



National Library of Canada
Collections Development Branch

Canadian Theses on
Microfiche Service

Bibliothèque nationale du Canada
Direction du développement des collections

Service des thèses canadiennes
sur microfiche

NOTICE

The quality of this microfiche is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us a poor photocopy.

Previously copyrighted materials (journal articles, published tests, etc.) are not filmed.

Reproduction in full or in part of this film is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30. Please read the authorization forms which accompany this thesis.

THIS DISSERTATION
HAS BEEN MICROFILMED
EXACTLY AS RECEIVED

AVIS

La qualité de cette microfiche dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de mauvaise qualité.

Les documents qui font déjà l'objet d'un droit d'auteur (articles de revue, examens publiés, etc.) ne sont pas microfilmés.

La reproduction, même partielle, de ce microfilm est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30. Veuillez prendre connaissance des formules d'autorisation qui accompagnent cette thèse.

LA THÈSE A ÉTÉ
MICROFILMÉE TELLE QUE
NOUS L'AVONS REÇUE

PREFACE

Immobilization techniques and the study of supported enzymes have developed rapidly over the past few years, mainly because of their manifold applications as well as their fundamental interest in enzymology and biology. Tubes with enzymes attached to their inner surfaces are particularly useful for these purposes, and kinetic studies of such systems are essential for any successful application and also for understanding the function of enzymes in vivo. This thesis describes a study of the reaction between ethanol and NAD catalyzed by yeast alcohol dehydrogenase attached to nylon tubing and in flow systems. The results have been discussed in terms of the various factors responsible for the difference in behaviour of the immobilized enzyme from that in free solution, particular attention being paid to the diffusional effects as treated by the Kobayashi-Laidler theory. Other effects resulting from immobilization have also been considered, and conclusions have been drawn as to the kinetic mechanism involved in the catalysis.

Some of the work described in this thesis has been published, and the remainder is under preparation for publication as follows:

1. Flow Kinetics of Yeast Alcohol Dehydrogenase Attached to Nylon Tubing, M.A. Mazid and K.J. Laidler, *Biochim. Biophys. Acta* 614, 225 (1980).
2. Temperature Effects with Immobilized Yeast Alcohol Dehydrogenase in Flow Systems, M.A. Mazid and K.J. Laidler, *Biochim. Biophys. Acta* 614, 237 (1980).
3. pH-Dependence of YADH Kinetics in Free Solution and in a Tubular Flow Reactor, M.A. Mazid and K.J. Laidler, manuscript in preparation.

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation and great admiration to Professor K.J. Laidler, my research supervisor, for the stimulating freedom and constant encouragement provided throughout the course of this work, which will inspire me always. I am especially indebted to him for many invaluable suggestions and critical comments, and for being available at any time for discussion, involving much of his valuable time, particularly in reviewing the manuscript of this thesis.

I am grateful to Mr. O. Faruque, formerly a graduate student in the Department of Civil Engineering at Carleton University, for his indispensable assistance in writing the computer programme, and to Dr. H. Teitelbaum for helpful suggestions in this regard. I am thankful to my fellow research colleagues, especially to N.J. Daka, for various important discussions, and to Mrs. F. Peat for some of the experiments on the enzyme in free solution.

Thanks are also due to Mrs. S. Ryan for typing this thesis in a short time, and to all members of this Department for their friendship. I gratefully acknowledge financial support for this work by the University of Ottawa through teaching assistantships, and by the National Research Council of Canada for research assistantships during 1977-1980.

Finally, and very importantly, with special gratitude to my wife Sanjida, I must acknowledge her exceptional patience and understanding in allowing me to devote sufficient time and the privilege of working mostly in the University, when she looked after the household and took care of the baby, Imrul.

TABLE OF CONTENTS

PREFACE	i
ACKNOWLEDGEMENTS	ii
TABLE OF CONTENTS	iii
LIST OF FIGURES	vi
LIST OF TABLES	ix
ABSTRACT	xii
CHAPTER 1 GENERAL INTRODUCTION	1
Preliminary Information and Choice of the Enzyme	2
Occurrence and Multiple Molecular Forms of the Enzyme	3
Comparative Features of Yeast and Liver Alcohol Dehydrogenase	4
Functional Aspects and Physiological Role of Alcohol Dehydrogenase	5
Objectives and Outline of the Present Work	8
General Aspects of Enzyme Immobilization	10
General Considerations in Immobilized Enzyme Kinetics	16
CHAPTER 2 IMMOBILIZATION OF YEAST ALCOHOL DEHYDROGENASE INSIDE A NYLON TUBE	28
Introduction: Choice of the Support Material	28
Use of Nylon-Supported Enzymes: Choice of the Tubular Configuration	29
Principles and Chemistry of Immobilization of Enzymes on Nylons	35
Previous Work on the Immobilization of Alcohol Dehydrogenases	45
The Present Method and the Materials Used	49

Covalent Attachment Procedure for YADH Inside a Nylon Tube	51
Stability of the Nylon-tube-supported YADH	52
CHAPTER 3 FLOW KINETICS OF YEAST ALCOHOL DEHYDROGENASE ATTACHED TO NYLON TUBING	57
Introduction	57
Theoretical Considerations	58
Kinetic Procedure	66
Results and Discussion	66
$K_{m,app}$ Values	66
Dimensionless Parameters	74
Product Concentrations	78
Inhibition by Products	83
Concluding Remarks	89
CHAPTER 4 TEMPERATURE EFFECTS WITH IMMOBILIZED YEAST ALCOHOL DEHYDROGENASE IN FLOW SYSTEMS	91
Introduction	91
Theoretical Considerations	92
Experimental Procedure	94
Results and Discussion	95
Temperature Dependence of Overall Rates	95
Temperature Dependence of Michaelis Parameters	113
Conclusions	122
CHAPTER 5 pH DEPENDENCE OF YADH KINETICS IN FREE SOLUTION AND IN A TUBULAR FLOW REACTOR	125
Introduction	125
Theoretical Principles	126

Experimental Procedure	129
Analysis of Data: Determination of Ionization Constants	131
Results for the pH-Dependence of YADH Kinetics	134
Discussion of Results for the Enzyme in Free Solution	142
The pH-Activity Profiles	142
pH-Dependence of Michaelis Parameters	143
Ionization Constants of Catalytically Important Groups	144
Discussion of Results for the Immobilized Enzyme	146
The Patterns of pH Behaviour	146
pH-Dependence of Michaelis Parameters	151
Ionization Constants of Catalytically Important Groups	152
Concluding Remarks	156
CHAPTER 6 GENERAL DISCUSSION AND CONCLUSIONS	160
Introduction	160
Stability of the Immobilized YADH	160
Kinetics and Mechanism	161
Problems to be Resolved	168
Suggestions for Further Work	171
CLAIMS TO ORIGINAL RESEARCH	173
REFERENCES	176
APPENDIX: FORTRAN IV PROGRAMME FOR THE DETERMINATION OF IONIZATION CONSTANTS OF CATALYTICALLY IMPORTANT GROUPS	186

LIST OF FIGURES

<u>Figure</u>		<u>Page</u>
1	Four basic approaches in the immobilization of enzymes.	13
2	A schematic diagram of a flow-type automated analysis system, involving the use of tube-supported enzymes.	31
3	Covalent binding of enzymes to nylon by methods involving partial hydrolytic cleavage of peptide bonds by acids and coupling through the liberated carboxyl groups.	37
4	Covalent binding of enzymes to nylon by methods involving peptide bond cleavage and coupling through the liberated primary amino groups.	39
5	O-alkylation of nylon with triethyloxonium salts and reaction of the imidate salt of nylon with acid hydrazides, enzymes and bis-alkyl amines.	42
6	Synthesis of polyisocyanide-nylon via four-component condensation reaction.	44
7	The activity of the nylon-tube-supported YADH, measured in terms of the absorbance at 340 nm of NADH, plotted against time in months.	53
8	Schematic diagram of a tube with enzyme attached to the inner surface, showing the diffusion layer and the concentration profile.	60
9	Lineweaver-Burk plots (a) with excess NAD at 2.0 mM, and (b) with excess ethanol at 100 mM. Temperature 25.5°C, pH 7.5, and the flow rates are shown.	67
10	Plot of apparent Michaelis constants, $K_{m,app}$, for ethanol against $v_f^{-1/3}$, obtained from experiments with excess NAD at 2.0 mM and at different flow rates.	71

FigurePage

- 11 : Double-logarithmic plots of the dimensionless parameters ϕ and ρ . The half-circles are typical points for constant NAD at 5.0 mM and variable ethanol (3.0-100 mM). Other symbols are due to the results with excess ethanol at 500 mM and variable NAD (0.10-2.0 mM) for the flow rates 0.30 (solid circles, ●), 0.50 (open circles, ○), 1.00 (triangles, Δ), 2.00 (crossed circles, ⊗), and 3.30 (squares, □) in cm s^{-1} . The theoretical regions are Region 1, little diffusional control (<5%); Region 2, moderate diffusional control (5-60%); Region 3, considerable diffusional control (>60%). 75
- 12 Double-logarithmic plots of the product concentration at the exit $[P]_e$, against flow rate v_f , for the results with ethanol in excess at 500 mM (circles) and for excess NAD at 5.0 mM (triangles). Variable NAD concentrations (0.10-2.0 mM) for the former, and variable ethanol concentrations (3.0-100 mM) for the latter (within brackets) are shown. 79
- 13 Lineweaver-Burk plots for constant ethanol at 100 mM and variable NAD concentrations with NADH added as indicated at 25.5°C, pH 7.5 and a flow rate of 1.15 cm s^{-1} . 84
- 14 Arrhenius plots for the rate of product formation at a flow rate = 0.50 cm s^{-1} , with excess $[\text{NAD}] = 5.0 \text{ mM}$ and at the ethanol concentrations as indicated. 106
- 15 Arrhenius plots for the rate of product formation at a flow rate = 0.50 cm s^{-1} , with excess $[\text{C}_2\text{H}_5\text{OH}] = 500 \text{ mM}$ and at the NAD concentrations as indicated. 107
- 16 Lineweaver-Burk plots with excess $[\text{NAD}] = 5.0 \text{ mM}$, at different flow rates as indicated, pH 7.5 and temperature 25.5°C. 114
- 17 pH-dependence of initial rates at 25.5°C and different substrate concentrations as indicated, for yeast alcohol dehydrogenase in free solution with (a) excess ethanol at 500 mM and (b) excess NAD at 5.0 mM. 135
- 18 pH-dependence of apparent rates at 25.5°C and different substrate concentrations as indicated, for YADH attached to nylon tubing and at a flow rate of 0.90 cm s^{-1} , with (a) excess ethanol at 500 mM and (b) excess NAD at 5.0 mM. 136

FigurePage

19

Lineweaver-Burk plots for the data with YADH attached to nylon tubing, at a flow rate of 0.30 cm s^{-1} and temperature 25.5°C , with excess ethanol at 500 mM and at various pH as indicated.

140

LIST OF TABLES

<u>Table</u>		<u>Page</u>
I	Michaelis parameters with YADH attached to nylon tubing, at pH 7.5 and temperature 25.5°C.	69
II	Michaelis constants for ethanol and NAD with alcohol dehydrogenase in static systems.	73
IIIA	Values of the dimensionless parameters ϕ and ρ with excess $[NAD]=2.0$ mM, at different ethanol concentrations and flow rates. All points fall in the theoretical Region 1 (see Figure 11).	76
IIIB	Values of the dimensionless parameters ϕ and ρ with excess $[C_2H_5OH]=100$ mM, at different NAD concentrations and flow rates. The numbers in brackets indicate the theoretical regions (see Figure 11).	77
IVA	Concentration of the product NADH (μM) measured at the exit of the tube with excess $[NAD]=2.0$ mM and variable ethanol at different flow rates.	80
IVB	Concentration of the product NADH (μM) measured at the exit of the tube with excess $[C_2H_5OH]=100$ mM and variable NAD at different flow rates.	81
V	Rates of reaction ($10^6 \times M s^{-1}$) for the product inhibition by added acetaldehyde with variable ethanol and constant $[NAD]=1.0$ mM at a fixed flow rate of 1.15 cm s^{-1} , pH 7.5 and temperature 25.5°C.	85
VIA	Effect of flow rate on product inhibition by added acetaldehyde with constant $[C_2H_5OH]=20.0$ mM and excess $[NAD]=5.0$ mM at 25.5°C and pH 7.5.	87
VIB	Flow rate dependence of product inhibition by added NADH with constant $[NAD]=0.50$ mM and excess ethanol at 100 mM.	88
VIIA	Rates of reaction ($10^6 \times M s^{-1}$) at various flow rates and ethanol concentrations, with excess $[NAD]=5.0$ mM and at temperature 5°C.	96
VIIB	Rates of reaction ($10^6 \times M s^{-1}$) at various flow rates and ethanol concentrations, with excess $[NAD]=5.0$ mM and at temperature 15°C.	97

<u>Table</u>		<u>Page</u>
VIIC	Rates of reaction ($10^6 \times \text{M s}^{-1}$) at various flow rates and ethanol concentrations, with excess $[\text{NAD}] = 5.0 \text{ mM}$ and at temperature 25.5°C .	98
VIID	Rates of reaction ($10^6 \times \text{M s}^{-1}$) at various flow rates and ethanol concentrations, with excess $[\text{NAD}] = 5.0 \text{ mM}$ and at temperature 35°C .	99
VIIE	Rates of reaction ($10^6 \times \text{M s}^{-1}$) at various flow rates and ethanol concentrations, with excess $[\text{NAD}] = 5.0 \text{ mM}$ and at temperature 25.5°C .	100
VIIIA	Rates of reaction ($10^6 \times \text{M s}^{-1}$) at various NAD concentrations and flow rates, with excess $[\text{C}_2\text{H}_5\text{OH}] = 500 \text{ mM}$ and at temperature 5°C .	101
VIIIB	Rates of reaction ($10^6 \times \text{M s}^{-1}$) at various NAD concentrations and flow rates, with excess $[\text{C}_2\text{H}_5\text{OH}] = 500 \text{ mM}$ and at temperature 15°C .	102
VIIIC	Rates of reaction ($10^6 \times \text{M s}^{-1}$) at various NAD concentrations and flow rates, with excess $[\text{C}_2\text{H}_5\text{OH}] = 500 \text{ mM}$ and at temperature 25.5°C .	103
VIIID	Rates of reaction ($10^6 \times \text{M s}^{-1}$) at various NAD concentrations and flow rates, with excess $[\text{C}_2\text{H}_5\text{OH}] = 500 \text{ mM}$ and at temperature 35°C .	104
VIIIE	Rates of reaction ($10^6 \times \text{M s}^{-1}$) at various NAD concentrations and flow rates, with excess $[\text{C}_2\text{H}_5\text{OH}] = 500 \text{ mM}$ and at temperature 45°C .	105
IX	Activation energies (kcal mol^{-1}) with excess NAD at 5.0 mM .	108
X	Activation energies (kcal mol^{-1}) with excess ethanol at 500 mM .	109
XIA	Values of V_{max} with excess $[\text{NAD}] = 5.0 \text{ mM}$, obtained from Lineweaver-Burk plots for 3-10 mM (within brackets) and 10-50 mM concentration ranges of ethanol, at different temperatures and flow rates.	115
XIB	Apparent Michaelis constants for ethanol with excess $[\text{NAD}] = 5.0 \text{ mM}$, at different temperatures and flow rates, obtained from Lineweaver-Burk plots (values in brackets for 3-10 mM and others for 10-50 mM concentrations of ethanol).	116

<u>Table</u>		<u>Page</u>
XIIA	Values of V_{max} with excess $[C_2H_5OH]=500$ mM, obtained from Lineweaver-Burk plots for variable NAD, at different temperatures and flow rates.	117
XIIB	Apparent Michaelis constants for NAD with excess $[C_2H_5OH]=500$ mM, obtained from Lineweaver-Burk plots at different temperatures and flow rates.	118
XIII	Michaelis parameters with yeast alcohol dehydrogenase in free solution, at various pH and temperature $25.5^\circ C$.	138
XIV	Michaelis parameters with immobilized YADH in a tubular flow reactor, at various pH and temperature $25.5^\circ C$.	139
XV	Values of pK under different conditions, as obtained by the least-squares treatment.	141

ABSTRACT

Yeast alcohol dehydrogenase (alcohol:NAD oxidoreductase, EC 1.1.1.1) was attached covalently to the inner surface of nylon tubing. The immobilized enzyme retained its activity over a period of about one year, and allowed consistent kinetic data to be obtained.

A study was made of the flow kinetics for the reaction between ethanol and nicotinamide adenine dinucleotide (NAD) in two limiting cases: one substrate in excess and the concentration of the other one varied. Rate measurements were made over a range of substrate concentrations and flow rates, under steady-state conditions, and the results were analyzed in various ways on the basis of the Kobayashi-Laidler treatment of flow systems for one-substrate reactions. With the ethanol held at saturating concentrations, there was partial diffusion control, the extent decreasing with increasing flow rate and increasing NAD concentration. On the other hand, with the NAD at saturating concentrations, there was no appreciable diffusion control. In general, the apparent Michaelis constants varied with flow rate v_f , being linear in $v_f^{-1/3}$, and extrapolation to infinite flow rate ($v_f^{-1/3}=0$) gave the diffusion-free inherent Michaelis constants.

There was inhibition by products of the reaction (NADH and acetaldehyde), being reflected in the smaller dependence of product concentration on flow rate compared to those predicted by the Kobayashi-Laidler theory. The results for both NADH and acetaldehyde showed mixed competitive and non-competitive inhibition, with a preponderance of the former. Acetaldehyde was found to be the stronger inhibitor, and this was consistent with the lack of diffusion control with variable ethanol.

Inhibition by acetaldehyde was not affected by flow rate, in contrast to that by NADH, because of the greater degree of diffusion control with variable NAD.

Activation energies were determined for the flow kinetics over a range of concentrations of the two substrates (NAD and ethanol) and at various flow rates. With NAD at saturating concentrations, the activation energies showed little dependence on ethanol concentration and flow rate. Under these conditions, the reaction showed hardly any diffusion control, and the activation energy for the chemical interaction at the surface was concluded to be about 9 kcal mol^{-1} . At saturating ethanol concentrations, the activation energies increased significantly with increasing NAD concentration and flow rate. The limiting value at high NAD concentration and flow rate was consistent with the 9 kcal mol^{-1} deduced for complete chemical control. Extrapolation to zero NAD concentrations and flow rates suggested a value of $4.2 \text{ kcal mol}^{-1}$ for the reaction under conditions of full diffusion control.

Temperature dependence of the Michaelis parameter V_{max} showed similar behaviour to that of the overall rates, and yielded activation energies corresponding to the chemical interaction at the surface. The effect of temperature on the Michaelis constant itself showed complications due to changes of mechanism with substrate concentration and flow rate. The activation energies for the Michaelis constants with variable NAD apparently decreased with increasing flow rate and gave a value of about 2 kcal mol^{-1} at high flow rate. This was similar to the results for the diffusion-free Michaelis constants with variable ethanol at different flow rates. The Michaelis parameters were obtained from Lineweaver-Burk

plots, which were non-linear particularly with variable ethanol, at different temperatures. This suggested microheterogeneity of the enzyme active site, resulting from immobilization.

pH dependence of the YADH kinetics was studied both for the enzyme in free solution and in the tubular flow reactor. The pH-activity profiles were different for the two types of systems. For the enzyme in free solution, the initial rate passed through a maximum at pH values of 7.5 and 8.0 with variable NAD and variable ethanol, respectively. With the immobilized enzyme in the presence of excess ethanol, when the NAD concentration was varied the apparent rate also passed through a maximum but the entire profile was shifted towards the right giving a pH optimum between 8.5 and 9.0. The pH optimum was shifted further upwards with variable ethanol and excess NAD, but its value could not be established because of the instability of the substrate NAD at pH values greater than 10.0 up to which the rate increased continuously. This behaviour was due to the diffusional limitations to the proton transport and the lack of buffer-mediated diffusion of protons generated in the reaction, the differences from the behaviour in free solution of the enzyme in general resulting from electrostatic and partitioning effects.

Michaelis parameters obtained from the pH studies were explained in terms of the binding and dissociation rate constants for a compulsory ordered mechanism. When the substrate ethanol is present in excess, the ratio $V_{\max}/K_m$ is an estimate of the binding constant for the enzyme reaction with the substrate NAD, while $V_{\max}$ itself reflects the dissociation rate constant for the breakdown of the enzyme-substrate ternary complex into products. Both $V_{\max}$ and $V_{\max}/K_m$ passed through maximum pH values corres-

ponding to the maximum activity, but the pH dependence of K_m was not simple. The effect of substrate diffusion was manifested in apparently constant values of K_m for NAD with the immobilized enzyme at low flow rate. There was stronger dependence of K_m on pH at higher flow rate or with the enzyme in free solution.

The dependence on pH of the Michaelis parameters was used to evaluate the ionization constants of catalytically important groups. This was done reliably by computerized curve-fitting involving a simple statistical least-squares treatment. In all cases, two different ionizing groups were implicated, except for the data with excess NAD and variable ethanol when there was no fall-off of rate on the alkaline side of the pH maximum. The ionization constant of an acidic group in the enzyme itself in the presence of either of the substrates in excess, and in the enzyme-substrate ternary complex, were significantly lower with the immobilized enzyme, i.e., the pK_a values were higher than the corresponding values found with the enzyme in free solution. This was indicative of the presence of some residual negative charges on the supporting nylon structure, after the immobilization of the enzyme. In the case of the enzyme in free solution, the ionization constants depended on which substrate was saturating the enzyme. The ionization of the basic group but not of the acidic group in the enzyme itself, and that of the acidic group but not of the basic group in the enzyme-substrate ternary complex, was affected. This was consistent with the compulsory order of substrate binding, which involves the addition of NAD prior to that of ethanol to the enzyme. It was suggested that the protonated form of a basic amino group and of the deprotonated form of an acidic carboxylic group are required for the binding of NAD and ethanol, respectively.

CHAPTER 1

GENERAL INTRODUCTION

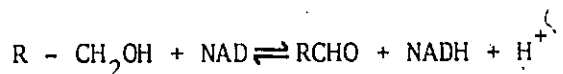
Tubes with enzymes attached to their inner surfaces are useful as open tubular heterogeneous enzyme reactors in industrial preparations (1-5). They are also being used in clinical medicine for the automated analysis of metabolites (6-9) and in extracorporeal shunts to remove undesirable substances from the body (10). Kinetic investigations of such systems are useful because of these practical applications, and also because they lead to models for understanding the function of enzymes in vivo, particularly of enzymes attached to the interior walls of blood vessels (11). Knowledge of the kinetic parameters and of the behaviour of an immobilized enzyme is necessary for the design of a reactor and for ensuring improved efficiency in any application. There is also an increasing interest in the investigation of immobilized enzymes in general as a promising tool for fundamental studies in enzymology and biochemistry (12).

Open tubular heterogeneous enzyme reactors are basically of two types, namely, diffusionally controlled and kinetically controlled, and these are superior in many ways to packed-bed enzyme reactors (13). For industrial applications, it is usually best to have diffusionally controlled reactors because these do not require the knowledge of enzyme-kinetic parameters for their design. The kinetically controlled types are more useful for analytical procedures since at low substrate

concentrations (less than the corresponding K_m values) the rate will be proportional to the concentration of the substance to be analyzed. It is therefore important to know the conditions under which an enzyme-catalyzed reaction in flow systems may be kinetically controlled or diffusionally controlled. This knowledge can only be obtained from a systematic and detailed kinetic study of the system, and this is the aim of this thesis which is concerned with yeast alcohol dehydrogenase.

Preliminary Information and Choice of the Enzyme

Alcohol dehydrogenases are oxidoreductases which catalyze the interconversion



where R can be a normal or a branched chain radical, either primary or secondary, for aliphatic or aromatic alcohols and carbonyl compounds. These enzymes were first recognized in elucidating the role of nicotinamide adenine dinucleotide (NAD) or coenzyme I in fermentation reactions (14). They can also react with NAD analogues and NADP or coenzyme II.

Alcohol dehydrogenase is of considerable interest in medicine, especially in relation to ethanol metabolism and alcoholism, but its physiological role is not clear (15). The enzyme has been used in forensic science (16-19) for determining ethanol in blood samples and organs, and in synthetic organic chemistry. In particular, the liver enzyme has found application for the stereospecific oxidoreduction of cyclic ketones and alcohols (20) and for the preparation of a pure

enantiomorph of ethanol-1-d (21). Yeast alcohol dehydrogenase was the first enzyme showing such specificity (22).

Hornby et al (23) described a method for immobilizing dehydrogenases, including alcohol dehydrogenase, and discussed applications to automated analysis. Other studies of immobilized alcohol dehydrogenase have been reported (24-28) but no detailed kinetic studies have previously been made for flow systems. It therefore seemed highly desirable to immobilize the enzyme inside a tube so as to stabilize it for a sufficiently long time, and to study the kinetics in the tubular flow reactor. Such systems may also be useful as cofactor regenerating systems (29,30) and as affinity media for NAD (31).

Occurrence and Multiple Molecular Forms of the Enzyme

Alcohol dehydrogenase occurs in a variety of organisms, both in the plant and animal kingdoms. The most widely studied alcohol dehydrogenase from the plant source is a cytoplasmic protein which is usually obtained from bakers' yeast. It is the first pyridine nucleotide-dependent dehydrogenase to be crystallized for which several purification methods have been reported (32), the first successful crystallization being made by Negelein and Wulff in 1937 (33). The other most studied alcohol dehydrogenase is that obtained from mammalian liver, which is abundant to the extent of about one gram per kg weight of liver (34). This was first crystallized from horse liver by Bonnichsen and Wassén in 1948 (35).

Commercially available crystalline yeast alcohol dehydrogenase (to be referred to as YADH later in the thesis) is known to have protein kinase activity due to impurity (36). There has been a report in the literature of homogeneous preparations (37), and there is no evidence for heterogeneity from amino acid sequence analysis (38-40). Two different forms have also been reported on the basis of polyacrylamide gel electrophoresis (41). Separation of YADH from simple enzyme mixtures has also been made by affinity chromatography (42-44). Apart from the classical YADH, a second soluble isozyme having a broader substrate specificity is also known (45-49). This isozyme is repressed by glucose (46-48) and is selectively produced by yeast grown on lactate or ethanol as the main carbon source. Another isozyme which is also sensitive to glucose repression is the mitochondrial YADH (48,50-54). Each of the isozymes has also shown a multiplicity of enzyme forms (53,54), but the relationship between the different forms is not well known. Similarly, the liver alcohol dehydrogenase is also found in multiple molecular forms which is considered to be the result of the synthesis of different types of subunits as well as of the occurrence of secondary modified forms (55).

Comparative Features of Yeast and Liver Alcohol Dehydrogenase

YADH is a tetramer of molecular weight 140,000-150,000 as determined by various techniques, while the subunit molecular weights are in the range of 36,000-37,000 which is consistent with the results of different structural studies (55). It is a zinc metalloenzyme; the metal content varies from 3.2 to 5.2 atoms per molecule, the most generally accepted

value being 4 zinc atoms per molecule or one zinc atom per subunit.

Each of the subunit chains can also bind one molecule of NAD. On the other hand, mammalian LADH is a dimer, consisting of two identical chains of molecular weight 40,000 each of which contains one binding site for NAD and two sites for Zn^{2+} , but only one of the zinc ions is now believed to be directly concerned with catalysis (56). There is strong evidence that the subunits of YADH and LADH have similar structures (55), and it is often assumed that the same overall reaction mechanism holds for both enzymes (56) although the mechanistic details are different.

LADH and YADH also differ in substrate specificity and in the rate of catalytic activity (32). The mammalian enzymes have much broader substrate specificity towards alcohols, aldehydes and ketones. It is interesting that LADH can oxidize cyclic secondary alcohols, particularly cyclohexanol, but not isopropanol. However, this enzyme does not show maximum activity even with primary alcohols such as ethanol, whereas the YADH is more specific for ethanol and acetaldehyde. The latter is completely inactive towards secondary alcohols having both alkyl groups larger than the methyl group, and also towards cyclohexanol. Further details about these aspects can be found in the reviews by Sund and Theorell (32) and Brändén et al (55).

Functional Aspects and Physiological Role of Alcohol Dehydrogenases

Much of the interest in the study of alcohol dehydrogenases, especially in the fields of medicine, is due to the fact that ethanol is one of the substrates. In a comprehensive review on these enzymes,

Brändén et al (55) noted that the great interest in the enzyme is due to the multiplicity of isozyme patterns of ADH in human liver, together with acquired and racial differences in ethanol metabolism. It has not, however, been possible to find any clear correlation between the patterns of ADH isozymes and ethanol metabolism or alcoholism (57). Also, the exact physiological role of the enzyme is very much debated (15).

There are many reports in the literature indicating that animals under their normal living conditions may become exposed to large amounts of alcohol by eating foods which are fermented to alcohol in the intestine (58). The physiological combustion or the oxidation of ethanol then takes place by the action of LADH (35,59-61). The enzyme is very powerful in that ethanol is oxidized, mainly in the human liver, at a rate of 7 g/hour, at 70 kg body weight (59). The oxidation of ethanol produces acetaldehyde which is converted to acetate or acetyl-CoA (55), the latter being metabolized by a number of routes such as biosynthesis of fatty acids, cholesterol and porphyrin, and oxidation by way of the citric acid cycle (15).

Alcohol dehydrogenase also plays an important role in the visual processes. Bliss (62) discussed the possibility that ADH present in liver extracts could convert the alcohol vitamin A to the corresponding aldehyde, retinene. It is known that retinene (vitamin A aldehyde in its all-trans form) is formed in a free state from the complex with opsin by the influence of light (32). The visual purple is produced again after its cis-isomerization and subsequent reoxidation by ADH to cis-retinene followed by recombination with opsin (63).

Apart from ethanol and the vitamin A alcohol, several other substrates of physiological interest have been discovered (55). The enzyme is involved in the degradation of fructose (64,65), and it reduces glyceraldehyde, which is formed through the cleavage of fructose-1-phosphate, to glycerol. Horse liver alcohol dehydrogenase has particularly been found to contain steroid-active isozymes for which 3-keto and 3 β -hydroxysteroids are substrates, although their functional significance is not clear (55), especially because mammalian livers contain different steroid specific dehydrogenases (66). However, the metabolism of steroids in the liver has been reported to be influenced in several ways by the activity of LADH during ethanol metabolism (67). It was shown that low doses of ethanol given to humans cause a marked increase in the plasma levels of certain steroid sulfates having a free 17 β -hydroxyl group, and simultaneously there is a decrease of the concentration of the corresponding 17-keto steroid (68). The magnitude of the changes was related to the blood ethanol level below a concentration of about 8-12 mM, and the effect was assumed to be due to the increase of the ratio of free NADH to NAD in the liver. This change in the NADH/NAD ratio has also been alleged to be responsible for many of the metabolic derangements seen in alcoholism (69). The oxidation of ethanol producing large amounts of NADH is therefore of prime importance in human physiology. Of no less significance is the greater specificity of YADH for ethanol and acetaldehyde, which was noted to be consistent with its recognized physiological significance to participate in alcohol fermentation at the end of glycolytic pathway (55), and also because there is some evolutionary link between YADH and

LADH_o (38). Several workers reported YADH to function in fermentation reactions (45-47). This enzyme also participates in the formation of carbohydrates from ethanol during oxidative metabolism (55).

Objectives and Outline of the Present Work

In a symposium on the structure and function of oxidation-reduction enzymes, Blomstrand (15) stated in 1970 that "Alcohol is perhaps the most universal hazard of every national culture, especially in a period of explosive population growth. This confrontation demands that we learn to control the effects of alcohol in different ways. One way to achieve this is to link together biochemistry and clinical investigative medicine ...". He reported such a study on the regulation of ethanol oxidation in man. Immobilization of the enzyme can also contribute something in this regard. This is because of the particular advantages due to the usually enhanced stability and ready availability of immobilized enzymes, which can be used repetitively for the automated estimation of their substrates, with a considerable economy in the enzyme utilization. The use of immobilized dehydrogenases supported in nylon tubing and in automated analysis has already been mentioned. Such an enzyme can also be useful in the forensic determination of ethanol and in stereospecific synthesis involving ADH as was also noted earlier. Moreover, their potential in controlling the effects of ethanol in human physiology, as in extra-corporeal shunts, is another possibility.

Efficient utilization of an immobilized enzyme requires the preparation to be of sufficient stability. Previous methods used by

different workers for the immobilization of ADH, particularly in nylon tubing, were not very successful since the enzyme lost its activity rapidly, with the result that consistent kinetic data could not be obtained. It is therefore an important objective to find or develop a suitable method for the stable attachment of yeast ADH inside nylon tubing. This will be described in the next chapter. Yeast alcohol dehydrogenase was particularly chosen in view of the economics and also because of its greater specificity towards ethanol.

A comprehensive experimental study of the kinetics of the reaction between ethanol and NAD, catalyzed by yeast alcohol dehydrogenase attached to nylon tubing and in flow systems, will be presented in subsequent chapters. The work was done at different flow rates and in two limiting cases of varying the concentration of one substrate with the other kept constant at a sufficiently high concentration so that it saturates the enzyme. This allows the evaluation of the apparent Michaelis constants at different flow rates, and also allows the data to be treated according to the Kobayashi-Laidler theory (70). It should be noted that this is the only theory available for the treatment of data for the flow kinetics of reactions catalyzed by enzymes when they are attached to the interior of tubes. This theory was developed for single substrate systems, and therefore the present studies provide experimental tests as to the extension for two-substrate systems, which present considerable difficulty. One particular reason for a possible deviation from the theoretical predictions can be due to inhibition by products of the reaction which will also be examined in the tubular flow reactor.

The work was done at several temperatures for each of the substrate concentrations and flow rates. This also provides mechanistic information, particularly about the diffusional control, and gives the activation energies under the conditions of full diffusion control and of complete chemical control. Finally, it is of interest to study the pH dependence of the kinetics, since hydrogen ions take part in the reaction catalyzed by the enzyme, and also because the pH-activity behaviour of an immobilized enzyme is often different from that of the same enzyme in free solution. A reliable analysis of such data will provide valuable information about the nature of the ionizing groups and their role in the catalytic behaviour. The results of the various studies will lead to the reaction mechanisms, which are of some physiological significance, and also the conditions suitable for the practical applications of the enzyme.

General Aspects of Enzyme Immobilization

According to the Commission on Biochemical Nomenclature (IUPAC-IUB 1974), the term immobilized enzyme is used to describe all enzyme preparations in which the enzyme is constrained one way or another within the limited confines of the supporting material. Other types of molecules that have been immobilized include antibodies, antigens, substrates and inhibitors. The practical applications of such systems in many areas and the recognition of their potential in basic research have led to a very intense activity in this field. As a result, immobilization itself has in the last few years rapidly emerged into a new technology. The technique of immobilization has been extensively reviewed by many workers of which

mention may be made of Goldstein and Manecke(71) and of several articles appearing in a complete volume of Methods in Enzymology devoted to Immobilized Enzymes (72). More recently, Klibanov (73) made a detailed evaluation of the potential of immobilization for the stabilization of enzymes against different kinds of inactivation. In this section, however, only a brief general description of enzyme immobilization will be given, enough to provide a background to the particular method used for the present study.

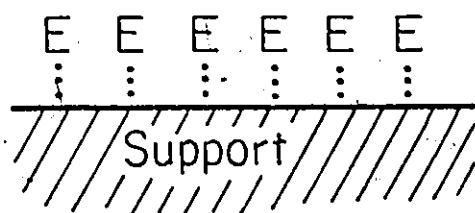
A very large variety of materials are being used for the immobilization of enzymes, the choice being dependent on the application of the particular derivative. There are some general characteristics which are desirable in the supporting materials aside from their commercial availability at a reasonable or moderate cost. It is necessary to have a certain degree of chemical and mechanical stability (rigidity), as well as permeability to substrates and resistance to microbial attack, in order to allow prolonged use of any preparation. The materials may possess potential chemical reactivity which, to some extent, determines the method of immobilization. A carrier should also be available in suitable geometrical shape and appropriate dimensions. Immobilized enzymes have been used in a variety of configurations such as packed beds, fluidized beds, stirred tanks, hollow fibers, enzyme-electrodes, open tubular reactors, enzyme pads, membrane reactors, fiber reactors, and several other more unusual configurations (74).

Immobilization of enzymes is subject to strict methodological constraints, since it must be compatible with the catalytic function of

the enzyme. A number of methods are available, and have been classified by different workers (75) into four basic approaches, as shown schematically in Figure 1. These are (i) adsorption on inert carriers, (ii) physical entrapment in gel lattices, (iii) microencapsulation within thin-wall spheres, and (iv) covalent binding to natural or synthetic polymers and intermolecular covalent cross-linking by multifunctional reagents into macroscopic particles. Broadly speaking, all these methods can be grouped into two major techniques, viz., chemical attachment of the enzyme by the introduction of insolubilizing groups, and physical attachment. The former technique results in a chemical "tying down" of the enzyme. This is sometimes difficult to achieve since the insolubilizing groups can attach across the active site destroying the intrinsic activity of the enzyme, while the latter offer the advantages of rapidity and ease of preparation (76). Despite the practical difficulties, however, covalent attachment is the most widely used since it offers many advantages. It allows a choice of the binding points on the enzyme, and the use of pre-designed carriers. Besides, the reproducibility of bonding to the carrier substances, and little or no elution of bonded enzyme by aqueous buffer systems, make them universally useful in different types of reactor systems. The covalently bonded enzyme is specially suited for the assay of large substrates such as proteins with proteolytic enzymes. Physical entrapment, on the other hand, renders the enzyme somewhat inaccessible in that large molecules cannot readily diffuse into the matrix.

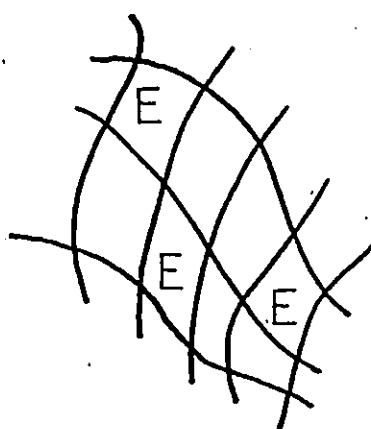
Figure 1. Four basic approaches in the immobilization of enzymes (10).

(i)



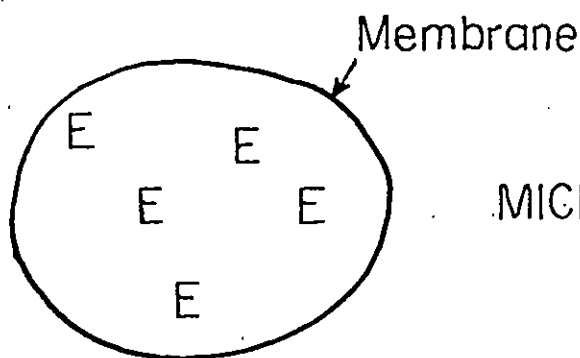
ADSORPTION

(ii)



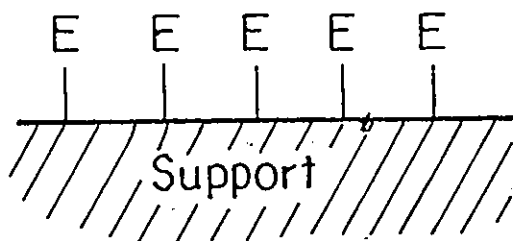
PHYSICAL
ENTRAPMENT
IN GEL LATTICE

(iii)



MICROENCAPSULATION

(iv)



COVALENT
ATTACHMENT

The chemical attachment of enzymes involves forming covalent bonds between enzymes and functional groups of supporting materials. The most frequently used carriers used range from water-insoluble synthetic polymers like polyacrylamide, polystyrene, maleic anhydride polymers, methacrylic acid polymers, and polypeptides, to water-insoluble natural carriers like agarose, cellulose, sephadex, glass and starch (10). Enzymes have also been covalently bonded to water-soluble macromolecules like CM-cellulose (77) or dextran conjugate (78) to form soluble immobilized enzymes which are useful for certain biomedical applications. A large number of reactions have been utilized for the covalent coupling of enzymes, sometimes involving the activation of the polymer by introducing additional groups to imprint the desired physicochemical properties. For example, the acylazide reaction, diazonium coupling, the cyanuric chloride reaction, the cyanogen bromide reaction, addition of isothiocyanates, and the use of condensation reagents such as carbodiimides are among the most frequently employed (71). Coupling reactions usually proceed under alkaline conditions and involve reactions with the amino groups of the enzyme. However, the use of organic isocyanides has allowed the fixation to occur via amino groups or carboxylic groups, and also at slightly acid, neutral, or slightly alkaline pH values (79). The alternative approach to covalent binding of enzymes is the intermolecular cross-linking between enzyme molecules by the use of multifunctional reagents like glutaraldehyde, bisdiazobenzidine-2,2'-disulfonic acid, and others (71). This method has also been used in combination with or after microencapsulation (80), and adsorption on the surface of a carrier (81), or impregnation in porous membranes (82,83).

Adsorption of enzyme in aqueous solution to the surface of carriers is the simplest of all the physical methods. The various types of carriers used for this purpose include carbon, alumina, collodion, clay, glass, cellulose, collagen and others. Ion-exchange resins have also been used for the adsorption of enzymes by electrostatic interaction (84). An important feature of the adsorbed enzymes is that they can be reversibly desorbed provided there is suitable change in an environmental factor like pH, ionic strength, or temperature. This is not possible with any of the other methods, particularly with matrix entrapment or microencapsulation. The former involves the entrapment of enzyme molecules within the interstitial space of a cross-linked network of water-insoluble polymer. The most common type of material used is polyacrylamide gel, to which the enzyme may be attached by mixing it with acrylamide and N,N'-methylenebisacrylamide. Other materials like starch, silica and siliconerubber (85) have also been reported for matrix entrapment. The relative activity of such enzyme preparations is usually low since a large amount of the polymeric network is required to entrap a rather small amount of enzyme. There are also diffusional limitations to the substrates and the problem of enzyme leakage from the polymeric network. A somewhat different situation exists with microencapsulated systems in which the enzyme is entrapped within thin walls of artificial membranes. The membrane materials vary from synthetic polymers like cellulose nitrate, polystyrene and nylon, to biological materials like lipids, lipoproteins and liposomes (10). The general procedure in microencapsulation is to emulsify the aqueous solution of enzyme with a water-immiscible organic



solution to form aqueous microdroplets. This is followed by the addition of a polymer to the stirred emulsion, resulting in the formation of a membrane around the surface of each microdroplet, thus producing the so-called artificial cells. The contents of the artificial cell can be varied at will, and any of the other immobilized enzyme systems can be used in combination with such preparations (86). These offer several advantages making them particularly valuable for biomedical applications.

General Considerations in Immobilized Enzyme Kinetics

The importance of the kinetic studies of immobilized enzymes can hardly be overemphasized in view of their growing employment in various technological applications and in fundamental research. Several recent reviews (87-91) have dealt with the subject, but the available literature in this area is relatively small compared to those on immobilization techniques. However, it is well known that the behaviour of an immobilized enzyme can differ significantly from that of the same enzyme in free solution. Besides, the same enzyme can behave differently according to the method of immobilization, and also a particular method can cause different changes in different enzymes. In general, there are two major factors responsible for the difference in kinetic behaviour between an immobilized enzyme and its free counterpart (89). The first involves changes in the enzyme molecule or in its immediate vicinity resulting directly from the attachment of the enzyme to the carrier, and the second arising from the heterogeneous nature of the local enzymic environment. These can manifest themselves in several ways such as conformational

(including steric), environmental, partitional and diffusional effects on the enzyme kinetic behaviour. Each of the effects can also be modified by the nature of the supporting material, and a clear separation into the individual factors is generally difficult to achieve. Nevertheless, it is important at least to be able to recognize and distinguish them for the interpretation of data as well as for theoretical modelling or the design of experiments. Various workers have presented theories incorporating the different effects, but their discussion is beyond the scope of this thesis and only a brief general description of the above mentioned effects will be given here.

It is well established that the kinetic behaviour of an enzyme depends primarily and strongly on its three-dimensional conformation. This is susceptible to alteration due to interaction between the enzyme and the support when the enzyme is immobilized by any method. The decrease in enzyme activity usually encountered upon immobilization has often been attributed to such conformational changes or to steric hindrance to the approach of the substrate, resulting mainly from the shielding effect of the matrix. It is difficult to distinguish between these two effects especially with low molecular weight substrates, whereas the possibility of reduced diffusivity with high molecular weight substrates creates a different problem. However, the shielding effect can be reduced by introducing a "spacer" to keep the enzyme at a certain distance from the matrix. Interaction between the bound enzyme and the matrix also depends on the nature and properties of the support. For example, hydrophobic interactions induced by contact with the support can cause

inactivation, and narrowing of pH-activity profile by lowering the dielectric constant in the surrounding. This effect can be more pronounced as a result of electrostatic interaction when the enzyme is immobilized on charged supports. It is therefore apparent that the conformational changes and matrix interactions can modify the catalytic activity as well as the selectivity and stability of the bound enzyme. In many cases, the stability of the supported enzyme is enhanced which is attributed to the stabilization of the three-dimensional structure and the prevention of autolysis, or simply because of less accessibility to denaturing agents and microbial attack (73,89). However, the improvement in stability as a result of immobilization is not a general rule, and there are examples of reduced or no change of stability. Whatever may be the effect, it is important that the stability of an immobilized enzyme preparation is ascertained before any consistent kinetic data can be obtained.

Immobilization of an enzyme can put it in a different environment, especially with respect to the concentration of substrates or other effectors, from that existing in free solution. Thus, in the immediate vicinity of the bound enzyme (microenvironment) the concentration of species influencing the rate of reaction may differ from those in the bulk solution (macroenvironment). This can also result from the electrostatic or hydrophobic interactions between the matrix and the substrate or other effectors, as well as from the presence of diffusional resistances, and is determined largely by the chemical nature of the support material. Such effects would be reflected in the concentration dependence of rates, and may have far-reaching kinetic consequences. For example, the intrinsic

kinetic parameters of an enzyme and its apparent specificity might be changed as a result of perturbations of the catalytic pathway of the reaction induced by the modified microenvironment (90). It is difficult to evaluate the intrinsic kinetic parameters when there are microenvironmental effects, since the experimental conditions usually permit measurement of the respective bulk concentration in the macroenvironment while the activity of the bound enzyme is determined by the local concentration in the microenvironment. However, studies of enzyme reactions in different solvents have shown that environmental effects can be very profound, and some of these can be satisfactorily explained in terms of the influence of the dielectric constant of the medium on electrostatic interactions (87). The environmental effect is more clearly observed in the shifting of the pH optimum of the enzyme since the local concentration of hydrogen ions in the microenvironment may either be more or less than that in the bulk solution depending on whether the support is negatively or positively charged. In order to achieve maximal activity, then, the required alkalinity of the bulk solution must be increased or decreased. Similar changes in the pH optimum can also be observed with immobilized enzyme systems producing or consuming acid during the reaction. Besides, the presence of fixed charges of the support in the neighbourhood of the active site can modify the dissociation of the catalytic groups due to electrostatic interactions, thus resulting in further changes of hydrogen ion concentration in the microenvironment (91). This effect can be profitably utilized to extend the active pH range of the enzyme, and to stabilize the enzyme at a pH where microbial growth can be highly discouraged.

It is possible that environmental changes can be due to the partitional effects arising from hydrophobic, hydrophilic and electrostatic interactions with the support. For instance, a relatively non-polar substance is more soluble in a hydrophobic support containing a number of non-polar groups than in aqueous solution. Conversely, a charged or a polar substance will have a lower concentration in the domain of the immobilized enzyme. In principle, this effect can be realized simply by measuring the partition coefficients between the solution and the support and in absence of the enzyme. These effects are likely to be small if the support as well as the substrate and/or other effectors are uncharged but profound changes can occur if they are charged. Microenvironmental partitioning effects owing to electrostatic interactions can be readily eliminated or cancelled at high ionic strength, while the effects due to hydrophobic interactions can be detected by the use of labelled materials (90). The effect of a hydrophobic microenvironment on the partitioning of substrate has particularly been observed with horse liver alcohol dehydrogenase coupled to cross-linked copolymers of acrylamide and methyl acrylate (24). Similarly, the observed changes in the degree of inhibition of β -fructofuranosidase by aniline and Tris when the enzyme is attached to polystyrene surfaces have been explained by partitional effects (92). Some workers also ascribe the pH optimum shifts for immobilized enzymes to the unequal partitioning of the hydrogen ions between the enzyme micro and macroenvironment arising from electrostatic interactions with the support (see 89). Detailed treatments of the partitioning of hydrogen ions and of charged substrates between a charged

immobilized-enzyme particle and the bulk solution have been given by Goldstein (90), and similar treatments based on electrostatic interactions have been extended to inhibitors by Royer (88). It is understandable that the electrostatic attraction or repulsion between the charged substrate or inhibitor and the support can increase or decrease their effective concentration within the microenvironment, and hence affect the activity of the enzyme. In addition, there can be no change of inhibitor potency if the substrate and the inhibitor possess like charges since the binding effect will be the same for both. On the other hand, a very large increase in the inhibitor effectiveness can be expected if the substrate carries the same charge as the support and the inhibitor carries the opposite charge (88).

Finally, and of particular importance in this thesis, is the effect of mass transfer or diffusional limitations on the kinetics of immobilized enzymes. The overall reaction catalyzed by such a system consists of three consecutive steps involving (i) the transport of the substrate from the bulk to the enzyme active site, (ii) the chemical transformation of the substrate into the product at the active site, and (iii) the transport of the product away from the active site to the bulk of the solution. The transport of the substrates and of the products, i.e., steps (i) and (iii), can occur both by molecular and convective diffusion, and these may be slower than the usually high catalytic activity of the enzyme, i.e., step (ii). Consequently, the observed rate of the reaction would be lower than that expected for a given amount of enzyme in solution, since not all enzyme molecules would be in contact with substrate at a

concentration level identical with that of the bulk solution. In other words, the overall reaction is what is known as diffusion-controlled. This is better understood by imagining that concentration gradients are established in the surrounding of the bound enzyme so that the concentration of either or both the substrate and product differ between the micro and macroenvironment. The effect is such that the concentration of the substrate or product decreases or increases gradually from the bulk solution towards the interior of the medium, and these can be distinguished from the partitional effects which result in steep concentration changes at the interface. It can be emphasized that the concentration differences due to diffusional resistances are caused by the respective depletion and accumulation of substrate and product as a result of chemical reaction in the enzyme microenvironment (89). The extent of these concentration changes usually depends on the size of the species involved which may be critical in the case of enzyme immobilized in a porous medium. Thus, if the substrate molecule is larger than the pore size of the medium containing the enzyme, it cannot diffuse to the catalytic site so that no reaction can take place even if the enzyme is fully active in the interior. Diffusional resistances can also give rise to an apparently high stability of the immobilized enzymes; the observed activity can remain constant in the limit of full diffusional control, although the enzyme may suffer extensive denaturation or irreversible inhibition.

There are two types of diffusional resistances, especially in the case of a porous medium, viz., the external diffusional resistances to the transport of the substrates and products between the bulk solution

and the outer surface of the supported enzyme across a boundary layer of liquid, and the internal diffusional resistances pertaining to the transport inside the porous catalytic medium. The concentration changes across the boundary layer can often be approximated by a linear gradient in the former case, but the latter effect normally gives rise to non-linear concentration gradients (90). Another difference between the two types of diffusional effects is that the chemical reaction occurs in series and only after the external transport, while the internal diffusion proceeds simultaneously or in parallel with the chemical reaction. Consequently, the external diffusion is independent of inherent kinetic parameters and is unaffected by the action of chemical inhibitors, in contrast to the internal diffusion for which the rate of enzymic reaction can never be determined by the rate of substrate diffusion alone (89). It is also possible that the latter is affected by substances other than chemical effectors which can modify the structure of the porous medium, thereby altering the internal diffusivity.

Both types of diffusional limitations can significantly influence the kinetic behaviour of immobilized enzymes. For example, the presence of diffusional inhibition can cause the chemical inhibition to manifest itself differently from that in free solution. In general, the effect of chemical inhibition is smaller in the presence than in the absence of diffusional effects since the former can lower not only the inherent activity of the enzyme but also the extent of substrate depletion. This means that the combined effect in decreasing the rate of the reaction is smaller than the sum of the effects that would be obtained if each of

them were acting independently. This is particularly true when the inhibitor is neither substrate nor product, but if the substrate is an inhibitor then the rate of the reaction can be higher in the presence than in the absence of diffusional limitations especially at relatively high substrate concentrations. The latter can also show multiple steady states in a certain concentration range of the substrate provided that internal diffusional resistances are sufficiently large. In the case of product inhibition, both substrate depletion and product accumulation must be taken into account. The effect is greatest at low product concentration, i.e., when the activity of the enzyme is the highest for a given system, since the magnitude of diffusional resistances would be reduced with increasing product concentration. The combined effect of diffusional and product inhibition can also be different for competitive and non-competitive inhibitors, particularly at high substrate concentrations (89). The effects of inhibition due to product accumulation is of particular interest when the product is either an acid or a base. The usual bell-shaped pH profile can be considered as the result of activation and inhibition of the enzyme by hydrogen ions at concentrations lower and higher than that at the pH optimum, respectively. Diffusional limitations not only can shift the pH optimum but also the entire pH-activity profile since the accumulation of acids or bases can make the local pH independent of the bulk pH so that the enzyme activity plateaus or rises monotonously with increasing pH (89). It is important to note that electrostatic factors can also affect the transport of various charged species, and may even have a greater influence on the overall kinetics

when diffusional limitations are considerable. Different workers have developed theoretical models with particular emphasis on diffusional limitations, but the only treatment available for open tubular flow reactors is that due to Kobayashi and Laidler (70) which will be discussed later (in Chapter 3).

There are several ways of demonstrating diffusional effects, the most direct method being concerned with the change of transport conditions for substrate and product. As mentioned before, external diffusion is affected by the efficiency of mixing or the flow rate in the heterogeneous enzyme reactor. Similarly, the effect of internal diffusion can be detected by varying the immobilized-enzyme particle size or the membrane thickness. The interaction between diffusional and chemical inhibition can also serve as a diagnostic tool for diffusional resistances. Another important method depends on the effect of temperature on reaction rates and the shape of the appropriate Arrhenius plots. Thus, when the temperature is low and the reaction is kinetically controlled, the true activation energy is obtained. At intermediate temperatures, internal diffusion becomes significant and the apparent activation energy is different. The result is non-linear Arrhenius plots, while at sufficiently high temperatures, the rate becomes practically independent of temperature when the reaction is bulk diffusion controlled (89,90). It is to be noted that non-linear Arrhenius plots can also be obtained for temperature-dependent conformational changes such as inactivation or other effects. Finally, and perhaps the most commonly employed method for the character-

ization and evaluation of diffusional effects is the analysis of conventional graphical plots used in enzymology. Diffusional limitations usually yield characteristic non-linear curves for the classical Michaelis-Menten kinetics, their usefulness being discussed by different workers, notably Engasser and Horvath (89) and Goldstein (90).

In general, the effects of various factors on the behaviour of immobilized enzymes are reflected in their kinetic and rate parameters such as K_m and V_{max} . The decrease in enzymic activity as a result of immobilization is inferred from increased K_m or decreased V_{max} , the latter being the direct result of the attachment which can lead to a total or partial modification of the active-site topology. This can be due to the reaction of an amino acid side chain necessary for catalysis or substrate binding, disruption of the three-dimensional structure of the enzyme, or simply steric hindrance to the approach of the substrate. On the other hand, the decrease in K_m can result from these as well as from environmental, partitional, diffusional and electrostatic effects. In any case, if the effect is such that it gives rise to a lower concentration of the substrate in the immediate vicinity of the active site, then a higher concentration will be required in the bulk solution to overcome the effect, i.e., to achieve the same rate as for the enzyme in free solution, thus increasing the apparent Michaelis constant. The same reasoning applies to inhibition constants or any other dissociation constant, the increased values simply meaning less binding or more dissociation.

It is apparent that the measured rate and kinetic parameters or the inhibition and dissociation constants for an immobilized enzyme

system do not reflect their intrinsic or inherent values. The experimentally obtained effective or apparent values are of limited usefulness since these are obtained under certain experimental conditions and cannot be compared directly with the intrinsic kinetic parameters of the native enzyme so that the effect of immobilization can be established. Three different types of rate and kinetic parameters are to be recognized in this context: the intrinsic parameters characteristic of the native enzyme in free solution, inherent parameters pertaining to the immobilized enzyme in the absence of diffusional limitations, and the effective or apparent parameters observed for an immobilized enzyme when diffusional effects are significant (89,90). The determination of intrinsic and inherent kinetic parameters has received great attention in the study of immobilized enzyme kinetics. The former is obtained from the experimental data only when the microenvironmental and diffusional effects are evaluated, while the latter requires only the separation of diffusional effects. It is not very difficult to demonstrate the presence of microenvironmental partitioning effects, but their exact evaluation is generally not easy. On the other hand, the techniques and methods for the characterization and evaluation of diffusional effects, and therefore of inherent kinetic parameters of immobilized enzyme systems, are well known, as discussed in the reviews by Engasser and Horvath (89) and Goldstein (90). In particular, the theory of Kobayashi and Laidler (70) also allows determination of Michaelis constants in the absence of diffusional limitations and in flow systems, with which this thesis is concerned.

CHAPTER 2

IMMOBILIZATION OF YEAST ALCOHOL DEHYDROGENASE INSIDE A NYLON TUBE

Introduction: Choice of the Support Material

Nylons are synthetic polyamides, typically formed either by the condensation of ω -aminocarboxylic acids or of a bis-aliphatic amine with a bis-aliphatic carboxylic acid. They represent a family of linear polymers consisting of repeating assemblies of methylene groups joined together by secondary amide linkages, and differing only in the number of repeating methylene groups in the alkane segments. These are designated according to the number of carbon atoms in their component monomeric units, e.g., the so-called nylon 6 is obtained from the polymerization of a six carbon compound caprolactam. Similarly, the polycondensation of hexamethylene diamine with adipic acid (both 6-carbon compounds) or with sebacic acid (a 10-carbon compound) yield nylon 66 and nylon 610, respectively.

Nylons are very attractive for the immobilization of enzymes because they possess all the characteristics desirable for a supporting material (93). They are mechanically strong and non-biodegradable which make them particularly useful for prolonged exposure to biological media without impairment of their structural integrity. Some of the nylons, especially those of shorter methylene chains such as nylon 6 and nylon 66, are considerably hydrophilic, thus offering support for an environment which is otherwise conducive to the stability of the enzyme. Finally,



nylons are readily available in a wide variety of configurations such as films, membranes, powders, hollow fibers and tubes. Thus, it is possible to prepare multifarious immobilized enzyme structures using a common method for a common supporting material so that the different modes of the immobilized enzyme may be entertained (93).

Use of Nylon-Supported Enzymes: Choice of the Tubular Configuration

The commercial availability of nylons in various forms has resulted in the preparation of different types of immobilized enzyme derivatives. Perhaps, the earliest use of nylons in the immobilization of enzymes is in microencapsulation. The preparation of immobilized enzyme microcapsules within semipermeable membranes of nylon 610, and their importance in clinical therapy, has been described by Chang (94). Several workers reported the immobilization by adsorption on the surface of nylon structures, such as nylon floc, pellicular nylon, nylon membranes, or nylon filaments, and then fixed by cross-linking with carbodiimides (95), bisimides or glutaraldehyde (96-98). However, the majority of immobilized enzymes using nylons to date have been made by covalently binding the enzyme to the nylon polymer itself. This allows a greater choice of support configurations which can be "tailor-made" to suit any operational requirement (93). Thus, enzymes have been covalently bonded to nylon powders, membranes and tubes which have been used in the form of packed beds, stirred tanks and open tubular reactors. A typical use of these systems is in the continuous flow analytical techniques for the automated determination of substrates as described by Campbell and Hornby (74).

In principle, any immobilized enzyme structure which can be operated in continuous flow mode can be employed in automated or semi-automated systems of analysis. This is, however, more conveniently done with the enzyme attached to the interior surface of a tube, and such systems have so far been used almost exclusively for the determination of substrates in automated analysis. Many automated procedures of analysis involve the use of tube-supported enzymes in flow systems as illustrated schematically in Figure 2. The sample, S, to be analyzed is allowed to react with the enzyme, E, normally used in free solution, but the latter can be replaced simply by a length of tubing, containing the enzyme attached to its interior wall, at T. The products of the enzyme catalyzed reaction then react with the reagents R_1 and R_2 to form, for example, a coloured product whose concentration may be determined at A, say, spectrophotometrically. The reactants are all sampled automatically and pumped through the separate pieces of tubing, allowing them to be mixed in the order required by the particular analytical procedure involved. Numerous samples may be analyzed sequentially in this way, the waste material being continuously collected in the discard vessel, D. Automated analytical techniques using immobilized enzymes have been in use for some years, and several instruments such as those pioneered by Technicon are commercially available for executing such operations in many routine analytical laboratories.

Historically, one of the first reports on the application of immobilized enzymes in automated analysis involved the use of open tubular reactors, in which glucose oxidase was covalently linked to the

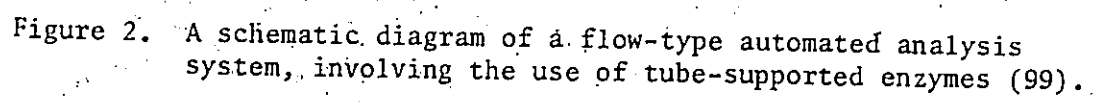
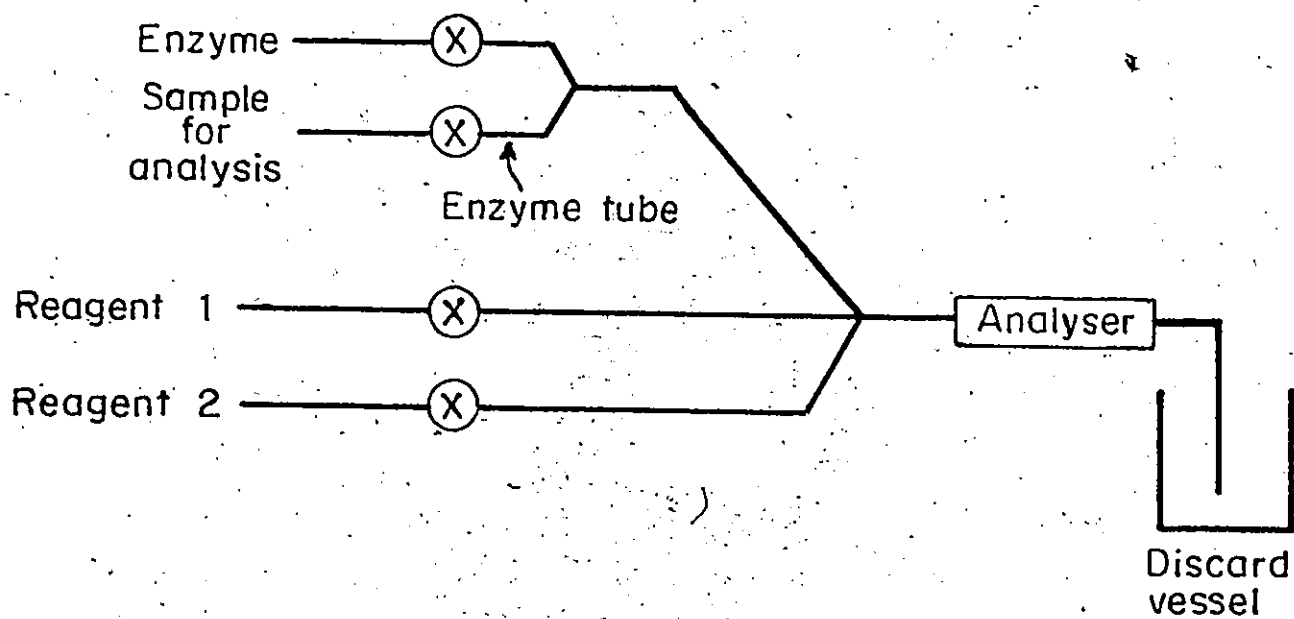


Figure 2. A schematic diagram of a flow-type automated analysis system, involving the use of tube-supported enzymes (99).



inside surface of a polystyrene tube (7). Subsequently, Sundaram and Hornby (8) developed a method for the covalent attachment of enzymes on the inside surface of type 6 nylon tubing. Filippusson et al (100) followed their method to attach urease inside a nylon tube and used the derivative in a Technicon AutoAnalyzer continuous-flow system for the automated determination of urea. Hornby et al (23) used different techniques for the immobilization of lactate dehydrogenase, yeast alcohol dehydrogenase, and malate dehydrogenase separately in nylon tubing and described their use for the automated determination of pyruvate, ethanol and oxaloacetate, respectively. Automated analyses of substrates requiring linked enzyme systems can also be performed by using tube-supported derivatives in which either the component enzymes are coimmobilized in the same tube, or they are immobilized individually and then operated in series. Thus, Inman and Hornby (9) demonstrated the utility of glucose oxidase linked invertase, β -galactosidase and amyloglucosidase systems in nylon tubing for the automated determination of the disaccharides sucrose, lactose and maltose, respectively. Similarly, Morris et al (101) immobilized hexokinase and glucose-6-phosphate dehydrogenase both singly and together on nylon tubing, and used them successfully in the automated determination of glucose. The versatility of nylon-tube supported enzymes has been emphasized for several other systems such as those with penicillinase and urease for the automated analysis of penicillin G in fermentation media (102) and for the determination of plasma urea (103), respectively. Another very important application of immobilized enzyme derivatives is in

the continuous generation of an expensive cofactor from a cheaper precursor, and for the in situ supply of specific reagents necessary for continuous flow automated systems of analysis. To this end, Hornby et al (23) used nylon-tube-supported alcohol dehydrogenase for the in situ production of NADH by perfusion with a solution containing ethanol and the less expensive coenzyme NAD, the NADH produced being delivered for the assay of pyruvate and oxaloacetate with lactate and malate dehydrogenases, respectively, in continuous flow analysis.

Several workers (104-107) have probed the use of open tubular enzyme reactors in high-speed continuous flow systems, in which the enzymes were not covalently bound to the wall of the tube but were trapped in an insoluble, porous annulus fixed to the inside wall. Thus, Leon et al (104) described a system using nylon tube-supported glycerol kinase for the assay of triglycerides. They have also studied the nylon-tube-supported hexokinase/glucose-6-phosphate dehydrogenase systems, both singly and together, for the assay of ATP formed by the enzyme creatine phosphokinase. Other immobilized enzyme configurations have been employed in continuous-flow automated analysis. For example, Inman and Hornby (98) cross-linked glucose oxidase and urease separately using glutaraldehyde within the matrix of nylon 6 membranes. Such derivatives have been operated in continuous flow modes by incorporating them in conventional dialyzer modules. The same workers also immobilized these enzymes on nylon 6 powders and utilized them in the form of packed beds for the automated analysis of their substrates. Similarly, Filippusson et al (100) coupled urate oxidase to nylon 66 powder using glutaraldehyde,

packed them into a column and used in a continuous flow process for the analysis of uric acid.

Inman and Hornby (98) evaluated the use of different nylon-supported derivatives as potential enzyme reagents for the automated determination of substrates. The application of powder-supported derivatives in the form of packed beds was considered unsatisfactory since air-segmentation must be removed as the sample stream is passed through the column. This necessitates the introduction of a debubbler preceding the reactor which causes spreading of samples and increases the possibility of carry over, hence limits the rate at which samples can be analyzed (74, 93). Membrane-supported derivatives also did not permit sufficiently high rate of analysis because they suffer from lateral diffusion problems. On the other hand, tube-supported derivatives showed sufficient activity for the required sensitivity, and could be perfused with an air-segmented stream such that an acceptable rate of sample analysis was achieved. In view of these advantages, Campbell and Hornby (74) concluded that the open tubular reactors are the best immobilized enzyme configuration for their direct incorporation in continuous flow automated analytical processes. Such systems permit an unobstructed flow of substrate solution, and as Ngo and Laidler (13) noted, their low pressure drop as well as favourable hydrodynamic properties render themselves very useful in biomedical and analytical experiments. These factors can also make them superior to other types of enzyme reactors in industrial preparative work such as mentioned previously (1-5). Besides, the use of a tube-supported enzyme is particularly valuable for any fundamental investigation

since the same enzyme can be used throughout a series of experiments, thus avoiding difficulties in the rationalization of results from different works which may be intrinsically different due to variation in the sources of the enzyme.

Principles and Chemistry of Immobilization of Enzymes on Nylons

Goldstein and Manecke (71) have reviewed the chemistry of enzyme immobilization in general, while Hornby and Goldstein (104) described the immobilization of enzymes on nylon itself. It has been pointed out that native nylons, generally of high molecular weight, suffer from the intrinsic drawback, as support for covalent immobilization of enzymes, of having relatively few end groups. This is evident from the relative inertness of the polyamide backbone in which only the terminal amino and carboxyl groups are left as the possible reactive centres. However, the problem can be alleviated by some pretreatments in which the characteristic secondary amide linkages of nylons are broken, thus liberating either the component groups or generating potentially reactive centres which can be used for the subsequent covalent binding of enzymes. In principle, there are three basic approaches that have been adopted to increase the binding capacity of nylons (71,104). These include (i) the controlled cleavage of amide bonds to increase the number of carboxyl and amino groups, (ii) the introduction of reactive centres via O-alkylation of the backbone peptide bonds, and (iii) the introduction of reactive side chains via N-alkylation of backbone peptide bonds.

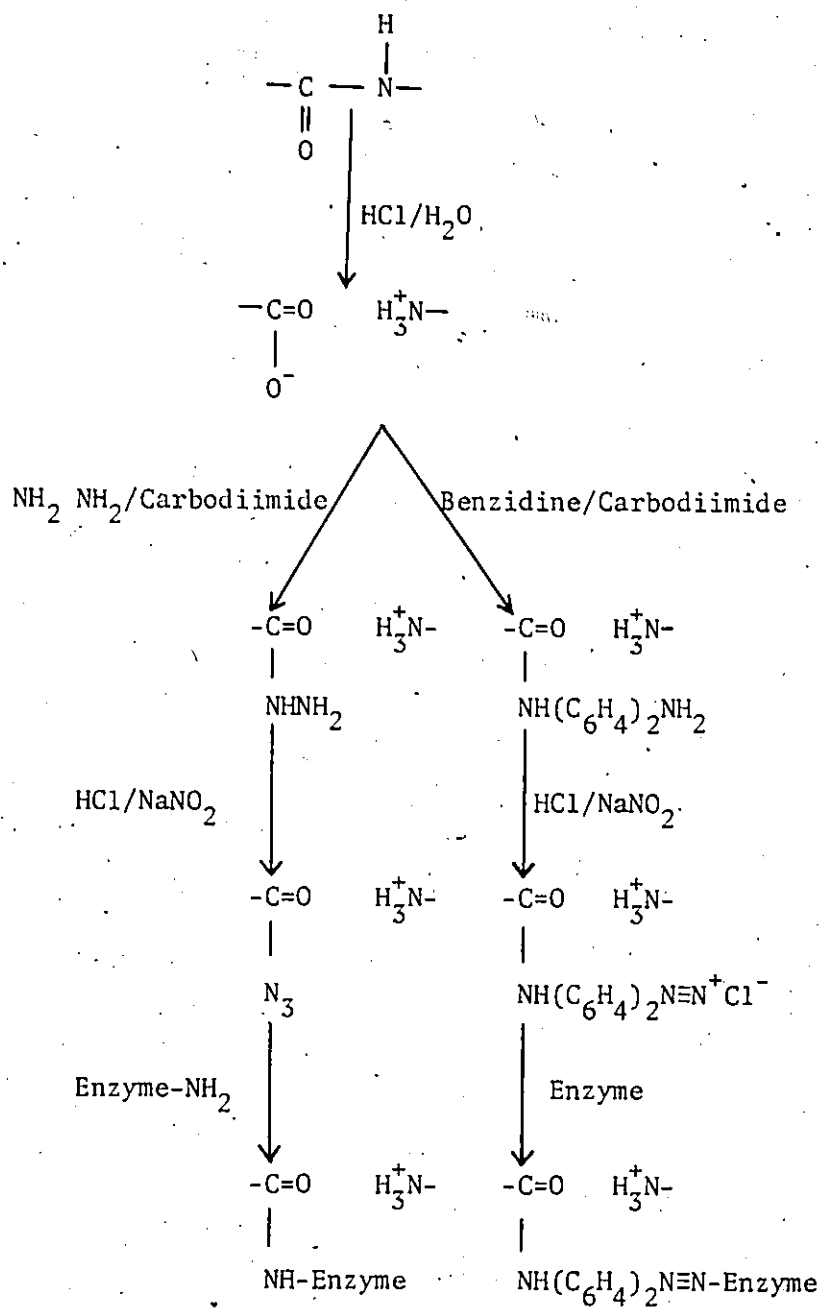
Covalent binding of enzymes to nylon by methods involving the first approach is essentially carried out by partial cleavage of the

nylon followed by activation of either of the released groups, and then the reaction of the enzyme with the activated nylon. The treatment adopted for affecting the release of the component groups of the peptide bonds must be sufficiently mild that neither the morphology nor the strength of the support is impaired. This is usually done by acidic hydrolysis, or non-hydrolytically under non-aqueous conditions with an amine such as N,N-dimethylpropylamine. Acid hydrolysis releases free amino groups together with a complement of free carboxyl groups, whereas treatment with an amine yields free primary amino groups and a complement of tertiary amino groups derived from the aminated carboxyl component of the peptide bond.

The carboxyl group derived from acidic hydrolysis can be activated by a variety of methods. For example, Hornby and Filippusson (105) coupled the free carboxyl groups to hydrazine or benzidine using carbodiimide as a condensation reagent, and then activated them to the corresponding acylazide derivative or the aryldiazonium salts, respectively, for reaction with the enzyme. The former allows chemical attachment of the enzyme directly with the carboxyl group of nylon, while the latter involves coupling of the enzyme through the amino group of benzidine as shown in Figure 3.

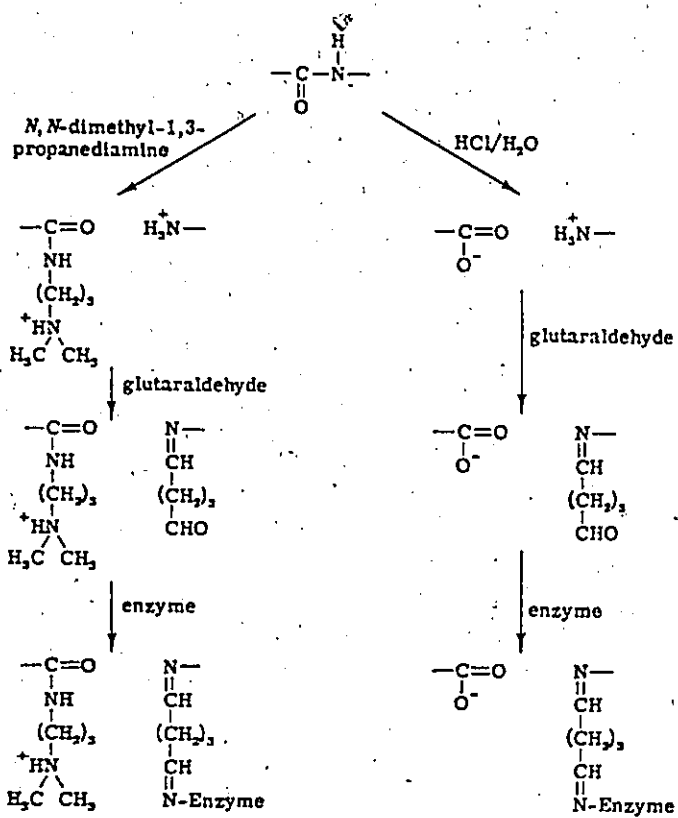
Alternatively, the chemical attachment of enzymes to nylon surfaces can be accomplished through the primary amino group released either in the acidic hydrolysis or in the non-hydrolytic cleavage. The activation of the amino group has been reported by different workers (8,106,107)

Figure 3. Covalent binding of enzymes to nylon by methods involving partial hydrolytic cleavage of peptide bonds by acids and coupling through the liberated carboxyl groups.



using the bifunctional reagent glutaraldehyde. In principle, the support is treated with the reagent under conditions which favour reaction of only one of its functional groups, while the other is used to attach the enzyme, as shown in Figure 4. The nature of the glutaraldehyde reaction has been discussed by several workers, notably by Quiocho (108) and Goldstein and Manecke (71), but the exact mechanism of this reaction and also the nature of the linkage is uncertain (109-111). Hornby and Morris (95) mentioned the possibility that glutaraldehyde reaction may modify lysine side chains of the enzyme without conserving the positive charge on these moieties, thus affecting the activity. These workers also suggested that some of the uncertainties associated with the use of glutaraldehyde can be obviated by using an alternative bifunctional agent such as bis-imido esters like ethyl adipimide. There are other methods for the activation and subsequent attachment of an enzyme through the amino group. For example, Horvath and Solomon (112) coupled trypsin to an isocyanate derivative of nylon prepared by reaction of the amino group with phosgene, and also uricase through the trifunctional reagent cyanuric chloride. It is to be noted that the coupling of an enzyme through the amino group of hydrolytically cleaved nylons leaves a residual negative charge due to the free carboxyl groups on the surface; this may have an adverse effect on the stability of some enzymes (23). On the other hand, the non-hydrolytic cleavage blocks the carboxyl group by amidating it, but this creates a net positive charge in the form of the protonated complement of tertiary amino groups.

Figure 4. Covalent binding of enzymes to nylon by methods involving peptide bond cleavage and coupling through the liberated primary amino groups (104).



Methods based on the controlled cleavage of peptide bonds are of limited usefulness since the mechanical strength of the support might be impaired by such treatments. In order to avoid this problem, techniques have been developed for the grafting of sidechains, by O-alkylation or N-alkylation, without causing any depolymerization of the support. It has long been known that secondary amides of general formula $R-CO-NH-R'$ can be alkylated by powerful alkylating reagents to yield the corresponding imidate salts (113). These are versatile intermediates which can afford several routes to enzyme immobilization either directly via the salt or after the introduction of a bifunctional spacer molecule, usually a nucleophile such as an amine or an acid hydrazide. The latter can react with the imidate salts or their free bases (imido esters) to yield the corresponding amidines and amidrazones. It has been found that the immobilization of an enzyme via spacer molecules yields a more active derivative (114). This method is preferable also because of the fact that the enzyme is attached at a point away from the surface, which reduces the possibility of steric hindrance between the enzyme and the substrate (93).

Campbell et al (114) used dimethyl sulfate for the O-alkylation of some of the peptide bonds inside a nylon tubing, and then allowed the imidate salt of the nylon to react with lysine or a polyethyleneimine such as hexamethylenediamine to which the enzyme glucose oxidase was attached by using a cross-linking agent such as diethyladipimide or glutaraldehyde. Morris et al (101) preferred triethyloxonium salts like tetrafluoroborate as the O-alkylating reagent, since these can be used

under much milder conditions giving a better yield of the immobilized enzyme derivative; also, it is much less toxic than the volatile dimethyl sulfate. Again, the enzyme can be coupled directly with the imidate salt through its free amino groups, or after the reaction of the salt with a bis-amine such as 1,6-diaminohexane and with a bis-acid hydrazide such as adipic acid dihydrazide under non-aqueous conditions. All these possibilities are summarized in Figure 5. The amine-substituted nylon can be activated for enzyme coupling by one of the conventional procedures using a bifunctional reagent (either glutaraldehyde or diethyl adipimidate), while the hydrazide-substituted nylon can additionally be utilized by converting it to an acid-azide through reaction with HNO_2 (see Figure 3) and then coupled to the enzyme. The particular advantage of the methods involving O-alkylation is that nowhere in the entire process is there any cleavage of the secondary amide bonds of the nylon structure so that the mechanical strength or the structural integrity of the support is preserved. However, the method leaves a residue of positive charge on the surface of the support in the form of the protonated amidine groups. This may elicit non-specific binding of anions present in the reaction mixtures, particularly in the case of amino-substituted derivatives, as noted by Hornby and Goldstein (104), or else can be more favourable as an environment for bound enzymes since the polarity, and therefore hydrophilicity, of the support is increased, as noted by Hornby and Morris (93).

The third approach to the generation of potentially reactive centres on the nylon surface, capable of interacting covalently with the enzyme, is a combination of the two approaches already described. It

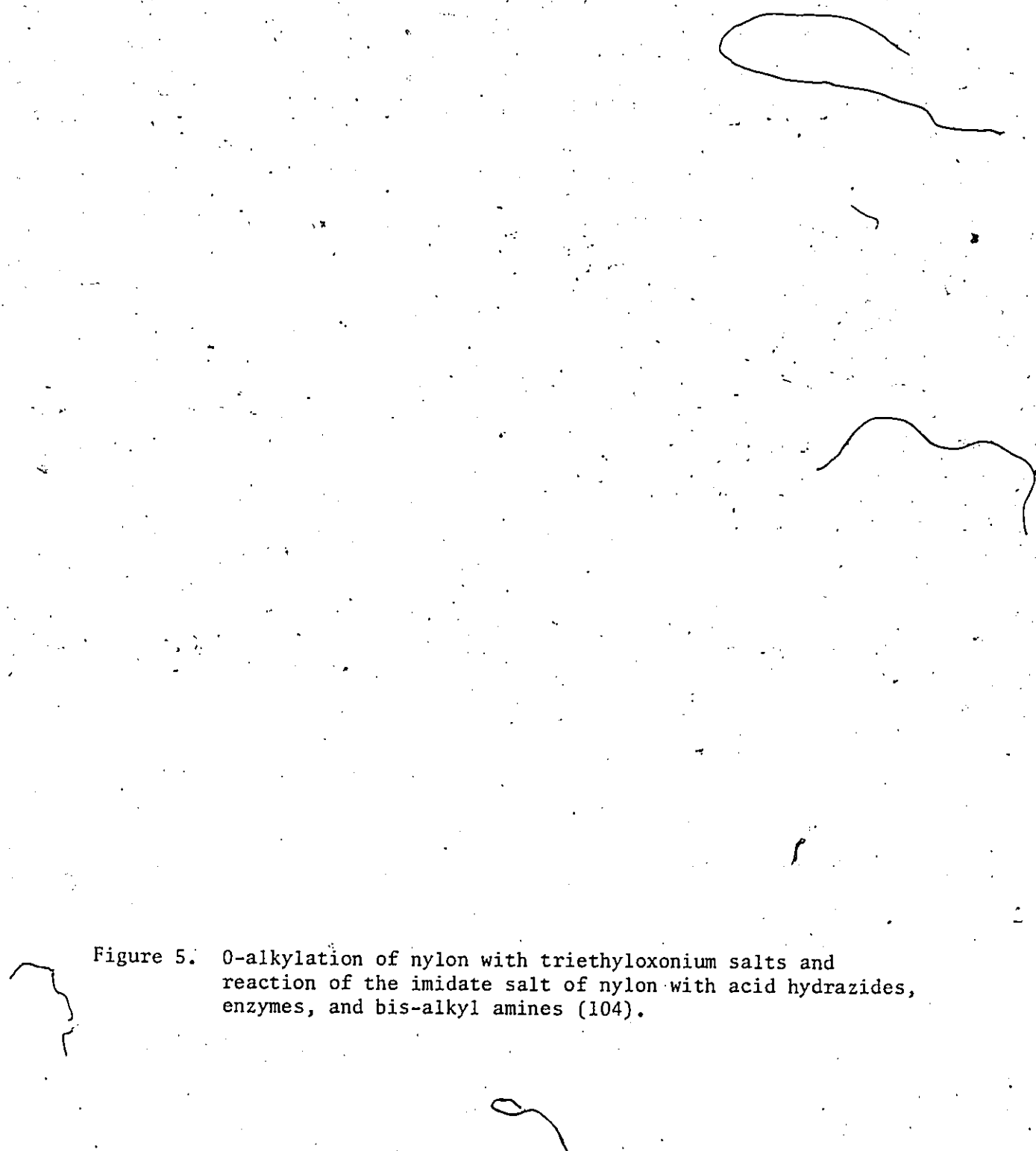
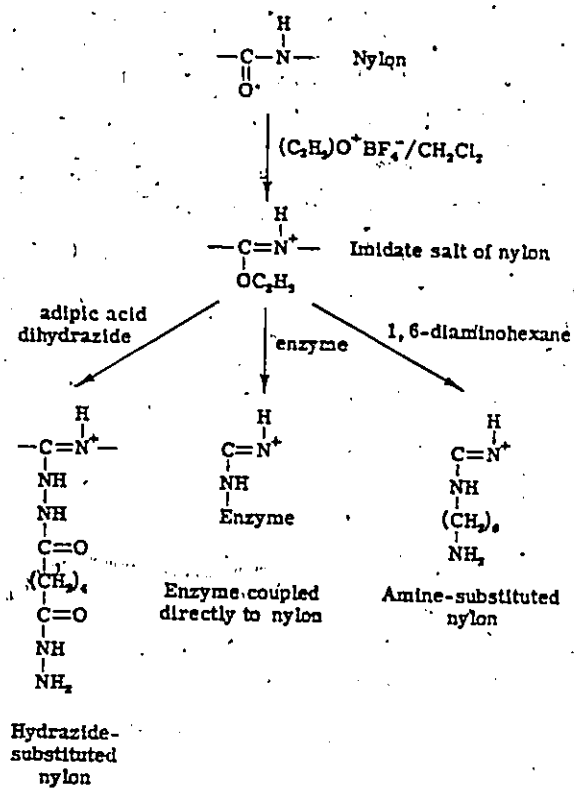
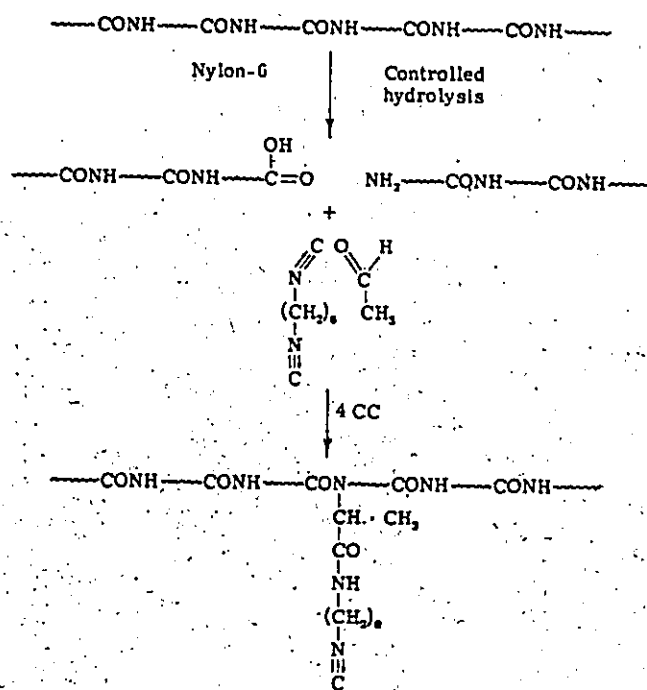


Figure 5. O-alkylation of nylon with triethyloxonium salts and reaction of the imidate salt of nylon with acid hydrazides, enzymes, and bis-alkyl amines (104).



involves initially partial hydrolysis of the nylon followed by grafting of reactive side chains via N-alkylation of backbone peptide bonds. The cleaved peptide bonds are subsequently reconstituted in a four-component condensation (4CC) that involves reaction of the liberated amino and carboxyl groups with an aldehyde and an isocyanide. In this process, the carboxyl and the amino components combine to form an N-substituted amide which carries the aldehyde and the isocyanide components as the side chain on the amide nitrogen. Goldstein et al (115,116) described a procedure in which acetaldehyde or isobutyral was used as the aldehyde component and the bifunctional 1,6-diisocyanohexane as the isocyanide component, thus leading to the production of polyisocyanide-nylon as shown in Figure 6. The isocyanide functional group of the nylon-derivative is then used to couple the enzyme by another 4CC reaction in which the enzyme provides either the amino or the carboxyl group (second component) in the presence of a water-soluble aldehyde such as acetaldehyde (3rd component) in aqueous buffers at neutral pH. The fourth component added in excess to the reaction medium can be an acetate if the enzyme utilizes one of its amino groups or an amine such as Tris if one of the carboxyl groups of the enzyme is used for its immobilization. It is also possible to modify the isocyanide group of the nylon derivative into other functional groups such as the diazotizable arylamino derivative, via similar 4CC reactions (115), to which the enzyme can be coupled through azo bonds and mainly with aromatic amino acid residues of the enzyme. This is particularly valuable in cases where coupling of enzymes to nylon via 4CC reactions is undesirable owing to sensitivity of the enzyme to aldehydes. On the

Figure 6. Synthesis of polyisonitrile-nylon via four-component condensation reaction (104).



Polyisocitrile-nylon

whole, the N-alkylation approach is considered to be versatile since it allows flexibility in the choice of the various components of the 4CC reactions so that different functional groups can be introduced on the N-alkyl side chains suitable for binding with specific groups of the enzyme. Besides, the method does not impair the mechanical strength of the polymer since the initially broken secondary amide linkages are ultimately resealed, and above all, it leaves no residual charge on the polyamide backbone or anywhere in the side chain.

Previous Work on the Immobilization of Alcohol Dehydrogenases

There are several reports in the literature about the immobilization of alcohol dehydrogenases, the earliest one being due to Hornby, Inman and McDonald (23). These workers immobilized several dehydrogenases, including the yeast alcohol dehydrogenase, inside nylon tubing and demonstrated their usefulness in the automated determination of substrates. Their method involved non-hydrolytic cleavage of the peptide bonds in nylon with N,N'-dimethylaminopropylamine in non-aqueous conditions, since it was considered that the hydrolytic cleavage produces free carboxyl groups which are unsuitable for the immobilization of dehydrogenases. The enzyme was attached covalently, using glutaraldehyde, through the primary amino group released during the cleavage of the peptide bond, while the carboxyl group was amidated, thus leaving the cationic dimethylamino group on the surface of nylon. The immobilized enzyme derivative was used over a period of twenty days "without incurring any loss in their activity" but no details of the latter were provided.

Johansson and Mosbach (24) covalently coupled horse liver alcohol dehydrogenase to cross-linked copolymers of acrylamide and methylacrylate, using the glutaraldehyde method. These workers studied the effect of a hydrophobic microenvironment on the enzyme reactions by varying the composition of the copolymer matrix in which acrylamide and methylacrylate served as the hydrophilic and hydrophobic components, respectively. It was found that increasing the hydrophobicity of the matrix caused a four-fold decrease of the apparent Michaelis constant for the more hydrophobic substrate n-butanol, whereas that for ethanol was practically unaffected. The assumption that the more hydrophobic matrix will preferentially adsorb the more hydrophobic substrate was substantiated by equilibrium studies with n-[^{14}C]-butanol; the more hydrophobic acrylamide-methylacrylate copolymer binding was about six times greater. The immobilized enzyme preparation was found to lose only 10% of its activity after storage for a period of three weeks at 4°C.

Gestrelus, Månsson and Mosbach (25) coimmobilized horse LADH and an NADH analogue, N^6 -[(6-aminohexyl)carbamoylmethyl]-NADH, to Sepharose 4B under conditions permitting binary complex formation between the enzyme and the cofactor derivative. The enzyme and the NADH-analogue was mixed prior to coupling to permit formation of the binary enzyme-cofactor complex during an equilibration time. The coupling of the enzyme and the cofactor-analogue to the same matrix was performed according to the cyanogen bromide method (117). It was claimed that both the enzyme and the NADH-analogue was covalently bonded in such a manner that the coenzyme is fixed in or near the active site of the enzyme. The efficiency

of the immobilized preparation was increased several times which was ascribed to the existence of the immobilized complex of the interacting enzyme and the coenzyme so that only minimal or zero amounts of the coenzyme were required to be added. Both thermal and storage stability of the enzyme was somewhat increased allowing a very high recycling rate, and this was considered to be due to the protection of the active site by coimmobilized cofactor molecules. It was confirmed that the formation of the enzyme-cofactor complex took place in the solution prior to coupling, and that after immobilization a number of these binary complexes were bound to the matrix, allowing them to interact more efficiently. The cofactor was immobilized in the reduced form because of its lower dissociation constant than for NAD (118), thus favouring the binary complex formation, while the analogue was chosen to provide a 'spater' as well as a functional group suitable for binding to the matrix. However, the regeneration of the cofactor was accomplished by the alcohol dehydrogenase activity per se, using the coupled oxidoreduction between the two alternative substrates, ethanol and lactaldehyde (119).

Kelly, Flynn and Johnson (26) reported a preliminary investigation on the immobilization of YADH on porous glass surfaces activated with 3-aminopropyltriethoxysilane (3-APTES) and then further treated separately with glutaraldehyde, benzaldehyde, stearoyl chloride, and p-nitrobenzoyl chloride followed by dithionite reduction and diazotization. The supports thus prepared were stirred gently with the enzyme in tetrasodium pyrophosphate buffer (0.03 M, pH 8.8) containing 0.2 mM cystein at 4°C for 2 hours. The benzaldehyde and the stearoyl chloride derivatives were

designed to bind the enzyme by hydrophobic bonding, while the other two derivatives were used to provide covalently bonded enzyme. Each of the methods provided active samples of immobilized YADH, the most promising being the p-nitrobenzoyl derivative, but neither the cause nor the extent of the stability was determined or evaluated. In continuing this work, Brougham and Johnson (27) studied the effect of the factors on the stability of the bound enzyme, including the support and the binding method used. The enzyme was immobilized on Enzyacryl-TiO (porous particles of TiO_2 , coated with polymerized and diazotized m-diaminobenzene), 1R-45 (standard amberlite resin) and on porous glass activated with 3-APTES followed by glutaraldehyde or azo-linkage. The stability of the immobilized enzyme preparations was found to depend on the support or the linkage method in the order Enzyacryl-TiO=porous glass (glutaraldehyde linkage)>1R-45>porous glass (azo-linkage). The stability was greater in the pH range of 6.5 to 8.0 than at 8.5 or 9.0. It was also observed that the stability was increased by increasing the quantity of the enzyme bound or by coimmobilizing albumin with the enzyme, but not by increasing the ionic strength of the storage solutions in contrast to that with the soluble enzyme.

Finally, Barry, Griffin and Johnson (28) described the immobilization of YADH on sepharose derivatives, such as the acetyl and phenylglycyl derivatives of agarose, by non-specific adsorption followed by cross-linking with glutaraldehyde. The enzyme was strongly adsorbed on the two non-specific gel derivatives which had acetylminohexyl and phenylglycylminohexyl groups as their respective ligands, the binding of the enzyme being stronger with the latter than that with the other.

Glutaraldehyde cross-linkage was required to eliminate enzyme desorption which occurred in the presence of NAD. Despite the cross-linkage, however, the immobilized enzyme was less stable than the soluble enzyme at 20°C, but both were more stable in the presence of 2-mercaptoethanol of concentration up to 20 mM. Coimmobilization of albumin with the enzyme improved the stability and ionic strength had no effect.

The Present Method and the Materials Used

Kinetic studies of an immobilized enzyme derivative as well as its practical application demand that the preparation should be stable for sufficient length of time. The method of Hornby et al (23) for the covalent attachment of dehydrogenases inside nylon tubing was particularly tried for the present purpose with YADH, but was not very successful in that the immobilized enzyme lost its activity rapidly. It was difficult to rationalize the failure since Hornby et al did not provide any activity vs time data nor did they justify the validity of their assay procedure to establish the stability. These led to the consideration that the generation of the cationic dimethylamino group by the non-hydrolytic cleavage of the nylon, which is left even after the covalent attachment of the enzyme with the primary amino group using glutaraldehyde, might be responsible for the loss of activity. Consequently, a method has been developed which leaves little or no charge on the surface of the nylon tube. It consists of partial acidic hydrolysis and cleavage of secondary amide bonds, followed by coupling of the carboxyl groups with benzidine in presence of dicyclohexylcarbodiimide and of amino groups with glutar-

aldehyde. The enzyme is then attached chemically to the surface through its polar groups. This is essentially a modification of the methods used by Hornby and coworkers (8,23,100,105) and by Allison et al (106), and of those used previously in this laboratory (13,107,120,121), and is similar to that described recently by Daka and Laidler (122) for lactate dehydrogenase inside a nylon tube.

The nylon tubing (0.206 cm internal diameter) was obtained from the Canus Plastics Company, Ottawa. The enzyme yeast alcohol dehydrogenase (EC 1.1.1.1) having 233 U/mg protein (lot 107C-8500), and the coenzyme NAD also from yeast (lot 106C-7140) type III were obtained from Sigma Chemical Company, USA. Benzidine used in the coupling processes was also available from Sigma Chemical Company, while dicyclohexylcarbodiimide and glutaraldehyde were purchased from Matheson Coleman and Bell Manufacturing Chemists, Ohio, and Eastman Kodak Company, Rochester, NY, respectively. Coupling buffers containing EDTA and β -mercaptoethanol were prepared by using sodium phosphates obtained from J.T. Baker Chemical Co., Phillipsburg, NJ. Analytical grade sodium borate and boric acid, used for another buffer, were obtained from Anachemia Chemicals Ltd. and British Drug Houses Ltd., respectively. The substrate ethanol was a product of Consolidated Alcohols, Toronto, and was more than 99.9% pure. Disodium salt of β -NADH from yeast (lot 117C-74801) grade III and acetaldehyde required for other studies were procured from Sigma Chemical Company, and Matheson Coleman and Bell Manufacturing Chemists, respectively.

Covalent Attachment Procedure for YADH Inside a Nylon Tube

The following procedure is essentially the same as that described by Mazid and Laidler (123).

A 1-metre portion of the tubing was used as a tightly wound coil. The tube was first filled with a mixture of 18% (w/v) calcium chloride in a solution of 18% (v/v) water in methanol, and was incubated for 30 min at 40°C. The available surface area was increased as a result of the removal of amorphous nylon by rinsing with water at a flow rate of $5 \text{ cm}^3 \text{ min}^{-1}$ for about 20 min. Partial hydrolysis of the inside of the tube was then accomplished at 40°C by pumping ~4 M HCl for 20 min at a flow rate of $5 \text{ cm}^3 \text{ min}^{-1}$. The hydrolysis was stopped by washing the tube for 30 min with ice-cold distilled water at $5 \text{ cm}^3 \text{ min}^{-1}$.

A mixture of 1% (w/v) benzidine with 1% (w/v) dicyclohexylcarbodiimide in methylene chloride was used at $1 \text{ cm}^3 \text{ min}^{-1}$ for 4 hours at 10°C to couple with the carboxyl groups liberated in the previous step. Free benzidine was then washed away with 50 cm^3 of methylene chloride, 50 cm^3 of acetone, and 50 cm^3 of ice-cold distilled water, successively, at a flow rate of $1 \text{ cm}^3 \text{ min}^{-1}$. The free amino groups were then blocked by reaction with a solution of 12.5% (v/v) glutaraldehyde in 0.2 M borate buffer (pH 8.5) perfused at $1 \text{ cm}^3 \text{ min}^{-1}$ for 1 hour and at 0-4°C. The excess of the bifunctional reagent was removed afterwards by washing at $5 \text{ cm}^3 \text{ min}^{-1}$ with 0.1 M phosphate buffer (pH 7.5) containing 1 mM EDTA and 1 mM β -mercaptoethanol.

The same phosphate buffer was used for making the enzyme solution (1 mg cm^{-3}), which was allowed to circulate through the tube at $0.5 \text{ cm}^3 \text{ min}^{-1}$

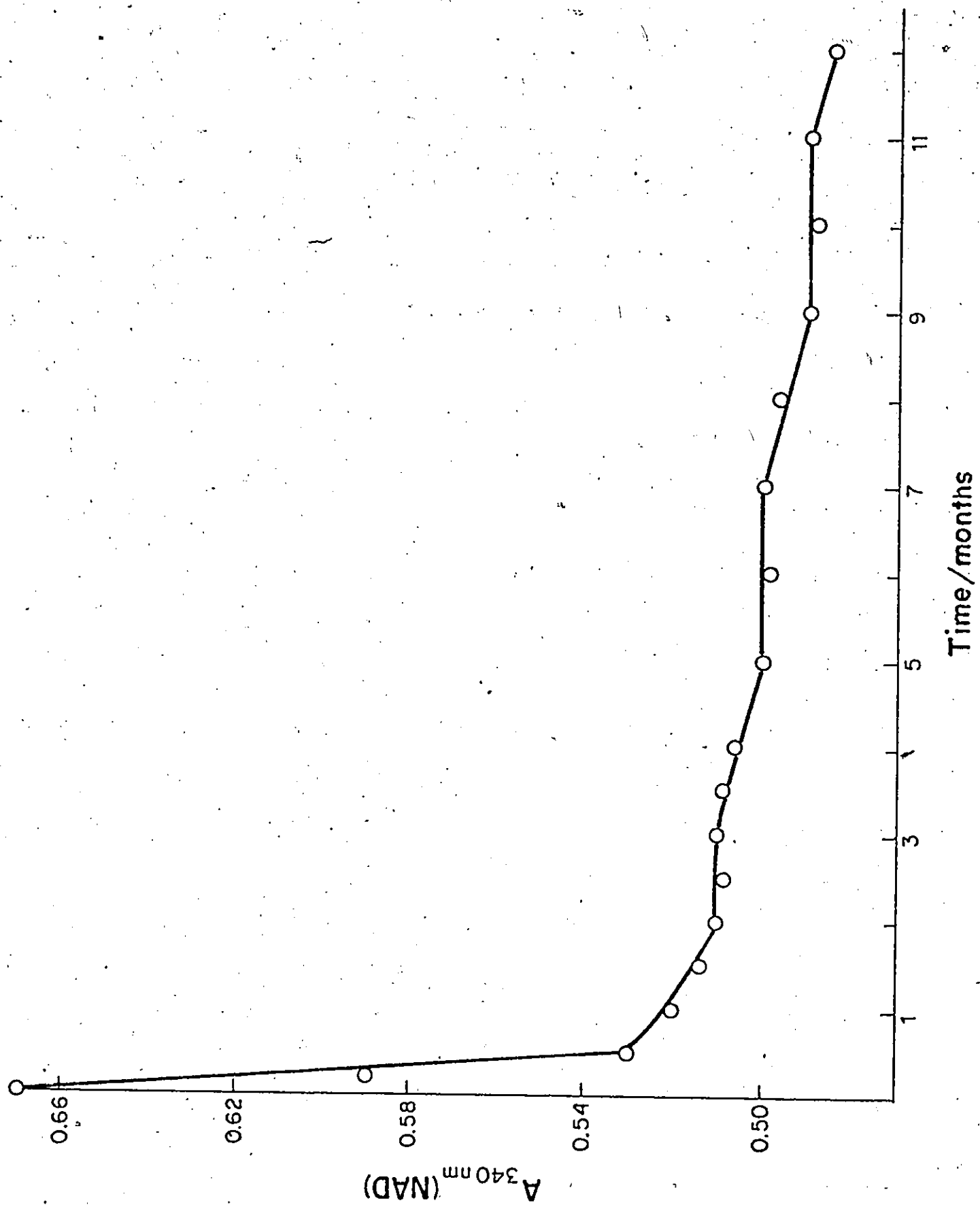
overnight to ensure complete reaction, the temperature being maintained within 0-5°C by the use of crushed ice. Immediately after the circulation, the absorbance at 280 nm due to enzyme protein was measured; a difference of 0.54 was noted and the initial absorbance showed that a considerable amount of the enzyme was bound to the tube. Any non-covalently attached enzyme was removed by washing the tube with 1 dm³ of 0.5 M NaCl solution in 0.1 M phosphate buffer (pH 7.0) at 5 cm³ min⁻¹. The tube was then filled with the assay buffer (pH 7.5) and stored below 5°C.

Stability of the Nylon-tube-supported YADH

The stability of the enzyme chemically attached to the nylon tube was checked by periodic rate measurements (to be described in Chapter 3) at a linear flow rate of 0.90 cm s⁻¹, temperature 25.5°C and pH 7.5, with [NAD]=1.0 mM and [C₂H₅OH]=100 mM. It was found that the rate decreased by about 20% during the first two weeks after the attachment of the enzyme, presumably because of the detachment from the surface of loosely-bound enzyme. After that period, the activity remained constant within ±4% over a period of about one year. The observed activity measured in terms of the absorbance at 340 nm of NADH against time in months are shown in Figure 7.

In discussing the activity vs time data such as those shown in Figure 7 and to justify the stability of an immobilized enzyme derivative, the validity of the assay procedure must be ascertained. It has been suggested by Ollis (124) that to compare validly the stability of an immobilized enzyme with its soluble counterpart steps should be taken to

Figure 7. The activity of the nylon-tube-supported YADH, measured in terms of the absorbance at 340 nm of NADH, plotted against time in months.



avoid diffusional limitations on mass transfer in the solid phase system. These included avoiding high assay temperature, high reaction rates and low ionic strength, as noted by Barry, Griffin and Johnson (28). Kelly et al (26) have drawn particular attention to the possibility that thermal stability studies on immobilized enzymes may give distorted or spurious results if an assay system is used in which excessive conversion of the substrate is achieved. In the present studies, however, neither the temperature nor the conversion was high enough to cast any doubt on the validity of the assay procedure. Besides, the buffer used was of sufficiently high ionic strength (0.1M phosphate buffer, pH 7.5) and, since Barry et al (28) showed that the activity of immobilized YADH is unaffected by ionic strength, this factor is of no consequence. Nevertheless, the question of diffusional limitations still remains since this is not reflected in the activity vs time curves and may conceal the true stability. This is because, in the limit of diffusion control, the observed activity of a bound enzyme may remain constant despite the fact that the enzyme may have undergone considerable denaturation. Hence, activity vs time or temperature data must be considered very carefully when used to deduce inherent stability parameters of an immobilized enzyme (90). Besides, the possible effect of diffusional phenomenon must be considered before drawing conclusions about the effect of immobilization on the stability of the enzyme (89).

Engasser and Coulet (125) suggested a simple graphical procedure for the removal of diffusional effects, based on the determination of substrate concentration differences between the micro and macroenvironment

of the enzyme. However, as discussed in the next chapter, our assay conditions are such that diffusional limitations were avoided, and therefore the conclusion about the high stability cannot be invalidated. We believe that the exceptional stability of our preparation is due to the production of a fairly neutral surface such as that discussed by Daka and Laidler (122) in the case of lactate dehydrogenase. It is also possible that the enzyme is immobilized considerably via the amino group of benzidine so that it is kept at a distance from the surface to avoid any steric or other interactions.

There are other limitations arising, for example, from chemical modification or partial inactivation effects in the interpretation of results on stability as discussed recently by Klibanov (73). Chemical modification may be regarded as a change in the primary structure, and this is expected to result in significant changes in stability. Zaborsky (126) suggested that effects due to immobilization must be separated from those related to modification by comparing stabilities of immobilized and free modified (but not native) enzymes. It is possible that the enzymes are partially inactivated during immobilization and as a result of the loss of activity by labile forms. As a result, the rate of immobilized enzyme inactivation will be diminished in comparison with the free enzyme; in other words, stabilization of the enzyme upon immobilization will be observed. Therefore, the stability of the immobilized enzyme must be compared with that of the free counterpart in which the degree of inactivation of the latter corresponds to the loss of its activity during immobilization (73). It is, however, not

the purpose of the present studies to establish the cause of stabilization or to evaluate the effect of immobilization on the stability. All that is needed to obtain consistent kinetic data is to ensure that the kinetic stability does not change with time and that the activity remains practically constant.

CHAPTER 3

FLOW KINETICS OF YEAST ALCOHOL DEHYDROGENASE ATTACHED TO NYLON TUBING

Introduction

The use of enzymes attached to the interior walls of tubes has been mentioned in Chapter 1, and has been discussed in more detail in Chapter 2, with particular reference to the automated determination of substrates in continuous flow systems of analysis. The importance of alcohol dehydrogenase in human physiology, as well as its practical applications, have been emphasized in Chapter 1. Studies of the immobilized alcohol dehydrogenases have been summarized in Chapter 2, and so far the only kinetic studies reported with this enzyme are those of Mazid and Laidler (123).

A number of experimental investigations of flow systems have been carried out in this laboratory (13,107,120,121) on the basis of a theoretical treatment developed by Kobayashi and Laidler (70) for single-substrate systems. Engasser and Horvath (89) have also developed a theory of single-substrate systems, and Engasser et al (127) considered an extension to two-substrate systems with special reference to aspartate aminotransferase. They found that diffusional limitations are entirely responsible for the differences between the behaviour of free enzyme and enzyme bound to collagen. A complete theoretical treatment of two-substrate systems present considerable difficulty and has not yet been developed for immobilized enzymes.

Daka and Laidler (122) carried out experimental work on the flow kinetics of lactate dehydrogenase, chemically attached to nylon tubing. The substrates were pyruvate and NADH (the reduced form of nicotinamide adenine dinucleotide, NAD), and the experiments dealt with the two limiting cases: one substrate present in excess and the concentration of the other varied. These conditions permit interpretation on the basis of the Kobayashi-Laidler one-substrate treatment. This chapter describes a similar investigation with yeast alcohol dehydrogenase, the substrates being ethanol and NAD. In the present study, particular attention is focussed on the kinetic aspects, especially, of the role of diffusion as described by the Kobayashi-Laidler theory, therefore testing its validity for a two-substrate system.

Theoretical Considerations

A general equation for two-substrate systems which applies to several mechanisms is given by (128)

$$v = \frac{k_c [E][A][B]}{K_{mB}[A] + K_{mA}[B] + [A][B] + K_{mA}K_{mB}} \quad (1)$$

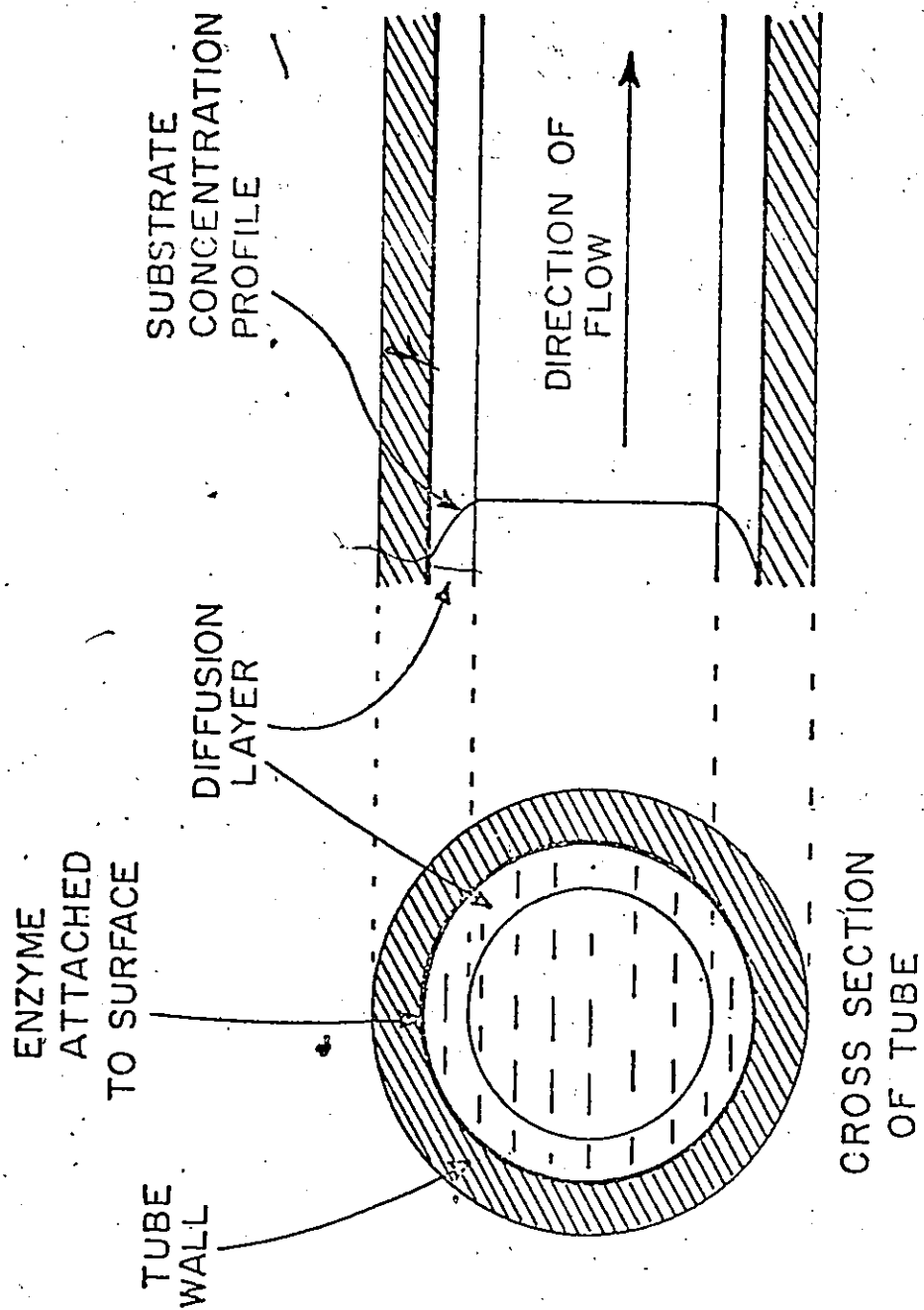
where in the present case A and B refer to the substrates ethanol and NAD, respectively. If one substrate such as A is in excess, the second and the last terms in the denominator may be neglected, and the equation reduces to the Michaelis-Menten form for a single substrate:

$$v = \frac{k_c [E][B]}{K_{mB} + [B]} \quad (2)$$

According to the theoretical treatment of Sundaram, Tweedale and Laidler (129), the rate of a single-substrate reaction catalyzed by an immobilized enzyme can be approximated by an equation of the Michaelis-Menten form (Eqn. 2) in which $[E]$ is the total concentration of the immobilized enzyme, the catalytic rate constant k_c is modified by conformational and environmental effects but not by partitional and diffusional effects, while the Michaelis constant is influenced by all these factors. The same is true for a two-substrate system if one of the substrates is in excess, and we can write the modified Michaelis constants as $K_{m,app}(A)$ and $K_{m,app}(B)$ corresponding to the two substrates.

The theory for the flow kinetics of a system in which the enzyme is attached to the inner surface of a tube has been presented by Kobayashi and Laidler (70). The mathematical treatment is quite complicated, and only the essential features of the theory as well as the main conclusions will be drawn here. It was assumed that the enzyme is present in a unimolecular layer at the surface so that internal diffusional effects within the support can be neglected, but external diffusion in the substrate solution flowing through the tube must be taken into account. This led to the suggestion that a diffusion boundary layer is established at the walls of the tube, and that the concentration of the substrate within the diffusion layer varies from its constant value in the central part of the tube, as shown schematically in Figure 8. The concentration gradient arises from the fact that the rate of disappearance of the substrate by chemical reaction at the surface is not necessarily the same as the rate of diffusion of the substrate through

Figure 8. Schematic diagram of a tube with enzyme attached to the inner surface, showing the diffusion layer and the concentration profile (91).



the boundary layer to the surface. The thickness of the diffusion layer depends on the magnitude of the mass transfer coefficient k_L which is in fact a measure of the rate at which the substrate diffuses through the layer. The theory also assumes that the flow of the substrate is laminar, and that the substrate solution is dilute so that its diffusion rate has a negligible effect on the velocity distribution. Then, taking into account the mass-balance both for the substrate and the product, and applying the appropriate boundary conditions, the theory leads to the following expression for the mass-transfer coefficient:

$$k_L = 1.29 \left[\frac{D^2 v_f}{rL} \right]^{1/3} \quad (3)$$

where $r(\text{cm})$ and $L(\text{cm})$ are the radius and length of the tube, $D(\text{cm}^2 \text{s}^{-1})$ is the diffusion coefficient for the substrate in solution, and $v_f(\text{cm s}^{-1})$ is the flow rate of the substrate through the tube.

Equation (3) shows that decreasing the flow rate of the substrate or its diffusion coefficient and increasing the diameter of the tube or its length decreases the mass-transfer coefficient, and therefore favours the diffusion control. The dependence of k_L on L arises due to the changing concentration patterns along the tube, whereas the dependence on v_f is due to the reduction of the effective thickness of the diffusion layer. It is apparent that increasing the flow rate increases the rate of substrate diffusion, i.e., the mass-transfer to and from the surface becomes large, and thus no longer controls the rate of the reaction. In other words, the substrate reaches the surface rapidly and the rate of product formation depends on the chemical interaction at

the surface. Diffusional effects are also unimportant at sufficiently high substrate concentrations, when the reaction involves the modified rate constant k_c but not the Michaelis constants (Eqn. 2), the latter being the one affected by diffusional limitations. Conversely, at low flow rates and low substrate concentrations, considerable diffusion control is expected so that the kinetic behaviour may deviate from that obtained with the enzyme in free solution.

The theory was developed for simple Michaelis-Menten kinetics as well as for substrate and product inhibition. In the simplest case, Eqn. (2) is obeyed to a good approximation but the apparent Michaelis constant is modified in that it increases with increasing diffusional control according to the equation

$$K_{m,app} = K'_m + \frac{V'_m}{2k_L} = K'_m + \frac{k'_c[E]}{2.58} \left[\frac{rL}{D^2} \right]^{1/3} v_f^{-1/3} \quad (4)$$

where V'_m is the maximal rate for the immobilized enzyme and K'_m is the inherent Michaelis constant in the absence of diffusion. $K_{m,app}$ thus approaches K'_m in the limit of very high flow rates when the second term is negligible, and the latter can therefore be determined from the plot of $K_{m,app}$ against $v_f^{-1/3}$.

There are other important results predicted by the theory, relating to the overall rate of reaction within the tube and the product concentration at the exit. Thus, under the limiting conditions of little diffusional control (e.g., at high $[S]$ and high v_f), the overall rate is equal to the product of the internal surface area $2\pi rL$ of the tube and the inherent rate v_r of the enzymic reaction per unit surface

area. The latter is given by an equation of the Michaelis-Menten form (Eqn. 2), and the rate v_o of product formation within the tube can be written as

$$v_o = 2\pi r L v_r = \frac{2\pi r L k'_c [E][S]}{K'_m + [S]} \quad (5)$$

The product concentration $[P]_e^o$ at the exit is this rate divided by the volume of solution $\pi r^2 v_f$ which emerges in unit time, and is therefore given by

$$[P]_e^o = \frac{2\pi r L v_r}{\pi r^2 v_f} = \frac{2L}{rv_f} v_r \quad (6)$$

At the other extreme, if the conditions are such that there is complete diffusional control (e.g., at low $[S]$ and low v_f), the overall rate v_D of reaction within the tube involves the product of the terms containing the internal surface area, mass-transfer coefficient and the substrate concentration, and the theory leads to

$$v_D = 2\pi r L \times 1.29 \left[\frac{D^2 v_f}{rL} \right]^{1/3} \times [S] = 8.06 (v_f D^2 r^2 L^2)^{1/3} [S] \quad (7)$$

The product concentration $[P]_e^D$ at the exit of the tube is now predicted by the expression

$$[P]_e^D = \frac{8.06 (v_f D^2 r^2 L^2)^{1/3} [S]}{\pi r^2 v_f} = 2.56 \left[\frac{DL}{r^2 v_f} \right]^{2/3} [S] \quad (8)$$

It can be seen that Eqns. (7) and (8) do not contain the concentration or the rate constant (activity) of the enzyme, in contrast to Eqns. (5) and (6), but Eqns. (7) and (8) do involve the diffusion

coefficient, and there is also a different dependence on flow rate. In the case of full diffusion control, this can be rationalized by the assumption that there is enough enzyme at the surface to convert all the substrate molecules that come in contact with it. Equations (5) to (8) are very useful because they permit the product concentration and rates to be calculated and compared with the experimental values. Alternatively, double-logarithmic plots can be made to obtain information about the extent of diffusion control. In particular, Eqns. (6) and (8) provide a convenient way in which the slope of the double-logarithmic plots of the product concentrations against the flow rates are predicted to be -1.0 and -0.67 corresponding to no or complete diffusion control, respectively.

A special procedure for determining the extent of diffusion control is the use of two dimensionless parameters, defined by the Kobayashi-Laidler theory as follows:

$$\phi = \frac{[P]_e}{[S]} \left[\frac{v_f r^2}{DL} \right]^{2/3} \quad (9)$$

and

$$\rho = \frac{K_{m,app}}{[S]} \quad (10)$$

Another parameter η called the utilization factor has also been defined as the ratio of the actual rate to that in the absence of diffusional effects. According to Eqns. (9) and (10), a double-logarithmic plot of ϕ against ρ can be divided into regions (an example is to be found in Figure 11) corresponding to different degrees of diffusion control. This can be determined for a particular experiment since all the quantities

in the equations are easily obtainable. A particular advantage of this type of plot is that it can be used even for a single experiment provided, of course, that $K_{m,app}$ is known, and therefore the conditions can be modified to alter the extent of diffusion control (91).

It is to be noted that the dimensionless parameter ρ (Eqn. 10) is changed if the simple Michaelis-Menten equation is not obeyed. Numerical and approximate solutions for this factor were given for different kinetic conditions including those for the inhibition by substrate and products. In the case of competitive inhibition by product, found in the present system, there is a factor of $1 + \frac{[I]}{K_i}$ where $[I]$ is the inhibitor concentration and K_i is the inhibition constant. The nature and the type of inhibition can be understood from the fact that the degree of inhibition decreases with increasing $[S]$ or is independent of $[S]$ for competitive and non-competitive inhibition, respectively. Alternatively, these can be ascertained from simple Lineweaver-Burk plots for different inhibitor concentrations and with variable substrate concentrations in which only the slope or the slope and intercept are both altered by the same factor depending on whether the product inhibition is competitive or non-competitive (128). The inhibition constant can be obtained from the secondary plot of the slope of Lineweaver-Burk plots against the inhibitor concentrations, where the ratio of the intercept to the slope of the secondary plot corresponds to the required inhibition constant.

Kinetic Procedure

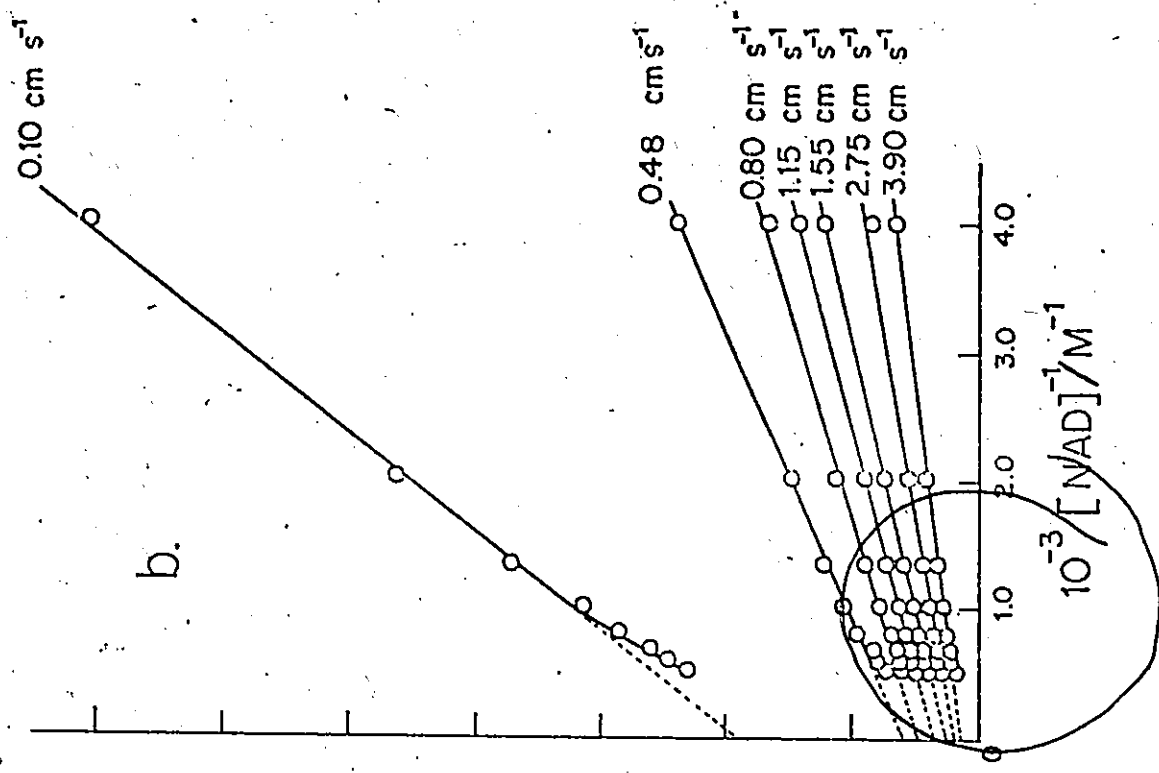
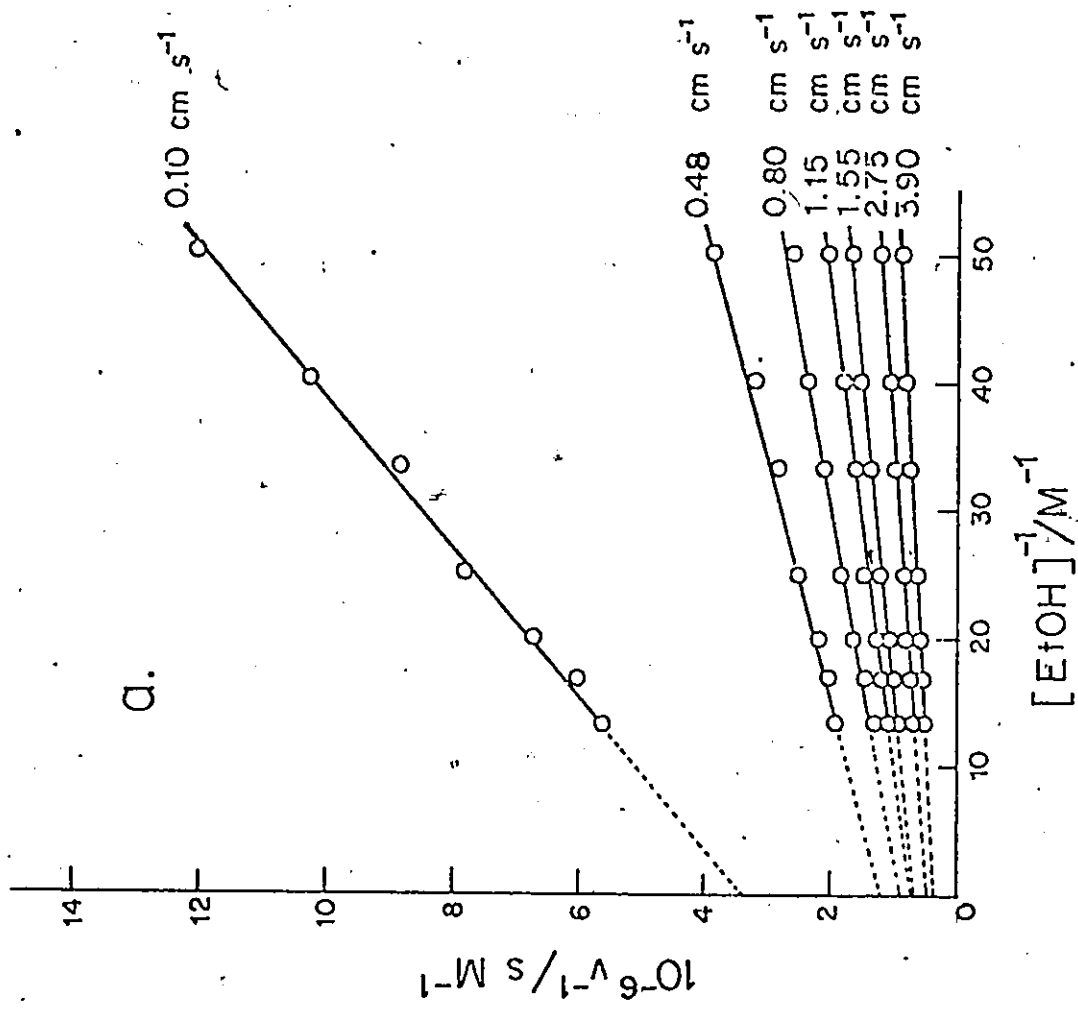
All solutions were made up in 0.1 M sodium phosphate buffer, at pH 7.50 ± 0.05 , with 1 mM EDTA and 1 mM β -mercaptoethanol. The kinetic runs were carried out at $25.5 \pm 0.1^\circ\text{C}$, and were followed spectrophotometrically by measuring the absorbance of NADH at 340 nm with a Pye Unicam SP 1800 UV Spectrophotometer. The enzyme tube was kept immersed in a temperature-controlled waterbath, one end being connected by means of tygon tubing to the thermostatted feed solution through a LKB Varioperpex II peristaltic pump. The product was delivered to a flow cell (1cm path length) in the spectrophotometer. The rates were calculated using $\epsilon_{340}^{1\text{cm}} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$ (130). A steady state was maintained throughout each kinetic run, and this was always confirmed by the fact that the amount of product remained practically constant at various time intervals for any run. For the present purpose, however, the data used were the average of several such measurements.

Results and Discussion

$K_{m,\text{app}}$ Values

The apparent Michaelis constants for NAD are substantially less than 1.0 mM, and a concentration of 2.0 mM was used to give complete saturation of the enzyme with respect to this substrate. Figure 9a shows Lineweaver-Burk plots at this NAD concentration, with ethanol concentrations varying from 20 to 75 mM, and with flow rates ranging from 0.10 to 3.90 cm s^{-1} . Another set of experiments were carried out with excess NAD at 5.0 mM in which the range of ethanol concentration was

Figure 9. Lineweaver-Burk plots (a) with excess NAD at 2.0 mM, and (b) with excess ethanol at 100 mM. Temperature 25.5°C, pH 7.5, and the flow rates are shown.



extended from 3.0 to 100 mM and the flow rate was varied from 0.30 to 3.30 cm s^{-1} . These showed non-linear Lineweaver-Burk plots at concentrations greater than 20 mM ethanol.

Some representative Lineweaver-Burk plots with ethanol in excess are shown in Figure 9b. The apparent Michaelis constants for ethanol are much larger, and a concentration of 100 mM was used for complete saturation. The results show significant deviations from linearity at high substrate concentrations and low flow rates, and this may be due to a change in the degree of diffusion control (90,91,99). Similar behaviour was also observed with excess ethanol at 500 mM, especially at higher NAD concentrations when this was varied from 0.10 to 2.0 mM, and with flow rates in the range of 0.30 to 3.30 cm s^{-1} .

The apparent Michaelis constants, $K_{m,\text{app}}$, for ethanol and NAD were obtained from the linear portions of the Lineweaver-Burk plots, and these are listed in Table I under different conditions. The values were found to decrease with increasing flow rate, and they varied linearly with $v_f^{-1/3}$, as shown typically in Figure 10 for excess NAD at 2.0 mM and variable ethanol. The linearity is consistent with Eqn. (4) and led to the extrapolated values of 4.6 mM and 4.2 mM for K'_m which relates to ethanol, corresponding to excess NAD concentrations of 2.0 mM and 5.0 mM, respectively. Similar plots for ethanol in excess at 100 mM and 500 mM gave values of 256 μM and 235 μM , respectively, for the K'_m relating to NAD.

A linear relationship between $K_{m,\text{app}}$ and $v_f^{-1/3}$, as predicted by the Kobayashi-Laidler theory, was also found with other systems (13,107,

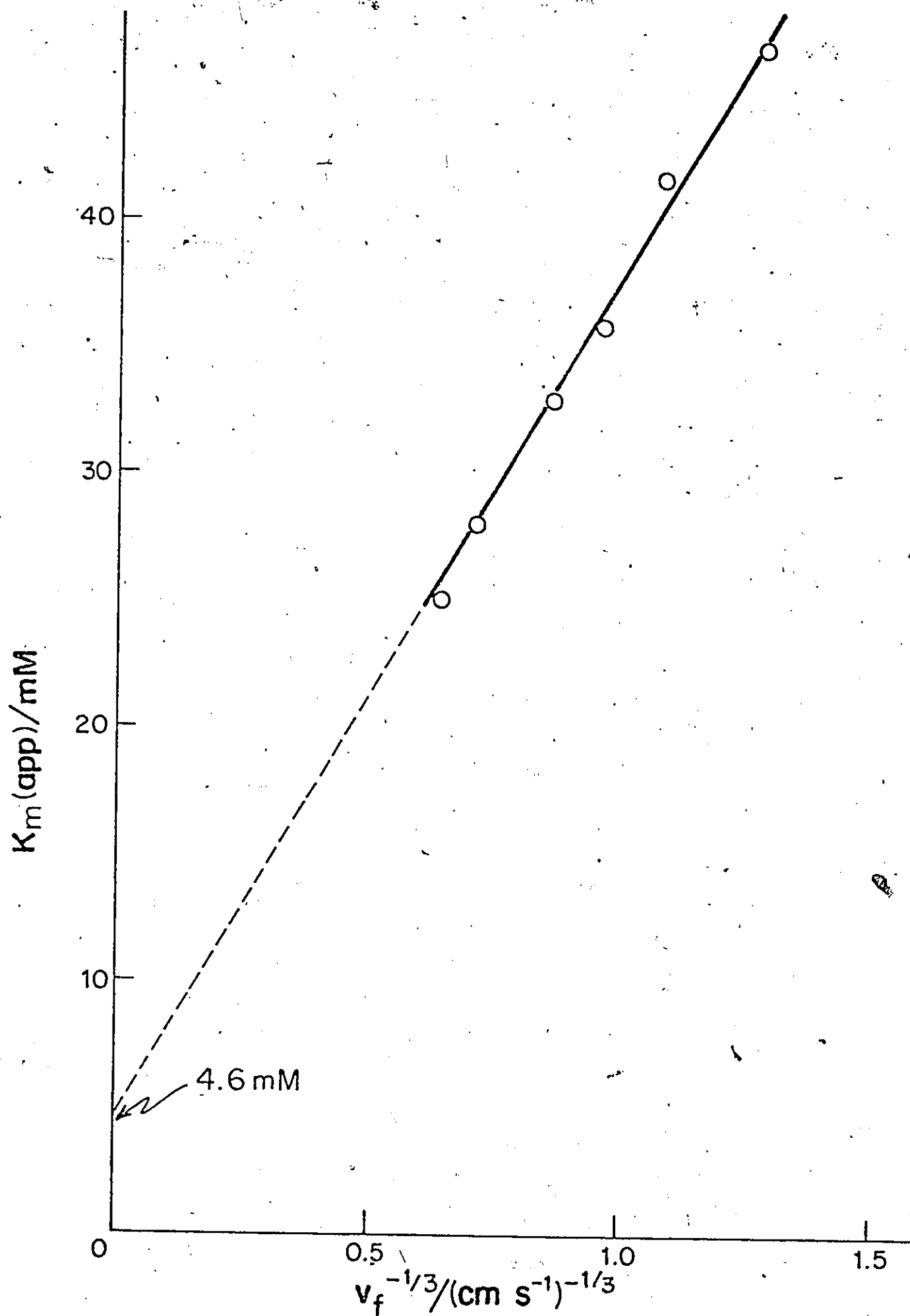
Table I. Michaelis parameters with YADH attached to nylon tubing, at pH 7.5 and temperature 25.5°C.

Flow Rate (cm s ⁻¹)	Excess [NAD]=2.0 mM		Excess [C ₂ H ₅ OH]=100 mM	
	10 ⁶ xV _{max,app} M s ⁻¹	K _{m,app} (C ₂ H ₅ OH) mM	10 ⁶ xV _{max,app} M s ⁻¹	K _{m,app} (NAD) μM
0.48	0.83	46.7	0.87	780
0.80	1.11	41.6	1.05	673
1.15	1.33	35.8	1.25	620
1.55	1.54	32.9	1.67	606
2.75	2.00	28.0	2.22	560
3.90	2.50	25.0	2.86	520
∞ (extrapolated)		4.6(K' _m)	—	256(K' _m)

Table I. (cont'd) Michaelis parameters with YADH attached to nylon tubing, at pH 7.5 and temperature 25.5°C.

Flow Rate (cm s ⁻¹)	Excess [NAD]=5.0 mM		Excess [C ₂ H ₅ OH]=500 mM	
	10 ⁶ xV _{max,app} M s ⁻¹	K _{m,app} (C ₂ H ₅ OH) mM	10 ⁶ xV _{max,app} M s ⁻¹	K _{m,app} (NAD) μM
0.30	0.46	10.4	0.95	810
0.50	0.56	9.5	1.18	747
1.00	0.71	8.6	1.33	613
2.00	0.91	7.5	1.67	550
3.30	1.05	7.1	1.82	500
∞ (extrapolated)		4.2(K' _m)		234(K' _m)

Figure 10. Plot of apparent Michaelis constants, $K_{m,app}$, for ethanol against $v_f^{-1/3}$, obtained from experiments with excess NAD at 2.0 mM and at different flow rates.



120,122), and in those cases the K'_m values were not far from the K_m with the enzyme in free solution. In the present case, the K'_m values of 4-5 mM for ethanol are comparable with the values found for the free enzyme and in static studies of the immobilized enzyme (see Table II). On the other hand, the K'_m (NAD) value of 234 μ M or 256 μ M is two orders of magnitude higher than that for free solution. It is possible that these values are somewhat in error since the extrapolation procedure is not very accurate, and the V'_m values used to obtain the apparent K_m results from the non-linear Lineweaver-Burk plots were also subject to error. Nevertheless, it is significant that both sets of experiments with different amounts of excess ethanol give approximately the same result. As will be discussed later, this is attributed to the substantial diffusion control found in this situation since ethanol cannot bind to the free enzyme but only to the enzyme-NAD complex.

However, one would expect similar values for K'_m and the intrinsic Michaelis constants in free solution if diffusional effects alone are responsible for the increased $K_{m,app}$ values found with the immobilized enzyme systems. This leads to the suggestion that other factors such as the conformational, environmental and partitional effects may be responsible for the difference between the K'_m values for NAD with those in the free solution, especially when diffusional effects have been separated. Conformational and environmental effects generally lead to relatively small changes in Michaelis constants, and these can be combined together since they are difficult to separate (99). It is also more likely that these effects are similar for both the substrates (ethanol and NAD), whereas partitional effects could affect the two substrates

Table II. Michaelis constants for ethanol and NAD with alcohol dehydrogenase in static systems.

Type of Enzyme	Matrix	pH	Temperature (°C)	$K_{m,app}$ (NAD) μ M	$K_{m,app}$ (C_2H_5OH) mM	Reference
Liver	Polyacrylamide	8.0	25.0	-	0.57	24
Liver	Copolymer of acrylamide and methyl acrylate	8.0	25.0	-	0.54	24
Liver	(free)	8.0	25.0	-	0.30	24
Yeast	(free)	8.2	room	-	14	49
Yeast	(free)	7.7	20.0	-	16	131
Liver	(free)	7.15	23.5	10	-	132
Liver	(free)	8.0	23.5	5	-	132

differently depending on the nature of the support. In terms of the hydrophilicity or hydrophobicity, the latter effects seem unreasonable since both the support and the substrates are of similar nature. Therefore, the most plausible explanation appears to be due to any electrostatic interaction between the positively charged NAD with similar charges, say, near the enzyme active site or on the support. This would give rise to a higher value of K_m^i compared to the intrinsic values in free solution. Electrostatic partitioning effects may be reduced or eliminated by carrying out the kinetic study at high ionic strength, but these investigations have not been pursued and more attention has been paid to the diffusional effects.

Dimensionless Parameters

A very convenient way to evaluate the extent of diffusional limitations is provided by the double-logarithmic plot of the dimensionless parameters ϕ and ρ defined by Eqns. (9) and (10), respectively, according to the Kobayashi-Laidler theory. Such a plot for the experiments with excess NAD at 5.0 mM and excess ethanol at 500 mM when the concentrations of ethanol and NAD were varied, respectively, is shown in Figure 11. In the calculation of ϕ , the diffusion coefficient was taken as $4 \times 10^{-6} \text{ cm}^2 \cdot \text{s}^{-1}$, which is appropriate for molecules of the kind we are dealing with (133). The plot has been divided into three theoretical regions, as explained in the caption. For constant ethanol and variable NAD, the points lie in Regions 2 and 3 corresponding to substantial diffusion control. On the other hand, with constant NAD and variable ethanol, all the results are well inside the diffusion-free region (Region 1). This arises from

Figure 11. Double-logarithmic plots of the dimensionless parameters Φ and ρ . The half-circles are typical points for constant NAD at 5.0 mM and variable ethanol (3.0-100 mM). Other symbols are due to the results with excess ethanol at 500 mM and variable NAD (0.10-2.0 mM) for the flow rates 0.30 (solid circles, ●), 0.50 (open circles, ○), 1.00 (triangles, Δ), 2.00 (crossed circles, ⊗), and 3.30 (squares, □) in cm s^{-1} . The theoretical regions are Region 1, little diffusional control (<5%); Region 2, moderate diffusional control (5-60%); Region 3, considerable diffusional control (>60%).

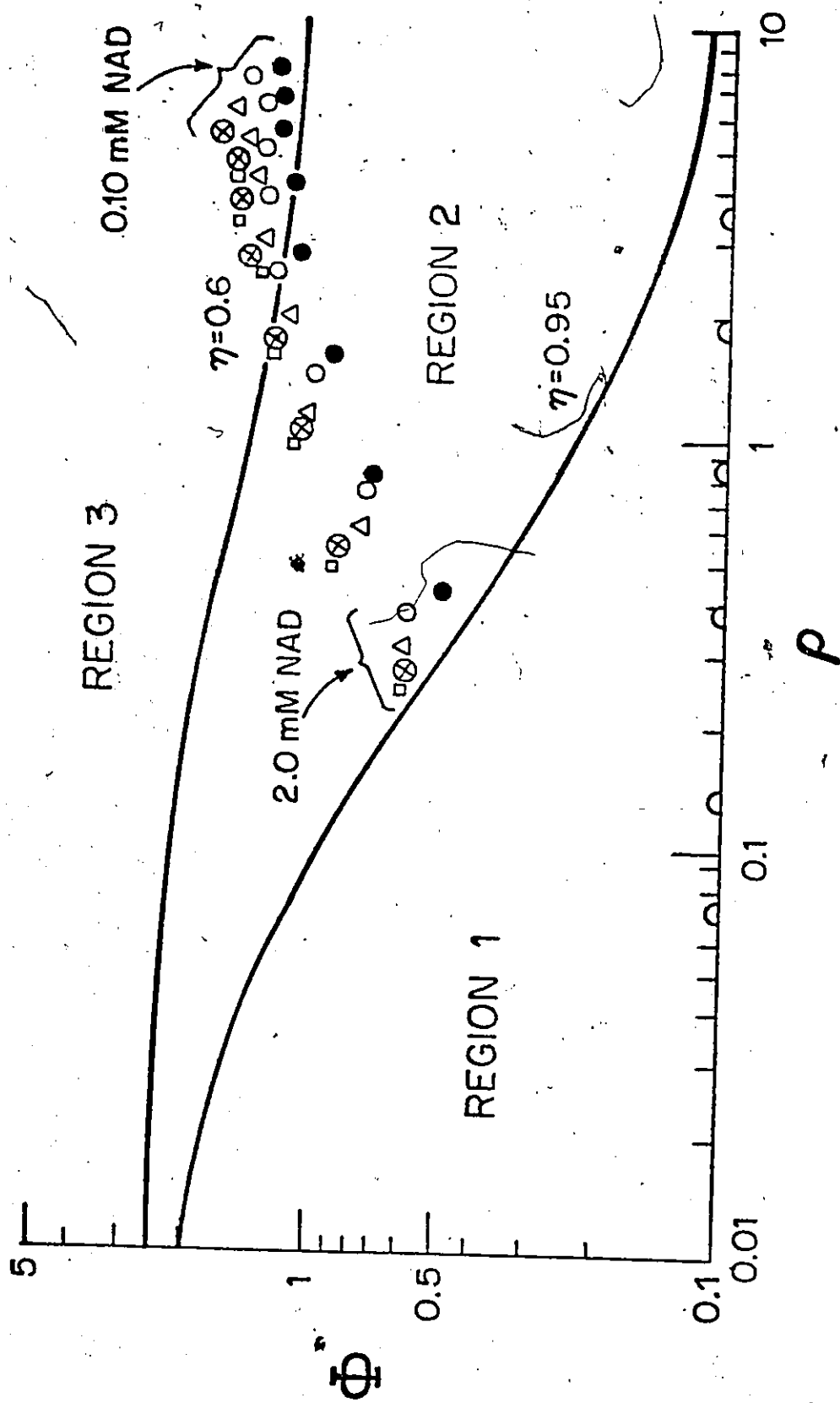


Table IIIB. Values of the dimensionless parameters ϕ and ρ with excess $[C_2H_5OH] = 100$ mM, at different NAD concentrations and flow rates. The numbers in brackets indicate the theoretical regions (see Figure 11).

[NAD] mM	Flow Rate, $v_f/\text{cm s}^{-1}$											
	0.48		0.80		1.15		1.55		2.75		3.90	
	ϕ	ρ	ϕ	ρ	ϕ	ρ	ϕ	ρ	ϕ	ρ	ϕ	ρ
0.25	0.95	3.12 (2)	1.15	2.69 (2)	1.17	2.48 (2)	1.23	2.42 (2)	1.48	2.24 (3)	1.66	2.08 (3)
0.50	0.75	1.56 (2)	0.83	1.35 (2)	0.92	1.24 (2)	0.98	1.21 (2)	1.13	1.12 (2)	1.26	1.04 (2)
0.75	0.61	1.04 (2)	0.69	0.90 (2)	0.74	0.83 (2)	0.77	-0.81 (2)	0.90	0.75 (2)	1.02	0.70 (2)
1.00	0.53	0.78 (2)	0.60	0.67 (2)	0.65	0.62 (2)	0.68	0.61 (2)	0.75	0.56 (2)	0.86	0.52 (2)
1.25	0.47	0.62 (2)	0.55	0.54 (2)	0.57	0.50 (2)	0.62	0.49 (2)	0.71	0.45 (2)	0.77	0.42 (2)
1.50	0.44	0.52 (2)	0.49	0.45 (2)	0.51	0.41 (2)	0.56	0.40 (2)	0.63	0.37 (2)	0.71	0.35 (2)
1.75	0.41	0.45 (2)	0.44	0.39 (2)	0.49	0.35 (2)	0.53	0.35 (2)	0.61	0.32 (2)	0.67	0.30 (2)
2.00	0.38	0.39 (1)	0.41	0.34 (1)	0.46	0.31 (1)	0.49	0.30 (1)	0.56	0.28 (2)	0.64	0.26 (2)

the very low ϕ values, which result from the fact that the product concentrations are very much smaller than the ethanol concentrations, the latter being large because of the large $K_{m,app}$ values. The results obtained for the experiments with excess NAD and excess ethanol at 2.0 mM and 100 mM are shown in Tables IIIA and IIIB, respectively, and these are also similar to those shown in Figure 11 except that there are now fewer points (only two) in Region 3 for variable NAD since the concentrations used were not sufficiently low.

In general, the theory predicts diffusion-free behaviour at high substrate concentrations. In the experiments with saturation by NAD and variable ethanol, the concentrations of the latter are high enough that there is no diffusion control at any of the flow rates employed. The trends found with constant ethanol and variable NAD are also consistent with less diffusion control at higher substrate concentrations. The points lying near Region 1 (little diffusional control) correspond to these conditions, while those in Region 3 (considerable diffusion control) are for low substrate concentrations.

Product Concentrations

Another method for the demonstration of diffusional effects uses the double-logarithmic plots of product concentration at the exit against flow rate. This is shown in Figure 12 for the experiments done with constant NAD and ethanol concentrations of 5.0 and 500 mM, respectively, and the data for excess NAD at 2.0 mM and excess ethanol at 100 mM are presented in Tables IVA and IVB. The slope of the plots in Figure 12

Figure 12. Double-logarithmic plots of the product concentration at the exit $[P]_e$, against the flow rate v_f , for the results with ethanol in excess at 500 mM (circles) and for excess NAD at 5.0 mM (triangles). Variable NAD concentrations (0.10-2.0 mM) for the former, and variable ethanol concentrations (3.0-100 mM) for the latter (within brackets) are shown.

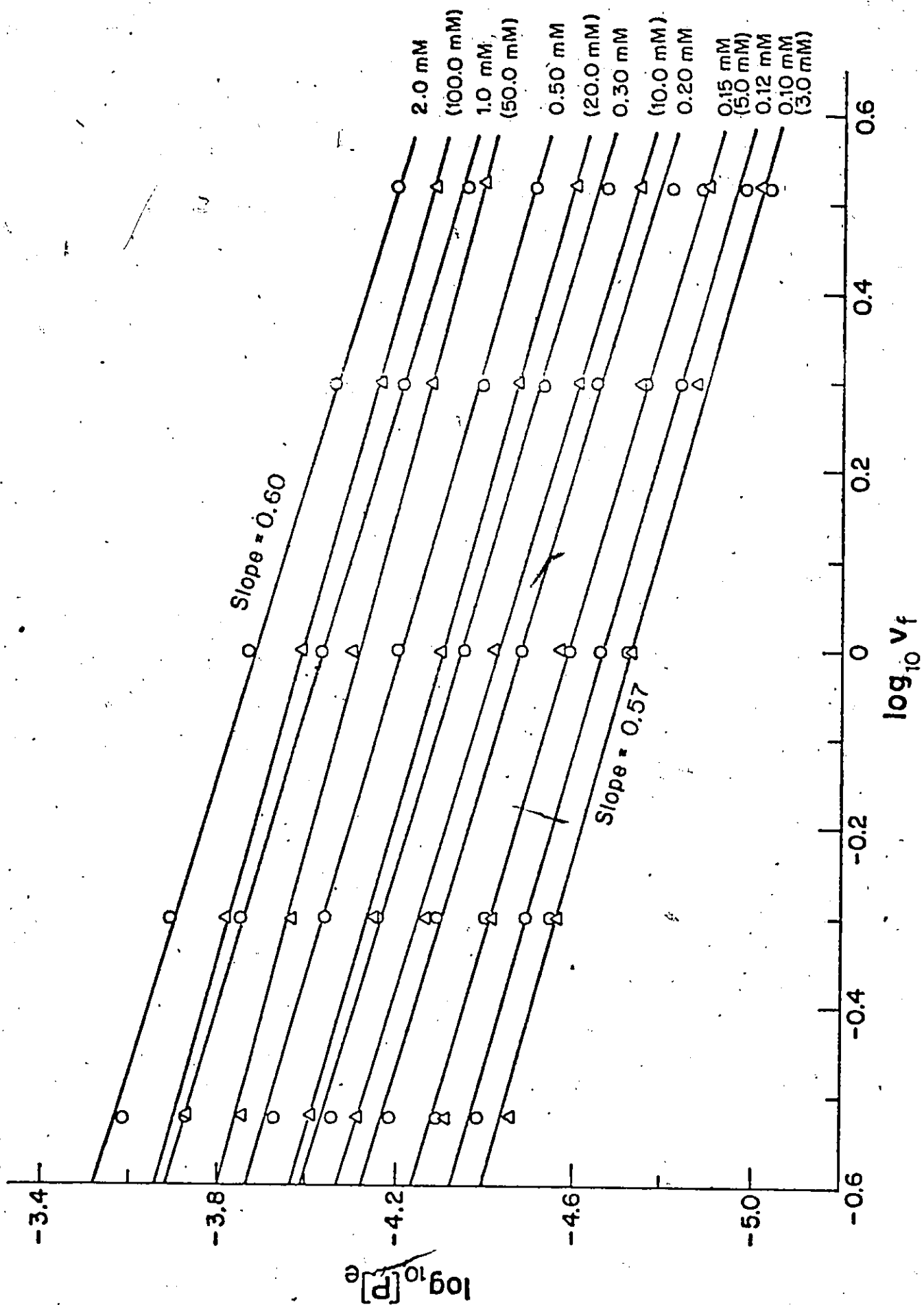


Table IVA. Concentration of the product NADH (μM) measured at the exit of the tube with excess $[\text{NAD}] = 2.0 \text{ mM}$ and variable ethanol at different flow rates.

$[\text{C}_2\text{H}_5\text{OH}]$ mM	Flow Rate, $v_f/\text{cm s}^{-1}$						
	0.10	0.48	0.80	1.15	1.55	2.75	3.90
20	82.8	53.9	47.3	41.6	36.5	28.6	26.4
25	97.0	65.6	52.1	46.5	40.0	32.0	28.3
30	113.3	73.2	58.7	51.8	45.0	35.2	31.2
40	128.1	83.1	66.4	58.2	51.0	39.1	35.1
50	149.0	94.9	74.8	65.6	58.4	43.9	40.0
60	164.8	101.6	86.3	71.5	63.8	48.4	44.7
75	178.3	109.2	94.2	78.5	69.0	53.7	49.2

Table IVB. Concentration of the product NADH (μM) measured at the exit of the tube with excess $[\text{C}_2\text{H}_5\text{OH}]=100 \text{ mM}$ and variable NAD at different flow rates.

[NAD] mM	Flow Rate, $v_f/\text{cm s}^{-1}$						
	0.10	0.48	0.80	1.15	1.55	2.75	3.90
0.25	70.9	43.9	37.5	30.1	25.9	21.2	18.8
0.50	108.0	69.5	54.2	47.0	41.3	32.5	28.5
0.75	134.4	84.6	67.7	57.1	48.4	38.6	34.7
1.00	159.0	97.1	78.1	66.9	57.4	42.8	38.9
1.25	174.3	108.8	89.1	73.0	64.6	50.6	43.4
1.50	191.3	120.6	94.9	78.8	70.4	54.2	48.1
1.75	201.8	131.8	99.7	88.1	77.3	60.8	53.5
2.00	213.8	140.0	107.9	95.0	82.3	64.6	57.9

varies from -0.57 to -0.60, while the corresponding plots for the data in Tables IVA and IVB gave slopes of -0.40 and -0.45, respectively, the points for the lowest flow rates being ignored in the latter case since these showed greater deviations.

The treatment of the Kobayashi-Laidler theory predicts that the slope of the above plots should be -1.0 for no diffusion control (Eqn. 6) and -0.67 for full diffusion control (Eqn. 8). The smaller dependence of product concentration on flow rate therefore indicates that there are some complications. Bunting and Laidler (107) found a similar low dependence of product concentration on flow rate with L-asparaginase, and concluded that it was due to complete conversion into products before the substrate had reached the tube outlet. In the present system, however, the conversion was much smaller and therefore this explanation is not tenable.

An alternative possibility is that there is inhibition by products which may remain attached to the enzyme and block further reaction. This will have a stronger effect at lower flow rates, when the residence times are longer and allow more blockage of reaction by products. As the flow rates are reduced, the product concentrations at the exit will therefore increase to a smaller extent than in the absence of inhibition, and the negative slopes of the $\log_{10}[P]_e$ vs $\log_{10}v_f$ plots will be smaller, as in Figure 12. We therefore tested for inhibition by products, and this is illustrated in the following section.

Inhibition by Products

Wratten and Cleland (134,135) found inhibition by products for liver and yeast alcohol dehydrogenase both in free solutions. In order to demonstrate this as well as to establish the type of inhibition with the immobilized yeast alcohol dehydrogenase, a number of experiments were carried out. Rate measurements were made at various substrate concentrations, and with different initial concentrations of the products, acetaldehyde and NADH. The products were added in amounts considerably greater than produced in the reaction so that the results would not be significantly affected by those formed in a particular run.

Figure 13 shows some Lineweaver-Burk plots at a fixed flow rate and variable NAD concentration with constant ethanol and different amounts of NADH added as indicated. All the plots are convergent near the $1/v$ axis, and therefore suggest mixed competitive and non-competitive inhibition, with a preponderance of the former. Secondary plots of the slopes from Figure 13 against the inhibitor NADH concentrations yielded a straight line. The ratio of the intercept to the slope in the latter plot led to an inhibition constant K_i of 120 μM . Data for the inhibition by added acetaldehyde with variable ethanol at a fixed flow rate and constant NAD are shown in Table V. Lineweaver-Burk plots of these data were similar to Figure 13 but now the lines were convergent almost on the vertical axis, thus suggesting even more competitive inhibition than with NADH. The inhibition constant for acetaldehyde was found to be 62 μM which means that this is a stronger inhibitor than NADH ($K_i=120 \mu\text{M}$).

Figure 13. Lineweaver-Burk plots for constant ethanol at 100 mM and variable NAD concentrations with NADH added as indicated at 25.5°C, pH 7.5 and a flow rate of 1.15 cm s⁻¹.

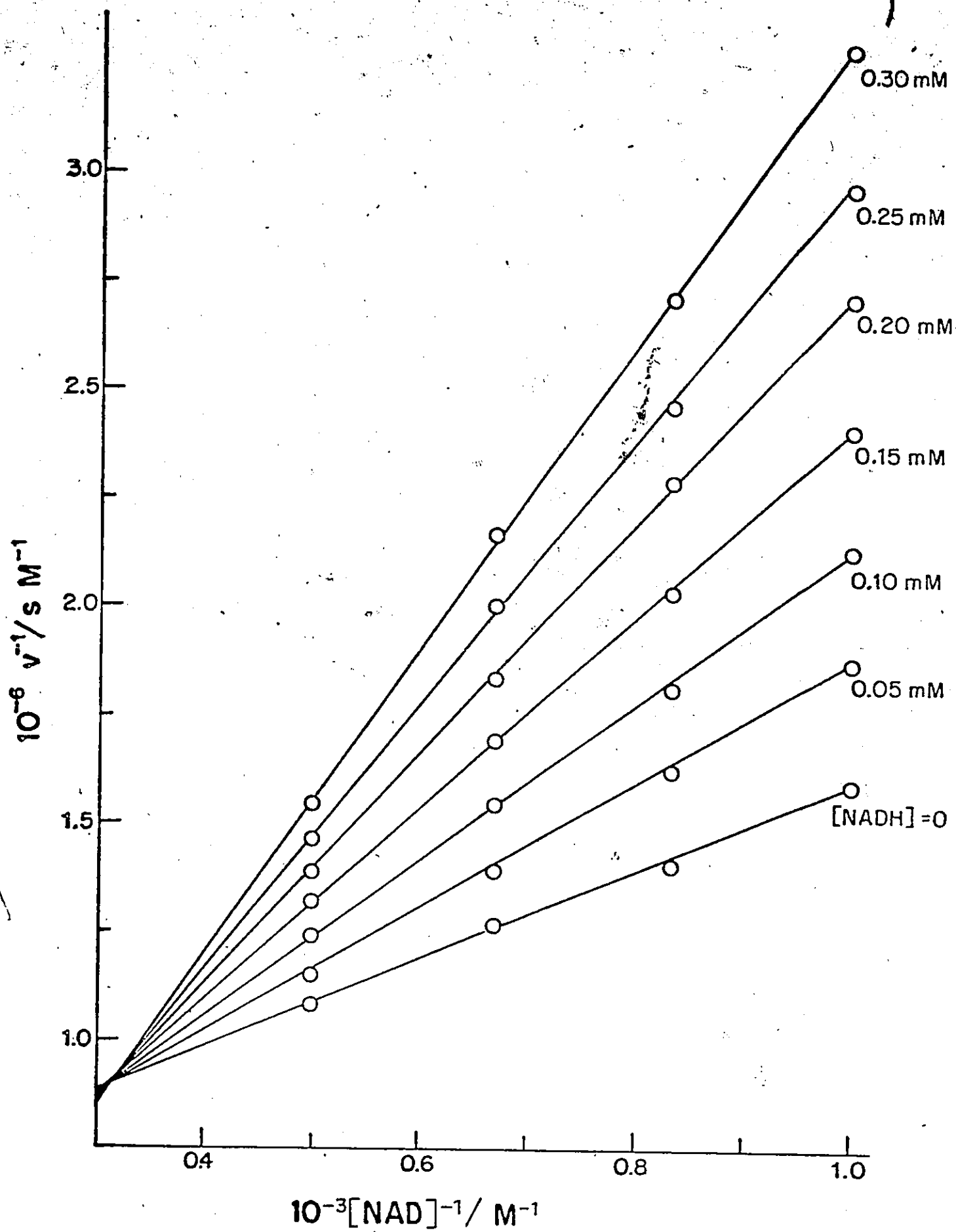


Table V. Rates of reaction ($10^6 \times \text{M s}^{-1}$) for the product inhibition by added acetaldehyde with variable ethanol and constant $[\text{NAD}] = 1.0 \text{ mM}$ at a fixed flow rate of 1.15 cm s^{-1} , pH 7.5 and temperature 25.5°C .

Concentration of added $[\text{CH}_3\text{CHO}]$ in mM	Concentration of Ethanol (mM)		
	30	50	100
0	0.375	0.468	0.660
0.10	0.205	0.283	0.483
0.20	0.133	0.205	0.346
0.30	0.104	0.159	0.277

Our results are to be compared with those for the free enzyme as studied by Wratten and Cleland (134,135) who found NADH to have a K_i of 108 μM and acetaldehyde to be a much weaker inhibitor with a K_i of 780 μM . Besides, the latter showed linear non-competitive inhibition with ethanol as the variable substrate, whereas the former showed linear competitive inhibition with NAD as the variable substrate. However, the differences are understandable in view of the fact that in our experiments diffusion control is much more important with variable NAD than with variable ethanol. Hence, the greater the amount of diffusional control the less the inhibition, since inhibitors affect the chemical interaction at the enzyme but not the diffusion process. The interplay of diffusional and chemical inhibition has been discussed in more detail in Chapter 1, and it is quite well known that the latter behaves differently in the presence of the other. There are also reports that the type of inhibition can be changed upon immobilization. For example, with membrane-bound enzymes, Thomas et al (136) found competitive inhibitors which tended to behave as non-competitive inhibitors, and non-competitive inhibitors which behaved as anti-competitive inhibitors.

In the present system, the amount of product formed depends significantly on the flow rate, as in Figure 12. We therefore studied the effect of flow rate on the product inhibition, and the results are shown in Tables VIA and VIB for different amounts of acetaldehyde and NADH added, respectively. In either case, it is observed that the degree of inhibition increases with increasing concentration of the inhibitor and at any flow rate. In the case of inhibition by acetaldehyde, the

Table VIA. Effect of flow rate on product inhibition by added acetaldehyde with constant $[C_2H_5OH]=20.0$ mM and excess $[NAD]=5.0$ mM at 25.5°C and pH 7.5.

Concentration of added $[CH_3CHO]$ in mM	Degree of Inhibition at Flow Rates			
	0.50 cm s^{-1}	1.00 cm s^{-1}	2.00 cm s^{-1}	3.30 cm s^{-1}
0.10	0.35	0.36	0.34	0.37
0.20	0.54	0.55	0.54	0.56
0.30	0.65	0.65	0.64	0.66
0.40	0.72	0.72	0.71	0.71

Table VIB. Flow rate dependence of product inhibition by added NADH with constant $[NAD]=0.50$ mM and excess ethanol at 100 mM.

Concentration of added [NADH] in mM	Degree of Inhibition at Flow Rates		
	0.48 cm s^{-1}	1.15 cm s^{-1}	2.75 cm s^{-1}
0.05	0.20	0.18	0.15
0.10	0.34	0.29	0.24
0.15	0.43	0.39	0.33
0.20	0.50	0.46	0.40
0.25	0.56	0.52	0.46
0.30	0.60	0.56	0.50

flow rate alone has no considerable effect on the degree of inhibition, as shown in Table VIA. With NADH, however, there is a significant decrease in the degree of inhibition with increasing flow rate and at any concentration of the inhibitor added (see Table VIB). This difference of behaviour is again consistent with the fact that there is essentially no diffusion control with variable ethanol, in contrast to substantial diffusion control with variable NAD. Consequently, the inhibition by products depends on the degree of diffusion control which in turn depends on the flow rate.

Concluding Remarks

The foregoing results and discussion for the flow kinetics of YADH attached to nylon tubing have shown that when the NAD concentration is held constant and the ethanol concentration varied, there is no appreciable diffusion control, but that the converse conditions lead to substantial diffusion control. It is important to note that the mechanisms involved with alcohol dehydrogenase in free solution are by no means clear, and the yeast and liver enzymes behave differently. There is some evidence (134,135,137) that with both yeast and liver enzymes there is an ordered mechanism, the NAD adding first and the product NADH leaving last. Various investigations have shown that NAD and NADH occupy the same binding sites (59,138-140), NADH being bound more tightly (56), and Dunn (141) has concluded that under steady-state conditions the rate of substrate turnover is limited by the rate with which NADH leaves. However, Silverstein and Boyer (142) for both the horse liver and the yeast enzyme showed that a random mechanism is important, and for the yeast

enzyme Dickinson and Monger (143) concluded that there is a partly random mechanism for addition of NAD and alcohol and a compulsory order of product dissociation. Dead-end complexes (144) and abortive enzyme-NADH-alcohol ternary complexes (142) have also been proposed.

The situation is therefore complicated even for the free enzyme. Our results are most satisfactorily explained if under our experimental conditions the mechanism is an ordered one, the NAD adding first and the alcohol reacting with the enzyme-NAD complex. Thus, in our experiments with NAD fixed at 2.0 mM or at 5.0 mM and the alcohol concentration varied, the enzyme will be saturated with NAD and the process will involve the diffusion of alcohol to the enzyme-NAD at the surface and the subsequent reaction. This diffusion can occur readily, and there is no appreciable diffusion control. When, on the other hand, the alcohol concentration is in excess of the $K_{m,app}$ the enzyme will not be saturated by alcohol; the NAD will first have to diffuse to the surface and form an enzyme-NAD complex, after which the alcohol must diffuse to the enzyme-NAD at the surface and form the ternary complex. Diffusion is therefore much more heavily involved, and the larger NAD molecules will diffuse more slowly; the diffusion control is therefore understandable.

CHAPTER 4

TEMPERATURE EFFECTS WITH IMMOBILIZED YEAST ALCOHOL
DEHYDROGENASE IN FLOW SYSTEMSIntroduction

It is well known that the overall rate of an enzyme-catalyzed reaction passes through a maximum as the temperature is increased. This is due to the fact that changing the temperature affects two independent processes, viz., the catalyzed reaction itself and the thermal inactivation of the enzyme. At sufficiently low temperatures, the rate of inactivation can be very slow so that this may have no appreciable effect on the rate of the catalyzed reaction. Thus, the overall rate will increase with rising temperature, as with ordinary chemical reactions. On the other hand, inactivation becomes more and more important at higher temperatures when the concentration of the active enzyme falls during the course of the reaction, resulting in a decrease in the overall rate.

Many enzymes are stabilized against different types of inactivation upon immobilization (73). This is sometimes true for thermal inactivation, and then the effect of temperature on the catalyzed reaction itself can be studied more conveniently and, perhaps, over a wider temperature range. Temperature studies on immobilized enzymes are very useful in a number of ways. For example, activation energies for diffusion in water fall within a certain range of values, and may differ from those for the chemical interaction. An analysis of the temperature dependence

under different conditions of concentration and flow rate can therefore provide mechanistic information. These studies also have a practical bearing on the design of enzyme-reactors for various purposes.

Most studies with alcohol dehydrogenases have been performed at single temperatures, or else the temperature effects were limited to the estimation of thermodynamic parameters such as the free energy changes (see 32). As mentioned above, temperature studies can otherwise be diagnostic of some general feature of the mechanisms, especially in the case of the immobilized enzyme since diffusional effects play an important role in the overall mechanism (123). It therefore seemed desirable to carry out such investigations with the immobilized yeast alcohol dehydrogenase in the tubular flow reactor. In the previous chapter, the flow kinetics of the immobilized enzyme have been described at a single temperature of 25.5°C. This chapter discusses the kinetic results obtained and analyzed at temperatures ranging from 5 to 45°C. Similar studies have previously been made in this laboratory with immobilized electric eel acetylcholinesterase (145) and with immobilized lactate dehydrogenase (146). Some of the results presented in this chapter have also been published recently (147).

Theoretical Considerations

Temperature dependence of an enzyme-catalyzed reaction is manifested in the various kinetic constants associated with a rate expression. In the simplest case of an enzyme in free solution with a single substrate S, the rate involves at least two kinetic constants, viz., the catalytic rate constant k_c which relates to the breakdown of

the enzyme-substrate complex into products, and the Michaelis constant K_m pertaining to the equilibrium of the complex, the latter being a composite of several rate constants. Each of these kinetic constants can vary with temperature according to the Arrhenius equation as follows:

$$k_c = A_c e^{-E_c/RT} \quad (11)$$

and

$$K_m = A_m e^{\Delta E_m/RT} \quad (12)$$

where A and E terms are the corresponding frequency factor and the activation energies, respectively. The observed activation energy E_c will relate to k_c if $[S] \gg K_m$. If, however, both constants vary simultaneously with temperature, especially at low substrate concentrations, the observed activation energy will be $E_c + \Delta E_m$. In the case of immobilized enzymes, the apparent Michaelis constants $K_{m,app}$ vary with the extent of diffusion control so that their temperature dependence will be different.

Many treatments have been reported of the kinetics of immobilized enzymes taking into account the diffusional effects, but little has been done for the temperature effects in such systems. In the previous chapter, the treatment of the Kobayashi-Laidler theory (70) have been outlined for the interpretation of the flow kinetics of yeast alcohol dehydrogenase attached to nylon tubing. This theory can also be extended to deal with the temperature effects, especially in some limiting cases (91). Thus, under the conditions of high substrate concentrations and high flow rates, the $K_{m,app}$ approaches K'_m , and the rate is given by Eqn. (5):

$$v = \frac{2\pi r L k'_c [E]_s [S]}{K'_m + [S]} = 2\pi r L k'_c [E]_s \quad (13)$$

where k'_c is the inherent rate constant at the surface. The temperature dependence of the rate therefore reflects only that of this chemical rate constant, and the activation energy will correspond to the breakdown of the enzyme-substrate complex. At the other extreme conditions, i.e., at low flow rates and low substrate concentrations, the rate is given by Eqn. (7)

$$v = 8.06 (v_f D^2 r^2 L^2)^{1/3} [S] \quad (14)$$

which involves diffusion but not the enzyme-catalyzed reaction. The overall rate of the reaction now depends on the flow rate, and the temperature dependence is controlled entirely by that of the diffusion coefficient D . This constant can also be expressed in terms of the Arrhenius equation

$$D = A_D e^{-E_D/RT} \quad (15)$$

where A_D and E_D are the respective frequency factor and activation energy. Under these circumstances, therefore, the observed activation energy will be two-thirds that for the diffusion process.

Experimental Procedure

The materials and methods used for the present studies are essentially the same as those given in the previous two chapters. The same enzyme-tube as that described in Chapter 2 was used throughout the experiments, and the kinetic procedure is to be found in Chapter 3.

This involved pumping of thermostatted substrate solution through the tubular reactor which was kept in a temperature-controlled waterbath having the same temperature as the substrate solution. Rates were measured at five (or, in some cases, four) temperatures ranging from 5 to 45°C, and at a variety of flow rates and substrate concentrations, all solutions being prepared in 0.1M phosphate buffer of pH 7.5.

Results and Discussion

Temperature Dependence of Overall Rates

The experimental data for the rates of reaction with excess NAD and excess ethanol, at different temperatures and flow rates, are listed in Tables VIIA-E and VIIIA-E, respectively. Figure 14 shows typical Arrhenius plots for NAD in excess and at a single flow rate, with various ethanol concentrations as indicated. Similar plots for excess ethanol and variable NAD concentration are shown in Figure 15. It can be noted that in all the experiments the Arrhenius law was obeyed within experimental error; there were no changes in slope, such as were found with immobilized acetylcholinesterase (145) and which were attributed to changes in mechanism.

Table IX shows the observed activation energies with excess NAD, and those with excess ethanol are given in Table X. These values were calculated using the method of least squares (148,149), the errors being estimated at the 90% confidence level. The activation energies listed are the apparent values, obtained directly from the Arrhenius plots,

Table VIIA. Rates of reaction ($10^6 \times \text{M s}^{-1}$) at various flow rates and ethanol concentrations, with excess $[\text{NAD}] = 5.0 \text{ mM}$ and at temperature 5°C .

Flow Rate (cm s^{-1})	Concentration of Ethanol (mM)		
	10.0	20.0	50.0
0.10	0.025	0.034	0.046
0.50	0.063	0.089	0.126
1.00	0.071	0.110	0.170
2.00	0.100	0.141	0.120
3.30	0.117	0.170	0.251

Table VIIB. Rates of reaction ($10^6 \times \text{M s}^{-1}$) at various flow rates and ethanol concentrations, with excess $[\text{NAD}] = 5.0 \text{ mM}$ and at temperature 15°C .

Flow Rate (cm s^{-1})	Concentration of Ethanol (mM)				
	3.0	5.0	10.0	20.0	50.0
0.10	0.026	0.032	0.043	0.062	0.087
0.50	0.062	0.083	0.112	0.157	0.229
1.00	0.076	0.097	0.132	0.193	0.305
2.00	0.090	0.129	0.178	0.263	0.347
3.30	0.110	0.148	0.214	0.309	0.437

Table VIIC. Rates of reaction ($10^6 \times \text{M s}^{-1}$) at various flow rates and ethanol concentrations, with excess $[\text{NAD}] = 5.0 \text{ mM}$ and at temperature 25.5°C .

Flow Rate (cm s^{-1})	Concentration of Ethanol (mM)				
	3.0	5.0	10.0	20.0	50.0
0.10	0.041	0.051	0.071	0.100	0.150
0.50	0.110	0.145	0.193	0.285	0.422
1.00	0.138	0.186	0.251	0.355	0.531
2.00	0.184	0.257	0.351	0.457	0.653
3.30	0.219	0.292	0.389	0.537	0.794

Table VIID. Rates of reaction ($10^6 \times M \text{ s}^{-1}$) at various flow rates and ethanol concentrations, with excess $[NAD]=5.0 \text{ mM}$ and at temperature 35°C .

Flow Rate (cm s^{-1})	Concentration of Ethanol (mM)				
	3.0	5.0	10.0	20.0	50.0
0.10	0.063	0.079	0.105	0.145	0.209
0.50	0.188	0.240	0.285	0.447	0.676
1.00	0.245	0.305	0.380	0.562	0.851
2.00	0.295	0.389	0.531	0.794	1.023
3.30	0.355	0.457	0.562	0.871	1.259

Table VIIE. Rates of reaction ($10^6 \times \text{M s}^{-1}$) at various flow rates and ethanol concentrations, with excess $[\text{NAD}] = 5.0 \text{ mM}$ and at temperature 45°C .

Flow Rate (cm s^{-1})	Concentration of Ethanol (mM)			
	3.0	5.0	10.0	20.0
0.10	0.105	0.132	0.174	0.257
0.50	0.263	0.355	0.473	0.708
1.00	0.398	0.513	0.668	0.891
2.00	0.501	0.700	0.891	1.189
3.30	0.603	0.794	1.000	1.413

Table VIIIA. Rates of reaction ($10^6 \times \text{M s}^{-1}$) at various NAD concentrations and flow rates, with excess $[\text{C}_2\text{H}_5\text{OH}] = 500 \text{ mM}$ and at temperature 5°C .

Flow Rate (cm s^{-1})	Concentration of NAD (mM)						
	0.10	0.12	0.15	0.20	0.30	0.50	1.00
0.10	0.031	0.033	0.035	0.042	0.046	0.056	0.086
0.30	0.037	0.042	0.045	0.050	0.059	0.071	0.100
0.50	-	0.063	0.069	0.085	0.098	0.132	0.166
1.00	-	0.083	0.097	0.112	0.130	0.162	0.209
2.00	-	0.095	0.112	0.129	0.151	0.200	0.257
3.30	-	0.107	0.127	0.157	0.186	0.251	0.339

Table VIIIB. Rates of reaction ($10^6 \times \text{M s}^{-1}$) at various NAD concentrations and flow rates, with excess $[\text{C}_2\text{H}_5\text{OH}] = 500 \text{ mM}$ and at temperature 15°C .

Flow Rate (cm s^{-1})	Concentration of NAD (mM)						
	0.10	0.12	0.15	0.20	0.30	0.50	1.00
0.10	0.036	0.042	0.046	0.055	0.062	0.078	0.126
0.30	0.051	0.059	0.066	0.078	0.091	0.112	0.162
0.50	0.083	0.100	0.112	0.141	0.178	0.240	0.309
1.00	0.107	0.126	0.151	0.182	0.224	0.302	0.398
2.00	0.138	0.155	0.182	0.224	0.275	0.363	0.490
3.30	0.148	0.178	0.229	0.263	0.316	0.447	0.575

Table VIIIC. Rates of reaction ($10^6 \times \text{M s}^{-1}$) at various NAD concentrations and flow rates, with excess $[\text{C}_2\text{H}_5\text{OH}] = 500 \text{ mM}$ and at temperature 25.5°C .

Flow Rate (cm s^{-1})	Concentration of NAD (mM)						
	0.10	0.12	0.15	0.20	0.30	~~0.50~~	1.00
0.10	0.045	0.056	0.063	0.078	0.089	0.117	0.204
0.30	0.076	0.089	0.100	0.119	0.145	0.186	0.316
0.50	0.129	0.151	0.182	0.224	0.288	0.385	0.513
1.00	0.162	0.188	0.224	0.275	0.355	0.468	0.631
2.00	0.224	0.251	0.282	0.355	0.447	0.603	0.804
3.30	0.245	0.288	0.324	0.417	0.501	0.716	1.000

Table VIIIID. Rates of reaction ($10^6 \times M s^{-1}$) at various NAD concentrations and flow rates, with excess $[C_2H_5OH]=500 \text{ mM}$ and at temperature $35^\circ C$.

Flow Rate ($cm s^{-1}$)	Concentration of NAD (mM)						
	0.10	0.12	0.15	0.20	0.30	0.50	1.00
0.10	0.059	0.071	0.083	0.102	0.122	0.164	0.272
0.30	0.095	0.123	0.136	0.166	0.211	0.288	0.468
0.50	0.162	0.200	0.248	0.302	0.380	0.556	0.813
1.00	0.204	0.240	0.288	0.363	0.479	0.716	1.023
2.00	0.295	0.327	0.385	0.490	0.646	0.912	1.288
3.30	0.316	0.380	0.427	0.575	0.692	1.047	1.496

Table VIIIIE. Rates of reaction ($10^6 \times \text{M s}^{-1}$) at various NAD concentrations and flow rates, with excess $[\text{C}_2\text{H}_5\text{OH}] = 500 \text{ mM}$ and at temperature 45°C .

Flow Rate (cm s^{-1})	Concentration of NAD (mM)					
	0.10	0.12	0.15	0.20	0.30	0.50
0.10	0.071	0.088	0.102	0.129	0.153	0.214
0.30	0.126	0.164	0.197	0.251	0.324	0.447
0.50	0.229	0.275	0.355	0.447	0.575	0.832
1.00	0.288	0.331	0.407	0.525	0.708	1.109
2.00	0.417	0.479	0.589	0.741	1.000	1.429
3.30	0.479	0.537	0.661	0.832	1.047	1.549

Figure 14. Arrhenius plots for the rate of product formation at a flow rate = 0.50 cm s^{-1} , with excess $[\text{NAD}] = 5.0 \text{ mM}$ and at the ethanol concentrations as indicated.

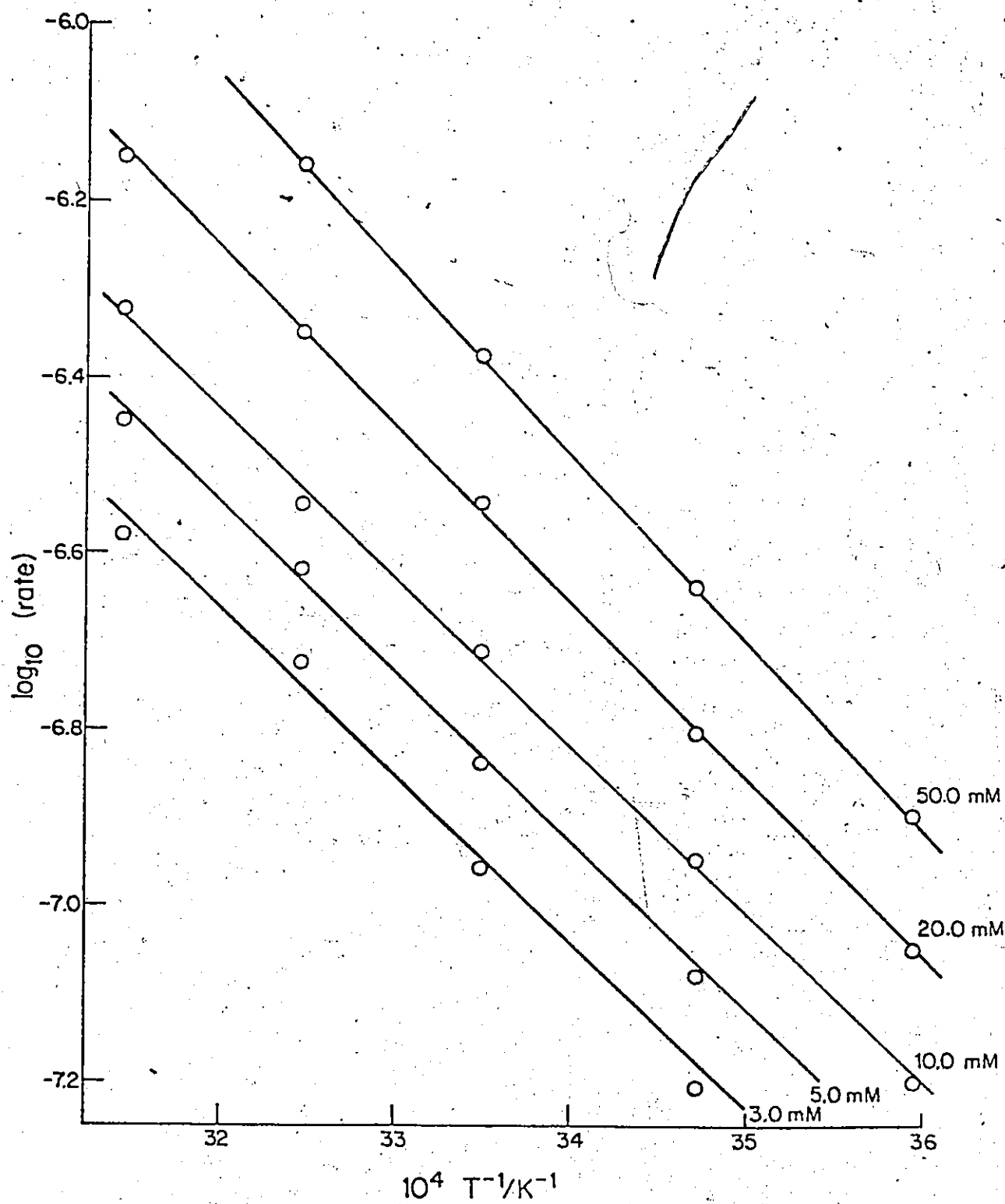


Figure 15. Arrhenius plots for the rate of product formation at a flow rate = $0.50 \text{ cm}^3 \text{ s}^{-1}$, with excess $[\text{C}_2\text{H}_5\text{OH}] = 500 \text{ mM}$ and at the NAD concentrations as indicated.

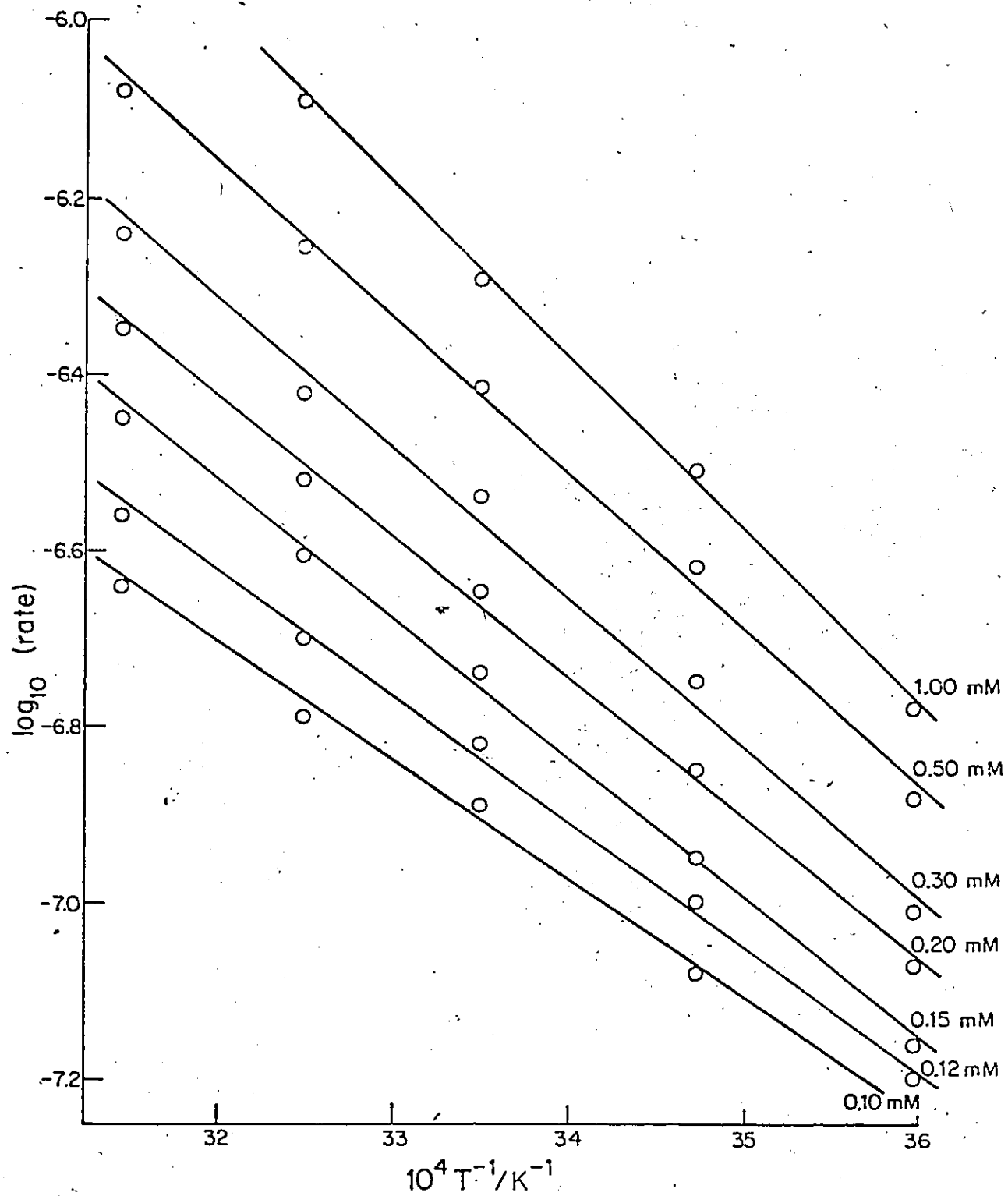


Table IX. Activation energies (kcal mol^{-1}) with excess NAD at 5.0 mM.

Concentration of $[\text{C}_2\text{H}_5\text{OH}]$ mM	Flow Rates in cm s^{-1}				
	0.10	0.50	1.00	2.00	3.30
3.0	8.5 ± 1.1	9.0 ± 1.6	10.2 ± 0.6	10.3 ± 1.0	10.3 ± 0.8
5.0	8.6 ± 0.9	8.9 ± 0.8	10.1 ± 0.5	10.1 ± 1.4	10.1 ± 1.0
10.0	8.5 ± 0.4	8.7 ± 0.4	9.8 ± 0.5	9.6 ± 0.5	9.2 ± 0.7
20.0	8.6 ± 0.8	9.1 ± 0.2	9.3 ± 0.2	9.4 ± 0.5	9.3 ± 0.1
50.0	8.7 ± 1.7	9.4 ± 0.3	9.1 ± 0.2	9.4 ± 0.7	9.2 ± 0.4
∞ (Extrapolated)	8.7	9.1	9.2	9.3	9.1

Table X. Activation energies (kcal mol^{-1}) with excess ethanol at 500 mM.

Concentration of [NAD] mM	Flow rates in cm s^{-1}					
	0.10	0.30	0.50	1.00	2.00	3.30
0 (extrapolated)	2.8	4.0	5.0	5.1	5.9	6.5
0.10	3.8 ± 0.5	5.4 ± 0.4	6.0 ± 1.1	5.9 ± 0.9	6.6 ± 1.0	6.9 ± 1.4
0.12	4.3 ± 0.3	6.1 ± 0.3	6.4 ± 0.5	6.0 ± 0.5	7.0 ± 0.6	7.0 ± 0.7
0.15	4.8 ± 0.3	6.5 ± 0.2	7.2 ± 0.5	6.2 ± 0.5	7.2 ± 0.4	6.9 ± 1.0
0.20	5.0 ± 0.3	7.1 ± 0.3	7.2 ± 0.5	6.7 ± 0.5	7.5 ± 0.6	7.3 ± 0.5
0.30	5.4 ± 0.4	7.5 ± 0.3	7.6 ± 0.9	7.3 ± 0.6	8.2 ± 0.6	7.5 ± 0.5
0.50	6.0 ± 0.4	8.1 ± 0.3	8.0 ± 0.6	8.3 ± 0.6	8.3 ± 0.4	7.9 ± 0.5
1.00	6.7 ± 0.9	9.0 ± 1.4	8.9 ± 0.7	8.9 ± 1.1	9.0 ± 0.9	8.5 ± 0.5
∞ (extrapolated)	7.0	9.2	9.1	9.4	9.4	8.6

without multiplication by the $3/2$ factor (see Eqn. (14)). The net result of the consideration of the errors leads to the conclusion that these are mostly less than $\pm 10\%$ or at worst within $\pm 2 \text{ kcal mol}^{-1}$ of the apparent values in a few cases where only four temperatures were used.

The results in Table IX, for NAD at saturating concentrations and with ethanol concentrations varied, show very little dependence of the activation energy on the ethanol concentration or on the flow rate. This result is consistent with the conclusion of the previous chapter that the kinetics under these conditions are essentially diffusion free. The activation energies, particularly at the higher concentrations of ethanol, therefore correspond to the chemical process occurring at the surface. Table IX also includes values extrapolated to $1/[S]=0$ and therefore corresponding to very high concentrations of ethanol. Thus, it appears that the activation energy for the chemical processes when there is saturation by both substrates is about 9 kcal mol^{-1} .

In contrast, the results with saturating concentrations of ethanol, listed in Table X, show a significant variation of activation energy with flow rate and with concentration of NAD. Increasing the flow rate and the NAD concentration, both produce an increase in activation energy. This result is also consistent with the conclusions of the preceding chapter, which showed that there is substantial diffusion control under these conditions, the extent of diffusion control being reduced as the flow rate and the substrate concentration are increased. The activation energies extrapolated to infinite concentrations and flow rates should therefore correspond to the diffusion-free value.

Table X shows the values extrapolated to $1/[S] = 0$. These values also increase with increasing flow rate, and at the higher flow rates are consistent with the 9 kcal mol^{-1} obtained from the results in Table IX. Both sets of results therefore suggest that the activation energy for the chemical interaction at the surface is about 9 kcal mol^{-1} .

Extrapolation of the results in Table X to low NAD concentration and flow rate leads to an activation energy for the fully diffusion-controlled process. The values extrapolated to $[NAD]=0$ are shown at the top of the columns in Table X. In accordance with expectations, these results fall as the flow rate is reduced, and suggest a value of about $2.8 \text{ kcal mol}^{-1}$ for the apparent activation energy for complete diffusion control. This value must be multiplied by $3/2$, in the light of Eqn. (14), to obtain the activation energy corresponding to the diffusion coefficient. This gives a value of $4.2 \text{ kcal mol}^{-1}$, which is very reasonable for diffusion in water.

The results described above are somewhat similar to those obtained by Buchholz and R  th (150) for immobilized trypsin. These workers also found a significant drop in activation energy (from 9.5 to $4.5 \text{ kcal mol}^{-1}$) with decreasing substrate concentration (from 30 to 0.1 mM), and concluded that the decrease was due to an increase in the extent of diffusion control. On the basis of a simplified model, it was suggested further that the apparent activation energy may be reduced to half its diffusion-free value at high substrate concentrations, or less if the activation energy for the Michaelis constants has a positive value. For alcohol dehydrogenase in free solution, Gierer (131) obtained

an activation energy of about 14 kcal mol^{-1} for V_{max} ; this is somewhat higher than the present value of about 9 kcal mol^{-1} for the immobilized enzyme. Gierer also found values of $3-8 \text{ kcal mol}^{-1}$ from the temperature coefficient of the Michaelis constant, these being for association processes. Similar results have been obtained from our data, but these were not very reliable (to be discussed later) because of the changes in mechanism with change of substrate concentration.

However, in the present studies as well as those of Buchholz and R  th (150), the activation energy for the chemical process is substantially greater than that for the diffusion process. In these systems, it is therefore easy to find conditions in which the processes are essentially diffusion-free. These results show an interesting contrast with those for lactate dehydrogenase attached to nylon tubing, studied by Daka and Laidler (146). They obtained an activation energy for the chemical process of about 1 kcal mol^{-1} , which is very much less than that of about 5 kcal mol^{-1} for the diffusion process. In this system, it was therefore much easier to find conditions favouring diffusion control, and diffusion-free behaviour was only found under the extreme conditions of high substrate concentration and of high flow rate. An important consequence of the situation existing in the present work with the immobilized yeast alcohol dehydrogenase is that, because of the high activation energy for the chemical process, the reactions are very much slower than found in the system studied by Daka and Laidler (122,146).

Temperature Dependence of Michaelis Parameters

The Michaelis parameters ($K_{m,app}$ and V_{max}) for the data with excess NAD and ethanol in Tables VIIA-E and VIIIA-E were obtained from Lineweaver-Burk plots, and these are summarized in Tables XIA-B and XIIA-B, respectively. It is to be noted that these plots were non-linear, particularly for variable ethanol, a typical example being shown in Figure 16. Similar behaviour was noted in Chapter 3 for both the substrates, and it was suggested that this might be due to a change in the degree of diffusion control. Such an explanation would seem to be more reasonable for variable NAD when there is substantial diffusion control, in contrast to appreciably no diffusion control with variable ethanol. However, the Michaelis parameters were evaluated from the linear portions of the Lineweaver-Burk plots at lower concentrations with variable NAD, while the results from both low (3-10 mM) and high (10-50 mM) concentration ranges of ethanol are listed.

With ethanol as the variable substrate, Tables XIA-B show two different sets of Michaelis parameters resulting from the non-linear kinetics. Similar behaviour was observed with native human liver alcohol dehydrogenase, and Dubied et al (151) discussed several possible mechanisms to interpret the apparent kinetic constants. One possibility is a random mechanism in which rapid binding steps are not assumed, but where the free enzyme can first bind either ethanol or NAD. This was ruled out on the basis of the fact that the non-linear kinetics was obtained with saturating concentrations of NAD, and so also in the present work, which

Figure 16. Lineweaver-Burk plots with excess $[NAD]=5.0$ mM, at different flow rates as indicated, pH 7.5 and temperature 25.5°C .

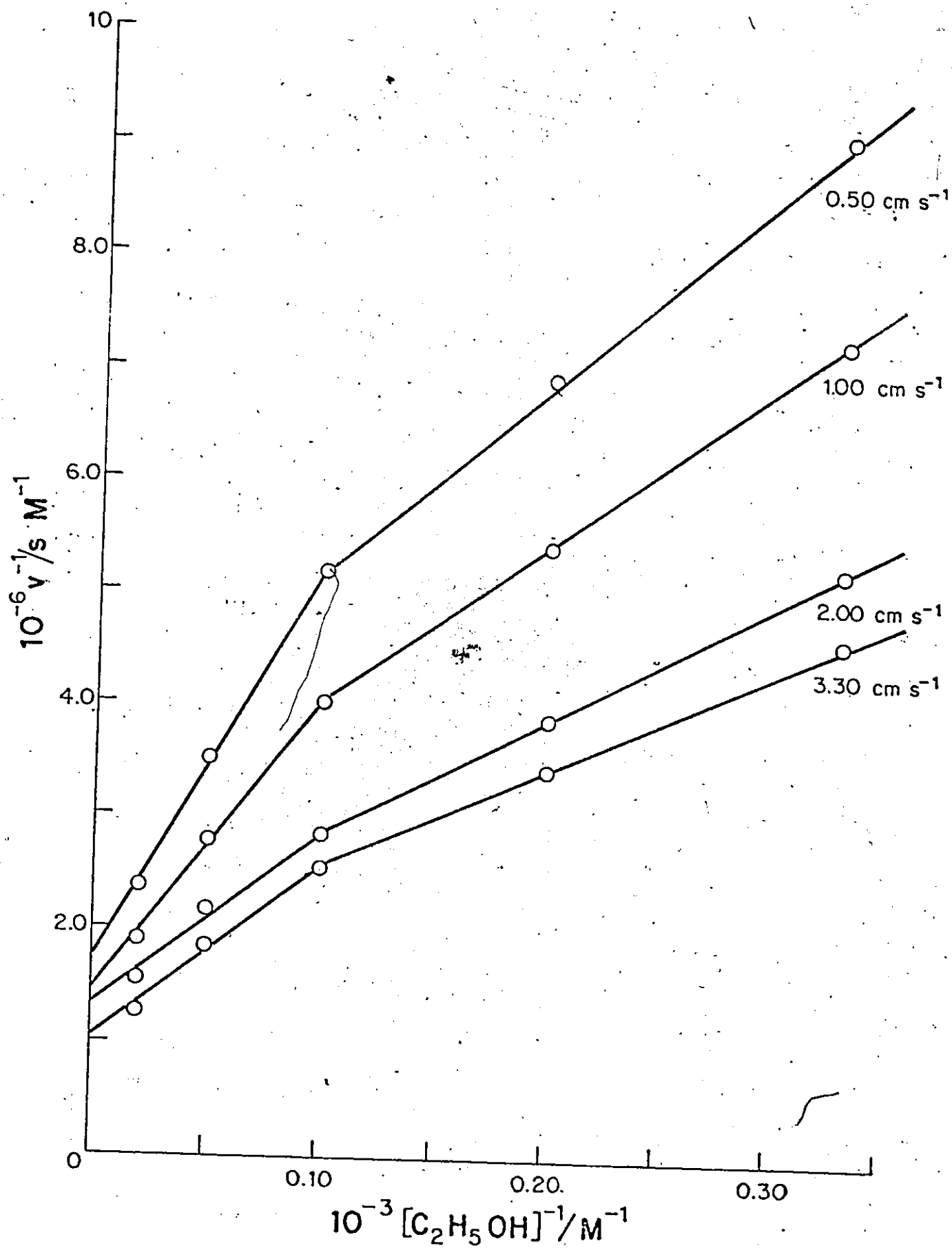


Table XIA. Values of $V_{\max}$ with excess $[NAD]=5.0$ mM, obtained from Lineweaver-Burk plots for 3-10 mM (within brackets) and 10-50 mM concentration ranges of ethanol, at different temperatures and flow rates.

Flow Rate, (cm s^{-1})	$10^6 \times V_{\max}$ (M s^{-1}) at Temperature ($^{\circ}\text{C}$)				
	5.0	15.0	25.5	35.0	45.0
0.10	0.057	0.117 (0.059)	0.197 (0.099)	0.265 (0.142)	0.500 (0.273)
0.50	0.164	0.293 (0.180)	0.588 (0.284)	1.014 (0.371)	1.370 (0.725)
1.00	0.257	0.421 (0.188)	0.708 (0.383)	1.213 (0.493)	1.351 (0.926)
2.00	0.256	0.464 (0.322)	0.773 (0.542)	1.447 (0.742)	1.667 (1.683)
3.30	0.342	0.518 (0.453)	1.028 (0.584)	2.089 (0.747)	2.381 (1.434)

Table XIB. Apparent Michaelis constants for ethanol with excess $[NAD]=5.0$ mM, at different temperatures and flow rates, obtained from Lineweaver-Burk plots (values in brackets for 3-10 mM and others for 10-50 mM concentrations of ethanol).

Flow Rate (cm s ⁻¹)	K _{m,app} [C ₂ H ₅ OH] (mM) at Temperature (°C)				
	5.0	15.0	25.5	35.0	45.0
0.10	13.3	17.5 (4.1)	18.0 (4.4)	15.7 (3.8)	19.0 (5.6)
0.50	16.3	16.3 (5.9)	20.6 (4.8)	25.5 (2.9)	18.9 (5.3)
1.00	26.5	22.1 (4.5)	18.6 (5.4)	22.3 (3.1)	10.3 (4.0)
2.00	15.8	16.1 (7.6)	12.2 (5.5)	18.0 (4.6)	8.7 (5.2)
3.30	19.3	12.0 (9.7)	16.8 (5.1)	26.8 (3.3)	13.8 (4.1)

Table XIIA. Values of $V_{\max}$ with excess $[C_2H_5OH]=500$ mM, obtained from Lineweaver-Burk plots for variable NAB, at different temperatures and flow rates.

Flow Rate (cm s^{-1})	$10^6 x V_{\max}$ (M s^{-1}) at Temperatures ($^{\circ}\text{C}$)				
	5.0	15.0	25.5	35.0	45.0
0.10	0.065	0.101	0.189	0.287	0.411
0.30	0.086	0.178	0.342	0.610	1.400
0.50	0.201	0.440	0.761	1.379	2.583
1.00	0.238	0.542	0.897	1.759	3.292
2.00	0.304	0.633	1.020	1.907	3.580
3.30	0.444	0.836	1.323	2.209	3.218

Table XIIB. Apparent Michaelis constants for NAD with excess $[C_2H_5OH]=500$ mM, obtained from Lineweaver-Burk plots at different temperatures and $^2.5$ flow rates.

Flow Rate ($cm\ s^{-1}$)	$K_{m,app}$ [NAD] (mM) at Temperatures ($^{\circ}C$)				
	5.0	15.0	25.5	35.0	45.0
0.10	0.142	0.177	0.303	0.375	0.463
0.30	0.133	0.254	0.354	0.519	0.962
0.50	0.275	0.433	0.486	0.721	0.997
1.00	0.226	0.397	0.451	0.787	1.051
2.00	0.264	0.366	0.367	0.570	0.767
3.30	0.380	0.444	0.443	0.590	0.582

would effectively give rise to a compulsory ordered mechanism by forcing the binding of NAD to the free enzyme. Alternatively, a random mechanism with an enzyme-NAD-alcohol complex that dissociates more rapidly than the enzyme-NADH-alcohol complex was considered, but then inhibition by higher concentrations of ethanol, which has obviously not been found in the present system, was not explained. Secondly, the enzyme may contain multiple forms differing in kinetic characteristics so that the observed activities would be the sum of the individual ones. Finally, the enzyme may exhibit cooperativity in that the binding of the first molecule of ethanol changes the affinity for the second or the subsequent molecules of the substrate.

In the case of yeast alcohol dehydrogenase, neither the non-linear kinetics nor any of the above possibilities have so far been reported. We have used a crystallized and lyophilized enzyme which is homogeneous at least chromatographically and electrophoretically, but microheterogeneity of the enzyme active-site may result from any modification due to the immobilization. The results in Table XIA-B show that both $V_{\max}$ and $K_{m,app}$ values differ in the limit of high and low concentration ranges of ethanol with saturating concentrations of NAD. This indicates that different dissociation rate constants are involved in the product formation (152). Differences due to intrinsic activity would seem unlikely as will be seen shortly, whereas the modification of the catalytic activity is possible in a number of ways. At one extreme, the enzyme may be attached to the support through the catalytically active groups in which

case complete loss of activity would be observed. Similarly, partial modification may occur if the enzyme is immobilized through the active group of one subunit while the others are free, or via different groups away from the active-site but which can influence the catalytic rate constant. This means necessarily that two different forms of enzyme are present in the immobilized system, thus implying microheterogeneity. The effect would be particularly reflected at high substrate concentrations when all the enzyme molecules may be involved in the catalysis. It is, however, difficult to conclude unequivocally which of the above possibilities are predominant, and therefore the explanation of the non-linear kinetics with ethanol as the variable substrate remains to be elucidated more clearly.

It can be seen from Tables XIA and XIIA that, in all cases, the apparent $V_{\max}$ values increase with increasing flow rate and with increasing temperature. This is reasonable since the extent of diffusion control decreases under these conditions, and the rate therefore approaches the diffusion-free values. The temperature dependence of the $V_{\max}$ showed generally linear Arrhenius plots and gave activation energies of 8-11 kcal mol⁻¹ with variable NAD and of 7-10 kcal mol⁻¹ with variable ethanol. These values are within ± 2 kcal mol⁻¹ of the activation energy of about 9 kcal mol⁻¹ as deduced earlier for the chemical interaction at the surface, and therefore are in reasonable agreement in view of the accuracy of the extrapolation procedure in obtaining the $V_{\max}$ values. The activation energies together with the linearity of the Arrhenius plots for both $V_{\max}$ and the overall rates (discussed before) suggest that the maximum

steady-state velocity, as have been measured in our experiments, is controlled by a single rate constant which, in accordance with the conclusions of the previous chapter, is due to the dissociation of the product NADH from the ternary complex.

The temperature dependence of apparent Michaelis constants indicated a complicated behaviour. In the case of variable NAD, Table XIIB shows that the values increase generally with increasing temperature, but these did not yield linear Arrhenius plots and gave somewhat unreliable activation energies of 2 to 8 kcal mol⁻¹. This is due to the fact that there is a change of mechanism with the change of substrate concentration and also because Michaelis constants are composite of several rate constants, each of which is a function of temperature. However, the values are similar to those of 3-8 kcal mol⁻¹ for the association constants obtained by Gierer (131) with alcohol dehydrogenase in free solution. Besides, it is noteworthy that with the immobilized enzyme the activation energies for the apparent Michaelis constants for NAD decreased generally with increasing flow rate, and a value of 2 kcal mol⁻¹ was obtained at the high flow rate of 3.30 cm s⁻¹ when diffusional effects are unimportant. This value is also consistent with the activation energies of 1-3 kcal mol⁻¹ found for the apparent Michaelis constants for ethanol (in Table XIB) for which there is no diffusion control at any of the flow rates employed. In general, activation energies for K_m may be positive or negative but are expected to be small (145) and therefore a value of 2 ± 1 kcal mol⁻¹ for the diffusion-free Michaelis constants is quite reasonable.

Finally, it may be noted that the apparent Michaelis constants for either of the substrates do not show a regular decrease with increasing flow rate and at different temperatures, as would be expected on the basis of the Kobayashi-Laidler theory (70). This is not surprising for variable ethanol when diffusional effects are insignificant. In the case of variable NAD, however, the apparent Michaelis constants are functions of both flow rate and temperature. The results are also subject to a certain degree of inaccuracy because of the limitations in the extrapolation procedure in deriving the Michaelis parameters from the non-linear Lineweaver-Burk plots. Besides, there is the possibility that other factors, such as the electrostatic partitioning effects, as discussed in Chapter 3, may be involved with the substrate NAD so that the variation of its Michaelis constants with temperature and/or flow rate becomes even more unpredictable.

Conclusions

Extensive theoretical and experimental studies of the diffusional behaviour of simple molecules have shown that diffusion-controlled reactions occur at very high rates (153). A number of enzyme-catalyzed reactions have also been observed to be fast enough for the diffusion limit to apply (153-155). Immobilized enzymes behave differently and these have been treated by the Kobayashi-Laidler theory (70) for the flow kinetics of enzymes attached to the interior surface of tubes. This theory has been extended to the temperature effects in such systems, and has been applied to experimental results, notably by Daka and Laidler (146)

who observed complete diffusion control with lactate dehydrogenase attached to nylon tubing. Our results are also consistent with the theory and showed that there is only partial or substantial diffusion control for NAD in contrast to no diffusion control for ethanol with yeast alcohol dehydrogenase in similar systems.

The activation energy for complete diffusion control was obtained by extrapolation to zero concentration of NAD, and was found to be $4.2 \text{ kcal mol}^{-1}$. At higher concentrations, the situation is complicated by the simultaneous processes of diffusion and chemical reaction with a preponderance of the latter for which an activation energy of about 9 kcal mol^{-1} was obtained by extrapolation to infinite concentrations of both substrates. As a consequence, the reactions are slower than the diffusion process so that it is easier to find conditions in which there is no diffusion control. It is also concluded that flow rates have no significant effect on chemical processes, but the extent of diffusion control decreases with increasing flow rate as well as with increasing substrate concentration.

Temperature dependence of Michaelis parameters obtained from Lineweaver-Burk plots were also discussed. In general, the Lineweaver-Burk plots were non-linear, and with ethanol as the variable substrate, two different sets of results were obtained but these could not be explained conclusively. Activation energies for $V_{\max}$ were similar to the value of 9 kcal mol^{-1} for the chemical interaction at the surface, showing that the same rate constant is involved in the product formation

from the ternary complex. The apparent Michaelis constants did not follow the Arrhenius equation and gave somewhat unreliable activation energies of 2-8 kcal mol⁻¹ which are similar to those obtained by Gierer (131) for the enzyme in free solution. The results with the $K_{m,app}$ for NAD, however, decreased with increasing flow rate and showed an activation energy of 2 kcal mol⁻¹ at a high flow rate when there is no diffusion control. This is also consistent with the activation energies of 1-3 kcal mol⁻¹ for the diffusion-free $K_{m,app}$ values for ethanol.

CHAPTER 5

pH DEPENDENCE OF YADH KINETICS IN FREE SOLUTION AND
IN A TUBULAR FLOW REACTORIntroduction

The pH dependence of the rate of an enzymic reaction usually shows a bell-shaped profile as a result of activation and inactivation of the enzyme by hydrogen ions (89). A systematic study of the effects of pH on the rates and kinetic parameters of the reaction is very important for establishing the mechanism, which requires a knowledge of the amino acid residues involved. This information can be obtained, at least partly, from pH studies which enable one to evaluate the dissociation constants of certain ionizing groups, at or near the active centre of the enzyme, participating directly or indirectly in the catalysis (128,156). All the participating groups are not, however, necessarily revealed by the pH dependence of the kinetics, and only those groups are implicated whose change in state of ionization has some effect on the rate of reaction. These are the groups which ionize in the initial state and whose ionization affects the ease of formation of the activated state (157). It is to be noted that with an immobilized enzyme, the results of the pH dependence are, in general, different from those in free solution because of microenvironmental effects arising from electrostatic, partitioning and diffusional effects (89,91).

A number of workers reported the pH-dependent kinetics of alcohol dehydrogenase catalyzed reactions in free solution, but there is no general agreement about the nature and identity of the ionizing groups

involved in the catalysis. This is possibly because the enzymes and the experimental conditions used were not identical so that the results cannot be rationalized on a common basis. An advantage of working with an immobilized enzyme is that the same preparation can be used repeatedly in a series of kinetic experiments. This is conveniently done when an enzyme tube is used. Few kinetic investigations of the immobilized YADH have been made, except those by ourselves (123,147) on the flow kinetics and temperature effects with the enzyme attached to nylon tubing. These have been described in the previous chapters, and the present chapter illustrates further studies with the immobilized enzyme, on the pH dependence at various substrate concentrations and at a temperature of 25.5°C. Studies have also been carried out with the enzyme in free solution and in static systems, in order to compare the results with those found in the tubular reactor so as to be able to distinguish the effects of immobilization and of flow.

Theoretical Principles

Various workers have presented the theory of pH effect in enzyme kinetics, and have dealt particularly with one-substrate reactions in free solution. A complete kinetic analysis of the pH effects on enzymic reactions involving two substrates is too cumbersome, and therefore a very high concentration of one of the substrates has often been used to permit interpretation on the basis of equations describing a single-substrate system. Under this condition, the classical Michaelis-Menten equation holds (as shown in Chapter 3) and the pH dependence of the

kinetics is reflected in the overall rate as well as in the kinetic parameters, viz., the $V_{\max}$, K_m and $V_{\max}/K_m$ values. Kaplan and Laidler (158) developed equations showing the pH dependence of the Michaelis parameters in a form which distinguishes between essential and non-essential ionizing groups. The pH dependence of the overall rates was discussed by Krupka and Laidler (159) in terms of the nature of ionizations of the free enzyme and the enzyme-substrate complexes, taking into account the various possible rate-determining steps. It was shown that the acidic and basic side of the pH optimum correspond to the dissociation of the basic and acidic groups, respectively. In a recent review, Tipton and Dixon (156) considered the effects of pH both as a kinetic parameter and also as a way to identify specific ionizing groups. The ionization constants were obtained from the pH-activity profiles or by utilizing the steady-state kinetic parameters.

Ionization constants in enzyme systems are usually obtained graphically from plots of the common logarithms of the kinetic parameters against pH, generally known as Dixon plots (160). According to this method, the values of pK are found from the intersections of tangent lines. From theoretical considerations, it can be shown that the ionization constants obtained from k_c/K_m or $V_{\max}/K_m$ correspond to the free enzyme, while those from k_c or $V_{\max}$ correspond to the enzyme-substrate complex. Similar results can be obtained from plots of pK_m against pH; these show many patterns of behaviour since K_m has, in general, a more complex dependence than either k_c or k_c/K_m . Nevertheless, the pH profile of pK_m is often found to be less complex than the profiles of either

$\log_{10} k_c$ or $\log_{10}(k_c/K_m)$, since the pK_m plot is the difference between the latter two plots. However, these plots are reliable only when the ionizations of the dissociating groups are well separated by several units of pH. In such cases, fairly reliable estimates of the pKs may also be obtained by plotting the kinetic parameter itself against pH and noting the pHs at which it has half its maximum value. If the ionizations are not well separated, the dissociation constants can be obtained by the method of Alberty and Massey (161). This method is based on the derivation that the hydrogen ion concentration at the maximum activity is simply the geometric mean of the two dissociation constants, the latter values being obtained from the pHs at which a kinetic parameter has half its maximum value.

The same general principles as above are expected to apply to immobilized enzymes, but since these behave differently from those in free solution, the results may not be the same. Laidler and Bunting (91) pointed out that the partitioning of hydrogen ions between the solution and the immobilized enzyme microenvironment can very much influence the pH dependence of rate. This is particularly important if the enzyme-catalyzed reaction produces or consumes protons. When acid is being produced, the pH in the vicinity of the enzyme active-site will be less than that in the bulk solution so that the pH profile will be shifted towards the right; the converse conditions will lead to a shift of apparent pH optimum to a lower value. Hence, the dissociation constants obtained for the specific ionizing groups will be appreciably different.

This is even more possible if the immobilizing support contains charged groups. In such cases, the pH-activity profile of bound enzymes has been thoroughly investigated both from the experimental and theoretical points of view, and it has been noted by Engasser and Horvath (89) that the general shape of the pH profile of the bound enzyme is not affected but that it is shifted towards higher or lower pH values depending on whether the matrix is negatively or positively charged. Thus, the dissociation constant of an essential acidic group in the active centre of the enzyme can be decreased due to mutual electrostatic interaction with a negatively charged group on the support, and vice-versa (91). The pH dependence with immobilized enzymes has also been found to be affected by diffusional limitations for the protons, and in such cases, unusual pH profiles can be observed especially if the buffer concentrations are not high enough to facilitate the transport of protons (89,162). All these factors must therefore be taken into consideration in the interpretation of results from the pH dependence with immobilized enzymes.

Experimental Procedure

Kinetic experiments for the reaction between ethanol and NAD were carried out under two limiting conditions, with the concentration of each substrate in excess and the other one varied. All measurements were made both in free solution (static system) and also with a tubular flow reactor of 1 m in length, at $25.5 \pm 0.1^\circ\text{C}$ and over a pH range of 6.5 to 10.0. The buffers used were 0.1 M NaH_2PO_4 - Na_2HPO_4 and 0.1 M Na_2HPO_4 - Na_3PO_4 , containing 1.0 mM EDTA and 0.1 mM β -mercaptoethanol.

The kinetic procedure for the rate measurements with the tubular reactor is essentially the same as described in Chapter 3, and again the same enzyme-tube was used throughout the experiments. The enzyme-tube and the solutions prior to entering the reactor were thermostatted in a waterbath. The product was delivered to a flow cell connected to one end of the reactor by means of tygon tubing, while the substrate solution entered the other end with the help of a LKB Varioperpex II peristaltic pump. The reaction was followed by determining NADH absorbance at 340 nm in a Pye Unicam SP 1800 UV Spectrophotometer, and the rates under steady-state conditions were calculated using the absorption coefficient of $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$ (130).

In the case of free solution experiments, initial rates were measured in static systems. Each kinetic run was initiated by the addition of 10 μl of the enzyme solution (1 mg/ml buffer) to a thermostatted cuvette containing 2.5 ml of the substrate solution at the same pH. Before the start of a particular run, the reaction and the reference cuvettes (1 cm path length), both containing the same concentration of the substrate solutions in 0.1 M phosphate buffers of the desired pH, were kept inside the spectrophotometer for sufficient time to allow the system to attain the temperature equilibrium, after which the reaction was initiated by the enzyme. The observed initial velocity was obtained from the slope of a continuous trace recorded by the spectrophotometer. The full scale of the recorder was varied from 0.2 to 2.0 absorbance ranges in order to obtain a trace with a slope as close to 45° as possible in different runs.

It can be noted that the traces of absorbance vs time were usually linear for the first few minutes so that accurate initial rates were obtained.

Analysis of Data: Determination of Ionization Constants

The ionization constants for catalytically important groups were evaluated from the Michaelis parameters, the latter being derived from Lineweaver-Burk plots. The analysis is based on the fact that the pH variation of a kinetic parameter y often follows an equation of the form

$$y = C \left(1 + \frac{K_a}{[H^+]} + \frac{[H^+]}{K_b} \right) \quad (16)$$

where C is a pH-independent constant, and K_a and K_b are the dissociation constants for acidic and basic groups, respectively, at the active centre of the enzyme (128,156,163). The kinetic parameter y can be $K_m/V_{\max}$, in which case the dissociation constants relate to the free enzyme. It can also be $1/V_{\max}$, when the constants are related to the enzyme-substrate addition complex or some other intermediate. The Michaelis constant K_m itself shows more complex behaviour, which has been analyzed in terms of Dixon's plot (160); however, this procedure sometimes leads to difficulty because of masking of the dissociation constants. We have therefore carried out the analyses of the data using $K_m/V_{\max}$ and $1/V_{\max}$. In general, any graphical method such as the logarithmic plots of the kinetic parameters against pH gives only approximate values for the dissociation constants, and for more reliable estimates, a statistical treatment is necessary.

Statistical analyses of the pH dependence of kinetic parameters have previously been made by Wilkinson (164), Hinberg (157) and by Hinberg and Laidler (165). The present treatment, also based on the method of least squares, is somewhat simpler. The deviation D_i of the experimental value y_i of the parameter y from the value calculated by using Eqn. (16) is given by

$$D_i = y_i - C - \frac{CK_a}{[H]_i} - \frac{C[H]_i}{K_b} \quad (17)$$

so that

$$\sum_{i=1}^n D_i^2 = \sum_{i=1}^n \left(y_i - C - \frac{CK_a}{[H]_i} - \frac{C[H]_i}{K_b} \right)^2 \quad (18)$$

In order to satisfy the least-squares criterion for the best fit, that the sum of the squares of the deviations be minimized, Eqn. (18) can be set equal to zero, and the partial derivative of this with respect to K_a can be written as

$$\frac{\partial \sum_{i=1}^n D_i^2}{\partial K_a} = 2 \sum_{i=1}^n \left(-\frac{C}{[H]_i} \right) \left(y_i - C - \frac{CK_a}{[H]_i} - \frac{C[H]_i}{K_b} \right) = 0 \quad (19)$$

Similarly, after partial differentiation with respect to K_b , we have

$$\frac{\partial \sum_{i=1}^n D_i^2}{\partial K_b} = 2 \sum_{i=1}^n \left(\frac{C[H]_i}{K_b^2} \right) \left(y_i - C - \frac{CK_a}{[H]_i} - \frac{C[H]_i}{K_b} \right) = 0 \quad (20)$$

Solving Eqns. (19) and (20) therefore yields

$$C = \frac{\sum_{i=1}^n (y_i/[H]_i)}{\sum_{i=1}^n (1/[H]_i) + n/K_b + K_a \sum_{i=1}^n (1/[H]_i)^2} = \frac{\sum_{i=1}^n (y_i[H]_i)}{\sum_{i=1}^n [H]_i + \frac{1}{K_b} \sum_{i=1}^n [H]_i^2 + nK_a} \quad (21)$$

where n is the number of data points. Equation (21) shows a relationship between K_a and K_b , and partial differentiation of Eqn. (17) with respect to C gives

$$\sum_{i=1}^n (y_i - C - \frac{C[H]_i}{K_b} - \frac{CK_a}{[H]_i}) (1 + \frac{K_a}{[H]_i} + \frac{[H]_i}{K_b}) = 0 \quad (22)$$

Substitution of the values of C and K_a (or K_b) from Eqn. (21) into Eqn. (22) gives a quadratic in K_b (or K_a) after re-arrangement, and this can be solved for K_b (or K_a); the two dissociation constants are thus obtained. A computer programme in FORTRAN IV language was used to perform the lengthy calculations (see Appendix).

The approximate 100(1-q)% confidence contours, as described by Draper and Smith (166), were determined by finding the values (K_a , K_b) which satisfy the equation

$$\sum_{i=1}^n D_i^2(K_a, K_b) = \sum_{i=1}^n D_i^2(\bar{K}_a, \bar{K}_b) [1 + \frac{p}{n-p} F(p, n-p, 1-q)] \quad (23)$$

Here, $\bar{K}_a$ and $\bar{K}_b$ are the dissociation constants obtained by the least-squares treatment, the parameter p is the number of variables, which is 2 in the present case, and C is the value corresponding to the least-square

$\bar{K}_a$ and $\bar{K}_b$ values. Standard statistical tables give the necessary information about the F-distribution. The right-hand-side of Eqn. (23) is therefore known, while the left-hand-side is given by Eqn. (18) into which the value of C has been substituted. Equation (23) therefore yields an expression involving only the two unknowns K_a and K_b , and this can be re-arranged to give a quadratic equation in either K_a or K_b . From the least-squares estimates of $\bar{K}_a$, the quadratic then yields the two limiting values of K_b , and vice-versa.

The analysis is simpler when, as with excess NAD and variable ethanol in the enzyme-tube, there is no fall-off of rate at high pH. In that case, $K_a = 0$ and Eqn. (16) reduces to a linear form; a simple least-squares treatment can then be applied.

Results for the pH-Dependence of YADH Kinetics

Figures 17(a) and (b) show plots against pH of initial rates at different substrate concentrations for the enzyme in free solution. Fig. 17(a) gives the results with ethanol at 500 mM, and Fig. 17(b) with excess NAD at 5.0 mM. In the case of the immobilized enzyme, two different sets of measurements were made at flow rates of 0.30 cm s^{-1} and 0.90 cm s^{-1} with the saturating concentration of ethanol, and with the NAD concentration varying from 0.10 to 1.00 mM for the pH range of 6.5 to 10.0. Another set of experiments was done at the flow rate of 0.90 cm s^{-1} with excess NAD, the alcohol concentration being varied from 5.0 to 50 mM and over the same pH range. The pH-activity profiles for the apparent rates with the immobilized enzyme are shown in Figs. 18(a) and (b). Figure 18(a) is for

Figure 17. pH-dependence of initial rates at 25.5°C and different substrate concentrations as indicated, for yeast alcohol dehydrogenase in free solution with (a) excess ethanol at 500 mM and (b) excess NAD at 5.0 mM.

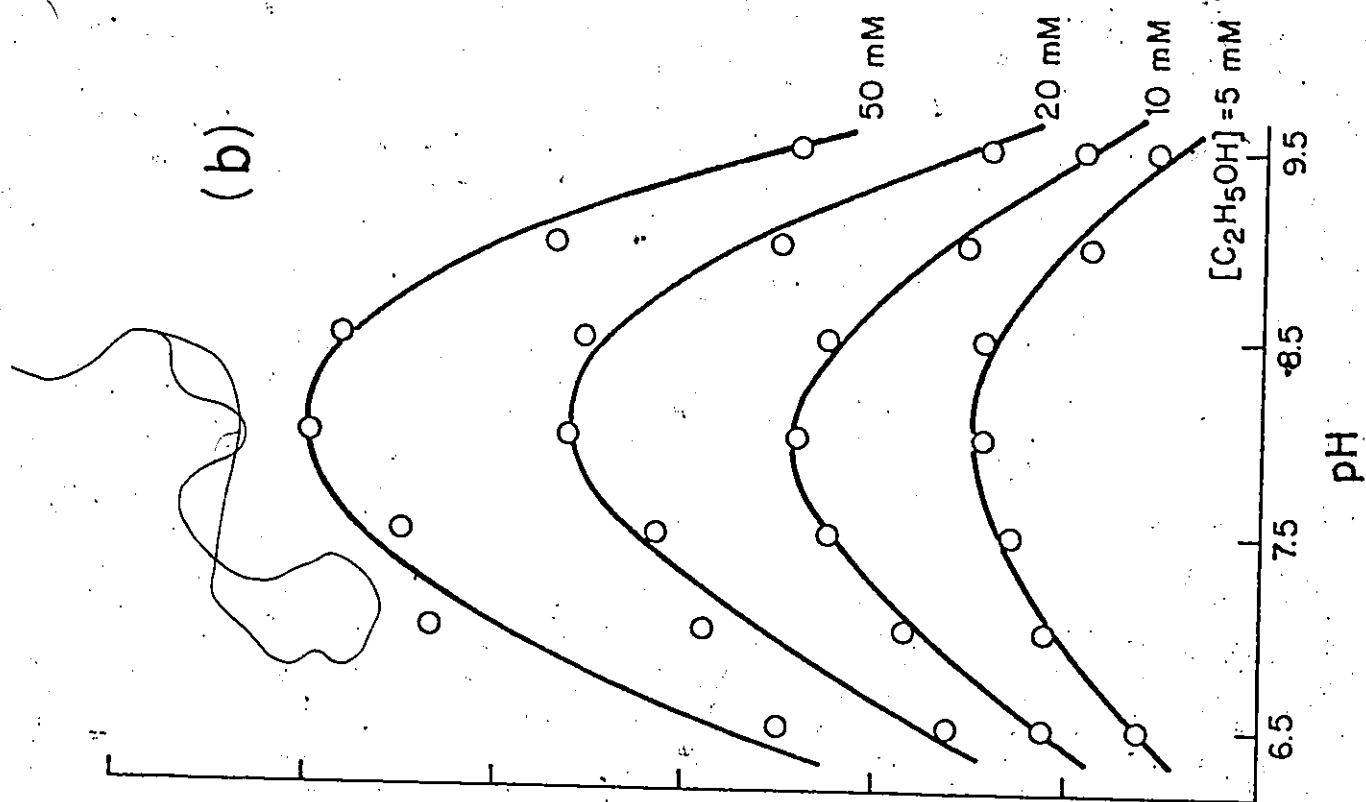
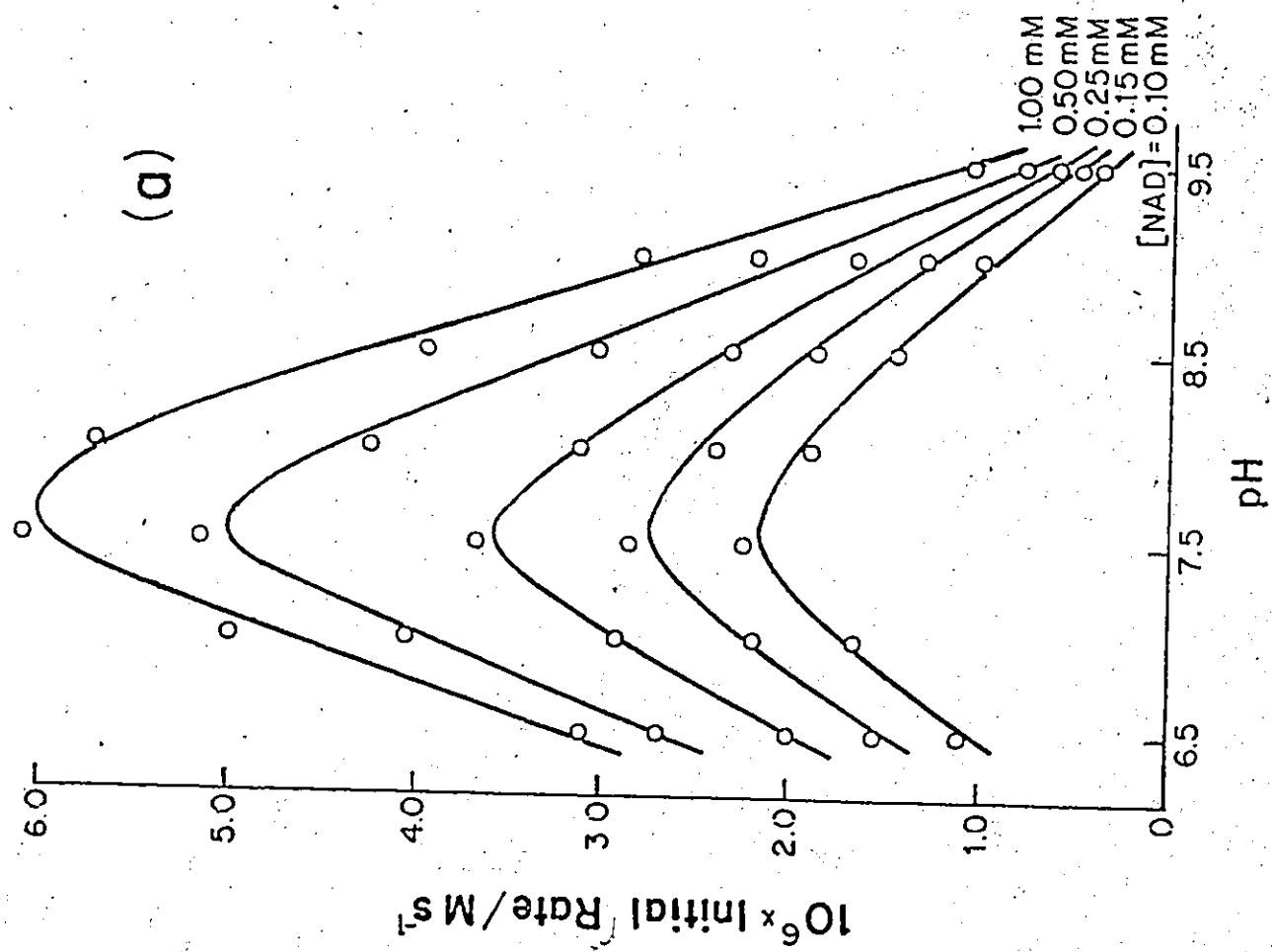
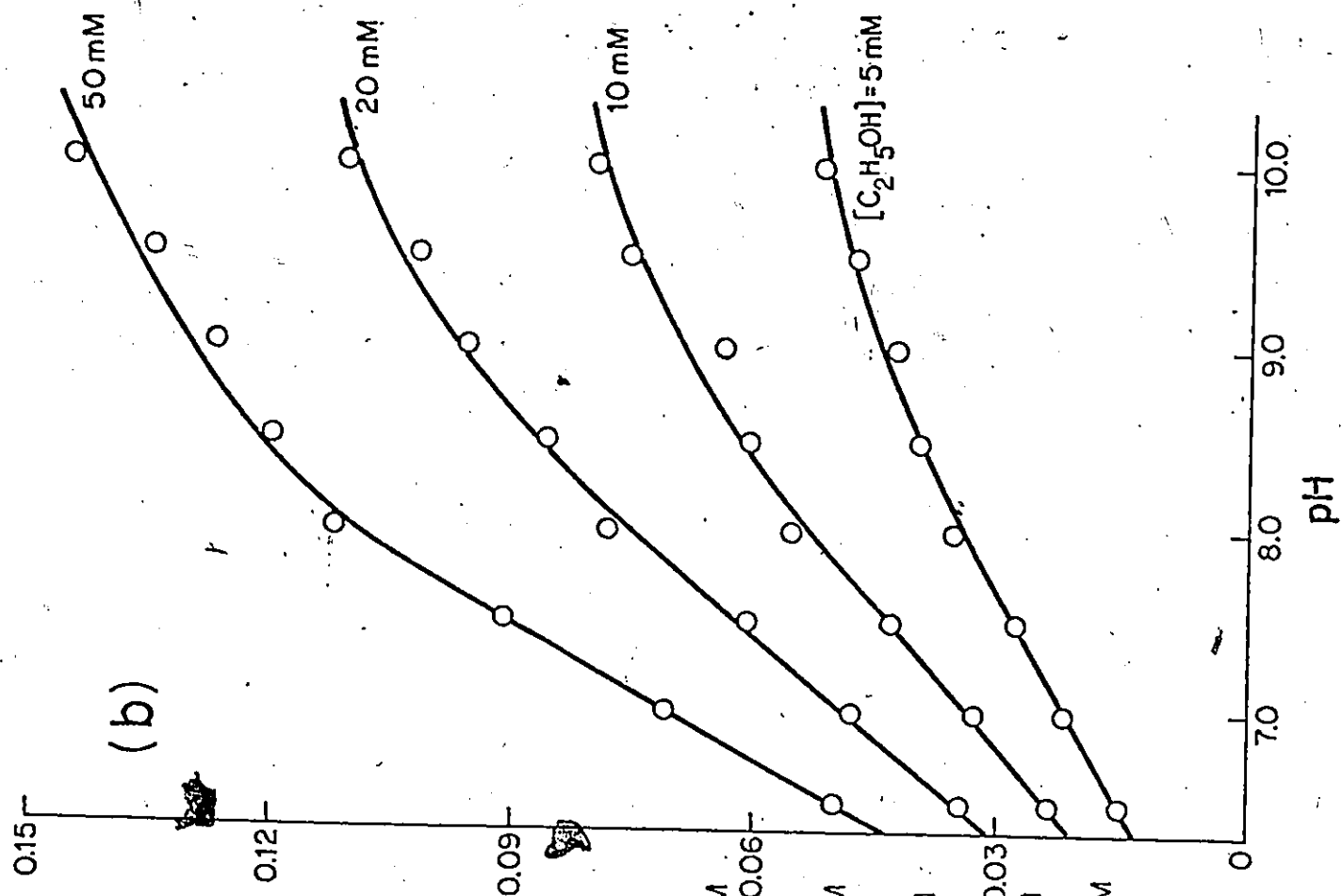
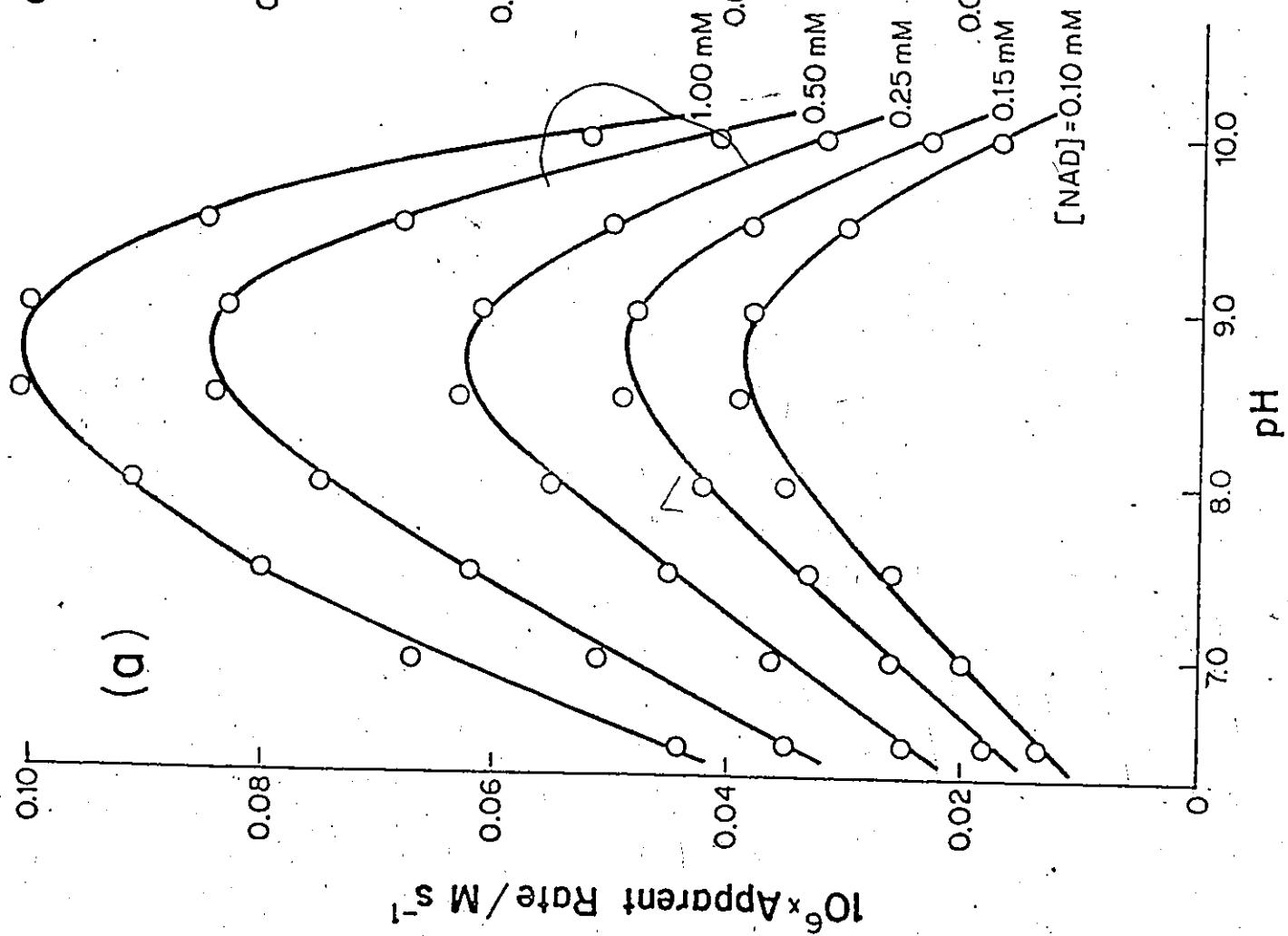


Figure 18. pH-dependence of apparent rates at 25.5°C and different substrate concentrations as indicated, for YADH attached to nylon tubing and at a flow rate of 0.90 cm s^{-1} , with (a) excess ethanol at 500 mM and (b) excess NAD at 5.0 mM.



the higher flow rate with excess ethanol, and Fig. 18(b) is for excess NAD.

The Michaelis parameters are listed in Tables XIII and XIV for the data with the enzyme in free solution and with the immobilized enzyme, respectively. These values were obtained from Lineweaver-Burk plots, a typical example being shown in Fig. 19 for the immobilized enzyme in the tubular reactor, at a flow rate of 0.30 cm s^{-1} and at various pH values as indicated. All these plots for the enzyme both in free solution and in the tubular flow reactor were linear over the ten-fold concentration range of 0.10-1.00 mM NAD when ethanol was held in excess at 500 mM, and with variable ethanol in the range of 5-50 mM when the saturating concentration of NAD was 5.0 mM. The Michaelis parameters noted are the average values based on the data for several runs covering the range of substrate concentrations.

The Michaelis parameters were used to obtain the ionization constants of catalytically important groups. Approximate values of the ionization constants were first obtained by graphical methods, viz., from the pHs at half maximal values of the rates and Michaelis parameters, or from the Dixon plot of the common logarithms of the Michaelis parameters against pH. In all cases, two different ionizing groups were implicated except for the immobilized enzyme with excess NAD and at a flow rate of 0.90 cm s^{-1} . More accurate and reliable values of the dissociation constants were, however, determined by computerized curve-fitting involving the statistical least-squares treatment already described, and these results are summarized in Table XV for different conditions. The pK values are the negative

Table XIII. Michaelis parameters with yeast alcohol dehydrogenase in free solution, at various pH and temperature 25.5°C.

pH	Excess $[C_2H_5OH]=500$ mM		Excess $[NAD]=5.0$ mM	
	$10^6 V_{max}/M\ s^{-1}$	$K_m(NAD)/\mu M$	$10^6 V_{max}/M\ s^{-1}$	$K_m(C_2H_5OH)/mM$
6.5	4.11	269	3.60	23.0
7.0	6.33	284	6.11	22.3
7.5	7.37	234	6.34	19.4
8.0	6.56	255	6.99	19.1
8.5	4.41	207	6.40	17.3
9.0	3.19	220	5.53	25.2
9.5	1.12	194	3.36	25.2

Table XIV. Michaelis parameters with immobilized YADH in a tubular flow reactor, at various pH and temperature 25.5°C.

pH	Excess ethanol, Flow rate=0.30 cm s ⁻¹		Excess ethanol, Flow rate=0.90 cm s ⁻¹		Excess NAD, Flow rate=0.90 cm s ⁻¹	
	10 ⁶ V _{max} /M s ⁻¹	K _{m,app} (NAD)/μM	10 ⁶ V _{max} /M s ⁻¹	K _{m,app} (NAD)/μM	10 ⁶ V _{max} /M s ⁻¹	K _{m,app} (C ₂ H ₅ OH)/mM
6.5	0.042	190	0.058	334	0.065	16.8
7.0	0.056	185	0.084	319	0.086	15.1
7.5	0.069	182	0.098	282	0.110	14.8
8.0	0.082	182	0.105	210	0.137	14.0
8.5	0.095	181	0.118	205	0.147	13.5
9.0	0.093	190	0.117	214	0.155	13.1
9.5	0.078	194	0.101	240	0.170	12.7
10.0	0.061	195	0.065	275	0.179	12.2

Figure 19. Lineweaver-Burk plots for the data with YADH attached to nylon tubing, at a flow rate of 0.30 cm s^{-1} and temperature 25.5°C , with excess ethanol at 500 mM and at various pH as indicated.

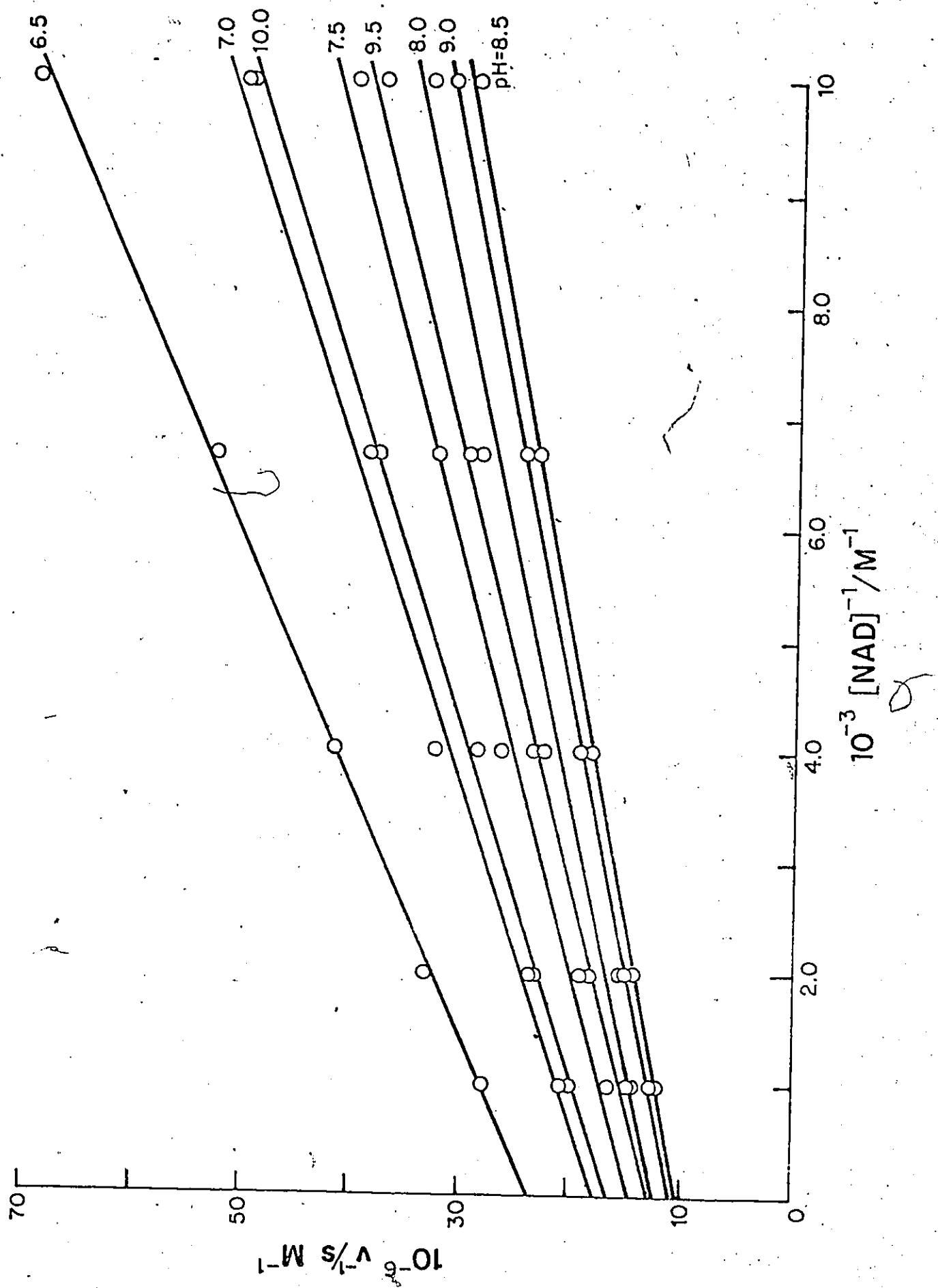


Table XV. Values of pK under different conditions, as obtained by the least-squares treatment.

$-\log_{10}$ (Dissociation Constant, K)	YADH in Free Solution		YADH Attached to Nylon Tubing	
	Excess C_2H_5OH (Static System)	Excess NAD (Static System)	Excess C_2H_5OH (Flow Rate=0.30 $cm\ s^{-1}$)	Excess NAD (Flow Rate=0.90 $cm\ s^{-1}$)
pK_a^E	8.8	8.9	9.9	9.6
pK_b^E	6.6	7.0	6.9	7.1
pK_a^{ES}	8.5	9.1	9.9	9.8
pK_b^{ES}	6.8	6.8	6.9	6.8
				6.7

(Note: The average values of the 95% confidence interval limits for each pair of pK values and in each column separately were less than or equal to ± 0.3 units).

logarithms of the respective dissociation constants, and are those for the enzyme itself in presence of saturating concentrations of either of the substrates, which are referred to as pK^E , and for the ternary complex of the enzyme with both substrates, as indicated by pK^{ES} . These are rounded-off at the first place after the decimal point and the average values of the 95% confidence interval limits are noted.

Discussion of Results for the Enzyme in Free Solution

The pH-Activity Profiles

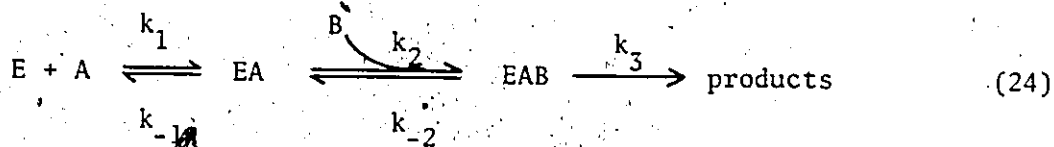
Figure 17 shows clearly that the rate passes through a maximum at each of the various substrate concentrations whether NAD or ethanol is held in excess with the enzyme in free solution. This gives a pH optimum of 7.5 with variable NAD and of 8.0 with variable ethanol. A broad optimum over the pH range of 8.4 to 9.5 was reported by Wenger and Bernofsky (52) for commercial yeast alcohol dehydrogenase, whereas Wallenfels and Sund (167) obtained a value of 8.6 for crystalline YADH. The difference can be ascribed to the purity of the enzymes, and our results suggest further that the experimental conditions are also important in determining the pH optimum.

For a particular enzyme, the difference in pH-activity profiles and in pH optimum can arise from a number of causes, as discussed earlier in this chapter and also in Chapter 1. In the case of the enzyme in free solution, there is no partitioning or diffusional effect, and the slightly higher pH optimum of 8.0 found with excess NAD compared to that of 7.5 with excess ethanol may be due to microenvironmental effects

resulting from electrostatic interaction, for example, with a negatively charged group in or near the enzyme active-site itself. This will give rise to differences in the dissociation constants of catalytically important groups, and may also result from the compulsory order of substrate binding, as will be discussed later.

pH-Dependence of Michaelis Parameters

It is important to note that the kinetic mechanism of the action of alcohol dehydrogenases is in general random, but becomes ordered when the enzyme is saturated with either of the substrates. For yeast alcohol dehydrogenase in free solution, Klinman (168) showed that the mechanism involves a ternary complex, and therefore the reaction can be represented as



where A is the first and B is the second substrate. Klinman (168) also concluded from various lines of evidence that under saturating conditions the rate-controlling step is the breakdown of the ternary complex EAB. In our experiments, the fixed concentration of either of the substrates when the other was varied was sufficiently high to maintain saturation at all pH values, and the above conclusions also apply.

The rate expression for the scheme (24) can be derived and the kinetic parameters can be utilized to obtain binding and dissociation rate constants. The second-order rate constant k_1 for the binding of the

enzyme and the first substrate can be determined from the observed $V_{\max}$ and K_m values, the latter being referred to as the Michaelis constant for the first substrate with respect to the infinitely high concentration of the second one. For compulsory ordered kinetics, Suhadolnik and Lennon (169) noted that $V_{\max}/K_m$ is, in fact, an estimate of the binding constant k_1 of the enzyme reaction with NAD while $V_{\max}$ itself reflects the dissociation rate constant k_3 . The Michaelis constant K_m for the first substrate is expressed by the ratio $(k_{-1} + k_3)/k_1$ which reduces to the dissociation constant k_{-1}/k_1 , provided k_3 is rate-limiting.

Table XIII shows considerable degree of pH dependence of the Michaelis parameters derived from the experimental data in free solution. In general, both $V_{\max}$ and K_m values are affected by pH, and therefore hydrogen ions can be regarded as mixed or non-competitive effectors of the enzyme (156). For both ethanol and NAD in excess, the $V_{\max}$ goes through a maximum at pH values 7.5 and 8.0, respectively, while the Michaelis constant shows more complex behaviour especially for those of NAD. This is reasonable since the K_m values are functions of several rate constants, each of which may be affected by pH. If, however, the ratio $V_{\max}/K_m$ is considered then this is also found to go through a maximum at similar pHs as those for the $V_{\max}$ results, thus indicating that the binding as well as the dissociation rate constants k_1 and k_3 are favoured approximately at the same pH, particularly with excess ethanol.

Ionization Constants of Catalytically Important Groups

For the enzyme in free solution, it can be seen from Table XV that the pK_a^E and pK_b^{ES} values are independent of whether NAD or ethanol is in

excess, whereas the pK_b^E and pK_a^{ES} values are considerably higher in the presence of excess NAD compared to those with excess ethanol. In general, the pK of a group responsible for the enzymic activity with respect to one substrate is expected to be perturbed as a result of the saturation by the other. The dissociation constants for a group in the free enzyme and in the enzyme-substrate complex, in the presence of a particular substrate in excess, may also differ either because the binding of the second substrate changes the dissociation of the relevant group, or because the constants refer to different ionizations. This is understandable since it is not necessary that the protonation or deprotonation of the same group that involves substrate binding by the free enzyme will be responsible for the reaction of the enzyme-substrate complex once formed (156). However, since pK_b^E but not pK_a^E , and pK_a^{ES} but not pK_b^{ES} , are altered it appears that the order of substrate binding is particularly important.

The mechanism of the YADH-catalyzed reaction is essentially an ordered one when one of the substrates is in large excess, but the order of substrate addition to form the ternary complex may be different. The results in Chapters 3 and 4 as well as those of other workers have shown that the substrate NAD is first added to the enzyme. Accordingly, if this binding occurs through the protonated form of a basic (e.g., amino) group, its dissociation constant will be decreased; this effect would be more significant with excess NAD than with excess ethanol. Once the enzyme-NAD addition complex is formed, the effect of the binding of ethanol on the dissociation of the acidic group (presumably a carboxyl group) will be similar for excess NAD and excess ethanol, as reflected in

almost identical pK_a^E values. This explains why the pK_b^{ES} values are also the same since the addition of the second substrate, i.e., ethanol, is now involved in the formation of the ternary complex, but not of NAD which is already attached to the enzyme through the basic group. As a result, however, the pK_a^{ES} values are significantly altered, the dissociation being favoured with excess ethanol and decreased with excess NAD since it is possible that the deprotonated form of the acidic group is required for the binding of ethanol. Similar arguments can be put forward for the binding of ethanol first with the enzyme in a way that pK_b^E and pK_a^{ES} are decreased, i.e., the corresponding dissociations are favoured, and that the pK_a^E and pK_b^{ES} values remain similar with either of the substrates in excess. Therefore, the results for the ionization constants of catalytically important groups involved in the free solution kinetics are again consistent with the fact that the mechanism is essentially ordered, but which of the substrates forms the binary complex first with the enzyme remains uncertain. It is, however, clear that two different ionizing groups, one with a pK of 6.6-7.0 and another of 8.5-9.1, are involved in the catalytic process.

Discussion of Results for the Immobilized Enzyme

The Patterns of pH Behaviour

Two different kinds of pH behaviour were observed for the immobilized enzyme, as shown in Figure 18. With saturating ethanol and variable NAD concentrations, when the rate also passes through a maximum and falls at both high and low pH values, as in free solutions, the entire profile is

shifted towards the right showing a pH optimum between 8.5 and 9.0. On the other hand, with saturating concentrations of NAD and variable ethanol, there is no fall-off at high pH values and the pH optimum appears to have been shifted further upwards. This optimum could not be ascertained since the instability of the substrate NAD at higher pH values (170) did not allow rate measurements to be carried out in order to establish any fall-off in the activity on the alkaline side of the maximum. However, differences in pH optimum and in the pH-activity profiles with the immobilized enzyme can result from electrostatic, partitioning and/or diffusional effects.

When the enzyme is immobilized, the presence of charged groups on the supporting material can produce marked and even more pronounced changes in the pH-activity profiles compared to those for the enzyme in free solutions. In the present system, this is possible if all the carboxyl groups released in the partial hydrolysis of the nylon peptide bonds were not blocked by the reaction with benzidine (see Chapter 2). However, the effect of electrostatic interaction with a negatively charged group either in the support or in the enzyme itself would result in a decrease in the apparent Michaelis constants, and the extrapolated K'_m values at infinite flow rates should correspond with the intrinsic values in free solution. The results obtained for the K'_m (NAD) were higher by two orders of magnitude (see Chapter 3) and therefore suggested different possibilities such as electrostatic interaction with a positively charged group in the enzyme itself. This point will be returned to later, and for the pH-activity profiles the diffusional effects, such as those discussed in Chapters 3 and 4, must be considered.

The effect of diffusional limitations is particularly indicated in the pH-activity profiles of the immobilized enzyme with excess NAD (Fig. 18b) which shows not only the shift but also some changes in shape of the profile in that it flattens or rises continuously with increasing pH. The experimentally observed flattening of the pH profile has been conveniently explained by proton accumulation in the microenvironment of the immobilized enzyme. For example, the kinetics of ester hydrolysis with immobilized enzymes have been studied by Goldman et al. (171), who postulated that proton accumulation is responsible for profound changes in catalytic behaviour. Similarly, Ngo and Laidler (145) observed a continuous increase in rate with increasing pH for acetylcholinesterase immobilized in polyacrylamide gels, in contrast to the bell-shaped behaviour with the free enzyme. Other workers also suggested that anomalies will arise as a result of local pH changes during substrate hydrolysis. Our results with excess NAD and variable ethanol found with the immobilized YADH are similar to those with papain in collodion (171,172) and with membrane-bound acetylcholinesterase (145,173).

The results obtained with excess ethanol and variable NAD show the ordinary bell-shaped profile (Fig. 18a) with the immobilized YADH. In terms of a theoretical model presented by Engasser and Horvath (162), this represents the pH profile of the enzyme without diffusional limitations and at a very low proton modulus, the latter characterizing the interplay between the rates of proton generation and transport, therefore expressing the relative importance of diffusion resistances for the proton. The results in Chapters 3 and 4 have shown that the overall rate is partly

diffusion controlled with respect to NAD. Engasser and Horvath (89,162), however, suggest that at the saturation rate, the diffusion of the substrate has no effect on the rate and that only the diffusion of protons formed in the reaction has to be considered. Under these conditions, buffers may also play a dynamic role in acting as carriers for the hydrogen ions, thus facilitating their transport. The transport facilitating effect of a proton acceptor such as a buffer manifests itself in a reduction of the diffusion resistance for protons and also by increasing the effective proton transport coefficient by several orders of magnitude. This ability of buffers to facilitate the molecular or convective diffusion of protons arises from their capacity for binding H^+ reversibly. The buffer-mediated transport of H^+ to or from the catalytic site can therefore attenuate the degree of proton accumulation or depletion, thus causing changes in the pH behaviour.

In our experiments with excess ethanol and variable NAD, it is possible that the proton transport is facilitated to some extent by the readily available buffer anions so that there is not much change of local pH. The intrinsic bell-shaped profile, unaffected by the diffusional resistance to the proton, is thus obtained. On the other hand, with excess NAD at 5.0 mM and variable ethanol, the buffer-mediated proton transport is relatively less important, since the concentration of the buffer anions, especially in the microenvironment of the enzyme active-site, can be significantly reduced, owing to the large excess of the positively charged substrate NAD, to affect the proton transport. In other words, the very

high concentration of the positively charged NAD makes the buffer anions much less available for the transport of protons. As a result, H^+ accumulates at the surface and the activity plateaus, indicating that the surface pH becomes independent of the bulk pH. This is equivalent to saying that as the external (bulk) pH is raised, the increased rate of enzymic activity causes a decrease in the local pH which tends to decrease the observed rate, thus leading to the flattening of the pH-activity curve.

A theoretical treatment by Ngo and Laidler (145) for the data with immobilized acetylcholinesterase has shown that there is a considerable lowering of local pH as a result of acid released in the hydrolysis. The existence of local pH gradients of several units generated by an enzyme-catalyzed reaction was directly demonstrated by Goldman and coworkers (171). It is therefore evident that the transport of the H^+ generated at the enzyme active-site to the bulk medium, and the role of the buffers in facilitating this transport, are very important in determining the pH-activity behaviour of immobilized enzymes. This is particularly so with charged substrates which can alter the effective concentration of buffers, although the local change of pH can be significant even with neutral substrates and in the presence of buffers. Hence, the continuous increase or flattening of the pH-activity profile with excess NAD and variable ethanol, in contrast to the bell-shaped profile with variable NAD and excess ethanol, can be accounted for by the diffusional resistances to the protons generated in the reaction as well as by the lack of buffer-mediated proton transport in the former case.

pH-Dependence of Michaelis Parameters

The Michaelis parameters for the immobilized enzyme as shown in Table XIV are remarkable. It was concluded in Chapters 3 and 4 that the degree of diffusion control with variable NAD is greatest at low substrate concentrations and at low flow rates. Changes of pH will not have much effect on diffusion constants, since it is the chemical reaction which is affected by pH but not the diffusion process. It is therefore significant that at low flow rates the K_m values are little affected by pH, as indicated by the results at 0.30 cm s^{-1} flow rate and with excess ethanol. Under these conditions, K_m values also represent k_{-1}/k_1 , and there is relatively less dependence on pH of $V_{\max}$ which reflects the dissociation rate constant k_3 . The results at higher flow rate, however, show a stronger pH dependence. Between pH 8.5 and 9.0, the rates are at a maximum, but the Michaelis constant passes through a minimum, because the enzyme-substrate ternary complex decomposes most readily into products. In this case, there is very little or no diffusion control, and the Michaelis constant is no longer affected by the diffusion constant and depends significantly on the relative magnitudes of the different rate constants, particularly on the dissociation rate constant k_3 . It is also notable that at pH between 8.5 and 9.0 the values of $V_{\max}/K_m$ and hence the binding constant k_1 go through a maximum with excess ethanol at both flow rates.

The results for the Michaelis parameters with variable ethanol and saturating concentrations of NAD are different from those discussed above. The value of $V_{\max}$ now increases and the Michaelis constant

decreases steadily with increasing pH, in contrast to the behaviour with variable NAD and excess ethanol. This indicates that when NAD is saturating the enzyme, the binding of the substrate as well as the dissociation of the ternary complex into products is favoured at more alkaline pH values than those required with excess ethanol. As explained earlier, the behaviour with excess NAD is due to the slowness of proton transport and the lack of buffer-facilitated diffusion. It can be noted that with the immobilized enzyme, the mechanism also involves the formation of the enzyme-substrate addition complex and that the rate-controlling step is the breakdown of the ternary complex. Therefore, the same general principles as those discussed for the pH dependence of the Michaelis parameters with the enzyme in free solution can also apply to the immobilized system.

Ionization Constants of Catalytically Important Groups

In the case of the immobilized enzyme, the results in Table XV. also indicate that there are two ionizing groups that play a role in the reaction mechanism. At the higher flow rate, the dissociation constants for the acidic group were not obtainable for excess NAD since there was no fall-off in the rate on the alkaline side of the pH maximum. The pK of the basic group for the free enzyme varies from 7.1 with excess ethanol to 6.9 with excess NAD, and that of the basic group is 9.6 with excess ethanol. Both with excess ethanol and excess NAD there appears to be a small downward shift of the pK_b values, but an upward shift for the pK_a with excess ethanol, when the ternary complex is formed. The pK value of 6.8 for the basic group of the enzyme-substrate ternary complex with

excess ethanol only changes to 6.7 with excess NAD, both at the higher flow rate. These differences are too small and are within experimental errors, but if significant they may be due to perturbation or saturation by one or the other substrates. However, the results obtained at the lower flow rate of 0.30 cm s^{-1} and with excess ethanol show that the dissociation constants for the groups in the free enzyme are not altered from those in the enzyme-substrate ternary complex. Hence, it is reasonable that the same groups are also involved at the higher flow rate. The lower pK_b of 6.8 for the ternary complex compared to that of 7.1 for the free enzyme in the presence of excess ethanol suggests that the dissociation has been favoured by the binding of the second substrate.

The difference in pK values for the acidic group is also not very significant. Only in the case of excess ethanol is there a considerably lower pK_a of 9.6 for the free enzyme at the higher flow rate, compared to 9.9 at the lower-flow rate, with the immobilized enzyme. The pK of this group changes from 9.6 for the free enzyme to 9.8 for the ternary complex at the higher flow rate. If this change is significant, it can be understood provided the group is involved in the binding of ethanol since the deprotonated form can be stabilized by interaction with the hydroxyl group of ethanol, which in turn can assist the release of a proton in the reaction. This interaction will be much less important at the lower flow rate, when diffusion control plays a significant role. Therefore, the ionization constants of the relevant groups are now unaffected as seen in Table XV.

The ionization constants obtained for the acidic and basic groups of the free enzyme in the presence of excess ethanol are slightly different at the two flow rates. While the pK of the basic group increases from 6.9 to 7.1, that of the acidic group decreases from 9.9 to 9.6, as the flow rate is increased from 0.30 cm s^{-1} to 0.90 cm s^{-1} . Again, the differences are quite small but can possibly be explained by the fact that as the rate approaches the diffusion-controlled limit the substrate binding can no longer be in equilibrium, as demonstrated by Brant et al. (174) for the rapid action of fumarate hydratase. Alternatively, Tipton and Dixon (156) suggested that if the enzyme contains more than one active form the mechanism will give two pK values for the free enzyme and also two distorted values. If, however, all forms of the enzyme combine with substrate to the same extent, then only the two distorted values will be observed but the shift will be symmetrical, with pK_a lowered as much as pK_b is raised. Thus, the dissociation constants for the enzyme itself in the presence of excess ethanol and variable NAD with the immobilized system are also consistent with the extent of diffusion control, as well as with the presence of microheterogeneity of enzyme forms, the latter being suggested in Chapter 4 to explain the non-linear Lineweaver-Burk plots especially with variable ethanol (see Fig. 16). For the present purpose, however, the dissociation constants for the acidic and basic groups in the immobilized enzyme may be considered to remain the same under different conditions.

The results for the enzyme in free solution and those for the immobilized enzyme show notable difference. It is evident from Table XV

that the pK_a^E and pK_a^{ES} values are significantly increased upon immobilization. The pK_b^E and pK_b^{ES} results for the immobilized enzyme are more or less the same as for the enzyme in free solution, particularly with excess NAD. The dissociation of the basic group is therefore affected by similar factors such as any electrostatic interaction of the substrate NAD or its compulsory binding with the enzyme prior to the binding of ethanol, as discussed previously. The effect on the acid dissociation constant as a result of immobilization indicates that there are some negatively charged groups in the supporting nylon structure. The presence of such charges in the close proximity of the active-site acidic group can repress the dissociation of the latter, thereby increasing the pK_a .

The interaction between a negative charge in the support and an acidic group in the active site of the enzyme can occur independently, and without affecting the basic group, but we are faced again with the problem that the K_m' values for NAD (as obtained in Chapter 3) were much higher than the intrinsic values of 5-10 μM obtained by Theorell et al. (132) with the liver enzyme in free solution. However, it is well known that the yeast and liver enzymes have different substrate specificities. With YADH in free solution, Wratten and Cleland (134) found a K_m (NAD) value of 74 μM , and our results in Table XIII give values of 200-300 μM which are similar to the K_m' values of 230-260 μM obtained in Chapter 3. It is therefore not difficult to rationalize the results for the acid dissociation constants of the immobilized enzyme in terms of electrostatic interaction with some negatively charged groups in the support. Such charges can exist if all the carboxyl groups released during the partial

acid hydrolysis of the nylon peptide bonds are not blocked by benzidine, as discussed earlier. This could also produce a deleterious effect on the stability of the immobilized enzyme preparation, but the introduction of benzidine which serves as a 'spacer' prevents any strong interaction with the support so that the stability and hence activity are not greatly reduced, as shown in Figure 7.

Finally, it is also possible that the presence of positively charged groups either in the support or in the active site itself can affect the dissociation of the catalytically important groups. The existence of positively charged groups in the supporting nylon structure is not likely, while that in the active site of the enzyme would result in changes in dissociation constants for both acidic and basic groups, even in free solutions. This interaction is therefore of negligible importance, and the differences in pK_b^E and pK_a^{ES} with saturating concentrations of either substrate in free solution confirm the compulsory ordered kinetics which, in view of the previous results, appears to be due to the binding of NAD prior to ethanol. However, the nature of the ionizing groups remains speculative, but it seemed reasonable that the protonated form of a basic amino group and of a deprotonated form of an acidic carboxylic group are required for the binding of NAD and ethanol, respectively.

Concluding Remarks

It follows from the above results and discussion that a basic group whose pK varies between 6.6 and 7.0 due to saturation by or addition of one or the other substrate is responsible for substrate binding as well

as for the subsequent reaction of the enzyme-substrate ternary complex into products in free solution. In addition, an essential acidic group of pK varying from 8.5 to 9.1 has been implicated under similar conditions. The pK of the basic group ranges from 6.7 to 7.1 and that of the acidic group from 9.6 to 9.9 for the enzyme attached to a nylon tube, the higher values of the latter compared to those in free solution being due to the presence of some negative charges in the immobilizing support. In the case of the immobilized enzyme, the effect of buffer-facilitated proton transport as well as of the substrate diffusion have been manifested in the anomalous pH-activity profiles with excess NAD and in Michaelis constants for NAD with excess ethanol and at a low flow rate, respectively.

Our results for the ionization constants are to be contrasted with those of Klinman (175) who demonstrated the participation of a single active-site basic group of pK 8.25 functioning in acid-base catalysis of a hydride transfer step. The effect of pH on the steady-state kinetic parameters for the YADH catalyzed reduction of aldehyde and oxidation of alcohols was studied by this worker. He showed that different enzyme forms, but consistent with the single active side chain residue, are responsible for the reactions in the opposite directions. This residue was suggested to be due to the participation of an active-site amino acid side chain, e.g., imidazole, cysteine or lysine. More recently, Klinman et al. (176) proposed the ionization of an active-site Zn-OH_2 , as revealed from solvent isotope effects and also in view of the similarity of the dissociation constant for zinc-bound water with a pK of 8.7 (177).

The oxidation of ethanol itself was studied by Wenger and Bernofsky (52) and was found to result in a four-fold decrease in rate between pH 6.5 and 9.5. These workers did not consider the participation in the enzymic catalysis of any particular group. Our results, however, clearly show that two different groups are involved in the mechanism; these groups have, to some extent, been identified from inhibition studies. Thus, Jeck and Woeckhaus (178) observed that the inhibition of dehydrogenases by NAD analogs involves histidine and cysteine residues. The reactivity of the active-site sulfhydryl group and the identification of Cys-43 in ADH was demonstrated by the effect of pH (179) as well as by inhibition experiments (180), while the role of an essential histidine residue was observed in inactivation studies (181). The participation of the active-site zinc atom at the substrate binding pocket was particularly invoked for the mechanism of action of liver alcohol dehydrogenase (182). Studies of the pH dependence of reactions catalyzed by the native enzyme also showed that the free enzyme has a group with pK of about 9.0 which changes to about 7.5 in the enzyme-NAD complex (137,183). Other workers suggested pH-dependent conformational change and, indeed, Shore et al. (184) found that the pK of 6.4 observed in the transient oxidation of ethanol could result from an isomerization step.

It is apparent that the identification of an essentially active group corresponding to a particular pK is not always unambiguous. An important reason for the disagreement between the various workers is that the enzyme used is not necessarily of the same form. The experimental conditions are also different so that the kinetic differences may not be

rationalized on the basis of intrinsic activities. Our results, particularly with the immobilized enzyme, are free from these difficulties since the same enzyme-tube has been used throughout the experiments. It is also necessary that the experiments be carried out over a sufficiently wide range of pH and of substrate concentrations. In these regards, the present work has provided a more reliable and reasonable estimate of the pK values of the groups involved in the catalysis by YADH. The exact nature and identity of the catalytically important groups, however, remain to be elucidated; this will require more detailed studies, probably involving isotopic labelling and crystallographic structure determinations.

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

Introduction

The procedure for immobilizing yeast alcohol dehydrogenase (YADH) inside a nylon tube has been described in Chapter 2, and the kinetic results for the reaction between ethanol and NAD in the tubular flow reactor have been presented in the subsequent chapters. In Chapter 5, the pH dependence of YADH kinetics in free solution has also been reported, and has been compared with the results obtained for the immobilized enzyme system. This final chapter is concerned with a general discussion and with drawing conclusions from the experimental data given in the previous chapters. The discussion centres mainly around the diffusional effects observed in the kinetics and mechanism of the reaction catalyzed by the immobilized enzyme.

Stability of the Immobilized YADH

It was shown in Chapter 2 that the method used for the chemical attachment of the enzyme on the interior surface of a nylon tube resulted in a preparation of considerable stability, there being little change in activity in about a year. This was believed to be due to the production of a fairly neutral surface, as discussed by Daka and Laidler (122) for lactate dehydrogenase in similar systems. However, the results in Chapter 3 suggested that electrostatic interaction due to some residual charges on the support gave rise to the disagreement between the K_m^1 (NAD)

values and the intrinsic values obtained by other workers in free solution. Finally, in Chapter 5, it was concluded that not all of the carboxyl groups released in the initial acidic hydrolysis of the nylon peptide bonds were blocked by reaction with benzidine. Nevertheless, the activity of the immobilized enzyme remained practically constant since the introduction of benzidine provided a 'spacer' to prevent any strong interaction between the enzyme and the support, thus allowing consistent kinetic data to be obtained.

Kinetics and Mechanism

The flow kinetics of YADH attached to nylon tubing has been described in Chapter 3, and the results have been interpreted on the basis of the Kobayashi-Laidler theory (70). This theory was developed for one-substrate systems, and the present work provided experimental tests as to the extension to a two-substrate reaction. In general, the apparent Michaelis constants were higher at low substrate concentrations and at low flow rates, when diffusion control is greatest. The $K_{m,app}$ values were derived from Lineweaver-Burk plots which suggested diffusional effects, especially for variable NAD and with excess ethanol. These results varied linearly with $v_f^{-1/3}$, as predicted by the theory, and gave extrapolated values of $K'_m(C_2H_5OH)=4-5$ mM and $K'_m(NAD)=230-260$ μ M at infinite flow rates. The former values are similar to, but the latter are two orders of magnitude higher than, the corresponding values found in the literature for alcohol dehydrogenases in free solution and in static systems of immobilized enzymes. In accordance with the theory, this indicated greater degree

of diffusion control with variable NAD than with variable ethanol. However, the results for $K'_m(\text{NAD})$ suggested some electrostatic interaction with the supporting medium.

Treatment of the data for the flow kinetics in terms of the dimensionless parameters defined by the Kobayashi-Laidler theory confirmed that there is a substantial or moderate degree of diffusion control with respect to the substrate NAD, in contrast to appreciably no diffusion control with variable ethanol at any of the flow rates employed. The theory also predicts the dependence of product concentration at the exit of the tube on the flow rate, and the results for the double-logarithmic plots showed deviations due to inhibition by products. Both acetaldehyde and NADH were found to be competitive and non-competitive inhibitors, with a preponderance of the former, the inhibition by acetaldehyde being stronger than that by NADH. The degree of inhibition decreased with increasing flow rate with added NADH, in contrast to little variation with added acetaldehyde at different flow rates. This is also consistent with the degree of diffusion control for the two substrates, the extent of which decreases with increasing flow rate and substrate concentration.

Temperature effects with the immobilized YADH in flow systems were discussed in Chapter 4. The Arrhenius plots for the overall rates were linear, suggesting that there is no change of mechanism over the temperature range of 5-45°C that was employed. With ethanol in excess and NAD as the variable substrate, the activation energies depended significantly on flow rate and substrate concentration, the values increasing

with increasing flow rate and NAD concentration. On the other hand, when ethanol was used as the variable substrate with excess NAD, the activation energies remained practically the same at different flow rates and substrate concentrations. The extrapolated values at high substrate concentrations and flow rates gave an activation energy of $\sim 9 \text{ kcal mol}^{-1}$ for the chemical interaction at the surface when both the substrates are saturating the enzyme. The activation energy for the diffusion process was obtained by extrapolation to zero NAD concentration and at low flow rates. The values decreased with decreasing flow rate and yielded an activation energy of $4.2 \text{ kcal mol}^{-1}$ for the reaction under the conditions of full diffusion control. The result is very reasonable for the diffusion in aqueous solution of the molecules we are dealing with, and again is consistent with the degree of diffusion control with variable NAD in contrast to no diffusion control with variable ethanol. As a consequence of the much higher activation energy for the chemical process, the reactions are very much slower than the diffusion process, and therefore it is easy to find conditions favouring the chemical interaction at the surface.

Temperature dependence of the Michaelis parameters were also discussed, the results for the V_{max} being similar to those for the overall rates, showing that the same rate constant is involved in the product formation, i.e., in the dissociation of the product from the enzyme-substrate ternary complex. Arrhenius plots for the apparent Michaelis constants were generally non-linear, thus suggesting complications due to changes of mechanism with substrate concentration and flow rate.

These plots gave somewhat inaccurate values of the activation energies of 2-8 kcal mol⁻¹ for $K_{m,app}$ (NAD), which are similar to those obtained for the association processes by Gierer (131) with the enzyme in free solution. Again, the results decreased with increasing flow rate and gave a value of ~2 kcal mol⁻¹ at high flow rate, similar to those for the diffusion-free $K_{m,app}$ values for ethanol at any flow rate. It is to be noted that the Michaelis parameters were obtained from Lineweaver-Burk plots which were, in general, non-linear at different temperatures, particularly with variable ethanol over the concentration range of 3-50 mM. This indicates that the enzyme active-site is microheterogeneous as a result of immobilization, which can modify the enzyme into different forms. The Michaelis parameters obtained from the limits of lower and higher concentration ranges of ethanol (within 3-10 mM and 10-50 mM, respectively) were different, but the activation energies for V_{max} were similar to those discussed earlier, thus confirming that the same rate constants are involved in the enzymic catalysis.

Finally, the pH dependence of YADH kinetics in free solution as well as in the tubular flow reactor were described in Chapter 5. The pH-activity profiles for the initial rate with the enzyme in free solution showed the ordinary bell-shaped behaviour giving a pH optimum of 7.5 and 8.0 for variable NAD and ethanol, respectively. Similar behaviour was observed for the immobilized enzyme with variable NAD, but the entire profile for the apparent rate was shifted towards the right showing a pH optimum between 8.5 and 9.0 at different flow rates. These changes were indicative of microenvironmental effects arising from electrostatic,

partitioning and/or diffusional effects. The pH optimum was shifted further upwards with variable ethanol in the tubular reactor, but this optimum could not be determined on account of the instability of the substrate NAD at higher pH; there was no fall-off in the apparent rate. The continuous increase of rate with pH up to 10.0 showed diffusional limitations to the transport of protons generated in the reaction, and is due to the lack of buffer-facilitated diffusion. The latter is relatively less important with excess ethanol when the effective concentration of the buffer anions is not much altered in the microenvironment of the enzyme, thus showing the bell-shaped pH profile.

The pH dependence of the Michaelis parameter $V_{\max}$ and of the ratio $V_{\max}/K_m$ showed similar behaviour to that of the initial rates with the enzyme in free solution, or of the apparent rates with the immobilized enzyme. The results for the Michaelis constant K_m itself showed complicated behaviour since these are functions of several rate constants, each of which may be affected by pH. In general, the K_m value passed through a minimum at a pH corresponding to the maximum activity. In the case of the immobilized enzyme with excess ethanol and at a low flow rate of 0.30 cm s^{-1} , when there is substantial diffusion control, the $K_m(\text{NAD})$ values were found to be independent of pH, because the Michaelis constants are not affected by diffusion coefficients which in turn do not depend on pH. At higher flow rates, diffusion control is less important and there is much stronger dependence of K_m on pH, as observed for the results at a flow rate of 0.90 cm s^{-1} and with excess ethanol. Similar results could

be expected with excess NAD and variable ethanol, when there was no diffusion control at any flow rate, but now the K_m decreased and V_{max} or V_{max}/K_m increased continuously with increasing pH since there was no fall-off in the apparent rate; this may be due to diffusional resistances to proton transport and the lack of buffer-facilitated diffusion of protons, as discussed earlier.

The Michaelis parameters $1/V_{max}$ and K_m/V_{max} were utilized to determine the ionization constants of catalytically important groups, which were obtained reliably by computerized curve-fitting involving a simple statistical least-squares treatment. In all cases, two different pK values were obtained, except for the data with excess NAD and variable ethanol in the tubular flow reactor. With the immobilized enzyme, the dissociation constant of the acidic group for the enzyme itself in the presence of excess ethanol and that for the enzyme-substrate ternary complex were significantly lower, i.e., the pK_a values were higher, compared to the corresponding values found in free solution. This was due to the presence of some negatively charged groups on the support which can decrease the ionization of the acidic group by electrostatic attraction to the leaving proton. The pK values for the acidic and basic groups in the immobilized enzyme with excess ethanol were slightly different at a low and a high flow rate, which appeared to be consistent with the varying degree of diffusion control.

In the case of the enzyme in free solution, the dissociation constants of catalytically important groups were dependent, to some extent,

on which substrate was used in excess. The pK_a^E and pK_b^{ES} results were not affected, but the pK_b^E and pK_a^{ES} values were considerably higher with excess NAD than with excess ethanol. This indicated the compulsory order of substrate binding which, in accordance with the previous results, involves the addition of NAD prior to the binding of ethanol with the enzyme to form the ternary complex. The binding of NAD with the protonated form of a basic group will decrease its dissociation constant (pK_b^E is higher with excess NAD), but will not change that of the acidic group which is required for the binding of ethanol, presumably in its deprotonated form. The binding of ethanol will, however, affect the pK_a^{ES} values, but the pK_b^{ES} will remain the same since NAD is already attached to the enzyme through the basic group.

In general, the mechanism of action of alcohol dehydrogenases is random but it becomes essentially ordered when one of the substrates is in excess. There are several reports in the literature indicating that the substrate NAD is added first to the enzyme, and that the product NADH leaves last. Our results in Chapters 3 and 4 showed clearly that there is substantial degree of diffusion control with variable NAD and excess ethanol in contrast to appreciably no diffusion control with variable ethanol and excess NAD. This is understandable if there is a compulsory order of binding, the substrate NAD being bound prior to ethanol. Since ethanol cannot bind to the free enzyme but only to the enzyme-NAD complex, and the larger NAD molecule will diffuse more slowly, diffusion control is important with excess ethanol. With excess NAD, however, the situation is different since the substrate ethanol can diffuse more readily and

become attached to the enzyme-NAD complex already formed, so that there is little or no diffusional control. The pH dependence of the YADH kinetics, particularly the results for the ionization constants of catalytically important groups in free solution, explains the compulsory ordered mechanism more clearly. Evidently, there are two important groups, one basic and the other acidic, involved in the catalytic process, a result that was not known earlier. However, the exact nature and the identity of the catalytically important groups remains to be elucidated.

Problems to be Resolved

Immobilized enzymes behave differently from those in free solution as a result of conformational, environmental, partitional and diffusional effects, each of which may also be modified or affected by electrostatic factors. This thesis was concerned with the kinetics of YADH attached to nylon tubing, and particularly with the diffusional effects in the kinetics of the reaction between ethanol and NAD in flow systems.

In general, the apparent Michaelis constant $K_{m,app}$ for both the substrates was higher than those found in free solutions. Treatment of the data in terms of the Kobayashi-Laidler theory gave the inherent Michaelis constant K'_m at infinite flow rates when there is no diffusional effect. The diffusion-free K'_m values for ethanol were not considerably different, but for NAD were substantially higher than the corresponding values in free solution. The latter suggested the possibility of electrostatic interaction of the positively charged substrate NAD with similar charges either in the support or in the enzyme active-site itself. It

would seem desirable to investigate the effect of other factors on the immobilized YADH kinetics.

The pH dependence of the immobilized enzyme kinetics showed that the support bears some negatively charged groups which reduce the ionization and hence increases the pK of the acidic group involved in the catalysis. Experimental tests as to the nature of residual charges on the support or the presence of any charge in the enzyme active-site itself should therefore be carried out. This would seem to be possible by studying the effect of ionic strength on the kinetics of the enzyme-catalyzed reaction.

The presence of residual charges on the support could affect the stability and therefore the activity of the immobilized enzyme. It was suggested that the introduction of benzidine as a 'spacer' prevents any strong interaction to induce substantial loss of enzymic activity. However, the exact cause of the stability remains to be determined. It may be possible to attach the enzyme directly to the carboxyl and/or the amino groups released in the acidic hydrolysis of the nylon peptide bonds, and then to determine the effect of the 'spacer' on the stability of the immobilized enzyme.

The dependence of Michaelis constants either on temperature or on pH showed complicated behaviour since the former is a composite of several rate constants; which of these is affected to what extent is not known with any certainty. More detailed studies are necessary to separate the individual rate constants; this requires the use of transient-kinetic techniques, which have, as yet, not been applied to immobilized enzymes.

In obtaining the Michaelis constants from Lineweaver-Burk plots, non-linear behaviour was observed particularly with excess NAD and variable ethanol at different temperatures. Several possibilities were discussed, and the most plausible interpretation appeared to be the presence of microheterogeneity as a result of immobilization. Again, the details are to be worked out, perhaps by the use of modification experiments.

Finally, the pH dependence of the YADH kinetics in free solution and in the tubular flow reactor showed different behaviour. The pH-activity profiles for the immobilized enzyme were explained in terms of the electrostatic partitioning and diffusional limitations to proton transport, resulting from the lack of buffer-mediation, particularly with excess NAD. The pH optima observed for the enzyme in free solution were somewhat different with either of the substrates in excess. The slightly higher pH optimum obtained with excess NAD appeared to be due to electrostatic interaction with the enzyme, but the nature of this interaction is not very well understood.

Both for the enzyme in free solution and with the immobilized enzyme, two different and catalytically important ionizing groups were implicated. The ionization constants of an essential acidic and a basic group were obtained under different conditions. The protonated form of the basic group was required for the binding of NAD, while the deprotonated form of the acidic group was involved in the subsequent binding of ethanol. Again, the exact nature and identity of the groups are to be determined. However, all of the results are consistent with the compulsory order of binding, NAD being bound prior to ethanol to form the enzyme-substrate ternary complex which ultimately decomposes into the products.

Suggestions for Further Work

Some suggestions have already been made with respect to the particular problems to be resolved in the preceding section. It would be of particular interest to look at the extent to which one or the other factors, viz., conformational, environmental, partitional, diffusional and electrostatic effects, contribute to the difference in behaviour of the immobilized enzyme compared to its free counterpart. Different immobilization techniques involving various configurations of the supporting nylon structure can be utilized in this regard. In particular, the immobilization of the enzyme by matrix entrapment in a gel may lead to models for understanding the function of the enzyme in vivo.

A very useful investigation would be to study the flow kinetics in a multistep system, i.e., in which both the enzyme and the coenzyme NAD are immobilized, either singly or together. Since the enzyme requires the participation of the readily dissociable coenzyme, its economic use would necessitate methods to retain them in the reaction mixture and to regenerate them. Such a study would be of practical interest in enzyme technology and enzymic analysis in general.

The use of enzymes as active elements in electrochemical probes or sensors for analytical purposes, especially of biologically important metabolites, is quite well known. The reaction catalyzed by alcohol dehydrogenases is pH-dependent, and therefore immobilizing the enzyme for use as an enzyme-electrode may also be desirable.

Kinetic studies of immobilized enzymes especially in flow systems have mostly been carried out with arbitrary concentrations of the enzyme,

which may be entirely unphysiological, or the concentration may not be optimum for practical purposes. In case of the enzyme attached to a tube, the effective concentration may not be known so that it is difficult to evaluate the exact rate constants. Experiments may therefore be designed to work with different amounts of the enzyme and to be able to determine the actual amount which is immobilized. The activity of YADH can also depend on its metal content which may be varied or substituted to see the effect.

More importantly, the YADH differs from LADH in its substrate (including the coenzyme) specificity and catalytic activity, and therefore the kinetics of both types of the enzyme when they are immobilized deserve comparison. The action of LADH, particularly in mammalian physiology, can be complicated by a number of inhibitors, apart from the products, of which berberine and some other related alkaloids have received considerable attention (185) since alkaloids are frequently used in medical sciences. Hence, the effect of different inhibitors can be studied, and the results may help to explore their physiological role, and relate to biomedical applications.

CLAIMS TO ORIGINAL RESEARCH

1. A method has been developed for the chemical attachment of yeast alcohol dehydrogenase inside a nylon tube. The immobilized enzyme retained its activity over a period of about one year, and allowed consistent kinetic data to be obtained in flow systems.
2. Kinetic studies of the reaction between ethanol and NAD were carried out in the tubular flow reactor. The apparent Michaelis constants were higher at different flow rates compared to the values in free solutions and in static systems of the immobilized enzyme. Extrapolation of the results at infinite flow rates, according to the Kobayashi-Laidler theory for the flow kinetics, yielded the diffusion-free values of the Michaelis constants.
3. The present work provided experimental tests as to the validity of the Kobayashi-Laidler one-substrate treatment, extended for a two-substrate reaction under the limiting conditions of one substrate present in excess while the other was varied. Treatment of the data in terms of the various methods suggested by the theory showed that there was substantial degree of diffusion control with excess ethanol and variable NAD, in contrast to appreciably no diffusion control under the converse conditions. The extent of diffusion control with variable NAD was found to be increased with decreasing flow rate and decreasing substrate concentration.
4. There was inhibition by products of the reaction, acetaldehyde and NADH, which showed deviations for the flow rate dependence of product

concentration at the exit of the tube, as predicted by the theory. Inhibition constants were determined and acetaldehyde was found to be the stronger inhibitor for the immobilized enzyme, in contrast to the behaviour observed in free solution by other workers. This, as well as the dependence of product inhibition by NADH on flow rates, was due to the greater degree of diffusion control with variable NAD.

5. The effect of temperature was studied at different flow rates and substrate concentrations. Activation energies for the overall rates varied significantly with flow rate and NAD concentration, in contrast to little variation for the other substrate ethanol. This was again the result of the varying degree of diffusion control. The activation energy for the reaction under the conditions of full diffusion control at zero NAD concentration and at low flow rates was found to be $4.2 \text{ kcal mol}^{-1}$, and that for the chemical interaction at the surface when there was saturation by both the substrates and at high flow rate was $\sim 9 \text{ kcal mol}^{-1}$.
6. Lineweaver-Burk plots at different temperatures were non-linear with variable ethanol over a concentration range of 3-50 mM, and gave two different sets of Michaelis parameters, indicating micro-heterogeneity of the enzyme active-site resulting from the immobilization. Temperature dependence of V_{max} gave activation energies similar to that for the chemical interaction at the surface, while the Michaelis constants showed complicated behaviour. The activation energies for the latter varied with flow rate and showed a value of $\sim 2 \text{ kcal mol}^{-1}$ for the diffusion-free Michaelis constants.

7. The pH-dependence of the YADH kinetics were studied both in free solution and in the tubular flow reactor. The pH-activity profiles for the immobilized enzyme showed electrostatic partitioning effects and/or diffusional limitations to the proton transport due to the lack of buffer-facilitated diffusion with excess NAD. The variations of Michaelis constants with pH were not simple. The effect of substrate diffusion was manifested in apparently constant K_m values for NAD at a low flow rate.
8. Dissociation constants for two different ionizing groups involved in the enzymic catalysis were obtained reliably by a computerized curve-fitting technique involving a simple statistical least-squares treatment. The pK values for the acidic group in the immobilized enzyme itself in the presence of either of the substrates in excess and also in the enzyme-substrate ternary complex were higher than the corresponding values in free solution. This was due to the presence of some residual negative charges on the support.
9. For the enzyme in free solution, there were considerable differences in the pK values of the basic group in the enzyme itself and for the acidic group in the ternary complex, in the presence of either of the substrates in excess. This was consistent with the compulsory ordered mechanism, in which the NAD is bound to a basic group prior to the binding of ethanol to an acidic group.

REFERENCES

1. K. Yamamoto, T. Tosa, K. Tamasita and I. Chibata, *Biotechnol. Bioeng.* 19, 1101 (1977).
2. T. Tosa, T. Mori, N. Fuse and I. Chibata, *Enzymologia* 32, 153 (1967).
3. I. Chibata and T. Tosa, in *Applied Biochemistry and Engineering* (L.B. Wingard, Jr., E. Katchalski and L. Goldstein, eds.), Vol. 1, p. 329, Academic Press, New York (1976).
4. D.Y. Ryu and S.H. Chung, *Biotechnol. Bioeng.* 19, 159 (1977).
5. S.S. Sofer, D.M. Ziegler and R.P. Popovich, *Biochem. Biophys. Res. Commun.* 57, 183 (1974).
6. T.M.S. Chang (Ed.), *Biomedical Applications of Immobilized Enzymes and Proteins*, Vol. 2, Plenum Press, New York (1977).
7. W.E. Hornby, H. Filippusson and A. McDonald, *FEBS Lett.* 9, 8 (1970).
8. P.V. Sundaram and W.E. Hornby, *FEBS Lett.* 10, 325 (1970).
9. D.J. Inman and W.E. Hornby, *Biochem. J.* 137, 25 (1974).
10. T.M.S. Chang, in *Immobilized Enzymes, Antigens, Antibodies and Peptides* (H.H. Weetall, ed.) Vol. 1, p. 245, Marcel Dekker, New York (1975).
11. J.E. Kirk, *Enzymes of the Arterial Wall*, Academic Press, New York (1969).
12. K. Mosbach (Ed.) *Methods in Enzymology*, Vol. 44, Section 8, Academic Press, New York (1976).
13. T.T. Ngo and K.J. Laidler, *Biochim. Biophys. Acta* 377, 317 (1975).
14. C. Neuberg, *Adv. in Carbohydrate Chem.* 4, 75 (1949).
15. R. Blomstrand, in *Structure and Function of Oxidation-Reduction Enzymes* (Å. Åkeson and E. Ehrenberg, eds.) p. 667, Pergamon Press, Oxford (1972).
16. R.K. Bonnichsen and H. Theorell, *Scand. J. Clin. Lab. Invest.* 3, 58 (1951).
17. T. Bucher and H. Redetzki, *Klin. Wochenschr.* 29, 615 (1951).

18. G. Dotzauer, H. Redetzki, K. Johannsmeier and T. Bucher, Dtsch. Z. Gesamte Gerichtl. Med. 41, 15 (1952).
19. N.G. Brink, R. Bonnichsen and H. Theorell, Acta Pharmacol. Toxicol. 10, 223 (1954).
20. B.J. Jones, Methods Enzymol. 44, 831 (1976).
21. H.R. Levy, F.A. Loewus and B. Vennesland, J. Am. Chem. Soc. 79, 2949 (1957).
22. F.H. Westheimer, H.F. Fisher, E.E. Conn and B. Vennesland, J. Am. Chem. Soc. 73, 2403 (1951).
23. W.E. Hornby, D.J. Inman and A. McDonald, FEBS Lett. 23, 114 (1972).
24. A.C. Johansson and K. Mosbach, Biochim. Biophys. Acta 370, 348 (1974).
25. S. Gestrelus, M. Mansson and K. Mosbach, Eur. J. Biochem. 57, 529 (1975).
26. N. Kelly, A. Flynn and D.B. Johnson, Biotechnol. Bioeng. 19, 1211 (1977).
27. M.J. Brougham and D.B. Johnson, Int. J. Biochem. 9, 283 (1978).
28. S. Barry, T. Griffin and D.B. Johnson, Int. J. Biochem. 9, 289 (1978).
29. J.R. Wykes, P. Dunhill and M.D. Lilly, Biotechnol. Bioeng. 17, 51 (1975).
30. R.W. Coughlin, M. Aizawa, B.F. Alexander and M. Charles, Biotechnol. Bioeng. 17, 515 (1975).
31. K. Das, P. Dunhill and M.D. Lilly, Biochim. Biophys. Acta 397, 277 (1975).
32. H. Sund and H. Theorell, in The Enzymes (P.D. Boyer, ed.) 2nd Ed., Vol. 7, p. 25, Academic Press, New York (1963).
33. E. Negelein and H.J. Wulff, Biochem. Z. 293, 351 (1937).
34. R.K. Bonnichsen, Acta Chem. Scand. 4, 715 (1950).
35. R.K. Bonnichsen and A.M. Wässén, Arch. Biochem. Biophys. 18, 361 (1948).
36. B.E. Kemp, M. Froscio and A.W. Murray, Biochem. J. 131, 271 (1973).

37. P.J.G. Butler and G.M.T. Jones, *Biochem. J.* 118, 375 (1970).
38. H. Jönrvall, *Proc. Natl. Acad. Sc. US* 70, 2295 (1973).
39. P.J.G. Butler, *Dissertation, University of Cambridge* (1967).
40. P.J.G. Butler and J.I. Harris, *Abstr. Fed. Eur. Biochem. Soc.* 5, 186 (1968).
41. F.M. Dickinson, *Biochem. J.* 120, 821 (1970).
42. C.R. Lowe, K. Mosbach and P.D.G. Dean, *Biochem. Biophys. Res. Commun.* 48, 1004 (1972).
43. M.K. Weibel, E.R. Doyle, A.E. Humphrey and H.J. Bright, *Biotechnol. Bioeng. Symp.* 3, 167 (1972).
44. R. Ohlsson, P. Brodelius and K. Mosbach, *FEBS Lett.* 25, 234 (1972).
45. L. Schimpfessel, *Arch. Int. Physiol. Biochim.* 75, 368 (1967).
46. L. Schimpfessel, *Biochim. Biophys. Acta* 151, 317 (1968).
47. U. Lutstorff and R. Megnet, *Arch. Biochem. Biophys.* 126, 933 (1968).
48. P.W. Fowler, A.J.S. Ball and D.E. Griffiths, *Can. J. Biochem.* 50, 35 (1972).
49. K. Ebisuzaki and E.S.G. Barron, *Arch. Biochem. Biophys.* 69, 555 (1957).
50. L. Schimpfessel, J. Sugar and R. Crokaert, *Arch. Int. Physiol. Biochim.* 77, 556 (1969).
51. H.M.C. Heick, J. Willemot and N. Bégin-Heick, *Biochim. Biophys. Acta* 191, 493 (1969).
52. J.I. Wenger and C. Bernofsky, *Biochim. Biophys. Acta* 227, 479 (1971).
53. J. Sugar, L. Schimpfessel, E. Rozen and R. Crokaert, *Arch. Int. Physiol. Biochim.* 78, 1009 (1970).
54. J. Sugar, L. Schimpfessel, E. Rozen and R. Crokaert, *Arch. Int. Physiol. Biochim.* 79, 849 (1971).
55. C.I. Brändén, H. Jönrvall, H. Eklund and B. Furugren, in *The Enzymes* (P.D. Boyer, ed.) 3rd Edn., Vol. 11, p. 103, Academic Press, New York (1975).
56. A. Fersht, *Enzyme Structure and Mechanisms*, W.H. Freeman and Company, Reading (1977).

57. J.A. Edwards and D.A.P. Evans, Clin. Pharmacol. Ther. 8, 824 (1967).
58. H.H. Krebs and J.R. Parkins, Biochem. J. 118, 635 (1970).
59. H. Theorell and R.K. Bonnichsen, Acta Chem. Scand. 5, 1105 (1951).
60. H. Theorell and B. Chance, Acta Chem. Scand. 5, 1127 (1951).
61. L. Ting-Kai and H. Theorell, Acta Chem. Scand. 23, 892 (1969).
62. A. Bliss, Biol. Bull. 97, 221 (1949).
63. G. Wald and R. Hubbard, in The Enzymes (P.D. Boyer, ed.) 2nd Edn., Vol. 3, p. 369, Academic Press, New York (1960).
64. H. Holzer, S. Schneider and K. Lange, Angew. Chem. 67, 276 (1955).
65. H. Holzer and S. Schneider, Klin. Wochschr. 33, 1006 (1955).
66. R.I. Dorfman and F. Ungar, Metabolism of Steroid Hormones, p. 394, Academic Press, New York (1965).
67. T. Cronholm, I. Makino and J. Sjövall, in Structure and Function of Oxidation-Reduction Enzymes (Å. Åkeson and E. Ehrenberg, eds.) p. 663, Pergamon Press, Oxford (1972).
68. T. Cronholm and J. Sjövall, Eur. J. Biochem. 13, 124 (1970).
69. C.S. Lieber, Ann. Rev. Med. 18, 35 (1967).
70. T. Kobayashi and K.J. Laidler, Biotechnol. Bioeng. 16, 99 (1974).
71. L. Goldstein and G. Manecke, in Applied Biochemistry and Bioengineering (L.B. Wingard et al, eds.) Vol. 1, p. 23, Academic Press, New York (1976).
72. K. Mosbach (Ed.) Methods in Enzymology, Vol. 64, Section 2, Academic Press, New York (1976).
73. A.M. Klibanov, Anal. Biochem. 93, 1 (1979).
74. J. Campbell and W.E. Hornby, in Biomedical Applications of Immobilized Enzymes and Proteins (T.M.S. Chang, ed.) Vol. 2, p. 3, Plenum Press, New York (1977).
75. P.V. Sundaram, E.K. Pye, T.M.S. Chang, V.H. Edwards, A.E. Humphrey, N.O. Kaplan, E. Katchalski, Y. Levin, M.D. Lilly, G. Manecke, K. Mosbach, J. Porath, H. Weetall and L.B. Wingard, Jr., Biotechnol. Bioeng. Symp. No. 3, 14, 15 (1972).

76. G.G. Guilbault and M.H. Sadar, in *Techniques in Protein and Enzyme Biochemistry - Part II* (H.L. Kornberg, J.C. Metcalfe, D.H. Northcote, C.I. Pogson and K.F. Tipton, eds.) B118, p. 1, Elsevier/North-Holland Biomedical Press (1978).
77. J.R. Wykes, P. Dunhill and M.D. Lilly, *Biochim. Biophys. Acta* 250, 522 (1971).
78. S.P. O'Neill, J.R. Wykes, P. Dunhill and M.D. Lilly, *Biotechnol. Bioeng.* 13, 319 (1971).
79. R. Axén, P. Vretblad and J. Porath, *Acta Chem. Scand.* 25, 1129 (1971).
80. T.M.S. Chang, *Biochem. Biophys. Res. Commun.* 44, 1531 (1971).
81. R. Haynes and K.A. Walsh, *Biochem. Biophys. Res. Commun.* 36, 235 (1969).
82. R. Goldman, O. Kedem, I.H. Silman, S.R. Caplan and E. Katchalski, *Science* 150, 758 (1965).
83. G. Brown, E. Selegny, S. Avrameas and D. Thomas, *Biochim. Biophys. Acta* 185, 258 (1969).
84. T. Tosa, T. Mori, N. Fuse and I. Chibata, *Enzymologia* 31, 214 (1966).
85. H.D. Brown, A.B. Patel and S.K. Chattopadhyay, *J. Biomed. Mater. Res.* 2, 231 (1968).
86. T.M.S. Chang, *Artificial Cell, Monograph*, Charles C. Thomas, Springfield, Illinois (1972).
87. K.J. Laidler and P.V. Sundaram, in *Chemistry of the Cell Interface, Part A*, p. 255, Academic Press, New York and London (1971).
88. G.P. Royer, in *Immobilized Enzymes, Antigens, Antibodies and Peptides* (H.H. Weetall, ed.), Vol. 1, p. 49, Marcel Dekker, New York (1975).
89. J.M. Engasser and C. Horvath, in *Applied Biochemistry and Bioengineering* (L.B. Wingard, Jr. et al, eds.) Vol. 1, p. 127, Academic Press, New York (1976).
90. L. Goldstein, in *Methods in Enzymology* (K. Mosbach, ed.) Vol. 44, p. 397, Academic Press, New York (1976).
91. K.J. Laidler and P.S. Bunting, in *Methods in Enzymology* (D.L. Purich, ed.) Vol. 64B, p. 227, Academic Press, New York (1980).
92. H. Filippusson and W.E. Hornby, *Biochem. J.* 120, 215 (1970).

93. W.E. Hornby and D.L. Morris, in *Immobilized Enzymes, Antigens, Antibodies and Peptides* (H.H. Weetall, ed.) Vol. 1, p. 141, Marcel Dekker, New York (1976).
94. T.M.S. Chang; *Science* 146, 524 (1964); *Science Journal* 3, 62 (1967); *Science Tools* 16, 35 (1969); *Nature (London)* 229, 117 (1971).
95. G.M. Edelman, U. Rutishauser, and C.F. Millette, *Proc. Natl. Acad. Sc. USA* 68, 2153 (1971).
96. C. Horvath, *Biochim. Biophys. Acta* 358, 164 (1974).
97. J.H. Reynolds, *Biotechnol. Bioeng.* 16, 135 (1974).
98. D.J. Inman and W.E. Hornby, *Biochem. J.* 129, 255 (1972).
99. P.S. Bunting, Ph.D. Thesis, University of Ottawa, Canada, 1973.
100. H. Filippusson, W.E. Hornby and A. McDonald, *FEBS Lett.* 20, 291 (1972).
101. D.L. Morris, J. Campbell and W.E. Hornby, *Biochem. J.* 147, 593 (1975).
102. W.E. Hornby, J. Campbell, D.J. Inman and D.L. Morris, in *Enzyme Engineering* (E.K. Pye and L.B. Wingard, Jr. eds.) Vol. 2, p. 401, Plenum Press, New York (1974).
103. D.R. James and B. Pring, *Clin. Chim. Acta* 62, 435 (1975).
104. W.E. Hornby and L. Goldstein, *Methods Enzymol.* 44, 118 (1976).
105. W.E. Hornby and H. Filippusson, *Biochim. Biophys. Acta* 220, 343 (1970).
106. J.P. Allison, A. Davidson, A. Gutierrez-Hartman and G.B. Kitter, *Biochem. Biophys. Res. Commun.* 47, 66 (1972).
107. P.S. Bunting and K.J. Laidler, *Biotechnol. Bioeng.* 16, 119 (1974).
108. F.A. Quijcho, in *Insolubilized Enzymes* (M. Salmons, C. Saronio and S. Garattini, eds.) p. 113, Raven Press, New York (1974).
109. F. Richards and J. Knowles, *J. Mol. Biol.* 37, 231 (1968).
110. J. Bowes and C. Carter, *Biochim. Biophys. Acta* 168, 341 (1969).
111. P.M. Hardy, A.C. Nicholls and H.N. Rydon, *Chem. Commun.* 1969, 565 (1969).
112. C. Horvath and B.A. Solomon, *Biotechnol. Bioeng.* 14, 885 (1972).
113. R.E. Benson and T.L. Cairns, *Org. Synth.* 31, 72 (1951).

114. J. Campbell, W.E. Hornby and D.L. Morris, *Biochim. Biophys. Acta* 384, 307 (1975).
115. L. Goldstein, A. Freeman and M. Sokolovsky, *Biochem. J.* 143, 497 (1974).
116. L. Goldstein, A. Freeman and M. Sokolovsky, in *Enzyme Engineering* (E.K. Pye and L.B. Wingard, Jr., eds.) Vol. 2, p. 97, Plenum Press, New York (1974).
117. R. Axén, J. Porath and S. Ernback, *Nature (London)* 214, 1302 (1967).
118. H. Theorell and A.D. Winer, *Arch. Biochem. Biophys.* 83, 291 (1959).
119. M.P. Schulman, N.K. Gupta, A. Omachi, G. Hoffman and W.E. Marshall, *Anal. Biochem.* 60, 302 (1974).
120. D. Narinesingh, T.T. Ngo and K.J. Laidler, *Can. J. Biochem.* 53, 1061 (1975).
121. T.T. Ngo, D. Narinesingh and K.J. Laidler, *Biotechnol. Bioeng.* 18, 119 (1976).
122. N.J. Daka and K.J. Laidler, *Can. J. Biochem.* 56, 774 (1978).
123. M.A. Mazid and K.J. Laidler, *Biochim. Biophys. Acta* 614, 225 (1980).
124. D.F. Ollis, *Biotechnol. Bioeng.* 14, 871 (1972).
125. J.M. Engasser and P.R. Coulet, *Biochim. Biophys. Acta* 485, 29 (1977).
126. O.R. Zaborsky, in *Enzyme Engineering* (L.B. Wingard, Jr., ed.) Vol. 1, p. 211, Wiley, New York (1972).
127. J.M. Engasser, P.R. Coulet and D.C. Gautheron, *J. Biol. Chem.* 252, 7919 (1977).
128. K.J. Laidler and P.S. Bunting, *The Chemical Kinetics of Enzyme Action*, Clarendon Press, Oxford (1973).
129. P.V. Sundaram, A. Tweedale and K.J. Laidler, *Can. J. Chem.* 48, 1498 (1970).
130. B.L. Horecker and A. Kornberg, *J. Biol. Chem.* 175, 385 (1948).
131. A. Gierer, *Biochim. Biophys. Acta* 17, 111 (1955).
132. H. Theorell, A.P. Nygaard and R. Bonnichsen, *Acta Chem. Scand.* 9, 1148 (1955).

133. P.S. Bunting and K.J. Laidler, *Biochemistry* 11, 4477 (1972).
134. C.C. Wratten and W.W. Cleland, *Biochemistry* 2, 935 (1963).
135. C.C. Wratten and W.W. Cleland, *Biochemistry* 4, 2442 (1965).
136. D. Thomas, C. Bourdillon, G. Brown and J.P. Kernevez, *Biochemistry* 13, 2995 (1974).
137. K. Dalziel, *J. Biol. Chem.* 238, 2850 (1963).
138. J. van Eys, F.E. Stolzenbach, L. Sherwood and N.O. Kaplan, *Biochim. Biophys. Acta* 27, 63 (1958).
139. J. van Eys, M.M. Ciotti and N.O. Kaplan, *Biochim. Biophys. Acta* 23, 581 (1957).
140. J.E. Hayes and S.F. Velick, *J. Biol. Chem.* 207, 225 (1954).
141. M.F. Dunn, *Structure and Bonding* 23, 61 (1975).
142. E. Silverstein and P.D. Boyer, *J. Biol. Chem.* 239, 3908 (1964).
143. F.M. Dickinson and G.P. Monger, *Biochem. J.* 131, 261 (1973).
144. J.T.-F. Wong and C.S. Hanes, *Nature* 203, 492 (1964).
145. T.T. Ngo and K.J. Laidler, *Biochim. Biophys. Acta* 525, 93 (1978).
146. N.J. Daka and K.J. Laidler, *Biochim. Biophys. Acta* 612, 305 (1980).
147. M.A. Mazid and K.J. Laidler, *Biochim. Biophys. Acta* 614, 237 (1980).
148. B. Ostle, *Statistics in Research*, 2nd Edn., Iowa State University Press, Ames, Iowa (1963).
149. E.S. Swinbourne, *Analysis of Kinetic Data*, Thomas Nelson and Sons Ltd., London (1971).
150. K. Buchholz and W. R  th, *Biotechnol. Bioeng.* 18, 95 (1976).
151. A. Dubied, J.-P. von Wartburg, D.P. Bohlken and B.V. Plapp, in *Alcohol and Aldehyde Metabolizing Systems* (R.G. Thurman, J.R. Williamson, H.R. Drott and B. Chance, eds.) Vol. 2, p. 63, Academic Press, New York (1977).
152. L.K. Ashman and D.B. Keech, *Aust. J. Biol. Sc.* 28, 379 (1975).
153. B. J  nsson and H. Wennerstr  m, *Biophys. Chem.* 7, 285 (1978).

154. M. Eigen and G.G. Hammes, *Adv. Enzymol.* 25, 1 (1963).
155. R.A. Alberty and G.G. Hammes, *J. Phys. Chem.* 62, 154 (1958).
156. K.F. Tipton and H.B.F. Dixon, in *Methods in Enzymology* (D.L. Purich, ed.) Vol. 63A, p. 183, Academic Press, New York (1979).
157. I. Hinberg, Ph.D. Thesis, University of Ottawa, Canada (1973).
158. H. Kaplan and K.J. Laidler, *Can. J. Chem.* 45, 539 (1967).
159. R.M. Krupka and K.J. Laidler, *Trans. Faraday Soc.* 56, 1467 (1960).
160. M. Dixon, *Biochem. J.* 55, 161 (1953).
161. R.A. Alberly and V. Massey, *Biochim. Biophys. Acta* 13, 347 (1954).
162. J.M. Engasser and C. Horvath, *Biochim. Biophys. Acta* 358, 178 (1974).
163. W.W. Cleland, *Adv. Enzymol.* 29, 1 (1967).
164. G.N. Wilkinson, *Biochem. J.* 80, 324 (1961).
165. I. Hinberg and K.J. Laidler, *Can. J. Biochem.* 51, 1096 (1973).
166. N.R. Draper and H. Smith, *Applied Regression Analysis*, Chaps. 1 and 10, John Wiley & Sons, Inc., New York (1966).
167. K. Wallenfels and H. Sund, *Biochem. Z.* 329, 17 (1957).
168. J.P. Klinman, *J. Biol. Chem.* 247, 7977 (1972).
169. R.J. Suhadolnik and M.B. Lennon, in *Alcohol and Aldehyde Metabolizing Systems* (R.G. Thurman, et al., eds.), Vol. 2, p. 123, Academic Press, New York (1977).
170. O.H. Lowry, J.V. Passonneau and M.K. Rock, *J. Biol. Chem.* 236, 2756 (1961).
171. R. Goldman, O. Kedem, I.H. Silman, S.R. Caplan and E. Katchalski, *Biochemistry* 7, 486 (1968).
172. R. Goldman, H.I. Silman, S.R. Caplan, O. Kedem and E. Katchalski, *Science* 150, 758 (1965).
173. H.I. Silman and A. Karlin, *Proc. Natl. Acad. Sc. USA* 58, 1664 (1965).

174. D.A. Brant, L.B. Barnett and R.A. Alberty, J. Am. Chem. Soc. 85, 2204 (1963).
175. J.P. Klinman, J. Biol. Chem. 250, 2569 (1975).
176. J.P. Klinman, K.M. Welsh and D.J. Creighton, in Alcohol and Aldehyde Metabolizing Systems (R.G. Thurman, et al., eds.) Vol. 2, p. 53, Academic Press, New York (1977).
177. S. Chaberek, Jr., R.C. Courtney and A.E. Martell, J. Am. Chem. Soc. 74, 5057 (1952).
178. R. Jeck and C. Woenckhaus, Z. Naturforsch, Part C, 29, 180 (1974).
179. C.J. Belke, C.C.Q. Chin and F. Wold, Biochemistry 13, 3418 (1974).
180. J.P. Klinman, Biochemistry 14, 2568 (1975).
181. C.J. Dickenson and F.M. Dickinson, Eur. J. Biochem. 52, 595 (1975).
182. C.I. Brändén, in Alcohol and Aldehyde Metabolizing Systems (R.G. Thurman, et al., eds.) Vol. 2, p. 17, Academic Press, New York (1977).
183. S. Taniguchi, H. Theorell and Å. Åkeson, Acta Chem. Scand. 21, 1903 (1967).
184. J.D. Shore, H. Gutfreund, R.L. Brooks, D. Santiago and P. Santiago, Biochemistry 13, 4185 (1974).
185. L. Skurský and J. Kovář, in Structure and Function of Oxidation-Reduction Enzymes (Å. Åkeson and E. Ehrenberg, eds.) p. 653, Pergamon Press, Oxford (1972).

APPENDIX

FORTRAN IV PROGRAMME FOR THE DETERMINATION OF IONIZATION
CONSTANTS OF CATALYTICALLY IMPORTANT GROUPS

```

      IMPLICIT REAL*8 (A-H,O-Z)
      DIMENSION Y(20),H(20)
      TOLN=.001
      NP=0
2    READ(5,100)I,YI,HI
      IF(I.EQ.0)GO TO 20
      Y(I)=1./YI
      H(I)=HI
      WRITE(6,105)I,YI,HI
      NP=NP+1
      GO TO 2
20   CONTINUE
      C1=0.
      C2=0.
      C3=0.
      C4=0.
      C5=0.
      C6=0.
      C7=0.
      C8=0.
      DEV=0.
      DO 25 I=1,NP
      C1=C1+Y(I)
      C2=C2+10.**( -H(I))
      C3=C3+Y(I)*(10.**( -H(I)))
      C4=C4+1./((10.**( -H(I)))
      C5=C5+1./((10.**( -H(I)))*2)
      C6=C6+(10.**( -H(I)))*2
      C8=C8+Y(I)*Y(I)
25   C7=C7+Y(I)/(10.**( -H(I)))
      WRITE(6,107)C1,C2,C3,C4,C5,C6,C7,C8
      XNP=NP
      U=C3*C4-C2*C7
      V=XNP*C3-C6*C7
      W=XNP*C7-C3*C5
      X=W*C3
      R=W*C2+XNP*U
      Z=W*C6+XNP*V
      WRITE(6,108)U,V,W,X,R,Z
      TA=C1*(W**2)*R-XNP*(W**2)*X-2.*C4*U*W*X-C5*(U**2)*X+C7*U*W*R
      TB=C1*(W**2)*Z-2.*C2*(W**2)*X-2.*XNP*U*W*X-2.*C4*V*W*X
      TC=C3*(W**2)*Z+C7*V*W*Z-C5*(V**2)*X-2.*XNP*V*W*X-C6*(W**2)*X
      WRITE(6,110)TA,TB,TC
      XKB=(-TB+DSQRT(TB**2-4.*TA*TC))/(2.*TA)
      XKB2=(-TB-DSQRT(TB**2-4.*TA*TC))/(2.*TA)
      XKA=(U+(V/XKB))/W
      XKA2=(U+(V/XKB2))/W
      A=C7/(C4+(XNP/XKB)+XKA*C5)
      WRITE(6,102)XKB,XKA,XKB2,XKA2,A

```

```

DO 30, I=1, NP
D=(Y(I)-A*(1.+(10.**(-H(I)))/XKB+XKA/(10.**(-H(I))))**2
30 DEV=DEV+D
SQ=2.7133*DEV
WRITE(6,111)DEV
T1=(A**2)*C5
T2=2.*A**C4+2.*XNP*A**A/XKB-2.*A*C7
T3=C8-2.*A*C1-2.*A*C3/XKB+XNP*A**A+A**A*C6/(XKB*XKB)
1+2.*A**A*C2/XKB-SQ
XKAH=(-T2+DSQRT(T2*T2-4.*T1*T3))/(2.*T1)
XKAS=(-T2-DSQRT(T2*T2-4.*T1*T3))/(2.*T1)
T4=C8-2.*A*C1-2.*A*XKA*C7+XNP*A**A
1+A**A*XKA*XKA*C5+2.*A**A*XKA*XKA*C4-SQ
T5=2.*XNP*A**A*XKA+2.*A**A*C2-2.*A*C3
T6=A**A*C6
XKBH=(-T5+DSQRT(T5*T5-4.*T4*T6))/(2.*T4)
XKBS=(-T5-DSQRT(T5*T5-4.*T4*T6))/(2.*T4)
WRITE(6,112)XKBH,XKBS,XKAH,XKAS
STOP
100 FORMAT(15,2F10.5)
102 FORMAT(/10X,'XKB.....=',D12.6/
110X,'XKA.....=',D12.6/
110X,'XKB2.....=',D12.6/
110X,'XKA2.....=',D12.6/
110X,'A.....=',D12.6)
105 FORMAT(1X,15,5X,F11.9,5X,F10.5)
107 FORMAT(/10X,'C1.....=',D12.6/
110X,'C2.....=',D12.6/
110X,'C3.....=',D12.6/
110X,'C4.....=',D12.6/
110X,'C5.....=',D12.6/
110X,'C6.....=',D12.6/
110X,'C7.....=',D12.6/
110X,'C8.....=',D12.6)
108 FORMAT(/10X,'U.....=',D12.6/
110X,'V.....=',D12.6/
110X,'W.....=',D12.6/
110X,'X.....=',D12.6/
110X,'R.....=',D12.6/
110X,'Z.....=',D12.6)
110 FORMAT(/10X,'TA.....=',D12.6/
110X,'TB.....=',D12.6/
110X,'TC.....=',D12.6)
111 FORMAT(/10X,'DEV.....=',D12.6)
112 FORMAT(/10X,'XKBH.....=',D12.6/
110X,'XKBS.....=',D12.6/
110X,'XKAH.....=',D12.6/
110X,'XKAS.....=',D12.6)
END

```