

Contents

Preface to the Second Edition XIII

Note to the Reader XIV

Abbreviations XV

1	Introduction	1
	Standard Books, Series, and Databases	3
2	General Aspects of Enzyme Analysis	5
2.1	Basic Requirements for Enzyme Assays	5
2.2	What Must Be Observed for an Enzyme Assay?	8
2.2.1	Order of Reactions	8
2.2.2	Significance of the Reaction Order for Enzyme Reactions	10
2.2.3	Determination of the Velocity of Enzyme Reactions	14
2.2.3.1	Enzyme Units	14
2.2.3.2	Progress Curves	16
2.2.3.3	Difficulties in Determination of Initial Velocities	17
2.2.3.4	A Short Discussion on Error	21
2.2.3.5	Preparation of Dilution Series	25
2.2.3.6	Considerations about Statistical Treatments	27
2.2.4	Michaelis–Menten Equation	28
2.2.4.1	General Considerations	28
2.2.4.2	Linear Representations of the Michaelis–Menten Equation	30
2.2.5	Enzyme Inhibition	33
2.2.6	Multisubstrate Reactions	39
2.2.7	Essential Conditions for Enzyme Assays	41
2.2.7.1	Dependence on Solvents and Ionic Strength	41
2.2.7.2	pH Dependency	42
2.2.7.3	Isoelectric Point	44
2.2.7.4	Buffers: What Must Be Regarded?	44
2.2.7.5	How to Prepare Buffers?	48
2.2.7.6	Temperature Dependency	49

2.2.7.7	Why Are Enzymes Unstable?	53
2.2.7.8	How Can Enzymes Be Stabilized?	55
2.2.7.9	How to Store Enzymes?	55
2.3	Instrumental Aspects	57
2.3.1	Spectroscopic Methods	57
2.3.1.1	Absorption (UV/Vis) Photometry	57
2.3.1.2	Cuvettes	68
2.3.1.3	Turbidity Measurements	70
2.3.1.4	Fluorescence Photometry	71
2.3.1.5	Luminometry	76
2.3.1.6	Polarimetry	77
2.3.2	Electrochemical Methods	78
2.3.2.1	pH Meter and Glass Electrodes	78
2.3.2.2	pH Stat	79
2.3.2.3	Potentiometry	79
2.3.2.4	Oxygen and Carbon Dioxide Electrodes	80
2.3.3	Radioactive Labeling	81
2.3.4	Diverse Methods	81
2.4	Theory of Coupled Enzyme Reactions	83
2.4.1	Two Coupled Reactions	83
2.4.2	Three Coupled Reactions	86
2.5	Substrate Determination	86
2.5.1	End Point Method	87
2.5.2	Substrate Determination by Coupled Enzyme Reactions	88
2.5.3	Kinetic Method for Substrate Determination	89
2.5.4	Enzymatic Cycling	90
3	Enzyme Assays	93
3.1	Enzyme Nomenclature	93
3.2	Practical Considerations for Enzyme Assays	97
3.3	Special Enzyme Assays	100
3.3.1	Oxidoreductases, EC 1	100
3.3.1.1	Optical Assay	100
3.3.1.2	Fluorimetric Assay	100
3.3.1.3	Alcohol Dehydrogenase, EC 1.1.1.1	101
	A. Reduction Assay	101
	B. Oxidation Assay	102
3.3.1.4	Alcohol Dehydrogenase (NADP ⁺), EC 1.1.1.2	103
3.3.1.5	Homoserine Dehydrogenase, EC 1.1.1.3	104
3.3.1.6	Shikimate Dehydrogenase, EC 1.1.1.25	105
3.3.1.7	L-Lactate Dehydrogenase, EC 1.1.1.27	106
	A. Spectrophotometric Reduction Assay	106
	B. Fluorimetric Reduction Assay	107
	C. Oxidation Assay	108
3.3.1.8	Malate Dehydrogenase, EC 1.1.1.37	108

- 3.3.1.9 Malate Dehydrogenase (Oxaloacetate-Decarboxylating) (NAD^+), EC 1.1.1.38, and Malate Dehydrogenase (Decarboxylating), EC 1.1.1.39 110
- 3.3.1.10 Malate Dehydrogenase (Oxaloacetate-Decarboxylating) (NADP^+), EC 1.1.1.40 111
- 3.3.1.11 Isocitrate Dehydrogenase (NAD^+), EC 1.1.1.41 112
- 3.3.1.12 Isocitrate Dehydrogenase (NADP^+), EC 1.1.1.42 113
- 3.3.1.13 Glucose-6-phosphate Dehydrogenase, EC 1.1.1.49 114
- 3.3.1.14 Glucose Oxidase, EC 1.1.3.4 115
- 3.3.1.15 Formate Dehydrogenase, EC 1.2.1.2 116
- 3.3.1.16 Glyceraldehyde-3-phosphate Dehydrogenase, EC 1.2.1.12 117
 - A. Oxidation Assay 117
 - B. Reduction Assay Coupled with 3-Phosphoglycerate Kinase (PGK) 118
- 3.3.1.17 Pyruvate Dehydrogenase (Acetyl-Transferring), EC 1.2.4.1 119
 - A. Ferricyanide as Electron Acceptor 120
 - B. Dichlorophenolindophenol as Electron Acceptor 120
- 3.3.1.18 Oxoglutarate Dehydrogenase (Succinyl-Transferring), EC 1.2.4.2 121
- 3.3.1.19 Pyruvate Ferredoxin Oxidoreductase, EC 1.2.7.1 123
 - Assay with Cytochrome *c* as Electron Acceptor 123
- 3.3.1.20 Alanine Dehydrogenase, EC 1.4.1.1 124
 - A. Oxidation of Alanine 124
 - B. Reduction of Pyruvate 125
- 3.3.1.21 Glutamate Dehydrogenase, EC 1.4.1.3 125
- 3.3.1.22 Leucine Dehydrogenase, EC 1.4.1.9 127
- 3.3.1.23 L-Amino Acid Oxidase, EC 1.4.3.2 128
- 3.3.1.24 D-Amino Acid Oxidase, EC 1.4.3.3 129
- 3.3.1.25 Monoamine Oxidase, EC 1.4.3.4 129
- 3.3.1.26 Primary Amine Oxidase, EC 1.4.3.21 130
 - A. Spectrophotometric Assay 131
 - B. Polarographic Assay of O_2 uptake with O_2 Electrode 131
 - C. Assays for Benzylamine Oxidase Activity 132
- 3.3.1.27 Diamine Oxidase, EC 1.4.3.22 132
- 3.3.1.28 Urate Oxidase, EC 1.7.3.3 133
- 3.3.1.29 Dihydrolipoamide Dehydrogenase, EC 1.8.1.4 134
 - A. Oxidation of Dihydrolipoamide 135
 - B. Reduction of Lipoamide 135
- 3.3.1.30 Glutathione Disulfide Reductase, EC 1.8.1.7 136
- 3.3.1.31 Catalase, EC 1.11.1.6 137
- 3.3.1.32 Peroxidase, EC 1.11.1.7 139
 - A. Assays with 2,2'-Azino-bis-3-ethylbenzothiazoline-6-sulfonic Acid (ABTS) 139
 - B. Assay with Guaiacol 140
 - C. Assay with Dianisidine 140
- 3.3.1.33 Luciferase, EC 1.13.12.7 141

3.3.2	Transferases, EC 2	143
3.3.2.1	Dihydrolipoamide Acetyltransferase, EC 2.3.1.12	143
	A. Spectrophotometric Assay	143
	B. Stopped Assay	145
3.3.2.2	Fatty Acid Synthase, EC 2.3.1.85	146
3.3.2.3	Phosphorylase a, EC 2.4.1.1	147
3.3.2.4	Aspartate Transaminase, EC 2.6.1.1	149
3.3.2.5	Alanine Transaminase, EC 2.6.1.2	150
3.3.2.6	Tyrosine Transaminase, EC 2.6.1.5, Tryptophan Transaminase, EC 2.6.1.27, Phenylalanine Transaminase, EC 2.6.1.58	151
3.3.2.7	Hexokinase, EC 2.7.1.1	153
3.3.2.8	Pyruvate Kinase, EC 2.7.1.40	155
3.3.2.9	Acetate Kinase, EC 2.7.2.1	156
3.3.2.10	Phosphoglycerate Kinase, EC 2.7.2.3	157
3.3.2.11	Aspartokinase, EC 2.7.2.4	159
3.3.3	Hydrolases, EC 3	160
3.3.3.1	Lipase, EC 3.1.1.3	160
	A. Assay with pH-Stat (Auto titrator)	161
	B. Fluorimetric Assay	162
3.3.3.2	Phospholipase A ₂ , EC 3.1.1.4	162
3.3.3.3	Acetylcholine-Esterase, EC 3.1.1.7	163
3.3.3.4	Choline Esterase, EC 3.1.1.8	164
	A. pH-Stat Assay	165
	B. Colorimetric Assay	166
3.3.3.5	S-Formylglutathione Hydrolase, EC 3.1.2.12	166
3.3.3.6	Alkaline Phosphatase, EC 3.1.3.1	167
	A. Mammalian Alkaline Phosphatase	167
	B. Bacterial Alkaline Phosphatase	168
3.3.3.7	Acid Phosphatase, EC 3.1.3.2	168
3.3.3.8	Ribonuclease (Pancreatic), EC 3.1.27.5	169
3.3.3.9	α -Amylase, EC 3.2.1.1	170
3.3.3.10	Amyloglucosidase, EC 3.2.1.3	171
	A. Coupled Assay with HK and G6PDH	172
	B. Photometric Assay with 4-Nitrophenyl-D-Glucose	172
	C. Fluorimetric Assay with 4-Methylumbelliferyl- α -D-Glucoside	173
3.3.3.11	Cellulases, β -1,4-Glucanase, EC 3.2.1.4, and β -Glucosidase, EC 3.2.1.21	174
	A. Orcinol Assay	174
	B. Activity Staining	175
3.3.3.12	Lysozyme, EC 3.2.1.17	177
3.3.3.13	Sialidase, EC 3.2.1.18	177
	A. Fluorimetric Assay	178
	B. Activity Staining	178
3.3.3.14	α -Glucosidase, EC 3.2.1.20	179
	A. α -Glucosidase Assay	180

	B. Glucose Determination	180
	C. Assay with 4-Nitrophenylglucopyranoside	181
3.3.3.15	β -Galactosidase, EC 3.2.1.23	182
3.3.3.16	β -Fructosidase, EC 3.2.1.26	183
3.3.3.17	β -Glucuronidase EC 3.2.1.31	184
	A. Fluorimetric Assay	184
3.3.3.18	Proteases, EC 3.4, General Assays	185
	A. Anson Assay	185
	B. Casein-Assay	187
	C. Azocasein Assay	188
	D. Ninhydrin Assay	189
3.3.3.19	Leucine Aminopeptidase, EC 3.4.11.1	190
	A. Assay with Leucineamide	191
	B. Assay with Leucine- <i>p</i> -nitroanilide	191
3.3.3.20	α -Chymotrypsin, EC 3.4.21.1	192
	A. Assay with SUPHEPA	192
	B. Assay with GLUPHEPA	193
3.3.3.21	Pancreatic Elastase, EC 3.4.21.35 (Previous EC 3.4.4.7)	194
	A. Assay with Succinyl-Ala-Ala-Ala- <i>p</i> -Nitronilide	194
	B. Esterase Activity of Elastase	194
3.3.3.22	Pepsin, EC 3.4.23.1	195
3.3.3.23	Trypsin, EC 3.4.21.4	196
3.3.3.24	Asparaginase, EC 3.5.1.1	197
3.3.3.25	Glutaminase, EC 3.5.1.2	198
	A. Determination of Ammonia with Nessler's Reagent	199
	B. pH-Stat Assay	199
3.3.3.26	Urease, EC 3.5.1.5	200
	A. pH Stat Assay	201
	B. Photometric Assay	201
3.3.3.27	Adenosinetriphosphatase, EC 3.6.1.3	202
3.3.4	Lyases, EC 4	203
3.3.4.1	Pyruvate Decarboxylase, EC 4.1.1.1	203
3.3.4.2	Glutamate Decarboxylase, EC 4.1.1.15	205
3.3.4.3	Aldolase, EC 4.1.2.13	206
3.3.4.4	Anthranilate Synthase, EC 4.1.3.27	207
3.3.4.5	Carbonic Anhydrase, EC 4.2.1.1	208
	A. pH-Stat Assay	208
	B. Esterase Assay with 4-Nitrophenylacetate	209
3.3.4.6	Fumarase, EC 4.2.1.2	210
3.3.5	Isomerases, EC 5	211
3.3.5.1	Glucose/Xylose Isomerase EC 5.3.1.5	211
	A. D-Xylose Isomerase Assay	211
	B. D-Xylose Isomerase Microplate Assay	212
	C. D-Glucose Isomerase Assay	213
	D. D-Glucose Isomerase Microplate Assay	214

3.3.5.2	Phosphoglucosmutase, EC 5.4.2.2	215
3.3.6	Ligases (Synthetases) EC 6	216
3.3.6.1	Tyrosine-tRNA Ligase, EC 6.1.1.1	216
	A. Fluorimetric Assay	216
	B. ATP – ³² PP-Exchange	217
3.3.6.2	Glutamine Synthetase EC 6.3.1.2	218
3.3.7	Assays for Multienzyme Complexes	220
3.3.7.1	Pyruvate Dehydrogenase Complex (PDHC)	220
	A. Overall Activity of PDHC by NAD ⁺ Reduction	221
	B. Overall Activity of PDHC by by Dismutation Assay	222
3.3.7.2	α-Oxoglutarate Dehydrogenase Complex (OGDHC)	224
	Overall Activity by NAD ⁺ Reduction	224
3.3.8	Substrate Determination	225
3.3.8.1	Determination of NADP(H) by Enzymatic Cycling	225
3.3.8.2	Determination of NAD(H)	227
3.4	Assays for Enzyme Characterization	229
3.4.1	Protein Determination	229
3.4.1.1	Biuret Assay	230
3.4.1.2	BCA Assay	232
	A. Assay for Soluble Proteins	232
	B. Modification for Immobilized Proteins	233
3.4.1.3	Lowry Assay	234
3.4.1.4	Coomassie Binding Assay (Bradford Assay)	235
3.4.1.5	Absorption Method	236
3.4.1.6	Fluorimetric Assay	238
3.4.1.7	Ninhydrin Assay	239
3.4.1.8	Modified Ninhydrin Assay without Hydrolysis	240
3.4.1.9	Protein Assay with 2-Hydroxy-1-naphthaldehyde	242
3.4.2	Phosphate Determination	243
3.4.3	Glycoprotein Assays	245
	A. Detection in Electrophoresis Gels	245
	B. Determination of Protein-Bound Hexoses	245
3.4.4	Cross-Linking of Proteins with Dimethylsuberimide	246
3.4.5	Concentration of Enzyme Solutions	247
3.4.5.1	Precipitation	248
3.4.5.2	Ultrafiltration and Dialysis	251
3.4.5.3	Ultracentrifugation	252
3.4.5.4	Lyophilization	252
3.4.5.5	Other Concentration Methods	253
3.5	Enzyme Immunoassays	254
3.5.1	Radioimmunoassays	254
3.5.2	Noncompetitive Solid-Phase Enzyme Immunoassay	256
3.5.3	Competitive Solid-Phase Enzyme Immunoassay	257
3.5.4	Methods for Enzyme Immunoassays and Immobilization Techniques	257

3.5.4.1	Protein Coupling to Cyanogen Bromide–Activated Agarose	257
3.5.4.2	Coupling of Diaminohexyl Spacer	259
3.5.4.3	Periodate Activation of Cellulose	259
3.5.4.4	Introduction of Thiol Groups into Proteins (Antibodies)	260
3.5.4.5	Conjugation of a Protein (Antibody) with an Enzyme (Peroxidase)	261
3.5.4.6	Conjugation of β -Galactosidase to Proteins (Antibodies) by MBS	262
3.5.4.7	Conjugation of Alkaline Phosphatase to Antibodies by Glutaraldehyde	263
4	Binding Measurements	265
4.1	Different Types of Binding	265
4.1.1	General Considerations	265
4.1.2	How to Recognize Specific Reversible Binding?	266
4.1.3	Experimental Aspects	268
4.2	Binding Measurements by Size Discrimination	271
4.2.1	Equilibrium Dialysis	271
4.2.1.1	Binding of Indole to Bovine Serum Albumin	273
4.2.2	Evaluation of Binding Experiments	276
4.2.3	Ultrafiltration	278
4.2.4	Gel Filtration	278
4.2.5	Ultracentrifugation	280
4.3	Spectroscopic Methods	281
4.3.1	Difference Spectroscopy	282
4.3.1.1	Difference Spectroscopic Titration of Ligands Binding to Catalase	283
4.3.1.2	Evaluation of Spectroscopic Binding Curves	287
4.3.2	Fluorescence Spectroscopy	289
4.3.2.1	Binding of ANS to Bovine Serum Albumin	290
4.4	Other Binding Methods	295
4.4.1	Radioactive Labeling	295
4.4.2	Surface Plasmon Resonance	295
5	Enzymes in Technical Applications	297
5.1	Modes of Enzyme Immobilization	297
5.1.1	Adsorption	298
5.1.2	Entrapment	300
5.1.3	Encapsulation	300
5.1.4	Cross Linking	301
5.1.5	Covalent Immobilization to Solid Supports	303
5.1.5.1	Supports	303
5.1.5.2	Spacer	303
5.2	Methods for Enzyme Immobilization	305
5.2.1	Microencapsulation in Nylon Beads	305
5.2.2	Entrapment in Polyacrylamide	306
5.2.3	Covalent Immobilization on Glass Surfaces	307
5.2.4	Covalent Immobilization on Controlled-Pore Glass	309

5.2.5	Covalent Immobilization to Polyamide	312
5.2.5.1	o-Alkylation with Triethyloxonium Tetrafluoroborate	313
5.2.5.2	Immobilization to Amino Groups after Partial Hydrolysis of Polyamide	315
5.2.5.3	Immobilization to Carboxyl Groups after Partial Hydrolysis of Polyamide	317
5.2.6	Immobilization to Polyester	318
5.2.7	Immobilization by Alkaline Hydrolysis and Activation with Tosylchloride	320
5.2.8	Alkaline Hydrolysis and Activation by Carbonyldiimidazol	321
5.3	Analysis of Immobilized Enzymes	322
5.3.1	General Principles	322
5.3.2	Continuous Photometric Assays for Immobilized Enzymes	324
5.3.3	Cofactors in Reactions with Immobilized Enzymes	325
5.4	Enzyme Reactors	326
5.4.1	Batch Reactor (Stirred-Tank Reactor)	327
5.4.2	Membrane Reactor	327
5.4.3	Solid Bed Reactor	328
5.4.4	Immobilized Cells	329
5.5	Biosensors	329
5.5.1	Enzyme Electrodes	329
5.5.2	Immunoelectrodes	333
5.5.3	Other Biosensors	334
5.6	Immobilized Enzymes in Therapy	335

Appendix 337

List of enzymes according to the EC numbers	337
---	-----

Index 353