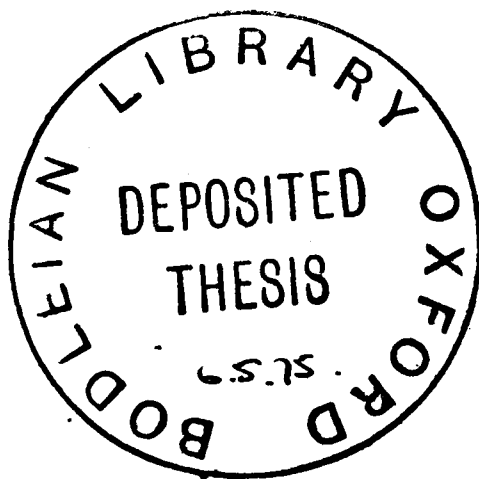


THE CONTROL OF THE CONCENTRATIONS OF
CYCLIC NUCLEOTIDES AND ADENOSINE
IN ANIMAL TISSUES

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CONTENTS

Acknowledgements	11
Contents	iii
Abbreviations	ix
Enzymes	xi
Abstract	xiii

Chapter 1 GENERAL INTRODUCTION

Mechanisms of control	1
Cyclic nucleotides and adenosine	2
The approach employed in the present work	3
A. <u>CYCLIC NUCLEOTIDE METABOLISM</u>	3
1. Pathways of cyclic nucleotide metabolism	3
(a) Cyclic AMP formation - adenylyl cyclase	4
(b) Cyclic nucleotide degradation - phosphodiesterase	7
2. The roles of cyclic nucleotides	14
(a) Cyclic nucleotides as mediators of hormone action	14
(b) Cyclic nucleotides and non-hormonal effects	15
3. The mechanism of action of cyclic nucleotides	16
(a) Protein kinase mediated actions	16
(b) Cyclic nucleotide effects which are not mediated by protein kinase	21
4. Compartmentation of cyclic AMP	22
Summary of Section A	26
B. <u>ADENOSINE METABOLISM</u>	27
Historical introduction	27
1. The concentration and location of adenosine in animal tissue	28
(a) Adenosine concentrations	28
(b) The location of adenosine	28
2. The control of adenosine concentrations	30
(a) Pathways of adenosine metabolism	30
(b) The significance of equilibria in understanding the control of adenosine concentrations	33
(c) Adenosine formation - 5'-nucleotidase	34
(d) Enzymes which utilize adenosine	36
(e) A theory of the control of intracellular adenosine concentrations	37

3. The output of adenosine from the cell	38
(a) Problems in the measurement of the transport of adenosine out of the cell	38
(b) Assessment of the transport of adenosine out of the cell	39
(c) Treatments which increase adenosine transport out of the cell	41
(d) Evidence for the theory of control of adenosine concentrations	42
(e) The localization of 5'-nucleotidase and the site of adenosine production	42
4. The uptake of adenosine by the cell	45
(a) The locations of adenosine output and metabolism	45
(b) Evidence that adenosine is largely metabolized within the cell	45
(c) The adenosine uptake mechanism	47
5. The physiological effects of adenosine	50
(a) The effects of exogenous adenosine	50
(b) The biochemical mechanisms which mediate the physiological effects of adenosine	53
(c) The role of adenosine in the living animal	58
Conclusions	61

Chapter 2 DEVELOPMENT OF RADIOCHEMICAL ASSAY METHODS

Criteria used in developing the assays	62
A. <u>A SUMMARY OF THE ENZYME ASSAY METHODS</u>	63
1. Dissection and homogenization of tissues	63
2. Summary of the adenyl cyclase assay	64
3. Summary of the cyclic AMP and cyclic GMP phosphodiesterase assays	66
4. Summary of the adenosine kinase and adenosine deaminase assays	67
5. Summary of the 5'-nucleotidase assay	69
6. Summary of the thin-layer chromatography method	69
B. <u>DEVELOPMENT OF THE ADENYL CYCLASE ASSAY</u>	71
1. The problem of substrate breakdown	71
2. The problem of product breakdown	73
3. The detection of cyclic AMP formed in the assay	75
4. Further components of the assay mixture	77
5. The extraction of adenyl cyclase	77

C. <u>DEVELOPMENT OF THE PHOSPHODIESTERASE ASSAY</u>	78
1. The estimation of product formation	78
2. Interference in the assay by other enzymes	80
3. Further components of the assay mixture	82
4. Extraction of the enzyme	83
D. <u>DEVELOPMENT OF THE ADENOSINE KINASE ASSAY</u>	84
1. Interference by contaminating enzymes	84
2. Estimation of product formation	86
3. The effects of buffers and pH	87
4. The effect of divalent metal ions	87
5. Validation of the assay	88
6. The extraction and stability of adenosine kinase	89
E. <u>DEVELOPMENT OF THE ADENOSINE DEAMINASE ASSAY</u>	91
1. Estimation of product formation	91
2. Interference by other enzymes	92
3. Further components of the assay mixture	92
4. The extraction and stability of adenosine deaminase	93
F. <u>DEVELOPMENT OF THE 5'-NUCLEOTIDASE ASSAY</u>	94
1. Interference by other enzymes	94
2. Estimation of product formation	95
3. Further components of the assay mixture	95
G. <u>CALCULATION OF RESULTS</u>	96
1. Graphical methods	96
2. Analyses of phosphodiesterase kinetics	96
3. Calculation of $V_{\max}$	98
H. <u>THE STATISTICAL SIGNIFICANCE OF THE RESULTS</u>	99

Chapter 3 STUDIES OF ADENYL CYCLASE AND PHOSPHODIESTERASE

A. <u>INTRODUCTION AND METHODS</u>	100
1. Variations in the procedure for extraction of adenylyl cyclase	100
2. Measurement of the activity of adenylyl cyclase	101
3. Substrate concentrations for enzyme assays	102
B. <u>RESULTS</u>	102
1. The effect of extraction procedure on the activity of adenylyl cyclase	102
2. The activities of adenylyl cyclase and phosphodiesterase from animal tissues	103
3. The stimulation of the activity of adenylyl cyclase by fluoride and Triton X-100	106
4. K_m values and deviations from Michaelis-Menten kinetics	108
C. <u>DISCUSSION</u>	110
1. The molecular nature of cyclic AMP and cyclic GMP phosphodiesterase	110
2. The application of the present data to a model of cyclic AMP turnover	111
3. The rate of turnover of cyclic AMP in the cell	115
4. The roles of high and low K_m phosphodiesterase in the control of cyclic nucleotide concentrations	119
5. The compartmentation of cyclic AMP	125
6. The role of cyclic AMP in various tissues	127

Chapter 4 STUDIES OF 5'-NUCLEOTIDASE, ADENOSINE KINASE AND ADENOSINE DEAMINASE

A. <u>INTRODUCTION</u>	133
B. <u>METHODS</u>	133
1. Effects of Na^+ and K^+	134
2. Effects of Ca^{2+}	134
3. Measurements of maximal activities and K_m values	134

C. <u>RESULTS</u>	135
1. The specificity of adenosine kinase for its phosphate donor	135
2. The effects of methyl xanthines, papaverine and cyclic AMP on the activities of adenosine kinase and adenosine deaminase	135
3. The effects of Na^+ and K^+ on the activities of adenosine kinase and adenosine deaminase	136
4. The effects of Ca^{2+} on the activities of adenosine kinase, adenosine deaminase and 5'-nucleotidase	137
5. Comparative studies of 5'-nucleotidase, adenosine kinase and adenosine deaminase	137
6. Activities of 5'-nucleotidase, adenosine kinase and adenosine deaminase in the brains of newborn and adult mice	142
D. <u>DISCUSSION</u>	142
1. The control of tissue adenosine concentrations	142
(a) The control of the activity of 5'-nucleotidase	142
(b) The control of the activities of adenosine kinase and adenosine deaminase	144
(c) The control of adenosine concentrations	149
2. The roles of adenosine kinase and adenosine in the autoregulation of blood flow	152
(a) The role of adenosine in muscle tissues	153
(b) The role of adenosine in nervous tissues	155
(c) The role of adenosine kinase in vertebrate livers and blood	157
(d) The role of adenosine in the lung	159
(e) The control of adenosine concentrations for the purpose of regulating blood flow	160
3. A possible role for adenosine as an inter-organ communicator	161
4. The roles of 5'-nucleotidase, adenosine kinase and adenosine deaminase in adenine nucleotide metabolism	162
5. A summary of the hypotheses presented	164
<u>Chapter 5 THE REGULATION OF LIPOLYSIS IN THE HEARTS OF STARVED AND DIABETIC RATS</u>	
A. <u>INTRODUCTION</u>	167
B. <u>METHODS</u>	168
C. <u>RESULTS AND DISCUSSION</u>	169

Chapter 6 SOME RELATIONSHIPS BETWEEN THE METABOLISM OF ADENOSINE AND CYCLIC AMP

A. <u>THE EFFECT OF ADENOSINE ON THE METABOLISM OF CYCLIC AMP</u>	173
1. The role of adenosine	173
2. The mechanism of action of adenosine	174
(a) Effects of adenosine on intact tissues	174
(b) Effects of adenosine in tissue homogenates	175
B. <u>A COMPARISON OF THE METABOLISM OF CYCLIC AMP AND ADENOSINE</u>	177
1. Similarities between the metabolism and roles of cyclic AMP and adenosine	177
2. Differences between the metabolism and roles of cyclic AMP and adenosine	179
(a) The significance of the structures of cyclic AMP and adenosine	179
(b) The control of messenger formation by precursor concentration	180
(c) The changes in free energy during the metabolism of cyclic AMP and adenosine	180
(d) Further differences between the control of the concentrations of adenosine and cyclic AMP	181
(e) Why should the concentration of adenosine vary more than that of cyclic AMP?	183
<u>Appendix A LIQUID SCINTILLATION COUNTING TECHNIQUES</u>	184
<u>Appendix B TECHNIQUES USED TO REDUCE THE BLANK OF THE ADENYL CYCLASE ASSAY</u>	185
<u>Appendix C ATTEMPTS TO MEASURE THE ACTIVITY OF GUANYL CYCLASE</u>	186
<u>Appendix D SOURCES OF ANIMALS AND MATERIALS</u>	189
<u>REFERENCES</u>	191

ABBREVIATIONS

ADP	Adenosine 5'-diphosphate
AMP or 5'-AMP	Adenosine 5'-monophosphate
ATP	Adenosine 5'-triphosphate
ACTH	Adrenocorticotrophic hormone
CAP	Catabolite gene-activating protein (Cyclic AMP receptor protein)
CoA	Coenzyme-A
Cyclic AMP	Adenosine 3',5'-cyclic monophosphate
Cyclic GMP	Guanosine 3',5'-cyclic monophosphate
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetra-acetic acid
EGTA	Ethylene glycol-bis(β -amino ethyl ether)N,N'-tetra-acetic acid
FDP	Fructose 1,6-diphosphate
ΔG°	Change in the standard free energy
GDP	Guanosine 5'-diphosphate
GMP	Guanosine 5'-monophosphate
GTP	Guanosine 5'-triphosphate
IMP	Inosine 5'-monophosphate
ITP	Inosine 5'-triphosphate
K_a	Activator constant
K_i	Inhibitor constant
K_m	Michaelis constant
M^{+} or M^{2+}	Mono- or divalent metal ion
Me6MPR	6-(Methylmercapto)purine ribonucleoside
Mg-ATP	The equimolar complex between Mg^{2+} and ATP. (In tables of reagents for enzyme assays: equimolar concentrations of $MgCl_2$ and ATP.)

NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced)
PEP	Phosphoenolpyruvate
P _i	Inorganic phosphate
PP _i	Inorganic pyrophosphate
PRPP	5-Phosphoribosyl-1-pyrophosphate
RNA	Ribonucleic acid
s	Substrate concentration
TCA cycle	Tricarboxylic acid cycle
Tris	Tris-(hydroxymethyl)-aminomethane
UV	Ultra-violet
v	Velocity of enzyme reaction
V _{max}	Maximal (extrapolated) velocity of enzyme reaction

ENZYMES DISCUSSED IN THE TEXT

<u>Enzyme</u>	<u>E. C. No.</u>	<u>Systematic name</u>
Acetoacetyl-CoA thiolase	2.3.1.9.	Acetyl-CoA:acetyl-CoA C-acetyltransferase
Acid phosphatase	3.1.3.2.	Orthophosphoric-monoester phosphohydrolase (acid optimum)
Adenine phosphoribosyl- transferase	2.4.2.7	AMP:pyrophosphate phosphoribosyltransferase
Adenosine deaminase	3.5.4.4.	Adenosine aminohydrolase
Adenosine kinase	2.7.1.20	ATP:adenosine 5'-phospho- transferase
Adenyl cyclase	4.6.1.1.	ATP pyrophosphate-lyase (cyclizing)
Adenylate kinase, myokinase	2.7.4.3.	ATP:AMP phosphotransferase
Adenylosuccinate lyase	4.3.2.2.	Adenylosuccinate AMP-lyase
Adenylosuccinate synthetase	6.3.4.4.	IMP:L-aspartate ligase (GDP-forming)
Alkaline phosphatase	3.1.3.1.	Orthophosphoric-monoester phosphohydrolase (alkaline optimum)
AMP deaminase	3.5.4.6.	AMP aminohydrolase
ATPase	3.6.1.3.	ATP phosphohydrolase
Carnitine palmitoyl- transferase	2.3.1.21	Palmitoyl-CoA:L-carnitine O-palmitoyltransferase
Creatine kinase	2.7.3.2.	ATP:creatine phosphotransferase
Diglyceride lipase	3.1.1.34.	Diacylglycerol acylhydrolase
FDPase	3.1.3.11.	D-fructose-1,6-diphosphate 1-phosphohydrolase
Glycogen synthetase	2.4.1.11.	UDP-glucose:glycogen 4- α - glucosyltransferase
Guanylyl cyclase	4.6.1.2.	GTP pyrophosphate-lyase (cyclizing)
Hypoxanthine phospho- ribosyltransferase	2.4.2.8.	IMP:pyrophosphate phospho- ribosyltransferase

Lactate dehydrogenase	1.1.1.27	L-lactate:NAD oxidoreductase
Lipase phosphatase (see protein phosphatase)		
Myokinase (see adenylate kinase)		
Nucleosidediphosphate kinase	2.7.4.6.	ATP:nucleosidediphosphate phosphotransferase
5'-Nucleotidase	3.1.3.5.	5'-Ribonucleotide phosphohydrolase
3-Oxo acid CoA-transferase	2.8.3.5.	Succinyl-CoA:3-oxo acid CoA-transferase
Phosphodiesterase	3.1.4.17.	3':5'-Cyclic-AMP (or cyclic GMP) 5'-nucleotidohydrolase
Phosphofructokinase	2.7.1.11.	ATP:D-fructose-6-phosphate 1-phosphotransferase
Phosphorylase	2.4.1.1.	1,4- α -D-glucan:orthophosphate glucosyltransferase
Phosphorylase <u>b</u> kinase	2.7.1.38	ATP:phosphorylase- <u>b</u> phosphotransferase
Protein kinase	2.7.1.37	ATP:protein phosphotransferase
Protein phosphatase	3.1.3.16	Phosphoprotein phosphohydrolase
Purine nucleoside phosphorylase	2.4.2.1.	Purine-nucleoside:orthophosphate ribotransferase
Pyrophosphatase	3.6.1.1.	Pyrophosphate phosphohydrolase
Pyruvate kinase	2.7.1.40	ATP:pyruvate phosphotransferase
RNA polymerase	2.7.7.6.	Nucleosidetriphosphate:RNA nucleotidyltransferase
Triglyceride lipase	3.1.1.3.	Triacylglycerol acyl-hydrolase

ABSTRACT

The aim of this thesis is to provide information concerning the control of the concentrations and the physiological roles of cyclic nucleotides and adenosine in animal tissues. For this purpose the activities, kinetics and some properties of adenyl cyclase, cyclic AMP and cyclic GMP phosphodiesterase, 5'-nucleotidase, adenosine kinase and adenosine deaminase have been studied in muscles, nervous tissues and livers from a number of vertebrates and invertebrates.

Chapter 1

Metabolic events within an organism are controlled and coordinated at a number of levels of complexity. Cyclic nucleotides and adenosine are messenger molecules concerned with the coordination of metabolism.

In Section A some aspects of cyclic nucleotide metabolism are considered and certain points are made which are relevant to the interpretation of the data presented in later chapters. Some factors which modulate the activities of adenyl cyclase and phosphodiesterase in vitro are described and the relevance of their effects to the in vivo situation is discussed. The significance of the basal and fluoride-stimulated activities of adenyl cyclase and the unusual kinetics of phosphodiesterase is considered.

Cyclic AMP, and possibly cyclic GMP, transmit hormonal stimuli to the interior of the cell but it is pointed out that they may have further roles. In particular, cyclic AMP may play an important role in nervous tissue. Some of the effects of cyclic AMP depend on the phosphorylation of enzymes or other proteins by a cyclic AMP-dependent protein kinase. The mediation of protein kinase means that there may be a low concentration of cyclic AMP in the cell but nevertheless a small change in the concentration of cyclic AMP may produce a large and rapid effect on the flux through a metabolic

pathway. The low concentration of cyclic AMP means that a high rate of turnover of cyclic AMP is associated with only a low utilization of energy. The high rate of turnover of cyclic AMP is necessary for a rapid response of the cell to hormonal stimulation of adenyl cyclase. It has frequently been claimed that the concentration of cyclic AMP available to protein kinase or phosphodiesterase is very different from that measured in the whole cell. It is shown that it is often possible to provide different explanations for the data which have led to this claim.

In Section B adenosine metabolism and the role of adenosine are discussed in some detail. It is pointed out that it does not now appear that adenosine is a metabolite on a main pathway of intermediary metabolism. There have been few measurements of tissue concentrations of adenosine and there is no consensus of opinion concerning the primary location of adenosine — intra- or extracellular. The simplest hypothesis is that adenosine equilibrates across the cell membrane. Evidence is presented to show that in vivo adenosine is produced by 5'-nucleotidase and utilized by adenosine kinase and adenosine deaminase. The regulation of the activities of these enzymes for the purpose of controlling the concentration of adenosine is discussed. A theory of the control of the concentration of adenosine by changes in the phosphorylation state of the cell is presented. This theory predicts variations in the rate of output of adenosine from the cell which are consistent with data in the literature. The output and uptake of adenosine by the cell are discussed in detail. Low concentrations of adenosine within a tissue are mostly phosphorylated within the cell; deamination of adenosine, which may be partly extracellular, becomes relatively more important when the concentration of adenosine is high.

Exogenous adenosine has a number of effects on the physiology of mammalian tissues. For example, it relaxes most smooth muscles. Adenosine

produces a large increase in the content of cyclic AMP in mammalian brains and it is suggested that a number of the physiological effects of exogenous adenosine are mediated by a change in the concentration of cyclic AMP. In the final section of Chapter 1, it is argued that adenosine plays a role in the regulation of blood flow in some vertebrate tissues. It may also play a role in neurotransmission.

Chapter 2

For comparative studies it is necessary that enzyme assays are applicable to enzymes in homogenates of very different tissues. In some cases very small quantities of tissue are available. Moreover, when maximal activities are being measured, optimal conditions for the assays are required. Radiochemical assays were developed to meet these requirements; they are described in Section A of Chapter 2. In the succeeding sections the development of each assay is described. A number of preliminary results are presented, including pH curves. Finally, the graphical methods which were used to analyze the results and the significance of the data obtained are discussed.

Chapter 3

As a preliminary to the discussion of the results of Chapter 3, it is reported that the extraction of adenyl cyclase using a hypotonic medium and a ground-glass homogenizer and pestle leads to higher basal activities and a greater degree of stimulation of enzyme activity by fluoride than extraction in isotonic medium using an ordinary glass homogenizer. The former method was used for a comparative study of basal and fluoride-stimulated activities of adenyl cyclase. The activities and K_m values of adenyl cyclase and cyclic AMP and cyclic GMP phosphodiesterase are reported for a number of animal tissues. In general the activities decrease in the order nervous tissues > hearts > muscles with a high oxidative capacity and livers > muscles with

a low oxidative capacity. The kinetic data for phosphodiesterase are analyzed on the assumption that at least two enzymes (high and low K_m) contribute to the kinetics. It is argued that the analysis supports this assumption. It is also argued that, in vertebrate tissues, one high K_m enzyme hydrolyzes both cyclic AMP and cyclic GMP.

The data are applied to a simple model of the turnover of cyclic AMP in the cell. Maximum and minimum tissue concentrations of cyclic AMP are predicted from the model. The predictions are consistent with much reported data. It is suggested that tissue concentrations of cyclic AMP do not vary greatly in vivo. The results, as interpreted by the model, indicate that the extrusion of cyclic AMP from the cell does not play an important role in the turnover of cyclic AMP in most tissues.

The roles of the high and low K_m phosphodiesterases are discussed. It is proposed that the high K_m enzyme prevents the accumulation of cyclic AMP in tissues where the activity of adenyl cyclase may be higher than the activity of low K_m phosphodiesterase. However, it is more efficient for the cell to synthesize the low K_m enzyme. Possible explanations for variations in the proportions of high and low K_m enzymes between tissues are suggested. One of these explanations is consistent with the present data only if the simple model of the turnover of cyclic AMP is modified. The modification of the model permits the explanation of some further data. According to the modified model adenyl cyclase and low K_m phosphodiesterase are located at the cell membrane, whereas high K_m phosphodiesterase is located in the cytosol. Nevertheless, it is claimed that, unless the local activity of adenyl cyclase is much greater than that of low K_m phosphodiesterase, the compartmentation of cyclic AMP is not great.

The roles of cyclic AMP in nervous tissue and muscle are discussed. It is argued that high activities of adenyl cyclase and phosphodiesterase

are found in those tissues in which the concentration of cyclic AMP must change rapidly, rather than in tissues which contain a large number of enzyme molecules whose activity is regulated by cyclic AMP-dependent protein kinase.

Chapter 4

The activities and K_m values of 5'-nucleotidase, adenosine kinase and adenosine deaminase are reported for a number of animal tissues. The activities of the enzymes are higher in nervous tissues and red muscles than in white muscles. The brain of the new-born mouse contains low activities of 5'-nucleotidase and adenosine kinase. The activity of adenosine kinase is very high in vertebrate livers.

It is reported that methylxanthines and papaverine activate adenosine kinase. Na^+ also activates adenosine kinase, whereas K^+ and Ca^{2+} inhibit. The significance of these effects in relation to the control of tissue concentrations of adenosine is discussed. During basal metabolism the concentration of adenosine is low; it is proposed that when the cell is under stress the activity of 5'-nucleotidase increases and the activity of adenosine kinase decreases so that the concentration of adenosine is increased. Some of this adenosine passes out of the cell. The data are consistent with the view that one of the functions of extracellular adenosine in muscle and brain is to cause the smooth muscle of the vasculature to relax so that blood flow increases. It is suggested that the liver, and possibly the lung and blood cells remove adenosine from the circulation. Further roles for adenosine are suggested.

By comparing measured K_m values with reported tissue concentrations of AMP and adenosine, it is deduced that the activities of 5'-nucleotidase, adenosine kinase and adenosine deaminase are partly regulated by their substrate concentration in vivo. The proportion of exogenous adenosine

which is deaminated rather than rephosphorylated is increased when the concentration of adenosine is high because the K_m value of adenosine deaminase is greater than that of adenosine kinase and the activity of adenosine kinase is inhibited by a high concentration of adenosine. Moreover, if a high concentration of adenosine is associated with the cell being under stress, the activity of adenosine kinase may be reduced by other factors. It is argued that reduced phosphorylation and increased deamination of adenosine help to maintain the tissue ATP/ADP concentration ratio constant when the cell is under stress.

Chapter 5

The control of lipolysis in the rat heart is discussed. It is pointed out that variations in the activities of triglyceride lipase, adenyl cyclase or phosphodiesterase could cause the increased lipolysis which has been measured for hearts from diabetic and starved rats. It is reported that no differences in the activities or kinetics of these three enzymes are found between normal, starved and diabetic rats. There is further speculation concerning the factors which increase lipolysis in the diabetic and starved conditions.

Chapter 6

The role of adenosine is discussed further. It is suggested that the control of the activity of adenosine kinase by Ca^{2+} , Na^+ and K^+ would be consistent with a role for adenosine in neurotransmission. It is proposed that adenosine stimulates the activity of adenyl cyclase in a number of tissues but usually the effect cannot be detected because it is limited to a small proportion of the cells. The difficulties involved in obtaining effects of adenosine on the activity of adenyl cyclase in tissue homogenates are discussed and some results are presented concerning this problem.

It is pointed out that there are a number of similarities between the metabolism of cyclic AMP and adenosine. These similarities derive from the similar roles of the two molecules as messengers. The mechanisms which the cell uses to metabolize messenger molecules are therefore discussed. However, there are also some differences between the metabolism of cyclic AMP and adenosine. It is argued that the differences can be accounted for in terms of the differing roles of the two messengers: adenosine functions extracellularly as a first messenger, whereas cyclic AMP functions intracellularly as a second messenger.

Chapter 1

GENERAL INTRODUCTION

MECHANISMS OF CONTROL

Within the cell metabolic events are controlled and coordinated through variations in the concentrations of messenger or regulator molecules. The earliest and simplest example of regulation is that of feedback inhibition, in which the end product of a metabolic pathway inhibits the first enzyme in the pathway. In this way excessive accumulation of the end product is prevented. Other kinds of regulation have been discovered in recent years. These "primitive" regulatory mechanisms are particular to the cell and do not depend on hormonal or nervous stimulation from outside.

Superimposed upon the primitive control mechanisms which exist within the cell, there are mechanisms which enable events within one cell to be coordinated with those in another. The level of interaction may vary from that of coordinating events within adjacent cells, to that of coordinating events between organs. For example, prostaglandins act hormonally on cells in the same tissue where they are produced, whereas many hormones act in tissues remote from their site of production.

The link between two cells or groups of cells may be electrical in nature, or it may involve the transfer of a messenger molecule between the cells. In either case the end result is that the concentration of a regulator molecule within the cell is changed. Thus the link may take the form of a nervous stimulus which produces a change in the distribution of calcium ions within the target cells, or it may take the form of a hormone which produces a change in the cyclic AMP content of the target cells.

CYCLIC NUCLEOTIDES AND ADENOSINE

This thesis is concerned with two messenger molecules: cyclic AMP and adenosine. Some consideration is also given to cyclic GMP. The role of cyclic AMP as a messenger molecule, in particular as the "second messenger" mediating the effect of a variety of hormones, is well known (see Sutherland et al., 1965). Cyclic GMP is presumed to play a similar, perhaps complementary, role (see Goldberg et al., 1973). However, the possible role of adenosine as a messenger molecule has not been clearly established and is not widely known. One of the objects of this thesis is to investigate the possible role of this compound in metabolic regulation.

There are a number of reasons why cyclic AMP and adenosine metabolism should be studied together. Firstly, effects which have been ascribed to one molecule may in fact be caused by the other; knowledge of the metabolism and role of both molecules should minimize such interpretative error. Thus exogenous cyclic AMP is degraded to adenosine (Kaukel et al., 1972). Therefore some effects of exogenous cyclic AMP may in fact be caused by adenosine. Erroneous interpretations of results may also arise because some compounds which are used to modify cyclic AMP metabolism also affect adenosine metabolism. Secondly, the metabolism of adenosine and cyclic AMP is closely interlinked. Exogenous adenosine can greatly affect cyclic AMP levels in some tissues and exogenous adenosine has been found to enter a pool of brain ATP which is selectively utilized by adenyl cyclase. Thirdly, it is useful to compare the mechanisms which control the concentrations of cyclic AMP and adenosine because there are a number of similarities between the metabolism of the molecules. Thus the tissue concentrations of cyclic AMP and adenosine, and the kinetic constants of the enzymes which are involved in their metabolism, are very similar. It is particularly interesting to consider how cyclic AMP and adenosine concentrations are controlled by a balance between their

production and metabolism. Moreover, the roles of the high and low K_m cyclic AMP phosphodiesterases may be compared with the roles of the two enzymes metabolizing adenosine.

THE APPROACH EMPLOYED IN THE PRESENT WORK

In this thesis the control of cyclic nucleotide and adenosine concentrations has been studied by comparing the activities and properties of the enzymes which produce and metabolize these molecules, in tissues from a wide variety of animals. Amongst the properties studied are the kinetics of the enzymes and their response to certain ions. In particular, the effects of Ca^{2+} on adenosine kinase and F^- on adenyl cyclase have been investigated. The approach also provides information on the use of cyclic nucleotide and adenosine messengers in animals widely distributed in the animal kingdom.

In this chapter some aspects of our present knowledge of cyclic nucleotides and adenosine are reviewed in order that the significance of the results recorded in later chapters may be more easily discussed. Reviews of cyclic nucleotide metabolism include one book (Robison et al., 1971), the series of Advances in Cyclic Nucleotide Research and various articles in the Annual Reviews series (for example, Hardman et al., 1971; Jost & Rickenberg, 1971). In view of this wealth of information covering every aspect of the subject, it is only necessary to bring out certain points which are essential for the understanding of the present work. However, there is no general review of adenosine metabolism to which the reader can be referred, and so a fuller treatment of this subject is presented.

A. CYCLIC NUCLEOTIDE METABOLISM

A.1 PATHWAYS OF CYCLIC NUCLEOTIDE METABOLISM

The enzyme reactions concerned in cyclic nucleotide metabolism and the structural formulae of the nucleotides are depicted in Figure 1.1.

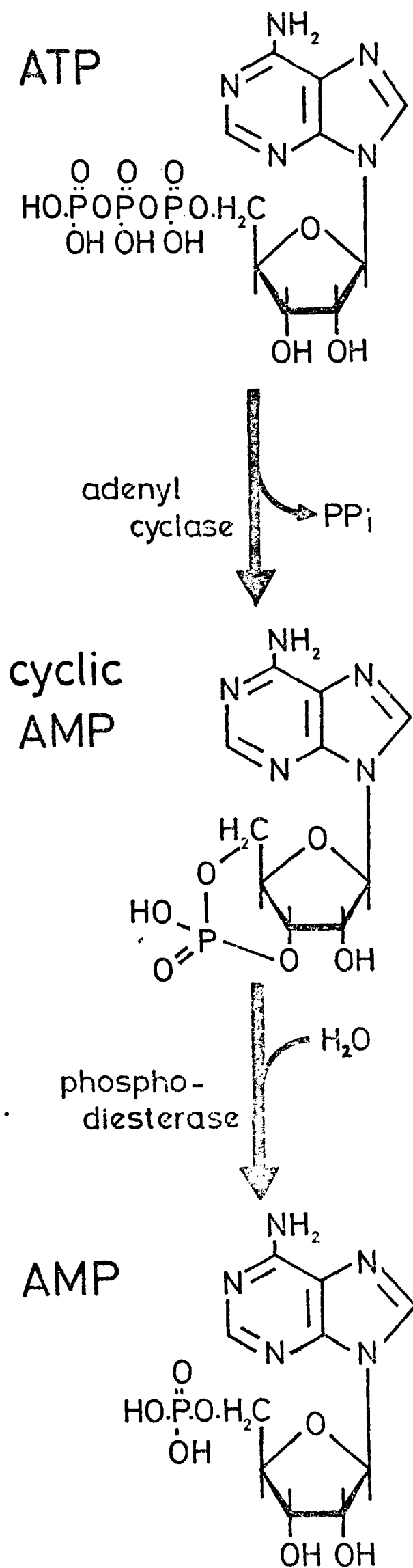
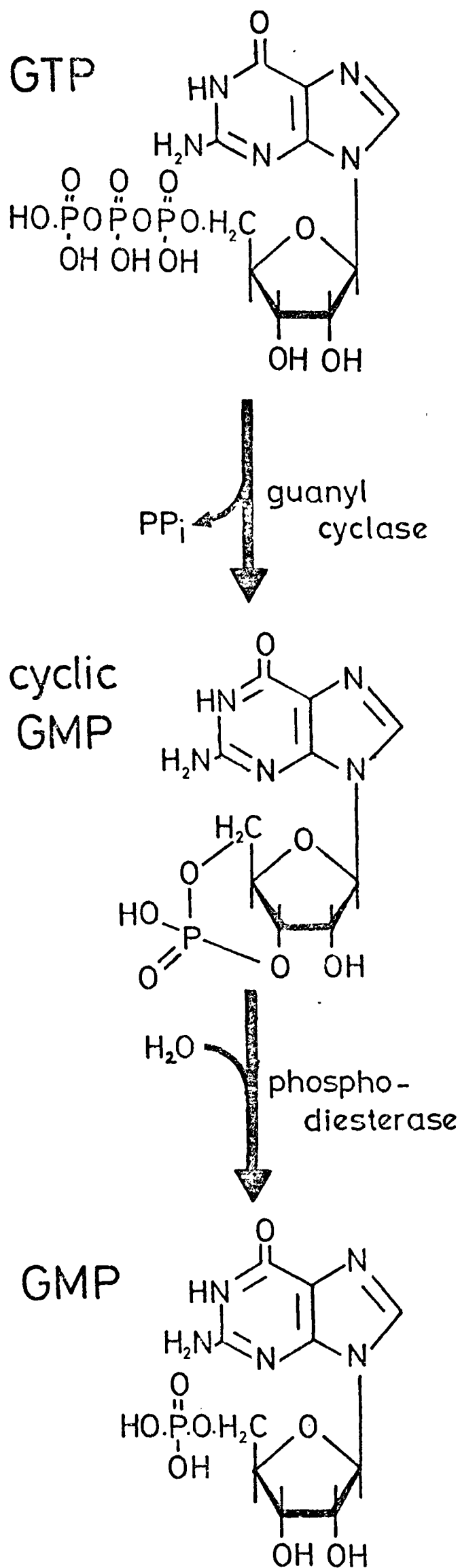
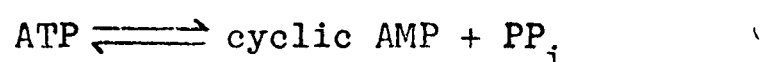


FIG 1.1 Pathways of cyclic nucleotide metabolism.

Cyclic AMP is produced from ATP by the action of adenyl cyclase; cyclic GMP is produced from GTP by the action of guanyl cyclase. Both cyclic AMP and cyclic GMP are metabolized by an enzyme or group of enzymes known as the phosphodiesterases. There are phosphodiesterases which have larger K_m values for one or other of the cyclic nucleotides, but they apparently constitute a closely related group of enzymes which all catalyze the same reactions. As far as it is known no other enzymes have a cyclic nucleotide as their substrate or product. Guanyl cyclase was not studied for this thesis and its properties will not be discussed.

(a) CYCLIC AMP FORMATION---ADENYL CYCLASE

The reaction catalyzed by adenyl cyclase is



The subject of adenyl cyclase has been reviewed recently by Perkins (1973). The standard free energy change of the adenyl cyclase reaction is +1.63 kcal/mol, which favours ATP formation (see Greengard & Kuo, 1970). However, in vivo concentrations of PP_i are between 10^{-6} and 10^{-5} M (because pyrophosphatase rapidly and irreversibly hydrolyzes PP_i to P_i — H.E. Morgan, personal communication to E.A. Newsholme). Moreover, ATP concentrations are very high (at least 1000-fold higher than cyclic AMP concentrations). Therefore, it may be assumed that in vivo the enzyme catalyzes a reaction which is far from equilibrium. One important point about a reaction which is far from equilibrium is that the flux through the reaction may be regulated either by substrate concentration or by any factor unrelated to the reaction (i.e. allosterically) (Newsholme & Start, 1973).

(i) Regulation of adenyl cyclase activity

The K_m of adenyl cyclase has usually been reported to be in the region of 0.1 to 0.2 mM ATP. Since intracellular ATP concentrations are about 5 mM, it might be expected that adenyl cyclase is not regulated by changes in

substrate concentration in vivo (i.e. it is nearly saturated with substrate). However, adenyl cyclase is located on membranes so that its K_m in vivo may differ from that measured in vitro. Alternatively, the concentration of ATP available to the enzyme may be far lower than that of the total ATP content of the cell.

Adenyl cyclase is inhibited in vitro by high concentrations of ATP. Its activity is increased by Mg^{2+} . Activation by Mg^{2+} is in part a consequence of the fact that the true substrate of the enzyme is Mg-ATP. However, when the Mg^{2+} concentration is greater than that of ATP, adenyl cyclase activity is increased. Consequently, it has been proposed that there is a site on the enzyme for Mg^{2+} other than the Mg-ATP site. Alternatively, activation of adenyl cyclase by Mg^{2+} may be explained entirely in terms of the replacement of the inhibitor, free ATP, by the substrate, MgATP, as the concentration of Mg^{2+} is increased (see Perkins, 1973; de Haen, 1974). There is no evidence that changes in ATP or Mg^{2+} concentrations play any part in regulating adenyl cyclase activity in vivo.

Undoubtedly many hormones activate adenyl cyclase in vivo and thereby elevate tissue cyclic AMP levels. Thus adenyl cyclase activity is increased in vitro by some hormones. The response of adenyl cyclase to a particular hormone depends on the source of the enzyme (see Jost & Rickenberg, 1971 for a table illustrating this point). Certain non-hormonal factors may modulate the hormonal response to adenyl cyclase. Ca^{2+} appears to be required for the response of adrenal cortical and adipose tissue adenyl cyclase to ACTH (Bar & Hechter, 1969b; Birnbaumer & Rodbell, 1969). GTP stimulates the response of liver adenyl cyclase to glucagon (Rodbell et al., 1971).

The role of adenosine as a physiological activator of adenyl cyclase is dealt with in Section B.5.

(ii) The significance of basal and fluoride-stimulated adenyl cyclase activities

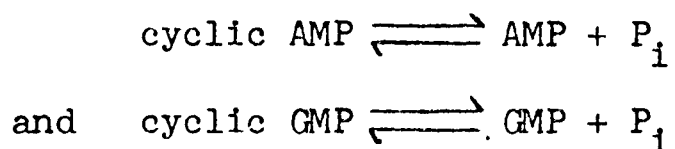
When adenyl cyclase activity is measured in tissue homogenates in the absence of hormones or fluoride, a "basal" activity is recorded. There has been some discussion as to whether basal activity represents anything other than an artefact of tissue homogenization. It is clear that there is some turnover of cyclic AMP in the cell in the absence of hormonal stimulation. Thus [^3H]-adenine is incorporated into cyclic AMP in the absence of an increase in total cellular cyclic AMP levels (see Perkins, 1973). When an analogue of ATP, which is known to inhibit adenyl cyclase, is added to fat cells, the concentration of cyclic AMP decreases rapidly (Krug et al., 1973). Furthermore, the maintenance of the phosphodiesterase reaction far from equilibrium is another indication of cyclic AMP turnover (see Section (b)). However, it is not clear whether basal adenyl cyclase activities are a measure of the "non-hormonal" minimal adenyl cyclase activity in vivo.

The fluoride ion is an important non-physiological activator of adenyl cyclase. Its effect is irreversible and it is not observed with intact tissues. It is a general observation that maximally effective concentrations of F^- or hormones do not, when used together, produce additive increases in adenyl cyclase activity. Moreover, the sensitivity of adenyl cyclase to hormonal stimulation is far more labile than its sensitivity to fluoride, so that fluoride-stimulated adenyl cyclase activities may be conveniently used to measure the maximal activity of the enzyme in vitro. However, there are situations in which hormones produce a greater stimulation of adenyl cyclase than fluoride (see Appleman et al., 1973; Pilkis et al., 1974).

Systematic measurements of basal and fluoride-stimulated adenyl cyclase activities may both indicate the significance of these activities and provide a great deal of information concerning the regulation of cyclic AMP levels in vivo.

(b) CYCLIC NUCLEOTIDE DEGRADATION—PHOSPHODIESTERASE

The reactions catalyzed by phosphodiesterase are



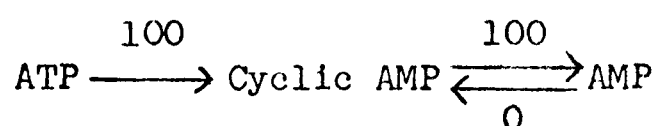
The subject of phosphodiesterase has been reviewed by Appleman et al. (1973). Goldberg et al. (1973) have dealt specifically with the subject of cyclic GMP hydrolysis. The latter authors point out that the concentration of cyclic nucleotides in the cell may be reduced by their extrusion from the cell, since appreciable quantities of cyclic nucleotides are found in the urine. The quantitative importance of this mechanism cannot be assessed until cyclic nucleotide turnover is compared with the release of cyclic nucleotides from individual tissues.

The standard free energy change of cyclic AMP hydrolysis is -11.9 kcal/mol (Greengard & Kuo, 1970). This corresponds to an equilibrium constant ($[\text{AMP}]/[\text{cyclic AMP}]$) of 22,000, whereas the mass-action ratio of the phosphodiesterase reaction in vivo is of the order of 100 to 1000. These values suggest that the cyclic AMP phosphodiesterase reaction is maintained far from equilibrium. However, there may be compartments of the cell where cyclic AMP levels are very different from those measured in the whole cell. If the phosphodiesterase reaction is maintained far from equilibrium in all situations, either adenylyl cyclase is always active (i.e. a steady-state concentration of cyclic AMP is established) or phosphodiesterase activity must be controlled in concert with that of adenylyl cyclase. (This point is referred to in Section (a)(ii)).

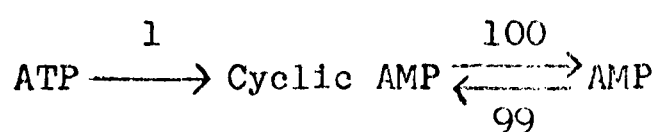
Before continuing the discussion on the assumption that the cyclic AMP phosphodiesterase reaction is maintained far from equilibrium, it may be noted that there are two related problems which would be explained if the reaction is near-equilibrium, at least under some conditions. One problem

is that hormones have frequently been observed to have little effect on tissue cyclic AMP levels unless phosphodiesterase is inhibited. Conversely, phosphodiesterase inhibitors have little effect on cyclic AMP levels unless adenylyl cyclase activity is increased by hormonal stimulation (see Robison et al., 1971, p.41; Butcher et al., 1965). Consider the following extreme situations concerning the equilibrium state of the phosphodiesterase reaction. In both examples it will be assumed that the adenylyl cyclase reaction is irreversible, the forward rate of the phosphodiesterase reaction is proportional to substrate concentration, and the backward rate of the phosphodiesterase reaction is constant (i.e. the AMP concentration is constant).

(a) The phosphodiesterase reaction is far from equilibrium



(b) The phosphodiesterase reaction is close to equilibrium



In both situations a steady-state will be established. One difference between the two situations is the response of cyclic AMP levels to a change in either adenylyl cyclase or phosphodiesterase activities. In situation (a), if the adenylyl cyclase activity is doubled, the cyclic AMP concentration must increase at least twofold in order for the flux through the phosphodiesterase reaction to double. In contrast, in situation (b), if the adenylyl cyclase activity is doubled, the cyclic AMP concentration need only increase by 1% for the net flux through the phosphodiesterase reaction to double (from 100 minus 99, to 101 minus 99). Similar considerations apply if the activity of phosphodiesterase is reduced.

Either stimulation of adenylyl cyclase or inhibition of phosphodiesterase (or both) decreases the mass action ratio of the phosphodiesterase reaction and could thereby convert the reaction from near-equilibrium to far-removed

from equilibrium. Therefore, stimulation of adenylyl cyclase and inhibition of phosphodiesterase will have greater than additive effects if, in the absence of either factor, the phosphodiesterase reaction is near-equilibrium. It should be stressed that there is no direct evidence to support the view that the phosphodiesterase reaction is near-equilibrium. Indeed there is some evidence against it. If the reaction was close to equilibrium any change in AMP concentrations should produce changes in the concentration of cyclic AMP. There are a number of situations in which the AMP level is known to change without a concomitant change in cyclic AMP level, e.g. electrical stimulation of muscle (Posner *et al.*, 1965). (For further discussion of this problem see Newsholme & Start, 1973, page 166; Kakiuchi & Rall, 1968; Rall & Sattin, 1970).

There are few data concerning GMP levels in animals tissues. Cha & Cha (1970) report that guanosine nucleotide concentrations are generally one-tenth of adenine nucleotide concentrations. Since cyclic GMP levels are generally a fiftieth to a hundredth of cyclic AMP levels it may be that the mass-action ratio of the cyclic GMP phosphodiesterase reaction is in some circumstances quite similar to the equilibrium constant of the reaction. If so cyclic GMP levels could be affected by changes in GMP levels.

(1) The kinetics of phosphodiesterase

Phosphodiesterase, or at least some phosphodiesterases, is not saturated with substrate in vivo, so that its activity may be regulated by changes in substrate concentration. The situation is complicated because in tissue homogenates cyclic AMP phosphodiesterase frequently exhibits two K_m values: one is in the region of $1 \mu M$ and the other is between 12 and $500 \mu M$ in different tissues. Even higher K_m values (up to $7000 \mu M$) have been observed when the enzyme has been studied using very high substrate concentrations. It seems probable that more than two K_m values would be detectable for the

enzyme(s) in tissue homogenates if phosphodiesterase were assayed over a wide range of substrate concentrations using a very accurate assay. This may explain the large variations in the reported values of the high K_m enzyme. With cyclic GMP as substrate, usually only one K_m value has been reported. However, the values reported are variable (1.6 - 180 μM).

The question arises whether the various K_m values of phosphodiesterase are the result of isoenzymes or a single polymeric enzyme with distinct active sites. In the former situation, there may be two different enzymes or one enzyme which is able to aggregate. In the latter situation, the active sites may be non-identical or the binding of one molecule of substrate may cause a decrease in the affinity of the second site for the substrate (i.e. the enzyme exhibits negative cooperativity). In addition to these models, it is possible to propose kinetic models to explain phosphodiesterase kinetics, in the same way that Ferdinand (1966) and Rabin (1967) have proposed kinetic models to explain sigmoid kinetics.

Few authors appear to realize that there are many possible explanations of phosphodiesterase kinetics. However, attempts have been made to distinguish between some of these possibilities. There is evidence that phosphodiesterase from bovine liver and from slime moulds can be interconverted in vitro between forms exhibiting different kinetic properties (Schröder & Rickenberg, 1973; Chassy, 1972). The bovine liver enzymes aggregate to form complexes with higher K_m values. Cyclic AMP is one factor which favours this aggregation. Russell et al. (1972) report isolation of a pure enzyme which displays two K_m values, but they do not appear to have ruled out aggregation as an explanation. Their results certainly do not prove that phosphodiesterase is negatively cooperative.

Another question, that may be more relevant to this thesis, may be asked: what is the physiological significance of these unusual phosphodiesterase kinetics? A number of answers are possible.

- a. Firstly, there may be no significance: the two K_m values may be an in vitro phenomenon, resulting from the removal of phosphodiesterase from its normal cellular environment. This seems unlikely since the high and low K_m enzymes have been found in different tissues from a wide variety of sources (see Appleman et al., 1973).
- b. Cyclic AMP may not be the physiological substrate for the high K_m enzyme: cyclic GMP may be the normal substrate. The K_m for cyclic GMP is indeed lower than that for cyclic AMP for some enzymes (Thompson & Appleman, 1971b) but not all (Schröder & Rickenberg, 1973).
- c. The high K_m cyclic AMP phosphodiesterase may be present in a small compartment of the cell where cyclic AMP concentrations are particularly high, whereas the low K_m enzyme is distributed throughout the cell. This explanation is unlikely since the high K_m enzyme is generally found in the soluble fraction of the cell, and the low K_m enzyme is frequently associated with a particulate fraction (Thompson & Appleman, 1971b).
- d. Chassy (1972) has suggested that the in vitro conversion of low K_m phosphodiesterase to the high K_m form represents a mechanism whereby phosphodiesterase is inhibited during phases of slime mould differentiation when cyclic AMP levels must be elevated. However, there is some evidence to support the role of an inhibitor of phosphodiesterase during these phases of development (Riedel et al., 1972, 1973). Furthermore, there are no reports of inter-conversions between low and high K_m enzymes in response to physiological stimuli in any species.
- e. Finally, there remains the possibility that the high and low K_m enzymes function in concert with adenylyl cyclase to provide a satisfactory mechanism for the regulation of cyclic AMP levels.

Russell et al. (1972) regard the role of the unusual phosphodiesterase kinetics to be a means whereby the hydrolysis of cyclic AMP is delayed

following a pulse of hormone-stimulated adenyl cyclase activity. In this way they claim that cyclic AMP may exist long enough to activate protein kinase. They consider that an enzyme obeying classical Michaelis-Menten kinetics may respond too sharply to a change in cyclic AMP concentration for the increased cyclic AMP level to be maintained for a suitable time; a negatively cooperative phosphodiesterase may respond less sharply. This explanation is far from adequate. Firstly, it takes no account of the fact that the response to changes in substrate concentration of an enzyme conforming to any type of kinetics depends on the part of the kinetic curve in which the change in substrate concentration occurs. Thus, given the same $V_{\max}$, a negatively-cooperative enzyme is more responsive at low substrate concentrations to changes in concentration than an enzyme exhibiting hyperbolic kinetics, whereas at high substrate concentrations the position is reversed. Secondly, Russell et al. consider that the response of adenyl cyclase to hormonal stimulation is only transient. Hormone concentrations vary over periods of minutes or even days and it would seem far more realistic to discuss changes in the steady-state level of cyclic AMP which are brought about by a change in adenyl cyclase activity. This problem will be discussed further in the light of the results presented in Chapter 3.

Before concluding this section it may be noted that phosphodiesterases which display sigmoid kinetics have been isolated from rat liver, bovine heart and Neurospora crassa (Russell et al., 1973; Bevers et al., 1974; Beavo et al., 1970; Scott & Solomon, 1973). Once again, more than one explanation is possible for both the physical and physiological significance of such kinetics, but there is little value in further speculation here.

(ii) Control of phosphodiesterase by factors other than substrate concentration

If it is assumed that the phosphodiesterase reaction is far from equilibrium, the flux through the reaction may be regulated by factors other

than substrate concentration. A few effectors of phosphodiesterase are known which may play a physiological role in controlling cyclic AMP concentration.

Cheung (1970a) has found that rat brain phosphodiesterase is inhibited by ATP and PP_i . In the absence of divalent metal ions half-maximal inhibition was obtained at 50 μM ATP and 35 μM PP_i . Other nucleoside triphosphates caused inhibition with a potency comparable to ATP, but nucleoside diphosphates were usually much less inhibitory. Citrate is inhibitory but, in the presence of metal ions, 50% inhibition was obtained only at a concentration of 12 mM (Cheung, 1967). There is no evidence that these metabolites regulate cyclic AMP levels in vivo (but see Section B.5b(ii)).

Various protein activators and inhibitors of phosphodiesterase activity have been isolated (Appleman et al., 1973; Ray & Forte, 1972; Riedel et al., 1972, 1973). It has been discovered that an inhibitor of phosphodiesterase is excreted by slime mould cells at the end of their growth phase. The inhibitor terminates the activity of previously excreted phosphodiesterase. In no other case has a specific physiological function for a phosphodiesterase effector been proposed (see Smoake et al., 1974; Lin et al., 1974).

Low concentrations of Ca^{2+} (10 μM) activate the low K_m enzyme from brain. A non-dialyzable, heat-stable factor increases the sensitivity to Ca^{2+} (Kakiuchi & Yamazaki, 1970; Kakiuchi et al., 1971). This factor is similar and possibly identical to one described by Cheung (1970b). An inhibitory protein whose effect is modified by Ca^{2+} has also been described (Miki & Yoshida, 1972).

Cyclic GMP and cyclic AMP have frequently been observed to inhibit each other's hydrolysis. The physiological relevance of these observations is questionable (Appleman et al., 1973; Goldberg et al., 1973). It seems more relevant to the in vivo situation that low concentrations of cyclic GMP

stimulate cyclic AMP hydrolysis in a variety of preparations (Beavo et al., 1971; Franks & McManus, 1971; Klotz & Stock, 1972; Manganiello & Vaughan, 1972), which may explain the observation that cyclic AMP and cyclic GMP frequently show reciprocal changes in concentration under the influence of physiological stimuli (Goldberg et al., 1973).

The only hormone which has been found to have a rapid effect (within minutes) on phosphodiesterase activity is insulin (see Appleman et al., 1973). House et al. (1972) have reported that low levels of insulin increase phosphodiesterase activity in cell free systems (a subfraction of rat liver plasma membranes). However, other workers have failed to confirm this effect (Thompson et al., 1973; Pilakis et al., 1974). There is little evidence that insulin directly stimulates phosphodiesterase in vivo. Other hormones have been found to affect phosphodiesterase but only after hours or days, which suggests that their effects are indirect (i.e. involving changes in concentration or distribution of the enzyme).

It should be noted that cyclic AMP hydrolysis could be regulated not only by modifying phosphodiesterase activity but also by regulating the proportion of cyclic AMP which is bound to protein kinase (see Section A.4), since bound cyclic AMP is not hydrolyzed by phosphodiesterase (Cheung, 1972).

A.2 THE ROLES OF CYCLIC NUCLEOTIDES

This section will attempt to show what roles cyclic nucleotides may be playing in the tissues which were investigated for this thesis.

(a) CYCLIC NUCLEOTIDES AS MEDIATORS OF HORMONE ACTION

Cyclic AMP was originally discovered as the agent which mediates the hyperglycaemic effects of adrenaline and glucagon. It stimulates the conversion of inactive glycogen phosphorylase to the active form (Sutherland & Rall, 1960). Since that time it has been implicated as the "second messenger" for a large number of hormones (see Jost & Rickenberg, 1971),

although not all hormone actions are mediated by cyclic AMP. The concept of the secondary messenger was developed as follows. The "first messenger" is a hormone. On reaching its target cell it interacts with a receptor which is linked to adenyl cyclase. Adenyl cyclase activity is increased with the result that the concentration of the second messenger, cyclic AMP, is increased. Cyclic AMP acts within the cell to mediate the effect of the hormone (Sutherland et al., 1965).

Of the tissues investigated for the work reported in this thesis, liver adenyl cyclase has been shown to be stimulated by adrenaline and glucagon; heart adenyl cyclase by adrenaline, glucagon, prostaglandins and triiodo-thyronine; skeletal muscle adenyl cyclase by adrenaline, prostaglandins and parathyroid hormone, and brain adenyl cyclases by adrenaline, noradrenaline and dopamine (see Jost & Rickenberg, 1971; Kebabian & Greengard, 1971). All these hormonal effects have been observed in cell free systems. In addition, brain cyclic AMP levels are elevated by some other neurohormones: adenosine, 5-hydroxytryptamine and histamine (McIlwain, 1972; Schultz & Daly, 1973a). The relatively high activities of adenyl cyclase and phosphodiesterase in nervous tissue and the response of cyclic AMP levels to various neurohormones and neurotransmitters suggest that cyclic AMP plays an important role in nerve function (Greengard & McAfee, 1972).

A role for cyclic GMP as a mediator of hormone action is not so easily established. Guanyl cyclase has not been shown to be affected by any hormone in a cell free system (see Goldberg et al., 1973). However, some hormones elevate tissue cyclic GMP levels. There is good evidence to suggest that cyclic GMP mediates some but not all the effects of acetylcholine (Goldberg et al., 1973).

(b) CYCLIC NUCLEOTIDES AND NON-HORMONAL EFFECTS

Cyclic nucleotide levels are affected by factors other than hormones. Variations in the growth conditions of bacteria affect both cyclic AMP and

cyclic GMP levels. Cyclic AMP is well established as an agent which regulates the expression of a number of bacterial genes (Pastan, 1972; Goldberg et al., 1973; Grodzicker et al., 1972). Cancer cells and other growing cells contain low levels of cyclic AMP compared to differentiated and non-growing cells (Pastan, 1972; Otten et al., 1972; Millis et al., 1972). Induction of DNA synthesis by mitogenic agents is preceded by a rise in cyclic GMP and a fall in cyclic AMP concentrations (Seifert & Rudland, 1974).

In nervous tissue, cyclic AMP levels may be elevated by electrical stimulation, although the latter effect is probably secondary to adenosine release by the nerve cells (Kakiuchi et al., 1969). Electrical stimulation does not affect cyclic nucleotide levels in rat and frog skeletal muscles (Posner et al., 1965), but there are reciprocal changes in cyclic AMP and cyclic GMP levels during the cycle of contraction and relaxation of the heart (Wollenberger et al., 1973; Brooker, 1973). In rod outer segments adenylyl cyclase is activated by light (Brown & Makman, 1972).

Thus it should be noted in way of summary that cyclic nucleotides may not only act as a second messenger mediating hormone action. They may in some tissues play a role in cell growth and division and/or mediate the effects of nervous stimulation.

A.3 THE MECHANISM OF ACTION OF CYCLIC NUCLEOTIDES

In order to more fully appreciate the role of cyclic nucleotides and assess the significance of changes in cyclic nucleotide concentrations, it is necessary to know the mechanism whereby cyclic nucleotides carry out their regulatory function. A brief description of this subject is provided below.

(a) PROTEIN KINASE MEDIATED ACTIONS

The majority of the actions of cyclic AMP are apparently mediated through the action of protein kinase. The latter consists of an aggregate of two types of subunit: the regulatory or R subunit, and the catalytic or

C subunit. In the absence of cyclic AMP, R and C subunits are combined with each other to form an inactive molecule. Cyclic AMP binds to the R subunit and causes dissociation of the two subunits. The free C subunit, in the presence of ATP and Mg^{2+} , phosphorylates a number of proteins (see Langan, 1973).

It is far from proven that cyclic GMP actions are mediated by protein kinases. Protein kinases have been isolated which are activated by concentrations of cyclic GMP lower than those of cyclic AMP. However, tissue cyclic AMP concentrations are usually for higher than cyclic GMP concentrations so that these protein kinases may not be affected specifically by cyclic GMP in vivo (Goldberg et al., 1973).

Cyclic nucleotide-dependent protein kinases have a wide substrate specificity: as yet, a protein kinase which is specific for one substrate has not been found. Some of the in vitro substrates of cyclic AMP-dependent protein kinase are listed in the table below.

Protein Kinase Substrates

Enzymes: Phosphorylase b kinase, Glycogen synthetase,
Triglyceride lipase.

Muscle proteins: Actin, Troponin.

Nuclear proteins: Protamines, Histamines, Other nuclear proteins.

Membrane proteins: A specific brain membrane protein,
Microtubule proteins.

\ Others: Casein, Ribosomal proteins, Connective tissue protein.

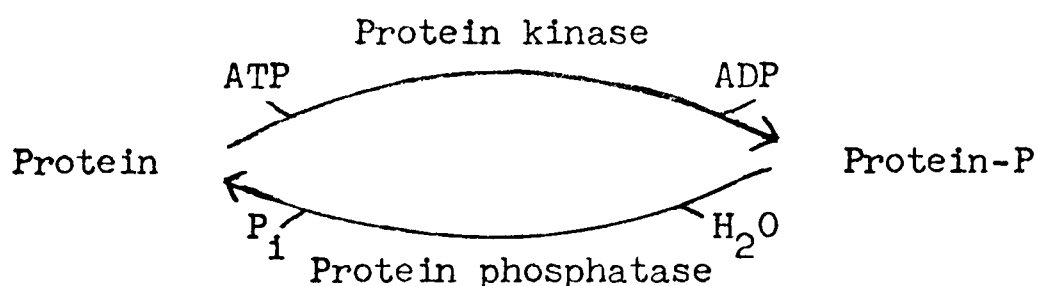
The fact that these substrates are phosphorylated by protein kinase does not in itself establish that cyclic AMP mediates any of its effects through their phosphorylation. It must also be demonstrated that the protein substrate is modified in vivo in response to cyclic AMP, and that modification of the protein substrate will produce the same response as cyclic AMP. (For

a full discussion see Langan, 1973). At present there are three effects of cyclic AMP which have been shown, by these criteria, to be mediated by protein phosphorylation: stimulation of glycogen breakdown, inhibition of glycogen synthesis and increased lipolysis in adipose tissue. The protein kinase substrates which are phosphorylated are phosphorylase b kinase, glycogen synthetase and triglyceride lipase, respectively (Langan, 1973).

(1) Protein kinases and amplification

It may be concluded that at least some of the effects of cyclic AMP on the flux through metabolic pathways do not result from the direct interaction of cyclic AMP with the enzyme controlling the flux, but rather the cyclic AMP effect is mediated indirectly by the activation of a separate protein (i.e. protein kinase). In this case what significance can be attached to small changes in cyclic AMP concentrations? The following discussion is intended to demonstrate that small changes in cyclic AMP concentrations may have a large effect on a metabolic flux. However, it should be noted that the effect of cyclic AMP may be modified by any other factors which affect protein kinase activity or the aggregation of the R and C subunits.

The extent to which a protein kinase substrate is phosphorylated probably depends on a balance between the activity of the kinase and the activity of a corresponding phosphatase. In other words there exists what may be termed an "interconversion-cycle", which can be considered as a logical extension of the substrate-cycle (Newsholme & Crabtree, 1973). The interconversion-cycle is illustrated below.



From this simple diagram it may be suggested that if protein kinase activity is greater than that of the phosphatase, most of the protein will be converted to the phosphorylated form. If protein kinase activity is less than that of the phosphatase, most of the protein will be converted to the dephosphorylated form. However, the situation is more complex since the activities of the kinase and phosphatase will be affected by the change in concentration of their substrates. Nevertheless, if the K_m of protein kinase is low compared to the concentration of its substrate, a small change in protein kinase activity may result in a large change in the ratio of protein to protein-P (since the protein kinase has a catalytic effect). A small change in protein kinase activity may be brought about by a small change in cyclic AMP concentration (Means et al., 1974; Spaulding & Burrow, 1974). Therefore, if one form of the protein substrate is an active enzyme and the other form is inactive (or far less active), a small change in cyclic AMP concentration may greatly affect the activity of the enzyme. The intervention of protein kinase may be said to "amplify" the response of a metabolic flux to cyclic AMP (Newsholme & Crabtree, 1973).

In addition to cyclic AMP there is a heat-stable protein inhibitor which may modify protein kinase activity (Walsh et al., 1971). It is not known whether the inhibitor plays any role in vivo.

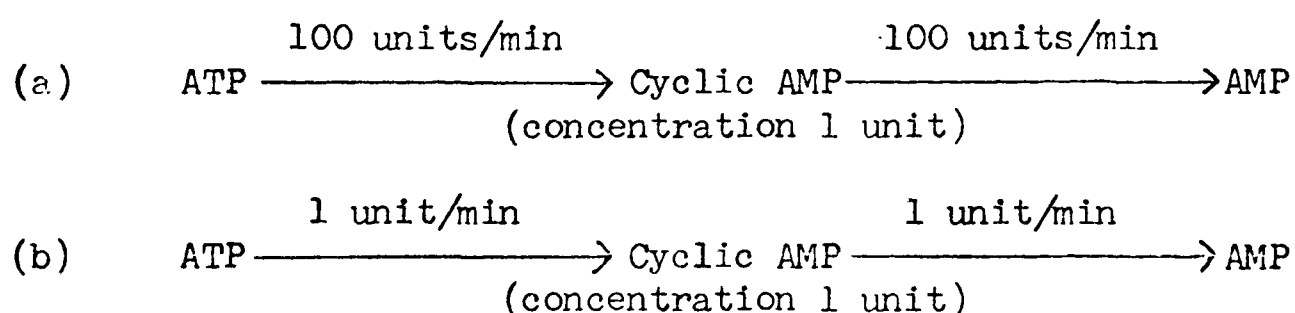
The high sensitivity of metabolic fluxes to changes in cyclic AMP concentration has an important consequence: hormones need not cause large changes in cyclic AMP concentration in order to exert large effects on metabolic fluxes. Therefore, both the response of adenyl cyclase activity to a hormone, and the response of intracellular cyclic AMP concentrations to increased adenyl cyclase activity need only be small in order to account for the known hormonal effects. These two conclusions are important to the evaluation of the results of Chapter 3.

(ii) The rate of response to changes in the activity of adenyl cyclase

The mediation of cyclic AMP effects by protein kinase has another advantage other than that of amplification. Cyclic AMP concentrations within the cell are low in relation to the concentration of the metabolic target enzymes, e.g. glycogen phosphorylase. If cyclic AMP interacted directly with these enzymes a considerable proportion of it would have to be bound in order to produce activation. This would result in a slow response of the target enzymes to changes in the activity of adenyl cyclase (Newsholme & Crabtree, 1973). Moreover, there would not be enough cyclic AMP for full activation.

One molecule of protein kinase, since it is a catalyst, interacts with many other enzyme molecules. Consequently, its concentration may be much lower than that of the metabolic target enzymes. In fact its concentration is similar to that of cyclic AMP (E.G. Krebs, personal communication). In addition, maximal activation of the metabolic target enzyme may be produced by a submaximal activation of protein kinase (see above). Therefore maximal activation of the target enzyme may be produced when a small proportion of the cyclic AMP is bound. Thus the target enzyme may respond rapidly to a change in the activity of adenyl cyclase.

The rate of turnover of cyclic AMP will also influence the rate at which metabolic target enzymes respond to changes in the activity of adenyl cyclase. Consider the following steady-state situations and the rate at which the cyclic AMP concentration will reach a new steady-state if the rate of the adenyl cyclase reaction is doubled.



In situation (a) the initial rate of change of the concentration of cyclic AMP in response to a doubling of the rate of the adenyl cyclase reaction is

10,000% per minute. In situation (b) the initial rate of change of the concentration of cyclic AMP in response to a doubling of the rate of the adenylyl cyclase reaction is only 100% per minute. In situation (a) the rate of turnover of cyclic AMP is 100 times that in situation (b). Turnover requires energy expenditure. However, since cyclic AMP concentrations are very low, the energy expenditure involved in cyclic AMP turnover is low.

(b) CYCLIC NUCLEOTIDE EFFECTS WHICH ARE NOT MEDIATED BY PROTEIN

KINASES

There is at least one cyclic nucleotide effect which is not mediated by protein kinase. Thus it is well established that cyclic AMP affects gene expression in bacteria by binding to a protein called CAP. Having bound CAP, the latter protein binds to DNA and causes the attachment of RNA polymerase to promoter sites of certain operons. Cyclic GMP can apparently inhibit the binding of CAP to DNA directly or inhibit the cyclic AMP effect on CAP binding. However, cyclic GMP is only effective at relatively high concentrations. It is possible that cyclic nucleotides affect gene expression in eukaryotic systems by similar mechanisms, but protein kinase mediated mechanisms have also been suggested (see Langan, 1973; Goldberg et al., 1973).

There may be other effects of cyclic nucleotides that are not mediated by protein kinase. Both cyclic nucleotides have been shown to modify the activities of enzymes other than protein kinase in vitro. The effect of cyclic GMP on cyclic AMP phosphodiesterase was referred to in Section A.1b(ii). Cyclic AMP has been shown to activate phosphofructokinase. Half maximal binding to the rabbit skeletal muscle enzyme occurs at a cyclic AMP concentration of $0.6 \mu\text{M}$ (Kemp & Krebs, 1967). This binding constant is similar to intracellular cyclic AMP concentrations and so it suggests that cyclic AMP might regulate phosphofructokinase in vivo. Unfortunately, it has been difficult to demonstrate that hormones which elevate cyclic AMP

levels stimulate phosphofructokinase. However, there is evidence that the activity of phosphofructokinase in extracts of rabbit skeletal muscle is increased by administration of adrenaline prior to extracting the muscle in the presence of caffeine (Mansour, 1972b; see also Karadsheh et al., 1974). It has also been shown that 5'-hydroxytryptamine stimulates phosphofructokinase in cell free extracts of liver flukes, apparently by stimulating adenylyl cyclase (Mansour, 1972a).

A.4 COMPARTMENTATION OF CYCLIC AMP

Measurements of tissue cyclic AMP levels have been used as evidence to demonstrate that many hormones act through this second messenger. However, it is not known how cyclic AMP is distributed throughout the cell. It is possible to suggest that the concentration of cyclic AMP available to protein kinase or phosphodiesterase is very different from that measured in the whole tissue. For example, a large proportion of the measured cyclic AMP may be bound to protein; phosphodiesterase may be located in vesicles which contain very high concentrations of cyclic AMP. The existence of compartmentation for cyclic AMP has been suggested for a number of reasons. Some of the arguments put forward appear convincing at first but, on deeper study and in the light of more recent knowledge, it will be seen that they are not conclusive.

a. Large changes in the flux through a particular metabolic pathway have often been found to be associated with very small changes in cyclic AMP levels, even though there is good reason to presume that cyclic AMP controls the flux in question. This problem has been noted particularly in the case of lipolysis in adipose tissue (Butcher et al., 1966; Kono & Barham, 1973; Khoo et al., 1973). The finding that insulin causes a large drop in lipolytic rate but only a small drop in cyclic AMP levels in rat epididymal fat pad led Butcher et al. (1966) to conclude that most cyclic AMP is inactive in

promoting lipolysis. The small pool of cyclic AMP which regulates lipolysis is, they suggested, affected by hormones such as insulin and catecholamines. This view has also been expressed by Kuo & De Renzo (1969), Fain et al. (1972) and Park & Exton (1972).

It was shown in Section A.3a(i) that small changes in the concentration of cyclic AMP may be amplified by protein kinase to produce large changes in the flux through a metabolic pathway. This argument alone can explain why hormones can greatly affect metabolic flux with only small changes in the concentration of cyclic AMP. There is no need to invoke the concept of compartmentation.

b. If adenyl cyclase or phosphodiesterase is not evenly distributed throughout the cell, it is probable that cyclic AMP is also unevenly distributed. Adenyl cyclase is associated with membranes, especially with the plasma membrane (Davoren & Sutherland, 1963; see Perkins, 1973). A large proportion of phosphodiesterase activity is also membrane-bound (see Appleman et al., 1973). These observations suggest that cyclic AMP may be largely located within or very close to certain membranes. In brain, high activities of adenyl cyclase and phosphodiesterase are found in synaptosomal fragments (cyclic AMP may play a role in synaptosomal transmission). Therefore, in the brain there is circumstantial evidence that cyclic AMP is compartmented.

c. Compartmentation of adenyl cyclase is also suggested by evidence which indicates that adenyl cyclase uses a pool of ATP which constitutes 20 to 30% of total intracellular ATP (see Section B.5b(ii)). In muscle cells the size of the pool could be altered by procedures which affected membrane structure and cell fusion. It was therefore suggested that adenyl cyclase uses an ATP pool which is associated with the cell membrane (Reporter, 1972). It is possible that adenyl cyclase releases cyclic AMP into the same region of the cell in which it reacts with ATP.

d. Such compartmentation would provide an explanation for the existence of high K_m phosphodiesterase. Thus cyclic AMP might be present in a membrane at a concentration which would saturate the low K_m enzyme. Unfortunately, the high K_m phosphodiesterase is usually found in the soluble fraction of cells rather than in a membrane fraction.

e. Hittelman & Butcher (1971) suggest that the far greater activity of phosphodiesterase than adenyl cyclase in the cell would reduce cyclic AMP accumulation, unless adenyl cyclase and phosphodiesterase are physically segregated. Thus cyclic AMP produced in one compartment of the cell by adenyl cyclase would not immediately be available to phosphodiesterase. The concentration of cyclic AMP available to phosphodiesterase would be less than that measured for the whole cell. The premise of the argument, that cyclic AMP accumulation should be prevented by high phosphodiesterase activity, lacks a rigorous mathematical basis. What is required is a calculation of the steady-state concentration of cyclic AMP concentration which would result from the activities and kinetics of adenyl cyclase and phosphodiesterase in the cell, assuming that these enzymes are not themselves compartmented. The calculated steady-state cyclic AMP concentration may then be compared with actual measurements of cyclic AMP concentrations. If the calculated value is much lower than the measured value there may be some substance in the argument of Hittelman & Butcher.

f. The C and R subunits of protein kinase are both present in the cell at a similar concentration to cyclic AMP (E.G. Krebs, personal communication). Therefore a substantial amount of cyclic AMP may be bound to the R subunit. The proportion of cyclic AMP bound to the R subunit will depend on the dissociation constant of the cyclic AMP-R complex. Attempts have been made to measure directly the proportion of protein kinase in the cell which is catalytically active (Soderling et al., 1973). Under basal conditions the

proportion is about 20%. If, under basal conditions, C and R subunits are present in the cell at similar concentrations to cyclic AMP, it follows that about 20% of the cyclic AMP is bound to the R subunit.

g. It has been suggested that protein kinase must always be fully active unless only a small proportion of the cyclic AMP of the cell is usually available to the enzyme. Thus, the dissociation constant of protein kinase for cyclic AMP was found in early studies to be approximately 1×10^{-8} M (Walsh et al., 1971; Garren et al., 1971). The activation of protein phosphorylation by protein kinase was half maximal at about 10^{-7} M cyclic AMP (Walsh et al., 1968). Since the measured cyclic AMP levels are much higher than this, it might be expected that protein kinase is always fully active or that a large proportion of the cyclic AMP is not available to the enzyme. However, more recent measurements have shown that binding and activation constants are increased 10-fold by prior incubation of the enzyme with Mg^{2+} and ATP (Haddox et al., 1972). Moreover, by using the physiological substrate phosphorylase b kinase and protein concentrations of the order of those found in the cell, the K_a of protein kinase activation may be increased to $1.5 \mu M$. In the presence of the protein kinase inhibitor the K_a is increased to $10 \mu M$ (E.G. Krebs, personal communication).

These recent experiments show that concentrations of cyclic AMP normally measured for whole tissues are of a suitable magnitude to regulate protein kinase in vivo; measured cyclic AMP levels are neither peculiarly high or peculiarly low in relation to the K_m for protein kinase activation. Therefore, it seems that there is little compartmentation of cyclic AMP, at least in muscle tissue. Nevertheless, some compartmentation may occur. A knowledge of tissue adenyl cyclase and phosphodiesterase activities, when compared with cyclic AMP levels may shed further light on some of the arguments put forward in favour of cyclic AMP compartmentation.

SUMMARY OF SECTION A

Cyclic AMP is produced by adenyl cyclase and hydrolysed by phosphodiesterase. Therefore variations in the activities of these two enzymes may regulate cyclic AMP concentrations in the cell. However, the precise mechanisms by which cyclic AMP concentrations are regulated are unclear. For example, there is not a consensus of opinion concerning the following problems.

Is adenyl cyclase regulated by substrate concentration?

What role does the high K_m phosphodiesterase play in regulating cyclic AMP concentration?

Are the activities of adenyl cyclase and phosphodiesterase regulated by factors other than hormones in the living animal?

Are the measured changes in cyclic AMP concentrations sufficient to account for the modified rates of metabolism in the cell?

How rapidly do cyclic AMP concentrations change from one steady-state to another, and are only transient changes caused by hormonal stimulation?

Finally, to what extent is cyclic AMP compartmented?

The role of cyclic AMP as the second messenger mediating some hormonal actions is well established. Cyclic AMP is also involved in gene expression and probably in synaptic transmission in some animal tissues. However, it is not known whether these are the only roles of cyclic AMP, or how important these roles are in different tissues from animals widely distributed in the animal kingdom. Little is known of the role of cyclic GMP.

Comparative quantitative information concerning the activities and properties of adenyl cyclase and phosphodiesterase may be useful in resolving some of these problems. The uses to which such information can be put are shown in Chapter 3. Attention has been centred upon muscle, nervous and liver tissues. In Chapter 5 one particular role of cyclic AMP is studied, i.e. the regulation of lipolysis in the heart of the diabetic rat.

B. ADENOSINE METABOLISM

HISTORICAL INTRODUCTION

Adenosine is the nucleoside which is produced by the partial hydrolysis of the nucleotide AMP. In the 1950's attempts were made to assess the role of adenosine in intermediary metabolism (see Chargaff & Davidson, 1955). It was found that exogenous adenosine is incorporated into adenine nucleotides by animal tissues (Lowy et al., 1952). However, there was no evidence that the conversion of adenosine to AMP constituted part of the main route by which adenine nucleotides are synthesized from small molecules. Instead, it appeared that in de novo adenine nucleotide synthesis, the nucleotide, AMP, is formed by way of an acyclic ribonucleotide, without the intermediary formation of the purine base, adenine, or the nucleoside, adenosine (Kornberg et al., 1955; Korn et al., 1955). Thus the nucleoside level of complexity could be bypassed. The significance of the incorporation of exogenous adenosine into adenine nucleotides remained to be explained.

Kornberg (1957) developed the concept that the conversion of adenosine to adenine nucleotides is a mechanism whereby the purine base is "salvaged" in order to spare the purine synthesizing pathways of the cell. Adenosine was presumed to be formed from the breakdown of more complex molecules (e.g. AMP), rather than by synthesis from small molecules.

Two problems are presented by this theory. Firstly, can it be shown that adenosine is actually present in animal tissues under normal circumstances? (All early experiments studied the metabolism of exogenous adenosine.) In the following section it is shown that, since the development of the salvage theory (Kornberg, 1957), adenosine has indeed been detected in animal tissues. Secondly, why does the organism permit the degradation of adenine nucleotides

to adenosine if the nucleoside is only to be reconverted to adenine nucleotides? It will be demonstrated in the remainder of this chapter that adenosine may have a far more important role than that of an intermediate in the nucleic acid degradation.

B.1 THE CONCENTRATION AND LOCATION OF ADENOSINE IN ANIMAL TISSUES

(a) ADENOSINE CONCENTRATIONS

Until recently it was not possible, using the techniques available, to measure the concentration of adenosine in animal tissues and body fluids. Only when a tissue was subjected to hypoxia or electrical stimulation did adenosine concentrations rise to detectable concentrations in the tissue or the fluid perfusing it. In the past five years, following the development of new techniques, adenosine concentrations which have not been deliberately elevated have been measured in heart, skeletal muscle and brain tissues, and in fluids perfusing and bathing these tissues. The concentrations of adenosine reported for various tissues are summarized in Tables 1.1 and 1.2. Concentrations of adenosine reported for body fluids (Table 1.3) are also of interest because it has been proposed that adenosine plays an important role outside the cell (see Section B.5b(iii)). The distribution of adenosine between the intracellular and extracellular spaces is now considered in more detail.

(b) THE LOCATION OF ADENOSINE

No experiments have been reported which directly measure the concentrations of adenosine in both the intracellular and extracellular spaces; nevertheless, it has often been assumed that adenosine is primarily located outside the cell. Somewhat surprisingly, the main argument put forward in favour of this assumption is that adenosine deaminase is found in the supernatant fraction after high speed centrifugation (Baer et al., 1966) (at least in the heart upon which most attention is centred). Consequently, it was argued that

TABLE 1.1 Adenosine levels in muscle tissues

ANIMAL	TISSUE	TREATMENT OF TISSUE	ADENOSINE CONCENTRATION (nmol/g wet wt)
Rat ¹	Heart	Well oxygenated	6
		20 min ischaemia	13
		90 min ischaemia	35
		180 min ischaemia	155
Dog ¹	Heart	Well oxygenated	6
		90 min ischaemia	12
		180 min ischaemia	12
Dog ²	Heart	Well oxygenated	0.28
		0.25 min ischaemia	1.61
Rabbit ³	Heart	Well oxygenated	n.d.
		20 min: 95% N ₂ -5% CO ₂	n.d.
		20 min uncoupling agent	410
Rat ¹	Skeletal muscle	Well oxygenated	4
		20 min ischaemia	7
		20 min contraction	7
		20 min ischaemia and contraction	12
		90 min ischaemia	8
		180 min ischaemia	8
Dog ¹	Skeletal muscle	Well oxygenated	4
		90 min ischaemia	20
Dog ⁴	Sartorius muscle	Well aggregated	0.7
		5 min ischaemia and contraction	1.5

n.d. = not detected

All values refer to in situ tissue preparations with the exception of the rabbit heart which was isolated and perfused. Adenosine was assayed enzymically following its isolation by adsorption onto charcoal and thin-layer chromatography.

¹ Rubio et al., (1973)

² Olson (1969)

³ Richman & Wyborny (1964)

⁴ Dobson et al. (1971)

TABLE 1.2 Adenosine levels in brain tissues

ANIMAL	TISSUE	TREATMENT OF ANIMAL OR TISSUE	ADENOSINE CONCENTRATION (nmol/g wet wt)
Rat ¹	Whole brain	No treatment	86 ^a
		Deprived paradoxical sleep 48 hr	108 ^a
		Deprived paradoxical sleep 72 hr.	147 ^a
Rat ²	Whole brain	Well oxygenated	22
		0.25 min ischaemia	30
		1 min ischaemia	64
		5 min ischaemia	79
		20 min ischaemia	53
Guinea- pig ³	Cortical slices? ^b	Well oxygenated? ^b	≤0.5 ^b

- a. These values are probably too high because the brain was not freeze-clamped but rather cooled to 0 to 4°C before homogenization.
- b. This is a preliminary report and the technical details are not recorded.

¹ Haulica et al. (1973)

² Berne & Curnish (1972)

³ Shimizu et al. (1972)

TABLE 1.3 Adenosine levels in body fluids

ANIMAL	BODY FLUID	TREATMENT OF ANIMAL OR TISSUE	ADENOSINE CONCENTRATION (nmol/ml)
Rabbit ¹	Coronary perfusate	Well oxygenated	n.d.
		20 min: 95% N ₂ -5% CO ₂	n.d.
		20 min uncoupling agent	350
		40 min uncoupling agent	70
Guinea-pig ²	Coronary perfusate ^a	Well oxygenated	n.d.
		Anoxic	0.27
Cat ²	Coronary perfusate ^a	Well oxygenated	0.015
		Anoxic	0.15
		Normal oxygenation,	2.6
		0.3 µg/ml adrenaline	
Dog ³	Coronary sinus blood	Well oxygenated	n.d.
		Following 30-60 sec ischaemia	0.13
Dog ⁴	Pericardial fluid	No treatment	1.09
Dog ⁵	Hind limb venous plasma	Well oxygenated	0.7
		5 min ischaemia and contraction	1.5
Rat ⁶	Cerebrospinal fluid	No treatment	0.22

n.d. = not detected

a. In these experiments the perfusion medium contained 1 mM
8-azaguanine, which is an inhibitor of adenosine deaminase.

¹ Richman & Wyborny (1964)	² Katori & Berne (1966)
³ Rubio <u>et al.</u> (1969)	⁴ Rubio & Berne (1969)
⁵ Dobson <u>et al.</u> (1971)	⁶ Berne & Curnish (1972)

any adenosine must be deaminated within the cell as soon as it is formed (Rubio & Berne, 1969; Dobson et al., 1971). This logic is totally inadequate. There must be a steady-state concentration of adenosine within the cell. The steady-state concentration depends on the activities and properties of the adenosine-forming and adenosine-metabolizing reactions of the cell, and cannot be deduced from the activity of adenosine deaminase alone.

However, there is other evidence to support the view that adenosine is primarily located outside the cell. Schrader et al. (1972) could not detect adenosine in human erythrocyte ghosts incubated in the presence of concentrations of adenosine up to 50 μM . Unfortunately, these results must be viewed with reservation because it was not adequately demonstrated that low intracellular concentrations of adenosine would have remained constant in the course of the separation of the ghosts from their surrounding medium. Moreover, the sensitivity of the adenosine assay was not recorded. Shimizu et al. (1972) found that the concentration of adenosine in guinea-pig brain slices was 26% of the concentration of adenosine in the medium bathing the slices, when the latter concentration ranged from 5 μM to 100 μM . Since the extracellular space of brain tissues can be as much as 30% of the total brain volume (McIlwain & Bachelard, 1971, pp.34-5), it is possible that adenosine was primarily located outside the cell in these experiments.

The experiments of Schrader et al. (1972) and Shimizu et al. (1972) suggest that intracellular adenosine concentrations are low in the presence of high extracellular concentrations of exogenous adenosine. However, there has been no measurement of intracellular and extracellular adenosine concentrations under physiological conditions. Kübler et al. (1970) maintain the contrary view to most workers, i.e. they believe that adenosine is primarily located within the cell under physiological conditions.

There is therefore controversy about the location of adenosine within tissues. The simplest hypothesis is that the transport of adenosine across the cell membrane is an equilibrium process. The direction of movement of adenosine will then depend upon the intra- and extracellular concentrations. Thus in some situations the intracellular adenosine concentration may rise leading to a net efflux of adenosine from the cell; in other situations the intracellular adenosine concentration may fall leading to a net influx of adenosine into the cell. In this case the direction of adenosine transport will depend on the concentration of adenosine within the cell. The enzymes which control adenosine concentrations and the properties of these enzymes are now discussed. However, before these enzymes can be selected for discussion, it is necessary to consider the various pathways of adenosine metabolism.

B.2 THE CONTROL OF ADENOSINE CONCENTRATIONS

The pathways by which adenosine can be formed and metabolized are illustrated in Figure 1.2. Murray et al. (1970) have reviewed these pathways and briefly discussed the enzymes involved. Not all of the illustrated pathways are of significance in animal tissues.

(a) THE PATHWAYS OF ADENOSINE METABOLISM

(i) Non-specific phosphatases

Acid and alkaline phosphatases can hydrolyze 5'-AMP to adenosine but their activity is very low at neutral pH's. It has been suggested, on the basis of substrate specificity studies, that 3% of the AMP-hydrolyzing activity of rat heart can be ascribed to non-specific phosphatases (Sullivan & Alpers, 1970). It is unlikely that the role of non-specific phosphatases is to form adenosine in normal metabolism (Morton, 1965; Fernley, 1971; Hollander, 1971). Thus 5'-nucleotidase is the only enzyme important in the production of adenosine.

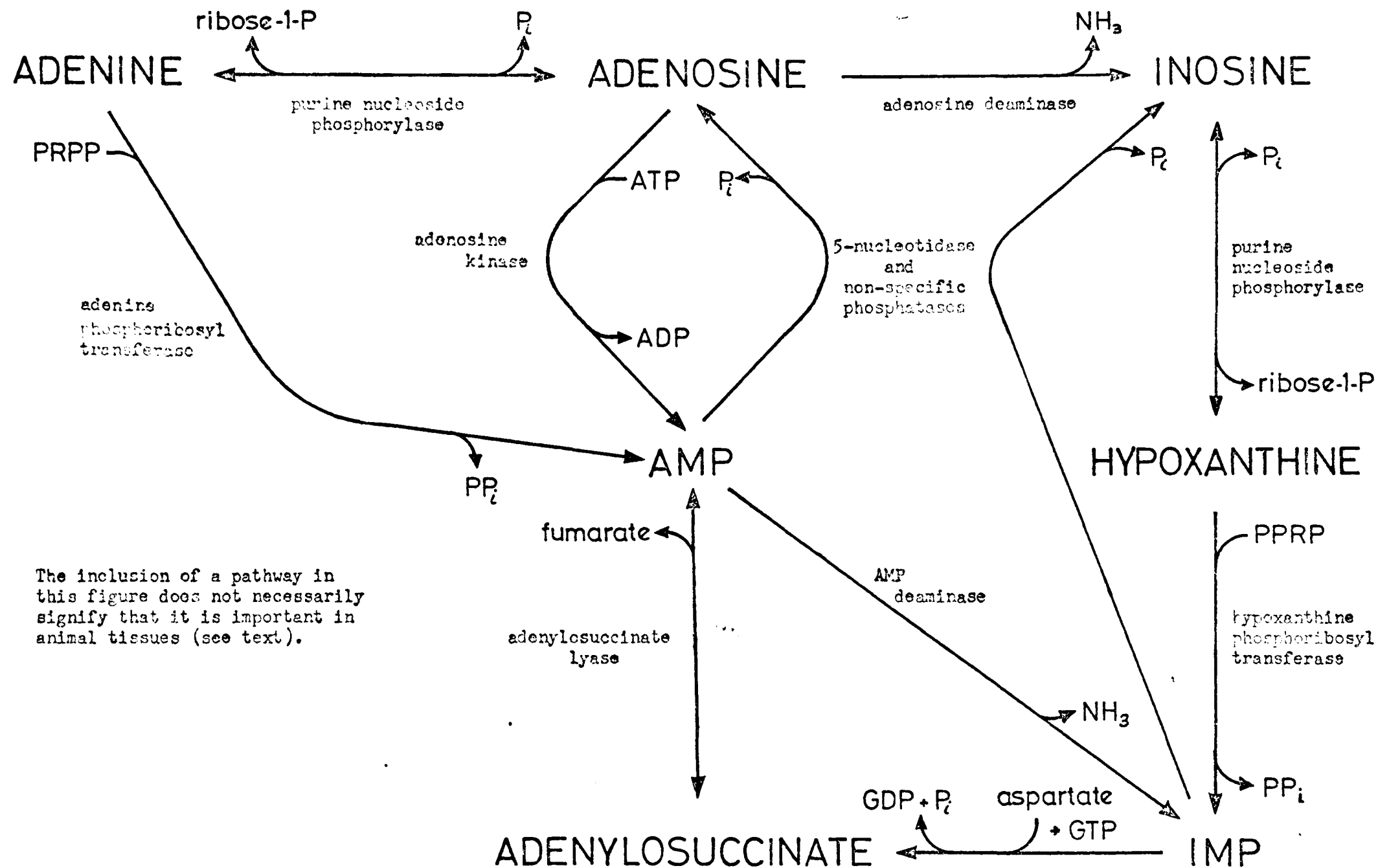


FIG 1.2 Pathways of adenosine metabolism

(11) The role of adenosine kinase in adenosine metabolism

There has been some controversy over the role of adenosine kinase in adenosine metabolism. Although exogenous adenosine is incorporated into adenine nucleotides, adenosine kinase may not be involved. Two other pathways of adenine nucleotide formation have been considered (see Figure 1.2). One of these pathways requires the phosphorolysis of adenosine to adenine followed by the reaction of adenine with PRPP. This pathway has always been hypothetical, since the phosphorolysis of adenosine catalyzed by purine nucleoside phosphorylase is undetectable or insignificant in animal tissues (see Murray et al., 1970; Zimmerman et al., 1971). The second pathway involves the intermediates inosine, hypoxanthine and IMP (Figure 1.2).

The adenosine kinase pathway was considered to be unimportant because the activity of adenosine kinase could not be detected whereas the activities of some of the enzymes of the other pathways were very high (Santos et al., 1968; Lowy et al., 1960; Lowy et al., 1962; Bartlett & Bucolo, 1968; Maguire et al., 1972). However, various lines of evidence have now shown that, in both tissue homogenates and in vivo, the adenosine kinase route is the major one by which adenosine is converted to adenine nucleotides. These lines of evidence are discussed below. (In many of the experiments cited the uptake of label from [^{14}C]-adenosine into adenine nucleotides is followed. Such experiments are discussed more fully in Section B.4.)

- a. The activities of adenosine kinase that have been measured in some tissues can account for the rates of conversion of adenosine to adenine nucleotides (De Jong & Kalkman, 1973; Chapter 4 of this thesis).
- b. Under conditions in which exogenous labelled adenosine is incorporated into adenine nucleotides, inosine and hypoxanthine are only slightly labelled. Similarly, there was no incorporation of labelled adenosine into adenine in heart, red blood cells and kidney, although the former compound was

taken up by these tissues (Liu & Feinberg, 1971; Whitton & Wiley, 1968; Lowy et al., 1952).

c. If $[^{14}\text{C}]$ -adenosine incorporation into adenine nucleotides proceeds via inosine, hypoxanthine or adenine, then a procedure which increases the size of the unlabelled pool through which the label passes should cause a reduction in the rate of incorporation of the label into adenine nucleotides. This is provided the larger pool size is not well below the K_m value of the enzyme acting upon the pool, in which case the decreased proportion of label in the pool will be compensated for by a corresponding increase in the rate at which the enzyme acts upon the pool. This proviso, together with the uncertainty of knowing whether the relevant pool size is really being increased, means that such an experiment is never definitive when no effect of the addition of an unlabelled intermediate is found. Goldthwaite (1957) found that the addition of unlabelled hypoxanthine or inosine to homogenates of pig heart did not decrease the rate of $[^{14}\text{C}]$ -adenosine incorporation into adenine nucleotides. Lowy & Williams (1966) similarly found no effect when unlabelled inosine or hypoxanthine were added to erythrocytes taking up $[^{14}\text{C}]$ -adenosine.

d. In the studies discussed so far, adenosine labelled in the eighth position of the purine base was used to follow adenosine uptake. If adenosine is split into purine and ribose moieties before the purine is incorporated into adenine nucleotides then, provided the ribose moiety is not equally vigorously incorporated, the label from U- $[^{14}\text{C}]$ -adenosine will appear more in the adenine than the ribose moiety of adenine nucleotides. In practice equal labelling of the base and ribose moieties has been shown for guinea-pig heart (Wiedmeier et al., 1972), erythrocytes (Miech & Santos, 1969), and guinea-pig cerebral cortex slices (Shimizu et al., 1972). Moreover, Miyazaki & Minaki (1972) showed that in mouse erythrocytes only the base moiety of

U- $[^{14}\text{C}]$ -inosine is converted into adenine nucleotides and so ribose phosphate derived from the phosphorolysis of the nucleoside is not rapidly incorporated into adenine nucleotides in this tissue.

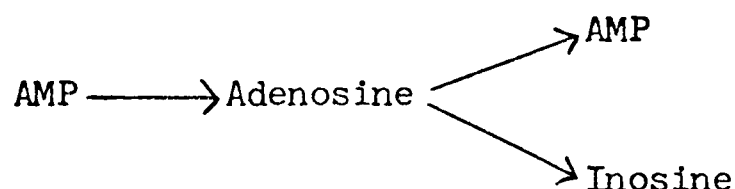
e. Lerner & Rubinstein (1970) have demonstrated that the γ -phosphate group of γ - $[^{32}\text{P}]$ -ATP is incorporated into AMP in the presence of adenosine and red blood cell haemolysates. The only reasonable mechanism for this is direct phosphorylation of adenosine.

(iii) Conclusions

From the evidence presented in this section it may be concluded that adenosine kinase plays an important role in adenosine metabolism. The acid and alkaline phosphatases and purine nucleoside phosphorylase are considered to be unimportant and so they will be omitted from the following discussion.

(b) THE SIGNIFICANCE OF EQUILIBRIA IN UNDERSTANDING THE CONTROL OF ADENOSINE CONCENTRATIONS

The steady-state concentration of adenosine in the cell depends upon the rates of its formation and utilization. Adenosine is formed from 5'-AMP by the enzyme 5'-nucleotidase and it is utilized by adenosine kinase or adenosine deaminase.



The extent to which the steady-state concentration of adenosine may be varied by alterations in the activities of these three enzymes depends upon a number of factors, including whether the enzymes catalyze equilibrium or non-equilibrium reactions. Thus it was shown in Section A.1(b) that the changes in concentration of an intermediary metabolite caused by variation in the activity of the producing enzyme may be small, if the enzyme utilizing it catalyzes a reaction which is close to equilibrium. It was also shown that

variations in the activity of the utilizing enzyme have little effect on the concentration of the metabolite, if this enzyme catalyzes a reaction which is close to equilibrium. By similar reasoning it may be shown that the concentration of the metabolite is little affected by variation in the activity of either enzyme, if the metabolite-producing enzyme catalyzes a reaction which is close to equilibrium. Therefore the most sensitive control of adenosine concentrations would be possible if all the enzymes (i.e. 5'-nucleotidase, adenosine kinase and adenosine deaminase) catalyze reactions which are far from equilibrium. Moreover, under such conditions, fluxes through these reactions may be regulated by factors other than substrate concentration.

It seems probable that 5'-nucleotidase catalyzes a non-equilibrium reaction in vivo. Thus the equilibrium of the reaction is in favour of adenosine formation ($\Delta G^{\circ} = -3$ to -4 kcal/mol — West et al., 1966, p.891), whereas intracellular AMP levels are an order of magnitude greater than adenosine levels. Unfortunately, it is not known whether adenosine kinase and adenosine deaminase catalyze non-equilibrium reactions in vivo, since intracellular concentrations of adenosine and inosine are not known. (The standard free energy of the adenosine kinase reaction is about -5 kcal/mol —from Benzinger et al., 1959; West et al., 1966, p.891—and the standard free energy of the adenosine deaminase reactions is -6 kcal/mol—Wolfenden, 1967). Despite these difficulties, in order to formulate a theory of control of adenosine concentrations in vivo, it is necessary to discuss the properties and regulation of the enzymes.

(c) ADENOSINE FORMATION—5'-NUCLEOTIDASE

The formation of adenosine from 5'-AMP catalyzed by the enzyme 5'-nucleotidase is generally accepted to be the only important pathway of adenosine formation in animal tissues. Studies of the properties and

cellular location of this enzyme have been reviewed by Drummond & Yamamoto (1971) and earlier, in great detail, by Bodansky & Schwartz (1968).

(i) Control of adenosine formation by changes in AMP concentration.

The extent to which an enzyme's activity increases in response to an increase in substrate concentration rapidly decreases as the substrate concentration rises above the K_m . Therefore, if the activity of 5'-nucleotidase is controlled by the concentration of AMP within the cell, the concentration of AMP available to the enzyme should be lower than the K_m of the enzyme. The reported K_m values of 5'-nucleotidase for AMP vary from 4 to 12,000 μM (Table 1.4), although in most reports they are less than 100 μM . The AMP concentration in the cell is about 50 μM . However, the K_m of 5'-nucleotidase is increased by ATP and ADP (see the following paragraph). This could mean that in vivo the apparent K_m of 5'-nucleotidase is raised well above the cellular concentration of AMP, thereby enabling adenosine formation to be regulated by AMP concentration.

(ii) Control of adenosine formation by factors other than substrate concentration.

The activities of 5'-nucleotidase from various vertebrate sources are strongly inhibited by ATP. The enzyme from rat heart is inhibited by ADP in addition to ATP. Other nucleoside triphosphates also inhibit the enzyme (Ipata, 1968; Kluge et al., 1972; Sullivan & Alpers, 1971; Nakatsu & Drummond, 1972; Gibson & Drummond, 1972). The K_i for ATP is about 1 to 40 μM . ADP is a more effective inhibitor than ATP for the enzyme from rat heart.

Inhibition is competitive (or mixed) and so the low K_i 's, in relation to ATP and ADP levels in the cell, do not signify that 5'-nucleotidase is totally inhibited in vivo. Moreover, inhibition by ATP and ADP is greatly reduced by Mg^{2+} , i.e. it is the free nucleotides which inhibit the enzyme. It is not clear whether Mg^{2+} activates the enzyme other than by forming

TABLE 1.4 The K_m values of 5'-nucleotidase from animal tissues

ANIMAL	TISSUE	SUBCELLULAR FRACTION	K_m FOR AMP (μM)
Rat ¹	Liver	100,000g supernatant	6800
Rat ²	Liver	"Plasma membranes"	12
Rat ³	Liver	Microsomes	12
Rat ⁴	Brain	Microsomes	200
Rat ⁵	Cerebellum	40,000g supernatant	80
Rat ^{6,7}	Heart	Membranes	18-21
Rat ⁸	Heart	— ^a	23
Calf ⁹	Intestinal mucosa	— ^b	50
Ox ¹⁰	Pituitary	— ^b	60
Ox ¹¹	Milk fat globules	Membranes	940
Pig ¹²	Intestine	16,000g supernatant	4
Sheep ¹³	Brain	27,000g supernatant	7.5
Dog ¹⁴	Cerebrum	Crude homogenate	20
Pigeon ¹⁵	Heart	100,000g supernatant	12000
Human ¹⁶	Liver	35,000g supernatant	39

a. The enzyme was purified from an acetone powder of the tissue.

b. The enzyme was purified from a crude homogenate of the tissue without any preliminary fractionation.

1. Fritszon (1969)	2. Widnell & Unkeless (1968)
3. Segal & Brenner (1960)	4. Kluge <u>et al.</u> (1972)
5. Bosman & Pike (1971)	6. Baer <u>et al.</u> (1966)
7. Nakatsu & Drummond (1972)	8. Sullivan & Alpers (1971)
9. Center & Behal (1966)	10. Lisowski (1966)
11. Huang & Keenan (1972)	12. Burger & Lowenstein (1971)
13. Ipata (1968)	14. Waked & Kerr (1955)
15. Gibson & Drummond (1972)	16. Song & Bodansky (1966)

complexes with free nucleotides (Sullivan & Alpers, 1971; Gibson & Drummond, 1972). Other inhibitors of 5'-nucleotidase include NADH, NAD, CoA, acetyl-CoA and glycogen (Sullivan & Alpers, 1971) and P_i (Nakatsu & Drummond, 1972).

On the basis of these properties it is possible to propose a theory for regulation of the activity of 5'-nucleotidase by variations in free ATP and ADP levels. From in vitro measurements of the dissociation constants of AMP-Mg, ADP-Mg and ATP-Mg complexes, it may be calculated that free ADP and ATP concentrations will be reduced in the cell when the phosphorylation state of the adenine nucleotides is reduced. Therefore, 5'-nucleotidase activity may be increased when the phosphorylation state of the cell is decreased (Sullivan & Alpers, 1971; Kluge et al., 1972).

(d) ENZYMES WHICH UTILIZE ADENOSINE

Adenosine can be metabolized in two main ways: adenosine deaminase converts it to inosine, and adenosine kinase phosphorylates it to AMP. Studies of adenosine deaminase have been reviewed by Zielke & Suetter (1971) and studies of adenosine kinase have been reviewed by Anderson (1973).

(i) Control of adenosine metabolism by changes in adenosine concentration.

Adenosine concentrations are well below the K_m for adenosine deaminase (Zielke & Suetter, 1971; Ma & Fisher, 1966; Baer et al., 1966) and under some circumstances they are less than the K_m for adenosine kinase (compare Tables 1.1 to 1.3 with 1.5). Therefore, an increase in the in vivo adenosine concentration may be expected to be accompanied by an increase in the rate of adenosine metabolism.

(ii) Control of adenosine metabolism by factors other than adenosine concentration.

Adenosine deaminase is notable in that very few modifiers of its

TABLE 1.5 K_m values of adenosine kinase for adenosine

<u>TISSUE</u>	<u>K_m FOR ADENOSINE (μM)</u>
Ehrlich ascites cells ¹	5.8
Tumour cells ²	2.8
Human H. Ep. No.2 cells ³	1.8
Sarcoma 180 cells ⁴	0.5
Human red blood cells ⁵	1.9
Human platelets ⁶	2.0
Rabbit liver ⁷	1.6
Dog or rabbit heart ⁸	0.91
Rat heart ⁹	5.9
Guinea-pig brain ¹⁰	20

1. Lindberg et al. (1967)

2. Murray (1968)

3. Schnebli et al. (1967)

4. Divekar & Hakala (1971)

5. Meyskens & Williams (1971)

6. Holmsen et al. (1969)

7. Lindberg (1969)

8. Alma & Feinberg (1971)

9. De Jong & Kalkman (1973)

10. Shimizu et al. (1972)

activity have been discovered, despite the fact that it is a much-studied enzyme. One exception is that 5 mM Mg^{2+} has been found to inhibit the supernatant enzyme from rat brain (Mustafa & Tewari, 1970b).

Few effectors of adenosine kinase other than Mg^{2+} and Mn^{2+} have been reported, but Meyskens & Williams (1971) found that adenosine kinase from human erythrocytes was inhibited by AMP, ADP, GDP, IMP, GMP and adenine, with respective K_i values of 0.4, 0.4, 1.4, 2.0 and 8 mM. In addition Mg^{2+} or ATP have often been found to inhibit the enzyme if the concentration of one greatly exceeds the concentration of the other (see also Chapter 2, Section D.4). The pH optimum of adenosine kinase from most mammalian sources is between pH 5 and 6 and the effect of pH is quite large (Table 1.6).

The properties of adenosine kinase and adenosine deaminase do not permit the formulation of a detailed theory of regulation of adenosine metabolism by any factor other than substrate concentration. One possible theory is that adenosine phosphorylation is regulated in some tissues by pH changes. Thus in anaerobic tissues there is a fall in pH when the rate of energy metabolism is high. The fall in pH may lead to increased adenosine kinase activity. It is also possible that an increase in AMP and ADP concentrations in vivo inhibits adenosine kinase activity.

(e) A THEORY OF THE CONTROL OF INTRACELLULAR ADENOSINE CONCENTRATIONS

From the properties of 5'-nucleotidase, adenosine kinase and adenosine deaminase, and from the theories of regulation of the activities of these enzymes, it is possible to construct a theory of the control of intracellular adenosine concentrations by adenine nucleotides. This theory is illustrated below. 5'-Nucleotidase, adenosine kinase and adenosine deaminase are all assumed to catalyze non-equilibrium reactions.

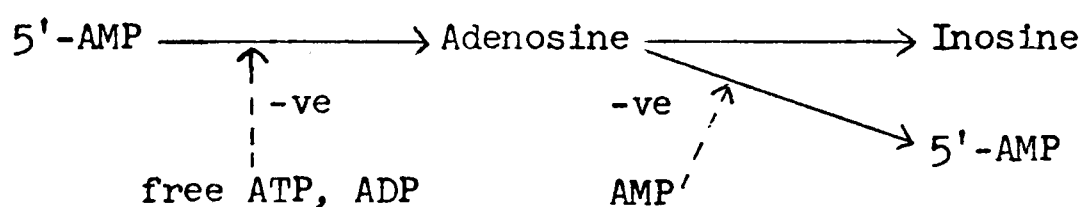


TABLE 1.6 pH Optima of adenosine kinase from animal tissues

TISSUE	pH OPTIMUM
Ehrlich ascites cells ¹	6.2 to 7.3
Sarcoma 180 cells ²	≤5.5
Human red blood cells ³	5.7
Human platelets ⁴	5.6
Rat and rabbit liver ⁵	4.9 to 5.0
Mouse liver ⁶	5.4
Rat and rabbit kidney ⁵	4.9 to 5.0
Rat heart ⁷	5.4 to 5.6

1. Murray (1968)

2. Divekar & Hakala (1971)

3. Meyskens & Williams (1971)

4. Holmsen & Rosenberg (1968)

5. Caputto (1951)

6. Ho et al. (1968)

7. De Jong & Kalkman (1973)

The theory predicts that when the phosphorylation state of the adenine nucleotides of the cell decreases, the adenosine content of the cell increases; the proposed mechanism is as follows.

- (a) 5'-Nucleotidase activity is increased by increased availability of substrate and/or by reduction in the inhibition by free ATP and ADP.
- (b) Adenosine kinase activity is inhibited by AMP. Since adenosine deaminase is not saturated with substrate, the adenosine concentration will not rise continuously but a new steady state concentration of adenosine will be established.

According to the hypothesis presented at the end of Section B.1(b), an increase in the concentration of adenosine within the cell should result in an increase in the efflux (or output) of adenosine from the cell. Therefore, in the following section the output of adenosine from the cell will be studied in order to test the theory of control of adenosine concentration in the intact cell.

B.3 THE OUTPUT OF ADENOSINE FROM THE CELL

The output of adenosine from heart and brain tissue has been closely studied. Skeletal muscle and fat cells have been studied to a lesser extent. The rate of adenosine output can vary greatly. In order to test the theory of control of adenosine concentrations, the factors which affect the rate of adenosine output will be discussed. However, certain problems must be considered first in order that the results presented can be properly interpreted.

(a) PROBLEMS IN THE MEASUREMENT OF THE TRANSPORT OF ADENOSINE OUT OF THE CELL

The problems of measuring the gross rate of adenosine output from the cell are illustrated in Figure 1.3 (following p. 48).

One problem is that a metabolite released from the cell may be degraded extracellularly. Consequently, if adenosine is released into the extra-

cellular space of a tissue, its rapid deamination to inosine may mean that no increase in the level of adenosine will be detected within the tissue or the fluid bathing the tissue. Similarly, if a rise in extracellular adenosine levels is observed, the possibility must be considered that this rise is a result of adenine nucleotide release from the cell followed by dephosphorylation to adenosine (see Pull & McIlwain, 1973, for evidence that adenine nucleotides are released by the cell). There is ample evidence that adenosine can be formed from adenine nucleotides and degraded extracellularly at rates comparable to adenosine output from the cell (Van Belle, 1964; Richman & Wyborny, 1964; Baer & Drummond, 1968; Pull & McIlwain, 1972a, 1973; also compare Tables 1.7 and 1.8).

A second problem in measuring gross adenosine transport out of the cell is that adenosine can be taken up by the cell. Therefore, the rate of adenosine output which is measured in most experiments will be the difference between the output and the uptake (i.e. a net rate).

The problems of measuring the transport of adenosine out of the cell can, to some extent, be accounted for, as described in the following section.

(b) ASSESSMENT OF THE TRANSPORT OF ADENOSINE OUT OF THE CELL

In paragraphs (i) and (ii) below the methods for measuring the net rate of transport of adenosine out of the cell are described. In paragraph (iii) the methods for measuring the gross rate of adenosine transport out of the cell are described.

(i) Corrections for extracellular adenosine breakdown

Three approaches have been used to correct for the breakdown of adenosine outside the cell.

a. Measurement of adenosine + inosine + hypoxanthine output is often taken to represent adenosine output on the assumption that the inosine and hypoxanthine are derived extracellularly from adenosine. It is possible,

TABLE 1.7 Rates of adenosine formation from extracellular
adenine nucleotides

ANIMAL	TISSUE PREPARATION	SUBSTRATE	RATE OF ADENOSINE FORMATION (nmol/min/g wet wt.)
Rat ¹	Perfused heart	AMP	30
		ATP	15
Guinea-pig ²	Cerebral cortex slices	ATP	0.15

Rates of adenosine formation were calculated using the equation,

$$\frac{\text{nmoles of adenine nucleotide added to tissue} \times \% \text{ breakdown}}{\text{time (min)} \times \text{wt. of tissue (g)}}$$

The weight of the rat heart was assumed to be 1g (Humphrey, 1961) and the weight of cerebral cortex slices 0.08g (see McIlwain & Snyder, 1970). The rates of adenosine formation are not maximal rates. However, comparison of this data with that presented in Table 1.8 shows that adenosine may be formed extracellularly from adenine nucleotides at rates comparable with measured rates of adenosine output from the cell.

1. Baer & Drummond (1968)
2. Pull & McIlwain (1972a)

TABLE 1.8 Net rates of adenosine output from animal tissues

ANIMAL	TISSUE	BASAL RATE OF OUTPUT ^a (nmol/min/g wet wt)		STIMULATED RATE OF OUTPUT		TREATMENT USED TO STIMULATE ADENOSINE OUTPUT
		ADENOSINE	CORRECTED RATE ^b	ADENOSINE	CORRECTED RATE ^b	
Guinea-pig ¹	Brain slices	0.1	-	6	16	Electrical
Guinea-pig ²	Perfused heart	0.016	-	-	-	—
Guinea-pig ³	Perfused heart	0-0.04	0.1-0.5	1.8	4.5	Severe hypoxia ^c
Cat ³	Perfused heart	0-0.001	0.1-0.3	0.3	1.7	Severe hypoxia ^c
Dog ⁴	<u>In situ</u> heart	0.0005-0.003	-	0.0014-0.005	-	Hypoxia ^d
Dog ⁵	<u>In situ</u> heart	-	-	0.06	0.08	Hypoxia ^e
Dog ⁶	<u>In situ</u> hind limb	0.0015	0.003	(Precise rates cannot be calculated)		Ischaemia + electrical stimulation
Rat ⁷	Fat cells	0.7	-	0.9	-	10 μ m Noradrenaline

When it was not recorded, the rate of output of adenosine was calculated by using the equation:

$$\frac{\text{rate of flow of perfusate (ml/min)} \times \text{concentration of adenosine in perfusate (nmol/ml)}}{\text{wt. of tissue (g)}}$$

Weights of tissues were obtained from Humphrey (1961) and Mr G.J.R. Hovell of the Physiology Department, Oxford

a. Rates for well oxygenated tissues supplied with all the metabolites necessary for stable metabolism.

b. See Section B3b(i) for calculation of corrected rates.

c. $O_2 \approx 40$ mm Hg

d. 4/5 Normal respiration.

e. Coronary blood flow occluded 30-60 sec.

1. Pull & McIlwain (1972a)

2. Wiedmeier et al. (1972)

3. Katori & Berne (1966)

4. Rubio & Berne (1969)

5. Rubio et al. (1969)

6. Dobson et al. (1971)

7. Schwabe et al. (1973)

however, that inosine or hypoxanthine is released per se.

- b. An estimate of net adenosine output from the heart has been made by taking into account the known rate of intravascular adenosine degradation (Rubio et al., 1969).
- c. The breakdown of adenosine outside the cell can be reduced by perfusing the tissue with 8-azaguanine, which is an inhibitor of adenosine deaminase (Richman & Wyborny, 1964).

The results of these methods are summarized in Table 1.8.

(ii) Correction for adenine nucleotide release

An assessment of the proportions of adenosine and adenine nucleotides released from electrically stimulated guinea-pig brain slices has been made by Pull & McIlwain (1973). It was assumed that adenine nucleotides are released by Ca^{2+} -dependent secretory processes, so that this release could be prevented by the omission of Ca^{2+} from the incubation medium. It was also assumed that the entry of Na^+ into the cell is an essential step in the effect of electrical stimulation on adenosine release, so that tetrodotoxin, which inhibits Na^+ entry into nerve cells (Kao, 1966), should prevent the effect of electrical stimulation on adenosine release. By this approach it can be concluded that, of the total adenosine observed in the brain perfusate, 70% was transported out of the brain cells as adenosine, and 30% was transported as adenine nucleotides and subsequently degraded.

(iii) Correction for adenosine reuptake

The gross rate of transport of adenosine out of the cell could be assessed by measuring the output of labelled adenosine into a pool of unlabelled adenosine. In order to do this, the adenine nucleotides of the cell must be pre-labelled. This can be done by incubating or perfusing a tissue with labelled adenine. Such an approach has been used, perhaps unwittingly, by Pull & McIlwain (1973). It was found that the presence of unlabelled adenosine

apparently increased the output of labelled adenosine from prelabelled guinea-pig brain slices by three- to four-fold. The results were interpreted, however, in terms of an increase in total adenosine output.

It is therefore possible to compile a table of corrected net rates of outward transport of adenosine by various tissues (Table 1.8). Unfortunately, a table of gross rates of adenosine output cannot be compiled, since in only one case has a gross rate been measured. The data will be compared with the maximal activities of 5'-nucleotidase obtained in this study in Chapter 4. At this stage, it is possible to assess the effect of various procedures on adenosine output in order to test the theory of control of intracellular adenosine concentration presented in Section B.2(e).

(c) TREATMENTS WHICH INCREASE ADENOSINE TRANSPORT OUT OF THE CELL

The lowest rates of adenosine output from the cell are obtained when tissues are adequately supplied with oxygen and an oxidizable substrate (Table 1.8). The output of adenosine from guinea-pig cerebral cortex is increased by anoxia, the omission of glucose from the superfusing medium, electrical stimulation or the addition of depolarizing agents such as K^+ (43 mM), ouabain or veratridine (Pull & McIlwain, 1972a; Shimizu et al., 1970). The output of adenosine by hearts is increased by ischaemia, hypoxia, uncouplers of oxidative phosphorylation and adrenaline (see Table 1.8; Richman & Wyborny, 1964). (The effect of adrenaline may merely be to increase the rate and force of heart contractions, and thereby increase the requirement of the tissue for oxygen and produce hypoxia.) Adenosine output from skeletal muscle can be increased by a combination of electrical stimulation and ischaemia.

Increases of 100-fold in the net adenosine output from heart and brain tissues can easily be induced in vitro (see Table 1.8) but there are no reports of measurements in the living animal.

(d) EVIDENCE FOR THE THEORY OF CONTROL OF ADENOSINE CONCENTRATIONS

A theory of the control of intracellular adenosine concentrations by adenine nucleotides was presented in Section B.2(e). It predicted that when the phosphorylation state of the cell decreases, the adenosine content of the cell leads to an increase in the transport of adenosine out of the cell so that the extracellular concentration of adenosine is increased.

Nearly all the conditions, such as the lack of oxygen or glucose, the presence of uncoupling agents and electrical stimulation, which have been found to elevate adenosine levels in tissues and body fluids and cause increased rates of release of adenosine from nervous and muscular tissues are known to elevate AMP and depress ATP levels within tissues. (For a discussion of the effect of electrical stimulation on adenine nucleotide levels see Pull & McIlwain, 1973). There is therefore some evidence to support the theory of control of intracellular adenosine concentrations by adenine nucleotides.

(e) THE LOCALIZATION OF 5'-NUCLEOTIDASE AND THE SITE OF ADENOSINE PRODUCTION

It has been suggested (Section B.1(b)) that adenosine efflux from the cell results from an increase in the concentration of adenosine within the cell followed by net outward transport down a concentration gradient. It is possible, however, that 5'-nucleotidase is predominantly located in the plasma membrane such that the adenosine it forms is released directly into the extracellular environment. This view is consistent with the suggestion that adenosine is primarily located outside the cell (see Section B.1(b)). The localization of 5'-nucleotidase is therefore of some consequence in understanding the factors which control intracellular adenosine concentrations, the release of adenosine from the cell and the further metabolism of adenosine.

Both cell fractionation and histochemical approaches have been used to determine the location of 5'-nucleotidase activity. Bodansky & Schwartz

(1968) have fully reviewed early studies of 5'-nucleotidase location and this subject will be discussed here, especially in relation to the tissues investigated in the present work.

(i) Brain

The localization of 5'-nucleotidase in brain tissue has not been well characterized. Guinea-pig brain myelin is reported to have a lower specific activity of 5'-nucleotidase than the whole brain homogenate (Coleman & Finean, 1966). However, 5'-nucleotidase activity has been reported to be greatly diminished in the demyelinated areas of the brains of patients with multiple sclerosis (Barron & Bernsohn, 1965). Moreover, Naidoo (1962) concluded from histochemical studies that adenosine production occurs largely in the region of the myelin sheath of rat brain cells.

(ii) Liver

It has been reported that the 5'-nucleotidase of rat liver exists solely in the membrane fraction and that probably much of it is associated with the endoplasmic reticulum (Song & Kappos, 1969). On the other hand, rat liver 5'-nucleotidases have been purified from lysosomal and cytosolic fractions of the cell, and there are reports that liver plasma membrane fractions are enriched in the enzyme (see Drummond & Yamamoto, 1971). A proportion (40%) of pigeon liver 5'-nucleotidase is reported to be present in the soluble fraction of the cell (Gibson & Drummond, 1972).

(iii) Heart and skeletal muscle

Much of the 5'-nucleotidase of rat heart has been shown to be associated with the membrane fraction of the tissue (Baer et al., 1966), but the distribution of the enzyme in the heart cells of various mammalian and avian species varies greatly (Gibson & Drummond, 1972; Nakatsu & Drummond, 1972). The histochemical studies of Rubio et al. (1973) suggest that the 5'-nucleotidase

of rat skeletal muscle is located only in those zones within selected muscle fibres which are in close proximity to blood vessels. On the other hand the 5'-nucleotidase of rat heart is found in all myocardial fibres and is associated with most myocardial membranes, e.g. transverse tubules, the sarcolemma, intercalated discs, sarcoplasmic reticulum. 5'-Nucleotidase of both rat heart and skeletal muscle is also found in capillary endothelial cells. The activity of 5'-nucleotidase in rat skeletal muscle is much lower than that in rat heart. However, the adenosine which is produced in skeletal muscle is more likely to enter the blood than that produced in the heart, since in the former case the adenosine has a shorter distance to diffuse to the blood stream.

Nakatsu & Drummond (1972), who studied histochemically the hearts of various mammals and birds, found that 5'-nucleotidase was located predominantly in the capillary endothelium, rather than the myocardial membranes. Borgers et al. (1971), on the other hand, concluded that in dog, rat and human hearts adenosine is only found in perivascular mesenchymal cells, which are cells located between the myocardium and the arterioles controlling the coronary blood supply.

(iv) Conclusion

In many mammalian tissues 5'-nucleotidase is preominantly associated with membranes. It is therefore possible that adenosine produced by 5'-nucleotidase is released into membrane-bound vesicles or the extracellular environment, rather than the cytosol. However, it is equally possible that adenosine is released into the cytosol by 5'-nucleotidase located at cell membranes. Therefore, the association of 5'-nucleotidase with membranes is not inconsistent with the view that adenosine is produced within the cell and can be transported outwards down a concentration gradient.

It should be noted that most investigations of 5'-nucleotidase location have used mammalian species. Avian muscle 5'-nucleotidase is mostly located

in the cytosol so that in these animals it is very likely that adenosine is formed in the cytosol (Gibson & Drummond, 1972). Thus it is possible that there are important species, if not tissue, variations in the localization of this enzyme.

B.4 THE UPTAKE OF ADENOSINE BY THE CELL

Adenosine is released from tissue preparations in vitro under some conditions which may be expected to pertain at times in vivo. The fate of this adenosine and the mechanisms controlling it are now discussed.

(a) THE LOCATIONS OF ADENOSINE OUTPUT AND METABOLISM

The rapid uptake of adenosine by animal tissues (Table 1.9) and the low or undetectable levels of adenosine in the blood and other body fluids (Table 1.3) suggest that extracellular adenosine is largely metabolized at a location near to the cell from which it is released. Nevertheless, some exchange of adenosine between tissues may occur, since the transfer of adenosine from rabbit liver to blood has been reported recently (Lerner & Lowy, 1974). There is some inverse correlation between the size of the tissue and the basal rates of net adenosine output, which may result from the differing surface:volume ratios of the tissues. Thus adenosine released by the individual cell of a tissue is more likely to be taken up by the tissue if the surface:volume ratio of the tissue is small. Therefore, if the total output by the individual cell is similar in all tissues, the net output of adenosine per gram of tissue will be greater for the smaller tissues. Even in the case of guinea-pig brain slices, which have a high surface:volume ratio, a considerable proportion (75 to 80%) of the released adenosine is taken up again and is not released into the perfusate (Section B.3b(iii)).

(b) EVIDENCE THAT ADENOSINE IS LARGELY METABOLIZED WITHIN THE CELL

Since adenosine is released from the cell it is possible that some of its metabolism is extracellular. However, evidence will be cited which shows

that adenosine may be taken up by tissues and metabolized within the cell (paragraph (i) below). In addition, phosphorylation of adenosine would not be expected to take place outside the cell because extracellular ATP levels are usually very low or undetectable (Dobson et al., 1971; Berne et al., 1971; Pull & McIlwain, 1973). Therefore if adenosine is phosphorylated by a tissue, it is likely that it is metabolized within the cell. On the other hand, deamination of adenosine may occur intra- or extracellularly (cf. Section B3a). For this reason the metabolism of exogenous adenosine is discussed below (paragraph (ii)).

(i) The uptake of adenosine by tissues

Experiments, in which labelled adenosine was injected into guinea-pigs, show that the lungs and liver have the largest capacities for the uptake of adenosine from blood (Pfleger et al., 1969). Where it has been possible, approximate rates of adenosine uptake have been calculated from data in the literature (Table 1.9). However, these values do not represent maximum rates of uptake, since usually very low adenosine concentrations were employed. In addition to the tissues listed in Table 1.9, adenosine has been shown to be taken up by skeletal muscle but quantitative data are not available (Lowy et al., 1952).

In the case of the guinea-pig heart, there is good evidence that adenosine uptake precedes adenosine metabolism because dipyridamole, which inhibits the uptake of exogenous adenosine, also inhibits its metabolism (Hopkins, 1973b).

(ii) The metabolic fate of adenosine

Low concentrations (less than 5 μM) of exogenous adenosine are phosphorylated, rather than deaminated, by most tissues (Jacob & Berne, 1960; Santos et al., 1968; Parker, 1970; Liu & Feinberg, 1971; Shimizu et al., 1972; Schrader et al., 1972). (Rat heart is an exception - see De Jong, 1972.)

TABLE 1.9 Rates of adenosine uptake by animal tissues

ANIMAL	TISSUE	CONCENTRATION OF ADENOSINE (μ M)	RATE OF UPTAKE (nmol/min/g wet wt.)
Guinea-pig ¹	Lungs	-	3.4
	Liver	-	0.87
Guinea-pig ²	Heart	<8	0.6
Guinea-pig ³	Heart	0.01	0.016 ^a
Guinea-pig ⁴	Heart	-	4.5 ^b
Cat ⁵	Heart	1.5	0.3
		25	7
Rabbit ⁶	Heart	0.3	0.47
Rat ⁷	Heart	500	350
Rat ⁴	Heart	-	12 ^b
Human ⁸	Blood	100	174
Rabbit ⁸	Blood	100	6670
Rat ⁸	Blood	100	28
Chicken ⁸	Blood	100	850
Guinea-pig ⁹	Brain	-	30 ^b
Rat ¹⁰	Brain	8.5	0.1

Where rates of uptake were given for the whole organ, the weight of the organ was obtained as described in the legend to Table 1.8.

Methods were as follows:

- lungs - adenosine injected into the jugular vein;
- liver - adenosine injected into the hepatic portal vein;
- heart - adenosine perfused through the isolated or in situ organ;
- blood - adenosine added directly to a blood sample;
- brain - adenosine added to medium perfusing the slice.

- a. The rate of blood flow through a guinea-pig heart was assumed to be 3.6 ml/min (Katori & Berne, 1966).
- b. V_{max} for adenosine uptake systems (see Table 1.10).

1. Pflieger et al. (1969)	2. Kolassa <u>et al.</u> (1970)
3. Wiedmeier <u>et al.</u> (1972)	4. Hopkins & Goldie (1971)
5. Jacob & Berne (1960)	6. Liu & Feinberg (1971)
7. De Jong (1972)	8. Van Belle (1964)
9. Shimizu <u>et al.</u> (1972)	10. Santos <u>et al.</u> (1968)

Since under most physiological conditions extracellular adenosine concentrations are less than 5 μM , its metabolism must be predominantly intracellular.

(Extracellular phosphorylation cannot occur.) At high concentrations of extracellular adenosine, when deamination preponderates, it is not clear whether adenosine metabolism is intra- or extracellular. However, some evidence suggests that adenosine deamination is intracellular (Schrader et al., 1972).

(c) THE ADENOSINE UPTAKE MECHANISM

The uptake of adenosine by the cell involves not only the transport of the molecule across the plasma membrane but also its further metabolism. Therefore the roles played by adenosine kinase and adenosine deaminase in the uptake of adenosine are now considered.

(i) Kinetics of the uptake mechanism

Guinea-pig erythrocytes, human erythrocytes ghosts and hepatoma cells take up adenosine by a saturable mechanism and also by a mechanism which has the characteristics of simple diffusion (Roos & Pflieger, 1972; Schrader et al., 1972; Plagemann, 1970). The latter process has not been identified for rat heart, guinea-pig heart, guinea-pig brain and dog erythrocytes, whereas a saturable uptake process is present (Hopkins & Goldie, 1971; Shimizu et al., 1972; Kübler & Bretschneider, 1963). The K_m values of the saturable process are presented in Table 1.10. It is inhibited by the adenosine analogues inosine (Pflieger et al., 1969; Kolassa et al., 1970; Jenkins et al., 1972) and 2-deoxyadenosine (Pull & McIlwain, 1972b).

The saturable uptake mechanism apparently does not involve an active transport mechanism because there is no evidence that adenosine can be concentrated within the cell (Schrader et al., 1972; Shimizu et al., 1972).

TABLE 1.10 K_m Values for adenosine of adenosine kinase and
adenosine uptake

ANIMAL	TISSUE	K_m VALUES (μM)	
		ADENOSINE KINASE	ADENOSINE UPTAKE
Guinea-pig	Brain	20^1	20^1
Guinea-pig	Heart	$0.9^{2,a}$	1^3
Rat	Heart	5.9^4	5^3
Rat	Hepatoma cells	$5.8^{5,b}$	10^6
Human	Erythrocyte ghosts	1.9^7	7.5^8
Dog	Erythrocytes	-	190^9
Guinea-pig	Erythrocytes	-	102^{10}

a. Value for dog or rabbit heart

b. Value for Ehrlich ascites tumour cells.

1. Shimizu et al. (1972)
2. Alma & Feinberg (1971)
3. Hopkins & Goldie (1971)
4. De Jong & Kalkman (1973)
5. Lindberg et al. (1967)
6. Plagemann (1970)
7. Meyskens & Williams (1971)
8. Schrader et al. (1972)
9. Kübler & Bretschneider (1963)
10. Roos & Pflieger (1972)

(ii) A simple kinetic interpretation of the roles of adenosine kinase and adenosine deaminase in adenosine uptake

Since there are two enzymes which can metabolize adenosine when it is taken up by the cell, the question arises as to what controls the direction of adenosine metabolism. A simple hypothesis of adenosine metabolism is as follows: adenosine enters the cell by means of a facilitated diffusion mechanism and within the cell it is deaminated or phosphorylated in proportion to the activities of adenosine deaminase and adenosine kinase (Figure 1.4). Since the enzymes have different K_m 's for adenosine, their relative activities will depend on the intracellular concentration of adenosine. (The K_m value of adenosine kinase for adenosine is about 5 μM and that of adenosine deaminase is about 50 μM .) This hypothesis predicts that the direction of metabolism of analogues of adenosine will depend on the V_{max} and K_m values of adenosine kinase and adenosine deaminase for the analogues. Parks & Brown (1973), who studied the direction of metabolism of adenosine analogues by human erythrocytes, confirmed this prediction.

A further prediction of the hypothesis is that by increasing the extracellular concentration of adenosine the ratio, rate of deamination/rate of phosphorylation should be increased. This effect has been demonstrated with rat heart (De Jong, 1972), guinea-pig brain (Shimizu *et al.*, 1972) and human erythrocytes (Meyskens & Williams, 1971; Schrader *et al.*, 1972). (However, it should be noted that alternative hypotheses can give rise to similar predictions - see below.)

(iii) Interpretations of the roles of adenosine kinase and adenosine deaminase based on their intracellular locations

The hypothesis presented in the previous section requires that adenosine kinase and adenosine deaminase metabolize intracellular adenosine. However, intracellular adenosine has been demonstrated only under conditions where the

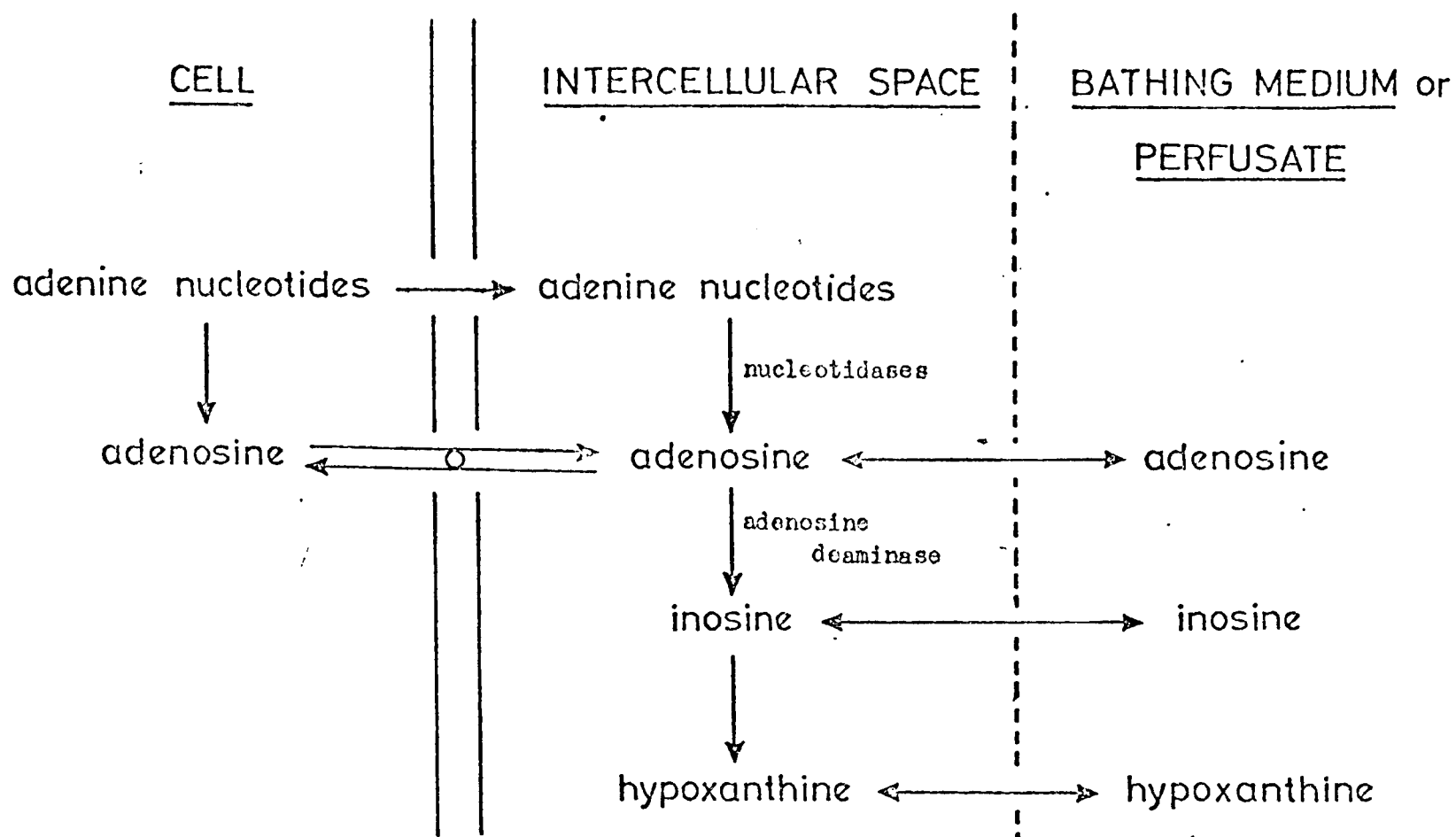


FIG 1.3 The sources and fate of extracellular adenosine.

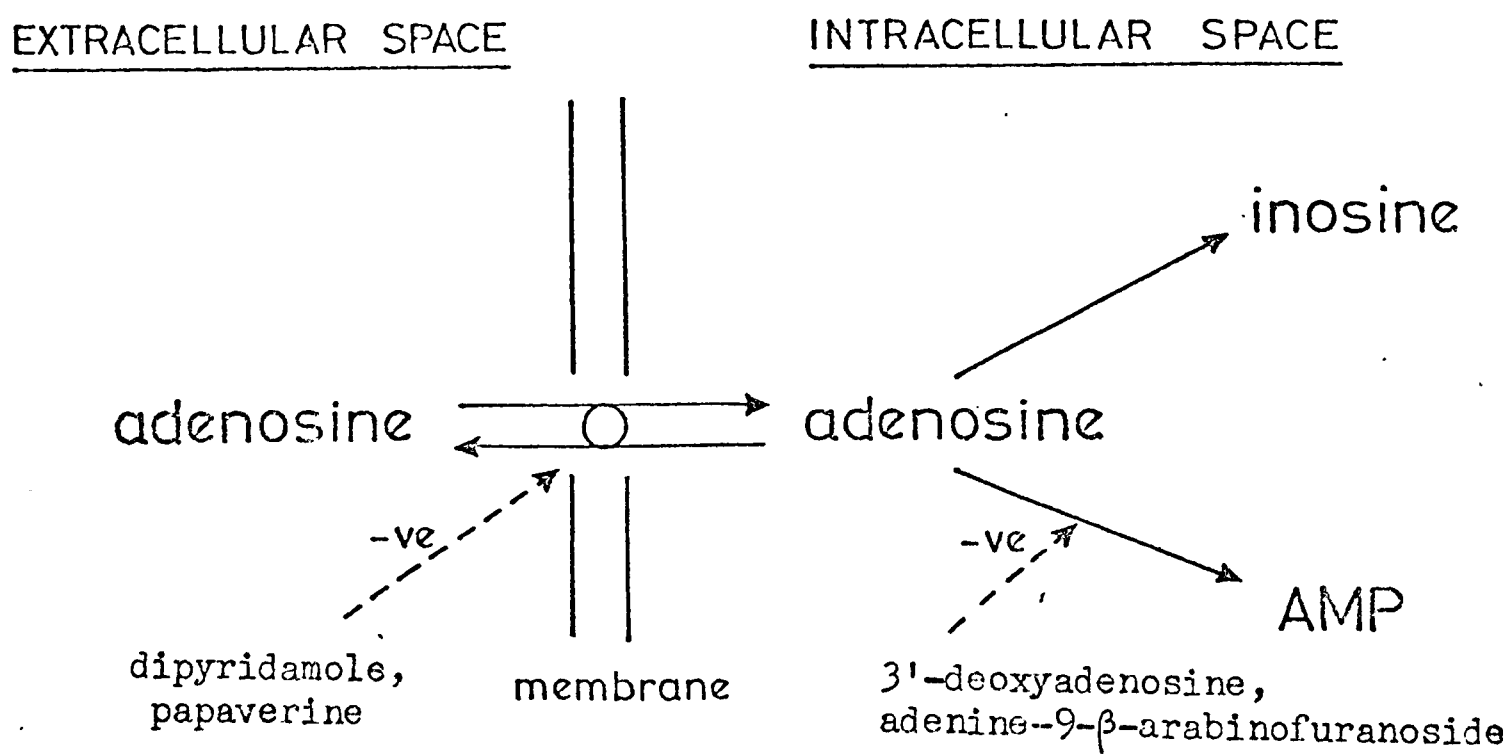


FIG 1.4 The proposed adenosine uptake mechanism.

concentration of extracellular adenosine is very high. It appears that the ratio, rate of deamination/rate of phosphorylation, rises rapidly as the extracellular (rather than the intracellular) concentration of adenosine is raised above the K_m for adenosine kinase. Therefore, adenosine kinase appears to respond to extracellular adenosine concentrations. It is possible that adenosine kinase responds to extracellular adenosine concentrations because it forms part of the transport mechanism. In some cases the saturable uptake mechanism and adenosine kinase have similar K_m values (Table 1.10), but this only suggests that there is a close functional relationship between the uptake mechanism and adenosine kinase. Adenosine kinase would be expected to have a membrane location if it responds to extracellular adenosine concentrations. In practice only a small proportion (about 10%) of the activity of adenosine kinase is associated with the membrane fraction; most of it is in the soluble fraction (De Jong & Kalkman, 1973; Schrader et al., 1972). (Similarly, adenosine deaminase is usually located primarily in the soluble fraction - Baer et al., 1966; Pull & McIlwain, 1974; but see Mustafa & Tewari, 1970a; Mustafa, 1972.) Various workers have suggested that the ratio, rate of deamination/rate of phosphorylation, is less than that which would be expected on the basis of the kinetic constants of adenosine deaminase and adenosine kinase in a tissue (Rubio & Berne, 1969; Liu & Feinberg, 1971; Shimizu et al., 1972). This means that adenosine kinase has access to exogenous adenosine prior to adenosine deaminase, which is consistent with the view that adenosine kinase responds to extracellular adenosine concentrations. However, the data upon which this suggestion is based does not include measurements of the maximal activities of adenosine kinase and adenosine deaminase in crude tissue homogenates.

(iv) Conclusions

The simple kinetic interpretation (Figure 1.4) of the roles of adenosine

kinase and adenosine deaminase in adenosine uptake adequately explains most of the data. The evidence against this interpretation is largely based on the fact that intracellular adenosine has not been demonstrated. However, this probably reflects the lack of a satisfactory method for measuring intracellular adenosine concentrations (see Section B1(b)). It may be noted that adenosine uptake may be inhibited at the level of adenosine transport by dipyridamole (see Schrader et al., 1972; but see Hopkins & Goldie, 1971) or papaverine (Plagemann & Sheppard, 1974) or at the level of adenosine metabolism (Shimizu et al., 1972). The hypothesis is similar to the situation of glucose transport in rat liver (Cahill et al., 1959). Thus the putative adenosine carrier is sufficiently active so that the concentrations on either side of the membrane are very similar. This proposal is necessary since the ratio, rate of deamination/rate of phosphorylation rises as the concentration of extracellular adenosine is increased above the K_m value of adenosine kinase. The roles of adenosine deaminase and adenosine kinase in the uptake of adenosine are discussed further in Chapter 4, Section D1b(ii).

B.5 THE PHYSIOLOGICAL EFFECTS OF ADENOSINE

Since adenosine is both released from and taken up by the cell, the question arises as to the significance of extracellular adenosine. There is evidence that adenosine is of great importance in regulating the physiological activity of a number of tissues. Some of the physiological and biochemical effects of adenosine are described below and their significance in the living animal is then discussed.

(a) THE EFFECTS OF EXOGENOUS ADENOSINE

Drury & Szent-Györgi in 1929 were the first to catalogue the main physiological effects of adenosine. Injection of 1.5 mg of adenosine into a guinea-pig dilated the coronary blood vessels, caused general arterial dilation, slowed the heart rate and thus lowered the arterial blood pressure.

Rabbits became sleepy when they were injected with adenosine and cat intestinal movements were inhibited. Adenosine had no effect on the contraction of tetanized cat skeletal muscle. More detailed studies have since been made of all these effects..

(i) Vascular smooth muscle

Adenosine has been shown to cause vasodilation of the blood vessels of mammalian heart, skeletal muscle, brain, skin and intestine (Wolf & Berne, 1956; Scott et al., 1965; Sutara, 1967; Haddy & Scott, 1968). The blood vessels of the kidney are exceptional in that they contract in response to adenosine (Scott et al., 1965). It is possible that adenosine inhibits the release of a vasodilatory substance, such as a prostaglandin, which is especially important in the kidney (Herbaczynska-Cedro & Vane, 1974; McGriff et al., 1970). No effect of adenosine was found on coronary blood flow through the heart of the turtle, which is the only poikilotherm which has been studied (Nakatsu & Drummond, 1972).

(ii) Non-vascular smooth muscle

Smooth muscle from sources other than blood vessels is also relaxed by adenosine. Thus adenosine inhibits the activity of the isolated taenia coli of guinea-pig caecum (Axelsson & Holmberg, 1969; Burnstock et al., 1970) and relaxes the isolated rabbit intestine (Stafford, 1966). Uterine smooth muscle is an exception, at least in the guinea-pig, since it contracts in response of adenosine (Bennet & Drury, 1931; Deuticke, 1932; Stafford, 1966).

(iii) Cardiac and striated muscle

No effect of adenosine upon the contraction of striated muscle has been reported. However, adenosine has pronounced effects on the rate and force of heart contraction. Thus adenosine temporarily slows or stops the heart beat of the cat, rabbit, guinea-pig and rat (Stafford, 1966; James, 1965; Hopkins, 1973a). Isolated oyster hearts beating in a medium at pH 8.1 or 9.4 are also

slowed or blocked by the presence of adenosine (Aikawa et al., 1967). In contrast rat heart cells in culture beat faster in the presence of adenosine (Seraydarian et al., 1972).

Buckley et al. (1959) have reported that adenosine reduces the force with which the isolated dog heart contracts.

(iv) Cell structure and behaviour

Adenosine has been shown to cause a change in the configuration of the membrane of cultured iris epithelial cells from newts (Ortiz et al., 1973), decrease the susceptibility of ATP-depleted red blood cells to deformation by small pressures (Weed et al., 1969) and inhibit the induction of platelet aggregation by ADP (Born & Cross, 1963; Born et al., 1964).

(v) Nervous tissue

Despite the startling effect of adenosine on brain cyclic AMP levels (see Section b(i)), little is known of the physiological effects of adenosine on nervous tissue. Recently, the sleep-inducing effect of adenosine has been rediscovered in dogs (Haulica et al., 1973), but there is no evidence to show that this is a result of adenosine acting directly on the nerve cells of the brain.

(vi) General metabolic effects

Chagoya de Sanchez et al. (1972, 1974) found that the injection of 200 mg/kg of adenosine into rats led to a large increase in the rate of [^{14}C]-glucose incorporation into liver glycogen without a comparable enhancement in the amount of glycogen. It was calculated that liver glycogen turnover was increased 23-fold. In addition, adenosine caused hyperglycaemia and a diminution of liver fatty acid catabolism.

(b) THE BIOCHEMICAL MECHANISMS WHICH MEDIATE THE PHYSIOLOGICAL EFFECTS OF ADENOSINE

The various physiological effects of adenosine require an explanation in biochemical terms. The changes in metabolite levels induced in a tissue when it is supplied with adenosine and the relevance of the changes to the physiological effects induced by adenosine are now considered.

(i) The effects of adenosine on metabolite levels

Exogenous adenosine may modify the tissue content of ATP, ADP, AMP or cyclic AMP. (In addition, pyrimidine nucleotide levels may be depleted - Ishii & Green, 1973; Green & Chan, 1973).

In the case of red blood cells (Gabrio et al., 1955), guinea-pig brain slices (Thomas, 1957), rabbit heart (Von Mäurer et al., 1969), rat cardiac cells in culture (Seraydarian et al., 1972) and rat liver cells (P. Lund, personal communication), it has been found that adenosine can elevate adenine nucleotide levels. However, De Jong (1972) and Shimizu et al. (1972) could detect no effect of extracellular adenosine on intracellular adenine nucleotide levels in the rat heart and guinea-pig brain slices. Moreover, the elevation of adenine nucleotide levels by adenosine in red blood cells is only seen when the adenine nucleotide levels have first been depleted (Gabrio et al., 1955).

Exogenous adenosine can cause cyclic AMP levels in guinea-pig brain slices to increase by a factor of 30-fold or more. The half-maximal effect is produced by 30 μ M adenosine (Satlin & Rall, 1970). Adenosine also elevates cyclic AMP levels in cultured mouse neuroblastoma cells (Blume et al., 1973), and possibly rat glial cells (Clark & Perkins, 1972; but see Schultz et al., 1972).

The effect of exogenous adenosine on cyclic AMP levels is best established for nervous tissue (see Rall, 1971; Clark et al., 1974). Adenosine (10 μ M)

has been shown to stimulate the conversion of adenine nucleotides to cyclic AMP in platelets (Mills & Smith, 1971). In addition, it has been reported that high concentrations (0.5 mM) of adenosine raise the cyclic AMP content of rabbit colon muscle by 12% (Andersson, 1972). There are also a number of situations in which exogenous adenosine has a similar effect to exogenous cyclic AMP and so it is possible that exogenous adenosine acts by raising cyclic AMP levels in a variety of tissues. Thus cyclic AMP (or dibutyryl cyclic AMP) has similar effects to adenosine in that it stimulates steroid production by adrenal cells (Kowal & Fiedler, 1969), increases the rate of beating of cardiac cells in culture (Seraydarian et al., 1972) and inhibits lipogenesis in liver slices (R.A. Harris, personal communication). Further similarities are described in Robison et al. (1971, Chapter 5). Unfortunately, cyclic AMP levels have not been measured in experiments with adenosine.

It has been suggested above that exogenous adenosine raises cyclic AMP levels in a variety of tissues. However, fat cells are an exception, since adenosine inhibits cyclic AMP accumulation (Fain et al., 1972; Fain, 1973).

(ii) The mechanism by which adenosine affects cyclic AMP levels

Exogenous adenosine may elevate cyclic AMP levels in a number of ways:

- (a) By directly inhibiting phosphodiesterase.
- (b) By directly activating adenyl cyclase.
- (c) A product of adenosine metabolism may inhibit phosphodiesterase.
- (d) A product of adenosine metabolism may activate adenyl cyclase.
- (e) Adenosine may elevate the concentration of the substrate for adenyl cyclase, ATP, and thereby increase adenyl cyclase activity.

Mechanisms (a) and (b) could operate through the action of adenosine on a receptor molecule.

There is little evidence from studies with cell-free systems to support the hypothesis that adenosine directly regulates cyclic AMP phosphodiesterase activity in vivo. Adenosine has been shown to inhibit phosphodiesterase in cell-free systems, but usually only when the concentration of adenosine is unphysiological (i.e. mM). Moreover, phosphodiesterase activity has often been studied at unphysiological substrate concentrations. Results of these studies are presented in Table 1.11.

However, Schultz & Daly (1973b) have found that, in brain slices, adenosine not only stimulates adenyl cyclase to produce more cyclic AMP but also reduces the rate at which phosphodiesterase utilizes it. This could result from an elevation of ATP levels in these slices, since ATP is an inhibitor of phosphodiesterase (Section A.1b(ii)).

When guinea-pig brain slices were pulse-labelled with [^{14}C]-adenine and then incubated with 0.1 mM [^3H]-adenosine, both labels were found in the cyclic AMP (Shimizu & Daly, 1970; Schultz & Daly, 1973a). The fact that a small proportion of cyclic AMP was derived from adenosine has been used as evidence that adenosine stimulates cyclic AMP formation because it is converted to, and thereby increases the concentration of, the adenyl cyclase substrate, ATP. However, the present author does not believe that this conclusion is necessary to explain the incorporation of adenosine into cyclic AMP. Indeed, a comparison of the K_m of adenyl cyclase and ATP levels (Section A.1(i)) indicates that the enzyme is nearly always saturated with substrate in vivo, so that it is not responsive to changes in substrate concentration. It is possible that the in vivo K_m of adenyl cyclase is much higher than that consistently measured in vitro, or that the ATP in the pool supplied by adenosine and supplying adenyl cyclase is at a relatively low concentration. There is evidence that adenosine (or adenine) enters a pool of ATP in the brain which constitutes 20 to 30% of the total ATP (Pull &

TABLE 1.11 Inhibition of phosphodiesterase by adenosine

ANIMAL	TISSUE	CONCENTRATION OF CYCLIC AMP IN ASSAY (μ M)	ADENOSINE CONCENTRATION (mM)	PERCENT INHIBITION OF PHOSPHO- DIESTERASE
Toad ¹	Bladder	30	5	52
Human ²	Platelets	20	3	27
Ox ³	Heart	1000	1	10
Mouse ⁴	Brain	300	10	19
Guinea-pig ⁵	Cerebral cortex	0.0055	0.5	26 ^a
			0.05	17 ^b
Rat ⁶	Epidydymal fat cells	0.001 to 0.005	0.07	26
			0.017	12

The inhibition of phosphodiesterase by adenosine was, in each case, studied in a cell free system.

a. Value for supernatant enzyme.

b. Value of particulate enzyme.

1. Gulyassy (1971)
2. Mills & Smith (1971)
3. Goren & Rosen (1971)
4. Roberts & Simonsten (1970)
5. Sattin & Rall (1967)
6. Fain et al. (1972)

McIlwain, 1972c; Shimizu et al., 1970; Schultz & Daly, 1973a,b,c; Shimizu & Okayama, 1973). However, if this pool is as large as 20% of the total ATP of brain, an increase in the pool size in the presence of adenosine should be detectable - particularly if these changes are to lead to a three- to four-fold increase in cyclic AMP production, as suggested by Shimizu & Daly (1970). (Other calculations have suggested that the pool size is about 5% of the total ATP - see McIlwain, 1971, 1972).

In the experiment described in the above paragraph adenosine stimulated the rate of conversion of $[^{14}\text{C}]\text{-ATP}$ to $[^{14}\text{C}]\text{-cyclic AMP}$. This is clear evidence that adenosine activates adenyl cyclase in guinea-pig brain slices. However, it has not been possible to demonstrate that adenosine activates adenyl cyclase in broken cell preparations of guinea-pig brain or any other tissue (Sattin & Rall, 1970; Morwaki & Foa, 1970; Weinryb & Michel, 1974).

There is no evidence that any product of adenosine metabolism or adenosine itself, activates adenyl cyclase in broken cell preparations. It has therefore been suggested that adenosine affects adenyl cyclase through a receptor, and that this receptor is separated from the enzyme when cells are disrupted. There have been no reports of the isolation of an adenosine receptor or of adenosine binding to a putative receptor but nevertheless it is widely believed that such a receptor is present in the brain (Sattin, 1971; Schultz & Daly, 1973c). The putative receptor is apparently blocked by theophylline and caffeine, which prevent the adenosine induced rise in cyclic AMP levels in guinea-pig cerebral cortex slices.

(iii) Correlation of the biochemical and physiological effects of adenosine

At this stage it is possible to consider whether the changes in metabolite levels which are caused by exogenous adenosine can explain the physiological effects of adenosine. If a physiological effect results from

the interaction of adenosine with an extracellular receptor, it cannot be mediated by the conversion of adenosine to adenine nucleotides. In this case the drugs dipyridamole, papaverine and hexobendine, which inhibit the uptake of adenosine by various tissues (Section B.4c(iv); Hopkins, 1973b), should accentuate the effect. Thus these drugs accentuate the effects of adenosine in dilating coronary blood vessels (Schaumann et al., 1970) and in inhibiting platelet aggregation induced by ADP (Jarret et al., 1972; Born & Mills, 1969). It is possible that the extracellular receptor of platelets with which adenosine interacts is simply the receptor for ADP. It has been suggested that adenosine influences platelet aggregation by elevating cyclic AMP levels (Mills & Smith, 1971).

Exogenous adenosine causes cyclic AMP levels to rise in smooth muscle and elevated levels of cyclic AMP have been correlated with smooth muscle (including vascular smooth muscle) relaxation (Andersson, 1972; Andersson et al., 1972; Somlyo et al., 1972; Pöck & Kukovetz, 1972; Dunham et al., 1974). It is therefore possible that the effect of adenosine on smooth muscle relaxation is mediated by elevated cyclic AMP levels. However, it must be remembered that this suggestion hinges upon the results of one study (Andersson, 1972) in which only small rises (12%) in cyclic AMP levels in response to exogenous adenosine were reported.

Uterine smooth muscle is different from other smooth muscles in that it contracts in the presence of adenosine (Section B5a(ii)) and in that it can be made to contract with little changes in cyclic AMP levels (Polacek et al., 1971; Vesin & Harbon, 1974).

Red cell deformability is only affected by exogenous adenosine when the ATP levels of the cell are previously depleted, and so the effect of adenosine presumably results from its repletion of the ATP. Similarly, the metabolic changes in a rat following the injection of adenosine are claimed to result

from increased ATP levels in the liver. It is not possible to suggest why adenosine should slow the heart beat or cause sleep. In view of the effect of the phosphodiesterase inhibitor caffeine to increase alertness, it seems unlikely that sleep results from an elevation of cyclic AMP levels in the brain.

(c) THE ROLE OF ADENOSINE IN THE LIVING ANIMAL

With one exception, that of the autoregulation of blood flow, there is little evidence that adenosine plays any role in the living animal which corresponds to the physiological effects described in Section (a) above. Surprisingly nothing is known of the formation of adenosine by platelets and other blood cells, so that it is not known whether adenosine regulates platelet aggregation in the living animal. The biochemical effect of exogenous adenosine in raising the level of cyclic AMP in the brain has led to the suggestion that adenosine plays a neurohormonal role (see below).

(i) The role of adenosine in regulating the structure of red blood cells

The finding that the adenosine moiety of adenine nucleotides can be transferred from liver to red blood cells (Lerner & Lowy, 1974) suggests that adenosine might regulate the structural properties of red blood cells in the living animal (see Section a(iv)).

(ii) The role of adenosine in sleep and coma

Glucose lack leads to increased rates of adenosine output by guinea-pig cerebral cortex slices (Pull & McIlwain, 1972a). The injection of adenosine into rabbits and dogs causes sleepiness. Since glucose lack leads to coma in vivo, it is possible to speculate, assuming coma and sleep are related phenomena, that the production of coma by glucose lack is mediated by elevated adenosine levels in the brain (Pull & McIlwain, 1972a). Similarly,

the deprivation of paradoxical sleep leads to increased levels of adenosine in rat brain (Haulica et al., 1973). Therefore the sleepiness caused by the deprivation of paradoxical sleep may be mediated by adenosine. These theories are very speculative.

(iii) The role of adenosine in the autoregulation of blood flow

When the supply of oxygen to a tissue is reduced or the requirements of the tissue for oxygen rise, the vasculature dilates with the result that the tissue receives an increased supply of blood and therefore of oxygen (see Haddy & Scott, 1968). This phenomenon is known as autoregulation. Berne (1963) proposed that a reduced supply of oxygen to a tissue results in the release of adenosine and that this adenosine in some way causes the tissue vasculature to dilate. The following evidence supports this theory.

- a. The quantities of adenosine released from the isolated perfused cat heart are roughly proportional to the degree of cardiac hypoxia imposed (Katori & Berne, 1966).
- b. When dog myocardial adenosine levels are varied by varying the heart's oxygen supply, coronary blood flow varies, and there is a quantitative relationship between the adenosine content of the heart and blood flow (Olson, 1969).
- c. The drug dipyridamole, which raises the adenosine content of the heart, causes vasodilation (Bunag et al., 1964).
- d. A degree of hypoxia which leads to maximum coronary artery dilation also leads to the release of adenosine such that the extracellular adenosine concentration is greater than that which is known to cause maximum coronary artery dilation (Rubio et al., 1969).
- e. Evidence has been presented that adenosine release from dog heart occurs under normal conditions, and small reductions in oxygen supply which occur in the normal course of an animal's existence lead to the release of vasodilatory

amounts of adenosine (Rubio & Berne, 1969; Berne et al., 1971).

f. It was pointed out in Section B3(a) that extracellular adenosine may be released from the cell as adenine nucleotides. AMP, ADP and ATP increase coronary blood flow in the perfused dog heart in situ (Wolf & Berne, 1956). Therefore, the evidence discussed above is consistent with the view that adenine nucleotides, rather than adenosine, mediate the autoregulation of blood flow. However, no release of adenine nucleotides by heart muscle has been reported and the release of adenine nucleotides by skeletal muscle is disputed (Dobson et al., 1971; Berne et al., 1971).

It is noticeable that most of the evidence discussed relates to mammalian hearts, in particular the dog. The role of adenosine in skeletal muscle is less well established and there is virtually no evidence to suggest that adenosine plays a role in the autoregulation of blood flow in non-mammalian tissues or in nervous tissues (but see Berne & Curnish, 1972).

Adenosine is not the only compound which has been proposed to play a role in the regulation of blood flow. Prostaglandins are also involved in this function (McGriff et al., 1970; Kadowitz et al., 1972). There are other vasoactive chemicals, such as H^+ and K^+ , which may play some role in the autoregulation of blood flow but quantitative studies indicate that their role is only minor (see Haddy & Scott, 1968).

(iv) The role of adenosine in neurotransmission

Electrical stimulation and depolarizing agents cause the accumulation of cyclic AMP in guinea-pig cortical slices. Adenosine appears to mediate some of these effects (see Huang & Daly, 1974). Since cyclic AMP plays an important role in the brain, it has been suggested that adenosine has a neurohormonal role in vivo. The neurohormonal role may be additional to a role in the regulation of blood flow (McIlwain, 1972).

Further insights into the role of adenosine may be provided by measuring the maximal activities, and examining the properties, of the enzymes which regulate its tissue concentration. In Chapter 4 of this thesis this approach is used with muscle, liver and nervous tissues.

CONCLUSIONS

The evidence presented in Section B of this chapter indicates that adenosine is produced within the cell and subsequently released from it, so that the physiological state of the cell may be communicated to other cells. It is unclear what responses adenosine elicits in receptive tissues of living animals except for the smooth muscle of mammalian heart vasculature, which is induced to relax. Nor is it clear how adenosine brings about any response in a receptive tissue. However, it is suggested that extracellular adenosine produces some effects by modifying the cyclic AMP metabolism of the tissue.

Chapter 2

DEVELOPMENT OF RADIOCHEMICAL ASSAY METHODS

A variety of methods have been employed previously to measure the activities of the enzymes investigated in the present work. None of these methods were considered to be entirely suitable for the present study. This chapter will contain a description of how these methods have been modified to provide satisfactory assays for the work in this thesis. The chapter also includes the results of preliminary tests of homogenization and assay methods (including pH curves).

CRITERIA USED IN DEVELOPING THE ASSAYS

Most enzyme assays described in the literature have been developed for the purpose of studying the properties of an enzyme from one particular tissue. The enzyme is frequently purified to reduce the activity of other enzymes which interfere with the assay. The present investigations impose limitations upon the types of assays which may be employed for three main reasons. Firstly, the approach is largely comparative so that assay methods are needed which are applicable to enzymes in homogenates of very different tissues. Secondly, in some cases the maximal activities of these enzymes are required so that they must be measured under optimal conditions. Thirdly, some of the tissues investigated are very small (weighing as little as one mg). For all these reasons, it is necessary that the enzymes are assayed in crude tissue homogenates. Any purification or fractionation of an enzyme could lead to the removal of cofactors or alter the structure of the enzyme in such a way as to alter its properties or activities. Furthermore, if only a particular fraction of the homogenate is assayed, variations in maximal catalytic capacities between tissues may merely reflect variations in the proportion of the enzyme in that fraction of a tissue. When only milligram

quantities of material are available, enzyme fractionation or purification is also difficult for technical reasons. In a comparative study, the time that would be needed to overcome these objections in the case of each tissue studied would be out of all proportion to the additional information acquired.

The approach to each method is as follows. The main problems to be overcome in each assay are briefly summarized. Each problem is then considered in turn and the methods used by other workers to overcome the problem are compared with the methods used in this study. Finally, the graphical methods used to analyze the results and the significance of the results obtained are discussed. The basic assay methods will, however, be summarized first, in order that the tests used during their development can be understood.

A. A SUMMARY OF THE ENZYME ASSAY METHODS

A.1 DISSECTION AND HOMOGENIZATION OF TISSUES

All animals were killed by decapitation or cervical dislocation. Rats and mice were anaesthetized and lobster and scallops were cooled in ice before killing. All tissues were immediately dissected from the dead animal and kept on ice before weighing.

The tissues were homogenized in hand-operated glass homogenizers of the Potter-Elvehjem type with 10-50 volumes of ice-cold medium. Any further dilution of the homogenate which was necessary before assay was brought about by the addition of extraction medium. The composition of the extraction media were as follows.

For the adenylyl cyclase and phosphodiesterase assays	Tris chloride, pH 7.5	40 mM
	MgCl ₂	8.7 mM
	Dithiothreitol	1 mM
	Bovine serum albumin	1mg/ml
For the adenosine kinase and adenosine deaminase assays	MgCl ₂ }	1 mM
	EDTA } pH 7.0	1 mM
For the 5'-nucleotidase assay	Tris-maleate, pH 7.0	10 mM
	EDTA	1 mM

In addition 0.1% Triton X-100 was included in the extraction medium for most assays of adenosine kinase, adenosine deaminase and 5'-nucleotidase, and for assays of phosphodiesterase in nervous tissues (see also Chapter 3, Section B?).

Adenosine may accumulate in ischaemic tissues following death (see Tables 1.1 and 1.2) and in tissue homogenates. This endogenous adenosine would dilute the specific radioactivity of the adenosine added for the assay of adenosine kinase and adenosine deaminase. These assays were therefore carried out as soon as possible following the death of the animal.

A.2 SUMMARY OF THE ADENYL CYCLASE ASSAY

It was essential that all possible products of side reactions were separated from the product, cyclic AMP, and the substrate, ATP, of the adenylyl cyclase reaction. This was made possible by the use of a thin-layer chromatography system which is described in Section A.6.

The details of the assay, as used in Chapters 3 and 5, are given below. The experiments carried out to test and develop the method are described in Section B of this chapter.

ASSAY MEDIUM

The concentrations of the components of the final incubation medium were as follows.

Tris chloride, pH 7.5	32 mM
MgCl ₂	7.6 mM
EDTA	0.6 mM
Cyclic AMP	2.5 mM
Dithiothreitol	1.8 mM
Creatine phosphate	16 mM
Creatine phosphokinase	0.8 mg/ml (about 14
[2- ³ H]-ATP, ammonium salt	80 μ Ci/ml Units/ml)
Mg-ATP	0.1 to 5 mM
Inorganic pyrophosphate	0.01 Units/ml
Bovine serum albumin	0.3 mg/ml

In some experiments ATP labelled in the 8 position was used as substrate. Inorganic pyrophosphatase was only added in assays where the homogenate was highly diluted.

THE METHOD

The assay was performed in a total volume of 20 μ l at 30°C in glass test tubes (50 x 6 mm). The assay was started by adding 6 μ l of tissue homogenate to 14 μ l of the assay medium. The time of the assay varied from 3 to 20 minutes. The shorter times were used at the lowest substrate concentrations. (At low ATP concentrations it is difficult to maintain ATP levels, but the rate at which radioactivity appears in the product is greatest because the specific activity of the ATP is highest.) The assay was terminated by adding 3 μ l of a 20% perchloric acid solution containing approximately 7 mM adenine, adenosine, inosine, ADP and AMP. These compounds acted as carriers for the labelled compound and also enable the position of the purine derivatives on the chromatogram to be visualized.

Two controls, in which the perchloric acid solution was added before the homogenate, were incubated for each chromatogram.

The tubes were centrifuged at 2000 g for three minutes and the supernatant was applied to the chromatogram within an hour (see Section A.6).

Modification of the method to purify [^{3} H]-ATP

It was found that some batches of [^{3} H]-ATP contained some labelled cyclic AMP. In these cases the blank was reduced by pre-incubating the [^{3} H]-ATP with a commercial phosphodiesterase preparation. Suitable conditions were as follows: 0.2 μ l of commercial phosphodiesterase (15 Units/ml) were incubated at 30°C with 100 μ l of assay medium from which cyclic AMP was omitted. After 30 to 45 minutes a concentrated cyclic AMP solution was added to give a final concentration of 3.5 mM cyclic AMP in the assay. Aliquots of the mixture were immediately added to each tube and the assay was started.

(The development of this technique is discussed in Appendix B.)

A.3 SUMMARY OF THE CYCLIC AMP AND CYCLIC GMP PHOSPHODIESTERASE ASSAYS

Phosphodiesterase was assayed by a coupled radiochemical method. The product of the phosphodiesterase reaction, AMP, was hydrolyzed to adenosine by excess 5'-nucleotidase. Adenosine was separated from the substrate, cyclic AMP, using an anion exchange resin.

ASSAY MEDIUM

The concentrations of the components of the final incubation medium were as follows.

Tris chloride, pH 7.5	50 mM
MgCl ₂	6 mM
Dithiothreitol	2.5 mM
<u>Crotalus atrox</u> venom or 5'-nucleotidase	0.05 mg/ml
[8- ³ H]-cyclic AMP or [8- ³ H]-cyclic GMP	1.2 μ Ci/ml
Cyclic AMP	0.044 to 460 μ M
or Cyclic GMP	0.050 to 770 μ M
Bovine serum albumin	0.23 mg/ml

The concentration of 5'-nucleotidase was reduced for assays of 30 minutes or longer duration.

THE METHOD

The assay was performed in a total volume of 130 μ l at 30°C in plastic test tubes (60 x 8 mm). The assay was started by adding 30 μ l of tissue homogenate to 100 μ l of the assay medium. The assay was terminated after 15 to 60 minutes. Two methods were used to terminate the reaction and separate the substrate from the products. These methods were as follows.

Separation Method (1)

The reaction was terminated by the addition of 75 μ l of a solution containing 50 mM EDTA, 5 mM adenosine and 2 mM inosine, pH 4.5 (cyclic AMP assays) (or 50 mM EDTA, 1 mM guanosine - cyclic GMP assays). The whole

mixture was applied to a column (2.5 cm x 0.5 cm) of Bio-Rad-AG1 ion exchange resin (chloride form) and washed through with 4 ml of water. Adenosine was eluted from the column whereas cyclic AMP remained bound. Radioactivity in the eluate was measured as described in Appendix A.

Separation Method (ii)

The reaction was terminated by adding 1.2 ml of a 1:1 slurry of Bio-Rad-AG1 (chloride form) to each test tube. The tubes were mixed three times over a period of about half an hour and they were then centrifuged briefly. Radioactivity in an aliquot (0.5 ml) of the supernatant was measured as described in Appendix A.

When the assay was performed at a number of substrate concentrations several controls were included, especially at the highest and the lowest substrate concentrations. (The control value varied with the substrate concentration.) Extraction medium, rather than the homogenate was added to the controls for the duration of the experiment. In this way it was possible to correct for any phosphodiesterase activity contaminating the 5'-nucleotidase.

The total radioactivity which would be measured in the product if all the substrate was converted to product was usually estimated by adding 2 μ l of a commercial phosphodiesterase preparation to a tube containing 0.2 μ M substrate, and incubating for 15 minutes. In this way it was possible to be certain that the reaction had reached completion. (Incubations for more than about an hour often resulted in an appreciable proportion of the product being degraded to compounds which bound to the resin.)

When separation method (i) was used an alternative measurement of total radioactivity was available: the radioactivity remaining on the column could be eluted with 0.2 N HCl.

A.4 SUMMARY OF THE ADENOSINE KINASE AND ADENOSINE DEAMINASE ASSAYS

The assays for adenosine kinase and adenosine deaminase were performed

simultaneously. Substrates and products were separated by thin-layer chromatography (Section A.6).

ASSAY MEDIUM

The concentrations of the components of the final incubation medium were as follows.

Citric acid	} pH 7.5 or pH 5.3	3.3 mM*
Na ₂ HPO ₄		77 mM*
MgCl ₂		4 mM
EDTA		0.4 mM
Creatine phosphate		8 mM
Creatine phosphokinase		0.2 mg/ml (about 3 Units/ml)
Myokinase		0.003 mg/ml (about 1 Unit/ml)
ATP		4 mM
[³ H]-Adenosine-(G)		40 μCi/ml
Adenosine		0.2 to 200 μM

* The concentrations of the components of the buffer depended upon the pH. The concentrations listed are for pH 7.5. At pH 5.3 the incubation medium contained 19 mM citric acid and 46 mM Na₂HPO₄.

THE METHOD

The assays were performed simultaneously in a total volume of 20 μl at 30°C in glass test tubes (50 x 6 mm). The assay was started by addition of 6 μl of tissue homogenate to 14 μl of the assay medium. Tubes were incubated for between 3 and 15 minutes. (The shortest times were used at the lowest adenosine concentrations.) The assay was terminated by addition of 3 μl of a 20% perchloric acid solution containing approximately 7 mM adenine, adenosine, hypoxanthine, inosine, cyclic AMP, ADP and AMP. The tubes were centrifuged at 2000 g for about 3 minutes and the supernatant was applied to the chromatogram within an hour (see Section A.6).

A.5 SUMMARY OF THE 5'-NUCLEOTIDASE ASSAY

A radiochemical assay of 5'-nucleotidase requires the separation of the substrate, AMP, from the product, adenosine, and any products of side reactions. Thin-layer chromatography was used for this purpose.

ASSAY MEDIUM

The concentrations of the components of the final incubation medium were as follows.

Tris-maleate buffer, pH 7.0	50 mM
MgCl ₂	10 mM
EDTA	0.4 mM
Dithiothreitol	1 mM
Adenosine	3 mM
[8- ¹⁴ C]-5'-AMP, ammonium salt	3.7 μ Ci/ml
5'-AMP	50 to 800 μ M

THE METHOD

The method was analogous to that used for the assay of adenosine kinase and deaminase, except that the reaction was usually terminated after 10 minutes and adenosine was omitted from the perchloric acid solution used to stop the reaction.

A.6 SUMMARY OF THE THIN-LAYER CHROMATOGRAPHY METHOD

Purine nucleotides were separated from adenine, adenosine, hypoxanthine, cyclic AMP and inosine by thin-layer chromatography. A silica-coated plastic sheet (10 x 20 cm), impregnated with a fluor absorbing at 254 nm, was used. The purine derivatives appeared as blue spots on a green background.

Assays were carried out as described in Sections A.2, A.4 and A.5. A 10 μ l portion of the supernatant was taken from each tube with a microcap and applied to a 9 to 11 mm line, 13 mm from the base of the chromatogram.

Each 10 μ L portion was applied in three to five stages, separated by a period of drying, in order to produce a thin line of solutes at the origin of the chromatogram. (Each spot was separated from the next by a distance of 8 mm.) In this way 10 or occasionally 11 spots were applied to each chromatogram.

The chromatograms were developed in a solvent containing isopropanol, ethyl acetate, water and 0.880 ammonia (58:24:10:8). Ascent of the solvent was complete in one to one and a half hours. In order to obtain completely separated and compact spots an initial development of 5 to 10 minutes was employed. The chromatogram was then dried in a stream of cold air before the main development of the chromatogram.

The chromatogram was dried with air and then at 50 to 80°C in an oven and was cooled in a dessicator in order to ensure that no quenching of the radioactivity by adsorbed water could occur.

Spots were visualized under ultraviolet (254 nm) light and cut out with scissors. The radioactivity in each spot was measured as described in Appendix A.

Precise r.f. values varied between experiments. Typical values are given below.

<u>Compound</u>	<u>r.f.</u>
ATP, ADP, AMP, ITP, IMP, GTP, GMP	0.03
Cyclic GMP*	0.12
Inosine	0.24
Cyclic AMP	0.35
Hypoxanthine	0.43
Adenosine	0.56
Adenine	0.64

* An attempt to use the chromatography system for the assay of guanyl cyclase is described in Appendix C.

QUENCHING

It was found that radioactivity in the spot which remained at the origin of the chromatogram was quenched by about 10% compared to radioactivity in other parts of the chromatogram. This difference resulted from the application of perchloric acid at the origin. Measurements of radioactivity were corrected for this variable quenching.

B. DEVELOPMENT OF THE ADENYL CYCLASE ASSAY

The methods used in the assay of adenylyl cyclase have been reviewed by Robison et al. (1971) and Perkins (1973) and the problems of the assay have been discussed in detail by Barr & Hechter (1969a) and Drummond & Duncan (1970). Two major problems are presented by the assay: very low activities of adenylyl cyclase must be detected in the presence of relatively large activities of enzymes which remove both substrate and product; detection of small amounts of cyclic AMP is difficult. The development of an assay which overcame each problem is presented below.

B.1 THE PROBLEM OF SUBSTRATE BREAKDOWN

Adenylyl cyclase is membrane bound and is relatively unstable, so that it has not been purified to any large extent. Thus adenylyl cyclase is usually studied in whole homogenates or washed membrane fractions. In these preparations the activities of ATPase are considerable, so that the concentration of ATP is rapidly reduced.

It is possible to overcome this problem by using very high initial ATP concentrations, so that adenylyl cyclase remains saturated with ATP throughout the assay. However, in radiochemical assays the commercially available [^3H]-ATP is often contaminated with [^3H]-cyclic AMP, so that the use of high ATP concentrations increases the amount of [^3H]-cyclic AMP in the assay, i.e. the radioactivity in the blank is large. Changes in the concentration of ATP

during the assay will cause large variations in the Mg:ATP ratio which interfere in the cyclase assay.

More commonly, ATP regenerating systems are used in an attempt to "buffer" the ATP concentration, e.g. creatine phosphate and creatine phosphokinase or phosphoenolpyruvate and pyruvate kinase. However, the pyruvate kinase system cannot be used under all conditions. (K^+ must be present to activate pyruvate kinase and high concentrations of Ca^{2+} (2 mM) inhibit the enzyme - Drummond & Duncan, 1970.)

Another approach has been to use the analogue of ATP, 5'-adenyl imidophosphate, which is a substrate for the adenylyl cyclase of rat liver plasma membranes but is a poor substrate for ATPase (Rodbell et al., 1971).

In the present study it was particularly important to minimize the breakdown of ATP, since the degradation products of ATP could interfere with the estimation of cyclic AMP. Moreover, the K_m of the enzyme was measured in many experiments and so it was not possible to use saturating ATP concentrations. The use of an analogue of ATP to measure adenylyl cyclase activities is not desirable in a comparative study: adenylyl cyclase from different sources may exhibit different activities towards the analogue. Consequently, a regenerating system for ATP was used. The creatine phosphate-creatine phosphokinase system was chosen.

The effectiveness of the regeneration system (16 mM creatine phosphate, 0.8 mg/ml creatine phosphokinase) was tested by measuring the concentrations of ATP, ADP and AMP during the course of a radiochemical assay for mouse brain adenylyl cyclase. The assay system was basically that described in Section A.2 except that the reaction was terminated by boiling for 2.5 minutes. ATP, ADP and AMP were separated from each other and nucleosides by ascending chromatography on PEI-cellulose thin layers using 0.4 M formic acid, 0.25 M LiCl as solvent (Leech, 1973). Radioactivity in each nucleotide was measured. The

effect of the regenerating system was compared with the effect of 6.5 mM NaF, an inhibitor of ATPase.

The time course of changes in ATP, ADP and AMP concentrations are shown in Figure 2.1. The regeneration system prevented reduction in the ATP concentration, although there was a small linear increase in AMP and ADP concentrations. Furthermore, the AMP and ADP impurities in the commercial ATP were removed by the regeneration system even before the assay was started. NaF was far less effective than the regenerating system in maintaining ATP levels.

It was clearly impractical to test the ATP-regenerating system during the assay of adenyl cyclase activity from all the different animals and tissues studied. Instead a simple alternative was introduced: the time course of cyclic AMP formation was checked at the lowest and highest ATP concentrations employed in the cyclase assay. Linear time courses at both concentrations indicated that ATP breakdown was not interfering in the assay.*

B.2 THE PROBLEM OF PRODUCT BREAKDOWN

It is difficult to achieve a complete separation of adenyl cyclase from phosphodiesterase, so that cyclase activity must be assayed in the presence of the enzyme that will hydrolyze cyclic AMP.

* An important technical point arose from these studies. If the activity of creatine kinase is in excess of the activity of the ATPases and if the activity of adenylate kinase in the assay system is high, the relative concentrations of adenine nucleotides in the assay are a function of the creatine phosphate:creatine ratio. If this ratio remains high throughout the assay, the concentration of ATP is not decreased and that of AMP is not increased. Under these conditions, the activities of AMP deaminase and 5'-nucleotidase become important in reducing the ATP levels. In this study the regenerating system was least efficient when cockroach flight muscle adenyl cyclase was assayed; this tissue has a very high activity of 5'-nucleotidase - see Table 4.5.

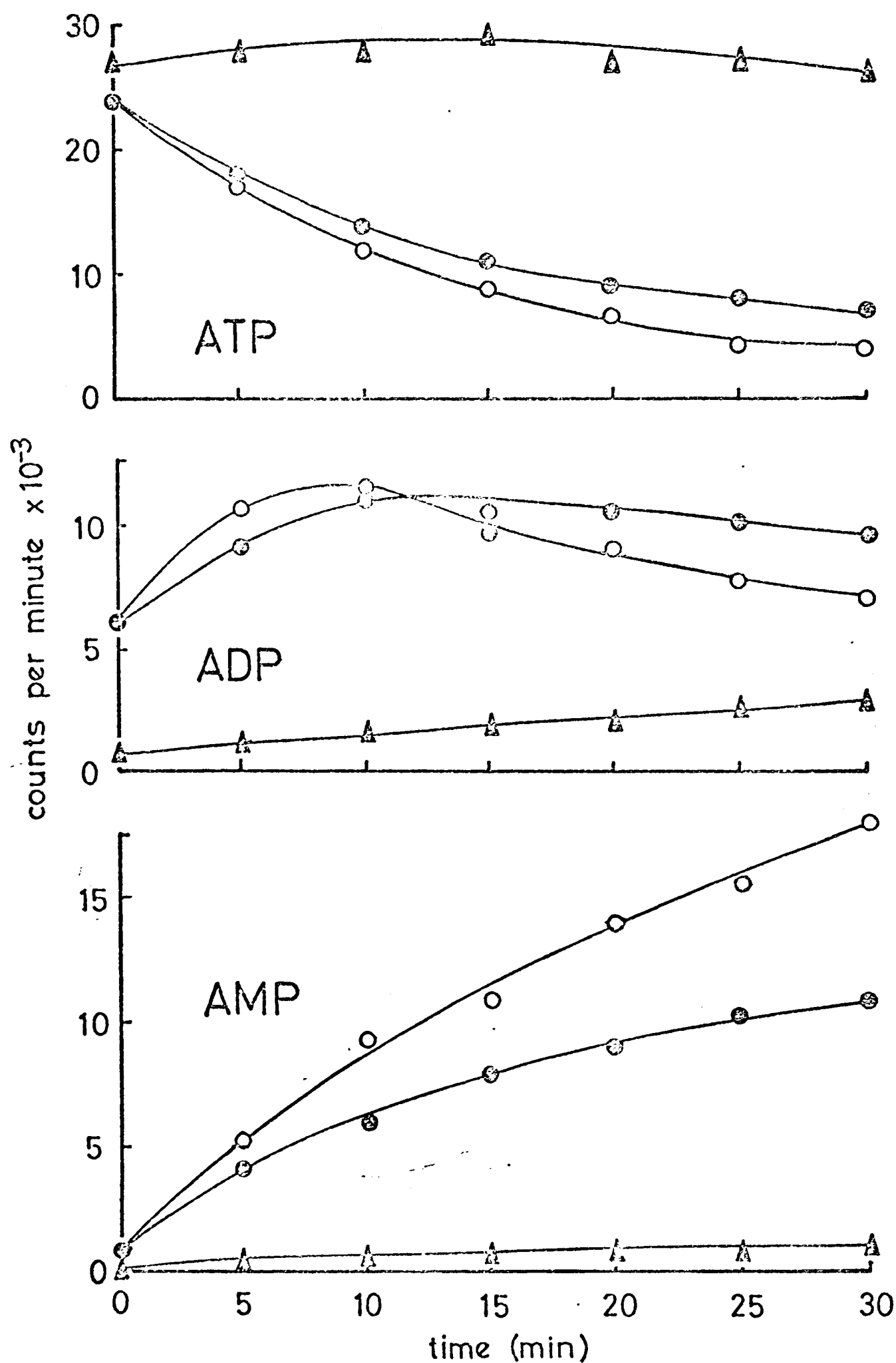


FIG 2.1 The effect of an ATP-regenerating system and NaF on the levels of adenine nucleotides during the adenyl cyclase assay.

Methods are described in section B1. The timecourses of changes in the concentrations of AMP, ADP and ATP are shown for assays performed in the presence of creatine phosphate and creatine kinase (▲), and NaF (●) and for assays performed in the absence of these compounds (○).

However, from the experience of the author, the magnitude of this problem is sometimes overstated in the literature. Although phosphodiesterase activity in tissue homogenates is one to two orders of magnitude higher than adenylyl cyclase (Perkins, 1973), the phosphodiesterase activities are measured at very high cyclic AMP concentrations. At the low concentrations of cyclic AMP produced in the cyclase assay the diesterase activity is much lower and the problem is much less severe. For example, from the data of Table 3.3 it can be calculated that in the assay of mouse brain adenylyl cyclase using 1 mM ATP as substrate, the rate of hydrolysis of product will only approach its rate of formation when about 1% of the ATP has been converted to cyclic AMP. This represents a very high percentage conversion of substrate to product in the adenylyl cyclase assay. In view of this it is not surprising that Albano et al. (1973) found no increase in adenylyl cyclase activities in the presence of a phosphodiesterase inhibitor. However, other workers have observed an effect of phosphodiesterase inhibitors (for example, Birnbaumer et al., 1969). Consequently, it is usual to include either methyl xanthines in adenylyl cyclase assays, or a high concentration of unlabelled cyclic AMP in a radiochemical assay (see Perkins, 1973). The latter measure has been claimed to be more effective (Birnbaumer et al., 1969).

Although high concentrations of methyl xanthines are sometimes included in the assays, they do not inhibit phosphodiesterase activity completely and, in some cases, adenylyl cyclase activity is inhibited (see Perkins, 1973). Furthermore, it is not known if phosphodiesterase from different tissues and a variety of species is inhibited by methyl xanthines. Therefore the inclusion of unlabelled cyclic AMP as a "radioactive trap" was preferred in the assay developed for this study. There is some evidence that cyclic AMP does not inhibit adenylyl cyclase activity (Weinryb et al., 1973).

B.3 THE DETECTION OF CYCLIC AMP FORMED IN THE ASSAY

In the early work on adenylyl cyclase the enzyme was assayed by measuring cyclic AMP using a biological assay. This was very tedious and non-reproducible. More recent studies have used the newer developments in the techniques for measurement of cyclic AMP (e.g. protein binding cyclic AMP assays; radioimmunoassays - see Albano et al., 1973). The advantage of such assays is that it is unnecessary to separate the product from the substrate. There are, however, a large number of reports in which the activity of adenylyl cyclase has been measured by following the formation of labelled cyclic AMP from labelled ATP. In these assays it is necessary to separate the product from the substrate as well as any labelled products of side reactions. The use of [^{32}P]-ATP labelled in the α -position limits the separation to that of cyclic AMP from adenine nucleotides. On the other hand if [^3H]-ATP (labelled in the adenine base) is used, cyclic AMP must be separated from adenine nucleotides, nucleosides and purines which can be formed from ATP degradation.

Cyclic AMP can be separated from these other labelled compounds by a variety of techniques (e.g. thin-layer and column chromatography, electrophoresis). A useful precipitation stage can be included in these separations: protein and all nucleotides except cyclic AMP are precipitated by $\text{Ba}(\text{OH})_2$ and ZnSO_4 (Krishna et al., 1968).

Assays in which cyclic AMP is measured directly by protein binding methods are unsuitable for comparative work for the following reasons. Firstly, it is not certain that interference by phosphodiesterase can be adequately prevented (see above). Secondly, interference in the saturation assay is caused by substances present in tissue extracts and the quantities of these can vary in a comparative study (Brown et al., 1973). Thirdly, radiochemical assays can be far more easily adapted for assaying very small quantities of enzyme (see below).

[α - ^{32}P]-ATP was not used in this investigation for a number of reasons. ^{32}P is a more dangerous isotope than ^{14}C or ^3H and so special facilities would have been needed when handling it. It also has a short half-life and so it is expensive and inconvenient to maintain a stock of the isotope ready for when suitable tissues are available. Therefore [^{14}C]- or [^3H]-ATP was used as substrate. Generally the latter isotope was preferred because it is relatively inexpensive.

Since [^3H]-ATP is labelled in the purine moiety it was necessary to devise a procedure which could separate cyclic AMP from adenine nucleotides, adenosine, inosine and preferably hypoxanthine and adenine - all compounds which may be formed from ATP. Thin-layer separation methods using plastic-coated sheets were investigated because such separations are rapid and spots can be cut directly from the sheets and their radioactivity measured without elution. A silica-coated sheet, developed with a solvent based on that of House et al. (1972), was found to separate all these compounds satisfactorily. Similar separations have been obtained electrophoretically (Verbert & Cacan, 1972). The details of the method have been given in Section A.6.

It was impracticable to validate the separation method with every tissue studied. However, it was shown that the omission of the unlabelled cyclic AMP from the assay and addition of commercial phosphodiesterase together prevented the incorporation of label into cyclic AMP. (In these experiments the reaction was terminated by boiling for two minutes, instead of acidification, since the phosphodiesterase was added after termination.) The product of a side reaction might interfere with the measurement of cyclic AMP when adenylyl cyclase from one tissue, but not another, is assayed. Therefore, the control was carried out for three very different tissues: mouse brain, locust flight muscle and scallop slow adductor muscle.

B.4 FURTHER COMPONENTS OF THE ASSAY MIXTURE

pH Optima for adenyl cyclases are between 7 and 8. The enzyme was assayed in Tris buffer at pH 7.5. These are the conditions used by most workers.

Optimal Mg^{2+} concentrations have usually been found to lie between 5 and 10 mM. Concentrations of Mg^{2+} above 15 mM may be inhibitory (Weinryb et al., 1973). Therefore a concentration of free Mg^{2+} of 7 mM was used.

Ca^{2+} generally inhibits adenyl cyclase but there is evidence that small quantities of Ca^{2+} are necessary for the full expression of the activity of adenyl cyclase from brain. Thus EGTA, but not EDTA, inhibits the activity of some adenyl cyclases (see Perkins, 1973; Poirier et al., 1974; von Hungen & Roberts, 1973; Johnson & Sutherland, 1973). A small amount of EDTA (but not EGTA) was included in the present assay system.

Sulphydryl reagents are frequently included in adenyl cyclase assays. Rosen et al. (1970) have reported that dithiothreitol enhances frog erythrocyte adenyl cyclase activity. Dithiothreitol was included in the present assay.

The adenyl cyclase reaction is reversible (see Chapter 1, Section A1a). An assay performed with 0.2 mM ATP in the presence of 2.5 mM cyclic AMP would reach equilibrium if PP_1 reached a concentration of 0.5 mM. It is generally assumed that endogenous pyrophosphatase is sufficient to maintain a low concentration of pyrophosphate. However, there is no direct evidence that this is so. Therefore, commercial pyrophosphatase was added to many assays. In some assays pyrophosphatase appeared to increase the activity. Since it did not increase the apparent activity in the controls, it was not contaminated with adenyl cyclase.

B.5 THE EXTRACTION OF ADENYL CYCLASE

The development of optimal conditions for the extraction of adenyl cyclase is discussed in this section. It is reported that, although the basal

adenyl cyclase activity is stable at 4°C for some hours, the F^- or adrenaline stimulated activity is not so stable (Robison et al., 1971). These findings were confirmed in the present work using mouse brain.

Low concentrations of detergents have sometimes been found to stimulate basal adenylyl cyclase activity (for example, Birnbaumer et al., 1971). Homogenization of pigeon brain adenylyl cyclase in the presence of 0.1% Triton X-100 approximately doubled the activity. Higher concentrations of detergent did not lead to any further increase in activity. However, the adenylyl cyclase activities from other pigeon tissues were unaffected by the presence of Triton X-100. Consequently, detergent was included in the homogenization of nervous tissues in some experiments.

Some experiments concerning the extraction of adenylyl cyclase are described in Chapter 3 (Sections A1, B1), where the significance of basal and fluoride-stimulated activities of adenylyl cyclase is discussed (Section C).

Sonication of mouse brain and liver homogenates did not increase adenylyl cyclase activities.

C. DEVELOPMENT OF THE PHOSPHODIESTERASE ASSAY

Assay methods for phosphodiesterase have been well reviewed (Cheung, 1970a; Robison et al., 1971; Appleman et al., 1973; Rutten et al., 1973). The major problems in this assay involve the measurement of the formation of product.

Most of the discussion which follows refers to cyclic AMP phosphodiesterase assays but is applicable to the assay of the cyclic GMP-hydrolyzing enzyme. (The details of the assay commonly used have been described in Section A.3).

C.1 THE ESTIMATION OF PRODUCT FORMATION

The formation of AMP may be measured spectrophotometrically in a sampling or continuous assay. For example, Drummond & Perrot-Yee (1961)

coupled the phosphodiesterase reaction with adenylic acid deaminase and followed the decrease in absorbancy at 265 nm which resulted from the deamination of AMP to IMP. Alternatively a nucleotidase is used to convert AMP to P_i and the latter is estimated.

Radiochemical methods are more common. [^3H]-Cyclic AMP may be incubated with the enzyme for a period of time and the formation of [^3H]-AMP is measured. This assay involves the separation of cyclic AMP and AMP by chromatography. However, a useful experimental method is the hydrolysis of AMP to adenosine with 5'-nucleotidase. The adenosine can be easily separated from the negatively-charged substrate, cyclic AMP, using an anion exchange resin.

There are two problems involved in the use of non-radiochemical assays of phosphodiesterase: firstly, there is invariably a large blank and secondly it is difficult to measure enzyme activity when a low concentration of substrate is used because the amount of product formed must be small.

Radiochemical assays in which the nucleotide product is hydrolyzed to a nucleoside are both sensitive and convenient. In this study such assays were used. In the method most commonly employed an anion exchange resin was added directly to the assay mixture so that the substrate was bound, whereas the product remained free in the supernatant (Thompson & Appleman, 1971a). The alternative method involved the separation of product from substrate on an anion exchange column (Loten & Sneyd, 1970). The direct addition of resin was less time-consuming and gave greater reproducibility within an assay than the separation on columns. However, there are disadvantages in the method. Firstly, the blank is high compared to the alternative method (6%, rather than 1%, of the cyclic AMP is not bound by the resin). Secondly, since the resin has some affinity for adenosine, only a certain proportion of the adenosine produced in the assay remains free in the supernatant. It was found that this proportion was independent of the concentration of adenosine but it was not possible to be sure that this

proportion was the same in each experiment. Therefore, in order to avoid any possible variation between experiments, the radioactivity which appeared in the supernatant when all the substrate was converted to product was measured in each assay (see Section A.3).

In some previous studies of purified phosphodiesterase it has been found that 5'-nucleotidase preparations activate the enzyme (Cheung, 1967). Consequently, the conversion of AMP to adenosine is frequently performed in a second stage, after the phosphodiesterase reaction has been terminated by boiling. However, in the present studies of crude phosphodiesterase it was found that it made no difference to the results obtained if 5'-nucleotidase was present for the duration of the assay. Therefore, two-stage assays were not employed.

C.2 INTERFERENCE IN THE ASSAY BY OTHER ENZYMES

The product of the phosphodiesterase assay, 5'-AMP, can be hydrolyzed to adenosine or deaminated to IMP by other enzymes in the tissue homogenate. Both adenosine and IMP may then be converted to inosine. This problem is usually overcome by measuring the formation of all non-nucleotide products or by measuring the formation of P_i in a coupled assay as described above. The problem is also avoided if an assay is used in which substrate removal is followed.

It has been suggested recently (Rutten et al., 1973) that the separation methods which employ anion exchange resins of Loten & Sneyd (1970) and Thompson & Appleman (1971a) are liable to produce errors. The reason for this is that in practice much of the adenosine is deaminated to inosine. Rutten et al. (1973) report that inosine is eluted from an anion exchange column after adenosine and so its formation is not measured by a technique which has been developed to separate cyclic AMP from adenosine. They also report that a greater proportion of inosine than adenosine remains bound to

an anion exchange resin when it is added directly to the assay mixture. Therefore, in such assays, the results will be erroneous if variable proportions of adenosine are converted to inosine. However, the difficulties encountered by Rutten et al., were not found in the present study. Thus inosine was eluted from an AGI-X2 column before adenosine and so any radioactivity appearing in inosine was measured in the assay which used anion exchange columns. Similarly, in the method in which resin was added directly to the assay mixture, the proportion of inosine which remained in the supernatant, after precipitation of either AGI-X2 or AGI-X8 resin, was identical with the proportion of adenosine which remained free under the same conditions. Therefore the conversion of adenosine to inosine was not a problem in these assays.

C.3 FURTHER COMPONENTS OF THE ASSAY MIXTURE

Phosphodiesterases generally have broad pH optima with maxima between 7.5 and 8.5. In this study phosphodiesterase was assayed in Tris buffer at pH 7.5, as in many other studies.

The enzyme requires Mg^{2+} or Mn^{2+} but a concentration of free M^{2+} above 10 mM can be inhibitory. In this study a concentration of free Mg^{2+} of 6 mM was used.

Brain phosphodiesterases have been reported to be activated by Ca^{2+} (see Appleman et al., 1973). The maximal effect of Ca^{2+} occurs at about 10 μM Ca^{2+} . In the present study EGTA, which chelates Ca^{2+} , was used to maintain a low concentration of free Ca^{2+} in some preliminary studies of phosphodiesterase. A concentration of 1 mM EGTA in the assay medium reduces the concentration of Ca^{2+} to 10^{-9} to 10^{-8} M (Portzehl et al., 1964). This inhibited phosphodiesterase from mouse brain by about 50%. Both the high and low K_m enzymes were inhibited. (This contrasts with the results of Kakiuchi et al. (1971) who found that only the low K_m enzyme from rat brain

was activated by Ca^{2+} .) The addition of $23 \mu\text{M}$ Ca^{2+} to the assay of phosphodiesterase from mouse brain was found to activate the enzyme by, at most, 20% compared to the normal assay conditions. Therefore brain phosphodiesterases were probably almost maximally stimulated by Ca^{2+} under the conditions used for these assays.

It has been reported that phosphodiesterase is activated by sulphhydryl agents (Cheung & Jenkins, 1969). Dithiothreitol was included in the present assay.

C.4 EXTRACTION OF THE ENZYME

In the present study tissues were stored for up to two hours on ice between isolation and homogenization. It was shown that the mouse brain enzyme was not affected by storage of the tissue for three hours, but after 24 hours the activity of the high K_m enzyme was reduced by about 20%.

Cheung & Salganicoff (1967) found that the extraction of rat brain for phosphodiesterase assays in isotonic sucrose did not produce maximal activities. Maximal activities were obtained by treating with 0.2% Triton X-100, but not by mechanical disruption or repeated freezing and thawing. It was suggested that the latent enzyme was embedded in the lipoprotein matrix of microsomes in such a manner that it could only be exposed by the detergent.

In the present study the presence of Triton X-100 during the homogenization procedure in hypotonic solution was found to double mouse brain and locust cerebral ganglion phosphodiesterase activities. Only the low K_m enzyme from the locust was activated, whereas both enzymes from mouse tissues appeared to be affected. The optimum Triton X-100 concentration was 0.1%. Triton X-100 either had no effect or inhibited phosphodiesterases from tissues other than brain (mouse liver and heart, locust and bumble-bee flight muscles). Sonication did not affect mouse phosphodiesterase activities. Therefore, Triton X-100 was included during the homogenization of nervous tissues but not other tissues.

D. DEVELOPMENT OF THE ADENOSINE KINASE ASSAY

Adenosine kinase assay methods have been briefly summarized by Anderson (1973). The problems involved in assaying adenosine kinase are similar to those involved in assaying adenyl cyclase in that both substrates and products are removed by enzymes which are usually present in homogenates in larger activities than adenosine kinase. In addition, in the present study it was found that adenosine kinase activity is greatly affected by the precise composition of the assay mixture.

D.1 INTERFERENCE BY CONTAMINATING ENZYMES

The enzyme reactions which can interfere with the assay of adenosine kinase are shown in Figure 2.2. The necessary cofactors for all these reactions will be present during an adenosine kinase assay. Therefore, it has been usual to partially purify adenosine kinase prior to study. Separation of adenosine kinase from ATPases and adenosine deaminase has been achieved but little attention has been paid to 5'-nucleotidase or AMP deaminase.

Another way of overcoming this problem is to use the adenosine analogue Me6MPR, which is not a substrate for adenosine deaminase or nucleoside phosphorylases, and the 5'-phosphate derivative formed by the action of the kinase is not further metabolized (Caldwell et al., 1966; Schnebli et al., 1967). The breakdown of the second substrate, ATP, could reduce the rate of the adenosine kinase reaction but ATP can be included in the assay at high initial concentration so that it saturates adenosine kinase throughout the assay. Alternatively, an ATP-regenerating system may be included in the assay (De Jong & Kalkman, 1973; Kornberg & Pricer, 1951).

A further method of preventing interference by the adenosine deaminase reaction is to inhibit the enzyme. Dipyridamole has been used for this purpose (De Jong & Kalkman, 1973). (In Chapter 1, Section B4c(iv) dipyridamole was referred to as an inhibitor of the adenosine uptake mechanism. Far

higher concentrations are needed to inhibit adenosine deaminase than to inhibit adenosine uptake - Hopkins, 1973b.)

Finally, some workers (for example, Meyskens & Williams, 1971) have taken none of these precautions. Instead a relatively small fractional conversion of adenosine to AMP was measured so that reduction of the substrate level or removal of the product was minimal.

In the present study adenosine kinase was not purified for reasons which were discussed at the beginning of this chapter. The introduction of compounds such as Me6MPR or dipyridamole into a comparative study is unwise, since their usefulness has only been tested for a limited number of systems.

An ATP-regenerating system was included in the assay at pH 7.5 in order to maintain ATP levels. The regenerating system was supplemented with myokinase so that AMP, in addition to ADP, would be converted to ATP. A further point is that, in red blood cells at least, AMP and ADP are competitive inhibitors of the adenosine kinase reaction with respect to ATP (Meyskens & Williams, 1971). Therefore the presence of a regenerating system will reduce the accumulation of possible inhibitors of the reaction and increase the time during which the reaction follows a linear time course.

A test of the effectiveness of the regeneration system has been described (Section B.1). In addition it was shown that the system supplemented with myokinase can reduce AMP concentrations to less than $0.5 \mu\text{M}$.

It was an advantage not to inhibit adenosine deaminase in the present study. In this way adenosine deaminase and adenosine kinase were conveniently assayed simultaneously. Preliminary experiments with the mouse brain enzyme showed that, even at concentrations of adenosine near the K_m of the enzyme, the rate of the adenosine kinase reaction was linear, provided that not more than 25% of the adenosine had been converted to products. Therefore, assays were designed to meet this requirement.

D.2 ESTIMATION OF PRODUCT FORMATION

In some assays of adenosine kinase the substrate and products have been separated by chromatographic or precipitation techniques and the products or the remaining adenosine have been assayed by means of their UV absorbance properties or enzymically (Caputto, 1951; Kornberg & Pricer, 1951; Franz et al., 1962). Since the products are adenine nucleotides, which are also produced by the breakdown of ATP (the second substrate of the assay), these methods require purification of the adenosine kinase from ATPases, in order to reduce the size of the blank. The same problem arises in a coupled enzymic assay following the rate of ADP formation with pyruvate kinase and lactate dehydrogenase (Lindberg et al., 1967; Lerner & Rubinstein, 1970).

Such problems are avoided if radiochemical assays are employed. Labelled adenosine or [γ ³²P]-ATP may be used as substrates. If the label is in adenosine its appearance in adenine nucleotides is followed; if it is in ATP its appearance in the α -position of adenine nucleotides is followed.

Most of the methods used to separate the labelled substrate and products are tedious. Some separation methods are less tedious but they suffer from the disadvantage that the conversion of adenosine to inosine is not followed (Murray, 1968; Shimizu et al., 1972; De Jong & Kalkman, 1973). Consequently, data which enable the activity of adenosine deaminase to be determined are lost. In addition the extent to which the substrate concentration has been reduced by the action of adenosine deaminase cannot be assessed.

In the present study generally-tritiated adenosine was used as substrate and was separated from the products of the reaction (adenine nucleotides, inosine and hypoxanthine) by thin-layer chromatography (see Section B.3). In the chromatographic separation AMP, ADP and ATP all remain close to the origin so that the radioactivity appearing in this position is a measure of the adenosine kinase reaction. (The measurement of the adenosine deaminase reaction is discussed in Section E.2).

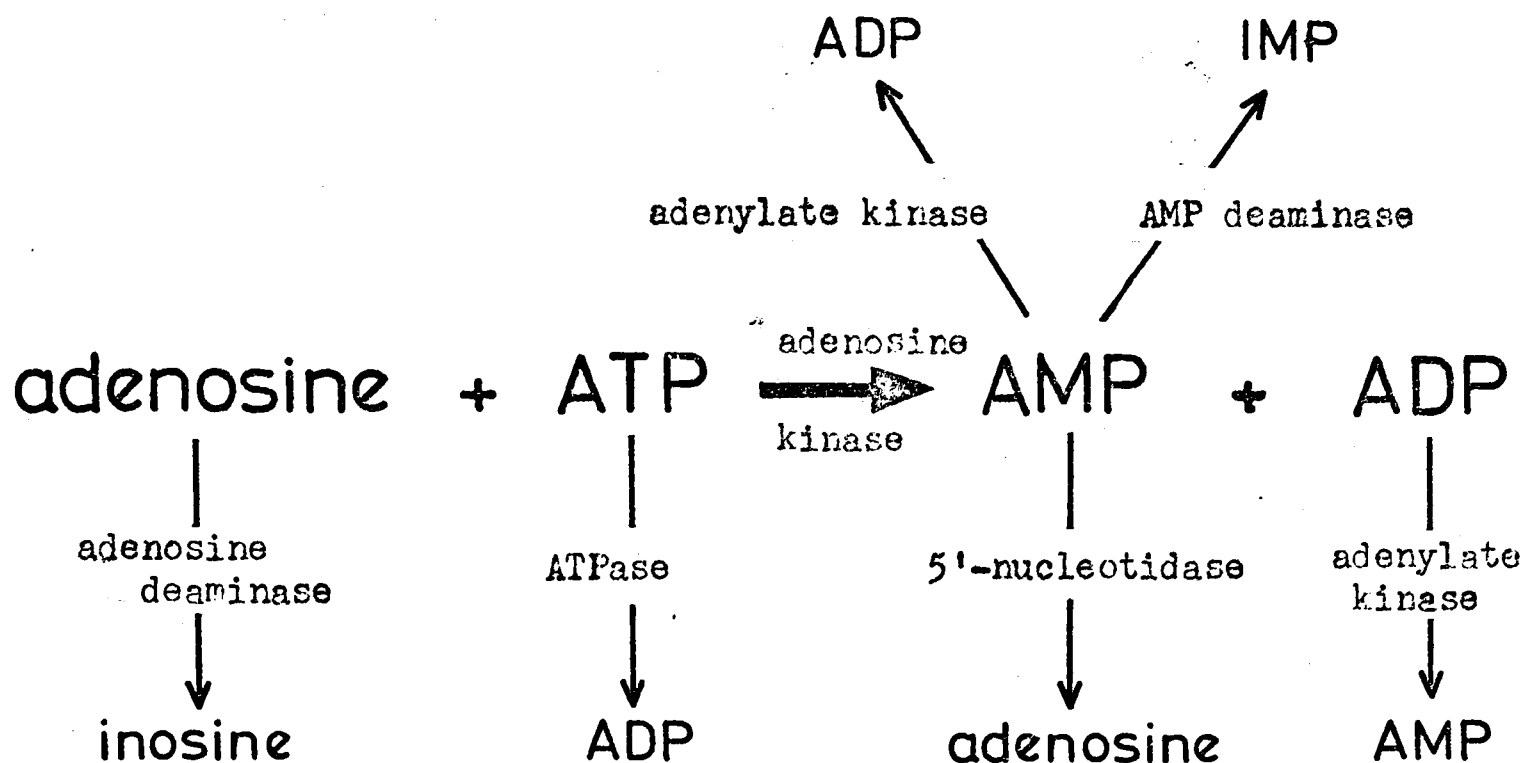


FIG 2.2 The reactions which interfere with the adenosine kinase assay.

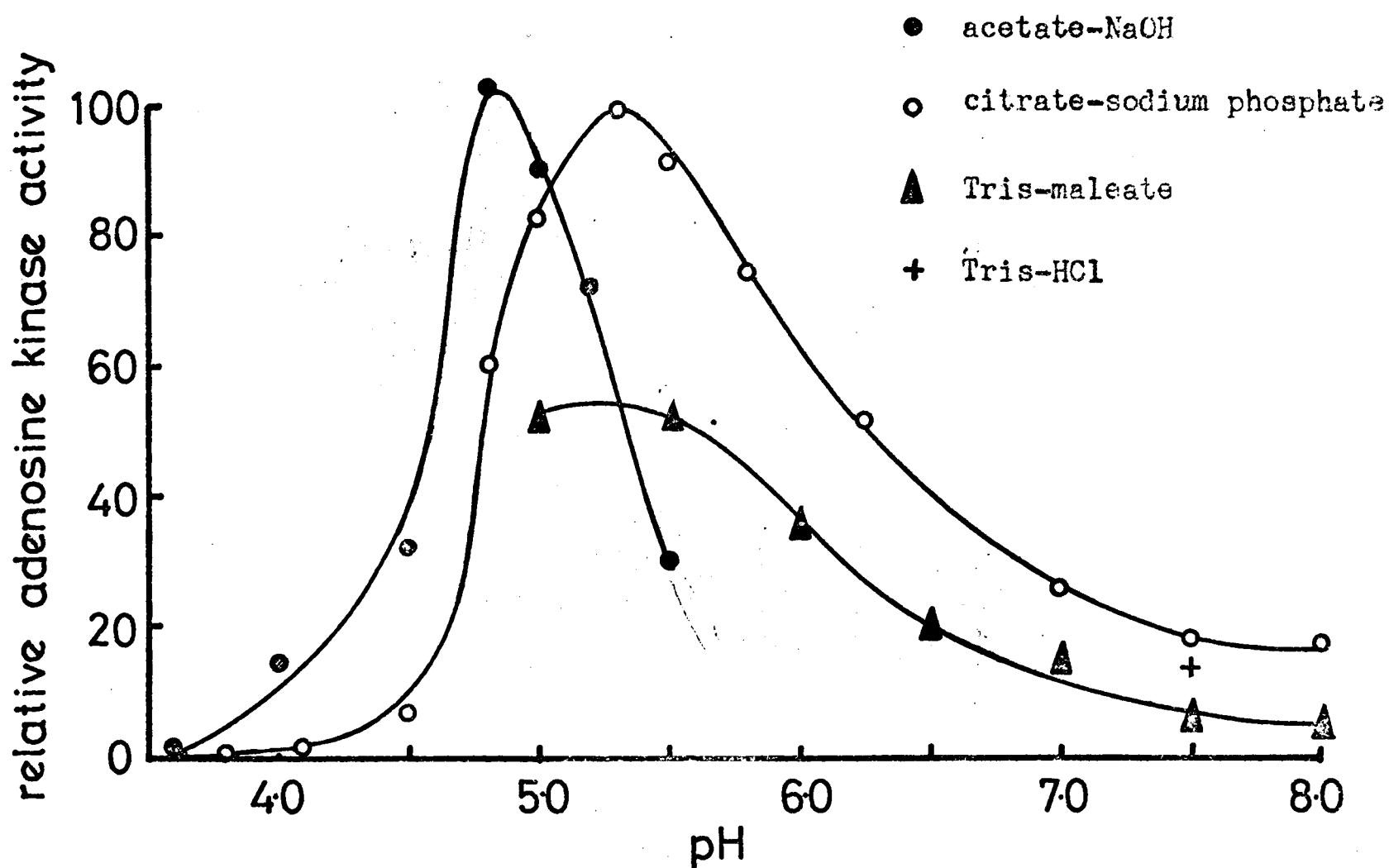


FIG 2.3 The activity of adenosine kinase from mouse brain in different buffers at various pH's.

Methods as described in sections A1 and A4. Concentration of acetate and Tris-maleate was 50mM. Adenosine concentration was 10 μM . A relative activity of 100 corresponds to the maximal activity of 148 nmol/min/g wet wt.

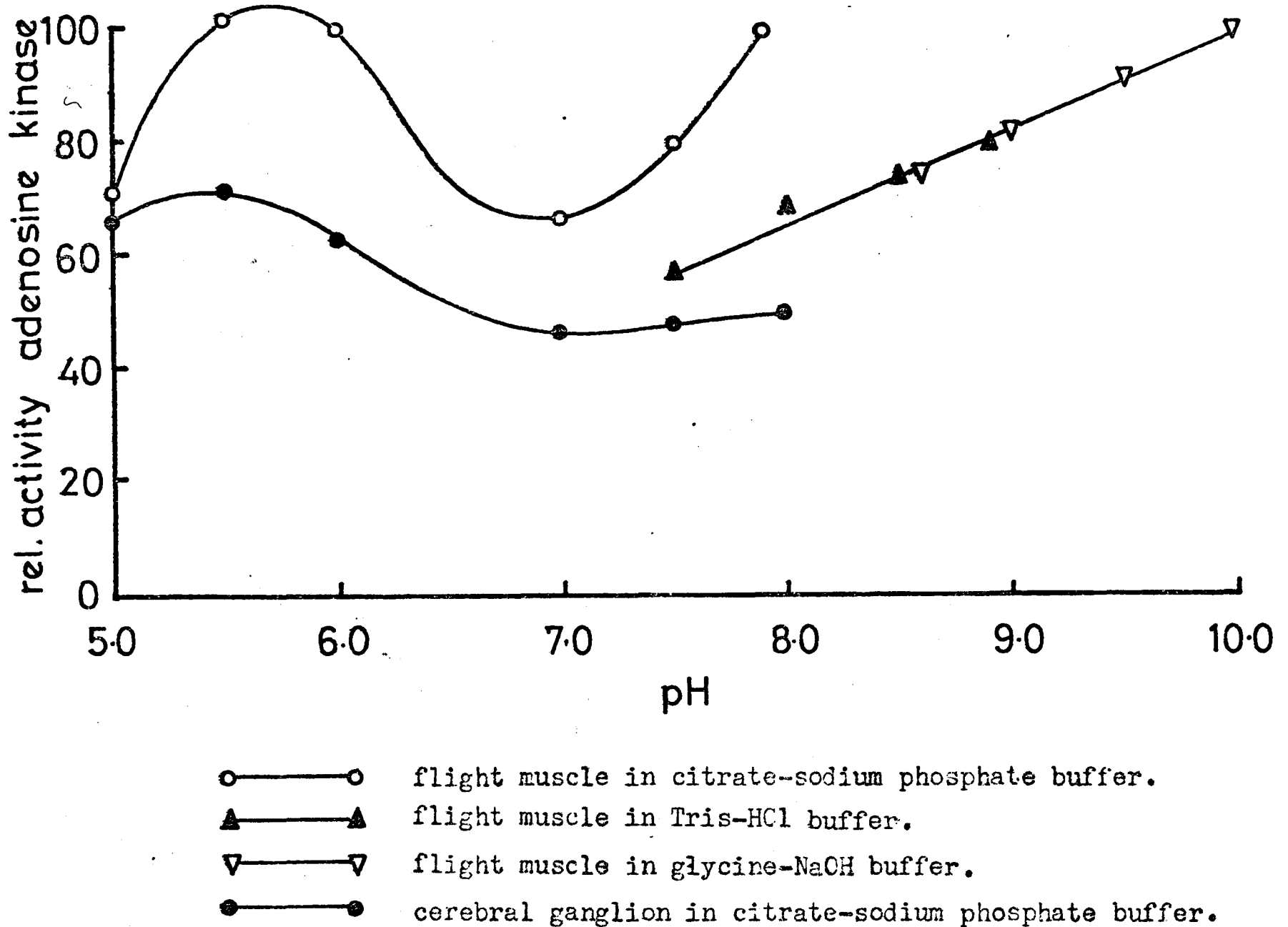


FIG 2.4 The activity of adenosine kinase from locust tissues in different buffers at various pH's.

Methods were as described in sections A1 and A4 except for the nature of the buffer. Tris-HCl and glycine were used at a concentration of 50 mM. Adenosine concentration was 10 μ M. For the assay in citrate-sodium phosphate buffer a relative activity of 100 corresponds to an activity of 61 nmol/min/g wet wt. For the other assays a relative activity of 100 corresponds to an activity of 25nmol/min/g wet wt.

D.3 THE EFFECTS OF BUFFERS AND pH

In mammalian tissues, with the possible exception of tumour cells, the pH optimum of the adenosine kinase reaction has been found to be between 5 and 6. Yeast enzyme has an optimum of about 7.0. Adenosine kinase has usually, but not always, been assayed at its pH optimum. A variety of buffers have been used in the assay.

In order to find optimal assay conditions for the enzyme its activity was measured in various buffers at different pH's: Tris-HCl, Tris-maleate, citrate-sodium phosphate, acetate-NaOH and glycine-NaOH, pH 3.5 - 10. Adenosine kinases from mouse brain and locust flight muscle and cerebral ganglion were used for this.

The results of these assays are presented in Figures 2.3 and 2.4. The mouse and locust enzyme exhibit very different pH curves. Adenosine kinase activities were far higher in some buffers than in others. Similar observations have been reported previously (Holmsen & Rosenberg, 1968; Ho et al., 1972). It is believed that these differences largely reflect differences in the nature of the buffer cation although the nature of the anion may also be important (see Chapter 4, Section C.5).

Adenosine kinase activity was frequently measured at both a pH near neutrality and a low pH in order to see whether the enzyme might be activated in vivo by a fall in pH. It was desirable to use a buffer which could be used at both pH's and in which the enzyme apparently expressed its full activity. Therefore citrate-sodium phosphate was chosen.

D.4 THE EFFECT OF DIVALENT METAL IONS

Most studies of the effect of divalent metal ions on the activity of adenosine kinase have led to the conclusion that Mg^{2+} or Mn^{2+} are essential for enzyme activity; Ca^{2+} or Co^{2+} may be a less efficient substitute (Caldwell et al., 1966; Ho et al., 1968; Lindberg et al., 1967; Holmsen & Rosenberg, 1968; Alma & Feinberg, 1971). However, Schnebli et al. (1967) concluded

that a divalent metal ion was not needed by a human tumour cell adenosine kinase. Murray's (1968) detailed study of adenosine kinase from Ehrlich ascites tumour cells suggested that a small number of Mg^{2+} ions must be bound to the enzyme for activity. Concentrations greater than 0.1 mM Mg^{2+} inhibited the enzyme. Other studies have revealed higher optimal Mg^{2+} concentrations. Most studies seem to agree with Lindberg *et al.* (1967) that maximal activity is obtained when the Mg^{2+} concentration is similar to the ATP concentration used for the assay.

A brief investigation of the effects of ATP and Mg^{2+} concentration on mouse brain adenosine kinase was carried out in the present study. The results of this investigation are shown in Table 2.1. Mg^{2+} was essential for enzyme activity and the presence of 5 mM Mg^{2+} in excess of ATP had no effect on enzyme activity in this assay system. 4 mM $MgCl_2$ -ATP gave maximal activity at both pH 7.5 and pH 5.3.

In the comparative study, 4 mM $MgCl_2$ -ATP was used as substrate. This concentration is well above the reported K_m values of adenosine kinase for ATP, which vary from 0.025 to 0.5 mM (Henderson *et al.*, 1972; Lindberg *et al.*, 1967). When new phyla or tissues were first studied, 8 mM $MgCl_2$ -ATP was tested but the activities obtained were always identical to those with 4 mM $MgCl_2$ -ATP.

No additional Mg^{2+} was included in the assay mixture. It may be noted that the citrate of the assay buffer as well as ATP chelates Mg^{2+} . Therefore the concentration of free Mg^{2+} in the assay is very low even if there is slightly more Mg^{2+} than ATP. Citrate has far less affinity for Mg^{2+} than ATP (O'Sullivan, 1969) so that nearly all the Mg^{2+} in the assay medium is chelated by ATP.

D.5 VALIDATION OF THE ASSAY

In view of the controversy which has surrounded the role of adenosine kinase in the incorporation of adenosine into adenine nucleotides in vivo

TABLE 2.1 The effect of variations in Mg^{2+} and ATP concentration on the activity of adenosine kinase from mouse brain

Adenosine kinase was assayed using 50 μ m adenosine as substrate. Otherwise the methods were as described in Sections A1 and A4, except for the variations indicated. A relative activity of 100 corresponds to an activity of 45 nmol/min/g wet wt. at pH 7.5 and 155 nmol/min/g wet wt. at pH 5.3.

CONCENTRATION OF ATP (mM)	CONCENTRATION OF Mg^{2+} (mM)	RELATIVE ENZYME ACTIVITIES	
		pH 7.5	pH 5.3
0.2	0.2	100	20
2.0	2.0	100	100
4.0	4.0	100	110
2.0	7.0	100	100
2.0	2.0*	2	-

* EDTA (5 mM) was also included in this assay

(Chapter 1, Section B2a(11)), it was considered necessary to show clearly that the conversion of adenosine to nucleotides in these experiments was catalyzed by adenosine kinase. The other possible routes for conversion of adenosine to nucleotides are via inosine and hypoxanthine, or via adenine (see Figure 1.2). These alternative routes require the cofactor PRPP; although it is not added to the assay mixture, some PRPP could be generated in the incubation.

The route via inosine and hypoxanthine was shown to be inoperative in the following way: labelled inosine was substituted for labelled adenosine in assays which were otherwise identical to the assay of adenosine kinase. (Adenosine at 5 μ M was present in the assays.) A range of tissues were used (mouse brain, locust flight muscle and waterbug flight muscle). There was no incorporation of inosine into nucleotides under these conditions, so that adenosine cannot be deaminated to inosine before it is incorporated into adenine nucleotides.

The adenine route of nucleotide formation was not ruled out directly in this way but rather by the finding that there was no detectable formation of adenine during the adenosine kinase assay. This observation was made with various tissues from various phyla. Moreover, the adenosine kinase reaction was shown to follow a linear time course. If phosphorylation required more than one catalytic step, the rate of appearance of radioactivity in product would be expected to be non-linear.

D.6 THE EXTRACTION AND STABILITY OF ADENOSINE KINASE

After extraction the activity of adenosine kinase has frequently been found to decrease rapidly. Results suggest that the heart enzyme is most stable and the liver enzyme least stable (De Jong & Kalkman, 1973; Lindberg et al., 1967). Glycerol partially protects adenosine kinase from rabbit liver from spontaneous inactivation but glutathione, 2-mercaptoethanol, Mg^{2+} , adenosine and 3'-deoxyadenosine are ineffective (Lindberg et al., 1967).

It is clearly important in a comparative study that enzyme activity is not lost during tissue preparation. Therefore, the stability of adenosine kinase in tissue extracts was studied. In addition, the effects of Triton X-100 and glycerol on the extraction of the enzyme were studied.

(a) STABILITY STUDIES

Studies of the stability of adenosine kinase from various sources in crude tissue homogenates are summarized in Table 2.2 and Figure 2.5. Preliminary studies indicated that, during incubation for periods of time at 30°C, the activity of adenosine kinase in homogenates of mouse brain increased rather than decreased. This may be due to the release of the enzyme from a bound location such as vesicles or membranes (cf. Section C.4). In subsequent experiments it was discovered that adenosine kinase rapidly loses activity in homogenates of lobster tissues and vertebrate livers. Homogenization and incubation of these tissues in the presence of various concentrations of glycerol or Triton X-100 reduced the rate of decrease in enzyme activity by as much as 50%.

(b) EXTRACTION STUDIES

(i) The Extraction Medium

In many cases assays were performed on the same homogenate at very different pH's, so that it was not possible to use a strong buffer for extraction. Indeed, it was found that suitable extraction could be achieved in a solution consisting of 1 mM MgCl₂ and 1 mM EDTA, adjusted to pH 7 with NaOH. The extraction of one half of a mouse brain in this medium and the other half in distilled water gave identical results. The addition of extraction medium to the assay buffer at pH 5.3 did not significantly alter the pH of the buffer.

TABLE 2.2 The effect of incubating tissue homogenates on the activities of adenosine kinase and adenosine deaminase

Tissues were extracted as described in Section A1 in the presence of Triton X-100 or glycerol as indicated. Adenosine kinase and adenosine deaminase were assayed immediately (see Section A4) and again after the homogenate had been incubated for the time indicated. The percentage change in the activity of the enzyme following incubation of the homogenate is recorded. Absolute activities of the enzymes at pH 7.5 and pH 5.3 are given in Tables 4.5 to 4.7. A dash (-) indicates that the activity was not measured.

ANIMAL	TISSUE	TRITON X-100 IN HOMOGENATE	INCUBATION TEMPERATURE (°C)	INCUBATION TIME (HR.)	PERCENTAGE CHANGE IN ENZYME ACTIVITY DURING INCUBATION OF HOMOGENATE			
					ADENOSINE	KINASE	ADENOSINE	DEAMINASE
					pH 7.5	pH 5.3	pH 7.5	pH 5.3
Lobster	Cerebral ganglion	-	30	1	-100	-	+54	-
		0.1%	30	1	-99	-	+53	-
	Abdominal muscle	-	30	1	-82	-	+19	-
		0.1%	30	1	-58	-	+9	-
	Claw muscle	-	30	1	-91	-	Zero	-
		0.1%	30	1	-69	-	+8	-
Frog	Liver	0.01%	30	1 $\frac{1}{4}$	-60	-65	+19	+12
Domestic fowl	Liver	0.02%*	4	24	-12	-34	-23	+14
Mouse	Brain	0.1%	20	1	+10	-2	+8	0
				2	-3	-16	+21	+10
				3 $\frac{1}{2}$	-18	-36	+23	+11

* Glycerol (20% v/v) was also present in this homogenate

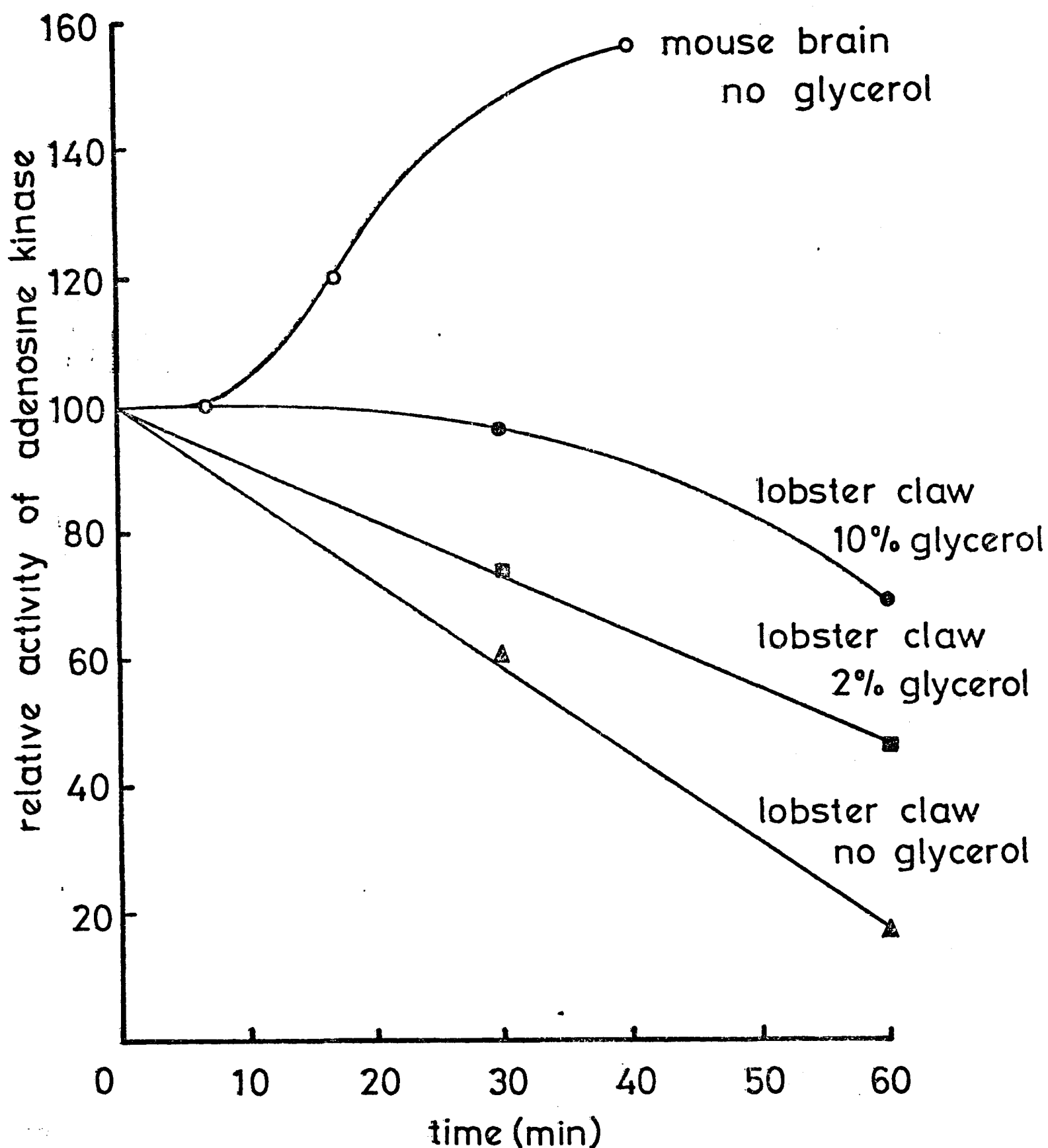


FIG 2.5 The effect of incubating tissue homogenates on the activity of adenosine kinase.

Tissues were extracted in the presence of Triton X-100 as described in section A1. Glycerol was added to the homogenates in the concentrations indicated. The homogenates were incubated at 30°C and aliquots were assayed for adenosine kinase at the times indicated as described in section A4. Activity was measured at pH 7.5 using 20 μ M adenosine. A relative activity of 100 corresponds to 10 nmol/min/g wet wt. for lobster tissues and 33 nmol/min/g wet wt. for mouse brain.

(11) Extraction in Triton X-100 and glycerol

Studies of the effect of Triton X-100 on the extraction of adenosine kinase are presented in Table 2.3. Triton X-100 (0.1%) increased the activity of adenosine kinase from all tissues (both nervous and non-nervous).

In view of the ability of glycerol to reduce the decrease in activity of adenosine kinase in tissue homogenates, it was decided to determine whether the use of glycerol in the extraction medium would increase adenosine kinase activity. Adjacent pieces of tissue were extracted with or without glycerol, which was usually present at a concentration of 20% (v/v), and adenosine kinase was assayed immediately. The results of this study are shown in Table 2.4. Glycerol increased the activity of adenosine kinase in those cases where the enzyme had been found to be unstable in tissue extracts (liver and lobster tissues). Therefore, glycerol was used in the extraction of these tissues. However, no quantitatively consistent effect of glycerol was observed.

E. DEVELOPMENT OF THE ADENOSINE DEAMINASE ASSAY

The assay of adenosine deaminase presents a few minor problems: the activity of the enzyme may be low; the product, inosine, may be removed to hypoxanthine, and suitable extraction methods for comparative studies have not been reported. In this study adenosine deaminase was assayed simultaneously with adenosine kinase and so many of the preliminary experiments on adenosine kinase also provided information for the adenosine deaminase assay (Tables 2.2 to 2.4).

E.1 ESTIMATION OF PRODUCT FORMATION

Adenosine deaminase can be assayed by following the fall in absorbance at 265 nm which occurs when adenosine is converted to inosine (Kaplan, 1955). Radiochemical assays have also been used (for example, Santos et al., 1968; Meyskens & Williams, 1971; Schrader et al., 1972). Ammonia production has

TABLE 2.3 The effect of Triton X-100 on the extraction of adenosine kinase and adenosine deaminase from tissues

Tissues were extracted as described in Section A1 but in the absence of Triton X-100. The homogenate was divided into two portions. One portion was rehomogenized in the same manner; the other portion was rehomogenized in the presence of 0.1% (v/v) Triton X-100. The activities of adenosine kinase and adenosine deaminase at pH 7.5 were measured for each portion, using 10 or 20 μ m adenosine as substrate (see Section A4). Absolute activities of the enzymes are given in Tables 4.5 to 4.7.

ANIMAL	TISSUE	PERCENTAGE CHANGE IN ENZYME ACTIVITY PRODUCED BY HOMOGENIZATION IN TRITON X-100	
		ADENOSINE KINASE	ADENOSINE DEAMINASE
Mouse	Brain ^a	+70	-10
Mouse	Brain ^b	+90	+59
Mouse	Brain ^{b,c}	+57	+50
Mouse	Liver	+5	-5
Lobster	Cerebral ganglia	+30	+25
Lobster	Claw muscle	+58	+34
Lobster	Abdominal muscle	+26	+38

a. Mean of triplicate assays.

b. Mean of duplicate assay.

c. In this experiment the enzymes were assayed using 0.5 μ M adenosine.

TABLE 2.4 The effect of glycerol on the extraction of adenosine kinase and adenosine deaminase from different tissues

Adjacent pieces of tissue were extracted as described in Section A1, except that glycerol was added to the extraction medium for one of the pieces of tissue. Adenosine kinase and adenosine deaminase were assayed immediately in each extract using 10 or 20 μ M adenosine as substrate (see Section A4). The effect of glycerol is expressed as a percentage change. Absolute activities and Latin names for the species used are given in Tables 4.5 to 4.7. A dash (-) indicates that the activity was not measured.

ANIMAL	TISSUE	CONCENTRATION OF GLYCEROL (% v/v)	PERCENTAGE CHANGE IN ENZYME ACTIVITY PRODUCED BY GLYCEROL			
			ADENOSINE AT pH 7.5	KINASE AT pH 5.3	ADENOSINE DEAMINASE AT pH 7.5	AT pH 5.3
Lobster	Claw muscle	2	+8	-	+20	-
		10	+19	-	+41	-
	Abdominal muscle	2	+23	-	0	-
		10	+67	-	+46	-
Locust	Flight muscle	20	0	-	-47	-
Cockroach (<u>Periplaneta americana</u>)	Flight muscle	20	+45	-	-27	-
Waterbug	Flight muscle	20	-7	-	-59	-
Rosechafer	Flight muscle	20	+28	-	-73	-
		20	-7	-	-32	-
Blowfly	Flight muscle	20	+7	-	-	-
		20	+45	-	-24	-
Cockroach (<u>Blaberus discoidalis</u>)	Cerebral ganglion	20	-4	-	-30	-
Domestic fowl	Liver	10	+45	+22	-21	+32
		20	+75	-	-37	-
		20	+12	+10	-22	-55
	Heart	10	-21	+9	-43	-47
	Pectoral muscle	10	+8	-5	-10	-
		20	-14	-	-47	-
	Sartorius muscle	10	-54	-28	-26	0
	Brain	10	0	-13	-33	-8
Mouse	Liver	20	+17	+44	-31	-35
	Brain	20	+15	+5	-13	-15

been followed in some cases (Brady & O'Donovan, 1965; Mustafa & Tewari, 1970a). In the present study it was most convenient to assay adenosine kinase radiochemically, simultaneously with adenosine kinase.

E.2 INTERFERENCE BY OTHER ENZYMES

The product of the adenosine deaminase reaction, inosine, can be further degraded to hypoxanthine by enzymes present in crude homogenates. Therefore, the rate of hypoxanthine formation was added to the rate of inosine formation when assessing adenosine deaminase activity. (Hypoxanthine formation was usually very small.)

The simultaneous assay of adenosine deaminase and adenosine kinase introduced a further interfering reaction. When the activity of the kinase was considerably greater than that of the deaminase, measurement of the latter activity became inaccurate, especially at low adenosine concentrations. This occurred with extracts of some livers and insect flight muscles.

E.3 FURTHER COMPONENTS OF THE ASSAY MIXTURE

The effects of various buffers and different pH's on the activity of adenosine deaminase from mouse brain and locust were studied simultaneously with identical studies of adenosine kinase (Section D.3). The results are shown in Figures 2.6 and 2.7.

Mouse brain adenosine deaminase has a very broad pH optimum at pH 6 to pH 7. In this respect it is similar to adenosine deaminase from other vertebrate sources (Zielke & Suetter, 1971; Brady & O'Donovan, 1965). In contrast, locust flight muscle adenosine deaminase exhibited a pronounced pH optimum at pH 7.5 to 8.5.

The nature of the buffer did not affect adenosine deaminase activity, in agreement with the results of Baer et al. (1966) on the enzyme from rat heart.

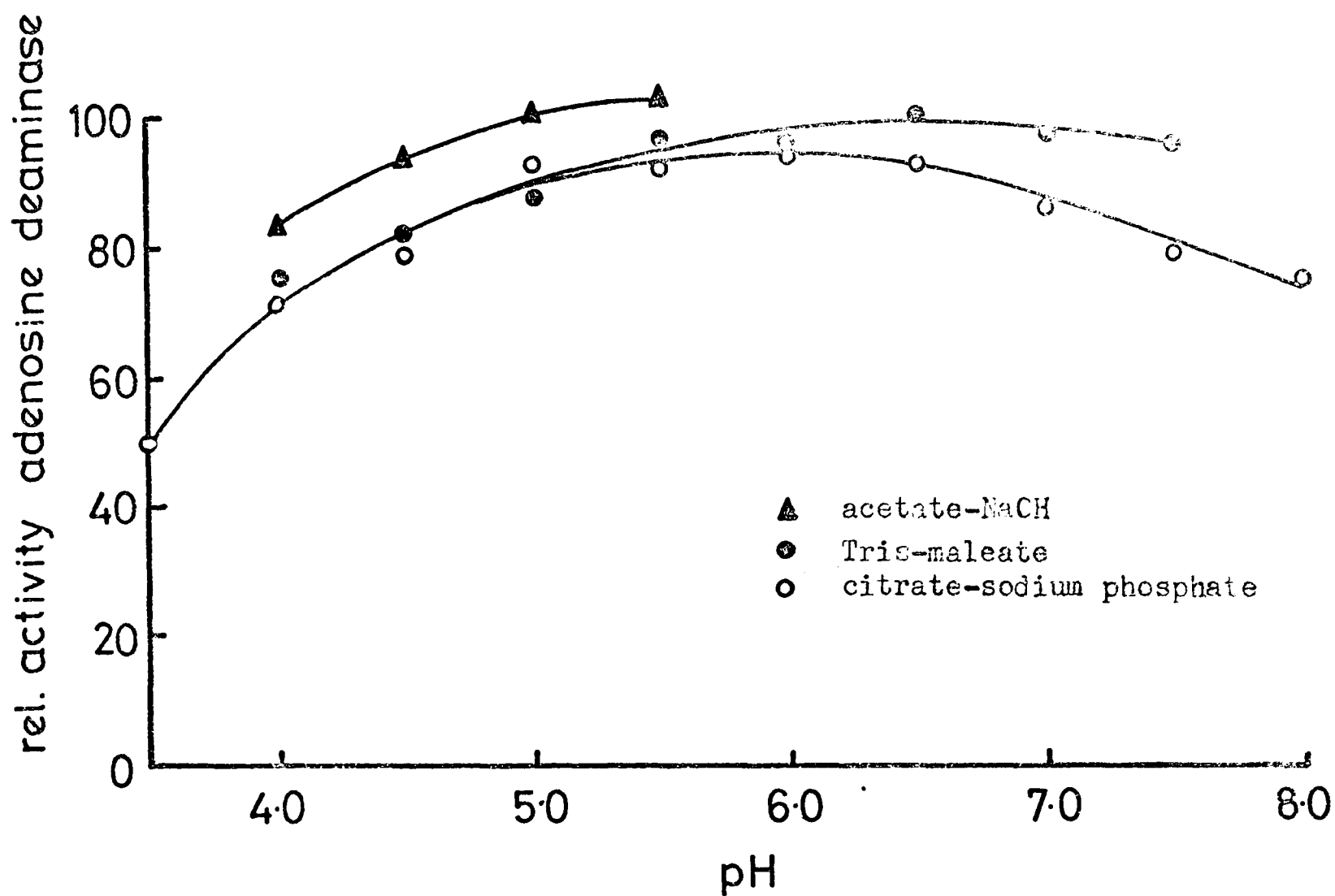


FIG 2.6 The activity of adenosine deaminase from mouse brain in different buffers at various pH's.

See legend to Fig. 2.5 for methods.

The maximum activity of the enzyme in Tris-maleate buffer (34 nmol/min/g wet wt.) corresponds to a relative activity of 100.

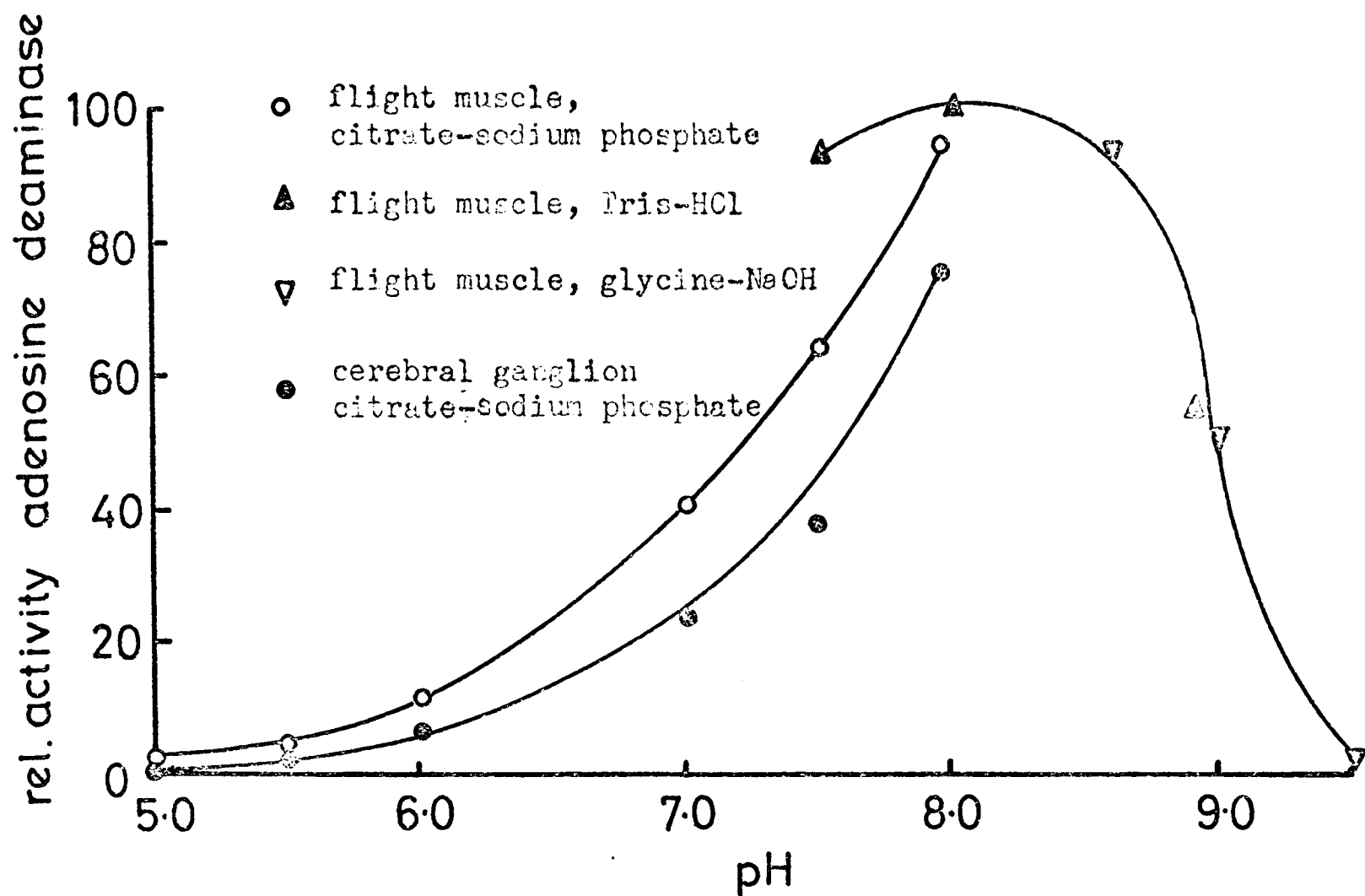


FIG 2.7 The activity of adenosine deaminase from locust tissues in different buffers at various pH's.

See legend to Fig 2.4 for methods.

For the assays in citrate-sodium phosphate buffer a relative activity of 100 corresponds to an activity of 51 nmol/min/g wet wt; for other assays a relative activity of 100 corresponds to an activity of 23 nmol/min/g wet wt.

Variations in ATP and Mg^{2+} concentration in the assay did not affect deaminase activity (see Section D.4).

It may therefore be concluded that the composition of the assay medium for adenosine kinase was adequate for measuring maximal adenosine deaminase activities.

E.4 THE EXTRACTION AND STABILITY OF ADENOSINE DEAMINASE

In the course of the studies on the extraction and stability of adenosine kinase (Section D.6), information was obtained which indicated the best techniques for obtaining maximal activities of adenosine deaminase. These findings are recorded in Tables 2.2 to 2.4 and Figure 2.8.

Incubation of crude tissue homogenates from mouse, chicken, frog and lobster usually resulted in an increase in adenosine deaminase activities (Table 2.2). (There was a 60% increase in the case of mouse brain homogenate stored for 40 minutes at 30°C in the presence of 0.1% Triton X-100.)

The inclusion of glycerol during the extraction of chicken, mouse and various insect tissues decreased the adenosine deaminase activity. In the case of lobster claw and tail muscle there was an increased enzyme activity (Table 2.4).

The extraction of lobster tissues in the presence of 0.1% Triton X-100 resulted in an increase of about 30% in the enzyme activity (Table 2.3). In the case of the extraction of mouse brain conflicting results were obtained. Mustafa & Tewari (1970a) showed that 15 to 20% of the adenosine deaminase of mouse brain is bound to mitochondria or a component of the mitochondrial fraction. The bound enzyme is inactive until released by incubation of the mitochondrial fraction with Triton X-100. Various other non-ionic, anionic and cationic detergents did not have this effect. Activation of the enzyme by Triton X-100 was not observed until after 15 minutes contact between detergent and mitochondria. It is feasible, therefore,

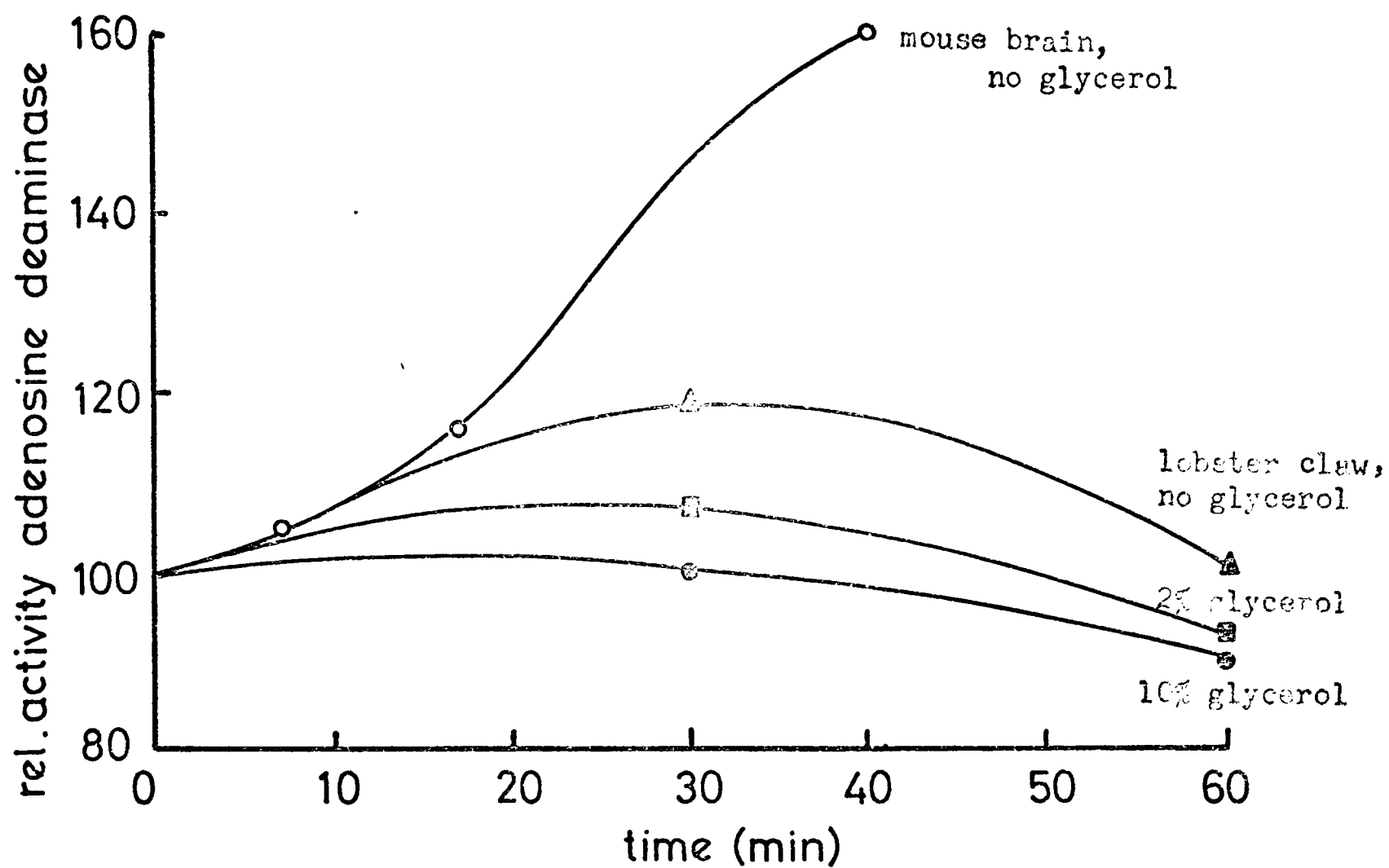


FIG 2.8 The effect of incubating tissue homogenates on the activity of adenosine deaminase.

See legend to Fig. 2.5 for methods.

A relative activity of 100 corresponds to 120 nmol/min/g wet wt. for lobster tissues and 35 nmol/min/g wet wt. for mouse brain.

that the conflicting results obtained in this study could reflect different degrees of activation of the bound enzyme.

In those cases where conditions were optimal for the extraction of adenosine kinase but not adenosine deaminase, the activity of the latter enzyme is not recorded in the results of the comparative study reported in this thesis.

F. DEVELOPMENT OF THE 5'-NUCLEOTIDASE ASSAY

There are no reviews available in the literature concerning the assay of 5'-nucleotidase. As in the assays of adenylyl cyclase and adenosine kinase, side reactions can increase the difficulty of assay of the enzyme.

F.1 INTERFERENCE BY OTHER ENZYMES

The substrate of the 5'-nucleotidase reaction, AMP, can be degraded by AMP deaminase; the product, adenosine, may be further degraded to inosine by adenosine deaminase.

There are no reports of specific inhibitors of AMP deaminase but this enzyme is not maximally active in the absence of ATP or at the low concentrations of AMP suitable for the assay of 5'-nucleotidase. The problem of adenosine degradation can be tackled in a number of ways: the 5'-nucleotidase assay may be performed at pH 9 where adenosine deaminase is inactive (Baer et al., 1966); labelled adenosine may be "trapped" in a pool of unlabelled adenosine in a radiochemical assay (Burger & Lowenstein, 1967); the rate of formation of both inosine and adenosine may be measured; the rate of formation of the second product, P_1 , may be measured. With respect to the last two approaches in this list, it must be noted that inosine and P_1 may be formed by another route: AMP deamination to IMP followed by the action of 5'-nucleotidase on IMP.

In the present work 5'-nucleotidase was not assayed at pH 9 because it is unlikely that it is fully active and adenosine deaminase totally

inactive at pH 9 for all the tissues investigated. Adenosine deaminase is far more active than 5'-nucleotidase in some tissues (Tables 4.5 to 4.8) and 5'-nucleotidase from some tissues hydrolyzes IMP faster than AMP (Itoh et al., 1967, 1968). Therefore, measurement of adenosine plus inosine, or P_1 , production could overestimate 5'-nucleotidase activities. Consequently, in the present study, the effect of adenosine deaminase was limited by including an adenosine trap in the assay: 3 mM adenosine was included in the incubation medium. This concentration of adenosine has little effect on 5'-nucleotidase activity from rat liver, brain and heart, sheep brain, calf intestinal mucosa and pigeon heart (Itoh et al., 1968; Bosmann & Pike, 1971; Burger & Lowenstein, 1967; Ipata, 1968; Center & Behal, 1966; Gibson & Drummond, 1972). In addition, it was shown in the present study that 3 mM adenosine had no effect on the activity of 5'-nucleotidase from the bumble-bee (B. pratorum). (AMP deaminase is absent from bumble-bee flight muscle (Leech, 1973) and so it was possible to omit adenosine from the 5'-nucleotidase assay and still obtain a maximal enzyme activity.)

F.2 ESTIMATION OF PRODUCT FORMATION

In early studies P_1 formation was commonly used as an estimate of 5'-nucleotidase activities. Continuous spectrophotometric assays in which the nucleotidase is coupled to adenosine deaminase have also been employed (for example, Widnell & Unkeless, 1968).

The sensitivity of these methods is rather low. Moreover, contaminating enzymes interfere with the assay. Therefore, in the present study, a radiochemical assay utilizing $[^{14}\text{C}]$ -AMP was employed. AMP was separated from the product, adenosine, and the products of side reactions, inosine and hypoxanthine, using the chromatography system previously described (Section A.6).

F.3 FURTHER COMPONENTS OF THE ASSAY MIXTURE

5'-Nucleotidases from different sources exhibit a variable dependency

upon divalent metal ion concentrations. Mg^{2+} (10 mM) usually gives optimal activity and so this was used in the present study (see Bodansky & Schwartz, 1968).

The pH dependency of the 5'-nucleotidase reaction depends on the source of the enzyme and the precise assay conditions, such as temperature and metal ion concentration. In all cases a pH optimum near 7.0 has been observed and so this pH was used for the present study (Bodansky & Schwartz, 1968).

G. CALCULATION OF RESULTS

G.1 GRAPHICAL METHODS

It has been pointed out (Dowd & Riggs, 1965; Walter, 1974) that Hofstee plots (1952) are the best method for presenting enzyme kinetic data (v is plotted against v/s). This method is least likely to magnify experimental errors in the measurement of V_{max} and K_m . It also provides a simple visual means of detecting deviations from Michaelis-Menten kinetics. In addition the Hofstee plot distributes data relatively evenly over a graph. This point is particularly relevant to the representation of phosphodiesterase activity data, since the reaction velocity was measured over a 1000-fold range of substrate concentration. Therefore Hofstee plots were used to analyze all the data obtained for this thesis.

G.2 ANALYSES OF PHOSPHODIESTERASE KINETICS

Hofstee and double-reciprocal plots of phosphodiesterase activity data usually appear as two straight line portions connected by a curve. Many workers have calculated the proportions of low and high K_m enzymes by simply extrapolating the straight line portions of the plots to infinite substrate concentration. Others have corrected the high substrate concentration region of the curve for the contribution of the low K_m enzyme but have not

recognized that even at the lowest substrate concentrations the high K_m enzyme may contribute appreciably to phosphodiesterase activity.

Spears et al. (1971) devised a procedure whereby the kinetic constants of two enzyme acting on the same substrate may be approximately estimated. This method was adapted for the purposes of the present study. The basic steps are as follows.

- (a) A v versus v/s graph is drawn for all the data.
- (b) V_{max} and K_m (the intercept on the ordinate and the slope) are calculated for the linear portion of the graph at high substrate concentration.
- (c) v/s is calculated from this V_{max} and K_m for each of the lower substrate concentrations. Each of these v/s values is subtracted from the actual values of v/s obtained at the low substrate concentrations to give v/s values corresponding to the low K_m enzyme.
- (d) From these v/s values, values of v are calculated and a new v versus v/s graph is plotted.
- (e) The linear portion of this graph is used to calculate V_{max} and K_m for the low K_m enzyme.
- (f) As in stages (c) and (d), the contribution of the low K_m enzyme to v/s values at high substrate concentration is calculated and a plot is produced for the high K_m enzyme.
- (g) The cycle (b) to (f) is repeated until the calculations converge to within the experimental error of the results. Often two or three cycles are sufficient.

It was not found to be useful to calculate initially V_{max} and K_m for the low K_m enzyme, whatever the proportions of the two enzymes were.

A computer programme was produced which dealt with all but the graph plotting aspects of this procedure.

The procedure was frequently complicated by the finding that the high K_m enzyme appeared to exhibit sigmoid rather than Michaelis-Menten kinetics. (A further complication arose since sometimes the sigmoid kinetics did not become apparent until the first correction had been applied to the high substrate concentration data.) In such cases the high substrate concentration data were extrapolated to the abscissa and the contribution of the high K_m enzyme to the value of v/s at low substrate concentration was estimated (see Figure 2.9). It was assumed that the low K_m enzyme obeyed Michaelis-Menten kinetics.

The Hill coefficient, n , was used as an estimate of the degree of cooperativity of the high K_m enzyme (Whitehead, 1970; Newsholme & Start, 1973).

G.3 CALCULATION OF V_{MAX}

V_{max} was calculated according to the formula:

$$V_{max} \text{ (nmoles/min/g wet wt.)} = \frac{(h + 1000w).a.s.c}{w.v.t.T}$$

where h is the volume, in μl , in which the tissue was homogenized;

w is the weight, in grams, of tissue homogenized;

a is the total volume, in ml, of the assay mixture;

s is substrate concentration (μM);

c is the counts which appeared in the product;

v is the volume, in μl , of homogenate added to the assay;

t is the time of the assay (minutes);

T is the total counts which could appear in the product.

In the assays which involved the thin-layer chromatography, T was the number of counts in substrate in the blank. Calculation of T for each individual tube did not improve the accuracy of the experiments.

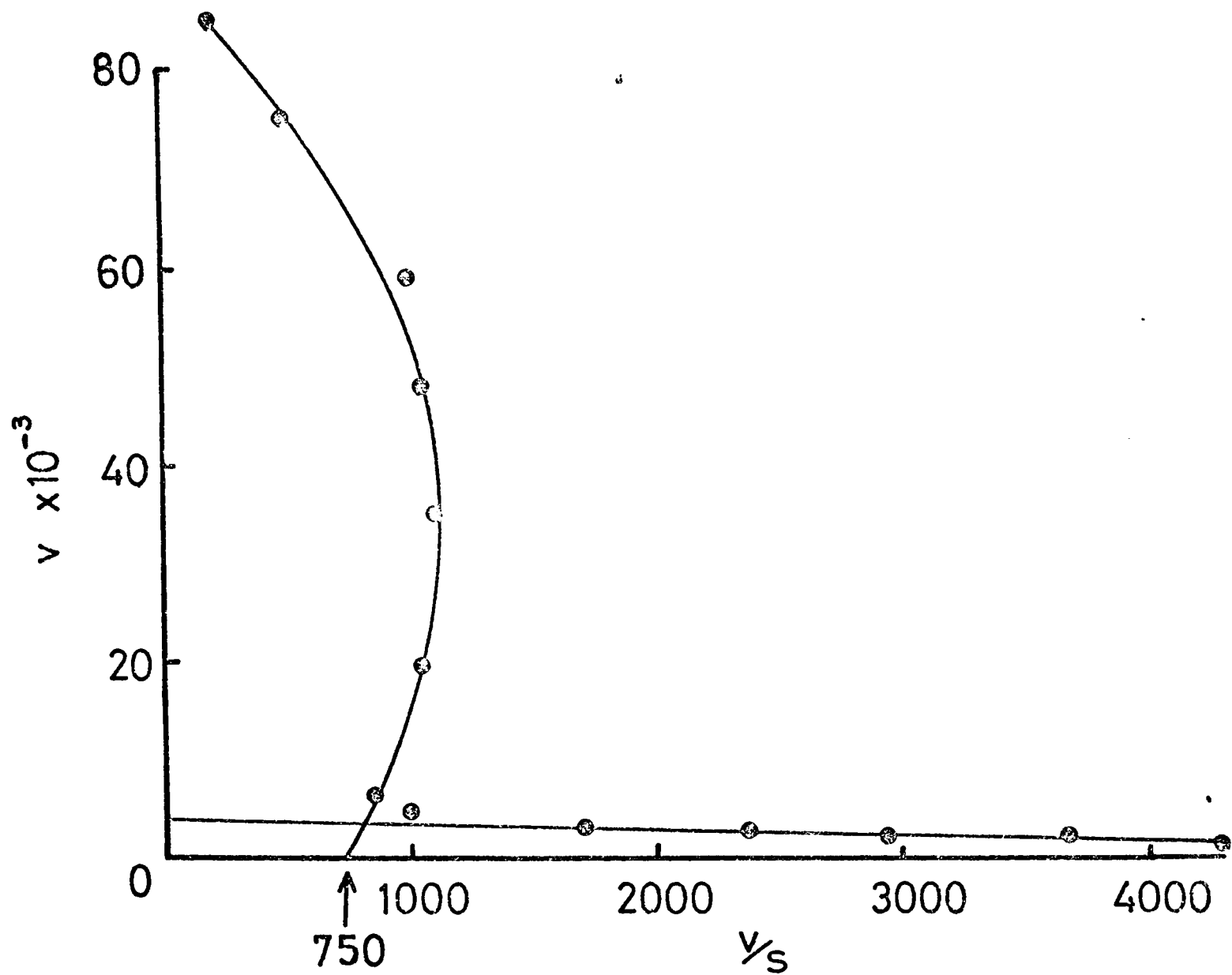


FIG 2.9 a The initial stages in the analysis of a complex phosphodiesterase kinetic plot.

Cyclic AMP phosphodiesterase was assayed as described in section A3.

The data are taken from an experiment on the rat liver enzyme.

The abscissa (v/s) is the counts per minute which appeared in the product; the ordinate (v) is this value multiplied by s (μM) (see section G3). On this page the initial plot is presented. Overleaf, the first stages in the analysis of the plot are illustrated (see text for details of the method.)

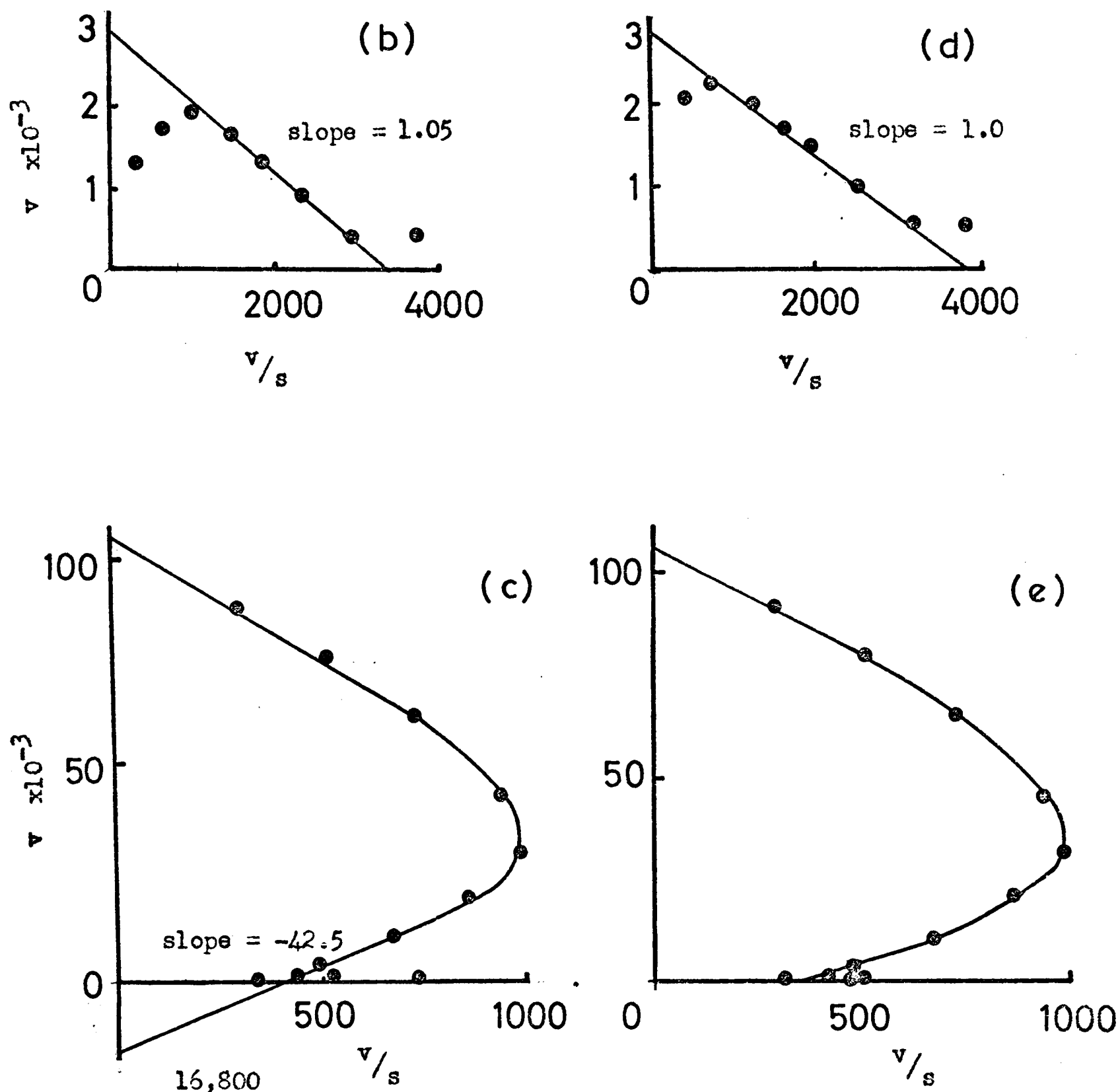


FIG 2.9(b-e) The initial stages in the analysis of a complex phosphodiesterase kinetic plot.

See legend to Figure 2.9a.

- (b) The first corrected low K_m enzyme plot. (750 has been subtracted from each value of v/s at low substrate concentrations.)
- (c) The first corrected high K_m enzyme plot. (The value of $2900/1.05 + s$ has been subtracted from each value of v/s .)
- (d) The second corrected low K_m enzyme plot. (The value of $16800/42.5 - s$ has been subtracted from each value of v/s .)
- (e) The second corrected high K_m enzyme plot. (The value of $2950/1.0 + s$ has been subtracted from each value of v/s .)

H. THE STATISTICAL SIGNIFICANCE OF THE RESULTS

Three factors may lead to variations in the maximal catalytic activities and K_m 's measured for an enzyme from the same tissue of animals of a given species. Firstly, the assay method may give different results when the activity of an enzyme in the same extract is measured a number of times. In the present study duplicate experiments performed on the same extract, or on two extracts of the same tissue prepared in parallel, always gave results which agreed within 10%. This is well within the variations of enzyme activity which were usually found for a tissue of a given species. Secondly, there may be differences in the activity or K_m of an enzyme between two animals of a given species owing to genetic differences, the dietary state of the animals, age, season, etc. In the comparative studies reported in this thesis (but not in the results of Chapter 5) no account has been taken of these factors and so they may account for many of the variations in the results. Thirdly, variations in the extraction method for an enzyme between two experiments may lead to large differences in the activities and K_m values measured. This has been shown to be an important problem for the enzymes assayed for this thesis, in particular for adenosine kinase (Section D.6).

Therefore, the variations in the activities and K_m 's of enzymes recorded in the comparative studies may reflect both "real" differences between animals and the difficulty of extracting the enzymes. For this reason no attempt has been made to interpret the precise quantitative data obtained in each experiment. However, where it is possible to show that a number of results for different species conform to a certain pattern, an attempt has been made to interpret the observation. The results of the comparative studies are presented as a mean with the range of activities. Standard deviations have not been calculated but, if they are required, they can be calculated from the range by the method of Dean & Dixon (1951).

Chapter 3

STUDIES OF ADENYL CYCLASE AND PHOSPHODIESTERASE

A. INTRODUCTION AND METHODS

This study of the activities and properties of the enzymes which produce and metabolize cyclic nucleotides provides further information concerning the roles of the enzymes in controlling cyclic nucleotide concentrations and the roles of cyclic AMP and cyclic GMP in different tissues and in animals widely distributed in the animal kingdom. The approach to these problems has been to measure the maximal activities and kinetics of adenylyl cyclase and of cyclic AMP and cyclic GMP phosphodiesterase. In addition, basal activities (in the absence of F^- or hormones) of adenylyl cyclase have been measured, since they may represent the in vivo activities of adenylyl cyclase when it is not stimulated by hormones. The regulation of cyclic nucleotide concentrations is examined by applying the data obtained to a model of the in vivo situation.

The standard methods used to homogenize tissues and assay the enzymes for the work presented in this chapter have been described in Chapter 2, Section A. Some additional details are described below.

A.1 VARIATIONS IN THE PROCEDURE FOR EXTRACTION OF ADENYL CYCLASE

The effect of the method used to extract adenylyl cyclase on the basal and fluoride - or hormone - stimulated activities of the enzyme was studied in the following manner. The tissue was gently extracted in a homogenizer made of smooth glass using a teflon pestle. An isotonic extraction medium was employed (250 mM sucrose, 20 mM Tris chloride, pH 7.5, 1 mg/ml bovine serum albumin). Any sediment was removed by very low speed (100g) centrifugation. In order to compare the effects of different homogenization procedures, one portion of the extract was rehomogenized in the manner

described above after dilution (one volume into ten volumes) into the isotonic extraction medium and another portion was rehomogenized using a ground-glass homogenizer and pestle after dilution into hypotonic extraction medium (Chapter 1, Section A.1). Basal and fluoride- and/or hormone-stimulated activities of adenylyl cyclase were measured for both portions of the extract as described below, except that 250 mM sucrose was present during the assay of the enzyme extracted in isotonic medium.

A.2 MEASUREMENT OF THE ACTIVITY OF ADENYLYL CYCLASE

For measurements of the basal activities of adenylyl cyclase, nervous tissues (and all other tissues) were extracted in a medium from which Triton X-100 was omitted (see Chapter 2, Section A.1). Maximal activities of adenylyl cyclase from nervous tissues were obtained by including Triton X-100 in the extraction medium or by stimulating the enzyme with F^- . (F^- does not stimulate the activity of the brain enzyme if it has been extracted in the presence of Triton X-100 - Perkins & Moore, 1971; see also Johnson & Sutherland, 1973. This was confirmed in the present study for the mouse brain enzyme.)

Fluoride-stimulated activities of adenylyl cyclase were measured as follows. Tissues were extracted in the absence of F^- as described in Chapter 2, Section A.1. One portion of the homogenate was diluted with the same extraction medium and another was diluted into a similar medium, except that it contained NaF and supplementary $MgCl_2$, so that the final concentrations were 12 mM NaF and 12.3 mM $MgCl_2$. (This procedure enabled the fluoride-stimulated activity to be compared with a basal activity measured for the same extract.) The diluted extracts were incubated at $30^{\circ}C$ for 10 minutes (unless otherwise indicated). They were then assayed for adenylyl cyclase as described in Chapter 2, Section A.2, except that the fluoride-stimulated activity was measured in the presence of 10 mM NaF and 8.7 mM $MgCl_2$.

A.3 SUBSTRATE CONCENTRATIONS FOR ENZYME ASSAYS

The activity of phosphodiesterase from each tissue was initially assayed using at least 12 different substrate concentrations (0.2 to 500 μM) so that its kinetic behaviour could be assessed. In the comparative study, the activity of adenylyl cyclase was usually assayed at 5 substrate concentrations (100 to 2000 μM) in order to increase the accuracy of V_{max} measurement. The method also permitted the determination of K_m values for the enzyme. When the measurement of V_{max} was not the purpose of an assay of the activity of adenylyl cyclase, 500 μM ATP was used as substrate.

B. RESULTS

B.1 THE EFFECT OF EXTRACTION PROCEDURE ON THE ACTIVITY OF ADENYLYL CYCLASE

The basal activity of adenylyl cyclase from mouse brain was consistently increased (52 to 57% in three experiments) after it had been rehomogenized in hypotonic medium (see Section A.1 for methods). In the case of mouse liver, the effect of rehomogenization in hypotonic medium on the basal activity was very variable (from a decrease of 20% to an increase of 130% in four experiments). However, there was a consistent increase in the effects of F^- , adrenaline and glucagon: the ratio, activity in the presence of effector/activity in the absence of effector, increased by about 60% in each case (Table 3.1). It may also be noted that, following rehomogenization in either isotonic or hypotonic medium, the adrenaline- and fluoride-stimulated activities were similar to each other, whereas the glucagon-stimulated activity was greater by about 70% (see also Chapter 1, Section Ala(ii) and Birnbaumer et al., 1971).

It was concluded that the extraction of adenylyl cyclase into a hypotonic medium releases the enzyme from environments where its in vitro activity is limited but does not disrupt the integrity of the adenylyl cyclase complex (hormone receptor, transducer(?), catalytic unit - see Birnbaumer et al., 1970;

TABLE 3.1 The effect of extraction procedure on the stimulation of the activity of adenyl cyclase from mouse tissues by fluoride and hormones

Methods are described in Section A1. The ratio of activities of adenyl cyclase, plus effector/minus effector, is given following rehomogenization of the tissue in both isotonic and hypotonic media. The mean basal activities of the brain enzyme following rehomogenization in isotonic and hypotonic media were 48 and 74 nmol/min/g wet wt., respectively. The corresponding activities for the liver enzyme were 0.8 and 1.0 nmol/min/g wet wt. Results are the means of three experiments, unless indicated otherwise.

TISSUE	EFFECTOR	RATIO OF ACTIVITIES	
		ISOTONIC REHOMOGENIZATION	HYPOTONIC REHOMOGENIZATION
Liver	F ⁻ (1x10 ⁻² M)	1.61	2.81
	Adrenaline (5x10 ⁻⁶ M)	1.61	2.50
	Glucagon (4x10 ⁻⁷ M)	2.82	4.35
Brain	F ⁻ (1x10 ⁻² M)	1.68	1.58
	Noradrenaline (1x10 ⁻⁴ M)*	0.97	1.10
	Dopamine (1x10 ⁻⁵ M)*	0.87	1.21

* Single experiments

Perkins, 1973). Therefore this method of extraction was used in all subsequent studies.

B.2 THE ACTIVITIES OF ADENYL CYCLASE AND PHOSPHODIESTERASE FROM ANIMAL TISSUES

The kinetic data for cyclic AMP and cyclic GMP phosphodiesterases indicated (with some exceptions - see Tables 3.2 to 3.4) that the enzyme had at least two K_m values. The data were analyzed as described in Chapter 2, Section G. In the following discussion it is assumed that each K_m value represented a distinct molecular species (see Chapter 1, Section Alb(i)).

The basal and fluoride-stimulated activities of adenylyl cyclase, and the activities of the low and high K_m enzymes (see Chapter 2, Section G) of phosphodiesterase for both cyclic AMP and cyclic GMP substrates in muscles of vertebrates and invertebrates are shown in Table 3.2. Equivalent data for nervous tissues are given in Table 3.3 and for livers and some other tissues in Table 3.4. All the activities recorded are V_{max} values. In this section the general features emerging from the study will be noted for each enzyme in turn.

(a) ADENYL CYCLASE

There is a distinct correlation between the activity of adenylyl cyclase in a muscle and its capacity for oxidative metabolism. (This correlation has been noted by Severson et al. (1972) on the basis of very limited data for only vertebrate muscles.) Thus the highest mean basal activities were obtained for insect flight muscles and vertebrate hearts; they range from 13 to 2 nmol/min/g wet wt. (pigeon and trout hearts). Only the activity of adenylyl cyclase from the flight muscle of the honey bee falls below this range. The next highest basal activities were obtained for "red" skeletal muscles (for a description of red muscles see Needham, 1926, 1971); these activities range from 1.4 to 1.1 nmol/min/g wet wt. (the red parts of mouse

TABLE 3.2 Activities of adenyl cyclase and phosphodiesterase in muscles from vertebrates and invertebrates

The enzyme assays are described in Chapter 2, Section A (and in Sections A2 and A3 of the present chapter). The calculation of the activities of the phosphodiesterase enzymes is described in Chapter 2, Section G2. All activities are $V_{\max}$ values; they are expressed as a mean, with the range of activities and the number of animals used given in parentheses. In those cases where the kinetics indicated that only one phosphodiesterase was present, this enzyme was classified as "low K_m " if its K_m value was below 5 μM . A dash (-) indicates that this activity has not been determined; n.d. indicates that the activity was not detectable.

ANIMAL	MUSCLE	ENZYME ACTIVITIES (nmol/min/g wet wt. of tissue, 30°C)					
		ADENYL CYCLASE		CYCLIC AMP PHOSPHODIESTERASE		CYCLIC GMP PHOSPHODIESTERASE	
		Basal Activity	F ⁻ -Stimulated Activity	Low K_m	High K_m	Low K_m	High K_m
Rat tapeworm (<u>Hymenolepis diminuta</u>)	Whole animal	-	-	116 (89-142)(2)	207 (199-215)(2)	37 (28-46)(2)	136 (134-138)(2)
Pig roundworm (<u>Ascaris lumbricoides</u>)	Body wall	<0.2 (2)	-	1.5 (1.2-1.7)(2)	7.2 (3.4-11)(2)	0.03 (1)	n.d. (2)
Earthworm (<u>Allolobophora longa</u>)	Body wall	0.4 (1)	-	-	-	-	-
Scallop (<u>Chlamys varia</u>)	Fast adductor	-	-	14 (1)	260 (1)	-	-
	Slow adductor	-	-	162 (1)	558 (1)	-	-
Scallop (<u>Pecten maximus</u>)	Fast adductor	0.17 (0.15-0.20)(2)	-	34 (27-41)(2)	143 (71-215)(2)	4 (1)	58 (1)
	Slow adductor	3.5 (1.1-5.9)(2)	-	393 (297-490)(2)	934 (1)	25 (1)	98 (1)
Lobster (<u>Homarus vulgaris</u>)	Abdomen	0.4 (0.3-0.5)(2)	-	8.0 (4.1-12)(4)	6.3 (4.0-8.6)(2)	0.7 (0.4-1.0)(2)	3.6 (3.0-4.3)(2)
	Chela	0.5 (0.5)(2)	-	5.2 (4.3-6.1)(2)	21 (13-29)(2)	0.6 (0.4-0.7)(2)	4.7 (3.0-6.4)(2)
Locust (<u>Schistocerca gregaria</u>)	Flight	6.3 (4.4-8.1)(6)	9.8 (8.6-11)(3)	116 (86-134)(4)	159 (84-209)(3)	12 (12)(2)	52 (42-64)(3)
	Hind leg femoral	0.90 (0.70-1.1)(4)	3.4 (2.3-4.5)(2)	20 (17-23)(3)	75 (67-88)(3)	4.9 (3.2-6.6)(2)	35 (35-36)(2)
Cockroach (<u>Periplaneta americana</u>)	Flight	4.3 (1)	-	-	-	-	-
Cockroach (<u>Blaberus discoidalis</u>)	Flight	5.7 (4.8-7.4)(3)	8.5 (1)	131 (105-172)(3)	83 (57-131)(3)	5.6 (4.4-6.8)(2)	23 (23)(2)
Rosechafer (<u>Pachnoda ephippiata</u>)	Flight	2.8 (2.6-3.3)(3)	-	70 (51-91)(3)	n.d. (4)	1.1 (0.1-1.5)(3)	27 (11-43)(2)

ANIMAL	MUSCLE	ENZYME ACTIVITIES (nmol/min/g wet wt. of tissue, 30°C)					
		ADENYL CYCLASE		CYCLIC AMP PHOSPHODIESTERASE		CYCLIC GMP PHOSPHODIESTERASE	
		Basal Activity	F ⁻ -Stimulated Activity	Low K _m	High K _m	Low K _m	High K _m
Honey-bee (<u>Apis mellifera</u>)	Flight	0.73 (0.42-1.0)(3)	3.1 (1.5-4.0)(3)	44 (40-53)(3)	n.d. (2)	3.6 (1)	30 (1)
Bumble-bee (<u>Bombus</u> sp) ^a	Flight	4.3 (3.0-6.9)(3)	9.3 (5.3-12)(3)	35 (32-39)(3)	47 (41-54)(2)	8.0 (1)	n.d. (1)
Blowfly (<u>Calliphora erythrocephala</u>)	Flight	5.2 (4.2-6.5)(3)	-	53 (43-64)(4)	123 (100-145)(2)	n.d. (3)	12.4 (8.3-20)(4)
Dogfish (<u>Scylliorhinus canicula</u>)	Red abdominal	-	-	2.9 (1)	4.5 (1)	-	-
	White abdominal	-	-	0.15 (0.10-0.20)(2)	0.48 (0.40-0.57)(2)	-	-
Rainbow trout (<u>Salmo gairdneri</u>)	Heart	2.0 (1.7-2.3)(2)	8.8 (8.1-9.5)(2)	107 (103-111)(2)	102 (75-130)(2)	55 (53-57)(2)	192 (164-220)(2)
	Red abdominal	1.1 (0.9-1.3)(2)	3.0 (1)	5.6 (4.3-6.9)(2)	28 (20-36)(2)	2.4 (1.3-3.5)(2)	43 (34-52)(2)
	White abdominal	<0.3 (3)	1.8 (1.7-2.0)(3)	1.5 (1.2-1.8)(2)	6.3 (5.1-7.5)(2)	0.30 (0.29-0.32)(2)	7.7 (6.7-818)(2)
Frog (<u>Rana temporaria</u>)	Heart	2.3 ^b (0.7-4.6)(5)	10.1 ^b (10.1-10.2)(2)	58 (53-65)(3)	1150 (1030-1360)(3)	43 (25-58)(2)	770 (604-938)(2)
	Gastrocnemius	1.1 ^c (0.94-1.3)(2)	0.33 ^c (0.31-0.35)(2)	6.0 (3.2-7.0)(3)	63 (33-49)(3)	2.1 (1.5-2.7)(2)	43 (25-59)(2)
Domestic pigeon (<u>Columba livia</u>)	Heart	13 (6.1-16)(6)	18 (16-20)(2)	308 (205-410)(2)	875 (750-1000)(2)	318 (259-377)(2)	392 (371-413)(2)
	Pectoral	1.2 (1.0-1.3)(5)	4.6 (1)	13 (12-14)(4)	130 ^d (66-240)(2)	n.d. (2)	275 (243-307)(2)
	Sartorius	0.5 (0.38-0.62)(2)	1.9 (1.6-2.2)(3)	12 (1)	173 (1)	n.d. (1)	118 (1)
Domestic fowl (<u>Gallus gallus</u>)	Heart	4.0 (2.9-4.8)(3)	-	71 (61-85)(3)	167 (74-217)(3)	57 (1)	115 (1)
	Pectoral	0.5 (1)	-	8.6 (8.5-8.7)(3)	13 (11-16)(3)	2.3 (2.2-2.4)(2)	16 (1)
	Sartorius	0.4 (1)	-	9.0 (7.9-11)(3)	11 (9-13)(2)	16 (11-21)(3)	13 (12-15)(2)
Pheasant (<u>Phasianus colchicas</u>)	Heart	4.3 (1)	-	114 (1)	115 ^e (1)	45 (1)	102 (1)
	Pectoral	0.15 (0.11-0.3)(3)	-	15 (14-16)(2)	27 (23-32)(2)	6.0 (1)	15 (1)
	Sartorius	1.3 (1)	-	6.2 (1)	7.8 (1)	4.3 (1)	13.5 (1)

ANIMAL	MUSCLE	ENZYME ACTIVITIES (nmol/min/g wet wt. of tissue, 30°C)					
		ADENYL CYCLASE		CYCLIC AMP PHOSPHODIESTERASE		CYCLIC GMP PHOSPHODIESTERASE	
		<u>Basal Activity</u>	<u>F⁻-Stimulated Activity</u>	<u>Low K_m</u>	<u>High K_m</u>	<u>Low K_m</u>	<u>High K_m</u>
Laboratory mouse	Heart	7.0 (5.9-9.5)(4)	18 (18-19)(2)	30 (20-39)(3)	341 (323-352)(3)	n.d. (2)	452 (429-476)(2)
	Gastrocnemius (white)	0.7 (0.5-1.0)(5)	2.0 (1)	10.7 (9.8-12.6)(3)	51 (40-77)(3)	n.d. (2)	42 (30-54)(2)
	Gastrocnemius (white and red)	1.4 (1.3-1.4)(2)	3.8 (1)	8.5 (1)	140 (1)	n.d. (1)	109 (1)
Laboratory rat	Heart	10.5 (6.3-11.4)(8)	17 (1)	12 (11-15)(3)	226 (151-277)(3)	n.d. (2)	296 (272-320)(2)

- Adenyl cyclase values are for Bombus terrestris males; cyclic AMP phosphodiesterase values are for B. agrorum or B. lapidarius workers; cyclic GMP phosphodiesterase values are for B. agrorum.
- Adenyl cyclase from the hearts of two different groups of frogs had a basal activity of about 0.8 in one case and 4.6 in the other. The F⁻-stimulated activities were obtained for the latter group.
- Adenyl cyclase from the gastrocnemius muscle of two different pairs of frogs had a basal activity of 1.1 in one case and 0.15 in the other. The F⁻-stimulated activities were obtained for the latter pair.
- The high K_m cyclic AMP phosphodiesterase enzyme from two different pairs of pigeons had a mean activity of 227 in the one case and 78 in the other.
- This enzyme had a very high K_m (>500 μM). The value recorded was the activity of the enzyme in the presence of 150 μM substrate.

TABLE 3.3 Activities of adenyl cyclase and phosphodiesterase in nervous tissues from vertebrates and invertebrates.

See legend to Table 3.2 for details

ANIMAL	NERVOUS TISSUE	ENZYME ACTIVITIES (nmol/min/g wet wt. of tissue 30°C)					
		Basal Activity	ADENYL CYCLASE Triton-stimulated Activity	F ⁻ -Stimulated Activity	CYCLIC AMP PHOSPHODIESTERASE Low K _m	High K _m	CYCLIC GMP PHOSPHODIESTERASE Low K _m High K _m
Earthworm (<u>Allolobophora longa</u>)	Nerve cord	-	1.8 (1)	-	-	-	-
Lobster (<u>Homarus vulgaris</u>)	Cerebral ganglion	-	3.0 (2.2-3.8)(2)	-	101 (88-119)(3)	151* (1)	130 (109-140)(3) 257 (232-283)(2)
Locust (<u>Schistocerca gregaria</u>)	Cerebral ganglion	46 (42-49)(2)	70 (42-94)(5)	171 (167-175)(2)	396 (322-521)(3)	1690 (1000-2080)(3)	65 (76-50)(3) 3730 (3500-3950)(2)
Cockroach (<u>Blaberus discoidalis</u>)	Cerebral ganglion	62 (48-77)(2)	62 (44-80)(2)	199 (185-210)(2)	770 (730-800)(3)	400 (400)(2)	560 (410-710)(2) 1370 (1180-1560)(2)
Rosechafer (<u>Pachnoda ephippiata</u>)	Cerebral ganglion	-	38 (25-53)(2)	-	223 (195-250)(2)	250 (250)(2)	42 (40-44)(2) 153* (1)
Rainbow Trout (<u>Salmo gairdneri</u>)	Whole brain	<5	21 (20-23)(2)	28 (1)	39 (20-58)(2)	284 (1)	62 (42-82)(2) 225 (150-300)(2)
Frog (<u>Rana temporaria</u>)	Whole brain	23 (22-24)(2)	24 (24-25)(2)	35 (32-37)(2)	34 (29-39)(2)	1290 (1080-1500)(2)	n. d. (1) 1240 (1)
Domestic pigeon (<u>Columba livia</u>)	Whole brain	38 (34-42)(2)	150 (94-223)(4)	73 (54-90)(2)	61 (54-94)(3)	15200 (9000-11,600)(3)	n. d. (1) 12,100 (1)
Domestic fowl (<u>Gallus gallus</u>)	Whole brain	-	152 (130-168)(3)	-	97 (81-113)(2)	8850 (8750-8950)(2)	n. d. (1) 11,800 (1)
Laboratory mouse	Whole brain	77 (63-91)(2)	71 (60-83)(5)	123 (85-161)(2)	95 (82-130)(3)	5550 (5520-5600)(3)	n. d. (1) 8250 (1)

* In some experiments these enzymes appeared to have a very high K_m (200-1000 μM) and a very high V_{max} (300-1300 nmol/min/g wet wt.).

TABLE 3.4 Activities of adenyl cyclase and phosphodiesterase in livers from vertebrates and some other mammalian tissues

See legend to Table 3.2 for details.

ANIMAL	TISSUE	ENZYME ACTIVITIES (nmol/min/g wet wt. of tissue, 30°C)					
		ADENYL CYCLASE		CYCLIC AMP PHOSPHODIESTERASE		CYCLIC GMP PHOSPHODIESTERASE	
		<u>Basal Activity</u>	<u>F⁻-Stimulated Activity</u>	<u>Low K_m</u>	<u>High K_m</u>	<u>Low K_m</u>	<u>High K_m</u>
Rainbow trout (<i>Salmo gairdneri</i>)	Liver	<0.5 (3)	2.9 (2.1-3.9)(3)	18 (11-25)(2)	40 (39-41)(2)	8.0 (7.0-8.9)(2)	70 (58-82)(2)
Frog (<i>Rana temporaria</i>)	Liver	3.8 (3.0-4.5)(4)	13 (13-14)(2)	12 (8-17)(3)	263 (200-340)(3)	21 (20-22)(2)	231 (216-246)(2)
Domestic pigeon (<i>Columba livia</i>)	Liver	2.8 (1.9-4.18)(5)	7.6 (6.1-9.1)(2)	19 (18-19)(3)	366 (335-426)(3)	n.d. (1)	640 (1)
Domestic fowl (<i>Gallus gallus</i>)	Liver	1.6 (1.5-1.7)(2)	-	5.6 (4.9-6.4)(2)	74 (68-80)(2)	n.d. (1)	126 (1)
Laboratory mouse	Liver	1.2* (0.4-2.6)(6)	5.9* (3.9-7.8)(2)	27 (25-29)(2)	555 (550-560)(2)	n.d. (1)	682 (1)
Laboratory rat	Liver	-	-	19 (15-25)(4)	481 (380-567)(4)	n.d. (4)	588 (444-691)(4)
Laboratory mouse	Lung	14.5 (1)	-	17 (1)	280 (1)	n.d. (1)	300 (1)
	Blood	-	-	5.9 (4.3-7.5)(2)	10.2 (9.4-11.1)(2)	n.d. (2)	16 (15-17)(2)
Laboratory rat	Epididymal fat pad	-	-	1.3 (0.9-1.7)(4)	60 (40-77)(4)	n.d. (3)	53 (50-55)(3)

* Adenyl cyclase from the livers of two different groups of mice had a mean basal activity of 0.7 in one case and 2.0 in the other. The F⁻-stimulated activities were obtained from the latter group.

gastrocnemius muscle and pigeon pectoral muscle). Most of the muscles examined in the present study appeared to be of uniform colour but the gastrocnemius muscle of the mouse could be easily dissected into white and reddish portions. When the red portion of the muscle was excluded, the activity of adenyl cyclase was typical of white skeletal muscles; the activities in the latter range from 1.1 to less than 0.3 nmol/min/g wet wt. (frog gastrocnemius and trout white muscles). The basal activities of adenyl cyclase from muscles varied over an overall range of greater than 40-fold. The fluoride-stimulated activities conformed to an identical pattern to that described for the basal activities. Thus they were higher in red than in white muscles.

The activities of adenyl cyclase from livers and nervous tissues varied over smaller ranges than those from muscle, except that the activities in lobster and earthworm nervous tissues were exceptionally low. Excluding these values, the ranges were 5-fold for livers and 7-fold for nervous tissues. The activities from livers were similar to those measured in red muscles from the same species. However, the activities of adenyl cyclase from nervous tissues were exceptionally high; the highest activity measured was 199 nmol/min/g wet wt. (fluoride-stimulated adenyl cyclase from the brain of the cockroach).

(b) CYCLIC AMP PHOSPHODIESTERASE

The total activity of cyclic AMP phosphodiesterase is highest in those muscles which have the greatest capacity for oxidative metabolism. However, this correlation is less distinct than in the case of adenyl cyclase. The sum of the mean activities of the high and low K_m enzymes ranges from 0.6 to 1208 nmol/min/g wet wt. (dogfish white abdominal muscle and frog heart) - a range of 2000-fold.

The individual activities of the low and high K_m enzymes from muscle tissues conform to a similar pattern to that described for the total activities,

although there are some exceptions. (For example, the activities of the low K_m enzyme from the hearts of the rat and mouse are similar to those for skeletal muscles.)

In nervous tissues the range of activities of low K_m , high K_m or total phosphodiesterase was no more than 20-fold (cf. the 2000-fold variation between different muscles, above). The activity of the low K_m enzyme was often similar to the activity of the enzyme in the heart (vertebrates) or flight muscle (insects) of the same species. In contrast the activities of the high K_m enzyme were very high in the brains of the mouse, domestic fowl and pigeon; the mean activity in the brain of the pigeon was 15,200 nmol/min/g wet wt.

In vertebrate livers the activities of the low K_m cyclic AMP phosphodiesterase were all very similar; they ranged from 5.6 to 27 nmol/min/g wet wt. (domestic fowl and mouse). The low K_m enzyme was consistently detected in homogenates of rat liver. This differs from the results of Thompson & Appleman (1971b), who found that the low K_m enzyme was only detectable after preparations of rat liver had been allowed to stand at 4°C for 24 hours (Russell et al., 1973). In the present study the kinetics of cyclic AMP and cyclic GMP phosphodiesterases were identical when the enzymes were extracted at 30°C and assayed immediately and when they were extracted at 4°C and assayed after the extract had been incubated for two hours at this temperature. The mean activities of the high K_m cyclic AMP phosphodiesterase from livers ranged from 40 to 555 nmol/min/g wet wt. (trout and mouse).

(c) CYCLIC GMP PHOSPHODIESTERASE

The pattern of cyclic GMP phosphodiesterase activities from vertebrate muscles is similar to that described for cyclic AMP phosphodiesterase. Thus the sum of the activities of the high and low K_m cyclic GMP phosphodiesterases was highest for vertebrate hearts; these activities ranged from 813 to 147

nmol/min/g wet wt. (frog and pheasant heart). Generally the redder skeletal muscles contained higher activities of the enzyme but the correlation was not clear-cut. Amongst the vertebrates, the lowest total activity of cyclic GMP phosphodiesterase measured was 8 nmol/min/g wet wt. (trout white abdominal muscle). However, in contrast to the activities of cyclic AMP phosphodiesterase, the total activities of cyclic GMP phosphodiesterase from insect flight muscle were low — similar to the activities of the enzyme from vertebrate skeletal muscles. The activities of the enzyme from the fly and bumble-bee were particularly low (8 nmol/min/g wet wt.).

The low K_m cyclic GMP phosphodiesterase was not detectable in many vertebrate tissues. With the exception of these tissues and invertebrate muscles (see above), the distributions of the activities of the individual cyclic GMP phosphodiesterases in livers, muscles and nervous tissues are similar to the distributions described for the individual cyclic AMP phosphodiesterases. The widespread distribution of a cyclic GMP phosphodiesterase with a low K_m value does not appear to have been recognized previously — probably because most studies have used mammalian tissues in which this enzyme cannot be detected.

B.3 THE STIMULATION OF THE ACTIVITY OF ADENYL CYCLASE BY FLUORIDE AND TRITON X-100

(a) THE EFFECT OF FLUORIDE

The stimulation of the activity of adenylyl cyclase by F^- is irreversible. The effect of F^- develops over a period of time, at a rate which is dependent upon the temperature and the concentrations of F^- and Mg^{2+} (Perkins & Moore, 1971; but see also Birnbaumer et al., 1971). Under the conditions used in the present work, preincubation of the homogenate with F^- and Mg^{2+} for 10 minutes at 30°C (the control homogenate was also incubated) was adequate to produce maximal stimulation of the activity of the enzyme by F^- (Figure 3.1).

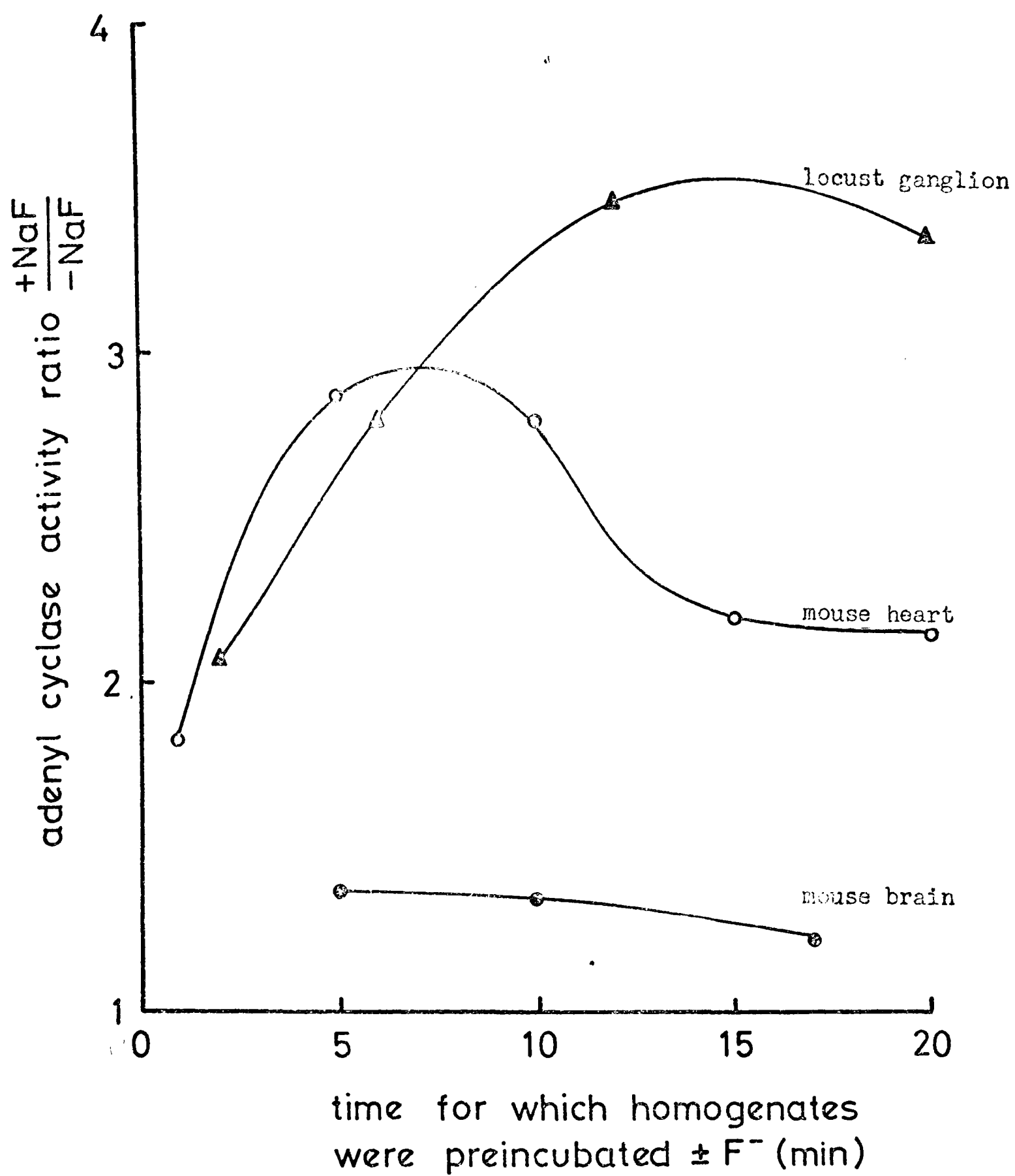


FIG 3-1 The timecourse of activation of adenylyl cyclase by F^- .

Methods are described in section A2.

Further incubation of the homogenates frequently resulted in a fall in both basal and fluoride-stimulated activities and in a reduced activity ratio, plus F^- /minus F^- .

The degree of stimulation by F^- of the activity of adenyl cyclase ranged from 1.4 to greater than 6 (Table 3.5). In most cases the activity in the presence of F^- was 2 to 4 times the activity in the absence of F^- but there was a consistently lower effect of F^- on the activity of adenyl cyclase from vertebrate brains (see also Sutherland et al., 1962). Similar degrees of stimulation by F^- (and hormones) of the activity of adenyl cyclase in crude homogenates have been measured in numerous other studies (see for example Murad & Vaughan, 1969; Birnbaumer et al., 1970; Triner et al., 1971; Severson et al., 1972). However, larger effects of F^- (up to 31-fold) have been measured when purified or fractionated adenyl cyclase from frog erythrocytes, rabbit skeletal muscles, rat liver and various hearts have been studied (see Perkins, 1973; Drummond & Duncan, 1970).

(b) THE EFFECT OF TRITON X-100

The relationship of detergent-stimulated activities of adenyl cyclase to the basal and fluoride-stimulated activities is difficult to evaluate. In some studies the activity of the detergent-treated enzyme could not be stimulated by F^- but the activity of the F^- -activated enzyme could be increased by detergent (Perkins & Moore, 1971). Similarly, in the present study, the enzyme from pigeon brain exhibited a greater activity when it was extracted in the presence of Triton X-100 and assayed in the absence of F^- than when it was extracted in the absence of Triton X-100 and assayed in the presence of F^- (Table 3.3). On the other hand adenyl cyclases extracted in the presence of detergents have on some occasions been found to be further activated by F^- (Levey, 1970; Ryan & Storm, 1974). In the present study, the fluoride-stimulated activities of adenyl cyclase from nervous tissues were, with the

TABLE 3.5 The stimulation of the activity of adenylyl cyclase by fluoride

Methods are described in Section A2. The ratio of activities of adenylyl cyclase, plus NaF/minus NaF, which is tabulated is the mean ratio obtained from a number of determinations of the ratio for each tissue. (It is not the ratio of the mean activities, plus NaF/minus NaF.) The individual activities are included in the data of Tables 3.2 to 3.4. The number of determinations of the ratio is given in parenthesis.

ANIMAL	TISSUE	RATIO OF ACTIVITIES OF ADENYL CYCLASE	
Locust	Cerebral ganglion	3.8	(2)
	Flight muscle	1.6	(3)
	Leg muscle	3.3	(3)
Cockroach	Cerebral ganglion	3.4	(2)
Bumble-bee	Flight muscle	2.5	(3)
Honey-bee	Flight muscle	4.1	(3)
Trout	Brain	>5	(1)
	Liver	>5	(3)
	Heart	3.5	(1)
	Red abdominal muscle	3.2	(1)
	White abdominal muscle	6	(1)
Frog	Brain	1.4	(2)
	Liver	3.5	(2)
	Heart	2.2	(2)
	Gastrocnemius muscle	>3	(2)
Pigeon	Brain	1.8	(2)
	Liver	3.9	(2)
	Heart	2.6	(2)
	Pectoral muscle	4.0	(2)
	Sartorius muscle	3.9	(2)
Mouse	Brain	1.6	(2)
	Liver	3.1	(3)
	Heart	2.4	(2)
	Gastrocnemius muscle	3.4	(2)

exception of pigeon brain, higher than the activities of the enzyme when it was extracted in the presence of detergent (Table 3.3). Thus detergent may release adenylyl cyclase from environments where its in vitro activity is limited, so that Triton-stimulated activity may be a better measure of the basal activity of adenylyl cyclase for most nervous tissues.

B.4 K_m VALUES AND DEVIATIONS FROM MICHAELIS-MENTEN KINETICS

The following discussion refers, in the case of phosphodiesterase, to kinetic data which have been analysed on the assumption that the unusual kinetics of phosphodiesterase result from the presence of two enzymes with different K_m values (see Chapter 2, Section G).

There was little variation in the mean K_m values for adenylyl cyclase or the phosphodiesterases from different species and tissues (Table 3.6). Thus the mean K_m values of adenylyl cyclase range from 80 to 660 μM ; those for low K_m cyclic AMP phosphodiesterase range from 0.67 to 4.1 μM , and those for low K_m cyclic GMP phosphodiesterase range from 0.45 to 5.3 μM . (However, when only one K_m value for cyclic GMP phosphodiesterase can be detected it is not always clear whether this value should be classified as high or low. Thus the cyclic GMP-hydrolyzing enzymes from mouse lung and blowfly flight muscle could be classified as low, rather than high, K_m .) The K_m values of the high K_m phosphodiesterases vary over a wider range but in general they are between 20 and 100 μM . All these K_m values are similar to previously reported values (see Appleman et al., 1973; Robison et al., 1971, p.90). In some species the K_m of an enzyme is similar for each tissue. Thus the low K_m cyclic AMP phosphodiesterase from trout tissues has a K_m value which is about 3-fold higher than in rat tissues. However, the results presented in Table 3.6 indicate that there is no correlation between the K_m value of adenylyl cyclase or the K_m values of the phosphodiesterases and the function of the tissue.

TABLE 3.6 K_m values of adenylyl cyclase and phosphodiesterase in tissues from various animals

K_m values were measured as described in Chapter 2, Section A (and in Section A.3 of the present chapter). The calculation of the K_m values of the phosphodiesterase enzymes is described in Chapter 2, Section G.2; if the kinetics of a phosphodiesterase enzyme did not produce a straight line on a Hofstee plot, the value recorded corresponds to the linear portion of the plot. The K_m values for phosphodiesterase which are recorded are those which were detectable over the range of substrate concentrations 0.2 to 500 μ M. The K_m values of adenylyl cyclase were measured for the enzyme exhibiting basal activity or for the enzyme which had been extracted in the presence of Triton X-100 (Nervous tissue). Mean values are presented, with the range and number of animals used given in parentheses.

ANIMAL	TISSUE	ADENYL CYCLASE	K _m VALUES (μM)			
			CYCLIC AMP PHOSPHODIESTERASE		CYCLIC GMP PHOSPHODIESTERASE	
			Low K _m	High K _m	Low K _m	High K _m
Rat tapeworm (<i>Hymenolepis diminuta</i>)	Whole animal	-	2.7 (2.4-3.1)(2)	63 (61-65)(2)	2.3 (2.2-2.4)(2)	151 (138-165)(2)
Pig roundworm (<i>Ascaris lumbricoides</i>)	Body wall	-	0.7 (0.55-0.82)(2)	24 (19-29)(2)	0.45 (1)	-
Scallop (<i>Chlamys varia</i>)	Fast adductor muscle	-	1.5 (1)	54 (1)	-	-
	Slow adductor muscle	-	3.0 (1)	78 (1)	-	-
Scallop (<i>Pecten maximus</i>)	Fast adductor muscle	200 (150-260)(2)	2.5 (2.3-2.6)(2)	90 (69-109)(2)	1.5 (1)	36 (1)
	Slow adductor muscle.	280 (1)	4.1 (3.2-4.9)(2)	107 (1)	1.1 (1)	24 (1)
Lobster (<i>Homarus vulgaris</i>)	Cerebral ganglion	280 (1)	1.3 (0.6-2.1)(3)	120 (75-165)(2)	3.0 (1.9-4.1)(2)	100 (1)
	Abdominal muscle	-	2.4 (1.2-3.1)(3)	120 (1)	1.9 (0.9-2.9)()	72 (52-93)(2)
	Claw muscle	390 (1)	1.3 (1.3-1.4)(2)	137 (1)	2.5 (2.0-3.0)(2)	123 (1)
Locust (<i>Schistocerca gregaria</i>)	Cerebral ganglion	170 (140-200)(3)	0.85 (0.60-1.1)(3)	77 (66-89)(3)	0.5 (0.4-0.7)(2)	84 (72-96)(2)
	Flight muscle	310 (1)	1.5 (0.7-2.8)(5)	22 (20-25)(2)	4.6 (4.4-4.7)(3)	59 (40-76)(3)
	Hind leg muscle	200 (110-300)(2)	1.4 (0.8-2.0)(2)	33 (30-35)(2)	2.0 (1.7-2.4)(2)	75 (43-107)(2)
Cockroach (<i>Blaberus discoidalis</i>)	Cerebral ganglion	180 (160-200)(3)	1.6 (1.6)(3)	40 (1)	6.3 (4.3-8.2)(2)	70 (60-80)(2)
Rosechafer (<i>Pachnoda ephippiata</i>)	Cerebral ganglion	170 (150-200)(2)	1.5 (1.1-1.9)(2)	- ^a	5.0 (4.7-5.4)(2)	- ^a
	Flight muscle	320 (240-390)(3)	1.7 (1.6-1.8)(3)	-	1.6 (1.5-1.8)(2)	- ^a

ANIMAL	TISSUE	K _m VALUES (μM)			
		ADENYL CYCLASE	CYCLIC AMP PHOSPHODIESTERASE		CYCLIC GMP PHOSPHODIESTERASE
			Low K _m	High K _m	
Honey-bee (<u>Apis mellifera</u>)	Flight muscle	130 (1)	2.0 (1.5-2.5)(2)	-	1.0 (1) 105 (1)
Bumble-bee (<u>Bombus</u> sp.)	Flight muscle	110 (1)	1.8 (1.0-2.8)(3)	12 (6-19)(2)	2.8 (1) -
Blowfly (<u>Calliphora erythrocephala</u>)	Flight muscle	370 (320-420)(3)	1.7 (1.7-1.8)(3)	36 (1)	- 7.1 (5.1-9.3)(3)
Dogfish (<u>Scylliorhinus canicula</u>)	Red abdominal muscle	-	1.6 (1)	24 (1)	- -
	White abdominal muscle	-	1.3 (1.1-1.5)(2)	11 (1)	- -
Rainbow trout (<u>Salmo gairdneri</u>)	Brain	250 (100-350)(3)	2.6 (2.0-3.2)(2)	60 (24-96)(2)	2.5 (2.4-2.6)(2) 32 (23-42)(2)
	Liver	160 (1)	2.0 (1.1-2.9)(2)	38 (27-48)(2)	1.3 (1.3-1.4)(2) 44 (39-49)(2)
	Heart	-	2.3 (1.7-2.8)(2)	-	0.83 (0.81-0.86)(2) 83 (83)(2)
	Red abdominal muscle	-	2.7 (2.4-3.0)(2)	50 (41-60)(2)	2.3 (2.1-2.6)(2) 24 (41-47)(2)
	White abdominal muscle	-	1.8 (1.6-2.0)(2)	78 (50-87)(2)	2.0 (1.9-2.1)(2) 70 (67-73)(2)
Frog (<u>Rana temporaria</u>)	Brain	200 (1)	1.0 (0.9-1.1)(2)	- ^b	- 13 (1)
	Liver	660 (518-820)(2)	0.85 (0.5-1.1)(3)	136 (133-140)(3)	4.7 (4.2-5.2)(2) 109 (85-133)(2)
	Heart	120 (70-190)(3)	0.9 (0.8-1.0)(3)	183 (170-200)(3)	1.9 (1.8-1.9)(2) 41 (28-55)(2)
	Gastrocnemius muscle	520 (260-790)(2)	1.7 (1.0-2.1)(3)	250 (135-400)(3)	2.3 (2.3)(2) 74 (73-75)(2)
Domestic pigeon (<u>Columba livia</u>)	Brain	290 (180-360)(5)	0.64 (0.58-0.70)(2)	- ^b	- 34 (1)
	Liver	210 (200-220)(2)	0.54 (1)	- ^b	- 57 (1)
	Heart	180 (160-220)(4)	140 (1.34-1.45)(2)	78 (76-81)(2)	1.2 (1.2-1.3)(2) 13 (11-16)(2)
	Pectoral muscle	360 (300-500)(3)	0.79 (0.66-0.92)(3)	95 (50-140)(3)	- 33 (32-33)(2)
	Sartorius muscle	-	0.85 (1)	98 (1)	- 20 (1)

ANIMAL	TISSUE	K _m VALUES (μM)			
		ADENYL CYCLASE	CYCLIC AMP PHOSPHODIESTERASE		CYCLIC GMP PHOSPHODIESTERASE
			Low K _m	High K _m	
Domestic fowl (<u>Gallus gallus</u>)	Brain	170 (110-200)(4)	0.8 (0.74-0.89)(2)	- ^b	- ^b
	Liver	160 (120-220)(3)	0.7 (0.48-0.85)(2)	73 (1)	22 (1)
	Heart	100 (60-160)(3)	1.3 (1.2-1.3)(2)	28 (21-56)(2)	13 (1)
	Pectoral muscle	610 (300-1100)(3)	1.7 (1.5-1.7)(2)	21 (21)(2)	1.0 (1)
	Sartorius muscle	280 (1)	0.8 (0.7-0.9)(2)	25 (1)	4.7 (4.1-5.9)(2)
Pheasant (<u>Phasianus colchicas</u>)	Heart	80 (1)	1.1 (1)	- ^a	10 (1)
	Pectoral muscle	80 (1)	1.4 (1.2-1.6)(2)	-	5.3 (1)
	Sartorius muscle	-	1.2 (1)	-	4.1 (1)
Laboratory mouse	Brain	170 (80-250)(6)	0.47 (0.2-0.9)(4)	68 (53-81)(3)	18 (1)
	Liver	230 (120-370)(4)	1.2 (1.1-1.4)(2)	61 (43-80)(2)	32 (1)
	Lung	100 (1)	0.67 (1)	55 (1)	8.6 (1)
	Blood	-	1.3 (0.77-1.8)(2)	43 (28-59)(2)	5.0 (2.6-7.5)(2)
	Heart	130 (70-200)(3)	1.7 (1.4-2.0)(3)	70 (58-78)(3)	24 (24-25)(2)
	Gastrocnemius muscle	410 (320-470)(3)	1.6 (1.3-2.0)(5)	41 (31-52)(3)	21 (13-29)(3)
Laboratory rat	Epididymal fat pad	-	0.67 (0.42-1.1)(3)	55 (32-78)(2)	17 (12-23)(5)
	Heart	110 (80-160)(13)	0.69 (0.42-1.0)(9)	40 (28-52)(8)	15 (1)
	Liver	-	0.75 (0.40-1.2)(3)	- ^b	24 (17-33)(4)

a. The K_m values for these enzymes were greater than 250 μM.

b. These enzymes displayed marked sigmoid kinetics and it was not possible to determine a K_m value from the Hofstee plot.

The high K_m phosphodiesterases frequently exhibited kinetics which were non-linear in the Hofstee plot and corresponded to sigmoid kinetics. These deviations from Michaelis-Menten kinetics were easily detected in tissues in which there was no detectable low K_m enzyme (e.g. the cyclic GMP-hydrolyzing enzyme from some mammalian and avian tissues - see also Sakai *et al.*, 1974). However, when the data were plotted according to the method of Hill ($\log \frac{v}{V_{\max} - v}$ versus $\log s$ - see Newsholme & Start, 1973), the value of the Hill coefficient rarely exceeded 1.2 - even if the Hofstee plot clearly indicated that the enzyme did not conform to Michaelis-Menten kinetics. Those tissues for which Hill coefficients greater than 1.2 were measured are listed in Table 3.7. The highest coefficient was measured for rat liver ($n = 2.0$). Sigmoid kinetics have previously been reported for purified phosphodiesterases (see Chapter 1, Section 1b(i)). It is possible that many workers have failed to recognize sigmoid kinetics for high K_m phosphodiesterase assayed in crude homogenates, either because they have used double-reciprocal kinetic plots, which compress data obtained at high substrate concentrations, or because the deviation from Michaelis-Menten kinetics is small (see, for example, Figure 1 of Bevers *et al.*, 1974).

It has been assumed in the above discussion that the kinetics of phosphodiesterase result from the presence of no more than two enzymes, which individually display Michaelis-Menten or sigmoid kinetics. However, there was evidence in some tissues of more than two enzymes, or enzymes which display kinetics characteristic of negative cooperativity. Thus high K_m cyclic GMP phosphodiesterase from pigeon liver had a Hill coefficient of 0.83 and low K_m cyclic AMP phosphodiesterase from rat liver had a coefficient of 0.95.

TABLE 3.7 The value of the Hill coefficient for high K_m phospho-
diesterases which display sigmoid kinetics

Data were analyzed as described in Chapter 3, Section G.

Only Hill coefficients greater than 1.2 are listed below.

The range of values measured and the number of determinations are given in parentheses.

ANIMAL	TISSUE	HILL COEFFICIENT (n)	
		CYCLIC AMP PHOSPHODIESTERASE	CYCLIC GMP PHOSPHODIESTERASE
Locust	Flight muscle	-	1.2 (1.1-1.3)(3)
Cockroach	Cerebral ganglion	-	1.6 (1.5-1.7)(2)
(<u>Blaberus</u> <u>discoidalis</u>)	Flight muscle	-	1.4 (1.2-1.6)(2)
Trout	Liver	1.3 (1.28-1.32)(2)	-
	Red abdominal muscle	1.5 (1.52-1.55)(2)	-
	White abdominal muscle	1.6 (1.56-1.67)(2)	-
Frog	Brain	1.2 (1.1-1.3)(3)	-
	Heart	1.3 (1.2-1.4)(2)	-
Domestic pigeon	Brain	1.5 (1.4-1.6)(3)	-
	Liver	1.2 (1.1-1.3)(2)	-
	Pectoral muscle	1.2 (1.1-1.3)(3)	-
Domestic fowl	Brain	1.3 (1)	1.2 (1)
	Pectoral muscle	1.3 (1.2-1.5)(2)	-
Laboratory rat	Liver	2.0 (1.7-2.3)(3)	-
	Heart	1.25 (1.17-1.35)(9)	-

C. DISCUSSION

C.1 THE MOLECULAR NATURE OF CYCLIC AMP AND CYCLIC GMP PHOSPHODIESTERASE

There are a number of accounts in the literature of attempts to isolate phosphodiesterases which have only one K_m and which are specific for cyclic AMP or cyclic GMP (for example, Beavo *et al.*, 1970; Thompson & Appleman, 1971a,b). Characterization of isolated phosphodiesterases may enable the roles of the individual enzymes to be determined. The comparative data obtained in the present study led to certain observations which suggest whether each activity measured in crude homogenates is that of an individual molecular species or whether one molecular species may have two kinds of activity (i.e. low and high K_m , cyclic AMP- and cyclic GMP-hydrolyzing). Since the enzymes were not purified, these suggestions are not conclusive. However, the data have the advantage that the molecular species present in crude homogenates of tissues are more likely to be those usually present in the cell. Thus phosphodiesterase may associate on purification (see Chapter 1, Section Alb(i)).

The relative activities of the low and high K_m enzymes for a particular substrate vary greatly between tissues (see Table 3.12 for cyclic AMP phosphodiesterase). This observation suggests that the low and high K_m values of phosphodiesterase reflect the activities of two molecular species whose concentrations vary independently between tissues - a conclusion which has been implicit up till now in the terminology "low K_m and high K_m enzymes". If an individual enzyme exhibited both K_m values it would be expected that the kinetics of the enzyme would be similar in different tissues.

The activities of the high K_m enzymes for cyclic AMP and cyclic GMP are similar, for all vertebrate tissues (Table 3.8) (see also Robison *et al.*, 1971, p.408ff.). This observation suggests that the same molecule hydrolyzes both substrates, which is consistent with the finding that one protein fraction

TABLE 3.8 Ratios of the maximal activities of phosphodiesterase when hydrolyzing cyclic AMP and cyclic GMP

Data are taken from Tables 3.2, 3.3 and 3.4. The ratios tabulated are, activity with cyclic GMP as substrate/activity with cyclic AMP as substrate. A ratio of zero indicates that the cyclic GMP-hydrolyzing enzyme was not detected; infinity indicates that the cyclic AMP-hydrolyzing enzyme was not detected.

ANIMAL	TISSUE	RATIOS OF MAXIMAL ACTIVITIES OF PHOSPHODIESTERASE	
		LOW K_m ENZYME	HIGH K_m ENZYME
Rat tapeworm	Whole animal	0.32	0.65
Scallop	Fast adductor muscle	0.12	0.40
	Slow adductor muscle	0.06	-
Lobster	Cerebral ganglion	1.29	1.70
	Abdominal muscle	0.09	0.57
	Claw muscle	0.12	0.22
Locust	Cerebral ganglion	0.16	2.20
	Flight muscle	0.10	0.33
	Hind leg muscle	0.24	0.47
Cockroach	Cerebral ganglion	0.73	3.43
	Flight muscle	0.04	0.28
Rosechafer	Cerebral ganglion	0.19	0.61
	Flight muscle	0.02	∞
Honey-bee	Flight muscle	0.08	∞
Bumble-bee	Flight muscle	0.23	Zero
Blowfly	Flight muscle	Zero	0.10
Trout	Brain	1.57	0.79
	Liver	0.44	1.75
	Heart	0.19	1.88
	Red abdominal muscle	0.43	1.53
	White abdominal muscle	0.20	1.22
Frog	Brain	Zero	0.96
	Liver	1.75	0.88
	Heart	0.74	0.67
	Gastrocnemius muscle	0.35	0.68

ANIMAL	TISSUE	RATIOS OF MAXIMAL ACTIVITIES OF PHOSPHODIESTERASE	
		LOW K_m ENZYME	HIGH K_m ENZYME
Pigeon	Brain	Zero	0.80
	Liver	Zero	1.90
	Heart	1.03	0.45
	Pectoral muscle	Zero	2.11
	Sartorius muscle	Zero	0.68
Domestic fowl	Brain	Zero	1.33
	Liver	Zero	1.70
	Heart	0.80	0.69
	Pectoral muscle	0.27	1.23
	Sartorius muscle	1.78	1.18
Pheasant	Heart	0.39	0.89
	Pectoral muscle	0.40	0.55
	Sartorius muscle	0.69	1.73
Mouse	Brain	Zero	1.48
	Liver	Zero	1.23
	Lung	Zero	1.07
	Blood	Zero	1.57
	Heart	Zero	1.32
	Gastrocnemius muscle	Zero	0.82
Rat	Epidydymal fat pad	Zero	0.89
	Heart	Zero	1.31
	Liver	Zero	1.22

from rat tissues hydrolyzes cyclic AMP and cyclic GMP with similar maximal activities (fraction D.II of Russell et al., 1973). Since the high K_m enzyme has a lower K_m value for cyclic GMP than for cyclic AMP, it has been suggested that its primary role in the cell is to hydrolyze cyclic GMP (Thompson & Appleman, 1971a; see Chapter 1, Section Alb(i)). As this is the only cyclic GMP-hydrolyzing enzyme which has been detected in some tissues, it may indeed function to hydrolyze cyclic GMP. However, the present data indicate that it contributes significantly to the rate of hydrolysis of cyclic AMP in many tissues (see Section C.4). Moreover, the present data show that at low concentrations of substrate, where v is proportional to V_{max}/K_m , the activity of the enzyme when it is hydrolyzing cyclic GMP is, at most, twice its activity when it is hydrolyzing the same concentration of cyclic AMP (see Tables 3.8 and 3.6). Since the concentration of cyclic GMP is usually far lower than the concentration of cyclic AMP (see Goldberg et al., 1973), the rate of hydrolysis of cyclic AMP by the high K_m enzyme must be faster than the rate of hydrolysis of cyclic GMP in vivo.

The relative activities of the cyclic AMP- and cyclic GMP-hydrolyzing low K_m phosphodiesterases vary widely (Table 3.8). This observation suggests that there is more than one molecular species with a low K_m for cyclic nucleotides. In many tissues from the mouse, pigeon and domestic fowl the low K_m enzyme is specific for cyclic AMP. The same finding has been reported for tissues of the rat (Thompson & Appleman, 1971a,b; but see Sakai et al., 1974).

C.2 THE APPLICATION OF THE PRESENT DATA TO A MODEL OF CYCLIC AMP TURNOVER

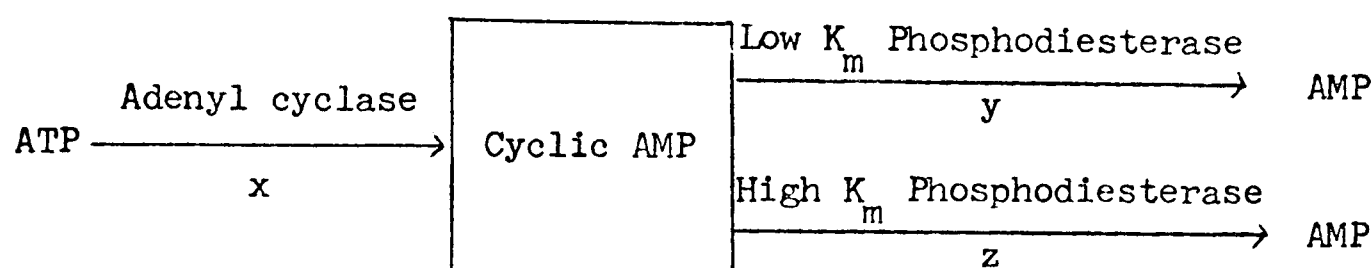
Since the method which was used to extract adenyl cyclase from tissues did not apparently disrupt the adenyl cyclase complex (Table 3.1), it will be

assumed, for the purposes of the present discussion, that the activities measured are similar to those pertaining in vivo, at least under some conditions. The basal activity of adenyl cyclase may represent its in vivo activity in the absence of hormones, whereas the maximal activity may (with certain reservations - see Chapter 1, Section Ala(ii)) represent its activity in the presence of optimal concentrations of hormones. The K_m value of adenyl cyclase (Table 3.6) is invariably less than the concentration of ATP in the cell. Therefore, it will be assumed that the activity of adenyl cyclase in vivo is not limited by substrate availability (i.e. the adenyl cyclase reaction is a flux-generating step - see Newsholme & Crabtree, 1973). Consequently, the V_{max} values measured in the present study will be assumed to represent in vivo rates of formation of cyclic AMP.

Although a number of factors modify the in vitro activity of phosphodiesterase, there is little evidence to suggest that the activity of phosphodiesterase is regulated in vivo by any factor other than substrate concentration (see Chapter 1, Section Alb(ii)). Therefore, it will be assumed that the activities measured in vitro at a particular substrate concentration are similar to the in vivo rate of hydrolysis of cyclic AMP for the same concentration of substrate.

On the basis of these assumptions, the data obtained in the present study have been applied to a simple model of in vivo cyclic AMP metabolism. According to this model, adenyl cyclase and both high and low K_m phosphodiesterases are not confined to compartments of the cell. It is assumed that cyclic AMP from a single pool is hydrolyzed by both the low and high K_m phosphodiesterases (see Section C.1). Thus the relative activities of the phosphodiesterases depend only upon their kinetic parameters and the concentration of cyclic AMP. The activity of adenyl cyclase can vary between the basal and maximal values measured in homogenates. For any activity of adenyl cyclase,

the concentration of cyclic AMP rises or falls to a steady-state level at which the sum of the activities of high and low K_m phosphodiesterases is equal to the activity of adenyl cyclase. The model is illustrated below.



The rates of the reactions are x , y and z ; in the steady-state $x = y + z$.

The model predicts that, if low and high K_m phosphodiesterases conform to Michaelis-Menten kinetics, the minimum (or basal) concentration of cyclic AMP (S_b) in intact tissues given by

$$\text{Basal } V_{\max}^a = \frac{V_{\max}^l}{K_m^l + S_b} = \frac{V_{\max}^h}{K_m^h + S_b}$$

where the superscript, a , refers to adenyl cyclase, l to low K_m phosphodiesterase and h to high K_m phosphodiesterase. The maximal concentration of cyclic AMP can be predicted by substituting the maximal activity of adenyl cyclase into the equation. It is possible to substitute mean values of $V_{\max}$ and K_m into the equation in order to estimate mean minimum and maximum values for the steady-state concentration of cyclic AMP. Unfortunately, this approach cannot be used for all tissues because the phosphodiesterases frequently do not obey Michaelis-Menten kinetics (see Section B.4). Therefore, it has been necessary to refer to the individual assays of phosphodiesterase which were performed for each tissue. From the initial kinetic plot (see Figure 2.9a), the concentrations of cyclic AMP have been deduced at which the total activity of phosphodiesterase is equal to the mean basal and mean maximal activities of adenyl cyclase from the same tissue. These concentrations of cyclic AMP are the minimum and maximum steady-state concentrations

of cyclic AMP which may be predicted by the model. The mean minimum and maximum values for each tissue are presented in Table 3.9. For some tissues the more direct approach described above has been used and shown to give identical results (see legend to Table 3.9).

In most cases the ratio, maximum concentration of cyclic AMP/minimum concentration of cyclic AMP, is little greater than the ratio, maximal activity of adenyl cyclase/basal activity of adenyl cyclase (see Tables 3.2 to 3.4). Therefore, the model predicts that, for most intact tissues, the effect of hormonal stimulation upon the concentration of cyclic AMP will be approximately the same as the effect of F^- upon the activity of adenyl cyclase extracted from the tissue (see Table 3.5). The extent to which this prediction is confirmed by data in the literature can be assessed by referring to Table 3.10 (see also Table 3.11). In the majority of studies, hormones have been found to elevate cyclic AMP concentration in intact tissues by less than 10-fold and frequently less than 5-fold. The stimulation of the activity of adenyl cyclase by F^- measured in the present study was generally 4-fold or less (Table 3.5). From the data of Birnbaumer et al. (1969), it may be deduced that the stimulatory effect of F^- should be increased by a factor of 1.4 in order to account for the fact that adenyl cyclase was assayed at 30°C in the present study, whereas hormonal effects upon cyclic AMP concentrations in tissues have been studied mostly at 37°C . Therefore the model predicts relative changes in cyclic AMP concentrations similar to those measured in whole tissues. (In general the predicted changes are a little low - particularly in the case of brain tissues. For this reason a slight modified model is suggested in Section C.4.) Moreover, the absolute concentrations of cyclic AMP predicted by the model are similar to reported concentrations, as Table 3.10 shows. Some discrepancies are discussed in Section C.5. (Unfortunately, there is a lack of information concerning cyclic AMP levels in non-mammalian tissues.)

TABLE 3.9 The concentrations of cyclic AMP which are predicted from
a model of cyclic AMP turnover

The model and the methods used to calculate the mean minimum and maximum steady-state concentrations of cyclic AMP are described in Section C2. For those tissues in which the maximal activity of adenyl cyclase was not measured it was assumed that this activity was four times the basal activity (see Section B3a). Calculations based on this assumption are indicated by an asterisk.

An example of the simpler method of derivation of cyclic AMP concentrations (see Section C2) is given below for white gastrocnemius muscle from the mouse. (The phosphodiesterases from this tissue conform to Michaelis-Menten kinetics.)

	ADENYL CYCLASE		PHOSPHODIESTERASE	
	<u>Basal</u>	<u>Maximal</u>	<u>High K_m</u>	<u>Low K_m</u>
V _{max} (nmol/min/g)	0.7	2.0	51	10.7
K _m (μM)	-	-	41	1.6

∴ Minimum concentration of cyclic AMP is given by

$$0.7 = \frac{10.7 s_b}{1.6 + s_b} + \frac{51 s_b}{41 + s_b} \qquad \therefore s_b = 0.10 \mu M$$

Maximum concentration of cyclic AMP is given by

$$2.0 = \frac{10.7 s_m}{1.6 + s_m} + \frac{51 s_m}{41 + s_m} \qquad \therefore s_m = 0.30 \mu M$$

The values of s_b and s_m may be compared with the values of 0.10 and 0.33 μM calculated by the longer method and recorded below.

ANIMAL	TISSUE	MEAN CONCENTRATIONS OF CYCLIC AMP INDICATED BY THE MODEL (μ M)	
		<u>Minimum</u>	<u>Maximum</u>
Scallop (<u>Pecten maximus</u>)	Fast adductor muscle	0.012	0.055*
	Slow adductor muscle	0.040	0.16*
Lobster	Cerebral ganglion	0.056	0.25*
	Abdominal muscle	0.27	1.31*
	Claw muscle	0.11	0.63*
Locust	Cerebral ganglion	0.11	0.63
	Flight muscle	0.076	0.13
	Hind leg muscle	0.057	0.22
Cockroach (<u>Blaberus discoidalis</u>)	Cerebral ganglion	0.17	0.84
	Flight muscle	0.083	0.13
Rosechafer	Cerebral ganglion	0.16	0.39*
	Flight muscle	0.074	0.34*
Honey-bee	Flight muscle	0.030	0.13
Bumble-bee	Flight muscle	0.27	0.75
Blowfly	Flight muscle	0.19	0.95*
Trout	Brain	<0.33	3.09
	Liver	<0.065	0.45
	Heart	0.047	0.22
	Red abdominal muscle	0.65	3.18
	White abdominal muscle	<0.52	1.90
Frog	Brain	0.64	1.22
	Liver	0.26	1.50
	Heart	0.034	0.16
	Gastrocnemius muscle	0.14	0.94*
Pigeon	Brain	0.31	1.50
	Liver	0.082	0.37
	Heart	0.079	0.12
	Pectoral muscle	0.077	0.37
	Sartorius muscle	0.037	0.15

ANIMAL	TISSUE	MEAN CONCENTRATIONS OF CYCLIC AMP INDICATED BY THE MODEL (μM)	
		<u>Minimum</u>	<u>Maximum</u>
Domestic fowl	Brain	0.42 ^a	2.08
	Liver	0.26	2.84*
	Heart	0.14	0.61*
	Pectoral muscle	0.11	0.52*
	Sartorius muscle	0.026	0.16*
Mouse	Brain	0.78	1.78
	Liver	0.050	0.30
	Lung	1.05	13.1*
	Heart	0.47	1.63
	Gastrocnemius muscle (white)	0.10	0.33
	Gastrocnemius muscle (white and red)	0.16	0.52
Rat	Heart	0.66	10.0*

a. The basal activity of adenyl cyclase in the brain of the domestic fowl was assumed to be 38 nmol/min/g wet wt. (the value for pigeon brain - see Table 3.3).

TABLE 3.10 The concentrations of cyclic AMP which have been predicted from a model and some concentrations reported for intact tissues

The predicted concentrations of cyclic AMP are taken from Table 3.9. These values are presented together with data from the literature. The methods used to raise the concentration of cyclic AMP for actual measurements in intact tissues may be found in Table 3.11. Some data for the rat are presented for comparison with the mouse, .References, indicated by a numeral, are given following Table 3.12.

ANIMAL	TISSUE	MINIMUM CONCENTRATION OF CYCLIC AMP		MAXIMUM CONCENTRATION OF CYCLIC AMP	
		PREDICTED (μM)	MEASURED (nmol/g wet wt.)	PREDICTED (μM)	MEASURED (nmol/g wet wt.)
Frog	Leg muscle	0.64	0.59 ¹ , 1.0 ²⁵	1.22	1.59 ¹
Mouse	Whole brain	0.78	1.1 ²⁶ , 1.3 ²⁷ , 4.6 ^{21,a}	1.78	37 ^{21,a}
Rat	Whole brain	-	-	-	5.0 ²²
Mouse	Liver	0.05	0.95 ²⁸ , 1.0 ²⁵	0.30	-
Rat	Liver	-	0.1 ¹⁵ , 0.4 ¹⁸ , 0.5 ^{16,17}	-	2.0 ¹⁶
Mouse	Heart	0.47	-	1.63	-
Rat	Heart	0.66	0.2 ¹⁰ , 0.3 ¹¹ , 0.4 ⁸ 0.5 ^{9,12} , 1.4 ²⁹ , 2.7 ³⁰	10.0 ^b	2.1 ¹¹ , 3.4 ⁸ , 3.6 ¹² 6.0 ⁹ , 8.6 ¹⁰ , 12 ³⁰
Mouse	Leg muscle	0.16	1.95 ³	0.52	4.8 ³
Rat	Leg muscle	-	0.3 ²⁵ , 0.4 ² , 0.6 ²⁹ , 0.7 ¹ , 1.0 ³¹	-	1.8 ^{1,2}

a. Data for cerebral cortex slices. b. Value derived from an estimated maximal activity of adenyl cyclase (see legend to Table 3.9).

TABLE 3.11 Reported concentrations of cyclic AMP in intact tissues before and following hormonal treatment of the tissue

The data tabulated refer only to experiments in which inhibitors of phosphodiesterase were not utilized. If the data in the literature were recorded as pmole/mg protein they have been divided by 10 for brains and 5 for other tissues to give nmol/g wet wt. of tissue. As far as possible, the stimulated content of cyclic AMP tabulated is the highest value which was recorded and corresponds to a steady-state concentration. References are given following Table 3.11.

ANIMAL	TISSUE PREPARATION	HORMONE ADDITION	CONTENT OF CYCLIC AMP (nmol/min/g wet wt. of tissue)		RATIO OF CONTENTS <u>Stimulated</u> Control
			<u>Control</u>	<u>Stimulated</u>	
Frog ¹	Leg muscle <u>in situ</u>	Adrenaline (5×10^{-8} mol/kg injected into heart)	0.59	1.59	2.7
Rat ¹	Gastrocnemius muscle <u>in situ</u>		0.69	1.82	2.6
Rat ²	Leg muscle <u>in situ</u>	Adrenaline (5×10^{-6} mol/kg injected into heart)	0.44	1.8	4.1
Mouse ³	Gastrocnemius muscle <u>in situ</u>	Adrenaline (2.5×10^{-8} mol/kg injected intravenously)	1.95	4.8	2.5
Rat ⁴	Isolated diaphragm	Adrenaline (6×10^{-5} M)	0.39	10	25
Rat ⁵	Isolated diaphragm	Adrenaline (5×10^{-5} M)	0.21	3.17	15
Rat ⁶	Isolated uterus	Isoproterenol (5×10^{-7} M)	1.1	2.2	2.0
Guinea-pig ⁷	Isolated taenia coli	Adrenaline (2.5×10^{-8} M)	0.27	0.38	1.4
Rat ⁸	Isolated perfused heart	Adrenaline (10^{-7} mol)	0.42	1.42	3.4
Rat ⁹	Isolated perfused heart	Adrenaline (2.5×10^{-7} M)	0.5	3.0	6.0

ANIMAL	TISSUE PREPARATION	HORMONE ADDITION	CONTENT OF CYCLIC AMP (nmol/min/g wet wt. of tissue)		RATIO OF CONTENTS <u>Stimulated</u> Control
			<u>Control</u>	<u>Stimulated</u>	
Rat ¹⁰	Isolated perfused heart	Adrenaline (1.5×10^{-8} mol)	0.21	0.48	2.3
		Adrenaline (0.5×10^{-8} mol)			
		+ glucagon (8.1×10^{-7} mol)	0.21	1.8	8.6
Rat ¹¹	Heart <u>in situ</u>	Adrenaline (10^{-8} mol/kg rat/min)	0.27	0.58	2.1
Rat ¹²	Heart <u>in situ</u>	Adrenaline (10^{-8} mol/kg rat/min)	0.5	1.8	3.6
Rabbit ¹³	Isolated perfused heart	Adrenaline (2.5×10^{-8} mol)	0.26	0.35	1.3
Rabbit ¹⁴	Liver slices	Adrenaline (4×10^{-5} M)	0.4	1.4	3.5
Rat ¹⁵	Liver <u>in situ</u>	Glucagon (4×10^{-9} mol/kg)	0.10	0.39	3.9
Rat ¹⁶	Perfused liver	Adrenaline (3×10^{-7} M)	0.5	2.0	4.0
		Glucagon (2×10^{-8} M)	0.5	38	76
Rat ¹⁷	Perfused liver	Glucagon (2.5×10^{-6} M)	0.5	27	54
Rat ¹⁸	Liver slices	Glucagon (1.4×10^{-6} M)	0.4	6.4	16
Rabbit ¹⁹	Cerebellar slices	Noradrenaline (1×10^{-4} M)	0.64	3.9	6.1
		Histamine (1×10^{-4} M)	0.64	1.9	3.0
		Serotonin (1×10^{-4} M)	0.64	3.2	5.0
Rabbit ²⁰	Cerebral cortex slices	Histamine (1×10^{-3} M)	2.0	25	12.5

ANIMAL	TISSUE PREPARATION	HORMONE ADDITION	CONTENT OF CYCLIC AMP (nmol/min/g wet wt. of tissue)		RATIO OF CONTENTS <u>Stimulated</u> Control
			<u>Control</u>	<u>Stimulated</u>	
Mouse ²¹	Cerebral cortex slices	Adrenaline (1×10^{-4} M)	4.6	37	8.0
		Noradrenaline (1×10^{-4} M)	4.6	33	7.2
		Adenosine (1×10^{-4} M)	4.6	78	17
Rat ²¹	Cerebral cortex slices	Adrenaline (1×10^{-9} M)	1.6	5.8	3.6
		Adenosine (1×10^{-4} M)	1.6	5.9	3.7
Rat ²²	Brain <u>in situ</u>	Adenosine (5×10^{-7} mol injected into cerebral ventricle)	1.9	5.0	2.6
Guinea-pig ²³	Cerebral cortex slices	Histamine (1×10^{-4} M)	1.0	7.0	7.0
		Adenosine (1×10^{-4} M)	1.0	20	20
		Adenosine (1×10^{-4} M)			
		+ histamine (1×10^{-4} M)	1.0	110	110
		+ noradrenaline (1×10^{-4} M)			

TABLE 3.12 Reported rates of increase of the content of cyclic AMP during hormonal stimulation of intact tissues

The rates of increase of the content of cyclic AMP are mostly maximal rates taken from time courses. Other details of how the data were obtained are described in the legend to Table 3.11.

ANIMAL	TISSUE PREPARATION	METHOD USED TO INCREASE CONTENT OF CYCLIC AMP	RATE OF INCREASE OF CONTENT OF CYCLIC AMP (nmol/min/g wet wt.)
Frog ¹	Leg muscle <u>in situ</u>	Adrenaline (5×10^{-8} mol/kg injected into heart	0.1
Rat ¹	Gastrocnemius muscle <u>in situ</u>		1.1
Rat ²	Leg muscle <u>in situ</u>	Adrenaline (5×10^{-6} mol/kg injected into heart)	1.4
Mouse ³	Gastrocnemius muscle <u>in situ</u>	Adrenaline (2.5×10^{-8} mol/kg injected intravenously)	1.0
Rat ⁴	Isolated diaphragm	Adrenaline (6×10^{-5} M)	10
Rat ⁶	Isolated uterus	Isoproterenol (5×10^{-7} M)	0.26
Guinea-pig ⁷	Isolated taenia coli	Adrenaline (2.5×10^{-8} M)	0.6
Rat ⁹	Isolated perfused heart	Adrenaline (2.5×10^{-7} M)	7.5
Rat ⁸	Isolated perfused heart	Adrenaline (10^{-7} mol)	20
Rat ¹⁰	Isolated perfused heart	Adrenaline (1.5×10^{-8} mol)	3.0
		Adrenaline (0.5×10^{-8} mol) + glucagon (0.1×10^{-7} mol)	4.8
Rat ¹¹	Heart <u>in situ</u>	Adrenaline (10^{-8} mol/kg rat/min)	1.24
Rat ¹²	Heart <u>in situ</u>	Adrenaline (10^{-8} mol/kg rat/min)	7.8

ANIMAL	TISSUE PREPARATION	METHOD USED TO INCREASE CONTENT OF CYCLIC AMP	RATE OF INCREASE OF CONTENT OF CYCLIC AMP (nmol/min/g wet wt.)
Rabbit ¹⁴	Liver slices	Adrenaline (4×10^{-5} M)	0.1
Rat ¹⁶	Perfused liver	Adrenaline (3×10^{-7} M)	2.0
		Glucagon (2×10^{-8} M)	10.0
Rat ¹⁷	Perfused liver	Glucagon (2.5×10^{-6} M)	23
Rat ¹⁸	Liver slices	Glucagon (1.4×10^{-6} M)	1.2
Rat ¹⁹	Cerebellar slices	Noradrenaline (1×10^{-4} M)	5.0
Rabbit ²⁴	Cerebral cortex slices	Histamine (5×10^{-4} M)	3.0
Rat ²¹	Cerebral cortex slices	Noradrenaline (1×10^{-4} M)	0.8
		Adenosine (1×10^{-4} M)	0.4
Mouse ²¹	Cerebral cortex slices	Noradrenaline (1×10^{-4} M)	2.8
		Adrenaline (1×10^{-4} M)	3.2
		Adenosine (1×10^{-4} M)	7.2
Rat ²²	Brain <u>in situ</u>	Adenosine (5×10^{-7} mol injected into cerebral ventricle)	2.3
Guinea-pig ²³	Cerebral cortex slices	Histamine (1×10^{-4} M)	3.0
		Adenosine (1×10^{-4} M)	5.0
		Histamine (1×10^{-4} M)	
		+ Noradrenaline (1×10^{-4} M)	12.5
		+ Adenosine (1×10^{-4} M)	

References to Tables 3.10, 3.11 and 3.12

1. Posner et al. (1965)
2. Brooker et al. (1968)
3. Lyon & Mayer (1969)
4. Craig et al. (1969)
5. Lundholm et al. (1967)
6. Dobbs & Robinson (1968)
7. Bueding et al. (1966)
8. Robison et al. (1965)
9. Cheung & Williamson (1965)
10. Mayer et al. (1970)
11. Namm & Mayer (1968)
12. Namm et al. (1968)
13. Hammermeister et al. (1965)
14. Sutherland et al. (1965)
15. Steiner et al. (1970)
16. Robison et al. (1967a)
17. Exton & Park (1968)
18. Christoffersen & Berg (1974)
19. Kakichi & Rall (1968)
20. Kuo et al. (1972)
21. Schultz & Daly (1973e)
22. Burkard (1972)
23. Schultz & Daly (1973a)
24. Palmer et al. (1972)
25. Walton & Garren (1970)
26. Breckenridge (1964)
27. Aurbach & Houston (1968)
28. Gilman (1972)
29. Ishikawa et al. (1969)
- 30 Lee et al. (1971)
31. Turtle & Kipnis (1967)

However, there are instances in which hormones elevate cyclic AMP concentrations far more than 10-fold (Table 3.11). Thus glucagon has a very large effect upon the concentration of cyclic AMP in the liver and adrenocorticotrophic hormone has a large effect in the adrenal gland.

(Note that the concentrations of glucagon used in the experiments listed in Table 3.11 are at least 10-fold higher than those measured in vivo—see Exton et al., 1970). It is possible that the activities of adenyl cyclase in the presence of these hormones are far greater than the activity in the presence of F^- (see Section B.1), or that the activity of phosphodiesterase is inhibited (see Pawlson et al., 1974). The effect of adenosine on the concentration of cyclic AMP in the brain may also be far more than 10-fold. It is possible that adenosine increases the concentration of cyclic AMP in the brain both by activating adenyl cyclase and inhibiting phosphodiesterase (Schultz & Daly, 1973a,b,c). Therefore the degree to which it stimulates the activity of adenyl cyclase may be far less than the degree to which it increases the concentration of cyclic AMP in the brain.

It may be concluded that a simple model of cyclic AMP turnover based upon the activities of adenyl cyclase and phosphodiesterase measured in extracts of tissues is consistent with much of the reported data for intact tissues. The model is also supported by observations which indicate that the concentrations of cyclic AMP in tissue extracts which are supplied with ATP and Mg^{2+} are similar to those found in vivo (Grahame-Smith et al., 1967; Robison et al., 1971, p.11). A slight modification of this model will be presented below (Section C.4). Meanwhile, since there are some data which support the model, it will be used to put the present data to some further uses.

C.3 THE RATE OF TURNOVER OF CYCLIC AMP IN THE CELL

If the model presented in the preceding section is correct, the basal activity of adenyl cyclase is a measure of the minimum rate of turnover of

cyclic AMP in the cell. Comparison of reported tissue concentrations of cyclic AMP (Tables 3.10, 3.11) with basal activities of adenyl cyclase (Tables 3.2 to 3.4) indicates that, in the absence of hormones, the cellular content of cyclic AMP in most vertebrate muscles and livers turns over in about 10 to 60 seconds. The turnover time for mammalian and avian hearts is about three seconds and in brain it is about one second.

The rate of turnover of cyclic AMP determines the rate at which the cyclic AMP content of the cell can respond to a change in the activity of adenyl cyclase (Chapter 1, Section A3a(ii)). Therefore, the present results indicate that the brain and heart respond more rapidly to a doubling of the activity of adenyl cyclase than do liver and skeletal muscle. In order to determine whether this deduction is valid, reported rates of increase of the concentration of cyclic AMP in intact tissues in response to hormonal stimulation have been compiled (Table 3.12). These data confirm that the brains and hearts of mammals are capable of greater rates of increase in their content of cyclic AMP than are skeletal muscles and livers (with the exception of glucagon-treated livers - see above). The significance of this conclusion in relation to the role of cyclic AMP in these tissues is discussed below (Section C.6).

A further observation may be made from the data in Table 3.12. The maximal activities of adenyl cyclase measured in the present study (Tables 3.2 to 3.4) are largely adequate to account for known rates of increase of tissue contents of cyclic AMP (see also McIlwain, 1971; but see Robison et al., 1970). Indeed, in the case of mammalian brain the maximal activities of adenyl cyclase are much greater than any reported rate of increase of the tissue content of cyclic AMP. Adenosine does not produce exceptionally high rates of increase in the content of cyclic AMP, whereas the final concentration of cyclic AMP may be much higher than that produced by neuro-hormones. This again indicates that the effect of adenosine upon the

cyclic AMP content of the brain cannot be explained entirely in terms of activation of adenyl cyclase.

Two consequences of a high rate of turnover of cyclic AMP in animal tissues may be mentioned. Firstly, the effect of exogenous cyclic AMP upon the content of cyclic AMP within the cell will be small, unless the molecule can enter the cell at a rate which is comparable with the normal rate of turnover of cyclic AMP. Therefore, if the permeability of the cell membrane to cyclic AMP is identical in two tissues, it will be the content of cyclic AMP in the tissue which has the lowest turnover rate which is most affected by exogenous cyclic AMP. The inability of exogenous cyclic AMP at normal intracellular concentrations to affect cellular processes probably is a consequence of the low permeability of the cell to cyclic AMP (Kaukel & Hilz, 1972; Szabo & Burke, 1972), and the high rate of turnover of cyclic AMP in the cell.

Secondly, the present data suggest that the release of cyclic AMP from the cell is unlikely to be a general mechanism for the control of the intracellular concentration of cyclic AMP (see Chapter 1, Section Alb and Broadus et al., 1971). Thus since most rat tissues contain at least 0.2 nmol/g of cyclic AMP (Table 3.10), a rat weighing 100 g must contain at least 5 nmol cyclic AMP (an average of 0.05 nmol/g in the tissues and body fluids of a 100 g animal). The present data suggest that this quantity of cyclic AMP is synthesized and degraded at least once every minute. On the other hand the average rate of excretion of cyclic AMP in the urine is 0.015 nmol/100 g rat/min (Hardman et al., 1966, 1969). Even if the rate of excretion of cyclic AMP in the urine is only 10% of its rate of extrusion into extracellular fluids (Blonde et al., 1972; Broadus et al., 1970), the rate of extrusion of cyclic AMP from the cell can be only 3% of its rate of hydrolysis by phosphodiesterase. A similar calculation may be applied to cyclic GMP turnover. The concentration of cyclic GMP in the tissues of the rat is

at least 0.02 nmol/g (Goldberg et al., 1973). The present data concerning the activities of cyclic GMP phosphodiesterase in mouse tissues suggest that this concentration of cyclic GMP is metabolized at a rate of 0.07 nmol/min/g (assuming a $V_{\max}$ of 70 nmol/min/g and a K_m of 20 μ M for phosphodiesterase). On the other hand, the rate of excretion of cyclic GMP is only 0.00011 nmol/min/g of rat (Hardman et al., 1966,1969). Therefore, the average rate of extrusion of cyclic GMP from the cell is probably only about 2% of its rate of turnover. Similar conclusions may be reached concerning cyclic nucleotide turnover in human tissues, even if peak rates of cyclic nucleotide excretion during the day are considered (Murad, 1973).

However, if the extrusion of cyclic nucleotides from the cell is the property of one particular tissue or organ, extrusion may be of considerable importance in regulating the intracellular concentration of the cyclic nucleotide. Thus Goldberg et al. (1969) have reported that a major fraction of urinary cyclic GMP in the dog comes directly from kidney tissue (but see Broadus et al., 1970 and Blonde et al., 1972). Similarly, Broadus et al. (1970) estimated that one third of urinary cyclic AMP is synthesized in the kidney. In addition, the extrusion of cyclic AMP may be faster than its hydrolysis in pigeon red blood cells (Davoren & Sutherland, 1963) and extrusion may play an important role in intercellular communication in lower organisms (see Makman & Sutherland, 1965; Broadus et al., 1971; Ashworth, 1974). There are few data available for the tissues studied for this thesis. Pilakis et al. (1974) have measured the release of cyclic AMP from the perfused liver of the rat. The basal rate of release was 0.006 nmol/min/g liver, which is 0.5% of the basal activity of adenylyl cyclase in mouse liver (1.2 nmol/min/g). Under the influence of glucagon the rate of release rose to 0.3 nmol/min/g liver, which is 20% of the fluoride-stimulated activity of adenylyl cyclase (5.9 nmol/min/g). However, the latter activity is probably less than the

activity of adenylyl cyclase in the presence of glucagon, so that it cannot be concluded that the extrusion of cyclic AMP is important for the regulation of its intracellular concentration in the liver cell.

The preceding discussion has demonstrated that the activities of adenylyl cyclase and phosphodiesterase in homogenates can account for a number of results obtained in experiments using intact tissues. It appears that hydrolysis by phosphodiesterase is quantitatively far more important than extrusion from the cell in the turnover of cyclic AMP, at least for the tissues studied for this thesis. It will now be discussed why the activities of the enzymes vary between tissues. In particular, attempts will be made to explain why there are both low and high K_m phosphodiesterases and why their relative activities vary.

C.4 THE ROLES OF HIGH AND LOW K_m PHOSPHODIESTERASES IN THE CONTROL OF CYCLIC NUCLEOTIDE CONCENTRATIONS

(a) A ROLE FOR HIGH K_m PHOSPHODIESTERASE

The roles played by the high and low K_m phosphodiesterases can be deduced by comparing the activities measured in the present study. The mean basal activity of adenylyl cyclase was invariably lower than the maximal activity of the low K_m cyclic AMP phosphodiesterase (Tables 3.2 to 3.4). Therefore, the activity of the low K_m enzyme is always adequate to degrade the cyclic AMP which is produced by adenylyl cyclase operating at its basal activity. However, in some vertebrate tissues, the maximal activity of adenylyl cyclase was higher than the maximal activity of the low K_m phosphodiesterase. In these tissues, when adenylyl cyclase is maximally stimulated by hormones, the content of cyclic AMP in the cell would rise indefinitely and would not reach a new steady-state were it not for the presence of high K_m phosphodiesterase. In order to prevent excessive accumulation of cyclic AMP, it may be expected that these tissues will have the highest activities

of high K_m cyclic AMP phosphodiesterase. (The accumulation of cyclic AMP might not be immediately harmful to the cell but it would take an unnecessarily long time to revert to a new steady-state when the hormone was no longer present. It is also possible that the adenyl cyclase reaction would approach equilibrium so that the concentration of cyclic AMP could not be controlled—see Chapter 1, Section B.2b). Accordingly, those tissues which have the lowest maximal activity ratio, low K_m phosphodiesterase/adenyl cyclase, also have much higher activities of high K_m phosphodiesterase than the low K_m enzyme (Table 3.13). This correlation is particularly obvious (with some exceptions - e.g. pheasant sartorius muscle) when ratios of maximal activities are compared for a number of tissues from one species. Thus there are the following two extreme situations.

1. The maximal activity of adenyl cyclase is less than the maximal activity of low K_m phosphodiesterase; therefore the activity of low K_m phosphodiesterase is sufficient to maintain a steady-state concentration of cyclic AMP; therefore the cell does not require high K_m phosphodiesterase in order to maintain the steady-state.
2. The basal activity of adenyl cyclase is similar to the activity of low K_m phosphodiesterase; the maximal activity of adenyl cyclase is greater than the activity of low K_m phosphodiesterase; therefore the activity of low K_m phosphodiesterase is not sufficient to maintain a steady-state concentration of cyclic AMP when the activity of adenyl cyclase is stimulated; therefore the cell requires high K_m phosphodiesterase in order to maintain the steady-state.

It may be concluded that one role of high K_m phosphodiesterase is to prevent the excessive accumulation of cyclic AMP in the cell.

(b) A ROLE FOR LOW K_m PHOSPHODIESTERASE

If this is the case, it may be asked whether it is of any advantage to the cell to synthesize an enzyme with a low K_m . One advantage is suggested

TABLE 3.13 Ratios of activities of adenylyl cyclase and low and high K_m cyclic AMP phosphodiesterase

Data are taken from Tables 3.2 to 3.4. For those tissues in which the maximal activity of adenylyl cyclase was not measured it was assumed that this activity was four times the basal activity (see Section B3a). The ratios of activities, low K_m phosphodiesterase/adenylyl cyclase, have been tabulated in ascending order for the tissues of each species.

ANIMAL	TISSUE	RATIOS OF MEAN MAXIMAL ACTIVITIES	
		LOW K_m PHOSPHODIESTERASE ADENYLYL CYCLASE	HIGH K_m PHOSPHODIESTERASE LOW K_m PHOSPHODIESTERASE
Scallop	Slow adductor muscle	28	0 - 2.4
	Fast adductor muscle	50	4.2
Lobster	Claw muscle	2.6	4.0
	Abdominal muscle	4.0	0.8
	Cerebral ganglion	8.4	1.5
Locust	Cerebral ganglion	2.3	4.3
	Leg muscle	5.9	3.7
	Flight muscle	12	1.4
Cockroach	Cerebral ganglion	3.9	0.5
	Flight muscle	15	0.6
Rosechafer	Cerebral ganglion	5.9	1.1
	Flight muscle	6.3	0
Honey-bee	Flight muscle	14	0
Bumble-bee	Flight muscle	3.8	1.3
Blowfly	Flight muscle	2.5	2.3

ANIMAL	TISSUE	RATIOS OF MEAN MAXIMAL ACTIVITIES	
		LOW K_m PHOSPHODIESTERASE	HIGH K_m PHOSPHODIESTERASE
		ADENYL CYCLASE	LOW K_m PHOSPHODIESTERASE
Trout	White abdominal muscle	0.84	4.2
	Brain	1.4	7.5
	Red abdominal muscle	1.8	5.0
	Liver	6.2	2.2
	Heart	12	0.95
Frog	Liver	0.9	22
	Brain	1.0	38
	Heart	5.7	20
	Gastrocnemius muscle	18	10
Domestic pigeon	Brain	0.4	250
	Liver	2.5	19
	Pectoral muscle	2.8	10
	Sartorius muscle	6.3	14
	Heart	17	3.5
Domestic fowl	Brain	0.6	89
	Liver	0.9	13
	Pectoral muscle	4.3	1.5
	Heart	4.4	2.4
	Sartorius muscle	5.6	1.2
Pheasant	Heart	6.6	1.0
	Pectoral muscle	25	1.8
Mouse	Brain	0.8	58
	Heart	1.6	11
	Red gastrocnemius muscle	2.2	16
	Liver	4.6	20
	White gastrocnemius muscle	5.3	4.8
Rat	Heart	0.7	19

by the following argument. In order to maintain a concentration of $1 \mu\text{M}$ cyclic AMP in the cell, the low K_m enzyme ($K_m = 1 \mu\text{M}$) must operate at one half of its V_{max} ; the high K_m enzyme ($K_m = 20 \mu\text{M}$), in contrast, must operate at only $1/21$ of its V_{max} . If the turnover numbers of the active sites of both enzymes are the same, ten molecules of the high K_m enzyme must be synthesized in order to metabolize cyclic AMP at a rate which can be achieved by one molecule of the low K_m enzyme. Since the synthesis of protein molecules requires energy, the cell will use least energy for enzyme synthesis, if it synthesizes enzymes which have a high affinity for their substrates. To recapitulate, it is necessary to synthesize fewer molecules of phosphodiesterase if the enzyme has a K_m which is close to the concentration of cyclic AMP suitable for basal conditions, but a higher K_m enzyme is necessary, in addition, in order to control the concentration of cyclic AMP when the activity of adenyl cyclase is stimulated.

(c) PROPOSED EXPLANATIONS FOR VARIATIONS IN THE KINETICS OF PHOSPHODIESTERASE BETWEEN TISSUES

However, it is unclear why the ratio of activities of phosphodiesterase, high K_m enzyme/low K_m enzyme, should vary between tissues. Thus vertebrate brains have a very high activity of high K_m compared to low K_m phosphodiesterase (situation 2 in Section (a) above), whereas avian and trout hearts have similar activities of the high and low K_m enzymes. (However, mammalian and frog hearts do not conform to this pattern.) Vertebrate and invertebrate skeletal muscles also tend to have similar activities of the high and low K_m phosphodiesterases but some skeletal muscles, notably the red pectoral muscle of the pigeon and the redder parts of the gastrocnemius muscle of the mouse have a far greater activity of the high K_m than the low K_m enzyme. These correlations between the kinetics of phosphodiesterase and the function of a tissue do not explain why the kinetics vary between tissues. Nevertheless,

three possible explanations for this variation are proposed below and some data supporting each explanation are described.

(i) The effect of the kinetics of phosphodiesterase on the basal concentration of cyclic AMP

One proposal that may be put forward to explain the variations in the kinetics of phosphodiesterase between tissues is that these variations bring about different basal concentrations of cyclic AMP in different tissues. Thus if the total activity of phosphodiesterase is maintained at the level necessary to prevent accumulation of cyclic AMP but the proportion of the high K_m enzyme is increased, the steady-state concentration of cyclic AMP will be increased (see Table 3.14). The observation that vertebrate brains contain higher concentrations of cyclic AMP than other vertebrate tissues is consistent with this proposal (Table 3.9). The higher concentrations may be necessary to activate protein kinase, which has a higher K_m in the brain than in most other tissues of the rat (Kuo et al., 1970). Unfortunately, there are not enough comparative data available concerning cyclic AMP levels in non-mammalian tissues for this proposal to be adequately tested.

(ii) The regulation of the activities of individual phosphodiesterases

Another proposal to account for differing relative activities of low and high K_m phosphodiesterase is that these enzymes are subject to regulation by different agents. For example, the activity of low K_m phosphodiesterase may be modified by insulin and the activity of high K_m phosphodiesterase by cyclic GMP (Sakai et al., 1974; Loten & Sneyd, 1970; Russell et al., 1973; Pilakis et al., 1974; Pawlson et al., 1974). Thus by varying the activities of low and high K_m phosphodiesterases between tissues the response of a tissue to a particular effector may be modified. Consistent with this suggestion is the fact that insulin has little direct effect on metabolism in the brain—a tissue which contains a low activity of the low K_m compared to the high K_m enzyme.

TABLE 3.14 The effect of varying proportions of low and high K_m phosphodiesterases on the steady-state concentration of cyclic AMP

The steady-state concentration of cyclic AMP has been calculated for varying proportions of low and high K_m phosphodiesterases by using the model described in Section C2. The sum of the V_{max} values of the phosphodiesterases is assumed to be 100 nmol/min/g in each case, the K_m values are 1 and 20 μM , and the activity of adenylyl cyclase is 10 nmol/min/g.

V_{max} VALUES OF PHOSPHODIESTERASE (nmol/min/g)		STEADY-STATE CONCENTRATION OF CYCLIC AMP
HIGH K_m ENZYME	LOW K_m ENZYME	(μM)
0	100	0.11
25	75	0.15
50	50	0.23
75	25	0.49
100	0	2.22

(iii) The sensitivity of the control of cyclic AMP

Finally, it may be suggested that by varying the proportions of high and low K_m enzymes in a tissue the sensitivity of the tissue content of cyclic AMP to changes in the activity of adenyl cyclase may be varied (cf. the discussion of Chapter 1, Section Alb(i)). Thus if the concentration of cyclic AMP in the cell were greater than the K_m of the phosphodiesterase controlling the concentration of cyclic AMP, the steady-state concentration of cyclic AMP would rise sharply in response to an increase in the activity of adenyl cyclase. (In the extreme situation, where the activity of adenyl cyclase is greater than that of phosphodiesterase, no steady-state concentration would be reached and cyclic AMP would accumulate, out of control - see Section (a) above.) The present results, interpreted by the model of Section C.2, indicate that the steady-state concentration of cyclic AMP in the cell is approximately proportional to the activity of adenyl cyclase for a wide variety of tissues. (The model predicts this proportionality because the activity of adenyl cyclase is always much less than the total activity of phosphodiesterase.) Therefore, it would not appear that the sensitivity of the concentration of cyclic AMP to changes in the activity of adenyl cyclase is varied between tissues by varying the proportions of high and low K_m enzymes. However, the predictions of the simple model are not entirely accurate. Thus, in the case of brain tissues in particular, reported changes in tissue cyclic AMP content are frequently greater (perhaps 2-fold) than those changes predicted from the present results on the assumption that there is no compartmentation of cyclic AMP (compare Tables 3.9 and 3.11). These discrepancies have led to the formulation of a modified model of the turnover of cyclic AMP. The modified model, unlike the simple model permits the sensitivity of the concentration of cyclic AMP to changes in the activity of adenyl cyclase to be varied between tissues by varying the kinetics of

phosphodiesterase. It also predicts larger effects of hormonal stimulation on the concentration of cyclic AMP than are predicted by the simple model.

(iv) A modified model of the turnover of cyclic AMP

In the modified model of the turnover of cyclic AMP (Figure 3.2), it is assumed that there is some degree of compartmentation of cyclic AMP (see Chapter 1, Section A.4). Both adenyl cyclase and low K_m cyclic AMP phosphodiesterase are associated with membranes, whereas high K_m phosphodiesterase is largely a supernatant enzyme. It is proposed that low K_m phosphodiesterase is situated near to adenyl cyclase so that it has access to newly-synthesized cyclic AMP prior to high K_m phosphodiesterase (see the discussion of adenosine utilization, Chapter 1, Section B4c(ii)). Consequently, the concentration of cyclic AMP is controlled primarily by the relative activities of adenyl cyclase and low K_m phosphodiesterase. The discussion in paragraph (ii) above shows that the concentration of cyclic AMP is then most sensitive to variations in the activity of adenyl cyclase in those tissues in which the basal activity of adenyl cyclase is similar to (rather than less than) the activity of low K_m phosphodiesterase.

Accepting that the model shows that the concentration of cyclic AMP is more sensitive to changes in the activity of adenyl cyclase in some tissues (those categorized under situation 2 in paragraph (a)) than in others, it is unclear why these tissues should include vertebrate brains, mammalian and frog hearts and some red muscles, but not avian and trout hearts, insect muscles, and white skeletal muscles. When the role of cyclic AMP in these tissues is fully understood the situation may become clearer.

The modified model can explain one problem that was discussed in Chapter 1, Section A1(b). The problem is that inhibitors of phosphodiesterase or hormones which stimulate the activity of adenyl cyclase may individually have little effect on the concentration of cyclic AMP in a tissue. However, together they may have far greater than additive effects.

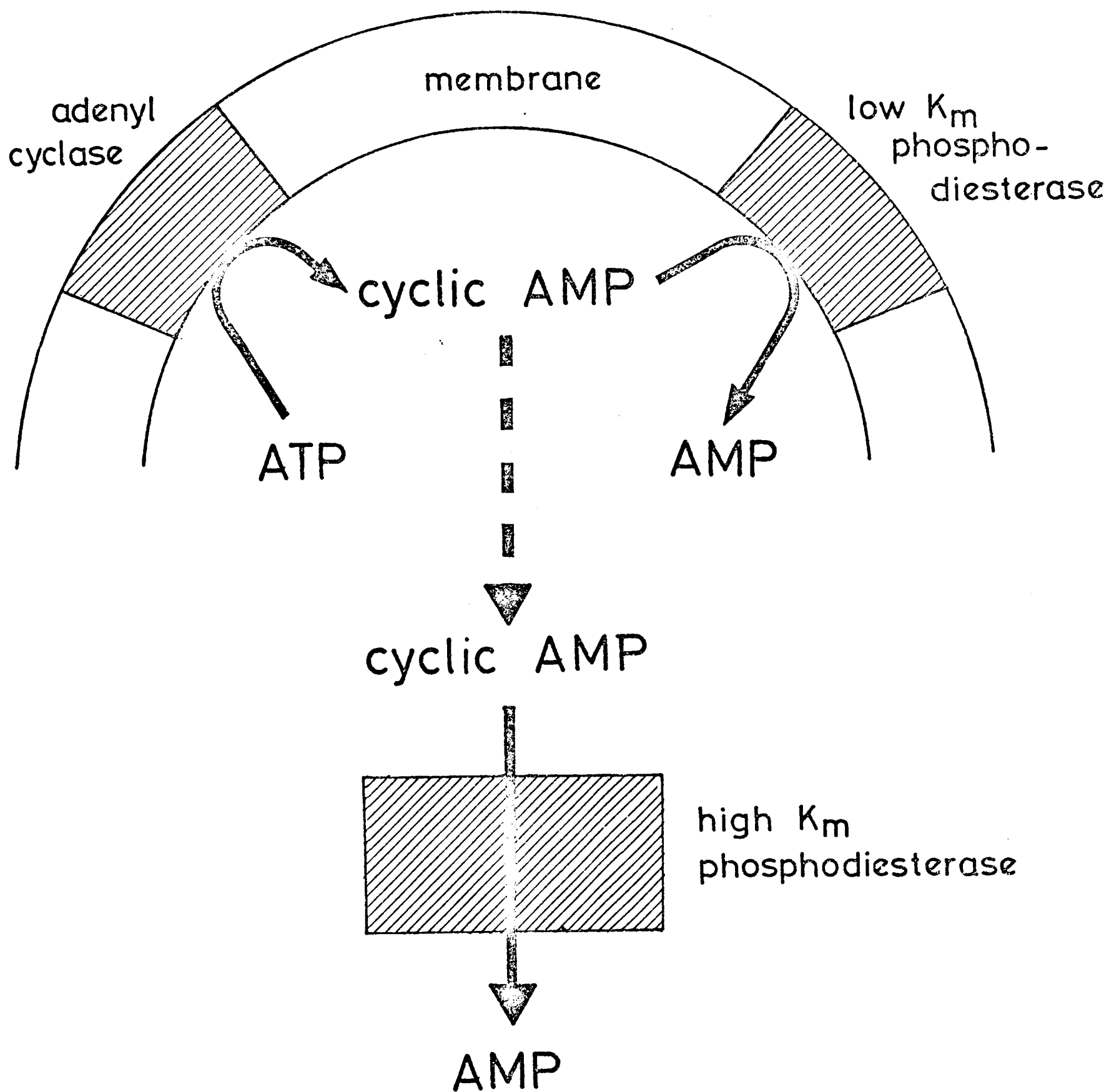


FIG 3.2 A model of the turnover of cyclic AMP in the cell.

The model is discussed in section C4.

The broken line represents the diffusion of cyclic AMP from its site of production.

It was previously suggested that this problem might indicate that the phosphodiesterase reaction is near equilibrium under basal conditions. The present discussion, on the other hand, suggests that the effect of the phosphodiesterase inhibitor and the hormone together is to convert a tissue from situation 1 to situation 2 (see paragraph (a) above) and thereby make its content of cyclic AMP more sensitive to any modification of the activity of adenylyl cyclase or phosphodiesterase.

According to the modified model, the explanation why the activity of the high K_m phosphodiesterase is high when the activities of the low K_m enzyme and adenylyl cyclase are similar is as follows (see Figure 3.2). Cyclic AMP is produced at the cell membrane. It is then either hydrolyzed by low K_m phosphodiesterase at the same location or it diffuses away to high K_m phosphodiesterase in the cytosol and is hydrolyzed. Thus there will be competition between hydrolysis by low K_m phosphodiesterase and diffusion: as the concentration of cyclic AMP near the cell membrane increases, the activity of the low K_m phosphodiesterase rises asymptotically towards a maximum but the rate of the diffusion process continues to rise linearly. In those tissues where the local activity of adenylyl cyclase is higher than the activity of phosphodiesterase, the rate of diffusion will be highest. Accordingly, these tissues require the highest activities of high K_m phosphodiesterase. The role of high K_m phosphodiesterase is therefore essentially the same according to both the simple and modified models.

C.5 THE COMPARTMENTATION OF CYCLIC AMP

(a) COMPARTMENTATION DUE TO THE LOCATIONS OF PHOSPHODIESTERASE AND ADENYLYL CYCLASE

The locations for the low and high K_m phosphodiesterases suggested above require that there is some compartmentation of cyclic AMP. However, the difference in the concentration of cyclic AMP between the region where

adenyl cyclase and low K_m phosphodiesterase are located and where high K_m phosphodiesterase is located may only be small when cyclic AMP is produced by adenyl cyclase at a rate which is well below the local maximal activity of low K_m phosphodiesterase. The concentration differences will be large when the activity of adenyl cyclase is greater than the activity of the low K_m enzyme (see above). Under these circumstances, the simple model presented in Section C.2 will underestimate the steady-state concentration of cyclic AMP, because it does not predict the accumulation of cyclic AMP which occurs in those compartments where the activity of adenyl cyclase is greater than the activity of phosphodiesterase. (The accumulation is limited by the diffusion of cyclic AMP out of the compartment.) Thus in those cases where there is a discrepancy between the predictions of the simple model and measured concentrations of cyclic AMP it is indeed found that the predicted concentration is too low (Table 3.10).

(b) COMPARTMENTATION DUE TO BINDING OF CYCLIC AMP

There is a further complication in understanding the control of the concentration of cyclic AMP: some cyclic AMP may be bound to proteins and unavailable to phosphodiesterase (Chapter 1, Section A.4). This is another classification of compartmentation. It also would cause the simple model to underestimate the concentration of cyclic AMP in a tissue, since in the simple model all the cyclic AMP is available to phosphodiesterase. The present results, as interpreted by the simple model, indicate that the proportion of cyclic AMP unavailable to phosphodiesterase cannot be very large; they are consistent with the finding that 20% of the cyclic AMP is bound to protein kinase when adenyl cyclase is not activated by hormones (Soderling et al., 1973).

C.6 THE ROLE OF CYCLIC AMP IN VARIOUS TISSUES

(a) THE REGULATION OF THE PERMEABILITY OF MEMBRANES BY CYCLIC AMP

It has been noted by many workers that the activities of adenyl cyclase and cyclic AMP phosphodiesterase are relatively high in the brain. This observation suggested that cyclic AMP plays an important role in nerve function (Robison et al., 1970). In the present study it has been found that the activities of adenyl cyclase and phosphodiesterase are also high in invertebrate nervous tissues compared to the activities in muscles from the same species. However, since the actions of cyclic AMP are mediated through a catalyst, protein kinase, high activities of adenyl cyclase and phosphodiesterase need not signify that cyclic AMP-mediated metabolic processes (or enzymes whose activity is modified by cyclic AMP-dependent protein kinase) are particularly abundant in a tissue. Instead it could be suggested that the number of enzyme molecules whose activity is modulated by cyclic AMP may be reflected in the activity of protein kinase, rather than adenyl cyclase or phosphodiesterase. It appears from the data of Kuo & Greengard (1969) that the activity of protein kinase is similar in a number of bovine, rat and invertebrate tissues (see also Kuo et al., 1970). The activity of cyclic AMP-stimulated protein kinase is about three times higher in rat skeletal muscle than in rat heart, liver and brain, whereas in the present study it has been found that the activities of adenyl cyclase and phosphodiesterase are higher in heart, liver and brain than in skeletal muscle. On the other hand, the activity of protein kinase in the cerebral ganglion of the lobster is 34-fold greater than its activity in abdominal muscle (Kuo et al., 1971), which is similar to the relative activities of cyclic AMP phosphodiesterase measured in the present study. High activities of adenyl cyclase and phosphodiesterase may indicate that steady-state levels of cyclic AMP must be increased and decreased very rapidly, i.e. transient times must be very small. Thus in nervous tissues cyclic AMP levels may

control changes in the permeability of membranes, which must be modulated rapidly for the transmission of nerve impulses (see Greengard & McAfee, 1972; Johnson et al., 1972; Maeno & Greengard, 1972).

The high activities of adenylyl cyclase and phosphodiesterase in the heart compared to skeletal muscle may be explained in a similar manner. One difference between the heart and skeletal muscles is that in the heart excitation is transmitted from one muscle fibre to another, whereas in skeletal muscle each fibre is innervated at one or more end-plates. In the heart there are bundles of primitive muscle fibres which are particularly concerned in the transmission of a wave of excitation throughout the heart. The rate at which the wave of excitation is transmitted is continuously regulated by the sympathetic and parasympathetic nervous systems. The action of one or both of these nervous systems may be modulated by cyclic AMP. (It seems probable that the sympathetic nervous system, which releases adrenaline and noradrenaline, will affect cyclic AMP levels, whereas the parasympathetic nervous system, which releases acetylcholine, will affect cyclic GMP levels - see Chapter 1, Section A.2.) Since the rate of heart contraction must be regulated rapidly at the onset of activity in order to maintain oxygen and fuel supplies to muscles, the modulation of cyclic AMP levels must be rapid. Therefore the activities of adenylyl cyclase and phosphodiesterase must be high. Indeed, it has been shown that cyclic nucleotide levels oscillate during the contraction-relaxation cycle of the heart (see Chapter 1, Section A2b). Thus changes in the concentrations of cyclic nucleotides may be necessary for the transmission of the wave of excitation between muscle cells, even in the absence of any autonomic regulation of the transmission. (Another possible explanation for the high activities of adenylyl cyclase and phosphodiesterase in the heart is discussed in section (c) below.)

(b) THE REGULATION OF THE FORCE OF MUSCLE CONTRACTION BY CYCLICAMP

In the above discussion it has been proposed that cyclic AMP is involved in the transmission of electrical excitation between the muscle cells of the heart. Another role for cyclic AMP in muscle has been suggested in the literature. Catecholamines modulate the force of contraction of both heart and skeletal muscle (see Sobel & Mayer, 1973; Gruber, 1922a,b; cf. Triner et al., 1971, for smooth muscle). This effect may be brought about through an increase in the concentration of cyclic AMP in the tissue in response to the hormone (Robison et al., 1965; Posner et al., 1965). However, the effect can occur independently of any increase in glycogenolysis in the muscle (see Sobel & Mayer, 1973). It is possible that cyclic AMP affects the force of contraction by increasing the rate of hydrolysis of ATP by actomyosin ATPase. Thus a component of troponin (which regulates the ATPase) from rabbit skeletal muscle is phosphorylated by phosphorylase b kinase (Stull, et al., 1972; Pratje & Heilmeyer, 1972). (It is unlikely that a change in the concentration of cyclic AMP is an obligatory link in the coupling of nervous stimulation to its contraction, since electrical stimulation does not affect the concentration of cyclic AMP in skeletal muscle - Posner et al., 1965.) If cyclic AMP mediates the effect of catecholamines in increasing the force of contraction of both heart and skeletal muscle, it may be asked why the activities of adenyl cyclase and cyclic AMP phosphodiesterase are low in white skeletal muscle compared to the heart. The reason may be that this effect of catecholamines does not need to be developed quickly compared to, for example, effects of hormones on the transmission of electrical excitation. Thus it may take more than a minute for adrenaline to develop its maximal effect on the force of muscle contraction (Gruber, 1922a,b; Robison et al., 1965). Consequently, this putative role of cyclic AMP would not necessitate

high activities of adenyl cyclase and phosphodiesterase. Nevertheless, the effect of adrenaline which would be ultimately developed may be large, because changes in the concentration of cyclic AMP are amplified through protein kinase.

(c) THE REGULATION OF ENERGY SUPPLY BY CYCLIC AMP

As yet there has been no attempt to explain why the activities of adenyl cyclase and phosphodiesterase are higher in red than in white skeletal muscles. One suggestion which has been put forward to account for this observation is as follows (see Severson et al., 1972). The major fuel store in red muscles (and hearts) is fat, whereas the major store in white muscles is glycogen. It appears that exercise can only induce lipolysis through hormonal mechanisms, whereas glycogenolysis can be induced by exercise independently of hormones. Thus adrenalectomy or adrenal blockage do not influence exercise-induced glycogenolysis but lipolysis is prevented (Gollnick et al., 1970). It is possible that the rapid increases in glycogenolysis which occur during the contraction of white skeletal muscle are brought about by the activation of phosphorylase b kinase by Ca^{2+} or by the activation of phosphorylase b by AMP (Ozawa et al., 1967; Lyon et al., 1967). In red muscles it might be important that the rate of lipolysis of endogenous triglyceride is rapidly increased at the onset of exercise. Since lipolysis is regulated by hormones, the need for rapid changes in lipolytic rate would explain why the activities of adenyl cyclase and phosphodiesterase are higher in red than in white muscles. (Moreover, since the heart also has large stores of triglyceride, this might explain why it too contains high activities of the enzymes.)

However, it has not been demonstrated that endogenous triglyceride is rapidly hydrolyzed by red muscles at the onset of exercise. The role of stored triglyceride is unclear. Since muscles require oxygen in order to

obtain energy from stored triglyceride, they must have an adequate blood supply in order to make use of the store. The blood will supply fuels such as glucose and fatty acids which should provide adequate energy for exercise. The store of triglyceride probably only supplements this supply.

Thus the inadequacy of the above argument suggests that cyclic AMP may have another, as yet unknown, role in red muscle. Red muscles contain a large number of mitochondria and high activities of the enzymes of the tricarboxylic acid cycle. Could cyclic AMP be involved in the control of the activity of one of the enzymes of the cycle or the transport of metabolites across the mitochondrial membrane? If cyclic AMP has one of these roles, the high activities of adenyl cyclase and phosphodiesterase in insect flight muscles may be explained, since these muscles also contain large numbers of mitochondria. The activities of the enzymes are similar in flight muscle from a number of species (except the honey-bee), and are not correlated with the physiology of the muscle (fibrillar or non-fibrillar) or the dependence of the muscle upon the oxidation of fat or carbohydrate during flight (see Crabtree & Newsholme, 1972a,b). It is possible that the TCA cycle is switched on at a similar rate in all the insects investigated for the present study (except the honey-bee) by a cyclic-AMP mediated process. However, at the present time there is no evidence to support such a role for cyclic AMP.

This discussion does not exclude a role for adrenaline in the stimulation of glycogenolysis in muscle. It is suggested that the action of the hormone in preparing an animal for action by mobilizing glycogen reserves does not need to be as rapid as the mechanisms which mobilize fuel reserves at the onset of muscular activity. Consequently in white muscles, where the main role of adrenaline is to prepare the animal for exercise, the activities of adenyl cyclase and phosphodiesterase are comparatively low. The effect of adrenaline on the concentration of cyclic AMP, though not as rapid as in

some tissues, is amplified both through protein kinase and phosphorylase b kinase, so that the rate of breakdown of glycogen can be greatly increased. The mediation of phosphorylase b kinase in this system not only increases the level of amplification but it also introduces a stage at which Ca^{2+} can influence glycogenolysis independently of any hormonal effects.

Chapter 4

STUDIES ON 5'-NUCLEOTIDASE, ADENOSINE KINASE AND ADENOSINE DEAMINASE

A. INTRODUCTION

This study of the enzymes which produce and metabolize adenosine was attempted in order to provide further information concerning and perhaps a better understanding of, some aspects of the metabolism of adenosine; the mechanisms by which adenosine concentrations are controlled; the functions of the enzymes in releasing adenosine from the cell and taking it up again; the role of adenosine within the organism; and the biochemical mechanisms whereby adenosine fulfils this role.

Because of this range of interests, the enzymes have been investigated in a number of ways. Firstly, the effects of some possible regulators of the enzymes have been examined; the effect of pH may be included under this heading. Secondly, the kinetics of the enzymes have been studied in numerous tissues. Thirdly, the effects on the enzymes of some compounds which are known to alter the physiological effects of adenosine have been studied. Finally, an attempt has been made to correlate the maximal activities of the enzymes with their proposed physiological functions in relation to the role of adenosine.

B. METHODS

The standard methods used to homogenize tissues and assay the enzymes for the work presented in this chapter have been described in Chapter 2, Section A. Additional details and variations from the standard methods are described below.

B.1 EFFECTS OF Na^+ AND K^+

In experiments where the ionic strength of the assay medium was held constant, citrate-sodium phosphate and citrate-potassium phosphate buffers were mixed in varying proportions, so as to give known concentrations of Na^+ and K^+ in the assay. In other experiments NaCl and KCl were dissolved in 21 mM Tris or imidazole buffers at pH 7.5. The adenosine concentration in the enzyme assays was 5 μM .

B.2 EFFECTS OF Ca^{2+}

Calcium-EGTA buffers were made up in Tris at pH 7.1 according to the specifications of Portzehl et al. (1964). EGTA was present in the assay medium at a concentration of 1.68 mM. Further variations from the standard assay methods were as follows: EDTA was excluded from the assays; NaCl or KCl (60 mM) was included in some assays; Mg^{2+} was present at a concentration of 21 mM in the assay medium for 5'-nucleotidase. The enzymes were assayed using 5 μM adenosine or 200 μM AMP as substrate.

It is assumed that 1.68 mM EGTA reduced Ca^{2+} concentrations to 3×10^{-9} M. However, the precise concentration of Ca^{2+} may have been lower if the amount of contamination of salts used to make up the assay medium was very low.

B.3 MEASUREMENTS OF MAXIMAL ACTIVITIES AND K_m VALUES

In the comparative study, K_m values were determined for each enzyme from each tissue in order that determinations of V_{max} could be performed at concentrations of adenosine which approached saturation but were not inhibitory. In other enzyme assays at least two concentrations of substrate were used.

C. RESULTS

C.1 THE SPECIFICITY OF ADENOSINE KINASE FOR ITS PHOSPHATE DONOR

PEP acts as a donor of high energy phosphate in some bacterial systems (Kaback, 1968). The ability of adenosine kinase to use PEP and creatine phosphate as phosphate donors has not been reported. The activity of adenosine kinase with these substrates was very low (less than 14% of that with ATP - see Table 4.1). Since crude extracts of mouse brain were used in this study, the small amounts of adenosine phosphorylation in the presence of PEP or creatine phosphate may have been due to low concentrations of endogenous adenine nucleotides and the presence of endogenous creatine kinase or pyruvate kinase. There is therefore no evidence that PEP or creatine phosphate can act directly as the phosphate donor for adenosine kinase.

C.2 THE EFFECTS OF METHYLYXANTHINES, PAPAVERINE AND CYCLIC AMP ON THE ACTIVITIES OF ADENOSINE KINASE AND ADENOSINE DEAMINASE

Theophylline activated adenosine kinase but inhibited adenosine deaminase from mouse tissues when the enzymes were assayed at pH 7.5 (Figure 4.1, Table 4.2). Adenosine kinase was inhibited by theophylline at pH 5.3 (Table 4.2). The percentage activation of adenosine kinase at pH 7.5 (about 75%) and the percentage inhibition at pH 5.3 (about 25%) produced by 10 mM theophylline were similar with each tissue, except gastrocnemius muscle (Table 4.2). It was not possible to classify the kinetics of these effects. A Dixon plot (1953) gave a K_i for adenosine deaminase inhibition by theophylline of 5 mM. Half-maximal activation of adenosine probably occurs at a similar concentration (Figure 4.2). Pre-incubation of homogenates of mouse brain with theophylline caused a decrease in the activity of adenosine kinase (Figure 4.3).

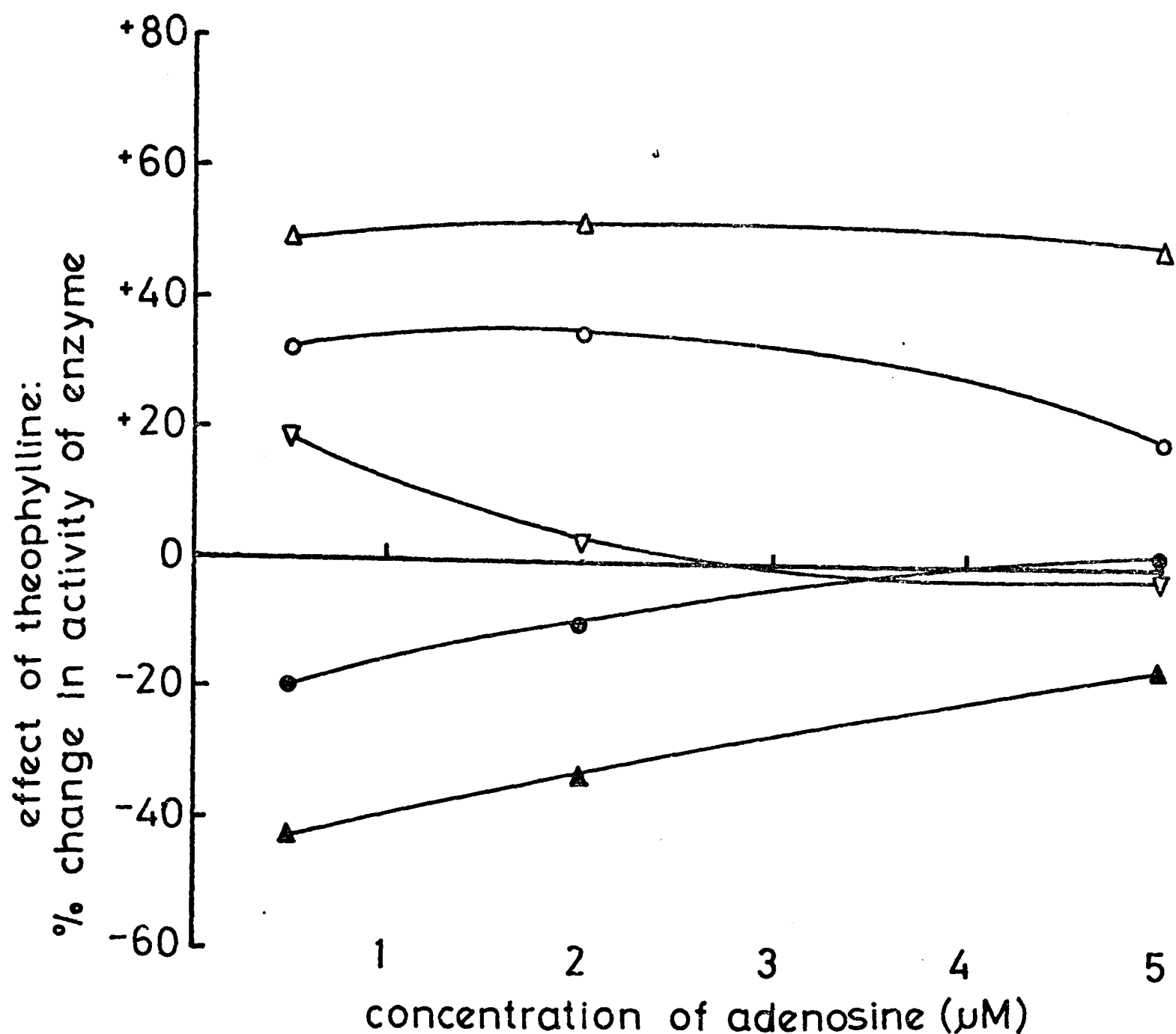
Papaverine (200 μ M) activated adenosine kinase from mouse brain and heart by 40-50% (Figure 4.4). Therefore the observed inhibition of adenosine

TABLE 4.1 The effect of replacing ATP by PEP and creatine phosphate
in the adenosine kinase reaction

Adenosine kinase was assayed as described in Chapter 2, Section A.4 except that ATP was replaced by PEP or creatine phosphate as indicated and creatine kinase was omitted from the incubation medium. Assays were performed on crude extracts of tissues at pH 7.5 using 5 μ M adenosine as substrate.

PHOSPHATE DONOR (4 mM)	ACTIVITY OF ADENOSINE KINASE (nmol/min/g wet wt.)	
	MOUSE BRAIN	DOMESTIC FOWL LIVER
ATP	120	594
PEP	21	0
Creatine phosphate	16	0
Creatine phosphate*	-	79

* Creatine kinase (0.2 mg/ml) was added to the incubation medium for this assay.



	adenosine kinase	adenosine deaminase
0.2 mM theophylline	▽	
1.0 mM theophylline	○	●
5.0 mM theophylline	△	▲

FIG 4.1 The effect of theophylline on the activities of adenosine deaminase and adenosine kinase at different concentrations of adenosine.

Mouse brain homogenate was used.

Activities measured in the absence of theophylline are represented by the central line. These were 47, 28 and 9.5 nmol/min/g wet wt. for the kinase, using 5, 2 and 0.5 μM adenosine respectively and 9.4, 3.9 and 1.1 nmol/min/g wet wt. for the deaminase, using the same substrate concentrations. Activation by theophylline is represented by points above the central line; inhibition by points below.

TABLE 4.2 The effect of theophylline upon the activity of adenosine kinase from mouse tissues

The activities of adenosine kinase from various mouse tissues were measured as described in Chapter 2, Section A.4, except that theophylline (10 mM) was included in some assays. The concentration of adenosine used for the assays was 5 μ M

TISSUE	ACTIVITY OF ADENOSINE KINASE AT pH 7.5 (nmol/min/g wet wt)		RATIO OF ACTIVITIES + THEOPHYLLINE - THEOPHYLLINE	ACTIVITY OF ADENOSINE KINASE AT pH 5.3 (nmol/min/g wet wt)		RATIO OF ACTIVITIES + THEOPHYLLINE - THEOPHYLLINE
	CONTROL	+ THEOPHYLLINE		CONTROL	+ THEOPHYLLINE	
Brain	36	60	1.68	145	106	0.73
Heart	85	152	1.79	194	147	0.76
Liver	1260	2290	1.82	2580	1960	0.76
Gastrocnemius muscle	26	34	1.30	44	28	0.64

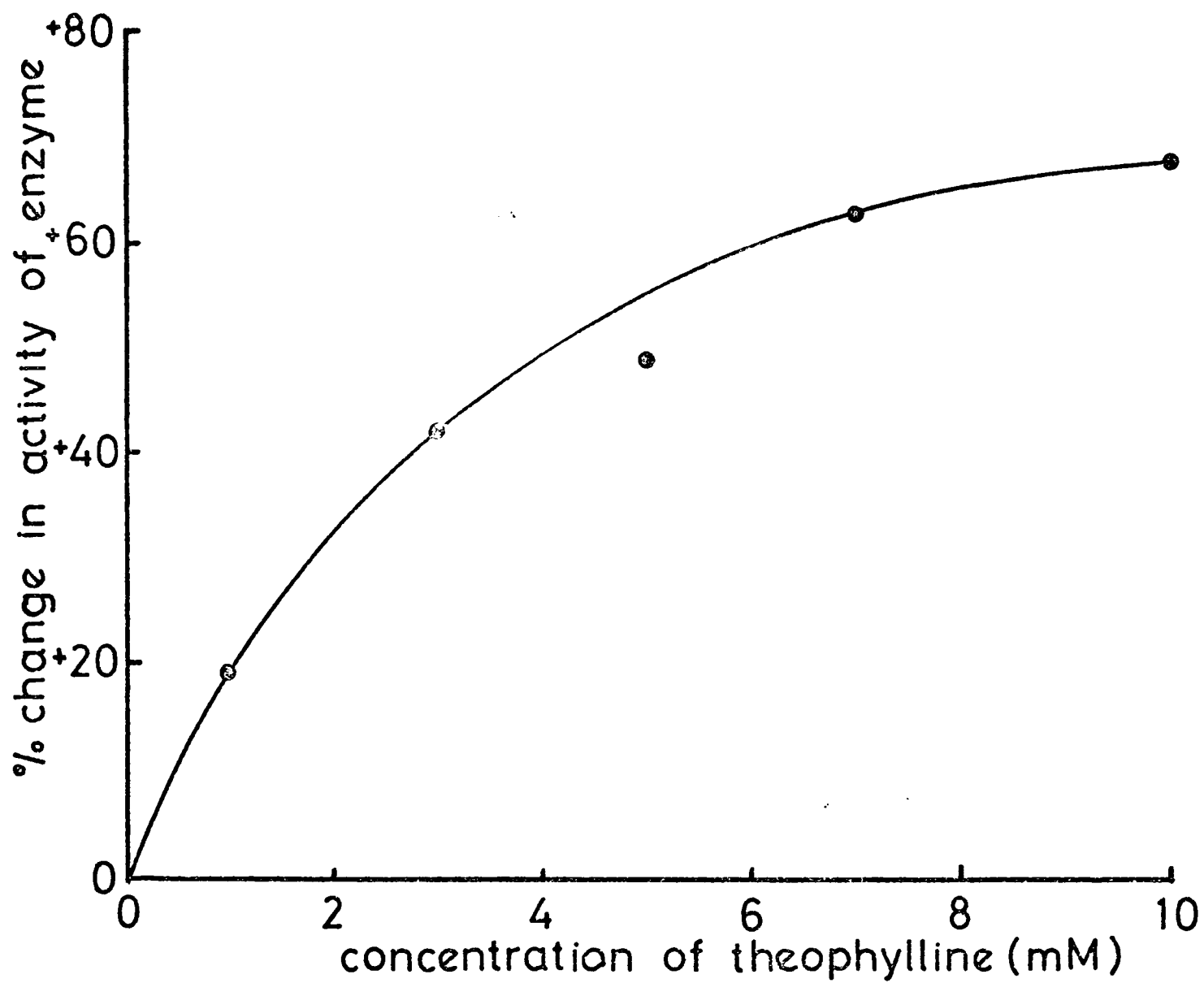


FIG 4.2 The effect of theophylline on the activity of adenosine kinase.

Adenosine kinase from mouse brain was assayed as described in Chapter 2, section A4. Activities are relative to the activity in the absence of theophylline (47 nmol/min/g wet wt.)

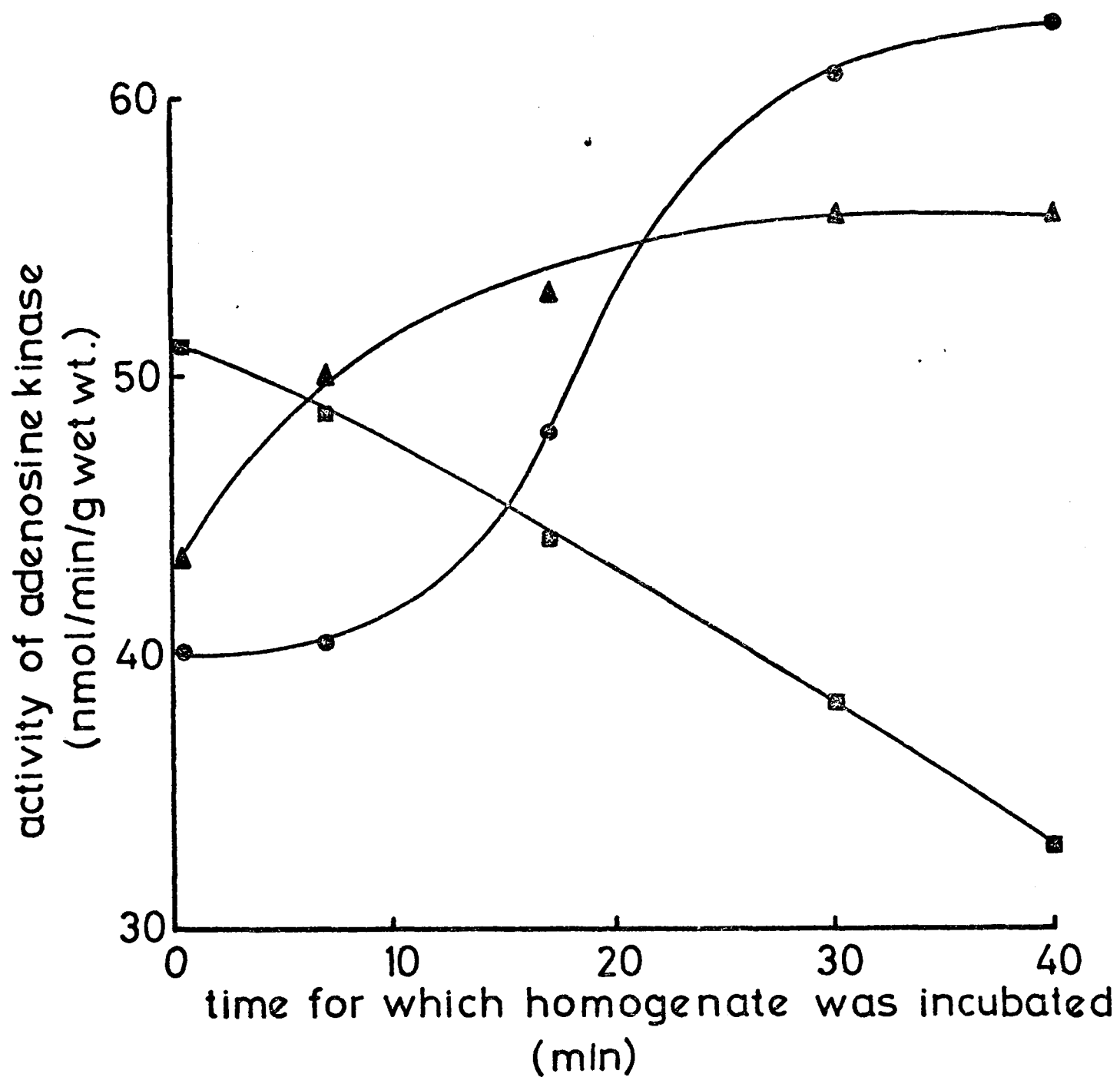


FIG 4.3 a The effect of incubating homogenates of mouse brain with methyl xanthines on the activity of adenosine kinase.

- Incubated in the absence of methyl xanthines.
- Incubated with 3.33mM theophylline.
- ▲ Incubated with 3.33mM caffeine.

Samples of a homogenate of mouse brain were incubated at 30°C with theophylline or caffeine. Aliquots from these incubations were taken at intervals and assayed for adenosine kinase as described in Chapter 2, section A4, using an adenosine concentration of 5 μ M. The concentration of methyl xanthines carried over into the assays was 1 mM.

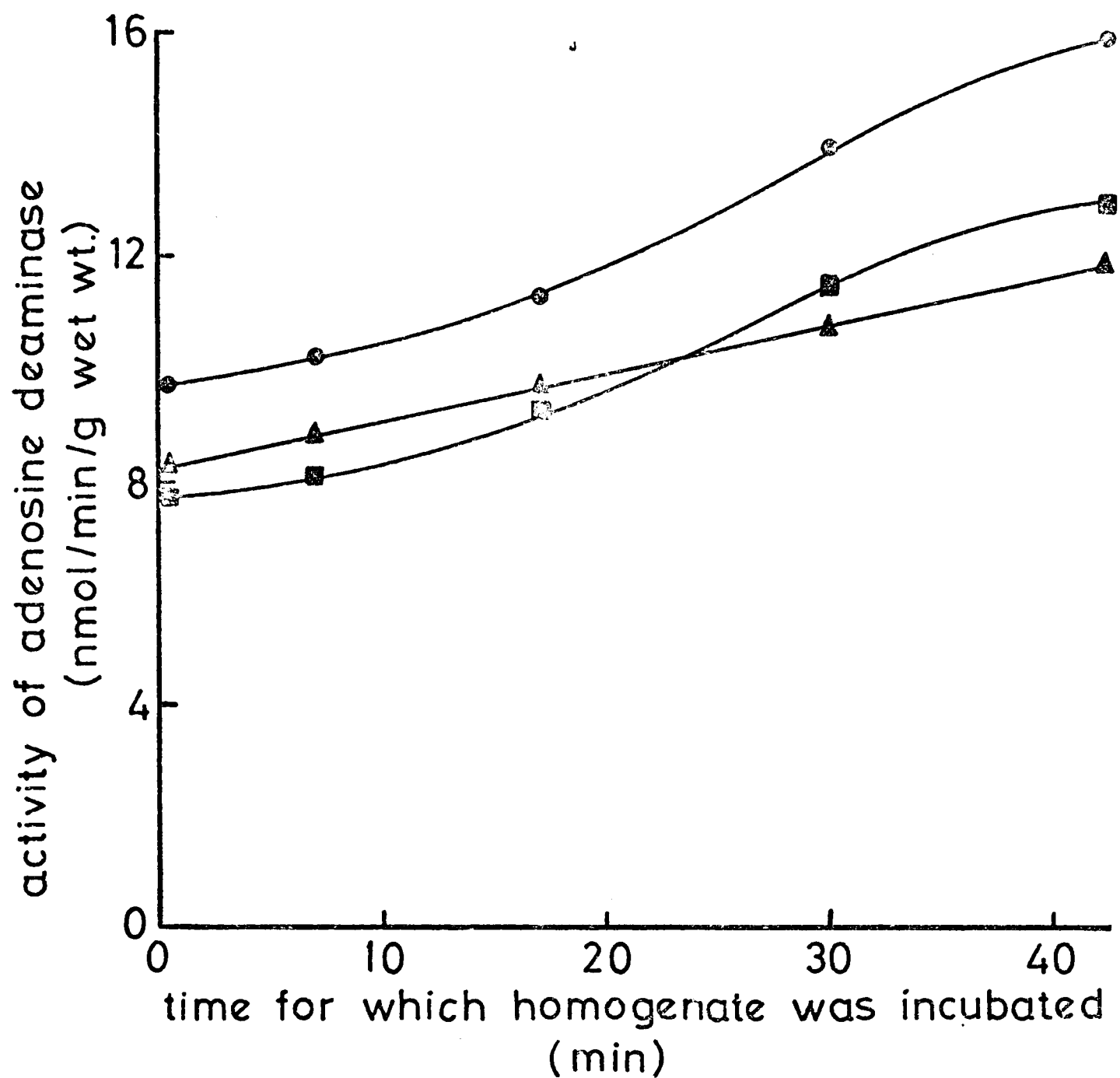


FIG 4.3b The effect of incubating homogenates of mouse brain with methyl xanthines on the activity of adenosine deaminase.

- Incubated in the absence of methyl xanthines.
- Incubated with 3.33 mM theophylline.
- ▲ Incubated with 3.33 mM caffeine.

Details as given in the legend for Figure 4.3a.

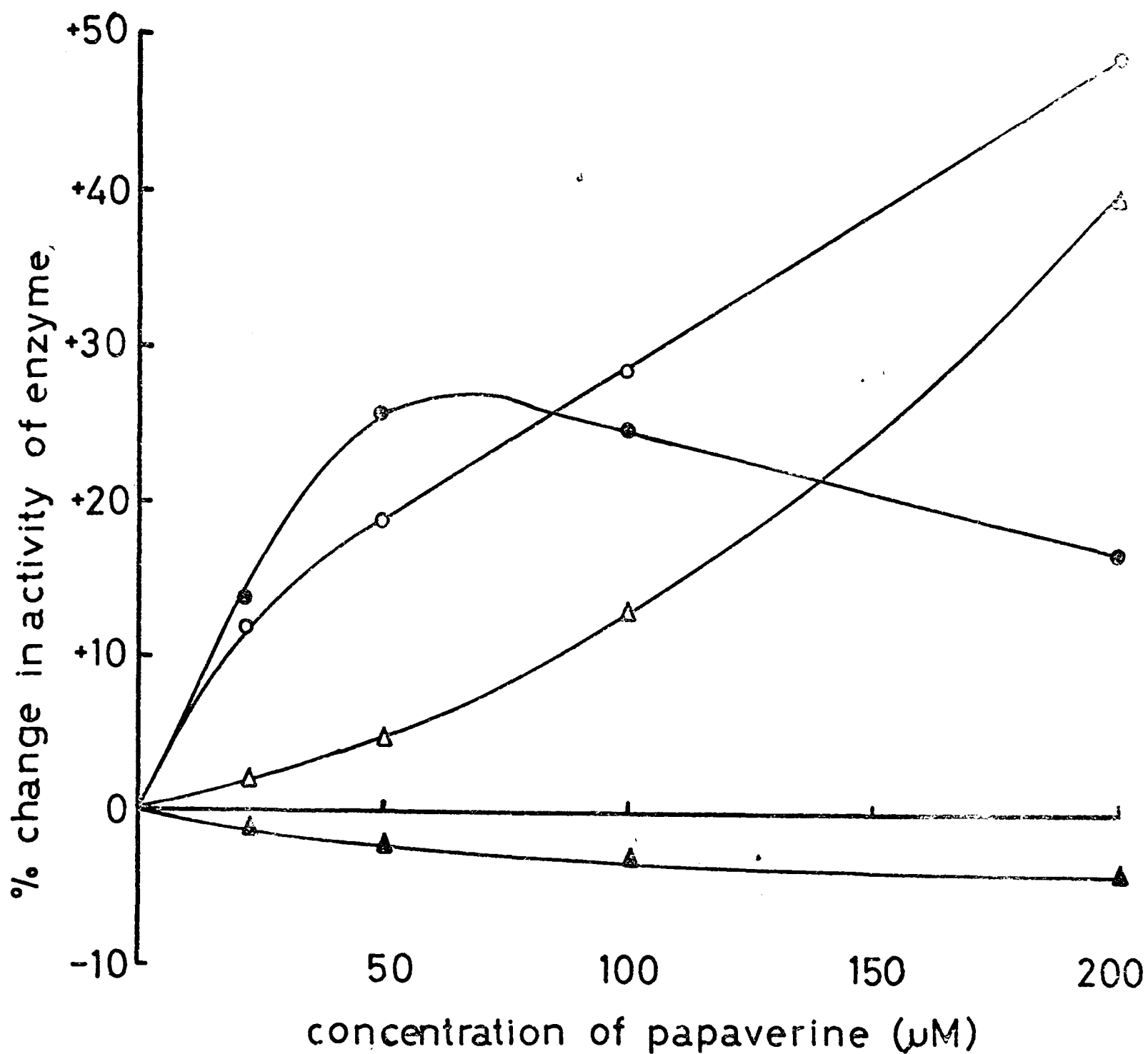


FIG 4.4 The effect of papaverine on the activities of adenosine kinase and adenosine deaminase from mouse tissues.

	TISSUE	ENZYME	ACTIVITY IN ABSENCE OF PAPAVERINE (nmol/min/g wet wt.)
○	brain	adenosine kinase	38
Δ	heart	adenosine kinase	102
●	brain	adenosine deaminase	11
▲	heart	adenosine deaminase	22

The assays were carried out as described in Chapter 2, section A4 using an adenosine concentration of 5 μM. The ordinate represents the percentage activation or inhibition of each enzyme by papaverine in comparison with the activity of the enzyme measured in the absence of papaverine.

uptake by papaverine (Koss et al., 1962; Markwardt et al., 1967; Born & Mills, 1969; Pflieger et al., 1969; Pull & McIlwain, 1972b) is not caused by inhibition of adenosine phosphorylation. Adenosine deaminase from brain (but not heart) was activated by 25%.

Methylxanthines and papaverine are inhibitors of phosphodiesterase, so that it is possible that they caused cyclic AMP to accumulate in the assay medium. However, adenosine kinase and adenosine deaminase were unaffected by cyclic AMP (0.33 mM).

C.3 THE EFFECTS OF Na^+ AND K^+ ON THE ACTIVITIES OF ADENOSINE KINASE AND ADENOSINE DEAMINASE

(a) MOUSE TISSUES

Na^+ activated adenosine kinase from mouse brain assayed at pH 7.5. Maximal activation (120%) occurred at about 400 mM Na^+ (Figure 4.5). On the other hand, K^+ (20-70 mM) slightly inhibited the enzyme. Adenosine kinase from mouse tissues was twice as active in the presence of 140 mM Na^+ than in the presence of 140 mM K^+ (Figure 4.6). (The effects of Na^+ and K^+ were similar in the presence of 10^{-6} or 10^{-9} M Ca^{2+} .)

Adenosine kinase from mouse brain, assayed at pH 5.3, was unaffected by variations in Na^+ concentration but it was inhibited by K^+ (Table 4.3, Figure 4.7).

Adenosine deaminase activity was only slightly affected by variations in Na^+ and K^+ concentrations (Figure 4.5).

(b) INVERTEBRATE TISSUES

The effects of Na^+ and K^+ on adenosine kinase and adenosine deaminase from invertebrate tissues (locust flight muscle and earthworm nerve cord) are very different from the effects observed with mouse tissues (Figures 4.8 and 4.9). There was little effect of the cations on adenosine kinase, whereas adenosine deaminase from locust flight muscle was activated by 50% by a small change in the proportions of ions in the assay medium.

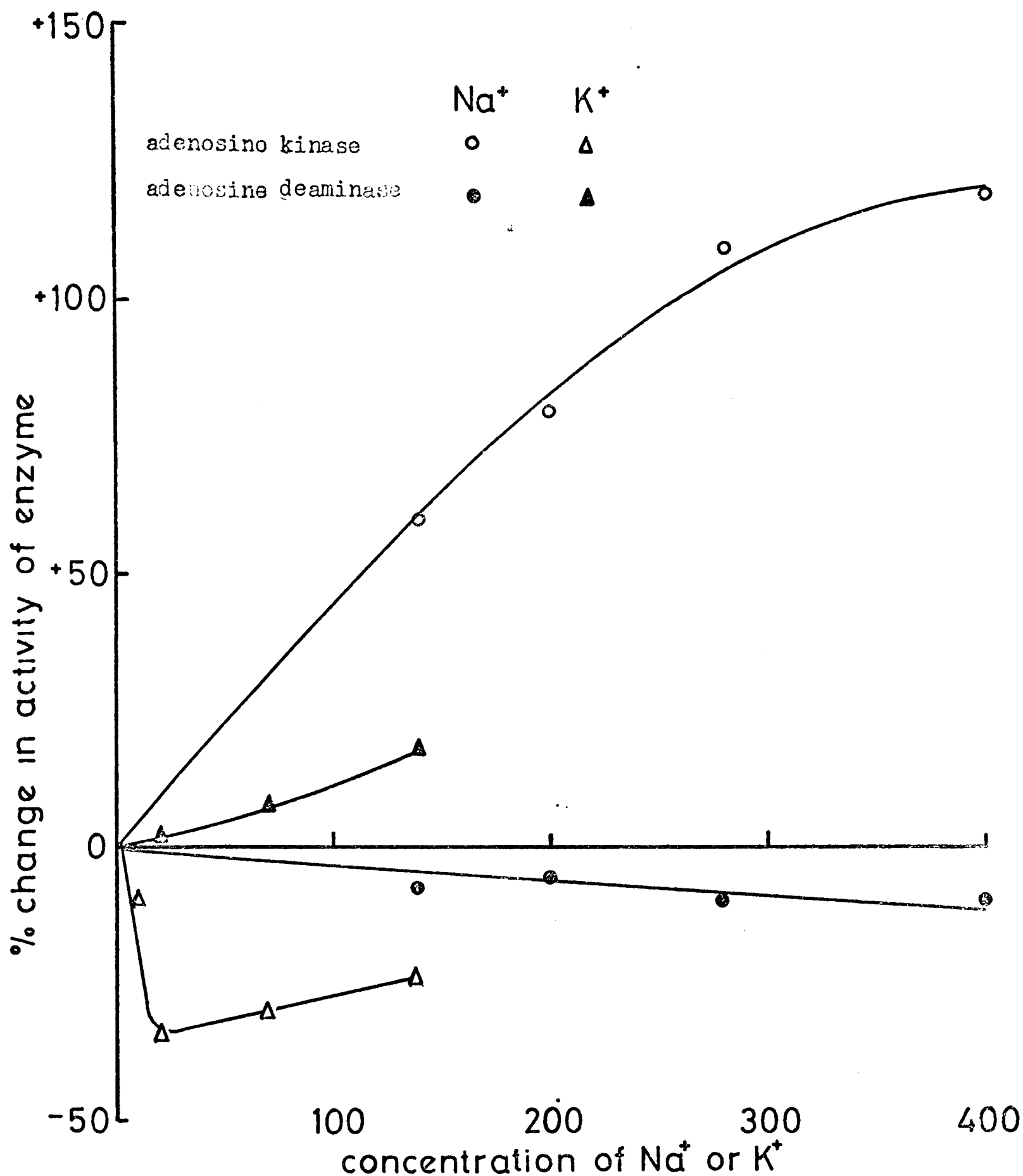


FIG 4.5

The effects of Na⁺ and K⁺ on the activities of adenosine kinase and adenosine deaminase from mouse brain.

The enzymes were assayed in Tris buffer (pH 7.5) in the presence of different concentrations of NaCl or KCl as described in section B1. (Similar results were obtained using imidazole buffer.) The enzyme activities in the absence of metal ions were 2.9 and 11.7 nmol/min/g wet wt. for adenosine kinase and adenosine deaminase respectively.

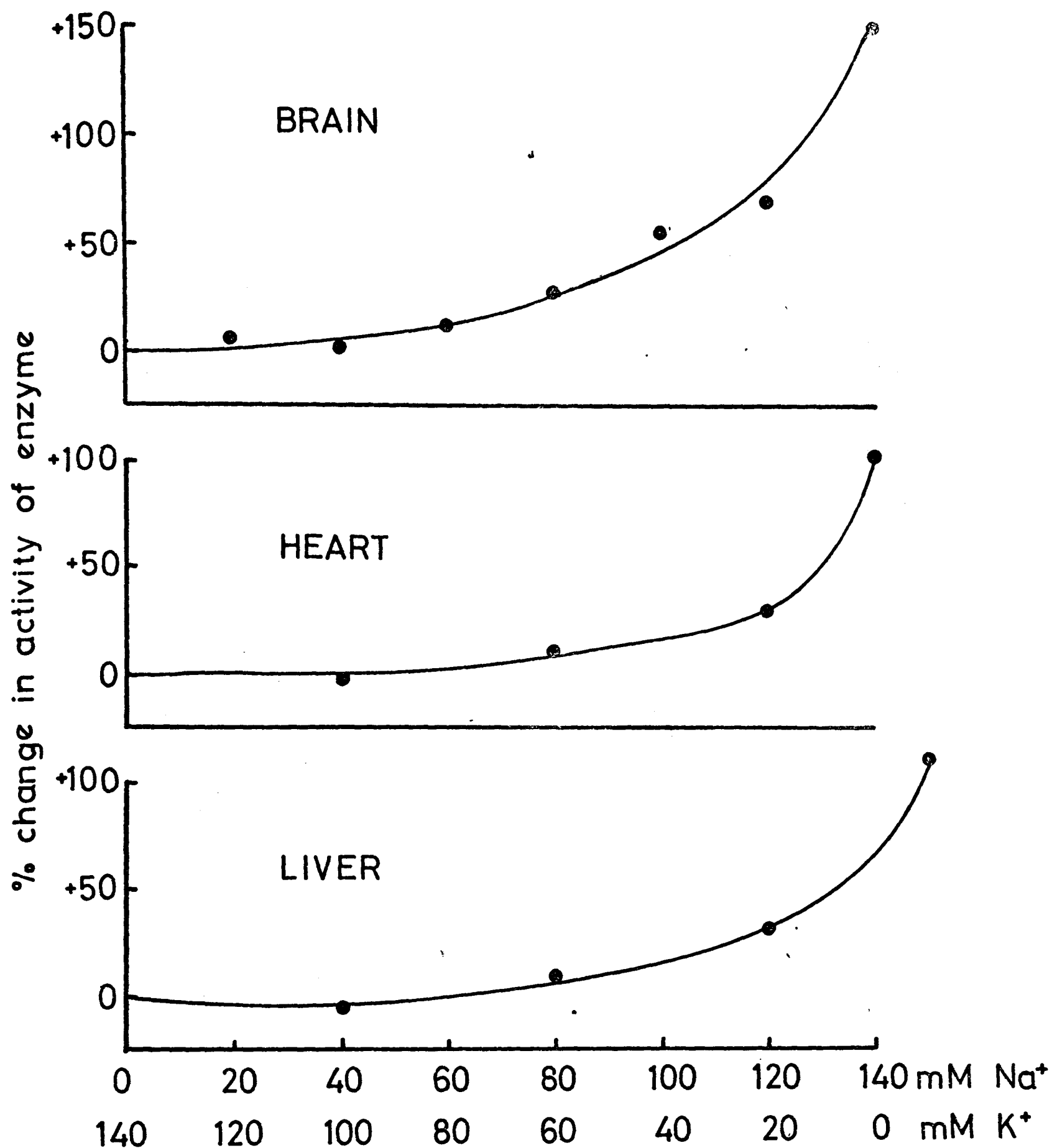


FIG 4.6

The effect of varying the proportion of Na⁺ and K⁺ on the activity of adenosine kinase from mouse tissues.

Adenosine kinase was assayed at pH 7.5 as described in section B1. The ordinates represent the percentage activation of the enzyme in relation to the activity in the presence of 140 mM K⁺ and zero Na⁺. The latter activities were 11, 50 and 593 nmol/min/g wet wt. for the enzymes from brain, heart and liver respectively.

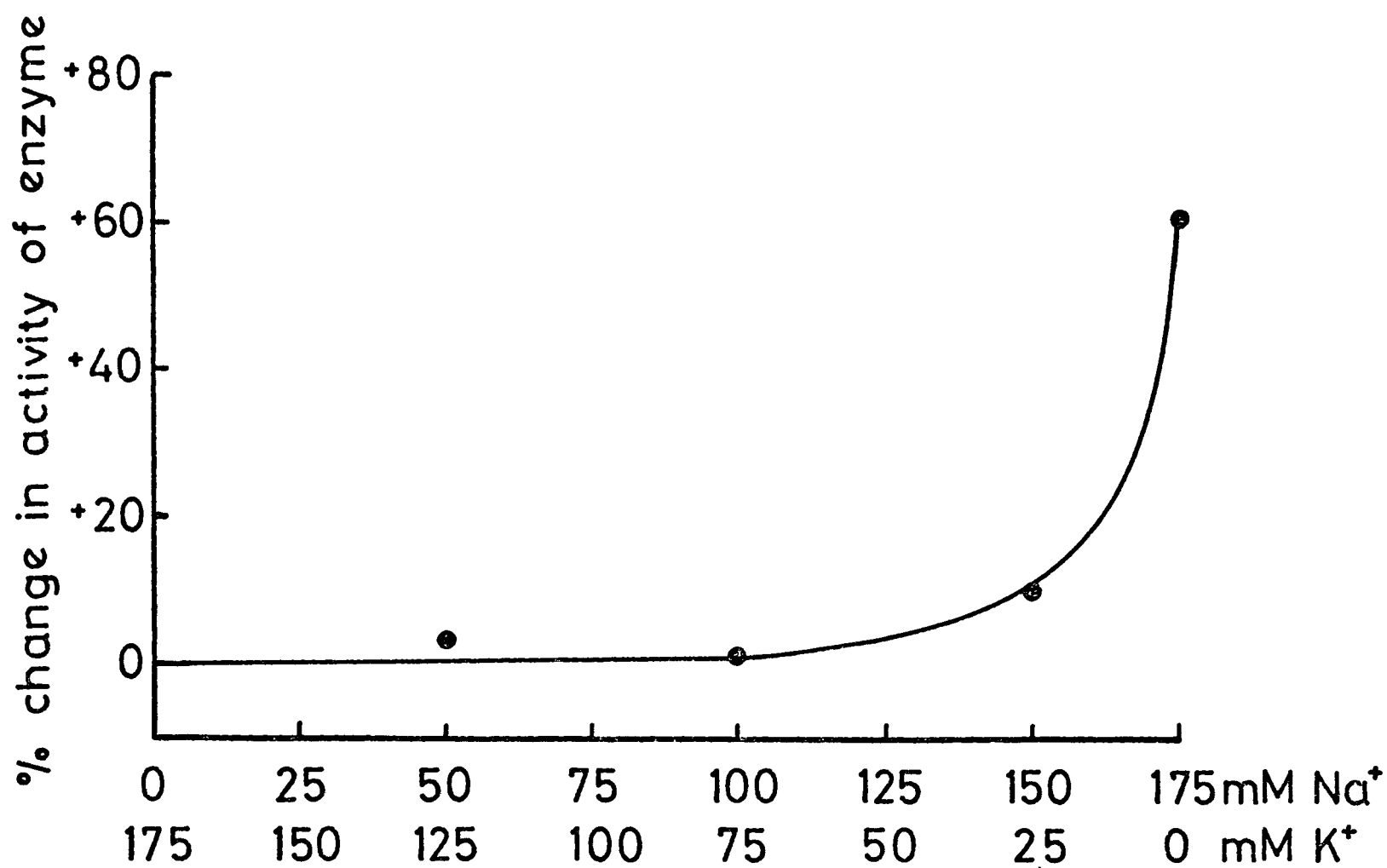


FIG 4.7 The effect of varying proportions of Na⁺ and K⁺ on the activity of adenosine kinase from mouse brain at pH 5.3.

Adenosine kinase was assayed at pH 5.3 as described in section B1. The ordinate represents the percentage activation of the enzyme in relation to its activity in the presence of 175mM K⁺ and zero Na⁺ which was 38 nmol/min/g wet wt.

TABLE 4.3 The effects of Na⁺ and K⁺ on the activity at pH 5.3 of
adenosine kinase from mouse brain

Adenosine kinase from mouse brain was assayed using citrate-phosphate buffers at pH 5.3 as described in Section B.1. The incubation medium was supplemented with NaCl to give concentrations of Na⁺ greater than 92 mM (the concentration of metal ion in the buffer).

CONCENTRATION OF METAL ION	ACTIVITY OF ADENOSINE KINASE (nmol/min/g wet wt.)
92 mM K ⁺	21
92 mM Na ⁺	37
154 mM Na ⁺	36
214 mM Na ⁺	40

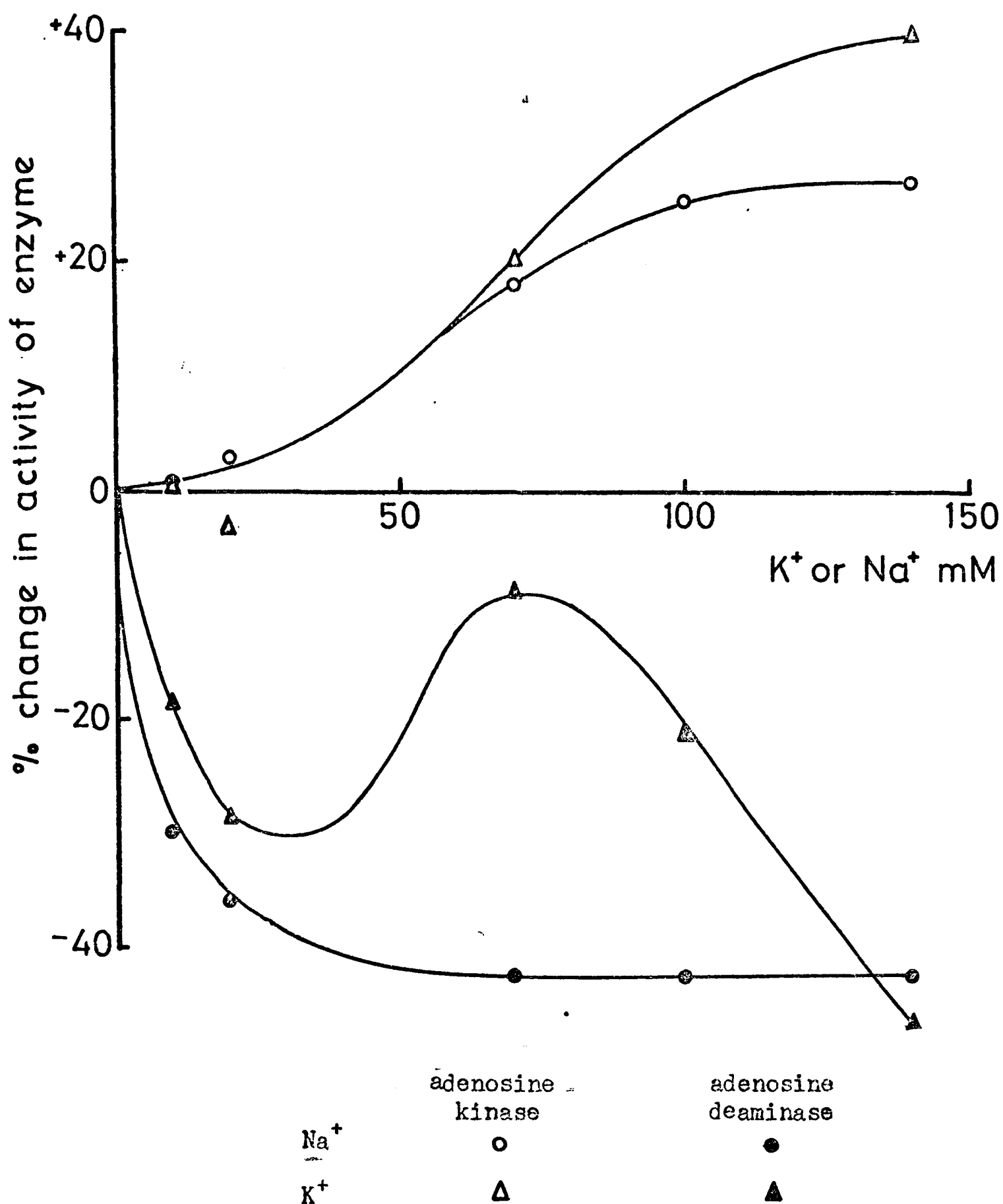


FIG 4.8 The effects of Na⁺ and K⁺ on the activities of adenosine kinase and adenosine deaminase from locust flight muscle.

The enzymes were assayed in imidazole buffer (pH 7.5) in the presence of different concentrations of NaCl or KCl as described in section B1. The ordinate represents the percentage change in the activity of these enzymes compared with the activities in the absence of these ions. The latter activities were 14 and 5.8 nmol/min/g wet wt. for adenosine kinase and adenosine deaminase respectively.

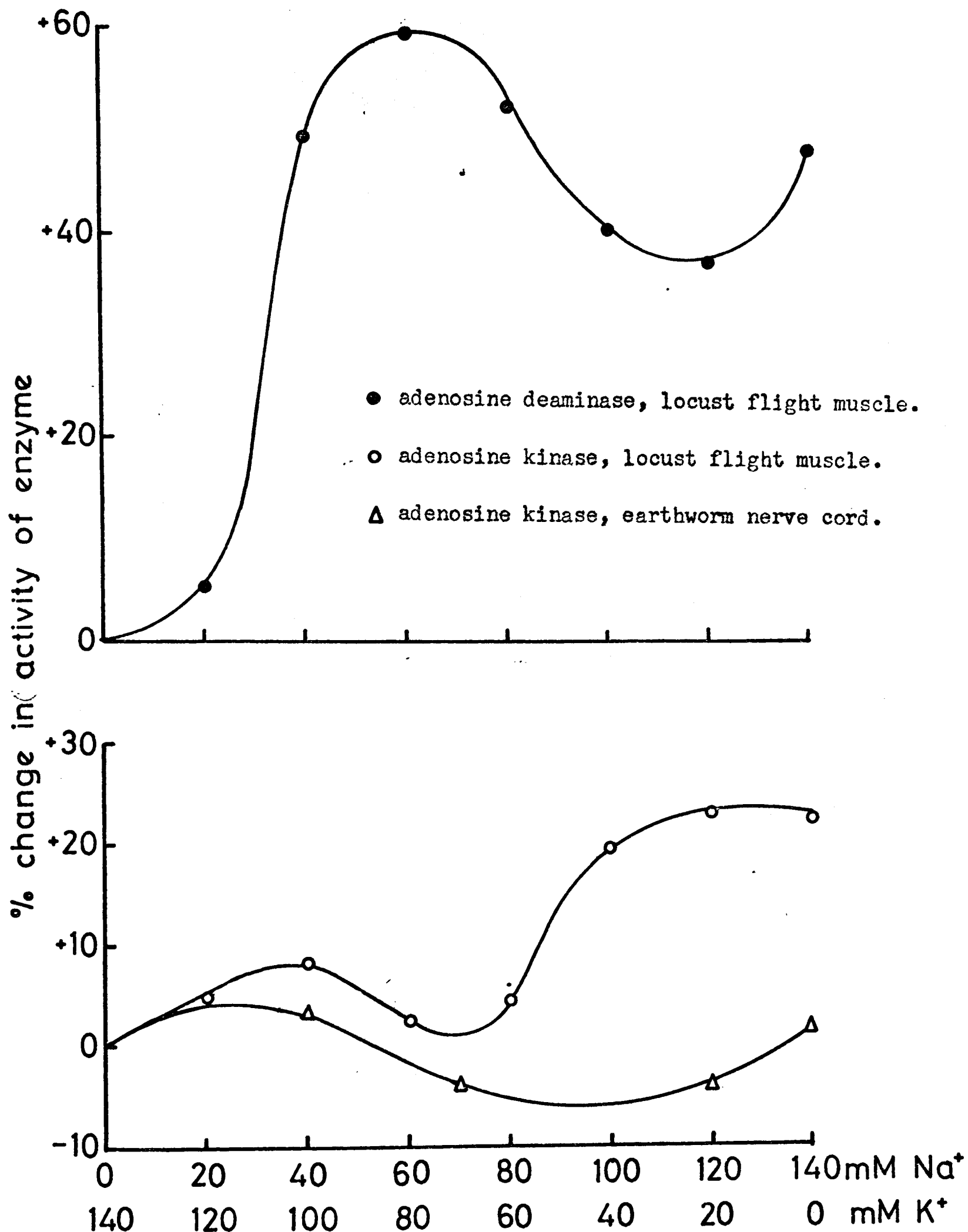


FIG 4.9 The effect of varying proportions of Na⁺ and K⁺ on the activity of adenosine kinase and adenosine deaminase from invertebrates.

For details see the legend to Figure 4.6. The activities of the enzymes in the presence of 140 mM K⁺ and zero Na⁺ were 9.6 nmol/min/g wet wt. for adenosine deaminase from locust muscle and 27 and 28 nmol/min/g wet wt. for adenosine kinase from locust muscle and earthworm nerve cord respectively.

C.4 THE EFFECTS OF Ca^{2+} ON THE ACTIVITIES OF ADENOSINE KINASE, ADENOSINE DEAMINASE AND 5'-NUCLEOTIDASE

Adenosine kinases from mouse and locust tissues were markedly inhibited by 10^{-7} M Ca^{2+} provided the assays were performed in the presence of NaCl (60 mM) (Table 4.4, Figures 4.10 to 4.12): this inhibition was not observed when NaCl was omitted from the assay or when KCl was substituted for NaCl. The activities of 5'-nucleotidase and adenosine deaminase were not affected by Ca^{2+} .

C.5 COMPARATIVE STUDIES OF 5'-NUCLEOTIDASE, ADENOSINE KINASE AND ADENOSINE DEAMINASE

Since EGTA was not included in the assay medium for adenosine kinase, maximal activities may not have been measured. However, this is unlikely since the assay method for adenosine kinase, which employed a citrate-phosphate buffer, was found to give enzyme activities which were approximately 50% higher than those obtained in Tris buffer containing EGTA (i.e. 3×10^{-9} M Ca^{2+}).

The maximal activities of adenosine kinase which are recorded in this chapter were obtained by extrapolating the linear portion of a Hofstee kinetic plot to infinite substrate concentration. Any substrate inhibition was ignored. This means that the V_{max} , a kinetic parameter rather than a measurable activity, is recorded. However, where substrate inhibition was most prevalent, the measurable maximal activity is at most 20% less than the V_{max} recorded. By use of the Hofstee plots, the need to find optimal substrate concentrations in each assay was avoided and enzyme activities can be calculated at specific substrate concentrations from the V_{max} value.

(a) MAXIMAL ACTIVITIES AT pH 7.5 OF 5'-NUCLEOTIDASE, ADENOSINE KINASE AND ADENOSINE DEAMINASE FROM MUSCLES

The maximal activities of 5'-nucleotidase, adenosine kinase and adenosine deaminase in muscle tissues of vertebrates and invertebrates are

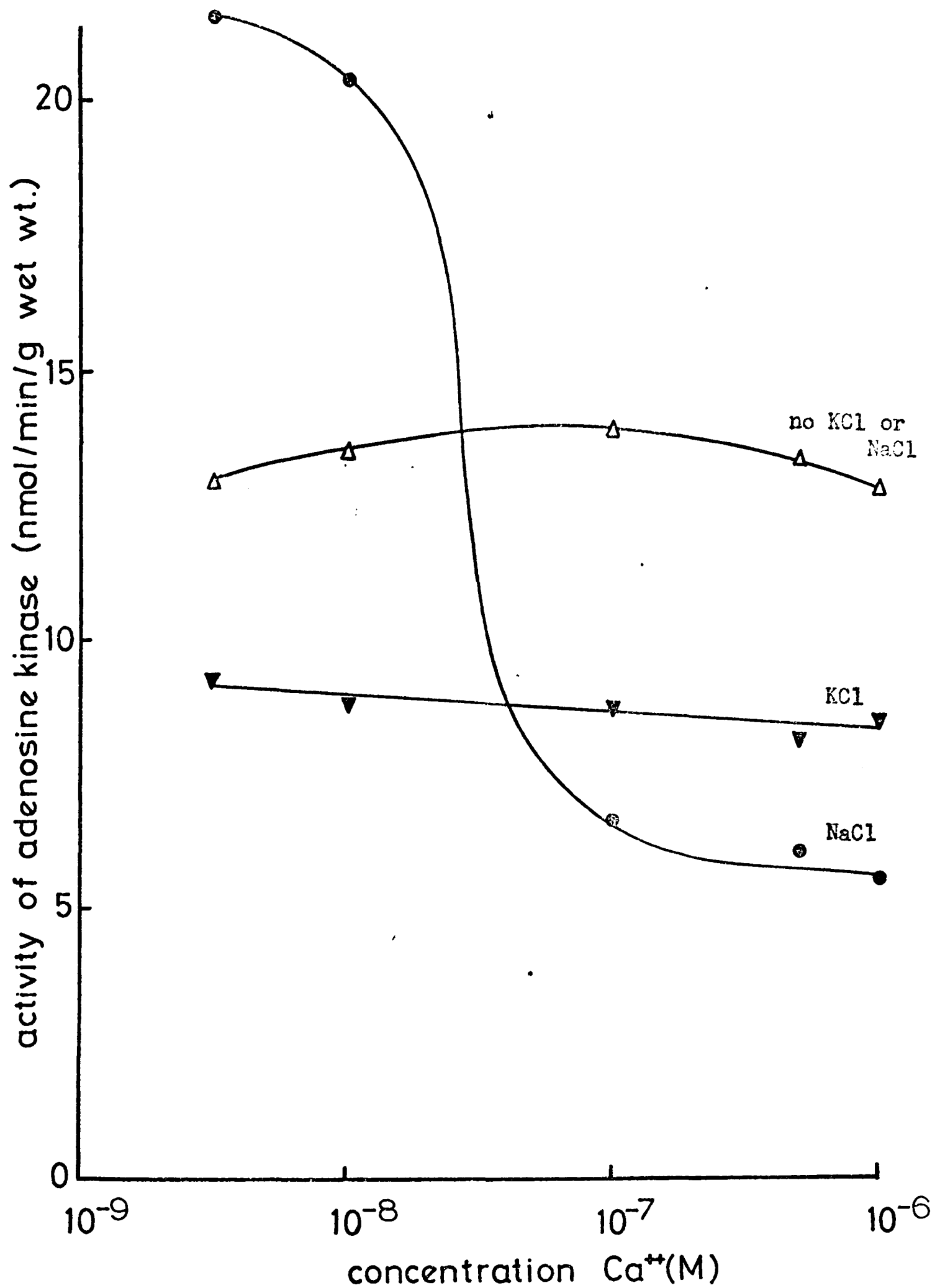


FIG 4.10 The effect of Ca^{++} on the activity of adenosine kinase from mouse brain.

Adenosine kinase was assayed as described in section B2. NaCl or KCl (60 mM) were included in the assay as indicated.

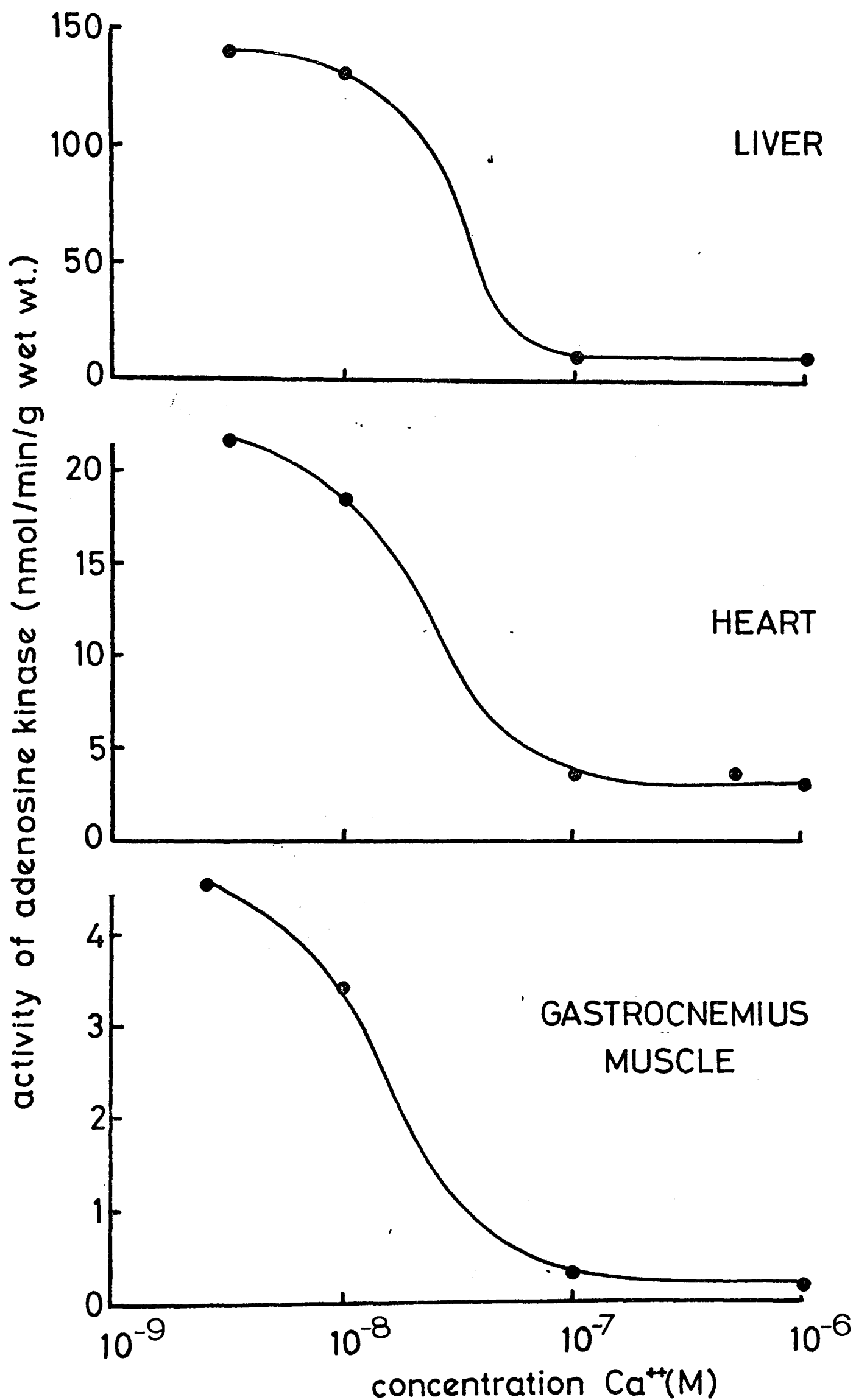


FIG 4.11 The effect of Ca^{++} on the activity of adenosine kinase from mouse tissues.

Adenosine kinase was assayed as described in section B2.
NaCl (60 mM) was included in each assay.

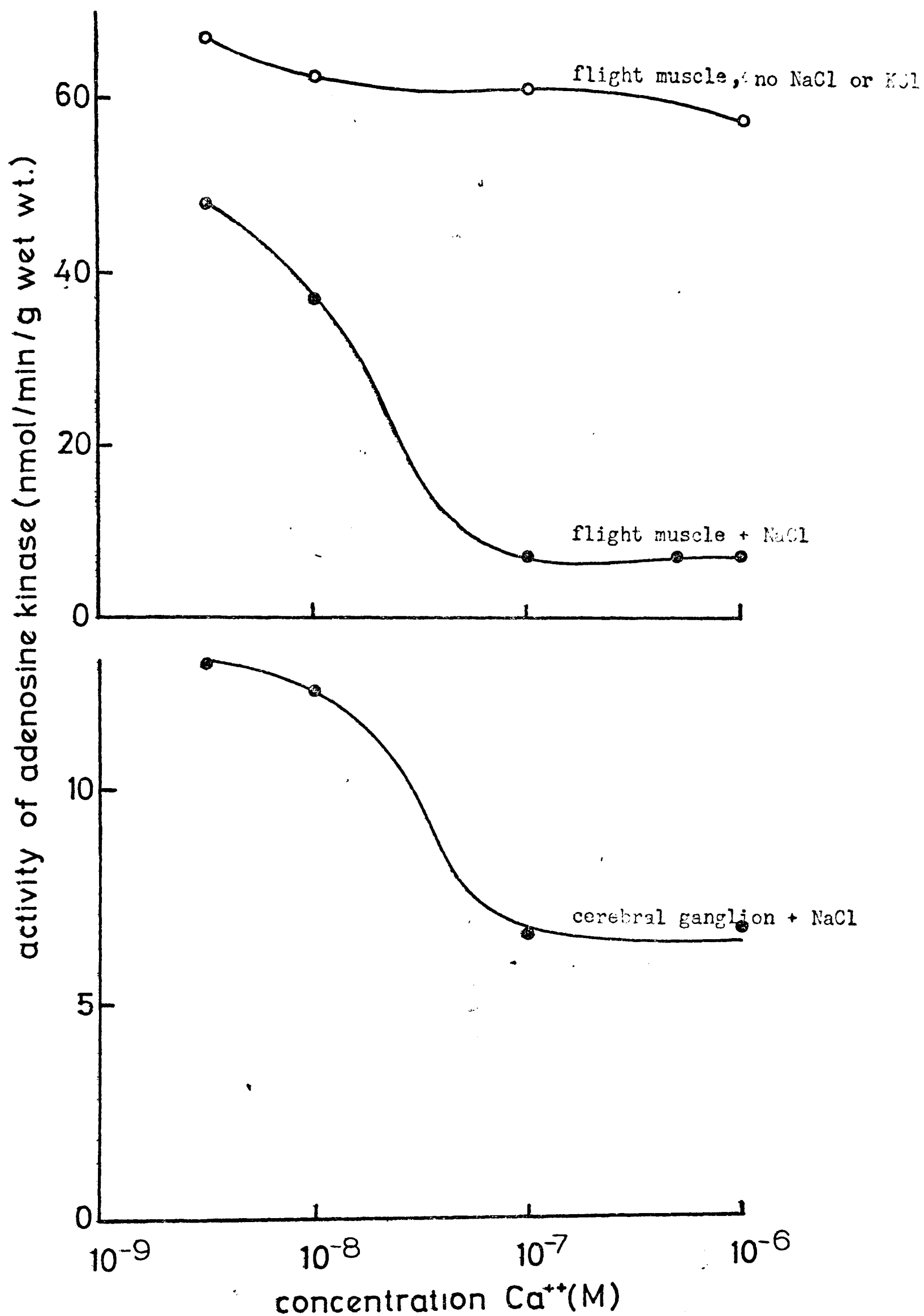


FIG 4-12 The effect of Ca^{++} on adenosine kinase from locust tissues.

Adenosine kinase was assayed as described in section B2 with the addition of NaCl (60 mM) as indicated.

TABLE 4.4 The effect of Ca²⁺ on the activity of adenosine kinase

Adenosine kinase was assayed as described in Section B.2. NaCl (60 mM) was included in the incubation medium and the concentration of Ca²⁺ was varied as indicated.

ANIMAL	TISSUE	ACTIVITY OF ADENOSINE KINASE (nmol/min/g wet wt.)		RATIO OF ACTIVITIES $\frac{10^{-8} \text{ M Ca}^{2+}}{10^{-7} \text{ M Ca}^{2+}}$
		$10^{-8} \text{ M Ca}^{2+}$	$10^{-7} \text{ M Ca}^{2+}$	
Mouse	Brain	20.4	6.50	3.12
Mouse	Heart	18.3	3.54	5.17
Mouse	Gastrocnemius muscle	3.40	0.28	12.00
Mouse	Liver	129	9.56	13.50
Locust	Flight muscle	36.9	7.15	5.17
Locust	Cerebral ganglion	12.3	6.55	1.88

shown in Table 4.5. Wide variations in maximal activity were observed. Thus, although the number of 5'-nucleotidase activity measurements are small, the range of mean activities is 200-fold (31-6070 nmol/min/g wet wt., chicken pectoral muscle and rat heart); adenosine kinase mean maximal activities at pH 7.5 vary over a 400-fold range (3.5-1410 nmol/min/g wet wt., pheasant pectoral muscle and honey-bee flight muscle); and adenosine deaminase activities vary over at least a 40-fold range (less than 40-1500 nmol/min/g wet wt., waterbug flight muscle and scallop slow adductor muscle). Amongst the vertebrates the range of adenosine kinase activities is considerably reduced: mouse heart, with a mean activity of 109 nmol/min/g wet wt., provides the highest activity.

Amongst the vertebrates the maximal activities of the adenosine deaminases are similar to those of 5'-nucleotidase from the same tissue, whereas the activities of adenosine kinase are generally far lower. In contrast, in insect flight muscles the activities of adenosine kinase are similar to or greater than those of adenosine deaminase.

(b) THE MAXIMAL ACTIVITIES AT pH 7.5 OF 5'-NUCLEOTIDASE, ADENOSINE KINASE AND ADENOSINE DEAMINASE FROM NERVOUS TISSUES

The maximal activities of 5'-nucleotidase, adenosine kinase and adenosine deaminase in nervous tissues of vertebrates and invertebrates are shown in Table 4.6. The mean maximal activities of each enzyme vary over at least a 10-fold range: 370-5060 nmol/min/g wet wt. for 5'-nucleotidase; 13-189 nmol/min/g wet wt. for adenosine kinase and less than 1.5-500 nmol/min/g wet wt. for adenosine deaminase. These ranges are very greatly reduced if only nervous tissues of vertebrates are compared. Maximal activities of adenosine deaminase are greater than maximal activities of adenosine kinase, except in the cases of the earthworm nerve cord and the waterbug pterothoracic ganglion, where the kinase exhibits far greater activity. It

TABLE 4.5 Maximal activities of 5'-nucleotidase, adenosine kinase and adenosine deaminase in muscles from vertebrates and invertebrates

The enzyme assays are described in Chapter 2, Section A (and in Section B.3 of the present chapter). 5'-Nucleotidase was assayed at pH 7.0; adenosine kinase and adenosine deaminase were assayed at pH 7.5 and, in some cases, at pH 5.3 as well. Activities are expressed as a mean, with the range of activities and the number of animals used given in parentheses. A dash (-) indicates that this activity has not been determined.

ANIMAL	MUSCLE	ENZYME ACTIVITIES (nmol/min/g wet wt. of muscle, 30°C)				
		5'-NUCLEOTIDASE	ADENOSINE KINASE		ADENOSINE DEAMINASE	
			AT pH 7.5	AT pH 5.3	AT pH 7.5	AT pH 5.3
Scallop (<i>Pecten maximus</i>)	Fast adductor	-	110 (75-148)(3)	31 (28-34)(2)	640 (513-849)(3)	257 (251-263)(2)
	Slow adductor	-	90 (68-110)(3)	21 (16-27)(2)	1500 (1340-1630)(3)	146 (110-182)(2)
Lobster (<i>Homarus vulgaris</i>)	Abdomen	-	14 (11-16)(3)	-	218 (202-229)(3)	-
	Chela	-	7.3 (6.3-8.5)(3)	-	290 (270-318)(3)	-
Locust (<i>Schistocerca gregaria</i>)	Flight	200 ^a (120-280)(2)	62 (49-77)(5)	60 (1)	105 (91-119)(5)	-
	Hind leg femoral	-	21 (17-24)(2)	-	51 (35-66)(2)	-
Cockroach (<i>Periplaneta americana</i>)	Flight	-	42 (34-49)(3)	-	35 (1)	-
Cockroach (<i>Blaberus discoidalis</i>)	Flight	3400 (2850-3960)(2)	155 (136-189)(3)	-	376 (2)	-
Waterbug (<i>Lethocerus cordofanus</i>)	Flight	1400 (1)	465 (382-548)(2)	-	n.d. ^b (2)	-
Rosechafer (<i>Pachnoda ephippiata</i>)	Flight	-	64 (55-80)(4)	-	113 (96-131)(3)	-
Wasp (<i>Vespa vulgaris</i>)	Flight	-	450 (1)	-	70 (1)	-
Honey-bee (<i>Apis mellifera</i>)	Flight	-	1410 (1310-1520)(2)	-	n.d. ^b (2)	-
Bumble-bee (<i>Bombus pratorum</i>)	Flight	180 (147-194)(2)	-	-	-	-
Bumble-bee (<i>Bombus agrorum</i>)	Flight	-	1110 (990-1240)(2)	-	55 (50-60)(2)	-
Solitary-bee (<i>Colletes cunicularius</i>)	Flight	-	964 (882-1060)(3)	-	n.d. ^b (4)	-
Blowfly (<i>Calliphora erythrocephala</i>)	Flight	203 (159-232)(3)	306 (274-369)(5)	-	164 (131-189)(3)	-

ANIMAL	MUSCLE	ENZYME ACTIVITIES (nmol/min/g wet wt. of muscle, 30°C)				
		5'-NUCLEOTIDASE	ADENOSINE KINASE		ADENOSINE DEAMINASE	
			AT pH 7.5	AT pH 5.3	AT pH 7.5	AT pH 5.3
Rainbow trout (<u>Salmo gairdneri</u>)	Heart	-	213 (1)	100 (1)	540 (405-674)(2)	62 (1)
	Red	-	101 (56-147)(2)	39 (1)	780 (625-932)(2)	165 (1)
	White	-	32 (25-38)(2)	14 (1)	146 (143-148)(2)	24 (1)
Frog (<u>Rana temporaria</u>)	Heart	-	26 (22-29)(2)	61 (59-62)(2)	150 (129-273)(2)	160 (137-182)(2)
	Gastrocnemius	-	15 (13-17)(2)	42 (1)	187 (175-199)(2)	182 (163-200)(2)
Domestic pigeon (<u>Columba livia</u>)	Heart	139 (128-150)(2)	15 (13-18)(2)	58 (1)	630 (614-643)(2)	390 (268-517)(2)
	Pectoral	98 (95-100)(2)	14 (11-18)(3)	47 (36-58)(2)	220 (218-223)(2)	135 (99-171)(2)
	Gastrocnemius	-	6.3 (1)	21 (1)	190 (181-200)(2)	80 (1)
	Sartorius	97 (93-100)(2)	9.5 (1)	29 (1)	192 (1)	-
Domestic fowl (<u>Gallus gallus</u>)	Heart	381 (1)	10 (8.3-11.7)(3)	16 (15-17)(3)	223 (187-246)(3)	177 (141-235)(3)
	Pectoral	31 (1)	6.0 (5.1-7.4)(3)	8.6 (8.5-8.7)(3)	43 (41-47)(3)	26 (19-35)(3)
	Sartorius	355 (1)	10 (6.7-12)(3)	13 (11-16)(3)	102 (91-108)(3)	79 (60-90)(3)
Pheasant (<u>Phasianus colchicas</u>)	Heart	-	16 (12-28)(3)	35 (24-36)(3)	191 (119-296)(3)	228 (138-338)(3)
	Pectoral	-	3.5 (3.2-4.0)(3)	11 (8.7-14)(2)	97 (89-103)(3)	112 (101-122)(2)
	Sartorius	-	6.9 (5.9-7.9)(3)	11 (8.5-17)(3)	107 (95-114)(3)	107 (85-122)(3)
Laboratory mouse	Heart	385 (355-415)(2)	109 (76-128)(3)	277 (267-292)(3)	138 (108-188)(5)	93 (91-95)(2)
	Gastrocnemius	49 (40-58)(2)	25 (21-35)(3)	66 (46-79)(4)	43 (39-46)(5)	27 (21-31)(5)
Laboratory rat	Heart	6070 (1)	25 (1)	-	1385 (1)	

a. The activity of 5'-nucleotidase from locust flight muscle is very low at substrate concentrations below 1 mM, which were used in the present assay. The value recorded would be obtained using a substrate concentration of 10 mM.

b. n.d. indicates not detectable. In these cases the activity was less than 100 nmol/min/g wet wt., using a substrate concentration of 100 μ M (see Chapter 2, Section E.2).

TABLE 4.6 Maximal activities of 5'-nucleotidase, adenosine kinase and adenosine deaminase in nervous tissue from vertebrates and invertebrates

See legend to Table 4.5 for methods. Activities are expressed as a mean, with the range of activities and the number of animals used given in parentheses.

ANIMAL	NERVOUS TISSUE	ENZYME ACTIVITIES (nmol/min/g wet wt. of nervous tissue, 30°C)				
		5'-NUCLEOTIDASE	ADENOSINE KINASE		ADENOSINE DEAMINASE	
			AT pH 7.5	AT pH 5.3	AT pH 7.5	AT pH 5.3
Earthworm (<u>Lumbricoides terrestris</u>)	Nerve cord	-	139 (1)	46 (1)	50 (1)	137 (1)
Earthworm (<u>Allolobophora longa</u>)	Nerve cord	352 (1)	148 (126-166)(4)	-	n.d.* (4)	-
Lobster (<u>Homarus vulgaris</u>)	Cerebral ganglion	-	24 (19-29)(3)	-	296 (291-300)(3)	-
Locust (<u>Schistocerca gregaria</u>)	Cerebral ganglion	370 (1)	44 (41-46)(3)	45 (1)	117 (94-140)(2)	-
Cockroach (<u>Blaberus discoidalis</u>)	Cerebral ganglion	5060 (3460-6650)(2)	69 (67-70)(2)	-	308 (306-310)(2)	-
Waterbug (<u>Lethocerus cordofanus</u>)	Pterothoracic ganglion	3000 (1)	189 (175-203)(2)	-	n.d.* (2)	-
Rainbow trout (<u>Salmo gairdneri</u>)	Whole brain	-	62 (48-75)(2)	29 (1)	500 (426-565)(2)	65 (1)
Frog (<u>Rana temporaria</u>)	Whole brain	-	14 (13-14)(2)	40 (36-48)(2)	221 (184-258)(2)	228 (286-290)(2)
Domestic pigeon (<u>Columba livia</u>)	Whole brain	910 (710-1110)(2)	13 (12-13)(2)	40 (37-43)(2)	140 (110-175)(2)	78 (50-106)(2)
Domestic fowl (<u>Gallus gallus</u>)	Whole brain	937 (1)	15 (11-19)(2)	50 (38-62)(2)	60 (59-60)(2)	53 (51-54)(2)
Laboratory mouse	Whole brain	480 (450-610)(3)	65 (48-85)(6)	156 (119-176)(6)	51 (44-60)(10)	88 (71-99)(10)

* n.d. indicates not detectable. In these cases the activity was less than 5 nmol/min/g wet wt., using a substrate concentration of 100 μ M (see Chapter 2, Section E.2).

TABLE 4.7 Maximal activities of 5'-nucleotidase, adenosine kinase and adenosine deaminase in livers from vertebrates and in two parasites

See legend to Table 4.5 for methods. For the assay of adenosine kinase, livers were extracted in the presence of 20% glycerol (see Chapter 2, Section D.6), unless otherwise indicated. Activities are expressed as a mean, with the range of activities and the number of animals used given in parentheses.

ANIMAL	TISSUE	ENZYME ACTIVITIES (nmol/min/g wet wt. of tissue, 30°C)				
		5'-NUCLEOTIDASE	ADENOSINE KINASE		ADENOSINE DEAMINASE	
			AT pH 7.5	AT pH 5.3	AT pH 7.5	AT pH 5.3
Rat tapeworm (<i>Hymenolepis diminuta</i>)	Whole animal	-	0.4 (0.2-0.6) (2)	-	310 (1)	-
Pig roundworm (<i>Ascaris lumbricoides</i>)	Body wall	-	n.d. ^a	-	-	-
Rainbow trout (<i>Salmo gairdneri</i>)	Liver	-	10,500 (10,200-10,800) (2)	2440 (1)	870 (710-1030) (2)	-
Frog (<i>Rana temporaria</i>)	Liver	-	250 (223-280) (2)	490 (405-585) (2)	1830 (1600-2080) (3)	1430 (1100-1740) (3)
Domestic pigeon (<i>Columba livia</i>)	Liver	2350 (1710-3000) (2)	330 (298-363) (2)	950 (730-1170) (2)	490 (344-635) (2)	238 (1)
Domestic fowl (<i>Gallus gallus</i>)	Liver	806 (1)	781 (772-790) (2)	1500 (1300-1690) (2)	323 (283-363) (2)	219 (150-287) (2)
Laboratory mouse	Liver	1290 (990-1580) (2)	2100 (1470-2670) (2)	3630 (3500-3880) (3)	1740 (1210-2400) (5)	1600 (1100-2000) (3)
	Liver ^b	-	1130 (880-1480) (4)	2670 (2320-3300) (4)	-	-

a. n.d. indicates non-detectable. The activity was less than 0.2 nmol/min/g wet wt.

b. Data for adenosine kinase extraction in the absence of 20% glycerol.

TABLE 4.8 Maximal activities of 5'-nucleotidase, adenosine kinase and adenosine deaminase in tissues from the mouse

See legend to Table 4.5 for methods. Activities are expressed as a mean, with the range of activities and the number of animals used given in parentheses.

MOUSE TISSUE	ENZYME ACTIVITIES (nmol/min/g wet wt. of tissue)			
	ADENOSINE KINASE		ADENOSINE DEAMINASE	
	AT pH 7.5	AT pH 5.3	AT pH 7.5	AT pH 5.3
Whole blood*	44 (34-50)(2)	63 (52-75)(2)	133 (82-184)(2)	152 (99-205)(2)
Testis	53 (48-59)(2)	-	693 (575-811)(2)	-
Kidney medulla	70 (50-91)(2)	-	1990 (1875-2100)(2)	-
Kidney cortex	136 (120-151)(2)	-	874 (867-881)(2)	-
Lung	235 (209-261)(2)	379 (1)	439 (1)	329 (1)
Brain	65 (48-85)(6)	156 (119-176)(6)	51 (44-60)(10)	88 (71-99)(10)
Liver	2100 (1470-2670)(2)	3630 (3500-3850)(3)	1740 (1210-2400)(5)	1600 (1100-2000)(3)
Heart	109 (76-128)(3)	277 (267-292)(3)	138 (108-188)(5)	93 (91-95)(2)
Gastrocnemius muscle	25 (21-35)(3)	66 (46-79)(4)	43 (39-46)(5)	27 (21-31)(5)

* Data expressed per ml of blood.

TABLE 4.9 K_m values of 5'-nucleotidase, adenosine kinase and adenosine deaminase in tissues from vertebrates and invertebrates

K_m values were measured as described in Section B.3 and Chapter 2, Section A. The K_m value of 5'-nucleotidase for AMP was measured at pH 7.0; the K_m values of adenosine kinase and adenosine deaminase for adenosine were measured at pH 7.5 and, in some cases, at pH 5.3 as well. K_m values are expressed as a mean with individual results in parentheses for those cases where more than one determination was performed.

ANIMAL	TISSUE	5'-NUCLEOTIDASE	K_m VALUES (μ M)		ADENOSINE DEAMINASE	
			pH 7.5	pH 5.3	pH 7.5	pH 5.3
Rat tapeworm (<i>Hymenolepis diminuta</i>)	Whole animal	-	2.9	-	33	-
Earthworm (<i>Allolobophora longa</i>)	Nerve cord	280	11(9.5,13)	43	-	27
Scallop (<i>Pecten maximus</i>)	Fast adductor muscle	-	113(106,120)	58(50,67)	29(20,30,36)	25
	Slow adductor muscle	-	27(20,34)	-	25(20,29)	21
Lobster (<i>Homarus vulgaris</i>)	Cerebral ganglion	-	3.6(3.2,3.6,3.9)	-	41	-
	Abdominal muscle	-	2.8(2.5,3.0,3.0)	-	48(47,48)	-
	Claw muscle	-	5.6(4.2,6.2,6.3)	-	50	-
Locust (<i>Schistocerca gregaria</i>)	Cerebral ganglion	3700*	8.4	-	30	-
	Flight muscle	4000*	2.4	-	45	-
	Leg muscle	-	3.0	-	35	-
Cockroach (<i>Periplaneta americana</i>)	Flight muscle	-	3.5	-	-	-
Cockroach (<i>Blaberus discoidalis</i>)	Cerebral ganglion	257(212,302)	9.0	-	49	-
	Flight muscle	212(211,212)	2.2	-	115	-
Waterbug (<i>Lethocerus cordofanus</i>)	Pterothoracic ganglion	211	34	-	-	-
	Flight muscle	117	25(20,29)	-	-	-
Rosechafer (<i>Pachnoda ephippiata</i>)	Flight muscle	-	4.0(3.0,5.0)	-	21	-
Wasp (<i>Vespa vulgaris</i>)	Flight muscle	-	8.0	-	-	-
Honey-bee (<i>Apis mellifera</i>)	Flight muscle	-	5.8(5.5,6.0)	-	-	-
Bumble-bee (<i>Bombus pratorum</i>)	Flight muscle	180	-	-	-	-
Bumble-bee (<i>B. agrorum</i>)	Flight muscle	-	8.7(7.7,9.6)	-	10	-
Solitary-bee (<i>Colletes cunicularius</i>)	Flight muscle	-	10	-	-	-
Blowfly (<i>Calliphora erythrocephala</i>)	Flight muscle	614(551,677)	6.8	-	17	-

ANIMAL	TISSUE	5'-NUCLEOTIDASE	K _m VALUES (μM)		ADENOSINE DEAMINASE	
			ADENOSINE KINASE		pH 7.5	pH 5.3
			pH 7.5	pH 5.3		
Rainbow trout (<u>Salmo gairdneri</u>)	Brain	-	7.6(7.6,7.6)	17	49(42,56)	17
	Liver	-	6.7(5.8,7.6)	13	10(11,9)	-
	Heart	-	6.6(6.3,6.8)	27	55(51,59)	10
	Red muscle	-	6.6	15	40(25,55)	27
	White muscle	-	5.1(4.9,5.3)	10	30(30,29)	11
Frog (<u>Rana temporaria</u>)	Brain	-	1.8	3.6	23	39
	Liver	-	1.7	2.8	23(20,25)	26
	Heart	-	2.9	5.9	20	25
	Gastrocnemius muscle	-	1.7	3.7	13	25
Domestic pigeon (<u>Columba livia</u>)	Brain	197	1.9(1.6,2.2)	3.5	18(14,23)	26
	Liver	214	1.2	4.5	24	32
	Heart	98	0.9	4.3	21(20,22)	31(29,32)
	Pectoral muscle	167	1.0	1.4(1.0,1.7)	14	25(18,31)
	Gastrocnemius muscle	-	2.1	8.4	17	37
	Sartorius muscle	93	2.0	4.2	19	-
Domestic fowl (<u>Gallus gallus</u>)	Brain	80	1.9	6.9	9.9	12
	Liver	97	1.0	2.5	10	7.7
	Heart	100	2.2	3.2	8.4	9.7
	Pectoral muscle	-	1.8	4.2	9.8	6.9
	Sartorius muscle	73	2.3	2.8	9.8	9.6
Pheasant (<u>Phasianus colchicas</u>)	Heart	-	2.7	6.7	13	21
	Pectoral muscle	-	2.0	5.8	8.1	11
	Sartorius muscle	-	1.8	2.0	10	11
Laboratory mouse	Brain	136	4.0(2.9,3.2,4.7,4.9)	8.0	12(10,14)	31
	Liver	122(92,152)	1.0(0.9,1.0)	3.5	20(17,21,21)	32
	Heart	143(140,145)	2.8	3.3	27	54
	Gastrocnemius muscle	82(77,87)	2.0(1.9,2.0)	3.1(2.0,4.2)	26(24,28)	16
	Whole blood	-	4.4	6.9	33	33
	Testis	-	3.7	-	24	-
	Kidney medulla	-	15(12,19)	-	31	-
	Kidney cortex	-	7.2	-	24	-
	Lung	-	4.3	8.1	26	20
Laboratory rat	Heart	158	6.2	-	39	-

* These values are approximate, since they were obtained by assaying 5'-nucleotidase using concentrations of AMP below 1 mM.

is interesting to note that these two tissues probably have high phosphorylase activities (Sugden, 1973).

(c) THE MAXIMAL ACTIVITIES AT pH 7.5 OF 5'-NUCLEOTIDASE, ADENOSINE KINASE AND ADENOSINE DEAMINASE IN VERTEBRATE LIVERS AND IN PARASITES

The maximal activities of 5'-nucleotidase, adenosine kinase and adenosine deaminase in some vertebrate livers and two parasites are shown in Table 4.7. Trout liver has a very high mean maximal activity of adenosine kinase at pH 7.5 (10600 nmol/min/g wet wt.) but, apart from this, the activities of the enzyme from each liver exhibit no more than a 6-fold range among the five species studied. The activities of all three enzymes vary over a similar range in vertebrate livers.

The two parasites examined possessed very low levels of adenosine kinase. Similar results have been found for other parasites (see Senft *et al.*, 1972, 1973).

(d) THE MAXIMAL ACTIVITIES OF ADENOSINE KINASE AND ADENOSINE DEAMINASE IN VARIOUS MOUSE TISSUES

The maximal activities of adenosine kinase and adenosine deaminase in various tissues from the mouse are shown in Table 4.8. The activity of adenosine kinase in the mouse lung (235 nmol/min/g wet wt.) is higher than in any other mouse tissue investigated except the liver. The activity of adenosine deaminase is considerably higher in the testis, lung, kidney medulla and kidney cortex than in muscle and nervous tissue.

(e) THE K_m VALUES OF 5'-NUCLEOTIDASE, ADENOSINE KINASE AND ADENOSINE DEAMINASE AT pH 7.5

The K_m values of 5'-nucleotidase, adenosine kinase and adenosine deaminase for all the tissues investigated are shown in Table 4.9. The values for the enzyme from vertebrates are similar to those previously reported, except that the reported value for guinea-pig brain is unusually

high (Table 1.5). The K_m value of each enzyme shows little variation between species. With some exceptions, the K_m values of each enzyme are similar within a species or in similar tissues among a group of related species. For example, the K_m values of adenosine kinases from the pigeon range from 2.9 to 3.8 μM , whereas in the case of the trout they range from 5.1 to 7.6 μM ; adenosine kinases from the cerebral ganglia of the locust and cockroach have K_m values which are different from those of the flight muscle enzymes, but they are very similar to each other.

The K_m values of 5'-nucleotidase range from 73 to 4000 μM (domestic fowl sartorius muscle and locust flight muscle). However, the latter value is so high that it suggests that the activity of a phosphatase, which is not specific for AMP, was measured (see Chapter 1, Section B2a). If the locust and blowfly tissues are excluded, the highest value for the K_m is only 280 μM (earthworm nerve cord).

The K_m values of adenosine kinase for adenosine range from 0.9 to 113 μM (pigeon pectoral muscle and scallop fast adductor muscle). If scallop and waterbug tissues are excluded, the highest value is only 15 μM (mouse kidney medulla).

The K_m values of adenosine deaminase for adenosine range from 8.4 to 115 μM (domestic fowl heart and cockroach flight muscle). These values are consistently higher than those for adenosine kinase with the exception of the scallop.

(f) DEVIATIONS FROM MICHAELIS-MENTEN KINETICS BY ADENOSINE DEAMINASE

It was frequently noticed that adenosine kinase was inhibited by concentrations of adenosine above the K_m of the enzyme. No specific study of the substrate inhibition of adenosine kinase was undertaken but nevertheless it is possible to reach some general qualitative conclusions (Table 4.10).

TABLE 4.10 A qualitative survey of substrate inhibition of adenosine kinase by adenosine in animal tissues

Adenosine kinase was assayed at pH 7.5 as described in Section B.3 and Chapter 2, Section A. The degree of substrate inhibition is graded by comparing the observed activity using 100 μ M adenosine with the activity estimated for this concentration of substrate by extrapolating the linear portion of the kinetic plot. 0-10% inhibition is graded -; 11-30% inhibited +; 31-50% inhibition ++; and greater than 50% inhibition is graded +++. The number of measurements for each tissue is apparent from Table 4.9.

ANIMAL	TISSUE	DEGREE OF INHIBITION BY SUBSTRATE
Earthworm (<u>Lumbricoides terrestris</u>)	Nerve cord	-
Scallop (<u>Pecten maximus</u>)	Fast adductor muscle	- *
	Slow adductor muscle	- *
Lobster (<u>Homarus vulgaris</u>)	Cerebral ganglion	- *
	Abdominal muscle	- *
	Claw muscle	- *
Locust (<u>Schistocerca gregaria</u>)	Cerebral ganglion	-
	Flight muscle	-
	Leg muscle	-
Cockroach (<u>Periplaneta americana</u>)	Flight muscle	+++
Cockroach (<u>Blaberus discoidalis</u>)	Cerebral ganglion	+
	Flight muscle	++
Waterbug (<u>Lethocerus cordofanus</u>)	Pterothoracic ganglion	-
	Flight muscle	-
Rosechafer (<u>Pachnoda ephippiata</u>)	Flight muscle	+++
Wasp (<u>Vespa vulgaris</u>)	Flight muscle	-
Honey-bee (<u>Apis mellifera</u>)	Flight muscle	-
Bumble-bee (<u>Bombus agrorum</u>)	Flight muscle	-
Solitary bee (<u>Colletes cunicularius</u>)	Flight muscle	-

ANIMAL	TISSUE	DEGREE OF INHIBITION BY SUBSTRATE
Blowfly (<u>Calliphora erythrocephala</u>)	Flight muscle	+
Rainbow trout (<u>Salmo gairdneri</u>)	Brain	+
	Liver	+
	Heart	+
	Red abdominal muscle	++
	White abdominal muscle	+
Frog (<u>Rana temporaria</u>)	Brain	-
	Liver	-
	Heart	-
	Gastrocnemius muscle	-
Domestic pigeon (<u>Columba livia</u>)	Brain	++
	Liver	++
	Heart	+
	Pectoral muscle	++
	Sartorius muscle	++
Domestic fowl (<u>Gallus gallus</u>)	Brain	+
	Liver	+
	Heart	+
	Sartorius muscle	++
Pheasant (<u>Phasianus colchicas</u>)	Heart	-
	Pectoral muscle	-
	Sartorius muscle	-
Laboratory mouse	Brain	+++
	Liver	+++
	Heart	+++
	Gastrocnemius muscle	+++
	Whole blood	+++
	Lung	+++
	Kidney medulla	-

* The kinetics of adenosine kinase from scallop and lobster tissues was suggestive of negative cooperativity or the presence of two enzymes with different K_m values, i.e. the Hofstee plots were concave upwards.

Firstly, even when substrate inhibition of adenosine kinase was marked at pH 7.5, it was scarcely detectable at pH 5.3. Secondly, if substrate inhibition was found in one tissue of a species it was usually found in all the other tissues from this species. (For example, adenosine kinase from mouse and pigeon tissues shows marked substrate inhibition, whereas the enzyme from frog tissues is not inhibited.) Adenosine kinase from mouse kidney medulla is an exception to this generalization since, unlike adenosine kinase from other mouse tissues, it exhibits no substrate inhibition. Thirdly, optimal adenosine concentrations for adenosine kinase activity at pH 7.5 may be classified as 5 to 10 μM for mouse, pigeon and chicken tissues, 10 to 20 μM for insect tissues and 50 to 100 μM for trout tissues.

Adenosine deaminase usually conformed to Michaelis-Menten kinetics.

(g) THE EFFECT OF pH ON ADENOSINE KINASE AND ADENOSINE DEAMINASE

The maximal activities of adenosine kinase and adenosine deaminase at pH 5.3 have been presented in Tables 4.5 to 4.8. The K_m values of adenosine kinase and adenosine deaminase at pH 5.3 have been presented in Table 4.9. In Table 4.11 ratios of activities of adenosine kinase and adenosine deaminase at pH's 5.3 and 7.5 are shown.

With some exceptions (for example, the domestic fowl brain) the effect of pH on the activities of adenosine kinase and adenosine deaminase is similar for all tissues from one species. Generally, the enzymes had a lower activity at pH 5.3 than at pH 7.5, but mouse, pigeon, pheasant, frog and domestic fowl adenosine kinases were more active at pH 5.3 than at pH 7.5. Since the K_m values of adenosine kinases from these species were also greater at pH 5.3 than at pH 7.5, there was no stimulation of activity at the lower pH when the concentration of adenosine was low.

TABLE 4.11 The effects of pH on the activities of adenosine kinase and adenosine deaminase from different animal tissues

The mean activities presented in Table 4.5 to 4.8 and the K_m values presented in Table 4.10 have been used to estimate the ratio of activities at pH 5.3/pH 7.5 for adenosine kinase and adenosine deaminase. This ratio is estimated as V_{max} , pH 5.3/ V_{max} , pH 7.5 for high concentrations of adenosine and as V_{max} , pH 5.3/ V_{max} , pH 7.5 $\times$ K_m , pH 7.5/ K_m , pH 5.3 for low concentration of adenosine (see Section D1b(11)).

ANIMAL	TISSUE	RATIO OF MEAN ACTIVITIES, pH 5.3/pH 7.5			
		ADENOSINE KINASE		ADENOSINE DEAMINASE	
		<u>High adenosine concentration</u>	<u>Low adenosine concentration</u>	<u>High adenosine concentration</u>	<u>Low adenosine concentration</u>
Earthworm (<u>Lumbricoides terrestris</u>)	Nerve cord	0.33	0.08	-	-
Scallop (<u>Pecten maximus</u>)	Fast adductor	0.28	0.55	0.40	0.47
	Slow adductor	0.23	-	0.10	0.13
Lobster* (<u>Homarus vulgaris</u>)	Cerebral ganglion	0.16	-	-	0.7
	Abdominal muscle	0.13	-	-	0.6
	Claw muscle	0.31	-	-	0.5
Locust* (<u>Schistocerca gregaria</u>)	Cerebral ganglion	1.5	-	-	0.1
	Flight muscle	1.2	-	-	0.1
	Leg muscle	-	-	-	0.1
Rainbow trout (<u>Salmo gairdneri</u>)	Brain	0.47	0.21	0.13	0.04
	Liver	0.23	0.12	-	-
	Heart	0.47	0.23	0.11	0.06
	Red muscle	0.39	0.17	0.21	0.03
	White muscle	0.44	0.22	0.16	0.04

ANIMAL	TISSUE	RATIO OF MEAN ACTIVITIES, pH 5.3/pH 7.5			
		ADENOSINE KINASE		ADENOSINE DEAMINASE	
		<u>High adenosine concentration</u>	<u>Low adenosine concentration</u>	<u>High adenosine concentration</u>	<u>Low adenosine concentration</u>
Frog (<u>Rana temporaria</u>)	Brain	3.0	1.5	1.3	0.8
	Liver	2.0	1.2	0.8	0.6
	Heart	2.3	1.1	1.1	0.9
	Gastrocnemius muscle	2.8	1.3	1.0	0.5
Domestic pigeon (<u>Columba livia</u>)	Brain	3.4	1.9	0.6	0.4
	Liver	2.9	1.2	0.5	0.4
	Heart	3.8	0.8	0.6	0.4
	Pectoral muscle	3.4	2.4	0.6	0.3
	Gastrocnemius muscle	3.3	0.8	0.4	0.2
	Sartorius muscle	3.0	1.4		
Domestic fowl (<u>Gallus gallus</u>)	Brain	3.3	0.9	0.9	0.7
	Liver	1.9	0.8	0.7	0.9
	Heart	1.5	1.0	0.8	0.7
	Pectoral muscle	1.4	0.6	0.6	0.9
	Sartorius muscle	1.3	1.1	0.8	0.8
Pheasant (<u>Phasianus colchicas</u>)	Heart	2.2	0.9	1.2	0.7
	Pectoral muscle	3.2	1.1	1.2	0.9
	Sartorius muscle	1.7	1.5	1.0	0.9
Mouse	Brain	2.4	1.2	1.7	0.7
	Liver	2.4	0.7	0.9	0.6
	Heart	2.5	2.1	0.7	0.4
	Gastrocnemius muscle	2.6	1.7	0.7	1.1
	Blood	1.4	0.9	1.1	1.1
	Lung	1.6	0.9	0.8	1.0

* These ratios are $\frac{\text{activity, pH 5.3}}{\text{activity, pH 7.5}}$, using 10 μM adenosine substrate.

C.6 ACTIVITIES OF 5'-NUCLEOTIDASE, ADENOSINE KINASE AND ADENOSINE DEAMINASE IN THE BRAINS OF NEWBORN AND ADULT MICE

The activities of 5'-nucleotidase, adenosine kinase and adenosine deaminase were measured in the brain of the new-born mouse (Table 4.12). The activities as percentages of those of the adult brain are as follows: 5'-nucleotidase 18%; adenosine kinase 63%; adenosine deaminase 264%. In each case the difference in enzyme activity between the new-born and adult brain was statistically highly significant ($p < 0.001$).

D. DISCUSSION

D.1 THE CONTROL OF TISSUE ADENOSINE CONCENTRATIONS

The control of tissue adenosine concentrations will be discussed by first considering the control of the three enzymes which produce and utilize adenosine. It will then be possible to discuss how these three enzymes together control the concentration of adenosine. Most of the discussion refers primarily to vertebrates, for which other data are available.

(a) THE CONTROL OF THE ACTIVITY OF 5'-NUCLEOTIDASE

The maximal activities of 5'-nucleotidase, adenosine kinase and adenosine deaminase indicate that 5'-nucleotidase activity rarely approaches its maximal value in vivo. Thus maximal activities of 5'-nucleotidases are far higher than net rates of adenosine output from tissues (Tables 4.5-4.7, 1.8). Unfortunately, only one gross rate of adenosine output can be calculated from reported data (see Chapter 1, Section B3b(iii)). It is 64 nmol/min/g wet wt. for electrically-stimulated guinea-pig cerebral cortex slices (deduced from Pull & McIlwain, 1973). Even this value is less than a tenth of the maximal catalytic activity of 5'-nucleotidase in any of the vertebrate brains investigated in the present work. Therefore, it is possible either that

TABLE 4.12 The activities of 5'-nucleotidase, adenosine kinase and adenosine deaminase in the brains of new-born and adult mice

Brains were dissected from five new-born mice less than twelve hours after birth and from four adult mice of the same strain. The brains were divided medianally. 5'-Nucleotidase was assayed in one half of each brain, using a substrate concentration of 200 μ M AMP; adenosine kinase and adenosine deaminase were assayed in the other half using 5 μ M adenosine.

	ENZYME ACTIVITY (nmol/min/g fresh wt.)		
	<u>5'-Nucleotidase</u>	<u>Adenosine kinase</u>	<u>Adenosine deaminase</u>
Adult mice	390 $\pm$ 83 (4)	12.0 $\pm$ 1.1 (4)	10.4 $\pm$ 1.4 (4)
New-born mice	70 $\pm$ 11 (5)*	7.6 $\pm$ 0.9 (5)*	27.4 $\pm$ 2.0 (5)*

* p < 0.001 compared with the activity for the adult.

5'-nucleotidase is maximally active but most of the adenosine which it produces is not released from the cell, or it operates at far less than its maximal activity. The latter situation is more probable. Thus in general the combined maximal activities of adenosine kinase and adenosine deaminase are less than the maximal activity of 5'-nucleotidase in muscle and nervous tissues (Tables 4.5, 4.6). Therefore, if 5'-nucleotidase operated at its maximal activity and the adenosine produced was not released from the cell, large quantities of adenosine would accumulate. In contrast, taking the particular example of mouse brain, if 5'-nucleotidase operates below its maximal activity at 64 nmol/min/g wet wt. (the value for electrically-stimulated guinea-pig brain suggested above) and adenosine kinase and adenosine deaminase operate with the activities measured in the present study (Tables 4.6 and 4.9), it may be calculated that adenosine will reach a steady-state concentration of 3 μ M. (This value was obtained by substituting the kinetic parameters measured in the present study into the equation

$$v \text{ for } 5'\text{-nucleotidase} = \frac{V_{\max} \text{ adenosine kinase} \times s}{K_m \text{ adenosine kinase} + s} + \frac{V_{\max} \text{ adenosine deaminase} \times s}{K_m \text{ adenosine deaminase} + s} .)$$

The estimate compares favourably with concentrations of adenosine which have been measured in tissues (Tables 1.1 and 1.2).

Since it appears likely that 5'-nucleotidase operates below its maximal activity, it is necessary to consider whether its activity is restricted by substrate concentration and/or by inhibitors. Many of the reported K_m values of 5'-nucleotidase (Table 1.4) are lower than normal intracellular AMP concentrations, which suggests that the enzyme is not greatly restricted by its substrate concentration. However, these values were measured using purified enzymes. In contrast, the K_m values for

5'-nucleotidase measured in the present study are higher than AMP levels in the same tissue (Table 4.13). Mammalian livers may be an exception to this generalization. However, the methods used to anaesthetize mammals prior to freeze-clamping livers may cause increases in AMP levels (Faupel et al., 1972). It may be concluded that the in vivo activity of 5'-nucleotidase is restricted by substrate concentration.

However, changes in AMP concentration alone cannot explain reported variations in adenosine output. Thus the data of Table 4.12 indicate that, if mouse brain 5'-nucleotidase activity is restricted by AMP concentration only, it operates at 25% of its maximal capacity under normal conditions. Therefore a very large increase in AMP concentration could only increase 5'-nucleotidase activity 4-fold. Moreover, AMP concentrations do not vary greatly even under very different physiological conditions. For example, ischaemia of mouse brain caused only a 1.5 fold increase in AMP concentration (Lowry et al., 1964), anaerobiosis of perfused rat heart for 15 minutes resulted in only a 3-fold increase in AMP concentration (Regen et al., 1964) and initiation of flight in the blowfly causes only a 2.5-fold increase in AMP concentration (Sacktor & Hurlbut, 1966). These small variations in AMP concentration contrast with measured variations in adenosine output of up to 100-fold (Table 1.8). It may be concluded that the net output of adenosine is controlled by factors in addition to the concentration of AMP. One factor may be the concentrations of free ATP and ADP, which may control the activity of 5'-nucleotidase (see Chapter 1, Sections B2c(ii) and B3d). Another factor controlling the net output of adenosine may be the rate of uptake of adenosine. This is discussed in Section (c) below.

(b) THE CONTROL OF THE ACTIVITIES OF ADENOSINE KINASE AND ADENOSINE DEAMINASE

The present data do not support the view that adenosine kinase has access to exogenous adenosine prior to adenosine deaminase (see Chapter 1,

TABLE 4.13 The K_m values of 5'-nucleotidase and levels of 5'-AMP
in different animal tissues

The K_m values for 5'-nucleotidase are taken from Table 4.9. Tissue levels of 5'-AMP are taken from Beis (1973), unless otherwise indicated. The levels tabulated are those found in vivo in resting animals, unless otherwise indicated.

ANIMAL	TISSUE	K_m (μ M) OF 5'-NUCLEOTIDASE	LEVEL OF AMP (μ M)
Locust (<u>Schistocerca gregaria</u>)	Flight muscle	4000	100
Cockroach (<u>Blaberus discoidalis</u>)	Flight muscle	212	40
Waterbug (<u>Lethocerus cordofanus</u>)	Flight muscle	117	40
Blowfly (<u>Calliphora erythrocephala</u>).	Flight muscle	614	60 ^a
Pigeon (<u>Columba livia</u>)	Pectoral muscle	167	50
Laboratory mouse	Brain	136	41 ^b , 70 ^c
	Skeletal muscle	82	30
	Liver	122	15 ^d , 130 ^e
Laboratory rat	Heart	158	60 ^f , 90 ^g

a. Data for Calliphora vicina.

b. Data for rat brain from Veech et al. (1973).

c. Data for slices of guinea-pig brain in oxygenated media from Rolleston & Newsholme (1967).

d. Data for rat liver deduced from levels measured after varying periods of anoxia, from Faupel et al. (1972).

e. Data for rat liver from Start & Newsholme (1968).

f. Data for the isolated perfused heart from Opie et al. (1971).

g. Data for the isolated perfused heart from Regen et al. (1964).

Section B4c). It will be suggested that the utilisation of adenosine is controlled by metal ions which modify the activity of adenosine kinase. Before discussing the control of adenosine utilization, some data will be presented, which indicate that the activities of adenosine kinase and adenosine deaminase measured in homogenates reflect their activities in vivo.

(i) Correlation of enzyme activities with data for intact tissues and the whole animal

Unfortunately, it is difficult to correlate the activities of adenosine kinase measured in the present investigation with reported data because the latter are available for only a limited number of mammals. However, it appears that the activities of adenosine kinase can account for rates of adenosine phosphorylation by intact tissues (see Table 1.9 and references for data in the literature).

The analogue of adenosine, 6-(methylmercapto)purine ribonucleoside (Me6MPR), is taken up by animal tissues and phosphorylated to its 5'-phosphate. Evidence has been presented to show that adenosine kinase catalyzes this reaction (Caldwell et al., 1966; see also Chapter 2, Section D.1). In Table 4.14 data from the present study for activities of adenosine kinase from mouse tissues using adenosine as substrate are compared with the data of Ho et al. (1968) using Me6MPR as substrate. The data indicate that the ratio, activity of adenosine kinase for adenosine/activity of adenosine kinase for Me6MPR, is the same for each tissue. The activities of adenosine kinase are also compared with the tissue distribution of Me6MPR following its injection into the living animal. There is some degree of correlation between adenosine kinase activity and the rate at which Me6MPR is phosphorylated by the mouse tissues. These results suggest that the maximal activities of adenosine kinase measured in vitro in the present investigation are a useful indication of the activities of the enzyme in vivo.

TABLE 4.14 The rate of uptake of Me6MPR and the activity of adenosine kinase in mouse tissues

The rate of accumulation of Me6MPR-5'-P by the tissues of the mouse in the 30 minutes following the intraperitoneal injection of radio-active Me6MPR into the animal is expressed per gram of tissue relative to skeletal muscle (1.0). The activity of adenosine kinase with Me6MPR as substrate is expressed per gram of tissue relative to liver (14.7); the activity with adenosine as substrate is expressed per gram of tissue relative to gastrocnemius muscle (1.0).

MOUSE TISSUE	DISTRIBUTION OF Me6MPR-5'-PHOSPHATE ^a	ACTIVITY OF ADENOSINE KINASE	
		SUBSTRATE:Me6MPR ^b	SUBSTRATE:ADENOSINE ^c
Liver	14.7	(14.7)	45.2
Heart	3.8	-	4.4
Brain	1.2	1.2	2.5
Skeletal muscle	(1.0)	-	(1.0)
Blood cells	14.0	0.2 ^d	1.8 ^e
Lung	10.0	-	8.0
Kidney	3.2	5.0	(medulla) 2.8 (cortex) 5.4

a. Data from Caldwell et al. (1966)

b. Data from Ho et al. (1968)

c. Data from the present study (Table 4.8)

d. Data for red blood cells only

e. Data expressed per ml of whole blood

(ii) The control of adenosine kinase, adenosine deaminase and adenosine uptake by substrate concentrations

The K_m values for adenosine kinase are similar to the concentrations of adenosine measured in animal tissues (compare Table 4.9 with Tables 1.1 and 1.2); the K_m values for adenosine deaminase are usually much higher (Table 4.9). These observations indicate that the activity of adenosine deaminase is always regulated by the concentration of adenosine in vivo, whereas that of adenosine kinase is only regulated when the concentration of adenosine is low.

The present results suggest that exogenous adenosine will be metabolized in very different ways in different tissues because the relative activities and K_m values of adenosine kinase and adenosine deaminase vary between tissues. The ratios, adenosine kinase activity/adenosine deaminase activity, for low and high concentrations of adenosine are shown in Table 4.15. (At low substrate concentrations - $s \ll K_m$ - the activity of an enzyme which obeys Michaelis-Menten kinetics is proportional to V_{max}/K_m . Therefore V_{max}/K_m has been used to compare enzyme activities under these conditions.) It may be predicted from the calculations in Table 4.15 that mouse brain will phosphorylate 65 to 80% of exogenous adenosine, according to the concentration of adenosine; the remainder is deaminated. These percentages correlate well with data obtained for guinea-pig cerebral cortex. Thus Shimizu et al. (1972) found that 70 to 88% of the adenosine supplied to guinea-pig cerebral cortex slices was phosphorylated. A similar correlation is found between the data of Table 4.15 and the metabolism of adenosine by rat heart (De Jong, 1972). Therefore these data support the view that exogenous adenosine is metabolized to AMP or inosine according to the relative activities of adenosine kinase and adenosine deaminase (see Chapter 1, Section B4c). Unfortunately, no data are available in the literature for the majority of species investigated in the present study.

TABLE 4.15 Ratios of the activities of adenosine kinase to those of adenosine deaminase

The mean activities presented in Tables 4.5 to 4.8 and the K_m values presented in Table 4.10 have been used to estimate the ratio, activity of adenosine kinase/activity of adenosine deaminase, at pH 7.5. This ratio is estimated as $V_{\max}$, adenosine kinase/ $V_{\max}$, adenosine deaminase for high concentrations of adenosine and as $V_{\max}/K_m$, adenosine kinase x $K_m/V_{\max}$, adenosine deaminase for low concentrations of adenosine (see Section D1b(ii)). A dash (-) indicates that data were not available.

ANIMAL	TISSUE	RATIOS OF MEAN ACTIVITIES, KINASE/DEAMINASE	
		High adenosine concen- tration	Low adenosine concen- tration
Earthworm (<u>Allolobophora longa</u>)	Nerve cord	>14	-
Scallop (<u>Pecten maximus</u>)	Fast adductor muscle	0.17	0.045
	Slow adductor muscle	0.06	0.06
Lobster (<u>Homarus vulgaris</u>)	Cerebral ganglion	0.083	0.93
	Abdominal muscle	0.06	1.1
	Claw muscle	0.03	0.22
Locust (<u>Schistocerca gregaria</u>)	Cerebral ganglion	0.37	1.3
	Flight muscle	0.67	1.2
Cockroach (<u>Blaberus discoidalis</u>)	Cerebral ganglion	0.22	1.2
	Flight muscle	0.42	22
Waterbug (<u>Lethocerus cordofanus</u>)	Pterothoracic ganglion	>120	-
	Flight muscle	>10	-
Rosechafer (<u>Pachnoda ephippiata</u>)	Flight muscle	0.57	-
Wasp (<u>Vespa vulgaris</u>)	Flight muscle	6.3	16
Honey-bee (<u>Apis mellifera</u>)	Flight muscle	>14	-
Bumble-bee (<u>Bombus agrorum</u>)	Flight muscle	20	23
Solitary-bee (<u>Colletes cunicularis</u>)	Flight muscle	>10	-

ANIMAL	TISSUE	RATIOS OF MEAN ACTIVITIES, KINASE/DEAMINASE	
		<u>High</u> <u>adenosine</u> <u>concen-</u> <u>tration</u>	<u>Low</u> <u>adenosine</u> <u>concen-</u> <u>tration</u>
Blowfly (<u>Calliphora erythrocephala</u>)	Flight muscle	1.85	4.7
Rainbow trout (<u>Salmo gairdneri</u>)	Brain	0.12	0.79
	Liver	12	20
	Heart	0.4	3.3
	Red muscle	0.05	0.5
	White muscle	0.1	1.3
Frog (<u>Rana temporaria</u>)	Brain	0.062	0.78
	Liver	0.14	1.8
	Heart	0.17	1.2
	Gastrocnemius muscle	0.83	0.63
Pigeon (<u>Columba livia</u>)	Brain	0.091	0.87
	Liver	0.68	14
	Heart	0.02	0.57
	Pectoral muscle	0.06	0.88
	Gastrocnemius muscle	0.03	0.27
	Sartorius muscle	0.05	0.48
Domestic fowl (<u>Gallus gallus</u>)	Brain	0.25	1.3
	Liver	2.4	24
	Heart	0.05	0.17
	Pectoral muscle	0.15	0.75
	Sartorius muscle	0.1	0.43
Pheasant (<u>Phasianus colchicas</u>)	Heart	0.39	0.83
	Pectoral muscle	0.04	0.15
	Sartorius muscle	0.06	0.35
Laboratory mouse	Brain	1.3	3.8
	Liver	0.65	14
	Heart	0.77	7.6
	Gastrocnemius muscle	0.61	7.6
	Whole blood	0.33	2.5
	Testis	0.076	0.50
	Kidney medulla	0.035	0.07
	Kidney cortex	0.16	0.52
	Lung	0.54	3.24
Laboratory rat	Heart	0.02	0.11

(iii) The control of the activity of adenosine kinase by Na^+ and K^+

The effect of varying the proportions of Na^+ and K^+ on the activity of mouse adenosine kinase does not immediately suggest a possible control mechanism. Thus intracellular ion concentrations in guinea-pig brain slices are reported to be 40 mM Na^+ , 114 mM K^+ , increasing to 80 mM Na^+ , 70 mM K^+ on stimulation (Keesey et al., 1965). Similar ion concentrations are found in many other vertebrate tissues (Davson, 1970). The presents results indicate that such ionic changes can result in, at most, a 35% stimulation of adenosine kinase activity. Therefore depolarizing conditions, which increase the concentration of intracellular Na^+ , should increase adenosine phosphorylation and result in a decreased concentration of adenosine. However, it is known that depolarizing conditions elevate adenosine concentrations (Chapter 1, Section B.3).

Nonetheless, the in vitro effects of Na^+ and K^+ on the activity of adenosine may not be irrelevant to the in vivo situation. Some adenosine kinase is associated with the membranes of the cell (Chapter 1, Section B4c(iii)) and, if this proportion of the cells complement of enzyme is responsive to monovalent cations in the extracellular environment, its activity may vary considerably. Body fluids contain about 140 mM Na^+ and 5 mM K^+ (Davson, 1970) and this presumably corresponds to the concentration of these ions surrounding most cells. When excitable tissues are stimulated they release K^+ and take up Na^+ (see Davson, 1970). It appears that little is known of the effect this has on the immediate external environment of the cell but, in view of the large changes in intracellular ion concentrations which may be evoked (see, for example, Keesey et al., 1965), it is reasonable to suggest that ion concentrations surrounding the cell may change to 120 mM Na^+ and 20 mM K^+ . In this case the activity of adenosine kinase would be reduced by 40%. Changes in the distribution of Na^+ and K^+ between intracellular and extracellular

compartments are not only brought about by nervous stimulation of excitable tissues: anaerobiosis also increases the loss of K^+ from tissues (Krebs et al., 1951; see Davson, 1970). Therefore, it is possible to suggest that membrane-associated adenosine kinase from non-excitabile tissues is also regulated by the concentrations of Na^+ and K^+ .

There is circumstantial evidence which suggests that adenosine kinase is partly controlled by the concentrations of Na^+ and K^+ in vivo. Ouabain inhibits Na^+ , K^+ -activated ATPase and thereby inhibits Na^+ and K^+ transport. Consequently, it causes a redistribution of Na^+ and K^+ between intracellular and extracellular compartments analogous to the redistribution effected by electrical stimulation of tissues or anaerobiosis. The present theory would predict that ouabain should indirectly inhibit adenosine kinase and therefore prolong any effect of exogenous adenosine. Accordingly, it has been found that the auriculoventricular block of guinea-pig heart caused by exogenous adenosine is increased in length tenfold by doses of ouabain (10^{-7} M) which are otherwise without effect upon the heart (Rand et al., 1955). Moreover, ouabain inhibits hormone-stimulated lipolysis in fat cells, apparently by reducing the formation of cyclic AMP (Jeanrenaud et al., 1967). One possible explanation of this effect is that ouabain indirectly inhibits adenosine kinase and therefore the concentration of adenosine in the medium bathing the cells is increased. This extracellular adenosine may inhibit the formation of cyclic AMP in fat cells (Fain et al., 1972; Fain, 1973).

The details of the mechanism whereby ouabain prolongs some physiological effects of exogenous adenosine have not been elucidated. The early view that ouabain inhibits the metabolism of adenosine has not been disputed (Rand et al., 1955) but it is extremely unlikely that ouabain directly inhibits adenosine kinase or adenosine deaminase (Rockwell & Maguire, 1966; Shimizu et al., 1972; Huang et al., 1973). The present hypothesis is that ouabain

inhibits the activity of adenosine kinase through changes in the concentrations of Na^+ and K^+ in the immediate external environment, but it could also exert its effects through changes in the concentration of cytosolic Ca^{2+} , as discussed below (see Lee & Klaus, 1971).

(iv) The control of the activity of adenosine kinase by Ca^{2+}

The effect of Ca^{++} on the activity of adenosine kinase from mouse and locust tissues suggests that the activity of the enzyme is regulated by Ca^{2+} in vivo. Adenosine kinase was most sensitive to changes in Ca^{2+} concentration between 10^{-8} and 10^{-6} M. This is precisely the range of concentration over which Ca^{2+} concentrations are believed to vary in resting and contracting muscle (see Ashley, 1971) and there is evidence that cytosolic Ca^{2+} concentrations may vary over this range in other tissues, particularly in resting and depolarized nervous tissue (Hodgkin & Keynes, 1957; Ramsey & McIlwain, 1970). Mitochondria from a number of vertebrate and invertebrate tissues (blowfly flight muscle is an exception) have been shown to be able to take up Ca^{2+} ions until the Ca^{2+} in their bathing medium is very low (Carafoli & Lehninger, 1971). The uptake of Ca^{2+} by mitochondria is dependent upon respiration (Vasington & Murphy, 1962). It may be suspected, therefore, that a reduction in oxygen or fuel supply to a tissue will lead to an increase in cytosolic Ca^{2+} concentration. Consequently, adenosine kinase may be inhibited during the contracted phase of the muscle cycle, during depolarization of nervous tissues, and under anaerobic conditions. The physiological significance of the resulting increase in the concentration of adenosine is discussed in Section D2e and Chapter 6, Section A.1.

(c) THE CONTROL OF ADENOSINE CONCENTRATIONS

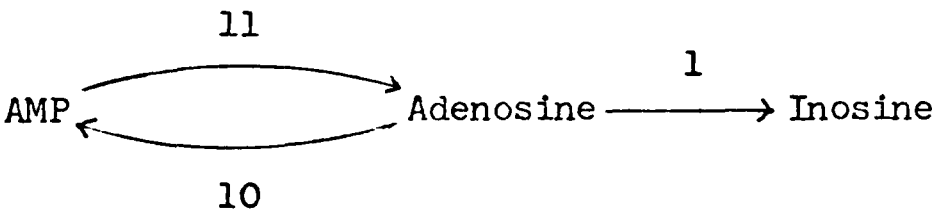
There appears to be some release of adenosine from the cell even when tissues are adequately supplied with oxygen and an oxidizable substrate (Chapter 1, Section B.3); adenosine is also taken up by all animal tissues

which have been studied (Chapter 1, Section B.4). Therefore, it seems probable that adenosine is continuously produced and utilized and that the concentration of adenosine in a tissue depends on a balance between production and utilization. Maximal activities of the enzymes involved in adenosine turnover are high compared with tissue adenosine concentrations (compare Tables 4.5 to 4.8 with Tables 1.1 and 1.2). Steady-state concentrations of adenosine may therefore be reached within a few seconds of a change in activity of any of the enzymes.

Adenosine is produced by 5'-nucleotidase and utilized by adenosine kinase and adenosine deaminase. It was pointed out in Chapter 1, Section B2b that, provided all three enzymes catalyze non-equilibrium reactions, the concentration of adenosine in a tissue may be controlled by regulating the activity of any of the three enzymes. Thus both an increase in the activity of 5'-nucleotidase, or a decrease in the activity of adenosine kinase or adenosine deaminase increases the steady-state concentration of adenosine. (The time required to reach the new steady-state will depend on the rate of turnover of adenosine - see Chapter 1, Section A3a(ii).) It is proposed in this thesis that concentrations of adenosine are controlled through the regulation of the activities of 5'-nucleotidase and adenosine kinase by changes in the concentrations of both substrate and effectors of the enzymes (e.g. adenine nucleotides and metal ions). The activity of adenosine deaminase is regulated only by changes in the concentration of substrate. Anaerobic conditions and depolarization of a tissue affect the concentrations of the effectors so that 5'-nucleotidase is activated and adenosine kinase is inhibited (see Chapter 1, Section B2e and above), with the result that intracellular adenosine concentrations are increased. Since adenosine equilibrates between intracellular and extracellular compartments, the extracellular concentration of adenosine also increases. These proposals are illustrated in Figure 4.13.

Since adenosine concentrations may be reduced by increased activity of adenosine kinase, it may be predicted from the results of Section C.2 that theophylline and caffeine will reduce adenosine concentrations in vivo. This could explain why theophylline and caffeine prevent the elevation of cyclic AMP levels in the brain by adenosine (Sattin & Rall, 1970); it could also explain why they prevent the relaxing effect of adenosine on vascular smooth muscle (Schaumann et al., 1970).

It can be demonstrated by using a simple example that, if adenosine kinase is almost saturated with adenosine, the concentration of adenosine in a tissue may be greatly affected by a relatively small change in the activity of the enzyme. Consider the following situation in which the rates of the enzyme reactions are as shown.



It will be assumed that the concentration of AMP is maintained at a constant level by those mechanisms which control the ATP:ADP:AMP concentration ratio, so that the activity of 5'-nucleotidase is not affected by changes in the rate of formation of AMP by adenosine kinase. The concentration of adenosine is well above the K_m of adenosine kinase so that adenosine kinase activity is unaffected by an increase in adenosine concentration; on the other hand it is well below the K_m of adenosine deaminase so that adenosine deaminase activity is proportional to adenosine concentration. If now adenosine kinase activity is halved by, for example, an increase in Ca^{2+} concentration, the adenosine concentration must increase 6-fold to reach a new steady-state in which the situation is as follows.

tissues. It has not proved possible to produce a satisfactory theory of the role of adenosine in invertebrate tissues.

(a) THE ROLE OF ADENOSINE IN MUSCLE TISSUES

An examination of the activities of adenosine kinase in various muscles suggested that there might be an interesting, and perhaps unexpected, correlation between the capacity of the muscle to utilize fat and the activity of adenosine kinase. Comparison of the activities of adenosine kinase with the known activities of lipid metabolizing enzymes in these muscles demonstrates that such a correlation may exist but, unfortunately, few data are available for white muscle (Table 4.16). However, preliminary data for ketone body-utilizing enzymes do suggest a correlation between the capacity of a muscle to utilize fat and its activity of adenosine kinase (A. Beis, personal communication). It is also interesting that there is an inverse correlation between the activities of glycogen phosphorylase and adenosine kinase (with the exception of lobster and possibly scallop muscles). Thus muscles with low capacities for glycogen utilization have the highest activities of adenosine kinase (see Table 4.16, Figure 4.14).

The activities of glycogen phosphorylase and lipid metabolizing enzymes are only available for some of the tissues studied. However, it is known that the capacity of a muscle to utilize fat is generally correlated with a high oxidative capacity, a red colour and the ability of the muscle to sustain its activity (Needham, 1926, 1971; Crabtree & Newsholme, 1974). Thus the correlation between the capacity of a muscle to utilize fat and its activity of adenosine kinase is extended by considering the colour of the muscle: red vertebrate muscles contain higher activities of the enzyme than white vertebrate muscles. For example, the heart always contains the highest activity of adenosine kinase of any muscle (from the same species). Similarly, comparing the same muscles from different species, the red pigeon

TABLE 4.16 The activities of adenosine kinase, glyceride lipases, carnitine palmitoyltransferase and glycogen phosphorylase in muscles from vertebrates and invertebrates

Mean maximal activities of adenosine kinase are taken from Table 4.5. The parameter, $V_{\max}/K_m$, pH 7.5, has been calculated for adenosine kinase from the data of Tables 4.5 and 4.9 in order that the activities of adenosine kinase at low concentrations of adenosine may be compared. Other data are from Crabtree & Newsholme (1972a,b) unless indicated. Activities of triglyceride lipase are given for vertebrates muscles, whereas activities of diglyceride lipase are given for insects, since insect muscles may oxidize exogenous diglyceride (see Crabtree & Newsholme, 1972b). Latin names are given in Table 4.5 and are only given in the present table for those cases where two species having the same common name have been studied. The muscles have been placed in the order of their $V_{\max}$'s for adenosine kinase (second column of figures).

ANIMAL	MUSCLE	ENZYME ACTIVITIES (nmol/min/g wet wt. of tissue)				
		ADENOSINE KINASE $V_{\max}$ (nmol/min/g wet wt.) K_m (μ M)	ADENOSINE KINASE	DIGLYCERIDE LIPASE (insects) or TRIGLYCERIDE LIPASE (vertebrates)	CARNITINE PALMITOYL- TRANSFERASE	GLYCOGEN PHOSPHORYLASE - 10^{-3}
Honey-bee	Flight	245	1410	300	200	4
Bumble-bee (<i>Bombus</i> sp.)	Flight	128	1110	200	700	8
Solitary-bee	Flight	96	964	-	-	-
Waterbug	Flight	19	465	900	3500	1
Wasp	Flight	56	450	200	-	3
Blowfly	Flight	45	306	200	200	55
Trout	Heart	32	213	-	-	-
Cockroach (<i>Blaberus discoidalis</i>)	Flight	71	155	-	-	-
Scallop	Fast adductor	1.0	110	-	-	8.5 ^a
Mouse	Heart	39	109	-	-	-
Scallop	Slow adductor	3.4	90	-	-	1.8 ^a
Rosechafer	Flight	16	64	300	100	14 ^b
Locust	Flight	25	62	700	2800	7.5
Cockroach (<i>Periplaneta americana</i>)	Flight	12	42	100	100	30
Trout	Red abdominal	10	39	20	200	14
Frog	Heart	9	26	-	-	-
Rat	Heart	4	25	60	2200	12
Mouse	Gastrocnemius	13	25	-	-	34 ^c

ANIMAL	MUSCLE	ADENOSINE KINASE		ENZYME ACTIVITIES (nmol/min/g wet wt. of tissue)			
		$V_{\max}$	(nmol/min/g wet wt.)	ADENOSINE KINASE	DIGLYCERIDE LIPASE (insects) or TRIGLYCERIDE LIPASE (vertebrates)	CARNITINE PALMITOYL- TRANSFERASE	GLYCOGEN ¹ PHOSPHORYLASE - 10 ⁻³
		K_m	(μ M)				
Locust	Hind leg femoral	7		21	-	-	20
Pheasant	Heart	5.9		16	-	-	-
Pigeon	Heart	17		15	-	-	-
Frog	Gastrocnemius	8.8		15	-	-	29 ^d
Lobster	Abdominal	4.9		14	-	-	8.5 ^a
Pigeon	Pectoral	14		14	70	3200	18
Trout	White abdominal	6.2		14	-	-	48
Domestic fowl	Heart	4.5		10	-	-	-
Domestic fowl	Sartorius	4.3		10	-	-	-
Pigeon	Sartorius	4.8		9.5	-	-	-
Lobster	Claw	1.3		7.8	-	-	4.6 ^a
Pheasant	Sartorius	3.8		6.9	-	-	-
Pigeon	Gastrocnemius	3.0		6.3	-	-	-
Domestic fowl	Pectoral	3.3		6.0	-	-	83
Pheasant	Pectoral	1.8		3.5	10	30	120

a. Data from Zammit (1974)

b. Data for cockchafer (Melolontha melolontha)

c. Data for quadriceps muscle

d. Data for sartorius muscle

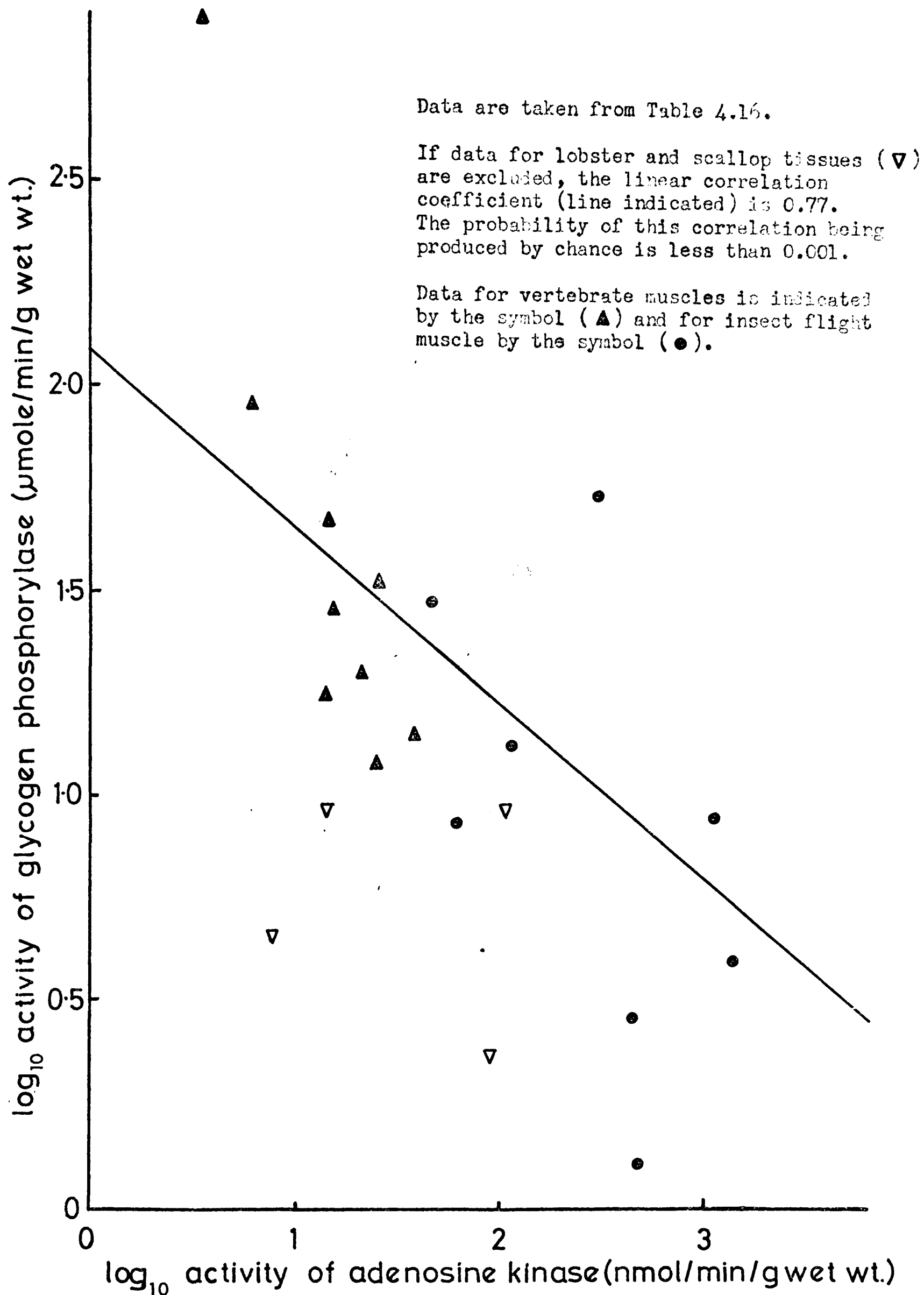


FIG 4.14 A regression plot of the activities of glycogen phosphorylase and adenosine kinase in vertebrate and insect muscles.

pectoral muscle has a higher activity of the enzyme than the white pectoral muscle of the pheasant or domestic fowl.

It is also useful to consider the function of the muscle. Thus insect flight muscles, which can sustain very high rates of energy metabolism for long periods of time have the highest activities of the enzyme. (Although data are only available for a few insects, it is noticeable that the highest activities of adenosine kinase were found in those insect muscles which have the highest oxidative capacities - see Crabtree, 1969; Crabtree & Newsholme, 1972a). Vertebrate heart muscles, which are continually contracting for the whole of an animal's life, but at lower rates of energy utilization than insect flight muscles, have the next highest activities of the enzyme. Pigeon pectoral muscle can also sustain a high level of energy utilization for a long time and has a similar activity of adenosine kinase to pigeon heart. In contrast, the pheasant, which can only fly for short periods of time, and the domestic fowl, which can scarcely fly at all, have very low activities of adenosine kinase in their pectoral muscles. The red muscle of the trout, which is electrically active during slow swimming, has a 3-fold higher activity than the white muscle, which is only used during vigorous movements (Bone, 1966).

When a muscle is adapted to use short bursts of energy it usually obtains this energy from the anaerobic degradation of glycogen to lactate. Such muscles generally have a low blood supply, since they do not require a supply of oxygen and glucose to provide energy for their contractile activity. These muscles have no requirement for control of their blood supply in relation to the contractile activity. On the other hand, muscles which are mechanically active for long periods of time depend exclusively upon a supply of oxygen and fuel (either glucose or fat), which is provided via the blood stream (Crabtree & Newsholme, 1974). The regulation of blood supply so

that sufficient fuel and oxygen reaches the muscle is fundamentally important. If adenosine is concerned in the autoregulation of blood flow, it is not surprising that adenosine kinase activities are highest in muscles that produce sustained mechanical activity.

Invertebrate muscles are always bathed with very high concentrations of fuel and it seems unlikely that the activity of these muscles are ever limited by their supply of fuel. It is also unlikely that the activity of insect muscle is limited by its supply of oxygen. Adenosine might play a very different regulatory role in insect, and other invertebrate, muscles to that which it plays in vertebrates (see Chapter 6, Section A.1 for further discussion).

(b) THE ROLE OF ADENOSINE IN NERVOUS TISSUES

Most nervous tissues depend for their supply of energy on the aerobic utilization of glucose (McIlwain & Bachelard, 1971). It is known that under some conditions the fuel supply to a nervous tissue, or to localized regions of it, must be greatly increased. For example, an increase in neural activity may require a considerable increase in glucose oxidation and thus blood supply. (In addition, disorders which lead to high plasma K^+ levels may increase the neural activity of the total nervous system, so that increased fuel utilisation and blood supply will be required.) Therefore, if adenosine concentrations regulate blood flow in vertebrate brains, it might be expected that 5'-nucleotidase, adenosine kinase and adenosine deaminase activities would be relatively high in brain. Accordingly, the activities of these enzymes are often similar in brain to the activities in the heart of the same species.

However, it is not possible to correlate activities of adenosine kinase in nervous tissues with activities of glycogen phosphorylase and fat metabolizing enzymes since data are not available for the latter two enzymes.

Nevertheless, data for ketone body utilizing enzymes and phosphofructokinase are available (Sugden & Newsholme, 1973), and these activities are probably indicative of rates of fat and glucose metabolism in nervous tissue. These data are compared with the activities of adenosine kinase measured in the present study in Table 4.17. It is noticeable that the activities of all the enzymes vary over very small ranges. Indeed, the activities of adenosine kinase at low substrate concentration are very similar for a variety of vertebrate and invertebrate brains. Therefore, the activities of adenosine kinase in nervous tissues are consistent with a role for adenosine in the regulation of blood flow.

In contrast, there is a marked difference between the activities of 5'-nucleotidase and adenosine kinase in the brains of new-born and adult mice: both enzymes have higher activities in the adult brain (Table 4.12). Adult mammals suffer brain damage within minutes if they are deprived of oxygen, whereas the new-born can withstand relatively long periods of anaerobiosis (Bullough, 1958; Mott, 1961). The new-born brain has large reserves of glycogen and a high glycolytic capacity (Krebs, 1972), which provide a source of energy during parturition (Dawes, 1968). Since the new-born is less dependent on oxygen supply, the low activities of 5'-nucleotidase and adenosine kinase are consistent with a role for adenosine in regulating blood flow in the brain.

Young rats lose their immunity to asphyxia at the age of about 11 days, which is when myelin formation begins (McIlwain & Bachelard, 1971, pp.412,429). It may be predicted that developmental studies would show that 5'-nucleotidase and adenosine kinase activities increase rapidly at this stage. Such a finding might also suggest that 5'-nucleotidase (and possibly some adenosine kinase) is located in the myelin sheet (see Chapter 1, Sections B3d(i) and B4c(iii)).

TABLE 4.17 The activities of adenosine kinase, 3-oxo acid CoA-transferase, acetoacetyl-CoA thiolase and phosphofructokinase in nervous tissues from vertebrates and invertebrates

Mean maximal activities of adenosine kinase are taken from Table 4.6. The parameter, $V_{\max}/K_m$, pH 7.5, has been calculated for adenosine kinase from the data of Table 4.6 and 4.9 in order that the activities of adenosine kinase at low concentrations of adenosine may be compared. Other data are from Sugden & Newsholme (1973). The tissues have been arranged in order of their $V_{\max}$'s for adenosine kinase (second column of figures).

ANIMAL	NERVOUS TISSUE	ADENOSINE KINASE		ENZYME ACTIVITIES (nmol/min/g wet wt. of tissue)			
		$V_{\max}$ (nmol/min/g wet wt.)	K_m (μM)	ADENOSINE KINASE	3-OXO ACID CoA-TRANSFERASE $\times 10^{-3}$	ACETOACETYL-CoA THIOLEASE $\times 10^{-3}$	PHOSPHOFRUCTOKINASE $\times 10^{-3}$
Waterbug	Pterothoracic ganglion	5.6		189	-	-	24
Earthworm	Nerve cord	13		148	1.3	1.5	18.4
Cockroach	Cerebral ganglion	7.7		69	4.0	3.8	6.0
Mouse	Whole brain	16		65	9.8	2.7	14.6
Trout	Whole brain	8.1		62	2.8	4.0	7.4
Locust	Cerebral ganglion	5.2		44	2.7	1.2	4.6
Lobster	Cerebral ganglion	6.7		24	1.1	1.4	9.9
Domestic fowl	Whole brain	8.0		15	2.9	2.1	13.7
Frog	Whole brain	7.5		14	1.5	4.0	8.2
Pigeon	Whole brain	6.6		13	3.2	2.3	12.2

In contrast to 5'-nucleotidase and adenosine kinase, the activity of adenosine deaminase in the brain of the new-born mouse is high. It is possible that this high activity reflects the role of the enzyme in DNA and RNA synthesis. Thus tissues which are synthesizing DNA rapidly have low levels of most catabolic enzymes but high levels of adenosine deaminase (see Zielke & Suetter, 1971). The brain of the new-born mammal synthesizes DNA rapidly up to parturition (McIlwain & Bachelard, 1971, p.418). It would be interesting to know whether the activity of adenosine deaminase decreases in the developing mouse at a stage when DNA synthesis is reduced.

It should be noted from the evidence above that adenosine regulation of blood flow in nervous tissues does not rule out the possibility of a combined role for adenosine including a role in nerve function, as suggested by McIlwain (1972).

(c) THE ROLE OF ADENOSINE KINASE IN VERTEBRATE LIVERS AND BLOOD

The role of adenosine kinase is often assumed to be to "scavenge" for the purine base and thereby spare the purine synthesizing activities of the organism (see Murray, 1971, and the introduction to Section B of Chapter 1). In this thesis it has been proposed that in muscle and brain adenosine kinase is regulated, together with 5'-nucleotidase, in order to produce changes in adenosine concentrations (Section D1c). However, it is possible that the primary function of adenosine kinase in some tissues (e.g. liver and blood) is to scavenge for adenosine in order to terminate its physiological effects. (The sparing of the purine synthesizing activities of these tissues would be of secondary importance.)

In nearly all the vertebrate tissues which have been investigated the maximal activity of 5'-nucleotidase is somewhat greater than the combined maximal activities of adenosine kinase and adenosine deaminase. (Adenosine deaminase has a higher maximal activity than adenosine kinase.) The enzyme activities in the liver are exceptions (Table 4.7). The high activities of

adenosine kinase in vertebrate livers suggest that in these tissues adenosine kinase functions to phosphorylate adenosine produced elsewhere. To support this suggestion, it may be pointed out that even the low levels of adenosine which are found in tissue perfusates (Table 1.3) may affect tissue metabolism (see Rubio et al., 1969). By removing this adenosine from the circulation, the liver would help to confine the effects of adenosine to the tissue where it was produced. (This is not a new role for the liver: it removes a number of other metabolites which would be deleterious to the organism if they were allowed to accumulate in the blood, for example, lactate produced by muscle and free fatty acids from adipose tissue.) In addition, adenosine is formed in the course of digestion and possibly reaches the liver via the hepatic portal vein. In view of the role of adenosine in the regulation of blood supply, it is particularly important that this adenosine is not allowed to enter the general circulation.

Since the activity of adenosine kinase is high in mouse blood, it is possible that the uptake of adenosine by the cells of the blood, like the uptake of adenosine by the liver, ensures that the effects of adenosine are localized to the tissue which produces it. Thus once adenosine diffuses from the intercellular space of a tissue into the blood stream, it is rapidly taken up and converted to AMP. Clearly the adenosine moiety does not accumulate within the cell; the purine base is probably degraded or released as adenine, inosine or hypoxanthine (see below).

An entirely different explanation for the role of adenosine kinase in blood cells may be suggested. It has been established that rabbit, mouse and human blood cannot synthesize purine de novo (Lowy & Williams, 1960; Fontenelle & Henderson, 1969); consequently any turnover of purine nucleotides in red cells must result from the continual entry and release of purines. Human red cell adenine nucleotides have a turnover time of 9 days (Murray, 1971). This turnover might be mediated by adenosine and be dependent on the

uptake of adenosine and its phosphorylation by adenine nucleotides. However, inhibition of nucleoside uptake by rabbit blood cells does not affect the turnover time of the nucleotide pool in these cells (see Murray, 1971). It is therefore more likely that adenine nucleotide turnover is mediated by adenine, inosine, or hypoxanthine than by adenosine.

(d) THE ROLE OF ADENOSINE IN THE LUNG

Mouse lung contains a very high activity of adenosine kinase (Table 4.8). This adenosine kinase may perform a similar "scavenging" function to that suggested above for the liver and blood. Thus the lung, situated between the left and right sides of the heart, is ideally placed to remove any adenosine from the venous circulation before it can affect the mechanical activity of the heart, via the coronary blood supply. (The role of the lung as a general filter is discussed by Heinemann & Fishman (1969)). It is interesting that the lung also has a high capacity to metabolize prostaglandins; these too are concerned in the regulation of blood flow (Ferreira & Vane, 1967).

Alternatively, it is possible that the activity of adenosine kinase is high in the lung because adenosine is concerned in the regulation of blood supply in the lung itself. As in muscles and brain, adenosine concentrations may be regulated by both 5'-nucleotidase and adenosine kinase activities. However, there is an important difference between muscles and brain, and the lung: the vasculature of the lung constricts in response to a reduced alveolar oxygen tension (Fishman, 1961). (Unlike muscle and brains cells, lung cells are probably never short of a supply of oxygen for their own metabolism. The effect of this unusual response is to divert the blood supply to areas of the lung where the alveolar oxygen tension is high and where the blood can be most efficiently oxygenated.) If adenosine mediates the response of the pulmonary vasculature to hypoxia, it must cause the vasculature to

constrict. Adenosine has been shown to have a vasoconstricting effect in the kidney; its effect on the lung has not been studied (Haddy & Scott, 1968; Tierney, 1974). (The inhibition of the activity of adenyl cyclase from guinea-pig lung by adenosine may be of relevance here - Weinryb & Michel, 1974; see also Chapter 6, Section A and footnote.)

(e) THE CONTROL OF ADENOSINE CONCENTRATIONS FOR THE PURPOSE OF REGULATING BLOOD FLOW

It has been discussed how a reduction in the supply of oxygen to a tissue may reduce the phosphorylation state of the cell and alter the distribution of Na^+ , K^+ and Ca^{2+} , so that 5'-nucleotidase is activated, adenosine kinase is inhibited and, consequently, the concentration of adenosine is increased (see Figure 4.13, Section D.1 and Chapter 1, Section B2e). Reduced fuel supply or electrical stimulation also increases the concentration of adenosine, in the brain at least (Pull & McIlwain, 1972a). In both cases the increased concentration of adenosine may be mediated by changes in the concentrations of adenine nucleotides and cations (Figure 4.18; see also Pull & McIlwain, 1973). If depolarization of a tissue leads, through changes in ion distribution, to increased concentrations of adenosine, an important consequence is that blood flow to the tissue may be increased even before the supply of oxygen and fuel to the tissue has begun to limit energy supply.

At this stage a further mechanism by which the concentration of adenosine may be controlled can be discussed (see Chapter 1, Section B2d). The pH of a tissue may fall under anaerobic conditions. In some species (the mouse, pigeon, pheasant, frog and domestic fowl) the activity of adenosine kinase may increase under these conditions, if the concentration of adenosine is high (Table 4.11). This would result in a decrease in the concentration of adenosine and, if adenosine regulates blood flow to the tissue, a decrease

in blood supply. It is difficult to see how this response could be of any advantage to the organism. It is therefore doubtful whether the effect of pH on the activity of adenosine kinase in homogenates of tissues is of any physiological significance.

D.3 A POSSIBLE ROLE FOR ADENOSINE AS AN INTERORGAN COMMUNICATOR

Adenosine is released extracellularly by some tissues and, although much of this adenosine is removed by the same tissue, it is probable that some of it escapes into the general circulation and finds its way into the liver (see Chapter 1, Section B4(a) and Section D2(c) above). This transport of adenosine from tissues such as muscle and brain to the liver may play a role as a specific inter-tissue regulatory mechanism. The largest store of carbohydrate in the body is found in the liver. Increased demand for glucose by muscle or brain must be met by mobilization of glucose from liver glycogen stores. Indeed, it is known that liver glucose is utilized during muscular exercise (Rohr et al., 1966). The question arises as to the messenger that causes increased glycogenolysis in the liver. It is unlikely to be blood glucose concentration, since this changes very little. Is it possible that adenosine provides the link? It is known that muscle (and brain) tissues release adenosine, it is present in the blood and it can be taken up by the liver. Although it has not been established that low concentrations of adenosine cause an increase in release of glucose from the liver, there are some indications to support the proposal. Hunter & Jefferson (1969) found that perfusion of the isolated rat liver with AMP (2 mM) causes increased glycogenolysis and glycolysis and decreased formation of glucose from lactate. Present theories can explain this effect if some of the AMP enters the liver cell but it is possible that the AMP effect is secondary to adenosine formation outside the cell.

D.4 THE ROLES OF 5'-NUCLEOTIDASE, ADENOSINE KINASE AND ADENOSINE DEAMINASE IN ADENINE NUCLEOTIDE METABOLISM

(a) THE ROLE OF 5'-NUCLEOTIDASE

There are two enzymes in animal tissues which catalyze the conversion of an adenine nucleotide (AMP) into a non-adenine nucleotide produce: 5'-nucleotidase and AMP deaminase. The latter enzyme catalyzes the formation of IMP and NH_3 from AMP. It is suggested in the following discussion that 5'-nucleotidase plays a similar role to one which has been suggested for AMP deaminase, i.e. the control of the tissue ATP/ADP concentration ratio. (Various reasons have been put forward to explain why the cell usually maintains this ratio at a constant value - Newsholme, 1970; Watts, 1971; Leech, 1973.) The mechanism by which AMP deaminase might help to maintain the tissue ATP/ADP concentration ratio constant is as follows (see Chapman & Atkinson, 1973, for a more detailed theory).

AMP deaminase is usually inhibited when the ATP/ADP concentration ratio is high in muscle (Ronca-Testoni *et al.*, 1970) and liver (Chapman & Stkinson, 1973). When the ATP/ADP concentration ratio falls, AMP deaminase is activated, owing to an increase in the concentration of the substrate, AMP, and possibly to a decrease in the concentration of the inhibitor, ATP. The consequent removal of AMP from the cell causes some ATP and AMP to be formed from ADP through the adenylate kinase reaction. The net result is that the ATP/ADP concentration ratio does not fall greatly, even though the number of high energy phosphate bands in the cell has fallen.

The results of the present study indicate that the maximal activity of AMP deaminase is low in those tissues which have a high activity of 5'-nucleotidase (Table 4.18). This suggests that 5'-nucleotidase may perform a similar function to AMP deaminase in some tissues. Thus the activity of 5'-nucleotidase, like the activity of AMP deaminase, increases when the

TABLE 4.18 The activities of 5'-nucleotidase and AMP deaminase in
different animal muscles

Activities of 5'-nucleotidase are taken from Table 4.5 and activities of
AMP deaminase are taken from Leech (1973).

ANIMAL	MUSCLE	ENZYME ACTIVITIES (nmol/min/g wet wt.)	
		5'-NUCLEOTIDASE	AMP DEAMINASE x 10 ⁻³
Waterbug (<u>Lethocerus cordofanus</u>)	Flight	1400	0.56
Bumble-bee (<u>Bombus pratorum</u>)	Flight	180	1.58
Pigeon (<u>Columba livia</u>)	Pectoral	98	60.2
Domestic fowl (<u>Gallus gallus</u>)	Pectoral	31	72.8 ^a
Rat	Heart	6070	1.25
Mouse	Gastrocnemius	49	76 ^b

a. Data for pheasant (Phasianus colchicas)

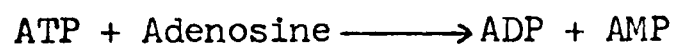
b. Data for rat.

ATP/ADP concentration ratio decreases (Chapter 1, Section B3d). Therefore, variations in the activity of 5'-nucleotidase may also serve to maintain the ATP/ADP concentration ratio. (The maximal activities of AMP deaminase are far higher than those of 5'-nucleotidase. However, the relative rates at which these two enzymes operate in vivo may not be reflected in their maximal activities.)

It is unlikely that the roles of 5'-nucleotidase and AMP deaminase are solely the stabilization of the ATP/ADP concentration ratio; in this case two separate systems would not be required. It is suggested that 5'-nucleotidase also plays a role in the control of adenosine concentrations and AMP deaminase might be involved in the control of tissue pH.

(b) THE ROLES OF ADENOSINE DEAMINASE AND ADENOSINE KINASE

Adenosine kinase catalyzes the reaction



Therefore, the rephosphorylation of adenosine by adenosine kinase lowers the phosphorylation state of the cell and decreases the ATP/ADP concentration ratio of the cell. When the cell is under stress the ATP/ADP concentration ratio falls and it is important that the rate of any reaction which reduces the ATP/ADP concentration ratio is not high. Indeed, the activity of 5'-nucleotidase cannot help to stabilize the ATP/ADP concentration ratio if all the adenosine which is produced from AMP is rephosphorylated (see above). The solution to this problem is that the activity of adenosine kinase is not sufficient to rephosphorylate all the adenosine which is produced under stress. Moreover, adenosine kinase may be inhibited in some species under these conditions by the high concentrations of adenosine which are produced (Table 4.10).

However, even when the cell is under stress and adenosine kinase is saturated with adenosine, the cell must continue to metabolize adenosine so

that it does not accumulate. Under these circumstances, adenosine deaminase, which has a higher K_m than adenosine kinase (Table 4.10), converts adenosine to inosine, which is physiologically inactive. Thus one role of adenosine deaminase is to remove adenosine under conditions of severe energy stress and another is that, by competing for adenosine with adenosine kinase under these conditions, it reduces changes in the ATP/ADP concentration ratio.

D.5 A SUMMARY OF THE HYPOTHESES PRESENTED

The following hypotheses or suggestions concerning the role of adenosine and the control of its concentrations were made in the present chapter (see also Chapter 1).

(a) THE ROLES OF ADENOSINE

It is proposed that, in vertebrate brain and muscle, adenosine causes vasodilation and mediates the autoregulation of blood flow.

The possibility is raised that, in the lung, adenosine causes vasoconstriction and takes part in a mechanism whereby blood is directed to those alveoli which are best supplied with oxygen.

It is suggested that adenosine might also function as a messenger which is released by muscle and nervous tissue in order to instruct the liver to increase its rate of glycogenolysis.

It is proposed that adenosine may have further roles, particularly in insect flight muscle.

(b) THE CONTROL OF THE CONCENTRATION OF ADENOSINE IN THE CELL

It is proposed that the enzymes 5'-nucleotidase, adenosine kinase and adenosine deaminase catalyze non-equilibrium reactions and that the concentration of adenosine in the cell is regulated by modulation of the activities of 5'-nucleotidase and adenosine kinase.

In some tissues, the kinetics of adenosine kinase and adenosine deaminase may be such that a large change in the concentration of adenosine is produced by a small change in the activity of 5'-nucleotidase or adenosine kinase.

It is proposed that the activity of 5'-nucleotidase is controlled by the concentrations of AMP and free ATP and ADP.

It is proposed that adenosine kinase is located in both the cytosol and the cell membrane, and it is possible that the activity of the enzyme is subject to different control mechanisms in the two locations: the activity of cytosolic adenosine kinase is regulated by Ca^{2+} ; the activity of membrane adenosine kinase is regulated, in vertebrates, by the concentrations of Na^+ and K^+ in the immediate external environment of the cell; the activity of both enzymes is regulated by the concentration of adenosine and in some tissues the activities are inhibited at high concentrations of substrate.

It is proposed that the concentration of adenosine in a tissue may rise in response to changes in the concentrations of Ca^{2+} , Na^+ and K^+ , before or independently of any reduction in the supply of oxygen to the tissue.

The activity of adenosine deaminase is regulated only by the concentration of adenosine.

(c) THE CONTROL OF THE CONCENTRATION OF ADENOSINE IN THE EXTRACELLULAR SPACE - ADENOSINE TRANSPORT

It is proposed that the movement of adenosine across the cell membrane takes place by diffusion down a concentration gradient.

The diffusion is a near-equilibrium process: under most conditions the rate of movement of adenosine into the cell is not greatly different from its movement out of the cell.

It is proposed that the metabolism of exogenous adenosine is dependent on the relative activities of adenosine kinase and adenosine deaminase; the saturable uptake mechanism reflects the activity of adenosine kinase, which maintains the concentration gradient by catalyzing the conversion of intracellular adenosine to AMP.

(d) THE CONTROL OF THE CONCENTRATION OF ADENOSINE IN THE BLOOD

It is proposed that the majority of the adenosine released from the cell is taken up again by the same or nearby cells.

The liver, and possibly the blood cells and lungs, take up adenosine which reaches the circulation.

The liver removes adenosine from the hepatic portal blood.

(e) SOME FURTHER SUGGESTIONS

It is proposed that the degradation of AMP via adenosine to inosine helps to maintain the ATP/ADP concentration ratio when the cell is under stress.

It is suggested that 5'-nucleotidase is located in the myelin sheath in the brain.

It is tentatively suggested that, since the concentration of adenosine may be very sensitive to changes in the activity of adenosine kinase, the small activation of adenosine kinase by methyl xanthines may account for their competitive inhibition of the effect of adenosine on tissue cyclic AMP levels and coronary vasodilation.

Chapter 5

THE REGULATION OF LIPOLYSIS IN THE HEARTS OF STARVED AND DIABETIC RATS

A. INTRODUCTION

The heart can oxidize a number of fuels which are supplied to it in the blood: glucose, free fatty acids, ketone bodies and triglyceride. In addition, the tissue contains triglyceride and glycogen, which can be degraded for energy formation - particularly during periods of stress. The rate of lipolysis is increased in the rat heart during starvation and alloxan diabetes (Garland & Randle, 1964; Randle et al., 1966). Increased lipolysis results in increased availability and consequent oxidation of fatty acids (see Fritz, 1961; Eaton & Steinberg, 1961); this increased oxidation of fatty acids causes decreased utilization of glucose by the heart. The mechanisms by which fatty acid oxidation decreases glucose utilization are as follows (see Newsholme & Start, 1973, p.100 ff., 124 ff., for a review). Fatty acid oxidation leads to elevated levels of citrate and acetyl-CoA and a depressed level of CoA; citrate inhibits phosphofructokinase, and an increase in the concentration ratio AcCoA/CoA inhibits pyruvate dehydrogenase. Moreover, fatty acid oxidation inhibits glucose transport into the cell by an unknown mechanism. In addition, the activity of pyruvate dehydrogenase is reduced in starvation and diabetes as the result of its phosphorylation to a less active form (Wieland et al., 1971). Since metabolism in the heart is profoundly affected by an increase in the rate of lipolysis, it is important to understand the mechanisms which control lipolysis.

Alloxan diabetes is due to a deficiency of circulating insulin; during starvation insulin levels are reduced. Therefore a knowledge of

the mechanisms which increase lipolysis in the heart during diabetes and starvation might lead to a fuller understanding of the mechanism of action of insulin. The administration of insulin to the isolated perfused heart from the diabetic rat does not reduce the rate of lipolysis. Moreover, injection of insulin into diabetic rats increases the rate of glucose utilization in subsequently perfused hearts only several hours after administration. The slow response to insulin administration suggests that the mechanism controlling lipolysis in the heart is subject to changes in enzyme activity via effects on protein synthesis, rather than changes in the specific activity of enzymes. The effect of insulin could be due to modification of the concentration of triglyceride lipase. Alternatively, insulin could modify the mechanism that controls the activity of the lipase, i.e. "the cyclic AMP system" (see Chapter 1, Section A3a). The enzymes which could be involved in the inhibition of lipolysis by insulin and their interrelationships are shown in Figure 5.1. These enzymes are triglyceride lipase, adenylyl cyclase, phosphodiesterase, protein kinase and lipase phosphatase. Since methods were available in this laboratory for the assay of the first three of these enzymes, their activities, and where possible their K_m values, were measured in homogenates of hearts from fed, starved and alloxan diabetic animals and the results are reported in this Chapter.

B. METHODS

ANIMALS

Male Wistar rats (approximately 250g body weight) were starved for 48 hr or made diabetic with alloxan as described by Newsholme & Randle (1964). Alloxan-treated rats were used only if the blood glucose concentration in the starved animal was greater than 175 mg/ml when tested with "Dextrostix".

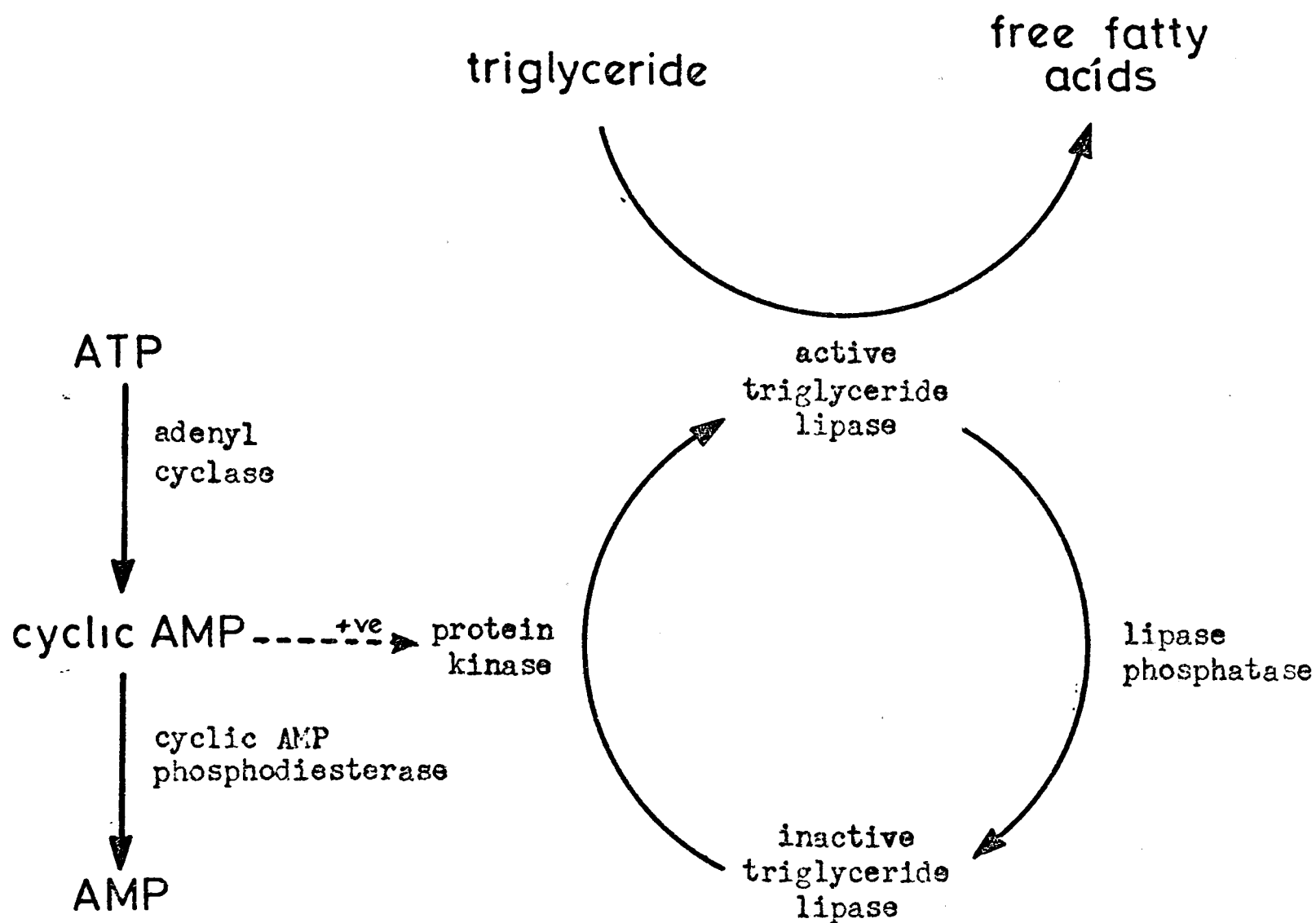


FIG 5.1 The enzymes which are involved in the control of lipolysis.

The dashed line represents the activation of protein kinase by cyclic AMP.

Further enzymes which may be involved in the control of lipolysis are considered in section C.

MEASUREMENT OF ENZYME ACTIVITIES

For the measurement of the activities of adenylyl cyclase and phosphodiesterase, hearts were homogenized as described in Chapter 2, Section A.1 and the enzymes were assayed as described in Chapter 2, Sections A.2 and A.3 (see also Chapter 3, Section B.3 for the measurement of K_m values). NaF was not present during the assay of adenylyl cyclase. Small variations in method used to extract these enzymes can lead to different activities of the enzymes in the homogenates (see Chapter 2, Section H), and so hearts from fed, starved, and/or diabetic hearts were extracted and assayed in parallel using identical methods.

For the measurement of the activity of triglyceride lipase, hearts were homogenized in 10 volumes of 200 mM triethanolamine containing 2% (w/v) defatted albumin at pH 7.5. The homogenates were assayed for lipase activity immediately after preparation. Preliminary experiments established that sonication of the homogenate did not increase the activity of the lipase. The lipase was assayed as described by Crabtree & Newsholme (1972b).

C. RESULTS AND DISCUSSION

There was no detectable effect of cyclic AMP (0.1-1 mM in the presence of 2 mM caffeine, 5 mM ATP and 5 mM $MgCl_2$) on the activity of triglyceride lipase from rat heart. This suggests that under the present extraction and assay conditions the lipase is present in its active form and that maximal activities are being measured (Löffler & Weiss, 1970; Jungas & Schwartz, 1970). This conclusion is supported by the observation that the total lipase activity is approximately 5-fold greater than the usual rate of lipolysis in the intact heart and similar to that in the alloxan diabetic state (Table 5.1). The activities of adenylyl cyclase which are given in Table 5.1 are basal activities (measured in the absence of F^-).

TABLE 5.1 The effect of starvation and alloxan diabetes on lipolysis and enzyme activities in the rat heart

Methods are described in Section B. All rates are expressed as nmol/min/g wet wt. of tissue $\pm$ standard deviation. The number of hearts used for separate determinations of each measurement is given in parentheses. Adenyl cyclase and phosphodiesterase were assayed at 30°C. Triglyceride lipase was assayed at 25°C. Rates of lipolysis in the intact heart (taken from Garland & Randle, 1964) were measured at 37°C but for the present table these rates have been divided by 2.4 to give the rate corresponding to a temperature of 25°C. (The Q_{10} of the lipase was determined and found to be approximately 2.) No variation could be detected in the kinetics of high K_m cyclic AMP phosphodiesterase (see Chapter 3, Section B.4).

CONDITION OF ANIMAL	RATE OF LIPOLYSIS IN THE INTACT HEART (glycerol release)	TRIGLYCERIDE LIPASE V_{max}	ADENYL CYCLASE		LOW K_m PHOSPHODIESTERASE		HIGH K_m PHOSPHODIESTERASE	
			V_{max}	K_m (μM)	V_{max}	K_m (μM)	V_{max}	K_m (μM)
Fed	12.3	60 $\pm$ 6 (6)	10.5 $\pm$ 0.7	120 $\pm$ 30 (5)	16 $\pm$ 5 (5)	0.68 $\pm$ 0.23 (5)	288 $\pm$ 34 (5)	36 $\pm$ 11 (5)
Starved 48 hr.	17.9	48 $\pm$ 4 (5)	10.5 $\pm$ 1.6 (4)	130 $\pm$ 34 (4)	18 $\pm$ 2 (4)	0.79 $\pm$ 0.09 (4)	393 $\pm$ 11 (4)	43 $\pm$ 7 (4)
Alloxan diabetic	28.5	56 $\pm$ 7 (5)	8.3 $\pm$ 1.3 (2)	100 $\pm$ 27 (2)	15 $\pm$ 4 (3)	0.81 $\pm$ 0.21 (3)	266 $\pm$ 48 (3)	32 $\pm$ 5 (3)

The results given in Table 5.1 show that neither starvation nor diabetes had any significant effect upon the total lipase, the basal adenyl cyclase or the phosphodiesterase activities of the rat heart. The kinetic properties of adenyl cyclase and phosphodiesterase were also unaffected. Furthermore, the activity of lipase is similar to the rate of lipolysis in the alloxan diabetic state. It is therefore concluded that the effects of starvation and diabetes upon the rate of lipolysis in rat heart are the result of an increased activation, rather than an increased concentration, of the lipase. Since the activities of adenyl cyclase and phosphodiesterase are similar in all conditions of the animal, it seems unlikely that increased activation of lipase results from an elevated concentration of cyclic AMP. However, one possibility is that the sensitivity of adenyl cyclase to some hormones is increased in the diabetic and starved states. (This explanation requires that some hormones are present in the isolated perfused heart.) In this case the level of cyclic AMP would be greater in these states than in the fed animal. Measurements of the content of cyclic AMP in hearts from fed, starved and diabetic rats might help to resolve this problem.

Since the activity of triglyceride lipase depends upon the balance between the activities of protein kinase and lipase phosphatase, increased activation of lipase may result from an increase in the total activity of the kinase or a decrease in the total activity of the phosphatase. It would be possible to measure these activities in hearts from fed, starved and diabetic rats.

Finally, it must be recognized that as yet unknown, physiological regulators of the activities of the enzymes which have been discussed may mediate the effect of insulin on lipolysis. Proteins which modulate the activities of protein kinase and phosphodiesterase have been described (see Chapter 1, Section A) but it has not been shown that changes in their

concentration are important in vivo. Cyclic GMP has been suggested as a regulator of protein phosphatase (see Goldberg et al., 1973) and cyclic AMP phosphodiesterase (see Appleman et al., 1973). Therefore it may be useful to measure the activities of guanyl cyclase and cyclic GMP phosphodiesterase and the concentration of cyclic GMP in hearts from fed, starved and diabetic rats.

Whilst there are few data in the literature concerning the metabolism of cyclic AMP in the heart of the diabetic rat, there are a number of data for the liver and adipose tissue (see Appleman et al., 1973 for a review). These data will be briefly summarized, since they may indicate which of the lines of approach in future work suggested above are likely to be most successful. The concentration of cyclic AMP is increased by about 2-fold in the in situ liver and adipose tissue of diabetic and fasted rats (Jefferson et al., 1968; Selawry et al., 1973; Pilkis et al., 1974). Some workers suggest that this increase in concentration of cyclic AMP results from a decrease in the total activity of phosphodiesterase (Schultz et al., 1966; but see Müller-Oerlinghausen et al., 1968) or the activity of the low K_m enzyme (Loten & Sneyd, 1970; Pilkis et al., 1974; but see Hepp, 1972). These findings differ from those reported in the present work for the heart. Moreover, the activity of phosphodiesterase can be rapidly (15 to 45 minutes) restored in adipose tissue by the in vitro addition of insulin and in liver by the in vivo injection of the hormone (Loten & Sneyd, 1970; Schultz et al., 1966). In contrast, it is some hours before the in vivo injection of insulin reduces lipolysis in the heart of the diabetic rat.

It has been reported that the hormone-stimulated activity of adenyl cyclase is increased in livers from diabetic rats (Bitenskey et al., 1972; Hepp et al., 1972) but there is no consensus of opinion on this point (see Pilkis et al., 1974). Finally, the activities of protein kinase in the livers of diabetic and fed rats were identical both in the presence and

absence of cyclic AMP (Pilis et al., 1974). It may be concluded that the long term effects of insulin deprivation upon metabolism in the alloxan-diabetic rat have not been explained in terms of changes in the activities or properties of adenyl cyclase or protein kinase. The elevation of the activity of low K_m phosphodiesterase may explain some of the long term effects of insulin in the liver and adipose tissue, but not the heart. It seems probable that the long term effects of insulin upon lipolysis in the heart will require the study of enzymes other than triglyceride lipase, adenyl cyclase, cyclic AMP phosphodiesterase and cyclic AMP-dependent protein kinase.

Chapter 6

SOME RELATIONSHIPS BETWEEN THE METABOLISM OF ADENOSINE AND CYCLIC AMP

The metabolism of adenosine and cyclic AMP were studied together for this thesis because it was known that exogenous adenosine affects the metabolism of cyclic AMP in some tissues, and because the roles and metabolism of the two molecules display some interesting similarities. These relationships between adenosine and cyclic AMP will be discussed further in the present Chapter.

A. THE EFFECT OF ADENOSINE ON THE METABOLISM OF CYCLIC AMP

A.1 THE ROLE OF ADENOSINE

Many hormones which have a rapid effect upon their target tissues have been shown to act by stimulating the activity of adenylyl cyclase. The role of adenosine is therefore of some relevance in a discussion of its mechanism of action. In the present thesis the role of adenosine in vertebrate tissues has been envisaged as that of a hormone, which acts primarily upon smooth muscle cells in the blood vessels of the organ where it is produced, in order to increase blood flow. Since adenosine is a primitive molecule (see McIlwain, 1972), it is unlikely that its role in the animal kingdom is restricted to a specialized tissue in the highest phylum of animals. It has been suggested that adenosine may play some role in communication between organs (Chapter 4, Section D3). In addition, it may, like other primitive, small and diffusible molecules (e.g. glycine, glutamate, 5-hydroxytryptamine, γ -aminobutyric acid) play a role in neurotransmission - it would be similar to adrenaline in being both a hormone and a neurohormone. The only persuasive evidence that adenosine has a

special function in nervous tissue is that it produces large changes in the concentration of cyclic AMP in brain slices and it mediates the effect of electrical stimulation in raising the concentration of cyclic AMP (see Huang et al., 1973). The present results neither support nor deny such a role. However, they show that the mechanisms which exist for the reuptake or destruction of adenosine are similar to those which exist for neurotransmitters such as acetylcholine or noradrenaline (see, Iversen, 1970). Thus the uptake system is saturable, it has a low (micromolar) K_m , it is energy-dependent and the present results indicate that it may be stimulated by Na^+ . Moreover, the present results suggest mechanisms, mediated by Ca^{2+} , Na^+ and K^+ , whereby adenosine may be released from a nerve cell following its depolarization. It seems probable that a role for adenosine in neurotransmission will be established in the near future.

Further roles of adenosine in animal tissues may remain to be discovered. The very high activities of adenosine kinase which were measured in the present study in some insect flight muscles, particularly those capable of very high rates of energy utilization, indicate that adenosine plays an important role in this tissue. Since adenosine can cross the cell membrane and all the roles which have been attributed to it require it to leave the cell, it seems probable that its function in insect flight muscle is also intercellular. It is possible to speculate that it plays some role in the transfer of electrical excitation throughout the muscle and/or in the control of energy supply to the muscle.

A.2 THE MECHANISM OF ACTION OF ADENOSINE

(a) EFFECTS OF ADENOSINE ON INTACT TISSUES

The question arises as to the mechanism whereby adenosine interacts with the cell in order to fulfil its hormonal or neurotransmitter role. In the brain adenosine interacts with an extracellular receptor - possibly

part of the adenylyl cyclase complex - and thereby stimulates the production of cyclic AMP (see Chapter 1, Section B5b(iii)). It was suggested (Chapter 1, Section B5b(iii)) that adenosine stimulates the production of cyclic AMP in other target tissues, such as the smooth muscle of some blood vessels. In the case of the heart, this suggestion is supported by the observation that theophylline, which blocks the effect of adenosine on the concentration of cyclic AMP in the brain, also blocks adenosine-induced coronary vasodilation (Sattin & Rall, 1970; Schaumann *et al.*, 1970). However, there is little evidence that adenosine raises the concentration of cyclic AMP in tissues other than the brain (see Rall, 1971; but see Andersson, 1972). One reason why attempts to detect such effects of adenosine have been unsuccessful may be that in the tissues which have been studied only a small proportion of the cells are responsive to adenosine. If the main role of adenosine in the tissue is to regulate blood flow, the responsive cells may be only those of the smooth muscle of the arterioles which regulate blood supply. Clearly, even a doubling of the concentration of cyclic AMP in these cells would not be detected against the high background provided by the bulk of the tissue.

(b) EFFECTS OF ADENOSINE IN TISSUE HOMOGENATES

A further difficulty in the elucidation of the mechanism of action of adenosine may be mentioned. It is that, despite the large effect which adenosine has on the concentration of cyclic AMP in slices of nervous tissue, it has not proved possible to reproduce this effect in tissue homogenates (see Sattin & Rall, 1970)*. In the present study, the effect of adenosine on

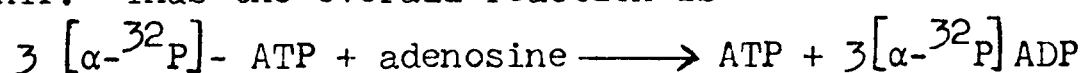
* There is need for caution in interpreting the data of some reports in which it is claimed that the activity of adenylyl cyclase is inhibited by adenosine (Morwaki & Foa, 1970; Weinryb & Michel, 1974). In these experiments [α - ^{32}P]-ATP was used as the substrate in a radiochemical assay for adenylyl cyclase. The incorporation of label into cyclic AMP was reduced by adenosine only when the nucleoside was present at high concentrations. (50% inhibition was obtained with approximately 1 and 0.15 mM adenosine in the case of rat liver and guinea-pig lung, respectively.) Firstly, these concentrations of adenosine are unphysiological, so that their effect may not be significant *in vivo* (but note the calculations of Pull & McIlwain (1972c), which assume some compartmentation of

the activity of adenyl cyclase was studied in a small number of experiments. Mild homogenization procedures (a Teflon pestle, isotonic media) were employed in an attempt to maintain the integrity of the putative adenosine-receptor—adenyl cyclase-catalytic unit complex. Homogenates of mouse brain cortex were studied, together with homogenates of mouse colon, since this is a tissue which contains a large proportion of smooth muscle.

The results of these experiments are presented in Table 6.1. In two experiments the activity of adenyl cyclase from mouse brain cortex was stimulated by 100 μ M adenosine, provided the tissue was extracted and assayed in an isotonic medium. However, the effect of adenosine was neither large (it was less than 2-fold) nor consistently reproduced. There was no effect of adenosine on the activity of adenyl cyclase from mouse colon.

The stimulation of the activity of adenyl cyclase by adenosine is easily destroyed by the disruption of the cell and precisely controlled conditions will be required in order to obtain an effect in tissue homogenates. Recently Chasin et al. (1974) have reported that the activity of adenyl cyclase is stimulated 2-fold by adenosine (100 μ M) in a cell-free preparation from the hippocampus of the guinea-pig. The preparation consisted of vesicles prepared in a Krebs-Ringer buffer. The same buffer was used for the assay of adenyl cyclase - Tris buffer prevented any effect

adenosine). Secondly, adenosine may have reduced the incorporation of label from $[\alpha\text{-}^{32}\text{P}]\text{-ATP}$ into $[\text{}^{32}\text{P}]\text{-cyclic AMP}$, without inhibiting adenyl cyclase, by the following mechanism. The γ -phosphate group, which is not labelled, of $[\alpha\text{-}^{32}\text{P}]\text{-ATP}$ is transferred to adenosine in the presence of adenosine kinase, which contaminates the adenyl cyclase preparations. (It has been shown in the present study - Table 4.8 - that both of the tissues studied - liver and lung - contain high activities of adenosine kinase.) The unlabelled AMP reacts with $[\alpha\text{-}^{32}\text{P}]\text{-ATP}$ through the myokinase and nucleoside diphosphate reactions to form unlabelled ATP. Thus the overall reaction is



Consequently, the concentration and specific activity of ATP is reduced and adenosine reduces the incorporation of label from $[\alpha\text{-}^{32}\text{P}]\text{-ATP}$ into cyclic AMP.

TABLE 6.1 The effect of adenosine on the activity of adenyl cyclase in homogenates of mouse tissues

Tissues were homogenized and adenyl cyclase was assayed using hypotonic or isotonic media, as described in Chapter 3, Section A.1. For the assay of adenyl cyclase in the mouse colon KCl (125 mM) replaced sucrose. Adenosine was included in the assays at the concentrations indicated. Each assay was performed in duplicate or triplicate.

EXPERIMENT	MOUSE TISSUE	TONICITY OF EXTRACTION AND ASSAY MEDIA	ACTIVITY OF ADENYL CYCLASE (nmol/min/g wet wt.)			
			CONCENTRATION OF ADENOSINE (μ M)..... <u>0</u>	<u>10</u>	<u>100</u>	<u>1000</u>
1.	Brain cortex	Hypotonic	27.5	-	26.4	-
		Isotonic	12.4	-	22.9	-
2.	Brain cortex	Isotonic	14.3	17.5	21.8	6.6
3.	Brain cortex	Isotonic	16.2	-	10.5(14.8*)	-
4.	Colon	Isotonic	4.58	4.70	4.75	-

* Theophylline (1 mM) was included in this assay

of adenosine. In one respect the preparation behaves like whole cells and not tissue homogenates: its activity of adenylyl cyclase is unaffected by F^- . (F^- was effective in the preparations used for the present study - see Chapter 3, Section B1). Nevertheless, the preparation of Chasin et al. may prove to be useful in studies of the mechanism of action of adenosine on the brain and other tissues.

B. A COMPARISON OF THE METABOLISM OF CYCLIC AMP AND ADENOSINE

Cyclic AMP and adenosine are both primitive molecules which act as messengers in mechanisms which regulate metabolic processes. A comparison of their metabolism may point to some general principles concerning the way in which the cell controls the concentrations of messenger molecules.

B.1 SIMILARITIES BETWEEN THE METABOLISM AND ROLES OF CYCLIC AMP AND ADENOSINE

There are a number of similarities between the metabolism of cyclic AMP and adenosine: both molecules are usually present in tissues at micromolar concentrations; they are each formed from an adenine nucleotide by an enzyme which has a K_m value of about 200 μM and they can be further metabolized by one of two enzymes. In each case one of the latter enzymes has a K_m value of less than 10 μM and it is only saturated with substrate when the concentration of substrate is high; the other has a K_m value of about 50 μM , it usually has a higher maximal activity than the enzyme which has the lower K_m value, and it is rarely, if ever, saturated with substrate. Probably, all the reactions of cyclic AMP and adenosine metabolism are far from equilibrium in vivo. These similarities (and some differences) are illustrated in Figure 6.1.

In the course of separate discussions of the metabolism of cyclic AMP and adenosine, explanations have been proposed for most of the features listed

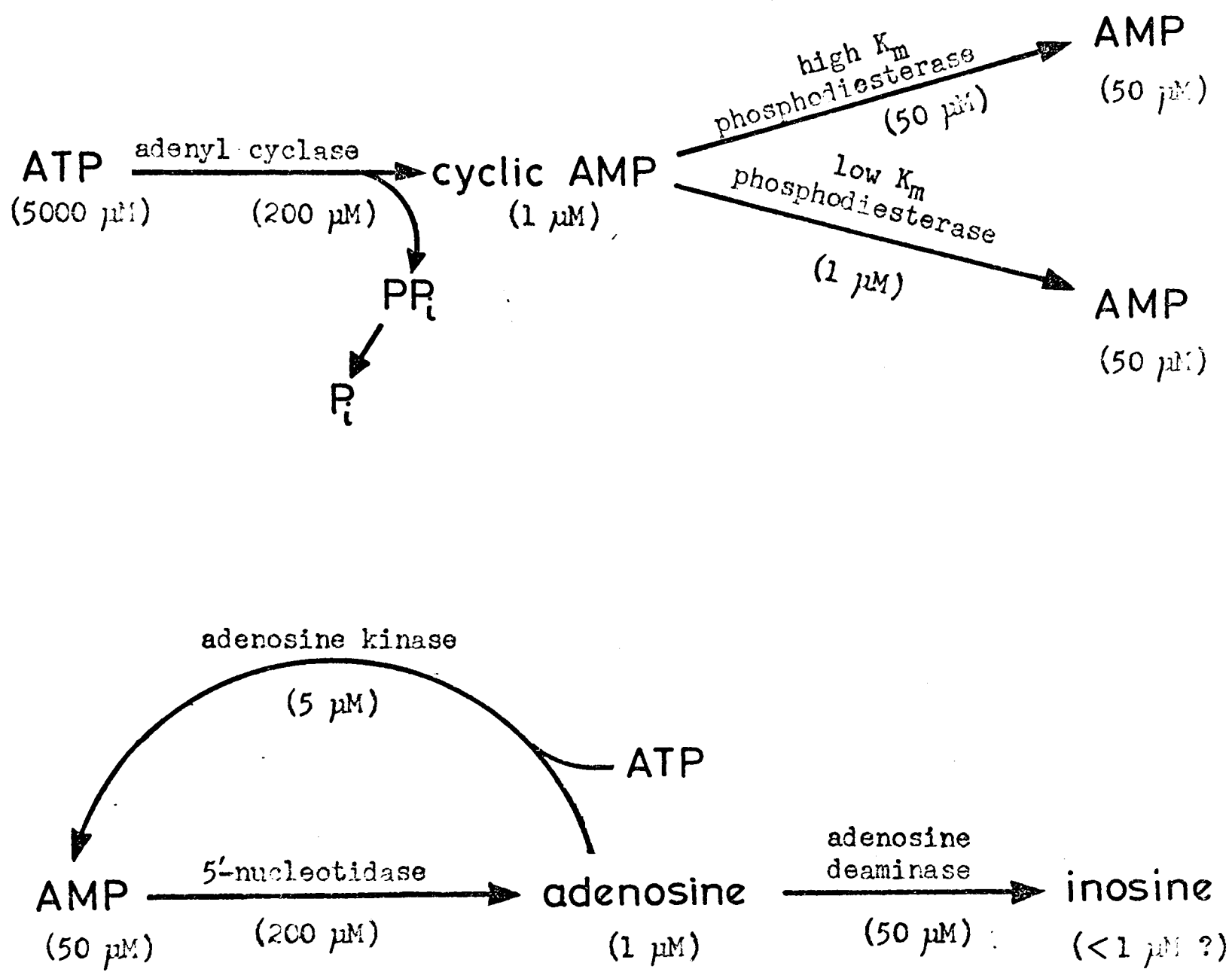


FIG 6.1 The metabolism of cyclic AMP and adenosine.

The figures below the names of the enzymes are typical K_m values, (see Tables 3.6 and 4.9) The figures below the metabolites are typical tissue concentrations.

above. Many of these explanations may be similar for both cyclic AMP and adenosine metabolism. Thus the displacement of the reactions from equilibrium means that a change in the activity of one of the enzymes results in at least a proportionate change in the concentration of the messenger molecule (see Chapter 1, Section B2b); the low concentrations of both metabolites permits their rapid turnover, with little utilization of energy; the high K_m metabolite-utilizing enzyme prevents uncontrolled accumulation of the metabolite, when the rate at which it is synthesized is high; a low K_m enzyme is synthesized by the cell because a small number of molecules of this enzyme have the same activity as a large number of molecules of the high K_m enzyme; the low K_m enzyme may be involved in a mechanism which allows the concentration of the metabolite to be sensitively controlled by changes in the rate of synthesis of the metabolite (see Chapter 3, Section C3c(iii), and Chapter 4, Section D1(c)).

It may be proposed that these similarities between the metabolism of cyclic AMP and adenosine arise because the molecules play very similar roles in the organism: they are both messenger molecules and they are not metabolites on the main pathways of intermediary metabolism. There are also similarities between the mechanism of action of the molecules which may help to explain their similar metabolism. Thus both molecules initiate a chain of events before their ultimate effects upon the cell are realized. Cyclic AMP interacts with protein kinase and adenosine interacts with an extracellular receptor - possibly a part of the adenylyl cyclase complex. The mediation of these interactions permits small changes in the concentration of the messenger to be amplified. In addition, since the messengers do not exert their effects directly, they may be present in the cell at a lower concentration than those of the molecules whose function they ultimately control, and yet still activate these molecules maximally and rapidly (for

a fuller discussion of this point see Newsholme & Crabtree, 1973). As was pointed out above, a low concentration of the messenger molecule is required if it is to be turned over rapidly without wastage of energy. Rapid turnover of the molecules permits a rapid modulation of their concentrations (see Chapter 1, Section A3a(ii)). It may be concluded that the metabolism of cyclic AMP and of adenosine is such that their concentrations can be modulated rapidly between controlled limits. The cell uses similar mechanisms, in the case of both molecules, to achieve this end.

B.2 DIFFERENCES BETWEEN THE METABOLISM AND ROLES OF CYCLIC AMP AND ADENOSINE

Nevertheless there are some differences between the metabolism of cyclic AMP and adenosine which deserve comment (Figure 6.1). The main differences lie in the concentrations of the precursors and products of the messenger molecules, the size of the free energy changes of the reactions involved and the nature of the mechanisms which control the concentrations of the messenger molecules.

A number of these differences may be accounted for if a fundamental difference between the roles of cyclic AMP and adenosine is noted: cyclic AMP transmits signals arriving (in the form of hormones) outside of the cell to the inside (but there are exceptions to this generalization - see Chapter 3, Section C3); adenosine transmits signals arising inside the cell to other cells - i.e. adenosine is a first and cyclic AMP a second messenger.

(a) THE SIGNIFICANCE OF THE STRUCTURES OF CYCLIC AMP AND ADENOSINE

The role of adenosine demands that it should be able to pass out of the cell, whereas the role of cyclic AMP demands that it is largely confined to the cell. This difference is reflected in the structures of the two molecules. Thus the presence of the phosphate group in cyclic AMP may prevent its passage through the cell membrane. In support of this argument,

it may be noted that monosaccharides enter the cell in the unphosphorylated form but they are immediately phosphorylated and remain in the cell until they are degraded to pyruvate and lactate, which lack a phosphate moiety.

(b) THE CONTROL OF MESSENGER FORMATION BY PRECURSOR CONCENTRATION

Since the role of cyclic AMP is to transmit signals arriving outside the cell to the inside, it is undesirable that its rate of formation should be modulated by changes arising within the cell alone. (Nevertheless, it is possible that the response of the cell to a hormone may be modulated by endogenous factors - see Perkins, 1973). Thus adenylyl cyclase is always saturated by ATP, so that the rate of formation of cyclic AMP is not affected by the concentration of ATP - i.e. adenylyl cyclase generates the flux through the metabolic pathway which includes cyclic AMP (see Newsholme & Crabtree, 1973).

On the other hand the rate of formation of adenosine is affected by changes arising within the cell, since it is the function of adenosine to inform other cells of these changes. Thus 5'-nucleotidase is not saturated by AMP, so that the concentration of AMP is one factor which controls the rate of formation of adenosine - i.e. 5'-nucleotidase catalyzes a substrate-dependent, non-equilibrium reaction (see Newsholme & Crabtree, 1973). (The flux-generating reactions of this pathway are the ATPase reactions of the cell, which are generally saturated with ATP so that their activities are not regulated by the concentration of the nucleotide.)

(c) THE CHANGES IN FREE ENERGY DURING THE METABOLISM OF CYCLIC AMP AND ADENOSINE

The difference in the standard free energy of the precursor of cyclic AMP, ATP, and the product of cyclic AMP hydrolysis, AMP, is 14 kcal/mol, i.e. two high-energy phosphate bonds. The difference in the standard free energy of the precursor of adenosine, AMP, and the products of adenosine

metabolism is 7 kcal/mol or 9 kcal/mol for the products AMP or inosine, respectively. These free energy changes are probably sufficient for all of the reactions concerned in the metabolism of the messenger molecules to be maintained far from equilibrium. A large change in standard free energy is probably required in the phosphodiesterase reaction in order to maintain it far from equilibrium, because the concentration of AMP in the cell may be 100-fold or more greater than the concentration of cyclic AMP. This explanation makes redundant the proposal of Greengard et al. (1969a,b) that the high-energy bond in cyclic AMP is necessary for the interaction of the molecule with protein kinase through the formation of a covalent bond.

(d) FURTHER DIFFERENCES BETWEEN THE CONTROL OF THE CONCENTRATIONS OF ADENOSINE AND CYCLIC AMP

One difference between the patterns of metabolism of adenosine and cyclic AMP is that adenosine can be directly converted back to its precursor, whereas cyclic AMP cannot. Unless only one of the enzymes, 5'-nucleotidase or adenosine kinase, is active at any one time, which seems unlikely, there must be a substrate cycle between AMP and adenosine. However, there is no such cycle between ATP and cyclic AMP.

The rephosphorylation of adenosine may be a mechanism which conserves the purine base in a tissue: any adenosine which is not rephosphorylated may be lost from the tissue, as may the non-phosphorylated products of adenosine metabolism, inosine and hypoxanthine. It was pointed out in Chapter 4, Section D4(b) that the conservation of the purine base must be sacrificed when the cell is under stress in order that the ATP/ADP concentration ratio may be maintained. Therefore it is proposed that adenosine kinase is active during resting metabolism but its activity is reduced during stress. (This is not unlike the situation in the substrate cycle between F6P and FDP

during energy stress. Thus the activity of FDPase is reduced by AMP when the glycolytic flux is high - see Newsholme & Crabtree, 1973). Since cyclic AMP remains within the cell, this explanation of the adenosine-AMP substrate cycle does not require that there is a similar cycle in cyclic AMP metabolism.

It has been suggested that the kinetics of adenosine kinase and adenosine deaminase may be such that a large but controlled change in the concentration of adenosine can be produced by a small change in the activities of 5'-nucleotidase and/or adenosine kinase (see Chapter 4, Section D1b(iv)). This suggestion is difficult to test. It was similarly proposed that the kinetics of high and low K_m phosphodiesterases might be such that a large change in the concentration of cyclic AMP can be produced by a small change in the activity of adenyl cyclase. However, by applying the present data to a model of the turnover of cyclic AMP, it was shown that a mechanism of this kind demands that there is some compartmentation of cyclic AMP (Chapter 3, Section C4c(iii)). A similar model cannot be applied to the data concerning the metabolism of adenosine, since the V_{max} values for 5'-nucleotidase, unlike those for adenyl cyclase, do not indicate the activity of the enzyme in vivo (see Chapter 4, Section D(a)). It has not been shown that large changes in the tissue concentration of adenosine occur in vivo (see Tables 1.1 and 1.2 for some in vivo and in vitro data). Nevertheless, there is some evidence that large changes in the concentration of adenosine occur in selected cells or parts of cells. Thus histochemical studies indicate that 5'-nucleotidase is unevenly distributed in some tissues. Moreover, large changes in the concentration of adenosine in cells which are close to the fluid perfusing a tissue may lead to the large changes (as much as 100-fold) in the rate of output of adenosine, which have been measured in vitro (see Rubio et al., 1973 for a full discussion; also Chapter 1,

Section B3). There are a number of mechanisms, acting through both 5'-nucleotidase and adenosine kinase, which may regulate the concentration of adenosine and it is possible that at times they act in concert to produce a large effect (see Figure 4.13).

(e) WHY SHOULD THE CONCENTRATION OF ADENOSINE VARY MORE THAN THAT OF CYCLIC AMP?

It would perhaps be surprising if some cells produce larger changes in their concentration of adenosine than cyclic AMP. Thus if adenosine produces its effects by activating adenyl cyclase, the change in the concentration of adenosine may be amplified through protein kinase, in the same way that changes in the concentration of cyclic AMP are amplified. However, there is a difference between the two systems: the maximum distance that cyclic AMP need diffuse is no further than from the cell membrane to the centre of the cell, whereas adenosine must diffuse out of the cell and perhaps into the circulation. Since the rate of diffusion is proportional to the concentration gradient, the concentration gradient for adenosine must not only be produced rapidly but it must also be large, if the production of adenosine by the cell is to influence rapidly the metabolism within other cells. In this respect adenosine may be similar to the hormones adrenaline and insulin, whose rates of secretion and concentrations in the blood may vary rapidly over a 10-fold range or more (Holzbauer & Vogt, 1954; Goldfien et al., 1961; Vendsalu, 1960; Cahill et al., 1966).

Appendix A

LIQUID SCINTILLATION COUNTING TECHNIQUES

All measurements of radioactivity were carried out in a Beckman LS-200 (or LS230 or LS233) liquid scintillation spectrometer, using low-potassium glass vials. The standard scintillation fluid used for counting all non-aqueous samples contained 3g 2,5-diphenyloxazole (PPO) and 0.1g 1,4-bis-(5-phenyloxazolyl-2)-benzene (POPOP) per litre of sulphur-free toluene. Sections carrying labelled compounds which were cut from thin-layer chromatograms were placed face-upwards in the scintillation vials and covered with 5 ml of this fluid. Radioactivity in aqueous samples obtained from the phosphodiesterase assay (Chapter 2, Section A.3) was measured in one of three ways.

(i) A sample (0.5 ml) was measured for radioactivity in 10 ml of toluene and 2-ethoxyethanol (625:375) containing 4 g/l PPO, 0.1 g/l POPOP.

(ii) The whole eluate from the anion exchange chromatography was dried down as described by Leech (1973). The residue was taken up in 0.4 ml of 1N HCl and 10 ml of the scintillation medium described in (i) was added.

(iii) A volume (15 ml) of toluene and Triton X-100 (2:1), containing 3.3 g/l PPO and 0.033 g/l POPOP was added to the whole eluate (4 ml). An emulsion was produced. Radioactivity in the emulsion was not measured for at least 4 hours since the counts recorded per minute decayed during this time.

Appendix B

TECHNIQUES USED TO REDUCE THE BLANK OF THE

ADENYL CYCLASE ASSAY

The activities of adenylyl cyclase in many tissues are very low (i.e. 1 nmol/min/g wet wt.). In the present work it was found that only about 0.05% of the substrate was converted to product during the assay. Therefore it was essential that the assay blank was both low and constant. Practical tips to obtain consistent, low blanks are as follows: the sections of the chromatogram carrying a labelled compound which are cut from each sheet are of a similar size; duplicate controls are included on each chromatography sheet, so that the sections carrying the control spots are of the same size as the sections with which they are compared; the radioactivity carried in the sections is measured in scintillation vials which have been checked for any variations in background.

THE PURIFICATION OF $[^3\text{H}]$ -ATP

The amount of $[^3\text{H}]$ -cyclic AMP in the labelled ATP was variable. Some batches of labelled ATP contained as much as 0.2% cyclic AMP. The blank was reduced to a low level by preincubating the assay mixture with commercial phosphodiesterase. (The adenine nucleotide concentration was only slightly affected. The effect of the procedure is shown in Figure B.1.)

The activity of phosphodiesterase added during the preincubation was no greater than that added to the assay due to the presence of phosphodiesterase in the extract. Since a large "radioactive trap" of unlabelled cyclic AMP was added to the incubation medium immediately before the start of the assay, phosphodiesterase was prevented from interfering with the assay (see Chapter 2, Section B.2). The details of the method are given in Chapter 2, Section A.2.

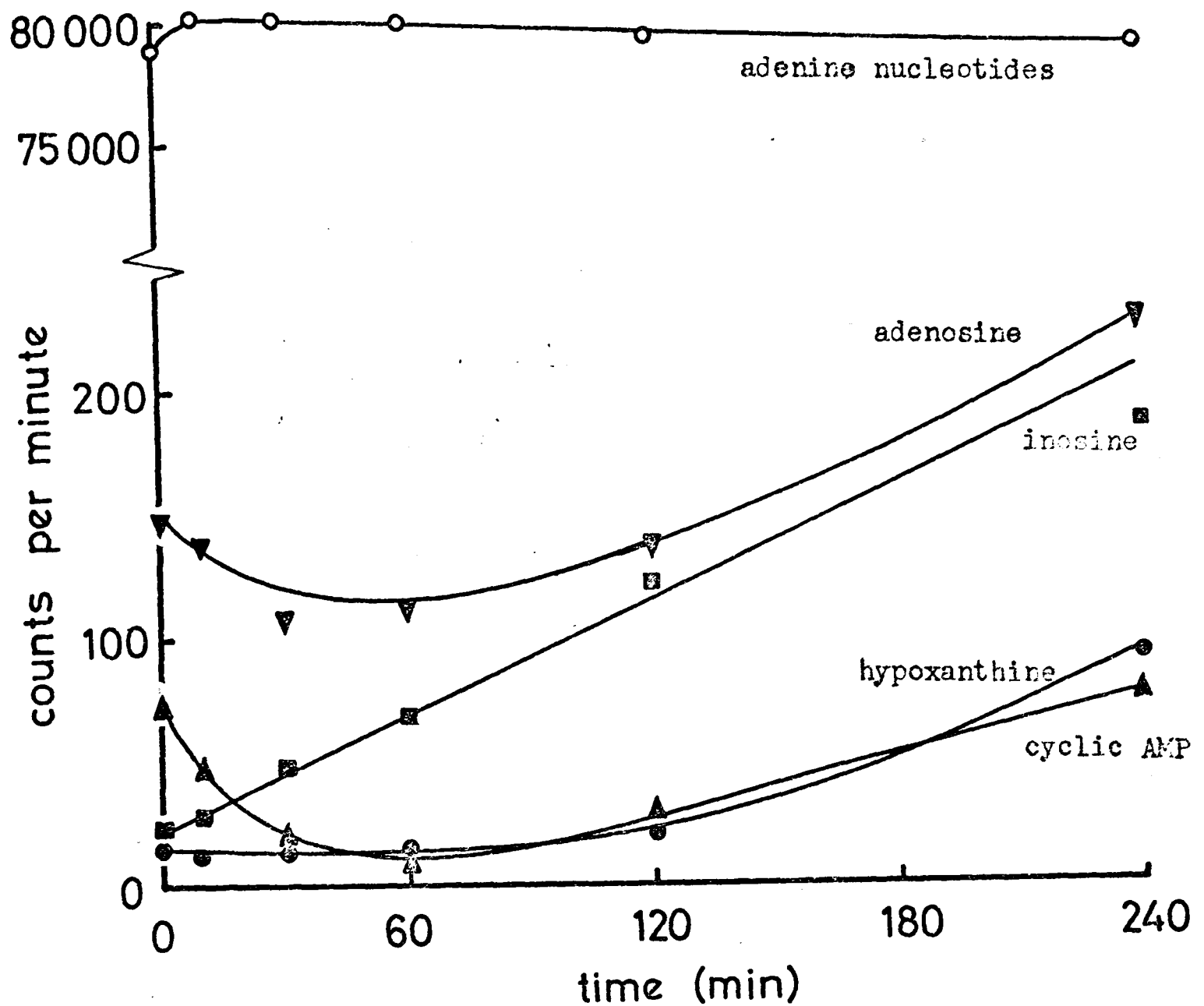


FIG B.1 The removal of ^3H -cyclic AMP from ^3H -ATP using commercial phosphodiesterase.

^3H -ATP was incubated at 30°C in 90 μl of solution containing phosphodiesterase (0.02mg/ml) and other constituents of the adenylyl cyclase assay medium, except for the cyclic AMP (see chapter 2, section A2). Aliquots were added to perchloric acid at the times indicated, as described in Chapter 2, section A7. The radioactivity in adenine nucleotides, cyclic AMP, adenosine, inosine and hypoxanthine was measured as described in Chapter 2, section A6.

Appendix C

ATTEMPTS TO MEASURE THE ACTIVITY OF
GUANYL CYCLASE

The measurement of guanyl cyclase activities would have permitted a study of the regulation of the concentration of cyclic GMP, similar to the study described for cyclic AMP (see Chapter 3). The chromatography system described in Chapter 2, Section A.6, separates cyclic GMP from other guanosine nucleotides. Therefore it was considered that an assay for guanyl cyclase could be devised that was analogous to that described for adenyl cyclase (Chapter 2, Section A.2). The methods which were used in an attempt to measure the activity of guanyl cyclase are summarized below.

Mouse lung, cerebellum or heart (tissues which have a relatively high activity of guanyl cyclase - Hardman & Sutherland, 1969) were extracted using the following medium

Tris chloride, pH 7.5	40 mM
MnCl ₂	4.33 mM
EDTA	1 mM
Dithiothreitol	1 mM
Bovine serum albumin	1 mg/ml

(MnCl₂ was added to the extraction medium immediately before the tissues were extracted in order to avoid oxidation of Mn²⁺.)

The components of the final incubation medium for the guanyl cyclase assay were as follows

Tris chloride, pH 7.5	25 mM
MnCl ₂	3.6 mM
EDTA	0.6 mM
Cyclic GMP	2 mM
Dithiothreitol	1 mM
Phosphoenolpyruvate	10 mM
Pyruvate kinase	0.02 mg/ml (about 4 Units/ml)
KCl	80 mM
[8- ³ H]-GTP, ammonium salt	80 µCi/ml
GTP	0.5 mM
Bovine serum albumin	0.3 mg/ml

MnCl₂ replaced the MgCl₂ used for the assay of adenylyl cyclase because the activity of guanylyl cyclase is greater in the presence of Mn²⁺ (Hardman & Sutherland, 1969). Creatine phosphokinase does not react with GDP and so phosphoenolpyruvate and pyruvate kinase were used to regenerate GTP from GDP. KCl was added in order to activate pyruvate kinase.

The assay was carried out in the same manner as described for adenylyl cyclase (Chapter 2, Section A.2), except that the reaction was terminated with a 20% perchloric acid solution containing approximately 7 mM guanine, guanosine, inosine, hypoxanthine and GTP. Chromatography was carried out as described in Chapter 2, Section A.6, except that longer times were used, so that the cyclic GMP spot was carried well away from the origin. Guanosine appeared on the developed chromatogram in the same position as inosine; guanine appeared in the same position as hypoxanthine but it tended to trail.

Unfortunately, the controls contained a high proportion of the radioactivity (>1.5%) in the cyclic GMP spots and this obscured any incorporation of radioactivity into cyclic GMP. In some experiments the radioactive GTP

was preincubated with commercial phosphodiesterase in an attempt to remove any radioactive cyclic GMP. The method was similar to that described for adenylyl cyclase, except that both Mg^{2+} and Mn^{2+} were used as the divalent cation during different preincubation experiments and various preincubation times were tested. However, no significant removal of radioactivity from the cyclic GMP spots in the controls was detectable.

The method may be useful when purer preparations of labelled GTP are available. (One batch of labelled ATP also contained large quantities of a contaminant which co-chromatographed with cyclic AMP but which could not be removed with phosphodiesterase.) Unfortunately, the expense involved in testing different batches of labelled GTP precluded any further progress.

Appendix DSOURCES OF ANIMALS AND MATERIALSAnimals

Pig roundworms were obtained from a local slaughter house by Dr J. Barrett.

Rat tapeworms were cultured in the Department of Zoology, Oxford, by Dr J. Barrett.

Scallops were obtained from the Marine Biological Laboratory, Plymouth.

Lobsters were obtained from Fisher Bros., 12 Billingsgate Street, London, E.C.3.

Waterbugs were sent from Uganda and maintained under tropical conditions in the Department of Zoology, Oxford.

Locusts, cockroaches, rosechafers, blowflies and mice were obtained from stocks in the Department of Zoology, Oxford.

Honey-bees were provided by Dr A.M. Anderson from a hive kept at the Department of Zoology, Oxford.

Earthworms, bumble-bees and wasps were caught locally.

Dogfish were obtained from the Marine Biological Laboratory, Plymouth.

Trout were obtained from the Trout Hatchery, Bibury, Glos.

Frogs were obtained from Gerrard & Haig Ltd., East Preston, Sussex.

Pigeons, pheasants and domestic fowl were obtained from Park Farm, Northmoor, Oxford.

Rats (Wistar strain) were obtained from Charles River Ltd., Cherry Garden Lane, Ash, Canterbury, Kent and kept in the animal house of the Department of Biochemistry, Oxford.

With the exception of some experiments described in Chapter 5, all animals had access to food and water for at least 24 hr before sacrifice.

Materials for chromatography

Ion exchange resins (AGI-X8 and AGI-X2; 200-400 mesh, chloride form) were supplied by Bio-Rad Laboratories Ltd., St. Albans, Herts.

Silica (Polygram SIL N-HR/UV₂₅₄) and PEI-cellulose (MN-Polygram, CEL 300 PEI) thin-layer sheets were supplied by Camlab Ltd., Cambridge.

Drummond Microcaps were supplied by Shandon Southern Instruments Ltd., Camberley, Surrey.

Chemicals and enzymes

All radiochemicals were obtained from The Radiochemical Centre Ltd., Amersham, Bucks.

Cyclic AMP, cyclic GMP, Tris-maleate, bovine serum albumin (Fraction V) and EGTA were obtained from Sigma London Chemical Co. Ltd., Kingston-upon-Thames, Surrey.

All nucleotides (except cyclic nucleotides), nucleosides, purines, PEP, creatine phosphate, Tris and enzymes were obtained from the Boehringer Corporation (London) Ltd., London, W5 2TZ.

PPO and POPOP were obtained from Koch-Light Laboratories, Ltd., Colnbrook, Bucks.

Triton X-100, alloxan, papaverine and dithiothreitol were obtained from BDH Chemicals, Ltd., Poole, Dorset.

All other inorganic chemicals and EDTA were supplied by Fisons Scientific Apparatus, Loughborough, Leics., and were of the highest grade of purity available.

Dextrostix reagent strips were obtained from Ames Company, Miles Laboratories Ltd., Stoke Poges, Slough, Bucks.

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