Studies on formate dehydrogenases on legumes

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STUDIES ON FORMATE DEHYDROGENASES ON LEGUMES

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STUDIES ON FORMIC DEHYDROGENASES OF LEGUMES

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A scheme has been developed for the purification of formic dehydrogenase from Phaseolus aureus seeds. Using this scheme 50 mg./kg. of formic dehydrogenase can be obtained with an estimated purity of 55% and in 9% yield. The final product contains one major and two minor impurities. Details are given of the stability of enzyme activity under various conditions and of each step in the purification scheme.

The steady state kinetic analysis of formic dehydrogenase, using initial velocity, product inhibition, and dead end inhibition analysis, indicate an ordered BiBi sequential mechanism with the probable absence of a rate limiting ternary complex. The variation of the kinetic parameters with pH and temperature is also reported.

Investigations into the changes which occur in the levels of both formic acid and formic dehydrogenase during germination of Phaseolus aureus seeds, suggest that the metabolic role of formic dehydrogenase is primarily the oxidation of formic acid produced during the first day of germination. The attempts to measure the equilibrium constant of the reaction, the estimated rate of the reverse reaction, and the results of in vivo experiments with labelled formic acid, all confirm that the enzyme is unlikely to carry out any incorporation of carbon dioxide into formic acid.

CONTENTS

		Page
INTRODUCTION		1
MATERIALS AND METHODS		
1.	Biological materials	9
2.	Chemicals and reagents	9
3.	Preparation of solutions	11
4.	Estimation of enzyme activity	12
	(a) Spectrophotometrically	12
	(b) Radiochemically	12
5.	Estimation of protein	13
6.	Extraction of formic dehydrogenase activity from various members of the Leguminosae	14
7.	Formic dehydrogenase of Phaseolus aureus	
	(a) Estimation of enzyme stability	
	(i) pH	14
	(ii) Heat	14
	(iii) Dialysis	14
	(iv) Metal ions	15
	(v) Low temperature storage	15
	(b) Purification	
	(i) Extraction	15
	(ii) Salt fractionation	15
	(iii) Ion exchange chromatography	16
	(iv) Hydroxyl apatite chromatography	17
	(v) Alumina gel chromatography	17
	(vi) Acrylamide gel electrophoresis	18
	(vii) Gel Filtration	18
	(viii) Purification scheme	18

Page

(c) Kinetics

(i) Purification of NAD 20

(ii) Estimation of initial velocity 20

(iii) pH optimum 20

(iv) Inhibitors 20

(v) Statistical analysis 21

(vi) Variation of the maximum velocity with temperature 22

(vii) Nomenclature and derivation of rate and kinetic equations 22

(d) Metabolic role

(i) Preparation of mitochondria 23

(ii) Estimation of oxygen uptake 23

(iii) Germination of Phaseolus aureus 23

(iv) Estimation of formic acid 24

(v) Separation of organic acids 24

(vi) Separation of amino acids 24

(vii) In vivo incubation with $[^{14}C]$- Formate 24

(e) Molecular weight 25

RESULTS

1. Formic dehydrogenase activity of extracts of various members of the Leguminosae 26

2. Formic dehydrogenase of Phaseolus aureus

(a) The stability of enzyme activity

(i) pH 26

(ii) Heat 27

(iii) Dialysis 27

(iv) Metal ions 32

(v) Low temperature storage 33

Page

(b) Purification

(i) Extraction 34

(ii) Salt fractionation 37

(iii) Ion exchange chromatography 38

(iv) Hydroxyl apatite chromatography 38

(v) Alumina gel chromatoglaphy 39

(vi) Gel filtration 39

(vii) Acrylamide gel electrophoresis 39

(viii) Purification scheme 41

(c) Kinetics

(i) The pH optimum 42

(ii) Inhibitors 42

(iii) Initial velocity analysis 43

(iv) Product inhibition 43

(v) Dead end inhibition 44

(vi) The effect of ionic strength on the velocity at low substrate concentrations 45

(vii) Variation of the kinetic parameters with pH 45

(viii) Variation of the maximum velocity with temperature 46

(ix) Attempts to measure the equilibrium constant 46

(x) Measurement of the reverse reaction 47

(d) Metabolic Role

(i) Mitochondrial content of formic dehydrogenase 48

(ii) Changes in enzyme activity during germination 50

Page

(iii) Changes in formic acid content during germination 50

(iv) In vivo utilisation of formic acid 50

(e) Molecular weight 51

DISCUSSION 52

BIBLIOGRAPHY 86

ACKNOWLEDGEMENTS

INTRODUCTION

Biological oxidations represent one of the most important groups of enzyme catalysed reactions. These oxidations are carried out by three basic classes of proteins; oxygenases and hydroxylases, dehydrogenases, and haemo-proteins. Dehydrogenases can be further subdivided into pyridinoproteins, flavoproteins and cuproproteins. Cuproprotein enzymes, which have a relatively high oxidative potential, are concerned with the dehydrogenation of substrates of similarly high oxidative potential, using oxygen as acceptor. These enzymes are therefore ideally suited as the terminal members of the respiratory chain. Pyridinoproteins on the other hand have a lower oxidative potential and catalyse the transfer of two reducing equivalents from substrates of low oxidative potential to coenzymes, the coenzymes being readily dissociable. Pyridinoproteins are ideally designed therefore for the initial dehydrogenation of most substrates. The coenzymes used by pyridinoproteins are either NAD* or NADP, and the reduced coenzyme is able to shuttle freely to a second site where it can either be utilised to reduce a second substrate via a linked reaction, provide reducing equivalents for biosynthetic sequences, or be reoxidized by the respiratory chain accompanied by the conversion of ADP to ATP. Flavoproteins occupy an intermediate position between pyridino-proteins and flavoproteins, being able to utilize substrates with both high and low oxidative potentials.

Investigations into the mechanism of enzyme reactions involving pyridine nucleotides, have been in progress for a number of years. A recent symposium (1965), demonstrates the intense interest shown

*The abbreviations and conventions used throughout this thesis are those recommended to contributors to "The Biochemical Journal". (Biochem. J., 66, 8)

in these proteins and of the variety of techniques being used to study their mechanism of action. There are four basic steps which have to be accomplished before any enzyme mechanism can be fully understood. It is necessary to know, (i) the pathway of the reaction; (ii) the amino acids involved in substrate binding, and in bond-making and bond-breaking steps; (iii) the three dimensional arrangements of the amino acids involved in the reaction; (iv) the specific roles of the various groups involved in the reaction. When all of these are known it should be possible to explain the reaction velocity within an order of magnitude, although this has yet to be achieved for any enzyme. Kinetic analysis has been used extensively in studying enzyme mechanisms and the pathway of a number of dehydrogenase reactions has been determined by this method; for alcohol dehydrogenase by Sund and Theorell (1963), Dalziel (1963), Wratten and Cleland (1963), Theorell and Chance (1951), Boyer and Silverstein (1963), Boyer (1959), Wong and Hanes (1964), Yonetani and Theorell (1964), Snyder, et al. (1965), Anderson, et al. (1965) and Van Eys (1961); for lactate dehydrogenase by Schwert and Winder (1963), Silverstein and Boyer (1964), Zewe and Fromm (1965 and 1962), Thompson et al. (1964); for malate dehydrogenase by Raval and Wolfe (1962 & 1963); for NAD: isocitrate dehydrogenase by Sanwal and Stachow (1965), Atkinson et al. (1965); for glyceraldehyde-3-phosphate dehydrogenase by Furfine and Velick (1964, Velick and Furfine (1963), Harting and Velick (1954), Toews (1967), Koshland et al. (1968), Bolotina et al. (1967), De Vijlder and Slater (1967);

for glutamate dehydrogenase by Frieden (1959), Fahien et al. (1965), Corman et al. (1967).

In addition to kinetics there are a number of physico-chemical methods capable of detecting enzyme reaction intermediates. Optical rotatory dispersion has been used to detect dehydrogenase complexes by Rosenberg, et al. (1964 & 1965), Furfine (1965), Magar (1965), Listowsky et al. (1965). Other techniques which have found similar applications for dehydrogenases include fluorescence used by McKinley-McKee (1963), Schwert and Winer (1963), Winer (1964), Anderson, Ihnen and Vestling (1964), Cassman Englard (1964), Czerlinski and Schreck (1964), Velick (1958); double difference spectroscopy, Theorell and Yonetani (1964), Friedrich (1965), Fisher Cross (1965); and X-ray diffraction used by Brander et al. (1965).

The detection of the amino acids involved in the reactions of dehydrogenase enzymes has largely been accomplished by the use of specific labelling techniques; Li and Drum (1964), Schellenberg (1965), Fondy and Everse (1964); Haris (1964), Olson and Park (1964), Matthew and Park (1965), Mathew et al. (1965) , Whitehead and Rabin (1964), Rabin et al. (1964), Anderson and Anderson (1964), Anderson and Reynolds (1965); Harris and Polgar (1965), Li and Vallee (1964).

The determination of the three dimensional arrangement of the amino acids involved in dehydrogenase reactions will undoubtedly be accomplished by X-ray crystallography. A start in the use of this technique has already been made in several cases, Watson, Banaszak (1964), Rossman et al. (1967), Zeppezauer et al. (1967), and Banaszak (1966).

In every instance so far cited the source of the enzyme has been a mammal or a micro-organism. No similar studies have yet been reported for a higher plant dehydrogenase. The present work was designed to gain preliminary information on a higher plant dehydrogenase of a comparable nature to that obtained from mammalian and microbiological sources.

Since no plant dehydrogenase has yet been obtained in a pure form, and as most of the techniques used in studying enzyme mechanisms require pure enzymes, the initial section of the present work was directed towards this end. The enzyme chosen for purification and study was formate: NAD oxidoreductase (abbreviated to formic dehydrogenase, FDH) enzyme No. 1.2.1.2.

FDH, however, is not restricted to the plant kingdom and its presence has been reported in bacteria by Malavolta et al. (1962), Stickland (1929), Takamiya (1953), Yamada & Asano (1954), Sasakawa, Kimura (1954), Lichstein and Boyd (1953), Gale (1939), Woods (1936); in protozoa by Lindblom (1961), Nermut and Ryc (1964); in insects by Agarwal et al. (1963); and in mammals by Venkataraman and Sreenivasan (1966), Elliott (1941), Mathews and Vennesland (1950). Two surveys on FDH within the plant kingdom have been conducted, one by Davison (1949a), the other by Thurman et al. (1967). The first survey examined the seeds of ninety-three species, in thirty-nine families distributed among twenty-five orders in both gymnosperms and angiosperms, FDH was detected in fifty-four species. Seeds of members of the Leguminosae were particularly rich in this enzyme, all twenty-eight species examined showed the same activity.

The second survey was restricted to the Favaceae (Leguminosae) and of the one hundred and three species selected from seventeen tribes only one lacked detectable FDH activity.

Formic dehydrogenase was first discovered in plants by Thunberg (1921, 1936), using Phaseolus vulgaris seeds. The coenzyme requirements of the enzyme was first reported in peas, by Fodor and Frankenthal (1930), and identified as coenzyme I, (NAD), by Anderson (1934) and Lichtenstein (1936). The strong inhibition of enzyme activity by cyanide was first reported by Alder and Sreenivasaya (1937), who used the pea enzyme. This inhibition was later confirmed by both Davison (1951) and Nason and Little (1955), who also reported the effectiveness of other metal complexing and -SH specific chemicals in causing inhibition. (Davison, in 1949, demonstrated that the dry pea seed contained the greatest amounts of the enzyme, and that during germination and growth these levels became greatly reduced, and only increased during the development of the new seeds in the pod).

Several procedures for the partial purification of FDH have been published, Alder and Sreenivasaya (1937), Mathews and Vennesland (1950), Davison (1951) and Nason and Little (1955). The most extensive procedure is the most recent (Nason and Little, 1955), and involves fractionation of the preparation with ammonium sulphate, calcium phosphate and alumina gel. The present work extends this purification scheme and uses hydroxyl apatite column chromatography, Levin (1962), in place of calcium phosphate fractionation, ion exchange chromatography, Peterson and Sober (1962), and gel filtration, Lathe and Ruthven (1956) and Porath and Flodin (1959).

Little is known of the mechanism of action of the plant enzyme, although it is known to differ from both the bacterial and mammalian enzymes. The bacterial enzyme does not require a coenzyme (Gale, 1939), (an exception being Clostridium thermoaceticum which has been shown to require NADP, Lan-Fun et al. (1966)) the animal enzyme which also does not require a coenzyme is activated by ATP, Mathews and Vennesland (1950). A deuterium isotope effect has been reported for the enzyme from Phaseolus multiflorus, Aebi (1956). 94% replacement of the carbon bound hydrogen atom caused a reduction in the velocity of one half to one third, and an increase in both the Michaelis constant from 2.7 mM to 3.1 mM, and the activation energy from 11,900 to 14,900 cal./mole. Apart from this deuterium isotope effect, the Michaelis constants for formate, and the effect of various inhibitors no more information is known about the mechanism of action of FDH from any source.

The mechanism of FDH using steady state kinetics was investigated in order to determine the kinetic mechanism of action, and originally it was hoped to use specific labelling techniques to determine some of the amino acids involved in the reaction. Present understanding of steady state kinetics allows the determination of (a) the order in which substrates and products are bound and released; (b) the composition of complexes between the enzyme and reactants, together with permissible interconversions; (c) the detection of isomerization of stable enzyme forms, and in some cases, isomerization of non-central transitory complexes. There are four main procedures with which this can be achieved. The first is the

interpretation of the initial velocity patterns, Cleland (1963c), which enables the distinction between sequential mechanisms (mechanisms where all substrates bind to the enzyme before any products are released) and Ping-Pong mechanisms. The second method involves the testing of numerical consistency with special relationships which are known to exist for certain mechanisms, Alberty (1953), Dalziel (1957), Cleland (1963a). The third, product inhibition, is able to distinguish between any mechanism with a different overall rate equation, Alberty (1958), Rainer (1959) and Cleland (1963b), Fromm and Nelson (1962). The last technique, isotope exchange, can provide a useful check on the mechanism proposed by the first three. This technique involves measurements on the rate of isotope exchange at equilibrium, Boyer (1959), Alberty, et al. (1962) and Boyer and Silverstein (1963).

One of the most successful techniques for identifying the amino acids in the region of the active centre of SH dehydrogenases has proved to be the use of $[^{14}C]$-iodoacetate as a label for the active site, Harris et al. (1963), Perham and Harris (1963), Harris (1964), Fondy and Everse (1964), Gold and Segal (1965), Holbrook et al. (1966), Holbrook et al. (1967), Li and Vallee (1964) and Harris (1967). As FDH is known to be sensitive to SH specific reagents, Davison (1951), Nason and Little (1955), it was planned to use this technique if it proved possible to isolate and completely purify formic dehydrogenase.

The metabolic role of FDH in the germinating seedling has only previously been investigated by Davison (1949b). In view of the greater

understanding of the biochemical pathways involved in living processes, and also of the biochemical changes involved in germination, since that time, it was felt that this subject could benefit from a re-examination.

It was, therefore, proposed to examine the changes in both the levels of formic acid and FDH within various tissues of the germinating seedling, and to attempt to correlate these findings with metabolic changes known to be occurring within the seedling. It was also proposed to examine the fate of formic acid during the initial period of germination.

MATERIALS AND METHODS

1. Biological materials.

The seeds were obtained from the following suppliers:

Arachis hypogea, from the 'Honey Pot', Durham City.

Canavalia ensiformis, from British Drug Houses (BDH) Ltd., Poole, Dorset.

Lathyrus ordoratus, Phaseolus vulgaris cv. The Prince, Pisum sativum cv. The Pilot, Vicia faba cv. Claudia Aquadulce, Trifolium alba, from Carters Tested Seeds Ltd., Norwich.

Phaseolus coccineus cv. Streamline, Toogoods Seeds Ltd., Southampton.

Phaseolus aureus, from Anglo-Continental Supplies Ltd., Grove Street, Newcastle.

2. Chemicals and reagents.

With the exceptions listed below, chemicals were obtained from British Drug Houses Ltd., Poole, Dorset, and were of analytical reagent grade when available.

Aldolase

Alumina gel Cγ

Bovine serum albumin (fraction V)

p-Chloromercuribenzoate

Digitonin

γ Globulin (grade III)

Hydroxylamine hydrochloride (grade I)

MTT tetrazolium

NAD (grade III)

NADH (grade III in preweighed vials)
were obtained from Sigma Chemical Co. Ltd., London.

ADP
N-methylphenazonium methosulphate
were obtained from Koch-Light Laboratories Ltd., Colnbrook.

Sephadex G-200
was obtained from Pharmacia Ltd., Uppsala, Sweden.

Chromotropic acid was obtained from EASTMAN Chemicals Ltd.

DEAE-cellulose (DE52)
was obtained from Whatman Ltd.

$[^{14}C]$ sodium bicarbonate
$[^{14}C]$ sodium formate
were obtained from the Radiochemical Centre, Amersham.

Hydroxyl apatite (Biogel HT)
Biogel P-150
Biogel P-300
were obtained from BIO-RAD Laboratories Ltd., London.

3. Preparation of Solutions

Assay medium for formic dehydrogenase (A)

NAD (2 μM/ml.)	0.2 ml.
Ammonium formate (2M)	0.2 ml.
Phosphate buffer (0.5M, pH 6.5)	1.6 ml.
Enzyme (diluted with water)	1.0 ml.

Assay medium for the radiochemical estimation of FDH (B)

30 mM $NaHCO_3$ in 0.2M phosphate buffer pH 8.0	2.0 ml.
NADH	0.8 mg.
$[^{14}C]$ -$NaHCO_3$ (52 mCi/mM)	0.1 ml. (100 μ Ci)
Enzyme	1.0 ml.

Assay medium for the radiochemical estimation of FDH (C)

0.2M phosphate buffer saturated with CO_2 (20 mM) pH 6.3)	2.0 ml.
NADH	0.8 mg.
$[^{14}C]$ -$NaHCO_3$ (52 mCi/mM)	0.1 ml. (100 μ Ci)
Enzyme	1.0 ml.

Scintillation fluid (D)

Naphthalene	100 g./lt.
P.P.O.	100 g./lt.
Dioxane	make up to 1 lt.

4. Estimation of enzyme activity.

(a) Spectrophotometrically.

The activity of formic dehydrogenase was determined by following the rate of NADH production spectrophotometrically as observed by the increase in optical density of 340nm, Nason and Little (1955). The solutions (see solution A), were kept at 25°C, and the reaction started by the addition of the enzyme. The increase in optical density was recorded continuously on a Vitatron chart recorder, from a SP800 spectrophotometer with the full scale deflection set at 0.1 optical density units. The initial linear portion of the trace was used to determine the initial velocity. A change of 0.001 optical density units/min. was defined as one unit of enzyme activity. The reaction velocity was found to be proportional to the enzyme concentration up to 300 enzyme units, Fig. 1.

A unit of specific activity (abbreviated SA) was defined as one enzyme unit/mg. of protein.

The activity of alcohol dehydrogenase (ADH) and malate dehydrogenase (MDH), was determined in a similar manner using the conditions described by Theorell and Bonnichsen (1951) and Lowry (1957) respectively.

(b) Radiochemically.

The radiochemical estimation of FDH activity was based upon the measurement of the increase of labelled formate after prior separation of bicarbonate, using concentrated HCl, followed by flushing with air.

The reaction media (B and C) were adjusted to 20°C and the reaction commenced by the addition of the enzyme. At timed intervals 0.4 ml. of the mixture was removed and 50 µL of concentrated HCl added. This stopped the reaction and converted all the bicarbonate to carbon dioxide, which was then flushed out by passing a stream of air through the solution. This displaced the labelled carbon dioxide left in solution by unlabelled carbon dioxide from the air stream. Formate was unaffected by the treatment, as is shown in Table 1.

After flushing the solution with air for two minutes 0.2 ml. was added to 10 ml. of naphthalene based scintillation fluid (solution D) and counted in a Beckman LS-200B scintillation counter. No correction was made for quenching.

Table 1. The effect of conc. HCl followed by flushing with air upon a solution of labelled HCOOH and HCO_3^-

Solution	Treatment	Total cpm.
HCO'_3^- + reaction medium pH = 8.0	none	16,900 ± 5%
	50 µL conc.HCl	2040 ± 5%
	50 µL conc. HCl + flushed with air	34 ± 20%
HCOOH + reaction medium pH = 8.0	none	16,800 ± 5%
	50 µL conc.HCl	17,400 ± 5%
	50 µL conc.HCl+ flushed with air	17,200 ± 5%

5. Estimation of proteins.

Protein concentrations were assayed by the method of Lowry et al. (1951), which is based on the measurement of the optical density at 280 mµ.

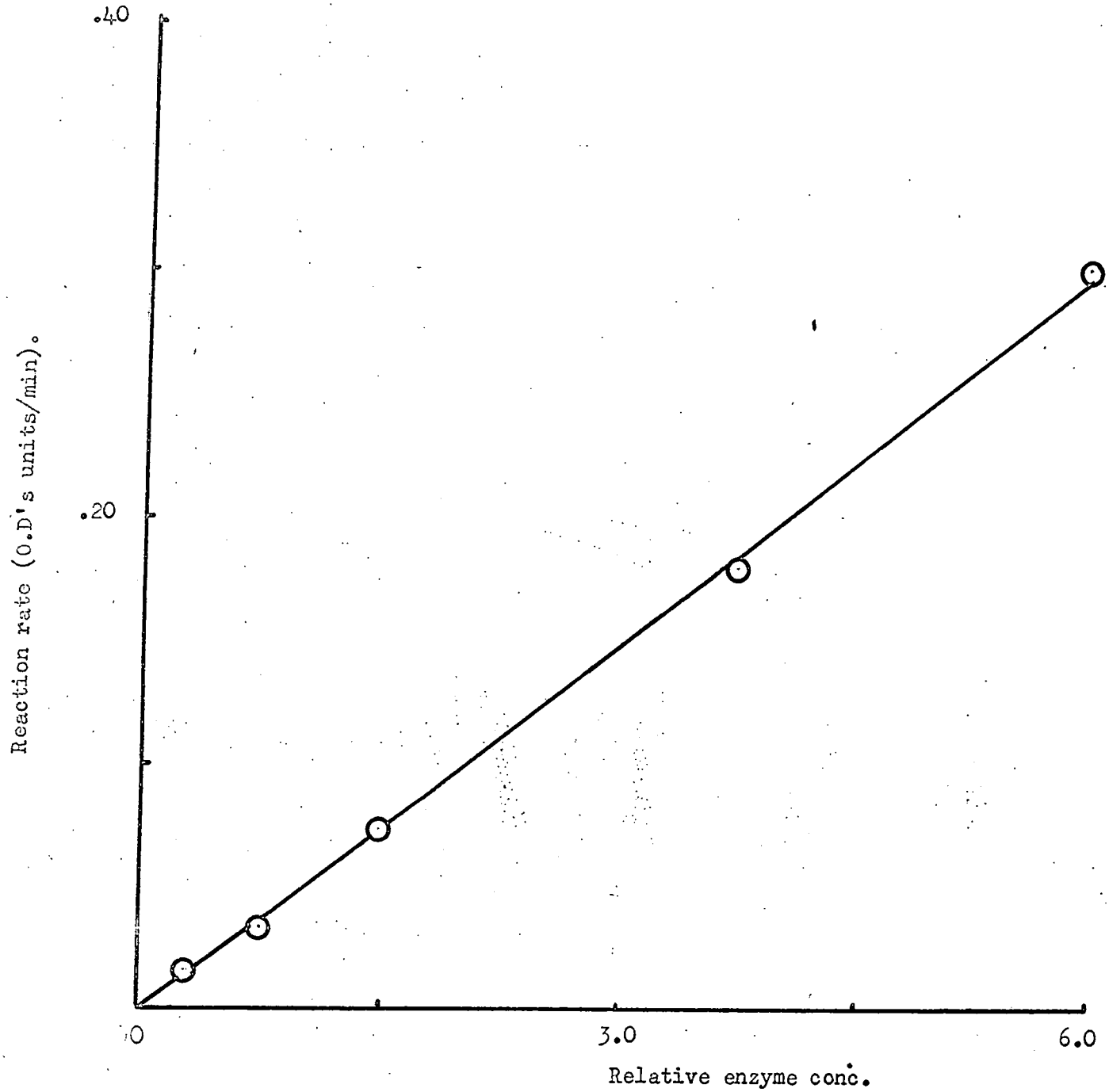


Fig. 1. The dependence of the reaction rate on the conc. of formic dehydrogenase.

6. Extraction of formic dehydrogenase activity from various members of the Leguminosae

The dry seeds were soaked in running tap water for two days and then blended with 5 vol. of 0.5M phosphate buffer adjusted to pH 6.5, in an M.S.E. overhead homogeniser for two minutes. The homogenate was allowed to solubilise for 20 min. before squeezing through one layer of fine terylene mesh and centrifuging for 10 min. at 4000 xg.

7. Formic dehydrogenase of Phaseolus aureus

(a) Estimation of enzyme stability.

(i) pH

Aliquots of fraction III, (Table 20), were adjusted to various pH values and incubated at 25°C. After 30 min. the pH was re-adjusted to 6.5 and the activity remaining was estimated.

(ii) Heat.

1 ml. aliquots of fraction III (Table 20), in thin walled glass tubes, were placed in a water bath at 65°C. The temperature of the enzyme was recorded at timed intervals when a 50 µL sample was withdrawn and immediately mixed with 2.95 ml. of assay medium at 20°C (method 4 (a)). The activity remaining was then measured. This procedure was repeated on fraction III with the addition of 1 mM NAD.

(iii) Dialysis.

The effect of the length of soaking of the beans before extraction, the salt used in extraction, the ionic strength of the salt used in extraction, the pH of the salt used in extraction, the pH of the dialysis medium, the salt used in the dialysis medium, upon the activity after dialysis was tested in the following way:

10 gm. of beans were extracted as in method 6. and dialysed against 10 l. of 0.005M potassium phosphate buffer at pH = 6.5. This method was repeated changing the conditions of one of the variables listed at a time keeping all the other variables constant. The activity of the enzyme was measured before and after dialysis.

(iv) Metal ions.

1 ml. of fraction III (Table 20),was incubated with 1 ml. of a metal ion adjusted to pH = 6.5, for 10 min. at 25°C. 100 µL samples was taken after incubation and assayed to determine the amount of activity remaining.

(v) Low temperature storage.

10 ml. aliquots of fraction III (Table 20), were subjected to various forms of low temp. storage and the amount of activity remaining determined.

(b) Purification.

(i) Extraction.

10 gm. samples of beans were extracted using method 6 except that one of the following:

length of homogenisation, ionic strength of extracting salt, volume of extracting buffer per weight of seed, length of solubilisation of the homogenate, extracting salt, length of soaking of the beans before extraction, pH of the extracting buffer were varied systematically one at a time. In each case the total yield of enzyme activity was estimated.

(ii) Salt fractionation.

Fraction I (table 20) was brought to the correct temperature and adjusted to the required pH by the addition of a solution of either

K_2HPO_4 or KH_2PO_4. Solid ammonium sulphate (low in heavy metals), was ground to a fine powder and added slowly with vigorous stirring to bring the solution up to the required ionic strength (see Dixon(1953) for the conversion tables from % saturation to gm. /l.) After 1 hr. equilibration, the solution was centrifuged at 20,000 x g. for 30 min. and the supernatant was decanted from the resulting precipitate. In measuring the solubility of the enzyme, the supernatant was assayed for activity.

(iii) Ion exchange chromatography.

The binding equilibria were determined by the following method. 1 g. of wet DEAE-cellulose equilibrated with 0.005M buffer was placed in a 10 ml. centrifuge tube. 9 ml. of buffer and 1 ml. of enzyme (fraction II, see Table 23) were added and mixed by inversion. After equilibrating for a set time the tube was centrifuged and the supernatant assayed. The amount of enzyme bound to the resin was assumed to be the difference between the enzyme remaining in the supernatant and that added originally. The supernatant was then decanted and 10 ml. of fresh buffer added, mixed by inversion, equilibrated for a set time, centrifuged and then assayed. All these procedures were carried out at $0^{\circ}C$. To determine the kinetics of absorbtion the procedure was repeated and the time of equilibration varied; the changes in binding efficiency with ionic strength were determined by varying the ionic strength of the buffer, and the distribution isotherm by varying the concentration of enzyme added.

Columns were packed by pouring a thick slurry of resin into the column and packing under a head of buffer. The columns were run at $0^{\circ}C$ with a head of buffer of approximately 30-60". The resin was regenerated as described in the Whatman technical bulletin IE2.

The gradient used in gradient elution was produced by the apparatus described by Bock and Ling (1954) and was a concave exponential gradient conforming to equation 1.

$$C_v = C_1 - (C_1 - C_2)\left(1 - \frac{v}{V_t}\right)^P \quad \text{..... eq. 1.}$$

where C_v = concentration of eluent after volume v has flowed through the apparatus

C_1 = conc. in resevoir A

C_2 = conc. in resevoir B

V_t = total volume in both containers

P = $\frac{\text{area of cross section of container A}}{\text{area of cross section of container B}}$

The apparatus consisted of two different sized beakers open to the atmosphere and connected by a siphon. The larger beaker fed into the column and contained a stirrer. In this case the beakers used were 4 l. (container B) which was filled with 2 l. of 0.005M buffer and a 1 l. beaker (A) which contained 800 ml. of 0.5M buffer. The ratio of the cross sectional areas is therefore 0.8/2.0 (P) = 0.40.

(iv) Hydroxyl apatite chromatography.

The binding equilibria were determined in a similar manner to those on DEAE (7b, (iii)), except that the buffer was phosphate at pH = 6.5.

The column was packed under pressure but was operated with a hydrostatic head of 6-10" only. Hydroxyl apatite was used at 0°C throughout.

(v) Alumina gel chromatography.

The wet gel was stirred into the enzyme solution and equilibrated for 15 min. It was then centrifuged and the supernatant

assayed. Further gel was added until only 10% of the original activity remained in the supernatant. The enzyme was then eluted by equilibration with successive portions of buffer of increasing ionic strength, allowing 15 min. equilibration with every portion, and pooling those supernatants which contained more than 8% of the activity. The entire operation was carried out at $0^{\circ}C$.

(vi) Acrylamide gel electrophoresis.

The analytical gel electrophoresis used was as described by Ornstein and Davis (1962). Protein was detected by staining with amido black and destaining electrolytically, enzyme was located in an incubation mixture containing MTT-tetrazolium. The bands of both enzyme and protein stain were recorded using a Joyce-Lobel chromoscan.

The preparative gel electrophoresis used was following the method of Lewis et al. (1968) using the Quickfit and Quartz apparatus and recording protein elution continuously with an Isco U.V. analyser.

The L.K.B. apparatus was operated as described in the manual of L.K.B. Ltd., with continuous protein monitoring using an Isco U.V. analyser.

(vii) Gel filtration.

The column was prepared by pouring a slurry of P-300 or P-150 (polyacrylamide) into the column (150 x 2 cm.) and packing under pressure. The hydrostatic head used for filtration was 20-30".

(viii) Purification scheme.

1 Kg. of beans were soaked in running tap water for 3 days, blended with 1 l. of 1M ammonium formate for 4 min. in a large capacity

waring blender, and allowed to solubilise for 20 min. The homogenate was squeezed through one layer of fine terylene mesh and centrifuged at 4000 x g. for 10 min. The resulting supernatant was subjected to ammonium sulphate fractionation at 20°C and pH 6.2 in the manner described in section 7b (ii) collecting the 30-50% saturation precipitate. The precipitate was re-dissolved in a minimum volume of distilled water (labelled fraction I for the sake of convenience) and dialysed against 10 l. of 0.005M tris/Cl pH 8.6. The volume of fraction I was usually 100-200 ml. After dialysis the volume had changed to 200-300 ml. and this fraction was labelled II. Fraction II was applied to a 60 x 4 cm. column of DEAE/Cl equilibrated to 0.005M with tris/Cl at pH 8.6. The column was developed with a concave exponential gradient (see section 7b (iii)) and 15 ml. fractions collected using a flow rate of 400 - 600 ml./hr. The tubes containing enzyme activity were pooled and the 40-60% saturation precipitate on ammonium sulphate fractionation was re-dissolved in the minimum volume of distilled water (fraction III). Fraction III was dialysed against 10 l. of 0.005M phosphate buffer at pH 6.5 (fraction IV). Fraction IV (approx. 40 ml.) was applied to a 15 x 2 cm. column of hydroxyl apatite equilibrated with 0.005M phosphate at pH 6.5. The column was developed in a stepwise manner by 2 - 3 column volumes, of phosphate buffer of increasing ionic strength. The enzyme activity in the 0.06M step was collected in 5 ml. fractions at a flow rate of 10 ml./hr. The enzyme was then dialysed against 10 l. of 0.005M phosphate buffer at pH 6.5 (fraction V). Fraction V was then fractionated on alumina as described in 4b (vi). Fractions II-VI were always kept at 0°C.

(d) Kinetics.

(i) Purification of NAD.

In all kinetic work NAD was purified by the method of Dalziel (1963).

(ii) Estimation of initial velocity.

The initial velocity was measured by following the change in optical density at 340 mu in a quartz cuvette of 4 cm. light-path. The incubation mixture had a total volume of 10 ml., contained phosphate buffer at 100 mM, and substrate and inhibitor at the concentrations shown in the various legends. The enzyme concentration was 2-4 x 10^{-8}M of fraction V or VI, all solutions were kept in a water bath at 25^{o}C which also circulated within the SP800 spectrophotometer. The initial velocity was taken as the tangent drawn to the initial portion of the continuous trace which was recorded on a Vitatron chart recorder which had a full scale deflection of 0.1 O.D. units (10"). The reaction was started by addition of the enzyme (100 µL) the trace was begun within 10 sec. of addition of the enzyme, and was continued for at least 2 min.

(iii) pH optimum.

Assuming that 200 mM HCOOH and 1 mM NAD to be saturating concentrations of substrate (assumption later proved correct, Figs. 51, 53), the velocity of the reaction at fixed enzyme concentration was measured at different pH's in 100 mM phosphate buffer.

(iv) Inhibitors.

The effect of various inhibitors was measured on the reaction rate, using saturating concentrations of substrate. (200 mM HCOOH and 1 mM NAD). The pH was kept constant at 6.5 with 100 mM phosphate buffer.

(v) Statistical analysis.

The kinetic data were analysed by making direct fits to the assumed rate equations, using the statistical procedure of Wilkinson (1961). To perform the lengthy calculations involved, the appropriate computer programmes of Cleland (1963) were used on an IBM 360/67 computer. The double reciprocal plots were drawn by fitting the points of each line to equation 2 with the HYPER computer program. The double reciprocal plots were then inspected to determine the pattern, and then having decided the pattern (Cleland 1963a) the data were fitted to one of the following equations (2 - 8):

$$v = \frac{Vm.A}{K + A} \qquad \ldots\ldots \text{eq. 2}$$

$$v = \frac{VAB}{Kia.Kb + Ka.B + Kb.A + AB} \qquad \ldots\ldots \text{eq. 3}$$

$$v = \frac{VA}{K(1 + I/Kis) + A} \qquad \ldots\ldots \text{eq. 4}$$

$$v = \frac{VA}{K + A(1 + I/Kii)} \qquad \ldots\ldots \text{eq. 5}$$

$$v = \frac{VA}{K(1 + I/Kis) + A(1 + I/Kii)} \qquad \ldots\ldots \text{eq. 6}$$

$$v = \frac{VA}{K(1 + I/Ki_1 + I^2/Ki_2) + A} \qquad \ldots\ldots \text{eq. 7}$$

$$v = \frac{VA}{K(1 + I/KiSA + I^2/KiSB) + A(1 + I/Kii)} \qquad \ldots\ldots \text{eq. 8}$$

Equation 2 is used when only one variable is analysed, equation 3 represents the initial velocity rate equation for a sequential mechanism, equation 4 represents competitive inhibition, equation 5 - uncompetitive inhibition, equation 6 - noncompetitive inhibition, equation 7 - parabolic competitive inhibition, equation 8 S-para, I-lin, noncompetitive inhibition.

(vi) Variation of the maximum velocity with temperature.

The maximum velocity (V_1) was measured at different temperatures by equilibrating both the constant temperature housing of the spectrophotometer and all the solutions to the required temperature using the same water bath. At the highest temperature measured the substrate concentrations were doubled from 200 mM HCOOH and 1 mM NAD to 400 mM HCOOH and 2 mM HCOOH and the velocity measured. No increase in velocity was noticed, the substrate concentrations were therefore saturating.

(vii) Nomenclature and derivation of rate and kinetic equations.

The nomenclature and equations are those proposed by Cleland 1963b. For the sake of convenience part of that work is repeated here. A and B are substrates designated in the order they bind to the enzyme. P and Q are products designated in the order in which they are released by the enzyme.

V_1 is the maximum velocity in the forward direction.

V_2 is the maximum velocity in the reverse direction.

Ka, Kb, Kp, Kq are the Michaelis constants.

Kia, Kib, Kip, Kiq are all inhibition constants.

k_1, k_2, k_3, etc. are all rate constants.

E, F represents free enzyme forms.

EA, EAB, EPQ represent enzyme substrate complexes.

Uni, Bi, Ter are the number of kinetically important substrates or products.

'Ordered' refers to a reaction where the substrates react in an obligatory order.

'Sequential' refers to reaction where all the substrates must bind to the enzyme before any product is released.

Random refers to reaction in which there is no obligatory order of addition or release of products.

(AAB), (EPQ) are transitory complexes which cannot undergo bimolecular reactions.

The initial rate equations were derived by the method of King and Altman (1956) and transformed into kinetic coefficients by Cleland (1963b).

(e) Metabolic Role.

(i) Preparation of mitochondria.

Beans were soaked for three days before the mitochondria were extracted by the method of Ikuma and Bonner (1967).

(ii) Estimation of oxygen uptake.

Oxygen uptake was measured using a Rank oxygen electrode at 30°C. The reaction medium used for mitochondrial oxygen uptake was that described by Ikuma and Bonner (1967). The reaction rate was converted into um O_2 per min by using the solubility of oxygen in aqueous solutions.

(iii) Germination of Phaseolus aureus.

The beans were soaked overnight in running tap water, then planted in small wooden seed trays in John Innes No. 1 compost. Germination was continued under glass with natural lighting.

(iv) Estimation of Formic acid.

Formic acid was estimated by the method of Grant (1948). A standard line was prepared using known amounts of ammonium formate (Fig. 2).

In estimating formic acid in plant tissues the acid was separated using low temperature distillation under reduced pressure (Grant 1948). The efficiency of this method was determined using a known amount of ammonium formate and found to be 95-100% recovery.

(v) Separation of organic acids.

Organic acids were separated on Whatman No. 3 chromatography paper using the solvents of Smith (1960). 100 μ L of sample was applied to each chromatogram. After separation the resulting spots were identified by comparison of their positions with those of known amino acids separated under identical conditions.

(vii) Separation of amino acids.

Amino acids were separated in two dimensions on Whatman No. 3 chromatography paper using the solvents of Smith (1960). 100 μL. of sample was applied to each chromatogram.

(viii) The in vivo incubation with $[^{14}C]$HCOOH.

1 g. of 3 day cotyledons were sliced and incubated in small tubes with 2 ml. of phosphate buffer 50 mM at pH 6.5 and 100 μL of sodium formate (44 mCi/mM) containing 0.1 mCi of $[^{14}C]$. The tubes were kept at room temperature in the dark with a stream of air bubbled through the solution and then led into 5 M KOH to absorb $[^{14}C]$ CO_2 produced. After a fixed period of time, cotyledons were separated from the solution and extracted in hot 80% ethanol. The volume of the extract was reduced to 1 ml. and 100 μ L samples were used in the separation and autoradiography.

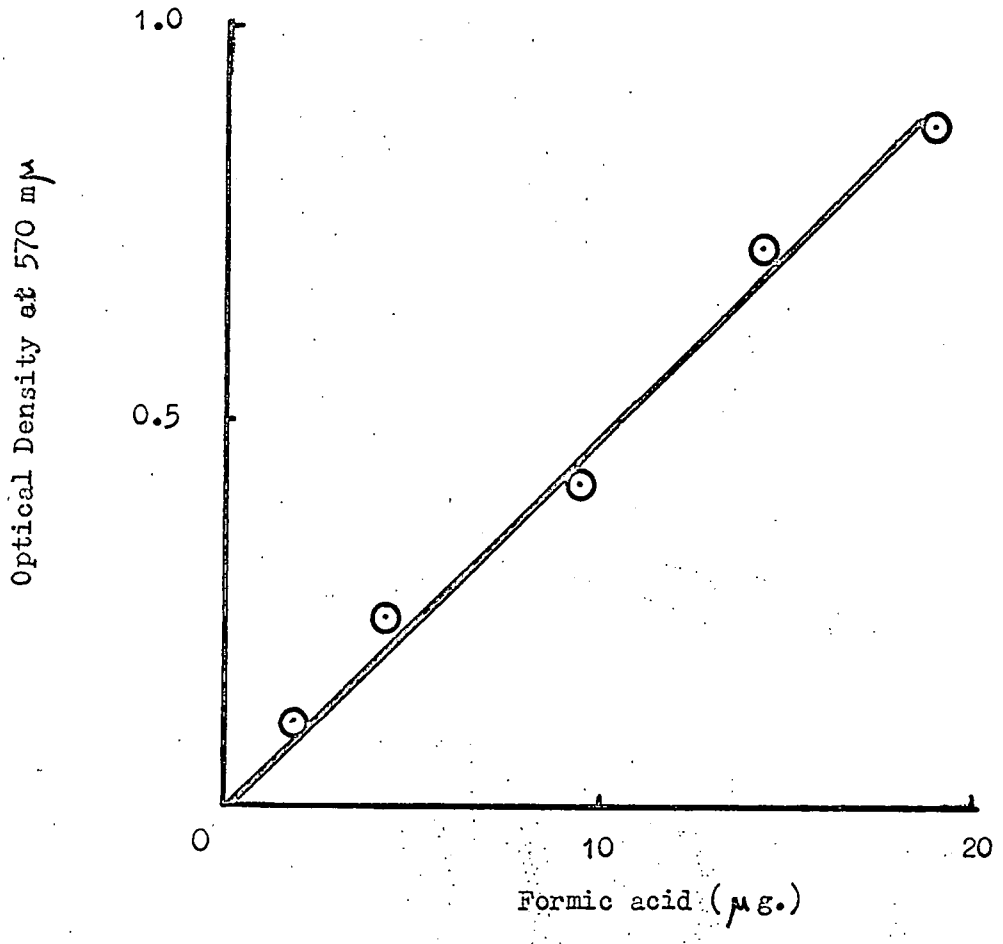


Fig. 2. The Colorimetric estimation of Formic acid.

(e) Molecular weight.

The molecular weight of FDH was determined by the method of Andrews (1965) on Sephadex G-200, equilibrated with 200 mM phosphate buffer at pH 6.5. The column dimensions were 100 x 2 cm., and it was operated at a flow rate of 10 ml./hr.

RESULTS

1. The activity of FDH in various members of the Leguminosae.

A large difference was found in the amount of enzyme activity that could be extracted from the soaked seeds of certain members of the Leguminosae (Table 2). Of those tested Phaseolus aureus was found to be the richest source, and Vicia faba the poorest with 60 times less activity than the former.

Table 2. The activity of formic dehydrogenase in various members of the Leguminosae

Species	enzyme activity/g.dry wt.
Phaseolus aureus	6000
Phaseolus coccineus	4500
Phaseolus vulgaris	2300
Pisum sativum	2300
Lathyrus ordoratus	1400
Arachis hypogea	1000
Canavalia ensiformis	450
Vicia faba	100
Trifolium alba	*

*The mucilaginous coating of this species prevented extraction.

2. Formic dehydrogenase of Phaseolus aureus.

(a) The stability of enzyme activity.

(i) pH.

The enzyme was stable to pH above 5.8, but below 5.8 increasing losses in enzyme activity were encountered with decreasing pH;at pH 5.2

no activity could be detected (Table 3). The addition of 1 mM NAD increased the stability at pH 5.4 and 5.6 but did not change the stability at pH 5.2.

(ii) Heat.

The activity of the enzyme was found to be sensitive to heat. At 60°C for 1 minute there was little loss of activity, but after 3 minutes the loss of activity was 100%. The addition of 1 mM NAD increased the stability at 60°C to 2 minutes with a 70% loss within 3 minutes (Table 4).

(iii) Dialysis.

The loss of activity encountered on the dialysis of formic dehydrogenase was affected by both the conditions of extraction and dialysis (Tables 5 - 11). The greatest influence on the activity recovered after dialysis was the length of soaking of the beans before extraction, and the salt used during extraction and dialysis (Tables 10, 5 and 8). Table 11 summarises the maximum variation that changes in the conditions of extractions and dialysis produced.

Table 3. The effect on the activity of FDH of 30 minutes incubation at various pH's

pH	% activity remaining -NAD	+ 1 mM NAD
8.5	100	100
7.5	100	100
6.5	100	100
5.8	100	100
5.6	73	100
5.4	20	57
5.2	0	0

The temperature was constant at 25°C.

Table 4. The effect on the activity of FDH on incubation at high temperatures

Temperature (°C)	Time (sec.)	% activity remaining -NAD	+ NAD (1mM)
5	0	100	100
50	45	100	110
60	120	40	102
62	180	0	75
62	240	0	60

The pH was constant at 6.5.

Table 5. The influence of the salt species used during extraction on the recovery of enzyme activity after dialysis

Extracting salt.	% recovery from dialysis
tris/maleate	63
ammonium formate	60
potassium phosphate	54
tris/Cl	51
KCl	51
distilled water	45
phosphate/citrate	43

Table 6. The influence of the pH of the extracting buffer on the recovery of enzyme activity after dialysis

pH	% recovery on dialysis
6.5	51
7.5	46
8.5	43

Table 7. The influence of the ionic strength of the extracting buffer on the enzyme recovery after dialysis

ionic strength (M./l.)	% recovery of dialysis
4.0	55
2.0	52
1.0	50
0.4	67

Table 8. The influence of the buffer salt used in dialysis on the recovery of enzyme activity after dialysis

buffer salt used in dialysis	% recovery on dialysis
cysteine/HCl	84
ammonium formate	82
distilled water	78
NAD	74
potassium phosphate	74
ascorbic acid	71
EDTA	44

Table 9. The influence of the pH used in dialysis on the recovery of enzyme activity after dialysis

pH	% recovery on dialysis
6.5	74
7.5	71
8.5	67

Table 10. The influence of the length of presoaking of the beans on the recovery of enzyme activity after dialysis

length of soaking (hr.)	% recovery on dialysis
24	46
45	56
70	64
94	68
118	70

Table 11. A summary of the optimum conditions for the dialysis of FDH.

variable	optimum	max. % variation
length of soaking of the beans	118 hr.	37
extraction salt	tris/maleate	33
ionic strength of extractant	0.4M	27
salt used during dialysis	cysteine/HCl	17*
pH used during extraction	6.5	16
pH used during dialysis	6.5	5

*The effect of EDTA was unique, and is not included in estimating this figure.

(iv) Metal ions

The activity of the enzyme was found to be affected by various metal ions over a period of 10 minutes. Ca^{++}, Cd^{++}, Co^{++}, Cu^{++}, Cr^{+++}, Hg^{++}, Fe^{++}, Zn^{++}, were found to cause loss of activity (Table 12).

Table 12. The effect on the activity of FDH of incubation with metal ions

Metal	conc. (M)	% activity remaining after 10 min.
Na^{+}	10^{-1}	100
Fe^{+++}	10^{-4}	100
Mg^{++}	10^{-2}	100
Mn^{++}	10^{-2}	100
Fe^{++}	10^{-3}	77
Zn^{++}	10^{-3}	77
Ca^{++}	10^{-3}	60
Co^{++}	10^{-2}	66
Cr^{+++}	10^{-2}	55
Cd^{++}	10^{-2}	43
Cu^{++}	10^{-2}	40
Hg^{++}	10^{-5}	0

(v) Low temperature storage.

Of the various forms of low temperature storage tried, storage in solution at 4°C with added chloroform caused no loss of enzyme activity over a period of one week. Over longer periods storage at -20°C was the most effective in reducing losses of enzyme activity. Freeze drying caused large losses of enzyme activity during the drying process but no losses in activity occurred during storage of the dry powder (Table 13).

Table 13. The effect on the activity of FDH of various forms of low temperature storage.

Storage conditions	period of storage in days	% activity remaining
4°C + 1:1000 v/v chloroform	7	100
-20°C	1	90
-20°C	14	84
4°C + 1:1000 v/v chloroform	14	70
4°C in solution	7	70
freeze drying	1	35
freeze drying	14	35

(b) Purification

(i) Extraction.

A series of pilot extractions were carried out under different conditions, and from the results shown in Tables 15 - 21, it can be seen that the yield of enzyme activity has an optimum with respect to length of homogenisation; ionic strength of the extractant; volume of extractant used per gram of tissue; length of the extraction; length of the soaking of the beans before extraction; and the buffer salt. Only a slightly greater yield obtained using different pH's of extraction.

Table 14 summarises the optimum conditions for the extraction of enzyme activity from soaked beans.

Table 14. A summary of the optimum conditions for the extraction of enzyme activity from pre-soaked beans

Variable	optimum	% max. variation in enzyme activity
length of homogenisation	60 secs.	100
ionic strength of extractant	4M	40
volume of buffer: wt of tissue	9	38
length of extraction	20	33
buffer salt	Ammonium formate	26
length of soaking	2 days	25
pH of extracting buffer	6.5	10

Table 15. The effect of pH of the extracting buffer on the yield of enzyme activity

pH	yield (enzyme units/g.dry wt.)
6.5	5000
7.5	4500
8.5	4500

Table 16. The effect of the extracting salt on the yield of enzyme activity

extracting salt	yield (enzyme units/g. dry wt.)
ammonium formate	5400
potassium chloride	5200
potassium phosphate	5000
tris/maleate	4900
K^+/phosphate/citrate	4000

Table 17. The effect of the length of homogenisation on the yield of enzyme activity

length of homogenisation (sec.)	yield (enzyme units/g.dry wt.)
0	0
5	2900
15	4800
60	5400
120	5400

Table 18. The effect of the ionic strength of ammonium formate on the yield of enzyme activity

Molarity	yield (enzyme units/g.dry wt.)
0	3300
0.1	3600
0.5	4200
1.0	4600
2.0	5000
4.0	5400

Table 19. The effect of the volume of extractant used per gram dry weight of tissue on the yield of enzyme activity

volume of extractant (ml./g. dry wt.)	yield (enzyme units/g. dry wt.)
2.0	4000
7.0	5000
10	6200
16	6200

Table 20. The effect of the length of extraction on the yield of enzyme activity

length of extraction (min.)	yield (enzyme units/g. dry wt.)
0	2800
20	4800
40	4800
60	4800

Table 21. The effect of length of soaking of the beans on the yield of enzyme activity

length of soaking (days)	yield (enzyme units/g.dry wt.)
0	4000
1	5000
2	5600
3	5400
4	5200

(ii) Salt fractionation.

The solubility of the enzyme was determined under different conditions (Figs. 3 and 4) and the results show the linear relationship between the log. of the enzyme solubility and the ionic strength of ammonium sulphate, predicted by Cohn (1925). Table 22 summarises the yield and specific activity obtained when the solubility of the enzyme is reduced by 90% under the same conditions used in the determination of the solubility curves.

Table 22. The effect of different conditions of pH and temperatures upon the salt fractionation of FDH

pH	temp. (^{o}C)	% yield after precipitation	S.A. of ppt.
6.2	20	72	52
6.5	20	40	80
6.5	4	45	40

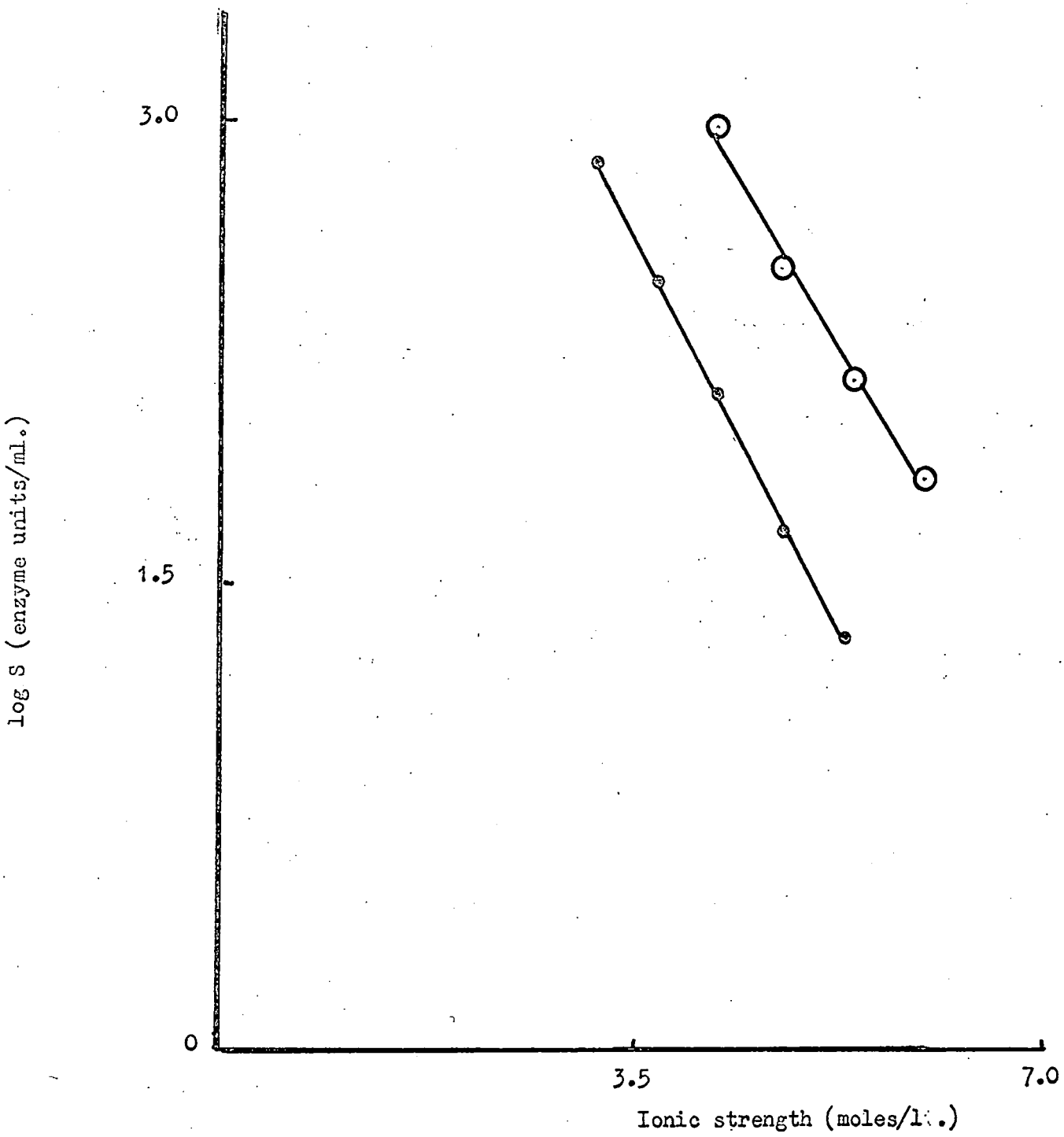


Fig. 3. Variation in the solubility of Formic dehydrogenase in ammonium sulphate at 20°C.

pH = 6.5

pH = 6.2

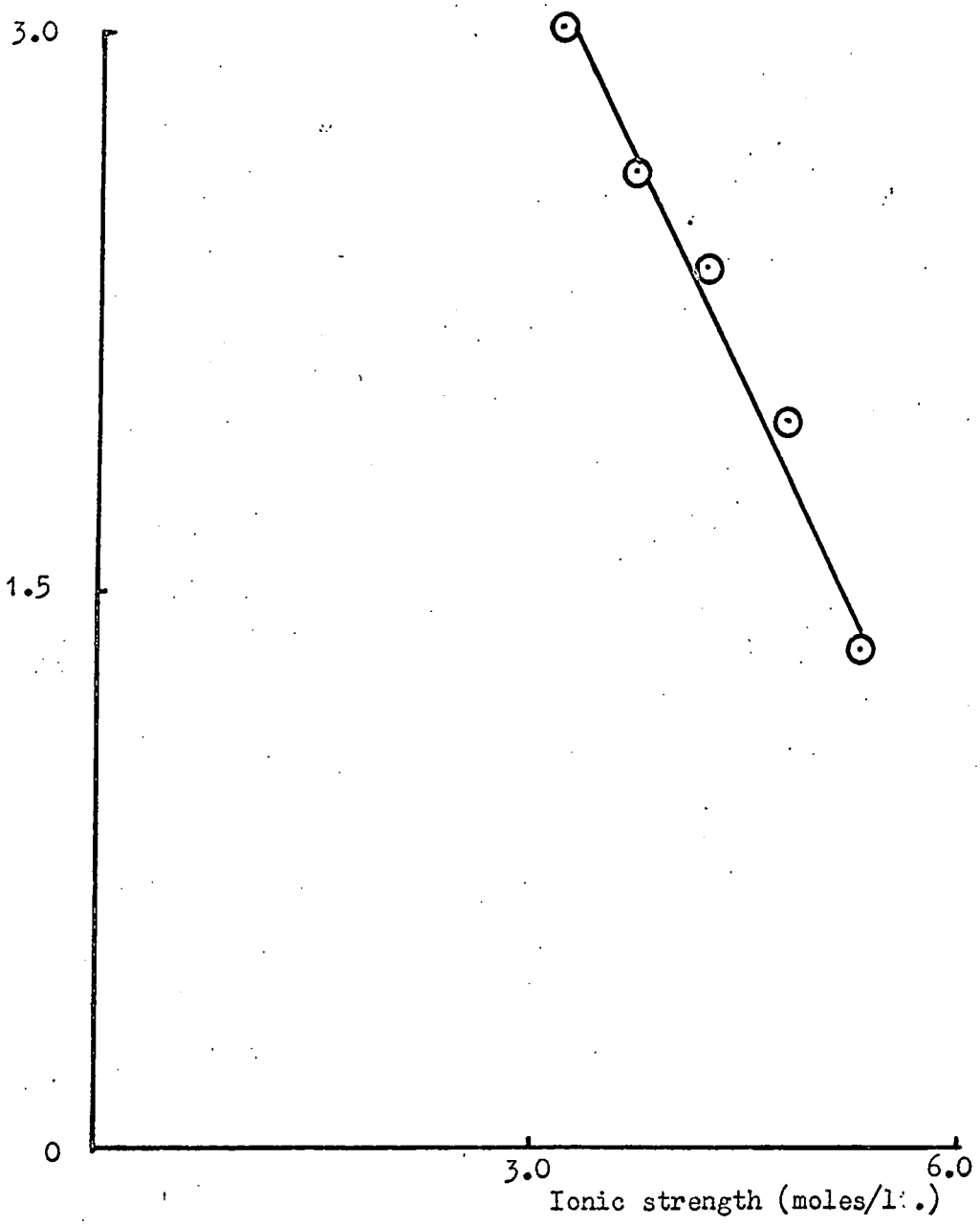


Fig. 4. Variation in the solubility of formic dehydrogenase in ammonium sulphate at 2°C.

The pH was adjusted to 6.5

(iii) Ion exchange chromatography.

The kinetics of absorbtion and desorbtion, the mixed distribution isotherm, and the changes in binding capacity with ionic strength on DEAE-cellulose were determined for the enzyme under differing conditions (Figs. 5 - 10).

The kinetics of absorbtion and desorbtion show that there is little qualitative difference between the two buffer systems (Figs. 5 & 6), and that 70% of the enzyme is absorbed or desorbed in 5 minutes.

The mixed distribution isotherms in the two buffer systems show differences, both are non linear but phosphate buffer produces an isotherm which is only slightly curved (Figs. 7 & 8).

The changes in binding capacity with ionic strength show little differences in the two buffer systems, except for the higher total capacity found with phosphate buffer (Figs. 9 & 10.)

The separation of enzyme activity from protein during zonal separation of fraction II (see Table 23) is shown in Figures 11 and 12. The purification achieved is greater using the tris/chloride buffer system even though the trailing of the enzyme peak is greater. The separation was found to be greater using gradient elution and Figure 12 is a typical elution profile.

(iv) Hydroxyl apatite chromatography.

The kinetics of the absorbtion/desorbtion of the enzyme to hydroxyl apatite show that although a large proportion of the enzyme is bound within 5 minutes there is still a measurable amount of binding occurring after 60 min. (Fig. 14).

The mixed distribution isotherm (Fig. 15) is linear and the changes in binding capacity with ionic strength are small compared to those on DEAE.

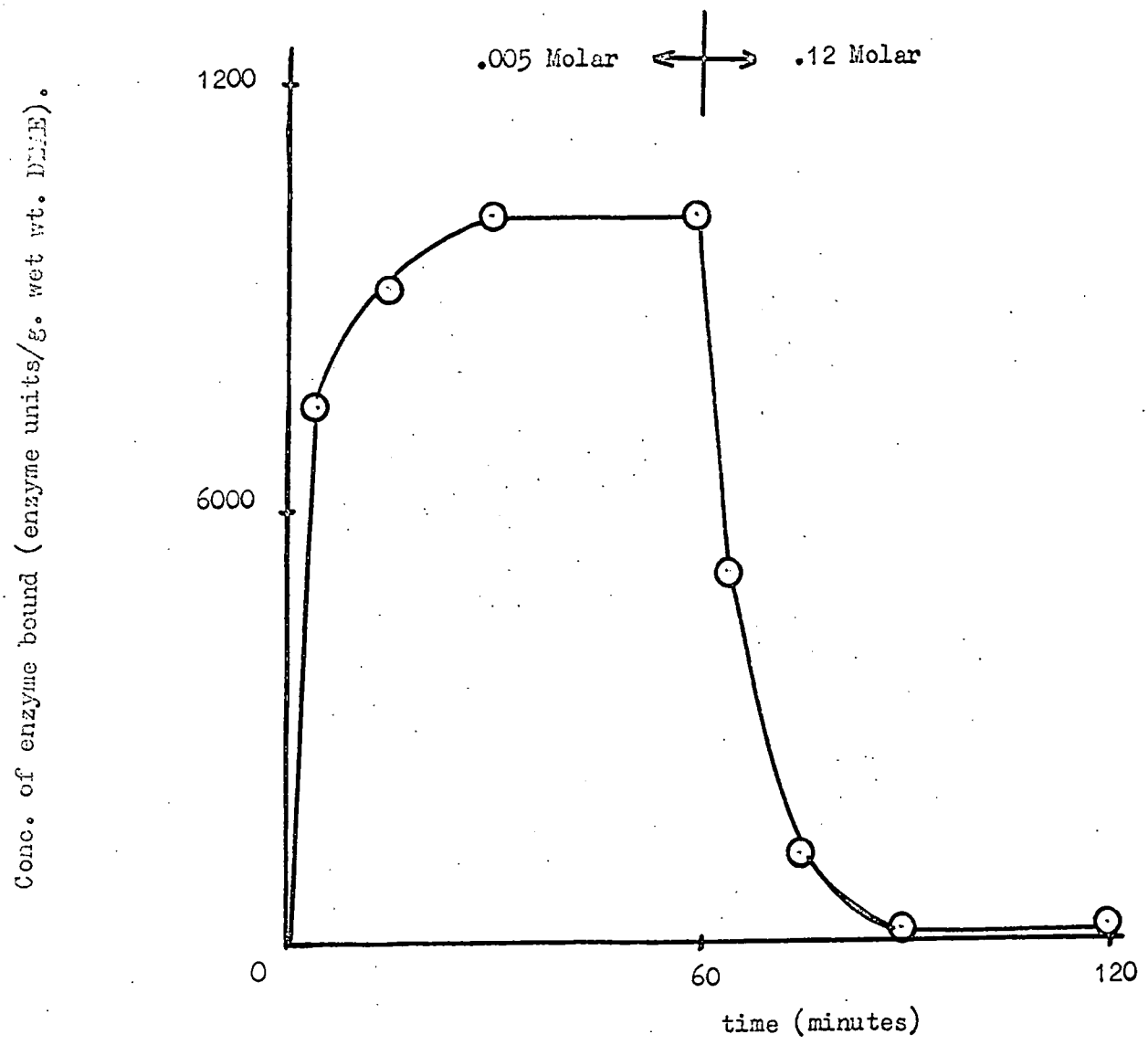


Fig. 5. The kinetics of absorbtion/desorbtion of formic dehydrogenase on DEAE/Phosphate.

pH = 6.5

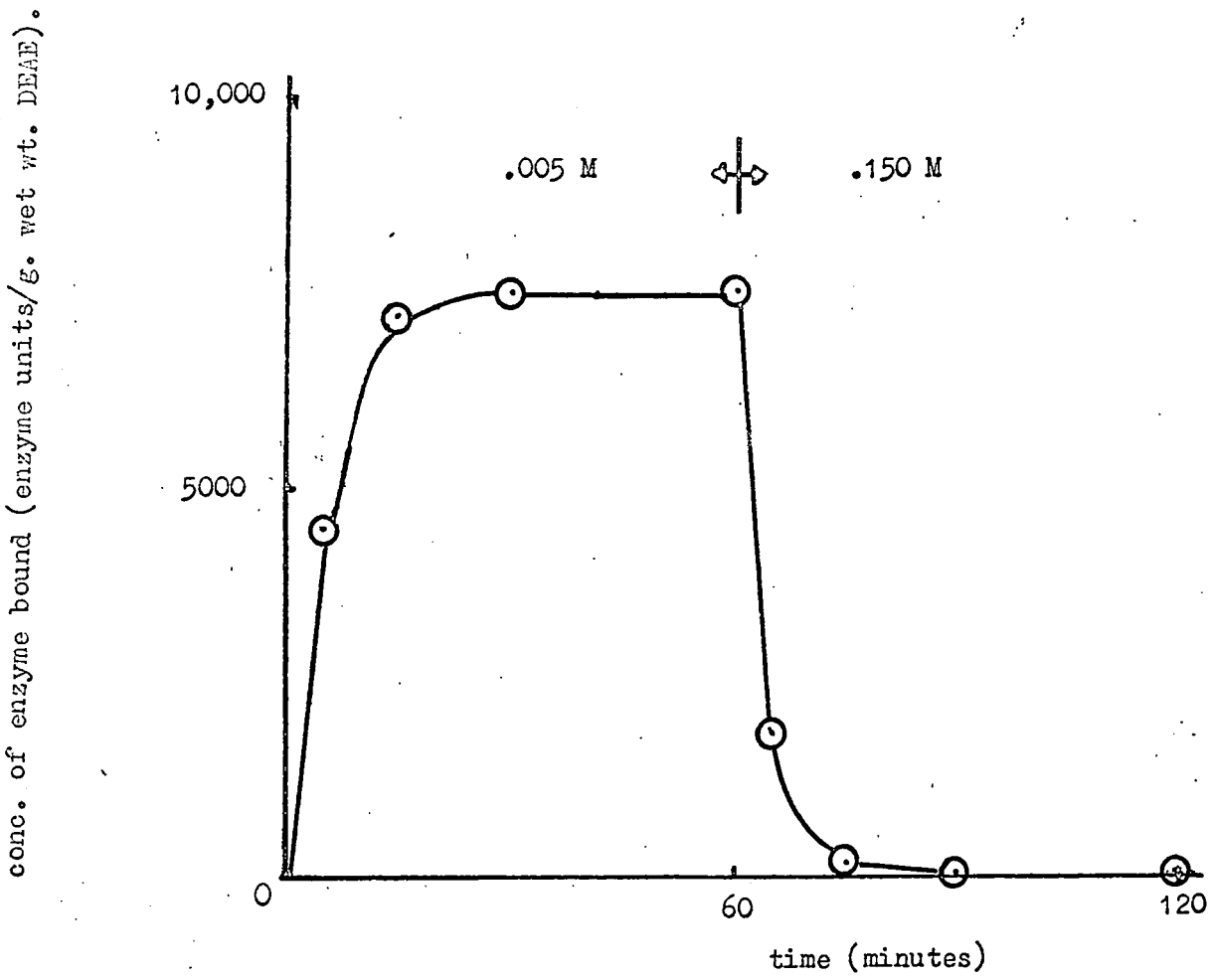


Fig. 6. The kinetics of absorbtion/desorbtion of formic dehydrogenase on DEAE/Chloride.

pH = 8.6

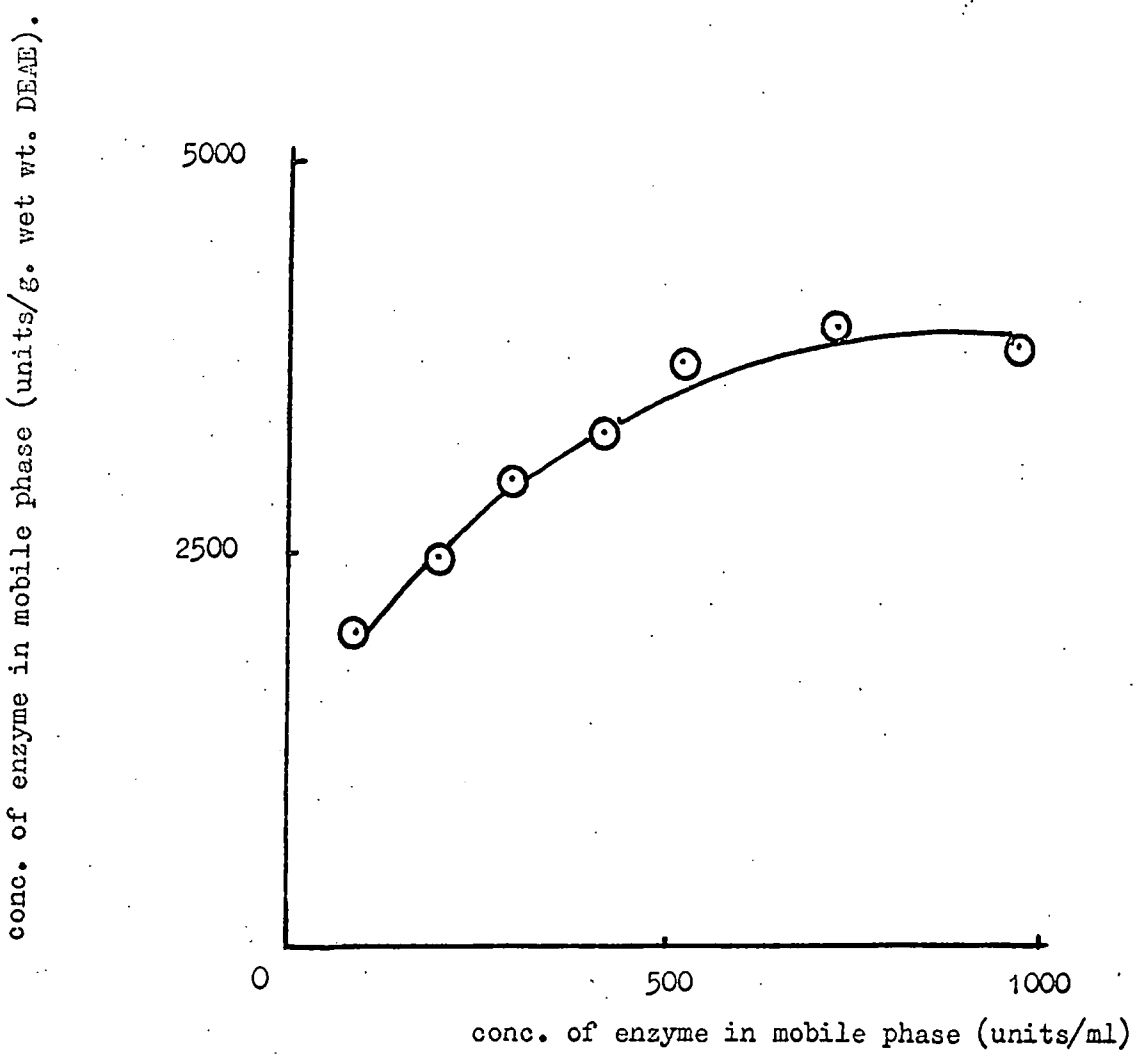


Fig. 7. The mixed distribution isotherm of FDH on DEAE/Chloride.

.01M tris/Chloride buffer at pH 8.6

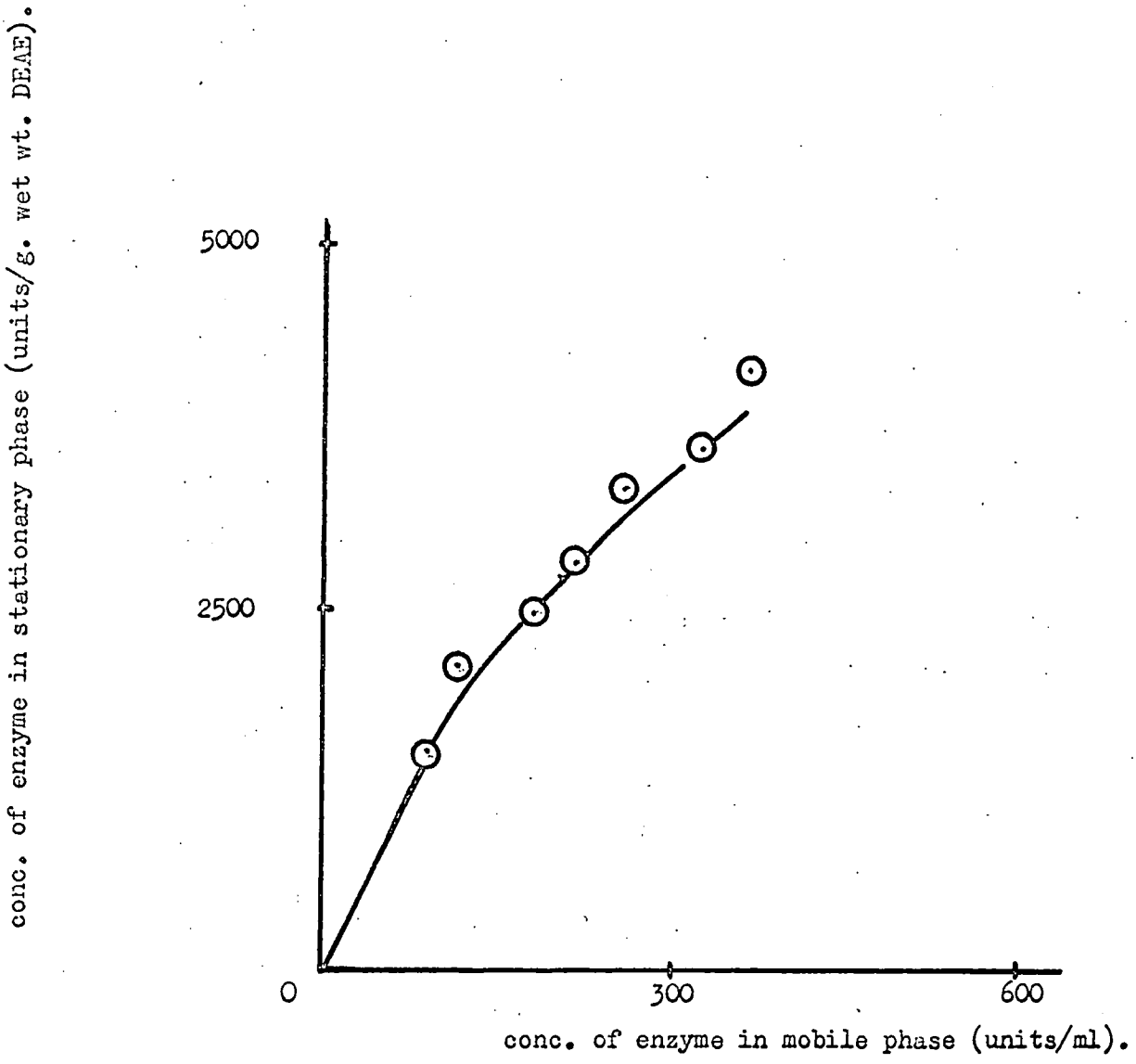


Fig. 8. The mixed distribution isotherm for FDH on DEAE/Phosphate.

.01M Phosphate buffer at pH 6.5

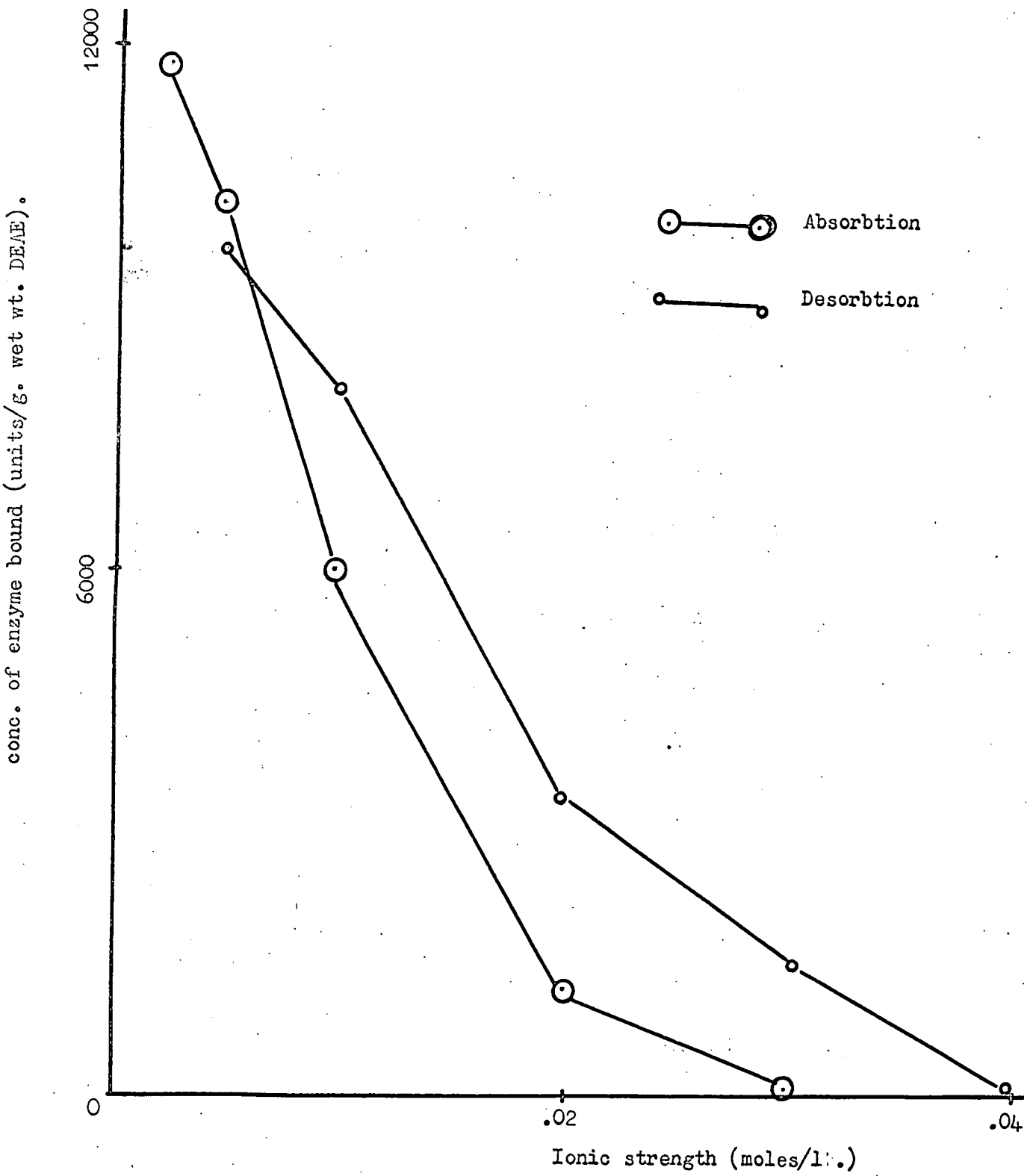


Fig. 9. Changes in the binding capacity of DEAE/phosphate for FDH with ionic strength.

pH = 6.5

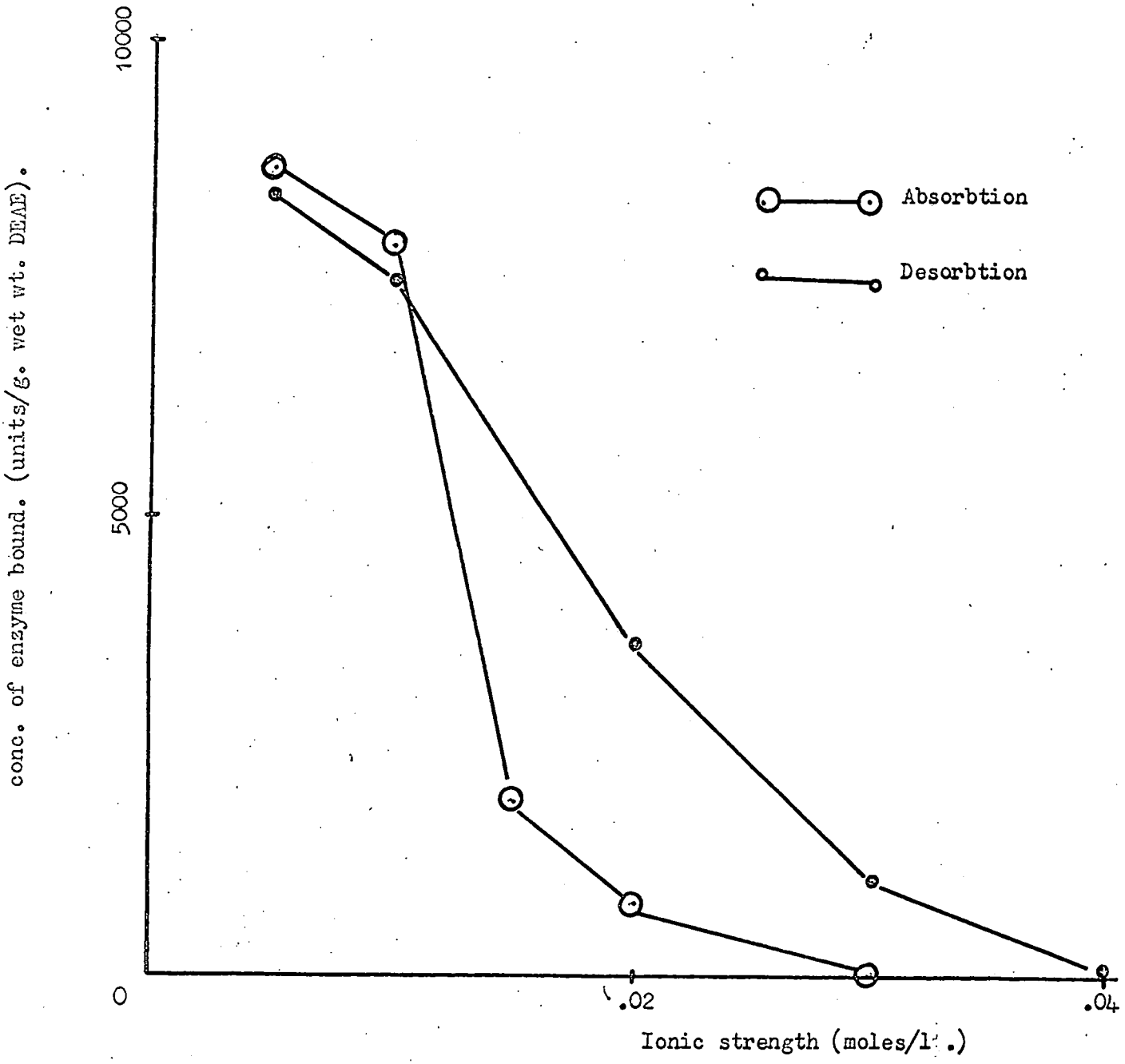


Fig. 10. Changes in the binding capacity of DEAE/Chloride for FDH with ionic strength.

pH = 8.6

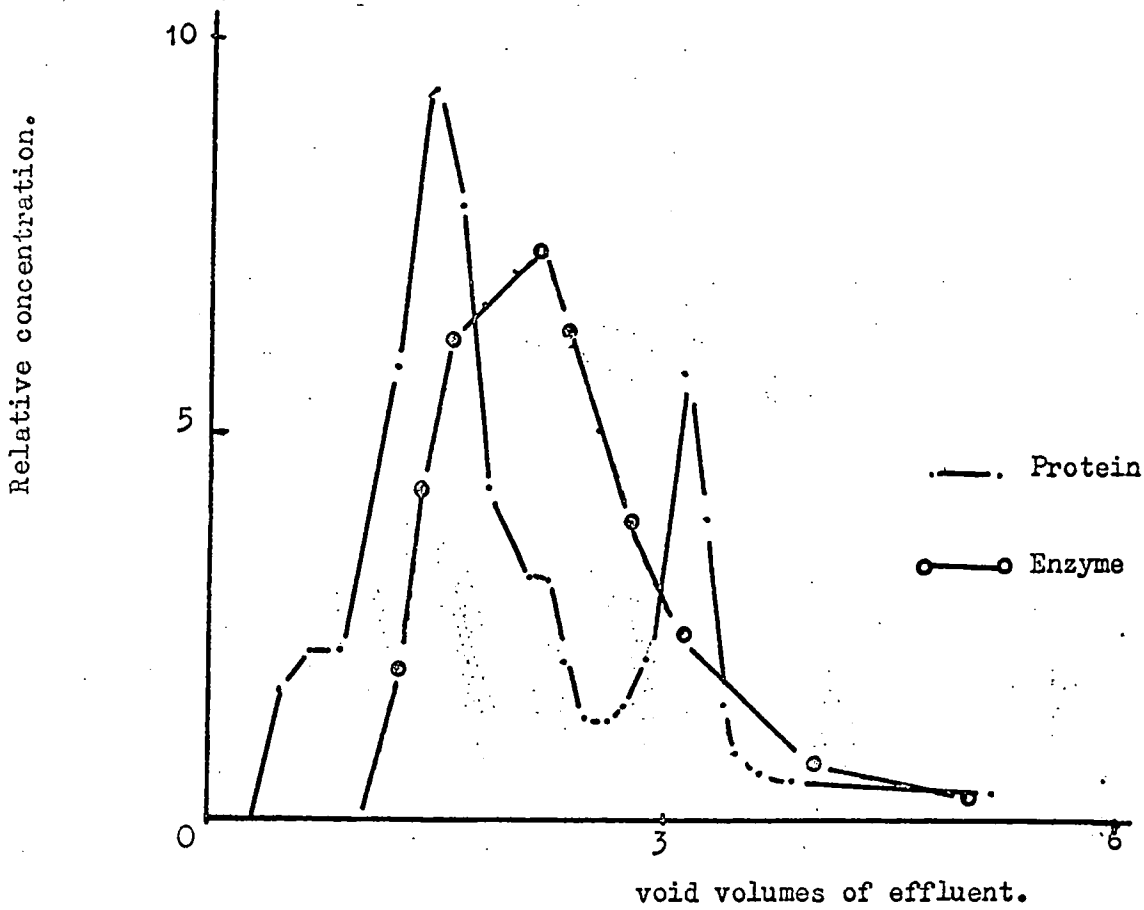


Fig. 11. The Zonal separation of fraction II on DEAE/phosphate. The column, 15cm. x 1.5cm. was eluted with .04M phosphate pH 6.5, and the effluent collected in 1.5 ml fractions with a flow rate of 20 ml/hr. (Fraction II, see table 23).

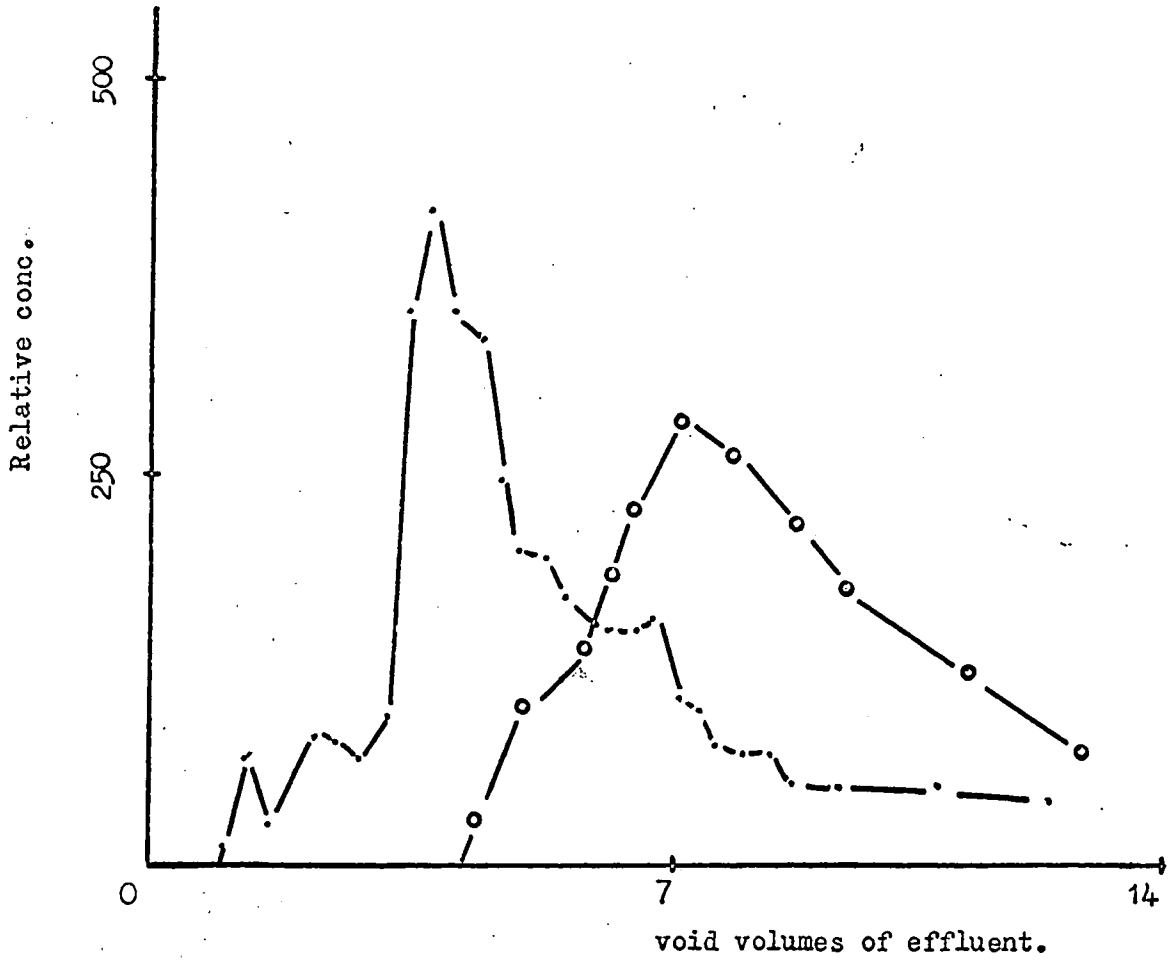


Fig. 12. The Zonal separation of fraction II on DEAE/Chloride.
The column, 15 x 1.5 cm, was eluted with .04M tris/chloride pH 8.6
The effluent was collected in 1.5 ml fractions at a flow rate of
20 ml/hr.

·——· Proteins

o——o Enzyme

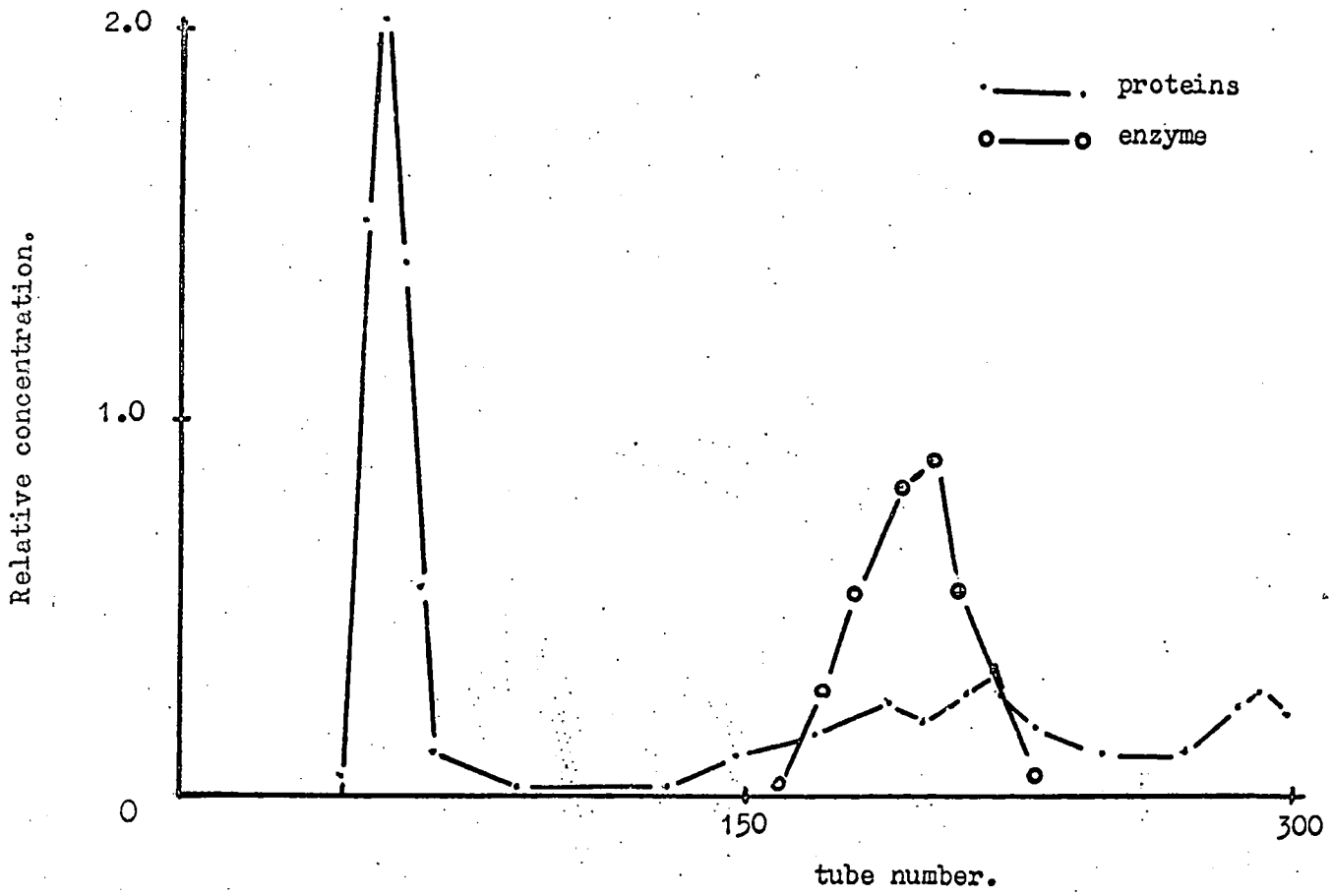


Fig. 13. The gradient elution profile of fraction II on DEAE/Chloride.

The column 60 x 4 cm, was eluted with a concave exponential gradient (see methods), and 15 ml fractions were collected using a Flow rate of 500 ml/hr.

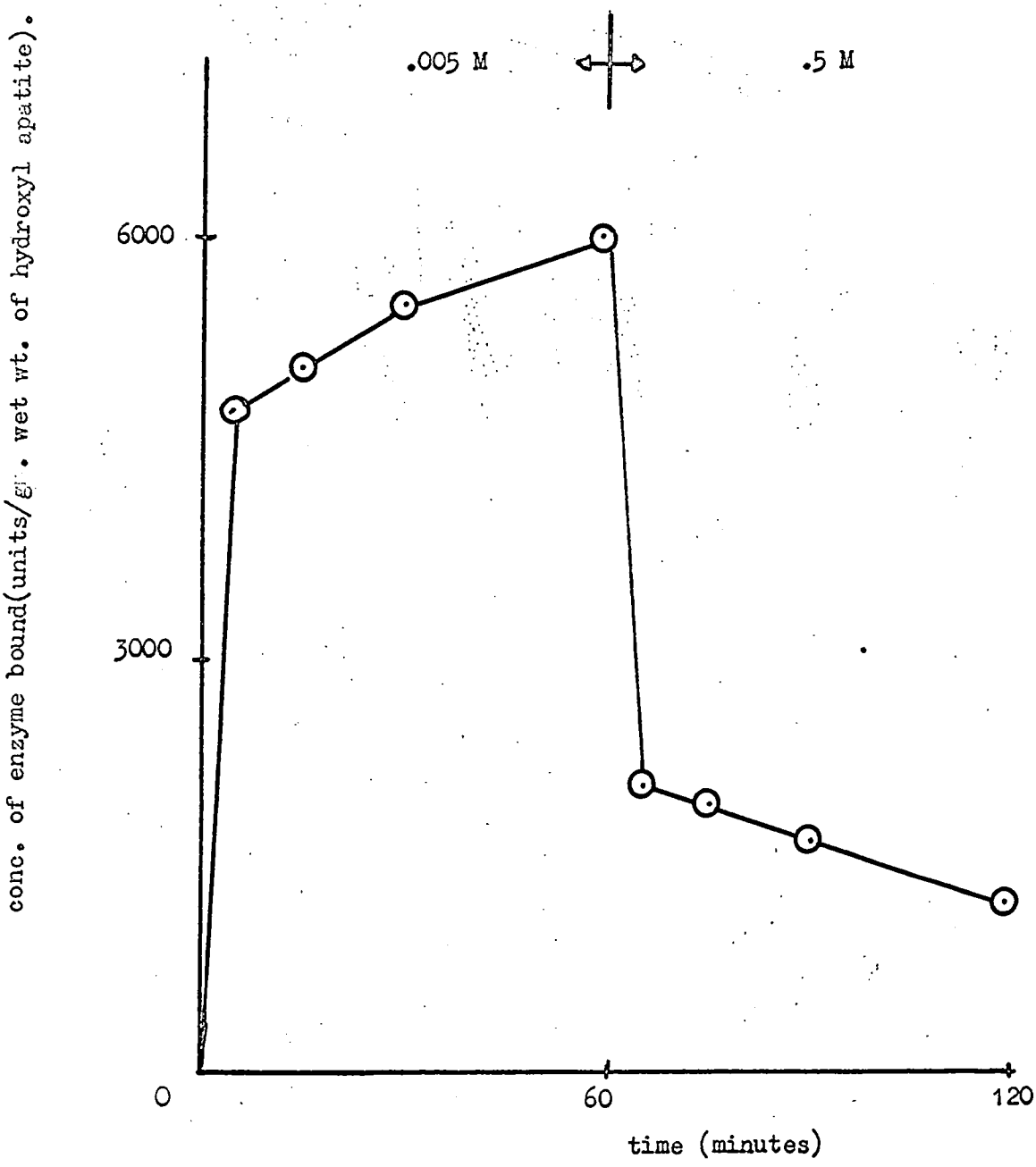


Fig. 14. The kinetics of absorbtion/desorbtion of FDH on hydroxyl apatite.

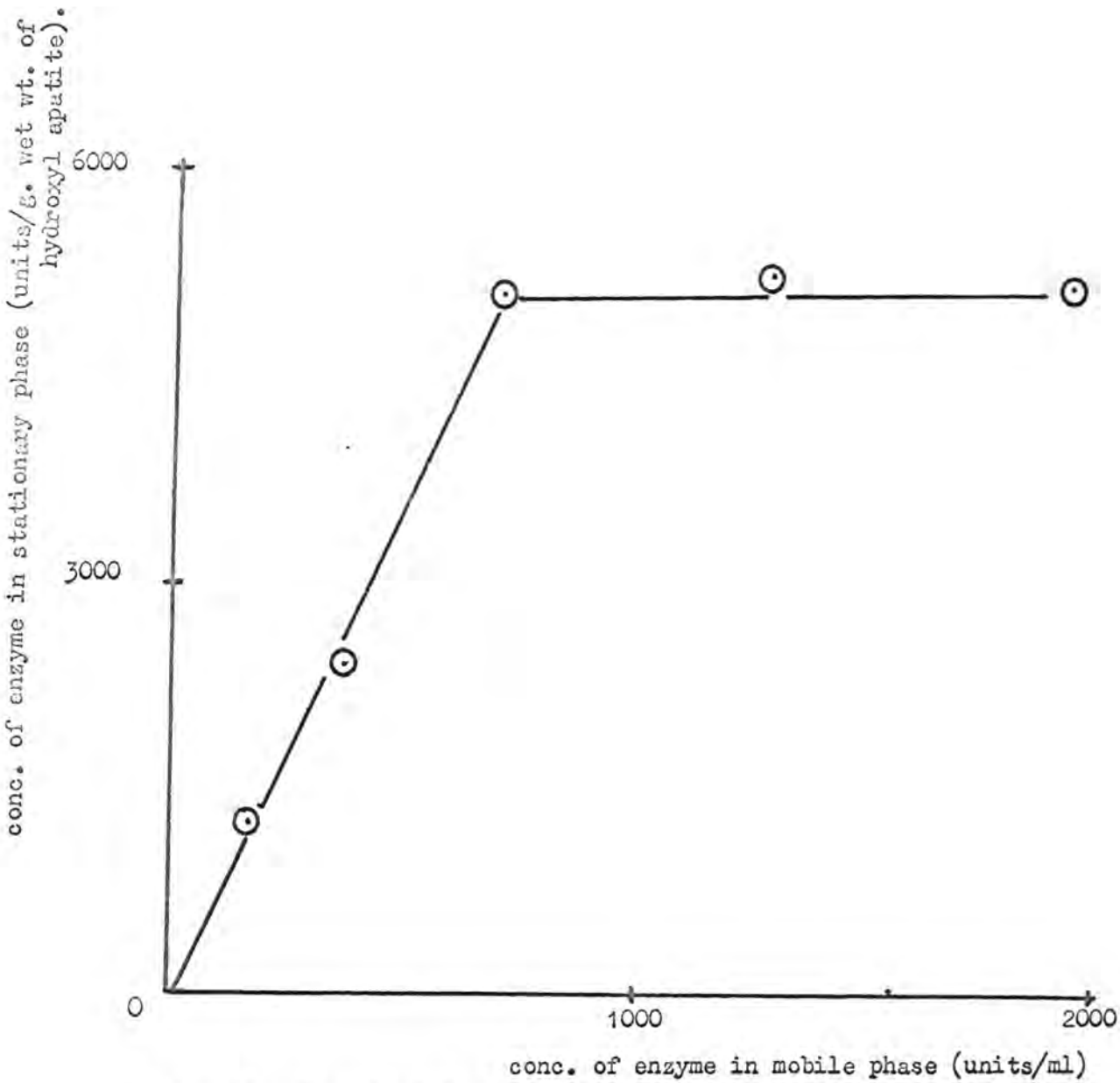


Fig. 15. The mixed distribution isotherm of FDH on hydroxyl apatite.

The column chromatography of fraction IV on hydroxyl apatite using stepwise elution profiles showed trailing of the back edge of each peak (Figs. 17 and 18) as well as multiple zoning of enzyme activity when the ratio of enzyme to bed volume was not correctly balanced (Fig. 18).

(v) Alumina absorption chromatography

Alumina gel was used in a batchwise process. The enzyme was found to be eluted with 0.06 to 0.10 M phosphate buffer at pH 6.5. Figure 19 shows the analysis of each portion of buffer for protein and enzyme activity. The purification of fraction V was x 2 with a yield of 75%. The concentration of enzyme was critical at this stage, with a concentration of 1500 enzyme units/ml. a yield of 75% was obtained, with a concentration of 15,000 units/ml. a yield of only 40% was obtained.

(vi) Gel filtration.

The gel filtration of fraction VI on P-150 and P-300 was found to yield no further purification only one peak of protein was eluted which coincided with the emergence of enzyme activity.

(vii) Acrylamide gel electrophoresis.

Analytical disc acrylamide gel electrophoresis was performed on each stage of the purification scheme as a means of assessing the degree of purity of each stage. Figure 20 shows a typical pattern obtained for samples of fraction VI. Fraction VI was estimated to be approximately 50% pure, with one major impurity and two minor impurities.

Attempts were made to use preparative gel electrophoresis, as an aid in purification, using differently designed apparatus. One apparatus was manufactured by LKB Ltd., and was tested using 7.5% gels with and without large pore gel on both bovine serum albumin V and formic

dehydrogenase fraction V. In all cases all that was eluted in 24 hr. of running was the bromophenol blue marker band. No protein or enzyme activity was ever recovered.

The second apparatus was manufactured by Quickfit and Quartz Ltd., and proved partially successful. On one occasion bovine serum albumin was successfully separated from its impurities, and the Rp values on the preparative gel for each component compared favourably with those obtained during analytical separation on the same percentage gel. Figure 21 shows the separation obtained with fraction IV of formic dehydrogenase which was recovered in 80% yield with a purification of x4. It was not possible, however, to reproduce these traces each time. In some cases all that was eluted was the bromophenol blue band. The elution cell was tested by passing bromophenol blue through it from the inlet of the elution buffer in an attempt to find the cause of the failure. It was found that in some cases the bromophenol blue did not cover the whole area of the base of the acylamide column and hence this can account for the failure of elution of the protein bands. In other cases it was not possible to draw the bromphenol blue through the elution cell with the peristaltic pump used. On checking the elution cell it was found that it was either blocked at the inlet with acrylamide or silicone grease, or it was leaking through one of the seals. Several modifications were tried such as silicone rubber seals between the main body and the elution cell as well as terylene mesh to hold the acrylamide in place; but all failed to remedy these recurring faults.

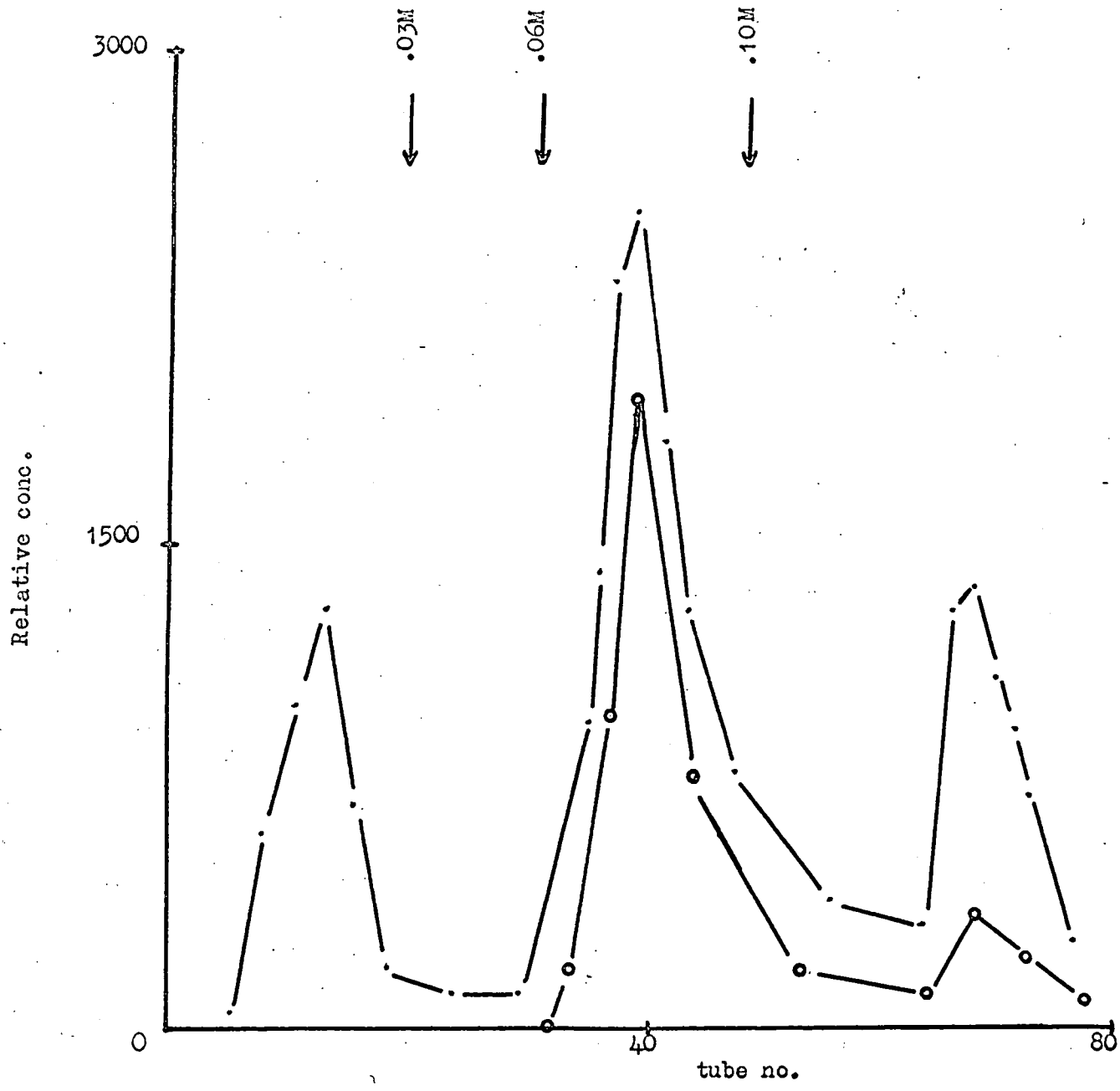


Fig. 17. The stepwise elution profile of FDH from hydroxyl apatite.

A column 2 x 12 cm. was eluted with steps of S, 30, 60, 100 mM phosphate buffer at a flow rate of 20 ml/hr collecting 5 ml fractions.

. ——— . proteins

o ——— o enzyme

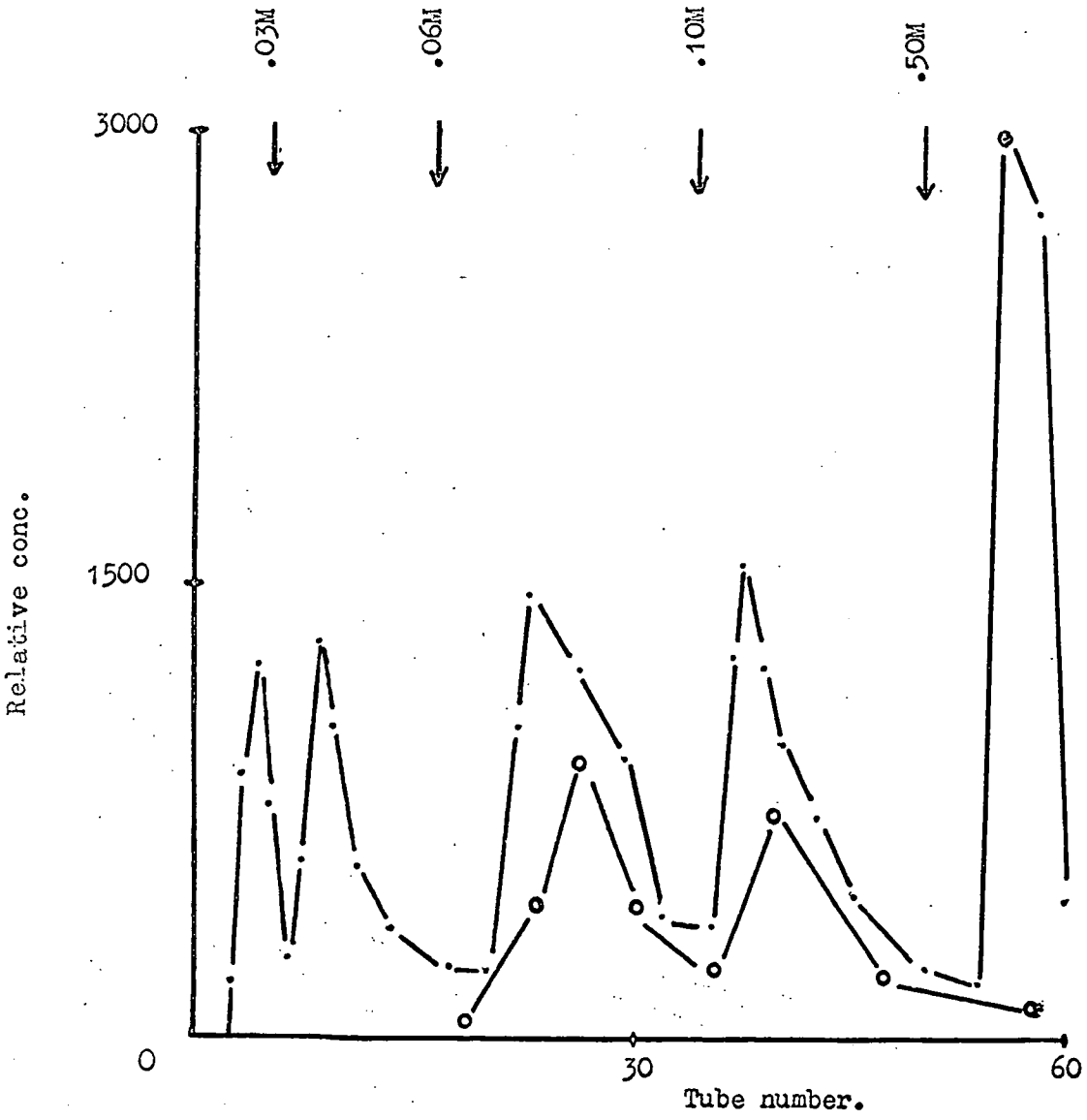


Fig. 18. The Stepwise elution profile of FDH from hydroxyl apatite.

A column 4 x 12 cms, was eluted with increasing steps in ionic strength, collecting 5 ml. fractions at a flow rate of 20 ml/hr.

·——— · proteins. o ———o enzyme.

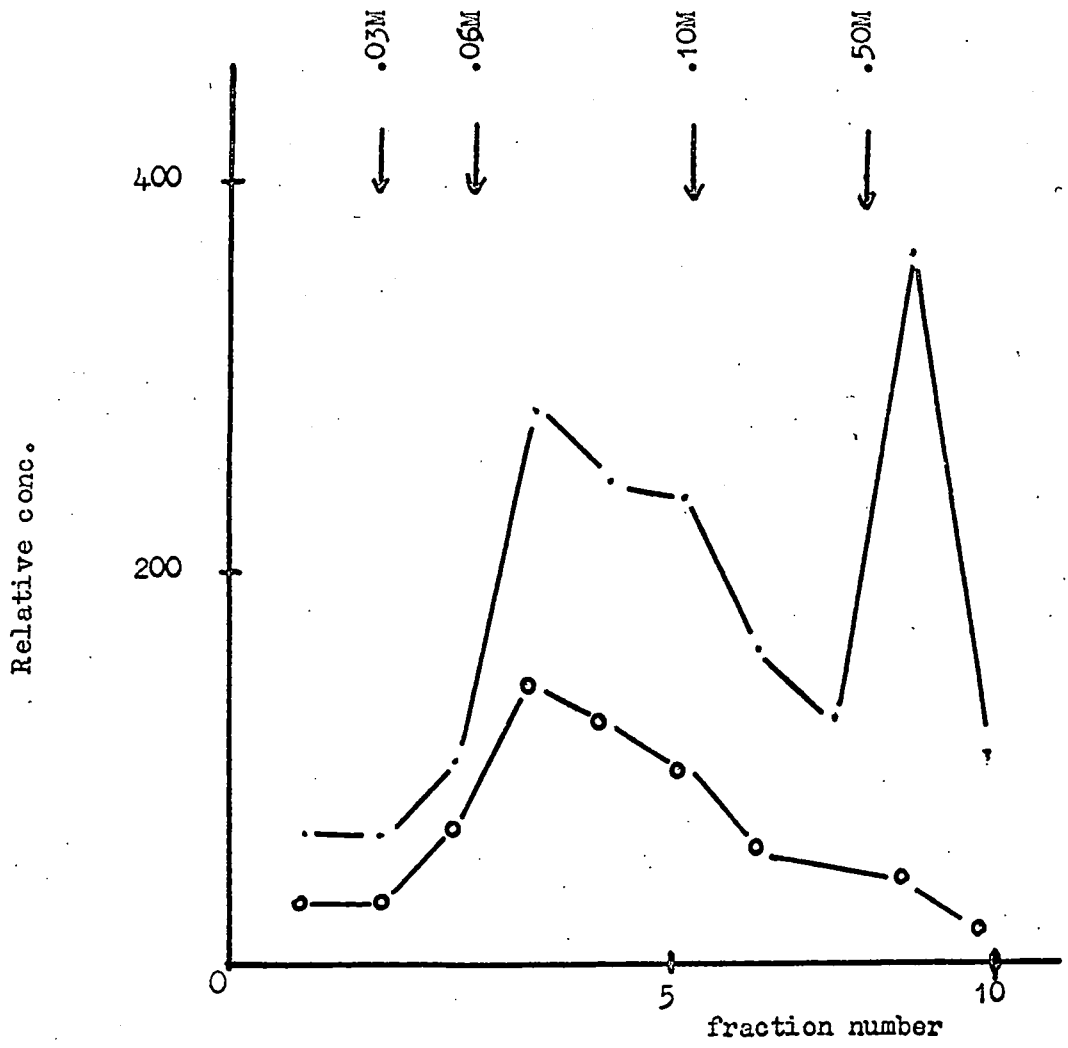


Fig. 19. The elution profile of FDH on Alumina gel.

FDH (fraction v see table 23) was eluted batchwise with 15 ml portions of phosphate buffer pH = 6.5

.———. proteins. o———o enzyme

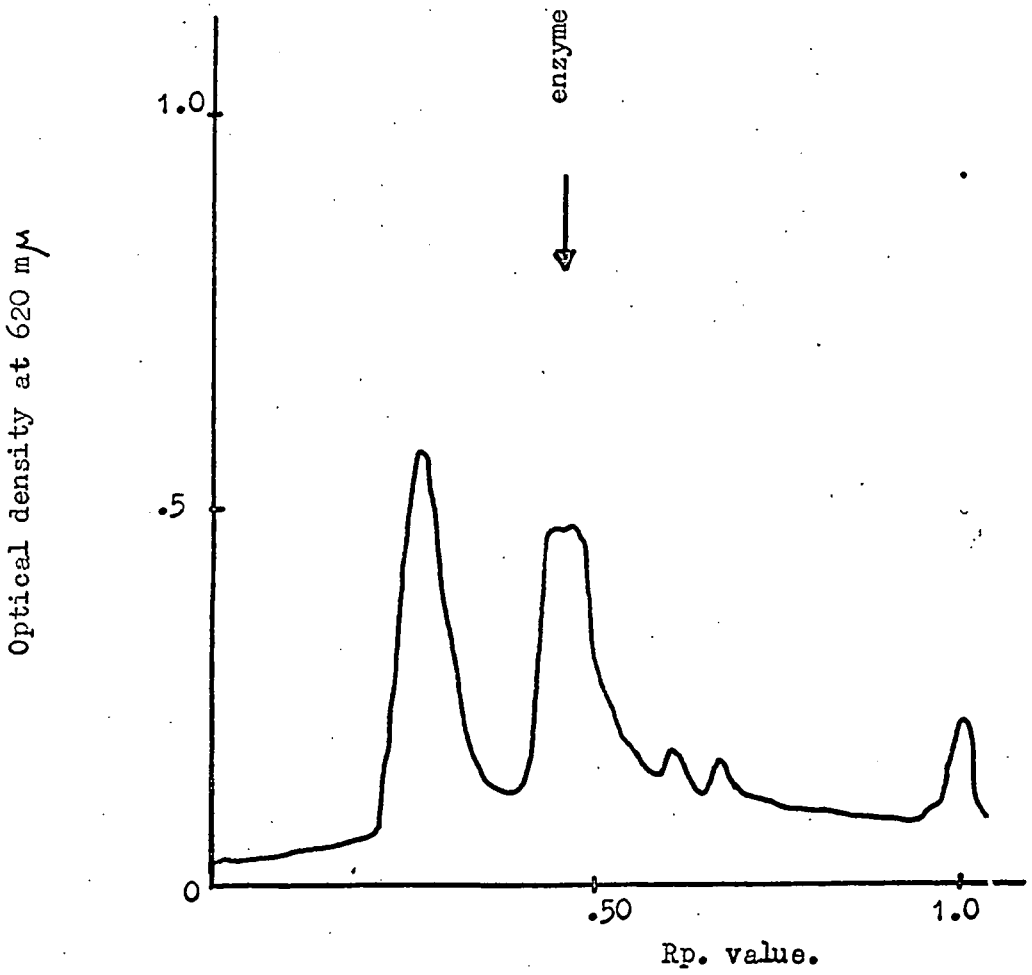


Fig. 20. Analytical acrylamide gel. separation of Fraction VI (see table 23).

Figure redrawn from 'Chromoscan' trace.

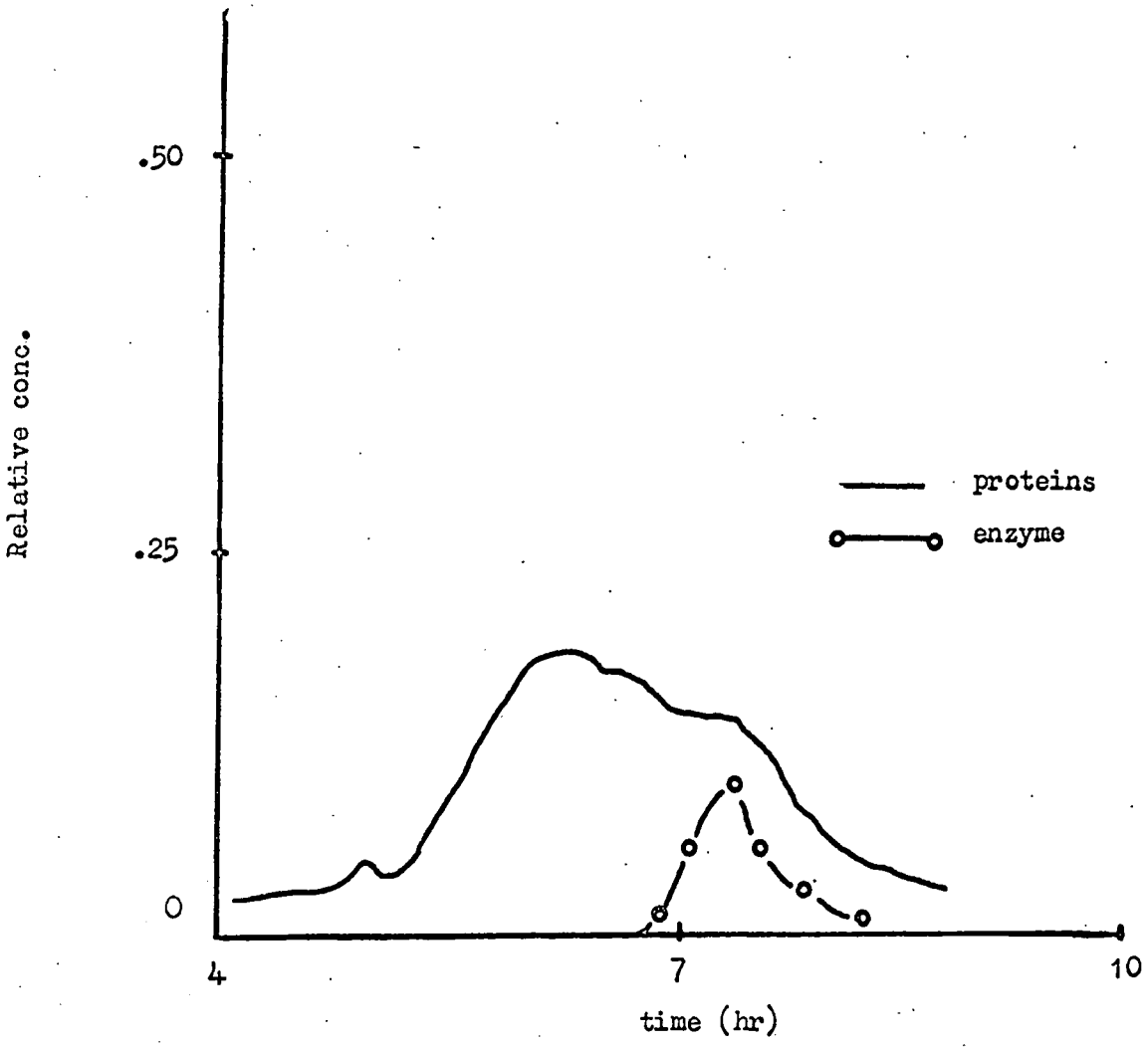


Fig. 21. The preparative scale acrylamide gel. separation of FDH (fraction IV, table 23).

The column of 7.5% acrylamide (5 cm) was loaded with 100 mg. of fraction IV, and run in .005M tris/glycine buffer at 300v and 50 A with a flow rate of 20 ml/hr. 5 ml. fractions were collected. The figure is redrawn from a continuous trace of an Isco U.V. analyser.

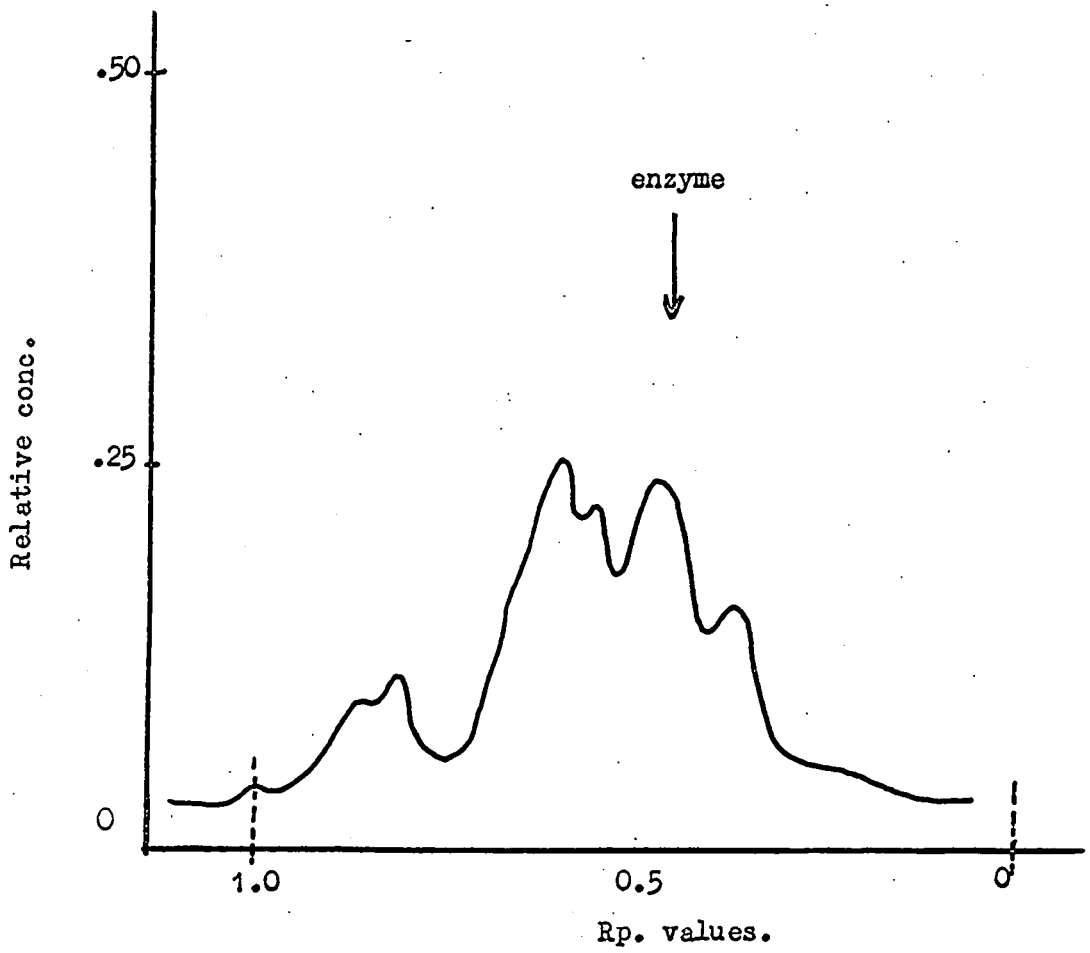


Fig. 22. Analytical acrylamide gel. separation of FDH (fraction IV, see table 23).

The fig. is redrawn from a 'Chromoscan' trace.

(viii) Purification scheme.

Table 23 summarises the average results obtained using the purification schedule given in section 7b (viii) of the methods, figures 20 and 22 show the degree of purity of fraction VI and IV as judged by acylamide gel electrophoresis. The final stage (VI) was estimated to be 55% pure.

Table 23. Summary of the purification of formic dehydrogenase

	Step	mg.protein /kg.	Enzyme units ($\times 10^{-5}$)	S.A.	Purification	Yield %
I	Extraction	300,000	20	6.7	0	100
	30-50% $(NH_4)_2SO_4$	27,000	14	52	8	72
II	Dialysis	27,000	11	42	6	58
III	DEAE-chromatography	3,800	8.2	220	30	43
	40-60% $(NH_4)_2SO_4$	1,470	7.4	500	74	39
IV	Dialysis	960	5.2	540	80	26
	Hydroxyl apatite chromatography	200	3.1	1500	220	15
V	Dialysis	140	2.5	1800	270	12
VI	C alumina	50	1.8	3600	540	9

The figures represent the average of 10 preparations, the order of variation on the figures for fraction VI is $\pm$ 50%.

(d) Kinetics.

(i) The pH optimum.

Formic dehydrogenase shows no sharp changes in the maximum velocity of the reaction with pH (Fig. 23). The curve is flat from pH 5.8 to pH 10.0 and the only sharp change is at pH 5.5 which is due to denaturation of the enzyme activity (see section 2a (i) of the results).

(ii) Inhibitors

Various known enzyme inhibitors were found to affect the maximum velocity of the reaction (Table 24). Sodium azide and para-chloromercuribenzoate were by far the most potent, inhibiting almost completely at 10^{-6}M. Cyanide and hydroxyquinoline showed significant inhibition but iodoacetic acid, iodoacetamide and hydroxylamine showed no inhibition.

Table 24. The effect of enzymic inhibitors on the max. velocity

Inhibitor	concentrations (M)	% inhibition
p-chloromercuribenzoate	10^{-6}	90
	10^{-7}	26
sodium azide	10^{-6}	73
8-hydroxyquinoline	10^{-3}	66
potassium cyanide	10^{-3}	60
Iodoacetic acid	10^{-3}	0
Hydroxylamine HCl.	10^{-3}	0
Iodoacetamide	10^{-2}	0

The reaction was measured at pH 6.5.

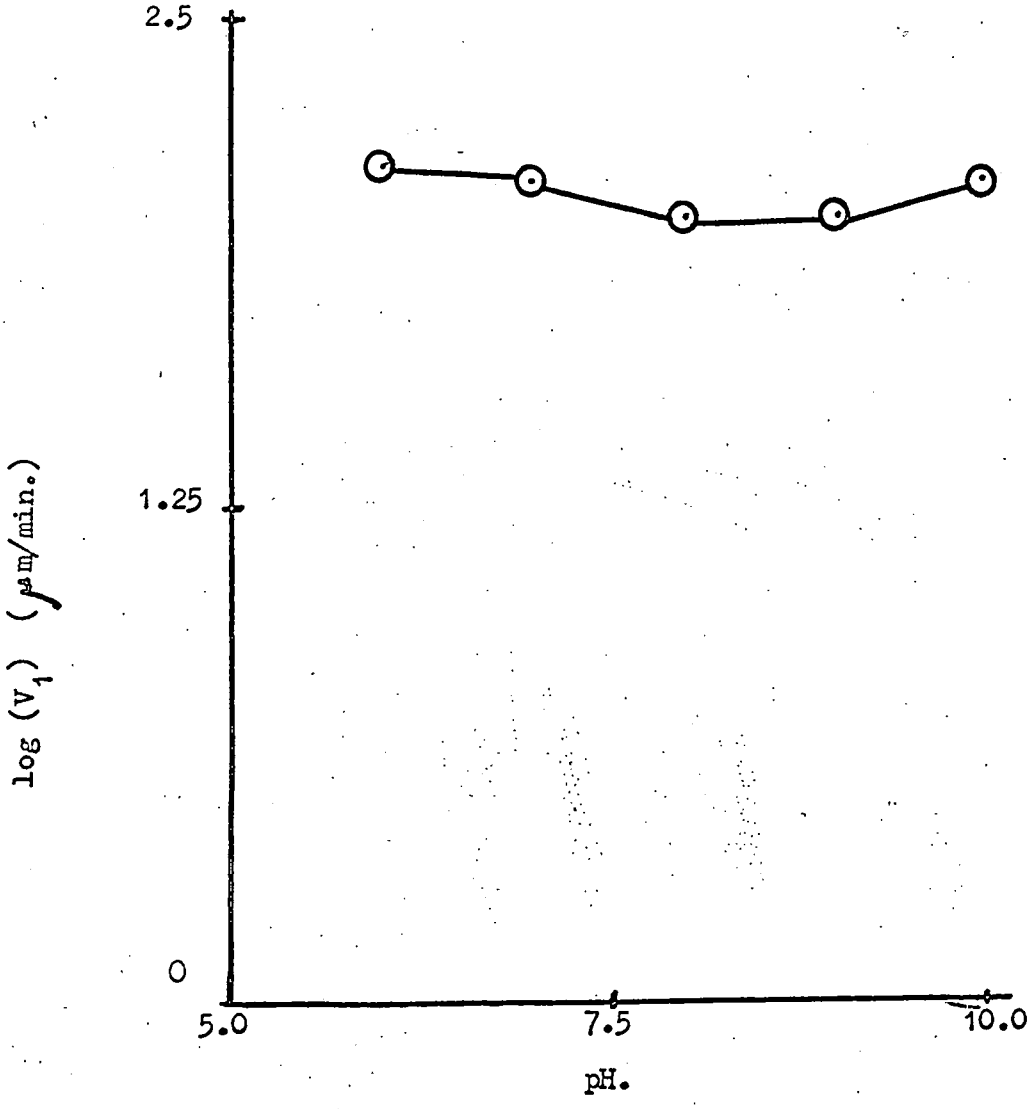


Fig. 23. The variation in the maximum velocity with pH.

(iii) Initial velocity analysis.

The initial velocity patterns in the forward direction are shown in Figures 24 and 27. When NAD was the variable substrate with different levels of HCOOH as the fixed substrate, an intersecting pattern indicating a sequential mechanism was obtained. With HCOOH as the variable substrate and different concentrations of NAD as the fixed substrate an intersecting pattern was again found. The replots of the slopes and intercepts of the primary data (Figs. 25, 26, 28 and 29) were linear. The values of the Michaelis constants for NAD and HCOOH (Ka and Kb), and the dissociation constant for NAD (Kia), are summarised in Table 25, and were obtained by fitting the data to equation 2.

$$V = \frac{VAB}{Kia.Kb + Ka.B + Kb.A + AB} \qquad \ldots\ldots\ldots \text{eq. 2}$$

(This equation is common to all sequential mechanisms).

Table 25. Average values of Ka, Kia, Kb

Ka	=	7.2 ± 1.0 μ M
Kia	=	4.2 ± 8.0 μ M
Kb	=	1.6 ± 0.3 mM

The values are the average of three experiments.

(iv) Product inhibition analysis.

The product inhibition patterns are shown in Figures 30-40, with NADH as inhibitor and NAD as variable substrate, competitive inhibition was obtained (Fig. 30); but with HCOOH as variable substrate the inhibition was non-competitive (Fig. 33). The replots were all

linear (Figs. 32, 34 & 35). With H_2CO_3 as inhibitor and HCOCH as variable substrate the inhibition was competitive (Fig. 36); and non-competitive with NAD as the variable substrate (Fig. 38). The replots were all non-linear (Figs. 37, 39 and 40).

(v) Dead end inhibition.

Two dead end inhibitors were used, ADPR and nitrate. The inhibition of ADPR against NAD was found to be competitive (Fig. 44); with ADPR against HCOOH non-competitive (Fig. 41). The replots were found to be linear (Figs. 42, 43 & 45). Nitrate was found to be competitive with HCOOH and uncompetitive versus NAD (Figs. 46 & 48). The replots were both non-linear (Figs. 47 and 49).

Table 26 summarises the patterns obtained for both product inhibition and dead end inhibition, with the Kis and Kii values from computer analysis summarised in Table 27.

Table 26. A summary of the inhibition patterns.

Inhibitor	Variable substrate	Fixed substrate	Pattern
NADH	NAD	non-saturating	comp.
NADH	NAD	saturating	comp.
NADH	HCOOH	non-saturating	non-comp.
H_2CO_3	HCOOH	"	comp.*
H_2CO_3	NAD	"	non-comp.*
ADPR	NAD	"	comp.
ADPR	HCOOH	"	non.comp.
Nitrate	HCOOH	"	comp.*
Nitrate	NAD	"	un-comp.

* non-linear replots

The pH of the reaction medium was 8.0

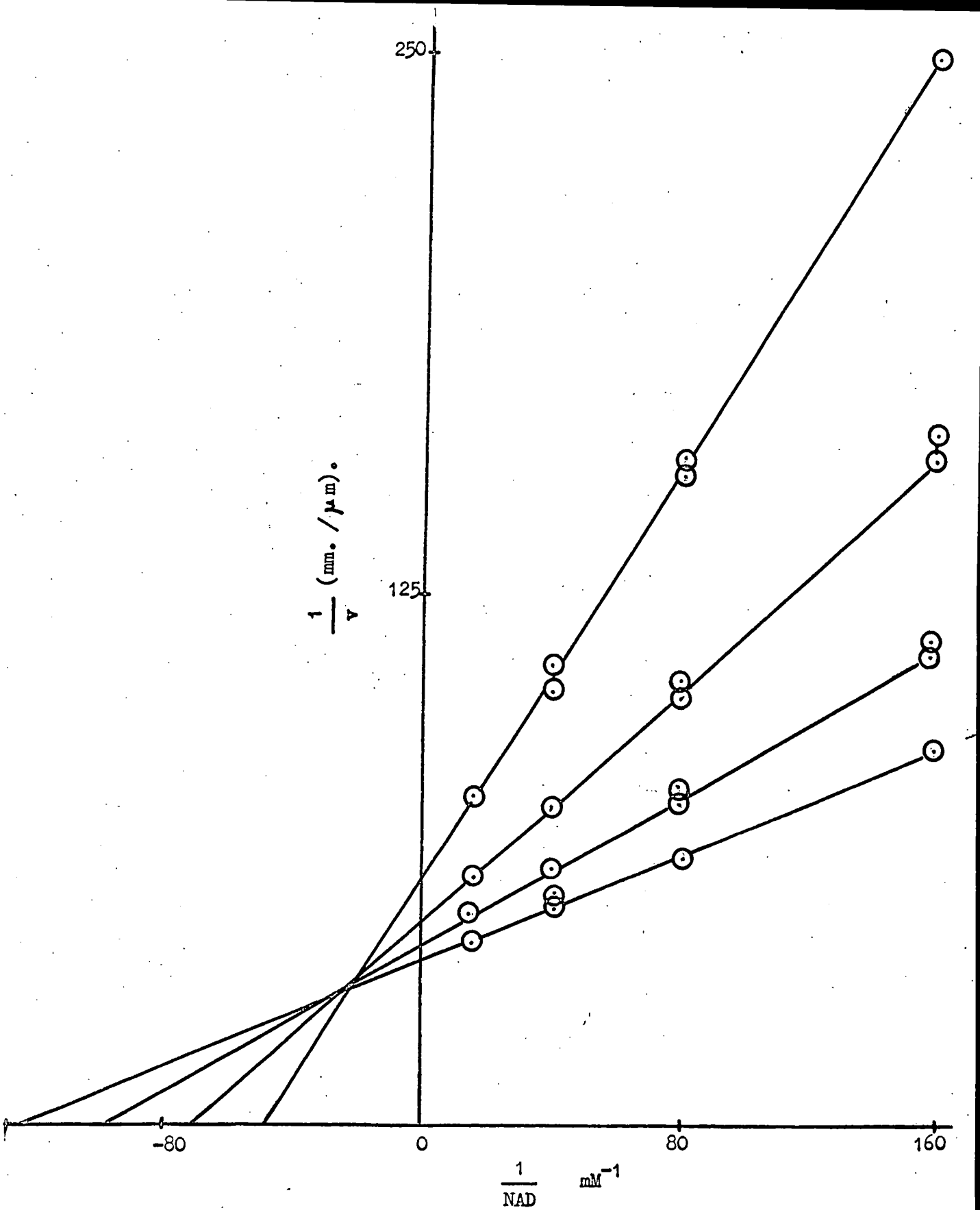


Fig. 24. The initial velocity analysis with NAD as the variable substrate. Formic acid conc. were held constant at 2.5, 5.0, 10.0, 25.0 mM.

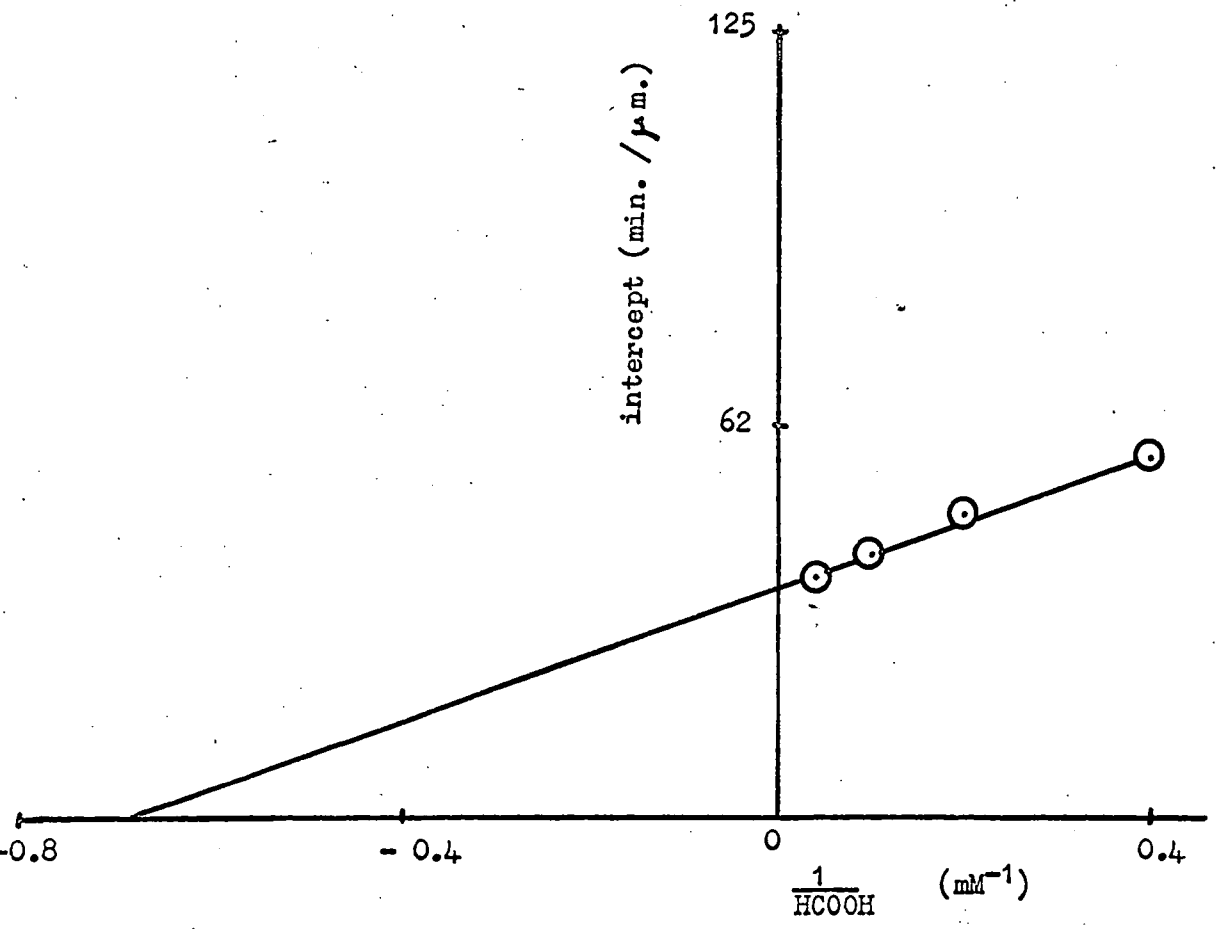


Fig. 25. The secondary plot of intercept against the Fixed Formic acid conc.

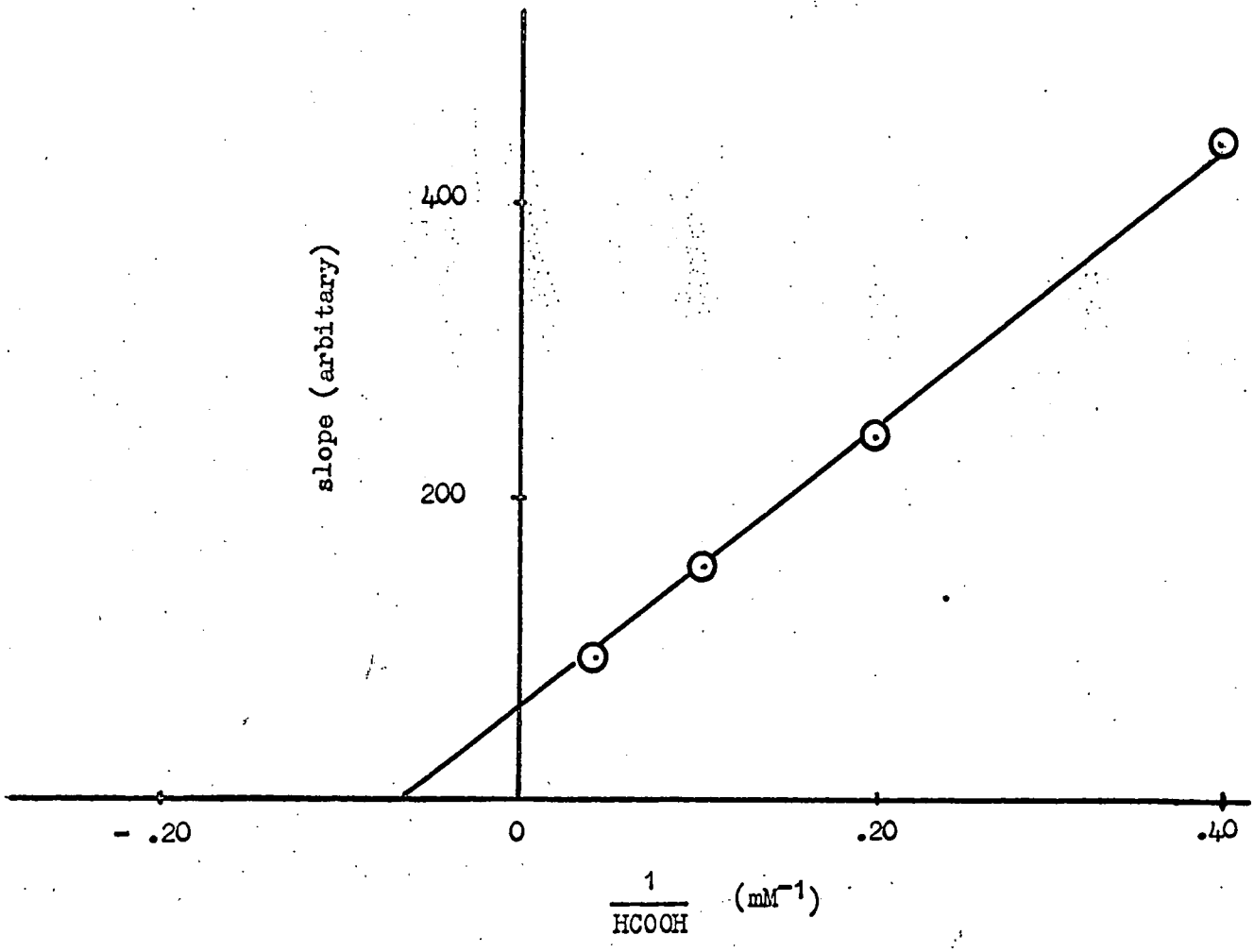


Fig. 26. The secondary plot of slope against the fixed formic acid conc.

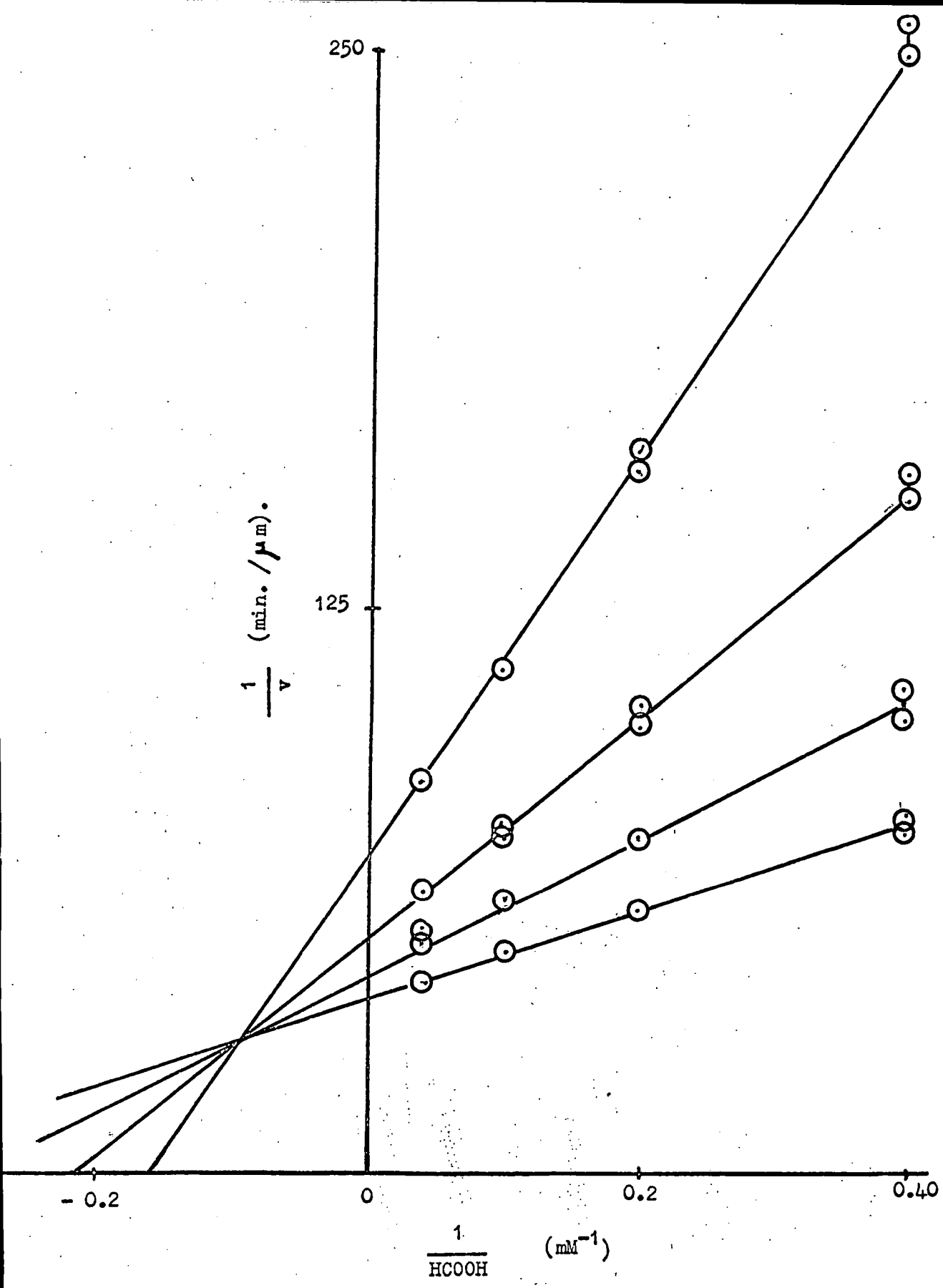


Fig. 27. The initial velocity analysis with formic acid as the variable substrate. NAD conc. were held constant at 6.25, 12.5, 25, 62 μM.

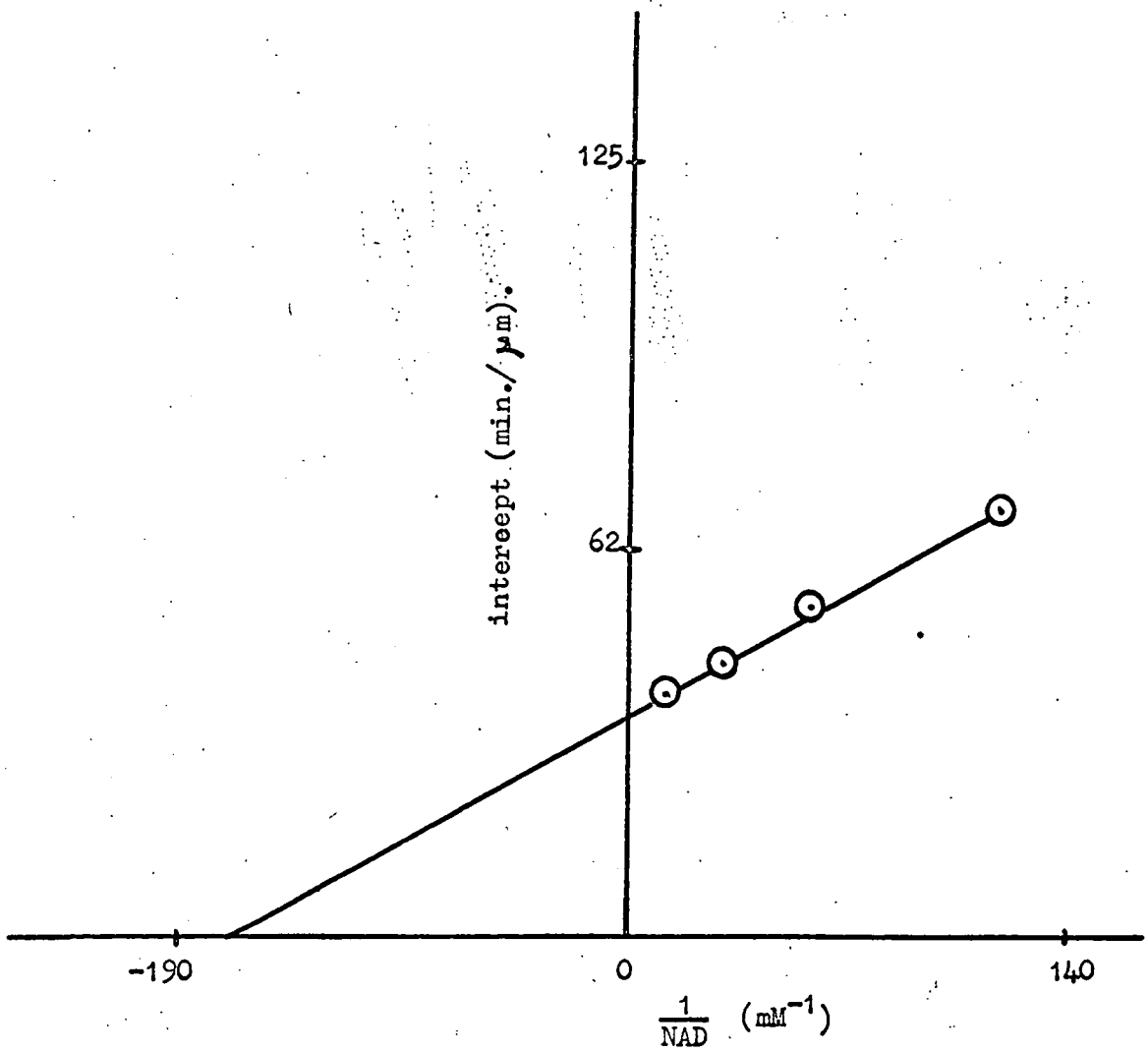


Fig. 28. The secondary plot of intercept against the fixed NAD conc.

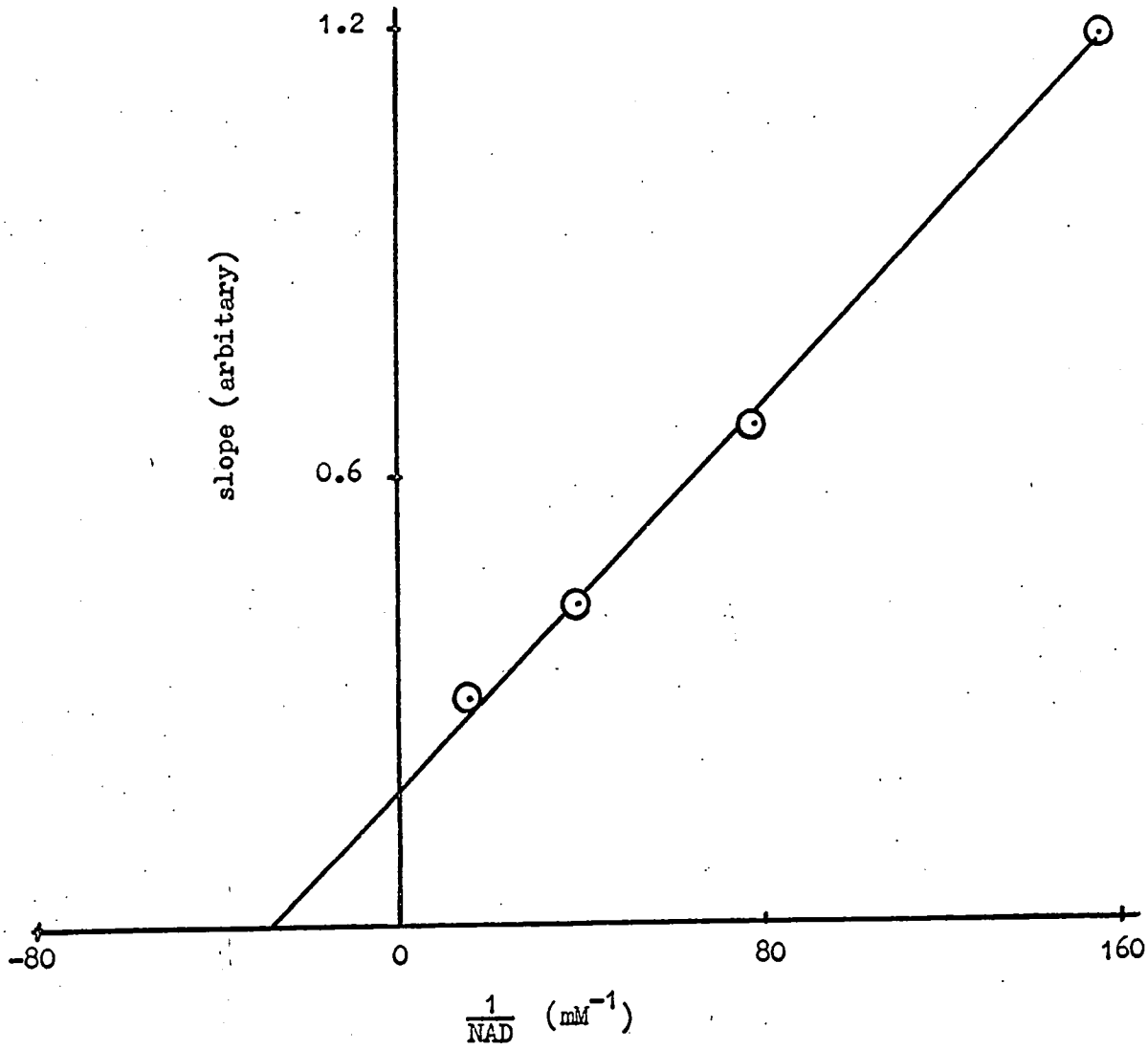


Fig. 29. The secondary plot of slope against the fixed NAD conc.

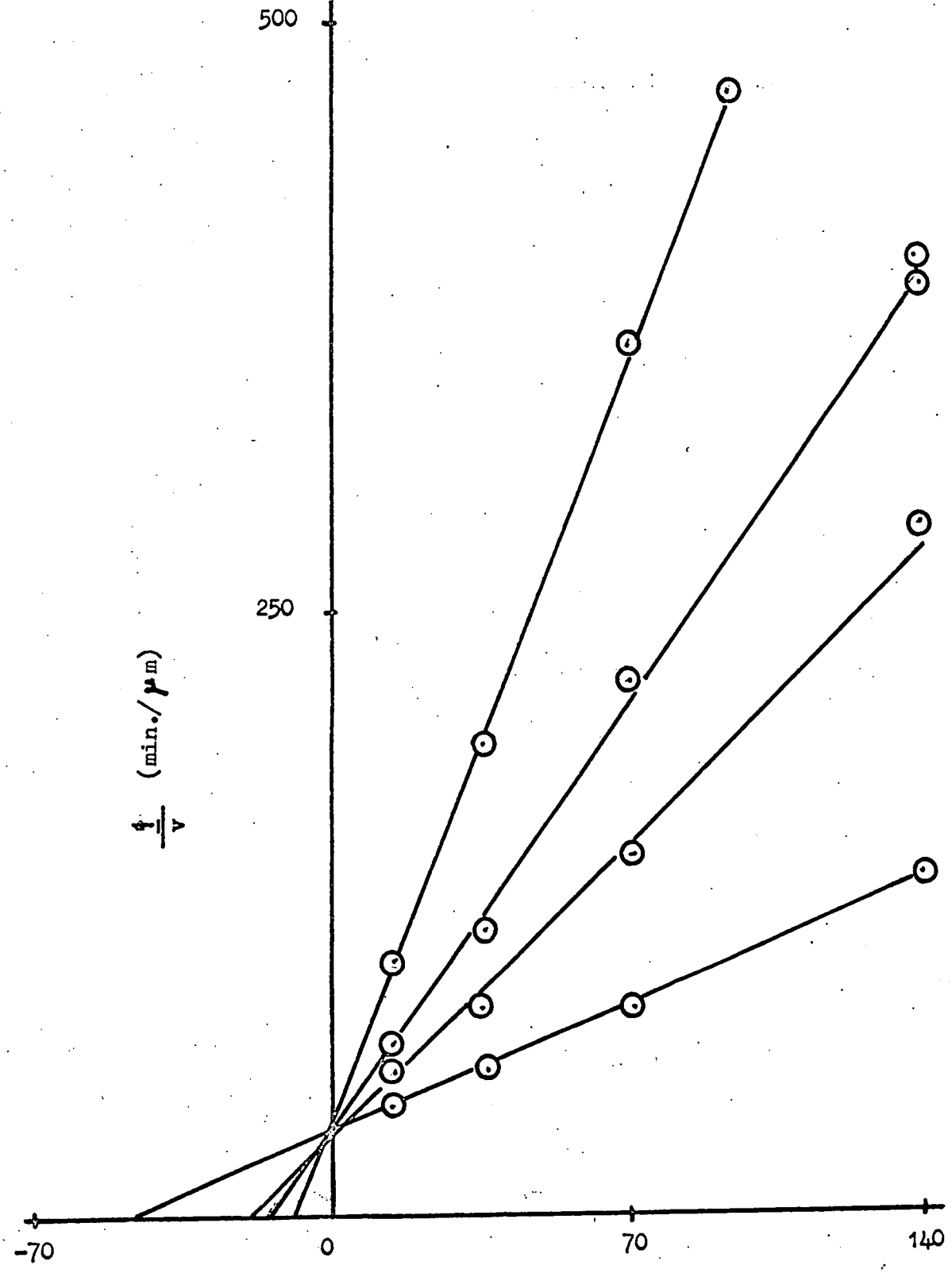


Fig. 30. The Products inhibition pattern of NADH with variable NAD.

NADH levels were held const. at 0, 2.5, 5.0, 10.0 μM with formate fixed at 2.5 mM.

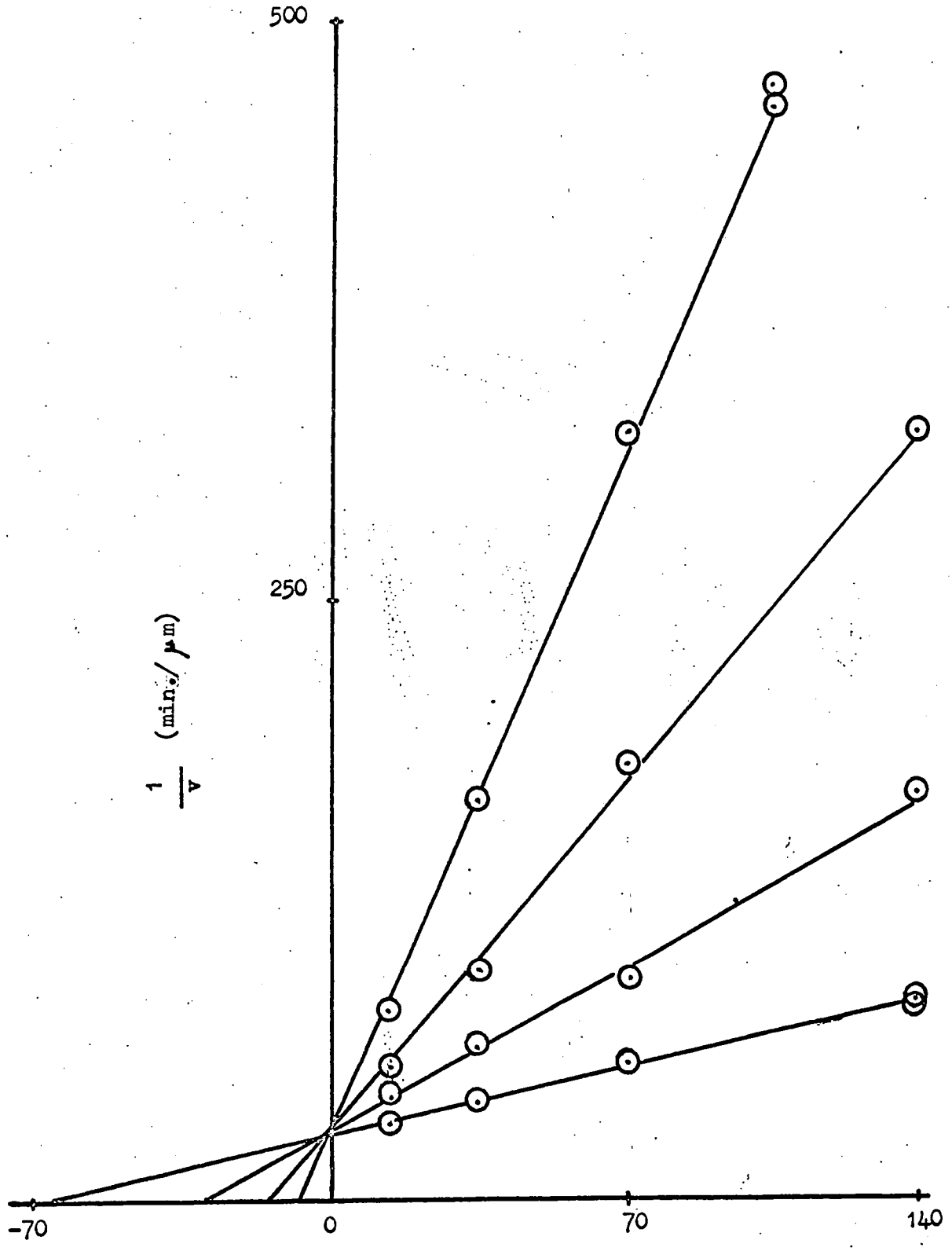


Fig. 31. The product inhibition pattern of NADH with variable NAD and high formate concentration.

NADH levels were held constant at 0, 5, 12.5, 25μM with Formate fixed at 10mM.

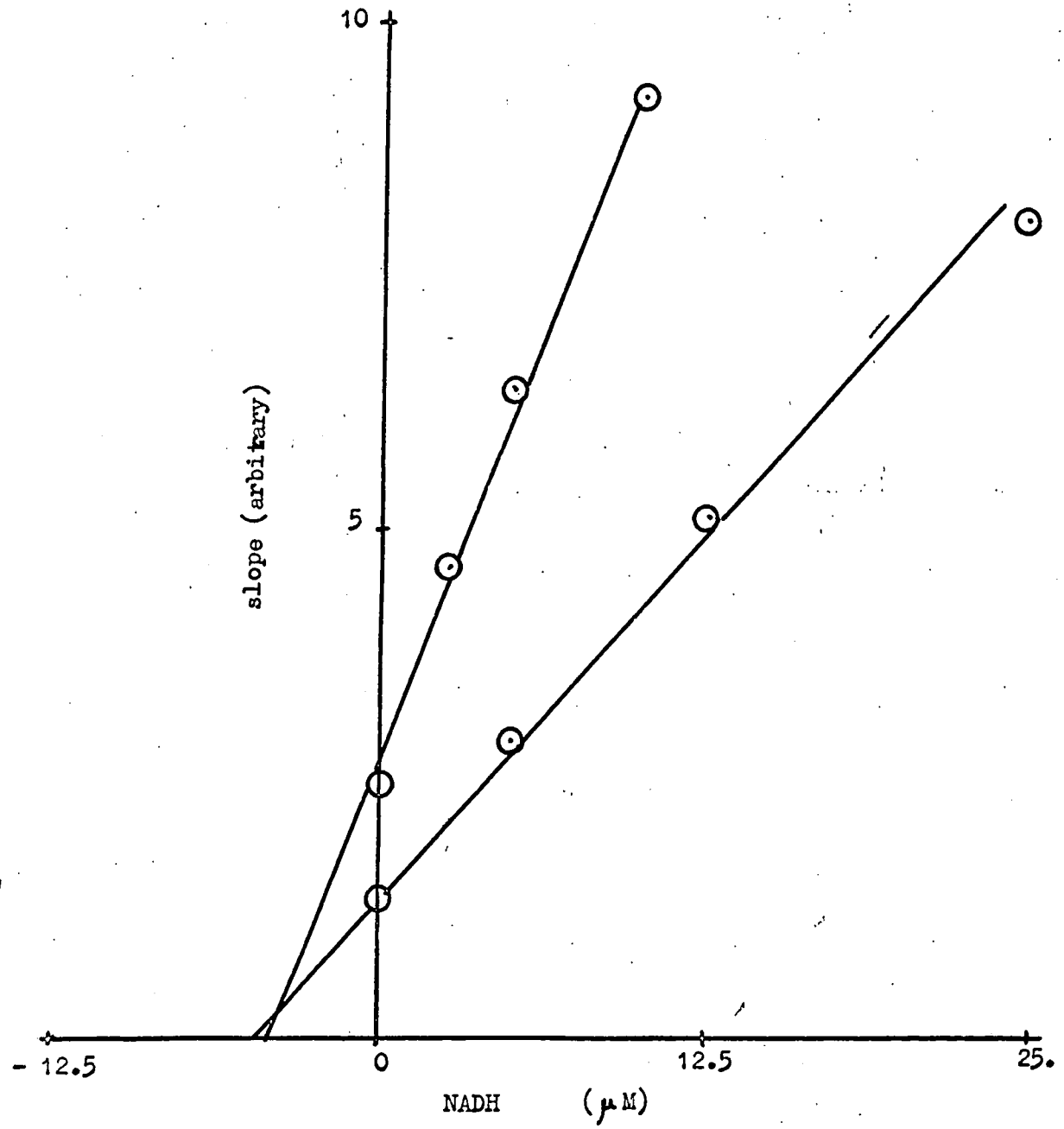


Fig. 32. The secondary plot of the slopes of the NADH versus NAD product inhibition.

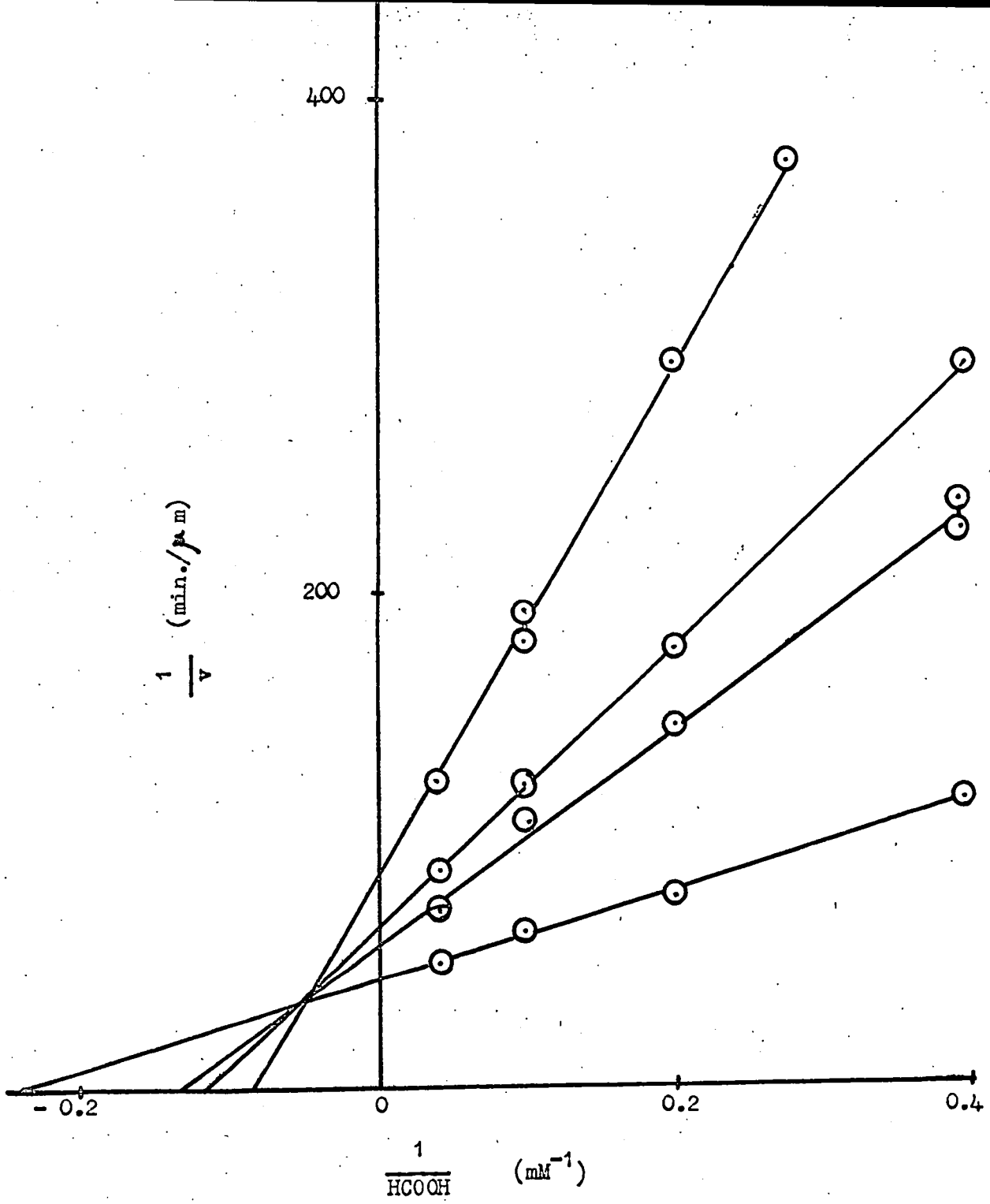


Fig. 33. The product inhibition of FDH by NADH with formate as the variable substrate.

The levels of NADH were held constant at 0, 2.5, 5.0, 10.0μM with NAD fixed at 25 μM.

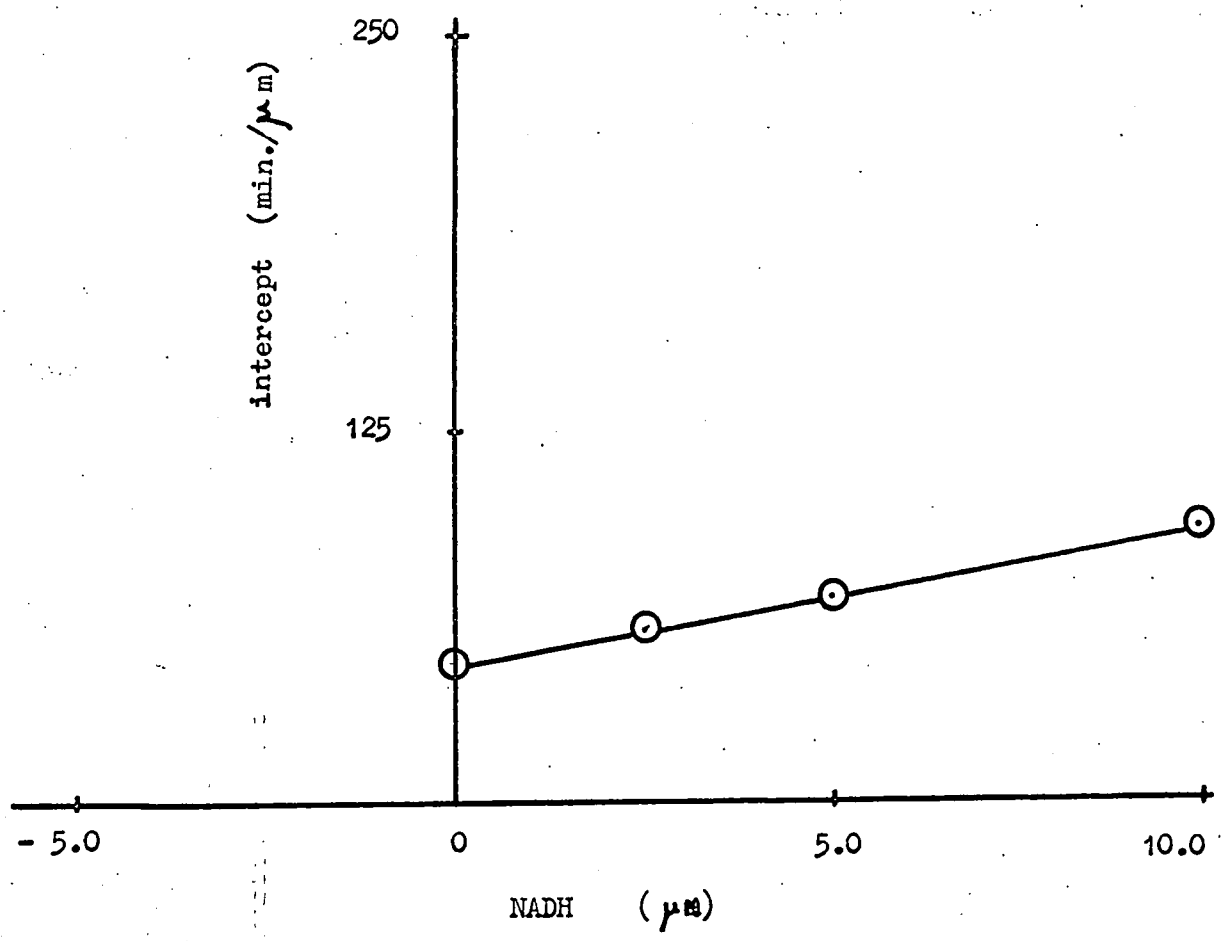


Fig. 34. The secondary plot of the intercept of the NADH versus formate product inhibition.

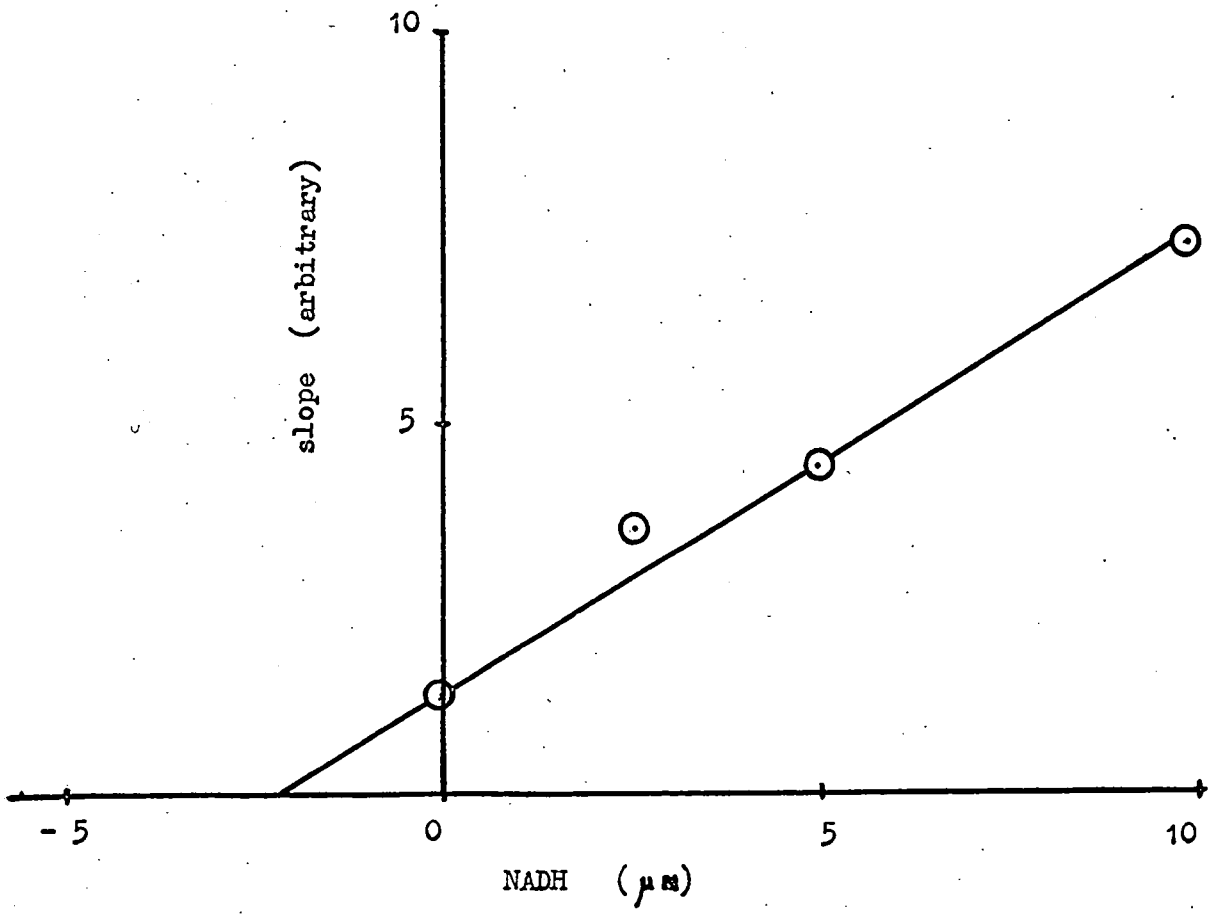


Fig. 35. The secondary plot of the slope of NADH versus formate product inhibition.

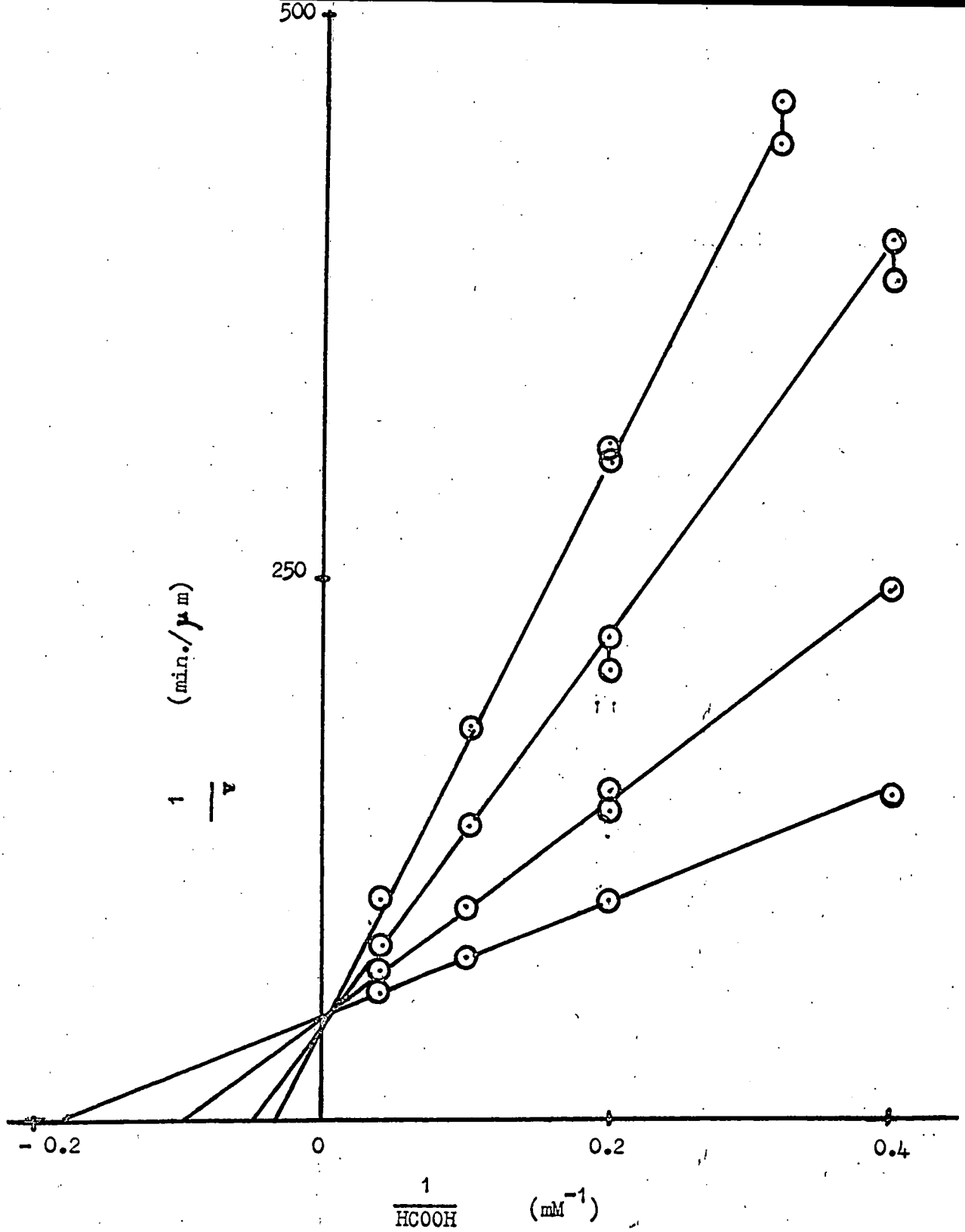


Fig. 36. The product inhibition of FDH by bicarbonate with formate as the variable substrate.

The levels of bicarbonate were held constant at 0, 225, 500, 675 mM with NAD fixed at 6.15 μM

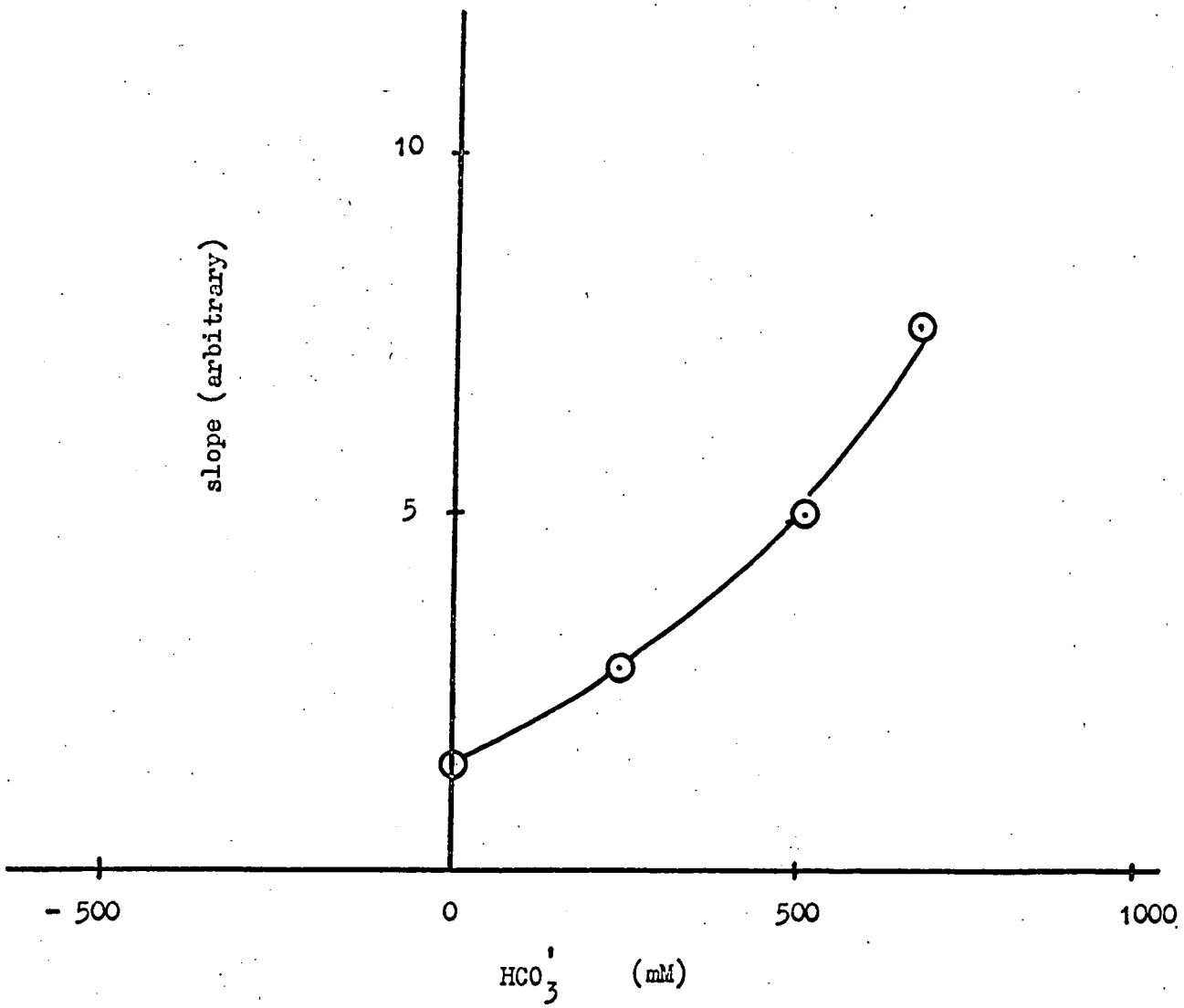


Fig. 37. The secondary plot of the slope of the bicarbonate versus formate product inhibition.

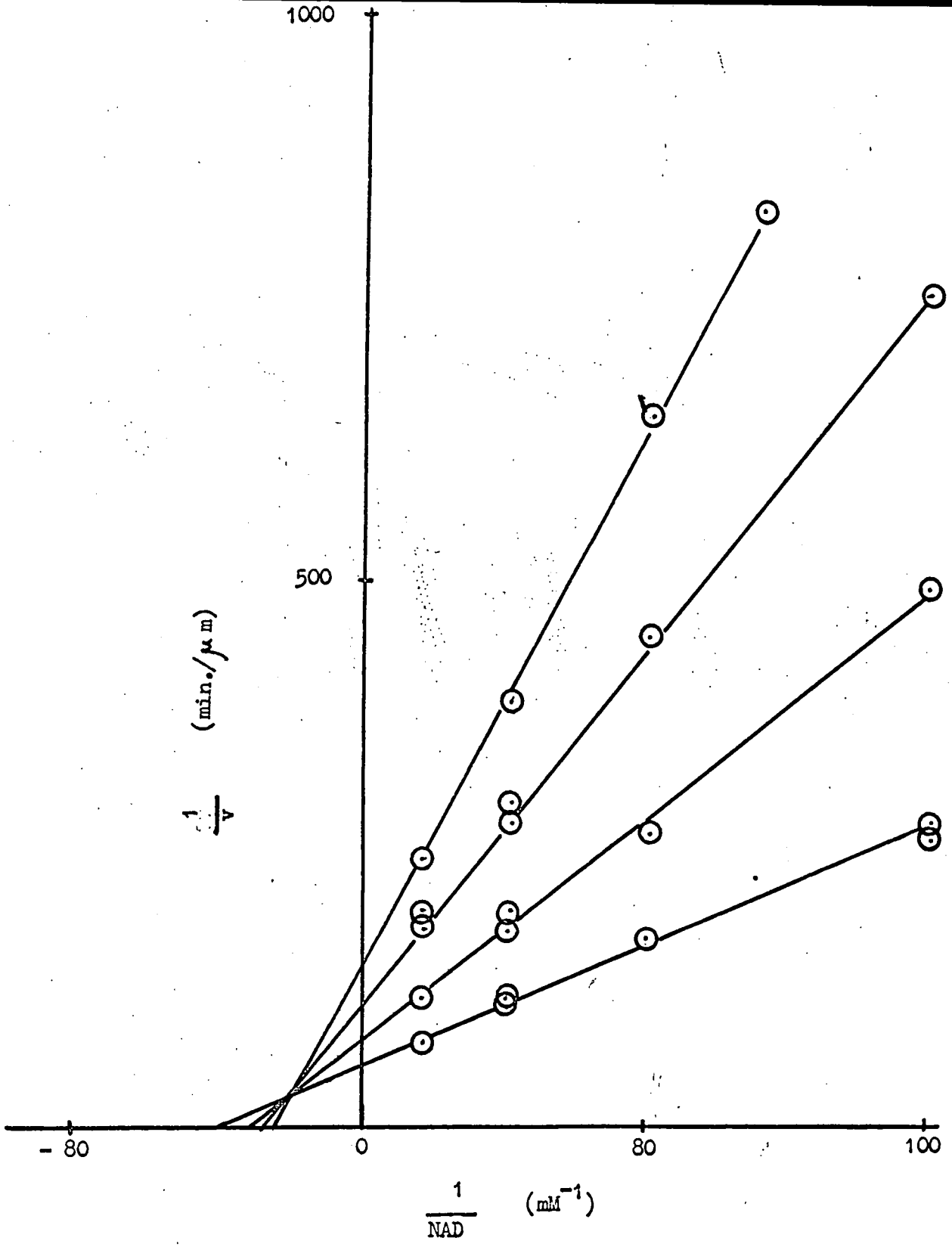


Fig. 38. The product inhibition of FDH by bicarbonate with NAD as the variable substrate.

The levels of bicarbonate were held constant at 0, 225, 500, 675 mM with formate fixed at 2.5 mM.

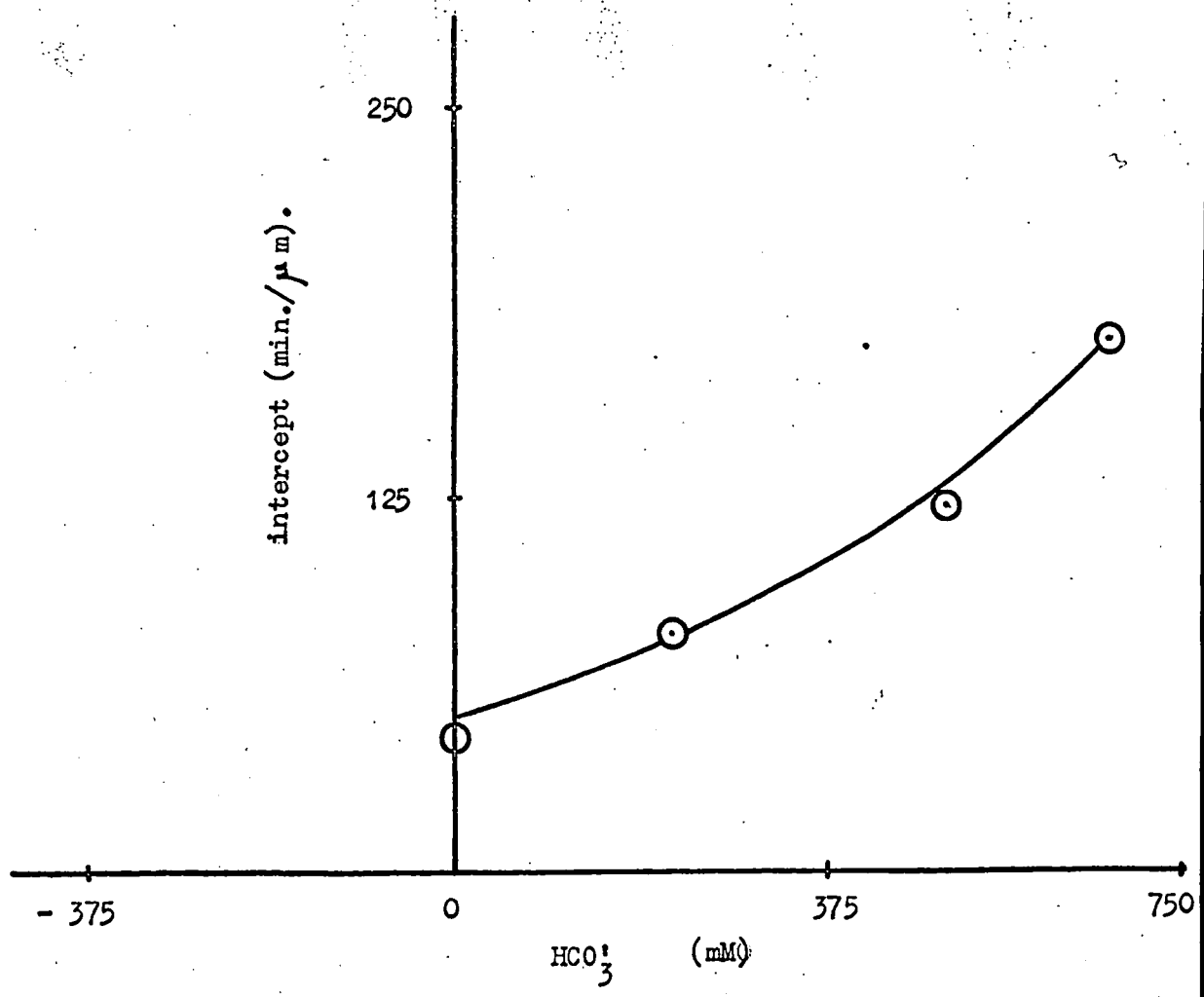


Fig. 39. The secondary plot of the intercept of the bicarbonate versus NAD product inhibition.

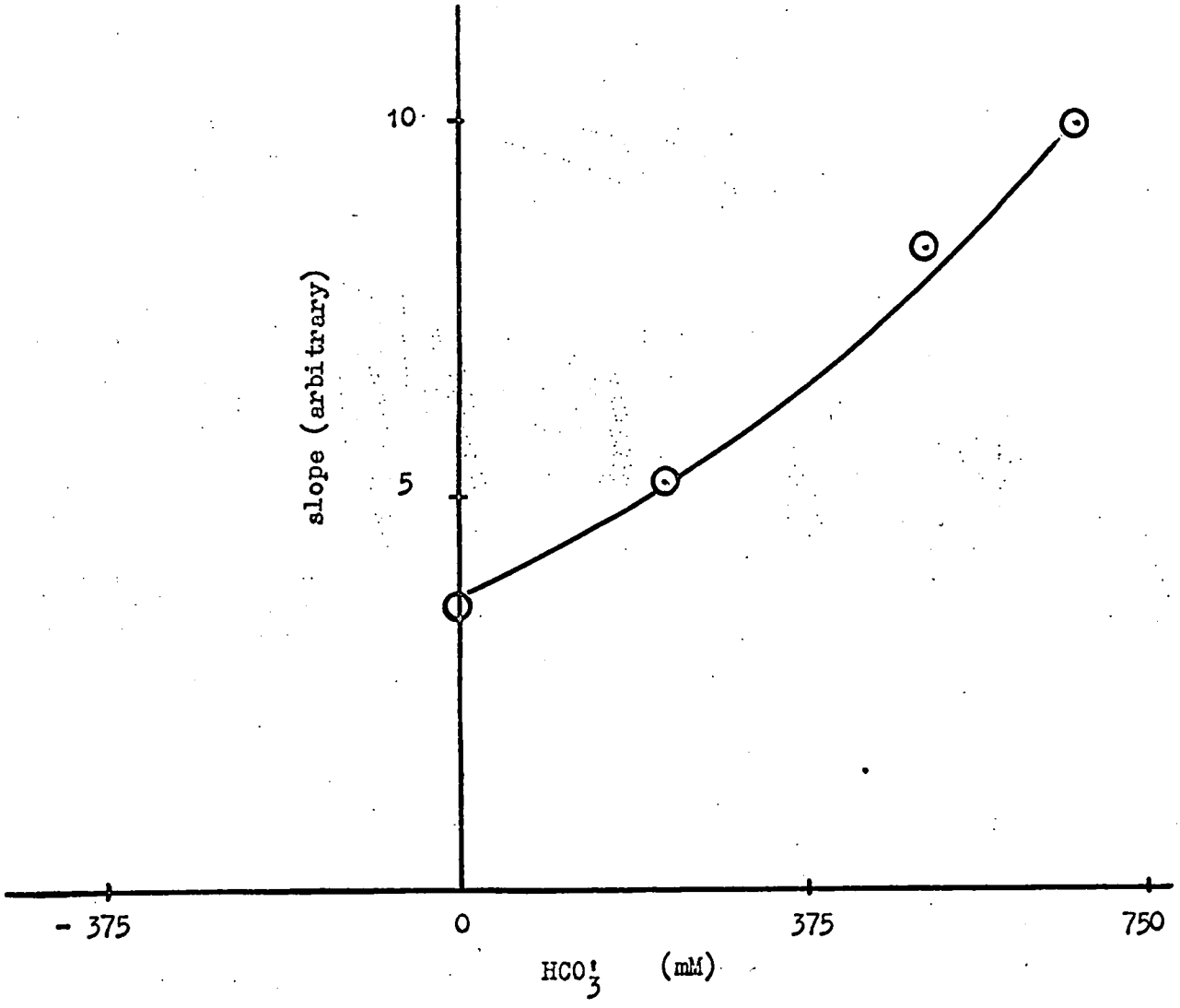


Fig. 40. The secondary plot of the slope of the bicarbonate versus NAD product inhibition.

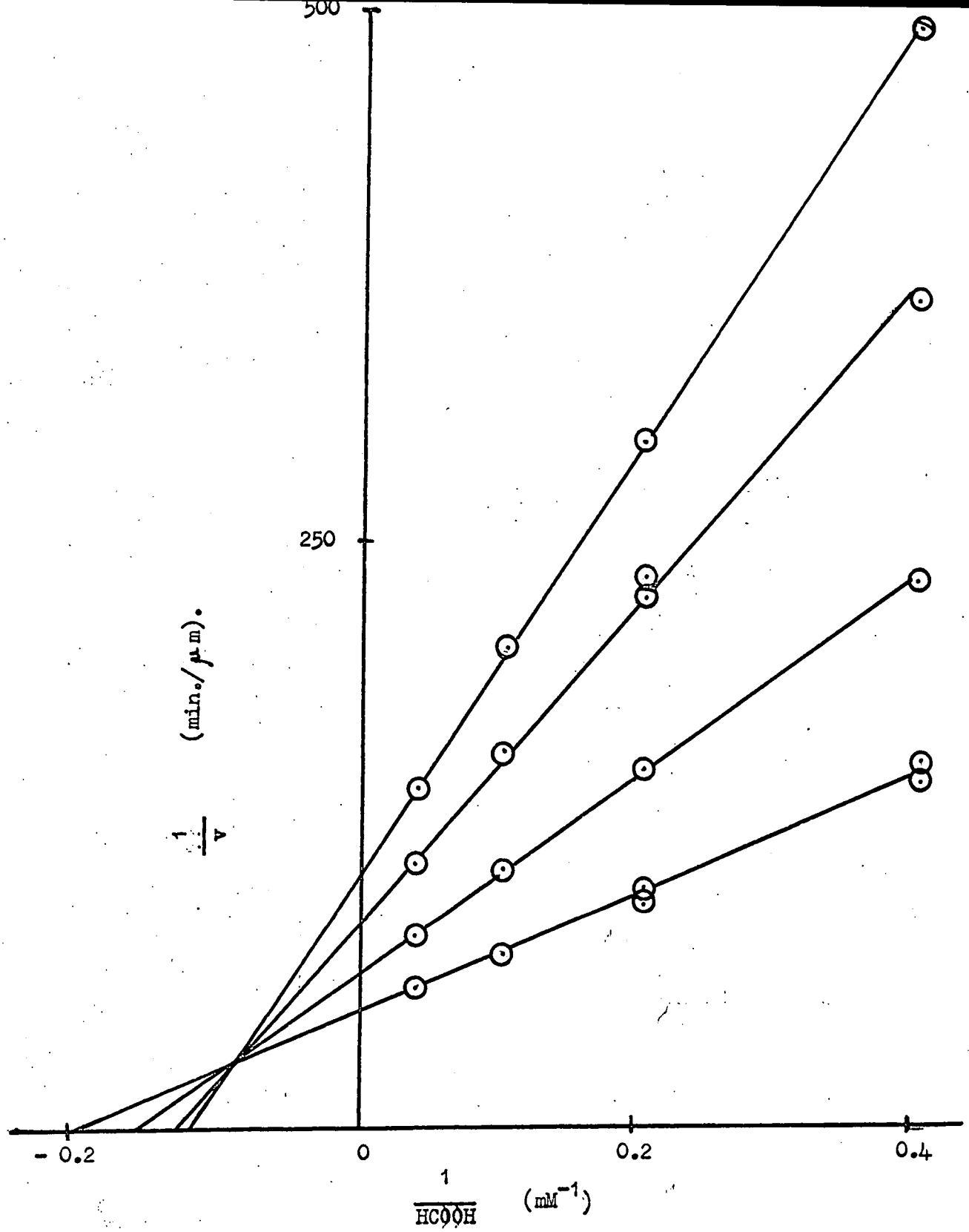


Fig. 41. The dead end inhibition of FDH by ADPR with formate as the variable substrate.

The levels of ADPR were held constant at 0, 12, 24, 48 μM with NAD fixed at 12.3 M.

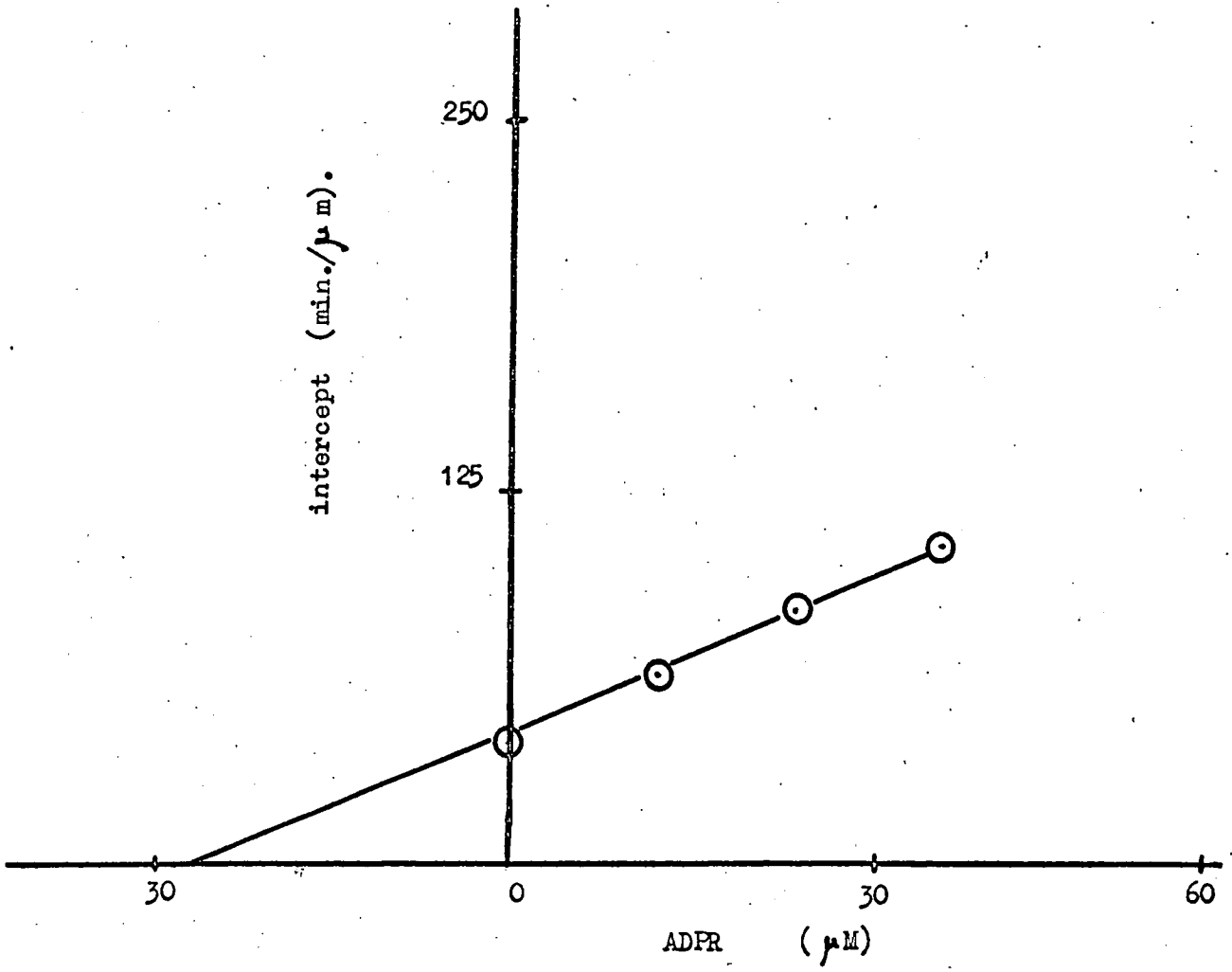


Fig. 42. The secondary plot of the intercept of the ADPR versus formate dead end inhibition.

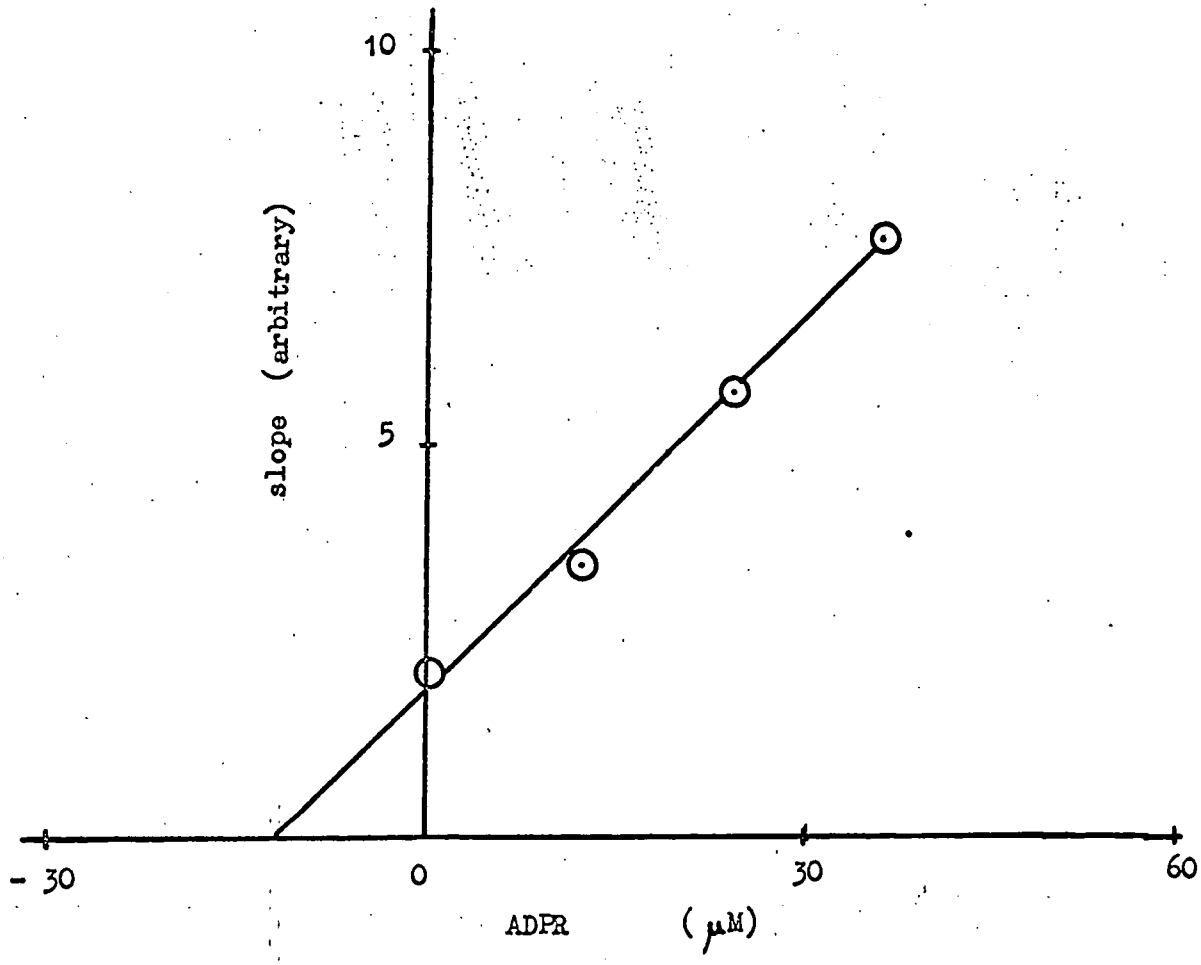


Fig. 43. The secondary plot of the slope of the ADPR versus formate dead end inhibition.

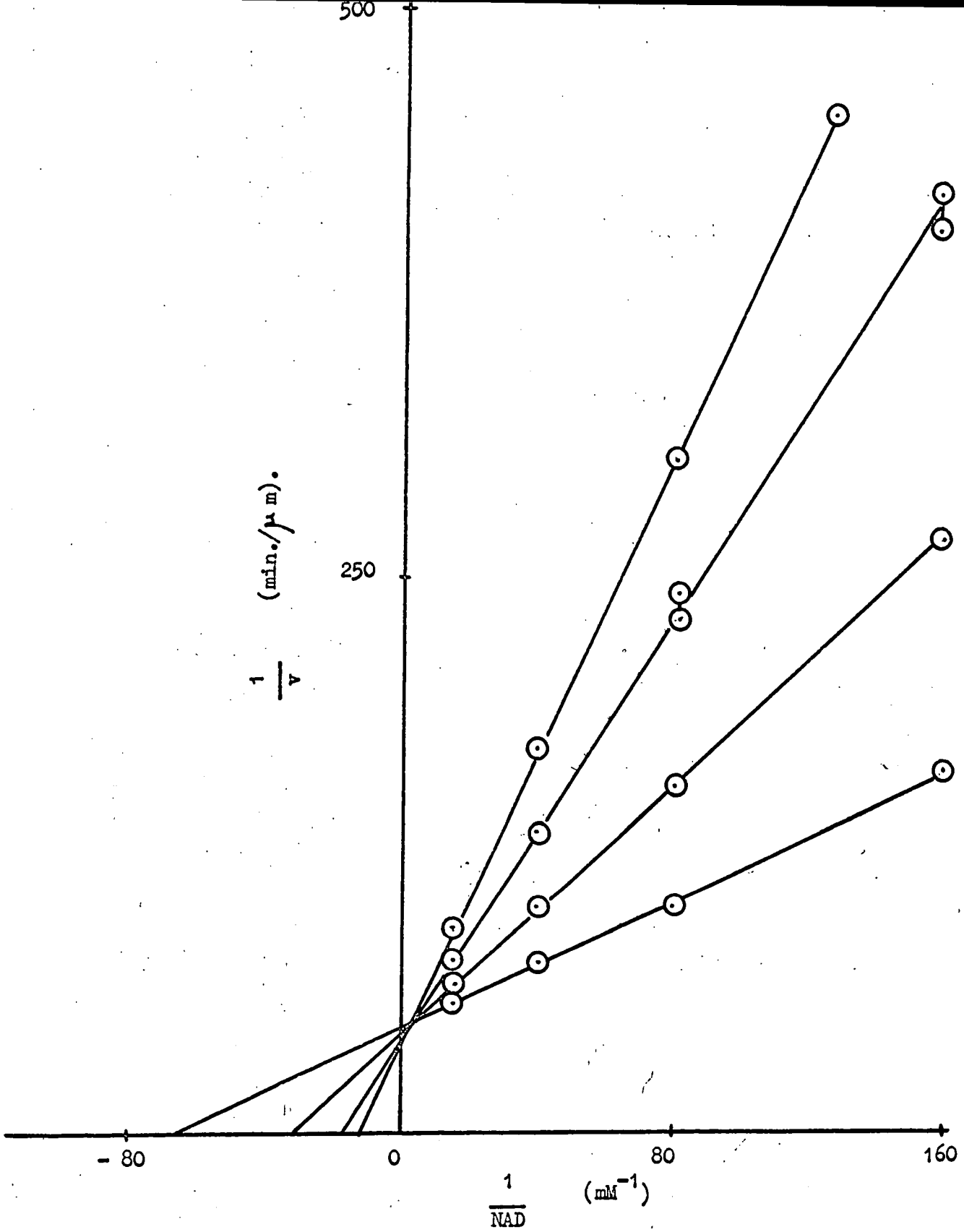


Fig. 44. The dead end inhibition of FDH by ADPR with NAD as the variable substrate.

ADPR levels held constant at 0, 12, 24, 48 μM with formate fixed at 5 mM.

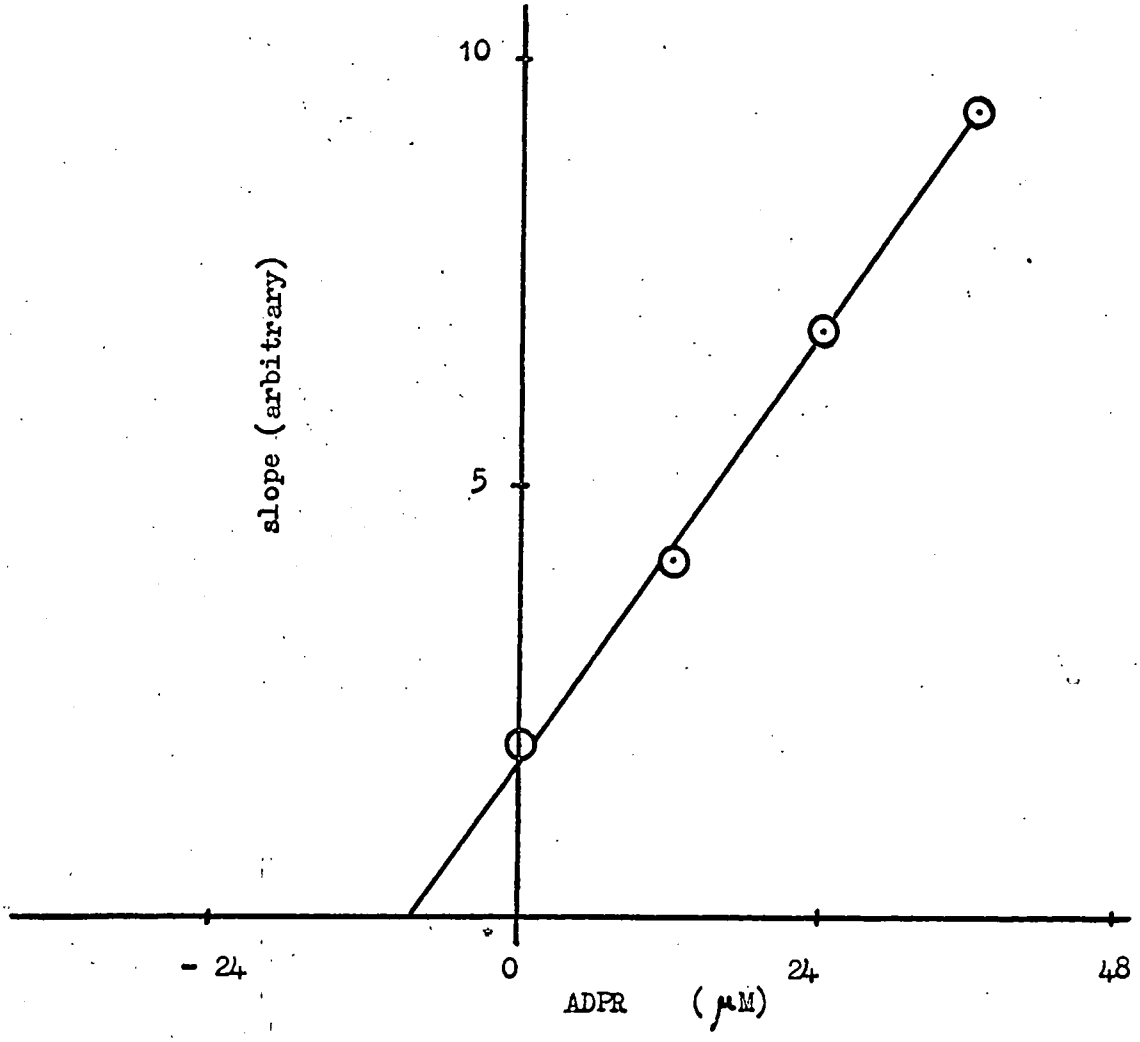


Fig. 45. The secondary plot of the slope of the ADPR versus NAD dead end inhibition.

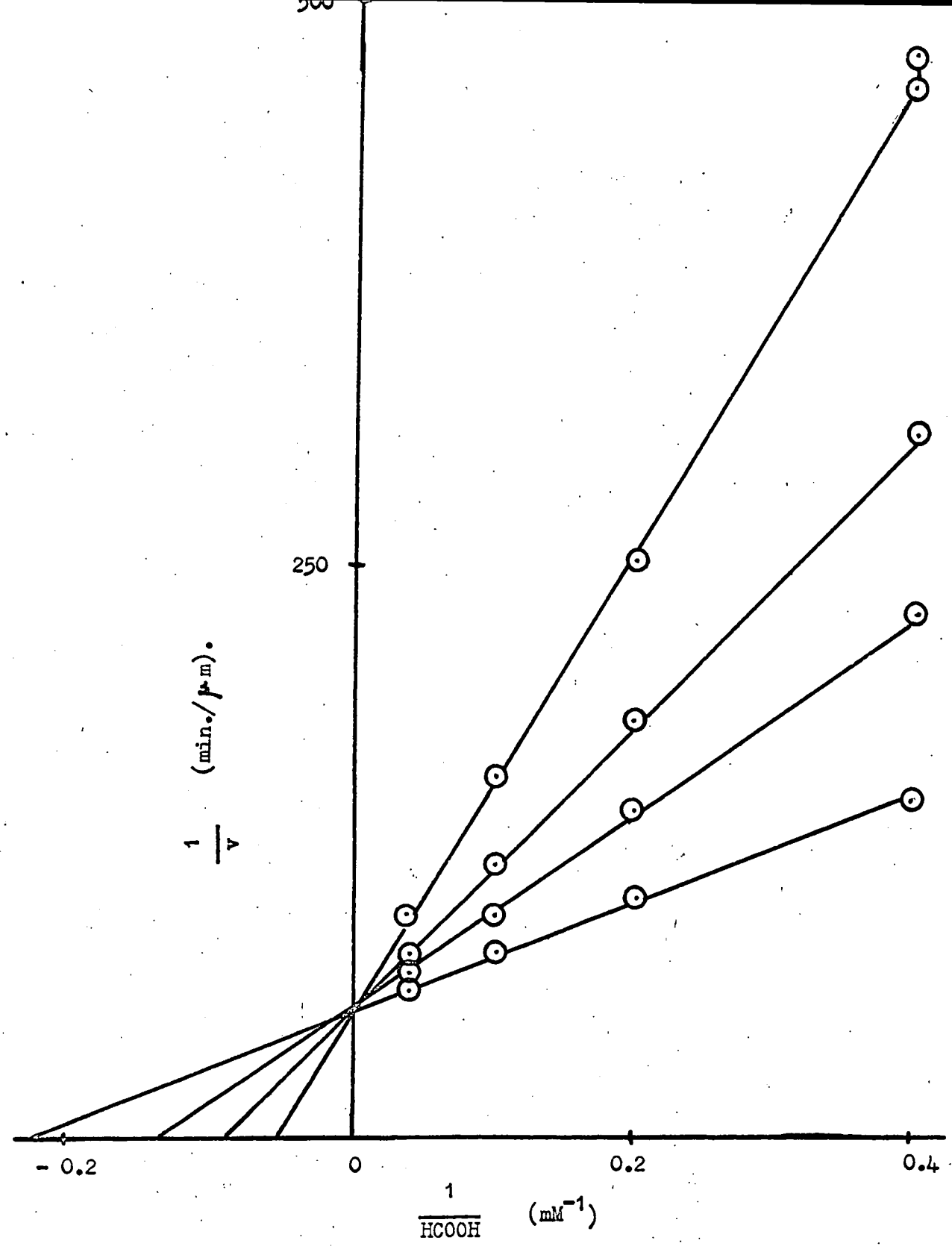


Fig. 46. The dead end inhibition of FDH by nitrate with formate as the variable substrate.

The nitrate levels were held constant at 0, 4.5, 15, 25 mM with NAD fixed at 12.3μM.

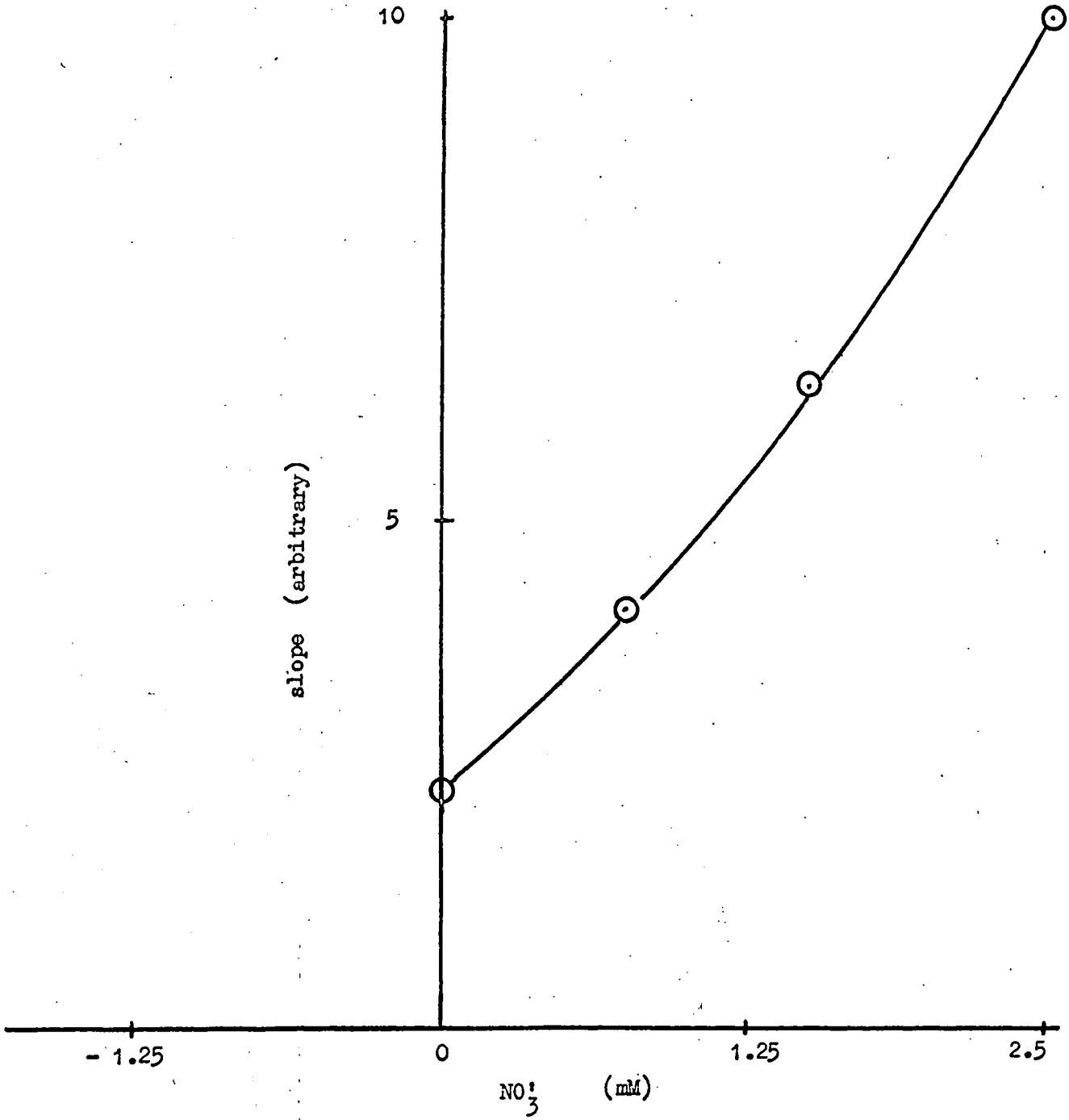


Fig. 47. The secondary plot of the slope of the nitrate versus formate dead end inhibition.

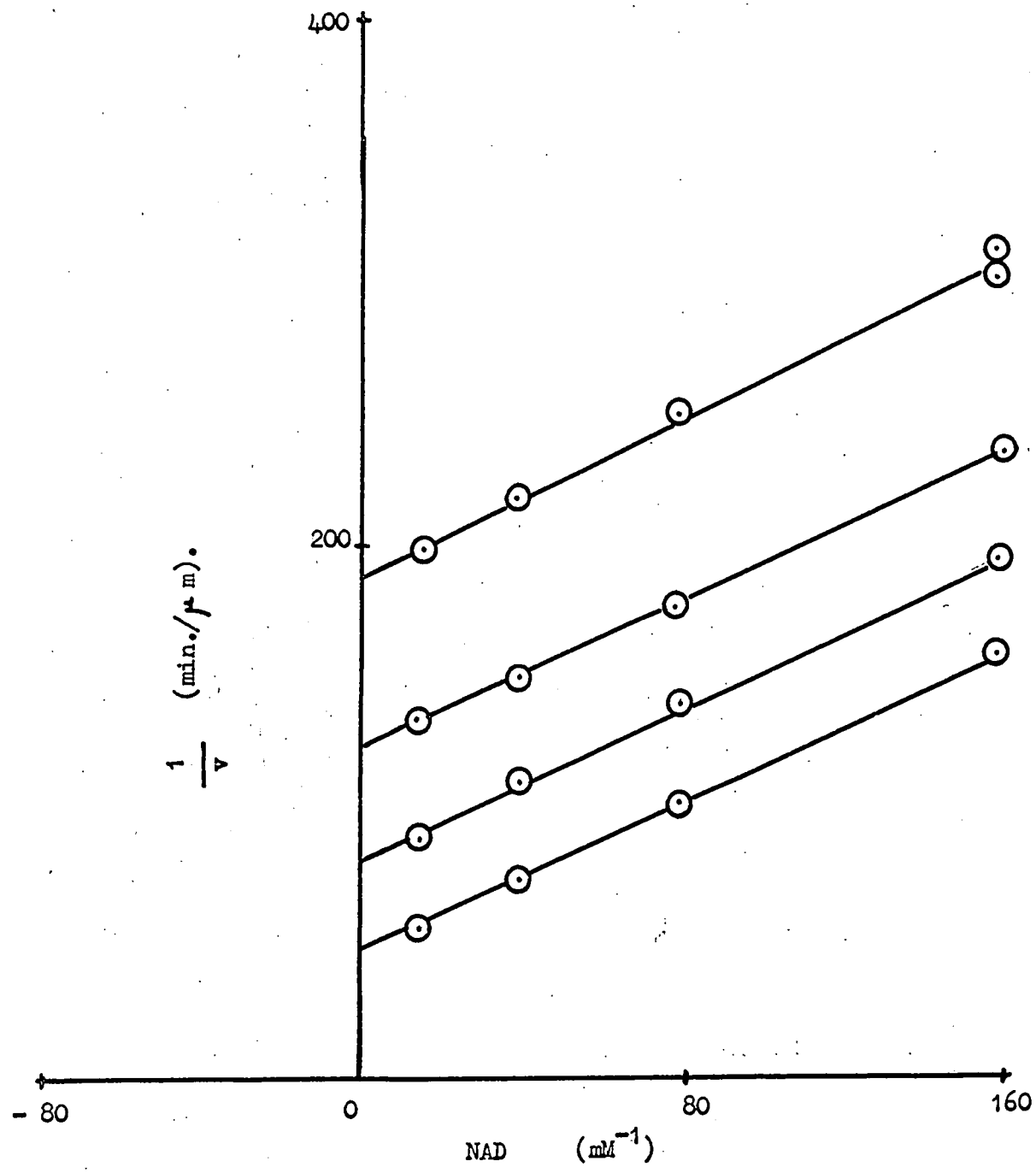


Fig. 48. <u>The dead end inhibition of FDH by nitrate with NAD as the variable substrate.</u>

The nitrate levels were held constant at 0, .45, 15, 25 mM with formate fixed 5mM.

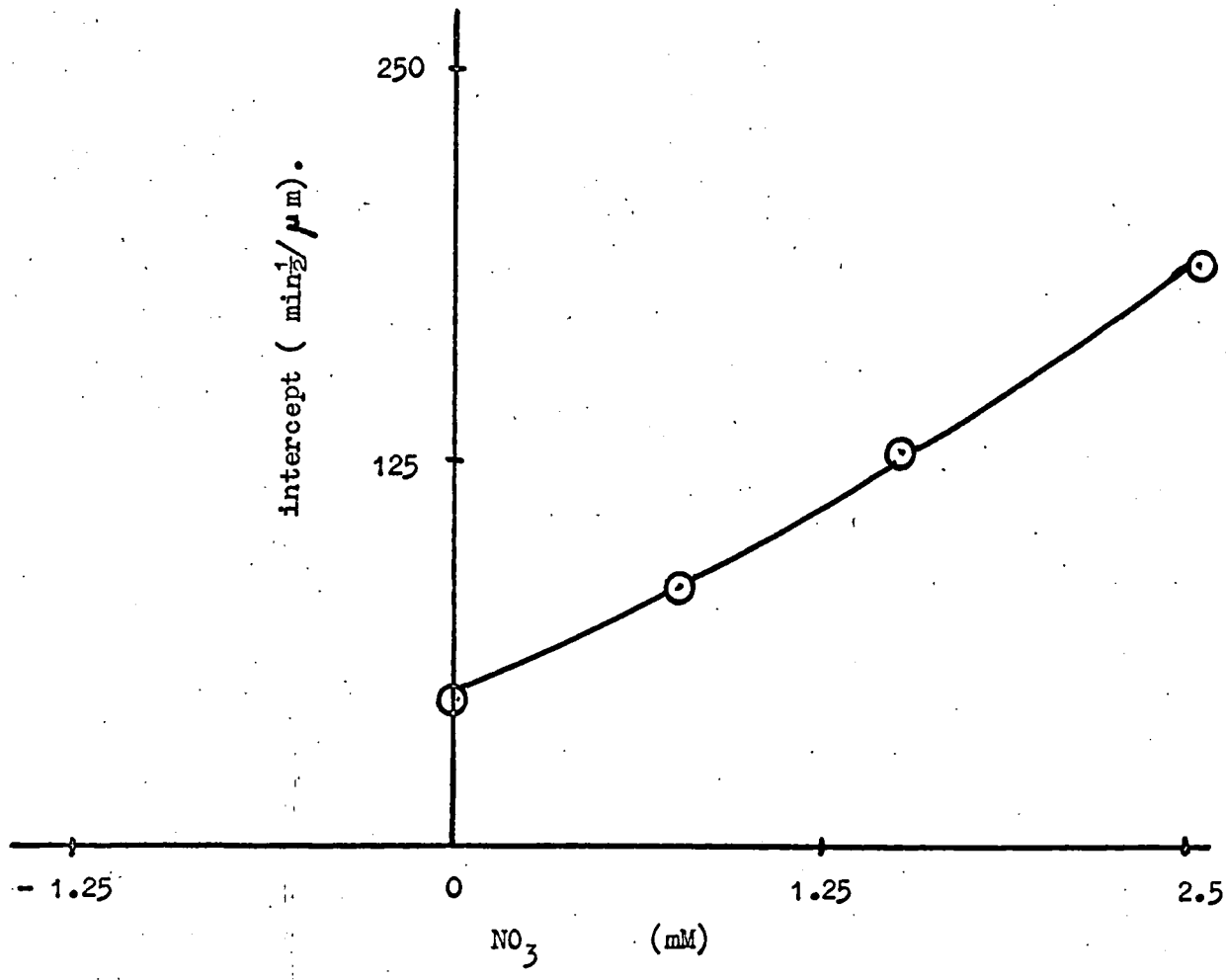


Fig. 49. The secondary plot of the intercept of the nitrate versus NAD dead end inhibition.

Table 27. A summary of the Kii and Kis values

Inhibitor	Variable substrate		Kis	Kii
NADH	NAD		2.5 ± 0.3 μM	-
NADH	HCOOH		2.1 ± 0.5 μM	10.1 ± 3.0 μM
H_2CO_3	NAD	KisA	530 ± 130 mM	445 ± 48 mM
		KisB	*******	
H_2CO_3	HCOOH	Ki1	570 ± 300 mM	-
		Ki2	*******	
ADPR	NAD		12.1 ± 1.3 μM	-
ADPR	HCOOH		12.5 ± 2.0 μM	33.7 ± 5.0 μM
Nitrate	HCOOH	Ki1	1.2 ± 0.14 mM	-
		Ki2	6.3 ± 1.8 mM	
Nitrate	NAD		-	0.92 ± 0.04 mM

******* Using the data of Table 26, the appropriate computer program was selected for each of those patterns and used to calculate the Kii and Kis values directly from the primary data.
Computer unable to calculate value.

(vi) The effect of ionic strength on the velocity at low substrate concentrations.

The effect of ionic strength on the velocity at low substrate concentrations was found to be small (Fig. 50). A difference only became apparent at ionic strengths above 2.3 moles/l.

(vii) Variation of the kinetic parameters with pH.

The effect of pH was determined on the values of Ka, Kia, Kb (Figs. 51, 52 & 53). The most noticeable feature in these results is the maximum in the pKb which corresponds to a minimum in the pKia curve.

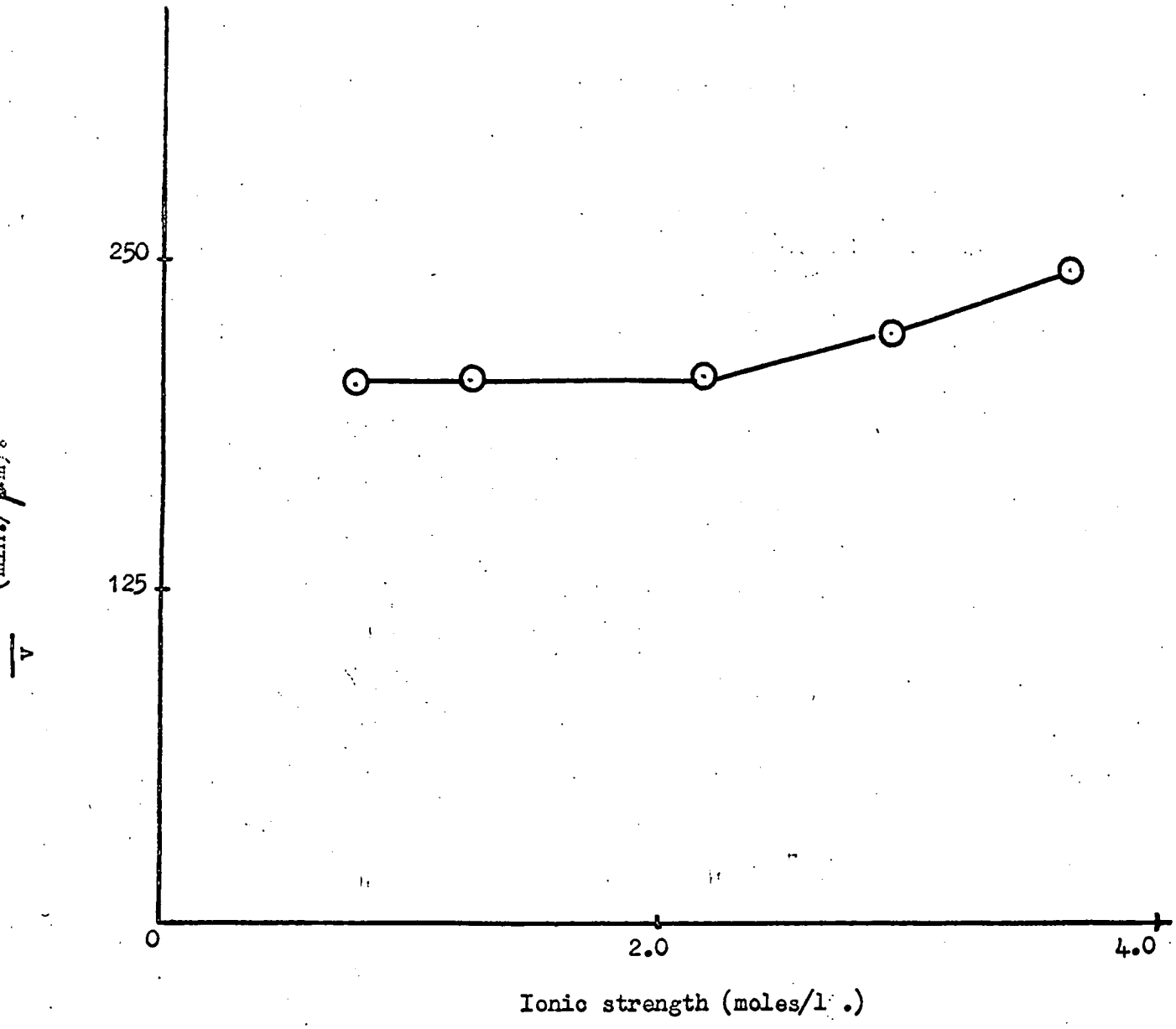


Fig. 50. The variation in the velocity of the reaction at low substrate concentrations with ionic strength.

Formate and NAD were fixed at 5.0 mM and 12 μM respectively.

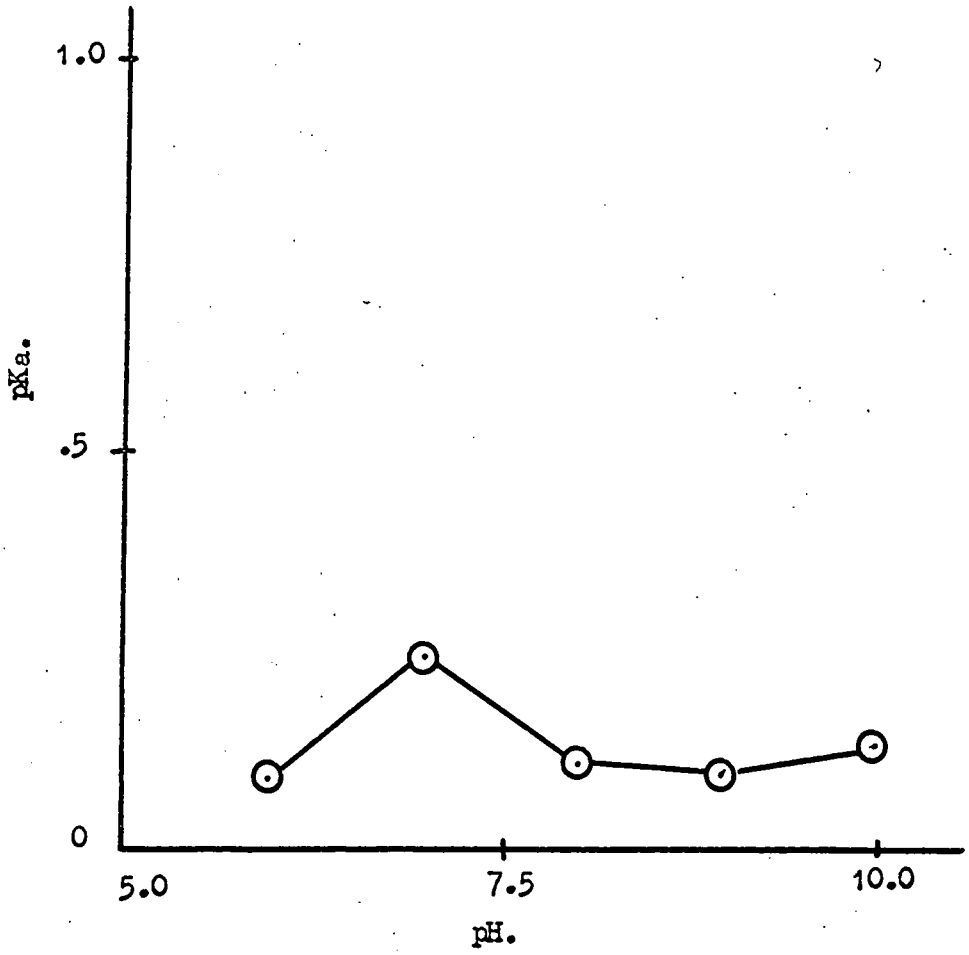


Fig. 51. The changes in pKa with pH.

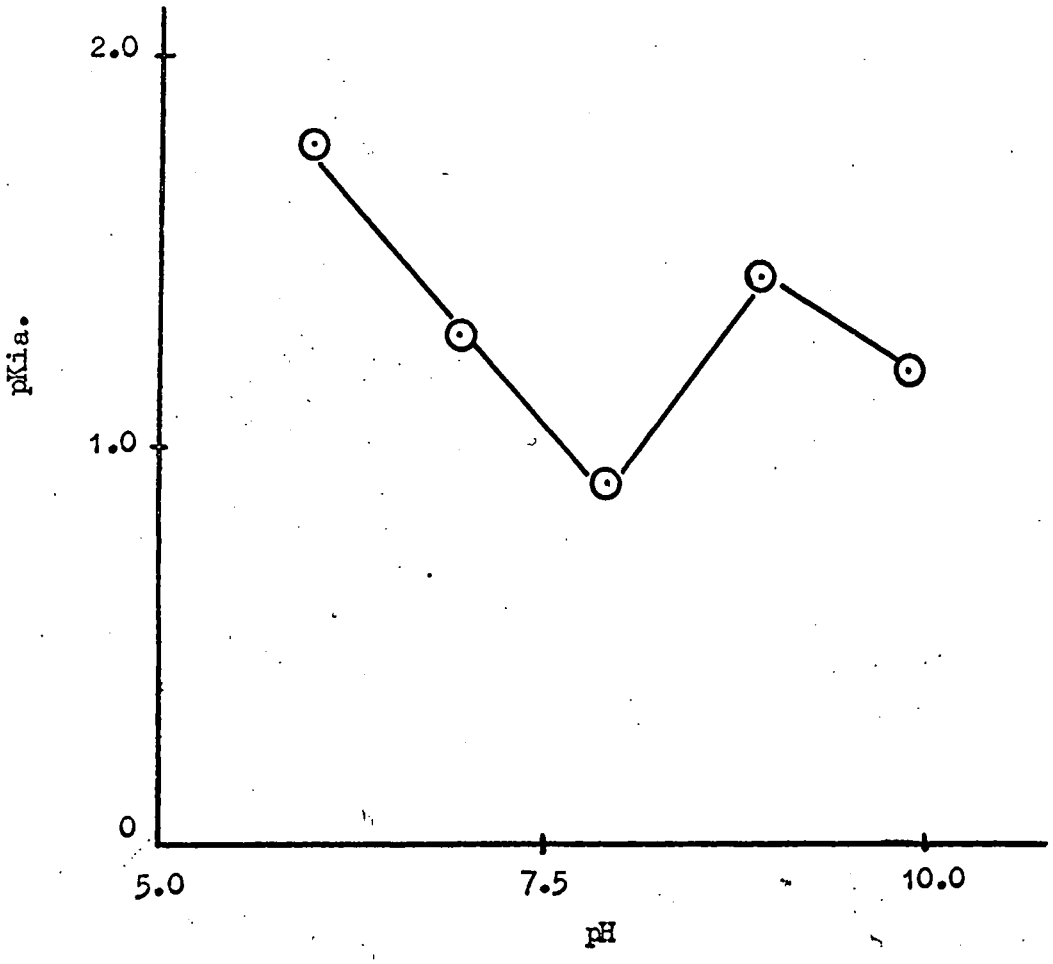


Fig. 52. The changes in pKia with pH.

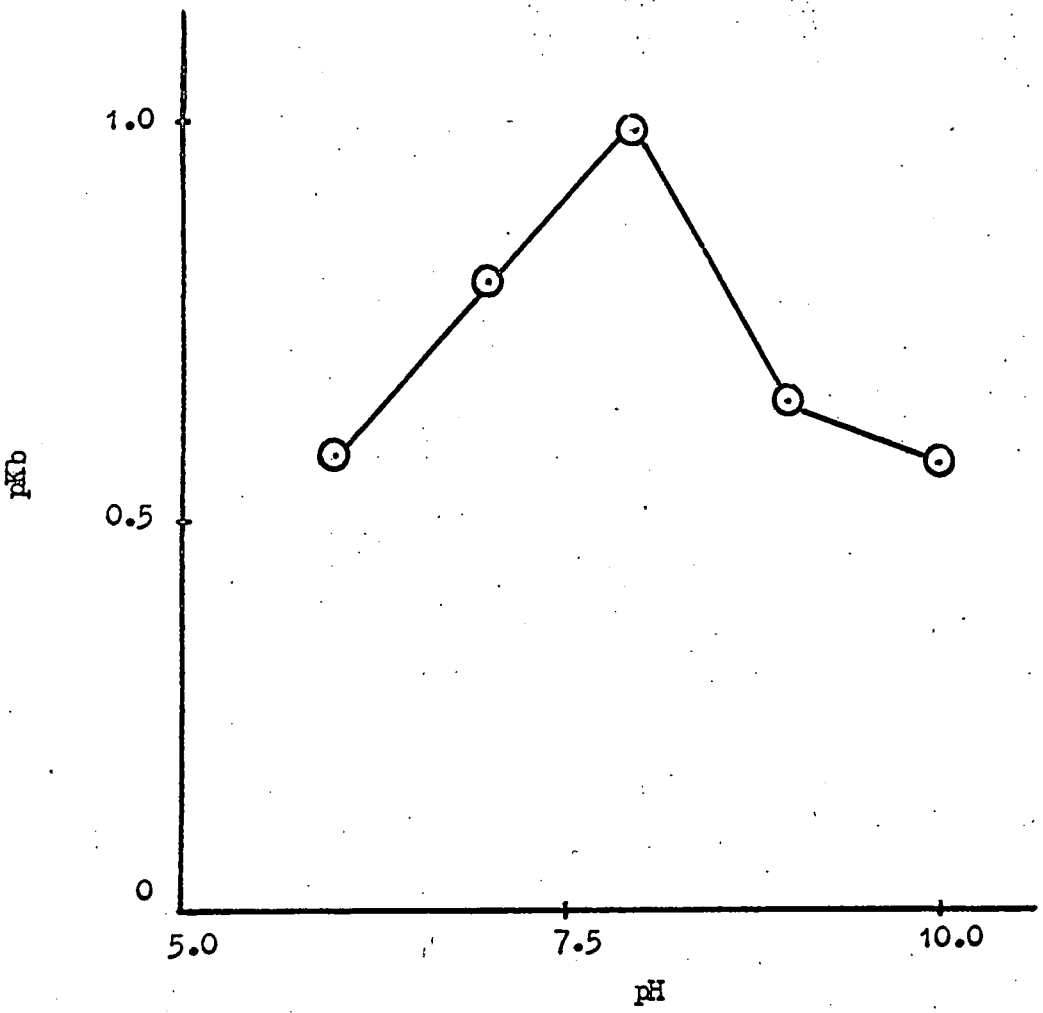


Fig. 53. The changes in pKb with pH.

Although other points are apparent they are so small as to be within the limit of experimental values for these constants were; Ka varied from 5.4 to 11.4 μM, Kb from 0.97 to 2.4 mM and Kia from 16 to 110 mM.

(viii) Variation of V_1 with temperature.

A linear relationship was found when the log. V_1 was plotted against 1/T in °A over the range of 6°C to 40°C (Fig. 54). The slope of this line was identical for pH 6.0, 8.0, 10.0 although the y intercept for pH 6.0 differed from the y intercepts for 8.0 and 10.0 which were the same.

(ix) Attempts at measuring the equilibrium constant.

Using various concentrations of the two substrates, the time taken to reach the half way stage to equilibrium was measured spectrophotometrically. It was found (Table 28) that the only possible combination of concentrations that permitted equilibrium to be reached within a finite time was when HCOOH was in much larger concentration than NAD. Although it only took 20 min. to reach an estimated half equilibrium with approx. 10 mM of both NAD and HCOOH, it can be seen from the progress curve (Fig. 55), that the time to reach equilibrium would be excessive.

Unfortunately, a large excess of HCOOH over NAD made the estimation of the equilibrium constant from measurements of the amount of HCOOH left at equilibrium, too inaccurate.

Table 28. The $T_{\frac{1}{2}}$ values for reaching equilibrium

conc. of NAD (mM)	conc. HCOOH (mM)	$T_{\frac{1}{2}}$ min.
0.66	0.66	greater than 60
3.0	100	2.0
8.0	10.0	20.0
100	100	greater than 600

The pH of the reaction medium was 8.0.

(x) Reversal of the reaction.

The rate of incorporation of $[^{14}C]\ H_2CO_3$ into HCOOH was measured at two pH's. At pH 8.0 (Fig. 56) the amount of incorporation is very small and does not reflect the decrease in optical density at 340 mu which was presumed to be due to the decrease of NADH. The rate of the backward reaction at pH 6.3 was 6x greater and more closely followed the decrease at 340 m (Fig. 56). Table 29 summarises the initial velocities as measured from $[^{14}C]$ incorporation and spectrophotometrically, as well as the conditions of the experiments.

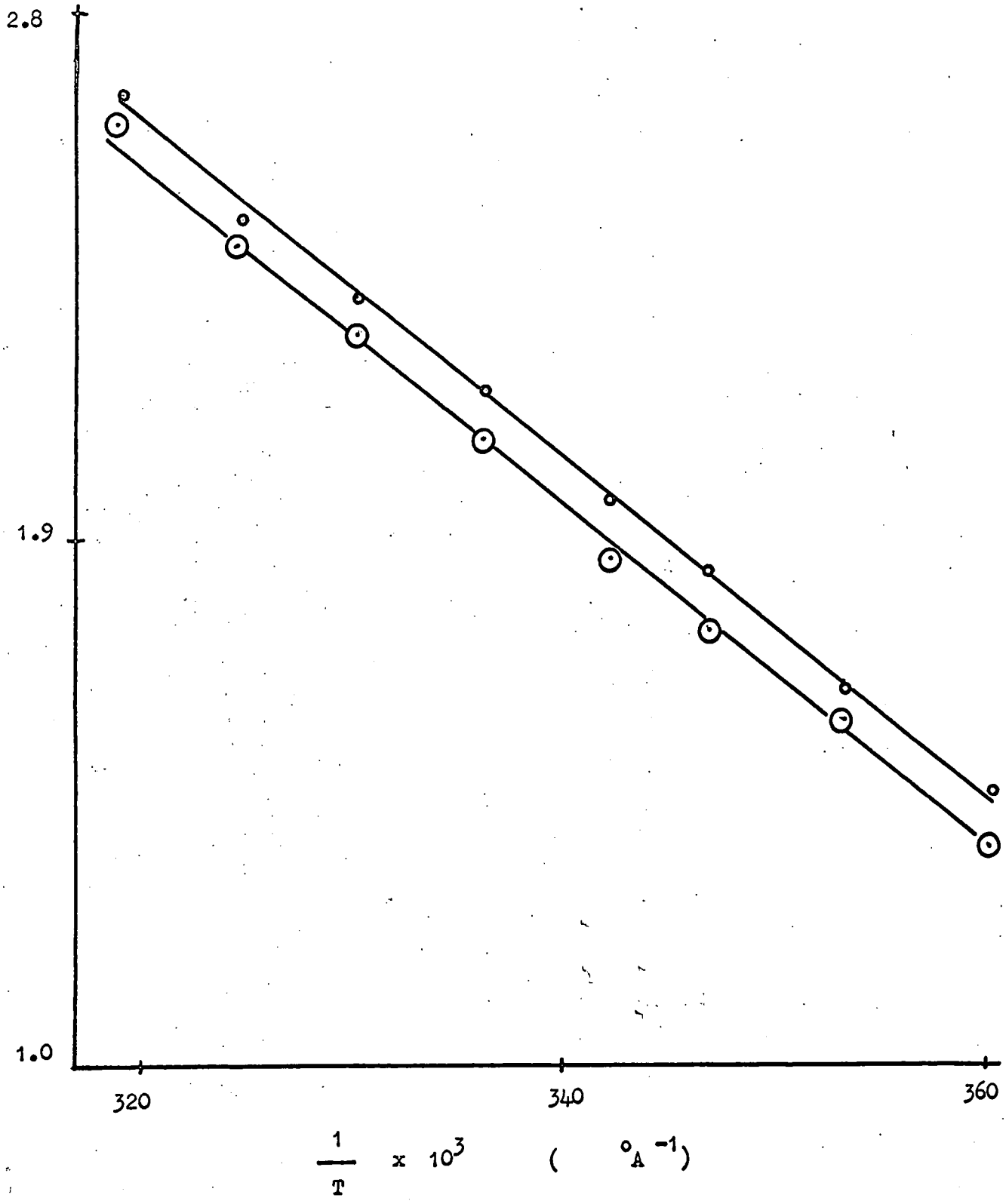


Fig. 54. The variation of V_1 with temperature.

The conc. of formate and NAD were fixed at 200 mM and 500 μM respectively.

pH = 6.0

pH = 8.0 and 10.0

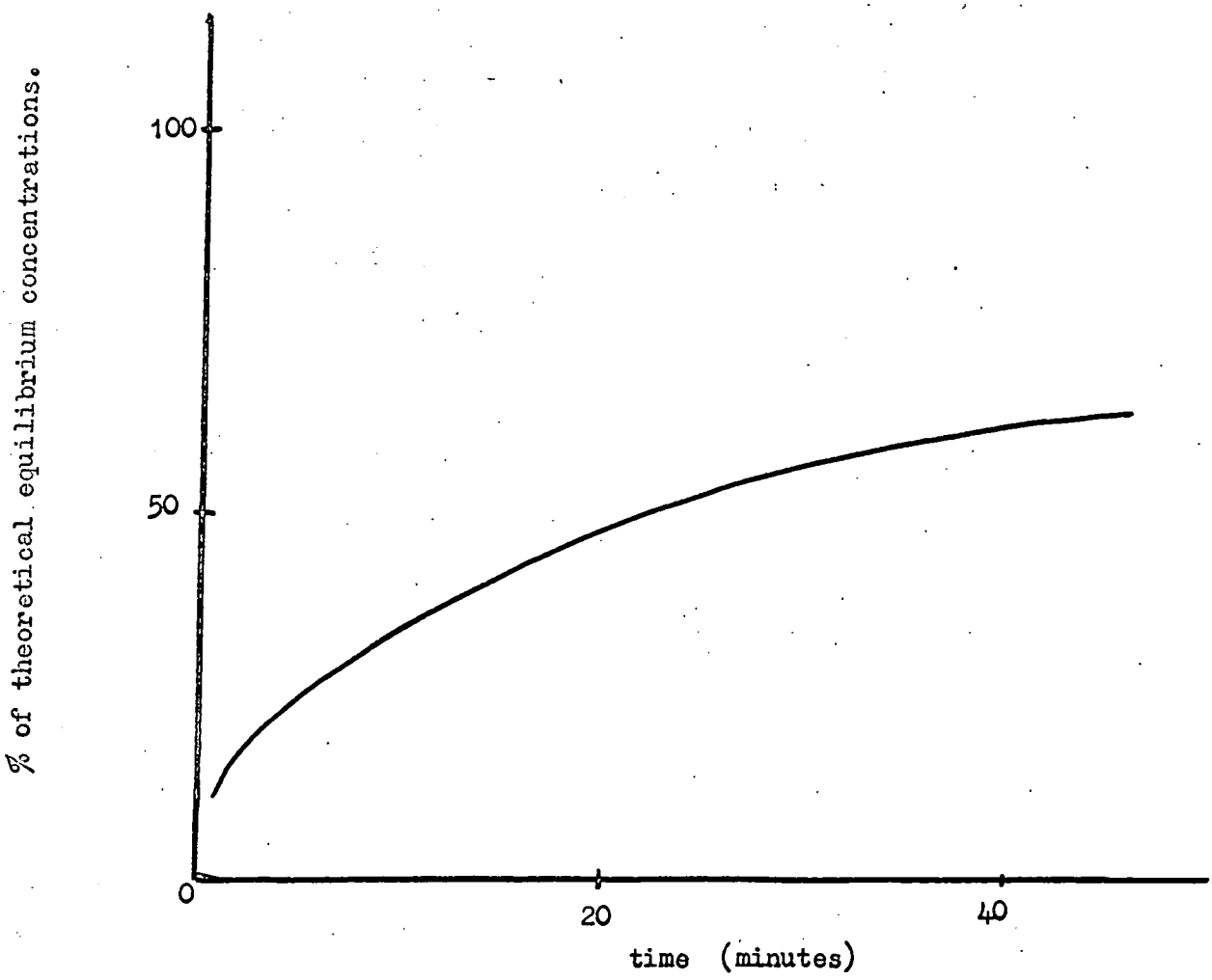


Fig. 55. The progress curve of the forward reaction towards equilibrium.

The levels of formate and NAD initially were 10 mM and 8 mM respectively. The trace was redrawn from a continuous recording of an SP800 spectrophotometer.

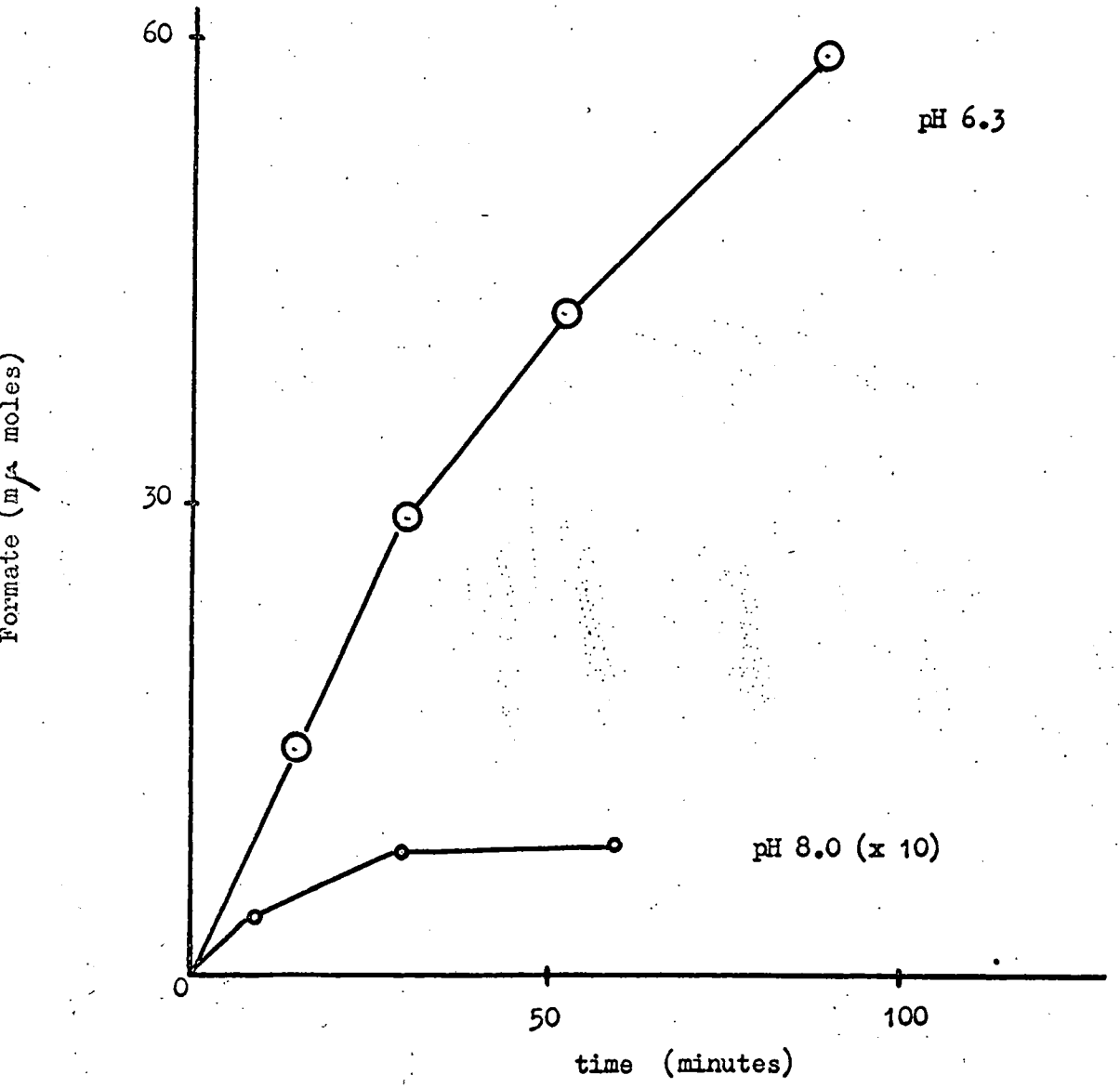


Fig. 56. The progress curve for the reverse reaction.
The levels of bicarbonate and NADH were 20 mM and 400 μM at pH 8.0, and 35 mM and 400 μM at pH 6.3, respectively.

Table 29. The reversal of the reaction

pH	conc. NADH μM	conc. HCO_3 mM	con. CO_2 mM	rate (HCOOH) μμm/min.	NADH μμm/min.
8.0	400	20	-	36	1200
6.5	400		35	710	1200

Ratio of forward to backward reaction = 45,000 pH 8.0

= 2,300 pH 6.3

The enzyme concentration used was 6 mg./ml. of fraction V. 0.1 mCi of HCOONa (44 mCi/mM) was added to 3.0 ml.

(e) Metabolic Role.

(i) Mitochondrial content of formic dehydrogenase.

The activity of the enzyme was estimated in the mitochondrial preparation and the 6000 x g. supernatant. The oxidation of succinate was used to test for mitochondrial activity; although the mitochondrial preparation was active in oxidising succinate and NADH, it was inactive in the oxidation of HCOOH with or without either NAD or ADP (Table 30).

The mitochondrial preparation was subjected to osmotic shock and 1% digitonin and the resulting preparation tested for enzyme activity. Table 31 gives as a percentage the proportion of enzyme in the various preparations.

Table 30 Mitochondrial Oxidation

Substrate	μm O_2 uptake/min./mg. protein
None	0
8mM succinate	100
8mM succinate + 150 M ADP	100
100 mM HCOOH	0
100 mM HCOOH + 150 M ADP	0
100 mM HCOOH + 1 mM NAD	0
100 mM HCOOH + 1 mM NAD + 100 g. FDH (V)	40
1 mM NADH	75

All reactions were measured at 30°C.

Table 31. The proportion of enzyme activity in the various functions of mitochondrial preparation

Preparation	Treatment	% of enzyme units
original extract	-	100
6000 x g. supernatant	-	100
mitochondrial pellet	-	0
" "	osmotic shock	0
" "	1% (wt./v.) digitonin	1

(iii) Changes in enzyme activity during germination.

The changes in formic dehydrogenase (FDH), alcohol dehydrogenase (ADH), and malate dehydrogenase (MDH), were followed during germination. The cotyledons contained the highest levels of all three enzymes but these levels disappeared around day 5. The leaves contained a fairly constant level of FDH and MDH without any detectable ADH. The shoot and root showed a decline of both FDH and ADH with age which closely followed that of the cotyledons (Figs. 57, 58 & 59). MDH on the other hand declined less rapidly in the shoot and root. The ratio of ADH activity to FDH activity was always about 10x greater.

(iii) Changes in the content of formate during germination.

The levels of formate rose to maximum on day 1 which contained 16 mg./100 g. fresh weight (Fig. 60). After this there is a decline until day 5 when there is no longer any detectable amounts. If the beans instead of being planted at day 1 are soaked for a second day the levels continue to rise instead of decreasing. 44 mg./100 gm. fresh wt. was recorded after two days soaking.

(iv) In vivo utilisation of formic acid.

Within 15 minutes 65% of the labelled formate has been oxidized to carbon dioxide (Table 32). None of the amino acids and organic acids located on the two dimensional chromatograms contained sufficient $[^{14}C]$ to be detected after two weeks autoradiographic development.

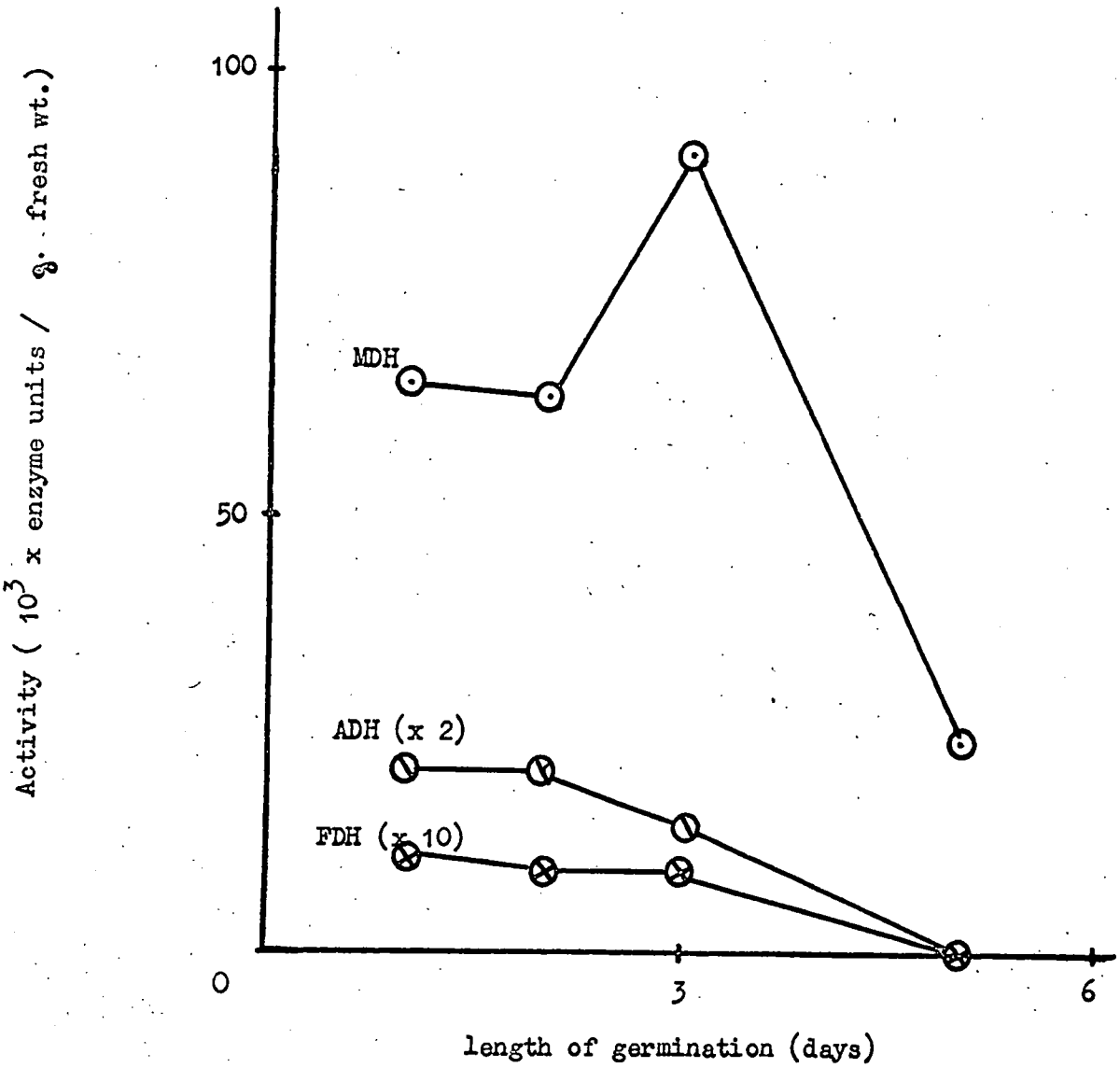


Fig. 57. The changes in enzyme activity during germination of the cotyledon.

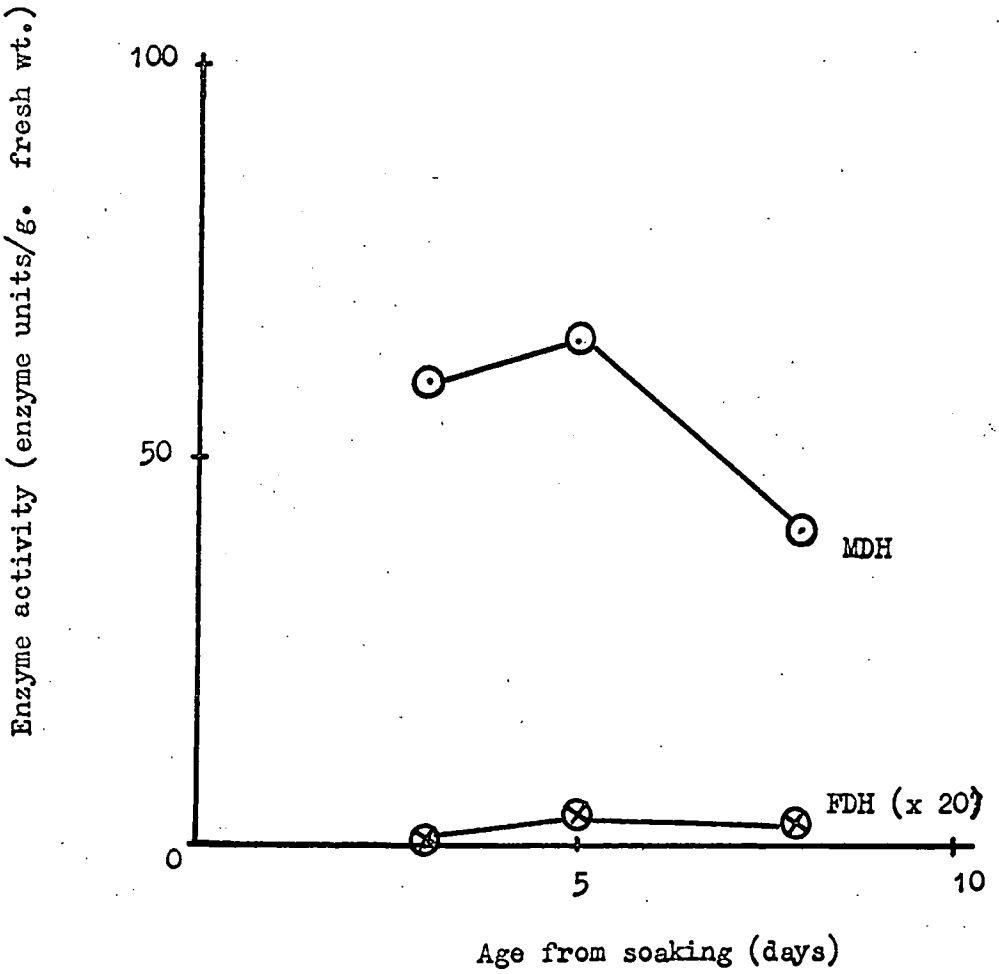


Fig. 58. The changes in enzyme activity during germination of the primary leaves.

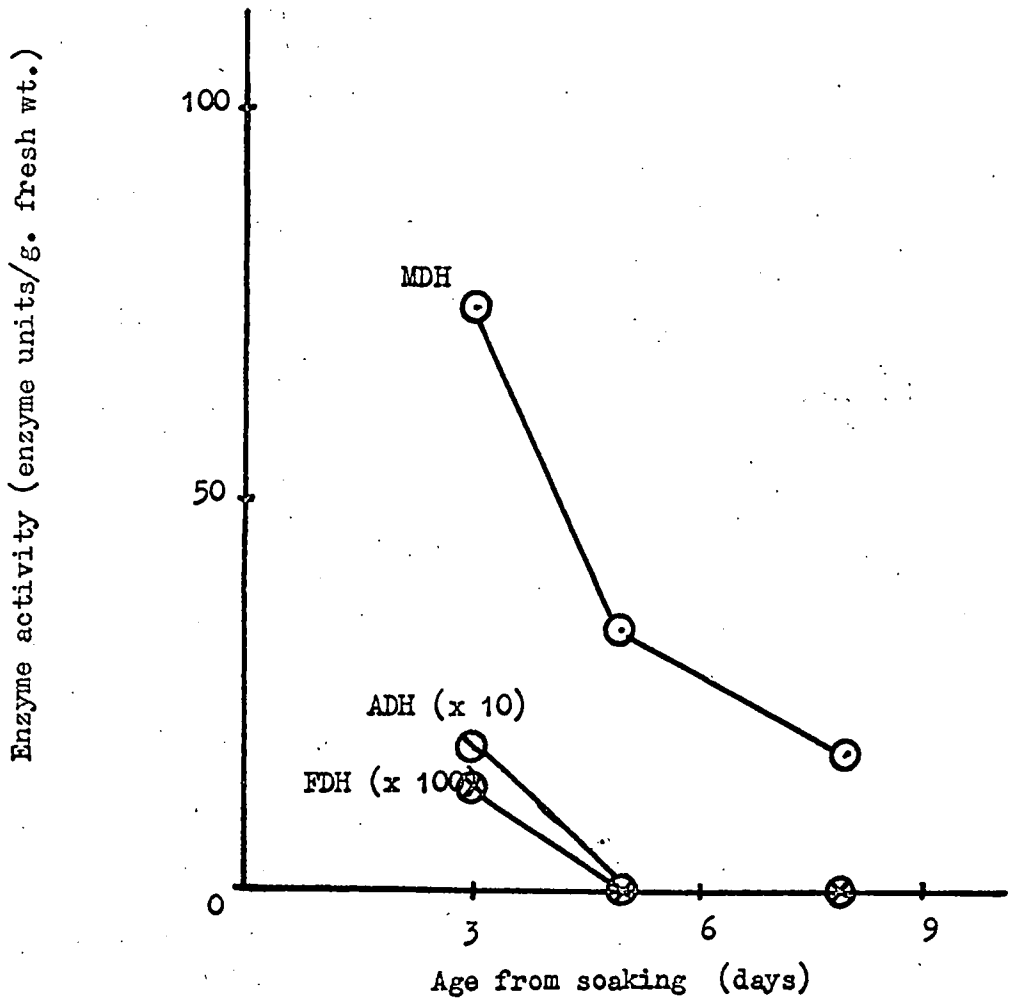


Fig. 59. The changes in enzyme activity during germination of the root.

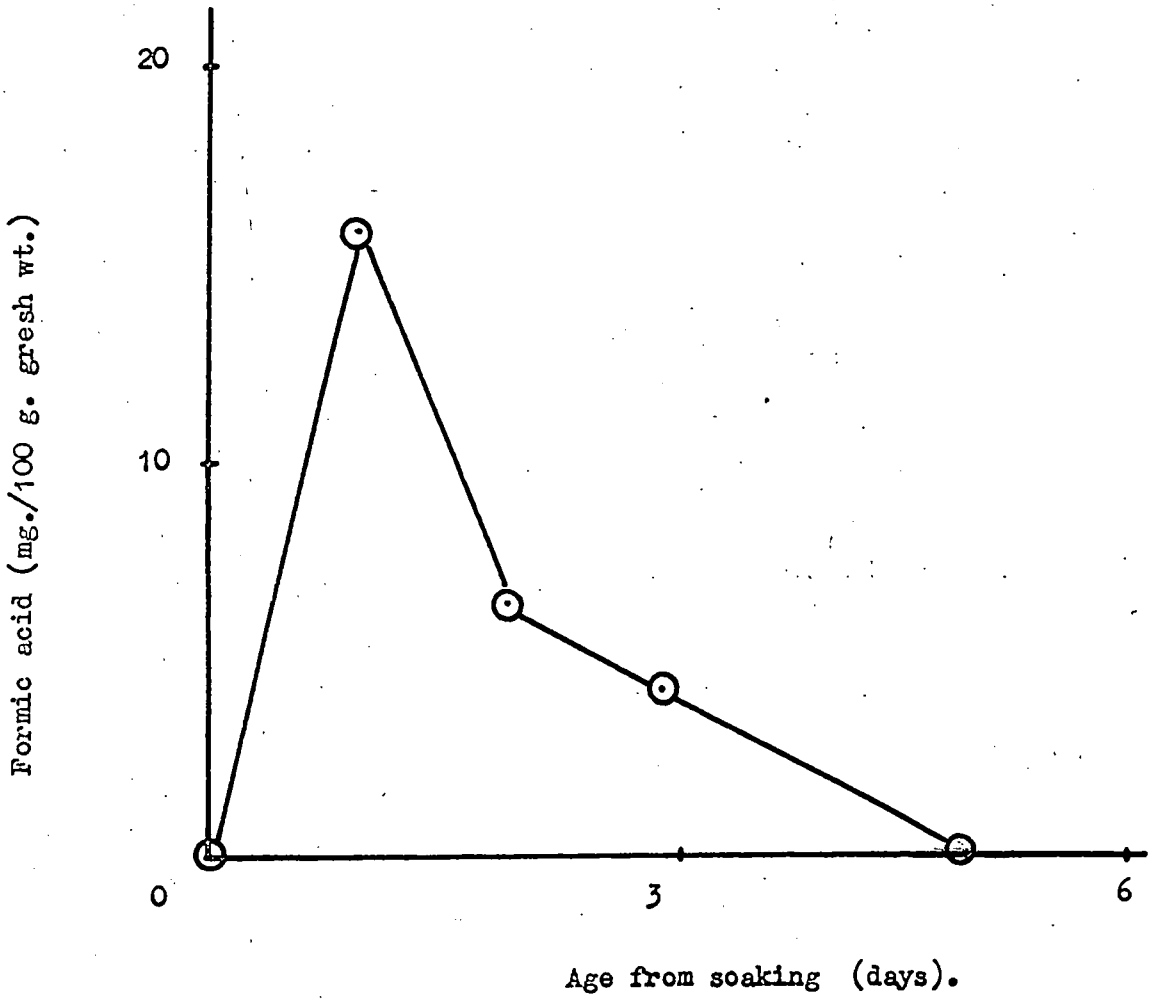


Fig. 60. The changes in the formic acid content during germination.

Table 32. The distribution of [^{14}C] after *in vivo* incubation of labelled formate with sliced cotyledons

Location	% of original radioactivity
aqueous supernatant after incubation	25
alcoholic extract of cotyledons	10
NaOH used for carbon dioxide absorption	65

(f) Molecular weight.

The elution volume of FDH was 233 ml. which corresponds to a molecular weight of 92,000 + 10,000 assuming an approximately spherical shape to the molecule. (Fig. 61).

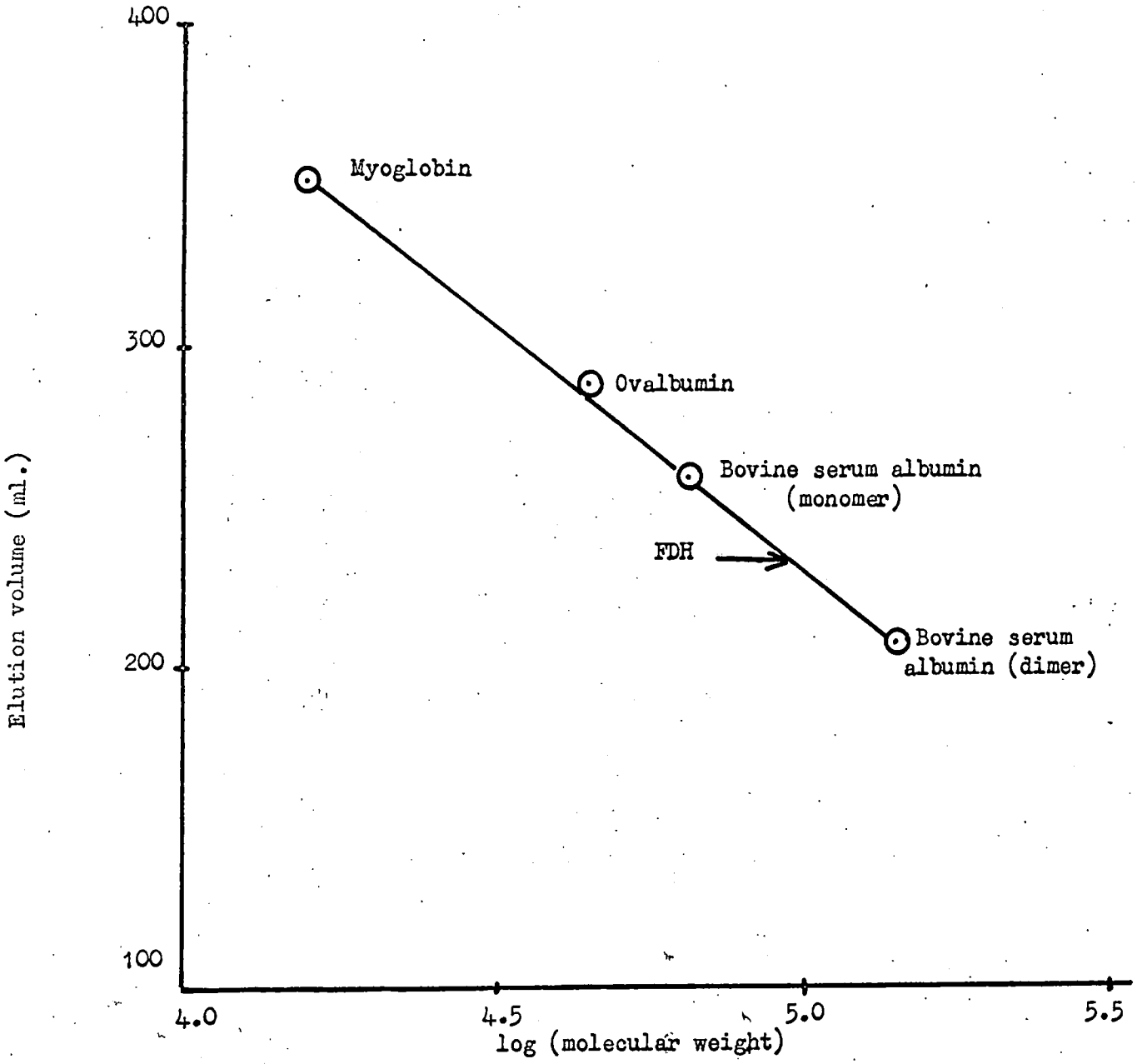


Fig. 61. The estimation of the molecular weight of FDH by gel filtration on sephadex G-200.

DISCUSSION

Purification

In order to purify an enzyme, particularly from a plant source in which proteins often occur in low concentrations, it is necessary to survey various plants to select the best source. For this reason a survey was conducted on leguminous seeds (since this family had been previously shown by Davison (1949a) to be the best plant family for this purpose). The survey was restricted to seeds commercially available in bulk. The results of this survey showed Phaseolus aureus to be the best source of FDH, the results of this survey agreed reasonably well with those of the past survey, (Davison 1949a) when species common to both were compared.

Having selected a source, it was purchased in quantities large enough (50 Kg.) to ensure that the whole investigation could be conducted upon the same batch of seeds.

The purification of any protein begins with its extraction into free solution. This is usually accomplished by mechanical disruption of the cells from which the enzyme is being extracted into an aqueous buffered salt solution. In this process there are a number of steps in which some variation is possible, which would lead to a change in the amount of enzyme solubilised. For example, the degree of mechanical stress employed in homogenization, the ionic species of the salt used in solubilization, etc., and it has not yet proved possible to predict the optimum conditions for the extraction of a particular enzyme, from either theoretical or comparative grounds. For this reason a series of experiments were conducted to find the optimum conditions of

extraction when the conditions under which the enzyme was extracted were changed systematically. The information gained in a previous extraction of FDH from peas, was used in the design of this experiment - Nason and Little (1955). The results obtained (Tables 14-21) established the optimum although due to certain characteristics of subsequent procedures, the conditions employed routinely were slightly below the optimum.

In selecting purification procedures likely to prove useful in the separation of FDH from contaminating proteins, a major consideration was the stability of the enzyme activity to the various procedures and in certain cases the stability of the enzyme activity determined under various conditions (Tables 3 - 12) was used to predict the usefulness of certain procedures for purification of FDH. For example, the loss of activity observed when the enzyme was exposed to pH's below 5.6 (Table 3) precludes the use of low pH as a fractionating procedure. Similarly the loss of enzyme activity observed when the enzyme was exposed to heat (Table 4) suggests that heat fractionation would not prove effective in purification. The enzyme isolated from pea, has also been reported to show a comparable lack of stability towards high temperature and low pH, Nason and Little (1957).

Usually an indispensable part of any purification scheme is a method of reducing salt concentrations prior to either absorption or ion exchange chromatography. Two methods are in common use, dialysis and gel filtration. In this investigation gel filtration was not used since impure preparations caused the column to become blocked, and it would also have taken a column of impracticable size to handle some of the volumes produced during the purification procedure.

However, dialysis of FDH always caused some loss of activity, and what effects the changes in the conditions of dialysis and extraction had upon the loss of activity caused by dialysis, was investigated so that these losses could be kept at a minimum. It is interesting to note that the greatest effects upon the loss caused by dialysis, were produced by changes in the extraction conditions used, and not in the subsequent dialysis conditions (Table 11). A possible explanation could be that other molecular species present in the dialysed mixture were causing loss of enzyme activity by their absorbtion to the enzyme which resulted in co-precipitation. Such absorbtions are favoured by low ionic strength and also different extraction conditions could change the proportion of these molecular species if this is in fact a correct explanation. Other possible causes for this loss on dialysis could have been a loss of a dialysable co-factor from the enzyme, or the possible denaturation of the enzyme at low ionic strength. No doubt the true cause was a combination of several effects. However, by using the appropriate conditions, these losses could be kept to 20%, and it was not considered worthwhile investigating the reasons for it any further. Other workers have also reported a similar loss on the dialysis of the pea enzyme (Davison, 1951, Nason and Little, 1955).

Ammonium sulphate fractionation was found to be one of the most useful steps in the purification procedure, achieving both an impressive separation of the enzyme from other proteins as well as reducing the volume of solution quite considerably. Dixon and Webb (1961) discussed the theory behind the use of salt fractionation in the separation of proteins. They point out that the amount of protein precipitated from solution by a given salt concentration is dependent upon the original concentration of

that protein. This means that to be able to predict the percentage of protein that will be salted out from solution at a given salt concentration, it is necessary to know both the concentration of the protein and its solubility at that particular salt concentration. It was for this reason that the results contained in Figures 3 & 4 were obtained and allowed the calculation of the amounts of ammonium sulphate needed to precipitate a given amount of enzyme.

When FDH was fractionated using these solubility curves under various conditions of temperature and pH, and it was found that lowering the temperature at the same pH had an adverse effect on the specific activity of the fractionated enzyme, without affecting the yield of enzyme activity recovered. On the other hand, lowering the pH at the same temperature reduced the specific activity but increased the yield (Table 22). The second fractionation carried out at a later stage in the purification procedure, involved high pH; it was decided to use low pH and high temperatures for the first ammonium sulphate fractionation. It is interesting to note that the second fractionation, although carried out at high pH, did not cause the same loss of activity observed in similar conditions for the first fractionation. It would therefore appear that the loss of activity was dependent on the state of purity of the enzyme prior to fractionation; the higher the state of purity, the higher the % yield of enzyme recovered after fractionation.

Ion exchange chromatography was chosen to follow ammonium sulphate fractionation in the purification scheme because of its large capacity for protein. The separation of proteins on DEAE-cellulose is brought about by making use of the different affinities they show for the fixed ionic groups

in the ion exchanger. The affinity any particular protein shows for DEAE is a characteristic property of the ionic charges, and their spatial separation within the protein. The separation of a mixture of protein molecules is brought about by opposing the forces which will tend to bind the proteins to the ion exchanger, with a second force which acts upon the separate protein species to the same degree. In this way, the difference between the opposing set of forces can be used to cause movement of the protein molecules. This movement is best thought of as occurring in one direction along a channel, the cross sectional area of which is small in comparison to its length. The volume of the channel is completely occupied by molecules of the solvent, and the solute, which are free to move, and also by molecules accessible to the solute and solvent, but which are not capable of movement (stationary phase). The impelling forces causing movement of the solute and solvent are hydrodynamic, and the retarding forces are of two kinds. The first are a group of forces generally acting on molecules in homogeneous solution, molecular frictional effects, electrostatic and dipole interactions with the solvent and other solute molecules. The second group of retarding forces, are those introduced by the interaction of the solute molecules with the stationary phase. Both these groups will be characteristic for each of the different solute molecules, and when these retarding forces reach equilibrium with the impelling forces, a specific molecule will move at a constant speed along the channel, characteristic of that molecule in these given environmental conditions.

The different migration speeds of the different molecular species will bring about their separation provided the distance of migration in the channel is adequate. There are three fundamental methods of

operation for the separation process depending on the initial boundary conditions employed. The first is frontal analysis in which the mixture to be separated is fed continuously into the operating channel. In this case complete separation of the various solutes does not occur and their presence can only be detected by a series of fronts which in an ideal case corresponds to each of the individual solutes. This method has only analytical applications and was not considered further. The second fundamental method of operation is that of elution analysis in which the mixture to be separated is introduced into the operating channel in a narrow zone so that it occupies only a small length of the channel. The zone is then induced to move along the channel and the differences in retarding forces cause the separation of the solutes. The essential nature of elution analysis is that in the ideal case all the components can be obtained in the pure state. It was for this reason that this was the only method of operation that was investigated. The third method of operation is that of displacement analysis which depends upon the relationship between the rate of movement of the zone on the solute concentration. A narrow band of the mixture to be separated is introduced into the column as in elution analysis. A solution of displacing substance which is more strongly absorbed than any of the components of the mixture to be separated is then fed continuously into the column. This substance as a consequence of its stronger affinity for the absorbent will displace all the components of the mixture which will begin to move along the column. Each component will act as a displacing agent for all other components of lower affinity for the absorbent. Thus after a steady state has been reached a system of continuous zones will move along

the column. The length of the column need only be long enough to reach this steady state for after this no further separation occurs. Distribution analysis is only possible when certain definite relationships exist between the distribution characteristics of the displacer and the displaced substances. The ultimate resolution is also inferior to elution analysis, so for these reasons it was not considered further.

Having decided upon elution analysis it was necessary to determine the conditions best suited for elution of FDH from DEAE-cellulose. The ultimate resolving power of the separation process depends upon the system reaching equilibrium throughout the column. The kinetics of reaching equilibrium are therefore essential for determining how dependent the resolution will be on the flow rate of the eluent through the column. Figs. 5 & 6 show that the majority of the enzyme is absorbed and desorbed within 5 min. Flow rates were therefore considered not critical for good resolution. The affinity of the enzyme for the column is dependent upon the ionic environment of the molecule, and this is shown clearly when the effect of ionic strength is plotted against total capacity of the resin for the enzyme (Figs. 9 & 10). From these results it can be seen that to obtain the maximum binding of the enzyme to the resin it is necessary to reduce the ionic strength to 5mM. In this way a very narrow starting zone of enzyme on the column is obtained and it is the width of this starting zone upon which the subsequent resolution achieved depends. These results also show that 40mM is sufficient to desorb the enzyme from the resin. Another factor which can affect resolution is the shape of the distribution isotherm. The distribution isotherm shows the relationship between the concentration of the solute at equilibrium and the concentration of

solute absorbed to the stationary phase. In an ideal case an increase of the solute concentration will cause a linear increase in the amount of solute absorbed to the stationary phase, until saturation is reached. Such a linear distribution isotherm can be used to predict the shape of the zone eluted during elution analysis. A linear isotherm will give a Gaussian elution profile but a non-linear isotherm will produce trailing of one of the boundaries. This trailing will adversely affect the resolution. The distribution isotherm for FDH on DEAE-cellulose was found to be convex (Figs. 7 & 8). This is not uncommon for polyelectrolytes such as proteins and the effect of this convex isotherm is to cause the rear boundary to trail. This trailing can be counteracted by using an elution system of increasing ionic strength, instead of fixed ionic strength. Increasing ionic strength reduces the affinity of the protein for the exchanger and hence more and more protein is desorbed as the ionic strength is increased. This counterbalances the effect of the convex isotherm which causes less and less protein to be desorbed as the concentration of the protein falls. A concave exponential gradient was used as the most efficient in counteracting the adverse effect of a convex distribution isotherm.

Two buffer systems were investigated, one at pH 6.5 and the other at pH 8.6, and there was little difference between them which indicated that there were only small changes in the nature and distribution of the charged groups involved in binding the enzyme to DEAE over this pH range. However, the higher pH was selected for the higher purification that could be achieved using it.

Hydroxyl apatite which was used as the third step in the purification scheme was investigated in a similar manner to DEAE and the theory discussed for DEAE applies equally well to absorbtion chromatography. The kinetics of absorbtion/desorbtion were very poor and so very slow rates were used in operating this column. In fact the speed towards equilibrium was so slow (Fig. 14) that it is doubtful that even with very slow flow rates, equilibrium was ever reached. This can be caused in the case of large molecules by diffusion being rate limiting, and if diffusion from the stationary phase to the mobile phase is slower than the reverse process it can produce rear boundary trailing. As the distribution isotherm was linear it is likely that this was indeed the case as there was excessive trailing of the rear boundary. It was for this reason that stepwise elution was employed which is even better in reducing trailing than gradient elution but unfortunately, it can introduce multiple zoning and this effect is indeed seen in Fig. 18.

Alumina gel completed the purification scheme but had to be used in a batchwise manner owing to its poor hydrodynamic properties. The final purification achieved was 540 fold with a 9% yield. This compares with a 50 fold purification and a 10% yield that was obtained by Nason and Little (1955) from peas. The estimated state of purity of this final fraction (VI), was 55% as judged by acrylamide gel electrophoresis. Fraction VI did not separate further on either Sephadex G 200 or polyacrylamide P-150 and P-300; and rechromatography on DEAE only increased the specific activity by 50%. Polyacrylamide was noticeably a better resin for gel filtration than Sephadex.

Sephadex columns had to be packed with great care to ensure that no irregularities occurred within the column which could cause disturbances in the migrating zones. Sephadex also could only be operated with flow rates less than 10 ml./hr. Bio-Gel (polyacrylamide), on the other hand packed evenly and allowed flow rates up to 40 ml./hr. without any of the bed shrinkages noticed when Sephadex was operated at high flow rates.

Acrylamide gel disc electrophoresis has undoubtedly a better resolution in the separation of proteins than any other single systems. So it is very likely that the estimate made of the state of purity of fraction VI is correct, and considerable additional effort was put into finding ways to obtain 100% pure enzyme. De scriptions of apparatus for using acrylamide gel electrophoresis on a preparative scale have been published over the past 5 years in an ever increasing number. Lewis and Clark (1963), Hjerten (1963), Joven et al. (1964), Racusen and Calvanico (1964), Raymond (1964), Radharkrishnamurthy et al. (1965), Maizel (1964), Duesberg, Reuckert (1965), Hjerten, et al. (1965) and (1969), Raymond and Jordan (1966), Sulitzeanu, et al. (1967), Bengt and Harlestam (1968), Groves and Sells (1968), Bront et al. (1969) and Brownstone (1969).

In view of the high resolution of acrylamide gel electrophoresis it was decided to use one or two of the latest apparatus being produced as an additional step in the purification scheme.

One apparatus tried was that produced commercially by L.K.B. Ltd. It only proved capable of eluting the bromophenol blue marker band. No protein or enzyme activity was ever eluted. No reference to this apparatus was available in the literature. The second apparatus produced commercially by Quickfit and Quartz Ltd., proved to be

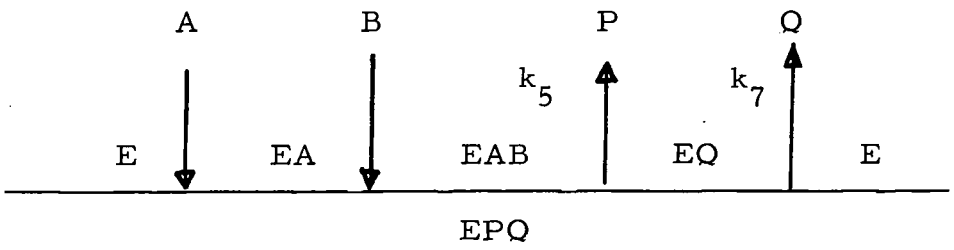
partially successful. Enzyme activity could be recovered from a successful experiment with a 80% yield. Bovine albumin was also separated with a resolution similar to that of an analytical gel. However, the few experiments that were successful were outnumbered by those that failed. Three reasons were found to explain the failures. The elution cell became blocked on some occasions, the elution cell leaked on certain occasions, and thirdly a gap between the elution cell and the main body of the apparatus caused the elution buffer to deviate from its route across the base of the gel and then to travel around the outside of the gel. All of these faults stem from the way the elution cell is connected to the main body of the apparatus. In the published literature Lewis, Cheever and Seavey (1968), Gordon and Louis (1967) the diagrams of the apparatus show differences in the design of the elution chamber. It is possible that these differences, which Quickfit and Quartz claim to be improvements, have in fact caused unforeseen problems. This apparatus is therefore considered to show great promise, only needing slight modifications to overcome the faults which developed in the elution chamber. Unfortunately, limited time did not allow any further attempts at personal modifications.

Having investigated in great detail all the purification techniques which have proved the most useful in purifying similar enzymes, no further attempts at the purifications were made. However, it is likely that one further purification procedure would prove sufficient to obtain the enzyme in a pure state, with yields of the order of 30 mg./kg which would represent a useful yield of enzyme for structural studies.

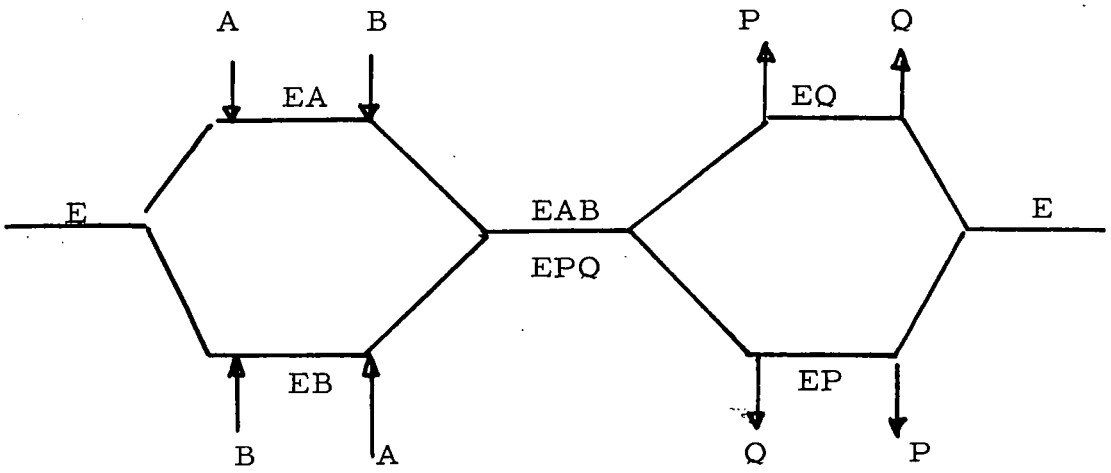
Kinetics

Studies on the action of the enzyme were begun by using known enzyme inhibitors as a means of indicating some of the groups necessary for activity. p-chloromercuribenzoate was the most effective inhibitor found and is known to be fairly specific for SH enzymes. Azide, cyanide and hydroxyquinoline also proved to be effective inhibitors, all of which are known to cause inhibition of metal containing enzymes. However, both cyanide and azide are known to inhibit some enzymes which do not contain metals. It is therefore unsafe to assume the presence of a metal in FDH from such inhibitions, especially as NO_3 also proved an effective inhibitor. The inhibition of NO_3 was due to its competition HCOOH for a site in the active centre (Fig. 46) and it is not unlikely that azide and cyanide which are both small negatively charged ions similar in size to NO_3 and HCOOH could also be acting as competitive inhibitors of HCOOH and not as metal complexing agents. Similar inhibitors to the above have also been reported for the pea enzyme, Davison (1951) and Nason and Little (1957).

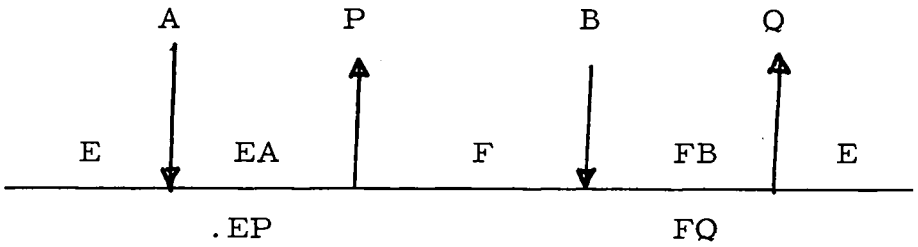
An enzyme mediated reaction involving two substrates and two products (BiBi) can follow three basic sequences. An ordered reaction sequence when substrate A must bind to the enzyme before substrate B is able to bind, and product P must be released before product Q can be released. This sequence can be graphically represented as follows:



A second possible basic sequence occurs when either substrate is capable of binding to the enzyme before the other, or both can bind simultaneously. This is called a random mechanism and can be represented as follows:



The third sequence occurs when one substrate binds to the enzyme and one product is released from the enzyme before the second substrate binds to this new enzyme form, releasing the second product. This sequence is called Ping-Pong and is represented:



The nomenclature used throughout is that of Cleland (1963a) and a shortened definition of the terms used is given in the methods page 22.

Although these three sequences are the only possible basic sequences for a BiBi reaction, there are three possible modifications to each which may or may not occur. Isomerisation of any of the enzyme forms may occur, or the enzyme may form dead end complexes with substrate and product, such as EAP which are not capable of participating further in the reaction sequence. It is also possible in some cases that more than one molecule of substrate or product adds to the same enzyme form.

For any combination of basic sequence and modification it is possible to derive a rate equation for the overall reaction (King and Altman, 1956) by assuming that the reaction has reached a state where the rate of change of any of the enzyme forms is small in comparison to the rate of change of the substrates and products (Steady state kinetics). Having derived the rate equation for any particular reaction sequence it is then possible to transform it into a form which only contains kinetic constants which can be experimentally measured (Cleland, 1963a) instead of rate constants which in most cases can

not be determined experimentally under steady state conditions. Comparing the kinetic equations it is possible to predict the effect of any one variable at any fixed level of the other variables. If the concentrations of the products are set to zero all the kinetic equations for Bi Bi mechanisms simplify into only two equations. One which is common to all Ping-Pong mechanisms and the other which is common to all sequential mechanisms (i.e. random or ordered). It is therefore possible to determine whether an unknown mechanism is Ping-Pong or sequential by determining the effect both substrate concentrations have upon the velocity of the reaction in the absence of products (initial velocity analysis). Experimentally this is carried out by plotting the variation of the reciprocal of the varied substrate concentration against the reciprocal of the initial velocity. This plot will be linear with very few exceptions.* If this is then repeated at different fixed levels of the second substrate these lines will intersect at one point showing a sequential mechanism, or will run parallel to each other showing a Ping-Pong mechanism.

There are several ways of elucidating the mechanism further. It is possible to use the overall kinetic equation to predict the time course of the reaction. However, this is the most difficult method and has been used mainly for Uni-Bi hydrolytic reactions.

Testing the consistency of the experimentally determined kinetic constants with the relationships that can be predicted for them,

*
Addition of two or more molecules of one substrate to the same enzyme form, and random mechanisms without rate limiting interconversions both give non linear reciprocal plots.

from the rate equation is theoretically a good method.

In practice it involves the combination of large numbers of constants each of which may be in error to some degree. As a result such comparisons have rarely enabled clear distinctions between mechanisms to be made. Product inhibition studies, however, will distinguish between any mechanism whose complete rate equations have differing forms, and is therefore the method with the greatest range of application.

Product inhibition studies are carried out experimentally by plotting the reciprocal of the variation in one substrate concentration, at fixed concentrations of the other substrate and one of the products, against the reciprocal of the velocity. The fixed concentration of the product is then changed and the process repeated. The product can change the double reciprocal plot in three ways. It can change the vertical intercept without changing the slope of the line. This inhibition is termed uncompetitive inhibition (uncomp.) The product can alter the slope of the line without altering the vertical intercept, this is termed competitive inhibition (comp.) Lastly, the product can alter both the slope and intercept of the line, and is termed noncompetitive inhibition (noncomp.) In predicting the inhibition patterns a given mechanism will have, it is only necessary to follow two fundamental rules:

(a) A compound affects the intercept of a reciprocal plot when it combines reversibly with an enzyme form other than the one the variable substrate combines with, thereby changing the reaction velocity in a manner which cannot be eliminated with the variable substrate.

(b) A compound affects the slope of a reciprocal plot when it and the variable substrate either combine with the same form of the enzyme or are separated in the reaction sequence by a series of reversible steps along which they can interact in such a manner that a change in the concentration of the compound specifically alters the net rate of the step involving the addition of the variable substrate in a manner which can be eliminated by a change in the concentration of the variable substrate. Release of a product at zero concentration or addition of a substrate at infinite concentration (saturation), are considered irreversible steps for purposes of this analysis (Cleland, 1963a).

Using initial velocity analysis and product inhibition analysis the following conclusions were reached concerning the mechanism of FDH. The initial velocity double reciprocal plots were intersecting (Figs. 24 and 27), a Ping-Pong mechanism can therefore be ruled out. The initial velocity of FDH in the absence of products must therefore conform to equation 2.

$$v = \frac{VAB}{Kia.Kb + Ka.B + Kb.A + AB} \qquad \ldots\ldots\ldots \text{eq. 2}$$

NADH was comp. versus NAD (change in slope) NAD and NADH must therefore bind to the same enzyme form. HCO_3 was comp. versus HCOOH, and so HCO_3 and HCOOH bind to the same enzyme form. NADH was noncomp. versus HCOOH (change in both slope and intercept), NADH must bind to a different enzyme form to HCOOH but be connected to the form of enzyme to which HCOOH binds

by a reversible sequence. HCO_3 was also noncomp. versus NAD and so binds to an enzyme form other than the one to which NAD binds, but the two enzyme forms are reversibly connected. If HCO_3 and NADH are both acting solely as product inhibitors, the only mechanism to fit these product inhibition patterns is an ordered BiBi without a rate limiting central ternary complex (Theorell-Chance mechanism) which is shown in Fig. 62.

Unfortunately, it is possible for products to act both in the role of product inhibitors and also as dead end inhibitors. In the case of such mixed inhibitions the fundamental rules still operate for both inhibitions separately so that the pattern produced is the combination of both inhibitions. For example: consider a random mechanism, page 64, (rapid equilibrium random) product P can combine to both EQ and E to form EQP and EP respectively. Substrate A also combines with enzyme E to form EA so that as both P and A combine with the same enzyme form only the slope is affected and inhibition patterns are comp. Although P also combines with EQ, Q would be initially at zero when examining product inhibition, therefore EQ is initially zero. If, however, P can also combine with EA to form an EAP complex, which can no longer undergo further reaction, it is now combining with an enzyme form with which A cannot combine, but with which A is in reversible connection through the combination with E to produce EA. The effect is therefore on both the slope and the intercept. As the slope is already affected by the combination of P to E, the combination of P to EA compounds the effect on the slope which now becomes parabolic in nature. The intercept, however,

only affected once, remains a linear effect. Thus the total effect is to change a comp. inhibition into a noncomp. S-para, I-lin. To find these possible non linear effects, the slope and intercepts of the double reciprocal plots are re-plotted against the fixed inhibitor concentrations. Of the product inhibitions only HCO_3 proved to be non linear. The comp inhibition of HCO_3 versus HCOOH was non linear so was the noncomp versus NAD, which had both nonlinear slopes and intercepts.. HCO_3 must therefore bind either to two separate enzyme forms, or bind more than one molecule to the same enzyme form.

The interpretation of the non linear HCO_3 inhibitions is easier if the second feature of dead end inhibitions is discussed first. Products are not the only compounds capable of dead end inhibitions. Molecules which are chemically similar to the substrates but which are incapable of undergoing reaction are often found to act as dead end inhibitors. The interpretation of these inhibitions is easier owing to the fact that the inhibitions are, in most cases, due to combination of the molecule to only one enzyme form, and therefore the inhibitions are simple and not mixed inhibitions. The effects of dead end inhibitors can be predicted in a similar manner to that of product inhibition. For a random mechanism a dead end inhibitor binding to the free enzyme at the same site as substrate A will be comp with A and noncomp with B which can bind to the EA complex (introducing both intercept and slope effect). For similar reasons a dead end inhibitor competing for the same site as B in a random mechanism will be comp with B and noncomp with A. An ordered mechanism on the other hand produces a different pattern. A dead

end inhibition binding to the EA complex will be uncomp with A as increasing the concentration of A will not change the reaction velocity. (In the case of a random mechanism, increasing the concentration of A would have the effect of changing the concentration of the EB complex and hence increasing the velocity). A dead end inhibitor combining to the free enzyme would be comp with A and noncomp with B as increasing the concentration of B would have the effect of decreasing the concentration of the EA complex and hence increasing the velocity. It is therefore the uncomp pattern of a dead end inhibitor competing with B in an ordered mechanism which can be used to distinguish an ordered from a random sequential mechanism. It also distinguishes which of the substrates is binding to the enzyme first in an ordered mechanism.

In considering possible dead end inhibitions NO_3 was chosen for its similarity in size and charge to HCOOH. It indeed proved to be a dead end inhibitor competing with HCOOH and giving an uncomp pattern with NAD. A second compound ADPR was chosen, since it is similar to NAD and again it was proved that it was in fact a dead end inhibitor which was comp with NAD and noncomp with HCOOH. These results together with the initial velocity analysis limit the choice of mechanism for FDH down to an ordered sequential BiBi mechanism. The first substrate (A) binding being NAD, the second (B) HCOOH, the first product released (P) being HCO_3, the second (Q) NADH. The non linear replots of the NO_3 inhibition can only be interpreted as the combination of more than one molecule of NO_3 to the same enzyme form. Any mixed inhibitions involving NO_3 would alter the uncomp pattern to noncomp.

Coming back to the interpretation of the product inhibition patterns it is now only necessary to consider them in the light of an ordered sequential mechanism. To recap, the only non linear inhibitions were those with HCO_3. The levels of HCO_3 needed to obtain inhibition were extremely high, and it is thought extremely likely that dead end inhibition as well as product inhibition is occurring. An ordered BiBi with a dead end EAP complex would give S-para, I-lin, noncomp of P versus B, and S-lin I-para, noncomp P versus A. With a parabolic slope effect and only a linear intercept effect it is just possible that this pattern could be difficult to distinguish from a comp pattern which has no intercept effects. For this reason it is just possible, even though the noncomp inhibition of HCO_3 versus NAD appear to be I-para, S-para that the mechanism of action of FDH is in fact ordered BiBi with a dead end EAP complex. (Increasing the ionic strength with ammonium sulphate only produced inhibitions at levels way above those produced by HCO_3 (Fig. 50), so it is thought unlikely that any non-specific ionic strength effects were causing any part of this inhibition).

Another possible explanation for the inhibition of HCO_3 is the Thorell-Chance mechanism in which HCO_3 is binding more than once to the same enzyme form. This is the only mechanism to fit the product inhibition patterns as they are interpreted in Table 26, and is thought the most likely mechanism. It is not possible using product inhibition studies to detect isomerisation, except isomerisation of the free enzyme if it had occurred would produce different inhibition patterns to those found, and so is thought an unlikely possibility.

The Theorell-Chance mechanism has a unique relationship between the kinetic constants (Dalziel 1957) which is given by equation 17.

$$\frac{V_2 \, Ka}{V_1 \, Kia} = 1 \qquad \ldots\ldots \text{ eq. 17.}$$

Ka, Kia and V_1 can all be obtained from the initial velocity analysis in the forward direction, but V_2 can only come from analysis of the reverse reaction. A measure of the velocity at fixed substrate concentrations, was obtained at two pH values (Table 26), using a concentration of enzyme approximately 20μM. The steady state rate equations are only obtainable on the assumption that the concentration of enzyme is very small compared with that of the substrates, so that it was not possible to do initial velocity analysis on the reverse reaction as the concentration of enzyme necessary to obtain a measurable rate was in the order of the substrate concentrations. It was therefore not possible to confirm the Theorell-Chance mechanism for formic dehydrogenase.

Other methods of confirming the Theorell-Chance mechanism such as pre-steady state kinetics and alternate substrate kinetics require a pure enzyme and so are as yet of no help.

The use of isotopic exchange studies was also found to be impossible as the time taken to reach equilibrium was so slow, and the reaction velocity in the reverse direction was unmeasurable using the concentrations of enzyme necessary for steady state kinetics (Table 28).

Assuming the Theorell-Chance mechanism to be the one operating for FDH the overall kinetic equation can be derived by the methods of

King and Altman (1956) and Cleland (1963a) and is shown in equation 8.

$$v = \frac{V_1 V_2 \left(AB - \frac{PO}{Keq}\right)}{KiaKbV_2 + KbAV_2 + KaBV_2 + ABV_2 + \frac{KqV_1P}{Keq} + \frac{V_1PQ}{Keq} + \frac{KqV_1AP}{KiaKeq} + \frac{KaV_2BQ}{Kiq}}$$

..... eq. 8.

From this equation can be derived the equations that describe the initial velocity analysis and the various product inhibitions. In the absence of products P = O, Q = O, equation 8 simplifies to equation 2 (page 21). The product inhibition of Q is described by equation 9 and 10 which are derived by setting P to zero in equation 8. Equation 9 predicts comp inhibition of A versus Q, and equation 10 predicts B is noncomp versus Q.

$$1/v = \frac{Ka}{V_1}\left(1 + \frac{KiaKb}{KaB}\right)\left(1 + \frac{Q}{Kiq}\right)\frac{1}{A} + 1/V_1\left(1 = \frac{Kb}{B}\right) \quad \text{..... eq. 9.}$$

$$1/v_o = \frac{Kb}{}\left(1 + \frac{Kia}{}\right)\left(1 + \frac{Q}{Kiq\left(1 + \frac{A}{}\right)}\right)\frac{1}{} + 1/V_1\left(1 + \frac{Ka}{}\right)\left(1 + \frac{Q}{Kiq\left(1 + \frac{A}{Ka}\right)}\right)$$

..... eq. 10.

With Q set to zero equations 11 and 12 are obtained which predict B; comp with P, and A noncomp with P respectively, and represent the inhibitions of HCO_3^- (ignoring the parabolic effect).

$$1/v = \frac{Kb}{V_1}\left(1 + \frac{Kia}{A}\right)\left(1 + \frac{P}{Kip}\right)\frac{1}{B} + \frac{1}{V_1}\left(1 + \frac{Ka}{A}\right) \qquad \ldots\ldots \text{ eq. 11}$$

$$1/v = Ka\,(1 + KiaKb)\left(1 + \frac{P}{Kip\left(1 + \frac{KaB}{KiaKb}\right)}\right)\frac{1}{A} + \frac{1}{V_1}\left(1 + \frac{Kb}{B}\right)\left(+ \frac{P}{Kip\left(1 + \frac{B}{Kb}\right)}\right)$$

$\ldots\ldots$ eq. 12.

The distribution equations derived by the method of King and Altman (1957) and Cleland (1963a) for a Theorell-Chance mechanism are shown in equation 13 and 14.

$$E/Et = \frac{KiaKbV_2 + \frac{Kq\,V_1P}{Keq} + KaV_2B}{\text{(denominator of rate equation)}} \qquad \ldots\ldots \text{ eq. 13.}$$

$$EA/Et = \frac{KbV_2A + \frac{KqV_1AP}{Kia\,Keq} + \frac{V_1PQ}{Keq}}{\text{(denominator of rate equation)}} \qquad \ldots\ldots \text{ eq. 14.}$$

To obtain the kinetic equations describing dead end inhibitions, all the terms in equation 8 which also appear in the numerator of the distribution equation relating to the proportion of the enzyme form to which the dead end inhibitor binds , are multiplied by the factor $(1 + I/Ki)$.

Thus the dead end inhibition of NO_3 is described by equation 15 and 16. Equation 15 predicting uncomp inhibition of NO_3 versus NAD, and equation 16 the comp inhibition of NO_3 versus HCOOH.

$$\frac{1}{v} = \frac{1}{B}\left(\frac{KiaKb}{B} + Ka\right)\frac{1}{A} + \frac{1}{V_1}\left(1 + \frac{Kb}{B}\right)\left(1 + \frac{I}{Ki\left(1 + \frac{B}{Kb}\right)}\right) \quad \text{..... eq. 15.}$$

$$\frac{1}{v} = \frac{Kb}{V_1}\left(1 + \frac{Kia}{A}\right)\left(1 + \frac{I}{Ki\left(1 + \frac{A}{Kia}\right)}\right)\frac{1}{B} + \frac{1}{V_1}\left(1 + \frac{Ka}{A}\right) \quad \text{..... eq. 16}$$

The dead end inhibition of ADPR is described by equation 17 and 18. Equation 17 predicting comp ADPR versus NAD and equation 18 predicting noncomp ADPR versus HCOOH.

$$\frac{1}{v} = \frac{1}{V_1}\left(\frac{KiaKb}{B} + Ka\right)\left(1 + \frac{I}{Ki}\right)\frac{1}{A} + \frac{Kb}{KB} + \frac{1}{V_1} \quad \text{..... eq. 17.}$$

$$\frac{1}{v} = \frac{Kb}{V_1}\left(1 + \frac{Kia}{A}\right)\left(1 + \frac{I}{Ki\left(1 + \frac{A}{Kia}\right)}\right)\frac{1}{B} + \frac{1}{V_1}\left(1 + \frac{Ka}{A}\right)\left(1 + \frac{I}{Ki\left(1 + \frac{A}{Ka}\right)}\right)$$

..... eq. 18.

The predictions arrived at from the equations are of course the same as those arrived at by using the two fundamental rules, and as already stated results were consistent with the predictions.

Using the appropriate equations the Kis and Kii values can now be related to the various kinetic constants:

with NADH inhibiting and NAD varying Kis = kiq

with NADH inhibiting and HCOOH varying

Kis = kiq(1 + a/Kia) Kii = Kiq (1 + A/Ka)

with ADPR inhibiting and HCOOH varying

Kis = Ki(1 + A/Kia) Kii = Ki(1 + A/Ka)

with ADPR inhibiting and NAD varying Kis = Ki

with HCO_3 inhibiting and HCOOH varying (assuming para)

Ki_1 = Kip

with HCO_3 inhibiting and NAD varying

KiSA = Kip(1 + KaB/KiaKb) Kii = Kip(1 + B/Kb)

with NO_3 inhibiting and HCOOH varying

Ki1 = Ki(1 + A/Kia)

with NO_3 inhibiting and NAD varying

Kii = Ki (1 + B/Kb)

and from table 27 some of the kinetic constants can be calculated. These are summarised in table 33.

Table 33. A summary of some of the values of the kinetic constants

Kinetic constant				
Kiq		A v Q	2.5 ± 0.3μM	-
Kiq		B v Q	1.2 ± 0.6μM	2.1 ± 0.7μM
Kip		B v P	1100 ± 600mM	-
Kip		A v P	870 ± 500 mM	180 ± 40 mM
Ki (ADPR)	ADPR	v A	12.1 ± 1.3μM	-
Ki(ADPR)	ADPR	v B	9.6 ± 2.5μM	12.5 ± 3μM
Ki (NO_3)	NO_3	v A	no computer programs	
Ki (NO_3)	NO_3	v B	1.8 ± 0.2μM	-

It is only the values of Kip which could be in error if equation 8 does not represent the mechanism of formic dehydrogenase. The two possible mechanisms are drawn schematically on the following page.

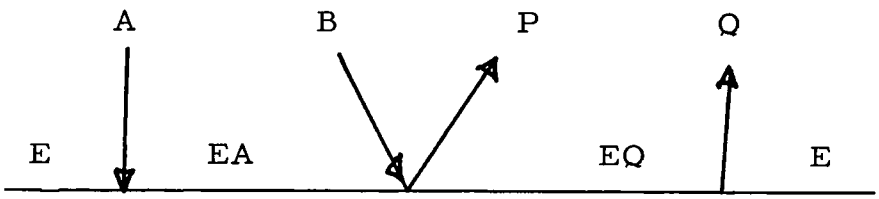


Fig. 62. The diagramatic representation of the Theorell-Chance mechanism

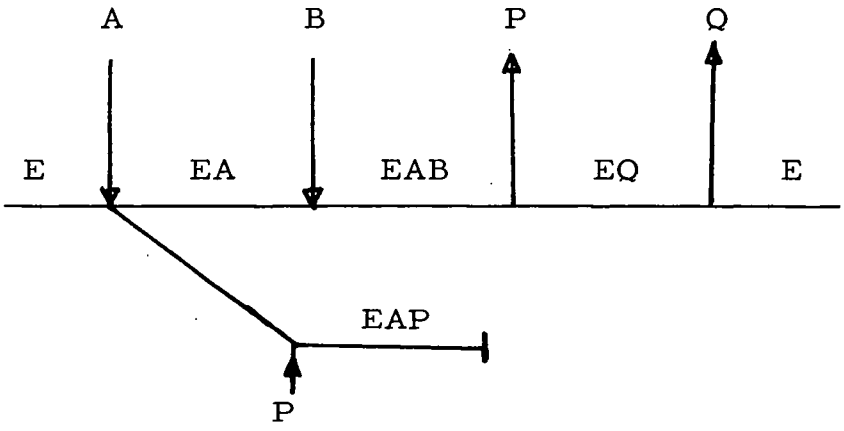


Fig. 63. The diagramatic representation of an ordered BiBi sequential mechanism with a dead end EAP complex

Two trends emerged when the variation in some of the kinetic constants with pH was determined. V_1 and Ka remain essentially constant, while Kia increases to a maximum, Kb decreases to a minimum (Figs. 51 - 53). Dixon (1953) laid down simple rules for the interpretation of the changes in pKm with pH, in terms of the pK's of some of the ionising groups involved in the reaction. The maximum of pKb and the minimum of pKia coincide at pH 8.0, and so it would appear that this represents the pK of one of the ionising groups involved. It is not possible to relate this to the individual pH's of the amino acids due to the large variation in pK caused by the influence of the surrounding groups. However, the small changes noted in these kinetic constants points to either a constancy in the ionic environment of the active centre, or that several groups in the active centre are changing their ionic states so as to have opposing effects causing the overall effect to remain constant.

The affect of temperature on the maximum velocity of the reaction gave a linear plot of Log. V against 1/T. The slopes of this line did not vary with pH from 6.0 to 10.0 although the absolute magnitude of the velocity did. (Fig. 54). In an ordered BiBi mechanism the slope of this plot gives a value for the energy of activation in the steps controlled by the rate constants k_5 and k_7 (page 46). If these rate constants had differing temperature coefficients it would have caused a break in plot at a point when one of the rate constants became limiting and the other ceased to be limiting. With a Theorell-Chance mechanism operating, k_5 would almost certainly be much greater than k_7 and so no break would be expected

from this cause. The Q_{10} and the energy of activation were both high at 2.2 and 12000 cals./mole respectively which could be a contributing factor in the low turnover number estimated at 120 min^{-1}.

Although the equilibrium constant was never measured as the approach to equilibrium was so slow (Table 25) from the measured rate of the reverse reaction there seems little doubt that it is at least as great as the theoretical figure of 3.5×10^4 calculated by Mathews and Venesnland in 1950), from thermodynamic data for the chemical reaction HCOOH + NAD = CO_2 + NADH.

The kinetic analysis of FDH has yielded the following information concerning the mechanism of action:

(i) The kinetic mechanism is an ordered BiBi without a rate limiting central ternary complex.

(ii) The binding of the coenzyme is much more efficient than that of formate.

(iii) The binding of HCO_3 is very inefficient and more than one molecule of HCO_3 can bind to the active centre.

(iv) CO_2 was a much more effective substrate for the reverse reaction than HCO_3.

(v) The maximum velocity in the forward direction was independent of pH.

(vi) The binding of the substrates was not greatly affected by pH.

(vii) An ionising group with an approx. pK of 8.0 is implicated in the mechanism.

(viii) The turnover number of the enzyme is extremely small.

Metabolism

It is now well established that some enzymes have distinct and discernable localisations in or on certain of the cellular organelles (Crook, 1959). The localisation of enzymes leads to the restriction of metabolic pathways to certain organelles. In establishing the position of an enzyme in the metabolism it is of importance to determine its localisation within the cell. There are two basic methods for doing this, one involves the fractionation of the subcellular components, and the other histochemical staining of the enzyme site in situ. The first method has been applied to the localisation of FDH in plants by Smillie (1955), Davies (1956) and Mazelis (1960), Linnane & Still (1955), who reported that the enzyme was localised predominantly in the mitochondrial fraction. It was therefore surprising that using P. aureus no enzyme could be detected in mitochondria isolated by the method of Ikuma and Bonner (1967) which was developed especially for this tissue. Again, using the method of Mazelis (1960), no enzyme could be detected in the mitochondrial fraction. These experiments were repeated several times using very gentle disruption of the cells but each time the results were the same.

de Duve (1964) has discussed in detail the principles and limitations of enzyme location by this method. He points out that it is possible for enzymes to be latent, in which case while in their native state, integrated in the particulate matrix, they are inaccessible to substrates and therefore do not show their full activity. Detection of these latent enzymes involves some method of exposing the enzyme. However, this was unlikely to be the case with FDH free P. aureus as there was so much enzyme already in solution.

A second possibility occurs when the enzyme is so loosely attached to a particle that the force necessary to disrupt the cell, is enough to cause the detachment of the enzyme from the particle. This is certainly a possible explanation for the recovery of FDH from P.aureus in the supernatant. A third possible pitfall of this method is that it is possible for an enzyme to become detached from its original particle during disruption and then become firmly bound to a second type of particle. In interpreting the distribution of enzymes amongst various isolated subcellular fractions these possible occurrences must be kept firmly in mind.

A second method of enzyme localisation has been applied to FDH from Proteus vulgaris by Nermut and Ryc (1964) who used the electron microscope to detect the regions of a dye that had been reduced by the localised action of FDH, and found the site of action to be the cytoplasmic membrane. Most of the evidence available therefore points to the particulate localisation of FDH in one manner or another. In the case of P. aureus FDH, however, its attachment to an organelle, if any, must be very loose.

Formic acid has been reported in potatoes by Stoklase et al. (1907) and in Vigna Sesquipedalis by Yamamoto (1954). Although the quantitative changes during germination were not discussed. In Phaseolus aureus the rapid disappearance of FDH from the seedling during germination (Fig. 57) suggests that the main metabolic function of FDH is concerned with the first few days of germination. This assumption is supported by the equally rapid changes in the formic acid content of the germinating seedling (Fig. 60). Both the enzyme and its substrate are concentrated within the cotyledons.

The biochemical changes accompanying germination are numerous, but one fact clearly emerges, and that is the anaerobic nature of the carbohydrate metabolism during the first two days before the root pierces the testa. The rate of carbon dioxide output and oxygen uptake both increase with germination, Evenari et al. (1955), Halvorson (1956), Spragg and Yem (1959), Hackett (1959), Seances (1966) and James (1953), and removal of the seed coat greatly accelerates the increasing rate of carbon dioxide output and oxygen uptake Seances (1966), showing that the barrier of the seed coat contributes towards the creation of the anaerobic conditions under which the seed begins to germinate. This situation changes once the seed coat is pierced by the germinating seedling. Likewise the metabolism of glucose proceeds via glycolysis with the end products alcohol or lactate, during the first few days, and only in later stages of germination does the TCA cycle begin to function (Leggat, 1948), Stumph (1952), Oota et al. (1956), Hatch and Turner (1958), Bartels (1960). Both the levels of alcohol and lactate in pea seedlings reach a maximum on day one, and then quickly fall off, Cossins and Turner (1959), Cossins (1964). In pea seedlings at the stage when the levels of both alcohol and lactate are normally falling Cossins (1962, 1964), showed that these seedlings possessed the ability to utilise in vivo, alcohol and lactate, incorporating these compounds into keto, carboxylic and amino acids. It would appear therefore that after the initial period of anaerobic fermentation, the products of this fermentation are metabolised back into the main pathways of the organism, when the seed coat is broken, and the oxygen is available for normal oxidative respiration. The similarity between the production of alcohol and lactate in pea seedlings and the

production of formate in P. aureus seedlings is striking. It is therefore likely that the production of formic acid during germination is caused by the anaerobic conditions prevailing in the first day or two of germination. This is supported by the increase in formic acid which can be produced if the seedlings are subjected to continuing anaerobic conditions.

The pathways involved in this production of formic acid were not determined, however there are three enzymes capable of producing formate under anaerobic conditions in other organisms. Pyruvate phosphoroclastase, Kalnitsky and Werkman (1943), which splits pyruvate to yield formic acid and acetic acid; oxalate decarboxylase Jakoby et al. (1956), which decarboxylates oxalic acid to yield carbon dioxide and formic acid and N^{10}-formyl tetrahydrofolate deacylase, producing formic acid and tetrahydrofolic acid, Osborn et al. (1957).

The fate of the formic acid produced was investigated and found to be almost entirely oxidised to carbon dioxide (Table 32). Other pathways are known to be capable of utilising formate, Cossins, Sinhar (1965), using various plants showed that formate could be utilised in several synthetic reactions, especially into the production of serine and methionine. However, these authors also report that the incorporation into serine was greater under anaerobic conditions. It therefore seems likely that apart from the small amount of formic acid which may be in biosynthetic pathways via THF, the acid is mainly oxidised to carbon dioxide producing NADH, which can either be re-oxidized via the electron transport chain or be used for providing reducing power in other linked reactions. To summarize, formic acid is produced in the first day of germination in a similar manner to lactate

and ethanol, when largely anaerobic conditions prevail. Unlike ethanol and lactate which are later shunted back into the metabolism, formic acid for some reason is largely oxidized to carbon dioxide, and only a small percentage used in various biosynthetic pathways.

Although these findings apparently pose more questions than they solve, they represent an advance on the only other investigation into the role of FDH in plants, Davison (1949b), in that the production and uses of formic acid have been studied in addition to the changes in enzyme levels. This previous work investigated the possible 'dismutations' that could occur between FDH and other dehydrogenases, and showed that glutamate dehydrogenase was easily linked to FDH, indicating a possible mechanism for linked reaction using the reducing power of NADH.

Further work is needed to find which enzymes are responsible for the production of formic acid, why formic acid is produced under anaerobic conditions, and why formic acid is oxidised to carbon dioxide in preference to other possible synthetic pathways.

The decline in the levels of the enzyme in the cotyledons is not thought to represent any economy measure on the part of the plant, as both alcohol and malate dehydrogenase also decline. The most likely explanation for both these declines is that they are due to the senescence of the cotyledons.

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