Studies on a respiration-dependent primary Na^+ pump and roles of the primary Na^+ and H^+ pumps in energy metabolism of a psychrophilic marine bacterium, Vibrio sp. strain ABE-1

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Contents

Results

Abbreviation

The following abbreviations were used in this thesis.

AIB; α-aminoisobutyric acid

CCCP; carbonylcyanide *m*-chlorophenylhydrazone

DCCD; *N,N'*-dicyclohexylcarbodiimide

DCIP; 2,6-dichlorophenolindophenol

HQNO; 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide

MES; 2-(*N*-morpholino)ethanesulfonic acid

MGK; 0.5 mM MES-KOH (pH 6.5) containing 10% (v/v) glycerol and 0.5 M KCl

MGN; 0.5 mM MES-NaOH (pH 6.5) containing 10% (v/v) glycerol and 0.5 M NaCl

MOPS; 3-(*N*-morpholino)propanesulfonic acid

M_r; molecular weight

NADH-DH; NADH dehydrogenase

Na_3VO_4; sodium orthovanadate

trans-parinaric acid; 9,11,13,15-*all*-*trans*-octadecatetraenoic acid

POPOP; 2,2'-*p*-phenylenebis(5-phenyloxazole)

PPO; 2,5-diphenyloxazole

Q-*n*; ubiquinone possessing isoprenyl side-chain unit(s) of which length is specified by *n*

TGK; 0.5 mM Tricine-KOH (pH 8.5) containing 10% (v/v) glycerol and 0.5 M KCl

TGN; 0.5 mM Tricine-NaOH (pH 8.5) containing 10% (v/v) glycerol and 0.5 M NaCl

TPP; tetraphenyl phosphonium

Tricine; *N*-tris(hydroxymethyl)methylglycine

Summary

An unique respiration-dependent primary Na^+ pump of a psychrophilic marine bacterium Vibrio sp. strain ABE-1 (Vibrio ABE-1) was characterized and roles of the Na^+ pump and a respiration-dependent primary H^+ pump in energy metabolism of this bacterium were studied.

At alkaline pH, this bacterium grew without being affected by a proton ionophore, carbonylcyanide m-chlorophenylhydrazone (CCCP). Anaerobic cell suspensions prepared with Na^+-containing buffers at pH 6.5 and 8.5 showed transient acidification by O_2-pulse, but in the presence of CCCP it showed transient alkalization. However, the suspensions with Na^+-free buffers showed the acidification at pH 6.5 by O_2-pulse and the CCCP-dependent alkalization at pH 8.5. Several experiments with some inhibitors such as tetraphenyl phosphonium (TPP), monensin, amiloride, and Na_3VO_4 demonstrated that the acidification is attributed to a respiration-dependent primary H^+ pump, and the CCCP-dependent alkalization is ascribed to a respiration-dependent primary Na^+ pump but not to a secondary Na^+ translocater driven by an electrochemical potential gradient of H^+ such as Na^+/H^+ antiporter and an ATP-dependent ion translocater such as Na^+/K^+ ATPase of eukaryotes. From these results and the absolute requirement of NaCl on the growth of Vibrio ABE-1, it was concluded that both the respiration-dependent H^+ and Na^+ pumps of this bacterium function over pH range of the

growth.

NaCl stimulated NADH oxidase activity at pH 6.5 but inhibited at pH 8.5. 2-n-Heptyl-4-hydroxyquinoline N-oxide (HQNO), which is known as a respiratory inhibitor and intensely inhibited the primary Na^+ pump activity at pH 6.5 and 8.5, inhibited strongly the NADH oxidase activity at pH 6.5, while faintly at pH 8.5. On the other hand, NADH:ubiquinone-1 oxidoreductase activity was stimulated by NaCl to similar extent at pH 6.5 and 8.5, but it was inhibited by HQNO at pH 8.5 less than at pH 6.5 in the presence of 0.425 M NaCl. These results indicate that the primary Na^+ pump at pH 6.5 is coupled at NADH: quinone oxidoreductase to the respiratory chain as similar as reported firstly in a marine bacterium Vibrio alginolyticus, and that the coupling site of the pump at pH 8.5 is different from the site at pH 6.5. Inhibition of succinate oxidase activity by NaCl and its HQNO-insensitivity at pH 8.5 verify that the coupling site at the same pH is also absent in the oxidase system. Identification of quinones of Vibrio ABE-1 was attempted to clarify the native electron and proton acceptor for NADH dehydrogenase in these experiments, and it was manifested that this bacterium grown aerobically contained ubiquinone-8 and a trace amount of ubiquinone-7.

Both at pH 6.5 and 8.5, Vibrio ABE-1 showed chemotactic motility on a soft agar plate with sodium succinate as an attractant and actively transported α-aminoisobutyric acid (AIB), a nonmetabolized amino acid deriv-

ative. NaCl-dependence of the chemotactic motility and the AIB uptake and the effects of inhibitors such as TPP and CCCP proved that these phenomena were driven by an electrochemical potential gradient of Na^+ ($\Delta\bar{\mu}_{Na^+}$) both at pH 6.5 and 8.5, and that the $\Delta\bar{\mu}_{Na^+}$ was produced at pH 8.5 by the primary Na^+ pump whereas at pH 6.5 mainly produced by a secondary Na^+-translocater such as Na^+/H^+ antiporter. However, the primary Na^+ at pH 6.5 may also partially participate to the formation of $\Delta\bar{\mu}_{Na^+}$ because of less sensitivity of the chemotactic motility at pH 6.5 to CCCP than the growth at the same pH. These results and the CCCP-resistant growth at pH 8.5 represent that Vibrio ABE-1 use Na^+, but not H^+, as a coupling ion at various steps of the energy metabolism at alkaline pH range.

The primary Na^+ and H^+ pumps and the membrane-bound NADH dehydrogenase of Vibrio ABE-1 operated very actively at low temperatures. In particular, the activities of the dehydrogenase of this bacterium and a mesophilic Vibrio parahaemolyticus at 5°C were about 70 and 30% of the maximum activities at 20 and 30°C, respectively, and specific activities of the former enzyme were higher than those of the later enzyme below 10°C. Therefore, these pumps and enzyme must play an important role for sustenance of active growth of this bacterium at low temperatures.

Fatty acid composition of total phospholipids in Vibrio ABE-1 cells was drastically changed by the bacterial cultivation in the presence of various fatty acids.

The primary Na^+ and H^+ pumps of such the cells were active at low temperatures, regardless of the compositional changes of fatty acids, but at pH 8.5 the H^+ pump activity at 25°C observed in normally grown cells was not present at all under the same conditions in the cells cultivated with oleic acid. Since no specific changes for the cells cultivated with oleic acid were detected in the membrane fluidity of the intact cells, it was concluded that the alterations in physical properties of the whole membranes were not responsible for the loss of the H^+ pump activity.

Introduction

Living organisms require enormous energy for operation and maintenance of their biological processes and intracellular reactions. For example, it has been estimated that adult man may consume over a half of the body weight of ATP per day even at rest (Erecińska and Wilson, 1978). Therefore, energy transduction system must be indispensable for sustenance of every aerobic organism's life.

According to chemiosmotic theory postulated by Mitchell (1961, 1976, 1979), electron transfer through respiratory or photoredox chain is accompanied with translocation of proton, and consequently form an difference in electrochemical potential of H^+ across membranes ($\Delta\bar{\mu}_{H^+}$, also designated as proton motive force (Δp)) which is composed of membrane potential ($\Delta\psi$) and concentration gradient of H^+ (ΔpH) across membranes. The proton motive force is used for synthesis of ATP by H^+-translocating ATPase (F_0F_1-ATPase) and for active transport of such solutes as amino acids, sugar and so on. The energy transduction mechanism employing H^+ as a coupling ion has been found generally in many organisms. Furthermore, the proton motive force has been recently reported to be utilized in other cellular functions and processes such as flagellar rotation in bacterial motility (Doetsch and Sjoblad, 1980; Macnab, 1987), genetic transformation (Santos and Kaback, 1981; Van Nieuwenhoven, et al., 1982),

and protein translocation across membranes on the occasion of settlement of membrane-bound or organellar proteins synthesized in cytoplasm to their native location (Douglas et al., 1986; Pfanner et al., 1988; Verner and Schatz, 1988).

On the other hand, it has been reported that Na^+ is a coupling ion of solute symport in several bacteria containing Escherichia coli (Lanyi, 1979; Maloney, 1987), and that a flagellar motor of alkalophilic Bacillus (Hirota et al., 1981; Hirota and Imae, 1983) and motility of a non-flagellated cyanobacterium (Willey et al., 1987) are driven by an electrochemical potential gradient of Na^+ ($\Delta\bar{\mu}_{Na^+}$). A membrane-bound and biotin-dependent oxaloacetate decarboxylase of facultative anaerobic Klebsiella pneumoniae (Dimroth, 1980, 1981, 1982) extrude Na^+ across the membrane during the decarboxylation of oxaloacetate. Similar Na^+ pump function has been found in methylmalonyl-CoA decarboxylases of anaerobic Veillonella alcalescens (Hilpert and Dimroth, 1982), Propionigenium modestum (Hilpert et al., 1984) and glutaconyl-CoA decarboxylase from anaerobic Acidaminococcus fermentans, Peptococcus aerogenes and Clostridium symbiosum (Buckel and Semmler, 1982, 1983). Furthermore, Hilpert et al. (1984) reported that P. modestum has a Na^+-stimulated and -transporting ATPase, which can synthesize ATP by $\Delta\bar{\mu}_{Na^+}$ generated upon the action of the methylmalonyl-CoA decarboxylase. The existence of similar ATPase has been implied in a methanogenic Methanobacterium thermoautotro-

phicum (Šmigáň et al., 1988). Tokuda and Unemoto have described in a series of their studies that a primary Na^+ pump links to a NADH:quinone oxidoreductase segment in the respiratory chain of a marine bacterium Vibrio alginolyticus and supports CCCP-insensitive growth of the bacterium (1981, 1982, 1983, 1984). These findings suggest that not only H^+ but also Na^+ plays important roles in energy metabolism of many bacteria as coupling ion.

Vibrio sp. strain ABE-1 (Vibrio ABE-1) is a psychrophilic marine bacterium isolated from sea water of the shore of Abashiri in Hokkaido in winter, and its optimum and maximum temperatures for growth are about 15°C and between 20 and 25°C, respectively (Takada et al., 1979). Furthermore, this bacterium has also halophilic characteristics, that is, NaCl is essential for its growth and the maximum growth was achieved in the presence of about 0.4 M NaCl. Considering its halophilic nature, it is possible that both H^+ and Na^+ may be involved in energy metabolism of this bacterium.

Many psychrophilic bacteria like Vibrio ABE-1, which can sufficiently grow even at low temperatures such as 0°C, have been isolated from various sources and environments (Morita, 1975; Herbert, 1986). Different subjects of investigations has been performed to clarify adaptation mechanisms of these bacteria to low temperatures so far (Morita, 1975; Inniss, 1975; Inniss and Ingraham, 1978). Also in my laboratories, following informations have been obtained. i) Homeoviscous adaptation found in

Vibrio ABE-1 between 0 and 20°C seems to be attributed to very high content of hexadecenoic acid in membrane phospholipids (Okuyama et al., 1986). Palmitoleic acid and cis-vaccinic acid contents of phospholipids in psychrotrophic Pseudomonas sp. strain E-3 were increased by lowering of the growth temperature, and these fatty acids are produced by aerobic desaturation of $C_{16:0}$ and anaerobic elongation of palmitoleic acid, respectively (Wada et al., 1987). ii) Protein synthesizing activities of psychrophilic and psychrotrophic bacteria are higher at 0°C than those of mesophilic Pseudomonas aeruginosa and Bacillus subtilis, and the activities of Vibrio ABE-1 and a psychrotrophic Pseudomonas sp. strain 351 (P-351) was hardly changed or rather increased by chilling at 0°C whereas the corresponding activity of P. aeruginosa was markedly decreased (Saruyama and Sasaki, 1980; Saruyama et al., 1979, 1980). Protein synthesizing activities with cell-free S-30 fraction of Vibrio ABE-1 are also higher below 25°C than those of P. aeruginosa, and 50S subunit of Vibrio ABE-1 ribosome seems to be responsible for the higher activities at low temperatures (Oshima et al., 1980, 1987). iii) Optimum induction temperature of isocitrate lyase, an inducible enzyme, of a psychrotrophic Flavobacterium strain G-3 (G-3) is lower than that of P. aeruginosa and Proteus vulgaris (Oshima and Sasaki, 1979). v) Purified isocitrate dehydrogenase (ICDH) of G-3 is more active and stable at 4°C than that purified from P. vulgaris (Ochiai et al., 1979a), and activation

energies of ICDH from G-3 and purified thermolabile isozyme II of ICDH from Vibrio ABE-1 are lower than that of P. vulgaris (Ochiai et al., 1979a, 1979b). vi) The adenylate energy charge of Vibrio ABE-1 is maintained in high level (about 0.85 to 0.9) between 0 and 20°C (Hakeda and Fukunaga, 1983).

In addition to these findings, energy transduction system must be sufficiently active at low temperatures to sustain growth of psychrophiles and psychrotrophs under this condition. In fact, NADH oxidation has been reported to be higher at 7°C in Vibrio ABE-1 and P-351 than in P. aeruginosa (Okuyama et al., 1979).

I present here the experimental evidences that, in addition to primary H^+ pump, a respiration-dependent primary Na^+ pump is existent in Vibrio ABE-1, and describe about several characteristics of the unique Na^+ pump and the coupling site(s) of the Na^+ pump to respiratory chain. Furthermore, in order to elucidate importance and contribution of the primary H^+ and Na^+ pump in energy metabolism of this bacterium, driving forces of two energy-consuming cellular functions, that is, chemotactic motility and active transport of amino acid was examined.

Also in this study, it was found that the respiration-dependent primary H^+ and Na^+ pumps was sufficiently active at low temperatures such as 5°C. Considering the functions of these pumps, they must be transmembrane proteins or proteins embedded in membranes and therefore their activities are presumed to be influenced by mem-

brane lipid constituents. Since fatty acid composition of membrane lipids was changed in many bacteria by the growth temperatures and was one of most remarkable qualitative differences detected between psychrophilic and other bacteria (Kogut, 1980; Russell, 1984; Herbert, 1981), effect of alteration in fatty acid composition of phospholipids on the pump activities was investigated to search why the pumps are active at low temperatures.

Materials and Methods

Bacterial strains and growth conditions

Unless otherwise stated, a psychrophilic marine bacterium, *Vibrio* sp. strain ABE-1 (*Vibrio* ABE-1; Takada *et al.*, 1979), was cultivated for 24 hr (up to late exponential phase of the growth) with vigorous shaking at 15°C in a synthetic Tris-salts medium (pH 7.5) containing 0.1 M sodium succinate as a carbon source (Hakeda and Fukunaga, 1983), or for 24 hr with vigorous shaking at 10°C in a peptone broth medium consisting of 1% peptone, 1% meat extract and 0.5 M NaCl (pH 7.0) in several experiments. Furthermore, for the experiments of chemotactic motility, the bacterium was grown at 10°C for 18 hr in the peptone broth medium in the same manner. In the case of the growth at acidic pH, 50 mM MES-NaOH was used instead of 50 mM Tris-HCl in the Tris-salts medium. *Vibrio parahaemolyticus* strain Y-3 (Kimura *et al.*, 1979), which was used to examine temperature-dependence of the activity of NADH-DH from mesophilic bacteria, was cultivated at 37°C for 2.5 hr with vigorous shaking in the peptone broth medium.

For the determination of the specific growth rate, cell growth was monitored by measuring the turbidity at 600 nm with a Hitachi Perkin-Elmer spectrophotometer Type 139. One ml of preculture was inoculated into 100 ml of fresh Tris-salts medium in a 500 ml Erlenmeyer flask. The specific growth rate, μ, was calculated from the for-

mula:

$$\mu = \frac{2.303(\log x_2 - \log x_1)}{t_2 - t_1} \quad hr^{-1},$$

where x_1 and x_2 are the turbidities at time t_1 and t_2, respectively. To examine the effect of CCCP on the growth, CCCP in ethanol (less than 1 ml) was added to a fresh medium when the bacteria was inoculated.

Preparation of membrane fraction

All procedures were carried out below 4°C. Cells of Vibrio ABE-1 or Vibrio parahaemolyticus were harvested by centrifugation at 4,300 x g for 10 min and washed three times with 0.5 M NaCl or 10 mM Tris-HCl (pH 7.5) containing 0.5 M NaCl and 2 mM $MgCl_2$. Membrane fractions of both bacteria were prepared as described previously (Takada et al., 1981) except that wash of crude membrane fraction by centrifugation at 105,000 x g for 1 hr was omitted in the purification of membrane-bound NADH-DH from Vibrio ABE-1.

Measurement of respiration-dependent proton flows across membranes

Respiration-dependent proton flows were measured as the pH change of an anaerobic cell suspension induced by O_2-pulse with a glass pH electrode. The harvested cells were washed three times by centrifugation at 4°C with one of the following buffers sufficiently bubbled with N_2 gas. The NaCl-buffer systems were 0.5 mM MES-NaOH (MGN

buffer; pH 6.5) and Tricine-NaOH (TGN buffer; pH 8.5), containing 10% glycerol and 0.5 M NaCl. The Na^+-free buffer systems were MGK (pH 6.5) and TGK buffer (pH 8.5) containing KCl and KOH instead of NaCl and NaOH in MGN and TGN buffers, respectively. The washed cells were suspended in the respective buffers at about 10 mg protein/ml of suspension. Two milliliters of the anaerobic cell suspension was transferred into a water-jacketed chamber connected to a temperature-controlled water circulator adjusted at 10°C unless otherwise stated. The chamber was sealed with a butyl-rubber stopper, and a glass electrode was inserted through the stopper. The atmosphere of the chamber was kept anaerobic with a continuous flow of N_2 gas. The cell suspension was thoroughly stirred with a magnetic bar and the pH was then readjusted with a small amount of HCl or NaOH. An O_2-pulse was given by injection of 50 µl of O_2-saturated 0.5 M NaCl or KCl. The pH changes in the cell suspension resulting from respiration-dependent H^+ flows across membrane were monitored with a glass electrode (Radiometer, GK2321C) connected to a pH meter (Radiometer, PHM84) and recorded with a recorder (Rikadenki Kogyo Co., Ltd., R-51). Various reagents used in the experiments were added to the cell suspensions as ethanolic solutions except that amiloride and Na_3VO_4 were as an aqueous solution.

Determination of the intracellular Na^+ and K^+ concentrations

Cells were harvested and washed three times with MGN, TGN or TGK buffer at 4°C. Intracellular concentrations of Na^+ and K^+ were calculated by subtraction of the amounts of Na^+ and K^+ in the extracellular buffer from the total amounts of the respective cations in the cell pellets.

For the determination of Na^+ and K^+ contents in the cell pellets, after the final washing of the cells by centrifugation at 4,300 x g for 10 min, plastic centrifuge tubes were rapidly cut off along the interface of the cell pellets to remove the excess buffer. The pellets were then suspended in distilled water and the final volume of the suspension was exactly adjusted to 10 ml. The volume of the cell pellets was estimated by weighing the amount of water required to make the suspension up 10 ml. Next, 25 ml of concentrated HNO_3 was added to the suspension and the mixture was heated to complete the hydrolysis of the cells. The Na^+ and K^+ contents of the hydrolysates were measured with a Hitachi 180-70 atomic absorption spectrophotometer.

Measurement of the extracellular space in the cell pellets

In order to evaluate intracellular Na^+ and K^+ concentrations, the occupancy ratio of extracellular space in the cell pellets were estimated from the radioactivi-

ties of ^{14}C and ^{3}H in the supernatants and pellets as follows. Cell pellets washed three times with MGN, TGN or TGK buffer at 4°C were resuspended in the same buffer at about 3 mg protein/ml. Next, 25 μCi $^{3}H_2O$ and 1.25 μCi [^{14}C]inulin were added to 2.5 ml of the cell suspension. The mixtures were gently shaken for 10 min at 4°C, then centrifuged at 4,300 x g for 10 min at 4°C, and the supernatants were saved. After wiping off residual buffer adhering to the sides of the centrifuge tubes with absorbent tissues, the pellets were suspended in 0.2 ml of the same buffer. Next, 10 μl of the saved supernatant or the pellet suspension was mixed with 6 ml of a liquid scintillation cocktail containing 0.6% (w/v) PPO and 0.05% (w/v) POPOP in the mixed solution of toluene and Triton X-100 (2:1, by volume). The radioactivity was determined with a Beckman liquid scintillation system LS-3801. The percentages of extracellular space in the cell pellets were calculated as described by Sadler et al. (1980) and were 48, 51 and 64% when cells were washed with MGN, TGN and TGK buffer, respectively.

Enzyme assay

Unless otherwise noted, all enzyme assays were carried out spectrophotometrically at 20°C with a Hitachi Perkin-Elmer spectrophotometer Type 139 equipped with a temperature-controlled cell.

a) NADH dehydrogenase (EC 1.6.99.3) activity was assayed by measuring the decrease in absorbance at 600 nm

due to the reduction of DCIP and, only in the experiments of temperature-dependence, at 420 nm due to the reduction of ferricyanide. The standard reaction mixture for DCIP reductase contained 0.1 M Tris-HCl (pH 7.5), 0.25 M NaCl, 134 μM NADH, 35 μM DCIP, 10 mM KCN and an appropriate amount of enzyme in a final volume of 2 ml. In the assay of ferricyanide reductase, 1.34 mM NADH and 1.215 mM ferricyanide were used instead of 134 μM NADH and 35 μM DCIP, respectively. The reaction was started by addition of DCIP or ferricyanide. Correction was made by subtraction of DCIP or ferricyanide reduction without NADH.

NADH:Q-1 oxidoreductase activity was assayed at 20°C by directly monitoring change of the difference of absorbance between 248 and 267 nm due to ubiquinol-1 formation, as reported by Unemoto and Hayashi (1979), with a dual wavelength spectrophotometer (Shimadzu Corp., UV-3000). The standard assay mixture contained 0.1 M MES-diethanolamine (pH 6.5) or Tris-HCl (pH 8.5), 134 μM NADH, 10 mM KCN, 100 μM Q-1 and an appropriate amount of enzyme in a final volume of 2 ml. After incubation of the reaction mixture for 2 min at 20°C, the reaction was started by addition of NADH.

b) NADH oxidase activity was assayed by measuring the decrease in absorbance at 340 nm due to the oxidation of NADH. The standard reaction mixture contained 0.1 M MES-diethanolamine (pH 6.5) or Tris-HCl (pH 8.5), 134 μM NADH and an appropriate amount of enzyme in a final volume of 2 ml. The reaction was started by addition of

NADH.

c) Succinate oxidase activity was measured polarographically with a Clark oxygen electrode (Beckman Instruments, Inc., Process oxygen analyzer model 778) equipped with a recorder (Hitachi, Ltd., 056) at 20°C as the rate of oxygen consumption. The standard reaction mixture containing 50 mM Tris-HCl (pH 8.5), 10 mM disodium succinate and approximately 1 mg of protein in the final volume of 3.5 ml was stirred thoroughly in the reaction vessel. After incubation of the reaction mixture for about 90 sec at 20°C, the reaction was started by addition of disodium succinate.

Specific activities of these enzymes were designated as units per mg protein. One unit of enzyme activity was defined as the amount of enzyme catalyzing the reduction of 1 μmol of DCIP or ferricyanide per min for NADH-DH and as that catalyzing the formation of 1 μmol of ubiquinol-1 per min for NADH:Q-1 oxidoreductase. In the calculation, the following molar extinction coefficients ($M^{-1}.cm^{-1}$) were used: DCIP, 20.6×10^3 (Armstrong, 1964); ferricyanide, 1×10^3 (Minakami et al., 1962); Q-1, 7.8×10^3 (Tokuda and Unemoto, 1984).

Protein determination

Protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard and, in the purification of membrane-bound NADH-DH, Bradford (1976) with bovine plasma gamma globulin as

a standard.

Assay of chemotactic motility

Motility in chemotactic response was assayed by swarm formation on a semisolid agar plate as reported by Adler (1966). The following procedure was carried out in sterile. Cells were harvested by centrifugation at 2,200 x g for 10 min at 4°C and washed twice with chemotactic buffer consisting of 1 mM $MgSO_4$, 0.1 mM EDTA and 0.5 M NaCl and 10 mM potassium phosphate (pH 6.5). In order to avoid severance of the flagella, the washed cells were gently suspended in a trace amount of the same buffer. The thick cell suspension (1 µl) was placed on center of a swarm agar plate consisting of 0.4 M NaCl, 10 mM KCl, 25 mM NH_4Cl, 3.3 mM K_2HPO_4, 3 mM trisodium citrate, 1 mM $CaCl_2$, 0.1 mM $FeSO_4$, 1 mM $MgSO_4$, 0.2% agar, 1 mM disodium succinate as a chemotactic attractant, and 50 mM MES-NaOH (pH 6.5). The agar plates were incubated for about 2 weeks at 15°C. For the assay at pH 8.5, the chemotactic buffer was adjusted to pH 8.5 and 50 mM MES-NaOH in the swarm agar plate was replaced by 50 mM Tricine-NaOH (pH 8.5).

Lipid extraction, isolation of phospholipid and analysis of fatty acid composition

Total lipids of Vibrio ABE-1 were extracted as described by Okuyama and Sasaki (1986) and stored in a chloroform solution under nitrogen at -20°C until use.

Phospholipids were separated from the total lipids by thin-layer chromatography on glass plates coated with 0.5 mm-thick silica gel (Merck Kieselgel 60H, Art. 7736). The plates were developed with either of the following solvent mixtures: chloroform/methanol/water (65:25:4, by volume) and hexane/diethyl ether/acetic acid (90:30:1, by volume). After the development was finished, all lipids on the plate were detected by iodine vapor and phospholipids were identified with spraying molybdenum blue reagent (Dittmer and Lester, 1964). All phospholipid spots on the plates were scraped off and the silica gel was boiled for 3 hr in 5% methanolic HCl solution or 10% (v/v) acetyl chloride in methanol. Fatty acid methyl esters were then extracted with hexane and analyzed by gas-liquid chromatography on a Shimadzu gas chromatograph GC-8APF equipped with a flame ionization detector. After injection of sample, column (Okuyama et al., 1984) was heated from 100 to 190°C at 4°C/min. The fatty acids were identified by comparing the retention times with methyl ester standards. Fatty acid composition was calculated from the area of each peak on the chromatogram.

Measurement of fluorescence anisotropy

Using trans-parinaric acid (Sklar et al., 1977) as a fluorescent probe, fluorescent anisotropy of the intact cells was measured as reported by Okuyama et al. (1986) with the following modifications. The harvested cells were washed three times with 20 mM MES-NaOH (pH 6.5) or

Tricine-NaOH (pH 8.5) containing 10% (v/v) glycerol and 0.5 M NaCl, which was previously bubbled with nitrogen gas for 5 min to remove air, and suspended in the same buffer at a protein concentration of about 60 μg/ml (absorbance at 600 nm is 0.1625). One μl of trans-parinaric acid in ethanol (1.5 mg/ml) was added to 3 ml of the dilute cell suspension and the mixture was incubated for 30 min at 15°C before the measurement of the fluorescence intensity.

Extraction and purification of quinones

Quinones were extracted according to the procedure described by Kröger et al. (1971) with a minor modification. 6.6 g Fresh weight of the cells washed three times with 0.5 M NaCl were suspended in 66 ml of 0.1 M triethanolamine-HCl (pH 7.2) containing 0.5 M NaCl. The suspension was mixed with 330 ml of a mixture of methanol and petroleum ether (boiling point of 30-60°C) (1:1, by volume) and then 132 ml of acetone was added. The mixture was vigorously stirred for 60 min and petroleum ether phase was collected after a brief centrifugation. The aqueous phase was re-extracted with 198 ml of petroleum ether. The collected petroleum ether extracts were evaporated and the remaining materials were dissolved in a small volume of ethanol.

Quinones in the ethanol solution were purified by thin-layer chromatography on a pre-coated silica gel plate (Merck Kieselgel $60F_{254}$, Art. 5808) with petroleum

ether (boiling point of 60-70°C)/diethyl ether (85:15, by volume) as a developing solvent. After the development, spots of quinones on silica gel plate were detected by irradiating ultraviolet light of wavelength 254 nm and then scraped off. The quinones were extracted from the silica gel with chloroform, evaporated to dryness and resuspended in a small volume of methanol/propane 2-ol mixture (8:2, by volume).

Identification of quinones

The purified quinones were separated according to the length of their polyisoprenyl side-chains by reverse-phase partition high performance liquid chromatography (HPLC) and identified by mass spectrometry. Reverse-phase partition HPLC was performed on a chromatographic system (Tosoh Corp.) with a pre-packed TSKgel ODS-80T_M column (4.6 x 250 mm, Tosoh Corp.). Sample was eluted with methanol/propane 2-ol mixture (8:2, by volume) at the flow rate of 1 ml/min. Quinones were detected with an ultraviolet monitor (Tosoh Corp., model UV-8000) at 270 nm.

Peak fractions of HPLC were analyzed with a JEOL mass spectrometer JMS-DX 300 by direct introduction of samples at an ionizing voltage of 70 eV and a temperature range of sample heater of 250-310°C.

Assay of AIB uptake

Cells were harvested and washed twice with MN buffer consisting of 50 mM MES-NaOH, 10% (v/v) glycerol and 0.5 M NaCl (pH 6.5). The washed cells were suspended in MN buffer to give a protein concentration of about 20 mg/ml. Twenty μl of the cell suspension was mixed with 1 ml of MN buffer and incubated for 10 min at 15°C. The reaction was then started by addition of 50 μl of [1-^{14}C]AIB (20 μCi/umol, final concentration of 0.1 mM). After incubation for given time interval, aliquot of the reaction mixture (50 μl) was diluted by 2 ml of MN buffer and immediately filtered with a cellulose nitrate filter (Toyo Roshi Kaisha, Ltd., pore size of 0.45 μm) to stop the uptake. The filter was washed by 2 ml of MN buffer, dried and transferred into a vial. Five ml of a liquid scintillation cocktail containing 0.4% PPO and 0.005% POPOP in toluene was added to each vial and the radioactivity was determined with a Aloka liquid scintillation system LSC-3500. For the assay at pH 8.5, 50 mM Tricine-NaOH (pH 8.5) was used instead of 50 mM MES-NaOH in MN buffer (designated as TN buffer).

AIB uptake of Na^+-loaded cells, which were prepared by the method of Tokuda et al. (1982) with minor modifications, was assayed as follows. The harvested cells were treated twice at 15°C for 30 min with 50 mM diethanolamine (pH 8.5) containing 0.5 M NaCl and washed twice with 0.5 M NaCl. The Na^+-loaded cells were suspended in MN buffer at a final concentration of about 20 mg pro-

tein/ml. Four μl of the cell suspension was diluted with 0.22 ml of MN buffer containing 20 mM disodium succinate and incubated for 10 min at 15 °C. The uptake was started by addition of 4 μl of 0.5 M KCl and [1-^{14}C]AIB (20 μCi/umol, final concentration of 0.1 mM). The termination of the uptake, determination of the radioactivity and the assay at pH 8.5 were carried out as described above except that 0.5 M NaCl was used instead of MN buffer to terminate the uptake and wash the filter.

Electrophoresis

A polyacrylamide disc gel electrophoresis was carried out at 4°C by the method of Davis (1964) using 7.5% polyacrylamide gel containing 0.1% (w/v) Triton X-100. Proteins were run at a constant current of 2 mA/gel tube. After electrophoresis, the band of NADH-DH was identified by staining for the enzyme activity as described elsewhere (Takada et al., 1988).

Chemicals

Tricine, MOPS, disodium succinate, n-capric acid, n-undecanoic acid, oleic acid and DCCD were obtained from Nakarai Tesque, Inc. TPP bromide and vitamin K_1 were obtained from Tokyo Chemical Industry Co., Ltd. CCCP, Na_3VO_4, monensin sodium salt, amiloride hydrochloride, HQNO, Q-6 from Saccharomyces cerevisiae and Q-10 from bovine heart were supplied by Sigma Chemical Co. Potassium ferricyanide, trans-parinaric acid and soybean asolectin

were the product of Wako Pure Chemical Industries, Ltd., Molecular Probes, Inc. and Nippon Pharmacy Co., respectively. MES was obtained from Nakarai Tesque, Inc. or Dojindo Laboratories. DCIP sodium salt was supplied by Wako Pure Chemical Industries, Ltd. or E. Merck. Triton X-100 was the product of Wako Pure Chemical Industries, Ltd. or Nakarai Tesque, Inc. NADH was supplied by Boehringer Mannheim or Kyowa Hakko Kogyo Co., Ltd. Q-1 was a kind gift of Eizai Co. $^{3}H_2O$ (5 mCi/ml), [carboxyl-^{14}C]-inulin (2.7 mCi/g) and [1-^{14}C]AIB (55 mCi/mM) were purchased from Amersham International, New England Nuclear and American Radiolabeled Chemicals, Inc., respectively. All other reagents used were of analytical grade.

Results

Effect of CCCP on the growth of Vibrio ABE-1

The proton conductor CCCP makes biological membranes permeable to proton. I examined the effect of various concentrations of CCCP on the growth of Vibrio ABE-1 in a synthetic Tris-salts medium containing 0.4 M NaCl. In the pH 6.5 medium (Fig. 1C), even a low concentration of CCCP (0.5 μM) completely blocked the bacterial growth, whereas at pH 8.5 (Fig. 1D), 2.5 and 5 μM CCCP hardly influenced it, and at last more than 10 μM of CCCP caused complete inhibition of the growth. Since pH values in the media were progressively elevated with the bacterial growth (Fig. 1A), specific growth rates were calculated from the growth during the period when the pH was scarcely changed: 0.143 h^{-1} for the growth without CCCP at pH 6.5; 0.135, 0.152, and 0.130 h^{-1} for the growth in a presence of 0, 2.5 and 5 μM CCCP at pH 8.5.

Fig. 2 shows the effect of pH in the medium on the specific growth rate in the presence or absence of 2.5 μM CCCP. In the absence of CCCP, this bacterium could not grow below pH 6.15, but grew well over the wide pH range of 6.5 to 8.5. On the other hand, in the presence of 2.5 μM CCCP, no growth was observed on the medium below pH 7.0. At pH 7.4, the bacteria began to grow after a long lag phase of about one day. Above pH 8.0, the specific growth rates in the presence of CCCP were rather fast than those without CCCP. These results indicate that

Vibrio ABE-1 is less sensitive to CCCP at alkaline pH and the growth under alkaline pH may be supported by other type(s) of energy than the proton motive force, as found with Vibrio alginolyticus by Tokuda and Unemoto (1983).

Respiration-dependent proton flows across the membrane

In order to ascertain this possibility, H^+ flows across the membrane induced by transient endogenous respiration were examined at pH 6.5 and 8.5, at which Vibrio ABE-1 exhibited CCCP-sensitive and -resistant growth, respectively. H^+ flows were monitored in anaerobic cell suspensions prepared with N_2-bubbled MGN or TGN buffer (referred to as Na^+-cell suspension). Furthermore, to examine the effect of Na^+ on the H^+ flows, similar experiments were performed with anaerobic cell suspensions prepared with N_2-bubbled Na^+-free MGK or TGK buffer to minimize the Na^+ content in the assay system (referred to as Na^+ free-cell suspension). The temporary respiration was achieved by an O_2 pulse that small amount of O_2-bubbled 0.5 M KCl or NaCl was injected into the cell suspension. Table 1 shows intracellular Na^+ and K^+ contents of the cells washed with MGN, TGN or TGK buffer. Because these buffers contained 500 mM NaCl or KCl, an artificial membrane potential seemed to be formed across the membranes of the cells suspended in the buffers. Under these conditions, precise evaluation for respiration-dependent H^+ flows was difficult. The dissipation of the artificial membrane potential by loading Na^+ or K^+ to the

cells with a diethanolamine treatment similar to that reported by Tokuda et al. (1982) was incomplete as described in the experiments of AIB transport. Therefore, TPP, a membrane permeable cation, was added to the cell suspension to abolish the artificial membrane potential.

At pH 6.5, with the Na^+-cell suspension, a transient acidification of the suspension was observed by adding 50 µl of O_2-saturated 0.5 M NaCl in the presence of TPP (Fig. 3A-a). However, this acidification was inhibited by CCCP (Fig. 3A-b,c,d). Essentially similar results were obtained with a Na^+ free-cell suspension by O_2-pulsing with either 0.5 M NaCl or KCl (Fig. 4A). These results demonstrate that the acidification of the cell suspensions results from H^+ extrusion by the action of a respiration-dependent primary H^+ pump at pH 6.5. Furthermore, at pH 8.5, the activity of a respiration-dependent primary H^+ pump was also observed with the Na^+-cell suspension (Fig. 3B), but not with the Na^+ free-cell suspension (Fig. 4B).

On the other hand, in the presence of CCCP at pH 8.5, a temporary alkalization of the Na^+-cell suspension occurred by O_2-pulsing and was inhibited by TPP (Fig. 3D). In the Na^+ free-cell suspension, this CCCP-dependent alkalization was detected by O_2-pulsing with 0.5 M NaCl, but not with 0.5 M KCl (Fig. 4B-b,d) and was inhibited by TPP (Fig. 4B-c). Furthermore, the CCCP-dependent alkalization did not occur by injection of a low concentration of O_2-saturated NaCl, but was gradually intensi-

fied by repeating O_2-pulsing with 0.5 M NaCl at pH 8.5 (Fig. 5). These results for the CCCP-dependent alkalization at pH 8.5 indicated that Na^+ appears to contribute to the phenomenon and agreed with those of Vibrio alginolyticus which has a respiration-dependent primary Na^+ pump (Tokuda and Unemoto, 1982). Therefore, the CCCP-dependent alkalization is considered to be a counter-influx of H^+ in the presence of CCCP to dissipate a membrane potential generated with the electrogenic primary Na^+ pump, suggesting that a respiration-dependent electrogenic primary Na^+ pump also functions in Vibrio ABE-1 at pH 8.5. At pH 6.5, such a CCCP-dependent alkalization was observed only with the Na^+-cell suspension (Figs. 3C and 4A-b,d).

These O_2-pulsing experiments suggest that the respiration-dependent primary H^+ and Na^+ pumps simultaneously function in Vibrio ABE-1 over the pH range of the growth at least in the presence of an appropriate amount of NaCl. The results of cell growth and the contributions of H^+ and Na^+ pumps estimated from O_2-pulsing experiments are summarized in Table 2.

Effect of inhibitors on the CCCP-dependent alkalization

To further characterize the above CCCP-dependent alkalization, the sensitivity to inhibitors was examined at pH 6.5 and 8.5. An ionophore for Na^+, monensin, inhibited the alkalization at both pHs, but not completely at pH 8.5 within the inhibitor concentrations tested (Fig. 6A

and B). Amiloride, which inhibits the Na^+/H^+ antiporter in many eukaryotes (Krulwich, 1983) and Escherichia coli (Mochizuki-Oda and Oosawa, 1985), showed no inhibitory effect (Fig. 6C and D). Furthermore, DCCD and Na_3VO_4 are known to be potent inhibitors of F_0F_1-ATPase (Bragg and Hou, 1976; Pedersen and Carafoli, 1987) and E_1E_2-ATPase such as Ca^{++}-ATPase of sarcoplasmic reticulum and Na^+/K^+ ATPase (Cantley et al., 1977; Macara, 1980; Pedersen and Carafoli, 1987), respectively. They only slightly inhibited the alkalization even if high concentrations of the inhibitors were added, suggesting that the phenomenon is not ascribable to ATP-dependent ion translocations (Fig. 7). On the other hand, the alkalization at both pHs was strongly inhibited by HQNO which is a respiratory inhibitor and has been reported to inhibit the respiratory-dependent primary Na^+ pump of Vibrio alginolyticus (Tokuda and Unemoto, 1984) (Fig. 8).

These results allow me to conclude that the CCCP-dependent alkalization is attributed to the respiration-dependent primary Na^+ pump as suggested above.

Effect of temperature on the respiration-dependent H^+ and Na^+ pumps

Since Vibrio ABE-1 is a psychrophilic bacterium, these H^+ and Na^+ pumps are presumed to function sufficiently to sustain active growth at low temperatures. Thus, I examined the effect of temperature on the activities of both H^+ and Na^+ pumps. As shown in Figs. 9 and

10, both pumps were functioning very actively at both pHs of 6.5 and 8.5 at low temperature (5°C), but were inactive at 40°C except that a little activity of Na^+ pump remained at pH 8.5. Relative changes of pH in the medium by H^+ pump at pH 6.5 and by Na^+ pump at pH 8.5 were as follows: 88 and 97% at 5°C, 100 and 100% at 15°C, 75 and 32% at 25°C, and 0 and 21% at 40°C, respectively.

Some properties of NADH oxidase

In Vibrio alginolyticus, it has been reported that NADH oxidase and NADH:quinone oxidoreductase were activated by Na^+ and inhibited by HQNO, and the primary Na^+ pump is coupled with the NADH:quinone oxidoreductase in the NADH oxidase (Tokuda and Unemoto, 1984). Because previous study indicated that NADH oxidase and NADH-DH (NADH:DCIP oxidoreductase) of the crude membrane fraction from Vibrio ABE-1 were also somewhat activated by NaCl at pH 7.5 (Takada et al., 1981), NADH oxidase of this bacterium was investigated to identify the coupling site of the respiratory-dependent primary Na^+ pump.

Fig. 11 shows dependence on NaCl and KCl of NADH oxidase activities at pH 6.5 and 8.5. NADH oxidase activity at pH 6.5 was continuously stimulated with elevation of NaCl concentration, but the activity at pH 8.5 was inhibited by NaCl at any concentration. Similar salt-dependence was observed with KCl except that maximum stimulation at pH 6.5 was achieved at 0.3 M of KCl. Furthermore, NADH oxidase exhibited maximum activity at

about pH 8 without the addition of salts (Fig. 12A), but the optimum pH was shifted to acidic region about 0.5 pH unit in the presence of 0.425 M NaCl (Fig. 12B) or 0.4 M KCl (Fig. 12C). On the other hand, HQNO which intensely inhibited the primary Na^+ pump activity of Vibrio ABE-1 at pH 6.5 and 8.5 (Fig. 8), severely inhibited the enzyme activity at pH 6.5 but only slightly at pH 8.5, regardless of NaCl concentration in the reaction mixture (Fig. 13).

These results indicate that NADH oxidase at pH 6.5 has the properties similar to that of Vibrio alginolyticus but the enzyme at pH 8.5 has quite different from those of the enzymes of Vibrio alginolyticus and at pH 6.5 of Vibrio ABE-1, suggesting that Vibrio ABE-1 might possess two kind of NADH oxidases systems which can operate at different pH range and that, at least, the primary Na^+ pump might be coupled with NADH oxidase at pH 6.5.

Identification of quinones

NADH-DH is one possible candidate for the coupling site of the primary Na^+ pump in NADH oxidase system because NADH:DCIP oxidoreductase has been found to be stimulated by NaCl (Takada et al., 1981). To evaluate this possibility, it is required to examine the properties of NADH:quinone oxidoreductase. Therefore, at first, I tried to identify quinone(s) functioning as the electron and proton acceptor of NADH-DH.

Fig. 14 shows thin layer chromatogram of quinones ex-

tracted from Vibrio ABE-1 cells grown aerobically in the Tris-salts medium on a silica gel plate. An analog of menaquinones, vitamin K_1, and Q-10 of bovine heart were employed as standard materials. In the solvent system used in this study, vitamin K_1 (R_f value of 0.47) migrated larger than Q-10 (R_f value of 0.21). A major spot had almost the same mobility as Q-10, and two other faint spots were also detected. Further analytic experiments by ultraviolet absorption and mass spectra showed that the two minor spots were not quinones.

Major quinones were extracted from the gel and purified with reverse-phase partition HPLC with a TSKgel ODS-80T_M column and a mixture of methanol and propane 2-ol as an eluting solvent. In this chromatographic system, the elution times of Q-6 of Saccharomyces cerevisiae and Q-10 of bovine heart were 8.3 and 26.2 min, respectively. Fig. 15 shows the elution pattern of the sample. A major peak was eluted at 14.1 min after application of the sample and several minor peaks were detected at about 4, 10 and 20 min. In ultraviolet absorption spectrum (Fig. 16), the major peak quinone represented characteristics of ubiquinones (Crane and Barr, 1971; Yamada et al., 1969), that is, maximum absorption was found at 275 nm, and it was decreased and sifted to 290 nm by reduction with sodium borohydride. Furthermore, in mass spectrometric analysis of the quinone, the parent mass was found at m/e 726, and intense peaks of m/e 69, 197 and 235 which seemed to correspond to C_5H_9 derived from polyiso-

prenyl side-chain, benzelium ion and pyrilium ion, respectively, were detected (Fig. 17). These have been known to be specific fragments observed in mass spectra of ubiquinones (Muraca et al., 1967; Yamada et al., 1969). From these results, the major peak quinone was identified as Q-8.

Mass spectra of other minor peak substances obtained by HPLC indicated that only the peak eluted at 10.5 min contained Q-7 and no quinones were present in the other peaks. Therefore, it is concluded that Vibrio ABE-1 contained Q-8 and a slight amount of Q-7 as quinones.

Salt-dependence and HQNO-sensitivity of NADH:Q-1 oxidoreductase

It is difficult to obtain high NADH-DH activity when the quinones with long isoprenyl side-chains are used as the electron acceptor. In fact, NADH-DH of this bacterium could not reduced Q-10 by NADH. Therefore, Q-1 was used as an acceptor, and its reduction was directly assayed with monitoring change of absorbance difference at a wave length pair of 248 and 267 nm.

Table 3 shows salt-dependence of NADH:Q-1 oxidoreductase activity. Differently from salt-dependence of NADH oxidase (Fig. 11), even 0.2 M KCl inhibited the activity at pH 6.5, 0.225 M NaCl stimulated the activity at pH 8.5, and salt-dependence of the activity were almost the same at both pHs. HQNO inhibited the oxidoreductase activity at pH 6.5 and the inhibition was somewhat inten-

sified by 0.425 M NaCl (Table 4). On the other hand, the activity in the presence of 0.425 M NaCl was inhibited by HQNO at pH 8.5 less than at pH 6.5, whereas almost the same inhibitions by HQNO were observed at both pHs without the addition of 0.4 M NaCl.

These results for NADH oxidase and NADH:Q-1 oxidoreductase suggest that the properties of these enzymes at pH 6.5 are in accord with those of Vibrio alginolyticus (Tokuda and Unemoto, 1984) and therefore the primary Na^+ pump at pH 6.5 seems to be coupled with NADH-DH of respiratory chain. However, the coupling site of the primary Na^+ pump at pH 8.5 could not be specified to NADH-DH.

Salt-dependence and HQNO-sensitivity of succinate oxidase

To clarify the possibility that the primary Na^+ pump functioning at pH 8.5 might be coupled with some components of respiratory chain other than NADH-DH in NADH oxidase, effects of salts and HQNO on the activity of succinate oxidase which contained sufficiently active succinate dehydrogenase (Takada et al., 1981) was examined. Similarly to the results for NADH oxidase at pH 8.5 (Figs. 11 and 13B), succinate oxidase activity was inhibited by NaCl and KCl (Fig. 18), and HQNO had little effect on the activity irrespective of NaCl concentration in the reaction mixture (Fig. 19). Furthermore, preliminary study indicated that succinate dehydrogenase was not be activated by NaCl (Takada et al., 1981). Therefore, the Na^+

pump did not appear to be coupled with succinate oxidase system at pH 8.5.

Temperature-dependence of the activities of membrane-bound NADH-DH from Vibrio ABE-1 and Vibrio parahaemolyticus

Since the primary Na^+ pump functioning at pH 6.5 was markedly active at low temperatures (Fig. 9) and found to be coupled with NADH-DH in the respiratory chain, temperature-dependence of the activity of NADH-DH (ferricyanide reductase) was examined (Fig. 20). Like the Na^+ pump at pH 6.5, the enzyme sustained very high activity at low temperature (5°C) (70% of the maximum activity at 20°C). On the other hand, its activity decreased markedly above 30°C.

In order to compare with the temperature-dependence of NADH-DH of this bacterium, the corresponding enzyme activity of a mesophilic bacterium Vibrio parahaemolyticus was assayed at various temperatures (Fig. 20). The enzyme was most active at 30°C and still preserved some activity at 5°C (30% of the maximum activity). However, specific activities of the enzyme of Vibrio ABE-1 were higher than those of Vibrio parahaemolyticus at lower temperatures below 10°C. These results indicate that NADH-DH of Vibrio ABE-1 is a psychrophilic enzyme.

Purification of membrane-bound NADH-DH

In order to clarify further detailed properties of the membrane-bound NADH-DH and the respiration-dependent Na^+ pump linked to the enzyme, I tried to purify the membrane-bound NADH-DH of Vibrio ABE-1.

Unless otherwise stated, the following purification procedure was carried out below 4°C.

The membrane of Vibrio ABE-1 were prepared from the cells grown in the peptone broth medium and suspended in 20 mM Tris-HCl (pH 7.5) containing 16% (v/v) glycerol and 2 mM 2-mercaptoethanol at a final concentration of 20 mg protein/ml. Fig. 21 shows the solubilization of NADH-DH by the treatment with various concentrations of a detergent, Triton X-100. By a final concentration of 1% Triton X-100, almost all membrane-bound NADH-DH was solubilized. Therefore, the membrane suspension was mixed with the equal volume of 2% Triton X-100 containing 20 mM EDTA (pH 7.5) and incubated for 30 min at 4°C. The mixture was then centrifuged at 27,000 x g for 40 min and the collected supernatant (referred as solubilized enzyme) was applied to a TEAE-cellulose column (4.5 x 36.5 cm) previously equilibrated with a buffer A consisting of 40 mM Tris-HCl, 10% (v/v) methanol, 0.5% (w/v) Triton X-100 and 2 mM 2-mercaptoethanol (pH 7.5). The column was then washed with about two bed volume of buffer A at a flow rate of 80 ml/hr and the adsorbed enzyme was eluted with a linear gradient of 0 to 0.6 M NaCl in 1,000 ml of buffer A at a flow rate of 50 ml/hr. NADH-DH was eluted at

about 0.4 M NaCl. Active fractions were combined and solid ammonium sulfate was added to give 30% saturation. The solution was adjusted to pH 7.5 with small volume of NH_4OH. After stirring for 30 min, the precipitate was removed by centrifugation and the supernatant was brought to 60% saturation with further addition of ammonium sulfate. Resultant precipitate was collected by centrifugation and dissolved in a small volume of buffer B lowered the Triton X-100 concentration in buffer A to 0.3% (w/v). Next, the enzyme sample was applied to a Sepharose CL-4B column (25 x 90 cm) previously equilibrated with buffer B and eluted with the same buffer at a flow rate of 20 ml/hr. Fractions containing NADH-DH activity were pooled and concentrated about 2.5 fold with polyethylene glycol No. 20,000. The concentrated sample was applied to a DEAE-Sephadex A-50 column (1.5 x 20 cm) previously equilibrated with buffer C lowered the Triton X-100 concentration in buffer A to 0.1% (w/v). The column was then washed with 130 ml of buffer C and the enzyme was eluted with a linear NaCl gradient of 0 to 0.6 M in 130 ml of buffer C at a flow rate of 10 ml/hr. The enzyme was eluted at about 0.4 M NaCl. Active fractions were combined and used as the purified NADH-DH in the following experiments.

Table 5 shows a summary of purification of membrane-bound NADH-DH. Specific activity of the enzyme was rather decreased with proceeding of the purification step and the recovery of enzyme activity in each step was also

markedly poor. The other means for purification such as blue-, phenyl-, octyl- and 5'AMP-Sepharose column chromatographies and so on were not successful because the enzyme was scarcely adsorbed to the gels or the enzyme was insufficiently recovered from the gels even if adsorbed. In addition to several sharp bands of the enzyme activity, a broad active band was observed beneath the stacking gel on polyacrylamide gel electrophoresis of the solubilized enzyme, while the purified enzyme preparation was composed of only one major and two additional active bands (Fig. 22). These results indicate that, in the solubilized enzyme, most of the enzyme protein formed irregular aggregates with each other and/or other membrane constituents and detergent, but many aggregates were broken during the purification, suggesting that the enzyme was practically somewhat purified. However, since specific activity of the purified enzyme was decreased, it is possible that the enzyme was inactivated by release of activating and/or stabilizing factor(s) during the purification.

Since several purified membrane-bound enzymes have been reported to be activated by detergents or phospholipids (Sandermann, 1978), I investigated whether these substances activated the partially purified enzyme or not. Low concentrations of Triton X-100 (up to 0.1%) was somewhat stimulated the enzyme activity (Fig. 23) but phospholipids extracted from cells of Vibrio ABE-1 grown at 10^{o}C and soybean asolectin have no effect within the

tested concentrations (Fig. 24). Furthermore, since it has been reported that NADH-DH was a flavoprotein (Dixon and Webb, 1979) and several flavoproteins lost flavin cofactors during the purifications (Dancey and Shapiro, 1976; Petitdemange et al., 1980; Borneleit and Kleber, 1983), effect of flavins on the enzyme activity was examined, but only a slight activation was observed (about 10% activation with 50 uM FMN or FAD). Although stabilization of the enzyme was also examined with various substances such as salts, detergents, organic solvents, flavins, protease inhibitor and so on, the poor recovery of the enzyme during the purification was not improved.

Properties of the enzyme

The partially purified NADH-DH was activated by salts. 0.5 M NaCl and 0.1 M $MgCl_2$ brought about maximum stimulation of the enzyme activity with similar extent but the stimulation by $MgCl_2$ was limited to its lower concentrations (Fig. 25). Furthermore, the enzyme was most active at 15°C and sustained high activity at low temperature (about 70% of the maximum activity at 5°C) but the activity was considerably decreased at higher temperatures above 25°C (Fig. 26). These results were fundamentally coincident with those of NADH-DH in the crude membrane of Vibrio ABE-1 (Takada et al., 1981), indicating that the properties of the purified enzyme has not been changed after the solubilization by Triton X-100.

Fatty acid compositions of total phospholipids of the cells grown with various fatty acids

The functions of many membrane-bound proteins such as the primary H^+ and Na^+ pumps of this bacterium have been reported to be influenced by physical state and constituents of the membranes, particularly the compositions of fatty acids and phospholipids (Coleman, 1973; Machtiger and Fox, 1973; Sandermann, 1978). Although reconstitution of the purified protein into liposome provides many useful informations for the interaction between the membrane-bound proteins and membrane lipids, identification of these pump proteins remains to be investigated and the partially purified membrane-bound NADH-DH was unsuitable for this study. Therefore, in order to approach the problem, I attempted to alter native fatty acid composition of total phospholipids in Vibrio ABE-1 by cultivating in the Tris-salts medium supplemented with various fatty acids.

Table 6 shows the fatty acid compositions of total phospholipids from the bacterial cells grown with various amounts of oleic acid at 15°C. As Okuyama et al. (1984) previously described for fatty acid compositions of the outer and inner membrane fractions of Vibrio ABE-1, total phospholipids extracted from this bacterium showed a simple fatty acid composition. Namely, hexadecenoic acid ($C_{16:1}$) as a major component comprised about 70% of total fatty acids and the content of C_{16} fatty acids attained to about 90%. On the other hand, the residual components

consisted of small amounts of several fatty acids with shorter acyl chains (C_{10} to C_{14}) and monounsaturated acids with longer acyl chains. Similar fatty acid composition was observed in total phospholipids from the cells grown with only a detergent, 0.4% Brij 58, used to disperse the added fatty acids. On the other hand, in the cells grown with oleic acid, contents of the both C_{16} fatty acids were decreased and, simultaneously, the corresponding amount of $C_{18:1}$ was increased without change of other fatty acid contents. Furthermore, the larger amount of oleic acid was added to the growth medium, the more the compositions were changed.

More drastic compositional change of fatty acid was observed when the bacteria were grown in the Tris-salts medium supplemented with 0.4% Brij 58 and 0.2 mg/ml n-undecanoic acid ($C_{11:0}$) at 15°C (Table 7). The content of odd carbon numbers of fatty acids in total phospholipids of such the cells was ascended to about 75% of total fatty acids, while these fatty acids were hardly detected (less than 1% of total fatty acids) in the cells grown without $C_{11:0}$. Furthermore, C_{16} fatty acids, particularly $C_{16:1}$, were considerably decreased in these cells. Similar fatty acid composition was obtained in total phospholipids of the cells grown with $C_{11:0}$ at 5°C (Table 8). On the other hand, irrespectively of the growth temperatures, the addition of 0.2 mg/ml n-capric acid ($C_{10:0}$) into the growth medium scarcely influenced on the fatty acid compositions (Tables 7 and 8).

The addition of various amounts of oleic acid into the growth medium had practically no effect on the bacterial growth at 5, 10 and 15°C (Figs. 27 and 28). Although remarkable increase of absorbance was observed at early stage of the growth at 5°C in the medium containing 0.5 mg/ml oleic acid (Fig. 27A), it seems to be attributed to overestimate the absorbance by insolubility of oleic acid under low temperature because this phenomenon was not found on the growth at a higher temperature, 10°C (Fig. 27B). Similar results were obtained on the growth with $C_{10:0}$ or $C_{11:0}$ at 5 and 15°C except that a short lag of the growth was observed at 5°C (Fig. 29).

Temperature-dependence of Na^+ and H^+ pump activities of the cells altered the fatty acid composition of total phospholipids

The respiration-dependent primary Na^+ and H^+ pump activities of the cells modified the fatty acid composition of total phospholipids by the growth with oleic acid or $C_{11:0}$ were assayed at various temperatures. Both pump activities of the cells grown at 15°C with Brij 58 alone at pH 6.5 and 8.5 (Figs. 30 and 31) had essentially the same temperature-dependence as those grown in the Tris-salts medium without the addition (Figs. 9 and 10). Interestingly, the drastic compositional change of fatty acid by the growth with $C_{11:0}$ also scarcely influenced the temperature-dependence of the both pump activities at pH 6.5 and 8.5 (Figs. 32 and 33). Similar results were

obtained with the cells grown at 5°C with Brij 58 alone or $C_{11:0}$ (Figs. 34 to 37). These results indicated that lowering of the growth temperature had no effect on the temperature-dependence of the both pump activities.

On the other hand, the cells cultivated with oleic acid were completely defect of the H^+ pump activity at 25°C at pH 8.5, while no change was detected in the temperature-dependence of Na^+ pump activity at pH 8.5 and both pump activities at pH 6.5 (Figs. 38 and 39).

Membrane fluidity of the intact cells

It was possible that change of the membrane fluidity resulted from the alteration of fatty acid composition of total phospholipids was responsible for the defect of H^+ pump activity at 25°C. Therefore, fluorescence anisotropies, which is a parameter for the membrane fluidity, of the intact cells grown at 5 or 15°C with various fatty acids were determined with trans-parinaric acid as a fluorescent probe (Table 9). In all cells tested, a large gaps of fluorescence anisotropies were observed between 15 and 25°C, indicating that alteration in physical state of the membrane occurred between these temperatures. However, no change of the membrane fluidity specific for the cells grown with oleic acid was detected at pH 8.5.

Motive forces for flagellar motor and amino acid uptake of Vibrio ABE-1

From above studies, it was found that Vibrio ABE-1 formed $\Delta\bar{\mu}_{Na^+}$ by the respiratory chain-linked primary Na^+ pump. In order to clarify the role of $\Delta\bar{\mu}_{Na^+}$ in energy metabolism, an attempt was made to identify the energy sources necessary to drive amino acid transport and motility in chemotactic response.

When flagellated bacteria such as Vibrio ABE-1 were spotted on such a soft agar containing an attractant of chemotactic response that they could swim, they have been reported to move about and spread on the agar (Adler, 1966). At first, NaCl-dependence of this phenomenon (called as swarm) was investigated with 0.2% agar plate containing 1 mM succinate as an attractant (Hagihara et al., unpublished data) and various concentrations of NaCl. Fig. 40 indicates that this bacterium can swim in response to the chemotactic attractant at both pHs of 6.5 and 8.5, and that the motility is more active at pH 8.5. The motility was scarcely observed on the agar containing 0.05 M NaCl at both pHs, but the more NaCl was supplemented in the agar, the higher motility was observed, indicating that it depended on the external NaCl concentration. Up to 10 μM of CCCP scarcely inhibited the motility at pH 8.5, but it was somewhat inhibited by 5 μM CCCP and was completely at 10 μM at pH 6.5 (Fig. 41). On the other hand, the motility was inhibited with similar extent by TPP at pH 6.5 and 8.5 (Fig. 42). These results

suggest that flagellar motor was driven by $\Delta\bar{\mu}_{Na^+}$ which was formed at pH 8.5 by the primary Na^+ pump but mainly at pH 6.5 by a secondary Na^+ translocation driven by proton motive force. However, $\Delta\bar{\mu}_{Na^+}$ formed by the primary Na^+ pump at pH 6.5 may also partially contribute to drive the motor, because the motility at pH 6.5 was less sensitive to CCCP than the growth of this bacterium at the same pH (Figs. 1 and 41).

Amino acid transport of this bacterium was examined by assaying uptake of non-metabolized amino acid analog AIB, which has been employed in studies for transport of several halophilic bacteria (Niven and MacLeod, 1980; Kushner et al., 1983; Tokuda et al., 1982). As shown in Table 1, since intracellular cation concentrations of Vibrio ABE-1 cells were changed by washing with different salt solutions, an artificial gradient of specified cation across the membrane could be formed by washing and suspending the cells with adequate salt solutions. AIB uptake driven by these artificial gradients of various cations across the membrane is shown in Fig. 43. A large Na^+ gradient is expected to be formed across the membrane by suspending the cells in TN and MN buffer after washing with TN or TK and MN or MK buffer, respectively (see Table 1). Indeed, these cells showed active AIB uptake at pH 6.5 and 8.5 in regardless of washing buffer (Fig. 43A and B). Whereas, AIB uptake was only slightly driven by Li^+ gradient at both pHs and by K^+ gradient at pH 8.5 when the cells were washed with TN or MN buffer (Fig. 43A

and B). Since Li^+ gradient did not drive any uptake at both pHs in the cells washed with the Na^+-free buffers (Fig. 43A and B), preceding AIB uptake appeared to be driven by Li^+ and K^+ gradients might depended on Na^+. These results suggest that only a membrane potential does not drive AIB uptake of Vibrio ABE-1 but $\Delta\bar{\mu}_{Na^+}$ does.

To ascertain whether the respiratory chain-dependent primary Na^+ pump contributes to drive the AIB uptake or not, Na^+ was loaded into the cells by treatment with diethanolamine to abolish the artificial gradient of Na^+ across the membranes and inhibitions of the AIB uptake of these Na^+-loaded cells by CCCP and TPP were investigated in the presence of succinate as a respiration substrate (Table 10). Although the procedure of diethanolamine treatment was modified to apply this bacterium, the artificial gradient was not completely dissipated because, particularly at pH 8.5, AIB was somewhat incorporated into the cells without succinate. Nevertheless, different sensitivities of the uptake to TPP and CCCP were obviously detected between pH 6.5 and pH 8.5, that is, 1 μM CCCP inhibited completely the uptake at pH 6.5 while the cells had a little uptake activity at pH 8.5 even in the presence of 10 μM CCCP. On the other hand, subtracting the uptake activity without succinate, which seemed to be driven by the artificial Na^+ gradient, from each uptake activity, the uptake activity at pH 8.5 was estimated to be almost completely inhibited by 50 μM TPP but about 50% of the uptake activity without TPP was observed in 50 μM

TPP at pH 6.5. These results suggest that drive force of AIB transport at pH 8.5 is $\Delta\bar{\mu}_{Na^+}$ formed by the primary Na^+ pump, but the drive force at pH 6.5 is mainly $\Delta\bar{\mu}_{Na^+}$ formed by a secondary Na^+ transporter such as Na^+/H^+ antiporter other than the primary Na^+ pump.

Discussion

CCCP-resistant growth similar to those reported with V. alginolyticus (Tokuda and Unemoto, 1983) was observed in Vibrio ABE-1 at the alkaline pH range. Hamaide et al. (1985) claimed that CCCP-resistant growth observed in the moderately halophilic Vibrio costicola at alkaline pH may be due to the inefficacy of CCCP by binding with amino-thiol compounds in a complex medium. Since the synthetic Tris-salts medium used in this study does not contain such the compounds, I concluded that the growth of Vibrio ABE-1 observed at alkaline pH in the presence of CCCP (Figs. 1 and 2) is not due to the inefficacy of the protonophore.

Several O_2-pulse experiments with anaerobic cell suspension of Vibrio ABE-1 (Figs. 3-5) indicated that CCCP-dependent alkalization of the cell suspension by the O_2-pulse was responsible for a respiration-dependent primary Na^+ pump, and not for a respiration-dependent primary K^+ pump and a cation antiporter such as Na^+/H^+ antiporter, because the alkalization did not cause in Na^+-free cell suspension by O_2-pulse with KCl and CCCP was present in the cell suspension. Furthermore, experiments using several inhibitors (Figs. 6-8) show that the CCCP-dependent alkalization is not attributed to Na^+/H^+ antiporter and an ATP-dependent ion translocation, and therefore confirm that the respiration-dependent primary Na^+ pump is present in this bacterium.

O_2-pulse experiments with the Na^+-cell suspension indicated that the activities of respiration-dependent H^+ and Na^+ pumps were detected both at pH 6.5 and 8.5 (Fig. 3). In the Na^+ free-cell suspension, however, the activities of the H^+ and Na^+ pumps strictly depended on the pH. That is to say, the activity of the H^+ and Na^+ pump can be observed only at pH 6.5 and 8.5, respectively (Fig. 4). These results suggest that extracellular NaCl may greatly affect the pH-dependence of the H^+ and Na^+ pumps, and my preliminary experiments indicate that the Na^+ pump may be activated by NaCl (data not shown). Although similar respiration-dependent primary Na^+ pump has been first reported in V. alginolyticus (Tokuda and Unemoto, 1981, 1982, 1983), Na^+ pumps of Vibrio ABE-1 and V. alginolyticus are different from each other in the functioning pH range, that is, the latter Na^+ pump functions only at alkaline pH.

Since NaCl is essential for the growth of Vibrio ABE-1 (Takada et al., 1979), respiration-dependent primary H^+ and Na^+ pumps must function simultaneously over a wide pH range. pH-dependence of the growth in the presence of CCCP (Fig. 2) indicates that the primary Na^+ pump is not sufficient to support the energy metabolism of the bacterial cells in the acidic pH region, because CCCP, which dissipates the H^+ gradient across membrane, completely inhibited the growth of Vibrio ABE-1 at below pH 7. On the other hand, the Na^+ pump alone may be enough for the energy metabolism of this bacterium at alkaline

pH, because this bacterium can grow in the presence of CCCP at alkaline pH. Several experiments for the chemotactic motility on a soft agar plate (Figs. 40-42) and for active transport of AIB (Fig. 43 and Table 10) suggest that both of flagellar rotation and uptake of several amino acids of this bacterium are driven by $\Delta\bar{\mu}_{Na^+}$ both at pH 6.5 and 8.5. The $\Delta\bar{\mu}_{Na^+}$ seems to be produced at pH 8.5 by the primary Na^+ pump whereas chiefly at pH 6.5 by a secondary Na^+-translocater such as Na^+/H^+ antiporter. However, it is likely that the primary Na^+ pump functioning at pH 6.5 also partially contribute to the generation of $\Delta\bar{\mu}_{Na^+}$ because the chemotactic motility at pH 6.5 is less sensitive to CCCP than the growth of the bacterium at the same pH (Figs. 1 and 41). These findings support the above presumption.

Na^+ pumps similar to those of V. alginolyticus and Vibrio ABE-1 have been also found in several other halotolerant and halophilic bacteria (Tsuchiya and Shinoda, 1985; Ken-Dror et al., 1984, 1986; Udagawa et al., 1986). These findings suggest that the respiration-dependent primary Na^+ pump may exist generally in halophilic and halotolerant bacteria. It has been reported that the transport of solutes are driven by $\Delta\bar{\mu}_{Na^+}$ in an extremely halophilic Halobacterium halobium (Lanyi, 1979) and in V. alginolyticus (Tokuda et al., 1982; Tokuda and Unemoto, 1982), and that a marine bacterium Alteromonas haloplanktis possesses a Na^+-substrate symporter (Niven and MacLeod, 1980). Furthermore, Dibrov et al. (1986a, 1986b)

found a flagellar motor driven by $\Delta\bar{\mu}_{Na^+}$ and ATP synthesis coupled with $\Delta\bar{\mu}_{Na^+}$ at alkaline pH in V. alginolyticus. These allow me to suppose that halophilic bacteria such as Vibrio ABE-1 probably utilize Na^+ as a coupling ion at various steps in the energy metabolism than other organisms or may use only Na^+ under some conditions, particularly at alkaline pH range. Such the Na^+-dependence in the energy metabolism might provide these bacteria with some advantages for living in Na^+-abundant environments. Furthermore, it is known that the intracellular pHs of most bacteria are kept about neutral, irrespective of external pH (Padan et al., 1981). If so, the ΔpH component of the proton motive force (Mitchell, 1976) will be completely dissipated or be formed in the reverse direction in the alkaline pH range. Under such the conditions, the primary Na^+ pump should play important roles.

Vibrio ABE-1 ought to synthesize ATP with $\Delta\bar{\mu}_{Na^+}$ at pH 8.5 at which it can grow in the presence of CCCP (Fig. 1). Thus, a Na^+-ATPase catalyzing this reaction, which corresponds to the well-known F_0F_1-ATPase, may be existent in this bacterium. Such the ATPase has been suggested to be present in P. modestum (Hilpert et al., 1984) and V. alginolyticus (Dibrov et al., 1986b) as mentioned above. In the former bacteria, the enzyme was recently purified and characterized (Laubinger and Dimroth, 1987, 1988). The enzyme contains soluble F_1 and membrane-bound F_0 parts which consist of 5 and 3 different subunits, respectively. Their molecular weights are similar to those

of the corresponding subunits of the E. coli F_0F_1-ATPase. In particular, it has been suggested that subunit composition of the F_1 part is the same as that of the F_1 of the E. coli ATPase. Sensitivities of the Na^+-ATPase to various inhibitors show that it is a F_0F_1-type but not an E_1E_2-ATPase. Interestingly, Na^+ stimulates ATP-hydrolyzing activity of the membrane-bound enzyme, but does not stimulate that of the purified F_1-ATPase. Furthermore, the Na^+-stimulation restores by reconstitution of the purified F_1 into membrane-bound F_0 part, implying that Na^+ is bound to F_0 of the enzyme complex. On the other hand, it was recently reported that H^+-ATPases of several archaebacteria were quite different from usual F_0F_1-ATPase (Nanba and Mukohata, 1987; Mukohata and Yoshida, 1987; Hochstein et al., 1987; Konishi et al., 1987; Lübben et al., 1987). These enzymes which resemble well each other, show simpler subunit compositions than F_0F_1-ATPase, and are rather similar to vacuolar type ATPase in sensitivities to various inhibitors. Furthermore, some of them are different from F_0F_1-type ATPase in immunological properties and the primary structure of several subunits deduced from the nucleotide sequence (Lübben et al., 1987; Mukohata et al., 1987; Denda et al., 1988a, 1988b). No similarity between the putative Na^+-ATPase of Vibrio ABE-1 and the unique H^+-ATPase was found in a double immunodiffusion test with the solubilized membrane proteins of Vibrio ABE-1 and antiserum against the purified H^+-ATPase of an archaebacterium, Sulfolobus acidocaldarius (data

not shown). It is very interesting to characterize the putative Na^+-ATPase of Vibrio ABE-1.

It is an important problem to determine the site of the respiratory chain to which the primary Na^+ pump is linked. Tokuda and Unemoto (1984) reported that the primary Na^+ pump of V. alginolyticus functioning at alkaline pH was inhibited strongly by HQNO, and that the bacterium possessed two different NADH oxidase systems. Namely, one was activated by Na^+ and showed the maximum activity at alkaline pH range and the other was independent of Na^+. The former was inhibited by HQNO whereas the later did not. Furthermore, they elucidated that NADH:quinone oxidoreductase was the site which was activated by Na^+ and inhibited by HQNO in the former NADH oxidase system, and concluded that the primary Na^+ pump was coupled to the NADH:quinone oxidoreductase of the respiratory chain. Furthermore, it has been suggested that the Na^+-dependent NADH oxidase translocated only Na^+ with NADH oxidation (Tokuda et al., 1985). In Vibrio ABE-1, both activities of the primary Na^+ pump at pH 6.5 and 8.5 were intensely inhibited by HQNO (Fig. 8). Furthermore, sensitivity to HQNO and salt-dependence of NADH oxidase and NADH:Q-1 oxidoreductase at pH 6.5 (Figs. 11-13 and Tables 3 and 4) indicated that NADH:quinone oxidoreductase was the Na^+-dependent and HQNO-sensitive site of NADH oxidase, and these also suggest that the primary Na^+ pump is coupled to the oxidoreductase at pH 6.5 similarly to that of V. alginolyticus. On the other hand, the coupling site of

the primary Na^+ pump at pH 8.5 could not be specified to NADH:quinone oxidoreductase from the properties of NADH oxidase and NADH:Q-1 oxidoreductase. Therefore, it is also likely that the coupling site is different from that at pH 6.5. If so, Vibrio ABE-1 has to possess two kinds of primary Na^+ pumps functioning different pH range. However, it was found that the coupling site was not present in succinate oxidase (Figs. 18 and 19). It is necessary for determination of the coupling site at pH 8.5 to identify and characterize the components of respiratory chain of Vibrio ABE-1.

How is the respiration-dependent primary Na^+ pump at pH 6.5 of Vibrio ABE-1 coupled to NADH:Q oxidoreductase and how does the pump extrude Na^+? Three types of decarboxylases in several facultative and obligately anaerobic bacteria described in "Introduction" have been purified, reconstituted into liposomes, and characterized in detail (Buckel and Liedtke, 1986; Dimroth, 1987). All decarboxylases (DCase) contains biotin and are remarkably activated by Na^+. Oxaloacetate DCase (ODCase) is somewhat activated by Li^+ but not by K^+, and is completely inactive without Na^+ or Li^+. Na^+-dependent step in each decarboxylation reaction is decarboxylation of a carboxybiotin-enzyme intermediate, but not carboxyl transfer from the substrates to biotin. These DCases consist of 3 or 4 different subunits; their molecular weights are 65 and 120 (α subunit), 34 and 60 (β), and 12 and 30 kDa (γ) in ODCase of K. pneumoniae and glutaconyl-CoA DCase (GDC-

ase) of A. fermentans, respectively. Biotin is contained in each a subunit in the both DCases. Carboxyltransferase activity resides in the α subunit of ODCase and the β subunit of GDCase, which are peripheral membrane proteins. On the other hand, decarboxylase activity of the intermediates seems to be contained in the β subunit or the $\beta+\gamma$ subunits of ODCase and the γ subunit of GDCase, and it is suggested that Na^+ may be extruded by the β and γ subunits of ODCase and GDCase, respectively, during the decarboxylation of the intermediates. These subunits are integral membrane proteins with similar molecular mass and are protected from tryptic digestion by Na^+.

On the other hand, Na^+-stimulated and HQNO-sensitive NADH:quinone oxidoreductase of V. alginolyticus was also purified and characterized (Hayashi and Unemoto, 1984, 1986, 1987). This enzyme contains three different subunits with the molecular weights of 52 (α subunit), 46 (β), and 32 kDa (γ). Since total mass of the enzyme is 254 kDa, its subunit composition appears to be a dimer of $\alpha\beta\gamma$ or $\alpha_2\beta_2\gamma_2$. This enzyme produces ubisemiquinone as an intermediate in the course of the reduction of ubiquinone to ubiquinol. The β subunit contains 1 molecule of FAD/subunit and catalyzes the reduction of ubiquinone to ubisemiquinone. This reaction step is Na^+-independent and HQNO-insensitive. On the other hand, the α subunit contains 1 molecule of FMN/subunit and catalyzes the formation of ubiquinol from ubisemiquinone (quinone reductase), which is Na^+-dependent and HQNO-sensitive. Furthermore,

it has been suggested that Na^+-translocation was coupled to the reaction catalyzed by this subunit. The γ subunit is essential for the quinone reductase and increases the affinity of the β subunit for Q-1. In spite of such extensive studies, the mechanisms of Na^+-translocation in these enzymes are still obscure.

To study these problems in Vibrio ABE-1, I tried to purify membrane-bound NADH-DH. However, in spite of the solubilization of the enzyme with several detergents and use of various purification techniques, the purification was quite incomplete possibly owing to an instability of the solubilized enzyme. Every efforts for the stabilization of the enzyme were not successful. Furthermore, it is also likely that content of NADH-DH in Vibrio ABE-1 is lower than those of other constituents in the respiratory chain as reported in mitochondria and Paracoccus denitrificans (Capaldi, 1982; Gupte et al., 1984; Albracht et al., 1980). Therefore, it may be necessary for conquest of these difficulties to overproduce the enzyme by cloning and amplification of its gene as performed in E. coli (Jaworowski et al., 1981a, 1981b; Young et al., 1981).

It has been reported that most of purified bacterial NADH-DHs were single polypeptides or composed of only one subunit species (Hochstein and Dalton, 1973; Mains et al., 1980; Walsh et al., 1983; Wakao et al., 1987; Bergsma et al., 1982; Kawada et al., 1981; Hisae et al., 1983). Therefore, they are much simpler than the corresponding enzyme complex (complex I) of mitochondria,

which is composed of more than 20 different polypeptides (Ragan 1987). However, it was recently found that NADH-DHs of Paracoccus denitrificans (Yagi, 1986) and V. alginolyticus (Hayashi and Unemoto, 1987) contained 10 and 3 different polypeptides, respectively.

In E. coli, Dancey et al. (1976) first purified NADH :DCIP oxidoreductase, which was a single polypeptide (M_r 38 kDa) and absolutely required FAD for the activity. They considered that the enzyme should be a component of respiratory NADH oxidase from its kinetic similarity to NADH oxidase and inhibition of the oxidase activity by antisera against the purified enzyme. Then, by cloning and amplification of the enzyme gene (ndh) and its in vivo overproducing, NADH:Q oxidoreductase was purified (Jaworowski et al., 1981a, 1981b). The purified enzyme was a single polypeptide of 47,304 Da deduced from the nucleotide sequence, contained 1 molecule of FAD/polypeptide and no iron-sulfur clusters, and could reconstitute membrane-bound, cyanide-sensitive NADH oxidase activity in membrane vesicles prepared from ndh mutant strains (Young et al., 1981; Jaworowski et al., 1981b). At almost the same time, similar NADH:quinone oxidoreductase (M_r 46 kDa) was purified by Thomson and Shapiro (1981). This enzyme contained about 1 atom of Fe/polypeptide and its activity was stimulated by FAD. However, two different NADH-DHs were recently found (Ohnishi et al., 1987; Matsushita et al., 1987) as previously expected from a crossed immunoelectrophoretic study of E. coli membrane

proteins (Owen and Kaback, 1979). One of the enzymes contained Fe-sulfur clusters and oxidized both of NADH and deamino-NADH, and the oxidation was coupled to H^+-translocation. Another enzyme exclusively oxidized NADH and did not lead to concomitant generation of $\Delta\bar{\mu}_{H^+}$. Furthermore, it was supposed from several enzymatic properties that the later enzyme was same as the 47,304 Da enzyme encoded by the ndh gene. Because of its inactivation by solubilization from the membranes, the former enzyme has not been purified yet. On the other hand, physiological roles of the remaining 38 k, 46 k and 47,304 Da enzymes is unknown at present. Similar existence of two different NADH-DHs has been expected in Micrococcus lysodeikticus by crossed immunoelectrophoretic studies of membrane proteins (Owen and Salton, 1975; Crowe and Owen, 1983) and was confirmed in Thermus thermophilus (Yagi et al., 1988). Simultaneously, these enzymes were purified from T. thermophilus. One of the enzymes was composed of 10 different polypeptides of molecular weights between 16 and 70 kDa, contained FMN, non-heme iron and acid-labile sulfide, and the authors supposed from similarities of several enzymatic properties to the known NADH:quinone reductases coupling to H^+-translocation that this enzyme involved an energy-coupling site. Another one was a single polypeptide (53 kDa), contained FAD and no Fe-sulfur clusters, and might not lead to generation of $\Delta\bar{\mu}_{H^+}$. Since all the above simpler NADH-DHs purified from several bacteria have not been examined for ability of H^+-

translocation, it is much likely that additional subunit(s) and/or other different enzyme(s) may be found in these bacteria as well as in E. coli and T. thermophilus.

Also in Vibrio ABE-1, two NADH-DHs differing in Na^+-dependence and HQNO-sensitivity should be present as mentioned above. This anticipation is supported by the fact that at least two immunoprecipitates showing NADH-DH activity are detected in preliminary crossed immunoelectrophoretic experiments of the solubilized membrane proteins with antisera against these proteins or partially purified NADH-DH (data not shown). Furthermore, a soluble NADH:DCIP oxidoreductase was purified from this bacteria and characterized (Takada et al., 1988). This enzyme exhibited different properties from those of the known diaphorases such as lipoamide dehydrogenase, NADH:cytochrome c reductase, NAD(P)H:quinone oxidoreductase and so on, and its physiological role is unknown at present. However, because NADH-DHs of a few thermophilic bacteria have been shown to be peripheral membrane proteins (Mains et al., 1980; Walsh et al., 1983; Wakao et al., 1987) and several enzymes of the mitochondrial matrix can bind to the inner mitochodrial membrane (D'souza and Srere, 1983; Sumegi and Srere, 1984), it is also likely that the soluble NADH-DH may be loosely bound to the membrane and associated with the respiratory chain. To resolve these possible complicated participations of several proteins into NADH-DH segment of respiratory chain of Vibrio ABE-1 should be also significant for understanding of many bac-

terial respiratory chains. The membrane-bound NADH-DH of Vibrio ABE-1 must be purified in this respect, too.

By the identification of natural electron and proton acceptor of NADH:quinone oxidoreductase, it was found that Vibrio ABE-1 grown aerobically contained predominantly Q-8 and a small amount of Q-7, and no menaquinones which exist in E. coli (Polglase et al., 1966; Haddock and Jones, 1977), Proteus rettgeri (Kroger et al., 1971) and V. alginolyticus (Unemoto and Hayashi, 1979). Because the menaquinone was much more contained in anaerobically grown E. coli cells than those grown aerobically (Polglase et al., 1966; Haddock and Jones, 1977) and the two kinds of quinones function differently from each other in the respiratory chain (Newton et al., 1971; Ingledew and Poole, 1984), some menaquinone(s) may be detected if this bacterium is grown anaerobically.

For active growth of psychrophilic bacteria at low temperatures, the energy transducing systems of these bacteria must actively operate at low temperatures. Okuyama et al. (1979) reported that the rate of ATP synthesis coupled with NADH oxidation was higher in Vibrio ABE-1 than in P. aeruginosa at 7°C. In this study, activities of the membrane-bound NADH-DHs from Vibrio ABE-1 and V. parahaemolyticus at 5°C were about 70 and 30% of the maximum activities at 20 and 30°C, respectively, and the specific activities of the former enzyme were higher than those of the later enzyme below 10°C, showing that the NADH-DH of Vibrio ABE-1 retained considerably high activ-

ity at low temperature (Fig. 20). Although urocanase and histidine ammonia-lyase of a psychrotrophic Pseudomonas putida known as typical psychrophilic enzymes exhibit 30% of the respective maximum activity at 0 and 1.5°C, respectively (Hug and Hunter, 1974a, 1974b), the activity of the NADH-DH of Vibrio ABE-1 at low temperature was comparable to or rather higher than those of the enzymes from P. putida. Moreover, the primary H^+ and Na^+ pumps of this bacterium were very active over the temperature range for growth. These findings all support cold-loving nature of this bacterium.

Estimations of the activities of the primary H^+ and Na^+ pumps and the membrane fluidities of Vibrio ABE-1 cells after alteration of the fatty acid composition with exogenous fatty acids (Figs. 30-39 and Tables 6-9) showed that both pumps were active at low temperatures in spite of the drastic changes of fatty acid compositions, and that the complete loss of the H^+ pump at 25°C was not due to the change in physical properties of whole membrane. However, these results does not completely exclude some effects of membrane lipids on the maintenance of the pump activities at low temperatures. Many membrane-bound enzymes require the specified lipid(s) for the activities (Coleman, 1973; Sandermann, 1978), and therefore lipid(s) bound to and/or close to these enzyme proteins should have critical effects on their activities. NADH-DH purified from an extremely thermophilic, Bacillus caldotenax shows a linear Arrhenius plot, whereas the membrane-bound

enzyme and the purified enzyme mixed with the extracted membrane lipids do two discontinuities in the plots (Kawada and Nosoh, 1981). Temperatures of the break points are substantially consistent with those in a fluorescence anisotropy of the membrane and the extracted membrane lipids of the bacteria. The discontinuity and break temperature in the plots are altered by mixing the enzyme with various lipids. Since the loss of H^+-pump activity at 25°C was only observed in the cells grown with oleic acid (Fig. 39), it might be attributed to the change of acyl chain(s) of phospholipids around the pump protein by the addition of oleic acid, but not to thermolability of the pump protein.

Another possibility for the maintenance of high activity of the pumps at low temperatures is that it may be due to high activeness of the pump proteins for themselves. However, the information for the mechanism that the enzyme proteins, particularly membrane-bound enzymes, of psychrophilic bacteria actively function at low temperatures is as good as nothing at present. At all events, the primary H^+ and Na^+ pumps and the membrane-bound NADH-DH of Vibrio ABE-1 must be suitable research materials for studying these mechanisms.

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Table 1 Intracellular Na^+ and K^+ concentrations of *Vibrio* ABE-1 cells washed with various buffers

Wash buffer	Buffer pH	Intracellular concentration	
		Na^+	K^+
Na^+-containing buffer		(mM)	(mM)
MGN	6.5	68	377
TGN	8.5	30	358
Na^+-free buffer			
TGK	8.5	16	675

The harvested cells were washed three times with one of the indicated buffers and intracellular Na^+ and K^+ concentrations were determined as described in Materials and Methods.

Table 2 Cell growth and contributions of H^+ and Na^+ pumps to the respiration-dependent pH change of cell suspension at pH 6.5 and 8.5

	pH 6.5		pH 8.5	
	H^+ pump	Na^+ pump	H^+ pump	Na^+ pump
Na^+-cell suspension				
Acidification	+[a]	-[b]	+	-
CCCP-dependent alkalization	-	+	-	+
Na^+ free-cell suspension				
Acidification	+	-	-	-
CCCP-dependent alkalization	-	-	-	+
Cell growth in the presence of CCCP[c]	Not grow		Grow	

a,b: + and - denote the presence or absence of H^+ or Na^+ pump contribution to the phenomena.

c: Cells were grown in the Tris-salts medium containing 0.4 M NaCl.

Table 3 Effects of NaCl and KCl on NADH:Q-1 oxidoreductase activity

Added salts	Specific activity at pH	
	6.5	8.5
	units/mg protein (%)	
No additions	0.155 (100)	0.272 (100)
NaCl 0.2 M	0.198 (128)	0.348 (128)
0.5 M	0.128 (83)	0.206 (76)
KCl 0.2 M	0.136 (88)	0.234 (86)
0.5 M	0.058 (37)	0.112 (41)

NADH:Q-1 oxidoreductase activity was assayed with the addition of the indicated concentrations of NaCl or KCl to the reaction mixture. Regardless of the addition of salts, final 25 mM of NaCl was carried over in all reaction mixtures with the enzyme sample. Values in parentheses represent relative activities to the respective control (no addition).

Table 4 Effect of HQNO on NADH:Q-1 oxidoreductase activity

Additions	Specific activity at pH	
	6.5	8.5
	units/mg protein (%)	
In the presence of 25 mM NaCl		
Ethanol	0.149 (100)	0.241 (100)
HQNO 1 μM	0.110 (74)	0.192 (80)
2.5 μM		0.160 (66)
5 μM	0.090 (61)	0.134 (56)
10 μM	0.069 (47)	
In the presence of 0.425 M NaCl		
Ethanol	0.101 (100)	0.173 (100)
HQNO 1 μM	0.065 (65)	0.150 (87)
2.5 μM		0.130 (75)
5 μM	0.032 (32)	0.086 (50)
10 μM	0.028 (28)	

NADH:Q-1 oxidoreductase activity was assayed in the presence of various concentrations of HQNO without or with the addition of 0.4 M NaCl to the reaction mixture. Regardless of the addition of NaCl, final 25 mM of NaCl was carried over in all reaction mixtures with the enzyme sample. Values in parentheses represent relative activities to those in the presence of ethanol.

Table 5 Purification of membrane-bound NADH-DH

Purification step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Purification (-fold)	Yield (%)
Solubilized enzyme	110	1,140	1,064	0.933	1.00	100
TEAE-cellulose	127	217	167.7	0.774	0.83	15.8
Ammonium sulfate fractionation	10	54.3	98.6	1.818	1.95	9.3
Sepharose CL-4B	74	41.4	25.9	0.624	0.67	2.4
DEAE-Sephadex A-50	35	17.7	9.24	0.522	0.56	0.9

NADH-DH activity was assayed with the standard reaction mixture. Details of each purification step are described in the text. About 65 g fresh weight of the cells was used for the purification.

Table 6 Fatty acid compositions of total phospholipids from the cells grown with various amounts of oleic acid

Fatty acid	No additions	0.4% Brij58	0.4% Brij58 +20mg C18:1	0.4% Brij58 +50mg C18:1	0.4% Brij58 +100mg C18:1
10:0	3.3±0.4	3.6±0.3	3.3±0.4	2.8±0.3	2.7±0.3
12:0	1.1±0.3	1.3±0.2	1.1±0.2	0.7±0.3	0.7±0.1
N.I.	0.1±0.1	0.1±0.1	0± 0.05	0	0
14:0	0.7±0.1	0.8±0.1	1.1±0.1	1.1±0.2	1.1±0.3
14:1	1.3±0.3	1.4±0.2	0.8±0.1	0.5±0.2	0.4±0.3
15:0	0± 0.06	0	0± 0.05	0	0
15:1	0± 0.07	0± 0.05	0± 0.05	0	0
16:0	20.1±0.9	20.3±0.7	19.9±0.8	17.4±0.7	15.3±1.0
16:1	68.7±2.0	67.6±1.2	61.1±2.8	60.7±3.1	58.4±1.0
17:1	1.4±0.4	1.5±0.1	1.5±0.3	1.1±0.2	1.0±0.2
18:1	3.2±0.5	3.2±0.4	10.9±2.6	15.1±2.1	19.7±0.9
19:0	0.1±0.1	0.2±0.2	0.3±0.1	0.6±0.2	0.7±0.1

All values are expressed as weight percentage of total fatty acids and show mean±standard deviation of six or nine determinations with three independent preparations. The amounts of oleic acid shown in the table represent that added into 100 ml of the growth medium.

N.I., Not identified.

Table 7 Fatty acid compositions of total phospholipids of the cells grown with different fatty acids at 15°C

Fatty acid	No additions	0.4% Brij58	0.4% Brij58 +20mg $C_{10:0}$	0.4% Brij58 +20mg $C_{11:0}$
9:0	0	0	0	0.3±0.1
10:0	2.8±0.3	3.1±0.5	2.8±0.3	0.5±0.3
11:0	0± 0.03	0	0	3.5±0.8
12:0	1.1±0.3	1.0±0.3	0.6±0.2	0.2±0.1
12:1	0± 0.1	0	0	0
13:1	0	0± 0.12	0.2±0.3	0.8±0.8
14:0	0.6±0.3	0.7±0.1	0.8±0.1	0.2±0.2
14:1	1.5±0.3	1.6±0.2	1.3±0.1	0.2±0.2
15:0	0± 0.08	0.1±0.1	0± 0.03	11.4±1.7
15:1	0.1±0.1	0.1±0.1	0	13.7±1.8
16:0	19.6±0.9	19.6±1.2	22.1±1.1	5.2±2.2
16:1	69.4±1.8	69.1±1.5	67.0±1.9	17.0±7.5
17.0	0	0	0	6.7±2.4
17:1	0.5±0.7	0.5±0.8	0.7±0.9	39.2±5.6
N.I.	1.1±0.9	0.8±0.8	0.8±1.2	0
18:0	0.2±0.3	0.2±0.2	0.2±0.3	0.2±0.3
18:1	2.8±0.6	2.9±0.8	3.2±0.5	0.8±0.4
19:0	0.3±0.3	0.3±0.3	0.3±0.3	0.1±0.1
19:1	0± 0.12	0± 0.17	0± 0.03	0± 0.08

All values are expressed as weight percentage of total fatty acids and show mean±standard deviation of seven to thirteen determinations from three or four independent preparations. The amounts of the fatty acids shown in the table represent that added into 100 ml of the growth medium.

N.I., Not identified.

Table 8 Fatty acid compositions of total phospholipids of the cells grown with different fatty acids at 5°C

Fatty acid	No additions	0.4% Brij58	0.4% Brij58 +20mg $C_{10:0}$	0.4% Brij58 +20mg $C_{11:0}$
9:0	0	0	0	0.3±0.04
10:0	3.4±0.5	3.8±0.4	5.3±0.3	0.8±0.4
11:0	0	0	0	3.1±0.3
12:0	1.3±0.3	1.2±0.4	0.4±0.3	0.2±0.2
12:1	0	0	0	0
13:1	0	0.1±0.2	0	0.3±0.4
14:0	1.0±0.1	1.0±0.2	0.7±0.3	0.4±0.2
14:1	3.6±0.2	3.4±0.5	2.3±0.3	0.8±0.4
15:0	0	0.1±0.1	0± 0.05	9.6±1.6
15:1	0.2±0.2	0.1±0.1	0.1±0.1	20.6±2.3
16:0	16.3±0.6	16.6±0.9	18.2±2.0	6.5±0.7
16:1	71.2±0.9	69.9±2.0	69.5±1.9	25.4±6.1
17:0	0	0	0	3.7±1.3
17:1	0.7±0.8	0.5±0.7	0.6±0.9	27.3±3.0
N.I.	0.4±0.6	0.9±0.6	0.5±0.5	0
18:0	0	0.1±0.1	0± 0.08	0.1±0.2
18:1	1.7±0.2	2.1±0.8	2.2±0.3	0.8±0.3
19:0	0.2±0.2	0.2±0.2	0.2±0.2	0.1±0.1
19:1	0	0	0	0± 0.04

All values are expressed as weight percentage of total fatty acids and show mean±standard deviation of seven to thirteen determinations with three or four independent preparations. The amounts of the fatty acids shown in the table represent that added into 100 ml of the growth medium.

N.I., Not identified.

Table 9 Fluorescence anisotropy of the intact cells grown with different fatty acids

<Cells prepared at pH 6.5>

Growth temperature (°C)	Growth media	Fluorescence anisotropy at 5°C	15°C	25°C	40°C
5	0.4% Brij58	0.113	0.113	0.047	0.065
	0.4% Brij58 + 20mg $C_{11:0}$	0.122	0.133	0.053	0.082
15	0.4% Brij58	0.094	0.088	0.040	0.047
	0.4% Brij58 + 20mg $C_{11:0}$	0.100	0.093	0.056	0.066
	0.4% Brij58 + 50mg $C_{18:1}$	0.141	0.151	0.096	0.118

<Cells prepared at pH 8.5>

Growth temperature (°C)	Growth media	Fluorescence anisotropy at 5°C	15°C	25°C	40°C
5	0.4% Brij58	0.091	0.091	0.052	0.035
	0.4% Brij58 + 20mg $C_{11:0}$	0.097	0.093	0.052	0.037
15	0.4% Brij58	0.100	0.102	0.054	0.049
	0.4% Brij58 + 20mg $C_{11:0}$	0.101	0.108	0.054	0.048
	0.4% Brij58 + 50mg $C_{18:1}$	0.124	0.127	0.057	0.052

The fluorescent anisotropy, $\underline{r}$, was calculated by the following equation (Shinitzky and Inbar, 1974):

$$\underline{r}=(I_{/\!/}-I_{\perp})/(I_{/\!/}+2I_{\perp}),$$

where $I_{/\!/}$ and $I_{\perp}$ was the emission intensity parallel and perpendicular to the excitation polarizer, respectively.

Table 10 Effects of TPP and CCCP on AIB uptake

Additions	AIB uptake at pH	
	6.5	8.5
	(nmol/20 min/mg protein)	
Control	10.11	15.54
50 μM TPP	5.44	3.78
1 mM TPP	0.18	0
Without succinate and KCl	0.51	3.44
Control	9.03	15.92
1 μM CCCP	0	18.58
10 μM CCCP	0	5.05
Without succinate and KCl	0.69	3.10

AIB uptake was assayed with Na^+-loaded cells prepared as described in Materials and Methods. Values in the table indicate the uptake for 20 min at 15°C. Non-specific attachment of AIB to the cells was corrected with subtracting radioactivity of the uptake in the presence of 50 μM CCCP and 2 mM TPP.

Legends to Figures

Fig. 1 Effect of CCCP on the growth of Vibrio ABE-1 at pH 6.5 and 8.5.

The growth of Vibrio ABE-1 at 15°C on the Tris-salts medium containing 50 mM of either MES-NaOH (pH 6.5; **C**) or Tris-HCl (pH 8.5; **D**) was monitored. The pH of the medium was simultaneously measured (**A** and **B**, respectively). At 0 time, various concentrations of CCCP were added to the medium as follows: 0 (○), 0.5 (△), 1.0 (□), and 2.5 (●) μM in the growth at pH 6.5; 0 (○), 2.5 (●), 5 (▲), 10 (■), and 20 (▽) μM in the growth at pH 8.5.

Fig. 2 Specific growth rate of Vibrio ABE-1 as a function of pH in the medium.

The growth of Vibrio ABE-1 at 15°C on the Tris-salts medium was monitored. At 0 time, 0 (○) or 2.5 μM CCCP (●) was added to the medium.

Fig. 3 Respiration-dependent proton flows induced by O_2 pulse.

The bacterial cells were washed and suspended in MGN (pH 6.5; **A** and **C**) or TGN (pH 8.5; **B** and **D**) buffer under anaerobic conditions. At the time points indicated by arrowheads, 50 μl of O_2-saturated 0.5 M NaCl was injected into the suspension. TPP (**A** and **B**) or CCCP (**C** and **D**) was added to the suspension at final concentrations of 2.5 mM and 62.5 μM, respectively. 1.25% ethanol (a), 2.5 μM

(b), 12.5 μM (c), 62.5 μM CCCP (d), 0.625 mM (e), 1.25 mM (f), or 2.5 mM TPP (g) was contained in the suspension. An upward change represents acidification of the suspension.

Fig. 4 Respiration-dependent proton flows in the cells prepared with Na^+-free buffers.

The bacterial cells were washed and suspended in MGK (pH 6.5; **A**) or TGK (pH 8.5; **B**) buffer under anaerobic conditions. At the time points indicated by arrowheads, 50 μl of O_2-saturated 0.5 M KCl (a and b) or NaCl (c and d) was injected into the cell suspension, which contained 2.5 mM TPP (a and c) or 62.5 μM CCCP (b and d). An upward change represents acidification of the suspension.

Fig. 5 Effect of NaCl on CCCP-dependent alkalization.

The bacterial cells were washed and suspended in TGK buffer (pH 8.5) under anaerobic condition. CCCP was added to the suspension at a final concentration of 62.5 μM. At the time points indicated by arrowheads, 50 μl of O_2-saturated 0.05 M (a), 0.2 M (b), or 0.5 M (c) was injected into the suspension. A downward change represents alkalization of the suspension.

Fig. 6 Effects of monensin and amiloride on CCCP-dependent alkalization.

The bacterial cells were washed and suspended in MGN (pH 6.5; **A** and **C**) or TGN (pH 8.5; **B** and **D**) buffer under

anaerobic conditions. At the time points indicated by arrowheads, 50 µl of O_2-saturated 0.5 M NaCl was injected into the suspension. All assays were performed in the presence of 62.5 µM CCCP. Amiloride was added to the cell suspensions as a water solution. 1.25% ethanol (a), 31.25 µM (b), 62.5 µM (c), 125 µM monensin (d), 0.625% ethanol (e), 125 µM (f), or 250 µM amiloride (g) was present in the suspension. A downward change represents alkalization of the suspension.

Fig. 7 Effects of DCCD and Na_3VO_4 on CCCP-dependent alkalization.

The bacterial cells were washed and suspended in MGN (pH 6.5; **A** and **C**) or TGN (pH 8.5; B and **D**) buffer under anaerobic conditions. Before the assay, the cell suspension was incubated for 30 min in an ice bath with the indicated concentrations of DCCD or Na_3VO_4. At the time points indicated by arrowheads, 50 µl of O_2-saturated 0.5 M NaCl was injected into the suspension. All assays were performed in the presence of 62.5 µM CCCP. Na_3VO_4 was added to the cell suspensions as a water solution. 1.25% ethanol (a), 0.1 mM (b), 0.3 mM (c), 1 mM DCCD (d), 0.625% ethanol (e), 0.1 mM (f), 0.3 mM (g), 0.5 mM (h), or 1 mM Na_3VO_4 (i) was present in the suspension. A downward change represents alkalization of the suspension.

Fig. 8 Effect of HQNO on CCCP-dependent alkalization.

The bacterial cells were washed and suspended in MGN (pH 6.5; A) or TGN (pH 8.5; B) buffer under anaerobic conditions. The assay was performed as described in the legend of Fig. 6 except that various concentrations of HQNO was added to the cell suspension instead of amiloride or monensin. 1.25% Ethanol (a), 10 μM (b), 25 μM (c), 50 μM (d), or 100 μM HQNO (e) was present in the suspension. A downward change represents alkalization of the suspension.

Fig. 9 Temperature-dependence of the activities of H^+ and Na^+ pumps at pH 6.5.

The bacterial cells were washed and suspended in MGN buffer (pH 6.5) under anaerobic condition. At the time points indicated by arrowheads, 50 μl of O_2-saturated 0.5 M NaCl was injected into the suspension. TPP and CCCP was added to the suspension at final concentrations of 2.5 mM and 62.5 μM in the left and right series of the assays, respectively. pH was monitored at 5 (a), 15 (b), 25 (c), or 40°C (d). An upward change represents acidification of the suspension.

Fig. 10 Temperature-dependence of the activities of H^+ and Na^+ pumps at pH 8.5.

Assays were performed as described in the legend to Fig. 9 except that the bacterial cells were washed and suspended in TGN buffer (pH 8.5).

Fig. 11 Effects of NaCl and KCl on NADH oxidase activity.

NADH oxidase activity was assayed with the standard reaction mixture containing the indicated concentrations of NaCl (**A**) or KCl (**B**) at pH 6.5 (○) and 8.5 (△).

Fig. 12 Optimum pH of NADH oxidase activity.

The enzyme activity was assayed at the indicated pHs without salts added (**A**) or with the addition of 0.4 M NaCl (**B**) or 0.4 M KCl (**C**). In any case, however, each reaction mixture contained final 25 mM of NaCl came from the enzyme solution. Three buffer systems, 0.1 M Tris-HCl (●), 0.1 M MES-diethanolamine (○) and 0.1 M MOPS-diethanolamine (△) were used. The enzyme activities are represented as relative values to the maximum activity obtained by the assay without the added salts (at pH 7.88 in **A**).

Fig. 13 Effect of HQNO on NADH oxidase activity.

The enzyme activity was assayed in the presence of various concentrations of HQNO without (○) or with the addition of 0.4 M NaCl (△) at pH 6.5 (**A**) and 8.5 (**B**). Regardless of the addition of NaCl, final 25 mM of NaCl was carried over in all reaction mixtures with the enzyme sample.

Fig. 14 Thin-layer chromatogram of quinones extracted from Vibrio ABE-1.

Thin-layer chromatography was carried out as described in Materials and Methods. **A**, vitamin K_1; **B**, Q-10; **C**, quinones extracted from Vibrio ABE-1.

Fig. 15 Reverse-phase partition high performance liquid chromatogram of quinones of Vibrio ABE-1.

Reverse-phase partition HPLC with a TSKgel ODS-80T_M column was carried out as described in Materials and Methods. Sample was injected into the column at 0 time. Arrowheads indicate the positions at which Q-6 (CoQ_6) and Q-10 (CoQ_{10}) used as a standard were eluted under the same chromatographic conditions.

Fig. 16 Ultraviolet absorption spectra of the purified quinone in methanol/propane 2-ol mixture.

Ultraviolet absorption spectrum of major peak quinone of Fig. 15 was analyzed with a Shimadzu spectrophotometer UV-180 (line **A**). Line **B** represents the spectrum of the quinone reduced by sodium borohydride.

Fig. 17 Mass spectrum of the purified quinone of Vibrio ABE-1.

Major peak quinone shown in Fig. 15 was analyzed as described in Materials and Methods.

Fig. 18 Effects of NaCl and KCl on succinate oxidase activity.

Succinate oxidase activity was assayed with the standard reaction mixture containing the indicated concentrations of NaCl (○) or KCl (△) at pH 8.5.

Fig. 19 Effect of HQNO on succinate oxidase activity.

Succinate oxidase activity was assayed at pH 8.5 without (○) or with the addition of 0.4 M NaCl (△) to the standard reaction mixture containing the indicated concentrations of HQNO. Regardless of the addition of NaCl, final 34 mM of NaCl was carried over in all reaction mixtures with the enzyme sample and sodium succinate.

Fig. 20 Effect of temperature on the activities of membrane-bound NADH-DHs of Vibrio ABE-1 and Vibrio parahaemolyticus.

NADH-DH activity was assayed at different temperatures and corrected by subtraction of non-enzymatic ferricyanide reduction and of enzymatic ferricyanide reduction without NADH at the respective temperatures. 0.25 M and 0.5 M NaCl was added to the reaction mixture for the assay of the enzymes of Vibrio ABE-1 (○) and Vibrio parahaemolyticus (△), respectively.

Fig. 21 Solubilization of membrane-bound NADH-DH by various concentrations of Triton X-100.

The membrane fraction of Vibrio ABE-1 was suspended

in 20 mM Tris-HCl (pH 7.5) containing 16% (v/v) glycerol and 2 mM mercaptoethanol at a concentration of 20 mg protein/ml. The membrane suspension was mixed with the equal volume of 20 mM EDTA containing the indicated final concentrations of Triton X-100 and stirred for 30 min at 4°C. The mixture was then centrifuged at 48,000 x g for 1 hr and the supernatant was collected. Protein content in the supernatant was determined by the method of Lowry et al. (1951). The solubilized NADH-DH activity (○) and proteins (△) are expressed as relative values to those of the membrane suspension before the solubilization, respectively.

Fig. 22 Polyacrylamide gel electrophoresis of the solubilized and partially purified NADH-DH in the presence of Triton X-100.

About 50 μg of the purified enzyme (A) and 100 μg of the solubilized (B) was applied to the gels, respectively.

Fig. 23 Effect of Triton X-100 on the activity of the purified NADH-DH.

NADH-DH activity was assayed as described in Materials and Methods except that NaCl was excluded from the standard reaction mixture, each of various concentrations of Triton X-100 was added to the mixture and the mixture was incubated for 1 min at 20 °C before start of the reaction. Each enzyme activity is expressed as a relative

value to that without the added Triton X-100 (final 0.0033% of Triton X-100 was carried over with the enzyme preparation).

Fig. 24 Effects of soybean asolectin and phopholipids of Vibrio ABE-1 on the purified NADH-DH activity.

The enzyme activity was assayed as described in the legend of Fig. 23 except that various concentrations of asolectin (△) or phopholipids of Vibrio ABE-1 (○) was added to the reaction mixture instead of Triton X-100 and the reaction was started without incubation. Phospholipids of Vibrio ABE-1 grown at 10°C were prepared as described in Materials and Methods. Asolectin and phospholipids were dispersed in distilled water with a brief sonication just before use.

Fig. 25 Effects of salts on the purified NADH-DH activity.

The enzyme activity was assayed with the standard reaction mixture containing the indicated concentrations of the following salts: ○, NaCl; △, KCl; ●, $MgCl_2$.

Fig. 26 Effect of temperature on the purified NADH-DH activity.

The enzyme activity was assayed at the indicated temperatures with ferricyanide as an electron acceptor as described in Materials and Methods.

Fig. 27 Effects of various amounts of oleic acid on the growth of Vibrio ABE-1 at 5 and 10°C.

The growth of Vibrio ABE-1 on the Tris-salts medium containing 0.4% Brij 58 and various amounts of oleic acid was monitored at 5 (**A**) and 10°C (**B**). 0.4% Brij 58 was used to disperse oleic acid. 0% (○), 0.4% Brij 58 (△), or 0.4% Brij 58 and 20 mg (□) or 50 mg oleic acid (●) was added to 100 ml of the medium.

Fig. 28 Effects of various amounts of oleic acid on the growth of Vibrio ABE-1 at 15°C.

Monitoring the growth at 15°C and symbols used are the same as Fig. 27. In addition, 0.4% Brij 58 and 100 mg oleic acid (▲) was added to 100 ml of the medium.

Fig. 29 Effects of n-capric acid and n-undecanoic acid on the growth of Vibrio ABE-1 at 5 and 15°C.

The bacterial growth was monitored at 5 (**A**) and 15°C (**B**) as described in the legend of Fig. 27. 0% (○), 0.4% Brij 58 (△), or 0.4% Brij 58 and 20 mg n-capric acid (□) or n-undecanoic acid (●) was added to the 100 ml of the medium.

Fig. 30 Effect of temperature on the H^+ and Na^+ pump activities at pH 6.5 of the cells grown at 15°C with Brij 58.

As described in the legend to Fig. 9, the both pump activities of the cells grown at 15°C in the Tris-salts

medium containing 0.4% Brij 58 were assayed at pH 6.5 and the same symbols were used.

Fig. 31 Effect of temperature on the H^+ and Na^+ pump activities at pH 8.5 of the cells grown at 15°C with Brij 58.

As described in the legend to Fig. 10, the both pump activities of the cells grown at 15°C in the Tris-salts medium containing 0.4% Brij 58 were assayed at pH 8.5 and the same symbols were used.

Fig. 32 Effect of temperature on the H^+ and Na^+ pump activities at pH 6.5 of the cells grown at 15°C with $C_{11:0}$.

As described in the legend to Fig. 9, the both pump activities of the cells grown at 15°C in the Tris-salts medium containing 0.4% Brij 58 and 0.2 mg/ml $C_{11:0}$ were assayed at pH 6.5 and the same symbols were used.

Fig. 33 Effect of temperature on the H^+ and Na^+ pump activities at pH 8.5 of the cells grown at 15°C with $C_{11:0}$.

As described in the legend to Fig. 10, the both pump activities of the cells grown at 15°C in the Tris-salts medium containing 0.4% Brij 58 and 0.2 mg/ml $C_{11:0}$ were assayed at pH 8.5 and the same symbols were used.

Fig. 34 Effect of temperature on the H^+ and Na^+ pump activities at pH 6.5 of the cells grown at 5°C with Brij 58.

As described in the legend to Fig. 9, the both pump activities of the cells grown at 5°C in the Tris-salts medium containing 0.4% Brij 58 were assayed at pH 6.5 and the same symbols were used.

Fig. 35 Effect of temperature on the H^+ and Na^+ pump activities at pH 8.5 of the cells grown at 5°C with Brij 58.

As described in the legend to Fig. 10, the both pump activities of the cells grown at 5°C in the Tris-salts medium containing 0.4% Brij 58 were assayed at pH 8.5 and the same symbols were used.

Fig. 36 Effect of temperature on the H^+ and Na^+ pump activities at pH 6.5 of the cells grown at 5°C with $C_{11:0}$.

As described in the legend to Fig. 9, the both pump activities of the cells grown at 5°C in the Tris-salts medium containing 0.4% Brij 58 and 0.2 mg/ml $C_{11:0}$ were assayed at pH 6.5 and the same symbols were used.

Fig. 37 Effect of temperature on the H^+ and Na^+ pump activities at pH 8.5 of the cells grown at 5°C with $C_{11:0}$.

As described in the legend to Fig. 10, the both pump

activities of the cells grown at 5°C in the Tris-salts medium containing 0.4% Brij 58 and 0.2 mg/ml $C_{11:0}$ were assayed at pH 8.5 and the same symbols were used.

Fig. 38 Effect of temperature on the H^+ and Na^+ pump activities at pH 6.5 of the cells grown at 15°C with oleic acid.

As described in the legend to Fig. 9, the both pump activities of the cells grown at 15°C in the Tris-salts medium containing 0.4% Brij 58 and 0.5 mg/ml oleic acid were assayed at pH 6.5 and the same symbols were used.

Fig. 39 Effect of temperature on the H^+ and Na^+ pump activities at pH 8.5 of the cells grown at 15°C with oleic acid.

As described in the legend to Fig. 10, the both pump activities of the cells grown at 15°C in the Tris-salts medium containing 0.4% Brij 58 and 0.5 mg/ml oleic acid were assayed at pH 8.5 and the same symbols were used.

Fig. 40 Effect of NaCl on chemotactic motility.

Chemotactic motility of the cells washed with the chemotactic buffer adjusted to pH 6.5 and 8.5 was observed on swarm agar plates containing various concentrations of NaCl at pH 6.5 (**A-D**) and 8.5 (**E-H**), respectively. 0.05 M (**A** and **E**), 0.1 M (**B** and **F**), 0.2 M (**C** and **G**) or 0.4 M NaCl (**D** and **H**) was added to the swarm agar plate.

Fig. 41 Effect of CCCP on chemotactic motility.

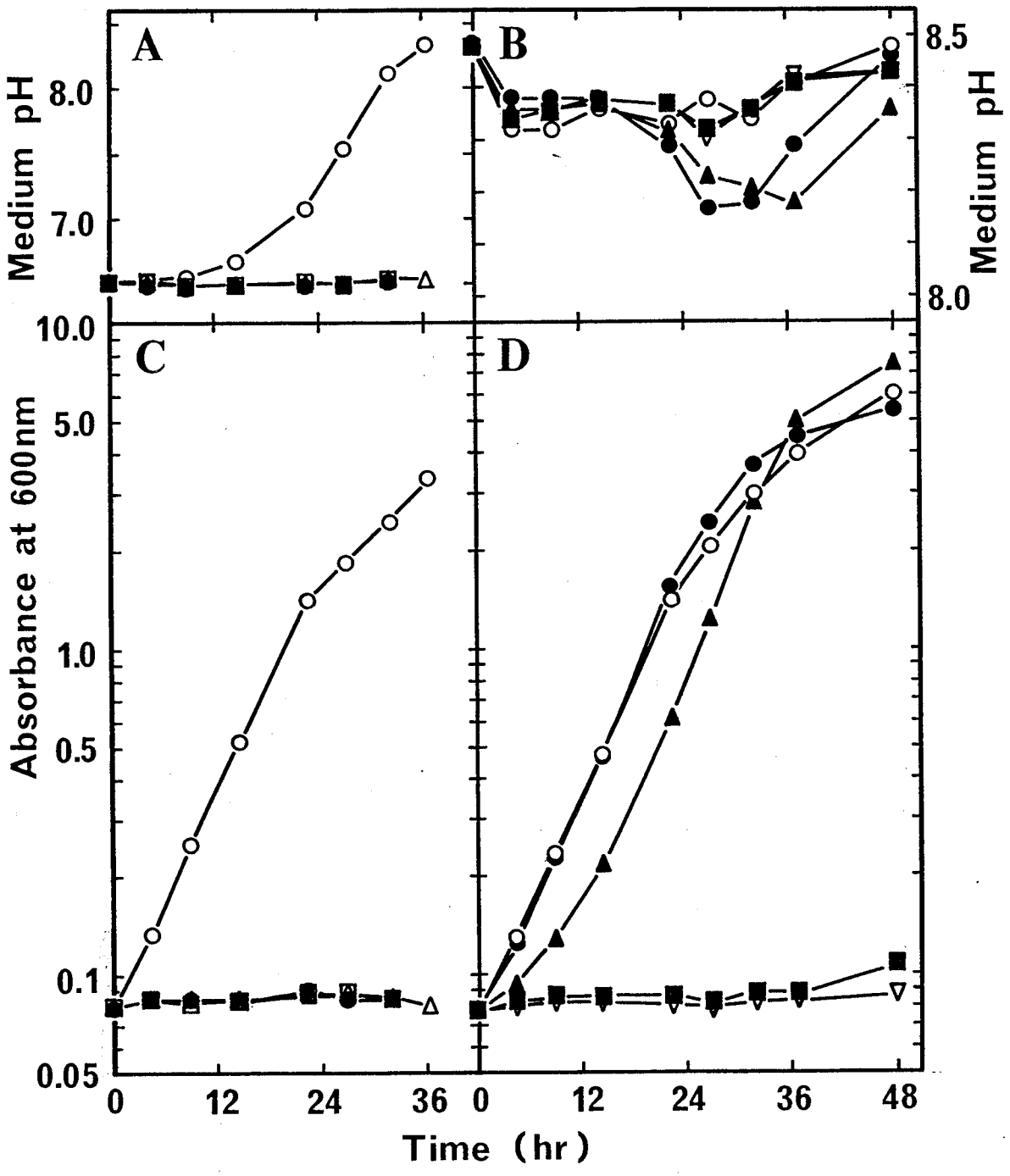
Chemotactic motility of the cells washed with the chemotactic buffer adjusted to pH 6.5 and 8.5 was observed on swarm agar plates containing various concentrations of CCCP at pH 6.5 (**A-D**) and 8.5 (**E-H**), respectively. 0 μM (**A** and **E**), 1 μM (**B** and **F**), 5 μM (**C** and **G**) or 10 μM (**D** and **H**) was added to the swarm agar plate.

Fig. 42 Effect of TPP on chemotactic motility.

Assay was performed as described in the legends to Fig. 41 except that various concentrations of CCCP in the swarm agar plate were replaced with 0 μM (**A** and **E**), 10 μM (**B** and **F**), 100 μM (**C** and **G**) or 1 mM (**D** and **H**).

Fig. 43 AIB uptake dependent on various cation gradients across the cell membrane.

AIB uptake was examined at pH 6.5 (**A**) and 8.5 (**B**). The harvested cells were washed twice with MN and TN buffer (open symbols), or 50 mM MES-KOH (pH 6.5) or Tricine-KOH (pH 8.5) containing 10% (v/v) glycerol and 0.5 M KCl (designated as MK or TK buffer, respectively; closed symbols). The washed cells were suspended in MN (○ and ● in **A**), TN (○ and ● in **B**), MK (△ in **A**), TK buffer (△ in **B**) or 50 mM MES-LiOH (pH 6.5; □ and ■ in **A**) or Tricine-LiOH (pH 8.5; □ and ■ in **B**) containing 10% (v/v) glycerol and 0.5 M LiCl. The uptake was started by the addition of [1-^{14}C]AIB to the cell suspensions.


A
B
C
D
Medium pH
8.0
7.0
8.5
8.0
Absorbance at 600nm
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5.0
1.0
0.5
0.1
0.05
0
12
24
36
48
Time (hr)

Fig. 1.

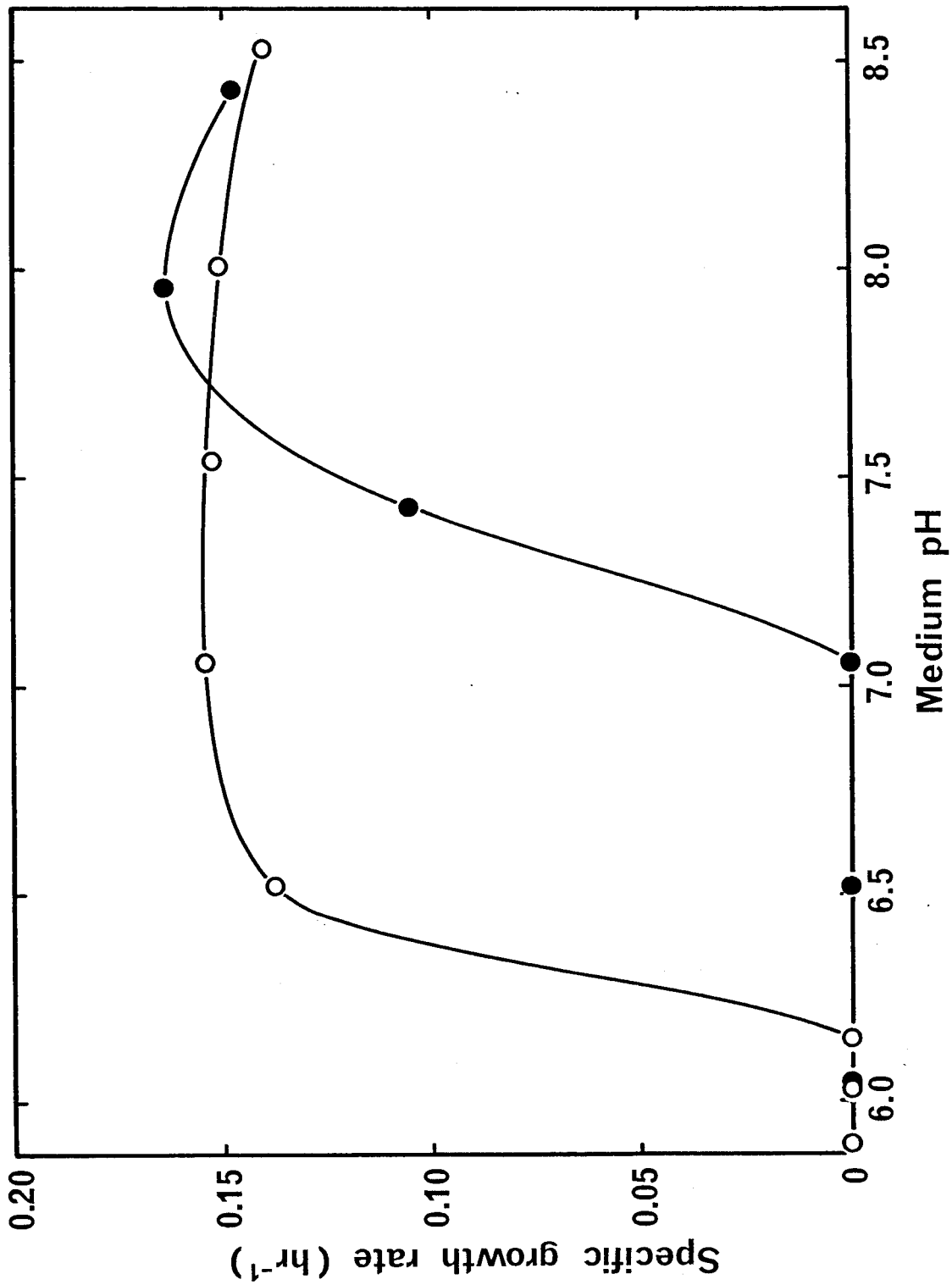
Specific growth rate (hr⁻¹)
0.20
0.15
0.10
0.05
0
6.0
6.5
7.0
7.5
8.0
8.5
Medium pH

Fig. 2.

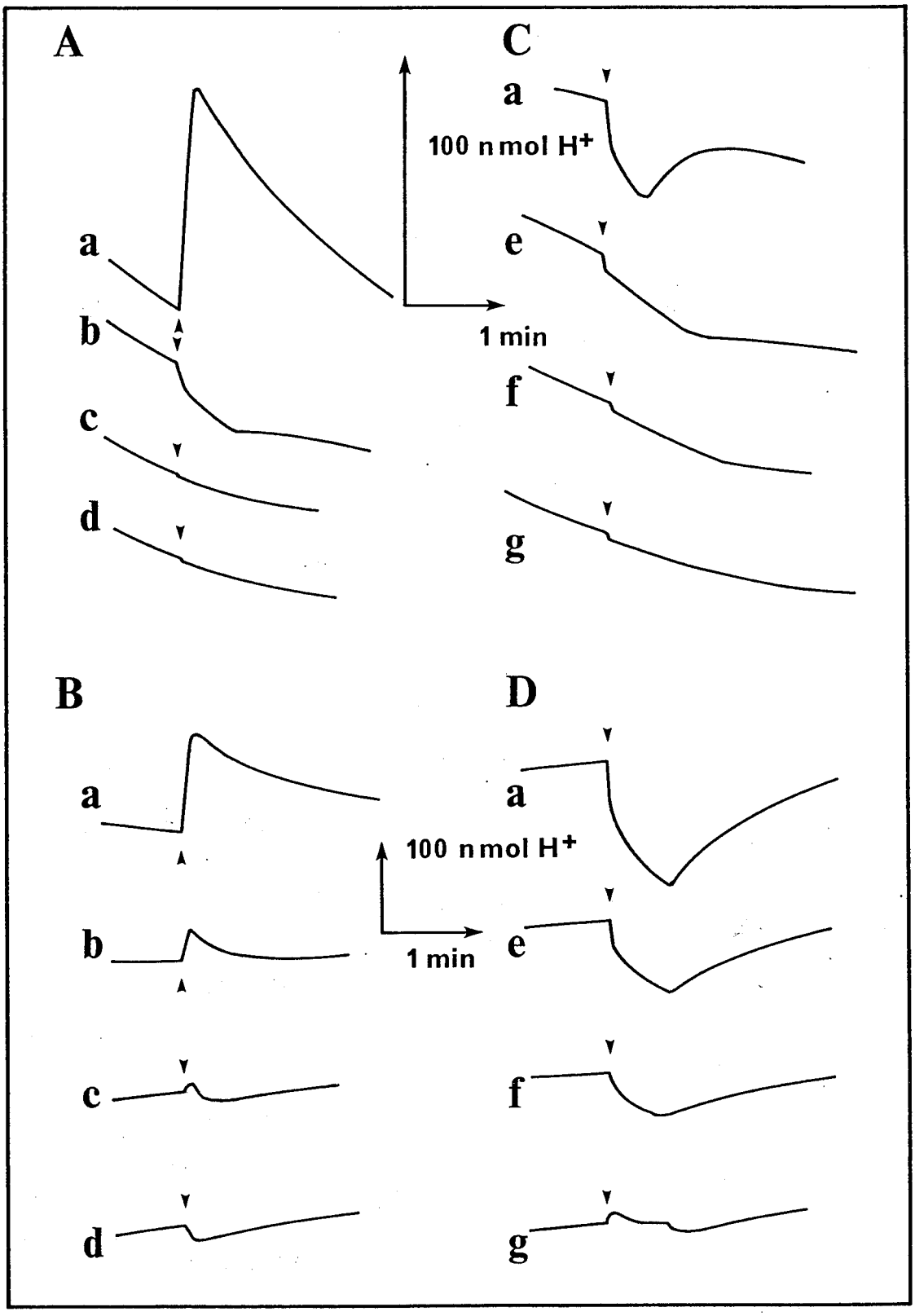

Fig. 3.

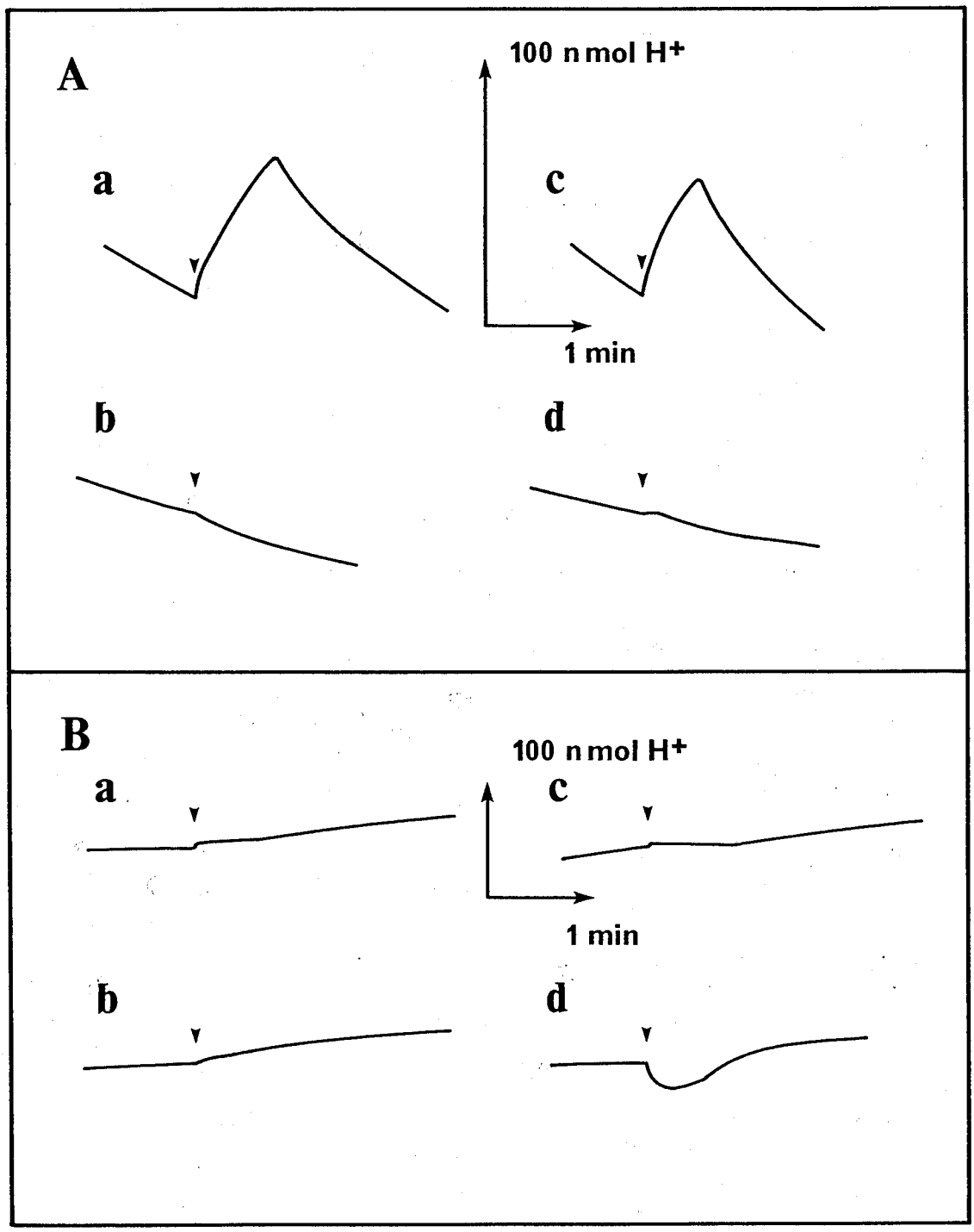

Fig. 4.

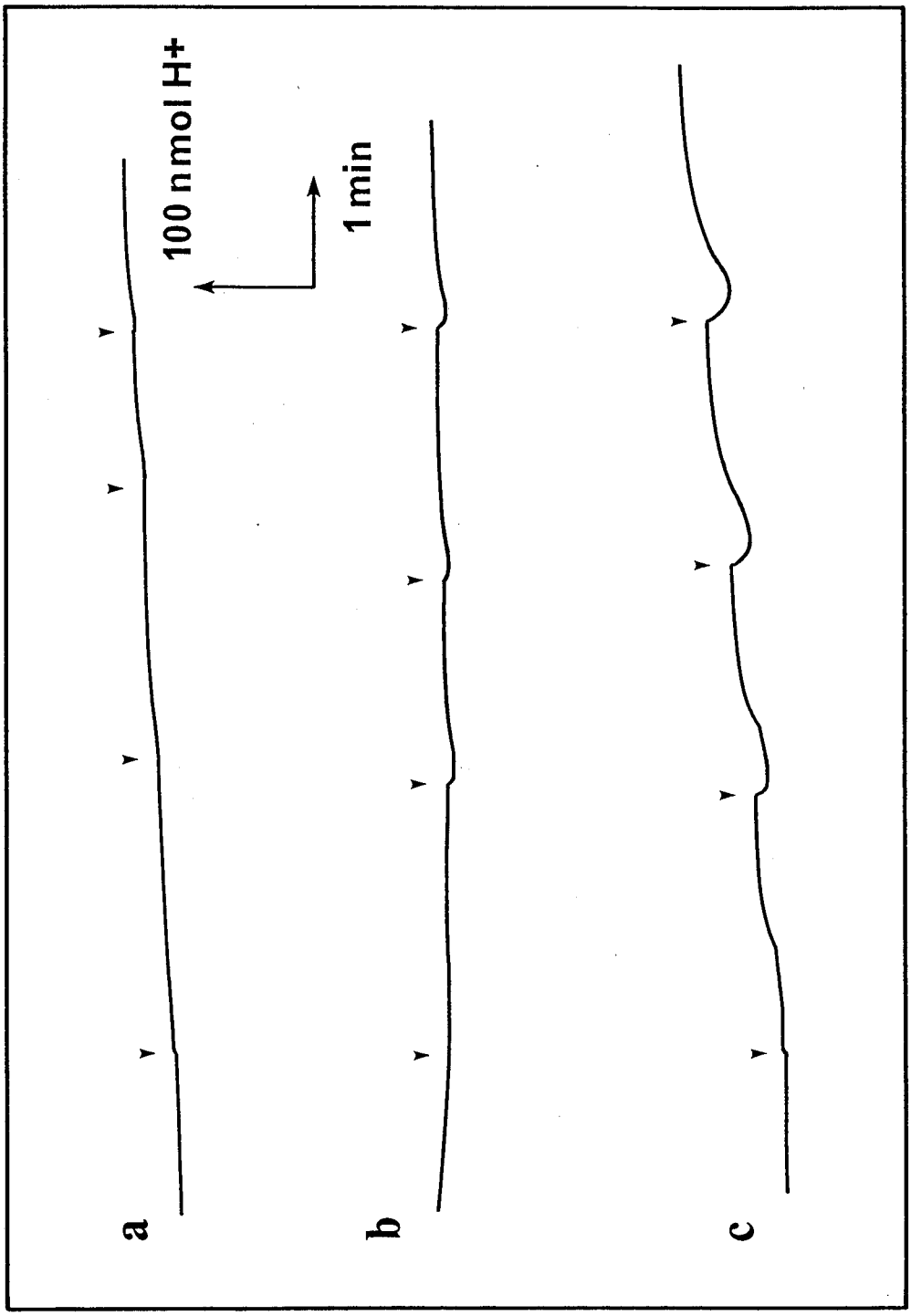

Fig. 5.

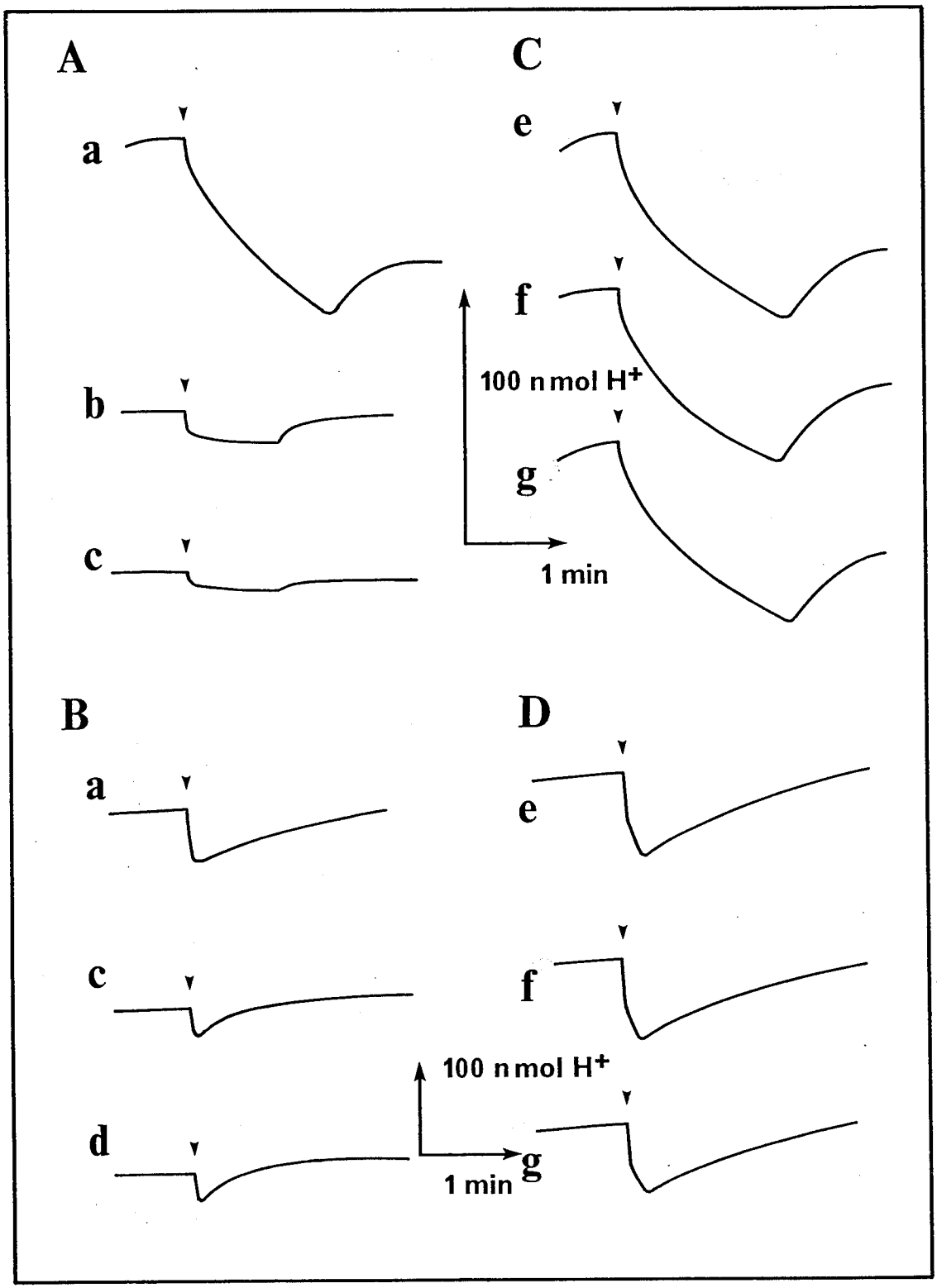

Fig. 6.

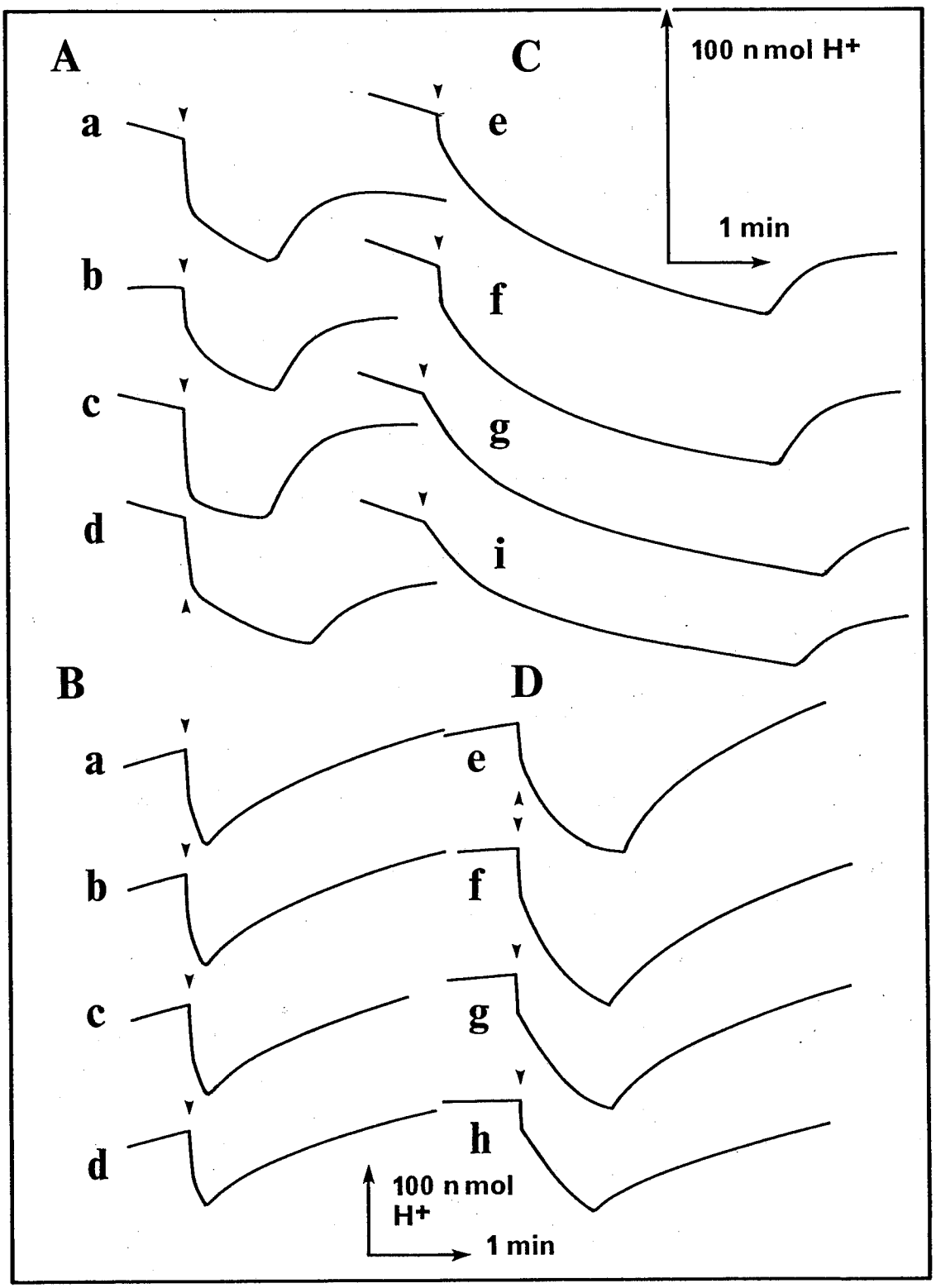

Fig. 7.

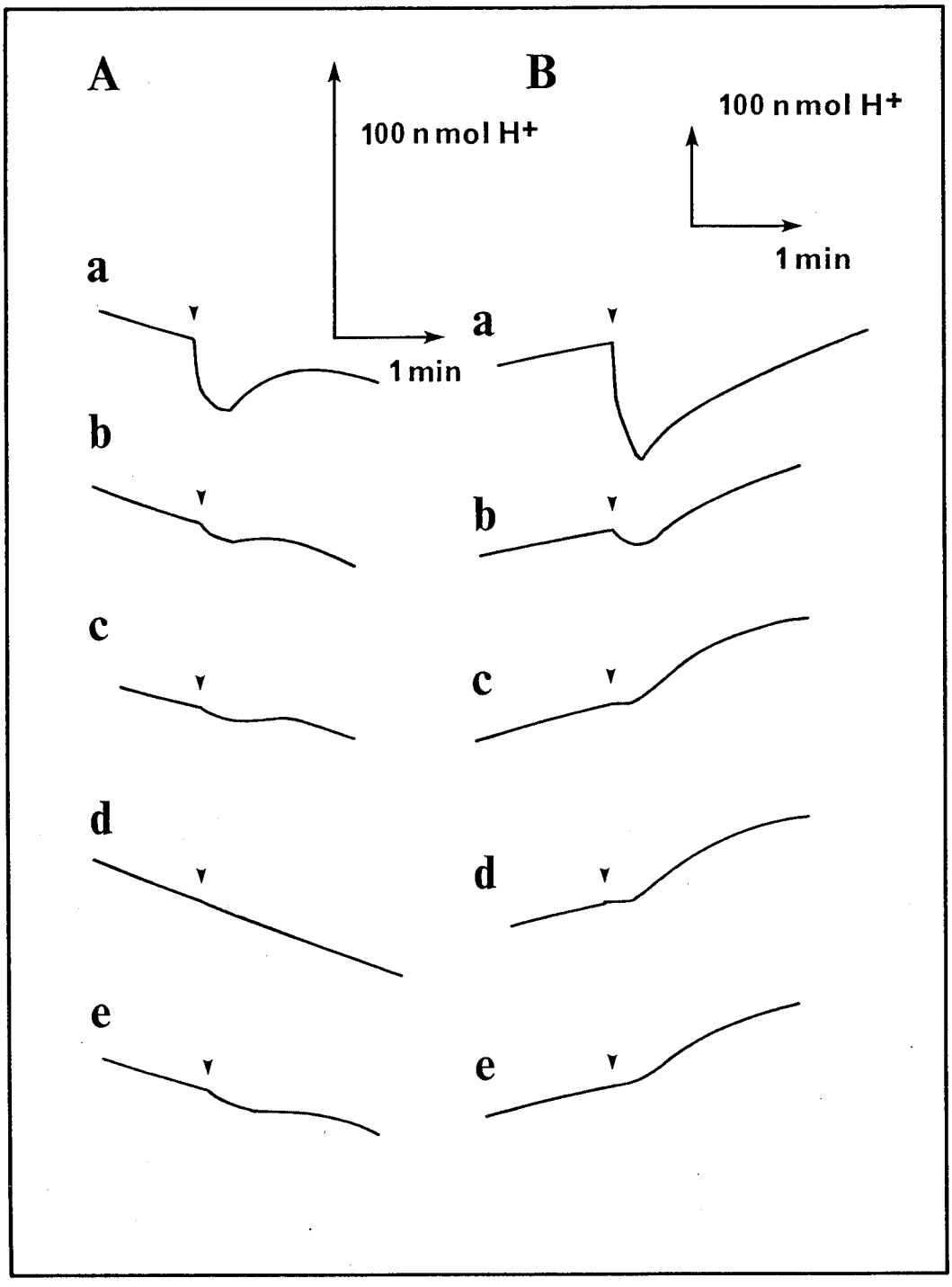

Fig. 8.

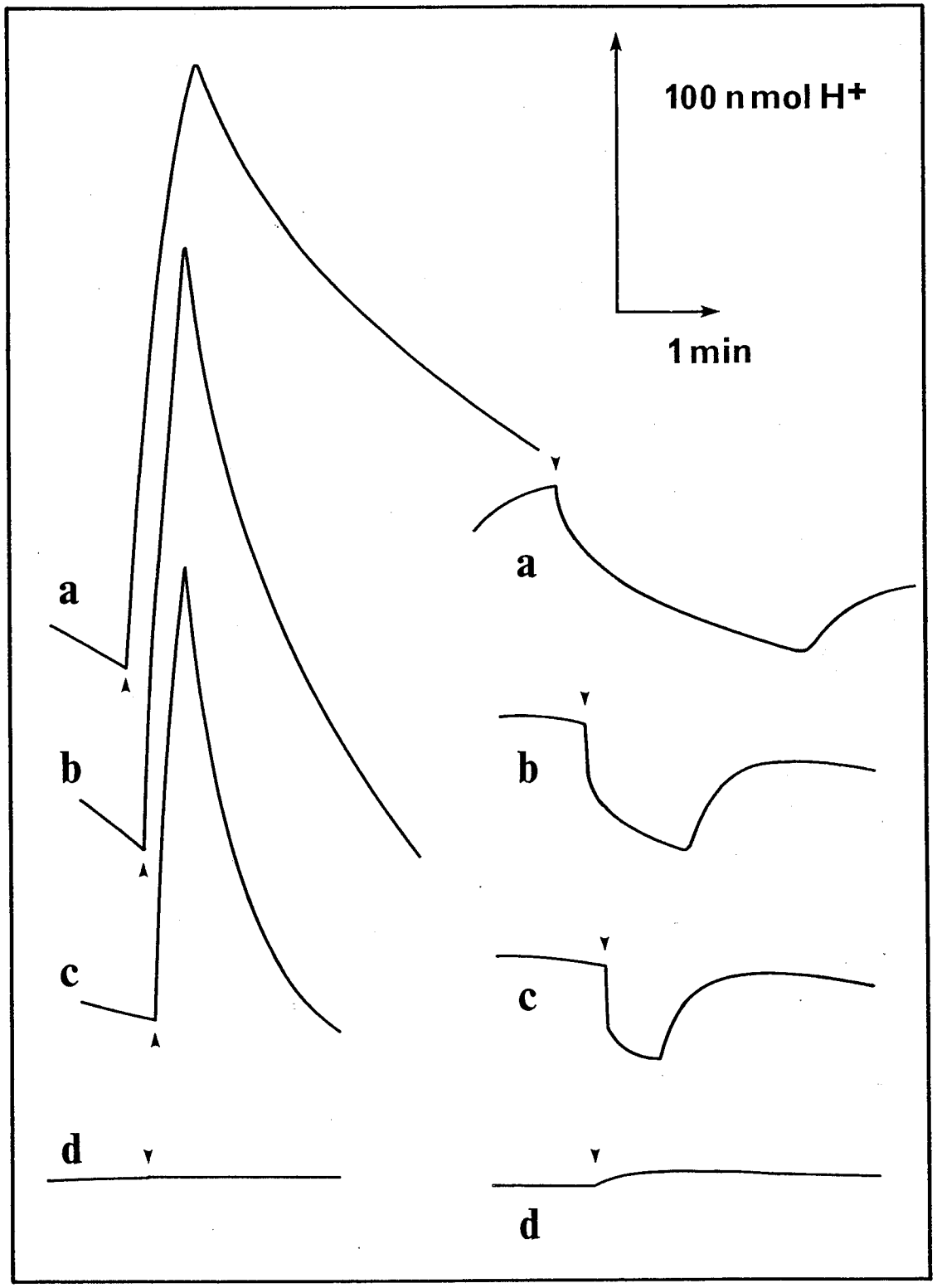

Fig. 9.

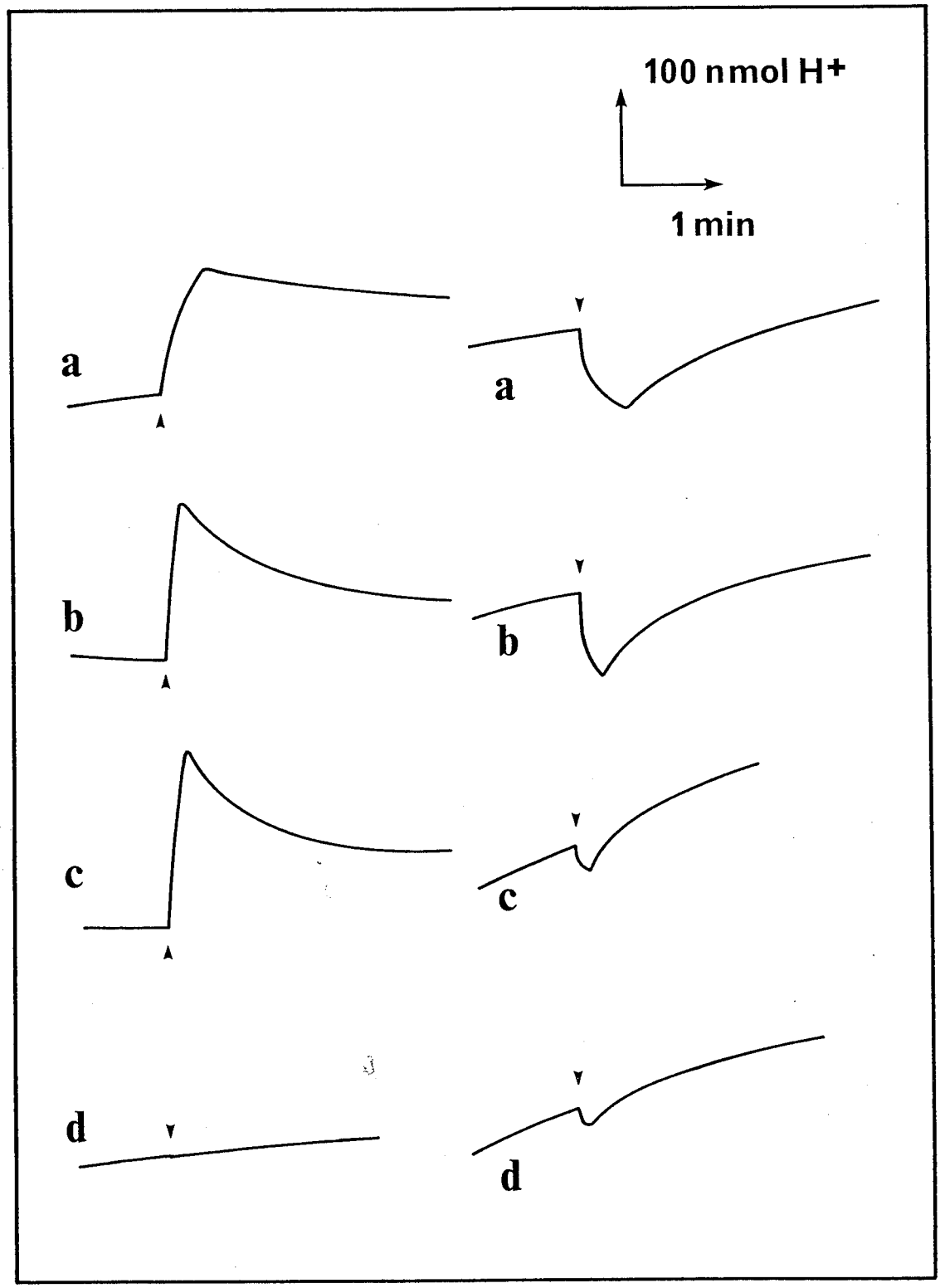

Fig. 10.

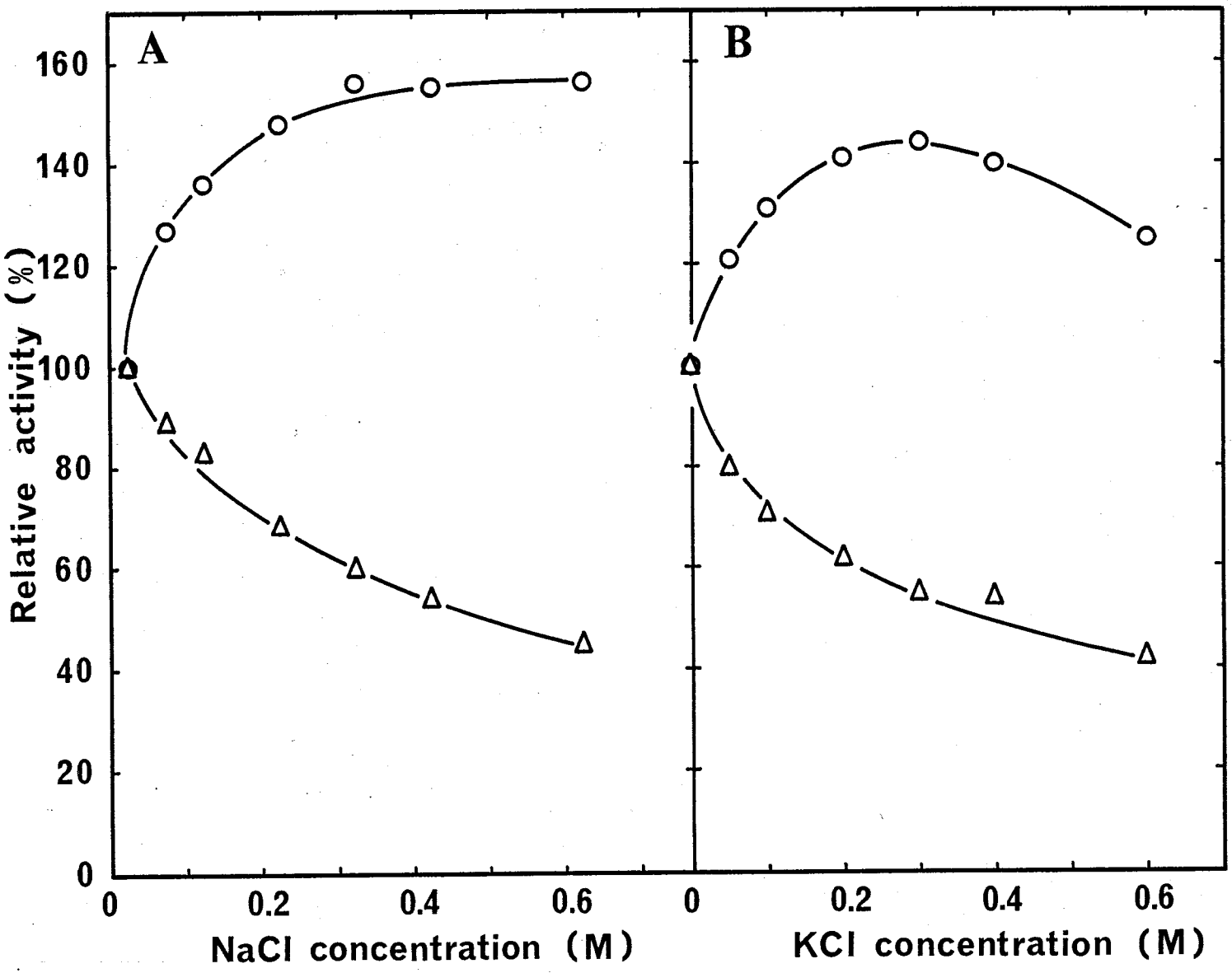
A
B
Relative activity (%)
160
140
120
100
80
60
40
20
0
0
0.2
0.4
0.6
NaCl concentration (M)
KCl concentration (M)

Fig. 11.

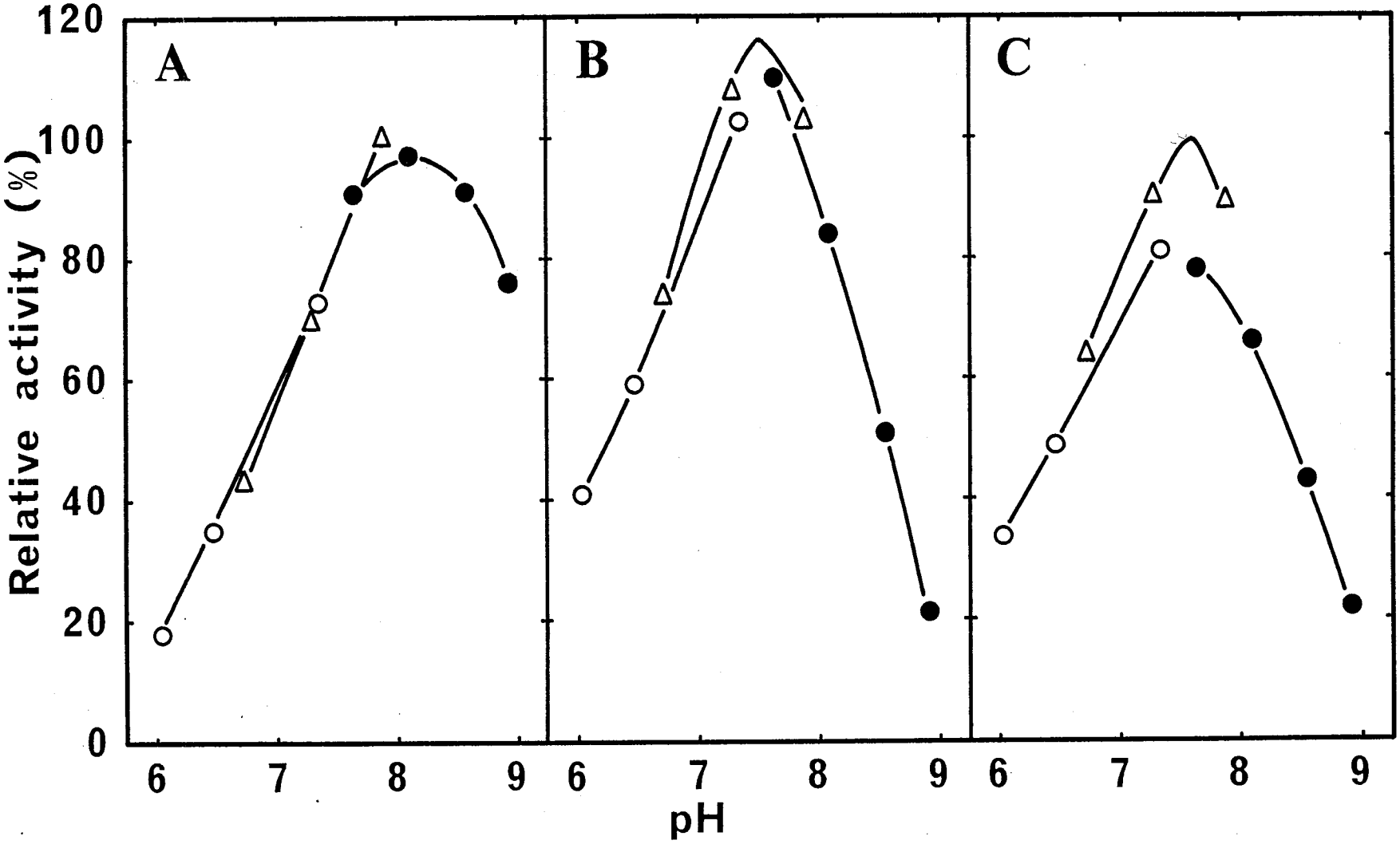

Fig. 12.

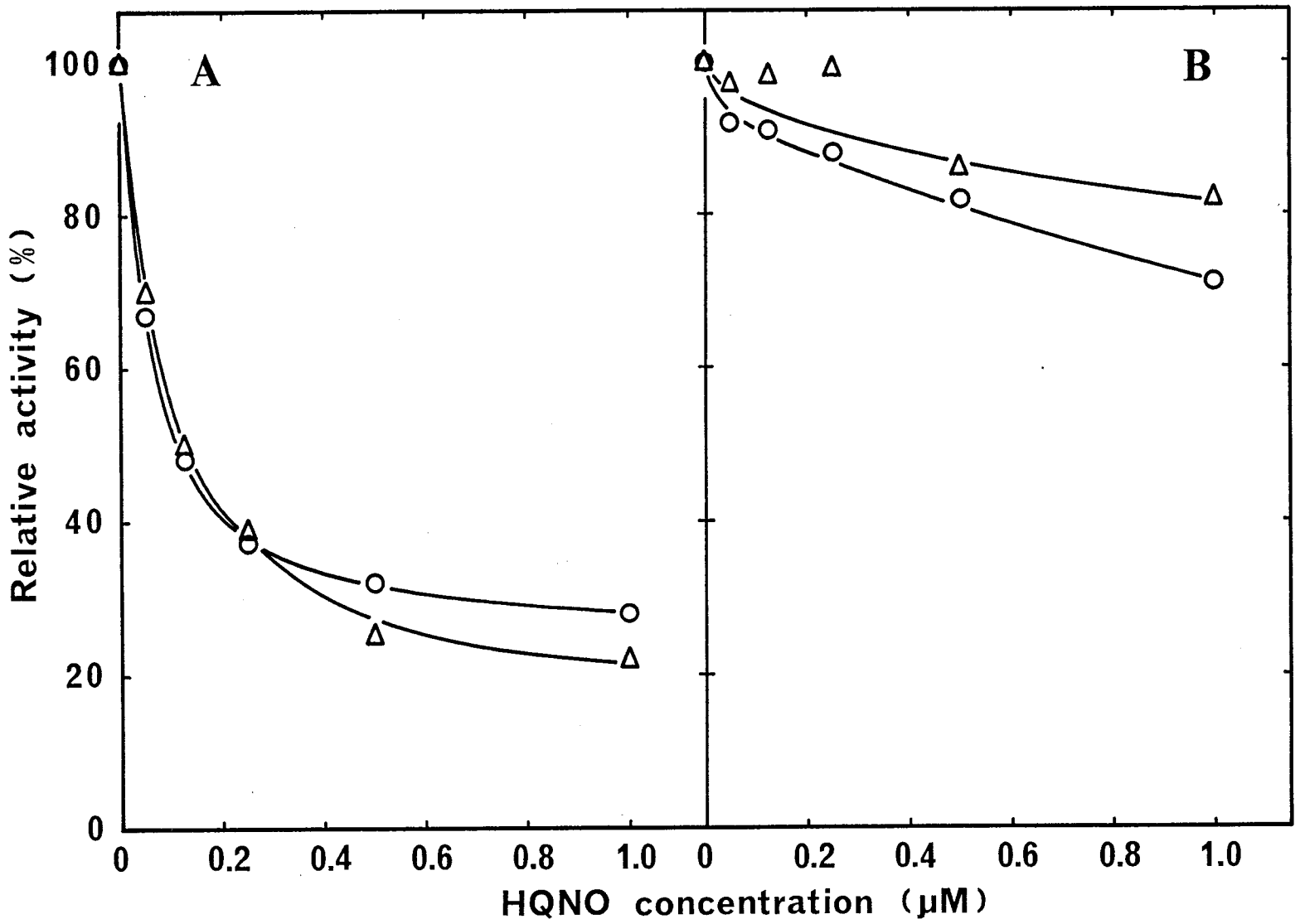
A
B
100
80
60
40
20
0
Relative activity (%)
0
0.2
0.4
0.6
0.8
1.0
HQNO concentration (μM)

Fig. 13.

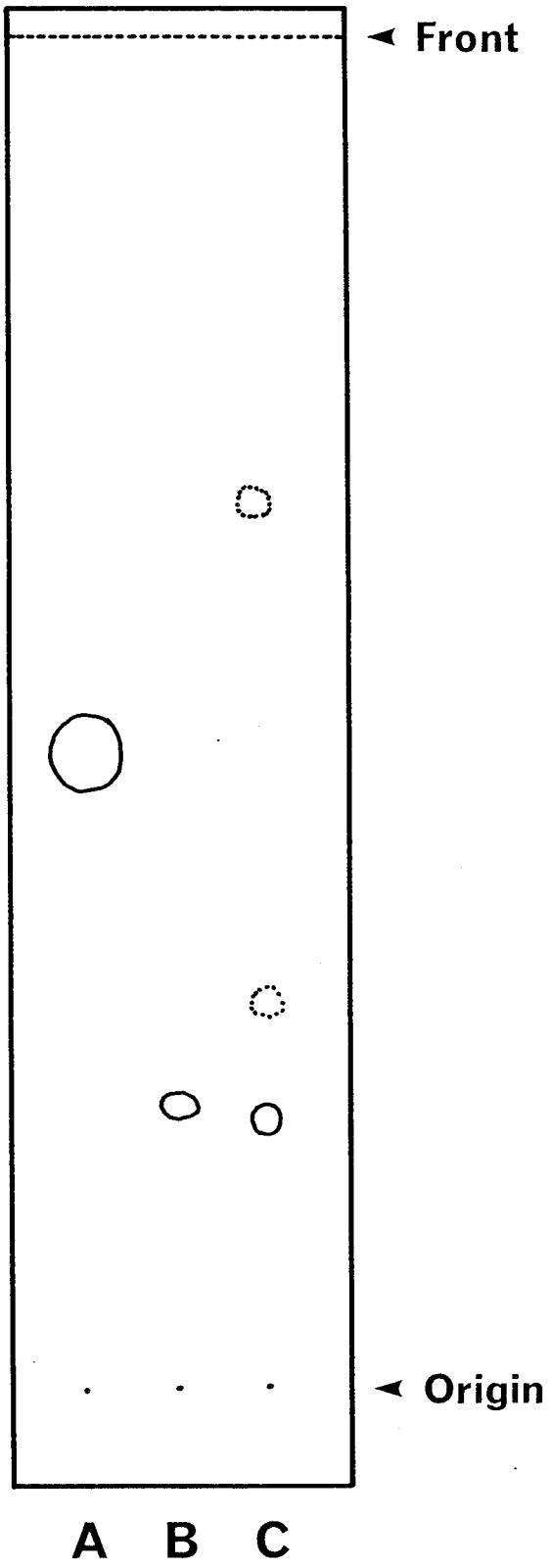

Fig. 14.

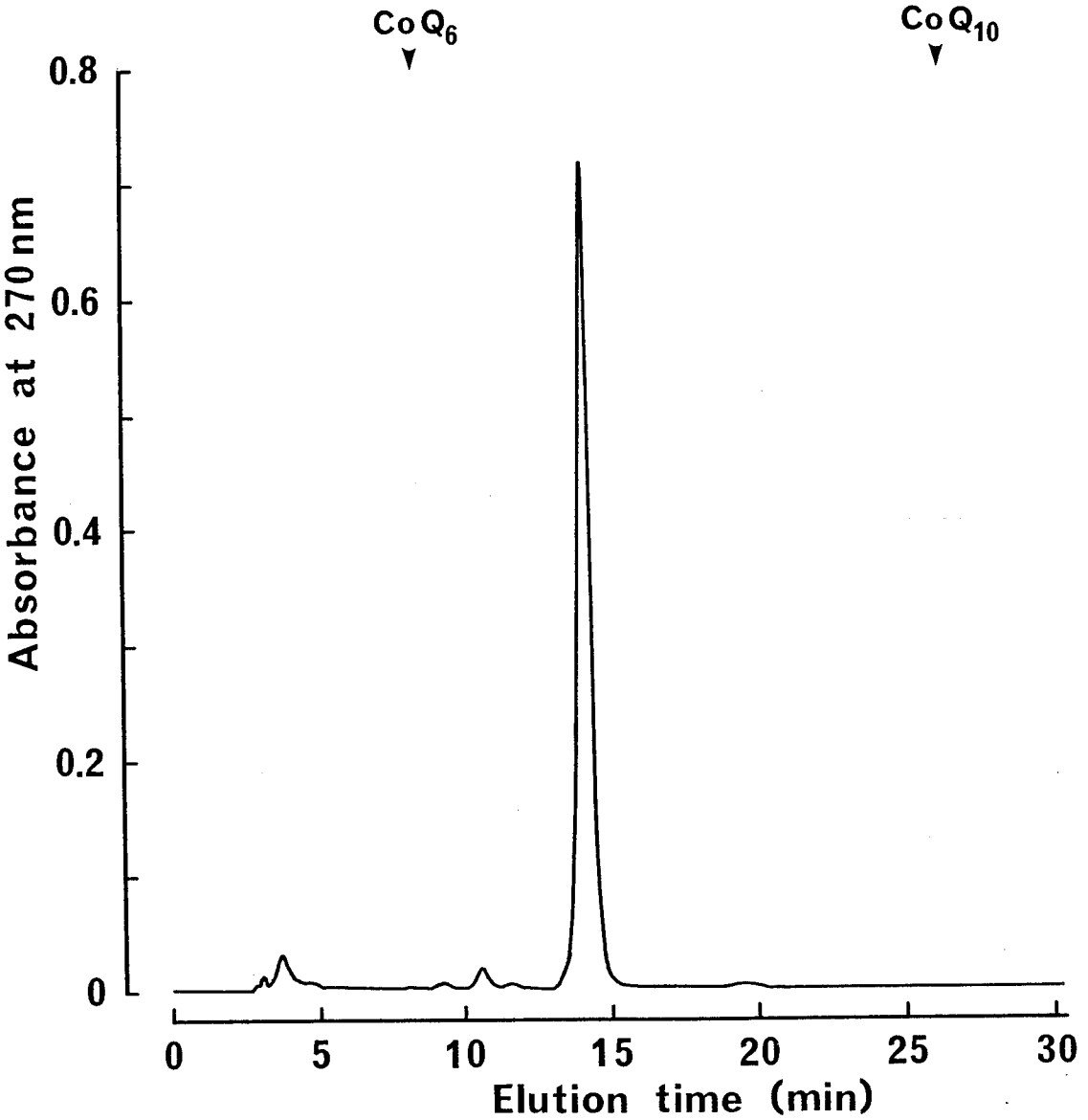
CoQ6
CoQ10
0.8
0.6
0.4
0.2
0
Absorbance at 270 nm
0
5
10
15
20
25
30
Elution time (min)

Fig. 15.

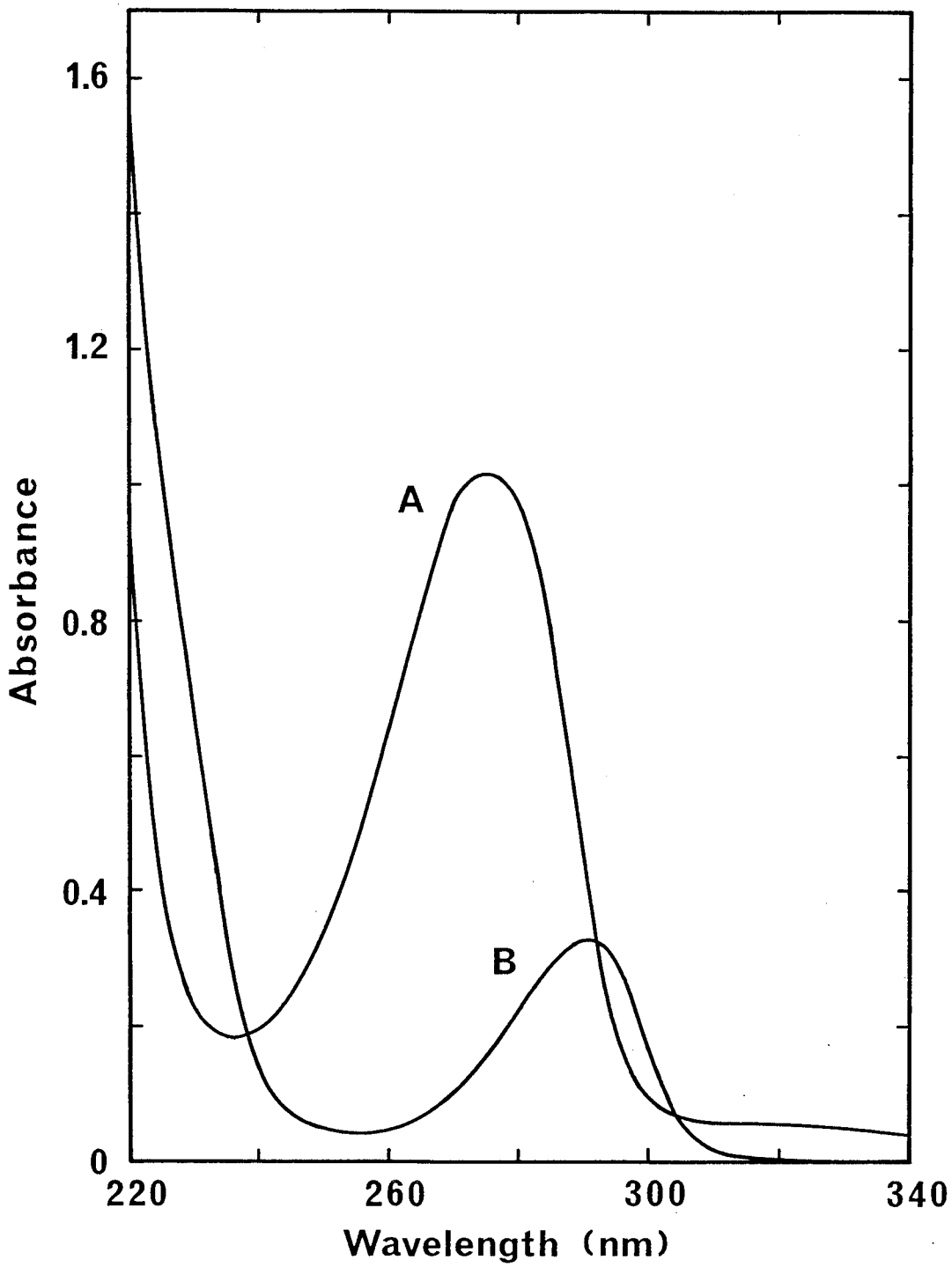

Fig. 16.

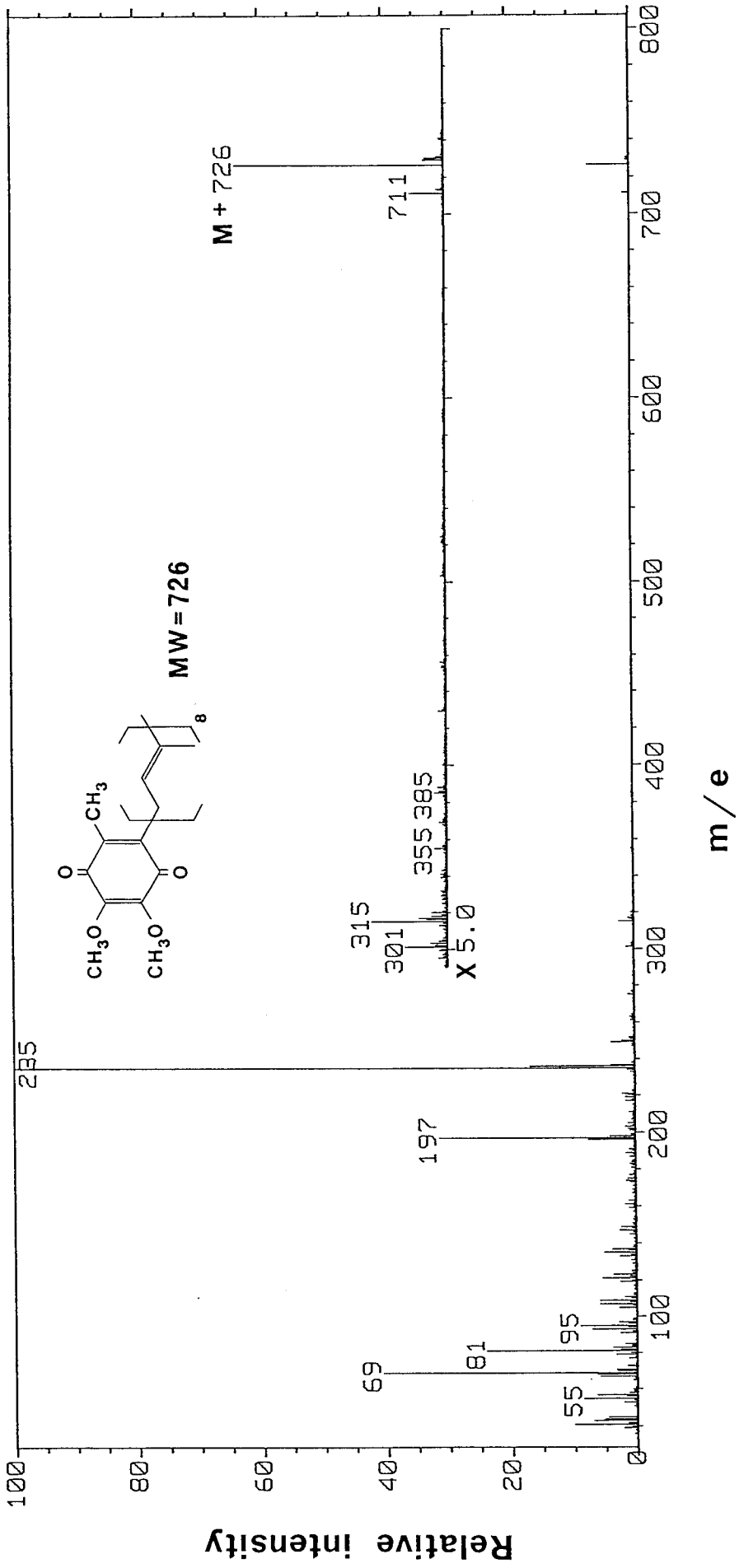
Relative intensity
m/e
MW=726
M+726
711
385
355
315
301
X 5.0
235
197
95
81
69
55
100
200
300
400
500
600
700
800
0
20
40
60
80
100
CH3O
CH3O
CH3
O
O
8

Fig. 17.

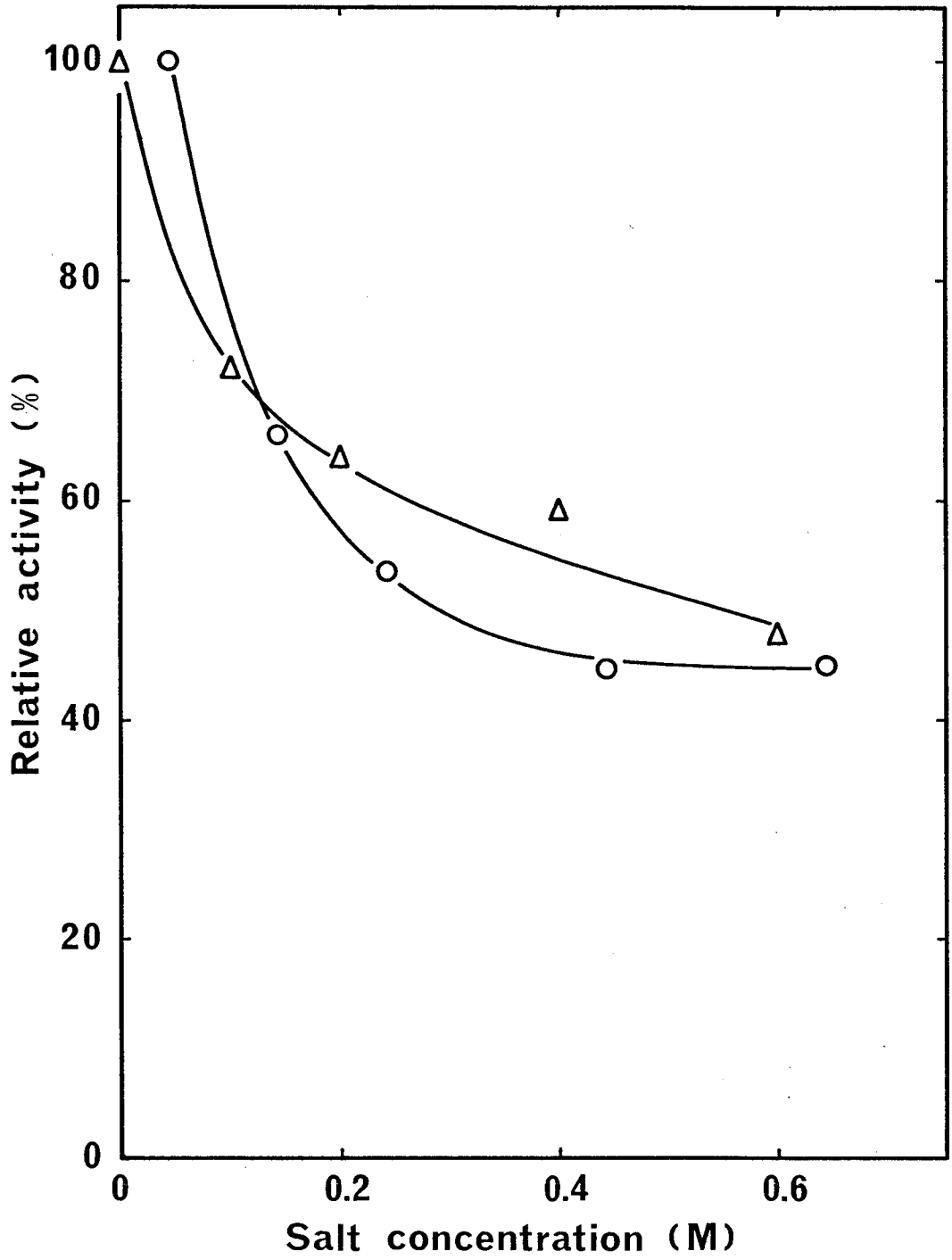
100
80
60
40
20
0
Relative activity (%)
0
0.2
0.4
0.6
Salt concentration (M)

Fig. 18.

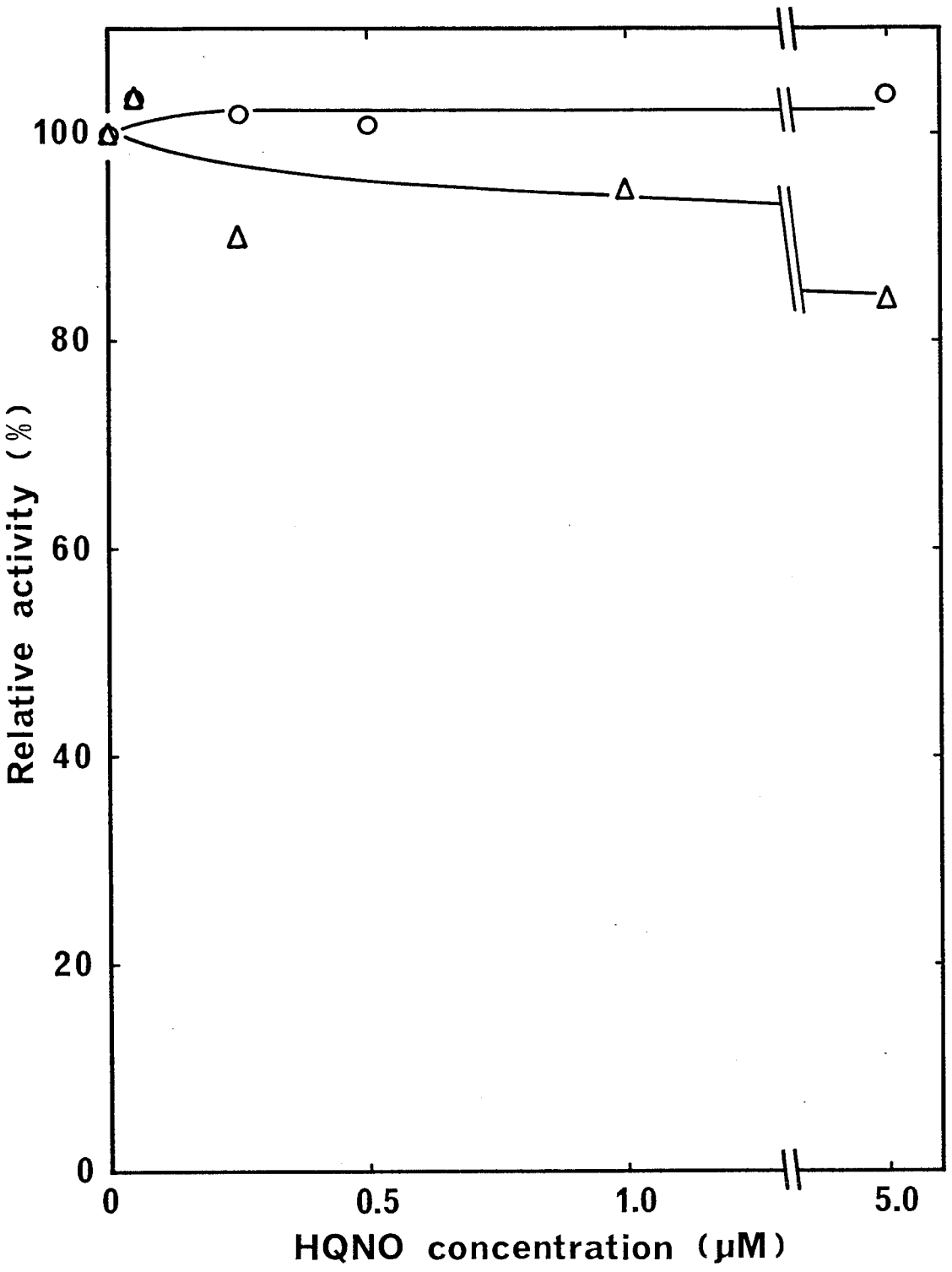
100
80
60
40
20
0
Relative activity (%)
0
0.5
1.0
5.0
HQNO concentration (μM)

Fig. 19.

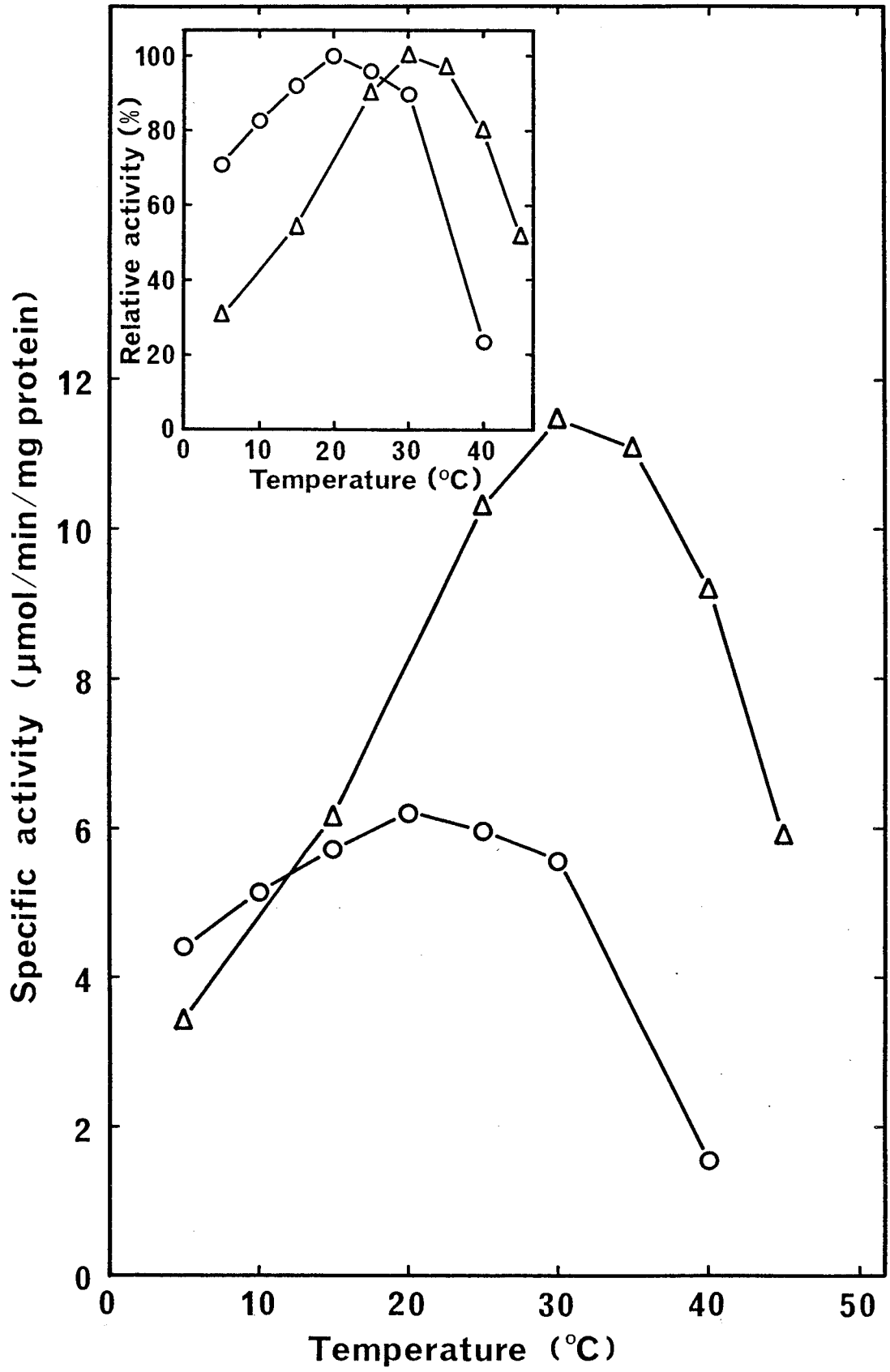
Specific activity (μmol/min/mg protein)
0
2
4
6
8
10
12
0
10
20
30
40
50
Temperature (°C)
Relative activity (%)
0
20
40
60
80
100
0
10
20
30
40
Temperature (°C)

Fig. 20.

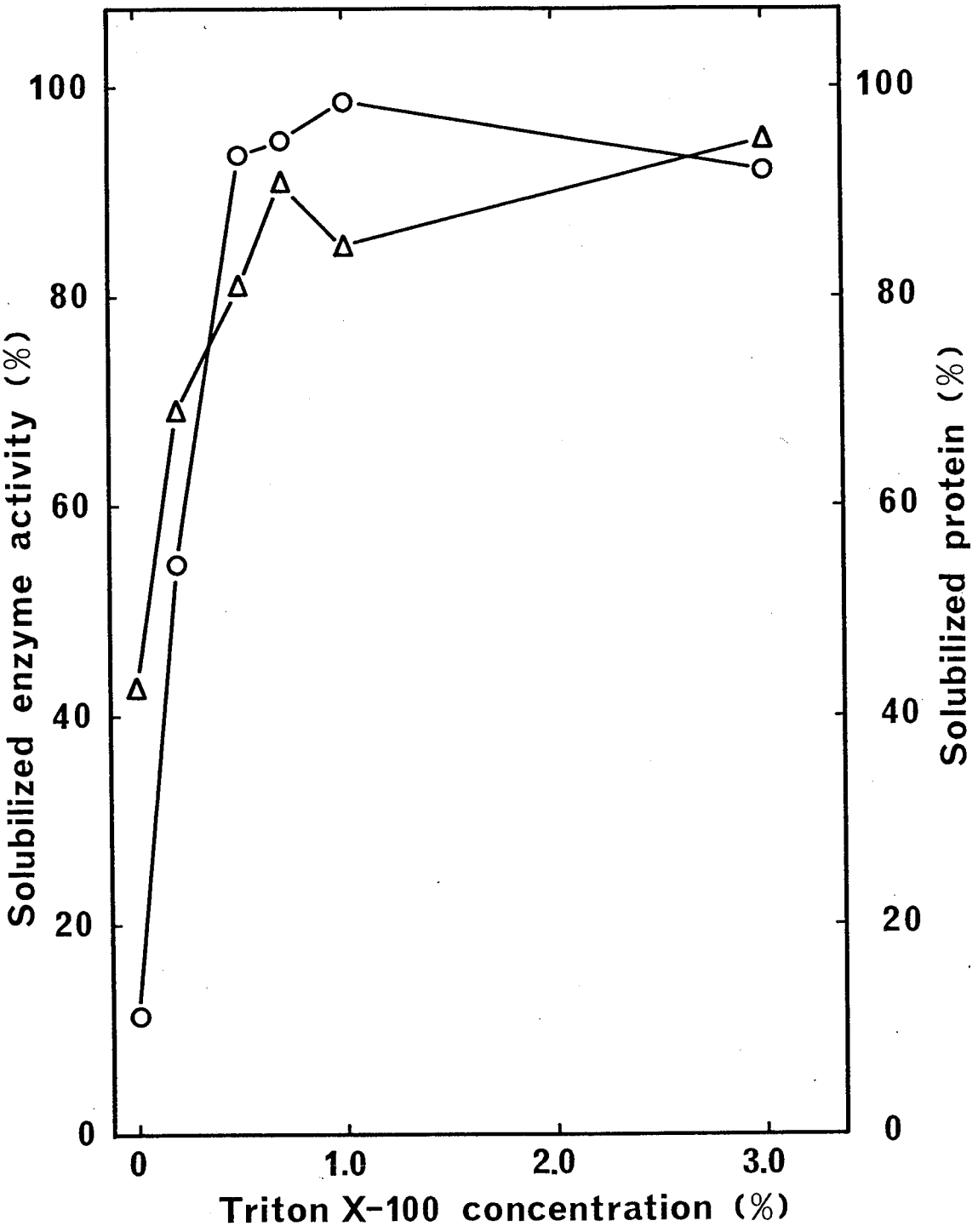
100
80
60
40
20
0
Solubilized enzyme activity (%)
100
80
60
40
20
0
Solubilized protein (%)
0
1.0
2.0
3.0
Triton X-100 concentration (%)

Fig. 21.

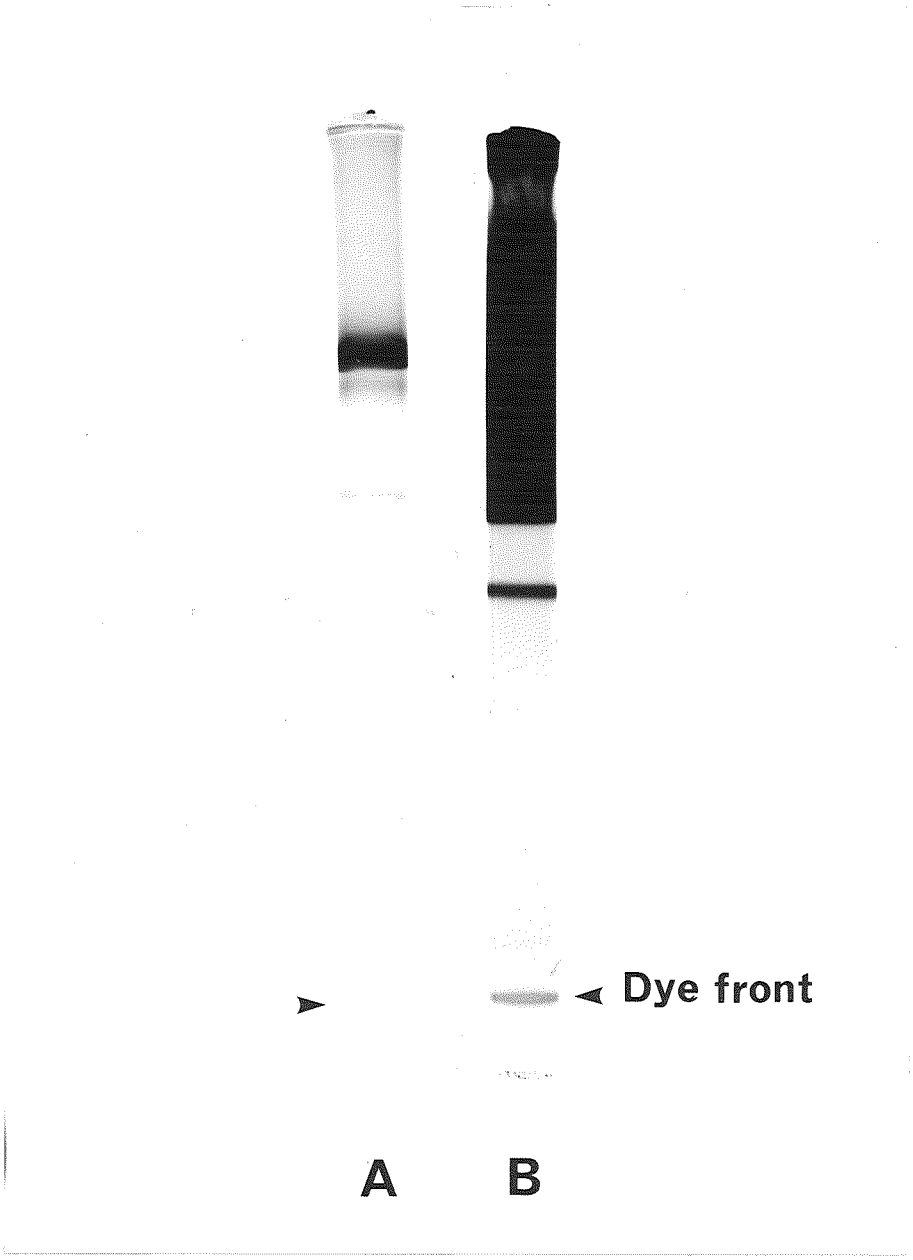

Fig. 22.

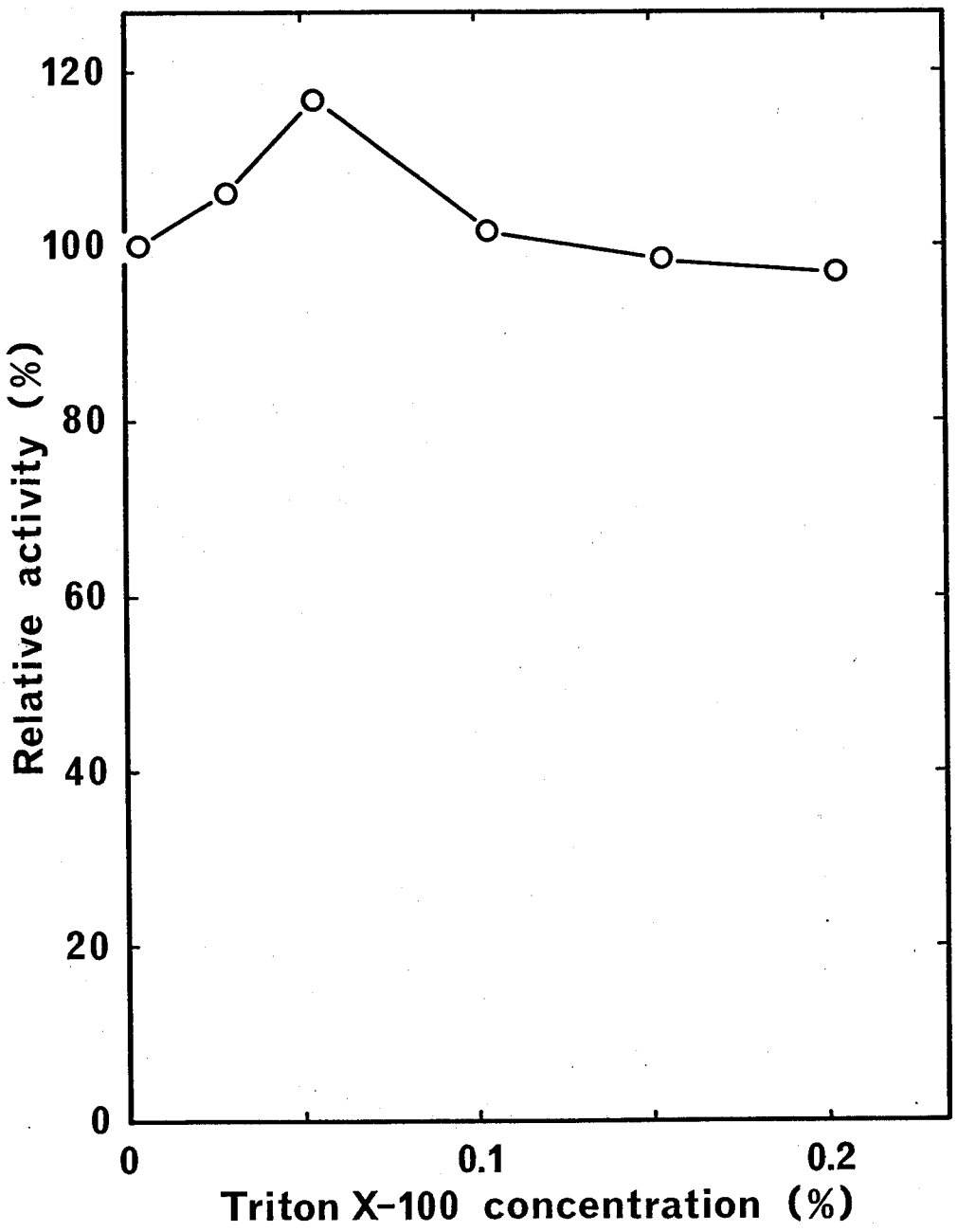
120
100
80
60
40
20
0
Relative activity (%)
0
0.1
0.2
Triton X-100 concentration (%)

Fig. 23.

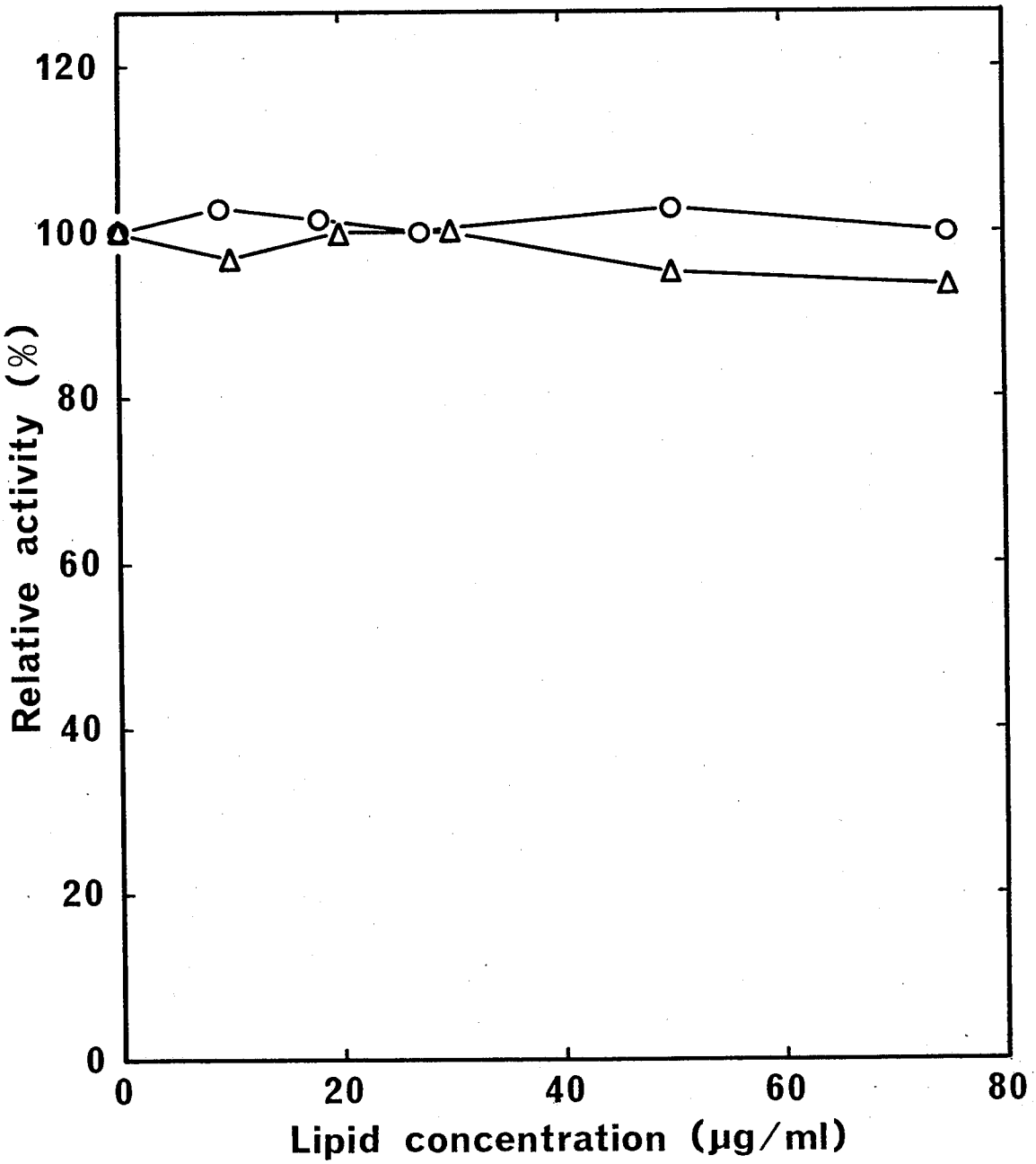
120
100
80
60
40
20
0
Relative activity (%)
0
20
40
60
80
Lipid concentration (μg/ml)

Fig. 24.

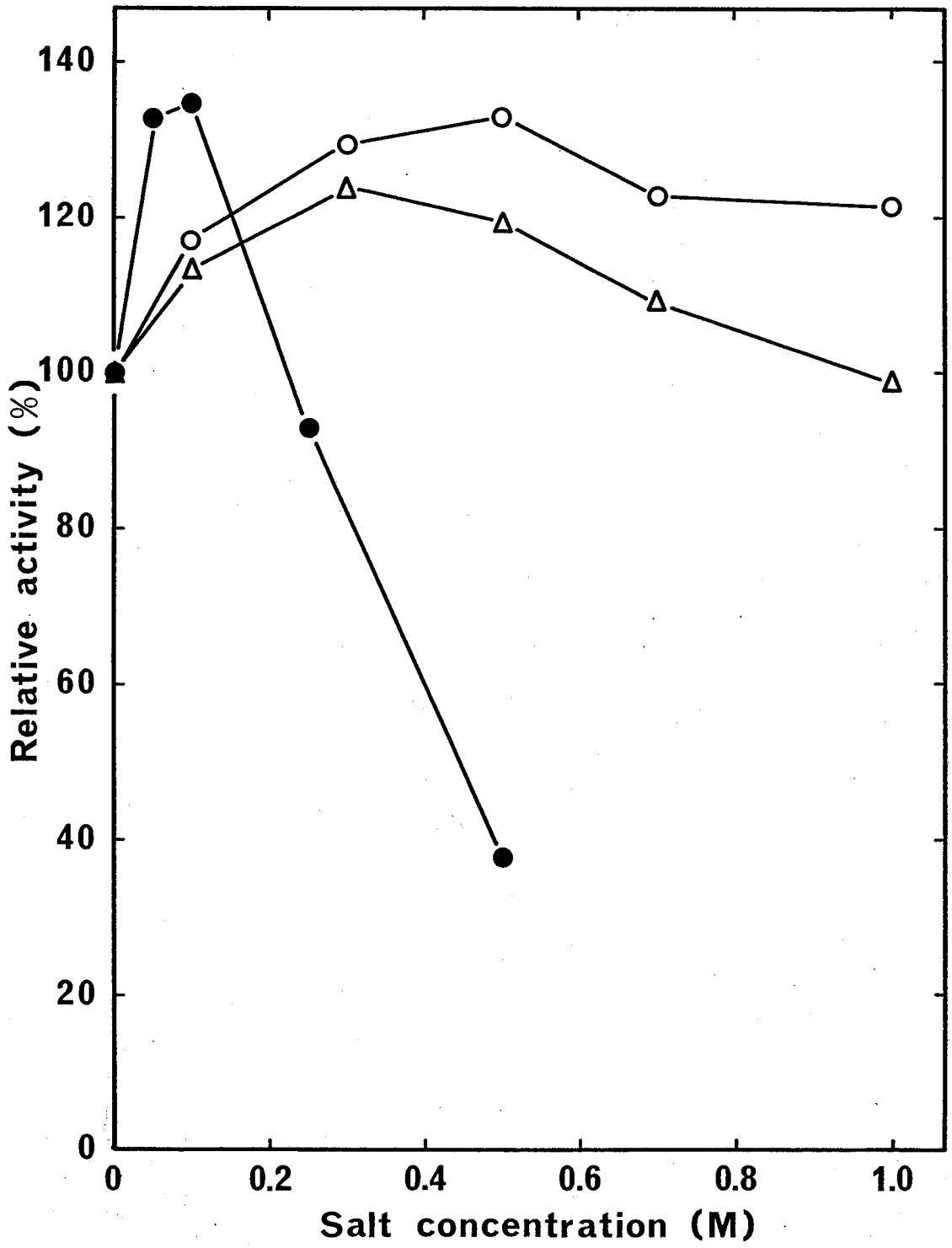
140
120
100
80
60
40
20
0
Relative activity (%)
0
0.2
0.4
0.6
0.8
1.0
Salt concentration (M)

Fig. 25.

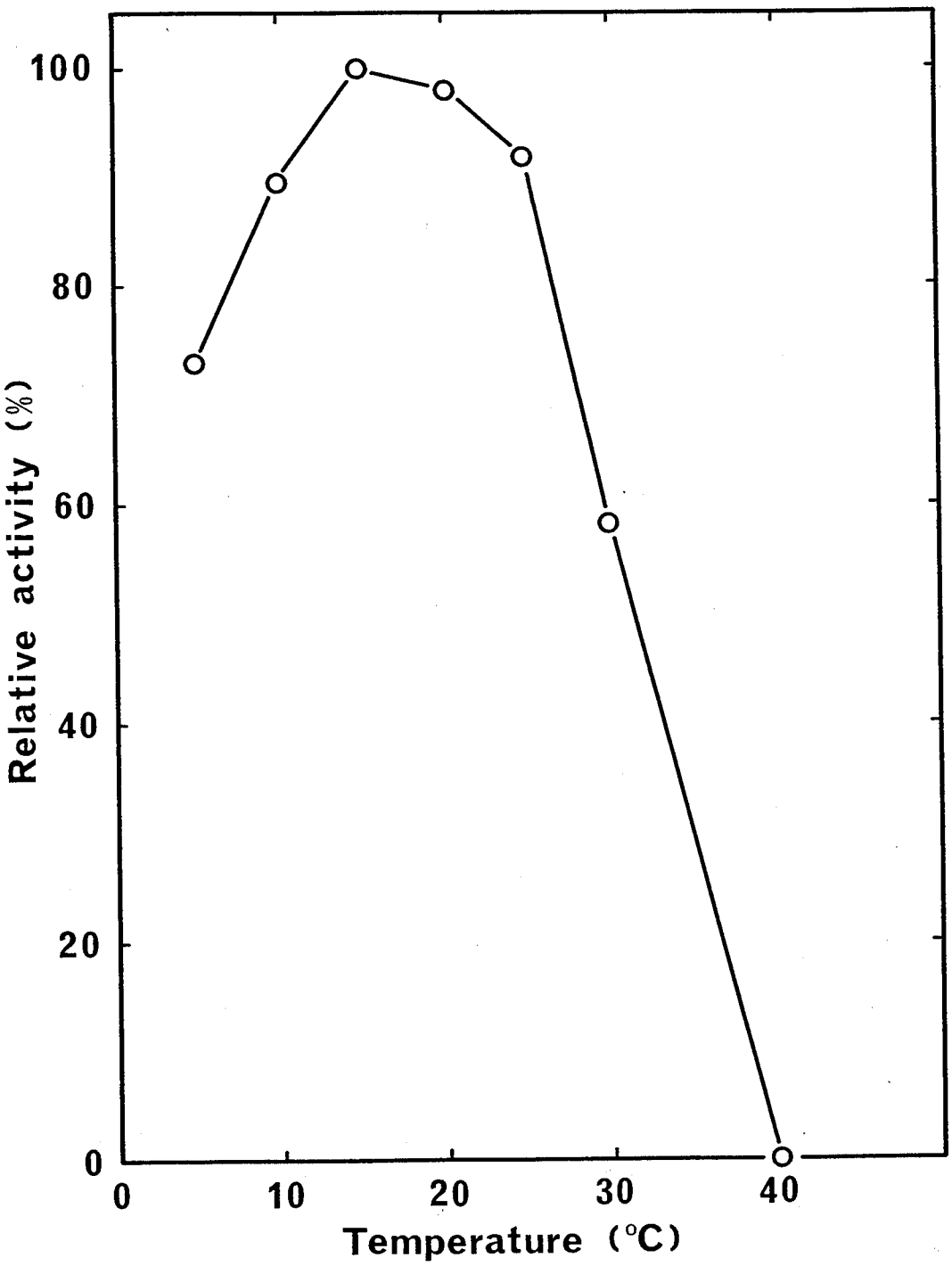

Fig. 26.

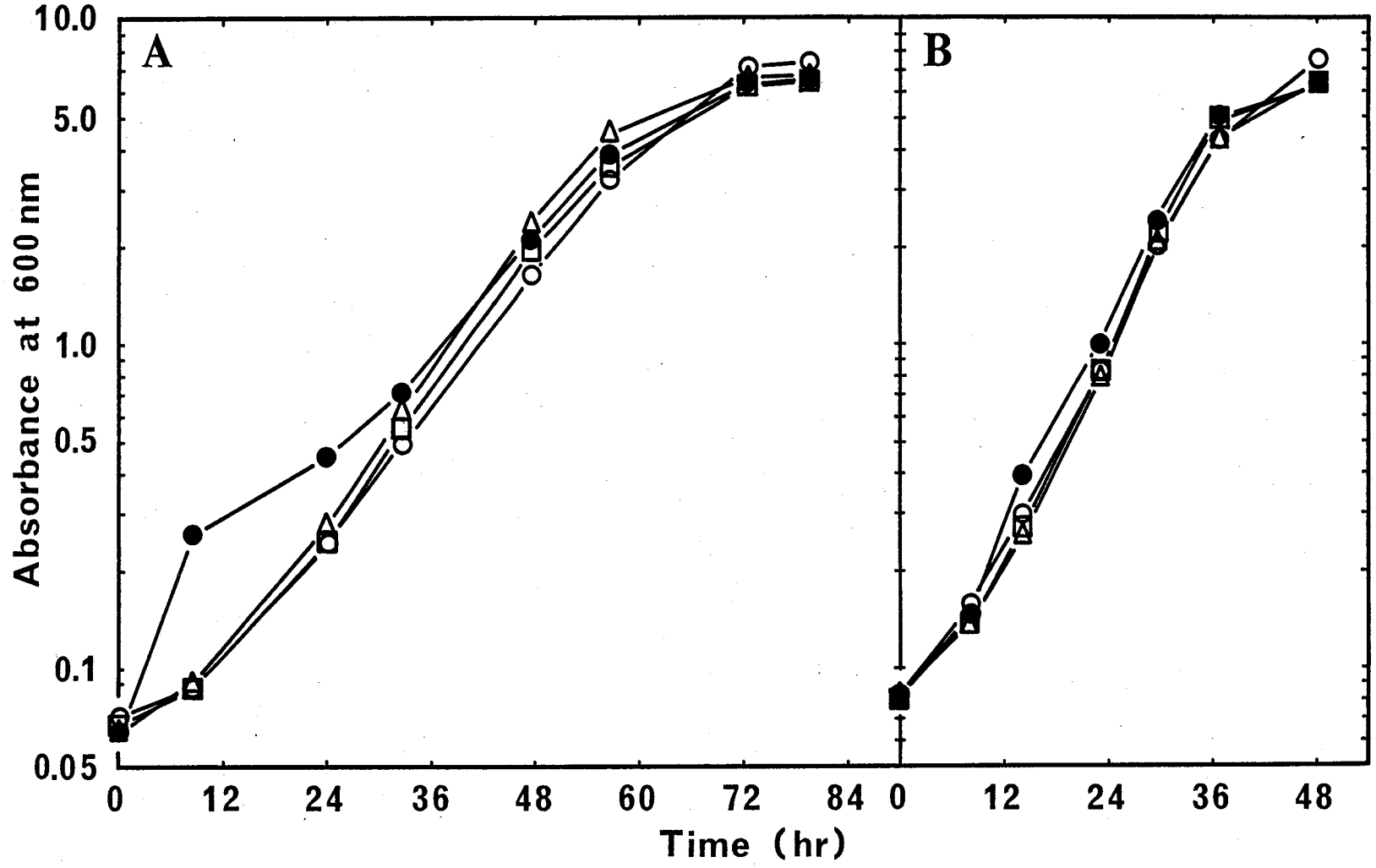
A
B
10.0
5.0
1.0
0.5
0.1
0.05
Absorbance at 600 nm
0
12
24
36
48
60
72
84
Time (hr)

Fig. 27.

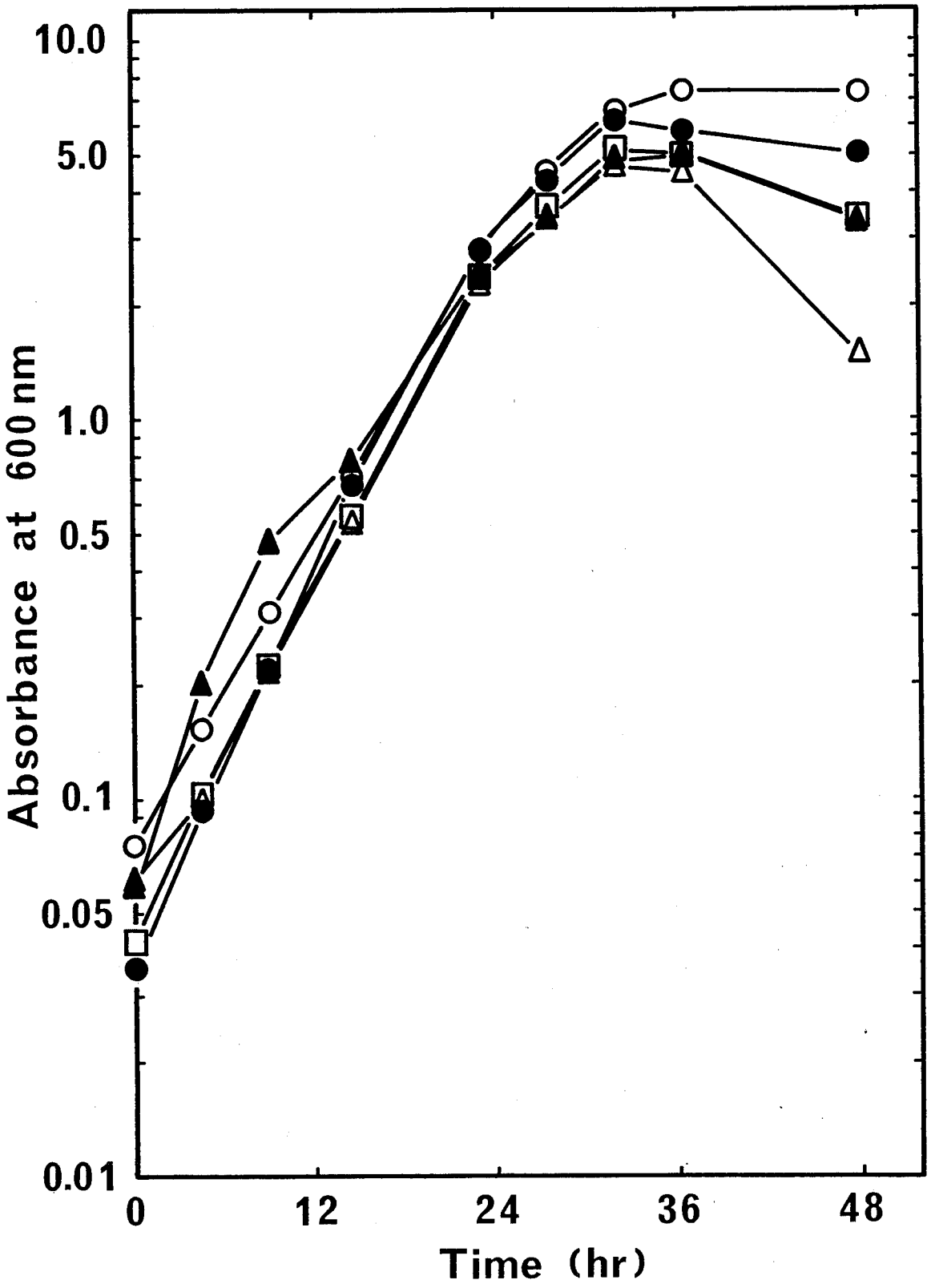

Fig. 28.

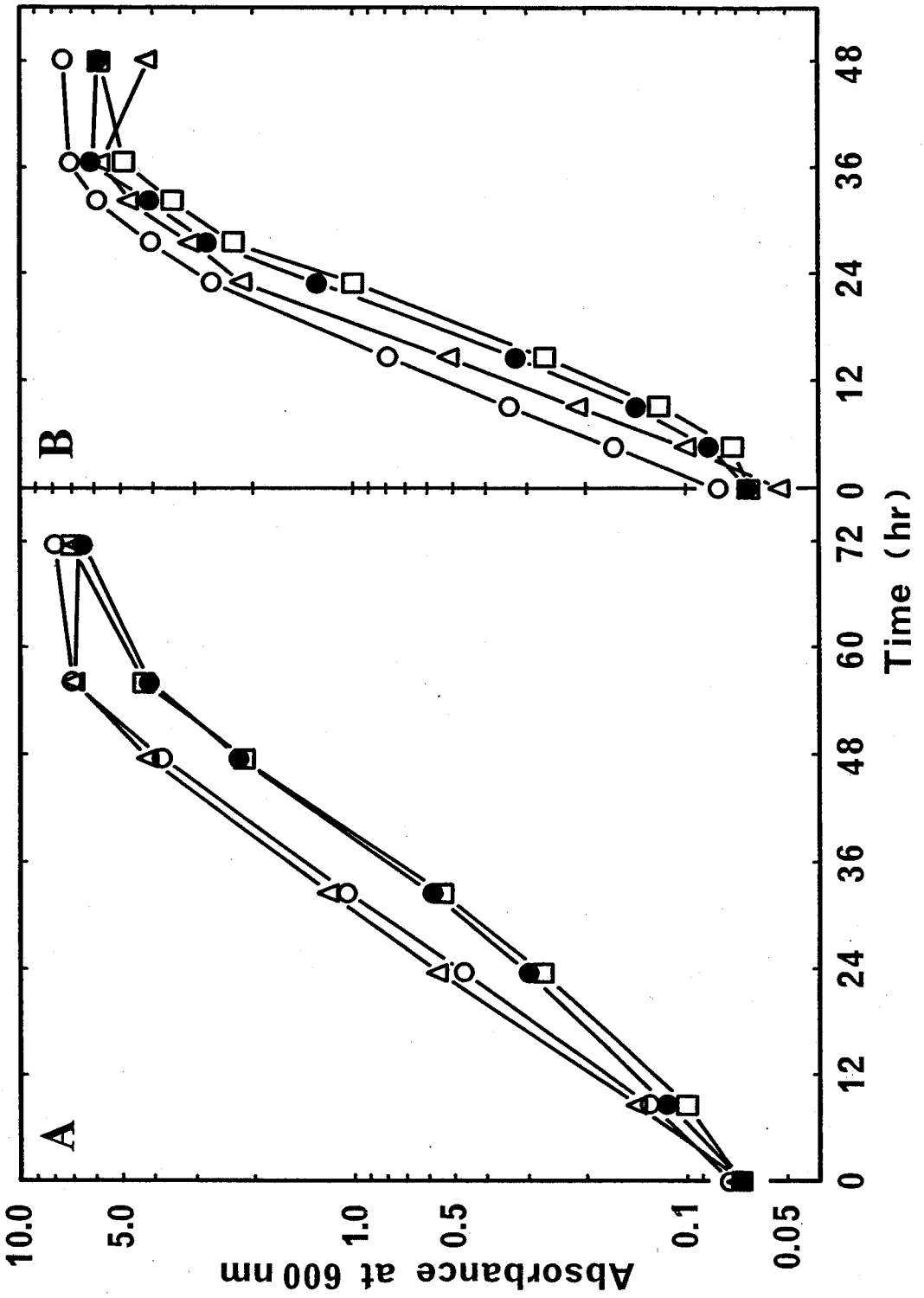
A
B
10.0
5.0
1.0
0.5
0.1
0.05
Absorbance at 600 nm
0
12
24
36
48
60
72
0
12
24
36
48
Time (hr)

Fig. 29.

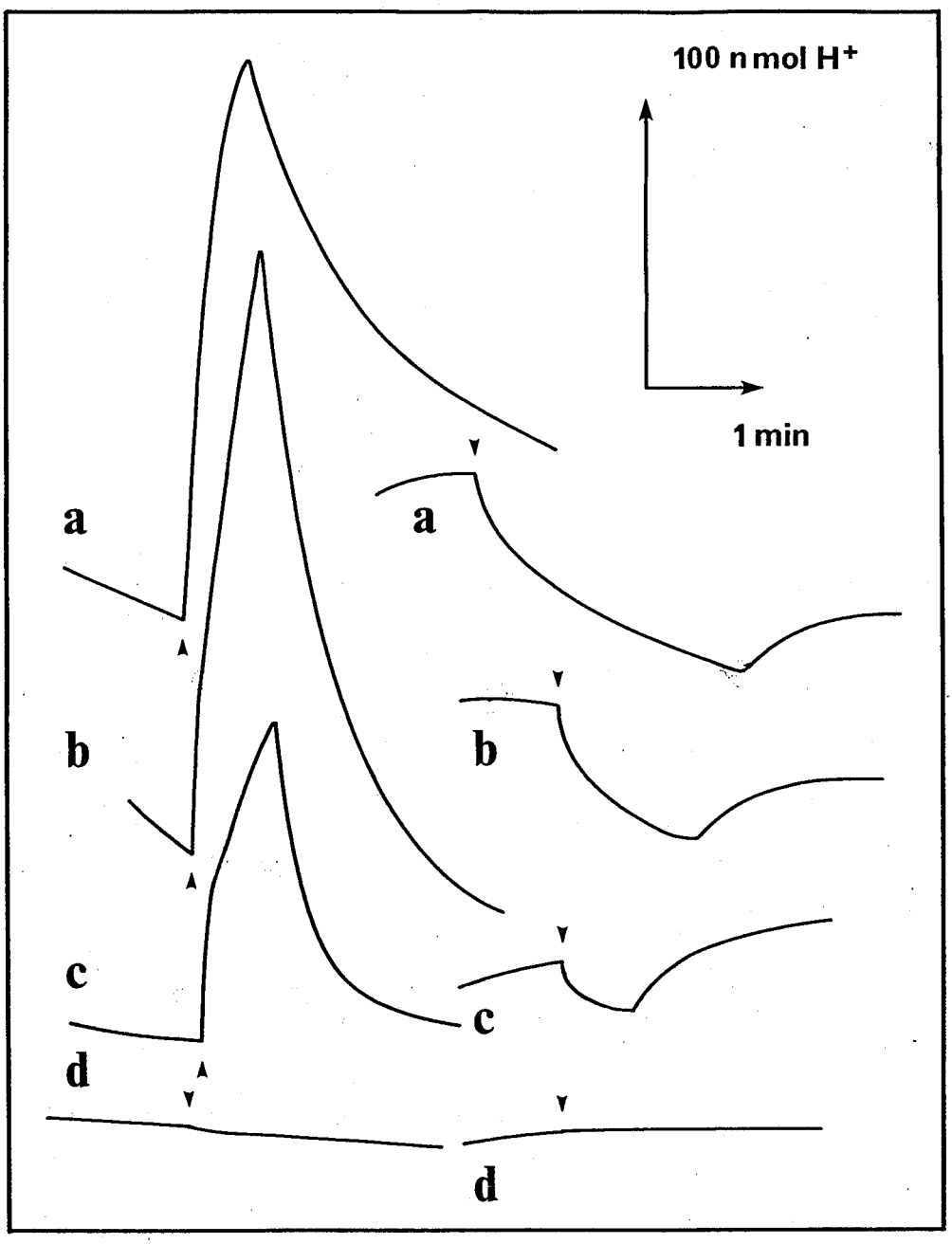

Fig. 30.

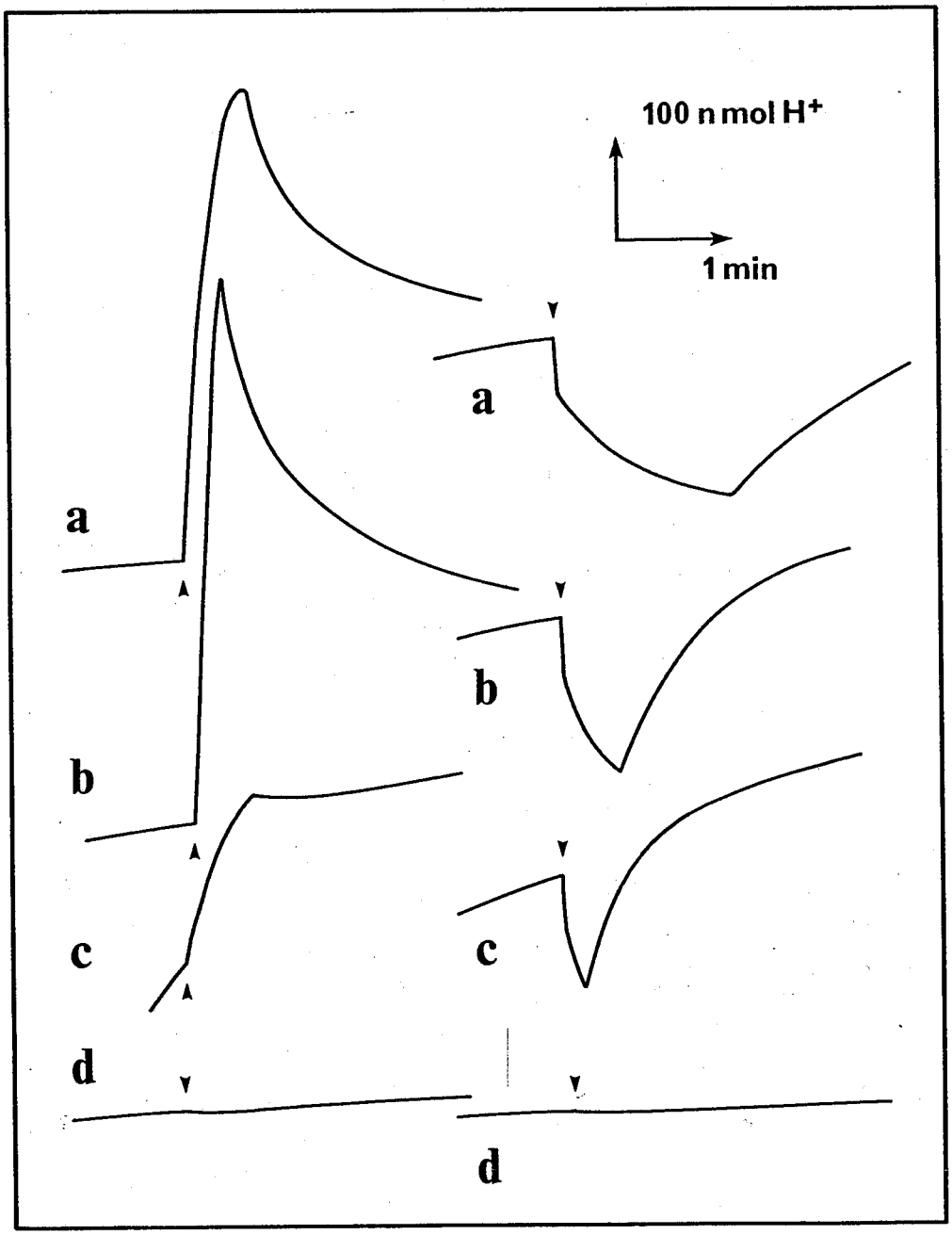

Fig. 31.

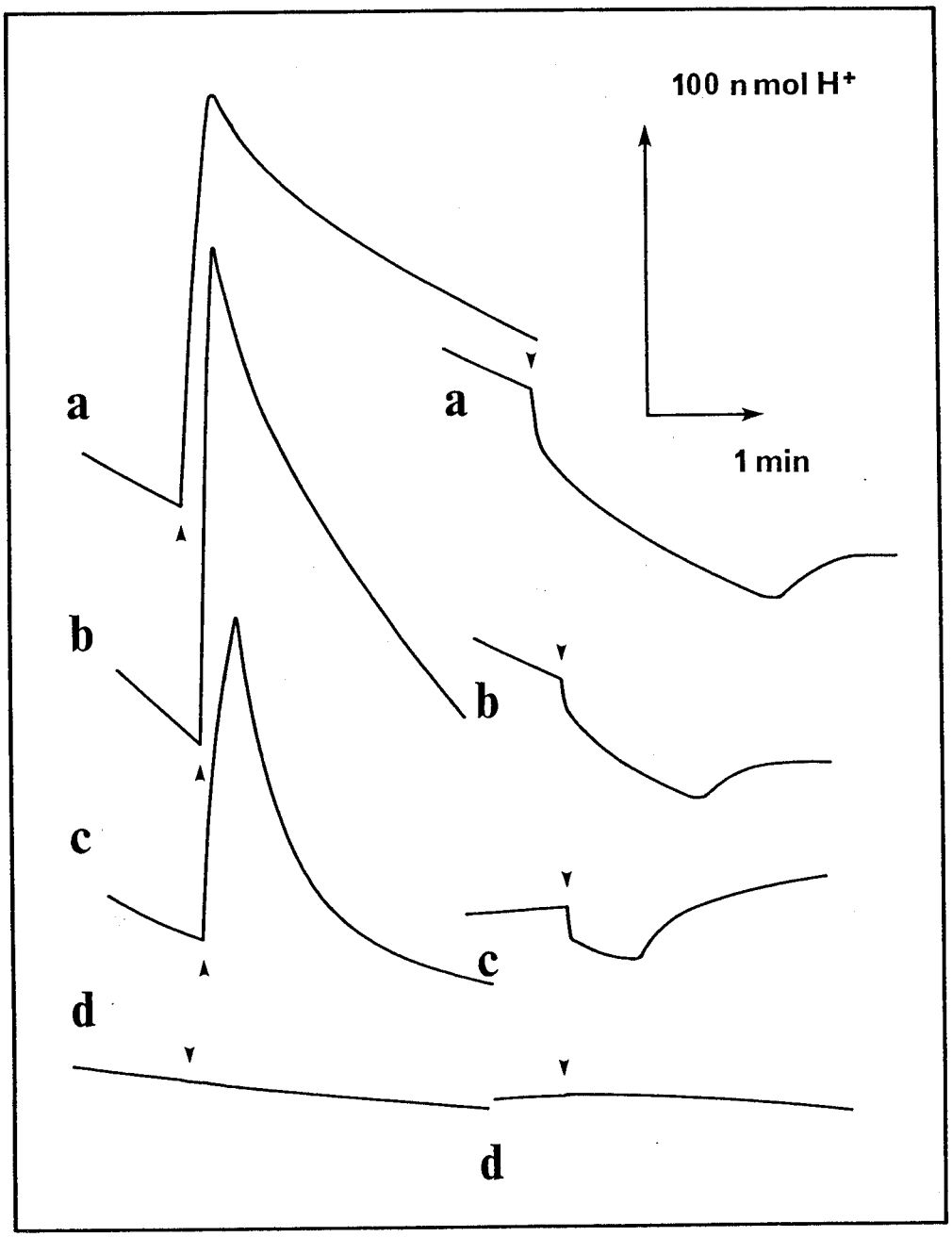

Fig. 32.

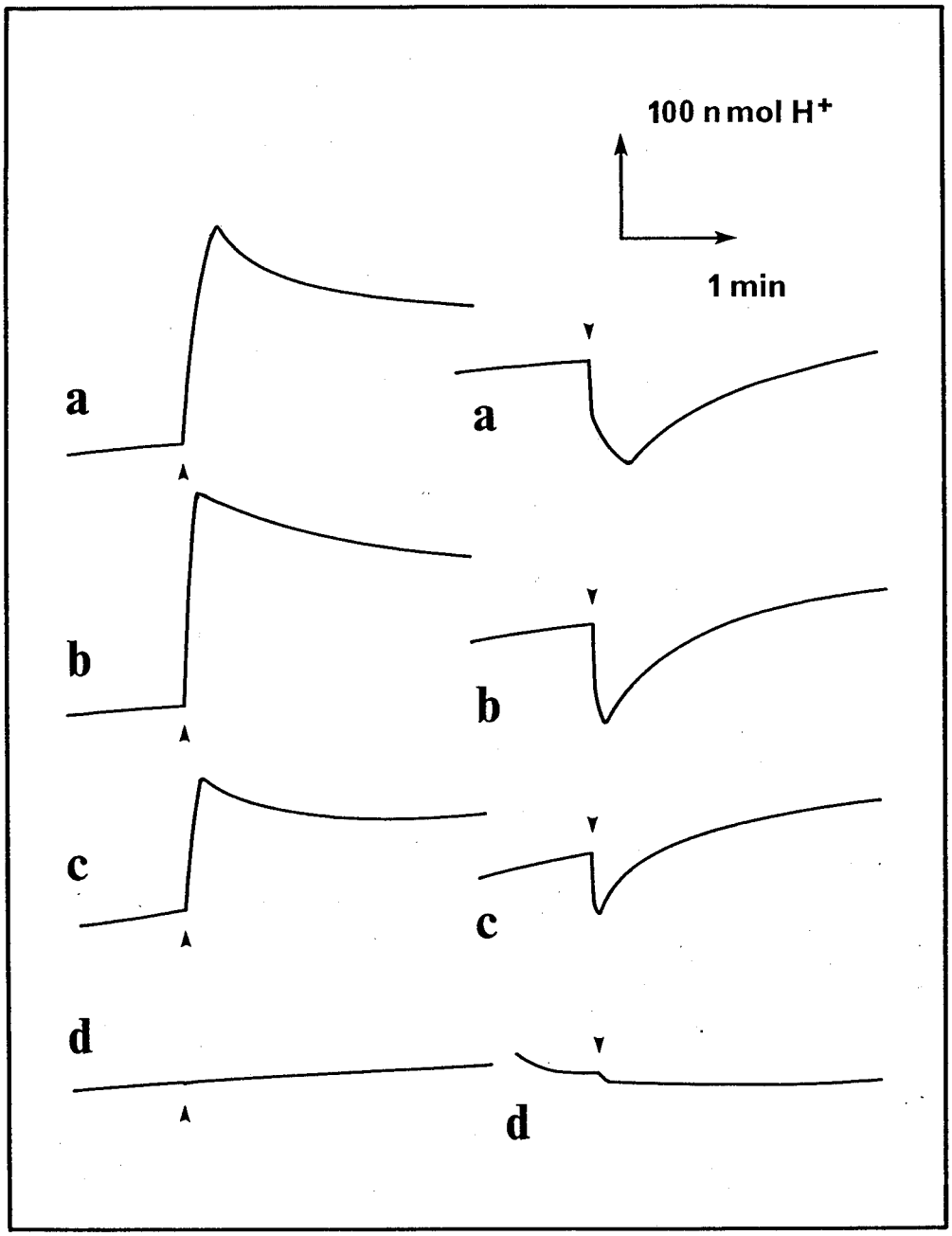

Fig. 33.

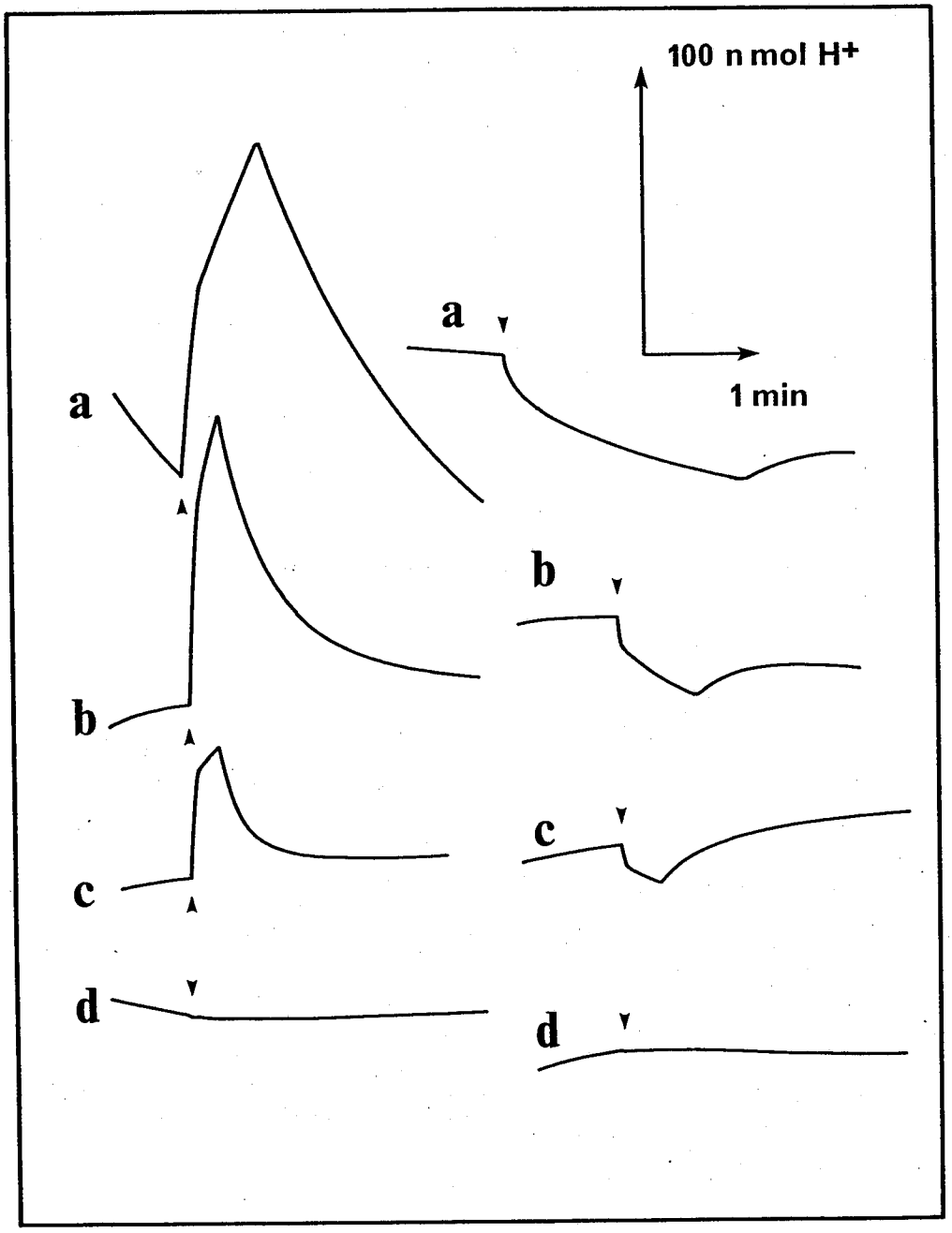

Fig. 34.

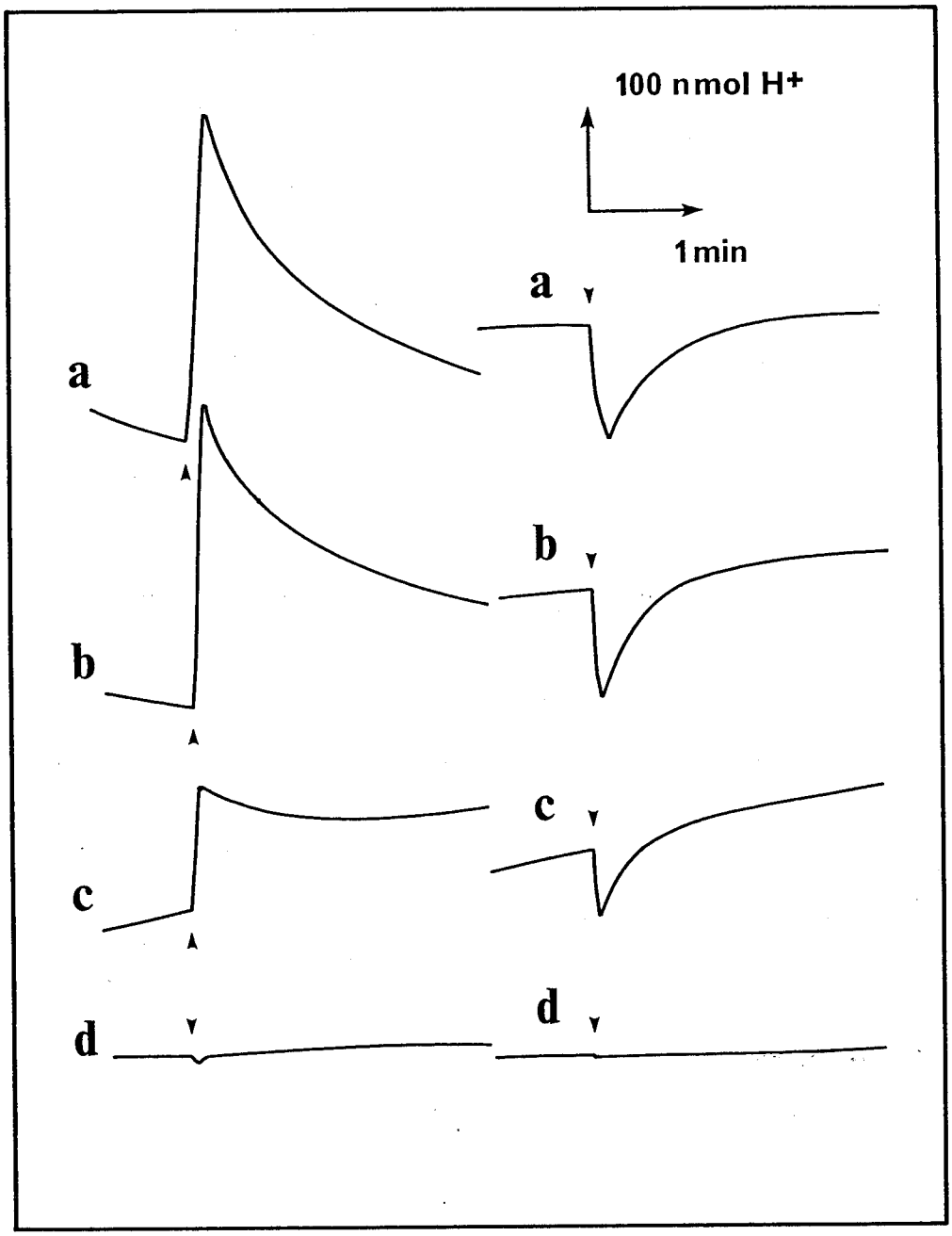

Fig. 35.

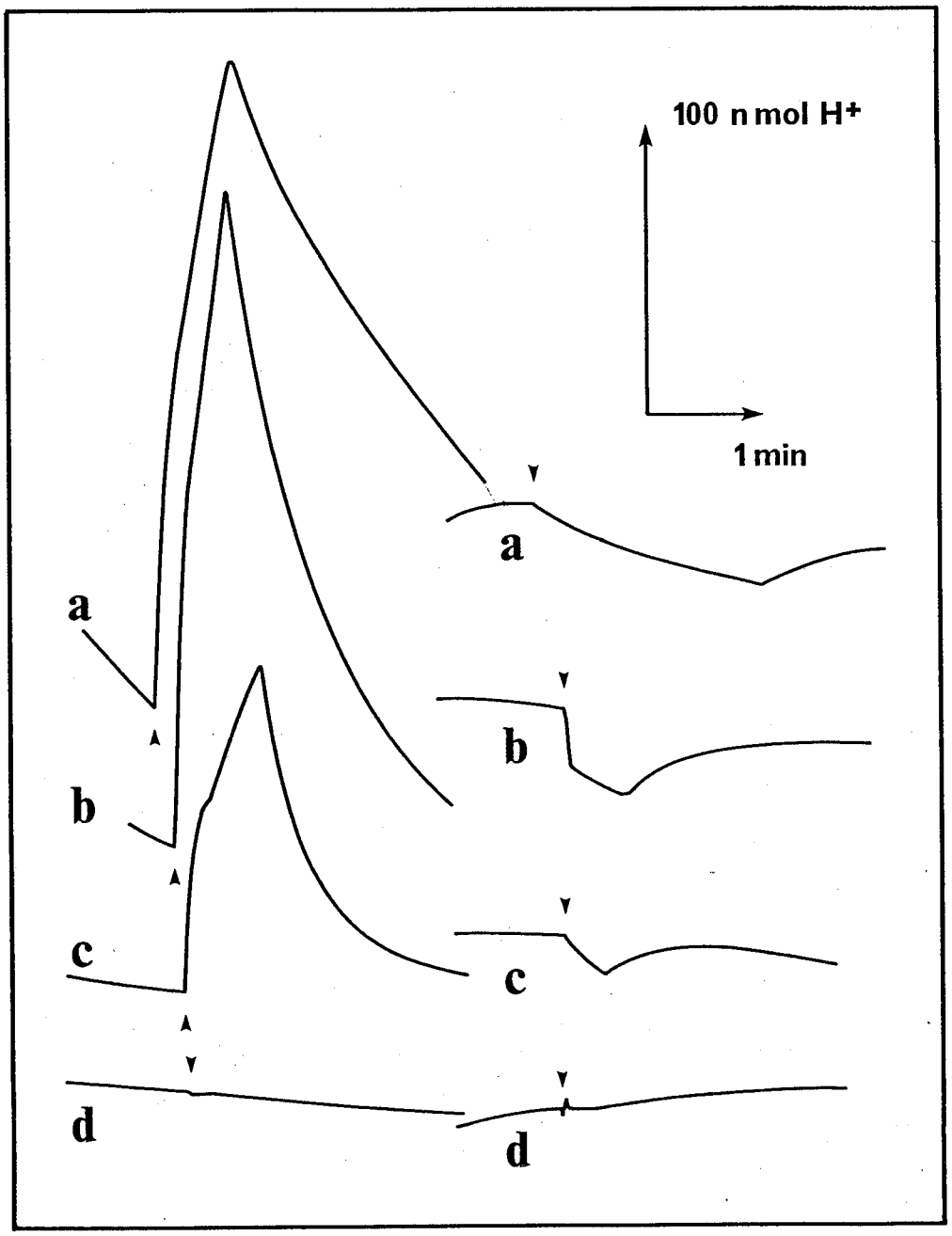

Fig. 36.

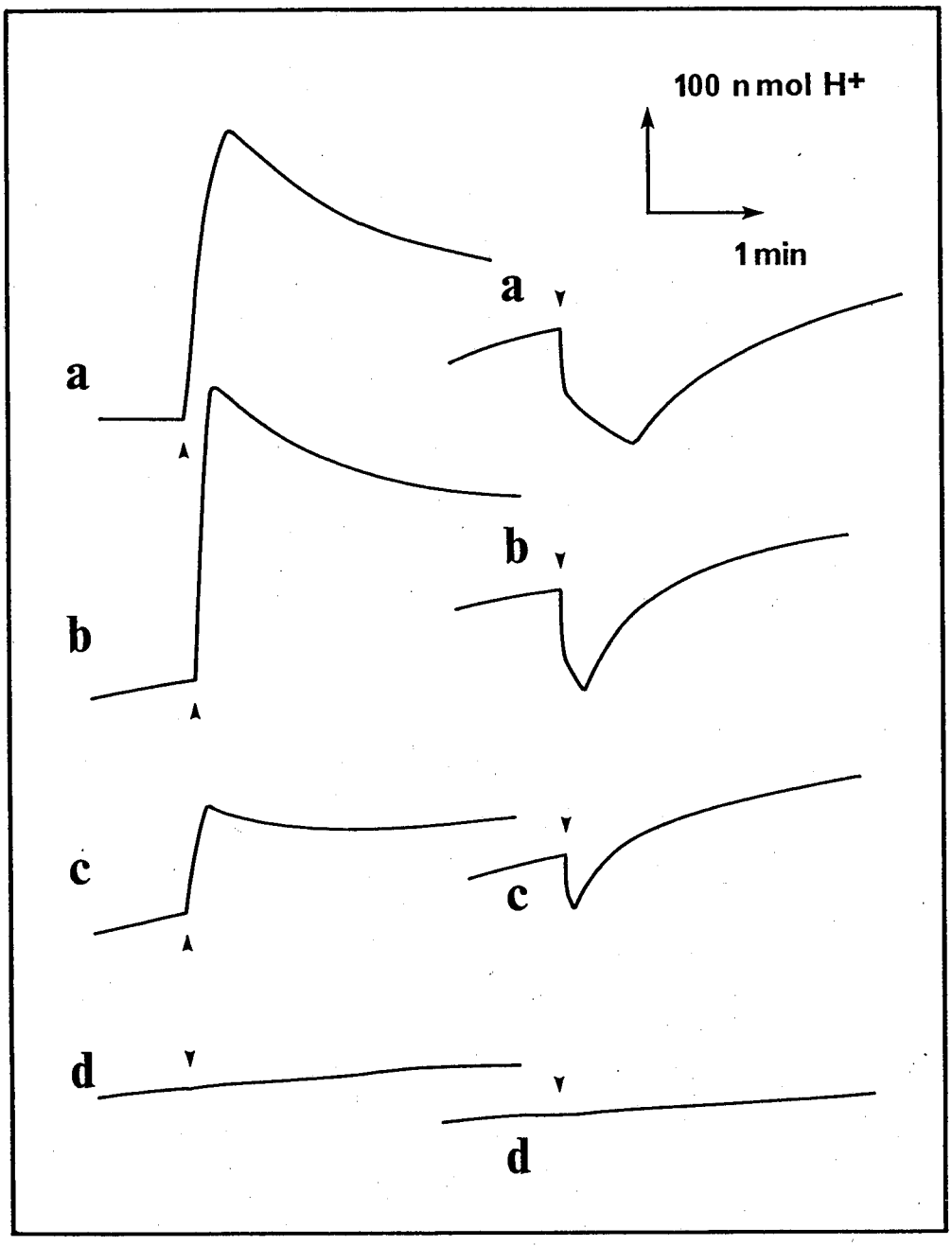

Fig. 37.

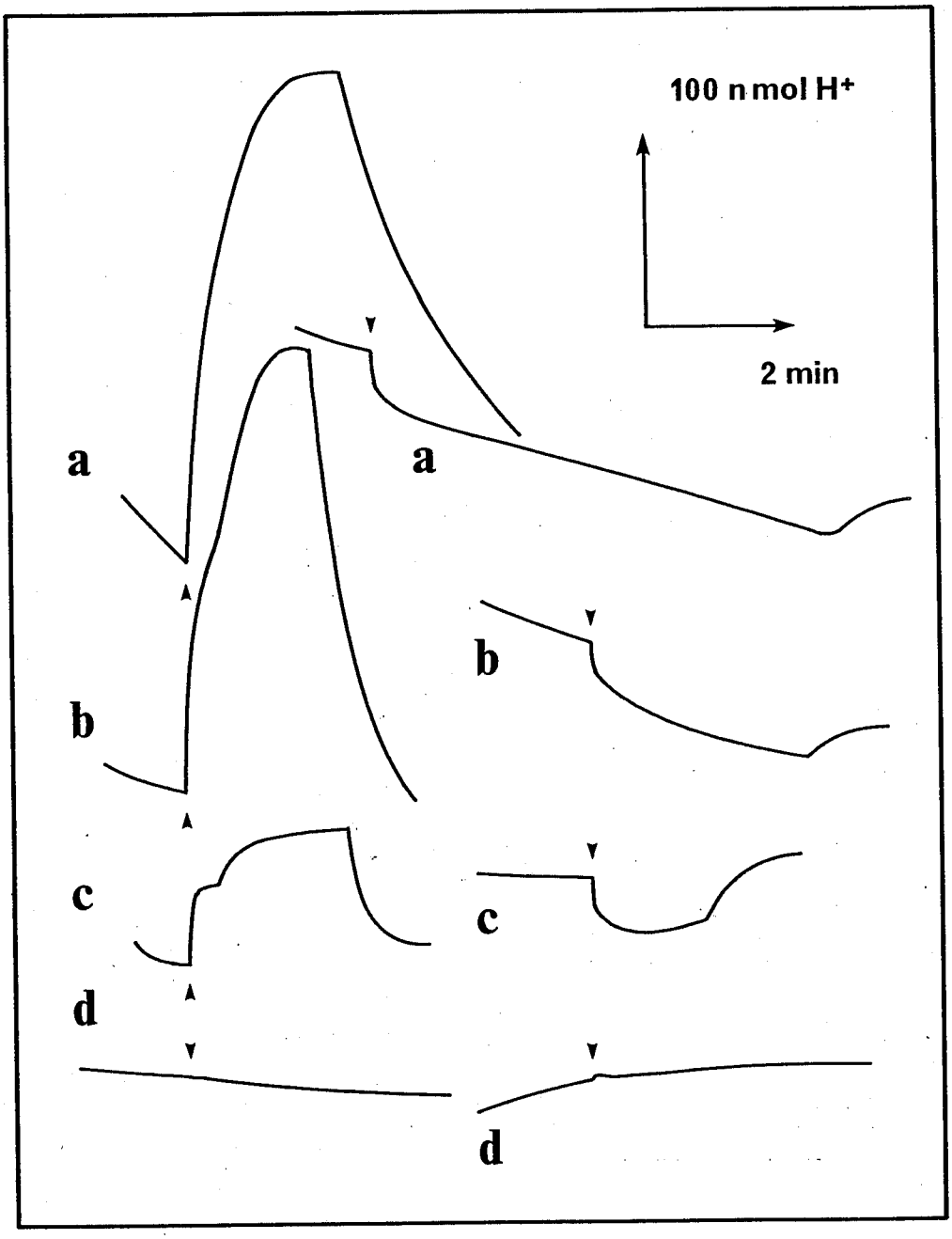

Fig. 38.

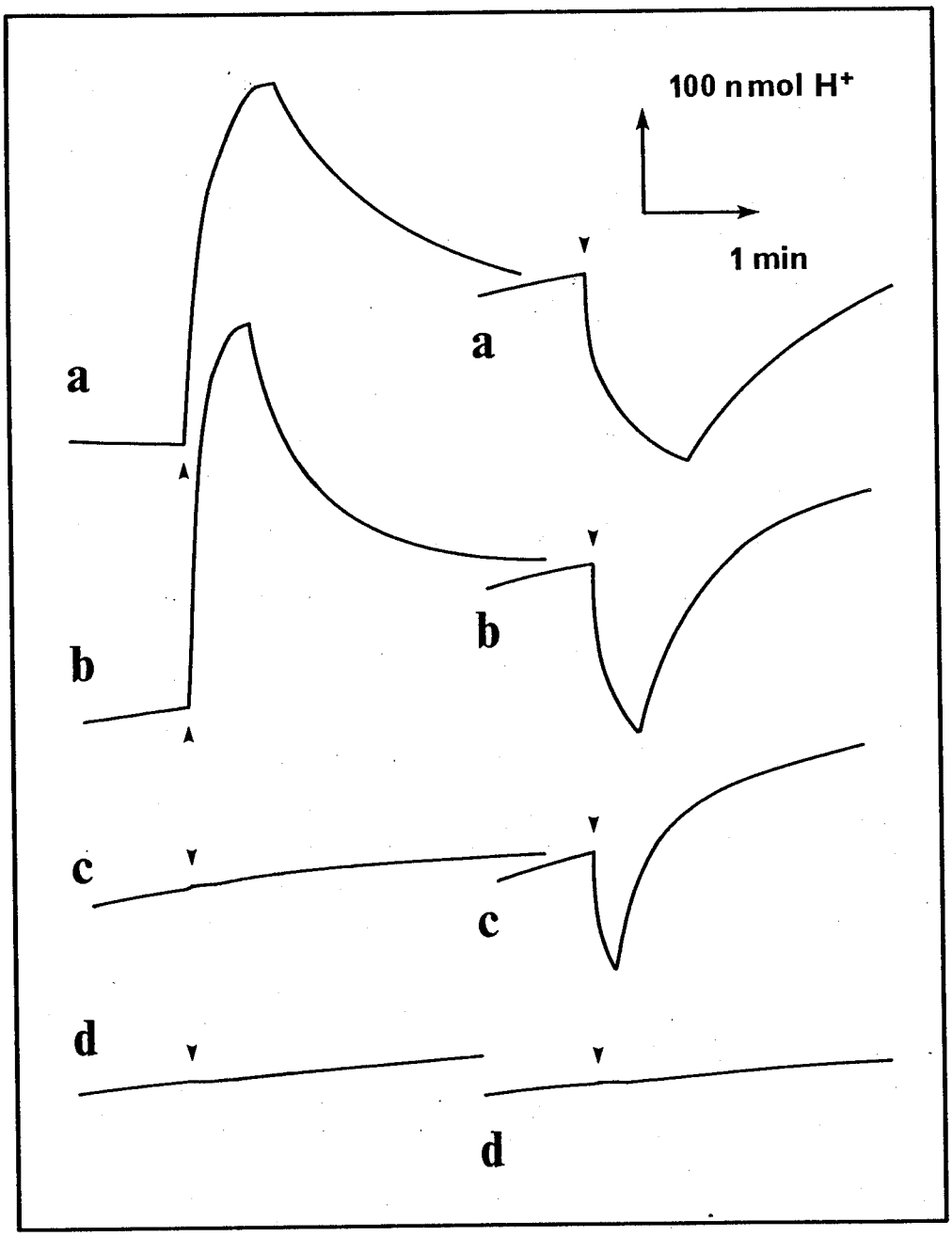

Fig. 39.

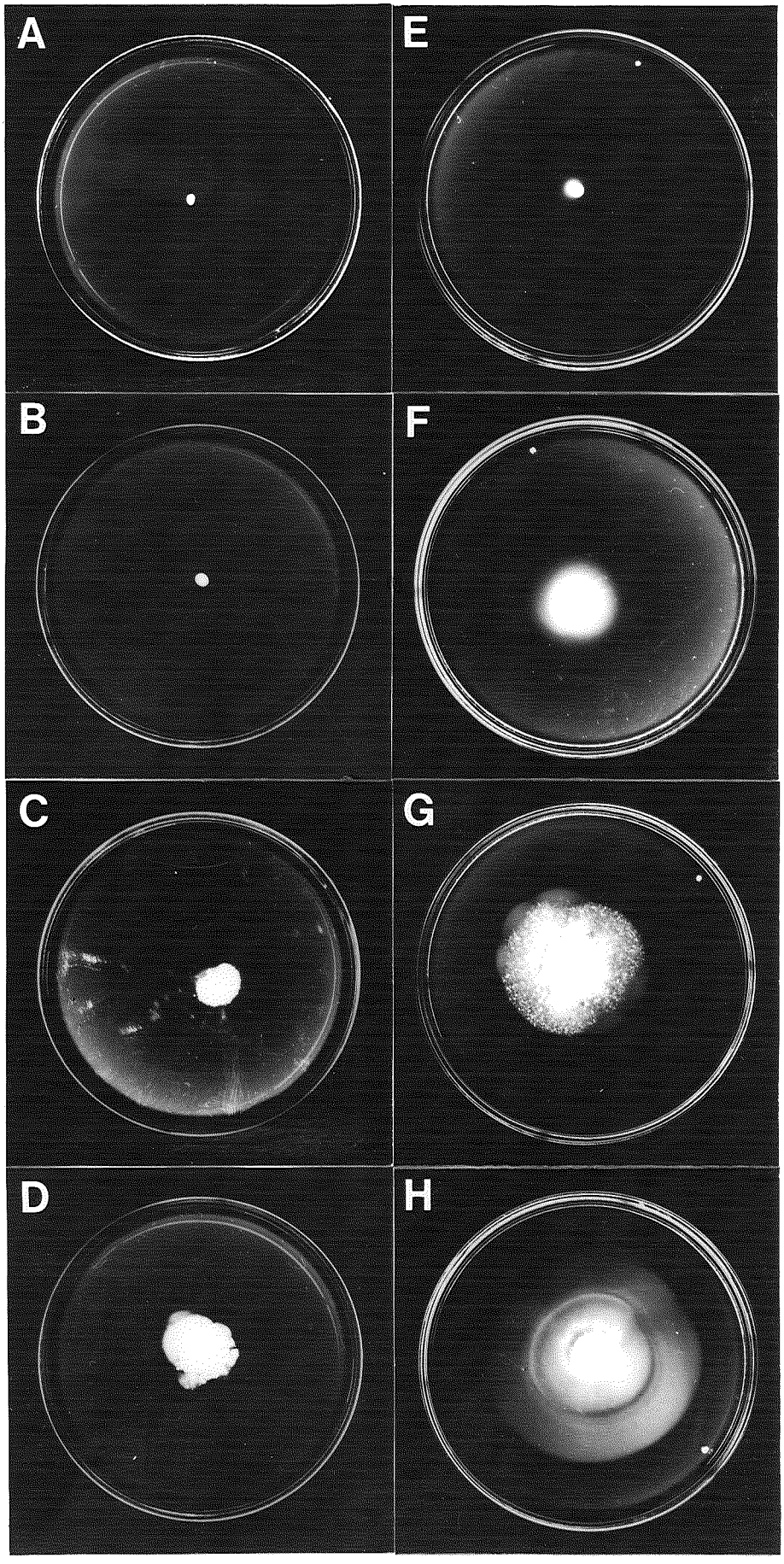

Fig. 40.

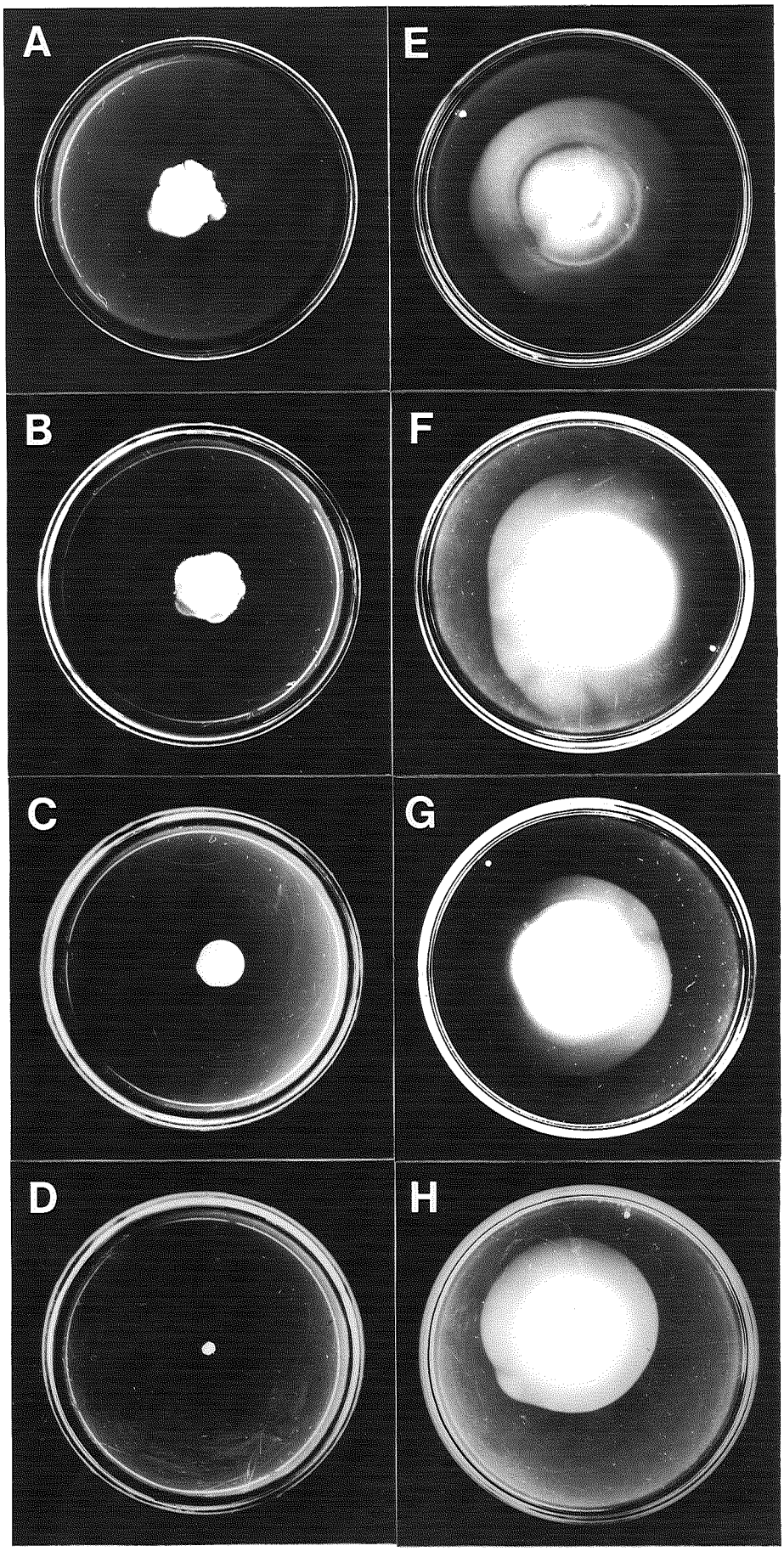

Fig. 41.

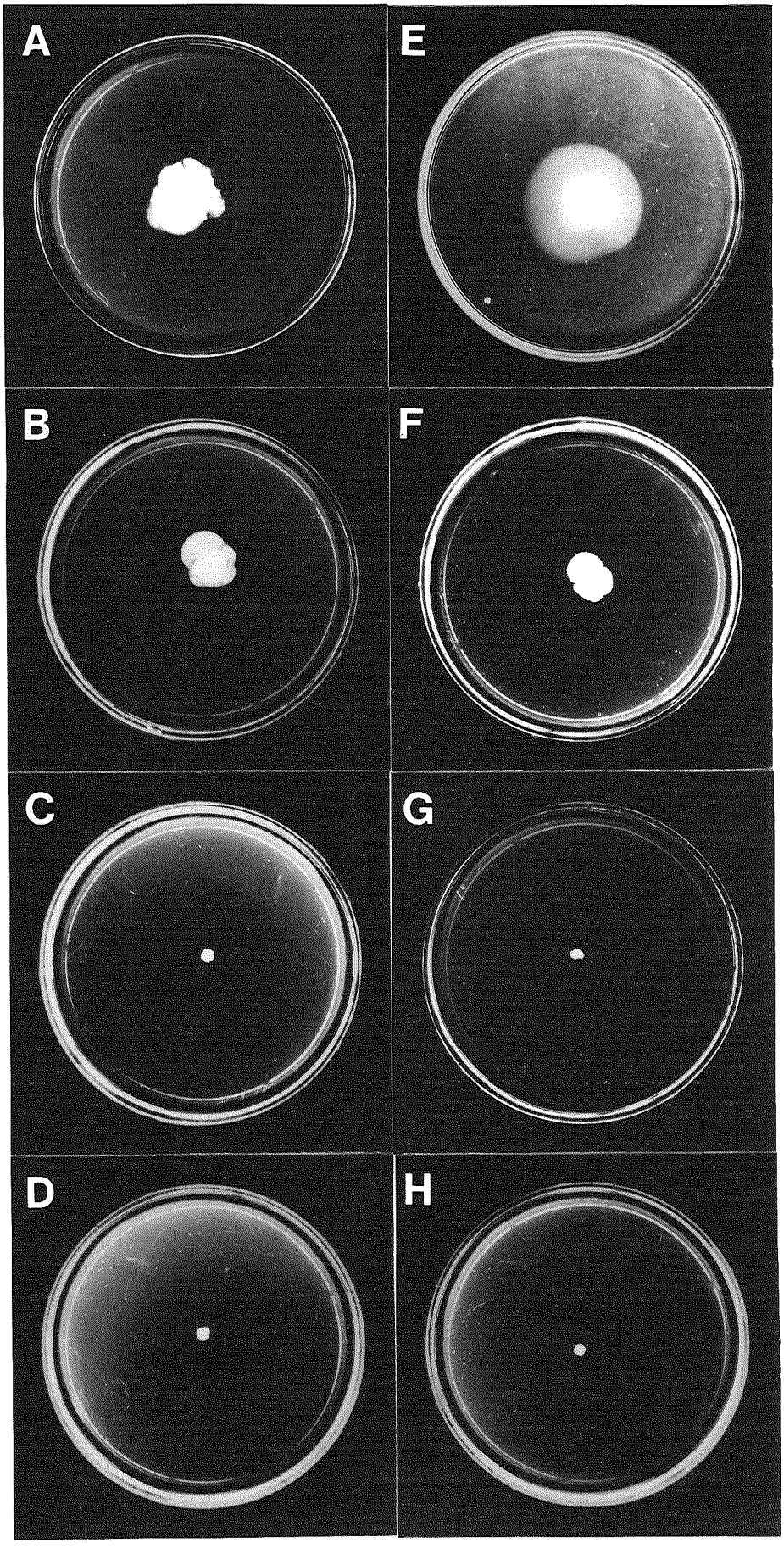

Fig. 42.

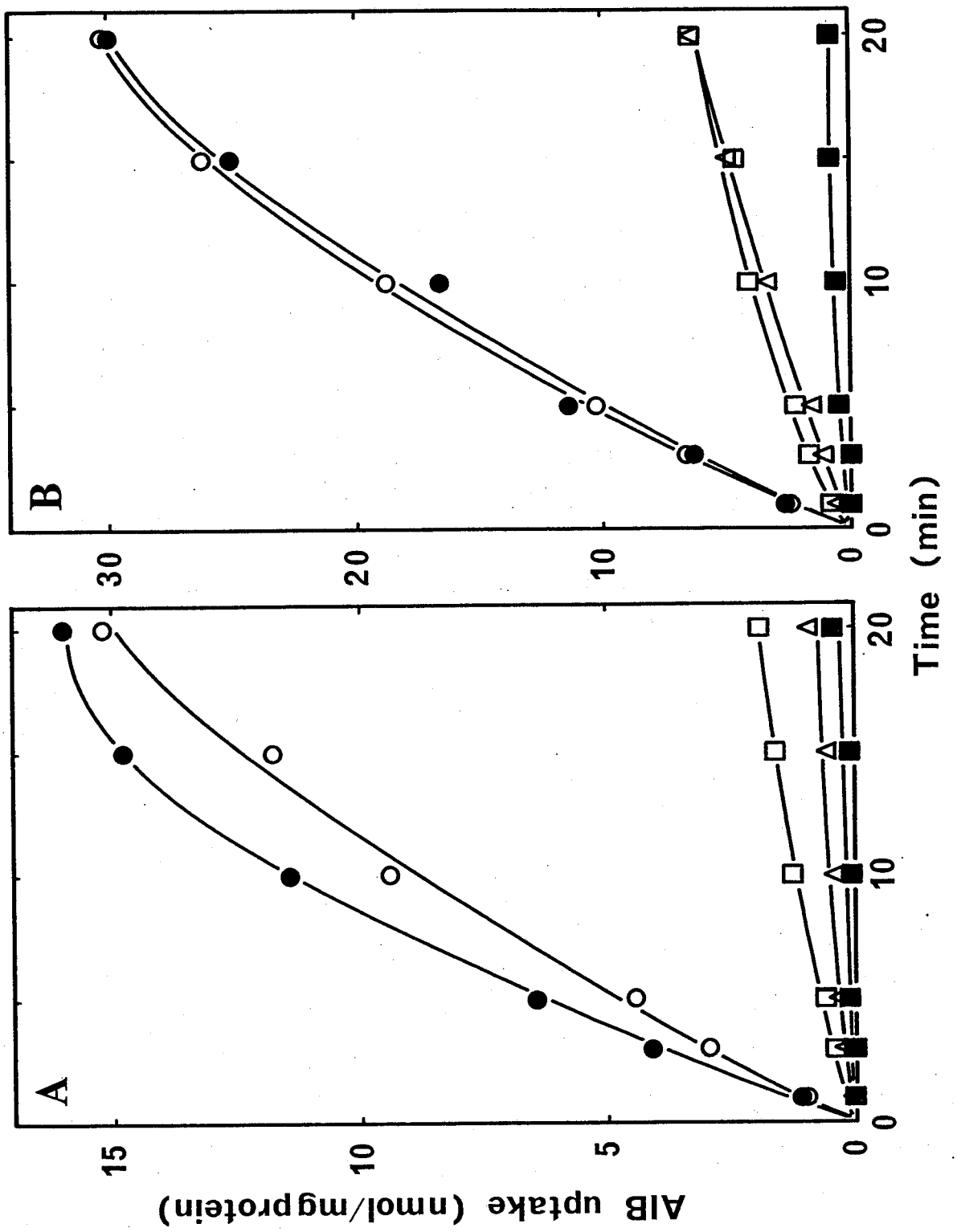

Fig. 43.