1

MICROD

National Library
of Canada

Bibliothèque nationale
du Canada

Canadian Theses Service

Services des thèses canadiennes

Ottawa, Canada
K1A 0N4

CANADIAN THESES

THÈSES CANADIENNES

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 an inferior 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.

**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 qualité inférieure.

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.

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

**ISOLATION, STRUCTURAL AND MECHANISTIC
STUDIES OF RAT LIVER MITOCHONDRIAL
ALDEHYDE DEHYDROGENASE**

by

David John Senior, B.Sc.

A thesis submitted to the Faculty
of Graduate Studies in partial fulfillment
of the requirements for the degree of

Doctor of Philosophy

Department of Chemistry
Carleton University
Ottawa, Ontario
27/02/87

© copyright
1987, David J. Senior

Permission has been granted to the National Library of Canada to microfilm this thesis and to lend or sell copies of the film.

The author (copyright owner) has reserved other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without his/her written permission.

L'autorisation a été accordée à la Bibliothèque nationale du Canada de microfilmer cette thèse et de prêter ou de vendre des exemplaires du film.

L'auteur (titulaire du droit d'auteur) se réserve les autres droits de publication; ni la thèse ni de longs extraits de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation écrite.

ISBN 0-315-35860-2

The undersigned recommend to the Faculty of
Graduate Studies and Research acceptance of the thesis

**"ISOLATION, STRUCTURAL AND MECHANISTIC STUDIES OF RAT
LIVER MITOCHONDRIAL ALDEHYDE DEHYDROGENASE"**

submitted by David J. Senior, B.Sc.

in partial fulfillment of the requirements for
the degree of Doctor of Philosophy

Chairman, Chemistry Department

Thesis Supervisor

External Examiner

Carleton University
February 27, 1987

ABSTRACT

Rat liver mitochondrial aldehyde dehydrogenase has been purified to electrophoretic and chromatographic homogeneity with respect to dehydrogenase and esterase activities. A partial characterization is presented and a comparison of physical and catalytic properties is made with other similar enzymes. The enzyme exists as a tetramer of four identical subunits and the enzyme does not exhibit Mg^{+2} initiated subunit dissociation yielding a half-of-sites to all-of-sites phenomenon.

Steady state kinetic studies have shown that the dehydrogenase reaction follows an Ordered Bi Bi mechanism using either NAD^{+} or $NADP^{+}$, with oxidized cofactor bound first followed by the aldehyde. Substrate inhibition is consistent with the formation of an abortive ternary complex.

Nicotinamide adenine dinucleotide is the preferred coenzyme as measured by steady state kinetics. However pre-steady state studies using stopped-flow spectrometry reveal that $NADP^{+}$ is bound and undergoes reaction faster than NAD^{+} . A proposed conformational change occurs prior to release of the reduced cofactor and results in the lower catalytic efficiency for $NADP^{+}$ as observed by steady state analysis. A multi-step product release, in which acid is released first followed by the reduced cofactor, is supported by the stopped-flow studies and steady state kinetic studies using *n*-alkanal substrates of increasing acyl chain length. The

observation of a burst of NAD(P)H formation suggests that reduced coenzyme release is rate limiting. A detailed mechanism is proposed.

The catalytically active residues in the active site were identified by pH kinetic studies and chemical modifications. Amino acid analyses of modified enzymes were done to correlate the effects of modification of specific residues with changes in activities. Up to two active site cysteine residues have been identified by these studies and one is likely involved in the formation of a thiohemiacetal intermediate by nucleophilic attack on the aldehyde substrate. This intermediate is likely hydrolyzed by an active site histidine. Similar studies have shown the active site to contain four essential histidine species, one arginine residue and a single essential lysine species (for esterase activity).

The difference in catalytic efficiency using NAD^+ and NADP^+ as cofactors was found to be due to an active site arginine and possibly an acidic species. The active site arginine likely provides an attractive influence on the 2'-phosphate of NADP^+ yielding an appreciable activity, as determined by chemical modification and ^{31}P NMR studies.

The esterase activity was shown to arise from catalytic residues in the same or an associated active site region as the dehydrogenase activity by kinetic data including pH and effector studies. Also, similar responses of esterase and dehydrogenase activities to chemical modifications suggest

the activities arise from the same or associated active site regions. An active site lysine has been proposed to function in a charge relay or acid assisted catalytic capacity to facilitate ester hydrolysis. It was apparent that the catalytically active cysteine required for the dehydrogenase activity was not involved in the esterase activity. Both activities have identical dependencies on histidine for activity. The esterase reaction was shown to involve the formation of an acyl-enzyme which is likely hydrolyzed by histidine.

The esterase activity was found to follow an Ordered Uni Bi mechanism with *p*-nitrophenol released first, followed by butyrate ions. Stopped-flow and kinetic studies indicated that the rates of deacylation and product release were comparable.

The esterase activity observed an activation effect by phosphatidyl ethanolamine and cardiolipin only when encapsulated into phospholipid vesicles, which suggests the enzyme exists in a more activated form *in vivo* than when purified.

ACKNOWLEDGEMENTS

It is a pleasure to acknowledge the guidance, instruction, encouragement and criticism and friendship I have received from Dr. C.S. Tsai throughout the course of this work. I also wish to thank Dr. P. Buist, Dr. K. Storey, Dr. H. Dettman, Dr. M. Kates, Dr. A. Storer, Dr. B. Hollebone, Dr. G. Buchanan, Bill Mills, J. Johns and Bruce Ramsey.

I am grateful to P. Bertels for his technical expertise, Keith Bourque for running NMR spectra, Chris Matson for amino acid analyses, Ernie Bond from Agriculture Canada for his help with the liposome electron microscopic photographs, Dr. Larry Holbrook of Agriculture Canada for use of his gel scanner, Dr. Trevor Bladen for use of his 2D-electrophoresis apparatus, Dr. P. Kruus for use of his sonicator and Karl Dietrich and Brian Picknell for technical support.

I extend a warm thank you to my good friend Loola for her help and encouragement. Thanks also to other members of our lab including Jim Godin, Dr. J. White, Dr. A.J. Wand, Ken Mitton, Karen Farrell, Leslie Thompson, Monica Narang. Thanks also to Lee Collier, Tony Mattioli, Shaole Wu and to Doug Stewart.

Thanks to Dr. D. Wigfield for the use of his stopped-flow spectrometer and to Dr. R. Barradas for the use of his graphics plotter.

Thanks to Dr. A. Storer for the use of his stopped-flow device, software and generous assistance.

Special thanks are extended to Karen McKernan for her excellent typing skills and good natured enthusiasm, Joanne Picknell for her friendship and skilled typing support, and also to Monik and Tim Wold for their typing skills and friendship.

Financial support was provided by the Faculty of Graduate Studies, the Department of Chemistry and Government of Ontario.

I am especially thankful for the love and support I have received from my mother, father and sister Kathy and to the entire Orr family and also to my friends for their support and encouragement.

This thesis is dedicated to Donna Senior

TABLE OF CONTENTS

	page
Title Page	ii
Acceptance Sheet	iii
Abstract	vi
Acknowledgements	
Table of Contents	vii
List of Tables	viii
List of Figures	x
List of Schemes	xix
List of Plates	xx
List of Abbreviations	xxi
Introduction	1
Materials and Methods	24
1 Purification and Characterization	38
2 Steady State Studies	43
3 Chemical Modifications	55
4 Pre-Steady State Kinetic Studies	58
5 Encapsulation of Aldehyde Dehydrogenase	
Results	
1 Purification and Characterization	64
2 Steady State Kinetic Studies- Part A	83
3 Steady State Kinetic Studies- Part B	173
4 Chemical Modifications	229
5 Pre-Steady State Kinetic Studies	283
6 Encapsulation of Aldehyde Dehydrogenase	297
Discussion	326
Conclusions and Claims of Originality	382
Appendix I	385
Appendix II	393
Appendix III	398
Appendix IV	400
Appendix V	402
Appendix VI	426
References	430

LIST OF TABLES

Table	Description	page
I	Comparison of the Distribution of Aldehyde Dehydrogenases in Sub-Cellular Fractions from Rabbit, Rat and Mouse Livers	3
II	Enzyme-Coenzyme Interactions in LDH, LADH and G3PDH	18
III	Volumetric Guide for Preparation of Sucrose Gradient for Isoelectric Focusing	32
IV	Summary of the Purification of Aldehyde Dehydrogenase	65
V	Physical Properties of Aldehyde Dehydrogenase	74
VI	Amino Acid Composition of Aldehyde Dehydrogenase	82
VII	Relative Retentions of PTH-Amino Acids	84
VIII	Kinetic Parameters For NAD^+ and NADP^+ Dependent Dehydrogenase Activities, pH 7.5.	91
IX	Summary of Kinetic Constants For NAD^+ and NADP^+ Analogues	109
X	pH Dependence of Kinetic Constants of NAD^+ Dependent Dehydrogenase Activity	124
XI	Substrate Specificity: Summary of the Kinetic Constants for NAD^+ Dependent Dehydrogenase Activity	140
XII	Comparison of Substrate Specificity with Respect to Dehydrogenase Activities	142
XIII	Summary of Product Inhibition Patterns for Bireactant Mechanisms	144
XIV	Product Inhibition Patterns of NAD^+ Dehydrogenase Activity of Aldehyde Dehydrogenase	152
XV	Product Inhibition Patterns of NADP^+ Dehydrogenase Activity of Aldehyde Dehydrogenase	153

XVI	Influence of Various Effectors on Dehydrogenase Activities	166
XVII	Summary of Product Inhibition Constants of Reduced Cofactors on Dehydrogenase Activities	170
XVIII	Substrate Specificity of Esterase Activity	179
XIX	Effect of pH on Esterase Activity	188
XX	Effect of Solvent Polarity on Esterase Activity	198
XXI	Product Inhibition Patterns For Uni Bi Mechanisms	201
XXII	Product Inhibition Patterns For Esterase Activity	204
XXIII	Effects of Inhibitors on Esterase Kinetic Parameters	208
XXIV	Effect of Nucleotides on Esterase Kinetic Parameters	222
XXV	Effect of Aromatic Substitution of Ester Substrates on Esterase Activity, pH 7.5	228
XXVI	Summary of Modification Effects on Dehydrogenase and Esterase Activities of Aldehyde Dehydrogenase	231
XXVII	Amino Acid Analysis of Modified Acetaldehyde Dehydrogenase	261
XXVIII	Summary of Modification Effects on Amino Acid Analyses of Aldehyde Dehydrogenase	262
XXIX	Summary of Properties of Rat Aldehyde Dehydrogenases	331
XXX	Comparison of Amino Acid Composition of Aldehyde Dehydrogenases From Various Sources	334

TABLE OF FIGURES

Figure	Description	page
1	Elution Profile of Aldehyde Dehydrogenase from AGNAD Affinity Column	67
2	Elution Profile from Sepharose 4B Column	71
3	Subunit Molecular Weight Determination of Aldehyde Dehydrogenase by SDS Gel Electrophoresis	75
4	Stokes Radius Determination of Aldehyde Dehydrogenase	77
5	Isoelectric Focusing of Mitochondrial Aldehyde Dehydrogenase	78
6	UV Scan of Aldehyde Dehydrogenase	79
7	Thermal Stability of Mitochondrial Aldehyde Dehydrogenase	81
8 a	Double Reciprocal Plot for NAD^+ Dependent Dehydrogenase Kinetics, pH 7.5, 25°C	86
8 b&c	Secondary Plots for NAD^+ Dependent Dehydrogenase Kinetics, pH 7.5, 25°C	87
9 a	Double Reciprocal Plot for NADP^+ Dependent Dehydrogenase Kinetics, pH 7.5, 25°C	88
9 b&c	Secondary Plots for NADP^+ Dependent Dehydrogenase Kinetics, pH 7.5, 25°C	89
10	Structures of NAD(P)^+ Analogues	93
11 a	Double Reciprocal Plot For 3-Acetylpyridine- NAD^+ Dependent Dehydrogenase Kinetics, pH 7.5, 25°C	95
11 b&c	Secondary Plots for acPy- AD^+ Kinetics, pH 7.5, 25°C	96
12 a	Double Reciprocal Plot for 3-Acetylpyridine- NADP^+ Dependent Dehydrogenase Kinetics, pH 7.5, 25°C	97
12 b&c	Secondary Plots for acPy- ADP^+ Kinetics, pH 7.5, 25°C	98

13 a	Double Reciprocal Plot for Thio-NAD ⁺ Dependent Dehydrogenase Kinetics, pH 7.5, 25°C	99
13 b&c	Secondary Plots for Thio-NAD ⁺ Kinetics, pH 7.5, 25°C	100
14 a	Double Reciprocal Plot for Thio-NADP ⁺ Dependent Dehydrogenase Kinetics, pH 7.5, 25°C	101
14 b&c	Secondary Plots for Thio-NADP ⁺ Kinetics, pH 7.5, 25°C	102
15 a	Double Reciprocal Plot for Deamino-NAD ⁺ Dependent Dehydrogenase Kinetics, pH 7.5, 25°C	103
15 b&c	Secondary Plots for Deamino-NAD ⁺ Depen- dent Dehydrogenase Kinetics, pH 7.5, 25°C	104
16 a	Double Reciprocal Plot for Pyridine Alde- hyde Adenine Dinucleotide Dependent Dehydrogenase Kinetics, pH 7.5, 25°C	106
16 b&c	Secondary Plots for Pyridine Aldehyde Adenine Dinucleotide Dependent Dehydro- genase Kinetics, pH 7.5, 25°C	107
17 a	Double Reciprocal Plot for Aldehyde Dehydrogenase Kinetics, pH 7.0, 25°C	112
17 b&c	Secondary Plots for Aldehyde Dehydro- genase Kinetics, pH 7.0, 25°C	113
18 a	Double Reciprocal Plot for Aldehyde Dehydrogenase Kinetics, pH 8.0, 25°C	115
18 b&c	Secondary Plots for Aldehyde Dehydro- genase Kinetics, pH 8.0, 25°C	116
19 a	Double Reciprocal Plot for Aldehyde Dehydrogenase Kinetics, pH 9.0, 25°C	118
19 b&c	Secondary Plots for Aldehyde Dehydro- genase Kinetics, pH 9.0, 25°C	119
20 a	Double Reciprocal Plot for Aldehyde Dehydrogenase Kinetics, pH 10.0, 25°C	121
20 b&c	Secondary Plots for Aldehyde Dehydrogenase Kinetics, pH 10.0, 25°C	122

21	pH Profile for NAD^+ Dependent Dehydrogenase Activity, 25°C	125
22	pH Profile for NADP^+ Dependent Dehydrogenase Activity, 25°C	126
23 a	Double Reciprocal Plot for Substrate Specificity of Dehydrogenase Kinetics Using Methanal, 25°C , pH 7.5	128
23 b&c	Secondary Plots for Substrate Specificity of Aldehyde Dehydrogenase Kinetics Using Methanal, 25°C , pH 7.5	129
24 a	Double Reciprocal Plot for Substrate Specificity of Dehydrogenase Kinetics Using Propanal, 25°C , pH 7.5	131
24 b&c	Secondary Plots for Substrate Specificity of Aldehyde Dehydrogenase Kinetics Using Propanal, 25°C , pH 7.5	132
25 a	Double Reciprocal Plot for Substrate Specificity of Dehydrogenase Kinetics Using n-Butanal, 25°C , pH 7.5	134
25 b&c	Secondary Plots for Substrate Specificity of Aldehyde Dehydrogenase Kinetics Using n-Butanal, 25°C , pH 7.5	135
26 a	Double Reciprocal Plot for Substrate Specificity of Dehydrogenase Kinetics Using n-Pentanal, 25°C , pH 7.5	137
26 b&c	Secondary Plots for Substrate Specificity of Aldehyde Dehydrogenase Kinetics Using n-Pentanal, 25°C , pH 7.5	138
27 a	Double Reciprocal Plot for Product (Acetate) Inhibition of NAD^+ Dependent Dehydrogenase Activity, 25°C , pH 7.5	148
27 b&c	Secondary Plots for Product (Acetate) Inhibition of NAD^+ Dependent Dehydrogenase Activity, 25°C , pH 7.5	403
28 a	Double Reciprocal Plot for Product (NADH) Inhibition of NAD^+ Dependent Dehydrogenase Activity, 25°C , pH 7.5	149
28 b&c	Secondary Plots for Product (NADH) Inhibition of NAD^+ Dependent Dehydrogenase Activity, 25°C , pH 7.5	405

29 a	Double Reciprocal Plot for Product (Acetate) Inhibition of NAD^+ Dependent Dehydrogenase Activity, 25°C , pH 7.5	150
29 b&c	Secondary Plots for Product (Acetate) Inhibition of NAD^+ Dependent Dehydrogenase Activity, 25°C , pH 7.5	406
30 a	Double Reciprocal Plot for Product (NADH) Inhibition of NAD^+ Dependent Dehydrogenase Activity, 25°C , pH 7.5	151
30 b	Secondary Plot for Product (NADH) Inhibition of NAD^+ Dependent Dehydrogenase Activity, 25°C , pH 7.5	408
31 a	Double Reciprocal Plot for Product (Acetate) Inhibition of NADP^+ Dependent Dehydrogenase Activity, 25°C , pH 7.5	154
31 b&c	Secondary Plots for Product (Acetate) Inhibition of NADP^+ Dependent Dehydrogenase Activity, 25°C , pH 7.5	409
32 a	Double Reciprocal Plot for Product (NADPH) Inhibition of NADP^+ Dependent Dehydrogenase Activity, 25°C , pH 7.5	155
32 b&c	Secondary Plots for Product (NADPH) Inhibition of NADP^+ Dependent Dehydrogenase Activity, 25°C , pH 7.5	410
33 a	Double Reciprocal Plot for Product (Acetate) Inhibition of NADP^+ Dependent Dehydrogenase Activity, 25°C , pH 7.5	156
33 b&c	Secondary Plot for Product (Acetate) Inhibition of NADP^+ Dependent Dehydrogenase Activity, 25°C , pH 7.5	412
34 a	Double Reciprocal Plot for Product (NADPH) Inhibition of NADP^+ Dependent Dehydrogenase Activity, 25°C , pH 7.5	157
34 b	Secondary Plot for Product (NADPH) Inhibition of NADP^+ Dependent Dehydrogenase Activity, 25°C , pH 7.5	414
35 a	Double Reciprocal Plot for Inhibition of NAD^+ Dependent Dehydrogenase Activity By Disulfiram, 25°C , pH 7.5	161

35 b	Secondary Plot for Disulfiram Inhibition of NAD^+ Dependent Dehydrogenase Activity, 25°C , pH 7.5	415
36 a	Double Reciprocal Plot for Inhibition of NADP^+ Dependent Dehydrogenase Activity By Disulfiram, 25°C , pH 7.5	162
36 b	Secondary Plot for Disulfiram Inhibition of NADP^+ Dependent Dehydrogenase Activity, 25°C , pH 7.5	416
37 a	Double Reciprocal Plot for Inhibition of NAD^+ Dependent Dehydrogenase Activity By Chloral Hydrate, 25°C , pH 7.5	164
37 b	Secondary Plot for Chloral Hydrate Inhibition of NAD^+ Dependent Dehydrogenase Activity, 25°C , pH 7.5	417
38 a	Double Reciprocal Plot for Inhibition of NADP^+ Dependent Dehydrogenase Activity By Chloral Hydrate, 25°C , pH 7.5	165
38 b	Secondary Plot for Chloral Hydrate Inhibition of NADP^+ Dependent Dehydrogenase Activity, 25°C , pH 7.5	418
39 a	Double Reciprocal Plot for Inhibition of NAD^+ Dependent Dehydrogenase Activity By NADPH , 25°C , pH 7.5	168
39 b	Secondary Plot for Inhibition of NAD^+ Dependent Dehydrogenase Activity By NADPH , 25°C , pH 7.5	419
40 a	Double Reciprocal Plot for Inhibition of NADP^+ Dependent Dehydrogenase Activity by NADH , 25°C , pH 7.5	169
40 b	Secondary Plot for Inhibition of NADP^+ Dependent Dehydrogenase Activity by NADH , 25°C , pH 7.5	420
41	Inhibition By Substrate, 25°C , pH 7.5	172
42 a-d	Double Reciprocal Plots for Substrate Specificity of Esterase Activity, 25°C , pH 7.5	175
43 a-g	Double Reciprocal Plots for pH Effect on Esterase Kinetics, 25°C	181

44	pH and Solvents Effects on Esterase Activity, 25°C	189
45 a-h	Double Reciprocal Plots for Solvent Effect on Esterase Kinetics, 25°C	190
46 a	Double Reciprocal Plot for Product Inhibition of Esterase Activity By Butyrate Ions, 25°C, pH 7.5	202
46 b	Secondary Plot For product Inhibition of Esterase Activity By Butyrate Ions, 25°C, pH 7.5	421
47 a	Double Reciprocal Plot for Product Inhibition of Esterase Activity By p-Nitrophenol, pH 7.5, 25°C	205
47 b&c	Secondary Plots for Product Inhibition of Esterase Activity By p-Nitrophenol, 25°C, pH 7.5	422
48 a	Double Reciprocal Plot For Esterase Inhibition By Acetaldehyde	206
48 b	Secondary Plot for Esterase Inhibition By Acetaldehyde, 25°C, pH 7.5	424
49 a	Double Reciprocal Plot for Esterase Inhibition By Chloral Hydrate, 25°C, pH 7.5	209
49 b	Secondary Plot for Esterase Inhibition By Chloral Hydrate, pH 7.5, 25°C	425
50 a	Double Reciprocal Plot for Esterase Inhibition By Disulfiram, 25°C, pH 7.5	210
50 b	Secondary Plot for Esterase Inhibition By Disulfiram, 25°C, pH 7.5	426
51 a	Double Reciprocal Plot for Esterase Activation By Low Concentrations of NAD ⁺ , 25°C, pH 7.5	212
51 b	Secondary Plot for Esterase Activation in The Presence of Low Concentrations of NAD ⁺ , 25°C, pH 7.5	215
52 a	Double Reciprocal Plot for Esterase Inhibition By High Concentrations of NAD ⁺ , 25°C, pH 7.5	217
52 b	Secondary Plot for Esterase Inhibition By High Concentration of NAD ⁺ , 25°C, pH 7.5	218

53 a,b&c	Double Reciprocal Plots for the Action of Nucleotides on Esterase Activity, 25°C, pH 7.5	219
54 a,b&c	Double Reciprocal Plots for the Effect of Aromatic Substitution of Ester Substrates on Esterase Activity, 25°C, pH 7.5	225
55	Carboxymethylation by Iodoacetic Acid	233
56a	Carboxymethylation by Iodoacetamide	234
56b	Carboxymethylation of Cysteine Residues	235
57	Modification with DTNB	237
58	Carboxymethylation with NAD ⁺ Protection by Iodoacetamide	238
59a	Dye-Sensitized Photooxidation with Rose Bengal	241
59b	Oxidative Destruction of Histidine Residues During Rose Bengal-Sensitized Photooxidation of Aldehyde Dehydrogenase	242
60	Acetamidination of Aldehyde Dehydrogenase	244
61 a	Phenyl Glyoxalation of Aldehyde Dehydrogenase	247
61 b	Phenyl Glyoxalation of Aldehyde Dehydrogenase-Esterase Activity	249
61c	Phenyl Glyoxalation of Arginine Residues of Aldehyde Dehydrogenase	250
62	Amidation of Aldehyde Dehydrogenase	253
63	NBS Oxidation of Aldehyde Dehydrogenase	256
64	Nitration of Aldehyde Dehydrogenase	258
65	³¹ P NMR Spectra of NAD ⁺ in the Presence of Aldehyde Dehydrogenase, pH 7.5, 4°C	264
66	³¹ P NMR Spectra of NADP ⁺ in the Presence of Aldehyde Dehydrogenase, pH 7.5, 4°C	266
67	Effect of Aldehyde Dehydrogenase on the 2'-phosphate ³¹ P NMR Resonance of NADP ⁺	271

68	Effect of Chemically Modified Aldehyde Dehydrogenase on the ^{31}P NMR Spectra of NADP^+ , pD 7.5, 20°C	273
69	Effect of Chemically Modified Aldehyde Dehydrogenase on the ^{31}P NMR Spectra of NAD^+ , pD 7.5, 20°C	276
70 a-d	Densitometer Scans of Chymotrypsin Digested Aldehyde Dehydrogenase	279
71	NADH -Enzyme Interactions By Difference Spectroscopy	284
72	NADPH -Enzyme Interactions By Difference Spectroscopy	285
73 a	Stopped-Flow Assay of Aldehyde Dehydrogenase	288
73 b	Stopped-Flow Control Assay of Aldehyde Dehydrogenase	209
74 a	Stopped-Flow Determination of NAD^+ Dependent Dehydrogenase Activity Rate Constants	292
74 b	Secondary Plot for Determination of NAD^+ Dependent Dehydrogenase Activity Rate Constants	293
75 a	Stopped-Flow Determination of NADP^+ Dependent Dehydrogenase Activity	294
75 b	Secondary Plot for Determination of NADP^+ Dependent Dehydrogenase Activity Rate Constants	295
76	Stopped-Flow Determination of NAD^+ Dependent Dehydrogenase Activity-Ethanal Utilization	298
77	Stopped-Flow Determination of NAD^+ Dependent Dehydrogenase Activity-n-Pentanal Utilization	300
78	Stopped-Flow Determination of NADP^+ Dependent Dehydrogenase Activity-Ethanal Utilization	302
79	Stopped-Flow Determination of NADP^+ Dependent Dehydrogenase Activity-n-Pentanal Utilization	304

80	Elution Profile of Sonicated [^{14}C]-Cholic Acid-Phospholipid Mixture From Sephadex G-25 Column	307
81 a-d	Effect of Cholesterol Concentration on β -Glucosidase Activity in Intact and Lysed Liposomes	313
82	Dependency of Effectiveness Factor on Liposome Composition	318
83	Effect of Lipid Composition on the Esterase Activity of Aldehyde Dehydrogenase in Intact and Lysed Liposomes	321
Apl	Theoretical Plot Demonstrating The Dependency of Velocity on Ionization of Catalytic Species	429

LIST OF SCHEMES

Scheme	Description	page
1	Schematic representation of an Ordered Uni Bi mechanism	199
2	Non-essential activation	213
3	Photooxidation by sensitizing dye	240
4	General mechanism for aldehyde dehydrogenases	286
5	Ordered Bi Bi mechanism of NAD(P) ⁺ dependent activity of aldehyde dehydrogenase	356
6	Simplified mechanism of product release from aldehyde dehydrogenase	358
7	Two step dissociation of NADH from sheep aldehyde dehydrogenase	359
8	Proposed detailed mechanism of product release from aldehyde dehydrogenase	361
9	Proposed mechanism for dehydrogenase activity of rat mitochondrial aldehyde dehydrogenase	363
10	Proposed mechanism for esterase activity of rat aldehyde dehydrogenase	365
11	Acyl enzyme formation	367
12	Proposed charge relay function of lysine in esterase activity of rat aldehyde dehydrogenase	371

LIST OF PLATES

Plate	Description	page
1	SDS Discontinuous Polyacrylamide Gel Electrophoresis of Aldehyde Dehydrogenase From AGNAD Column.	70
2	SDS Discontinuous Gel Electrophoresis of Purified Mitochondrial Aldehyde Dehydrogenase	73
3	X-Ray Microscopic Photographs of Liposomes	309

ABBREVIATIONS

acPy-AD(P) ⁺	3-acetyl pyridine adenine dinucleotide (phosphate), oxidized form
ADH	Alcohol Dehydrogenase
ADP	Adenosine diphosphate
ADPR	Adenosine diphosphoribose
AGNAD	Agarose-hexane NAD ⁺ (Type 1) affinity chromatographic medium
AldDH	Aldehyde dehydrogenase
AMP	Adenosine monophosphate
Å	Angstroms
Bis	N, N'-methylene-bis-acrylamide
BSA	Bovine serum albumin
cm	centimeter
cmc	critical micelle concentration
DEAE	Diethylaminoethyl Sephadex ion-exchange medium
Deamino-NAD ⁺	Deamino-analogue of NAD ⁺ (see NAD ⁺)
DES	Diethylstilbestrol
DPG	Cardiolipin, diphosphatidyl glycerol
DTNB	5, 5'-Dithiobis(2-nitrobenzoic acid), Ellman's reagent.
DTT	Dithiothreitol, Clelands Reagent
EDC	1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide
E _t	Total enzyme concentration
EtOH	Ethanol
GDH	Glutathione reductase
G3PDE	Glyceraldehyde-3-phosphate dehydrogenase

g	Gram
x g	Centrifugal force (x unit gravitational field)
h ν	Light radiation
K _{AV}	Relative partition coefficient (gel filtration)
L	Litre
LADH	Horse Liver alcohol dehydrogenase
LDH	Lactate dehydrogenase
LUV	Large unilamellar vesicle(s) (liposome)
M	Molar
MeOH	Methanol
min	Minute
MLV	Multilamellar vesicle(s) (liposome)
MW	Molecular weight
N	Avogadro's number
n	Number of trials
NAD(P)H	Nicotinamide adenine dinucleotide (phosphate), reduced form
NAD(P) ⁺	Nicotinamide adenine dinucleotide (phosphate), oxidized form
NBS	N-Bromosuccinimide
NMN	Nicotinamide mononucleotide
NMR	Nuclear magnetic resonance
pNP	p-Nitrophenol
pNPA	p-Nitrophenyl acetate
pNPB	p-Nitrophenyl butyrate
pI	Isoelectric point
p.s.i.	Pounds per square inch

PyA-AD ⁺	3-Pyridine aldehyde adenine dinucleotide, oxidized form
R _f	Relative distance
r	Correlation coefficient
s	Second
s.d.	Standard deviation
SDS	Sodium dodecyl sulfate
SUV	Small unilamellar vesicle(s) (liposome)
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-Tetramethylethylenediamine
Thio-NAD(P) ⁺	Thio-NAD(P) ⁺ analogue of NAD(P) ⁺ (See NAD(P) ⁺)
TLC	Thin layer chromatography
tris	Tris(hydroxymethyl)aminomethane
TNM	Tetranitromethane
U	Enzyme activity unit
uv	Ultraviolet radiation
V _{max}	Maximum Velocity
V _{max} /E _t	Turnover number
v	Initial reaction rate (velocity)
° 1	Primary
° 2	Secondary
λ	Wavelength (in nanometers)
δ	Chemical shift (in ppm)

INTRODUCTION

The oxidation of aliphatic alcohols to aldehydes is a common metabolic process which is widely distributed in nature. Enzymes which catalyze this process are found in plants, fungi and yeasts (1), bacteria (2) and mammalian systems (3).

The metabolism of ethanol in mammals can occur by any of three enzyme systems: pyridine nucleotide linked alcohol and aldehyde dehydrogenase (4), the microsomal drug-metabolising system linked to P450 (5), and catalase (4).

The alcohol and aldehyde linked enzyme systems have wide cellular distribution. Both enzymes are found predominantly in the liver (6). Ethanol metabolism involves two steps. The first is the conversion of ethanol to acetaldehyde by alcohol dehydrogenase (ADH, EC.1.1.1.1) followed by the oxidation of acetaldehyde to acetic acid by aldehyde dehydrogenase (AldDH EC.1.2.1.3):

The cellular localization of each enzyme has also been the subject of numerous studies. Alcohol dehydrogenase predominates in the cytosol (6, 7) whereas aldehyde dehydrogenase is a more widely distributed enzyme. Estimates have placed the human mitochondrial content of AldDH as ranging from 13 to 80% (8, 9). Recently the AldDH distribution was compared in rat, mouse and rabbit (10). The activity of

AldDH varied substantially in the various organelles, as is indicated below in Table I.

The physiological roles of the LADH and AldDH are uncertain. A role has been proposed by Krebs and Perkins (11) suggesting ethanol and other alcohols produced by intestinal bacteria are transported via the hepatic portal vein to the liver where they are oxidized. A physiological role was proposed for the conversion of retinol to retinal, a reaction important in the visual process (12). Other roles have been suggested including an involvement in steroid biosynthesis (13) and bile acid metabolism (14).

The acute effects of ethanol consumption are, of course, inebriation arising from a combination of several factors. Predominant among these are the alteration of steady state levels of biogenic amines by alteration in rates of AldDH metabolism of biogenic aldehydes (15, 16). Acetaldehyde, the first product of ethanol metabolism, has also been shown to cause release of norepinephrine. In vitro studies have shown acetaldehyde reacts with norepinephrine to produce tetrahydroisoquinolines which are important psycho-active compounds. These are implicated as having an involvement in the addiction phenomenon (17). Davis and Walsh (18) have identified similarities in the withdrawal symptoms of alcoholics and opium addicts, particularly with regard to the biological compounds involved.

TABLE I

Comparative Distribution of Dehydrogenase Activity
in Subcellular Fractions in the Rat, Mouse and Rabbit

Fraction	% Total Activity		
	<u>Rat</u>	<u>Mouse</u>	<u>Rabbit</u>
Mitochondria	66	81	35
Microsomes	21	8	38
Cytosol	0.1	1.6	28

Taken from (10).

Acetaldehyde is more toxic than ethanol, which itself is comparatively fairly inert (17). The toxicity may be due to effects on metabolic pathways (as above) and on cellular organelles. Chronic ethanol ingestion has been associated with striking ultrastructural changes of the hepatic mitochondria, increased mitochondrial fragility and compromised integrity of the inner membrane, all leading to the injurious effect of chronic alcohol consumption (19, 20).

Four isozymes of aldehyde dehydrogenase have been identified in the human liver (21). These are named E₁, E₂, E₃, and E₄ based on their isoelectric points (22). The isozymes have distinct K_m values for acetaldehyde. The isozyme named E₂ (human liver AldDH, pI 4.9) has a micromolar K_m for acetaldehyde (high aldehyde affinity). Many people of oriental persuasion exhibit a "facial flush reaction" after the consumption of small amounts of alcohol. Symptoms of the reaction include facial flushing, increased skin temperature, elevated pulse rate and nausea. This reaction has been attributed to the lack of isozyme E₂ in susceptible individuals (23). These individuals may be exposed to elevated blood acetaldehyde levels until the aldehyde is removed by isozymes such as E₁, which act at a slower rate due to their lower aldehyde affinities (higher K_m for acetaldehyde) (17).

Alcoholism is an important health problem and major efforts have been made to gain insight into the pathophysiology of the disease. The involvement of aldehyde dehydro-

genases in the clearance of acetaldehyde is of particular interest, especially in light of the acute and chronic toxic effects of this compound. Liver aldehyde dehydrogenases have also been isolated to various degrees from many mammalian sources. These include sheep (24, 25, 26), cow (27, 28), horse (29, 30), rabbit (31, 32), pig (16), mouse (33), and rat (7, 34-41).

Substantial efforts have been made in understanding the localization, characteristics and catalytic properties of the isozymes. Human E_1 and E_2 isozymes (cytoplasmic and mitochondrial derived respectively) were first identified by Blair and Bodely (42). These have subsequently been purified to homogeneity (43, 44). Tryptic digests of these preparations reveal less than 40% structural homology thereby suggesting major differences in amino acid sequences (6). A structural comparison was made of the human cytoplasmic isozyme (E_1) and the analogous isozyme from horse (45a, b 46). The primary structures of the two enzymes revealed 91% homology. Primary sequence analysis of the human mitochondrial enzyme has been done (45b).

As many as five isozymes of aldehyde dehydrogenase have now been identified in rat liver. These have all been shown to be membrane associated, being located in the mitochondria or microsomes (39). Two of the rat isozymes were shown to have similar kinetic properties to the human E_1 and E_2 enzymes (7). The low K_m (acetaldehyde) rat isozyme (micromolar K_m) was found to exist almost exclusively in the mito-

chondria. This was corroborated by the subsequent fractionation studies of Horton and Barrett (35), Koivula and Koivusalo (36) and Siew et al. (41). This enzyme has similar properties to those of the human (E₂) and horse (F₂) mitochondrial enzymes. The predominant isozymes differ in their higher K_m for acetaldehyde (millimolar). Since the levels of acetaldehyde produced during ethanol metabolism are very low (ca 25 μ M in blood (47)), the function of this enzyme in the clearance of acetaldehyde in ethanol administered rats has been suggested to be insignificant (48). In light of these kinetic properties, the physiological significance of the enzyme is in question. The role of the various isozymes in ethanol oxidation in general has also been in question. The use of enzyme inhibitors such as chloral hydrate and disulfiram, for example, has helped to establish roles for other isozymes. Disulfiram (shown below) acts as an inhibitor of aldehyde dehydrogenases but the susceptibility of the various isozymes to inhibition varies greatly.

Disulfiram (antabuse) is used for "aversion therapy" in the treatment of alcoholism. When an individual has an alcoholic drink while being treated with disulfiram, an

accumulation of acetaldehyde results from the inability of the aldehyde dehydrogenases to oxidize this compound. The ensuing toxic effects such as nausea occur (49). This unpleasant experience is used to decrease the desire to drink by behavior modification (50).

Chloral hydrate, a central nervous system depressant, has also been found to act in an inhibitory manner on aldehyde dehydrogenases (30, 52, 53). This has been attributed to the similarity in structure between chloral hydrate compound and acetaldehyde.

It has been shown that the human E_1 enzyme is very susceptible to the inhibitory effects of disulfiram whereas E_2 is essentially unaffected (44). The human E_2 isozyme has been shown to be required for bulk removal of ethanol (6). Since a disulfiram effect is observed in a treated individual but the E_2 enzyme is unaffected, a role for E_1 in ethanol clearance is suggested (6). Thus the differential responses of the various isozymes to inhibitors can be used to help classify and compare an isozyme preparation to other preparations. For example the sheep (25, 49), horse (48) and rat (37, 39, 40) mitochondrial isozymes which show similar physical and catalytic properties are all partially inhibited by disulfiram whereas the cytoplasmic enzymes of horse (48) and sheep (49) are dramatically inhibited by disulfiram. On this basis the roles of the isozymes in acetaldehyde metabolism have been proposed. Eckfeldt et al. (29) claim that the cytoplasmic enzymes F_1 and E_1 are

responsible for acetaldehyde metabolism because these are inhibited by disulfiram and a visible toxic reaction results. In rat, the mitochondrial rather than cytosolic enzymes have been attributed for acetaldehyde clearance (7, 35). Mitochondrial enzymes from rat have subsequently been shown to be susceptible to inhibition by disulfiram (39).

Another feature of aldehyde dehydrogenases is that they exhibit a second catalytic activity, namely the ability to hydrolyze *p*-nitrophenyl esters (39, 40ab, 51-55). This enzyme bifunctionality was first reported in 1961 for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) by Park et al. (56). Kinetic studies on G3PDH were important in the elucidation of the reaction mechanism for the dehydrogenase reaction. The esterase reaction of G3PDH has been shown to arise from the same active site as the dehydrogenase reaction. Detailed kinetic studies of the esterase activity were used to demonstrate the nature of an acyl intermediate formed during the dehydrogenase reaction. Much of the work done in the elucidation of the mechanism of the aldehyde dehydrogenase has been done by exploiting the nature of the esterase activity as was done for G3PDH (51-53). There is at present a controversy regarding the nature of the active sites of the esterase and aldehyde dehydrogenase reactions. Evidence has been presented which suggests the activities arise from separate active sites (55, 57, 58). Recently the evidence for and against this proposal has been reviewed, as is discussed below (59).

The purification of the enzyme has been achieved using a variety of biochemical techniques. Partially pure enzyme preparations have been obtained using routine ion exchange, ammonium sulphate fractionation techniques and gel filtration (27-30, 60). Affinity media such as 5'-AMP-Sepharose-4B (39, 40, 45, 60) and Cibacron blue F3G-A-Sepharose 4B (62) have also been used to attain high purity with shortened preparation times. It would seem obvious that these NAD(P)⁺ requiring enzymes could be purified on a gel containing a covalently linked NAD⁺, however, this has not yet been attempted.

The ability to oxidize aldehydes in the presence of NADP⁺ as cofactor has been reported in horse (29,30), cow (27), yeast (61, 62) and rat (7, 35, 39, 40ab). However none of these studies follow the purification of the enzymes with respect to NADP⁺ activity or provide detailed kinetic studies. As mentioned above, Lindahl and Evces recently provided a comparison of the localization of NAD⁺ and NADP⁺ dependent activities in subcellular organelles of rat, rabbit and mouse (10). These authors reported that the NADP⁺ activity was found predominantly in the microsomes rather than in the mitochondria, as observed for the NAD⁺ activity.

It is important that NAD⁺ and NADP⁺ activities be carefully monitored throughout purification procedures and that accurate kinetic similarities be established. It has simply been assumed that the NAD⁺ and NADP⁺ dependent dehy-

drogenase activities arise from the same active sites. It is quite possible that the two activities arise from separate enzymic forms of the enzyme as has been observed for glucose-6-phosphate dehydrogenase (63). In addition, there have been absolutely no attempts to account for the differences in activities from a mechanistic viewpoint, using the two cofactors. For example in the rat enzymes, Horton and Barrett (35) and Lindahl and Evces (39) report a decreased catalytic efficiency with NADP^+ as cofactor. However, the reasons responsible for this are not mentioned. Enzymes such as alcohol dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, and lactate dehydrogenase are NAD^+ specific (64). Detailed structural studies, particularly the X-ray evidence of Rossmann et al. (65), have demonstrated the repulsive interactions of certain active site acidic species with the 2'-phosphate of NADP^+ and have therefore provided an explanation as to the NAD^+ specificity. Similarly, Matthews et al. have identified an active site arginine in the active site of dihydrofolate reductase and shown that this species provides an attractive interaction with the 2'-phosphate of NADPH (66). This interaction provides an explanation for the unique specificity of the enzyme for this coenzyme rather than for NADH . The ability of aldehyde dehydrogenase to utilize both NAD^+ and NADP^+ suggests that a unique combination of repulsive and attractive interactive effects are present between the active site and the two coenzymes.

Much work has been involved in determining the mechanism of the dehydrogenase reaction. The mechanism is generally considered to follow a sequential-ordered pathway with coenzyme binding first, followed by aldehyde to the enzyme-coenzyme binary complex. The acid product is released first, followed by the reduced coenzyme (6, 65). This mechanism has been established in pig (67), horse (53, 68) and for a limiting case of the sheep cytoplasmic enzyme (69).

The human enzyme has been shown to operate by a random mechanism (52). Similarly the sheep enzyme is reported to utilize a random mechanism at high concentrations of acetaldehyde substrate (69). Several different techniques have been employed to determine the mechanisms above. For example, the horse P_1 isozyme was determined to follow an ordered pathway by limited product inhibition evidence (68). Feldman and Weiner were able to suggest an ordered mechanism based on the effect of NAD^+ on the esterase reaction (53). They measured dissociation constants for NAD^+ and aldehyde by the dehydrogenase reaction and subsequently measured the same constants when both were used as effectors of the esterase reaction. The comparable dissociation constants for NAD^+ were interpreted such that NAD^+ was bound first to the enzyme. Also NAD^+ was found to protect the enzyme from thermal inactivation whereas the aldehyde substrate had no effect.

MacGibbon observed non-linear double reciprocal plots at high concentrations of acetaldehyde (69), as did Hart and Dickinson for the sheep enzyme (70). This was interpreted as the result of a Random Bi Bi mechanism. Roberts suggests that product inhibition studies can be used to distinguish the mechanism and order of substrate and product addition and release in enzymes (71). These techniques have generally been limited with aldehyde dehydrogenases which has been considered irreversible (53).

The key catalytic species in G3PDH was first identified by Krimsky and Racker as a cysteine residue (72) which was involved in the formation of an acyl-enzyme. They were able to prepare an acetyl enzyme with acetyl phosphate that was reducable by NADH to give acetaldehyde and NAD^+ . The acetyl enzyme was inhibited by iodoacetate, suggesting involvement of the thiol. Harris et al. (73) were able to confirm the involvement of thioester formation in this reaction by carboxymethylation of the enzyme. ^{14}C -iodoacetic acid was found to be incorporated into the enzyme via sulfhydryl linkages concomitant with inactivation of the enzyme activity. Similar conclusions were drawn by analogy for aldehyde dehydrogenase. Susceptibility of the dehydrogenase activities of aldehyde dehydrogenase to the inactivating effects of iodoacetic acid have been attributed to thiol inactivation in enzymes from pig (67), rabbit (59), and sheep (60). A rat enzyme was found to be inhibited by p-chloromercuribenzenes (34), suggesting the involvement of active site

thiol species. The formation of an acyl enzyme intermediate was shown by Feldman and Weiner (53). They demonstrated the electropositive nature of the carbonyl carbon of the substrate by use of a series of aldehyde substrates (RCHO) with a progressive increase in the electron-withdrawing power of the R group (discussed in next section).

It is also possible to identify catalytically essential ionizable species by the use of a pH profile of the enzyme (71, 74). A plot of $\log V_{\max}$ versus pH can be used to identify the ionizing species by extrapolation of the appropriate regions of the curve to the abscissae.

The involvement of an active site sulfhydryl species was confirmed in the horse cytoplasmic enzyme by von Bahr-Lindstrom et al. (75) using coenzyme analogues. They observed a specific thiol (Cys-302) was modified with the radio-labelled coenzyme analogue [5-[3-(bromoacetyl) pyridinio]-pentyl] diphosphoadenosine. This reactive coenzyme was reacted with the active site cysteine to give a covalently linked product. A tryptic digest was performed, the peptide was isolated, and sequence analysis of the labelled peptide revealed the radiolabel to be located at Cys-302. Their modification and identification of Cys-302 as a reactive species occurred simultaneously to the inactivation of the dehydrogenase activity as determined by Jeck (76). This confirmed their previous work which showed disulfiram protected Cys-302 from specific labelling with iodoacetamide (77).

Jencks has proposed that the hydrolysis of all acyl intermediates occurs by a general base reaction (78). Histidine exists as an excellent general base because of its inherent low pK and, has been demonstrated to act as such in chymotrypsin, papain and glyceraldehyde-3-phosphate dehydrogenase (79). This same species has been proposed by Takahashi et al. in the thiohemiacetal hydrolysis of aldehyde dehydrogenase on the basis of the pH profile for the horse enzyme (80). They also observed a second ionizable species of low pK arising from the biphasic nature of the profile. It was hypothesized this was possibly the result of a "charge relay" system with a second species such as tyrosine. A similar "charge relay" system involving the tyrosine phenol occurs in G3PDH and is discussed by Garavito (79).

The order of the release of products after thiohemiacetal hydrolysis was determined in the pig enzyme by product inhibition studies and shown to be NADH followed by the acid (67). Feldman and Weiner were able to show that the thioester hydrolysis was not the rate limiting step (53) for the reaction. They found no measurable kinetic isotope effect when deuterated and normal (protonated) aldehyde was used as substrate which suggested the hydride transfer was a rapid event. An identical observation was made by MacGibbon et al. in the sheep enzyme (69). These studies were corroborated by Eckfeldt and Yonetani with the horse enzyme (68). They observed a burst of enzyme-NADH complex formation by

pre-steady state analysis in a stopped-flow spectrometer. These results clearly indicated that release of NADH from the enzyme was the rate determining step. A similar burst was observed by Hart and Dickinson (70)

MacGibbon et al. were able to detect a two step release of NADH from sheep cytosolic AldDH by use of stopped-flow techniques (81). They used NAD^+ to displace NADH from an enzyme-NADH binary complex and monitored the release by stopped-flow spectrometry. A biphasic release of NADH was observed and was interpreted as being the result of a two step dissociation as described below:

The first step involved either a change in the conformation of NADH in the binding site or a conformational change of the enzyme-NADH complex, which is followed in either case by the dissociation of NADH from the enzyme. The slow rate limiting step was calculated to have a rate constant equal to that of the turnover number measured by steady state analysis. The concept of a two step dissociation would explain any possible acid product kinetic effects based on structure (ie. chain length), however this has not been investigated.

It was mentioned previously that the mechanism of the sheep cytosolic aldehyde dehydrogenase was thought to be random at high concentrations of substrate due to the non-linearity of the double reciprocal plots (69). A similar observation was made by Hart and Dickinson for the same enzyme (70). However, they suggested such behavior was possibly the result of the formation of an enzyme-NADH-aldehyde abortive complex in which high concentrations of aldehyde bound to the enzyme-NADH complex. This would provide an activated species and thus an alternative pathway for substrate addition, thus leading to an apparent increase in activity by steady state analysis. They also observed inhibition of activity in the presence of high concentrations of aldehyde and low levels of NAD^+ .

We have seen above that detailed structural studies of aldehyde dehydrogenases have been limited to human (43, 45a, b), and horse (46) systems. Amino acid analysis of the cytosolic enzyme from cow has also been done (28) and appreciable structural homology exists between this enzyme and the above (with respect to this parameter). The human and horse cytoplasmic enzymes exhibit 91% structural homology as determined by sequence analysis (45a, b, 46) and most of the amino acid changes are compatible with a single base mutation. Of the total 500 amino acids present in the peptide chain, only 43 are different between the two enzymes. Secondary structures were also calculated according to the method of Chou and Fasman (82) and only four

regions of apparent difference were estimated. Both enzymes were identified to have a catalytically active cysteine (Cys-302) to be identically positioned in the protein chains.

A second cysteine was also identified in both enzymes and was suggested to be involved in the active site events.

Earlier studies suggested that Cys 455/463 were close to the coenzyme binding site as it was specifically labelled by the coenzyme analogue [4-(3-acetylpyridinio)butyl] diphosphoadenosine. This reactive coenzyme analogue was covalently bound to an active site cysteine in a manner similar to the pentyl analogue we have seen above. The specificity of the two analogues is such that the pentyl analogue was bound to the catalytically active cysteine and the butyl was bound to another cysteine. This second cysteine was also shown to be located in the active site but it was not susceptible to the inhibitory effects of iodoacetamide. (83).

Structural studies of aldehyde dehydrogenases have not been sufficiently advanced to outline specific interactions between active site amino acids and the coenzyme. X-Ray analysis of crystallized enzymes have identified some of these specific interactions in lactate dehydrogenase, liver alcohol dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase. A summary of these interactions, as determined by Rossmann et al. (65) is found below in Table II (taken from Branden and Eklund (64). Jeffery (84) has commented

TABLE II

Enzyme - coenzyme interactions in LDH, LADH and GAPDH.

Underlined residues on the same line are structurally equivalent according to the alignment by Rossmann et al. (1975).

Coenzyme moiety	LDH	Residues LADH	GAPDH	Type of contact
Adenine	<u>Val 27</u>	<u>Phe 198</u>	<u>Asn 6</u>	Interior of pocket
	<u>Val 52</u>	<u>Val 222</u>	<u>Asn 31</u>	---
	<u>Asp 53</u>	<u>Asp 223</u>	<u>Asp 32</u>	H-bond to ring N
	<u>Val 54</u>	<u>Ile 224</u>	<u>Phe 34</u>	Hydrophobic
	<u>Tyr 85</u>			H-bond to ring N
	<u>Ile 96</u>			Hydrophobic
	<u>Ala 98</u>	<u>Ile 269</u>	<u>Thr 96</u>	---
	<u>Ile 119</u>		<u>Phe 99</u>	---
	<u>Ile 123</u>			---
		<u>Arg 271</u>		H-bond to N6
Adenosine ribose	<u>Gly 28</u>	<u>Gly 199</u>	<u>Gly 7</u>	
	<u>Asp 53</u>	<u>Asp 223</u>	<u>Asp 32</u>	H-bond to O-2'
	<u>Lys 58</u>	<u>Lys 228</u>	<u>C=O 8</u>	H-bond to O-3'
	<u>Asp 30</u>			H-bond to O-3'
	<u>Met 55</u>	<u>Ile 200</u>		Hydrophobic
			<u>Leu 187</u> <u>Pro 188</u>	Hydrophobic from adjacent subunit
Pyrophosphate	<u>N 31</u>	<u>N 202</u>	<u>N 10</u>	H-bond to main chain
	<u>N 32</u>	<u>N 203</u>	<u>N 11</u>	---
	<u>Arg 101</u>	<u>Arg 47</u>	<u>Asn 180</u>	Charged or polar
	<u>Lys 58</u>	<u>Arg 369</u>		---
			<u>Gly 99</u> <u>Ala 31</u>	
Nicotinamide ribose	<u>C=O 98</u>	<u>C=O 269</u>	<u>C=O 96</u>	H-bond to O-3'
	<u>N 140</u>	<u>N 294</u>	<u>anion</u>	H-bond to O-2'
		<u>His 51</u>	<u>Ser 119</u>	H-bond to O-3'
	<u>Gly 99</u>	<u>Gly 270</u>	<u>Gly 97</u>	
	<u>Val 32</u>	<u>Val 203</u>		Hydrophobic
	<u>Thr 97</u>			
	<u>Pro 139</u>	<u>Gly 293</u>	<u>Ala 120</u>	---
		<u>Val 294</u>		---
Nicotinamide	<u>Val 32</u>	<u>Val 203</u>	<u>Ile 11</u>	Hydrophobic
	<u>Val 138</u>	<u>Val 292</u>		---
	<u>Asn 140</u>	<u>Val 294</u>		
	<u>Leu 167</u>	<u>Thr 178</u>	<u>Tyr 317</u>	
	<u>Thr 246</u>			
	<u>Ile 250</u>			
Carboxamide			<u>Cys 149</u>	Charge transfer
	<u>C=O 139</u> <u>Ser 163</u>	<u>C=O 292</u> <u>N 319</u>	<u>Asn 313</u>	H-bond ---

(above) on the involvement of specific residues in the coenzyme specificity of several enzymes such as ADH, LDH and G3PDH (NAD^+ specific) and dihydrofolate reductase (NADP^+ specific). The various specificities are attributed to specific interactions of the coenzymes with active site residues. The only attempts that have been made to address specific coenzyme active site interactions in aldehyde dehydrogenases have been with respect to the cysteine species, as described above.

Detailed structural studies were preceded by the use of coenzyme analogues in alcohol dehydrogenase (85-89). Various NAD^+ analogues, differing in the substitution of functional groups, were used to examine the effects of the substitutions on kinetic parameters and to make inferences as to the specific functional groups required for activity. This was done recently for lactate dehydrogenase (90) where 18 NAD^+ analogues with alterations to the nicotinamide moiety, were used to study the effects on the kinetic properties of the enzyme. These workers were able to show that substitution to the C-3 position of the pyridinium had little effect on coenzyme whereas substitutions at the C-4 position caused over-crowding of the carboxamide group, prohibiting normal hydrogen bonding. Thus these studies are useful to gain information regarding the active site environment. This is particularly true when detailed structural information about the enzyme is not available to establish, in a general sense, the nature of the active site. There

have been no similar studies performed on aldehyde dehydrogenases.

The use of ^{31}P NMR has been useful in providing an understanding of the nature of binding of coenzymes to the active site of isocitrate dehydrogenase (91) and dihydrofolate reductase (92, 93).

Significant downfield shifts of the 2'-phosphate resonance of NADP^+ were observed when the ^{31}P NMR spectrum of this coenzyme was taken in the presence of dihydrofolate reductase (93). The same experiments observed upfield shifts of the 5'-pyrophosphate resonances in the presence of enzyme. The 2'-phosphate shifts were attributed to strong electronic interactions with an active site cationic species on the enzyme (92). Dihydrofolate reductase is the only NAD(P)H binding enzyme for which both ^{31}P NMR and detailed X-ray structure for the ternary complex with the reduced coenzyme are available (91). Matthews et al. have identified from their X-ray data that this species is an arginine, as we have seen above (66). The 5'-pyrophosphate shifts were primarily attributed to conformational changes in one of the pyrophosphates.

Thus ^{31}P NMR can play a useful role in the identification of interactions between active site species and the coenzymes.

The bulk of the evidence suggesting that the esterase and dehydrogenase activities arise from the same active site has been provided by Duncan and his colleagues. Duncan was

able to cause a reversal of the esterolytic pathway by placing the rabbit enzyme in the presence of NADH and *p*-nitrophenyl acetate (32). He observed the formation of the esterase product *p*-nitrophenol and a stoichiometric amount of acetaldehyde and NAD^+ . This suggested the involvement of the ester, enzyme and aldehyde at the same active site. Duncan also demonstrated that the esterase and dehydrogenase activities exhibited the same susceptibilities to the inactivation by iodoacetamide. Hart and Dickinson (94) were able to demonstrate with the sheep cytosolic enzyme that aldehydes could be produced if the corresponding anhydride was added to enzyme in the presence of NADH. The procedure would be expected to involve the formation of an enzyme-thioester-NADH complex (94). These authors also showed that not only did NAD^+ stimulate the esterase activity but the maximum rate of stimulated esterolysis was equal to the maximum rate for aldehyde oxidation. This suggested that the rates of both reactions were limited by the catalytic efficiency of the same active site (25).

Evidence against the concept of the esterase and dehydrogenase activities as arising from the same active site has been provided primarily by Blackwell, MacGibbon and their colleagues. It has been demonstrated in the sheep enzyme that high concentrations of NAD^+ inhibit the esterase activity but not the dehydrogenase activities (26). Kitson has observed selective inhibition of the dehydrogenase activity of sheep but not the esterase activity in the presence

of disulfiram (95). Blackwell observed non-competitive inhibition of the esterase activity but competitive inhibition of the dehydrogenase activity with chloral hydrate in the sheep cytosolic enzyme (57). From the above evidence it is obvious that the subject of similar or dual active sites for the two activities has not been satisfactorily addressed. It may be possible that the nature of the active site(s) is dependent on the enzyme source.

The hydrolysis of the proposed thioester intermediate of the cytosolic horse enzyme was shown by Takahashi and Weiner to result in a burst of p-nitrophenol formation (96). The burst size was variable on the concentration of NAD^+ used to pre-incubate the enzyme prior to addition of ester substrate. Thus the rate limiting step was concluded to be either deacylation or product release depending on any NAD^+ present. These authors proposed that the action of NAD^+ was to increase the magnitude of the rate constant of the acylation step. This could be the result of an increase in the nucleophilicity of the active site nucleophile in the presence of NAD^+ , thus increasing the velocity of the attack of the nucleophile on the ester carbonyl group.

Magnesium has been shown to cause dissociation of horse mitochondrial aldehyde dehydrogenase from a tetramer into dimers with a concomitant doubling of the dehydrogenase steady state activity and pre-steady burst of NADH formation (97). This was termed as a half-of-sites to all-of-sites phenomenon. A similar effect was not observed in the sheep

cytosolic enzyme but rather Mg^{+2} effected the inhibition of dehydrogenase activity (98). Stopped-flow experiments have recently shown in the sheep enzyme that Mg^{+2} accelerates the burst of NADH formation in the dehydrogenase activity. Magnesium also accelerated the hydrolysis of the acyl-enzyme using *p*-nitrophenyl propionate in the presence of NADH, as indicated by increased burst rates (99). These workers have proposed that acyl hydrolysis from the enzyme-NADH- Mg^{+2} complex provides evidence that the esterase and dehydrogenase activities occur on the same active site.

It is therefore obvious that there is no general consensus as to whether or not the dehydrogenase and esterase activities arise from the same active sites. It would appear that the nature of this effect varies depending on the source of the enzyme.

It is apparent that aldehyde dehydrogenases vary significantly in physical and catalytic properties depending on the source of the enzyme and the subcellular location. The limited amount of knowledge concerning these properties has been essentially provided from studies on human, sheep and horse enzymes. We have undertaken the first purification of a rat isozyme to homogeneity to allow detailed structural and mechanistic studies to be carried out. This will contribute greatly to the understanding of the inter-relationships of the dehydrogenase and esterase activities in aldehyde dehydrogenases in general.

MATERIALS AND METHODS

1. PURIFICATION AND CHARACTERIZATION

1. Materials.

Sprague-Dawley rat livers were obtained from Pel-Freez Biologicals Inc. The following were obtained from Sigma Chemical Company: Triton X-100; tris buffer salts; catalase; acrylamide; NADH; NADPH; thio-NAD⁺; deamino-NAD⁺; acPy-AD⁺; acPy-ADP⁺; PyA-AD⁺; disulfiram; diethylstilbestrol; ADP; electrophoresis protein molecular weight standards; yeast aldehyde dehydrogenase and the *p*-nitrophenyl esters of acetate, butyrate, caproate, caprylate, caprate and laurate. NAD⁺, NADP⁺ and NMN were from Boehringer-Mannheim. Agarose-hexane-NAD⁺ (Type 1) and ADPR were from P.L. Biologicals Inc. Anachemia supplied pH buffers and propanal and *n*-pentanal which were distilled before use. DEAE-A50 Sephadex and Sepharose-4B were purchased from Pharmacia Fine Chemicals. Ampholines in the range of pH 5.0-8.0 were the products of LKB. Bovine serum albumin and Bio gel P6 were from Bio Rad Laboratories. Phenyl isothiocyanate, hydrazine (95%) and chloral hydrate were from Eastman Organic Chemicals. *N*-Bromosuccinimide and benzaldehyde were from the Aldrich Chemical Company. Phenylthiohydantoin amino acid standards were provided by Mann Research Labs Inc. Silica 60-F254 plates were from EM Labs. Fisher supplied *n*-butanal, methanal, phenol reagent, glycerol, ammonium persulfate and 1,4-dioxane. Dithiothreitol was from Calbiochem-Behring. Sodium phosphate and borate was from Baker. Ferritin was from Polysciences Inc.

Phenyl octanoate and *p*-methylphenyl octanoate were synthesized as described by Tsai (100).

1.2. Buffers.

Protic buffers were prepared from appropriate weights of basic and acidic components. Specific buffers used are described in the text. All buffers were prepared with ultrapure deionized water from a Millipore Milli-Q water purification system. pH determinations were made using a Fisher Accumet model 825MP pH meter standardized with Anachemia standard buffers and read to ± 0.01 pH units with temperature corrections where necessary.

1.3. Preparation of Enzyme.

All procedures were performed at $0-4^{\circ}\text{C}$ with 10 mM phosphate buffer, pH 7.5 unless stated otherwise. Rat livers (50 g) were suspended in 100 mL of 0.23 M sucrose, 5 mM phosphate buffer medium, pH 7.3 (101). This mixture was then homogenized in a glass tissue homogenizer driven by a Fisher Dynamix variable speed drill mixer at position 5 for three strokes. Cellular debris was pelleted by centrifugation at $1100 \times g$ for 20 minutes in a Sorval RC2-B refrigerated centrifuge. Mitochondria were subsequently collected by centrifugation at $10,800 \times g$ for 40 minutes. The mitochondrial pellet was washed three times by repeated centrifugation and stored frozen at -20°C until needed.

To extract the enzyme, the mitochondrial pellet was thawed and suspended in 10 mM phosphate buffer containing 10 mM DTT and was made up to 0.1% with Triton X-100. It was sonicated on ice in 30 second intervals for a total of 3 minutes using a Fisher-Artek Sonic Dismembrator Model 300 at a power setting of 85% relative output. Membrane fragments and debris were removed by centrifugation at 65,000 x g in a Beckman L8-70M Ultracentrifuge. The supernatant was applied to a DEAE-A50 column (12.0 cm x 2.6 cm) equilibrated with the buffer described above, and 30 drop fractions were collected. The peak fractions (determined by measurement of absorbancy at 280 nm) were pooled and dialyzed against 0.20 M phosphate buffer, pH 7.5 for 1.5 hours. The enzymic sample was then heated to 45°C for 5 minutes followed by a rapid cooling to 0-1°C on ice. The denatured proteins were removed by centrifugation at 10,800 x g for 30 minutes. The supernatant was applied to an AGNAD column (0.9 cm x 8.0 cm) equilibrated with 10 mM phosphate buffer, pH 7.5. After extensive buffer washing the active enzyme was eluted immediately on addition of buffer containing 10 mM NAD⁺. The active fractions were pooled and concentrated with Millipore CX-10 Ultrafiltration Units to a volume of approximately 5 mL. Cofactor was then removed by 24 hour dialysis against three 1.0 L buffer changes. The enzyme was then applied to a Sepharose 4B column equilibrated with 10 mM phosphate buffer. The eluted enzyme was located by measurement of

absorbancy at 280 nm and the peak fractions were pooled and concentrated as described above.

1.4. Protein Determinations.

Protein concentrations were determined by the method of Lowry et al. (102) as modified by Miller et al. (103) using the following procedure. A solution labelled mixture I was prepared by mixing 98 mL of 2% (w/v) sodium carbonate with 1.0 mL of 2% (w/v) sodium potassium tartarate and 1.0 mL of 1% (w/v) cupric sulfate pentahydrate in the specified order. To 5 mL of mixture I was added 1 mL of enzyme preparation (usually diluted about 100 fold from any given step above) or stock BSA standard in a range of 5-100 μ g/mL. The solution was left to stand for 10 minutes after which time 0.5 mL of freshly prepared phenol reagent (104) (diluted 1:1 with water) was added. The samples were mixed well using a vortex mixer and were left to stand 30 minutes. Absorbancies were measured at 750 nm and protein concentrations were determined relative to the standard curve obtained from BSA standards.

1.5. SDS Discontinuous Gel Electrophoresis.

For discontinuous SDS polyacrylamide gel electrophoresis, a 10% separating gel and 5% stacking gel were prepared according to the tris/glycine method of Davis (105). A 1 mm slab gel apparatus was supplied by Bethesda Research Laboratories and the following solutions were used:

Running Gel: 12.5 mL of separating gel buffer (0.4% (w/v) SDS, 1.5 M tris, pH 8.2) + 16.7 mL acrylamide solution (30% (w/v), 0.8% (w/v) Bis) + 25 μ L TEMED + 20.8 mL water + 50 mg ammonium persulfate.

This was poured into the base of the apparatus, a fine layer of water was laid on top and the gel was allowed 1 hour to polymerize. After polymerization, the water layer was removed and the following stacking gel solution was added.

Stacking gel: 5 mL of stacking gel buffer (0.2% SDS, 0.25 M tris, pH 6.8) + 1.7 mL acrylamide solution (30% (w/v) acrylamide + 0.8% (w/v) Bis) + 5 μ L TEMED + 3.3 mL water + 10 mg ammonium persulfate.

After polymerization, the gel was assembled into the reservoir box and the electrode reservoirs were filled with a 1:4 dilution with water of 0.125 M tris, 0.46 M glycine, 0.5% (w/v) SDS, pH 8.3. Purified enzyme and molecular weight standards (1 mg/mL) were dissolved in the following sample buffer and heated for 2 minutes in a boiling water bath.

Sample buffer: 3% (w/v) SDS + 0.062 M tris + 10% glycerol.

The samples (1 μ L) were layered onto the tops of the gel and electrophoresis was run for 5 hr at 200 volts, 0-2°C using an Ortec 4100 power supply.

Proteins were visualized using the silver staining procedure of Nielson and Brown (106). The gel was fixed for 30 minutes in 10% (w/v) TCA. This was washed out with 3 x 10 minute washes of 10% EtOH + 5% acetic acid. This was then replaced with 3.4 mM potassium dichromate solution for 6 minutes. The oxidant was removed and the gels were washed 3 x 5 minutes with water. A solution of 0.012 M silver ni-

trate was then added and allowed to remain for 20 minutes after which time it was removed and two water washes were done each lasting one minute. A developer solution of 0.28 M sodium carbonate + 0.0185% (w/v) formaldehyde was then added and allowed to remain until the protein bands were visualized as black-brown spots. Development was arrested on replacement of the developer with 5% acetic acid. Determination of the subunit molecular weight was made relative to the standard proteins (BSA, ovalbumin, trypsinogen, β -lactoglobulin and lysozyme where indicated) using a plot of log molecular weight against mobility (107).

1.6. Molecular Weight Determination.

The molecular weight of the native enzyme was determined by sucrose gradient centrifugation (108). Aldehyde dehydrogenase and standard proteins (2 mg/mL) were dissolved in 10 mM phosphate buffer, pH 7.5 containing 14% (w/v) sucrose. Linear sucrose gradients (15-30%) were prepared using a Dekastaltic peristaltic pump, with $Rate_{in}/Rate_{out}$ preset to equal 0.5. The gradients were formed in Beckman polyallomer tubes (1/2" x 2") and were pre-chilled to 2-3°C for 2 hours prior to use. Enzyme aliquots (200 μ L) were layered onto the tops of the gradients with a syringe and the gradients were centrifuged for 5 hr at 150,000 x g and 2°C in a Beckman L8-70M ultracentrifuge equipped with a SW 55TI rotor. After centrifugation was completed, the tubes were punctured at the bottom with a syringe and 10 drop

fractions were collected. Protein bands were located by measurement of absorbancy at 280 nm against a 14% sucrose-buffer reference. Aldehyde dehydrogenase was located by enzyme assay of the peak fractions. Ferritin, catalase, yeast aldehyde dehydrogenase and horse liver alcohol dehydrogenase were used as standard proteins and molecular weights were calculated according to Martin and Ames (109). The ratio R of the distance travelled by two species (d_1 and d_2) is inversely proportional to both their elution volume ratio (V_2/V_1) and the ratio of sedimentation coefficients (S_1/S_2) as shown below:

$$R = d_1/d_2 = V_2/V_1 = S_1/S_2 = (MW_1/MW_2)^{2/3}$$

1.7. Isoelectric Focusing.

Isoelectric focusing was performed using a LKB 8100-1 (110 mL) column employing an ampholyte range of pH 5.0-8.0 (LKB Ampholines) according to the directions of the manufacturer. A dense cathode solution (0.4 mL monoethanolamine + 14 mL deionized water + 12 g sucrose) was poured into the base of the column. A 10% working solution of Ampholines was prepared by dilution of 2.5 mL of stock 40% Ampholines to 10 mL with deionized water. A sucrose gradient was established in the column (to prevent convection mixing) in the following manner. A heavy solution (7.5 mL of working Ampholine solution + 34.5 mL deionized water + 28 g sucrose) and a light solution (2.5 mL of working Ampholine solution + 57.5 mL deionized water) were prepared and added to separate burets. Twenty-four tubes were then prepared with varying

amounts of heavy and light solutions according to Table III to establish a sucrose gradient series. Each tube was then added in succession to the column, taking due care to avoid agitation of layers. Midway through the procedure (tube 12), the light solution was replaced by an equivalent volume of purified enzyme (approximately 5 mg/mL). When all fractions were applied, a light (anode) solution was layered on the top of the gradient (0.1 mL H_3PO_4 (85%) + 10 mL deionized water). The preparation was focused at 700 volts for 35 hr at 0-2°C. After focusing, fractions were eluted from the column, pH of the fractions was determined and protein was located by determination of absorbancy at 280 nm. Enzyme was identified by dehydrogenase assay of the peak fraction.

1.8. Stokes Radius.

Stokes radius was determined by comparative elution of purified enzyme against protein standards from a Sepharose 4B column (43.2 cm x 0.9 cm) equilibrated with 10 mM phosphate buffer, pH 7.5 (110).

The frictional ratio, f/f_0 was calculated from the effective hydrodynamic radius using the following equation:

$$f/f_0 = \left(\frac{r}{\frac{30M}{4\pi N}} \right)^{1/3} \quad (111)$$

where r is the Stokes radius, v is the partial specific volume (0.73 mL/mg (112)), M is the molecular weight and N is Avogadro's number.

TABLE III

VOLUMETRIC GUIDE FOR THE PREPARATION OF CONSTANT
SUCROSE GRADIENT IN A 110 mL COLUMN

Tube No.	Dense Solution (mL)	sum (mL)	Light Solution (mL)	sum (mL)
1	4.6	4.6	0.0	0.0
2	4.4	9.0	0.2	0.2
3	4.2	13.2	0.4	0.6
4	4.0	17.2	0.6	1.2
5	3.8	21.0	0.8	2.0
6	3.6	24.6	1.0	3.0
7	3.4	28.0	1.2	4.2
8	3.2	31.2	1.4	5.6
9	3.0	34.2	1.6	7.2
10	2.8	37.0	1.8	9.0
11	2.6	39.6	2.0	11.0
12	2.4	42.0	2.2	13.2
13	2.2	44.8	2.4	15.6
14	2.0	46.2	2.6	18.2
15	1.8	48.0	2.8	21.0
16	1.6	49.6	3.0	24.0
17	1.4	51.0	3.2	27.2
18	1.2	52.2	3.4	30.0
19	1.0	53.2	3.6	34.2
20	0.8	54.0	3.8	38.0
21	0.6	54.6	4.0	42.0
22	0.4	55.0	4.2	46.2
23	0.2	55.2	4.4	50.6
24	0.0	55.2	4.6	55.2

1.9. Extinction Coefficient.

Purified enzyme was extensively dialyzed against H₂O and lyophilized. The extinction coefficient was determined from a uv scan (320-250 nm) of the enzyme (0.240 mg/ml) dissolved in 10 mM phosphate buffer, pH 7.5, on a Cary 14 spectrophotometer at 25°C.

1.10. Thermal Stability.

The thermal stability of all activities were determined by incubating 600 µL of purified enzyme preparation (3.4 mg/mL) at 55°C in a water bath. At fixed time intervals 60 microLitre aliquots were withdrawn, placed into microfuge vials, and chilled on ice. Dehydrogenase and esterase assays were performed on these aliquots (see below).

1.11. Amino Acid Analysis.

Purified enzyme was dissolved in 6 M HCl (1.0 mg/mL) and placed in a reacti-therm vessel. The tube was evacuated with a mechanical pump, sealed, and heated at 110°C for 22 hours. The tubes were then allowed to cool, opened, and the contents were evacuated to dryness. The residue was dissolved in 10% methanol. Ortho-phthalaldehyde derivatives were prepared (113, 114) and these were subsequently analyzed on a Waters WISP 710B amino acid analyzer. To determine proline or cystine, samples were prepared separately and analyzed on a Beckman 119 BL amino acid analyzer. Cysteine residues were determined using the chromophore formation

method described by Scoffone and Pontana (115). Cysteine residues were modified using DTNB (see chemical modification section) in the presence of 6 M urea and cysteine content was determined by following the formation of 2-nitro-5-thio-benzoic acid at 412 nanometers on a Beckman model 25.

The tryptophan content was determined using the NBS method of Spande and Witkop (116). Purified enzyme (1.00 mg) was dissolved in 2.00 mL of 0.10 M acetate buffer, pH 4.0 including 8.0 M urea and centrifuged at 10,800 x g for 15 minutes, after which time no pellet was observed. The sample was scanned from 350 to 250 nm in a Cary 14 spectrophotometer. N-Bromosuccinimide (10 μ L of 7.5 mM NBS) was added and five minutes was allowed for the reaction to reach completion. The scan was repeated and the number of tryptophan residues (n) was determined using the following equation:

$$n = \frac{\Delta OD \times 1.31 \times MW \times V}{W \times 5500}$$

where ΔOD is the change in optical density at 280 nm corrected for dilution effects, V is the initial volume of titrated solution (mL), W is the weight of protein titrated (mg), MW is the molecular weight, 5500 is the molar extinction coefficient at 280 nm for tryptophan and 1.31 is an empirical factor which corrects for absorption at 280 nm of the oxidation product oxindol (this allows the optical

density decrease at 280 nanometers to be related to optical density contributions of tryptophan in the sample.

Determination of cysteine content in the native enzyme by radiolabelling techniques is described below in Section (3.4.)

1.12. N-Terminal Analysis

For N-terminal analysis, the isothiocyanate degradation method described by Edman and Henschen (117) was followed. Purified enzyme was dialyzed against 50 mM tris buffer, pH 8.0 and subsequently lyophilized. This preparation (0.8 mg) was dissolved in 5.0 mL of aqueous pyridine solution (1:1 by volume). The pH was adjusted to 8.6 with 2.86 mL of 0.10 M NaOH. The temperature was increased to 40°C on a hot plate with concomitant stirring and 0.3 mL of phenylisothiocyanate was added. An additional 0.78 mL of 0.1 M NaOH was added over the course of 0.5 hr at which time the reaction was considered complete. The preparation was cooled to room temperature and extracted with 3 volumes of benzene to remove excess reagents and side products. The benzene layers were washed once with 1 volume of aqueous pyridine solution. The aqueous fractions were pooled and acidified to pH 1.0. The white precipitate was collected by centrifugation at 10,800 x g for 10 minutes. After heating, the solution was cooled to room temperature and extracted with 3 volumes of ethyl acetate. This was then washed with 1 volume of 1M HCl. Recrystallization was not attempted due to

the very small quantity of material available. The preparation was filtered and stored at -20°C for TLC analysis on Silica 60-F254 plates. The solvent system used for TLC was 60 parts diethylene chloride to 7 parts acetic acid as described by Inglis and Nicholls (118). The PTH-amino acid standards and sample were located under an uv lamp.

1.13. C-Terminal Analysis.

The hydrazinolytic method of Narita et al. (119) was adapted for analysis of the C-terminal residue. A sample of lyophilized enzyme (6.0 mg) was placed into a 5 mL reaction vial. To this was added 0.50 mL of freshly prepared anhydrous hydrazine under N_2 and the tube was evacuated with a mechanical pump and sealed. This was then heated to 100°C for 10 hr with occasional shaking. The tube was cooled, opened and the reagent was then removed by rotary evaporation. The remaining material was then dissolved in 2.0 mL of H_2O and the contents were transferred to a small test tube. To this was added 50 μL of freshly distilled benzaldehyde and the mixture was rapidly shaken for 15 minutes on a vortex mixer followed by centrifugation in a microfuge. The supernatant was transferred to a second test tube. The oily layer was washed twice with 1.0 mL of H_2O and the washings were combined. The preparation was then passed through a Waters C_{18} Sep Pak cartridge and freeze dried. Amino acid analysis was subsequently performed as described above on a Waters WISP 710B amino acid analyzer.

1.14. Enzyme Assays.

Nicotinamide adenine dinucleotide and NADP^+ dependent dehydrogenase activities were monitored spectrophotometrically at 340 nm for the formation of NADH ($\epsilon_{340}=6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$) and NADPH ($\epsilon_{340}=6.20 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$) in a Beckman model 25 spectrophotometer. The assay mixture typically contained 1.0 mM NAD(P)^+ , 10 mM acetaldehyde and was initiated with 50.0 μL of the enzyme preparation (no less than 0.05 mg/ml) in 10 mM phosphate buffer, pH 7.5 at 25°C . One unit of dehydrogenase activity (U) is defined as that amount of enzyme which produces one micromole of NAD(P)H per minute at 25°C .

Esterase activity was monitored spectrophotometrically at 25°C by the formation of p-nitrophenol ($\epsilon_{400}=16.2 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$) at 400 nm (pH 7.5). p-Nitrophenyl butyrate (pNPB) was used as the substrate unless otherwise indicated. A stock solution of 5 mM p-nitrophenyl butyrate was prepared in 1,4-dioxane to limit spontaneous hydrolysis. All rates were corrected for this. The final concentration of pNPB for the assay was 25.0 μM in 10^{-4} M phosphate buffer, pH 7.5. A typical assay was initiated with 5.0 μL of enzyme preparation (no less than 5 $\mu\text{g/mL}$) Esterase activity is expressed in units where one unit is the amount of enzyme which produced one micromole of p-nitrophenol per minute at 25°C .

1.15. Reduced Cofactor-Enzyme Interaction.

Difference spectra were obtained of free NADH and NADPH versus NAD(P)H in the presence of enzyme in a Beckman model 25 spectrophotometer. The free cofactor spectra were obtained by placement of NADH (6.99×10^{-2} mM) or NADPH (9.35×10^{-2} mM) into side 1 of the sample cell and purified enzyme (7.48 mg/mL) into side two of the same cell. The cell was referenced to a second difference cell with enzyme (7.48 mg/mL) and buffer in the two respective compartments and a scan from 400-310 nm was obtained. The solutions in the sample cell were then replaced with an identical combination of cofactor-enzyme; however these were mixed prior to the scan. The reference cell was corrected for dilution effects and the scan was repeated.

2. STEADY STATE KINETIC STUDIES

2.1.i. Dehydrogenase Kinetics.

All kinetic studies were carried out in 100 mM phosphate buffer, pH 7.5 at 25°C. Stock buffered solutions of cofactors were prepared fresh daily. In general, assay mixtures contained cofactor and substrate concentrations in variable amounts as indicated in figure legends and axes. Enzyme concentrations ranged typically from 60-220 µg/mL. Reaction mixtures were made up to 1.00 mL with buffer. All kinetic assays were performed on a Beckman model 25 spectrophotometer as described in section 1.14. Specific kinetic experiments are described below.

The ability of the enzyme to utilize NAD^+ and NADP^+ and various analogues of these was investigated. Stock solutions ($10.0 \pm 0.2 \text{ mM}$) of NAD^+ and the analogues 3-acetylpyridine adenine dinucleotide (acPy-AD^+), thio- NAD^+ , 3-pyridine aldehyde adenine dinucleotide (PyA-AD^+) and deamino adenine dinucleotide were used in kinetic assays in the amounts described in the figure legends and axes. Similarly, stock solutions of NADP^+ , thio- NADP^+ and 3-acetylpyridine adenine dinucleotide phosphate (acPy-ADP^+) were employed as specified. Velocities were calculated as described previously using the following extinction coefficients: acPy-AD(P)H , $\epsilon_{363\text{nm}} = 9.1 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$; PyA-ADH , $\epsilon_{358\text{nm}} = 9.3 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$; deamino- NADH , $\epsilon_{338\text{nm}} = 6.2 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$; Thio- NAD(P)H , $\epsilon_{395\text{nm}} = 11.3 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ (120).

The effects of variation in substrate chain length on dehydrogenase kinetics was investigated. Kinetic assays were performed using a series of *n*-alkanal substrates of increasing chain length from one to five carbons. Stock solutions of methanal, ethanal, propanal (5.0 mM each) and *n*-butanal and *n*-pentanal (1.0 mM each) were prepared and used in the kinetic assays in concentrations as indicated in figure legends and axes.

The effect of variation in pH on dehydrogenase kinetic properties was investigated by performing kinetic experiments under assay conditions of varied pH. A series of 100 mM phosphate buffers ranging from pH 5.5-10.5 were used in

the kinetic assays along with cofactor and substrate concentrations as indicated in the figure legends and axes.

2.1.ii. Dehydrogenase Effector Studies.

Product inhibition studies were carried out in an effort to deduce the mechanism by which the dehydrogenase reactions are catalyzed (121). Kinetic experiments were performed in the presence of either acetate or reduced coenzyme products by variation in either substrate or cofactor concentration while holding the other at a fixed concentration. The specific product inhibition experiments are described in figure legends and axes. All assays were performed in buffered solution (100 mM phosphate buffer) at pH 7.5, 25°C. Stock solutions of reduced cofactors (1.00 $\pm$ 0.02 mM) and acetate (1.0 M sodium acetate, pH 7.5) were also prepared in buffered solutions and used in concentrations as indicated in figure legends and axes.

Cross product inhibition studies by NADPH on NAD⁺ dependent dehydrogenase activity and NADH on NADP⁺ dependent dehydrogenase activity were performed to demonstrate the duality of active sites for both activities. Kinetic assays were performed as described above using a stock solution of NADPH (1.00 $\pm$ 0.02 mM) to inhibit NAD⁺ dependent dehydrogenase activity (in concentrations described in figure legends and axes) and a stock solution of NADH (1.00 $\pm$ 0.02 mM) to inhibit NADP⁺ dependent dehydrogenase activity.

The influence of various effectors on dehydrogenase kinetics was performed in an effort to further characterize the enzyme, to aid in the determination of mechanism and to investigate the possibilities of a shared active site with the esterase activity. Stock solutions of chloral hydrate (100 mM) and disulfiram (10.0 mM in absolute ethanol) were used in kinetic assays in concentrations as indicated in figure legends and axes.

Initial velocity assays were performed using a stock diethylstilbestrol solution (10 mM in absolute ethanol) in a concentration range from 0-50 μ M. Similarly, the effect of the cations Mg^{+2} and K^{+} in the range 0-20 mM were investigated by initial velocity assays under assay conditions described above.

2.2.1. Esterase Kinetics.

All esterase kinetic studies described below were carried out in 100 mM phosphate buffer, pH 7.5 at 25°C. Stock solutions of *p*-nitrophenyl ester substrates (5.0 $\pm$ 0.1 mM) were prepared in 1,4-dioxane (5.0 mM) to limit spontaneous hydrolysis. Kinetic assays were performed as specified in section 1.14. above using 5 L of aldehyde dehydrogenase preparation (ranged from 5-50 μ g/mL) per assay. Specific kinetic experiments are described below.

The effect of variation in substrate chain length on esterase kinetics was investigated. Kinetic assays were performed using a series of *p*-nitrophenyl esters from two to

twelve carbons in length over a range of assay concentrations as specified in the figure legends and axes.

The effect of pH variation on esterase kinetics was also investigated by performing kinetic experiments under assay conditions of varied pH. A series of 100 mM phosphate buffers with a range of pH 6.0-11.0 were used in the kinetic assays with substrate concentrations specified in the figure legends and axes.

In an effort to assist in the elucidation of the mechanism of esterase activity, pH kinetics were repeated in an environment of increased hydrophobicity. Kinetic assays over the pH range 6.5-10 were performed as described above in the presence of 2.5% dioxane.

2.2.ii. Esterase Effector Studies.

Kinetic experiments were repeated in the presence of various nucleotide cofactors to investigate any relationships between esterase and dehydrogenase active sites. Stock solutions of ADP, ADPR, NMN (50.0 mM) in buffer were prepared and used in concentrations indicated in figure legends and axes. Similarly the action of NAD^+ on esterase activity over an assay range of 0.194 to 0.322 mM (low range) and 1.04-2.08 mM (high range) was investigated.

The effect of variation of electron withdrawing groups within the esterase substrate molecule was investigated to aid in the determination of the mechanism of esterase activity. Stock solutions (5.0 mM in 1,4-dioxane) of *p*-nitro-

phenyl octanoate, phenyl octanoate and *p*-methylphenyl octanoate were used in kinetic assays described above over a concentration range of 5.0 to 10.0 μ M.

Product inhibition studies were performed on the esterase activity in order to elucidate the esterase reaction mechanism. Esterase kinetics were carried out as described above in the presence of butyric acid in the concentration range of 0-2.0 mM (pH 7.5). Similarly, inhibition of esterase activity by *p*-nitrophenol was examined. Kinetic assays were performed in the presence of *p*-nitrophenol over the range of 0-0.20 mM.

Inhibition of esterase activity by acetaldehyde, chloral hydrate and disulfiram was carried out by performing kinetic assays as described above (including inhibitor preparations) in the presence of the effectors over a range of 0-30.0 mM, 0-0.75 mM and 0-0.080 mM respectively.

Initial velocity assays were performed in the presence of diethylstilbestrol and the cations Mg^{+2} and K^{+} as described previously in the dehydrogenase effector studies (2.1.ii.)

3. CHEMICAL MODIFICATIONS

Purified aldehyde dehydrogenase was chemically modified by a number of reagents intended to target specific amino acid residues. The modified enzymes were used in several different studies. Specific modifications and relevant studies are described below.

3.1. Materials.

Iodoacetic acid, DTNB, iodoacetamide were obtained from Sigma Chemical Co. Fisher supplied Scintiverse II, D-glucose, rose bengal, ammonium chloride and 1,4-dioxane. Phenyl glyoxal, N-bromosuccinimide and selenious acid were the products of Aldrich Chemical Co. Tetranitromethane was from the Nutritional Biochemical Corp. and methyl acetamidinate and 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide were from Pierce Chemical Co. [^{14}C]-Iodoacetamide (250 μCi total) and [$2\text{-}^{14}\text{C}$]-methylphenyl ketone (1.0 $\mu\text{Ci}/\text{mmol}$) were from ICN. Cellex-T was from Bio-Rad.

3.2.i. General

The modifications described below are specifically for activity studies. Aliquots removed for activity assay during the course of a reaction were used directly in the assay cell only if sufficient controls indicated no change in activity over the assay period as a result of a residual reaction. Otherwise, the reaction was arrested by passage of the aliquot through a P6 column (8 cm x 2.6 cm) and re-determination of protein concentration by Lowry assay.

The modifications were adapted for further studies described in sections 3.3 to 3.6.

3.2.ii. Carboxymethylation.

A stock solution (10.0 μL) of 0.20 M iodoacetic acid or iodoacetamide in 50 mM tris buffer, pH 8.0 was mixed with

0.50 mL of purified enzyme preparation (7.17 mg/mL, dialyzed 24 hr against 3 x 1 L of the same buffer) with stirring at 0-1°C (122-125). Aliquots were removed at time intervals specified in figure legends and axes and assayed for NAD⁺ and NADP⁺ dehydrogenase and esterase activities as described above.

Alternatively, sulfhydryl groups were modified with DTNB (126-130). Stock DTNB (100 μ L of 4.0 mM in 20% phosphate buffer, pH 7.5 and 5% absolute ethanol) was added to 0.90 mL of purified enzyme (1.47 mg/mL) while stirring on ice. At fixed time intervals, 100 μ L aliquots were removed and desalted through a P6 column (15 cm x 0.9 cm) and assayed for both dehydrogenase activities and esterase activities and protein concentration by Lowry assay.

3.2.iii Cysteine Modification With NAD⁺ Protection.

The modification with iodoacetamide was repeated as above in the presence of 1.33 mg/mL NAD⁺. Aliquots (100 μ L) removed during the course of the reaction were desalted through a P6 column and assayed for dehydrogenase activities and for protein concentration following the Lowry procedure.

3.2.iv. Dye-Sensitized Photooxidation.

A stock solution (20 μ L) of rose bengal (20.0 mM in phosphate buffer, pH 7.5) was added to 0.50 mL of purified enzyme solution (1.45 mg/mL) in a glass vessel surrounded with a water jacket circulating at 0-1°C. The reaction was

initiated by simultaneous addition of the rose bengal and application of a 250 watt tungsten lamp at a distance of 30 cm with stirring (131-133). Samples were removed over 40 minutes and assayed for all activities as previously described.

3.2.v. Acetamidination.

A stock solution (10.0 mL) of methylacetamidinate HCl (200 mg/mL neutralized in phosphate buffer, pH 7.5) was added with mixing to 0.5 mL of purified enzyme preparation (8.33 mg/mL) on ice (134). Aliquots were removed over 20 minutes and assayed for both NAD^+ and NADP^+ dehydrogenase and esterase activities.

3.2.vi. Phenyl Glyoxalation

The modification was initiated by addition of 50 μL of phenyl glyoxal (23 mg/mL in H_2O) to 1.0 mL of purified enzyme (9.04 mg/mL, dialyzed 24 hr in 3 x 1 L of 0.10 M borate buffer, pH 8.0) with stirring at $0-1^\circ\text{C}$ (135-137). Aliquots were removed over 30 minutes and assayed for dehydrogenase and esterase activities.

3.2.vii. Phenylglyoxalation With NAD(P)^+ Protection.

The modification was repeated as above with the exception that 2.5 mL of enzyme preparation was used and 2.0 $\mu\text{mol/mL}$ of NAD(P)^+ was added prior to initiation of reaction. The reactions were initiated on addition of 0.125 mL of the stock phenyl glyoxal. Aliquots (0.5 mL) were removed over

the time course of reaction, desalted through a P6 column (8 cm x 2.6 cm) and assayed for NAD(P)⁺ dehydrogenase and esterase activities and protein concentration by Lowry assay described above.

3.2.viii. NBS Oxidation.

A stock solution of 13.3 mg/mL of NBS in 50 mM tris buffer, pH 8.0 was prepared and 15.0 μ L of this was added with stirring at 0-1°C to 0.5 mL of purified enzyme (1.45 mg/mL, dialyzed for 24 hr in 3 X 1 L of the same buffer) to initiate the reaction (116, 138). Aliquots were removed over 26 minutes and assayed for both dehydrogenase and esterase activities.

3.2.ix. Nitration.

A stock solution of 1% (w/v) TNM in absolute ethanol was prepared and 1.0 μ L of this was added with stirring at 0-1°C to 0.5 mL of purified enzyme (1.45 mg/mL in 50 mM tris buffer, pH 8.0) to initiate the reaction (139-141). Aliquots were removed over 30 minutes and assayed for dehydrogenase and esterase activities.

3.2.x. Amidation.

This was performed by addition of 20 μ mol NH₄⁺Cl⁻ and 20 μ mol EDC to 1.0 mL of enzyme preparation (6.38 mg/mL in 0.10 M borate buffer, pH 7.0) with stirring at 0-1°C (142, 143). Aliquots were removed at times specified in figure legends

and axes and assayed for dehydrogenase and esterase activities.

3.2.xi. Amidation With NAD^+ Protection.

To 3.0 mL of the above enzyme preparation was added 3.99 mg NAD^+ . The reaction was initiated by addition of 6.42 mg NH_4Cl and 22.62 mg EDC with stirring at $0-1^\circ\text{C}$. After fixed times of reaction, 0.5 mL aliquots were desalted through a P6 column (8 cm x 2.6 cm) equilibrated with the same buffer. The aliquots were then assayed for both dehydrogenase and esterase activities and for protein concentration.

3.3. Amino Acid Analyses of Modified Enzyme.

Enzymes were modified with specific reagents (described above) for the reaction times which produced the observed inactivation effects. The modifications which were followed up with amino acid analyses, during the course of reaction, included carboxymethylation, phenyl glyoxalation and photo-oxidation. Samples of modified enzyme (300 μL) were removed during the course of reaction, stopped by passage through a P6 column (8 cm x 2.6 cm) equilibrated with deionized water and prepared for amino acid analysis as above. Rose bengal was removed by passage of the enzyme-reagent mixture through a Cellex-T column (1 cm x 0.9 cm) (132).

Lysine modified enzyme was prepared as described above and subjected to amino acid analysis after 1 minute of reaction.

3.4. Active Site Cysteine Determination

[¹⁴C]-Iodoacetamide (250 μ Ci total) was diluted with cold iodoacetamide to a specific activity of 1.430×10^{12} dpm/mol. Activity of this working solution was determined by counting a measured quantity of diluted reagent in a Beckman LS-8000 Liquid Scintillation Counter.

The diluted working solution was delivered (15.0 μ L) to 1.0 mL of purified enzyme preparation (0.480 mg/mL in 50 mM tris buffer, pH 8.0) and the modification was allowed to proceed as described previously. After 13 minutes of reaction at 0-1°C, 0.01 mL-0.10 mL aliquots were delivered to Whatman 3MM disks mounted on straight pins. The disks were collected in 200 mL of ice cold 10% TCA for 30 minutes. Following this the pins were removed and the disks were resuspended in 5% TCA and heated to 80°C for 30 minutes. The disks were transferred to a large Büchner funnel and rinsed with 3 x 200 mL of 5% TCA followed by 3 x 200 mL of ether:ethanol (1:1). The disks were air dried and then placed into scintillation vials with 10 mL of Scintiverse II cocktail and counted in a Beckman LS-8000 pre-set for total [¹⁴C] counting (automatic program #4).

3.5. ^{31}P NMR Studies

Phenyl glyoxalated and amidated enzymes were prepared as described above with the reactions being arrested by passage through a P6 column. Purified native and the modified enzymes above were dialyzed extensively against water and freeze-dried. The dried enzymes (about 20-30 mg each) were then dissolved in D_2O and lyophilized. This cycle was repeated twice more and the final lyophilized enzymes were stored under vacuum at -20°C . Prior to ^{31}P NMR spectroscopy, the enzymes were dissolved in 10 mM tris buffer, pD 7.50. This buffer was made by adding the required volume of D_2O to the appropriate amounts of deuterated tris buffer salts. Adjustments of pH were made by adding DCl or NaOD and apparent pH readings in D_2O were converted to pD according to $\text{pD} = \text{pH} + 0.44$ (144-146). Deuterated NAD^+ , NADP^+ and tris salts were prepared by repeated (three cycles) dissolution in D_2O and lyophilization. ^{31}P NMR spectra were recorded of free NAD^+ and NADP^+ . Two main studies were carried out. The first involved comparison of the ^{31}P NMR spectra of NAD^+ and NADP^+ as free cofactors (5.0 mM) and then over a concentration range of 0.50 mM to 4.0 mM in the presence of 4.2 mg of deuterated enzyme in 1.0 mL of deuterated tris buffer, pD 7.5. In the second study, similar spectra were obtained of the free cofactors; however modified enzymes were used to replace the native enzyme. ^{31}P NMR spectra were obtained of free NAD(P)^+ (1.0 mM) and again in the presence of native, phenyl glyoxalated and amidated

enzymes (10.3 mg/mL each). Similarly, spectra were obtained of free NAD^+ (1.0 mM) and in the presence of native, phenyl glyoxalated and amidated enzymes (11.8 mg each) as described above. ^{31}P Nuclear magnetic resonance spectra were obtained in a Varian XL-200 NMR spectrometer using a Nicolet TT-100 Data system to acquire the spectra. The following system parameters were used:

Transmitter nucleus	31.000	
Sweep width	4000.0	(Hz)
Aquisition time	0.500	(s)
Pulse width	10	(μs)
Delay	1.000	
Transmitter offset	-300	
Decoupler nucleus	1.700	
Decoupler mode	YYY	
Line broadening	5.000	(Hz)

^{31}P NMR spectra were taken in a 5 mm tube with respect to the 80% H_3PO_4 external reference. The first studies were taken at 4°C . The modified enzyme studies were taken at room temperature (20°C). An average of 1500 transients were accumulated for each spectrum. Enzyme activities were monitored on enzyme subjected to the same conditions of temperature and time as a control to ensure no changes in enzyme properties occurred during the NMR scans.

Binding constant between NAD(P)^+ and the enzyme were calculated according to:

$$K = \frac{1}{\left([E^0] - \frac{[N^0]}{1 + \Delta}\right)\Delta} \quad (147)$$

where $[E^0]$ is the starting enzyme concentration (4.2 mg/mL) $[N^0]$ is the starting NADP⁺ concentration (0.50-4.0 mM) and Δ is given by:

$$\Delta = \frac{\delta_b - \delta}{\delta - \delta_f}$$

where δ_b and δ_f are the observed chemical shifts for the bound and free coenzymes respectively and δ is the observed chemical shift.

3.6. Peptide Studies

Aldehyde dehydrogenase was modified with specific reagents as before. These however were labelled with the respective [¹⁴C] reagent. The reaction times were chosen to be sufficiently long ~~as~~ to permit only the observed activity effect to be completed. Exhaustive modification was not the intention. The modified enzymes were then subjected to chymotryptic cleavage and the peptides were analyzed by electrophoresis and radiolabel quantitation by scintillation counting. The specific procedures are described below.

[2-¹⁴C]-Phenyl glyoxal was prepared by Ken Mitton following the synthesis of Riley & Gray (148). [2-¹⁴C]-Phenylmethyl ketone (0.86 mmol, 250 μ Ci) was refluxed with a stoichiometric amount (0.86 mmol) of selenious acid in 15 mL of absolute ethanol for 3 hours. intervened by an overnight stirring. The selenium precipitate was decanted off and the phenyl glyoxal was collected by a vacuum distillation at 55°C (1.0 mm Hg) and recrystallized from five volumes of

water which yielded white needle crystals (specific activity 2.21×10^{10} dpm/mol), (m.p. 96°C).

[^{14}C]-Phenyl glyoxalated enzyme was prepared by addition of 100 μL of a stock solution of [^{14}C]-phenyl glyoxal (14.0 mg/mL in H_2O) to 1.0 mL of purified enzyme (1.5 mg/mL) in 50 mM tris buffer, pH 8.0 and the reaction was allowed to proceed for 40 minutes as previously described. The reaction was stopped by desalting through a P6 column (8.5 cm x 2.6 cm) and the preparation was freeze-dried and stored at -20°C .

[^{14}C]-Carboxymethylated enzyme was prepared as described above (3.4.) using 60 μL of 0.2 M [^{14}C]-iodoacetamide (specific activity 1.43×10^{12} dpm/mol) and 1.0 mL of purified enzyme (1.5 mg/mL). After 30 minutes of reaction at $0-1^{\circ}\text{C}$ the reaction was stopped by passage through a P6 column (8.5 cm x 2.6 cm) and the modified enzyme was freeze dried and stored at -20°C .

4

[^{14}C]-Amidated enzyme was prepared by addition of 100.0 μL of [^{14}C]-methyl amine (specific activity 2.40×10^{11} dpm/mol) to 1.0 mL of purified enzyme (1.5 mg/mL in 0.10 M borate buffer, pH 8.0) while stirring on ice followed by addition of 2.0 mg of EDC. The reaction was allowed to proceed for 1 hr and stopped by passage through a P6 column. The collected enzyme was freeze-dried and stored at -20°C .

Native enzyme and the [^{14}C]-amidated, [^{14}C]-phenyl glyoxalated and [^{14}C]-carboxymethylated enzymes (1.5 mg each) were each dissolved in 5.0 mL of deionized H_2O and the pH

was adjusted to pH 8.0 with 0.01 N NaOH. To each enzyme was added 20 μ L of a chymotrypsin solution (1.0 mg/mL in H₂O, pH 8.0) and digestion was allowed to proceed for 2 hr at 25°C. The chymotryptic digests were stopped by rapid freezing in a cryocool at -60°C and the enzymes were freeze-dried. The preparations were prepared for electrophoresis (1.0 mg/mL in electrophoresis sample buffer) as described in section 1.5. Discontinuous electrophoresis was performed on the samples as presented previously. The gels were stained with silver stain as above and scanned in a Beckman (DU-8) Gel Scanner. The protein regions corresponding to peaks on the gel scan were cut from the gel and placed into scintillation vials each containing 200 μ L of 30% H₂O₂. Digestion was allowed to continue for 2 hours after which time 5.0 mL of scintillation cocktail was added. The vials were counted as before after 24 hr dark pretreatment.

4.0 PRE-STEADY STATE KINETICS

Pre-steady state kinetic studies were performed on the purified enzyme using a Cantech Stopped-Flow Spectrophotometer. A schematic diagram of the spectrometer, an end-stopping type device, is seen below:

Schematic diagram of a stopped flow apparatus. A, B, are driven syringes; A' and B' are reservoir syringes; T1-T3 are 3-way valves; M, mixing chamber; C, observation cell; LS, light source; MN, monochromator; PM, photomultiplier; SS, stopping syringe; MS, microswitch for triggering the recording device; CRO, transient recorder, oscilloscope and computer; TR, trigger circuit; I, input to the transient recorder from the photomultiplier.

The basic principle of operation is as follows. The reagents, which are stored in separate syringes, are driven into the mixing chamber when force (compressed gas at 20-30 p.s.i.) is applied to the piston. The reagents are rapidly mixed and flow into the observation cell. The detection system, a photomultiplier interfaced to a transient recorder,

der, oscilloscope and computer, is activated when a sufficient volume (dead volume) of liquid displaces the stopping syringe which strikes the trigger switch. Changes in optical density at a preset selected wavelength are detected by the photomultiplier and the data is digitized and collected in the transient recorder. Data is sent to the oscilloscope for viewing and can be directed to the computer for processing and storage.

The flow system consists of two 2.0 mL syringes backed up by two 10 mL reservoir syringes all completely immersed in a thermostatted water bath. The dead time for this instrument is 3 milliseconds. The system employs a Cantech 1024B, 4 channel transient recorder which accumulates 256 digitized data points for a given experiment. The transient recorder has an adjustable time base enabling the worker to select the acquisition time (in milliseconds) per data point. Data accumulated from each experiment was stored on floppy diskette on a Commodore CBM computer interfaced to the transient recorder. Software for data accumulation, storage and processing was provided by Dr. A. Storer of the Division of Biological Sciences, National Research Council of Canada. Data from a given experiment was visualized using a Tektronix oscilloscope and printed in hard copy with Hewlett-Packard 7470A graphics plotter.

The program employed on our spectrometer was "expfit 256/09" which is a data fitting program allowing the operator to fit the data to a single first order exponential

event according to: $-\ln [A] = kt + I$ (150) — A copy of "exp-fit 256/09" can be found, written in basic, in Appendix I.

Stopped-flow kinetics were performed on purified enzyme in 100 mM phosphate buffer, pH 7.5 (10.0 mg/mL) which was placed in drive syringe 1. The second drive syringe was filled with a series of NAD^+ (0.74-1.99 mM), NADP^+ (0.61-1.63 mM) and acetaldehyde (0.35-3.0 mM) preparations in amounts specified in figure legends and axes. The stopped-flow reactions were measured at 340 nm for the formation of reduced cofactor at 25°C. The reaction was initiated by firing the drive syringes with a 20 p.s.i. compressed air source. The monochromator was set with no slit and the photomultiplier was set to a gain of 700 Volts and a gain of 280 with a response of 40 ns. The time base for the collection of data was 5-10 ns per point. Data collection and flow stopping was begun when the stopping syringe (pre-set dead volume of 0.40 mL) contacted a switch triggering the transient recorder. The first order rate constants obtained from the observed burst in the dehydrogenase reaction were used to calculate the enzyme kinetic constants as described in Appendix II.

The esterase reaction was investigated in a similar manner as above. The drive syringe 2 was filled with 0.5 mM *p*-nitrophenyl butyrate in buffer and the monochromator was set to 400 nm.

A comparison was made of the relative burst rate constants for NAD^+ and NADP^+ dependent activities using ethanal

and *n*-pentanal as substrates. The concentrations of NAD^+ and NADP^+ used in the assays were 0.98 and 0.92 mM respectively. The ethanal and *n*-pentanal concentrations used were both set at 0.4 mM.

ENCAPSULATION OF ALDEHYDE DEHYDROGENASE

5.1. Materials.

Phosphatidyl choline and ethanolamine, cholesterol, cholic acid, cardiolipin *p*-nitrophenyl- β -glucopyranoside, Triton X-100 and β -glucosidase were obtained from Sigma Chemical Co. [Carboxyl- ^{14}C]-Cholic Acid (52.0 mCi/mmol) and aquasol scintillation cocktail were from New England Nuclear. Silica gel 60 plates (0.25 mm) were from BDH. Chromatographic grade solvents were from Caledon. *N*-Methyl-*N*-nitro-*N'*-nitrosoguanidine was from Aldrich Chemical Co. Fatty acid methyl ester standards were purchased from Polyscience Corp. Sephadex G-25 was from Pharmacia Fine Chemicals.

5.2. Liposomes

5.2.1. Lipids.

The purity of the lipids purchased were checked by TLC analysis. Phosphatidylcholine was chromatographed using the following two solvent systems: (65:35:5 of CHCl_3 :MeOH: H_2O (151) and 25:15:4:1 of CHCl_3 :MeOH:Acetic Acid: H_2O (P.L. Biochemicals solvent system XIX (152)). The plates were developed using a 50:50 H_2SO_4 :MeOH spray followed by char-

ring over a hot plate (153) and also using a simple H₂O spray which was more sensitive than the charring method. Both methods yielded a single spot on the TLC plate.

Cholesterol purity was confirmed by TLC analysis using the solvent system of Kates described above (151). Phosphatidyl ethanolamine and cardiolipin purity were confirmed using the P.L. Biochemicals solvent system. Best results were achieved when plates were spotted, run and developed under N₂(g).

The fatty acid content of the lipids was determined by hydrolysis and methyl ester preparation of the fatty acids (154, 155). The lipid in question (1.00 mg) was dried under an N₂ stream in a 50 mL round bottom flask. An aqueous ethanol solution (10 mL of 50%) and KOH pellets to make 1.0 N were added. The lipids were refluxed for 1 hour, cooled and the ethanol was removed with a rotary evaporator. The solution was neutralized with 50% H₂SO₄ to pH 3.0 with congo red paper. The fatty acids were extracted with 2 x 10 mL of ether. The ethereal layer was washed with water (2 x 5 mL) and dried over MgSO₄. The ether was removed by rotary evaporation. Diazomethane used for methyl esterification was prepared using a diazomethane generator (a simple distillation device uses a solvent cooled condenser). Ether (3 mL) was layered over 3 mL of 50% (w/v) KOH in H₂O in a 25 mL round bottom flask and a small spatula of *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (extremely carcinogenic) was added while swirling. Several milliliters of ether and diazomethane were distilled by gentle heating directly into the flask.

containing the free fatty acids above. The reagent was quenched on addition of 1 mL of concentrated acetic acid. Removal of ether by rotary evaporation yielded the methylated fatty acids. Gas chromatographic analysis was performed on a Perkin Elmer 990 gas chromatograph using an 11'7" OV-17 column at 218°C.

5.2.iii. Liposomes.

Preparations of large unilamellar vesicles were made following a scaled up adaptation of the procedure described by Enoch and Strittmatter (156). Mixtures of lipids consisting of cholesterol, phosphatidyl choline, phosphatidyl ethanolamine and cardiolipin (100 μ mol total) as described in the figure legends, were dissolved in 1 mL of anhydrous ether in a 15 mL Corex centrifugation tube. The lipids were dried to a thin film under an N₂ stream in a glove bag and residual ether was removed by vacuum using a water aspirator for 45 minutes. A 20 mM tris buffer solution, pH 7.5 containing 50 mM NaCl (5.0 mL) or an equivalent volume of purified enzyme preparation (10.5 mg/mL) in the same buffer was mixed with 21.5 mg of cholic acid. The enzyme-detergent solution was then added, at room temperature, to the dried lipid mixture. This preparation was sonicated for a total of 3 minutes in 30 second intervals to prevent heating, at 35% relative output with a Fisher-Artek Sonic Dismembrator (equipped with a micro-probe) under an N₂ stream. The sonicated mixture was then passed through a G-25 column (40 cm

x 0.9 cm). The cloudy fractions were centrifuged for 15 minutes at 22°C in a Beckman swinging bucket rotor (SW 55.1) at 40,000 x g. The supernatant was decanted (assayed for enzyme activity when enzyme was to be entrapped) and the pellets were resuspended in 5 mL of 20 mM tris buffer, pH 7.5. This procedure was repeated 3 times to ensure the supernatants were free of all unbound enzyme. The final pellet was dissolved in 1.5 mL of buffer and kept on ice. Phospholipid content was determined by the method of Hallam (157) for aqueous phospholipid dispersions. To 2.00 mL of lipid dispersion (maximum of 2 μ mol lipid) was added 1 mL of molybdenum reagent and this was heated at 100°C for 2 minutes. This was allowed to cool to room temperature followed by addition of 3.50 mL of 9:1 CHCl_3 :MeOH. The sample was mixed and allowed to separate for 20 minutes. The organic layer was removed and absorbancy at 730 nm was determined against a pure chloroform reference. Phospholipid content was established from a standard curve prepared using accurately weighed phosphatidycholine standards.

To confirm successful removal of detergent on these scaled up preparations, [^{14}C]-cholic acid (0.104 mCi/ μ mol) was used in place of cold cholic acid above. The liposome preparation was passed as before through the G-25 column and 30 drop fractions were collected. The absorbancy at 450 nm was determined for all fractions eluted and 0.50 mL aliquots of each fraction were placed into scintillation vials containing 5 mL of scintillation cocktail and counted in a

Beckman LS 8000 scintillation counter. The remaining cloudy fractions from the column were pooled and centrifuged as before. After each spin 0.50 mL and 50.0 μ L of the resuspended pellet were used for scintillation counting and phospholipid determination respectively to determine the ratio of lipid to detergent remaining.

5.3. Encapsulation Studies.

Liposomes of various compositions as described in the results section were prepared by replacing buffer above using β -glucosidase (10 mg/mL) and purified aldehyde dehydrogenase (7.48 mg/mL) in separate experiments. β -Glucosidase activity was determined using varied concentrations of p-nitrophenyl- β -glucopyranoside as enzyme substrate in concentrations indicated in figure axes and legends. Relative β -glucosidase activity was determined by formation of p-nitrophenol at 400 nm on a Beckman model 25 recording spectrometer. Relative esterase activity was monitored as described previously (section 1.14.). Assays were repeated on encapsulated enzyme samples which were allowed 3 minutes for lysis with 5 μ L of an 8% Triton X-100 solution in buffer.

5.4. Electron Microscopy

The final pellet recovered from the final centrifugation during liposome preparation above was resuspended in 0.8 mL buffer and 0.2 mL glycerol. The sample was placed on a copper stage and rapidly frozen using a Balzers Cryojet

with high speed liquid propane jets at -150°C . The replica was further cooled with liquid nitrogen and then inserted into a BAF 400D freeze cleavage device and cleaved at 2×10^{-6} torr using a knife pre-chilled with liquid nitrogen. The samples were shadowed with Pt/C at 45° . The samples were then removed from the instrument and the replica recovered by floating it in water away from the stage. The replica was picked up onto a copper electron microscopy grid and residual lipids were removed by a wash in CHCl_3 . The grids were air dried and examined and photographed by Ernie Bond of Agriculture Canada in a Philips model 300 transmission electron microscope.

RESULTS

1. Purification and Characterization of Aldehyde Dehydrogenase.

Aldehyde dehydrogenase was purified from rat liver mitochondria. The enzyme was found to have both NAD^+ and NADP^+ dependent dehydrogenase activities and an esterolytic activity. No ethanol oxidizing activity was observed.

Table IV summarizes the purification procedures with respect to dehydrogenase and esterase activities. Extraction of the enzyme was facilitated in the presence of the non-ionic detergent Triton X-100. Similar preparation excluding the detergent realized a 50% decrease in yield during the solubilization step.

Active enzyme passed unbound through the anion ion-exchange (DEAE-A50) column. Initial preparation in which the system was equilibrated well above the pI of the enzyme (pI 6.40) resulted in tight binding to the ion exchange gel and this could not be reversed using a sodium chloride concentration gradient. Conditions for ion-exchange, including choice of buffer salt, were chosen to facilitate free passage of the enzyme through the gel and maintain a high activity. Other chromatographic media, assessed at this stage over a range of buffer and pH conditions included carboxymethyl cellulose CM-C50 and phosphocellulose. These were rejected on the basis of irreversible (CM-C50) or no binding (phosphocellulose) and substantially decreased flow rates. The higher flow rate of the DEAE-A50 column and

TABLE IV
Summary of Enzyme Purification Procedures for Activities of Aldehyde Dehydrogenase

Procedure	Vol. (mL)	Protein (mg/mL)	Specific Activity ^a NAD ⁺ NADP ⁺	Enzyme	Purification NAD ⁺ NADP ⁺	Yield ^b NAD ⁺ NADP ⁺	(%)
Crude	140	183	1.29×10^{-4}	1.04×10^{-4}	—	—	1.24 100
Mitochondrial Homogenate	38	40	6.27×10^{-4}	4.84×10^{-4}	6.95×10^{-4}	4.88 4.66	3.8 1.30 —
Mitochondrial Supernatant	34	18	3.68×10^{-3}	1.73×10^{-3}	—	5.87 3.57	— 2.13 69
DEAE -A50 Fractions	32	16.2	4.04×10^{-3}	1.99×10^{-3}	2.64×10^{-3}	1.10 1.57	1.30 2.03 64
Dialysis Resting	15.7	9.5	8.08×10^{-3}	3.98×10^{-3}	3.43×10^{-3}	2.0 2.0	4.89 2.03 36
AGNAD Fractions	8.0	0.15	2.86×10^{-1}	1.47×10^{-1}	5.66×10^{-2}	35.4 36.9	16.5 1.95 10
4B Sepharose Fractions	4.0	0.11	3.78×10^{-1}	2.12×10^{-1}	8.04×10^{-2}	1.32 1.44	1.42 1.78 5
NET					2900 2000	660	

^a Specific Activity in Units/mg
^b Yield based on NAD⁺ Activity

the highest increases in specific activity over the other media led to selection of this ion exchanger. Sephadex G-200 was discounted for similar reasons. All activities were co-eluted from the ion exchange gel.

The AGNAD column was found to be an extremely effective medium for affinity chromatography. Appreciable esterase activity, which was eluted during the initial addition of enzyme (Figure 1), may indicate the presence of other esterases; however, these were not investigated further. Co-elution of dehydrogenase and esterase activities was evident on addition of 10 mM NAD^+ to the column. This procedure was the most significant step throughout the purification scheme. Appreciable increases in activity of 35.4, 36.9 and 16.5 fold were realized for NAD^+ and NADP^+ dependent dehydrogenase and esterase activities respectively. Alternative affinity media were also investigated. Dye matrix chromatography using Amicon and Bio-Rad Dextran Blue dyes linked to insoluble gels afforded only minor increases in specific activity. Such dyes have been shown to bind strongly to many proteins including dehydrogenases (158-161) and other enzymes requiring adenylyl-containing substances. Binding is presumed to be via the nucleotide binding pocket (162). Red dye, 5'-AMP and Agarose-hexane NAD^+ (Type 3) linked gels resulted in no binding whatsoever. Chromatofocusing also failed to increase the purity of the enzyme (162).

Figure 1 Elution Profile of Aldehyde Dehydrogenase From AGNAD⁺ Affinity Column. Bound enzyme was released from the column on addition of 10 mM NAD⁺ where indicated. The absorption at 280 nm (---), NAD⁺ (●), NADP⁺ (○) and esterase (■) activities were determined.

Discontinuous polyacrylamide electrophoresis indicated that this preparation contained, in addition to aldehyde dehydrogenase, a high molecular weight contaminant (Plate 1). This contaminant was removed by final passage of the eluted affinity enzyme through a Sepharose 4B column (Figure 2). Co-elution of all activities throughout the various procedures and electrophoresis of the final isolated enzyme (Plate 2) demonstrated chromatographic and electrophoretic homogeneity. A reasonably constant $\text{NAD}^+/\text{NADP}^+$ activity ratio maintained throughout all steps of the purification reveals the ability of the enzyme to use either cofactor with nearly equal utility. Net purification of NAD^+ and NADP^+ activities was 2,900 and 2,000 fold respectively. A significantly decreased purification³ of the esterase activity (660 fold) may indicate the presence of other esterases with respect to this activity. The pure enzyme can survive up to one week in storage at 0-1°C. A summary of some of the physical properties of the purified mitochondrial aldehyde dehydrogenase discussed below, is found in Table V.

Molecular weight determination of the native enzyme was done by sucrose gradient centrifugation. The molecular weight was found to be 240,000 $\pm$ 2,000 Daltons ($n=2$). The subunit weight of aldehyde dehydrogenase was determined by SDS gel electrophoresis. The mobility of the SDS treated aldehyde dehydrogenase was compared to a linear regressed standard curve prepared from similarly treated standards (Figure 3). The molecular weight of the aldehyde dehydro-

Plate 1

SDS Disc Polyacrylamide Gel Electrophoresis of Aldehyde Dehydrogenase Eluted From AGNAD Column. Lane 1 contains enzyme eluted from AGNAD column. Lane 2 contains the following standards shown in order of decreasing molecular weight; BSA, ovalbumin, β -lactoglobulin, lysozyme.

1

2

Figure 2

Elution Profile From Sepharose 4B Column. Chromatographic homogeneity is indicated by co-elution of NAD⁺ dependent (▲) NADP⁺ dependent (△) and esterase (■) activities and absorbance at 280 nm (●). Peak fractions from 8-11 mL were pooled and used as homogeneous enzyme. Fractions to the left of the peak center contained a high molecular weight contaminant and were discarded.

Plate 2

SDS Discontinuous Gel Electrophoresis of Mitochondrial Aldehyde Dehydrogenase. Lane 1 contains standard proteins in the following order of decreasing molecular weight: Bovine serum albumin, ovalbumin; trypsinogen; β -lactoglobulin. Lane 2 contains purified enzyme from the 4B column.

Figure 3

Subunit Molecular Weight Determination of Aldehyde Dehydrogenase. Standard proteins indicated are: A, bovine serum albumin; B, ovalbumin; C, trypsinogen; D, β -lactoglobulin. AldHase, aldehyde dehydrogenase as indicated. Mobilities were measured on a SDS-polyacrylamide gel electrophoresis apparatus as described in experimental section. Samples were 1 μ g.

2

1.0

1.1

1.25

1.4

1.6

1.8

2.0

2.2

2.5

MICRO

1 2

TABLE V

Physical Properties of Aldehyde Dehydrogenase

Native Molecular Weight	240,000 Daltons
Sub-Unit Molecular Weight	60,000 Daltons
Stokes Radius	55.6 Å
pI	6.40
ϵ M	$2.79 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$
f/f_0	1.36

Figure 3

Subunit Molecular Weight Determination of Aldehyde Dehydrogenase. Standard proteins indicated are: A, bovine serum albumin; B, ovalbumin; C, trypsinogen; D, β -lactoglobulin. AldHase, aldehyde dehydrogenase as indicated. Mobilities were measured on a SDS-polyacrylamide gel electrophoresis apparatus as described in experimental section. Samples were 1 μ g.

genase subunits was determined to be 60,000 $\pm$ 1,000 Daltons ($n=2$).

The effective hydrodynamic radius was determined by comparative elution of purified enzyme and standard proteins from a Sepharose 4B column (110). Stokes radii of known standard proteins were plotted against the relative partition coefficient $[-(\log K_{AV})^{1/2}]$. From the standard curve obtained (Figure 4), the Stokes radius of purified aldehyde dehydrogenase was established as 556 $\pm$ 11 nm. The determination of molecular weight by gel filtration is valid only for a series of homologous polymers shown to have the same density and shape (164). The effective hydrodynamic volume can be used to estimate the molecular size, reflecting both the radius and molecular asymmetry through the frictional ratio f/f_0 (110, 165) which was calculated to be 1.36. This ratio corresponds to the shape of a flattened disc according to Tanford (111), with an axial ratio $(a/b) = 7.5$.

Isoelectric focusing on separate preparations of purified enzyme revealed an isoelectric point of pI 6.40 $\pm$ 0.02. Focusing revealed a single peak (Figure 5) suggesting a single molecular form of aldehyde dehydrogenase from rat liver mitochondria using the specified procedure.

An ultraviolet absorption spectrum was obtained on a Cary 14 spectrophotometer (Figure 6) and the extinction coefficient calculated for the wavelength of maximum absorption ($\lambda_{277\text{nm}}$) was found to be $2.79 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$.

Figure 4 Stokes Radius Determination of Aldehyde Dehydrogenase. Standard proteins include: a, thyroglobulin; b, ferritin; c, catalase; d, aldolase. Aldehyde dehydrogenase as indicated. All protein concentrations were 1 mg and applied to a Sepharose 4B column (43.2 cm x 0.9 cm).

Figure 5 · Isoelectric Focusing of Mitochondrial Aldehyde Dehydrogenase. A pH gradient was established with Ampholines in sucrose and run as described in the methods section. Eluted protein was measured for optical density at 280 nm and activity was measured on the peak fraction.

Figure 6 UV Scan of AldDHase. Scan obtained at 25°C, enzyme concentration was 0.240 mg/mL.

Thermal inactivation profiles were established for the NAD^+ and NADP^+ dependent dehydrogenase and esterase activities of a purified preparation of aldehyde dehydrogenase (Figure 7). Rapid inactivation of dehydrogenase activities occurred within 4 minutes of heat treatment at 55°C . A small percentage of activity remained after 8 minutes followed by total inactivation by 20 minutes. Both NAD^+ and NADP^+ activities followed biphasic heat inactivation profiles. The esterolytic activity was much less sensitive to heat treatment. A linear inactivation profile for esterase activity was observed, with 68% activity remaining after 25 minutes of heat exposure and suggests separate active sites.

A summary of the amino acid composition of the enzyme, expressed in moles amino acid per mole of subunit enzyme, is found in Table VI. A particularly high content of acidic residues was observed in addition to a high content of glycine, alanine and leucine in comparison to the human mitochondrial enzyme (45b).

Hydrazinolytic preparation of the C-terminal residue with subsequent amino acid analysis revealed this terminal residue was likely blocked. It was not possible to identify a specific C-terminal residue. Thin layer chromatographic analysis of the N-terminal PTH-amino acid derived from a single cycle of Edman degradation yielded a single spot with a relative distance (R_f) value corresponding closely to that of PTH-lysine. The relative distance observed for the PTH-amino acid standards used are found in Table VII. The iso-

Figure 7

Thermal Stability of Mitochondrial Aldehyde Dehydrogenase. Both NAD(P)⁺ (●) dehydrogenase and esterase (■) activities were monitored during heat exposure as outlined in the procedures section. Enzyme concentration was 3.4 mg/mL and was incubated at 55°C.

TABLE VI
Amino Acid Composition of Aldehyde Dehydrogenase

	# Moles	% Error		# Moles*	% Error
Asp	62	17	Phe	17	9
Glu	63	14	Ile	15	34
Ser	59	14	Leu	42	10
His	9	20	Lys	31	12
Gly	97	23	Trp	4	6
Thr	32	16	Pro	10	
Arg	18	21	Cysteine	16	
Ala	82	11	Met	13	24
Tyr	11	10	Val	23	23

* Expressed as moles per mol of enzyme sub-unit. Errors as indicated.

lated N-terminal residue was observed as a single spot by TLC. The retention of the N-terminal residue was slightly lower than that for PTH-lysine. This may have been due to the fact that PTH-amino acid standards have been observed to run slightly fast if not fresh (118).

2. STEADY STATE KINETIC STUDIES-PART A

2.1.i. General

Steady state dehydrogenase kinetics were performed using NAD(P)^+ and various analogues as cofactors and short chain aldehydes as substrates. Double reciprocal plots were prepared of initial rates over a range of substrate or cofactor concentrations while holding the other variable constant. Kinetic data and constants were initially obtained by fitting the data to the following bisubstrate rate equation:

$$v = \frac{V_{\max} [A][B]}{K_{ia}K_b + K_b[A] + K_a[B] + [A][B]}$$

where $V_{\max}$ is the maximum velocity, $[A]$, $[B]$ are the concentrations of the substrates, K_a , K_b , and K_{ia} are Michaelis constants for cofactor, substrate and the inhibition constant for cofactor respectively (166). Steady state derivation of the rate equations for bisubstrate mechanisms produces exclusive equations for both Random and Ping Pong mechanisms thereby allowing these mechanisms to be distinguished by initial-velocity studies. However the Ordered, Rapid Random and Theorell-Chance mechanisms cannot be dis-

TABLE VII
Relative Retentions Of PTH-Amino Acids

Residue	R _f (± 0.002),	Residue	R _f (± 0.002)
Lys	0.593	Leu	0.731
Tyr	0.527	Arg	0
Glu	0.419	Ile	0.750
Trp	0.652	His	0
Gly	0.667	Cys	0.574
Met	0.681	Thr	0.321
Ala	0.632	Asn	0.122
Phe	0.721	Asp	0.231
Pro	0.775	Gln	0.119
Val	0.710	Ser	0.689
N-Terminal PTH	0.590		

Samples were applied to Silica 60-F254 plates and run using a solvent mixture of 60 parts diethylene chloride to 7 parts acetic acid.

tinguished from each other because they have the same form of initial-velocity equation (71). The kinetic studies were initially fitted assuming an Ordered Bi Bi mechanism. This was later confirmed by product inhibition studies. For an explanation of rate constant determination using the above bisubstrate equation see Appendix III.

2.1.ii. NAD^+ and NADP^+ Dehydrogenase Kinetics

Initial rate data for NAD^+ dependent activity was plotted against substrate concentration in a double reciprocal plot as shown in Figure 8a. Secondary plots were obtained (Figures 8b & c) and used to determine the kinetic constants. Similarly, a double reciprocal plot was obtained for NADP^+ activity (Figure 9a) and kinetic constants were determined from the secondary plots (Figures 9b & c). A summary of the kinetic constants obtained can be found in Table VIII. The enzyme was found to have micromolar dissociation constants for both cofactors. That NAD^+ is found to bind tighter to the enzyme is evidenced by the lower dissociation constant. Tighter binding of this cofactor may be responsible for the slightly higher V_{max} and turnover numbers. An interesting feature to note is the relative sizes of K_{i2} . This constant was found to be smaller for NADP^+ . One might expect such a result to contradict the general kinetic behavior described above. The implication of such a result may be that NADP^+ may in fact be a more desirable cofactor at certain points within the mechanism. Specifically, the initial binding of NADP^+ to the free enzyme is

Figure 8a Double Reciprocal Plot For NAD^+ Dependent Dehydrogenase Kinetics, pH 7.5. Each line represents variation in $1/\text{initial rate}$ plotted against $1/\text{acetaldehyde concentration}$ at a fixed concentration of NAD^+ . The $1/\text{NAD}^+$ concentrations shown top to bottom are 3.00, 1.80, 1.20 and 0.90 mM^{-1} . Enzyme concentration was 0.22 mg/mL . All assays were at 25°C , pH 7.5.

Figure 8b&c Secondary Plots For NAD⁺ Dependent Dehydrogenase Kinetics, pH 7.5. Plots of primary intercepts (Figure 8b) and primary slopes (Figure 8c) were plotted against 1/NAD⁺ concentration and used to calculate kinetic parameters. Enzyme concentration was 0.22 mg/mL. All assays were at 25°C, pH 7.5.

Figure 9a Double Reciprocal Plot For NADP⁺ Dependent Dehydrogenase Kinetics, pH 7.5. Each line represents variation of 1/initial rate plotted against 1/[NADP⁺] concentration at a fixed concentration of acetaldehyde. The 1/acetaldehyde concentrations shown, top to bottom are 0.500, 0.286, 0.200 and 0.111 mM⁻¹. Enzyme concentration was 0.22 mg/mL. All assays were at 25°C, pH 7.5.

Figure 9 b&c Secondary Plots For $NADP^+$ Dependent Dehydrogenase Kinetics, pH 7.5. Plot of primary intercepts (Figure 9b) and primary slopes (Figure 9c) were plotted against $1/\text{acetaldehyde}$ concentration and used to calculate kinetic parameters. Enzyme concentration was 0.22 mg/mL. All assays were at 25°C, pH 7.5.

Figure 9c

TABLE VIII
Kinetic Constants For NAD^+ and NADP^+ Dependent
Dehydrogenase Activities, pH 7.5

	$V_{\max}$ ($\mu\text{M}/\text{min}$)	K_a (mM)	K_b^r (mM)	K_{ia} (mM)	$V_{\max}/E_t$ (sec^{-1})
NAD^+	2.86	7.05×10^{-2}	3.61	10.30	0.17
NADP^+	2.57	5.69×10^{-1}	2.28	4.52	0.15

NOTE: $V_{\max}$, K_a , K_b , K_{ia} and $V_{\max}/E_t$ are maximum velocity, Michaelis constants for NAD(P)^+ , acetaldehyde, inhibition constant for NAD(P)^+ , and turnover number respectively. Errors were determined from the correlation coefficients of secondary plots and found to be $V_{\max} \pm 7.0\%$, $K_a \pm 7.1\%$, $K_b \pm 9.1\%$, $K_{ia} \pm 15.3\%$, $V_{\max}/E_t \pm 7.5\%$.

faster than the binding of NAD^+ . This anomaly will be further investigated below.

Throughout the purification procedure the ratio of specific activities of $\text{NAD}^+/\text{NADP}^+$ was monitored. The ratio remained essentially constant at a value between 1.25 and 2.10. As a check of the accuracy of the kinetic constants determined, the ratio can be calculated by inserting the experimentally obtained kinetic values into the bisubstrate rate equation above. This was done using typical assay conditions of 10.0 mM acetaldehyde and 1.0 mM NAD(P)^+ . The ratio $v_{\text{NAD}^+}/v_{\text{NADP}^+}$ obtained was 1.5 which is in good agreement with the ratios obtained throughout the purification procedures.

2.1.iii. NAD(P)^+ Analogue Dehydrogenase Kinetics

Dehydrogenase kinetics were repeated as above replacing NAD^+ and NADP^+ as cofactors with NAD^+ and NADP^+ analogues.

The structures of the various analogues can be found in Figure 10. The 3-acetylpyridine- NAD(P)^+ structure (Figure 10c, d) differs from the NAD(P)^+ structure (Figures 10a, b) by replacement of the amine functionality of the carboxamide moiety with a methyl residue. 3-Pyridine aldehyde adenine dinucleotide (Figure 10e) has an identical structure as NAD^+ except for complete removal of the carboxamide amine. The deamino- NAD^+ analogue (Figure 10f) has the adenine amine (C_6) replaced by an hydroxyl species. The thio- $\text{NAD}^+(\text{P})$

Figure 10 Structures of NAD(P)^+ Analogues. Representative structures are provided for the NAD(P)^+ analogues. The analogues shown are: (a), NAD^+ ; (b), NADP^+ ; (c), acPy-AD^+ ; (d), acPy-ADP^+ ; (e), PyA-AD^+ ; (f), deamino-NAD^+ ; (g), thio-NAD^+ ; (h), thio-NADP^+ .

analogues (Figure 10g, h) have a thio carbonyl substitution at the nicotinamide carbonyl (167).

Kinetic data using acPy-AD⁺ as cofactor can be found in the double reciprocal plot Figure 11a. Secondary plots of primary intercepts (Figure 11b) and primary slopes (Figure 11c) plotted against cofactor concentration were used to determine kinetic constants. Similarly, double reciprocal plots and secondary plots were obtained for acPy-ADP⁺ (Figure 12a and b, c respectively), thio-NAD⁺ (Figures 13a and 13b, c respectively), thio-NADP⁺ (Figures 14a and b, c respectively), deamino-NAD⁺ (Figures 15a, and b, c respectively), PyA-AD⁺ (Figures 16a and b, c respectively). The kinetic parameters obtained from the above plots are summarized in Table IX. Replacement of the pyridine amide moiety of NAD⁺ with an acetyl species (acPy-AD⁺) resulted in a dramatic decrease in binding affinity as indicated by an 8 fold increase in Michaelis constant (5.6×10^{-1} mM). A concomitant 7 fold decrease in turnover number was also observed. The PyA-AD⁺ analogue experienced a less dramatic 2 fold increase in Michaelis constant (1.59×10^{-1} mM). The most significant decrease in maximum velocity was observed for this analogue. Thio-carbonyl replacement of the carboxamide carbonyl also resulted in a slight decrease in binding affinity of the enzyme for thio-NAD⁺ ($K_m = 9.42 \times 10^{-1}$ mM). A concomitant 1.6 fold decrease in maximum velocity was observed. Replacement of the adenine amine with an hydroxyl species in deamino-NAD⁺ also seemed to have a very dramatic

Figure 11a Double Reciprocal Plot For ̢-Acetylpyridine-NAD⁺ Dependent Dehydrogenase Kinetics, pH 7.5. A double reciprocal plot is shown for initial rates plotted against substrate concentrations at fixed concentrations of acPy-AD⁺. The $1/\text{acPy-AD}^+$ concentrations shown top to bottom are 2.07, 1.38, 1.04 and 0.69 mM^{-1} . Enzyme concentration was 84 $\mu\text{g/mL}$. All assays were at 25°C, pH 7.5.

Figure 11b&c Secondary Plots For acPy-AD⁺ Kinetics, pH 7.5. Plot of primary intercepts (Figure 11b) and primary slopes (Figure 11c) versus $1/[acPy-AD^+]$ were used to calculate kinetic parameters. Enzyme concentration was 84 $\mu\text{g/mL}$. All assays were at 25°C, pH 7.5.

Figure 12a Double Reciprocal Plot For 3-Acetylpyridine-NADP⁺ Dependent Dehydrogenase Kinetics, pH 7.5. A double reciprocal plot is shown for initial rates plotted against substrate concentration for fixed concentrations of acPy-ADP⁺. The 1/acPy-ADP⁺ concentrations shown top to bottom are 5.38, 3.07, 2.15 and 1.43 mM⁻¹. Enzyme concentration was 84 uq/mL. All assays were at 25°C, pH 7.5.

Figure 12b&c Secondary Plots for acPy-ADP⁺ Kinetics, pH 7.5. Plot of primary intercepts (Figure 12b) and primary slopes (Figure 12c) versus $1/\text{acPy-ADP}^+$ concentration were used to calculate kinetic parameters. Enzyme concentration was 84 $\mu\text{g/mL}$. All assays were at 25°C, pH 7.5.

Figure 13a Double Reciprocal Plot For Thio-NAD⁺ Dependent Dehydrogenase Kinetics, pH 7.5. A double reciprocal plot is shown for initial rates plotted against substrate concentrations, for fixed concentrations of Thio-NAD⁺. The lines represent 1/Thio-NAD⁺ concentrations shown top to bottom correspond to 3.32, 2.25, 1.50 and 1.12 mM⁻¹ respectively. Enzyme concentration was 84 μg/mL. All assays were at 25°C, pH 7.5.

Figure 13b&c

Secondary Plots For Thio-NAD⁺ Kinetics, pH 7.5. Plot of primary intercepts (Figure 13b) and primary slopes (Figure 13c) plotted against $1/\text{Thio-NAD}^+$ concentrations were used to calculate kinetic parameters. Enzyme concentration was 84 $\mu\text{g/mL}$. All assays were at 25°C, pH 7.5.

Figure 14 a

Double Reciprocal Plot For Thio-NADP⁺ Dependent Dehydrogenase Kinetics, pH 7.5. Each line represents a variation in $1/\text{initial rate}$ plotted against $1/\text{acetaldehyde concentration}$ for fixed concentrations of Thio-NADP⁺. The lines corresponding to $1/\text{Thio-NADP}^+$ concentration are (shown top to bottom), 5.62, 3.21, 2.25 and 1.50 mM⁻¹. Enzyme concentration was 84 $\mu\text{g}/\text{ml}$. All assays were at 25°C, pH 7.5.

Figure 14b&c Secondary Plots For Thio-NADP⁺ Kinetics, pH 7.5.
 Primary intercepts (Figure 14b) and primary slopes
 (Figure 14c) plotted against $1/\text{Thio-NADP}^+$ concen-
 tration were used to calculate kinetic parameters.
 Enzyme concentration was 84 $\mu\text{g/mL}$. All assays
 were at 25°C, pH 7.5.

Figure 15a Double Reciprocal Plot For Deamino- NAD^+ Dependent Dehydrogenase Kinetics, pH 7.5. Each line represents variation in $1/\text{initial rate}$ with $1/\text{acetaldehyde concentration}$ for fixed concentrations of deamino- NAD^+ . The lines corresponding to $1/\text{deamino-}\text{NAD}^+$ concentration are (shown top to bottom) 1.49, 1.12, 0.75, 0.56. Enzyme concentration was 84 $\mu\text{g/mL}$. All assays were at 25°C, pH 7.5.

Figure 15b&c Secondary Plots For Deamino- NAD^+ Dependent Dehydrogenase Kinetics, pH 7.5. Primary intercepts (Figure 15b) and primary slopes (Figure 15c) plotted against $1/Deamino-NAD^+$ concentration were used to calculate kinetic parameters. Enzyme concentration was 84 $\mu g/mL$. All assays were at 25°C, pH 7.5.

Figure 15c

✓

Figure 16 a

Double Reciprocal Plot For Pyridine Aldehyde Adenine Dinucleotide Dependent Dehydrogenase Kinetics, pH 7.5. Each line represents a variation in 1/initial rate with 1/acetaldehyde concentration for fixed concentrations of PyA-AD⁺. The lines corresponding to 1/PyA-AD⁺ concentration are (shown top to bottom) 4.69, 2.68, 1.88 and 1.25 mM⁻¹. Enzyme concentration was 84 µg/mL. All assays were at 25°C, pH 7.5.

Figure 16 b&c

Secondary Plots for Pyridine Aldehyde Adenine Dinucleotide Dependent Dehydrogenase Kinetics, pH 7.5. Primary intercepts (Figure 16b) and primary slopes (Figure 16c) plotted against $1/PyAD^+$ concentrations were used to calculate kinetic parameters. Enzyme concentration was 84 $\mu g/mL$. All assays were at $25^{\circ}C$, pH 7.5.

Figure 16 c

TABLE IX

Summary of Kinetic Constants for NAD^+ and NADP^+ Analogues, pH 7.5

	$V_{\max}$ ($\mu\text{M}/\text{min}$)	K_a (mM)	K_b (mM)	K_{ia} (mM)	$V_{\max}/E_t$ (sec^{-1})
NAD^+	2.86	7.05×10^{-2}	3.61	10.30	0.17
acPy-AD $^+$	0.42	5.55×10^{-1}	1.50	18.9	0.025
Thio-NAD $^+$	1.81	9.42×10^{-1}	6.90	2.33	0.11
PyA-AD $^+$	0.27	1.59×10^{-1}	2.91	4.20	0.016
Deamino-NAD $^+$	1.61	2.25	2.25	9.66	0.094
NADP^+	2.57	5.69×10^{-1}	2.28	4.52	0.15
Thio-NADP $^+$	0.11	2.38×10^{-2}	3.11	3.03	0.012
acPy-ADP $^+$	0.21	4.06×10^{-1}	20.5	3.50	0.0065

NOTE: $V_{\max}$, K_a , K_b , K_{ia} and $V_{\max}/E_t$ are maximum velocity, Michaelis constant for cofactor, acetaldehyde and inhibition constant for cofactor and turnover number respectively. Errors were determined by regression analysis and

found to be: $V_{\max} \pm 12.1\%$, $K_a \pm 12.9\%$, $K_b \pm 11.6\%$, $K_{ia} \pm 14.8\%$, $V_{\max}/E_t \pm 17\%$.

effect on enzyme-cofactor binding. Approximately a 32 fold increase in the Michaelis constant for deamino-NAD⁺ was observed in comparison to NAD⁺ indicating a significant decrease in binding affinity. However the maximum velocity value was only affected about 2 fold.

Unlike the thio counterpart above, presence of the 2'-orthophosphate in thio-NADP⁺ resulted in an increase in enzyme-cofactor binding efficiency in comparison to NADP⁺ (Michaelis constants are 2.38×10^{-2} mM and 5.69×10^{-1} mM for thio-NADP⁺ and NADP⁺ respectively). An appreciable 13 fold decrease in turnover number was observed. When acPy-ADP⁺ was used as cofactor, there was also a decrease in Michaelis constant in comparison to NADP⁺. This change however was very small. The maximum velocity observed for acPy-ADP⁺ was 23 fold lower than for NADP⁺. The presence of the 2'-orthophosphate in NADP⁺ seems to exert a small effect with respect to the steady state reaction velocity in comparison to NAD⁺. However the effect is accentuated on modification of the cofactor structure.

2.1.iv. Effect of pH on Dehydrogenase Kinetics.

The effect of pH on NAD⁺ and NADP⁺ dependent dehydrogenase activities was investigated over the range of pH 5.0-10.5. Kinetic assays were performed as described previously with the sole exception being a variation in the pH. Figures 17a-20a are double reciprocal plots of initial rates plotted against acetaldehyde concentration for fixed

concentrations of NAD^+ with the pH variation corresponding to pH 7.0, 8.0, 9.0 and 10.0 respectively. Kinetic constants were determined as above at each pH from secondary plots derived from primary intercepts (Figures 17b-20b) and primary slopes (Figures 17c-20c) plotted against $1/\text{NAD}^+$ concentration. For kinetics at pH 7.5 see Figures 8a, b and c. Estimates of V_{max} values at the limiting pH values of 5.5, 6.0 and 10.5 were made from measurement of initial velocities at high substrate concentrations. A summary of the kinetic constants obtained at various pH values is tabulated in Table X. As the pH is increased, V_{max} values experience a progressive increase to a maximum value between pH 8.5-9.0. Further increases in pH result in a decrease in V_{max} . A slightly asymmetric pH profile is obtained when one plots $\log V_{\text{max}}$ against pH (Figure 21). Concomitant with the progressive increase in V_{max} at pH 9.0 is an increase in enzyme-cofactor binding efficiency. The Michaelis constants for NAD^+ decrease to a minimum value at pH 9.0 when approached from either high or low pH values. The pH profile can be used to identify the ionizable species involved in the catalytic activity (71, 74, 168). Two such ionizable species can be identified, one with pK 6.1 and the other with pK 9.5 (see Appendix VI for explanation).

A cursory pH dependency was obtained when NADP^+ was used as cofactor. The pH profile was generated from initial rate data determined over the pH range 7.5-10.0 (Figure 22).

Figure 17a Double Reciprocal Plot For Aldehyde Dehydrogenase Kinetics, pH 7.0. Each line represents a variation in $1/\text{initial rate}$ with $1/\text{acetaldehyde concentration}$ for fixed concentrations of NAD^+ . The lines corresponding to $1/\text{NAD}^+$ concentrations are (shown top to bottom) 5.06 , 3.37 , 2.02 and 1.35 mM^{-1} respectively. Enzyme concentration was $73 \text{ } \mu\text{g/mL}$. All assays were at 25°C .

Figure 17b&c

Secondary Plots ^a For Aldehyde Dehydrogenase Kinetics, pH 7.0. Primary intercepts (Figure 17b) and primary slopes (Figure 17c) plotted against $1/[NAD^+]$ concentrations were used to determine kinetic parameters. Enzyme concentration was 73 $\mu\text{g/mL}$. All assays were at 25°C .

Figure 17c

Figure 18a Double Reciprocal Plot For Aldehyde Dehydrogenase Kinetics, pH 8.0. Each line represents a variation in $1/\text{initial rate}$ with $1/\text{acetaldehyde concentration}$ for fixed concentrations of NAD^+ . The lines corresponding to $1/\text{NAD}^+$ concentrations are (shown top to bottom) 5.06, 3.37, 2.02 and 1.35 mM^{-1} respectively. Enzyme concentration was 73 $\mu\text{g/mL}$. All assays were at 25°C.

Figure B b&c Secondary Plots For Aldehyde Dehydrogenase Kinetics, pH 8.0. Primary intercepts (Figure 18b) and primary slopes (Figure 18c) plotted against $1/[NAD^+]$ concentrations were used to determine kinetic parameters. Enzyme concentration was 73 $\mu\text{g/mL}$. All assays were at 25°C .

Figure 18 c

Figure 19a Double Reciprocal Plot For Acetaldehyde Dehydrogenase Kinetics, pH 9.0. Each line represents a variation in $1/\text{initial rate}$ with $1/\text{acetaldehyde concentration}$ for fixed concentrations of NAD^+ . The lines corresponding to $1/\text{NAD}^+$ concentrations are (shown top to bottom) 5.06, 3.37, 2.02 and 1.35 mM^{-1} respectively. Enzyme concentration was 73 $\mu\text{g/mL}$. All assays were at 25°C .

Figure 19b&c Secondary Plots For Aldehyde Dehydrogenase Kinetics, pH 9.0. Primary intercepts (Figure 19b) and primary slopes (Figure 19c) plotted against $1/[NAD^+]$ concentrations were used to determine kinetic parameters. Enzyme concentration was $73 \mu\text{g/mL}$. All assays were at 25°C .

Figure 19c

Figure 20a Double Reciprocal Plot For Aldehyde Dehydrogenase Kinetics, pH 10.0. Each line represents a variation in $1/\text{initial rate}$ with $1/\text{acetaldehyde concentration}$ for fixed concentrations of NAD^+ . The lines corresponding to $1/\text{NAD}^+$ concentrations are (shown top to bottom) 5.06, 3.37, 2.02 and 1.35 mM^{-1} respectively. Enzyme concentration was 73 $\mu\text{g/mL}$. All assays were at 25°C .

Figure 20b&c

Secondary Plots For Aldehyde Dehydrogenase Kinetics, pH 10.0. Primary intercepts (Figure 20b) and primary slopes (Figure 20c) plotted against $1/[NAD^+]$ concentrations were used to determine kinetic parameters. Enzyme concentration was 73 $\mu\text{g/mL}$. All assays were at 25°C.

Figure 20c

pH Dependence of Kinetic Constants of
NAD⁺ Dependent Dehydrogenase Activity

pH	V _{max} (μM/min)	K _a (mM) × 10 ²	K _b (mM)	K _{ia} (mM)	log V _{max}
5.5	0.78	--	--	--	-0.108
6.0	1.46	--	--	--	0.164
7.0	2.51	9.60	4.06	5.31	0.400
7.5	2.86	7.05	3.61	10.3	0.456
8.0	2.92	2.63	3.40	8.27	0.465
9.0	2.94	2.92	2.68	6.52	0.468
10.0	2.31	6.01	1.86	5.74	0.364
10.5	0.70	--	--	--	-0.155

-- Not determined.

NOTE: V_{max}, K_a, K_b, K_{ia} and log V_{max} are maximum velocity, Michaelis constants for NAD⁺, acetaldehyde inhibition constant for NAD⁺ and log of maximum velocity respectively. Errors were determined by regression analysis to be V_{max} ± 19.8%, K_a ± 22.7%, K_b ± 25.5%, K_{ia} ± 7.8%.

Figure 21 pH Profile for NAD⁺ Dependent Dehydrogenase Activity.
All assays were at 25°C.

Figure 22 pH Profile For NADP⁺ Dependent Dehydrogenase Activity.
All assays were at 25°C.

It is not possible to assign pK values with this data but the profile does follow the shape obtained from NAD^+ data.

2.1.v. Substrate Specificity of Dehydrogenase Kinetics.

Kinetic parameters were obtained for the NAD^+ dehydrogenase reaction using a series of *n*-alkanal substrates of variable chain length. Solutions of methanal, ethanal, propanal, *n*-butanal and *n*-pentanal were each used as substrates. Initial rates were determined with respect to variation in *n*-alkanal concentration for several different, fixed concentrations of NAD^+ . The double reciprocal and secondary plots for ethanal have been described above (Figures 8a, b and c). Double reciprocal plots of initial rate and alkanal concentration were made for each aldehyde (23a-26a). Secondary plots of intercepts and slopes against $1/\text{NAD}^+$ concentration (Figs 23b-26b & Fig 23c-26c respectively) were made in order to obtain kinetic constants as described previously. Kinetic constants were determined as outlined in Appendix III and these are listed in Table XI. One trend which is particularly evident is that as chain length increases ($\text{C}_2\text{-C}_5$), there is a progressive increase in maximum velocity and turnover numbers. Michaelis constants for NAD^+ remain largely unchanged. Binding affinity of the enzyme for substrate exhibits no particular trends concomitant with increasing chain length. This is manifested by a random dependency in Michaelis constants for aldehyde substrates.

Figure 23a

Double Reciprocal Plot For Substrate Specificity of Dehydrogenase Kinetics. Each line represents a variation in $1/\text{initial rate with } 1/\text{methanal concentration for fixed concentrations of } \text{NAD}^+$. The lines corresponding to $1/\text{NAD}^+$ concentrations are (shown top to bottom) 2.01, 1.34, 1.01 and 0.67 mM^{-1} respectively. Enzyme concentration was 68 $\mu\text{g/mL}$. All assays were at 25°C , pH 7.5.

Figure 23b&c

Secondary Plots For Substrate Specificity of Aldehyde Dehydrogenase Kinetics. Primary intercepts (Figure 23b) and primary slopes (Figure 23c) plotted against $1/[NAD^+]$ concentrations were used to determine kinetic parameters. Enzyme concentration was 68 $\mu g/mL$. All assays were at $25^\circ C$, pH 7.5.

Figure 23c

Figure 24 a Double Reciprocal Plot For Substrate Specificity of Dehydrogenase Kinetics. Each line represents a variation in 1/initial rate with 1/propanal concentration for fixed concentrations of NAD⁺. The lines corresponding to 1/NAD⁺ concentrations are (shown top to bottom) 2.01, 1.34, 1.01 and 0.67 mM⁻¹ respectively. Enzyme concentration was 68 µg/mL. All assays were at 25°C, pH 7.5.

Figure 24b,c Secondary Plots For Substrate Specificity of Aldehyde Dehydrogenase Kinetics. Primary intercepts (Figure 24b) and primary slopes (Figure 24c) plotted against $1/[NAD^+]$ concentrations were used to determine kinetic parameters. Enzyme concentration was 68 $\mu\text{g/mL}$. All assays were at 25°C , pH 7.5.

Figure 24 c

Figure 25 a Double Reciprocal Plot For Substrate Specificity of Dehydrogenase Kinetics. Each line represents a variation in 1/initial rate with 1/n-butanol concentration for fixed concentrations of NAD⁺. The lines corresponding to 1/NAD⁺ concentrations are (shown top to bottom) 2.01, 1.34, 1.01 and 0.67 mM⁻¹ respectively. Enzyme concentration was 68 µg/mL. All assays were at 25°C, pH 7.5.

Figure 25b&c

Secondary Plots For Substrate Specificity of Aldehyde Dehydrogenase Kinetics. Primary intercepts (Figure 25b) and primary slopes (Figure 25c) plotted against $1/[NAD^+]$ concentrations were used to determine kinetic parameters. Enzyme concentration was 68 $\mu\text{g/mL}$. All assays were at 25°C , pH 7.5.

Figure 25 c

Figure 26a Double Reciprocal Plot for Substrate Specificity of Dehydrogenase Kinetics. Each line represents a variation in $1/\text{initial rate}$ with $1/\text{n-pentanal}$ concentration for fixed concentrations of NAD^+ . The lines corresponding to $1/\text{NAD}^+$ concentrations are shown (top to bottom) 2.01, 1.39, 1.01 and 0.67 mM^{-1} respectively. Enzyme concentration was 68 $\mu\text{g}/\text{mL}$. All assays were at 25°C , pH 7.5.

Figure 26 b & c Secondary Plots For Substrate Specificity of Aldehyde Dehydrogenase Kinetics. Primary intercepts (Figure 26b) and primary slopes (Figure 26c) plotted against $1/\text{NAD}^+$ concentrations were used to determine kinetic parameters. Enzyme concentration was $68 \mu\text{g/mL}$. All assays were at 25°C , pH 7.5.

Figure 26 c

TABLE XI

Substrate Specificity: Summary of Kinetic Constants for NAD^+ Dependent Dehydrogenase Activity

	$V_{\max}$ ($\mu\text{M}/\text{min}$)	K_a (mM), $\times 10^2$	K_b (mM)	K_{ia} (mM)	$V_{\max}/E_t$ (s^{-1}), $\times 10^1$
Methanal	4.17 ± 0.54	1.67 ± 0.46	0.560 ± 0.119	3.67 ± 0.69	2.44 ± 0.80
Ethanal	2.86 ± 0.20	7.06 ± 0.50	3.61 ± 0.32	10.30 ± 1.58	1.67 ± 0.10
Propanal	2.92 ± 0.05	7.07 ± 0.24	3.04 ± 0.26	6.63 ± 0.32	1.71 ± 0.30
n-Butanal	2.96 ± 0.50	8.91 ± 2.40	3.08 ± 0.134	5.75 ± 1.3	1.73 ± 0.40
n-Pentanal	4.95 ± 2.03	7.10 ± 2.50	9.06 ± 3.10	7.85 ± 2.50	2.90 ± 1.4

NOTE: $V_{\max}$, K_a , K_b , K_{ia} , $V_{\max}/E_t$ are maximum velocity, Michaelis constant for NAD^+ and n-alkanal, inhibition constant for NAD^+ and turnover number respectively.

A comparison was made of initial velocities for a variation in substrate chain length while using NAD^+ and NADP^+ as cofactors. Initial velocity data is listed in Table XII. An analogous trend is observed for the alkanal series using NAD^+ as cofactor as was observed in the kinetic studies. The initial velocities obtained using NADP^+ as cofactor reveal a similar trend as observed for NAD^+ .

2.2. Dehydrogenase Effector Studies.

2.2.i. General.

Dehydrogenase kinetics were performed in the presence of inhibitors. These included products, substrates and several effectors. The information obtained is used to help establish the mechanism of the reaction, probe active site similarities for dehydrogenase and esterase reactions and compare kinetic data obtained by other workers on similar systems.

Inhibition kinetics can be performed while maintaining the concentration of one of the substrates at a constant value. In this case the reaction is simplified to that of a single substrate reaction and the kinetic constants can be readily evaluated according to equations derived for single substrate inhibition kinetics. Determination of kinetic constants in the presence of an inhibitor is outlined in Appendix IV.

TABLE XII

Comparison of Substrate Specificity With
Respect to Dehydrogenase Activities

	Initial Velocities	
	NAD ⁺	NADP ⁺
Methanal	1.48	5.25×10^{-1}
Ethanal	3.78×10^{-1}	2.12×10^{-1}
Propanal	4.57×10^{-1}	2.41×10^{-1}
<u>n</u> -Butanal	3.08	6.39×10^{-1}
<u>n</u> -Pentanal	2.30×10^1	7.06×10^{-1}

Initial velocities in (Units/mg).

NOTE: The assay mixtures contained 84.0 mg/mL AldDH,
1.0 mM NAD(P)H and 0.10 mM n-alkanal.

2.2.11. Product Inhibition.

Inhibition of bisubstrate enzymes by their products was originally done by Alberty (169) to differentiate between the possible mechanisms that the enzyme could employ for its catalytic function. Rate equations describing product inhibition for the four major bisubstrate mechanisms can be found in Rudolph (121) and Roberts (71). Terms which accommodate inhibition effects are seen as factors which affect slopes and intercepts of the double reciprocal plots. These equations allow one to predict the type of inhibition pattern expected for a given mechanism when kinetics are performed under certain specified conditions of substrates and products. A summary of product inhibition patterns predicted by these equations is found in Table XIII. The evidence to be presented for aldehyde dehydrogenase supports an Ordered Bi Bi mechanism. The following equations apply to the general ordered system, according to Cleland nomenclature, in Appendix III.

The double reciprocal form of the bisubstrate rate equation above (section 2.1.1.) can be re-written to consider presence of inhibitors, including products as inhibitors. Different forms can be obtained that consider which substrate is varied, which is fixed and which product is present as an inhibitor (71). In the presence of product P, when the concentration of substrate A is varied and B is fixed, the reciprocal rate equation becomes:

TABLE XIII

Product Inhibition Patterns for Bireactant Mechanisms

Mechanism	Varied substitute			
	A		B	
	Product	Unsaturated, Saturated, with B	Unsaturated, Saturated, with A	Saturated, with A
Ordered Bi Bi	P	N	U	N
	Q	C	C	N
Theorell-Chance Bi Bi	P	N	-	C
	Q	C	C	N
Ping Pong Tetra Uni	P	N	-	C
	Q	C	C	N
Rapid Equilibrium	P	C	-	C
Random Bi Bi	Q	C	-	C

Table adapted from Rudolph (121).

N, C, U refer to non-competitive, competitive and uncompetitive inhibition respectively.

$$\frac{1}{v} = \frac{K_a}{V_{\max}} \left[1 + \frac{K_{ia}K_b}{K_a[B]} \left(1 + \frac{K_q[P]}{K_pK_{iq}} \right) \frac{1}{[A]} + \frac{1}{V_{\max}} \left[1 + \frac{[P]}{K_{ip}} + \frac{K_b}{[B]} \left(1 + \frac{K_q[P]}{K_pK_{iq}} \right) \right] \right]$$

This equation only differs from the inverse rate equation described previously by the presence of the inhibition factor

$$\left(1 + \frac{K_q[P]}{K_pK_{iq}} \right)$$

in terms corresponding to slopes and intercepts of the inverse rate equations. Thus, at non-saturating concentrations of B, the inhibition is non-competitive as both slope and intercept are affected. This appears on a double reciprocal plot as a series of converging lines which fail to meet on the ordinate axis. At saturating levels of substrate B the reaction is first order in A and thus $B \rightarrow \infty$ and $1/B \rightarrow 0$ and the inhibitor P acts only to affect the intercept term. This uncompetitive inhibition is graphically depicted as a series of parallel lines showing no indication of convergence on a double reciprocal plot.

Conversely, if substrate A is fixed and B is varied, the inverse rate equation in the presence of product P becomes:

$$\frac{1}{v} = \frac{K_b}{V_{\max}} \left(1 + \frac{K_{ia}}{[A]} \right) \left(1 + \frac{K_q[P]}{K_pK_{iq}} \right) \frac{1}{[B]} + \frac{1}{V_{\max}} \left(1 + \frac{K_a}{[A]} + \frac{[P]}{K_{ip}} \right)$$

Both slope and intercept terms are affected at both unsaturating and saturating concentrations of B. Therefore non-competitive inhibition is observed for both situations.

An analogous inverse rate equation can be obtained in the presence of product Q as inhibitor. When $[P]=0$, $[A]$ is varied and $[B]$ is fixed, the equation is written as:

$$\frac{1}{v} = \frac{K_a}{V_{\max}} \left(1 + \frac{K_{ia}K_b}{K_a[B]} \right) \left(1 + \frac{[Q]}{K_{iq}} \right) \frac{1}{[A]} + \frac{1}{V_{\max}} \left(1 + \frac{K_b}{[B]} \right)$$

The lack of an inhibition factor in the intercept term causes only the slope term to be affected. Thus, competitive inhibition is observed at all concentrations of B. On the double reciprocal plot a series of converging lines meet on the ordinate axis. Conversely, when $[B]$ is varied and $[A]$ is fixed,

$$\frac{1}{v} = \frac{K_b}{V_{\max}} \left[1 + \frac{K_{ia}}{[A]} \left(1 + \frac{[Q]}{K_{iq}} \right) \right] \frac{1}{[B]} + \frac{1}{V_{\max}} \left[1 + \frac{K_a}{[A]} \left(1 + \frac{[Q]}{K_{iq}} \right) \right]$$

both slope and intercept terms are affected. At unsaturating concentrations of A, inhibition will be non-competitive. However at saturating levels of A, no inhibition is observed.

Identical product inhibition studies were performed on aldehyde dehydrogenase using both NAD^+ and NADP^+ as cofactors. Initial rates were calculated for variations in substrate, cofactor and products as described above.

Initial rates were determined for a variation in acetaldehyde (at fixed NAD^+ concentration) at various acetate concentrations. Very high acetate concentrations were used. The irreversibility of the enzyme as commented on by Feldman and Weiner (53) is likely due to the high acetate require-

ments for the reverse reaction. The double reciprocal plot (Figure 27a) obtained is indicative of non-competitive inhibition. Secondary plots of the intercepts (Figure 27b) and slopes (Figure 27c) of the double reciprocal plot were used to determine the kinetic parameters. All secondary plots for product inhibition studies are found in Appendix V. The specific values obtained for the kinetic constants for each inhibition experiment are listed in Table XIV. Similarly, acetaldehyde concentrations were varied (using fixed concentrations of NAD^+) in the presence of variable amounts of NADH. Non-competitive inhibition was observed (Figures 28a, b & c). Non-competitive inhibition was also observed for kinetics in which NAD^+ was varied, acetaldehyde was fixed and acetate was present as an inhibitor. The double reciprocal (Figure 29a) and secondary plots (Figure 29b & c) are shown. The double reciprocal plot obtained at fixed unsaturated levels of acetaldehyde while varying NAD^+ in the presence of NADH as inhibitor, clearly shows (Figure 30a) competitive inhibition. The secondary plot of slopes (Figure 30b) versus inhibitor concentration yields only a single inhibition constant ($K_{is} = 2.45 \times 10^2 \text{ mM}$) which is indicative of competitive inhibition.

An identical set of experiments were performed using NADP^+ as cofactor (Figures 31-34). The kinetic constants obtained are listed in Table XV. The double reciprocal and secondary plots used to determine these constants are also present. Table XV should be referred to for identification

Figure 27a Double Reciprocal Plot For Product Inhibition of NAD^+ Dependent Dehydrogenase Activity. Acetaldehyde concentrations were varied in the presence of acetate as inhibitor at a fixed concentration of NAD^+ (1.0 mM). Each line corresponds to different acetate concentrations and these are (shown top to bottom) 70, 50, 30, 0 mM. Enzyme concentration was 63 $\mu\text{g/mL}$. All assays at 25°C, pH 7.5.

Figure 28 a

Double Reciprocal Plot For Product Inhibition of NAD⁺ Dependent Dehydrogenase Activity. Acetaldehyde concentrations were varied in the presence of NADH as inhibitor at a fixed concentration of NAD⁺ (1.0 mM). Each line corresponds to different NAD⁺ concentrations and these are (shown top to bottom) 3.60 $\times 10^{-3}$, 2.70 $\times 10^{-3}$, 1.80 $\times 10^{-3}$ and 0 mM. Enzyme concentration was 63 $\mu\text{g/mL}$. All assays at 25°C, pH 7.5.

Figure 29a Double Reciprocal Plot For Product Inhibition of NAD⁺ Dependent Dehydrogenase Activity. NAD⁺ concentrations were varied in the presence of acetate as inhibitor at a fixed concentration of acetaldehyde (7.5 mM). Each line corresponds to different acetate concentrations and these are (shown top to bottom) 75, 50, 25, 0 mM. Enzyme concentration was 63 $\mu\text{g/mL}$. All assays at 25°C, pH 7.5.

Figure 30a Double Reciprocal Plot For Product Inhibition of NAD⁺ Dependent Dehydrogenase Activity. NAD⁺ concentrations were varied in the presence of NADH as inhibitor at a fixed concentration of acetaldehyde (7.5 mM). Each line corresponds to different NADH concentrations and these are (shown top to bottom) 4.65 x 10⁻³, 2.34 x 10⁻³, 1.56 x 10⁻³ and 0 mM. Enzyme concentration was 63 µg/mL. All assays at 25°C, pH 7.5.

TABLE XIV
Product Inhibition Patterns of NAD^+ Dependent
Activity of Aldehyde Dehydrogenase

Varied Substrate ^a					
Acetaldehyde					
Product	K _{ii}	K _{is}	Figures ^b		Figures ^b
			K _{ii}	K _{is}	
Acetate	2.84 x 10 ⁻²	1.04 x 10 ¹	(27)	9.59 x 10 ¹	2.45 x 10 ² (29)
NADH	1.70 x 10 ⁻⁴	2.46 x 10 ⁻³	(28)	--	9.84 x 10 ⁻⁴ (30)

NOTE: Inhibition constants are given in mM. When both constants are obtained, non-competitive inhibition is observed. When either K_{ii} or K_{is} only are obtained, uncompetitive and competitive are observed respectively. Average errors for inhibition constants were determined from correlation coefficients of linearly regressed data are $K_{ii} \pm 23\%$, $K_{is} \pm 33\%$.

^a When acetaldehyde was varied over concentrations as indicated in figure legends and axes, NAD^+ concentration was fixed at 1.0 mM. For variation of NAD^+ as indicated in figure legends and axes, acetaldehyde was fixed at 7.5 mM.

^b For specific plots refer to the figure number stated (a) for double reciprocal plots and; (b) primary intercept versus inhibitor concentration to obtain K_{ii} and (c) primary slopes versus inhibitor concentration to obtain K_{is} .

-- No value observed.

TABLE XV
Product Inhibition Patterns of NADP^+ Dependent
Activity of Aldehyde Dehydrogenase

Product	Varied Substrate ^a				
	Acetaldehyde			NADP ⁺	
	K ₁₁	K ₁₂	Figures ^b	K ₁₁	K ₁₂
Acetate	4.92 x 10 ⁻²	4.89 x 10 ⁻¹	(31)	1.61 x 10 ²	6.20 x 10 ¹
NADPH	7.10 x 10 ⁻³	2.46 x 10 ⁻²	(32)	--	1.03 x 10 ⁻³

NOTE: Inhibition constants are given in mM. When both constants are obtained, non-competitive inhibition is observed. When either K_{11} or K_{12} only are obtained, uncompetitive and competitive are observed respectively. Average errors for inhibition constants were determined from correlation coefficients of linearly regressed data are $K_{11} \pm 23\%$, $K_{12} \pm 18\%$.

^a When acetaldehyde was varied over concentrations as indicated in figure legends and axes, NADP^+ concentration was fixed at 3.2 mM. For variation of NADP^+ as indicated in figure legends and axes, acetaldehyde was fixed at 10 mM.

^b For specific plots refer to the figure number stated (a) for double reciprocal plots and; (b) primary intercept versus inhibitor concentration, to obtain K_{11} and (c) primary slopes versus inhibitor concentration to obtain K_{12} .

- No value observed.

Figure 31a

Double Reciprocal Plot For Product Inhibition of NADP⁺ Dependent Dehydrogenase Activity. Acetaldehyde concentrations were varied in presence of acetate as inhibitor at fixed NADP⁺ concentration (3.2 mM). Each line corresponds to different acetate concentrations and these are (shown top to bottom) 5.95×10^{-1} , 4.25×10^{-1} , 255×10^{-1} and 0 mM. Enzyme concentration was 63 $\mu\text{g/mL}$. All assays at 25°C, pH 7.5.

Figure 32a

Double Reciprocal Plot for Product Inhibition of NADP⁺ Dependent Dehydrogenase Activity. Acetaldehyde concentrations were varied in the presence of NADPH as inhibitor, at a fixed concentration of NADP⁺ (3.2 mM). Each line corresponds to different NADPH concentrations and these are (shown top to bottom) 1.45×10^{-2} , 7.26×10^{-3} , 3.63×10^{-3} and 0 mM. Enzyme concentration was 63 $\mu\text{g/mL}$. All assays at 25°C, pH 7.5.

Figure 33a Double Reciprocal Plot For Product Inhibition of NADP⁺ Dependent Dehydrogenase Activity. NADP⁺ concentrations were varied in the presence of acetate as inhibitor at a fixed concentration of acetaldehyde (20 mM). Each line cor-

Figure 34a Double Reciprocal Plot For Product Inhibition of NADP⁺ Dependent Dehydrogenase Activity. NADP⁺ concentrations were varied in the presence of NADPH as inhibitor at a fixed concentration of acetaldehyde (20 mM). Each line corresponds to a different NADPH concentration and these are (shown top to bottom) 1.45×10^{-2} , 1.09×10^{-2} , 7.26×10^{-3} and 0 mM. Enzyme concentration was 63 $\mu\text{g/mL}$. All assays at 25°C, pH 7.5.

of the kinetic constants with the specific experiments and respective plots.

In comparing the Michaelis constants for NAD^+ of aldehyde dehydrogenase ($K_m = 7.05 \times 10^{-2} \text{ mM}$) with the concentration of NAD^+ used in the product inhibition studies where the NAD^+ concentration was fixed (1.0 mM), one observes a 14 fold difference. That non-competitive inhibition was observed for all cases where the NAD^+ concentration was fixed, indicates the enzyme had not been saturated. High levels of NAD^+ were necessary to obtain consistently accurate velocities. Initial velocities were determined in the presence of as high as 6.0 mM NAD^+ . No indication of substrate inhibition was observed at these levels. Attempts to saturate enzyme at levels beyond 6.0 mM failed to yield acceptable initial rates. Analyses were precluded by a high inherent absorbancy by high levels of cofactor.

The level of acetaldehyde used as a fixed level for inhibition kinetics (7.5 mM) was slightly above the Michaelis constant ($K_m = 2.86 \text{ mM}$). Attempts to perform product inhibition kinetics at fixed, saturated levels of acetaldehyde were precluded by the observation of inhibition by substrate above 12 mM. Product inhibition kinetics were therefore not carried out at saturating levels of either NAD^+ or acetaldehyde. The product inhibition patterns that were obtained did provide sufficient information to identify the mechanism as Ordered Bi Bi according to Table XIII.

This is supported by a lack of variation of the values for K_{12} determined for substrate specificity (Table XI).

Similarly, high levels of NADP^+ (3.2 mM) were used to obtain consistently high activities, for fixed NADP^+ assays. The level was 5.6 fold higher than the Michaelis constant for NADP^+ ($K_a = 5.69 \times 10^{-1}$ mM). As above, no indication of saturation was detected based on possible inhibition patterns for saturated enzyme listed in Table XIII. Similarly, the level of acetaldehyde used for fixed acetaldehyde assays was 4.4 fold higher than the Michaelis constant ($K_D = 2.28$ mM). Again no saturation was detected at this concentration. Levels above 15-20 mM acetaldehyde resulted in apparent substrate inhibition which precluded attempts to saturate the enzyme. As was observed for product inhibition kinetics employing NAD^+ as cofactor, the NADP^+ dependent product inhibition kinetics did provide sufficient information to establish the mechanism as Ordered Bi Bi.

Several other generalizations can be obtained from these studies. Very high levels of acetate were necessary to observe inhibition by this product at low levels of cofactors. Where NAD(P)^+ is varied, the acetate exhibits high millimolar dissociation constants which are suggestive of poor enzyme-acetate affinity. This may lead to the irreversibility of the enzyme (as is generally assumed for this enzyme (53)). Complete saturation can likely be ruled out as the inhibition patterns described in Table XIII for sat-

urated systems were not altered at the levels used. Unlike acetate, NAD(P)H does not exhibit any major variations in Michaelis constants at low (variable) or high (fixed) levels of either NAD(P)⁺ or acetaldehyde. The reduced cofactors appeared to have a stronger binding affinity than the oxidized form at all levels of substrate and cofactor. The Michaelis constants began to approach micromolar levels for the reduced cofactors. This might suggest that the enzyme may exhibit reversibility in the presence of appropriate levels of acetate.

2.2.iii. Other Effectors.

The action of disulfiram on NAD⁺ and NADP⁺ dehydrogenase activities was investigated. Kinetic assays performed in the presence of disulfiram using NAD⁺ as cofactor (acetaldehyde concentration fixed at 10 mM) clearly exhibit competitive inhibition properties. The double reciprocal plot obtained over a range of disulfiram concentrations converges at a single point on the ordinate axis (Figure 35a). A plot of slopes obtained from the double reciprocal plot versus disulfiram concentration (Figure 35b) was used to obtain the competitive inhibition constant ($K_{is} = 6.93 \times 10^{-2}$ mM). All secondary plots for dehydrogenase effector studies are found in Appendix V. A similar study was repeated using NADP⁺ as cofactor. Double reciprocal and secondary plots (Figures 36a & b respectively) were used to obtain the competitive inhibition constant ($K_{is} = 1.24 \times 10^{-1}$ mM).

Figure 35a Double Reciprocal Plot For Inhibition of NAD⁺ Dependent Dehydrogenase Activity By Disulfiram. Initial rates were determined over a series of NAD⁺ concentrations for a range of disulfiram concentrations (acetaldehyde concentration fixed at 10 mM). Each line corresponds to a different disulfiram concentration and these are (shown top to bottom) 0.100, 0.080, 0.050 and 0 mM. Enzyme concentration was 0.151 mg/mL. All assays at 25°C, pH 7.5.

Figure 36a

Double Reciprocal Plot For Inhibition of NADP⁺ Dependent Dehydrogenase Activity By Disulfiram. Initial rates were determined over a series of NADP⁺ concentrations for a range of disulfiram concentrations (acetaldehyde fixed at 10 mM). Each line corresponds to a different disulfiram concentration and these are (shown top to bottom) 0.100, 0.080, 0.050 and 0 mM. Enzyme concentration was 0.151 mg/mL. All assays at 25°C, pH 7.5.

Chloral hydrate was also investigated to assess its inhibitory effect on the enzyme. Kinetic assays performed over a range of 0-0.8 mM of chloral hydrate were used to obtain the double reciprocal plots for NAD^+ and NADP^+ dehydrogenase activities (Figures 37a & 38a respectively). A clear indication of competitive inhibition with respect to NADP^+ is revealed by convergence to a single point on the ordinate scale. The inhibition constants were obtained from secondary plots (Figure 37b for NAD^+ and Figure 38b for NADP^+) and are listed in Table XVI. Of the two inhibitors used above, disulfiram proved to be slightly greater in its inhibitory power as reflected by slightly lower inhibition constants.

Diethylstilbestrol (DES) has been reported to exhibit various inhibitory and activatory effects on some isozymes of rat liver aldehyde dehydrogenase (39). Inhibition was not observed at levels up to 0.05 mM for our enzyme.

Magnesium (61) and potassium (97, 170) ions have been found to affect aldehyde dehydrogenase. A 2 fold stimulation in activity was observed for the horse liver aldehyde dehydrogenase in the presence of 50 μM Mg^{+2} ions (97). The increase was attributed to a half-of-sites to all-of-sites phenomenon. In the absence of Mg^{+2} the enzyme is believed to exist as a tetramer. Inclusion of Mg^{+2} results in dissociation of the enzyme into two equally active dimers thereby doubling the activity. Yeast aldehyde dehydrogenase has been shown to be unstable in the absence of high

Figure 37a

Double Reciprocal Plot For Inhibition of NAD^+ Dependent Dehydrogenase Activity By Chloral Hydrate. Initial rates were determined over a series of NAD^+ concentrations for a range of chloral hydrate concentrations (acetaldehyde fixed at 7.5 mM). Each line corresponds to a different chloral hydrate concentration and these are (shown top to bottom) 0.80, 0.50, 0.30 and 0 mM. Enzyme concentration was 0.63 mg/mL. All assays were at 25°C, pH 7.5.

Figure 38a

Double Reciprocal Plot For Inhibition of NADP⁺ Dependent Dehydrogenase Activity By Chloral Hydrate. Initial rates were determined over a series of NAD⁺ concentrations (acetaldehyde fixed at 7.5 mM). Each line corresponds to a different chloral hydrate concentration and these are (shown top to bottom) 0.80, 0.50, 0.30, 0 mM. Enzyme concentration was 0.63 mg/mL. All assays were at 25°C, pH 7.5.

TABLE XVI

Influence of Various Effectors on Dehydrogenase Activities,
pH 7.5.

	K_{11} (mM)	K_{1s} (mM)
Chloral Hydrate		
NAD ⁺	-	$1.87 \times 10^{-1} \pm 2.2 \times 10^{-2}$
NADP ⁺	-	$2.00 \times 10^{-1} \pm 1.32 \times 10^{-2}$
Disulfiram		
NAD ⁺	-	$6.93 \times 10^{-2} \pm 1.32 \times 10^{-2}$
NADP ⁺	-	$1.24 \times 10^{-1} \pm 2.6 \times 10^{-2}$
Other Effectors		
0-20 mM Mg^{+2} , No effect		
0-20 mM K^+ , No effect		
0-0.05 mM DES, no effect		

K_{1s} - Inhibition constant with respect to cofactor was obtained from a plot of slopes (of double reciprocal plot of velocity versus cofactor concentration) versus inhibitor concentration. Specific concentrations of substrates and effectors are as outlined in the procedures section. Errors for inhibition constants were calculated from the correlation coefficients of linear regressed data.

concentrations of K^+ ions. However, neither inhibitory nor activatory effects were observed for our enzyme when assays were performed in the presence of up to 20 mM Mg^{+2} or K^+ ions.

2.2.iv. Cross Product Inhibition.

The action of NADPH as a product inhibitor on NAD^+ dependent dehydrogenase activity was investigated. Initial rates were determined over a series of NAD^+ concentrations for a variety of NADPH concentrations (acetaldehyde concentration was fixed at 7.5 mM). The double reciprocal plot obtained (Figure 39a) shows a family of lines convergent on the ordinate axis suggesting competitive inhibition. This is analogous to the product inhibition previously described for NAD^+ (Figure 30). The competitive inhibition constant was obtained from a secondary plot (Figure 39b) as previously described and found to be $K_{is} = 1.42 \times 10^{-2}$ mM. Secondary plots are found in Appendix V. Similarly, NADH was found to competitively inhibit $NADP^+$ dependent dehydrogenase activity. The inhibition constant obtained from the double reciprocal (Figure 40a) and secondary plots (Figure 40b) was $K_{is} = 1.02 \times 10^{-2}$ mM.

The cross product inhibition constants above and the inhibition constants obtained for each reduced cofactor acting as an inhibitor on the activity which produces it (i.e. NADH inhibition of NAD^+ activity and NADPH inhibition of $NADP^+$ activity) are presented for comparison in Table XVII. Each reduced cofactor inhibits competitively both NAD^+ and

Figure 39a

Double Reciprocal Plot For Inhibition of NAD⁺ Dependent Dehydrogenase Activity By NADPH. Initial rates were determined over a series of NADPH concentrations and these are (shown top to bottom) 4.69×10^{-3} , 3.29×10^{-3} , 1.64×10^{-3} and 0 mM NADPH. Enzyme and ethanol concentrations were 0.22 mg/mL and 75 mM respectively. All assays were at 25°C , pH 7.5 .

Figure 40a Double Reciprocal Plot for Inhibition of NADP⁺ Dependent Dehydrogenase Activity by NADH. Initial rates were determined over a series of NADH concentrations. Each line corresponds to a different NADH concentration and these are (shown top to bottom) 86×10^{-2} , 1.40×10^{-2} , 9.3×10^{-3} and 0 mM NADH. Enzyme and ethanol concentrations were 0.22 mg/mL and 75 mM respectively. All assays were at 25°C, pH 7.5.

3

METRO

TABLE XVII

Summary of Product Inhibition Constants (K_{is}) by Reduced
Cofactors on Dehydrogenase Activities

Inhibiting Cofactor	Dehydrogenase Activity	
	NAD ⁺	NADP ⁺
NADH	9.84×10^{-4} ($\pm$ 18%)	1.02×10^{-2} ($\pm$ 6.8%)
NADPH	1.42×10^{-2} ($\pm$ 30%)	1.03×10^{-3} ($\pm$ 18%)

Inhibitor constants reported are all competitive (K_{is}) and have units of mM. Acetaldehyde was held at a fixed concentration of 7.5 mM for all assays.

NADP⁺ dependent dehydrogenase activities. Since both reduced cofactors compete with either oxidized cofactor for the free enzyme, this may imply that both NAD⁺ and NADP⁺ co-enzymes are reduced in the same active site and supports the idea of a single molecular form of the enzyme. The inhibition constants obtained for NADH and NADPH are lowest for their own respective activities.

2.2.v. Inhibition by Substrate

Earlier kinetic studies revealed that acetaldehyde levels exceeding 12-15 mM resulted in inhibition of NAD⁺ dependent dehydrogenase activity. The double reciprocal plot obtained using higher concentrations shows upward curvature of those assays performed at higher substrate levels (Figure 41). A similar study using assays performed at NAD⁺ levels up to 6.0 mM failed to exhibit an inhibitory trend. Assays beyond this level of cofactor were precluded by the high inherent absorbancy of the cofactor. Thus even though substrate inhibition by cofactor is not observed up to 6.0 mM, one cannot rule out the possibility of inhibition beyond this level.

Substrate inhibition observed for acetaldehyde is highly suggestive of the formation of a substrate-enzyme-product complex. This ternary complex is discussed in detail in following sections.

Figure 41 Inhibition By Substrate. The initial rates were determined over a range of acetaldehyde concentrations from 2.5 to 150 mM. The concentration of NAD^+ was fixed at 1.0 mM for all assays. Enzyme concentration was 0.22 mg/mL. All assays were at 25°C , pH 7.5.

3. STEADY STATE KINETICS-PART B

3.1. Esterase Kinetics

3.1.1. General

Esterase kinetics were performed using the assays described in the procedures section. The effects of pH, substrate chain length and solvent are presented in this section. The action of various effectors and specialized substrates are found in following sections. The kinetic studies help characterize the enzyme, elucidate the mechanism and probe similarities in active sites between dehydrogenase and esterase activities.

3.1.ii. Substrate Specificity

Initial rates were determined for *p*-nitrophenyl esters ranging in chain length from C₂ (*p*-nitrophenyl acetate) to C₁₂ (*p*-nitrophenyl laurate). Double reciprocal plots were obtained from initial rate data determined over a range of *p*-nitrophenyl ester concentrations. The double reciprocal plots prepared for ester substrates of various carbon chain lengths (2 to 12 carbons inclusive) are found in Figures 42a-d respectively. Each curve was obtained by an average of duplicate or triplicate measurements. A summary of the kinetic constants determined from these plots is found in Table XVIII. Errors indicated were calculated from the correlation coefficients obtained by regression analysis.

p-Nitrophenyl butyrate was observed to be the optimal substrate having both the highest turnover number and strongest affinity for the enzyme (manifested by the lowest dissociation constant ($K_m = 2.52 \times 10^{-2}$ mM)). As the chain length is increased from butyrate to laurate the K_m values progressively increase, indicating that the longer chain substrates are less favorably bound in the esterase active site. This appears to be reflected in turnover number data. As chain length increases, there is a concomitant decrease in V_{max} and turnover number.

3.1. iii. Effect of pH on Esterase Kinetics.

The effect of pH on esterase kinetics was investigated over the range pH 6.0-11.0. Kinetic assays were performed

Figure 42a-d Double Reciprocal Plots For Substrate Specificity of Esterase Activity. The plots were constructed from initial rate data obtained over a range of concentrations of the following p-nitrophenyl-ester substrates: a) acetate (■) and butyrate (●); b) caproate; c) caprylate; d) caprate (●) and laurate (■). Enzyme concentration was 44 $\mu\text{g/mL}$. All assays were at 25°C, pH 7.5.

Figure 42b

Figure 42c

Figure 42d Substrates shown are p-nitrophenyl caprate (●) and p-nitrophenyl laurate (■).

TABLE XVIII

Substrate Specificity of Esterase Activity

p-Nitrophenyl Ester	Chain Length	$V_{\max}$ ($\mu\text{M}/\text{min}$)	K_m (mM), $\times 10^2$	$V_{\max}/E_t$ (s^{-1}), $\times 10^{-2}$
Acetate	C ₂	1.14	3.24	1.05
Butyrate	C ₄	1.59	2.52	1.47
Caproate	C ₆	1.50	3.17	1.38
Caprylate	C ₈	1.28	3.54	1.18
Caprate	C ₁₀	1.12	3.84	1.03
Laurate	C ₁₂	0.13	0.65	0.12

Kinetic data was calculated from an average of duplicate or triplicate measurements. Errors in kinetic constants were determined by regression analysis of rate data. Average errors are $V_{\max} \pm 4.1\%$, $K_m \pm 6.9\%$ and $V_{\max}/E_t \pm 10.9\%$. C_n refers to the carbon chain length of the ester.

under identical conditions with the exception being a variation in pH. Figures 43a-g are double reciprocal plots obtained from initial rate data determined over a range of substrate concentrations at a specific pH. The double reciprocal plots (Figures 43a-g) correspond to variations in pH of pH 6.0-11.0 respectively. The kinetic constants determined from the plots are summarized in Table XIX. Errors indicated were calculated by regression analysis of duplicate data.

A plot of $\log V_{\max}$ versus pH indicates a broad pH profile (Figure 44). Maximal esterase activity was observed at approximately pH 8.0-8.5 with a slightly asymmetric decrease between pH 9.0-10.5. This profile suggests that esterolytic activity is based on two ionizable species, one with pK 7.3 and the other pK 10.35.

3.1.iv. Solvent Effect

A similar pH profile as described above was generated from initial rate data obtained over a range of pH (Figures 45a-h) in the presence of 2.5% dioxane (Figure 44). The kinetic constants determined from the plots are summarized in Table XX. The change in solvent polarity effected a slight shift in pK from 7.35 to 7.90. An increase in maximum velocity values was also observed above pH 8.0. The profile does not reveal the traditional shape observed in the absence of dioxane. Rather, the $\log V_{\max}$ values appear to reach an asymptote at high pH values. The lower pH

Figure 43a-g Double Reciprocal Plots For pH Effect on Esterase Kinetics. Assays were performed using p-nitrophenyl butyrate as indicated over the following range of pH conditions: a) pH 6.0; b) pH 7.0; c) pH 7.5; d) pH 8.0; e) pH 9.0; f) pH 10.0; g) pH 11.0. Enzyme concentration was 7.3 $\mu\text{g/mL}$. All assays were at 25°C.

Figure 43b

Figure 43c

Figure 43 d

Figure 43e

Figure 43f

Figure 43 g

TABLE XIX.

Effect of pH on Esterase Activity

pH	$V_{\max}$ ($\mu\text{M}/\text{min}$)	K_m (mM) $\times 10^2$	$V_{\max}/K_m$ (s^{-1}), $\times 10^{-2}$	$\text{Log } V_{\max}$ $\times 10^1$
6.0	0.094	4.60	0.087	-10.3
7.0	0.669	3.37	6.17	-1.75
7.5	1.14	3.24	1.05	0.569
8.0	1.60	4.56	1.47	2.04
9.0	1.77	3.28	1.63	2.48
10.0	1.21	1.81	1.15	0.828
11.0	0.400	2.24	0.368	-3.98

Errors were determined by regression analysis and found to be

$V_{\max} \pm 6.9\%$, $K_m \pm 10.1\%$, $V_{\max}/E_t \pm 10.9\%$.

Figure 44 pH and Environment Effects on Esterase Activity. Esterase kinetics over a pH range reveals the broad, slightly asymmetric pH profile (●). A similar profile generated in the presence of 2.5% of 1,4-dioxane reveals an upward shift at higher pH values (■). Enzyme concentration was 7.3 $\mu\text{g/mL}$. All assays were at 25°C.

Figure 45a-h Double Reciprocal Plots For Effect of Hydrophobic Environment On Esterase Kinetics. Assays were performed using *p*-nitrophenyl butyrate as substrate over a range of pH from pH 6.5 to 9.9. Assays were performed in the presence of 2.5% dioxane. The plots correspond to the following pH conditions: a) pH 6.5; b) pH 7.0; c) pH 7.5; d) pH 8.0; e) pH 8.6; f) pH 9.0; g) pH 9.5; h) pH 9.9. Enzyme concentration was 8.9 $\mu\text{g/mL}$. All assays were at 25°C.

Figure 45b

Figure 45c

Figure 45 d

Figure 45e

Figure 45f

Figure 45.g

Figure 45h

TABLE XX

Effect of Solvent Polarity* on Esterase Activity

pH	$V_{\max}$ ($\mu\text{M}/\text{min}$)	K_m (mM) $\times 10^2$	$V_{\max}/E_t$ (s^{-1}), $\times 10^{-2}$	$\text{Log } V_{\max}$ $\times 10^1$
6.5	5.9×10^{-2} ($\pm 25.6\%$)	39.4 ($\pm 26.6\%$)	0.054 ($\pm 30.6\%$)	-12.3
7.0	9.14×10^{-1}	3.00	0.842	-0.391
7.5	1.59	2.52	1.47	2.01
8.0	4.70	7.21	4.33	6.72
8.6	6.92	9.70	6.37	8.40
9.0	8.66	11.6	7.98	9.38
9.5	9.14	9.14	8.42	9.61
9.9	9.40	9.0	8.65	9.73

Errors were determined by regression analysis of duplicate measurements and found to be $V_{\max} \pm 4.8\%$, $K_m \pm 5.8\%$ and $V_{\max}/E_t \pm 9.8\%$ unless specified otherwise.

* Performed in 2.5% 1,4-dioxane

region retains the curvature inherent of typical pH profiles and permitted the determination of the pK of a single ionizable species (above).

3.2.1 Product Inhibition

Esterase kinetics were performed in the presence of products to investigate the inhibitory effects of each, thereby enabling some inference to be made about the mechanism. Enzymes employing Uni Bi kinetics can typically follow one of three possible treatments (171). One of these, the Ordered Uni Bi mechanism, is depicted in Scheme 1:

Scheme 1. Schematic Representation of an Ordered Uni Bi Mechanism

The rate equation describing this mechanism in the presence of P ($[Q]=0$) is:

$$\frac{1}{v} = \frac{1}{V_1} \left(1 + \frac{[P]}{K_{ip}} \right) + \frac{1}{V_1} \left(K_a + \frac{V_1 K_a [P]}{V_2 K_{eq}} \right) \frac{1}{[A]}$$

where V_1 and V_2 are $V_{\max}$ values in forward and reverse directions, $[P]$ and $[A]$ are concentrations of species P and A respectively, K_a and K_p are the Michaelis constants for A and P and K_{eq} is the equilibrium constant. Similarly it can

be shown that the rate of equation in the presence of Q ([P]=0) is:

$$\frac{1}{v} = \frac{1}{V_1} + \frac{1}{V_1} \left(K_a + \frac{V_1 K_p [Q]}{V_2 K_{eq}} \right) \frac{1}{[A]}$$

As can be implied from the equations, product P will affect both slope and intercept terms of the double reciprocal plot. Thus P will act as a non-competitive inhibitor relative to A. The second product Q only affects the slope term and will therefore inhibit competitively with respect to A. Similar treatment of the rate equations for the remaining two treatments (Rapid Equilibrium Random Uni Bi and Steady State Random Uni Bi) can be done to predict the product inhibition patterns for these patterns. A summary of the product inhibition patterns for Uni Bi mechanisms can be found in Table XXI. Therefore, product inhibition studies can reveal, for Uni Bi mechanisms, the type of mechanism and the ordered of products released as well.

Esterase kinetics performed in the presence of butyrate ions clearly indicate this product inhibits competitively with respect to A. An explanation of inhibition kinetic constant determination for single substrate reactions can be found in Appendix IV. The double reciprocal plot (Figure 46a) shows a family of lines obtained over a range of butyrate concentrations which are convergent on the ordinate axis indicating only the slope term was affected. The secondary plot of slopes from the above plot versus inhibitor concentration (Figure 46b) was used to determine the inhibi-

TABLE XXI

Product Inhibition Patterns for Uni Bi Mechanisms

Mechanism	Product	Type of Inhibition
Ordered Uni Bi	P	N*
	Q	C
Rapid Equilibrium	P	C
Random Uni Bi	Q	C
Steady State Random	P	N
Uni Bi	Q	N

*Abbreviations are C(competitive) and N(non-competitive).

Taken from (121).

Figure 46a Double Reciprocal Plot For Product Inhibition of Esterase Activity By Butyrate Ions. Initial rates were determined over p-nitrophenyl butyrate concentrations in the presence of butyrate ions. The lines each represent a fixed butyrate concentration and are (shown top to bottom) 2.0, 1.5, 1.0 and 0 mM. Enzyme concentration was 8.9 μ g/mL. All assays were at 25°C, pH 7.5.

tion constant which can be found in Table XXII. Secondary plots are found in Appendix V.

Esterase kinetics were also performed in the presence of *p*-nitrophenol as a product inhibitor. The double reciprocal plot obtained over a range of *p*-nitrophenol concentrations shows both slope and intercept terms are affected (Figure 47a). Non-competitive inhibition constants for *p*-nitrophenol obtained with respect to substrate were determined from secondary plots of primary intercepts (Figure 47b) and primary slopes (Figure 47c) plotted against *p*-nitrophenol concentration and are compiled in Table XXII. The values for K_{is} and K_{ii} are very similar indicating that *p*-nitrophenol binds with almost equal affinity to both free and binary complex enzyme.

The summary of product inhibition therefore reveals, according to Table XXI, that the esterase activity follows an Ordered Uni Bi mechanism with *p*-nitrophenol being the first product released followed by butyrate from the enzyme-product binary complex (EQ).

3.2.ii. Action of Other Effectors on Esterase Activity

The action of acetaldehyde, the substrate for the dehydrogenase reaction, on esterase activity was investigated to observe the relationships of the active sites. A double reciprocal plot of esterase activity versus *p*-nitrophenyl butyrate concentration was drawn for several acetaldehyde concentrations (Figure 48a). A series of parallel lines

TABLE XXII
Product Inhibition Patterns for Esterase Kinetics

Product	K_{ii} (mM)	K_{is} (mM)
Butyrate	-	8.94×10^{-1}
p-Nitrophenol	8.0×10^{-2}	8.12×10^{-2}

- No value determinable.

NOTE: K_{ii} and K_{is} refer to the inhibition constants obtained from plots of intercepts and slopes (respectively) of the double reciprocal plot plotted against inhibitor concentration. Average errors were determined by regression analysis of duplicate measurements found to be $K_{ii} \pm 16.2\%$, $K_{is} \pm 40.9\%$.

Figure 47 a Double Reciprocal Plot For Product Inhibition of Esterase Activity By p-Nitrophenol. Initial rates were determined at various p-nitrophenyl butyrate concentrations in the presence of p-nitrophenol. The lines each represent a fixed p-nitrophenol concentration and are (shown top to bottom) 0.20, 0.15, 0.10 and 0 mM. Enzyme concentration was 8.9 $\mu\text{g/mL}$, all assays at 25°C, pH 7.5.

Figure 48a Double Reciprocal Plot For Esterase Inhibition By Acetaldehyde. Initial rates were determined over a range of *p*-nitrophenyl butyrate concentrations in the presence of acetaldehyde. Each line represents a fixed acetaldehyde concentration and these are (shown) 13.3, 10.0 and 0 mM. Enzyme concentration was 34 $\mu\text{g/mL}$, all assays at 25°C , pH 7.5.

indicate that only intercept terms of the rate equation are affected by acetaldehyde. This indicates that acetaldehyde inhibits uncompetitively with respect to substrate. The inhibition constant ($K_{ii} = 9.50 \pm 1.68$ mM, see Table XXIII) was obtained from a secondary plot of primary intercepts versus acetaldehyde concentration (Figure 48b). Secondary plots for esterase effector studies are found in Appendix V.

Esterase kinetics were found to be competitively inhibited in the presence of chloral hydrate. A double reciprocal plot of esterase kinetics measured over a range of chloral hydrate concentrations (Figure 49a) indicates convergence to a single ordinate intercept. The slopes of each line are plotted against chloral hydrate concentration (Figure 49b) from which the competitive inhibition constant was obtained ($K_{is} = 2.61 \times 10^{-1} \pm 1.07 \times 10^{-1}$ mM, see Table XXIII). A comparison of inhibition constants of the effectors for esterase and dehydrogenase activities (see Table XVI) reveals all constants to be identical within experimental error. The enzyme-inhibitor affinity is the same when measured by either assay. Since both activities are competitively inhibited, the active sites for esterase and dehydrogenase activities must be located close together (51).

A similar kinetic study using disulfiram as an inhibitor (Figures 50a & b) also showed competitive inhibition which is similar to that obtained for the dehydrogenase activities. The inhibition constant and those of all the

TABLE XXIII

Effects of Inhibitors on Esterase Kinetic Parameters

Effector	K_{11} (mM)	K_{1s} (mM)
Acetaldehyde	9.50 ± 1.68	-
Chloral Hydrate	-	$2.6 \times 10^{-1} \pm 1.07 \times 10^{-1}$
Disulfiram	-	$1.41 \times 10^{-2} \pm 0.45 \times 10^{-2}$
Other Effectors		
0-20 mM Hg^{+2} , No effect		
0-20 mM K^+ , No effect		
0-50 μ M DES, no effect		

Note: K_{11} is the inhibition constant and was obtained from a secondary plot of slopes (of the double reciprocal plot) plotted against inhibitor concentration. Similarly, the K_{1s} constant was obtained from intercepts of the double reciprocal plot.

- No constant observed.

Figure 49a. Double Reciprocal Plot For Esterase Inhibition By Chloral Hydrate. Initial rates were determined over a range of p-nitrophenyl butyrate concentrations in the presence of chloral hydrate. Each line represents a fixed chloral hydrate concentration and these are (shown top to bottom) 0.70, 0.50, 0.30, 0.20 and 0 mM. Enzyme concentration was 34 $\mu\text{g/mL}$, all assays at 25°C, pH 7.5.

Figure 50a Double Reciprocal Plot For Esterase Inhibition By Disulfiram. Initial rates were determined over a range of p-nitrophenyl butyrate concentrations in the presence of disulfiram. The lines represents a fixed disulfiram concentration and these are (shown top to bottom) 0.08, 0.05, 0.03 and 0 mM. Enzyme concentration was 25 $\mu\text{g/mL}$, all assays at 25°C, pH 7.5.

inhibitors used can be found in Table XXIII. Again the similarity in competitive inhibition constants suggests that esterase and dehydrogenase active sites are proximally located. The K_{is} for the esterase activity is $1.41 \times 10^{-2} \pm 0.45 \times 10^{-2}$ mM which is within an order of magnitude of that obtained for NAD^+ and NADP^+ dependent dehydrogenase activities ($K_{is} = 6.93 \times 10^{-2} \pm 1.3 \times 10^{-2}$ mM and $1.24 \times 10^{-1} \pm 2.6 \times 10^{-2}$ mM respectively).

The esterase activity was unaffected by up to 20 mM Mg^{+2} and K^+ and 50 μM DES.

3.2.iii. Nucleotide Effects

It has been observed that the pyridine nucleotide NAD^+ can function as an activator for the esterase activity of aldehyde dehydrogenases. Such has been the case for the horse (53), human (51) and two of the isozymes of the rat enzyme (39). We investigated the effects of low concentrations of NAD^+ on esterase kinetics. A double reciprocal plot was made of esterase initial velocities measured over a range of *p*-nitrophenyl acetate concentrations in the presence of fixed concentrations of NAD^+ (Figure 51a). The figure indicates slight activation as the NAD^+ concentration is increased to 0.322 mM. This was the maximum concentration observed to elicit the activation and can be considered the apparent saturating level of NAD^+ with respect to esterase activity. As can be seen from the plot the lines tend to converge to this maximum activity. The level of NAD^+ re-

Figure 51a Double Reciprocal Plot For Esterase Activation By Low Concentrations of NAD^+ . Initial rates were determined over a range of p-nitrophenyl acetate concentrations in the presence of NAD^+ . Each line represents a different NAD^+ concentration and corresponds to 0, 0.104, 0.156, 0.260 and 0.322 mM NAD^+ listed top to bottom. Enzyme concentration was 34 $\mu\text{g/mL}$, all assays at 25°C, pH 7.5.

The values of α and β can be evaluated at the apparent saturating concentration of NAD^+ which is determined to be 0.32 mM. At the saturating activator concentration the y-intercept of the double reciprocal plot is given as $y\text{-intercept} = 1/\beta V_{\max}$. Similarly the $x\text{-intercept} = -1/\alpha K_s$. Values of $V_{\max}$ and K_s are determined from the curve in the absence of any activator. The factors α and β were determined to be 0.97 and 1.11 respectively. Thus we see that in the presence of 0.32 mM NAD^+ a net increase in activity of 1.11 fold is observed and the substrate Michaelis constant is 0.97 fold lower. This indicates that the saturating level of NAD^+ results in a slight increase in *p*-nitrophenyl acetate-enzyme binding affinity. The Michaelis constant K_a can be evaluated from a secondary plot of $1/\Delta$ intercepts versus $1/\text{activator}$ concentration where Δ intercepts is the difference in the intercepts obtained in the absence of activator minus the intercept obtained from the double reciprocal plot in the presence of a specified concentration of activator. The $x\text{-intercept}$ of the secondary plot is used to evaluate the value for K_a by employing the relation $x\text{-intercept} = -\beta/\alpha K_a$. The secondary plot obtained for the activation effect of NAD^+ on this enzyme is shown in Figure 51b. The plot consists of a linear region which was extrapolated to determine K_a . The value for K_a was determined to exist in the range 1.67×10^{-1} mM to 6.8×10^{-2} mM. This range includes the value obtained for the Michaelis constant of NAD^+ in the dehydrogenase reaction and supports therefore

Figure 51b Secondary Plot For Esterase Activation In The Presence of Low Concentrations of NAD^+ . The inverse of the difference in intercepts from the double reciprocal plot (intercept for 0 mM NAD^+ minus intercept for specified amount of NAD^+) were plotted against $1/NAD^+$ concentration. The plot was used to obtain an estimate of $K_{\text{Activator}} (K_a)$. Enzyme concentration was $34 \mu\text{g/mL}$, all assays at 25°C , pH 7.5.

the idea that the active sites may be the same. More interesting however is the observation that the curve has a non-linear region. This suggests that the constants α and β are variable with activator concentration. Other workers have observed that the ability of NAD^+ to activate esterase activity is lost at high concentrations. In fact, NAD^+ inhibits esterase activity of rat mitochondrial and microsomal isozymes when present in levels above 0.10 mM (39). The effect of higher NAD^+ levels on esterase activity of our enzyme was determined. A double reciprocal plot of esterase activity determined using *p*-nitrophenyl acetate as substrate in the presence of higher NAD^+ concentrations is shown in Figure 52a. Levels of NAD^+ from 1.04 to 2.08 mM are seen to competitively inhibit esterase activity as indicated by convergence of all lines to a single point on the ordinate axis. The inhibition constant K_{is} was determined from a secondary plot (Figure 52b) and found to be $K_{is} 1.29 \pm 0.34$ mM.

The nucleotide study was extended to observe the effects of various NAD^+ fragments on esterase activity. Double reciprocal plots were prepared for esterase activity measured in the presence of 0.5 mM of ADP, ADPR and NMN. The plots are shown as Figures 53a, b and c respectively. The kinetic constants obtained and those for absence of nucleotide (Figures 42a) and for 0.32 mM NAD^+ are listed in Table XXIV. In the presence of NAD^+ the esterase kinetics observed both an increase in turnover number and a decrease

Figure 52 a Double Reciprocal Plot For Esterase Inhibition By High Concentrations of NAD^+ . Initial rates were determined over a range of p-nitrophenyl acetate concentrations in the presence of NAD^+ . Each line represents a different NAD^+ concentration and corresponds to 2.08, 1.56, 1.04, 0 mM listed top to bottom respectively. Enzyme concentration was 34 $\mu\text{g/mL}$, all assays at 25°C, pH 7.5.

Figure 52 b

Secondary Plot For Esterase Inhibition By NAD^+ . The slopes obtained from the primary plot were plotted against NAD^+ concentration and this was used to evaluate K_{is} . Enzyme concentration was $34 \mu\text{g/mL}$, all assays at 25°C , pH 7.5.

Figure 53 a,b & c Double Reciprocal Plots For The Action of Nucleotides on Esterase Activity. Initial rates were determined over a range of *p*-nitrophenyl acetate concentrations in the presence of 0.5 mM of a) ADP, b) ADPR, and c) NMN. These plots were used to determine kinetic constants. Enzyme concentration was 50 $\mu\text{g/mL}$, all assays at 25°C, pH 7.5.

Figure 53b

Figure 53c

TABLE XXIV
Effect of Nucleotides on Esterase Kinetic Parameters

Nucleotide	$V_{\max}$ ($\mu\text{M}/\text{min}$)	K_m (mM)	$V_{\max}/E_t$ (s^{-1})
None	1.14	3.24×10^{-2}	105
ADP	1.11	3.02×10^{-2}	102
ADPR	1.11	2.92×10^{-2}	102
NMN	1.21	2.89×10^{-2}	112
NAD^+	1.27	2.44×10^{-2}	117

NOTE: $V_{\max}$, K_m and $V_{\max}/[E_t]$ refer to maximum velocity, Michaelis constant for p-nitrophenyl acetate and turnover number respectively. The concentrations used were ADP (0.5 mM), ADPR (0.5 mM), NMN (0.5 mM), NAD^+ (0.32 mM). The average errors determined by regression analysis of duplicate measurements are $V_{\max} \pm 4.6\%$, $K_m \pm 7.2\%$, $V_{\max}/E_t \pm 8.6\%$.

in K_m (117 s^{-1} and $2.44 \times 10^{-2} \text{ mM}$ respectively.) When esterase kinetics were performed in the presence of NMN a similar trend was observed as for NAD^+ . However, both the increase in turnover number and the decrease in Michaelis constant were not as large as for NAD^+ . Using ADP as the nucleotide effected a slight increase in Michaelis constant ($3.02 \times 10^{-2} \text{ mM}$) but the turnover number was decreased (102 s^{-1}) with respect to kinetics performed in the absence of nucleotide (105 s^{-1}). The Michaelis constant dropped even lower in the presence of ADPR ($2.92 \times 10^{-2} \text{ mM}$), however the turnover number remained depressed as for ADP.

3.3. Effect of Substrate Inductive Substitution on Esterase Reaction.

Three differently substituted phenyl octanoate esters were employed to investigate the effects of electron withdrawing groups within the substrate on the rate of reaction. *p*-Nitrophenyl octanoate was used as a substrate in which the substituent bound to the aromatic ring acts to withdraw electron density from the ring. Conversely, *p*-methylphenyl octanoate represents a substrate in which electron density is donated into the ring. Phenyl octanoate is a substrate used as an intermediate between the two above. Esterolysis by the enzyme is assumed to be via nucleophilic attack of the ester by an appropriate species in the esterase active site (96). The relative rates of hydrolyses also yield information about the order of products being released in

the esterase reaction. This is discussed in detail in the next chapter.

Esterase kinetics were performed by determination of initial velocities over a range of concentrations of the three substituted phenyl octanoate esters. Double reciprocal plots of activities plotted against substrate concentrations are found in Figure 54 (a) *p*-nitrophenyl octanoate, (b) phenyl octanoate and (c) *p*-methylphenyl octanoate esters respectively. Kinetic constants obtained from the plots are listed in Table XXV. The Michaelis constants for all three substrates proved to be almost identical. The values of K_m were 3.54×10^{-2} , 3.73×10^{-2} and 3.01×10^{-2} for *p*-nitrophenyl, phenyl and *p*-methylphenyl octanoate esters respectively. The V_{max} and turnover numbers however exhibited dramatic variations with the three substrates. The substrate which had an electron withdrawing substituent, *p*-nitrophenyl octanoate, had an appreciable 24 fold higher turnover number than phenyl octanoate. The nitro substrate had a dramatic 153 fold increase in turnover number with respect to *p*-methylphenyl octanoate, the substrate in which the *para* substituted species acts to donate electrons to the aromatic ring. The effects on esterase activity by the substrates is likely the result of the relative stability of the substituted phenoxalate produced during esterolysis. This is discussed in the next chapter.

Figure 54a, b & c Double Reciprocal Plots For The Effect of Aromatic Substitution of Substrate Esters on Esterase Activity. Initial rates were determined over a range of substrate concentrations. The substrates employed in each figure were a) p-nitrophenyl octanoate, b) phenyl octanoate and c) p-methyl phenyl octanoate. Enzyme concentration was 5.0 $\mu\text{g/mL}$, all assays at 25°C, pH 7.5.

Figure 54 b

Figure 54c

TABLE XXV
Effect of Aromatic Substitution of Substrate Esters on
Esterase Kinetics, pH 7.5

Substrate	$V_{\max}$ ($\mu\text{M}/\text{min}$)	K_m (mM)	$V_{\max}/E_t$ (s^{-1})
$\text{CH}_3(\text{CH}_2)_6-\overset{\text{O}}{\parallel}{\text{C}}-\text{O}-$ <div style="display: inline-block; vertical-align: middle;"> $\left[\begin{array}{c} \text{---} \text{C}_6\text{H}_4 \text{---} \text{NO}_2 \\ \text{---} \text{C}_6\text{H}_4 \text{---} \text{H} \\ \text{---} \text{C}_6\text{H}_4 \text{---} \text{CH}_3 \end{array} \right]$ </div>	.1.29	3.54×10^{-2}	119
	3.57×10^{-2}	3.73×10^{-2}	5.05
	8.44×10^{-3}	3.0×10^{-2}	0.78

NOTE: $V_{\max}$, K_m , and $V_{\max}/E_t$ are maximum velocity, Michaelis constant for substrate and turnover number respectively. Errors were determined by regression analysis of duplicate measurements and found to be $V_{\max} \pm 9.5\%$, $K_m \pm 11.7\%$, $V_{\max}/E_t \pm 13.5\%$.

4. CHEMICAL MODIFICATIONS

4.1.1. General

Chemical modifications of specific amino acids within an enzyme can provide insight into the mechanism. This is accomplished by measurement of enzyme activity during modification of specific residues and observation of the susceptibility of the activity to the specific modification. Theoretically, if one chemically modifies those residues essential to enzymatic activity, a concomitant eradication of activity is likely. Monitoring enzyme activity during a series of chemical modifications performed on specific amino acids can be useful in identifying these essential residues. In practice however, this technique often suffers from a lack of specificity of the reagents towards a specific amino acid. The result of an inherent lack of specificity of certain agents is that often a clear determination of the essential residues cannot be made. It is useful to attack a given residue with several different modifying agents to provide supportive evidence.

A number of chemical modifications have been performed on aldehyde dehydrogenase isolated from rat liver mitochondria. Both dehydrogenase and esterase activities were monitored throughout the modifications in an effort to identify catalytically essential amino acids of each activity. In addition, amino acid content was monitored during the course of the reaction, for several of the modifications, in order to correlate the catalytic effects with the change in number

of specific residues. A conceptual picture of dehydrogenase and esterase active sites will be developed using evidence presented here and from other studies.

The chemical modifications were designed to measure the relative effects of the modifications on the three enzymatic activities. Conditions were chosen which effected the predominate changes within a time span of up to 1 hour. For each modification, the activity curve is a composite of the measured activity minus a control curve. The control curve follows enzyme activity of free, unmodified enzyme subjected to identical conditions of pH and temperature over an identical time span as the modified enzyme curve. The specific modifications are described in each section. Table XXVI provides a summary of the results of chemical modifications with respect to dehydrogenase and esterase activities.

4.1.ii. Carboxymethylation

Cysteine residues are particularly sensitive to carboxymethylation by haloacetates as alkylating agents. The nucleophilic displacement of iodine by an enzymatic sulfhydryl species is shown below:

TABLE XXVI
Summary of Effects of Chemical Modifications on the Activities of
Aldehyde Dehydrogenase

Residue	Reagent	pH	Reaction Time (min)		% Activity of Control		
			NAD ⁺	NADP ⁺	NAD ⁺	NADP ⁺	ESase
Tyrosine	TNM	8.0	21	---	0	---	50
Arginine	Phenyl Glyoxal	8.0	37	2	66	0	35
Tryptophan	NBS	6.5	7	---	0	---	60
Cysteine	Iodoacetic Acid	8.0	15	15	43	90	100
	DTNB	7.5	70	70	0	0	100
	Iodoacetamide	8.0	13	13	0	0	100
Histidine	Rose Bengal, hv	8.0	35	35	0	0	0
Lysine	Methyl- acetamidinate	7.5	18	18	100	100	0
	EDC, NH ₄ Cl ⁺	7.0	60	60	118	175	---

---- not determined

Modification of aldehyde dehydrogenase with iodoacetic acid resulted in a 57% decrease in NAD^+ dependent dehydrogenase activity after 10 minutes of reaction (Figure 55). Curiously, only a 10% decrease in NADP^+ dependent activity was observed over the same time span. The esterase activity was completely unaffected. The different sensitivities of the NAD^+ and NADP^+ dependent activities to the alkylating agent was thought to be possibly linked to a charge effect, possibly a repulsive interaction between an active site species and the anionic alkylating agent. Such a repulsive interaction could prevent the reaction from taking place to the same extent as when no repulsive interaction was in effect. In order to test this hypothesis, the modification was repeated under identical conditions and concentrations of reagents, however, the iodoacetic acid was replaced with iodoacetamide. This reagent performs a similar carboxymethylating function but has an amide functionality in place of the acidic moiety. It would be expected that this substitution of reagents would relieve the proposed repulsive interactive effects described above. After 13 minutes of reaction with iodoacetamide, both NAD^+ and NADP^+ dependent dehydrogenase activities were completely eliminated. Replacement of the acidic reagent with iodoacetamide appeared to overcome the repulsive effect as hypothesized (Figure 56). Again the esterase activity was unaffected by cysteine modification. A correlation of loss of the dehydrogenase activity with modification of cysteine residues is discussed in the next section. That the dehydrogenase activity was

Figure 55 Carboxymethylation By Iodoacetic Acid. The activities of aldehyde dehydrogenase were monitored during the course of reaction with iodoacetic acid and expressed as % activity relative to the unmodified enzyme. The activities measured are: NAD⁺ (○) and NADP⁺ (●) dependent dehydrogenase and esterase (■). Enzyme concentration was 7.2 mq/mL, iodoacetic acid concentration was 3.9 mM. Reaction was at 0°C, pH 8.0.

Figure 56a Cysteine Modification By Iodoacetamide. The activities of aldehyde dehydrogenase were monitored during the course of reaction with iodoacetamide and expressed as % activity relative to the unmodified enzyme. The activities measured are: NAD⁺ (○), NADP⁺ (●), dependent dehydrogenase and esterase (■). Enzyme concentration was 7.2 mg/mL, iodoacetamide concentration was 3.9 mM. Reaction was carried out at 0-10°C, pH 8.0.

Figure 56b Carboxymethylation of Cysteine Residues. Aldehyde dehydrogenase (7.2 mg/mL) was carboxymethylated with iodoacetamide (3.9 mM) at 0-10°C, pH 8.0. Samples were removed at time intervals, desalted through a P6 column and subjected to amino acid analysis.

dependent on a catalytically active cysteine(s) was further tested by modification with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB-Ellmans Reagent). This reagent is very specific for cysteine residues and subjects the sulfhydryl residues to the following modification:

The enzyme was modified with DTNB and NAD^+ dependent dehydrogenase and esterase activities were measured throughout the reaction. Although the rates of inactivation by DTNB differ from those of the haloacetates, the relative effects of the modifications are the same (Figure 57). After 70 minutes of reaction the dehydrogenase activities were totally inactivated. The esterase activity remained completely active and independent of any effects of cysteine modification.

4.i.iii. Cysteine Modification With NAD^+ Protection.

The iodoacetamide modification was repeated with enzyme in the presence of 500 fold excess NAD^+ added prior to reaction. As can be seen in Figure 58, the NAD^+ serves to protect the enzyme from the inactivation effects observed in the absence of cofactor. After 20 minutes of reaction 94%

Figure 57

Cysteine Modification By DTNB. The activities of aldehyde dehydrogenase were monitored during the course of reaction with DTNB and expressed as % activity relative to the unmodified enzyme. The activities measured are: NAD⁺ dependent dehydrogenase (●) and esterase (■). Enzyme concentration was 1.47 mg/mL, DTNB concentration was 0.90 mM. Reaction was carried out at 0-1°C, pH 7.5.

Figure 58

Cysteine Modification With NAD^+ Protection By Iodoacetamide. The activity of NAD^+ dependent aldehyde dehydrogenase was measured during the course of reaction with iodoacetamide in the presence of 2.0 mM NAD^+ . The change in activity is expressed as % activity relative to the unmodified enzyme (O). The NAD^+ activity during modification in the absence of NAD^+ is also shown (●). Enzyme concentration was 7.2 mg/mL , iodoacetamide concentration was 3.9 mM , NAD^+ concentration was 1.33 mg/mL . Reaction was carried out at $0-10^\circ\text{C}$, pH 8.0.

of the NAD^+ dependent dehydrogenase activity remained whereas the enzyme modified in the absence of NAD^+ was inactivated by 13 minutes (Figure 56). An active site cysteine residue(s) likely exists which is (are) protected from the inactivation effects of iodoacetamide when NAD^+ is bound to the active site.

4.1.4. iv. Dye-Sensitized Photooxidation

The imidazole group of histidine has been shown to be particularly sensitive to modification in the presence of a sensitizing dye (138, 172-177). This modification is useful in assessing the necessity of histidine for enzyme function (172).

Singlet oxygen is believed to be the species responsible for the photooxidation of proteins (174-177). Rose bengal was the dye-sensitizer chosen for its particular sensitivity to histidine species (138). It has the following structure:

Singlet oxygen is produced on absorption of light by the dye. This reacts with the substrate (Sub) in question to produce the oxidized product described in Scheme 3:

Scheme 3. Photooxidation by sensitizing dye.

When histidine is the substrate the oxidized products are aspartic acid and urea (176). These products are used to quantitate the residues modified in Sections 4.2.

The NAD^+ and NADP^+ dehydrogenase activities were found to be very sensitive to photooxidation. The inactivation observed during the modification is shown in Figure 59a. The NADP^+ activity was completely lost by 35 minutes whereas the NAD^+ activity was eliminated by 40 minutes. The esterase activity followed an identical inactivation profile as the dehydrogenase activities, suggesting a similar dependency on photooxidizable residues. The involvement of histidine residues in the photooxidation response is discussed below.

4.1.v. Acetamidination

The possible role of lysine residues in the active site of aldehyde dehydrogenase was investigated by selective modification of this species. The lysine residues were modified with methyl acetamidinate and the enzyme activities were monitored throughout the modification to illustrate the activity dependency on lysine residues.

Imido esters such as methyl acetamidinate react specifically with amine groups to form amidine (imido-amine) derivatives (134). Both α - and ϵ -amino groups are modified

Figure 59 a Dye-Sensitized Photooxidation With Rose Bengal. The dehydrogenase and esterase activities were monitored during the reaction of aldehyde dehydrogenase with rose bengal. The activities are expressed as % activity relative to the unmodified enzyme. The activities are NAD⁺ (●), dehydrogenase (○) and esterase (■) as indicated. Enzyme concentration was 1.45 mg/mL, rose bengal concentration was 0.77 mM. Reaction was carried out at 0-10°C, pH 7.5.

Figure 59b Oxidative Destruction of Histidine Residues During Rose Bengal-Sensitized Photooxidation of Aldehyde Dehydrogenase. Aldehyde dehydrogenase (1.45 mg/mL) was photooxidized with rose bengal (0.77 mM) at pH 8.0, 0-1°C. Samples were removed at time intervals, dye was removed with Cellex-T and subjected to amino acid analysis.

with the products being protonated at physiological pH. The α -product is acid labile but the ϵ -product is stable and can therefore be quantitated by amino acid analysis (134). This will be dealt with later. The reaction proceeds according to the following mechanism:

The sensitivities of dehydrogenase and esterase activities to lysine modifications are shown in Figure 60. Both NAD^+ and NADP^+ dehydrogenase activities are completely unaffected by the modification. The esterase activity exhibits a dramatic sensitivity to lysine modification. Esterase activity is entirely lost after about 45 seconds of reaction. A dependency of esterase activity on active site lysine residue(s) is suggested. Dehydrogenase activity appears to have no lysine requirement. A correlation between esterase inactivation on methylacetamidination with loss of lysine residues (as determined by amino acid analysis) is evident and is discussed below.

Figure 60 Acetamidination of Aldehyde Dehydrogenase. Both NAD^+ ($\bullet$) and NADP^+ ($\circ$) dependent dehydrogenase and esterase ($\blacksquare$) activities were monitored during the reaction of the enzyme with methyl-acetamidinate. The activities are expressed as % relative to the unmodified enzyme. Enzyme concentration was 8.33 mg/mL, methyl acetamidinate concentration was 3.9 mg/mL. Reaction was carried out at 0-10°C, pH 7.5.

4.1.vi. Phenyl Glyoxalation.

Arginine residues have been shown to be essential for the binding of coenzymes through the pyrophosphate moiety. Specific arginine-pyrophosphate interactions have been identified for LDH and LADH (64). NADP^+ dependent dehydrogenase activity is absent from LADH and GAPDH because of an identified repulsive interaction between the 2'-phosphate and acidic species in the active site (178, 179). However the NADP^+ activity present in dihydrofolate reductase is attributed to the attractive interaction between an arginine and the 2'-phosphate of the cofactor (66). The presence of such a residue in aldehyde dehydrogenase was investigated based on the appreciable NADP^+ activity observed.

The reaction of arginine residues is relatively specific with α -dicarbonyls (136). The α -dicarbonyls react with the guanidino functionality of the arginine residue as described by the following mechanism:

Phenyl glyoxal was employed as the α -dicarbonyl reagent (179, 180) in an effort to identify arginine residue(s) at the nucleotide binding site of aldehyde dehydrogenase.

All activities of aldehyde dehydrogenase were monitored during the phenyl glyoxalation reaction. As can be seen in Figure 61a, all activities were rapidly affected. The NADP⁺ dependent dehydrogenase activity is eradicated after 2 minutes of reaction. However, both the esterase and NAD⁺ dependent dehydrogenase activities retain appreciable activities. Sixty-five percent of the NAD⁺ and 37% of the esterase activities remain after thirty-five minutes of reaction. It would seem apparent that active site arginine(s) are required for all activities. Since NAD⁺ and esterase activities are affected to nearly the same extent, the two activities may share the arginine(s) in the same or juxtaposed active sites. An active site arginine might also explain the existence of NADP⁺ dehydrogenase activity. Loss of the arginine causes the enzyme to retain only NAD⁺ dependent dehydrogenase activity. This may imply the existence of possible acidic residues in the active site. This possibility will be investigated further below. Correlation of the inactivation of activities on phenyl glyoxalation of AldDH with loss of arginines (as determined by amino acid analysis) is discussed below.

Figure 61 a Phenyl Glyoxalation of Aldehyde Dehydrogenase. Phenyl glyoxal was used to modify aldehyde dehydrogenase and NAD⁺ (●), NADP⁺ (○) dependent dehydrogenase and esterase (■) activities were monitored throughout the reaction. The dehydrogenase activities were also monitored during the reaction of enzyme with phenyl glyoxal in which the active site is protected with NAD⁺ and NADP⁺ (separate experiments). Protection experiments (where indicated) gave identical results with either cofactor. Enzyme concentration was 9.04 mg/mL, phenyl glyoxal concentration was 1.1 mg/mL. Reaction was carried out at 0-10°C, pH 8.0. For protection experiments, 2.0 μmol/mL of NAD(P)⁺ was used.

4.1.vii Phenyl Glyoxalation With Cofactor Protection

To help confirm further the presence of an arginine(s) species in the active site of aldehyde dehydrogenase the modification was repeated in the presence of cofactors. As can be seen in Figure 61a, both NAD^+ and NADP^+ dehydrogenase activities are fully retained when the modification is performed in the presence of 2.0 mM NAD^+ or 2.0 mM NADP^+ . Esterase activity is appreciably protected during arginine modification in the presence of NAD^+ . Only 18% of the esterase activity is lost during modification in the presence of NAD^+ whereas 52% is lost when NADP^+ is present (Figure 61b). This is still a higher retention of activity than when no cofactors are present (63% activity lost). The study furnishes more evidence supporting the existence of an active site arginine species.

4.1.viii. Amidation

The observation in the previous section that loss of an active site arginine residue(s) resulted in a dramatic loss of NADP^+ activity prompted further investigation of this effect. As was briefly mentioned above, several enzymes which exhibit absolute NAD^+ specificity have been compared. Specific enzyme-coenzyme interactions which give rise to the NAD^+ specificity were compared for lactate dehydrogenase and horse liver alcohol dehydrogenase by Dalziel (182). A comparison of these two enzymes with glyceraldehyde-3-phosphate dehydrogenase is given by Branden et al. (183).

Figure 61b Phenyl Glyoxalation of Aldehyde Dehydrogenase-Esterase Activity. The esterase activity was monitored during the reaction of the enzyme with phenyl glyoxal in the presence of NAD^+ and NADP^+ as active site protectors. The NADP^+ protected enzyme (■) retains a higher activity than the unprotected enzyme (●). The NAD^+ protected enzyme (□) retains the highest activity. The activities are expressed as % activity relative to unmodified enzyme. Enzyme concentration was 9.04 mg/mL, phenyl glyoxal concentration was 1.1 mg/mL. Reaction was carried out at 0-10°C, pH 8.0. For protection experiments, 2.0 $\mu\text{mol/mL}$ of NAD(P)^+ was used.

Figure 6lc Phenyl Glyoxalation of Arginine Residues of Aldehyde Dehydrogenase. Aldehyde Dehydrogenase (9.04 mg/mL) modified with phenyl glyoxal (1.1 mg/mL) at pH 8.0, 0-1°C. Samples were removed and desalted through a P6 column and then subjected to amino acid analysis.

Present in each case is the hydrogen bond interaction of the 2'-hydroxyl of the adenosine-ribose with an aspartic acid residue in the active site of each enzyme. It has been suggested by Rossmann et al. (65) that the extra phosphate group of NADP^+ will cause both a steric and charge repulsion with this aspartate residue thereby weakening the binding of this coenzyme considerably. It was therefore of interest to attempt to modify acidic residues and observe any changes in the relative specificities for NAD^+ and NADP^+ . The loss in NADP^+ dependent activity on modification of arginine residues might be construed as the removal of an attractive interaction. If this attractive effect has been removed, it may allow other active site species to exert their own effects. In particular, if an active site acidic residue exists, as has been identified as a common dehydrogenase property above, the potential for repulsive effects by this species with the 2'-phosphate of NADP^+ may now become realized.

Acidic residues can be converted into amides using a water soluble carbodiimide and an amine in a two step reaction described by the following mechanism:

The reaction was performed in the presence of *N*-ethyl, *N'*-(3-dimethylaminopropyl)-carbodiimide HCl (EDC) and ammonium chloride. The NAD^+ and NADP^+ dependent dehydrogenase activities were monitored throughout the course of the reaction (Figure 62). The activities plotted were expressed as percent activity with respect to the NAD^+ activity. Over the course of 66 minutes of reaction, the NAD^+ dependent activity observed only a slight increase as a result of the modification. However, the NADP^+ dependent activity increased dramatically and the activity observed for this cofactor became equal to that of NAD^+ after 66 minutes. Thus the distinction between the two cofactors made by the enzyme is lost when the acidic residue(s) are removed.

The same reaction was performed in the presence of NAD⁺ as an active site protector. As is indicated in Figure 62 inset, the NADP⁺ activity is unchanged. That is to say that

Figure 62 Amidation of Aldehyde Dehydrogenase. EDC and ammonium chloride were used to amidate aldehyde dehydrogenase. NAD⁺ and NADP⁺ dependent dehydrogenase activities were monitored during the reaction ((●) and (○) respectively). The activities were also followed during modification in the presence of NAD⁺ as an active site protector (inset). Activities are expressed as % activity relative to NAD⁺ dependent dehydrogenase activity. Enzyme concentration was 6.38 mg/mL, concentration of NH₄⁺Cl⁻ and EDC were 20 μmol/mL each. Reactions were carried out at 0-1°C, pH 7.0. For protection studies, 1.33 μg NAD⁺ was used.

when the active site acidic species are protected from amidation, the distinction between the two cofactors is retained. Possibly the repulsive effect of active site acidic residues is still present.

4.1.ix. NBS Oxidation

The function of tryptophan residues was probed by modification with *N*-bromosuccinimide (NBS). This reagent has been shown useful in the cleavage of tryptophanyl peptide bonds (138, 184). NBS provides a potent source of electrophilic bromonium ions (Br^+) which are added to the carbon-carbon double bonds of tryptophan resulting in a disruption of the peptide bonds as described below:

The reaction suffers from an inherent lack of specificity. In addition to tryptophan, tyrosine and histidine are also modified and cysteine residues are even faster to react.

Thus any changes in activity on modification by NBS cannot be solely attributed to reaction with tryptophan. The esterase and NAD^+ dependent dehydrogenase activities were monitored throughout the modification (Figure 63). Dehydrogenase activity is lost by seven minutes whereas the esterase activity still retains 67% of its initial activity after 26 minutes of reaction. Although the inactivation of the dehydrogenase activity cannot be entirely attributed to modification of tryptophan, it is unlikely the inactivation is due to cysteine modification. The esterase activity which is insensitive to cysteine modification shows a 33% decrease in activity. This may be due to tryptophan modification and/or also histidine and tyrosine residues as well. The high retention of esterase activity on NBS oxidation is highly suggestive of separate active sites.

4.1.x. Nitration

The function of tyrosine residues can be probed using tetranitromethane-(TNM) as a nitrating agent. The reagent is reasonably specific under mild conditions (141) with cysteine being the only other residue modified. The phenolic group is susceptible to attack by electrophilic reagents such as TNM. The reaction which is more rapid at elevated pH's (where the phenolic group is increasingly more ionized) is described below (185):

Figure 63 NBS Oxidation of Aldehyde Dehydrogenase. The enzyme was modified with NBS and the activities monitored during the course of reaction. The activities are expressed as % relative to the unmodified enzyme. The activities are NAD⁺ dependent dehydrogenase (●) and esterase (○) as shown. Enzyme concentration was 1.45 mg/mL, NBS concentration was 1.29 mg/mL. Reaction was carried out at 0-10°C, pH 8.0.

Both NAD^+ dependent dehydrogenase and esterase activities showed appreciable sensitivities to tyrosine modifications (Figure 64). The dehydrogenase activity was completely lost after 21 minutes of reaction, whereas the esterase activity observed a 50 percent decrease after 3 minutes of reaction which remained constant for the rest of the modification period.

Tyrosine has been shown to provide a hydrophobic linkage with the nicotinamide ring of NAD^+ in GAPDH (65) and has been shown to provide an electron sink function in a charge relay system in this enzyme (186). The dependency of this species on aldehyde dehydrogenase may imply a similar function.

4.2. Amino Acid Analyses of Modified Enzymes.

The chemical modifications were performed on aldehyde dehydrogenase and the effects of the individual modifications on the enzyme activities were observed. However, most of the modifications suffer from an inherent lack of specificity towards a target amino acid. Thus it is not correct to simply correlate the loss of a specific amino acid to the

Figure 64 Nitration of Aldehyde Dehydrogenase. The enzyme was modified with TNM and the NAD⁺ dependent dehydrogenase (●) and esterase (○) activities were monitored during the reaction. The activities are expressed as a percentage relative to the unmodified enzyme. Enzyme concentration was 1.45 mg/mL, TNM concentration was 0.002%. Reaction was carried out at 0-1°C, pH 8.0.

activity of an enzyme without confirming the success and specificity of the modification. Four amino acids which appear to be essential to the enzyme activities are cysteine, arginine and histidine and lysine. The enzyme was modified separately with respect to each residue as described previously. During the course of each modification samples of enzyme were removed, desalted and prepared for amino acid analysis. Thus a direct correlation between inactivation by a specific modification with the loss of a specific residue can be made.

As is indicated in Figure 56b, two cysteines were modified after 13 minutes of reaction with iodoacetamide. This also corresponds to the time required for inactivation of NAD^+ and NADP^+ dehydrogenase activities (Figure 56a). Similarly, four histidine residues were modified after 40 minutes of photooxidation with rose bengal (Figure 59b). This directly corresponds to the inactivation of dehydrogenase and esterase activities by the same time (Figure 59a). The loss of four histidine residues is corroborated in part, by the formation of three aspartic acid residues after the same time of reaction.

Amino acid analyses were also performed during the phenyl glyoxalation of aldehyde dehydrogenase (Figure 61c). After one minute of reaction a single arginine is modified. Concomitant with this is the loss of NADP^+ dependent dehydrogenase activity. The NAD^+ dehydrogenase and esterase

activities are also significantly affected by the loss of this residue but not entirely lost.

Amino acid analysis was also performed on methylacetamidinated enzyme after one minute of reaction. The amino acid results for all of the modifications are presented in Table XXVII. A single lysine residue is modified in the same time required to inactivate the esterase activity.

Thus amino acid analyses correlate the loss of activities to the modification of specific residues. A summary of the residues modified during the inactivation events is found in Table XXVIII. The enzyme requires two cysteines for dehydrogenase activity, one arginine for NADP⁺ dependent dehydrogenase, four histidines for both dehydrogenase and esterase activities and a lysine for the esterase activity.

4.3. Active Site Cysteine Determination.

Aldehyde dehydrogenase was modified using [¹⁴C]-iodoacetamide for the exact time required to eradicate dehydrogenase activity (13 minutes). The reaction was then stopped and the enzyme was prepared for radiolabel counting free of the hot reagent. The amount of radiolabel incorporated during the modification was determined and this was correlated with the amount of enzyme modified to yield a ratio of moles of cysteine modified per mole of monomeric enzyme. From this study, it was determined that one ($\pm 5\%$) cysteine residue per monomer of enzyme was required for dehydrogenase activity. This result confirms the determination for cys-

TABLE XXVII

Amino Acid Composition of Modified Aldehyde Dehydrogenase

Amino Acid	No. Residues/Mole Subunit Enzyme				
	Unmodified	^a Methylacetamidinated	^b Phenyl Glyoxalated	^c Photo-oxidized	^d Carboxymethylated
Asp	62	72	60	60	55
Glu	63	61	57	59	48
Ser	59	70	42	41	43
His	9	6	11	5	6
Gly	97	118	89	95	95
Thr	32	27	31	30	21
Arg	18	15	17	17	12
Ala	82	97	79	82	81
Tyr	11	9	12	9	8
Met	13	15	11	11	10
Val	23	21	55	57	21
Phe	17	16	20	19	16
Ile	15	12	25	34	11
Leu	42	38	53	53	37
Lys	31	30	15	17	-
*CMC	0	-	-	-	2

^aAverage of two preparations. Enzyme prepared for amino acid analysis after one minute of reaction.

^bPhenyl glyoxalated enzyme preparation prepared after 1 minute of reaction.

^cPhotooxidized enzyme prepared for amino acid analysis after 50 minutes of reaction.

^dCarboxymethylated enzyme prepared for amino acid analysis after 13 minutes of reaction.

*CMC - carboxymethylcysteine

TABLE XXVIII

Summary of Modification Effects of Aldehyde Dehydrogenase

Modified Residue	Residue/Monomer		Δ Residue Monomer	Activity Lost
	Native	Modified		
Arginine	18	17	1	a(p),b,c(p)
Histidine	9	5	4	a,b,c
Lysine	31	30	1	c
Cysteine (as CMC*)	0	2	2	a,b

a NAD^+ dependent dehydrogenase

b NADP^+ dependent dehydrogenase

c Esterase

Activities lost are complete unless specified as (p), partial loss of activity.

*CMC Carboxymethylcysteine

teine made above by amino acid analyses of carboxymethylated enzyme.

4.4.1. ^{31}P NMR. Influence of Enzyme on ^{31}P NMR Spectra of Reduced Coenzymes.

The ^{31}P NMR spectrum of free NAD^+ (5.0 mM) is shown in Figure 65a. The resonance at -9.12 ppm is attributed to the 5'-pyrophosphate moiety. The two phosphorus resonances are accidentally coincidental, as was observed by Feeney et al. (93). An expansion of this region (Figure 65b) shows the peak is broad and poorly resolved. A series of spectra were obtained of variable concentrations of NAD^+ (0.5-4.0 mM) in the presence of aldehyde dehydrogenase (4.2 mg/mL). Expansion plots of the 5'-pyrophosphate resonance over the concentration range (Figures 65c-f correspond to 0.5, 1.0, 2.0 and 4.0 mM NAD^+ respectively) reveal slight resolution of the individual phosphate resonances. It is clear that no chemical shift effects or broadening of the resonances has resulted when the spectra of NAD^+ were taken in the presence of aldehyde dehydrogenase.

A similar series of spectra were obtained for the coenzyme NADP^+ . A spectrum of 5.0 mM free NADP^+ (Figure 66a) reveals, in addition to the broad 5'-pyrophosphate resonance at -9.23 ppm, a 2'-phosphate singlet at 1.93 ppm. An expansion of the 5'-pyrophosphate region (Figure 66b) reveals a similar broad resonance as was observed for free NAD^+ . A spectrum taken of 0.5 mM NADP^+ in the presence of enzyme reveals a substantial downfield shift of the 2'-phos-

Figure 65

^{31}P NMR Spectra of NAD^+ in the Presence of Aldehyde Dehydrogenase. ^{31}P NMR Spectra of NAD^+ were obtained at 4°C as free cofactor and in the presence of purified enzyme. (a) Free NAD^+ (5.0 mM) full spectrum; (b), Expansion of 5'-pyrophosphate region (c) free, 5.0 mM NAD^+ ; (c-f), Expansion spectra of 5'-pyrophosphate region of NAD^+ in the presence of enzyme; (c), 0.5 mM NAD^+ ; (d), 1.0 mM NAD^+ ; (e), 2.0 mM NAD^+ ; (f), 4.0 mM NAD^+ . Enzyme concentration was 0.08 mM for all spectra. pD of all preparations were 7.5.

Figure 66

^{31}P NMR Spectra of NADP^+ in the Presence of Aldehyde Dehydrogenase. ^{31}P NMR Spectra were obtained at 4°C as free cofactor and in the presence of purified enzyme. (a), Full spectrum of 5.0 mM free NADP^+ ; (b), Expansion of 5'-pyrophosphate region of 5.0 mM free NADP^+ ; (c), Full spectrum of 0.5 mM NADP^+ in presence of enzyme; (d), Expansion of 5'-pyrophosphate region of (c); (e), Full spectrum of 1.0 mM NADP^+ in presence of enzyme; (f), Expansion of 5'-pyrophosphate region of (e); (g), Full spectrum of 2.0 mM NADP^+ in the presence of enzyme; (h), Expansion of 5'-pyrophosphate region of (g); (i), Full spectrum of 4.0 mM NADP^+ in presence of enzyme; (j), Expansion of 5'-pyrophosphate region of (i). Enzyme concentration was 0.08 mM for all spectra. pH of all preparations were 7.5.

4

MICRO

phate resonance to 2.65 ppm (Figure 66c). The expansion plot of the 5'-pyrophosphate peak (Figure 66d) indicates, as was the case for NAD^+ , no effect of the enzyme on the pyrophosphate resonance. An increase in the NADP^+ concentration to 1.0 mM effected a shift in the 2'-phosphate resonance back toward that of the free cofactor. Figure 66e reveals the 2'-phosphate chemical shift to be 2.15 ppm. The 5'-pyrophosphate species remains unaffected (Figure 66f). The 2'-phosphate resonance of 2.0 mM NADP^+ in the presence of aldehyde dehydrogenase continued a shift back towards that of the free cofactor. A chemical shift of 1.98 was observed for this resonance (Figure 66g). The expansion of the 5'-pyrophosphate resonance region (Figure 66h) again shows no indication of an enzyme-5'-pyrophosphate interaction.

A final decrease in the enzyme/coenzyme ratio again resulted in a 2'-phosphate shift towards the resonance obtained by the free coenzyme. A spectrum taken of 4.0 mM NADP^+ reveals a 2'-phosphate resonance of 1.96 ppm (Figure 66i) which is close to the value obtained for the free coenzyme (1.93). The expansion spectrum of the 5'-pyrophosphate resonance (Figure 66j) again indicates no significant effects were occurring.

The 2'-phosphate resonance therefore demonstrated an enzyme concentration dependence. A maximal downfield shift was observed at the highest enzyme/coenzyme ratio used. This decreased progressively with a decrease in the ratio. A single resonance observed over the concentration range

employed suggests a fast exchange between enzyme and cofactor (92). A plot was prepared of the observed 2'-phosphate shifts plotted against NADP^+ concentration (Figure 67). At high coenzyme concentration (lowest enzyme/coenzyme ratio), the 2'-phosphate shift is approximately equal to that of the free cofactor. As the concentration of NADP^+ is decreased (increase in enzyme/cofactor ratio) the plot appears to reach an asymptote, which suggests that a limiting concentration of cofactor which elicited the maximum 2'-phosphate shift was used in the experiment. Similar 2'-phosphate shifts were observed by Hyde et al. (92) for NADP^+ binding to dihydrofolate reductase and by Feeney et al. (93) for isocitrate dehydrogenase. The shift is attributed to the interaction of the dianionic form of the 2'-phosphate with cationic group(s) of the enzyme and/or resulting distortion of the O-P-O bond angle (93, 187).

The dependency of the 2'-phosphate chemical shift on enzyme concentration is essentially a titration curve and can be used to obtain the binding constant K_A of NADP^+ and the enzyme. In order to employ the equations outlined in the methods section, an estimate of δ_b (chemical shift of the completely bound cofactor) must be made. The asymptotic region shows an estimate of δ_b to be about 3 ppm. A more exact estimate was made by iteration of the titration curve and δ_b was found to be $\delta_b = 3.82 \text{ ppm} \pm 0.80 \text{ ppm}$. Using this value the binding constant was determined to be $K_A = 9.71 \times 10^2 \pm 2.03 \times 10^2 \text{ M}^{-1}$. This is about 10 fold lower than was

Figure 67 Effect of Aldehyde Dehydrogenase on 2'-Phosphate ³¹P NMR Resonance of NADP⁺. The 2'-phosphate ³¹P NMR resonances of NADP⁺ were measured in the presence of a fixed concentration of enzyme. The chemical shifts (in ppm) were plotted against NADP⁺ concentration to ascertain the concentration dependency of enzyme on the 2'-phosphate peak shifts. The asymptotic region suggests an appropriate cofactor/enzyme ratio was used to demonstrate the shift dependency. pD of all solutions were 7.5. Enzyme concentration was 0.08 mM for all spectra.

observed by Feeney et al. for the binding of NADP^+ to dihydrofolate reductase (93).

The striking similarities in the ^{31}P NMR spectra for dihydrofolate reductase and isocitrate dehydrogenase have permitted analogies to be drawn with respect to the active sites of these enzymes (91). Matthews et al. (66) have provided X-ray evidence for the interaction of the 2'-phosphate species with active site arginine residues.

In order to further probe specific residue-cofactor interactions, aldehyde dehydrogenase was subjected to several chemical modifications prior to ^{31}P NMR.

4.4.ii. Effect of Modified Enzyme on ^{31}P NMR Spectra of Reduced Coenzymes.

The ^{31}P NMR spectrum of 1.0 mM NADP^+ was repeated at 20°C and compared to that obtained for the same concentration of NADP^+ in the presence of 10 mg aldehyde dehydrogenase (Figures 68a & b respectively). In contrast to the spectra previously obtained, both 2'-phosphate and 5'-pyrophosphate resonances are shifted downfield at the higher temperature. The 2'-phosphate signal shift is dramatically more significant than the latter at the higher temperature. The 2'-phosphate signal for NADP^+ containing native enzyme has a chemical shift of 5.60 ppm whereas the free cofactor observes a 2.29 ppm signal. The expansion plots of the 5'-pyrophosphate region of free NADP^+ (Figure 68c) and NADP^+ in the presence of enzyme (Figure 68d) indicate no chemical

Figure 68

Effect of Chemically Modified Aldehyde Dehydrogenase on ^{31}P NMR Spectra of NADP^+ . ^{31}P NMR Spectra of NADP^+ were obtained at 20°C as free NADP^+ and in the presence of enzyme. (a) Full spectrum of 1.0 mM free NADP^+ ; (b), Full spectrum of 1.0 mM NADP^+ in presence of native enzyme; (c), Expansion spectrum of 5'-pyrophosphate of 1.0 mM free NADP^+ ; (d), Expansion spectrum of 5'-pyrophosphate region of 1.0 mM NADP^+ in presence of enzyme; (e), Full spectrum of 1.0 mM NADP^+ in presence of phenyl glyoxalated enzyme; (f) Expansion spectrum of 5'-pyrophosphate region of 1.0 mM NADP^+ in presence of phenyl glyoxalated enzyme; (g), Full spectrum of 1.0 mM NADP^+ in presence of amidated enzyme; (h), Expansion spectrum of 5'-pyrophosphate region of 1.0 mM NADP^+ in presence of amidated enzyme. Enzyme concentration was 0.2 mM for all spectra. pD of all preparations was 7.5.

Figure 68

shift or broadening effects as was observed in the previous study (4.4.i.).

A spectrum was obtained for NADP^+ in the presence of phenyl glyoxalated enzyme (Figure 68e). On modification of arginine residues, the 2'-phosphate signal shifts upfield to 2.79 ppm, much less than was observed for the native enzyme. No effects were observed for the 5'-pyrophosphate peak of free NADP^+ as is evident in the expansion plot (Figure 68f).

A spectrum was also obtained of NADP^+ in the presence of amidated enzyme (Figure 68g). The 2'-phosphate signal was not affected as was observed with the phenyl glyoxalated enzyme. In fact, the 2'-phosphate signal was shifted slightly downfield ($\delta_{\text{ppm}} = 5.96$) in comparison to the free cofactor. Again no changes in the 5'-pyrophosphate signal was observed, as can be seen in the expansion of this resonance (Figure 68h).

Similar spectra were obtained for NAD^+ in the presence of modified enzyme at 25°C. Free NAD^+ exhibited two 5'-pyrophosphate peaks centered at -8.82 ppm (Figure 69a) which are downfield shifted with respect to free cofactor at 4°C. The spectrum of NADP^+ obtained in the presence of native enzyme (Figure 69b) reveals no shift or broadening effects. Similar 5'-pyrophosphate resonances were observed for the spectrum of NAD^+ in the presence of amidated enzyme (Figure 69c). The 5'-pyrophosphate resonance is also unaffected in the presence of phenyl glyoxalated enzyme (Figure 69d).

Figure 69 a-d

Effect of Chemically Modified Aldehyde Dehydrogenase on ^{31}P NMR Spectra of NAD^+ . ^{31}P NMR spectra of NAD^+ were obtained at 20° as free NADP^+ and in the presence of enzyme. All spectra shown here are expansions of the 5'-pyrophosphate region. (a), Free, 1.0 mM NAD^+ ; (b) NAD^+ (1.0 mM) in presence of native enzyme; (c) NAD^+ (1.0 mM) in presence of amidated enzyme; (d) NAD^+ (1.0 mM) in presence of phenyl glyoxalated enzyme. Enzyme concentration was 0.2 mM for all spectra. pD of all preparations was 7.5.

Thus, active site arginine residue(s) are implicated in the binding of NADP^+ to the enzyme. The 2'-phosphate peak is shifted to the value obtained for the free coenzyme when the coenzyme spectrum is obtained in the presence of phenyl glyoxalated enzyme. When the arginine residue is removed by modification, the 2'-phosphate species no longer see the effects of the enzyme and yields a spectrum similar to that obtained for the free coenzyme in the absence of enzyme.

The presence of acidic residues appear to play a small role in the binding of the NADP^+ . The small downfield shift of the 2'-phosphate peak on modification of the acidic residues does suggest some interaction between the coenzyme and an active site acidic residue(s).

The general observation was made that the 5'-pyrophosphate resonances were unaffected by the presence of enzyme. This does not preclude the existence of possible interactions but rather simply reflects the sensitivity of the spectra to the conditions employed. Hyde et al. (92) were able to detect pyrophosphate interactions with dihydrofolate reductase as well as similar 2'-phosphate effects as we have observed.

4.5. Peptide Studies

Native and chemically modified aldehyde dehydrogenases were subjected to peptide digestion and electrophoresis in an attempt to identify an active site containing peptide. The modifications which were performed were done according

to the previously described procedures. However the reagents used were [^{14}C] radiolabelled. The modifications performed were targeted at cysteine, arginine and acidic residues, based on the significance of these residues revealed from modification-activity studies and the availability of [^{14}C]-reagents. The enzyme was separately modified with each labelled reagent and then cleaved into peptides with chymotrypsin. The peptides were then separated by electrophoresis, visualized by silver staining and scanned with a densitometer in order to quantitate each of the resolved peaks. An attempt to correlate the amount of incorporated radiolabel with the amount of protein in each peptide was made by scintillation counting of the individual peptide bands cut from the gel. The reaction times were chosen, based on the modification-activity profiles, such that the reaction time was sufficiently long only to elicit the observed change in activity. Thus over-incorporation of radiolabel into the entire protein could be avoided. Incorporation could be limited to active site regions.

The peptide digest of native aldehyde dehydrogenase is seen in Figure 70a. A large number of peptide bands are visualized and the poor resolution of some of these precluded any attempts at quantitation. Unfortunately the scans for the modified enzymes suffered from the same lack of resolution. Thus a quantitative comparison of the active site amino acids in question was not possible. However a qualitative comparison of the peptides was possible. As can

Figure 70 a-d

Densitometer Scans of Chymotrypsin Digested Aldehyde Dehydrogenase. Enzyme was digested with chymotrypsin and subjected to electrophoresis. The silver stained gels were scanned with a Beckman DU-8 densitometer to obtain the tracings. The bands were removed and radiolabels were quantitated by scintillation counting. Radiolabel peaks are drawn emanating from the abscissae. The scans correspond to native, carboxymethylated, amidated and phenyl glyoxalated enzymes (a-d) respectively.

be seen in Figures 70a-d (corresponding to native, carboxymethylated, amidated and phenyl glyoxalated enzymes respectively) the densitometric tracings are very reproducible with respect to number and location of peptide band peaks.

Superimposed on each scan are blackened peaks revealing the relative radioactivity counted over the length of the scan. The scan of carboxymethylated enzyme reveals a band at R_f 0.55 which contains a substantial quantity of radiolabel (Figure 70b). The same peak is also identified in the amidated tracing as having a proportionately large amount of radiolabel. It would therefore seem apparent that this peptide contains active site cysteine and acidic species since the modifications were stopped after inactivation of the enzyme was observed. Unfortunately the bulk of the radiolabel was incorporated into a different peak in the phenyl glyoxalated preparation (Figure 70d). This would imply that an arginine(s) species required for enzyme activity is not located in the same peptide. This certainly does not preclude the possibility that the residue(s) does not exist in the active site.

It is a poor assumption that modification would be limited to active site regions if the reaction times were limited to the time required to manifest the activity effect. However, the peptide-radiolabel profiles revealed significant specificity in the attack of the enzymes. This specificity has already been demonstrated for carboxymethylation and phenyl glyoxalation reactions. Both the amidated

and phenyl glyoxalated enzymes indicate the target residue(s) was located in a single peptide. The carboxymethylated enzyme exhibits likewise relative specificity of the reagents towards the target residue within the allotted reaction time.

The peptide studies therefore reveal that cysteine and /acidic residue(s) required for enzyme activity are located within the same region of the polypeptide chain. The arginine residue required for dehydrogenase activity is not within the same relative region of the polypeptide chain (this may depend on the cleavage sites of chymotrypsin as well) and the residue(s) must therefore exert its catalytic presence through the ternary and/or quaternary properties of the enzyme (i.e. folding).

The possibility of cross-linking of acidic residues during the amidation reaction is not likely. The peptide digest of the amidated enzyme reveals good reproducibility of band patterns with the other digests. Also the 1000 fold excess of amidating reagent (methyl amine) would significantly reduce the possibility of a cross-linking reaction.

4.6. Reduced Cofactor Binding

UV spectra were taken by difference spectroscopy of the reduced cofactors NADH and NADPH as free coenzymes and in the presence of aldehyde dehydrogenase. The spectrum obtained for free NADH and in the presence of enzyme were identical. No shifts in the location of the peak maxima (340

nm) were observed (Figure 71). Unlike the previous spectra, there was a hypochromic shift observed for NADPH in the presence of enzyme. Figure 72 reveals the peak maximum to be located at 340 nm for the free cofactor (Scan b) and at 336 nm for NADPH in the presence of enzyme (Scan a). The uv scan information above only observes a spectral change with NADPH. If such a shift corresponds to either a coenzyme or coenzyme-enzyme conformational change then a possible explanation for a lower V_{max} using $NADP^+$ than for NAD^+ as cofactor may be found.

5. PRE-STEADY STATE KINETIC STUDIES

Stopped-flow studies were performed on aldehyde dehydrogenase by monitoring the production of reduced cofactor from variable concentrations of acetaldehyde and oxidized cofactor. The most predominant observation was a burst of reduced cofactor release prior to the establishment of the steady state.

The rate constant (k_2) was determined for NAD^+ and $NADP^+$ conversion to the corresponding reduced cofactors. The reader was previously directed to Appendix II for an explanation of the mathematical method of the determination of k_2 from the kinetic data. The method is based on the following Rapid Random Bi Bi mechanism which is illustrated in Scheme 4:

Figure 71

NADH-Enzyme Interactions By Difference Spectroscopy. An UV spectrum was obtained of free NADH by placement of NADH and enzyme in separate compartments of the cell. The spectrum of NADH in the presence of enzyme was taken by addition of an equivalent concentration of NADH to the enzyme compartment. The cells were referenced against an enzyme blank adjusted for dilution effects. Both the free and NADH-enzyme scan gave identical spectra as shown above. Enzyme concentration was 7.48 mg/mL, NADH concentration was 6.99×10^{-2} mM. Spectra were taken at 25°C, pH 7.5.

Figure 72

NADPH-Enzyme Interactions By Difference Spectroscopy. An UV spectrum was obtained of free NADPH by placement of NADPH and enzyme in separate compartments of the cell (scan b). The scan of NADPH-enzyme was taken by adding an equivalent concentration of NADPH to the enzyme compartment (Scan a). Both scans were referenced against an enzyme blank adjusted for dilution affects where necessary. Enzyme concentration was 7.48 mg/mL, NADPH concentration was 9.35×10^{-2} mM. Spectra were taken at 25°C, pH 7.5.

Scheme 4: General mechanism for aldehyde dehydrogenases (shown above for NAD^+ activity)

In order for a pre-steady state burst of product, the relative sizes of k_2 and k_3 must be such that $k_2 > k_3$. When this is true, an excess of the products of the k_2 reaction accumulate at the rate given by k_2 until all the enzyme has been converted to ENADH . This species then degrades at the steady state rate (k_3). Thus k_3 is the rate constant determined from steady state kinetic studies and is rate limiting. The k_3 is called the turnover number and the values for k_3 for NADH and NADPH formation are tabulated in Table VIII. The observed burst of reduced cofactor corresponds to the production of ENAD(P)H . This species is the enzyme- NAD(P)H binary complex (188). The release of reduced cofactor from the enzyme must therefore be a slower process. This is not unexpected on consideration of the micromolar Michaelis constants obtained from product inhibition studies.

A curious observation obtained from steady state kinetics was that K_{12} for NADP^+ was smaller than for NAD^+ (4.52 and 10.30 mM respectively). Since $K_{12} = k_{-1}/k_1$ then the smaller K_{12} for NADP^+ implies faster binding to the en-

zyme. This would suggest that the use of NADP^+ could impart a faster catalytic rate. Interestingly, the burst of reduced cofactor production (given by k_2) for NADP^+ was also greater than for NAD^+ . These anomalies appear to contradict steady state studies where NAD^+ is the preferred cofactor.

First order rate constants were obtained from the stopped-flow spectrometer using a range of NAD^+ concentrations at a fixed concentration acetaldehyde. The assays were repeated over acetaldehyde concentrations ranging from 0.35 to 2.0 mM. A representative assay obtained from the stopped-flow device is shown in Figure 73a. Superimposed on the plot is the fitted line generated by the program and used to estimate k_2 . Also included is a control trace (Figure 73b) in which the assay was run in the absence of coenzyme. The control shows the initial decrease in optical density on dilution of the enzyme which levels to a constant value after about 0.05 seconds. The rate constants were plotted against NAD^+ concentration (Figure 74a). The rate constant k_2 was determined from a plot of acetaldehyde concentration/slopes of the above plot versus acetaldehyde concentration (Figure 74b) according to the method described in the appendix. The rate constant k_2 was determined to be $k_2 = 1.5 \pm 0.2 \text{ s}^{-1}$. The determination of the rate constant k_2 for NADP^+ was made by similar stopped-flow studies. A plot of rate constants observed at specific fixed concentrations of acetaldehyde for NADPH production over a range of NADP^+ concentrations is seen in Figure 75a. The secondary plot

Figure 73a

Stopped-Flow Assay of Aldehyde Dehydrogenase. This is a typical result from a single stopped-flow experiment. After the piston is fired and the reactive species are mixed and transported into the observation cell, the detection system is actuated. The figure shows the end of the dead time (far left) where the mixed solutions have just entered the observation cell. The initial spike is due to the lack of absorption of the oxidized cofactor. The exponential curve corresponds to the burst of reduced cofactor production. This is followed by a steady state release of cofactor which appears as the linear section. The digitized data appears as individual points. The solid line superimposed on the data curve is a computer fitted line obtained from data in the exponential region. Enzyme concentration was 10.0 mg/mL. NADP⁺ and acetaldehyde concentrations for the trace shown are 1.63 and 3.0 mM respectively. All spectra were taken at 25°C, pH 7.5.

Figure 73b

Stopped-Flow Control Assay of Aldehyde Dehydrogenase. An assay was performed using 2.0 mM acetaldehyde in syringe one and an identical concentration of enzyme in syringe two was used in the stopped-flow studies described in the experimental section. NAD^+ and NADP^+ were excluded. The trace reveals successful rapid mixing indicated by the steep decrease in relative absorbancy followed by a constant (flat) section which shows the absence of a steady state reaction. Enzyme concentration was 10.0 mg/mL. Spectrum was taken at 25°C, pH 7.5.

Figure 74a Stopped-Flow Determination of NAD^+ Dependent Dehydrogenase Activity Rate Constants. Rate constants (k) were calculated from the exponential regions observed from pre-steady state release of NADH from aldehyde dehydrogenase. The individual rate constants were obtained over a variable concentration of NAD^+ at fixed acetaldehyde concentrations. The acetaldehyde concentrations are shown top to bottom as 2.0, 1.0, 0.75 and 0.35 mM. Data points are averaged from 6-12 separate assays. Enzyme concentration was 10.0 mg/mL, all spectra were taken at 25°C, pH 7.5.

Figure 74b Secondary Plot For Determination of NAD^+ Dependent Dehydrogenase Activity Rate Constants. Slopes of the primary plot (74a) were plotted as above in order to obtain the rate constants as described in Appendix II. The errors indicated were obtained by regression analysis. Enzyme concentration was 10.0 mg/mL, all spectra were taken at 25°C, pH 7.5.

Figure 75a Stopped-Flow Determination of NADP^+ Dependent Dehydrogenase Activity Rate-Constants. Rate constants (k) were calculated from the exponential regions observed from pre-steady state release of NADPH from aldehyde dehydrogenase. The individual rate constants were obtained over a variable concentration of NADP^+ at fixed acetaldehyde concentrations here shown top to bottom as 3.0, 2.0, 1.0 and 0.50 mM. Each rate constant is an average of 6-12 assays. Enzyme concentration was 10.0 mg/mL, all spectra were taken at 25°C, pH 7.5.

Figure 75b Secondary Plot For The Determination of NADP^+ Dependent Dehydrogenase Activity Rate Constants. Slopes of the primary plot (75a) were plotted as above in order to obtain the rate constants. The errors indicated were determined by regression analysis. Enzyme concentration was 10.0 mg/mL, all spectra were taken at 25°C, pH 7.5.

(Figure 75b) of acetaldehyde concentration/slopes plotted against acetaldehyde concentration was used to obtain the k_2 value for NADP^+ as cofactor. This rate constant was determined to be $17 \pm 8 \text{ s}^{-1}$. Thus a comparison of the rate constants for NAD^+ and NADP^+ dependent activities reveals the k_2 for NADP^+ to be up to a full order of magnitude greater than for NAD^+ . Based on this evidence above, the binding and reaction involving NADP^+ is more favored than for NAD^+ . Although this evidence supports the general proposal above (Scheme 4), certain inadequacies arise from other data which are addressed in the next section.

The esterase reaction was also probed by stopped-flow studies. Aldehyde dehydrogenase and *p*-nitrophenyl butyrate were co-injected and the production of *p*-nitrophenol was monitored. No burst of activity was observed over the range of substrate concentrations attempted. The release of *p*-nitrophenol is therefore likely to be rate limiting.

Steady state evidence above has shown that *n*-pentanal is a more favorable substrate than ethanal with approximately a 2-3 fold greater turnover number than that substrate. Since the production of reduced cofactor may be coupled to oxidation of the substrate (corresponding to $\text{EAB} \rightleftharpoons \text{EPQ}$ in Appendix III), it is conceivable that the substrate preference could be exerted at this step. In order to assess this effect, burst rates were measured for identical concentrations of each substrate using both NAD^+ and NADP^+ as cofactors. The rate constants determined for NAD^+ dependent

activity using identical concentrations of ethanal and *n*-pentanal were 4.71 and 4.76 s⁻¹ respectively (Figures 76 and 77 respectively). Similarly, the rate constants obtained for NADP⁺ dependent activity using identical concentrations of ethanal and *n*-pentanal were 7.47 and 8.44 s⁻¹ respectively (Figures 78 and 79 respectively). The kinetic traces show the slight increase in burst size that was previously observed for NADP⁺ over NAD⁺. More importantly however is the observation that, within experimental error, there is no difference in the observed bursts for either substrate. Thus it is apparent that the substrate preference is not exerted during the burst event.

6. ENCAPSULATION OF ALDEHYDE DEHYDROGENASE

1. Lipids.

The successful formation of large vesicles depends in part on the exclusion of surfactants and detergents from the lipid mixtures. Typically, these compounds act to lower the critical micellar concentration of the phospholipids (189) and amphiphiles such as lysolecithins which can interact with bilayer membranes and alter the curvature of the bilayer, resulting in changes in physical properties (190). Phospholipid preparations are susceptible to autoxidation, particularly those with high contents of polyunsaturated fatty acids (191), resulting in the formation of contaminants. In order to ensure reproducible liposome preparations, the purchased commercial lipids were checked for

Figure 76

Stopped-Flow Determination of NAD^+ Dependent Dehydrogenase Activity-Ethanal Utilization. The trace provides a determination of the burst rate constant obtained for NAD^+ dependent activity in the presence of ethanal as substrate. Enzyme, NAD^+ and ethanal concentrations were 8.4 mg/mL, 0.98 mM and 0.4 mM respectively. The trace was obtained at 25°C, pH 7.5.

Figure 77

Stopped-Flow Determination of NAD^+ Dependent Dehydrogenase Activity-n-Pentanal Utilization. The trace provides a determination of the burst rate constant obtained for NAD^+ dependent activity in the presence of n-pentanal as substrate. Enzyme, NAD^+ and n-pentanal concentrations were 8.4 mg/mL, 0.98 mM and 0.4 mM respectively. The trace was obtained at 25°C, pH 7.5.

Figure 78

Stopped-Flow Determination of NADP^+ Dependent Dehydrogenase Activity-Ethanal Utilization. The trace provides a determination of the burst rate constant obtained for NADP^+ dependent activity in the presence of ethanal as substrate. Enzyme, NADP^+ and ethanal concentrations were 8.4 mg/mL, 0.92 mM and 0.4 mM respectively. The trace was obtained at 25 °C, pH 7.5.

Figure 79

Stopped-Flow Determination of NADP^+ Dependent Dehydrogenase Activity-n-Pentanal Utilization. The trace provides a determination of the burst rate constant obtained for NADP^+ dependent activity in the presence of n-pentanal as substrate. Enzyme, NADP^+ and n-pentanal concentrations were 8.4 mg/mL, 0.92 mM and 0.4 mM respectively. The trace was obtained at 25°C, pH 7.5.

purity by thin layer chromatographic analysis. All lipids used in the preparations were shown to be free of any contaminants as indicated by a single spot on the TLC plate. The reagent prepared for phospholipid assay was also used successfully as a spray to develop the TLC plates.

Liposomes were prepared by an adaptation of the detergent removal method of Enoch and Strittmatter (156). Sodium deoxycholate was replaced by sodium cholate because of its lower pK and its higher critical micellar concentration (189). In order to ensure the accuracy and uniformity of experimental results, residual detergent levels of liposome preparations must be minimized. A level of residual detergent of 1 mole percent with respect to phospholipid is considered adequate for most experiments (192). Enoch and Strittmatter demonstrated successful removal of radiolabeled detergent from liposomes by passage of their preparation through a gel filtration column.

In order to check that the maximum detergent level was maintained in our scaled-up preparation, a lipid-detergent mixture was sonicated using [^{14}C] sodium cholate. The mixture was then passed through a G-25 column and the eluted fractions were measured for turbidity (optical density at 450 nm) and radiolabel content. The elution profile from the G-25 column (Figure 80) indicates the successful incorporation of [carboxyl- ^{14}C]-cholic acid into the liposomes. A single absorbance peak and radiolabel peak suggests the tracer was incorporated into the vesicles. The pooled

Figure 80

Elution Profile of Sonicated [^{14}C]-Cholic Acid-Phospholipid Mixture From Sepharose G-25 Column. A sonicated mixture of phosphatidyl choline and [^{14}C]-cholic acid was eluted from the G-25 column. The initial peaks correspond to radiolabelled large unilamellar vesicles as indicated by a high absorbance and radiolabel content. Unincorporated detergent is eluted as a large band containing the bulk of the radiolabel.

fractions of this peak were routinely used for the liposomal preparation. Confirmation of this as a successful liposomal preparation is given below. The bulk of the labelled cholic acid is eluted as unbound detergent at a higher elution volume.

The liposomal eluant was pelleted and washed as indicated in the experimental section. The phospholipid concentration was measured by the method of Hallam. By pre-determination of the specific activity of [^{14}C]-cholic acid it was possible to determine the molar ratio of lipid/cholate. The above preparation routinely yielded about 1.5 mole percent which is considered acceptable for reproducible work (192).

Successful formation and size determination of large unilamellar liposomes was confirmed by electron microscopy. A sample of liposomes were prepared for freeze-cleavage as described in the experimental section. The transmission electron microscopic photographs obtained show two large unilamellar vesicles (Plate 3) which were representative of the preparations. The vesicles were about 10,000 Å in diameter. In photo 3a the cleavage occurred across the face of the liposome. One can easily see the aqueous environment trapped inside the vesicle.

The preparation of liposomes by the above procedure is ideal for entrapment of enzymes. A homogeneous size distribution was observed and the single lamellar structure minimizes the complex transport processes which occur with mult-

Plate 3

X-Ray Microscopic Photographs of Liposomes. A preparation of liposomes were freeze-fractured and observed by transmission electron microscopy. The two photographs (a,b) are representative of the preparation. The large unilamellar vesicle shown in a) clearly depicts the fracture face across the liposome and the aqueous environment contained therein. The bar scale is 1000 Å.

a

b

iple lamellar vesicles (ie. systems which are very dependent on internal diffusion (193)). Preparation of the latter also suffers from a non-uniform size distribution. Similarly, small unilamellar vesicles are not ideal for entrapment studies because of poor entrapment volumes. Also the high degree of curvature of these vesicles affects some of the physical properties of the phospholipids (194, 195) such as phase transition temperature. As well, mixtures of phospholipids are more susceptible to lateral phase separations in small vesicles (196). Thus, our preparation boasts a large entrapment volume, homogeneous size distribution and a consistency of physical properties. In addition, the preparation is very gentle on proteins. An ether infusion preparation was initially attempted but was found to be too destructive to be of any practicality.

One of the effects of cholesterol on bilayer properties is to reduce the permeability of the bilayer to small polar molecules and ions (197). The intention of the encapsulation studies was to reconstitute aldehyde dehydrogenase into liposomes of identical composition as the mitochondrial membrane. However the mitochondrial membrane contains about 10% cholesterol (198). Because of the low inherent activity of aldehyde dehydrogenase, β -glucosidase was chosen for initial encapsulation experiments to investigate whether or not the cholesterol would seriously affect the kinetics of the small substrate molecules.

β -Glucosidase was encapsulated in a liposome preparation composed of 100% phosphatidyl choline. A series of activity assays were performed over a range of substrate concentrations on intact vesicles and again on vesicles which were lysed by detergent prior to the assay. As can be seen in Figure 81a, the intact vesicles display a marked decrease in activity with respect to free enzyme (lysed vesicles). The difference in activities is not great at high substrate concentrations but the upward curvature at low concentrations suggests a significant diffusion effect. A similar activity plot was obtained for liposomes composed of 10% cholesterol and 90% phosphatidyl choline (Figure 81b). As above, the activity of the lysed vesicles displays a linear dependency on substrate concentration. The intact vesicles display a near linear dependency at high substrate levels but at lower substrate levels a non-linear decrease in activity with concentration is observed. This effect is not as pronounced as was observed for the 100% phosphatidyl choline liposomes. The effect of a further increase in cholesterol content is observed in Figure 81c. Liposomes prepared from 80% phosphatidyl choline and 20% cholesterol show a further decrease in the non-linear dependency of the enzyme activity in intact vesicles on low substrate concentrations. At high substrate concentrations the activities of lysed and intact vesicles are almost identical. However, at low substrate concentrations, the intact liposomes still display a decreased activity with respect to the lysed prep-

Figures 81 a-d

Effect of Cholesterol Concentration on β -Glucosidase Activity in Intact and Lysed Liposomes. The inverse of initial velocities were determined over a range of substrate concentrations for enzyme in intact liposomes (●) and lysed liposomes (■). The composition of the vesicles were: (a), 100% phosphatidyl choline; (b), 90% phosphatidyl choline, 10% cholesterol; (c), 80% phosphatidyl choline, 20% cholesterol; (d), 70% phosphatidyl choline, 30% cholesterol.

Figure 81b

Figure 81c

Figure 81 d

aration) The highest cholesterol content used for liposome preparations was 30% (70% phosphatidyl choline). At low substrate concentrations this preparation displays the least difference in activities between intact and lysed vesicle preparations (Figure 81d). In general it is therefore apparent that the intact vesicles display a decrease in activity at low substrate concentrations in comparison to lysed vesicles. The decrease is much more apparent at low cholesterol levels. Since the only difference between the intact and lysed preparations is the absence of a single barrier in the latter, it is likely the observed effect is a mass-transfer (diffusional) effect. Since this effect is most pronounced at low substrate concentration, the calculation of effectiveness factor was done at this level. The effectiveness factor (n) is the ratio of initial substrate conversion rates at the same substrate concentration for the same amount of encapsulated and free enzyme (193, 199). The effectiveness factors for each liposome preparation of different composition were determined. A plot of the variation of effectiveness factor with cholesterol content reveals (Figure 82) that as cholesterol content increases, $1/n$ approaches unity. That is to say as cholesterol concentration increases, the apparent effects of diffusion are eliminated (When $1/n = 1$ there is no diffusion effect).

A full kinetic study of encapsulated enzymes requires the use of modifications to traditional steady state treatments. The Michaelis equation for enzymes in solution re-

Figure 82 Dependency of Effectiveness Factor on Liposome Composition. The effectiveness factor (n) was determined for the lowest substrate concentration for each different composition of liposome. A linear dependency on cholesterol content is observed.

veals a linear dependency on substrate concentration, as is illustrated by the kinetic assays of the lysed vesicles. However the Michaelis equation for encapsulated enzymes is non-linear because n is a function of the substrate concentration. Therefore a full kinetic description of an encapsulated enzyme system must be described in terms of dimensionless quantities, independent of substrate concentration (193). The study described here was limited to the determination of n at low substrate levels. Detailed activity calculations also require, in addition to the effectiveness factors for all assays, the knowledge of biocatalyst concentration. Because of the inherent interference by lipids, the concentration of enzyme could not be determined.

Colbeau et al. have made an estimation of the relative composition of the outer mitochondrial membrane (198). The cholesterol content has been determined to be 12 mole percent of the total phospholipid content. The relative content of phospholipids are:

Phosphatidyl choline	49.4%
Phosphatidyl ethanolamine	34.9%
Phosphatidyl inositol	9.2%
Cardiolipin (DPG)	4.2%

Liposomes were prepared of encapsulated aldehyde dehydrogenase using several combinations of the above lipids. All of the liposome preparations made contained 10 mole percent cholesterol. Unfortunately, the dehydrogenase activity was too low to be detected over the high inherent light

scattering of the vesicles. However it was possible to detect the esterase activity.

A liposome preparation of 100% phosphatidyl choline reveals a similar mass-transfer dependency as was observed for β -glucosidase studies. As is evident in Figure 83a, low concentrations of *p*-nitrophenyl butyrate observe very low activities in the intact vesicles. An effectiveness factor of 0.5 was measured at low ester concentrations. However, with the inclusion of 10% phosphatidyl ethanolamine, the apparent effect of mass-transfer effects are eliminated. Identical activities were observed for intact and lysed vesicles over the entire range of *p*-nitrophenyl butyrate investigated (Figure 83b). As the content of phosphatidyl ethanolamine is increased to 20% (80% phosphatidyl choline) there is a dramatic reversal of the diffusion effects previously observed. As is evidenced in Figure 83c, the intact vesicles experience 123% higher activities than the lysed vesicles. Controls failed to indicate inhibition of esterase activity due to the detergent so that the increase in activity in the intact liposomes must be the result of either a partitioning effect or an activation of the enzyme in the particular three dimensional lipid environment. When liposomes were prepared from 96% phosphatidyl choline and 4% cardiolipin, a dramatic 153% increase in activity was observed for the intact vesicles (Figure 83d). It is therefore obvious that the *in vivo* enzyme exists in a more active state than in the purified form. This could be a parti-

Figure 83 a-d. Effect of Lipid Composition on the Esterase Activity of Aldehyde Dehydrogenase in Intact and Lysed Liposomes. Activities of Intact (●) and lysed (○) vesicles were determined over a range of p-nitrophenyl butyrate concentrations for a variety of phospholipid compositions which include: (a) 100% phosphatidyl choline; (b) 90% phosphatidyl choline, 10% phosphatidyl ethanolamine; (c) 80% phosphatidyl choline, 20% phosphatidyl ethanolamine; (d) 96% phosphatidyl choline, 4% DPG

Figure 83 b

Figure 83c

Figure 83 d

tioning effect and/or activation of the enzyme. Further studies are suggested to determine compositional and concentrational dependencies of the enzyme on its lipid environment. Similarly substrate diffusion studies could be performed to ascertain mass-transfer effects on substrate availability and product removal. The latter could be expected to elicit interesting product inhibition effects.

DISCUSSION

Liver aldehyde dehydrogenases have been isolated to various degrees of purity from many mammalian sources. These include sheep (24-26), cow (27, 28), horse (29, 30), rabbit (31, 32), human (42-44, 52), pig (16) mouse (33), and rat (7, 34-36, 39, 41).

Earlier studies of rat aldehyde dehydrogenases have been concerned mainly with partial purification and cellular localization (7, 10, 35, 36, 39, 48). As a result, a number of isozymes have been identified (6, 10). The rat mitochondrial enzyme has been shown to possess NADP^+ activity (7, 10, 39), but no detailed account has been given. Esterase activity has been co-identified for a number of sources including horse (30), human (52), sheep (26) and rat (39). A controversy exists concerning whether or not the esterase and dehydrogenase activities share common active sites. Evidence supporting both sides of the controversy have been reviewed recently by Duncan (59).

Based on our experimental work, this discussion will include a comparison of the properties of our homogeneous preparation to those of other isozymes that have been isolated. The majority of the work has provided evidence to develop specific mechanisms for the dehydrogenase and esterase activities. We have also probed the active site using chemical modifications, kinetic studies and ^{31}P NMR in an effort to identify catalytically important residues. This has permitted several conclusions to be drawn concern-

ing the relationships between dehydrogenase and esterase activities. We have also attempted to address the reasons for differences in activities between NAD^+ and NADP^+ dehydrogenase and esterase activities.

The isozyme that we have studied was most readily purified in the presence of the non-ionic detergent Triton X-100. This suggests the enzyme exists in a membranous environment in its *in vivo* state. Lindahl and Evces (39) have achieved appreciable levels of purification for the 4-5 rat isozymes they have identified. These authors used a similar detergent system to solubilize the isozymes. Similarly, Koivula and Koivusalo (36) observed increased solubilization of the microsomal enzyme in the presence of Triton X-100 and digitonin.

DEAE-A50 Sephadex was chosen as the ion exchange medium. This exchanger was used to remove contaminants from a homogenate of purified mitochondria. Although the pH of the purification buffer used was above the pI of the enzyme, no binding was observed. On application of the homogenate to the column, aldehyde dehydrogenase was eluted directly. As the initial concentration of the buffer was low (10 mM), the failure of the enzyme to bind might have arisen as a result of the type of buffer salt employed (phosphate) (200). Attempts to bind the enzyme to an ion exchanger followed by release using a salt gradient were unsuccessful. The enzyme was found to bind tightly (irreversibly) using

tris buffer or low pH (6.0). Once bound, the enzyme could not be removed.

The use of AGNAD as an affinity medium afforded the most significant purification step. This medium has not been used in the preparation of other aldehyde dehydrogenases. It is this step which permitted our isozyme to be purified to homogeneity unlike other preparations. Aldehyde dehydrogenase was bound to the column and washed free of contaminating proteins. The enzyme was released on addition of 10 mM NAD^+ which was removed by dialysis. This preparation was found to be near homogeneity. A high molecular weight contaminant was removed by gel filtration through a Sepharose 4B column. Discontinuous SDS electrophoresis of the final preparation revealed the enzyme to be homogeneous. Both NAD^+ and NADP^+ dependent dehydrogenase and esterase activities were co-eluted throughout all purification steps thereby indicating chromatographic homogeneity. Interestingly, an appreciable esterase activity failed to bind to the affinity column. In light of this evidence and in consideration of the lower purification achieved with respect to this activity, the possible existence of other esterases or isozymes in the mitochondria is supported. The existence of esterases has yet to be addressed in the literature and this concept is suggested as a topic for further investigation.

The ratio of specific activities of $\text{NAD}^+/\text{NADP}^+$ remained constant throughout the purification procedures. The mito-

chondrial preparation maintained a constant ratio between 1.75-2.0 throughout the later steps of the procedure. The maintenance of a constant ratio is suggestive of a single molecular form.

The purified enzyme yielded a single peak by isoelectric focusing. The isoelectric point was determined to be 6.40. The single peak provides evidence for a single molecular form of the enzyme.

Both NAD^+ and NADP^+ dependent dehydrogenase activities followed identical heat inactivation profiles. This again supports the concept of a single molecular form. The esterase activity does not demonstrate the same heat sensitivity as the dehydrogenase activities. This evidence strongly suggests that the dehydrogenase and esterase activities arise from separate active sites. It even suggests the esterase activity arises from the simple presence of nucleophilic residues. The esterase activity may not have the same specific structural requirements that are characteristic of the dehydrogenase activities (discussed below).

The molecular weight of the native and subunit enzymes were determined to be 240,000 and 60,000 Daltons respectively suggesting the enzyme exists as a tetramer. A unique N-terminal residue was detected suggesting that the subunits are identical.

Steady state kinetic studies were carried out with respect to the NAD(P)^+ dependent dehydrogenase activities. The Michaelis constants for the coenzymes are in the micro-

molar range. Michaelis constants for acetaldehyde are in the millimolar region (see Table VIII).

Our preparation constitutes the first homogeneous preparation of the mitochondrial enzyme. The high degree of purity was achieved primarily due to the very effective affinity step. Lindahl and Evces (39) have provided the most pure preparations prior to our work using a 5'-AMP-Sepharose affinity column. We were unable to demonstrate any binding to this medium.

A comparison of recent rat liver aldehyde dehydrogenase preparations is provided in Table XXIX. Our enzyme most closely resembles the partially purified Mito-II isozyme of Lindahl and Evces (39). The enzymes exhibit similar pH optima, molecular weights, isoelectric points and kinetic parameters. Both enzymes display NADP^+ activities, esterase activities and similar sensitivities to the inhibitory effects of chloral hydrate and disulfiram. The esterase activities of both are stimulated by NAD^+ (low levels) and are inhibited at high concentrations of NAD^+ . Both systems showed no response to Mg^{+2} ions. Our enzyme also shows similarities to the Enz-II of Siew et al. (41), the Mito-II enzyme of Koivula and Koivusalo (36), both ALDH-I and II enzymes of Horton and Barrett (35), and the enzymes of Tottmar et al. and Shum and Blair (34).

Aldehyde dehydrogenases have been purified to homogeneity from several other liver sources. These include human (43, 44), horse (29, 30, 48), sheep (60, 201, 202), pig

TABLE XXIX

Summary of Properties of Rat Liver Aldehyde Dehydrogenases

Dehydrogenase Activity												
Authors	Enzyme	Liver Source	pH Optima	MW	pI	NAD ⁺		NADP ⁺		Purification	Comments	
						K _A	K _B	K _A	K _B			
Shum & Blair (34)	I	Homogenate	8.0	180,000	-	34 μ M	-	-	-	-		
	II	Homogenate	8-9.5	180,000	-	40 μ M	-	-	-	-		
Totter et al. (7)	I	Mitochondria	9-10	-	-	20-30 μ M	10 μ M	-	-	partial		
	II	Mitochondria & Microsomes	9-10	-	-	20-30 μ M	1.0 μ M	2.3mM	0.6mM	partial		
Morton & Bartlett (35)	ALDH-I	Mitochondria	8.8-9.0	-	-	22 μ M	5 μ M	0.3mM	0.6mM	partial		
	ALDH-II	Mitochondria & Microsomes	8.8-9.0	-	-	33 μ M	1.5 μ M	0.5mM	2.7mM	partial		
Kolva & Kalvase (36)	Cytoplasm-I	Cytoplasm	-	-	-	-	millimolar	-	-	partial	Cyto-I inhibited by chloral hydrate and disulfiram. All other enzymes inhibited by disulfiram. Micro-I enzyme is membrane bound.	
	Cytoplasm-II	Cytoplasm	-	-	-	-	micromolar	-	-	partial		
	Mito-I	Mitochondria	-	-	-	-	micromolar	-	-	partial		
	Mito-II	Mitochondria	-	-	-	-	micromolar	-	-	partial		
	Micro-I	Microsomes	-	-	-	-	millimolar	-	-	partial		
	Micro-II	Microsomes	-	-	-	-	micromolar	-	-	partial		
Siew et al. (37)	Enz-I	Mitochondria	-	320,000	6.06	-	micromolar	present	-	partial	Both enzymes are inhibited by chloral hydrate and are membrane bound.	
	Enz-II	Mitochondria	-	67,000	6.64	-	millimolar	present	-	partial		
Lindahl & Evans (38)	Mito-I	Mitochondria	8.8	170,000	6.70	36 μ M	-	910 μ M	-	partial	Mito-I enzyme is inhibited by disulfiram (NAD ⁺ activity). The NADP ⁺ activity is even more susceptible to disulfiram. Chloral hydrate affects mito-I & II and micro-II. No effect by Mg ²⁺ . All isoenzymes have esterase activity.	
	Mito-II	Mitochondria	8.8	250,000	6.40	22 μ M	-	440 μ M	-	partial		
	Mito-III	Mitochondria	-	-	-	-	micromolar	-	-	partial		
	Micro-I	Microsomes	8.5	250,000	6.40	97 μ M	2.4mM	440 μ M	-	partial		
	Micro-II	Microsomes	8.5	250,000	5.60	95 μ M	-	270 μ M	-	partial		
Senior & Tsai	Mito	Mitochondria	8.5-9.0	240,000	6.40	71 μ M	3.6mM	569 μ M	2.3mM	homogeneous	Enzyme is membrane bound and has appreciable esterase activity. Inhibited by chloral hydrate and disulfiram. No effect by Mg ²⁺ .	

K_A, K_B are Michaelis constants for co-factor and acetaldehyde respectively. The specific values or approximate values are given.

(16), cow (27, 28) and rat microsomes (38). However, structural work has been limited to the human (43, 45a, b) and horse (46) enzymes.

Although the mechanism of the aldehyde dehydrogenase activity has been studied in several sources, the majority of the mechanistic work has been done on the sheep (26, 69, 81, 203) and horse (53) enzymes. Many of the discussions concerning structural and functional relationships make generalizations which bridge conclusions drawn from several sources (26, 52, 53, 69, 81) and in general these appear valid. However there is often a significant difference in the physical properties of the enzymes from different sources and one should be careful about making generalizations.

Some of these discrepancies are discussed below. One particularly noticeable fact is that, with the exception of two publications (24, 54) and our own work, no evidence is provided to show the purified enzymes are free of ethanol oxidizing activity. In light of the fact that alcohol dehydrogenase is capable of acetaldehyde oxidation (100), one should be particularly wary of cytosolic preparations which could easily be contaminated by alcohol dehydrogenase.

Aldehyde dehydrogenase from human (E-I & E-II, (44)), horse (F₁ & F₂ (48)), sheep (60), bovine (28), rabbit (54), and rat (39) are all typically tetrameric. The human (E₂), horse, rat enzymes have nearly identical molecular weights (245,000 Daltons). The cow, sheep, human (E₁) and rat (mito-

1) enzymes have molecular weights of 220,000, 198,000, 225,000, 170,000 Daltons respectively. The mitochondrial enzymes from different species differ only slightly in their molecular weight, subunit molecular weight, electrophoretic mobility and kinetic properties (6).

A comparison of the amino acid content of human, horse, cow and rat enzymes is given in Table XXX. As is evidenced in the table, there are a number of close similarities between the five sources and in general the amino acid compositions are comparable. There are several residues in the rat enzyme which show rather dramatic differences from the other enzyme sources. These residues include Asx, Ser, Gly and Ala which are all in greater abundance and Pro and Val which are less abundant. A comparison of the human and rat mitochondrial enzymes reveals the closest similarities. Good agreement is observed with the essential residues such as arginine, histidine, and lysine. The primary structure of the human mitochondrial enzyme has been published. Cytosol and mitochondrial are two contrasting environments would obviously demand unique structural requirements of the enzymes. This is exemplified in the tryptic digests of E1 and E2 isozymes of the human enzyme. The tryptic digest of each suggests significant differences in the respective primary structures (204). One must also bear in mind that the apparent structural homologies implied by amino acid compositions may be misleading. For example, horse and human amino acid sequences reveal that although the net

TABLE XXX

Comparison of Amino Acid Composition
of Aldehyde Dehydrogenase From Various Sources

Amino Acid	Horse F ₁ (46)	Bovine (28)	Human E ₁ (45a)	Human E ₂ (45b)	Rat (a)
Cys	10	8	11	8	16
Asx	43	44	46	49	62
Thr --	28	20	28	27	32
Ser	31	28	29	23	59
Glx	51	47	50	54	63
Pro	25	26	24	24	10
Gly	47	45	48	44	97
Ala	41	39	38	48	82
Val	39	34	38	45	23
Met	10	10	8	9	13
Ile	25	23	35	25	15
Leu	40	35	37	35	42
Tyr	15	14	16	17	11
Phe —	26	22	22	24	17
Trp	7	8	7	4	4
Lys	38	37	38	27	31
His	7	7	8	7	9
Arg	17	19	17	20	18

* Amino acid analysis expressed as # moles/mole monomeric enzyme.

^aThis work.

difference in threonine content is zero, there are 8 (of 28 residues) differences in the placement of this amino acid in the sequences of the two enzymes. Thus the concealment of differences emphasizes the potential danger in attempts to present exact structural relationships by ordering residues in peptides from compositions only (46).

The horse and human enzymes are believed to have serine residues at both the C-terminals (45a, 46). The amino terminus was blocked and an amino residue could not be detected. The bovine enzyme was found to have an Ala and Glx-Ala at the N-terminal amino and carboxy-terminals respectively. We were able to detect a lysine at the N-terminal of the rat mitochondrial enzyme. It was not possible to detect the C-terminal residue.

There is a general consensus that aldehyde oxidation by aldehyde dehydrogenases follows a compulsory-order reaction pathway (59). The oxidized cofactor is bound first followed by the aldehyde. An essentially irreversible release of the acid product is then followed by the dissociation of the reduced cofactor from the enzyme (48, 53, 67, 69).

In order to establish the exact mechanism for the rat enzyme, product inhibition studies were carried out with respect to NAD^+ and NADP^+ dependent activities. Identical product inhibition trends were observed for these activities. This is indicative of a single molecular form. The product inhibition patterns observed clearly identify the

mechanism as following an Ordered Bi Bi pathway according to the possible product inhibition patterns described in Table XIII.

Thus the evidence does confirm that the oxidized coenzyme is bound first, followed by acetaldehyde. The acid product is the first to be released, followed by the reduced coenzyme.

The inhibition constants using acetate as an inhibitor are, in general, very high (millimolar). This indicates that enzyme-acetate binding affinity is very poor and likely leads to the irreversibility of the enzyme.

Cross product inhibition studies were investigated to observe the inhibitory effects of NADH on NADP^+ activity and vice versa. That identical cross product inhibition patterns were identified suggests the coenzymes bind to the same nucleotide binding site.

The mechanism of the dehydrogenase activity has been drawn in part by analogy from the mechanism of glyceraldehyde-3-phosphate dehydrogenase (205). The requirement of thiol groups has been demonstrated in a semi-purified pig brain extract (59), rat (34), rabbit (59) and sheep (60) liver preparations based on the sensitivity of the dehydrogenase activity to thiol reagents. Two active site thiol groups were initially identified in the porcine enzyme on the basis of enzyme inactivation by *o*-iodosobenzoic acid which forms disulfide bridges (206). This reagent has also been subsequently shown to cleave tryptophan bonds (includ-

ing tryptophan-cysteine bonds) (207, 208) and therefore the qualitative determination above cannot be substantiated on that evidence alone. Crystallographic studies of Buehner (209) and Harris and Waters (205) demonstrated an interaction of the nicotinamide ring of NAD^+ with a cysteine residue in the active site of G3PDH. The requirement of -SH groups for activity was demonstrated by Shum and Blair (34) in a rat liver preparation. These authors were able to show that the rat enzymes were equally inhibited by p-chloromercuribenzene sulfonic acid, a thiol reagent (210). This inhibition was reversible in the presence of dithiothreitol.

Racker suggested that all aldehyde dehydrogenases form a thiohemiacetal as a reactive intermediate in the reaction of an aldehyde with aldehyde dehydrogenases (211). Feldman and Weiner provided supportive evidence for the horse enzyme (53). A Taft plot was prepared for a series of aldehydes (RCHO) with differing electron-withdrawing character of the R group. As the carbonyl was made more electropositive, by increasing the electron-withdrawing power of the R group, the reaction rates were increased. Their evidence supports the concept of nucleophilic attack of the electropositive carbonyl carbon to form the thiohemiacetal (shown below).

The pH profiles that we obtained for both NAD^+ and NADP^+ dependent dehydrogenase activities both reveal a slightly asymmetric profile as was observed by Shum and Blair (34). Both profiles identify an ionizable species of $\text{pK } 9.5$ present in the enzyme which is suggestive of an active site cysteine, lysine or tyrosine residue(s) giving rise to both activities. On the basis of this identification, a series of chemical modifications were performed which were intended to demonstrate the identity of this active site group.

The modification of the enzyme with carboxymethylating agents such as DTNB, iodoacetic acid and iodoacetamide clearly shows the necessity of intact active site thiol species for both dehydrogenase activities. The modification (using iodoacetamide) was also repeated on enzyme in the presence of NAD^+ . The NAD^+ , which binds to the active site of the enzyme, was able to protect the active site cysteine group from modification by the reagent. This provided evidence that not only was a cysteine species necessary for the reaction, it was also located in the NAD^+ binding site of the enzyme.

It was apparent from the inactivation profile of aldehyde dehydrogenase by iodoacetamide that, after 13 minutes of reaction under the specified conditions, the thiol(s) needed for dehydrogenase activity was (were) inactivated. In an effort to quantitate these requisite thiols, we modified a known quantity of the enzyme with radiolabelled

[^{14}C]-iodoacetamide for the specific time required to remove any dehydrogenase activity (13 minutes). By pre-determining the specific activity of the reagent, it was possible to establish that one active site cysteine was required per monomeric enzyme for both dehydrogenase activities. In order to confirm that the loss of activity was due to the modification of cysteine residues, samples of the enzyme were removed during the modification and were prepared for amino acid analysis. It was clearly shown that the loss of dehydrogenase activities corresponded to the modification of two cysteine species. Earlier studies by von Bahr-Lindstrom *et al.* (83), Hempel *et al.* (77) and Hart and Dickinson (60) indicated two different cysteine residues were located in the active site of aldehyde dehydrogenase. One was suggested to be close to the coenzyme binding site (83). The other (Cys-302) was thought to be directly involved in catalysis (77). Subsequent sequence analysis of the human and horse enzymes (45, 46) confirmed that Cys-302 was directly involved in catalysis at the active site. Recently, the same residue was identified in the horse mitochondrial enzyme by specific labeling of the coenzyme binding region with brominated [5-(3-acetylpyridinio)pentyl] diphosphoadenosine (75). Thus our enzyme contains two active site cysteines which are essential for NAD^+ and NADP^+ dehydrogenase activities, as has been identified in other aldehyde dehydrogenases. Although we were only able to identify the two thiols, the existence of other close cysteine neighbors can-

not be discounted. The direct requirement of the second thiol for catalytic function is unknown and is apparently not of major consequence. Although sequence analysis has not been performed on our enzyme, the active site cysteine which we have identified, yields catalytic properties similar to those imparted by Cys-302 in the cytoplasmic human and horse enzymes (77, 83) and the mitochondrial horse enzyme (46).

As a final confirmation as to the specificity of the carboxymethylation of the active site cysteine residue, a peptide digest of radiolabeled enzyme was prepared. Carboxymethylation using [^{14}C]-iodoacetamide was performed identically as above (13 minutes reaction). The labelled enzyme was subjected to chymotryptic digestion and the peptides were separated by SDS polyacrylamide gel electrophoresis. The peptide digest of this enzyme (Figure 70b) reveals three peptides containing a radiolabeled cysteine species, one of which is identified in a peptide that is coincident with a radiolabelled amidated peptide. The observation of two residually labelled peptides must reflect a certain lack of proteolytic specificity of the chymotrypsin because we have established by two other methods that only 1-2 thiols are modified after 13 minutes, under the specified conditions.

From the pH profiles obtained for NAD^+ and NADP^+ dependent dehydrogenase activities, a second ionizable species of pK 6.1 was identified. This value is very close to that of histidine (pI 6.0). In order to confirm the requirement of

the dehydrogenase activities for histidine residue(s), dye-sensitized photooxidation was carried out on the purified enzyme. Both NAD^+ and NADP^+ activities were seen to be highly dependent on the presence of intact histidine residues. After 35 minutes of reaction only 6% of the NAD^+ activity remained and the NADP^+ activity was completely lost. In order to quantitate the requirement of the enzyme for histidine residues, and show that the loss of activity during photooxidation was due to the modification of histidines, amino acid analyses were performed on photooxidized enzyme withdrawn during the course of the modification. A direct correlation was observed between the loss of the dehydrogenase and esterase activities and the modification of histidine residues, thus showing histidine residues to be essential. It was determined that 2 histidine residues per monomeric enzyme were required for activity (Table XXVIII). This was corroborated by simultaneous determination of aspartic acid formation during the modification. Aspartic acid is produced in stoichiometric amounts with the decomposition of histidine residues. Two aspartic acid residues were produced per monomeric enzyme concomitant with the loss of the two histidine residues (per monomeric enzyme).

The hydrolysis of acyl intermediates by all enzymes is believed to be via a general base type reaction (78). Histidine has been identified as the general base in chymotrypsin, papain and glyceraldehyde-3-phosphate dehydrogenase (79). The low pK of histidine makes this a likely residue

for a base in such a mechanism. The hydrolysis by histidine of the thiohemiacetal intermediate in aldehyde dehydrogenase has been suggested by Takahashi et al. (80), as described below:

Our pH kinetic and chemical modification data support the involvement of cysteine and histidine groups in the dehydrogenase reaction, which are in accord with the mechanism above.

It has been proposed that histidine exists in a "charge relay" system (79) whereby an electron sink facilitates the hydrolysis of the thiohemiacetal similar to that described below:

Here species A acts as an electron sink. Tyrosine-phenol has been identified as such a sink in glyceraldehyde-3-phosphate dehydrogenase (186), the aspartate-carboxylate has been identified in chymotrypsin (212) and the asparagine-carboxyl oxygen in papain (79). We have identified two analogous residues in aldehyde dehydrogenase which could function in a "charge relay" system. Chemical modification of tyrosine residues suggests that dehydrogenase activity is

dependent on the presence of intact tyrosine residue(s). Dehydrogenase activity was completely lost after 21 minutes of reaction. The tyrosine effect could be through such a "charge relay" complex as above and/or could be the result of destruction of a hydrophobic linkage between the enzyme and the nicotinamide ring of the coenzyme as has been identified in glyceraldehyde-3-phosphate dehydrogenase. However, the nitration of the enzyme was not followed up with amino acid analysis, so it is not conclusive to suggest the tyrosine modification is solely responsible for the inactivation effect. In addition we did not observe a biphasic pH profile as did Takahashi et al. (80) which lends support to the involvement of a tyrosine in a "charge relay" function. These authors also pointed out that the involvement of such a charge relay function in the hydrolysis of acyl intermediates would be of dubious importance at physiological pH regions. A second possible residue which could function in "charge relay" system is an acidic species. As will be discussed in detail below, an acidic residue(s) is suggested as being present in the active site of our rat aldehyde dehydrogenase.

The modification of tryptophan species in our enzyme reveals that dehydrogenase activity is dependent on this residue(s). Modification of this residue results in rapid and complete loss of dehydrogenase activity. The modification with NBS is rather non-specific and therefore the loss of activity should not be considered entirely due to modifi-

cation of tryptophan. It is unlikely that tryptophan plays a major role in the oxidation of aldehydes. Rather, the contribution of tryptophan(s) to the activities of aldehyde dehydrogenase is likely structural. This cannot be confirmed until more detailed structural information is known.

As will be discussed below, the active site of aldehyde dehydrogenase likely contains a lysine residue. However, modification of lysines has absolutely no effect on the integrity of dehydrogenase activity.

An interesting observation was made concerning the relative abilities of iodoacetic acid and iodoacetamide to inactivate the NAD^+ and NADP^+ dependent dehydrogenase activities. Both activities were only partially inactivated upon modification with iodoacetic acid. However, when the same modification was performed using iodoacetamide, under exactly the same conditions as above, total inactivation of both activities was observed after 13 minutes of reaction. It was hypothesized that the different sensitivity of the dehydrogenase activities to the two reagents might arise from a charge effect. The presence of an active site acidic species could result in a repulsive interaction with similarly charged iodoacetic acid thereby preventing an efficient reaction. In comparison, the iodoacetamide reagent would not experience a repulsive effect with the active site because of the lack of a similar negative charge. Thus the reaction with iodoacetamide would be more efficient. This was indeed observed by rapid inactivation of the enzyme.

The presence of an active site acidic species has been shown to have important effects in the differential ability of several dehydrogenases to utilize NAD^+ and NADP^+ . Brändén et al. prepared a comparison of lactate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase and alcohol dehydrogenase (183). These enzymes utilize NAD^+ as the sole coenzyme for dehydrogenase activity. Each enzyme has a hydrogen bond interaction between the 2'-hydroxyl of the adenosine-ribose (of NAD^+) with an aspartic acid residue in the active site of the enzyme. Rossmann et al. (65) have suggested that the extra phosphate of NADP^+ experiences a steric and charge repulsion with this aspartate residue. This repulsion would result in the decreased binding affinity of the coenzyme considerably. A comparison of the kinetic parameters for NAD^+ and NADP^+ activities for our enzyme reveals that NADP^+ does experience a higher Michaelis constant (weaker binding). A concomitant decrease in catalytic efficiency is observed. In order to establish the presence of an active site acidic species, the enzyme was amidated and the effect of loss of acidic species on both dehydrogenase activities was determined. After 60 minutes of amidation the NAD^+ activity observed a slight increase. However, the NADP^+ dependent activity made a dramatic increase over the same time period. After this time, the NADP^+ activity became equal to that of the NAD^+ activity. Thus it appears that the loss of the acidic species removes the ability of the coenzyme to distinguish between the two

cofactors. In order to establish that this residue(s) is located in the active site of the enzyme, the modification was repeated in the presence of NAD^+ as an active site protector. The NADP^+ activity remained essentially unchanged at an activity like that observed for the native enzyme. When the active site acidic species were protected from amidation, the enzyme retains the ability to distinguish between the two cofactors. The reactions of carbodiimides with proteins is reasonably specific for carboxylic acids, but reaction with other acidic proton bearing groups can also occur. The amidation reaction could not be followed up with amino acid analyses so it is not possible to attribute the activation effect to the acidic residues alone. It is suggested that future work could monitor the incorporation of radiolabelled amidating reagent into the protein as a means for monitoring the involvement of acidic residues in the activating event.

The absence of any NADP^+ dependent dehydrogenase activity from lactate, alcohol and glyceraldehyde-3-phosphate dehydrogenases was attributed to the repulsion of the 2'-phosphate of NADP^+ with an active site acidic group. However, although NADP^+ is not the favored coenzyme, its activity is still appreciable. Therefore the repulsion described above cannot be absolute with respect to the two coenzymes. There must be an active site species present which counteracts, in part, this repulsive effect in order that NADP^+ can be used as a coenzyme. A likely candidate

for this species is arginine. This amino acid has a high pK (12.4) which ensures it will be protonated at the physiological pH. The presence of a cationic arginine in the active site could provide the source for an attractive interaction with $NADP^+$. This could allow the enzyme to overcome the repulsive effect arising from the acidic species described above.

Brändén has discussed specific arginine-pyrophosphate interactions in lactate and alcohol dehydrogenases (64). Using X-ray crystallography, Matthews et al. (66) have attributed the $NADP^+$ specificity of dihydrofolate reductase to an attractive interaction between an active site arginine and the 2'-phosphate of $NADP^+$.

The $NADP^+$ activity was confirmed to have a dramatic dependence on the presence of an active site arginine residue(s). Modification of aldehyde dehydrogenase with phenyl glyoxal showed that $NADP^+$ activity was rapidly and totally lost whereas 65% of the NAD^+ activity was retained.

The same reaction repeated in the presence of coenzymes as active site protectors (both NAD^+ and $NADP^+$ gave identical results) failed to elicit any change in either NAD^+ or $NADP^+$ activity. Both coenzymes were equally useful in preventing the modification of arginine residue(s). This implies that the arginine residues are located within the active site of the enzyme and provide an attractive center towards $NADP^+$, thereby permitting its use as a coenzyme.

The observation that the NADP^+ activity is lost on modification of an active site arginine(s) provides corroborative evidence for the effects of the acidic residues discussed above. When the attractive effects of the arginine for the 2'-phosphate are lost on phenyl glyoxalation, the repulsive interaction between the 2'-phosphate and the active site acidic species may predominate. As a result, NADP^+ experiences a repulsive effect from the active site and is no longer a desirable coenzyme. This is also supported by ^{31}P NMR evidence presented below.

In order to conclusively show the involvement of arginine(s) in the active site events, aliquots of enzyme were removed during the course of phenyl glyoxalation and subjected to amino acid analysis. It was shown that a single arginine was modified in the time required to completely remove NADP^+ dependent dehydrogenase activity and partially remove NAD^+ dependent dehydrogenase and esterase activities.

Chymotryptic digests of native and radiolabelled carboxymethylated, amidated and phenyl glyoxalated enzymes were prepared and the peptides were separated by gel electrophoresis. We have shown above that aldehyde dehydrogenase contains active site cysteine, arginine and likely an acidic species. The carboxymethylated and amidated digests reveal that a single peptide contains active site cysteine and acidic residue(s). The active site arginine is found in a different peptide. Therefore the involvement of the arginine residue in the active site must arise from tertiary or

quarternary structures in which the arginine is brought into the active site by the folding patterns of the polypeptide chain. The digests also provide confirmation that the reaction time periods are essentially limited to reaction with catalytically essential residues. The phenyl glyoxalated and amidated digests contain a single modified peptide which was prepared in the time required to remove NADP⁺ activity (phenyl glyoxalation) and to activate NADP⁺ activity to the same level as NAD⁺ activity (amidation).

In order to further probe the nature of the binding of NAD⁺ and NADP⁺ to aldehyde dehydrogenase, ³¹P NMR was employed. The 5'-pyrophosphate of NAD⁺ and NADP⁺ and the 2'-phosphate of NADP⁺ give rise to characteristic resonances when investigated by ³¹P NMR.

It is possible to examine and compare similar resonances of the two cofactors and investigate the effects of the enzyme on these resonances. The cofactors, on binding to the active site of the enzyme, will be influenced by those residues responsible for interaction with the specific cofactor. Presumably, if the interactions involve the phosphate species, we should observe changes in these resonances. It should therefore be possible to observe the different effects of the enzyme on the phosphate signals and make inferences as to the nature of the active site.

Under the conditions employed, the 5'-pyrophosphate of free NAD⁺ and NADP⁺ (5.0 mM) give rise to a rather broad peak. In the presence of enzyme there is no change in the

chemical shifts or resolution of these resonances. No apparent distinction is made by the enzyme, on this moiety, in either of the two coenzymes under the conditions employed.

We have established above that an active site arginine and likely acidic residue(s) were involved in coenzyme binding. In an effort to identify particular interactions between these residues and the phosphate moieties of the coenzymes, we studied the changes in the ^{31}P NMR resonances of these species in the presence of enzyme in which the arginine and acidic residues were modified. As was previously observed (at 4°C), the 5'-pyrophosphate signals experienced no chemical shift or broadening effects in the presence of native, amidated or phenyl glyoxalated enzyme.

The effects of the above modifications on the 2'-phosphate signal were also observed. This resonance was previously seen to experience a downfield shift (with respect to free NADP^+) of 0.72 ppm in the presence of native enzyme. This shift was also observed when the spectrum was obtained at the higher temperature (spectra were taken at room temperature for modified enzyme trials). However the downfield shift was much more dramatic under these conditions (3.31 ppm). When the spectrum was obtained in the presence of phenyl glyoxalated enzyme, the 2'-phosphate downfield shift was absent. The resonance experienced only a very minor downfield shift of 0.50 ppm. Therefore on removal of the arginine residue from the active site, the enzyme appears to be unable to recognize the presence of NADP^+ .

^{31}P NMR has shown that the 2'-phosphate moiety of NADP^+ has a strong dependence on presence of active site arginine residue. This interaction is likely similar to that present in dihydrofolate reductase in which the NADP^+ activity is attributed to the attraction between an active site arginine and the 2'-phosphate of NADP^+ (93). A similar NADP^+ , 2'-phosphate shift was observed by Peeney et al. (93) in the presence of that enzyme. The influence of an active site arginine on the 5'-pyrophosphate resonances cannot be ruled out entirely but were of no consequence under the conditions we employed. Hyde et al. were able to demonstrate the involvement of arginine residues with this resonance in dihydrofolate reductase (92). The arginine species could overcome repulsive effects resulting from the presence of an active site acidic species (which is discussed next) and impart NADP^+ binding thereby leading to a successful reaction with this cofactor.

The 2'-phosphate peak of NADP^+ experienced a slight downfield shift of 0.36 ppm to 5.96 ppm in the presence of amidated enzyme in comparison to the shift observed for native enzyme (5.60 ppm). That the large downfield shift, as seen for the 2'-phosphate peak of NADP^+ in the presence of native enzyme was still observed suggests the attractive effects for NADP^+ by the amidated enzyme are still maintained. Since there is a further increase in the 2'-downfield shift on amidation of the enzyme, a possible explanation is that the removal of active site acidic species per-

mits an even greater attractive interaction. The attractive interaction between arginine is manifested by a downfield 2'-phosphate shift. The shift observed with the amidated enzyme suggests an increase in attractive interactions has resulted through the removal of repulsive effects caused by the acidic residue(s).

It is also possible to obtain information regarding the active site by kinetic analysis. Analogues of NAD^+ and NADP^+ have been used to elucidate the nature of the active site of alcohol dehydrogenase (85-89) and lactate dehydrogenase (90). Recently, kinetic studies using 18 NAD^+ analogues were performed on LDH (90). Changes in analogue structure were compared with kinetic responses and thus permitted crude correlations to be made relating cofactor binding to the known structures within the active site of the enzyme. For example, substitutions at positions C-4 and C-5 of the pyridinium ring prevented proper positioning of the ring for hydride transfer and hindered binding of the analogues to the enzyme. Substitution of groups of the C-3 position of the pyridinium ring could be quite extensive without loss of coenzyme activity. V_{max} and K_m values found using acPy- AD^+ in dogfish LDH were about one-third the values observed for NAD^+ . Similar results were obtained for thio- NAD^+ .

In rat mitochondrial aldehyde dehydrogenase, the use of deamino- NAD^+ resulted in a significant decrease in enzyme-coenzyme binding affinity (with respect to NAD^+). However

little variation in catalytic efficiency was observed. This would suggest that the cofactor is bound to the enzyme, in part, through the adenine ring but does not take part in the reaction as is expected. The N-6 amino species of adenine in NAD^+ has been shown to be hydrogen bonded to an arginine (Arg 271) in LADH (64). An analogous interaction may be present in rat aldehyde dehydrogenase.

It is unlikely that steric effects account for decreased binding affinity of thio- NAD^+ . The phosphorylated analogue (thio- NADP^+) observes tighter binding than NADP^+ . The decreased binding affinity of thio- NAD^+ and slightly decreased turnover number may illustrate the effect of replacement of the carbonyl oxygen with a less electronegative thio group. Hydrogen bonds have been identified between the carboxamide and several residues in LDE, LADH and G3PDH (64). The decreased electronegativity of the thio species may result in weaker hydrogen bonds (if these exist in our enzyme) thereby contributing to a decreased binding affinity and turnover number. Similarly, a decreased turnover number was observed with thio- NADP^+ , possibly the result of the same electronegative effect as observed for thio- NAD^+ . However the binding affinity is greater than for NADP^+ . Perhaps a steric effect is now observed as the thio and 2'-phosphate groups have larger spatial requirements than the cofactor containing either alone. It may be possible that the steric effect due to the thio species forces the 2'-phosphate into

a position of increased interaction with the previously identified arginine group.

Disruption of similar carboxamide-active site hydrogen bonds is likely responsible for the decrease in binding affinity and turnover number observed using PyA-AD⁺ as cofactor. The binding affinity of acPy-AD⁺ is reduced, likely for similar reasons. A slight increase in binding affinity for acPy-ADP⁺ might be explained by a similar argument as for thio-NADP⁺. As hydrophilic residues have been identified in the carboxamide binding regions of LDB, LADB and G3PDB, the presence of an hydrophobic residue (the 3-position acetyl) could create a repulsive effect which causes the analogue position to change in the coenzyme binding pocket. The change in position due to this repulsion could be similar to the steric shift caused by the thiol proposed for thio-NADP⁺. This shift in position could force the enzyme into a more favourable position to increase the 2'-phosphate-arginine interaction. These arguments require that the active site be large enough to accommodate changes in position. In support of this it is common, in dehydrogenases, to have the nicotinamide positioned in a hydrophobic cavity which is sufficiently large to allow different orientations of the pyridine ring rather than being rigidly fixed (89). Such an active site supports our data but, admittedly, this is highly speculative. Detailed X-ray structural information, which is at present not available, is required to confirm the presence of these interactions.

The active site of aldehyde dehydrogenase appears to have an active site acidic species which imparts a repulsive effect towards NADP^+ . This acidic species is likely analogous to Asp 53, 32 and 223 (hydrogen bonded to 2'-hydroxyl of adenosine-ribose of NAD (65)) in LDH, G3PDH and LADH respectively giving rise to the NAD^+ specificity. The repulsive effect is partially overcome by the presence of an arginine residue (analogous to Arg-43 in dihydrofolate reductase (89) which interacts with the 2'-phosphate of NADP^+ and gives rise to enzyme activity with this cofactor.

It has been established previously, by product inhibition kinetic studies, that rat aldehyde dehydrogenase can be described by an Ordered Bi Bi mechanism. The mechanism, shown using the Cleland method below in Scheme 5, involves the binding of an oxidized cofactor followed by the aldehyde. A rearrangement takes place ($\text{EAB} \rightarrow \text{EPQ}$) which corresponds to the hydride transfer and thiohemiacetal hydrolysis. The acid product is then released followed by release of the reduced cofactor.

Scheme 5. Ordered Bi Bi mechanism of NAD(P)^+ dependent activity of aldehyde dehydrogenase.

Although the rate equation derived for the above bisubstrate mechanism can adequately describe our kinetic re-

suits, it is possible to obtain more detailed information with respect to the mechanism.

There has been some difference of opinion as to the mechanism of aldehyde dehydrogenases in general. An Ordered Bi Bi mechanism, in which the same order of substrate binding and product release as we have presented, has been identified for the horse liver (48, 53), pig brain (67), and sheep liver cytoplasmic enzymes (69, 70). The sheep enzyme has also been reported to follow a Random Bi Bi mechanism (69) as has been the human enzyme (52). A resolution to this controversy has been proposed by Dickinson (203) which is supported, in part, by our data. This will be discussed below.

An observation has been made from NAD^+ and NADP^+ kinetic studies that although NAD^+ had an apparent greater affinity for the enzyme and a greater catalytic efficiency, the binding of NADP^+ was a kinetically faster process. The presence of an active site arginine(s) was seen to be responsible for much of the NADP^+ activity as previously discussed and it seemed possible that a NADP^+ -arginine interaction could give rise to this kinetic anomaly. In order to probe this effect, pre-steady state kinetics using stopped-flow spectrometry were performed.

The rate equation for pre-steady state analysis was derived for practical reasons using a Rapid Random Bi Bi mechanism as the model. This model is suitable for our application. It is kinetically indistinguishable by steady

state analysis from the Ordered Bi Bi mechanism that we have identified for rat aldehyde dehydrogenase. The mechanism assumes that the events leading up to the formation of EAB (see Appendix II) are rapid and thereby allows the experimental data to be interpreted in terms of the rate constants for product formation.

The most important observation from the pre-steady state analysis of NAD(P)H formation was a burst of product formation prior to the establishment of the steady state. This implies that the dissociation of NAD(P)H is a slower process than the formation of E^{NADH} (ie. $k_3 > k_4$) as described in Scheme 6.

Scheme 6. Simplified mechanism of product release from aldehyde dehydrogenase.

The values for k_3 that we have measured for NADH formation compare favorably with similar bursts observed by Hart and Dickinson (70, 203), Eckfeldt and Yonetani (68) and MacGibbon et al. (81), all on the sheep cytoplasmic enzyme.

Both the initial binding of $NADP^+$ to the enzyme (from steady state studies) and the burst of E^{NADPH} formation were found to be greater than the binding of NAD^+ and formation of E^{NADH} . Thus the phosphorylated coenzyme appears to be favoured up to the formation of E^{NADPH} . We have discussed possible reasons for this above. However, steady state analysis reveals that NAD^+ gives rise to the highest catalytic

efficiency. Thus it would appear that the step encompassing the dissociation of reduced NAD(P)H must also involve an inactivating event, such as a conformational change, with respect to NADP⁺ activity. It would therefore appear that the product release described in Scheme 6 is insufficient to explain the data.

MacGibbon et al. (81) observed a two step dissociation of NADH from the sheep enzyme by stopped-flow studies which was interpreted by the following mechanism (Scheme 7)

Scheme 7: Two step dissociation of NADH from sheep aldehyde dehydrogenase.
^{*}E^{NADH} is E^{NADH} prior to conformational change

It was suggested that the step encompassing k_1/k_{-1} involves either a change in the conformation of NADH in the binding site, or a conformational change of the enzyme-NADH complex. This is followed in either case by dissociation of NADH from the enzyme. Our steady state kinetic studies, performed using a variety of *n*-alkanals of increasing chain length, provide evidence for a similar two step dissociation in the rat enzyme.

Turnover numbers exhibited a progressive increase as carbon chain length of the aldehyde substrate was increased from C₂ to C₅ (ethanal to *n*-pentanal). Methanal gave an unexpectedly high turnover number which was an anomaly in the

series. This same higher catalytic efficiency was also observed by Abeles and Lee (213) who attributed the effect to the difference in hydration of the two substrates (214).

The manifestation of the substrate effect can arise from two possible sources. First, the hydride species involved in the hydride transfer from aldehyde to NAD(P)^+ is assumed to arise from the aldehyde. The possibility exists that this transfer could be dependent on substrate chain length. The hydride transfer and thiohemiacetal hydrolysis constitutes the step described by k_3 in Scheme 6. Since this involves the burst of product formation, we could observe the substrate effect by stopped-flow. Bursts were measured for NAD^+ and NADP^+ using ethanal and *n*-pentanal as substrates (represent minimum and maximum substrate effects respectively). There were essentially no differences between the bursts for NAD^+ using ethanal and *n*-pentanal and no differences between the bursts for NADP^+ using either substrate. Therefore the hydride transfer and thiohemiacetal hydrolysis is not affected by substrate chain length.

Second, the burst of $\text{E}^{\text{NAD(P)H}}$ formation as described in Scheme 6 assumes concomitant release of the acid product. However this release may be influenced by environmental effects such that short chain (or long chain) products have a different affinity for the enzyme than the long chain (or short chain) and thus the release will depend on the relative affinities of chain length of the products for the enzyme. Since stopped-flow analysis has discounted the

first possibility, the substrate chain length effect on kinetics must be manifested through dissociation of the substrate from the enzyme. Therefore a more accurate description of product release from rat aldehyde dehydrogenase is described in Scheme 8.

Scheme 8. Proposed detailed mechanism of product release from aldehyde dehydrogenase.

In the scheme above, k_4 and k_5 are not distinguishable by steady state analysis and they constitute together the turnover number determined by kinetics. The combined k_3 , k_4 process permits substrate chain length to influence turnover numbers as observed by steady state analysis. This mechanism also provides a possible basis for an explanation of an inactivating event, such as a conformational change, with respect to NADP^+ activity. Ultraviolet scans were prepared of free NADH and NADPH and in the presence of enzyme. A small blue shift was observed when the NADPH was bound in the enzyme. This shift could be due to an analogous enzyme-NADH conformational change as observed by MacGibbon et al. (81). A conformational change may result in which the positioning of NADPH in the active site changes such that NADPH release is not favored. Therefore, although NADP^+ is an apparently "better" coenzyme than NAD^+ , it is in fact not as efficient as NAD^+ in the net oxidation of aldehydes. A

similar blue shift was observed by Dickinson using NADH (203).

The *n*-alkanal effect above was less pronounced when steady state assays were done in the presence of NADP^+ . This would suggest the conformational change does not occur until after the acid product is released.

Dickinson (203) also observed the biphasic dissociation of NADH from sheep cytosolic aldehyde dehydrogenase. However he provided evidence that an abortive complex was formed of enzyme-aldehyde-NADH. He proposed that this complex was more capable of releasing the reduced cofactor than was the enzyme-NADH binary complex described by MacGibbon et al. (69) and that this species was responsible for the faster dissociation event. In providing this explanation, Dickinson was able to support his belief that the mechanism followed an Ordered Bi Bi mechanism rather than a Random Mechanism. We also have evidence which supports the abortive complex described by Dickinson. A double reciprocal plot obtained over a range of acetaldehyde concentrations (fixed NAD^+) reveals that acetaldehyde concentrations above 12 mM resulted in substrate inhibition. Thus the formation of an abortive complex of substrate with the species $\text{E}^{\text{NAD(P)H}}$ would result in the net decrease in concentration of $\text{E}^{\text{NAD(P)H}}$ from the reaction solutions and a decrease in activity would be expected (as observed).

Therefore our rat aldehyde dehydrogenase shows many similarities in its mechanistic properties as to those from aldehyde dehydrogenases^s from other sources.

It is possible to propose a detailed mechanism based on all our kinetic evidence. This is supported by similar proposals from other authors (Scheme 9).

Scheme 9. Proposed mechanism for rat mitochondrial aldehyde dehydrogenase

The esterase activity of aldehyde dehydrogenase has been reported in human (51), horse (53), rabbit (54), sheep (55) and rat preparations (39, 40b). The esterase activity has been routinely reported as a secondary activity of the enzyme. No attempts to monitor the purification of the enzyme with respect to this activity have been reported. The mechanism is generally assumed to occur via acylation of the same active site thiol species as those that are involved in the aldehyde dehydrogenase activity (51, 53, 54, 68). This has been the subject of heated controversy in recent literature. Evidence in support of a common active site and

counter evidence against this have been recently reviewed by Duncan (59). This topic will be considered in detail below.

We have monitored the esterase activity throughout the purification procedure of aldehyde dehydrogenase. An esterase activity was co-purified with both NAD^+ and NADP^+ dehydrogenase activities through all steps leading to homogeneity. Chromatographic and electrophoretic homogeneity were observed and therefore demonstrated, unequivocally, the bifunctionality of the enzyme.

The final purification achieved for the esterase activity was 660 fold. This is significantly less than that obtained for the dehydrogenase activities. It was observed during the affinity procedure that a significant esterase activity was not bound to the AGNAD column. This would suggest the presence of another esterase(s) and could account for the lower purification of esterase activity. The failure of this esterase(s) to bind to the AGNAD column would suggest that its activity is not dependent on the dehydrogenase active site. It is possible that the arguments concerning separate active sites, presented by the above authors, are for distinctly different esterases. Since there is no report of other esterases in the literature, it is possible that arguments for and against a common site may have been improperly founded (the possibility of other esterases in aldehyde dehydrogenases are suggested as an excellent topic for future research).

5 of/de 5

METRO

As was done for aldehyde dehydrogenase activity, inhibition kinetics were performed on the esterase activity using the products butyrate and *p*-nitrophenol as product inhibitors. The difference in the inhibition patterns (Table XXI) obtained with different products can be used to determine the mechanism and order of addition of substrates and release of products (71). Butyrate and *p*-nitrophenol were found to act as competitive and non-competitive inhibitors respectively. Thus the product inhibition pattern is consistent with the esterase activity as following an Ordered Uni Bi mechanism with *p*-nitrophenol being the first product released followed by butyrate from the enzyme-product binary complex. This mechanism is identical to the three step mechanism originally proposed by Bender for chymotrypsin (215) and adapted by Takahashi and Weiner (96) for the esterase activity of horse aldehyde dehydrogenase. The mechanism shown in Scheme 10 implies the formation of an acyl intermediate on release of *p*-nitrophenol followed by deacylation in the third step (k_3).

Scheme 10: Proposed mechanism for esterase activity by aldehyde dehydrogenase. The species pNPA and pNP are *p*-nitrophenyl acetate and *p*-nitrophenol respectively and A is the free acetate.

Weiner et al. (216) were not able to observe a burst of *p*-nitrophenol release for aldehyde dehydrogenase when pre-steady state kinetics were performed and concluded, on that ~~basis~~, that the acylation step was rate limiting. However a very small burst was observed by MacGibbon et al. (26) for the same enzyme and it was concluded by these authors that deacylation was the rate limiting step. We were unable to detect any burst activity which suggested that deacylation was not rate limiting. We attempted to confirm this by performing steady state kinetics using *p*-nitrophenyl esters of increasing chain length. There was a very small dependency of turnover number on carbon chain length. A small but detectable progressive decrease in turnover number was observed as chain length was increased from C₄ to C₁₂. This would imply that neither acylation nor deacylation are rate limiting but rather they are within the same order of magnitude of each other (i.e. $k_2 \approx k_3$). A large V_{max} effect would have suggested k_3 was clearly rate limiting but this was not observed. This, and the lack of a detectable burst, led us to conclude k_2 is not larger than k_3 . Absolute independence of V_{max} on substrate chain length would imply $k_3 > k_2$. Although all substrates (C₂ to C₁₀ inclusive) have similar V_{max} values (within experimental error) the observation of a trend indicates some chain length dependency.

The Michaelis constants for C₂-C₁₀ reveal very small but detectable progressive decreases in binding affinity for

an enzyme with increasing chain length. Thus we must stand by the conclusion that $k_2 \approx k_3$.

The turnover number and Michaelis constant for *p*-nitrophenyl laurate (C_{12}) are both unusually low. One would expect from the Michaelis constant trend for substrates with chain length C_2 - C_{10} inclusive that, concomitant with the reduced Michaelis constant for the C_{12} substrate, an increase in V_{max} would result, but this was not observed. This would imply that a solubility or partitioning effect is occurring which influences the value of k_3 .

The formation of an acyl enzyme complex during the esterase reaction would likely require attack by a nucleophilic center within the enzyme on the esterase bond. The susceptibility of the ester bond to hydrolysis would depend on the relative stability of the phenoxide leaving group. When we performed kinetics using substrates which yield phenoxide products of various stability, we found such a dependency did exist (Scheme 11).

Scheme 11: Acyl enzyme formation. The release the phenoxide on formation of the acyl enzyme is dependent on the stability of the phenoxide ion. Since *p*-nitrophenoxide is more stable than phenoxide and *p*-methylphenoxide, the reaction rate should be fastest for *p*-nitrophenoxide > phenoxide > *p*-methylphenoxide (adapted from Walsh (217)).

Three substrates were employed which afforded products of various stability. The most stable phenoxide product was from *p*-nitrophenyl octanoate. The electron withdrawing nature of the nitro substituent would give resonance stability to the phenoxide intermediate. Thus this leaving group is the most stable and would yield the highest reaction rate. Conversely, *p*-methylphenyl octanoate would yield a *p*-methylphenoxide intermediate which is inductively destabilized due to the electron donating nature of the methyl substituent. The phenyl octanoate substrate would be expected to have intermediate activity as there are no electron donating or withdrawing substitutions. As predicted, the *p*-nitrophenyl octanoate showed a 24 fold higher turnover number than phenyl octanoate and a 153 fold higher turnover number than *p*-methylphenyl octanoate. Therefore, the relative stabilities of the phenoxide products supports their production by nucleophilic attack by the enzyme and provides evidence for the formation of an acyl-enzyme complex. Despite the dramatic variation in V_{max} , the Michaelis constants showed no variation at all (within experimental error). Thus the acyl chain has a significant role in ester substrate binding to the enzyme but no direct affects in catalysis.

A pH velocity profile was generated for the esterase activity in an effort to identify active site ionizable species. Two ionisable groups were identified, one with pK 7.3 and the other with pK 10.35. The higher pK species would

appear to be lysine whereas the low pK species could be histidine or cysteine.

It is doubtful that lysine would act as a nucleophile because of its high inherent pK. The pH of the assay solutions would demand this group to exist in a protonated state which is not at all nucleophilic. In addition, if lysine was a nucleophile then V_{max} would be expected to increase with increasing pH (corresponds to increasing nucleophilicity), but this was not observed.

The effects of chemical modifications on esterase activity were determined in order to further elucidate catalytically essential residues for esterase activity. Surprisingly, none of the cysteine modifications had any effect on esterase activity whereas dehydrogenase activity was totally dependent on the presence of an intact cysteine species. Modification of tyrosine, arginine and tryptophan residues (separate studies) resulted in moderate inactivation of the esterase activity (50, 35 and 60% of control activity respectively). A significant modification effect on esterase activity was observed for lysine. The lysine modification resulted in rapid and total inactivation of esterase activity. The same modification showed no effect on dehydrogenase activity whatsoever. Amino acid analysis of methylacetamidinated enzyme revealed a single lysine residue per mole of monomeric enzyme was responsible for the inactivation effect. The esterase activity was eliminated on modification of histidine residues. The modification of

four histidine residues was previously shown to result in the inactivation of dehydrogenase and esterase activities. It would therefore appear that the dehydrogenase and esterase activities share common active site histidine(s). However the identification of the active site nucleophile responsible for acyl-enzyme formation would appear to be different for the two activities. The opposite dependencies on cysteine and lysine suggest the dehydrogenase and esterase activities do not employ the same catalytically active residues.

A pH profile was generated for the esterase activity in the presence of 2.5% of 1,4-dioxane. In this more hydrophobic environment, a slight shift in the low pK species (from pK 7.35 to 7.90) was observed. The profile revealed a loss in identification of a high pK species. At high pH values, log V_{max} values increase above those observed in the absence of dioxane and an apparent asymptote is obtained. It is possible that lysine serves in a charge relay or acid assisting function. The inherent basicity of lysine may function as an electron sink during formation of the tetrahedral adduct as described below (Scheme 12).

Scheme 12: Proposed charge relay function of lysine in the esterase activity of rat aldehyde dehydrogenase.

Two tetrahedral adducts are likely formed. One during the nucleophilic attack of the enzyme on the substrate and the second during the acyl-enzyme hydrolysis. Both would be destabilized in the presence of a more hydrophobic solvent. However, kinetic results indicated that both steps were of approximate equal magnitude in the absence of dioxane. In the presence of dioxane it appears that only the acyl-enzyme tetrahedral adduct is destabilized because the reaction rate is increased. If the tetrahedral adduct formed during acyl-enzyme formation were destabilized, it is unlikely the reaction would progress and an increase in velocity would not be expected. In a solvent of increased hydrophobic character, the stability of the second tetrahedral adduct would be decreased and therefore the reaction would tend towards a more concerted form. The decreased stability of this adduct would also be more sensitive to pH. This adduct would be charge repulsed at higher pH's. Thus at higher pH's and in the presence of dioxane, the lifetime of the

adduct would be shorter and hence an increase in $V_{\max}$ could be expected (as observed).

Evidence has been presented for and against the presence of esterase and dehydrogenase activities as having originated from the same active site. Duncan (59) has recently reviewed arguments in favor and against this. We have shown above that the esterase and dehydrogenase activities likely follow the same mechanism for product release (acyl enzyme formation and hydrolysis) however the catalytic residues may differ. Histidine is likely a common catalytic residue. Amino acid analysis of photooxidized enzyme reveal four histidines are responsible for the inactivation of the dehydrogenase and esterase activities. It is not possible to speculate whether either or both residues are required for esterase and dehydrogenase activities. It would appear that the remaining residues involved in the two activities are not the same. The two species identified by the pH profiles are not identical thus the higher pK species are probably influenced by neighboring residues. The roles of cysteine and lysine have been proposed in acyl enzyme formation for dehydrogenase and esterase activities respectively.

In order to probe the relationships of the active sites of the two enzyme activities, we have performed a series of nucleotide and effector studies on the enzyme. In all cases the responses of the activities are essentially identical. A discussion of these effector studies is presented with regard to the distinction of separate catalytic sites within

aldehyde dehydrogenase. This also provides additional evidence in support of a single molecular form of the enzyme.

Chloral hydrate has been observed to act as a non-competitive and a competitive inhibitor with respect to esterase and dehydrogenase activities in the sheep enzyme by MacGibbon et al. (26). This was the basis of their support for separate sites for the two activities. One major flaw in their study was the high degree of impurity of their enzyme preparation. The possibility of other esterase enzymes, which has already been discussed, could have led to errors in these studies. Chloral hydrate has been observed to inhibit several of the rat isozymes (39) (including mito-II) but no detailed studies have been provided. We have observed that chloral hydrate is a competitive inhibitor to NAD^+ and NADP^+ dependent dehydrogenase activities and esterase activities. Moreover, the inhibition constants for the activities are nearly identical (within experimental error).

The differential action of disulfiram on dehydrogenase and esterase activities has also been used in support of separate active sites. Both Kitson (95) and MacGibbon et al. (26) have found that dehydrogenase activity is more susceptible than esterase activity to the effects of disulfiram. They use this evidence in support of separate active sites. Both NAD^+ and NADP^+ activities of the rat isozymes were reported to be inhibited by disulfiram, but no detailed effects (including effect on esterase) were provided (39).

We therefore examined the action of disulfiram on NAD^+ and NADP^+ dehydrogenase and esterase activities. The inhibitor acted competitively for all activities. The inhibition constants were also quite comparable.

Diethylstilbestrol failed to inhibit any activity of our enzyme. An inhibitory effect was observed for the rabbit preparation of Duncan (54) and a mixed inhibitory/activatory effect was observed for the various rat isozymes of Lindahl and Evces (39).

We also examined the inhibitory effect of acetaldehyde on esterase activity and found that acetaldehyde acted as an uncompetitive inhibitor. This suggests that acetaldehyde (the dehydrogenase substrate) is binding to the esterase active site and that it does so after the ester substrate has bound. This would suggest that the ester-enzyme complex is analogous to the NAD(P)^+ -enzyme complex of the dehydrogenase reaction. If so, then support for the formation of an acyl-enzyme intermediate in the dehydrogenase reaction can be drawn by analogy from the esterase reaction (which was shown to involve an acyl-enzyme intermediate).

Therefore, similar responses of the esterase and dehydrogenase to chloral hydrate and disulfiram suggests that the esterase and dehydrogenase activities share a common or overlapping active site. The inhibition exerted by the dehydrogenase substrate on esterase activity also supports the hypothesis of the dehydrogenase and esterase activities as having arisen from similar active sites.

Magnesium ions have been shown to have dramatic effects on the dehydrogenase (80, 97, 99) and esterase activities (203). The horse enzyme was found to undergo a 2 fold increase in dehydrogenase activity in the presence of Mg^{+2} . The molecular weight of the enzyme was also seen to decrease from 260,000 to 130,000 Daltons during the same activation effect. Thus the enzyme was suggested to have exhibited a half-of-sites to all-of-sites activation in the presence of Mg^{+2} . Concomitant with this was the dissociation of the tetrameric enzyme into dimers. It was concluded that the action of Mg^{+2} on the dehydrogenase was to increase the number of functioning catalytic sites from 2 to 4 per molecule of tetrameric enzyme. The esterase activity which was not affected by Mg^{+2} led the authors to suggest that the esterase reaction is independent of the degree of aggregation of the enzyme. Dickinson and Haywood (99) have found Mg^{+2} to accelerate both dehydrogenase and esterase activities in the sheep cytosolic enzyme. This evidence is in favor of the esterase and dehydrogenase activities occurring at the same active site on the enzyme. Both studies are in contrast to our own. We were unable to demonstrate any Mg^{+2} effect with respect to either activity. Similarly, Lindahl and Evces (39) were unable to demonstrate this affect in any of their rat isozymes as well. It would therefore appear that the rat enzyme is significantly

different from the enzyme (particularly cytosolic) prepared from other sources and caution is advised when using discussions of the rat enzymes to make statements about aldehyde dehydrogenases in general.

✓
Additional evidence supporting a single active site has come from observation of the effects of nucleotides on the two activities. Feldman and Weiner (53) and Takahashi and Weiner (96) observed that NAD^+ and NADH exerted a two fold enhancement of the rate of ester hydrolysis in the horse enzyme. A similar enhancement using NADH was observed by Sidhu and Blair (51) for the human enzyme but a 5 fold enhancement was noted when NAD^+ was present. Conversely, Duncan (54) found NADH to exert an inhibitory effect in a rabbit enzyme. Hart and Dickinson (25) found that NAD^+ and NADH stimulated esterase activity to a maximum level equal to the maximum rate for aldehyde oxidation. Using a homogeneous rabbit liver enzyme, Duncan observed the hydrolysis of p-nitrophenyl acetate in the presence of NADH and found NAD^+ and a stoichiometric amount of aldehyde were produced. MacGibbon et al. (26) also observed stimulation of the esterase activity of sheep cytosolic aldehyde dehydrogenase by relatively low concentrations of NAD^+ and NADH but found inhibition by these cofactors resulted at higher concentrations.

The evidence presented above is taken to support the concept of a single active site for the two activities. Lindahl and Eyles (39) have looked at the effects of NAD^+ on

fore increase the rate of nucleophilic attack on the ester carbonyl during acyl enzyme formation. This argument provides a logical basis to explain the observed Ordered Bi Bi mechanism identified for the dehydrogenase activity of aldehyde dehydrogenase. Binding of the coenzyme forms a chemically reactive adduct which favours aldehyde binding. The tetrahedral adduct formed during acylation is favored in the presence of NAD^+ .

The activation constant we calculated for NAD^+ (assuming non-essential activation) was within the range of the Michaelis constant calculated for NAD^+ (by dehydrogenase kinetics) and therefore supports our assumption. The non-linear dependency of the secondary plot of $1/\Delta$ intercepts versus $1/[\text{NAD}^+]$ suggests the values of α and β are variable with $[\text{NAD}^+]$ and therefore provides an explanation for activation and inhibition by NAD^+ by simple interactive events. This idea is supported by Duncan (59). At low levels of NAD^+ a ternary complex of increased activity is formed thereby increasing the rate of esterolysis. At higher concentrations of NAD^+ , the coenzyme competes with the ester for the active site and therefore competitive inhibition is observed. Also in support of this non-essential activation is the previous observation that acetaldehyde acts as an uncompetitive inhibitor. Acetaldehyde was seen to bind to the enzyme only after an acyl enzyme was formed. Thus the ester can adequately bind to the enzyme independent of coenzyme.

Esterase kinetics performed in the presence of activating amounts of NAD^+ revealed an increase in V_{max} and a decrease in Michaelis constant for the substrate. Thus the presence of NAD^+ appears to increase ester-enzyme binding affinity as discussed above. A similar effect on V_{max} and K_m were observed when NMN was employed, however both effects were not as pronounced. This fragment, which contains the pyridine moiety of NAD^+ , is able to produce the activated ester-enzyme-ternary complex above and stimulate activity. However, the lack of an adenine moiety prevents the complete activation effect to be realized. The adenine species is likely involved only in the binding of the cofactor. This was confirmed using ADP and ADPR as coenzymes. No increase in esterase activity was observed using either, which is expected, because no pyridine ring is available to form an activated ternary complex. However, a decrease in K_m for the ester in the presence of each, suggests that the fragments do bind to the active site and exert their effect on the active site in favour of an increased esterase activity. The K_m effect using ADPR is greater than for ADP. This is expected as there are more functional groups available (in ADPR) for interaction with active site species than ADP and therefore would experience a greater interactive effect.

Therefore, the use of NAD^+ fragments provide further evidence supporting the esterase and dehydrogenase activities as having arisen from the same or overlapping active site(s). The pyridine moiety is directly involved in the

activation event likely by increasing the nucleophilicity of the active site nucleophile as described by Takahashi and Weiner (96). The remaining sections of the nucleotide fragment are involved in binding to the active site and likely exert a partial conformational change type of effect. Specific interactions between coenzyme and active site species such as those described by Rossmann (65) using X-ray data, cannot be assigned because of a lack of structural information for this enzyme.

An effort was made to encapsulate rat aldehyde dehydrogenase into lipid vesicles to observe the properties and kinetics of a reconstituted system. Attempts to encapsulate dehydrogenase activity were, for the most part, unsuccessful. It was very difficult to detect aldehyde dehydrogenase activity because of the low levels of specific activity of the enzyme encapsulated, and the high inherent noise of the liposomes when spectrophotometric assays were performed.

We were successful in producing very large unilamellar vesicles containing a sufficiently low quantity of residual detergent. This makes the preparation ideal for encapsulation and kinetic studies of biomacromolecules. The electron microscopic photograph shown in Plate 3a clearly reveals the aqueous environment encapsulated. This photograph is the "nicest" picture of a LUV ever seen by the author.

Successful encapsulation of β -glucosidase was achieved. A cursory investigation was made using this enzyme to determine the effect of cholesterol on the transport of substrate

molecules through the liposomal membrane. Cholesterol was observed to decrease an apparent diffusion effect at low concentrations of substrate.

Encapsulation of rat aldehyde dehydrogenase failed to yield an observable dehydrogenase activity. However, it was possible to monitor the esterase activity. A limited study was made to investigate the influences of several, known mitochondrial membrane phospholipids on the activity. The action of phosphatidyl ethanolamine and cardiolipin appeared to activate the enzyme. We have shown above that the enzyme is membrane bound in its *in vivo* state. Appreciable activation of esterase activity was observed to be concentration dependent in the case of phosphatidyl ethanolamine. Synergistic effects of phospholipids were not investigated. The appreciable activation observed separately for near *in vivo* levels of phosphatidyl ethanolamine and cardiolipin, suggest that combinations of the lipids may yield interesting synergistic effects. This is suggested as a topic for future study.

CONCLUSIONS AND ORIGINAL CLAIMS

We have achieved the first homogeneous preparation of a rat liver aldehyde dehydrogenase. A partial characterization of the physical and structural properties of the enzyme was presented and a comparison was made with other liver preparations. This provides further knowledge into the basis of aldehyde dehydrogenase action and also furthers the knowledge of dehydrogenase enzymes in general. It is apparent that the mechanism of aldehyde dehydrogenases varies slightly from source to source. A detailed kinetic study was done with respect to NAD^+ and NADP^+ dependent dehydrogenase activities to provide an understanding of the mechanism of this enzyme. We have provided the most comprehensive mechanistic information regarding the NAD^+ and NADP^+ reactions of any of the aldehyde dehydrogenase preparations to date.

The dehydrogenase mechanism was shown to follow Ordered Bi Bi kinetics. Stopped-flow and steady state kinetic studies were used to measure and compare kinetic constants for NAD^+ and NADP^+ dependent dehydrogenase activities. The NADP^+ reaction was found to be the most catalytically favourable up to the release to the reduced cofactor. A conformational change was proposed to cause the effective lower catalytic efficiency for NADP^+ , as determined by steady state measurements. A detailed mechanism was proposed for the dehydrogenase activities which support the formation of an abortive ternary complex, the conformational change

above, and the burst of reduced cofactor formation as measured by stopped-flow analysis. We are the first to provide evidence which accounts for the difference in the activities between the two cofactors. The difference in activities is based on the unique active site structures. Chemical modifications and ^{31}P NMR were used to identify those essential amino acids responsible for the activities and to provide an explanation for the differences in the activities. An active site arginine is essential for all activities and provides a necessary interaction for NADP^+ activity. Two histidine residues and one cysteine are shown to be catalytically essential for dehydrogenase activity.

The enzyme was shown to be bifunctional. An esterase activity was identified and has been shown to arise from either the same or an associated active site as the dehydrogenase activities. The subject of independent active sites for the two activities is presently the topic of heated debate. We provided evidence which shows that, although the esterase and dehydrogenase activities may share the same active site, and in some cases the same active site residues, it is unlikely that cysteine plays a role in the acyl-enzyme formation of the two activities. The esterase activity was shown to be unlikely to involve the use of the active site cysteine. The role of an essential lysine was proposed in the esterase activity.

The esterase reaction was shown to involve the formation of an acyl-enzyme. Histidine residues, which are like-

ly involved in the acyl-enzyme hydrolysis, are shared by the dehydrogenase activity. An Ordered Uni Bi mechanism was proppsed for the esterase activity.

We have successfully reconstituted aldehyde dehydrogenase into phospholipid vesicles. The esterase activity was shown to be activated when the enzyme was encapsulated. This shows that a suitable hydrophobic environment is necessary for maximum *in vivo* activity.

APPENDIX I

EXPFIT256/09 PROGRAM

The following pages contain a hardcopy of the data acquisition, plotting and curve fitting program used by the Commodore CBM computer to fit digitized data obtained from stopped-flow experiments

REMOT.

```

10 DIM:OBS(1,256),YSMOOTH(256),P(5),YCALC(1,256),PB(5),E(5,5)
12 DIMF(256,5),H(5),C(5),S(5),U(5,5),AB(5,5),SF(5),PS(5)
14 INPUT "TODAYS DATE IS " DATE$
16 FOR I=1 TO 256:YOB(1,I)=0:NEXT I
18 FOR I=1 TO 5:P(I)=0:NEXT I
20 PRINT "DATA SOURCE "
22 PRINT "TRANSIENT RECORDER....1" PRINT "DISKETTE .... 2"
24 GETA:IF A$="" THEN 24
26 IF A$="2" THEN 162
28 IF A$="1" THEN 24
30 PRINT "DATA DESCRIPTION UP TO 255"
32 INPUT "CHARACTERS " IDE$
34 NAV=0:INPUT "TIME PER POINT IN MILLISECOND " T$
36 PRINT "MAKE SURE THAT THE RESET BUTTON"
38 PRINT "HAS BEEN PRESSED AND THAT TIME "
40 PRINT "BASE SELECT IS IN EXTERNAL MODE"
42 PRINT "WHEN READY TO TRANSFER DATA " "PRESS Q KEY"
44 GETA:IF A$="" THEN 44
46 IF A$="Q" THEN 44
48 PP=59471:PIN=59458:FLOW=59467:PHI=59468:POKE PIN,0
50 PRINT "COLLECTING DATA IN BUFFER 0"
52 FOR I=1 TO 256
54 POKEFLOW,PEEK(PFLOW)AND277
56 POKEPHI,PEEK(PHI)AND310R182
58 YOB(0,I)=PEEK(PP)
60 POKEPHI,PEEK(PHI)OR244
62 NEXT I
64 PRINT "DATA TRANSFER COMPLETE"
66 PRINT "ADD BUFFER 0 TO BUFFER 1 ?"
68 PRINT "Y OR N ?"
70 GETA:IF A$="" THEN 70
72 IF A$="N" THEN 80
74 IF A$="Y" THEN 70
76 PRINT "ADDING"
78 FOR I=1 TO 256:YOB(1,I)=(YOB(1,I)+(YOB(0,I)+(NAV+YOB(0,I)))/(NAV+1)):NEXT I:NAV=NAV+1
80 PRINT "DO YOU WANT TO COLLECT MORE DATA"
82 PRINT "USING THE SAME TIME/POINT " PRINT "Y OR N ?"
84 GETA:IF A$="" THEN 84
86 IF A$="Y" THEN 86
88 IF A$="N" THEN 84
90 PRINT "OPTIONS (2-8 ARE PERFORMED ON BUFFER 1)"
92 PRINT "COLLECT A NEW DATA SET.....0"
94 PRINT "COLLECT MORE DATA FROM T.R. TO ADD "
96 PRINT "TO THAT ALREADY IN BUFFER 1.....1"
98 PRINT "STORE ACQUIRED DATA ON DISKETTE....2"
100 PRINT "CHECK DATA FOR BAD POINTS.....3"
102 PRINT "SMOOTH DATA.....4"
104 PRINT "FIT DATA.....5"
106 PRINT "PLOT DATA.....6"
108 PRINT "PLOT DATA AND DRAW FITTED LINE....7"
110 PRINT "ADD FITTED LINE TO DATA PLOT.....8"
112 PRINT "STOP PROGRAM.....9"
114 GETA:IF A$="" THEN 114
116 IF A$="0" OR A$="9" THEN 114
118 A=VAL(A$):J=1:IFA=8 THEN STOP
120 ON A TO 36,710,622,604,138,216,224
122 PRINT "BUFFER 1 WILL BE ZEROED,SO DO YOU"
124 PRINT "WANT TO SAVE THE DATA IT CONTAINS"
126 PRINT "FIRST ? Y OR N ?"
128 GETA:IF A$="" THEN 128

```

```

130 IFQ$="1" THEN 16
132 IFQ$="2" THEN 128
134 AA=1:GOTO 710
136 AA=0:GOTO 16
138 PRINT "*****SINGLE (1) OR DOUBLE (2) EXPONENTIAL?*"
140 GETA$ IF A$="" THEN 140
142 IF A$="1" OR A$="2" THEN 140
144 PRINT "***** IF +VE GOING CURVE HAS A -VE AMPLITUDE*"
146 IE=VAL(A$):Z1=1:Z2=1:IF IE=2 THEN 158
148 PRINT "***** INPUT SIGN OF AMPLITUDE I.E. + OR - *"
150 GETA$ IF A$="" THEN 150
152 IF A$="+" THEN 168
154 IF A$="-" THEN 150
156 Z1=-1:GOTO 168
158 PRINT "***** INPUT SIGN OF AMPLITUDE FOR SLOW AND*"
160 PRINT "***** FAST EXPONENTIALS I.E. +,+,+,-,-,-"
162 INPUT Z2, Z3, Z4 OR Z1, Z2, Z3, Z4
164 IF Z3="" THEN Z1=-1
166 IF Z4="" THEN Z2=-1
168 FOR I=1 TO 5: P(I)=0: NEXT I
170 GOSUB 220
172 P(2)=P(2)/Z1: P(4)=P(4)/Z2
174 PRINT "***** DO YOU WANT THE RESULTS PRINTED*"
176 PRINT "***** Y OR N ?*"
178 GETA$ IF A$="" THEN 178
180 IF A$="Y" THEN 190
182 IF A$="N" THEN 178
184 OPEN 13, 4: PRINT#3, CHR$(147)
186 PRINT#3, "*****"
188 PRINT#3, "DATE DATA ACQUIRED : " : DATE$: PRINT#3
190 PRINT#3, "DATA DESCRIPTION : " : DES$: PRINT#3
192 PRINT#3, "TIME/POINT (MSEC) : " : TP$: PRINT#3
194 PRINT#3, "NUMBER OF SCANS AVERAGED : " : NAV$: PRINT#3
196 PRINT#3, "CONSTANT : " : P(1): PRINT#3, "AMPLITUDE (1) : " : P(2)
198 PRINT#3, "RATE CONSTANT (1) : " : P(3)
200 PRINT#3, "AMPLITUDE (2) : " : P(4): PRINT#3, "RATE CONSTANT (2) : " : P(5)
202 PRINT#3
204 PRINT#3, "*****"
206 PRINT#3, CHR$(19): CLOSE 3
208 GOTO 90
210 GOSUB 734
212 GOSUB 780
214 GOTO 90
216 GOSUB 734
218 GOSUB 780
220 GOSUB 114
222 GOTO 90
224 GOSUB 802
226 GOTO 90
228 PRINT "***** METHOD TO BE USED FOR A LINEAR*"
230 PRINT "***** LEAST SQUARES FIT OF THE DATA*"
232 IF IE=2 THEN PRINT "***** [O THE SLOW EXPONENTIAL*"
234 PRINT "***** (LOG A) VS TIME....." : PRINT "***** SWINBOURNE'S METHOD : 2*"
236 PRINT "***** ( METHOD 1 REQUIRES A GOOD ESTIMATE OF A AT INFINITY"
238 GETA$ IF A$="" THEN 238
240 IF A$="1" OR A$="2" THEN 238
242 IE=VAL(A$)
244 PRINT "***** RANGE OF DATA POINTS TO*"
246 PRINT "***** BE USED IN THE FITTING*"
248 IF IE=2 THEN PRINT "***** [O THE SLOW EXPONENTIAL*"
250 PRINT "***** THE FULL RANGE IS 1 TO" : PRINT "***** (256 POINTS)*"
252 IF IE=2 THEN 258: PRINT "***** A GOOD LAST POINT IS*"
254 PRINT "***** A GOOD LAST POINT IS*"
256 PRINT "***** = 3*HALF-LIFE*"
258 INPUT "***** FIRST AND LAST POINTS" : FP, LP
260 IF IE=2 THEN 272

```

```

262 AV=0:51+0:82+0:83+0:84+0:85+0:1N:0
264 FOR I=207 TO 256:AV=AV+Y0BS(1,1):NEXT I:P(1)=AV/30
266 FOR I=FP TO LP:Y=LOG(ABS(P(1)-Y0BS(1,1)))/X:(1-1)*TP/1000:S1=S1+X
268 S2=S2+X*Y:84=84+Y:83=83+X*Y:85=S5+Y*Y:NN=NN+1:NEXT I
270 P(3)=-((N*82-S1*84)/(N*83-S1*81)):P(2)=EXP((84+P(3)*S1)/N):GOTO 286
272 PRINT "-----"
274 INPUT "SUGGESTED VALUE IS 0.5*HALF-LIFE/DT"
276 S1=0:82=0:83=0:84=0:85=0:NN=0
278 FOR I=FP TO LP-DT:Y=Y0BS(1,1):X=Y0BS(1,1+DT):S1=S1+X:S2=S2+X*Y:84=84+Y
280 83=83+X*Y:85=S5+Y*Y:NN=NN+1:NEXT I
282 Q1=((N*82-S1*84)/(N*83-S1*81)):P(3)=LOG(Q1)/(DT*TP/1000)
284 Q2=((84-Q1*81)/81):P(1)=Q2/(1-Q1):P(2)=-((P(1)-Q2-Q1*Y0BS(1,DT))/Z1)
286 PRINT "-----"
288 IF N=2 THEN PRINT "FOR THE SLOW EXPONENTIAL THE"
290 PRINT "PARAMETER ESTIMATES ARE:"PRINT "CONSTANT" *P(1)
292 PRINT "AMPLITUDE" *P(2)/Z1:PRINT "RATE CONSTANT" *P(3)
294 PRINT "-----"
296 PRINT "DO YOU WANT TO DO A NON-LINEAR LEAST"
298 PRINT "SQUARES FIT USING THESE ESTIMATES? Y OR N"
300 GETA:IF A$="" THEN 308
302 IF A$="Y" THEN 318
304 IF A$="N" THEN 308
306 IF N=1 THEN RETURN
308 PRINT "DO YOU WANT TO REDETERMINE THE SLOW EXP. ESTIMATES Y OR N?"
310 GETA:IF A$="" THEN 310
312 IF A$="N" THEN 332
314 IF A$="Y" THEN 310
316 GOTO 244
318 PRINT "-----"
320 PRINT "USED IN THE BEST FIT (THE FULL RANGE"PRINT "IS 1 TO 256)"
322 SR=INT(4*1000/(P(3)*TP)):IF SR>256 THEN SR=256
324 PRINT "THE SUGGESTED LAST POINT IS"SR
326 INPUT "FIRST AND LAST POINTS"FP,LP:N=3
328 GOSUB 396
330 IF N=1 THEN RETURN
332 PRINT "-----"
334 PRINT "USED IN ESTIMATING THE PARAMETERS OF"
336 PRINT "THE FAST EXPONENTIAL (THE FULL RANGE"PRINT "IS 1 TO 256)"
338 INPUT "FIRST AND LAST POINTS"FP,LP
340 S1=0:82=0:83=0:84=0:85=0:NN=0
342 FOR I=FP TO LP:Y=LOG(ABS(P(1)-Y0BS(1,1))/Z2-P(1)/Z2-(Z1/Z2)*P(2)*EXP(-P(3)*X)))
344 82=S2+X*Y:84=84+Y:83=83+X*Y:85=S5+Y*Y:NN=NN+1:NEXT I
346 P(3)=-((N*82-S1*84)/(N*83-S1*81)):P(4)=EXP((84+P(3)*S1)/N)
350 PRINT "-----"
352 PRINT "FOR THE DOUBLE EXPONENTIAL FIT THE"
354 PRINT "ROUGH FIT PARAMETER ESTIMATES ARE:"
356 PRINT "CONSTANT" *P(1):PRINT "AMPLITUDE (1)" *P(2)/Z1
358 PRINT "RATE CONSTANT (1)" *P(3):PRINT "AMPLITUDE (2)" *P(4)/Z2
360 PRINT "RATE CONSTANT (2)" *P(5)
362 PRINT "-----"
364 PRINT "DO OTHER ESTIMATES? Y OR N?"
366 GETA:IF A$="" THEN 366
368 IF A$="N" THEN 382
370 IF A$="Y" THEN 366
372 PRINT "INPUT NEW PARAMETERS MANUALLY Y OR N?"
374 GETA:IF A$="" THEN 374
376 IF A$="N" THEN 380
378 IF A$="Y" THEN 374
380 FOR I=1 TO 5:PRINT "PARAMETER":INPUT PX:P(1)=ABS(PX):NEXT I
382 PRINT "-----"
384 PRINT "INPUT RANGE OF DATA POINTS TO BE USED IN"
386 SR=INT(3*1000/(P(3)*TP)):IF SR>256 THEN SR=256
388 PRINT "BEST FIT, SUGGESTED LAST POINT IS"SR
390 INPUT "FIRST AND LAST POINTS"FP,LP
392 N=5:IF LP>256 THEN LP=256
394 GOSUB 396

```

```

394 RETURN
396 INPUT "INPUT INTERVAL FOR FIRST ROUGH FIT: " IRF
398 I1=0:D6=.866:D5=10:D7=.002:D4=1:SS=0:PM=1
400 PRINT "*****" "THINKING"
402 FOR I=1 TO N:SF(I)=P(I):P(I)=1:NEXT I
404 FOR I=1 TO N:PS(I)=P(I):C(I)=0:SC(I)=0:E(1,I)=1
406 FOR I1=1 TO N:AB(1,I1)=0:UC(1,I1)=0:E(1,I1)=0:NEXT I1:NEXT I
408 D9=2:D8=1.7:I1=1+I:D2=0:S1=0:S2=0:S3=0
410 FOR IJ=1 TO N:PS(IJ)=P(IJ):SF(IJ):NEXT IJ
412 KS=PS(3)*TP/1000:KF=PS(5)*TP/1000
414 FOR I=FPTOLPSTEPF
416 YCALC(0,I)=PS(I)+Z1*PS(2)*EXP(-KS*(I-1))
418 IF N=3 THEN 422
420 YCALC(0,I)=YCALC(0,I)+Z2*PS(4)*EXP(-KF*(I-1))
422 S3=YOBSC(1,I)-YCALC(0,I):S2=S2+S3:S3=NEXT I
424 S1=S2:IF I1) THEN 430
426 PRINT "INITIAL SUM OF SQUARES=" S2
428 PRINT "*****" "STILL THINKING"
430 FOR I1=1 TO N:BI=P(I1)*P(I1):P(I1)=D7*P(I1)
432 FOR IJ=1 TO N:PS(IJ)=P(IJ):SF(IJ):NEXT IJ
434 KS=PS(3)*TP/1000:KF=PS(5)*TP/1000
436 FOR I=FPTOLPSTEPF
438 YCALC(1,I)=PS(I)+Z1*PS(2)*EXP(-KS*(I-1))
440 IF N=3 THEN 444
442 YCALC(1,I)=YCALC(1,I)+Z2*PS(4)*EXP(-KF*(I-1))
444 F(1,I1)=YCALC(1,I)-YCALC(0,I)/D7:NEXT I1:P(I1)=BI:NEXT I1
446 FOR I1=1 TO N:S5=0
448 FOR I=FPTOLPSTEPF:S2=YOBSC(1,I)-YCALC(0,I):S5=S5+S2*F(1,I1):NEXT I
450 C(I1)=S5
452 FOR IJ=1 TO I1:S5=0
454 FOR I=FPTOLPSTEPF:S5=S5+F(1,I1)*F(1,IJ):NEXT I
456 AB(1,IJ)=S5:NEXT IJ:NEXT I1
458 FOR I=1 TO N:IF AB(1,I) > 0 THEN 462
460 SC(I)=1:GOTO 464
462 SC(I)=SQR(AB(1,I))
464 NEXT I
466 FOR I=1 TO N:C(I)=C(I)/SC(I)
468 FOR I1=1 TO N:AB(1,I1)=AB(1,I1)/SC(I)/SC(I1):AB(1,I1)*AB(1,I1):NEXT I1
470 NEXT I
472 D4=D4/D5
474 FOR I=1 TO N:H(I)=C(I)
476 FOR I1=1 TO N:UC(1,I1)=AB(1,I1):E(1,I1)=AB(1,I1):NEXT I1
478 UC(1,I)=UC(1,I)+D4:NEXT I
480 FOR I=1 TO N:D=UC(1,I):D1=E(1,I)
482 FOR K=1 TO N:E(1,K)=E(1,K)/D1:UC(1,K)=UC(1,K)/D:NEXT K
484 H(1)=H(1)/D
486 FOR I1=1 TO N:IF (I-11)=0 THEN 494
488 F1=UC(11,I):F2=E(11,I)
490 FOR K=1 TO N:E(11,K)=E(11,K)-E(1,K)*F2:(K11,K)=UC(11,K)-UC(1,K)*F1:NEXT K
492 H(11)=H(11)-H(1)*F1
494 NEXT I1
496 NEXT I
498 S6=0:S7=0:S8=0
500 FOR I=1 TO N:S6=S6+H(1)*C(I):S7=S7+H(1)*H(1):S8=S8+C(1)*C(1):NEXT I
502 S9=S6/SQR(S7*S8):IF 9=1
504 FOR I=1 TO N:P(I)=P9(I)+F9*H(1)/SC(I):D1=10
506 IF P(I) > 0 THEN 510
508 P(I)=P9(I)+F9*H(1)/(D1*SC(I)):D1=D1*2:GOTO 506
510 NEXT I
512 S2=0
514 FOR IJ=1 TO N:PS(IJ)=P(IJ):SF(IJ):NEXT IJ
516 KS=PS(3)*TP/1000:KF=PS(5)*TP/1000
518 FOR I=FPTOLPSTEPF
520 YCALC(0,I)=PS(I)+Z1*PS(2)*EXP(-KS*(I-1))
522 IF N=3 THEN 526
524 YCALC(0,I)=YCALC(0,I)+Z2*PS(4)*EXP(-KF*(I-1))

```

```

520 S3=OBS(1,1)-YCALC(0,1):S2=S2+S3+S3*NEXT1
528 IF S2<S1 THEN GOTO 538
530 IF O2<O1 THEN GOTO 538
532 O2=O2+1:IF (S9-O6)<O6 THEN GOTO 536
534 F9=F9/O9:O9=O9+O8:GOTO 534
536 O4=O4+O5:GOTO 474
538 FOR J=1 TO N
540 FOR I=1 TO N:O3=S(1)+S(1):E(1,1)=E(1,1)/O3:NEXT I:NEXT J
542 PRINT "SUM OF SQUARES=";S2;" " IF O1=1 TO 38:PRINT " " :NEXT I:PRINT
544 PRINT "STILL THINKING?"
546 FOR J=1 TO N:O1=P(1)+S(1):NEXT J
548 IF ABS(S5-S2)<.001+S2 THEN GOTO 552
550 S5=S2:IF I<5 THEN GOTO 552
552 PRINT "-----"
554 IF N=3 AND O9=2 THEN PRINT "FOR THE SLOW EXPONENTIAL THE"
556 IF P(1) THEN PRINT "NEW ROUGH ESTIMATES ARE:" GOTO 560
558 PRINT "NEW ESTIMATES ARE:"
560 PRINT "CONSTANT " P(1):PRINT "AMPLITUDE " P(2)/2
562 PRINT "RATE CONSTANT " P(3):IF N=3 THEN GOTO 566
564 PRINT "AMPLITUDE " P(4)/2:PRINT "RATE CONSTANT " P(5)
566 PRINT "-----"
568 PRINT "DO YOU WANT TO CONTINUE THE SEARCH? Y OR N?"
570 GETAS:IFAS="" THEN GOTO 578
572 IFAS="Y" THEN GOTO 578
574 IFAS="N" THEN GOTO 576
576 RETURN
578 PRINT "BEFORE CONTINUING DO YOU WANT TO DEAF?"
580 PRINT "THE CURRENT FITTED LINE (Y OR N)?"
582 GETAS:IFAS="" THEN GOTO 582
584 IFAS="N" THEN GOTO 582
586 IFAS="Y" THEN GOTO 582
588 P(2)=P(2)/2:IF P(4)=P(4)/2:GOTO 588
590 P(2)=P(2)/2:IF P(4)=P(4)/2
592 PRINT "DO YOU WANT TO CHANGE TO A SMOOTH FIT?"
594 PRINT "USING ALL DATA POINTS FROM " P(1) " TO " P(4) " Y OR N?"
596 GETAS:IFAS="" THEN GOTO 596
598 IFAS="N" THEN GOTO 598
600 IFAS="Y" THEN GOTO 596
602 RE=1:GOTO 398
604 PRINT "INPUT SMOOTHING INTERVAL. A SHORT"
606 PRINT "INTERVAL REMOVES HIGH FREQUENCY NOISE"
608 PRINT "WHILE A LARGER INTERVAL REMOVES LOWER"
610 PRINT "FREQUENCY NOISE. SUGGESTED INTERVALS ARE:"
612 PRINT "FOR SINGLE EXPONENTIAL DATA: 1, 3, 10, AND 40"
614 PRINT "AND FOR DOUBLE EXPONENTIAL DATA: 1, 3, AND 10"
616 INPUT "CHOICE OF INTERVAL =":I:PRINT "SMOOTHING="
618 FOR I1=(3+1) TO (256-3+1)
620 S1=6*(OBS(1,I1+1)+OBS(1,I1-1)):S2=3*(OBS(1,I1+2+1)+OBS(1,I1-2+1))
622 S3=2*(OBS(1,I1+3+1)+OBS(1,I1-3+1))
624 YSMOOTH(I1)=(7*OBS(1,I1)+S1+S2+S3)/21:NEXT I1
626 FOR I1=(3+1) TO (256-3+1):OBS(1,I1)=YSMOOTH(I1):NEXT I1:GOTO 590
628 RBP=0:IFBP=0:PRINT "CHECKING"
630 FOR I=1 TO 254
632 IF ABS(OBS(1,I+1)-(OBS(1,I)+OBS(1,I+2))/2)<10 THEN GOTO 538
634 IFBP=IFBP+1:IF RBP<1 THEN GOTO 538
636 OBS(1,I+1)=(OBS(1,I)+OBS(1,I+2))/2
638 NEXT I
640 IF IFBP=0 THEN GOTO 538
642 IF RBP=1 THEN GOTO 538
644 PRINT "NUMBER OF BAD POINTS DETECTED IS=" IFBP
646 PRINT "REMOVE BAD POINTS Y OR N?"
648 GETAS:IFAS="" THEN GOTO 548
650 IFAS="N" THEN GOTO 548
652 IFAS="Y" THEN GOTO 548
654 PRINT "REMOVING BAD POINTS="
656 GOTO 538

```

```

856 ABF=10070660
858 PRINT "*****"
860 GOTO 900
862 PRINT "*****"
864 GETDIR: IF DIR#="" THEN 864
866 IF DIR#="0" OR DIR#="1" THEN 864
868 INPUT "*****" INPUT FILENAME: IF F#=""
870 OPEN 1,2,DIR#+"*"+F#+"",SEQ,READ
872 IF DSK<>62 THEN 878
874 PRINT "*****" FILE NOT FOUND *****
876 DCLOSE: 10070662..
878 IF DSK<>62 THEN PRINT DSK: STOP
880 INPUT 1,DATE,DESS
882 PRINT "*****"
884 PRINT "DATE DATA ACQUIRED " DATE: PRINT "DATA DESCRIPTION"
886 PRINT "*****" PRINT DESS: INPUT 1,TP,INV
888 PRINT "*****" POINT "*****" SCANS AVERAGED
890 FOR I=1 TO 5: INPUT 1,PC:
892 IF PC<>63 THEN PRINT "PARAMETER " PC:
894 NEXT I
896 PRINT "*****" IS THIS THE DATA YOU WANT Y OR N?
898 GET A: IF A#="" THEN 898
900 IF A#="Y" THEN 906
902 IF A#="N" THEN 898
904 DCLOSE: 10070662
906 FOR I=1 TO 256: INPUT 1,YOBS(1,1): NEXT I: DCLOSE:
908 PRINT "*****" DATA HAS BEEN PLACED IN BUFFER 10070700
910 PRINT "*****" INPUT DRIVE NUMBER:
912 GETDIR: IF DIR#="" THEN 912
914 IF DIR#="0" OR DIR#="1" THEN 912
916 INPUT "*****" INPUT FILENAME: IF F#="" OPEN 1,2,DIR#+"*"+F#+"",SEQ,WRITE
918 IF DSK<>63 THEN 924
920 PRINT "*****" FILE EXISTS *****
922 FOR I=1 TO 60: NEXT I: DCLOSE: 10070710
924 IF DSK<>63 THEN PRINT DSK: STOP
926 CR$=CHR$(13): PRINT 1,DATE,CPS,DESS,CR$,TP,CR$,INV
928 FOR I=1 TO 5: PRINT 1,PC: NEXT I
930 FOR I=1 TO 256: PRINT 1,YOBS(1,1): NEXT I: DCLOSE: IF A#="N" THEN 936
932 GOTO 900
934 PRINT "*****" INPUT THE RANGE OF DATA TO BE PLOTTED:
936 PRINT "*****" STARTING AT TIME ZERO:
938 INPUT "*****" RANGE = 1 TO N WHERE N (= 256)
940 IF R<>256 THEN R=256
942 OPEN 129,6
944 PRINT 129,"INI 1000,800,8000,7400"
946 PRINT 129,"SC0,1000,0,1000/PAC,0/PO"
948 PRINT 129,"PA0,1000,1000,1000,1000,0,0,0/PUT,1"
950 TR=TP*(R0-1)
952 FC=10*(INT(LOG(2*TR)/LOG(10))-1)
954 TF=TR/FC: IF TF<10 THEN DX=FC: GOTO 958
956 TF=TF/2: IF TF<10 THEN DX=2*FC: GOTO 958
958 TF=TF/2: 51DX=5*FC
960 TF=INT(TF): PRINT 129,"S1,19,,27/PA0,0/CP-1,5,-.9/LB0*CH0*03"
962 TS=1: IF FC>1 THEN TS=1000
964 FOR I=1 TO TS: XX=INT(1+DX*1000/TP): LWB$=STR$(1+DX/TS)
966 PRINT 129,"PA*XX",0/XT: CP-1,5,-.9/LB*/RIGHT$(LWB$,LEN(LWB$)-1) CH0*03)
968 NEXT I
970 XL$="TIME (HSEC)*IF FC>1 THEN XL$="TIME (SEC)"
972 PRINT 129,"S1,27,.4/PA150,-63/LB",XL$,CHR$(03)
974 INPUT "*****" INPUT PLOT LABEL: IT0
976 PRINT 129,"PA25,1025/LB*/RIGHT$(IT0,LEN(IT0)),CHR$(03)
978 PRINT 129,"PA0,0/S1,19,,27" : CLOSE 129: RETURN
980 PRINT "*****" INPUT NUMBER OF DATA POINT AT WHICH
982 INPUT "*****" PLOT IS TO START: SP: Y1=YOBS(1,SP): Y2=Y1
984 FOR I=SP+1 TO R0
986 IF YOBS(1,I)>Y1 THEN Y1=YOBS(1,I): GOTO 978

```


rat isozymes. Although their study is limited, they were able to show that all isozymes were inhibited by levels of NAD^+ above $100 \mu\text{M}$. They found only Mito-I and Micro-I isozymes to be activated by low levels of NAD^+ ($20 \mu\text{M}$). The isozymes Mito-II and Micro-II were unaffected by low concentrations of NAD^+ .

We investigated the activatory and inhibitory effects of NAD^+ on our enzyme in order to provide additional support for the existence of the dehydrogenase and esterase activities as arising from the same active site. In addition, we employed NAD^+ fragments to attempt to pin-point specific NAD^+ -active site interactions.

We observed a very slight stimulation of esterase activity in the presence of $322 \mu\text{M}$ NAD^+ . The activation effect was small with the activation factor equal to 1.11. Because the activation showed a dependency on NAD^+ (albeit small) as was observed by Hart and Dickinson, it was concluded that the activation event was of a non-essential nature involving the formation of an ester-enzyme- NAD^+ complex. Such an activated ternary complex has been proposed for the horse enzyme by Feldman and Weiner (53), the human enzyme by Sidhu and Blair (51), the rabbit enzyme by Duncan (54) and the sheep enzyme by Hart and Dickinson (25). Stopped-flow experiments of Takahashi and Weiner showed the NAD^+ activation is manifested during the burst event. They therefore concluded that NAD^+ acted to increase the nucleophilicity of the active site amino acid nucleophile. This would there-

```

788 IF YOSS(1,1)Y2 THEN Y2=YOSS(1,1)
790 NEXT I
792 IF Y1=INT((Y2-Y1)/10)Y2=Y2+INT((Y2-Y1)/5)
794 OPEN 129,6:PRINT#129,"LT0:PD"
796 FOR I=SP TO P0:XX=INT((I-1)*1000/(P0-1)):YY=INT((YOSS(1,1)-Y1)+1000*(Y2-Y1))
798 PRINT#129,"PA:XX",":YY":NEXT I
800 PRINT#129,"PU:PA1000,1000/LT:ICLOSE 129:RETURN
802 PRINT "***** HAVE YOU PLOTTED THE DATA YET ***** OR N ?"
804 GETAB:IFAB="":THEN 804
806 IFAB="Y":THEN 814
808 IFAB="N":THEN 804
810 GOSUB 734
812 GOSUB 780
814 IE=2:IF P(5)=0 THEN IE=1
816 OPEN 129,6:COUNT=0
818 FORX=0 TO 1000 STEP 5:XX=X*(P0-1)/1000:IFXX((SP-1) THEN 836
820 XX=XX+TP/1000:IF IE=2 THEN 824
822 YY=P(1)+P(2)*EXP(-P(3)*XX):GOTO 826
824 YY=P(1)+P(2)*EXP(-P(3)*XX)+P(4)*EXP(-P(5)*XX)
826 Y=INT((YY-Y1)+1000*(Y2-Y1))
828 IF YY=Y THEN N1=0:GOTO 832
830 IF YY>Y2 THEN N1=1000
832 IF COUNT=0 THEN PRINT#129,"PA:XX",":YY":PD:COUNT=1:GOTO 836
834 PRINT#129,"PA:XX",":YY":
836 NEXT X
838 P1=INT(P(1)+100)/100:P2=INT(P(2)+100)/100:P4=INT(P(4)+100)/100
840 FF=10*(3-INT(LOG(P(3))/LOG(10))):P3=INT(P(3)+FF)/FF
842 PRINT#129,"PA25,950/CP,1.27/LB:CONSTANT (1) ":P1,CHR$(63)
844 PRINT#129,"PA25,950/CP,1.27/LB:AMPLITUDE (1) ":P3,CHR$(63)
846 PRINT#129,"PA400,950/CP,1.27/LB:RATE CONSTANT (1) ":P3,CHR$(63)
848 IF IE=1:GOTO 856
850 FF=10*(3-INT(LOG(P(5))/LOG(10))):P5=INT(P(5)+FF)/FF
852 PRINT#129,"PA25,950/CP,1.27/LB:AMPLITUDE (2) ":P5,CHR$(63)
854 PRINT#129,"PA400,950/CP,1.27/LB:RATE CONSTANT (2) ":P5,CHR$(63)
856 PRINT "***** HAVE YOU PLOTTED THE RESIDUALS Y OR N ?"
858 GETAB:IFAB="":THEN 858
860 IFAB="N":THEN 860
862 IFAB="Y":THEN 858
864 UNS=STR$(P0)
866 PRINT#129,"PU:PA1100,1010/CP-1.5,0/LB:RIGHTS(UNS,LEN(UNS)-1),CHR$(63)
868 PRINT#129,"PA1100,1000/TL5,5:YT:PD:PA1100,0:YT:PU"
870 UNS=STR$(SP)
872 PRINT#129,"PA1100-25/CP-3.0/LB:RIGHTS(UNS,LEN(UNS)-1),CHR$(63)
874 XD=1000/(P0-SP):YM=0
876 PRINT "***** THINKING *****"
878 FORX=SP TO P0:XX=(X-1)*TP/1000:YY=P(1)+P(2)*EXP(-P(3)*XX) IF N1=1 THEN 882
880 YY=YY+P(4)*EXP(-P(5)*XX)
882 YSHOOT(X)=YOSS(1,X)-YY:IFABS(YY)(YSHOOT(X) THEN N1=YSHOOT(X)
884 NEXT X
886 FORX=SP TO P0:YY=INT((1100-YSHOOT(X)+50)/YM)
888 XI=INT((X-SP)*XD):IFX)SP THEN 892
890 PRINT#129,"PA:XX",":XX":PD:GOTO 894
892 PRINT#129,"PA:YY",":XX":
894 NEXT X
896 PRINT#129,"PU:PA1200,1000"
898 CLOSE 129:RETURN

```

APPENDIX II

DETERMINATION OF RATE CONSTANTS
BY PRE-STEADY STATE ANALYSIS

The Rapid Random Bi Bi mechanism provides a suitable model for the derivation of a pre-steady state rate equation for aldehyde dehydrogenases. The mechanism is kinetically indistinguishable from the Ordered Bi Bi mechanism by steady state analysis. The mechanism shown below, assumes rapid binding of substrates and concomitant rapid establishment of equilibrium.

$$\text{Let } E' = E + EA + EB + EAB$$

The dissociation constants for EA, EB, and EAB are:

$$[EA] = \frac{[E][A]}{K_1} \quad (a), \quad [EB] = \frac{[E][B]}{K_3} \quad (b), \quad [EAB] = \frac{[E][A][B]}{K_1 K_2} \quad (c)$$

Then

$$[E'] = [E] \left[1 + \frac{[A]}{K_1} + \frac{[B]}{K_3} + \frac{[A][B]}{K_1 K_2} \right]$$

If we assume all events encompassing E_{EQ} are rapid (ie. rapid equilibrium) then

and

$$k_{\text{measured}} = k_2' + k_3$$

For the reaction in the steady state

$$\frac{d[EP_2]}{dt} = [EAB]k_2 - [EP_2]k_3 = 0 \quad (d)$$

or

$$[EAB]k_2 = [EP_2]k_3$$

and

$$[EP_2] = [EAB] \frac{k_2}{k_3} \quad (e)$$

Substituting for $[EAB]$ into (e) we obtain

$$[EP_2] = \frac{[E][A][B]}{K_1 K_2} \frac{k_2}{k_3}$$

and therefore

$$[EAB]k_2 = \frac{[E][A][B]}{K_1 K_2} \frac{k_2}{k_3} k_3 \quad (f)$$

Substituting for $[E']$ into (f) gives

$$[EAB]k_2 = \frac{[E'] [A] [B] k_2}{K_1 K_2 \left[1 + \frac{[A]}{K_1} + \frac{[B]}{K_3} + \frac{[A][B]}{K_1 K_2} \right]} \quad (g)$$

Say

$$k_2' = \frac{[A] [B] k_2}{K_1 K_2 \left[1 + \frac{[A]}{K_1} + \frac{[B]}{K_3} + \frac{[A][B]}{K_1 K_2} \right]}$$

then

$$[EAB]k_2 = [E']k_2' \quad (h)$$

If we let

$$[E_{tot}] = [E'] + [EP_2]$$

then equation (d) can be rewritten as:

$$\begin{aligned} \frac{d[EP_2]}{dt} &= [E']k_2' - [EP_2]k_3 \\ &= ([E_{tot}] - [EP_2])k_2' - k_3[EP_2] \end{aligned}$$

Rearranging gives

$$dt = \frac{d[EP_2]}{[E_{tot}]k_2' - [EP_2](k_2' + k_3)} \quad (i)$$

This equation is of the form:

$$\int \frac{dx}{a + bx} = \frac{1}{b} \ln(a + bx)$$

Integration of (i) yields

$$-\frac{1}{(k_2' + k_3)} \ln([E_{\text{tot}}]k_2' - [EP_2](k_2' + k_3)) = t + \alpha \quad (j)$$

where α is a constant of integration. To solve for α at the start of the reaction (t=0 seconds) assume $[EP_2]=0$ and therefore

$$\alpha = -\frac{1}{(k_2' + k_3)} \ln([E_{\text{tot}}]k_2')$$

Substituting into (j)

$$-\frac{1}{(k_2' + k_3)} \ln([E_{\text{tot}}]k_2' - [EP_2](k_2' + k_3)) = t - \frac{1}{(k_2' + k_3)} \ln([E_{\text{tot}}]k_2')$$

Rearranging this yields

$$\ln([E_{\text{tot}}]k_2' - [EP_2](k_2' + k_3)) = -t(k_2' + k_3) + \ln([E_{\text{tot}}]k_2')$$

and therefore

$$\ln([E_{\text{tot}}]k_2' - [EP_2](k_2' + k_3)) = t(k_2' + k_3)$$

$$\ln \frac{[E_{\text{tot}}]k_2'}{([E_{\text{tot}}]k_2' - [EP_2](k_2' + k_3))} = (k_2' + k_3)t$$

or

$$\frac{[E_{\text{tot}}]k_2'}{([E_{\text{tot}}]k_2' - [EP_2](k_2' + k_3))} = e^{(k_2' + k_3)t}$$

Inverting this gives

$$\frac{[E_{\text{tot}}]k_2' - [EP_2](k_2' + k_3)}{[E_{\text{tot}}]k_2'} = e^{-(k_2' + k_3)t}$$

This rearranges to give an expression for $[EP_2]$ production

$$[EP_2] = \frac{[E_{\text{tot}}]k_2'(1 - e^{-(k_2' + k_3)t})}{(k_2' + k_3)}$$

Recalling

$$k_2' = \frac{k_2 [A] [B]}{K_1 K_2 \left[1 + \frac{[A]}{K_1} + \frac{[B]}{K_3} + \frac{[A][B]}{K_1 K_2} \right]}$$

and

$$k_{\text{measured}} = k_2' + k_3$$

therefore

$$k_{\text{measured}} = \frac{k_2 [A] [B]}{K_1 K_2 \left[1 + \frac{[A]}{K_1} + \frac{[B]}{K_3} + \frac{[A][B]}{K_1 K_2} \right]} + k_3$$

$$k_{\text{measured}} = \frac{k_2 [A] [B]}{K_1 K_2 \left[1 + \frac{[B]}{K_3} \right] + [A] (K_2 + [B])} + k_3$$

$$= \frac{\frac{k_2 [B] [A]}{K_2 + [B]}}{\frac{K_1 K_2 (K_3 + [B])}{K_3 (K_2 + [B])} + [A]} + k_3$$

Say $\frac{k_2 [B]}{K_2 + [B]} = k_2^0$ $K_S^0 = \frac{K_1 K_2 (K_3 + [B])}{K_3 (K_2 + [B])}$

Then

$$k_{\text{measured}} = \frac{k_2^0 [A]}{K_S^0 + [A]} + k_3$$

At low [A], $k_{\text{measured}} = \frac{k_2^0 [A]}{K_S^0} + k_3$

At high [A], $k_{\text{measured}} = \frac{k_2^0 [A]}{K_S^0} + k_2^0 + k_3$

For a fixed [B], a plot of k_{measured} versus [A] gives

at low [A]: slope = $\frac{k_2^0}{K_S^0}$, intercept = k_3

at high [A]: slope = $\frac{k_2^0}{K_S^0}$, intercept = $k_2^0 + k_3$

Substituting for k_2^0 and K_S^0

$$\begin{aligned} \text{slope} &= \frac{k_2^0}{K_S^0} = \frac{k_2 K_3 [B]}{K_1 K_2 (K_3 + [B])} \\ &= \frac{k_2 [B]}{K_1 K_2} + \frac{k_2 K_3}{K_1 K_2} \end{aligned}$$

A plot of $[B]/\text{slopes}$ versus $[B]$ is given by the following equation:

$$\frac{[B]}{\text{slope}} = \frac{K_1 K_2 [B]}{k_2 [B]} + \frac{K_1 K_2}{k_2 K_3} [B]$$

and has

$$\text{slope} = \frac{K_1 K_2}{k_2 K_3}$$

$$\text{intercept} = \frac{K_1 K_2}{k_2}$$

If it is possible to obtain a plateau region from the plot of k_{measured} versus $[A]$ then the intercept is $k_2^0 + k_3$.

Since

$$\begin{aligned} k_2^0 &= \frac{k_2 [B]}{K_2 + [B]} \\ &= \frac{k_2 [B]}{K_2} + k_2 \end{aligned}$$

Therefore a plot of k_2^0 versus $[B]$ has

$$\text{slope} = \frac{k_2}{K_2} \qquad \text{intercept} = k_2$$

APPENDIX III

METHODS OF KINETIC ANALYSIS

A bisubstrate reaction can be represented by the following equation:

$$v = \frac{V_{\max} [A] [B]}{K_{ia}K_b + K_b[A] + K_a[B] + [A][B]}$$

where $V_{\max}$ is the maximum velocity, $[A]$ and $[B]$ are substrate concentrations and K_a , K_b and K_{ia} are Michaelis constants for A and B and inhibition constant for A respectively. For an Ordered Bi Bi mechanism as described below, the inhibition constant K_{ia} is given by Cleland nomenclature (218) as

$$K_{ia} = \frac{k_{-1}}{k_1}$$

Ordered Bi Bi Mechanism

For initial steady state rate studies the concentration of products P and Q is equal to 0 and the above is valid.

Initial rates (v) are measured and plotted against variation of one substrate (say A) in a double reciprocal plot. The second substrate (B) is held constant. The inverse of the above equation becomes:

$$\frac{1}{v} = \frac{K_a}{V_{\max}} \left(1 + \frac{K_{ia}K_b}{K_a[B]} \right) \frac{1}{[A]} + \frac{1}{V_{\max}} \left(1 + \frac{K_b}{[B]} \right)$$

For an enzyme following bisubstrate kinetics in which the above relations are true, the double reciprocal plot will yield a family of converging lines, each being obtained for a different fixed concentration of B. The slopes of each line are given by:

$$\text{slope} = \left(\frac{K_a}{V_{\max}} + \frac{K_{ia}K_b}{V_{\max}} \frac{1}{[B]} \right)$$

and the intercepts are given by:

$$\text{intercepts} = \frac{1}{V_{\max}} \left(1 + \frac{K_b}{[B]} \right)$$

A secondary plot of intercepts above plotted against the inverse of the concentration of B yields a plot with:

$$\text{intercept} = \frac{1}{V_{\max}}$$

$$\text{slope} = \frac{K_b}{V_{\max}}$$

Similarly a plot of slopes from the primary plot plotted against the inverse of the concentration of B yields a plot with:

$$\text{intercept} = \frac{K_a}{V_{\max}}$$

$$\text{slope} = \frac{K_{ia}K_b}{V_{\max}}$$

Kinetic data for primary plots is averaged from duplicate or triplicate experiments. Kinetic constants from secondary plots are obtained using regression analysis as described by Cornish-Bowden (219). Standard errors are calculated from correlation coefficients determined by regression.

APPENDIX IV

INHIBITION STUDIES

For a single substrate reaction such as the one below, the inhibitor Q is seen to have two possible sites of interaction with the enzyme.

The inverse rate equation below accomodates these two sites

$$\frac{1}{v} = \frac{K_a}{V_{max}} \frac{1 + [Q]/K_{is} + [A] + [Q]/K_{ii}}{[A]}$$

of inhibition by inclusion of inhibition factors as above.

Double reciprocal plots yield:

$$\text{intercepts} = \frac{1}{V_{max}} + \frac{[Q]}{K_{ii}V_{max}}$$

from which a secondary plot of intercepts versus the concentration of Q can be used to obtain K_{ii} . Similarly, slopes obtained from the double reciprocal plots are:

$$\text{slopes} = \frac{K_a}{V_{max}} + \frac{K_a[Q]}{K_{is}V_{max}}$$

from which K_{is} is obtained from the slope of a secondary plot of double reciprocal plot slopes versus concentration of Q.

Competitive inhibition results when the inhibitor binds only to the free enzyme and therefore only a value for K_{is} is obtained. Uncompetitive inhibition results when the inhibitor interacts with the binary complex only. As a result only the intercept term is affected and therefore only K_{ii} is obtained. Non-competitive or mixed inhibition results when both slope and intercept terms are affected. If $K_{is}=K_{ii}$ then inhibition is considered pure non-competitive. Otherwise inhibition is considered mixed.

APPENDIX V

The following appendix contains secondary plots of product inhibition and effector kinetic studies obtained for dehydrogenase and esterase activities (refer to text).

Figure 27 b&c

Secondary Plots For Product Inhibition of NAD⁺ Dependent Dehydrogenase Activity: Variation in acetaldehyde in presence of acetate at fixed concentrations of NAD⁺. Primary intercepts (Figure 27 b) and primary slopes (Figure 27 c) plotted against acetate concentration were used to obtain kinetic constants.

Enzyme concentration was 63 µg/mL. All assays at 25°C, pH 7.5.

Figure 28 b&c

Secondary Plots For Product Inhibition of NAD^+ Dependent Dehydrogenase Activity: Variation in acetaldehyde in presence of NADH at a fixed NAD^+ concentration (1.0 mM). Primary intercepts (Figure 28b) and primary slopes (Figure 28c) plotted against NADH concentrations were used to obtain kinetic constants.

Figure 29b&c Secondary Plots For Product Inhibition of NAD^+ Dependent Dehydrogenase Activity: Variation in NAD^+ in presence of acetate at a fixed acetaldehyde concentration (7.5 mM). Primary intercepts (Figure 29b) and primary slopes (Figure 29c) plotted against acetate concentrations were used to obtain kinetic constants. Enzyme concentration was 63 $\mu\text{g/mL}$. All assays at 25°C, pH 7.5.

Figure 29c

Figure 30 b

Secondary Plot For Product Inhibition of NAD⁺ Dependent Dehydrogenase Activity: Variation in NAD⁺ in presence of NADH as inhibitor at fixed acetaldehyde concentration (7.5 mM). Primary slopes were plotted against NADH concentration (Figure 30b) and this plot was used to determine K_{is} . Enzyme concentration was 63 μ g/mL. All assays at 25°C, pH 7.5.

Figure 31b&c

Secondary Plot For Product Inhibition of $NADP^+$ Dependent Dehydrogenase Activity: Variation in acetaldehyde in presence of acetate as inhibitor at a fixed $NADP^+$ concentration (3.2 mM). Primary intercepts (Figure 31b) and primary slopes (Figure 31c) plotted against acetate concentrations were used to obtain kinetic inhibition constants. Enzyme concentration was 63 $\mu\text{g/mL}$. All assays at 25°C, pH 7.5.

Figure 32 b&c Secondary Plot For Product Inhibition of NADP⁺ Dependent Dehydrogenase Activity: Variations in acetaldehyde in presence of NADH as inhibitor at a fixed concentration of NADP⁺ (3.2 mM). Primary intercepts (Figure 32b) and primary slopes (Figure 32c) plotted against NADPH concentrations were used to obtain kinetic constants. Enzyme concentration was 63 μ g/mL. All assays at 25°C, pH 7.5.

Figure 32c

Figure 33b&c

Secondary Plot For Product Inhibition of NADP⁺ Dependent Dehydrogenase Activity: Variations in NADP⁺ in presence of acetate as inhibitor at a fixed concentration of acetaldehyde (20 mM). Primary intercepts (Figure 33 b) and primary slopes (Figure 33c) plotted against acetate concentrations were used to obtain kinetic constants. Enzyme concentration was 63 $\mu\text{g/mL}$. All assays at 25°C, pH 7.5.

Figure 33 c

Figure 34b

Secondary Plot For Product Inhibition of NADP⁺ Dependent Dehydrogenase
 Activity: Variations in NADP⁺ in presence of NADPH as inhibitor at fixed concentration of acetaldehyde (20 mM). Primary intercepts were plotted against NADPH concentration (Figure 34b) and this was used to determine K_i s. Enzyme concentration was 63 μ g/mL. All assays at 25°C, pH 7.5.

Figure 35 b

Secondary Plot For Disulfiram Inhibition of NAD^+ Dependent Dehydrogenase Activity. Slopes of the double reciprocal plot were plotted against disulfiram concentration from which K_{is} was determined. Enzyme concentration was 0.151 mg/mL. All assays at 25°C, pH 7.5.

Figure 36b

Secondary Plot For Disulfiram Inhibition of NADP⁺ Dependent Dehydrogenase Activity. Slopes of the double reciprocal plot were plotted against disulfiram concentration from which K_{is} was determined. Enzyme concentration was 0.151 mg/mL. All assays at 25°C, pH 7.5.

Figure 37 b

Secondary Plot For Chloral Hydrate Inhibition of NAD^+ Dependent Dehydrogenase Activity. Slopes of the double reciprocal plot were plotted against chloral hydrate concentration from which K_{is} was determined. Enzyme concentration was 0.63 mg/mL. All assays were at 25°C, pH 7.5.

Figure 38 b

Secondary Plot For Chloral Hydrate Inhibition of NADP⁺ Dependent Dehydrogenase Activity. Slopes of the double reciprocal plot were plotted against chloral hydrate concentration from which K_{is} was determined. Enzyme concentration was 0.63 mg/mL. All assays were at 25°C, pH 7.5.

Figure 39 b

Secondary Plot For Inhibition of NAD^+ Dependent Dehydrogenase Activity By NADPH. Slopes of the double reciprocal plot were plotted against NADPH concentration from which K_{is} was determined. Enzyme and ethanal concentrations were 0.22 mg/mL and 75 mM respectively. All assays were at 25°C, pH 7.5.

Figure 40 b Secondary Plot For Inhibition of NADP⁺ Dependent Dehydrogenase Activity by NADH. Slopes of the double reciprocal plot were plotted against NADH concentration from which K_{is} was determined. Enzyme and ethanal concentrations were 0.22 mg/mL and 75 mM respectively. All assays were at 25°C, pH 7.5.

Figure 46b Secondary Plot For Product Inhibition of Esterase Activity By Butyrate Ions. Slopes of the double reciprocal plot were plotted against butyrate concentration and from this the competitive inhibition constant (K_{i5}) was obtained. Enzyme concentration was 8.9 $\mu\text{g/mL}$. All assays were at 25°C, pH 7.5.

Figure 47b&c Secondary Plots For Product Inhibition of Esterase Activity By p-Nitrophenol. Slopes (Figure 47 b) and intercepts (Figure 47 c) of the double reciprocal plot were plotted against p-nitrophenol concentration and from these the inhibition constants K_{is} and K_{ii} were obtained. Enzyme concentration was 8.9 $\mu\text{g/mL}$, all assays at 25°C, pH 7.5.

Figure 47 c

Figure 48b

Secondary Plot For Esterase Inhibition By Acetaldehyde. The intercepts of the double reciprocal plot are plotted against acetaldehyde concentration and the kinetic constant (K_{ij}) was determined from this. Enzyme concentration was 34 $\mu\text{g/mL}$, all assays at 25°C, pH 7.5.

Figure 49b

Secondary Plot For Esterase Inhibition By Chloral Hydrate. The slopes of the double reciprocal plot are plotted against chloral hydrate concentration and this was used to determine K_{is} . Enzyme concentration was 34 $\mu\text{g/mL}$, all assays at 25°C, pH 7.5.

APPENDIX VI

An enzyme catalyzed reaction involving two ionizable species can be described by the following mechanism (74):

In rat mitochondrial aldehyde dehydrogenase, the step $\text{EH} \rightleftharpoons \text{EAH}$ encompasses the burst of reduced coenzyme formation and release of acid product. The mechanism assumes the horizontal processes are dead-end complexes. The rate equation is given as:

$$v = \frac{k_2 [E_t] [A]}{K_m \left[\frac{K_a}{[H]} + 1 + \frac{[H]}{K_b} \right] + [A] \left[\frac{K_{a'}}{[H]} + 1 + \frac{[H]}{K_{b'}} \right]}$$

where $[E_t]$ is the total enzyme concentration. If conditions are arranged such that $[A] \gg K_m$ then the equation simplifies to:

$$v = \frac{k_2 [E_t]}{\frac{K_{a'}}{[H]} + 1 + \frac{[H]}{K_b}}$$

- i) When $[H]$ is high (low pH) the equation becomes :

$$v = k_2 [E_t] K_b' \frac{1}{[H]}$$

which can be written as $\log v = \log (k_2 [E_t] K_b') + pH$.

A plot of $\log v$ versus pH has slope = 1.

- ii) When $[H]$ is approximately neutral the equation becomes:

$$v = k_2 [E_t]$$

which can be written as $\log v = \log (k_2 [E_t])$

A plot of $\log v$ versus pH has slope = 0.

- iii) When $[H]$ is low (high pH) the equation becomes:

$$v = \frac{k_2 [E_t]}{K_b'} [H]$$

which can be written as $\log v = \log \left(\frac{k_2 [E_t]}{K_b'} \right) - pH$

A plot of $\log v$ versus pH has slope = -1.

When the plots given by i) and ii) intersect then

$$v = k_2 [E_t] K_b' \frac{1}{[H]} = k_2 [E_t]$$

or

$$K_b' = [H]$$

and therefore $pK_b' = pH$ as described in the figure below.

Similarly it can be shown that when ii) and iii) intersect

then $pK_a' = pH$

In order to demonstrate this pH dependence on the ionization of these species a theoretical plot is prepared below using pK values for cysteine and histidine (catalytic species for AldDH). Values for k_2 and $[E_t]$ have been chosen from experimental data (see Figure A_{p1}).

$$v = \frac{k_2 [E_t]}{\frac{K_a'}{[H]} + 1 + \frac{[H]}{K_b'}}$$

where $k_2 = 10.2 \text{ min}^{-1}$

$[E_t] = 3.46 \times 10^{-1} \text{ M}$

$K_a' = 10^{-8.3}$ (Cysteine)

$K_b' = 10^{-6.0}$ (Histidine)

Figure Apl. Theoretical Plot Demonstrating The Dependency of Velocity on Ionization of Enzyme Catalytic Species. The profile above is prepared for cysteine and histidine as the reactive species.

REFERENCES

- 1 Banthorpe D.V., Cardemil, E., Contreras, M.Del.C.
(1976) *Phytochem.* 15, 391-394.
- 2 Sperle, G.T., Forrest, H.S., Gibson, D.T. (1974)
J. Bacteriol. 118, 541-550.
- 3 Sund, H., Theorell, H. (1963) in "The Enzymes" (Boyer,
P.D., Lardy, H.A, Myrback; K. eds.) Vol 7, Academic
Press, N.Y. pp. 25-83.
- 4 Leiber, C. in "Metabolic Aspects of Alcoholism"
(Leiber, C.S ed.) MTP Press, Eng., pp. 1-29.
- 5 Roach, M. (1975) in "Biochemical Pharmacology of
Ethanol" (Majchrowicz, E. ed.) Plenum Press, N.Y.,
pp. 33-35.
- 6 Pietruszko, R. (1980) in *Isozymes: Current Topics
in Biological and Medical Research* 4, 107-130.
- 7 Tottmar, S.O.C., Pettersson, H., Kiessling, K.-H.
(1973) *Biochem. J.* 135, 577-586.
- 8 Dietrich, R.A. (1976) *Biochem. Pharmacol*, 15, 1911-
1922.
- 9 Marjanen, L. (1972) *Biochem. J.* 127, 633-639.
- 10 Lindahl, R., Evces, S. (1984) *Biochem. Pharmacol.*
33, 3383-3389.
- 11 Krebs, H.A., Perkins, J.R. (1970) *Biochem. J.* 118,
635-644.
- 12 Zachman, R.D., Olsen, Y.A. (1961) *J. Biol. Chem.*
236, 2309-2313.
- 13 Christophe, J., Popjack, G. (1961) *J. Lip. Res.* 2,
244-257.
- 14 Bjorken, I. (1972) *J. Biochem*, 30, 441-451.
- 15 Noble, E.P., Tewari, S. (1977) in "Metabolic Aspects of
Alcoholism" (Leiber, C.S. ed.) MTP Press, Eng.,
pp. 149-185.
- 16 Duncan, R.J., Tipton, K.F. (1971) *Eur. J. Biochem.* 22,
257-262.
- 17 Goedde, H.W., Meir-Tackmann, D., Agarwal, D., Harada,
S. (1982) *Clin. Biol. Res.* 114, 347-362.

- 18 Davis, V.E., Walsh, M.J. (1970) *Science* 167, 1005-1006.
- 19 Cederbaum, A.I., Leiber, C.S., Rubin, E. (1974) *Arch. Biochem. Biophys.* 165, 560-569.
- 20 Truitt, E.B., Walsh, M.J. (1971) in "Biology of Alcoholism" (Kissin, B., Begleiter, H. eds.) Vol 1, Plenum Press, N.Y., pp. 161.
- 21 Harada, S., Agarwal, D.P., Goedde, H.W. (1978) *Hum. Genet.* 44, 181.
- 22 Weiner, H., (1985) *Alcoholism: Clin. Exp. Res.* 9, 541-542.
- 23 Goedde, H.W., Agarwal, D.P. (1981) *J. Clin. Chem. Clin. Biochem.* 19, 179.
- 24 Crow, K.E., Kitson, T.M., MacGibbon, A.K.H., Batt, R.D. (1974) *Biochim. Biophys. Acta* 350 121-128.
- 25 Hart, G.J., Dickinson, F.M. (1978) *Biochem.J.* 175, 899-908.
- 26 MacGibbon, A.K.H., Haylock, S.J., Buckley, P.D., Blackwell, L.P. (1978) *Biochem. J.* 171, 533-538.
- 27 Takahashi, N., Kitabatake, N., Sasaki, R., Chiba, H., (1979) *Agric. Biol. Chem.* 43, 1973-1982.
- 28 Leicht, W., Heinz, F., Freimuller, B. (1979) *Eur. J. Biochem.* 83, 189-196.
- 29 Ekfeldt, J.H., Mope, L., Takio, K., Yonetani, T. (1976) *J. Biol. Chem.* 251, 236-240.
- 30 Feldman, R.I., Weiner, H., (1972) *J. Biol. Chem.* 247, 260-266.
- 31 Maxwell, E.S., Topper, Y.I. (1961) *J. Biol. Chem.* 236, 1032-1037.
- 32 Duncan, R.J.S. (1979) *Biochem. J.*, 183, 459-462.
- 33 Sheppard, J.R., Albersheim, P., McClearn, G. (1970) *J. Biol. Chem.* 245, 2876-2882.
- 34 Shum, G.T., Blair, A.H. (1972) *Can. J. Biochem.* 50 741-748.
- 35 Horton, A.A., Barrett, M.C. (1975) *Arch. Biochem. Biophys.* 167, 426-436.

- 36 Koivula, T., Koivusalo, M. (1975) *Biochim. Biophys. Acta* 397, 9-23.
- 37 Racker, E., (1955) in *Methods of Enzymology* (Colowick, S.P., Kaplan, N.O. eds.) Vol 1, Academic Press, N.Y. pp. 514-517.
- 38 Nakayasu, H., Mihara, K., Sato, R. (1978) *Biochem. Biophys. Res. Comm.* 83, 697-703.
- 39 Lindahl, R., Evces, S. (1984) *J. Biol. Chem.* 259, 11986-11990.
- 40a Senior, D.J., Tsai, C.S. (1982) Purification of Rat Liver Mitochondrial Aldehyde Dehydrogenase, Presentation at the 25th C.F.B.S. Conference, Edmonton, Alta.
- 40b Senior, D.J., Tsai, C.S. (1986) "Bifunctionality of Aldehyde Dehydrogenase From Rat Liver Mitochondria, Presentation at the 29th C.F.B.S. Conference Guelph, Ontario.
- 41 Siew, C., Deitrich, R.A., Erwin, V.G. (1976) *Arch. Biochem. Biophys.* 176, 638-649.
- 42 Blair, A.H., Bodley, F.H. (1969) *Can. J. Biochem.* 47, 265-272.
- 43 Hempel, J.D., Reed, D.M., Pietruszko, R. (1982) *Alcoholism: Clin. Exp. Res.* 6, 417-425.
- 44 Greenfield, N.J., Pietruszko, R. (1977) *Biochim. Biophys. Acta.* 483, 35-45.
- 45a Hempel, J.D., von Bahr-Lindstrom, H., Jornvall, H. (1984) *Eur. J. Biochem.* 141, 21-35.
- 45b Hempel, J., Kaiser, R., Jornvall, H. (1985) *Eur. J. Biochem.* 153, 13-28.
- 46 von Bahr-Lindstrom, L.H., Hempel, J.D., Jornvall, H. (1984) *Eur. J. Biochem.* 141, 37-42.
- 47 Korsten, C.S., Matsuzaki, S., Feinman, L., Leiber, C.S. (1975) *N. Eng. J. Med.* 292, 386-389.
- 48 Ekfeldt, J., Yonetani, T. (1975) *Arch. Biochem. Biophys.* 175, 717-722.
- 49 Kitson, T.M. (1975) *Biochem. J.* 151, 407-412.
- 50 Zimbaro, P.G., Ruch, P.L., (1975) in *"Psychology and Life"*, 9th ed., Scott Foresman and Co., Ill. pp. 469.
- 51 Sidhu, R.S., Blair, A.H. (1975) *J. Biol. Chem.* 250, 7894-7898.

- 52 Sidhu, R.S., Blair, A.H. (1975) J. Biol. Chem: 250, 7899-7904.
- 53 Feldman, R.I., Weiner, H. (1972) 247, 267-272.
- 54 Duncan, R.J.S., (1977) Biochem. J. 161, 123-130.
- 55 MacGibbon, A.K.H. (1978) Biochem. J. 171, 533-538.
- 56 Park, J.H., Meriwether, B.P., Clodfelder, P., Cunningham, L.W. (1961) J. Biol. Chem. 236, 136-141.
- 57 Blackwell, L.F., Bennett, A.F., Buckley, P.D. (1983) Biochem. 22, 3784-3791.
- 58 Blackwell, L.F., Bennett, A.F., Crow, K.E., Buckley, P.D., Deady, L.W. (1983) Pharmacol. Biochem. Behav. 18, Suppl. 1, 83-87.
- 59 Duncan, R.J.S. (1985) Biochem. 230, 261-267.
- 60 Hart, G.J., Dickinson, M.F. (1977) Biochem. J. 163, 261-267.
- 61 Bostian, K.A., Betts, G.F. (1978) Biochem. J. 173, 773-786.
- 62 Tamaki, N., Nakamura, M., Kimura, K., Hama, T. (1977) J. Biochem. 82, 73-79.
- 63 Olive, C., Geroch, M.E., Levy, R.H. (1971) J. Biol. Chem. 246, 2047-2057.
- 64 Brändén, C.-I., Eklund, H. (1980) in "Dehydrogenases Requiring Nicotinamide Coenzymes" (Jeffery, J. ed.) Experientia Supplementum 36, Birkhauser, Basel, pp. 40-84.
- 65 Rossmann, M.G., Liljas, A., Brändén, C.-I., Banaszek, L.J. (1975) in "The Enzymes" (Boyer, P.D. ed.) 3rd ed., Vol XI, Academic Press, N.Y., pp. 61-102.
- 66 Matthews, D.A., Alden, R.A., Bolin, J.T., Pilman, D.J., Freer, S.T., Hamlin, R., Hol, W.G., Kisliuk, R.L., Pastore, E.J., Plante, L.T., Xuong, N., Kraut, J. (1978) J. Biol. Chem. 253, 6946-6954.
- 67 Duncan, R.J.S., Tipton, K.P. (1971) Eur. J. Biochem. 22, 538-543.
- 68 Ekfeldt, J.H., Yonetani, T. (1976) Arch. Biochem. Biophys. 173, 273-281.

- 69 MacGibbon, A.K.H., Blackwell, L.F., Buckley, L.F. (1977) *Eur. J. Biochem.* 77, 93-100.
- 70 Hart, G.J., Dickinson, M.P. (1982) *Biochem. J.* 203, 617-627.
- 71 Roberts, D.V. (1977) in "Enzyme Kinetics", Cambridge University Press, Cambridge, pp. 107-134.
- 72 Krimsky, I., Racker, E. (1955) *Science* 122, 319.
- 73 Harris, J.I., Meriwether, B.P., Park, J.H. (1963) *Nature (London)* 198, 154-159.
- 74 Laidler, K.J., Bunting, P. (1973) in "The Chemical Kinetics of Enzyme Action", Clarendon Press, Oxford.
- 75 von Bahr-Lindström, H., Jeck, R., Woenckhaus, C., Sohn, S., Hempel, J., Jornvall, H. (1985) *Biochem.* 24, 5847-5851.
- 76 Jeck, R. (1977) *Z. Naturforsch., C: Biosci.* 32C, 550-556.
- 77 Hempel, J., Pietruszko, R., Pietzek, P., Jornvall, H. (1982) *Biochem.* 21, 6834-6838.
- 78 Jencks, W.P. (1969) in "Catalysis in Chemistry and Enzymology", McGraw-Hill, N.Y.
- 79 Garavito, R.M., Rossmann, M.G., Argos, P., Eventoff, W. (1977) *Biochem.* 16, 5065-5071.
- 80 Takahashi, K., Weiner, H., Filmer, D.L. (1981) *Biochem.* 20, 6225-6230.
- 81 MacGibbon, A.K.H., Buckley, P.D., Blackwell, L.F. (1977) *Biochem. J.* 165, 455-462.
- 82 Chou, P.Y., Fasman, G.D. (1974) *Biochem.* 13, 222-245.
- 83 Von Bahr-Lindström, H., Sohn, S., Woenckhaus, C., Jeck, R., Jornvall, H. (1981) *Eur. J. Biochem.* 117, 521-526.
- 84 Jeffery, J. (1980) in "Dehydrogenases Requiring Nicotinamide Coenzymes" (Jeffery, J. ed.), *Experientia Supplementum* 36, pp. 85-125.
- 85 Eys, J.V., Kaplan, N.O. (1957) *Biochim. Biophys. Acta* 23, 574-581.
- 86 Eys, J.V., Ciotti, M.M., Kaplan, N.O. (1958) *J. Biol. Chem.* 231, 571-582.

- 87 Eys, J.V., Ciotti, M.M., Kaplan, N.O. (1957) *Biochim. Biophys. Acta* 23, 581-587.
- 88 Kaplan, N.O., Ciotti, M.M., Stolzenbach, F.E. (1957) *Arch. Biochem. Biophys.* 69, 441-457.
- 89 Pullman, M.E., Colowick, S.P., Kaplan, N.O. (1952) *J. Biol. Chem.* 194, 593-6082.
- 90 Samama, J.-P., Marchal-Rosenheimer, N., Biellmann, J.-F., Rossmann, M.G. (1981) *Eur. J. Biochem.* 120, 563-569.
- 91 Mas, M.T., Colman, R.F. (1984) *Biochem.* 23, 1675-1683.
- 92 Hyde, E.I., Birdsall, B., Roberts, G.C.K., Feeney, J., Burgen, A.S.V. (1980) *Biochem.* 19, 3746-3754.
- 93 Feeney, J., Birdsall, B., Roberts, G.C.K., Burgen, A.S.V. (1977) in "NMR in Biology", (Dwek, R.A., Campbell, I.D., Richards, R.E., Williams, R.J.P. eds.), Academic Press, N.Y., pp. 111-124.
- 94 Hart, G.J., Dickinson, M.F. (1978) *Biochem. J.* 175, 753-756.
- 95 Kitson, T.M. (1978) *Biochem. J.* 175, 83-90.
- 96 Takahashi, K., Weiner, H. (1981) *Biochem.* 20, 2720-2726.
- 97 Takahashi, K., Weiner, H. (1980) *J. Biol. Chem.* 255, 8206-8209.
- 98 Dickinson, F.M., Hart, G.J. (1982) *Biochem. J.* 205, 443-448.
- 99 Dickinson, F.M., Haywood, G.W. (1986) *Biochem. J.* 233, 877-883.
- 100 Tsai, C.S. (1982) *Arch. Biochem. Biophys.* 213, 635-642.
- 101 Pederson, P.L., Greenawalt, J.W., Reynefarje, B., Hulihan, J., Decker, G.L., Soper, J.W., Bustamente, E. (1978) *Meth. Cell Biol.* 20, 411-481.
- 102 Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. (1951) *J. Biol. Chem.* 193, 265-275.
- 103 Miller, G.L. (1959) *Anal. Chem.* 31, 964.

- 104 Polin, O., Ciocalteau, V (1927) J. Biol. Chem. 27, 627-650.
- 105 Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404-421.
- 106 Nielson, B.L., Brown, L.R. (1984) Anal. Biochem. 141, 311-315.
- 107 Priefelder, D. (1976) in "Physical Biochemistry, Applications to Biochemistry and Molecular Biology", 1st Ed., W.H. Freeman and Co., San. Fransisco, pp. 218-220.
- 108 Priefelder, D. (1973) Meth. Enzymol. (Hirs, C.H.W., Timasheff S.N. eds.), Vol 27, Academic Press, N.Y., pp. 140-150.
- 109 Martin, R.G., Ames, B.N. (1961) J. Biol. Chem. 236, 1372-1379.
- 110 McGuinness, E.T. (1973) J. Chem. Ed. 50, 826-830.
- 111 Tanford, C (1961) in "Physical Chemistry of Macromolecules", J. Wiley and Sons, N.Y., pp. 323-359.
- 112 Edsall, J.T. (1953) in "The Proteins", (Neurath, H., Bailey, K. eds.), Vol 1, Part B, Academic Press, N.Y.
- 113 Simons, S.S. Jr., Johnson, D.P. (1976) J. Am. Chem. Soc. 98, 7098-7099.
- 114 Roth, M., Hampai, A. (1973) J. Chromatog. 83, 353-356.
- 115 Scofone, E., Fontana, A. (1975) in "Protein Sequence Determination, Molecular Biology, Biochemistry and Biophysics", (Needleman, S.B. ed.), Vol 8, 2nd ed., Springer-Verlag, Berlin, pp. 184.
- 116 Spande, T.K., Witkop, B. (1967) Meth. Enzymol. (C.H.W. Hirs ed.), Vol 11, Academic Press N.Y. pp. 498-506.
- 117 Edman, P., Henschen, A. (1975) in "Protein Sequence Determination, Molecular Biology, Biochemistry and Biophysics", (Needleman, S.B. ed.), Vol 8, 2nd ed., Springer-Verlag, Berlin, pp. 232-236.
- 118 Inglis, A.S., Nicholls, P.W. (1973) J. Chromatog. 79, 344-346.
- 119 Narita, K., Matsuo, H., Nakajima, T. (1975) in "Protein Sequence Determination, Molecular Biology, Biochemistry and Biophysics" (Needleman, S.B. ed.), Vol 8, 2nd ed., Springer-Verlag, Berlin, pp. 30-103.

- 120 Siegal, J.M., Montgomery, G.A., Bock, R.M (1959) Arch. Biochem. Biophys. 82, 288-294.
- 121 Rudolph, F.B (1979) Meth. Enzymol. (Purich, D.A. ed.), Vol 63 Part A, Academic Press, N.Y., pp. 411-436.
- 122 Jornvall, H. (1973) Biochem. Biophys. Res. Commun. 53, 1096-1101.
- 123 Evans, N., Rabin, B.R. (1968) Eur. J. Biochem. 4, 548-554.
- 124 Li, T.K., Vallee, B.L., (1965) Biochem. 4, 1195-1202.
- 125 Means, G.E., Feeney, R.E. (1971) in "Chemical Modifications of Proteins", Holden-Day Inc., San Francisco, pp.106.
- 126 Butterworth, P.H.W., Baum, H., Porter, J.W. (1967) Arch. Biochem. Biophys. 118, 716-723.
- 127 Damjanovich, S., Kleppe, K. (1966) Biochim. Biophys. Acta. 122, 145-147.
- 128 Kastenschmidt, L.L., Kastenschmidt, J., Helmreich, E. (1968). Biochem. 7, 3590-3608.
- 129 Colman, R. (1969) Biochem. 8, 888-898.
- 130 Means, G.E., Feeney, R.E. (1971) in "Chemical Modifications of Proteins", Holden-Day Inc., San Francisco, pp. 155.
- 131 Ray, W.J. (1967) Meth. Enzymol. (Hirs, C.H.W. ed.) Academic Press, N.Y. Vol 11, pp.490.
- 132 Tsai, C.S., Godin, J.R.P., Wand, A.J. (1985) Biochem. J. 225, 203-208.
- 133 Means, G.E., Feeney, R.E. (1971) in "Chemical Modifications of Proteins", Holden-Day Inc., San Francisco, pp.165-169.
- 134 Means, G.E., Feeney, R.E. (1971) in "Chemical Modifications of Proteins", Holden-Day Inc., San Francisco, pp.89-93.
- 135 Lange, G.L. III, Riordan, J.P., Vallee, B.L. (1974) Biochem. 13, 4361-4370.
- 136 Takahashi, K. (1968) J. Biol. Chem. 243, 6171-6179.

- 137 Means, G.E., Feeney, R.E. (1971) in "Chemical Modifications of Proteins", Holden-Day Inc., San Francisco, pp. 195-196.
- 138 Means, G.E., Feeney, R.E. (1971) in "Chemical Modifications of Proteins", Holden-Day Inc., San Francisco, pp. 169-171.
- 139 Riordan, J.F., Vallee, B.L. (1972) Meth. Enzymol. (Hirs, C.H.W., Timasheff, S.N. eds.) Vol 25, Academic Press, N.Y., 515-521.
- 140 Sokolovsky, M., Riordan, J.F., Vallee, B.L. (1966) Biochem. 5, 3582-3589.
- 141 Means, G.E., Feeney, R.E. (1971) in "Chemical Modifications of Proteins", Holden-Day Inc., San Francisco, pp. 183-186.
- 142 Tsai, C.S., Wand, A.J., Templeton, D.M., Weiss, P.M. (1983) Arch. Biochem. Biophys. 225, 554-561.
- 143 Hoare, D.G., Koshland, D.E. Jr. (1967) J. Biol. Chem. 242, 2447-2453.
- 144 Rule, C.K., La Mer, V.K. (1938) J. Am. Chem. Soc. 60, 1974-1981.
- 145 Mikkelsen, K., Nielson, S.O. (1960) J. Phys. Chem. 64, 632-637.
- 146 Glasoe, P.K., Long, F.A. (1960) J. Phys. Chem. 64, 188-189.
- 147 Dettman, H. Personal Communication.
- 148 Riley, H.A., Gray, A.R., (1943) in "Organic Synthesis" (Blatt, A.H. ed.) Coll. Vol. 2, J. Wiley and Sons, N.Y., pp. 509-511.
- 149 Roberts, D.V. (1977) in "Enzyme Kinetics", Cambridge University Press, Cambridge, pp. 138.
- 150 Capellos, C., Bielski, B.H.J. (1972) in "Kinetic Systems, Mathematical Description of Chemical Kinetics in Solution", J. Wiley Interscience, N.Y. pp., 9-13.
- 151 Kates, M. personal communication.
- 152 P.L. Biochemical Product Guide (1984) Pharmacia P.L. Biochemicals Inc., Uppsala, Sweden.
- 153 Papahadjopoulos, P (1980) Ann. Rev. Biophys. Bioeng. 9, 467.

- 154 P.H. Buist, personal communication
- 155 Kates, M. (1972) in "Techniques in Lipidology, Laboratory Techniques in Biochemistry and Molecular Biology, (T.S. Work ed.) Vol 3, North Holland.
- 156 Enoch, H.G., Strittmatter, P. (1979) Proc. Natl. Acad. Sci., USA, 76, 145-149.
- 157 Hallam, M.R. (1982) J. Biophys. Biochem. Meth.
- 158 Lamkin, G.E., King, E.E (1976) Biochem. Biophys. Res. Commun. 72, 560-565.
- 159 Easterday, R.L., Easterday, I. (1974) in "Immobilized Biochemicals and Affinity Chromatography, (Dunlop, R.B ed.) Plenum Press, N.Y. pp. 123-134.
- 160 Stellewagon, E., Cass, R., Thompson, S.T. (1975) Nature 257, 716-717.
- 161 Affinity Chromatography, Principles and Methods (1979) Pharmacia Fine Chemicals Publication, Uppsala, Sweden. pp. 74-79.
- 162 Brändén, C.-I., Eklund, H., Zeppezaur, E. (1978) Proc. 12th F.E.B.S. Meeting, Dresden.
- 163 Chromatofocusing, Principles and Methods, Pharmacia Fine Chemicals Publication, Uppsala, Sweden.
- 164 Andrews, P. (1970) in "Methods of Biochemical Analysis" (Glück, D. ed.) Vol. 18, Wiley-Interscience, N.Y. pp.1.
- 165 Winzor, D.J. (1969) in "Physical Principles and Techniques of Protein Chemistry, Part A" (Leach, S.J. ed.) Academic Press, N.Y., pp. 451.
- 166 Tsai, C.S. (1978) Biochem. J. 173, 483-496.
- 167 Circular OR-18 P.L. Biochemicals Publication (1971) 5th Printing, P.L. Biochemicals Inc.
- 168 Dixon, M., Webb, E.C. (1979) in "Enzymes" Longman, G.B. pp. 154.
- 169 Alberty, R.A. (1958) J. Am. Chem. Soc. 80, 1777.
- 170 Sorger, G.J., Evans, H.J. (1966) Biochim. Biophys. Acta 118, 1-8.

- 171 Segal, I.H. (1975) in "Enzyme Kinetics, Behavior and Analysis of Rapid Equilibrium and Steady State Enzyme Systems", Wiley-Interscience, N.Y., pp. 227-231.
- 172 Krimsky, N.I. (1977) TIBS 2, 35-38.
- 173 Nilsson, R., Merkel, P.B., Kearns, D.R. (1972) Phytochem. Phytobiol. 16, 117.
- 174 Tsai, C.S., Wand, A.J., Godin, J.R.P., Buchanan, G.W. (1982) Arch. Biochem. Biophys. 217, 721-729.
- 175 Nakamura, S., Kaziro, Y. (1981) J. Biochem. 90, 1117-1124.
- 176 Yomiya, M., Irie, M. Ukita, T. (1969) Biochem. 8, 5149-5160.
- 177 Oster, G., Bellin, J.S., Kimball, R.W., Schrader, M.E. (1959) J. Am. Chem. Soc. 81, 5095-5099.
- 178 Dalziel, K., Dickinson, F.M. (1965) Biochem. J. 95, 311-320.
- 179 Fawcett, C.P., Kaplan, N.O. (1962) J. Biol. Chem. 237, 1709-1715.
- 180 Kantrowitz, E.R., Lipscomb, W.N., (1976) J. Biol. Chem. 251, 2688-2695.
- 181 Schloss, J.V., Norton, I.L., Stringer, C.D., Hartman, F.C. (1978) Biochem, 17, 5621-5631.
- 182 Dalziel, K. (1975) Phil. Trans. R. Soc. London B272, 109-122.
- 183 Brändén, C.-I., Jornvall, H., Eklund, H., Furugren, B. (1975) in "The Enzymes" (Boyer, P.D. ed.) 3rd ed., Vol XI, Academic Press, N.Y., pp. 103-190.
- 184 Viswanatha, T., Lawson, W.B., Witkop, B. (1960) Biochim. Biophys. Acta. 40, 216.
- 185 Bruice, T.C., Gregory, M.J., Walters, S.L. (1968) J. Am. Chem. Soc. 90, 1612.
- 186 Moras, P., Olsen, K.W., Sabesan, M.N., Buehner, M., Ford, G.C., Rossmann, M.G. (1975) J. Biol. Chem. 250, 9137-9162.
- 187 Gorenstein, D.G. (1981) Ann. Rev. Biophys. Bioeng. 10, 355-386.

- 188 Hart, G.J., Dickinson, M.F. (1985) *Biochem. J.* 225, 159-165.
- 189 Helenius, A., Simons, K. (1975) *Biochim. Biophys. Acta.* 415, 29-79.
- 190 Haydon, D.A., Taylor, J. (1963) *J. Theoret. Biol.* 4, 281-296.
- 191 Sunamoto, J., Baba, Y., Iwamoto, K., Kondo, H. (1985) *Biochim. Biophys. Acta* 833, 144-150.
- 192 Allen, T.M. (1984) in "Liposome Technology" (Gregoriadis, G. ed.) Vol 1, CRC Press, Boca Raton, Fla, pp. 110-122.
- 193 Goldstein, L. (1977) *Meth. Enzymol.* (Mosbach, K. ed.) Vol. 44, Academic Press, N.Y., pp. 397-443.
- 194 Suurkuusk, J., Lentz, B.R., Barenholz, Y., Biltonen, R.L., Thompson, T.E. (1976) *Biochem.* 15, 1393-1401.
- 195 Lentz, B.R., Barenholz, Y., Thompson, T.E. (1976) *Biochem.* 15, 4521-4528.
- 196 Lentz, B.R., Barenholz, Y., Thompson, T.E. (1976) *Biochem.* 15, 4529-4537.
- 197 Szoka, F. Jr., Papahadjopoulos, D. (1980) *Ann. Rev. Biophys. Bioeng.* 9, 467.
- 198 Colbeau, A., Nachbaur, J., Vignais, P.M. (1971) *Biochim. Biophys. Acta.* 249, 462-492.
- 199 Kasche, V. (1983) *Enz. Microb. Technol.* 5, 2-13.
- 200 Ion Exchange Chromatography, Principles and Methods" (1980) Pharmacia Fine Chemicals, Uppsala, Sweden, pp. 33.
- 201 Dickinson, M.F., Hart, G.J., Kitson, T.M. (1981) *Biochem. J.* 199, 573-579.
- 202 Allanson, S., Dickinson, F.M. (1984) *Biochem. J.* 223, 163-169.
- 203 Dickinson, M.F. (1985) *Biochem. J.* 225, 159-165.
- 204 Hempel, J.D., Pietruszko, R. (1978) *Ped. Proc.* 37, 350 (abstract).
- 205 Harris, J.I., Waters, M. (1976) in "The Enzymes" (Boyer, P.D. ed.) Academic Press, N.Y., pp. 1-49.

- 206 Means, G.E., Feeney, R.E. (1971) in "Chemical Modifications of Proteins", Holden-Day Inc., San Francisco, pp. 157.
- 207 Mahoney, W.C., Smith, P.K., Hermodson, M.A. (1981) Biochem. 20, 443-448.
- 208 Mahoney, W. Hermodson, M.A. (1979) Biochem. 18, 3810-3814.
- 209 Bhner, M., Ford, G.C., Moras, D., Olsen, K.W., Rossmann, M.G. (1974) J. Mol. Biol. 90, 25-49.
- 210 Means, G.E., Feeney, R.E. (1971) in "Chemical Modifications of Proteins", Holden-Day Inc., San Francisco, pp. 198-204.
- 211 Racker, E. (1955) Physiolog. Rev. 35, 1-56.
- 212 Henderson, R., Wright, C.S., Hess, G.P., Blow, D.M. (1971) Cold Spring Harbor Symp. Quant. Biol. 36, 63-70.
- 213 Abeles, R.H., Lee, H.A. Jr. (1960) J. Biol. Chem. 235, 1499-1503.
- 214 Hine, J.S. (1956) in "Physical Organic Chemistry", McGraw-Hill Book Co., N.Y., pp. 240.
- 215 Bender, M.L., Kezdy, P.J., Wedler, P.C. (1967) J. Chem. Educ. 44, 84-88.
- 216 Weiner, H., Hu, J.H.J., Sanny, C.G. (1976) J. Biol. Chem. 251, 3853-3855.
- 217 Walsh, C. (1979) in "Enzymatic Reaction Mechanisms, W.H. Freeman and Co., San Francisco, pp. 82.
- 218 Cleland, W.W. (1963) Biochim. Biophys. Acta. 67, 104-137.
- 219 Cornish-Bowden, A. in "Principles of Enzyme Kinetics" Butterworths, London.

END

0|2·01·88

FIN