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Title STUDIES ON PARTIALLY PURIFIED MALIC DEHYDROGENASE FROM THE MARINE PSYCHROPHILE, VIBRIO MARINUS

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The thermolability of malic dehydrogenase in whole cells of Vibrio marinus MP-1 grown at 15 C was compared with that of cell-free extracts and partially purified fractions.

A 20-fold purification of the enzyme was effected by ammonium sulfate fractionation and column chromatography on Sephadex G-200 gel. The intracellular enzyme was found to be stable between 0 C and the organism's optimum growth temperature, 15 C. In cell-free extracts considerable lability was noted even at 0 C, and this lability did not increase further until the enzyme was exposed to temperatures above the organism's maximum growth temperature, 20 C. Purified enzyme was stable between 15 C and 20 C, but both above and below this temperature range there was considerable loss of activity with a 30 minute exposure.

Both cold- and heat-inactivated enzyme were re-activated, although to a different degree, when placed at 15 C for five minutes.

A stimulation in activity was found when the enzyme was exposed to ammonium sulfate. This salt would also re-activate heat inactivated enzyme, and the presence of ammonium sulfate even at 40 C gave complete protection.

STUDIES ON PARTIALLY PURIFIED MALIC DEHYDROGENASE FROM THE MARINE PSYCHROPHILE, VIBRIO MARINUS

by

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My fiance whose belief in my ability to produce such a thesis greatly exceeded my own and whose encouragement inspired me to do so.

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INTRODUCTION

Several reports describing the physiological characteristics and growth of psychrophilic bacteria have appeared in the literature in recent years. Until recently few of the organisms studied exhibited a maximum growth temperature of 20 C or less, and therefore could not be considered to be 'obligate' in their low temperature requirement (9, p. 106).

The importance of using bacteria which are intrinsically obligately psychrophilic in nature must be emphasized if the studies are to give any true indication of the physiological nature of psychrophily. Colwell and Morita (2) describe the isolation and characterization of a series of psychrophiles from the marine environment. These have been designated as strains of Vibrio marinus, several of which have maximum growth temperatures of less than 20 C.

Ninety percent of the marine environment is at a temperature of less than 5 C (32), and studies of marine microorganisms which have a growth range allowing proliferation at these temperatures, are essential if we are to make any estimations of the activity of such organisms in their natural environment (6, 8, 22, 29, 30). Morita and Albright (12) have shown that this organism grows well at 4 C, that

is, at the normal environmental temperature, and produces 10^9 cells per ml in 24 hours. Studies using V. marinus MP-1, (15) have shown that this bacterium has an optimum growth temperature of 15 C. It will not grow above 20 C, and preincubation above this temperature causes extreme loss of viability.

Further studies (25) were initiated to establish the nature of this heat induced lethal effect. Oxygen uptake of whole cells at 15 C in the presence of glucose was completely inhibited by preincubation at 29.4 C for 30 minutes. Loss of integrity of the cell membrane was suggested, as leakage of materials which absorb at 260 mμ occurred at these temperatures. As membrane damage could not account for the complete loss of oxygen uptake, the possibility existed that there was a concomitant denaturation of one or more enzymes associated with this uptake.

That a single enzyme could in part be responsible for loss of cellular function at increased temperatures has been shown by Burton and Morita (1). These studies were performed with a facultative psychrophile, V. marinus PS-207, with an optimum growth temperature of 24 C. Malic dehydrogenase (MDH) activity decreased to 20 percent of its original value when cell-free extracts were heated to 30 C for 40 minutes.

Heat inactivation of formic hydrogenlyase in whole cells of a facultatively psychrophilic strain of Escherichia coli was shown to be

far greater than in a mesophilic strain under the same condition (29).

The above studies therefore resulted in the present investigation into the thermostability of MDH in partially purified preparations from the obligate psychrophile V. marinus MP-1.

REVIEW OF LITERATURE

Several theories have been postulated on the nature of optimal growth temperatures in microorganisms. Ingraham and Bailey (8) suggested that the differences in temperature optima probably reside in the intra-cellular organization. Rose (26) proposed that the accumulation of toxic metabolic substances formed at higher temperatures may be the cause of death at these temperatures.

That cell lysis is involved in loss of viability is shown by Hagen et al. (5). They state that the highest temperature at which growth occurs is two degrees below the temperature at which the cells lyse. However, it is suggested that the primary cause of death is not cell wall or membrane rupture, as release of substances associated with these structures occurs after the onset of lysis.

The majority of the literature in this field, however, is concerned with the effect of temperature on enzyme systems which themselves control viability and growth. Edwards and Rettger (3) demonstrated that several enzymes from species of Bacillus were inactivated at temperatures just above the maximum for growth. Nashif and Nelson (16) showed that although lipase activity in Pseudomonas fragi was optimal at 40 C, no enzyme was produced above 30 C, suggesting a heat sensitive enzyme forming system.

A similar effect was found by Upadhyay and Stokes (30) who

examined formic hydrogenlyase in both mesophilic and facultatively psychrophilic strains of Escherichia coli. This enzyme in the psychrophilic strain was optimal at 30 C but inactive at 45 C. The enzyme forming system was inactive above 20 C.

A dramatic difference in the MDH activity of psychrophilic and thermophilic bacteria is observable in the articles by Burton and Morita (1) and Morita and Haight (14). The former showed that in cell-free extracts of a marine psychrophile, the enzyme activity was decreased to twenty percent of the original value at 30 C for 40 minutes. However, in the latter case, MDH in extracts from Bacillus stearothermophilus exhibited optimal activity at 55 C and was completely inactivated at 78 C. An increase in hydrostatic pressure above 700 atm. permitted enzyme activity at 101 C.

In 1940 ZoBell and Conn (33) examined the thermal sensitivity of marine bacteria and found a pronounced decrease in oxygen uptake at 30 C compared with the activity at optimal growth temperatures, and suggested that the respiratory enzymes were important in the inability to grow at higher temperatures. Hagen and Rose (6) in studies on a psychrophilic Cryptococcus found that at the maximum growth temperature there was a rapid utilization of the intracellular amino acid pool. This was shown to be related to the inability to synthesize α-oxo glutarate at this temperature.

An apparent increase in MDH activity was found when whole cells

of a marine psychrophile were subjected to temperatures above the maximum for growth (13). However, this effect was reversed in cell free systems and so permeability, as well as heat lability of the enzyme, were suggested as partial reasons for the maximum growth temperatures.

As early as 1932, Northrup (17) showed that crystalline trypsin which was inactivated at 70 C could be renatured by slow cooling. Hence there is a need to differentiate between irreversible denaturation and reversible inactivation of enzymes. An excellent review on the denaturation and inactivation of enzyme proteins by Okunuki (19) discusses also the process of renaturation.

The renaturation of thermolabile MDH from a marine psychrophile, under various conditions in cell-free extracts was examined by Burton and Morita (1). They demonstrated that after heating at 40 C the inactivation was completely reversible. Stability of the enzyme in the whole cells was far greater than in extracts, and as at least two components of the synthetic renaturation system were normal cellular constituents, it was suggested that the cell may have a similar mechanism for the in vivo maintenance of the enzyme.

Several workers have found that the presence of certain salts would protect enzyme activity against heat and chemical inactivation (19, 24). The effect of several ionic species on both the activity and stability of MDH has been examined (10).

Many examples of purification procedures of MDH from both plant and animal sources exist in the literature (4, 7, 10, 18), and wide differences have been noted in the degrees of purity reached in each case. The existence of multiple forms of this enzyme which differ in heat stability and other properties has been reported (12). The probable existence of at least three forms of MDH in a marine psychrophile was suggested on the basis of the kinetics of the denaturation and renaturation processes (1).

METHODS AND MATERIALS

Growth Medium

The growth medium consisted of 1.0 g succinic acid, 15.0 g sodium chloride, 5.0 g proteose peptone, 2.0 g glucose, 2.0 g yeast extract, 5.0 g Rila marine salts (Rila Products, Teaneck, N. J.), and 0.01 g ferrous sulfate brought to a total of one liter with tap water. The pH was adjusted to 7.4 and sterilization was effected by autoclaving at 15 psi for 20 minutes.

Rila salts at a concentration of 26.25 g per liter in distilled water and pH 7.4, was used for washing and resuspending the cells after harvesting unless otherwise stated, and will be referred to as 75 percent sea water.

Microbiological Methods

The marine psychrophile V. marinus MP-1 isolated by Morita and Haight (15) was used in these studies. Stock cultures were maintained at 15 C and transferred twice at 12 hour intervals prior to mass culturing of cells.

For the preparation of the stock cell suspension, 36 liters of medium in a large carboy were inoculated with 3.6 liters of a 12 hour culture of the organism. The culture was incubated with shaking and aeration at 15 C for 21 hours. The cells were harvested by

centrifugation in a Sorvall RC-2 refrigerated centrifuge at 0 C and 20,000 x g. They were washed twice in 75 percent sea water and divided into six portions each of 2.6 g wet weight and stored at -20 C for preparation of cell-free extracts.

For whole cell studies, 300 ml of the above medium were inoculated with a 5.0 ml of 12 hour inoculum. The cells were incubated for 18 hours in a New Brunswick Psychrotherm at 15 C and aerated by shaking. They were harvested as above and suspended in 75 percent sea water so that a 1:40 dilution gave an optical density (OD) of 0.26 at 525 mμ in the Bausch and Lomb Spectronic 20 spectrophotometer. These cells were used immediately for heat inactivation studies and were kept on ice between procedures.

Enzyme Preparation

Each of the stored cell preparations was made up to 200 ml in 0.2 M tris-(hydroxymethyl)-aminomethane (Tris-) buffer which was dissolved in distilled deionized water and brought to pH 7.4 with sulphuric acid. The suspension was treated for 15 minutes in 10-kc Raytheon sonic oscillator which was cooled using an ethanol and ice mixture. Cell debris was removed by centrifugation at 35,000 x g for 60 minutes and the supernatant fractionated with ammonium sulfate (4). The fractionation was carried out at 0 C. The precipitates occurring between 0-60 percent saturation were kept and stored at

-20 C.

Column chromatography was performed on Sephadex G-200 gel in 0. 2 M Tris-sulfate pH 8. 6 buffer containing ammonium sulfate at a concentration of 0. 75 M A Pharmacia column of one inch diameter and 18 inches height was used together with a Buchler Polystaltic pump to control the flow rate. This system was adjusted to deliver between one and two ml per hour. The gel was placed in the buffer at least three days before packing the column, and immediately prior to packing, the gel was placed under a vacuum to remove trapped air bubbles. The column was prepared so that the hydrostatic head never exceeded ten cm. Buffer was allowed to flow through the column for 24 hours before sample application, to allow settling of the gel. Enzyme samples were applied to the top of the gel beneath the surface of the buffer, using a syringe with an elongated tip. Fractions were collected using an ISCO automatic fraction collector. The void volume of the column was determined using blue colored, two million molecular weight dextran (Dextran 2000) produced by Pharmacia Company.

Following chromatography the active enzyme fractions were pooled and frozen until there was a total of 250 ml. This enzyme solution was re-fractionated at pH 8. 6 and 0 C with ammonium sulfate. The active fractions were stored at -20 C until required. These fractions (referred to as purified enzyme preparation) were

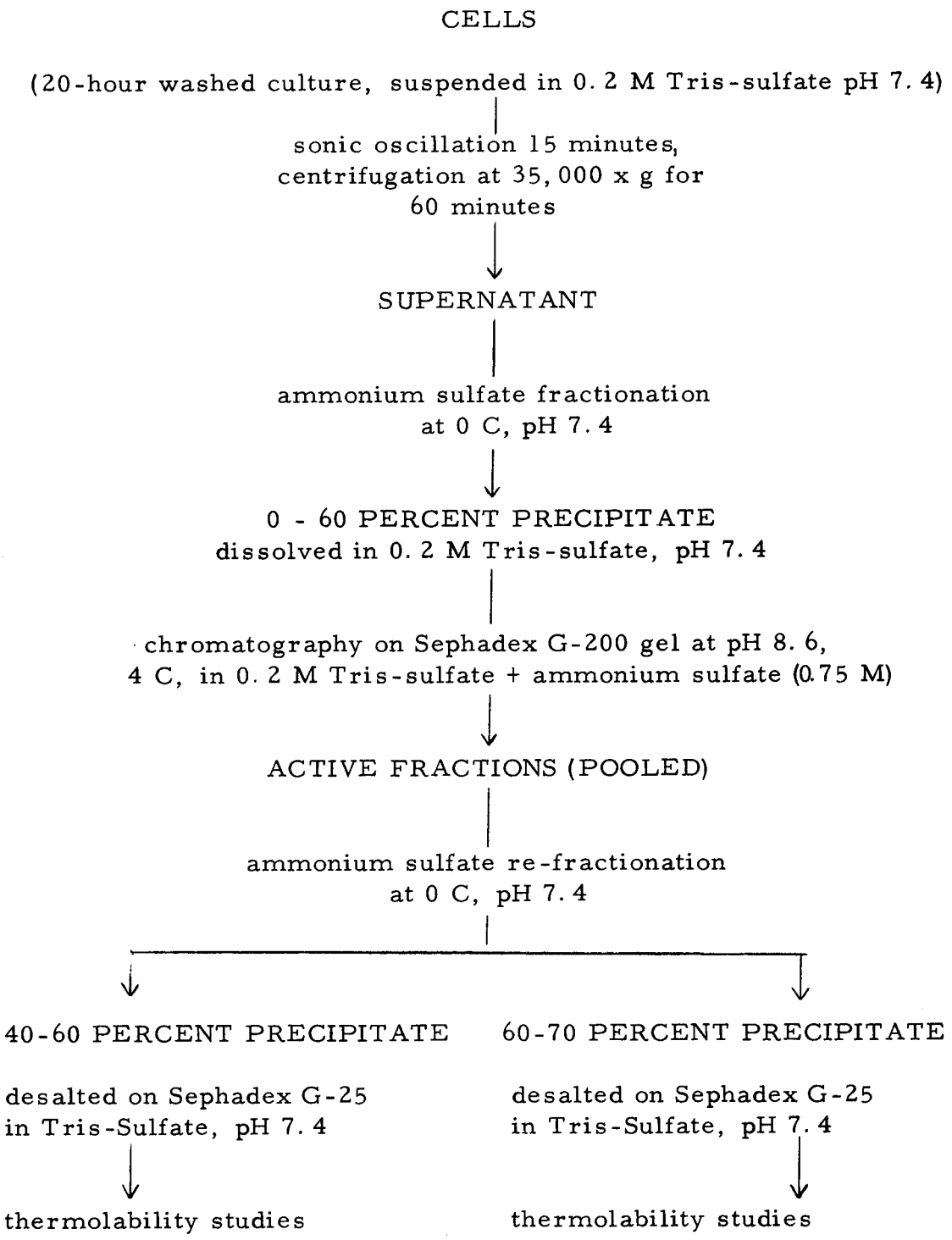

Figure 1. Flow diagram of MDH purification.

used in the heat inactivation studies. Immediately prior to use they were desalted on a short column (six inches x one inch) of Sephadex G-25 gel using Tris-sulfate buffer pH 7.4.

Methods of Enzyme Assay

The assay used throughout these studies unless otherwise stated was in the direction of oxidation of reduced nicotinamide adenine dinucleotide (NADH) as used by Ochoa (18). The decrease in OD at 340 mμ observed when NADH is oxidized to nicotinamide adenine dinucleotide (NAD) was measured using a Beckman DU spectrophotometer fitted with a thermospacer connected to a circulating water-bath to control the reaction temperature.

The reaction mixtures contained in 3.0 ml: 1.6 μmoles oxalacetic acid (OAA), 0.2 μmoles NADH, 520 μmoles Tris adjusted to pH 7.4 with either hydrochloric acid (Tris-chloride) or sulfuric acid (Tris-sulfate). Any additions to the assay are stated in the respective tables and figures. The protein concentration used depended on the enzyme fraction being assayed. All assays were performed at 20 C. Controls omitting either NADH, OAA or enzymes showed no activity. The enzyme units used are as stated by Ochoa (18), that is, one unit of activity is that amount which causes a decrease in optical density of 0.01 per minute. Specific activity is expressed as units per mg of protein.

The reverse-direction assay with malic acid as substrate was performed when the enzyme fractions were tested for activity with the analogues of NAD. The same assay was used for a qualitative spot plate check for the presence or absence of MDH following column chromatography.

The reaction mixture contained in 3.0 ml: 0.3 μmoles NAD, 20 μmoles malic acid, 0.5 μmoles sodium cyanide, 1.0 μmoles dichlorophenol indophenol, 0.3 μmoles phenazine methosulfate, 488 μmoles Tris-sulfate. The reaction was followed at 605 mμ in a Beckman DU spectrophotometer at 20 C, and was initiated by the addition of enzyme. Controls were performed for the non-specific decolorization of the dye in the absence of malate. When the analogues of NAD were substituted for the coenzyme the same molarity was used in the assay as given above for NAD.

The assay was modified for detecting enzyme in the chromatography fractions using a spot plate. The reaction mixture (0.1 ml) was pipetted into the compartments of the spot plate and 0.01 ml of the enzyme fractions added to each. Decolorization of the mixture indicated the presence of MDH in the fraction, which was then assayed quantitatively as stated in the forward-direction enzyme assay procedure.

Protein Determination

Protein was estimated by the method of Warburg and Christian (30).

Heat Inactivation

A polythermostat similar to that used by Oppenheimer and Drost-Hansen (21) and constructed by Morita and Haight (15) was used. The range of temperatures was adjusted between 0 C and 40 C depending on the particular requirements of the experiment.

The enzyme sample under consideration was pipetted into thermally equilibrated test tubes containing amounts of buffer to give the required dilution. At given time intervals, samples were withdrawn and assayed directly.

Lysed-Cell Preparation

Heat treated whole cells (0.2 ml), were lysed prior to assay by the addition of 1.8 ml of solution containing 180 mg per ml Triton X-100, 360 μmoles Tris-sulfate, pH 8.6, 0.8 μmoles ethylenediaminetetracetic acid. The mixture was incubated for five minutes at 0 C and assayed.

RESULTS

Preliminary studies were performed to show, both intracellularly and in cell-free extracts, the thermolability of MDH in V. marinus.

Washed whole cells suspended in 75 percent sea water were treated for one hour at various temperatures. They were lysed with Triton X-100 and assayed after five minutes. Microscopic examination showed the suspension to be completely lysed within this time at 0 C. There was no change in enzyme activity in the cells held between 0 C and 14 C (Figure 2). Above this temperature, enzyme activity decreased. At the maximum growth temperature, 20 C, the enzyme activity was reduced to 75 percent of its original value.

A large quantity of cells was grown and stored at -20 C as given in Methods and Materials. A portion of these cells was treated in the sonic oscillator to give a cell-free suspension in Tris-sulfate (pH 7.4). To test the thermolability of MDH in this solution, suitable dilutions were made of the enzyme into the same buffer to give readable activity when assayed directly. The enzyme activity was assayed with time after heat treatment at 0 C and 30 C (Figure 3). Protein determinations were performed on the cell free extract. Dilutions of the enzyme were also made into 75 percent sea water Tris-sulfate buffer at the same pH to show whether the inactivation was due to temperature, ionic strength, or both. Considerable protection of activity

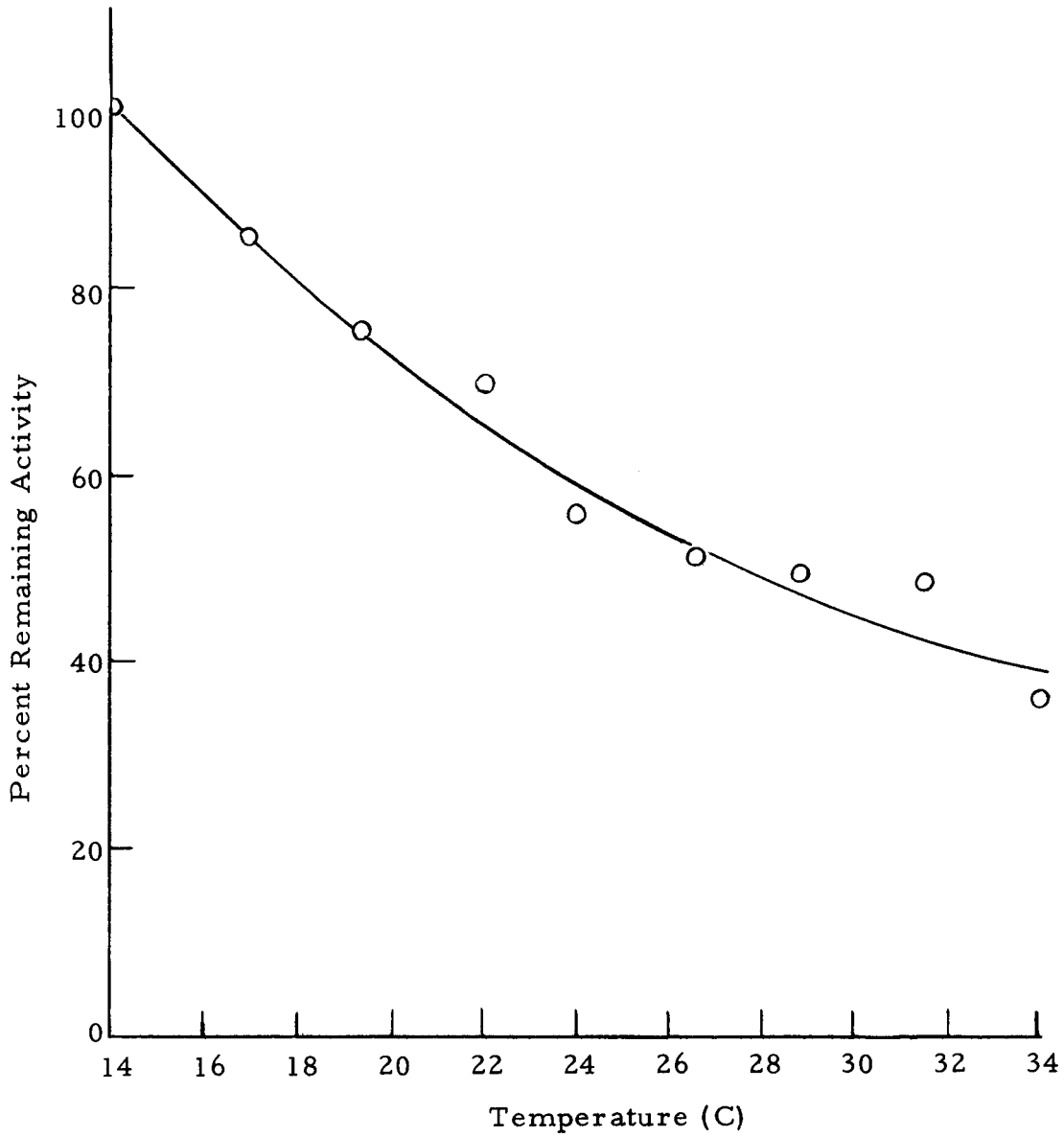

Figure 2. The effect of 60-minute treatment of whole cells to increasing temperature. Reaction mixtures contained in 3.0 ml: OAA 1.6 μmoles, NADH 0.2 μmoles, Tris-sulfate 520 μmoles, protein 0.4 mg. Cells were lysed with Triton X-100 prior to assay at 20 C.

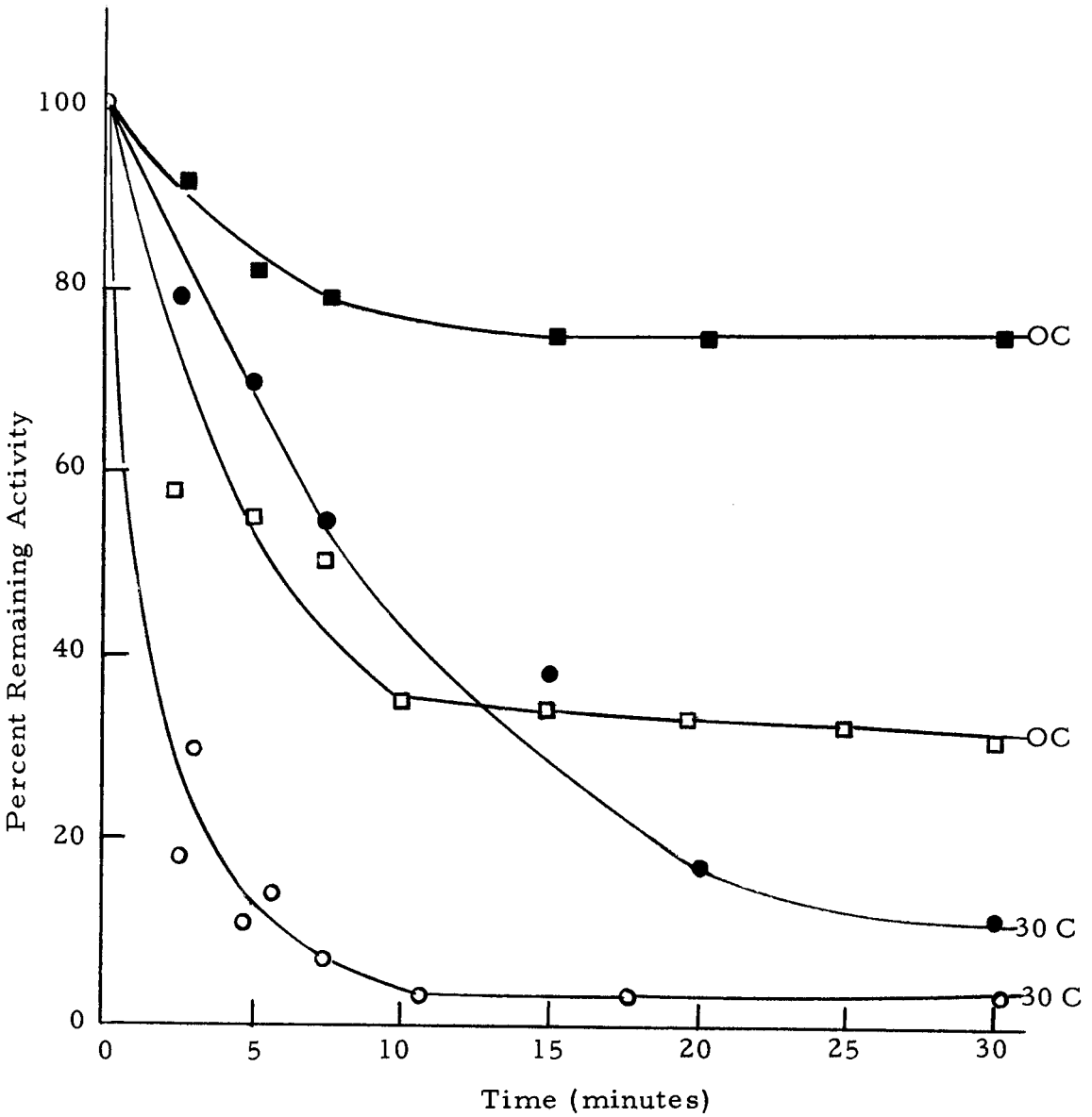

Figure 3. The effect of temperature on MDH activity of cell-free extracts. Reaction mixtures were as in Figure 2 and contain 0.44 mg protein. Inactivation temperatures are shown on graph, open figures represent inactivation in 0.2 M Tris-sulfate, closed figures represent inactivation in 75 percent sea water Tris-sulfate (pH 7.4). Protein concentration in inactivation mixture is 0.89 mg/ml.

was observed in the higher ionic strength buffer at 0 C, but this was not permanent at the higher temperature. Inactivation patterns were also obtained at 15 C and 20 C which corresponded identically to the 0 C curves in both environments.

Fractionation of the enzyme with ammonium sulfate was carried out at 0 C (Table I). The highest specific activity was observed in the fraction precipitating between 40-60 percent saturation, an almost four-fold increase over the original extract. It can be seen that a large amount of enzyme precipitated in the 0-40 percent region even though this fraction had a low specific activity (Table I). For this

TABLE I. SPECIFIC AND TOTAL ACTIVITIES OF MDH AFTER FRACTIONATION WITH AMMONIUM SULFATE.

Fraction	Total activity units	Specific activity units/mg protein
Extract	1,555,400	284
0 - 40 percent saturation	707,200	200
40 - 60 " "	986,000	1,074
60 - 70 " "	86,400	300
70 - 100 " "	29,000	36
0 - 100 Total	1,808,400	

reason both the 0-40 percent and the 40-60 percent precipitates were

combined and used for column chromatography. The fact that the combined fractions, and the 40-60 percent fraction alone, gave similar specific activity values on chromatography, further supported this action. Examination of Table I also shows that there was an increase in the total number of units of enzyme following fractionation.

The reason for this increase in activity may be due in part to increasing amounts of ammonium sulfate on the enzyme system. It can be seen that increasing the concentration of the salt to 0.75 M had little effect. To find whether this effect was due to the salt itself or to increased ionic strength, the assay was performed in buffer containing sodium chloride. A decrease in activity was noted at all of the concentrations used (Figure 5). The effects of several different ionic environments on the enzyme activity were found (Table II), showing that only ammonium sulfate and sodium sulfate stimulated over the activity observed in buffer alone.

The optimum conditions for stability of MDH prior to column chromatography were determined (Table III). The enzyme was incubated in the various systems at 4 C for 24 and 45 hours, each at the same protein concentration. System 4 was the only one which gave reasonable stability for the required length of time. The table also shows that the pH for optimum stability (8.6) was different to the pH for optimum activity, which was found to be 7.4, both in the presence or absence of ammonium sulfate.

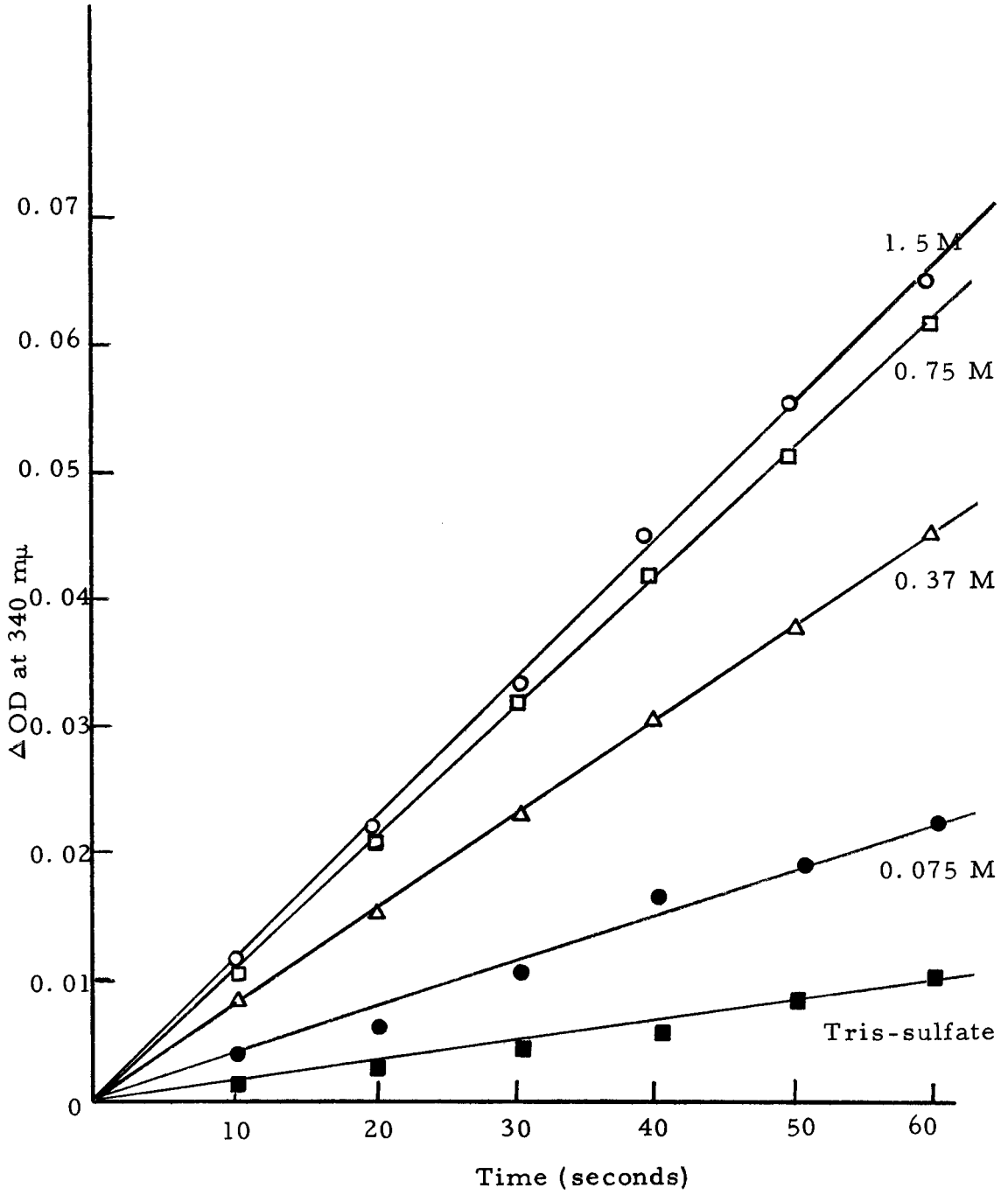

Figure 4. The effect of ammonium sulfate on the activity of MDH. The enzyme fraction used was the 40-60 percent fraction from Table I. Reaction mixtures were as in Figure 2 and contained 0.34 mg enzyme protein. The concentrations of ammonium sulfate in the assay buffer are shown above.

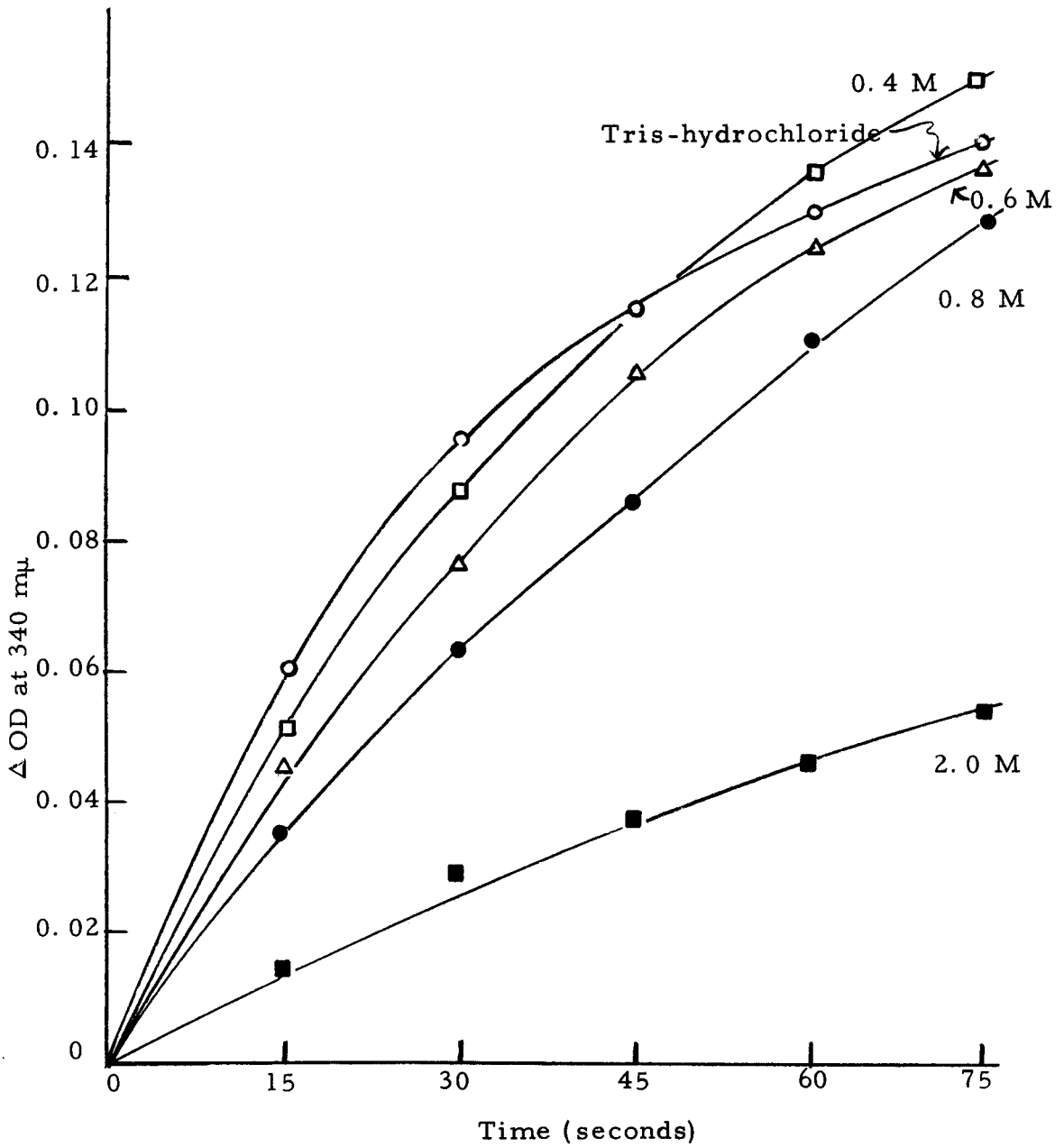

Figure 5. The effect of sodium chloride on the activity of MDH. All reaction mixtures are as in Figure 4, but contained 0.68 mg enzyme protein. The concentrations of sodium chloride in the assay buffer are shown in the above diagram.

TABLE II. THE EFFECT OF DIFFERENT SALTS ON THE ACTIVITY OF MDH.

Buffer	pH	Δ OD/minute
0.2 M Tris-chloride, 0.75 M ammonium sulfate.	7.7	0.185
0.2 M Tris-chloride, 0.70 M sodium sulfate.	7.8	0.088
0.2 M Tris-chloride.	7.55	0.066
0.2 M Tris-chloride.	7.8	0.033
0.2 M Tris-phosphate.	7.4	0.050
0.2 M Tris-chloride, 2.0 M ammonium chloride.	7.5	0.028

Reaction mixtures as given in Figure 2. Suitable dilutions were made to assay the enzyme under the different conditions. The above values for Δ OD were calculated on the basis of 0.102 mg protein/3.0 ml assay mixture.

TABLE III. STABILITY OF MDH AT 4 C.

Additions to System*	Percent Activity Remaining		
	0 hours	24 hours	45 hours
1. None	100	38.0	13.0
2. None pH 8.6	"	7.5	5.0
3. 75 μmoles ammonium sulfate	"	94.5	75.5
4. 75 μmoles ammonium sulfate pH 8.6	"	110.0	99.5
5. 0.6 μmoles NADH	"	39.5	11.0
6. 0.6 μmoles NADH pH 8.6	"	10.0	00.0
7. 0.6 μmoles NADH, 75 μmoles ammonium sulfate. pH 8.6	"	74.0	43.0
8. 0.3 μmoles NAD	"	36.5	12.0
9. 0.001 g Serum Albumin	"	53.5	16.0
10. 3.0 μmoles 2-mercapto-ethanol	"	40.0	15.0
11. 60 mmoles malate	"	41.0	30.0
12. Tris adjusted to pH with HCl	"	25.0	12.0

*All systems contained in 1.0 ml: 200 μmoles Tris-sulfate, 0.1 ml of the 0-60 percent enzyme fraction (3.4 mg protein). The pH unless otherwise stated was 7.4.

The elution pattern for MDH on Sephadex G-200 gel is shown in Figure 6. The void volume of the column used was 46 ml and the enzyme was eluted between 83 and 102 ml. The specific activities of the most active fractions eluted, varied from 2, 200 to 2, 800 units per mg protein. The flow rate affected these values, the greatest activities being obtained with rates of between one and two ml per hour.

Fractions from column chromatography were stored at -20 C and finally pooled. They were re-fractionated with ammonium sulfate at 0 C and pH 8. 6. The enzyme was precipitated in separate fractions as shown in Table IV.

Heat inactivation studies were performed on both of the most active fractions, following the removal of ammonium sulfate by passage through Sephadex G-25 gel (Figure 7). Similar patterns were found for both fractions. The results of this inactivation are more clear when Figure 7 is re-plotted as remaining activity against increasing temperature (Figure 8). They show that the enzyme was most stable between the optimum and maximum growth temperatures for the organism, but was unstable at temperatures both above and below this.

Attempts were made to re-activate the enzyme by placing it at 15 C following inactivation at both 0 C and 33 C (Figure 9). The re-activation is much greater in the former case, giving activity values of 15 percent above those at zero time. At 33 C there is a 30 percent

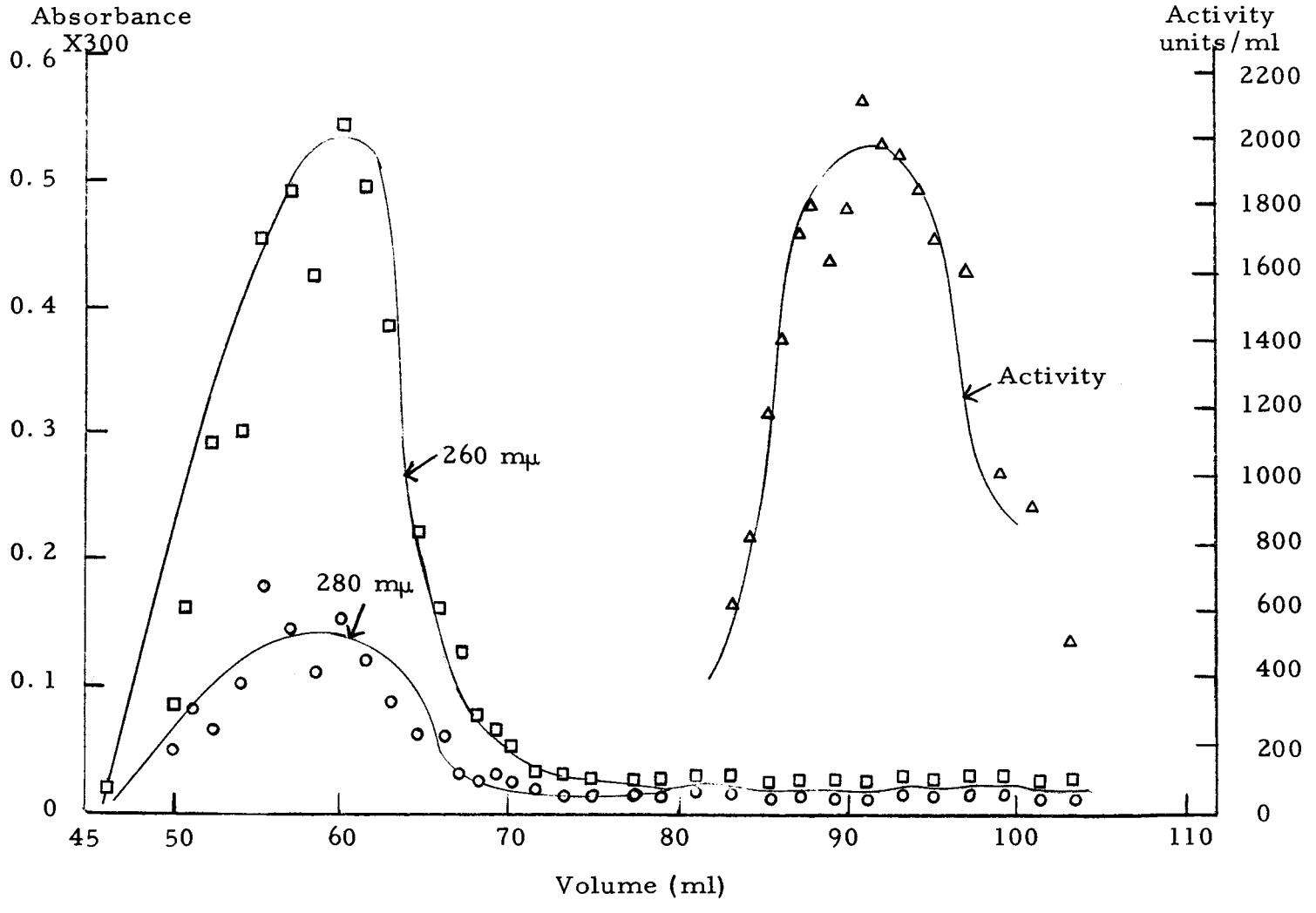

Figure 6. Chromatography of MDH on Sephadex G-200 gel in Tris-sulfate buffer (pH 8.6) containing ammonium sulfate (0.75 M), at 4 C.

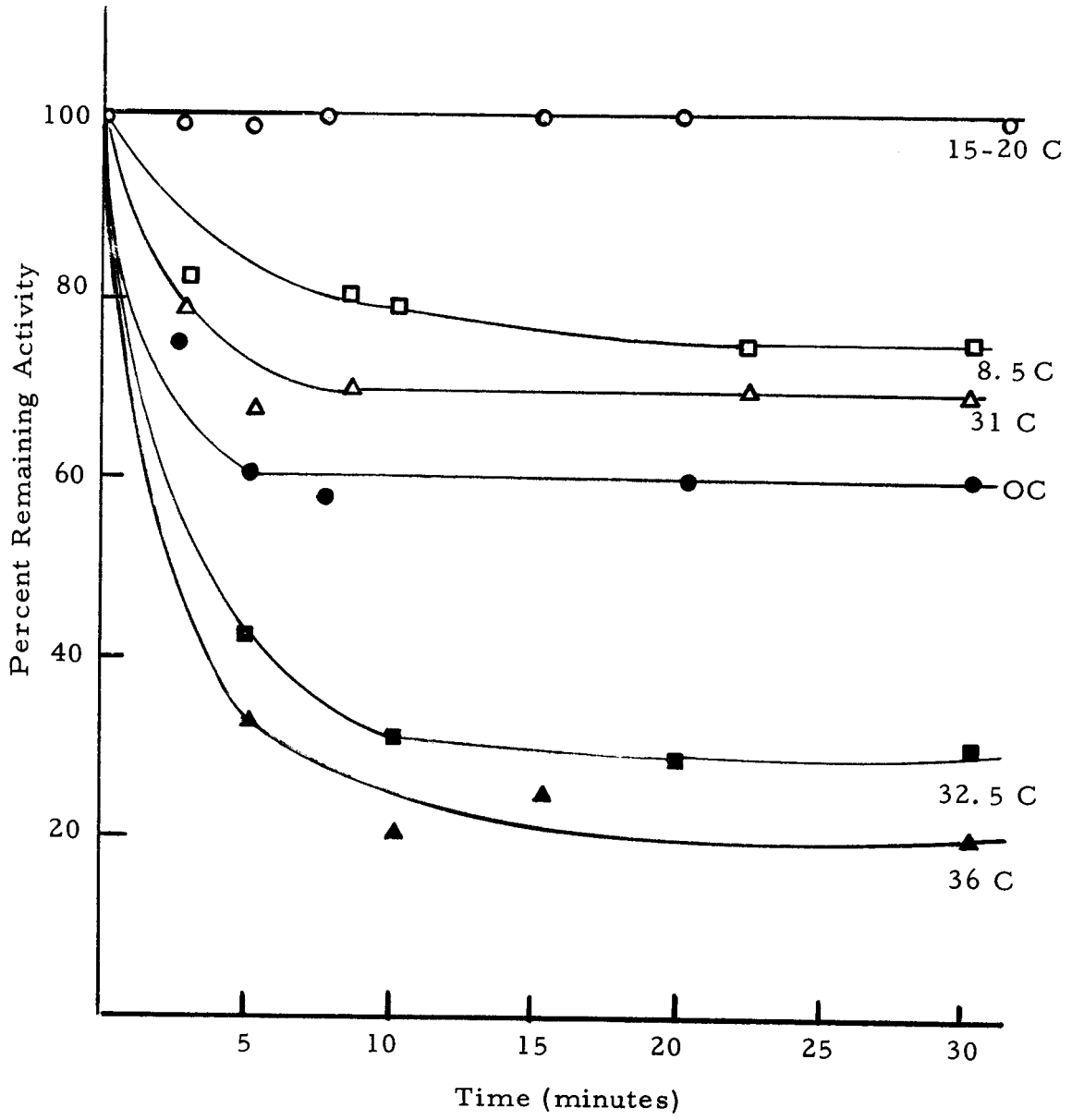

Figure 7. The effect of temperature on partially purified MDH activity. Reaction mixtures were as in Figure 2 and contained 0.004 mg protein. Inactivation performed in 0.2 M Tris-sulfate (pH 7.4) and 0.42 mg protein/ml.

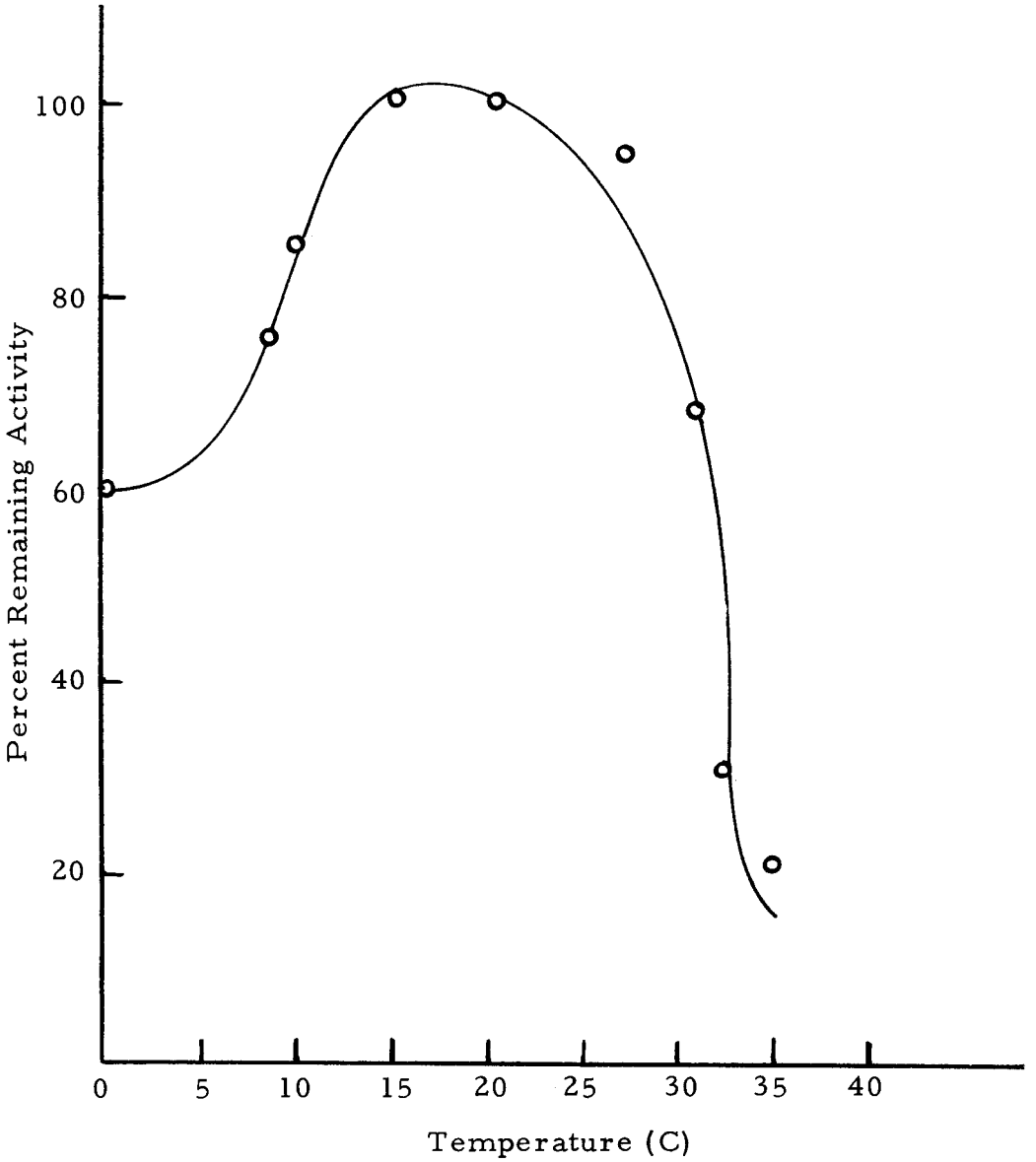

Figure 8. Replot of Figure 7 showing the effect of 30 minute exposure of partially purified MDH to increasing temperatures.

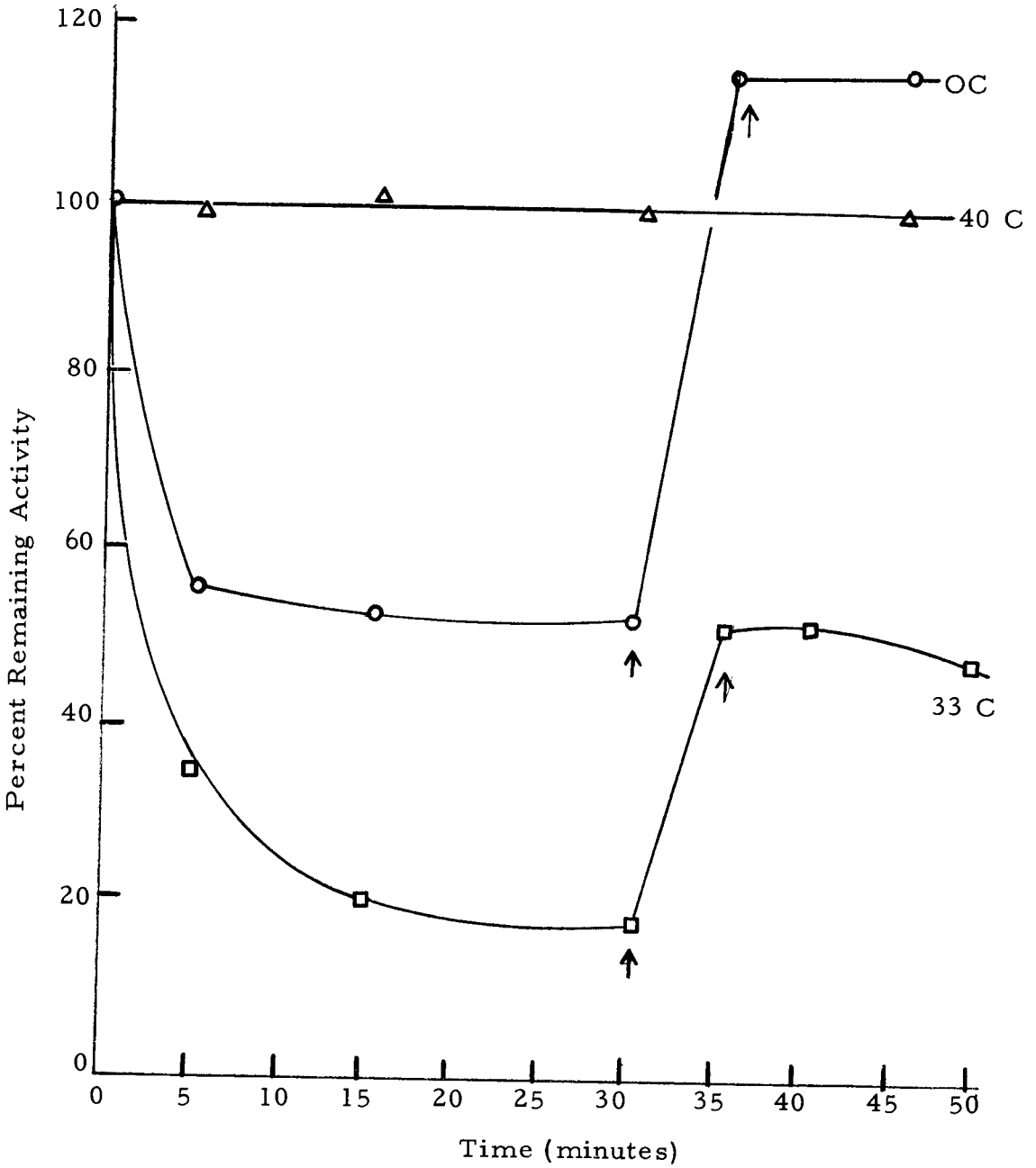

Figure 9. Temperature re-activation. Reaction mixtures and protein as in Figure 7. At times indicated the enzyme mixtures were placed at 15 C (period shown between arrows) and then assayed.
-Δ-Δ- enzyme heated in the presence of 1, 250 μmoles ammonium sulfate/mg protein.

TABLE IV. THE SPECIFIC AND TOTAL ACTIVITIES OF MDH AFTER AMMONIUM SULFATE RE-FRACTIONATION OF THE POOLED FRACTIONS FROM COLUMN CHROMATOGRAPHY.

Fraction	Protein mg/ml	Total activity units	Specific activity units/mg
Pooled fractions from column	0.9	580,000	2,550
0-40 percent	-	3,100	-
40-60 percent	5.7	110,400	1,680
60-70 percent	2.4	144,600	5,124
70-100 percent	-	50,000	-

re-activation to only 50 percent of the zero time value. The effect of the addition of 3,000 μmoles of ammonium sulfate per mg protein, to the enzyme inactivated at 33 C is also shown. In this case complete renaturation is observed showing that this is not an irreversible process. No inactivation of the enzyme was found when it was heated in the presence of the same amount of ammonium sulfate at 40 C.

Both of the active fractions from the second ammonium sulfate precipitation were assayed with analogues of NAD to investigate the possibility of isozymic forms of MDH, having different coenzyme specificities. The results are shown in Table V. It can be seen that there was no difference in the activities of the two fractions with any of the analogues but NAD itself.

TABLE V. THE ACTIVITY OF DIFFERENT FRACTIONS OF MDH, WITH ANALOGUES OF NAD, FOLLOWING RE-FRACTIONATION WITH AMMONIUM SULFATE.

Analogue of NAD	Activity ΔOD/minute	
	40-60 percent	60-70 percent
NAD	0.082	0.070
Deamino NAD	0.006	0.006
Acetyl pyridine NAD	0.035	0.038
Thio nicotinamide NAD	0.001	0.003

Reaction mixtures are as given in Methods and Materials. Protein concentrations used per 3.0 ml assay mixture were 0.0057 and 0.0024 mg/ml for the 40-60 and 60-70 percent fractions respectively.

DISCUSSION

Although the study of cell-free extracts is necessary if an attempt is to be made to elucidate the nature of enzyme systems without interference from permeability factors, care should be taken in the interpretation of these results when relating them to the activities of the whole cell. However, the closest approximation to whole cells which allows study of the individual components is a cell-free system. When a bacterial cell is disintegrated by sonic oscillation, there is a loss of cellular organization as well as the more obvious morphological changes. Purification of individual enzymes therefore permits the examination of the system under more strictly defined conditions.

During preliminary studies, an apparent increase occurred in MDH activity when whole cells of V. marinus were subjected to increasing temperature. This effect was also noted by Burton and Morita (1) working with the facultatively psychrophilic strain of the same bacterium and has since been shown to be due to leakage of the enzyme from the cells at the elevated temperatures and not to temperature induced stimulation. Study of the effect of heat on whole cell enzyme content therefore necessitated lysing of the cells prior to assay with Triton X-100, previously shown to affect the MDH activity very little (13).

At all temperatures above the optimum for growth, there was a

loss of activity when the cells were heat treated for one hour (Figure 2). However, the level of activity was not affected at all at the normal environmental temperatures of 0 C to 15 C. This intracellular heat lability may in part explain the reason for the psychrophilic nature of this bacterium.

The effect of temperature on the MDH activity in cell free extracts was far more drastic. Heating for ten minutes at 30 C gave an almost complete inactivation, and at 0 C there was considerable instability (Figure 3). The possibility that the decrease was due to low ionic strength was investigated by the inclusion of 75 percent sea water in the inactivation mixture. Considerable protection occurred but was only permanent in the case of the sample at 0 C. Further increase in salt concentration had no effect in either case. The implications of the effect of salt concentration on protein stability will be discussed later.

It appears therefore that MDH in cell-free extracts is more unstable than in the intact cell. Again the low temperature of thermal inactivation points to the existence of a psychrophilic enzyme. The MDH in cell-free extracts employed by Morita and Haight from a thermophile (14) was inactivated at 78 C.

The increase in total activity following ammonium sulfate fractionation (Table I) is not a unique phenomenon. Joyce and Grisolia (10) showed a similar effect in the purification of MDH from pig heart.

They stress the importance of employing caution in the interpretation of purification studies, and state that although this type of effect is generally attributed to the removal of inhibitors, few workers have investigated the possibility of enzyme activation. Ravel et al. (24) suggest the possibility of actual introduction of the sulfate ion into the enzyme molecule. Rutter (27), in studies on glucose-6-phosphate dehydrogenase, states that at the higher ionic strength there is a greater tendency for the dissociation of the enzyme and its products which would increase the reaction rates.

The stimulation of MDH activity with increasing ammonium sulfate concentrations up to 1.5 M (Figure 4), would tend to substantiate Rutter's hypothesis. However the increase in total activity following fractionation is difficult to explain on this basis, as the maximum amount of salt occurring in the actual assay was very low. A more likely theory, therefore, is that stabilization or re-activation of the enzyme molecule is occurring, but whether there is actual binding of an inorganic ion to the protein is difficult to say.

The depression of enzyme activity (Figure 5) in the presence of sodium chloride would also seem to refute the theory of Rutter (27) in this particular case. Hiatt and Evans (7) examined the influence of many salts on a 98-fold purified MDH from spinach leaves. With sodium salts of monovalent anions at concentrations of up to 0.04 M, they found a stimulation of two to three-fold. One peak of increased

activity was found with sodium chloride at a concentration of 0.04 M and another at 0.07 M. Between these two peaks, the activity was still at a level higher than that at zero salt concentration. An obvious difference exists, therefore, between this enzyme and that from V. marinus, as no stimulation with sodium chloride occurred in the latter case at any salt concentration.

Stimulation of MDH at pH levels above the optimum (7.4) was observed with both ammonium sulfate and sodium sulfate (Table II). When MDH was assayed in the presence of ammonium chloride as compared to assay in Tris-chloride alone, a decrease in activity was observed. These results would therefore implicate the sulfate ion in enzyme stimulation and possibly an inhibitory effect due to the chloride ion. Further studies did show that enzyme activity in Tris-sulfate was higher than in Tris-chloride and so this buffer was used for all future enzyme assays.

The investigations which were conducted to find the optimum conditions for stability of the enzyme prior to column chromatography (Table III) revealed interesting data. The only useful fact was that in the presence of ammonium sulfate at pH 8.6 the enzyme would remain stable up to 45 hours. This was less pronounced at pH 7.4 although in the absence of the salt the reverse was true and pH 7.4 gave greater stability than pH 8.6. Explanation of these facts is difficult. However if the sulfate ion does become somehow bound to the

enzyme molecule it is possible that it is, in some way, protecting the active sites at the higher pH. As sulfhydryl groups are believed to constitute these active sites (28) (the enzyme is inactivated by para-chloromercuribenzoate, a known inhibitor of these groups), and as such groups are subject to oxidation at high pH values, such protection is possible. The salt could also cause conformational changes in the enzyme itself which although they protect the active centres, still allow activity.

The addition of another protein such as bovine serum albumin does afford some protection. The protection of a dehydrogenase enzyme by its cofactors is discussed by Okunuki (19). In the case of MDH from V. marinus, the reduced cofactor decreases the stability even in the presence of ammonium sulfate, but NAD itself does give some protection. The involvement of both cofactor and substrate in these systems (Table III) may be complicated by the reaction kinetics of MDH as shown in studies by Raval and Wolfe (23). They have shown that there is a compulsory binding order mechanism involving at least one ternary complex between the enzyme, coenzyme, and substrate.

No significant protection was found when 2-mercaptoethanol was included in the system to keep any sulfhydryl groups involved in a reduced state. This would show that at least at a pH of 7.4 the inactivation of MDH is not related simply to sulfhydryl oxidation.

Attempts to chromatograph the enzyme on diethylaminoethyl

cellulose or carboxymethyl cellulose were unsuccessful. Sephadex G-200 gel, which allowed elution in buffers of high ionic strength, was therefore of great value in the chromatography of this enzyme.

Following this purification procedure, combined fractions from the column were re-precipitated with ammonium sulfate and the 40-60 percent fraction and 60-70 percent fraction retained separately (Table IV). The latter fraction had a specific activity of 5,000, a 20-fold purification over the original supernatant. This may be compared with final values of 13-, 98- and 130-fold in the respective studies of Joyce and Grisolia (10), Hiatt and Evans (7) and Ochoa (18).

Both of the fractions containing activity were tested separately for heat stability and activity with the analogues of NAD. This was done because of the wide spread noted in both column chromatography and salt precipitation. Very little difference was noted in activities with the NAD analogues (Table V) when the assays were performed as described by Moore and Villee (11), and no conclusive evidence for the existence of isozymic forms of MDH was shown. The temperature inactivation studies on the two fractions showed no differences in their thermostability patterns. The possibility that aggregate formation is causing the spread in fractionation of the enzyme does exist. The addition of fairly high concentrations of salt may be increasing the protein-protein interactions and decreasing the solvation of the molecules to such a point that although no precipitation is occurring,

aggregates are being formed.

The difference between the heat stability pattern of the enzyme in the purified form and that in whole cells is of considerable significance. It shows that in whole cells, normally found at temperatures near zero, MDH is protected from inactivation below 15 C although not above. This does not occur in the purified enzyme (Figure 8). If this phenomenon is universal for other enzymes in the organism, it may provide an adequate explanation of the psychrophilic nature of V. marinus. Tests for re-activation of heat inactivated enzyme in whole cells are complicated by the leakage of the enzyme from the cells at higher temperatures, but such data would be of value.

Burton and Morita (1) using a system of malate, NADH and 2-mercaptoethanol in Tris buffer, showed extensive renaturation of heat inactivated MDH in V. marinus PS-207. With this enzyme, slow cooling of the renaturation mixture was necessary to give complete renaturation.

In the psychrophilic strain, although a similar system to the above has not been employed, the 0 C inactivated enzyme can be spontaneously re-activated by returning it to 15 C either slowly or quickly (Figure 9). The enzyme which had been inactivated by exposure to 33 C would re-activate to only 50 percent of its original value. This would indicate that the two inactivation processes are different and may explain why whole cells are able to protect the enzyme from cold

inactivation. This differs from the results of Pullman et al. (22) in studies on adenosine tri-phosphatase. They showed a remarkable cold inactivation of the enzyme which could not be reversed unless precipitated by ammonium sulfate. However, the heat inactivation process could be prevented by the presence of adenosine triphosphate.

Re-activation of MDH from V. marinus occurred on addition of 3,000 μmoles ammonium sulfate per mg protein following exposure to 33 C. Complete protection from inactivation occurred even when the enzyme was heated to 40 C in the presence of this salt. This is in agreement with the results of Ravel et al. (24), who showed that only ammonium sulfate would protect carbamyl phosphate synthetase against heat and dialysis-inactivation, and would renature heat inactivated enzyme. One cannot imagine a similar process operating in vivo.

These studies therefore indicate the presence of an enzyme which, in a purified form, is unstable at temperatures both above and below the optimum for growth. The enzyme is protected to a degree by the intact cell although the mechanism of this protection is not understood. A relationship may exist between the growth temperature of the organism and the stability of enzymes formed at that temperature. If the organism were grown at 4 C, the temperature of its normal environment, the thermostability of its enzymes might differ accordingly. If this were true it would indicate a mechanism existing

in the cell, controlled by temperature, for the production of different proteins which allows adaptation to the environment.

Although there appears to be a universality in the MDH reaction from different sources (7, 10, 14, 18), the data also indicate that differences exist in the nature of the proteins themselves. This diversity is evidenced by several physical and chemical properties, which would indicate inherent differences in the structure of the enzyme. The wide differences noted in the response of MDH from thermophiles and from this psychrophile to heat suggest that different conformational changes are occurring. The data presented in this study definitely shows that obligate psychrophiles do exist and that they are a distinct class of organism when compared to mesophiles and thermophiles.

SUMMARY

When V. marinus MP-1 was subjected to temperatures above its optimum for growth (15 C) for 60 minutes, the MDH content of the cells decreases. Below 15 C the enzyme content of the cells was constant.

When cell free extracts were subjected to higher temperatures there was a drastic loss of activity which could be delayed but not prevented by use of a higher ionic strength.

A 20-fold purification of MDH was made following ammonium sulfate fractionation and chromatography on Sephadex G-200 gel. The properties of this purified preparation differed from those of the cell free extract.

When partially purified MDH was subjected to increases in temperature, the enzyme was shown to be stable between the organism's optimum and maximum growth temperatures, but both above and below this range there was considerable loss of activity. The cold-inactivated enzyme could easily be re-activated by placing it at 15 C. The heat-inactivated enzyme was only partially re-activated by this procedure.

The effect of ammonium sulfate on this enzyme was very marked. It allowed increased stability on storage at several temperatures. Its presence in the assay buffer at levels of 1.5 M stimulated activity

8-fold. Heating of MDH at 40 C with ammonium sulfate in the inactivation mixture gave no loss of activity after 60 minutes. The addition of the salt to heat- or cold-inactivated enzyme gave 100 percent reactivation instantaneously.

Comparison of MDH from V. marinus with the same enzyme from other sources, with respect to thermolability, helps to emphasize the psychrophilic nature of this organism.

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