The Isoenzymes of Isocitrate Dehydrogenase in *Acinetobacter lwoffi*

A Thesis submitted for the degree of Ph.D.

by

COLIN HENRY SELF B.Sc.

Department of Biochemistry, University of Leicester

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INTRODUCTION

INTRODUCTION

This thesis stems from the observation of an unusual property of the isocitric dehydrogenase (IDH) from the bacterium Acinetobacter lwoffi 4B. Assays of the NADP-linked IDH activity of crude sonic extracts of acetate-grown A. lwoffi showed a gradual increase in rate during the course of measurement. As such extracts contained the contaminating enzyme isocitrate lyase (ICL) and the assays were performed with isocitrate the effect of the products of the reaction catalysed by ICL on the IDH reaction was investigated. It was found that one of the products - glyoxylate - stimulated the IDH activity (Weitzman, unpublished). Such an activation had not previously been reported. Furthermore, as the activation was caused by a metabolite it was thought that it might have some physiological significance. Indeed, in higher organisms, IDH has been implicated in metabolic regulation. (see Atkinson, 1966).

Moreover, when growth of microorganisms occurs on acetate isocitrate is at a metabolic branch-point being metabolised either by IDH or ICL. Umbarger (1955) and Yates & Pardee (1956) pointed out the advantages of controls at such branch points in preventing the wasteful over-production of metabolites. It is

now well documented that branch-points are often centres of metabolic regulation. Therefore, IDH or ICL might be expected to be sensitive to regulation. There have been extensive studies on ICL which have indicated that it may be subject to both coarse control at the level of enzyme synthesis and fine control at the level of enzyme activity (Kornberg, 1965b). In addition to the stimulatory effects of glyoxylate on the IDH activity of A. lwoffi the initial studies had also shown that on addition of this effector the IDH activity was stabilised against inactivation by either heat or urea. Thus it appeared that a distinct physical change of the enzyme attended the activation.

As a result of these interesting findings I set out to investigate further the nature of the activation and its possible physiological significance.

The studies, reported in this thesis, revealed that pyruvate had a similar effect to that of glyoxylate. Furthermore, it was found that two isoenzymes of IDH existed in A. lwoffi, only one of which was sensitive to either effector. These isoenzymes were separated by a variety of techniques and shown to be markedly distinct in their molecular and kinetic properties. Moreover, the activation initially found in crude extracts was seen to be

a feature of only one of the isoenzymes. Other properties of this enzyme also changed upon activation.

Studies of both isoenzymes, including the changes induced in the sensitive enzyme by either effector, are described together with experiments performed to investigate the possible physiological role of either isoenzyme.

MATERIALS AND METHODS

MATERIALS

Most chemicals were purchased from the Sigma London Chemical Company or British Drug Houses Ltd., and were either the finest grade available or analytical grade. Ammonium sulphate (BDH) was a grade especially low in heavy metal ions; the trisodium salt of threo-$D_s L_s$-isocitrate (93-98% pure) was obtained from Sigma. Other materials were: Sephadex gels and blue dextran, Pharmacia Fine Chemicals Ltd.; diethyl amino ethyl-cellulose, H Reeve Angel Ltd.; Folin-Ciocalteu reagent, Fisons Chemical Company; ammonia-free glycine, Eastman Kodak Ltd.; N,N'methylene-bis-acrylamide and N,N,N',N'-tetramethyl-ethylene-diamine and protamine sulphate, Koch-Light Laboratories. Ampholines for electrofocussing were purchased with the electrofocussing column from LKB.

METHODS

Source and Maintenance of Organisms

Acinetobacter lwoffi 4B was isolated from distilled water supplies by Dr. P.D.J. Weitzman and *Escherichia coli* B was a stock culture originally obtained from Professor H. L. Kornberg.

Cultures were maintained on agar slopes containing either nutrient broth or salts-acetate of composition as below, and periodically sub-cultured.

Salts-Acetate Medium

Composition: 50 mM sodium/potassium phosphate buffer pH 7.2; 50 mM ammonium chloride; essential salts (4 mg $CaCl_2.6H_2O$; 8 mg $MgSO_4.7H_2O$; 0.4 mg $MnSO_4.4H_2O$; 0.4 mg $FeSO_4.7H_2O$ per 100 ml of medium) and 50 mM sodium acetate.

Sonication of Cell Suspensions

Chilled suspensions (up to 50 ml) were sonicated with a M.S.E. 100 watt disintegrator at full power.

Assays of Enzyme Activities

Enzyme activities are expressed throughout in units

of μmole catalysed/hr/ml and specific activities as μmole catalysed/hr/mg of protein.

1. Isocitrate dehydrogenase - NADP linked (IDH)

Isocitrate dehydrogenase (EC 1.1.1.42) was assayed by following the formation of NADPH at 340 mμ. Measurements were made with a Unicam SP800 spectrophotometer fitted with a thermostated cuvette holder and recorded on a Unicam SP21 recorder. Assay mixtures contained 20 mM Tris-HCl, pH 8.0, 1 mM EDTA and either 10 mM $MgCl_2$ or 10 mM $MnCl_2$; these are referred to as 'MET 8' or 'MnET 8' respectively. Unless otherwise stated NADP was present at a concentration of 0.2 mM, and threo-D_s-isocitrate at 2.0 mM. Enzyme solution was added to give a final volume of 1.0 ml. The mixtures were made up in 1.5 ml quartz semi-micro cuvettes, the reaction being initiated by the addition of the enzyme or one of the substrates. Assays were normally performed at 25°. The molar extinction coefficient of NADPH was taken as 6.2×10^3. NAD-linked IDH was assayed in the same manner but with NAD in place of NADP in the assay mixture.

2. Isocitrate lyase (ICL)

Isocitrate lyase (EC 4.1.3.1) was assayed by the continuous spectrophotometric assay of Kornberg (1965) but with the omission of glutathione.

A stock buffer was prepared to contain: 125 mM imidazole-HCl at pH 6.8, 25 mM $MgCl_2$, 5 mM EDTA and 20 mM phenylhydrazine hydrochloride. This was kept in ice and made fresh every four hours. Assay mixtures were prepared in cuvettes (as above) with 0.2 ml of this stock solution, 2.0 mM threo-D_s-isocitrate, distilled water and enzyme to a final volume of 1.0 ml. Determinations were made with the same equipment as above by measuring the rate of increase in absorbance at 324 mμ resulting from the formation of the phenylhydrazone of glyoxylate. The molar extinction coefficient of this product was taken as 16.8×10^3.

3. Glutamate dehydrogenase (GDH)

Glutamate dehydrogenase (EC 1.4.1.2) was assayed by following the oxidation of NADPH at 340 mμ in MET 8 containing 0.1 mM NADPH, 2 mM α-keto-glutarate and 2 mM NH_4Cl.

4. Lactic dehydrogenase (LDH)

Lactic dehydrogenase (EC 1.1.1.27) was assayed by following the oxidation of NADH at 340 mμ, in MET 8, in the presence of 2 mM pyruvate and 0.1 mM NADH.

5. Haemoglobin and Catalase

Both were determined by their absorbance at 405 mμ.

6. Blue Dextran

Blue dextran was determined by its absorbance at 605 mμ.

Protein Estimation

Protein concentrations were measured by the method of Lowry et al., (1951) and, on the occasions when a non-destructive determination was required, by the method of Warburg & Christian (1942).

pH Measurements

Determinations of pH values were carried out using a Radiometer TTT1 pH-meter and a combined glass-calomel electrode.

Ion-Exchange Chromatography

Diethylamino ethyl - (DEAE) - cellulose (Whatman, DE11), (150 g) was precycled with acid and alkali by the manufacturer's recommended procedure and then stirred into about 1 litre of 0.2M Tris-HCl or phosphate buffer, the pH adjusted to 8.0 and the mixture left overnight. The clear supernatant was then decanted to leave a thick slurry which could be poured easily without trapping air bubbles. A chromatography column was poured with this slurry and packed and equilibrated by passing through it about five column volumes of the buffer to be used initially in

the chromatography.

After use, the exchanger was collected and subsequently regenerated by the same method as above.

Sample Application

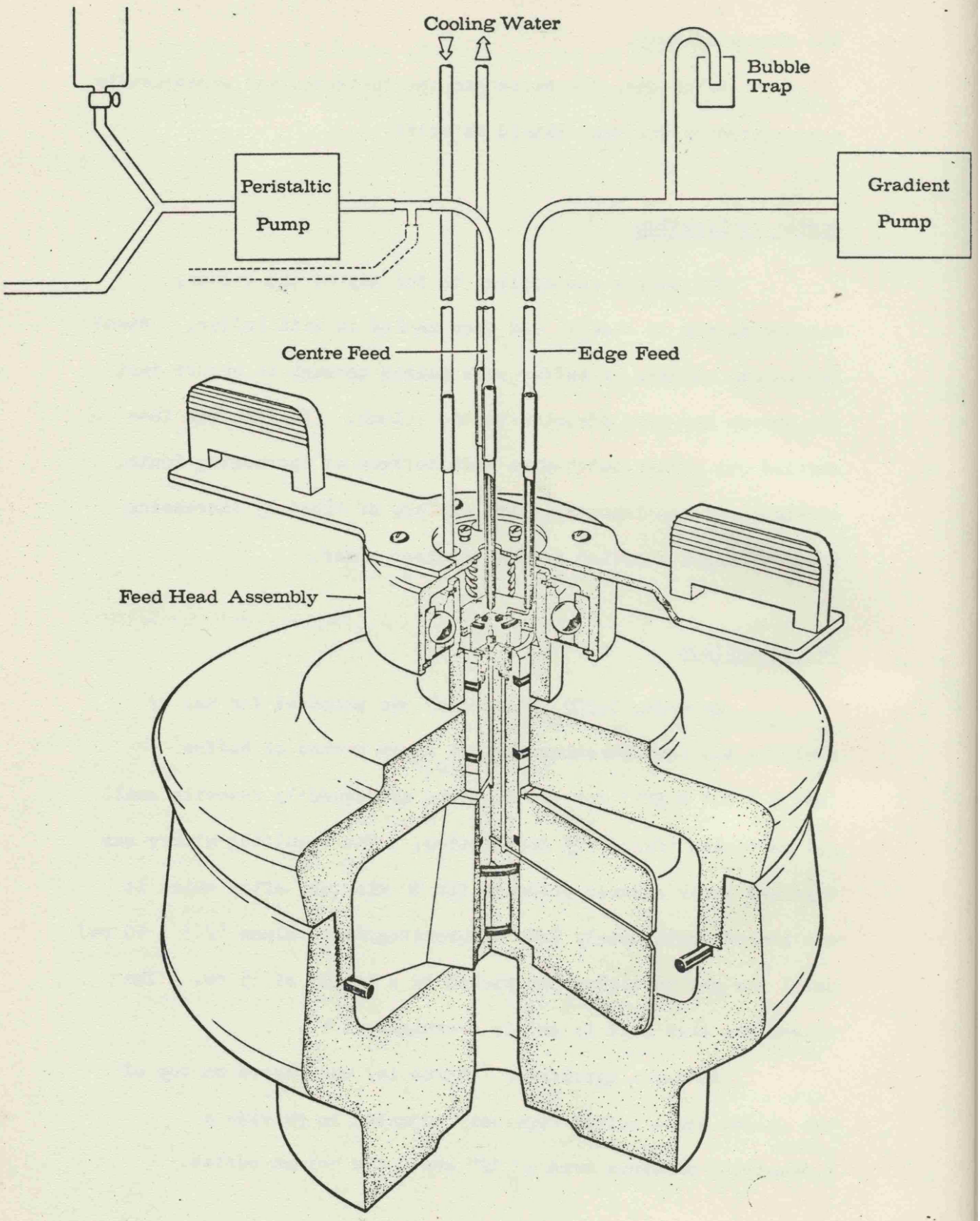
The sample was applied to the top of the column, allowed to run in slowly, and then washed in with buffer. About two column volumes of buffer were passed through to ensure that the enzyme had been adsorbed to the column. Elution was then carried out either batch-wise with buffers of increasing ionic strength, or continuously, with buffers of linearly increasing ionic strength supplied from a gradient mixer.

Gel Filtration

Sephadex G-200 (Pharmacia) was prepared for use by swelling and equilibrating the gel in an excess of buffer (MET 8 $\pm$ 0.1 M KCl) for two days and subsequently removing small gel particles (fines) by decantation. The resulting slurry was degassed under reduced pressure for 10 minutes, after which it was poured continuously into a chromatography column (2.5 x 40 cm) until the gel particles had packed to a height of 35 cm. The column was then left to settle overnight at 4°.

A sample applicator (Pharmacia) was placed on top of the column and a buffer reservoir adjusted to provide a hydrostatic pressure head of 12" above the column outlet.

Figure 2 - A B XIV Zonal Rotor with Feed Head Assembly
(Reproduced by kind permission of M.S.E.)



Further packing and equilibration were then achieved by passing about 5 column volumes of buffer through the gel.

The packing of the column was examined by passing 2 ml of a solution of blue dextran through the gel bed. If the blue band was seen to travel unevenly the column was emptied and repacked.

Sample Application

Flow through the column was stopped and the sample (made 10% w/v in sucrose) was carefully layered onto the top of the gel bed under a 3 inch head of buffer. Even addition of the sample was achieved by delivering it into a sample applicator with a pasteur pipette, the end of which was bent at right angles. The sample was run into the gel and the pressure head and flow restored.

As blue dextran had been found to reduce the elution volume of both LDH and IDH whenever it was used as a marker, it was instead applied in the same manner and volume as the protein sample but 6 ml in advance.

Preparative Zonal Ultracentrifugation

For a zonal separation the rotor was assembled as shown in Figure 2 and placed on the centrifuge spindle. A plastic guard tray and rubber splash ring were fitted and the

rotor accelerated to 2500 rpm. The centrifuge thermostat was set to maintain 10° and the feed head assembly was lowered onto the rotor. With the use of a gradient former the rotor was filled with a sucrose solution of linearly-increasing concentration from 5-20% (w/w) in buffer of composition 5 mM Tris-HCl, pH 8.0, and 1 mM EDTA at a flow rate of 20 ml/minute. The sucrose solution was fed, through the core and ducts in the vane-like septa, to the rotor wall and continuously displaced inwards by more concentrated solution. The flow was stopped when sucrose solution issued from the centre of the rotor core.

Taking care to exclude air bubbles, the protein sample was then slowly applied by injecting it to the face of the rotor core; it was then pushed well into the centrifugal field with about 20 ml of sucrose-free buffer solution.

The feed head assembly and the rubber splash ring were removed, the vacuum tight rotor cap fitted and the rotor accelerated to 47,000 rpm. The duration of centrifugation at this speed was taken as the time of the run, as quoted in the 'Experimental' section. At the end of this time the rotor was decelerated to 2500 rpm, the vacuum cap removed and the rubber splash ring and feed head assembly replaced. A dense solution of sucrose (30%) in distilled water, coloured by the addition of a small quantity of blue dextran, was then pumped to the periphery of the rotor chamber at a flow rate of about 30 ml/min,

to displace the contents of the rotor through the central core to a fraction collector. Twenty-ml fractions were collected until tubes were seen to contain blue dextran.

Cellulose-acetate Electrophoresis

Cellulose-acetate electrophoresis was performed with the Millipore "Phoroslide" apparatus. The cellulose-acetate strip was soaked in the electrophoresis buffer of composition: 100 mM Tris, 45 mM diethylbarbiturate at pH 8.6; blotted, air dried for 1 minute and fitted into the electrophoresis chamber. The top of the chamber was fixed in place, the sample applied with the special applicator provided, and the top closed. Electrophoresis was carried out at 100 volts for 20 minutes. The strip was then removed, blotted and air dried for 1 minute. It was then floated, porous side down, onto a microscope slide layered with a staining solution for IDH after Barron & Bell (1962). This consisted of MET 8 containing 2 mM isocitrate, 0.2 mM NADP, 0.3 mg/ml nitro-blue tetrazolium and 0.1 mg/ml phenazine methosulphate. Excess staining solution was removed by blotting and the strip incubated at 35° in a dark humid chamber until the enzyme stain had developed (usually about 5 to 15 minutes).

Polyacrylamide Gel Electrophoresis

Polyacrylamide gels (7% and pH 8.9) were prepared as

described by Davis (1964) in small glass tubes (95 x 5 mm) to 10 mm from one end. Polymerisation was achieved either with ammonium persulphate or riboflavin by exposure to a "Photoflood" light source. Spacer (large pore) and applicator gels were not included as they were found to be unnecessary for the subsequent sharp resolution of protein bands. The sample, made dense with 10 mg sucrose per 0.1 ml and coloured by the addition of bromo-phenol-blue, was applied under a layer of the electrode buffer (Davis, 1964) directly onto the top of the gel with a micro-syringe.

The apparatus was of the same basic design as that described by Davis (1964) and preparations for a separation were made in the same way.

Electrophoresis was carried out at 0.5 mA per gel until the sample had entered the gel completely at which point the current was increased to 2 mA per gel and the voltage then maintained at a constant value during the rest of the separation.

On completion of electrophoresis the gels were eased from the glass tubes by gently forcing distilled water between gel and tube with a syringe. The gel was rinsed in distilled water.

Removal of proteins from the Gel

The gels were cut into 2 mm segments with a razor

blade and each slice was crushed in 0.5 ml of MET 8 and left overnight at 4°.

Staining the (intact) gel

(a) Proteins

The gel was immersed in a solution of napthalene black (1%) in 7% acetic acid for about 45 minutes. Destaining was effected by soaking the gel in several changes of 7% acetic acid.

(b) Isocitrate dehydrogenase

Each gel was immersed in 10 ml of MET 8 containing 10 µmoles DL-isocitrate, 1 µmole NADP, 3 mg nitroblue tetrazolium and 1 mg phenazine methosulphate and incubated in the dark, at 37°, until a clear enzyme stain had appeared or interfering precipitation had become too pronounced. The staining mixture was not used after storage for more than 4 hours in the dark.

Thermal Inactivation

MET 8 buffer solution was equilibrated at the appropriate temperature in a large, thin-walled, test-tube. Enzyme solution of no more than one fifth the final volume was quickly added and mixed. If prolonged heating was required, the tubes were sealed with parafilm. Aliquots were removed

periodically into chilled tubes which were cooled further by swirling in an ice-water slurry and the enzyme assayed. Compounds which were tested for their effect on the thermal inactivation were added to the buffer before equilibration.

Urea Inactivation

Normal assay mixtures were made up containing urea from a freshly prepared stock solution, and well mixed. Enzyme solution was added to make the final volume 1.0 ml and the reaction followed in the usual way. When assays were carried out with pyruvate or glyoxylate the effector was added to the assay mixture and well mixed before addition of the enzyme.

EXPERIMENTAL SECTION

EXPERIMENTAL SECTION

Experiments were carried out to investigate the original observations of Dr. Weitzman that glyoxylate both activated and stabilised the IDH activity in crude extracts of A. lwoffi.

Preliminary Studies

The following preliminary studies were all performed with crude cell-free sonic extracts of acetate-grown A. lwoffi.

It was confirmed that glyoxylate stimulated the IDH activity. Furthermore, it was also found that the degree of activation produced by glyoxylate depended on whether Mg^{2+} or Mn^{2+} was supplying the necessary divalent cation requirement for isocitrate dehydrogenase activity. These results are presented in Table 1.

Throughout the text the "activation" is defined to be the extra activity on stimulation divided by the unstimulated activity.

As the results in Table 1 show, although the IDH activity is greater in the presence of Mn^{2+} than Mg^{2+}, the activation produced by glyoxylate is more marked in the presence of Mg^{2+} than Mn^{2+}. Therefore, to enhance the activation,

TABLE 1

Divalent cation	IDH activity	Activity + 0.2 mM glyoxylate	Activation by glyoxylate
Mg^{2+}	51	87	71%
Mn^{2+}	76	92	21%

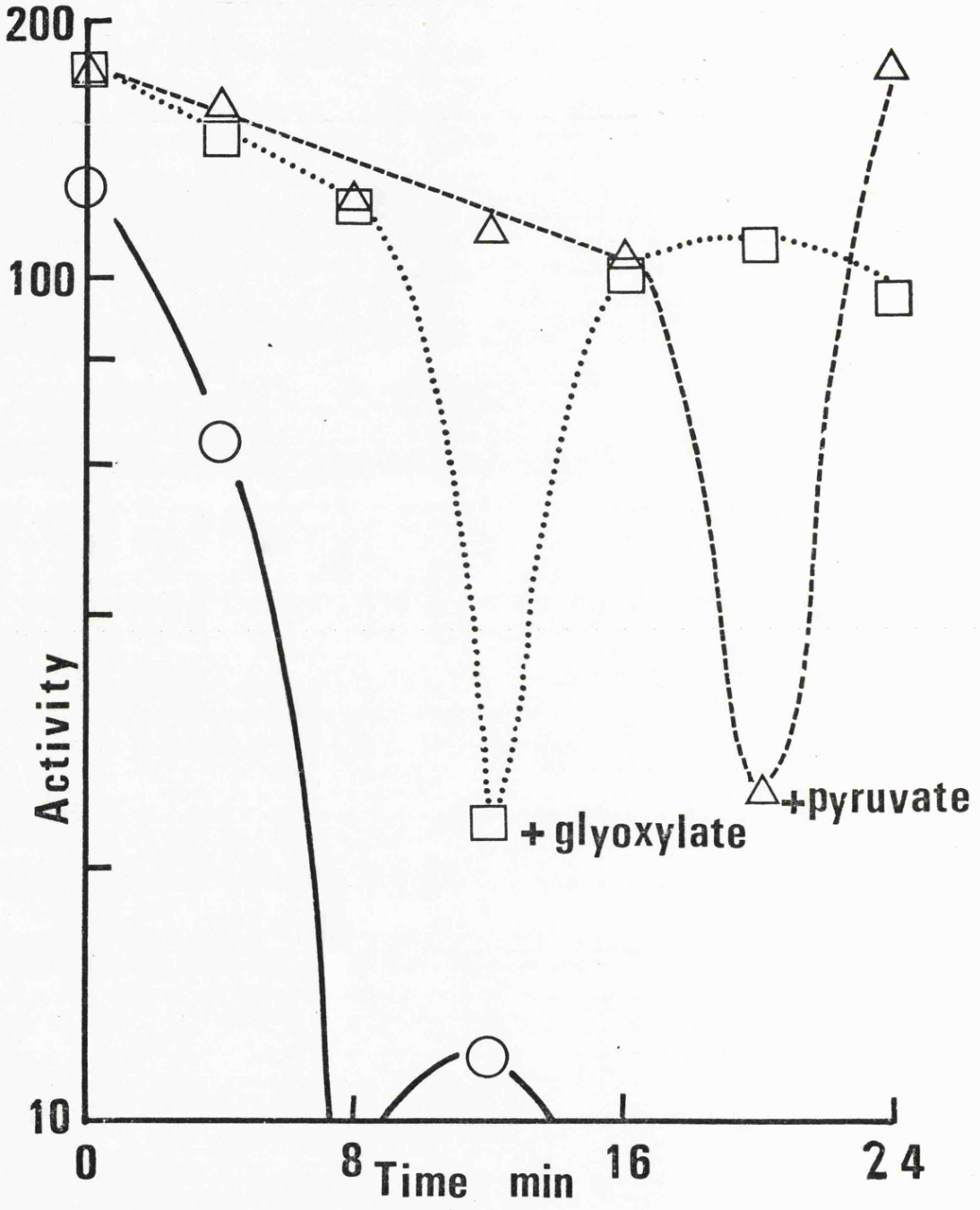


Figure 3 - The Thermal Inactivation at 40° of IDH in crude extracts of A. lwoffi with and without pyruvate or glyoxylate

where possible, further studies were carried out in the presence of Mg^{2+}. This was not, however, always possible because of interference from the enzyme isocitrate lyase (ICL). This enzyme was found in cell-free extracts of A. lwoffi, from many carbon sources, and interfered with IDH estimations by giving rise to glyoxylate, which could stimulate the IDH activity. Because ICL from this source appears to have an absolute requirement for Mg^{2+}, assays of IDH were conducted with Mn^{2+} where the contamination by ICL was particularly severe.

Of several other keto-compounds tested, pyruvate alone was found to activate the IDH in crude extracts and to the same extent as glyoxylate at comparable concentrations. In subsequent studies the effects of both glyoxylate and pyruvate were investigated.

Thermal Inactivation

The effect of glyoxylate or pyruvate on the thermal inactivation of IDH was investigated. The extract was incubated in MET 8 at 40° and aliquots periodically removed as described in "Methods". When added, pyruvate or glyoxylate were present in the incubation mixture at 0.2 mM concentration. The results are presented in Figure 3 as the residual activity after incubation. Both pyruvate and glyoxylate were seen to protect

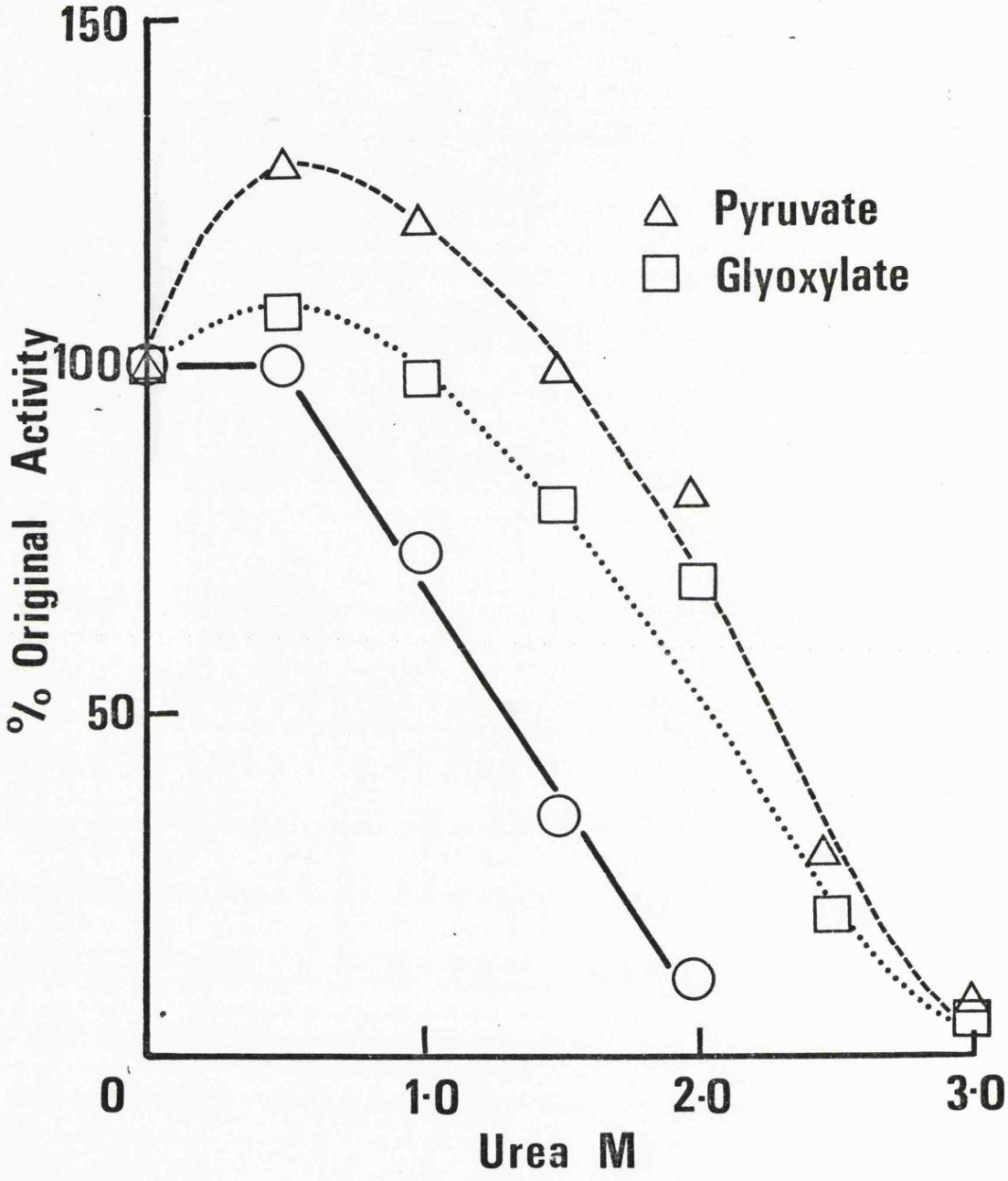


Figure 4 - Inactivation of IDH in crude extracts of A. lwoffi by urea with and without pyruvate or glyoxylate

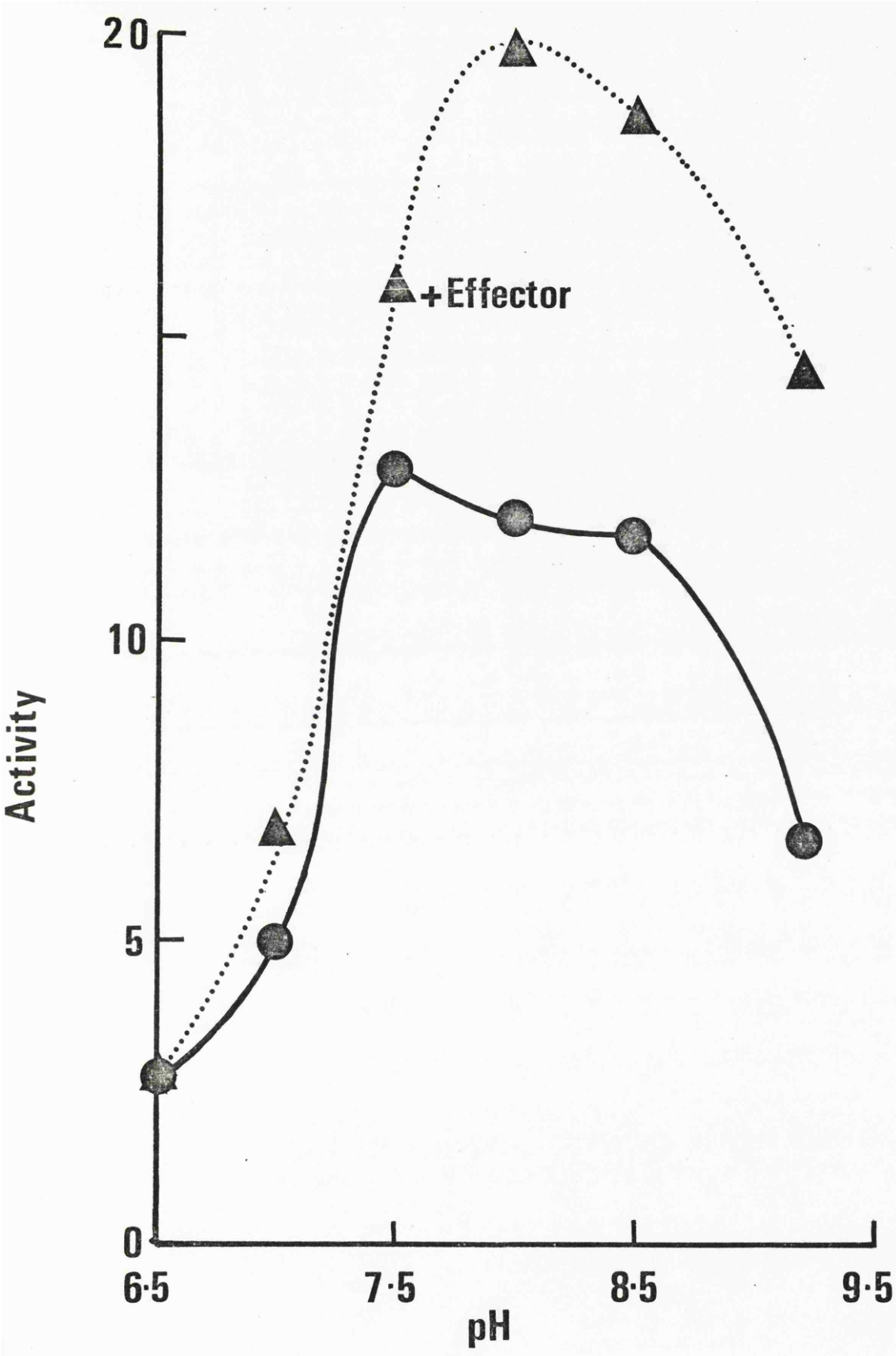


Figure 5 - The dependence of the IDH activity of crude extracts of A. lwoffi on pH and the effect of pyruvate or glyoxylate

the activity; however, in all cases the rate of decay is seen to be more complex than that often found with other enzymes which show a linear plot when presented in the same way.

Inactivation by Urea

Experiments were carried out to examine the effects of glyoxylate or pyruvate on the inactivation of the IDH by urea. Enzyme assays were performed with various concentrations of urea and, when added, glyoxylate or pyruvate was present at 0.2 mM concentration as described in "Methods". As is shown in Figure 4, the results confirm that the enzyme is somewhat protected by glyoxylate or pyruvate.

Dependence of Activity on pH

The IDH activity was measured over a pH range of 6.5 to 9.2. The measurements were repeated with pyruvate or glyoxylate. Figure 5 shows the results, from which it was apparent that without either effector the IDH activity had a complex dependence on pH - two pH optima being seen. However, in the presence of 0.2 mM glyoxylate or pyruvate, only one peak of activity was observed.

The preliminary results suggested that crude sonic extracts of A. lwoffi contained interfering agents and/or multiple forms of IDH. I therefore attempted some purification of the IDH.

Initial attempts to purify IDH

An inoculum of A. lwoffi was taken from an agar slope into 200 ml of sterile nutrient broth and then aerated at 30° by shaking. Towards the end of logarithmic growth the whole culture was transferred to 1 litre of sterile "salts-acetate" medium and shaken overnight at 30°. This culture was then inoculated into a further 15 litre of sterile "salts-acetate" medium (contained in a 20 l carboy) which was aerated at 30° with compressed air until the suspension reached an optical density at 680 mμ of about 1.6 corresponding to the end of the logarithmic phase of growth.

Collection and Disruption

The cells were collected with a Sharples continuous centrifuge, and then washed with distilled water. The cells were resuspended in MET 8 to a density which on 30-fold dilution had an O.D. at 680 mμ of about 1.3. With such a suspension the optimum time of sonication of 50 ml aliquots for liberation of IDH had been shown to be about 4 minutes (Table 2). Therefore, sonication was carried out for this time with 50 ml batches. The aliquots were pooled and centrifuged at 25,000 g and 4° for at least one hour to remove cell debris. The protein concentration of the resulting slightly turbid supernatant

TABLE 2

Liberation of IDH and protein with time of sonication.

Time (Min)	IDH units (Mn^{2+})	Protein (mg/ml)	Specific Activity
0.0	0	0.24	-
1.0	36	4.1	9
2.0	72	5.6	13
3.0	113	7.2	16
4.0	123	8.0	15
5.0	141	10.2	14
6.0	152	10.6	14

solution was determined by the method of Lowry et al. (1951).

Removal of Nucleic Acid

Nucleic acid was removed by precipitation with protamine sulphate.

An aqueous solution of protamine sulphate (2% w/v) was added dropwise to the supernatant solution with constant stirring until 1.3 mg of protamine sulphate had been added per 10 mg of protein in the extract. The mixture was stirred for twenty minutes and then centrifuged at 25,000 g for 5 minutes. If the decanted supernatant solution was turbid, a further 0.2 mg protamine sulphate per 10 mg protein was added slowly and the mixture again stirred for twenty minutes and centrifuged. Unless the extract could be clarified at this stage, it was found that subsequent purification attempts were both irreproducible and of little use. The amount of protamine sulphate needed varied with preparations. Occasionally the extract could be clarified after the addition of only 1.3 mg protamine sulphate per 10 mg protein, but sometimes it required as much as 1.9 mg. However, it was not advisable to add this high level initially as it would sometimes precipitate the enzyme. With some preparations the extract could not be clarified before the enzyme had been precipitated by protamine

sulphate. When this happened the extract was discarded as the enzyme activity could not be usefully recovered. Therefore, careful titration of protamine sulphate at this stage was crucial to a successful purification. Table 3 shows that as well as removing nucleic acid, treatment with protamine sulphate may give an apparent increase in specific activity with little loss of activity. This is most probably due to the removal of some protein on clarification of the extract.

The extract thus obtained was then initially fractionated by precipitation with ammonium sulphate.

Ammonium Sulphate Fractionation

Finely powdered ammonium sulphate was slowly stirred into the chilled clear extract to a final concentration of 45% saturation ~~(w/w)~~. The solution was stirred for a further $\frac{1}{2}$ hour at 4° and then centrifuged at 25,000 g for 5 minutes. The supernatant was poured off and the pellet dissolved in a small volume of MET 8. This procedure was repeated, increasing the ammonium sulphate concentration of the solution in 5% steps until the supernatant solution showed no IDH activity.

The results of the fractionation are shown in Table 3. From this it is apparent that ammonium sulphate fractionation gives a very good recovery of total enzyme units and the enzyme from the 55-60 and 60-65% saturation fractions is considerably purified

TABLE 3

Fractionation by Protamine Sulphate and Ammonium Sulphate

Stage	Vol.ml	Activity	Activation	Specific Activity	Enzyme Units	Yield (% initial)
Crude	265	152	0.26	13.6	41,000	-
Post protamine sulphate	245	154	0.17	22.7	37,800	90
Am. sulphate (%)						
0-45	-	142	0.44	9.6	-	-
45-50	5.0	228	0.49	15	1,140	3
50-55	3.9	1912	0.33	14	7,460	18
55-60	3.7	3356	0.40	114	12,420	30
60-65	3.5	2172	0.21	123	7,600	19
65-70	3.0	158	0.00	75	476	12

(5-fold). It is also evident that there is a trend of decreasing sensitivity of the fractions to glyoxylate. However, because the dehydrogenase activity was spread over a wide range and there was no clear cut fractionation, the fractions between 45 and 70% saturation were pooled and used to investigate the resolution of other fractionation methods.

The 45-70% fraction was dialysed overnight against 50 mM potassium orthophosphate, 1 mM EDTA at pH 8.0 and 4° and the precipitated protein removed by centrifugation. The resulting preparation was found to be stable to storage at 4° for at least one month with no change in its sensitivity to glyoxylate. Preparations made in this way but by taking a single 45-75% ammonium sulphate fraction were used in subsequent studies on further methods of fractionation.

The Separation of Isoenzymes of IDH

i) DEAE-cellulose Chromatography

A sample of the enzyme solution prepared as above was applied to a DEAE-cellulose column (2.5 x 30 cm) which had been equilibrated with 50 mM potassium (ortho) phosphate at pH 8.0, 1 mM EDTA and the column further eluted with two more column volumes of this buffer. The elution was continued with a buffer solution of linearly increasing phosphate concentration from 50 mM to 250 mM with a concentration gradient of 4.0 mM

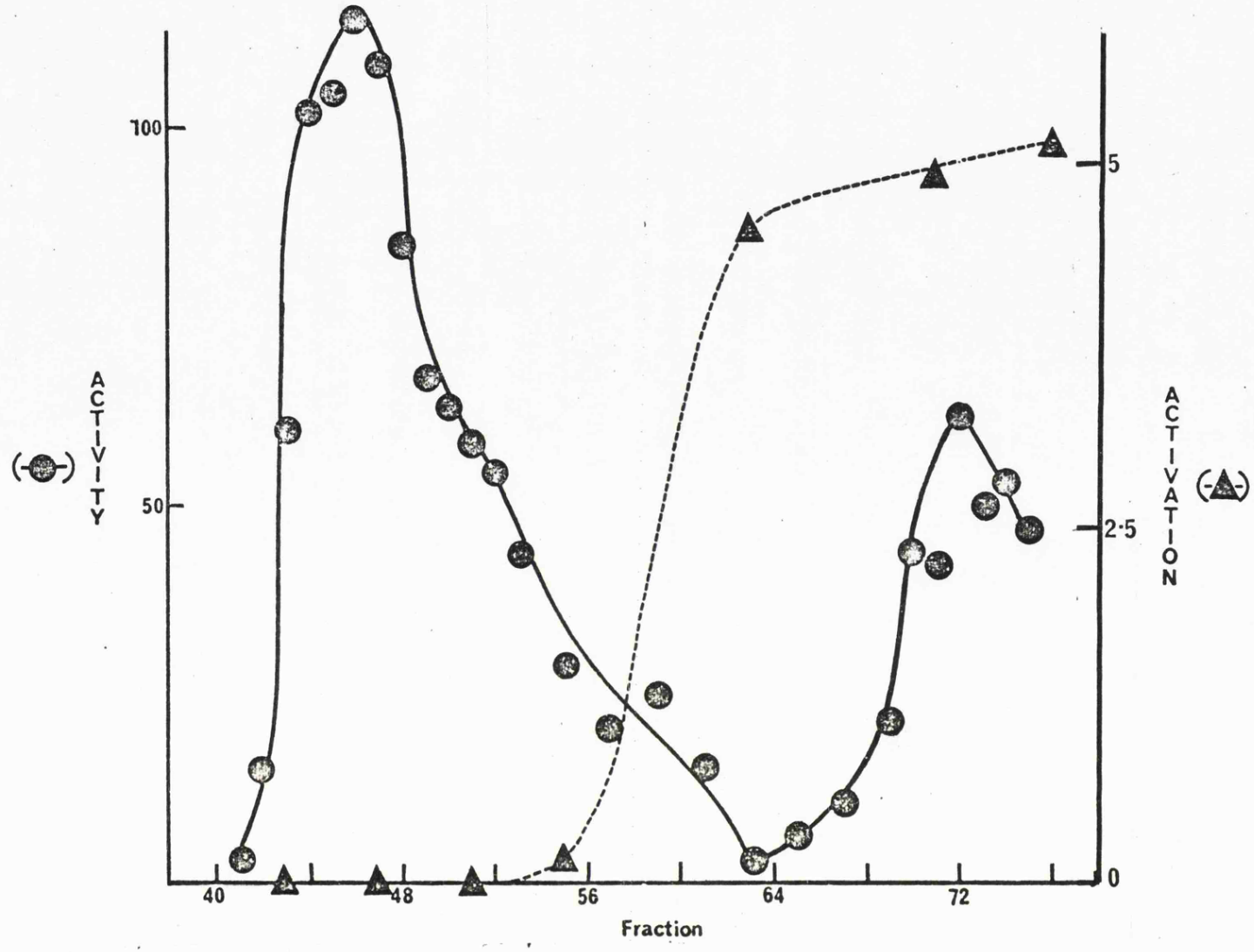


Figure 6 - DEAE-cellulose chromatography of an extract of A. lwoffi

phosphate/10 ml at pH 8.0. Five-ml fractions were collected and the fractions assayed for IDH with and without glyoxylate. Such assays had to be conducted with Mg^{2+} because of the high concentration of phosphate in the effluent which caused the formation of a precipitate with Mn^{2+}.

As is shown in Figure 6, this IDH activity was split into two components; the first to be eluted was termed IDH-I and the second IDH-II. IDH-I was found to be unaffected by glyoxylate or pyruvate but IDH-II was stimulated about five-fold by 0.2 mM glyoxylate or pyruvate.

Encouraged by this clear separation, I decided to investigate other separation methods in an attempt to demonstrate that the isoenzyme separation was not an artefact of DEAE-cellulose chromatography.

ii) Electrofocussing

Every protein has a characteristic isoelectric point. As a protein at its isoelectric point will not migrate in an electric field it should be possible to separate proteins in a pH gradient which is subject to a polarised electric field. This is the basis of "electrofocussing", the theoretical and practical aspects of which have been discussed by Vesterberg et al. (1967). I decided to investigate its usefulness in the separation of the IDH isoenzymes.

The apparatus was set up as described by Vesterberg et al. (1967). In operation it consisted of a vertical cooled sucrose gradient stabilising a pH gradient which had been formed by the differential migration of ampholines (amino-acids and small peptides) under the influence of an electric current passed through the solution. Each electrode was surrounded by a solution which repelled the ions attracted to it, thus causing an equilibrium to be set up. In such an environment a large protein slowly migrates to the pH region of its isoelectric point. The band is self-sharpening and stabilised by the sucrose gradient. The method is suitable only for proteins which can be kept in solution at their isoelectric points.

The column was set up with the ampholine (2%) in the sucrose gradient to form a pH gradient of 3-10. The manufacturers of the apparatus (LKB) recommended that electrofocussing be carried out for two days to achieve equilibrium with such a pH range. In initial attempts, a protein loading of 20 mg was applied but gross precipitation occurred after only 16 hours. In subsequent experiments such precipitation could not be prevented either by raising the ampholine concentration to 4% or by reversing the polarity of the electrodes. Precipitation was eventually avoided by reducing the protein loading to 1 mg. However, only a very small recovery of IDH activity was obtained in one pH region (5.00-5.05). This activity was found not to be stimulated by glyoxylate.

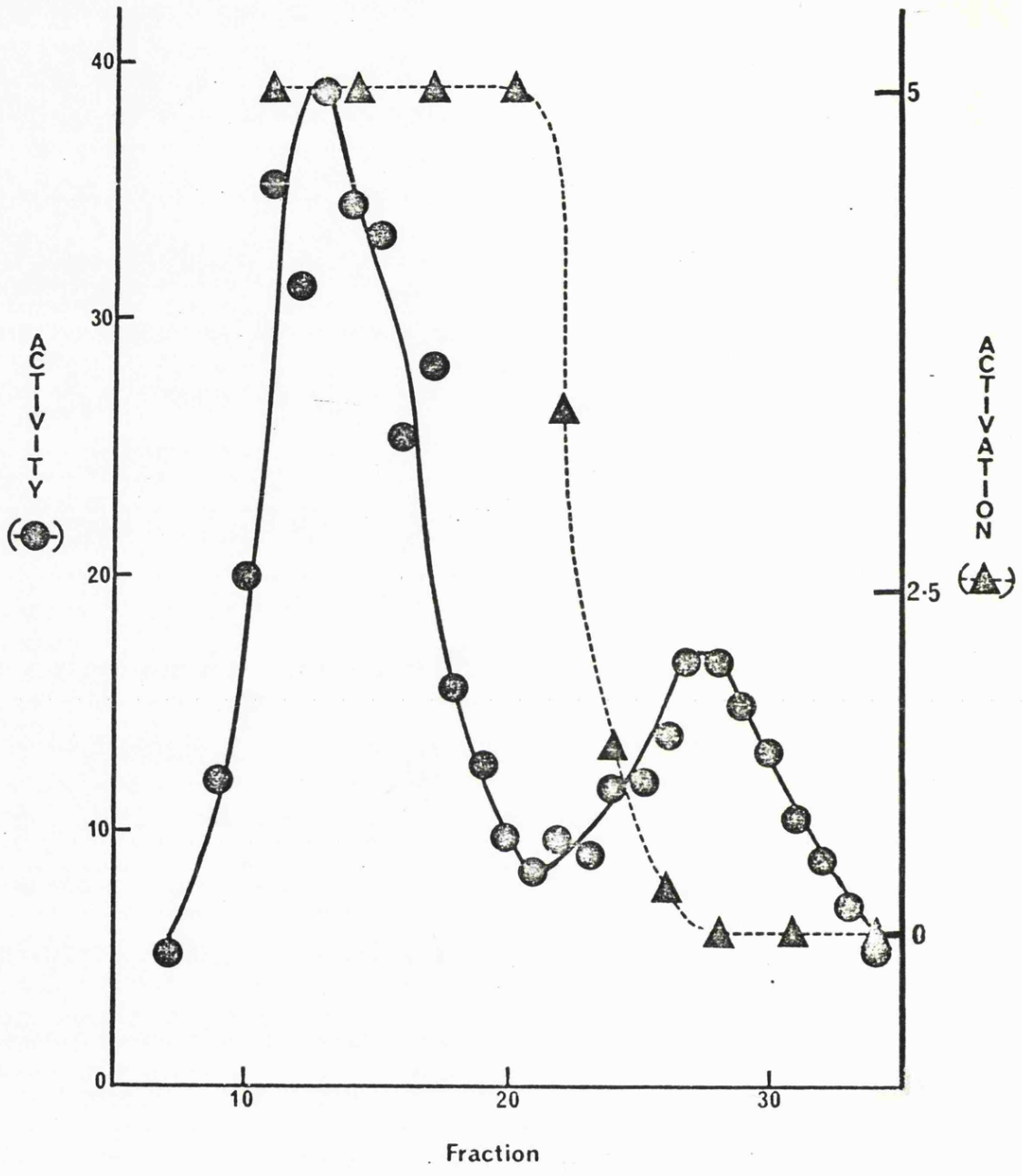


Figure 7 - Separation of IDH-I and IDH-II with Sephadex G-200

iii) Separation by Gel Filtration

A column of Sephadex G-200 (2.5 x 35 cm) was prepared, equilibrated with MET 8 and 2 ml of the enzyme solution applied. The column was eluted with MET 8 and 2 ml fractions were collected and assayed for IDH, with and without 0.2 mM glyoxylate. Figure 7 shows the elution pattern of IDH activity. The two isoenzymes are clearly separated. The first one to travel through the column is activated about five-fold by 0.2 mM glyoxylate thus appearing to be the IDH-II of the DEAE-cellulose separation, and the second is insensitive to either glyoxylate or pyruvate (IDH-I). The dotted line in Figure 7 shows the activation produced by 0.2 mM glyoxylate. Extracts of acetate-grown E. coli when prepared and chromatographed in the same way gave only a single peak of IDH activity (Figure 45 , page 101).

Because the isoenzymes were so well separated by a method which selects for molecular size, separation by zonal ultracentrifugation was attempted.

iv) Separation by Zonal Ultracentrifugation

The separation was carried out using a titanium BXIV zonal rotor containing a linear density gradient of sucrose (5-20% w/w) at 10°. A typical fractionation achieved by

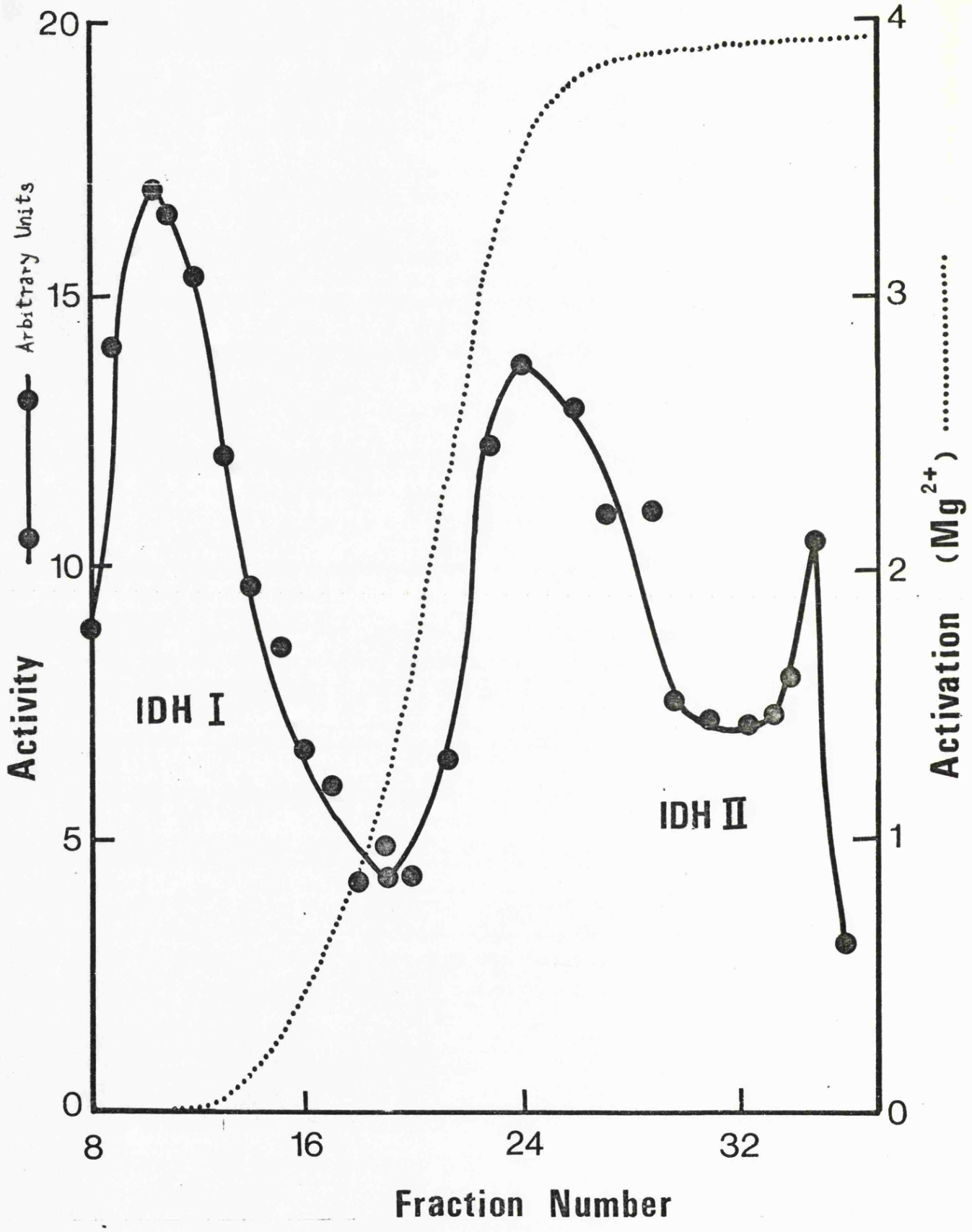


Figure 8 - Separation of IDH-I and IDH-II by zonal ultracentrifugation

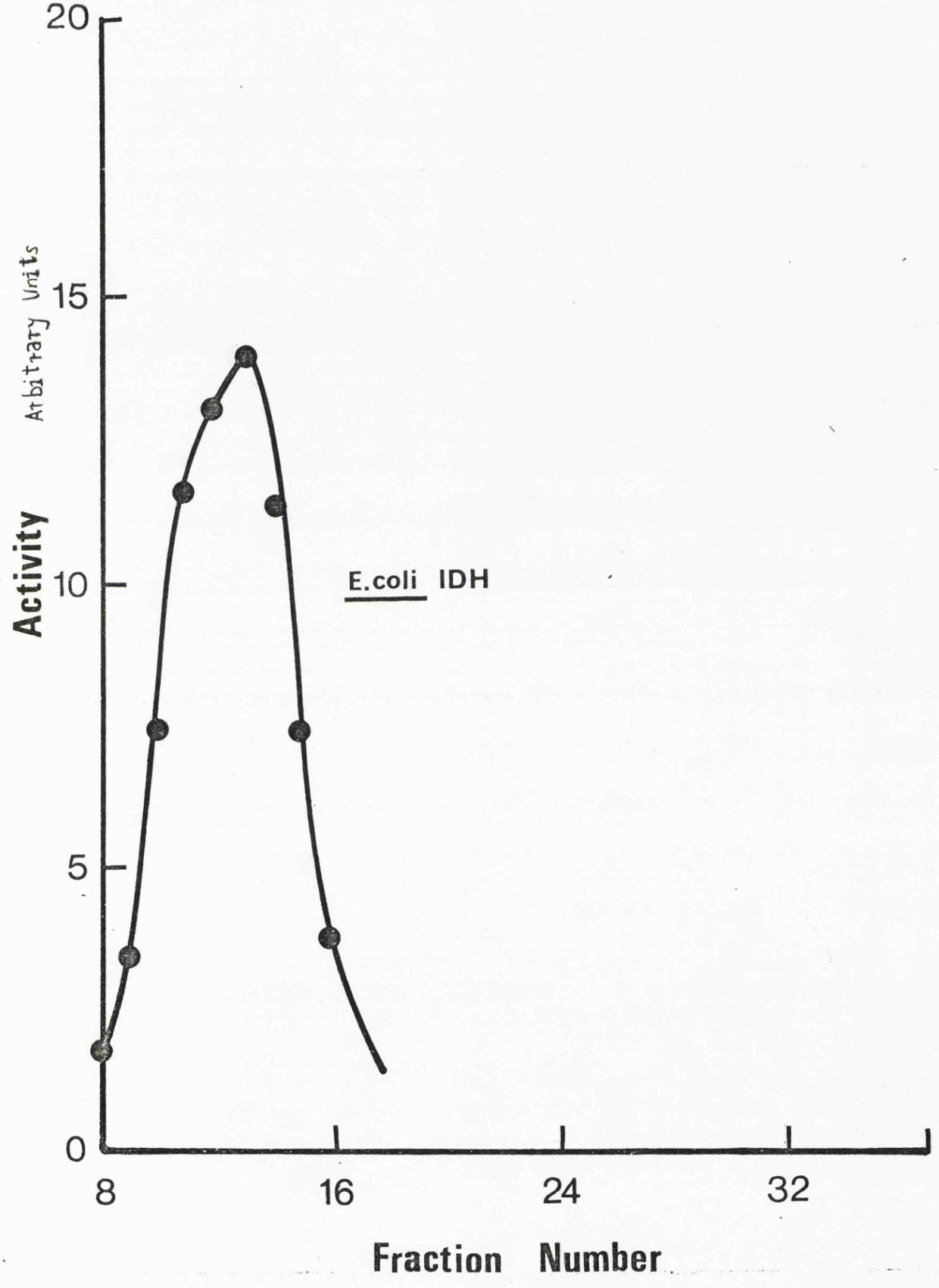


Figure 9 - Zonal ultracentrifugation of IDH from E. coli

centrifugation at 47,000 rpm for 20 hours is shown, Figure 8. Again, there is very good separation of the two species and IDH-II is seen to sediment much faster than IDH-I. The activation profile is again depicted by a dotted line and rises quite sharply between the two forms indicating a sharp fractionation. The small peak of activity has the same characteristics as IDH-II and represents IDH-II which has sedimented to the rotor wall. In contrast, a preparation from acetate-grown E. coli shows only a single peak of IDH activity even when centrifuged for a longer time (Figure 9).

Preparative Studies

In order to study the molecular and kinetic properties of the IDH isoenzymes it was necessary to make stable preparations of each isoenzyme. However, complications arose from the presence of the enzymes glutamate dehydrogenase and isocitrate lyase. Both of these interfered with accurate kinetic and molecular studies on IDH. As IDH was assayed by measuring the rate of formation of NADPH, GDH interfered, when ammonium ions were present, by oxidising the NADPH. ICL interfered by producing glyoxylate which could activate IDH-II and by lowering the concentration of the isocitrate in kinetic experiments. Interference was apparent from these sources even though "initial"

TABLE 4

The effect of sonication time on the relative liberation of IDH, GDH and ICL.

Time (Min)	IDH (Mn^{2+})	GDH	ICL
1.0	35.7	20.4	9.1
2.0	72	35.1	12.8
3.0	113	48.2	20.0
4.0	123	62.4	22.2
5.0	141	62.6	25.6
6.0	152	63.6	26.1

reaction rates were measured.

It was not found possible to repress completely the synthesis of either contaminating enzyme by growing the organism on carbon sources on which their synthesis might be unnecessary, nor was it possible to reduce the contamination by either enzyme by changing the duration of sonication (Table 4). It was, therefore, necessary to devise a purification scheme which would not only separate the IDH isoenzymes but also remove GDH and ICL. In the light of this the preparative usefulness of ion exchange and gel filtration chromatography and rate zonal ultracentrifugation were reinvestigated. For these studies preparations containing both IDH isoenzymes were made as described in the previous section by treatment of sonic extracts with protamine sulphate followed by fractionation and concentration with ammonium sulphate (protein precipitated between 45 and 75% saturation being used) and, finally, dialysed against 40 mM phosphate and 1 mM EDTA at pH 8.0.

i) DEAE-cellulose chromatography

DEAE-cellulose chromatography was carried out as previously described employing the same phosphate concentration gradient elution. The effluent from the column was collected in 5 ml fractions and assayed for IDH (with Mg^{2+}, $\pm$ glyoxylate), GHD, ICL and protein.

TABLE 5

The efficiency of DEAE chromatography in separating IDH-I and IDH-II

IDH type	Activation (Cation Mg^{2+})	Specific Activity	Yield %
Sample applied	0.8	25	(100)
I	0.0	105*	25
II	5.0	130*	15

* peak specific activity

Figure 10 - Purification with DEAE-cellulose

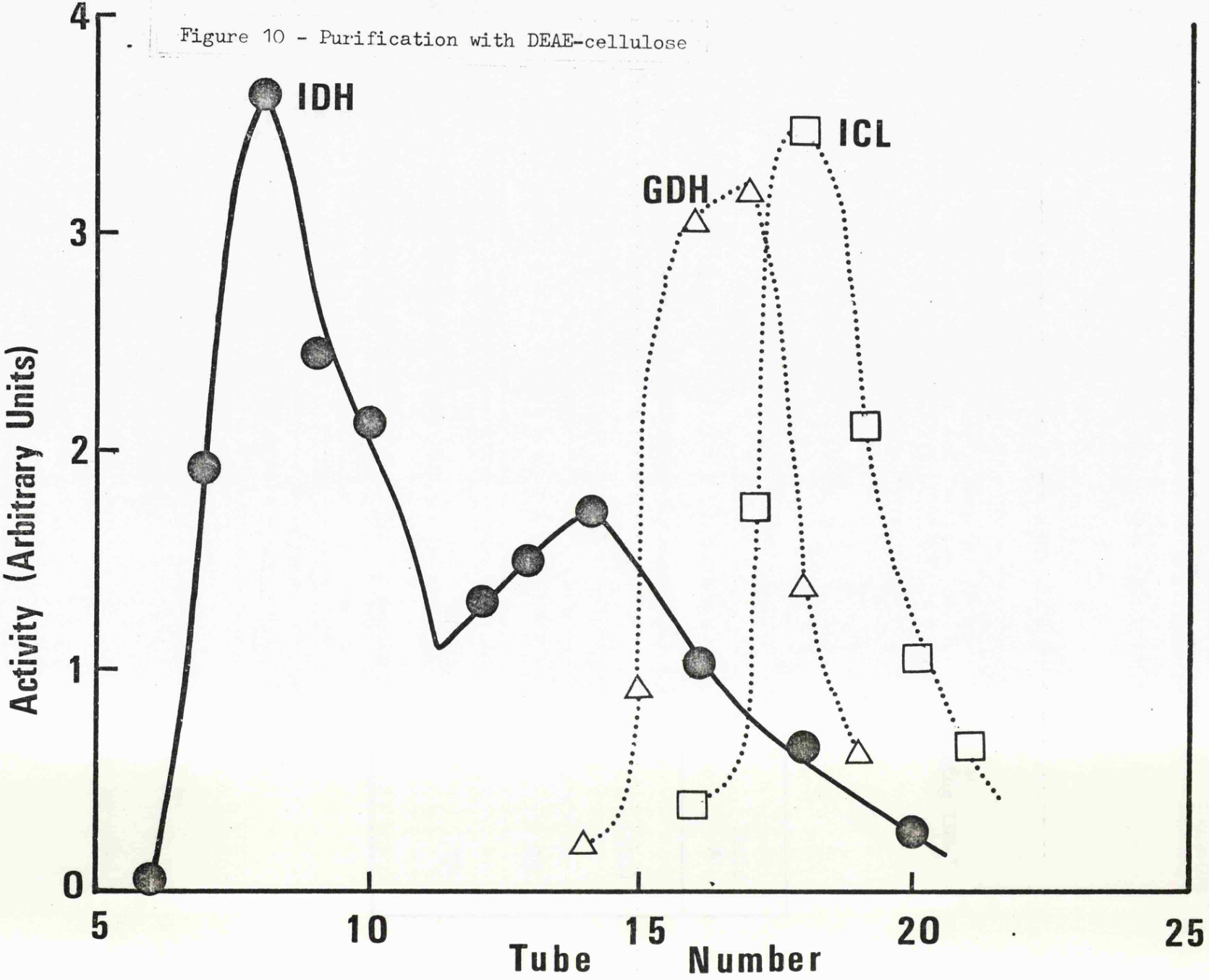


Figure 10 again shows the good separation of IDH-I and IDH-II possible with DEAE-cellulose chromatography. It is clearly shown that IDH-I may be obtained free of IDH-II as well as from GDH and ICL. Table 5 shows that good recovery of IDH-I was obtained. However, much of the IDH-II eluted free from IDH-I was still contaminated with GDH and ICL.

ii) Gel Filtration

Good separation of the isoenzymes of IDH can be achieved by chromatography through Sephadex G-200 prepared and eluted with MET 8. Although the separation is interesting as an index of molecular size, the method gives very little separation of GDH and ICL from IDH-II (Figure 11) and causes much greater inactivation of the isoenzymes than other, equally effective, separation methods.

iii) Rate-Zonal Ultracentrifugation

A linear concentration gradient of 5-20% sucrose in 5 mM Tris, 1 mM EDTA at pH 8.0 was chosen and subsequent separations were carried out at 10^{o} with this gradient. It was found necessary to include EDTA in the density gradient buffer to maintain the IDH activity; this may have been necessary because of heavy metal ions present in the sucrose. No

Figure 11 - Purification with Sephadex G-200

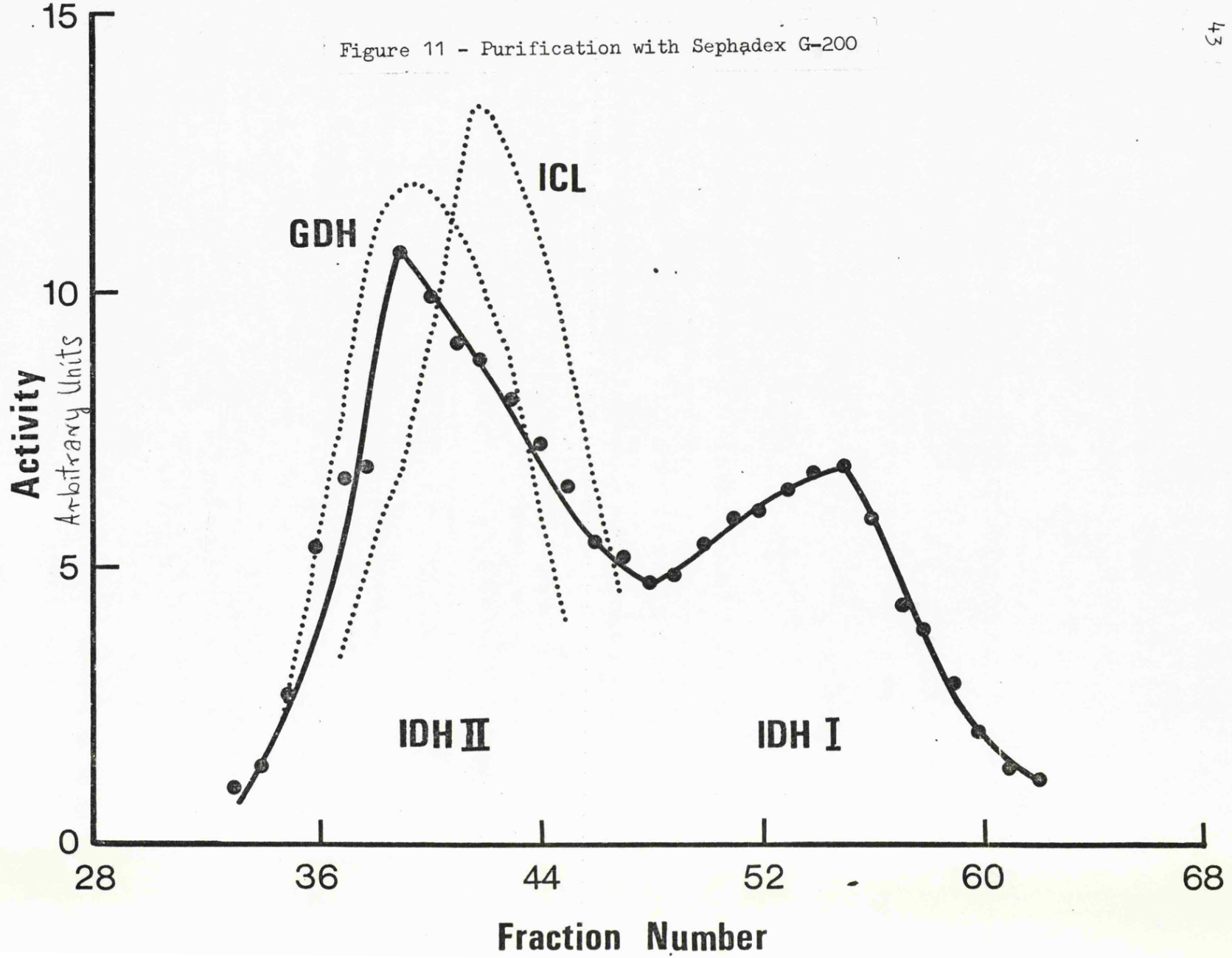


TABLE 6

The efficiency of zonal ultracentrifugation in separating IDH-I and IDH-II

IDH Type	Activation (Cation Mg^{2+})	Specific Activity	Yield %
Sample applied	1.0	33	(100)
I	0.2	35*	45
II	4.0	87*	25

* peak specific activity

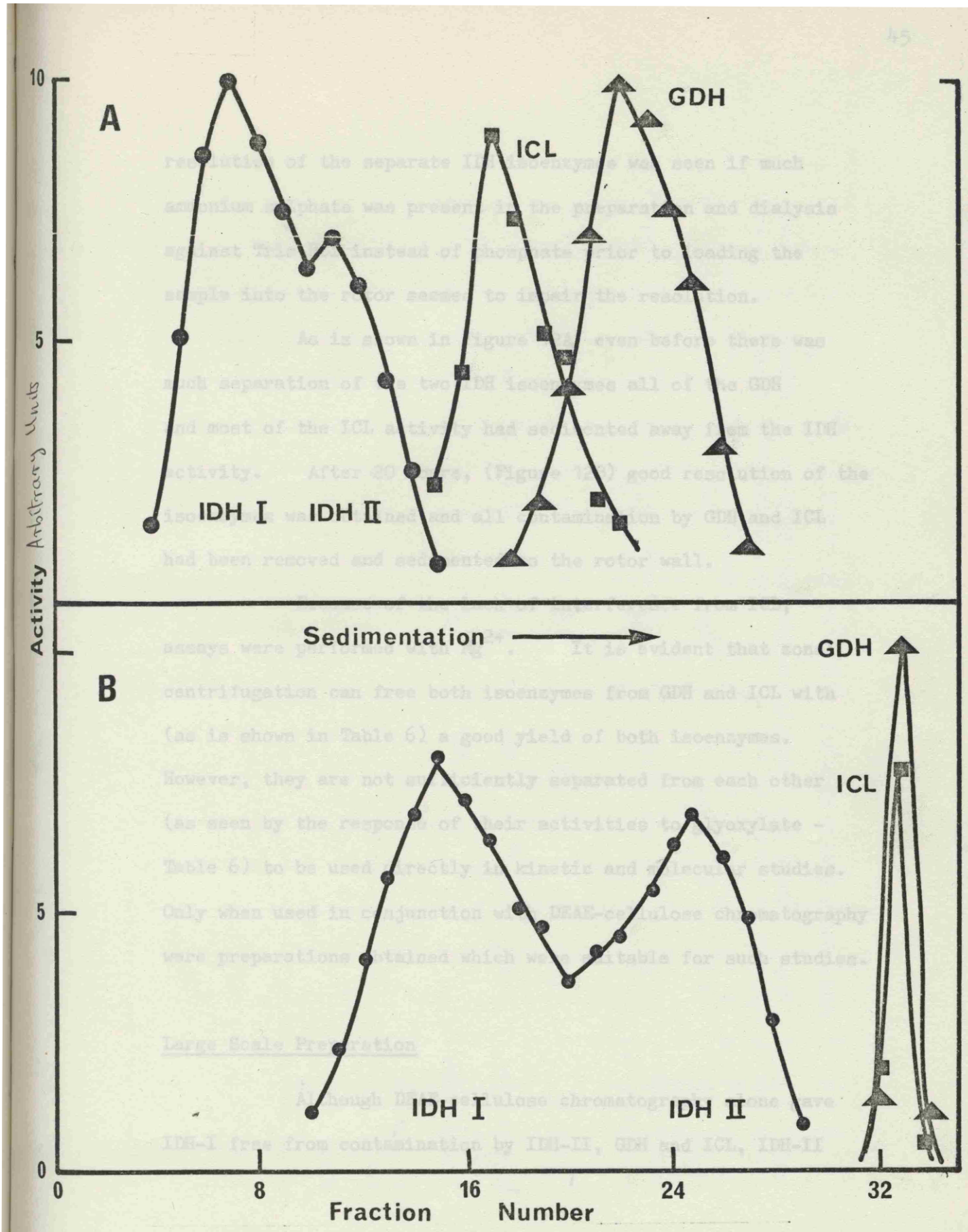


Figure 12 - Purification by zonal ultracentrifugation

resolution of the separate IDH isoenzymes was seen if much ammonium sulphate was present in the preparation and dialysis against Tris-HCl instead of phosphate prior to loading the sample into the rotor seemed to impair the resolution.

As is shown in Figure 12A, even before there was much separation of the two IDH isoenzymes all of the GDH and most of the ICL activity had sedimented away from the IDH activity. After 20 hours, (Figure 12B) good resolution of the isoenzymes was obtained and all contamination by GDH and ICL had been removed and sedimented to the rotor wall.

Because of the lack of interference from ICL, assays were performed with Mg^{2+}. It is evident that zonal centrifugation can free both isoenzymes from GDH and ICL with (as is shown in Table 6) a good yield of both isoenzymes. However, they are not sufficiently separated from each other (as seen by the response of their activities to glyoxylate - Table 6) to be used directly in kinetic and molecular studies. Only when used in conjunction with DEAE-cellulose chromatography were preparations obtained which were suitable for such studies.

Large Scale Preparation

Although DEAE-cellulose chromatography alone gave IDH-I free from contamination by IDH-II, GDH and ICL, IDH-II

TABLE 7

Preparation from a 32 litre culture of A. lwoffi

Stage	Vol. ml.	Metal ion	Activity	Activation	Specific Activity	Enzyme Units	Yield %
Crude	755	Mn^{2+}	150	0.26	13.4	113,000	-
Post protamine sulphate	760	"	131	0.29	25.2	99,500	88
Am. sulphate 45-75%	38	"	2275	0.40	43	86,400	76
Post Dialysis	44	Mg^{2+}	1280	0.81	31.4	56,500	50
- precipitate	44	"	882	1.09	33	38,800	38
Zonal IDH-I	290	"	62	0.21	35*	17,900	16
DEAE IDH-I	110	"	20	0.0	53*	2,200	2
Zonal IDH-II	430	"	22	3.0	87*	9,500	8
DEAE IDH-II	155	"	8	5.5	125*	1,260	1

* Peak specific activities

was contaminated with both GDH and ICL. Further fractionation of this IDH-II by zonal ultracentrifugation was thus desirable but as the material from DEAE chromatography was present in a large volume ($\sim$ 100 ml), it would have required concentration before application to the zonal rotor. However, as the presence of ammonium sulphate interferes with the separation by ultracentrifugation and the separated isoenzymes are unstable to dialysis, the solution could not easily be concentrated with ammonium sulphate prior to zonal separation. However, these concentration problems could be avoided if the material was first fractionated by ultracentrifugation and the separated isoenzymes then further fractionated with DEAE-cellulose chromatography. Table 7 shows the results of such a purification from 32 litre culture.

Although the separated isoenzymes were found to be stable when stored for many months at 4°, they were both very labile to (i) electrophoresis, (ii) dialysis, and (iii) passage through ion exchangers even in the presence of 1 mM mercaptoethanol. Hence the low yield shown in Table 7 resulting from DEAE-cellulose chromatography of the separated isoenzymes. Although it was necessary to fractionate IDH-II by both zonal ultracentrifugation and DEAE chromatography resulting in a small final yield, IDH-I could be prepared quite adequately by fractionating the mixture

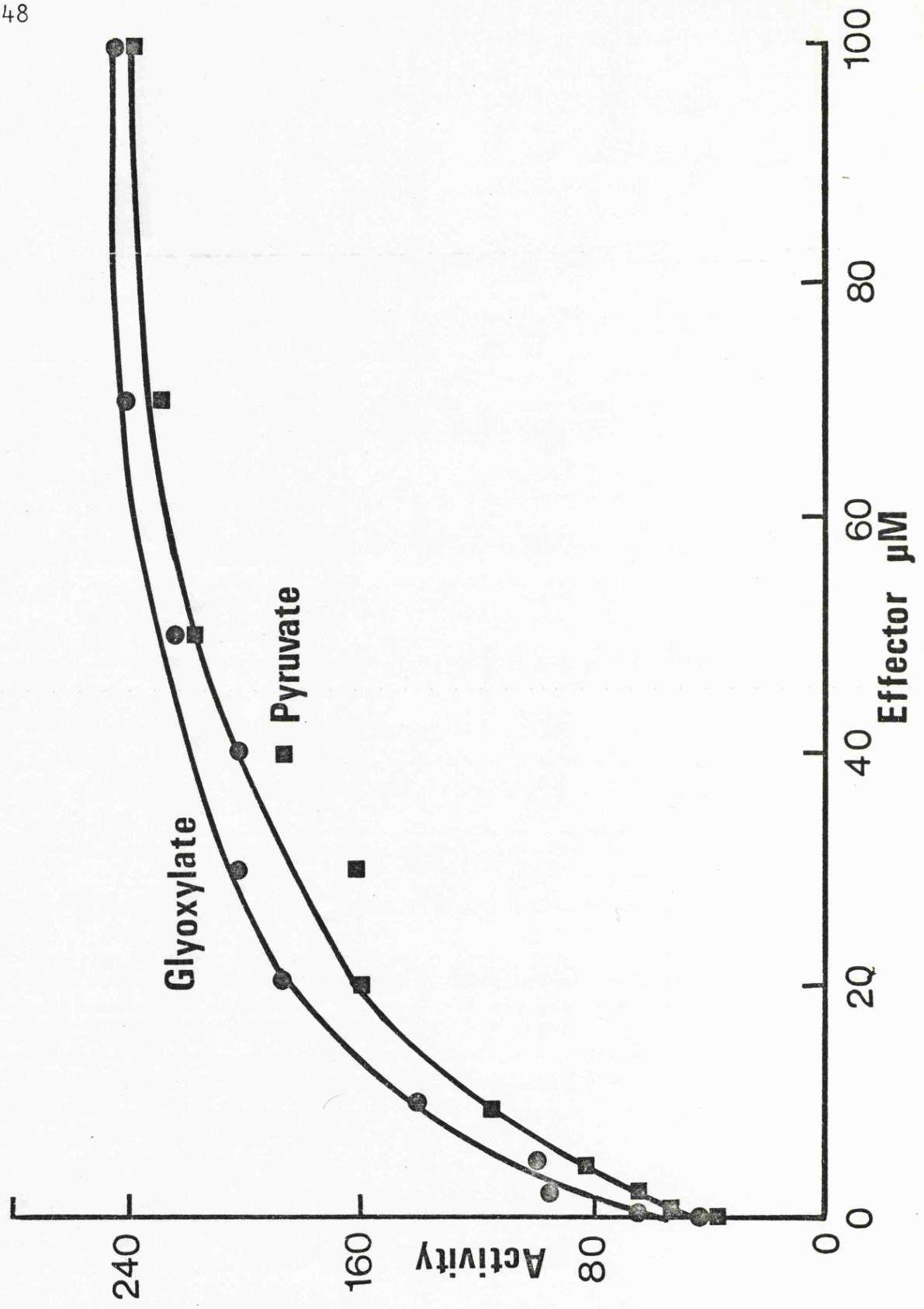


Figure 13 - The effect of pyruvate or glyoxylate on IDH-II

with DEAE cellulose, omitting the zonal centrifugation step. Therefore, while IDH-II was prepared by using the two steps, IDH-I was prepared using only DEAE-cellulose chromatography.

Comparative Studies of IDH-I and IDH-II

Studies on the partially purified preparations of IDH-I and IDH-II have revealed considerable differences in their response to glyoxylate or pyruvate, changes in pH and inactivation by heat or urea. Similar differences have also been found between activated and non-activated IDH-II. Both of the isoenzymes were found to be absolutely specific for NADP, no activity being observed with NAD even in the presence of pyruvate or glyoxylate.

The Activation

Of the two isoenzymes only IDH-II was found to be activated by glyoxylate or pyruvate. Figure 13 shows the effect of including different concentrations of either pyruvate or glyoxylate on the activity. With either effector almost complete activation is achieved at a concentration of 0.1 mM when assayed in MET 8.

TABLE 8

The influence of metal ion on activity and activation of IDH

	IDH-I	IDH-II			
Assayed with:	-	-	P	G	P.Actn.	G.Actn
Mg^{2+}	11.6	12.4	64.8	61.0	4.2	3.9
Mn^{2+}	13.9	26.4	71.8	74.0	1.7	1.8
Mn/Mg	1.2	2.1	1.1	1.2		

P and G assays carried out in the presence of pyruvate or glyoxylate respectively. P.Actn. and G.Actn. is the activation achieved by the addition of pyruvate or glyoxylate.

IDH-I was found not to be stimulated from pH 6.5 to 9.0 over a wide range of pyruvate or glyoxylate concentrations. The activity was, however, found to be slightly inhibited ($\sim$ 5%) by 0.2 mM glyoxylate in the normal assay.

Metal Requirement

Both isoenzymes exhibit greater activity with Mn^{2+} in the normal assay than with Mg^{2+}. However, as is shown in Table 8, the response of IDH-II to either pyruvate or glyoxylate is more marked with Mg^{2+} than with Mn^{2+}.

A more complete (kinetic) analysis is presented later.

Reversal of the Activation

The activity of a sample of IDH-II was measured in the absence of both pyruvate and glyoxylate. The remainder of the sample was then divided into two, one half being made 1.0 mM in glyoxylate and the other the same concentration in pyruvate. Each portion was assayed for IDH activity with 0.2 ml aliquots in the final 1.0 ml assay mixture, which thus contained effector at 0.2 mM concentration. Similar assay mixtures were made excluding isocitrate but with the addition of a slight excess ($\sim$ 0.02 μmole) of NADH above the stoichiometric concentration of the effector. Lactic dehydrogenase was added and the

mixture left until no further reduction of effector was evident by decrease in absorbance at 340 mμ. After the effector had thus been removed from the solution, the IDH reaction was initiated by the addition of 2 μmole isocitrate and the formation of NADPH followed in the usual way. Complete reversal of activation was seen for both glyoxylate and pyruvate.

Other Activity Modifiers

Mixtures of glyoxylate and oxaloacetate at 0.2 mM concentration have been found to inhibit various IDH's (Adinolfi, 1969; Ozaki & Shiio, 1968). Such mixtures were also found to inhibit both IDH-I and IDH-II completely. However, mixtures of OAA and pyruvate did not inhibit either enzyme, nor did OAA on its own.

Although ATP and ADP at 2 mM concentration did not affect either IDH-I or IDH-II, 2 mM AMP activated IDH-II by 1.5-fold. However, enzyme five-fold activated by 0.2 mM glyoxylate was unaffected by AMP as well as by ADP and ATP.

Reversal of the IDH-I and IDH-II Reactions

In the absence of glutamate dehydrogenase, it was possible to demonstrate that both IDH-I and IDH-II could catalyse the conversion of α-ketoglutarate, CO_2 and NADPH to

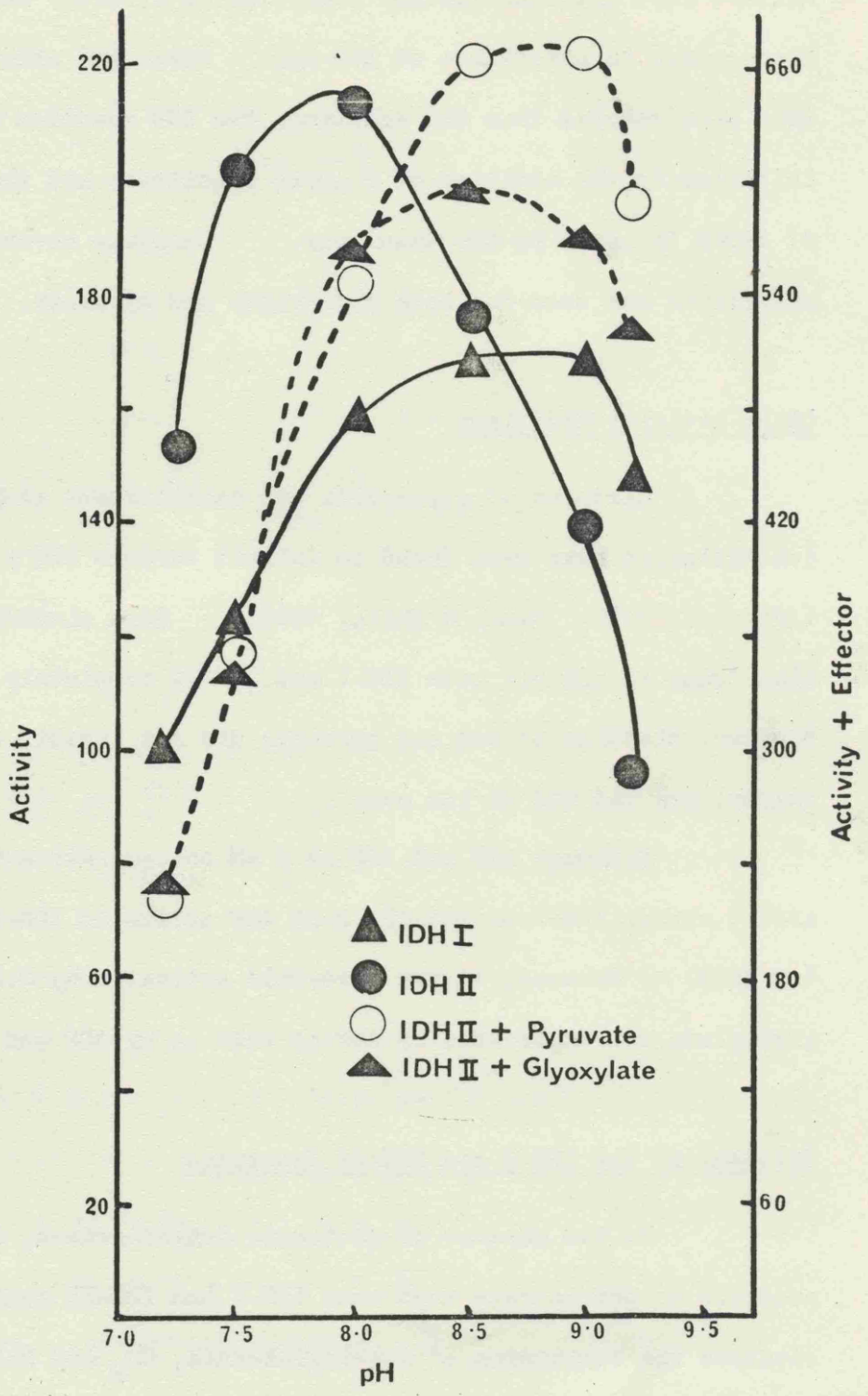


Figure 14 - The dependence of the activity of IDH-I and IDH-II with and without pyruvate and glyoxylate on pH

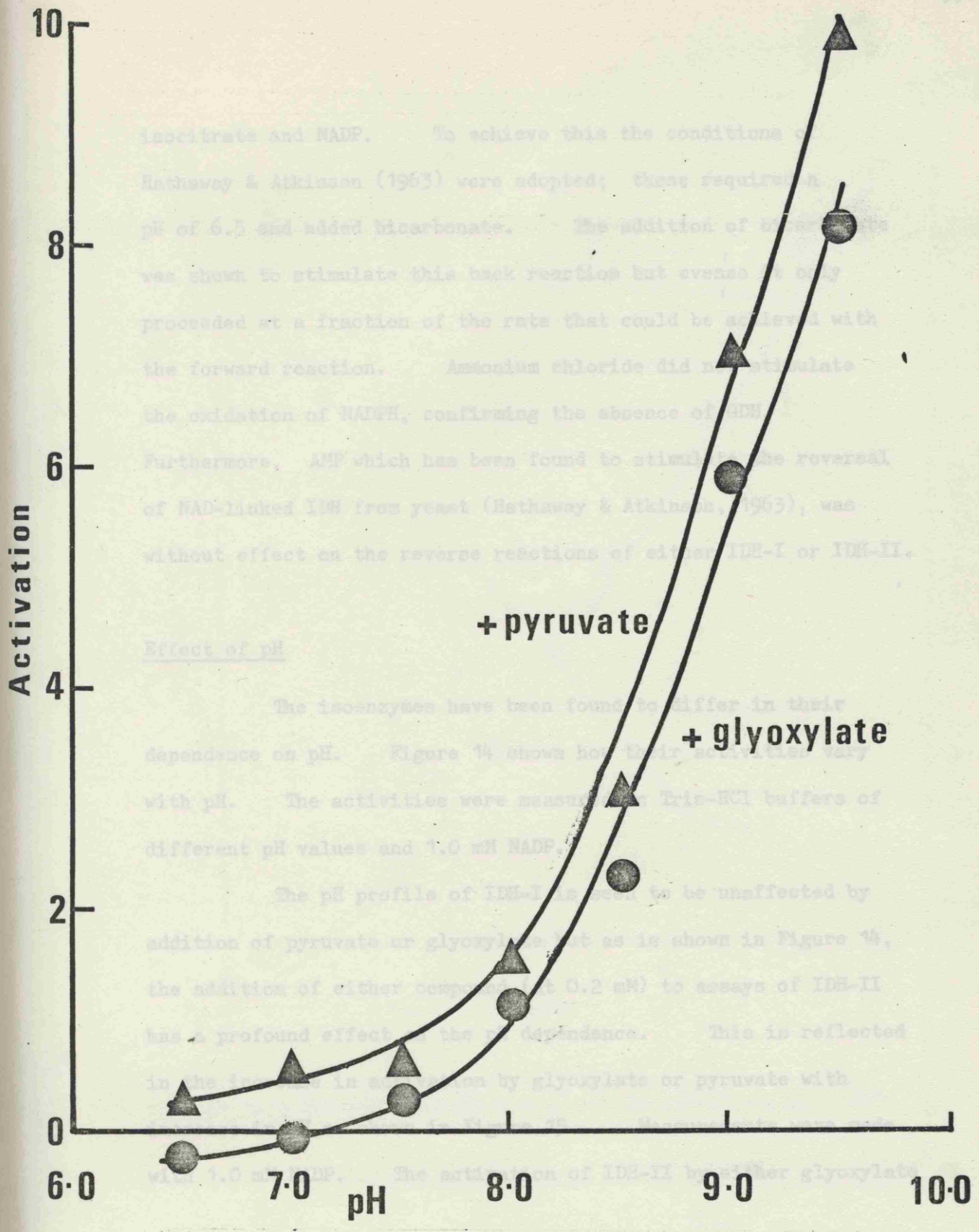


Figure 15 - The effect of pH on the activation of IDH-II

isocitrate and NADP. To achieve this the conditions of Hathaway & Atkinson (1963) were adopted; these required a pH of 6.5 and added bicarbonate. The addition of bicarbonate was shown to stimulate this back reaction but evenso it only proceeded at a fraction of the rate that could be achieved with the forward reaction. Ammonium chloride did not stimulate the oxidation of NADPH, confirming the absence of GDH. Furthermore, AMP which has been found to stimulate the reversal of NAD-linked IDH from yeast (Hathaway & Atkinson, 1963), was without effect on the reverse reactions of either IDH-I or IDH-II.

Effect of pH

The isoenzymes have been found to differ in their dependence on pH. Figure 14 shows how their activities vary with pH. The activities were measured in Tris-HCl buffers of different pH values and 1.0 mM NADP.

The pH profile of IDH-I is seen to be unaffected by addition of pyruvate or glyoxylate but as is shown in Figure 14, the addition of either compound (at 0.2 mM) to assays of IDH-II has a profound effect on the pH dependence. This is reflected in the increase in activation by glyoxylate or pyruvate with increase in pH as shown in Figure 15. Measurements were made with 1.0 mM NADP. The activation of IDH-II by either glyoxylate

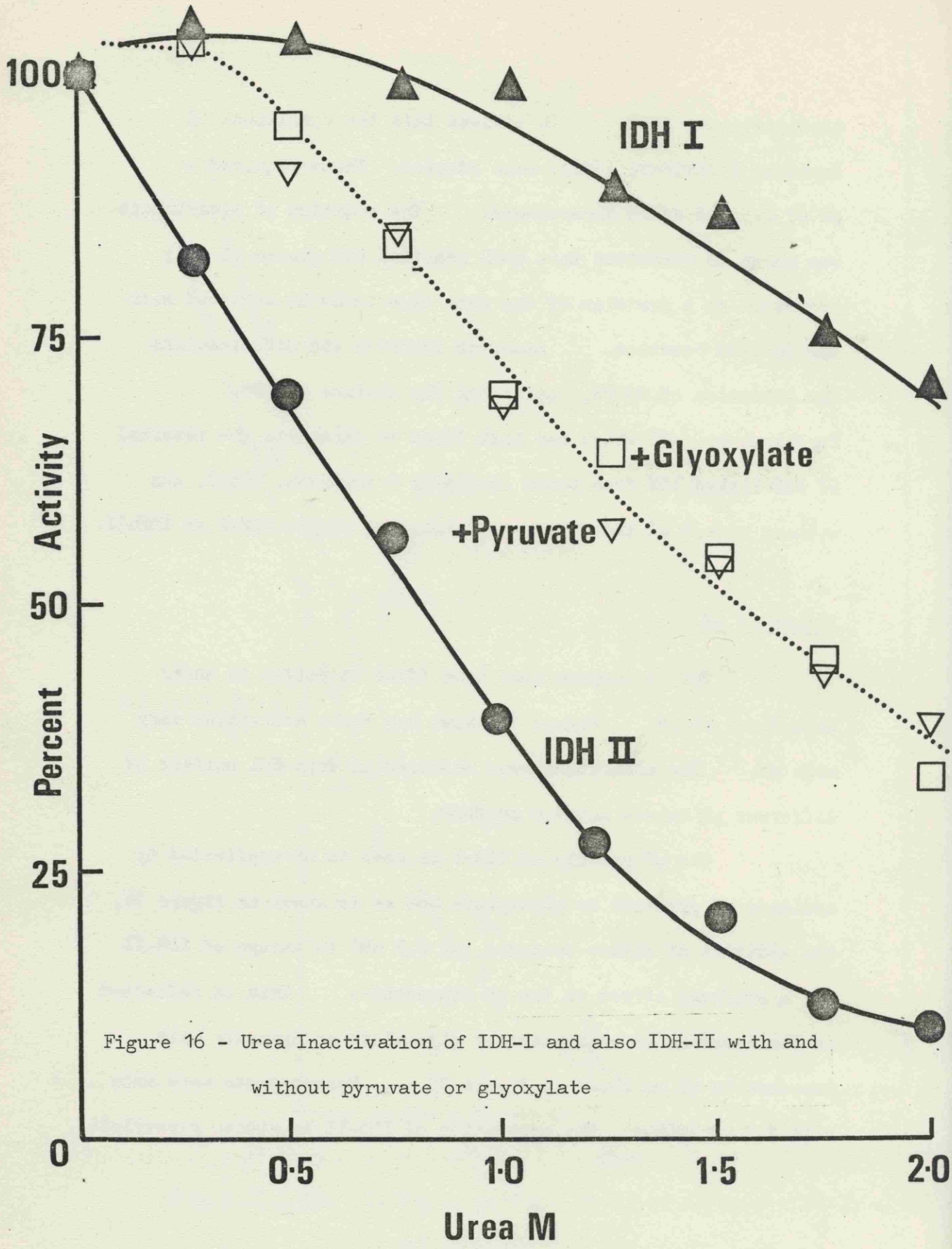


Figure 16 - Urea Inactivation of IDH-I and also IDH-II with and without pyruvate or glyoxylate

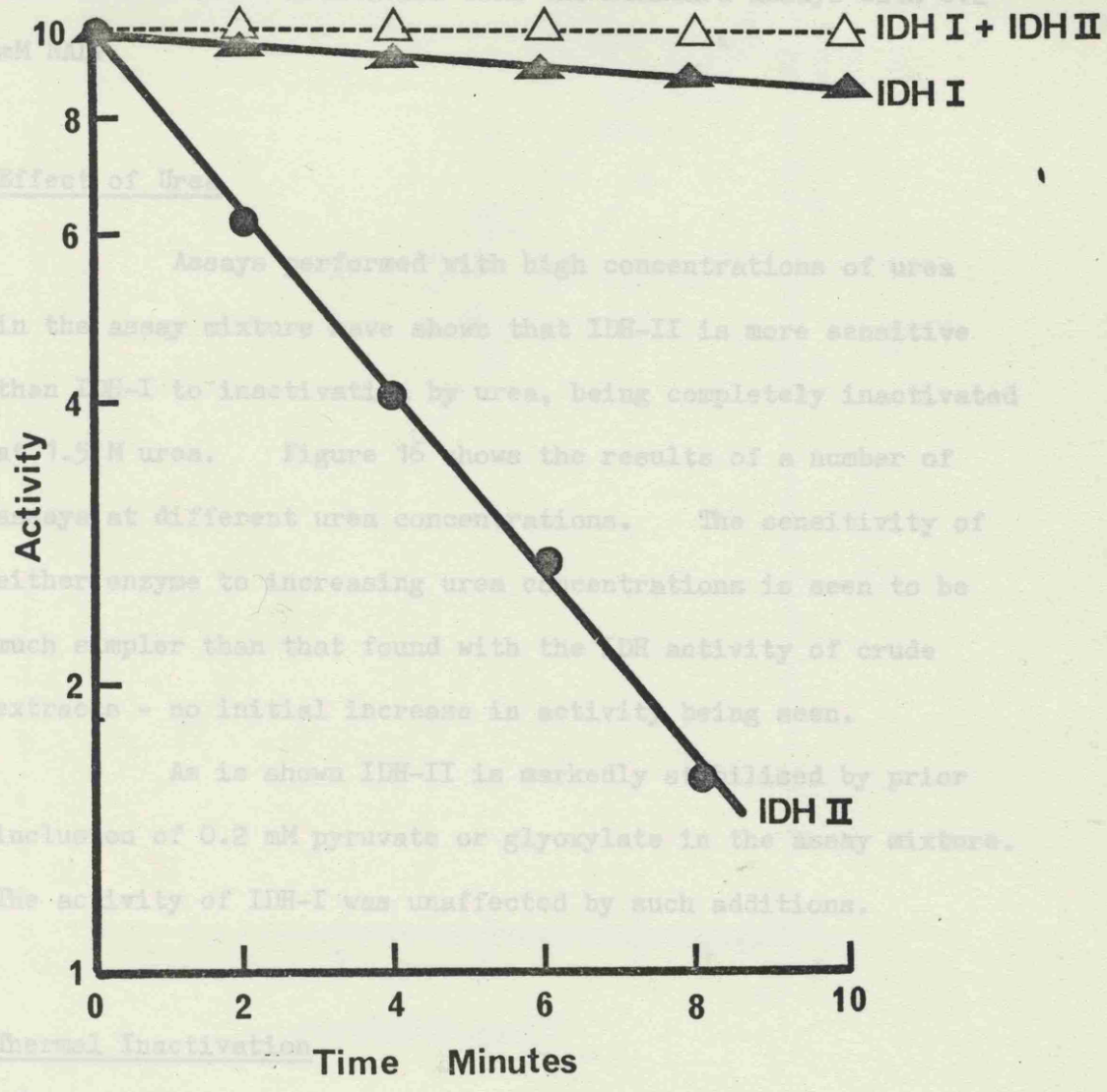


Figure 17 - Thermal Inactivation of IDH-I, IDH-II and a mixture of the two

or pyruvate has been shown to vary inversely with NADP concentration (page74). Thus assays undertaken with 1.0 mM NADP show a lower activation than the standard assays with 0.2 mM NADP.

Effect of Urea

Assays performed with high concentrations of urea in the assay mixture have shown that IDH-II is more sensitive than IDH-I to inactivation by urea, being completely inactivated at 1.5 M urea. Figure 16 shows the results of a number of assays at different urea concentrations. The sensitivity of either enzyme to increasing urea concentrations is seen to be much simpler than that found with the IDH activity of crude extracts - no initial increase in activity being seen.

As is shown IDH-II is markedly stabilised by prior inclusion of 0.2 mM pyruvate or glyoxylate in the assay mixture. The activity of IDH-I was unaffected by such additions.

Thermal Inactivation

As with inactivation by urea, IDH-I is much more stable to thermal inactivation than is IDH-II. Figure 17 shows the effect of inactivating the isoenzymes at 43° for

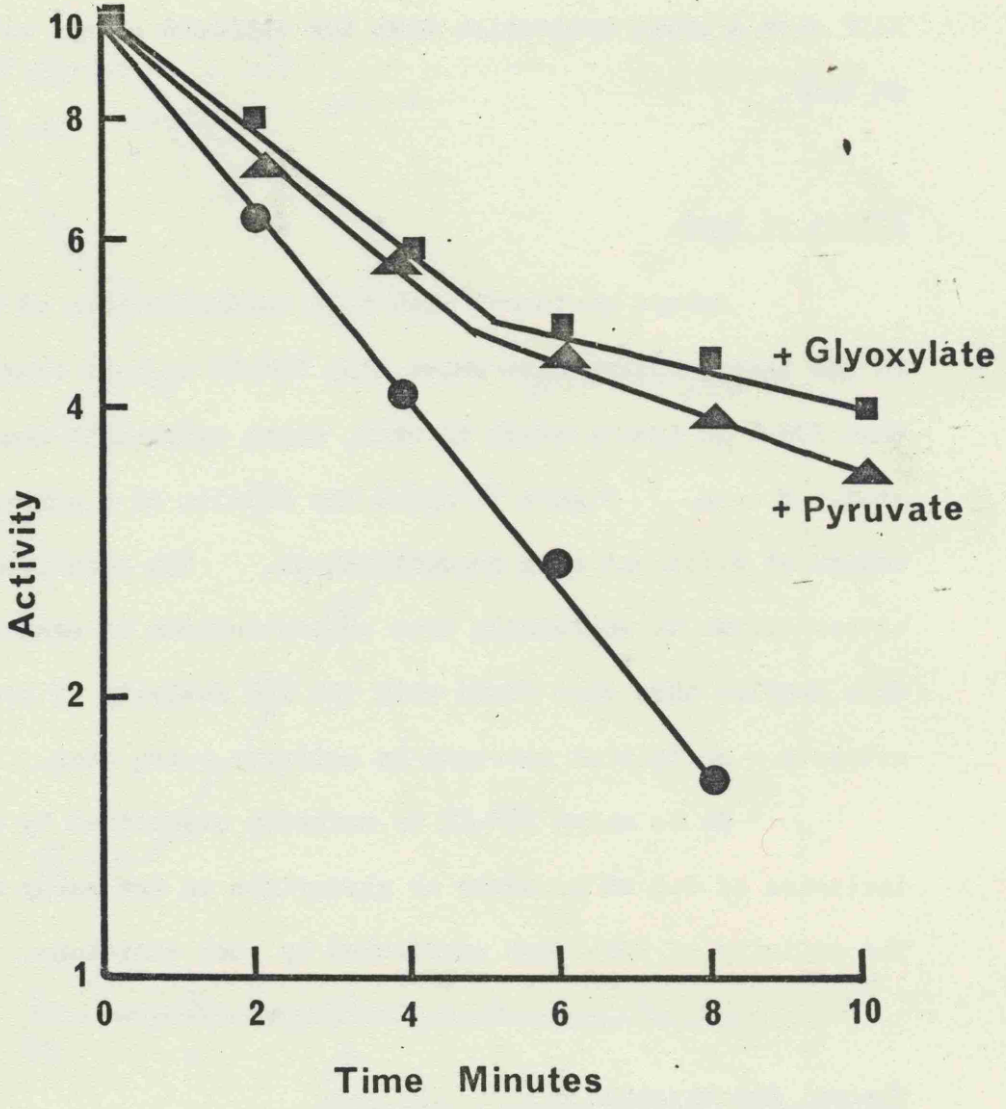


Figure 18 - Protection of IDH-II against thermal inactivation by pyruvate or glyoxylate

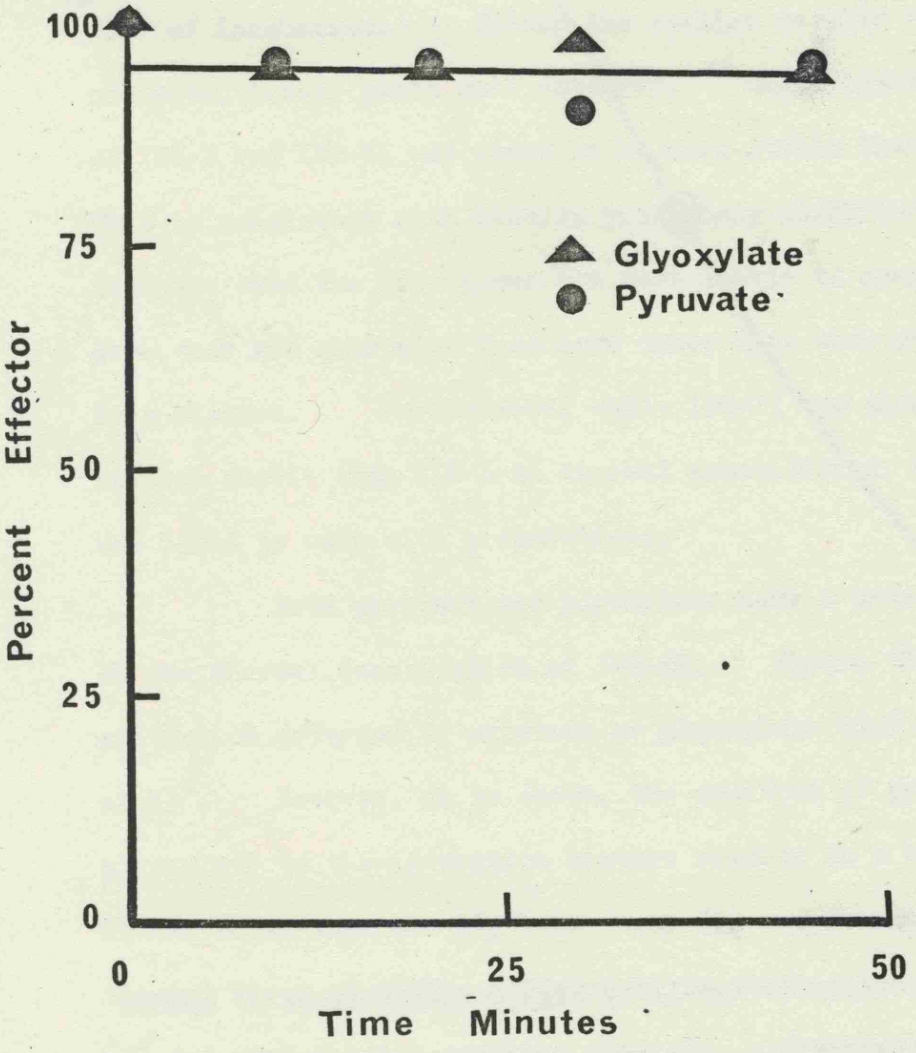


Figure 19 - The thermo-stability of pyruvate and glyoxylate

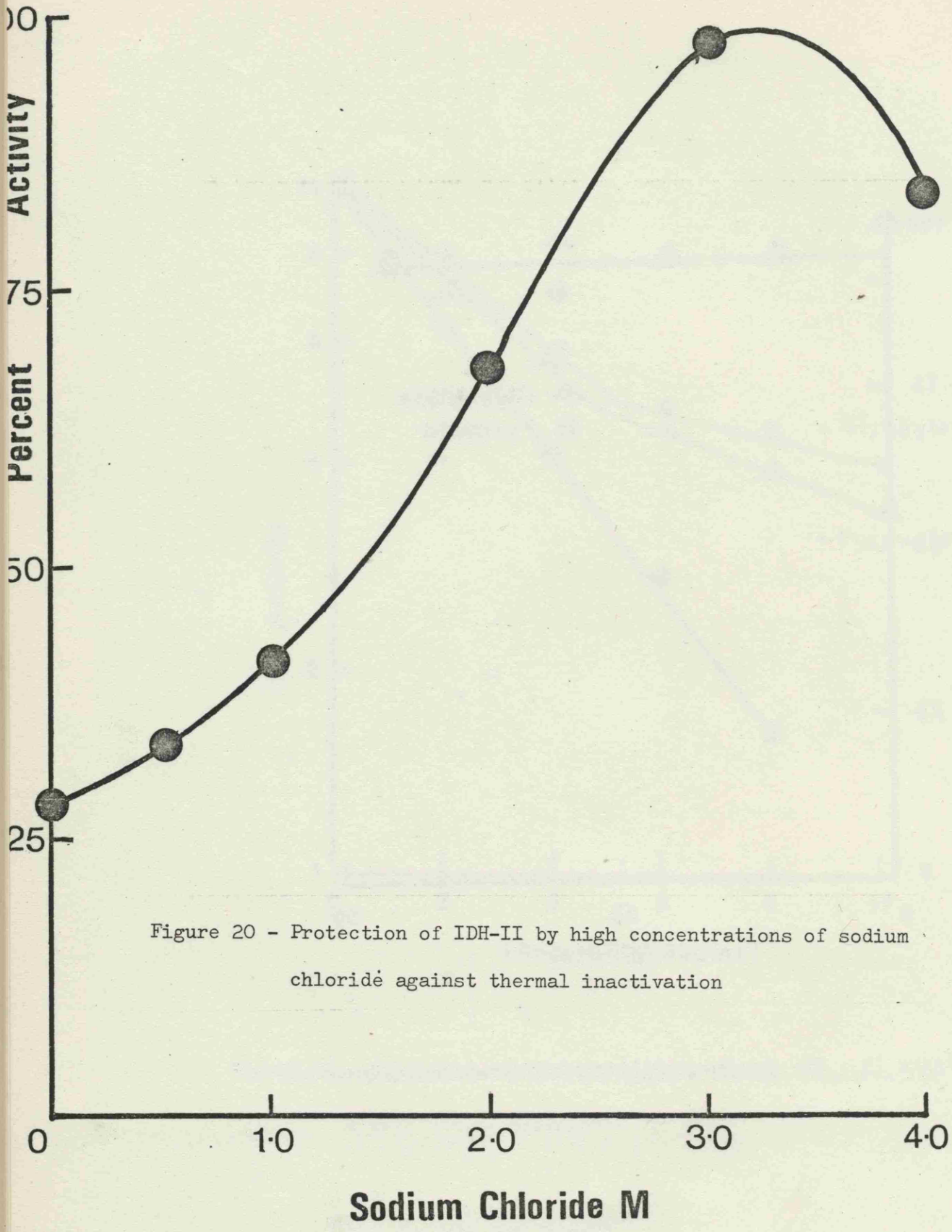


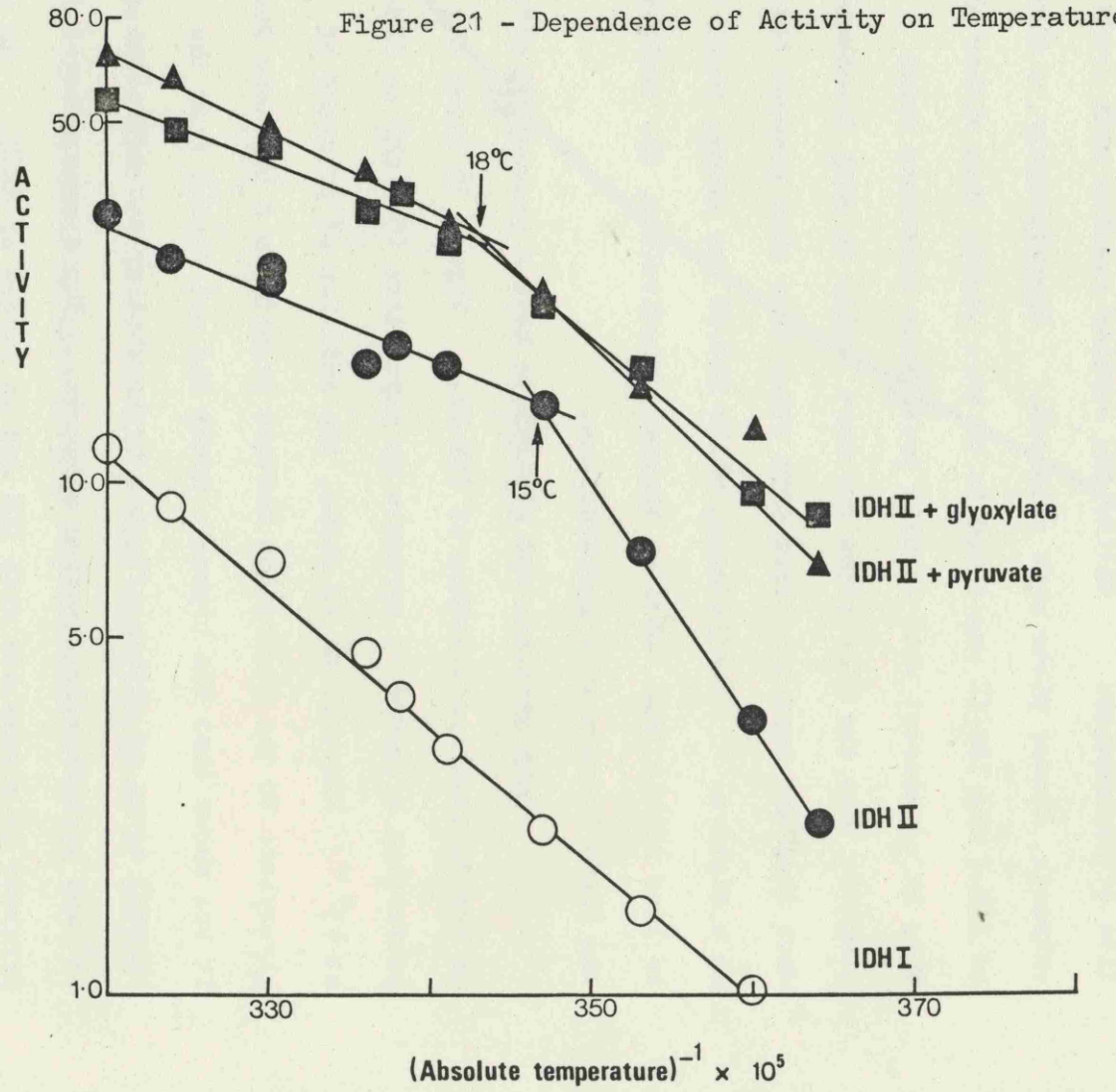
Figure 20 - Protection of IDH-II by high concentrations of sodium chloride against thermal inactivation

various times. After incubation, the enzyme solution was rapidly cooled and assayed for IDH activity at 25°. The logarithm of the measured activity was then plotted against the time of incubation. Unlike the earlier results with crude extracts, linear plots were obtained. Significantly, a mixture of IDH-I and IDH-II was found to be more stable than either alone. This is consistent with results previously described which indicate that the isoenzymes are more labile to many treatments when they are separated from each other than when present together in a mixture. Furthermore, while IDH-II was always found to be less stable than IDH-I to thermal inactivation, the stability was found to vary with preparations.

Both pyruvate and glyoxylate have a pronounced effect on the thermal inactivation of IDH-II. Figure 18 shows the protection afforded by pyruvate or glyoxylate (0.2 mM) to IDH-II at 43°. However, as is shown, the addition of pyruvate or glyoxylate to the incubation mixture results in a biphasic decay. It was shown that the biphasic decay did not result from the thermal decay of either effector by incubating the effectors at 43° and periodically removing aliquots. These were assayed for pyruvate or glyoxylate with LDH and an excess of NADH. As is shown in Figure 19 no decay of either effector was seen over the maximum period of incubation.

IDH-II is also protected against thermal inactivation

Figure 21 - Dependence of Activity on Temperature

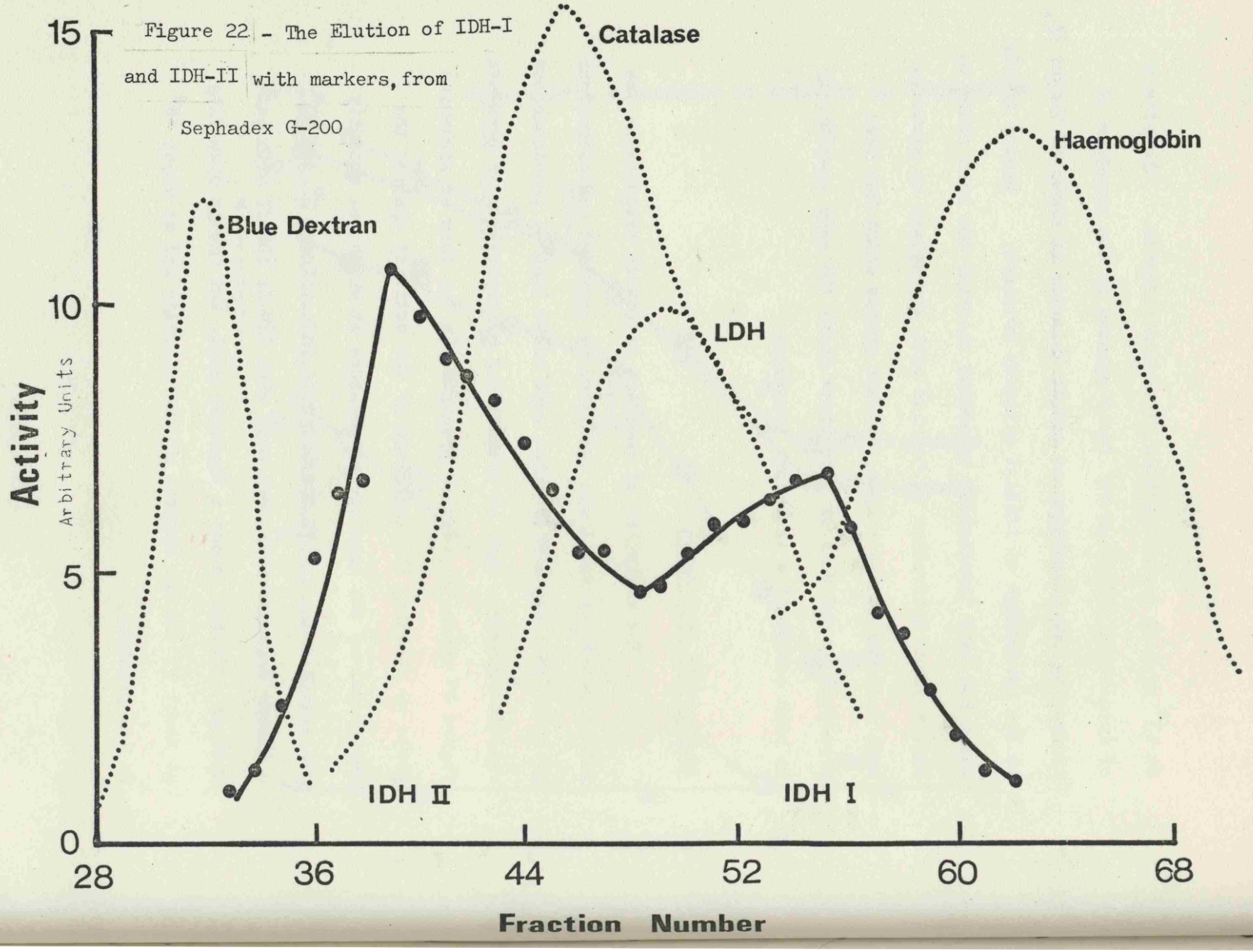


at 43° by high concentrations of sodium chloride. The effect of incubating IDH-II at 43° for 5 minutes in the presence of increasing concentrations of sodium chloride is shown in Figure 20, as the percentage of initial activity retained. Heated IDH-II which has been "protected" by sodium chloride has been found to show a lower activation (1.5-fold) with glyoxylate or pyruvate than does the native enzyme, whereas enzyme which has been protected by pyruvate or glyoxylate shows the same sensitivity to both metabolites as before treatment.

Temperature Dependence

The variation of reaction rate with temperature was investigated for reactions catalysed by constant concentrations of both IDH-I and IDH-II which were added last to pre-incubated reaction mixtures with or without 0.2 mM glyoxylate or pyruvate. Figure 21 shows the results presented in the form of Arrhenius plots by plotting the logarithm of the activity against the reciprocal of the absolute temperature at which the activity was determined. With measurements made between 2° and 40° a linear dependence is seen only with IDH-I; IDH-II with and without effector, shows a biphasic plot, the change occurring at about 15° in the absence of the effector and at about 18° in its presence.

Figure 22 - The Elution of IDH-I and IDH-II with markers, from Sephadex G-200



Molecular Size

(i) Gel Filtration

A column of Sephadex G-200 was prepared and equilibrated with MET 8 containing 0.1 M KCl. To 1 ml of a concentrated and partially purified extract of A. lwoffi prepared as described on page 38 to contain both isoenzymes, was added haemoglobin (4 mg), catalase (4 mg) and lactic dehydrogenase (0.2 mg). Blue dextran was run six ml in advance. Application of both solutions was carried out as previously described and the column further eluted with MET 8 containing 0.1 M KCl while 2 ml fractions were collected from the column until the haemoglobin had been eluted. The enzyme activities and proteins were determined as described uner "Methods". The elution pattern obtained is shown in Figure 22. IDH-II is seen to be excluded from the gel to a very much greater extent than IDH-I. IDH-II was eluted in advance of catalase, whereas IDH-I was eluted after lactic dehydrogenase but before haemoglobin.

(i) Zonal Ultracentrifugation

Zonal ultracentrifugation carried out in the manner previously described served to confirm the results of gel filtration - IDH-II sedimenting faster than IDH-I.

Electrophoretic Mobilities

The electrophoretic mobilities of IDH-I and IDH-II have been examined by:

(i) Polyacrylamide Gel Electrophoresis

Because the isoenzymes had not been purified to a sufficient degree of homogeneity it was not possible to indicate their positions in a gel after electrophoresis with the Amido-Schwartz protein stain because many sharp discrete bands resulted. Therefore, the gels were stained with a preparation which was selective for IDH (composition under "Methods"). While on occasions the stain did indicate the existence of two forms of IDH with distinct mobilities it gave very irreproducible results, the stain usually not developing after many hours at 35° in the dark. The isoenzymes did not seem to be sensitive to any consituent of the gel and changing from polymerising the gel with ammonium persulphate to photopolymerisation with riboflavin had no effect. The inclusion of 1 mM mercaptoethanol in both the gels and the electrophoresis buffers also did not improve the results.

When the gels were sliced after electrophoresis and the slices crushed in small volumes (0.5 ml) of MET 8 no IDH activity was detectable. It was, therefore, thought that the lack of activity might be due to inactivation during the long

Figure 23 - Electrophoresis of IDH-I and IDH-II on Cellulose-Acetate

period of electrophoresis (over 2 hr) and so a more rapid procedure was sought. This was provided by electrophoresis on cellulose-acetate.

(ii) Cellulose-acetate electrophoresis

Cellulose-acetate electrophoresis was performed with the Millipore Phoroslide apparatus (see "Methods"). This arrangement required electrophoresis to be carried out for only 20 minutes on a cellulose-acetate supporting matrix. After electrophoresis, the strips were stained with the same solution used with the polyacrylamide gels. As is shown in Figure 23 the existence of separated IDH activities was indicated.

When IDH-I and IDH-II were separately electrophoresed only one band resulted from either. The slower component of Figure 23 was found to have the same mobility as that of single IDH-I and the faster component as that of IDH-II.

Kinetic Studies

The dependence of the activity of each isoenzyme on the concentration of each substrate was studied at 25° with Mg^{2+} or Mn^{2+} as the divalent cation. The data are presented graphically in the double reciprocal form due to Lineweaver & Burk (1934).

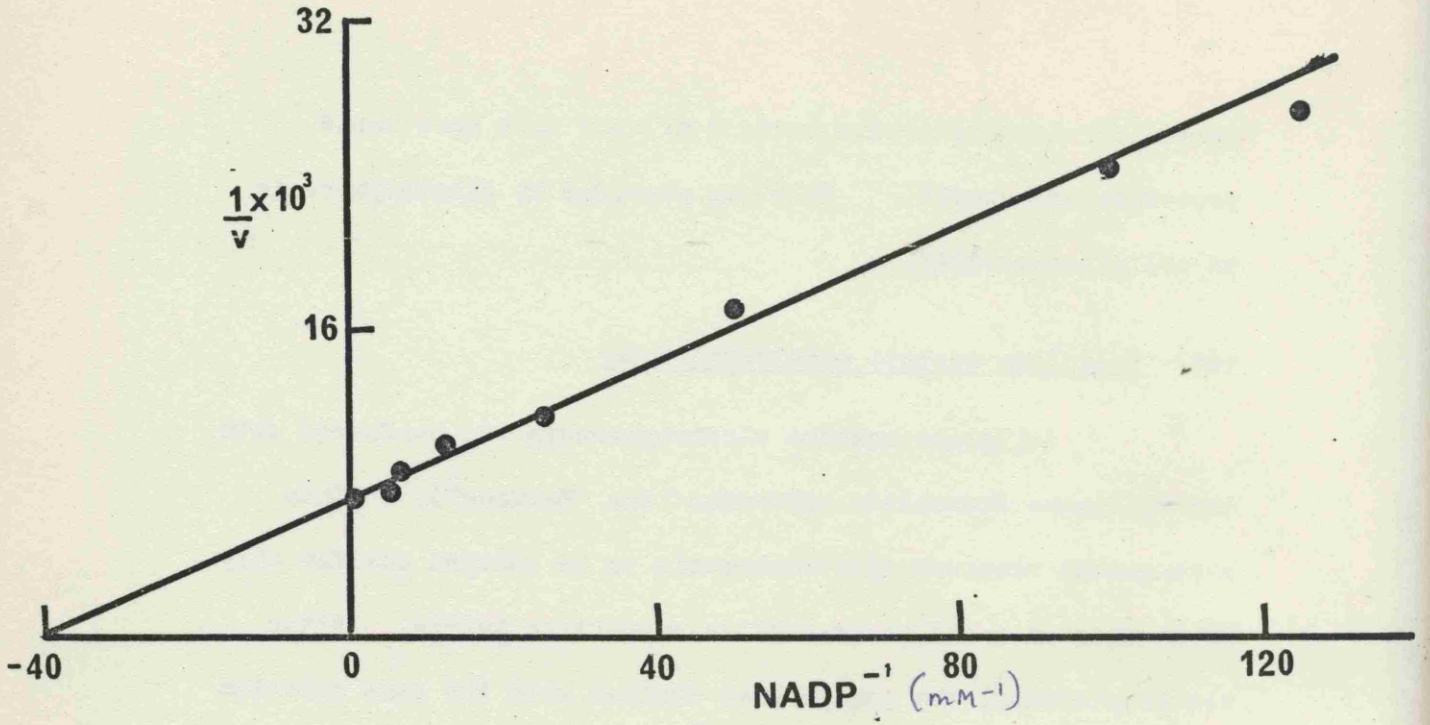


Figure 24 - Dependence of IDH-I with Mg^{2+} on NADP

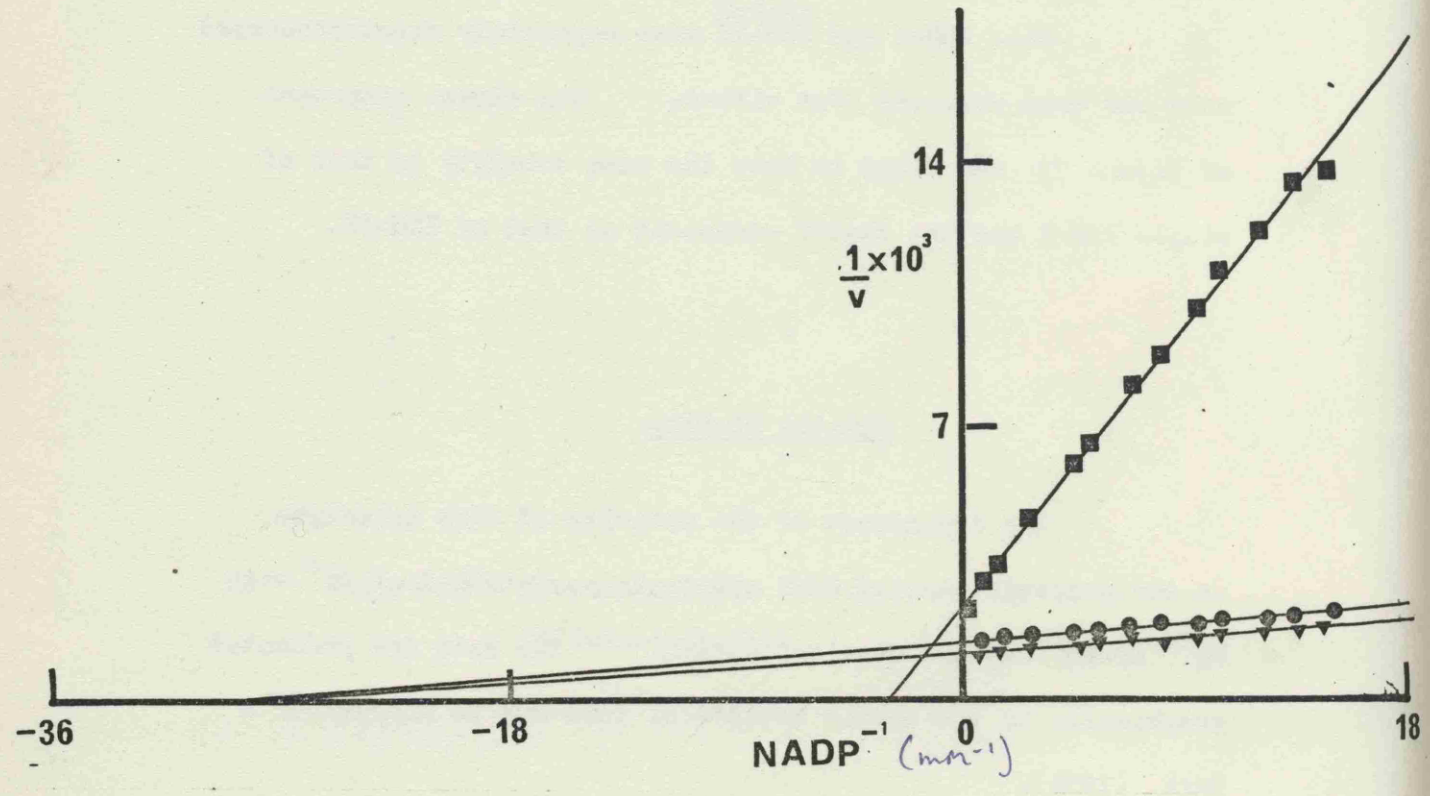


Figure 25 - Dependence of IDH-II with Mg^{2+} on NADP, with and without effector

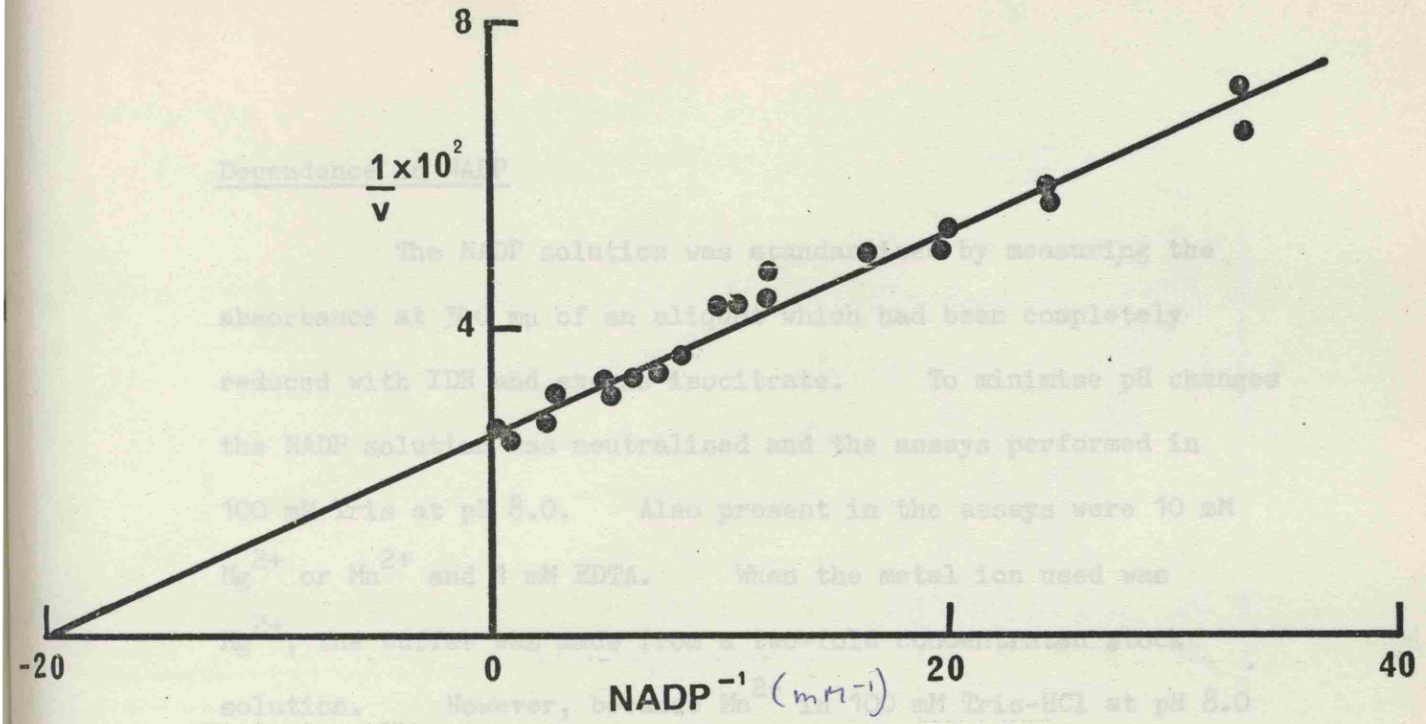


Figure 26 - Dependence of IDH-I with Mn^{2+} on NADP

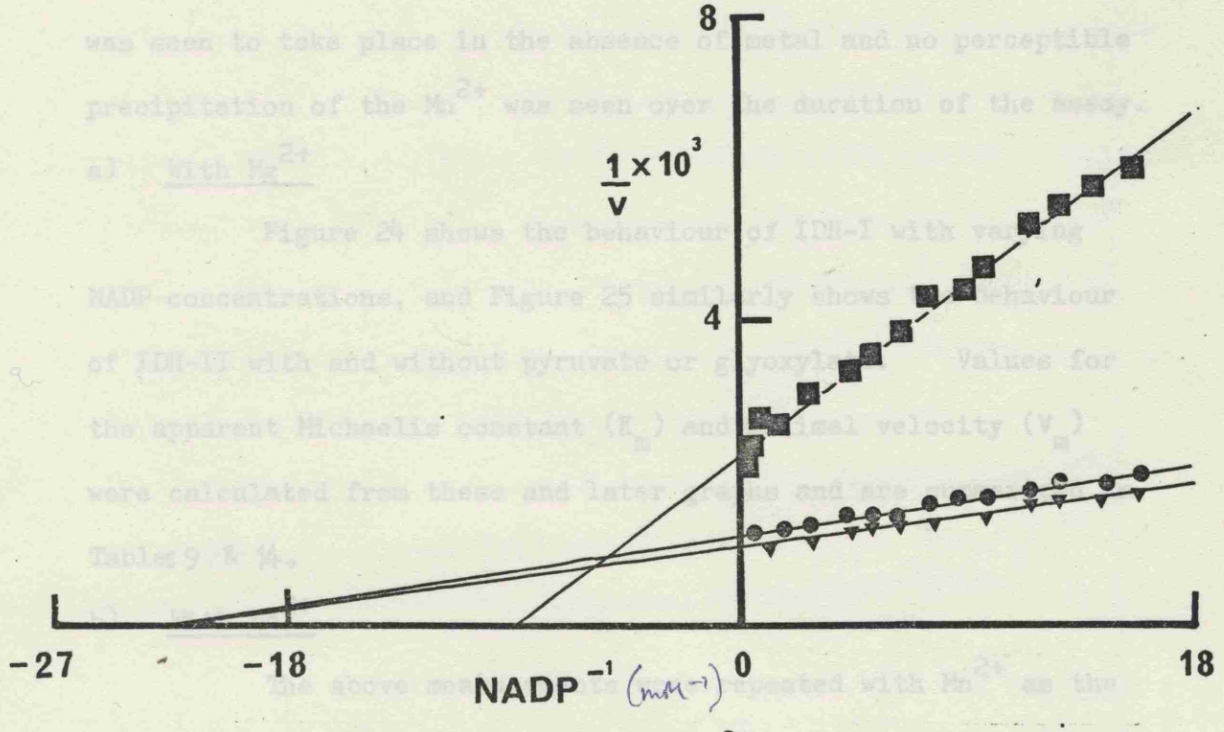


Figure 27 - Dependence of IDH-II with Mn^{2+} on NADP with and without pyruvate or glyoxylate

Dependence on NADP

The NADP solution was standardised by measuring the absorbance at 340 mμ of an aliquot which had been completely reduced with IDH and excess isocitrate. To minimise pH changes the NADP solution was neutralised and the assays performed in 100 mM Tris at pH 8.0. Also present in the assays were 10 mM Mg^{2+} or Mn^{2+} and 1 mM EDTA. When the metal ion used was Mg^{2+}, the buffer was made from a two-fold concentrated stock solution. However, because Mn^{2+} in 100 mM Tris-HCl at pH 8.0 and 25° rapidly precipitated as manganous hydroxide, a stock buffer without metal ion was used and reactions were initiated by the addition of Mn^{2+} to a final concentration of 10 mM. No reaction was seen to take place in the absence of metal and no perceptible precipitation of the Mn^{2+} was seen over the duration of the assay.

a) With Mg^{2+}

Figure 24 shows the behaviour of IDH-I with varying NADP concentrations, and Figure 25 similarly shows the behaviour of IDH-II with and without pyruvate or glyoxylate. Values for the apparent Michaelis constant (K_m) and maximal velocity (V_m) were calculated from these and later graphs and are summarized in Tables 9 & 14.

b) With Mn^{2+}

The above measurements were repeated with Mn^{2+} as the

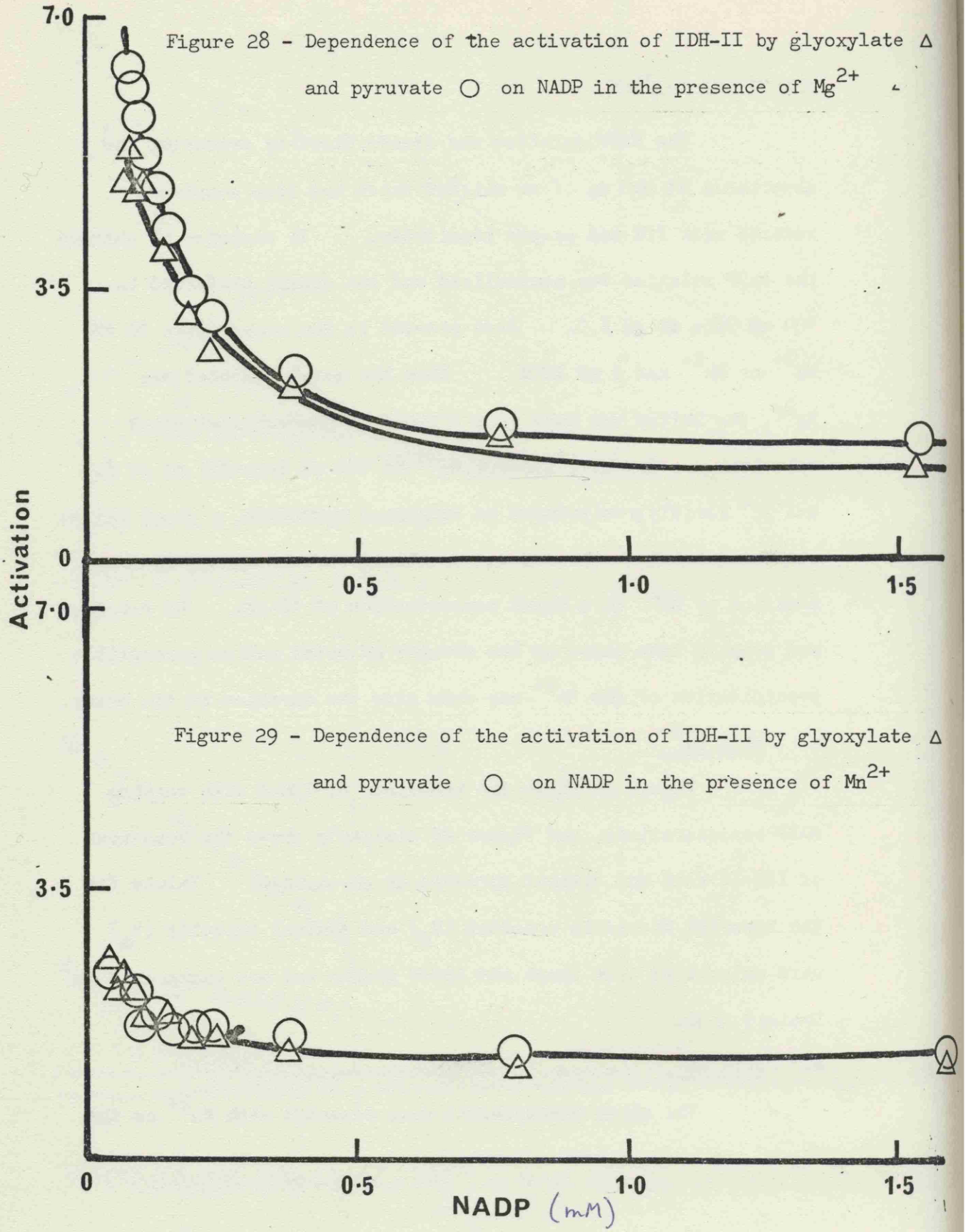


Figure 28 - Dependence of the activation of IDH-II by glyoxylate Δ and pyruvate ○ on NADP in the presence of Mg^{2+}

Figure 29 - Dependence of the activation of IDH-II by glyoxylate Δ and pyruvate ○ on NADP in the presence of Mn^{2+}

divalent cation; the results are shown in Figures 26 and 27.

The Effect of NADP on Activation of IDH-II

As shown in Figure 28, the activation of IDH-II by glyoxylate or pyruvate in the presence of Mg^{2+} falls with increasing NADP concentrations. Figure 29 shows that a similar effect is observed in the presence of Mn^{2+}. Therefore, when investigating the effect of glyoxylate or pyruvate on the K_m and V_m for isocitrate, it appeared desirable to conduct measurements with low concentrations ($\sim$ 0.2 mM) of NADP where IDH-II would be very sensitive to the effectors. However, with such low concentrations of NADP the K_m of IDH-II for isocitrate with Mg^{2+} and effector is much lower than the lowest concentration at which initial velocity measurements could be made. Therefore, determinations under these conditions were bound to have a very high experimental error. In view of this it was decided to carry out determinations with higher concentrations (1.0 mM) of NADP in an attempt to achieve greater accuracy, while losing some of the sensitivity to the effectors. However, to indicate that marked changes do occur on lowering the pyridine nucleotide concentration, certain measurements were repeated with 0.1 mM NADP.

Dependence on Isocitrate

Over one hour at room temperature, freshly-made isocitrate solution was found to decay in its capacity to reduce NADP with IDH to about 4/5ths of its initial activity. After such a time the lower activity of the solution was retained indefinitly. With IDH's of both bacterial and mammalian origin the effect was found to be absolutely reproducible with fresh solutions of isocitrate. Moreover, the effect was demonstrated to the same extent when the effective isocitrate concentration was measured with the enzyme isocitrate lyase.

No way was found of preventing this decay. Chilling the solution slowed down, but did not stop, the loss of isocitrate. However, as the products of the decay did not appear to affect the IDH activity, fresh solutions of isocitrate were aged overnight at room temperature prior to use and then assayed for isocitrate content with IDH and excess NADP. Their concentration did not then change appreciably during a series of measurements and thus their concentration could be determined accurately.

Isocitrate Dependence of Activity

a) With Mg^{2+}

As is shown in Figure 30, IDH-I with Mg^{2+} shows

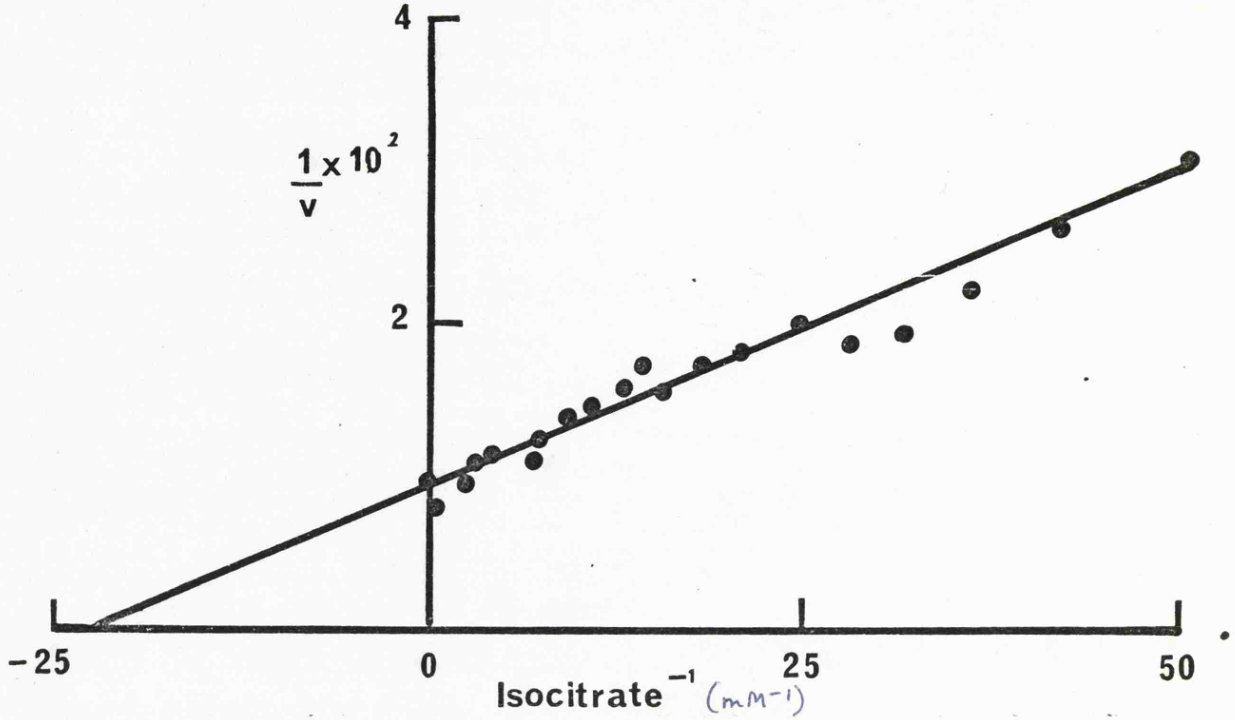


Figure 30 - Isocitrate dependence of IDH-I in the presence of Mg^{2+}

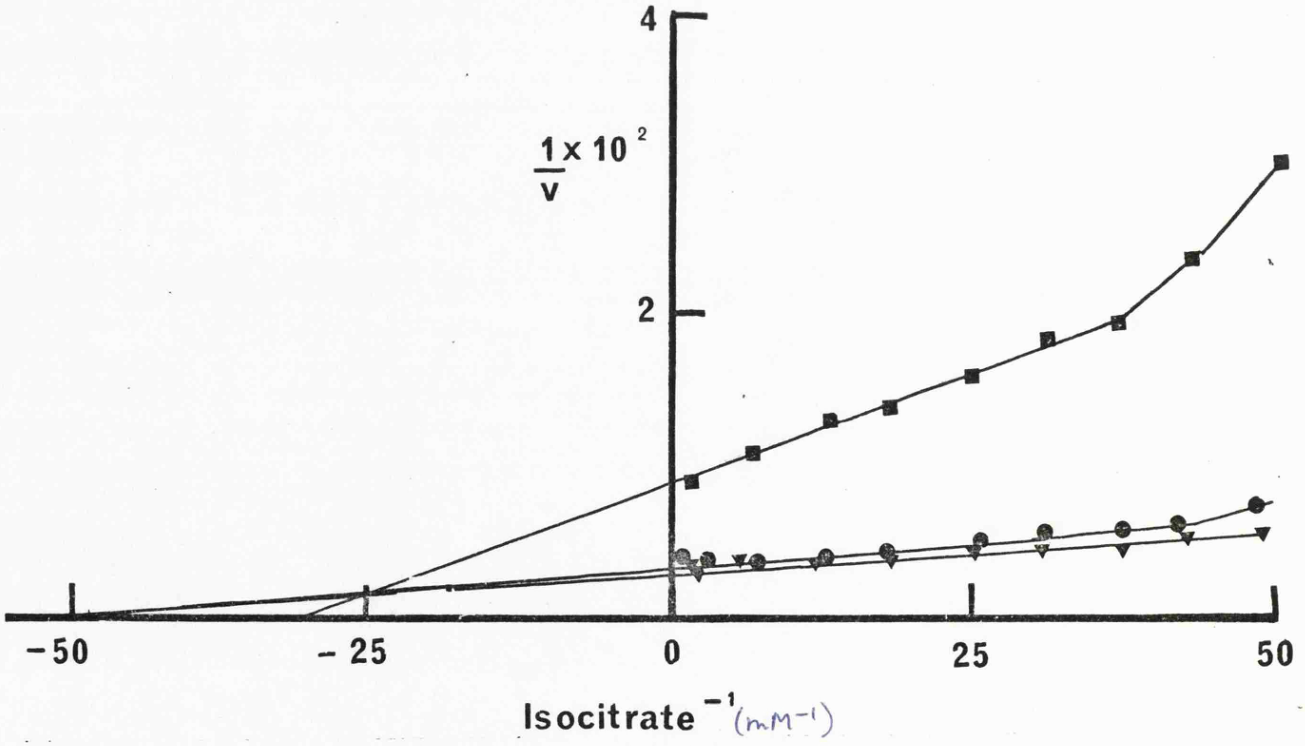


Figure 31 - Isocitrate dependence of IDH-II, with and without pyruvate or glyoxylate in the presence of Mg^{2+}

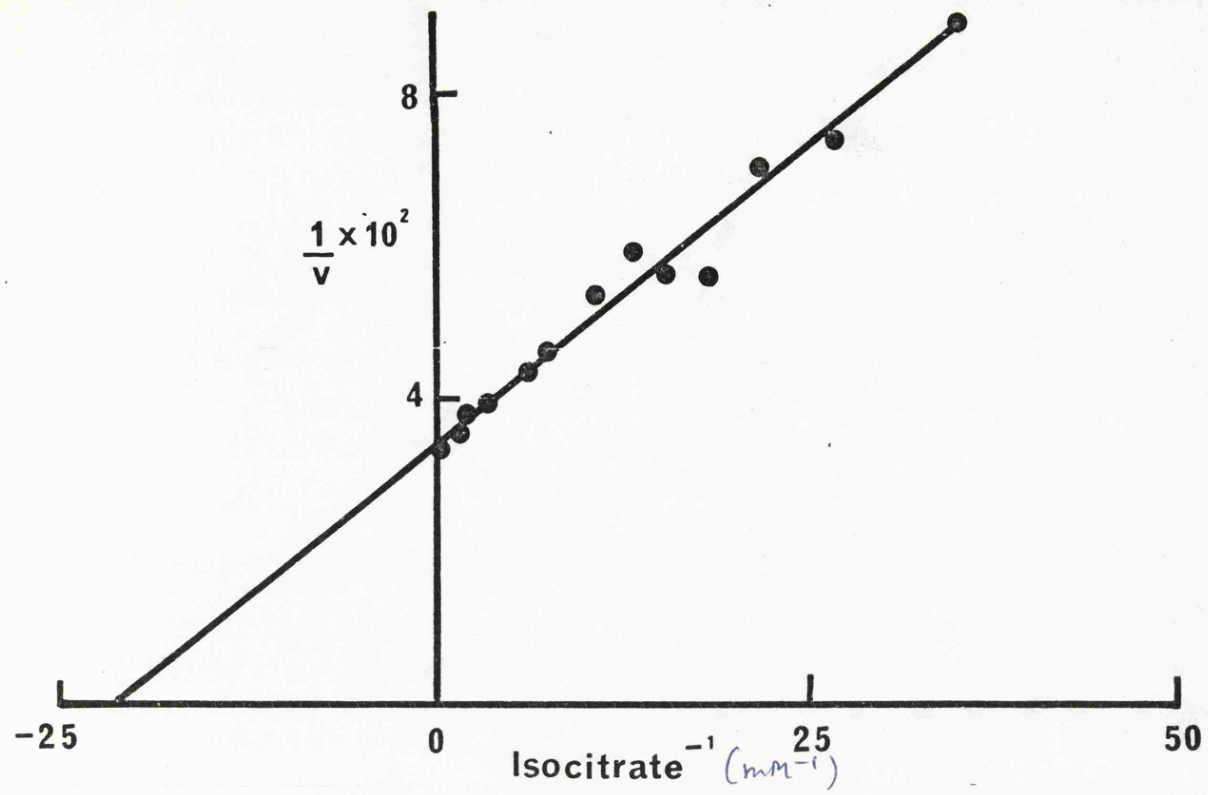


Figure 32 - Isocitrate dependence of IDH-II in the presence of Mn^{2+}

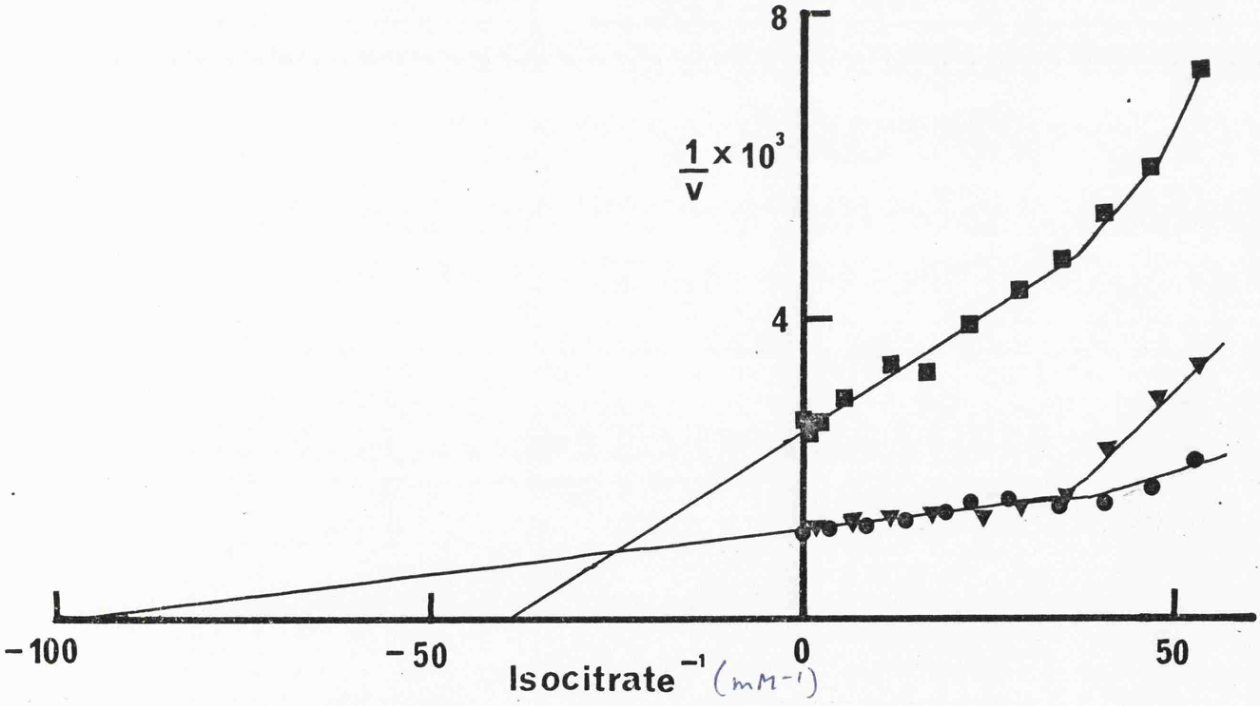


Figure 33 - Isocitrate dependence of IDH-II with and without pyruvate or glyoxylate in the presence of Mn^{2+}

Figure 34 - Effect of pyruvate or glyoxylate on the isocitrate dependence of IDH-II in the presence of Mg^{2+} and 0.1 mM NADP

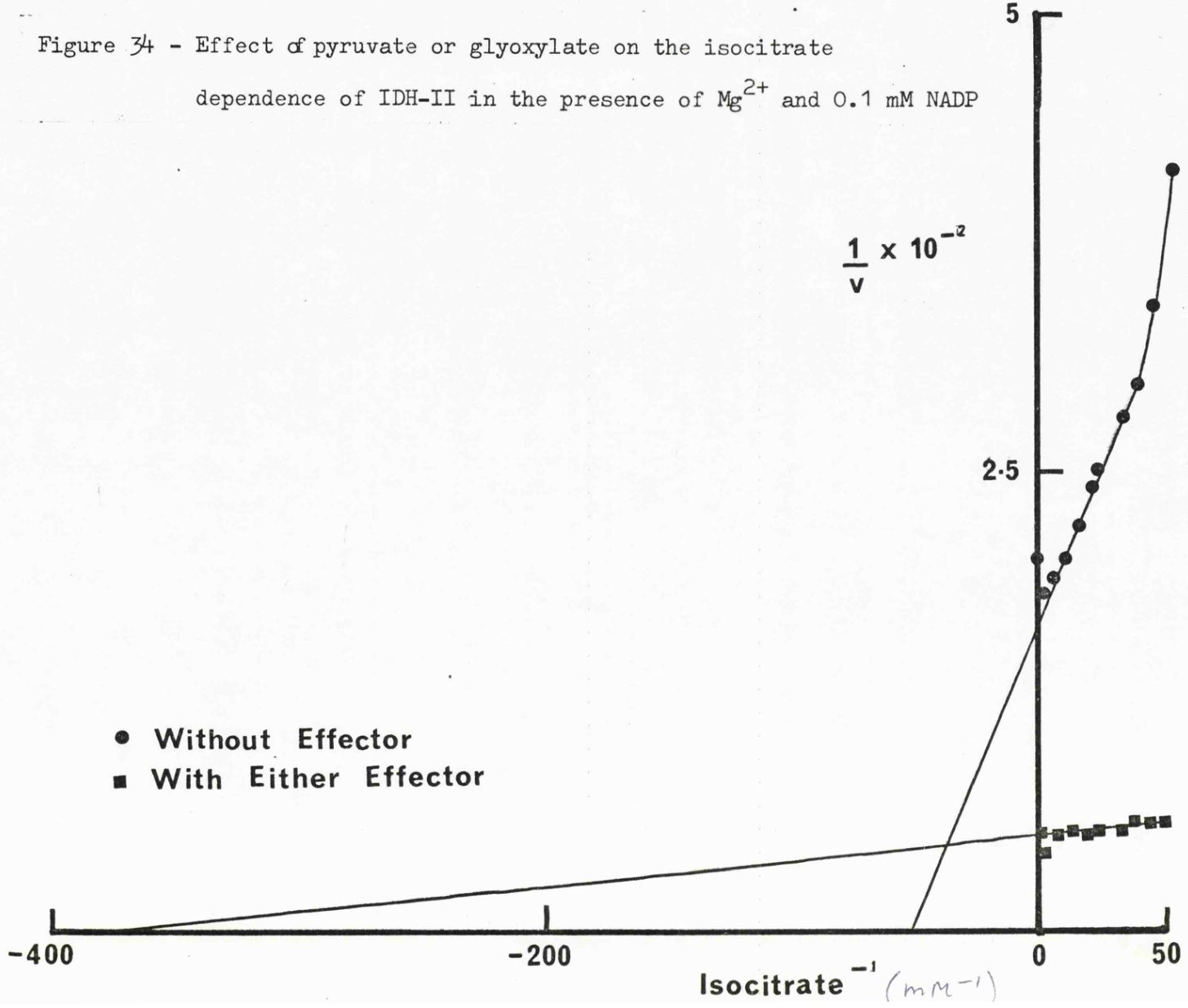
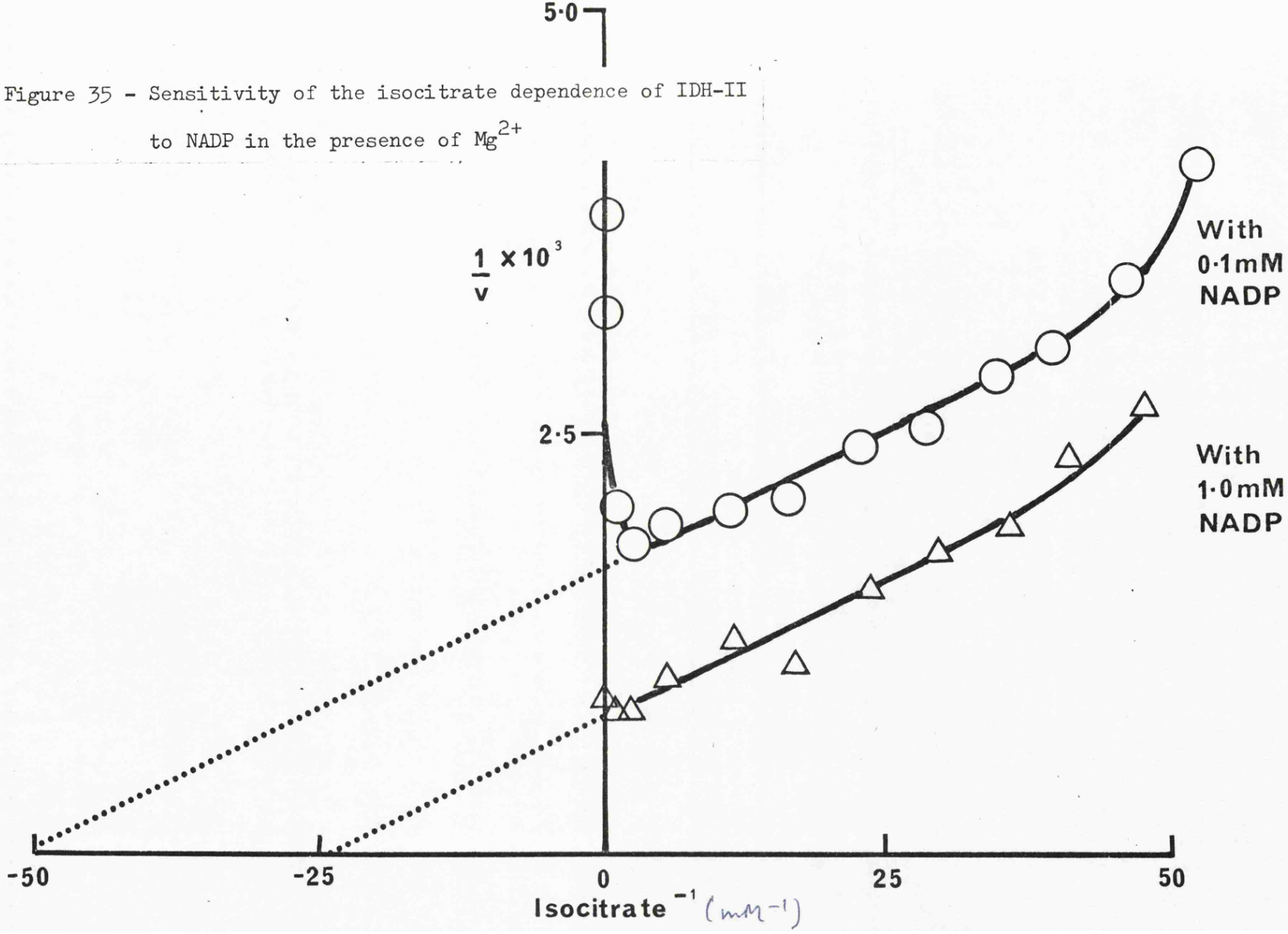


Figure 35 - Sensitivity of the isocitrate dependence of IDH-II to NADP in the presence of Mg^{2+}



"classical" Michaelis-Menten kinetic behaviour in response to varying isocitrate concentration. Essentially, the same behaviour was found for IDH-II with and without pyruvate or glyoxylate (Figure 31), the slight curvature of the reciprocal plot at low substrate concentrations most probably being introduced by limitations of the assay system.

b) With Mn^{2+}

Similar results as above were obtained with Mn^{2+} for the isocitrate dependence of IDH-I (Figure 32) and IDH-II (Figure 33).

IDH-II at low NADP concentration with Mg^{2+}

Figure 34 shows the marked change elicited by either pyruvate or glyoxylate on the isocitrate dependence of IDH-II with Mg^{2+} in the presence of only 0.1 mM NADP.

As is shown in Figure 35, the NADP concentration itself may be responsible for a certain type of kinetic behaviour. As well as the apparent K_m and V_m for isocitrate being different for the two concentrations of NADP, there is pronounced substrate inhibition with the lower concentration. However, the absence of this inhibition with higher NADP concentration may simply be a consequence of the relative concentrations of NADP to isocitrate.

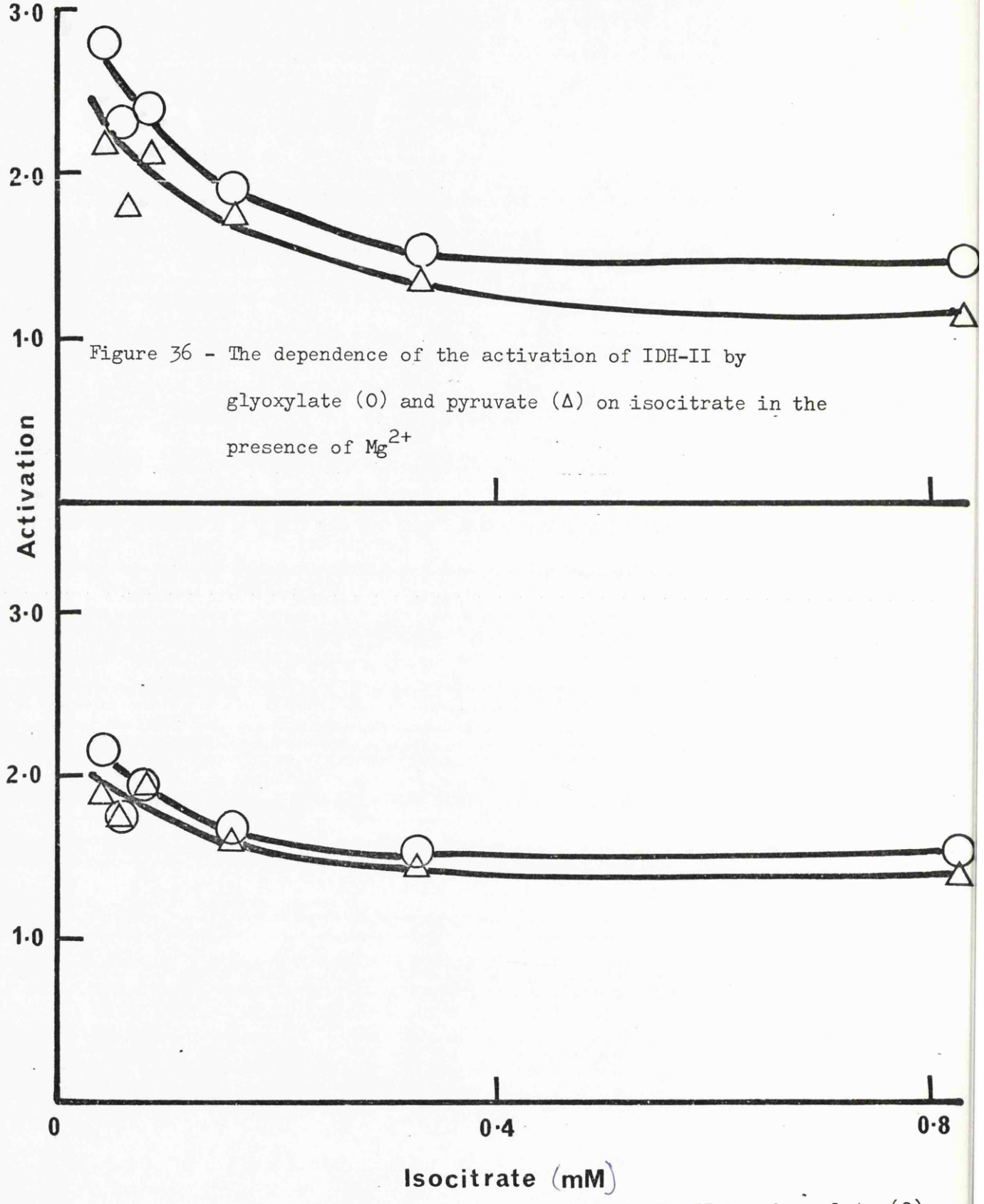


Figure 36 - The dependence of the activation of IDH-II by glyoxylate (O) and pyruvate (Δ) on isocitrate in the presence of Mg^{2+}

Figure 37 - The dependence of the activation of IDH-II by glyoxylate (O) and pyruvate (Δ) on isocitrate in the presence of Mn^{2+}

TABLE 9

The apparent K_m and V_m values of IDH-II for its substrates, with and without pyruvate or glyoxylate.

Substrate	Metal ion	Effector	K_m	V_m
NADP	Mg^{2+}	-	3.1×10^{-1} mM	445
"	"	pyruvate	3.5×10^{-2} "	820
"	"	glyoxylate	3.2×10^{-2} "	870
"	Mn^{2+}	-	1.0×10^{-1} "	425
"	"	pyruvate	4.9×10^{-2} "	950
"	"	glyoxylate	4.9×10^{-2} "	925
Isocitrate	Mg^{2+}	-	3.4×10^{-2} "	114
"	"	pyruvate	1.25×10^{-2} "	290
"	"	glyoxylate	1.4×10^{-2} "	264
"	Mn^{2+}	-	2.5×10^{-2} "	392
"	"	pyruvate	1.1×10^{-2} "	1000
"	"	glyoxylate	1.1×10^{-2} "	950

Assays for the isocitrate dependence were here conducted with 1.0 mM NADP and assays for the NADP dependence were conducted with 2 mM isocitrate

Dependence of IDH-II Activation on Isocitrate

The stimulation of IDH-II by either pyruvate or glyoxylate in the presence of Mg^{2+} or Mn^{2+} was found to increase with decreasing isocitrate concentration (in a similar manner as with NADP) when assays were conducted with 1 mM NADP (Figures 36 and 37).

Table 14 shows the parameters K_m and V_m calculated from the above data.

Physiological Role

The following experiments were designed to probe the physiological role of the activation of IDH-II and also of the occurrence of the two isoenzymes.

As IDH-II seems to be both a more complex and larger molecule than IDH-I, it is tempting to speculate that IDH-I may merely be a break-down product of IDH-II. The breakdown could have resulted either from a physiological process *in vivo* or as a consequence of extraction from the cell. As the latter would not be of physiological importance, it was necessary to see if it exerted any influence on the production of IDH-I.

Because both crude extracts of *A. lwoffi* and purified

TABLE 10

The Effect of Sonication on IDH-II

	Activation	
	Before	After
Crude extract (Mn^{2+})	0.4	0.4
Purified II (Mg^{2+})	3.6	3.5

IDH-II did not lose any sensitivity to glyoxylate or pyruvate on standing, spontaneous breakdown, *in vitro*, of IDH-II to give IDH-I was discounted.

This was also shown by the lack of appearance of "extra" IDH-I when techniques which resolve the isoenzymes were used in series on IDH-II, as with the large-scale preparations. However, it was still necessary to demonstrate that the ultrasonication regularly used to break the cells did not cause the formation of IDH-I from IDH-II.

i) Other methods of cell disruption

Treatment of *A. lwoffi* with either toluene or lysozyme + EDTA (Repaske, 1958) gave insufficient IDH activity to perform accurate experiments. The use of a French pressure cell, however, gave an extract with the same properties as found after sonication.

ii) Sonication of IDH-II

Both a cell-free crude extract of A. lwoffi and a sample of partially purified IDH-II were subjected to further ultrasonication for 5 minutes. As shown in Table 10, there was no change in sensitivity to

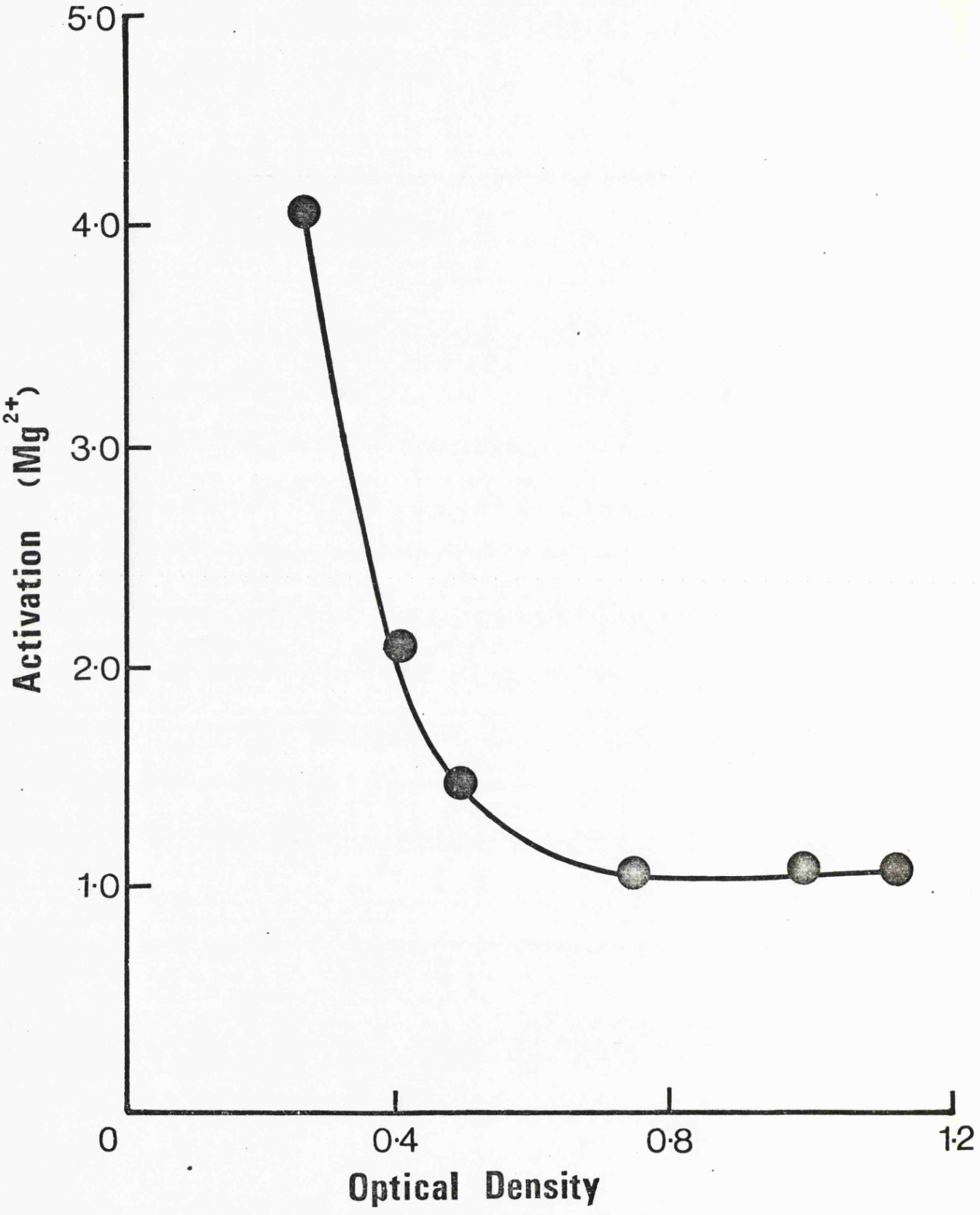


Figure 39 - Variation of the IDH-I : IDH-II ratio during growth

glyoxylate.

Therefore, it seems probable that the isoenzymes exist *in vivo* in the sort of proportions that have been found *in vitro*. Having established this I investigated the ratio of IDH-I to IDH-II of cells grown and harvested under a variety of conditions.

i) The Ratio of Isoenzymes during growth

Acetate culture medium (1 litre) was inoculated with organisms from an agar slope and aerated at 30° by shaking. Aliquots were taken during growth, harvested, washed and resuspended in MET 8 to the same cell density which on 30-fold dilution had an O.D. at 680 mμ of 0.35. Two-ml samples were sonicated at maximum power with a small probe for 1½ minutes. Figure 39 shows how the glyoxylate sensitivity of the IDH activity of these extracts varied with the growth of the culture (expressed as the O.D. at 680 mμ at which the aliquots were taken).

An initial decay was apparent. Such a decay in activation with growth would be consistent with IDH-II being broken down *in vivo* to IDH-I. However, the effect was not seen when cells were taken from an actively growing culture and greatly diluted into fresh media. The effect, therefore, seems to be specifically related to recently dormant cells and seems not to be of general importance.

ii) Growth on Various Substrates

In an attempt to obtain some insight into the physiological nature of the activation of IDH-II *A. lwoffi* was grown on a variety of carbon sources. Extracts of the organisms from these sources were made by sonication and centrifugation and the activation of the IDH activity by 0.2 mM glyoxylate examined. Assays were carried out with Mn^{2+} to offset possible interference by ICL. From the results of the growth studies, shown in Table 11, it was seen that within the limits of experimental variation, the ratio of IDH-I to IDH-II did not change appreciably with growth on a variety of carbon sources.

Citrate, aspartate, glycine and glucose did not support the growth of this organism.

iii) Effect of Sodium Chloride

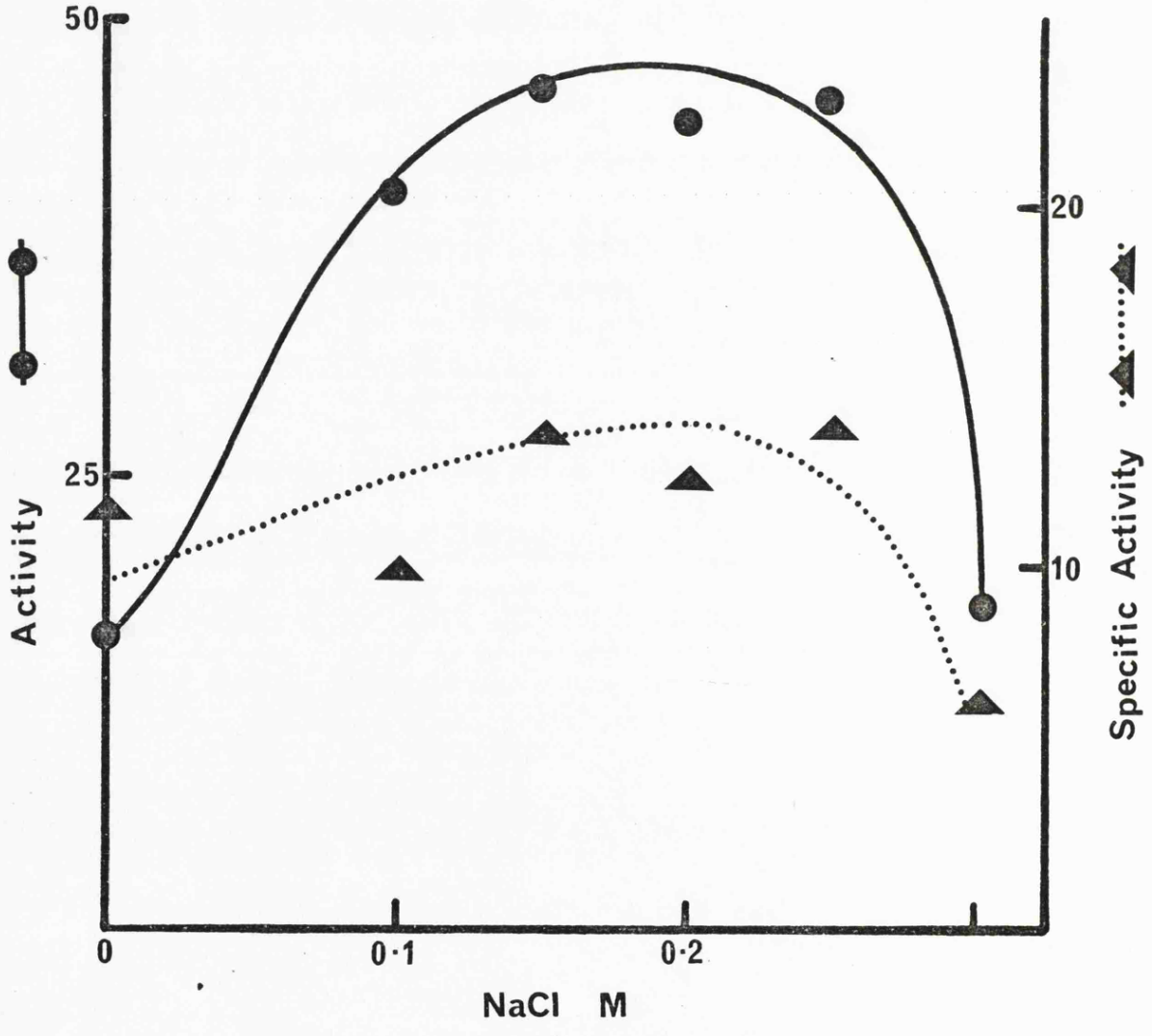
Vorgues & Brison (1966) stated that *A. lwoffi* may be

TABLE 11

The effect of carbon source on the glycxylate sensitivity of the IDH activity produced

Substrate	Activation (Mn^{2+})
Nutrient	0.5
Acetate	0.3
Gluconate	0.3
Succinate	0.3
Glutamate	0.4

Figure 40 - The Effect of sodium chloride in the growth medium of A. lwoffi on the total IDH produced



classified as a "moderate halophile". These authors further described experiments which suggested that the presence of 0.15-0.2 M sodium chloride in the growth medium and/or the sonication buffer greatly enhanced the IDH activity of subsequent extracts - the activity in the absence of NaCl being "very low". Because of the possible physiological significance and preparative value of such an effect, I investigated it with our strain of A. lwoffi.

a) Growth Experiments

Effect on the total IDH Synthesis

A.lwoffi was grown at 30° in 200 ml batches of "salts-acetate" medium containing different concentrations of sodium chloride. Sodium chloride was seen to have no effect on the generation time of the organism. The cells were harvested towards the end of logarithmic growth, washed with MET 8 and each batch resuspended to a fixed cell density in MET 8, and sonicated for 2 minutes. The sonicates were then centrifuged for fifteen minutes at 25,000 g and the supernatants carefully decanted. IDH was assayed in MnET 8 and protein was estimated by the Folin method. Figure 40 shows both the change in activity and specific activity (expressed in the usual units) with sodium chloride concentration, A maximum of both activity and specific activity is seen around 0.15 M NaCl.

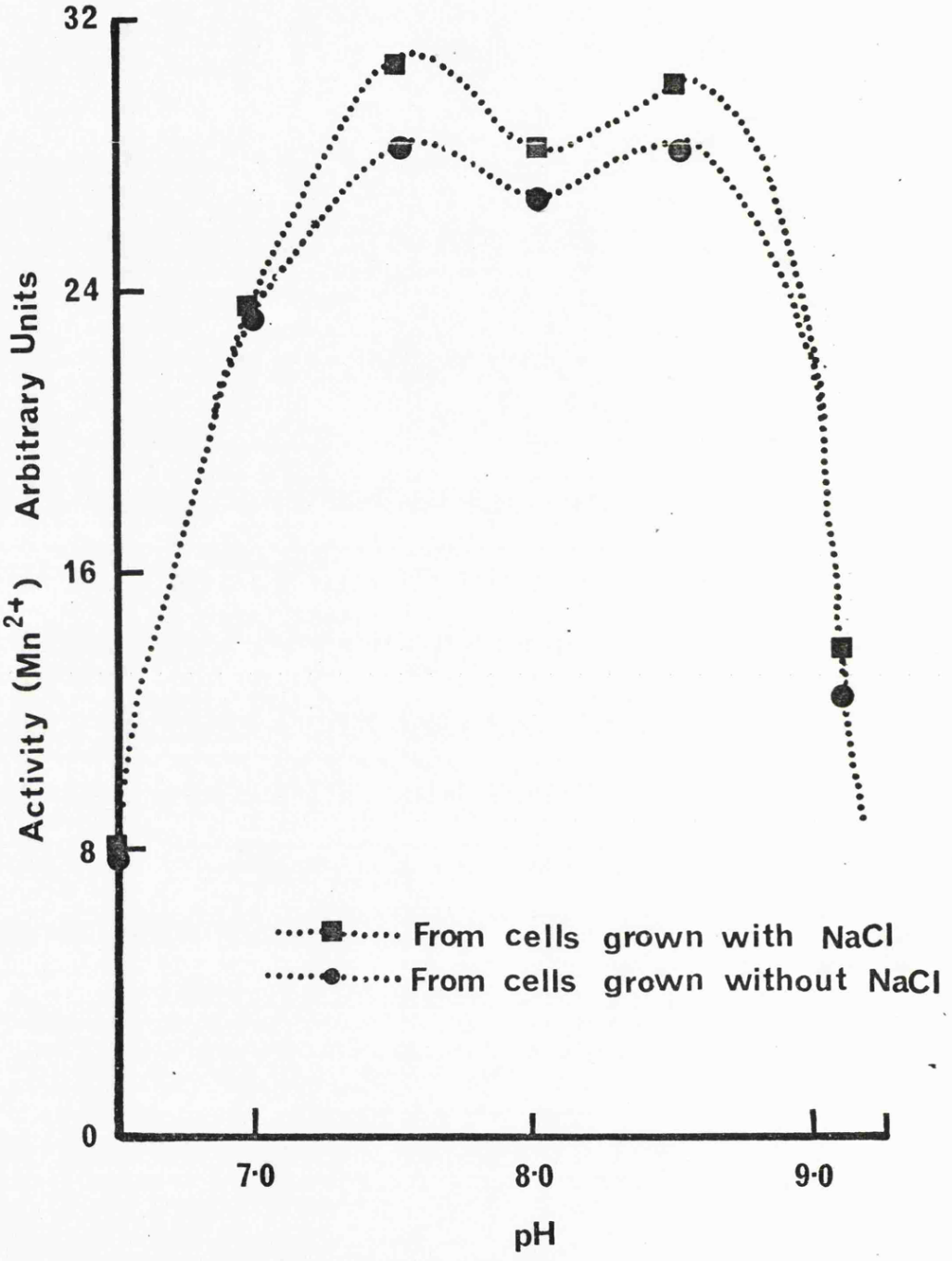


Figure 41 - The pH profiles of extracts of A. lwoffi grown with and without sodium chloride

Similar results were obtained with cells which were grown on nutrient-broth with added sodium chloride.

Effect on the Ratio of IDH-I to IDH-II

As IDH-I and IDH-II have distinct pH profiles, with different optimum pH values the relative proportion of IDH-I to IDH-II in mixtures of the two may be gauged from a pH profile of the mixtures. To see if the presence of NaCl in the growth medium affected the relative proportion of the isoenzymes, A. lwoffi was grown in "salts-acetate" medium with and without 0.2 M NaCl. The cultures were harvested, washed, resuspended in MET 8 to the same density and sonicated for 2 minutes. The extracts were centrifuged as before and the pH dependence of IDH activity in both of the extracts examined in MnET 8. The results are depicted in Figure 41. It appears that the presence of NaCl does not affect the proportion of IDH-I to IDH-II. This result was supported by the activation by 0.2 mM glyoxylate of the IDH activities of the two extracts. As is shown in Table 12, the stimulation of IDH activity is identical in extracts from cells grown with and without sodium chloride.

b) Effect on Sonication

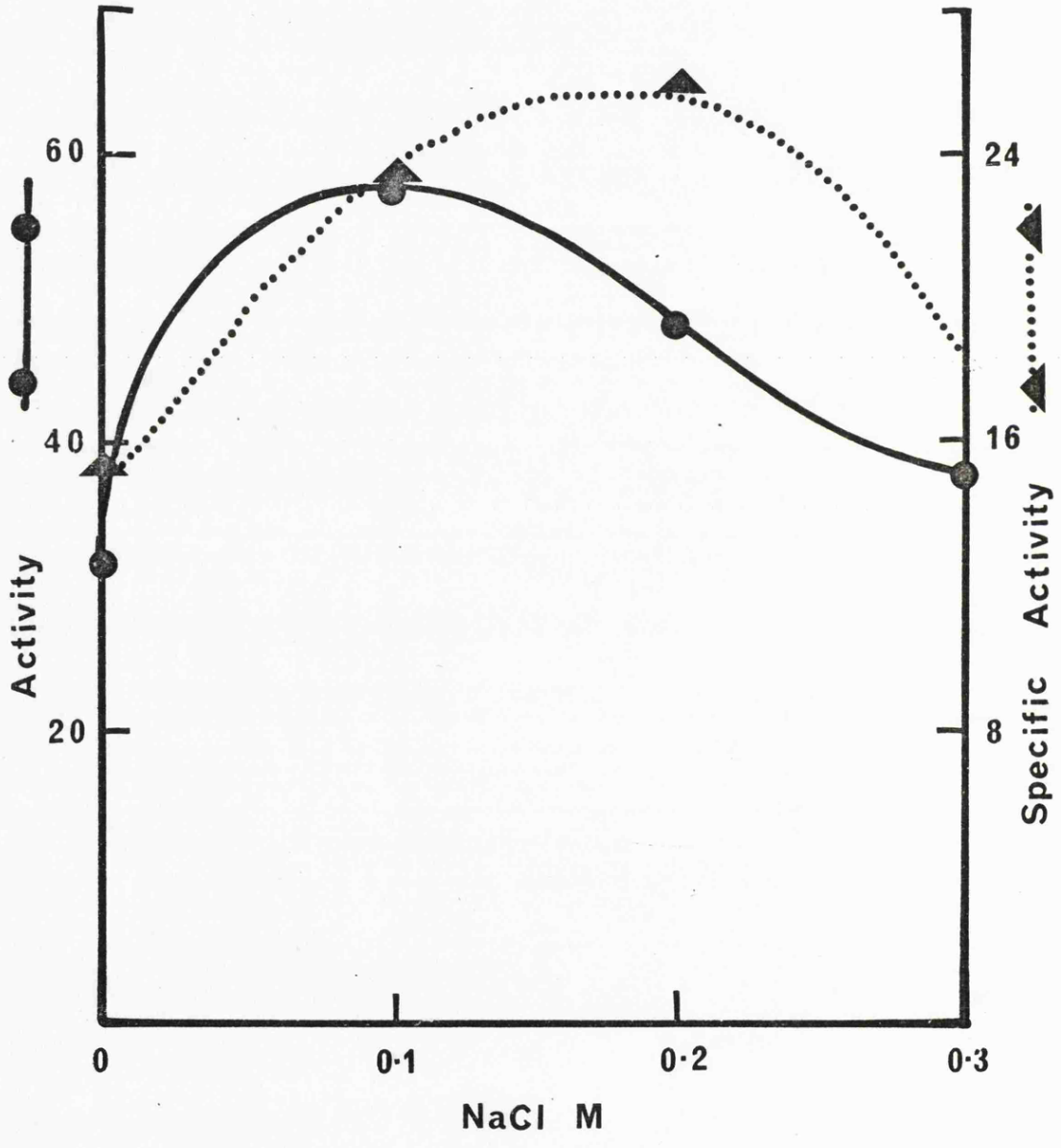
A 1 litre culture of A. lwoffi grown on "salts-acetate"

TABLE 12

The effect of sodium chloride on the glyoxylate sensitivity of the IDH activity produced

Extract from cells	Activation (Mn^{2+})
i) Grown without NaCl	0.3
ii) Grown with 0.2M NaCl	0.3

Figure 42 - Effect of the presence of sodium chloride during sonication on the total IDH liberated



medium (without added sodium chloride) was aerated at 30°, the cells harvested towards the end of logarithmic growth, washed and resuspended to a thick suspension in MET 8. This was divided into five equal portions which were then diluted to the same final optical density (at 680 mμ) with solutions of MET 8 of varying NaCl concentrations to give five suspensions with final sodium chloride concentrations of 0 to 0.4 M. These were separately sonicated for 2 minutes and centrifuged at 25,000 g for 15 minutes. Figure 42 shows the results of this experiment. Again the addition of certain concentrations of sodium chloride appear to give higher yields of IDH. Similar results were also obtained from cells grown on nutrient broth.

The Occurrence of IDH-II

A survey was conducted to investigate the incidence

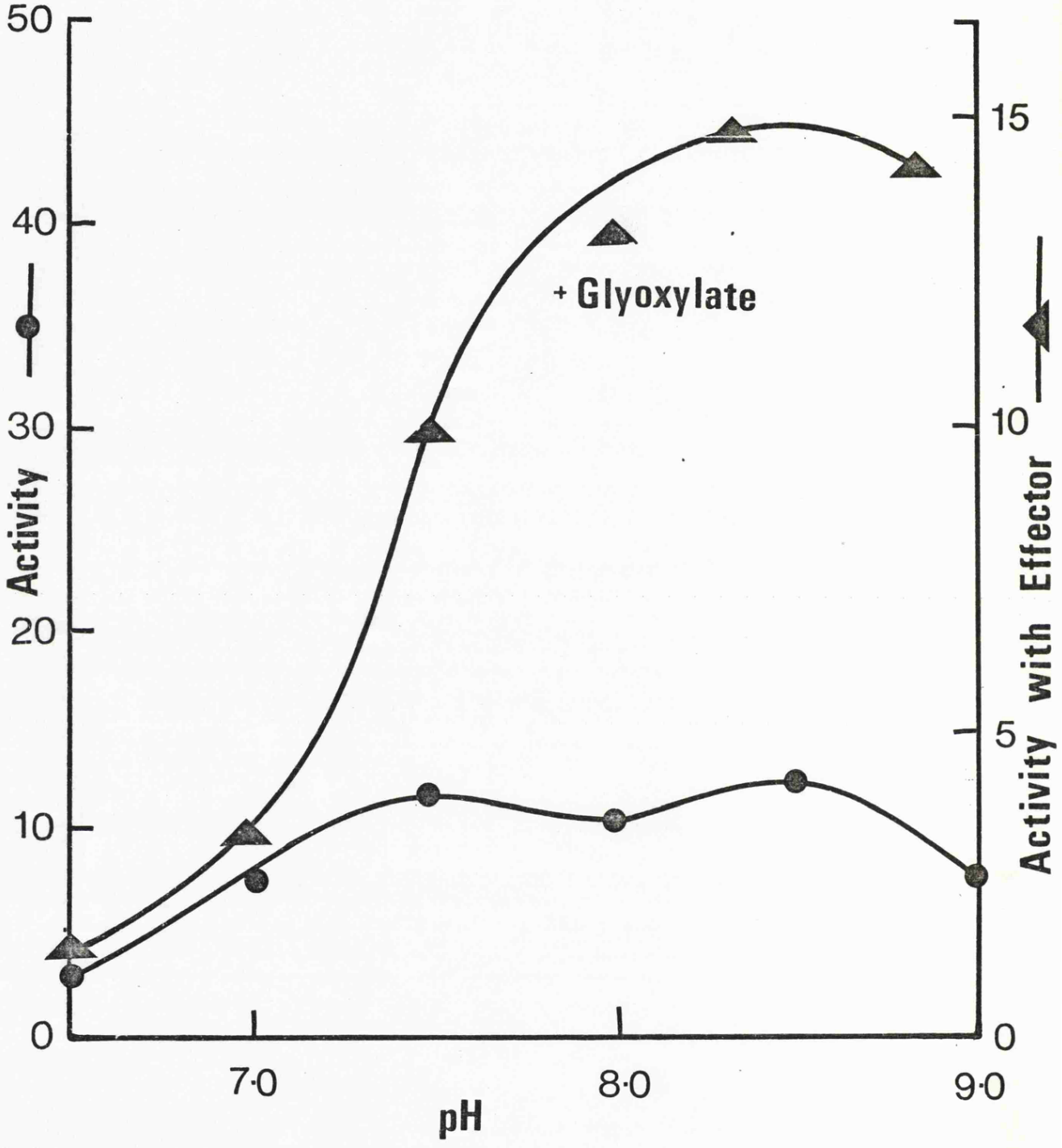


Figure 43 - pH profiles of the IDH of Mima polymorpha with and without glyoxylate

TABLE 13

The incidence of glyoxylate activatable IDH among bacteria

Organism	Strain	Effect of glyoxylate
Gram-negative		
Mima polymorpha	ATCC 9957	✓
Moraxella calcoacetica	73*	✓
Moraxella sp. (Vibrio O-1)	94*	✓
Pseudomonas aeruginosa	NCIB 8295	✓
Pseudomonas ovalis	NCIB 8296	X
Pseudomonas fluorescens	CCEB 488	X
Pseudomonas fluorescens	D 1086*	X
Pseudomonas fluorescens	D 1087	X
Azotobacter vinelandii	NCIB 8660	X
Proteus vulgaris	NCTC 4175	X
Serratia marcescens	NCTC 10211	X
Serratia marcescens	D 106*	X
Aerobacter aerogenes	NCTC 10006	X
Arizona arizonae	NCTC 8297	X
Salmonella anatum	Uetake 293*	X
Gram-positive		
Bacillus megaterium*		X
Kurthia zopfii	C5*	X

(✓ - positive X - negative)

* Cultures from the culture collections of MRC Microbial Systematics Research Unit and the Department of Biochemistry, University of Leicester.

of activatable IDH in a variety of bacteria. The results of this survey are presented in Table 13. A few other organisms were found to produce IDH which was activated by glyoxylate. Furthermore, on testing the dependence of the IDH activity on pH of one of these - Mima polymorpha - it was found to have the same dependence as that of A. lwoffi, with two maxima. (Figure 43) Therefore, it would appear that the production of isoenzymes like IDH-I and IDH-II is not confined to A. lwoffi.

The Singular Existence of IDH-I

The IDH activity of E. coli grown on acetate resisted all fractionation attempts and was not stimulated by either glyoxylate or pyruvate. To see how the A. lwoffi enzymes compared in molecular size with the IDH from E. coli similarly prepared extracts from both organisms were subjected to gel filtration on Sephadex G-200 with protein markers.

The extracts were separately applied to the gel column which was thoroughly washed between the applications by the passage of about 3 column volumes of buffer. Application, elution and subsequent enzyme and protein assays were conducted as previously described.

Figure 45 shows the elution patterns obtained. It is apparent that IDH-I and the IDH from E. coli were not

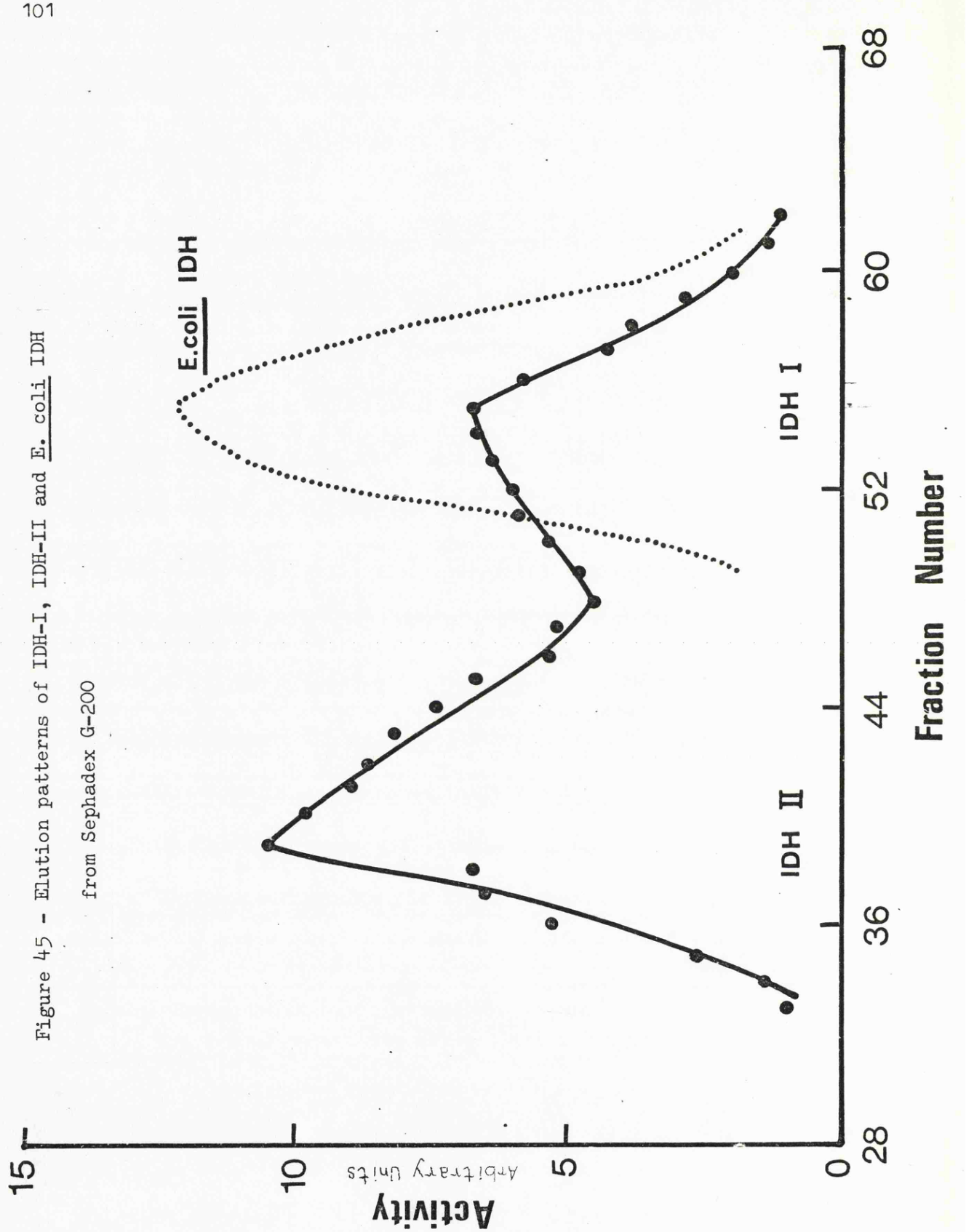


Figure 45 - Elution patterns of IDH-I, IDH-II and E. coli IDH from Sephadex G-200

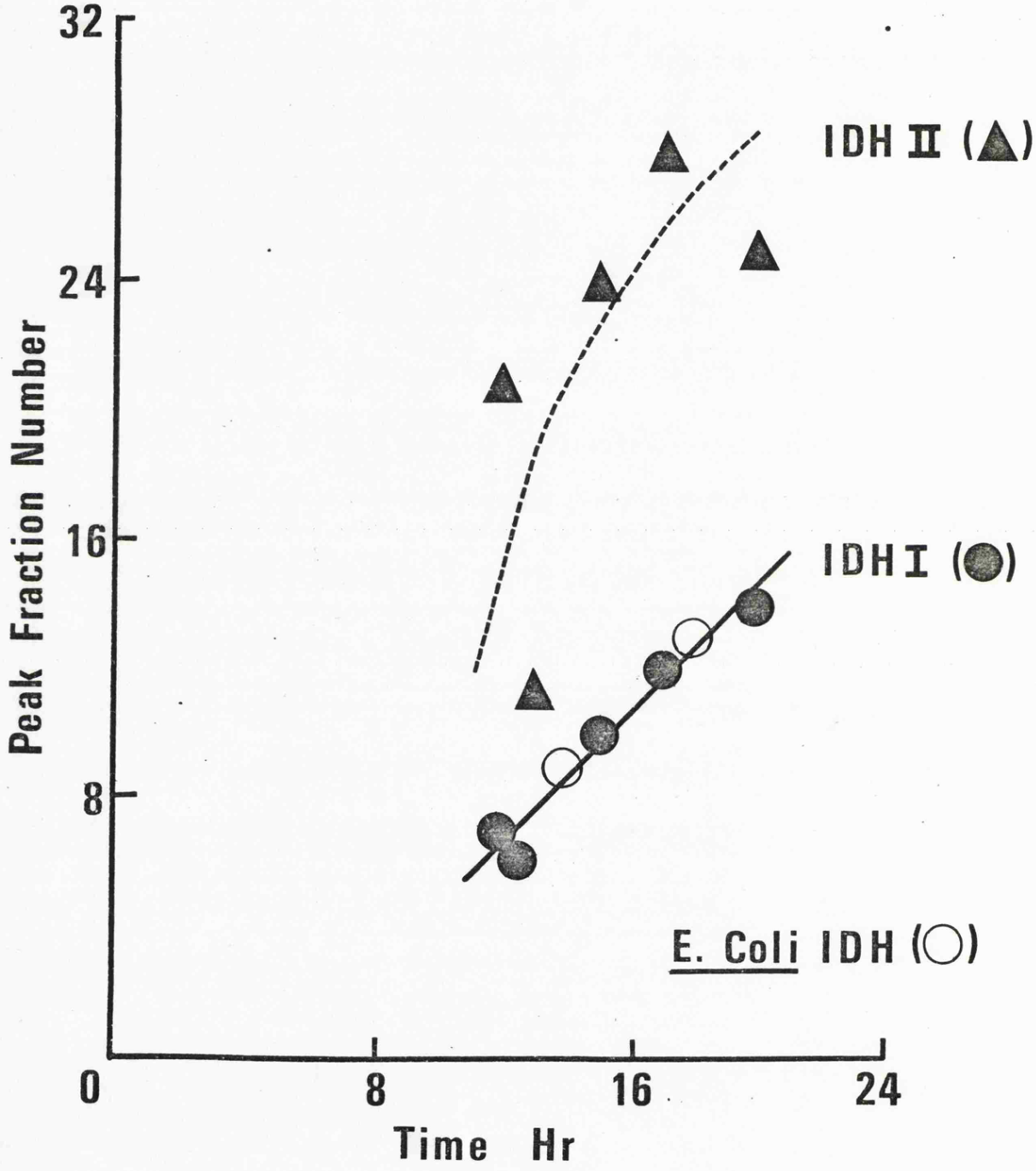


Figure 46 - Sedimentation of IDH-I, IDH-II and E. coli IDH in the zonal ultracentrifuge

separated by Sephadex G-200 in their elution volumes, even though these are in a range in which good resolution may be expected with this gel.

The similarity of the two enzymes, gauged by gel filtration, prompted a comparison of their sedimentation in the zonal ultracentrifuge. The many sedimentation experiments employing a linear 5-20% w/w sucrose density gradient made up in 5 mM Tris-HCl, 1 mM EDTA at pH 8.0 and carried out at 10^{o} in the B XIV zonal rotor at 47,000 rpm have allowed the sedimentation behaviour of the isoenzymes to be characterised and used as a "finger-print". When the time of sedimentation of IDH-I was plotted against the position of the peak activity in the fractions, a linear dependence was observed. Moreover, similar plots with the IDH of E. coli showed that this species had similar sedimentation properties to IDH-I (Figure 46).

DISCUSSION

DISCUSSION

My preliminary studies confirmed the original findings of Dr. P.D.J. Weitzman. It was evident that glyoxylate, at low concentration, activated the isocitrate dehydrogenase activity of crude extracts of *Acinetobacter lwoffi* and furthermore, offered some protection against inactivation by urea or heat. Moreover, it was found that pyruvate had a similar effect, both increasing the IDH activity and protecting it against these inactivating treatments.

However, the preliminary results were complex and suggested that the extracts might contain multiple IDH activities or agents which interfered with IDH assays. Because of this, purification of the IDH activity was undertaken.

Identification and Separation of the Isoenzymes

i) Fractionation with Ammonium sulphate

The initial fractionation was performed by selective precipitation with ammonium sulphate. A partial separation was achieved - the fractions showing a trend of decreasing sensitivity to glyoxylate.

Isoenzymes of aspartokinase have similarly been separated by fractionation with ammonium sulphate (Stadtman et al., 1961). A partial separation was demonstrated by the different sensitivity of this enzyme in different fractions to inhibition by homoserine.

The existence of multiple IDH activities was thus further suggested. However, the resolution was not good enough to offer more than a slight separation of the isoenzymes and was of no preparative value. Therefore, further fractionation methods were investigated.

ii) DEAE-cellulose chromatography

Different proteins with the same activity have often been separated by DEAE-cellulose chromatography (for example, Crawford & Yanofsky, 1958).

Fractionation of an extract of A. lwoffi by DEAE-cellulose chromatography resulted in a clear separation of two proteins both with IDH activity. The ~~first~~ second to be eluted was found to be stimulated about five-fold by either glyoxylate or pyruvate and was termed IDH-II. The ~~second~~ first was not stimulated by either metabolite and was termed IDH-I. Thus the low activation of crude extracts by either effector was seen to result from the presence of IDH-I with IDH-II.

As an initial step in the investigation of the molecular characteristics of the isoenzymes and to make certain that they were not formed as a result of the DEAE-cellulose chromatography, other procedures for separating them were tried.

iii) Electrophoretic Separation

The development of both analytical gel electrophoresis and specific enzyme and protein stains has provided a very powerful technique for the demonstration of enzyme multiplicity (Markert, 1968).

However, both isoenzymes of IDH were found to be inactivated during polyacrylamide gel electrophoresis over a period of two hours. Because of this, the shorter time required to effect a separation on a cellulose-acetate matrix with the Millipore "Phoroslide" system made it preferable to electrophoresis on polyacrylamide gel.

After electrophoresis of a partially purified extract of A. lwoffi on cellulose-acetate, two bands could be seen on staining the strip for IDH activity indicating the distinct electrophoretic mobilities of the two isoenzymes. It was demonstrated that the bands did not arise from interactions of a single species with the electrophoresis buffer or matrix or from isomerisation as described by Cann & Coad (1968), by

demonstration of the appearance of only one band when either enzyme was run separately.

Because of the lability of both enzymes to electrophoresis, large scale separation by this method, as reported by Barrera & Jurtshuk (1969) with IDH from Azotobacter vinelandii, was not attempted.

Electrofocussing

Although electrofocussing has been used to separate different myoglobins by Vesterberg & Svensson (1966), and isoenzymes of cellulase by Ahlgren et al. (1967), the technique was found to be of little use in studies on the isoenzymes of IDH from A. lwoffi. Initial investigations were performed on a preparative scale in the hope of obtaining separate isoenzymes for further studies; however, with large samples, gross precipitation could not be prevented during the electrofocussing. When the technique was used on an analytical scale to determine the isoelectric points of both isoenzymes, precipitation was not apparent but very little activity could be recovered. This activity had no sensitivity to either pyruvate or glyoxylate and was found in a single pH region. As this could have been IDH-I or desensitised IDH-II with or without IDH-I, no useful data were obtained.

Gel Filtration

As pointed out by Flodin (1962), molecules of different dimensions may often be separated by gel filtration. The technique depends upon the careful selection of the gel pore size so that some molecules may enter the pores more freely than others and thus be more retarded in their passage through the gel. Because there is often a good correlation between molecular size and molecular weight the technique has been used with much success in the determination of molecular weights (Andrews, 1964; 1965). However, because the axial ratios of macro-molecules may differ quite considerably there is not always such a simple relationship between the rate of diffusion through a gel (measured as the quantity of solvent required to carry a species through a gel bed, i.e. elution volume) and molecular weight (Squire, 1964). A wide variety of gels has been used to achieve such separation and many multiple enzymes and isoenzymes have been separated in this way. With Sephadex G-200, Hopkinson et al. (1963) separated two isoenzymes of acid phosphatase, Harris & Robson (1963) showed isoenzymes of cholinesterase to have different molecular sizes and Goebelsman & Beller (1965) separated two amylamidases. Furthermore, Plaut & Aogaichi (1967) showed that filtration of a rabbit liver extract through Sephadex G-100 resulted in the

separation of two forms of IDH, one requiring NAD and the other NADP.

Filtration of a concentrated and partially purified extract of A. lwoffi through a column of Sephadex G-200 resulted in clear separation of two forms of IDH. The first to be eluted was sensitive to glyoxylate, being activated some 5-fold, and was thus IDH-II. The second (IDH-I) was eluted long after the first and thus appeared to have a much smaller molecular size. Similar experiments were carried out on the IDH of acetate-grown E. coli where no evidence of isoenzymes or of glyoxylate or pyruvate stimulation has been found. It is interesting to note that the gel-filtration behaviour of this E. coli enzyme is very similar to that of IDH-I from A. lwoffi.

Many reports have shown the NAD-linked IDH's from mammalian sources to have molecular weights around 60,000 (Magor & Robbins, 1969). Studies on the NADP-linked IDH from Azotobacter vinelandii indicated a molecular weight of 80,000 (Chung & Frazer, 1969). A larger NADP-linked IDH has not been reported and indeed, from gel filtration studies performed with marker proteins, it appears that IDH-I could well have a molecular weight around the reported values. However, IDH-II would appear to be a considerably larger molecule as it

is eluted from Sephadex G-200 before catalase, which has a molecular weight of about 250,000 (Samejima & Yang, 1963).

Thus, while precise molecular weights could not be obtained from these measurements, the much greater elution volume of IDH-I compared with that of IDH-II almost certainly shows IDH-I to be a smaller molecule than IDH-II.

The apparent large difference in the molecular sizes of IDH-I and IDH-II indicated by gel filtration encouraged the examination of zonal centrifugation as a means of separating the isoenzymes.

Zonal Ultracentrifugation

The zonal ultracentrifugation as developed by N.G. Anderson at Oak Ridge, affords a powerful method for the preparative separation of biological macromolecules. The new zonal rotors fully exploit the method of Brakke (1951) in which zones of particles or macromolecules, separated in a centrifugal field, are stabilised by carrying out the separation in a density gradient. Brakke devised this for swing-out bucket rotors which have to be loaded and unloaded with sample and gradient while the tubes are out of the rotor; it is, therefore, very easy to disturb the gradient when forming it, applying the sample or starting the centrifuge, and it is also

difficult to collect the separated zones. To overcome these difficulties, the zonal rotor has been designed so that the gradient and sample may be introduced and subsequently removed while the rotor is spinning. This allows the operation to be performed easily and reproducibly. The design of the rotor has considerably reduced the side wall effects which can lower the resolution of separations carried out with swing-out buckets - sedimentation occurring along radii of the buckets' motion. However, the main advantage of the zonal rotor is its much greater capacity: 50 ml of sample can be fractionated by rate-zonal sedimentation in the smaller B XIV rotor of 650-ml capacity.

The technique has found application almost exclusively with very large molecules and particles such as subcellular fractions (Price & Brown, 1965), viruses (Anderson & Cline, 1965), nucleic acid (Hastings, et al., 1966) and globulins (Rankin et al., 1967). Setlow & Lowenstein (1967) have purified adenylate deaminase by large scale zonal ultracentrifugation but the use of the technique with free soluble enzymes has been very limited. It does not appear to have been previously used for the preparative separation of isoenzymes.

When a preparation containing both IDH-I and IDH-II was subjected to zonal centrifugation, the two isoenzymes were

found to separate from each other very well and were recovered in good yield. Moreover, the sedimentation pattern unequivocally showed IDH-II to have a much higher molecular weight than IDH-I. This is consistent with the previous results of gel-filtration.

As Tomkins & Yielding (1961) pointed out, the large size of a regulatory enzyme may be a consequence of the necessity to accommodate both catalytic and regulatory functions on the one protein. Moreover, recent studies by Weitzman & Dunmore (1969) of citrate synthases from various organisms have shown that those which are sensitive to metabolite regulation are much larger molecules than those which are insensitive.

It was thus demonstrated that the two isoenzymes were distinct readily-separable proteins. Moreover, the results afforded by gel filtration and ultracentrifugation were particularly useful in that they also gave information pertinent to molecular studies of the separate isoenzymes. For further studies preparations of the separated isoenzymes were required which did not contain glutamate dehydrogenase or isocitrate lyase which were present in extracts of A. lwoffi and had been found to interfere with IDH assays. Purification schemes to yield both separated enzymes free from GDH or ICL were thus required. The investigations described above were of great use in devising such schemes. In particular zonal ultracentrifugation proved most useful in removing GDH and ICL both of which sedimented much

faster than either IDH isoenzyme.

After removal of the nucleic acid of a cell-free sonic extract of A. lwoffi, the extract was fractionated and concentrated with ammonium sulphate and then dialysed. From this solution, preparations of IDH-I were easily obtained by DEAE-cellulose chromatography. For IDH-II, however, the solution had to be subjected to zonal ultracentrifugation before the chromatography in order to remove GDH and ICL. Many differences were subsequently seen to exist between IDH-I and IDH-II with such preparations of the two forms.

Studies on the Separated Isoenzymes

i) Dependence on pH

The dependence of the IDH activity of crude extracts of A. lwoffi had been found to show two pH maxima. On separation of the isoenzymes this was found to be a consequence of the different pH dependences of the two forms. A plot of pH against reaction rate for IDH-I showed a maximum at pH 8.0 and for IDH-II, at about pH 8.8.

ii) Temperature dependence

Arrhenius derived an empirical relation between the

reaction rate (k), activation energy (E) and the absolute temperature (T) at which the reaction occurred, thus:

$$\frac{d\ln k}{dT} = \frac{E}{RT^2}$$

which may be rewritten as:

$$d\log_{10}k = -\frac{E}{2.303R}\, d\left(\frac{1}{T}\right).$$

The activation energy of an enzyme catalysed reaction is often found to be constant over the temperature range in which the enzyme is stable. Thus, logarithmic plots of reaction rates catalysed by such enzymes at different temperatures against the reciprocal of the absolute temperature at which the rates were measured, gives rise to linear plots of gradient E (Arrhenius plots).

However, many enzymes have been described in which temperature dependent changes in activation energy are evident by polyphasic Arrhenius plots. Massey (1953) showed that while the backward reaction of fumarase in acid solution gave a continuous linear plot, a biphasic plot was obtained in alkaline solution and also for the forward reaction in either acid or alkali.

Arrhenius plots of IDH-I and IDH-II have indicated marked differences between the isoenzymes. Not only do they appear to differ in activation energy but while IDH-I gave rise to a continuous plot, IDH-II gave rise to a biphasic one.

Massey et al. (1966) and later Koster & Veeger (1968) ascribed such discontinuities to conformational flexibility of the protein which allowed thermally induced changes in protein structure. Indeed, such changes are more often observed with large proteins than small ones, presumably because the larger a protein, the greater the probability of a change which is dependent on conformational flexibility. It thus appears significant that the larger protein, IDH-II, shows a biphasic Arrhenius plot whereas IDH-I shows no such discontinuity.

iii) Kinetic Characteristics

Both IDH-I and IDH-II have been found to have an absolute requirement for NADP. NAD is not reduced even in the presence of pyruvate or glyoxylate. Like most of the other IDH's reported from many sources, both enzymes had higher activities with Mn^{2+} as the divalent cation than Mg^{2+}. However, the activation of IDH-II was found to be more pronounced with Mg^{2+} than Mn^{2+}. Differences were apparent between the substrate dependences of the isoenzymes.

TABLE 14

The Substrate Dependence of IDH-I and IDH-II

Isoenzyme	Cation in Assay	Substrate	Apparent K_m
IDH-I	Mg^{2+}	NADP	2.5×10^{-2}
IDH-II	"	"	3.3×10^{-1}
IDH-I	Mn^{2+}	"	5.4×10^{-2}
IDH-II	"	"	1.1×10^{-1}
IDH-I	Mg^{2+}	Isocitrate	4.7×10^{-2}
IDH-II	"	"	3.5×10^{-2}
IDH-I	Mn^{2+}	"	5.1×10^{-2}
IDH-II	"	"	2.4×10^{-2}

Assays for the NADP dependence were here performed with 2.0 mM threo-D_s-isocitrate, and for the isocitrate dependence with 1.0 mM NADP.

Substrate Dependence

The dependence of activity of both IDH-I and IDH-II on NADP and isocitrate in the presence of Mg^{2+} or Mn^{2+} was investigated. The apparent Michaelis constants (K_m) which resulted from the studies were compared.

As is shown in Table 14 the K_m values of the two isoenzymes for NADP in the presence of Mg^{2+} are very different, that for IDH-I being some 13 times smaller than that for IDH-II. However, the K_m values of both isoenzymes for isocitrate were not significantly different.

With Mn^{2+}, ~~the pattern was found to be somewhat reversed.~~ the difference in K_m for NADP of the two isoenzymes was small, that of IDH-I being only about two-fold lower than that of IDH-II, whereas the K_m value of IDH-I for isocitrate was two-fold higher than that of IDH-II. It should be emphasised that the K_m values are apparent values, and comparisons may be valid only for the one concentration of fixed substrate.

iv) Inactivation Studies

(a) Thermal Inactivation

From preliminary studies it appeared that the rate of decay of IDH activity of crude extracts of A. lwoffi was very complex, the activity appearing to be regenerated after an

initial decay. This enigma was found to be due to contaminating activities and to the existence of two isoenzymes of IDH. The separated and purified isoenzymes were shown to decay "normally" upon heating. Moreover, they differed markedly in inactivation by such treatment, IDH-I being more stable than IDH-II; thus they would appear to have distinct physical constitutions. It is not surprising to find that IDH-II, the apparently more complex enzyme, is less stable than IDH-I. Similarly Weitzman (unpublished) has shown that citrate synthases which are sensitive to metabolic regulators are more labile to heat and urea than the smaller insensitive ones and also that the NAD-linked IDH from yeast is more labile to heat than the smaller NADP-linked one.

Similar differences in stability have been shown with other isoenzymes. Stadtman et al. (1961) showed that isoenzymes of aspartate kinase differed in their heat sensitivities and Plagemann et al. (1961) showed differences with dog LDH isoenzymes. Indeed, from this latter observation have stemmed techniques for the selective histochemical visualisation of particular LDH isoenzymes after electrophoresis. Dubach (1962) showed that the isoenzymes could be distinguished by assaying after controlled thermal inactivation.

From these studies it was seen that IDH-I and IDH-II

were more stable to thermal inactivation when together than when separate. This finding was in agreement with the observation that, although when mixed, the isoenzymes were completely stable to dialysis against 50 mM phosphate, 1 mM EDTA at pH 8.0 and 4° for many days, when separate, such dialysis destroyed most of the activity after only a few hours. Furthermore, it had been observed that fractionation procedures which did not reduce the activity of mixtures of the enzymes caused a gross loss of activity when applied to the separated isoenzymes. This could be due to the existence of a mutually stabilising interaction between IDH-I and IDH-II.

(b) Inactivation by Urea

The purified isoenzymes were also found to differ in their sensitivities towards urea. Again, IDH-II was found to be less stable than IDH-I. Similar differences between the urea lability of other isoenzymes have been reported. Richterich et al. (1962) used such differences between the isoenzymes of LDH to visualise them selectively after polyacrylamide gel electrophoresis and Emerson & Wilkinson (1965) distinguished between LDH isoenzymes in assay mixtures by selective inactivation with urea.

The greater lability to urea of IDH-II compared with IDH-I is again consistent with IDH-II being a more complex

molecule than IDH-I. Although as Monod et al.(1965) pointed out that with large protein molecules the relative spatial position of groups participating in the active site can be more easily maintained than with smaller molecules, it is likely that with an increase in size there is an increase in urea-labile areas.

Studies on the Activation of IDH-II

The most interesting feature which distinguishes the two IDH isoenzymes is their response to low levels of pyruvate or glyoxylate. Both of these compounds were found to stimulate the activity of IDH-II only. Although assays were routinely performed in Tris-HCl buffer the stimulation was seen to the same extent in buffers of imidazole-HCl and phosphate. Neither compound was found to stimulate IDH-I at pH values between 6.5 and 9.5.

For such an enzyme modulation to have any physiological import it would need to be freely and completely reversible. The reversibility of both the pyruvate and glyoxylate activation was demonstrated by the removal of those effectors with lactic dehydrogenase and NADH.

The activation by either effector was found to be more pronounced in the presence of Mg^{2+} than Mn^{2+}.

On investigating the dependence of the activity of IDH-II on effector concentration an ordinary hyperbolic relationship was found between IDH-II activity and effector concentration. Thus no evidence of co-operative interactions was obtained. In MET 8 almost complete activation was achieved by the addition of 0.1 mM effector. Activation of IDH-II was found to produce a number of changes in the protein and its catalytic behaviour.

i) Dependence of Activity on pH

The activation of IDH-II was found to have a strong pH dependence, increasing with increasing pH to the limit of the measurements at pH 9.5. As a consequence, the activation brought about a marked change in the dependence of activity of IDH-II on pH. Without effector, a dependence showing an optimum value at pH 8.0 was obtained, and on addition of the effector, this dependence approached that of IDH-I, with a maximum activity at pH 8.8. Thus, in crude extracts, where the isoenzymes are mixed, two maxima are seen without the effector, while in the presence of effector only one is found.

From current ideas of enzyme structure, which imply

that there might be more than one active conformation that a protein may adopt, it is not surprising that such changes as above do occur on the addition of enzyme modifiers. Tomkins et al. (1963) described similar changes with glutamate dehydrogenase upon the addition of the activator diethylstilbestrol, and Chen & Plaut (1963) demonstrated a similar change in pH optimum upon the addition of activating ADP (or dADP) to NAD-linked IDH from bovine-heart. It does not necessarily follow, however, that because an enzyme is activated a change in pH optimum must occur. Setlow & Lowenstein (1967) showed that adenylate deaminase had the same pH optimum with and without the activator (ATP).

ii) Dependence on Temperature

On the addition of low levels of pyruvate or glyoxylate no gross changes in the temperature dependence of IDH-II were observed. It appeared that the temperature dependent change which gave rise to the biphasic Arrhenius plot of IDH-II and that which was responsible for the activation were at least partially discrete phenomena.

Changes in the temperature dependence of regulatory enzymes on the addition of effectors have been reported. Koster & Veeger (1968) observed such changes with the enzyme D-amino-acid oxidase.

iii) Kinetic behaviour of IDH-II

The effect of pyruvate or glyoxylate on the kinetic parameters apparent K_m and V_m of IDH-II for either substrate in the presence of Mn^{2+} or Mg^{2+} was investigated.

Both the K_m and V_m for NADP were changed on the addition of pyruvate or glyoxylate, when assays were carried out with either Mn^{2+} or Mg^{2+}. The K_m was lowered and the V_m increased.

Differences were also found with the isocitrate dependence upon the addition of either effector. When NADP was present at 1.0 mM concentration, changes in both the K_m and V_m for isocitrate were similar to those described above for NADP. However, when NADP was present at 0.1 mM concentration, the change in K_m with either effector, in the presence of Mg^{2+} was much more pronounced than that found at the higher NADP concentration. With either effector, the K_m was found to be about one sixth of that in its absence. The change in V_m was only slightly greater than before (rising by about 3-fold with effector).

Thus significant changes in both K_m and maximal velocity were seen on the addition of either effector and in the presence of either Mg^{2+} or Mn^{2+}. Atkinson (1968) pointed out that effectors are more often found to change the

K_m for a substrate rather than the maximal velocity of a regulatory enzyme. The probable importance of K_m values to metabolic sequences was first suggested by Trevelyan (1958). However, the V_m of IDH-II has been seen to be as sensitive as the K_m to either pyruvate or glyoxylate for both substrates with Mn^{2+}. Only with low concentrations of NADP were much greater changes in the K_m for isocitrate seen.

The activation by either effector with either metal ion is most pronounced at low isocitrate and NADP concentrations. Physiologically, this may be the prevailing situation. Even if it is not, it is clear that as the concentration of a branch point metabolite falls, the more crucial are controls which are involved in the utilisation of the metabolite, and thus these controls might be expected to function more actively at low substrate levels.

Other effectors

i) Glyoxylate with Oxaloacetate

Both IDH-I and IDH-II were found to be completely inhibited by mixtures of glyoxylate and oxaloacetate. Such multivalent inhibition had been previously reported by Ruffo et al. (1959) with aconitase from pigeon-heart extracts. Shiio

& Ozaki (1968) later demonstrated similar inhibition with bacterial NADP-linked IDH from Brevibacterium flavum. Hampton & Hanson (1969) have shown that the NAD-linked IDH from Thiobacillus thiooxidans is affected in the same way.

Shiio & Ozaki (1968) ascribed the role of facilitating a balance between the TCA and glyoxylate cycles to the inhibition. However, both glyoxylate and oxaloacetate can be considered to stem from the activity of the glyoxylate shunt; it seems strange, therefore, that when one pathway is working well it should give rise to inhibition rather than activation of a competing pathway. Such an arrangement is made even more unlikely by the fact that in strict aerobes, such as A. lwoffi, the glyoxylate shunt appears to be dependent upon the activity of the TCA cycle for energy. The demonstration of the effect with IDH from sources which do not have glyoxylate shunts (Adinolfi et al., 1969) also tends to make such a role unlikely.

ii) Adenosine Nucleotides

(a) Adenosine monophosphate (AMP)

IDH-II was found to be sensitive to AMP. In the presence of 2 mM isocitrate, 2 mM AMP produced an activation of 1.6-fold. However, enzyme activated 4.8-fold by 0.2 mM glyoxylate was insensitive to AMP. The effect appeared to be

specific: ADP and ATP were without any effect on the enzyme activity or on activation by glyoxylate or pyruvate.

Stimulation of IDH activity by AMP was first reported by Kornberg (1950), with pig-heart NAD-linked IDH. After this initial report describing the absolute requirement for AMP, other workers described a similar requirement of IDH from a variety of sources - pea seedling mitochondria (Davies, 1955), Aspergillus niger (Ramakrishnan & Martin, 1955) and Pig-heart (Moyle & Dixon, 1955). However, reports by Plaut & Sung (1954) and Chen & Plaut (1962) described an NAD-linked IDH from bovine heart mitochondria without an absolute requirement for AMP. This confusion was resolved by Hathaway & Atkinson (1963) who showed that the AMP requirement was dependent on isocitrate concentration (diminishing with increasing concentration). Hathaway & Atkinson (1963) considered that because of its specificity, the AMP effect was important and not a coincidence or artefact. Apropos of this, Sanwal & Cook (1966) presented kinetic evidence which indicated that the mechanism of the reaction changed from random to ordered on the addition of AMP.

Cennamo et al. (1968) have suggested that the activating effect of AMP is due to an antagonism of inhibition of IDH by small anions. However, that the activation is of

physiological importance was indicated from the elegant polarographic measurements of IDH activity *in vivo* by Bernofsky & Utter (1966). The experiments with intact mitochondria showed that there was no detectable IDH reaction with even relatively high concentrations of isocitrate (up to 0.2 mM) in the absence of AMP. The stimulation of IDH-II by AMP might, therefore, be of similar importance.

(b) Adenosine Diphosphate (ADP)

ADP was found to be without effect on either IDH-I or IDH-II, in contrast with the NAD-linked IDH from bovine heart which Chen & Plaut (1962) reported to be activated by ADP.

(c) Adenosine triphosphate (ATP)

ATP was similarly found to have no effect on IDH-I or IDH-II. This nucleotide has been found to inhibit the NAD-linked IDH from swede (Dennis & Coultate, 1967).

Change in Stability

It had been originally observed by Dr. Weitzman that the IDH activity of crude extracts of *A. lwoffi* was protected against urea and thermal inactivation by glyoxylate. Thus a physical change was seen to occur on the addition of the effector. On the separation and partial purification of both isoenzymes of IDH this increased stability was found to be a feature only

of IDH-II.

Thermal Inactivation

Pyruvate and glyoxylate were both found to stabilise IDH-II but not IDH-I against thermal inactivation. However, logarithmic kinetic plots of the thermal inactivation of IDH-II in the presence of effector were found to be biphasic. The initial phase was followed by a slower phase of decay.

The production of two phases is usually attributed to two distinct forms with the same sort of catalytic activity. Thus, as two phases are only produced in the presence of the effector it is tempting to speculate that they are caused by two forms of a single enzyme which differ in the amount of bound effector. Distinct phases are still evident in the presence of 1.0 mM effector while the enzyme appears to be completely activated with 0.1 mM effector.

The stabilisation of many enzymes by their substrates against inactivation has long been known (Baylis, 1904; Ter Menten, 1905; Wohl & Glimm, 1910). Often the protection was demonstrated both by the retention of biological activity and gross physical characteristics (Harris, 1956). Such stabilisation has been explained in terms of the Fischer template

hypothesis (1894): it was proposed that the binding of the substrate caused a localised freezing of part of the protein, rendering it more stable. This still can explain many results.

More recently, it has become evident that this sort of stabilisation is not confined to substrates. Many enzymes have been found to associate specifically with small molecules which can modify their activity. Marked changes of sensitivity to chaotropic environments have been shown to occur on the addition of these modifiers. These reports stem from the initial finding of Changeux (1961) that threonine deaminase is protected against inactivation by an inhibitor - isoleucine. Later studies by Changeux showed inactivation by both heat (Changeux, 1963) and urea (Changeux, 1965) to be reduced by isoleucine. Moreover, as was pointed out by Sawas & Dandoroff (1965) some enzymes are stabilised by their modifiers while their substrates have no effect. However, it is by no means necessary for the modifier to cause such stabilisation (Jensen & Nester, 1966). Furthermore, some enzymes show a decreased stability with effector. Koshland (1963) with phosphoglucomutase, and McClintock & Markus (1968) with aspartate transcarbamylase, both showed that on the addition of effector to these enzymes less stable forms resulted.

In the wake of the two major recent theories of enzyme modulation (Monod et al., 1963; Koshland, 1959; 1963) which consider that a protein may have more than one physiologically determined conformation, it has become popular to describe such stabilisations as above in terms of conformational changes.

Rowe & Weitzman (1969) have shown by electron microscopy and analytical ultracentrifugation that the molecular shape of citrate synthase from A. lwoffi is changed upon addition of the effectors NADH and AMP. Moreover, Weitzman (in preparation) has shown that these effectors also stabilise the enzyme against thermal inactivation. Chen et al. (1964) have demonstrated conformational changes occurring with NAD-linked IDH on the addition of various effectors by changes in sedimentation characteristics. Also, the stabilisation of NAD-linked IDH by citrate (an activator of the enzyme) has been ascribed by Sanwal & Stachow (1965) to a possible conformationalchange on the binding of the ligand.

However, it appears that few published results unambiguously separate such an effect from that of direct or protein mediated stabilisation of a catalytic site by ligand binding - leading to a freezing of protein structure.

Thus it is apparent that while the results from IDH-II are of interest in that they show that the binding of

either pyruvate or glyoxylate produces a distinct change in physical characteristics of the protein, it is not possible to ascertain whether this is a direct consequence of a change in conformation. Even though it appears from the results of the thermal inactivation experiments that the catalytic and regulatory sites are distinct, stabilisation could be considered to occur as a result of (gross) freezing of structure. It is even conceivable that the effectors take part in the catalysis in a similar way to that of pyruvate in covalently bound D-proline reductase reported by Hodgins & Abeles (1967). These authors have indicated that the catalytic mechanism of this enzyme proceeds via an electron shift in a pyruvate moiety which is part of the catalytic site. A similar involvement could explain the results presented here. However, an exact parallel is made unlikely by the ease of removal of either effector.

Protection by Sodium Chloride

It is often found that enzymes which are produced by halophilic organisms require high concentrations of sodium chloride for activity and are more stable in its presence. Hubbard & Miller (1968) have described an NADP-linked IDH from

Halobacterium cutirubrum - an "extreme halophile" - which requires sodium chloride for both activity and protection against inactivation. As A. lwoffi is a "moderate halophile" (Vorgues & Brison, 1966) the effect of sodium chloride on the thermal inactivation was investigated.

IDH-II was found to be protected against thermal inactivation by high concentrations of sodium chloride. This could explain the differences in thermolability found previously with preparations of different ionic strengths.

Furthermore, IDH-II which had been heated with sodium chloride was found to be partially desensitised to either pyruvate or glyoxylate. Thus it would appear that under such conditions the activator site is more sensitive to thermal disruption than the catalytic site. Therefore, it is possible that the effect of sodium chloride was not to afford a gross stabilisation of the protein but rather a more localised stabilisation of the region of the catalytic site.

Physiological Role

The Isoenzymes

The existence of isoenzymes in diverse biological systems has been found to be so widespread that Kaplan (1968) observed, "It now appears that enzymes existing in only one

molecular form are exceptions". It is almost certain that the existence of isoenzymes is not a reflection of any lack of specificity before or during enzyme formation but rather that they fulfil a definite physiological need.

To limit wasteful overproduction, many metabolic pathways are inhibited at their first step by the end-product of the pathway (Umbarger, 1955; Yates & Pardee, 1956). However, this is complicated in the case of a divergent metabolic pathway which gives rise to more than one distinct product. If the very first step of the pathway were largely inhibited by any one of the products then conditions could arise whereby the cell could be starved of the products of the other divergent pathways. To offset this, many complex feed-back control systems have been elaborated by cells. These have been excellently reviewed by Stadtman (1966) and Datta (1969). Isoenzymes have been shown to play an important part in these schemes and Stadtman (1968) has more fully reviewed the multiple roles of isoenzymes.

Further evidence for the physiological involvement of isoenzymes in control systems has been provided by the demonstration of induction of different isoenzymes by different growth substrates (Canovas & Stanier, 1967).

However, such growth studies have shown no indication

of any difference in the precise metabolic roles of IDH-I and IDH-II in A. lwoffi. It was shown that IDH-II was not a feature of growth on acetate. Furthermore, as it was thought that as IDH is the immediate metabolic step before the branch point leading from α-keto-glutarate to succinate or glutamate, one of the isoenzymes might be specifically synthesised for one of the routes. Growth on succinate or glutamate might, therefore, result in the repression of that enzyme. No such repression was seen. Alternatively, it was thought that the presence of a second NADP-linked IDH might result from the need for NADPH. Growth on gluconate, when the organism would produce much NADPH as a result of the dehydrogenation of gluconate-6-phosphate showed no significant change in the isoenzyme ratio. Consideration has been given to the possibility of the artefactual production of the two forms. To this end it has been shown that they are unaffected by prolonged sonication and present in the same proportion when extracted by either sonication or by use of the French press. These results indicate the likely existence of the isoenzymes in vivo.

It has been shown that multiple forms of macromolecules can be produced artefactually by techniques widely used in protein separation. In a theoretical treatment Gilbert (1955) showed that even with systems where equilibrium is instantaneously

established as

$$n\Delta \rightleftharpoons \Delta n$$

if $n \geqslant 3$, then two forms could be discerned in moving boundary electrophoresis. Later Bethune & Kegeles (1961) showed that complexing of the sort $A + B \rightleftharpoons C$ between macromolecules can give 1, 2 or 3 zones, even with rapid equilibrium. Conn & Goad (1968) pointed out that these effects may give rise to the resolution of "multiple forms" upon ultracentrifugation, ion-exchange chromatography or gel filtration. However, it is unlikely that such effects are responsible for IDH-I and IDH-II as both isoenzymes appear to have very stable characteristics when separated, show only single bands in electrophoresis, and do not give rise to each other when stored or further purified. Moreover, the pH profiles of the separated isoenzymes account for the two maxima found with such profiles of crude extracts. Therefore, it appears that they are distinct entities before separation.

During the early stages of growth of a culture of A. lwoffi from a dormant inoculum the ratio of IDH-II to IDH-I was seen to fall to a constant level. As IDH-II has been shown to be very stable in crude extracts of A.lwoffi the fall

was most probably not due to the breakdown of IDH-II. Thus it would seem likely that the ratio was a direct consequence of the rate of synthesis of both IDH-I and IDH-II. If IDH-II were composed of IDH-I units with other (perhaps regulatory) units, the ratio of IDH-I to IDH-II could be a reflection of the availability of the "other" units. However, if this were not the case ratio changes must indicate that the mechanisms of formation of each are distinct.

The similarity between IDH-I and the IDH of E. coli (shown by gel-filtration and ultracentrifugation) suggested that IDH-I was not some chance fragment of a more complex unit but had a distinct phylogeny, perhaps coincident with that from E. coli. Furthermore, IDH-II has been found to be more labile to both heat and urea than IDH-I. Although this greater lability could be considered a consequence of a changed exposure of IDH-I units when in a more complex state (IDH-II), it is likely that such a difference in lability is a feature of some distinct differences in the protein around the region of the catalytic site.

The Activation of IDH-II

As has been previously mentioned, when a metabolic

pathway branches, there is competition between the first enzyme of each branch for the branch-point metabolite. Mechanisms allowing the modulation of either or both enzymes by inhibition or activation confer a great efficiency and hence selective advantage on the cell. Occurring at such a branch-point IDH occupies a key position. It is not surprising, therefore, that many postulated controls of the TCA cycle have been centred on IDH; for example, Hathaway & Atkinson (1963), Sanwal et al. (1964), Dennis & Coultate (1967), Adinolfi et al. (1969). However, few previous reports consider the possible regulatory roles of bacterial IDH. Within this context, the possible role of the activation of IDH-II is immediately apparent. Isocitrate lyase and IDH both compete for substrate - isocitrate; if isocitrate is cleaved by ICL then one of the products (glyoxylate) acts as a positive signal to facilitate the channeling of isocitrate to the other branch, i.e. to IDH (II). Presumably the pool size of glyoxylate in the cell and thus the activation of IDH-II depends not only on the formation of glyoxylate but also on its removal by the malate synthase reaction. The activation by pyruvate is a little more difficult to explain. It could be an example of "precursor activation", a type of enzyme regulation considered to be complementary and similar to

feed-back inhibition by Sanwal et al. (1963) who described such an activation of NAD-linked IDH by citrate. Alternatively, as phosphoenolpyruvate may be considered to be a product of the glyoxylate shunt (Kornberg, 1965) so may pyruvate be thus considered. In this light it is similar to glyoxylate, being produced as a result of the activity of the glyoxylate cycle. It is possible, therefore, that its role in stimulating IDH-II may be similar to that of glyoxylate. However, because of the close structural resemblance of glyoxylate and pyruvate it is also possible that the effect of pyruvate results from the inability of the protein to distinguish it from glyoxylate. This is seen with lactic dehydrogenase, for as well as catalysing the hydrogenation of pyruvate, a similar reaction proceeds with glyoxylate (albeit more slowly).

However, the precise elucidation of the biological roles of both IDH-I and IDH-II must await further studies. These might show (i) important differences in the cellular localisation of IDH-I and IDH-II, (ii) the modulation of either isoenzyme by other metabolites (Sanwal [1963] has emphasised that multiple controls are often exerted on "pacemaker" enzymes), and (iii) the easier reversal of either enzyme when assayed under more physiological conditions - it being conceivable that one of the enzymes is solely concerned

with isocitrate formation. It is, of course, also possible that when the enzymes can be studied under more physiological conditions, some of the differences between the isoenzymes may appear quite changed as has been found to be the case with LDH (Vesell, 1965). Thus as is always the case, much care must be exercised in relating measurements made *in vitro* to *in vivo* mechanisms.

The IDHs of Other Organisms

Most previous studies of IDH have been concerned with mitochondrial NAD-linked IDH which appears larger and more complex than the extramitochondrial NADP-linked IDH in eucaryotes (Plaut & Aogaichi, 1967). Indeed in such cells it appears that the regulation of IDH activity occurs exclusively within the mitochondria with the NAD-linked enzyme.

In metabolism NADH and NADPH have somewhat distinct roles, NADH generally being used through oxidative phosphorylation to provide ATP and NADPH being used by anabolic routes for reductive biosynthesis. Thus in eucaryotes the compartmentation of NAD-linked IDH in the mitochondria is

consistent with the major energy producing role of this organelle. It is also not surprising that the NADPH producing enzyme is located in the cytoplasm - where the bulk of biosynthetic activity is thought to occur. The activation of the NAD-linked enzyme by AMP has been suggested by Hathaway & Atkinson (1963) to be a regulation for control of the relative production of NADPH and NADH. In a comparative study of many microorganisms Ragland et al. (1966) showed that most of them appeared to have only a NADP-linked IDH. Indeed only a few bacteria have been reported to have a NAD-linked IDH, e.g. Streptococcus bovis (Burchall et al., 1964), Thiobacillus thiooxidans (Hampton & Hanson, 1969), and Acetobacter peroxydans which has been reported to have both NAD and NADP-linked IDHs (Hathaway & Atkinson, 1963). Moreover, Ragland et al. (1966) also showed that many of the organisms which produced only NADP-linked IDH also produced a transhydrogenase capable of reducing NAD at the expense of NADPH. Thus in these organisms the regulation of the NADH : NADPH ratio produced as a result of the dehydrogenase activity might be controlled by another enzyme - the transhydrogenase.

From my studies with a wide variety of organisms it was evident that only a few micro-organisms produced an IDH-II-like enzyme. Moreover, as is shown in Table 12, it was not found

in any of the Gram-positive organisms tested, nor was it found in preparations from mammalian sources. Its production appeared to be limited to a few Gram-negative organisms. However, no obvious major biochemical or taxonomic distinction could be drawn between organisms which did or did not produce it. Hanson & Hampton (1969) have recently described an 80% activation of the NADP-linked IDH in crude extracts of a strain of Pseudomonas fluorescens by glyoxylate. In the light of the results presented in this thesis it would appear likely that their organism produced isoenzymes similar to IDH-I and IDH-II. However, in the present work the strains of Ps. fluorescens tested showed no indication of an IDH activated by glyoxylate. Thus it appears than even between different strains of the same organism there are differences.

The Possible Phylogeny of IDHs

Most procaryotes appear to produce only one NADP-linked IDH of low molecular weight and similar to IDH-I of A. lwoffi. The production of IDH-II is likely to have been a sophistication brought about by either convergent or divergent evolution. In the former the nature of a protein initially

evolved for another role is changed by mutation so that further protein synthesised has the facility to catalyse the reaction. In the latter, a selected part of the genome is duplicated and the independent original and copy can further evolve in different ways, leading to distinct enzymes. Gross mutation of either may occur as intermediate, otherwise lethal, mutations of either are protected by the "normal" synthesis of the other enzyme.

IDH-II is a much larger enzyme than IDH-I. Monod et al.(1965) discussed the advantages of large enzymes over smaller ones. They have the increased capacity to maintain specific conformations as well as having a smaller surface to volume ratio, thus reducing unwanted reactions. They further suggested that polymerisation of similar units could occur co-operatively by mutation. If a mutation leads to the production of a changed unit, an area of which has gained an affinity for a distinct and different area on an identical unit then a dimer could result from this single mutation with two bonds joining the units. Therefore, for one mutation of the enzyme two bonds could be formed. Thus, as has previously been mentioned, it is likely that large molecules like IDH-II may be composed to some extent of identical units. Moreover, it is possible that these may have originated from IDH-I units. Eucaryotes produce a mitochondrial NAD-linked IDH. Like IDH-II, this is a large protein and also responds to AMP. It

is intriguing to speculate that this may have originated from IDH-II by changes which altered the specificity for pyridine nucleotides. This would have rendered unnecessary the production of the transhydrogenase. It would be of interest to compare the intramitochondrial NADP-linked IDH reported by Bernofsky & Utter (1966) with IDH-II as this change may have occurred through divergent evolution. Furthermore, the extramitochondrial NADP-linked IDH is a small protein unstimulated by glyoxylate, pyruvate or AMP and thus appears similar to IDH-I.

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ABSTRACT

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Preliminary results had indicated that the isocitric dehydrogenase (IDH) of the bacterium Acinetobacter lwoffi 4B was activated by low levels of glyoxylate. Furthermore, in the presence of glyoxylate the enzyme had been found to be more stable to heat or high concentrations of urea.

Subsequent studies have shown that two isoenzymes of NADP-linked IDH exist in A. lwoffi and that only one of these is stimulated and protected by glyoxylate. It has also been shown that pyruvate has a similar activating and protecting effect as that of glyoxylate. On addition of either of these effectors the activity of the sensitive enzyme is markedly changed in its dependence on substrate concentration and pH (the former resulting from changes in both the apparent K_m and V_m for the substrates).

The isoenzymes have been separated by DEAE-cellulose chromatography, cellulose acetate electrophoresis, gel-filtration and preparative rate zonal ultracentrifugation. Investigations using the latter two techniques have shown the sensitive enzyme to be a much larger molecule than the insensitive one.

The use of preparative zonal ultracentrifugation for the separation of isoenzymes was novel and these studies have shown

it to be a potentially powerful technique for both the separation and purification of isoenzymes.

Studies on the separated isoenzymes have shown them to differ in their sensitivities to heat and urea and to have different dependences on pH, temperature and substrate concentration.

Experiments undertaken with the aim of elucidating the physiological role of both isoenzymes are also described and discussed.

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