THE ROLE OF ADENINE NUCLEOTIDES AND THEIR DERIVATIVES IN THE CONTROL OF LIVER METABOLISM

A thesis submitted for the degree of

Master of Philosophy

in the

University of London

by

Ravi Janak Sharma

Department of Biochemistry

Imperial College of Science and Technology

London, SW7 2AZ, UK.

July, 1979

ABSTRACT

Adenine nucleotides and their degradation products are important in the control of many cell functions. The production and turnover of these components has been investigated in liver, in relation to the responses of carbohydrate metabolism to variables such as anoxia and hormones.

A technique for prelabelling the intracellular adenine nucleotide pool, in the rat liver, using radioactive precursors and subsequent separation of the above as well as the relevant nucleosides and bases, was developed. The nucleotide pool was labelled using ^{14}C-(U)-adenine which is converted to AMP by adenine p-ribosyl-transferase. The adenine nucleotides and their products were purified from PCA extracts using activated charcoal and separated using two systems of thin layer chromatography.

During ischaemia (cessation of blood flow) adenine nucleotide degradation was increased 10 fold, involving intracellular increases of 10-14 fold in XMP, inosine, xanthosine and xanthine and 3-5 fold in IMP, adenosine and hypoxanthine. During anoxia (oxygen deprivation accompanied by normal flow) the increase in AMP degradation was considerably less than that observed during ischaemia. In contrast to the effects observed in ischaemia, hepatic adenosine concentration did not increase in anoxia while IMP and inosine concentrations were elevated. These changes indicate that the increase in AMP degradation observed during anoxia is primarily due to increased activity of AMP deaminase, while 5'nucleotidase is also stimulated during ischaemia.

The influence of ischaemia on glycogen metabolism was also studied. The above changes in the nucleotides ATP, ADP and AMP were confirmed by direct chemical analysis. The increased glycogen breakdown was accompanied by an increase in the glycogen phosphorylase activity and a decrease in the glycogen synthetase activity. Of the above described changes in tissue purines, only the rise in AMP can (so far) be implicated in the increase in phosphorylase activity.

The intracellular levels of ^{14}C-labelled purine pool constituents and their selective release into the perfusate in response to various hormones was studied, to determine the role of purine nucleotides in hormone effects. The stimulatory effect of glucagon on adenyl cyclase was confired by increased intracellular and extracellular concentration of ^{14}C-cyclic AMP. Other effects of glucagon included increased intracellular concentration of adenosine and inosine (inosine increasedmore than adenosine) whilst the extracellular concentration of inosine was greatly reduced. The effect of insulin on hepatic purine metabolism was small and consisted of a larger increase in the intracellular concentration of adenosine than inosine, while the concentration of purine compounds in the perfusion medium was lowered. Vasopressin and angiotensin II stimulated adenine nucleotide degradation by 3-4 fold. Vasopressin caused an increase in the intracellular concentration of adenosine and inosine while the perfusate concentration of AMP, adenosine, xanthosine and hypoxanthine were also increased. The effects of angiotensin II were similar to those of vasopressin except that an increase in tissue concentration of IMP was also observed. These changes are discussed with reference to the activities of the key enzymes of purine

metabolism.

Taken together, these data suggest that during regulatory responses in liver to anoxia or hormones, a wide range of changes in purine metabolism are set in train. These are likely to be relevant in enzymic control processes, although many of the detailed mechanisms still have to be elucidated.

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation for guidance and encouragement afforded by the late Dr Douglas Hems throughout his supervision of this study.

I would also like to express my sincere thanks to Dr P Whitton and Dr M J Clemens for many stimulating discussions and their invaluable assistance. I am also indebted to Loreta Rodrigues for her generous help in the assay of glycogen metabolising enzymes.

My thanks are due to all my friends and colleagues at Imperial College. I would also like to thank Dr Chris Gove and other past and present members of the Laboratory who have made the past years so enjoyable.

My manuscript has been speedily and efficiently translated into polished typescript by Mrs H Barrett.

Financial support from the Medical Research Council in the form of a research studentship is greatly appreciated.

Finally I would like to express my gratitude to my parents for their support and to my wife, Neelam for her constant support and encouragement.

CONTENTS

	Page
Abstract	2
Acknowledgements	5
Detailed contents of chapters	7
Abbreviations and Enzyme Nomenclature	11
List of Tables	13
List of Figures	15
Chapter 1: Introduction	18
Chapter 2: Animals, materials and methods	52
Chapter 3: Results	96
Chapter 4: Discussion	182
References	233

CHAPTER ONE

Page

INTRODUCTION

1.1 Pathways of purine metabolism 19

1.1.1 Purine nucleotide biosynthesis 19

1.1.2 Purine nucleotide metabolism 22

1.1.3 Enzymes of purine metabolism 26

1.1.4 Formation and fate of hepatic purines 33

1.1.5 Purine nucleotide cycle 36

1.2 Hepatic glycogen and purine nucleotide metabolism during ischaemia and anoxia 39

1.2.1 Glycogen metabolism during ischaemia and anoxia 39

1.2.2 Purine nucleotide metabolism during ischaemia and anoxia 42

1.3 Short term hormonal control of hepatic carbohydrate metabolism and role of purine nucleotides 45

1.3.1 Rapid effect of hormones on hepatic carbohydrate metabolism 45

1.3.2 Cyclic purine nucleotides and Ca^{2+} as second messenger 46

1.3.3 Other purines as mediators of hormonal effects 48

1.4 Scope and aims of the present study 50

CHAPTER TWO

ANIMALS, MATERIALS AND METHODS

2.1 Animals 53

2.2 Sources of materials 54

2.3 The technique of liver slicing 56

		Page
2.4	The technique of liver perfusion	58
	2.4.1 Perfusion apparatus	58
	2.4.2 Operative procedure	60
	2.4.3 Perfusion medium	64
	2.4.4 Non-recirculating perfusion	65
	2.4.5 Sample preparation	67
2.5	Analytical methods	69
	2.5.1 Purification of acid soluble extracts & separation of purines	69
	2.5.2 Determination of adenine nucleotides	79
	2.5.3 Lactate determination	83
	2.5.4 Glycogen and glucose determination	85
	2.5.5 Assay of glycogen metabolizing enzymes	87
	2.5.6 Oxygen measurement	91
2.6	Determination of carbon-14 radioactivity by liquid scintillation counting	92

CHAPTER THREE

RESULTS

3.1	The effect of anoxia and ischaemia on hepatic glycogen and adenine nucleotide metabolism	99
	3.1.1 Carbohydrate metabolism in aerobic perfused livers	99
	3.1.2 Effect of graded ischaemia on hepatic carbohydrate metabolism	100
	3.1.3 Short term effects of total ischaemia on enzymes of hepatic glycogen metabolism	108
	3.1.4 Effect of total ischaemia on hepatic adenine nucleotide concentrations	113

		Page
3.2	Validation of labelling of the intracellular adenine nucleotide pool using ^{14}C-adenine	119
	3.2.1 Validation of technique in isolated liver slices	120
	3.2.2 ^{14}C-adenine uptake by the red blood cells of the medium and by the perfused liver.	125
3.3	Adenine nucleotide metabolism in the perfused liver prelabelled with ^{14}C-adenine	134
	3.3.1 ^{14}C-adenine uptake and release by the perfused liver under the modified experimental conditions	134
	3.3.2 ^{14}C-adenine metabolism in the perfused liver	138
	3.3.3 Release of ^{14}C-labelled material into perfusate under normal aerobic conditions	143
3.4	Effect of oxygen lack on ^{14}C-adenine nucleotide metabolism in perfused liver prelabelled with ^{14}C-adenine	146
	3.4.1 Effect of total ischaemia on hepatic ^{14}C-labelled adenine nucleotide metabolism	146
	3.4.2 Effect of anoxia on ^{14}C-labelled adenine nucleotide metabolism in perfused liver	150
3.5	Effect of hormones on adenine nucleotide metabolism in liver prelabelled with ^{14}C-adenine	160
	3.5.1 Effect of insulin on ^{14}C-labelled adenine nucleotide metabolism in the perfused liver	163
	3.5.2 Effect of glucagon on hepatic ^{14}C-labelled adenine nucleotide metabolism	167

		Page
3.5.3	Effect of vasopressin on hepatic ^{14}C-labelled adenine nucleotide metabolism	171
3.5.4	Effect of angiotensin II on hepatic ^{14}C-labelled adenine nucleotide metabolism.	175

CHAPTER FOUR

4.1	Mechanism of increase in hepatic glycogenolysis in response to anoxia	184
4.1.1	Control of hepatic glycogen phosphorylase activity during anoxia	184
4.1.2	Changes in adenine nucleotides in anoxic liver	187
4.1.3	Hepatic glycogenolysis during shock	191
4.2	^{14}C-adenine nucleotide metabolism in liver prelabelled with ^{14}C-adenine	196
4.2.1	Prelabelling of the adenine nucleotide pool using ^{14}C-adenine	196
4.2.2	Metabolism of intra-hepatic ^{14}C-adenine nucleotides under normal conditions	198
4.2.3	Release of ^{14}C-labelled purine material by the liver	206
4.2.4	Effect of anoxia on ^{14}C-adenine nucleotide metabolism	213
4.3	Role of purines in rapid hormonal effects on hepatic carbohydrate metabolism	220
4.3.1	Effect of glucagon and insulin on hepatic adenine nucleotide metabolism	221
4.3.2	Effect of vasopressin and angiotensin on hepatic adenine nucleotide metabolism	229

ABBREVIATIONS and ENZYME NOMENCLATURE

ADP - adenosine 5'-diphosphate

AMP - adenosine 5'-monophosphate

ATP - adenosine 5'-triphosphate

Butyl-PBD - 5-(4-bi-phenyl)-2-(4-t-butyl-phenyl)-1-oxa-3-4-diazole

Ci - Curie, 3.7×10^{10} disintegrations/sec

CO_2 - carbon dioxide

CPM - counts per minute

cyclic AMP - adenosine 3':5'-monophosphate

cyclic GMP - guanosine 3':5'-monophosphate

DPM - disintegrations per minute

EDTA - ethylenediamine-tetra-acetic acid

GMT - Greenwich mean time

IMP - inosine 5'-monophosphate

NAD - nicotinamide-adenine dinucleotide

NADH - nicotinamide-adenine dinucleotide, reduced

NADP - nicotinamide-adenine dinucleotide phosphate

NADPH - nicotinamide-adenine dinucleotide phosphate, reduced

O_2 - oxygen

OD - optical density

PCA - perchloric acid

PEI-cellulose - polyethyleneimine cellulose thin layer impregnated with fluorescent indicator on plastic sheets

pO_2 - partial pressure of oxygen

Silica gel GF_{254} - fine silica powder containing fluorescent indicator

TEA - triethanolamine

TLC - thin-layer chromatography

Tris - 2-amino-2 hydroxymethyl-propane-1, 3-diol

UV - ultra violet

ENZYME NOMENCLATURE

The names of the enzymes are those recommended by a IUPAC and IUB Commission, 1972. In a few instances, trivial names are used and the Commission's recommendations are included in brackets.

	EC Number
adenine-p-ribosyl-transferase (AMP: pyrosphosphate phosporibosyl transferase)	2.4.2.7
adenosine deaminase (adenosine aminohydrolase)	3.5.4.4
adenosine kinase (ATP-adenosine-5'-phosphatransferase)	2.7.1.20
adenylate kinase	2.7.4.3
AMP deaminase (AMP aminohydrolyase)	3.5.4.6
glycogen phosphorylase	2.4.1.1
glycogen phosphorylase kinase	2.7.1.38
glycogen phosphorylase phosphatase	3.1.3.17
glycogen synthetase (glycogen synthase)	2.4.1.11
hypoxanthine-p-ribosyl-transferase (IMP: pyrophosphate phosphoribosyl-transferase)	2.4.2.8
IMP dehydrogenase (IMP: NAD oxidoreductase)	1.2.1.14
5'-nucleotidase (5'ribonucleotide-phosphohyrolase)	3.1.3.5
purine nucleoside phosphorylase (purine-nucleoside: orthophosphate ribosyl transferase)	2.4.2.1
xanthine oxidase (xanthine oxidoreductase)	1.2.3.2

LIST OF TABLES

		Page
1.	Recovery of individual purine compounds after adsorption onto charcoal.	72
2.	Levels of adenine nucleotides in aerobic perfused livers.	101
3.	Glycogen phosphorylase a activity in partially ischaemic livers.	107
4.	Influence of total ischaemia on dephosphorylation of endogenous phosphorylase a in liver.	112
5.	Effect of total ischaemia on the activity of glycogen synthetase.	114
6.	Time course of changes in adenine nucleotides in anoxic liver in vivo.	116
7.	Hepatic content of purine nucleoside cyclic monophosphates in total ischaemia.	117
8.	Accumulation and distribution of ^{14}C-adenine radioactivity into other purines in liver slices.	123
9.	Effect of hormones and anoxia on ^{14}C-labelled purine pool intermediates in liver slices.	124
10.	Removal of ^{14}C-adenine by the red blood cells during prelabelling of the liver.	128
11.	Hepatic ^{14}C-content after prelabelling as a function of time.	131
12.	^{14}C-adenine incorporation into the different lobes of the liver.	133
13.	Effect of glucose concentration in the second perfusion medium on hepatic glycogen phosphorylase a activity.	136
14.	Release of ^{14}C-labelled material into the perfusate under normal conditions.	137

		Page
15.	Adenine nucleotide metabolism in the perfused liver prelabelled with ^{14}C-adenine.	139
16.	Adenine nucleotide specific activity after perfusion with labelled adenine.	140
17.	Effect of ischaemia on adenine nucleotide metabolism in liver prelabelled with ^{14}C-adenine.	147
18.	Effect of anoxia on adenine nucleotide metabolism in liver prelabelled with ^{14}C-adenine.	152
19.	Anoxia and adenine nucleotide specific activity in liver prelabelled with ^{14}C-adenine.	153
20.	Release of ^{14}C-labelled material into perfusate under anoxic conditions.	155
21.	Release of ^{14}C-labelled material into perfusate in the presence of hormones.	161
22.	Effect of insulin on adenine nucleotide metabolism in liver prelabelled with ^{14}C-adenine.	164
23.	Effect of glucagon on adenine nucleotide metabolism in liver prelabelled with ^{14}C-adenine.	168
24.	Effect of vasopressin on adenine nucleotide metabolism in liver prelabelled with ^{14}C-adenine.	172
25.	Effect of angiotensin II on adenine nucleotide metabolism in liver prelabelled with ^{14}C-adenine.	176
26.	Effect of mild ischaemia on adenine nucleotide metabolism in liver prelabelled with ^{14}C-adenine.	180
27.	Calculated hepatic concentration of purine pool metabolites.	203

LIST OF FIGURES

		Page
1.	De novo pathway of purine nucleotide synthesis.	20
2.	Salvage pathways of nucleotide synthesis.	20
3.	Pathway of purine nucleotide biosynthesis, interconversion and degradation.	23
4.	The purine nucleotide cycle.	37
5.	Apparatus for perfusion of rat liver.	59
6.	Liver perfusion operation.	62
7.	Modified arrangement of apparatus for rat liver perfusion enabling changeover of perfusion medium.	66
8.	Recovery of ^{14}C-labelled purine material during purification of hepatic extract.	71
9.	Separation of purine compounds using silica gel thin layer chromatography.	74
10.	Separation of purine nucleotides using PEI-cellulose thin layer chromatography.	76
11.	Energy spectrum of ^{14}C and discriminator settings used to calculate counting efficiency.	93
12.	Calibration curve for determination of ^{14}C by liquid scintillation spectrometry.	95
13.	Oxygen consumption of the perfused liver.	102
14.	Relationship between glycogen breakdown and production of glucose and lactate.	103
15.	Glycogen breakdown and glucose and lactate production during partial ischaemia.	105
16.	Adenine nucleotide levels in partially ischaemic livers.	106

		Page
17.	Time course of change in hepatic phosphorylase a activity in *in vivo* anoxic liver.	109
18.	Rapid effect of anoxia on phosphorylase a activity in *in vivo* and perfused liver.	111
19.	Effect of ischaemia (cessation of flow) on adenine nucleotide content of the perfused liver.	115
20.	Liver slice incubation in the presence of ^{14}C-adenine.	121
21.	Removal of ^{14}C-adenine from the perfusate by the liver at two haematocrits.	126
22.	^{14}C-adenine removal by the red blood cells.	129
23.	Time course of release by the liver of ^{14}C-labelled purine pool metabolites under normal conditions.	144
24.	Redistribution of radioactivity from ATP and ADP degradation during total hepatic ischaemia.	149
25.	Time course of release by the liver of ^{14}C-labelled purine pool metabolites during anoxia.	157
26.	Time course of release by the liver of ^{14}C-labelled purine pool metabolites in the presence of insulin.	166
27.	Time course of release by the liver of ^{14}C-labelled purine pool metabolites in the presence of glucagon.	170
28.	Time course of release by the liver of ^{14}C-labelled purine pool metabolites in the presence of vasopressin.	174

Page

29. Time course of release by the liver of ^{14}C-labelled purine pool metabolites in the presence of angiotensin II. 178

30. Metabolism of ^{14}C-adenine precursor in the perfused liver. 199

31. Mechanism of increase in adenine nucleotide metabolism during anoxia. 219

Chapter 1

INTRODUCTION

1.1 Pathways of purine metabolism

1.1.1 Purine nucleotide biosynthesis.

(a) de-novo pathways.

(b) Salvage pathways.

1.1.2 Purine nucleotide metabolism.

1.1.3 Enzymes of purine metabolism.

1.1.4 Formation and fate of hepatic purines.

1.1.5 Purine nucleotide cycle

1.2 Hepatic glycogen and purine nucleotide metabolism during ischaemia and anoxia.

1.2.1 Glycogen metabolism during ischaemia and anoxia

1.2.2 Purine nucleotide metabolism during ischaemia and anoxia.

1.3 Short term hormonal control of hepatic carbohydrate metabolism and role of purine nucleotides.

1.3.1 Rapid effect of hormones on hepatic carbohydrate metabolism

1.3.2 Cyclic purine nucleotides and Ca^{2+} as second messenger

1.3.3 Other purines as mediators of hormonal effects

1.4 Scope and aims of the present study.

1.1 Pathways of purine metabolism.

1.1.1 Purine nucleotide biosynthesis.

Nucleotide synthesis can occur via many alternative pathways. The various pathways involved in nucleotide synthesis, more than one of which may exist in a single cell, were grouped into two main categories by Kornberg (1957). These are (a) de novo pathways in which the cell requires only ammonia, phosphate and simple carbon sources and (b) salvage pathways in which some of the component parts (e.g. purines, pyrimidines, nucleosides) are derived from the external medium or from degradation process within the cell.

(a) de novo pathways.

Steps of the de novo pathways have been worked out by Buchanan (see Hartman & Buchanan, 1959) and various other workers (Figure 1). So far there is no evidence for synthesis de novo of free purine. The actual rate of purine nucleotide biosynthesis de novo in mammalian cells is less than its potential maximum velocity indicating that the total enzyme activity is not an important regulatory factor. The various substrates of the pathway (phosphoribosyl pyrophosphate (PRPP), glutamine, glycine, aspartate and tetrahydrofolate co-enzymes) when added to the incubation medium of cells incubated in vitro stimulate purine nucleotide biosynthesis (Henderson, 1972) indicating that they may be limiting. The availability of PRPP appears to be critical in the regulation of purine nucleotide biosynthesis in vitro since it is the most limiting substrate.

PRPP is formed by the donation of a pyrophosphate group by ATP to carbon one of ribose 5-phosphate through the action of the enzyme ribose-P-pyrophosphokinase, according to the equation:

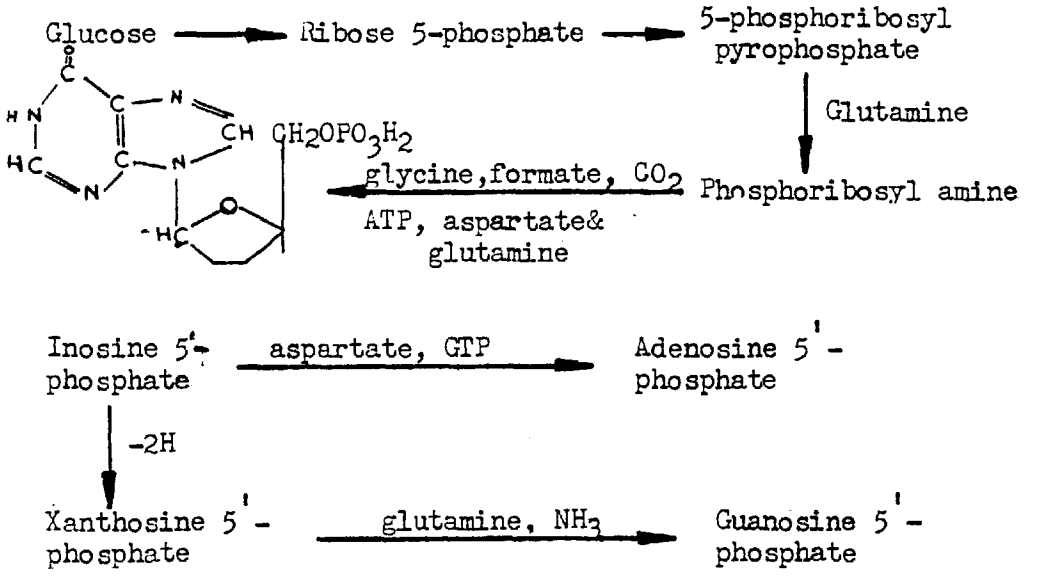


Figure 1 : De novo pathway of purine nucleotide synthesis

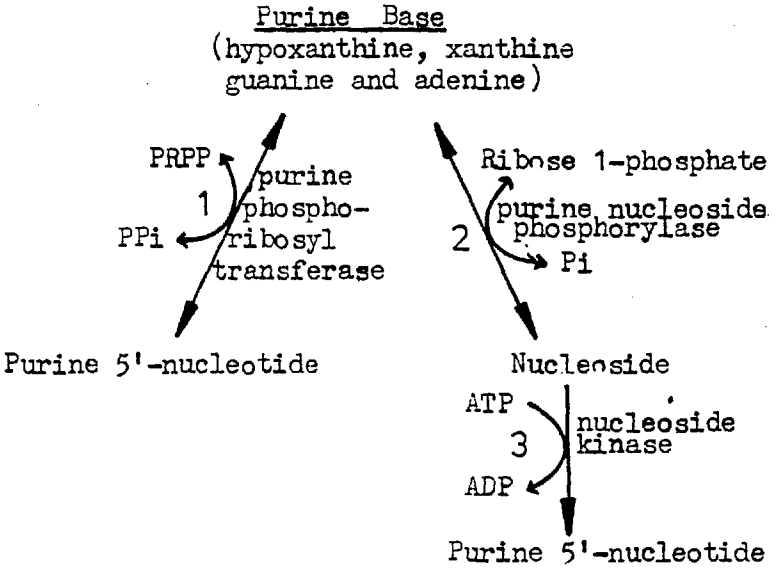


Figure 2 : Salvage pathways of nucleotide synthesis

ATP + R-5-P ⟶ PRPP + adenosine 5-P

PRPP is the activated pentose phosphate which reacts with glutamine to form 5-phosphoribosylamine, the ribotide precursor of purine nucleotide. Ribose 5-phosphate is synthesised from glycolytic intermediates via the pentose phosphate pathway . Factors that effect glycolysis and the pentose phosphate pathways (e.g. glucose concentration, rate of oxidation of NADPH, oxygen partial pressure and phosphate concentration), also affect the synthesis of PRPP (Henderson and Khoo, 1965a; Henderson et al., 1975).

Further control of the de novo purine biosynthesis is by feedback inhibition of the first enzyme of the pathway, p-ribosyl-pp-amidotransferase, by some of the products, mainly AMP, ADP, GMP, GDP and IMP. The enzyme ribose-p-pyrophospho-kinase responsible for synthesis of PRPP is also susceptible to inhibition by GDP and ADP (Murray, 1971).

(b) Salvage pathways.

The term purine salvage has been used to describe the phenomenon whereby nucleotide synthesis occurs via a pathway in which the purine ring of the nucleotide molecule is supplied in whole by either bases or nucleosides (i.e. hypo-xanthine, guanine and adenine or the nucleoside adenosine). The pathways involved are summarized in Figure 2 and will be discussed again in more detail in Section 1.1.2 . Reaction one outlined in Figure 2 is one of the other major consumers of PRPP as compared with de novo synthesis. The enzyme purine phosphoribosyl transferase catalyses the direct reaction between a purine base and PRPP to form the corresponding 5'-phosphoribosyl derivative and pyrophosphate (PPi). One form of the enzyme from most animal tissues has

a high affinity for adenine while another shows high specificity for hypoxanthine and guanine. The process of nucleotide synthesis from purine bases, however, increases the rate of phosphoribosyl pyrophosphate synthesis (Baguara et al., 1974), while high levels of both adenine and guanine nucleotides inhibit this reaction.

Reaction 2 (Figure 2) is catalyzed by the enzyme purine nucleoside phosphorylase. Although incomplete information is available on the substrate specificity of enzymes from different tissues, hypoxanthine, xanthine and guanine have been reported to be substrates. The enzyme, however, does not react with adenine.

The enzyme purine nucleoside kinase, which catalyses reaction 3 (Figure 2) is very specific and only carries out phosphorylation of adenosine by ATP to form AMP and ADP. At this stage it is worth pointing out that there is a slight anomaly between the salvage reactions 2 and 3 (Figure 2) in that products of the reaction 2 are not converted into nucleotide and that the substrate for reaction 3 is not formed by reaction 2. Thus products of reaction 2, namely xanthosine, inosine and guanosine are not converted into nucleotides and remain as such. The role of reaction 2 in salvage thus remains obscure.

1.1.2 Purine nucleotide metabolism.

The pathway of purine nucleotide metabolism known to occur in the mammalian liver are summarized in Figure 3. The reaction leading up to the synthesis of IMP (inosine-5-phosphate) and some of the other nucleotides have been discussed in Section 1.1.1. IMP is at a key branch

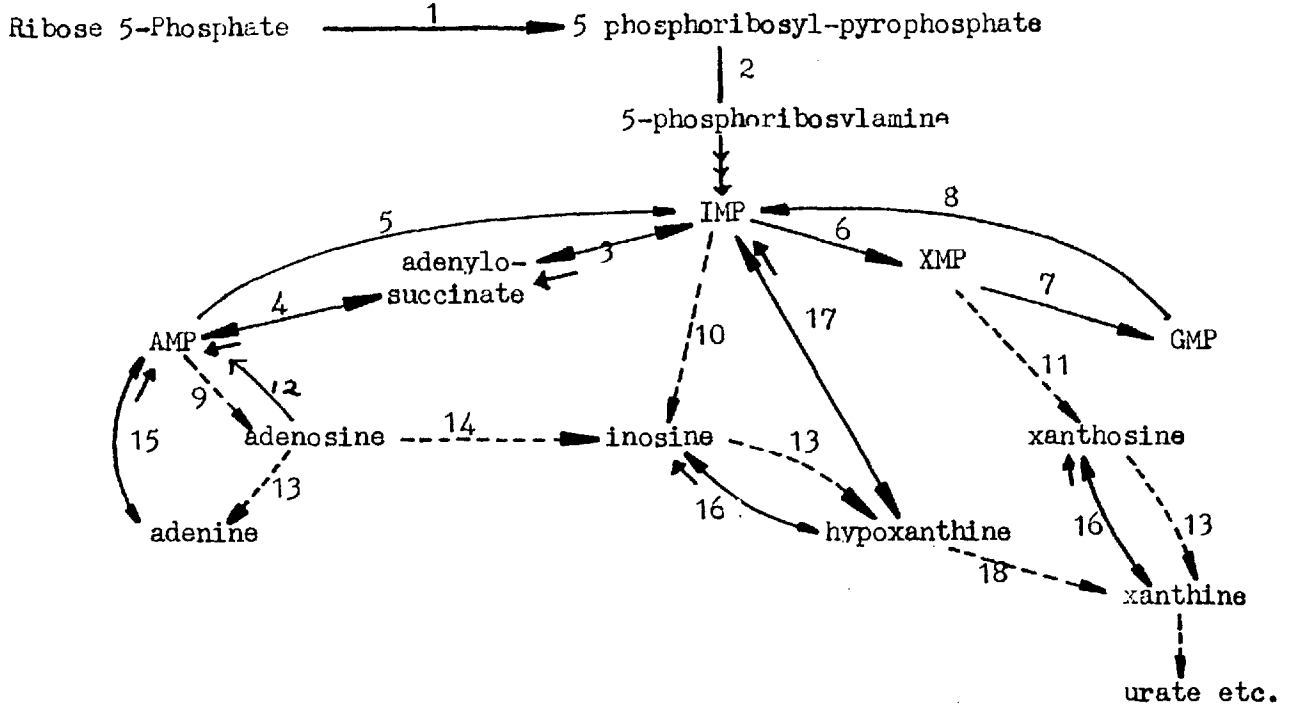


Figure 3 : Pathway of purine nucleotide biosynthesis, interconversion and degradation

Dashed arrows are used for catabolic pathways, continuous line arrows for synthetic pathways. Points on both ends of arrows indicate reversibility, a short arrow alongside it shows the preferred direction. Enzymes: 1, Ribose-p-pyrophospho kinase; 2, p-ribosyl-pp-amidotransferase; 3, adenylosuccinate synthetase; 4, adenylosuccinate lyase; 5, AMP deaminase; 6, IMP dehydrogenase; 7, GMP synthetase; 8, GMP reductase; 9,10,11. 5'nucleotidase; 12, adenosine kinase; 13, purine nucleosidase; 14, adenosine deaminase; 15, adenine-p-ribosyl-transferase; 16, purine nucleoside phosphorylase; 17, hypoxanthine-p-ribosyl-transferase; 18, xanthine oxidase.

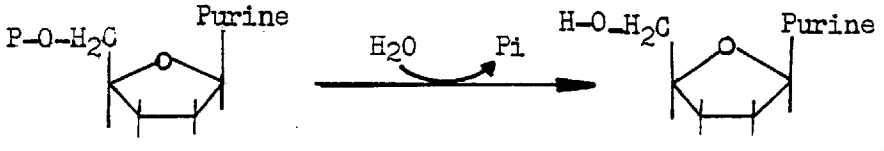
point in the biosynthesis of purine nucleotides as well as being a prominent intermediate in the inter-conversion between adenine and guanine nucleotides. The enzymes required for the above interconversion are present in most mammalian cells, but the relative proportions of IMP that are converted into AMP and into GMP vary considerably among different cell types. Cultured human lymphoblasts convert almost equal amounts of IMP into adenine nucleotides and guanine nucleotides (Fields and Brox, 1974), in slices of mouse brain 90% of the IMP is converted into AMP (Wong and Henderson, 1972). Factors that influence the relative rates of AMP and GMP synthesis from IMP include the rate of IMP synthesis de novo or from hypoxanthine, and intracellular concentrations of aspartate and glutamine (Crabtree and Henderson, 1971). Enzyme studies have shown that some of the enzymes of purine nucleotide interconversion are inhibited by nucleotides in such a manner that high levels of one species leads to an increased proportion of the IMP being converted to the other species. The reverse is also true in that a decrease in the level of one leads to its formation being increased. The enzyme IMP dehydrogenase is strongly inhibited by elevated concentrations of guanine nucleotides in tumour cells (Snyder and Henderson, 1973a), while being stimulated by raised ATP levels. The enzyme adenylsuccinate synthetase, the first enzyme involved in the biosynthesis of AMP from IMP is inhibited by AMP, GMP and GDP (Muirhead and Bishop, 1974).

High levels of adenine nucleotides and low levels of guanine nucleotides stimulate the enzyme AMP deaminase,

thus increasing the availability of IMP for the synthesis of guanine nucleotides. The rate of reductive deamination of GMP to IMP is low in most mammalian cells, the amount of GMP reductase considered to be the limiting factor (Crabtree and Henderson, 1971; Fields and Brox, 1974; Henderson et al., 1974).

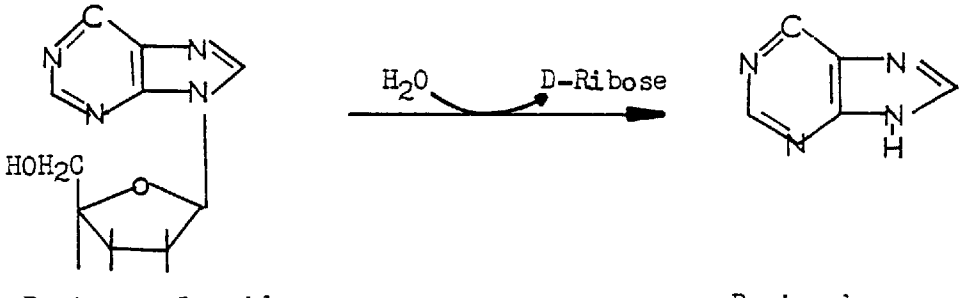
Degradation of the purine nucleotides occurs via the breakdown of the purine 5' monophosphates of adenosine, guanosine, inosine and xanthosine. Some of the reactions including those responsible for interconversion of GMP and AMP have already been discussed. Although these reactions are described as being degradative, some of the products, mainly nucleosides and bases can be reutilized by the salvage pathways.

Purine 5'-mononucleotides follow a common degradative pathway. The first reaction is catalyzed by the enzyme 5'-nucleotidase which cleaves the phosphate group by hydrolysis from the 5'-position of nucleoside monophosphate.



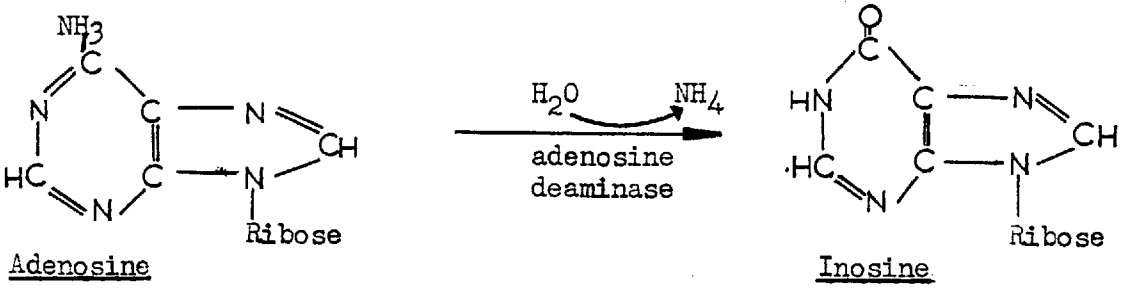
Purine 5'-nucleotide Purine nucleoside

The second step involves the enzyme nucleosidase which is responsible for removal of the ribose moiety from the nucleoside by hydrolysis as shown below.



Purine nucleoside Purine base

The enzyme purine nucleoside phosphorylase is also capable of carrying out this reaction in the reverse direction as mentioned above (Section 1.1.1). The bases guanine and hypoxanthine undergo deamination and reduction respectively to form the product xanthine which is converted to urate by the enzyme xanthine oxidase, where further degradation of the purine ring structure takes place, leading to the product allantoin in rats. In the case of adenine no further breakdown occurs in animals. An alternative pathway involves deamination of adenosine by the enzyme adenosine deaminase converting it to inosine. Further degradation of inosine is by removal of the ribose moiety as described above.



Adenosine Inosine

1.1.3 Enzymes of purine metabolism.

As mentioned in the previous section there are more than twenty enzymes involved in the interconversion and degradation of the purine nucleotides. In this section a few of the enzymes relevant to the work reported in this thesis will be considered in detail. Evidence concerning location of the enzymes and possible vectorial manner in which they may function will also be presented. This information will be useful in our understanding of the events occurring regarding purine nucleotide metabolism.

Since the work reported here is chiefly concerned with prelabelling of the hepatic adenine nucleotide pool

with ^{14}C-adenine and monitoring of its metabolism, enzymes likely to be important in this role will be dealt with here in detail.

5'nucleotidase

The enzyme 5'-nucleotidase (EC 3.1.3.5) catalyzes the cleavage of the phosphate group by hydrolysis from purine-5'-monophosphate acids to give the corresponding nucleosides (Section 1.1.2). It has been reported that several kinds of 5'-nucleotidase are present in rat liver (Segal and Brenner, 1960; Song and Bodansky, 1967; Itoh et al., 1968; Toda et al., 1975; Widnell and Unkeless, 1968; Arsenis and Touster, 1968; Fritzson and Smith, 1971; Drummond and Yamamoto, 1971); the most active one is bound to the cell membrane, another is lysosomal and a third is present in the cytosol. In rat livers 80% of the total IMP hydrolysing activity and 90% of the total AMP hydrolysing activity was found to be membrane bound (Naito et al., 1974). Gurd and Evans (1974) used anti plasma membrane serum (which inhibited the 5'-nucleotidase in a noncompetitive manner) to establish that 80-90% of the membrane bound 5'-nucleotidase was located on the outward facing surface of the plasma membrane. This report agreed with the previous studies of Widnell (1972) and the histochemical studies of Benedetti and Delbanffe (1971), which indicated that 5'-nucleotidase was located on the outer surface of the plasma membrane lining the bile spaces.

In addition intact adipocytes (Newby et al., 1975) and the external surface of skeletal muscle (Woo and Manery, 1975) have been shown to be capable of degrading 5' nucleotides, further substantiating the view that the membranous enzyme may be an ectoenzyme.

Vectorial nature of the enzyme as documented in human lymphocytes by Fleit and co-workers (1975) led them to suggest that the primary function may be the uptake of adenosine from AMP. This role, however, is not compatible with the release of adenosine by muscle where extracellular AMP has been shown to be converted to extracellular adenosine (Frick and Lowenstein, 1976). In reality it is most likely that both mechanisms are present.

The orientation of the membrane bound enzyme and its complete inhibition by ATP at physiological concentration (Evans and Gurd, 1973) indicates a minimal role in the metabolism of intracellular purine nucleotides. This role has been assigned to the cytoplasmic enzyme. Itoh et al., (1968) and Fritzson (1969) have reported that the cytoplasmic 5'-nucleotidase has a higher affinity for IMP than for AMP. Van den Berghe et al., (1977a) studied the kinetics of the enzyme in detail at physiological concentrations of its substrates and reported that the low affinity of the soluble 5'-nucleotidase towards AMP was accompanied by sigmoidal kinetics at low substrate concentrations, making the enzyme totally inactive under physiological conditions. In contrast, however, they also reported that the enzyme displayed nearly hyperbolic kinetics with IMP and other nucleoside 5'-monophosphates and allowed the degradation of these substrates to proceed.

Thus it has been proposed that in the liver cells at

normal physiological concentration, AMP hydrolysis by 5'-nucleotidase is not a major pathway of AMP breakdown, whereas, kinetic studies of the enzyme reveal that IMP is a better substrate for the enzyme and that the role of intracellular 5'-nucleotidase may be one of hydrolysis of IMP to inosine.

AMP Deaminase

The enzyme AMP deaminase (AMP aminohydrolase, EC 3.5.4.6) catalyses the hydrolytic deamination of AMP to IMP and is believed to be an intracellular enzyme (Kelckar, 1947). It is widely distributed in mammalian tissues and the purified form isolated from several sources has been extensively studied, namely in the brain (Cunningham and Lowenstein, 1965; Setlow and Lowenstein, 1968; Lee and Wang, 1968), the muscle (Smiley and Suelter, 1967; Ronca-Testoni et al., 1970) and the liver (Smith and Kizer, 1969; Chapman and Atkinson, 1973; Moss, 1977; Van den Berghe, et al., 1977b).

Kinetic studies of Smith and Kizer (1969) indicated that the hepatic enzyme, had regulatory properties characteristic of allosteric enzymes (see Monod et al., 1965). Allosteric nature was also suggested by the isolation of isoenzymes from rat tissues (Ogasawara et al., 1975). Sigmoidal property of the substrate-affinity curve was confirmed by Chapman and Atkinson (1973) and Van den Berghe et al., (1977b). These studies have also revealed that the liver enzyme is stimulated by ATP and inhibited by Pi and GTP.

The action of these effectors at physiological concentrations, on the enzyme activity was studied by Van den Berghe et al., (1977b). They found the enzyme to be inhibited by over 90% and concluded that the enzyme activity was poised to compensate for an increase in AMP production.

Adenosine kinase

The enzyme adenosine kinase (EC 2.7.1.20) catalyzes conversion of adenosine to adenosine monophosphate. Adenosine kinase has generally been purified from the cell cytosol and has been localized in the soluble fraction in rat heart (Lomax and Henderson, 1972). Membrane association of adenosine kinase has been demonstrated in the erythrocyte membrane (Schrader et al., 1972). Enzyme activity of 2210-3730 n mol/min/gm fresh tissue has been reported for the rat liver enzyme (Arch and Newsholme, 1978a).

Adenosine phosphorylation may be regulated in a complex manner that includes the Km for adenosine, the intracellular concentration of Mg^{2+}, cell energy charge, and the nucleotide pool. The Km for adenosine has been reported to be 0.4 to 6.0μM, with the Km of the enzyme from rat liver being 0.5μM at pH 7.5 and 2.4μM at pH 5.3 (Arch and Newsholme, 1978a; Fox and Kelley, 1978). In contrast, the Km for ATP is well below its normal intracellular concentration (Murray, 1968) suggesting that it may not be an important physiological regulator of adenosine phosphorylation under normal circumstances. Murray in his study also reported that an increase in ATP "charge" stimulated adenosine phosphorylation. This effect may be due to the decreasing concentration of ADP, since the Ki of ADP for adenosine kinase is in the range of its known intracellular concentrations. In these studies, there is a sigmoidal response of adenosine kinase to increasing ATP concentrations with 1-2mM Mg^{2+}, but a hyperbolic response in the absence of Mg^{2+}.

Adenosine deaminase

The enzyme adenosine deaminase (EC 3.5.4.4) degrades

adenosine to inosine. Activity for the rat liver enzyme has been reported to be in the range of 725-1590 n mol/min/g fresh tissue. The Km for adenosine ranges from 6 to 60μM with the value for rat liver varying between 35-58μM (Arch and Newsholme, 1978a). The enzyme does not appear to have many potentially physiological modifiers of its activity despite extensive investigations. The rate of the reaction appears to be regulated by the availability and concentration of the substrate and product, the level of enzyme activity in various tissues appears to be related to the molecular form of the enzyme that predominates. Very little is known about the prevalent form of the rat liver enzyme.

Hypoxanthine phosphoribosyl-transferase.

The enzyme hypoxanthine phosphoribosyl transferase (EC 2.4.2.8) catalyzes IMP and GMP synthesis from the corresponding base (Natsumeda et al., 1977). It may be predominantly cytoplasmic in location (Partsch et al., 1977). The enzyme has very low Km for the substrate suggesting that conversion of even low concentrations of the base to nucleotide can occur in the cells. Inhibition of the reaction by AMP, other adenine nucleotides, and GMP may be important as a regulatory mechanism.

IMP dehydrogenase.

The enzyme IMP dehydrogenase (EC 1.2.1.14) irreversibly catalyzes oxidation of IMP at position 2. The enzyme from rat liver exhibits hyperbolic saturation kinetics and is subject to inhibition by a number of purine nucleotides. Km for the substrates IMP and NAD^+ are 12 and 24μM respectively (Jackson et al., 1977).

Purine nucleoside phosphorylase.

The enzyme purine nucleoside phosphorylase (EC 2.4.2.1), reversibly catalyzes the interconversion between inosine and hypoxanthine (Divekar, 1976; Lewis & Glantz, 1976; Zannis et al., 1978). Km_{app} for rat erythrocyte enzyme is lower for hypoxanthine at 13μM than 83μM for inosine (Agarwal et al., 1975), thus favouring inosine formation. Plasma membrane located form has been demonstrated to be an ectoenzyme (Li and Hochstadt, 1976a, b), producing intracellular ribose 1-phosphate while converting extracellular inosine to hypoxanthine.

1.1.4 Formation and fate of hepatic purines

The rate of de novo purine synthesis in the liver is considered to be fairly high. This is indicated by the high activity of the enzyme PRPP amidotransferase (Caskey et al., 1964), the first enzyme of the de novo synthetic pathway, and rapid incorporation of ^{14}C-formate into purine nucleotides both in vivo (Smith and Salmon, 1969) and in vitro (Smellie et al., 1958). A rapid turnover of nucleotide material is also suggested by the observations that labelled nucleotide pools of rat liver show a marked decrease in specific activity with time (Smith and Salmon, 1969; Henderson and Le Page, 1959; Pritchard et al., 1970).

This rapid de novo synthesis of the purine nucleotides is supplemented by the equally active salvage pathways. High activity of the purine salvage enzymes adenosine kinase, nucleoside phosphorylase and hypoxanthine phosphoribosyl transferase have been recorded in the liver (Shenoy and Clifford, 1975). Furthermore efficient functioning of the salvage pathways is indicated by rapid assimilation of ^{14}C-labelled bases into the intracellular nucleotide pool. Pritchard and co-workers (1970) perfusing livers in situ, in non-recirculating manner, found that 80% of radioactive adenine (perfusate concentration of 5×10^{-6}M) was removed from the perfusate in one passage through the liver. Analysis of the liver sample taken immediately after perfusion showed that over 75% of the label was present as AMP, and the remainder was ADP or ATP, with none of the isotope remaining in the free base. Lerner and Lowry (1974) in a similar study using rabbit liver found that when labelling with ^{3}H-adenine, ^{3}H-hypoxanthine or ^{3}H-adenosine 60-80% of the radio-

activity in the liver was found as purine nucleotides.

The active de novo and salvage pathways in the liver as well as the high activity of the degradation enzymes (Shenoy and Clifford, 1975) combine to give the picture of an organ that is active in synthesis and turnover of the purine pool constituents. The ability of the liver to handle purines serves as a foundation for explaining its role which concerns the supply of purines to tissues incapable of meeting their purine requirments by de novo synthesis (Henderson and Le Page, 1959; Lajtha and Vane, 1958; Mager et al., 1967). This is analogous to the liver's role in supplying glucose and amino acids for utilization by other organs, e.g. brain. There is evidence to suggest that the liver may share this role with the skeletal and cardiac muscles which are known to release purines under anoxic conditions (e.g. Berne et al., 1971). The release of purine material by muscle tissue under these conditions, however appears to be primarily for the purpose of controling blood flow through the tissue.

Thus tissues which show no de novo pathway and have an absolute requirement for externally supplied purines are the prime candidates as the users of the released bases. This, however, does not rule out usage by tissues that exhibit a balance between de novo and salvage pathways. Tissues for which a complete absence of the de novo pathway has been suggested include rabbit bone marrow (Smellie et al., 1958; Lajtha and Vane, 1958; Thompson et al., 1960), human leukocytes (Scott, 1962; Williams, 1962) and blood platelets (Holmsen and Rozenberg, 1968). The conclusions from experiments using leukocytes and platelets were reached after the cells failed to incorporate

glycine or formate into purine nucleotides in vitro; however, some care in interpretation of these results is required, as substrates necessary for de novo synthesis may be lacking. It has, however, been clearly established that erythrocytes from rabbit (Lowry and Williams, 1960), mouse and humans (Fontenelle and Henderson, 1969), cannot synthesize purines via the de novo pathway.

Lajtha and Vane (1958) were the first workers to obtain evidence, although indirect, implicating the liver in mammals as a purine source for bone marrow. They showed that incorporation of ^{14}C-formate into DNA purines of bone marrow was specifically depressed by partial hepatectomy or portal occlusion, implying a role for the liver in purine supply. Similarly when bone marrow was incubated in vitro with substrates likely to be limiting for de novo synthesis (aspartic acid, glycine, glucose and glutamine), there was little incorporation of ^{14}C-formate into DNA purines (Smellie et al., 1958; Thompson et al., 1960).

Most detailed study however has been made concerning the supply of purines by the liver to erythrocytes where some advances as to the nature of the released purine moiety have been made. Pritchard et al., (1970), in their studies using the perfused liver, demonstrated the transfer of liver purines to erythrocytes in vivo and suggested that there is a continuous transfer of purines from liver to erythrocytes and then, in turn, to other tissues. Further insight into release of purine material by liver was obtained by Lerner and Lowry (1974) in similar but acute perfusion experiments. They found that liver labelled with [^{3}H] adenine or [^{3}H] hypoxanthine released [^{3}H] adenosine. This

[^{3}H] adenosine was detected after perfusion with unlabelled adenosine, which could have exchanged with a small quantity of adenosine of high specific activity from intracellular fluid. This release of adenosine was confirmed by Pritchard et al., (1975) who in addition measured the adenosine level of portal and hepatic venous blood and found the venous adenosine levels to be 12-fold higher than the portal levels.

In the experiments reported here this aspect of purine metabolism will be investigated by labelling the adenine nucleotide pool with radioactive precursor with emphasis on the perfusate levels of other nucleosides and bases as well as adenosine.

1.1.5 Purine nucleotide cycle.

The term purine nucleotide cycle (Lowenstein, 1972) is applied to the cyclical process in which conversion of aspartate to fumarate and ammonia occurs. The cycle consists of reactions catalyzed by adenylate deaminase, adenylosuccinate synthetase, and adenylosuccinate (Figure 4).

The reaction sequence IMP $\longrightarrow$ adenylosuccinate $\longrightarrow$ AMP $\longrightarrow$ IMP etc has been demonstrated in the liver (Moss and McGivan, 1975; Lowenstein, 1972). Several roles have been attributed to the purine nucleotide cycle, which include: (i) alternative pathway for the liberation of ammonia from amino acids; (ii) means of adjusting the levels of citric acid cycle intermediates; (iii) makes available certain amino acids as a source of carbon for energy production; (iv) regulator of relative levels of the adenine nucleotides AMP, ADP and ATP; and (v) aid in the control of PFK

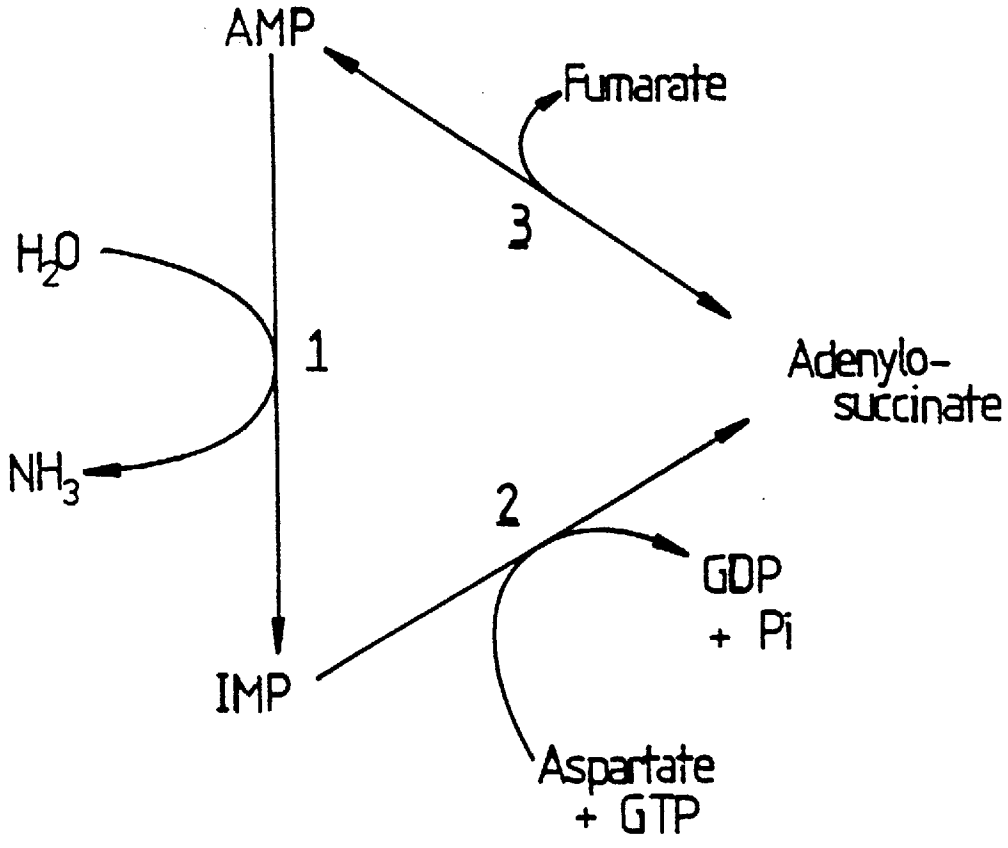


Figure 4 : The purine nucleotide cycle

Enzymes involved in reactions of the cycle are 1. AMP deaminase; 2. adenylosuccinate synthetase; and 3. adenylosuccinate lyase. One turn of the cycle results in the net reaction:

aspartate + GTP + $H_2O \longrightarrow$ fumarate + GDP + Pi + NH_3

activity and hence glycolysis.

Which aspect of the cycle is of particular significance depends on the nature of tissue in question.

The significance of the purine nucleotide cycle in the liver in the production of NH_3 is the subject of current research and results so far do not give a clear cut picture. Since this cycle incorporates reactions which have a central role in the turnover of adenine nucleotides, significance of the operation of this cycle in the liver was investigated.

1.2 Hepatic glycogen and purine nucleotide metabolism during ischaemia and anoxia.

1.2.1 Glycogen metabolism during ischaemia and anoxia.

Glycogen is stored in the liver in the form of granules which are located mainly in the extramitochondrial phase of the cells (Cardell, 1977; Lindberg and Falkama, 1977). The primary function of this store is to provide a temporary carbohydrate reserve for the maintenance of blood glucose levels. Rapid mobilization of hepatic glycogen to produce glucose is important in conditions where glucose utilization by other organs has increased (e.g. stress, exercise, etc).

Glycogen degradation occurs mainly via phosphorolysis catalyzed by glycogen phosphorylase. The product of degradation by phosphorolysis is G-I-P, so that in addition to glucose production the other fates of glycogen-derived carbon in livers are those of the hexose-phosphates, i.e. end-product of glycolysis (e.g. lactate, alanine) and products of acetyl unit metabolism (e.g. carbon dioxide, fatty acids, cholesterol, perhaps ketone bodies, etc). Details of metabolic balance between net rates of glycogenolysis, glucose release, glycolysis (and its various end-products) have not yet been fully documented.

In anoxic liver there is acceleration of glycogen degradation, to produce glucose and lactate which are released to the blood (Woods and Krebs, 1971; Walli et al., 1974; Glinsmann et al., 1969). Hypoxia will result from low rates of hepatic blood delivery which may occur during various stresses, starvation and exercise, as there is less splanchnic blood flow in these states. Also, as the hepatic

artery carries a substantial portion of the oxygen which the liver receives, then circulatory (arterial) shock will cause hepatic hypoxia. In all these conditions glucose required by organs such as muscle and brain could be supplied via increased hepatic glycogenolysis.

Production of lactate indicates that "anaerobic glycolysis" makes its contribution to sustaining ATP turnover. Glycolysis (consuming glycogen but not blood glucose) is clearly accelerated during anoxia (Hems and Brosnan, 1970; Woods and Krebs, 1971; Seglen, 1974), i.e. the liver exhibits a Pasteur effect. This implies a qualitative changeover from aerobic lactate uptake ("Cori Cycle") to anaerobic release.

Glycogen phosphorylase constitutes one control site in glycogenolysis in anoxic livers, as indicated by the increase in hepatic content of glucose 6-phosphate (Hems and Brosnan, 1970). On the evidence so far all control of glycogen degradation by the phosphorolysis route is exerted at the phosphorylase step. Phosphorylase in liver exists in at least two forms, designated a and b interconverted by a phosphorylation-dephosphorylation system. Depending on substrate and cofactor conditions, the velocity of reaction (calculated per mole of enzyme) catalyzed by a appears to exceed that for b by a factor of about 2-20 (Stalmans and Hers, 1975; Tan and Nuttall, 1975). In vivo, (i.e. in conditions identical for each form) a is likely to be much more active than b (Stalmans, 1976). Thus control of glycogenolysis in liver, in aerobic conditions at least, is achieved mainly by altering the available amount of the a form of phosphorylase.

Since hepatic AMP content rises in anoxia (Hems and

Brosnan, 1970; Faupel et al., 1972), any acceleration in glycogenolysis may be due to modifier responses of the a (and perhaps b) form of the enzyme, as a result of the increase in hepatic content of AMP during hypoxia; thus phosphorylase a (or b) in liver is stimulated (allosterically) by AMP (Tan and Nuttall, 1975; Stalmans and Hers, 1975). However, it is not clear whether the amount of available phosphorylase a increases in hypoxic liver. Such a response might be expected, as glycogenolysis in other states (e.g in response to hormones or glucose lack) is always associated with increases in phosphorylase a content. There is however no documentation that such a change in hypoxic liver exists, although it has been reported that the amount of assayable phosphorylase increases in liver perfused with cyanide or iodoacetate (Jacob and Diem, 1974).

Thus experiments were designed to study the control of hepatic glycogenolysis in liver during hypoxia and anoxia.

1.2.2 Purine nucleotide metabolism during ischaemia and anoxia.

In normal conditions the relative concentration of adenine nucleotides is constant. It is very important to maintain these nucleotides within a particular concentration range for a number of reasons. In particular they have profound effect on enzyme activity and thus metabolic processes. For example, the role of adenine nucleotides in control of carbohydrate metabolism is well documented; a high ATP/ADP ratio promotes gluconeogenesis and thus decreases ATP concentration, whereas a low ratio promotes glycolysis and increases ATP concentrations (Newsholme and Gevers, 1967).

Anoxia leads to a rapid decrease in hepatic ATP concentration, accompanied by increases in AMP and ADP concentrations. The time course of these changes has been well documented (e.g. Hems and Brosnan, 1970; Brosnan et al., 1970; Faupel et al., 1972; Weber et al., 1977). These changes are a consequence of failure of oxidative phosphorylation, and occur in parallel with increased glycogenolysis and glycolysis (see previous section 1.2.1). Cytoplasmic adenine nucleotides are kept at equilibrium by the adenylate kinase enzyme even during ischaemia, as illustrated by the constancy of the ratio of $[ATP][AMP]/[ADP]^2$ although the concentration of ATP and AMP change markedly (e.g. Brosnan et al., 1970).

Acceleration of anaerobic glycolysis as measured by an increase in lactate production at an estimated rate of 1.3μmol/min/g (Brosnan et al., 1970) is, however, not sufficient to maintain the adenine nucleotides at their normal relative concentrations. Chaudry and coworkers (1976c & 1977) have reported from in vivo studies

that haemorrhagic shock which is accompanied by reduced blood flow through the liver caused a more pronounced decrease in adenine nucleotide content than anoxia and hypoxia (with arterial PO_2 at 18mm Hg).

Hems and Brosnan (1970) demonstrated a decrease in total adenine nucleotide content of the liver during ischaemia. Increased hepatic concentration of IMP and XMP in ischaemic samples (Weber et al., 1977) indicates increase in AMP degradation, presumably in response to elevation of tissue AMP content. Increase in hepatic AMP level in the presence of fructose also results in decrease in the total adenine nucleotide content, due to increased AMP degradation (Woods et al., 1970). This phenomenon was investigated by Van den Berghe and associates (1977a, b) who concluded that AMP was predominantly deaminated to IMP.

It has been demonstrated in other tissues that anoxic-induced stimulation of adenine nucleotide degradation is accompanied by release of breakdown products. Berne and associates (Rubio and Berne, 1969; Berne et al., 1971) demonstrated that ischaemic heart released adenosine. In addition increased adenosine release in response to ischaemia has also been demonstrated for the skeletal muscle (Bockman et al., 1975 and 1976; Dobson et al., 1971) and the brain (Berne et al., 1974; Newman and McIlwain, 1977).

Although gross changes in adenine nucleotides have been documented in the liver in response to anoxia and ischaemia, little is known with respect to changes in hepatic content of other purine compounds. The release of these compounds has been documented in a number of tissues, but this aspect of nucleotide metabolism is not clear with

respect to liver. Experiments were designed to monitor stimulation of hepatic AMP degradation during anoxia in order to answer some of these questions.

1.3 Short term hormonal control of hepatic carbohydrate metabolism and role of purine nucleotides.

1.3.1 Rapid effect of hormones on hepatic carbohydrate metabolism.

A wide range of hormones can exert a rapid stimulatory effect on hepatic glycogenolysis, which may be demonstrated as an increase in net glycogen depletion or inferred from an increase in the amount of assayable phosphorylase a in the liver. Increased hepatic glycogenolysis demonstrated for glucagon (Glinsmann and Mortimore, 1968; Exton and Park, 1972), vasopressin (Hems et al., 1976; Hems and Whitton, 1973) and angiotensin (Hems et al., 1976), is usually associated with increased glucose release. Stimulation of glycogenolysis and stimulation of gluconeogenesis have been demonstrated for glucagon (Hutson et al., 1976; Fain et al., 1975), and vasopressin (Hems and Whitton, 1973), suggesting that the overall effect on hepatic carbohydrate metabolism of these hormones may be promotion of glucose release by the liver, from both glycogen and glucogenic substrates.

Insulin does not appear to have a direct effect on hepatic glycogen metabolism (Seglen, 1973; Whitton and Hems, 1975; Walker, 1977) but can suppress the effect of glucagon on stimulation of glycogen breakdown (Exton and Park, 1972).

Stimulation of hepatic glycogenolysis occurs in parallel with an increase in phosphorylase a activity in response to glucagon (Van den Heede et al., 1976), vasopressin (Hems et al., 1975 and 1976; Keppens and De Wulf, 1975) and angiotensin II (Hems et al., 1976; Keppens and De Wulf, 1976), which may

be mediated by alteration in the relative activities of phosphorylase kinase and phosphatases.

1.3.2 Cyclic purine nucleotides and Ca^{2+} as second messengers

Stimulation of hepatic glycogenolysis by glucagon, by an increase in glycogen phosphorylase a activity has been well documented (see Exton & Park, 1972). This stimulation occurs by raised cyclic AMP concentration leading to increased protein kinase activity and consequently increased phosphorylase kinase activity. However, phosphorylase a dephosphorylation to the b form catalyzed by phosphorylase a phosphatase could also serve as a possible control point, although none has been documented. Increased hepatic cyclic AMP content in response to glucagon is accompanied by its release into the effluent blood (Exton et al., 1972; Kirk and Hems, 1974), perhaps suggesting an extracellular role.

Vasopressin and angiotensin II, however do not exert their catabolic effect on liver through cyclic AMP. Vasopressin does not increase the cyclic AMP content of liver (Kirk and Hems, 1974) and neither does angiotensin II (Hems et al., 1978a). In parallel no increase in the assayable activity of cyclic AMP-dependent protein kinase has been observed in hepatocyte suspension in the presence of vasopressin or angiotensin II (Keppens and De Wulf, 1975 and 1976).

Since cyclic GMP can stimulate glycogenolysis in the liver (Exton et al., 1971a) it has been proposed as a possible intracellular mediator of vasopressin and angiotensin II mediated stimulation of hepatic glycogenolysis. However the content

of this nucleotide in perfused liver does not alter in response to vasopressin or angiotensin II (Hems et al., 1978), suggesting that cyclic GMP is not involved in the hepatic effect of these hormones.

Current interest in the role of Ca^{2+} in biological systems has led to the proposal that extracellular Ca^{2+} may be implicated in the glycogenolytic effect of vasopressin and angiotensin II (Keppens et al., 1977; Van de Werve et al., 1977). This may also be the case for vasopressin effect on hepatic glucose release since extracellular Ca^{2+} is critical for this effect (Stubbs et al., 1976). Extracellular Ca^{2+} is also critical in the activation of phosphorylase by vasopressin and angiotensin II (Hems et al., 1978b; Keppens et al., 1977) and in the glycogen-derived glucose release due to vasopressin and angiotensin II (Hems et al., 1978b; Whitton et al., 1978).

Thus it has been proposed that the mechanism of the rapid glycogenolytic effects of vasopressin and angiotensin involves an increase in the intracellular Ca^{2+} concentration. The raised Ca^{2+} concentrations in turn could stimulate phosphorylase b kinase directly, thus achieving phosphorylation of phosphorylase b without involving phosphorylation of phosphorylase kinase (Keppens et al., 1977; Van de Werve et al., 1977). There is, however, no direct evidence for the existence of hormone-induced Ca^{2+} transport across the hepatocyte plasma membrane, due to the problem of Ca^{2+} binding to the plasma membrane (Foden and Randle, 1978; Keppens et al., 1977). In addition it has been reported that vasopressin may cause net release of Ca^{2+} from hepatocytes in suspension (Blackmore et al., 1978; Chen et al., 1978). Thus the role of Ca^{2+}

in mediation of vasopressin and angiotensin effects on hepatic glycogenolysis is not clear.

1.3.3 Other purines as mediators of hormonal effects

Since the role of Ca^{2+} as mediator of hepatic effects of vasopressin and angiotensin is not clear and cyclic AMP and cyclic GMP are not involved, possible roles of other purine nucleotides and nucleosides may be considered in the control of hepatic metabolism. This would be analogous to glucagon stimulated formation of cyclic AMP.

Adenosine may fulfill such a role on the basis of evidence concerning its involvement in various aspects of metabolism. When added to hepatocyte suspensions it increases the intracellular concentration of ATP and total adenine nucleotides (Lund et al., 1975). Adenosine under these conditions is preferentially incorporated into ATP in comparison with its incorporation into AMP and ADP (Rapaport and Zamecnik, 1976). Adenosine has been shown to inhibit gluconeogenesis from lactate (Lund et al., 1975) and inhibit the stimulation of glycogenolysis by glucagon or adrenalin (Fain and Shepherd, 1977) in isolated rat liver cells. Adenosine has been shown to cause vasoconstriction in the liver which was accompanied by increased glucose release (Ismail and Hems, 1978). Adenine nucleotides have also been shown to increase glucose release (Hunter and Jefferson, 1969).

Involvement of other purines in modulation of hepatic metabolism is suggested by the large number of enzymes associated with purine nucleotide metabolism that are present at the plasma membrane (see Section 1.1 for details).

There are reports of stimulations of membrane-bound phosphodiesterase from rat epididymal fat cells by insulin (Kono et al., 1975), stimulation of AMP deaminase from rabbit skeletal muscle by angiotensin II (Sullivan, 1976) and stimulation of 5-phosphoribosyl 1-pyrophosphate formation in isolated hepatocytes by glucagon (Hisata et al., 1978).

1.4 Scope and aims of the present study

In the present study an attempt has been made to elucidate the role of adenine nucleotides in the control of hepatic carbohydrate metabolism in response to variables such as anoxia and hormones.

Onset of anoxia in the liver is accompanied by stimulation of glycolysis and glycogenolysis (Hems and Brosnan, 1970), in response to failure of oxidative phosphorylation. The mechanism of stimulation of glycogenolysis which is poorly understood has been investigated, with emphasis being placed on the possible role of changes in concentration of adenine nucleotides (which also occur in response to anoxia). Complete sudden cessation of hepatic blood flow provided a simple model for the study of hypoxic events (Hems and Brosnan, 1970) and the liver perfused at suboptimal rates was used as a model for circulatory shock (Mangnall and Clark, 1976).

Mechanisms involved in the increase in degradation of adenine nucleotides observed during anoxia were also investigated. This involved prelabelling the intracellular adenine nucleotide pool of the perfused liver using radioactive precursor. The adenine nucleotide pool was labelled using ^{14}C-adenine which is converted intracellularly to AMP by adenine p-ribosyl transferase.

It was thus possible to assess the role of the various pathways of AMP degradation which can be stimulated during anoxia.

As stated earlier, there is considerable evidence to suggest a role for purines in mediation of the effect of hormones on the liver. This aspect was investigated using the above technique of prelabelling of the adenine nucleo-

tide pool, which enabled both intracellular and extracellular events to be monitored at the same time.

Chapter 2

ANIMALS, MATERIALS and METHODS

2.1 Animals

2.2 Sources of materials

2.3 The technique of liver slicing

2.4 The technique of liver perfusion
- 2.4.1 Perfusion apparatus
- 2.4.2 Operative procedure
- 2.4.3 Perfusion medium
- 2.4.4 Non-recirculating perfusion
- 2.4.5 Sample preperation
  - (a) Liver samples
  - (b) Perfusion medium samples

2.5 Analytical methods
- 2.5.1 Purification of acid soluble extracts and seperation of purines
  - (a) Purification of extracts
  - (b) Seperation of ^{14}C-labelled purine material using thin layer chromatography
  - (c) Construction of ^{14}C-labelled purine pool intermediate profile
- 2.5.2 Determination of adenine nucleotides
  - (a) Adenosine triphosphate
  - (b) Adenosine diphosphate & adenosine monophosphate
- 2.5.3 Lactate determination
- 2.5.4 Glycogen and glucose determination
- 2.5.5 Assay of glycogen metabolising enzymes
  - (a) Phosphorylase a
  - (b) Phosphorylase a phosphatase
  - (c) Glycogen synthetase
- 2.5.6 Oxygen measurement

2.6 Determination of carbon-14 radioactivity by liquid scintillation counting

CHAPTER TWO

ANIMALS, MATERIALS AND METHODS

2.1 Animals

Albino, male, Sprague-Dawley rats of the CFY strain derived from Carworth, Europe were bred in the Biochemistry Department of Imperial College; the male stock were renewed every three months, ensuring a close genetic relationship to the foundation stock. Animals weighing between 190 and 220 gm. and allowed free access to a standard (Thompson's) cereal diet and water were used. They were maintained on a light/dark cycle of 12 hr. (daylight period 06.00 hr. - 18.00 hr. GMT), in a relative humidity of 55% and in a temperature of 19-23°C.

2.2 Sources of materials

Solvents, chemicals and reagents ('Analar' grade or of the highest purity available) were obtained from BDH Chemicals Ltd, (Poole, Dorset, UK), Fisons Ltd, (Loughborough, Leics, UK), Hopkins and Williams (Romford, Essex, UK), or May and Baker (Dagenham, Essex, UK), unless otherwise stated. Activated charcoal (Norit A) was also obtained from Hopkins and Williams.

Adenine nucleotides, 8-arginine vasopressin (synthetic grade VI), and trizma base were from Sigma (London) Chemicals Co Ltd (London, SW6, UK). Enzymes and substrates for analytical purposes, were from CF Boehringer Corporation (London) Ltd. All radiochemicals were from the Radiochemical Centre (Amersham, Bucks, UK). Bovine serum albumin (Pentex, Fraction V) was from Miles Laboratories (Kankakee, Illinois, USA). Insulin was the highest grade commercial ox preparation from Burroughs Wellcome (Dartford, Kent, UK) and glucagon was obtained from Eli Lilly (Indianapolis, USA). Synthetic [5-valine] angiotensin II was provided by the Medical Research Council National Institute of Biological Standards (Hampstead, London, NW3, UK).

The activity of each batch of 8-arginine vasopressin was determined by bioassay of its antidiuretic action, by Dr M Forsling (Department of Physiology, Middlesex Hospital, London, UK). The activity was consistently about 90% of that stated by Sigma (London) Chemical Co Ltd, so the true activity of each batch was calculated using this factor.

Silica gel GF254 was from Anderman Ltd, (London, SE1, UK) and was manufactured by Merck Ltd. Pre-coated sheets of Polygram CEL 300PE1/UV254 were from Camlab (Cambridge) and

manufactured by Macherey-Nagel and Company.

Surgical instruments were purchased from Holborn Surgical Instrument Co Ltd (London, EC1, UK) and John Weiss and Sons Ltd, (London, W1, UK). Metal intravenous cannulae (West Middlesex Hospital pattern) for rat liver perfusions were from Downs Surgical Ltd, (London, W1, UK) or AC Daniels Ltd, (London, W1, UK). Silicone rubber tubing ('Silescol') was from Esco, (Rubber) Ltd, (London, W1, UK).

2.3 The technique of liver slicing

The rats were stunned and killed by dislocation of the cervical vertebrae. The liver was quickly removed and placed in ice-cold Krebs-Ringer bicarbonate buffer (Krebs & Henseleit, 1932). Ionic composition of Krebs-Ringer bicarbonate buffer was as follows: 170 mM sodium chloride; 25 mM sodium hydrogen carbonate; 4.8 mM potassium chloride; 1.2 mM potassium dihydrogen phosphate; 1.2 magnesium sulphate; and 2.5 mM calcium chloride. The liver was cut into thin slices by hand using a microtome blade with the aid of a glass slide (one liver producing 20-30 good slices). The tissue was kept cold at all stages. Slices were quickly transferred for preincubation to a 100 ml flask containing 30 ml of Krebs-Ringer bicarbonate buffer, with potassium ion concentration raised to 30 mM, equilibrated with 5% CO_2 - 95% O_2 and incubated at 37°C for 20 min. After preincubation the slices were transfered to small flasks (4-6 slices, 0.2-0.4 gm. tissue) containing 2 ml of incubation medium and 0.5 mM (^{14}C)-adenine (sp.act. 0.4 μCi/μ mol). All incubation mediums were equilibrated at 37°C prior to the addition of the slices and the incubation was carried out in an atmosphere of a mixture of 5% CO_2-95% O_2.

After 30 min. incubation the hormone was added (volume not more than 0.1 ml) and the slices were removed at various times and rapidly frozen in liquid Nitrogen. The tissue was homogenized in 5 volumes of ice-cold 6% (v/v) perchloric acid (PCA) using a Vortex homogenizer. Samples

from the incubation medium were prepared by mixing with an equal volume of 6% PCA. The precipitates were removed by centrifugation and the supernatant was used for further purification and isolation of the labelled compounds as described in Section 2.5.

2.4 The technique of liver perfusion

2.4.1 Perfusion apparatus

The design of the apparatus used, with slight modification, was as described by Hems et al. (1966) based on the designs of Miller et al. (1951) and of Schimassek (1963). The apparatus was housed in a cabinet with a sash perspex window at the front and was heated by a thermostatically controlled fan heater attached to the rear of the cabinet. The temperature of the cabinet and medium was kept at 37°C. An additional fan was also placed in the cabinet to both maintain even temperature and assist in rapid restoration after procedures which involved opening of the window. The size of the cabinet and arrangement of the apparatus was such that two perfusions could be carried out simultaneously. Magnetic stirrers were an integral feature of the cabinet to ensure adequate mixing of the perfusion medium in the collecting vessels. Small openings at the sides of the cabinet provided access for gas and electrical equipment inside the cabinet. The assembly thus described provided a constant temperature environment for perfusions.

The arrangement of the glassware and tubing was as shown in Figure 5 . The multi-bulb glass oxygenator was attached to a stainless-steel plate which was mounted on a ball and socket joint and clamped to a horizontal rod, which could be moved through the vertical plane. The joint made it possible to adjust the position of the oxygenator vertically to ensure complete spreading of the perfusion medium over the surface. The perfusion medium

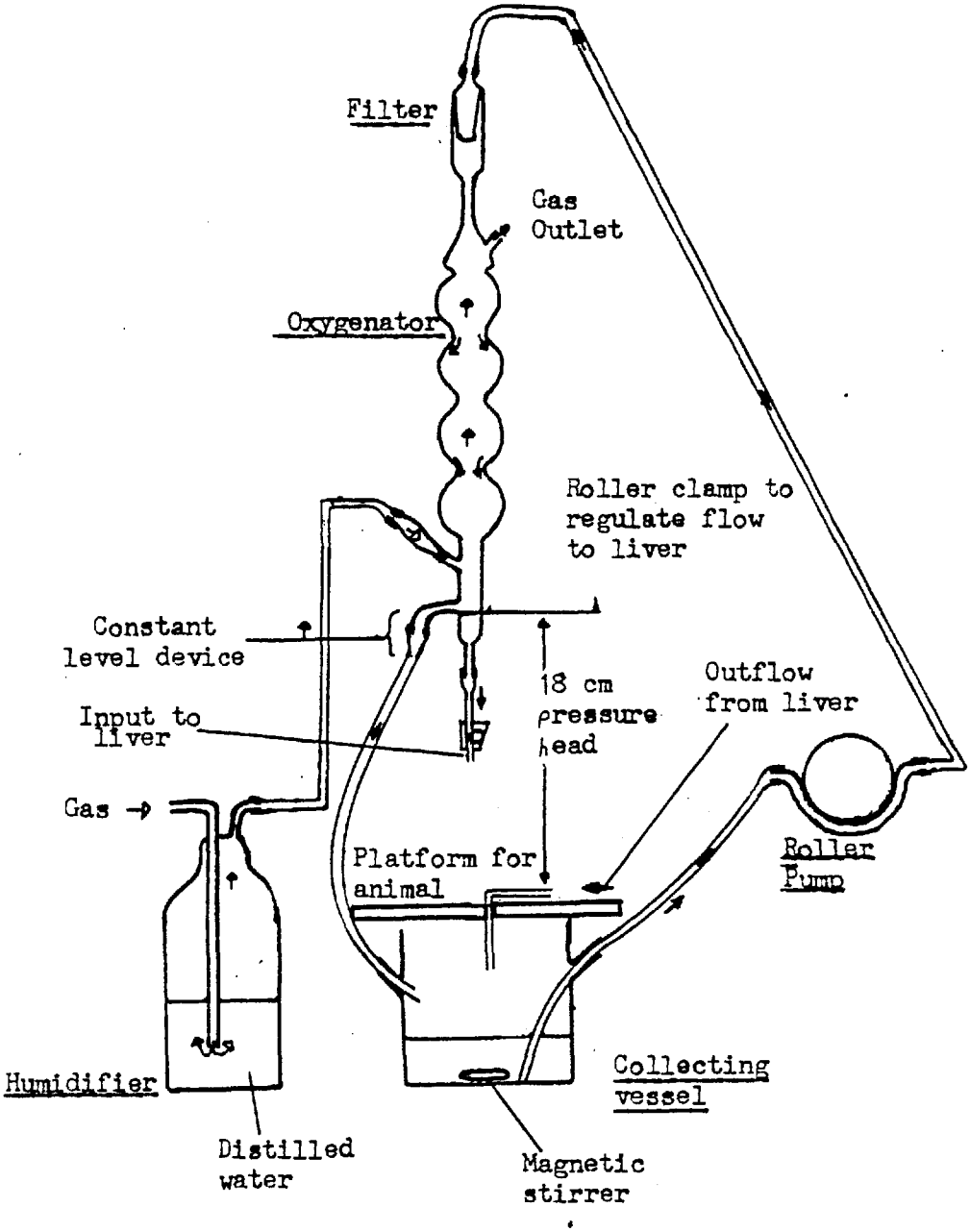


Figure 5: Apparatus for perfusion of rat liver

was pumped from the collecting vessel by a MHRE roller pump (supplied by Watson Marlow Ltd., Cornwall, U.K.) and passed through a plastic mesh filter, taken from a disposable blood transfusion set. From the filter the fluid flowed to the top of the oxygenator, the bulbous nature of which increased the surface area for gas exchange. The gas, usually CO_2+O_2 (5:95), was saturated with water by bubbling through a wash bottle fitted with as sintered glass distributor, and entered the oxygenator at the bottom and left through an outlet at the top. At the bottom of the oxygenator was a resevoir containing oxygenated medium, which was kept at a constant level by an overflow tube leading to the collecting vessel. The height of the reservoir could be adjusted to give a hydrostatic pressure for optimum flow rates without the liver swelling. The height was kept at 18 centimetres.

The input to the liver led from the bottom of the oxygenator via a length of silicone tubing supplied with a roller clamp, enabling fine adjustment of flow. The perfusion medium from the liver was then returned to the collecting vessel, and the recirculation continued.

2.4.2 Operative procedure

The operation was carried out essentially as described by Hems et al. (1966). The rat was anaesthetized by placing it in an environment saturated with diethyl ether. The animal was placed on the operating platform

and taped into position. A beaker containing diethyl ether-soaked cotton wool was kept over the animal's head during the operation procedure to maintain anaesthesia. The abdomen was opened by a horizontal cut and the intestines deflected to the animal's left on to layers of tissue soaked in Krebs-Ringer bicarbonate, so that the liver, hepatic portal vein, right kidney, inferior vena cava and the bile duct became exposed. Heparin (0.2 ml = 200 units) was injected into the inferior vena cava and the injection point covered with a tissue paper. The thin strands of connective tissue between the right lobe of the liver and the vena cava were cut and a loose ligature of silk (size 3/o) placed around the vena cava above the right renal vein. Two loose ligatures were passed around the hepatic portal vein, at intervals of 3-4 mm. below the point where the vein divides to enter the separate lobes of the liver. The ligature closest to the liver also included the hepatic artery the portal vein was cannulated with a no. 17 Frankis-Evans needle (Trocar and cannula, luer fitting), the needle removed and the ligature distal from the liver tied to hold the cannula in place (Figure 6). A rapid back flow was usually observed which indicated a good and rapid operation.

The thorax was than quickly opened by a transverse incision just below the pectoral girdle and by two deep longitudinal cuts-towards the diaphragm. This flap was then removed above the heart. A loose ligature was placed around the anterior vena cava and a cannula of Portex

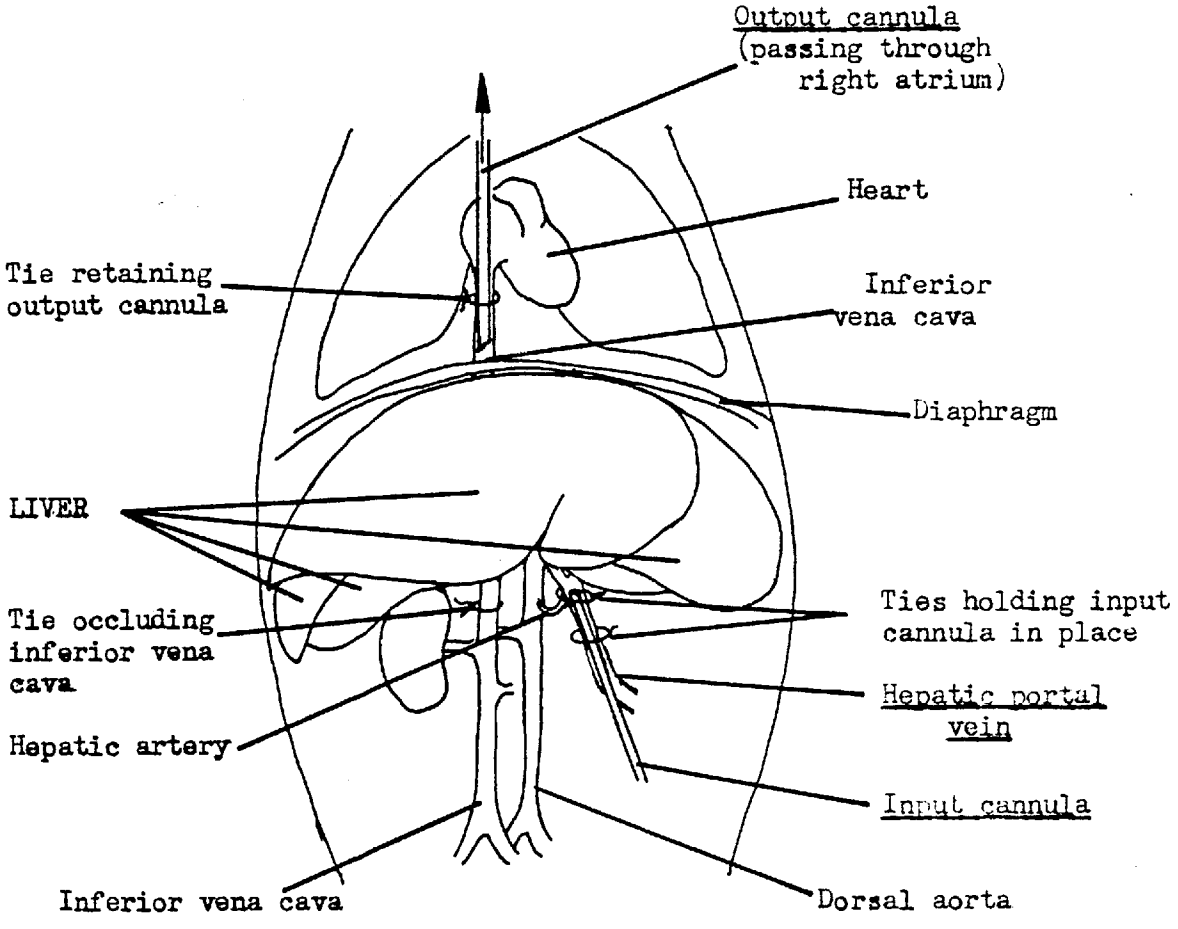


Figure 6: Liver perfusion operation in the rat
from Gove, C.D. PhD Thesis (1979)

tubing (3.00 x 2.00/2.42 x 1.67 mm drawn out to a bevelled point) inserted through the right atrium into the vena cava and as far as the diaphragm. The cannula was tied in place.

The preparation was then connected up to the perfusion apparatus and the circulation started at 15-20 ml/min. The cannula lying in the portal vein was usually filled with blood but if this was not the case perfusion medium was carefully injected with a syringe to exclude air bubbles prior to the above connection. The first 20 ml of venous blood was discarded. During this time the abdominal vena cava ligature and the second ligature around the portal vein were tied.

After discarding the venous blood the platform plus the animal was placed on the collecting vessel, the recirculation of medium started and the washed red cells added. The whole operation took about 10 minutes but the time from the insertion of the hepatic portal vein cannula to the connection to the perfusion medium was never more than 2 minutes. An indication of the success of the operation was the uniform olive-green colour of the liver obtained during the washout and the even red-brown colour observed on addition of the red cells. An extended interruption of the liver circulation caused a patchy liver seen either during the washout or when the red cells were added and these preparations were discarded.

A wire cage covered with tissues soaked in Krebs-Ringer bicarbonate buffer was placed over the liver, preventing

it from drying. The duodenum was cannulated to allow free flow of bile, impairment of which might result in a dysfunctional liver.

2.4.3 Perfusion medium

In all experiments the perfusion medium used contained (1) Krebs-Ringer bicarbonate buffer (Krebs & Henseleit, 1932), (2) bovine serum albumin and (3) washed rodent red blood cells. The albumin (Pentex: Fraction 5 from Miles-Seravac Ltd., Berks) was dialysed for three days against four changes of gassed Krebs-Ringer bicarbonate and a 15% (w/v) solution then prepared which was stored at -20°C until use . The ratio of albumin to Krebs-Ringer bicarbonate buffer was constant with albumin concentration being 2.5% (w/v). The red blood cells, where necessary, were added to this solution. Normally 4-5 mM glucose was also present in the medium.

Red blood cells were obtained from a fed donor rat weighing more than 600 g . The animal was bled from the aorta under ether anaesthesia and the blood defibrinated on glass beads (Baron & Roberts, 1963) by a rotary motion of a siliconised flask. After a period of 30 min., allowed for clot contraction, cells in the supernatant were washed twice with 20 volumes of Krebs-Ringer bicarbonate buffer containing 5 mM glucose and gassed with CO_2+O_2 mixture (5%:95%). The red blood cells were separated from the washing fluid by centrifugation (5 min. at 2000 g). The red blood cells

were then made up to the original blood volume with bicarbonate buffer, except in experiments where the effect of high haematocrit in the medium was being studied.

Perfusions carried out under these conditions have the following features: (1) red cell glycolysis in the absence of a liver and in the presence of 6 mM glucose is negligible, (2) the pH of the medium before and during the perfusion was in the range of 7.3-7.5 and (3) haemolysis during perfusion was about 1%/hr. as determined using Drabkin's reagent (0.05 gm. KCl and 0.2 gm. $K_3Fe(CN)_6$/l). 20 ml of sample was added to 4 ml Drabkin's reagent mixed and allowed to stand for 5 min. The optical density of the resultant coloured solution was read against water at 540 nm,. Standard 18% haemoglobin gave an optical density of 0.618.

2.4.4 Non-recirculating perfusion

In a few of the experiments it was necessary to change the conditions of the perfusion after equilibration from one with recirculating medium to non-recirculating medium. In these perfusions the arrangement of the perfusion glassware was as shown (Figure 7). This arrangement had the feature, that while one half of the apparatus was used for perfusing the liver until a 'steady-state' had been reached, the other half maintained the 'change-over' medium at the same temperature. The outflow from the reservoir of the two oxygenators was joined by a 3-way tap to a length of silicon tubing which served as

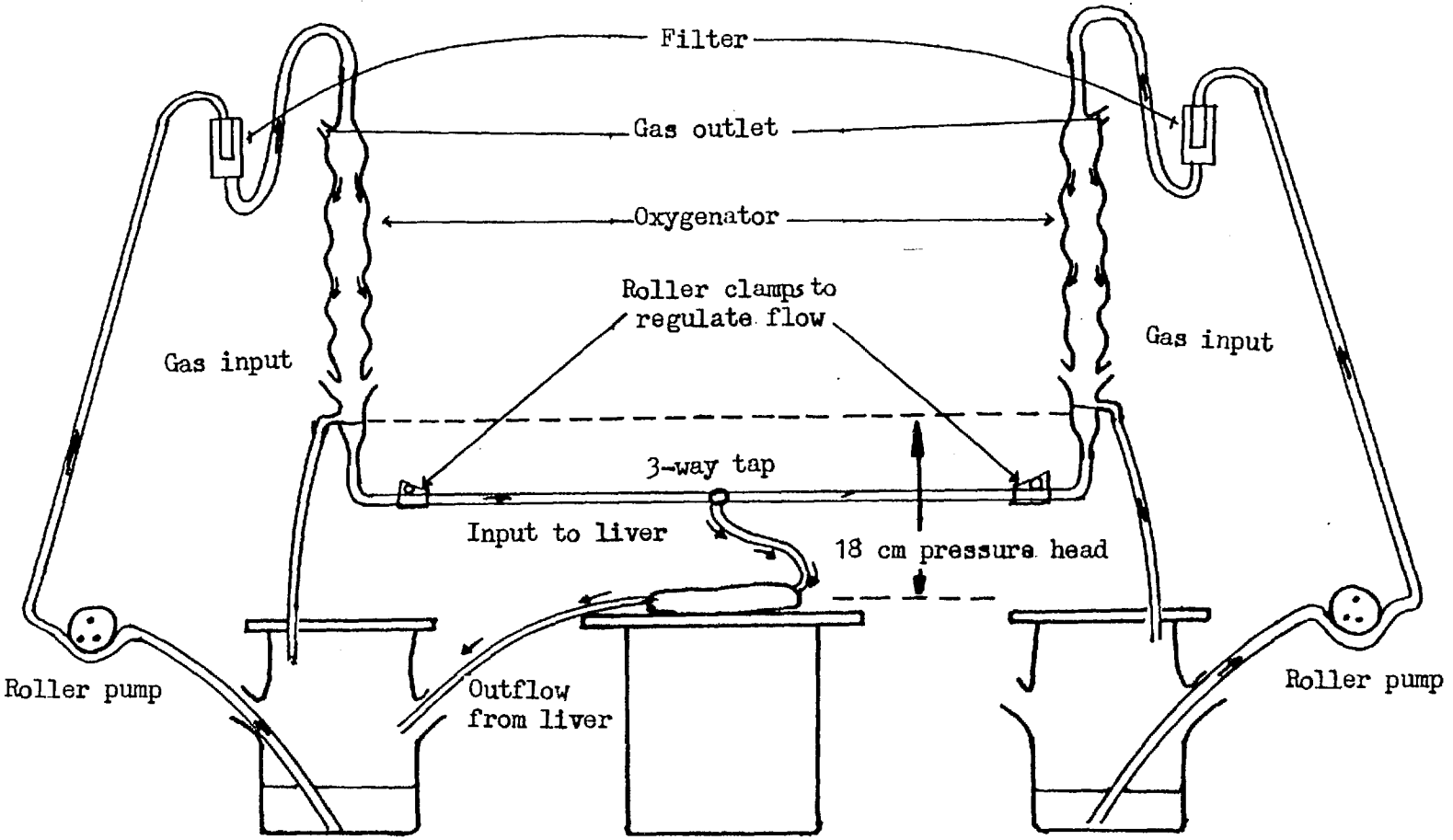


Figure 7: Modified arrangement of apparatus for rat liver perfusion enabling changeover of perfusion medium.

the single input to the liver. The platform instead of resting on the collecting vessel was supported at the same height by other means. This displacement of the platform also enabled collection of medium samples with minimum of disturbance when the perfusion was changed over to the non-recirculating mode. Outflow from the liver was returned to the collecting vessel as before, when in the recirculating mode.

In these perfusions the first medium was as described above (Section 2.4.3), with 12-50% haematocrit. The second medium consisted of Krebs-Ringer bicarbonate buffer containing albumin (final concentration of 2.5%), but no red cells.

2.4.5 Sample preparation

(a) Liver samples: Liver samples for various analyses were removed rapidly and quickly frozen using aluminium tongs pre-cooled in liquid nitrogen. The liver was stored at -20°C until used. Glycogen level of the liver and activity of the glycogen metabolizing enzymes were determined as described in Sections 2.5.5 and 2.5.6 using the frozen liver. Adenine nucleotide concentrations were determined in neutralized perchloric acid (PCA) extract using enzymatic assay procedures described in Section 2.5.3. 0.8-1.0 gm of frozen liver was homogenized with exactly 5 volumes of 6% PCA (v/v). The precipitate was removed by centrifugation. pH of the supernatant was adjusted to 7 with 30% KOH using universal indicator.

The mixture was left in ice for 30 min to allow completion of the potassium perchlorate precipitate. The 200µl samples were used for the adenine nucleotide assays. Analysis of hepatic ^{14}C-distribution amongst the purine compounds was done on the non-neutralized PCA liver extracts prepared as described above. 4.0 ml of extract was used for purification and separation of the ^{14}C-labelled purine compounds as described in Section 2.5.1.

(b) Perfusion Medium Samples: Perfusion medium analysis of glucose, lactate and ^{14}C-radioactivity was done after deproteination by perchloric acid. Medium samples for glucose and lactate determination were removed from the collecting vessel and mixed with an equal volume of 6% perchloric acid (usually 0.5 ml). Supernatant after centrifugation was neutralized using 30% KOH to remove perchloric acid before determination of glucose and lactate in the sample as described in Sections 2.5.3 and 2.5.4.

Preparation of perfusate sample for analysis of ^{14}C-purine compunds in the second perfusate in the recirculating/non-recirculation modification of the perfusion procedure was carried out in the following manner. The perfusion medium was collected over periods of 30 sec, the red blood cells sometimes present in the initial samples were removed by centrifugation and 2 ml of 60% perchloric acid was added to the supernatant. Protein precipitate was removed by centrifugation and the supernatant was used for isolation and separation of ^{14}C-labelled purine compounds as described in Seciton 2.5.1.

2.5 Analytical methods

2.5.1 Purification of acid soluble extracts and seperation of purines

(a) Purification of extract.

Perchloric acid extracts from liver and perfusion medium samples containing ^{14}C-labelled acid soluble purine compounds were purified using the technique of adsorption onto charcoal with subsequent elution, essentially according to the procedure of Tsuboi and Price (1959) with minor modifications (Walaas et al., 1969). All the purification steps were carried out at 4°C. The main steps were as follows: 0.1 ml of a "cold" carrier solution containing 40 µg each of adenine, adenosine, inosine, hypoxanthine, xanthosine, xanthine, ATP, ADP and AMP was added to either 4 ml of perchloric acid liver extract or 4-8 ml of perfusion medium extract followed by the addition of 40 mg of activated charcoal. The activated charcoal had been previosuly washed twice with 0.1N HCl and three times with distilled water, and stored as charcoal suspension containing 20 mg/ml. 2 ml of charcoal suspension was added to the PCA extract and shaken for 30 min at 4°C to allow adsorption of purine nucleotides. The charcoal after adsorption of purine material was washed three times with 4 ml deionized water to remove perchloric acid and perchlorate, and the pH was adjusted by a final washing with 4 ml 0.05M acetate buffer, pH 4.5. Elution of purine material from the charcoal was achieved by shaking with 4 ml 5% (v/v) pyridine solution in 50% (v/v) ethanol for 1 hr at 4°C. This procedure was repeated and followed by elution for 30 min at room temperature. The elutants were pooled and charcoal removed

by centrifugation and filtration using glass fibre filters (Whatman, GF/G). Ethanol was removed by rotary evaporation prior to freeze drying. Freeze dried material was dissolved in 0.1 - 0.2 ml of 0.01 M HCl and stored at -20°C until separated.

65% of the radioactive material present in the supernatant was recovered from the pyridine fraction (Figure 8). Total level of radioactivity in the supernatant and homogenate indicated that the radioactive material was predominantly acid soluble. Only 95% of this was adsorbed onto charcoal. Loss of material during the two water washes, and one buffer wash was under 2%. Thus nearly 30% of radioactive material was still associated with charcoal after elution with the pyridine solution. Studies involving recovery of individual radioactive purine compounds from a mixture (Table 1) revealed that individual recovery of the various compounds from the mixture was similar at approximately 70-80%. This level of binding of the individual purine compound to charcoal even after three pyridine washes adequately explains the nature of material lost on charcoal when liver samples were purified. 17% loss of radioactive material occurred during concentration of the pyridine eluent on the rotary evaporation and during freeze-drying. Thus only 54% of the material was recovered after purification. The loss of material was approximately equal for the various purine compounds. Design of the experiments using this technique was such that control and test samples were always treated identically so that the effect of disproportionate loss of a particular compound would be minimized.

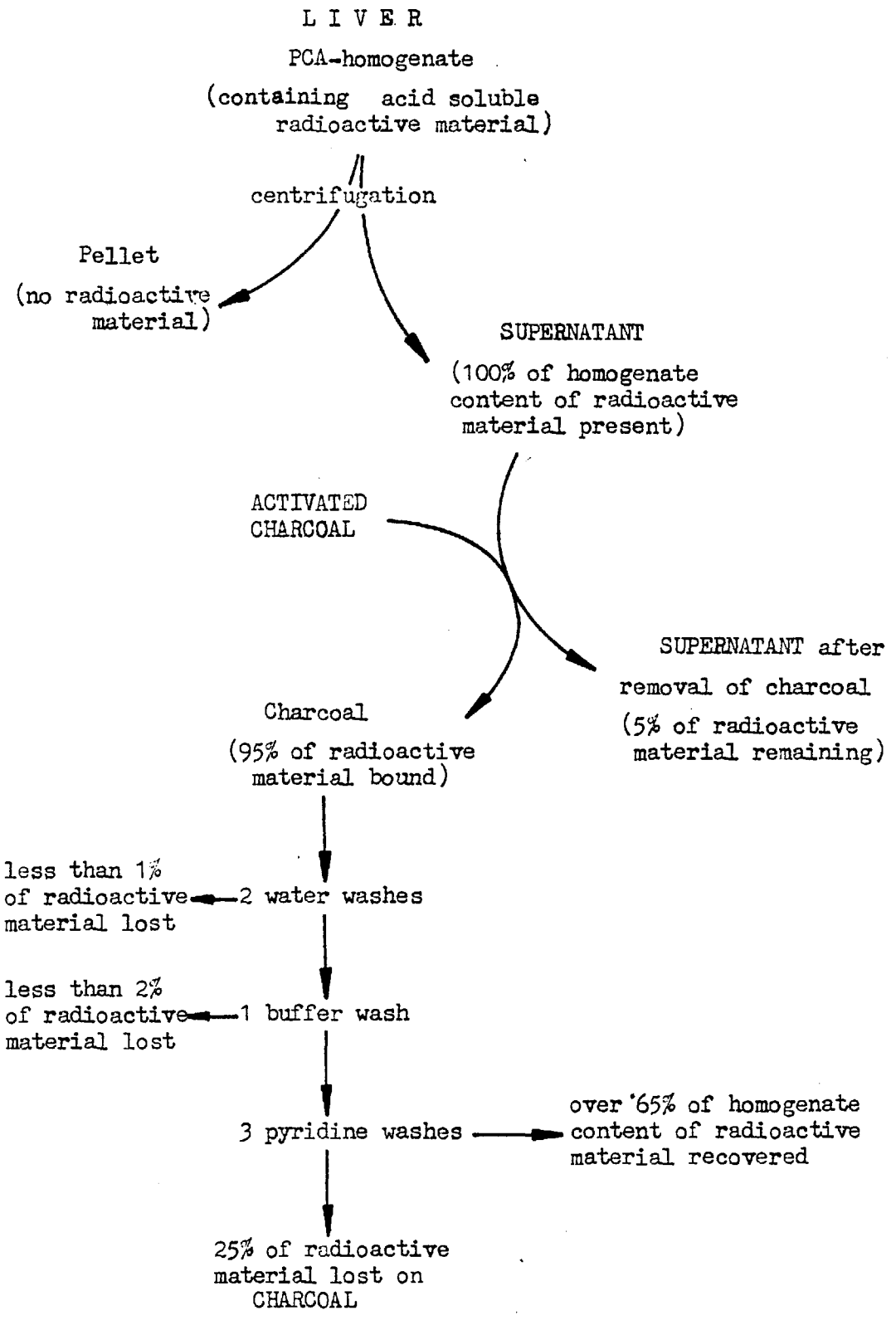


Figure 8: Recovery of ^{14}C-labelled purine material during purification of hepatic extract

Table 1: Recovery of individual purine compounds after adsorption onto charcoal.

In this study 40ug of ^{14}C-labelled purine compound was adsorbed onto charcoal (20ug) in the absence or presence of other purine compounds (40ug each of adenine, adenosine, inosine, hypoxanthine, AMP and ATP). The charcoal was washed with water and pH adjusted by acetate buffer before elution of purine material by pyridine mixture. Recovery of ^{14}C-labelled material from the pyridine washes was documented. Results are expressed as percent recovery of the original amount added to the charcoal mixture.

	Recovery of individual purine compound when adsorbed onto charcoal separately	Recovery of individual purine compound when present in a mixture of other purine compounds.
	(Percent of the original material recoverd)	
Adenine	95	86
Adenosine	88	67
Inosine	87	70
Hypoxathine	91	80
Adenosine 5'-monophosphate	90	68
Adenosine 5'-triphosphate	88	71

(b) Separation of ^{14}C-labelled purine material using thin-layer chromatography.

^{14}C-labelled purine compounds after initial purification by charcoal were separated by thin-layer chromatography using silica and PEI-cellulose plates.

Silica plate : Complete separation of major components of the purine pool was carried out using silica plates with 2-dimensional development of the chromatogram with solvents described by Pull & McIlwain (1972) and Haslam and McClenaghan (1974). The plates were prepared using a 30% (w/v) slury of silica gel GF 254 (Merck) spread to a thickness of 0.25 mm on glass plates (20 cm x 20 cm). The plates were initially air dried and then heated to 120-140^{o}C for 1 hour, to completely dry the plates, before chromatography. 20-40 µl of the purified extract was carefully applied to a spot 2.5 cm from two of the sides and dried using cold air. The plate was developed in the first direction using solvent system consisting of butanol:ethyl acetate:methanol:0.88 (7:4:3:4) until 2 cm from the top and then dried in cold air. The plate was then developed in the second direction using solvent mixture of butanol:acetone: acetic acid: 2M ammonium hydroxide: water (45:15:10:4:26) again until 2cm from the top of the plate. The plates were air dried, and the purine compounds were visualized as dark spots, against the fluorescent background provided by the indicator, using ultra-violet light of 254 nm. Separation of a test mixture by this system is illustrated in Figure 9 . Separation of purine compounds using similar methods with similar profiles has been well documented (Crabtree & Henderson, 1971 ; Das, 1978; Smith et al., 1977). Silica from each spot was removed and placed in a scintillation vial and 12 ml of

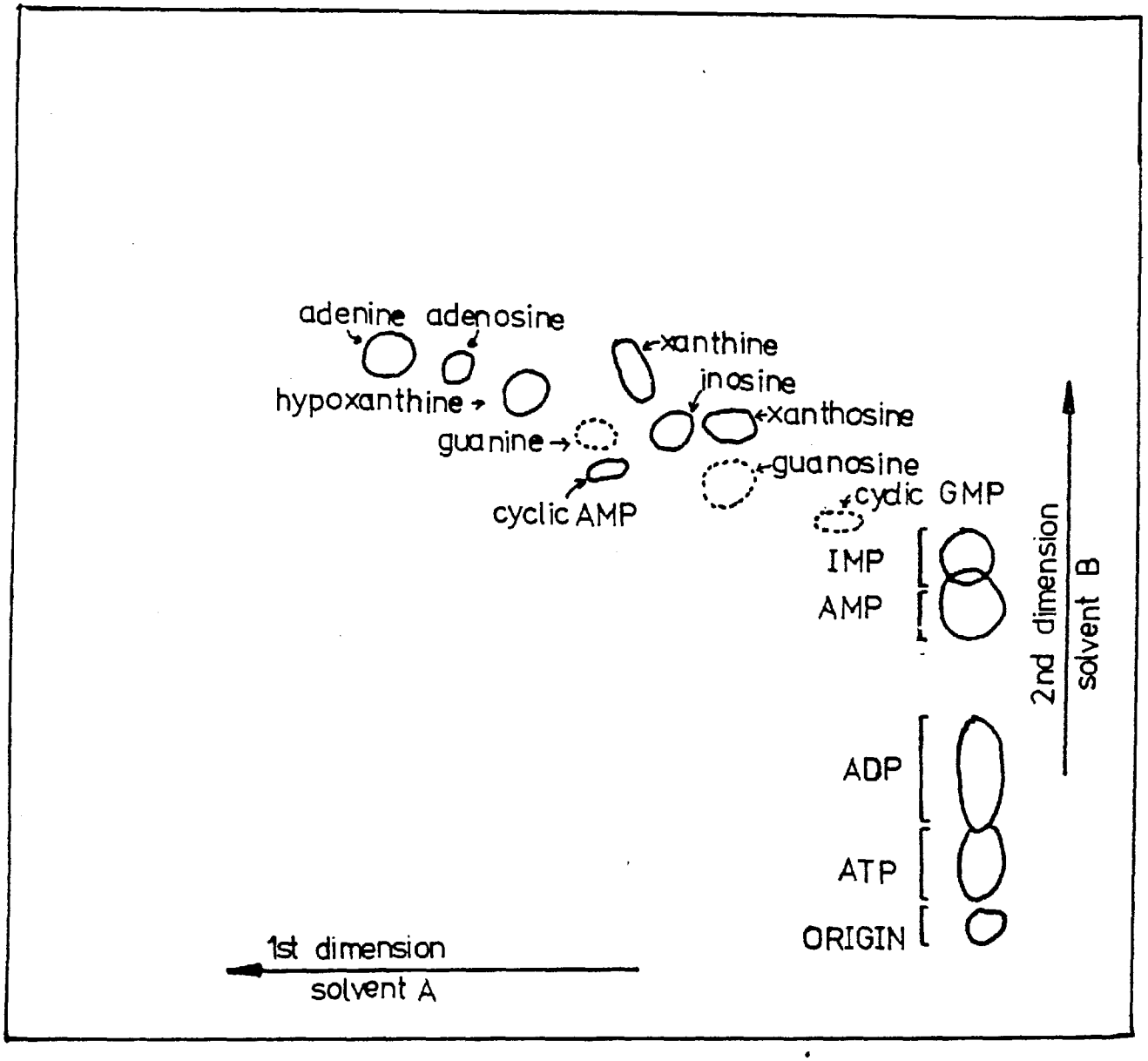


Figure 9: Seperation of purine compounds using silica gel thin layer chromatography

A standard mixture containing 4µg each of compound was developed two dimensionally in solvent A butan-1-ol/ethyl acetate/methanol/0.88 ammonia (7:4:3:4 by vol.) and solvent B butan-1-ol/acetone/acetic acid/2M ammonium hydroxide/water (45:15:10:4:26 by vol.) , as described in the text.

toluene containing butyl PBD added. The radioactivity was determined in a Packard scintillation counter as described in Section 2.6.

PEI-cellulose plates: Separation of the purine mononucleotides by silica thin layer plates as described above was found to be unsatisfactory. Polyethyleneimine cellulose thin layer impregnated with fluorescent indicator on plastic sheets were used to separate purine nucleotides by 1-dimensional chromatography based on the principle of ion exchange chromatography essentially as described by Randerath & Randerath (1964) with modification by Crabtree & Henderson (1971). A wick of Whatman No. 1 paper was stapled to the top of the sheet, which was developed for 5 hours with 4 M sodium formated buffer, pH 3.4, air dried and then developed overnight with methanol:water (1:1). After drying 10-20 µl of extract plus about 10 µg each of purine nucleotide was applied as a spot 2.5 cm from the bottom of the sheet. This was developed overnight with methanol:water (1:1) at 4°C to wash salts, purine bases and nucleosides onto the paper wick. For separation of the purine nucleotides the sheets were developed with increasing concentrations of sodium formate buffers, pH 3.4, as follows: 0.5M formate buffer to a line 2.9 cm above the origin, then 2.0M formate buffer to a line 7.0 cm above the origin, and finally 4.0M formate buffer to the top of the plate. The sheets were dried and the purine nucleotide spots were visualized as dark spots under 254 nm UV light as before (Figure 10). The cellulose area corresponding to the spot was cut out and placed in a scintillation vial and the radioactivity determined as described in Section 2.6., using toluene containing butyl PBD.

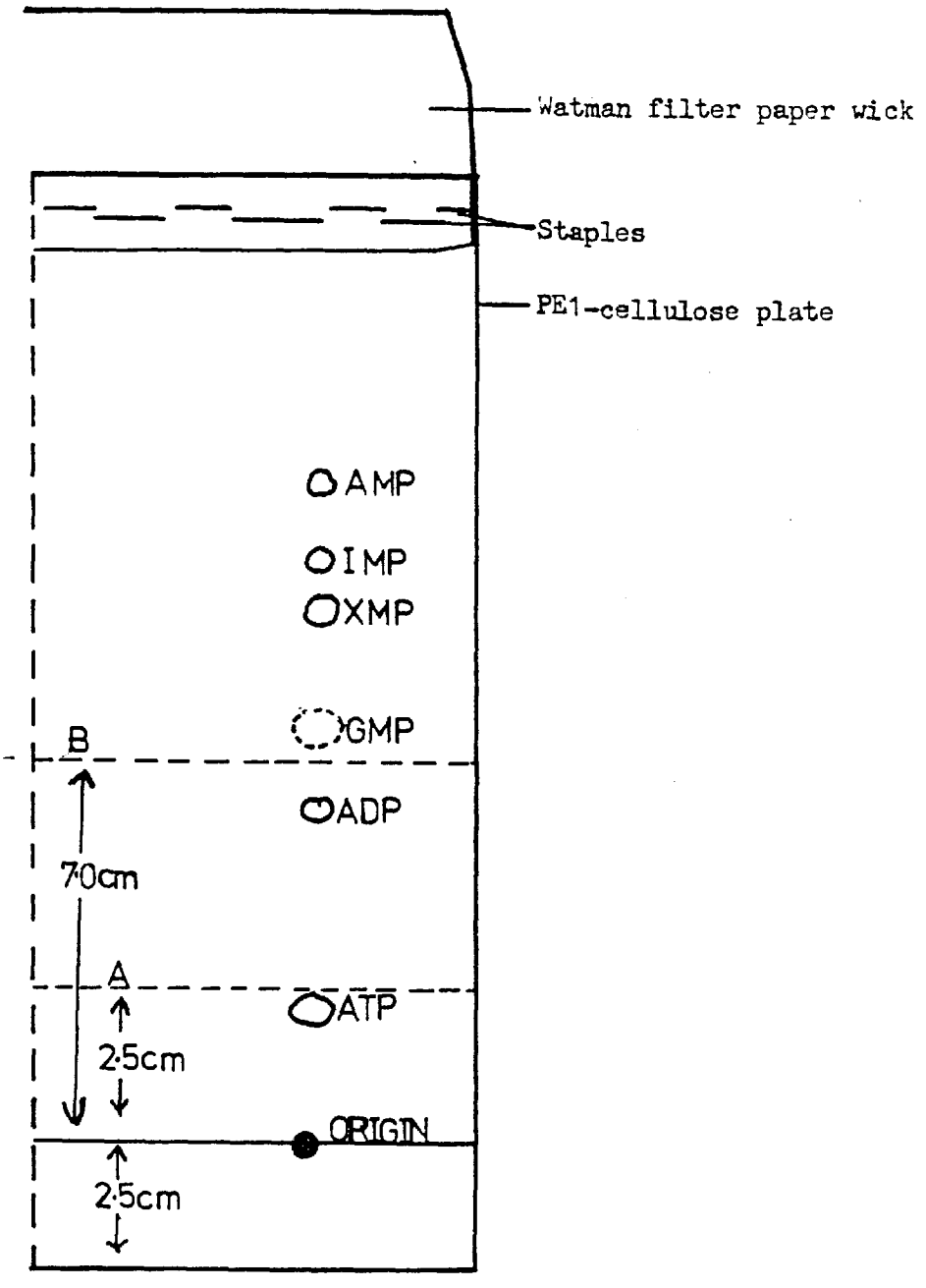


Figure 10: Seperation of purine nucleotides using PEI-cellulose thin layer chromatography

A standard mixture containing 4 µg of each nucleotide was developed by ascending chromatography in one dimension using increasing concentration of sodium formate buffers, pH 3.4. The chromatogram was developed to a line 2.5 cm above origin using 0.5 M buffer, then 7.0 cm above origin using 2.0 M buffer and then to the top using 4.0 M buffer, as described in the text.

Determination of radioactivity in the above two systems (silica powder at the bottom of the vial or a thin cellulose layer with variable orientations) lowered counting efficiency by 8-10%. The radioactive compounds remained bound to the chromatography material used for separation. This type of quenching was different from the normal chemical quenching observed since the channels ratio method used to determine chemical quenching (Section 2.6) could not detect it. In order to assess whether efficiency of counting could be improved, the silica and the cellulose sheet (after cutting it into small pieces) was suspended in the scintillant with carbosil. This did not improve counting efficiency. Thus the drop in efficiency of isotope determination resulted in an effective loss of 10%.

(c) Construction of ^{14}C-labelled purine pool intermediate profile

Loss of radioactive material during charcoal purification was similar for the individual purine pool intermediates tested in the presence of other purine compounds. The loss of these compounds by remaining adsorbed onto the charcoal was similar to that seen during purification of the liver extract where similar amounts of 'cold carriers' compounds were used. Thus it was concluded that under the conditions of the extraction, recovery of individual purine compounds was similar (i.e. the loss of material during purification of experimental sample was due to equal losses of the various constituents).

Therefore in estimating the hepatic content of the ^{14}C-labelled purine compounds the losses during purification,

freeze-drying and ^{14}C-isotope counting were taken into consideration. The levels were calculated in terms of the ^{14}C-adenine originally used for prelabelling the liver.

2.5.2. Determination of adenine nucleotides

Adenosine nucleotides ATP, ADP and AMP were determined spectrophotometrically using enzymatic procedures which involved pyridine nucleotides.

(a) Adenosine triphosphate (ATP)

ATP was determined using hexokinase and glucose-6-phosphate dehydrogenase according to the method of Lamprecht & Trantsehold (1963).

It is a double step procedure first involving phosphorylation of glucose and then oxidation, with NADP as the final electron acceptor, and the NADPH produced was measured spectrophotometrically.

$$\text{ATP} + \text{glucose} \xrightarrow{\text{hexokinase}} \text{ADP} + \text{glucose-6-phosphate}$$

$$\text{Glucose-6-phosphate} + \text{NADP} \xrightarrow{\text{G-6-P dehydrogenase}} 6\text{-phosphogluconate} + \text{NADPH}^{+} + \text{H}^{+}$$

Assay procedure: The neutralized hepatic perchloric acid extract (usually 0.2 ml) was added to a cuvette containing Tris-HCl buffer pH 7.5 (67 mM), magnesium chloride (33 mM), glucose (1.7 mM) and NADP (0.45 mM) in a final volume of 3.0 ml.

5 ul glucose-6-phosphate dehydrogenase (1 mg/ml) was added to the cuvette to oxidize any endogenous glucose-6-phosphate present in the extract. Optical density (O.D. 1) at 340 nm was determined after 5 min.

or until the value was steady. 5 µl of hexokinase (10 mg/ml) was then added to initiate phosphorylation and the new steady optical density value (O.D. 2) determined.

Under the stated conditions the reactions are stoichiometric. The amount of ATP present, was calculated from the optical density change as illustrated below.

$$\frac{O.D._{ATP} \times V_A \times V_E}{\varepsilon_{340} \times d \times V_p} = \text{umoles ATP/g tissue}$$

where:

$O.D._{ATP} = O.D._2 - O.D._1$

V_A = volume of tissue extract in the cuvette (3.0 ml)

V_E = total volume of tissue extract (6.6 ml/gm liver)

V_p = volume of extract added to the cuvette (0.2 ml)

d = light path of the cuvette (1 cm)

ε_{340} = extinction coefficient of NADH at 340 nm (6.22 cm^2/umol at 25°C)

(b) Adenosine diphosphate (ADP) and adenosine monophosphate (AMP)

ADP and AMP levels in tissue extracts were measured using the enzymatic method developed by Adams (1963). In this method ADP is phosphorylated by phosphoenolpyruvate (P.E.P.) in the presence of pyruvate kinase (PK). The pyruvate formed is reduced using NADH by the enzyme lactate dehydrogenase (LDH) and the fall in NADH concentration was measured spectrophotometrically. AMP was

phosphorylated with ATP and myokinase (MK) in the same cuvette and the resulting two equivalents of ADP determined as above.

$$AMP + ATP \xrightarrow{\text{myokinase}} 2\ ADP$$

$$2\ ADP + 2\ \text{phosphoenolpyruvate} \xrightarrow{PK} 2\ ATP + 2\ \text{pyruvate}$$

$$2\ \text{pyruvate} + 2\ NADH + H^+ \xrightarrow{LDH} 2\ \text{lactate} + 2\ NAD^+$$

Assay procedure: The neutralized hepatic perchloric acid extract (usually 0.2 ml) was added to a cuvette containing triethanolamine (TEA) buffer with EDTA pH 7.5 (80 mM TEA, 0.5 mM EDTA), potassium chloride (83 mM), magnesium chloride (33 mM), $NADH_2$ (0.125 mM), phosphoenolpyruvate (0.67 mM) and ATP (33 µM) in a final volume of 3.0 ml.

5 µl of lactate dehydrogenase (10 mg/ml) was added to reduce the endogenous pyruvate present in the sample. Optical density at 340nm was determined after 5 min (O.D. 1) or until the value was steady.

5 µl of pyruvate kinase (2 mg/ml) was then added to initiate ADP phosphorylation and the pyruvate produced was reduced as above. The new steady optical density value (O.D. 2) determined.

Finally 5 µl of myokinase (5 mg/ml) was added to convert AMP to ADP and the new steady optical density value (O.D. 3) determined.

The amounts of ADP and AMP present were determined from the optical density changes $O.D._2 - O.D._1$ and $O.D._3 - O.D._2$ respectively. The calculation was as above for ATP (2.5.2.a) and the results expressed as umoles/g fresh liver.

2.5.3 Lactate determination

Lactate was measured spectrophotometrically essentially as described by Hohorst (1963). The assay is based on the conversion of lactate to pyruvate in the presence of lactate dehydrogenase, and the $NADH_2$ produced, which is proportional to lactate present in the sample, is measured spectrophotometrically.

$$NAD + \text{lactate} \xrightarrow{\text{lactate dehydrogenase}} \text{pyruvate} + NADH_2$$

The neutralized perfusate extract (0.1 - 0.2 ml) was added to a cuvette containing hydrazine-glycine-EDTA buffer pH 9.3 (hydrazine sulphate 150 mM, glycine 100 mM, EDTA 10 mM and NAD 15 mM in a final volume of 3.0 ml.

The sample was added last, contents of the cuvette mixed and the O.D. at 340 nm measured. 5 µl of L.D.H. were added and the new steady O.D. was measured.
Calculation: The increase in optical density observed is due to the production of NADH in the reaction and is proportional to the lactate present in the cuvette since the reaction is stoichiometric under the stated conditions. The amount of lactate present was calculated as follows:

$$\frac{\text{O.D.} \times V_A \times V_E \times D_f}{\varepsilon_{340} \times d \times V_p} = \mu\text{mol lactate/ml perfusion medium}$$

where:

V_A = volume of test mixture in the cuvette (3.1 ml)

V_E = volume of perfusate sample taken (0.5 ml)

D_f = dilution factor (deproteination with PCA and neutralization)

d = light path of cuvette

V_p = volume of extract added to cuvette (0.2 ml)

ε_{340} = extinction coefficient at 340 nm (6.22 $cm^2/\mu mol$)

2.5.4 Glycogen and glucose determination

(a) Glycogen

About 0.5 gm of frozen liver was ground to a powder and boiled for 1 hr. in 9 volumes of aqueous 30% (w/v) KOH. The glycogen was precipitated with 3 volumes of ethanol (modification of the method by Good et al. 1933) and left overnight at 4°C. The precipitate was than sedimented by centrifugation at 4°C at 14,000 r.p.m. for 20 min., the supernatant discarded and the pellet dispersed in 10 ml water, using a motor driven pestle. A suitably sized sample (usually 0.15 ml containing 0.05-0.3 µmol of glycogen-glucose was then hydrolysed to glucose with 30 µl amyloglucosidase in 25 mM sodium acetate pH 4.8 (final volume 1 ml) for 1 hr. at 37°C (Lee & Whelan 1966). The glucose produced was determined as described below.

(b) Glucose

Glucose in the neutralized perchloric acid extract of the perfusion medium or after the hydrolysis of glycogen was determined enzymatically using glucose oxidase and peroxidase with the oxygen acceptor aminophenazone (Trinder, 1969).

Principle:

$$\text{Glucose} + O_2 \xrightarrow[\text{oxidase}]{\text{glucose}} \text{gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{aminopenazone} \longrightarrow \text{coloured a.p.} + H_2O$$

Assay procedure:

Glucose reagent composition

10 ml phenol solution (0.334% phenol in 3% NaCl solution)

1 ml 3.33% (w/v) aminophenozone solution

89 ml 0.5 M phosphate buffer pH 7.3

12 mg glucose oxidase

4 mg peroxidase.

The appropriate volume of the test sample (0.1 - 0.3 µmol) was made up to 1 ml with distilled water. 3 ml of freshly prepared glucose reagent was then added. The mixture was incubated at 37°C for 45 min. Optical density was read against reagent blank at 515 nm. Accurate glucose determination were made with the use of calibration graph.

2.5.5 Assay of glycogen metabolising enzymes

(a) Phosphorylase a

This assay, designed to measure the a form only, was based on the incorporation of ^{14}C-glucose, from labelled glucose 1-phosphate, into glycogen originally described by Cornblath et al. (1963), and essentially as described by Das & Hems (1974), with minor modification (Whitton & Hems, 1976; Stalmans & Hers, 1975).

Assay: 0.3-0.5 gm of liver sample was powdered at liquid nitrogen temperature and homogenized with exactly 3.5 volumes of 50 mM glycylglycine buffer pH 7.0 containing 100 mM NaF. 20 µl of homogenate was added to 200 µl of substrate mixture and incubated for 10 min. at 30°C.

The final assay mixture consisted of glycylglycine buffer (5 mM) glycogen (1% w/v) ^{14}C glucose-1-phosphate (50 mM, sp. act. 16,000DPM/µmol), EDTA (10 mM), NaF (150 mM) and caffeine (0.5 mM), but not AMP (Stalmans & Hers, 1975), the final pH was 6.5.

Reaction was stopped with 1 ml of ice-cold 6% (w/v) TCA containing glycogen (1 mg/ml) and lithium bromide (2 mg/ml).

Glycogen was precipitated by 2 volumes of 95% (v/v) ethanol and left overnight at 4°C. The sample was filtered on a glass filter (Millipore), and washed 6 times with 3 ml 66% (v/v) ethanol to remove contaminating

radioactive material. The filter with precipitate was put in a scintillation vial and 1 ml of 0.1 M sodium acetate pH 5.0 containing 5 µl amylo 1,4-1,6 glucosidase was added. The vial was left overnight at room temperature. 16 ml scintillation fluid (2.5 l 2-methoxyethanol, 2.5 l toluene and 20 gm butyl PBD) were added and the radioactivity determined in a Packard scintillation spectrometer. Phosphorylase _a_ rates were calculated as µmol of G-1-P incorporated into glycogen/min/g liver.

(b) Phosphorylase _a_ phosphatase

This enzyme which catalyzes conversion of phosphorylase _a_ to _b_ , was measured by determining the rate of phosphorylase _a_ conversion essentially as described by Whitton & Hems (1977). The assay was carried out under two conditions: (i) where conversion of the endogenous phosphorylase _a_ was measured, and (ii) where conversion of externally added muscle phosphorylase _a_ was measured.

Assay

(i) Endogenous: 0.3 - 0.5 gm of powdered tissue were homogenized with 3.5 volumes of 50 mM glycylglycine buffer. Homogenate was incubated for various times at 30°C and 20 µl was removed for phosphorylase _a_ assay as described above (Section 2.5.5.a)

(ii) Exogenous: 0.3 - 0.5 gm of powdered tissue were homogenized with 19 volumes of 50 mM glycylglycine buffer.

0.5 ml of homogenate was incubated with 20 µl of muscle phosphorylase *a* (100 units/ml) for various times at 30°C, and 20 µl sample was removed for phosphorylase *a* determination (2.5.5.a).

(c) Glycogen synthetase

The assay was based on incorporation of labelled glucose from ^{14}C-uridine diphospho glucose into glycogen originally outined by Villar-Palasi et al. (1966) and essentially as described by Das & Hems (1974), with minor modifications (Whitton & Hems, 1975; Whitton & Hems, 1976). The assay was carried out in the presence or absence of glucose 6-phosphate, which measured total and synthetase *a* activities respectively. For the assay of the *a* form sodium sulphate, which inhibits synthetase *b* , was also present (De Wulf et al., 1968).

Assay: 0.3 - 0.5 gm of powdered liver sample was homogenized with exactly 5 volume of glycylglycine buffer pH 7.0 (50 mM), containing 100 mM sodium fluoride. 50 µl of homogenate was added to 100 µl of substrate mixture and incubated for 15 minutes at 30°C. The final assay mixture contained Tris-HCl buffer pH 7.4 (50 mM), glycogen (1% w/v), ^{14}C-UDP glucose (4.5 mM, sp. activity 130,000 D.P.M./µmol), either with glucose 6-phosphate (7mM) for total estimation or with sodium sulphate (10 mM) for synthetase *a* estimation.

The reaction was stopped by adding 1 ml of ice cold 6% (w/v) TCA containing glycogen (1 mg/ml) and lithium bromide (2 mg/ml).

Remaining procedure as for the assay of phosphorylase a (section 2.5.5a)

2.5.6 Oxygen measurement

Uptake of O_2 by the perfused liver was measured in some perfusions by connecting the output tube to a flow-through cell, containing a pO_2 electrode connected to a Beckman gas analyser. The pO_2 of the input medium was 450 mm Hg., which is less than the pO_2 of the gas mixture because of inequilibration of the medium with the gas mixture in the oxygenator and the porosity to O_2 of the silicone tubing carrying the medium to the liver. The pO_2 of the output medium was 40-60 mmHg. The amount of O_2 bound to Hb was calculated from the O_2-dissociation curve of rat erythrocytes at pH 7.3 (Gray & Steadman, 1964). Other parameters necessary for calculating Hb bound O_2 were the Mol. wt. of haemoglobin (taken to be 67,000), haemoglobulin concentration (32 gm/100 ml R.B.C.) and that 1 mole of Hb at 100% saturation combines with 4 moles of O_2. The amount of O_2 in solution was calculated from a solubility coefficient in serum at 37°C of 0.021 ml gas/ml liquid at 760 nm pressure and 1 ml of O_2 at 37°C was taken to be 39.6 µmole. Oxygen uptake was expressed as µmol/min/g liver.

2.6 Determination of carbon-14 radioactivity by liquid scintillation counting

β-particle emission from carbon-14 present in the samples was measured in a Packard Tri-Carb liquid scintillation spectrometer. The energy from the emitted beta-particle was captured by the organic scintillator butyl-P.B.D. 5-(4-bi-phenylyl)2-(4-t-butyl phenyl)-1-oxa-3,4 diazole dissolved in toluene.
Dry samples were dissolved directly in 12 ml of scintillation fluid (8 gm butyl P.B.D./litre toluene) and placed in a glass scintillation vial ready for counting. Aqueous samples (never more than 0.1 ml of water) were mixed with 12 ml of scintillation fluid and sufficient 2-methoxyethanol to produce a single phase system.

Radioactivity in the vials was measured at 7°C in the Packard Tri-Carb liquid scintillation spectrometer. The D.P.M. present in each vial were calculated from the recorded C.P.M. by a channel ratio method (Baillie, 1960). The method used two channels, one recording the counts over the whole energy spectrum of the radioisotope, and the other only recording counts at the lower energy. The channels were set so that the ratio of counts from an unquenched sample was 0.3 (Fig 11).

i.e. $\frac{\text{CPM in channel B}}{\text{CPM in channel A}} = 0.3$

A series of standard vials containing a known amount of ^{14}C-labelled hexadecane and scintillation fluid, quenched with various amounts of water with sufficient

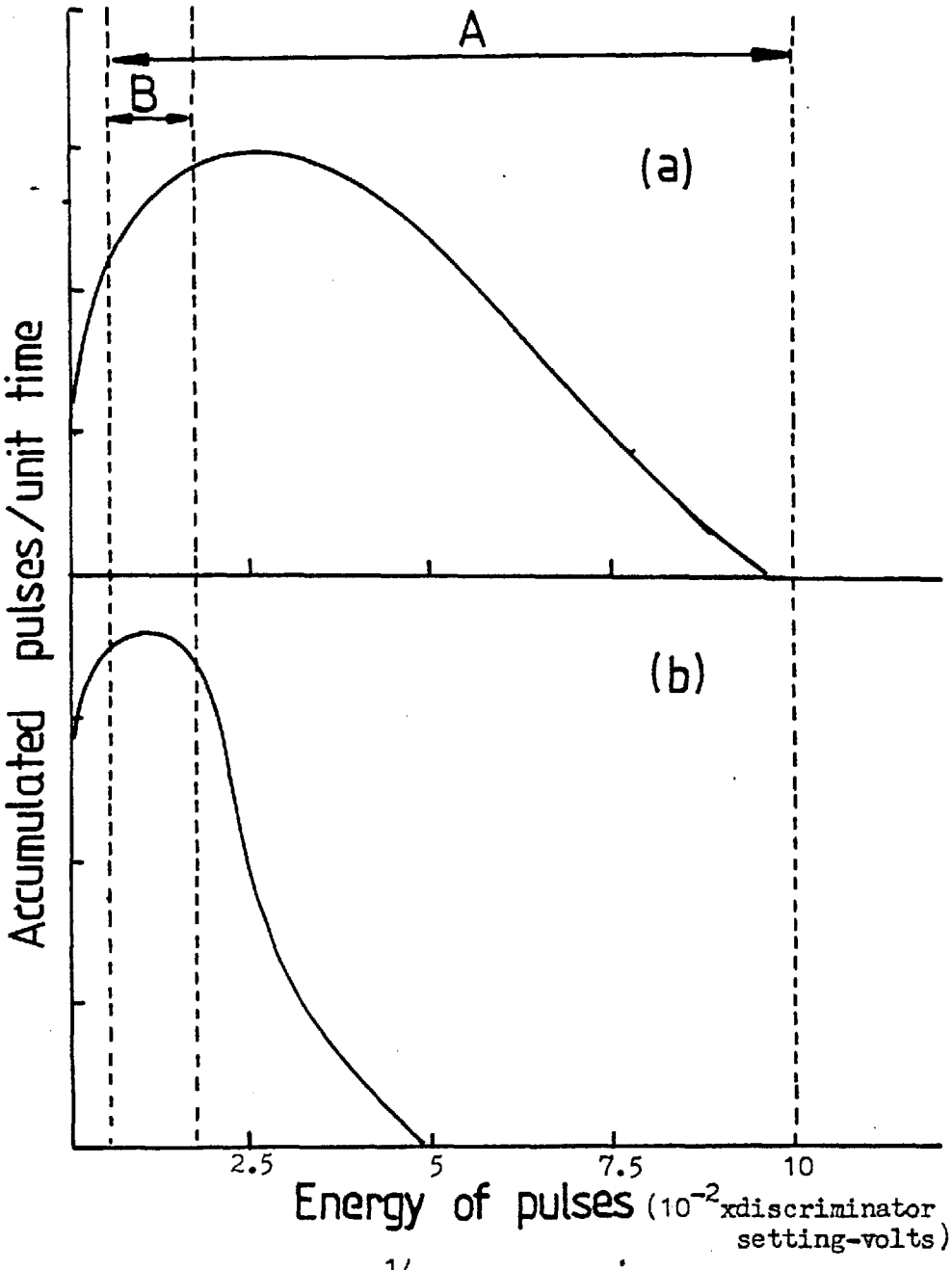


Figure 11: Energy spectrum of ^{14}C and discriminator settings used used to calculate counting efficiency.

(a) Shows the unquenched energy spectrum of ^{14}C and the discriminator settings used to determine the efficiency of counting. (b) Quenching of the sample with water caused the spectrum to be compressed into the lower energy range. This led to an increase in the proportion of counts in channel 'B' and a decrease in the total number of counts recorded (channel 'A'). From Gove, C.D. PhD Thesis (1979).

2-methoxyethanol to produce a single phase system, were used to produce a graph of counting efficiency vs. channels ratio (Fig.12). The counting efficiencies of experimental vials were determined from this standard curve using the channels ratio of the counts obtained for each sample. The efficiency of ^{14}C counting was 55-70% for aqueous and dry samples.

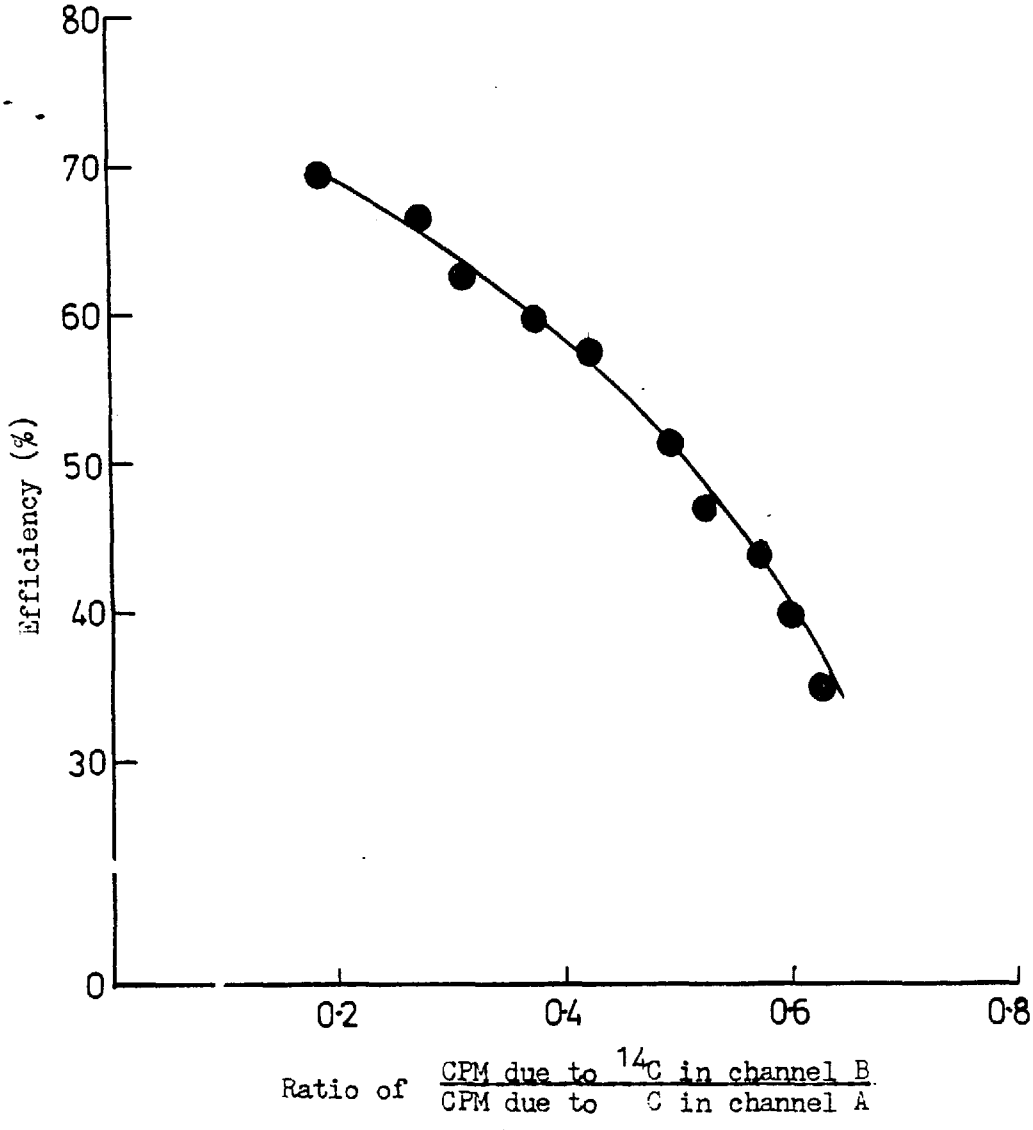


Figure 12: Calibration curve for determination of ^{14}C by liquid scintillation spectrometry.

Chapter 3

RESULTS

3.1 The effect of anoxia and ischaemia on hepatic glycogen and adenine nucleotide metabolism.

3.1.1 Carbohydrate metabolism in aerobic perfused livers.

3.1.2 Effect of graded ischaemia on hepatic carbohydrate metabolism.

3.1.3 Short term effects of total ischaemia on enzymes of hepatic glycogen metabolism.

(a) Activity of phosphorylase a

(b) Activity of phosphorylase a phosphatase

(c) Activity of glycogen synthetase a.

3.1.4 Effect of total ischaemia on hepatic adenine nucleotide concentrations.

3.2 Validation of labelling of the intracellular adenine nucleotide pool using ^{14}C-adenine.

3.2.1 Validation of technique in isolated liver slices.

(a) ^{14}C-adenine uptake by liver slices

(b) Effect of hormones on intracellular ^{14}C-adenine distribution.

3.2.2 ^{14}C-adenine uptake by the red blood cells of the medium and by the perfused liver

(a) ^{14}C-adenine uptake by the perfused liver

(b) ^{14}C-adenine uptake by the red blood cells

(c) Stability and distribution of the ^{14}C-content of the liver

(d) Validation of sequential liver sampling during the perfusion

3.3. Adenine nucleotide metabolism in the perfused liver prelabelled with ^{14}C-adenine.

3.3.1 ^{14}C-adenine uptake and release by the perfused liver under the modified experimental conditions.

3.3.2 ^{14}C-adenine metabolism in the perfused liver.

3.3.3 Release of ^{14}C-labelled material into perfusate under normal aerobic conditions.

3.4. Effect of oxygen lack on ^{14}C-adenine nucleotide metabolism in perfused liver prelabelled with ^{14}C-adenine.

3.4.1 Effect of total ischaemia on hepatic ^{14}C-labelled adenine nucleotide metabolism.

3.4.2 Effect of anoxia on ^{14}C-labelled nucleotide metabolism in perfused liver.

(a) Effect of anoxia on tissue ^{14}C-adenine metabolite levels

(b) Release of ^{14}C-labelled material into perfusate under anoxic conditions

3.5 Effect of hormones on adenine nucleotide metabolism in liver prelabelled with ^{14}C-adenine.

3.5.1 Effect of insulin on ^{14}C-labelled adenine nucleotide metabolism in the perfused liver.

3.5.2 Effect of glucagon on hepatic ^{14}C-labelled adenine nucleotide metabolism.

3.5.3 Effect of vasopressin on hepatic ^{14}C-labelled adenine nucleotide metabolism.

3.5.4 Effect of angiotensin II on hepatic ^{14}C-labelled adenine nucleotide metabolism.

3.1 The effect of anoxia and ischaemia on hepatic glycogen and adenine nucleotide metabolism.

It is well established that lack of oxygen in the liver directly causes rapid acceleration of glycogenolysis manifested by increased degradation of glycogen, and formation of glucose and lactate. There may be activation of glycogen phosphorylase in hypoxic tissue as shown by the increase in content of glucose 6-phosphate (Hems and Brosnan, 1970). Activation of glycogen phosphorylase might be expected to occur in hypoxic liver, since glycogenolysis in general is controlled by the amount of active phosphorylase (the a form). Perhaps, surprisingly, there are no reports of a rapid increase in the amount of phosphorylase a in liver, in response to lack of oxygen, although an increase after a period of cyanide treatment has been shown (Jakob and Diem, 1974). The other equally well known effect of oxygen lack is a rapid increase in the content of AMP (e.g. Hems and Brosnan, 1970; Faupel et al., 1972).

It was thus of interest to study the effect of anoxia on hepatic carbohydrate metabolism and on adenine nucleotide concentrations. In particular it was of interest to assess the role of adenine nucleotides in anoxia.

3.1.1 Carbohydrate metabolism in aerobic perfused livers.

Livers were perfused under standard conditions (Section 2.3), with perfusate containing 2% albumin, 5mM glucose and 25% haematocrit. Under normal aerobic conditions hepatic levels of adenine nucleotides showed very little

change (Table 2) over a period of 60 min as determined by serial biopsy of the perfused liver at 40 and 100 min. The individual levels of adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP) were similar to reported in vivo values (Brosnan and Hems, 1970; Faupel et al., 1972). Stability of the adenine nucleotide pool and the steady oxygen uptake by the liver (Figure 13) over the 100 min perfusion period indicated a metabolically functional liver.

Glycogen breakdown over the 60 min period was about 50 µmol glycogen-glucose degraded per hour per g of wet liver (Figure 14). In these perfusions, performed by Dr. P.D.Whitton, the glycogen level at the end of the perfusion was still 370 umol glycogen-glucose per g, a relatively high value indicating maintenance of glycogen store. Glucose and lactate concentrations in the perfusion medium stabilized at about 8 mM and 2 mM respectively (Figure 14), indicating autoregulation.

Hepatic phosphorylase a activity showed no significant change during perfusion. Its activity, measured in terms of glycogen synthesis from labelled glucose 1-phosphate, being 5 and 7 µmol of glucose transferred from glucose 1-phosphate per g per min at 40 and 100 min respectively.

Since none of the parameters measured changed significantly over 100 min perfusion this was a satisfactory system to assess changes due to ischaemia.

3.1.2 Effect of graded ischaemia on hepatic carbohydrate metabolism.

Livers were perfused at four different flow rates (and constant 12% haematocrit) to assess the effects of

Table 2: Levels of adenine nucleotides in aerobic perfused livers.

Livers of fed rats were perfused with 100 ml perfusate in standard conditions, at a haematocrit of 25%. Liver samples were removed at 40 and 100 min for analysis. Results are mean ± SEM of the number of observations in parenthesis.

Adenine nucleotides (µmol/g fresh livers)	Perfusion time (min) 40	100
ATP	2.3 ± 0.1 (10)	2.4 ± 0.2 (4)
ADP	1.0 ± 0.1 (8)	1.1 ± 0.1 (4)
AMP	0.25 ± 0.02 (10)	0.38 ± 0.04 (4)

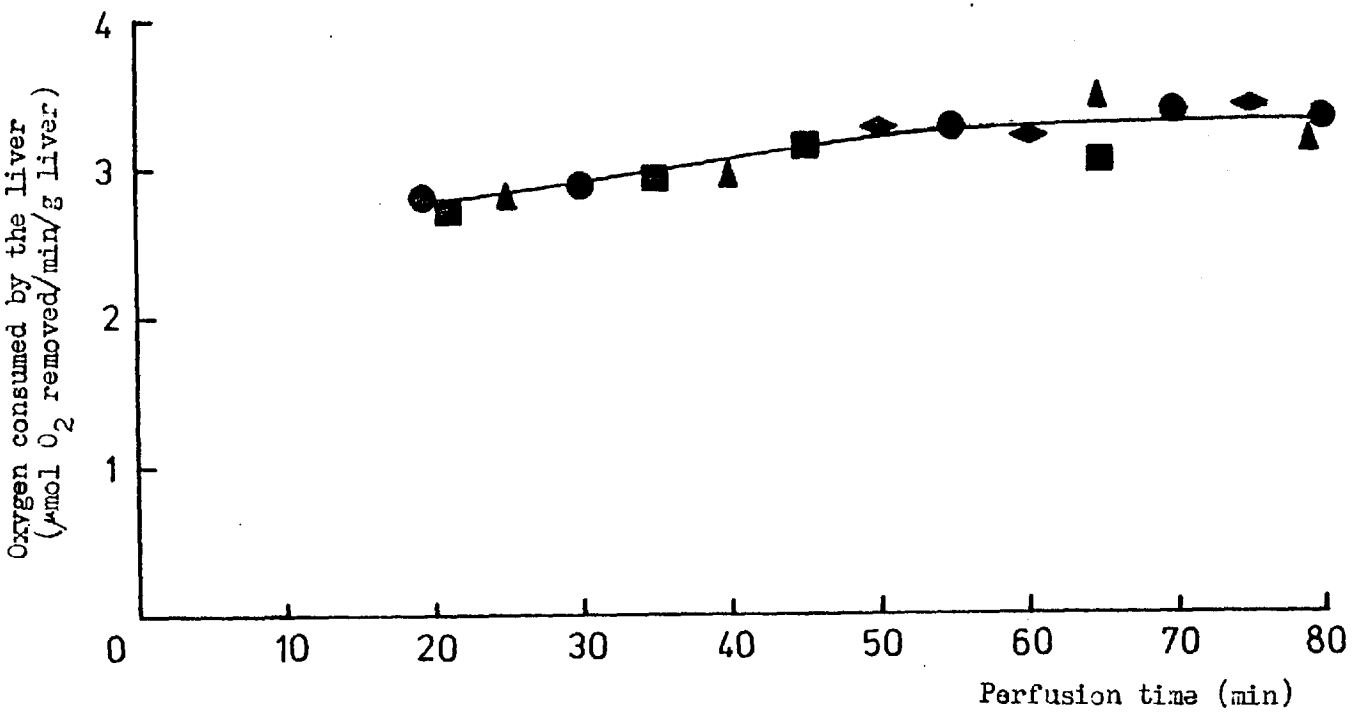


Figure13: Oxygen consumption of the perfused liver

Livers of fed rats were perfused with 60 ml perfusate in standard conditions at 11% haematocrit. Oxygen content of the input and the output perfusate was measured. Each point represents an individual measurement. Results are from four perfusions.

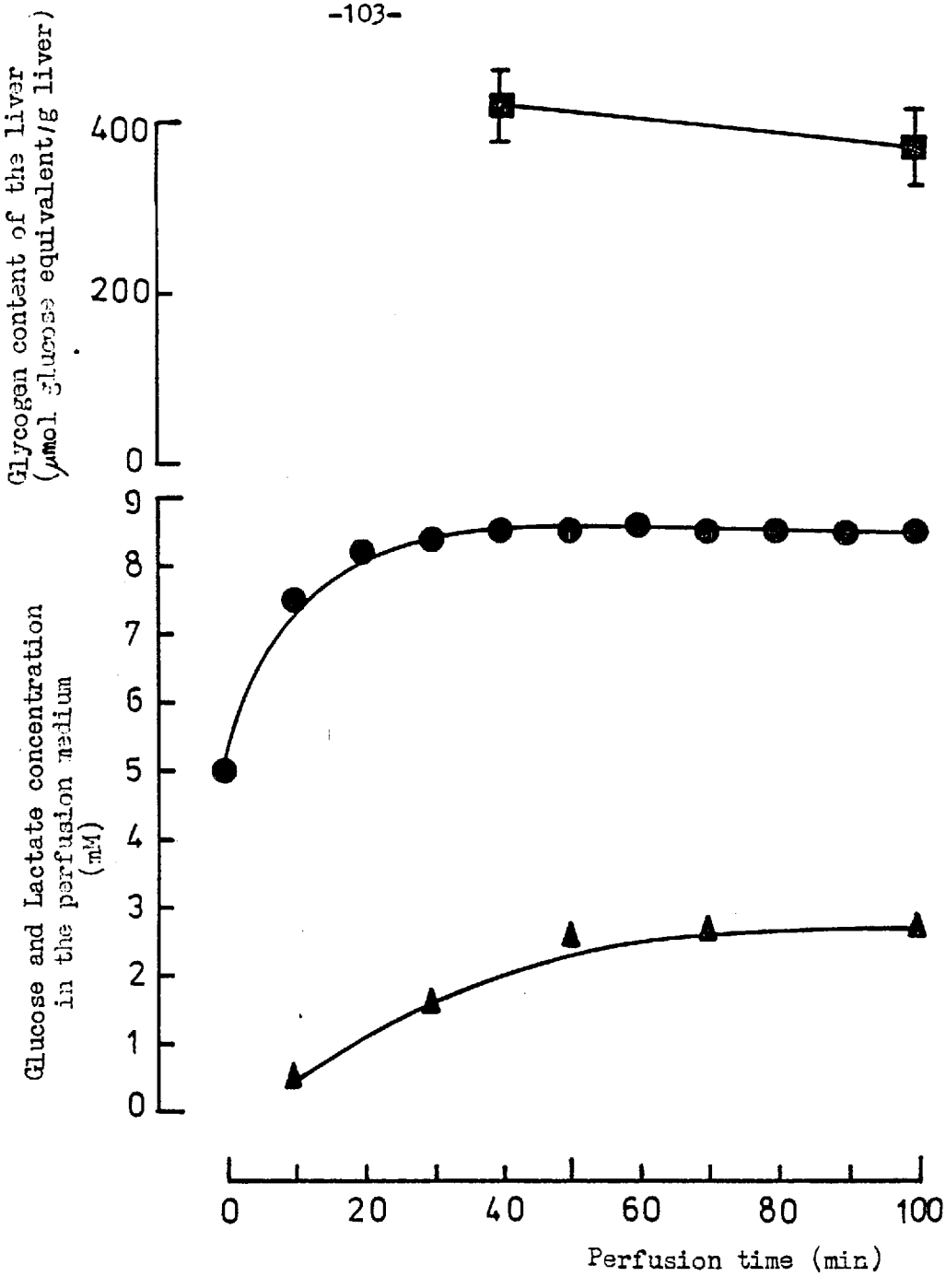


Figure 14 : Relationship between glycogen breakdown and production of glucose and lactate.

Liver of fed rats were perfused as described for Table 2. Initial glucose level in the perfusate was 5.0 mM. Glucose (●) and lactate (▲) were measured in perfusate. Liver samples were removed at 40 min and 100 min for analysis of glycogen (■). Average liver weight after removal of the initial liver sample was 5.6 gm. Results are from three perfusions.

graded ischaemia on metabolism. The flow was regulated by changing the input perfusate pressure in the portal vein. Basal rate of glycogenolysis (Figure 15) and the adenine nucleotide levels (Figure 16) were maintained, at the normal flow rate (i.e. normal input pressure) throughout the 100 min of perfusion. No significant change in the activity of phosphorylase a level was observed between the 38 and 100 min liver samples at all flow rates (Table 3).

Changes in perfusate flow did not produce any major change in any parameter, unless flow was reduced to one quarter of normal (Figure15). In this situation glycogenolysis was accelerated, and the extra glycogen breakdown of 46 µmole of glucose equivalent per h per g accounted for the extra release of glucose (36 µmol) and lactate (9 µmol) expressed as glucose equivalents.

The adenine nucleotide content of the livers exhibited marked changes in the most ischaemic group of perfused livers (Figure 16). The ATP content displayed a greater dependence on flow than the other two nucleotides, as it decreased in the perfusions at intermediate perfusion flow rates. Hepatic AMP content on the other hand rose 3-fold when the flow was reduced to 0.6 ml per min per gm while the rise was much smaller at the other flow rates. The hepatic energy change value (Atkinson, 1966) was lowered to 0.5 at the slowest flow rate from a relatively steady value of 0.76 at the other flow rates.

Thus it was observed that increased hepatic glycogenolysis in response to ischaemia was accompanied by changes in the adenine nucleotide levels. The mechanism of acceleration of glycogenolysis was investigated by studying the

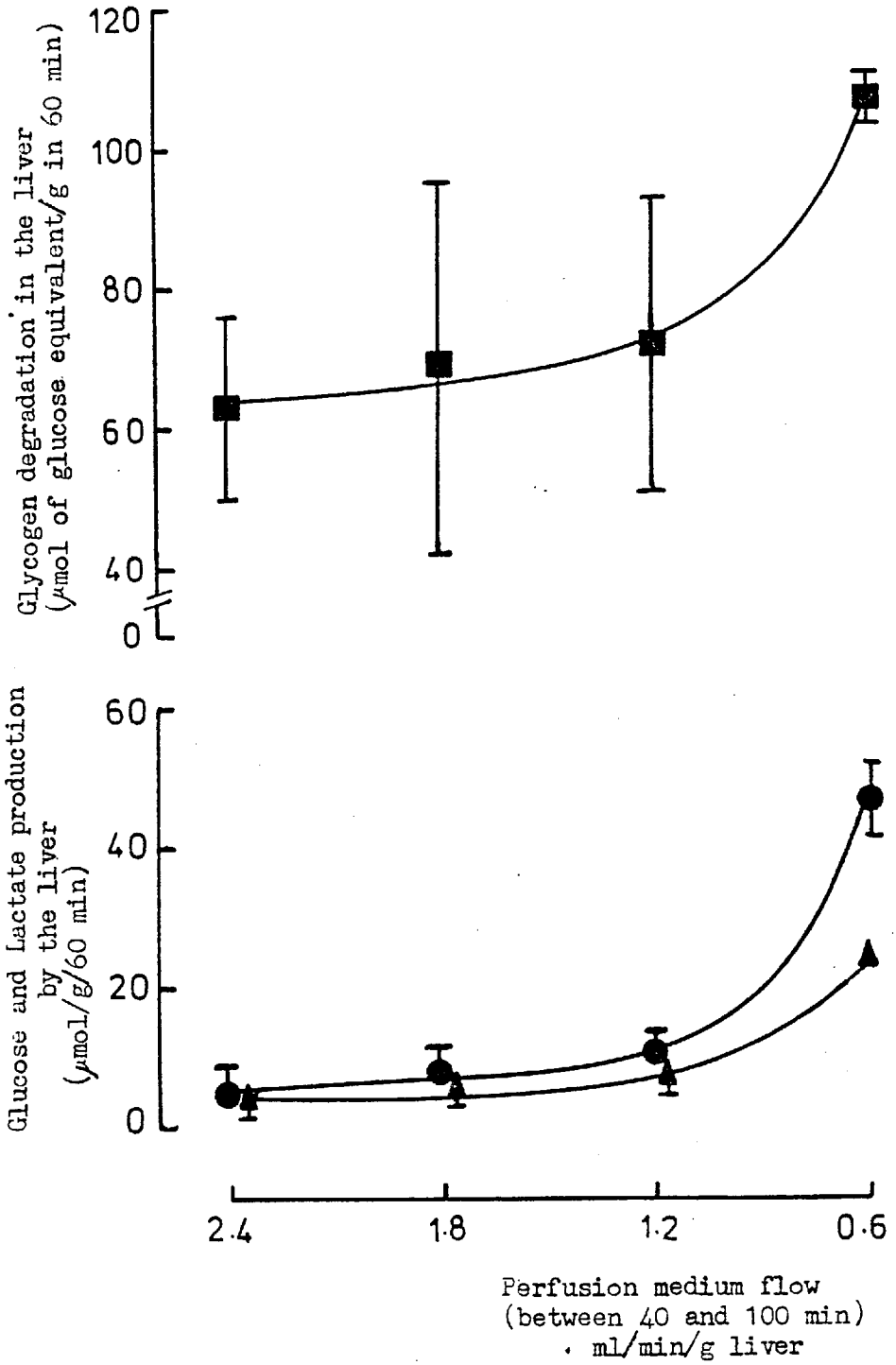


Figure 15: Glycogen breakdown and glucose and lactate production during partial ischaemia.

The livers of fed rats were perfused under standard conditions with 12% haematocrit. Normal flow was maintained for 40 min and then adjusted if necessary by changing the input perfusate pressure in the portal vein. Liver samples were removed at 40 min and 100 min for analysis of glycogen. Results are from three perfusions at perfusate flow of 2.4 and 0.6 ml/min/g and seven perfusions at perfusate flow of 1.8 and 1.2 ml/min/g: glycogen ■; glucose ●; lactate ▲. Bars indicate the SEM.

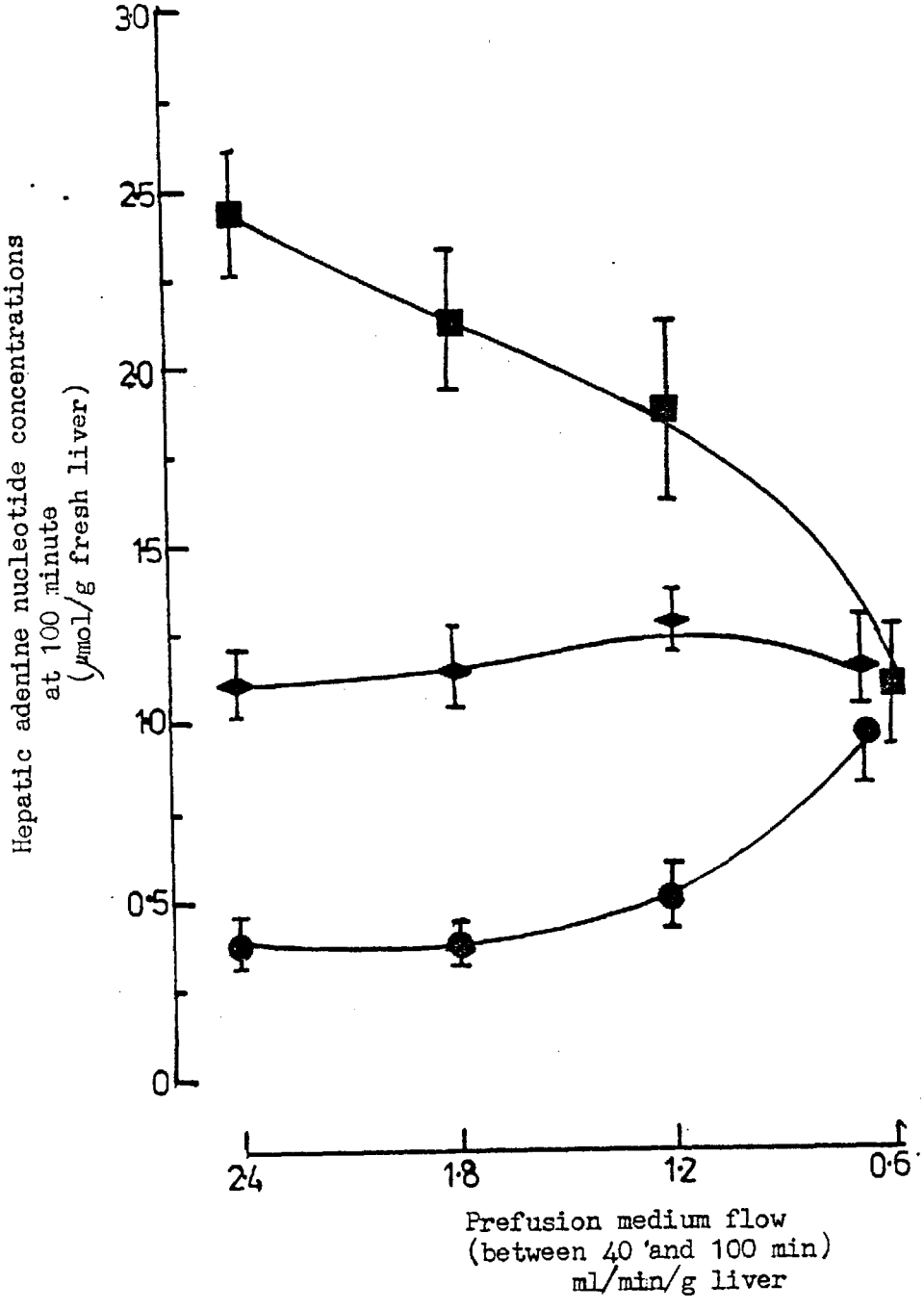


Figure16: Adenine nucleotide levels in partially ischaemic livers.

Experimental detail as in Figure15. Neutralized PCA extract from the final liver sample was used for ATP (■), ADP (◆) and AMP (●) analysis. Bars indicate SEM.

Table 3: Glycogen phosphorylase *a* activity in partially ischaemic livers.

Experimental detail as in Figure 15. Glycogen phosphorylase *a* activity was determined in the liver samples removed at 40 and 100 min. Perfusion medium flow 2.4 ml/min/g was maintained for initial 40 min of the perfusion and then adjusted where necessary by changing the input perfusate pressure, for the remaining time of perfusion. Results are mean ± SEM of the number of observations indicated.

PERFUSION MEDIUM Flow after 40 min (ml/min/g liver)	NO OF PERFUSION	Phosphorylase *a* activity (μmol glucose/min/g wet wt liver) SAMPLING TIME 40 min	100 min
2.4	3	6.7 ± 0.1	5.3 ± 0.2
1.8	7	5.3 ± 0.4	7.6 ± 0.6
1.2	7	4.4 ± 0.4	6.8 ± 0.6
0.6	3	5.4 ± 0.2	7.4 ± 0.8

short term effect of total ischaemia on activity of the enzymes involved in glycogen metabolism.

3.1.3 Short term effects of total ischaemia on enzymes of hepatic glycogen metabolism.

One explanation for the increase in hepatic glycogenolysis seen in response to ischaemia (Section 3.1.2) would be an increase in the activity of the enzyme glycogen phosphorylase. The active form of this enzyme, glycogen phosphorylase *a*, however did not show any significant change over the 60 min period of partial ischaemia (Section 3.1.2). However, it is possible that the response of phosphorylase *a* to ischaemia is very rapid and that after 60 min, the enzyme activity is normalized. In order to evaluate the rapid response of the enzymes of glycogen metabolism to ischaemia, experiments were designed to study the short term effects of changes in blood flow.

(a) Activity of phosphorylase *a*.

Initially the phosphorylase *a* activity was measured in livers from rats killed by cervical dislocation and then made anoxic by cessation of flow . The initial value of phosphorylase *a* activity of 12 µmol of glucose transferred per min per g fresh liver was nearly doubled within one min of total ischaemia (Fig 17). A steady decrease in the phosphorylase *a* activity was seen after the first minute reaching a minimum value of about 4 µmol glucose transferred per min per g at 40 min. This may explain the lack of effect seen in phosphorylase *a* activity in Section 3.1.2.

Phosphorylase *a* activity in perfused livers and

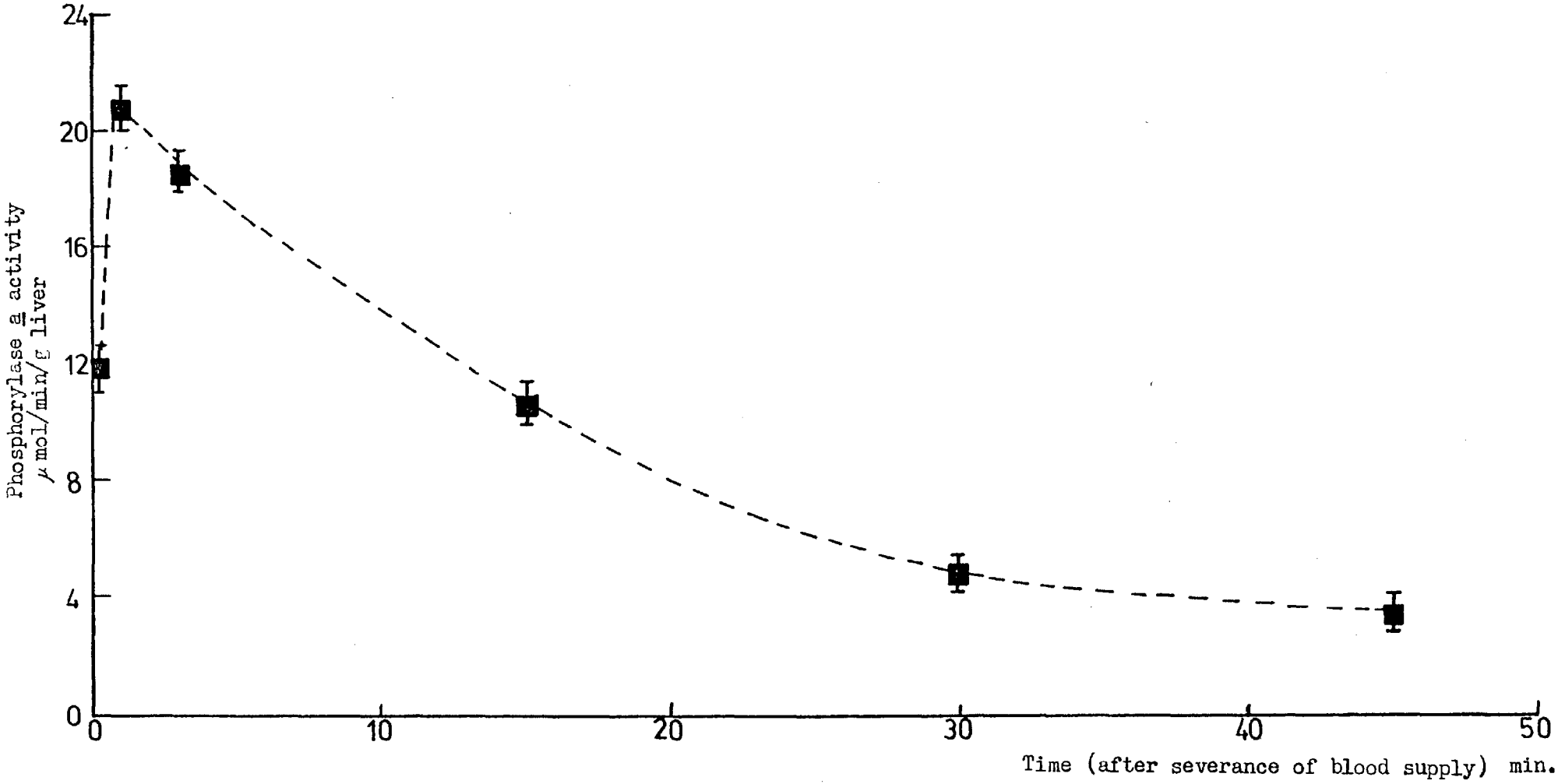


Figure 17: Time course of change in glycogen phosphorylase a activity in anoxic liver in vivo.

Livers in vivo were made anoxic by severance from blood vessels after rapid sacrifice by cervical dislocation. After various periods of total ischaemia, livers were rapidly frozen, and phosphorylase a activity measured. Initial liver sample was removed within 15 sec of cervical dislocation. Each value is mean of 3-6 observations. Bars indicate SEM.

in livers from anaesthetized rats rose by over 100% in the first minute of total ischaemia induced by flow cessation (Figure 18). Initial value of phosphorylase a activity was lower in comparison with those when the rat was killed by cervical dislocation, probably due to faster sampling time.

(b) Activity of phosphorylase a phosphatase.

The increase in phosphorylase a activity in anoxic liver must reflect either increased activity of phosphorylase b kinase, or decreased activity of phosphorylase a phosphatase. From the known properties of the phosphatase, this enzyme would be inhibited during total ischaemia, by the fall in ATP and rise in AMP. It is also possible that a stable inhibition of the phosphatase could occur, e.g. by covalent modification. This was investigated by following the decline in activity of endogenous phosphorylase a in liver during incubation at 30° of homogenates from both aerobic and anoxic livers (Table 4). In livers which were anoxic for 3 or 5 min, the activity of phosphatase in the homogenate was sufficient to destroy more phosphorylase a (than in aerobic livers) if the decrement was expressed in absolute units, whereas the decrement in anoxic liver homogenates was less if expressed as a percentage change. Thus there was no clearcut indication of stable change in phosphorylase a phosphatase activity in anoxic liver.

(c) Activity of glycogen synthetase a.

There are complex inter-relationships between the enzymes glycogen synthetase and glycogen phosphorylase. Hence it was of interest to follow changes in synthetase activity in anoxic tissue. Livers were perfused (to permit freeze-clamping within 2 sec) and then made anoxic through cessation of perfusate flow. A decrease in the proportion

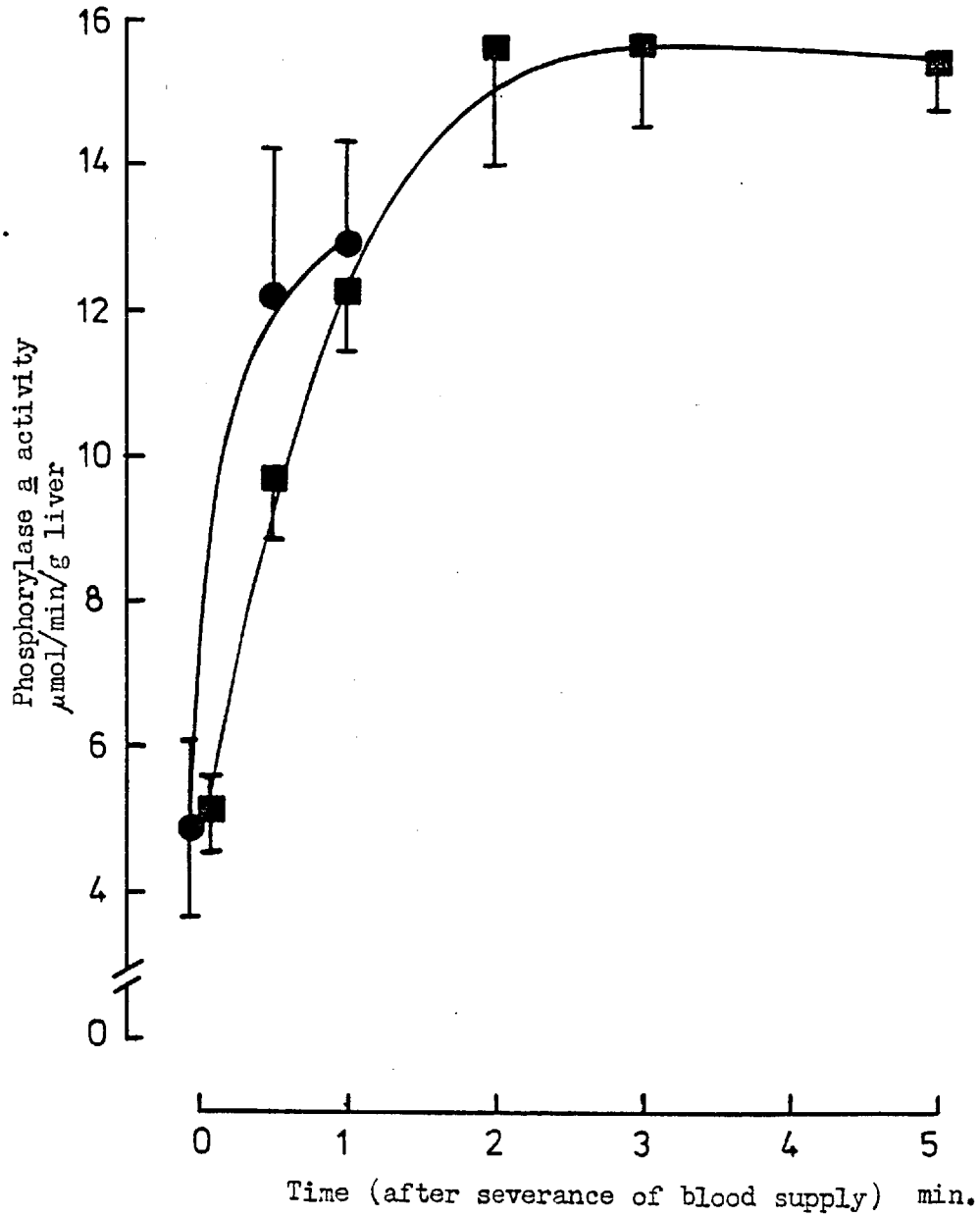


Figure 18: Rapid effect of anoxia on phosphorylase a activity in in vivo and perfused liver

Liver were made anoxic by stopage of flow through perfused livers (■) and livers in vivo from anaesthestized rats (●) were made anoxic by cessation of flow. Initial liver sample was removed within 5 sec and rapidly frozen. Further ischaemic liver samples were removed as indicated. Results are from 4 in vivo experiments and 10-15 perfusions. Bars indicate ±SEM.

Table 4: Influence of total ischaemia on dephosphorylation of endogenous phosphorylase a in liver.

Perfused livers (from the experiments described in Figure 18) were freeze-clamped after various periods of total ischaemia. In each liver, the initial phosphorylase a activity was determined (in the standard assay including fluoride) and the decline in activity during 10 min in a homogenate at 30° (lacking fluoride) was also followed, as a measure of phosphorylase a phosphatase activity. Other details are in the text. Results are means ± SEM of the number of observations in parenthesis.

Duration of total ischaemia	Phosphorylase a activity (μmols glucose/min per g wet wt. of original liver)			
	0 min	"0 min" at 30°	After 5 min at 30°	After 10 min at 30°
	(plus fluoride)	←	(no fluoride)	→
2 sec	5.1 ± 0.7 (11)	4.2 ± 0.5 (3)	1.5 ± 0.2 (9)	0.6 ± 0.1 (10)
3 min	15.6 ± 1.0 (14)	11.2 ± 1.2 (5)	6.9 ± 0.8 (3)	2.1 ± 0.7 (5)
5 min	15.4 ± 0.5 (5)	12.1 ± 0.9 (3)	5.9 ± 2.0 (3)	2.0 ± 0.7 (3)

of synthetase in the a form was observed within one minute of the onset of ischaemia (Table 5).

3.1.4 Effect of total ischaemia on hepatic adenine nucleotide concentrations.

Time courses of changes in adenine nucleotide content were followed in perfused livers which were made anoxic following sudden cessation of blood flow (Figure 19). The most rapid changes occurred in the contents of adenosine monophosphate (AMP) and adenosine diphosphate (ADP), which increased substantially within 0.5 min. The ADP content then declined during the 1-2 minute period of total ischaemia. The adenosine triphosphate (ATP) content of liver fell consistently during 5 min of total ischaemia.

For comparison with the above changes observed in the perfused liver, changes in adenine nucleotides in the in vivo anoxic livers were documented (Table 6). These resembled those reported previously (Hems and Brosnan, 1970; Faupel et al., 1972), and were similar to those in anoxic perfused liver, although ATP fell faster in vivo. The changes in levels of ATP and ADP in response to total ischaemia were more pronounced in liver from starved rats than fed rats (Table 6).

Cyclic-AMP has been implicated in the hormonal control of hepatic glycogen metabolism (Exton et al., 1971b). The possible role of purine nucleoside cyclic monophosphates in increased hepatic glycogenolysis in response to ischaemia was investigated. The contents of the nucleotides cyclic-AMP and cyclic-GMP were measured in control and anoxic liver,

Table 5: Effect of total ischaemia on the activity of glycogen synthetase

Perfused livers (see Figure 18 and Table 4) were freeze-clamped after various periods of total ischaemia. Results are means ± SEM of three measurements.

Duration of total ischaemia	Glycogen synthetase activity (μmols glucose/min/g wet wt.)		
	a	Total	% a
2 sec	0.21 ± 0.02	0.68 ± 0.05	33 ± 1
1 min	0.15 ± 0.01	0.63 ± 0.08	23 ± 1*
3 min	0.13 ± 0.02	0.69 ± 0.03	20 ± 5*

* $P < 0.05$, cf with control value

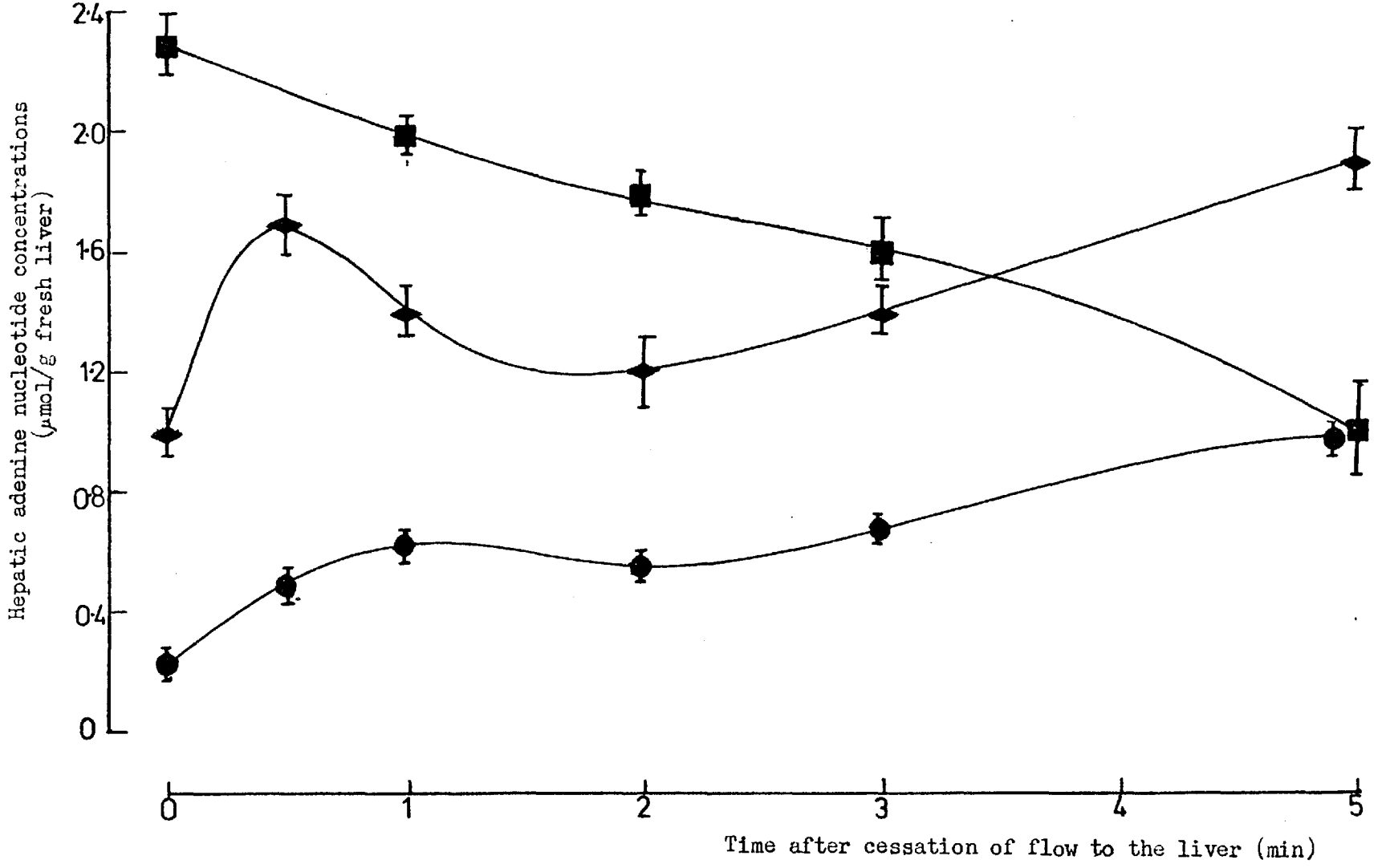


Figure 19: Effect of ischaemia (cessation of flow) on adenine nucleotide content of the perfused liver.

Livers perfused in the normal manner were made anoxic by sudden cessation of flow. Initial liver sample was removed within 3 sec and rapidly frozen using aluminium blocks, pre-cooled in liquid nitrogen. Further anoxic liver samples were removed as indicated. The symbols are (■) ATP; (◆) ADP; (●)AMP. Results are from 10-15 perfusions. Bars indicate ±SEM.

Table 6: Time course of changes in adenine nucleotides in anoxic liver in vivo.

Livers in vivo were made anoxic by severance from blood vessels, either after rapid sacrifice by cervical dislocation or after anaesthesia with Nembutal. After various periods of total ischaemia, livers were rapidly frozen, and adenine nucleotide contents were determined. Results are means ± SEM of the number of measurements in parenthesis.

Rat	Treatment	Time of ischaemia (min)	Adenine nucleotide content (µmol/g of fresh liver) ATP	ADP	AMP	Total
Fed	Cervical dislocation	0.2	2.77 ± 0.17 (4)	1.48 ± 0.04 (4)	0.38 ± 0.04 (4)	4.63
Fed	Cervical dislocation	1.0	2.05 ± 0.32 (3)	1.54 ± 0.10 (3)	0.63 ± 0.08 (3)	4.22
Fed	Cervical dislocation	3.0	1.05 ± 0.15 (3)	1.56 ± 0.27 (3)	0.84 ± 0.16 (3)	3.45
Fed	Nembutal	0.1	3.10 ± 0.19 (3)	0.73 ± 0.07 (3)	0.26 ± 0.05 (3)	4.09
Fed	Nembutal	0.5	2.22 ± 0.03 (3)	1.50 ± 0.09 (3)	0.59 ± 0.10 (3)	4.31
Fed	Nembutal	1.0	1.92 ± 0.13 (3)	1.67 ± 0.07 (3)	0.80 ± 0.10 (3)	4.39
Starved	Nembutal	0.1	2.60 ± 0.14 (6)	1.20 ± 0.09 (6)	0.43 ± 0.04 (6)	4.23
Starved	Nembutal	1.0	1.20 ± 0.11 (6)	2.10 ± 0.09 (6)	1.40 ± 0.10 (6)	4.70
Starved	Nembutal	3.0	0.33 ± 0.93 (6)	1.43 ± 0.10 (6)	2.25 ± 0.12 (6)	4.01

Table 7: Hepatic content of purine nucleoside cyclic monophosphates in total ischaemia.

Control and anoxic liver samples were prepared from rats sacrificed by cervical dislocation, as described in Table 6 and Figure 17. Purine nucleoside cyclic monophosphates were assayed in TCA extracts. Results are means ± SEM of three measurements.

Duration of total ischaemia	Content of nucleoside (pmol/g of wet liver)	
	Cyclic-AMP	Cyclic-GMP
10 sec	1420 ± 50	40 ± 4
2 min	900 ± 30*	19 ± 5*
5 min	1030 ± 50*	26 ± 6

*$P < 0.05$, cf with control value.

and were found to decrease within 2 min of the onset of total ischaemia (Table 7). Assay of the cyclic monophosphates based on the antibody binding principle were performed by Dr C J Davis and Dr K Siddle at the Welsh National School of Medicine, Cardiff. (Ref : Siddle et al., 1973 and Siddle et al., 1976).

3.2 Validation of labelling of the intracellular adenine nucleotide pool using ^{14}C-adenine.

In the previous section the effect of ischaemia and anoxia on hepatic glycogenolysis and adenine nucleotide metabolism was investigated using enzymatic methods for determining the nucleotide concentrations. The metabolic events occurring during ischaemia were accompanied by marked changes in the levels of the three adenine nucleotides. These findings suggest that such changes may be linked to the mechanism of increased hepatic glycogenolysis. However, it is likely that other nucleotide metabolite levels will also change in response to a decrease of over 50% in ATP concentration and a more than 5 fold increase in the AMP concentration. The assay methods for these metabolites, however, are not sensitive enough to permit chemical measurements of the low intracellular levels.

One alternative is to label the adenine nucleotide pool of the liver with a radioactive precursor, a technique which has been extensively used in other tissues (e.g. McIlwain, 1974; Kuo et al.,1974; Mah & Daly, 1975). Hepatic abundance of enzymes of the purine salvage pathway will allow rapid uptake of a number of purine pool metabolites when added externally. After allowing for the distribution of the radioactivity within the various "purine pool" intermediates a radioactive profile would be established and changes in the profile due to different effectors such as hormones and oxygen lack can be assessed.

Radioactively labelled adenine was chosen from the various radioactive precursors available (including adenosine, inosine and hypoxanthine) for the prelabelling of the adenine nucleotide pool. Adenosine and inosine were found to be unsuitable because both are known to affect

hepatic carbohydrate metabolism when added externally (P.D. Whitton, unpublished results) while adenosine is also known to cause hepatic vasoconstriction (Ismail & Hems, 1978). Inosine, will also be converted to guanine nucleotides as well as adenine nucleotides because the initial reaction of inosine incorporation will involve convertion to IMP which will lead to synthesis of both adenine and guanine nucleotides. Hypoxanthine shares the second problem encountered with inosine because the initial step in its incorporation is again one of conversion to IMP. Thus adenine was chosen because of the high specific labelling of the adenine nucleotide pool via the reaction catalyzed by the enzyme adenine-p-ribosyl-transferase and also because it has not been reported to affect hepatic metabolism.

Initial experiments involved labelling of the hepatic adenine nucleotide pool using ^{14}C-adenine, so that it could be elucidated whether the technique was valid for further studies with respect to the effects of hormones and ischaemia on nucleotide metabolism.

3.2.1 Validation of technique in isolated liver slices

(a) ^{14}C-adenine uptake by liver slices.

Incubation of liver slices in the presence of 0.5mM ^{14}C-adenine was carried out as described in section 2.3. Time course of accumulation of radioactivity in the liver slices was determined (Figure 20). Under the conditions of the experiment the increase in the tissue level of radioisotope (i.e. taken up by the slices) reached a plateau at about 25 min. The time course of radioisotope accumulation

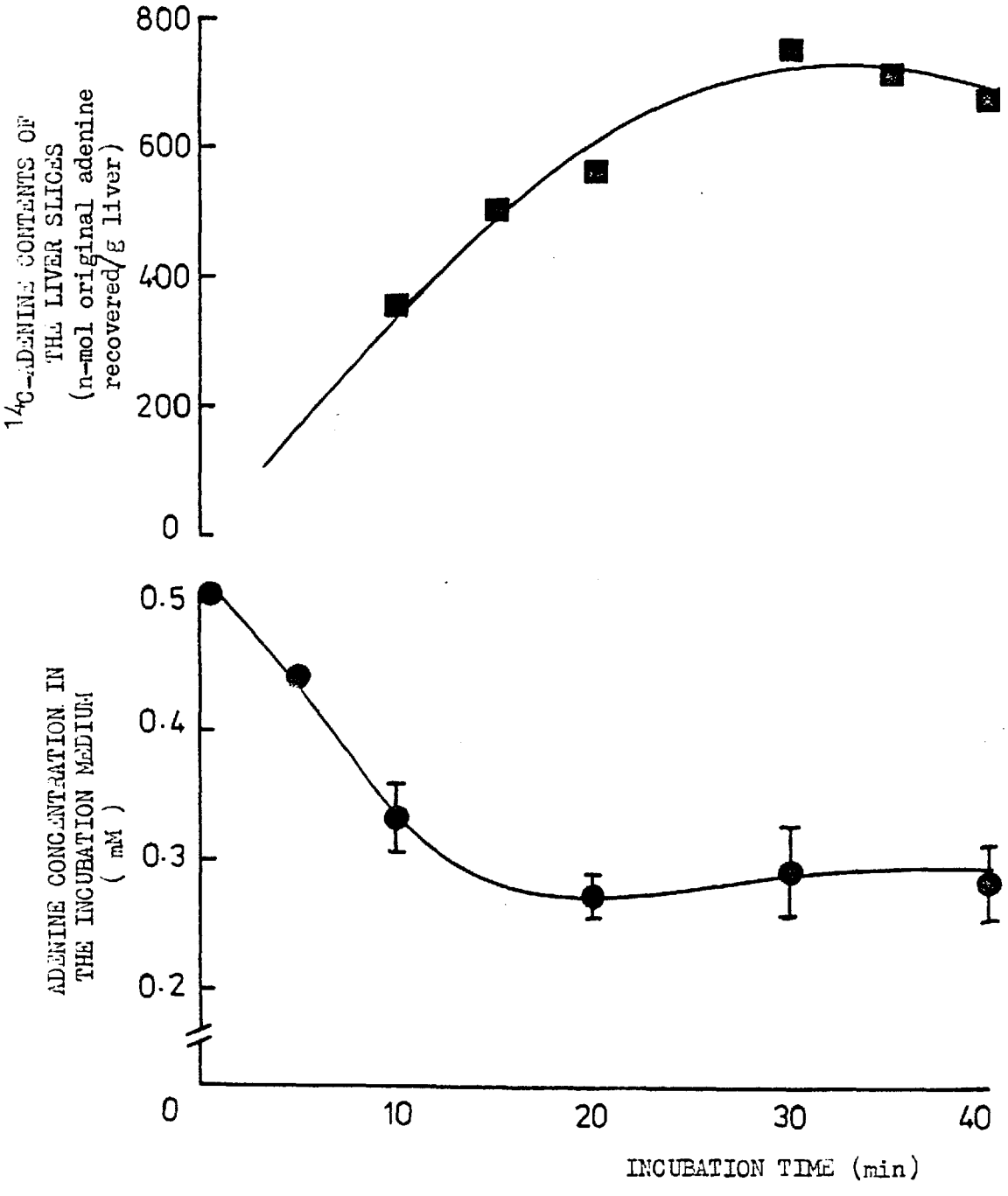


Figure 20: Incubation of liver slices in the presence of ^{14}C-adenine

Liver slices (total net wt. about 0.4g) were incubated in 2 ml of Krebs-ringer bicarbonate buffer with elevated K^+ (30 mM) containing 30 mM glucose and 0.5 mM adenine (specific activity 0.4 µCi/µmol).

(■) Total ^{14}C-radioactivity level in the liver slice. Each observation represents a pair of slices from each incubation flask (only one observation per flask). Results are from 3-5 observations.

(●) ^{14}C-adenine level in the incubation medium during liver slice incubation.

coincided with and accounted for the loss of radioactive material observed in the incubation medium (Figure 20). Analysis of the incubation medium after 40 min incubation revealed that the radioactive material in the medium was predominantly adenine.

Further analysis of the acid soluble ^{14}C-labelled material (Section 2.5) associated with the liver slices (Table 8) showed that after 30 min over 50% of the radioisotope was found in the phosphorylated form (i.e. as adenine nucleotide). More than 90% of the remaining radioactivity was still as adenine with only traces of radioactivity present in the breakdown products being monitored. The proportion of the radioisotope present in the nucleotide fraction rose while that of adenine fell suggesting continued incorporation of adenine into the nucleotide fraction.

(b) Effect of hormones on intracellular ^{14}C-adenine distribution.

A detailed profile of the distribution of radioactivity amongst adenine nucleotide degradation products was prepared and the effect of various hormones tested (Table 9). The level of radioactivity in each fraction was expressed as percentage of total radioactivity recovered because of the variation in total level of radioisotope assimilated in different liver slice preparations. Elevation of the cyclic AMP level in response to glucagon was similar to that reported by Daly (1972). Glucagon also raised intracellular levels of hypoxanthine, inosine, xanthine and xanthosine. Vasopressin and adrenalin lowered the level of labelled adenosine. Anoxia lowered the level of radioactivity of adenine nucleotides while raising the level of the

Table 8 : Accumulation and distribution into other purines of ^{14}C-adenine in liver slices.

Experimental details as in Figure 20. Perchloric acid extract from the liver slices were analysed using the 2-D TLC system described in Section 2.5.1. Results are mean ± SEM of three observations. Levels of the individual metabolites are expressed as % of the total in parenthesis.

Time of incubation	µmol of original adenine present in the incubation medium recovered per g liver			
(min)	Total ^{14}C adenine equivalent	Adenine nucleotide	Adenine	Other purine products
10	353	113 ± 16 (32%)	234 ± 5 (66%)	6 ± 1 (1.7 %)
20	575	260 ± 30 (45%)	302 ± 45 (53%)	13 ± 4 (2.2 %)
30	763	376 ± 17 (49%)	357 ± 18 (47%)	30 ± 5 (3.9%)
35	716	430 ± 40 (60%)	265 ± 45 (37%)	21 ± 4 (2.9%)
40	666	427 ± 55 (65%)	211 ± 29 (32%)	28 ± 6 (4.2%)

Table 9 : Effect of hormones and anoxia on ^{14}C-labelled purine pool intermediates in liver slices.

Liver slices prepared as described in Section 2.3. were pre-labelled by incubation in pairs for 35 min in the presence of ^{14}C-adenine (0.5mM). The hormone was then added to the flasks and after 5 min the slices were rapidly frozen in liquid nitrogen. Anoxia was induced by gassing the flask at 35 min with nitrogen:carbondioxide (95%:5%) gas mixture and the incubation stopped at 10 min. Each observation represented a pair of slices from each incubation flask (only one observation per flask). Concentrations of purine pool intermediates are expressed as percentage of total radioactive material recovered from the liver slices. Results are means of 2 observations.

	ADENINE NUCLEOTIDES (ATP, ADP, AMP)	cyclic AMP	adenine	adenosine	inosine	xanthosine and xanthine	hypoxanthine
	(^{14}C-labelled metabolite concentration expressed as percent of total ^{14}C-material recovered per g liver)						
CONTROL	46.8	0.33	45.8	1.5	0.4	0.4	0.5
GLUCAGON (20µg/ml)	55.6	0.9	38.9	1.6	0.7	1.1	1.0
VASOPRESSIN (2.5 units/ml)	52.4	0.3	44.3	1.2	0.5	0.5	0.7
ADRENALIN (0.1 mg/ml)	53.1	0.32	47.2	0.8	0.5	0.4	0.5
ANOXIA	37.2	0.45	47.3	4.8	←—6.7—→		3.5

metabolites.

3.2.2 ^{14}C-adenine uptake by the red blood cells of the medium and by the perfused liver.

In the previous section (3.2.1) conditions for labelling the adenine nucleotide pool in liver slices were established. The level of ^{14}C-adenine taken up by the slices was however found to be variable. In addition unsuitability of liver slices in general, made the liver slice preparation unsuitable for detailed study of adenine metabolism. In this section uptake of ^{14}C-adenine by the perfused liver in the presence of red blood cells, and other features concerning ^{14}C-adenine assimilation by different lobes of the liver and stability of the labelled purine profile was investigated.

(a) ^{14}C-adenine uptake by the perfused liver.

A perfused liver preparation with normal medium containing red blood cells (17 & 24% haematocrit) as described in Section 2.4 was used for this study. Carbon-14 labelled adenine was added to the perfusate 20 min after the start of the perfusion. Perfusate adenine concentration of 16.7 µM (specific activity 2.5 µCi/µmol) with perfusate volume of 60 ml was found to be sufficient for adequate and rapid uptake of ^{14}C-adenine by the liver, as measured by the reduction of radioisotope level in the perfusion medium (Figure 21). Perfusate radioactivity level stabilized within 10 min of ^{14}C-adenine addition.

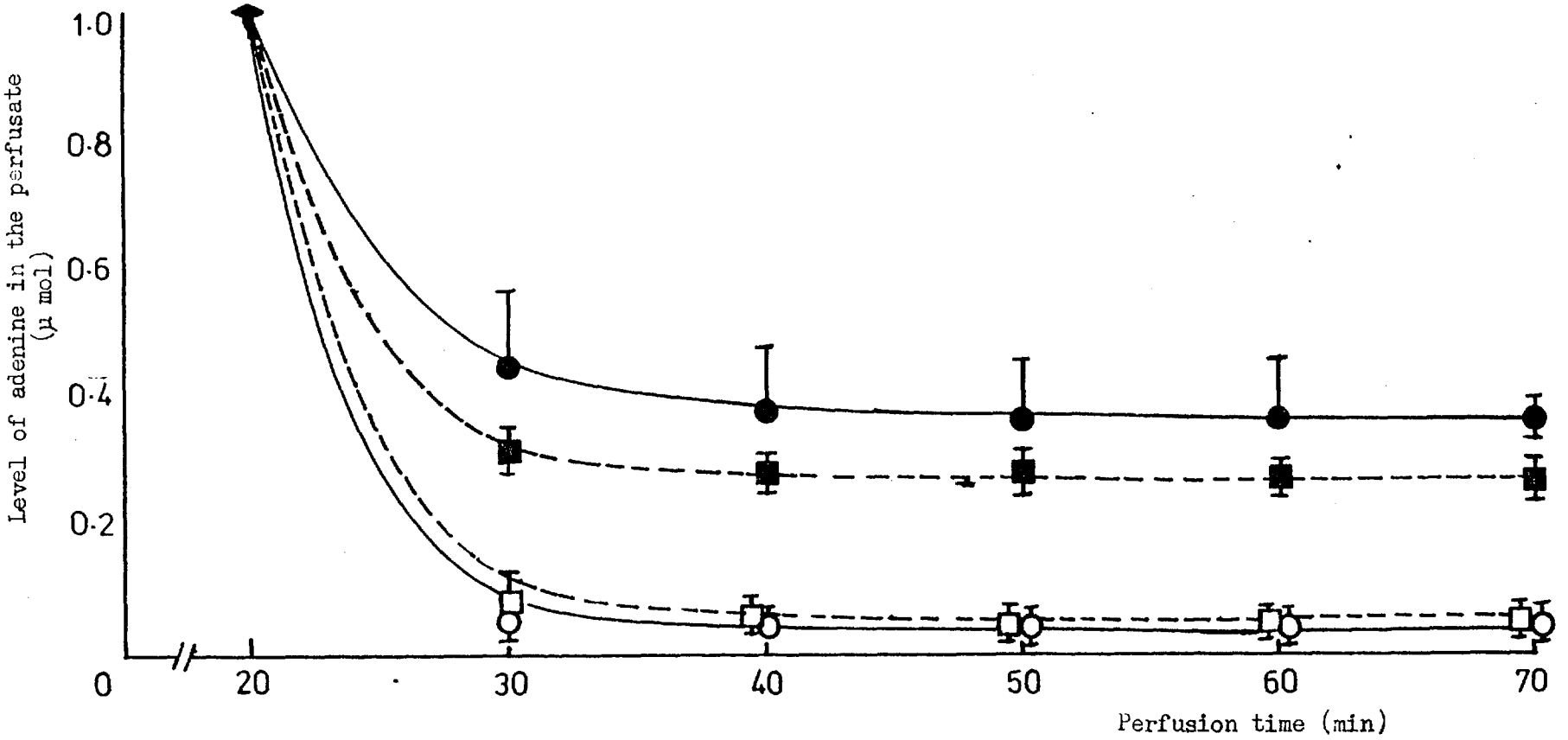


Figure 21: Removal of ^{14}C-adenine from the perfusion medium by the liver at two haematocrits.

Livers of fed rats were perfused with 60 ml perfusion medium in standard conditions at haematocrits of 17% (■&□) and 24% (●&○). 1μmol of ^{14}C-adenine (specific activity 2.5 μCi/μmol) was added at 20 minute. Total ^{14}C-adenine (■&●) of the medium was measured by determining radioactivity of 0.1 ml of the perfusion medium at the time indicated. Free ^{14}C-adenine (□&○) was measured by determining radioactive material present in the cell-free supernatant. The absolute amount of adenine remaining in the medium was calculated from the specific activity of the added adenine. Results are mean ± SEM of three observations at 17% haematocrit and four observations at 25% haematocrit.

(b) ^{14}C-adenine uptake by the red blood cells.

Since livers were being perfused by medium containing red blood cells, serving as oxygen carriers an assessment of adenine uptake by red blood cells under these conditions was made.

Total amount of radioactive adenine remaining in the perfusate after equilibration differed at the two haematocrits studied (Figure 21). Nearly 40% of the added adenine radioactivity was still present in the perfusate after 50 min when the haematocrit was 24%, and about 30% when the haematocrit was 17%. The level of free adenine in the perfusate was about 5% of the total added at both haematocrits. The total adenine taken up by the red blood cells was thus more in the perfusions with higher haematocrit and vice-versa. The level of radioactivity accumulated in the red blood cells was equivalent to 23 nmol of added adenine (1 µmol) per ml red blood cells at the two haematocrits (Table 10).

Incubation of red blood cells in perfusion medium with 11% haematocrit and 19 µM adenine, lowered the free adenine concentration to about 16 µM (Figure 22). Accumulation of adenine in the red blood cells was found to have reached a plateau at a level of about 30 nmol adenine/ml red blood cells (Figure 22). Thus it can be concluded that red blood cell uptake of adenine is limited to around 30 nmol/ml red blood cells, with the remainder being available for uptake by the liver.

(c) Stability and distribution of the ^{14}C-content of the liver.

A prerequisite for the investigation of ^{14}C-labelled

Table 10: Removal of ^{14}C-adenine by the red blood cells during prelabelling of the liver.

Experimental details as in Figure 21. Perfusate samples were analysed for total and plasma ^{14}C-adenine activity and the amount of adenine taken up by the red blood cells calculated. The results are mean ± SEM of three observations at 17% haematocrit and four observation at 24% haematocrit.

Time	Adenine taken up by the red blood cells (n mol/ml RBC)	
	17% haematocrit	24% haematocrit
10	21.8 ± 0.9	26.9 ± 6.4
20	23.2 ± 1.3	24.1 ± 6.0
30	23.8 ± 1.3	22.8 ± 5.7
40	23.0 ± 1.0	22.8 ± 5.9
50	22.7 ± 1.0	22.2 (2)

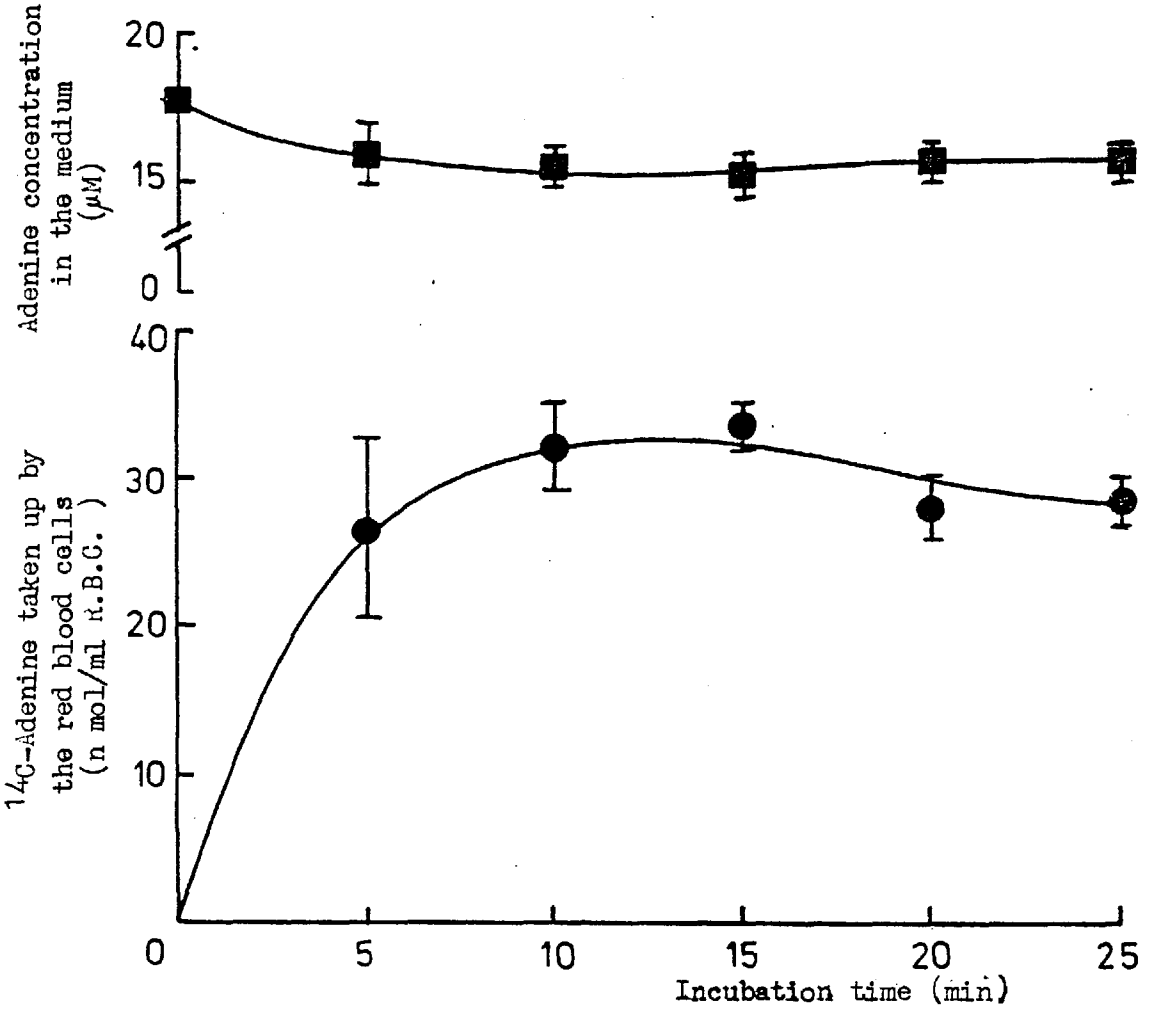


Figure 22: ^{14}C-adenine removal by the red blood cells.

2ml of normal perfusion medium (11% haematocrit) was incubated with 19 μM adenine (specific activity 1.33 μCi/μmol) at 37°C. Free adenine concentration (■) in the medium was monitored by measuring the radioactive material present in 50μl of supernatant. Adenine taken up by the red blood cells (●) was calculated from the difference between total added and that found in supernatant at a given time. Results are mean ± SEM of four observation.

adenine nucleotide metabolism was a study concerning stability of hepatic radioactivity level and its distribution amongst adenine nucleotides and their metabolites as a function of time, following adenine incorporation as described above. The level of radioisotope present in the liver was measured between 20 and 50 min after the addition of ^{14}C-adenine to the perfusion medium. There was a fall with time in the radioactivity level in the supernatant of PCA homogenized liver (Table 11). The rate of fall was approximately 0.6 nmol of original adenine entering the liver, per min per gm of liver. The total amount of ^{14}C-adenine taken up by these livers was 68 ± 6 nmol/g liver. The above loss of radioactive material from the acid soluble fraction of the livers represented loss of 0.7 % per min.

Analysis of the liver sample removed 20 min after the addition of ^{14}C-adenine to the perfusate, revealed that nearly 95% of the radioactivity was recovered from the nucleotide fraction. The remainder of the radioactivity was found to be roughly evenly distributed among the various purine ring containing breakdown products of nucleotide metabolism being monitored. This profile will be described in greater detail in Section 3.3. The perfused livers radioactivity distribution profile however differed from the liver slice findings where free adenine formed a substantial proportion of the recovered radioactivity.

Further analysis of radioactivity of PCA extracts from liver samples removed between 20 to 50 min after addition of ^{14}C-adenine to the perfusate, revealed that despite the fall in total radioactivity of the liver the relative levels of

Table 11 : Hepatic ^{14}C-content after prelabelling as a function of time

Livers of fed rats were perfused with 60 ml perfusion medium in standard conditions at haematocrit of 17%. ^{14}C-adenine (16.7 µM, sp.act. 2.5µCi/µmol) was added at 20 min and liver samples were removed at 40 and either 55 or 70 min . The liver samples were homogenized using 6% (v/v) perchloric acid and ^{14}C-adenine content determined from the level of radioactivity present in the supernatant. Results are mean ± SEM from 7 perfusions.

Time after addition of ^{14}C-adenine to the perfusion medium (min)	^{14}C-adenine content of the perfused liver (nmol of ^{14}C-adenine recovered/g liver)
20	68±7
35	57±10
50	50±5

radioactivity of the three adenine nucleotides did not alter significantly.

(d) Validation of sequential liver sampling during the perfusion.

In order to measure ^{14}C-adenine nucleotide metabolism in the liver by a sequential sampling procedure it was necessary that the total ^{14}C-adenine contents of different lobes of the liver could be validly related to each other, i.e. level of radioactivity in one area of the liver should resemble those in other areas. Biopsies could not be removed in sequence from within a single lobe, for technical reasons, and also because once the first liver biopsy had been taken, the remaining part of that lobe would have an interrupted blood circulation due to the ligature, and would not represent what may have occurred had it been left intact.

When different lobes of the liver were sampled simultaneously in perfused livers labelled with ^{14}C-adenine there was no significant difference in the level of radioactivity between different lobes (Table 12).

Table 12 : ^{14}C-adenine incorporation into the different lobes of the liver.

Livers were perfused in the normal manner and labelled with ^{14}C-adenine (16.7µM, specific activity 2.5µCi/µmol) at 20 min and the liver samples removed at 50 min. These were homogenized using 6% (v/v) perchloric acid and radioactivity of the supernatant was determined. Average level of ^{14}C-adenine incorporation per g liver was taken to be 100. Incorporation into the different lobes was expressed in terms of this value. Results are mean ± SEM of three perfusions.

	Liver lobes			
	Left half of median	Right half of median	Left lateral	Caudate
Adenine incorporation (taking average value as 100 per g liver)	89 ±10	105 ±12	99 ±10	108 ±8

3.3. Adenine nucleotide metabolism in the perfused liver prelabelled with ^{14}C-adenine.

In this section hepatic adenine nucleotide metabolism, using the technique of pre-labelling, was studied under normal conditions of perfusion. The aim was to quantify the events that take place with respect to the metabolism of ^{14}C-labelled nucleotides and their derivatives. The study also included monitoring of the perfusate to determine the release from liver of ^{14}C-labelled material under these conditions.

3.3.1 ^{14}C-adenine uptake and release by the perfused liver under the modified experimental conditions.

In the experiments described here livers of fed rats were perfused as described in the methods with Krebs-Ringer bicarbonate buffer containing 5mM glucose, 2% albumin and 12% haematocrit. The nucleotide pool was labelled using ^{14}C-adenine concentration of 17μM (specific acivity 25μCi/μmol) in the perfusate. The labelling mixture made up in 2.0 ml of 0.9% NaCl solution was added slowly (taking over 1 min) to the reservior, at the base of the oxygenator, from which oxygenated medium was supplied to the liver.

In labelling the liver in this manner, however, approximately 25% of the added ^{14}C-adenine again remained in the perfusion medium. A large proportion of this would be associated with, and consequently metabolized by the red blood cells, as seen in Section 3.2.2. Presence of large levels of ^{14}C-labelled material in the perfusate however would make it difficult to document release from the liver of comparatively small amounts of ^{14}C-labelled purine material.

Loss of radioactivity from the liver measured, as described in Section 3.2.2.C. over 30 min was found to be 0.6 n mol per min per g of liver under these conditions. Clearly such a small increment in perfusate concentration would be difficult to measure against the background of perfusate ^{14}C-adenine content of approximately 0.25μmol in 60 ml (4μM).

In the short term study documented here this problem was overcome by use of the recirculating/non-recirculating modification of the perfusion technique described in Section 2.4.4. In this technique the normal recirculating mode of perfusion was interrupted at the desired moment in a manner which enabled the liver to be perfused by fresh medium with minimum of disturbance. Composition of the second medium was similar to the first medium except that glucose concentration had been elevated to 8 mM and that red blood cells were not included. Glucose concentration was raised in the second medium, because under normal conditions of perfusion with initial perfusate glucose concentration of 5mM the liver was found to raise it to a stable value of 8mM within 20 min (see Section 3.1.1). Glucose concentration of 5mM in the second perfusion medium stimulated glycogenolysis as indicated by raised glycogen phosphorylase *a* activity (Table 13). A small rise in phosphorylase *a* activity was still observed however in response to the changeover (Table 13) when glucose concentration in the second medium was 8 mM. Red blood cells were omitted from the second medium to stop further metabolism of '^{14}C-labelled purine material' released by the liver (Table 14). The average rate of release was over 0.8 nmol of adenine equivalents (of 1μmol adenine added originally to the perfusate) per min per g. liver over a period of 3 min.

Table 13: Effect of glucose concentration in the second perfusion medium on hepatic glycogen phosphorylase a activity

Livers of fed rats were perfused in the recirculating/non-recirculating mode as described in the text and Section 2.4.4 . Standard perfusion medium containg 5mM glucose, 2.5% albumin and 12% haematocrit was used during the recirculating phase of the perfusion. The second perfusion medium for the non-recirculating phase contained either 5mM or 8mM glucose, 2.5% albumin but no red blood cells. The two perfusion media were equilibrated with O_2:CO_2 (95%:5%) gas mixture . The first liver sample was removed after 30min of perfusion was followed by changeover of the perfusion medium. The second liver sample was removed 4 min after the medium changeover. Results are mean ± SEM of 4 perfusions at the two glucose concentrations.

	Phosphorylase a activity (μmol glucose/min/g wet liver)	
Glucose conc. in second perfusion medium (mM)	Initial Sample	Second Sample
5	5±1	10±1
8	5±1	7±1

Table 14: Release of ^{14}C-labelled material into the perfusion medium under normal conditions

Livers of fed rats were perfused in the recirculating/non-recirculating mode as described in the text and section 2.4.4. Standard perfusion medium containing 5mM glucose, 2.5% albumin and 12% haematocrit was used during the recirculating phase of the perfusion. The second perfusion medium for the non-recirculating phase contained 8mM glucose, 2.5% albumin but no red cells. The two perfusion media were equilibrated with O_2:CO_2 (95%:5%) gas mixture. The nucleotide pool was labelled using ^{14}C-adenine (17μM, sp.act.25μCi/μmol). the first liver sample was removed 30 min after the addition of ^{14}C-adenine, and was followed by the changeover of the medium. Second perfusion medium was collected over 30sec periods and the radioactivity measured. Specific activity of adenine added to the first perfusion medium was used to calculate the adenine equivalents released. Average weight of the liver perfused by the second medium was 6.6 g at an average flow of 1.9 ml/g liver. Results are mean ± SEM of four perfusions.

Time (sec)	Vol of perfusate collected in 30 sec (ml)	Total radioactive material present in the perfusate x 10^6 DPM	Level of perfusate ^{14}C-labelled material (μmol of adenine equivalents released per gm liver/min)
30	5.9 ± 0.6	0.31 ± 0.04	1.52 ± 0.20
60	5.9 ± 0.4	0.23 ± 0.02	1.13 ± 0.09
90	6.3 ± 0.4	0.16 ± 0.02	0.77 ± 0.09
120	6.2 ± 0.4	0.13 ± 0.03	0.62 ± 0.14
150	6.3 ± 0.4	0.10 ± 0.01	0.50 ± 0.07
180	6.3 ± 0.6	0.09 ± 0.01	0.39 ± 0.01

3.3.2 ^{14}C-adenine metabolism in the perfused liver.

The '^{14}C-purine pool profile' of the liver labelled in the above manner was constructed to assess the state of ^{14}C-adenine metabolism in the basal aerobic condition (Table 15). This profile was constructed by expressing the level of each radioactive metabolite in terms of original perfusate adenine (total 1 µmol) converted to product. Thus hepatic metabolite concentrations are expressed as p-mol of original adenine present in the perfusate converted per g liver.

The first liver sample was removed 30 min after the addition of ^{14}C-adenine to the perfusion medium when ^{14}C content of the liver had stabilised as measured by the total hepatic radioactivity level (Section 3.2.2.). Under these conditions over 95% of the recovered radioactivity was in adenine nucleotides (Table 15). Labelled purine pool metabolites accounted for the remaining 5% of ^{14}C-adenine. Hepatic ^{14}C-adenine concentrations of 0.9 ng/g liver was 1.5% of the total hepatic level indicating nearly total conversion of the ^{14}C-adenine precursor to adenine nucleotides.

The intracellular concentration of labelled adenosine diphosphate was the highest, accounting for nearly 50% of the total hepatic nucleotides. Labelled adenosine triphosphate was second highest at 30% followed by adenosine monophosphate at 20%. This distribution of label amongst the nucleotides, however, was not proportional to the hepatic chemical levels as seen from the data in Table 16 . Specific activity of the nucleotides AMP and ADP was similar while that of ATP was 25% of the other two. The amount of radio-

Table 15 : Adenine nucleotide metabolism in the perfused liver prelabelled with ^{14}C-adenine.

Livers of fed rats were perfused in the recirculating/non-recirculating mode as described in the text and Section 2.4.4. Standard perfusion medium containing 5mM glucose, 2.5% albumin and 12% haematocrit was used during the recirculating phase of the perfusion. The second perfusion medium for the non-recirculating phase contained 8mM glucose, 2.5% albumin but no red blood cells. The two perfusion media were equilibrated with O_2:CO_2 (95%:5%) gas mixture . The nucleotide pool was labelled using ^{14}C-adenine at final concentrations of 17μM (specific activity 2.5μCi/μmol). The first liver sample was removed 30 min after the addition of ^{14}C-adenine, and was followed by the changeover of the medium. Average weight of the liver perfused by the second medium was 6.6 gm at an average flow of 2.1 ml/min/g liver. The second liver sample was taken 4 min after the medium changeover. The two liver samples and the second perfusate were analysed for ^{14}C distribution amongst the purine pool constituents. Hepatic levels are expressed as p-mol of adenine, originally added to the perfusate (1μmol), recovered per g liver. Average perfusate concentration over the 3 min collection period is also expressed in terms of adenine originally added to the perfusate. Results are mean ± SEM of 4 observations.

	ATP	ADP	AMP	IMP	XMP	ADENOSINE	INOSINE	XANTHOSINE	HYPOXANTHINE	XANTHINE
INITIAL SAMPLE (p mol adenine equivalents recovered per g liver)	15880 ±1060	25110 ± 640	9350 ±920	250 ±10	710 ±140	540 ±130	60 ±15	30 ±5	170 ±20	20 ±5
SECOND SAMPLE (p mol adenine equivalents recovered per g liver)	17900 ±1030	26070 ±1920	9370 ±970	270 ±40	820 ±390	150 ±20	50 ±10	40 ±15	90 ±10	15 ±5
PERFUSATE LEVEL (nM)	13 ±3	12 ±3	40 ±4	17 ±2	7 ±1	22 ±5	16 ±1	43 ±3	45 ±5	10 ±1

Table 16 : Adenine nucleotide specific activity after perfusion with labelled adenine.

Experimental detail as in Table 15. The levels of radioactivity present in the adenine nucleotides ATP, ADP and AMP were expressed as a function of the chemical concentrations. Labelled nucleotides are measured as n mol of ^{14}C-adenine originally added to the perfusion medium recovered per g liver. Results are from 3-4 experiments.

		Labelled nucleotide conc (nmol of adenine equivalent recorded g liver)	Chemical concentration µ mol/g liver	Specific activity (nmol ^{14}C adenine equivalent present per mol)	Ratio of specific activity relative to AMP
INITIAL SAMPLE	ATP	15.9	2.7	5.9	0.24
	ADP	25.1	1.04	24.1	0.98
	AMP	9.4	0.38	24.6	1.0
SECOND SAMPLE	ATP	17.9	2.8	6.4	0.28
	ADP	26.1	1.02	25.6	1.11
	AMP	9.4	0.41	22.9	1.0

activity incorporated into ADP and AMP indicated that ^{14}C-adenine distribution was proportional to their relative chemical concentrations. Similarly, lower specific activity of ATP indicated that a large proportion of intracellular ATP was unable to exchange with the newly labelled AMP and ADP.

The adenine nucleotide specific activity values although giving an indication of relative distribution of radioactivity, however, cannot give an accurate estimate of the size of the hepatic adenine nucleotide pool labelled because of compartmentalization of the adenine nucleotides into distinct pools (e.g. mitochondrial versus cytoplasmic compartments), as well as the fact that metabolically different pools may exist (Rapaport and Zamecnik , 1976). This phenomenon coupled with the additional problem of protein binding of the adenine nucleotides, thereby considerably reducing the effective free concentration, may be responsible for the non-equilibrium of the label within the three nucleotides despite a rapid turnover in the adenine nucleotides.

Cytoplasmic location of the enzyme adenine phosphoribosyl transferase (Partsch et al., 1977), however, suggests that the initial reaction of ^{14}C-adenine incorporation would be cytoplasmic. Considering the above problem of protein binding and compartmentalization, a considerable proportion of the newly synthesized radioactive adenosine monophosphate will remain in the cytoplasm. Since most of the enzymes responsible for AMP degradation are also located in the cytoplasm monitoring of the degradation products of ^{14}C-AMP will give an accurate

measure the rate of AMP degradation.

The intracellular content of radioactive inosine monophosphate, inosine and adenosine was low at 6%, 3% and 1% of labelled AMP respectively.

Labelled xanthosine monophosphate concentrations was also low at 0.7 nmol/g. No significant amounts of radioactive guanosine nucleotides were detected in the liver. This supports the view that the rate of inter-conversion between the adenine and guanine series is very low under normal conditions (Henderson, 1975). Thus the presence of radioactivity in the various purine breakdown products enables isolation of adenine nucleotide breakdown from that of guanine nucleotides, where some of the degradation products are common.

The levels of the labelled purine metabolites were low indicating that adenine nucleotide breakdown occurs at a very slow rate.

Removal of the first liver sample, after 30 min of prelabelling was followed by changing the perfusion medium to Krebs-Ringer bicarbonate buffer equilibrated with O_2:CO_2 (95%:5%) gas mixture containing 2% albumin and 8 mM glucose. A second liver sample was removed after 4 min of perfusion. No significant differences in the hepatic levels of the various ^{14}C-labelled purine pool constituents were seen (Table 15) except for a decrease of 70% and 50% in the levels of labelled adenosine and hypoxanthine respectively during 4 min. There was no difference in the specific acitivity of the adenine nucleotides between the two liver samples (Table 16).

Thus it can be concluded that perfusate changeover

had minimal effect on both the chemical concentration of adenine nucleotides and on the levels of ^{14}C labelled purine pool metabolites.

3.3.3 Release of ^{14}C-labelled material into perfusate under normal aerobic conditions.

The radioactive material present in the second perfusion medium (Table 14) was further analysed with the aim of identifying the nature of material being released by the liver. 75% of the released material during 3 min was recovered as constituents of the purine pool (Table 15). The remaining radioactive material may constitute further breakdown products of the purine ring such as urate, allantoin, etc. The decrease in the individual levels of the ^{14}C-labelled purine metabolites released into the perfusate (Figure 23) was consistent with the decrease in total perfusate radioactivity (Table 14) suggesting a reduction in release. A significant decrease in AMP, adenosine and hypoxathine was observed with time which may be due to reduced release or to increased uptake. The former is the more likely explanation since the tissue concentrations of these compounds were not increased.

The purine nucleotide fraction accounted for nearly 40% of the released radioactive purine material, half of which was AMP. The level of labelled AMP in the perfusate at 40 nM was 0.5% of the mean tissue level. Levels of the other nucleotides ATP, ADP and IMP were roughly equal at just over 10nM and were 0.08%, 0.04% and 5% of mean tissue levels respectively. XMP level at 0.8% of the mean

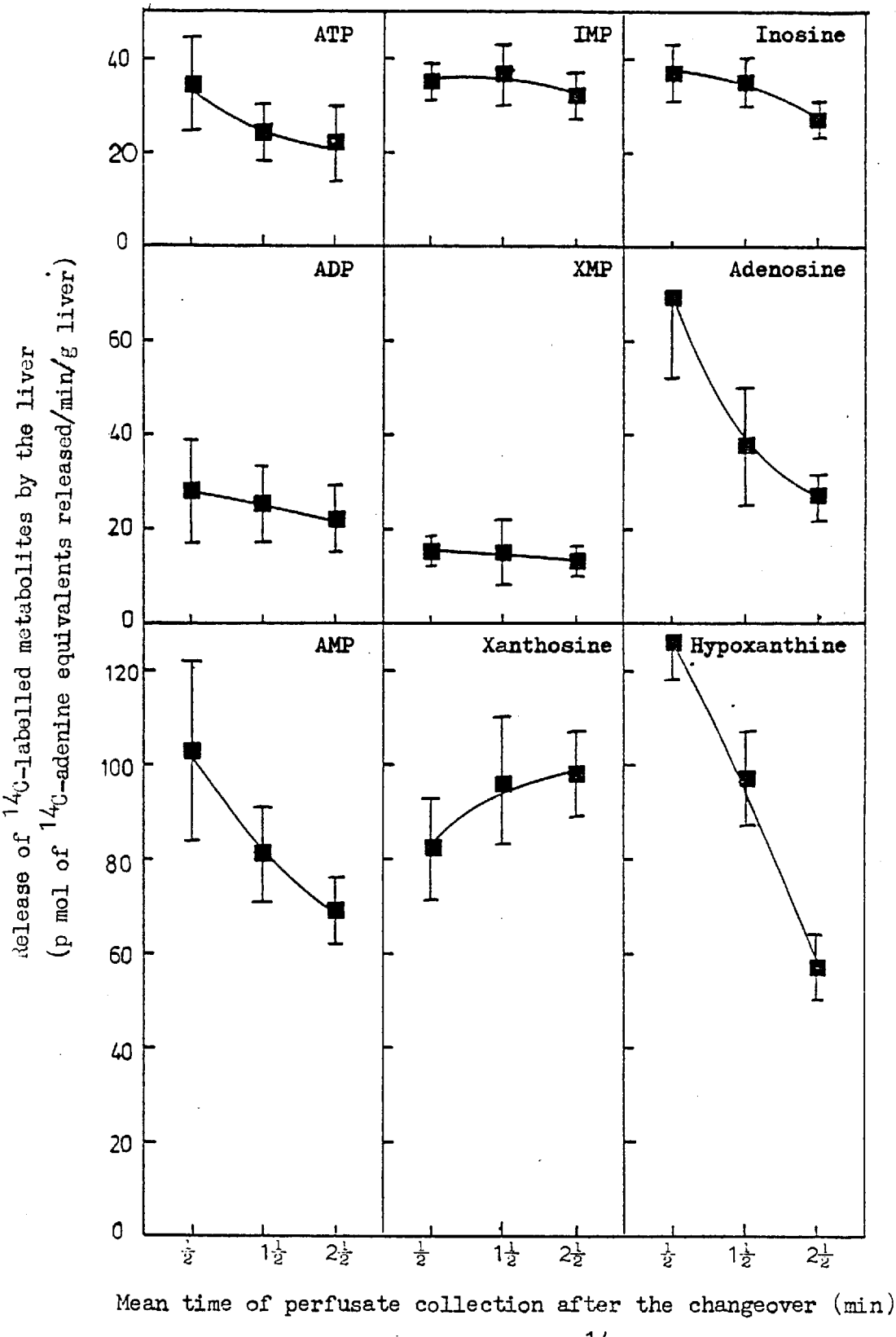


Figure 23: Time course of release by the liver of ^{14}C-labelled purine pool metabolites under normal conditions.

Experimental detail as in Table 14 and 15. Perfusion medium collected after the changeover was pooled into three fractions and each was analysed as described in section 2.5.1. Perfusate level of the individual purine metabolite was calculated as p mol of adenine equivalents (of the original 1 mol ^{14}C-adenine added) released per g over the first, second and third minute of perfusion. Results are mean ± SEM of four perfusions.

hepatic level was the lowest in absolute terms at 7 nM.

Hypoxanthine and xanthosine at concentrations of 45 and 44 nM accounted for over 60% of the released non-nucleotide purine metabolites. Hypoxanthine and xanthosine levels in the perfusate were over 30 and 100 percent of the mean intrahepatic concentrations respectively. The remaining purine metabolites of adenosine, inosine and xanthine at perfusate concentrations of 22, 15 and 10 nM were 6, 10 and 60% of the mean tissue levels.

Thus in summary it can be stated that the liver releases into the perfusate a wide range of purine containing compounds. There have been no previous reports of purine nucleotide release by the liver while selective release of purine compounds such as adenosine, inosine, hypoxanthine, and xanthine has been previously reported (Pritchard et al., 1970; Lerner and Lowry, 1974; Pritchard et al., 1975).

3.4. Effect of oxygen lack on ^{14}C-adenine nucleotide metabolism in perfused liver prelabelled with ^{14}C-adenine.

In Section 3.1 the effect of partial and total oxygen deprivation on hepatic adenine nucleotide metabolism was studied. However, that investigation was restricted to studying the gross chemical changes in hepatic adenine nucleotide levels in response to hypoxia and anoxia. The procedure for labelling hepatic adenine nucleotides (Section 3.2), used for documenting ^{14}C-adenine nucleotide metabolism under normal conditions (Section 3.3), was used to investigate adenine nucleotide metabolism in response to total ischaemia and anoxia.

3.4.1 Effect of total ischaemia on hepatic ^{14}C-labelled adenine nucleotide metabolism.

In the experiments described in this section the livers were perfused and the adenine nucleotide pool labelled using ^{14}C-adenine as described in Section 3.3. The first liver sample was removed 30 min after the addition of ^{14}C-adenine and rapidly frozen in liquid nitrogen to serve as the initial control. The remaining liver was then made ischaemic by sudden cessation of flow and the second anoxic liver sample was removed 5 min after the onset of total ischaemia.

The hepatic concentration of labelled ATP and ADP decreased by 60 and 30% during 5 min of total ischaemia while the AMP concentration rose by 80% (Table 17). These changes in

Table 17 : Effect of total ischaemia on adenine nucleotide metabolism in liver prelabelled with ^{14}C-adenine.

Livers of fed rats were perfused in standard conditions at a haematocrit of 28%. The nucleotide pool was labelled using ^{14}C-adenine at final concentration of 17μM (specific activity 1μCi/μmol). The first sample was removed 30 min after the addition of ^{14}C-adenine. The liver was made ischaemic by stoping the flow and the second sample was removed after 5min. The two liver samples were analysed for ^{14}C-distribution amongst the purine pool constituents as described in Section 2.5.1. Hepatic levels are expressed as p mol of adenine originally added to the perfusate (1 μmol), recovered per g liver. Results are mean ± SEM of 4 observations.

	ATP	ADP	AMP	IMP	XMP	ADENINE	ADENOSINE	INOSINE	XANTHOSINE	HYPO-XANTHINE	XANTHINE
	(p mol of original adenine present in the perfusate recovered per gm liver)										
INITIAL SAMPLE	18180 ±960	28080 ±420	10500 ±720	760 ±200	350 ±70	840 ±80	300 ±60	260 ±60	70 ±30	300 ±30	40 ±5
FINAL ISCHAEMIC SAMPLE	7770 ±900	19450 ±1260	19030 ±2220	2620 ±610	1500 ±270	890 ±50	1470 ±220	3920 ±740	890 ±230	1270 ±200	590 ±140

the concentration of labelled ATP and ADP in response to total ischaemia reflect the chemical level shifts seen under identical conditions (Section 3.1.4), but are much less pronounced. The decrease in labelled ADP concentration in response to total ischaemia, however, was not consistent with the doubling of the chemical level seen in Section 3.1.4 under similar conditions. These discrepancies between the two observations are due to compartmentation, since the chemical measurements recorded total changes in the tissue regardless of location whereas radioactive measurements are predominantly concerned with behaviour of the pool that contains the radioactive precursor. As stated in Section 3.3.2 ^{14}C-adenine may predominantly label the cytoplasmic adenine nucleotides with the result that the changes seen in the levels of labelled nucleotides will disproportionally reflect the cytoplasmic events. It is thus concluded that the overall response of the labelled adenine nucleotide pool to total ischaemia was in agreement with other related events taking place since the decrease in ^{14}C-ATP concentration was accompanied by a rise in ^{14}C-AMP concentration. Concentrations of the other labelled purine pool constituents rose by between three and ten fold (Table 17). Total loss of 19 nmol of adenine equivalents from the ATP and ADP fraction was fully accounted for by the extra rise of 18.8 n mol of adenine equivalents amongst the purine monophosphates and other breakdown products. Redistribution of the radioactivity lost from the ATP and ADP fraction is illustrated in Figure 24. Accumulation of 45% of the mobilized radioactivity in the AMP fractions indicated control of further AMP degradation. The second largest accumulation of 19% was

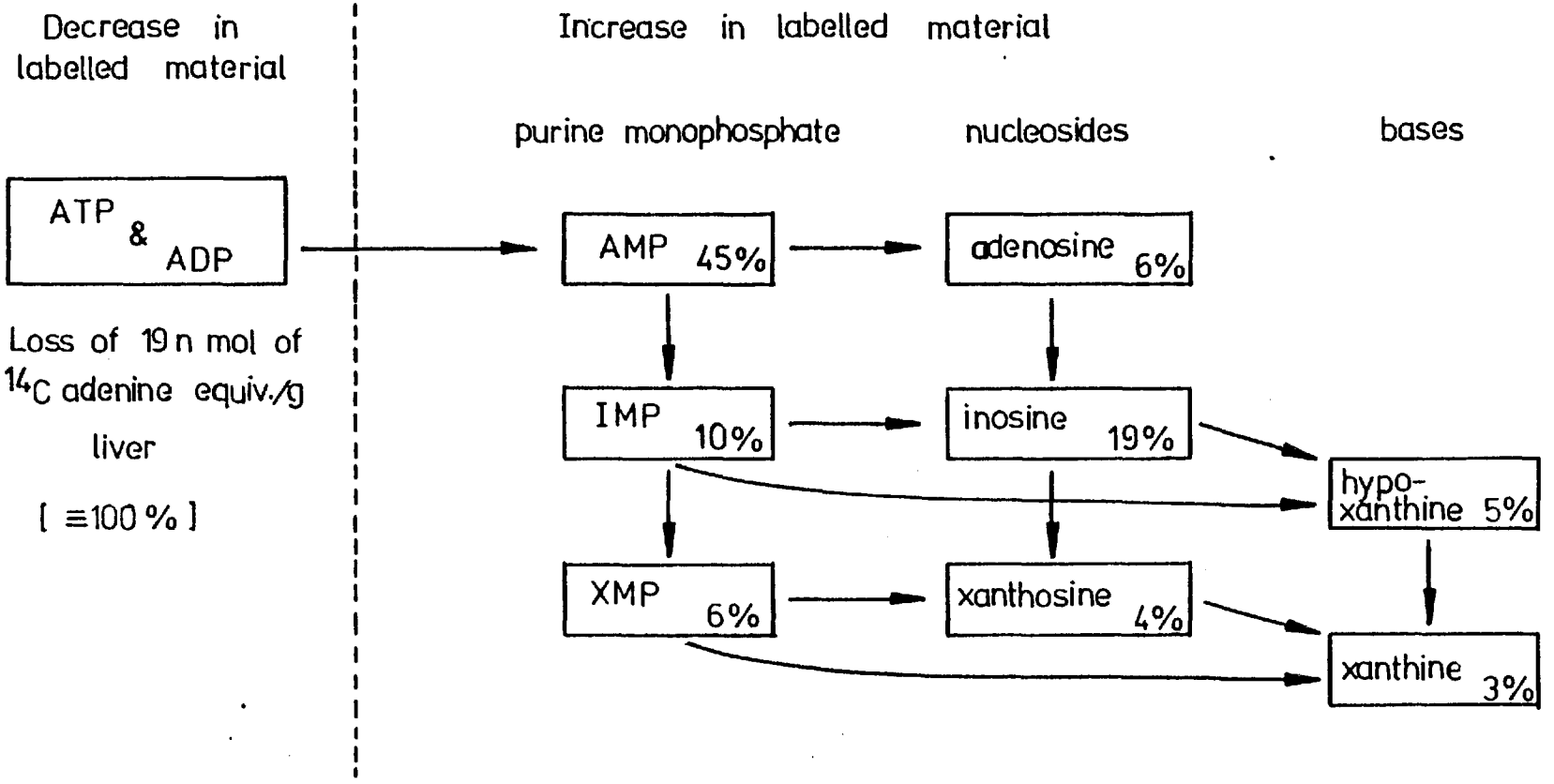


Figure 24: Redistribution of radioactivity from ATP and ADP degradation during total hepatic ischaemia. Details in the text.

observed in the inosine fraction suggesting inosine degradation to be limiting as well. Smaller depositions of radioactive material amongst the remaining metabolites of hypoxanthine, xanthosine and xanthine were seen.

Thus it can be concluded that anoxia stimulated adenosine triphosphate breakdown which was reflected in raised hepatic levels of breakdown products. This accumulation occurs at various points depending on the extent of metabolism such that maximum possible conservation tion of the purine ring was indicated under conditions of stimulated purine nucleotide degradation.

3.4.2 Effect of anoxia on ^{14}C-labelled adenine nucleotide metabolism in perfused liver.

As was noted in the previous Section (3.4.1) degradation of ^{14}C-labelled ATP during total ischaemia was accompanied by increases in all of the breakdown products being monitored except ^{14}C-ADP. Total ischaemia, however, involves stagnation in the tissue with the consequence of problems associated with the resultant build up of various products. Thus it was of interest to study ^{14}C-adenine nucleotide metabolism during anoxia without the accompanying stagnation associated with these studies.

In the experiments designed to study the effect of anoxia the recirculating/non-recirculating modification described in Section 3.3 was used. The liver was perfused and the nucleotide pool was labelled as described previously in Section 3.3. The initial liver sample was taken 30 min after the addition of ^{14}C-adenine to the perfusate (50

min after the start of the perfusion). Perfusion medium to the remaining liver was changed to one equilibrated with nitrogen:carbon dioxide (95%:5%) gas mixture to induce rapid anoxia. The second perfusate was collected after passage through the liver. The second liver sample was removed 3 min after the onset of anoxia and rapidly frozen. Stimulation of glycogenolysis was indicated by the raised hepatic glycogen phosphorylase *a* activity from a value of 5 ± 1 to 12 ± 1 (4) μ mol glucose transferred per min per g liver in response to anoxia.

(a) Effect of anoxia on tissue ^{14}C-adenine metabolite levels.

Pronounced differences existed in the response of ^{14}C-labelled adenine nucleotide metabolism to the two different types of anoxia. Anoxia induced by changing the gases with maintenance of perfusate flow through the liver resulted in smaller reductions in labelled hepatic ATP and ADP content of 40% and 20% respectively while labelled AMP did not alter significantly (Table 18), in contrast to the situation in total ischaemia. Once again the changes in the concentration of labelled adenine nucleotides were at odds with the chemical concentration changes of ATP which decreased 10% and ADP and AMP which increased 20 and 40% respectively. The specific activity of the three nucleotides decreased in response to anoxia (Table 19). Ratio of the specific activity of ATP: ADP: AMP was relatively similar between the two liver samples and thus did not reflect the complex changes that were taking place (Table 19).

The fall of nearly 10 nmol of adenine equivalents/g in the levels of ATP and ADP was accompanied by either no change or small rises in the remaining metabolite levels

Table 18: Effect of anoxia on adenine nucleotide metabolism in liver prelabelled with ^{14}C-adenine.

Livers of fed rats were perfused in the recirculating/non-recirculating mode as described in Section 2.4.4. First medium for the recirculatory phase was equilibrated with O_2:CO_2 (95%:5%) gas mixture and contained 5 mM glucose, 2·5% albumin and 12% haematocrit. Second perfusion medium containing 8 mM glucose and 2·5% albumin was equilibrated with N_2:CO_2 (95%:5%) gas mixture. The nucleotide pool was labelled using ^{14}C-adenine at final concentration of 17μM (specific activity 15μCi/μmol). The first liver sample was removed 30 min after the addition of ^{14}C-adenine, followed by the changeover of the medium which induced anoxia. Average weight of the liver perfused by the second medium was 6.8 g. at an average flow rate of 2.1 ml/min/g liver. The second liver sample was taken 4 min after the perfusate changeover. The two liver samples and the second perfusate were analysed for ^{14}C distribution amongst the purine pool constituents (Section 2.5.1). Hepatic levels are expressed as p mol of adenine, originally added to the perfusate (1μmol), recovered per g liver. Average perfusate concentration over the 3 min collection period is also expressed in terms of adenine originally added to the perfusate. Results are mean ± SEM of 4 observations.

	ATP	ADP	AMP	IMP	XMP	ADENOSINE	INOSINE	XANTHOSINE	HYPOXANTHINE	XANTHINE
INITIAL SAMPLE (p mol adenine equivalents recovered per g liver)	10080 ±1210	28510 ±1920	13580 ±750	280 ±30	710 ±100	590 ±180	150 ±60	50 ±15	200 ±70	70 ±25
SECOND SAMPLE (p mol adenine equivalents per g liver)	5970 ±1720	23040 ±2980	14900 ±1300	1330 ±270	790 ±100	450 ±160	640 ±310	120 ±30	230 ±60	150 ±70
PERFUSATE LEVEL (nM)	47 ±8	13 ±5	80 ±12	44 ±5	13 ±3	13 ±3	47 ±16	84 ±14	134 ±23	22 ±4

Table 19 : Anoxia and adenine nucleotide specific activity in liver prelabelled with ^{14}C-adenine

Experimental detail as in Table 18. The levels of radioactivity present in the adenine nucleotides ATP, ADP and AMP were expressed as a function of the chemical level. Results are from 3-4 perfusions.

		Labelled nucleotide conc (nmol of adenine equivalent/g liver)	Chemical concentration (µmol/g liver)	Specific activity (n mol ^{14}C-adenine equivalent present per µmol)	Ratio of specific activity relative to AMP
INITIAL SAMPLE	ATP	10.1	2.6	3.9	0.12
	ADP	28.5	1.04	27.4	0.85
	AMP	13.6	0.42	32.3	1.0
SECOND SAMPLE	ATP	6.0	2.4	2.5	0.1
	ADP	23.0	1.22	18.9	0.75
	AMP	14.9	0.59	25.3	1.0

so that a net loss of 7 nmol/g from the liver was observed during 3 min of anoxia. Hepatic levels of the metabolites XMP, hypoxanthine and xanthine were amongst those that did not change significantly when compared with aerobic control tissue levels. Inosine monophosphate rose by over 3 fold and xanthosine was more than doubled in response to anoxia. Since under normal conditions hepatic adenosine concentration decreased (Table 15), the lack of a significant change in tissue adenosine under anoxic conditions (i.e. similar ^{14}C-adenosine concentration in initial and anoxic liver sample) indicates a rise in cellular adenosine during anoxia.

All the hepatic purine metabolite levels were raised in the anoxic liver but were much lower than those observed in totally ischaemic liver (Table 18). The results reported here indicate that increased adenosine monophosphate degradation occurs by stimulation of the enzyme AMP deaminase because IMP and further products of degradation accumulate. The decrease in the intracellular level of adenosine seen under normal conditions (Section 3.3.2) was less acute suggesting that this might be due to inhibition of the adenosine deaminase (e.g. by accumulation of inosine from IMP) instead of stimulation of the enzyme 5'-nucleotidase.

(b) Release of ^{14}C-labelled material into perfusate under anoxic conditions.

Radioactive material was released by the perfused liver under anoxic conditions at a rate of 1.75 nmol of adenine equivalents per g liver per min (Table 20). This average level of release was twice that observed under

Table 20: Release of ^{14}C labelled material into perfusate under anoxic conditions.

Experimental details as in Table 18. Second perfusate was collected over 30 sec periods and the radioactivity measured. Specific activity of adenine added to the first perfusion medium (15μCi/μmol) was used to calculate the adenine equivalents released. Average weight of the liver perfused by the second medium was 6.8 g. Results are mean ± SEM of 4 perfusions.

Time (sec)	Vol of perfusate collected in 30 sec (ml)	Total radioactivity present (x 10^6 DPM)	Level of perfusate ^{14}C labelled material (n mol of adenine equivalent released/min/g liver)
30	6.7 ± 0.1	0.28 ± 0.04	2.2 ± 0.4
60	6.9 ± 0.1	0.27 ± 0.03	2.1 ± 0.2
90	7.0 ± 0.1	0.22 ± 0.03	1.7 ± 0.2
120	7.0 ± 0.1	0.23 ± 0.06	1.5 ± 0.2
150	7.1 ± 0.2	0.21 ± 0.03	1.6 ± 0.2
180	7.0 ± 0.2	0.19 ± 0.03	1.4 ± 0.2

normal conditions when the perfusion medium was fully oxygenated. However, it was observed that the rate of release was different between the two states, so that during the third minute 1.5 nmol of adenine equivalents were released per g liver during anoxia compared to the normal release of 0.45 nmol of adenine equivalents per g. Labelled purine pool constituents accounted for nearly 60% of the total amount released, in comparison with 75% recovered under normal aerobic conditions. This may be due to increased further breakdown products of xanthine since an increase in hepatic purine nucleotide degradation had been observed. Finally the total of 5.2 nmol of adenine equivalents recovered after purification and separation from the perfusate collected during three minutes accounted for most of the 6.6 nmol decrease in tissue level observed during anoxia (both values refer to 1g of liver).

The increased level of perfusate radioactivity in response to anoxia was reflected in raised perfusate levels of all intermediate with the exception of adenosine which decreased despite the raised average intracellular level (Figure 25). The perfusate changes observed in the levels of radioactive metabolites will be dealt with individually where they will be correlated with the tissue levels and compared with changes seen under normal conditions.

Adenosine triphosphate: The perfusate concentration of ^{14}C-ATP was steady during 3 min of anoxia (Figure 25). The average perfusate level of ATP rose 4-fold while the intracellular concentrations of ^{14}C-ATP fell (Table 18) thus indicating that ATP release was being stimulated under

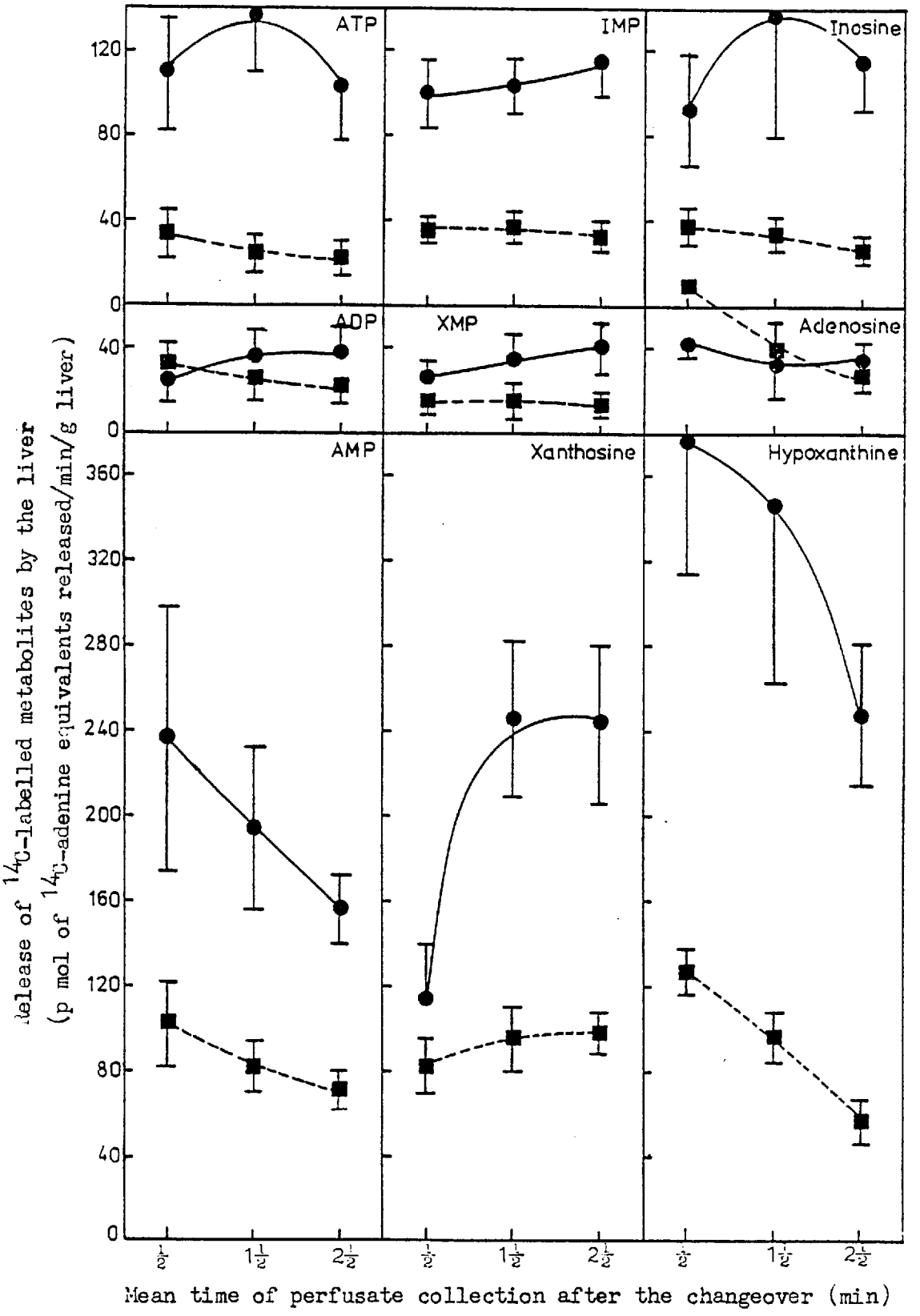


Figure 25: Time course of release by the liver of ^{14}C-labelled purine pool metabolites during anoxia.

Experimental detail as in Table 18 and 20. Second perfusion medium collected after the changeover was pooled into three fractions and each was analysed as described in section 2.5.1. Perfusate level of the individual purine metabolite was calculated as p mol of adenine equivalent (from the original 1 mol ^{14}C-adenine added) released per g over the first, second and third minute perfusion. Results are mean ± SEM of four perfusions under anoxic (●) and four perfusions under normal conditions (■).

conditions when the total tissue level was decreasing.

Adenosine diphosphate: No significant change in ADP perfusate level was observed.

Adenosine monophosphate: Two points to be noted, firstly a small rise in tissue level was observed and secondly there was a doubling in the average perfusate ^{14}C-AMP concentration (Table 18) even though the rate of release declined with time (Figure 25). However, since hepatic ^{14}C-AMP is also elevated during anoxia (Table 18) the perfusate level expressed as a ratio of mean tissue level remained unaltered.

Inosine monophosphate: A rise of nearly 4-fold in the average perfusate concentration (Table 18) was reflected in the steady increase in perfusate concentration during anoxia (Figure 25). Although this was accompanied by a similar rise in the tissue level, the perfusate level rose to 15% of the mean tissue level during anoxia compared to the value of 5% under normal conditions.

Xanthosine monophosphate: Perfusate concentration steadily rose during 3 min of anoxia so that the average perfusate concentration of ^{14}C-XMP was twice that observed under normal conditions of perfusion. The tissue concentration remained unaltered.

Adenosine: Average perfusate adenosine concentration during anoxia was lower than the value under control conditions

(Table 18). The rate of release during anoxia was also below the normal rate (Figure 25). Since the concentration in anoxic liver did not fall in the manner observed in normal conditions, the implication is that its release by the liver was being inhibited.

Inosine: An increase of nearly 4-fold in perfusate concentration accompanied the 3-fold increase in intracellular concentration. The perfusate concentration of ^{14}C-inosine was relatively steady during 3 min of anoxia (Figure 25)

Xanthosine: A 4-fold increase in perfusate concentration was accompanied by an increase in tissue concentration (Table 18). The perfusate concentration increased with the time of anoxia (Figure 25).

Hypoxanthine and xanthine: Rises in perfusate and tissue concentrations of hypoxanthine and xanthine were observed (Table 18). The perfusate level of both, although still elevated in comparison with normal release, decreased after the onset of anoxia (Figure 25).

Thus as seen above complex changes in purine nucleotide metabolism and purine metabolite release take place when liver is deprived of oxygen. These will be considered in detail in Section 4.3.

3.5. Effect of hormones on adenine nucleotide metabolism in liver prelabelled with ^{14}C-adenine.

Characterization of hepatic ^{14}C-labelled adenine nucleotide metabolism under normal and anoxic conditions has been carried out (see Section 3.3 and 3.4 respectively). These results contribute to our understanding of nucleotide metabolism in the liver. Thus it was of interest to test the effect of various hormones on the processes occurring in an effort to elucidate whether purine nucleotides are involved in mechanism of hormone action. The four hormones tested were insulin, glucagon, vasopressin and angiotensin II. These were added separately to the second perfusion medium, of the recirculating/non-recirculating perfusion arrangement (for details see Section 3.3). The hormone angiotensin II at the concentration used was found to reduce flow of medium through the liver. Control perfusions with similar flow reductions, produced by reducing the perfusate input pressure, were also carried out to distinguish between direct hepatic effects of angiotensin II and the effects of vasoconstriction. This is necessary since it is already known that flow reductions alter nucleotide metabolism (see Section 3.1.2).

Initial analysis of the perfusate revealed that there was wide variation in the level of release of radioactive material by the liver in response to hormones (Table 21). The initial release of radioactive material by the liver in the presence of insulin and glucagon was similar to the normal release, but in the case of insulin the decrease with time in the amount being released was more pronounced, so that during the fourth minute the level of

For table 21 see over leaf

n mol adenine equivalents released/g /min

Perfusion time (min)	Release of ^{14}C labelled material in response to hormones				Release during flow reduction	Normal level of release
	Insulin (10 mU/ml)	Glucagon (0.7µgm/ml)	Vasopressin (5 mU/ml)	Angiotensin II (0.1µgm/ml)		
1	0.70 ±0.19	1.06 ±0.08	1.15 ±0.11	0.84 ±0.16	0.68 ±0.07	1.13 ± 0.2
2	0.48 ±0.06	0.62 ±0.05	1.06 ±0.04	1.04 ±0.10	0.55 ±0.02	0.7 ± 0.1
3	0.41 ±0.04	0.53 ±0.10	0.88 ±0.08	1.13 ±0.06	0.53 ±0.01	0.45 ± 0.04
4	0.34 ±0.02	0.46 ±0.1	0.74 ±0.05	1.07 ±0.12	0.48 ±0.02	-
Wt. of liver perfused by the second medium (g)	6.7 ±0.5	5.7 ±0.2	5.7 ±0.3	5.2 ±0.3	6.2 ±0.3	6.6 ±0.1
Average flow of the second medium ml/min/g liver	2.4 ±0.1	2.4 ±0.2	2.3 ±0.2	1.8 ±0.2	1.8 ±0.2	2.1 ±0.2
Number of observations	3	3	3	4	3	4

release was significantly below the normal value. The hormones vasopressin and angiotensin II significantly raised the amount of radioactive material released by the liver. The effect of vasopressin was maximal within 1 min followed by a decrease in the rate of release, while the effect of angiotensin II was slower in onset but more sustained. Perfusate flow reduction of the magnitude seen in the presence of angiotensin II showed there was no difference in the release of radioactive material from these perfusions when compared with controls.

Due to small variations in the incorporation of ^{14}C-adenine, the level of incorporation into the initial liver sample was normalized to the average value of 60 n mol/g liver. Concentration of the individual metabolites in the second hormone treated liver sample were then calculated with reference to this initial value from the observed experimental values. The profile of labelled material in the initial liver samples were similar to those already observed (Sections 3.3 and 3.4), with over 95% of the radioactivity being associated with the nucleotide fraction. The amount of ^{14}C-label incorporated into the other purine pool metabolites, however, was found to be elevated to about 2-3 times the previously observed values of control samples (see sections 3.3 & 3.4.2). The effect of each hormone on the intracellular pool of ^{14}C-labelled purine compounds and their release into the perfusate will be described in detail individually.

3.5.1. Effect of insulin on ^{14}C-labelled adenine nucleotide metabolism in the perfused liver.

There were no significant differences in the ^{14}C-labelled hepatic purine pool metabolite levels between the initial and insulin treated liver samples (Table 22). However, it was noticeable that a slight lowering in ATP and ADP was accompanied by general elevation of the other purine metabolite

Table 22: Effect of insulin on adenine nucleotide metabolism in liver prelabelled with ^{14}C-adenine.

Experimental detail as described in Table 21. Insulin was present in the second medium at final concentration of 10 mU/ml. Average weight of the liver perfused by the second medium was 6.7 gm at an average flow of 2.4 ml/min/g liver. The second liver sample was removed 5 min after the perfusate changeover. The two liver samples and the second perfusion medium collected over 4 min were analysed for ^{14}C distribution amongst the purine pool constituents (Section 2.5.1). Hepatic levels are expressed as p mol of adenine, originally added to the first perfusion medium recovered per gm liver. Average concentration of the radioactive purine metabolites in the perfusate collected over 4 min is also expressed in terms of adenine originally added for pre-labelling. Results are mean ± SEM of 3 perfusions.

	ATP	ADP	AMP	IMP	XMP	ADENOSINE	INOSINE	XANTHOSINE	HYPOXANTHINE	XANTHINE
INITIAL SAMPLE (p mol adenine equivalents recovered/g liver)	16160 ±610	24240 ±920	14200 ±520	630 ±20	940 ±30	340 ±80	150 ±50	90 ±15	200 ±20	45 ±10
SECOND SAMPLE (p mol adenine equivalents recovered/g liver)	15660 ±230	23490 ±350	15980 ±350	590 ±50	950 ±50	480 ±80	230 ±110	140 ±30	260 ±90	50 ±10
PERFUSATE LEVEL (nM)	13 ±4	18 ±6	37 ±5	14 ±4	6 ±2	14 ±5	6 ±1	19 ±4	32 ±14	1.3 ±0.4

levels. Prominent amongst the latter were the 50% increases in the intracellular levels of adenosine, inosine and xanthosine. Under comparable control conditions (see Section 3.3.2) there was no change in inosine and xanthosine concentrations while adenosine fell by 70% compared to its own initial tissue level.

The total amount of labelled material being released into the perfusate in response to insulin was similar to that released under normal conditions (Table 21) despite the level being lower at 4 min in the presence of insulin. Despite the overall similarity small variations in the perfusate levels of individual purine pool metabolites and the rate at which the levels decreased with time (suggesting decreased release or increased uptake), were noticeable in insulin treated livers (Figure 26). Perfusate ATP, ADP and IMP concentrations decreased faster, while those of adenosine and hypoxanthine were steadier in comparison with normal livers. The most pronounced effect of insulin was on xanthosine, since the initial rate of release was markedly reduced and decreased in the presence of insulin despite a rise in the intracellular level. This is unlike the higher steady perfusate level maintained in the absence of insulin with no rise in the intracellular level.

The average perfusate concentration of the various metabolites were, however, more revealing in that insulin had lowered the combined nucleoside and base level by 48% while the purine nucleotide level remained unaltered at 88 nM (Table 22). Perfusate concentrations of the nucleosides, adenosine, inosine and xanthosine and the bases, hypoxanthine and xanthine all decreased by 40, 60, 60, 30 and 90% respectively when compared to normal levels. Lower

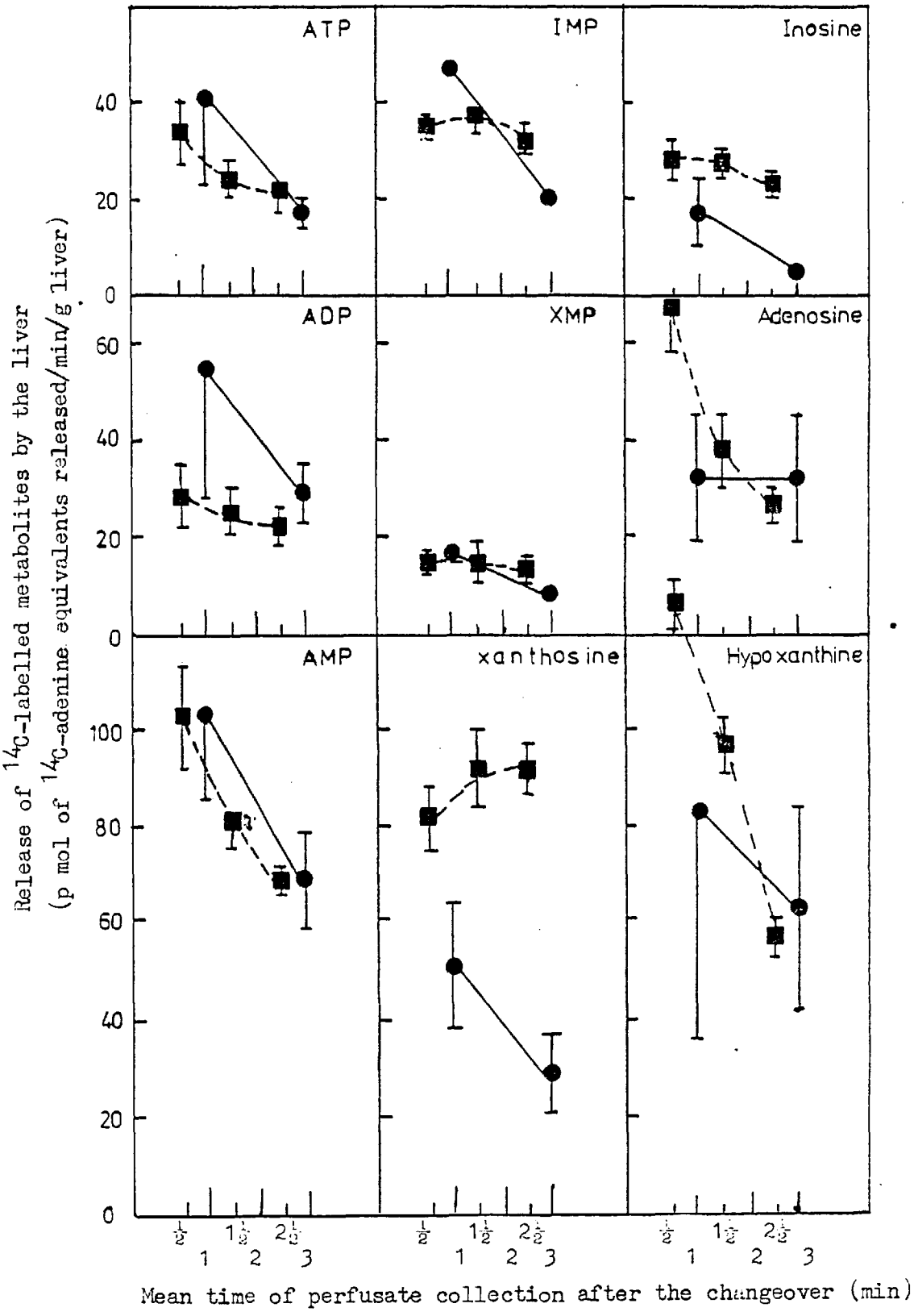


Figure 26: Time course of release by the liver of ^{14}C-labelled purine pool metabolites in the presence of insulin.

Experimental details as in Table 21 and 22. Perfusion medium containing the hormones insulin (●) collected over 4 min after the changeover was pooled into two fractions and the ^{14}C-distribution amongst the purine pool constituents in each fraction analysed as described in section 2.5.1. perfusate level of the individual purine metabolite was calculated as p mol of adenine originally added to the first perfusion medium released per g liver per min during the 2 min periods of perfusate collection. Results are mean ± SEM of four perfusions. Data illustrating normal level of release (■) was taken from section 3.3.3.

rates of release of the nucleosides and bases (Figure 26) also confirm the above observation. This decrease in perfusate levels was observed despite the increases varying between 30 and 60% in the intracellular concentration of all of these metabolites.

Thus in conclusion it can be stated that while there is no major change in purine nucleotide metabolism in the liver in response to insulin, the hepatic content of the labelled nucleosides adenosine, inosine and xanthosine was increased while their release into the perfusate had been inhibited.

3.5.2 Effect of glucagon on hepatic ^{14}C-labelled adenine nucleotide metabolism.

The effect of glucagon on ^{14}C-labelled intracellular levels of purine pool metabolites, like that of insulin, was minimal. Purine nucleotide levels were unaltered while the nucleosides and bases were generally raised in response to glucagon (Table 23). Inosine and xanthine increased the most with nearly 3-fold changes; there was a 40% increase in adenosine. These increases, like those seen with insulin, should be contrasted with the normal situation where decreases in hepatic levels of adenosine, hypoxanthine and inosine were observed. The level of cyclic AMP also rose to become detectable at 13 pmol/g liver in response to glucagon.

The total level of radioactive material released by the liver in response to glucagon was identical with that observed under normal conditions. Levels of release of most of the purine pool constituents were also similar to

Table 23: Effect of glucagon on adenine nucleotide metabolism in liver prelabelled with ^{14}C-adenine.

Experimental details as described in Table 21. Glucagon was present in the second medium at final concentration of 0.7µg/ml. Average weight of the liver perfused by the second medium was 5.7 g at an average flow of 2.4 ml/min/g liver. The second liver sample and the second perfusion medium collected over 4 min were analysed for ^{14}C distribution amongst the purine pool constituents (Section 2.5.1). Hepatic levels are expressed as p mol of adenine, originally added to the first perfusion medium (1µmol) recovered per g liver. Average concentration of the radioactive purine metabolites in the perfusate collected over 4 min is also expressed in terms of adenine originally added for pre-labelling. Results are mean ± SEM of 3 perfusions.

	ATP	ADP	AMP	IMP	XMP	ADENOSINE	INOSINE	XANTHOSINE	HYPOXANTHINE	XANTHINE	cyclic AMP
INITIAL SAMPLE (p mol adenine equivalents recovered per g liver)	15980 ±880	23970 ±1320	15300 ±590	470 ±60	770 ±100	190 ±70	130 ±60	70 ±20	260 ±100	70 ±10	<5
SECOND SAMPLE (p mol adenine equivalents per g liver)	15290 ±420	22930 ±620	14800 ±660	460 ±50	760 ±100	270 ±60	380 ±110	80 ±30	300 ±110	190 ±60	13 ±4
PERFUSATE LEVEL (nM)	15 ±2	31 ±6	31 ±4	17 ±3	7 ±1	17 ±4	-	22 ±8	36 ±9	4 ±2	2 ±0·4

the normal levels and followed the same time course (Figure 27). The rates of release of ADP, inosine and xanthosine were the exceptions with the former being increased while the latter two were decreased in the presence of glucagon. A similar effect was seen in the presence of insulin, where release of adenosine was also inhibited.

Analysis of the average perfusate concentration figures (Table 23) revealed that the elevated ADP was almost exclusively responsible for the 25% increase in the purine nucleotide concentration. The fall of 40% in the levels of nucleosides and bases being due to decreases of 30,50, 50 and 20% in the average perfusate levels of adenosine, xanthosine, xanthine and hypoxanthine respectively and complete absence of inosine in the perfusate. A level of 5nM for cyclic AMP in the glucagon treated perfusate was also recorded. The time-course of release of purine pool metabolites (Figure 27) revealed that the perfusate levels of nucleosides and bases decreased while the intracellular levels were increasing.

Thus it appears that glucagon is similar in action to insulin in that it raised intracellular levels of nucleosides while inhibiting their release into the perfusate. Most prominent of these changes was the 3-fold increase in hepatic level of inosine while its release into the perfusate was inhibited from 44 to below 5 p mol per min per liver, while insulin action was less acute. The other clear cut effect of glucagon was stimulation of the adenylate cyclase as indicated by the accumulation of cyclic AMP in the tissue as well as in the perfusate.

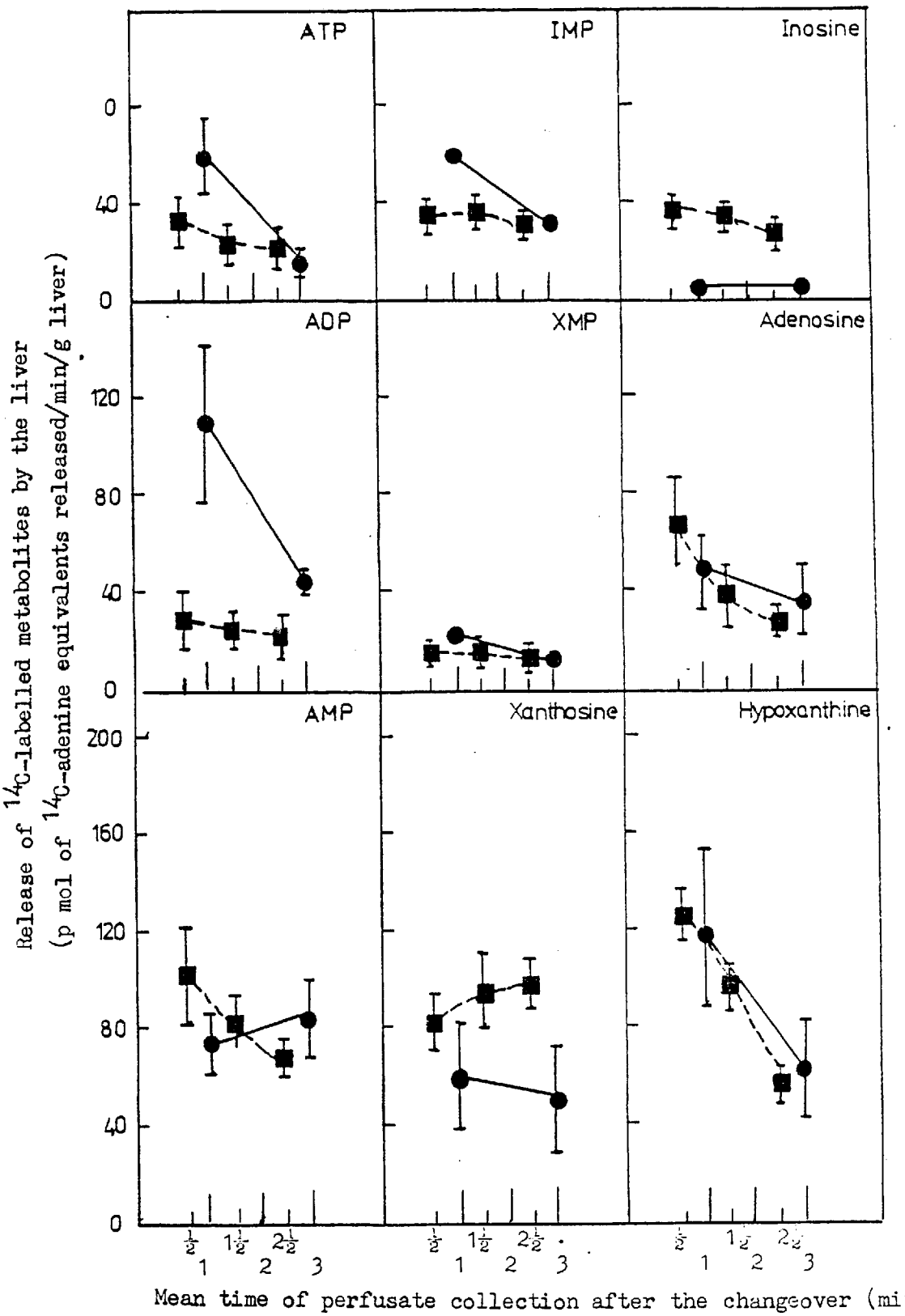


Figure 27: Time course of release by the liver of ^{14}C-labelled purine pool metabolites in the presence of glucagon.

Experimental details as in Table 21 and 23. For other details see Figure 26. (●) Rate of release in the presence of glucagon results are mean ± SEM from three perfusions; (■) normal rate of release taken from section 3.3.3.

3.5.3 Effect of vasopressin on hepatic ^{14}C-labelled adenine nucleotide metabolism.

Vasopressin decreased hepatic adenine nucleotides diminishing ATP and AMP levels by 10% so that the total size of the labelled purine pool in the liver was reduced by 5% (Table 24). Increases of various degrees were seen in the purine pool metabolites with the nucleosides adenosine, inosine and xanthosine increasing by 60, 120 and 70% respectively and the bases xanthine and hypoxanthine also increasing 150 and 50% respectively (Table 24). In contrast most metabolites decreased in level under normal conditions while the increases were smaller in response to insulin and glucagon (see Sections 3.5.1 and 3.5.2). Total increase in hepatic nucleoside and base levels of 840 p mol per g, however, only accounted for 25% of the reduced hepatic nucleotide content.

The total level of labelled material in the perfusate from vasopressin treated liver decreased but at a slower rate than under normal conditions (Table 21). Consequently the level of release in the vasopressin treated liver was twice the normal level of release after 4 min. The increased rate of release of a number of the metabolites is shown in Figure 28. Perfusate concentrations of the nucleotides ATP, ADP and AMP were elevated at all times although they increased at the same rate as in normal livers. The only other metabolite to show significantly increased release was adenosine although this also decreased with time. The remaining metabolite levels were similar to those seen under normal conditions. The rate at which hypoxanthine

Table 24: Effect of vasopressin on adenine nucleotide metabolism in liver prelabelled with ^{14}C-adenine.

Experimental details as described in Table 21. Vasopressin was present in the second medium at final concentration of 5 mU/ml. Average weight of the liver perfused by the second medium was 5.7 g at an average flow of 2.3 ml/min/g liver. The second liver sample was removed 5 min after the perfusate changeover. The two liver samples and the second perfusion medium collected over 4 min were analysed for ^{14}C-distribution amongst the purine pool constituents (Section 2.5.1). Hepatic levels are expressed as p mol of adenine, originally added to the first perfusion medium (1 µmol), recovered per g liver. Average concentration of the radioactive purine metabolites in the perfusate collected over 4 min is also expressed in terms of adenine originally added for pre-labelling. Results are mean ± SEM of 3 perfusions.

	ATP	ADP	AMP	IMP	XMP	ADENOSINE	INOSINE	XANTHOSINE	HYPOXANTHINE	XANTHINE
INITIAL SAMPLE (p mol adenine equivalents recovered /g liver)	17310 ±530	25970 ±800	12500 ±960	420 ±30	690 ±50	270 ±60	260 ±60	140 ±30	240 ±30	110 ±10
SECOND SAMPLE (p mol adenine equivalents recovered/g liver)	15830 ±2350	25750 ±1490	1150 ±610	370 ±20	620 ±30	420 ±100	570 ±100	240 ±60	350 ±50	280 ±120
PERFUSATE LEVEL (nM)	30 ±5	30 ±2	77 ±13	21 ±5	9 ±4	44 ±6	10 ±2	52 ±11	42 ±5	-

decreased in perfusate from vasopressin treated liver was slower than under normal conditions.

Average perfusate concentrations of these metabolites confirmed the above observations (Table 24). Levels of ATP, ADP and AMP were elevated by 140, 150 and 90% respectively, in comparison with normal perfusate levels. The increases in IMP, XMP and xanthosine were generally much lower at around 25% while hypoxanthine remained unaltered. Doubling of adenosine compared with the normal concentration of 20nM was accompanied by decrease of 40% in inosine and lowering of xanthine below the detections threshold of 1nM. This lowering of perfusate concentration of inosine and xanthine in response to vasopressin was also seen with insulin and glucagon. Perfusate level of the isolated and separated purine pool metabolites of 320nM averaged over the 4 min period meant release of 2900 p mol of ^{14}C-adenine labelled material per g of liver. When added to the intracellular increase of nucleoside and base levels of 840 p mol/g, this change adequately accounted for the decrease in the hepatic purine nucleotide level.

Categorization of the average metabolite levels into nucleotide and non-nucleotide fractions revealed that nucleotide and non-nucleotide levels went up by 75 and 15% respectively in vasopressin containing perfusate. Distribution of radioactivity between the two groups in response to the hormone was equal, in contrast to the distribution of 40 and 60% between the nucleotide and non-nucleotide fractions under normal conditions.

Thus the main effects of the hormone vasopressin can be summarized as lowering of the hepatic purine nucleotide

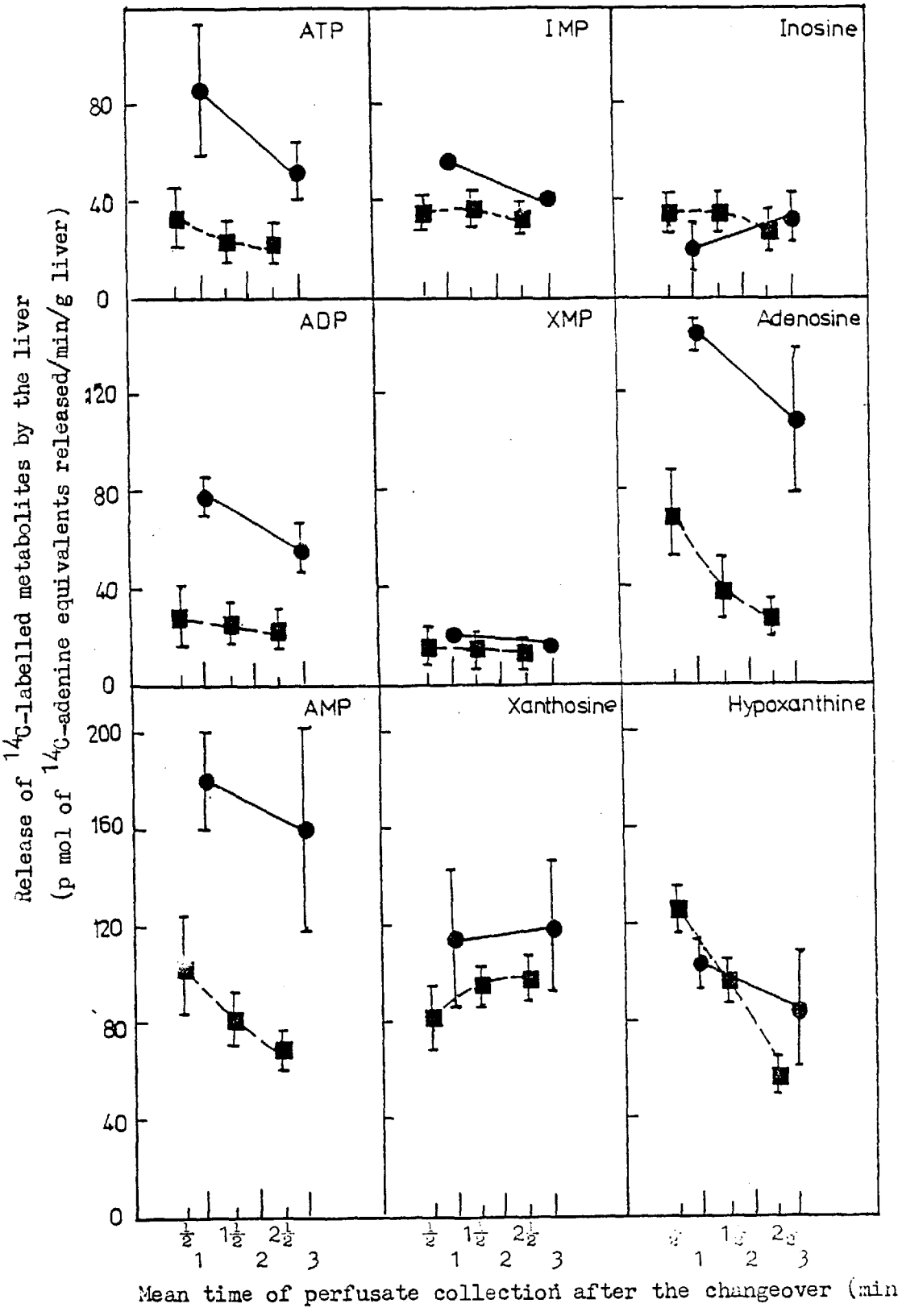


Figure 28 : Time course of release by the liver of ^{14}C-labelled purine pool metabolites in the presence of vasopressin.

Experimental details as in Table 21 and 24. For other details see Figure 26. (●) Rate of release in the presence of vasopressin, results are mean ± SEM of three perfusions; (■) normal rate of release taken from section 3.3.3.

level and raising of the intracellular level of other metabolites; increases in perfusate nucleotide levels were accompanied by lower purine metabolite levels (with the exception of adenosine which went up in both tissue and perfusate).

3.5.4 Effect of angiotensin II on hepatic ^{14}C-labelled adenine nucleotide metabolism.

Amongst the four hormones tested the effect of angiotensin on ^{14}C-labelled purine nucleotide metabolism was the most acute. Labelled purine nucleotide content of the liver fell by 13%. The decrease in the levels of ATP and ADP were the largest at 15% each while AMP and XMP fell by under 10% (Table 25). Intracellular ^{14}C-IMP content was however raised by 30% in response to the hormone. Acuteness of the angiotensin effect was further highlighted by the raised hepatic levels of nucleosides and bases. Adenosine, inosine, xanthosine and xanthine were amongst those which doubled whilst hypoxathine rose by only 50% (Table 25). The increases in hepatic levels of purine metabolites accounted for nearly 20% of the decrease in the intracellular level of labelled purine metabolites. Release of 4.1 n mol of adenine equivalent per g by the liver during 4 minute of perfusion, when combined with the intracellular purine metabolite increase accounted for 80% of the decrease in labelled purine nucleotides when the liver was exposed to angiotensin II (Table 21).

Release of radioactive material into the perfusate from angiotensin treated livers rose from the initial value

Table 25: Effect of angiotensin II on adenine nucleotide metabolism in liver prelabelled with ^{14}C-adenine.

Experimental detail as described in Table 21. Angiotensin II was present in the second medium at final concentration of 0.1µg/ml. Average weight of the liver perfused by the second medium was 5.2 g at an average flow of 1.8 ml/min/g liver. The second liver sample was removed 5 min after the perfusate changeover. The two liver samples and the second perfusion medium collected over 4 min were analysed for ^{14}C distribution amongst the purine pool constituents (Section 2.5.1). Hepatic levels are expressed as p mol of adenine, originally added to the first perfusion medium (1µmol), recovered per g liver. Average concentration of the radioactive purine metabolites in the perfusate collected over 4 min is also expressed in terms of adenine originally added for prelabelling. Results are mean ± SEM of 4 perfusions.

	ATP	ADP	AMP	IMP	XMP	ADENOSINE	INOSINE	XANTHOSINE	HYPOXANTHINE	XANTHINE
INITIAL SAMPLE (p mol adenine equivalents recovered/g liver)	16940 ±740	25540 ±1170	12620 ±1580	610 ±30	880 ±110	430 ±70	300 ±65	120 ±35	300 ±60	80 ±30
SECOND SAMPLE (p mol adenine equivalents recovered/g liver)	14360 ±1420	21540 ±2130	11500 ±940	800 ±60	830 ±110	920 ±220	630 ±100	230 ±60	460 ±140	170 ±45
PERFUSATE LEVEL (nM)	37 ±9	39 ±4	95 ±24	47 ±8	20 ±5	41 ±9	15 ±5	70 ±23	87 ±16	6 ±2

of 0.84 n mol of adenine equivalents per min per g liver to over 1 nmol within 2 minutes and was held steady at that level for the remainder of the 4 minutes of the study period (Table 21). Consequently the level of material released by the liver in the presence of angiotensin II was 2.5 times the normal level by the third minute of perfusion. This was reflected in elevated rates of release for virtually all of the purine pool constituents (Figure 29).

The change in the perfusate level of released purine pool constituents in response to angiotensin varied between the different metabolites (Figure 29). ATP and AMP content of the perfusion medium decreased with time while ADP remained steady. IMP and XMP in the perfusate increased. Inosine concentration also increased from a value initially lower than normal. A steady elevated adenosine level was maintained. A steeper increase in xanthosine was observed. Finally unlike the effect of other hormones angiotensin caused the hypoxanthine release into the perfusate to be increased with time.

Perfusate concentrations averaged over the 4 minute period of second perfusion confirmed and quantified the above observations of raised perfusate levels of virtually all the purine pool metabolites (Table 25). Total concentration of the purified and isolated purine pool components was twice that observed in the normal state. 60% of this increase was due to raised purine nucleotide levels, the remaining 40% due to increases in nucleosides and bases. These disproportionate rises again raised the proportion of the perfusate radioactivity due to the labelled nucleotide fraction to 60% from the normal level of 40%.

Thus the effect of angiotensin II on the liver appears

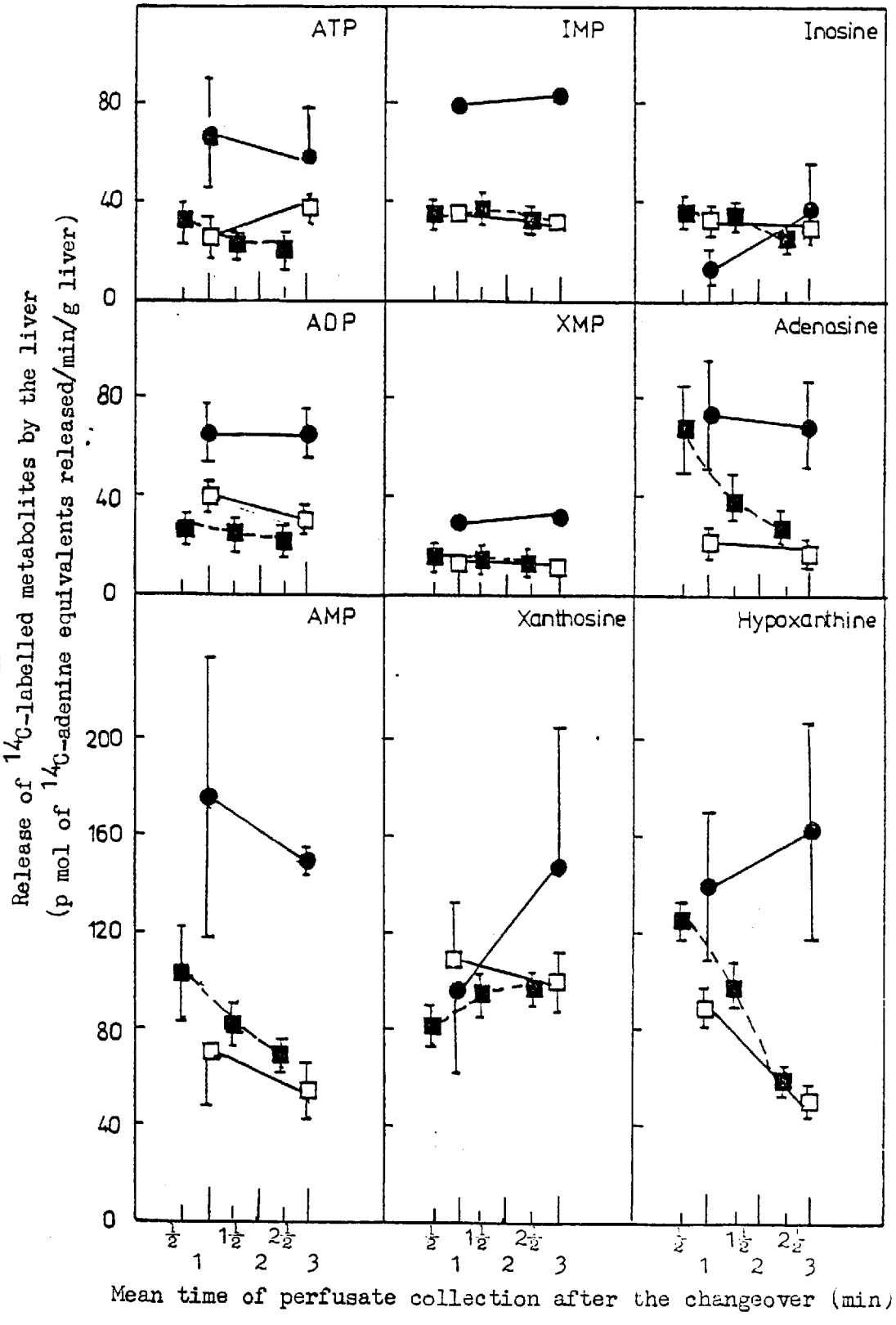


Figure 29: Time course of release by the liver of ^{14}C-labelled purine pool metabolites in the presence of angiotensin II

Experimental details as in Table 21 and 25. For other details see Figure 26. (●) Rate of release in the presence of angiotensin II results are mean ± SEM from four perfusions; (□) rate of release when the second perfusion medium flow rate was reduced by the amount seen in the presence of angiotensin II, results are mean ± SEM from three perfusions; (■) normal rate of release taken from section 3.3.3.

to be one of promoting intracellular AMP degradation as indicated by raised levels of the immediate breakdown products IMP and adenosine, as well as the other degradative products. This occurs in conjunction with an increase in the rate of release of nucleotides and some of the degradative products.

The hormone angiotensin II when present in the second medium at a concentration of 100 ng/ml reduced the perfusate flow by 25% (Table 23) due to vasoconstriction of the hepatic vasculature (Whitton et al., 1978). Thus control perfusions with similar reductions in flow were carried out to distinguish a direct effect on hepatic nucleotide metabolism from the possible effects due to hypoxia resulting from vasoconstriction.

The effect of flow reduction on hepatic levels of labelled purine pool constituents was minimal with no significant changes between the initial and the partially ischaemic liver sample (Table 26). Xanthosine and xanthine levels were the exception, their levels in the ischaemic samples being below the sensitivity of the analysis system, of 50 p mol/g (Figure 29).

The total level of radioactive material present in the perfusate from the partially ischaemic liver sample (Table 21) was relatively steady with the rate of release decreasing from the one minute value of 0.7 n mol per min per g to 0.5 n mol per min per g, so that it was 20% above the normal level by the third minute. There were no clear differences in the rates of release of individual purine metabolites with time between the partially ischaemic and normal liver (Figure 29). In perfusion medium from partially ischaemic

Table 26: Effect of mild ischaemia on adenine nucleotide metabolism in liver prelabelled with ^{14}C-adenine.

Experimental detail as in Table 21. The second perfusion medium did not contain any hormones. First liver sample was removed as before, followed by changing of the perfusion medium. Input perfusate pressure was adjusted to reduce the flow through the liver. Average weight of the liver perfused by the second medium was 6.2 g at a mean flow rate of 1.8 ml/min/g liver. Hepatic levels are expressed as p mol of adenine, originally added to the first perfusion medium, recovered per g liver. Average concentration of the radioactive purine metabolites in the perfusate collected over 4 min is also expressed in terms of adenine originally added for prelabelling. Results are mean ± SEM of 3 perfusions.

	ATP	ADP	AMP	IMP	XMP	ADENOSINE	INOSINE	XANTHOSINE	HYPOXANTHINE	XANTHINE
INITIAL SAMPLE (p mol adenine equivalents recovered/g liver)	16390 ±560	24580 ±840	15760 ±620	520 ±20	880 ±40	270 ±60	260 ±60	140 ±30	240 ±30	110 ±10
SECOND SAMPLE (p mol adenine equivalents recovered/g liver)	15840 ±180	23760 ±270	16270 ±520	540 ±20	910 ±30	290 ±60	280 ±45	-	240 ±20	-
PERFUSATE LEVEL (nM)	17 ±4	19 ±1	34 ±7	18 ±4	8 ±3	11 ±1	17 ±2	58 ±8	38 ±5	7 ±0.4

livers the level of 230 nM for the isolated purine pool metabolites averaged over the time of the perfusion (Table 26) was identical with the level in perfusion medium from normal livers. The proportion of radioactivity between the nucleotide and non-nucleotide fractions of the 'partially ischaemic perfusate' was the same as that seen in the normal perfusate at 40 and 60% respectively. Despite these similarities some variations in the perfusate levels of some of the purine pool components were observed (Table 26). A small elevation in the ATP and ADP levels was accompanied by lowered AMP level. A lowered level of release of adenosine under ischaemic conditions was accompanied by raised levels of some of the other metabolites.

Since the changes observed in adenine nucleotide metabolism in partially ischaemic livers are small, it is likely that the changes observed with angiotensin II are the result of a direct hormonal effect and not of vasoconstriction. Thus in conclusion the effect of angiotensin II on hepatic adenine nucleotide metabolism can be summed up as one of lowering the levels of intracellular purine nucleotides by promoting their release into the perfusate as well as stimulating their breakdown.

Chapter 4

DISCUSSION

4.1 Mechanism of increase in hepatic glycogenolysis in response to anoxia.

4.1.1 Control of hepatic glycogen phosphorylase activity during anoxia.

4.1.2 Changes in adenine nucleotides in anoxic liver.

4.1.3 Hepatic glycogenolysis during shock.

(a) Vasoconstriction and the liver.

(b) Fate of glycogen derived carbon in liver.

4.2 ^{14}C-adenine nucleotide metabolism in liver prelabelled with ^{14}C-adenine.

4.2.1 Prelabelling of the adenine nucleotide pool using ^{14}C-adenine.

4.2.2 Metabolism of intra-hepatic ^{14}C-adenine nucleotides under normal conditions.

(a) Distribution of ^{14}C-isotope in the hepatic adenine nucleotide pool.

(b) Estimate of chemical concentrations of ^{14}C-labelled metabolites.

(c) Purine nucleotide cycle.

(d) Interconversion of purine nucleotides.

(e) Adenine nucleotide degradation in aerobic perfused liver.

4.2.3 Release of ^{14}C-labelled purine material by the liver.

(a) Purine salvage.

(b) Origin of labelled material in the perfusion medium.

4.2.4 Effect of anoxia on ^{14}C-adenine nucleotide metabolism.

4.3 Role of purines in rapid hormonal effects on hepatic carbohydrate metabolism.

4.3.1 Effects of glucagon and insulin on hepatic adenine nucleotide metabolism.

4.3.2 Effects of vasopressin and angiotensin II on hepatic adenine nucleotide metabolism.

4.1 Mechanism of increase in hepatic glycogenolysis in response to anoxia.

Liver, which serves as a major store of utilizable carbohydrates, is the primary organ involved in maintenance of blood glucose level (Glinsmann et al., 1969). Glucose release by the liver is increased under conditions where flow is reduced to the liver (e.g. stress, shock and certain hormone effects). Hypoxia resulting from this reduction in blood flow is considered to have a role in this increased release of glucose. Glycogen phosphorylase may be a possible control site since its activity is known to increase in response to hormones (e.g. glucagon, vasopressin and angiotensin II) which also stimulate glucose release (Hems et al., 1976). However, there has been no report of such an increase in glycogen phosphorylase activity in response to hypoxia or anoxia. Here, a rapid increase in hepatic glycogen phosphorylase a content in response to lack of oxygen has been established and it is suggested that this action may be due to inhibition by AMP of the dephosphorylation of the glycogen phosphorylase a form.

4.1.1 Control of hepatic glycogen phosphorylase activity during anoxia.

The present results (see Section 3.1.1) clearly demonstrate that in livers made anoxic by total ischaemia the amount of available phosphorylase a activity is increased. This is a rapid control response, occurring within 0.5 min of the onset of ischaemia, in both perfused liver and in vivo.

Such an increase in the amount of phosphorylase a in liver during anoxia must reflect either decreased activity of phosphorylase a phosphatase, or increased activity of phosphorylase b kinase.

Phosphorylase a dephosphorylation in liver is inhibited by AMP (Stalmans et al., 1974b) and accelerated by ATP (Goris and Merlevede , 1974). Since AMP rises and ATP falls in hypoxia, the changes in both nucleotides would decrease dephosphorylation of phosphorylase a. However, there was no sign of a stable decrease in phosphatase activity in hypoxic tissue.

Phosphorylase b kinase activity seems unlikely to be increased as a result of phosphorylation (by other protein kinases) in anoxic tissue. Although there are no direct studies of this aspect, it is already known that there is no such increase in livers taken from stressed rats (Van de Werve et al., 1977), or following sympathetic nerve stimulation (Shimazu and Amakawa, 1975) which would produce ischaemia via vasoconstriction. Also, cyclic AMP-dependent protein kinase activity would not accelerate during anoxia; rather, this activity might decrease, as cyclic AMP content decreased in ischaemic liver. The possibility of non-covalent modulation of phosphorylase kinase activity (e.g. by allosteric modifiers) during anoxia remains open, but no modifiers which could fill such a role in anoxic liver have yet been identified.

Thus phosphorylase a phosphatase is likely to play the key role in regulating the availability of phosphorylase a as a function of oxygen tension.

During 15-60 min of total ischaemia in vivo, the amount of phosphorylase a in liver fell to normal. This is

associated with a decline in the rate of anaerobic glycogenolysis (Birch et al., 1974) and is likely to be due to products of glycogenolysis (e.g. glucose) accumulating in the stagnant tissue.

Phosphorylase a from liver responds to a range of modifiers and is for example stimulated by AMP (Stalmans et al., 1974b; Tan and Nuttall, 1975) especially in the presence of glucose (Stalmans et al., 1974b). As an increase in content of AMP in hypoxic liver is one of the earliest responses (Hems and Brosnan, 1970; Faupel et al., 1972) it is likely that in hypoxic tissue this nucleotide plays a key part, not only in the rapid inhibition of the phosphorylase a phosphatase reaction but also by rapid (non-covalent) stimulation of phosphorylase a.

Hepatic phosphorylase b can also be stimulated by AMP (Stalmans and Hers, 1975; Tan and Nuttall, 1975). Although the Ka for AMP (0.5-1.0 mM: ref. Stalmans and Hers, 1975) is higher than for the a form (0.01 - 0.2 mM: ref. Stalmans et al., 1974 b)the AMP content of anoxic liver increases to levels higher than 0.5 mM (Faupel et al., 1972; Hems and Brosnan, 1970), so it is possible that b is active in causing hepatic glycogenolysis, during anoxia.

Glycogen synthetase a activity declined rapidly in totally ischaemic tissue, implying that either the b phosphatase was inhibited, or the a kinase activated. The synthetase b phosphatase in liver may be the same enzyme as the phosphorylase a phosphatase (Killilea et al., 1976) so inhibition by AMP could explain the synthetase inhibition. Also, phosphorylase a can inhibit synthetase b phosphatase (Stalmans et al., 1971; Stalmans et al., 1974a); this effect

would also bring about reciprocal changes in activity of the a forms of synthetase and phosphorylase, such as occur in ischaemic tissue.

4.1.2 Changes in adenine nucleotides in anoxic liver.

Adenine nucleotides comprise one of the most important groups of modifiers of enzyme activity in cells (Newsholme and Gevers, 1967). It would be expected that they would be implicated in control responses during anoxia, as the maintenance of adenine nucleotide status is a vital requirement in anoxic tissue.

During 5 min of anoxia induced by flow cessation in (previously aerobic) perfused liver there was no decrease in the total content of the major adenine nucleotides, (ATP + ADP + AMP) which remained at about 4 μmol/g throughout. This is a difference from totally ischaemic tissue in vivo, which loses adenine nucleotides between 2 and 5 min of ischaemia, in agreement with observations of Hems and Brosnan (1970). The explanation for this could involve the less precipitous decline in ATP in the perfused ischaemic liver compared to that in vivo. This is true whether the decrement in ATP content is expressed in absolute units (μmol/g) or as a percentage change. A possible explanation for this is that the perfused liver might be more highly oxygenated than the liver in vivo (in the pre-ischaemic state), as the perfusate was in near-equilibrium with 95% oxygen; in our normal perfusion conditions, oxygen tension in the input perfusate is about 450 mm Mg.

In totally ischaemic liver the decrease in ATP content was accompanied by rapid increases in ADP and AMP contents.

This initial effect of anoxia is due to failure of oxidative phosphorylation with the consequences that ADP accumulates at the expense of ATP in the mitochondria. Under normal conditions phosphorylation of ADP produced in the cytoplasm and mitochondria takes place in the mitochondrial matrix. Transport of ADP across the inner mitochondrial membrane is via an exchange carrier mechanism (Klingenberg, 1970). Although the carrier system does not discriminate between ADP and ATP, experiments using radioactive tracers indicate that ADP transport into the mitochondria is 10 times as fast as outward movement (Klingenberg, 1970). This however may be due to the relative adenine nucleotide concentration gradients between cytoplasm and the mitochondria. Adenylate kinase activity would preserve the cytoplasmic ATP content at the expense of this ADP, and simultaneously produce AMP. Since adenylate kinase is located on the outer mitochondrial membrane (Chappel and Crofts, 1965) the discrimination in the outward movement of mitochondrial ADP could explain the initial rapid rise of total tissue ADP content followed by fall as the ADP distribution is equilibrated. Also because the enzyme has access only to the cytoplasmic nucleotide pool, and the inner mitochondrial membrane is impermeable to AMP (Adelman, et al., 1968) the increase in AMP content by this reaction will be confined to the cytoplasm.

Cytoplasmic AMP is also formed from ATP during activation of free fatty acids, and exogenous free fatty acids can increase the AMP content of liver (Hulsmann and Kurpershoek—Davidov, 1974). However, it seems unlikely that the rate of activation of endogenous free fatty acids would accelerate in hypoxic liver, as the utilisation of

free fatty acids is not altered (Mayes and Felts, 1976). Decelerated consumptionof AMP in liver during hypoxia could in theory also contribute to an increase in hepatic content. Slowing of the adenylate deaminase reaction does not occur in hypoxia, as ischaemic liver contains more IMP than aerobic liver (Weber et al., 1977). If deceleration of any reaction which uses AMP occurs during hypoxia, in liver, this reaction still has to be identified.

The failure of hepatic oxidative phosphorylation is accompanied by an increase in hepatic glycogenolysis (as indicated by rapid rise in glycogen phosphorylase activity) and glycolysis (e.g. Hems and Brosnan, 1970) to increase the tissue supply of anaerobically produced ATP. Thus the overall response to anoxia is that the site of phosphorylation of ATP moves from mitochondria to the cytoplasm.

The rapid decrease in ATP content of liver made anoxic by total flow stoppage indicates that substrate phosphorylation is not sufficient to maintain adenine nucleotide levels in normal aerobic liver. Lowering of the energy charge as defined by Atkinson (1966) within 5 min also indicates failure of glycogenolysis and glycolysis to be able to furnish hepatic energy needs. This failure, however, is not due to depletion of the hepatic glycogen store since under similar conditions tissue glycogen content decreased from 6% to 4% during 60 min of total ischaemia (Birch et al., 1974). The ability of liver to recover after long periods of total ischaemia has been demonstrated (Zhivkov and Gavazova, 1976).

Induction of anoxia by changing perfusion medium to one containing no oxygen only resulted in small changes

in the hepatic adenine nucleotide content (Section 3.4.2). The rise in hepatic AMP content was still observed. The maintenance of ATP level at 2.3μmol per g after 4 min of anoxia suggests that despite lack of oxygen adequate generation of ATP is occurring. Increased hepatic content of glycogen phosphorylase a under these conditions indicates increased glycogenolysis and glycolysis which will promote ATP generation via substrate phosphorylation. The possibility that a small amount of oxygen in the "anoxic perfusion medium" (gassed with nitrogen:carbon dioxide, 95%:5%) may be responsible for the maintenance of high tissue ATP content, is considered to be unlikely since the oxygen content of the perfusion medium at low oxygen partial pressure will not be sufficient to maintain oxidative phosphorylation. The steeper decline in hepatic ATP content during total ischaemia may be explained by inhibition of glycogenolysis in acidic conditions (Seglen, 1973) resulting from generation of lactate. Inhibition of glycogenolysis of similar magnitude is unlikely in anoxic livers with maintained flow since tissue lactate accumulation will be slow.

Cyclic AMP, produced in tissue from ATP, is a powerful activator of glycogenolysis, and is critically implicated in the activation of glycogenolysis by glucagon (Robinson et al., 1968). Cyclic-GMP can also stimulate glycogenolysis in liver (Exton et al., 1971a). However these nucleotides are not involved in the acceleration of glycogenolysis by hypoxia, as their content decreased in ischaemic tissue. This was presumably due to decline in availability of the precursor triphosphates.

Thus in conclusion the increase in hepatic AMP

content in response to anoxia is largely cytoplasmic and likely to originate from ADP via the adenylate kinase reaction. This rise in hepatic cytoplasmic AMP content occurs in parallel with the increase in glycogen phosphorylase a activity. Since phosphorylase a dephosphorylation in liver is inhibited by AMP (Stalmans et al., 1974b) the above results are thus compatible with the hypothesis that during anoxia, glycogen phosphorylase activity is increased as a result of inhibition by AMP of the dephosphorylation of the a form.

4.1.3 Hepatic glycogenolysis during shock

Under-perfusion of the liver is likely to occur during circulatory collapse ("shock"). The present perfusion experiments at a flow of 0.6 ml/min/g suggest that in this situation the main purpose of the accelerated glycogenolysis is to produce glucose for release to blood. Although this may well result in hyperglycaemia, it would serve to preserve the total quantity of glucose available in the bloodstream, for maintenance of cerebral function.

(a) Vasoconstriction and the liver.

The present data shed light on metabolic events during vasoconstriction in the liver. Vasoconstriction can cause a reduction in liver blood flow in several situations. First, the portal vasculature responds to e.g. adrenalin or angiotensin II in rat (Hems et al., 1976) and also to adrenalin or vasopressin in dog (Richardson and Withrington, 1977 & 1978). Also, a decrease in portal venous flow will occur in any state where splanchnic flow is reduced by intestinal constriction. Finally, there is hepatic vasoconstriction

during sympathetic discharge to the liver (Shimazu and Amakawa, 1975).

In all these states, vasoconstriction would cause hypoxia, and hence glycogenolysis. The present results suggest that this would be mainly for the purpose of releasing glucose to blood, and that AMP acting on the phosphorylase system would provide the main regulatory device.

During sympathetic discharge to the liver, there is an increase in the amount of available phosphorylase a which is not associated with an increase in tissue content of cyclic AMP, i.e. does not seem to involve adenyl cyclase activation by noradrenaline released from nerves (Shimazu and Amakawa, 1975). The present results suggest that this response could be a result of vasoconstriction-induced hypoxia, i.e. that during sympathetic discharge, a rise in hepatic AMP content would account for the increase in available phosphorylase a.

(b) Fate of glycogen-derived carbon in liver.

In aerobic perfusions, at a haematocrit of 25%, glycogen degradation amounted to 50 µmol glycogen-glucose per g of fresh liver, in one hour. This reflects the rate of glycogenolysis in a well-oxygenated liver, receiving an input perfusate containing plenty of erythrocytes, and oxygen at a tension of about 450 mm Hg. The degree of net glycogenolysis resembles that determined previously by analysis of serially-removed samples in livers of fed rats perfused at similar haematocrits (Glinsmann et al., 1969; Hems and Whitton, 1973).

From this data, an assessment of the fate of glycogen-derived hexose-phosphates (in basal non-hormonal conditions) may be made. Glucose and lactate concentrations in perfusate were constant during the one hour perfusion period, at about 8mM and 2mM respectively. These concentrations resemble those in blood in vivo, and this "autoregulation" by liver of the circulatory concentration of these metabolites confirms previous reports (Glinsmann et al., 1969). The autoregulation depends on the presence of sufficient erythrocytes in blood (Glinsmann et al., 1969).

Glycogen-derived carbon was therefore not supplying glucose and lactate to blood in these aerobic livers. There are no other likely major fates of glycogen as released carbon, (i.e. as products in blood). Although in hypoxic liver small quantities of amino-acids such as alanine may be released (Parrilla et al., 1977) these are likely to be derived from proteolysis. Hence all the glycogen degraded in these standard aerobic conditions must meet intra-hepatic fates. As glucose and lactate did not undergo net hepatic uptake in these conditions, glycogen probably supplied all the carbon for lipogenesis. It is now well established that glycogen can supply significant carbon for lipogenesis in liver, whereas glucose does not, even at supra-normal concentrations (Clark et al., 1974; Salmon et al., 1974). Under similar perfusion conditions, the rate of fatty acid synthesis (measured with ^3H_2O) was about 18 μmol C_2 units per g in one hour (Dr C Kirk, unpublished results). Fatty acid synthesis therefore would account for 9 of the 50 μmol intra-hepatic glycogen-derived glucose which were metabolised/hr.

The remainder of the glycogen-derived hexose (41 μmol

glucose/hr per g of wet liver) is likely to have been oxidised to carbon dioxide. The oxygen uptake corresponding to such complete oxidation would be (6 x 41), i.e. 246 µmol/g/h. During perfusion in the present conditions, oxygen uptake is 3.75 µmol/min/g, i.e. 225/h/g. This very close agreement suggests that glycogen entirely sustains the tricarboxylic acid cycle and ATP turnover during fully aerobic basal conditions in the liver of the fed rat, i.e. where there is no utilization of exogenous substrate.

These characteristics of the liver were maintained even during reduction of flow rate to half the standard rate (in perfusion with 12% haematocrit). This was true despite a slight decline in hepatic ATP content in perfusions with decreased flow rates. There were no increases in hepatic content of AMP and ADP however, consistent with the concept that glycogenolysis is more dependent on regulation by these nucleotides, than ATP. The same inference was drawn in the previous section, from the time courses of events in acutely ischaemic liver.

If perfusate flow was reduced to one-quarter of the standard rate (0.6 ml/min/g of fresh liver), glycogenolysis was accelerated. The extra glycogenolysis (46 µ mol glucose/g/h, over the control rate of 62 in the perfusions, which were at haematocrit 12%) exactly accounted for the extra glucose and lactate which were released into perfusate (18µmols lactate and 36 µmols glucose per g in one hour). Thus in mildly hypoxic conditions, simulating circulatory shock in vivo ("under perfusion", with erythrocytes still present), there is acceleration of glycogenolysis and glycolysis in the liver with release of glucose and lactate into the blood.

It should be noted that the regulatory mechanisms described above are likely to be continually operative in liver in vivo. Rather than being saturated with oxygen in most states, the liver is probably "poised" at a particular oxygen tension at any one moment (but which can vary as a function of time), implying that glycogen metabolism and the controlling nucleotide contents would be continually varying in response to small changes in oxygen tension.

4.2 ^{14}C-adenine nucleotide metabolism in the liver prelabelled with ^{14}C-adenine

4.2.1 Prelabelling of the adenine nucleotide pool using ^{14}C-adenine.

Incorporation of adenine into hepatic intracellular nucleotides is thought to involve passive diffusion of the adenine across the cell membrane, followed by conversion to 5'-AMP by the action of adenine phosphoribosyl transferase considered to be located intracellularly (Partsch et al., 1977) with 5-phosphoribosyl pyrophosphate (PRPP) as the co-substrate. Results from experiments described in Section 3.2.1 indicate a gradual incorporation into the adenine nucleotide pool. This is in agreement with the observations that in liver slices exposed to ^{14}C-adenine most of the ^{14}C-isotope not in its original form was found as adenine nucleotide (Daly, 1972).

Synthesis of adenosine monophosphate from the precursor ^{14}C-adenine was estimated to occur at a rate of 10 n mol/min/g, from the steady rise in the amount of labelled adenine nucleotides. Since incorporation of adenine into nucleotides is via the enzyme adenine phosphoribosyl transferase requiring the substrate PRPP, the above rate of adenine utilization also gives an estimate of PRPP production in the liver slices, i.e. they are capable of producing at least 10 n mole of PRPP/min/g. An intracellular concentration range for PRPP of 10-300 µM has been reported for mammalian cells (Henderson and Khoo, 1965a & b; Lalanne and Henderson, 1974). However the amount of PRPP immediately available for reaction with adenine was low as indicated by extrapolation of the above linear time course of AMP formation back to zero time.

The possibility that availability of phosphoribosyl pyrophosphate may be limiting during incorporation of externally added adenine is also indicated by the observation that PRPP synthesis and purine nucleotide synthesis are stimulated by increasing the concentration of phosphate in the incubation medium (Henderson et al., 1975). In addition it has been observed that inhibition of de novo purine biosynthesis was accompanied by an increase of 60-80% in the rate of nucleotide formation from added purines (Henderson et al., 1975). A Km value in the order of 20μM for the enzyme adenine phosphoribosyl transferase for the substrate adenine has been calculated by Shimizu and Daly (1972). Their kinetic studies involved measuring the initial rate of accumulation of ^{14}C-adenine nucleotide from different concentrations of ^{14}C-adenine. In human lymphocytes and cultured lymphoblasts the rate of nucleotide synthesis from adenine is much lower than the total activity of purine phosphoribosyl transferase (Fields and Brox, 1974). Thus it may be concluded that despite the enzyme being inhibited under physiological conditions phosphoribosyl pyrophosphate availability may be a controlling factor.

Assimilation of ^{14}C-adenine by the perfused liver was rapid and near completion within 10 min. The presence of only 1% of the intra-hepatic ^{14}C-isotope as the precursor adenine again indicates an estimated rate of adenine incorporation of at least 10 n mol per min per g. This rate of metabolism of adenine is similar to that observed in liver slices. The increase in the hepatic nucleotide pool is only 2% at 60-70 n mol per g liver. The effect of prelabelling of the hepatic adenine nucleotide pool in this manner on the nucleotide content of the liver was minimal since no differences in the presence of

adenine were apparent. Uniform labelling of the adenine nucleotides within the cell will depend on the intracellular location of the enzymes and on time dependent distribution of adenine nucleotides within cellular compartments, such as mitochondria and cytoplasm and the ease and rates with which they are transported between the two. Thus the use of the prelabelling technique to monitor nucleotide metabolism must be used with caution, since specific activity of the nucleotides cannot be used to calculate endogenous concentrations of other metabolites from the levels of radioactivity incorporated into that metabolite.

4.2.2 Metabolism of intra-hepatic ^{14}C-adenine nucleotides under normal conditions.

(a) Distribution of ^{14}C-isotope in the hepatic adenine nucleotide pool.

Analysis of the results in Section 3.2.2 revealed that the ^{14}C-isotope was predominantly present in the adenine nucleotides. As mentioned previously (Section 4.2.1) this incorporation of ^{14}C-adenine is mediated via the enzyme adenine-phosphoribosyl transferase forming the product AMP. The newly synthesised radioactive AMP can undergo one of many reactions as outlined in Figure 30. Redistribution of the ^{14}C-isotope amongst the other adenine nucleotides, catalyzed by adenylate kinase, as shown by the results, predominates in comparison with the AMP degradation reaction. This redistribution was rapid and relatively stable, between 20 and 40 min of perfusion, since the relative levels of labelled adenine nucleotides were unchanged during this period.

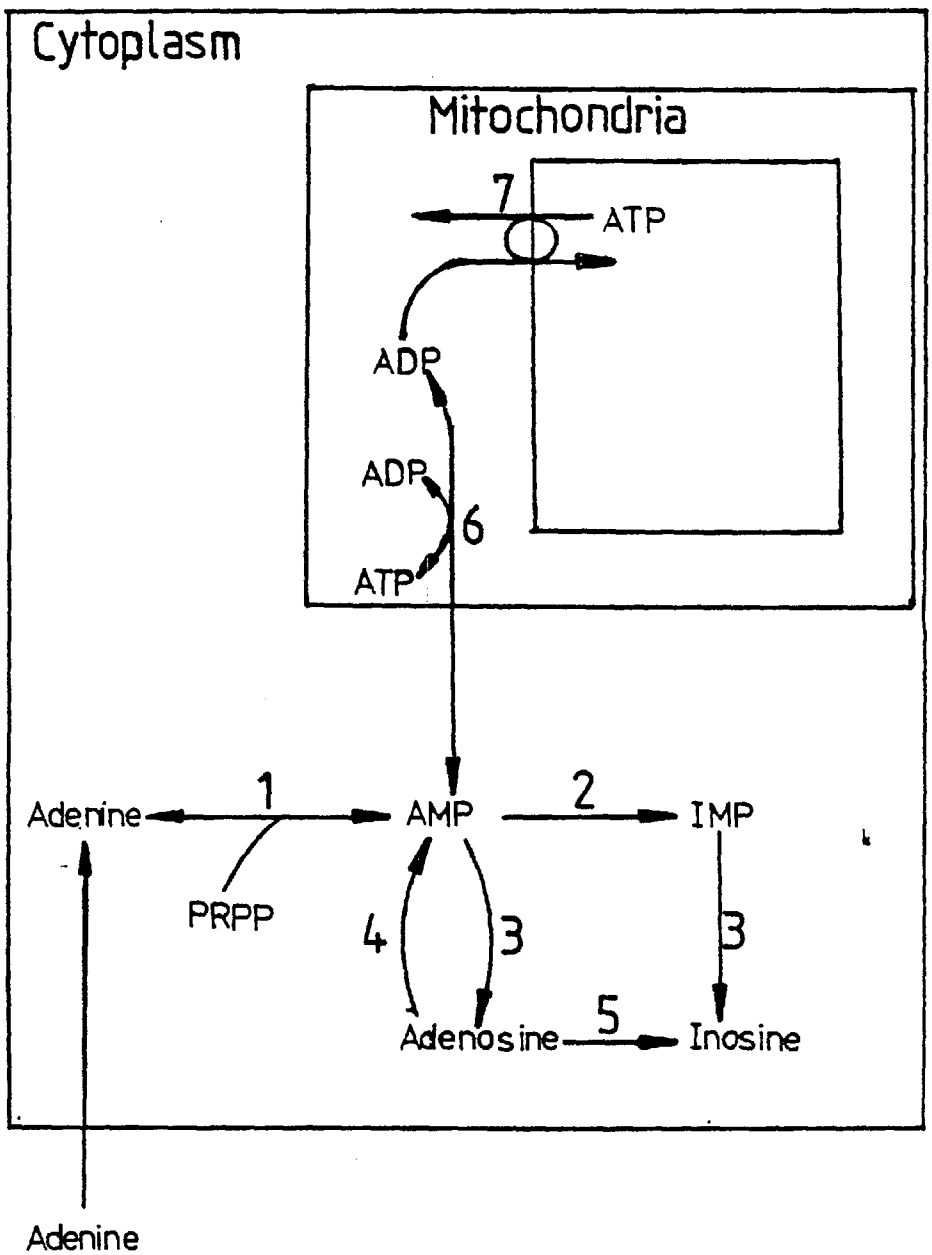


Figure 30: Metabolism of ^{14}C-adenine precursor in the perfused liver.

The enzymes involved are: 1. adenine-p-ribosyl-transferase; 2. AMP deaminase; 3. 5'nucleotidase; 4. adenylosuccinate lyase; 5. adenosine deaminase; 6. adenylate kinase; 7. adenine nucleotide translocase.

This distribution of radioactivity amongst the adenine nucleotides showed more labelling of the ADP fraction than the other two nucleotides. Similar profiles have been reported for the labelling of the perfused liver in this manner (Pritchard et al., 1970; Lerner and Lowry, 1974). In hepatocytes, however, incubation with ^{14}C-adenine results in more isotope being incorporated into the ATP fraction (Smith et al., 1977), showing better equilibration of the isotope amongst the adenine nucleotides. This may be due to the longer time of incubation.

Ratios of the specific activity of ATP, ADP and AMP indicated that distribution of radioactivity between the nucleotides ADP and AMP was proportional to their chemical concentrations while disproportionally less labelling of ATP occurred. Distribution of the radioactivity in this manner may be partially due to the presence of two major pools of adenine nucleotide, mitochondrial and cytoplasmic, with restriction in movement of nucleotides between the pools while some enzymes are restricted to particular pools. Notably AMP is impermeable to the inner mitochondrial membrane and its phosphorylation to ADP is catalysed by adenylate kinase located in the intramembrane space of the mitochondria. ADP is capable of crossing the inner membrane in exchange for ATP, using nucleotide translocase, into the matrix where phosphorylation to ATP takes place via the oxidative phosphorylation system.

The compartmentation phenomenon does not, however, explain the maintenance of the disproportionate labelling of the adenine nucleotides since normal rapid turnover in nucleotide metabolism should ensure equilibration of

radioactivity amongst the three nucleotides despite the barriers. The answer may be in binding of adenine nucleotides by the intracellular proteins. For the case of skeletal muscle it is estimated that nearly 90% of the cytoplasmic AMP and ADP is protein bound while ATP is effectively free (Seraydarian et al., 1962). If this was also true for the liver than most of the labelled AMP and ADP would be sequestered by the proteins. Consequently distribution of radioisotope into the ATP fraction involving transport of labelled ADP into the mitochondrial matrix would be slow. This would explain the slow equilibration of radioactivity with the ATP pool observed in the perfusion experiments.

(b) Estimate of chemical concentrations of ^{14}C-labelled metabolites.

In prelabelling experiments of this type it is difficult to assess distribution of labelled adenine nucleotides between the two main subcellular compartments of mitochondria and cytoplasm since the rate at which equilibration might take place is not known. Knowledge of distribution of labelled AMP is important since that can be used to calculate specific activity of the cytoplasmic pool, where degradation is localized, and thus enable quantification of the whole process of degradation and release by the liver.

Cytoplasmic AMP concentration in hepatocytes has been estimated, by the use of the digitonin technique to be approximately 25% of the total intracellular AMP (Akerboom et al., 1978; Siess et al., 1977). Thus ^{14}C-AMP will be dispersed in a pool whose size will vary between 25% and 100% of the total cellular AMP level. For the

purpose of estimating the chemical levels of the various labelled purine metabolites the specific activity of AMP is calculated assuming that the chemical AMP is evenly labelled. Thus using this AMP specific acitivity it is possible to estimate the chemical levels of the various labelled purine metabolites The chemical level range of the purine pool metabolites is calculated from the amount of radioactive material present in that particular metabolite (Table 27).

These calculated values compare within an order of magnitude with the reported intracellular concentrations of IMP and XMP at 8 and 3 n mol per g liver respectively (Weber et al., 1977) and adenosine at 5-20 n mol per g (Arch and Newsholme, 1978). Woods and colleagues (1970) have however reported hepatic IMP values of 0.1µmol per g liver. This high value cannot be explained by anoxia since the hepatic AMP content reported by the latter two groups of workers was similar and since anoxia has been shown to raise hepatic IMP content (Weber et al., 1977). The usefulness of these values is limited since only the concentrations of the various metabolites produced from AMP are measured, while other non-radioactive purine metabolite sources such as degradation of guanine nucleotides, and de-novo synthesis of IMP, are not taken into consideration. However, hepatic guanine nucleotides are relatively insignificant as suppliers of degraded purine material because their chemical concentration is only 14% of the adenine nucleotide concentration (Weber et al., 1977).

Thus, care should be taken when referring to Table 27 because the concentration of some of the metabolites will be underestimated, by a factor which will depend

Table 27: Calculated hepatic concentrations of purine pool metabolites.

Hepatic concentrations of the purine pool metabolites were calculated from the amount of ^{14}C-adenine incorporated into the metabolite and the estimated specific activity of the hepatic AMP pool (see text for detail). Results expressed as the range observed in the various perfusions.

Purine metabolite	Calculated hepatic chemical level (nmol/g liver)
IMP	10-20
XMP	30-36
adenosine	12-22
inosine	2-10
xanthosine	1-4
hypoxanthine	7-10
xanthine	0.5-5

on the rate at which a product is being produced from an additional source.

(c) Purine nucleotide cycle.

The purine nucleotide cycle comprising the reactions catalyzed by AMP deaminase, adenylosuccinate synthetase and adenylosuccinate lyase has been demonstrated in the liver (Moss and McGivan, 1975; Lowenstein, 1972). Several roles have been attributed to the operation of the cycle (Section 1.3.3), the main being the provision of an alternative pathway for the liberation of ammonia from amino acids. Rognstad (1977) in investigating the relative roles of glutamate dehydrogenase and the purine nucleotide cycle in NH_3 production in isolated rat liver cells came to the conclusion that the cycle was not a major source of NH_3 production in the liver.

In the perfused liver, prelabelled with ^{14}C-adenine, labelled IMP was less than 5% of the labelled AMP concentration. This low level of conversion coupled with the view that AMP deaminase is likely to be inhibited under physiological conditions (Van den Berghe et al, 1977b) indicates that the purine nucleotide cycle may not operate at a significant rate in the liver, in agreement with the conclusions reached by Rognstad (1977). The low level of operation of the purine nucleotide cycle in the liver is reconcilable with conservation of adenine nucleotides since the function of cytoplasmic 5'-nucleotidase is considered to be one of hydrolysis of IMP (Van den Berghe et al., 1977a).

(d) Interconversion of purine nucleotides.

Enzymes required for the interconversion of adenosine, inosine and guanosine mononucleotides exist in the liver.

The reactions involved in conversion of AMP to GMP are initial deamination to IMP and reduction to XMP followed by reamination to GMP. Levels of radioactive IMP and XMP were about 4% and 5% of labelled AMP concentrations, with very low levels of radioactivity associated with guanosine nucleotides. The results thus illustrate that formation of GMP from AMP occurs at an insignificant rate.

Inhibition of the AMP deaminase would be the main control mechanism in the interconversion as discussed above with further control being indicated at the reamination step because of the accumulation of radioactive xanthosine monophosphate. Operation of the purine nucleotide cycle would also restrict conversion into guanine nucleotide by recycling IMP back to adenine nucleotide.

(e) Adenine nucleotide degradation in aerobic perfused liver.

The hepatic adenine nucleotide pool, consisting of ATP, ADP and AMP maintained at equilibrium by adenylate kinase is subject to continual catabolism by the AMP metabolizing enzymes 5'-nucleotidase and AMP deaminase. The existence of a steady state in the hepatic concentrations of labelled IMP, adenosine and other purine breakdown products signals a continuous breakdown of adenine nucleotides. Under these conditions of steady hepatic purine metabolite concentrations, release of the degraded purine material gives a measure of AMP degradation.

This rate of release amounted to 4% of the hepatic labelled AMP content per minute. From the above specific activity of intracellular AMP the estimated rate of total AMP degradation as measured by release of material into

the perfusate is 14 n mol per minute per g liver. The size of the cytoplasmic AMP pool in contact with the AMP degradative enzymes is calculated to be 95 n mol per g liver. This calculation is based on the work of Siess et al., (1977) and Akerboom et al., (1978) on AMP distribution between cytoplasmic and mitochondrial compartments. Thus using these estimates it is calculated that 10-15% of cytoplasmic AMP is being degraded per minute. The depletion of the adenine nucleotide pool from this estimate is occurring at a rate of 1% per minute.

The role of 5'-nucleotidase in intracellular AMP degradation is thought to be minimal since a large proportion of the enzyme is located on the outer plasma membrane (Gurd and Evans, 1974) while the intracellular enzyme is virtually inactive at physiological AMP concentration (Van den Berghe et al., 1977b). AMP deaminase which is considered to control the breakdown of AMP is also greatly inhibited under physiological conditions (Van den Berghe et al., 1977b). Thus from these enzymic studies a very low rate of AMP degradation is suggested under normal physiological conditions. The presence of labelled adenosine and IMP, however, suggests that both of the enzymes are active to some extent. From the present data it is difficult to distinguish whether either of the two paths is favoured since the tissue levels of IMP and adenosine are similar, and further breakdown products are common.

4.2.3 Release of ^{14}C-labelled purine material by the liver.

(a) Purine salvage

The results presented show that a whole range of purine containing compounds were released into the perfusion medium by the liver (Section 3.3.3). This release of purine

material is essential to the concept of supply of purine material for use by other tissues. Lajtha and Vane (1958) were amongst the first workers to implicate liver in the supply of purine to bone marrow. Further work using perfused rabbit liver revealed that the released material was predominantly adenosine (Lerner and Lowry, 1974; Pritchard et al., 1975). Hypoxanthine and inosine were also detected (Pritchard et al., 1975) but they were considered to be breakdown products of adenosine. The postulated reason for the predominant release of adenosine was that it was supplied by the liver for the use of red blood cells to maintain their adenine nucleotide turnover. This was because they could only rapidly utilize adenosine due to their low capacity to convert IMP to AMP. According to estimates of Pritchard and co-workers (1975), the amount of adenosine being released at a concentration of 12μM was just sufficient to supply the needs of red blood cells. The remaining organs requiring purine presumably utilize purine material discarded by the red blood cells.

The results reported however show that the whole range of purine pool components labelled in the liver was being released into the perfusate. The purine material being released was fairly evenly shared between the various compounds, amongst which AMP, hypoxanthine and xanthosine concentrations were the highest. The level of adenosine only accounted for 10% of the total purine material.

Calculated requirements of the rat red blood cells, based on assumptions of Pritchard et al.,(1975) reveal it to be 10-20 n mol of purine material per min for a 200 g rat. This requirement is amply catered for by the 70 n mol/min supply of purine material by the liver. At least 50% of this can be readily

utilized by the red blood cells since it is present as either adenine nucleotide or adenosine. The presence of AMP and the lower levels of ATP and ADP all combine to lower dependence of red blood cells on adenosine. Presence of other purine containing compounds indicates that release of purines is not exclusively for utilization by red blood cells as suggested by Pritchard et al., (1975) and that material for use by other organs may also be released.

(b) Origin of labelled material in the perfusion medium.

Results reported in Section 3.3 reveal that the perfusion medium contains a number of purines. Elucidation of their origin in the perfusate may enable us to locate the site of action of a hormone if it is found to influence the type of purine material being released. This alteration in the type of material being released may be relevant to the concept of intracellular messengers.

Distribution of the various enzymes of adenine nucleotide metabolism has been described in Section 2.2. One problem that is prevalent when considering plasma membrane enzymes is their orientation and the possibility of vectorial function. Detailed information is available concerning the enzyme 5'-nucleotidase, which has been described as a plasma membrane marker. Amongst the properties to be characterized are its location, the enzyme being predominantly membrane bound (Naito et al., 1974; Lelievre et al., 1977); and orientation, 80-90% of the membrane bound fraction being on outward facing (Gurd and Evans, 1974). There are conflicting reports on the vectorial nature of the enzyme with reports of extracellular AMP leading to extracellular

production of adenosine in perfused rat heart (Frick and Lowenstein, 1976) and intracellular production in human lymphocytes (Fleit et al., 1975). Membrane bound enzymes which may function as ectoenzymes include ATP pyrophosphatase (Evans, 1974) and purine nucleoside phosphorylase (Li and Hochstadt, 1976b). Other enzymes of purine metabolism have also been found to be plasma membrane associated.

Thus it is apparent that the origin of the ^{14}C-labelled purine metabolites poses a problem since a number of possible routes for the production of a particular metabolite are present. In this section the origins of the released purine compounds will be considered individually.

Nucleotides: ATP, IMP and XMP can be assumed to be released from the cell as intact compounds since the enzymes leading to their production are not membrane located. Permeability of hepatic plasma membrane to ATP has been demonstrated by Chaudry et al., (1976b). AMP present in the perfusate could be either released directly or be produced by ATP degradation, since membrane bound ATP pyrophosphatase (Bischoff et al., 1975) is known to be an ecto-enzyme (Evans, 1974). The perfusate levels however suggest that the majority of the AMP would be released from the cell because extracellular ATP levels are always much lower than AMP and secondly AMP would be easier to transport across the cell membrane than ATP. The plasma membrane enzyme Na^+/K^+-ATPase probably has no role in the production of extracellular ADP since it is located on the cytoplasmic face of the plasma membrane and is not known to have vectorial properties concerning the adenine nucleotide substrates (i.e. ADP produced remains in the cytoplasm). Thus ADP present in the perfusate may have originated inside the cell and been transported across the plasma membrane.

Adenosine: Intracellularly formed adenosine can be released into the perfusion medium, since adenosine transport across the plasma membrane is thought to be by facilitated diffusion (Arch and Newsholme, 1978). Under anoxic conditions as well as in response to glucagon and insulin, it was observed that raised intracellular levels of adenosine were not accompanied by corresponding rises in perfusion medium level suggesting that adenosine release is not a simple matter of diffusion across the plasma membrane and that there may be interplay with other mechanisms. Presence of the enzyme 5'-nucleotidase at the plasma membrane with an 'outward' facing active site has also been suggested to lead to formation of extracellular adenosine from external AMP (Frick and Lowenstein, 1976). Fleit and associates (1975) have however reported that the membrane located 5'-nucleotidase may function in a vectorial manner by transporting the product across the membrane, thus suggesting that cytoplasmic AMP may be the precursor for external adenosine. Conversely, the role of the 'outward' facing 5'-nucleotidase is likely to be one of salvaging external AMP by transporting the product adenosine into the cytoplasm. The low extracellular ^{14}C-AMP concentration indicates that intracellular AMP may be the major contributor of extracellular adenosine.

Inosine: Origins of inosine present in the perfusion medium are even more complex than adenosine. Mechanisms discussed for extracellular formation of adenosine also apply to extracellular inosine formation since its permeability across the plasma membrane may be similar to adenosine and because the enzyme leading to adenosine production (5'-nucleotidase)

will also hydrolyse IMP to inosine. The plasma membrane does not allow easy passage of inosine, as indicated by the observation that the increase in intracellular concentration in response to insulin, glucagon, vasopressin and angiotensin II was not accompanied by a similar increase in extracellular concentration. A minimal role of 5'-nucleotidase in the production of extracellular inosine was suggested by two observations: firstly, in the presence of glucagon when 5'-nucleotidase is still active complete absence of inosine was observed; and secondly, under conditions where 5'-nucleotidase appears to be stimulated (e.g. in the presence of vasopressin and angiotensin II) inosine level was unaltered. Both these observations indicate an alternative source of external inosine. Good correlation between perfusate concentrations of hypoxanthine and inosine in the control and partially ischaemic set of perfusions suggests that extracellular hypoxanthine may be the predominant precursor of extrahepatic inosine. Li and Hochstadt (1976b) demonstrated that membrane bound purine nucleoside phosphorylase may be an ectoenzyme. Agarwal et al., (1975) have reported that the Km app of the rat erythrocyte purine nucleoside phosphorylase for hypoxanthine is 13μM while that for inosine is 83μM. Thus extracellular formation of inosine from hypoxanthine is possible.

Xanthosine: Formation of perfusate xanthosine is similar in mechanism to that for inosine since similar reactions are involved. Unlike inosine, however, extracellular xanthosine is not exclusively formed from xanthine but may also originate, either by the action of 5'-nucleotidase on the intracellular XMP or by diffusion of intracellular xanthosine.

Hypoxanthine: Presence of hypoxanthine in the perfusate may be largely by diffusion of intracellularly formed hypoxanthine, despite its participation in a number of reactions. Hypoxanthine is produced by the enzyme nucleotidase while three other enzymes (purine nucleoside phosphorylase, hypoxanthine p-ribosyl-transferase and xanthine oxidase) are responsible for its metabolism. Combination of these reactions may explain the decrease in the initially high perfusion medium concentration of hypoxanthine during the experiments. The enzyme purine nucleoside phosphorylase would elevate perfusate inosine concentrations while hypoxanthine phosphoribosyl transferase and xanthine oxidase would raise intracellular concentrations of IMP and xanthine.

Xanthine: The presence of this compound in the perfusion medium is again likely to be from intracellular sources where it is produced by oxidation of hypoxanthine. Its presence in perfusion medium may lead to formation of extracellular xanthosine by purine nucleoside phosphorylase.

4.2.4 Effect of anoxia on hepatic ^{14}C-adenine nucleotide metabolism.

The changes in chemical levels of hepatic adenine nucleotides in response to different types of anoxia have been discussed in Section 4.1.2. Experiments reported in Section 3.4 studied the effect of anoxia on adenine nucleotide metabolism in liver prelabelled with ^{14}C-adenine in an effort to supplement the above study and elucidate which is the major pathway of AMP degradation in anoxia.

Ischaemia

Results reported in Section 3.4.1 illustrate that total ischaemia induced depletion of labelled ATP in the liver which was accompanied by accumulation of AMP. These observations are in agreement with the changes in chemical levels of the nucleotides reported in Section 3.1.4. A decrease in ^{14}C-ADP concentration was observed despite an increase in the chemical concentration. This may be a reflection of the low specific activity of the ATP fraction and the problem of compartmentalization discussed earlier (see Section 4.2.2). Increased accumulation of ADP due to failure of oxidative phosphorylation would occur in the mitochondria, predominately from ATP of low specific activity. Thus whilst the majority of the non-radioactive ADP accumulates in the mitochondria, recycling of the cytoplasmic ADP by substrate rephosphorylation and adenylate kinase would promote re-equilibration of the radioisotope. The net results of these processes would be an increase in non-radioactive proportion of ADP while preferential removal of radioactive ADP was occurring.

An increase in the concentration of AMP breakdown products (measured using the pre-labelling technique) indicated stimulation of AMP degradation, presumably to maintain a low AMP concentration. The enzymes 5'-nucleotidase and AMP deaminase, involved in AMP degradation, are greatly inhibited under normal physiological conditions (Van den Berghe et al., 1977a, b; Moss, 1977). They are thus capable of being stimulated to lower the intracellular AMP concentration. In the experiments reported in Section 3.4 involving cessation of hepatic flow for 5 min, the proportion of AMP degraded via AMP deaminase was marginally more than via 5'-nucleotidase, because more AMP had been converted into IMP and XMP than into adenosine. But no clear cut conclusions can be made as to the relative prominence of the two enzymes since the amount of inosine (which is produced from both adenosine and IMP) was nearly equal to the total amount of adenosine, IMP and XMP. Thus the mechanism involved in stimulation of AMP degradation by flow cessation is unclear since elevation of all the purine metabolites was observed.

Anoxia

The onset of anoxia, by changing the perfusion medium to one lacking oxygen, reduced the hepatic concentration of labelled and un-labelled ATP and was accompanied by an increase in AMP concentration. The magnitude of the changes involved were considerably smaller than those observed when anoxia was induced by flow cessation. This offers further support to the view that maintenance of flow through the liver even during anoxia enables it to maintain a more normal adenine nucleotide profile.

Anoxia, also, resulted in an increase in the perfusion medium concentration of AMP and ATP. The increase in AMP concentration can be explained on the basis of increased cytoplasmic production. The increase in ATP release, however, is paradoxical in that it occurs under conditions where its synthesis has been impaired. One possible explanation may be that since ATP is being generated in the cytoplasm by substrate phosphorylation, there may be elevation of the cytoplasmic concentration of free ATP, thus resulting in more ATP being available for transportation out of the cell.

Stimulation of adenosine monophosphate degradation by anoxia is evident from the raised hepatic levels of ^{14}C-labelled AMP breakdown products. The rate of AMP degradation was however much lower than that observed in totally ischaemic samples. This is presumably due to the lower AMP content of anoxic tissue. Thus under these conditions where the rise in tissue AMP concentration has been less acute, the increase in the concentration of IMP, while adenosine concentration decreased, indicates that the increase in AMP degradation was primarily due to stimulation of AMP deaminase. Lack of an increase in 5'-nucleotidase activity can be explained by the properties of the cytoplasmic 5'-nucleotidase which is inhibited by Pi and stimulated by ATP (Van den Berghe, 1977a); since anoxia increases Pi and decreases ATP concentrations (Faupel et al., 1972; Brosnan et al., 1970). In addition the cytoplasmic 5'-nucleotidase has a sigmoidal substrate-saturation curve for AMP, making the enzyme almost totally inactive at physiological and slightly raised concentrations of AMP (Van den Berghe, 1977a). The activity

of AMP deaminase is also under complex allosteric control; ATP stimulates the enzyme while GTP and Pi inhibit so that under physiological conditions it is greatly inhibited (Van den Berghe et al., 1977b). The observed increase in activity, under conditions which decrease ATP and GTP and increase Pi concentration (Weber et al., 1977), suggests possible de-inhibition resulting from lower GTP concentration.

Hydrolysis of IMP by 5'-nucleotidase, however, was not affected since the increase in intracellular inosine concentration in response to anoxia was proportional to the rise in IMP. This maintenance of activity indicates a balance between the decrease in 5'-nucleotidase activity by changes in modifiers and the increase in activity in response to increased concentration of the substrate IMP.

The decrease in adenosine content of the liver during anoxia was less than that observed under normal conditions. The kinetic properties of the cytoplasmic 5'-nucleotidase predict inhibition of this enzyme with AMP as substrate (Van den Berghe et al., 1977a). An increase in the hepatic concentration of inosine (due to increased production from IMP) to a level above that of adenosine during anoxia will result in feed back inhibition of adenosine deaminase. Thus the reduction in cytoplasmic adenosine concentration must reflect an active adenosine kinase reaction even though this will lead to increased AMP concentration.

A reduction in the adenosine concentration of the perfusion medium by anoxia may indicate possible inhibition of membrane bound 5'-nucleotidase since the indications so far are that perfusate adenosine is predominantly formed from cytoplasmic

AMP via this enzyme. However, an increase in ATP concentration in the locality of the plasma membrane, as indicated by increased release, would have this effect since ATP has an inhibitory effect on the plasma membrane bound 5'-nucleotidase (Evans and Gurd, 1973).

An increase in the concentration of hypoxanthine and xanthine in the perfusion medium reflect the increased hepatic concentrations which are a direct consequence of increased nucleotide degradation.

The low perfusion medium adenosine concentration is at odds with the raised inosine and xanthosine concentrations since they are all products of the enzyme 5'-nucleotidase and may be expected to behave in a similar manner.
The raised perfusion medium concentration of hypoxanthine and xanthine may make a large contribution by extracellular formation of inosine and xanthosine, respectively, especially since the enzyme purine nucleoside phosphorylase has been demonstrated to be located on the plasma membrane, catalyzing extracellular purine conversion (Li and Hochstadt, 1976b).

Thus in conclusion, stimulation of AMP degradation accompanies anoxia induced by either flow cessation or oxygen deprivation. The increases in hepatic content of IMP, XMP, adenosine, hypoxanthine and other breakdown products suggest a non-specific manner of stimulation. This may be a result of the massive increase in tissue AMP content which may maximally stimulate the various degradative reactions, and thus may have less relevance in control of AMP degradation under physiological conditions. In anoxia where the increase in hepatic AMP concentration is smaller, the increase in AMP degradation occurs via stimulation of the AMP deamination

reaction. The likely pathway involved in anoxic stimulation is summarized in Figure 31.

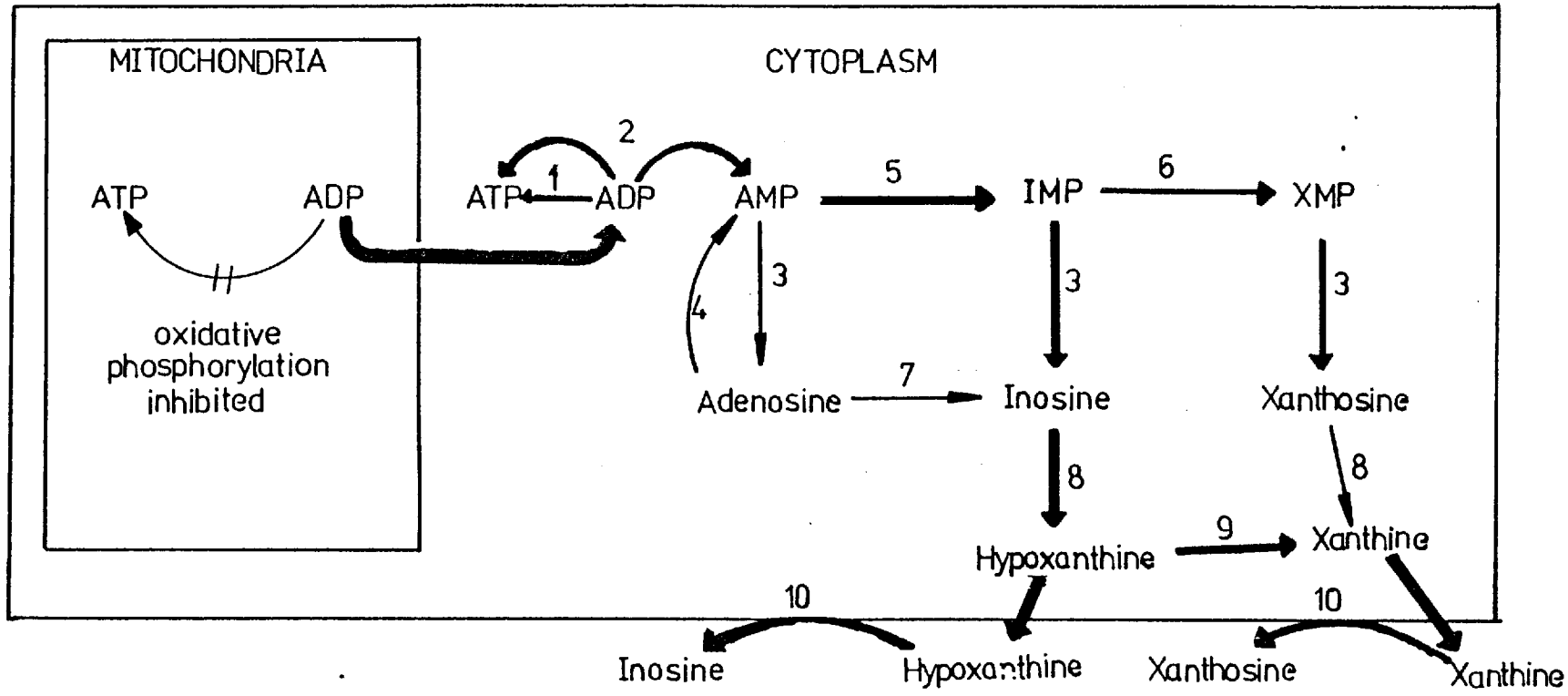


Figure 31: Mechanism of increase in adenine nucleotide metabolism during anoxia.

Thick arrows indicate the reaction stimulated by anoxia. The numbers refer to: 1. substrate phosphorylation; 2. adenylate kinase; 3. 5'-nucleotidase; 4. adenosine kinase; 5. AMP deaminase; 6. XMP dehydrogenase; 7. adenosine deaminase; 8. nucleosidase; 9. xanthine oxidase; 10. purine nucleoside phosphorylase.

4.3 Role of purines in rapid hormonal effects on hepatic carbohydrate metabolism.

The role of cyclic AMP has been well established as a mediator in the stimulation of hepatic glycogenolysis by glucagon. The hormones vasopressin and angiotensin which have a similar effects on hepatic glycogenolysis, however, do not involve changes in cyclic AMP or cyclic GMP (Hems et al., 1978a), the latter had been proposed as a mediator because of its stimulatory effect on hepatic glycogen degradation (Exton et al., 1971a). As mentioned in Section 1.3.2 the role of Ca^{2+} in the hepatic effects of vasopressin and angiotensin is still unclear.

A possible role for other purines in hormone action is suggested by the existence in the hepatic plasma membrane of a series of enzymes capable of handling purine nucleotides and nucleosides whose function is not clear. The explanation that enzymes are present 'merely' to degrade extracellular nucleotides for 'salvage' purposes is not sufficient to explain this ability of liver cells to handle purine nucleotides which are outside the cell. Further evidence for their possible involvement in the effects of hormone is provided by reports that the activity of the enzymes phosphodiesterase (Kono et al., 1975) and AMP deaminase (Sullivan, 1976) can be affected by hormone action. Increased synthesis of phosphoribosyl pyrophosphate in the liver in response to glucagon (Hisata et al., 1978) suggests additional involvement of purine nucleotides.

There are numerous other facets of purine nucleotide metabolism which may be implicated in control of cellular

metabolism. Chief amongst these are the Mg^{2+}/Ca^{2+} and Na^{+}/K^{+} ATPases. Susceptibility of these enzyme activities to hormonal effect would provide a means of altering the ionic nature of the cytoplasm thus providing fine control of a number of metabolic processes.

The discussion of short term hormonal effect on purine metabolism has been divided into two sections: I: those that may involve cyclic AMP; II: those that act via other mechanisms.

4.3.1 Effect of glucagon and insulin on hepatic adenine nucleotide metabolism

Glucagon

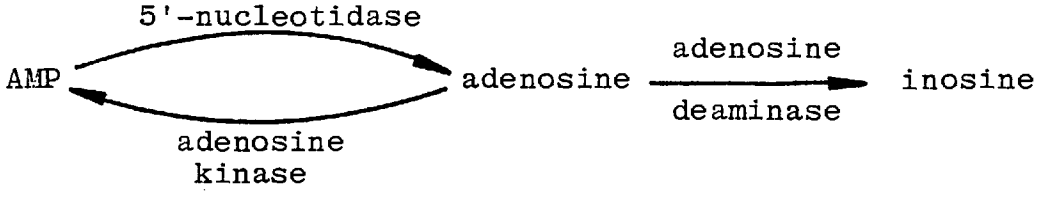
The experiments described in Section 3.4 illustrate that the effect of glucagon on the liver included stimulation of adenyl cyclase, as measured by raised intracellular levels of ^{14}C-cyclic AMP. This observation is in agreement with the accepted mechanism whereby glucagon stimulates glycogenolysis and gluconeogenesis (see recent review by Hems, 1977). The increase in the extracellular concentration of labelled cyclic-AMP is in agreement with the findings of Kirk and Hems (1974) who demonstrated its release into the perfusion medium. In addition, the increase in hepatic content of labelled purine metabolites, accompanied by a decrease in perfusion medium concentration, indicated that glucagon may also lower plasma membrane permeability to purine compounds. One exception to this rule was the increase in ADP content of the perfusion medium, which may be due to an increase in activity of the membrane bound ATPase.

The total amount of metabolites of AMP degradation

produced during 4 min of exposure to glucagon increased by over 80% relative to controls. The possibility that rapid degradation of increased cyclic AMP may account for this seems unlikely since the resultant increase in AMP concentration would be small, and any increase in the rate of breakdown resulting from this very small rise in AMP concentration would be insignificant.

Analysis of the data revealed that the total hepatic production (i.e. intracellular and extracellular) of adenosine, inosine, hypoxanthine and xanthine had been stimulated while xanthosine production had been reduced.

Accumulation of adenosine in the tissue could result from either increased production or decreased utilization.



Under normal conditions a large proportion of adenosine produced by the 5'-nucleotidase is reconverted to AMP because the Km of adenosine kinase for adenosine is an order of magnitude lower than that of adenosine deaminase (Arch and Newsholme, 1978a). Since there is no indication that conversion of adenosine to inosine is inhibited (indeed the increased hepatic content of inosine suggests the opposite), either stimulation of adenosine formation from AMP by 5'-nucleotidase or inhibition of its conversion to AMP by adenosine kinase must take place.

Stimulation of 5'-nucleotidase may occur via three different mechanisms. Firstly, the adenyl cyclase may

deplete membrane pools of ATP, which would result in lowering of the ATP concentration in the proximity of 5'-nucleotidase, the enzyme being largely located on the plasma membrane (Naito et al., 1974; Gurd and Evans, 1974). This would result in stimulation of 5'-nucleotidase since under physiological conditions ATP is an inhibitor (Gurd and Evans, 1974). Secondly, the local concentration of the substrate AMP may be increased since the increased concentration of cyclic AMP will lead to increased AMP production via membrane located phosphodiesterase. Thirdly, the increase in hepatic calcium uptake induced by glucagon at high concentration (Keppens et al., 1977) may lead to elevated AMP production in the proximity of the membrane since plasma membrane located ATP pyrophosphohydrolase has been shown to be activated by Ca^{2+} (Flodgaard and Torp-Pedersen, 1978).

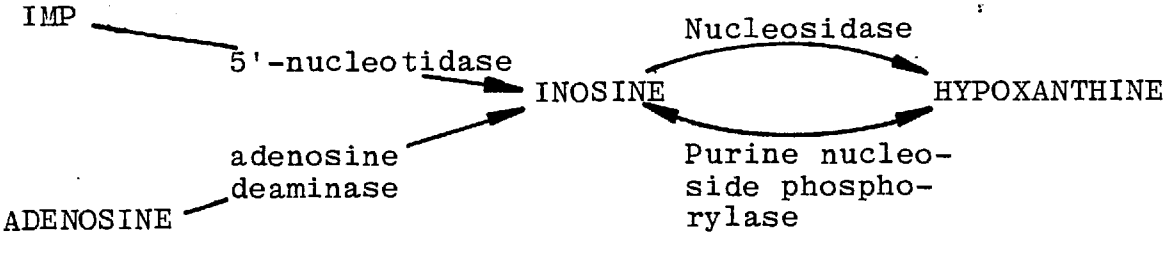
Inhibition of the enzyme adenosine kinase may also be expected if the ATP concentration is lowered since the nucleotide is a co-reactant in the phosphorylation of adenosine. The Km for ATP, however, is below its intracellular concentration (Murray, 1968) suggesting that ATP may not be an important regulator of adenosine phosphorylation under normal circumstances. Inhibition of adenosine kinase, however, may occur via a raised cytoplasmic ADP concentration (Murray, 1968) since the Ki of this enzyme for ADP is in the normal range of ADP levels.

Regulation of adenosine kinase by the ATP/ADP ratio may be relevant since it has been demonstrated that glucagon reduces the cytoplasmic ATP concentration (Siess et al., 1977).

Intracellular adenosine as discussed earlier (see Section 4.2.5) does not appear to be a major contributor of

extracellular adenosine. Thus the level of extracellular adenosine may reflect the activity of the enzyme 5'-nucleotidase, this being the only enzyme leading to formation of extracellular adenosine. Since there is no significant change in perfusate adenosine concentration in response to glucagon, it is unlikely that the membrane located 5'-nucleotidase is stimulated, which suggests that the increase in the intracellular concentration of adenosine observed may be predominantly due to inhibition of the adenosine kinase reaction.

The inosine concentration in the tissue as in the case of adenosine, will depend on the balance between reactions leading to its production and utilization. As outlined below it can be formed from both adenosine and IMP and degraded to hypoxanthine. In addition the enzyme purine nucleoside phosphorylase catalyzes reversible conversion between inosine and hypoxanthine. One feature of the glucagon stimulated increase in inosine concentration



is that it is exclusively confined to the liver, none being released into the perfusion medium.

Intracellular inosine is the product of the reactions catalyzed by the enzymes adenosine deaminase, 5'-nucleotidase and purine nucleoside phosphorylase. Activity of the enzyme adenosine deaminase which appears to be regulated by availability and concentration of both substrate and product, would

be expected to increase in the event of a raised adenosine concentration. The absence of inosine in the perfusion medium indicates that membrane bound 5'-nucleotidase does not participate in hydrolytic dephosphorylation of IMP to an appreciable extent. The kinetic properties of the soluble form of 5'-nucleotidase indicate that IMP is a better substrate than AMP (Van den Bergh et al., 1977a). Thus an increase in IMP concentration would be expected to result in increased inosine production. Steady levels of hepatic IMP, however, discount this possibility.

The role of purine nucleoside phosphorylase, which catalyses the reversible conversion between inosine and hypoxanthine, in maintenance of intracellular inosine concentration is not clear. This enzyme, however, probably has a role in the maintenance of extracellular inosine, since intracellular inosine and IMP appear to have a minimal contribution to the maintenance of extracellular inosine. Low levels of release of the bases xanthine and hypoxanthine in the presence of glucagon would also result in a decrease in the perfusion medium concentration of inosine and xanthosine. In view of these events, however, the absence of inosine in the perfusion medium from liver exposed to glucagon probably indicates complete inhibition of purine nucleoside phosphorylase. The concentration of ribose 1-phosphate which is a co-reactant of this enzyme with hypoxanthine would be expected to increase in response to inhibition by glucagon. Although such a rise has not been reported, it may explain the increased formation of phosphoribosyl pyrophosphate in the presence of glucagon (Hisata, 1978), since a reduction in the utilization of ribose 1-phosphate which is interconvertable with ribose

5-phosphate, would mean a larger reserve of ribose 5-phosphate for synthesis of phosphoribosyl pyrophosphate.

The hepatic concentration of xanthosine, which is formed by the reactions catalyzed by 5'-nucleotidase and purine nucleoside phosphorylase, remained unaltered in the presence of glucagon. Since the enzyme purine nucleoside phosphorylase may be involved in the formation of extracellular xanthosine, the decrease in xanthosine in the perfusion medium is consistant with the view that this enzyme may be inhibited by glucagon.

The effect of glucagon on purine metabolism, supplementary to its well known stimulation of cyclic AMP production, thus can be summarized to include a decrease in the membrane permeability to purine compounds and an inhibitory effect on the enzymes adenosine kinase and purine nucleoside phosphorylase. Thus apart from stimulation of adenyl cyclase there is no direct stimulation of purine nucleotide degradation.

The increase in the level of breakdown products is observed because phosphorylation of adenosine is inhibited resulting in a bigger proportion of adenosine being available for degradation. This is compatible with lowering of the cytoplasmic concentration of ATP by glucagon (Siess et al., 1977).

The net effect of the complex interrelationships between the various reactions is that intracellular inosine levels are raised in preference to adenosine. This may be significant in the stimulation of gluconeogenesis since it has been shown to stimulate this process in the perfused liver

(Haeckel, 1977). Lund et al., (1975) however, did not observe a significant increase in gluconeogenesis in the presence of inosine while adenosine decreased it in hepatocyte suspension.

Insulin

Insulin which has been shown to suppress the glucagon stimulation of glycogen breakdown (Exton and Park, 1972), despite a failure to exert any direct rapid effect on hepatic glycogen metab sm (Seglen, 1973; Whitton and Hems, 1975; Walker, 1977), was found to produce a very small effect on purine nucleotide metabolism. This included an increase in the concentration of labelled purine nucleosides and bases of 40% while there was a decrease in the amount of material being released into the perfusion medium. Thus, as with glucagon, stimulation of AMP degradation is restricted to the cytoplasm.

A detailed examination of the data reveals that the initial mechanism involved in stimulation of purine nucleotide degradation may be similar to that of glucagon (i.e. inhibition of the enzyme adenosine kinase).

Accumulation of the purine metabolites adenosine, inosine and hypoxanthine in the liver may be due to either stimulation of the enzyme 5'-nucleotidase or inhibition of the enzyme adenosine kinase. Since low perfusion medium concentration of nucleosides adenosine and xanthosine indicate possible inhibition of the plasma membrane located 5'-nucleotidase it is unlikely that the cytoplasmic 5'-nucleotidase

would be stimulated. Thus the accumulation of adenosine and subsequent breakdown products in the cytoplasm in the presence of insulin may be due to inhibition of the enzyme adenosine kinase.

There was, however, no indication of an increase in adenosine deaminase activity in the presence of insulin since the increase in the intracellular concentration of adenosine was not accompanied by an increase in inosine concentration. In addition inhibition of purine nucleoside phosphorylase is also indicated by the low levels of inosine and xanthosine in the perfusion medium.

Thus the net effect of insulin appears predominantly to be one of increasing the intracellular concentration of adenosine since the 40% rise in the level of purine nucleotide breakdown products can be totally accounted for by the intracellular increase in adenosine concentration.

The antagonistic nature of the actions of glucagon and insulin may be explained by the differences in the effects of these two hormones on the relative intracellular concentration of inosine and adenosine. Adenosine when added to hepatocyte suspension inhibits gluconeogenesis (Lund et al., 1975). Elevation and release of adenosine by insulin has been reported in isolated fat cells by Schwabe et al., (1974) which facilitated the effect of insulin. In addition the effect of adenosine is anticatabolic with respect to inhibition of glucagon stimulated glucose release (Fain and Shepherd, 1977). An inhibition

of gluconeogenesis is compatable with this view since this will result in a lower amount of glucose being available for release. Thus adenosine may have an overall 'anabolic' role in carbohydrate metabolism. However, the effect of inosine on carbohydrate metabolism is not well documented and does not appear to be similar to that of adenosine. The indications are that it may have an opposite effect to that of adenosine, since stimulation of gluconeogenesis has been demonstrated in perfused liver (Haeckel, 1977), while results from Lund et al., (1975) were inconclusive. These observations indicate that inosine may have an intracellular role in stimulation of catabolic processes. The results thus indicate possible involvement of purine nucleosides in the hepatic effects of the hormones glucagon and insulin which may explain their antagonistic nature.

4.3.2 Effect of vasopressin and angiotensin on hepatic adenine nucleotide metabolism

The results reported in Section 3.4 illustrate that the hormones vasopressin and angiotensin II, unlike glucagon did not stimulate formation of cyclic AMP. This observation is in agreement with other reports which indicate that stimulation of adenyl cyclase is not involved in the hepatic effect of vasopressin (Kirk and Hems, 1974; Hems et al., 1978) or the effects of angiotensin II (Hems et al., 1978). The results, however, indicate other profound changes in adenine nucleotide metabolism in response to these hormones. These involved stimulation of adenine nucleotide breakdown as

indicated by increases in the intracellular and extracellular concentrations of the degradation products. In addition a stimulation in the release of adenine nucleotides is also indicated.

The initial hepatic effect seen in response to angiotensin and vasopressin is the mobilization of the adenine nucleotides so that their release into the medium is stimulated . This increase in release is apparently not due to an increase in the transportation processes since the rise in perfusion medium concentration of IMP in the presence of angiotensin II is directly proportional to the intracellular rise. Thus this increase in perfusate concentrations of adenine nucleotides may be due to elevation of free purine nucleotide (i.e. non-protein bound) concentrations in the cytoplasm. In the case of ATP this would have to involve stimulation of dissociation of the protein bound ATP. The increased release of AMP and ADP may also involve stimulation of their formation. The enzymes ATPase and ATP pyrophosphohydrolase are located at the plasma membrane surface and any increase in their activity might result in raised adenine nucleotide concentration in the vicinity of the membrane. The increases in the perfusion medium concentrations of ATP, ADP and AMP were similar in response to both vasopressin and angiotensin, suggesting that the stimulatory effects on the enzymes ATPase and ATP pyrophohydrolase, were similar.

The rate of adenine nucleotide degradation was increased by 4-fold in the presence of angiotensin II and by 3-fold in the presence of vasopressin. This greater effect of angiotensin II was also apparent from the raised intracellular and extracellular concentrations of the purine nucleosides and bases. The release of

adenine nucleotides was also greater in the presence of angiotensin II than of vasopressin. Although the concentrations of the two hormones were chosen so as to have quantitatively similar effects on carbohydrate metabolism, the suggestion of apparently greater potency of angiotensin II in affecting adenine nucleotide metabolism is only tentative, since a dose response curve was not carried out.

The increased intracellular degradation of adenine nucleotides in the presence of angiotensin II and vasopressin was accompanied by stimulation of AMP hydrolysis, while angiotensin in addition stimulated deamination of AMP, as was shown by the increase in the products of these reactions (e.g. IMP, adenosine, inosine and xanthosine). Failure to observe stimulation of AMP deaminase as indicated by a lack of an increase in IMP concentration in the presence of vasopressin indicated that the angiotensin II stimulation of the enzyme AMP deaminase may not be due to changes in the cytoplasmic concentration of the various modifiers of this enzyme since both of the hormones appear to have similar effects on these concentrations. Sullivan (1976) on the basis of a stimulation of purified muscle AMP deaminase when exposed to angiotensin II, suggests that angiotensin may affect the enzyme directly.

The increase in the release of adenine nucleotides as seen in the presence of angiotensin II and vasopressin would have the effect of increasing the cytoplasmic concentration of free Mg^{2+}. This increase in Mg^{2+} would explain the stimulation of 5'-nucleotidase observed in the presence of these hormones since it has been reported to decrease the inhibitory effect of ATP (Burger and Lowenstein, 1970). Adenosine

kinase activity would also decrease since Mg^{2+} has been shown to inhibit the enzyme (Murray, 1968).

Thus in the presence of angiotensin II stimulation of IMP formation and possible stimulation of IMP dehydrogenase (Jackson et al., 1977) would result in a higher proportion of the nucleosides inosine and xanthosine being formed by the 5'-nucleotidase reaction. Increased inosine formation via this pathway may cause feed back inhibition of adenosine kinase and thereby explain the proportionately large increase in cytoplasmic adenosine concentration. The overall effect of angiotensin II which may be relevant in assessing control processes was the elevation of cytoplasmic concentration of adenosine and inosine while increasing the amount of AMP, adenosine and xanthosine being released.

The effect of vasopressin however, appears to be mainly on the mechanisms that control purine nucleotide degradation via adenosine. The overall effect on purine metabolism was again one of increased release of AMP, adenosine and xanthosine while inosine was the only metabolite to register a considerable increase in its cytoplasmic concentrations.

Thus vasopressin and angiotensin II have been demonstrated to affect hepatic purine metabolism, which results in alterations of purine concentration in both intracellular and extracellular compartments.

REFERENCES

Adams, H. (1963). In Methods of Enzymatic Analysis, 2nd edition, pp. 573-577. (ed. H.V. Bergmeyer).

Adelman, R.C., Lo, C.H. & Weinhouse, S. (1968). Adv.Enz. Reg. 6, 425-436.

Agarwal, K.C., Agarwal, R.P., Stoeckler, J.D. and Parks, R.E. Jn. (1975). Biochemistry 14, 79-84.

Akerboom, T.P.M., Bookelman, H., Zourendonk, P.F., Van der Meer, R. & Tagen, J.M. (1978). Eur.J.Biochem. 84, 413-420.

Arch, J.R.S. & Newsholme, E.A. (1978a). Biochem.J. 174, 965-977.

Arch, J.R.S. & Newsholme, E.A. (1978b). In Essays in Biochemistry 14, 82-123 (eds. T.N. Campbell & G.D. Greville).

Arsenis, C. & Touster, O. (1968). J.Biol.Chem. 243, 5702-5708.

Atkinson, D.E. (1966). Ann.Rev.Biochem. 35, 85-124

Badenoch-Jones, P. & Buttery, P.J. (1975). Biochem.J. 148, 599-601.

Badenoch-Jones, P. & Buttery, P.J. (1976). Biochem.J. 158, 549-556.

Baguara, A.S., Letter, A.A. & Henderson, J.F. (1974). Biochem.Biophys.Acta 374, 259-270.

Ballie, L.A. (1960). Int.J.Applied Radiation and Isotopes 8, 1-7.

Baron, D.N. & Roberts, P.M. (1963). J.Physiol. 165, 219-226.

Benedetti, E.L. & Delbanffe, D. (1971). In Cell Membrane (eds. G.L. Richter and D.G. Scarpelli) pp. 54-83. Williams & Wilkins, Baltimore.

Berne, R.H., Rubio, R., Dobson, J.G. and Curnish, R.R. (1971). Circulation Res. Supp. I, pp 115-119.

Berne, R.M., Rubio, R. & Curnish, R.R. (1974). Circ.Res. 35, 262-271.

Blackmore, P.F., Brumley, F.T., Marks, J.L. & Exton, J.H. (1978). J.Biol.Chem. 253, 4851-4858.

Birch, G.G., Lee, E.Y.C. & Hems, D.A. (1974). Int.J. Biochem. 5, 867-873.

Bischoff, E., Tran-Thi, T-A. & Decker, K.F.A. (1975). Eur. J.Biochem. 51, 353-361.

Bockman, E.L., Berne, R.M. & Rubio, R. (1975). Pflugers Arch. 355, 229-241.

Bockman, E.L., Berne, R.M. & Rubio,R. (1976). Amer.J. Physiol. 230, 1531-1537.

Brosnan, J.T., Krebs, H.A. & Williamson, D.H. (1970). Biochem.J. 117, 91-96.

Burger, R.M. and Lowenstein, J.M. (1970). J.Biol.Chem. 245, 6274-6280.

Cardell, R.R. Jr. (1977). Int.Rev.Cytol. 48, 221-279.

Caskey, C.T., Ashton, D.M. & Wyngaarden, J.B. (1964). J.Biol.Chem. 239, 2570-2579.

Chapman, A.G. & Atkinson, D.E. (1973). J.Biol.Chem. 248, 8309-8312.

Chappel,J.B. & Crofts,A.R.(1965). Biochem.J. 95, 378-386.

Chaudry, I.H., Sayeed, M.M. & Baue, A.E. (1976a). Can.J. Physiol.Pharmacol. 54, 736-741.

Chaudry, I.H., Sayeed, M.M. & Baue, A.E. (1976b). Can.J. Physiol.Pharmacol. 54, 742-749.

Chaudry, I.H., Sayeed, M.M. & Baue, A.E. (1976c). Can.J. Physiol.Pharmacol. 54, 750-756.

Chaudry, I.H., Sayeed, M.M. & Baue, A.E. (1977). Am.J. Physiol. 233, R83-R88.

Chen, J-L.J., Babcock, D.F. & Lardy, H.A. (1978). Proc. Natl.Acad.Sci. USA., 75, 2234-2238.

Clark,D.G.,Rognstad,R. & Katz,J. (1974), J.Biol.Chem.249, 2028-2036.

Cornblath, M., Randle, P.J., Parmeggiani, A. & Morgan, H.E. (1963). J.Biol.Chem. 238, 1592-1597.

Crabtree, G.W. & Henderson, J.F. (1971). Cancer Research, 31, 985-991.

Cunningham, B. & Lowenstein, J.M. (1965). Biochem.Biophys. Acta 96, 535-537.

Daly, J.W. (1972). In Methods in Cyclic Nucleotide Research (ed. M. Chasin) pp. 255-300. Marcel Dekker Inc.

Das, I. (1978). Biochem.Soc.Trans. 6, 1047-1048.

Das, I. & De Belleroche, J.S. (1978). Experientia, 34, 312-313.

Das, I. & Hems, D.A. (1974). Horm.Metab.Res. 6, 40-44.

Davis, J.O. & Freeman, R.H. (1977). Biochem.Pharm. 26, 93-97.

Dean, B.M. & Perrett, D. (1976). Biochem.Biophys.Acta 437, 1-15.

De Wulf, H., Stalmans, W. & Hers, H.-G. (1968). Eur.J. Biochem. 6, 545-551.

Divekar, A.Y. (1976). Biochem.Biophys. Acta. 422, 15-28.

Dobson, J.G., Rubio, R. & Berne, R.M. (1971). Circ.Res. 29, 375-384.

Drummond, G.I. & Yamamoto, M. (1971). Enzymes (3rd ed.) 4, 337-352.

Durbin, R.P. & Kircher, A.B. (1975). Biochem.Biophys.Acta 413, 298-308.

Exton, J.H. & Park, C.R. (1972). In Handbook of Physiol. Section 7, Vol.1, pp 437-455 (Amer.Physiol.Soc).

Exton, J.H., Hardman, J.G., Williams, T.F., Sutherland, E.W. & Park, C.R. (1971a). J.Biol.Chem. 246, 2658-2664.

Exton, J.H., Robinson, G.A., Sutherland, E.W. & Park, C.R. (1971b). J.Biol.Chem. 246, 6166-6177.

Exton,J.H.,Robinson,G.A. & Sutherland,E.W. (1972). In Handbook of Physiol. Section 7, Vol.1 pp 425-436(Amer.Physiol.Soc).

Evans, W.H. (1974). Nature 250, 391-394.

Evans, W.H. & Gurd, J.W. (1973). Biochem.J. 133, 189-199.

Fain, J.N. & Shepherd, R.E. (1977). J.Biol.Chem. 252, 8066-8070.

Fain, J.N., Tolbert, M.E.M., Pointer, R.H., Butcher, F.R. & Arnold, A. (1975). Metabolism. 24, 395-407.

Faulkner, A. & Jones, C.T. (1978). Biochem.Biophys.Acta. 538, 106-119.

Faupel, R.P., Seitz, H.J., Tarnowski, W., Thiemann, V. & Weiss, Ch. (1972). Arch.Biochem.Biophys. 148, 509-522.

Fields, T. & Brox, L. (1974). Can.J.Biochem. 52, 441-446.

Fleit, H., Conklyn, M., Stebbins, R.D. & Silber, R. (1975). J.Biol.Chem. 250, 8889-8892.

Flodgaard, H. & Torp-Pedersen, C. (1978). Biochem.J. 171, 817-820.

Foden, S. & Randle, P.J. (1978). Biochem.J. 170, 615-625.

Fontenelle, L.J. & Henderson, J.F. (1969). Biochem.Biophys. Acta 177, 175-176.

Fox, I.H. & Kelley, E.N. (1978). Ann.Rev.Biochem. 47, 655-686.

Fredholm, B.B. (1976). Acta.Physiol.Scand. 96, 422-430.

Frick, G.P. & Lowenstein, J.M. (1976). J.Biol.Chem. 251, 6372-6378.

Frick, G.P. & Lowenstein, J.M. (1978). J.Biol.Chem. 253, 1240-1244.

Fritzson, P. (1969). Biochem.Biophys.Acta 178, 534-541.

Fritzson, P. & Smith, I. (1971). Biochem.Biophys.Acta 235, 128-141.

Glinsmann, W.H. & Mortimore, G.E. (1968). Amer.J.Physiol. 215, 553-559.

Glinsmann, W.H., Hern, E.P. & Lynch, A. (1969). Amer.J. Physiol. 216, 698-703.

Good, C.A., Kramer, H.R. & Somogyi, M.(1933). J.Biol.Chem. 100, 485-491.

Goris, J. & Merlevede, W. (1974). FEBS Lett. 48, 184-187.

Gray,L.H. & Steadman,J.M.(1964). J.Physiol. 175, 161-171.

Gurd, J.W. & Evans, W.H. (1974). Arch.Biochem.Biophysics. 164, 305-311.

Haeckel, R. (1977). Adv.Exp.Med.Biol. 76A, 488-499.

Haslam, R.J. & McClenaghan, M.D. (1974). Biochem.J. 138, 317-320.

Hems, D.A. (1977). FEBS Lett. 80, 237-245.

Hems, D.A. & Brosnan, J.T. (1970). Biochem.J. 120, 105-111.

Hems, D.A. & Whitton, P.D. (1973). Biochem.J. 136, 705-710.

Hems, D.A., Whitton, P.D. & Ma, G.Y. (1975). Biochem. Biophys.Acta. 411, 155-164.

Hems, D.A., Rodrigues, L.M. & Whitton, P.D. (1976). Biochem.J. 160, 367-374.

Hems, D.A., Davies, C.J. & Siddle, K. (1978a). FEBS Lett. 87, 196-198.

Hems, D.A., Rodrigues, L.M. & Whitton, P.D. (1978 b). Biochem.J. 172, 311-317.

Hems, R., Ross, B.D., Berry, M.N. & Krebs, H.A. (1966). Biochem.J. 101, 284-292.

Henderson, J.F. (1972). In Regulation of Purine Biosynthesis, pp. 44-162. American Chemical Society, Washington.

Henderson, J.F. (1975). Biochem.Soc.Trans. 3, 1195-1198.

Henderson, J.F. & Khoo, M.K.Y. (1965a). J.Biol.Chem. 240, 2349-2357.

Henderson, J.F. & Khoo, M.K.Y. (1965b). J.Biol.Chem. 240, 2358-2362.

Henderson, J.F. & Le Page, G.A. (1959). J.Biol.Chem. 234, 3219-3223.

Henderson,J.F.,Fraser,J.H. & McCoy,E.E.(1974). Clin. Biochem. 7, 339-358.

* Hartman,S.C.& Buchanan,J.M.(1959). Ann.Rev.Biochem. 28,365-410.

Henderson, J.F., Baguara, A.S., Crabtree, G.W., Lomax, C.A., Shantz, G.D. & Snyder, F.F. (1975). Adv.Enzyme. Regul. 13, 37-64.

Hisata, T., Katsufuji, N. & Tatibana, M. (1978). Biochem. Biophys.Res.Commun. 81, 704-709.

Hohorst, H.J. (1963). In Methods of Enzymatic Analysis (ed. H.V. Bergmeyer) pp. 266-277, Academic Press, New York.

Holmsen, H. & Rozenberg, M.C. (1968). Biochem.Biophys. Acta 157, 266-279.

Housset, P. & Nagy, M. (1977). Eur.J.Biochem. 73, 99-105.

Hulsmann, W.C. & Kurpershoek-Davidov, R. (1974). Biochem. Biophys.Acta. 354, 39-48.

Hunter, A.R. & Jefferson, L.S. (1969). Biochem.J. 111, 537-545.

Hutson, N.J., Brumley, F.T., Assimacopoulos, F.D., Harper, S.C. & Exton, J.H. (1976). J. Biol. Chem. 251, 5200-5208.

Ismail, N. & Hems, D.A. (1978). Biochem.Pharmacol. 27, 1341-1345.

Itoh, R., Mitsui, A. & Tsushima, K. (1968). J.Biochem. (Tokyo) 63, 165-169.

Jackson, R.C., Morris, H.P. & Weber, G. (1977). Biochem.J. 166, 1-10.

Jain, K. & Logothetopoulos, J. (1978). Biochem.J. 170, 461-467.

Jakob, A. & Diem, S. (1974). Biochem.Biophys.Acta 362, 469-474.

Kalckar, H.M. (1947). J.Biol.Chem. 167, 461-475.

Keppens, S. & DeWulf, H.(1975). FEBS Lett. 51, 29-32.

Keppens, S. & De Wulf, H. (1976). FEBS Lett. 68, 279-282.

Keppens, S., Vandenheede, J.R. & De Wulf, H. (1977). Biochem.Biophys.Acta 496, 448-457.

Killilea, S.D., Brandt, H., Lee, E.Y.C. & Whelan, W.J. (1976). J.Biol.Chem. 251, 2363-2368.

Kirk, C.J. & Hems, D.A. (1974). FEBS Lett. 47, 128-131.

Kirk, C.J., Verrinder, T.R. & Hems, D.A. (1976). Biochem.J. 156, 593-602.

Kiss, Z. (1978). FEBS Lett. 92, 29-32.

Klingenberg, M. (1970). Essays in Biochemistry 6, 119-159.

Kono, T., Robinson, F.W. & Sarver, J.A. (1975). J.Biol. Chem. 250, 7826-7835.

Kornberg, A. (1957). In The Chemical Basis of Heredity (ed. W.D. McElray & B. Glass) pp. 579-608. Baltimore, Maryland: Johns Hopkins.

Krebs, H.A. & Henseleit, K. (1932). Hoppe-Seyler's Z. Physiol.Chem. 210, 33-36.

Kuo, W-N., Hodgins, D.S. & Kuo, J.F. (1974). Biochem. Pharm. 23, 1387-1391.

Lajtha, L.G. & Vane, J.R. (1958). Nature 182, 191-192.

Lalanne, M. & Henderson, J.F. (1974). Anal.Biochem. 62, 121-133.

Lamprecht, W. & Trantsehold, I. (1963). In Methods of Enzymatic Analysis. 2nd edition, pp. 543-551 (ed. H.V. Bergheyer).

Lee, Y-P. & Wang, M.-H. (1968). J.Biol.Chem. 243, 2260-2265.

Lee, E.Y.C. & Whelan, W.J. (1966). Arch.Biochem.Biophys. 116, 162-167.

Lelievre, L., Zachowski, A., Maget-Dana, R., Aubry, J. & Jonkman-Bark, G. (1977). Eur.J.Biochem. 80, 185-191.

Lerner, M.H. & Lowry, B.A. (1974). J.Biol.Chem. 249, 959-966.

Lewis, A.S. & Glantz, M.D. (1976). J.BiolChem. 251, 407-413.

Li, C-C. & Hochstadt, J. (1976a). J.Biol.Chem. 251, 1175-1180.

Li, C-C. & Hochstadt, J. (1976b). J.Biol.Chem. 251, 1181-1187.

Lindberg, L.A. & Falkama, A. (1977). Histochem.J. 9, 277-284.

Lomax, C.A. & Henderson, J.F. (1972). Can.J.Biochem. 50, 423-427.

Londos, C. & Preston, M.S. (1977). J.Biol.Chem. 252, 5951-5956.

Lowenstein, J.M. (1972). Physiological Reviews 52, 382-414.

Lowry, B.A. & Williams, M.K. (1960). J.Biol.Chem. 235, 2924-2927.

Lund, P., Cornell, N.W. & Krebs, H.A. (1975). Biochem. J. 152, 593-599.

Mager, J., Hershko, A., Zeilin-Beck, R., Shoshani, T. & Razin, A. (1967). Biochem.Biophys.Acta 149, 50-58.

Mah, H.D. & Daly, J.W. (1975). Biochem.Biophys.Acta 404, 49-56.

Mangnall, D. & Clark, R.G. (1976). Int.J.Biochem. 7, 301-307.

Mayes, P.A. & Felts, J.M. (1976). Biochem.J. 156, 686-686.

Miller, L.L., Bly, C.G., Watson, M.L. & Bale, W.F. (1951). J.Exp.Med. 94, 431-453.

Miyazaki, M., Nambu, K., Minaki, Y., Nashimoto, M. & Nakamura, K. (1974). J.Biochem. 76, 409-417.

Monod, J., Wyman, J. & Changeux, J-P. (1965). J.Mol.Biol. 12, 88-118.

Moss, K.M. (1977). FEBS Lett. 75, 141-144.

Moss, K.M. & McGivan, J.D. (1975). Biochem.J. 150, 275-283

Muirhead,K.M. & Bishop,S.H.(1974). J.Biol.Chem.249, 459-464,

Murray, A.W. (1968). Biochem.J. 106, 549-555

Murray, A.W. (1971). Ann.Rev.Biochem. 40, 811-826.

McIlwain, H. (1974). Biochem.Soc.Trans. 2, 379-382.

Naito, Y., Itoh, R. & Tsushima, K. (1974). Int.J.Biochem. 5, 807-810.

Natsumeda, Y., Yoshino, M. & Tsushima, K. (1977). Biochem. Biophys.Acta 483, 63-69.

Newby, A.C., Luzio, J.P. & Hales, C.N. (1975). Biochem.J. 146, 625-633.

Newman, M. & McIlwain, H. (1977). Biochem.J. 164, 131-137.

Newsholme, E.A. & Gevers, W. (1967). J.Biol.Chem. 245, 1717-1726.

Ogasawara, N., Goto, H. & Watanabe, T. (1975). Biochem. Biophys.Acta 403, 530-537.

Okajima, F. & Ui, M. (1976). Arch.Biochem.Biophys. 175, 549-557.

Parrilla, R., Goodman, M-N. & Toews, C.J. (1977). Pflugers.Arch. 369, 167-175.

Partsch, G., Sandtner, I. & Eberl, R. (1977). Adv.Exp.Med. Biol. 76A, 153-156.

Posternak, J. (1974). Ann.Rev.Pharm. 4, 23-33.

Potter, V.R. & Ono, T. (1961). Cold Spring Harbor Symp. Quant.Biol. 26, 355-362.

Pritchard, J.B., Chavez-Peon, F. & Berlin, R.D. (1970). Am.J.Physiol. 219, 1263-1268.

Pritchard, J.B., O'Connor, N., Oliver, J.M. & Berlin, R.D. (1975). Am.J.Physiol. 229, 967-972.

Pull, I. & McIlwain, H. (1972). Biochem.J. 126, 965-973.

Randerath, K. & Randerath, E. (1964). J.Chromatog. 16, 111-125.

Rao, G.S., George, R. & Ramasarma, T. (1976). Biochem.J. 154, 639-645.

Rapaport, E. & Zamecnik, P.C. (1976). Proc.Natl.Acad.Sci. 73, 3122-3125.

Richardson, P.D.I. & Withrington, P.G. (1977). Br.J.Pharmacol. 59, 293-301.

Richardson, P.D.I. & Withrington, P.G. (1978). Circulation Res. 43, 496-503.

Rivard, G.E., McLaren, J.D. & Brunst, R.F. (1975). Biochem. Biophys.Acta 381, 144-156.

Robinson, G.A., Butcher, R.W. & Sutherland, E.W.(1968). Ann.Rev.Biochem. 37, 149-174.

Rognstad, R. (1977). Biochem.Biophys.Acta 496, 249-254.

Ross, B.D., Hems, R., Freedland, R.A. & Krebs, H.A. (1967). Biochem.J. 105, 869-875.

Ronca-Testoni, S., Raggi, A. & Ronca, G. (1970). Biochem. Biophys. Acta 236, 409-415.

Rubio, R. & Berne, R.M. (1969). Circulation Res. 25, 407-415.

Salmon, D.M.W., Bowen, N.L. & Hems, D.A. (1974). Biochem. J. 142, 611-678.

Sato, F., Ito, T., Akiyoshi, H. & Fujii, S. (1978). J.Biochem. 83, 1-7.

Schimassek, H. (1963). Biochem. Z.(Ger) 336, 460-467.

Schrader, J., Berne, R.M. & Rubio, R. (1972). Am.J.Physiol. 223, 159-166.

Schrader, J., Nees, S. & Gerlach, E. (1977). Pflugers. Arch. 369, 251-257.

Schwabe, U., Schonhofer, P.S. & Ebrt, R. (1974). Eur.J. Biochem. 46, 537-545.

Scott, J.L. (1962). J.Clin.Invest. 41, 67-79.

Segal, H.L. & Brenner, B.N. (1960). J.Biol.Chem. 235, 471-474.

Seglen, P.O. (1973). FEBS Lett. 36, 309-

Seglen, P.O. (1974). Biochem.Biophys.Acta 338, 317-336.

Seraydarian,K.,Mommaerts,W.F.H.M. & Wallnes,A.(1962). Biochem. Biophys. Acta. 65, 443-460.

Setlow, B. & Lowenstein, J.M. (1968). J.Biol.Chem. 243, 6216-6221.

Shenoy, T.S. & Clifford, A.J. (1975) Biochem.Biophys. Acta 411, 133-143.

Shimazu, T. & Amakawa, A. (1975). Biochem.Biophys.Acta 385, 242-256.

Shimizu,H. & Daly,J.W.(1972). Eur.J.Pharmacol.17, 240-252.

Siddle, K., Kane-Maguire, B. & Campbell, A.K. (1973). Biochem.J. 132, 765-773.

Siddle, K., Davis, C.J., Shetty, K.J. & Elkeles, R.S. (1976). Clin.Sci.Mol.Med. 50, 487-491.

Siess, E.A., Brocks, D.G., Lattke, H.K. & Wieland, O.H. (1977). Biochem.J. 166, 225-235.

Smiley, K.L., Jr. & Suelter, C.H. (1967). J.Biol.Chem. 242, 1980-1981.

Smellie, R.M.S., Thomson, R.Y. & Davidson, J.N. (1958). Biochem.Biophys.Acta 29, 59-74.

Smith, L.D. & Kizer, D.E. (1969). Biochem.Biophys.Acta 191, 415-424.

Smith, C.M., Rovamo, L.H., Kekomaki, M.P. & Raivio, K.O. (1977). Can.J. Biochem. 55, 1134-1139.

Smith, R.C. & Salmon, W.D. (1969). Arch.Biochem.Biophys. 129, 554-559.

Snyder, F.F. & Henderson, J.F. (1973a). Can.J.Biochem. 51, 943-948.

Snyder, F.F. & Henderson, J.F. (1973b). J.Biol.Chem. 248, 5899-5904.

Song,C.S. & Bodansky,O.(1967). J.Biol.Chem.242, 694-699.

Stalman, W. (1976). Curr.Topics Cell Regul. 11, 51-97.

Stalmans, W. & Hers, H.G. (1975). Eur.J.Biochem. 54, 341-350.

Stalmans, W., De Wulf, H. & Hers, H.G. (1971). Eur.J. Biochem. 18, 582-587.

Stalmans, W., De Wulf, H., Hue, L. & Hers, H.G. (1974a), Eur.J.Biochem. 41, 127-134.

Stalmans, W., Laloux, M. & Hers, H.G. (1974b). Eur.J. Biochem. 49, 415-427.

Streeter, D.G., Simon, L.N., Robins, R.K. & Miller, J.P. (1974). Biochemistry 13, 4543-4549.

Stubbs, M., Kirk, C.J. & Hems, D.A. (1976). FEBS Lett. 69, 199-202.

Sullivan, J.M. (1976). Proc.Soc.Exp.Biol. and Med. 151, 203-206.

Tan, A.W.H. & Nuttall, F.Q. (1975). Biochem.Biophys.Acta 410, 45-60.

Thompson, R.Y., Ricceri, G. & Peretta, M. (1960). Biochem. Biophys.Acta 45, 87-100.

Toda, G., Oka, H., Oda, T. & Ikeda, Y. (1975). Biochem. Biophys.Acta 413, 52-64.

Trinder, P. (1969). Ann.Clin.Biochem. 24, 24-27.

Tsuboi, K.K. & Price, T.D. (1959). Arch.Biochem.Biophys. 81, 223-237.

Van den Berghe, G., Van Pottelsberghe, C. & Hers, H.G. (1977a). Biochem.J. 162, 611-616.

Van den Berghe, G., Bronfman, M., Vanneste, R. & Hers, H.G. (1977b). Biochem.J. 162, 601-609.

Van den Heede, J.R., Keppens, S. & DeWulf, H. (1976). FEBS Lett. 61, 213-217.

Van de Werve, G., Hue, L. & Hers, H.G. (1977). Biochem.J. 162, 135-142.

Van der Weyden, M.B. & Kelley, W.N. (1976). J.Biol.Chem. 251, 5448-5456.

Van Lelyveld, P.H. & Hommes, F.A. (1978). Biochem.J. 174, 527-533.

Villar-Palasi, C. & Larner, J. (1966). Methods in Enzymology 8, 374- 384.

Walaas, O., Walaas, E. & Wick, A.N. (1969). Diabetologia 5, 79-87.

Walli, A.K., Siebler, G., Zepf, E. & Schimassek, H. (1974). Hoppe-Seyler Z. Physiol. Chem. 355, 353-362.

Walker, P.R. (1977). Biochem. Biophys. Acta. 496, 255-263.

Waud, W.R. & Rajagopalan, K.V. (1976a). Arch.Biochem. Biophys. 172, 354-364.

Waud, W.R. & Rajagopalan, K.V. (1976b). Arch.Biochem. Biophys. 172, 365-379.

Weber, G., Jackson, R.C., Williams, J.C., Goulding, F.J. & Eberts, T.J. (1977). Adv.Enz.Reg. 15, 53-77.

Whitton, P.D. & Hems, D.A. (1975). Biochem.J. 150, 153-165.

Whitton, P.D. & Hems, D.A. (1976). Biochem.J. 156, 585-592.

Whitton, P.D. & Hems, D.A. (1977). FEBS Lett. 74, 195-200.

Whitton, P.D., Rodrigues, L.M. & Hems, D.A. (1978). Biochem.J. 176, 893-898.

Widnell, C.C. (1972). J.Cell Biol. 52, 542-558.

Widnell, C.C. & Unkeless, J.C. (1968). Proc.Nat.Acad.Sci. (U.S.A.) 61, 1050-1057.

Williams, A.M. (1962). Cancer Res. 22, 314-321.

Williams, J.F., Irving, M.G., Clark, M.G. & Rienits, K.G. (1973). Int.J.Biochem. 4, 195-206.

Wisher, M.H. & Evans, W.H. (1975). Biochem.J. 146, 375-388.

Wright, G.H. (1977). Biochem.Biophys.Acta 470, 368-381.

Wong, P.C.L. & Henderson, J.F. (1972). Biochem.J. 129, 1085-1094.

Woods, H.F., Eggleston, L.V. & Krebs, H.A. (1970). Biochem. J. 119, 501-510.

Woods, H.F. & Krebs, H.A. (1971). Biochem.J. 125, 129-139.

Woo, Y.-T. & Manery, J.F. (1975). Biochem.Biophys.Acta 397, 144-152.

Zannis, V., Doyle, D. & Martin, D.W. (1978). J.Biol.Chem. 253, 504-510.

Zhivkov, V. & Gavazova, E. (1976). Int.J.Biochem. 7, 497-500.

Zylka, J.M. & Plagemann, P.G.W. (1975). J.Biol.Chem. 250, 5756-5767.