acid sequence : NH_2-Glu (32) Gly Asp Gly Ile Gly Val Asp Val (40)-COOH

synthetic DNA : 5'-GAR GGI GAY GGI ATH GGI GTI GAY GT -3'

IDH-II

amino acid sequence : NH_2-Lys (6) Ile Ile Tyr Thr Ile Thr Asp Glu Ala Pro Ala (17)-COOH

synthetic DNA : 3'-TTY TAD TAD ATY TGI TAD TGI CTY CTR CGI GGI CG -5'

Figure 15

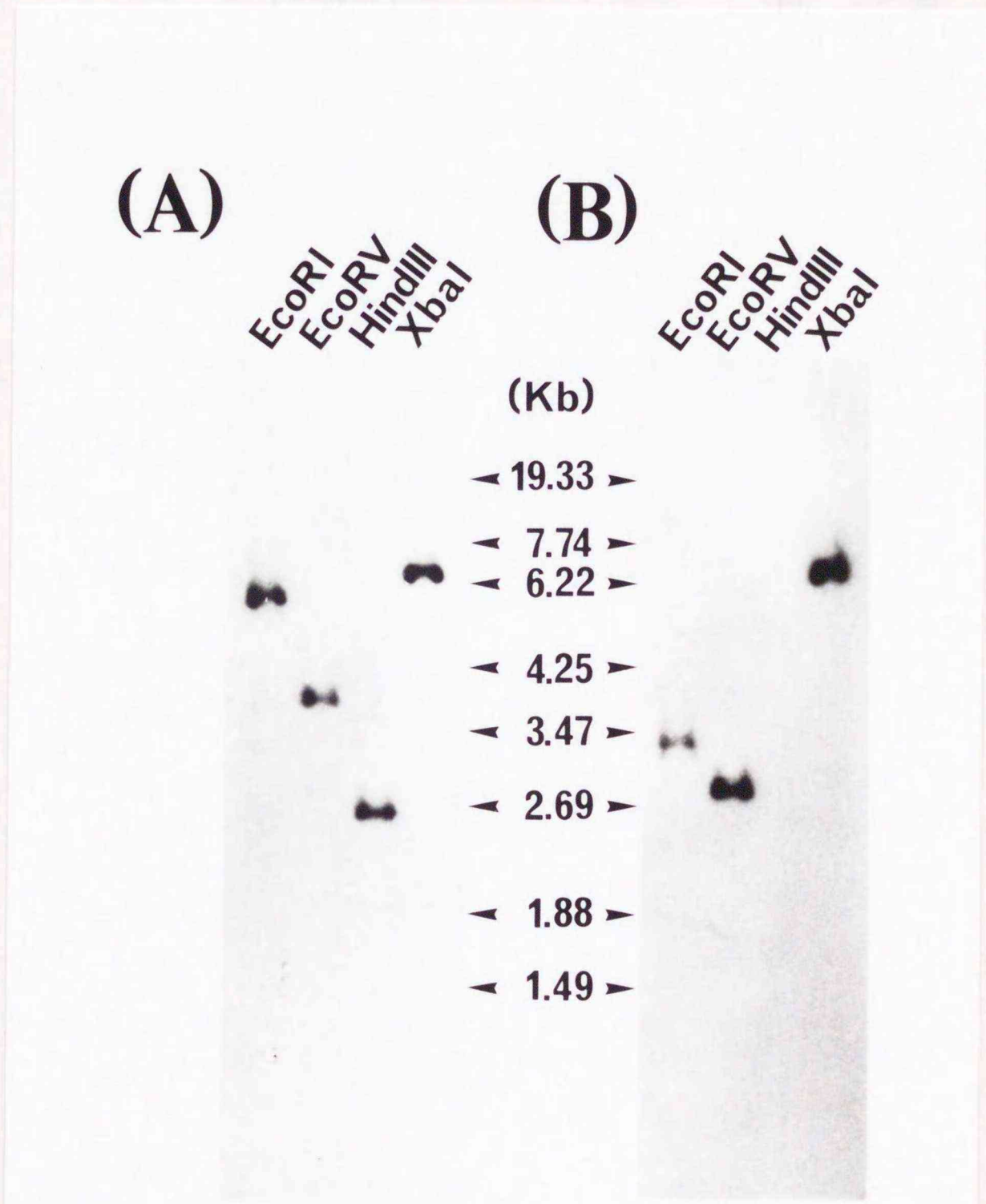

(A)
(B)
EcoRI
EcoRV
HindIII
XbaI
EcoRI
EcoRV
HindIII
XbaI
(Kb)
19.33
7.74
6.22
4.25
3.47
2.69
1.88
1.49

Figure 16

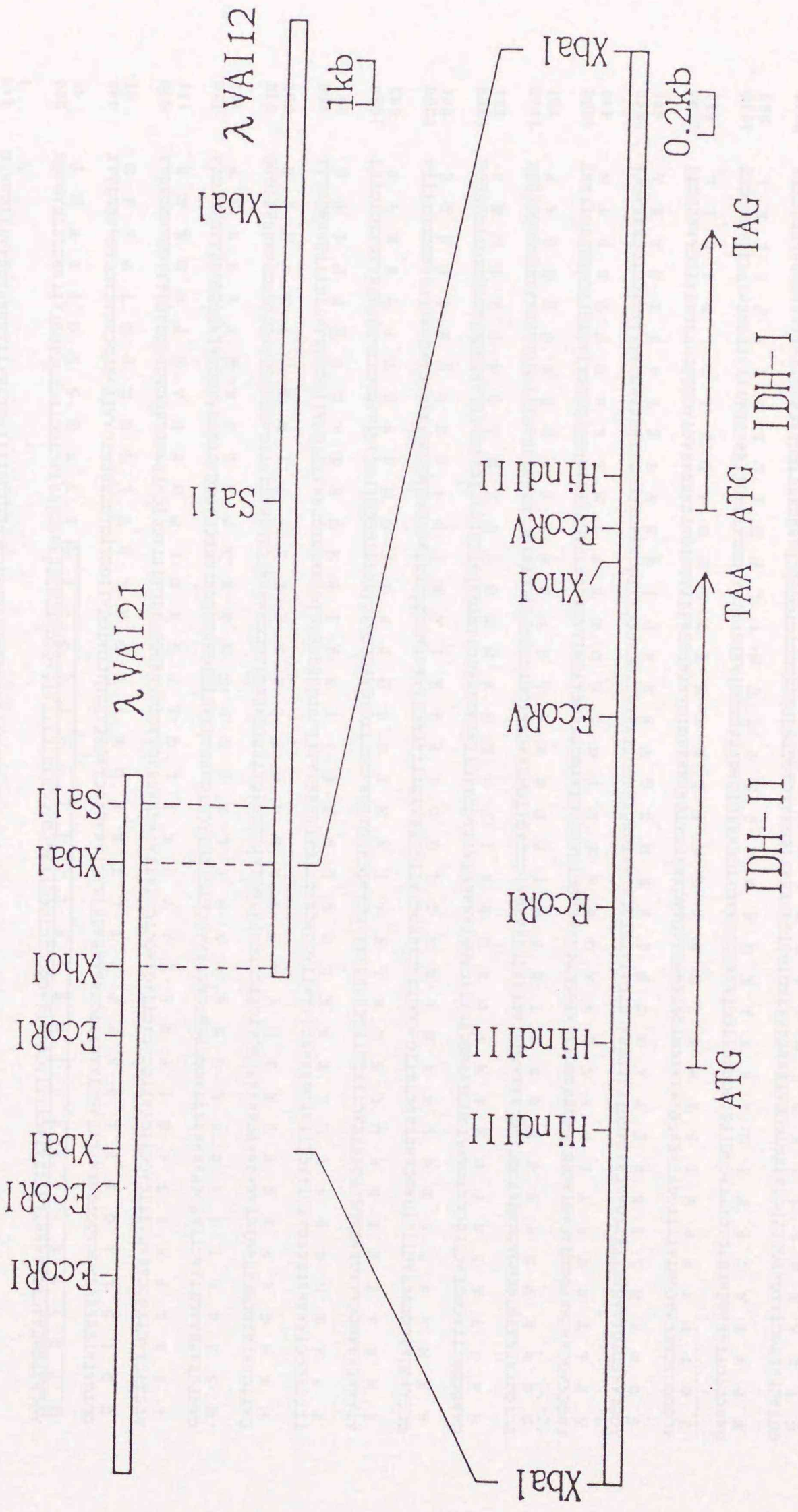

λVAI12
1kb
XbaI
SalI
λVAI21
SalI
XbaI
XhoI
EcoRI
XbaI
EcoRI
EcoRI
XbaI
HindIII
EcoRV
XhoI
EcoRV
EcoRI
HindIII
HindIII
XbaI
0.2kb
XbaI
TAG
ATG
IDH-I
TAA
ATG
IDH-II

Figure 17

```
AAGCTTGCTTATATAATTTAGAAATATCACTTAATTTTTTATAGCATCCTACAGTAAATATTTTCATTTCTAAGTGTTTTTTAGAAATATTGTGTAGGCCAATAATTTATAGGGTTTGGT   120

AAGTTTTCTAACTATACCTTTACTAAGGTGCCCGATTTGTTCAATTACCGAACATGAGGGTCGTATTATTCAGAGTAGAATCACCGGAGCTTTTAGCTAATTAAAATAGGAATTTCAATG   240
                                                                                                                        M     1

AGCACTGATAACTCAAAAATCATTTATACTATTACCGATGAGGCCCCTGCCTTAGCGAGGTATTCTTTATTACCGATTATTCAAGCTTATACTGCTTCTTCAGGTATTAACGTTGAAACA   360
S T D N S K I I Y T I T D E A P A L A T Y S I E P I Q A Y T A S S G I N V E T    41

CGTGATATTTCTTTAGCAGGTCGTATCTTAGCTAACTTTCCAAAATACTTAACTAAAGAGCAACGCATTGACGATGCATTGGCTGAGTTAGGTGAATTAGCGCAGACACCAGAAGCTAAT   480
R D I S L A G R I L A N F P K Y L T K E Q R I D D A L A E L G E L A Q T P E A N    81

ATCATCAAGTTGCCAAATATTTCAGCTTCTATTCCACAGTTAGAAGCTGTTATTAAAGAATTACAAGCAAAAGGCTATGATTTACCTCATTACCCTGCAGAGCCACAAAACGAAGCAGAA   600
I I K L P N I S A S I P Q L E A V I K E L Q A K G Y D L P H Y P A E P Q N E A E   121

GAGTCTATTAAGTTAACTTATGCTAAAATTTTAGGCTCGGCGGTTAACCCTGTTCTACGTGAAGGTAACTCTGATCGTCGTGCGCCAGCGTCTGTTAAACAATATGCGCGTAACAATCCA   720
E S I K L T Y A K I L G S A V N P V L R E G N S D R R A P A S V K Q Y A R N N P   161

CATTCAATGGGCGCTTGGTCTAAAGAATCAAAATCGCATGTTGCTCATATGGCATCAGGTGATTTCTACGGTAGCGAAAAATCAGTAACTATTGATGGTGCAACCAGTGTAAATATTGAG   840
H S M G A W S K E S K S H V A H M A S G D F Y G S E K S V T I D G A T S V N I E   201

TTTGTCGCTAAAAATGGTGATGTAACCTTATTGAAATCAAAATTACCACTACTTGATAAGGAAATTATTGATGCGTCAGTGATGAGTAAATCTGCATTAGTAGAATTCTTTGAAACTGAA   960
F V A K N G D V T L L K S K L P L L D K E I I D A S V M S K S A L V E F F E T E   241

ATAAATAAAGCGAAAGAAGAGGATGTTTTACTTTCATTGCATTTAAAAGCAACCATGATGAAGGTTTCAGATCCGGTCATGTTTGGCCATGCAGTAAGAGTTTTTTATAAAGATGTCTTT  1080
I N K A K E E D V L L S L H L K A T M M K V S D P V M F G H A V R V F Y K D V F   281

GCCAAGCATGCCGCTACTTTTGAGCAACTAGGTGTTGACGCTGACAATGGTATTGGTGATGTTTACGCTAAAATAGCCCGTTTACCGGCAGCGCAAAAAGAAGAAATTGAAGCCGATTTA  1200
A K H A A T F E Q L G V D A D N G I G D V Y A K I A R L P A A Q K E E I E A D L   321

CAAGCGGTTTACGCTACTCGCCCTGAAATGGCGATGGTTGATTCAGATAAAGGTATTACTAACTTACACGTACCTAGTGATGTCATTATTGATGCCTCAATGCCTGCTGCTCTTCGTGCC  1320
Q A V Y A T R P E M A M V D S D K G I T N L H V P S D V I I D A S M P A A L R A   361

TCTGGTATGATGTGGGGACCAGATGGCAAGCAAAAAGATACTAAGTTTATGATCCCAGATCGTAACTATGCTGGTGTTTTCTCTGCAGTAGTAGACTTTTGTCGTGAAAATGGCGCTTTT  1440
S G M M W G P D G K Q K D T K F M I P D R N Y A G V F S A V V D F C R E N G A F   401

AACCCAGCAACAATGGGTACAGTACCCAATGTTGGCTTAATGGCTCAAAAAGCTGAAGAGTATGGTTCACATGATAAAACATTTACCATGAAAGCCGCGGGTACTGTTCGTGTTGTTAAT  1560
N P A T M G T V P N V G L M A Q K A E E Y G S H D K T F T M K A A G T V R V V N   441

AGCCAAGGTGAAAGACTTATTGAGCAAGAGGTTGCTCAAGGTGATATTTACAGAATGTGTCAGGTTAAAGATGCACCCATTCAAGACTGGGTTAAGTTAGCAGTAACTCGTGCTAGAGCA  1680
S Q G E R L I E Q E V A Q G D I Y R M C Q V K D A P I Q D W V K L A V T R A R A   481

ACGGGCACGCCAACGGTATTTTGGTTAGATGAAAATCGTGGTCATGATGAACAAATGATCAAAAAAGTGAATACGTATTTAGCTGATCATGATACTACCGGCTTAGATATCCAAATTCTT  1800
T G T P T Y F W L D E N R G H D E Q M I K K V N T Y L A D H D T T G L D I Q I L   521

GAACCTGTTAAAGCATGTGAGTTTACGCTTGCCCGTGTTGCTAAAGGGGAAGATGCAATCTCAGTTACGGGTAATGTATTACGTGATTACTTAACTGATTTATTCCCAATTTTAGAGCTT  1920
E P V K A C E F T L A R V A K G E D A I S V T G N V L R D Y L T D L F P I L E L   561

GGTACTAGTGCTAAGATGCTTTCTATTGTGCCATTAATGAATGGTGGTGGTTTATTCGAAACAGGTGCTGGTGGCTCTGCGCCTAAGCATGTGCAACAGTTTGAAAAAGAAAACCACTTA  2040
G T S A K M L S I V P L M N G G G L F E T G A G G S A P K H V Q Q F E K E N H L   601

CGTTGGGATTCTTTAGGTGAGTTTTTAGCTCTTGCAGCATCACTTGAACATGTAGCGGTAACAACAGGAAATGCTAGAGCACAAATACTGGCAGATACATTAGATGCAGCGACAGGTAAG  2160
R W D S L G E F L A L A A S L E H V A V T T G N A R A Q I L A D T L D A A T G K   641

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F L D T N K S P S R K V G E L D N R V V T S I L Q C I G R N V A A Q T T D T E L   681
```
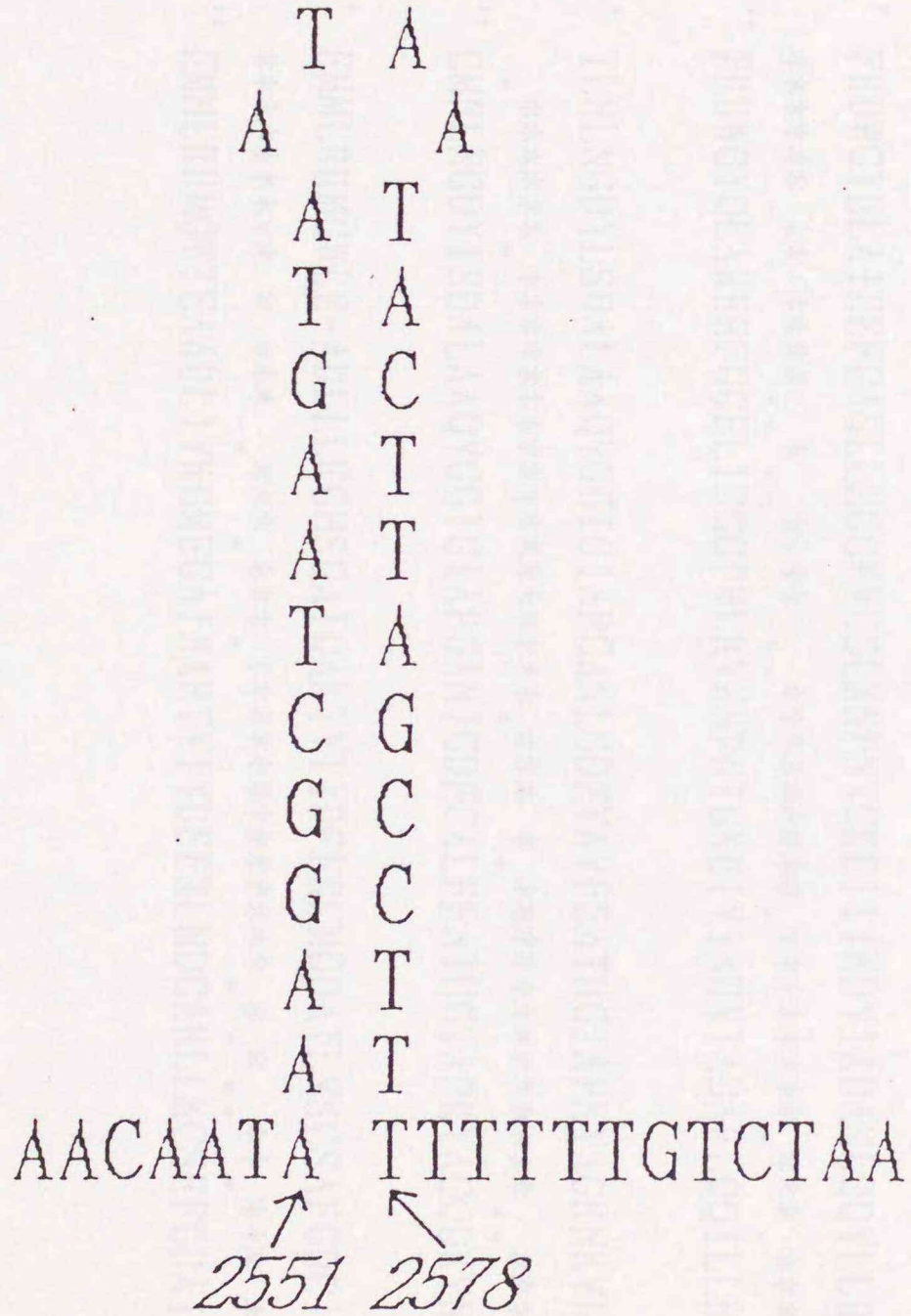

Figure 18

```
CAAGCAAGTTTTAGTAGTGTTGCTCAAGCGCTAACTAAGCAAGAAGAAAAAATTGTTGCTGAATTAAATGCTGCTCAAGGTCCTGCTATTGATCTTAATGGTTATTATTTTGCCGATACT  2400
 Q  A  S  F  S  S  V  A  Q  A  L  T  K  Q  E  E  K  I  V  A  E  L  N  A  A  Q  G  P  A  I  D  L  N  G  Y  Y  F  A  D  T   721

AAACTTGCAGAAAAAGCAATGCGACCAAGCGAAACATTTAATACCATTTTATCTGCATTACTTTAAAGCGACATACTCGCTGCTTAGCGGCAAGTTAGTAGACTCGAGGGTTAGTCGAAT  2520
 K  L  A  E  K  A  M  R  P  S  E  T  F  N  T  I  L  S  A  L  L  *                                                          742

AAAAGGTTGTTGAATAAAGCGCTGAACAATAAAGGCTAAGTAATAATACTTAGCCTTTTTTTTTGTCTAATTGTTGACTTTTGTCTTACTAGAGATCAACTTAAGTATGATACTTGATTTT  2640

GATGATAGTCGATATCACTAATTAATGTTTAGTTACTCGTTCTTTTTGCATTACTTTAGTAGTGCAATTTCTTTTATATCTGTTTAATGTGGAGCTCGCCCTATGACCAATAAAATCATC  2760
                                                                                                  M  T  N  K  I  I              6

ATTCCAACGACTGGAGATAAAATTACCTTTATCGACGGTAAGTTATCAGTACCTAACAATCCCATTATTCCTTATATTGAAGGTGACGGTATAGGCGTTGATGTTACTCCTCCTATGCTC  2880
 I  P  T  T  G  D  K  I  T  F  I  D  G  K  L  S  V  P  N  V  P  I  I  P  Y  I  E  G  D  G  I  G  V  D  V  T  P  P  M  L   46

AAAGTTGTTAATGCAGCGGTTGCTAAAGCTTATGGTGGCGATAGAAAAATAGAATGGTTGGAAGTGTATGCGGGTGAGAAAGCAACCAAAATGTATGATAGTGAAACATGGTTGCCAGAA  3000
 K  V  V  N  A  A  V  A  K  A  Y  G  G  D  R  K  I  E  W  L  E  V  Y  A  G  E  K  A  T  K  M  Y  D  S  E  T  W  L  P  E   86

GAAACACTTAACATTCTTCAAGAATATAAAGTGTCTATAAAAGGGCCGCTGACAACCCCAGTAGGTGGTGGTATGAGCTCTTTAAATGTTGCTATACGACAAATGCTTGACCTTTATGTG  3120
 E  T  L  N  I  L  Q  E  Y  K  V  S  I  K  G  P  L  T  T  P  V  G  G  G  M  S  S  L  N  V  A  I  R  Q  M  L  D  L  Y  V   126

TGTCAACGACCAGTTCAATGGTTTACTGGTGTACCTAGTCCGGTAAAAAGACCTTCAGAAGTTGATATGGTTATCTTTCGTGAAAATACTGAAGATATTTATGCGGGTATTGAGTATAAA  3240
 C  Q  R  P  V  Q  W  F  T  G  V  P  S  P  V  K  R  P  S  E  V  D  M  V  I  F  R  E  N  T  E  D  I  Y  A  G  I  E  Y  K   166

GCAGGTAGTGACAAGGCAAAGTCGGTTATTAAGTTTCTAATTGAAGAAATGGGTGCCAGTAATATTCGCTTTACTGAAAATTGTGGTATCGGCATAAAACCGGTATCAAAAGAAGGCTCA  3360
 A  G  S  D  K  A  K  S  V  I  K  F  L  I  E  E  M  G  A  S  N  I  R  F  T  E  N  C  G  I  G  I  K  P  V  S  K  E  G  S   206

CAACGCTTGGTAAGACAAGCCATTCAATACGCCATTGATAATAATAAAGACTCAGTAACGTTAGTTCATAAAGGTAATATTATGAAGTTTACTGAAGGCGCTTTTAAAGATTGGGGCTAT  3480
 Q  R  L  V  R  Q  A  I  Q  Y  A  I  D  N  N  K  D  S  V  T  L  V  H  K  G  N  I  M  K  F  T  E  G  A  F  K  D  W  G  Y   246

GAACTTGCGATAGAAGAATTTGGTGCAAGTTTACTGCACGGTGGGCCTTGGTGCTCACTTAAAAACCCTAATACCGGTAAAGAAATCATTATTAAAGATGTTATCGCTGATGCTATGTTG  3600
 E  L  A  I  E  E  F  G  A  S  L  L  H  G  G  P  W  C  S  L  K  N  P  N  T  G  K  E  I  I  I  K  D  V  I  A  D  A  M  L   286

CAACAAGTATTATTACGTCCTGCGGAATATAGTGTTATAGCAACGTTAAACTTAAATGGTGATTATTTATCGGATGCACTGGCCGCTCAAGTAGGTGGTATAGGTATTGCACCAGGCGCA  3720
 Q  Q  V  L  L  R  P  A  E  Y  S  V  I  A  T  L  N  L  N  G  D  Y  L  S  D  A  L  A  A  Q  V  G  G  I  G  I  A  P  G  A   326

AATTTAGGTGATGAAGTTGCTGTGTTTGAAGCAACTCATGGAACCGCACCTAAATATGCAGGTAAAAATAAAGTAAACCCAGGCTCGGTAATTCTTTCAGCGGAAATGATGTTAAGACAT  3840
 N  L  G  D  E  V  A  V  F  E  A  T  H  G  T  A  P  K  Y  A  G  K  N  K  V  N  P  G  S  V  I  L  S  A  E  M  M  L  R  H   366

ATGGGCTGGCTTGAAGCTGATTTATTACTTAAAGGTATGTCAGGAGCAATTCAAGCAAAAACAGTTACCTATGATTTTGAGCGACTTATGGATGATGCAACCTTAGTTTCTTGTTCTGCA  3960
 M  G  W  L  E  A  D  L  L  L  K  G  M  S  G  A  I  Q  A  K  T  V  T  Y  D  F  E  R  L  M  D  D  A  T  L  V  S  C  S  A   406

TTTGGCGATTGTATTATTGATCATATGTAGTTAACTGAAATATAGATATAAAGAGCATAAAAAACACAGCTAAATTGCTGTGTTTTTATGTTTTTAGCGCAACGTTTTATAGTTATACCA  4080
 F  G  D  C  I  I  D  H  M  *                                                                                              415

AGTCCATTAAGTTATTGACCACTCAGCGAGAATTAAAAGGCTTAGAGGCAAGGCATTGATTGATGATAATGGTTCTTCCCTTATCAAAATCAATAACGCAGCATATAAACCTTTTAAACT  4200

TGCCCTTTGGTAGTTTATTAGCAAACTAATAACTGTGCAAAATTAATGAAAGATAGCCAACTATGTTTAACCAATTTTACTTGTTCTAAGCTCGTTAATATAGCTCTG              4308
```

Figure 18 (Continued)


AACAATA TTTTTTGTCTAA
2551 2578

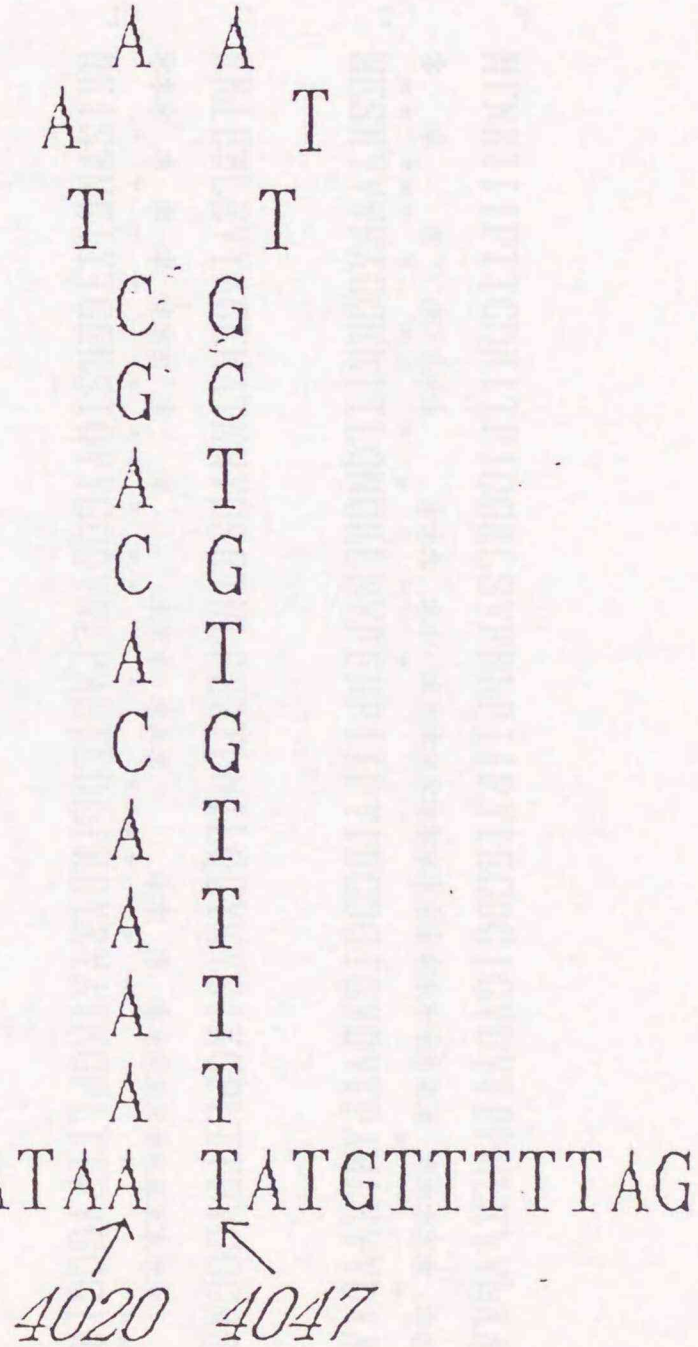

AGCATAA TATGTTTTTAG
4020 4047

Figure 19

```
1'  MTNKIIIPTTGDKITFIDGKLSVPNNPIIPYIEGDGIGVDVTPPMLKVVNAAVAKAYGGD
    *.*...*.*.***.*.***.**.******************.*****.***.*** *.
1'' MESKVVVPAQGKKITLQNGKLNVPENPIIPYIEGDGIGVDVTPAMLKVVDAAVEKAYKGE

61'  RKIEWLEVYAGEKATKMYDSETWLPEETLNILQEYKVSIKGPLTTPVGGGMSSLNVAIRQ
     ***.*.*.*.***.*..*...***.***....**.*.************..*****.**
61'' RKISWMEIYTGEKSTQVYGQDVWLPAETLDLIREYRVAIKGPLTTPVGGGIRSLNVALRQ

121'  MLDLYVCQRPVQWFTGVPSPVKRPSEVDMVIFRENTEDIYAGIEYKAGSDKAKSVIKFLI
      ****.* ***... *.*****.*. .********.********.**.*..*..*****
121'' ELDLYICLRPVRYYQGTPSPVKIIPELTDMVIFRENSEDIYAGIEWKADSADAEKVIKFLR

181'  EEMGASNIRFTENCGIGIKPVSKEGSQRLVRQAIQYAIDNNKDSVTLVIIKGNIMKFTEGA
      ****...***.*.******* *.**..****.**.***.*..******************
181'' EEMGVKKIRFPEIICGIGIKPCSEEGTKRLVRAAIEYAIANDRDSVTLVIIKGNIMKFTEGA

241'  FKDWGYELAIEEFGASLLIIGGPWCSLKNPNTGKEIIIKDVIADAMLQQVLLRPAEYSVIA
      ******.** ****..*..**** ..********.********.***.*******.***
241'' FKDWGYQLAREEFGGELIDGGPWLKVKNPNTGKEIVIKDVIADAFLQQILLRPAEYDVIA

301'  TLNLNGDYLSDALAAQVGGIGIAPGANLGDEVAVFEATIIGTAPKYAGKNKVNPGSVILSA
      .******.******************.*** *.**********..******.****
301'' CMNLNGDYISDALAAQVGGIGIAPGANIGDECALFEATIIGTAPKYAGQDKVNPGSIILSA

361'  EMMLRIIMGWLE-ADLLLKGMSGAIQAKTVTYDFERLMDDATLVSCSAFGDCIIDIIM
      ********* * ***..***.***.**********.*.*..**.*** **..*
361'' EMMLRIIMGWTEAADLIVKGMEGAINAKTVTYDFERLMDGAKLLKCSEFGDAIIENM
```

Figure 20

```
IDH-I[a]      282   DAMLQQVLLRPAEYS-VIATLNLNGDYLSDA--LAAQVGG
E. coli[b]    283   DAFLQQILLRPAEYD-VIACMNLNGDYISDA--LAAQVGG
IPDH[c]       206   DAMAMHLVRSPARFD-VVVTGNIFGDILGNL--RADLPGS
IDH-II[d]     513   KACEFTLARVAKGEDAISVTGNVLRDYLTDLFPILELGTS

IDH-I         323   IGIAPGANLGDEVAVFE-ATHGTAPKYAGKNKVNPGSVIL
E. coli       324   IGIAPGANIGDECALFE-ATHGTAPKYAGQDKVNPGSIIL
IPDH          247   LGLLPSASLGRGTPVFE-PVHGSAPDYAG-KGRNPTAAIL
IDH-II        554   AKMLSIVPLMNGGGLEETGAGGSAP---KHVQQFEKENHL

IDH-I         364   SAEMMLRHMG
E. coli       365   SAEMMLRHMG
IPDH          281   SAAMMI-EQL
IDH-II        595   RWDS-LGEFL
```

Figure 21

inches 1 2 3 4 5 6 7 8

cm 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

Kodak Color Control Patches

Blue Cyan Green Yellow Red Magenta White 3/Color Black

Kodak Gray Scale

C Y M

A 1 2 3 4 5 6 M 8 9 10 11 12 13 14 15 B 17 18 19