MEDIATION OF HEXOSE TRANSPORT

BY THE LIVER CELL MEMBRANE.

BY

JOHN ODO IBU, B.Sc., M.B.B.S., M.I.BIOL., M.R.S.H.
DEPARTMENT OF PHYSIOLOGY AND PHARMACOLOGY,
UNIVERSITY HOSPITAL AND MEDICAL SCHOOL,
NOTTINGHAM, ENGLAND,

BEING

THESIS SUBMITTED TO THE UNIVERSITY OF NOTTINGHAM
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

APRIL 1980

ACKNOWLEDGEMENTS

I am greatly indebted to my Supervisor, Dr. A. H. Short, for his guidance, encouragement and personal interest throughout the period of the study reported in this Thesis. Any successes that might have accrued from this work were largely due to his effort, but all faults are mine. I would very much like to thank Dr. I. A. MacDonald for his great help as my supervisor when Dr. Short was away on sabbatical leave.

May I also thank Professor A. D. M. Greenfield, Professor of Physiology and Dean of the Faculty of Medicine; Professor P. H. Fenten, Professor of Physiology and Head of Department; and Professor A. T. Birmingham, Professor of Pharmacology and former head of this Department for giving me the opportunity to do the Research in this Department. I wish to thank particularly Professor Birmingham for his useful advice and suggestions throughout the various stages of this work especially during the draft stage of the Thesis.

I would like to thank Dr. J. M. Patrick for showing me how to use Cunningham Trombone to mix gases, and other members of the academic and research staff of this Department for their useful criticisms and comments at various times throughout my stay in this Department. Also I would like to thank the technical staff of this Department, particularly Mr. J. Pierrepont (Head Technician) and Mr. I. Topharm who have been most helpful to me in this work. Similarly I owe much gratitude to Miss Susan Dillon and her secretarial staff of this Department for the immense help rendered to me.

I wish to register my profound gratitude to Mr. J. H. Kugler (Senior Lecturer in the Department of Human Morphology) for his permission to reproduce his original electron micrograph used in

figure 5.8 of this thesis and for his useful comments on my own electron microscopic work. Also I wish to thank Mrs. A. Tomlinson and other technical staff of the electron microscopy suite (Department of Human Morphology) for the final preparation of the liver specimens and production of electron micrographs used in this thesis.

Furthermore, I wish to thank Professor H. F. Woods and Dr. G. D. Broadhead, both of the Department of Pharmacology and Therapeutics, University of Sheffield for demonstrating the Hems type of liver perfusion technique to me, and for lending me a sample of the multi-bulb glass oxygenator.

The funds for this Research were provided by a study fellowship from Ahmadu Bello University, Zaria, Nigeria under the auspices of the Nigerian Federal Government Scholarship. I am grateful to both bodies for this grant.

Also I wish to thank Miss Barbara Andrews for her excellent secretarial duties in the typing and preparation of this thesis.

Now, may I thank my wife and children for their patience and endurance during the long years of separation in pursuance of this research.

Finally, I wish to register my thanks to Dr. R. H. Hooper of the University of Technology, Loughborough, for introducing me to the indicator dilution technique in his sheep livers, and for allowing me to use the extensively modified form of his computer programme.

TABLE OF CONTENTS

		Page
PART A - INTRODUCTION		
Chapter One		
1.	THE CELL MEMBRANE	**1**
1.1	Introduction and Definition	1
1.2	The Structure of Cell Membrane	5
1.3	Chemical Composition of Cell Membrane	7
1.4	Transport of Monosaccharides in different mammalian tissues	22
1.4.1	Transport in erythrocytes	26
1.4.2	Transport in placenta	31
1.4.3	Transport in the liver	34
1.4.4	Some other tissues having facilitated diffusion transport mechanism	36
1.4.5	Sodium-coupled active transport of sugars in the intestine and kidney	40
Chapter Two		
2	ISOLATED ORGAN PERFUSION	44
2.1	Historical review of liver perfusion	44
2.2	The choice of animal species	46
2.2.1	Introduction	46
2.2.2	Advantages of using the rat	47
2.2.3	Limitations of the rat for liver perfusion	48
2.3	The role of organ perfusion in research	49

Page

PART B - GENERAL METHODOLOGY

Chapter Three

3	GENERAL METHODOLOGY	53
3.1	Introduction	53
3.2	The perfusion apparatus	55
3.3	Ancilliary apparatus	60
3.4	The perfusion medium	61
3.4.1	The composition of the perfusion medium	61
3.4.2	Preparation of the perfusion medium	61
3.4.2.1	Collection and washing of bovine erythrocytes	61
3.4.2.2	Preparation of Krebs bicarbonate buffer solution	62
3.4.2.3	Preparation and dialysis of albumin	63
3.4.2.4	The mixture	63
3.5	Surgical operation of the rat for liver perfusion	65
3.5.1	The technique	65
3.5.2	Surgical operation	65
3.6	Preparation of samples for analysis	68
3.6.1	Infusion of test substances and collection of effluent venous samples	68
3.6.2	Processing of samples for β-scintillation counting	69
3.6.3	Preparation of liquid scintillation cocktail	70
3.7	Counting of radioisotopes in the samples	74
3.7.1	The theory of double indicator dilution technique	74
3.7.2	Loading the samples and β-counting	75
3.7.3	Computation	76
	a) Calculation of percentage efficiency in channels	77

		Page
	b) Conversion of cpm to dpm	78
	c) Calculation of percentage extraction	79

PART C - PRELIMINARY INVESTIGATIONS

Chapter Four

4	STUDIES ON THE PERFUSION MEDIUM	80
4.1	Washing of the blood	80
4.2	Haemolysis of the stored washed erythrocytes	89
4.3	Evidence of erythrocyte destruction during experiment	92
4.4	Tonometry of whole ox-blood	98
4.5	Tonometry of perfusate	101
4.6	Oxygen dissociation curve of fresh bovine erythrocytes in perfusate	104
DISCUSSION		110

Chapter Five

5	VALIDATION OF PROTOCOL	113
5.1	Viability of the preparation	113
5.1.1	Period of Hypoxia	113
5.1.2	Morphological appearance	116
5.1.3	Monitoring and control of flow rate	119
5.1.4	Temperature control	125
5.1.5	Oxygen consumption	126
5.1.6	Potassium and Haemoglobin release	128
5.1.7	Changes in pH during perfusion	131
5.1.8	The choice of gas mixture	134
5.2	Electron Microscopy of the rat liver	141
5.2.1	Introduction	141

		Page
5.2.2	Methods	144
5.2.3	Results and discussion	147

Chapter Six

6	VARIABILITY AND SOURCES OF ERROR	
6.1.1	Measurement Accuracy	157
6.1.2	The handling of the isotopic materials by the liver	161
6.1.3	Reproducibility	165
6.2	Sucrose as an extracellular reference substance	169
6.2.1	Introduction	169
6.2.2	Methods	171
	a) Compartmental behaviour of Sucrose	171
	b) Distillation experiments	172
6.2.3	Results	177
6.2.4	Discussion and mathematical formulation	187

PART D - TRANSPORT OF GLUCOSE, GALACTOSE AND FRUCTOSE BY THE LIVER

Chapter Seven

7	GLUCOSE TRANSPORT BY THE PERFUSED RAT LIVER	
7.1	Introduction	200
7.2	Method	203
7.3	Results	204
7.4	Discussion	237

Chapter Eight

8	GALACTOSE TRANSPORT BY THE PERFUSED RAT LIVER	
8.1	Introduction	239

		Page
8.2	Method	241
8.3	Results	243
8.4	Discussion	263

Chapter Nine

9	FRUCTOSE TRANSPORT BY THE PERFUSED RAT LIVER	
9.1	Introduction	266
9.2	Method	270
9.3	Results	271
9.4	Discussion	290

PART E - GENERAL DISCUSSION

Chapter Ten

10	GENERAL DISCUSSION	
10.1	Introduction	293
10.2	Molecular Structures of the sugars	296
10.3	Effects of the sugars on hexose transport	309

APPENDICES

Appendix 1	Computer programme Listing	327
Appendix 2	List of Publications	330
Appendix 3	List of Experiments	331
REFERENCES		339

LIST OF FIGURES

Figure		Page
1.1	Davson-Danielli model of the cell membrane	12
3.1	Diagram of the perfusion apparatus	57
3.2	Diagram of surgical operation for in situ liver perfusion	65a
3.3	Diagram of how isotopic mixture is used to measure extraction	73
4.1	Washing of fresh ox-blood	83
4.2	Washing of one-day old ox-blood	85
4.3	Potassium and Haemoglobin concentrations in washing seven-days old ox-blood	87
4.4	Storage of blood in days	91
4.5	Effect of Watson Marlow pump on perfusate	94
4.6	Potassium and Haemoglobin concentraion in perfusate of perfused liver	95
4.7	Tonometry of fresh whole heparinised ox-blood with 13.5% O_2, 6% CO_2, balance N_2 gaseous mixture	100
4.8	Tonometry of perfusate (PCV = 12%) with 13.5% O_2, 6% CO_2, balance N_2, gaseous mixture	103
4.9	Oxygen dissociation curve of fresh ox erythrocytes (12% haematocrit)	109
5.1	Comparative transport of D-glucose by normal and inadequately gased livers	115
5.2	Comparative transport of D-glucose by normal and oedematous livers	118
5.3	A typical recording during an experimental run	121
5.4	Comparison of extraction of D-galactose at different flow rates	123
5.5	Changes in pH during perfusion	133
5.6	D-glucose extraction using ^{14}C-Sucrose as reference	137

Figure		Page
5.7	D-glucose extraction in the presence of 95% O_2: 5% CO_2 mixture	139
5.8	Electron micrograph of a normal rat liver - low power	148
5.9	Electron micrograph of an unperfused rat liver - low power Liver A	149
5.10	Electron micrograph of a perfused liver - low power. Liver B	150
5.11	Electron micrograph of an unperfused liver - high power. Liver A	152
5.12	Electron micrograph of a perfused liver - high power. Liver B	153
6.1	Distribution and computation of isotopic materials	163
6.2	D-glucose and D-fructose extraction using ^{14}C-L-glucose as reference	167
6.3	Diagram of low temperature distillation apparatus	174
6.4	Distribution of isotopic materials in venous effluent	179
6.5	^{3}H-D-glucose extraction with ^{14}C-Sucrose as reference	181
6.6	Comparison of L-glucose and sucrose as references for ^{3}H-sucrose	183
6.7	Effect of 25 mM sugars on extraction of ^{3}H-sucrose	186
6.8	Relationship between extraction of reference (E_r) and measured fractional change or inhibition (ΔE)	196
6.9	Normogram for converting measured inhibition (ΔE) to true inhibition (ΔE_t)	
7.1	D-glucose transport in the presence of 25 mM D-glucose	210
7.2	D- and L-glucose extractions in four livers	212
7.3	Comparison of hepatocellular extractions of D-glucose and L-glucose	214
7.4	D-glucose transport in the presence of D-galactose	217

Figure		Page
7.5	D-glucose transport in the presence of 25 mM D-fructose	219
7.6	D-glucose transport in the presence of 25 mM α-M-D-glucoside	221
7.7	Molecular structure of phloretin and phlorizin	225
7.8	Effect of phlorizin on transport of hexoses	227
7.9	Effect of phloretin on hexose transport	229
7.10	Comparative inhibition by phloretin and phlorizin	231
7.11	D-glucose transport in the presence of 25mM lactose	233
7.12	D-glucose transport in the presence of Maltose	235
8.1	D-galactose transport in the presence of 25 mM D-galactose	245
8.2	D-galactose transport in the presence of 25 mM glucose	248
8.3	D-galactose transport in the presence of 25 mM D-fructose	251
8.4	D-galactose transport in the presence of α-M-D-glucoside	253
8.5	D-galactose transport in the presence of 25 mM lactose	256
8.6	D-galactose transport in the presence of 25 mM maltose	258
8.7	Effect of temperature on galactose transport	260
9.1	D-fructose transport in the presence of 25 mM D-fructose	273
9.2	D-fructose transport in the presence of 25 mM D-glucose	277
9.3	D-fructose transport in the presence of 25 mM D-galactose	279
9.4	D-fructose transport in the presence of 25 mM α-M-D-Glucoside	281

Figure		Page
9.5	D-fructose transport in the presence of 25 mM lactose	284
9.6	D-fructose transport in the presence of maltose	286
9.7	Comparative inhibition by phloretin and phlorizin	288
10.1	*Three-dimensional drawings of glucose in the (a) linear and (b) cyclic forms*	298
10.2	Ring structures of the monosaccharides (hexoses) - the glucose isomers	301
10.3	Ring structures of the monosaccharides (hexoses) - *galactose, fructose and α-methyl-D-glucopyranoside*	305
10.4	Ring structures of the dissacharides	307
10.5	Effects of 25 mM hexoses and disaccharides on (A) glucose transport (B) galactose transport and (C) fructose transport	311
10.6	The proposed binding site architecture	318
10.7	Effect of temperature on galactose transport - see also fig. 8.7	321

LIST OF TABLES

Table		Page
1.1	Transport of sugars in various mammalian tissues	23-24
4.1	Computed data to illustrate how to mix the gases to give the required partial pressures	107
5.1	Oxygen uptake by the rat liver	131
6.1	Volumetric Accuracy	159
7.1	Change in glucose extraction with time	206
7.2	Inhibition of glucose transport by different sugars	208
7.3	Effects of phlorizin or phloretin on glucose transport	223
8.1	Inhibition of galactose transport by different sugars	249
8.2	Effects of phlorizin or phloretin on galactose transport	254
9.1	Inhibition of fructose transport by different sugars	274
9.2	Effects of phlorizon or phloretin on fructose transport	282

ABSTRACT

1. Transport of D-glucose, D-galactose and D-fructose across the liver cell membrane was studied using an in situ rat isolated liver perfusion technique.

2. A bench-top liver perfusion apparatus which maintains the liver temperature constant for several hours of perfusion is described. This apparatus has the advantages that it is simple, cheap, and effective.

3. The perfusion medium used was Krebs bicarbonate buffer to which were added washed bovine erythrocytes and bovine albumin. This medium was found to meet the requirements for a viable liver perfusion.

4. Stringent viability criteria were adopted and livers which failed these criteria were rejected. Electron microscopic studies of perfused and unperfused livers were undertaken as adjunct to viability assessment of these livers.

5. The suitability of radioactively labelled sucrose as an extracellular reference material in a double indicator dilution technique in the perfused rat liver was investigated.

6. The hepatocellular transport of each of D-glucose, D-galactose and D-fructose was saturable.

All the three hexoses showed mutual competition.

The single pass extraction values for the hexoses were consistently different such that D-glucose>>D-galactose»D-fructose»»L-glucose.

7. D-glucose transport is by far greater than L-glucose transport suggesting stereospecificity of glucose transport mechanism, confirming the finding of the only previous study (WILLIAMS et al, 1968).

8. Both phlorizin and phloretin inhibited uptake of these hexoses. Of these two phloretin was by far the better inhibitor, a finding suggesting that the hepatic uptake of these sugars is analogous to

known passive (e.g. human erythrocyte) rather than active (e.g. e.nterocyte) types of hexose transport.

9. Some dissaccharides were found to inhibit the uptake of these hexoses. Lactose inhibited the uptake of all three hexoses. Maltose inhibited uptake on D-glucose and D-fructose only (it had no effect on D-galactose uptake). Sucrose had no effect on any of the three hexoses.

10. Galactose transport was found to be temperature dependent. On an Arrhenius plot there was a discontinuity of this temperature dependence suggesting a transition temperature of about 31 C. A relatively high activation energy (67 Kj mole at 37°C) was calculated from these findings.

11. It was concluded, on the basis of the findings of saturability, competition, stereospecificity and greater chemical inhibition of hexose transport by phloretin than phlorizin, that these hexoses are transported across the liver cell membrane by a facilitated diffusion (carrier mediated) transport mechanism similar to that existing in human erythrocytes. On the basis of dissacharide effects, it was inferred that the D-galactose carrier mechanism is distinct at the external surface of the membrane from the D-glucose/fructose carrier mechanism. This implies an asymmetry between the external and internal carrier sites, the latter probably being common to all three hexoses whereas the former is distinct. An architecture for these carrier sites in the liver cell membrane is proposed.

PART A

INTRODUCTION

CHAPTER ONE

1. THE CELL MEMBRANE

1.1 Introduction and definition

"Just as chemistry could not have developed without test tubes to hold reacting substnaces, so organisms could not have evolved without relatively impermeable membranes to surround the cell constituents. This barrier between the inside and the outside, the inner and external world of each living unit, has been and always must be considered one of the fundamental structures of a cell. No one can fail to be impressed with the great difference in properties of living and dead cells. The dead are completely permeable to diffusible substances, while the living retain one material and pass another. This difference, selective permeability, is so marked that it becomes the surest test to distinguish the living from the dead, holding where all other methods fail. It can truly be said of living cells, that by their membranes ye shall know them."

E. N. HARVEY 1943

What now seems conspicuously absent in this quotation is a mention of the parallel importance of barrier action within the cell such as the mitochondrial membrane. The essential role of membranes in energy transduction was, of course, not yet understood in 1943, although the necessity of internal compartmentalisation had been perceived (*CHRISTENSEN* 1975).

The maintenance of this selective permeability requires the presence of barriers to keep such environments, and steps leading to separation apart. These barriers are conspicuous and can be very effective indeed. At one point in history, the presence of a barrier between the cell and its environment was doubted. About 50 years ago a school of colloid chemists and their biological disciples proposed that the periphery of

of the cell was marked by no more than a sol-to-gel transformation, a coagulation process which they supposed would occur very simply on exposure of a new cytoplasmic surface to extracellular concentrations of calcium ion (cited by CHRISTENSEN 1975). It is perfectly clear, however, that the molecular changes that repair a defect in a broken cell membrane are much more complex than they supposed.

So, a strong, selective barrier action of cellular membranes is now believed to exist, and it applies particularly to hydrophilic molecules, a class to which all the substrates used in this study belong. The very high electrical resistance (about 10^7 to 10^8 ohms cm^{-1}) and capacitance [about 1 microfarad $(cm^2)^{-1}$] associated with the plasma membrane (COLE 1968) underscores the strength of this barrier action as far as ions are concerned. The action applies much less to hydrophobic, oil soluble molecules. Noting the comparative ease with which lipid-soluble substances penetrate cells, OVERTON (1899) proposed the concept of the lipoid character of the barrier. Overton's hypothesis constituted one of the main tenets of DAVSON-DANIELLI's proposal of the molecular structure of the cell membrane in 1943. This model dominated the field of membranology for a long time. But new findings coupled with newer hypotheses such as the fluid mosaic matrix hypothesis by SINGER and NICHOLSON (1972) have made the DAVSON-DANIELLI model (1943, 1952) or indeed its offspring the DANIELLI-ROBERTSON model (1960) not to be held as the complex structure of the cell membrane as we know it today.

But the very definition of the term "cell membrane" is a matter of great contention. A precise and complete definition of the word "membrane" is difficult to make, any complete definition given to cover all the facets of membrane behaviour will be inexact and any precise statement will be incomplete. It is defined as a "dynamic, plastic, fluid structure which has no fixed state" (O'BRIEN 1970). It was stated that "an outstanding characteristic of cells is that the relationship between

their internal activities and the external environment is regulated by a barrier; this essential barrier at the cell surface is the plasma membrane *or cell-surface membrane*" (FINEAN, COLEMAN & MICHELL 1978). It has also been defined "as a phase, usually heterogenous, acting as a barrier to the flow of molecular and ionic species present in the liquid and/or vapour contacting the two surfaces" (LAKSHMINARAYANAIAH 1969). The term heterogenous has been used to indicate the internal physical structure and external physicochemical performance.

Even these definitions are not all embracing. In fact the very definition of the term "cell membrane" is a matter of contention. The cell biologists use the term "cell membrane" or "plasma membrane" or "plasmalemma" in at least three quite different senses. In the anatomical sense the cell membrane is the external limiting region of the cell visible occasionally as a darkly staining region in the light microscope and with more certainty in the electron microscope as a layer (or a pair of layers) of osmiophilic material. In the biochemical sense, the cell membrane is a "fraction" of the cell prepared by the now classical techniques of selective disintegration of the whole cell, followed by differential centrifugation. A preparation is obtained which can be analysed chemically and which can by electron microscopy, be compared with the "cell membrane" seen in the whole cell. Finally, in the physiological sense, the "cell membrane" is an hypothetical structure invented to explain certain data on the "permeability of cells" (that, on the rate of entry into these cells of certain other substances and to explain other data on the distribution of metabolites and other molecules between the cell and the fluid in which this cell is immersed), STEIN (1967). Such data often suggest the presence of a "permeability barrier" between the cell and its environment. Perhaps, the simplest definition of "plasma membrane" is that by NEVILLE (1974) stating that "the cell surface or plasma membrane is the most peripheral membranous

portion of a cell". This is a morphological definition which excludes cytoplasmic vesicles which may have been derived from the surface membrane by pinocytosis or other forms of membrane recycling. The reason for this distinction is that we are interested in quantitating membrane components at the crucial cell-environment interface. The surface membrane thus defined is structurally heterogenous for many cells. The hepatocyte surface membranes, for example, exist in three morphologies:- a simple membrane exhibiting small protrusions and invaginations bordering on the sinusoid known as the blood front; a membrane exhibiting numerous desmosomes known as the cell front; and a rich profusion of long villous processes forming the bile canaliculus known as the bile front. Evidence for biochemical heterogeneity paralleling the structural heterogenity exists for liver (EVANS 1970) but is best documented in the intestinal epithelium (FUJITA et al 1972) and in renal proximal convoluted tubule epithelium (WILFONG & NEVILLE 1970; MARX et al 1973).

1.2 The Structure of Cell Membrane

The science of membrane biology is no longer a simple subject. Membranes vary in composition, structure and function from species to species, from cell to cell and even from one cell component or organelle to another. They are dynamic, plastic, fluid structures which have no fixed state (O'BRIEN 1970). O'Brien believes that one falls into the trap of naievete when one describes the molecular anatomy of membranes with simple molecular models. Such models, he contended fall short of reality; those who believe that they are devinely revealed are misled. Nonetheless, molecular models are useful in understanding the nature of membranes since they provide a framework upon which to shape, reshape and embellish our thoughts about these interesting biological structures.

Membranes may be broadly classified into natural and artificial membranes. Artificial membranes are man-made such as highly cross linked resins, dried collodion, oil impregnated porous filters, silicone, black films and reconstituted vesicles. But the use of artificial membranes has tremendously increased our knowledge of membrane function. LIPPERT and PETICOLAS (1971) studied the effects of cholesterol on membrane fluidization at different temperatures. They found that the apparent effect of cholesterol on lipid multi-layers is different above and below the transition temperature. In the case of dipalmitoyl lecithin, which they used, this temperature was 38°C. Fluidization occured at lower temperature and gel formation occurred at higher temperatures when lecithin was added to the lipid multilayers preparation. This study thus showed us the stabilizing effects of cholesterol on lipid membranes. It also gave us an insight to membrane fluidity, a concept used by SINGER and NICHOLSON (1972) to build up their fluid mosaic model of the cell membrane. Recently, artificial membrane (reconstituted black lipid membrane) has been prepared and shown to possess monosaccharide transport function (NICKSON & JONES, 1977). They reconstituted a monosaccharide

transport system in a black lipid membrane formed from egg lecithin/cholesterol/n-decane in the presence of Triton X-100-Solubilized fractions of the erythrocyte membrane. They found that D-glucose transport function was retained by this reconstituted membrane (black film), and using cytochalasin-B they were able to show that the retained sugar transport function was not just simple diffusion but the known facilitated diffusion transport mechanism that exists in the natural erythrocyte membrane. This paper was significant in view of the fact that earlier attempts to reconstitute facilitated diffusion system for monosaccharides in planar black lipid membranes was unsuccessful (JUNG 1971, LIDGARD & JONES 1975). They were able to pinpoint the transport function to the major intrinsic polypeptide component known as Band 3 in the polyacylamide-gel pattern of the solubilized membrane proteins. Band 4.2 is known to consist of polypeptides of cytoplasmic origin (STECK 1974) and to increase with age of the cell, so NICKSON and JONES (1977) argued that Band 3 transmembrane polypeptides must be the transport one. This was an important contribution to the identification of the sugar transport proteins of the cell membrane. Furthermore, reconstituted vesicles have helped us to understand more about sugar transport and membrane fluidity. MELCHIOR and CZECK (1979) found that reconstituted phospholipid vesicles containing adipocyte intrinsic membrane proteins and a 1:1 ratio of egg phosphatidylcholine (PC) and egg phosphotidyl ethanolamine (PE) exhibited a crystalline to fluid lipid phase transition between the temperatures of about -15°C and 17°C. They found that these vesicles retained facilitated diffusion transport system for D-glucose when in the fluid state, but not when in the gel state. This led them to the conclusion that the vesicles have a transport system which does not operate as a rigid channel-forming pore, but rather requires molecular motion in the bilayer. This conclusion will tie up very well with the findings that led to the proposition of the type of binding site architecture put

forward in this thesis (Chapter 10). So studies on artificial membranes are not only necessary adjuncts, but could help to initiate, validate or even substantiate findings and propositions on natural membranes.

However, natural membranes are biological membranes and exist in biological systems. This study is primarily concerned with this type of membrane, the "cell membrane" and this will be described further.

The biological membranes are considered to have a fundamental unit membrane called "the cell membrane", "plasma membrane", or "plasmalemma". The cell membrane was born as a functional necessity in the second half of the last century. That was a period in which the attention was focused in whether a substance penetrated or not into the cell and, in case it did not, what prevented it from entering. The observation that cells swell in hypotonic solutions and shrink in hypertonic ones led to the comparison of the cell with an osmometer. The analogy required a membrane. One of the pioneers in this field was OVERTON (1899). He pointed out that the cell was more permeable to substances which dissolved easily in lipids than to substances that have poor lipid solubility. Therefore, in a first approximation the cell membrane was considered to be a lipid film that substances have to traverse in order to get into the cell. A few decades later GORTER and GRENDEL (1925) carried out a very important experiment, perhaps one of the most important experiments in membrane science. They extracted red blood cells with acetone, and placed measured samples of the lipid extract on to the surface of water, in a Langmuir trough. The Langmuir trough is a rectangular vessel about one centimetre deep filled with an aqueous medium. It is fitted with a floating barrier having platinum brushes at the ends to prevent leakage of surface material past the barrier. The applied extract which floats on the surface of the water is confined between three fixed sides of the trough and the floating barrier. The barrier is now moved so as to confine the surface material within an ever-decreas-

ing, and ultimately a coherent mono-molecular film forms on the surface of the water. This force required to maintain this surface film is measured. This was originally done with a torsion wire and optical level system which was sensitive to changes of the order of 0.01 dyne.cm^{-1}. Initially there is little resistance to movement of the barrier, but as the area occupied by the surface film decreases, the force needed to move the barrier increases. The area occupied by the surface film is plotted against the force required to maintain it. GORTER and GRENDEL showed that the area occupied by the film at the point of the first detectable increment of pressure was about twice the surface area of the red blood cells from which the lipid extract had been prepared. On the basis of this evidence, they suggested that the lipids in red cell membranes are arranged as a continuous double molecular layer, a hypothesis which has been at the centre of every discussion of membrane structure from that day to this. This not withstanding, the experiments upon which their ideas were based are open to criticism from three points of view:-

1. Although it is probably true that all the lipid of the red cell is contained within the membrane, it would be essential in experiments of this sort to achieve something approaching 100% extraction: the method used by GORTER and GRENDEL was extraction with acetone, which gives significantly less than the total.
2. The surface area of the extracted lipids as a monolayer was compared with the surface area of red cells in dried film preparations ($99\mu^2$ instead of $145\mu^2$ in wet films). Thus the original experiments were based on an underestimate of both the amount of lipid and the surface area to be covered, and fortuitously provided the 2:1 ratio between the extract monolayer and red cell surface areas.
3. The outcome of experiments of this sort depends upon the more serious assumption that the area occupied by the lipids in the membrane is the same as the area occupied by lipids on a spread mono-

layer in a Langmuir trough at the point at which the first increment in surface pressure due to the presence of the lipids becomes detectable.

It must be admitted, however, that the great importance of the original experiment was that it led GORTER and GRENDEL on to the germinal principle which has dominated all thinking and controversy about membrane structure ever since them.

But the acceptance of the cell membrane as a mere lipid bilayer was not to be held for long. HARVEY (1931) studied the surface tension of cells subjected to a centrifugal force. Under the influence of this force the light and the heavy components of the cell separated producing an elongation of the cell. As the speed of the centrifuge was increased the separation was more pronounced until a centrifugal force was reached (0.2 dyne.cm^{-1}) that broke the cells into two halves. Since the cell membrane has to be broken in order to separate the two pieces of cell, the force necessary to split the cell is a measure of the surface tension of the membrane. This surface tension proved to be too low as the one expected for a simple oil-water interface (some 35 dynes.cm^{-1}). The finding that the surface tension of oil droplets coated with protein (actually, the oil droplets which cause buoyancy in mackerel eggs) is similar to that of some membranes, led DANIELLI and HARVEY (1935) to propose that the tension was lowered because of the absorption of proteins to the lipid bilayer. Other findings in support of this evidence were those of COLE (1932), HARVEY and DANIELLI (1938), ASKEW and DANIELLI (1940). Also an important support for this model was provided by the works of SCHMITT, BEAR and PONDER (1936) who studied the refrigence of cell membranes before and after the extraction of lipids and concluded that the lipids are oriented in a perpendicular position to both the surface of cell and the protein component of the membrane.

The foregoing contributions narrated from the time of OVERTON (1899)

to the work of ASKEW and DANIELLI (1940) provided DAVSON and DANIELLI with sufficient evidence to propose a model of cell membrane structure called the "paucimolecular" model of DAVSON and DANIELLI (1943, 1952). This model (shown in fig. 1.1) is now accepted by many to mimic the true cell membrane structure in its simplest form. It is considered to be a bimolecular leaflet of lipids with their polar groups oriented toward the two aqueous, extracellular and intracellular phases of the cell. On the polar heads on both sides is protein coat of the cell membrane. The existence of such bimolecular leaflet of lipids has been supported by the recent findings using the electron microscopy. The electron microscopy has demonstrated the presence at the limiting surface of almost all cell types studied, a layer of material some 75Å (range 50-100Å) wide ($75Å = 75 \times 10^{-8}$ cm) which binds and is thus made visible by electron-dense compounds such as osmium tetroxide, potassium permanganate, and uranyl acetate. The use especially of potassium permanganate as a stain has shown clearly that this limiting layer is in fact composite, being made up of two dark (that is heavy metal absorbing layers each about 25Å wide and separated by a light layer (one which does not take up the electron-dense stain) also some 25Å wide. Thus at the interface between two cells, four dense layers (two contributed by each cell) and three light layers (one from each cell and from the space between the two cells) can be seen. Each set of a pair of dense layers separated by a light layer is referred to as a "unit membrane" a term first introduced by ROBERTSON (1960). The bimolecular leaflet is further proved by the rather new freeze-cleaving methods combined with selective sublimation of water, or freeze-etching adequately reviewed by McNUTT and WEINSTEIN (1970), MORR (1971), and BRANTON (1971). To carry out this process, first the cells are frozen to -180°C, under conditions allowing their live recovery. They are then split mechanically, the fractured surfaces are shadowed with metal vapour (both at -180° in vacuo) and the resulting

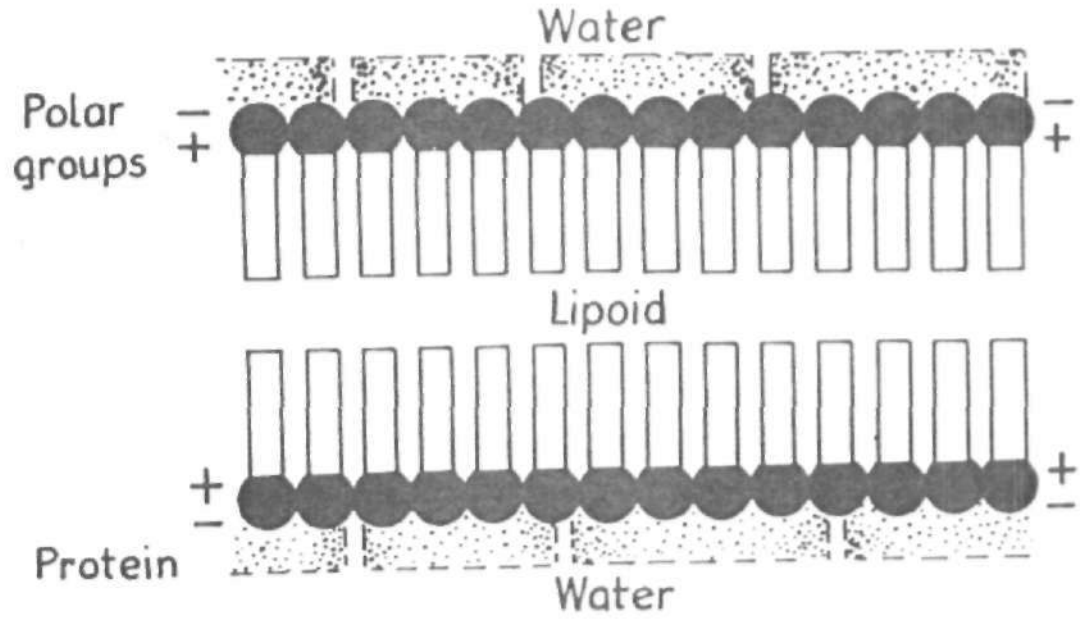
LEGEND TO FIG. 1.1

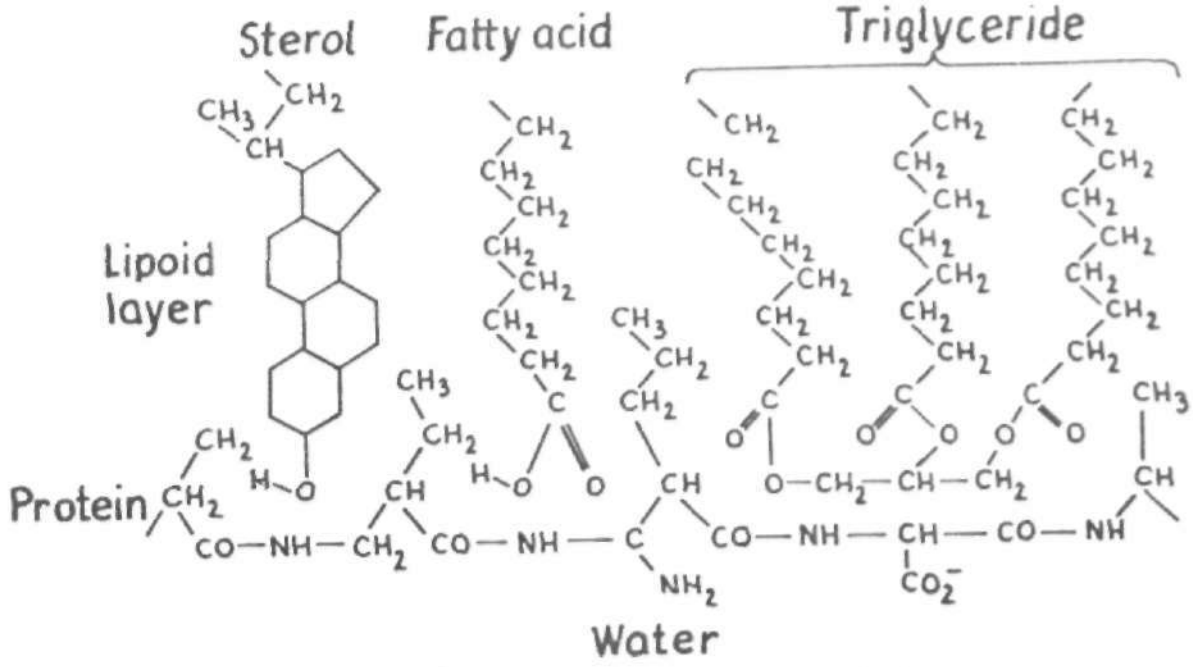
FIG. 1.1A - THE "PANCIMOLECULAR" MODEL OF CELL MEMBRANE STRUCTURE AS PROPOSED BY DAVSON AND DANIELLI 1952. NOTE THE ORIENTATION OF HYDROPHILIC (POLAR) GROUPS TOWARDS THE AQUEOUS INTERIOR AND EXTERIOR OF THE CELL MEMBRANE.

FIG. 1.1B - THE CHEMICAL STRUCTURE OF THE DAVSON-DANIELLI MODEL OF THE CELL MEMBRANE.

FIG. 1.1

A. THE "PANCIMOLECULAR" MODEL OF CELL MEMBRANE STRUCTURE, (AFTER DAVSON AND DANIELLI 1952).





B. CHEMICAL STRUCTURE OF DAVSON-DANIELLI MODEL OF THE CELL MEMBRANE.

replicas are examined electron microscopically. Sometimes, some water is sublimed off (etched) before shadowing. Etching exposes "true" external surface (WALLACH 1972). The splitting (fracturing process) seems to expose the internal membrane face (STEIN 1967). This internal membrane face of STEIN appears to be the "true" external surfaces referred to by WALLACH (1972).

It is tempting to accept the present DAVSON-DANIELLI model, the "unit membrane" concept, later on referred to as DANIELLI and ROBERTSON model (ROBERTSON 1964) because of its simplicity. But the evidence cited in its support in each instance has multiple interpretations, and there can thus be no detailed conclusions as to the molecular organisation of membranes (WALLACH 1972). The basis for such contention has been summarized by WALLACH in his book. However, a recent review by SINGER (1974) has clearly indicated that our present knowledge of the molecular organization of membranes is not all that fragmentary. Similarly, the structural organization of biological membranes was described in detail in a contemporary review by LEE (1974).

The concept of fluidity in the plane of the bilayer has become one of the main tenets of modern membrane biology. And, certainly, a limited state of lateral fluidity in the bilayer of membranes is necessary for the support of life since it was shown to be necessary for growth of bacteria - Acholesplasma laidlawii (McELHANEY 1974). He grew A. laidlawii in lipid-poor growth medium at 2° to 48°C with addition of various single exogenous fatty acids to a final concentration of 100μM as sterile ethanolic solutions. Cell growth was measured by turbidometry as a change in absorbency at 450nm. He found that the minimum growth temperature under certain conditions was clearly defined by the lower boundary of gel-to-liquid crystalline phase transition of the membrane lipids. The physical state of the membrane lipids could also influence the optimum and maximum growth temperature. Growth absolute rate and coefficient

both increase in a predominantly liquid crystalline state. But absolute growth rates decreased rapidly when most membrane lipids became solidified. But he found that some cells could grow even when only 10% liquid crystalline state existed. Conversion of membrane lipid from fluid to gel state was accompanied by a progressive aggregation of intra-membranous protein particles. He stated that transition arises from co-operative melting of the hydrocarbon chains in the interior of the bilayer.

The thermal characteristics of a number of activities associated with membranes, such as transport (KIMELBERG & PAPAHADJOPOULOUS, 1972; LINDEN, WRIGHT, McCONNEL & FOX 1973), assembly of membrane proteins (TSUKAGOSHI & FOX 1973), and other enzymes (MACHTIGER & FOX 1973) all show abrupt changes in rate at temperatures which may be related to the phase behaviour of the membrane lipids. In considering the concept of fluidity in membranes, it must be stressed that it refers only to the movement of molecules laterally in the plane of the membrane. The transfer of phospholipid across the membrane to a position in the opposing monolayer ("flip-flop") would be a most improbable event (GOMPERTS 1977). In this respect, the term "fluidity" as applied to membranes does not have the same meaning as when it is applied to bulk phases. The reason why the movement is restricted in two dimensions lies in the amphipathic nature (molecules having groups with different properties like having both hydrophilic and hydrophobic groups) of the component phospholipids and the intrinsic proteins found in membranes, and is due to the very favourable interaction between the polar head groups and the external aqueous environment and the great difficulty of transferring polar groups into the low dielectric medium of the interior of the membrane (GOMPERTS 1977). Evidence for the fast diffusion of the lipid molecules in the plane of the bilayer besides the fast internal motions within the lipid molecules in the liquid crystalline phase comes from electron spin

resonance (esr) studies of lipid bilyaers and monolayers containing spin labelled lipids and steroids (DAVAUX & McCONNELL 1972; TRAUBLE & SACKMANN 1972) and from proton nuclear magnetic resonance (nmr) studies of unperturbed lipid bilayers (LEE, BIRDSALL & METCALF 1973). In a nuclear magnetic resonance experiment, the sample to be studied is placed in a magnetic field. Those nuclei of the sample which have magnetic moments, such as protons and ^{31}P, precess in the magnetic field much as a spinning top precesses in the earth's gravitational field. There are three types of nmr experiments that are of importance for membrane studies. One is the absorption experiment, in which the frequencies at the various nuclei precess are measured by observing the absorption of radio frequency radiation. In practice, the experiment is usually performed by keeping the frequency fixed and measuring the absorption as a function of the magnetic field strength, but it is usually easier to think about the experiment as if it were the frequency that is varied at constant field strength. The second type of experiment is a measurement of the spin-lattice relaxation time, T_1. The inverse of T_1 is a measure of the rate at which a nuclear magnet or spin can exchange energy with its surroundings. The third type of experiment is a measurement of the transverse relaxation time, T_2. The inverse of T_2 is a measure of the amplitude of fluctuations in the precessional motion of the spins caused by, among other things, the interaction of the spin with other spins in its surroundings. Each of these three measurable properties, the absorption spectrum (including line positions, shapes, and widths), the values of T_1, and the values of T_2, are affected by the structural properties of the sample on a molecular level and by molecular motions. One of the most fruitful methods for studying membranes is to introduce stable free radical molecules into them and study the electron spin resonance (esr) spectrum of the radical in the presence of an external magnetic field. The term "spin label" is often used to describe such molecules. In a

spin label esr experiment, the spin label molecule is incorporated into the membrane sample, and the sample is placed in a magnetic field. The absorption spectrum for microwave radiation is measured. Usually, as in the case of nmr, the frequency is held fixed and the magnetic field is varied. If oriented membrane samples are used, the spectrum can be measured as a function of the angle between the magnetic field and the direction normal to the membrane surfaces.

1.3 Chemical Composition of Cell Membranes

The chemical composition of the cell membranes varies from one cell to the other in the mammalian tissues, and more so between the micro-organisms and mammalian cells, or even plant cells. Most chemical analyses of the cell membrane have been performed on fractions isolated from mammalian red blood cells. These cells are readily available in large quantities as a pure cell type and can be induced by a variety of simple techniques (such as their suspension in media of low osmolarity, the repeated freezing and thawing of cells and their subjection to the action of detergents) to lose the greater part of their haemoglobin, the major internal component. This is the process of "haemolysis". Light and electron microscopy confirm that these "ghost" preparations are indeed the cell membranes (PONDER 1955), and in a number of cases (TEORELL, 1952; STEIN 1956; LeFEVRE 1961) such membranes isolated by gentle haemolytic procedures have been shown to retain to a surprising degree their capacity to act as a permeability barrier. The "ghosts" can be washed by centrifugation, isolated, and analysed chemically. With the various methods available for preparing and analysing cell membranes, such as the various haemolytic processes of preparing enythrocyte "ghosts" the chemical composition is bound to vary over a wide range.

The major components of the membranes are proteins, lipids and water (KONO & COLOWICK, 1961; RAZIN, ARGAMAN & AVIGAN 1963; AUTILIO, NORTON & TERRY 1964; SALTON & FREER 1965; MADDY 1966). The weight of protein is more than that of the combined lipids in most mammalian cell membranes, except in the nerve myelin where this is reversed. The protein:lipid proportion is 85:15% in rat liver membrane, and 20:80% in Ox nerve myelin (KORN 1969). In the endoplasmic reticulum they are equally distributed (KORN 1969). A number of different protein fractions have been identified and some studies on their physical properties performed. A protein having enzymatic activity that has been identified in red cell ghosts is

the sodium/potassium-activated adenosine triphosphatase, another membrane-bound enzyme is acetylcholinesterase. MITCHELL, MITCHELL and HANAHAN (1965), chose five representative enzymes present in whole human red cells, namely carbonic anhydrase, adenosine deaminase, aldolase, glyceraldehyde phosphate dehydrogenase, and acetylcholinesterase, in order to discover by analyses of membrane which, if any, of these enzymes is an integral part of the cell membrane. Carbonic anlydrase and adenosine deaminase were never found bound to membrane. By a correct choice of the pH and the ionic strength during haemolysis, membrane could be prepared very largely free of both aldolase and glyceraldehyde phosphate dehydrogenase, but acetylcholinesterase was almost wholly retained by the membrane at all values of pH and ionic strength used. Thus, acetylcholinesterase would seem to be a real constituent of the membrane. Aldolase and glyceraldehyde phosphate dehydrogenase can, however, be bound by electrostatic linkages and may or may not be attached to the membrane under physiological conditions, while carbonic anhydrase and adenosine deaminase must be present in the cell water. It is the difficulty in solubilizing the membrane proteins that has hampered their further study.

There is a large body of evidence that the components of biological membranes are asymmetrically disposed across the membrane. Although membrane asymmetry has been discussed for many years, it is only recently that direct evidence has been obtained confirming asymmetry at the molecular level, and its fuller implications in membrane structure, function and biosynthesis have been appreciated (see SINGER, 1974 for review). It was suggested (SINGER 1971; SINGER & NICHOLSON 1972) that the proteins of membranes may usefully be considered in two categories, integral and peripheral. The molecules of integral proteins are postulated to be generally amphipathic (LEONARD & SINGER 1966; WALLACH & AZHLER 1966), and to be embedded by their hydrophobic portions into the lipid bilayer matrix

of the membrane while their hydrophilic portions protrude from the matrix into the aqueous phase. If the lipid is in the fluid state, as stated in the preceeding section of this chapter, the integral proteins are generally free to diffuse laterally in the membrane - this is the fluid mosaic model of SINGER and NICHOLSON (1972). It is proposed (SINGER & NICHOLSON 1972; SINGER 1974) that the molecules of peripheral proteins, on the other hand, are not inserted into the membrane matrix, but instead are generally attached to the membrane at the exposed portions of integral proteins. The erythrocyte membrane is so far the most thoroughly investigated membrane with respect to the composition and asymmetrical distribution of its protein components.

Also it has been suggested that the major phospholipids of the erythrocyte membrane are asymmetrically distributed across the two halves of the bilayer (BRETSCHER 1973). But there is no evidence at present for an asymmetrical distribution of phospholipids in cell membranes other than in erythrocytes. If an asymmetrical distribution of phospholipids is a general phenomenon of cell membranes, then its implications for the structure and function of membranes are considerable. For example, lipid asymmetry has not previously been considered a factor in lipid phase separation experiments (TRAUBLE & OVERATH 1973; LINDEN et al 1973). In E. coli membranes, with which most of these experiments were carried out, the major phospholipid (70-80%) is phosphotidyl-ethanolamine and the only other important phospholipids are phosphotidyl-glycerol (5-15%) and cardiolipin (5-15%). If the two latter anionic lipids were concentrated in one layer of the bilayer, with the phosphatidylethanolamine distributed in both layers, then the two layers and surfaces could have distinctly different properties. Lipid asymmetry would also play a role in the kinds of direct short range lipid-protein interactions that some integral enzymes of membranes may require to express their activities (ROTHFIELD 1971). Obviously, lipid asymmetry would also have to be

taken into account in reconstitution experiments between isolated proteins and lipids.

The existence of a stable asymmetrical distribution of proteins and lipids across a membrane bilayer implies that these components do not rotate from one surface of the membrane to the other at significant rates i.e. there is little "flip-flop" motion. Such rates have been examined directly for phospholipids in synthetic bilayers (KORNBERG & McCONNELL 1971) and have indeed been shown to be very slow. Where such transmembrane rotations of integral proteins have been sought (STECK 1972; BENNETT & CUATRECASAS 1973), they have also been found to be undetectable. This is especially remarkable in view of the rapid translational and in-plane rotational mobilities of protein (FRYE & EDIDIN 1970; BROWN 1972; CONE 1972) and lipids (KORNBERG & McCONNELL 1971) in many membranes under physiological conditions, and the conclusive evidence that the bulk of the lipid in a membrane is physically independent of the membrane protein (SINGER 1974). It is therefore clear that the negligibly slow rates of transmembrane rotations cannot be attributed to the presence of a rigid structure preventing such rotations. Rather it has been suggested (SINGER & NICHOLSON 1972) that the passage through the hydrophobic membrane interior of the polar head groups and ionic residues of the lipids and proteins is thermodynamically a highly unfavourable process. The free energies of activation required are so large that the rates of such rotations are neglibibly slow (SINGER & NICHOLSON 1972). The thermodynamic arguments are those which led originally to the suggestion that the molecules of integral membrane proteins are generally amphipathic (SINGER 1971; LEONARD & SINGER 1966). These considerations bear on the molecular mechanisms involved in the facilitated diffusion or active transport of small hydrophilic molecules through membranes. It is virtually certain that these processes are mediated by a set of proteins associated with the membrane, each with a

characteristic binding site capable of specifically binding a small molecule. These are what membrane physiologists call membrane transport proteins (KOTYK 1970). These transport proteins have actually been isolated using double labelling of inducible carriers - the inducer being thiomethyl B-galactoside in bacteria (KOLBER & STEIN , 1966, 1967) or from baker's yeast (HASKOVEC & KOTYK 1969). Other methods of identification of membrane transport proteins include ^{3}H-labelling of sugar transport inhibitors such as dinitrofluorobenzene (BNFB) by STEIN (1964), BOBINSKY and STEIN (1966), BONSALL and HUNT (1966), or specific labelling of transport system (or of its part) by FOX and KENNEDY (1965), and FOX et al (1967). Some of the isolated transport proteins (membrane carriers) have been crystallized (PARDEE 1966, 1967; PARDEE & PRESTIGE 1966; PARDEE et al 1966), their molecular weight determined, and even their amino acid composition estimated (PARDEE 1968).

The foregoing description of the structure and composition of the cell membrane indicates that although general membrane structure is apparently similar in widely varying tissues, there are variations in the type and distribution of hexose transport processes.

1.4 Transport of Monosaccharides in different mammalian tissues

Apart from the movement of substances across capillary walls between the intravascular plasma and extravascular interstitial fluids through intercellular passages, translocation of biological substances from one body compartment to another occurs through one or more cell (plasma) membranes. The common observation in a very wide variety of cell types is that while disaccharides are usually excluded, some degree of transmembrane passage of many monosaccharides is effected by way of selective transport mechanism at the cell surface. There is no class of animal cells totally lacking in this monosaccharide transport apparatus, unless perhaps it is in bone alone. To this end, a description of a few tissues whose transport of monosaccharides have been well documented in literature is given in this section. A summary of the transport mechanisms have been stated in column 3 of the table as either facilitated diffusion or active transport types. These terms may be briefly explained. The active transport system is one which operates against a concentration gradient (uphill transport), and it requires expenditure of energy. But the facilitated diffusion system can be distinguished from active transport and simple or free diffusion by the characteristics laid down by STEIN (1967) summarised here:-

1. The facilitated diffusion system operates on an existing electrochemical gradient of the permeant. The system requires no imput of energy.
2. If the permeant is optically active, its optical enantiomorph is likely to have a very different rate of penetration if it occurs by facilitated diffusion - by simple diffusion these rates should not differ - this is stereospecificity.
3. The rate of penetration may be expected not to be directly proportional to concentration but to reach a limiting (saturation) value. This is a special case of competition where the competitor and the

Cell or Tissue	Substrate	Type of Transport	Reference
Erythrocytes	Monosaccharides	facilitated diffusion most completely characterised	Widdas 1954 Lefevre 1954, 1961 Rosenberg & Wilbrandt 1957 Bowyer 1957 Sen & Widdas 1962 Stein 1967 Baker & Widdas 1973 Barnett et al 1973, 1975
Erhlich's Ascites Tumor cells	Hexoses	facilitated diffusion	Crane et al 1957
Blood Brain Barrier & CSF Barrier	Glucose Hexoses	facilitated diffusion	Fishman 1963, 1964 Oldendorf 1971
Placenta	Hexoses	facilitated diffusion	Huggett, Warren & Warren 1951 Widdas 1952 Nixon 1963 Dawes & Shelley 1968 Eaton, Liechtwiess, Short & Yudilerich 1978 Shelley 1979
Liver	Hexoses	facilitated diffusion	Cahill, Ashmore, Earle & Zottu 1958 Goresky 1965, 1967 Goresky, Bach & Nadeau 1973 Williams, Exton, Park & Regen 1968 Hooper & Short 1975, 1977 Pardridge & Jefferson 1975 Baur & Heldt 1977

Cell or Tissue	Substrate	Type of Transport	Reference
Skeletal Muscle & Cardiac Muscle	Monosaccharides	facilitated diffusion Insulin sensitive	Gemmill & Hamman 1941 Levine & Goldstein 1955 Park, Bornstein & Post 1955 Park & Johnson 1955 Randle & Smith 1958 Zierler 1959 Park et al 1961 Morgan, Post & Park 1961 Randle 1961 Morgan et al 1964
Adipose Tissue	Glucose	facilitated diffusion Insulin sensitive	Crofford, Jeanrenaud & Renold 1965 Kuo, Dill & Holmlund 1967
Intestinal Epithelium	Sugars	Active Transport Sodium-coupled	Crane, Miller & Bihler 1961 Crane 1965 Stein 1967 Schultz & Curran 1970
Renal tubular Epithelium	Sugars	Active Transport Sodium-coupled	Krane & Crane 1959 Schultz & Curran 1970

Table 1.1: Summary of Transport of Sugars in various mammalian cells

permeant are the same substrate.

4. The rate of penetration may be markedly decreased by presence of molecules structurally analogous to the permeant - this is competition.

5. The rate of penetration may also be reduced markedly and specifically by chemically different substances - inhibitors - which may also be active as enzyme poisons.

6. It may be possible in certain cases to link the facilitated movement of molecule down its electrochemical gradient with movement in opposite direction of structurally analogous molecule - this is the phenomenon of counterflow.

In any facilitated diffusion system there is a small amount of concurrent simple diffusion as well. In fact SHELLEY (1979) recently reiterated that all the factors controlling simple diffusion also control facilitated diffusion - the only difference being that the facilitated diffusion has other factors in addition. The factors controlling the transfer of water-soluble compounds across the cell membrane by simple diffusion are pore size, surface area of exchange, membrane thickness, molecular size and charge, concentration gradient, hydrostatic pressure and temperature. In addition to all these factors, facilitated diffusion system is controlled by these other factors:- chemical and stereospecificity, competition between isomers and analogues, uphill transport, saturation phenomenon and chemical inhibition, SHELLEY (1979).

1.4.1 Transport in erythrocytes

The transport of sugar into erythrocytes is by facilitated diffusion. This means that sugar transport here requires no expenditure of energy (like simple diffusion process), and differs also in that the system is quite insensitive to metabolic poisons and various "unphysiological" conditions which might well terminate any pump activity. Serious incompatibilities were noted when compared with simple diffusion in that this system shows "saturation" characteristics in the proportional diminution of rates as concentration gradients were increased, and in apparent "competitive" interactions when two monosaccharides were presented concurrently. Most detailed analysis of facilitated diffusion sugar transport has been done on human erythrocytes. It was established (KOZAWA 1914) that the erythrocytes of man and monkey were vastly more sugar permeable than those of assorted rodents, carnivores, and ungulates - the pig, ox, sheep, dog and cat having been studied. The fetal erythrocytes of rabbits, guinea pigs, pigs, sheep and deer all show glucose permeabilities of the same order (and comparable kinetics) as seen in adult human erythrocytes, a fairly abrupt loss of this capacity developing in the nonprimate species approximately at the time of birth.

The facilitated diffusion transport system shows a high degree of stereospecificity for hexose and pentose sugars in the pyranose ring form (LeFEVRE & MARSHALL 1958; NAFTATIN & HOLMAN 1977). The specificity of the sugar-membrane interactions was first systematically investigated by LeFEVRE and MARSHALL (1958). More recently this systematic approach was extended by BAKER and WIDDAS (1973b) to propose asymmetry of the sugar carrier mechanism in their studies with ethylidene glucose. They first demonstrated that 4-6-O-ethylidene-α-D-glucopyranose (ethylidene glucose) does not cross the red cell membrane on the hexose transfer system, nor can it induce uphil transfer by counterflow (BAKER & WIDDAS 1973a). Therefore, their subsequent finding that ethylidene glucose

blocked glucose transport in erythrocytes more effectively when present outside the cells than when present inside the cells led them to the conclusion that the carrier component inside the cell membrane is different from that present externally (BAKER & WIDDAS 1973b). The asymmetry of the carrier was supported by BARNETT, HOLMAN and MUNDAY (1973) who drew similar conclusions from their finding that 6-O-alkyl-D-galactoses which are non-transported competitive inhibitors competively inhibited erythrocyte sugar transport system from outside while relatively ineffective when present inside the cells. On the other hand, they found n-prophy-B-D-glucopyranoside inhibited the sugar transport when present inside the cells but has a negligible effect when present outside. By using modified sugars in positions C_1, C_2, C_3, C_4 and C_6, BARNETT, HOLMAN, CHALKLEY & MUNDAY (1975) were able to identify the carrier binding sites at C_4 and C_6 being in contact with solvent on the outside, but C_1 to be inside the cell.

The evidence that sugars interact in a stereospecific manner implies at least a three-point attachment with some membrane constituent. Stereognosis implies a three-dimensional geometry, so the minimum number of attachments to effect a stereospecific action would be three. This implication is developed further in Chapter 10 on the proposal for the binding-site architecture of the hexose transport mechanism. Also counterflow first described by ROSENBERG and WILBRANDT (1957), where uphill flow of labelled sugars into cells from an external solution containing sugars at low concentration is driven by the net downhill movement of unlabelled sugar present within the cells initially, at high concentrations or vice versa, demonstrates that there may be at least two sugar-binding sites within the transport pathway; one facing inwards on the inner surface and another facing outwards at the outer surface. However, some people think that the counterflow phenomenon does not necessarily imply an inward and an outward site. Rather, the proponents

of carrier hypothesis think that the carrier-sugar complex moves from one site and unloads the sugar at the other, and the effect of counter-flow stimulation is to accelerate this recycling of the carrier.

The existence of carriers in membranes responsible for the transfer of molecules across the membranes was proposed by OSTERHOUT (1930), and SHANNON and FISHER (1939), using the accumulation of electrolytes in Valonia and renal tubular reabsorption of glucose in dogs respectively. The carrier proposition was that the transported substance combines with the carrier and the complex either diffuses through the membrane or swings, as though hinged, from one side of the membrane to the other. In either case, it is proposed that once the complex reaches the other side of the membrane, it dissociates, releasing the transported substance. The carrier hypothesis has been advanced by many (LeFEVRE 1954, WIDDAS 1954, ROSENBERG & WILBRANDT 1957, and LeFEVRE & McGINNIS 1960). The data which have been interpreted as requiring postulation of a carrier system are of three kinds: first, specificity of penetration of the membrane. The fact that some molecules penetrate membranes more readily than do closely related molecules has suggested that there must be stereospecific combinations between the penetrating molecules and a carrier. Secondly, saturation - a point is reached beyond which the rate of penetration of the membrane is constant despite increases in concentration of the penetrating substance. Data of this sort have been interpreted as indicating that there is a limiting quantity of carrier. Once all carrier molecules are occupied, the transport rate becomes maximum. Thirdly, competition - when two related molecules, both of which can penetrate the membrane, are present simultaneously, the rates at which they penetrate the membrane are less than if either were present in the solution of the two molecules. These data have suggested, again, that there is a finite quantity of carrier, any molecule of which can transport either substance but not both simultaneously.

The question then arose as to whether there may be an alternative physical model compatible with those observations, leading to the postulation of the carrier hypothesis. The pore hypothesis was proposed as an alternative by ZIERLER (1961). He devised a mechanical membrane model with which he was able to demonstrate that the flux was saturable, and was increased with increased velocity by raising the temperature. He also found that the flux decreased with increased size of spheres but increased with increased size of the pore. He then gave a mathematical treatment on the basis of which he proposed the pore hypothesis for glucose entry into the muscle cells down a chemical potential of about 5mM in the extracellular medium bathing the muscle cell to nearly zero intracellularly. He showed for the first time that saturation can also occur in a passive phenomenon of entry through the pore. The pore model was supported and advanced by NAFTALIN (1970) who used the term lattice pore model. His arguments derived mainly from the work by ZIERLER (1961) and the fact that the anomaly of K_M being lower than K_S was a reverse of what would obtain in a case of a carrier. His work on the pore hypothesis was an advance on ZIERLER's work because he (NAFTALIN) studied the transport of sugars across the enythrocyte membrane instead of the mechanical model used by his predecessor. Also, more recently, studies on the pore model on sugar transport were carried out on human erythrocytes by BOWMAN and LEVITT (1977). They predicted that the permeability should be proportional to the volume of distribution of the solute in the pore and should therefore decrease with increasing molecular size. In order to test these predictions, the glucose-dependent permeability of a series of 4-Carbon (erythritol), 5-Carbon (D-arabitol, L-arabitol and Xylitol) and 6-Carbon (D-mannitol, D-sorbitol and myo-inositol), polyols was measured. The permeability of all the polyols was decreased by the presence of glucose and the K_i (inhibition constant) of this "inhibitable" component was found to be similar to that of D-sorbose, suggesting that

this component was associated with the glucose transport system. Since these observations could be explained entirely in terms of a specific affinity for a carrier binding site, they do not exclude a carrier mechanism. However, as predicted for the pore model, this "inhibitable" permeability decreased with increasing molecular size and the calculated geometric pore volume was of a size that would be expected for a cell membrane pore. They subjected their data to kinetic equations and computer analysis. But the kinetic equations describing transport through a pore that has a binding site and that undergoes a conformational change are identical to those of a carrier model. So at the moment it is difficult to choose between the carrier and the pore hypothesis and say one is in operation and the other is not. The two might well co-exist. The current light thrown on this as a result of the findings of hepatocellular handling of the hexoses and sucrose might as well be the explanation of co-existence as explained further in Chapter 10.

A number of other models have been proposed based on numerous studies on erythrocytes, but the most widely accepted are the carrier and the pore models.

1.4.2 Transport in Placenta

It is generally agreed that glucose of maternal origin is the principal substrate for oxidative metabolism in the fetus. It is also an important precursor for the synthesis of fetal glycogen and lipids both of which accumulate as reserves for use after birth, (DAWES & SHELLEY 1968). Thus, there is need for glucose transfer from mother to fetus, and this occurs across the placenta. Such a transfer of glucose from mother to fetus is essential both for fetal survival and for adaptation to extra-uterine life. During the last thirty years, evidence has accumulated that transplacental transfer of glucose is more rapid than would be predicted from its physico-chemical properties, regardless of species. The observation by HUGGETT, WARREN and WARREN (1951) that the sheep placenta was more permeable to glucose than to fructose, led WIDDAS (1952) to postulate the existence of a special mechanism (a carrier mechanism) for placental glucose transport, analogous to that being studied in human erythrocytes. HUGGETT et al (1951) established that loading of either the maternal or the fetal circulation with glucose (which readily passed the placenta in either direction) resulted in increased fetal fructose level, but no fructose appeared in the maternal blood. Placenta appeared to be the site of glucose converstion to fructose. WIDDAS supported his theoretical postulation derived from the observations of HUGGETT et al (1951) with his own findings (WIDDAS 1952) of a systematic saturation in the ready transfer of glucose from mother to fetus in sheep. He suggested that a "carrier" presumably a protein, in the cell membrane combined with the water soluble, polar, glucose molecule to form a lipid-soluble complex which would diffuse readily across the membrane and release glucose into the cell cytoplasm. The kinetics of glucose transfer across the sheep placenta were compatible with this hypothesis but, as pointed out by WIDDAS himself, the carrier-glucose complex need not be mobile. Similar kinetics would also apply

to transfer along a chain of carrier molecules or across a pore formed by a double layer of carrier molecules extending across the membrane, or alternatively the complex could transfer glucose by oscillating or rotating within the membrane (DANIELLI 1954; WHITTAM 1964; MILLER 1969; BOLIS & GOMPERTS 1972; and SELWYN & DAWSON 1977). Specificity of hexose transport was demonstrated by HUGGETT et al (1951) for the differential permeability of the sheep placenta to glucose and fructose. And this was confirmed in other species: guinea pig (KARVONEN & RÄIHÄ 1954), rabbit and rat (DAVIES 1955), monkey and man (CHINARD et al 1956), goat (WALKER 1960), and the observations were extended to include other sugars. As might be expected, the placenta was virtually impermeable to the disaccharides sucrose, maltose and lactose (WALKER 1960; DAVIES 1957), but certain other monosaccharides appeared to cross it as freely as glucose (WALKER 1960). Evidence for competitive inhibition of L-sorbose transfer in rabbit by D-glucose and D-fructose transfer in the guinea pig placenta by D-glucose was provided by ELY (1966). Early attempts to demonstrate phloridzin inhibition of glucose transport across the rat, rabbit and guinea-pig placenta were unsuccessful (DAVIES 1955; ELY 1966), possibly because this competitive inhibitor was studied in the presence of very high glucose concentrations. Later studies at physiological glucose concentrations showed that phloretin, the aglycone of phloridzin, had a profound inhibitory effect on the transport of D-glucose and D-mannose across the guinea-pig and human placenta (SCHRODER, LEICHTWEISS & MADEE 1975; CARSTENSEN, LEICHTWEISS, MOLSEN & SCHRODER 1977; EATON, LEICHTWEISS, SHORT & YUDILEVICH 1978), but did not affect the much lower transfer of L-glucose (SHELLEY 1979). Using dual closed-circuit perfusion of the isolated human placenta RICE, ROURKE and NESBITT (1976) elucidated the saturable characteristics of glucose transport system in the human placenta.

The evidence presented above suggests that the mechanism responsible

for carbohydrate transfer across the placenta resembles that of entry of sugars into the human erythrocyte as authoritative reviews have reiterated (WHITTAM 1964; MILLER 1969; BOLIS & GOMPERTS 1972). But whereas the entry of glucose into the erythrocyte merely involves its passage across the cell membrane, transport across the placenta necessitated movement through at least one complete cell layer in addition to the fetal capillary endothelium, in the haemo-monochorial placenta of the guinea-pig, and may involve as many as six layers in the epitheliochorial placentas of ruminants. The human placental membrane consists of three layers (trophoblast, connective tissue and capillary endothelium) but it becomes progressively thinner and may lose the connective tissue component towards term. Thus, there are species and temporal differences in the dimensions of the placental barrier. Such structural differences might reconcile the discrepancies in the transport phenomena observed in the placentae of the different mammals studied; for example sucrose was found to cross human placenta (CHESLEY et al 1964) but does not cross rabbit placenta (BRANDSTRUP 1930; DAVIES 1957), nor goat placenta (WALKER 1960). Or indeed, experimental damage to prepared placenta may be responsible for sucrose entry in those preparations. This alternative reason for the differences in sucrose entry is particularly plausible when it is remembered that the other layers of placenta act as free diffusion barriers and only the trophoblast acts by facilitated diffusion mechanism. However, the experiments of Chesley and his co-workers were performed on human subjects during parturition, so there could not have been any experimental damage to the placenta which was still being studied up till the third stage of labour (the delivery of placenta).

1.4.3 Transport in the Liver

Most of the studies on sugar transport were carried out on the erythrocytes, in which a great deal about facilitated diffusion mechanism is now known. Less work has been done on the transport by hepatocytes. Liver cells allow execptionally rapid intake and output of sugar (SACKS & BAKSHY 1957; CAHILL, ASHMORE, EARLE & ZOTTU 1958). Such rapid entry of sugar into hepatocytes led CAHILL et al to conclude that glucose entry into the liver cell was by free diffusion. In fact ASHMORE and WEBER (1968) stated that "an important basic fact is that in the liver there is no permeability barrier for glucose". But WILLIAMS, EXTON, PARK & REGEN (1968) found that D-glucose uptake into perfused rat liver cells was nearly two orders of magnitude faster than that of L-glucose and it inhibited the latter process. They concluded that this was a highly stereospecific action and could not be accounted for by simple (free) diffusion but that it was an evidence of existence of a facilitated diffusion system in the liver cells. The movement of D-glucose is inhibited by phlorizin but is not sensitive to insulin (WILLIAMS, EXTON, PARK & REGEN 1968; BAUR & HELDT 1977). Though sugar turnover in the liver is closely governed by the circulating insulin level, the essential action appears to be not on a transmembrane process, but on the glycogenesis-glycogenolysis balance as a function of plasma glucose level. Hepatocellular transport of hexoses in the dog (GORESKY 1965, 1967; GORESKY, BACH & NADEAU 1973), the rat (WILLIAMS et al 1968; BAUR & HELDT 1977), and the sheep (HOOPER & SHORT 1975, 1977) have all indicated that the transport cannot be by simple diffusion, although as stated in section 1.4 above, some amount of free diffusion occurs in all facilitated diffusion systems. Evidence has been provided that the sugar transport in the liver shows saturable characteristics (HOOPER & SHORT 1975, 1977; BAUR & HELDT 1977). Chemical inhibition by phlorizin (WILLIAMS et al 1968; BAUR & HELDT 1977), by phloretin and cytochalasin-E

B (BAUR & HELDT 1977) have been shown. But sugar transport by the liver cell membrane was shown not to be sodium coupled (BAUR & HELDT 1977) nor insulin sensitive (WILLIAMS et al 1968, BAUR & HELDT 1977). But the transport shows stereospecificity and competition between the analogues (WILLIAMS et al 1968; HOOPER & SHORT 1977). So sugar transport by the liver cell appears to be similar to sugar entry into human erythrocytes.

1.4.4 Some other tissues having facilitated diffusion transport mechanism

Facilitated diffusion has been demonstrated for sugar transport in tumour cells, blood-brain and blood-cerebrospinal fluid barriers, skeletal muscle, cardiac muscle, adipose tissue and aqueous tissues of the eye. Of these, the transport in tumour cells and that across blood-brain and blood cerebrospinal fluid barriers are non-insulin sensitive, but the transport in the other tissues mentioned here are insulin-dependent.

It was found that the equilibrium of monosaccherides into mouse EHRLICH ascites tumour cells to be extremely rapid, to show saturation kinetics and competition phenomena, symmetrical entry and exit, and inhibition by phlorhizin but indifference to insulin or lack of oxygen (HELMREICH & CORI 1956; CRANE, FIELD & CORI 1957).

Fishman's thorough study (FISHMAN 1963, 1964) of the sugar transfer between blood and cerebrospinal fluid (CSF) in intact dogs has provided clear demonstration of this system of essentially all the kinetic criteria for a facilitated diffusion; saturation (with glucose); stereo-selectivity; competitive inhibition (between glucose and 2-deoxyglucose) symmetrical operation in the two directions; and counterflow-induced uphill movement (entry of glucose being enhanced by efflux of 2-deoxyglucose). The blood-brain barrier was also found to be stereospecific with respect to amino acid transport when OLDENDORF studied twelve amino acids in rat by rapid injection (OLDENDORF 1973a). He also demonstrated that a facilitated diffusion transport mechanism existed across the blood-brain barrier for the transport of short-chain monocarboxylic organic acids (OLDENDORF 1973b).

Although the "uptake" of glucose by muscles had been studied from various points of view, little attempt had been made to distinguish membrane traversal from the subsequent metabolic events. PARK, BORNSTEIN

and POST (1955) first demonstrated the rise in free glucose within the diaphragm upon addition of insulin, verifying the site of action as at the membrane rather than on the hexokinase step. They concluded that insulin increased glucose uptake by accellerating the entry of glucose into the muscle cells. This observation was confirmed and view upheld by LEVINE and GOLDSTEIN (1955); PARK and JOHNSON (1955). Hence, the original observation of GEMMILL and HAMMAN (1941) that insulin enhanced glucose uptake by the in vitro preparation of the rat diaphragm which puzzled early workers in this research area was explained in terms of membrane effect rather than increase in metabolic sequestration. Using rat diaphragm preparation, RANDLE and SMITH (1958) showed that glucose entry into the muscle was accompanied by K^+ entry into the cell, in bicarbonate buffer medium. They showed that this effect of insulin on glucose entry into the muscle cells would not be demonstrated when phosphate buffer medium was substituted for the bicarbonate buffer. They concluded that failure to demonstrate this effect by earlier workers was due to the fact that such workers used phosphate buffer medium. They also showed that the uptake of glucose by muscle cells was increased by anoxia and by a number of substances such as 2,4-dinitrophenol (DNP), salicylate, arsenite and cyanide which have in common property of inhibiting oxidative phosphorylation. An accelerating effect of these factors on glucose uptake by muscle was overlooked for many years for the same reason as insulin that a wrong buffer was used. Since the rate-limiting step in uptake of glucose by muscle is transport of glucose across the muscle cell membrane this observation suggested that anoxia and substances such as DNP accelerate monosaccharide transport in muscle. In keeping with this view it has been shown that anoxia and substances such as DNP accelerate the intracellular accumulation of non-utilisable sugars such as D-Xylose in isolated rat diaphragm and that anoxia accelerates the outward transport of L-arabinose in the perfused isolated rat heart. Because factors which inhibit oxidative phosphoryla-

tion can very greatly impair synthetic processes in cellular metabolism, it seemed important to exclude the possibility that these factors might increase the permeability of muscle cells to monosaccharides not by accelerating transport but by damaging the cell membrane in some non-specific way. Although these factors increase the intracellular accumulation of sugars such as D-Xylose by muscle they do not allow penetration of the cell by substances such as D-Sorbitol or maltose or thiosulphate which are normally confined to extracellular water. The effects of anoxia on transfer of glucose across the muscle cell membrane was shown to be readily reversed by restoration of aerobic conditions (RANDLE & SMITH 1958). Furthermore, they showed that insulin can still accelerate uptake of glucose by diaphragm and heart muscle under anaerobic conditions or in the presence of substances such as DNP and the effect of insulin is generally held to depend upon the integrity of the cell membrane. Moreover competition for transport between different monosaccharides and inhibition of transport by substances such as phlorizin can still be demonstrated under anaerobic conditions (RANDLE & SMITH 1958; MORGAN, RANDLE & REGEN 1959). These observations appear to show that monosaccharide transport in skeletal and cardiac muscles is accelerated by factors which inhibit oxidative phosphorylation. MORGAN, POST and PARK (1961) demonstrated that glucose transport in the perfused normal and alloxan diabetic rats was saturable and followed Michaelis-Menten kinetics. They concluded the transport of glucose into the cardiac muscle was by a facilitated diffusion mechanism. Further work was done to elucidate the association of K^+ with glucose entry into the muscle cells in the presence of insulin by ZIERLER (1959). Using isolated rat skeletal muscle preparation (extensor digitorum longos), he found that there was a rise in membrane potential to the magnitude of 5mV when insulin was added, and that there was no increase in intracellular K^+ for the first one hour, but there was increased intracellular

K^+ after two hours. From this he concluded that the hyperpolarization was responsible for K^+ entry into the cells, not the potassium entry causing hyperpolarization in the presence of insulin. Hence it was stated that the K^+-associated glucose transport in adipocyte and muscle is a phenomenon of insulin sensitivity of this facilitated diffusion transport system. Although the transport of sugars into the cardiac muscle is very similar to that in the skeletal muscle, it is much faster than that in skeletal muscle (HENDERSON 1961; RANDLE 1961). Thus in perfused rat hearts, FISHER and LINDSAY (1956) found that galactose (not locally metabolized) rather rapidly attained a larger proportional tissue level than did sorbitol or inulin and this apparently intracellular penetration of galactose was enhanced by supplying insulin at as little as 20mU.Litre^{-1}. Studies on adipose tissue preparations (rat or mouse epididymal fat pad fragments or cells separated from them by treatment with collagenase) were initially concerned with the mechanism of insulin action rather than with transport mechanism as such. The uptake effects were found to be blocked by phlorizin (at about 1mM), KUO, DILL and HOLMLUND (1967) or phloretin (0.2mM) by CROFFORD, JEANRENAUD and RENOLD (1965), and presumably on a competitive basis by 3-O-methyl glucose (CROFFORD et al 1965).

Although sugar transport by the skeletal muscle, cardiac muscle and adipocyte is by facilitated diffusion system, it differs from those of erythrocyte, placenta and liver in being insulin-sensitive.

1.4.5 Sodium-coupled Active Transport of Sugars in the Intestine and Kidney

The transport of sugars across the brush border of the intestinal epithelium and renal tubular epithelium is an "active transport" mechanism which is sodium-coupled. This means the substrates are transported against a concentration difference (or gradient) - "uphill transport" (CRANE 1960; SCHULTZ & CURRAN 1970).

The concept of "selective permeability" of the small intestine was developed through the early works or experiements of HOBER (1899), HEDON (1900), CORI (1925), and VERZAR and McDOUGALL (1936), in which it was found that various sugars were absorbed at widely different rates and were differently influenced in their absorption by such inhibitors as phlorizin and iodoacetate. In 1939, BARANY and SPERBER demonstrated, using rabbit intestinal loop, that glucose absorption was against a concentration gradient (BARANY & SPERBER 1939). Further support was lent to these findings by the experiments of CAMPBELL and DAVSON (1948) in which it was found that 3-O-methyl-D-glucose, a compound which is not appreciably metabolized, was absorbed against its own concentration difference from intestinal loop in the cat. It was argued that this meant that the movement of sugars against their concentration gradient was a reflection of their actual transport uphill rather than due to metabolic sequestration. CORI (1925), and VERZAR and McDOUGALL (1936) separated sugars into two classes on the basis of their relative rates of absorption and the influence on the rate of alterations in the concentration of sugar in the lumen of the intestine or of the addition of metabolic poisons. They found that the rates of absorption of glucose and galactose were found to be maximal at relatively low luminal concentrations whereas the rates of absorption of the other sugars increased nearly proportionately to increases in their concentration (CORI 1925; VERZAR & McDOUGALL 1936). It was also found (VERZAR & McDOUGALL 1936) that the

addition of iodoacetate to the luminal contents markedly decreased the rates of absorption of glucose and galactose but was substantially without influence on the rates of absorption of fructose, mannose and pentoses. They contended that only glucose and galactose are absorbed against a concentration difference. But CRANE is of the opinion that "it would seem potentially most fruitful to assume that the respective sugars are absorbed by the same process whether or not there is an actual concentration difference across the intestinal mucosa." (CRANE 1960). A review of fourteen actively absorbed and thirty-five sugars and their analogues which are not transported against concentration gradient revealed that sugars are transported in the pyranose rig conformation with a specific C_1 chair form of the sugars (CRANE 1960). This is in agreement with a similar suggestion for erythrocyte sugar transport by LeFEVRE and MARSHALL (1958).

The possibility that glucose and galactose are absorbed by separate processes has been suggested on the basis of apparent differences in the degree to which their absorption is inhibited by croton oil (DURUISSEAU & QUASTEL 1959) and in the location of their maximal absorption along the intestine (FISHER & PARSONS 1953). However, it was suggested (CORI 1926) that amino acids and sugars share a common pathway in absorption (CORI 1926) as observed by mutual inhibition in the absorption of mixtures of glycine and glucose administered to rats by stomach tube. But the observations that phlorizin, at concentrations which profoundly inhibited glucose absorption did not inhibit amino acid transport in vivo (WILSON 1932) nor in vitro (FRIDHANDLER & QUASTEL 1955) have cast doubts on this proposition. Disaccharides are not absorbed, per se, by the gut but sucrose, lactose and maltose are hydrolyzed by intestinal brush border enzymes and the products, glucose, galactose and fructose are then absorbed in their usual way (FRIDHANDLER & QUASTEL 1955; WILSON & VINCENT 1955).

Phlorizin exerts a strong and relatively specific inhibitory action in intestinal and renal absorption of glucose. Renal glycosuria can be produced (McKEE & AHWKINS 1945) without influencing adversely the active reabsorption of a number of other substances. Active absorption of glucose from intestine can be inhibited in vivo and in vitro by phlorizin but it does not inhibit the absorption of fructose, arabinose or sorbose (CRANE 1960). The inhibitory ection of phlorizin was concluded to be on a Na^+-dependent, energy independent process at the brush border surface, not on the reactions responsible for accumulation (ALVARADO & CRANE 1962).

The role of Na^+ in the uptake of sugars by various preparations of hamster small intestine has been studied extensively by CRANE and his co-workers. CRANE, FORSTNER & EICHHOLZ (1965) examined the relation between extracellular Na and α-deoxy-D-glucose uptake by segments of hamster small intestine and found that lowering the external Na concentration markedly increased the K_t (the transport constant analogous to K_M of enzyme kinetics) of the uptake process but has no effect on the maximal rate of uptake. This relation was observed when either Tris or K was used to replace Na, but the K_t was increased more by K than by Tris, suggesting that K is a competitive inhibitor of Na in the Na-sugar interaction. BIHLER (1969), using segments of hamster small intestine observed that the uptake of both actively transported and non-actively transported sugars conform to Michaelis-Menten kinetics with a common maximum velocity and he concluded therefrom that they shared a common carrier mechanism. Furthermore he found that the sugars that are not subject to active transport were not influenced by Na and were characterized by very high K_ts ranging from 150mM to 500mM. In contrast, the sugars subject to active transport had K_t's that were comparatively high in the absence of Na but were reduced several hundredfold by the presence of physiological Na concentrations. Thus, according to BIHLER (1969) all of these sugars are capable of being bound by a common site but only

some of them bring about necessary conformational change for the binding and stabilizing effect of Na; it is these latter sugars that are actively transported. KLIENZELLER, KOLINSKA and BEMES (1967a,b) have examined the effect of Na on the kinetics of α-methyl-D-glucoside uptake by rabbit kidney cortex slices. Complete replacement of external Na with Tris resulted in a ten-fold decrease in the maximal rate of uptake but had no effect on the K_t. This observation is similar to that reported by VOGEL, LAUTERBACH and KROGER (1965) for the effect of luminal Na concentration on the rate of glucose absorption by isolated perfused frog kidney. These observations, coupled with the well-known asymmetric distribution of Na across most animal cell membranes, prompted the suggestion that the Na asymmetry could provide the energy required for the active transport of these solutes. This concept was first suggested by CHRISTENSEN and his co-workers (RIGGS, WALKER & CHRISTENSEN 1958), who felt that the "migration of potassium from the cell (down an electrochemical potential difference) may stimulate the simultaneous entrance of amino acid." They clearly pointed out, however, that "a propelling function might instead be assigned to the Na movements, which are reciprocal to the K movements. In the same way, consideration may be narrowed to a bound form of Na influx; that is, the excess of Na influx could occur in the form of a complex between the carrier, glycine and sodium ion." This appears to be the first statement about the concept now referred to as the "Na-gradient hypothesis" that was explicitly proposed as a mechanism for active intestinal sugar transport by CRANE (1960, 1962, 1965). It was thought that the energy required for the active transport of sugars derived from the transmembrane Na^+ gradient (SCHULTZ & CURRAN 1970). But recent evidence suggests that transmembrane gradient for Na^+ represents an energy source which is not sufficient to account for observed cellular sugar or amino acid gradients; the membrane potential provides the additional driving force (KIMMICH & CARTER-SU 1978).

CHAPTER TWO

2. ISOLATED ORGAN PERFUSION

2.1 Historical review of liver perfusion

The history of the technique for isolated liver perfusion dates back over 100 years. Claude BERNARD (1855) is usually credited with the first liver perfusion in his classical observations on the conversion of glycogen to glucose. He described a tube, filled with water connected to the portal vein at one end and to the fountain of the laboratory of the College de France at the other end; perfusion was started with tap water! With this rather crude set up, Claude BERNARD was able to demonstrate the glucose output from the liver.

A great deal of progress has been made since these early days and numerous methods of liver perfusion have been used throughout. Later, Carl LUDWIG and A. SCHMIDT (1868) recorded the unique possible advantages of isolating an organ (in their case, the dog skeletal muscle) and seeking to keep it alive by circulating artificially a stream of fresh oxygenated blood. In 1873, ASP described apparatus for isolated perfusion of rabbit liver, using it to study bile secretion. This apparatus was improved by VON FREY and GRUBER (1885) mainly aimed at improving oxygen supply. JACOBI (1890, 1895) introduced an extra circuit using a perfused animal lung.

In the nineteen-twenties, advances in our knowledge of liver physiology and biochemistry came from the liver slice preparation, developed by WARBURG. Also in the twenties, sanguineous perfusions were undertaken (BAER & ROESSLER 1926; SIMMONDS & BRANDES 1929). It was shown that vasoconstrictor substances were impeding the perfusion rate and evidence was given to suggest that histamine caused the hepatic venous constriction in the dog (SIMMONS & BRANDES 1929). This is in concordance with the explanation for the observed diminished portal venous flow rate owing to progressive increase in vascular resistance for which histamine was

said to be responsible in the perfusions described by DALE and BAUER (1928). It may be recollected that the dog veins have very active smooth muscle hence venoconstriction is marked. Such perfusions with blood where portal venous flow rate decreased progressively with time were criticised by TROWELL (1942). He described a method of rat liver perfusion with Ringer's solution at a constant flow rate maintained by a pump. This was the origin of the pumping in the circuitry of liver perfusion. Although radioisotopes were characterized and proposed for use in animal experimentation since 1933 by NIELSON, they were not applied to the study of liver perfusion techniques until 1951 when BRAUER, PESSOTTI and PIZZOLATO (1951) first used them to study bile secretion by the liver of the rat. It was the introduction of plastic tubing, improvement in anaesthesia, and monitoring techniques by the forties that helped in the firm establishment of perfusion techniques. This ushered in the technique of BRAUER et al (1949, 1951, 1953a, 1953b) which has come to be recognised by many as standard for isolated liver perfusions. About the same time, MILLER & BLY (1949) began to develop their apparatus and technique for perfusion of isolated rat liver with heparinised rat bolld. MILLER, WATSON and BALE (1951) succeeded, for the first time, in elucidating the dominant role of the liver in the biosynthesis of plasma proteins.

It is worthy of note that efforts were made by MILLER and his co-workers to popularise the technique of isolated liver perfusion (MILLER, BURKE & HAFT 1955; MILLER et al 1956; MILLER 1961), but it was not widely accepted by Biochemists or Physiologists until the early sixties when its effectiveness was demonstrated by recognised authorities such as KREBS (HEMS, ROSS, BERRY & KREBS 1966) whose type of perfusion medium was used in this study - with slight modifications as stipulated in Chapter 3.

2.2 The Choice of Animal Species

2.2.1 Introduction

The isolated perfused liver of rat has long been established (section 2.1). Whilst the perfusion of isolated organs has been an established scientific method of studying visceral physiology since the classical studies of Henry Dale in the early 1930s (BAUER, DALE, POULSSON and RICHARDS 1932), little effort has been made up to date to apply these techniques to the examination of problems more relevant to clinical work.

Physiological investigations are not usually confined to one species of animals. To this end, the choice of animal species for experiment is a matter of concern to the investigator. Liver perfusion studies have been carried out on the dog (BERNARD 1855; SIMMONS & BRANDES 1929; GORESKY 1965, 1967; GORESKY, NADEAU & BACH 1973; KESTENS & LAMBOTTE 1973; SERROU et al 1973); rabbit (ASP 1873; ZIMMERMAN et al 1973); pig (EISEMAN et al 1961; HARDCASTLE & RITCHIE 1968; TAUBER et al 1971; WEBER et al 1973); and indeed a small mammal, the rat (BRAUER et al 1951; MILLER et al 1951; MORTIMORE 1961; HEMS et al 1966; WILLIAMS, EXTON, PARK & REGEN 1968; BLOXAM 1971, 1973). Of all these the rat was chosen for these experiments. But the perfusion of the isolated rat liver has both advantages and its limitations. The choice of the rat in these experiments was therefore decided upon by the tilting of the balance in favour of the advantages.

2.2.2 Advantages of using the rat

There are many advantages of using the rat in these experiments. Firstly, one can substantially reduce the variability of behaviour among the individual animals since closely in-bred colonies can be readily obtained. Secondly, the use of rats is clearly less expensive than that of larger animals. Becuase the rat is small, the volume of perfusion medium required is also small, and this makes the use of expensive reagents such as isotopically labelled compounds more economical. Thirdly the operative procedure in setting up a rat liver preparation takes much shorter time than that of larger animals. Thus, if the preparation has been for some reason unsatisfactory it is very practicable to set up another one. Furthermore, the liver in the rat is divided for the most part into entirely discrete lobes, and thus excision of tissue during the course of an experiment does not give rise to problems of bleeding. Also, one lobe can be clamped and the rest perfused. And, finally, a very much larger amount of biochemical and physiological information is available for rat liver than for the livers of larger animals. Thus it is easier to make useful comparisons between results from isolated liver perfusion experiments and those from studies of the liver in intact animals, or of liver slices, disaggregated hepatocytes, homogenates and subcellular fractions.

2.2.3 Limitations of the rat for liver perfusion

But the use of the rat for liver perfusion studies is not without its own limitations. Firstly, when compared with larger animals, it is found that studies on bile are much more practicable in larger animals than in the rat due to volume considerations. Also studies on hepatic lymph are easier in larger animals, but can be carried out in the rat (BOLLMAN et al 1948). But these types of studies were never intended in this work, so these could hardly be regarded as limitations for this work. Secondly, it is difficult to take a large number of liver biopsies in the rat. But this is not a strong reason since it is unnecessary, except in exceptional cases, to take more than a few biopsies in one rat liver. In the case of this study it is not even applicable. Thirdly, it is very difficult to perfuse rat liver through the hepatic artery as well as the portal vein. But such a need does not arise in this type of study and circumstances under which this facility might be important have not been elucidated yet. Furthermore, studies of perfused livers relevant to hepatic support and transplantation in human liver disease are clearly better done in larger animals than in rat. Finally, the larger animals can provide their own perfusate, whereas many rats have to be sacrificed to provide even the priming volume of the perfusate required. This seems to be the most cogent of all the apparent limitations in the use of rat. But in the present circumstances of the experiments carried out in this study, where bovine blood (washed bovine erythrocytes) was used, even this important serious limitation does not apply.

2.3 The Role of Organ Perfusion in Research

Organs have been isolated and perfused for different purposes. Almost all branches of medicine have their application of perfusion of an organ. Organs are perfused sometimes in order to preserve them for subsequent transplantation; sometimes for therapeutic purposes as when cytotoxic drugs are circulated or in the treatment of liver failure; but basically to increase our knowledge of their physiology, biochemistry and pharmacology. It is for the purposes of increasing our knowledge of physiology regarding the handling of the sugars by the liver that these studies were undertaken.

Organ perfusion studies offer a half-way house between studies of the whole animal and in vitro experiments. In vitro experiments such as liver slices preparation and isolated liver cell preparation suffer the disadvantage of mechanical and chemical trauma during slicing or disaggregating of the liver cells (BAUR & HELDT 1977). Moreover these procedures are less convenient than the whole organ preparation. But it is not from such conveniences that the chief scientific value of organ perfusion studies arises. Rather it is from the precision and specificity of the responses which can be observed. For example, when gastrin is given to an intact animal it can be shown that the bile flow is markedly increased, but few conclusions can be drawn about the mechanism of this response from such a study. Gastrin stimulates acid secretion - acid in the duodenum promotes secretin release - secretin is a potent stimulant of bile flow. Hence it is difficult to tell in the intact animal whether this is the mechanism which is operating or whether, for example, gastrin itself is acting directly on the liver without secretin as the intermediary. Only in the isolated perfused liver can it be demonstrated that gastrin induces increased bile flow.

Another major advantage of the perfused organ preparation is that its blood flow, gaseous exchange and temperature can be controlled by

the experimenter. This enabled the quantitative studies of the effects of changes in perfusate flow rate and gaseous supply (Chapter 5) and changes of temperature (Chapter 8) to be made in these studies.

Besides these clear cut advantages of isolated organ perfusion in physiological investigations, it also has some advantages in pharmacological investigations, The isolated perfused liver has been widely used for studies concerning drug metabolism, and in general, for a number of investigations on transformation of a given chemical in other compounds, usually called, at least in the slang of biochemical pharmacologists, metabolites. GARATTINI, GUAITANI and BARTOSEK (1973) gave the following reasons why the perfusion of isolated liver is a useful technique for this type of study:-

1. The complex metabolism of drugs occurs in various tissues, but the liver should be considered the most important site. It is therefore necessary to establish what the role of the liver is without the interference of other organs that contribute to the general metabolism of the drug.
2. Many different metabolic processes that take place in drug transformation involve the action of mixed oxygenases, reductases, various types of conjugation, acetylation etc. Whereas various in vitro preparations (e.g. tissue homogenates, slices, microsomes) may be useful for the observation of single steps, the isolated liver preserving integral cell structure permits following the overall metabolism of the drug.
3. The metabolites are usually transformed in vivo in water soluble compounds that can be easily excreted. In fact, the metabolites found in higher quantities in urine represent the "terminal" step. Blood is a difficult place to look for metabolites, normally present in very low concentrations. Higher blood levels of drugs may represent a limitation because of their side pharmaco-dynamic and

toxic effects in vivo. These difficulties can be overcome using isolated perfused liver. The concentrations of the added drug may be higher, and therefore the chance of detecting many metabolites increases, particularly in the cases of products formed only in small quantities or as intermediary metabolites. KVETINA, SIMKOVA, CITTA and DEMI (1973) studied the influence of pathological processes on the pharmacological activity and metabolism of drugs, namely the influence of digitoxin intoxication on the activity, excretion and biotransformation of hexobarbital in isolated perfused liver. They also studied the influence of acute post-irradiation disease on the activities, levels and biotransformation of amidopyrine and pethidine in isolated perfused liver. They concluded (KVETINA et al 1973) that the study of pharmacokinetics using the isolated perfused liver did not only permit the application of more exact simulation of human therapy in animal experimentation, but also could lead to the modification of the dosage scheme, for example in the course of a pathological process. With these obvious advantages, it is no wonder then that many investigators have used isolated organ perfusion studies, particularly the liver, to increase our knowledge of the physiology, pathophysiology, biochemistry and other branches of medical science. Isolated liver perfusion studies have revealed the bile secretory function of the liver (BRAUER, PESSOTTI and PIZZOLATO 1951), protein metabolism in the liver (GREEN & MILLER 1960; MILLER et al 1965), synthesis of prothrombin and fibrinogen and some clotting factors (factors V, VII, OX, X) by MILLER et al (1951, 1964) and MILLER and JOHN (1970). Since the publication of the study on gluconeogenesis in the perfused rat liver by KREBS and his co-workers (HEMS et al 1966) biochemical studies using the perfused liver have been undertaken more frequently than ever before. Glucose and urea synthesis by the liver (HEMS et al 1966), protein metabolism in the liver

(MILLER et al 1951) and plasma protein synthesis (JOHN & MILLER 1969) are among the more important biochemical processes elucidated by liver perfusion techniques.

Clinically, the perfusion of isolated organs has been a well established therapeutic procedure. Liver perfusion has been used in the therapy of acute hepatic failure and hepatic coma (CODON et al 1970), or merely as a hepatic support (ABOUNA et al 1970). Whole limb perfusion with cytotoxic drugs has been used to treat malignant melanoma. Haemoperfusion has been used in the treatment of acute drug intoxication (ROSENBAUM 1972). And chronic renal failure has been successfully managed by isolated jejunal loop perfusion (PARISI 1969). Perfusion studies have been employed to find the state of the liver in shock where the effects of anoxia, hypothermia and injection of ammonia were elucidated (HARDCASTLE & RITCHIE 1968; RITCHIE & HARDCASTLE 1973). Various methods of perfusion studies have been extensively studied for use in organ transplantation (ZIMMERMANN et al 1971, 1973). At the moment the most important surgical application of the knowledge in organ perfusion is directed towards organ transplantation (ABOUNA 1968). Placental perfusion studies have been used to study drug transfer across the placenta (GINSBURG 1971). The clinical importance of perfusion of placenta in obstetrics was recently reiterated by SHELLEY (1979).

PART B

GENERAL METHODOLOGY

CHAPTER THREE

3. GENERAL METHODOLOGY

3.1 Introduction

Numerous methods for the preparation of isolated rat liver perfusion have been studied. Most methods in current use were adapted from either that of BRAUER et al (1951) or that of MILLER et al (1951). Liver perfusion is by far the commonest of all the organs perfused as surveyed for 1968 and 1969 by ROSS (1972). Most techniques described in literature differ only in details, but are fairly similar in generality. The method used in this study is a modification of that of HEMS et al (1966) which itself is essentially derived from the MILLER et al (1951) technique. But the operative procedure for in situ isolated perfusion of MORTIMORE (1961) was adopted in this study instead of complete isolation of the liver by removal from the rat carcass.

The MILLER technique consists of perfusing under gravity feed through portal vein of an isolated liver in a petri dish using diluted heparinized rat blood with additions of antibiotics and amino acids. The apparatus was enclosed in a heated cabinet at 38-40^{o}C. The Baruer technique consists of perfusing the liver in isolated state also, but using complex medium including rat blood (heparinized), bovine plasma albumin, salts, antibiotics and glucose. This perfusion method was by a mixture of gravity feed and pulsatile pressure. The apparatus was also enclosed in a heated cabinet. Worthy of mention are modifications of these methods by SCHIMASSEK (1962). SCHIMASSEK described the use of a semi-synthetic medium (tyrode solution) to which were added washed bovine erythrocytes and bovine semin albumin. Also MORTIMORE (1961) described the method in which the surgical operation was simplified by leaving the liver in situ, but isolated from the rest of the organs in the body of the rat by cannulation of the portal vein (inflow) and inferior vena cava (outflow). This operative procedure was adopted by HEMS et al

(1966), EXTON and PARK (1967), WILLIAMS et al (1968). It is also used in the present study.

However, TROWELL (1942) described a method of reverse perfusion. In reverse perfusion the inflow cannula is placed in the inferior vena cava and outflow in the portal vein. The liver is then perfused in that direction (opposite to the direction of blood flow in life). TROWELL used this method in an attempt to circumvent the vasoconstrictor effects that impeded perfusion flow rate at that time. But the "benefits" of reverse perfusion have been largely superseded by advances in the technique of forward perfusion. Not many workers have used this method. And no objective comparison using metabolic parameters seem to have been made by any one.

A further modification was that of perfusion through the hepatic artery. It is estimated that hepatic artery carriers only about 20% of the blood supply to the liver, and oxygenation is adequate via the portal vein supply. Metabolic experiments have confirmed that the liver functions normally without perfusion of the hepatic artery. There are two classical methods describing hepatic arterial perfusion; they are those of ROSS et al (1967), and POWIS (1970). The Ross method is perfusion under hydrostatic pressure by raising the oxygenator to a height of 120cm above the liver to provide the necessary pressure to perfuse the hepatic artery, and a second lower oxygenator (15-20cm above the liver) supplies the portal vein. The POWIS technique is that of pulsatile perfusion by direct pumping into both portal vein and hepatic artery, with dialysed whole rat blood. It is not yet evident what advantage acrues from using the arterial perfusion since oxygen demand of the liver can be met via the portal route. The main disadvantages of using arterial perfusion are that the apparatus is complex and it is more difficult to cannulate the hepatic artery than portal vein.

3.2 The perfusion apparatus

A closed circuit system was used in this perfusion technique. The circuit diagram of the set up is shown in fig. 3.1. The perfusion medium (perfusate) was contained in a conical flask (lower reservoir) with a rubber bung. The rubber bung has three holes - one for the outflow tube, one for inflow tube and a third for the overflow tube. The inflow tube was connected to a roller pump (WATSON MARLOW). This pumped the perfusate to the top of the oxygenator (a multibulb glass chamber) the lower segment of which acts as the upper reservoir. The perfusate filmed down the oxygenator and flowed out through two outlets - the outflow and the overflow tubes - from both of which tubes it passed into the lower reservoir and thereby completing the circuit. When the rat liver was in circuit, the outflow tube supplied perfusate to the liver via the portal vein cannula and effluent from the liver via the inferior vena caval cannula flow into the lower reservoir to complete the closed circuit or into sample cups in an open circuit which occurs during an experimental run.

The multibulb glass oxygenator is similar to that of MILLER et al (1951). It has the following features similar to Miller's oxygenator:-

a) inlet for perfusate, on top,

b) gaseous escape outlet also on top,

c) one conical bulb near the perfusate inlet to aid filming down of perfusate,

d) four cylindrical bulbs,

e) reservoir at the bottom of the bulbs (upper reservoir),

f) overflow outlet,

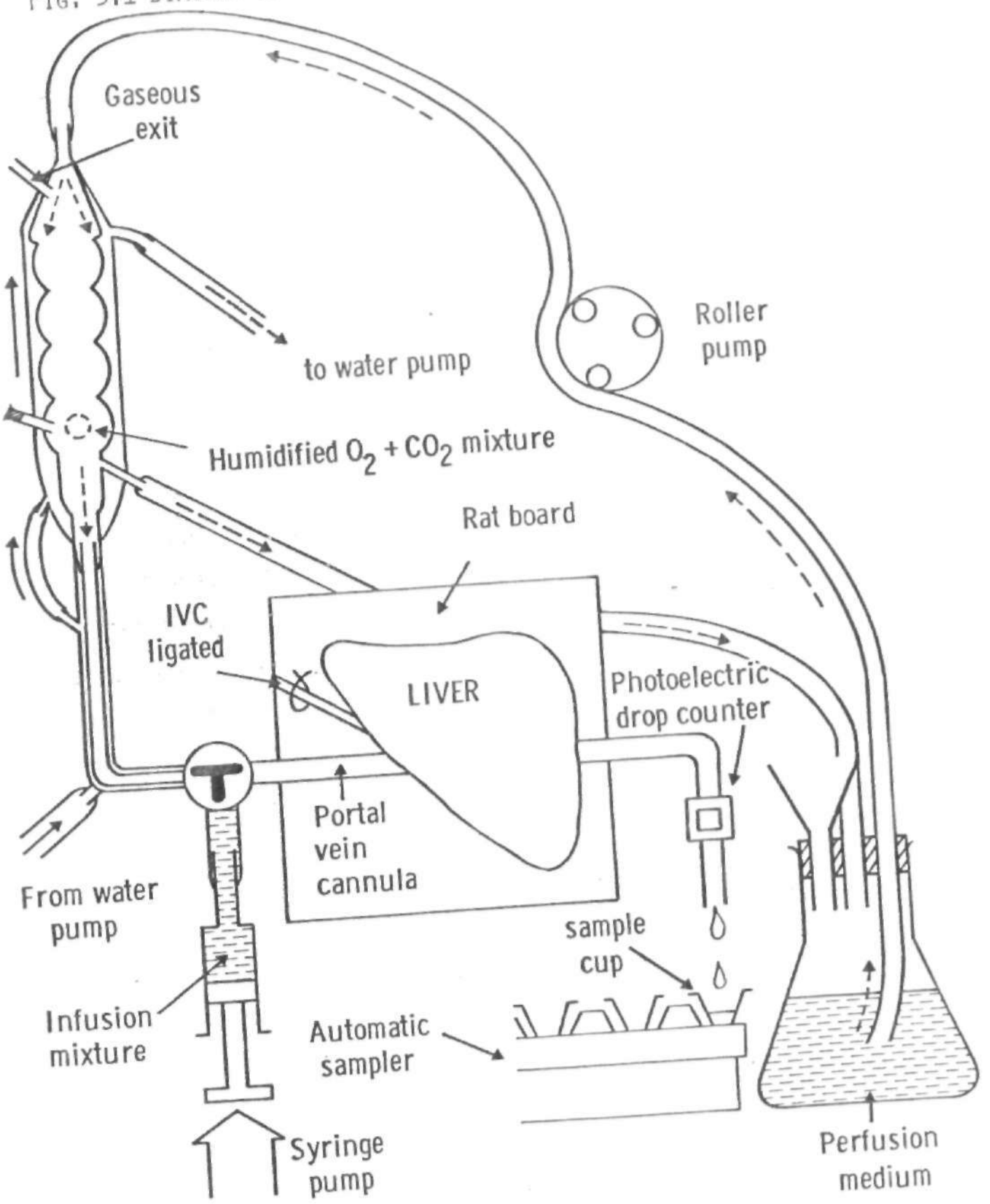
g) inlet for O_2:CO_2 mixture,

h) spout for sampling of perfusate when necessary. This was used for automatic pH control (auto-burette and autotitrator connection) by

LEGEND TO FIG. 3.1

FIG. 3.1 IS THE DIAGRAMATIC REPRESENTATION OF THE PERFUSION APPARATUS. THERE ARE TWO CIRCUITS - THE PERFUSATE CIRCUIT AND THE HOT WATER CIRCUIT. THE PERFUSATE IS CIRCULATED (- - - - →) BY PUMPING WITH A ROLLER PUMP IN A DIRECTION OPPOSITE TO THE FLOW OF THE OUTER JACKET HOT WATER (——→) WHICH KEEPS THE PERFUSATE FLOW TO THE LIVER AT A CONSTANT TEMPERATURE.

FIG. 3.1 DIAGRAM OF THE PERFUSION APPARATUS



Miller,

i) Outlet for perfusate - this leads perfusate into the liver, or directly into the conical flask (lower reservoir) when the rat liver is not in circuit.

However, it differs from the Miller oxygenator by having an outer jacket for the circulation of prewarmed water to keep the temperature of the perfusate constant at 37°C before delivery to the liver. This modification was necessary because of the bench top set up used in this study as opposed to the thermostatically controlled cabinet used by MILLER et al (1951) and HEMS et al (1966). The outer jacket for the warmed water had an inlet at the bottom (fig. 3.1) from a thermostatically controlled water heater/water pump (CHURCHILL), and an outlet on top. This warm water jacket was recirculated in a closed circuit by connecting the pump outflow tube to the inflow of the oxygenator, and the outflow from the oxygenator to the inflow of the pump. This preheated water jacket was made continuous in series with a similar plastic (PVC = polyvinylchloride) tubing which surrounded the cannula leading from the oxygenator outflow to the liver through a luer fitting from the Frankis Evans needle (1½ inches guage) which was inserted into the portal vein of the rat liver. By this means, the maintenance of constant liver temperature at 37°C was achieved.

The pumping of the tubes consists of three separate circuits. The three circuits are: the perfusate circulation in one direction, the hot water jacket circulation in the opposite direction and the third is the gaseous mixture tubing from the gas cylinder via the humidifier. The following connections of the tubes weredone (fig. 3.1):-

a) Perfusate inflow tube from the lower reservoir through the Watson Marlow roller pump to the upper part of the oxygenator,

b) perfusate overflow tube to the lower reservoir,

c) perfusate outflow tube from the oxygenator to the lower reservoir

(via the liver when the liver is in circuit), or to the sample cups during sampling procedure,

d) inflow tube from the water pump to the lower part of the oxygenator,

e) outflow tube to the water pump from the upper part of the oxygenator,

f) inflow tube for 95%O_2:5%CO_2 mixture.

3.3 Ancilliary apparatus

In addition to the above perfusion apparatus, a number of ancilliary pieces of apparatus were required for the experiment.

One of these ancilliary pieces of apparatus is a constant infusion syringe pump (Sage Model 351). All test substances were delivered to the liver with this syringe pump. The approximate rate of infusion and size of syringe used were set on the front panel dials. But the exact rate of infusion was calibrated for each setting used for the experiment.

Another of these is the automatic sampler. The effluent samples were collected from the inferior vena caval cannula at 1.5 second intervals using an automatic sample changer (Hook and Tucker Model A40), carrying the required number of auto-analyser cups - 24 cups in each experimental run. The sample changer was calibrated and adjusted until it rotated at a speed to change samples at 1.5 second intervals.

Also the temperature of the liver was continuously monitored. The temperature was monitored using a twelve channel thermistor with one probe and one disc ends. The disc end was inserted between the liver lobes and the temperature of the liver was observed in two ways - a visual display on the thermometer and a paper recorder in one of the recording channels of Grass Instrument recorder (Model 79D). Another recording channel of this recorder was used to record the flow rate connected from the photoelectric drop counter. The recorder was calibrated to give the flow rate measured with the drop counter.

3.4 The Perfusion Medium

3.4.1 The composition of the perfusion medium

The perfusion medium (perfusate) used for this study was a semi-synthetic medium made up of 180 mls Krebs bicarbonate buffer to which were added 40 mls of washed bovine erythrocytes and 80 mls of dialysed 10% bovine albumin fraction V (Sigma).

The Krebs solution was made up in the following concentrations:-

Salt	Conc. mM	Conc. $g.Litre^{-1}$
NaCl	119	6.90
KCl	4.7	0.35
Mg $SO_4.7H_2O$	1.2	0.29
Na $H_2PO_4.2H_2O$	0.9	0.14
Na HCO_3	25	2.10
Glucose	5.50	1.00
$CaCl_2$	2.5	0.28

Distilled water was added to make up one litre.

For each liver a total of 300 ml perfusate was thus made up.

3.4.2 Preparation of the perfusion medium

3.4.2.1 Collection and washing of bovine erythrocytes

A one litre wide-mouthed plastic bottle was washed thoroughly and rinsed twice with distilled water and then once with Normal Saline (0.9% NaCl). Into this bottle was then put 50 mls of normal saline to which had been added 10,000 i.u. heparin (Pularin-Evans medical). The bottle was then taken to the abattoir early in the morning. With the help of the slaughterhouse attendant a midstream specimen of blood (MSB) was collected into the bottle from the gush of blood coming out of the cut throat of the ox. The midstream blood was collected by allowing the first gush of blood to flow out before the bottle was held in front

of the cut throat to obtain the blood specimen. This precaution was taken to ensure that as far as possible a sterile blood specimen was obtained with the minimum of contamination. The blood was then mixed thoroughly with the heparinised saline to prevent clotting, and transported to the laboratory and kept in the refrigerator until the Krebs buffer was prepared. The freshly prepared Krebs buffer was used for washing the blood in the following way:-

The blood was filtered through glass wool into 200ml centrifuge bottles. This filtration was to help remove any possible micro-clots that might have formed. Four centrifuge bottles with screw caps were used and each pair was weighed on an MSE pan balance (MSE balance Model High Speed 18) so that the centrifuge arms were balanced in pairs when the centrifuge bottles were placed in the centrifuge buckets. The blood was centrifuged for ten minutes at 5,000 rpm at $4^{o}C$ using MSE refrigerated compressor centrifuge (MSE High Speed Model 18). The plasma and the buffy coat was sucked out. The Krebs ringer solution was added three volumes to one volume of packed cells and mixed thoroughly by tilting the screw capped bottle for a few times. The bottles were then centrifuged after weighing and balancing as above. This was the first wash. The washing was done two more times (three washings) and the packed cells stored in the refrigerator for use, when required.

3.4.2.2 Preparation of Krebs bicarbonate buffer solution

The salts were weighed out in concentrations stated in 3.4.1 above, and dissolved in the order arranged in that section. Either one or five litre volume was freshly prepared before use according to the day's requirements. No stock solutions were made because of possible growth of fungi, for example, which might block the microcirculation in the liver. The freshly prepared solution was then gased with $95\%O_2$:$5\%CO_2$ mixture for one hour to keep the pH of the solution at about 7.4 before use.

3.4.2.3 Preparation and Dialysis of Albumin

A 24 inches length of visking dialysis tubing size 2 (medium) was cut and boiled in distilled water for thirty minutes to restore its normal consistency and porosity before dialysis. Then 8.0g serum albumin fraction V (sigma) were weighed and dissolved in 80 ml of distilled water by slow stirring using a magnetic stirrer. The boiled visking tubing was cooled and the dissolved albumin poured into it after sealing one end by a double tie. The second end was then also tied and the albumin in the tube was put in a beaker of 2 litres freshly prepared Krebs solution bicarbonate buffer, and left to dialyse for 24 hours. The Krebs ringer was changed for a fresh one and dialysed for another 24 hours - so that the albumin was used for preparing the perfusate after 48 hours of dialysis. This dialysis was necessary to repurify the albumin by dialysing out any free-fatty acids left and also to make the acidic alubmin solution come to about the pH of the gased Krebs solution - usually pH 7.4.

3.4.2.4 The mixture

The perfusion medium (perfusate) was made up by mixing the following:-

a) 40 mls washed bovine erythrocytes

b) 80 mls dialysed albumin, and

c) 180 mls freshly prepared gased Krebs bicarbonate buffer, in that order.

This mixture was then stirred thoroughly using a magnetic stirrer (with a small "flea") at low setting for a slow speed of stirring in order to reduce frothing of the perfusate. The haematocrit (packed cell volume - PCV) of the perfusate was determined using the Hawksley Haematocrit centrifuge. Then the pH was measured with Corning EEL pH meter and pH adjusted to 7.40, if need be. The haemoglobin

concentration was determined by a spectrophotometer (Model SP5000), when required.

3.5 Surgical operation of the Rat for liver perfusion

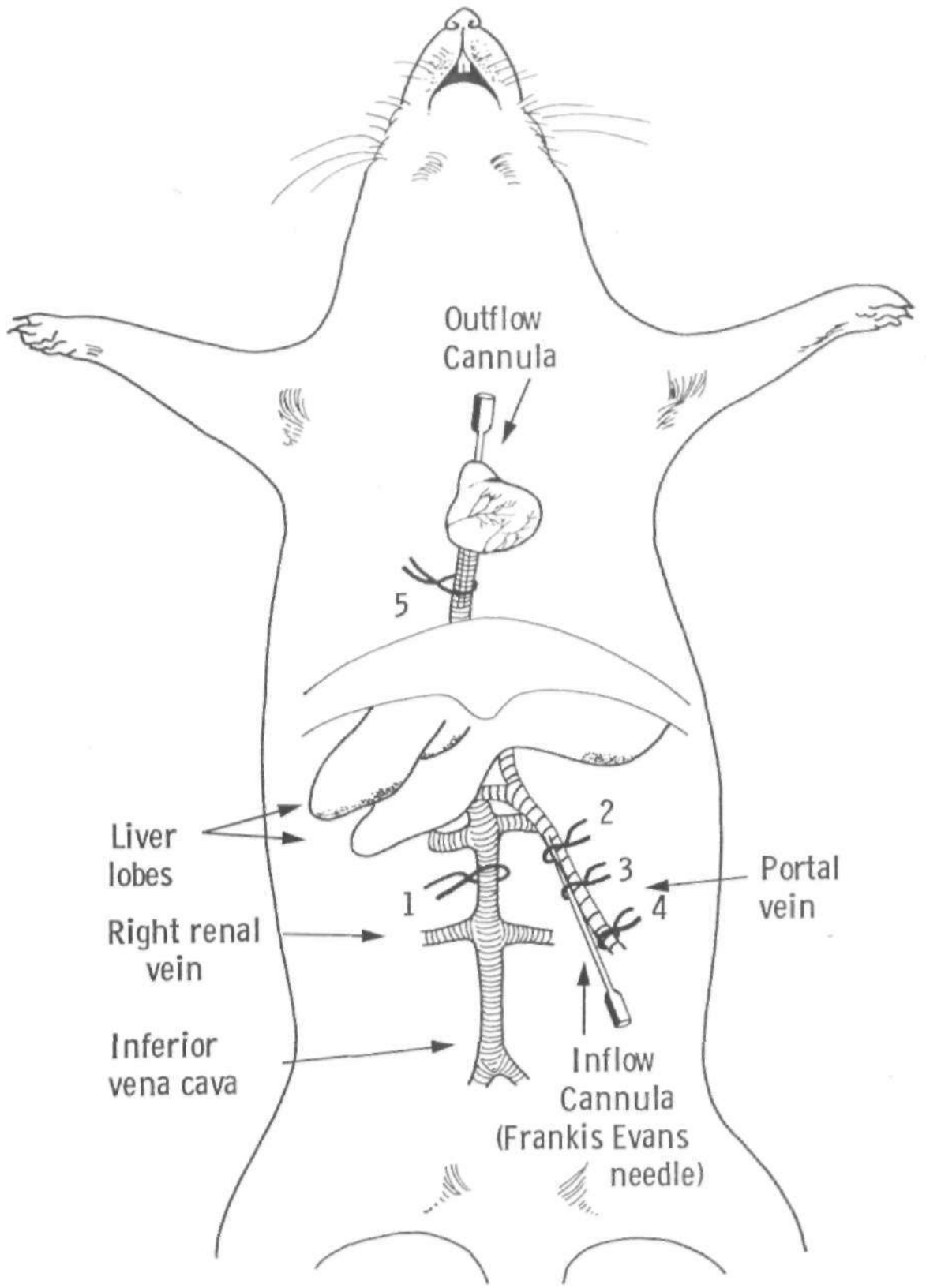
3.5.1 The technique

The rat was operated for the perfusion of its liver in situ after the method of MORTIMORE (1961), as carried out by HEMS et al (1966).

3.5.2 Surgical operation

Albino rats, well fed, both sexes, weighing between 300g and 400g body weight were anaesthetized with sodium pentobarbital (Sagatal - May & Baker) at 4.8 mg.100g^{-1} body weight by intraperitoneal route. Then 100 i.u. (0.1 ml) heparin (Pularin - Evans Medical) was injected into the saphenous vein. The abdomen was opened through a midline incision, and mid-transverse incision to right and left of the midline were made avoiding the larger vessels. Bleeding was minimized by clamping the major vessels of the abdominal wall with four artery forceps. The intestines were then placed to the animal's left side, on tissue paper wetted with saline so that the liver, portal vein, right kidney, inferior vena cava and the bile duct were exposed. The connective tissue was dissected out and a loose ligature of silk thread (ligature 1) was placed around the inferior vena cava above the right renal vein (fig. 3.2). Then two ligatures (ligatures 2 and 3) were passed round the portal vein at intervals of one to two inches below the point where the vein divides to enter the separate lobes of the liver and a third ligature (ligature 4) was placed around the vein at a point distal to the liver (fig. 3.2). Then a bull-dog artery clip was placed between ligature 2 and the liver in order to allow blood from the viscera to flow into the portal vein to distend it ready for easy cannulation. The ligature (ligrature 4) was tied, shutting off the blood supply from the viscera to the portal vein, before the insertion of the needle into the vein. The portal vein was cannulated with a No. 16 Frankis Evans needle - a double

Fig. 3.2 DIAGRAM OF SURGICAL OPERATION FOR IN SITU LIVER PERFUSION



cannula with a trocar which has a sharp end for puncturing the vein in the process of cannulation. This trocar was withdrawn with minimal loss of blood. The two ligatures (3 and 4) were tied securing the cannula in place.

Then the thorax was opened by a transverse incision just above and along the line of the insertion of the diaphragm and by two longitudinal incisions towards the head from the two ends of the transverse incision. The chest was then cut off to allow room for easy manouvre of the effluent cannula. A loose ligature was placed round the inferior vena cava close to the heart (ligature 5). The cannula for insertion into the inferior vena cava (I.V.C.) was made up of two parts - the caval component made up of polyvinylchlorice (P.V.C.) tubing of internal diameter of (1.5 mm) and external diameter of (2.5 mm) and the second part which connects to the effluent tubing of external diameter of (3.5 mm). The inserting end of this cannula was cut with a bevelled edge to provide a sharp tip. This sharp end was used to puncture the right atrium from where the I.V.C. was cannulated more easily. This cannula was then tied in position. At this stage the loose ligature around the abdominal vena cava (ligature 1) was tied to prevent reflux of the perfusate into the abdominal I.V.C. during perfusion. The rat operating table (rat board fig. 3.1) was lifted with the rat and liver in situ and the liver (in situ) was connected to the circuit of the perfusion apparatus and circulation started. The first five or six millilitres of venous blood (mainly rat blood) were discarded before the circuit was switched to a closed one.

The whole operation normally took seven to twelve minutes. And the period of partial ischaemia - when the portal vein was tied and circulation was maintained by only hepatic arterial supply - was two to three minutes. There was a period of absolute ischaemia (usually one minute) between the cessation of breathing (caused by opening the thorax) and the

start of perfusion.

3.6 Preparation of samples for Analysis

3.6.1 Infusion of test substances and collection of effluent venous samples

The substrate (hexose) whose uptake into the liver was being studied in any particular experiment was isotopically labelled, and mixed with a second substance which was also labelled. This infusion mixture contained [^{3}H]-hexose as test material and [^{14}C]-substance as reference material in most cases, and [^{14}C]-test substance mixed with [^{3}H]-reference substance in a few other cases. The isotopic substances used were 10uCi of [^{14}C]-labelled and 20 uCi of [^{3}H]-labelled materials for experiment of up to 10 runs. This means that for each experimental run, 1uCi of [^{14}C]-labelled, and 2 uCi of [^{3}H]-labelled substances were infused for the usual 24 samples collected. The [^{14}C]-labelled isotopic quantity was usually less because of the greater efficiency of counting of [^{14}C]-substance than [^{3}H]- substance in their respective counting channels.

Before the infusion of the mixture, 1uCi of [^{14}C]-substance (0.1 ml) and 2uCi [^{3}H]-substance (also 0.1 ml) were taken in a 10 ml syringe into which 1 ml of the potential competitor was added and mixed. The syringe was then connected with the perfusion circuit through a 3-way tap and clamped down on the sage infusion pump (Model 351). The sage pump was set to deliver 20 mls. min^{-1} using a 10 ml syringe. But this setting could not deliver exactly 20 ml. min^{-1}, so it was calibrated before use to know the exact quantity infused per minute. This calibration was done in conjunction with the flow rate calibration for the pen recorders on the Grass multi-channel recorder. Immediately before the infusion was started, the 3-way tap was switched to connect the syringe to the perfusion circuit on the oxygenator site (upper reservoir), and the perfusate was rapidly withdrawn into the syringe to reach the 10 ml mark. This rapid withdrawal of perfusate from the circuit into the

syringe caused turbulence which helped to mix the perfusate, isotopic mixture, and potential competitor before the infusion. The 3-way tap was then switched to connect the syringe in line with the inflow cannula to the liver. The infusion pump was then started and infusion commenced after collecting four (blank) samples, and continued until about 0.5 ml of the mixture in the syringe remained. The infusion was stopped and sampling, using automatic sample changer (Hook and Tucker sampler model A40), was continued until all the 24 samples were collected.
The remaining isotopic mixture in the syringe was sampled into infusate auto-analyser cup for processing of the isotopic mixture (ratio) in the infusion mixture (infusate).

Usually eight to ten runs were done in a single good liver preparation using different potential competitors to find their effects on the uptake of the isotopic substrate being studied.

3.6.2 Processing of samples for B-scintillation counting

For processing of samples, 12 ml centrifuge tubes were arranged in a test tube rack and numbered (1-24) according to the numbering on the auto-analyser cup holder. Then 0.5 ml of 8% perchloric acid W/V was pipetted using constant volume 500 ul oxford pipette. Then 0.2 ml of the samples was sucked and washed into the perchloric acid with 0.8 ml normal saline (0.15 M NaCl) using a variable diluter (Model H/T) which had previously been calibrated and set to sample and deliver these required volumes. The samples were then deproteinized by precipitating the proteins with perchloric acid. This was thoroughly mixed using a whirlimixer (Fissons Instrument Co. England). When the samples were so deproteinized, they were centrifuged, using bench centrifuge at 2,000 xg rpm for ten minutes. After centrifugation, 0.5 ml of the clear supernatant (which now contains the isotopically labelled materials) was pipetted using the 500 ul oxford pipette into 9.5 ml of scintillation

cocktail contained in a 20 ml scintillation vial with screw caps.

3.6.3 Preparation of liquid scintillation cocktail

The liquid scintillation cocktail consists of one volume of Triton X-100 plus two volumes of Xylene in which 4 $g.L^{-1}$ PPO (diphenyloxazide) were dissolved. The PPO was the active scintillation chemical which was hydrophobic and therefore insoluble in equeous media. It was therefore dissolved in an organic solvent. Although it is soluble in many other organic solvents such as chloroform, carbon tetrachloride, ether and benzene, xylene was chosen in this study because it has a lower quenching effect on the B-particles than the other organic solvents mentioned above. And where little quenching effect was desired as in this case, the solvent with the lowest quenching effect was chosen (xylene). But toluene also has low quenching effect. However, Xylene was preferred to toluene because it is less toxic. Xylene with a variable boiling point (137.2-145^{o}C) depending on the grade has less fire risks than toluene (boiling point 110.7^{o}C). While Xylene is toxic only by ingestion and inhalation, toluene is toxic by ingestion, inhalation and skin absorption. Above all toluene has explosive limits in the air of 1.27 to 7% and using it to propare the scintillation mixture in a laboratory common to other users can be a real hazard.

Triton X-100 is a detergent which acts as a vehicle for the suspension of the PPO dissolved in an organic solvent, which would otherwise be immiscible with the supernatant of the samples after precipitation and any other aqueous samples needed for the test in an experiment.

The composition of the scintillant was:-

a) 8 grams PPO (diphenyloxazide)-Koch light lab - the Scintillation chemical,

b) 1.34 Litres of Xylene (Koch Light Lab.) an organic solvent,

c) 660 ml Triton X-100 - a detergent.

The cocktail was made by first spinning down the Triton X-100 at 5,000xg rpm for three hours using the refrigerated centrifuge with 200 mls screw capped bottles (Model MSE 18) at 4°C. At the end of centrifugation the triton solidified and was allowed to thaw at room temperature. The 8g PPO were weighed and dissolved in 1.34 L Xylene using a magnetic stirrer to mix it thoroughly. To this was added the triton (660 ml) with continuous stirring to mix. The resultant final mixture is the liquid scintillation cocktail (scintillant). This was later replaced by fisons "fiso-flour" a commercial preparation which gives similar performance.

LEGEND TO FIG. 3.3

FIG. 3.3 DIAGRAMATIC REPRESENTATION OF HOW ISOTOPIC MIXTURE CAN BE USED TO CALCULATE HEPATIC EXTRACTION.

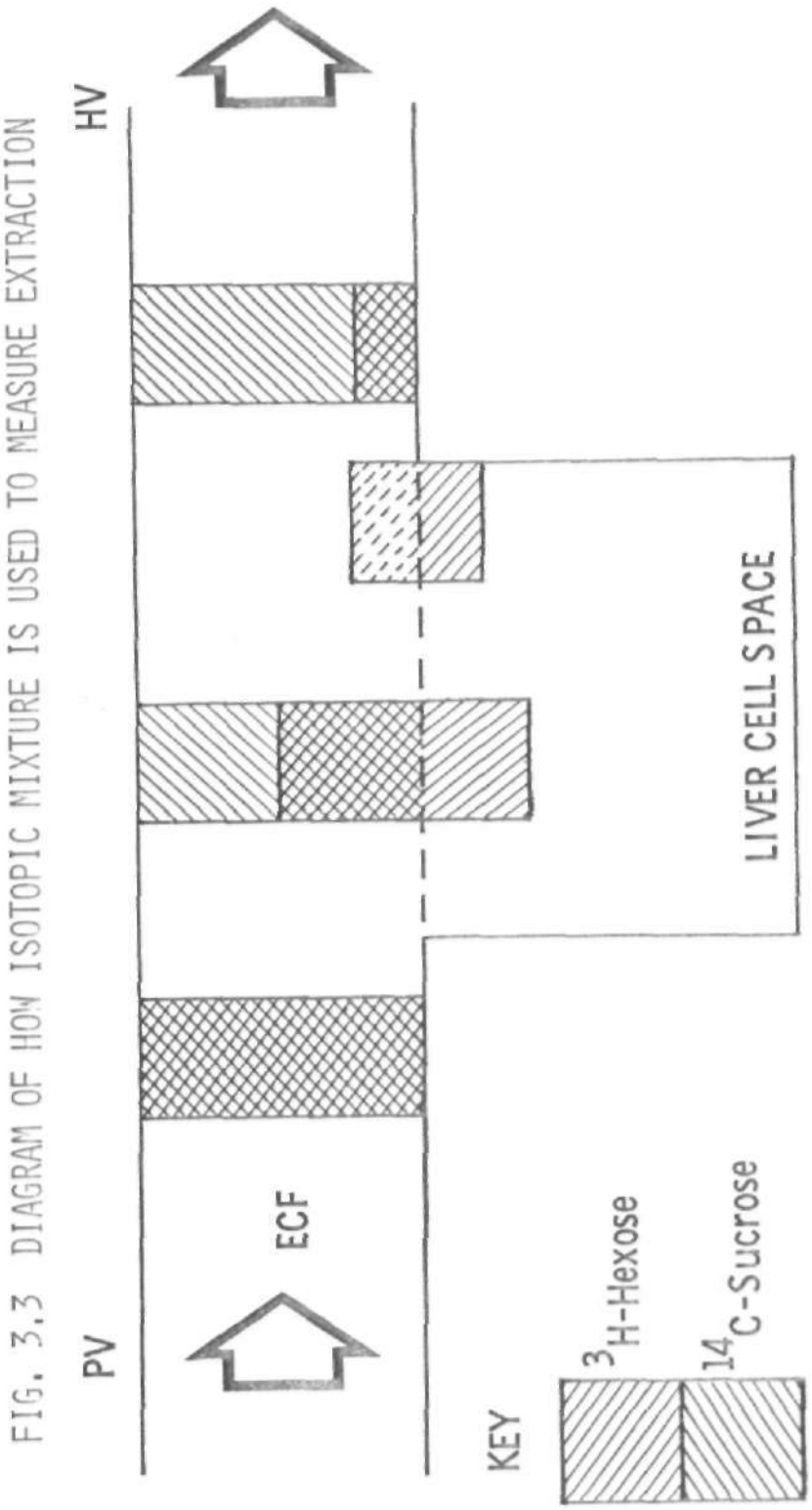


FIG. 3.3 DIAGRAM OF HOW ISOTOPIC MIXTURE IS USED TO MEASURE EXTRACTION

3.7 Counting of Radioisotopes in the samples

3.7.1 The Theory of double indicator dilution technique

The theory of the indicator-dilution method for measuring flow and volume was studied in different organs (MEIER & ZIERLER 1954). But the method of double indicator dilution technique as adopted in this study was after the method of CHINARD (1955). The theoretical basis of this technique was explained by ZIERLER (1962, 1965). Basically, the technique involves the use of two substances (indicators) which can be identified and measured independent of each other. In this way both can be measured simultaneously. In this present study two different isotopically labelled substances were used - usually one [^{3}H]-labelled and the other [^{14}C]-labelled. The efficient counting of the two isotopes in different channels of the liquid scintillation spectrometer confers the advantage of accuracy on this isotopic double indicator dilution technique. Fig. 3.3 illustrates how the isotopic mixture can be used to work out the extraction of one substrate by the liver. The rectangular box with cris-cross hatchings represents an aliquot of a mixture of [^{3}H]-hexose (right diagonal hatchings) as test material and [^{14}C]-sucrose (left diagonal hatchings) as extracellular reference material. When this mixture is infused into the liver via portal venous (PV) Cannula and it reaches the hepatic exchange area, some of the [^{3}H]-hexose may penetrate into the liver cell space thereby changing the ratio between the [^{3}H]-hexose and [^{14}C]-sucrose in this mixture compared with the ratio of these two isotopes in the original isotopic mixture because the [^{14}C]-sucrose does not penetrate the liver cell and is confined almost entirely to the extracellular fluid compartment (ECF). However, some of the [^{3}H]-hexose that penetrated the cell space might efflux back into the ECF thereby lowering the [^{3}H]-hexose component of this effluent ratio with consequent picture of a lower liver uptake of the hexose when this happens. Hence the percentage extraction, as will be seen in the

case of D-glucose extraction, shows a decline with time.

This double indicator dilution method has been used in the study of blood-brain transfer of glucose (YUDILEVICH & DeROSE 1971), to study liver extraction and distribution of many molecules (BRAVO & YUDILEVICH 1971), and to study first-passage extraction in the gastric wall, liver and skeletal muscle in anaesthetized dogs (YUDILEVICH, RENKIN, ALVAREZ & BRAVO 1968).

3.7.2 Loading the samples and B-counting

The prepared samples in the scintillation cocktail were counted for their isotopic activity in the B-scintillation spectrophotometer (Packard Tricarb Model 3375) with computerized digital count display and teleprinter connected. The counts were punched on a computer paper tape as well as being printed out on the teleprinter paper.

The samples were loaded into the counter in this order:-

standards (made up of toluene, PPO and dimethy/POPOP)

infusates,

blanks, and

test vials in serial numbers.

The [^{14}C]-standards (Packard) were first loaded in the following order [^{14}C]= 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1, 0.0, and [^{3}H]-standards were then loaded [^{3}H]= 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1, 0.0. The decimal figures represent the degree of quenching. The counter was programmed to use these quenched standards of variable degrees of quenching and their external standard ratio (E.S.R.) to assert appropriate ESR, to each of the test vials to be counted subsequently and hence the effeciency of counting in each channel. These standards were followed by all the infusate vials loaded sequentially. The computer programme was written such that the computer expected all the infusates for all the experimental runs being counted to follow the standards. Then

the blank vials for each run came at the beginning of each run and were thus loaded in that order. For each run the test vials were then loaded after the blanks.

The B-scintillation counter was set such that one channel counted [^{3}H] and the other channel counted [^{14}C] predominantly, with slight overlap of one in another channel.

The channel which predominantly counted the tritium [^{3}H] emissions was set with an energy window of 50-350V and a 50% pulse amplification. The [^{14}C]-channel was set with an energy window of 50-1,000V and a 4% pulse amplification. The external standard activity source was a radium pellet. Radium activity is in an energy range greatly in excess of ^{14}C- or ^{3}H- activities. The activity caused by the radium source was counted over a set time period in two channels, the energy windows being set to exclude simultaneous detection of ^{14}C- or ^{3}H- emissions. The windows were of different widths so that the ratio of counts detected in the two channels provided a measure of the quench level.

3.7.3 Computation

Using the information provided by the standard series, calculations were done to convert the counts per minute (cpm) to actual activities which is stated as nuclear disintegrations per minute (dpm). This was the first stage of computation. This was done by calculating the ^{14}C- and ^{3}H- efficiencies in each channel where they were counted. Before the efficiencies were calculated the actual radioactivity in the Packard standards should be known at the time they were used for counting with the test samples - that is the actual nuclear disintegrations per minute (dpm) at that date. Thus ^{14}C- standards were stated to contain 104,000 dpm in January 1975 (by the manufacturers). But half life of ^{14}C ($t_{/2}\,^{14}C$) = 5,100 years.

In January 1978 (3 years later) the decay is negligible.

But the half life of 3H ($^{t}/_{2}{}^{3}H$) = 12.1 years.

In three years the decay will be substantial.

As stated by the manufacturers, the (^{3}H) tritium activity in these standards was 10^{6} dpm.

Using the formula of percentage isotope remaining after a set time

$$= \frac{100}{\text{antilog}\ \{(0.3010)[^{t}/_{h}]\}}$$

where t = time of decay (3 years in this case)

h = half life or time taken for radioactivity to decay to 50% of original activity

and Actual decay is $\log_{10} \frac{No}{Nt} = 0.3010\ (^{t}/_{h})$

where No = radioactivity at zero time

Nt = radioactivity at time t

Substituting, for tritium after 3 years, the activity was

842,000 dpm

a) Calculation of percentage efficiency in channels

The efficiency of the isotopic count in a channel is the actual counts in that channel divided by the activity or nuclear disintegrations per minute

$$= \frac{cpm}{dpm}$$

Thus with ^{14}C- standard having an external standard ratio (ESR) of 0.9519 and cpm of 89766.6 in channel 2, and 9046.6 in channel 1,

^{14}C Efficiency in Channel 2 = $\frac{89,7666}{104,000} \times 100$ = 86.31%

and ^{14}C Efficiency in Channel 1 = $\frac{9,046.6}{104,000} \times 100$ = 8.7%

So ^{14}C was predominantly counted in Channel 2, its efficiency was so low in channel 1, that the energy window settings could be said to have been well set for Channel 2 to be counting mainly ^{14}C.

The percentage efficiency for each of the ^{14}C- standards and each

of the 3H- standards was calculated in a similar way, for each of the two channels as above.

b) Conversion of cpm to dpm

The following calculation is an example of how to convert cpm to dpm:-

The ESR was read off from the counted data sheet (from the B-counter). In this case ESR of infusate was 0.52.

% Efficiency could be calculated as in section 3.7.3a above.

Efficiency 14C in channel 1 = 22.0%

Efficiency 14C in channel 2 = 62.5%

Efficiency 3H in channel 1 = 22.5%

Efficiency 3H in channel 2 = <0.5% (ignore)

cpm of infusate in channel 1 = 290697

cpm of infusate in channel 2 = 209889

Channel 2 counts 14C mainly with efficiency of 62.5% (since only less than 0.5% 3H was counted by this channel).

100% efficiency in channel 2. = $209889 \times \frac{100}{62.5} = 335822$

14C dpm = $\underline{335822}$

But channel 1 contains 3H at 22.5% efficiency plus 14C at 22.0% efficiency.

dpm 3H = Total Count in Channel 1 minus the count in channel 1 due to 14C.

3H dpm = $[290697 - (335822 \times \frac{22}{100})] = \underline{985528}$

In this example, the 14C was the extracellular reference substance, and the 3H- was the test substance. The ratio of dpm of test substance to the dpm of reference substance was calculated in the infustion mixture as well as in the venous effluent samples, and the percentage extraction was computed from the two ratios thus:-

c. Calculation of percentage extraction

The percentage extraction (E) of the test substance is

$$E = (1 - \frac{Rv.e.}{R_i}) \times 100$$

where $R_{v.e.}$ = $\frac{\text{dpm test substance}}{\text{dpm extracellular reference}}$ in venous samples

i.e. the ratio of activity of the test substance to reference substance in the venous effluent samples.

and R_i = $\frac{\text{dpm test substance}}{\text{dpm extracellular reference}}$ in infusion mixture

i.e. the ratio of activity of the test substance to reference substance in the infusion mixture.

All these calculations were performed by a Digital Equipment Corporation PDP 11/05 computer which read the data used for the computation directly from the punched paper tape produced by the teleprinter connected to the B-counter. The computer programme is a modification of that used by HOOPER (1975) - see Appendix 1 for the programme listing used in this study.

PART C

PRELIMINARY INVESTIGATIONS

CHAPTER FOUR

4. STUDIES ON THE PERFUSION MEDIUM

In order to assess the suitability of the perfusion medium used in these studies, experimental studies were carried out on the medium thus:-

4.1 Washing of the blood

Method - fresh heparinised bovine (ox) blood was collected from the abattoir and centrifuged as described in section 3.4.2.1 above. The supernatant was sucked out, using Pasteur pipette, into scintillation bottle and capped. This was then put away in the freezer compartment of the refrigerator until time for determination of K^+ and haemoglobin (Hb) concentration. Then the rest of the supernatant was pipetted out and the erythrocytes washed with Krebs ringer bicarbonate buffer. The supernatant was again sampled using the Pasteur pipette and stored away in the refrigerator. The cells were washed twice more and the supernatant collected as second and third wash samples. After all the samples were collected the K^+ was determined using a clinical flame photometer (Cornign EEL) and the haemoglobin concentration was determined by spectrophotometer (Model SP 500) using Drabkin's reagent. This was done for fresh blood collected on eight different days (N = 8). The washed cells were stored in the refrigerator for 24 hours and then centrifuged. The supernatant was collected and stored away for K^+ and haemoglobin (Hb) determination. The cells were then rewashed and the supernatant collected similarly. The perfusate Hb and K^+ were measured in the whole perfusate. The K^+ concentration of the Krebs ringer was measured.

Furthermore, the washed cells were stored for seven days and spun down. The supernatant was again sampled and the cells rewashed thrice

and the supernatant sampled on each occasion. The perfusate made out of these stored cells was used to perfuse a rat liver, and at the end of a three hour perfusion, the supernatant of the perfusate was also sampled. These samples were measured for K^+ and Hb concentration.

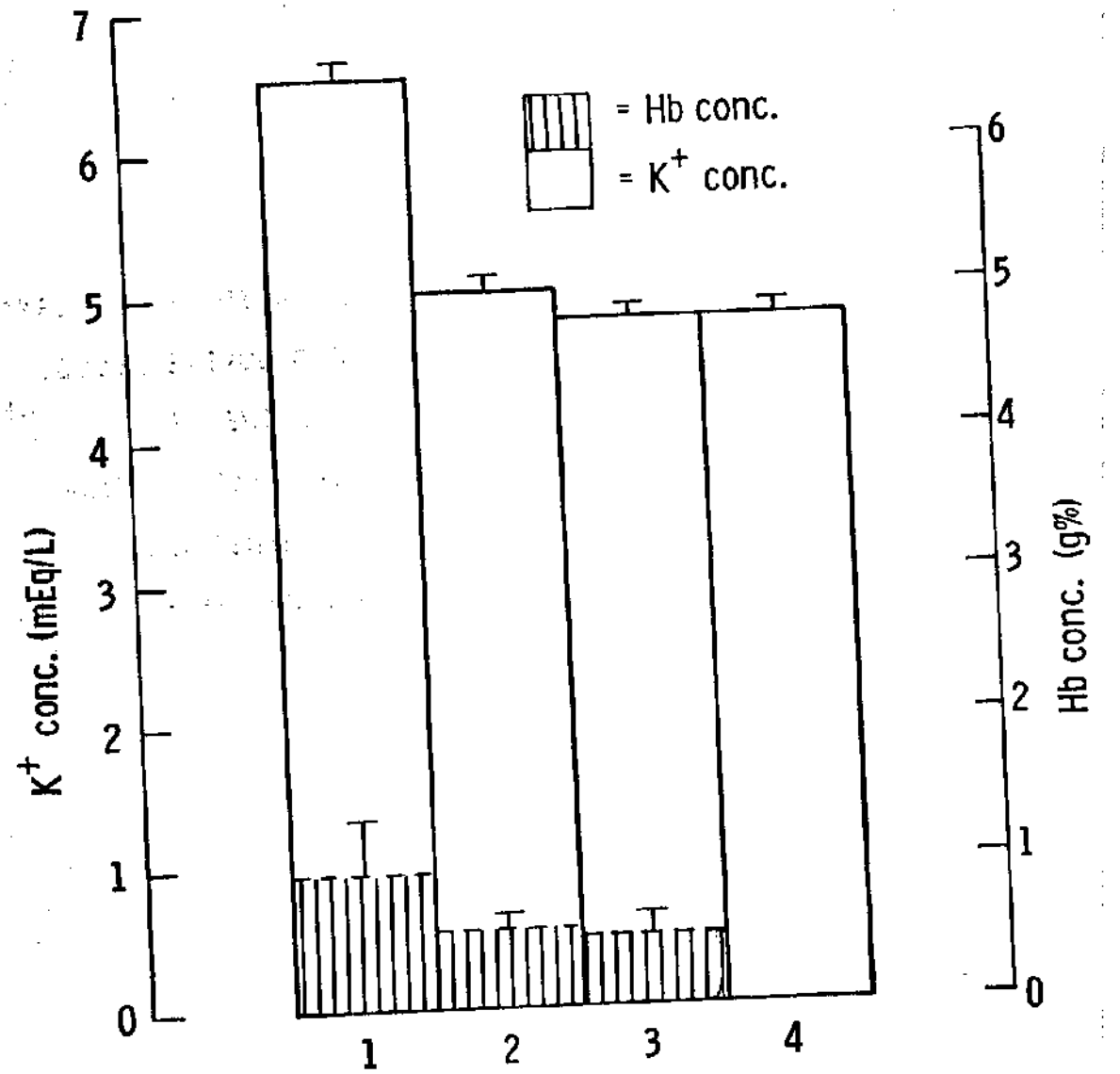
Results - The supernatant of unwashed heparinised fresh bovine blood (Column 1 of fig. 4.1) has a haemoglobin concentration of 1g%. It also has K^+ in excess of that present initially in the Krebs bicarbonate buffer used for the washing of the blood. This could be due to some haemolysis of the erythrocytes before the washing commenced. At least two washings were necessary before a clear supernatant could be obtained. The bars represent S.E.M. (N = 8).

Storage of the washed erythrocytes for one day caused some haemolysis as indicated by 0.5g% concentration of haemoglobin and 6.5mM K^+ concentration in the supernatant (fig. 4.2). However, one wash of the stored cells was enough to give a clear supernatant containing no haemoglobin. The total haemoglobin concentration in the perfusion medium was 5.0 ± 0.2g% (Column 4 of fig. 4.2), N = 8. Figure 4.3 shows that storing the blood for seven days caused a similar level of haemoglobin in the supernatant as in the one day old stored blood with (0.5g%) haemoglobin, but the K^+ concentration had risen very much above the one day old stored blood. This tends to suggest that the K^+ release into the medium precedes the Hb release presumably during the weakening of cellular membrane integrity. Also it was found (fig. 4.3) that one wash was no longer sufficient to obtain a clear supernatant (free of haemoglobin). At least two washings were required. This tends to suggest that, since the level of haemolysis was similar to that in the one-day old cells, a certain fraction of the cells were vulnerable and the removal of these in the first wash left the next

LEGEND TO FIG. 4.1

FIG. 4.1 - THE K^+ AND THE HB CONCENTRATION IN THE SUPERNATANT OF WASHINGS OF FRESH HEPARINISED BOVINE BLOOD. THE HORIZONTAL DOTTED LINE REPRESENTS THE LEVEL OF K^+ CONCENTRATION IN THE KREBS BUFFER USED FOR WASHING THE CELLS (4.7 mM). AT LEAST TWO WASHINGS ARE NECESSARY BEFORE A CLEAR SUPERNATANT CAN BE OBTAINED.

FIG. 4.1 WASHING OF FRESH OX-BLOOD

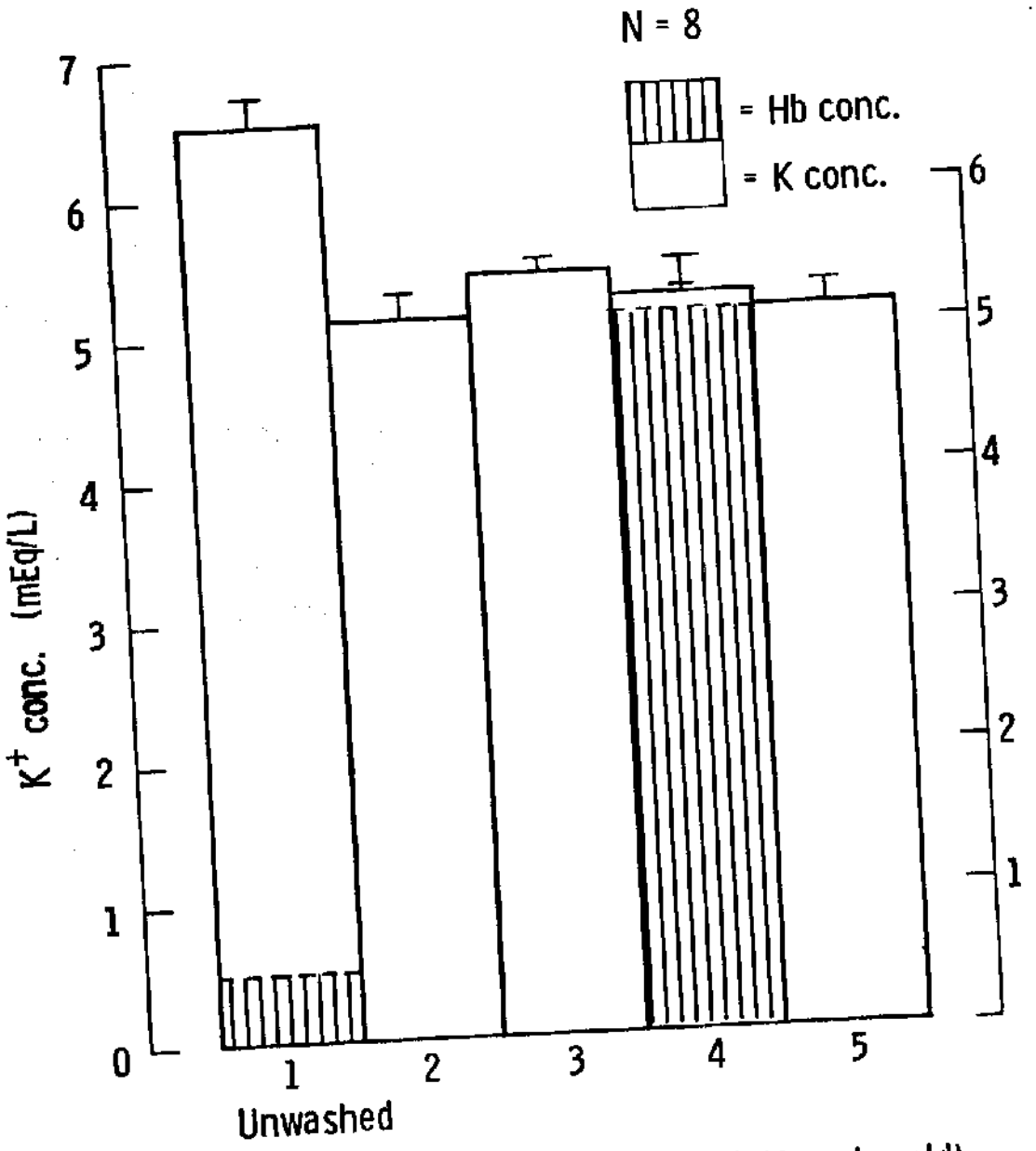


1 = Unwashed Heparinised blood (fresh)
2 = 1st wash
3 = 2nd wash
4 = 3rd wash

LEGEND TO FIG. 4.2

SUPERNATANT K^+ AND HB CONCENTRATIONS ON REWASHING OF ONE DAY OLD WASHED BOVINE ERYTHROCYTES. NOTE ALSO THE TOTAL HAEMOGLOBIN CONCENTRATION IN THE PERFUSATE OF 5.0G% AND DREBS RINGER K^+ CONCENTRATION OF 5 MM.

FIG. 4.2 WASHING OF ONE-DAY-OLD OX -BLOOD

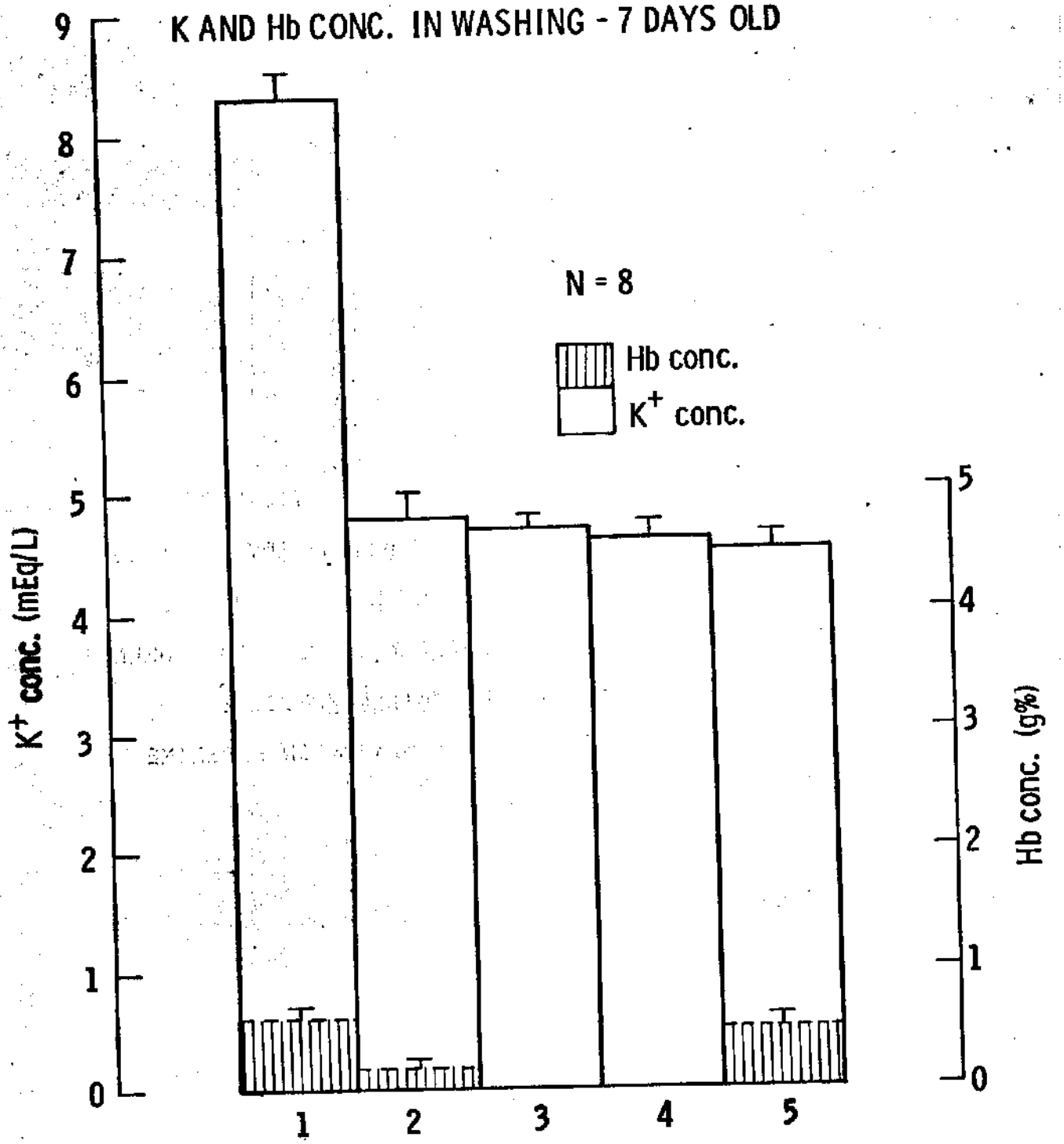


1 = Unwashed blood = supernatant (One day old)
2 = Rewashed blood A "
3 = Rewashed blood B "
4 = Perfusate (whole)
5 = Krebs bicarbonate buffer

LEGEND TO FIG. 4.3

FIG. 4.3 SUPERNATANT K^+ AND HB CONCENTRATIONS ON REWASHING OF 7 DAYS OLD STORED WASHED BOVINE ERYTHROCYTES. IT SHOWS THAT AT LEAST ONE WASH OF THE BLOOD SHOULD BE CARRIED OUT ON THE DAY OF USE, PREFERABLY TWO WASHES WHEN STORAGE IS AS LONG AS 7 DAYS. THE TOTAL PERFUSATE SUPERNATANT HB CONCENTRATION AFTER A PERFUSION EXPERIMENT IS SHOWN IN THE HISTOGRAM COLUM 5.

FIG. 4.3



1= Unwashed supernatant (7 days old)
2 = 1st wash "
3 = 2nd wash "
4 = 3rd wash "
5 = Perfusate "

vulnerable group to haemolysis, to a lesser degree. It was observed (Column 5, fig. 4.3) that the supernatant of the perfusate contained 0.5g% Hb after perfusion experiment, representing a 10% haemolysis during the circulation. This implies that some cells were haemolysed during the perfusion process. It was thought that this could be due to the mechanical effects of the Watson Marlow roller pump on the perfusion medium, hence a latter experiment was designed to test the effects of this pump. (fig. 4.5)

4.2 Haemolysis of the stored washed erythrocytes

The washed bovine erythrocytes were stored in the centrifuge screw-capped bottles in the refrigerator until the day of use. In order to ascertain that the cells have not been substantially haemolysed on storage, K^+ and Hb concentrations were measured in the supernatant.

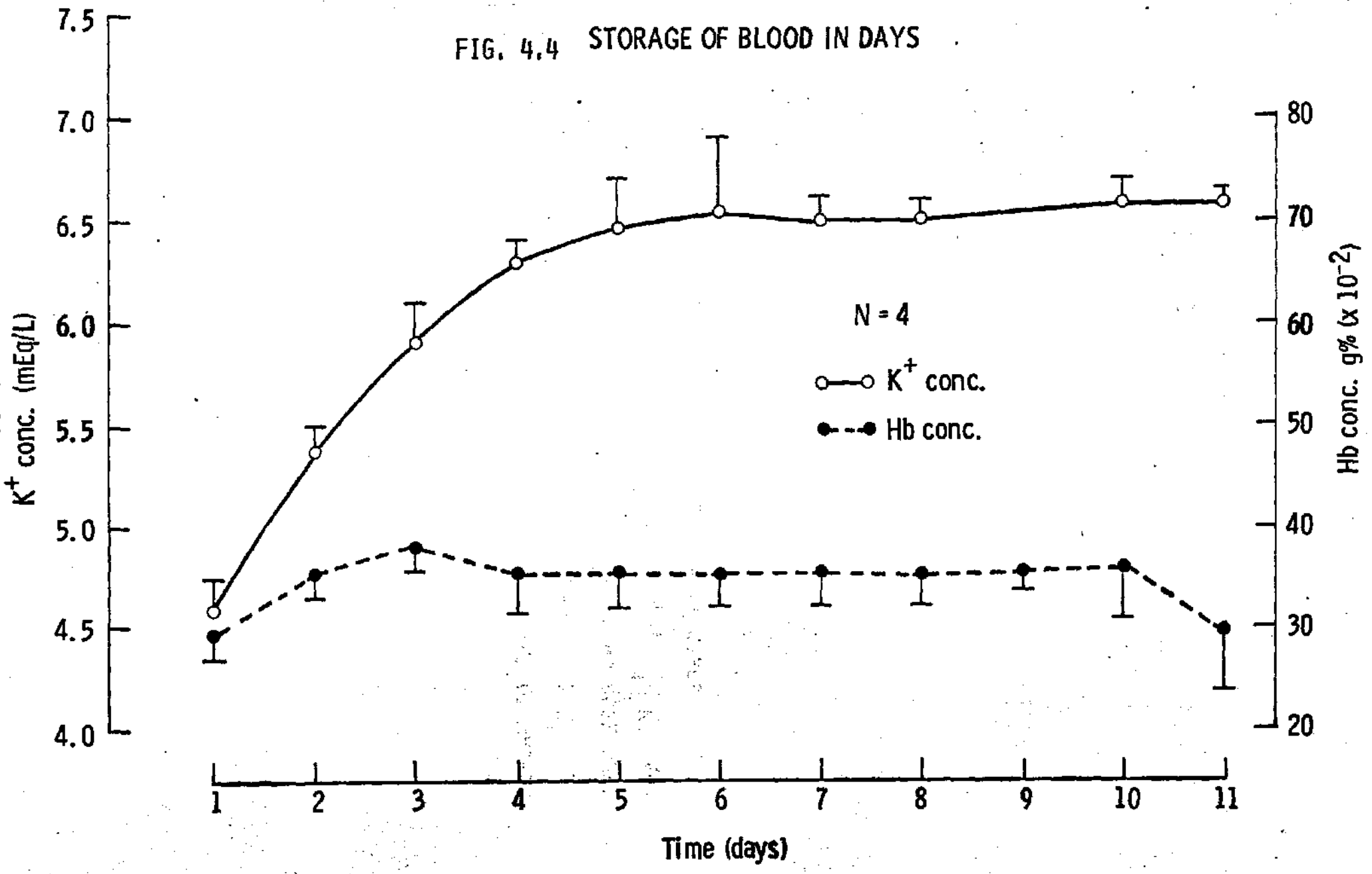
Method - The blood was collected from the abattoir in a heparinised saline bottle and thrice washed as described in section 3.4.2.1. The washed cells were then stored in the refrigerator at $4^{\circ}C$. The cells were rewashed once each day and the K^+ and Hb concentrations in the supernatant were measured in the samples collected daily for eleven days, taking the day of collection as day zero. Such measurements were made on four different occasions with different blood samples collected from four different cows in the same abattoir (N = 4).

Results - The effects of storage of the washed cells for eleven days on the Hb and K^+ concentrations in the supernatant of the daily washings are shown in fig. 4.4. The Haemoglobin rose for three days and K^+ level rose for the first four days and stabilized after that. This means the first three or four days of storage are the more delicate periods for the cell haemolysis. After that a stable and constant quantities of Hb and K^+ were released into the medium.

LEGEND TO FIG. 4.4

K^+ AND HB CONCENTRATION IN THE SUPERNATANT OF WASHED BOVINE ERYTHROCYTES STORED FOR ELEVEN DAYS. THE K^+ AND HB LEVELS IN THE SUPERNATANT FROM EACH WASHING ROSE DURING THE FIRST 3 DAYS FOR HB AND THE FIRST 5 DAYS FOR K^+, AND AFTER THAT REACHED A PLATEAU LEVEL IN BOTH CASES. BARS REPRESENT STANDARD ERRORS OF MEANS, (N = 4).

FIG. 4.4 STORAGE OF BLOOD IN DAYS



4.3 Evidence of Erythrocyte destruction during experiment

As evidence of red cell destruction during the experiment, potassium will be released and haemoglobin concentration in the supernatant would increase. So these parameters were measured in the perfused liver experiments, and in the control experiments when the liver was not in the circuit but all other conditions were the same.

Method - The perfusate was pumped round with the Watson Marlow roller pump. The sheering effects of this pump could result in considerable destruction of the erythrocytes in the course of an experiment. So it was decided to see the effects of the pump alone on the K^+ and Hb concentration in the perfusate as compared with when the liver was being perfused. In the pump effect alone, the perfusate was circulated in the usual way but no liver was in the circuit, and samples of perfusate were collected at 30, 60, 90, 120, 150, 180, 210 minutes from the start of recirculation. These samples were centrifuged and the supernatant kept for determination of K^+ and Hb concentration. In the next series of experiments, the liver was perfused and samples collected at the same time intervals for up to 180 minutes. Six experiments for each were performed (N = 6).

Results - The effects of Watson Marlow pump on the K^+ and Hb concentration are shown in fig. 4.5. It shows a sharp rise in the K^+ and Hb concentration in the supernatant within the first sixty minutes. It can also be seen that the rise in K^+ concentration continued for another sixty minutes before stabilising. After sixty minutes the Hb concentration was at a plateau level for the rest of the experiment - in the Watson Marlow alone. In the combined experiment with the pump and liver in circuit (fig. 4.6) the rise was within the first thirty minutes only and reached a plateau level thereafter. In comparing fig. 4.5 with fig. 4.6, it was seen that Hb concentrations measured at the start

4.2 Haemolysis of the stored washed erythrocytes

The washed bovine erythrocytes were stored in the centrifuge screw-capped bottles in the refrigerator until the day of use. In order to ascertain that the cells have not been substantially haemolysed on storage, K^+ and Hb concentrations were measured in the supernatant.

Method - The blood was collected from the abattoir in a heparinised saline bottle and thrice washed as described in section 3.4.2.1. The washed cells were then stored in the refrigerator at $4^{\circ}C$. The cells were rewashed once each day and the K^+ and Hb concentrations in the supernatant were measured in the samples collected daily for eleven days, taking the day of collection as day zero. Such measurements were made on four different occasions with different blood samples collected from four different cows in the same abattoir (N = 4).

Results - The effects of storage of the washed cells for eleven days on the Hb and K^+ concentrations in the supernatant of the daily washings are shown in fig. 4.4. The Haemoglobin rose for three days and K^+ level rose for the first four days and stabilized after that. This means the first three or four days of storage are the more delicate periods for the cell haemolysis. After that a stable and constant quantities of Hb and K^+ were released into the medium.

LEGEND TO FIG. 4.4

K^+ AND Hb CONCENTRATION IN THE SUPERNATANT OF WASHED BOVINE ERYTHROCYTES STORED FOR ELEVEN DAYS. THE K^+ AND Hb LEVELS IN THE SUPERNATANT FROM EACH WASHING ROSE DURING THE FIRST 3 DAYS FOR Hb AND THE FIRST 5 DAYS FOR K^+, AND AFTER THAT REACHED A PLATEAU LEVEL IN BOTH CASES. BARS REPRESENT STANDARD ERRORS OF MEANS, (N = 4).

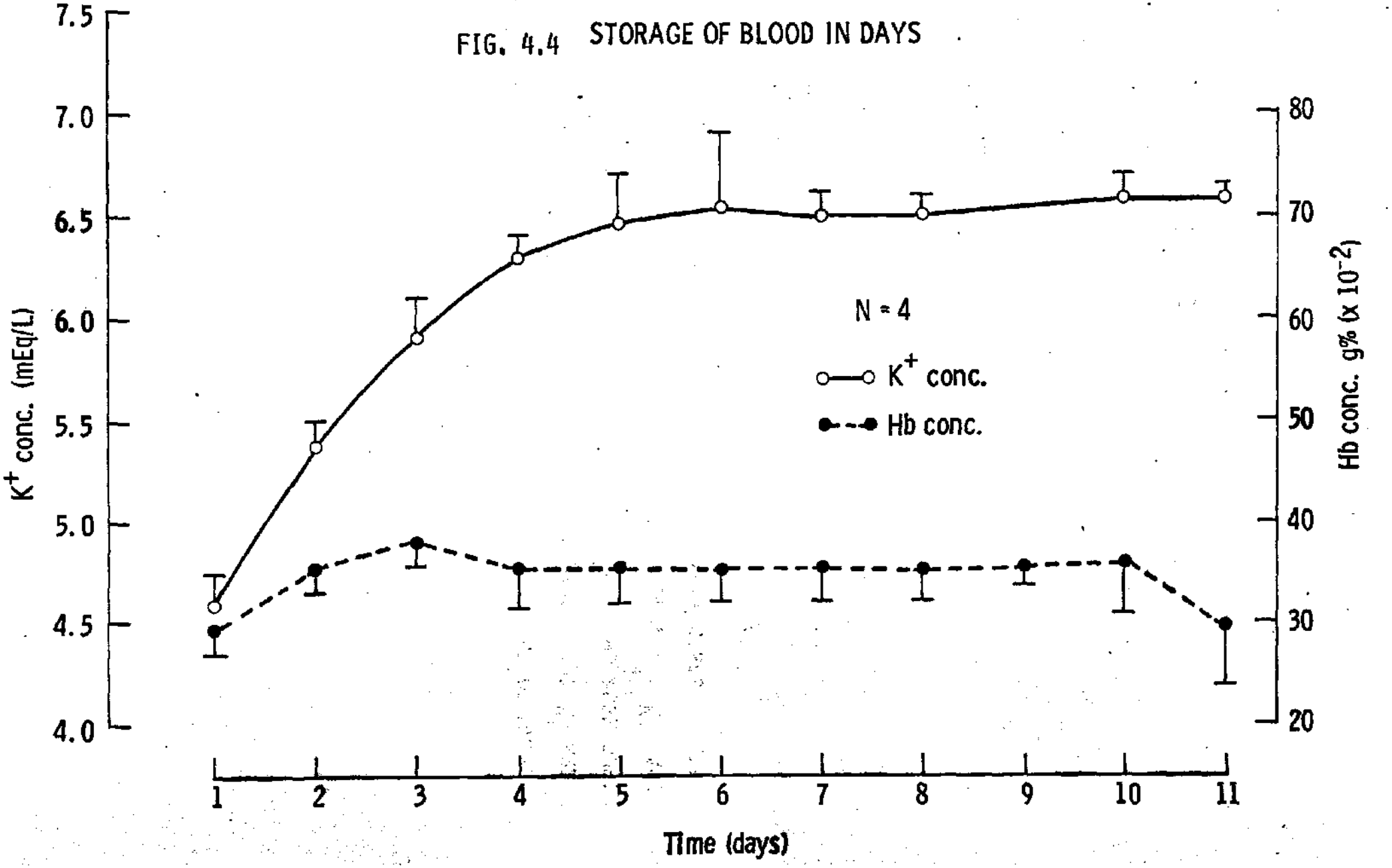
4.3 Evidence of Erythrocyte destruction during experiment

As evidence of red cell destruction during the experiment, potassium will be released and haemoglobin concentration in the supernatant would increase. So these parameters were measured in the perfused liver experiments, and in the control experiments when the liver was not in the circuit but all other conditions were the same.

Method - The perfusate was pumped round with the Watson Marlow roller pump. The sheering effects of this pump could result in considerable destruction of the erythrocytes in the course of an experiment. So it was decided to see the effects of the pump alone on the K^+ and Hb concentration in the perfusate as compared with when the liver was being perfused. In the pump effect alone, the perfusate was circulated in the usual way but no liver was in the circuit, and samples of perfusate were collected at 30, 60, 90, 120, 150, 180, 210 minutes from the start of recirculation. These samples were centrifuged and the supernatant kept for determination of K^+ and Hb concentration. In the next series of experiments, the liver was perfused and samples collected at the same time intervals for up to 180 minutes. Six experiments for each were performed (N = 6).

Results - The effects of Watson Marlow pump on the K^+ and Hb concentration are shown in fig. 4.5. It shows a sharp rise in the K^+ and Hb concentration in the supernatant within the first sixty minutes. It can also be seen that the rise in K^+ concentration continued for another sixty minutes before stabilising. After sixty minutes the Hb concentration was at a plateau level for the rest of the experiment - in the Watson Marlow alone. In the combined experiment with the pump and liver in circuit (fig. 4.6) the rise was within the first thirty minutes only and reached a plateau level thereafter. In comparing fig. 4.5 with fig. 4.6, it was seen that Hb concentrations measured at the start

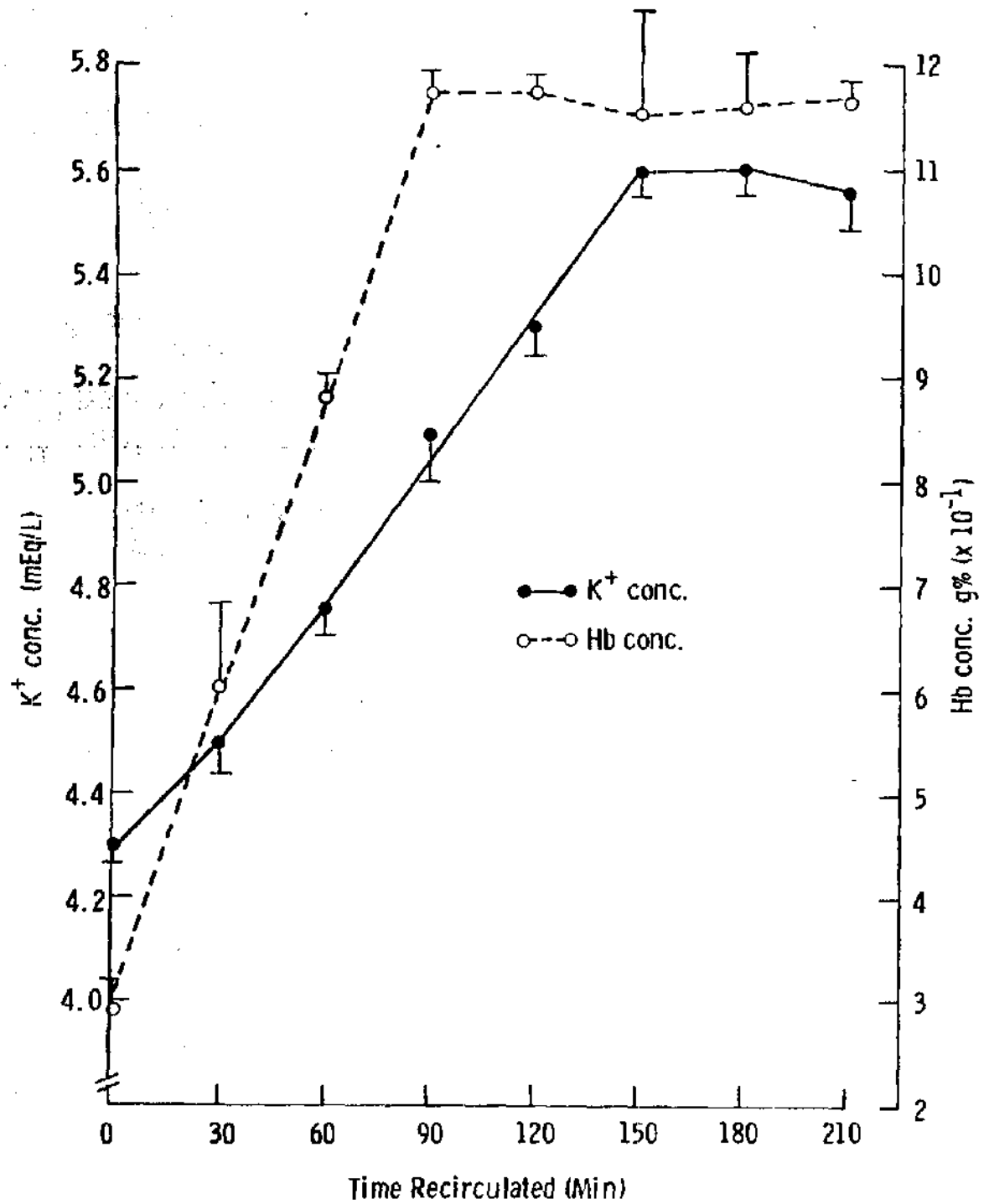
FIG. 4.4 STORAGE OF BLOOD IN DAYS



LEGEND TO FIG. 4.5

FIG. 4.5 - THE EFFECT OF WATSON MARLOW ROLLER PUMP ON THE K^+ AND HB CONCENTRATION IN THE PERFUSATE SUPERNATANT DURING A RECIRCULATION EXPERIMENT WITHOUT THE LIVER IN THE CIRCUIT. THE BARS REPRESENT STANDARD ERROR OF MEANS (N = 4).

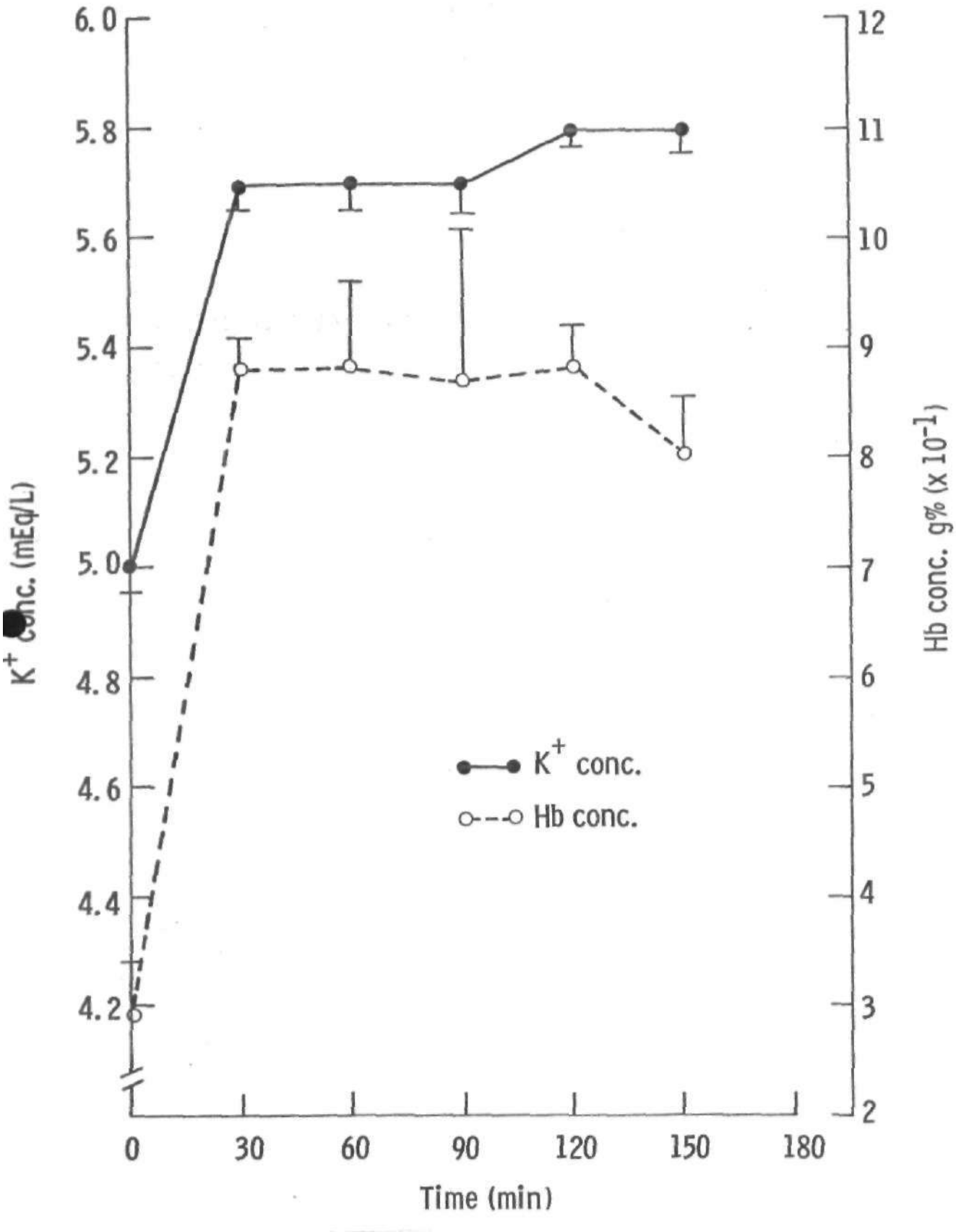
FIG. 4.5 EFFECT OF WATSON MARLOW PUMP ON PERFUSATE



LEGEND TO FIG. 4.6

FIG. 4.6 SHOWING THE K^+ AND HB CONCENTRATION IN THE PERFUSATE SUPERNATANT DURING LIVER PERFUSION EXPERIMENT. THE BARS REPRESENT THE ±S.E.M., N = 6.

FIG. 4.6 K^+ Hb CONC. IN PERFUSATE OF PERFUSED LIVER



(zero time) were 4.0 ± 0.3 and 4.20 ± 0.5g% (± S.E.M., N = 6) respectively. This discrepancy represents only the level of difference that could exist between different perfusate preparations due to the difference in the original concentrations of the erythrocytes added. The haematocrit of the packed cells added in making the medium, and indeed the accuracy of the measurements of 20 mls of washed packed cells to be added could also affect this basal Hb level. But more striking were the differences between Watson Marlow effect alone and with liver in circuit in the time course of increase and plateau level of this increase. In the pump experiment (fig. 4.5) there was a steady increase in Hb concentration for the first ninety minutes which rose to a plateau level of 5.75 ± 0.4g%. But in the experiment with liver in circuit (fig. 4.6), the Hb concentration rose only for the first thirty minutes and stabilised at a plateau level of 5.35 ± 0.5g%.

Also there was a difference in the basal level of K^+ concentrations in the perfusate at the starting (zero) time between the 4.3 ± 0.02 mM in the Watson Marlow pump experiments and 5.0 ± 0.02 mM (N = 6) in the experiments with liver in circuit. This discrepancy cannot be explained easily. But similar plateau levels of K^+ were reached at 5.6 ± 0.02 mM in fig. 4.5 and 5.65 ± 0.02 mM in fig. 4.6. Although the plateau levels were similar in both cases, but the rise to this peak was slower in the Watson Marlow effect alone rising to this peak over 120 minutes while the peak was attained in thirty minutes with the liver in circuit.

4.4 Tonometry of whole ox-blood

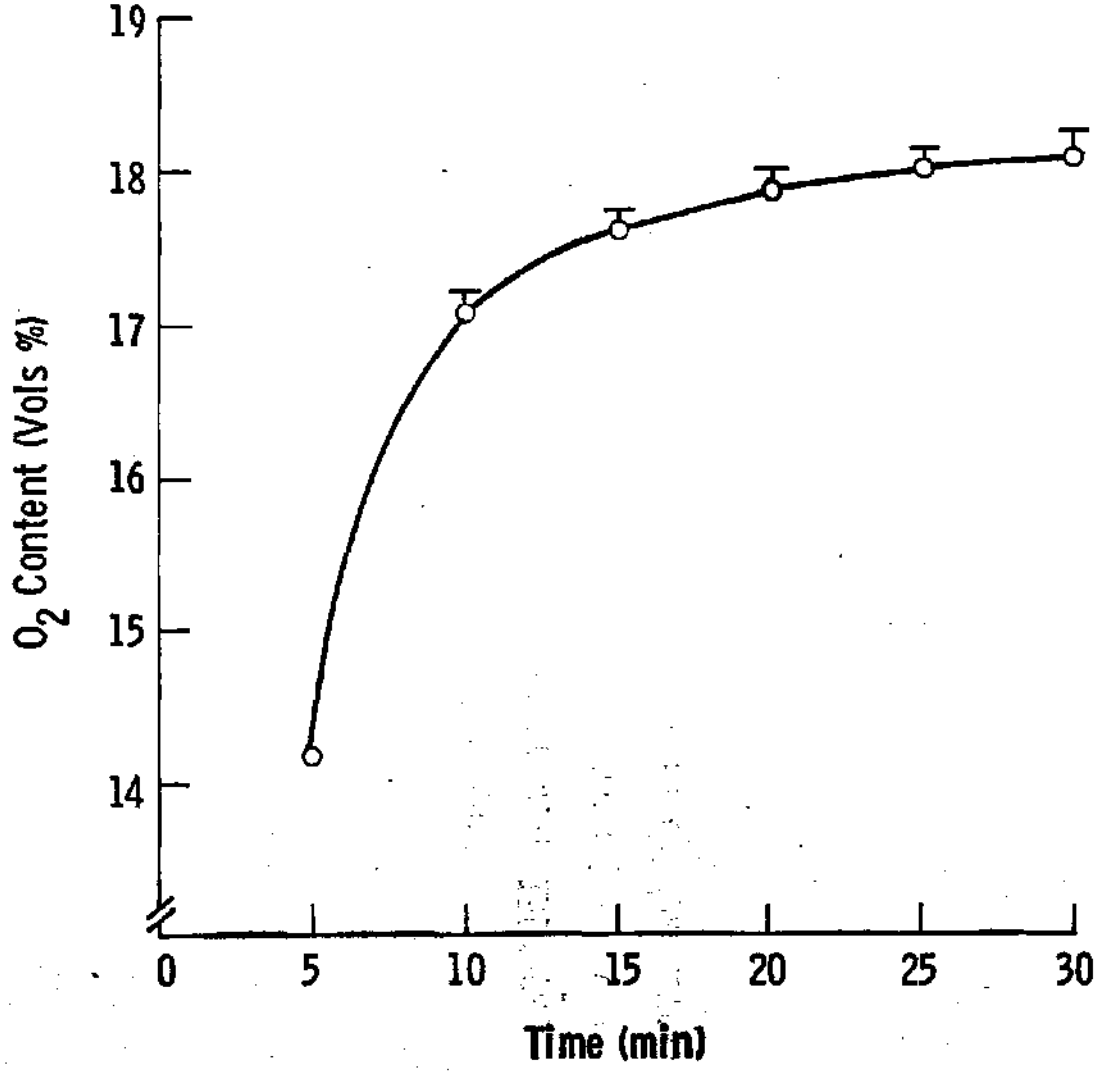
Method - Fresh whole heparinised bovine (ox) blood was tonometered for thirty minutes to assess the oxygen content of this blood. Five millilitres of fresh blood were withdrawn with a syringe and placed in the sample vessel of the tonometer (Model 237), and tonometered for 5, 10, 15, 20, 25, or 30 minutes as required on each occasion. After tonometry, 1 ml of blood was withdrawn into the syringe and its oxygen content measured with Les-O_2-Con. Six different blood samples were tonometered in this way using 13.5%O_2, 6%CO_2, balance N_2 (the approximate composition of alveolar air) and the means and standard errors of the means plotted on the graph.

Results - The time sequence of tonometry of fresh whole heparinised ox-blood is illustrated in fig. 4.7. It can be seen that the blood became almost fully saturated at ten minutes. The maximum oxygen content was 18.0 ± 0.1 vol.% (N = 6), at fifteen minutes. This represented 95% saturation, 100% saturation being calculated from the known normogram that 1 gm of haemoglobin would combine with 1.34 volumes of oxygen in the presence of such a gaseous mixture as this. The haematocrit and haemoglobin concentration had been determined in order to find out this saturation.

LEGEND TO FIG. 4.7

FIG. 4.7 SHOWS THE OXYGEN CONTENT OF FRESH WHOLE HEPARINISED BOVINE BLOOD WHEN TONOMETERED WITH 135% O_2, 6% CO_2, BALANCE N_2. NOTE THAT A MAXIMUM SATURATION WAS REACHED IN THE FIRST TEN TO FIFTEEN MINUTES OF TONOMETRY.

FIG. 4.7 TONOMETRY OF FRESH WHOLE HEPARINISED OX BLOOD WITH 13.5% O_2, CO_2, bal N_2



N = 6

4.5 Tonometry of Perfusate

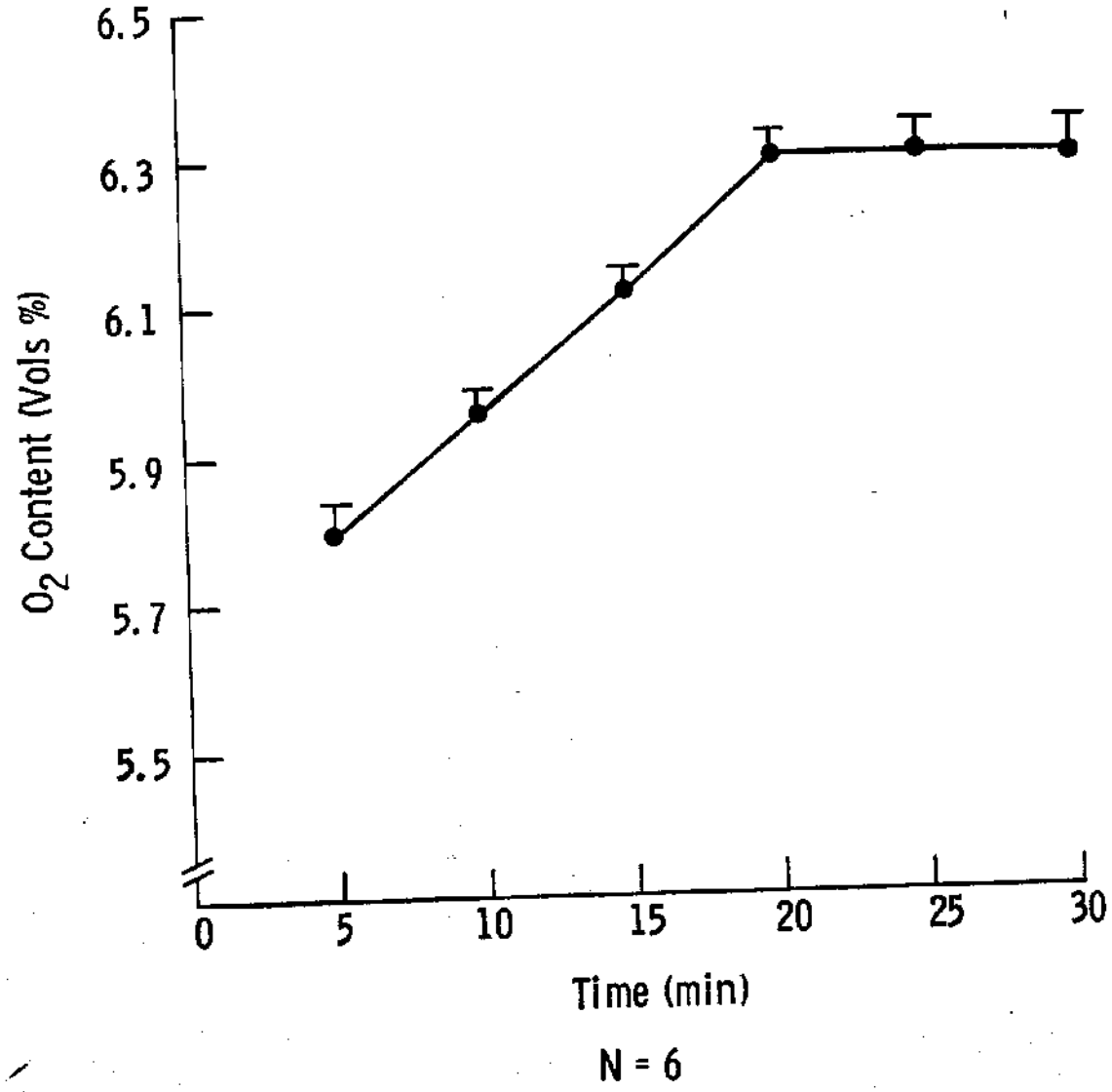
Method - The perfusate (12% haematocrit) was tonometered with 13.5% O_2, 6% CO_2 and balance N_2 as described in 4.4 above.

Results - The results of the perfusate tonometered with 13.5% O_2, 6% CO_2 and balanced with N_2 gaseous mixture are plotted in fig. 4.8. The perfusate reached its maximum oxygen carrying capacity in twenty minutes, this being 6.3 ± 0.1 vol.% (N = 6). This represented 94% saturation with the haematocrit of 12% (and Haemoglobin concentration of 5.0g%).

LEGEND TO FIG. 4.8

FIG. 4.8 SHOWS THE TONOMETRY OF PERFUSATE (WITH 12% HEAMATOCRIT) WITH 13.5% O_2, 6% CO_2, BALANCE N_2. A PLATEAU WAS REACHED IN FIFTEEN TO TWENTY MINUTES IN THIS CASE.

FIG. 4.8 TONOMETRY OF 12% PERFUSATE WITH 13.5% O_2, 6% CO_2, bal N_2



N = 6

4.6 Oxygen dissociation curve of fresh bovine erythrocytes in perfusate

The oxygen dissocation curve of fresh bovine erythrocytes in the perfusate of 12% haematocrit (PCV) was determined.

Method - To plot the graph of oxygen dissociation curve, one needs to measure the oxygen content (and from this the percentage saturation) in the perfusate at different partial pressures of oxygen (PO_2). In order to mix the gases to give the different partial pressures of oxygen needed, it was necessary to calculate from the known volumes in percentage composition of the gases, since the partial pressures of the gases in a mixture of gases must be directly proportional to their percentage volumes in the gaseous mixture (synthesis of Dalton's law and Boyle's law of gases).

The gases were mixed in a Douglas bag using the Cunningham's Trombone to deliver 200 litres per minute of the gaseous mixture. The Douglas bag was filled for thirty seconds in order to get about one hundred litres of gas in the bag for tonometry.

The following calculation of the gaseous mixture was used to make a table for the mixture:-

Calculation

Of the 200 litres, suppose we pump 40 litres of air per minute.

But air contains 20.93% O_2

200 litres contain $\frac{40 \times 20.93}{100}$ O_2

$= 8.72\ O_2$ in 200L

$= 4.36\% O_2$

Partial pressure of O_2 = 4.36% of 760

$= \frac{4.36 \times 760}{100}$

$= \underline{33.1}$ mmHg

But we want the partial pressure of CO_2 to be constant in all the mixtures at 40 mmHg

i.e. $\frac{40 \times 200}{760} = \underline{10.6}$L in 200L

In this mixture, the balance N_2 must be

200 - (40 + 10.6) = $\underline{149.4}$ Litres/min.

The calculation was done for each partial pressure of oxygen required and Table 4.1 was composed.

When the gases had been mixed in the Douglas bags, as indicated in Table 4.1, 5 mls of perfusate were withdrawn with a syringe and tonometered for 15 minutes and the oxygen content was measured using Lex-O_2-Con. The haemoglobin concentration in the perfusate was measured and the percentage saturation was calculated for each partial pressure. The percentage saturation was plotted against the partial pressures and the resultant graph is the oxygen dissociation curve for the perfusate (bovine erythrocyte haemoglobin) in fig. 4.9.

Results - The oxygen dissociation curve of the perfusate containing bovine erythrocytes at 12% haematocrit is shown in fig. 4.9. Note that there is a shift to the right when compared with the oxygen dissociation curve of the human erythrocyte haemoglobin. Also note that 50% saturation in this curve is 42 mmHg (P_{50} = 42 mmHg) whereas it is 27 mmHg (P_{50} = 27 mmHg) in the human erythrocytes. The corresponding values are, for bovine blood P_{50} = 32 mmHg and for albino rats blood P_{50} = 43 mmHg (ALTMAN & DITTMER, 1971).

TABLE 4.1

TABLE 4.1. THIS TABLE SHOWS THE CALCULATED VOLUMES OF AIR, N_2 AND CO_2 TO BE MIXED IN LITRES PER MINUTE WITH CUNNINGHAM'S TROMBONE IN ORDER TO DELIVER GASEOUS MIXTURE OF THE REQUIRED DIFFERING PARTIAL PRESSURES OF OXYGEN FOR TONOMETRY. THESE DIFFERENT GASEOUS MIXTURES WERE USED TO TONOMETER THE PERFUSATE FOR THE OXYGEN DISSOCIATION CURVE.

TABLE 4.1 COMPUTED DATA TO ILLUSTRATE HOW TO MIX THE GASES TO GIVE THE REQUIRED OXYGEN PARTIAL PRESSURES

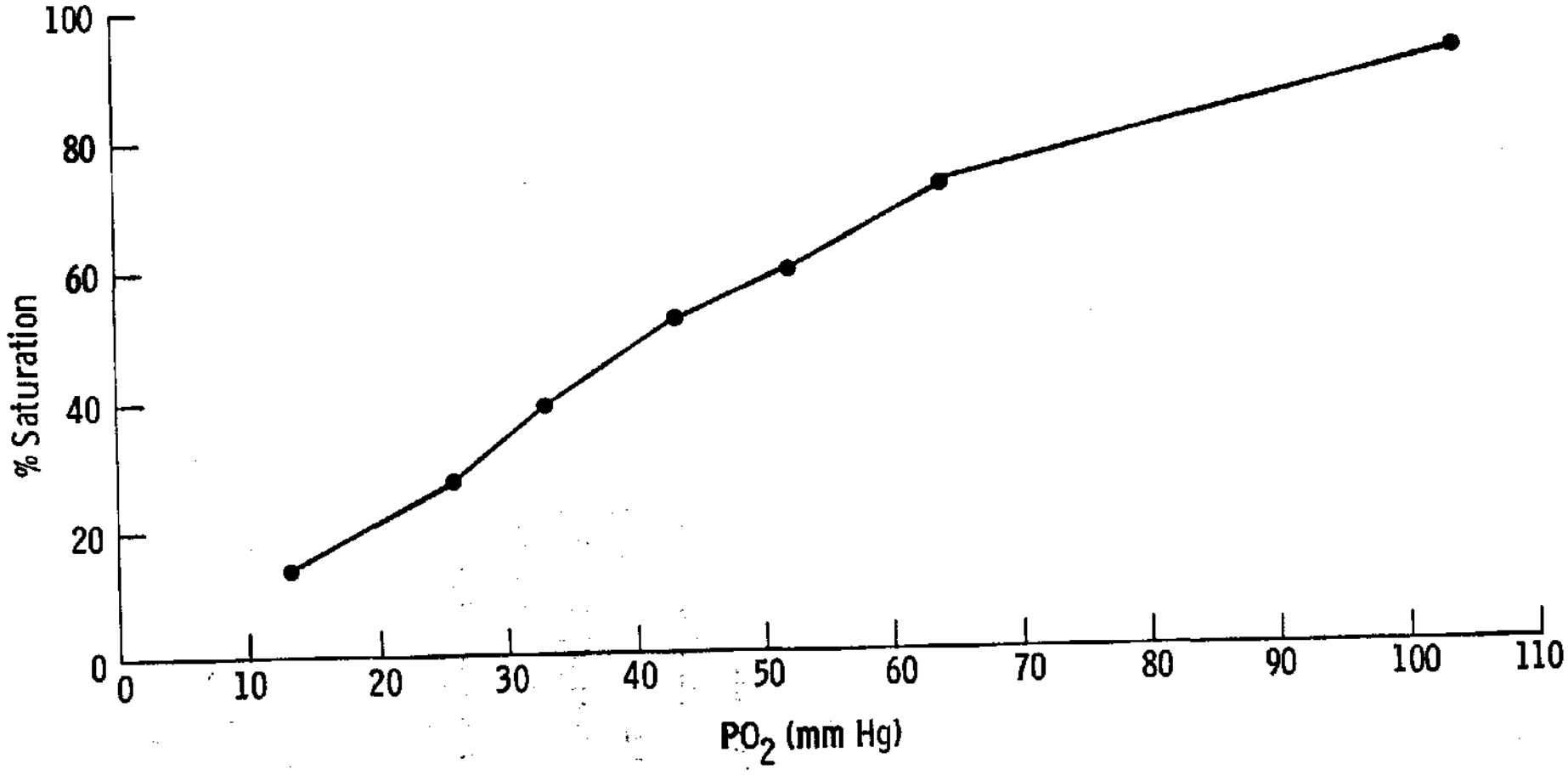
Approx. Partial Press. (mmHg)		Vol. of gases to mix (Litres/min)			Calculated Partial Press. (mmHg)	
PO_2	PCO_2	AIR	N_2	CO_2	PO_2	PCO_2
10	40	15	174.4	10.6	13	40
20	40	30	159.5	10.6	26	40
30	40	40	149.4	10.6	33	40
40	40	55	134.4	10.6	43	40
50	40	60	129.4	10.6	52	40
60	40	80	109.4	10.6	64	40
100	40	120	69.4	10.6	104	40

LEGEND TO FIG. 4.9

FIG. 4.9 SHOWING THE OXYGEN DISSOCIATION CURVE IN THE PERFUSATE PREPARED WITH FRESH WASHED BOVINE ERYTHROCYTES AT 12% HAEMATOCRIT. NOTE A RELATIVE SHIFT TO THE RIGHT WHEN THIS CURVE IS COMPARED WITH THE OXYGEN DISSOCIATION OF THE HUMAN ERYTHROCYTE (HAEMOGLOBIN). EACH POINT IS A MEAN OF TWO SAMPLES.

FIG. 4.9

O_2 DISSOCIATION CURVE OF FRESH CELLS IN 12% Ht



DISCUSSION

In these studies, the perfusion medium containing bovine erythrocytes was used. The ideal perfusion medium would be the homologous whole blood (in this case, the rat whole blood). But the whole blood as a perfusate suffers from certain major disadvantages thus:-

a) the viscosity is high,

b) it is likely to contain vasoconstrictor substances released during the process of obtaining the blood,

c) in the case of rat, the priming volume would necessitate sacrifice of many animals for one experiment.

The erythrocytes of man and macaque (monkey) are vastly more permeable to sugars than those of assorted rodents, carnivores and ungulates (HEDIN 1897; KOZAWA 1914; ULRICH 1934; WIDDAS 1955; DAWSON & WIDDAS 1964). So the choice of bovine erythrocytes for use in this study was based on the non-permeability of the hexoses and the ready availability of large quantities of this blood from the abattoir. It was also found later, that there was a more ready release or surrender of oxygen to the tissues by the bovine haemoglobin at the same or comparable oxygen partial pressures (see the results of oxygen dissociation curve in the immediate previous section to this), to those of human erythrocytes. This gives an extra advantage to this study over those of HEMS et al (1966) in which washed aged human erythrocytes were used.

But the use of bovine erythrocytes brought the problem of washing, Fig. 4.1 showed that at least two washings were required before a clear supernatant could be obtained. But in Fig. 4.2 only one further wash was necessary in a previously washed stored bottle of cells. However, if blood was washed and the cells stored, without daily rewashing, for seven days then one wash was not sufficient, and at least two washes

were mandatory. It was shown (fig. 4.4 that the daily rate of escape of Hb and K^+ to the washings was approximately constant from the fourth to the eleventh day of storage with daily washing. The total Hb liberated would correspond to about 10% of that originally present in the cells, disregarding any cells discarded during the daily washing procedure. It was considered that 90% survival of cells in storage was adequage if the washing before use removed the products of haemolysis. In practice, it was necessary after the initial separation and triple wash of the erythrocytes within the first four hours of collection from the abattoir, to store the cells only up to 72 hours. The cell stock was rewashed once at 24 hours and once at 72 hours, immediately before use.

The total haemolysis due to the effect of Watson Marlow roller pump on the recirculating perfusate amounted to about 10% of the cells over two and a half hours. There was apparently no further cell destruction from two and a half to three and a half hours of continuous pumping in the absence of the liver, which suggests that all of the vulnerable fraction of cells had been damaged by two and a half hours. In the presence of a liver (fig. 4.6), the maximum level of Hb in the supernatant of the perfusate was significantly lower (paired t-test, $N = 6$, $0.01 < P < 0.02$), though it was earlier in time (30 minutes compared with 90 minutes) than in the absence of the liver. These findings are not easily explained. The liver would appear both to accelerate some haemolysis, and either to prevent higher level being reached or sequestrate some of the Hb released later. Nevertheless, the K^+ concentration was somewhat higher when the liver was in circuit for the first sixty minutes of perfusion that when the pump was used alone. This could be due to release of K^+ from the liver during the first thirty minutes of liver equilibration phase. Indeed, it was

stated (MILLER et al 1973) that the perfusion medium exists as a defined medium only at the start of the experiment. Thereafter the release of substances such as K^+ ions and enzymes by the liver makes the composition unknown.

The ability of the perfusate to take up oxygen was determined by tonometry. The whole blood attained maximum oxygen content within the first 15 minutes and the perfusate twenty minutes (figs. 4.7 and 4.8). The time for equilibration in the tonometer is much longer than the transit time of perfusate through the falling film oxygenator. This means that during perfusion the reservoir cannot be completely equilibrated with the gas flowing past the film. The use of 95% oxygen shortens the equilibration time, and a pumping rate sufficient to maintain a rapid overflow limits the fall of oxygen content during perfusion. For example data from early experiments showed that after four experimental runs the oxygen content in the inflow cannula was 92% of the content at the start of the experimental run one. But to ascertain the ease of release of such combined oxygen to the tissues, the oxygen dissociation curve was determined (fig. 4.9). The possibility of a ready release of oxygen to the liver is shown by the shift to the right manifest in this graph. This is a situation analogous to the Bohr effect of CO_2 on the oxygen dissociation curve. This means that the same proportion of oxygen would be released at PO_2 42 mmHg as at 27 mmHg in the human erythrocyte or 43 mmHg in albino rats. This shift to the right also means a more ready surrender of oxygen in the organ, provided the upper reservoir blood (perfusate) was effectively saturated. The present experimental conditions achieve this.

CHAPTER FIVE

5. VALIDATION OF PROTOCOL

5.1 Viability of the preparation

5.1.1 Period of Hypoxia

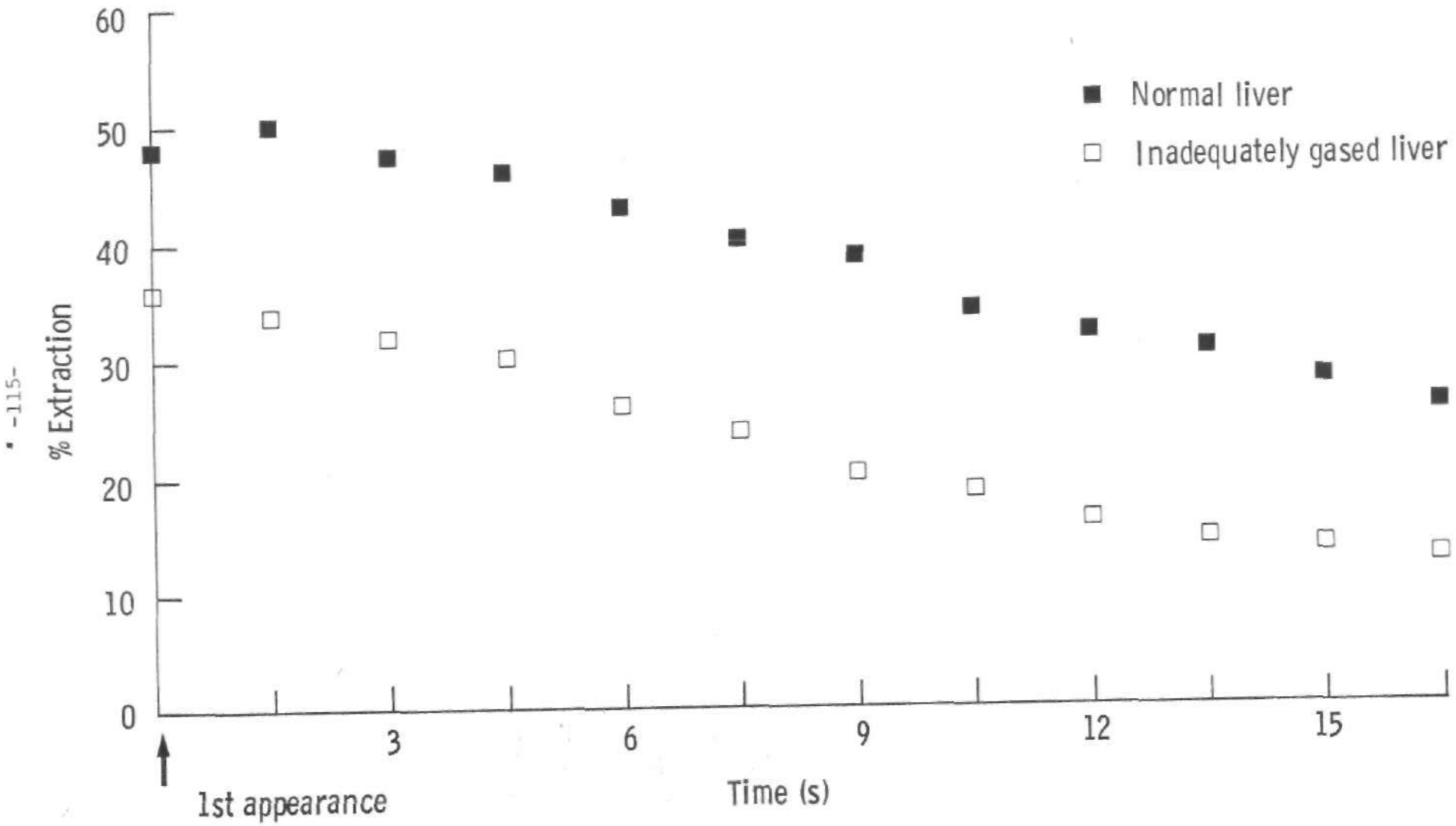
The first assessment of whether the liver preparation will be viable for perfusion or not is the time taken for the operative procedure to be carried out. If the surgical operation took unduly prolonged time, the liver was assumed not to be suitable (not viable) and the preparation was discarded. To this end, the surgical period was timed. Also the period of relative hypoxia was distinguished from the period of absolute hyposia; the former is the time from the occlusion of portal vein through the cannulation of the portal vein to the time the thorax is opened with consequent cessation of breathing, and the latter is the time from the cessation of breathing to the start of perfusion. During the period of relative hypoxia, blood flow was maintained by hepatic arterial flow which was still oxygenated as breathing under anaesthesia continued. But opening of the thoracic cage for the cannulation of the inferior vena cava via the right atrium caused cessation of breathing with attendant hypoxia. This period usually lasted one to two minutes. Any operation that lasted up to five minutes was suspect and any longer than five minutes was abandoned. The hepatic extraction of D-glucose in a properly aerated liver was compared with the extraction by the liver that was inadequately aerated (fig. 5.1). In the normally aerated liver, gassing with 95:5%O_2:CO_2 mixture was commenced promptly after the surgical operation, but in the inadequately gassed liver, the gassing with the

LEGEND TO FIG. 5.1

FIG. 5.1. COMPARATIVE TRANSPORT OF D-GLUCOSE BY NORMAL AND INADEQUATELY GASSED LIVERS. THE LATTER SHOWED A GREAT REDUCTION IN ITS CAPACITY FOR TRANSPORT OF D-GLUCOSE COMPARED WITH THE FORMER (THE NORMAL LIVER). THE DIFFERENCE IS HIGHLY SIGNIFICANT, PAIRED T-TEST, ($P < 0.001$, $N = 12$, THE NUMBER OF SAMPLES IN EACH LIVER).

Figure 5.1

COMPARATIVE TRANSPORT OF D-GLUCOSE BY NORMAL AND INADEQUATELY GASED LIVERS



same gas mixture was delayed for ten minutes from the time of operation. The result was a marked decrease in the transport capacity of the liver (fig. 5.1) from a peak extraction of 49.6 ± 1.6 to 34.8 ± 0.9 (± S.E.M., N = 4) in the normal liver compared with the inadequately gassed liver. This suggests that the poorly oxygenated liver is 8 S.E.'s below the oxygenated mean peak E, a highly significant result. The serial extractions with time showed a similar pattern - downward trend indicating efflux, hence the two curves ran parallel (fig. 5.1). The differences between the two liver extractions were highly significant ($P_{\angle}$ 0.001, N = 12, paired t-test).

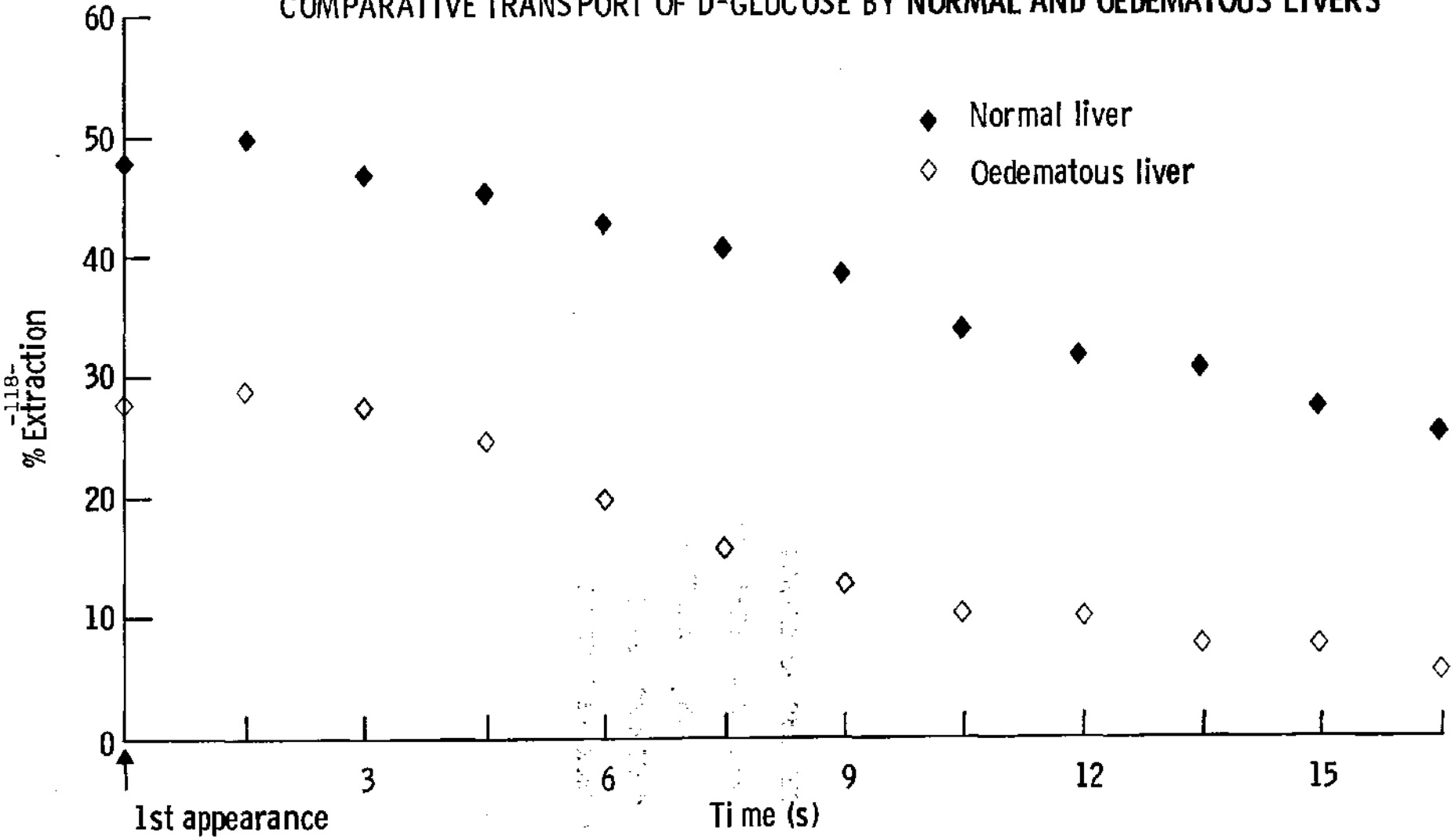
5.1.2 Morphological appearance

The morphological appearance of the liver was carefully noted during the surgical period and thereafter since it helped in the first instance to indicate deterioration of the preparation. To this end colour of the liver is very important. Blueness of the liver was an obvious indication of severely anoxic or damaged liver and such livers were discarded. Also mottling of the liver was not acceptable and such livers were also discarded. Furthermore, dryness and wrinkling of the liver reflecting underperfusion or oedema of the liver with gross distension or exudation of ascitic fluid from the liver surface reflecting overperfusion rendered the livers unviable and were discarded. These morphological criteria such as colour, mottling, wrinkling, and oedema were recorded at the time of experiments and a decision to eliminate such an experiment as being non-viable was always taken before the results were known. Oedematous livers were found in these studies not to perform well, they showed marked reduction in transport capacity compared with normal livers (fig. 5.2).

LEGEND TO FIG. 5.2

FIG. 5.2. COMPARATIVE TRANSPORT OF D-GLUCOSE BY NORMAL AND OEDEMATOUS LIVERS. THE OEDEMATOUS LIVER SHOWED A MARKED REDUCTION IN THE TRANSPORT CAPACITY. THE DIFFERENCE IN EXTRACTIONS BY THE TWO LIVERS WAS HIGHLY SIGNIFICANT ($P < 0.001$, $N = 12$, THE NUMBER OF SAMPLES IN EACH LIVER, PAIRED T-TEST).

Figure 5.2
COMPARATIVE TRANSPORT OF D-GLUCOSE BY NORMAL AND OEDEMATOUS LIVERS



The peak extraction of D-glucose in a normal liver was 50.0% compared with 29.1% in an oedematous liver. The patterns of extraction were similar in the two livers with only a difference in the level of extraction. The difference was highly significant ($P_{\angle}$ 0.001, N = 12, paired t-test).

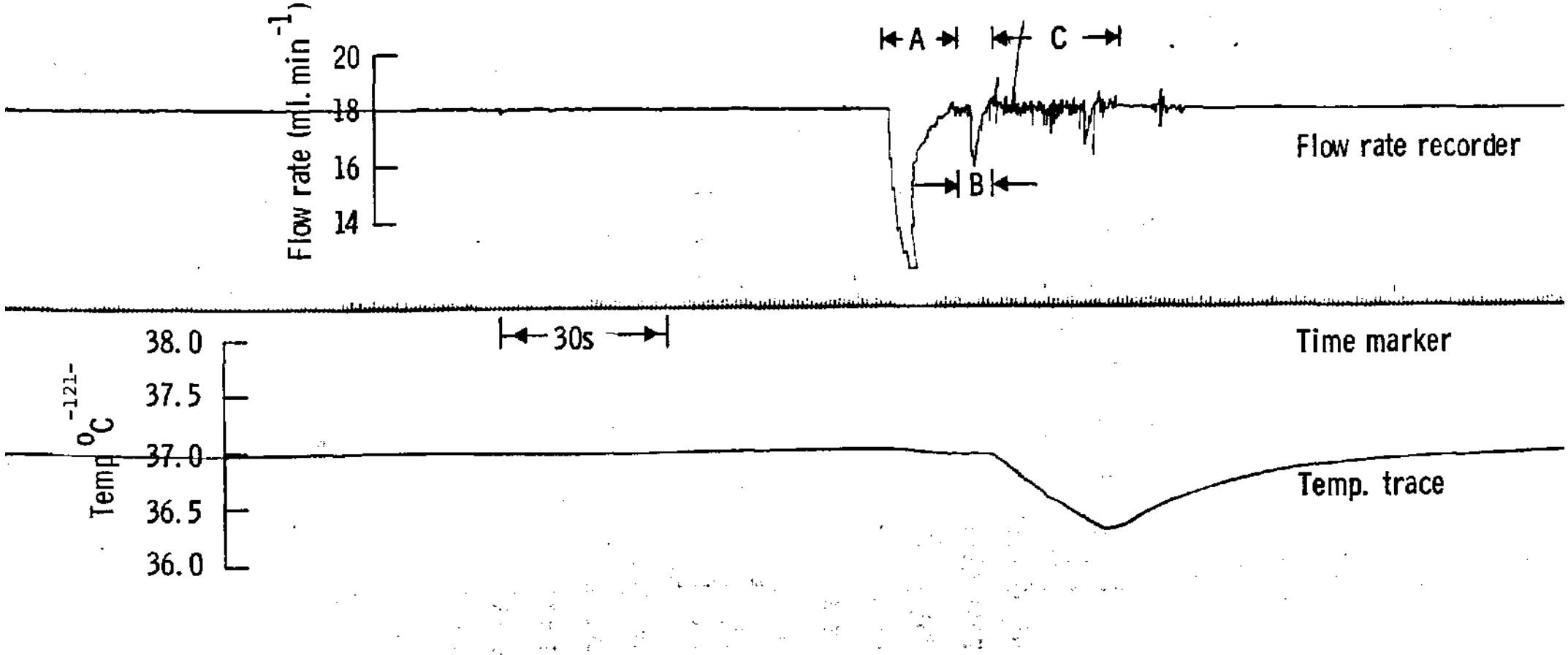
5.1.3 Monitoring and Control of flow rate

Since oedema and dryness or wrinkling of the liver is a function of the flow rate of the perfusate through the liver, as well as perfusion pressure and outflow resistance like a kinked cannula, it was necessary to monitor the flow rate accurately and throughout the whole perfusion period. The flow rate was monitored by a photoelectric drop counter connected to Grass recorder which recorded the flow rate on a paper tracing (fig. 5.3). The flow rate was normally maintained at 18 ml. min^{-1}. per liver giving a flow rate of 1.8 to 2.25 $ml.g^{-1}$. min^{-1} (wet weight of liver) - the liver wet weights lying between eight and ten grammes per 300-400 g rat. Constant flow was maintained with adjustable pressure of perfusion by raising or lowering of the upper reservoir. The perfusion pressures were usually between ten and twenty cm. of perfusate. This is within the normal portal venous pressure (KUNKEL & EISENMENGER 1949; RITCHIE & HARDCASTLE 1973). During the infusion of test substances, the flow rate was also maintained constant at the same rate by using a syringe pump thereby ensuring a constant flow rate both during each of the experimental runs, and normal perfusion without test infusion (fig. 5.3). This precaution was necessary because it was found that extraction was dependent on the flow rate (fig. 5.4). HOOPER and SHORT (1977) found no consistent relationship between extraction and flow rate in

LEGEND TO FIG. 5.3

FIG. 5.3. A TYPICAL RECORDING DURING AN EXPERIMENTAL RUN. THE TEMPERATURE AND FLOW RATES ARE MAINTAINED CONSTANT EXCEPT FOR BRIEF INTERRUPTIONS DURING ACTUAL EXPERIMENTAL RUN. A, B AND C ARE BRIEF PERIODS WHEN THE STOPCOCK WAS TURNED AND THE PERFUSATE WAS WITHDRAWN INTO THE INFUSION SYRINGE TO MIX WITH THE ISOTOPIC MATERIAL, AND ANY POTENTIAL COMPETITOR ADDED (A), AND SWITCHING BACK TO ONLINE CIRCUIT (B), AND FINALLY DURING CONSTANT INFUSION WITH A SYRINGE PUMP (C). THERE WERE BRIEF INTERRUPTIONS OF FLOW TO THE LIVER DURING THESE CHANGE-OVER PERIODS (A AND B). NOTE THE SLIGHT DROP IN TEMPERATURE DURING INFUSION (C) DUE TO THE COOLING OF THE INFUSION MIXTURE IN THE SYRINGE. THE EFFECT OF LIVER TEMPERATURE ON TRANSPORT WAS STUDIED (SEE CHAPTER 8), AND THIS CHANGE OF -0.75°C WAS TRIVIAL IN ITS EFFECT ON EXTRACTION.

FIG. 5.3 A TYPICAL RECORDING DURING AN EXPERIMENTAL RUN



KEY

A = withdrawal of perfusate from upper reservoir into infusion syringe

B = switching from withdrawal to on - line circuit

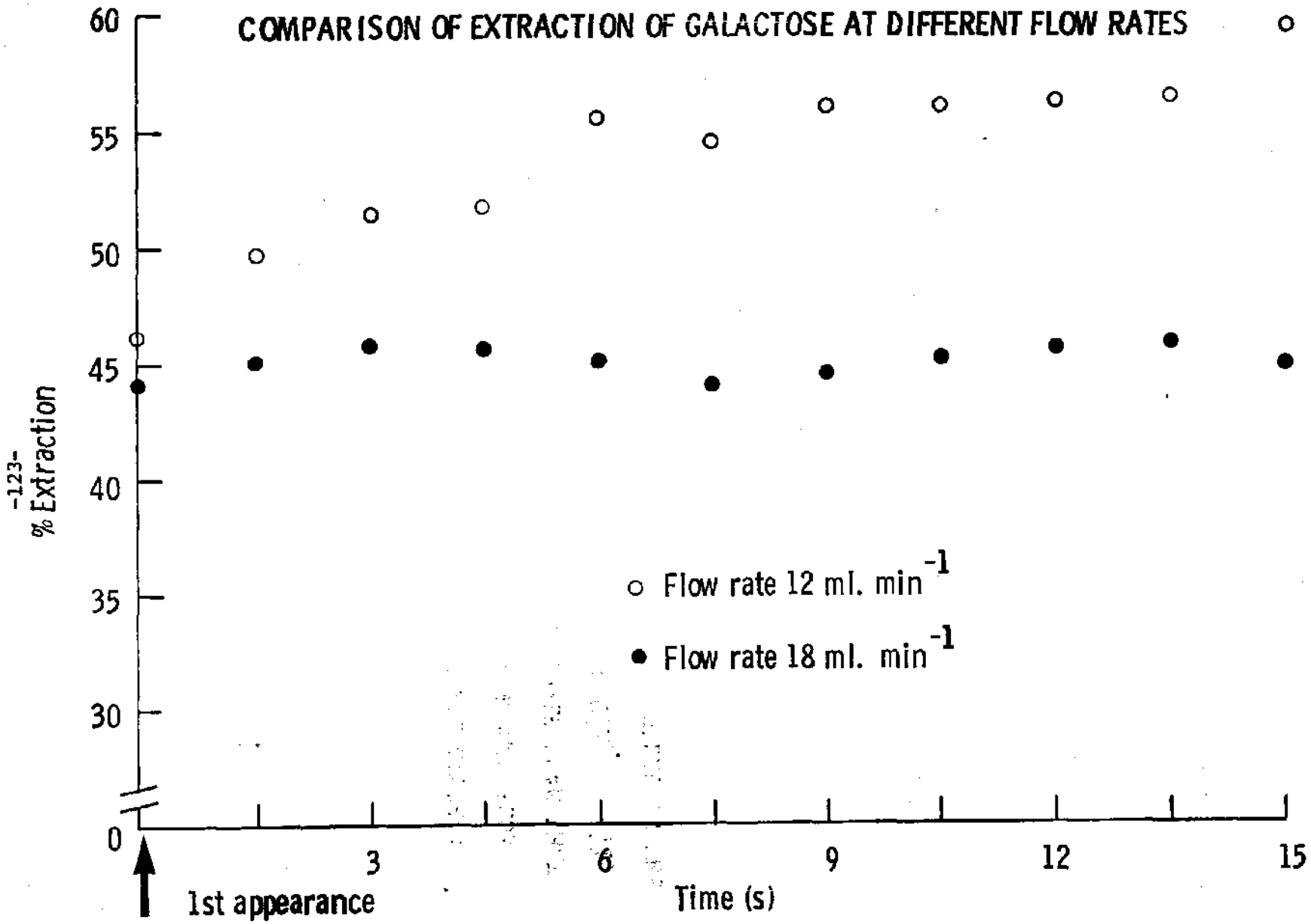
C = constant infusion

LEGEND TO FIG. 5.4

FIG. 5.4. COMPARISON OF EXTRACTION OF D-GALACTOSE AT DIFFERENT FLOW RATES. THE EXTRACTION AT NORMAL FLOW RATE OF 18 ML.MIN^{-1}. WAS SIGNIFICANTLY LOWER THAN THAT AT SLOWER FLOW RATE OF 12 ML.MIN^{-1} ($P < 0.001$, $N = 11$, WHERE N IS THE NUMBER OF OBSERVATIONS OR SAMPLES IN THE SAME LIVER, PAIRED T-TEST).

FIG. 5.4

COMPARISON OF EXTRACTION OF GALACTOSE AT DIFFERENT FLOW RATES



conscious sheep livers. Extraction of D-galactose at the normal flow rate of 18 ml. min^{-1} was significantly different from that at slower flow rate of 12 ml. min^{-1} (fig. 5.4) P_L 0.001, N = 11 (paired t-test where N = number of samples in the same liver). The two flow rates were performed in the same liver, one fifteen minutes after the other. This flow rate of 18 ml. min^{-1} was chosen firstly because higher rates tended to cause oedema of the liver (Section 5.2 above), and slower rates gave small quantities of effluent samples in the sample pots resulting in errors in volumetric sampling which would give erratic transport values. This erratic volumetric sampling might be responsible for the less uniform extraction values for the slow flow rate (open circles in fig. 5.4) compared with the normal flow rate (closed circles). Secondly, this flow rate is very near normal flow rate in the portal vein reported by other workers; 1.0 - 3.0 ml. min^{-1}. g^{-1} of liver by ROSS (1972), MILLER and JOHN 1960; 1-2 ml. min^{-1}. g^{-1} of liver by RITCHIE and HARDCASTLE (1973). But this flow rate was very much lower than those used by BLOXAM (1971) which were 5.8 ± 0.3 ml. min^{-1}. g^{-1} after 15 minutes and 10.8 ± 0.4 ml. min^{-1}. g^{-1} after 100 minutes in halothane anaesthesized rats. But these high flow rates were required by BLOXAM in order to meet the oxygen requirements of the liver since he was perfusing his livers with Krebs-ringer bicarbonate buffer, without any other additions. Also the viscosity of the cell-free perfusate he used was lower, so flow would be higher at a given perfusion pressure.

The difference in extraction at different flow rates observed here could be calculated to agree closely using the permeability/surface area produce thus:-

If (1) 18 ml. min^{-1}. gives E = 0.45 or 45%

and (2) 12 ml. min^{-1}. gives E = 0.55 or 55%

then we can estimate the value of PS (the permeability/surface area product):

$$PS = Ql_n(1-E) \quad \ldots\ldots\ldots 1 \quad \text{(RENKIN 1955, 1959).}$$

where Q = blood flow

E = fractional extraction

P = permeability

S = surface area

$$1 - E = \frac{PS}{Ql_n} \quad \ldots\ldots\ldots 2$$

$$PS = (1 - E)Ql_n \quad \ldots\ldots\ldots 3$$

Then PS (1) = $18 \times l_n (0.55) = -10.761$

and PS (2) = $12 \times l_n (0.45) = -9.58$

If PS (1) is constant, expected E at 12 ml. min^{-1}. would be calculated from $(1 - E) = \exp(\frac{PS}{Q})$

$$= \text{Exp}(\frac{-10.761}{12})$$

$I - E = 0.408$

$E = \underline{0.592}$ instead of .55

This value was in fact approached at the later time of 15 sec. The data certainly corresponds approximately to a PS of 10 ml. min^{-1} for galactose uptake.

5.1.4 Temperature Control

It was important to maintain the liver temperature constant except when experiments were done to alter the temperature as in Chapter 8. Since the flow rate of the perfusate controlled the temperature of the liver, the temperature was recorded concurrently with

the flow rate using a thermistor temperature probe inserted between the liver lobes. The output was continuously monitored via the Grass recorder and a paper trace was obtained simultaneously with the flow rate recording (fig. 5.3). This simultaneous recording helps to show the slight fall in temperature 0.5°C - 0.75°C during the infusion of the isotopic mixture from the syringe. This slight drop in temperature was due to the fact that the withdrawal of the perfusate into the syringe exposes the syringe mixture to laboratory temperature (A) a short time indicated in fig. 5.3, before the actual infusion (C) of the mixture into the liver. The transport of these hexoses by the liver is temperature dependent - and this has been shown for D-galactose in Chapter 8. So it was important to ensure that the temperature was maintained constant and this was monitored accurately to show when there was a temperature change of significant level to merit correction. The results for D-galactose show that the 1°C change has very modest effects on the measured extraction (E).

5.1.5 Oxygen consumption

It has been stated (RITCHIE & HARDCASTLE 1973) that the most easily monitored criterion of adequate perfusion flow rate is that of oxygen uptake by the preparation.

In order to measure the oxygen consumption by the liver, the oxygen content in the portal venous and hepatic venous cannulae were first measured using the Lex-O_2-con O_2 content meter. The inflow portal venous perfusate was sampled immediately before the outflow hepatic venous perfusate for determination of the oxygen content. The Lex-O_2-Con with electronic digital readout of oxygen content in volumes percent was first calibrated with room air, corrections being

made for the barometric pressure, temperature, relative humidity and hence vapour pressure (STPD). Then the arteriovenous O_2 difference (the difference between the portal venous and hepatic venous sample readings) was used to calculate the oxygen consumption after the liver weight and flow rate were known. The calculation was done thus:-

Barometric Pressure = 765

Less correction factor 2.35

Corrected B. Pressure = 762.65

Readings on the wet and dry-bulb hygrometer were:

Wet Bulb 17.5

Dry Bulb 25.0

Setting slide rule 25 at 100%, reading off at 17.5,

Relative Humidity = 46%

At $25^{o}C$, Water vapour pressure = 11.5

So 46% of 25 vapour pressure = 11.5

$\therefore$ Corrected pressure (S.T.P.D.) = 762.6 - 115 = 751.1

Using chart, read 25^{o} at 750 (approx) = 18.9

O_2 in Air = 18.9 volumes %

The Lex-O_2-Con was therefore calibrated to read this figure when air was injected. After this calibration, blood or perfusate samples were injected for O_2 determination. After every six injections of blook, the scrubber was flushed with distilled water. And after one hour, the Lex-O_2-Con was re-calibrated before use.

The results of the oxygen consumption by the livers used in these experiments are compared with results of oxygen consumption by livers used by other workers in Table 5.1. The oxygen consumption in these experiments was in the range of 2.30 to 3.68 $\mu Moles.g^{-1}.min^{-1}$ at thirty minutes and 2.21 to 2.8 $\mu Moles.g^{-1}.min^{-1}$ at sixty minutes.

These values compare very favourably with those reported by other workers (Table 5.1) except the findings of BRAUER (1963) which seem very much higher than all other findings. But it must be remembered that BRAUER (1963) used whole rat blood while the rest of the workers reported in this table used either semi-synthetic medium (which contained erythrocytes) or just synthetic medium (saline). In the present study, the oxygen consumption reported here represents 38.25 - 49.68% removal of the available oxygen at sixty minutes and 45.55 - 54.28% removal at thirty minutes. These values compare favourably with those of BLOXAM (1971) where his livers removed 40% at fifteen minutes and 35% at 100 minutes.

5.1.6 Potassium and Haemoglobin Release

During the time of perfusion of isolated liver, K^+ and Hb may be released into the perfusion medium. The degree of this release is a reflection of the amount of damage done to the liver or to the erthrocytes in the medium. Of these two, Hb comes from the haemolysis of erythrocytes, whereas K^+ comes from RBC and liver cells and may appear without lysis. The potassium release is particularly important because it is a manifestation of the trauma to the liver during surgical manipulation. K^+ level therefore rises to a peak during the first thirty minutes of perfusion (fig. 4.6), so did the haemoglobin. The implications of these rises were discussed in Chapter 4 above. But the rise of K^+ in the medium to 5.65 $mEq.L^{-1}$ (or 5.65mM) within the first thirty minutes of perfusion is consistent with earlier findings of RITCHIE and HARDCASTLE 1973. K^+ concentration was shown to rise due to anoxic conditions imposed on the perfused liver even for periods as short as thirty seconds of exclusion of oxygenator from

TABLE 5.1 OXYGEN UPTAKE BY THE RAT LIVER

Preparation	Perfusion medium	Time	O_2 consumption $\mu Moles.g^{-1}.min^{-1}$	Reference
Isolated perfused liver	Krebs-ringer bicarbonate buffer with added washed bovine erythrocytes and bovine plasma albumin	30 min. 60 min.	2.30 ~ 3.68 (N = 10) 2.21 ~ 2.85 (N = 8)	Present Study
Isolate perfused liver, perfused backwards	Krebs saline with haemoglobin	30 min.	1.78 ~ 2.38 (N = 5)	D'Silva & Neil 1954
Isolated perfused liver	Saline medium with glucose, lactate, pyruvate, bovine plasma albumin and erythrocytes	45-160 min.	2.53 (N not stated)	Schimassek, 1962
Isolate perfused liver	whole rat blood		5.72 (N not stated)	Brauer 1963
Isolated liver, perfused backwards	Krebs-ringer bicarbonate buffer with no additions	15 min. 30 min.	0.60 ~ 0.62 (N = 4) 0.62 ~ 0.71 (N = 4)	Bristow and Kerley 1964
Isolated perfused liver	Krebs-ringer bicarbonate buffer with added bovine plasma albumin and aged human erythrocytes	30 min.	2.53 ± 0.22 (N = 12)	Hems et al 1966
Isolated perfused liver	Krebs-ringer bicarbonate buffer with no additions	15 min. 100 min.	2.19 ± 0.25 1.91 ± 0.34 (N = 5)	Bloxam 1971

the system (RITCHIE & HARDCASTLE 1973). So excessive release, or continued rise of K^+ levels in the medium was used as one of the viability parameters of these preparations. It is therefore of importance that the K^+ concentrations in the medium stabilized after the initial sudden rise (fig. 4.6) suggesting that the initial rise was largely due to surgical manipulations and anoxic state of the liver before connection to the perfusion circuit, and no further damage was being done to the liver in the course of perfusion.

5.1.7 Changes in pH during perfusion

Maintenance of correct pH could be important to the survival and normal function of the perfused liver. So the pH of the freshly prepared perfusate was always checked and adjusted to 7.4 (if need be) before perfusion was started. Thirty minutes after perfusion and subsequently, the pH of the medium from the inflow reservoir was measured. There was no attempt to measure the pH in the portal venous perfusate different from that of hepatic venous effluent, because, at a recirculation rate of 18 ml.min^{-1} used in these experiments, any difference between this and hepatic venous level was expected to be negligible as was found in the perfused dog liver (RITCHIE & HARDCASTLE, 1973), the difference being less than 0.05 both in the normally perfused liver and in one with induced hypoxia.

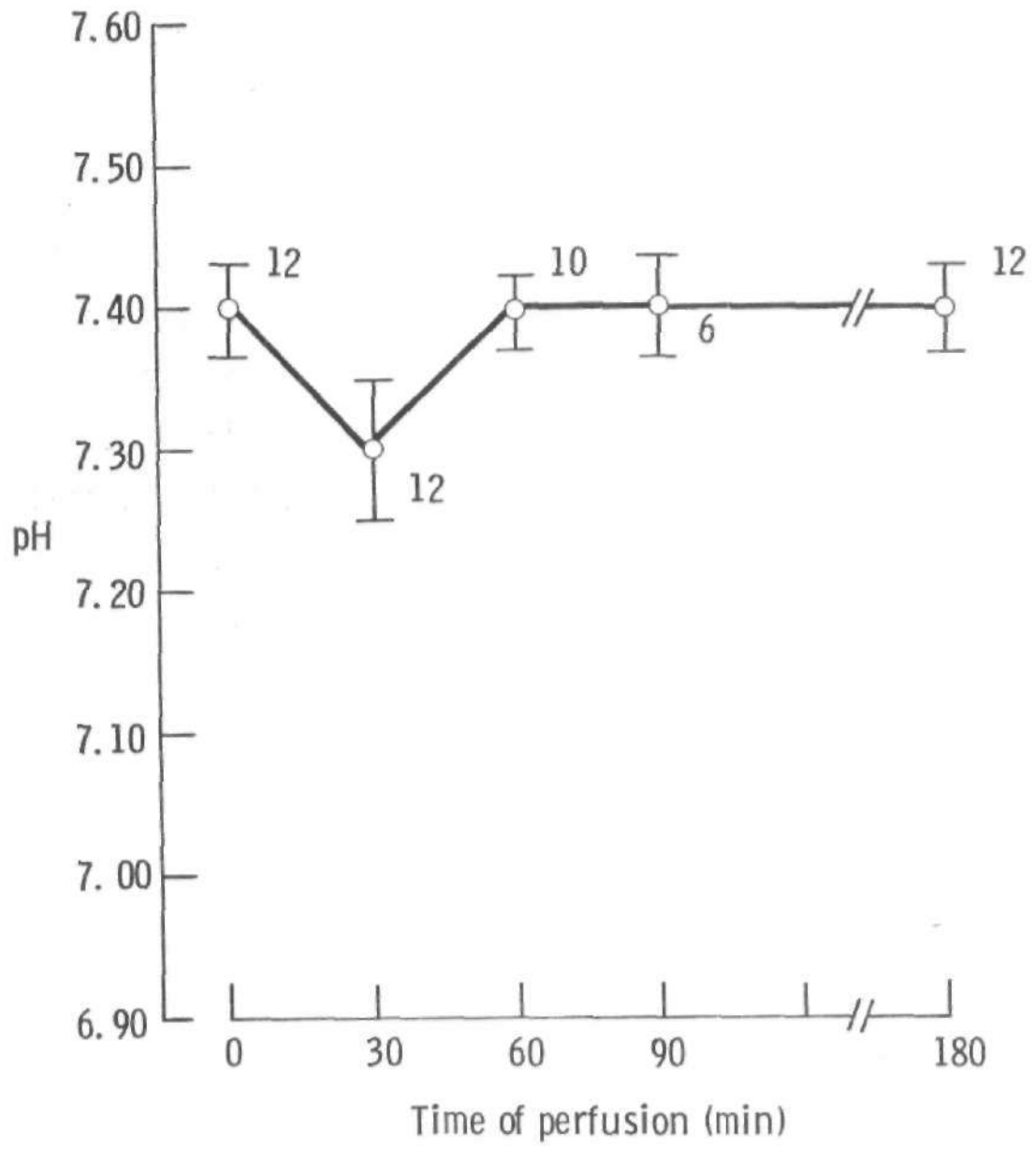
The results of pH changes in the perfusion medium are plotted in fig. 5.5. It was found that the pH dropped to 7.30 ± 0.05 within the first thirty minutes of perfusion (N = 12 livers) but rose to the original 7.40 thereafter and remained steady throughout the experiment. But this slight decrease in pH was not sufficient to be detrimental to the liver function. Indeed it has been shown that isolated

LEGEND TO FIG. 5.5

FIG. 5.5. CHANGES IN pH OF THE PERFUSATE DURING ISOLATED LIVER PERFUSION. THERE WAS A DROP BY 0.1 UNIT DURING THE FIRST 30 MINUTES BUT THIS WAS QUICKLY BUFFERED TO ITS ORIGINAL pH OF 7.40 IN THE NEXT 30 MINUTES. THE NUMBERS 12, 10 OR 6 NEAR THE POINTS OF THE PLOT INDICATE THE NUMBER OF LIVERS USED IN EACH CASE. THE BARS REPRESENT STANDARD ERROR OF MEANS (± S.E.M.).

Figure 5.5

CHANGES IN pH DURING PERFUSION



12, 10 or 6 = number of livers

perfused canine liver can function adequately when maintained at a pH of 7.25 (portal venous) and 7.20 (hepatic venous) perfusate for more than 200 minutes (RITCHIE & HARDCASTLE, 1973). Also BAUR and HELDT (1977) found that pH changes between 7.0 and 7.8 had no effect on D-glucose transport in rat isolated hepatocytes. It was found (RITCHIE & HARDCASTLE 1973) that perfused livers recovered remarkably well within thirty minutes after causing a drop in the perfusate pH from 7.35 to 6.9 by induction of hypoxia for twenty minutes (excluding the oxygenator from the perfusion circuit). Therefore the pH changes observed in these experiments were quite in order, and the drop within the first thirty minutes of the perfusion could be explained as being due to the anoxic period just before the liver was connected to the perfusion circuit. The recovery is not surprising since the bicarbonate buffer continuously gased with the $5\%CO_2$ in $95\%O_2$ was expected to be able to buffer such minor changes in pH in a recirculating medium as used in these experiments. Moreover, since the liver can recover fully from greater drop in pH changes such as when relative acidotic states were induced, like exclusion of oxygenation, it would be expected to recover satisfactorily when only minor change of the type reported here were encounted.

5.1.8 The Choice of gas mixture

The possibility of a lethal effect of high partial pressure of oxygen on the liver was thought of. So it was decided to do experiments to assess the best gaseous mixture to use for gassing the medium (perfusate). The mixtures compared in these experiments were $5\%CO_2$ in air and $5\%CO_2$:$95\%O_2$ mixture. The latter mixture was readily available as a commercial pressurized cylinder of gas ($95\%O_2$:$5\%CO_2$).

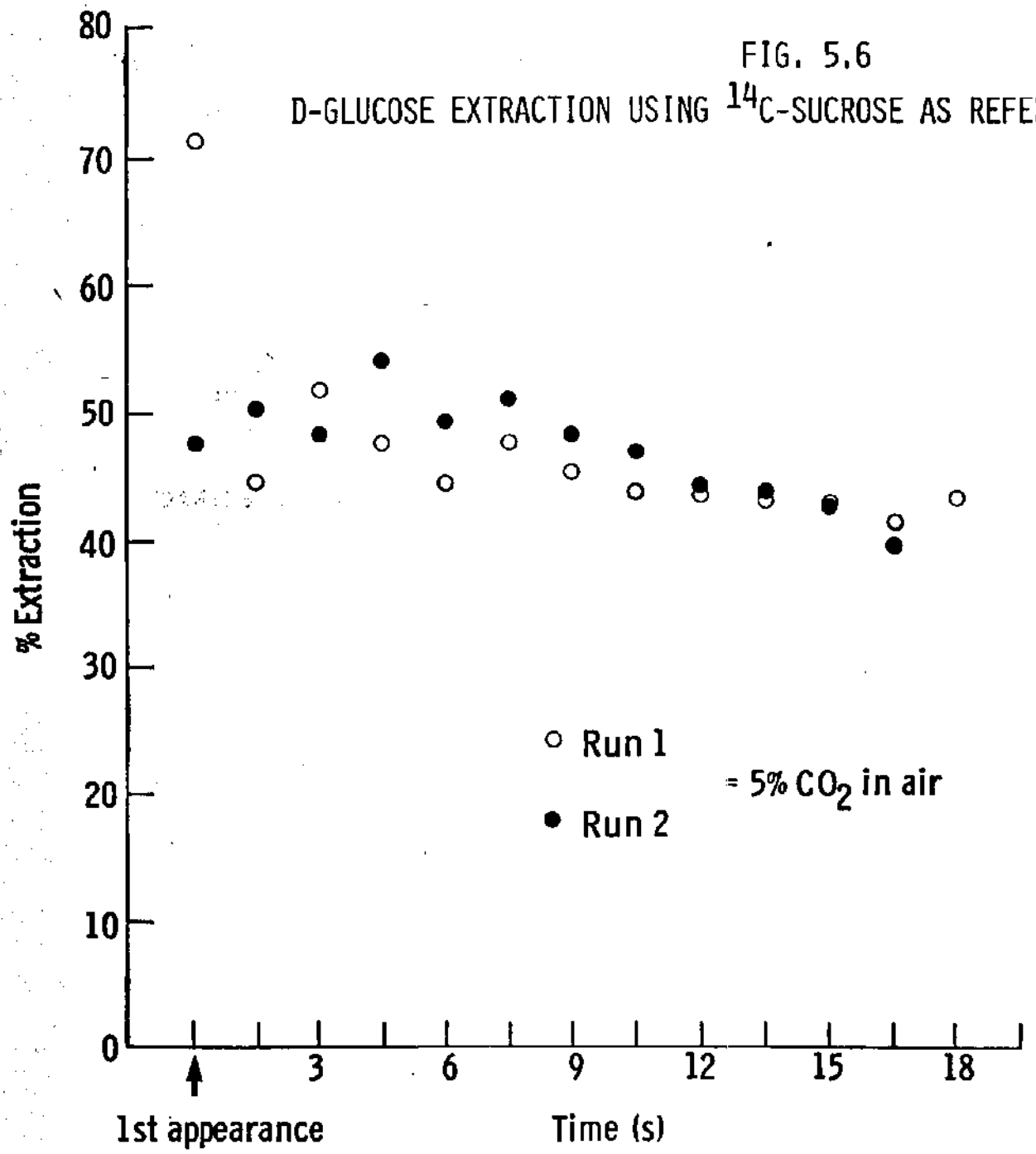
But the 5%CO_2 in air was mixed in our laboratory using a Cunningham Trombone for mixing the gases of the required composition, as described in Chapter 4 (section 4.6). The gaseous mixture (5%CO_2 in air) contained in a Douglas bag was used to aerate the perfusate by passing the gas into the oxygenator chamber with a small gas pump. The spout of the gaseous exchange was reconnected to the Douglas bag so that the escaping gas was recirculated between the bag and the oxygenator thereby making it possible for one Douglas bag containing 100 litres of gaseous mixture to suffice for the two experiments in the same liver (fig. 5.8). For experimental runs three and four, 95%O_2:5%CO_2 mixture was delivered from the cylinder to the oxygenator chamber via a pressure guage.

The results of the two experiments done in the presence of 5%CO_2 in air are shown in fig. 5.8. The extraction of ^{3}H-D-glucose was being measured here with ^{14}C-sucrose as the extracellular reference material. The extraction in run one was 47.58 ± 0.54% (± S.E.M., N = 12) and run two was 49.0 ± 0.58% (± S.E.M., N = 12). There was no significant difference between these two runs ($P > 0.05$, paired t-test). The extraction in run three was 50.16 ± 0.51%, and run four was 48.3 ± 0.62%, (± S.E.M., N = 12, each). There was no significant difference between runs three and four ($P > 0.05$, paired t-test). Also there was no difference between the means of extractions in fig. 5.8 and those of fig. 5.9 ($P > 0.05$, paired t-test). This means there was no significant difference between the extraction of D-glucose in the presence of 5%CO_2 in air and its extraction in the presence of 95%O_2:5%CO_2 mixture. So it was concluded that 5%CO_2 in air did not confer any advantage on the liver, as judged by its performance in transport function, over 95%O_2:5%CO_2 mixture. Since no advantage was

LEGEND FOR FIG. 5.6

FIG. 5.6 SHOWS THE EXTRACTION OF D-GLUCOSE IN THE PRESENCE OF 5% CO_2 IN AIR. EXPERIMENTAL RUNS 1 AND 2 WERE PERFORMED AT 15 MINUTES INTERVAL AND THE EXTRACTION LEVELS WERE SIMILAR IN BOTH RUNS.

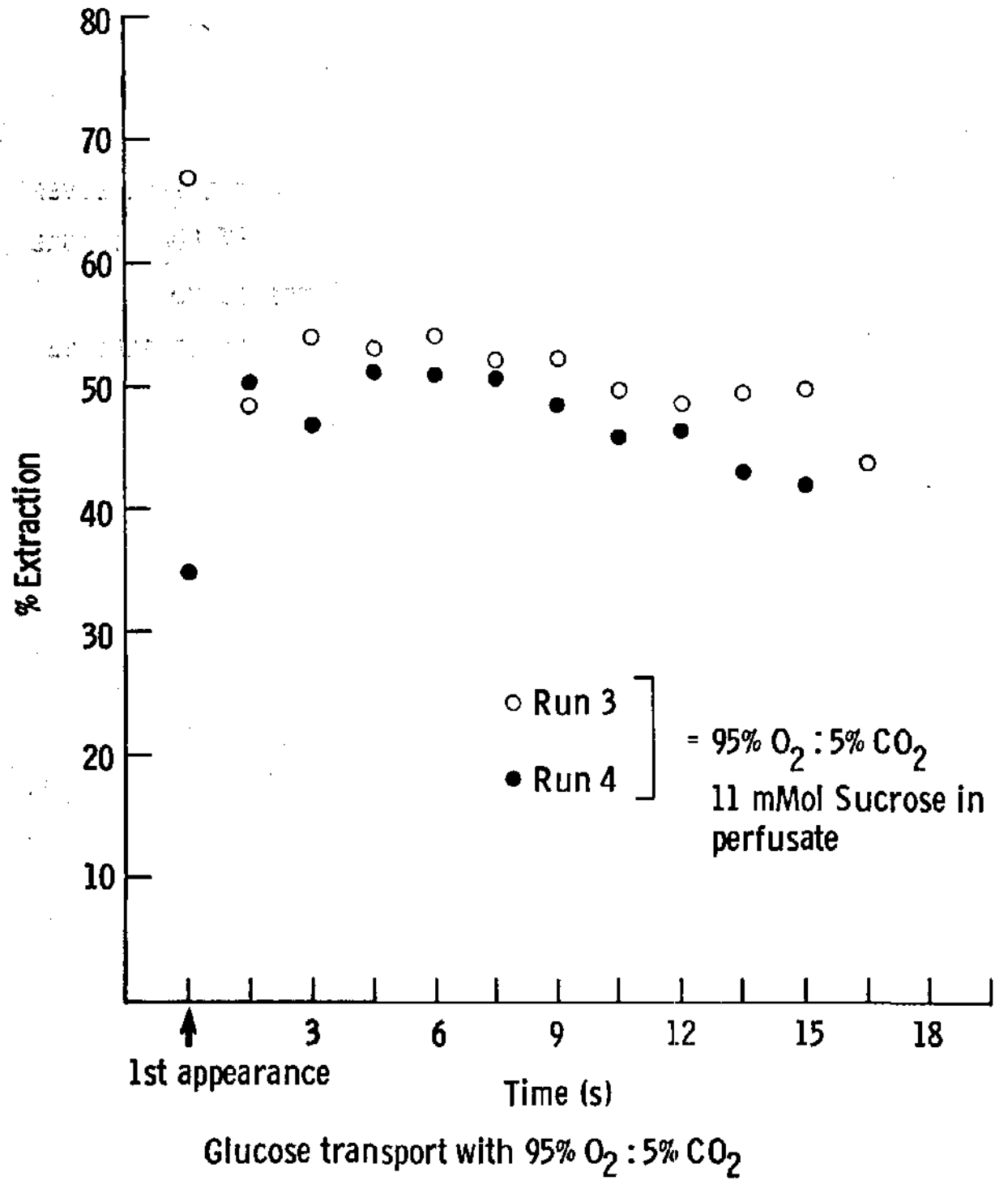
FIG. 5.6
D-GLUCOSE EXTRACTION USING ^{14}C-SUCROSE AS REFERENCE



LEGEND TO FIG. 5.7

FIG. 5.7 SHOWS EXTRACTION OF D-GLUCOSE IN THE SAME LIVER AS IN FIG. 4.4 IN THE PRESENCE OF 95% O_2 : 5% CO_2 MIXTURE AND 11 MM SUCROSE IN THE PERFUSATE. THERE IS NO SIGNIFICANT DIFFERENCE BETWEEN THE EXTRACTION OF GLUCOSE IN THIS FIGURE AND THAT OF FIGURE 4.4.

FIG. 5.7 D-GLUCOSE EXTRACTION IN THE PRESENCE OF 95% O_2:5% CO_2 MIXTURE



Glucose transport with 95% O_2 : 5% CO_2

found for the troublesome procedure of gassing the oxygenator at physiological PO_2, the orthodox use of $95\%O_2$:$5\%CO_2$ from a cylinder was adopted for all subsequent perfusion experiments.

These studies demonstrate that a viable perfused liver preparation is one which was exposed to only a brief period of anoxia, retained its normal morphological appearance without oedema or wrinkling, was maintained on a constant flow rate of about 18ml.min^{-1} with a perfusion pressure of 10-20 cm of perfusate, retained a constant temperature of about 37^{o}C, with a normal oxygen consumption, constant pH, K^{+} and Hb in the perfusate. Livers which failed any of these measured viability criteria were noted down at the time of the experiment and were excluded before the results were known.

5.2 Electron Microscopy of the rat liver

5.2.1 Introduction

The first electron microscope was constructed in 1932 (KNOLL & RUSKA 1932). But it took almost twenty years after that before the application of electron microscope began to yield many results. At present its application is considered indispensable for the study of morphology in many widely divergent fields of research, including cytology and biochemistry. Especially in the latter fields, electron microscopy offers a means to extend descriptive morphology to an almost molecular level, and makes it possible to demonstrate structures whose existence can be established by biochemical methods but which are too small for study by the light microscope.

The electron microscope (EM) has a wide scope of application in research. Its application in the present study was to elucidate morphological changes (if any) in rat liver histology during normal perfusion or during perfusion with medium containing phlorizin as compared to the non-perfused liver.

In its first period of application to biology and pathology, electron microscopy appeared to be less than fully adequate to fulfil its fundamental possibilities for the relation of fine structure to function - relating morphology to biochemistry. NOVIKOFF (1959) argued justifiably that "at best electron microscopy alone remains purely descriptive and essentially morphological. It needs to be supplemented by techniques providing biochemical information." To this may now be added techniques providing physiological information, which is the main object of this section. The morphological findings of the perfused liver in this section were supplemented by physiological parameters as presented in section 5.1 and the transport

phenomena in Chapters 7, 8 and 9. Although the electron microscope is, thanks to the use of electrons, the only instrument available for the direct observations of cellular fine structures, it has definite limitations because the penetrating power of electrons is limited. In the first place, the physical properties of electrons necessitate the maintenance of a vacuum in the microscope column, which, makes the study of living cells impossible. The necessity to observe the specimen in a vacuum requires dehydration of the biological specimens (as was done in these liver specimens - see methods section following this). Prior to dehydration they must be subject to fixation to ensure preservation of cellular organisation during further preparation. It must be kept in mind that eventually fixation leads to transformation of the chemical characteristics of cellular substances and may change the size, shape and spatial interrelationships of cell components. The way in which the fixative is applied, the chemical nature of the fixing compound, the osmolarity and pH of the fixative solution, and the temperature and duration of fixation, can all have a profound influence on the appearance of a cell or cell components (SABATINI, BENSCH & BARRNETT 1963; MAUNSBACH 1966a, 1966b; SJOSTRAND 1967). As a consequence of fixation, moreover, only a certain moment in the dynamic aspect of the living cell can be observed, and therefore an important restriction with respect to time must be accepted. The systematic ultrathin sectioning was used in the present study, as this method was preferred to other types. For this purpose, the specimen is normally infiltrated with a liquid plastic or epoxy resin, which after hardening by polymerization gives a specimen from which sections with the required thickness can be cut. Both the embedding of the specimen and the subsequent sectioning can lead to distortion

or displacement of its components. In addition, during fixation, dehydration or embedding, soluble components of the specimen may be extracted. The specimens must generally be subjected to an augmentation of their mass density by electron staining to obtain adequate image contrast because only structures able to scatter electrons can be located by the electron microscope. Unfortunately, however, only a limited number of specific or even selective, staining methods are available for this purpose as exemplified in the method section immediately following this section. As a consequence, only a limited number of components known from chemical analysis can be present in the cell can actually be localised on the level of fine structure. On this basis it was argued (DAEMS, WISSE & BREDEROO, 1970) that the appearance of the cell in the electron microscope only partially represents the actual composition of the living cell.

5.2.2 Methods

Three different types of liver slices (A, B, C) were prepared; the first liver (A) fixed as unperfused liver, liver B as a normal perfused liver and liver C as a perfused liver with addition of phlorizin.

For liver slices of group A, the rat was anaesthesized and operated in the usual manner (see Chapter 3) short of cannulation for perfusion. Then the right major lobe was removed, cut in small pieces and immersed in 2% glutaraldehyde fixative in 0.1M phosphate buffer for three hours. It was then taken out and immersed in 0.1M phosphate buffer with 10% Sucrose, to wash out any excess fixative from the tissue. The Sucrose buffer was changed five times to give six washings. The liver was taken out and cut in a petri dish into about $1mm^3$ slices which were reimmersed in 10% Sucrose buffer and transported to electron microscopy suite for post fixation preparation for electron microscopy.

The liver B was operated and perfused normally for three hours. At the end of normal perfusion, the liver was perfusion fixed by gravity feed from a 500ml aspirator bottle containing the sucrose buffer by a three-way tap.

The fixative buffer was made up in the following way:

To 110ml of 0.2M KH_2PO_4 and 90ml of 0.2M Na_2HPO_4 were added 50ml of 25% glutaldehyde (fixative) and 150ml distilled water making a total of 400ml.

To make up the 10% Sucrose buffer, 72ml KH_2PO_4 (0.2M) and 90ml of 0.2M Na_2HPO_4 were mixed with 162ml of 20% sucrose giving a total volume of 324ml.

For perfusion fixation, the three-way tap was switched to on-line

with the fixative reservoir and the liver was perfused with the fixative by gravity feed from a column height of about 20cm at the start. The perfusion flow rate decreased as the hydrostatic pressure decreased corresponding to the fall in the reservoir column height. When all fixative has been used up in the perfusion, the three-way tap was switched to on-line with the sucrose buffer and perfusion was carried out with the latter in order to wash out any excess fixative from the liver.

At the end of perfusion with the sucrose buffer, the right major lobe of the liver was removed and $1mm^3$ slices cut from the well fixed (hard) portion of the lobe and transported in sucrose buffer to the electron microscopy suite for further preparation. This was labelled liver B.

The method of preparation of liver C was the same except that the initial perfusion of the liver for the first three hours was done with normal perfusion medium to which has been added 1mM phlorizin. This was done to see if phlorizin had any deleterious effect on the liver histology. After perfusion with the phlorizinised medium, the liver was perfusion fixed and washed as described above for liver B. Then the right major lobe of the liver was removed and $1mm^3$ slices were cut from well perfused (hard) part of the lobe and transported in sucrose buffer to the electron microscopy suite for further preparation. This was labelled liver C.

I am indebted to Mr. J. H. Kugler, Mrs. A. Tomlinson and the electron microscopy technical staff of the Department of Human Morphology, University of Nottingham Medical School, for the further preparation and sectioning of the tissue and production of the electron micrographs. The liver pieces were post-fixed for one hour

in 1% osmium tetroxide in water or Millonigs buffer. Then the material was dehydrated by passing it through a series of increasing strength of alcohol (50% ethanol to absolute alcohol-dried). The specimen was then infiltrated with epoxy propane by immersing it in this liquid for two successive fifteen minute periods. This hardens by polymerization. This was then placed in a 50/50 Epoxypropane/embedding medium containing accelerator overnight in an uncovered pot. The embedding medium was made up of 50% araldite resin (CY212) and 50% D.D.S.A. (dodecenylsuccinic anhydride) a hardener. The accelerator or catalyst used was Benzyldimethylamine (B.D.M.A.). The material was then transferred to full strength embedding medium plus accelerator overnight in a covered pot. The material was then embedded in numbered capsules for flat blocks in fresh full strength araldite with accelerator, and polymerised for 48 hours at 60^{o}C. Ultrathin sections were cut with a microtome and electron micrographs were prepared from representative portions of the speciments as illustrated in the results.

5.2.3 Results and Discussion

The results of the liver histology done by electron microscopy are shown in figs. 5.9 to 5.13 inclusive.

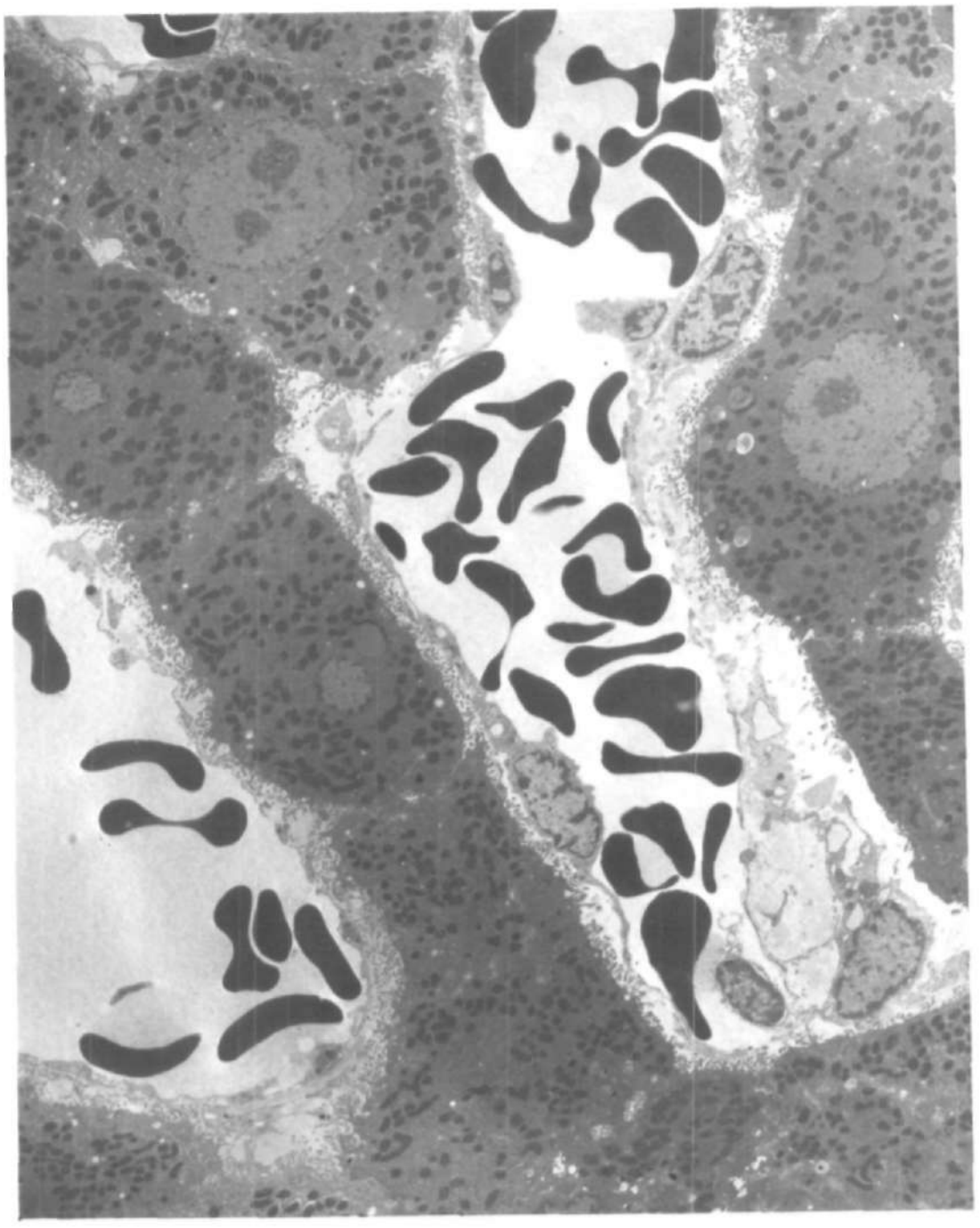
First of all, fig. 5.8 is an exemplary electron micrograph (low powered) of rat liver reproduced with kind permission of J. H. Kugler. It is one of the rare very good electron micrographs of liver section. It is used here as a standard for comparison with the normal unperfused liver histology in this study (fig. 5.9).

Figure 5.9 is the electron micrograph of the unperfused liver A in this study. In comparison with fig. 5.8, it is taken under a higher power than fig. 5.8 (x5,8000) although both are relatively low power micrographs. Also the quality of the micrograph is very much inferior to the prototype (fig. 5.8) one. Furthermore the micrographs represent essentially different portions of the liver - one of the problems due to very high magnification as a limitation of the use of electron microscopy. All these limitations notwithstanding, it appears as if there is no morphological change between figs. 5.8 and 5.9. So fig. 5.9 can be used as a good standard for comparing with the electron micrographs in subsequent figures.

The electron micrograph of a normally perfused liver B after three hours perfusion is shown in fig. 5.10. This compares very favourably with that of fig. 5.9, both of which are at a magnification of x5,800. The following features were notable in comparing the two figures. Figure 5.10 would appear to be from a thinner section than the micrograph in fig. 5.9. However both micrographs have been taken from apparently similar site of the liver lobule. There is no disorganisation of cellular organelles in either of them. There is neither mitochondnal swelling nor lysosomal ruptures in either of

FIG. 5.8

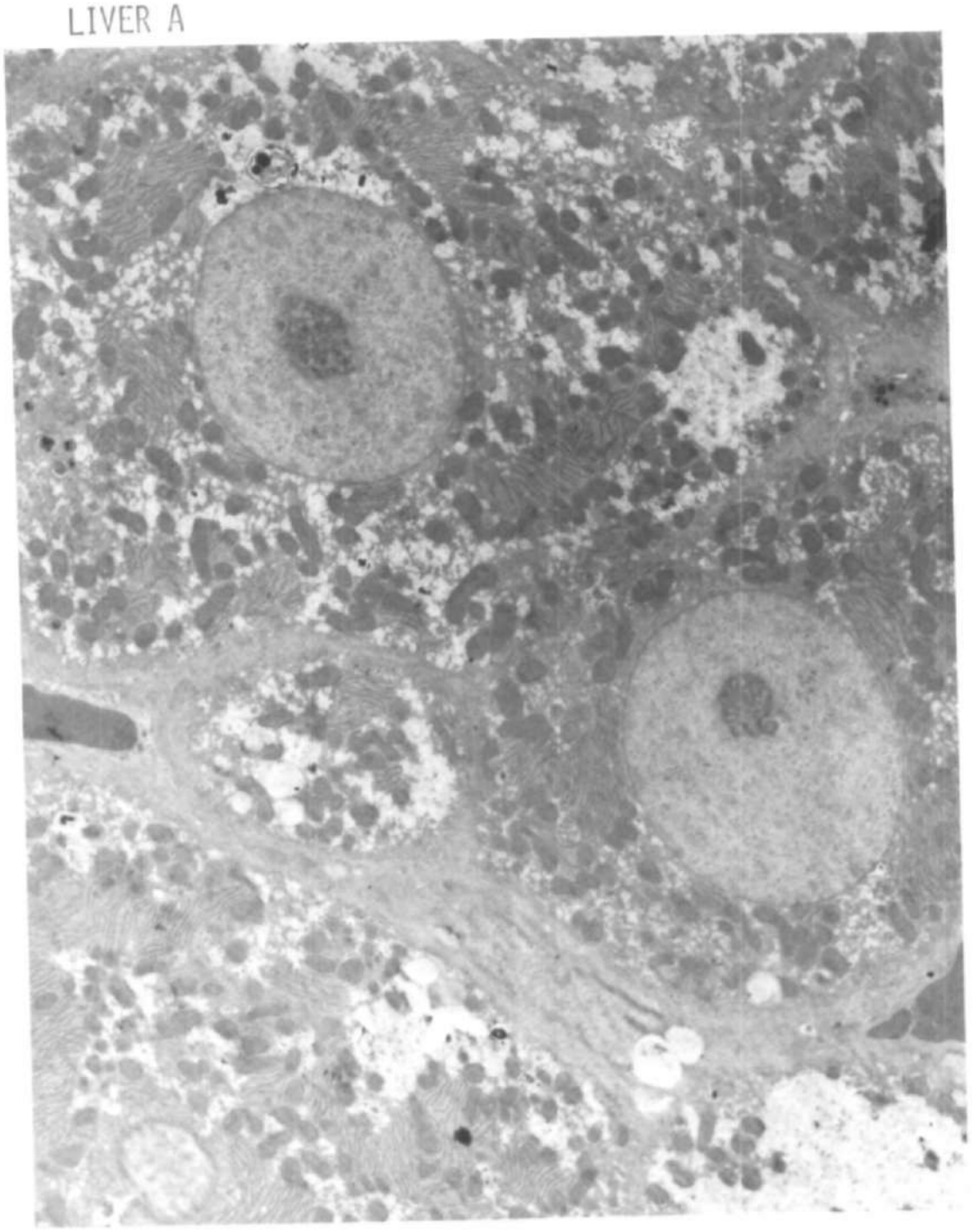
ELECTRON MICROGRAPH OF A NORMAL RAT LIVER - LOW POWER



UNPERFUSED LIVER

(REPRODUCED WITH KIND PERMISSION OF MR. J. H. KUGLER)

FIG. 5.9
ELECTRON MICROGRAPH OF AN UNPERFUSED RAT LIVER - LOW POWER
LIVER A

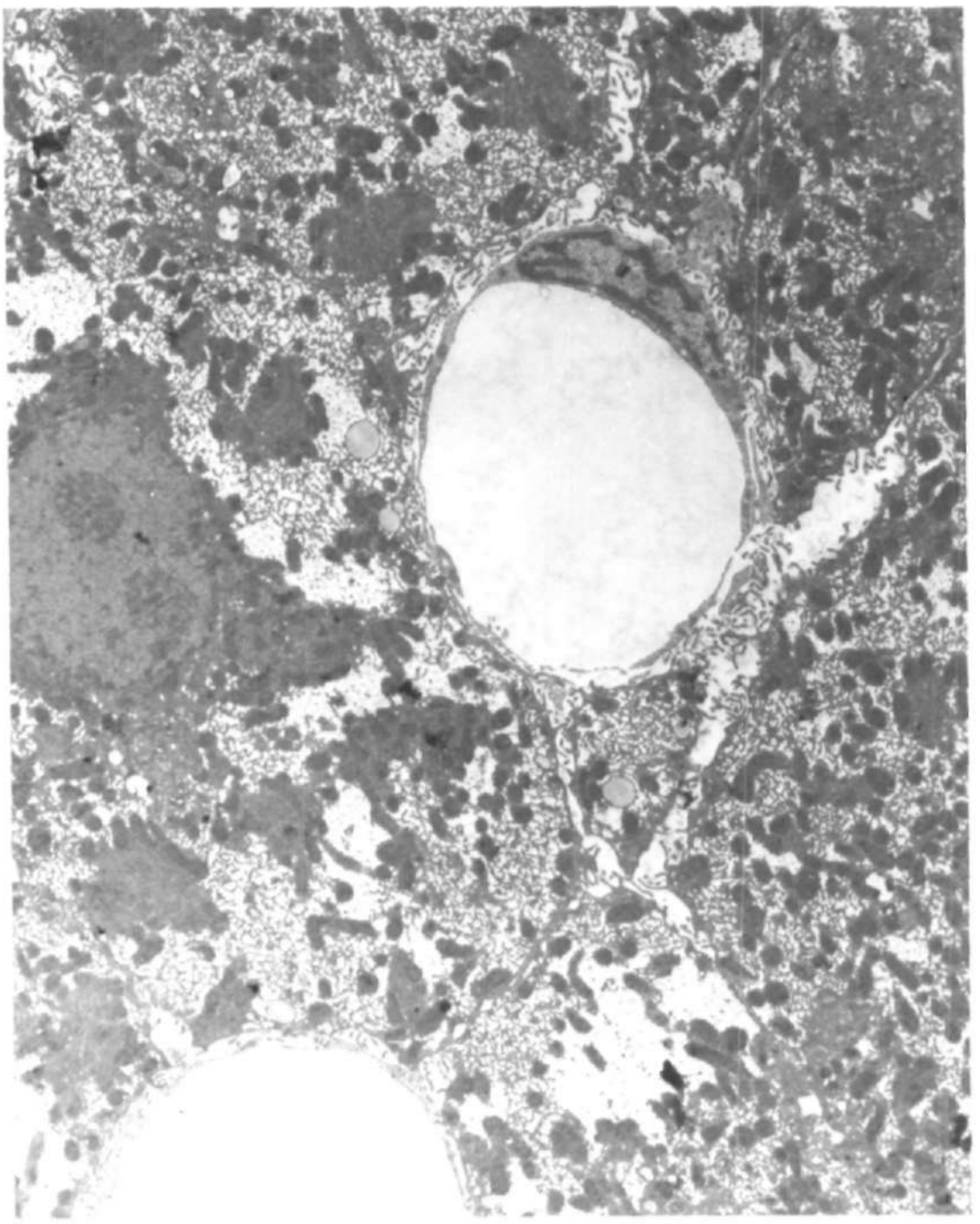


UNPERFUSED LIVER X 5,800

FIG. 5.10

ELECTRON MICROGRAPH OF A PERFUSED LIVER - LOW POWER

LIVER B



PERFUSED LIVER X 5,800

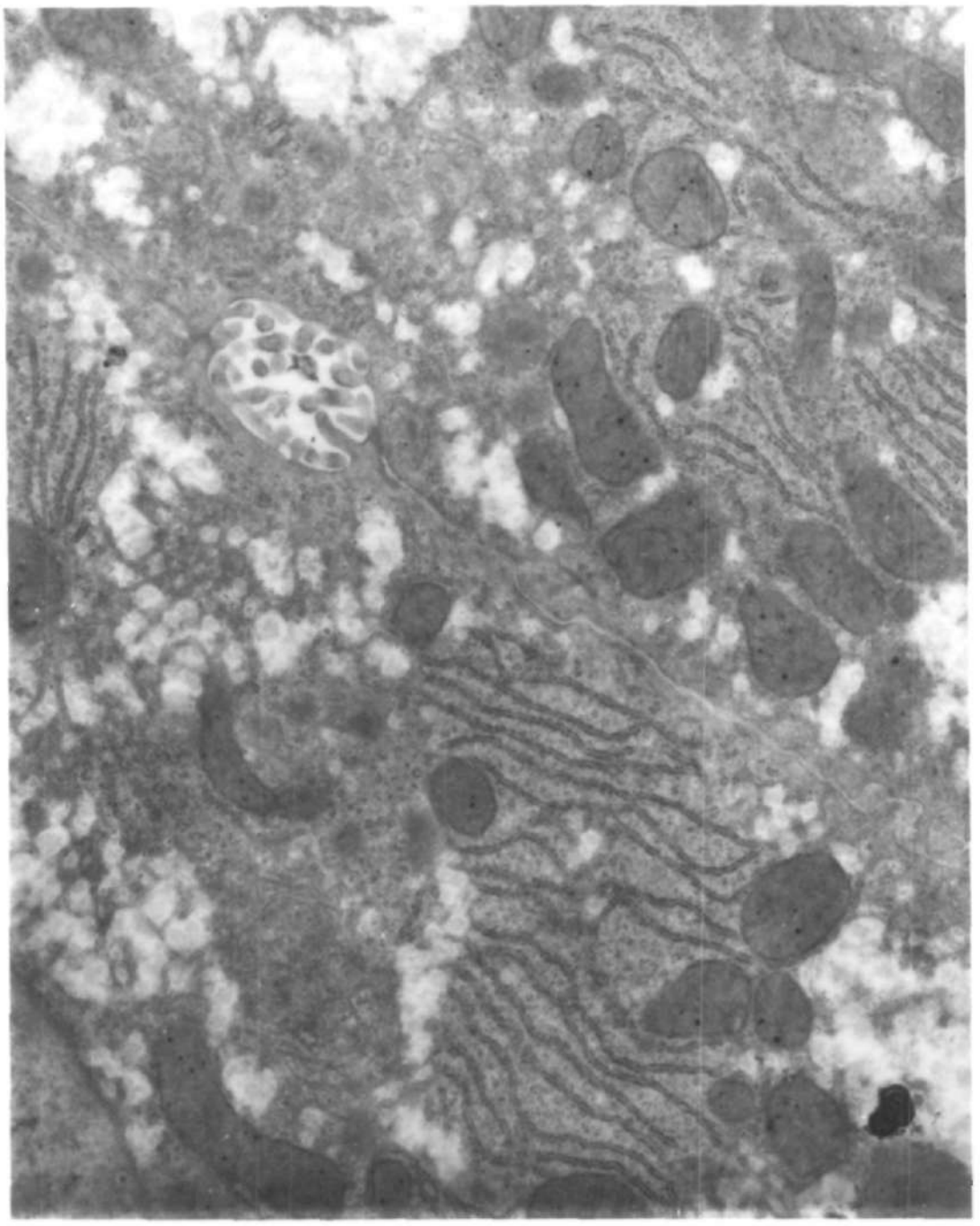
the micrographs, which would otherwise indicate liver damage. These findings indicate that the morphological appearance of the perfused rat liver is as good and unperturbed as that of normal rat liver when compared electron microscopically. This finding supports the proposition that this method of perfusion maintains the liver adequately, and that functions such as sugar transport (Chapters 7, 8 and 9) are likely to be comparable to those in the intact organ in situ.

Figure 5.11 is an electron micrograph of the unperfused liver A at high magnification x 23,000 compared with the micrograph of a perfused liver B (fig. 5.12) at the same magnification. The two photographs have been taken from similar sites in the respective liver lobules. These two become easier to note the fine details at this higher magnification when compared. Hence it has been possible to note that there is a greater preponderance of endoplasmic reticulum in the unperfused liver (fig. 5.11) compared to the perfused liver (fig. 5.12). Apart from this, other structures appear to be the same. Furthermore, the endoplasmic reticulum in the perfused liver (fig. 5.12) is predominantly the smooth type whereas that in the unperfused liver (fig. 5.11) is predominantly the rough type. For transport function which this study is mainly concerned with, these differences are not of grave concern. These findings would be of greater importance if protein synthesis was the main object of this study. Moreover, electron microscopy can show only a stage in the dynamic aspect of a living cell, therefore the level of protein synthesis by ribosomes and hence the preponderance of either rough or smooth endoplasmic reticulum over the other in a particular liver section will vary from one section to the other. So it is not even possible nor judicious to attribute these differences to perfusion of the liver B on the one

FIG. 5.11

ELECTRON MICROGRAPH OF AN UNPERFUSED LIVER - HIGH POWER

LIVER A

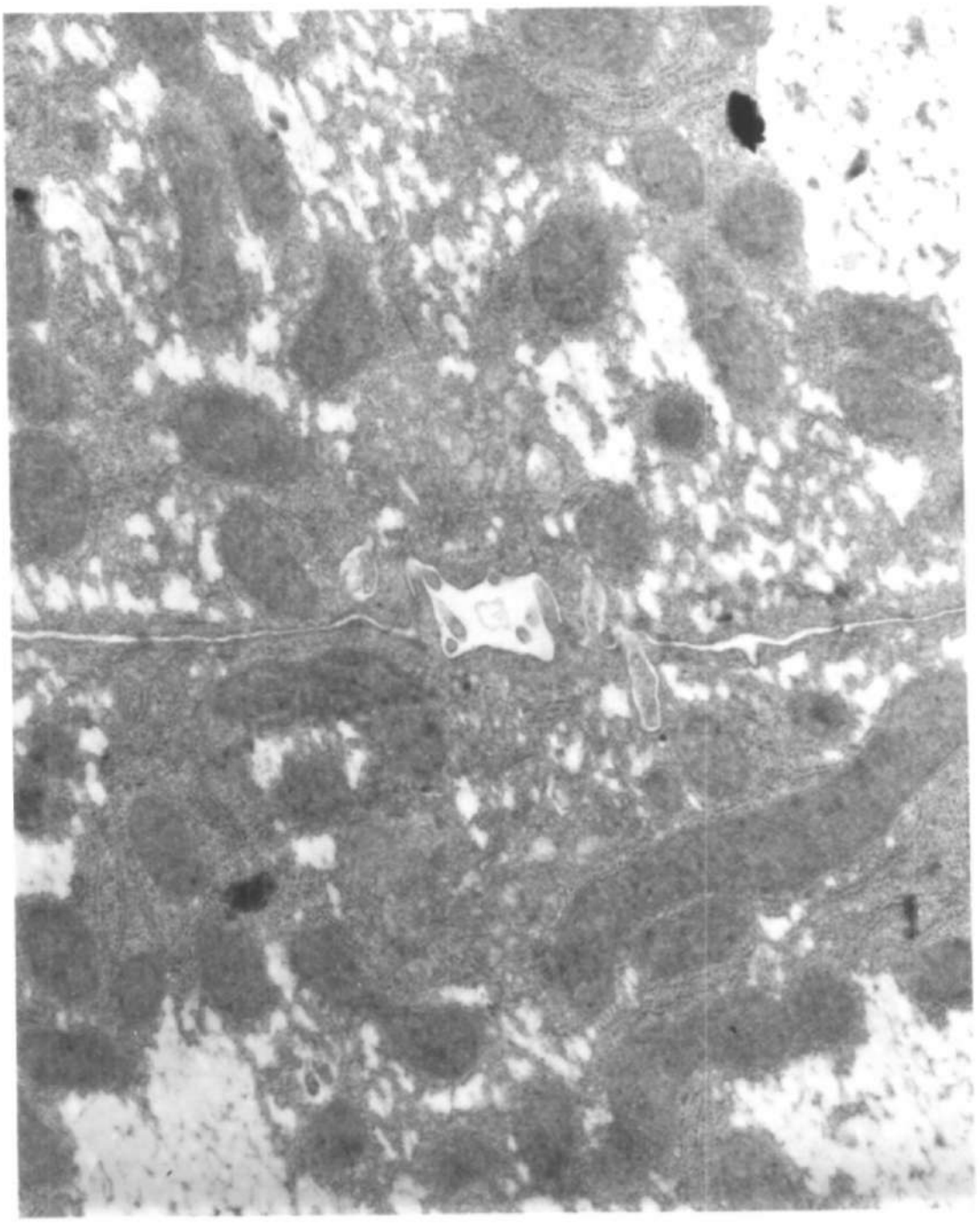


UNPERFUSED LIVER X 23,200

FIG. 5.12

ELECTRON MICROGRAPH OF A PERFUSED LIVER - HIGH POWER

LIVER B



PERFUSED LIVER X 23,200

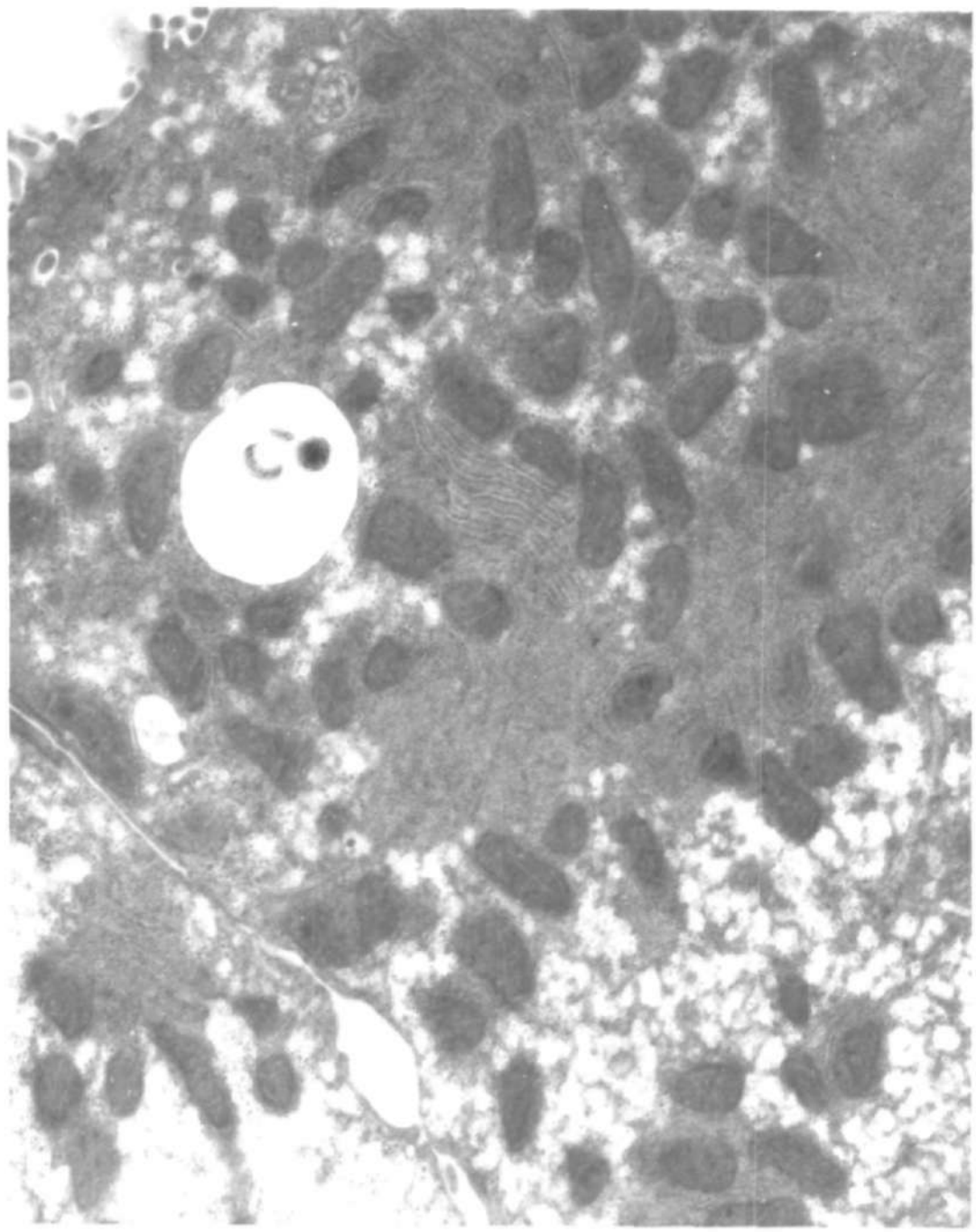
hand and nonperfusion of the liver A on the other hand.

Figure 5.13 is the electron micrograph of the liver perfused with the perfusion medium to which was added phlorizin at a concentration of 1mM. This figure was compared with figs. 5.11 and 5.12 and there was no morphological difference between it and the other two. It was therefore concluded that the phlorizin did not cause any morphological damage or change to the liver. This was an important finding because the effects of phlorizin is interpreted as a specific chemical inhibition of transport and not a general depression of cell metabolism. The morphological appearance did not show any appreciable change from the control which would otherwise suggest damage to the liver, and this finding was supported by the lack of functional deterioration when 1mM phlorizin was present in the medium. The functional integrity of the liver was manifested by the observation that the transport function of alpha-aminoisobutyric acid (AIB), which was not affected by presence of phlorizin, was retained to the same magnitude at the end of the experiment as at the start. In other words, AIB transport remained unaltered at the experimental run 8 as at run 1 although phlorizin significantly inhibited the transport of hexoses added in runs two to seven (between the two AIB runs) - see Chapters 7, 8, and 9. AIB is a non-metabolised analogue of the neutral amino acids and was used as representative of another class of substrates which the transport might be expected to reflect any non-specific metabolic damage to the cell.

AIB transport studies have not been further reported in this study but are mentioned here because of the importance of these findings to the validation of the inhibitory effects of phlorizin on the hexoses, and the fact that its effects show functional recovery of the liver in

FIG. 5.13

ELECTRON MICROGRAPH OF A PERFUSED LIVER WITH ADDED PHLORIZIN - HIGH POWER LIVER C



PHLORIZIN PERFUSED LIVER X 23,200

transport function.

It is therefore concluded that neither perfusion itself nor addition of phlorizin caused significant structural damage to the liver.

CHAPTER SIX

6. VARIABILITY AND SOURCES OF ERROR

6.1.1 Measurement Accuracy

The precision of sampling and pipetting techniques, and the accuracy of counts by the β-counter were measured using varying concentrations of isotopic mixtures sampled into ten vials for each experimental concentration. Four experiments were done with ten sample vials in each. Each of the four experiments had a different concentration of the isotopic mixture, experiment one having the lowest, and experiment four the highest. The samples were withdrawn and flushed into the vials using an autodiluter (Chapter 3) the spout of which is the tube that was washed one, two or three times in these experiments. The sample vials were counted and the activity in each vial calculated (nuclear disintegrations per minute, dpm) for [^{14}C]-L-glucose (reference) and [^{3}H]-D-glucose (test substances). In experiments one and two the sampling tube was washed once between samples, and twice in experiment three, and thrice in experiment four. The results of the activity in each experimental group are tabulated (Table 6.1). There was high volumetric accuracy and low variability as manifest in the low standard error of means (S.E.M.) and low coefficient of variation (C.V.%) respectively for the ^{14}C- and ^{3}H-activities in each experiment. However, the C.V. for experiments three and four were lower than those of experiments one and two. Assuming equal counting efficiency by the β-counter for all the experiments (which was in fact the case as shown by the efficiency, ^{14}C-overlap, and ^{3}H-overlap in the different channels obtained in the computer print-out of the dpm results), the lower C.V. for experiments

LEGEND TO TABLE 6.1

TABLE 6.1 - VOLUMETRIC ACCURACY. SAMPLING AND PIPETTING TECHNIQUE CAN BRING A WIDE RANGE OF VARIABILITY IF NOT CAREFULLY DONE, AND THE NUMBER OF WASHINGS OF THE SAMPLING NOZZLE BETWEEN SAMPLES CAN AFFECT RESULTS. IN THIS TABLE, THE LOW COEFFICIENT OF VARIATION IN EXPERIMENTS 3 AND 4 (2 AND 3 WASHES RESPECTIVELY) FURTHER STRENGTHEN THIS ARGUMENT.

TABLE 6.1 Volumetric Accuracy

	Experiment			
	1	2	3	4
Number of Samples (N)	10	10	10	10
No. of washes	1	1	2	3
^{14}C-$\frac{dpm}{\bar{X}}$	2,031	2,904	4,888	6,265
S.E.M.	4.5	12.9	4.3	8.8
C.V.%	2.2	4.4	0.9	1.4
^{3}H-$\frac{dpm}{\bar{X}}$	7,125	13,704	13.729	12,011
S.E.M.	21.5	59.4	15.7	14.5
C.V.%	3.0	4.3	1.1	1.2

three and four, can be looked at as some other source of variability, in this case in experimental technique, not the counter. The only differences in the experimental technique were the number of times the sampling tube was washed in between the samples and the dose in each experiment. In experiment three, it was washed twice, and in experiment four thrice, so it can be conjectured that washing the tube at least twice reduces the possible contamination of the new samples with unwashed isotopic material from the previous sample. In experiments one and two, the washing was once only but the dose in experiment two was higher than in experiment one. Also the C.V. was higher in two than one. If the increase in dosage was responsible for the higher C.V. in two than one (both have equal number of washes) then experiments three and four would have correspondingly higher C.V.'s than one and two. But the reverse was true. And the only variation between the first two and last two groups of experiments was the number of washes. So the low C.V.'s in three and four could be attributable to the washing. Hence it was decided from these results to wash the tube twice between samples in each experimental run, and at least six times between runs. By doing so the rising level of blank samples from one run to another was kept to the barest minimum. Since the first two blanks at the beginning of each experimental run were used for the calculation of extraction in all subsequent samples in that run, ensuring correct blank counts, and hence actual activity (dpm), by this means is of paramount importance to getting good results.

6.1.2 The handling of the isotopic materials by the liver

It was necessary to find out how the infused isotopic materials behaved with regard to the quantities detected in the effluent samples collected serially at 1.5 second intervals, so that an idea of the liver handling of these materials could be built up. To this end, the activities of ^{14}C-Sucrose and ^{3}H-D-glucose (extracellular reference and test materials respectively) were determined and plotted as in fig. 6.1, A, B, C, D. The plot of absolute activities of the ^{14}C- and ^{3}H- (fig. 6.1A) shows a sharp and steady rise of both isotopes for the first nine seconds, thereafter the extracellular reference material [^{14}C-] attained a plateau level till the 18 second sample. After 18 seconds there was another rise in the ^{14}C-dpm indicating a recirculation of the isotopic material. The ^{3}H-dpm attained no plateau but showed a steady rise during the period the ^{14}C-dpm showed a plateau. This difference in the handling of the two substances by the liver made it possible for the extraction of one of them (^{3}H-D-glucose in this case) by the liver to be worked out from the difference in the ratio between the two substances in the infusate and the samples collected at the venous end. Such ratio to be used is the $^{3}H/^{14}C$ activity in the effluent samples as plotted in fig. 6.1B. However, this ratio in fig. 6.1B called the relative activity of the one isotope to the other (in this case $^{3}H/^{14}C$) was worked out in both the infusate and in the effluent samples called R_i and R_e respectively. The ratio between these ratios R_e/R_i usually called the throughput (GORESKY, BACH & NADEAU 1973) is the material which swept pastthe liver cell membrane without entering at that time (fig. 6.1C). It is a mirror image of the material that actually penetrated the liver, called extraction (fig. 6.1D) because the extraction is the

LEGEND TO FIG. 6.1

FIG. 6.1. THE HANDLING OF THE ISOTOPIC MATERIALS BY THE LIVER.

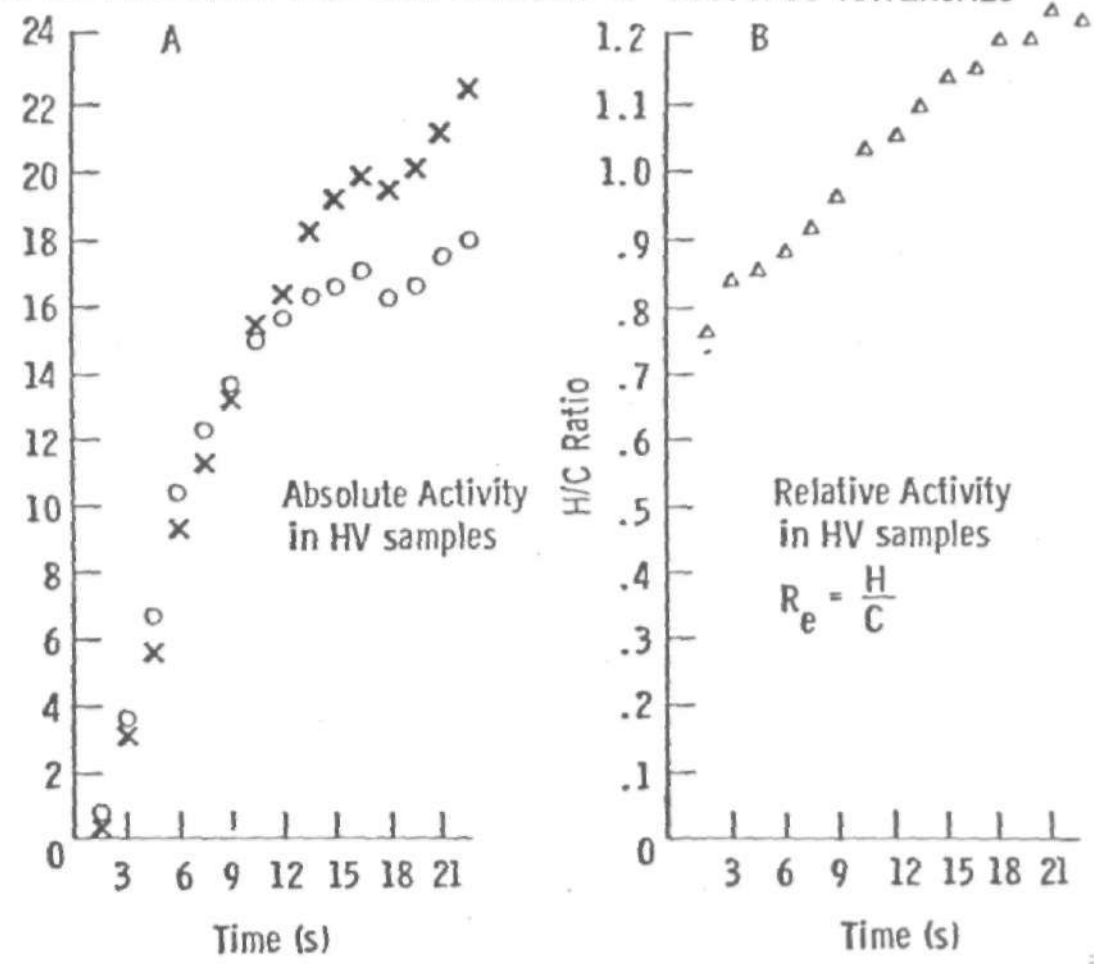
A: THE ABSOLUTE ACTIVITY OF EACH ISOTOPIC MATERIAL IN THE HEPATIC VENOUS SAMPLES (^{14}C- AND ^{3}H-DPM).

B: RELATIVE ACTIVITY IN THE HEPATIC VENOUS EFFLUENT SAMPLES IN THE RATION OF ^{3}H-/^{14}C-DPM).

C: THE THROUGHPUT - THIS SHOWS THE PROPORTION OF THE ISOTOPIC MATERIAL THAT WAS INITIALLY PUT IN THE INFUSATE THAT WENT PAST THE LIVER CELL SURFACE WITHOUT ENTERING THE CELL AND HENCE RECOVERABLE IN THE VENOUS EFFLUENT SAMPLES.

D: THE ACTUAL PERCENTAGE EXTRACTION AS A MEASURE OF THE AMOUNT PENETRATING THE HEPATOCELLULAR SPACE IN A SINGLE PASS. LIKE THE THROUGHPUT, THIS IS AFFECTED BY NOT ONLY THE MATERIAL THAT ENTERED THE CELL OR REMAINED UNENTERED BUT ALSO BY THE MATERIAL THAT FIRST ENTERED AND LATTER REFLUX BACK INTO THE MEDIUM. THIS IS REFLECTED BY THE INCREASING LEVEL OF THROUGHPUT IN FIG. 6.1C AND MIRROR IMAGE DECREASING LEVEL OF EXTRACTION IN FIG. 6.1D.

FIG.6.1 DISTRIBUTION AND COMPUTATION OF ISOTOPIC MATERIALS



difference between the whole number (or the total isotopic material put in at first) and this throughput is shown by the equation

$$\text{Extraction, } E = 1 \frac{R_e}{R_i}$$

$$\text{and \% Extraction} = (1 - \frac{R_e}{R_i} \times 100)$$

This is the basis for the computer calculation of the extraction of the text substance by the liver in each sample of an experimental run.

6.1.3 Reproducibility

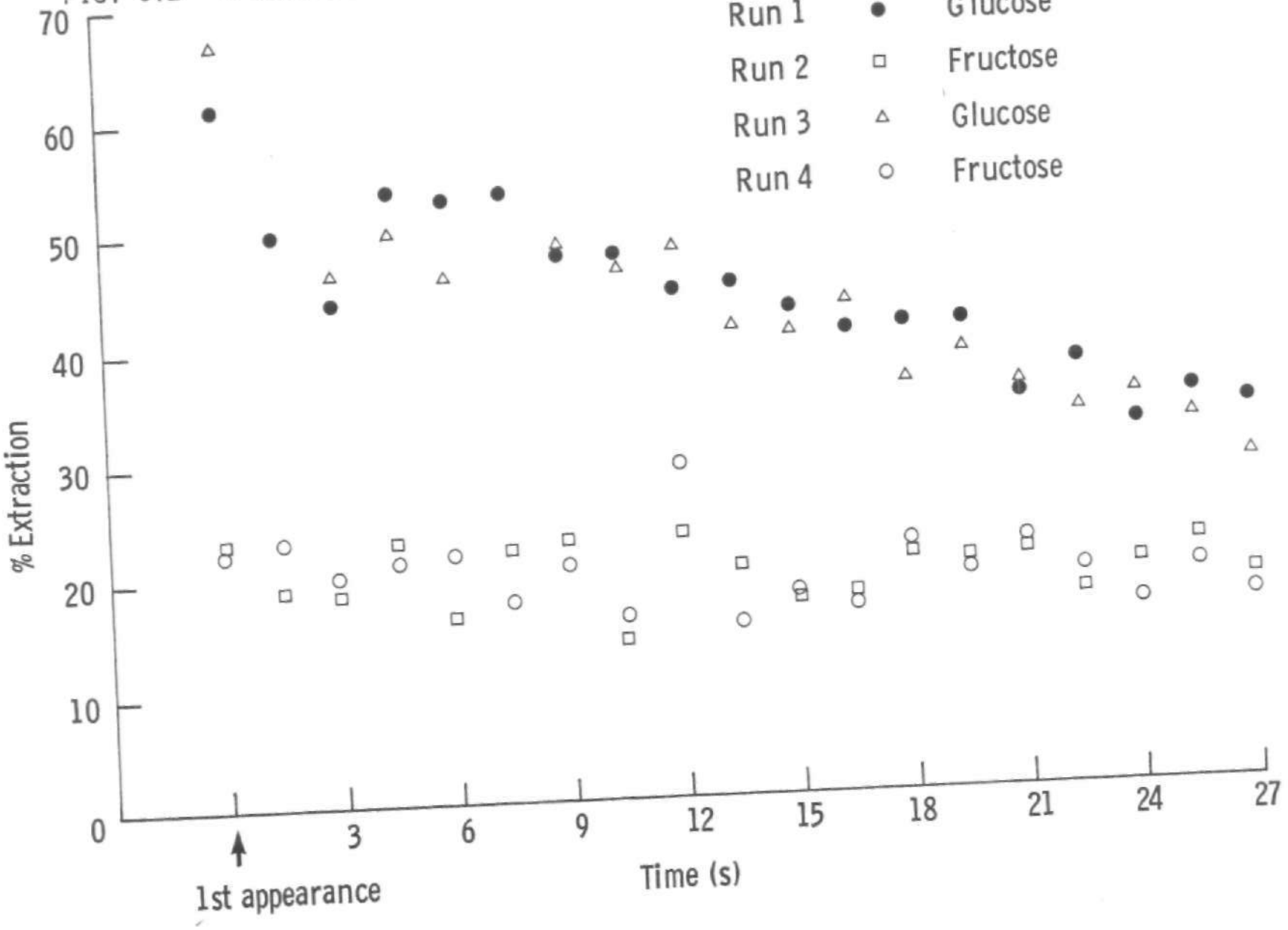
Experiments were done to assess whether reproducible results could be obtained with this preparation. A mixture of isotopes, ^{3}H-D-glucose or ^{3}H-D-fructose as test materials plus ^{14}C-L-glucose in each case as extracellular reference material, was infused into the rat liver for 40 seconds and venous effluent samples were collected for a total of 40 samples at 1.5 second intervals. Four experimental runs were performed during which the glucose run was alternated with fructose run, so that the first and third runs were glucose runs, while the second and last runs were fructose. The runs were performed at 30 minute intervals so that one hour elapsed between one glucose run and the other, and similarly for fructose runs which were done at the intervening periods. The whole time of experiment from run one to four lasted for two hours, following the first thirty minutes of the equilibration period.

The results of extraction of D-glucose and D-fructose are shown in fig. 6.2. There was good agreement between the extractions in runs one and three (D-glucose extraction) although the two experimental runs were performed in the same liver at 60 minutes apart. Also the extractions in runs two and four (D-fructose) show good agreement with each other, although they were also separated by 60 minutes. What is more interesting is that during the intervening 60 minutes, the alternate sugar was infused, and yet this did not alter the level of extraction of either sugar, nor did the time lag. If the glucose run (3) done after the first fructose run (2) did not show the same level of extraction as the first glucose run (1), then it could be suggested that the lower extraction of fructose found in run two was a manifestation of deterioration in the liver transport

LEGEND TO FIGURE 6.2

FIG. 6.2 SHOWS REPRODUCIBILITY USING D-GLUCOSE AND D-FRUCTOSE INFUSION REPEATED 60 MINUTES AFTER THE FIRST INFUSION OF THE PARTICULAR SUGAR. THE SUGARS WERE ALTERNATELY INFUSED AND THE WHOLE EXPERIMENT LASTED 120 MINUTES, BUT THE RESULTS WERE SIMILAR AT THE BEGINNING TO THOSE AT THE END OF THIS PERIOD.

FIG. 6.2 D-GLUCOSE AND D-FRUCTOSE EXTRACTION USING ^{14}C-L-GLUCOSE AS REFERENCE



function. But this is not the case, since run three showed comparably similar level of extraction as run one (both being D-glucose extractions) and run four showed similar level as run two (both being D-fructose extraction). These results suggest that the experiments can give reproducible results in the same liver which are dependable. It also indicates that the liver transport performance with these hexoses can be maintained for at least 120 minutes. Experiments done in four different livers following exactly the same protocol showed little variability between the livers resulting in low standard error values for D-glucose transport of 49.58 ± 1.58, D-galactose 45.34 ± 0.93, and D-fructose 27.25 ± 0.91 (Mean ± S.E.M., N = 4).

6.2 Sucrose as an extracellular reference substance

6.2.1 Introduction

Labelled sucrose has been used as an extracellular reference material in the liver by many workers. CAHILL, ASHMORE, EARLE and ZOTTU (1957) showed that sucrose and maltose were restricted to the extracellular fluid compartment of wistar rat liver. Also GORESKY (1963) demonstrated that sucrose does not enter the liver cell of anaesthetized dogs to any significant level. He went further to show that entry of inulin into liver cells of anaesthetized dogs was intermediate between those of labelled albumin-T-184 and labelled Sucrose-C^{14} (GORESKY 1965). The entry of labelled sodium coincided with that of Sucrose. Inulin, therefore, seems to be better retained within the extracellular fluid compartment than sucrose. He showed that none of this group of substances was retained in the liver during a single circulation. Their recoveries in the outflow (venous effluent) were complete in a study using rapid single-injection multiple-indicator dilution technique. On the basis of these findings he concluded that sucrose was a suitable extracellular reference material for indicator dilution studies in the liver. Consonant on this, he went on to use sucrose as the extracellular reference substance in the transport of galactose by the liver of anaesthetized dogs (GORESKY, BACH & NADEAU, 1973). Also HOOPER and SHORT (1977) used sucrose as the extracellular reference material in their study of liver uptake of glucose, galactose and fructose in the conscious sheep. Although these latter workers had shown that sucrose did not penetrate sheep erythrocytes to any measurable extent and was only slightly inferior to inulin in this respect, no attempt was made to assess the penetrability of sucrose into the liver cell. However,

WILLIAMS, EXTON, PARK and REGEN (1968) did not use sucrose as the extracellular reference substance in their study of glucose transport in the perfused rat liver; they used inulin as their reference substance. From their fractional retention curve of D-glucose, L-glucose and inulin, one could say that inulin is a better reference than L-glucose, although these workers obviously had no intention, and did not compare these two substances in this perspective. They were primarily concerned with the differential distribution of D- and L-glucose in rat liver after different times of equilibration of the sugars. BAUR and HELDT (1977) compared the uptake of L-glucose and sucrose by the isolated hepatocytes and found that L-glucose was taken up more than sucrose. So sucrose would appear to be a better extracellular reference substance than L-glucose. In fact they contended that the liver cell appears to be virtually impermeable to sucrose. This notwithstanding they still used inulin as the reference substance as this was thought to be a better reference substance than sucrose - there had been no experimental evidence in their work for this assumption. But earlier work by FORKER (1970) seems to be in contradistinction to the finding of virtual hepatocellular impermeability of sucrose. He found 15% sucrose, 5% inulin and 30% mannitol to appear in bile after one to five hours of experiment (FORKER 1970). In support of these findings, GLASINOVIC, DUMONT, DUVAL and ERLINGER (1975) found that erythritol and mannitol were taken up by the dog liver. Furthermore, PARDRIDGE and JEFFERSON (1975) found mannitol, inulin and sucrose to be taken up by the rat liver. They showed that the liver uptake indices (LUI's) were sucrose 24.3, inulin 27.7, and mannitol 80.2. This appears to be the only report in literature where inulin uptake by the liver is greater than that of sucrose. But no

proper studies have been done to compare sucrose with L-glucose or inulin as an extracellular marker in the liver. No method has been validated for the measurement of the extracellular-fluid volume either. And if measured, such a volume may be an intellectual abstraction rather than an anatomical entity. The volumes of distribution ("spaces") for inulin, sucrose and mannitol are somewhat similar to one another, averaging about 16-18% of the body weight (CHESLEY, UICHANCO, LENOBEL & KELLERMAN 1964); for this reason they have been taken as estimates of the extracellular fluid volume. Actually they may be underestimates, for sucrose, mannitol and inulin all enter the liver to variable extent. Against this background, it became necessary to validate the use of labelled sucrose as an extracellular reference substance in this study.

6.2.2 Methods

a) Compartmental behaviour of sucrose

In order to ascertain that the reference used in these experiments diffuses as freely in the extracellular space as the test material, the absolute activity of both the reference and the test materials (that is, dpm of ^{14}C or ^{3}H) in the hepatic effluent venous samples was determined. The method is the same as previously described in Chapter 3. In two identical distribution experiments ^{14}C-L-glucose was used as the reference material in one case and ^{14}C-sucrose in another together with ^{3}H-D-glucose in each case. The behaviour of the reference materials in the two cases was compared.

The extraction of the test material was calculated from the above experiments and these values were compared when ^{14}C-L-glucose was the reference in the one case and ^{14}C-sucrose was the reference

in the other.

Furthermore it was decided to find out experimentally if the sucrose itself penetrated into the liver. In order to find this out, ^{3}H-sucrose was chosen as the test material and ^{14}C-L-glucose or ^{14}C-sucrose as reference material in four experiments with each reference. The "apparent" extractions of ^{3}H-sucrose using either reference were compared. The term "apparent" is explained in the discussion section immediately following the results of this section (i.e. 6.2.4).

Since ^{3}H-sucrose appeared to have penetrated the liver, it was decided to see if any of the sugars (potential competitors) would affect this apparent extraction. So ^{3}H-sucrose as a test material with ^{14}C-L-glucose as reference was infused in the presence of Krebs bicarbonate buffer (as control run), and in the presence of unlabelled either 25mM sucrose, D-glucose, Lactose, D-galactose, Maltose or D-fructose in that order. The effects of these potential competitors on the "apparent" extraction of the ^{3}H-sucrose were compared with the control run.

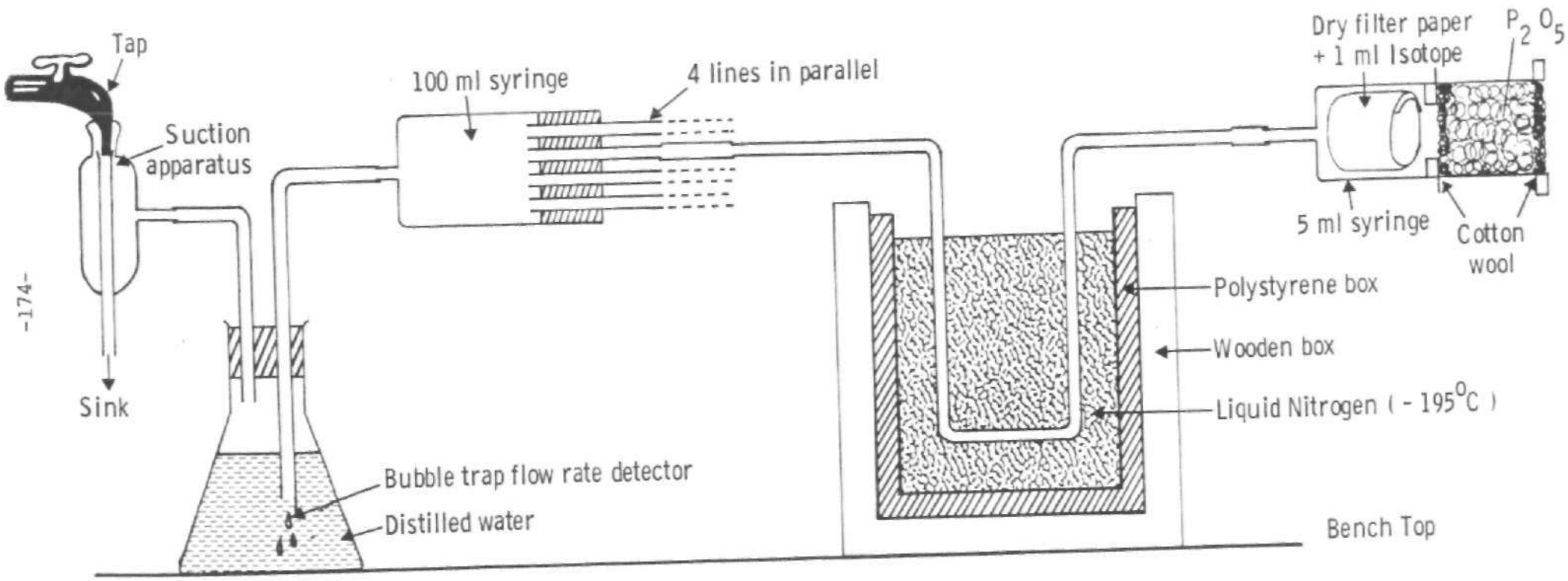
b) Distillation experiments

Having found some hepatic penetration of ^{3}H-sucrose, it was necessary to find out if this was due to a possible exchange of ^{3}H-label on the sucorse onto water thereby giving tritiated water (THO). So a low temperature distillation apparatus was set up as in fig. 6.3. Phosphorus pentoxide, with blue indicator, was tightly packed inside the plunger of a 5ml syringe between layers of cotton wool at both ends of the plunger. The rubber end of the plunger was borred with 1cm hole. The barrel of the 5ml syringe was packed with

LEGEND TO FIG. 6.3

FIG. 6.3. DIAGRAM OF THE LOW TEMPERATURE DISTILLATION APPARATUS. FOR DESCRIPTION SEE TEXT (SECTION 6.2.2B).

Figure 6.3 DIAGRAM OF LOW TEMPERATURE DISTILLATION



a rolled dry filter paper (Whatman No. 1, which has been dried in a dessicator overnight). The syringe was connected, with silicone rubber tubing, to a U-shaped glass tubing of 5mm internal diameter. This glass tubing was immersed in a liquid nitrogen bath contained in a polystyrene box which was itself placed in a wooden box. This is the distillation chamber. This U-tubing was connected at the other end, in parallel with three other similar lines, to 100ml syringe via a rubber bung with holes to admit the four parallel lines. The other (single exit) end of this syringe was then connected to a suction apparatus (Water Tap Suction) via a distilled water bubble flow rate detector in a conical flask. At the commencement of experiment, the plunger of the 5ml syringe was withdrawn and 1ml of the isotope (^{3}H-sucrose) was added onto the dry filter paper in the syringe barrel, and plunger replaced. Then a stream of atmospheric air was drawn through the apparatus from this end by applying tap suction at the opposite end. A clip was fixed on each line to regulate the flow rate. The water vapour from the atmosphere was absorbed by the phosphorous pentoxide (P_2O_5) which then changed colour due to the presence of an indicator. The stream of dry air reaching the filter paper evaporated the water off the filter paper and this was blown into the U-tube. Due to the low temperature of the liquid nitrogen (-195^{o}C) in which the U-tube was immersed, the water vapour reaching it was immediately condensed and solidfied to form a small layer of ice in the bend of the tube. This happened simultaneously in all the four parallel lines. After 30 minutes distillation, the U-tubes were disconnected from the 100ml syringe and carefully lifted out of the liquid nitrogen bath. The ice pellets in the tubes were allowed to thaw at room temperature. Then four different 1ml syringes each with

a 21 guage needle were connected to 1mm internal diameter polyethene tubing of 15cm length each and marked with corresponding numbers to the lines. Each syringe was thus used to withdraw the thawed liquid from the bend of the U-tube corresponding to the numbering. The distillation was repeated as well as thawing and fluid withdrawal until about two to three hundred µl of distillate was obtained in each syringe. This usually took three or four distillations. Each syringe was weighed with its contents, and after the liquid content had been dispensed into a vial containing 9.5ml liquid scintillation cocktail it was whirl-mixed. Another two to three hundred µl. of ordinary distilled water (not from this distillate) were added to the cocktail and mixed in order to attain suitable proportion of the distilled water to the scintillating liquid so as to prevent immiscibility. Two blank vials were prepared in which ordinary 500µl distilled water was added to the cocktail in each vial, and two control vials containing 500µl of the isotope (^{3}H-sucrose), from the same stock as the one distilled, in each cocktail vial. All the vials were counted and the activity (dpm) in each vial calculated as described in Chapter 3. From the weight of each of the syringes before and after dispensing the contents, the weight and hence the volume of the distilled water (since specific gravity of water = 1) dispensed was accurately known. By simple proportion, the isotopic activity which would have been present in 500µl of this distillate was calculated for each tube. This activity was then computed as a percentage of the mean measured activity in the 500µl non-distilled isotope in the two control vials. This represents the percentage of ^{3}H-label that transferred from sucrose to water in THO.

6.2.3 Results

The distribution of both the reference (^{14}C-L-glucose) and test (^{3}H-D-glucose) can be seen in fig. 6.4. This is compared with part A of fig. 6.1. The reference in fig. 6.1A was ^{14}C-sucrose and the test was ^{3}H-D-glucose. The behaviour of the two reference materials is identical as seen in these two figures. The only apparent difference is the slower rise in the activity of both the reference and test materials in fig. 6.4, compared to fig. 6.1A. The rise is due to the fact that the isotopic mixture was infused at a slow rate of 320µl.min^{-1} into perfusate flowing at the normal rate for fig. 6.4 instead of the direct fast infusion rate of 18 ml.min^{-1} in fig. 6.1A. The pattern of distribution of the isotopic materials was similar in the two figures. The portions marked A in both figures indicate the first pass circulation period of nine seconds, and B the recirculation period, also nine seconds.

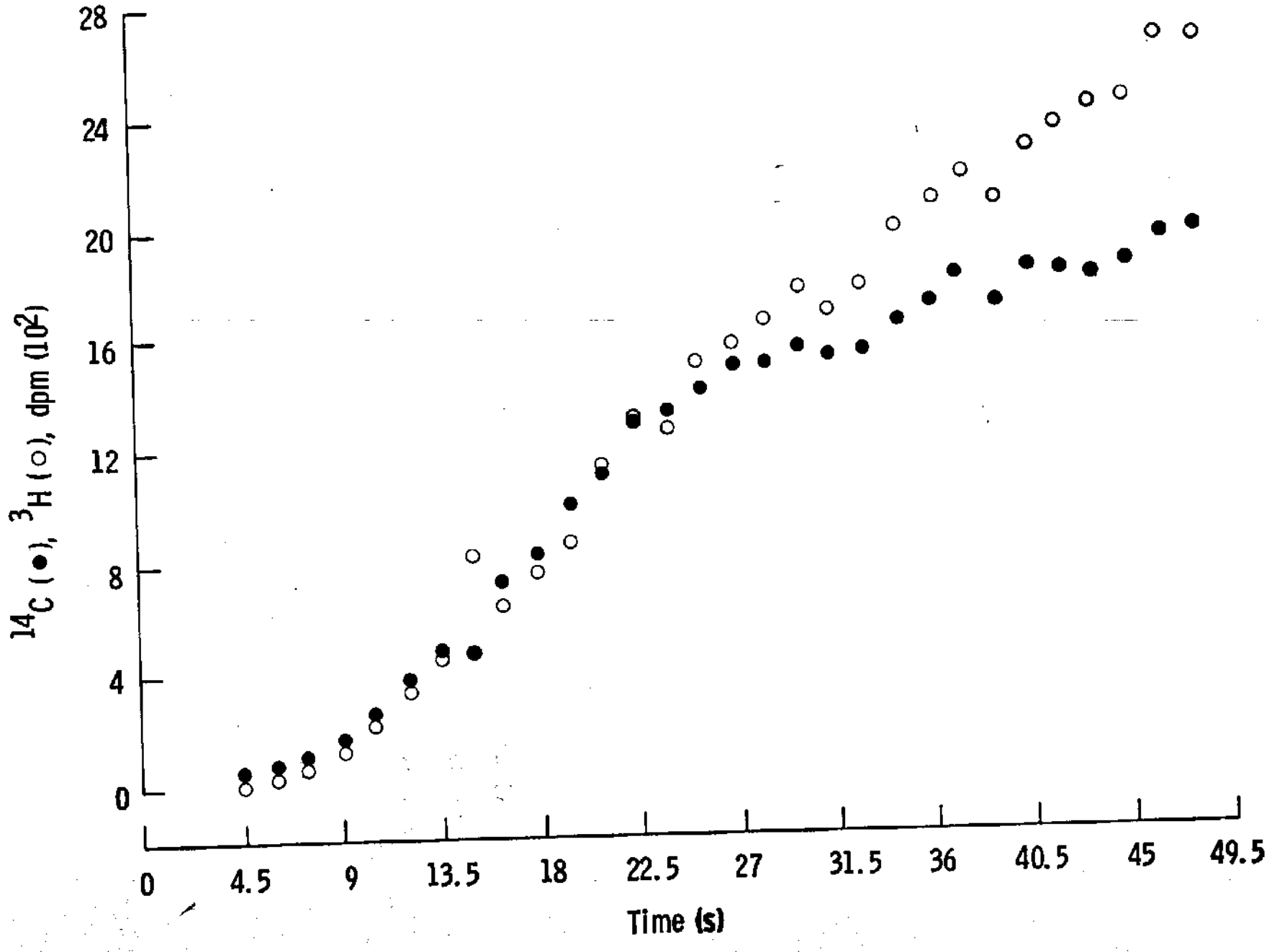
Figure 6.2 shows the extraction of ^{3}H-D-glucose using ^{14}C-L-glucose as reference (Runs 1 and 3). This showed a mean extraction of 50% in the two runs. This compares very favourably with the extraction of ^{3}H-D-glucose with ^{14}C-sucrose as the extracellular reference material in fig. 6.5 which also gave 50% mean extraction in the two runs.

In fig. 6.6 the "apparent" extraction of ^{3}H-sucrose when ^{14}C-L-glucose was used as the reference material was compared with its "apparent" extraction when ^{14}C-sucrose was the reference material. The mean extractions during the first six seconds after the first appearance were 13.26 ± 0.56% for ^{14}C-L-glucose reference and 16.89 ± 0.50% for ^{14}C-sucrose as reference. This difference was statistically significant, $0.001 < P < 0.01$ (paired t-test, N = 4). This would tend

LEGEND TO FIG. 6.4

FIG. 6.4 - DISTRIBUTION OF ISOTOPIC MATERIALS IN HEPATIC VENOUS EFFLUENT SAMPLES. NOTE THE GRADUAL RISE IN THE ACTIVITY OF BOTH ^{3}H- AND ^{14}C- ISOTOPES DUE TO SLOW RATE OF INFUSION IN THIS EXPERIMENT COMPARED WITH THAT IN FIG. 6.1.

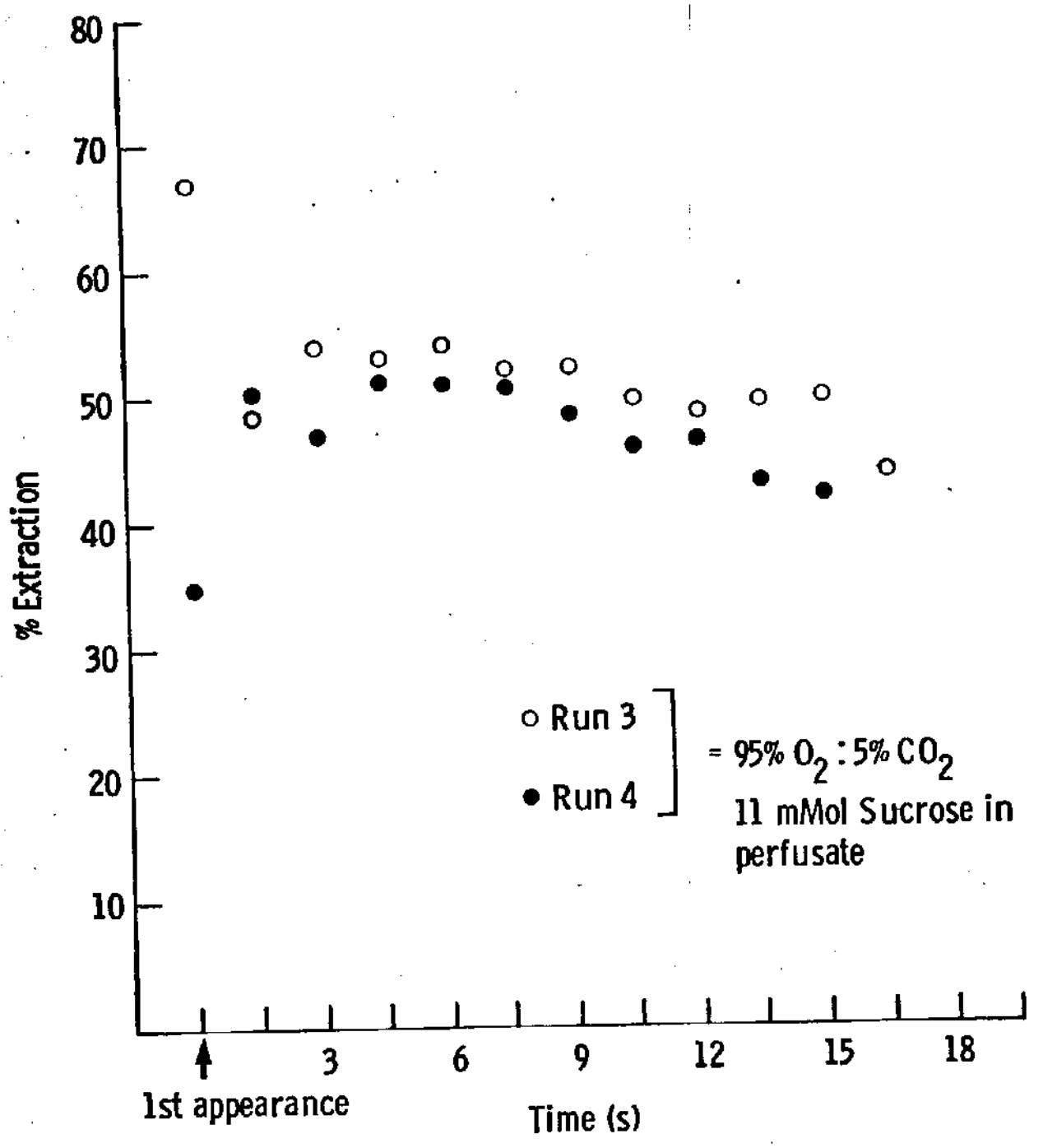
FIG. 6.4 DISTRIBUTION OF ISOTOPIC MATERIALS IN VENOUS EFFLUENT



LEGEND TO FIG. 6.5

FIG. 6.5 ^{3}H-D-GLUCOSE EXTRACTION USING ^{14}C-SUCROSE AS THE EXTRACELLULAR REFERENCE MATERIAL. NOTE ALSO A MEAN 50% EXTRACTION FOR THE SECOND TO FIFTH SAMPLES FOR THE TWO RUNS.

FIG. 6.5 ^{3}H-D-GLUCOSE EXTRACTION WITH ^{14}C-SUCROSE AS REFERENCE



Glucose transport with 95% O_2 : 5% CO_2

LEGEND TO FIG. 6.6

FIG. 6.6 - COMPARISON OF ^{14}C-L-GLUCOSE AND ^{14}C-SUCROSE AS EXTRACELLULAR REFERENCE MATERIALS FOR ^{3}H-SUCROSE. NOTE THE HIGHER "APPARENT" EXTRACTION OF ^{3}H-SUCROSE WHEN ^{14}C-SUCROSE WAS THE REFERENCE COMPARED TO WHEN ^{14}C-L-GLUCOSE WAS THE REFERENCE.

COMPARISON OF L - GLUCOSE AND SUCROSE AS REFERENCES FOR ^{3}H - SUCROSE

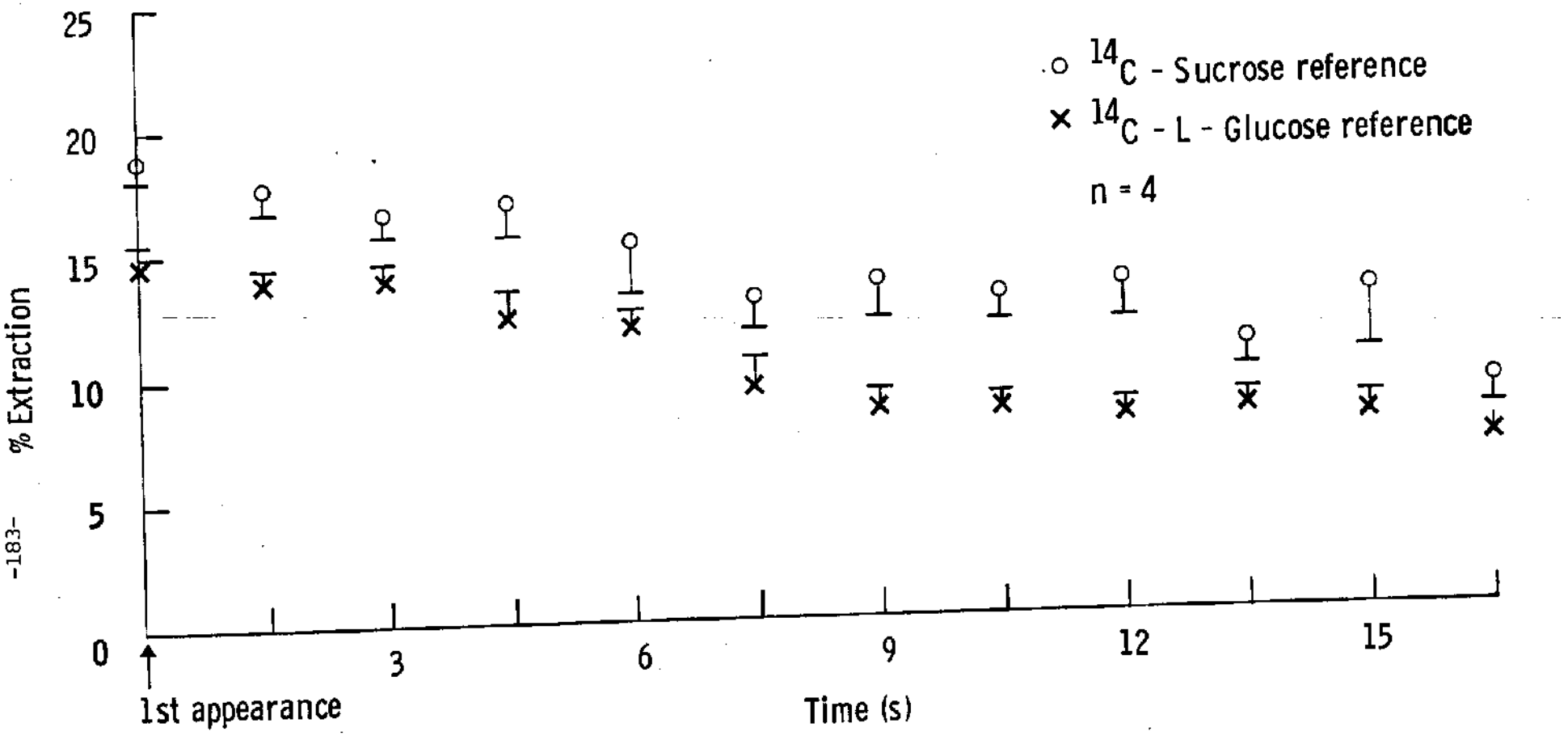


FIG. 6.6

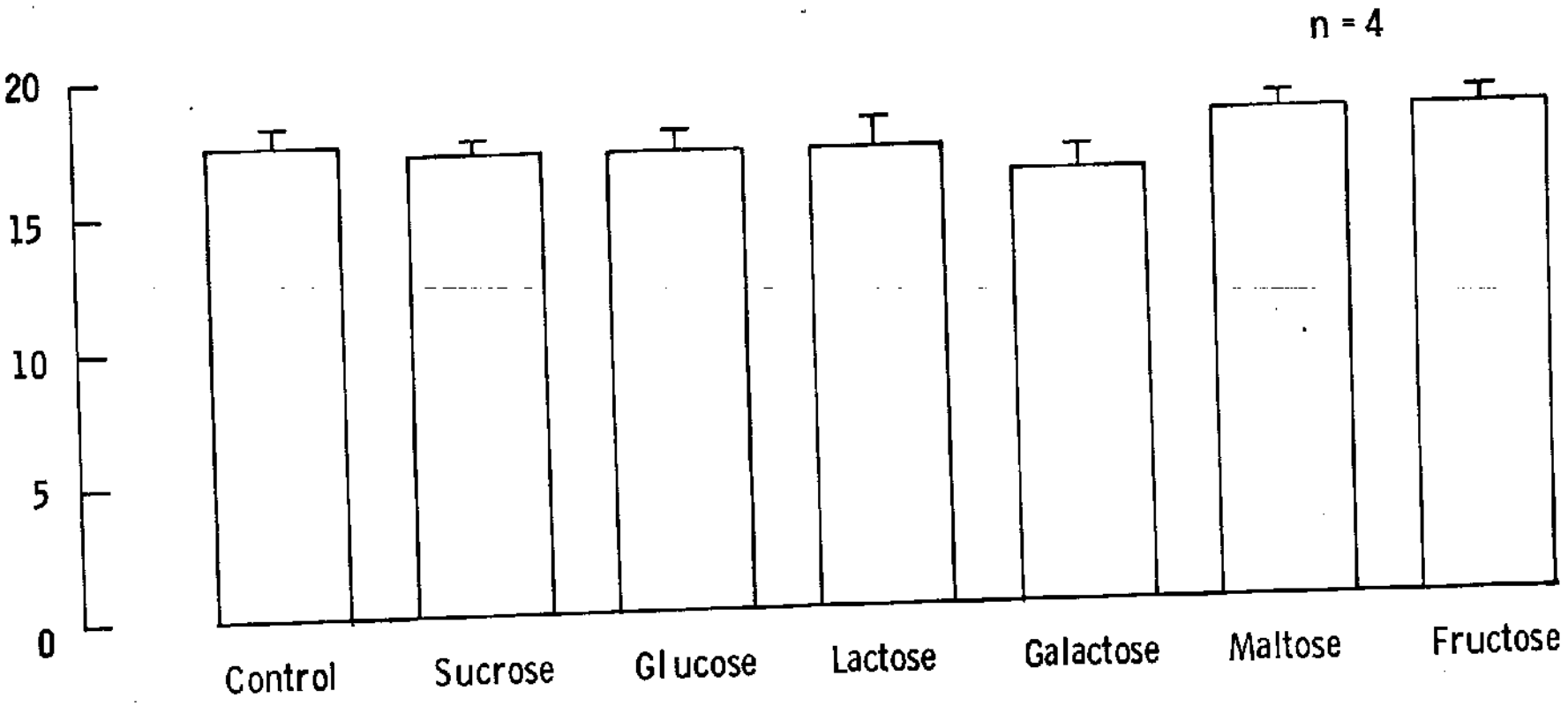
to imply that the sucrose was a better reference than L-glucose since the former gave a significantly higher extraction that the latter. But the findings in figs. 6.2 and 6.5 would rather seem to suggest that there is no difference between the two reference substances. Furthermore, fig. 6.6 suggests that an "apparent" ^{3}H-sucrose extraction of 13-17% exists in the rat liver.

In order to identify the nature of this "apparent" sucrose extraction by the liver, the effects of potential competitors were studied. Figure 6.7 shows the results of such a study - indicating that there was a mean "apparent" sucrose extraction of 17.73 ± 1.3% for the control run. The extracellular reference material was ^{14}C-L-glucose. Furthermore, this figure indicated that neither glucose, lactose, galactose, maltose, fructose nor sucrose itself added to the infusion mixture at 25mM concentration caused any significant change ($P > 0.05$) in this extraction. This means that the entry of the sucrose into the liver must be a purely passive, non-specific phenomenon. Even sucrose itself did not alter the extraction significantly from the control, thus further substantiates the proposal that there is no specific transport mechanism for the entry of this sugar into the rat liver - hence the term "apparent" extraction was introduced. It was therefore thought that this non-specific entry of sucrose into the liver could be due to exchange of ^{3}H-label on the sucrose for the H-atom of water to form a tritiated water (THO). The results of the low temperature distillation experiments showed that this did occur to some extent. The distillation showed that in four experiments, 8.30 ± 0.06% of the ^{3}H label had transferred from ^{3}H-sucrose to THO during storage for four months.

LEGEND TO FIG. 6.7

FIG. 6.7 - THE EFFECT OF 25mM UNLABELLED GLUCOSE, LACTOSE, GALACTOSE, MALTOSE, FRUCTOSE OR SUCROSE ITSELF ON THE HEPATIC ENTRY OF ^{3}H-SUCROSE. NONE OF THE SUGARS CAUSED ANY SIGNIFICANT CHANGE OF SUCROSE ENTRY INTO THE LIVER.

FIG. 6.7 EFFECT OF 25 mM SUGARS ON EXTRACTION OF ^{3}H - SUCROSE



6.2.4 Discussion and Mathematical formulation

This study has shown that both ^{14}C-L-glucose and ^{14}C-sucrose are well distributed in the extracellular compartment, and there is little difference in this respect between the two. The study further indicated that extraction of ^{3}H-D-glucose is similar when either reference was used. About 8% of the ^{3}H-label transferred from sucrose to THO within four months storage. This is higher than initial ^{3}H-label exchange from sucrose to THO reported by HOOPER (1975) where only 5% was thus exchanged initially, but found a much higher value after prolonged infusion. EVANS, REYNOLDS, SAUNDERS and SEGAL (1974) have found that the isotopic label remained attached to sucrose for at least three hours period of the experiment. The lower distillation value obtained by HOOPER (1975) could be due to a shorter storage period than the sample used in these experiments although the period of storage before the distillation was not stated by him. What is difficult to reconcile is the increase in the ^{3}H-label exchange with the length of time between infusion and effluent samples, since EVANS et al (1974) found the label to remain attached through three hour period. HOOPER (1975) explained this increase as a transfer from ^{3}H-D-glucose implying metabolism. Furthermore, the 8% ^{3}H-label exchange into THO would not wholly account for the 13-17% sucrose extraction measured in these experiments. So some amount of sucrose entry per se into the liver must be accepted from these results. This sucrose entry is thought to be into the more remote parts of the extracellular fluid compartment of the liver, possibly the intercellular gap adjacent to the canalicular space. ROUILLER (1969) has stated that this is the route of transport into bile. Indeed FORKER, (1970) found that 15% of sucrose injected appeared in bile after one

to five hours has elapsed. This figure agrees very closely with the findings in this study. His findings were supported by those of PARDRIDGE and JEFFERSON (1975) that sucrose was taken up by the rat liver. But BAUR and HELDT (1977) found that the rat liver cell was virtually impermeable to sucrose. This discrepancy can be resolved by the proposition that the hepatic extraction of sucrose measured is not a hepatocellular uptake but rather an entry into the liver most probably into the bile spaces. This proposition is strengthened by the findings in this study firstly that in fig. 6.6 sucrose extraction appeared to be better with sucrose reference than L-glucose reference; and secondly that none of the sugars was able to compete with this sucrose extraction, not even sucrose itself. It is likely, therefore, that this sucrose entry must be by a passive diffusion mechanism. A free diffusion alone would not be a suitable explanation, because L-glucose being a smaller molecule than sucrose would have penetrated more easily. Among the substances tested, FORKER (1970) found that the penetration of these substances - urea, malonamide, erythritol, mannitol, sucrose and polyethylene glycol - was inversely proportional to their molecular weight and molecular radius. For the substances he chose the law was obeyed. But this law fails when L-glucose and sucrose are compared. L-glucose has both a smaller molecular weight and molecular radius than sucrose, and consequently would have been expected to penetrate more than sucrose, but the converse was the case. Therefore the entry of sucrose could not be by a simple, free diffusion alone. So a second proposition of sucrose entry into the liver is put forward, namely, that the entry is by a pore. If entry by a pore is conjectured, it would be clear that the size of the pore could be such that the orientation of the bulky

-OH radicals of L-glucose in the axial direction is prohibitive whereas the equational orientation of these radicals in sucrose permits an easier entry. BOWMAN and LEVITT (1977) interpreted their findings of 4-carbon, 5-carbon and 6-carbon polyols permeability of the human erythrocytes in terms of a pore. They contended that the kinetic equations describing transport through a pore that has a binding site and that undergoes a conformational change are identical to those of a carrier model.

By whatever means sucrose enters the liver, the question remains to be answered as to what effects this entry of sucrose as the reference material could have on the estimate of extraction of the test material, and its extraction in the presence of a potential competitor. To this end, the following mathematical derivation was formulated and the ensuing graphs (figs. 6.8 and 6.9) have attempted to answer these questions.

MATHEMATICAL NOTATIONS

A_t = Activity of test substance in dpm (nuclear disintegrations per minute = dpm)

A_r = Activity of reference substance

A_{t_i} = Activity of test substance in infusate

A_{t_o} = Activity of test substance in effluent sample

A_{r_i} = Activity of reference substance in infusate

A_{r_o} = Activity of reference substance in effluent sample

R_i = $\frac{A_t}{A_r i}$, the ratio of test to reference materialssin the infusate

R_o = $\frac{A_t}{A_r o}$, the ratio of test to reference materials in the effluent sample

E = Measured Extraction of test substance

E_t = Actual hepatic extraction of test substance

E_r = Actual hepatic extraction of reference substance

E_t' = Apparent extraction of test substance in the presence of a potential competitor C_1, C_2, C_3, or C_4 etc.

E_{c_1} = Measured extraction of test substance in the presence of a potential competitor C_1, C_2, C_3 or C_4 etc. in the presence of extracted reference ($E_r \neq 0$)

ΔE = Measured fractional change in extraction (inhibition) due to the addition of a potential competitor C_1, C_2, C_3 or C_4 etc. in the presence of extracted reference ($E_r \neq 0$)

ΔE_t = True fractional change in extraction (inhibition) due to

the addition of a potential competitor C_1, C_2, C_3, or C_4 etc. in the presence of a reference which is not itself extracted ($E_r = O$)

E_a = Energy of activation

S = Slope of Arrhenius plot.

Let $R_i = \left(\frac{A_t}{A_r}\right)_i$ 1

and $R_o = \left(\frac{A_t}{A_r}\right)_o$ 2

$$E = \left(1 - \frac{R_o}{R_i}\right) \times 100 \quad . . . . . .3$$

or simply

$$E = 1 - \frac{Ro}{R_i} \quad . . . . . .4$$

But when reference is extracted (E_r), Equation 4 becomes E_t instead of E;

Also $$E_t = \frac{A_{t_i} - A_{t_o}}{A_{t_i}} \quad . . . . . .5$$

and

$$E_r = \frac{A_{ri} - A_{ro}}{A_{r_i}} \quad . . . . . .6$$

Substituting equations 1 and 2 in equation 4.

$$E = 1 - \frac{\left(\frac{A_t}{A_r}\right)_o}{\left(\frac{A_t}{A_r}\right)_i} \quad . . . . . .7$$

Rearranging equation 7 becomes

$$E = 1 - \left(\frac{A_t}{A_r}\right)_o \times \left(\frac{A_r}{A_t}\right)_i \quad . . . . . .8$$

or

$$E = 1 - \frac{A_{t_o} \cdot A_{r_i}}{A_{r_o} \cdot A_{t_i}} \quad . . . . . 9$$

Now substituting E_t and E_r in equations 5 and 6 respectively, equation 9 becomes:

$$E = 1 - \frac{(1 - E_t)\, A_{t_i} \cdot A_{r_i}}{(1 - E_r)\, A_{r_i} \cdot A_{t_o}} \quad \ldots\ldots\ldots 10$$

$$E = 1 - \frac{(1 - E_t)}{(1 - E_r)} \quad \ldots\ldots\ldots\ldots 11$$

opening the brackets,

$$E = \frac{1 - E_r - 1 + E_t}{1 - E_r} \quad \ldots\ldots\ldots\ldots 12$$

$$E = \frac{E_t - E_r}{1 - E_r} \quad \ldots\ldots\ldots\ldots 13$$

Using cross multiplication, equation 13 becomes:

$$E(1 - E_r) = E_t - E_r \quad \ldots\ldots\ldots\ldots 14$$

$$E_t = E - E.E_r + E_r \quad \ldots\ldots\ldots\ldots 15$$

$$E_t = E + E_r\,(1 - E) \quad \ldots\ldots\ldots\ldots 16$$

from equation 16, actual hepatic extraction of test substance (E_t) can be calculated since the values of E and E_r have been obtained from the experimental results.

From this equation also the absolute amount to be added to the measured extraction (E) to give the true or actual extraction (E_t) is $E_r(1 - E)$.

Now, to find the apparent extraction (E_t) of test substance in the presence of a potential competitor C_1, when the measured extraction due to the competitor's presence is E_{C_1}, and to find the true fractional change in extraction (inhibition) if the reference was not extracted (ΔE_t) compared with when reference was extracted (ΔE) as measured experimentally, from equation 16.

$$E_t = E + E_r (1 - E) \quad \ldots\ldots\ldots 16(b)$$

and by definition

$$E_t = E_{C_1} + E_r (1 - E_{C_1}) \quad \ldots\ldots\ldots 17$$

Also by definition,

$$\Delta E = \frac{E - E_{C_1}}{E} \quad \ldots\ldots\ldots 18$$

and

$$\Delta E_t = \frac{E_t - E_t}{E_t} \quad \ldots\ldots\ldots 19$$

Substituting the values of E_t and E_t from equations 16(b) and 17 in equation 19, we get

$$\Delta E_t = \frac{\{E + E_r (1 - E)\} - \{E_{C_1} + E_r (1 - E_{C_1})\}}{\{E + E_r (1 - E)\}} \quad \ldots\ldots 20$$

$$\Delta E_t = \frac{E - E.E_r - E_{C_1} + E_r.E_{C_1}}{E + E_r (1 - E)} \quad \ldots\ldots 21$$

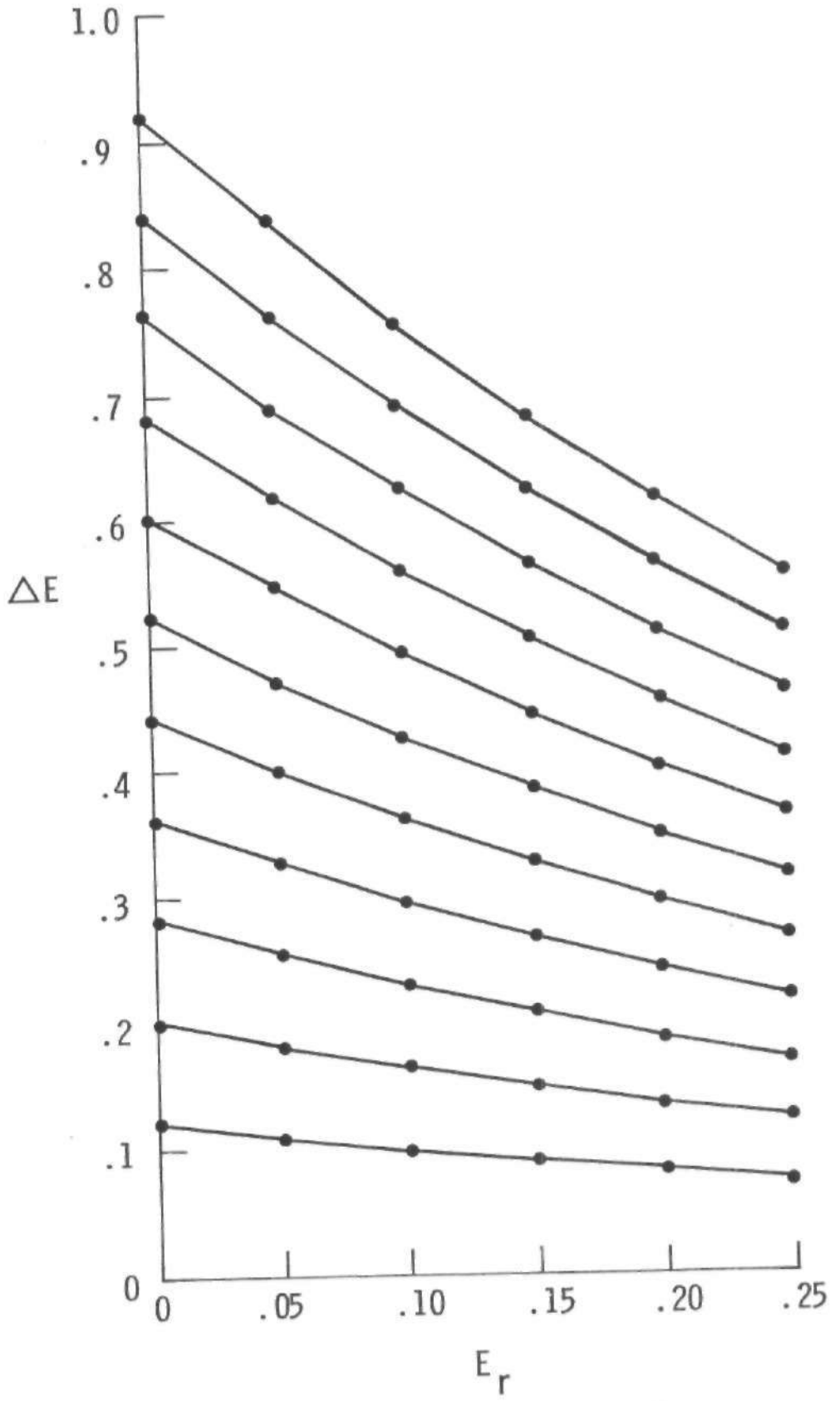
$$\Delta E_t = \frac{E - E_{C_1} + E_r(E - E_{C_1})}{E (1 - E_r) + E_r} \quad \ldots\ldots 22$$

$$\Delta E_t = \frac{(E - E_{C_1})(1 - E_r)}{E (1 - E_r) + E_r}$$

LEGEND TO FIG. 6.8

FIG. 6.8. THE RELATIONSHIP BETWEEN EXTRACTION OF REFERENCE (E_R) AND MEASURED FRACTIONAL CHANGE OR INHIBITION (ΔE). NOTE THAT FOR THE INHIBITION LEVELS MEASURED IN THE EXPERIMENTS REPORTED IN THIS STUDY, THE 15% APPARENT EXTRACTION OF REFERENCE ($E_R = 0.15$) HAS MINIMAL EFFECT. THIS NORMOGRAM CAN BE USED FOR ANY REFERENCE THAT HAS AN APPARENT EXTRACTION OF UP TO 25% AND FOR INHIBITION OF EXTRACTION OF OVER 90%. SO IT HAS A WIDE RANGE OF USE.

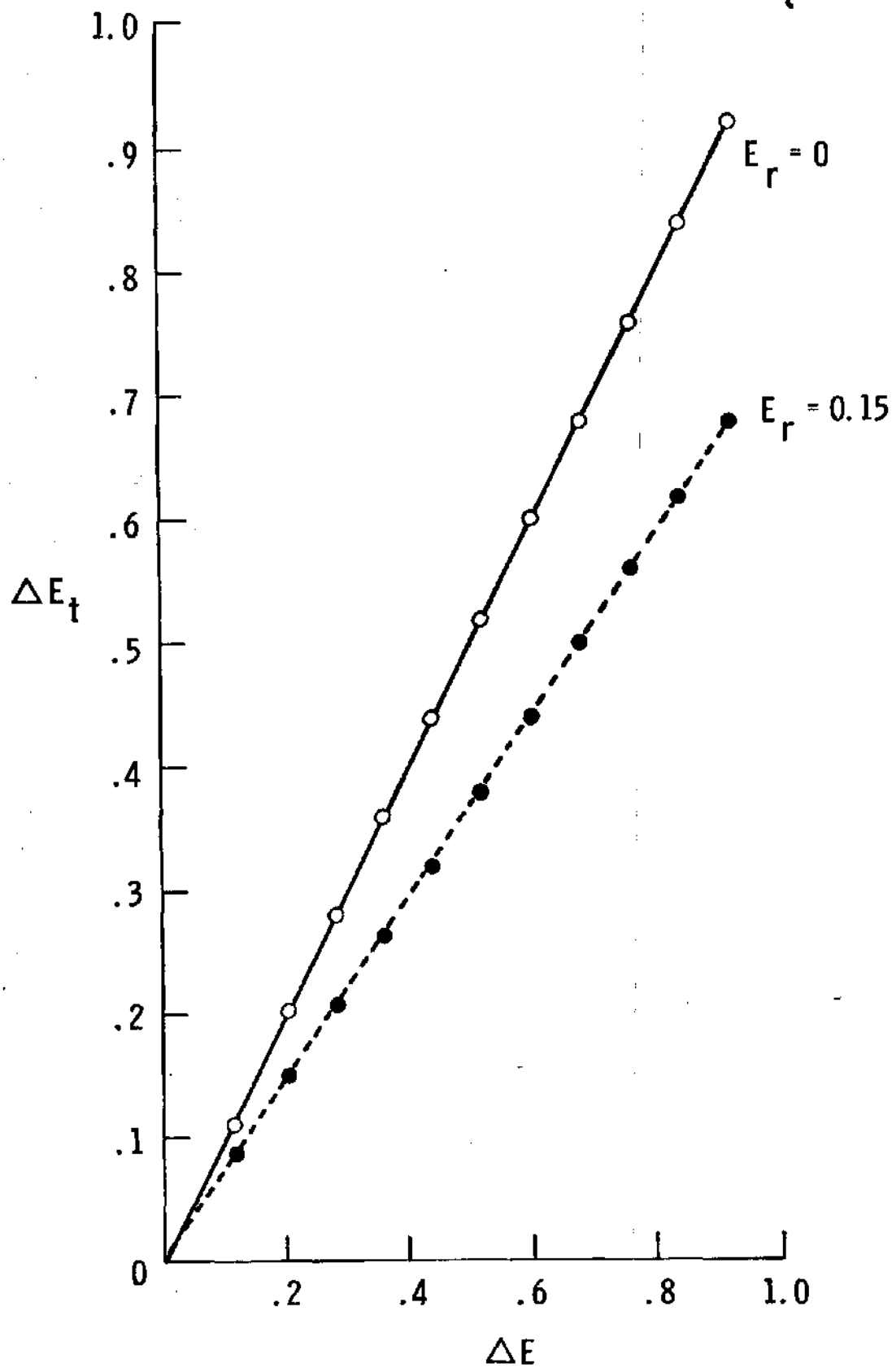
Fig. 6.8 RELATIONSHIP BETWEEN EXTRACTION OF REFERENCE (E_r) AND MEASURED FRACTIONAL CHANGE OR INHIBITION ($\triangle E$)



LEGEND TO FIG. 6.9

FIG. 6.9. NORMOGRAM FOR CONVERTING MEASURED INHIBITION (ΔE) TO TRUE INHIBITION (ΔE_T). THE VALUE FOR THE TRUE INHIBITION CAN BE EXTRAPOLATED ON $E_R = 0$ CURVE FOR ANY CORRESPONDING MEASURED INHIBITION ΔE. ONCE MORE FOR THE INHIBITIORY LEVELS OBTAINED IN THIS STUDY, THERE IS LITTLE DIFFERENCE BETWEEN THE TWO.

Fig. 6.9 NORMOGRAM FOR CONVERTING MEASURED INHIBITION (ΔE) TO TRUE INHIBITION (ΔE_t)



from equation 23, it is obvious that $(1 - E_r)$ is a common factor, which multiply the expressions $(E - E_{C_1})$ and E as numerator and denominator respectively. But this relationship is complicated by the error factor (E_r) in the denominator due to the extraction of the reference. Therefore there is no fixed single factor to be used (added, multiplied, divided by or subtracted from) in computing from the measures ΔE to give ΔE_t.

Hence it was necessary to compute the values to cover a wide range from $\Delta E = 0$ to 1.0 for the various levels of reference extraction from $E_r = 0$ to 0.25. Figure 6.8 shows the plot of such computation. It could be observed from this graph that at the 10 to 20% inhibition range that were obtained in the experiments reported in Chapters 7, 8 and 9 using the Sucrose reference whose extraction $(E_r = 0.15)$ was 15% approximately, the difference between $E_r = 0$ and $E_r = 0.15$ is not very great. In order to notice this more clearly and to compute the true fractional change in extraction (inhibition) ΔE_t due to the addition of a potential inhibitor in the presence of a referench which is not itself extracted $(E_r = 0)$ a normogram was computed and plotted in the graph shown in Fig. 6.9. With this normogram, all values of ΔE_t can be read off the graph for a given value of the measured inhibition ΔE since $E_r = 0$ would now be the read off point.

PART D

TRANSPORT OF GLUCOSE, GALACTOSE AND FRUCTOSE BY THE LIVER

CHAPTER SEVEN

7. GLUCOSE TRANSPORT BY THE PERFUSED RAT LIVER

7.1 Introduction

The liver regulates the concentration of glucose in the blood at any particular moment. In the presence of hyperglycaemia the liver removes glucose from the blood, and conversely in the presence of hypoglycaemia it releases glucose into the blood. Glucose thus removed from the blood is stored in a polymeric form, glycogen by the liver and when the need arises this polymer is converted by the liver into glucose monomers to be released into the blood stream. In most tissues, including muscle, the role of glycogen is to be a glycolytic fuel that provides energy locally when glucose or oxygen becomes scarce. In contrast, glycogen is of little use for the liver, which consumes mostly fatty acids (KREBS 1972) but stores glucose as glycogen when it is abundant and liberates it for the benefit of other tissues, especially the brain and the erythrocytes, during fasting. Indeed, during the alternation of feeding and short fasting, the level of glycaemia is maintained within normal limits because of very large changes in the rates of glycogen synthesis and breakdown in the liver (HERS 1976). These changes are tightly controlled through homeostatic and hormonal mechanisms by the availability of glucose in the body. This homeostatic role of the hepatic cell was first conclusively demonstrated by the classic experiment of SOSKIN, ESSEX, HERRICK and MANN (1938). SOSKIN and his coworkers demonstrated, using perfused dog livers that the liver extracted glucose when the perfusate was glucose-rich, and produced glucose when the perfusate was glucose-poor. Following this, HLAD, ELRICK and WITTEN (1956) demonstrated in humans that the rate of glucose uptake was directly proportional to the level of hyperglycaemia and the rate

of production was also directly related to the level of hypoglycaemia. The term hyper- or hypo-glycaemia is a relative one. SOSKIN and LEVINE (1946) defined the hepatic threshold for glucose as the glucose concentration at which the liver is converted from an organ of glucose output into an organ of glucose uptake. This threshold corresponds to the level of glycaemia that the animal usually maintains and may vary according to the endocrinological condition. This concept was based on a series of experiments performed on hepatectomized, pancreatectomized, or normal dogs and has received general acceptance (BERGMAN & BUCOLO 1974). From this, it became obvious that the liver is central to the mechanism of glucostasis. Interest began to be developed in liver handling of glucose. GEY (1956) using chromatographic technique studied glucose concentration in eight different tissues of the rat, including the liver. He found that the rat liver water contained glucose at a concentration similar to that of plasma when the liver was rapidly frozen after death and analysed. His work showed that the rat liver glucose concentration was higher than the glucose concentration in cell water from other tissues. Using rabbit liver slices, BERTHET, JACQUES, HERS and DEDURE (1956) found ^{14}C-glucose penetrated 0.5 ml of water per gram of liver. It seems that not all the water contained in the liver is likely to be accessible to glucose, as indeed BAUR and HELDT (1977) later showed that not the whole of the inulin impermeable tritiated water space was available to glucose. On the other hand, CAHILL, ASHMORE, EARLE and ZOTTU (1958) showed that the glucose concentration in rat liver slices was slightly higher than in the bathing medium. CAHILL et al (1958) found the entry of glucose into their liver slices to be so ready that they concluded that glucose must be entering the liver by free diffusion. But their results in liver slices of the penetration of inulin were approximately 50% compared with their 20%

results of in vivo inulin entry in anaesthetised rats. So the excessive leakiness could be attributable to the unphysiological state of the liver slices compared with in vivo state. However, HETENYI and ARBUS (1962) using similar techniques did not support the conclusions of CAHILL and his group based on the finding that insulin did not affect the distribution of glucose in the liver. The former found that in rats injected with insulin the concentration of glucose in the liver falls to a lesser extent than in plasma thereby altering the original ratio of distribution in normal fasted rats. Moreover, WILLIAMS, EXTON, PARK and REGEN (1968) argued from their finding of a stereospecific glucose transport in rat liver that glucose entry was not by free diffusion. They contended that it must be by some carrier-mediated transport mechanism. This idea has been supported by HOOPER and SHORT (1975, 1977), and BAUR and HELDT (1977) on the basis of their findings of glucose handling by the conscious sheep liver and isolated rat hepatocytes respectively.

It was because of the importance of the liver in regulation of glucose concentration coupled with the interest stirred up by these early investigations on liver handling of glucose that these studies were undertaken. The aim was, therefore, to elucidate the nature of glucose handling by the liver, using isolated liver perfusion technique.

7.2 Method :

Most of the method is as described in the general methodology (Chapter 3). But this section will further describe the fine details related to this Chapter which are different from that narrated in Chapter 3.

After surgical operation and cannulation of the anaesthetised rat, infusion of test substance was done by preparing the following infusion mixture. The test material (^{3}H-D-glucose, usually) and the reference material (^{14}C-Sucrose, in this case) were drawn up at 2μCi (100μl) and 1μCi (100 μl) respectively into a 10 ml syringe. The concentrations of the isotopes were Glucose = 5.5×10^{-3}M and Sucrose = 3.8×10^{-8}M. To this was added 1 ml of either Krebs bicarbonate buffer (Control) or 1 ml of 250mM of unlabelled Sucrose, glucose, galactose, fructose, lactose or maltose and mixed thoroughly. The syringe was then connected to the perfusion circuit on a sage syringe pump and the stop cock turned to allow rapid withdrawal of perfusate into the syringe to make a 10 ml volume thereby bringing the final concentration of the potential competitor (the added unlabelled sugar) to 25mM when presented to the liver. When uptake of L-glucose was studied, ^{14}C-L-glucose (1μCi of 100μl) as test substance was mixed with 2μCi (100μl) of ^{3}H-Sucrose as the extracellular reference material, for infusion.

To find out the effect of phlorizin or phloretin on the uptake of D-glucose, 1mM of either phlorizin or phloretin was made up in a 150 ml perfusate used for perfusing the liver. And the glucose transport measured with perfusate containing no phlorizin or phloretin was compared with the transport of the same sugar in the same livers when any of these chemicals was added. The percentage inhibition caused by these chemicals was compared and the results plotted on the same graph.

7.3 Results

Extraction of D-glucose by the liver is shown in fig. 7.1. In this graph and subsequent ones, except where otherwise stated, the ordinate (Y axis) represents percentage extraction and the abscissa (X axis) is time scale in seconds. Arrow indicating the "first appearance" points to the first extraction value when the extracellular reference isotope first appeared in the hepatic venous sample with isotopic activity of at least 1,000 dpm. This extraction at first appearance was not used in the calculation of the means in any experiment. In this and subsequent ones, except where stated, the second to fifth extraction values (after first appearance) were meaned in each liver. The means of these four samples were taken from each of four livers so that the total number of samples used for the calculation was 16. But N = 4 as stated in these results refers to the number of livers rather than number of observations (samples) which would have been 16.

In fig. 7.1, there are four curves plotted on the graph - the glucose control extraction, and the extraction when sucrose at 25mM concentration was added during the experimental run immediately following the control run (Sucrose 1), or as the last run (Sucrose 2), and the fourth curve represents the glucose extraction when 25mM D-glucose was added. The control and the two Sucrose runs in this graph are the same as those plotted for similar graphs in this chapter (that is, figs. 7.4, 7.5, 7.6, 7.11, and 7.12). The Sucrose 1 run was performed to find out if the presence of 25mM solute per se would have any significant osmotic effect on the extraction as compared with the control run. The sucrose 2 run was performed to compare the extraction in this run with that of sucrose 1 in order to ascertain if there was any significant difference between the two, which would indicate functional

LEGEND TO TABLE 7.1

TABLE 7.1. CHANGE IN GLUCOSE EXTRACTION WITH TIME IN THE PRESENCE OF 25mM UNLABELLED SUGARS AS POTENTIAL COMPETITORS. THE REGRESSION EQUATION USED IN THIS TABLE WAS:-

$$Y = A + BX$$

WHERE Y IS THE DEPENDENT VARIABLE PLOTTED ON THE ORDINATE (% EXTRACTION OF D-GLUCOSE), X IS THE INDEPENDENT VARIABLE PLOTTED ON THE ABSCISSA (TIME IN SECONDS), A REPRESENTS THE DISTANCE ABOVE THE BASELINE AT WHICH THE REGRESSION LINE CUTS THE VERTICAL (Y) AXIS, I.E. A IS THE INTERCEPT. B SIGNIFIES THE AMOUNT BY WHICH A CHANGE IN X MUST BE MULTIPLIED TO GIVE THE CORRESPONDING AVERAGE CHANGE IN Y.

NOTE THAT THE UPPER PART OF THE REGRESSION LINE CUTTING THE Y AXIS (INTERCEPT = A) WOULD GIVE THE MAXIMUM EXTRACTION (E_{MAX}). AND THE LOWERMOST CUT OF THE REGRESSION LINE ON X AXIS WOULD GIVE THE TIME WHEN EXTRACTION WOULD BE ZERO ($E = 0$), IF SAMPLING WERE CONTINUED LONG ENOUGH AFTER THE INFUSION.

TABLE 7.1

Change in Glucose Extraction with time

Competing Sugar at 25 mM	Regression Coefficient (b)	Intercept (a) %	Correlation Coefficient (r)	Significance P
Control (Krebs only)	-1.897	57.29	-0.994	<0.001
Sucrose-1	-2.079	58.19	-0.999	<0.001
Glucose	-1.297	47.65	-0.995	<0.001
Galactose	-1.471	49.89	-0.989	<0.001
Fructose	-1.224	41.25	-0.971	<0.001
α-M-D-G	-1.497	51.10	-0.991	<0.001
Lactose	-1.570	50.02	-0.994	<0.001
Maltose	-1.538	50.71	-0.996	<0.001
Sucrose-2	-1.438	52.60	-0.996	<0.001

N = 9

deterioration with time.

The control glucose extraction was 49.58 ± 1.58% (Mean ± S.E.M., N = 4) - Table 7.2. However, this extraction began to fall progressively with time as depicted in fig. 7.1. The fall in extraction was strongly correlated with time showing a regression coefficient of -1.897 and a correlation coefficient of -0.994 (Table 7.1). The Sucrose 1 and Sucrose 2 runs also fell with time and showed correlation coefficients of -0.999 and -0.996 respectively.

The effect of 25mM unlabelled D-glucose was a decrease of extraction from the control value (fig. 7.1). The mean extraction in the presence of glucose was 42.75 ± 2.63 representing an inhibition from the control value of 13.76 ± 0.25%. This difference from the control was statistically significant ($P < 0.01$), in this and subsequent P values, N = 4, paired t-test. This effect of glucose on its own transport means that the glucose transport is saturable.

In fig. 7.2, the Y and X axes are similar to those of fig. 7.1 except that the Y axis is not a broken axis in fig. 7.2. This figure shows the extractions of ^{3}H-D-glucose and ^{14}C-L-glucose. The D-glucose is shown to be better extracted by the same livers than L-glucose. ^{14}C-Sucrose was used as the extracellular reference substance for D-glucose extraction, while ^{3}H-Sucrose was the reference substance for the L-glucose. This use of two different labels as references arose from the fact that the two glucose isomers were differently labelled and it is obligatory to tag the reference with a different isotopic label from the test substance in a double indicator dilution method used here (the principle has been explained in Chapter 3). But this differential labelling of reference has resulted in the particular shape of the curves in this figure. The extraction of D-glucose continued to fall with time as in fig. 7.1. This fall is due to reflux

TABLE 7.2

Inhibition of Glucose Transport by Different Sugars

Competing Sugar 25mM	D-Glucose Extraction % E ± S.E.M.	ΔE% ± S.E.M.	Significance P
Control	49.58 ± 1.58	-	-
Sucrose 1	48.82 ± 1.15	-1.5 ± 0.86	P>0.05
D-Glucose	42.75 ± 2.63	-13.76 ± 0.25	P<0.01
D-Galactose	43.90 ± 1.89	-11.4 ± 0.32	** P<0.01
D-fructose	28.12 ± 1.39	-21.8 ± 0.49	** P<0.001
α-M-D-G	46.32 ± 1.10	-6.5 ± 0.42	P<0.05
Lactose	44.46 ± 0.28	-10.32 ± 0.30	P<0.01
Maltose	42.40 ± 2.42	-14.4 ± 0.48	P<0.01
Sucrose 2	46.19 ± 2.7	-6.8 ± 1.17	* P>0.05

N = 4 livers

α-M-D-G = α-methyl-D-glucopyranoside

* = Significance of difference between Sucrose 1 and Sucrose 2.
Other P values were compared with Control only.

All probability values were obtained using paired t-test

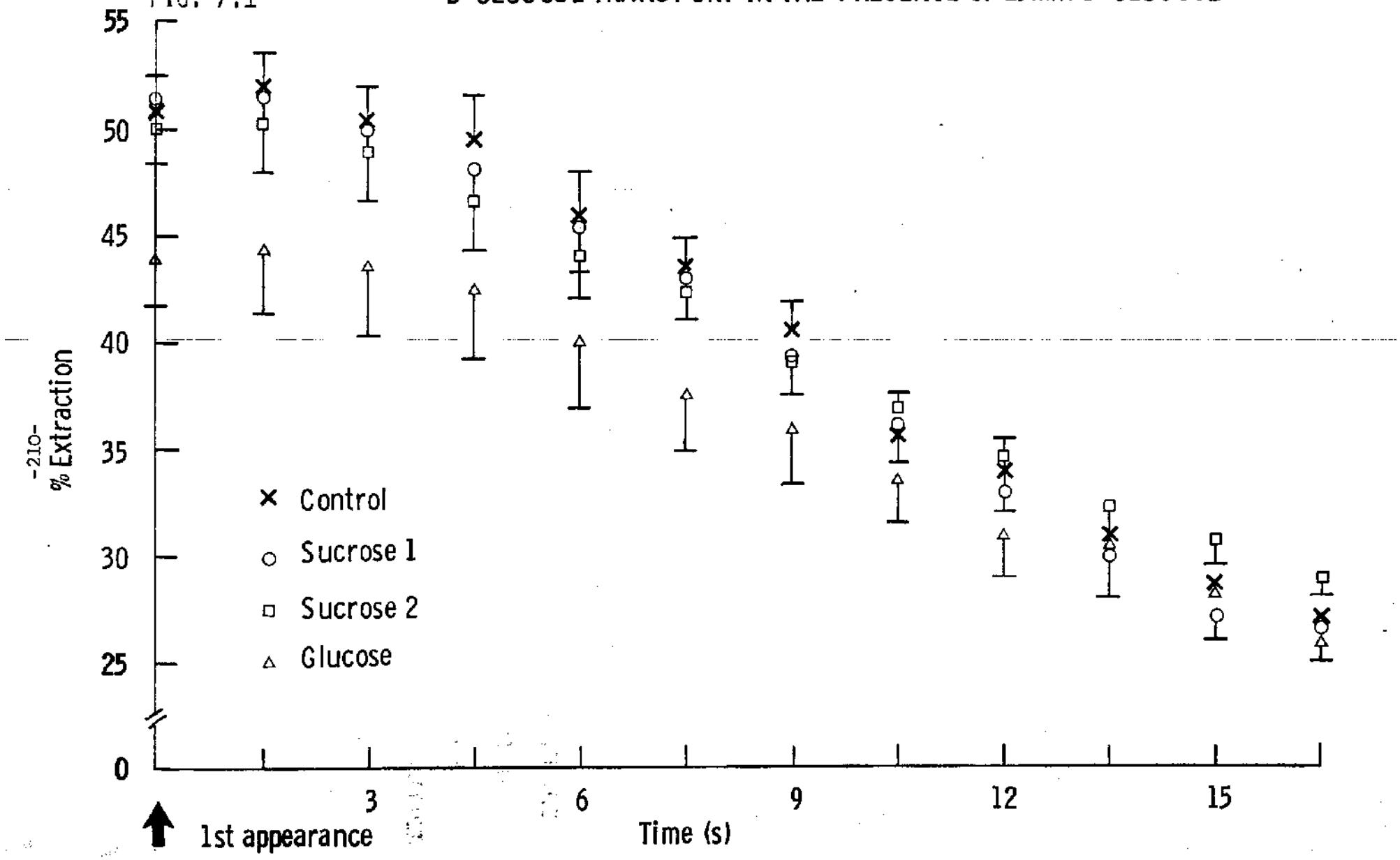
ΔE is change in extraction from control value expressed as percentage of control.

** = The difference between the inhibition by fructose and inhibition by galactose is highly significant (P<0.01, paired t-test).

LEGEND TO FIG. 7.1

FIG. 7.1 - D-GLUCOSE TRANSPORT IN THE PRESENCE OF 25mM UNLABELLED D-GLUCOSE. THE FIRST AND LAST SUCROSE RUNS (SUCROSE 1 AND SUCROSE 2 RESPECTIVELY) HAVE BEEN INCLUDED FOR EASY COMPARISON. THIS IS DONE IN SIMILAR FIGURES FOLLOWING THIS. NOTE GLUCOSE EXTRACTION WAS SIGNIFICANTLY DECREASED BY THE ADDITION OF 25mM GLUCOSE IN THE INFUSATE.

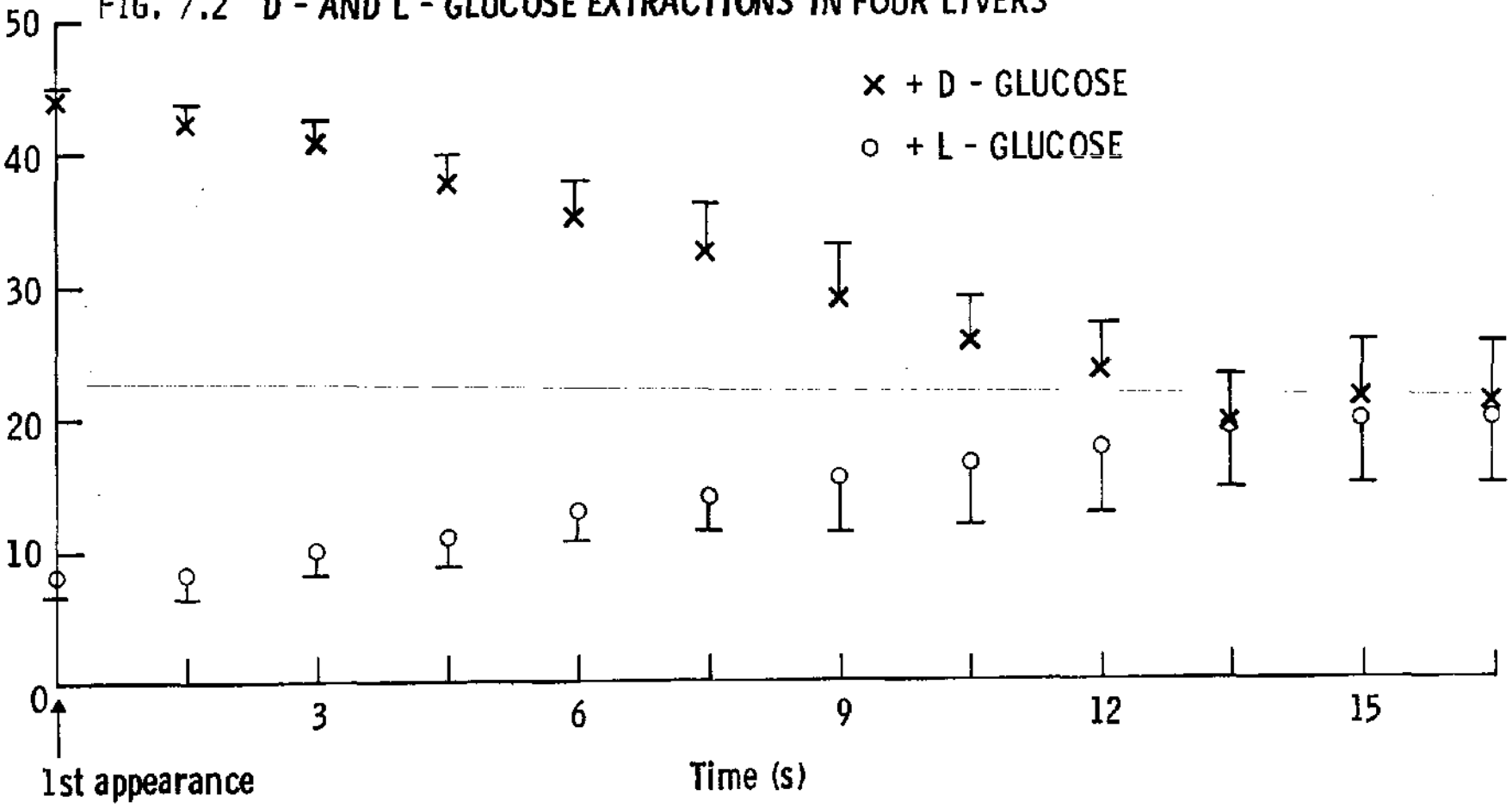
FIG. 7.1 D-GLUCOSE TRANSPORT IN THE PRESENCE OF 25mM D-GLUCOSE



LEGEND TO FIG. 7.2

FIG. 7.2. D-GLUCOSE AND L-GLUCOSE EXTRACTIONS IN FOUR LIVERS. NOTE THE FALLING LEVEL OF EXTRACTION OF D-GLUCOSE WITH TIME AND RISING LEVEL OF L-GLUCOSE EXTRACTION WITH TIME DUE TO THE USE OF ^{3}H-TEST MATERIAL IN THE FORMER AND ^{3}H-REFERENCE MATERIAL IN THE LATTER CASE. L-GLUCOSE EXTRACTION IS VERY MUCH LOWER THAN THAT OF D-GLUCOSE, EVEN IN THE SAME LIVERS.

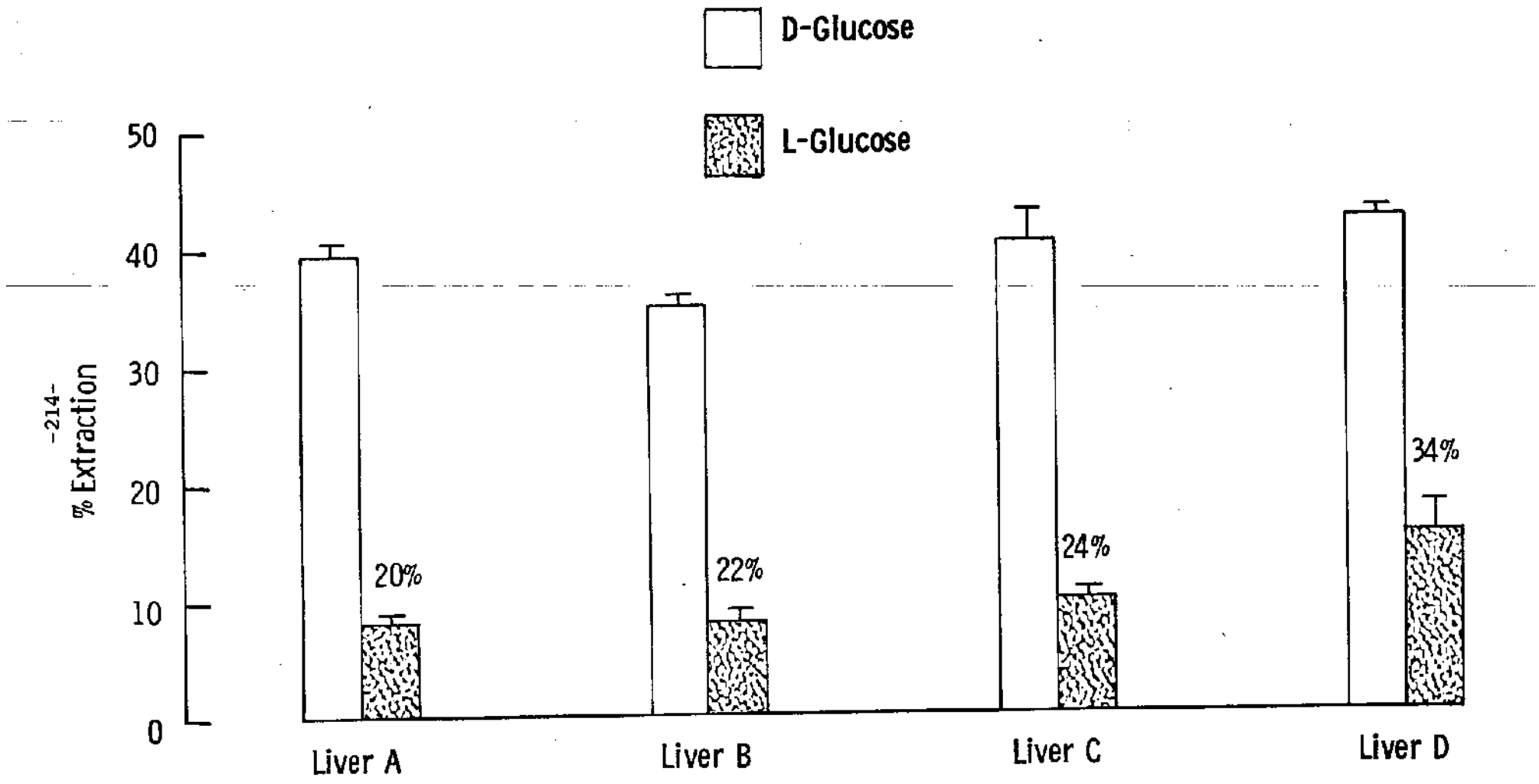
FIG. 7.2 D - AND L - GLUCOSE EXTRACTIONS IN FOUR LIVERS



LEGEND TO FIG. 7.3

FIG. 7.3 - COMPARISON OF HEPATOCELLULAR EXTRACTIONS OF D-GLUCOSE AND L-GLUCOSE. NOTE THAT L-GLUCOSE EXTRACTION IS ONLY 20-34% OF THE D-GLUCOSE EXTRACTION.

FIG. 7.3 COMPARISON OF HEPATOCELLULAR EXTRACTIONS OF D-GLUCOSE AND L-GLUCOSE



of the labelled ^{3}H-D-glucose back into the hepatic venous effluent, after entry into the liver, thereby modifying the ratio between the test and reference isotopes in subsequent venous effluent samples compared with initial ones. This was fully explained and illustrated in Chapter 5, and will not be discussed further here. This reflux of the test substance, bringing about the decreasing extraction contrasts with the gradual increasing extractions of L-glucose. This apparent increase in extraction of L-glucose is due to the reflux of the reference instead of test substance in this case. The quantitative difference between the D-glucose and L-glucose extractions is shown in fig. 7.3 in four livers. The L-glucose extraction is only 20% of D-glucose extraction in liver A, 22% in liver B, 24% in liver C and 34% in liver D. These findings suggest that glucose transport by the liver shows strong stereospecificity since the two stereo-isomers of glucose are transported to a markedly different extent.

Addition of D-galactose also decreased the extraction of D-glucose (fig. 7.4), and extraction also fell with time with a correlation coefficient of -0.989 (Table 7.1). The galactose decreased the glucose extraction to 43.90 ± 1.89 representing an inhibition of 11.4 ± 0.32%. This depression was also significant ($P<0.01$).

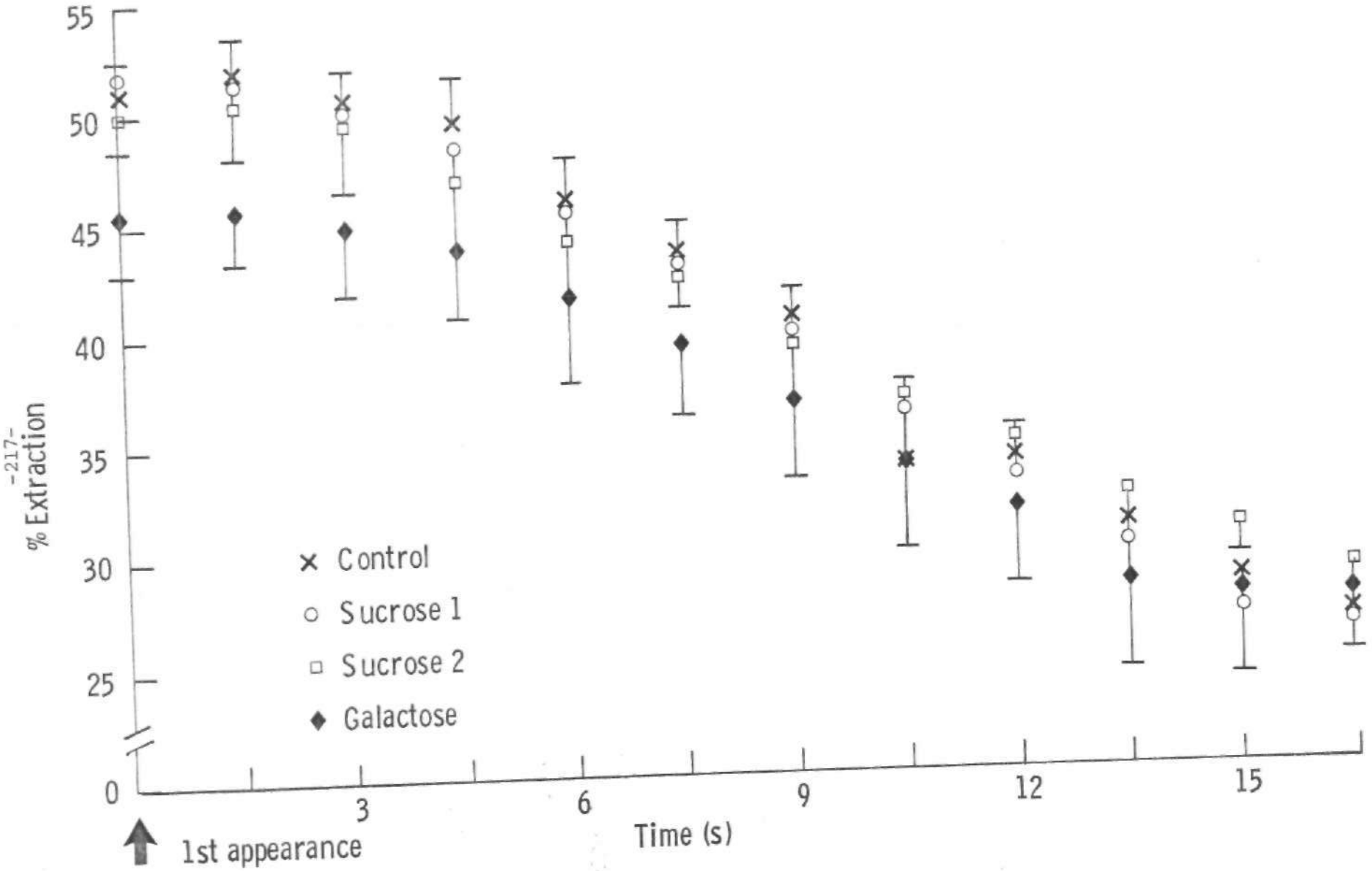
Also the addition of fructose decreased the glucose extraction (fig. 7.5) and the extraction fell with time having a correlation coefficient of -0.971 (Table 7.1). Fructose decreased the extraction to 28.12 ± 1.39%, which is an inhibition of 21.8 ± 0.49%. Thus there is a highly significant difference between the inhibitory effect of fructose and that of galactose ($P<0.01$).

Furthermore, the addition of a non-metabolizable methyl substituted sugar, α-methyl-D-glucopyranoside (α-M-D-G) decreased glucose extraction to 46.32 ± 1.10% representing an inhibition of 6.5 ± 0.42%

LEGEND TO FIG. 7.4

FIG. 7.4. D-GLUCOSE TRANSPORT IN THE PRESENCE OF 25mM D-GALACTOSE. THE PRESENCE OF GALACTOSE CAUSED A SIGNIFICANT DECREASE IN EXTRACTION OF GLUCOSE. THIS IS AN EVIDENCE OF COMPETITION BETWEEN GALACTOSE AND GLUCOSE.

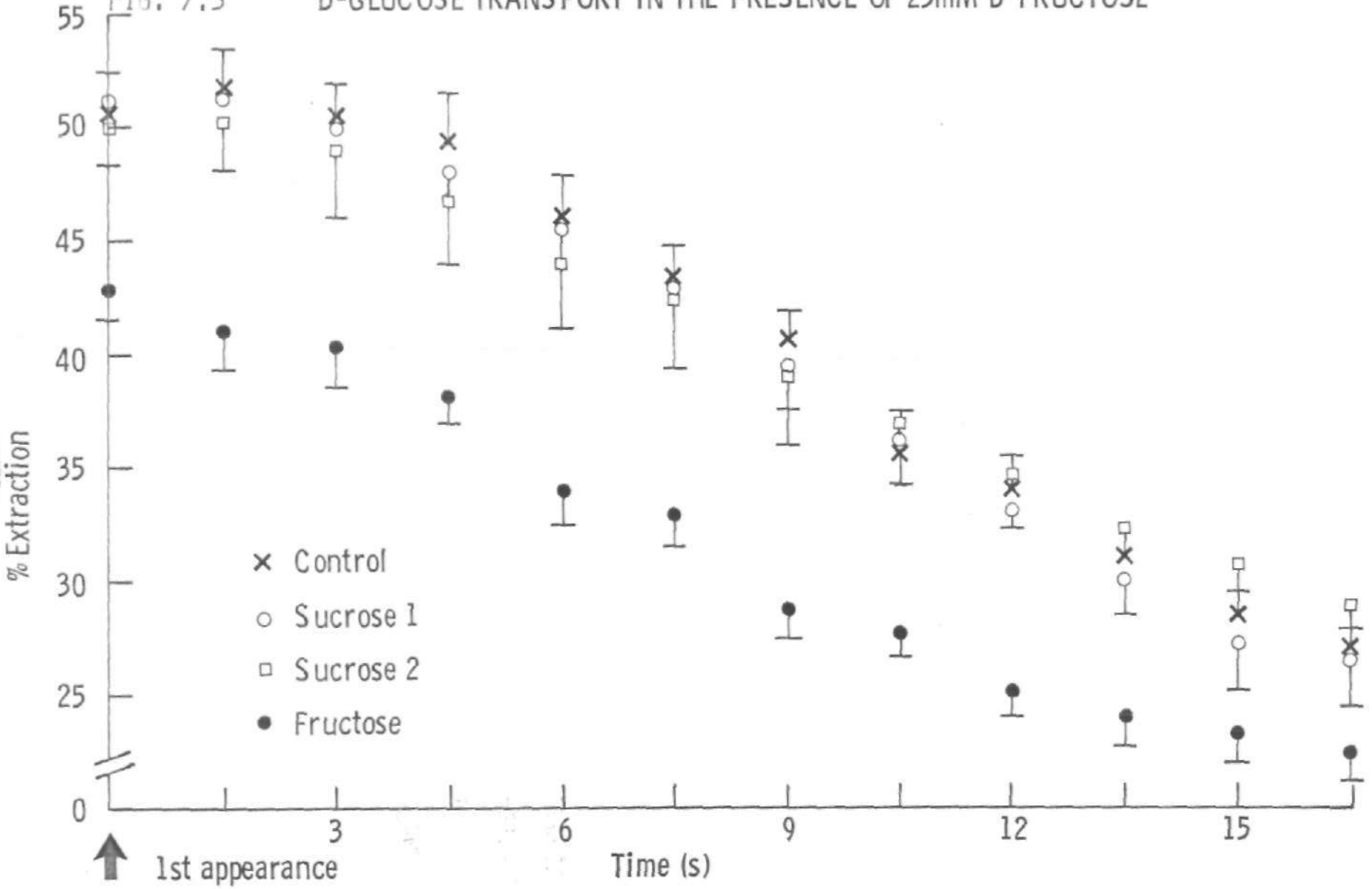
FIG. 7.4 D-GLUCOSE TRANSPORT IN THE PRESENCE OF D-GALACTOSE



LEGEND TO FIG. 7.5

FIG. 7.5. D-GLUCOSE TRANSPORT IN THE PRESENCE OF 25mM D-GRUCTOSE. FRUCTOSE CAUSED A CONSIDERABLE FALL IN GLUCOSE EXTRACTION. THIS IS ALSO EVIDENCE OF COMPETITION BETWEEN GLUCOSE AND FRUCTOSE. THE FRUCTOSE EFFECT IS SIGNIFICANTLY GREATER THAN THAT OF GALACTOSE.

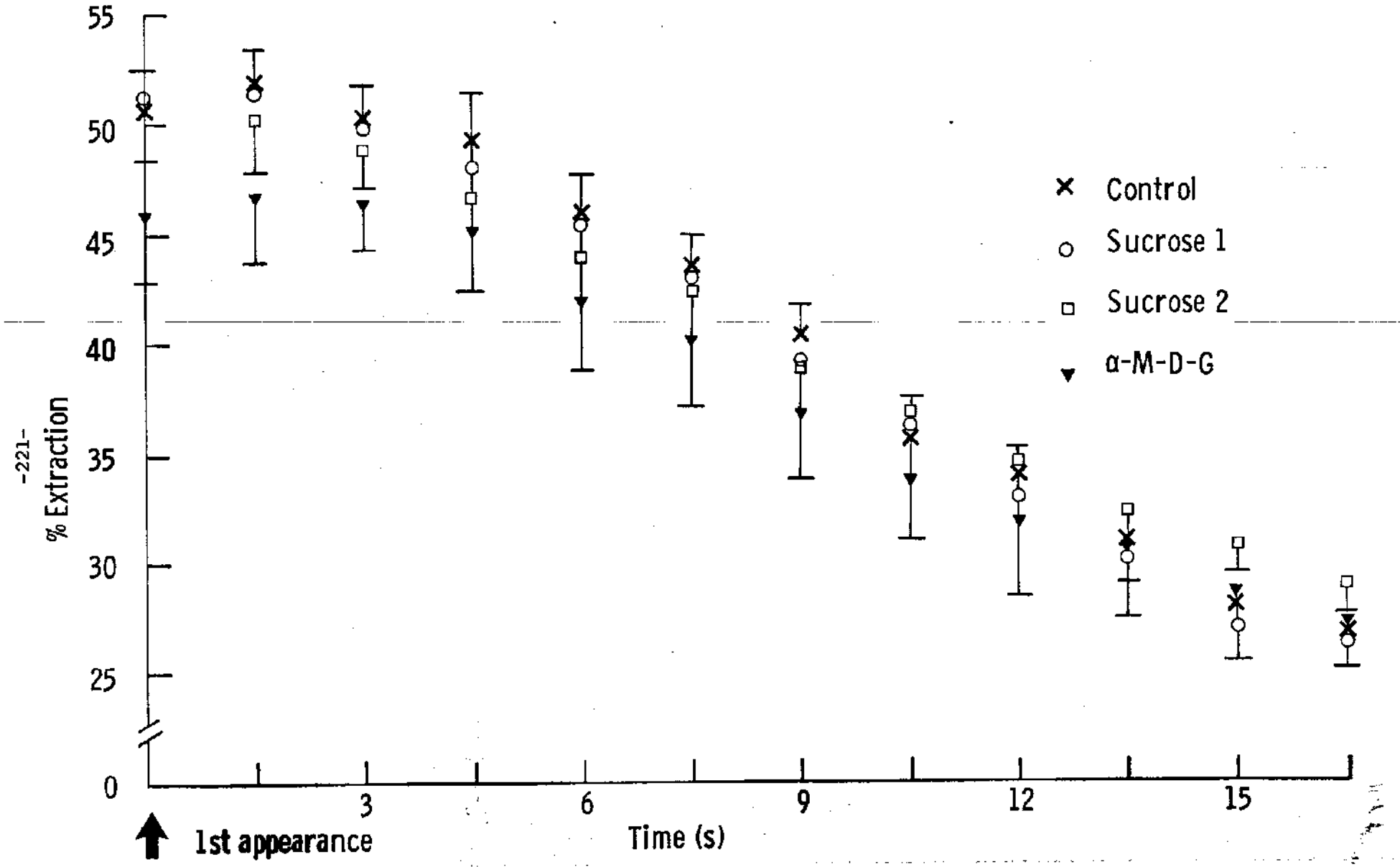
FIG. 7.5 D-GLUCOSE TRANSPORT IN THE PRESENCE OF 25mM D-FRUCTOSE



LEGEND TO FIG. 7.6

FIG. 7.6 - D-GLUCOSE TRANSPORT IN THE PRESENCE OF 25MM α-METHYL-D-GLUCOPYRANOSIDE (α-M-D-G). THE GLUCOSE TRANSPORT WAS ALSO SIGNIFICANTLY DECREASED BY α-M-D-G.

FIG. 7.6 D-GLUCOSE TRANSPORT IN THE PRESENCE OF 25mM α-M-D-GLUCOSIDE



(fig. 7.6, and Table 7.2). The extraction also fell with time and showed a correlation coefficient of -0.991 (Table 7.1). Although the inhibition is less than those of galactose and fructose, it is significantly lower than Control ($P<0.05$). So galactose, fructose and α-M-D-G all decrease D-glucose extraction depicting competition.

The chemical structures of phlorizin (variously spelt as phlorhizin, phloridzin etc.) and phloretin are shown in fig. 7.7. Note here that phloretin is an aglycone of phlorhizin (that is phlorhizin without the glucose molecule). Phlorizin normally exists as a dihydrate, the additional two molecules of water are not shown in the figure in order to facilitate comparison with phloretin structure.

The presence of 1mM phlorizin in perfusate caused a reduction in D-glucose extraction as shown on the left hand panel of fig. 7.8. The phlorizin decreased the glucose extraction from a mean control of 40.93 ± 2.62 to 29.36 ± 1.43 (Table 7.3), this representing an inhibition of 28.2 ± 2.06%. Also 1mM phloretin decreased glucose extraction (fig. 7.9, left hand panel). But phloretin decrease was greater, having been decreased from a mean control value of 42.22 ± 0.90% to 12.73 ± 0.27, giving an inhibition of 69.8 ± 2.34% (Table 7.3). The inhibition of phlorizin and phloretin were significant ($P<0.01$, and $P<0.001$ respectively). The inhibition by phloretin was highly significantly greater than that by phlorizin ($P<0.001$). The inhibitions by both phloretin and phlorizin are compared on the left hand panel of fig. 7.10, as a percentage decrease from the control value in each case ($N = 4$, for each chemical).

Although Sucrose had no significant effect on D-glucose transport, the addition of 25mM lactose decreased the extraction of glucose significantly (fig. 7.11). Lactose decreased glucose transport from 49.58 ± 1.58% to 44.46 ± 0.28% which represents an inhibition of

TABLE 7.3

Effects of Phlorizin or Phloretin on

Glucose transport

Type of Experiment	Extraction		Significance
	% E ± S.E.M.	ΔE% ± S.E.M.	P
1. Control	40.93 ± 2.62	-	
2. Added Phlorizin	29.36 ± 1.43	-28.2 ± 2.06	* P<0.01 ** P<0.001
3. Control	42.22 ± 0.90	-	
4. Added Phloretin	12.73 ± 0.27	-69.84 ± 2.34	* P<0.001 ** P<0.001

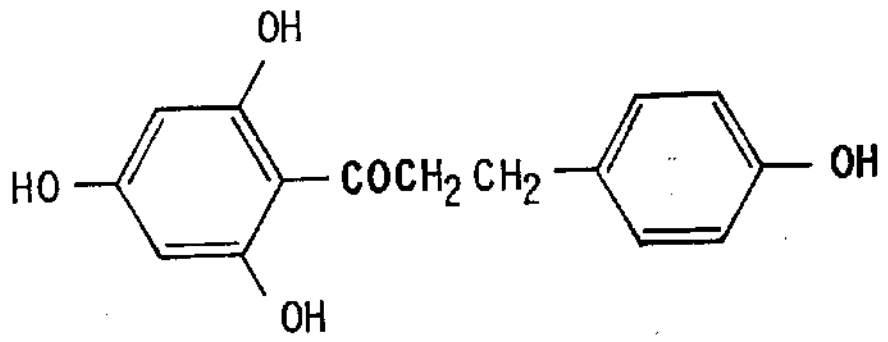
Key: * Significance of the difference between this and the control (i.e. between 1 and 2, and 3 and 4)

** Significance of the difference between 2 and 4.

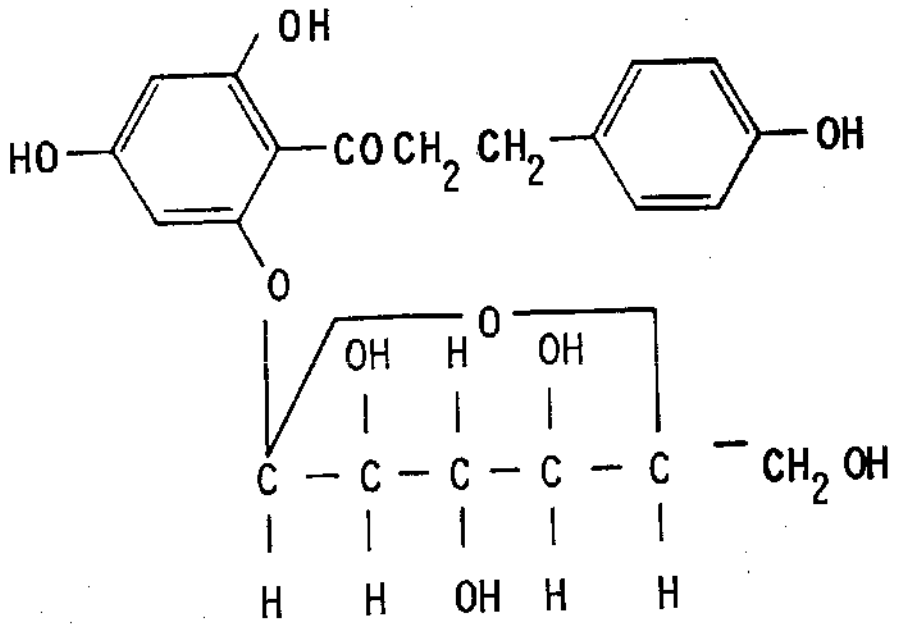
LEGEND TO FIG. 7.7

FIG. 7.7 MOLECULAR STRUCTURES OF PHLORETIN AND PHLORIZIN. NOTE THAT THE ONLY DIFFERENCE IN THE TWO STRUCTURES IS THE ABSENCE OF GLUCOSE MOIETY IN THE PHLORETIN (HENCE IT IS SAID TO BE THE AGLYCONE OF PHLORIZIN).

FIG. 7.7 MOLECULAR STRUCTURE OF PHLORETIN AND PHLORHIZIN



Phloretin



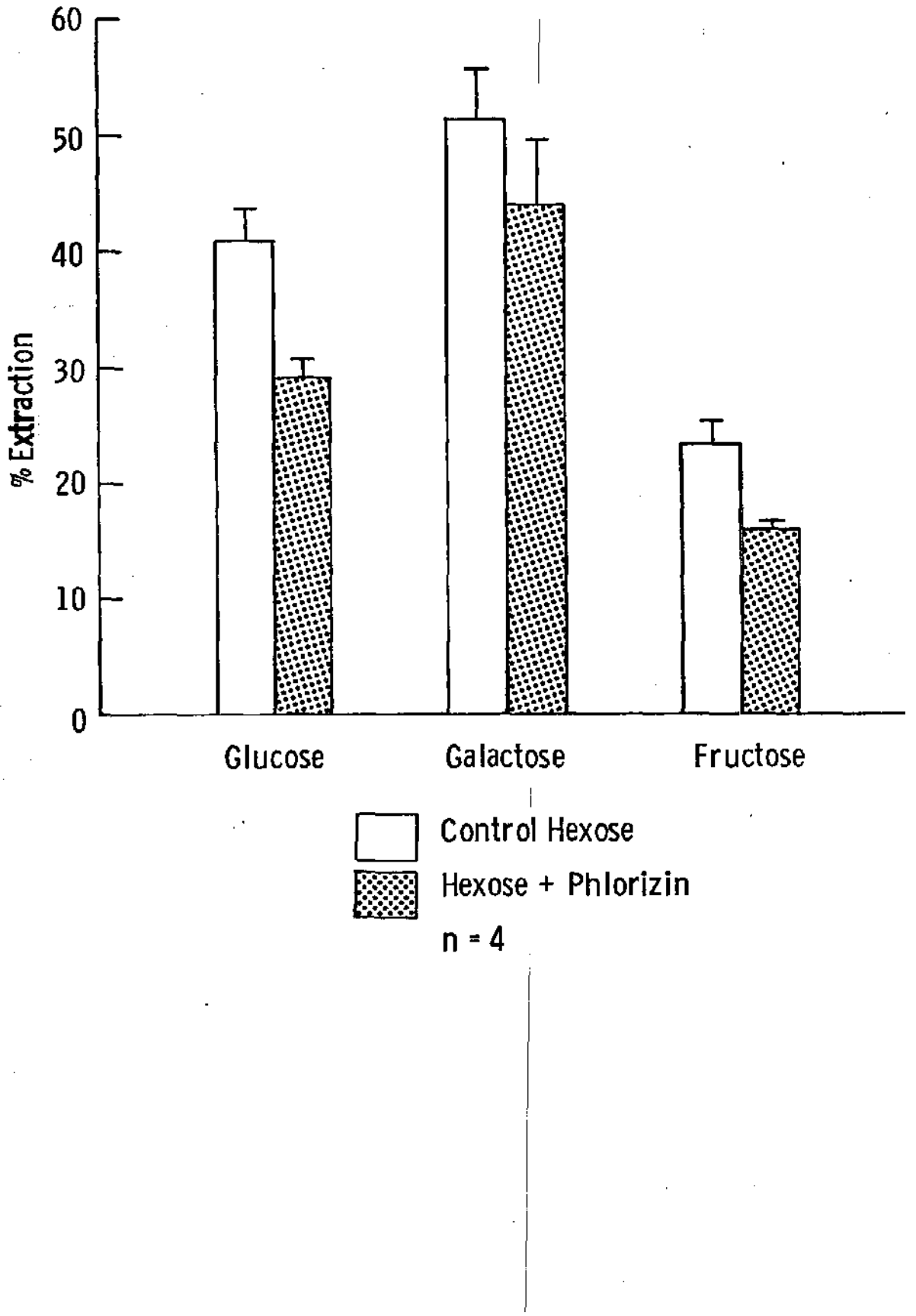
Phlorhizin (dihydrate)

LEGEND TO FIG. 7.8

FIG. 7.8. EFFECT OF PHLORIZIN ON TRANSPORT OF D-GLUCOSE, D-GALACTOSE OR D-FRUCTOSE. IT INHIBITED THE TRANSPORT OF ALL THE THREE HEXOSES.

FIG. 7.8

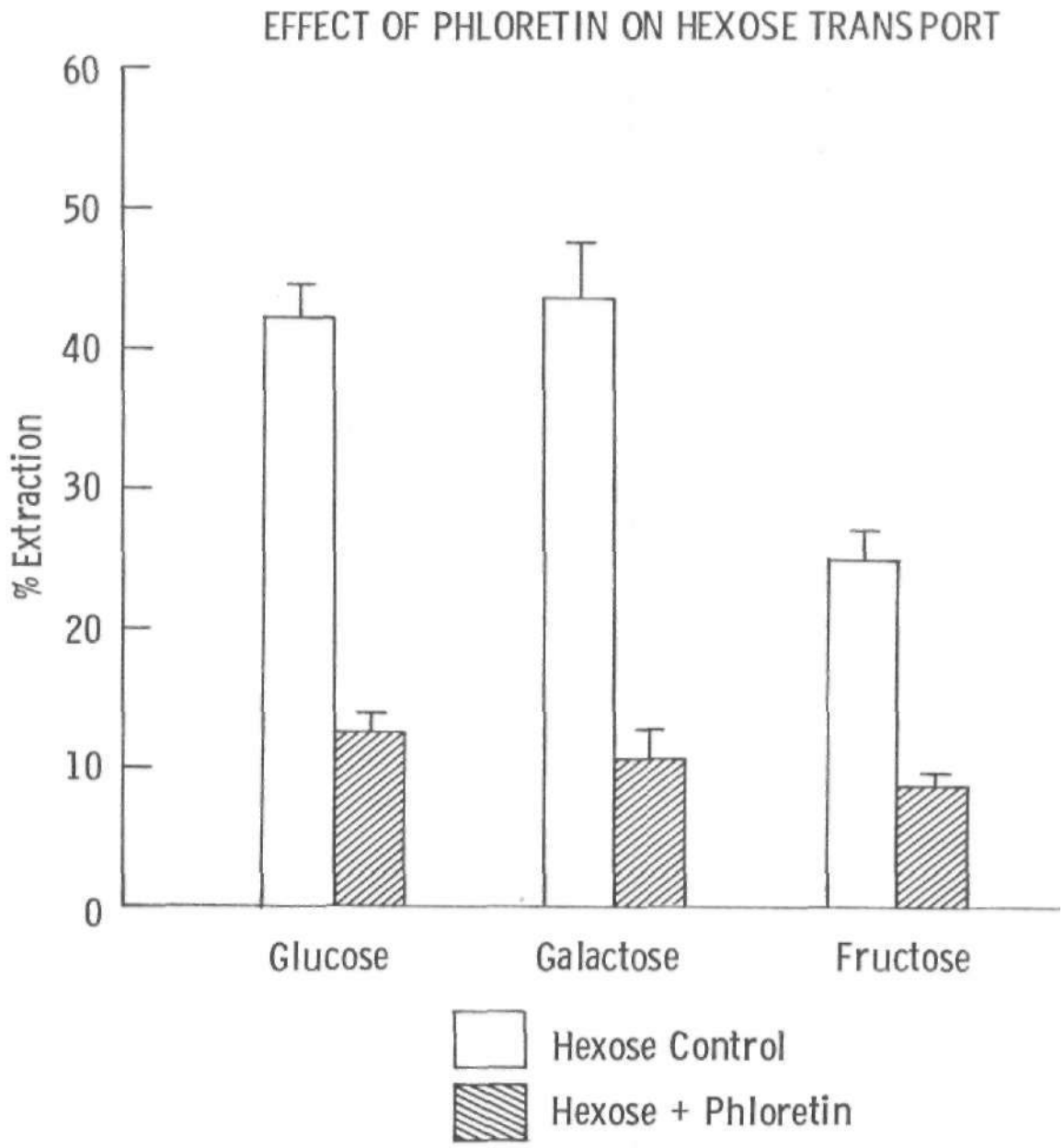
EFFECT OF PHLORIZIN ON TRANSPORT OF HEXOSES



LEGEND TO FIG. 7.9

FIG. 7.9. EFFECT OF PHLORETIN ON TRANSPORT OF D-GLUCOSE, D-GALACTOSE OR D-FRUCTOSE. IT HAS A PROFOUND INHIBITORY EFFECT ON THE TRANSPORT OF ALL OF THESE HEXOSES.

FIG. 7.9

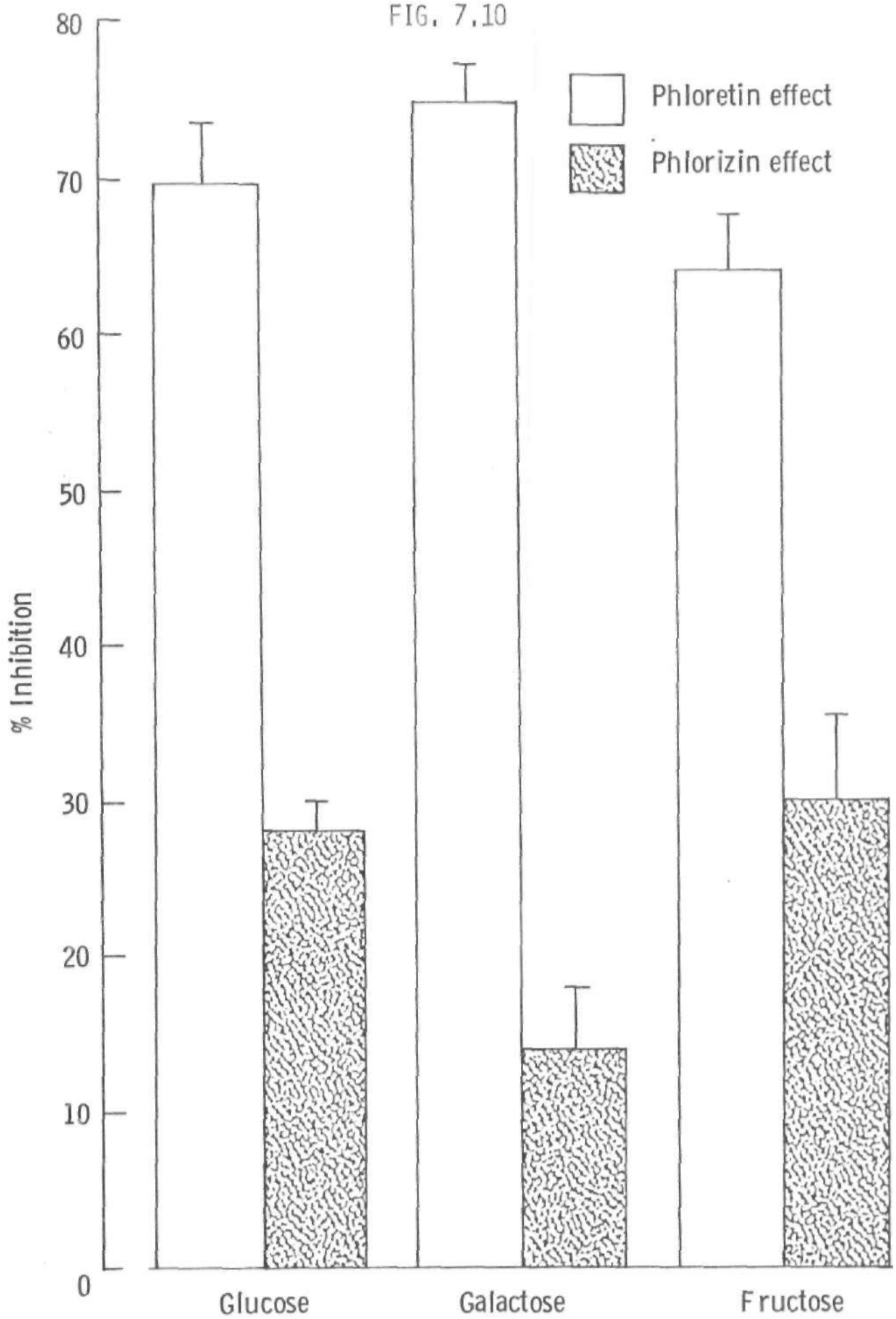

EFFECT OF PHLORETIN ON HEXOSE TRANSPORT
% Extraction
60
50
40
30
20
10
0
Glucose
Galactose
Fructose
Hexose Control
Hexose + Phloretin

LEGEND TO FIG. 7.10

FIG. 7.10. COMPARISON OF THE INHIBITION OF TRANSPORT OF D-GLUCOSE, D-GALACTOSE, AND D-FRUCTOSE BY PHLORETIN IS BY FAR A MORE POTENT INHIBITOR OF THE TRANSPORT OF ANY OF THESE HEXOSES THAN PHLORIZIN.

COMPARATIVE INHIBITION BY PHLORETIN AND PHLORIZIN

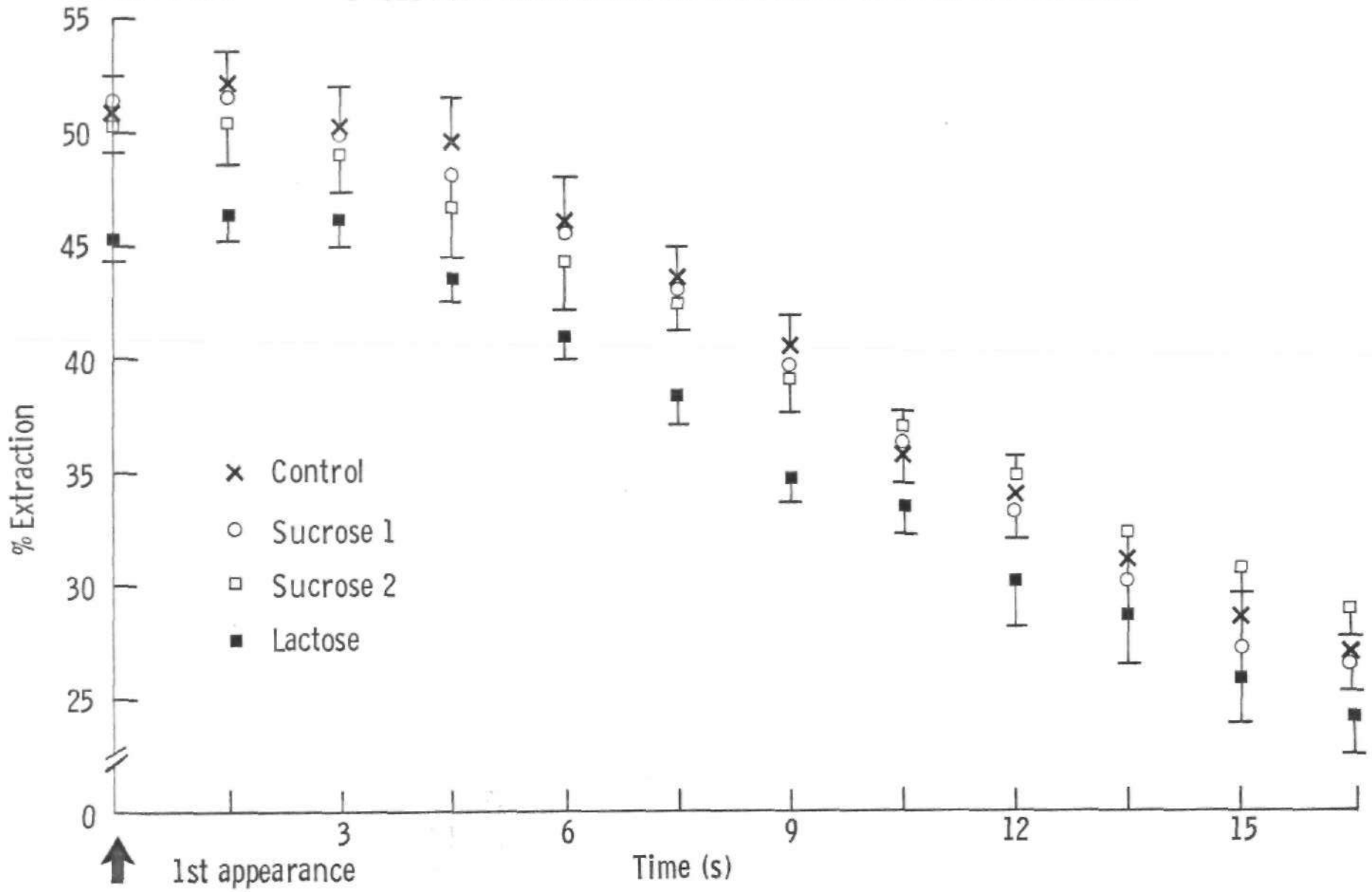
FIG. 7.10



LEGEND TO FIG. 7.11

FIG. 7.11. D-GLUCOSE TRANSPORT IN THE PRESENCE OF 25mM LACTOSE. LACTOSE SIGNIFICANTLY DECREASED THE GLUCOSE TRANSPORT.

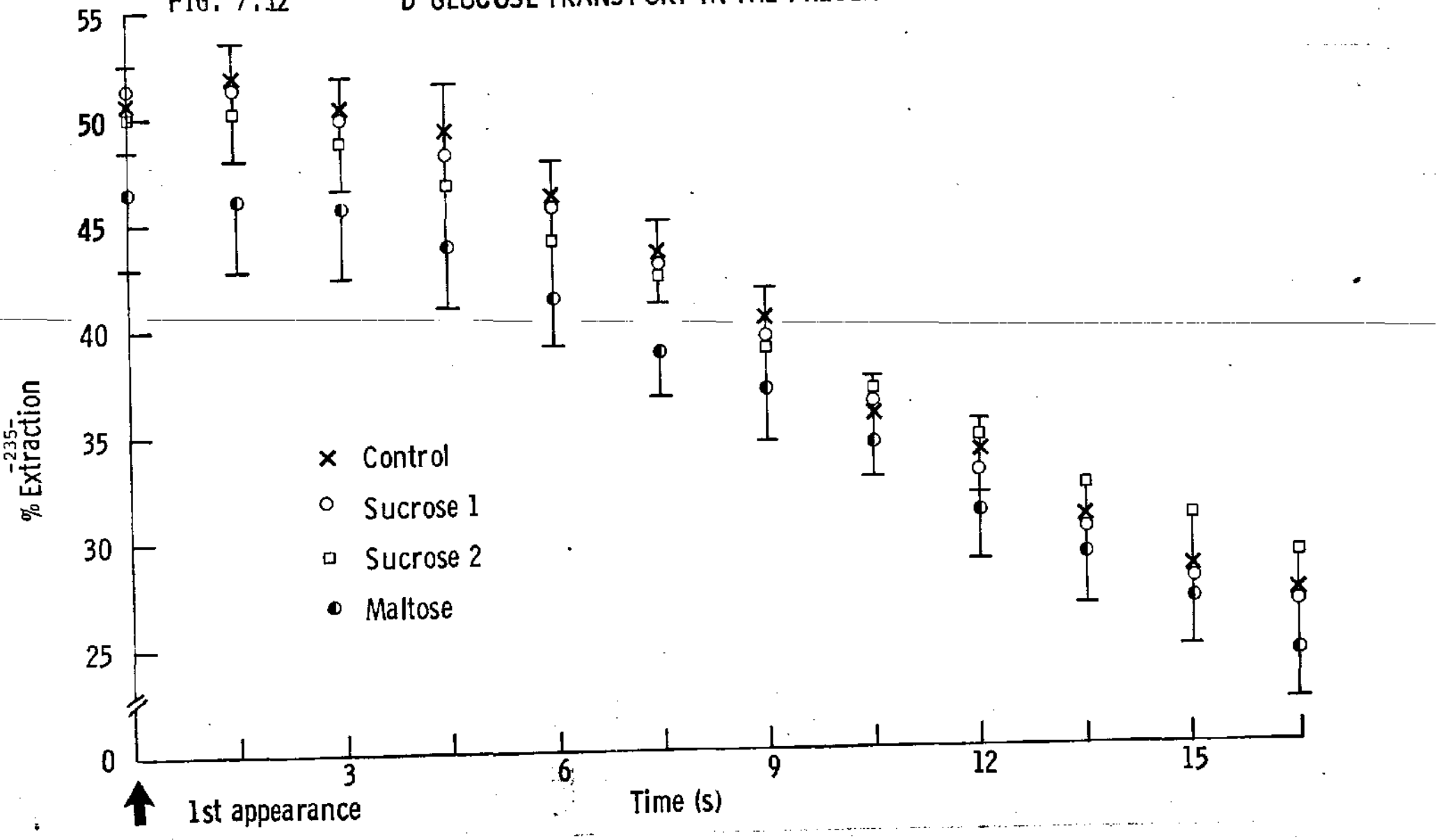
FIG. 7.11 D-GLUCOSE TRANSPORT IN THE PRESENCE OF 25 mM LACTOSE



LEGEND TO FIG. 7.12

FIG. 7.12. D-GLUCOSE TRANSPORT IN THE PRESENCE OF 25MM MALTOSE. MALTOSE SIGNIFICANTLY DECREASED THE GLUCOSE TRANSPORT.

FIG. 7.12 D-GLUCOSE TRANSPORT IN THE PRESENCE OF MALTOSE



10.32 ± 0.30%. This was a significant inhibition (P<0.01). Similarly, 25mM Maltose decreased glucose extraction (fig. 7.12) to 42.40 ± 2.42 representing an inhibition of 14.46 ± 0.48%. This was also a significant depression in extraction from the control (P<0.01).

7.4 Discussion

The role of the liver in the homeostatic control of glucose level in the blood has been spelt out in section 7.1 (introduction) above. Because of the importance of liver in this respect, the study of glucose transport in it is worth while. Glucose is the "sugar" of the blood, hence its handling by the liver was investigated by the early workers as narrated in section 7.1. But its exact nature of transport characteristics have not been elucidated. In the present study, more light was thrown on the glucose transport characteristics in the rat liver.

Although CAHILL et al (1958) concluded that D-glucose entered the liver by free diffusion, the results in the present study do not support this proposition. These results rather point to a special and specific transport mechanism which is analogous to the facilitated diffusion mechanism that is known to exist for glucose entry into the erythrocytes (WIDDAS 1954; WILBRANDT & ROSENBERG 1957; LEFEVRE 1961, 1972; STEIN 1967). The glucose transport in this study showed "saturation" characteristics by exhibiting a significant reduction in extraction as the glucose concentration was increased by addition of 25mM glucose. D-glucose transport was shown here to be very much higher than that of L-glucose when tested in the same livers, suggesting strong "stereospecifity" of glucose transport in the liver. Also it was shown that D-galactose, D-fructose and a non-metabolizable methyl-substituted sugar, α-methyl-D-glucopyranoside all reduce glucose extraction when present at 25mM concentration each. This means that there is a "competition" by these sugars for entry by the same mechanism as D-glucose, Furthermore, chemically unrelated substances, phlorizin and its aglycone, Phloretin inhibited transport of glucose significantly. Of these two, "chemical inhibition" by phloretin was greater than that

by phlorizin. Phlorizin has been known for a long time now to cause experimental glycosuria in mammals by inhibition of glucose reabsorption in the renal tubules, but its aglycone, phloretin, scarcely has any such effect on the kidney tubules (FRANCK 1914; FROMM 1917). The converse was true for the erythrocyte sugar transport system where WILBRANDT (1950) discovered that phloretin was many times more effective than phlorizin as inhibitors of sugar transport in the erythrocyte system. This was the dividing line separating the sugar transport mechanism in the erythrocytes from the active transport mechanism of sugar by the kidneys. On this basis, the erythrocyte sugar entry is said not to be by active transport. On similar basis, the findings of a greater phloretin effect than phlorizin inhibition of glucose transport by the liver can be interpreted as distinguishing the hepatocellular glucose transport mechanism from active transport as exists in the renal tubules and the gut, since all the other characteristics of saturation, stereospecificity, competion and chemical inhibition reported here are common to both active transport and facilitated diffusion mechanisms.

Furthermore, Lactose and Maltose inhibited glucose transport, while sucrose did not. This appears to be a case of species variation because HOOPER (1975), and HOOPER and SHORT (1977) found sucrose to inhibit glucose transport in the sheep liver. Since these disaccharides are confined to the extracellular compartment (CAHILL et al 1958; LEFEVRE 1972), they must be exerting their influence externally. The full implications of these findings are discussed in Chapter 10.

CHAPTER EIGHT

8. GALACTOSE TRANSPORT BY THE PERFUSED RAT LIVER

8.1 Introduction

Interest in the hepatic handling of hexoses first centred on glucose, but this widened to include galactose, and fructose. D-galactose is a hexose which is removed in a net fashion from the circulation by the liver. When the plasma level of galactose is high, the removal rate is constant, and so long as the hepatic blood flow is constant, a arterial-hepatic vein difference results; and when the plasma concentration is low (less than 30 mg. 100 ml^{-1}) the hepatic venous blood is almost completely cleared of galactose (GORESKY, BACH & NADEAU 1973). The extraction of galactose in the lower concentration range is large enough (an average of 88% in normal man) as reported by TYGSTRUP and WINKLER (1954) for its constant infusion to be used as a basis for the estimation of hepatic blood flow (TYGSTRUP & WINKLER, 1958). This net removal of galactose from the circulation by the liver depends on both membrane penetration and metabolic sequestration of galactose in the liver. Galactose sequestration is achieved by a process of phosphorylation, of conversion via galactokinase, into galactose-1-phosphate, a compound which does not freely pass through cell membranes. This compound, in turn, is ordinarily rapidly converted to glucose-1-phosphate by the enzyme galactose-1-phosphate uridyl transferase - the enzyme usually deficient in galactosaemia (KALCKAR, ANDERSON & ISSELBACHER, 1956; ISSELBACHER, ANDERSON, KURAHASHI & KALCKAR, 1956).

It would therefore be important to find out whether this net removal of galactose by the liver was a function of membrane penetration or the subsequent metabolic events in the liver. Galactose uptake

by the intestinal cells has been studied; and it shares the same transport system with glucose, merely having a different affinity for the carrier. The major distinction between the intestinal and hepatic systems seems to be that the hepatic hexose transport system is by facilitated diffusion, energy independent (PARK, CROFFORD & KONO 1968), whereas that on the brush border of the intestinal cells is by active transport (energy dependent) mechanism. It was therefore necessary to study the nature of the transport mechanism of galactose as well as those of glucose and fructose to elucidate whether they share a common transport system or not. The studies reported in this chapter and in the preceding and succeeding ones aimed at doing this. Earlier workers have confirmed that facilitated diffusion transport mechanism in the liver exists for galactose (GORESKY, BACH & NADEAU, 1973; HOOPER & SHORT, 1975; 1977; BAUR & HELDT, 1977) as well as the other two hexoses. Apart from the reported difference in affinity between these hexoses, these reports did not suggest duplicity or multiplicity of the hexose carrier mechanism in the liver of the various species used (dog, sheep and rat) although HOOPER (1975) proposed that galactose might be transported on a common monosaccharide carrier but that a certain specific fraction of the glucose carrier would not be available to galactose for its transport. This was a pointer to a distinction between glucose and galactose hepatocellular transport mechanisms. The studies reported here advanced this proposition and this chapter describes the findings.

8.2 *Method*

The method used for the study of galactose transport by the perfused rat liver was essentially the same as that reported for glucose transport studies (Section 7.2). Only changes made that distinguish this study from that reported in Chapter 7 will be described here.

The infusate mixture for each experimental run was made up of ^{3}H-D-galactose (as test material) and ^{14}C-Sucrose (as extracellular reference material) and 1 ml of 250 mM potential competitor (unlabelled sugar) and perfusate withdrawn rapidly to make a total volume of 10 ml thereby bringing the final concentration of the potential competitor to 25 mM. Infusion and sampling were done as previously described (Chapter 3). The concentrations of the isotopes were galactose = 2.5×10^{-6} M and Sucrose = 3.8×10^{-6} M.

The effect of phlorizin or phloretin on galactose transport was studied as described in section 7.2 for glucose transport.

Furthermore, the effect of temperature on galactose extraction by the liver was studied in five livers using the same perfusion method as in other experiments, the only differences being the variation of temperature from one run to the other, and that no potential competitors were added. The Churchill water pump/heater was switched on at its normal 40°C temperature rating. When the liver temperature had equilibrated at 37°C as usual, the first run was performed. Then the Churchill water pump/heater temperture control dial was turned down to a setting for 10°C, (thus ensuring that the heater would remain inoperative) and cold water was circulated through the Churchill from a water tap. The liver temperature was continuously monitored as described in Chapter 3. As the temperature of the liver was falling, a second run was performed when the temperature came down to

28°C, and a third run when a temperature of 22°C was reached. The Churchill was turned to 40°C mark and the cold water running from the tap was stopped. The liver temperature started rising, and as the temperatures of 25°C, 31°C, 34°C and 37°C were reached the fourth, fifth, sixth and seventh runs were performed respectively. The results in the five livers were pooled and plotted as a graph.

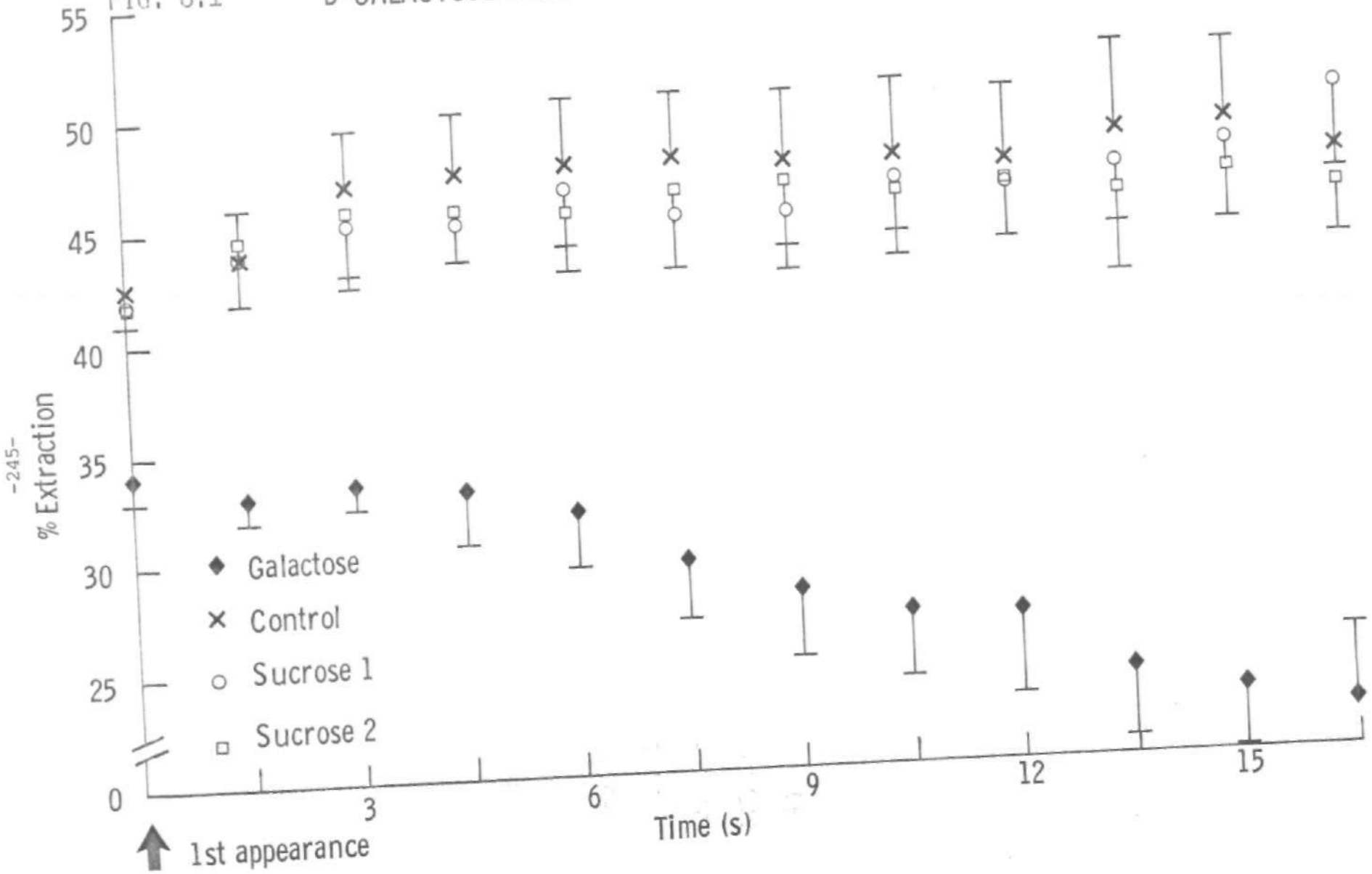
8.3 Results

Control D-galactose transport is shown in fig. 8.1. The axes in this figure and the next five are the same. The ordinate (Y axis) is a broken axis representing the percentage hepatic extraction of D-galactose, and the abscissa (X axis) is time scale in seconds. Control galactose extraction when no potential competitor was added in the infusate mixture has been compared with sucrose 1 and Sucrose 2 runs in this figure. The mean control galactose extraction was 45.35 ± 0.93% in four livers. The mean extraction in the presence of first 25mM sucrose addition (Sucrose 1 run) was 45.16 ± 1.63% and that of the last sucrose addition (Sucrose 2 run) was 45.38 ± 2.98% (Table 8.1). There was no significant difference between either of these sucrose runs and the control ($P > 0.05$, $N = 4$, using paired t-test in this and for all other P values reported in this Chapter). Also there was no siginficant difference between Sucrose 1 and Sucrose 2 runs ($P > 0.05$, $N = 4$) suggesting that there was no functional deterioration between the time of Sucrose 1 (run 2) and that of Sucrose 2 (last run). The lack of significant difference between Sucrose 1 and control means firstly that the presence of 25 mM solute does not alter the extraction per se, and secondly that 25mM sucrose has no significant effect on galactose transport. Hence the two sucrose runs were plotted in each figure to compare with effects of other potential competitiors. Also in fig. 8.1, the effect of presence of 25 mM unlabelled galactose is shown to be that of drastically reducing the galactose transport from 45.35 ± 0.93% to 33.08 ± 1.36% representing 27.0 ± 1.46% inhibition from the control value. This reduction was highly significant ($P < 0.02$, $N = 4$). This means that galactose entry or inward transport by the livers showed saturable characteristics.

LEGEND TO FIG. 8.1

FIG. 8.1. D-GALACTOSE TRANSPORT IN THE PRESENCE OF 25MM GALACTOSE. GALACTOSE REDUCED ITS OWN TRANSPORT SIGNIFICANTLY, AND SUBSEQUENT EXTRACTIONS FELL PROGRESSIVELY DUE TO REFLUX OF LABELLED GALACTOSE. THIS DEPRESSION OF THE INITIAL UPTAKE IS EVIDENCE OF THE SATURABILITY OF INWARD TRANSPORT. THE PROVOCATION OF REFLUX OF GALACTOSE LABEL BY ADDITIONAL GALACTOSE IS EVIDENCE OF SPECIFIC GALACTOSE BINDING WITHIN THE LIVER CELL, PRESUMABLY ON GALACTOKINASE, WHICH PHOSPHORYLATES VIRTUALLY ALL THE LABELLED GALACTOSE IN THE ABSENCE OF ADDITIONAL COLD GALACTOSE.

FIG. 8.1 D-GALACTOSE TRANSPORT IN THE PRESENCE OF 25mM D-GALACTOSE



Addition of 25 mM D-glucose reduced galactose transport (fig. 8.2) from the control value to 36.92 ± 2.23% which is an inhibition of 18.5 ± 0.99% (Table 8.1). This difference from control extraction was significant ($P < 0.05$, $N = 4$) although not as highly significant as that of galactose itself. Also the addition of 25 mM fructose reduced the galactose extraction (fig. 8.2) from the control value to 41.26 ± 1.36 giving an inhibition of 9.0 ± 0.70% (Table 8.1). This decrease was also significant ($P < 0.05$, $N = 4$) although the fructose effect on galactose extraction was highly significantly less profound than the effect of glucose on the same ($P < 0.01$). The addition of 25 mM α-methyl-D-glucopyranoside (α-M-D-G) significantly decreased galactose extraction (fig. 8.4) from control value to 33.93 ± 1.50% (Table 8.1) representing an inhibition of 25.72 ± 0.33% ($P < 0.05$). The effects of these sugars thus show competition for uptake.

Both 1 mM phlorizin and 1 mM phloretin inhibited galactose transport in these livers (Middle panel of figs. 7.8 and 7.9 respectively). 1 mM phlorizin decreased galactose transport from 51.57 ± 2.32% to 44.24 ± 1.55 (Table 8.2) giving a highly significant inhibition of 14.20 ± 2.48% ($P < 0.01$). However, 1 mM phloretin effect was more profound, causing a decrease from 43.30 ± 0.30% to 10.49 ± 0.18% in galactose extraction (Table 8.2) which gave a very highly significant inhibition of 75.76 ± 1.20% ($P < 0.001$). The inhibitory effect of phloretin was significantly greater than that of phlorizin ($P < 0.001$).

The effects of the disaccharides on galactose extraction differed from their effects on glucose extraction. The presence of 25 mM lactose caused a significant decrease in galactose extraction (fig. 8.5). Lactose decreased the extraction from a control value of 45.35 ± 0.93% to 34.83 ± 0.68 representing an inhibition of 23.18 ± 1.57%.

LEGEND TO FIG. 8.2

FIG. 8.2. D-GALACTOSE TRANSPORT IN THE PRESENCE OF 25MM GLUCOSE. GLUCOSE CAUSED A SIGNIFICANT DECREASE IN GALACTOSE EXTRACTION. THIS IS EVIDENCE OF COMPETITION FOR UPTAKE OF GALACTOSE. NOTE THE LACK OF GALACTOSE EFFLUX DUE TO GLUCOSE, DEMONSTRATING THE SPECIFICITY OF THE GALACTOSE EFFECT.

FIG. 8.2 D-GALACTOSE TRANSPORT IN THE PRESENCE OF 25mM GLUCOSE

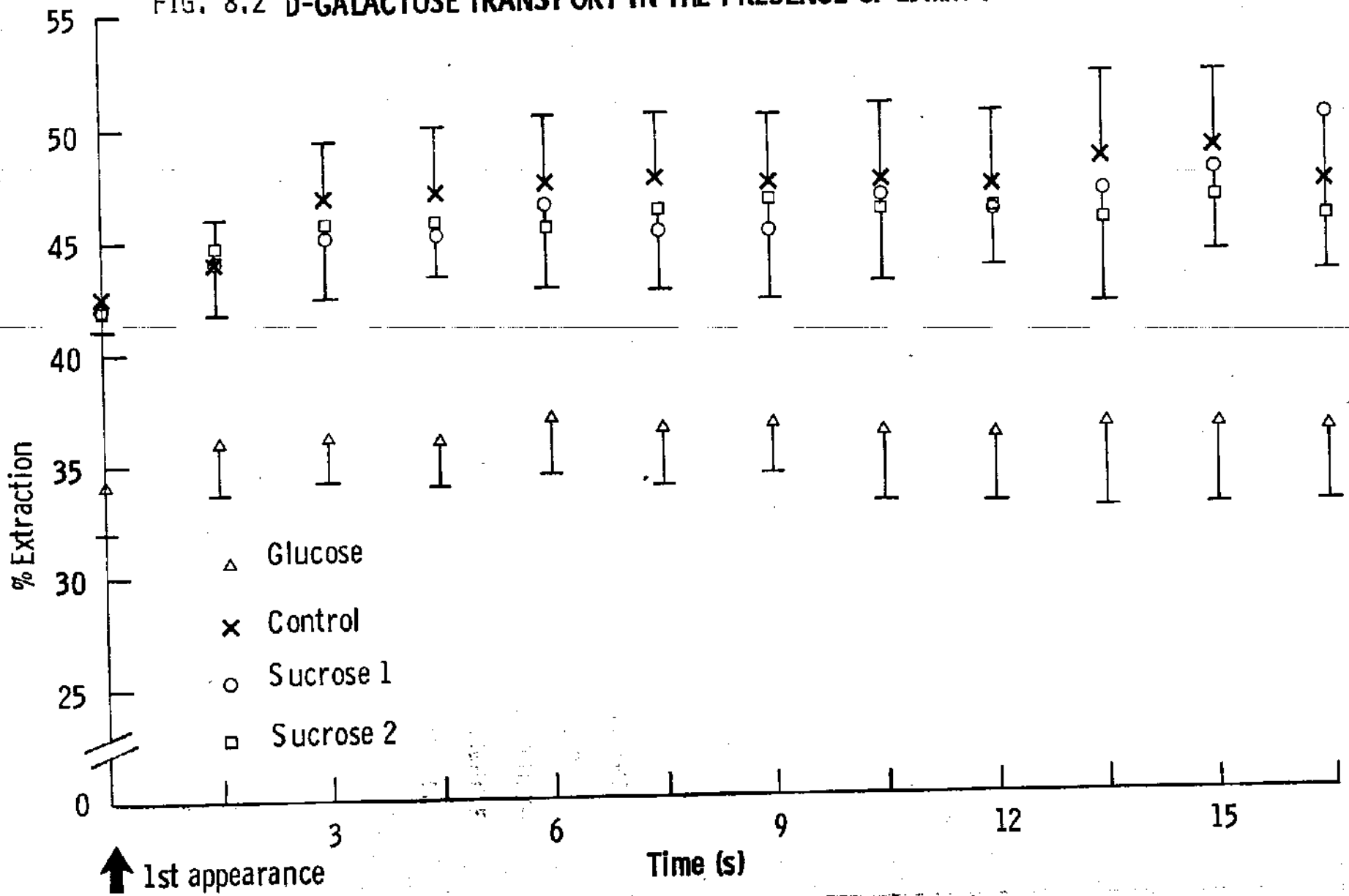


TABLE 8.1 INHIBITION OF GALACTOSE TRANSPORT BY DIFFERENT SUGARS

Competing Sugar 25 mM	D-Galactose Extraction %E ± S.E.M.	ΔE% ± S.E.M.	Significance P
Control	45.35 ± 0.93	-	-
Sucrose 1	45.16 ± 1.63	-0.03 ± 0.80	P > 0.05
D-Glucose	36.92 ± 2.23	-18.5 ± 0.99	**P < 0.05
D-Galactose	33.08 ± 1.36	-27.0 ± 1.46	P < 0.02
D-fructose	41.26 ± 1.36	- 9.0 ± 0.70	**P < 0.05
α-M-D-G	33.93 ± 1.50	-25.72 ± 0.33	P < 0.05
Lactose	34.83 ± 0.68	-23.18 ± 1.57	P < 0.01
Maltose	44.94 ± 1.35	- 0.9 ± 2.15	P > 0.05
Sucrose 2	45.38 ± 2.98	+0.006 ± 1.04	*P > 0.05

N = 4 livers

α-M-D-G = α-methyl-D-glucopyranoside

* = significance of difference between Sucrose 1 and Sucrose 2

Other P values were compared with control only.

All probability values were obtained using paired t-test.

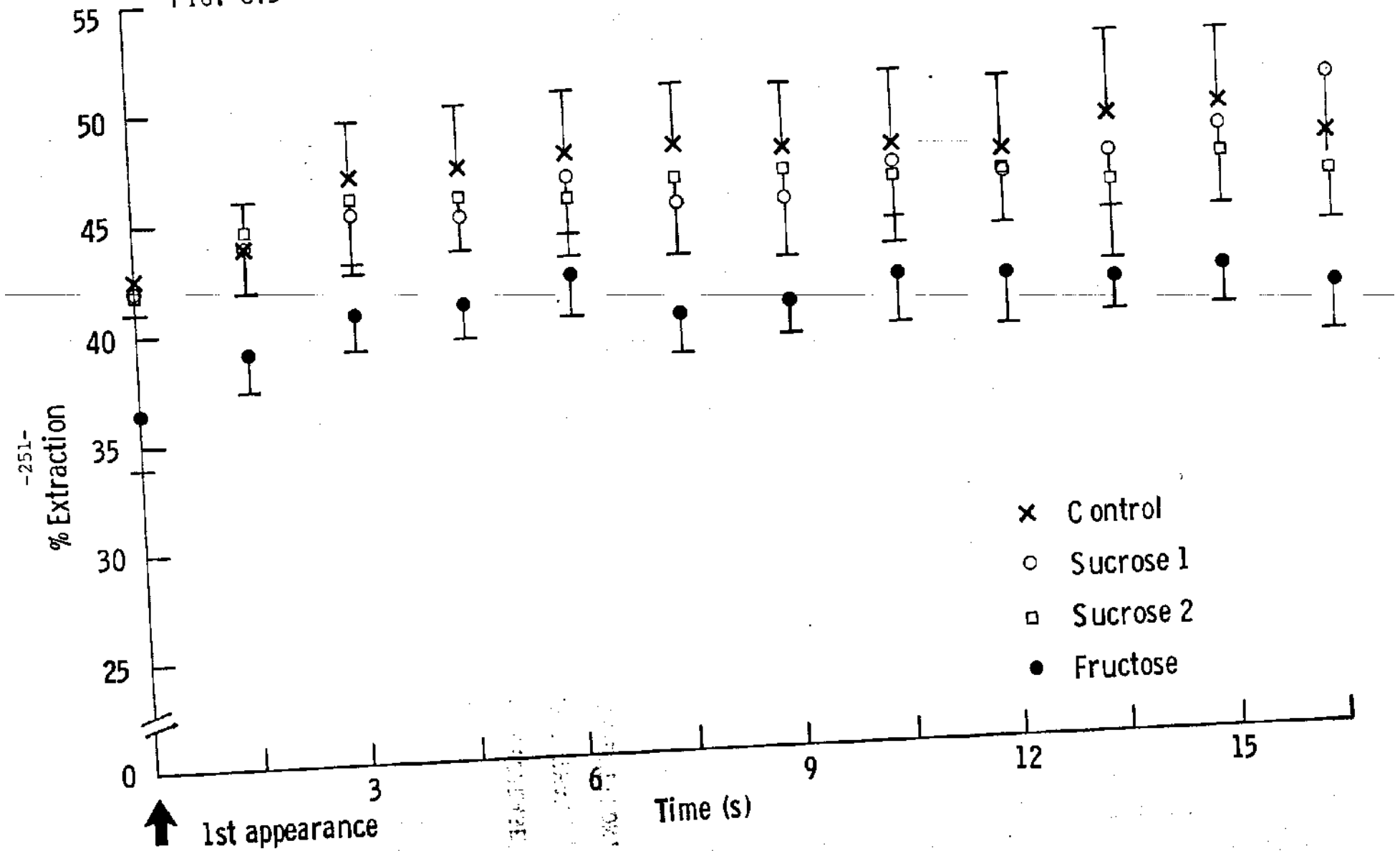
ΔE is change in extraction from control value expressed as percentage of the control.

** The difference between the inhibition by fructose and inhibition by glucose is highly significant (P < 0.01, paired t-test).

LEGEND TO FIG. 8.3

FIG. 8.3. D-GALACTOSE TRANSPORT IN THE PRESENCE OF 25MM D-FRUCTOSE. FRUCTOSE CAUSED A SIGNIFICANT DECREASE IN GALACTOSE EXTRACTION. BUT THIS EFFECT IS LESS THAN THAT CAUSED BY GLUCOSE. THIS IS EVIDENCE OF COMPETITION.

FIG. 8.3 D-GALACTOSE TRANSPORT IN THE PRESENCE OF 25mM D-FRUCTOSE



LEGEND TO FIG. 8.4

FIG. 8.4. D-GALACTOSE TRANSPORT IN THE PRESENCE OF α-METHYL-D-GLUCOPYRANOSIDE (α-M-D-G). IT CAUSED A SIGNIFICANT DECREASE IN GALACTOSE TRANSPORT.

FIG. 8.4 D-GALACTOSE TRANSPORT IN THE PRESENCE OF α-M-D-G

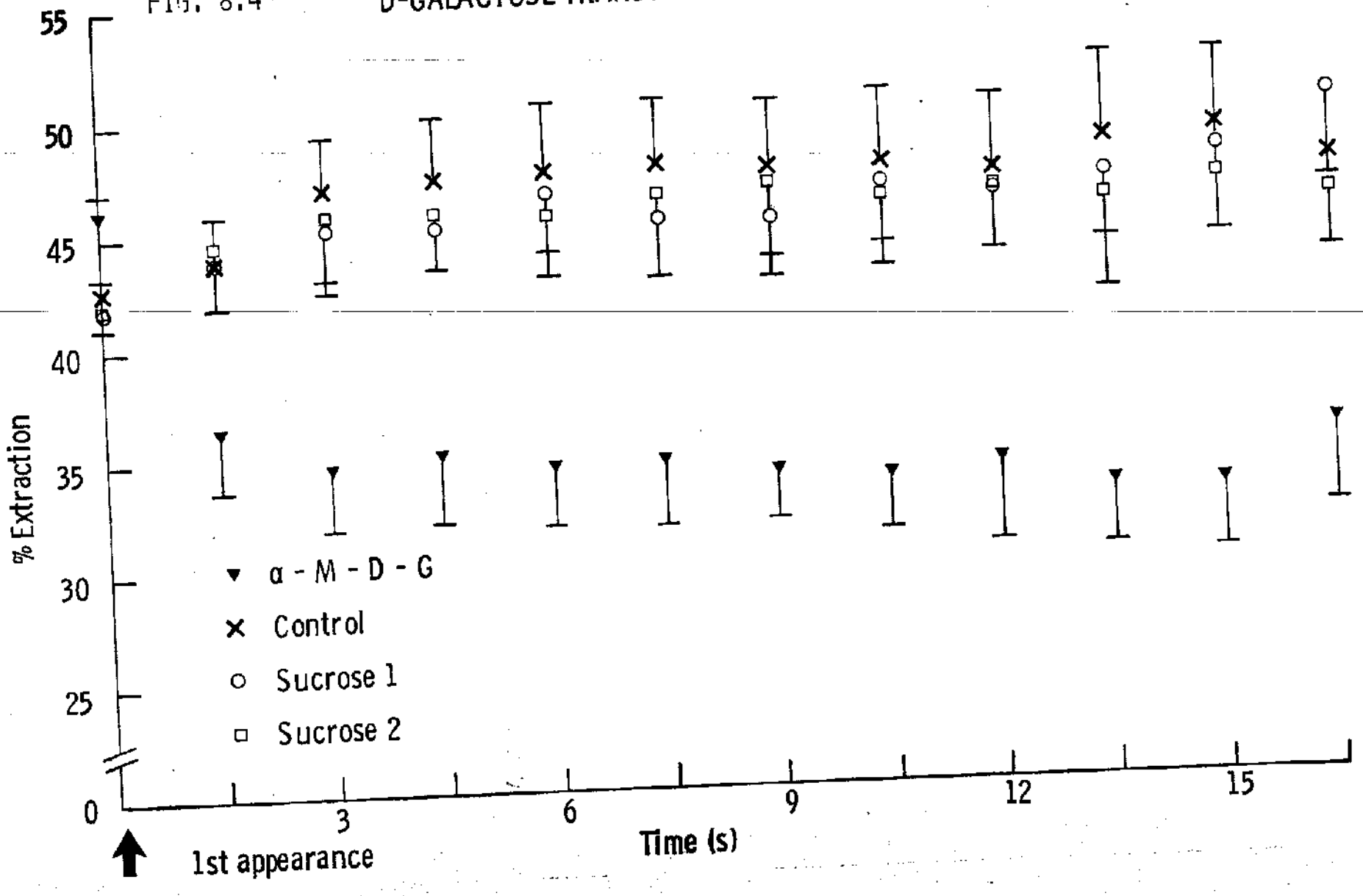


TABLE 8.2

EFFECTS OF PHLORIZIN OR PHLORETIN ON GALACTOSE TRANSPORT

Type of Experiment	Extraction		Significance
	% E ± S.E.M.	ΔE% ± S.E.M.	P
1. Control	51.57 ± 2.32	-	-
2. Added Phlorizin (1 mM)	44.24 ± 1.55	-14.20 ± 2.48	*P < 0.01 **P < 0.001
3. Control	43.30 ± 0.30	-	-
4. Added Phloretin (1 mM)	10.49 ± 0.18	-75.76 ± 1.20	*P < 0.001 **P < 0.00.

KEY

* Significance of the difference between this and the control

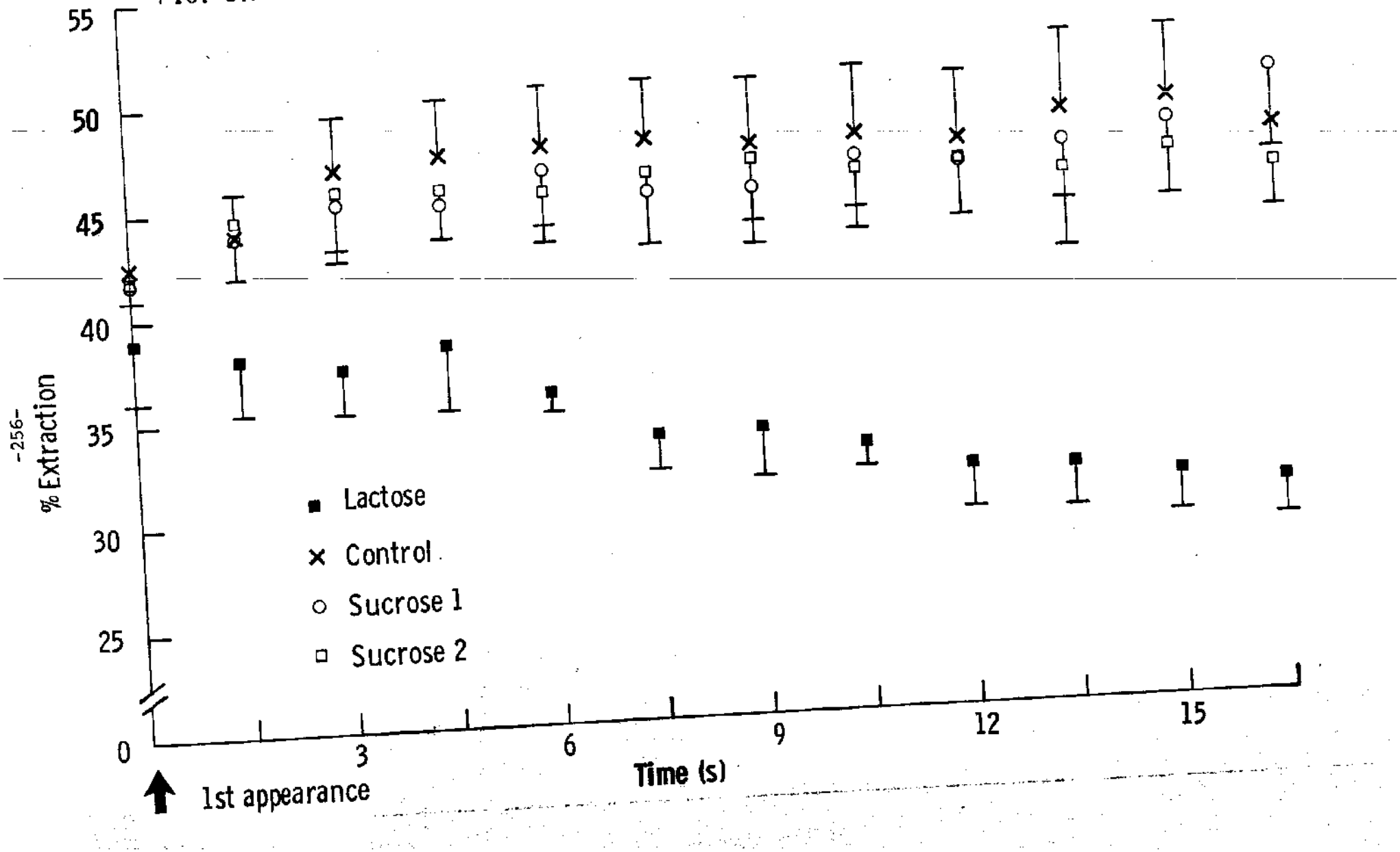
** Significance of the difference between 2 and 4

N = 4 livers in each case.

LEGEND TO FIG. 8.5

FIG. 8.5. D-GALACTOSE TRANSPORT IN THE PRESENCE OF 25MM LACTOSE. LACTOSE HAS A SIGNIFICANT INHIBITORY EFFECT ON GALACTOSE TRANSPORT. THERE WAS ALSO A PROGRESSIVE DECREASE IN EXTRACTION WITH TIME, DUE TO REFLUX OF LABELLED GALACTOSE.

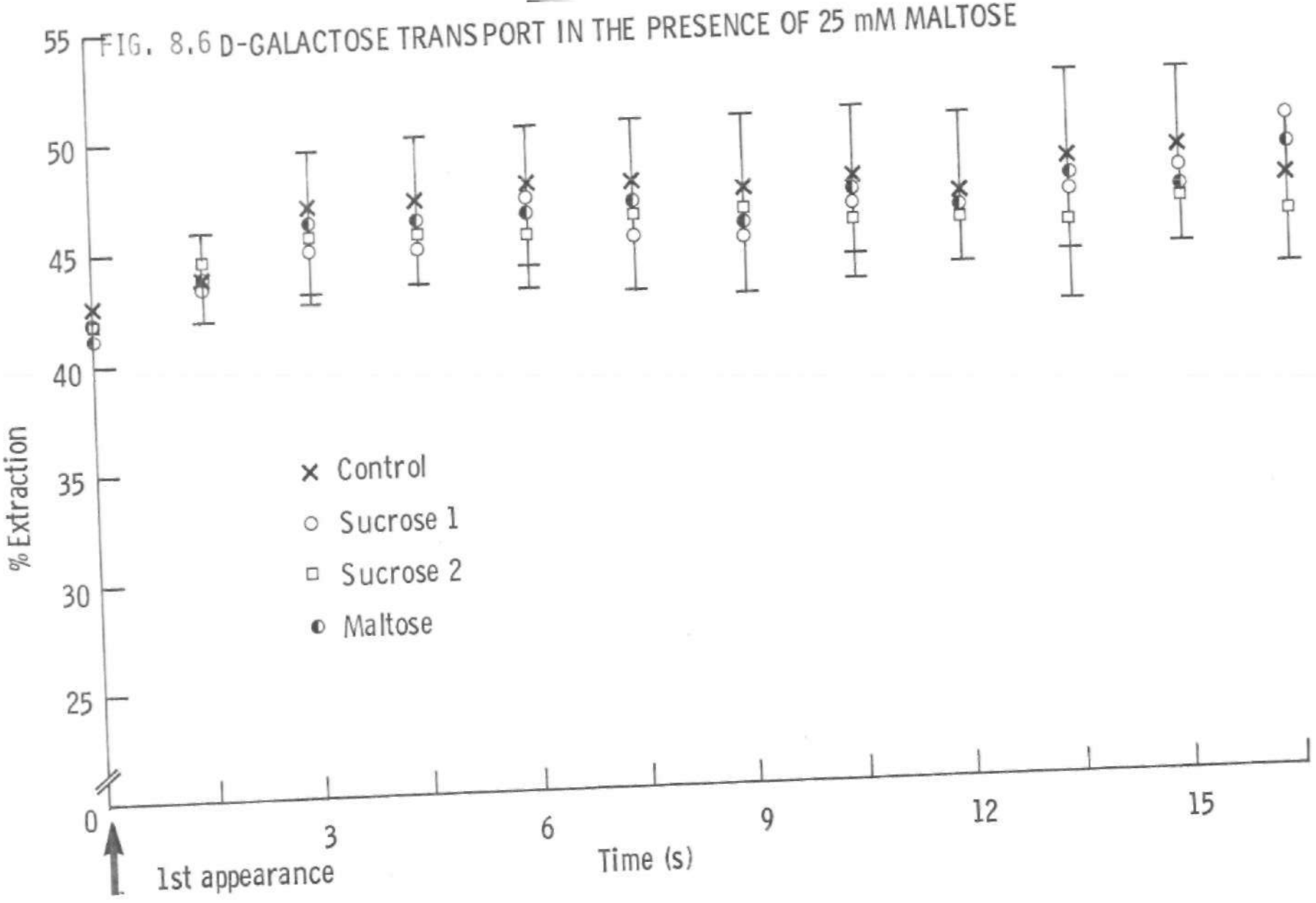
FIG. 8.5 D-GALACTOSE TRANSPORT IN THE PRESENCE OF 25mM LACTOSE



LEGEND TO FIG. 8.6

FIG. 8.6. D-GALACTOSE TRANSPORT IN THE PRESENCE OF 25MM MALTOSE. NOTE THAT MALTOSE HAS NO SIGNIFICANT EFFECT ON GALACTOSE TRANSPORT.

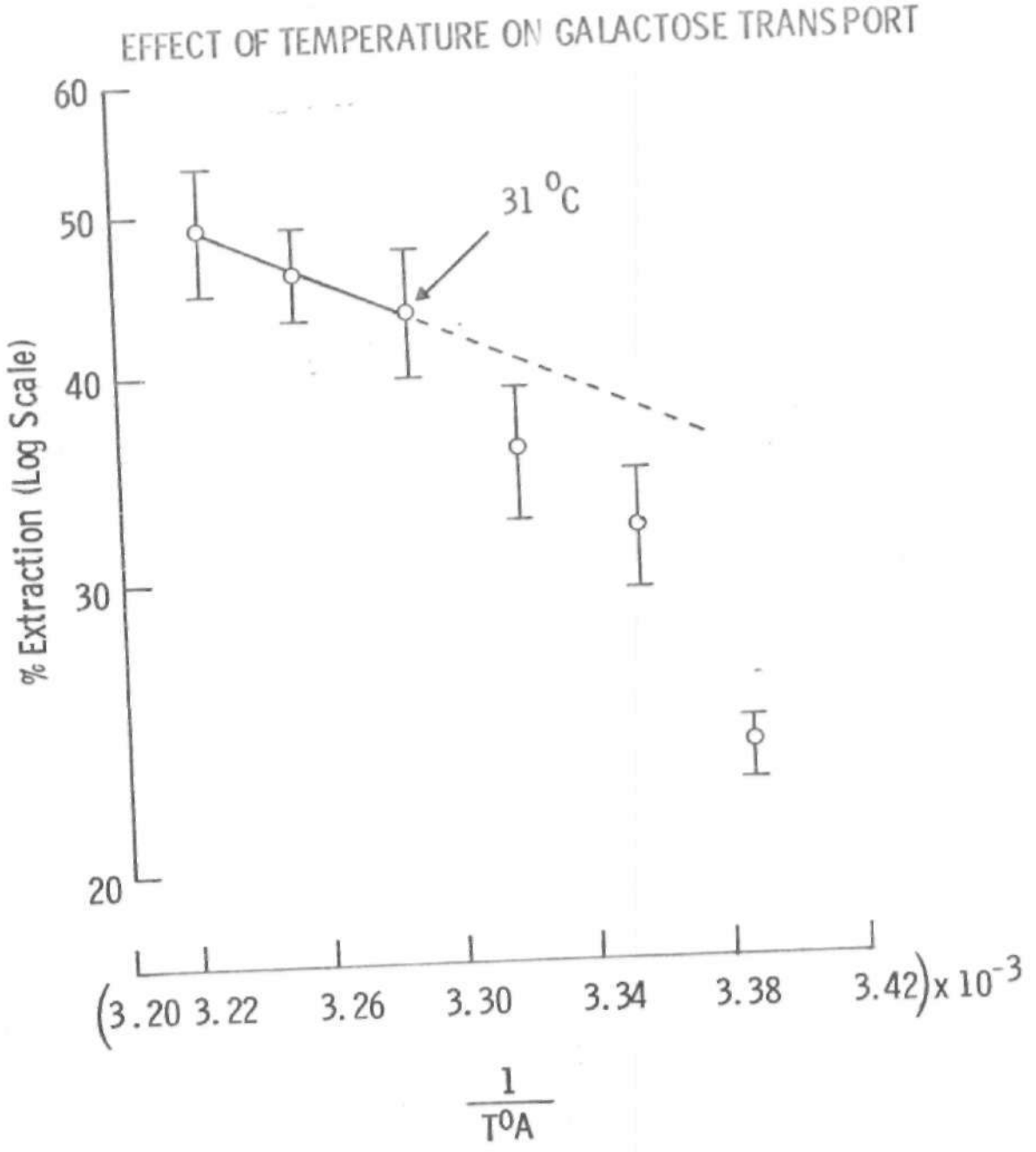
FIG. 8.6 D-GALACTOSE TRANSPORT IN THE PRESENCE OF 25 mM MALTOSE



LEGEND TO FIG. 8.7

FIG. 8.7. THE EFFECT OF TEMPERATURE CHANGES ON D-GALACTOSE TRANSPORT BY THE PERFUSED RAT LIVER. THE LOG OF EXTRACTION OF GALACTOSE IS PLOTTED AGAINST THE RECIPROCAL OF ABSOLUTE TEMPERATURE (ARRHENIUS PLOT). THERE IS A DISCONTINUITY OF TEMPERATURE DEPENDENCE OF GALACTOSE TRANSPORT AT ABOUT 31°C BELOW WHICH THERE WAS A MORE RAPID FALL IN EXTRACTION WITH DECREASE IN TEMPERATURE.

FIG. 8.7


EFFECT OF TEMPERATURE ON GALACTOSE TRANSPORT
% Extraction (Log Scale)
60
50
40
30
20
31 °C
3.20 3.22 3.26 3.30 3.34 3.38 3.42) x 10⁻³
1/T°A

This lactose effect is highly significant ($P < 0.01$). On the other hand, 25 mM Maltose (fig. 7.6) had no significant effect on galactose transport ($P > 0.05$) as shown on Table 8.1. This finding contrasts with that of glucose transport in which both maltose and lactose caused significant decrease in extraction. A further point to note here is that the addition of either galactose (fig. 8.1) or lactose (fig. 8.5) caused not only an initial decrease in extraction of galactose, but caused a reflux of labelled galactose into the hepatic venous effluent samples as depicted by the progressive fall in extraction level analogous to that reported for glucose transport in the presence of all potential competitors, and even with the control (Chapter 7). This reflux was not seen with galactose control or with any of the other competitors for galactose transport.

The effects of temperature changes on galactose transport are shown in fig. 8.7. The logarithm of galactose extraction (Y axis) was plotted against the reciprocal of the Absolute temperature (Arrhenius plot). This plot shows a biphasic characteristic of the temperature dependence of galactose transport in the rat liver with a transition (discontinuity) at about 31^{o}C. Above this temperature there was a more gradual decrease in galactose transport as the temperature was lowered. But below this transition temperature the decrease in extraction of galactose was more rapid for a corresponding lowering of the temperature, hence the discontinuity of the curve at this point (the transition temperature). On measuring the temperature quotient (Q_{10}) for galactose transport, it was found that the value for the Q_{10} was different at low temperature than high temperature range. The galactose extraction between 37^{o}C and 28^{o}C had a Q_{10} of 1.33, while that between 31^{o}C and 22^{o}C had a Q_{10} of 2.02.

For glucose transfer in the human erythrocyte, SEN and WIDDAS (1962) found the Q_{10} of 1.6 for a temperature range of 27°C - 37°C, and 1.8 for a temperature range of 7°C - 47°C. They found an overall Q_{10} 2.9 in their experiments, and this compared very well with an overall Q_{10} of 2.8 reported by FAUST and PARPART (1961). In the present study with galactose the overall Q_{10} for the temperature range of 22°C - 37°C in the rat perfused liver was 2.55. BAUR and HELDT (1977) found the Q_{10} of approximately three for glucose transport in isolated liver cells in the range of 5 - 18°C and Q_{10} of 1.5 in the range of 18-37°C. Although these latter workers found a transition in temperature dependence of glucose transport, they reported that there was no such transition with fructose transport, and Q_{10} for fructose transport was 2.0 over the whole range. No report seems to be available for galactose temperature dependence studies, but these glucose reports are similar to the galactose studies reported here. The findings of all workers are agreed on the fact that the Q_{10} is higher at lower temperatures than high ones. This is discussed further in Chapter 10.

8.4 Discussion

Hepatocellular uptake of D-galactose was studied using perfused rat livers. Certain characteristics of the galactose transport phenomenon have been identified. Galactose transport showed saturable characteristics by a significant reduction in its transport when its concentration was raised by 25 mM. It also showed competition between itself and D-glucose, D-fructose, or α-M-D-G when 25 mM of one of the competitors was added to the infusion mixture. Besides, chemical inhibition of galactose transport was manifest when 1mM of either phlorizin or phloretin was added to the perfusate. By these characteristics, galactose transport is believed to be by similar mechanism as that of glucose in these studies - that is by a carrier mediated facilitated diffusion mechanism as described in erythrocytes (WIDDAS 1954; WILBRANDT & ROSENBERG 1957; LEFEVRE 1961, 1972; STEIN 1967). The transport of D-galactose by the liver has been studied in anaesthetized dogs (GORESKY, BACH & NADEAU 1973), conscious sheep (HOOPER & SHORT 1975, 1977), and in rat isolated hepatocytes (BAUR & HELDT 1977). But nobody has shown, hitherto, that galactose carrier in the liver cell membrane is distinct from that of glucose. The present investigations have attempted to demonstrate this. It was first realized that a distinction could exist between glucose and galactose transports in these studies when the mutual competition between glucose, galactose and fructose was closely examined (IBU & SHORT 1979a). Glucose was a better competitor for galactose transport than fructose was, but fructose was by far a better competitor than galactose for glucose transport. So it was conjectured that glucose transport might be distinct from galactose transport. This conjecture received confirmation when it was found that lactose sig-

nificantly inhibited both glucose and galactose transport, but Maltose inhibited only glucose transport without effect on galactose (IBU & SHORT, 1979b). So it was concluded that the glucose carrier was distinct from galactose carrier in certain respects. The full arguments and explanation of this multiplicity of the hexose carrier mechanism is discussed in Chapter 10.

However, galactose uptake by intestinal cells is said to share the transport system with glucose, merely having a different affinity for the carrier (CRANE 1960). This does not appear to be the case in the liver, as the findings in these experiments have demonstrated. The major distinction between the intestinal and hepatic transport mechanisms seems to be that the hepatic monosaccharide transfer system is energy-independent (PARK, CROFFORD & KONO, 1968), whereas that on the brush border of the intestinal cells is by active mechanism. The findings in the present study also support this idea since it was shown that phloretin inhibition of galactose transport was highly significantly greater than that by phlorizin - a situation contrary to that existing in the intestine and the kidneys, where active transport of the monosaccharides is known to exist (SCHULTZ & CURRAN 1970).

Galactose transport was shown to be temperature dependent (fig. 8.7), a finding similar to that reported for glucose entry into erythrocytes (SEN & WIDDAS 1962), and glucose uptake by isolated hepatocytes (BAUR & HELDT 1977). There was a definite transition temperature (about 31^{o}C) at which some changes on the transport functions of the liver cell membrane appeared to occur. This is interpreted as being due to phase changes of liquid-crystalline to crystalline (gel) in the cell membrane. This interpretation is discussed in Chapter 10. The Q_{10} for galactose transport differed from those of glucose trans-

port reported by SEN & WIDDAS (1962) and BAUR and HELDT (1977) for both low and high temperature ranges. (See comparative values in section 8.3.) This difference is attributable to lower affinity of galactose for the carrier than glucose. This is consistent with the finding that the fructose overall Q_{10} is found to be the lowest reported for the three hexoses (BAUR & HELDT 1977), fructose also being the least well transported.

CHAPTER NINE

9 FRUCTOSE TRANSPORT BY THE PERFUSED RAT LIVER

9.1 Introduction

Hepatocellular uptake of fructose has been studied in the conscious sheep (HOOPER & SHORT 1977) and in the disaggregated heaptocytes of rat (BAUR & HELDT 1977). In both studies, fructose was found to be very much less well transported into the liver than either glucose or galactose. In fact fructose entry into erythrocytes had been found to be very much lower than that of glucose or galactose (BOWYER, 1957; STEIN 1967). Interest in hepatic handling of fructose has recently increased due to the finding that fructose raised alcohol removal in slices of human and rat livers (THIEDEN, GRUMMET, DAMGAARD & SESTOFT, 1972) and also in perfused pig livers (DAGMAARD, LUNDQUIST, TONNESEN, HANSEN & SESTOFT, 1973). This is of both clinical and nutritional importance in humans who ingest both fructose and alcohol in their diets. It is a vogue nowadays to have both of these materials in high society dinner parties. Moreover, fruity tasting drinks and sweets contain fructose. This broadens the dimensions of interest for studying the fructose handling by the liver. It has been suggested that there is significant hapatic retention of fructose on its first passage through the liver following infusion into the portal vein when compared to a systemic infusion in anaesthetised dogs (McINTYRE, TURNER & HOLDSWORTH 1970). Fructose has a high rate of metabolism, and it has been suggested that this rate is limited by the rate of fructose transport into the liver (BAUR & HELDT 1977). Its rate of metabolism is very much greater than that of glucose or galactose whose transport far exceeds the metabolic requirements and the low rate of glucose conversion is due to the limiting activities of

glucokinase (LUNDQUIST, 1969; NEWSHOLME & START 1973; SESTOFT & FLERON 1974). Using rat liver slices, PEREIRA and JANGAARD (1971) found that fructose conversion to various intermediates of carbohydrate and lipid metabolism was far greater than that of glucose. They found the ratio of the rate of fructose metabolism to the rate of glucose metabolism (F/G) ranged from a low of three for lactic acid, pyruric acid, carbon dioxide and fatty acids, to a high of 19 for glyceride-glycerol. These results suggest that fructose-induced hyperlipidaemia may result, at least in part, from the relatively efficient conversion of fructose to glycerophosphate in the liver. It has been reported that feeding of fructose or sucrose produced hyperlipidaemia in man (MacDONALD & BRAITHWAITE 1964; RIFKIND, LAWSON & GALE, 1966; AKINYANJU, QURESHI, SALTER & YUDKIN 1969). The feeding of these sugars also caused hyperlipaemia in animals (NIKKILA & OJALA, 1965; BAILEY, TAYLOR & BARTLEY, 1968; BAR-ON & STEIN, 1968; COHEN & TEITELBAUM 1968). This carbohydrate-induced hyperlipidaemia has been implicated in the aetiology of occlusive atherosclerotic disease (YUDKIN 1964; YUDKIN & MORLAND 1967; YUDKIN & RODDY 1964). The fructose portion of sucrose has been postulated as being responsible for the hyperlipidaemic effect by many groups of workers (NIKKILA & OJALA 1965; BAILEY, TAYLOR & BARTLEY, 1968; BAR-ON & STEIN 1968; COHEN & TEITELBAUM, 1968). These workers have given various reasons for the greater fructose metabolism, one of their reasons being a more rapid rate of uptake from the gut or the bloodstream. But this certainly does not include hepatocellular uptake from the bloodstream. In fact it has been found (HOOPER & SHORT 1977; BAUR & HELDT 1977) that fructose is very much less well transported into the liver than either glucose or galactose. SESTOFT et al (1972, 1974) have found that fructose

elimination rate increased with increasing extracellular fructose concentration. But they contended that the cell membrane tries to shut out entry of fructose to prevent severe depletion of adenine nucleotides - which would have been the case if metabolic sequestration by phosphorylation were allowed to go on unimpaired. How the liver achieves this has not been elucidated. But it was thought that identification of membrane transport mechanism by the liver would solve this problem. It was therefore necessary to study this mechanism as it applies to fructose, and the findings in this chapter are the results of such a study.

Besides, the study of fructose transport by the liver is of paramount importance because D-fructose has been used clinically as a source of energy during intravenous nutrition (ALLEN & LEE 1969) in the post operative state, as an antiketogenic agent in uncontrolled diabetes mellitus (ROSECAN & DAUGHADARY, 1954), as a sweetening agent in the diet of patients with diabetes mellitus (MARKS 1971), and in the treatment of acute alcoholic intoxication (BROWN, FORREST & ROSCOE 1972). But fructose infusions have no striking effects on the blood glucose level in normal men. FELBER, RENOLD and ZAHND (1959) reported a transient and moderate hypoglycaemia after a short-term (10 minutes) infusion in normal men. But prolonged infusion of fructose (30-60 minutes) increased glucose level initially and minimally, followed by a moderate decrease after the cessation of the infusion (WEICHSELBAUM, MARGRAF & ELMAN 1953; PAPPER, SAXON, PROUT & ALPERT, 1956; CRAIG, MILLER, MACKENZIE & WOODWARD 1958). It is surprising that fructose infusion did not cause a definite hyperglycaemia contrary to expectation since fructose is known to be partly converted into glucose by the human liver (MENDELOFF & WEICHSELBAUM, 1953) and

up to 70% of fructose load is converted to glucose and the remaining 30% converted to lactate and pyruvae in isolated perfused rat liver (WOODS, EGGLESTON & KREBS 1970). This notwithstanding fructose has been given to patients in preference to glucose because the former is metabolised much faster than the latter (PEREIRA & JANGAARD 1971). However, the high rate of metabolism is a primary cause of harmful side-effects of fructose administration (WOODS & ALBERFT, 1972). During fructose infusion the rate of lactate formation exceeds the rate of lactate disposal, resulting in lactate accumulation in blood. Hence one may now recommend that intravenous infusion of fructose should be absolutely contraindicated in clinical situations where there may be impairment of normal lactate metabolism such as shock, liver disease and uncontrolled diabetes mellitus.

With the foregoing reported findings, it was thought that a study of the hepatocellular handling of fructose should be undertaken, hence this study.

9.2 Method

Essentially, the method used in the study of fructose transport by these rat livers was the same as that described for glucose transport in section 7.2, except a few modifications which are described here. For this study, the test substance was ^{3}H-D-fructose and the reference material was ^{14}C-Sucrose. For each experimental run, 2µCi in 100µl of the test substance (concentration, fructose = 3.2 x 10^{-7}M) and 1µCi in 100µl of the reference substance (concentration Sucrose = 3.8 x 10^{-8}M) were drawn into a 10 ml syringe containing 250 mM of the potential competitor (unlabelled sugars) in 1 ml. This syringe was connected to the perfusate circuit and perfusate withdrawn rapidly to the 10 ml mark thereby bringing the final concentration of the potential competitor to 25 mM immediately before infusion.

To find out the effect of either phlorizin or phloretin on fructose transport, the experiments were done as described in section 7.2 for glucose, except that fructose transport was measured instead of glucose.

9.3 Results

For control D-fructose extraction (when potential competitor was not added) is shown in fig. 9.1. In this figure and subsequent ones, the ordinate (Y axis) represents percentage extraction of fructose by the liver, and the abscissa (X axis) represents the time scale in seconds. The Y axis is a broken axis, hence the fructose extraction which is 27.25 ± 0.91% (means ± S.E.M., N = 4) would appear to be near zero extraction, which is not the case. This scale is maintained in conformity with the scale used for the glucose and galactose extraction studies in Chapters 7 and 8 respectively for easy comparison. So it is easy to see at a glance that fructose extraction by the liver is several orders less than that of glucose or galactose. The effects of addition of 25 mM sucrose near the start (Sucrose 1) and at the end of all experimental runs (sucrose 2) are compared with the control extraction (fig. 9.1). There was no significant difference between Sucrose 1 extraction (26.66 ± 0.15%) and control (27.25 ± 0.91%), $P > 0.05$ (N = 4, paired t-test in this and all other subsequent P values stated). This Sucrose 1 effect represented an inhibition of 2.1 ± 0.45% from the control value. This effect of Sucrose 1 means that Sucrose has no significant effect on D-fructose transport. It also means that the presence of 25 mM solute per se does not have any significant effect on fructose extraction by the liver. Also, there was no significant difference between the Sucrose 1 and Sucrose 2 runs with mean extractions of 26.66 ± 0.15% and 26.16 ± 0.72% respectively ($P > 0.05$). This means that there was no significant depression in liver performance between the second run (Sucrose 1) and last run (Sucrose 2). This is an important point since all other potential competitors were added in runs between Sucrose 1 and Sucrose

LEGEND TO FIG. 9.1

FIG. 9.1. D-FRUCTOSE TRANSPORT IN THE PRESENCE OF 25mM D-FRUCTOSE. THE FRUCTOSE % EXTRACTION WAS PLOTTED AS THE ORDINATE (Y AXIS) AGAINST THE TIME SCALE IN SECONDS AS THE ABSCISSA (X AXIS), IN THIS AND THE OTHER FOLLOWING FIGURES IN THIS CHAPTER. NOTE THAT Y AXIS IS A BROKEN AXIS AND THE FRUCTOSE EXTRACTION IS NOT AS CLOSE TO ZERO AS IT APPEARS IN THE FIGURE, BUT THE SCALES WERE KEPT THIS WAY TO FACILITATE COMPARISON WITH THE RESULTS OF GLUCOSE AND GALACTOSE EXTRACTIONS IN CHAPTERS 7 AND 8 RESPECTIVELY. THE ADDITION OF 25mM FRUCTOSE CAUSED A SMALL BUT SIGNIFICANT DECREASE IN FRUCTOSE EXTRACTION. THIS IS EVIDENCE OF SATURABLE CHARACTERISTIC OF FRUCTOSE TRANSPORT BY THE LIVER.

FIG. 9.1 D-FRUCTOSE TRANSPORT IN THE PRESENCE OF 25mM D-FRUCTOSE

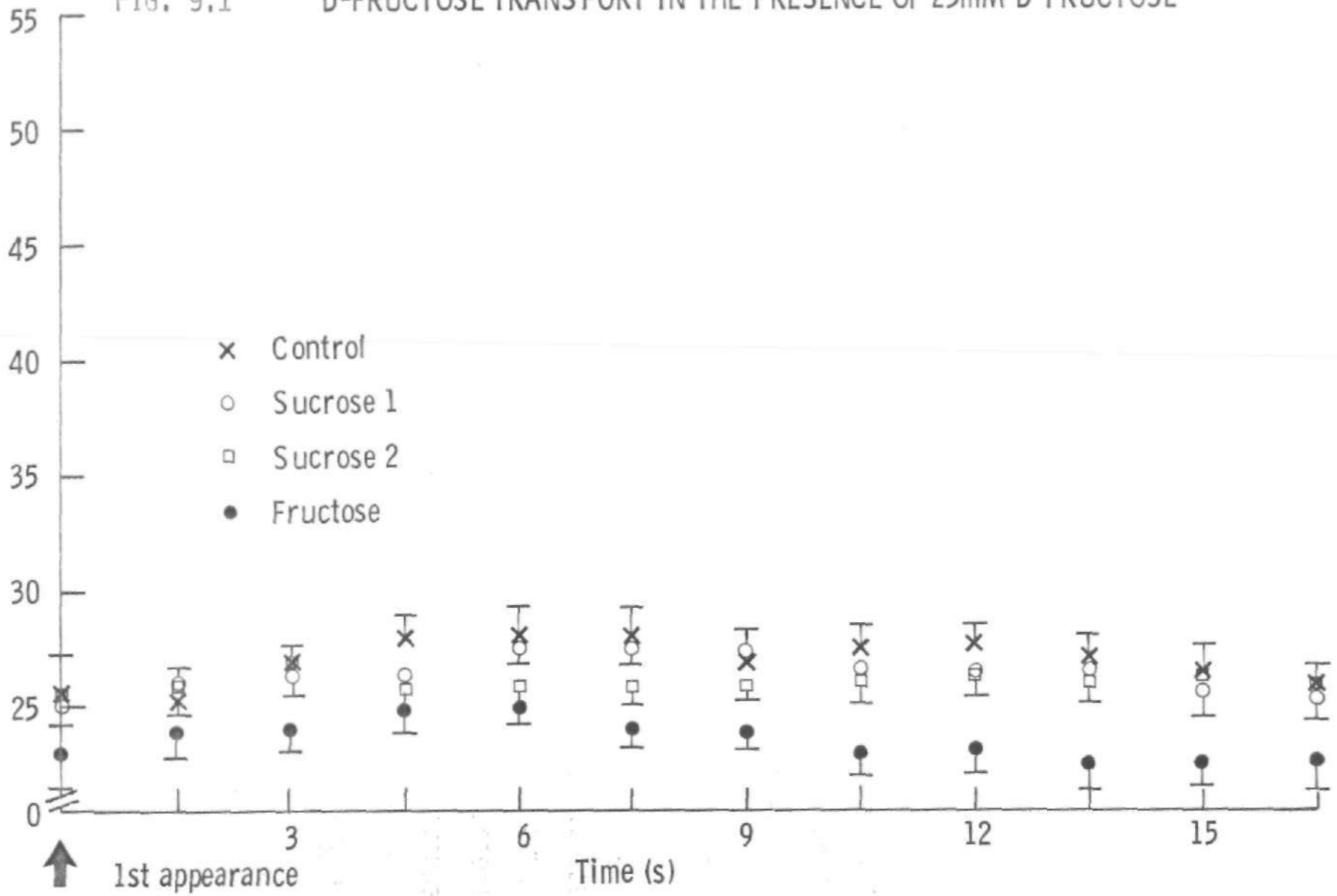


TABLE 9.1 INHIBITION OF FRUCTOSE TRANSPORT BY DIFFERENT SUGARS

Competing Sugar	D-Fructose Extraction		Significance
25 mM	%E ± S.E.M.	ΔE% ± S.E.M.	P
Control	27.25 ± 0.91	-	-
Sucrose 1	26.66 ± 0.15	-2.1 ± 0.45	*P > 0.05
D-Glucose	25.37 ± 0.89	-6.8 ± 0.39	**P < 0.02
D-Galactose	25.40 ± 0.22	-6.7 ± 0.54	**P < 0.05
D-Fructose	24.86 ± 0.42	-8.7 ± 0.53	P < 0.05
α-M-D-G	25.85 ± 0.35	-5.0 ± 0.30	P < 0.01
Lactose	25.66 ± 0.47	-5.8 ± 0.26	P < 0.02
Maltose	24.25 ± 0.44	-10.94 ± 0.27	P < 0.01
Sucrose 2	26.16 ± 0.72	-4.1 ± 0.55	*P > 0.05

N = 4 livers

α-M-D-G = α-Methyl-D-glucopyranoside

* = Significance of difference between Sucrose 1 and Sucrose 2. Other P values were compared with control only. All probability values were obtained using paired t-test.

ΔE is change in extraction from control value expressed as percentage of the control.

** = There was no significant difference between the effects of glucose and galactose on fructose transport (P > 0.05).

2 runs, so any significant change when any of these potential competitors was added could justifiably be attributed to the addition of such a competitor. The presence of fructose itself at 25 mM concentration decreased fructose extraction (fig. 9.1) from control value to 24.86 ± 0.42% representing an inhibition of 8.7 ± 0.53%, Table 9.1. This effect was significant ($P < 0.05$). This means that fructose transport in these livers was saturable.

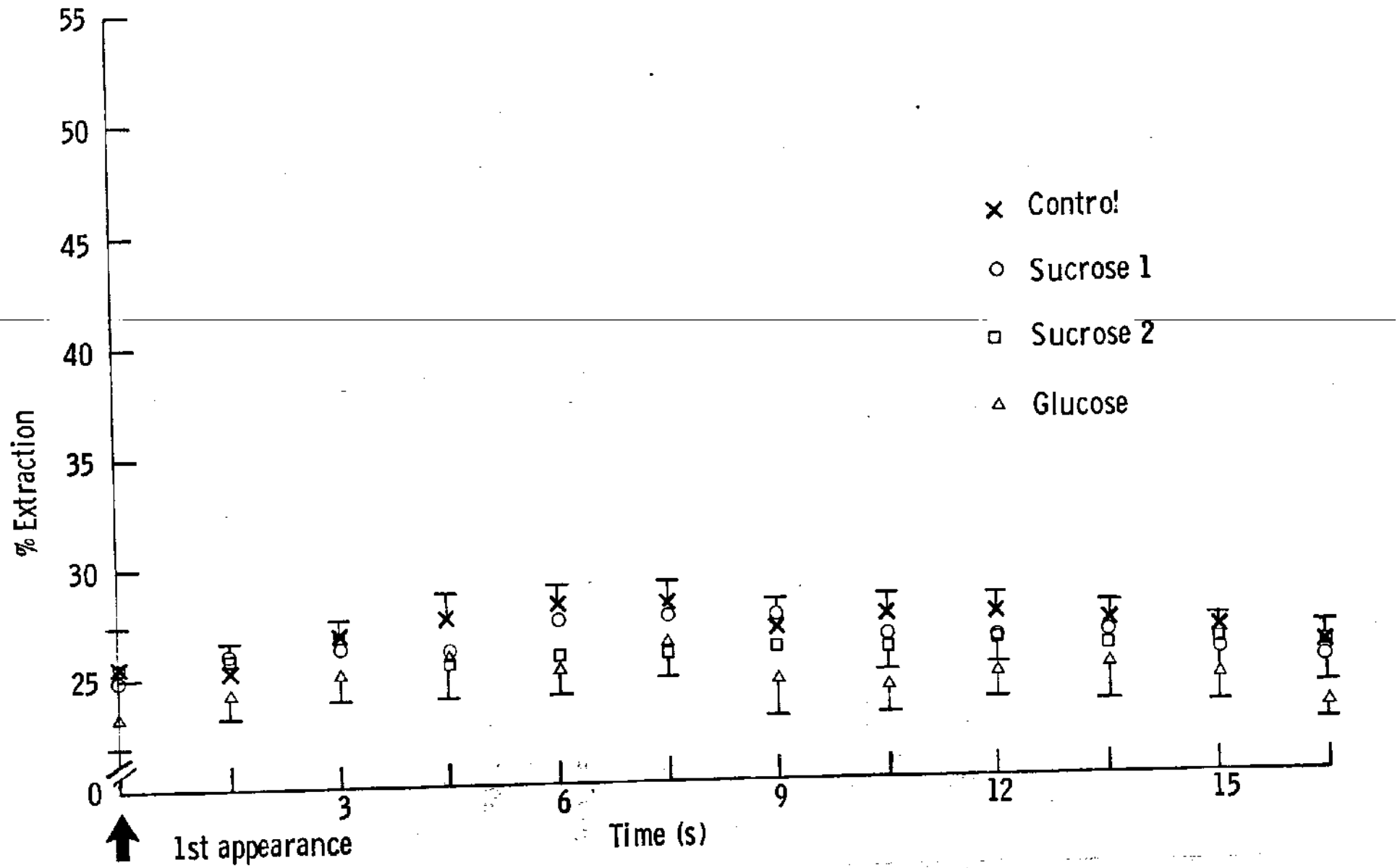
The addition of 25 mM unlabelled D-glucose caused a small but significant decrease in fructose extraction (fig. 9.2); the extraction in the presence of glucose was reduced from the control value to 25.37 ± 0.89% representing a significant inhibition of 6.8 ± 0.39% ($P < 0.02$), (Table 9.1). And the addition of 25mM D-galactose caused a similar decrease in fructose extraction to 25.40 ± 0.22% (fig. 9.3) which gave a significant inhibition of 6.7 ± 0.54% ($P < 0.05$). Also 25 mM α-methyl-D-glucopyranoside (α-M-D-G), a non-metabolizable methyl-substituted glucose analogue, significantly decreased fructose extraction (fig. 9.4) to 28.85 ± 0.35% giving an inhibition of 5.0 ± 0.30% ($P < 0.01$). These results indicate competition between fructose and these sugars.

When 1mM phlorizin was added to the perfusate, fructose extraction was highly significantly decreased from 28.37 ± 0.30% to 16.21 ± 0.34% which is an inhibition of 30.61 ± 2.54% ($P < 0.001$). Also when 1mM phloretin was added to the perfusate, fructose extraction was decreased from 24.95 ± 0.28% to 9.00 ± 0.29% which is an inhibition of 63.91 ± 2.14% (Table 9.2). This difference was very highly significant ($P < 0.001$). But the inhibition by phloretin was significantly greater than that by phlorizin ($P < 0.01$). So fructose extraction exhibits chemical inhibition like those of glucose (Chapter 7) and

LEGEND TO FIG. 9.2

FIG. 9.2. D-FRUCTOSE TRANSPORT IN THE PRESENCE OF 25mM D-GLUCOSE. THE AXES AND SCALES ARE THE SAME AS IN FIG. 9.1. GLUCOSE CAUSED A SMALL BUT SIGNIFICANT DECREASE IN FRUCTOSE EXTRACTION. THIS IS EVIDENCE OF COMPETITION.

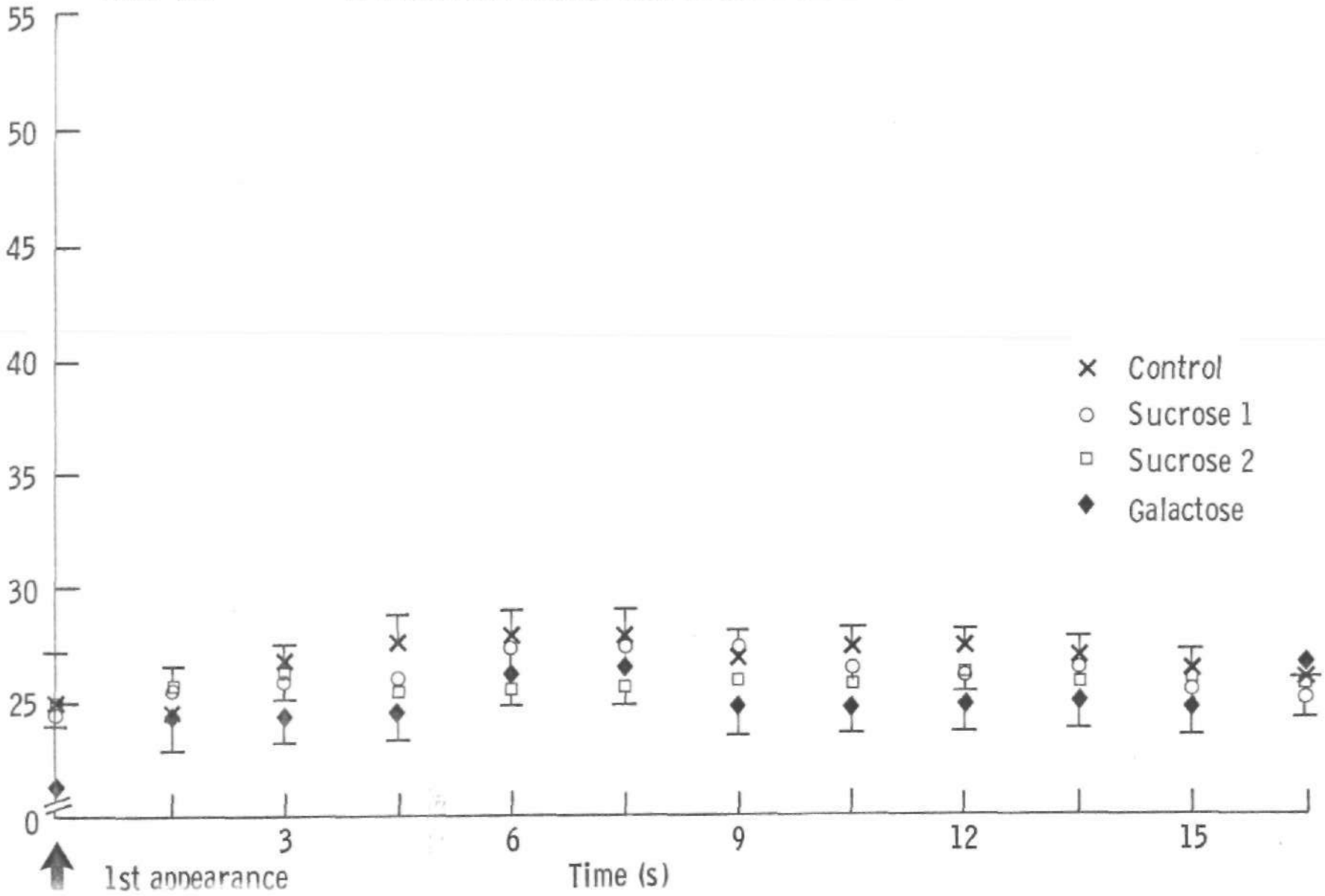
FIG. 9.2 D-FRUCTOSE TRANSPORT IN THE PRESENCE OF 25mM D-GLUCOSE



LEGEND TO FIG. 9.3

FIG. 9.3. D-FRUCTOSE TRANSPORT IN THE PRESENCE OF 25MM D-GALACTOSE. GALACTOSE CAUSED A SIGNIFICANT DECREASE IN FRUCTOSE TRANSPORT. THIS IS ALSO EVIDENCE OF COMPETITION.

FIG. 9.3 D-FRUCTOSE TRANSPORT IN THE PRESENCE OF 25mM D-GALACTOSE



LEGEND TO FIG. 9.4

FIG. 9.4. D-FRUCTOSE TRANSPORT IN THE PRESENCE OF 25MM α-METHYL-D-GLUCOPYRANOSIDE (α-M-D-G). α-M-D-G CAUSED SIGNIFICANT DECREASE IN FRUCTOSE TRANSPORT. THIS IS ANOTHER EVIDENCE OF COMPETITION.

FIG. 9.4 D-FRUCTOSE TRANSPORT IN THE PRESENCE OF 25mM α-M-D-G

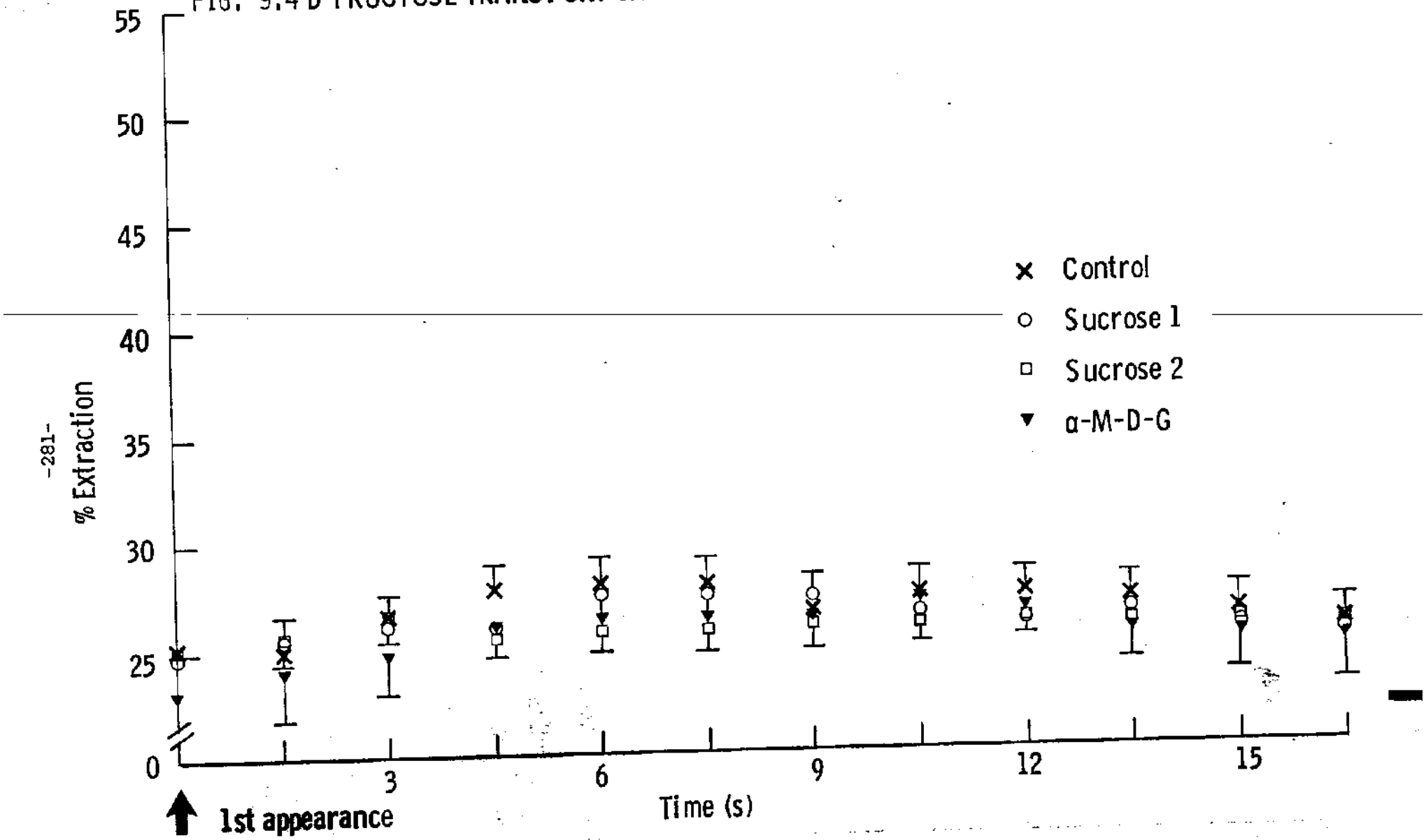


TABLE 9.2

EFFECTS OF PHLORIZIN OR PHLORETIN ON FRUCTOSE TRANSPORT

Type of Experiment	Extraction		Significance
	%E ± S.E.M.	ΔE% ± S.E.M.	P
1. Control	23.37 ± 0.30	-	-
2. Added Phlorizin	16.21 ± 0.34	-30.61 ± 2.54	*P < 0.001 **P < 0.01
3. Control	24.95 ± 0.28	-	-
4. Added Phloretin	9.00 ± 0.29	-63.91 ± 2.15	*P < 0.001 **P < 0.01

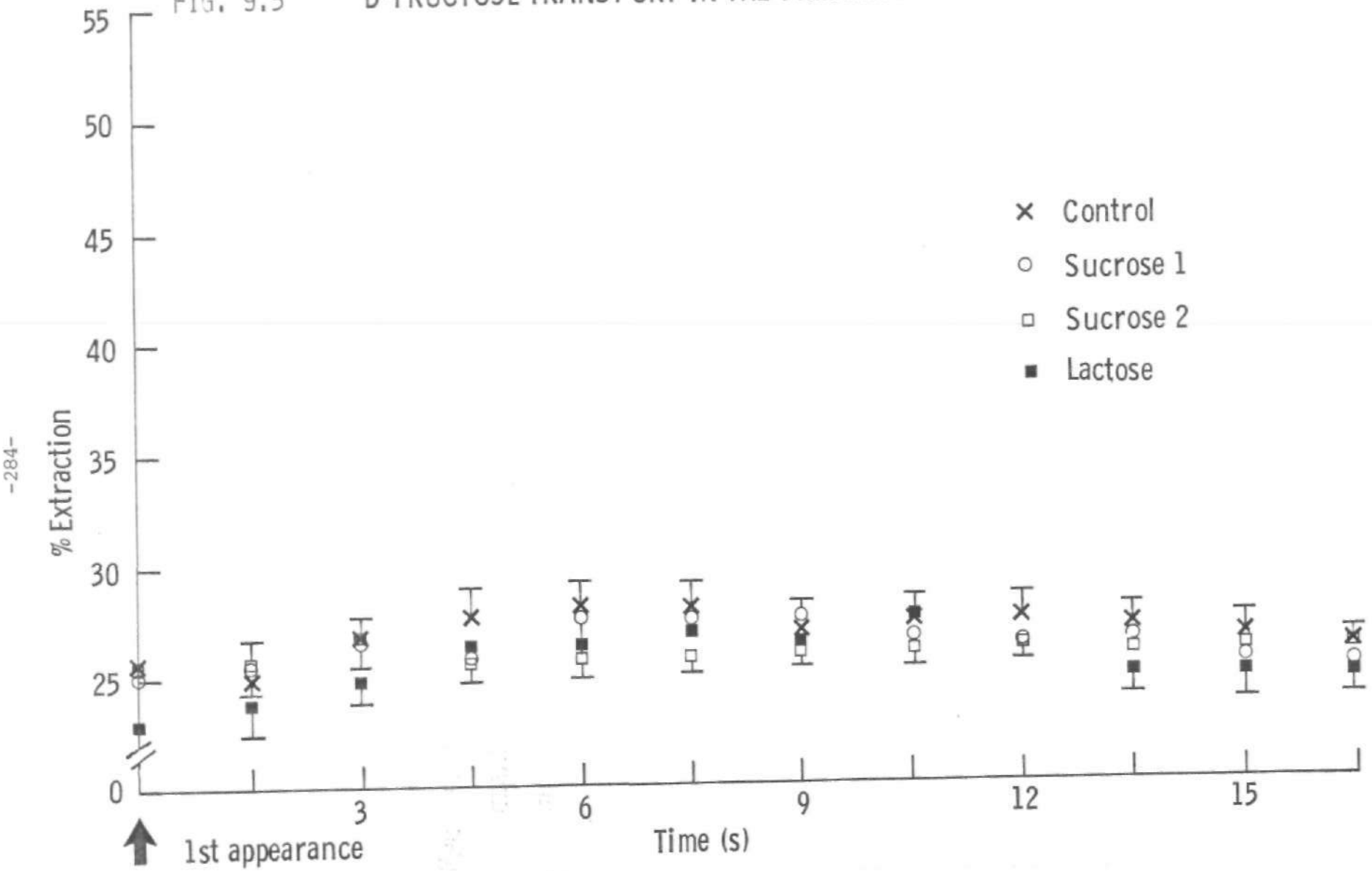
KEY: * Significance of the difference between this and the control.

** Significance of the difference between 2 and 4.

LEGEND TO FIG. 9.5

FIG. 9.5. D-FRUCTOSE TRANSPORT IN THE PRESENCE OF 25MM LACTOSE. THIS DISACCHARIDE SIGNIFICANTLY DECREASED FRUCTOSE TRANSPORT, LIKE IT DID TO BOTH GLUCOSE AND GALACTOSE.

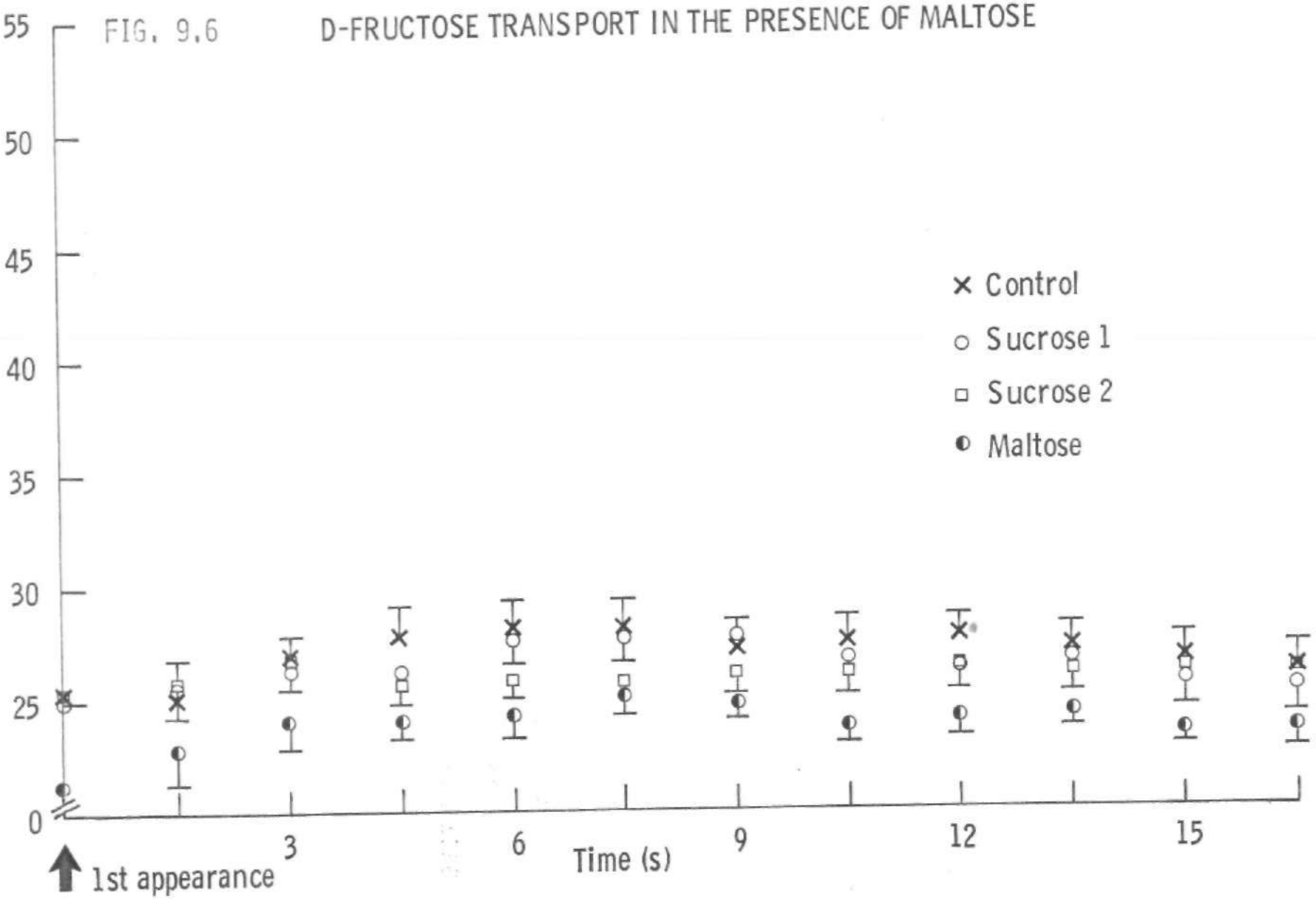
FIG. 9.5 D-FRUCTOSE TRANSPORT IN THE PRESENCE OF 25mM LACTOSE



LEGEND TO FIG. 9.6

FIG. 9.6. D-FRUCTOSE TRANSPORT IN THE PRESENCE OF MALTOSE. MALTOSE CAUSED SIGNIFICANT DECREASE IN FRUCTOSE EXTRACTION. THIS FINDING IS IN CONFORMITY WITH ITS EFFECT ON GLUCOSE TRANSPORT BUT AT VARIANCE WITH ITS EFFECT ON GALACTOSE TRANSPORT.

FIG. 9.6 D-FRUCTOSE TRANSPORT IN THE PRESENCE OF MALTOSE

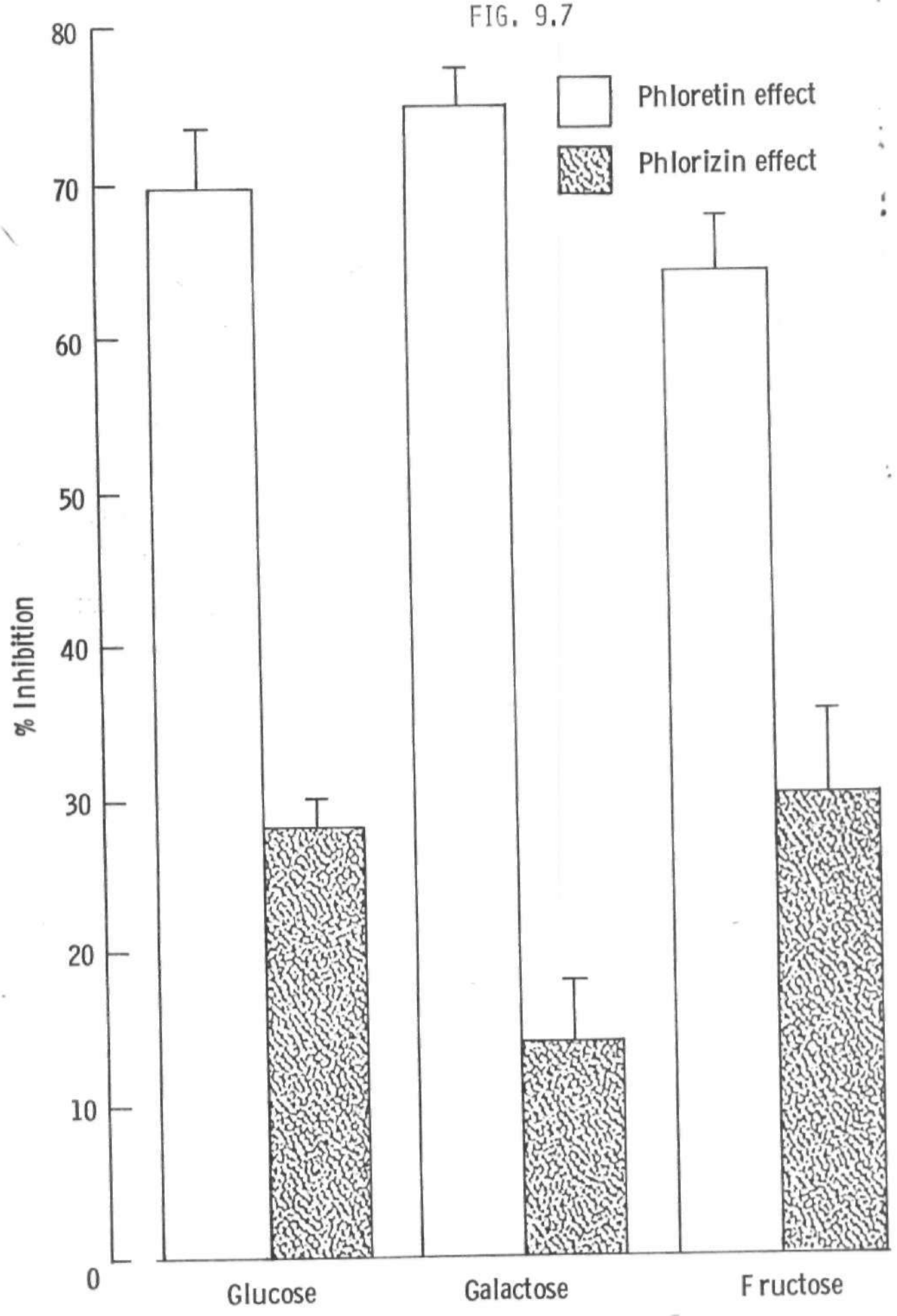


LEGEND TO FIG. 9.7

FIG. 9.7. COMPARATIVE INHIBITION BY PHLORETIN AND PHLORIZIN OF HEXOSE TRANSPORT. NOTE THAT PHLORETIN SIGNIFICANTLY INHIBITED TRANSPORT OF ALL THREE HEXOSES MORE THAN PHLORIZIN DID. ALSO NOTE THAT PHLORIZIN EFFECT ON GLUCOSE AND FRUCTOSE ARE OF SIMILAR MAGNITUDE BUT ITS EFFECT ON GALACTOSE TRANSPORT IS LESS THAN IN THE CASE OF EITHER GLUCOSE OR FRUCTOSE. THE EFFECTS OF PHLORETIN ARE IDENTICAL IN ALL THREE HEXOSES.

COMPARATIVE INHIBITION BY PHLORETIN AND PHLORIZIN

FIG. 9.7



galactose (Chapter 8). The pattern of inhibition by phloretin and phlorizin is similar in all three hexoses (fig.9.7). However, the mutual competition among the hexoses seem to differ slightly. While there is mutual competition among all the three hexoses, the competitive effect of glucose and galactose on fructose transport are not significantly different from each other (Table 9.1). This was not the case with either glucose or galactose transport reported in sections 7.3 and 8.3 respectively.

Furthermore, addition of 25 mM Lactose or Maltose significantly decreased fructose extraction, although Sucrose had no significant effect. Lactose significantly decreased the fructose extraction (fig. 9.5) from the control value of 27.25 ± 0.91% to 25.66 ± 0.44 which is an inhibition of 5.8 ± 0.26% ($P < 0.02$) - Table 9.1. SESTOFT has argued the saturability of fructose uptake from his computer evaluation of infusion data. Maltose significantly decreased the extraction (fig. 9.6) from the same control value to 24.25 ± 0.44% representing an inhibition of 10.94 ± 0.27% ($P < 0.01$). The last result is in contradiction to that found with galactose transport but in agreement with that of glucose.

9.4 Discussion

Fructose handling by the liver has been most difficult to study. Not many reports seem to have been given on the saturability of fructose transport into the liver. But SESTOFT (1974) using computer simulation of infusion data has put forward an argument of saturability of fructose uptake. It was possible to demonstrate in this study that fructose transport was saturable when 25 mM fructose was added to the infusate. However, BAUR and HELDT (1977) using isolated rat hepatocytes found that fructose transport was not saturable even at over 100 mM concentration. The main point of digression between their findings and the one reported here would appear to centre on the type of preparation which they used. The procedure used for the isolation of the hepatocytes itself could hamper a great deal of physiological phenomena. Although their procedure was better than the previously known liver slice preparations, it can hardly be expected to give results as good as the perfused liver preparation used in this study. In fact the authors themselves marshalled strong arguments against this isolated hepatocyte preparation, namely, that by isolating these cells, large areas of the cell membrane which normally are not in contact with the extra-cellular fluid are exposed to the medium. Furthermore, they contended, that "there is a possibility that transport properties may be altered by the isolation procedure, e.g. by treatment with collagenase". So it is not surprising that they were unable to find saturable characteristics of fructose transport. It would be a biological disaster if fructose were to enter liver cells entirely by free diffusion, because it is known that fructose metabolism in the liver far exceeds that of glucose (PEREIRA & JANGAARD, 1971). And SESTOFT & FLERON (1974) found

that fructose was rapidly taken up by the liver, and its elimination rate increased with increasing extracellular concentration of fructose. If this were allowed to continue unimpeded, calamity would ensue as the rapid fructose uptake would be met with rapid metabolism which could easily deplete the adenine nucleotides because of fructose phosphorylation. But this is not the case as the cell membrane tries to prevent free diffusion of the fructose - thanks to the facilitated diffusion transport mechanism.

Besides the saturability, fructose transport exhibited competition with glucose, galactose and α-M-D-G. This finding further supports the proposition of facilitated diffusion rather than free diffusion. The competition of glucose and galactose with fructose transport were of the same magnitude, whereas fructose effect was significantly greater than that of galactose on glucose transport, and glucose effect was significantly greater than that of fructose on galactose transport. So all the three hexoses would appear to share a common carrier (all being mutually competitive on one another's transport), with slight individuality between them since the pattern of this mutual competition differs slightly in all three of them.

But this was not the case with the chemical inhibition found with phlorizin or phloretin. Either of the chemicals inhibited transport of any of the three hexoses significantly. Of the two, phloretin was always a better inhibitor - and this pattern was common to all the three hexoses. It would therefore tend not to separate the hexoses in any way. But this finding has distinguished the type of transport that exists in fructose (as well as glucose and galactose) as being not the active transport mechanism that is known to exist in the kidney and the gut as phlorizin would have been a better inhibitor than

phloretin if this were the case.

However, the dissaccharide effects on fructose transport are qualitatively similar to their effects on glucose transport though smaller in magnitude, which is differenct from their effects on galactose. Both Lactose and Maltose significantly inhibited fructose transport (like glucose transport) so the fructose carrier may be the same as the glucose carrier, and this may be distinct from galactose carrier. This proposition and the arguments and deductions leading to this conclusion are discussed fully in Chapter 10.

PART E

GENERAL DISCUSSION

CHAPTER TEN

10. GENERAL DISCUSSION

10.1 Introduction

"I believe that the most useful path for physiology and medicine to follow now is to seek to discover new facts instead of trying to reduce to equations the facts which science already possesses. This does not mean that I condemn the application of mathematics to biological phenomena, because the science will later be established by this alone . . . ."

Claude Bernard 1865.

Certainly this was the situation over one hundred years ago, and Bernard was right at the time. In those early days of physiology, the speciality had a great deal of rudimentary factual and qualitative knowledge to be discovered. It was therefore not surprising that Bernard saw a greater need for discoveries at that stage of qualitative information rather than mathematical analysis of the already established facts. But mathematical analysis cannot be regarded as a subsidiary in a scientific investigation nowadays. Indeed in the field of biological transport, the modern emphasis is very much upon attempting to measure transport maximum (T_{max}) and apparent Michaelis' constant (K_m) to estimate the rate and affinity of transport process respectively. To this end a great deal of mathematical formulation and kinetic analysis similar to those of enzyme kinetics (DIXON & WEBB, 1964) have been adapted for use in membrane transport phenomena (WIDDAS 1952; STEIN 1967; GORESKY & BACH 1970). WIDDAS (1952) moved from hypothesis to mathematical analysis to account for the qualitative and quantitative findings of competitive inhibition of membrane transport phenomena.

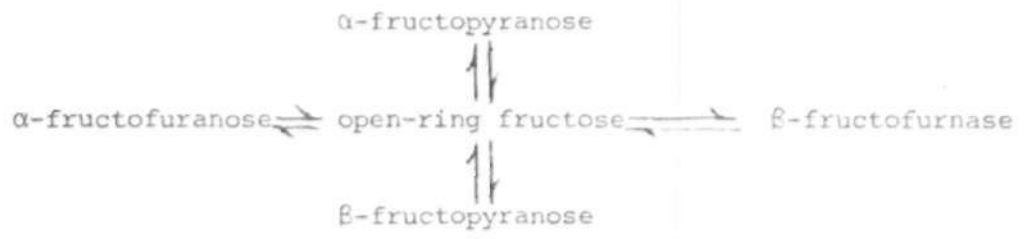
But one is confronted with serious difficulties when applying enzyme concepts to transport. Transport is inseparable from large molecules associated with unstirred layers where large concentration gradients exist. Furthermore, one cannot pretend that one is achieving the desired degree of transport quantitatively because the different experimental methods used attain varying degrees of steady state. For example the assessment of the percentage extraction by taking the mean of the first four samples, after the first appearance of the isotopically labelled extracellular reference, and before any significant efflux of the test substance into the medium occurred, as used in this study (Chapters 7, 8 and 9) would more closely resemble the true inward transport into the liver than the more prolonged time interval allowed for attainment of steady state, as in the studies of WILLIAMS et al (1968) and BAUR and HELDT (1977). In fact the aim of the study reported here is a quantitative analysis of transport of three hexoses with a view to achieving a qualitative statement. There has been no attempt to assess T_{max} or K_m in this study. It would have been possible to do so, as did GORESKY, BACH and NADEAU (1973), at the cost of being able to use the wider variety of potential competitors by which it was hoped to cast light on the nature of the transport process. In this study, therefore, attempts were made to elucidate whether the presence of one sugar influenced the transport of the other or not. There is enough reason to doubt the validity of such figures even when conducted with the best methods available. Assigning values to these kinetic parameters (T_{max} and K_m) could only be approximate because of the problems of extrapolating the Lineweaver-Burk plot (a double reciprocal plot), the effects of unstirred layers and large divergencies in concentration gradients that accompany them.

This is exemplified by the large variations in the values of apparent K_m for D-fructose transport; found to be 67mM in rat perfused liver cells (SESTOFT and FLERON, 1974), 14mM in rat isolated liver cells SESTOFT & FLERON 1974), and over 100mM in rat isolated liver cells (BAUR & HELDT 1977). The finding by SESTOFT and FLERON (1974) of such a difference between the perfused liver and isolated liver cells could be due to the effects of unstirred layer or the effects of enzymatic isolation of the hepatocytes on membrane transport phenomena. But the great difference between the apparent K_m of D-fructose transport in the isolated hepatocytes used by SESTOFT and FLERON (1974) and those used by BAUR and HELDT (1977) could be due to the uncertainty of the results of extrapolation from a Lineweaver-Burk plot. So parameters such as apparent K_m obtained from such a plot should be regarded as rough estimates only.

Although it has been argued that some complex mathematical treatments may be inappropriate to this type of transport study, this is not to say that important inferences cannot be made by careful analysis. For example, the analysis of the validation experiments in Chapter 6 (section 6.2.4) has yielded valuable information as to the behaviour of the extracellular reference material used and the magnitude of its influence on the transport parameters measured. Since the conclusion of the comparative study of the transport characteristics of these hexoses and the effects of the various sugars on them necessarily relate to the structural or molecular forms of these carbohydrates it is important to study the molecular structures of these carbohydrates with a view to finding out if there exists a structure-activity relationship among them as had been found for sugar entry into the erythrocytes (LEFEVRE, 1961).

10.2 Molecular Structures of the Sugars

The molecular structures of the sugars used in this study are described in this section. The existence of these sugars in ring (cyclic) forms rather than the open-chain (linear) structures was proposed in 1927 by HAWORTH. The evidence has been reviewed by GERIG (1974). Most Monosaccharides exist as either a five-membered or six-membered hemiacetal ring. D-glucose, for example, is found in cyclohexane-like ring form (fig. 10.2) and one form of D-fructose exists as a cyclopentane-like ring (fig. 10.3b). HAWORTH suggested that the type of hemiacetal ring formed by a monosaccharide be designated by reference to the two oxygen-containing hetocyclic compounds, furan (five-membered ring) and pyran (six-membered ring). The sugars with six-membered rings are, therefore, said to be pyranoses while those with five-membered rings are furanoses. The furanose form of fructose exists in equilibrium with a form which is characterized by a six-membered hemiacetal ring (i.e. fructopyranose). The schema of the anomeric equilibria in D-fructuse has been given by GERIG (1974) thus:-



Anomeric equilibria in D-fructose (after GERIG 1974).

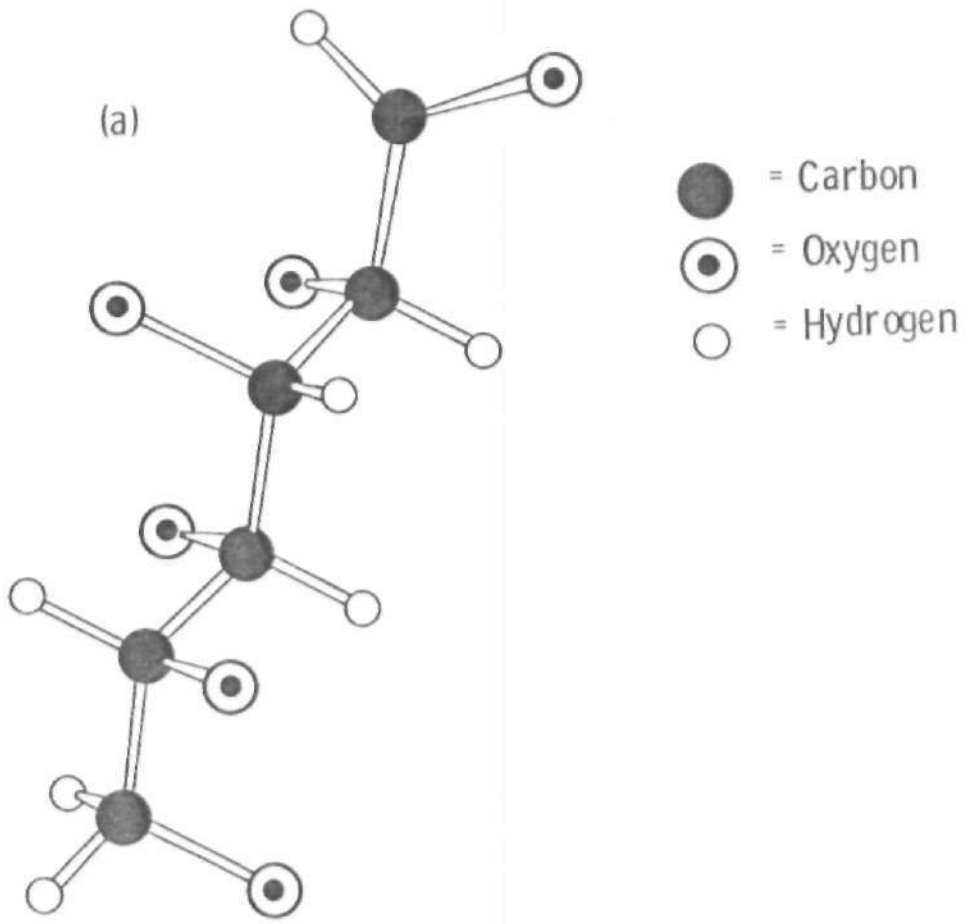
Pyranose-furanose interconversions have been reported for galactose, glucose, levulose and other sugars at varying pH from one to seven (ISBELL & PIGMAN, 1938). They contented that since the mutarotation of levulose is like that of fructofuranose set free from sucrose by invertase, but in the opposite direction, the mutarotation

LEGEND TO FIG. 10.1

FIG. 10.1. THREE DIMENSIONAL DRAWINGS OF GLUCOSE IN THE (A) LINEAR AND (B) CYCLIC FORMS. NOTE THAT -OH RADICALS ARE SIMPLY REPRESENTED BY THE OXYGEN ATOM. THE CYCLIC OR RING FORM IS DRAWN AS THE CHAIR (C_1) CONFORMATION OF THE PYRANOSE RING USING THE OXYGEN OF THE FORMER ALDEHYDE GROUP AS THE SIXTH MEMBER OF THE RING (THE MOST STABLE STATE OF ITS EXISTENCE).

Figure 10.1

THREE-DIMENSIONAL DRAWINGS OF GLUCOSE IN THE
(a) LINEAR AND (b) CYCLIC FORMS



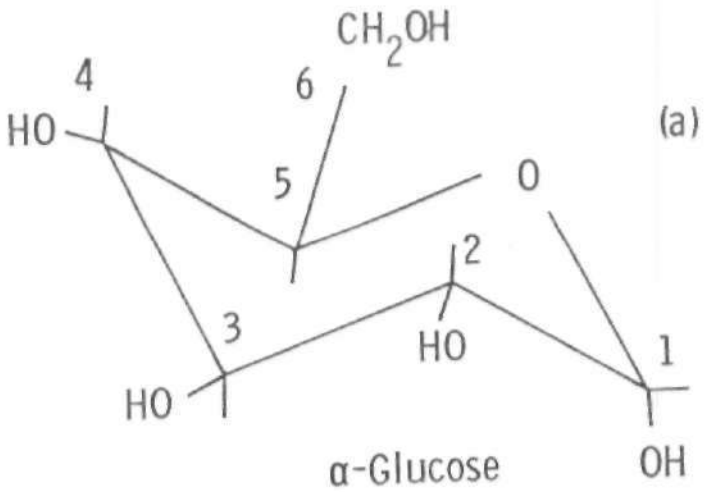
of levulose must consist of a pyranose-furanose interconversion. Similar arguments were developed for galactose and glucose mutarotations. Besides the pyranose-furanose or furanose-pyranose mutarotations, these sugars also show interconversions in the optically isomeric (α and β) forms. It is possible to isolate two crystalline forms of D-glucose which have different melting points and optical rotations. The most common form, α-D-glucose (fig. 10.2a) has an initial optical rotation in water of + 113°, but the rotation slowly changes to a steady value of + 52.5°. The other form, β-D-glucose (fig. 10.2b), has an initial rotation of + 19°, but this rotation also changes to + 52.5° as the solution stands. These materials are probably isomeric, but it is difficult to see how the (linear) open-chain structure that has been drawn here (fig. 10.1a) can be consistent with this. GERIG (1974) has summarised the evidence that gives support to this statement. He stated that attempts to form a dimethyl acetal of glucose by treating the compound with methanol and an acid catalyst were unsuccessful. This gave two isomeric products which do not react with FEHLING's or TOLLEN's reagents, thus suggesting that the carbonyl function, (i.e. the reducing activity of the aldehyde group) is no longer present. PMR (proton magnetic resonance) spectroscopy, however, shows that each isomer contains only one methoxyl group (CH_3O-) rather than two, as would be expected if an acetal had been formed. Thus it will be stated that glucose exists as a cyclic hemiacetal and the two isomeric forms observed differ only in the arrangement of the hydroxyl group at the anomeric carbon (which is the new asymmetric carbon of the ring structure derived from the former aldehyde carbon of the linear structure). The changing optical rotation that is observed when either of the pure isomeric forms is

LEGEND TO FIG. 10.2

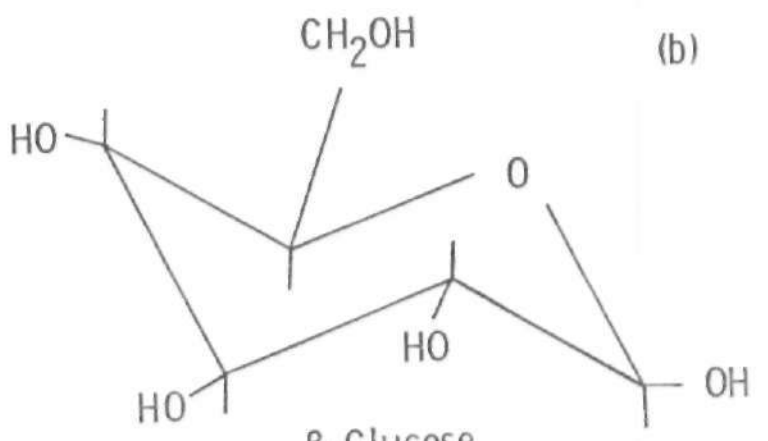
FIG. 10.2. RING STRUCTURES OF THE MONOSACCHARIDES (HEXOSES). THE HEXOSES DRAWN IN THIS FIGURE ARE THE TWO FORMS OF D-GLUCOSE (α- AND β-) AND THE STEREO-ISOMER OF THESE, THE L-GLUCOSE. NOTE THAT THE HEAVY LOH GROUPS OF THE L-GLUCOSE ARE ORIENTATED IN EXCATLY OPPOSITE POSITION TO THE CORRESPONDING -OH GROUPS OF THE D-GLUCOSE.

Figure 10.2

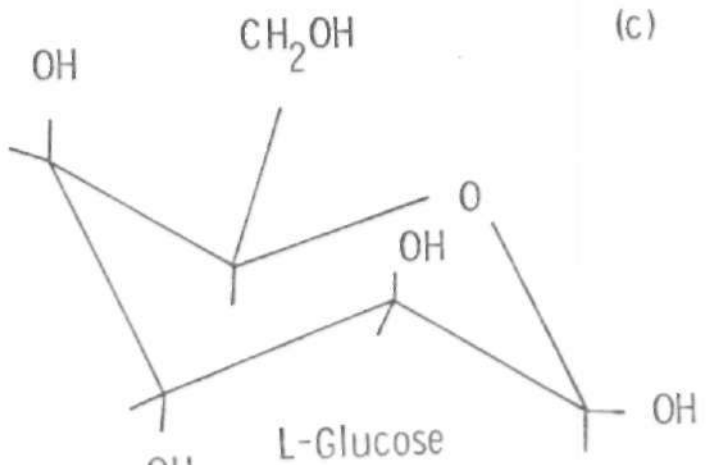
RING STRUCTURES OF THE MONOSACCHARIDES (HEXOSES)



α-Glucose
α-D-Glucopyranose



β-Glucose
β-D-Glucopyranose



L-Glucose
β-L-Glucopyranose

dissolved in water indicates that the two forms are approaching equilibrium. The rate of change of the rotation observed experimentally is a reflection of the rate at which equilibrium between the α and β forms is established. The rotation of + 52.5° that is observed when equilibrium is achieved is attributable to a mixture of the α and β isomers of the sugar. This process of equilibration is what is called mutarotation; it involves breaking and reforming the hemiacetal linkage. The open-chain form of glucose is present only in small amounts in solution. However, because the reactions comprising the equilibria are rapid in acid solution, glucose gives many of the reactions to be expected of an aldyhyde. Like D-glucose, other monosaccharides can exist as α and β anomeric isomers (or anomers) that are in equilibrium with each other via corresponding open-chain forms (ISBELL & WADE, 1967).

The structure of glucose is shown in fig. 10.1 as a linear (a) and cyclic (b) form. The cyclic form is shown in the chair (C.1) conformation which is known to be the most stable state of its existence (LEFEVRE 1961). This chair conformation, rather than its counterpart, the boat conformation, is used in the structural drawings of all the other pyranose ring forms in the following figures in this chapter. Figure 10.2 shows the glucose structures in the different isomeric forms, namely (a) α-D-glucose, (b) β-D-glucose and (c) β-L-glucose. When the ring structure is formed, the numerical nomenclature is depicted as in fig. 10.2a. The carbon atom No. 1 (the new asymmetric center in the glucose molecule in the ring form), and it is the stereochemistry at this carbon atom - formerly the aldehyde carbon in the linear form - that differentiates the α (fig. 10.2a) and β (fig. 10.2b) anomers of the sugar, and its methyl-D-glycoside.

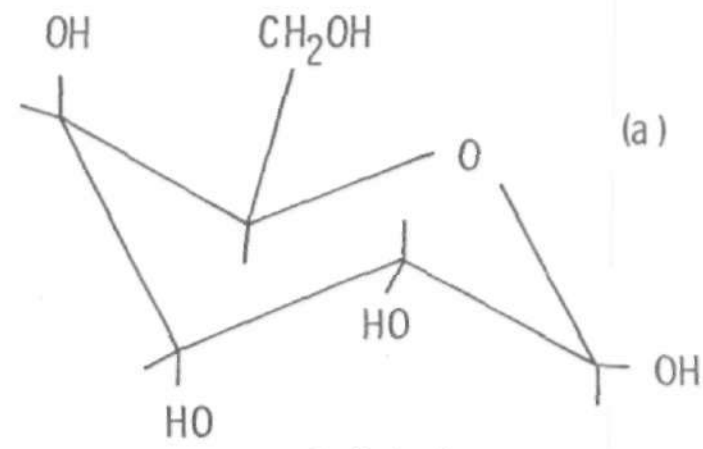
However, the L-glucose (fig. 10.2c) is a mirror image of the D-glucose (fig. 10.2a) - the -OH and -H groups are orientated in exactly opposite directions to each other in the two isomers. This is the only structural difference between the two isomers of glucose and hence the marked difference between the hepatocellular uptake of the two (Chapter 7) is explicable as a strong evidence of stereospecificity of glucose transport by the liver - a finding similar to that reported by WILLIAMS et al (1968). This is also an evidence of structure activity relationship in the hepatocellular sugar uptake, a finding similar to that in the erythrocytes (LEFEVRE 1961). A major difficulty in considering structure -bioactivity relations for hexoses is the prodigality of the conformational changes of which their molecules are capable. The use of disaccharides in which some of these structural changes are forbidden, has proved helpful. Figure 10.3 shows the ring structures of the other hexoses used in this study. In fig. 10.3a it will be seen that the only difference between D-glucose and D-galactose is the axial orientation of the -OH radical at carbon atom No. 4 in D-galactose instead of its equatorial orientation in D-glucose (fig. 10.2b). The ring structure of fructose exists mainly as a five membered ring (furanose) as depicted in fig. 10.3b. The numerical nomenclature of the furan ring is shown in this figure too. The methyl substituted glucose analogue (in alpha position) is also illustrated (fig. 10.3c). The structures of the disaccharides, sucrose, lactose and maltose are shown in fig. 10.4. Sucrose (fig. 10.4a) 1-2-α-D-glucopyranoside-β-D-fructofuranoside, is a disaccharide made up of glucose and fructose. The 1-2 glycosidic linkage in sucrose prohibits ring opening of both monosaccharide moieties of sucrose. This linkage is responsible for the lack of reducing reactions of

LEGEND TO FIG. 10.3

FIG. 10.3. RING STRUCTURES OF THE MONOSACCHARIDES (HEXOSES), OTHER THAN GLUCOSE, USED IN THIS STUDY. NOTE THAT FRUCTOSE IS A FIVE MEMBERED RING (FURANOSE) FORM, THE FIFTH MEMBER BEING AN OXYGEN ATOM.

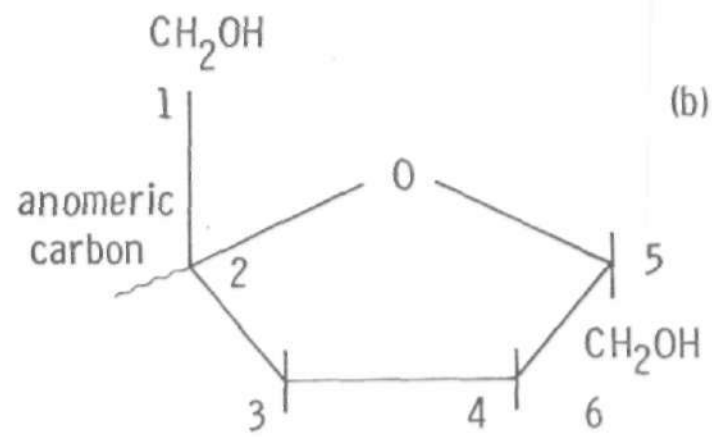
Figure 10.3

RING STRUCTURES OF THE MONOSACCHARIDES (HEXOSES)



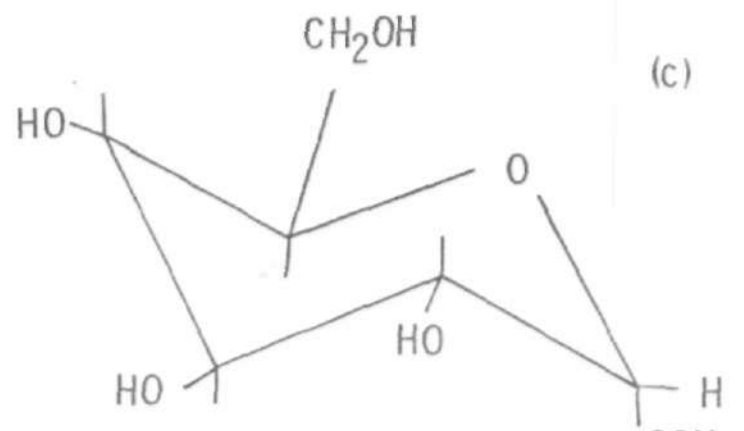
(a)

D-Galactose
β-D-Galactopyranose



(b)

D-Fructose
α-D-Fructofuranose



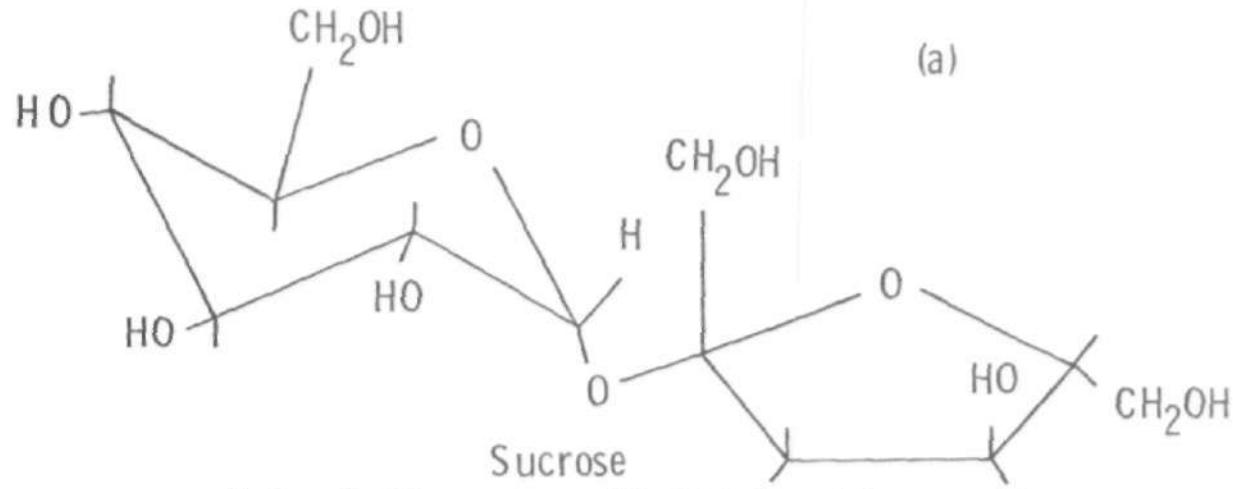
(c)

Methyl α-D-Glycoside
α-Methyl-D-Glucopyranoside

LEGEND TO FIG. 10.4

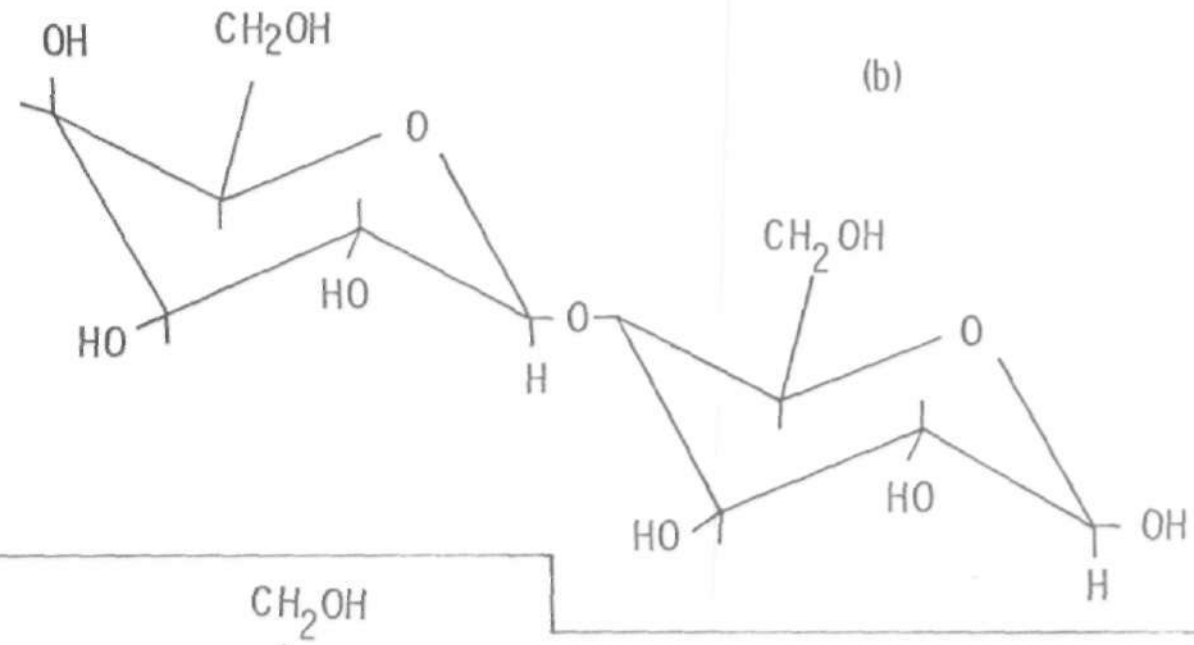
FIG. 10.4. RING STRUCTURES OF THE DISACCHARIDES USED IN THIS STUDY. BOTH MALTOSE AND LACTOSE (WHICH SHOWED EFFECTS) HAVE A 1 - 4 GLYCOSYL LINKAGE WHILE SUCROSE HAS A 1 - 2 LINKAGE.

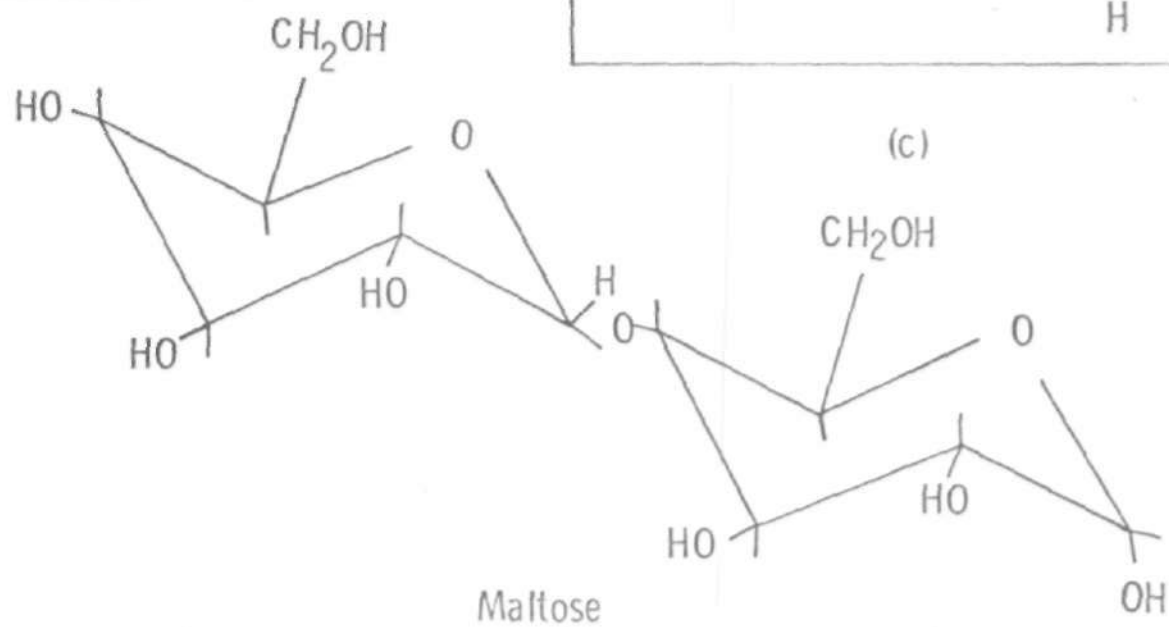
RING STRUCTURES OF THE DISACCHARIDES



Sucrose
1-2-α-D-Glucopyranoside-β-D-Fructofuranoside

Lactose
4-1-β-D-glucopyranoside-β-D-Galactopyranoside





Maltose
1-4-α-D-Glucopyranoside-α-D-glucopyranoside

sucrose. This may also be important in the difference in the effect of sucrose compared with maltose or lactose, as reported in Chapters 7, 8 and 9. Lactose, (fig. 10.4b), 4-1-α-D-glucopyranoside-β-D-galactopyranoside, is a disaccharide made up of glucose and galactose. The 4-1-glycosidic linkage allows ring-opening to occur in the glucose moiety since this anomeric carbon is free to react with water in solution. The galactose moiety cannot do this because its anomeric carbon participates in the linkage. Maltose (fig. 10.4c), 1-4-α-D-glucopyranoside-α-D-glucopyranoside, is a dimer of glucose. Like lactose, its 1-4-glycosidic linkage allows ring-opening to occur in one glucose moiety and the ring of the second glucose moiety cannot open for the same reason as the galactose moiety of lactose.

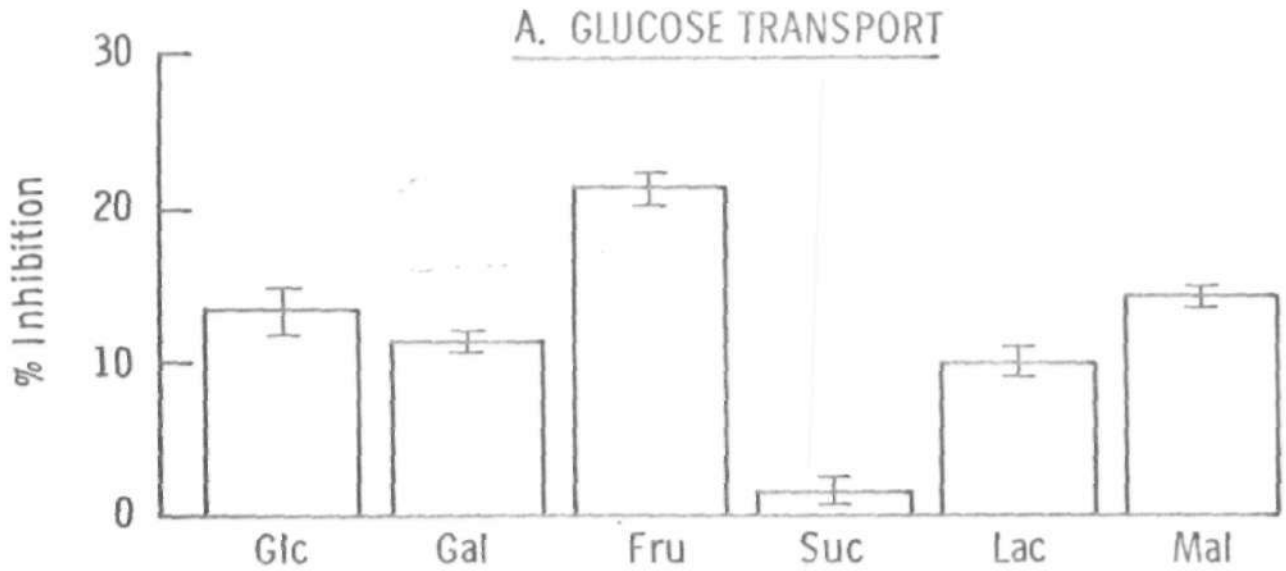
10.3 Effects of the Sugars on Hexose transport

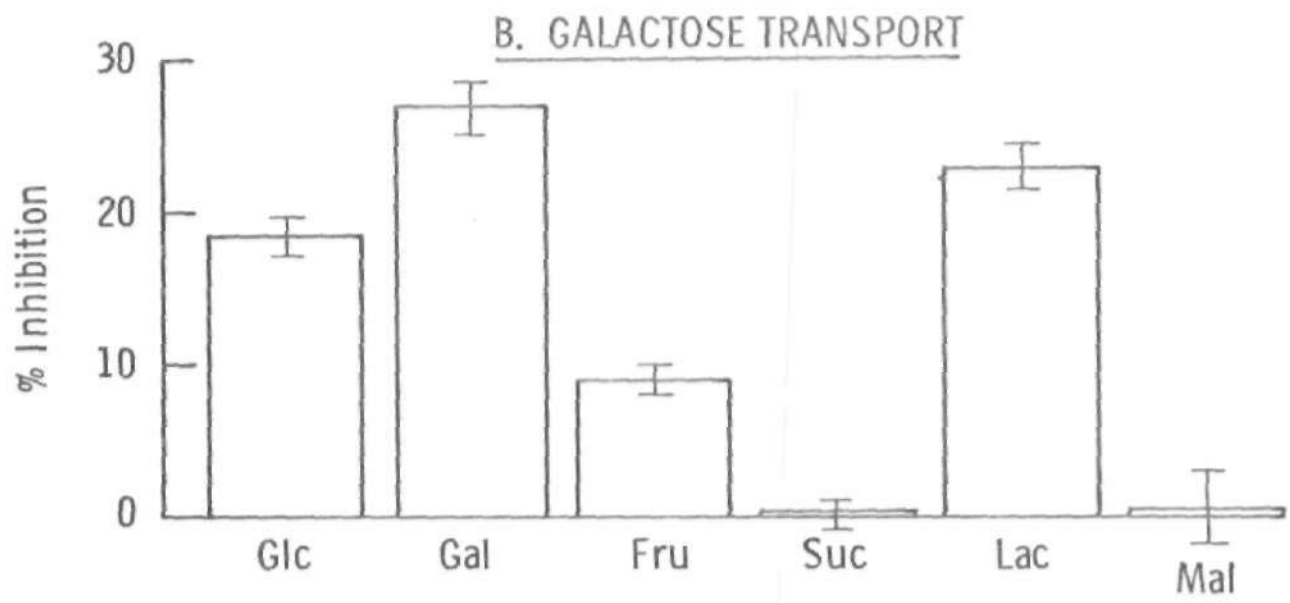
The D-stereoisomers of glucose, galactose and fructose all show saturable characteristics in this study (Chapters 7, 8 and 9). Hepatocellular uptake of both glucose and galactose have been found to be saturable (WILLIAMS et al 1968; HOOPER & SHORT 1977; BAUR & HELDT 1977) but not that of fructose. In fact BAUR and HELDT (1977) stated that fructose transport into isolated hepatocytes was not saturable up to 100mM concentration. This conflict may be due to the difference in experimental technique discussed in chapter 9. In this study all three hexoses show mutual competition among one another as summarized in fig. 10.5. Furthermore, both phlorizin and phloretin inhibited the uptake of these hexoses significantly (Chapters 7, 8 and 9), although the effect of phloretin was highly significantly greater than that of phlorizin on all three hexoses. This is interpreted as meaning these hexoses were transported across the liver cell membrane by a facilitated diffusion mechanism rather than by an active transport of the type found to be specifically sensitive to phlorizin. With respect to these competitors the hepatocellular transport of the hexoses is similar to their entry into human erythrocytes (WILBRANDT & ROSENBERG 1957; LEFEVRE 1961; 1972; STEIN 1967) as distinct from that by the brush border cells of the intestine or renal tubule cells (SCHULTZ & CURRAN 1970). This, coupled with the mutual competition observed among these hexoses, would tend to make one think that there was no selectivity among these hexoses for their transport by the liver cell and that a common hexose-carrier would be a plausible explanation. But a closer look at the pattern of mutual competition among the hexoses (fig. 10.5) reveals that some degree of discrimination is apparent. The competitive actions of the hexoses on D-glucose trans-

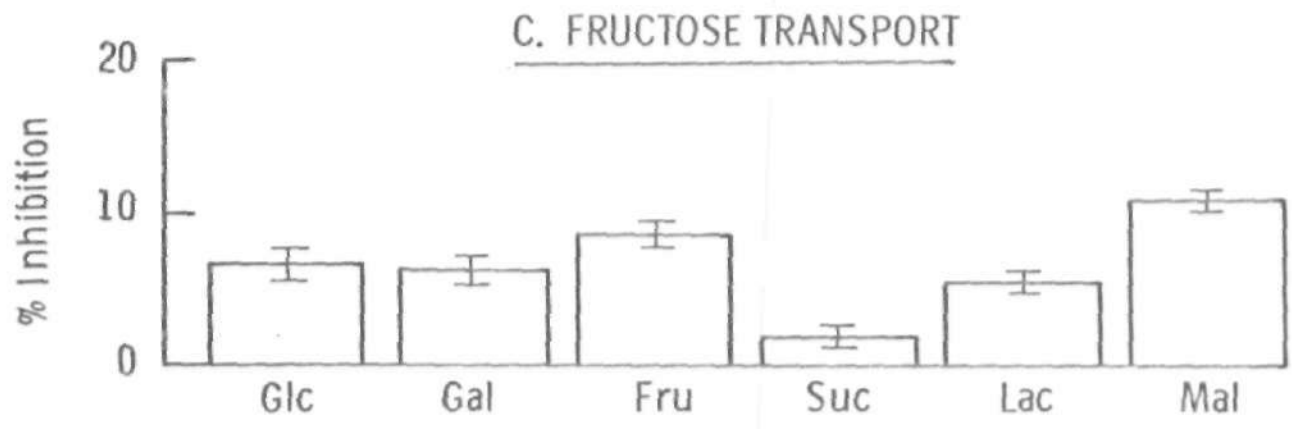
LEGEND TO FIG. 10.5

FIG. 10.5. SUMMARY OF THE EFFECTS OF 25mM HEXOSES AND DISACCHARIDES ON THE TRANSPORT OF D-GLUCOSE, D-GALACTOSE AND D-FRUCTOSE. NOTE THAT THE EFFECTS OF THESE SUGARS ON GLUCOSE AND FRUCTOSE TRANSPORTS ARE QUALITATIVELY SIMILAR BUT DIFFERENT FROM THOSE OF GALACTOSE TRANSPORT.

FIG. 10.5 EFFECTS OF 25mM HEXOSES AND DISACCHARIDES ON:







port (fig. 10.5a) are similar to those on fructose transport (fig. 10.5c). Although all the fructose extraction fractions were lower than those of D-glucose in these experiments, the competitor potency series fructose>glucose>galactose was identical. Both differ from the case of galactose transport where the order of competitive efficacy is galactose>glucose>fructose, that is, a reversal of the order. This qualitative difference ushered in the proposition that there might be a distinction between the glucose and galactose carriers, while fructose would appear to share a common carrier with glucose, although with less affinity for the carrier than glucose. But the evidence for such a proposition would not be complete at this stage. However conclusive evidence seems to have emerged from the disaccharide effects on transport of these three hexoses. Sucrose has no significant effect on any of these hexoses. Lactose has significant inhibitory effect on all three hexoses, but maltose has significant inhibitory effect on only glucose and fructose and has no effect on galactose transport (fig. 10.5a, b, c). In the case of the disaccharide competitors also, therefore, glucose and fructose transports show an identical pattern of competitive effects, while galactose is distinct because maltose has no effect on its transport. It is furthermore significant that the disaccharides are known not to penetrate the liver cells (CAHILL et al 1958). Disaccharides have previously been found to interfer with membrane transport of monosaccharides (LACKO & BURGER, 1962), but in this case the cells concerned were erythrocytes. The evidence for nonpenetrability of disaccharides into the hepatocytes has been discussed in Chapter 6. On this evidence, the competitive effects of these disaccharides on hexose transport must be exerted at the exterior of the cell membrane. But it is the

effect of disaccharides that distinguishes between galactose and glucose/fructose carrier. This means that there is a distinction between these two groups of carriers at the external border of the cell membrane. The mutual competition among all three hexoses means they must be sharing a common carrier which is not at the external surface. The simplest explanation, that the shared transport site is on the interior surface of the membrane, has great attractions. Although the effects of the penetrant hexoses on one another's transport considered alone could be at internal or external carrier sites, the action of glucose on galactose not shared by maltose must be at this distinct category of internal site. This is to assert asymmetry of the internal and external populations of carrier sites. Similar asymmetry of a sugar carrier mechanism has been reported for human erythrocytes (BAKER & WIDDAS 1973b; EDWARDS 1973), based on the differential inhibitory effect on glucose transport of ethylidene glucose inside and outside cells (BAKER & WIDDAS 1973a). Similarly the measurements of entry, exit and binding of various alkyl-substituted sugars (BARNETT, HOLMAN & MUNDAY 1973; BARNETT, HOLMAN, CHALKLEY & MUNDAY 1975)imply asymmetry in the erythrocyte. BARNETT et al 1973 drew their conclusions from the finding that 6-O-alkyl-D-galactoses which are non-transported competitive inhibitors (like the disaccharide effects reported here) competitively inhibited erythrocyte sugar transport system from outside while relatively ineffective when present inside the cells. On the other hand, they found n-propyl-β-D-glucopyranoside inhibited the sugar transport when present inside the cells but has a negligible effect when present outside. By using modified sugars in positions C_1, C_2, C_3, C_4 and C_6, BARNETT et al (1975) were able to identify the carrier binding sites as C_4 and C_6

being in contact with solvent on the outside, but C_1 to be inside the cell. This gave more support to the findings of BAKER and WIDDAS (1973).

It is considered that the competitive data reported here narrow down the range of possible binding mechanisms for these sugars. Hepatocellular entry by the pore has been discussed (section 6.2.4). It has been stated (BOWMAN & LEVITT 1977) that the kinetic equations describing transport through a pore that has a binding site and that undergoes a conformational change are identical to those of a carrier model. When a hydrophilic substrate (such as the hexoses used here) has to pass from a medium of high dielectric constant such as water to and through one of low dielectric constant such as a lipid phase (such as the cell membrane) a considerable electrostatic energy barrier has to be overcome. A simple way of overcoming such barrier in the lipid phase is for the lipid phase to be interrupted by a molecule of sufficient size, such as an integral membrane protein, spanning the membrane and forming an aqueous pore. Indeed such integral membrane proteins have been identified as the transport proteins (STEIN 1964; KILBER & STEIN 1966;1967; BOBINSKI & STEIN 1966; BONSALL & HUNT 1966; HASKOVEE & KOTYK 1969; FOX & KENNEDY 1965, and FOX et al 1967). These transport proteins have been called membrane carriers (KOTYK 1970). These carriers have been isolated from erythrocyte membrane extracts and crystallized (PARDEE 1966, 1967; PARDEE & PRESTIGE 1966; PARDEE et al 1966), their molecular weight determined, and even their amino acid composition estimated (PARDEE 1968). It seems likely that carrier proteins form pores of this nature in order to overcome this energy barrier. But such pores are unlikely to be water filled because mid-portions of the aggregating components of the pore must be

hydrophobic and associate hydrophobically. The sugar would then enter by forming hydrogen-bonds with the specific binding site at the pore entrance and the rest of the water molecules have some likelihood of desorption. The de-watered sugar would then enter the pore. The sugar transport is not likely to be through a water-filled pore, (SHORT 1980) (personal communication). In their studies of D-glucose transport across guineapig placenta, YUDILEVICH, EATON ans SHORT used low concentrations (5mM) and high concentrations (115mM) of L-glucose as an osmotic control for high competitor levels of D-glucose. They found no change in extraction due to either level of L-glucose although a high osmotic water flow due to the 115mM L-glucose had been induced. Had water movement in the D-glucose transport pore been possible such a finding would be most surprising. The hydrogen bond implicated in the transport phenomenon needs further clarification. Whenever hydrogen is covalently bound to a strong electronegative atom (F,O,N) it can hydrogen bind another electronegative atom within its vicinity. This interaction which is not electrostatic nor covalent, but an intermediate interaction is called hydrogen bond (ORGEL 1959; PAULING 1960; PIMENTEL & McCLELLAN 1960). It is a common occurrence among water molecules, but it is not an exclusive property of water molecules. Water molecules contain both the required donor and acceptor characteristics. Even when the hydrogen bond is a weak bond (4.5K Cal.mole), there are so many water molecules participating in the membrane structure that hydrogen bonds are believed to constitute an important factor in the protein-lipid association (VANDENHEUVEL 1965). Further support for the idea that the transport of hexoses studied here might be by a pore is from the temperature dependence of galactose transport and the relatively low activation energy (see below) obtained in

this study. Similar low activation energies associated with transport of ions have been advanced as evidence that hydrophilic solutes are transported via pores (MACEY 1979).

The sharp contrast between the effects of maltose and of lactose on β-galactose transport indicates high specificity involving the orientation of the hydroxyl group on C_4. This means, on the basis that hydrogen bonding is responsible for reversible binding of these structures, specific orientation of not less than two other adjacent groups (fig. 10.6). This derives from the finding of strong stereospecificity of glucose transport, which implies a minimum of three points of attachment. The spatial configuration in the region of C_5 must be highly variable because of rotation of the C_6 group and the ring-opening reactions involved in the α-β-anomeric equilibrium at C_1.

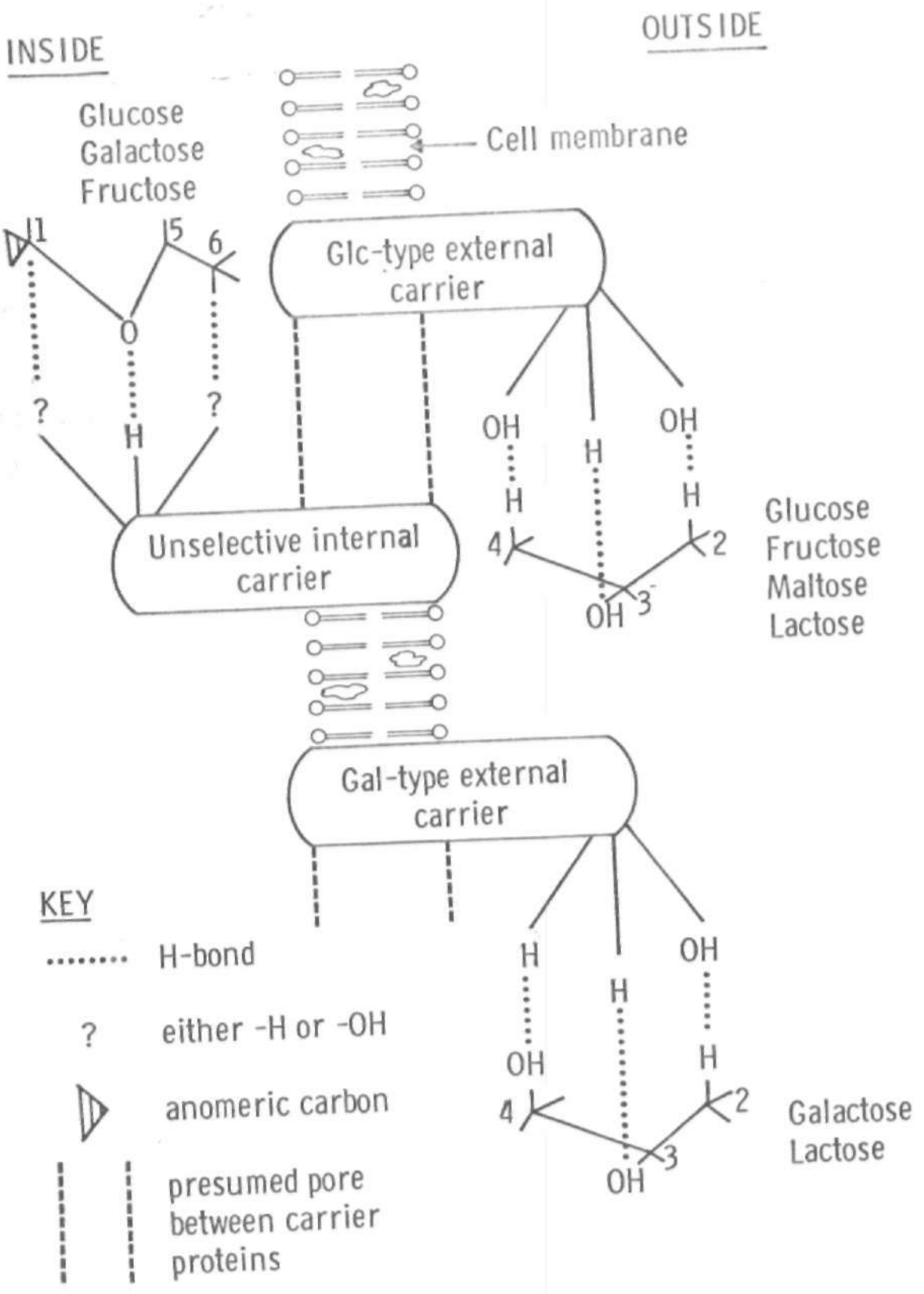
It seems most likely therefore, that the orientation of H-atom and -OH group at C_2, C_3, and C_4 (i.e. for the D-galactopyranose molecule -OH α-axial, -OH equatorial, H α-axial) is the means by which selectivity at the external galactose site is shown. It is therefore proposed that the external membrane binding site for galactose has -H and -OH arrangements complementary to those on C_2, C_3 and C_4 of the galactose molecule, and shared by the lactose molecule in which the chair conformation of the galactopyranose ring is stably present. The corresponding arrangement of the glucose/fructose site would then be complementary to the D-glucopyranose orientations at C_2, C_3 and C_4 (H α-axial, -OH equatorial, H α-axial).

An architecture for the supposedly common internal site cannot be so closely specified from these data, but configurations common to major forms of all three of these hexoses exist in the region of C_1,

LEGEND TO FIG. 10.6

FIG. 10.6. THE PROPOSED BINDING SITE ARCHITECTURE FOR THE HEXOSES BY THE RAT LIVER CELL MEMBRANE. IT IS BELIEVED THAT THE GALACTOSE EXTERNAL CARRIER IS DISTINCT FROM THE GLUCOSE/FRUCTOSE ONE WHICH IS THOUGHT TO BE A COMMON ONE.

FIG. 10.6
THE PROPOSED BINDING SITE ARCHITECTURE



C_5 and C_6 (fig. 10.6). This proposal seems to be consistent with the erythrocyte findings of BARNETT et al (1973, 1975), and has the merit that substrate transfer between the external and the internal sites would not require rotation of the transferred molecule. That sucrose has little effect on glucose transport in the perfused liver of rat suggests that the fructofuranose ring of sucrose both offers steric hindrance to binding of the glucopyranose ring, and is itself prevented from binding to the extent that fructose does, because ring-opening of fructose may be required to bring the configuration of the groups at C_2, C_3, and C_4 into correspondence with that of glucopyranose. Binding only of the opened or pyranose ring forms of fructose would account elegantly for the apparently low affinity of fructose for the hexose transport systems found in other tissues and seen also in these studies.

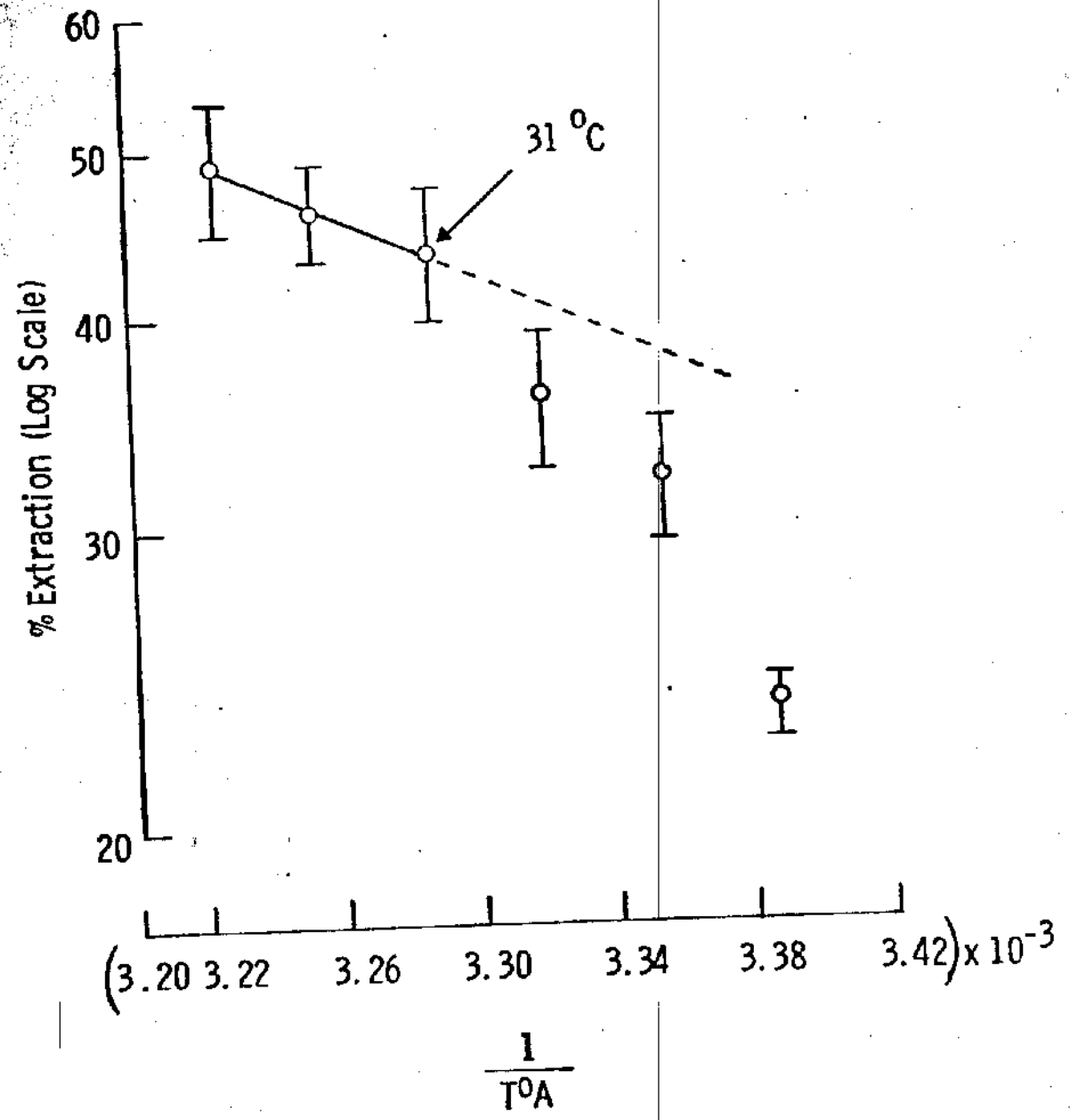
The question arises as to whether these distinct internal/external carriers are linked together as one unit or exist as separate components. The results of the temperature dependency of galactose transport may offer some answer to this questions. A transition temperature appears to exist at 31°C for D-galactose transport, at pH 7.4 (fig. 10.7). Above and below this transition temperature, the change in extraction with temperature was more uniform rather than the sudden break. Similar temperature dependence for glucose entry into erythrocytes was found by SEN and WIDDAS (1962) and glucose uptake by hepatocytes (BAUR & HELDT 1977). The transition temperature for glucose transport was found at 18°C by BAUR and HELDT (1977). This discrepancy from the transition temperature reported in this study might be due to possible changes in transport phenomenon in the isolated hepatocytes, which the enzymatic treatment (with collagenase) might cause.

LEGEND TO FIG. 10.7

FIG. 10.7. EFFECT OF TEMPERATURE ON GALACTOSE TRANSPORT. THIS IS THE SAME AS FIG. 8.7, REPRODUCED HERE FOR EASY REFERENCE.

FIG. 10.7

EFFECT OF TEMPERATURE ON GALACTOSE TRANSPORT



It was postulated (OVERATH, SCHAIRER & STOFFEL 1970) that the breaks in the temperature dependence of carrier mediated transport processes are related to the crystalline-liquid crystalline phase transition in the membrane. The transition is in the lipid phase of the membrane. LIPPERT and PETICOLAS (1971) stated that the apparent effect of cholesterol on lipid multilayers is different above and below the transition temperature. In the case of the dipamitoyl lecithin, this was found to be 38°C. Fluidization at lower temperature and gel formation occurred at higher temperature when egg lecithin was added. BLAZYK and STEIN (1972) found thermotrophic phase transitions in rat liver mitochondria and microsomes. They believe that the phenomenon appears to be the same as the gel-liquid crystalline transition of synthetic phospholipids in the lamellar phase, it provides strong evidence for an extensive bilayer in the membranes. They concluded that the membrane transitions indicate melting of the fatty acid chains of the phosphloipids, that the majority of the lipids in both mitochondria and microsomes is in bylayers and that at physiological temperatures the interior of the bilayers is liquid-like. The temperature of the lipid phase changes depends upon the length and saturation of the fatty acid chains, and upon the polar head group. The bilayer membrane transition temperatures were found to be slightly higher than those of the corresponding bulk free lipids (BLAZYK & STEIN 1972). The transition temperature found in this study suggests the same phenomenon of gel-liquid crystalline transition in the lipid component of the liver cell membrane. TRAUBLE and OVERATH (1973) have stated that 80% of the membrane lipids take part in the lipid phase transition. It would not therefore be surprising that transport functions should be altered in association with lipid phase changes of the cell mem-

brane.

The proposition of the involvement of hydrogen bonds in the type of binding site architecture proposed in this Thesis is consistent with the finding of the Energy of activation involved. The rate constants have a temperature dependence given by the Arrhenius equation (DAWSON & WIDDAS (1964)) thus:-

$$k = A_e^{-E_a/RT} \quad \ldots\ldots\ldots\ldots \quad 1$$

$$\text{or} \quad \log_e k = \log A - (E_a/RT) \quad \ldots\ldots\ldots \quad 2$$

where k is the rate constant, R the gas constant, T the absolute temperature and E_a the energy of activation. Equation (2) reduces to

$$E_a = RT\,(1_n A - 1_n k) \quad \ldots\ldots\ldots \quad 3$$

$$E_a = RT 1_n \left(\frac{A}{k}\right) \quad \ldots\ldots\ldots \quad 4$$

But the transport parameter is the change

$$1_n A - 1_n k = \Delta 1_n \quad \ldots\ldots\ldots \quad 5$$

substituting $\Delta 1_n$ in equation 3 and rearranging

$$\Delta 1_n = \frac{E_a}{R} \cdot \frac{1}{\Delta T} \quad \ldots\ldots\ldots \quad 6$$

$$\text{i.e. } E_a = R.\left(\frac{\Delta 1_n \text{ (transport)}}{\Delta 1/T}\right) \quad \ldots\ldots \quad 7$$

Taking the linear portion of the temperature dependence curve of galactose transport (fig. 10.7),

$$\text{the slope of the curve} = \frac{\Delta 1_n \text{ (transport)}}{\Delta 1/T},$$

The slope gives the Energy of activation similar to enzyme kinetics

(DIXON & WEBB, 1964).

Let this slope be S,

So $E_a = R.S$ 8

In computing,

from $37^{o}C$ to $31^{o}C$

$$S = \frac{1_n(49.12) - 1_n(43.79)}{\frac{1}{(31 + 273)} - \frac{1}{(37 + 273)}}$$

$$= \frac{0.11486}{6.3667 \times 10^{-5}}$$

$$= \underline{1804\ K}$$

And from $31^{o}C$ to $22^{o}C$,

$$S = \frac{1_n(43.79) - 1_n(23.58)}{\frac{1}{(22 + 273)} - \frac{1}{(31 + 273)}}$$

$$= \frac{0.619}{1.0036 \times 10^{-4}}$$

$$= \underline{6.68\ K}$$

Now, R is $8.31432 \pm 0.00034\ J.K^{-1}.mol^{-1}$ where $1J = 10^{7}$ erg.

From Equation (8), $E_a = R.S$,

At $37^{o}C$, $E_a = 217\ J.mole^{-1}.K^{-1}$

or $\underline{67\ kJ.mole^{-1}}$ at $37^{o}C$

And at $31^{o}C$, $E_a = 742\ J.mole^{-1}.k^{-1}$

or $\underline{226kJ.mole^{-1}}$ at $31^{o}C$

These activation energies are higher than those reported for glucose transport by the isolated hepatocytes (22 keal or 97 kJ.mole^{-1} in the range of 4 - 18^{o}C and 7 kcal or 29 kJ.mole^{-1} in the range of 18 - 37^{o}C) by BAUR and HELDT (1977). SEN and WIDDAS (1962) found the activation energy of glucose transfer into human erythrocytes was 10 kcal.mole^{-1}, and they postulated that this represented the energy of dissociation of the complex which glucose formed with the membrane component (now called carrier). Values for energy of activation for galactose transport in the liver have not been reported previously. That the values are higher than glucose values in hepatocytes or erythrocytes would be expected from the higher apparent K_m of galactose transport than glucose transport. Moreover, the high activation energy levels of galactose agrees with the proposition by SEN and WIDDAS (1962) that activation energies of the order of 10 Kcal or more indicate involvement of hydrogen bonds in the association or dissociation of substrate-carrier complex. The change in the slope on the Arrhenius plot is consistent with impairment below 31^{o}C of lateral diffusion of pore components in the plane of the membrane.

In conclusion, therefore, a proposal for a 3-prong binding site architecture is presented for distinct external and internal hexose carriers. The galactose carrier is distinct from the glucose/fructose carrier. The internal and external carriers are probably separate proteins capable of lateral mobility in the membrane plane. The transport of these hexoses involves the passage of a hydrophilic substance (hexose) through a hydrophobic barrier (the cell membrane). The resultant considerable energy barrier amounts to about 67 kJ.mole^{-1} in physiological conditions. To achieve the high glucose fluxes through the membrane under such a thermodynamic disadvantage the

membrane must contain very considerable numbers of the proposed pore-forming molecules (carrier proteins). On these, it can be said, depends the glucostatic function of the liver.

APPENDIX 1

THE LISTING OF THE COMPUTER PROGRAMME FOR CALCULATION OF PERCENTAGE EXTRACTION FROM THE SAMPLES. PAGES 327-329.

```
1.01 O K;O L;X FCHR(17;
1.04 C-- RAT LIVER DUALAB(JOI version,amended 29/11/79
1.05 C-DUALAB WITH SUPERFAILSAFE-MOD 12/5/77 H.P.
1.09 E
1.10 T "PACKARD 3H/14C DUAL LABELPROG FOR 3375 & 3255 "
1.12 T !,"PLEASE LOAD DATA TAPE & THEN SAY"
1.13 A !,"WHICH COUNTER (3255=1, 3375=2) ?   ",CI
1.14 S QQ=FITR(22-CC)
1.15 I (CC-2)1.17,1.17,1.16
1.16 T "TRY AGAIN ";G 1.13
1.17 I (CC+0)1.16,1.16,1.18
1.18 T !!
1.20 A "EXPT NO ",Z,"  DATE ",Z,"/",Z,"/",Z,!!!
1.30 A "C ST FROM ",S1,"TO ",S2,":C DPM ",D1,!;S CT=S1-1
1.32 I (CC-2)1.4,1.38,1.4
1.38 S CT=S1;G 1.4
1.40 A "H ST FROM ",S3,"TO ",S4,":H DPM ",D2,!!
1.45 T "STANDARD TABLE",!
1.47 T "VIAL    ESR    ZEFF   O/LZ",!
1.50 S S1=S2-S1;S S2=S1+1;S S3=S4-S3+S1+1
1.55 T "CARBON-14",!
1.60 F I=0,S1;D QQ;S O(I)=N3/N4;S E(I)=N4/D1;S Q(I)=N1;D 38
1.65 T "TRITIUM",!
1.70 F I=S2,S3;D QQ;S O(I)=N4/N3;S Q(I)=N1;S E(I)=N3/D2;D 38
1.80 D 40;C-INFUSATE READING,VARIABLE DESCRIPTION,DETAILS FOR PRO

2.01 C-DRIVING ROUTINE
2.10 F I=1,NO;D 45;D 3;D 50
2.99 Q

3.05 T "RUN NO. ",I
3.10 T !!" SN  BN    C-DPM    H-DPM  CZE HZE HOLZ COLZ    RATIO"
3.20 T "   EXTRACN."

4.10 D 25;D 7;D 8
4.50 T !,Z3.00,N2,"  "
4.55 S N1=(N4-L2*N3)/(E1*(1-L1*L2))-V1
4.56 S N3=((N3-L1*N4)/(E2*(1-L1*L2)))-V2
4.58 T Z6.01,N1,N3,Z2," ",E1*100,E2*100,L2*100,L1*100

7.10 S J=0;S Y=S1;S Z=1;D 9;S E1=E(J)-G*(E(J)-E(J+1))
7.20 S L1=O(J)+G*(O(J+1)-O(J)

8.30 S J=S2;S Y=S3;S Z=2;D 9;S E2=E(J)-G*(E(J)-E(J+1))
8.40 S L2=O(J)+G*(O(J+1)-O(J)

9.10 I (Q(J)-N1) 9.5;I (Q(J+1)-N1) 9.59
9.30 S J=J+1;I (J-Y) 9.1,9.1,9.5
9.50 I (Z-2) 9.52,9.54
9.52 T !,Z3.00,N2,"    14C-TOP =  "!;G 9.56
9.54 T !,Z3.00,N2,"   3H-TOP =  "!
9.56 S G=0
9.58 R
9.59 I (Q(J)-N1)9.6,9.56

9.60 S G=(Q(J)-N1)/(Q(J)-Q(J+1))
```

```
10.20 T !

20.01 C-3375 SUPERFAILSAFI
20.05 D R;A N1,N2
20.06 I (N1) 20.1,20.08,20.1
20.08 S CT=CT+1;C THIS SHOULD SKIP SAMPS WITH 0 ESR
20.09 T !"SAMPLE NO. ",CT-1,"HAD ZERO ESR",
20.10 A Z
20.15 I (CT-N2)20.2,20.5
20.20 I (N2-FITR(N2))20.3,20.4
20.30 S N1=N2;S N2=2
20.35 G 20.1
20.40 S MS=N2-CT;I (MS)20.99;S CT=N2
20.45 T Z2,!,MS," SAMPS MISSING"!!;S S4=S4+3
20.50 D 32
20.60 A N3,A1,N4,A2;S CT=CT+1
20.65 I (N3-A1)20.8
20.70 I (N4-A1)20.85
20.75 A Z,Z,;D 32;D K
20.79 R
20.80 S N3=A1;S A1=N4
20.85 S N4=A2;T " REDUNDANT CHARAC. CORRECTION ATTEMPTED"!
20.90 A A2
20.95 G 20.65
20.99 D 32;D 32;G 20.05

21.01 C-3255 SUPERFAILSAFE
21.02 D R;S Z=FCHR(-1);D 24
21.03 S Z=FCHR(-1);D 23
21.05 A N1,N2,Z
21.18 S CT=CT+1
21.20 S MS=N2-CT;I (MS)21.45,21.6,21.45
21.45 T Z3,!,MS," SAMPS MISSING",!
21.46 S CT=N2
21.60 A Z,Z,N3,N4,Z
21.75 D K
21.79 R
21.99 D 32;D 32;G 21.05

23.10 I (Z-160)23.2,23.3,21.03
23.20 I (Z-32)21.03,23.3,21.03
23.30 R

24.10 I (Z-255)24.2,24.3
24.20 I (Z-127)21.02,24.3,21.02
24.30 R

25.10 I (1-CC)25.2;D 26;R
25.20 S J=0;I (CT-201)25.4;S CT=CT-200;S J=1
25.40 D 20;I (J-1)25.5;S CT=CT+200;S N2=N2+200
25.50 R

26.20 S J=0;I (CT-301)26.4;S CT=CT-300;S J=1
26.40 D 21;I (J-1)26.5;S CT=300; S N2=N2+300

26.50 R
```

```
32.20 S L=FCHR(-1)
32.30 I (L-129)32.4;S L=L-128
32.40 I (L-13)32.2,32.5,32.:
32.50 R

38.10 T %3,N2,%5.04,N1,%5.02,E(I)*100,0(I)*100,!

40.01 C-VARIABLE DESCRIPTIONS
40.10 A !,?TOTAL:NOS:INFUSATES ?
40.30 A !!,?NOS:RUNS ,   SAMP:INTERVALS ,   RATIO:H'C'1:C'H'2
40.35 A !,?   DUPLICATES:YES'1:NO'2  ,?
40.40 S RA=1-RA
40.50 F I=1,TO;D 4;D 42

42.01 C-INFUSATE ARRAY
42.10 I (RA)42.50
42.20 S IR(N2)=N3/N1
42.30 R
42.50 S IR(N2)=N1/N3

45.01 C-INFUSATE SORTING&SAMPLE NOS FOR EACH RUN
45.10 T !!!,'          RUN NO. ',I
45.20 A !,' NO. OF INFUSATES ',Z
45.30 T !,'VIAL NOS. OF INFUSATES ',!
45.40 F I1=1,Z;A Z1,!;S IN(I)=IR(Z1)+IN(I)
45.50 S IN(I)=IN(I)/Z;T 'MEAN RATIO = ',%5.04,IN(I),!
45.60 A 'LAST SAMPLE NO. ',LA(I),!

50.01 C-EACH RUN, READ, CALCULATE & STORE
50.10 S V3=0;S V4=0;S V1=0;S V2=0
50.20 F I1=1,2;D 4;D 51
50.25 S V1=V3/2;S V2=V4/2
50.30 F I1=N2,LA(I)-1;D 55

51.20 T "BLANK SAMPLE"
51.40 S V3=(V3+N1);S V4=(V4+N3)

55.20 D 4
55.40 I (RA)55.8
55.50 S Z=N3/N1
55.60 G 55.9
55.80 S Z=N1/N3
55.90 T %5.02,'   ',Z;S SE=(1-Z/IN(I))*100;T SE
```

APPENDIX 2

LIST OF PUBLICATIONS

1. IBU, J. O., MACDONALD, I. A., & SHORT, A. H., (1978).
A technique for measuring first pass extraction in the rat perfused liver. Brit. J. Pharmacol. 64, p.469.

2. IBU, J. O., & SHORT, A. H. (1979a).
Competition among hexoses for hepatocellular uptake in the perfused liver of rat. J. Physiol. (Lond) 295, pp.18-19.

3. IBU, J. O. & SHORT, A. H. (1979b).
Disaccharide competition with hexose transport in the rat perfused liver. J. Physiol. (Lond) 296, p.31.

APPENDIX 3

LIST OF EXPERIMENTS

Date	Experiment	Notes
7.11.77	Collection of heparinised ox-blood Attempt at filming blood down glass oxygenator.	Trial
8.11.77	As above.	As above.
14.11.77	As above.	As above.
15.11.77	First attempt at washing of the blood.	Trial
16.11.77	Washing of blood	Successful
17.11.77	Making up perfusate with the washed bovine erythrocytes. Attempt at first liver cannulation	Unsuccessful cannulation
18.11.77	2nd attempt at liver cannulation	Marginal success fluid leakage from liver (damaged site).
20.11.77	Learning how to use Natelson's Microgasometer for measuring blood gases.	Trial
21.11.77	As above	As above
23.11.77	As above	As above
28.11.77	Learning how to use pH, PO_2, and PCO_2 autoanalyser	Trial
30.11.77	Collection of heparinised bovine blood	--
1.12.77	Washing of blood. Dialysis of albumin. Measurement of pH of various solutions made up	--
2.12.77	Connection of a new trial set of apparatus. Learning how to use Lex-O_2-Con	No perfusion
5.12.77	Operation and Cannulation of a rat liver. Effect of Watson Marlow pump on the perfusate	Successful cannulation RBC haemolysed
9.12.77	Washing of blood. Cannulation of liver. Measuring oxygen content with Lex-O_2-con .O_2 consumption	Successful cannulation Table

6.1.80	Collection and washing of blood Measuring haematocrit (PCV) of washed stored blood for 21 days starting now	No figure
7.1.78	Dialysis of albumin Measurement of PCV	--
8.1.78	As above	As above
9.1.78	Measurement of haemoglobin concentration	Trial
10.1.78	Cannulation of rat liver Measurement of PCV of inflow and outflow perfusate after one hour perfusion	Successful No significant haemolysis
11.1.78	Dialysis of albumin	
12.1.78	Re-operation of sheep No. 52 at Sutton Bonington	--
13.1.78	Cannulation of rat liver	Bleeding
14.1.78	Measurement of PCV of stored blood	--
15.1.78	As above	--
16.1.78	Calibration of variable autodiluter	--
17.1.78	Calculation of radioactivity in standard solutions and manual conversion of cpm to dpm	
18.1.78	Experiment on Sheep No. 52	Trial
20.1.78	Cannulation and perfusion of rat liver. Measurement of Hb conc. Measurement of O_2 consumption	Successful Table
21.1.78	Measurement of PCV	--
22.1.78	As above	Haemolysis
23.1.78	Collection and washing of blood	--
24.1.78	Dialysis of albumin. Measurement of Hb conc.	
25.1.78	Re-operation of Sheep No. 52 at Sutton Bonington	--
26.1.78	Measurement of Temperatures of perfusate and aeration chamber. Measurement of Hb conc.	

Date	Experiment	Notes
	Measurement of O_2 consumption	Table
27.1.78	Washing and sterilization of glassware in hypochlorite solution. Calibration of drop counter	--
30.1.78	Measurement of O_2 consumption	Table
31.1.78	Measurement of O_2 consumption	Table
3.2.78	Measurement of O_2 consumption	Table
6.2.78	Preparation liquid scintillation cocktail	Trial
7.2.78	Calibration of variable auto-diluter	--
8.2.78	Calibration of constant infustion syringe pump.	--
	Measurement of O_2 consumption	Table
15.2.78	Washing of one day old blood Washing of seven day old blood Washing of ten day old blood Measurement of Hb conc.	Figure
	Measurement of O_2 consumption	Table
23.2.78	Washing of stored blood.	--
	Measurement of O_2 consumption	
8.3.78	Manual converstion of cpm to dpm	--
14.3.78	As above	--
21.3.78	1st experiment of D-glucose uptake in the perfused liver	Successful
4.4.78	Glucose and fructose uptake with ^{14}C-L-glucose as reference.	Figure
	Expts. with 5.5mM unlabelled glucose and OmM glucose in Krebs.	
	Measurement of osmolarity of Krebs and of perfusate reproducibility.	
7.4.78	Uptake of glucose and fructose by the liver at OmM and 11.1mM glucose conc. in Krebs.	Figure
8.4.78	As above	Figure
10.4.78	Manual calculation of dpm from cpm, and % extraction worked out.	

Date	Experiment	Notes
18.4.78	Uptake of glucose and fructose in 5.5mM and 11.1mM	Unsuccessful
19.4.78	Manual calculation of results	--
26.4.78	To find saturability of glucose transport at 11.1mM	Unsuccessful experiment
27.4.78	Manual calculation of results	--
23.5.78	Expt. 1. Sources of error. Washing of sampling tip.	Table
2.6.78	Expt. 2. Comparing two infusion lines constructed	--
5.6.78	Expt. 3. Immipramine extraction by the liver	--
6.6.78	Expt. 4. Immipramine solution and pH changes	--
7.6.78	Expt. 5. Immipramine extraction by the liver	--
13.6.78	Expt. 6. Pardridge single bolus injection and immipramine uptake	Chimical imminosity
14.6.78	Expt. 7. Repeat Expt. 6.	As above
22.6.78	Expt. 8. Repeat Expt. 6.	As above
23.6.78	Expt. 9. Repeat Expt. 6.	As above
6.7.78	Expt. 10. Saturability of immipramine uptake by the liver.	--
14.7.78	Expt. 11. As above	--
8.8.78	Determination of O_2 content using lex-O_2-con.	familiarization
9.8.78	Learning to use tonometer. Tonometry of perfusate $\bar{c}$ 13.5% O_2. Measurement of O_2 content after tonometry	Figure
10.8.78	Tonometry of perfusate $\bar{c}$ 95% O_2	Figure
14.8.78	Measurement of Hb conc. with Sp 500	--
15.8.78	Learning to use pH, PO_2, PCO_2 automatic blood gas analyser	--

Date	Experiment	Notes
16.8.78	Learning how to use Cunningham's Trombone to mix gases	Table
17.8.78	Flame photometry - K^+ determination	Figure
18.8.78	Flame photometry	Figure
19.8.78	Hb conc. measurement - SP 500	Figure
21.8.78	Washing of fresh ox-blood	Figure
22.8.78	Determination of O_2 dissociation curve of perfusate	Figure
24.8.78	Hb conc. measurement - SP 500	Figure
25.8.78	Effect of Watson Marlow pump of recirculating perfusate	Figure
1.9.78	Effect of liver on recirculating perfusate	Figure
4.9.78	Measurement of K^+ levels in stored samples.	Figure
20.9.78	Making up stock solution of immipramine hydrochloride	--
25.9.78	Mixing of gases - 5% CO_2 in air, and 95%:5% O_2:CO_2 mixture Counting empty vials to detect background noise due to vial glass	--
26.9.78	To compare liver extraction with 5% CO_2 in air and 5% CO_2 in 95% O_2 mixtures. Sucrose reference oxygen consumption measurement	Figure Table
10.10.78	As above	As above
13.10.78	As above	Unsuccessful jammed portal cannula
27.10.78	Expt. 101. To find effect of different sugars on galactose uptake. 8 runs	Trial Successful Figure
3.11.78	Expt. 102. As Expt. 101.	Figure
10.11.78	Expt. 103. As Expt. 101.	Figure
14.11.78	Expt. 104. As Expt. 101.	Figure
28.11.78	Effect 50mM different sugars on galactose uptake	Successful

Date	Experiment	Notes
1.12.78	As above.	As above
5.12.78	As above	As above
7.12.78	As above	As above
6.1.79	Expt. 201. Effect of 25mM sugars on glucose uptake by the liver. 8 runs.	Successful. Figure
8.2.79	Expt. 202. As above	Figure Oedematous
13.2.79	Expt. 203. As above	Figure
15.2.79	Expt. 204. As above	Sample changer problem
20.2.79	Expt. 205. As above	Figure
22.2.79	Expt. 301. Effect of 25mM sugars on fructose extraction	Successful Figure
7.3.79	Expt. 206. Effect of 25mM sugars on glucose transport	Successful. Tight ligature. Figure
9.3.79	Expt. 207. As above	Inadequate gassing. Figure
13.3.79	Expt. 208. As above	First 2 livers unsuccessful. 3rd operation successful Figure
15.3.79	Expt. 302. Effect of 25mM sugars on fructose transport	Figure 1st liver unsuccessful 2nd liver successful
20.3.79	Expt. 303. As above	stored blood partly haemolysed
22.3.79	Expt. 304. As above	1st operation unsuccessful. 2nd operation successful. Figure
28.3.79	Expt. 305. As above	Figure
19.4.79	Expt. 401. Effect of phlorizin on uptake of AIB, glucose, galactose and fructose. 10 runs. Perfusion fixation with glutaraldehyde. Specimen of liver taken for electron microscopy.	Successful Figure

Date	Experiment	Notes
24.4.79	Expt. 402. As above. Perfusion fixation with glutaraldehyde Specimen of liver taken for electron microscopy	Figure
26.4.79	Expt. 403. As above No perfusion fixation after experiment	As above
2.5.79	Expt. 404. As above	As above
4.5.79	Expt. 405. As above Haemolysed stored blood	As above
15.5.79	Expt. 501 Expt. 502. Effect of 25mM maltose on galactose transport	Figure
16.5.79	Expt. 503 Expt. 504. As above	Figure
25.5.79	Expt. 406. Electron microscopy of unperfused liver. Glutaraldehyde fixation.	Figure
	Expt. 407 - E.M. of perfused liver with no phlorizin added	Figure
30.5.79	Expt. 601. Effect of phloretin on uptake of AIB, glucose, galactose and fructose	Successful Figure
31.5.79	Expt. 602. As above	Figure
6.6.79	Expt. 603. As above	Figure
8.6.79	Expt. 604. As above	Figure
12.6.79	Expt. 605. As above	Figure
26.6.79	Expt. 701. Effect of Temperature changes on galactose transport	Figure
28.6.79	Expt. 702. As above	Figure
3.7.79	Expt. 703. As above	Figure
6.7.79	Expt. 704. As above	Figure
7.7.79	Expt. 705. As above	Figure
19.7.79	Expt. 801. Comparison of D- and L-glucose extractions	Figure
	Expt. 802. As above	Figure
20.7.79	Expt. 803 Expt. 804. As above	Figures

Date	Experiment	Notes
24.7.79	Expt. 900. Extraction of ^{3}H-sucrose and the effects of 25mM sugars on it	Figure
26.7.79	Expt. 1000. Extraction of ^{3}H-sucrose using ^{14}C-sucrose or ^{14}C-L-glucose as reference	Figure
17.9.79	Expt. 1101. Low temperature distillation. Expt. 6 parallel lines	Trial
1.10.79	Expt. 1102. As above	Results
21.11.79	Expt. 1201. Effect of Low Sodium (tris buffer) on transport of D-glucose, D-galactose and AIB. Comparison of D- and L-glucose uptake with dried ^{3}H-sucrose. Effect of 2-deoxy-glucose on D-glucose extraction	Tris addition after first 5 runs caused reduced flow, and temperature of the liver. Liver oedematous
23.11.79	Expt. 1202. As above. Osmolarity measured and corrected before runs commenced.	Same problems. Expt. stopped Mottled liver
11.12.79	Expt. 1301. To find effect of 25mM sugars on dried ^{3}H-D-glucose and dried ^{3}H-D-galactose with ^{14}C-L-glucose reference. 14 runs	No figure
13.12.79	Expt. 1302. As above	As above
18.12.79	Expt. 1303. As above	As above
20.12.79	Expt. 1304. As above	As above
3.1.80	Comparison of extraction of dried and undried glucose or galactose	No figure
8.1.80.	Expt. 1501. To find extraction of dried ^{3}H-D-glucose and ^{3}H-D-galactose in presence of 25mM disaccharides	No figure
10.1.80.	Experiment 1502. As above	As above
	THE END	

REFERENCES

ABOUNA, G. M. (1968).

Extracorporeal liver perfusion using a new perfusion chamber.

Lancet 2 1216-1218

ABOUNA, G. M., ASHCROFT, T., MUCKLE, T. J., SKILLEN, A. W., HULL, C. J. KIRKLEY, J. R. & HUDSON, A. W. (1970).

Heterologous extracorporeal hepatic support: haemodynamic, biochemical and immunological observations.

Brit. J. Surg. 57: 213-220

AKINYANJU, P. A., QURESHI, R. U., SALTER, A. J. and YUDKIN, J. (1969)

Effect of an "atherogenic" diet containing starch or sucrose on the blood lipids of young men.

Nature 218: 975-977

ALLEN, P. C. and LEE, H. A. 1969

A clinical guide to intravenous nutrition. Oxford Univ. Press, Oxford 1969, p.27.

ALLMAN, D. W., BACHMAN, E., ORME-JOHNSON, N, TAN, W. C. and GREEN, D.E. 1968.

Membrane systems of mitochondria VI Membranes of liver mitochondria.

Arch. Biochem. Biophys. 125, 981-1012

ALTMAN and DITTMER (1971)

Blood and other body fluids.

Published by Federation of American Societies for Experimental Biology. Bethesda, Maryland, U.S.A. 1971. pp.151-161.

ALVARADO, F. and CRANE R. K. 1962.

Phlorizon as a competitive inhibitor of the active transport of sugars by hamster small intestine in vitro.

Biochim. Biophys. Acta 56 170-172

ANDRES R. A., and ZIERLER K. L. 1956

Effect of insulin on glucose uptake, lactate production and R.Q. in the forearm of man.

Am.J.Physiol. 187 583-589

ASHMORE, J. and WEBER, G. (1968)

In "Carbohydrate metabolism and its disorders" Ed. F. Dickens, P. J. Randle, and W. J. Whelan, Vol. 1. p.335. Acad. Press. New York, 1968.

ASKEW, F. A. AND DANIELLI J. F. (1940).

Measurements of the pressures due to monolayers at oil-water interfaces.

Trans.Faraday Soc. 36: 785-788

ASP, G.

Arbeiten aus der physiologischen Anstalt

Zu Leipzig 8: 124

ANTIKO, L. A., NORTON, W. T. and TERRY, R. D. 1964.

The preparation and some properties of purified myelin from the central nervous system.

J.Neurochem. II, 7-27.

BAER, R. and ROESSLER, R. (1926).

Beitrage zur Pharmakologie der Lebergefasse.

Arch.Exper.Path.Pharmakol. 119: 204 cited by Ritchie and Hard-castle 1973.

BAILEY, E., TAYLOR, C. B. and BARTLEY, W. (1968).

Effect of dietary carbohydrate on hepatic lipogenesis in the rat.

Nature 217: 471-472

BAKER, G. F. and WIDDAS, W. F. (1973)

The permeation of human red cells by 4-6-O-ethyledene-α-D-glucopyranose (ethylidene glucose).

J.Physiol. 231: 129-142

BAKER, G. F. and WIDDAS, W. F. (1973b)

The asymmetry of the facilitated transfer system for hexoses in human red cells and the simple kinetics of a two component model.

J.Physiol. 231:143-165.

BARANY, E. and SPERBER, E. 1939.

Absorption of glucose against a concentration gradient by the small intestine of rabbit.

Skand.Arch.Physiol. 81: 290-299

BARNETT, J. E. G., HOLMAN, G. D. and MUNDAY, K. A. (1973a)

Structural requirements for binding to the sugar-transport system of the human erythrocyte.

Biochem.J. (1973) 131: 211-221

BARNETT, J. E. G., HOLMAN, G. D. and MUNDAY, K. A. (1973b)

An explanation of the asymmetric binding of sugars to the human erythrocyte sugar-transport system.

Biochem.J. (1973). 135: 539-541

BARNETT, J. E. G., HOLMAN, G. D., CHALKLEY, R. A. and MUNDAY, K. A. 1975

Evidence for two asymmetric conformational states in the human erythrocyte sugar transport system.

Biochem.J. (1975). 154: 417-429.

BAR-ON, and STEIN, Y. (1968)

Effect of glucose and fructose administration on lipid metabolism in the rat.

J.Nutr. 94: 95-105.

BARTOSEK, I., GUAITANI, A, and GARATTINI, S. 1973.

Prolonged perfusion of isolated rat liver in isolated liver perfusion and its applications. Eds. I. Bartosek, A. Guaitani and L. L. Miller, pp.63-72.

Raven Press, New York.

BAUER, W., DALE, H. H. POULSSON, L. T., and RICHARDS, D. W. (1932)

The control of circulation through the liver.

J.Physiol. 74: 343-375

BAUR, H., and HELDT, W. H. 1977

Transport of Hexoses across the liver-cell membrane

Eur.J.Biochem 74, 397-403.

BENNETT, V. and CANTRECASAS, P. (1973)

Preparation of inverted plasma membrane vesides from isolated adipocytes.

Biochim.Biophys.Acta. 311: 362-380

BERGMAN, R. N. and BUCOLO, R. J. (1974).

Interaction of insulin and glucose in the control of hepatic glucose balance.

Am.J.Physiol. 227: 1314-1322

BERNARD, C. (1853)

Nouvelle fonction du foie. Bailiere, Paris.

1855 cited by Kestens and Lambotte 1973.

BERNARD C, 1855

Comptes Rendus Academie Sci. 41: 461.

BERNARD, C. (1865)

An introduction to the study of experimental medicine.

(republication by Dover, New York, 1957. pp.129-134.

BERTHET, J., JACQUES, P., HERS, G. and DEDUVE, C. (1956)

Influence de Uinsuline et du glucagon sur la synthesa du glucogene

hepatique.

Biochim.Biophys.Acta 20: 190-200

BIHLER, I.

Ltestinal sugar transport: ionic activation and chemical specificity

BBA 183 169-181

BLAZYK, J. F. and STEIN, J. M. 1972

Phase transitions in mammalian membranes.

Biochim.Biophys.Acta. 266: 737-741.

BLOXAM, D. L. 1971

Nutritional aspects of amino acid metabolism 1. A rat liver perfusion method for the study of amino acid metabolism.

Nutr. 26: 393-421.

BLOXAM, D. L. 1973

Condition and performance of the rat liver perfused with Kregs-ringer solution, with particular reference to amino acid metabolism.

In isolated liver perfusion and its applications, pp.147-153. Eds. I. Bartsek, A. Guaitani, and L. L. Miller, Raven Press, New York.

BOBINSKI, H. and STEIN, W. D. (1966)

Isolation of a glucose binding component from human erythrocyte membranes.

Nature, Lond. 211: 1366-1368

BOLIS, L. and GOMPERTS, B. A. (1972).

In transport and accumulation in biological systems. Ed. E. J. Harns. Butterworth and Co. Pub. Ltd., Lond. p.93.

BOLLMAN, J. L., CAIN, J. C. and GRINDLAY, J. H. (1948).

Techniques for collection of lymph from liver, small intestine or thoracic duct of rat.

J.Lab.Clin.Med. 33: 1349-1352

BONSALL, R. B. and HUNT, S. (1966)

Solubilization of a glucose-binding component of the red cell membrane.

Nature, Lond. 211: 1368-1370

BOWMAN, R. J. and LEVITT, D. G. (1977)

POlyol permeability of the human red cell. Interpretation of glucose transport in terms of a pore.

Biochimica et Biophys.Acta. 466: 68-83

BOWYER, F.

The kinetics of the penetration of non-electrolytes into the mammalian erythrocyte.

Intern.Rev.Cytol. 6, 469-511

BOWYER, F. and WIDDAS: W. F.

The action of inhibitiors on the facilitated hexose transfer system in erythrocytes.

J.Physiol.(Lond.) 141: 219-232

BRANDSTRUP, E.

Uber die diffusion der glukose und des harnstaffes durch die placenta.

Acta.Obst.Gynec.Scand. 8: 490-516

BRANTON, D. 1971

Freeze-etching studies of membrane structue.

Phil.Trans.Roy.Soc. 261: 133-138

BRAUER, R. W. 1963

Liver circulation and function.

Physiol. Rev. 43 115-213

BRAUER, R. W. PESSOTTI, R. L. and PIZZOLATO, P. 1951

Isolated rat liver preparation. Bile production and other basic

properties.

Proc.Soc.Exper.Biol. & Med. 78: 174-181

BRAUER, R. W. LEONG, G. F., and PESSO Hi. R. L. (1953)

Vasomotor activity in the isolated perfused rat liver.

Am.J.Physiol. 174: 304-312

BRAVO, I. R., and YUDILEVICH, D. L. (1971)

Liver distribution and uptake of molecules studied by rapid indicator-dilution technique.

Am.J.Physiol. 221(5): 1449-1455

BRETSCHER, M. S. 1973

Membrane structure: Some general principles.

Science 181: 622-629

BRIGGS, F. N., BRAUER, R. W., TAUROG, A., and CHAIKOFF, I. L. (1953)

Metabolism of I^{131}-labelled thyroxine-studies with isolated perfused rat liver.

Am.J.Physiol. 172 561-564

BROWN, S. S., FORREST, J. A. H., ROSCOE, P. 1972

A controlled trial of fructose in the treatment of acute alcoholic intoxication.

Lancet 1972 2 898-899

CAHIL, G. F., ASHMORE, J., EARLE, A. S., ZOTTU, S. 1958

Glucose penetration into liver.

Am.J.Physiol. 192(3) 491-496

CAMPBELL, P. N., and DAVSON, H. (1948)

Absorption of 3-methylglucose from the small intestine of the rat and cat.

Biochem.J. 43: 426-429

CARSTENSEN, M., LEICHWEISS, H. P., MOLSEN, G., and SCHRODER (1977)
Evidence for a specific transport of D-hexose across the human term placenta in vitro.
Arch.Gynaekol. 222 187-196

CEREIJIDO, M. and ROTUNDO, C. A. 1970
Introduction to the study of biological membranes. Gordon and Breach Science Publishers, New York, 1970.

CHESLEY, L. C. and UICHANCO, L., LENOBEL, A., and KELLERMAN, H. (1964)
Placental permeability to sucrose a source of error in measuring volumes of sucrose distribution in gravidas.
Obst.Gynae. 23(5) 795-798

CHINARD, F. P., VOSBURGH, G. J. AND ENNST, (1956)
Transcapillary exchange of water and of other substrates in certain organs of the dog.
Am.J.Physiol. 183: 221-234

CHRISTENSEN, H. N. 1975
Biological transport.
2nd. edn. W. A. Benjamin, Massachusetts.

CODON, R. E. and BOMBECK, C. T. (1970)
Liver perfusion in hepatic coma.
Surg.Clin.N.Amer. 50: 257-259

COHEN, A. M. and TEITELBAUM, A. (1968)
Effect of glucose, fructose, sucrose and starch on lipogenesis in rats.
Life Sci. 7: 23-29

COLE, K. S. 1932
Surface forces of the arbacia egg.
J.Cellular Comp.Physiol. 1: 1-7

COLE, K. S. and CURTIS, H. J. (1938)

Electrical impedance of nerve during activity.

Nature 142: 209-210

CONE, R. A. (1972)

Rotational diffusion of rhodopsin in the visual receptor membrane.

Nature, New.Biol. 236: 39-42

CORI, C. F. (1925)

Fate of sugar in the animal body. 1. Rate of absorption of hexoses and pentoses from the intestinal tract.

J.Biol.Chem. 66: 691-715

CORI, C. F. (1926)

Rate of absorption of a mixture of glucose and galactose.

Proc.Soc.Exptl.Biol.Med. 23 290

CRAIG, J. W., MILLER, M. MACKENZIE, M. S. and WOODWARD, H. 1958

The influence of dietary carbohydrate deprivation on the metabolism of intravenously administered fructose and glucose in man.

J.Clin.Invest.37: 118-126

CRANE, R. K. (1960)

Intestinal Absorption of Sugars.

Physiol. Rev. 40 789-825

CRANE, R. K. 1962

Hypothesis for mechanism of intestinal active transport of sugars.

Fed.Proc. 21 891-895

CRANE, R. K. 1965

Na^+-dependent transport in the intestine and other animal tissues

Fed.Proc. 24 1000-1006

CRANE, R. K., FIELD, R. A., and OORI, C. F. 1957

Studies of tissue permeability. 1. The penetration of sugars into the Ehrlich ascites tumour cells.

J.Biol.Chem. 224 649-662

CRANE, R. K., FORSTNER, G. and EICHHOLZ, 1965

Studies on the mechanism of intestinal absorption of sugars X. An effect of Na^+ concentration on the apparent Michaelis constants for intestinal sugar transport, in vitro.

Biochim.Biophys.Acta. 109 467-477

CRANE, R. K., MILLER, D., and BIHLER, I. (1961)

Restrictions on the possible mechanisms of intestinal active transport of sugars. In "Membrane transport and metabolism" Ed. A. Kleinzeller and A. Kotyk, pp.439-449.

Acad.Press. New York.

CROFFORD, O. B., JEANRENAUD, RENOLD, A. E. (1965)

Effect of insulin on the transport and metabolism of sorbitol by incubated rat epididymal adipose tissue.

Biochim.Biophys.Acta. 111 429-439

DAEMES, W. Th., WISSE, E. and BREDEROO, P. (1970)

Electron microscopy of the vacuolar apparatus.

In Lysosomes - a laboratory handbook. Ed. J. T. Dingle, North Holland Pub.Co., Amsterdam, London. 1972 pp.150-199

DALE, H. and BAUER (1928)

cited by L. L. Miller, 1973

DAMGAARD, S. E., LUNDQUIST, F., TONNESEN, K., HANSEN, F. V. and SESTOFT, L. (1973)

Metabolism of ethanol and fructose in the isolated perfused pig liver.

Eur.J.Biochem. 33: 87-97

DANIELLI, J. F. (1954)

The present position in the field of facilitated diffusion and selective active transport.

Proc.Symp.Colston.Res.Soc. 7 1-4

DANIELLI, J. F. and HARVEY, E. N. 1935

The tension at the interface of mackerel egg oil, with remarks on the nature of the cell surface.

J.Cell.Comp.Phusiol. 5: 483-486

DAVIES, J. (1955)

Permeability of rabbit placenta to glucose and fructose.

Amer.J.Physiol. 181 532-538

DAVIES, J. 1957

Differential permeability of the rabbit placenta to various sugars.

Am.J.Physiol. 188:21-26

DAVSON, H. and DANIELLI, J. F. 1943

"The permeability of Natural membranes. Camb. Univ. Press. Cambridge and New York.

DAVSON, H. and DANIELLI, J. F., 1952

"The permeavility of natural membranes"

2nd ed. Cambridge Univ.Press. Cambridge and N. York.

DAWES, G. S. and SHELLEY, H. J. 1968

In carbohydrate metabolism and its disorders.

Ed. F. Dickens, P. J. Randle and W. J. Whelan, Acad. Press. Lond. Vol. 2, p.87

DAWSON, A. C. and WIDDAS, W. F. 1964

Variations with temperature and pH of the parameters of glucose transfer across the erythrocyte membrane in the foetal guinea

pig.

J.Physiol. 172 107-122

DIXON, M. and WEBB, E. C. 1964

Enzymes. Chapter IV Enzyme kinetics p.54-166. Longmans, London

DRABKIN, D. L.

Spectrophotometric studies; crystallographic and optical properties of haemoglobin of man in comparison with those of other species.

J.Biol.Chem164: 703-723

D'SILVA, J. L. and NEIL, N. W. (1954)

The potassium, water and glycogen contents of the perfused rat liver.

J.Physiol. 124: 515-527

DURUISSEAU, J. P. and QUASTELL, J. H. (1959)

Effects of croton oil on sugar absorption by isolated surviving guinea pig intestine.

Proc.Soc. Exptl.Biol.Med. 100: 711-714

EATON, B. M., LEICHTWEISS, H. P., SHORT, A. H., and YUDILEVICH, D. L. (1978)

Transport of hexoses across the trophoblast in the dually perfused isolated placenta of the guinea-pig.

J.Physiol.(Lond.) 284:60-61P

EDWARDS, P. A. (1973a)

The inactivation by fluorodinitrobenzene of glucose transport across the human erythrocyte membrane. The effect of glucose inside or outside the cell.

Biochim.Biophys.Acta. 307: 415-518

Edwards, P. A. 1973b

Evidence for the carrier model of transport from the inhibition by N-ethylmaleimide of choline transport across the human red cell membrane.

Biochim.Biophys.Acta 311: 123-140

EISEMAN, B. Knipe, P., KOH, Y. NORMELL, L., McCALL, H. A. and ORLOFF, J. J., (1961)

Isolated liver perfusion in treatment of hepatic failure.

Arch.Surg.83: 356-361

ELY, D. A.

The placental transfer of hexoses and polyols in the guinea-pig as shown by umbilical perfusion of the placenta.

J.Physiol. 184: 255-271

EVANS, W. H.

Fractionation of liver plasma membranes prepared by zone centrifugation.

Bioch.J. 116: 833-842

EVANS, C. A. N., REYNOLDS, J. M., REYNOLDS, M. L., SAUNDERS, N. R. and SEGAL, M. B. (1974)

The development of a blood-brain barrier mechanism in foetal sheep.

J.Physiol. 238(2): 371-386

EXTON, J. H. and PARK, C. R. (1967)

Control of Gluconeogenesis in liver. 1. General features of gluconeogenesis in the perfused livers of rats.

J.Biol. Chem. 242(11): 2622-2636

FAUST, R. G., and PARPART, A. K. (1961)

Permeability studies on the red blood cell of the ground-hog,

Marmota monax.

J.Cell.Comp.Physiol. 57: 1-3

FINEAN, J. B. 1966

The molecular organisation of cell membranes.

Progr.Biophys.Molec.Biol. 16: 143-170

FINEAN, J. B., COLEMAN, R., and MICHELL, R. H. (1978)

Membranes and their cellular functions.

Blackwell Pub. Oxford, 1978

FINEAN, J. B. and ROBERTSON, J. D. 1958

Lipids and the structure of myelin.

Brit.Med.Bull., 14, 267-273

FISHER, R. B. and LINDSAY, D. B. (1956)

The action of insulin on the penetration of sugars into the perfused heart.

J.Physiol. 131: 526-541

FISHER, R. B. and PARSONS, D. S. (1953)

Glucose movement across the wall of the rat small intestine.

J.Physiol. 119: 210-223

FISHER, R. B. and PARSONS, D. S. (1953)

Galactose absorption from the surviving small intestine of the rat.

J.Physiol. 119: 224-232

FISHMAN, R. A. 1964

Carrier transport of glucose between blood and cerebrospinal fluid.

Am.J.Physiol. 206: 836-844

FORKER, E. L. 1968

Bile formation in guinea pigs: analysis with inert solutes of graded molecular radins.

Am.J.Physiol. 215(1): 56-62

FORKER, E. L. (1977)

Mechanisms of hepatic bile formation.

Ann.Rev.Physiol. 39: 323-347

FOX, C. F., CARTER, J. R. and KENNEDY, E. P., (1967)

Genetic control of the membrane protein component of the lactose transport system of Escherichia coli.

Proc.Nat.Acad.Sci.U.S.A., 57: 698-705

FOX, C. F. and KENNEDY, E. P. (1965)

Specific labelling and partial purification of the M protein, a component of the beta-galactoside transport system of Escherichia coli.

Proc.Nat.Acad.Sci.U.S.A., 54: 891-899

FRANCK, H. H. 1914

Agents producing glycosuria. 1. The identity of hydronaringenin and phloretin.

Beitr.Physiol. 1: 179-186, 1914 as abstracted in Chem.Abstr. 8: 3597

FRIDHANDLER, L. and QUASTEL, J. H. 1955

Absorption of sugars from isolated surviving intestinal.

Arch.Biochem.Biophys.56 412-423

FROMM, S. 1917

The smallest amounts of the substances phlorizin, phloretin, phloringlucuromic acid and phlorin belonging to the phlorhizin group which are capable of producing glycosuna.

Beitr.Physiol. 1: 287-312, 1917. as abstracted in Chem.Abstr. 14: 3724.

FRYE, L. D. and EDIDIN, M. (1970)
The rapid intermixing of cell surface antigens after formation of mouse-human heterokaryons.
J.Cell.Sci. 7:319-335

FUJITA, M. OHTA, H., KAWAI, K., MATSUI, H., AND NAKAS, M. (1972)
Differential isolation of microvillous and baso-lateral plasma membranes from intestinal mucosa: Mutually exclusive distribution of digestive enzymes and ouabain-sensitive ATPase.
B.B.A. 274:336-347

GARATTINI, S., GUAITANI, A., AND BARTOSEK, I. (1973)
Use of isolated perfused liver in the study of drug metabolism in isolated liver perfusion and its applications. Ed. I. Bartosek, A., Guaitani, L. L. Miller. Raven Press, New York, 1973, pp.225-234

GECK, P. (1971)
Eigenschaften eines asymmetrischen carrier modells fur den Zuckertransport am menschlichen erythro-zyten.
Biochim.Biophys.Acta. 241:462-472 cited by Wallach 1972

GEMMILL, C. L., AND HAMMAN L. Jr. (1941)
Effect of insulin on glycogen deposition and on glucose utilization by isolated muscles.
Bull.Johns Hopkins Hosp. 68:50-57

GERIG, J. T. 1974

Introductory organic chemistry.

Acad. Press, New York and London, Chapter 10.

Carbohydrates, pp.250-260

GEY, K. F. (1956)

The concentration of glucose in rat tissues.

Biochem.J. 64:145-150

GINSBURG, J. (1971)

Placental drug transfer.

Ann.Rev.Pharmacol. 11:387-408

GLASINOVIC, J. C., DUMONT, M., DUVAL, M., AND ERLINGER, S. (1975)

Hepatocellular uptake of bile acids in the dog: Evidence for a common carrier mediated transport system. An indicator dilution study.

Gastroent. 69: 973-981

GOMPERTS, B. D. 1977

The plasma membrane: models for structure and function. Acad. Press, Lond. New York

GORESKY, C. A. (1963)

A linear method for determining liver sinusoidal and extravascular volumes.

Am.J.Physiol. 204(4): 626-640

GORESKY, C. A. 1965

The nature of capillary exchange in the liver.

Can.Med.Ass.J. 92:517-522

GORESKY, C. A. AND BACH, G. G. 1970

Membrane transport and the hepatic circulation.

Ann.N.Y.Acad.Sci. 170:18-47

GORESKY, C. A., BACH, G. G., AND NADEAU, B. E. 1973
On the uptake of materials by the intact liver.
The transport and net removal of galactose.
J.Clin.Invest. 52:991-1009

GORLER, E. AND GRENDEL, F. (1925)
On bimolecular layers of lipoids on the chromocytes of the blood.
J.Exptl.Med. 1925, 41:439-443

GREEN, M. AND MILLER, L. L. (1960)
Protein catabolism and protein synthesis in perfused livers of normal and alloxan-diabetic rats.
J.Biol.Chem. 235(11):3202-3208

HASKOVEC, C. AND KOTYK, A. (1969)
Attempts at purifying the galactose carrier from galactose-induced baker's yeast.
Eur.J.Biochem. 9:343-347

HARDCASTLE, J. D. AND RITCHIE, H. D. (1968)
The liver in shock: a comparison of some techniques used currently in therapy.
Br.J.Surg. 55:365-368

HARVEY, E. N. (1931)
The tension at the surface of marine eggs especially those of the sea urchin.
Bull.Marine Biol.Lab. 61:273-279

HARVEY, E. N. (1943)
Forward to: Permeability of Natural Membranes by Davson, H., and Danielli, J. F. Cambridge Univ. Press. Cambridge 1943

HARVEY, E. N. AND DANIELLY, J. F. 1938

Properties of the cell surface.

Biol.Rev.Cambridge Phil.Soc. 13:319-322

HAWORTH (1927)

cited by Gerig 1974

HEDIN, S. G. (1897)

Über die permeabilität der blutkörperchen.

Arch.Ges.Physiol. 68:229 cited by Cereijido, M. and Rotundo, C. A. 1970.

HEDON, M. E. 1900

Sur la resorption intestinale des sucres en solutions isotoniques.

Compt.rend.soc.biol. 52: 87. cited by R. K. Crane 1960

HELMREICH, E. AND CORI, C. F. (1956)

Ciba found. Colloq. Endocrinol. Proc. 9:227 cited by Lefevre 1972

HEMS, R., ROSS, B. D., BERRY, M. N., AND KREBS, H. A. 1966

Gluconeogenesis in the perfused rat liver.

Biochem.J. 101:284-292

HENDERSON, M. J. (1964)

Uptake of glucose into cells and the role of insulin in glucose transport.

Cand.J.Biochem. 42:933-944

HERS, H. G. (1976)

The control of glycogen metabolism in the liver.

Ann.Rev.Biochem. 45:167-189

HEPTENYI, G. AND ARBUS, G. S. (1962)

The effect of insulin on the distribution of glucose between the blood plasma and the liver.

J.Gen.Physiol. 45:1049-1063

HILL, T. L. AND KENDEM, O. 1966

Studies in irreversible thermodynamics. 3. Models for steady state, and active transport across membranes.

J.Theoret.Biol. 10:399-441

HLAD, C. J., ELRICK, H. AND WITTEN, T. A. (1956)

Studies in the kinetics of glucose utilisation.

J.Clin.Invest. 35:1139-1149

HOBER, R. (1899)

Uber resorption in dunndarm.

Arch.ges Physiol.Pfluger's 74:246.1899 cited by Crane 1960

HOOPER, R. H. 1975

The hepatocellular uptake of monosaccharides in conscious sheep.

Ph.D. thesis, University of Nottingham 1975

HOOPER, R. H. AND SHORT, A. H. 1975

Hepatic uptake of glucose in well-fed conscious sheep.

J.Physiol. 244:

HOOPER, R. H. AND SHORT, A. H. (1977)

The hepatocellular uptake of glucose, galactose and fructose in conscious sheep.

J.Physiol.(Lond.) 264:523-539

HUGGETT, A. ST. G., WARREN, F. L. AND WARREN, N. Y. (1951)

The origin of blood fructose in the foetal sheep.

J.Physiol. 113: 258-295

IBU, J. O., MACDONALD, I. A., AND SHORT, A. H. 1978

A technique for measuring first pass extraction in the rat perfused liver.

Brit.J.Pharmacol. 64:469P

IBU, J. O. AND SHORT, A. H. 1979(a)

Competition among hexoses for hepatocellular uptake in the perfused liver of rat.

J.Physiol. 295:18-19P

IBU, J. O. AND SHORT, A. H. 1979(b)

Disaccharide competition with hexose transport in the rat perfused liver.

J.Physiol. 296:31P

ISBELL, H. S. AND PIGMAN, W. W. (1938)

Pyranose-furanose interconversions with reference to the mutarotations of galactose, levulose, lactulose and turanose.

J.Res.Natl.Bur.Stand. 20:773-798 cited in Chem. Abstr. 32:7022

ISBELL, H. S. AND WADE, C. W. R. (1967)

Deuterium isotope effects in α-β-pyranose and in pyranose-furanose interconversions.

J.Res.Nat.Bur.Stand. A72(2), 137-148 cited in Chem. Abstr. 67: 108858H

ISSELBAICHER,K. J. ANDERSON, E. P., KURAHASHI, K., AND KAICKAR, H. M. (1956)

Congenital galactosemia, single enzymatic block in galactose metabolism.
Science 123:635-636

JACOBJ, C. (1890)
Arch.Exp.Pharm.Path. 26:388 cited by L. L. Miller, 1973

JACOBJ, C. (1895)
Arch.Exp.Pharm.Path.36: 330 cited by L. L. Miller 1973

JOHN, D. W. AND MILLER, L. L. (1969)
Regulation of net biosynthesis of serum albumin and acute phase plasma proteins. 1. Induction of enhanced net synthesis of fibrinogen, α-acid glycoprotein, α_2-(acute phase)-globulin, and haptoglobin by amino acids and hormones during perfusion of the isolated normal rat liver.
J.Biol.Chem. 244(22):6134-6142

JUNG, C. Y. (1971)
Evidence of high stability of the glucose transport carrier function in human red cell ghosts extensively washed in various media.
Arch.Biochem.Biophys. 146:215-226

KALCKAR, H. M., ANDERSON, E. P., ISSELBAKHER,K. J. (1956)
Galactosemia, congenital defect in nucleotide transferase.
Biochim.Biophys.Acta. 20: 262-268

KARVONEN, M. J. AND RAIHA, N. (1954)
Permeability of placenta of guinea pig to glucose and fructose.
Acta.Physiol.Scand. 31:194-202

KESTENS, P. J. AND LAMBOTTE, L. 1973
Surgical preparation of the liver for perfusion and its applications. Ed. I. Bartosek, A. Guaitani and L. L. Miller, p.53-61. Raven Press, New York

KIMELBERG, H. K. AND PAPAHADJOPOULOS, D. 1972
Phospholipid requirements for (Na^+ + K^+) - ATPase activity: head group specificity and fatty acid fluidity.
Biochim.Biophys.Acta. 282:277-292

KIMMICH, G. A. AND CARTER,-SU, C. 1978
Membrane potentials and the energetics of intestinal Na^+-dependent transport systems.
Am.J.Physiol. 235(3): C73-C81 or
Am.J.Physiol.Cell Physiol. 4(2): C73-C81

KLEINZELLER, A., KOLINSKA, J. AND BENES, I. 1967a
Transport of glucose and galactose in kidney cortex cells.
Biochem.J. 104: 843-851

KLEINZELLER, A., KELINSKA, J. AND BENES, I. 1967b
Transport of monosaccharides in kidney cortex cells
Biochem.J. 104:852-860

KOLBER, A. R. AND STEIN, W. D. (1966)
Identification of a component of a transport "carrier" system: isolation of the permease expres-

sion of the LAC operon of Escherichia coli.
Nature, Lond. 209:691-694

KOLBER, A. R. AND STEIN, W. D. (1967)
The isolation of a membrane-bound protein associated with the beta-galactoside-permease of Echerichia coli.
Curr.Mod. Biol. 1:244-248

KONO, T. AND COLOWICK, S. P. 1961
Isolation of skeletal muscle cell membrane and some of its properties.
Arch.Biochem.Biophys. 93: 520-533

KORN, E. D. 1969
In theoretical and experimental Biophysics, A. Cole ed. New York, Marcel Dekker 1969, p.1

KORNBERG, R. D. AND MCCONNEL, H.M. 1971
Inside-outside transitions of phosphlolipds in vesicle membranes.
Biochem. 10: 1111-1117

KOTYK, A. (1970)
Present situation in the identification of membrane transport proteins in single cells. In Membranes - structure and function. FEBS symposium Vol. 20: pp99-107. Ed. J. R. Villanueve and F. Ponz. Acad. Press. London.

KOZAWA, S. 1914
Beitrage zum arteigenem verhalten der roten blut-korperchen. III. Artchiffenzen in der durchlassig-keit der roten blutkorperchen.
Biochem. Z 60: 231-256

KRANE, S. M. AND CRANE, R. K. 1959
The accumulation of D-galactose against a concentration gradient by slices of rabbit kidney cortex.
J.Biol.Chem. 234:211-216

KREBS, H. A. (1972)
Some aspects of the regulation of fuel supply in omnivorous animals.
Adv. Enzyme Regul. 10:397-420

KUNKEL, AND EISENMENGER (1949)
cited by Bloxam 1971

KUO, J. F., KILL, U. I. K., AND HOLMLUND, C. E. (1967)
Comparison of the effects of Bacillus subtilis protease, type 8 (subtilopeptidase A), and insulin on isolated adipose cells.
J.Biol.Chem. 242: 3659-3664

KVETINA, J., SIMKOVA, M., CITTA, M., AND DEMLF. (1973)
Relation between biotransformation of drugs in isolated perfused liver and their supply in perfusion liquid. In isolated liver perfusion and its applications. Ed. I. Bartosek, A. Guaitani, L. L. Miller. Raven Press, New York, 1973. pp.235-240

LACKO, L. AND BURGER, M. 1962
Interaction of some disaccharides with the carrier system for aldoses in erythrocytes.
Biochem.J. 83:622-625

LAKSHMINARAYANAIAH, N. 1969
Transport phenomena in membranes.
Acad.Press. New York, 1969

LEE, A. G. 1974

Functional properties of biological membranes. A physical-chemical approach.

Prog.Biophys.Mol.Biol. 29(1):3-56

LEE, A. G., BIRDSALL, N. J. M., METCALF, J. C. 1973

Measurement of fast lateral diffusion of lipids in vesicles and in biological membranes by ^{1}H nuclear magnetic resonance.

Biochem. 12:1650-1659

LEFEVRE, P. G. 1954

The evidence for active transport of monosaccharides across the red cell membrane.

Symp.Soc.Exptl.Biol. 8:118-136

LEFEVRE, P. G. 1961

Sugar transport in the red blood cell: Structure-activity relationships in substrates and antagonists.

Pharmacol. Rev. 13:39-70

LEFEVRE, P. G. 1972

Transport of carbohydrates by animal cells. In Metabolic Pathways Vol. VI - Metabolic transport. Ed. L. E. Hokin, pp.385-454. Acad. Press, New York and London. 1972

LEFEVRE, P. G. AND MARSHALL, J. K. 1958

Conformational specificity in a biological sugar transport system.

Am.J.Physiol. 194: 333-337

LEFEVRE, P. G. AND MCGINNISS, G. F. (1960)

Tracer Exchange vs Net Uptake of glucose through human red cell surface. New evidence for carrier

mediated diffusion.
J.Gen.Physiol. 44:87-103

LENARD, J. AND SINGER, S. J. (1966)
Protein conformation in cell membrane preparations as studied by optical rotary dispersion and circular dichroism.
Proc.Nat.Acad.Sci. 56: 1828-1835

LEVINE, R. AND GOLDSTEIN, M. S. (1955)
On mechanism of action of insulin.
Recent.Progr.Horm.Res. 11:343-375

LIDGARD, G. P., AND JONES, M. N. (1975)
D-glucose permeability of black lipid membranes modified by human erythrocyte membrane fractions.
J.Memb.Biol. 21:1-10

LINDEN, C. D., WRIGHT K. L., MCCONNEL, H. M. AND FOX, C. F. (1973)
Lateral phase separations in membrane lipids and the mechanism of sugar transport in Echerichia coli.
Proc.Nat.Acad.Sci.U.S.A. 70:2271-2275

LIPPERT, J. L. AND PETICOLAS, W. L. (1971)
Laser raman investigation of the effect of cholesterol on conformational changes in Dipalmitoyl lecithin multilayers.
Proc.Nat.Acad.Sci.U.S.A. 68:(7):1572-1576

LUDWIG, C. AND SCHMIDT. A. (1868)
Leipzig Ber. 20:12 cited by Miller 1973

LUNDQUIST, F. (1969)
cited by Damgaard et al 1973

MACDONALD, I. AND BRAITHWAITE, D. M. (1964)
The influence of dietary carbohydrates on the lipid pattern in serum and in adipose tissue.
Clin.Sci. 27:23-25

MACEY, R. I. 1979
Transport of water and non-electrolytes across red cell membranes. In Membrane transport in biology, Eds. Giebisch, G., Tosleson, D. C., and Ussing, H. H. Vol. 2, pp.1-57. Springer-Verlag Berlin, Heidelberg, New York, 1979

MACHTIGER, N. A. AND FOX, C. F. (1973)
Biochemistry of bacterial membranes.
Ann.Rev.Biochem. 42:575-600

MADDY, A. H. 1966
The chemical organization of the plasma membrane of animal cells. In
Int.Rev.Cytol. (Eds. Bourne, G. H. and Danielli, J. F.) Acad. Press. New York.

MARKS, V. 1971
Fructose as sweetening agent in normal and diabetic nutrition.
Acta.Diabet.Lac. 8:335-346

MARX, S. J., FEDEK, S. A., AURBACH, G. D. 1973
Preparation and characterization of a hormone-responsive renal plasma membrane fraction.
J.Biol.Chem.247:6913-6918

MAUNSBACH, A. B. (1966a)
The influence of different fixatives and fixation

methods on the ultrastructure of rad kidney proximal tubule cells. I. Comparison of different perfusion fixation methods and of glutaraldehyde, formalderhyde and osmium tetroxide.
J.Ultrastruct.Res. 15:242-282

MAUNSBACH, A. B. (1966b)
The infouence of different fixatives and fixation methods on the ultrastructure of rat kidney proximal tubule cells. II. Effects of varying osmolality, ionic strength buffer system and fixative concentration on glutaraldehyde solutions.
J.Ultrastruct.Res. 15:283-309

MCELHANEY, R. N. (1974)
The effect of membrane-lipid phase transitions on membrane structure and on the growth of Acholesplasma aidlawii.
J.Supramolecular Structure 2:617-628

MCINTYRE, N., TURNER, D. S. AND HOLDSWORTH, C. D. (1970)
The role of the portal circulation in glucose and fructose tolerance.
Diabetologica 6:593-596

MCNUTT, J. S., HERSHBERG, B. H. AND WEINSTEIN, R. S. 1970
Further observations on the occurance of hexuses in benign and malignant human cervical epitherlium.
J.Cell Biol. 51:805-825

MENDELOFF, A. I., AND WEICHSELBAUM, T. E. (1953)
Role of the human liver in the assimilation of intravenously administered fructose.
Metabolism, 2:450-458

MEIER, P. AND ZIERLER, K. L. (1954)
On theory of indicator-dilution method for measurement of blood flow and volume.
J.Appl.Physiol. 6:731-744

MELCHIOR, D. L. AND CZECH, M. P. (1979)
Sensitivity of the Adipocyte D-glucose transport system to membrane fluidity in reconstituted vesicles.
J.Biol.Chem. 254:8744-8747

MILLER, D. M. (1964)
Sugar uptake as a function of cell volume in human erythrocytes.
J.Physiol. 170:219-225

MILLER, D. M. (1969)
In red cell membrane structure and functions. Ed. G. A. Jamieson and T. J. Greenwalt. Lippincott Co. Philadelphia and Toronto, p.246

MILLER, L. L. AND BLY, C. G. 1949
cited by Miller 1973

MILLER, L. L. (1961)
Some direct actions of insulin, glucagon, and hydrocortisone on the isolated perfused rat liver.
Recent.Prog. Hormone Res. 17:539-568

MILLER, L. L. (1965)
Direct actions of insulin, glucagon, and epinephrine on the isolated perfused rat liver.
Fed.Proc. 24:737-744

MILLER, L. L. (1973)
History of isolated liver perfusion and some still

unsolved problems. In Isolated Liver Perfusion and its applications. Eds. I. Bartosek, A. Guaitani and L. L. Miller. Raven Press. New York. 1973, pp.1-9

MILLER, L. L., BLY, C. G., WATSON, M. L., BALE, W. F. (1951)

The dominant role of the liver in plasma protein synthesis. A direct study of the isolated perfused rat liver with the aid of lysine-E-C^{14}. J.Expt.Med. 94:431-453

MILLER, L. L. BURKE, W. T., AND HAFT, D. E. (1955)

Interrelations in amino acid and carbohydrate metabolism. Studies of the nitrogen sparing action of carbohydrate with the isolated perfused rat liver.

Fed. Proc. 14:707-716

MILLER, L. L., BURKE, W. T. AND HAFT, D. E. (1956)

In some aspects of amino acid. Supplementation. Ed. W.H. Cole, Rutgers Univ. Press. New Brunswick New Jersey. 1956

MILLER, L. L. AND GRIFFIN, E. E. 1973

Some aspects of perfusate pH on amino acid and protein metabolism in the isolated perfused rat liver. In Isolated liver perfusion and its applications, pp.139-145. Eds. I. Bartosek, A. Guaitani and L. L. Miller. Raven Press, New York

MILLER, L. L. AND JOHN D. W. (1970)

Nutritional, Hormonal, and Temporal factors regula-

ting net plasma protein biosynthesis in the isolated perfused rat liver. 1. Effect of feeding or fasting liver donors and of supplementation with amino acids, insulin, cortisol and growth hormone. In. Plasma protein metabolism - Regulation of Synthesis, distribution and degradation, pp.208-223. Eds. Marcus A. Rothschild and Thomas Waldmann, Acad. Press N.Y., London, 1970

MITCHELL, C. D., MITCHELL, W. B. AND HANAHAN, D. J. (1965)
Enzyme and haemoglobin retention in human erythrocyte stroma.
Biochim.Biophys.Acta. 104:348-358

MOOR, H. 1971
Recent progress in the freeze-etching technique.
Phil.Trans.Roy.Soc.B. 261:121-131

MORGAN, H. E., POST, R. L. AND PARK, C. R. (1961)
Glucose transport and phosphorylation in the perfused heart of normal and diabetic rats.
In Membrane transport and metabolism. Eds. A. Kleinzeller and A. Kotyk, Symposia Csav. 1961, pp.433-437

MORGAN, H. E., RANDLE, P. J. AND REGEN, D. M. (1959)
Regulation of glucose uptake by muscle. 3. The effects of insulin, anoxia, salicylate and 2-4-dinitrophenol on membrane transport and intracellular phosphorylation of glucose in the isolated rat heart.
Biochem.J. 73:573-579

MORGAN, H. E., REGEN, D. M., AND PARK, C. R. (1964)
Identification of a mobile carrier-mediated sugar transport system in muscle.
J.Biol.Chem. 239:369-374

MORTIMORE, G. E. 1961
Effect of insulin on potassium transfer in isolated rat liver.
Am.J.Physiol. 200(6):1315-1319

NAFTALIN, R. J. 1970
A model for sugar transport across red cell membranes without carriers.
Biochim.Biophys.Acta. 211:65-78

NAFTALIN, R. J. AND HOLMAN, G. D. 1977
Transport of sugars in human red cells.
In Membrane transport in red cells. Eds. J. C. Ellory and V. L. Lew. Acad.Press. London 1977, pp.257-300

NELSON, G. J. 1967
Lipid composition of erythrocytes in various mammalian species.
Biochim.Biophys.Acta. 144:221-232

NEVILLE, D. M. (1968)
Problems in quantitating surface membrane components in normal and pathologic cells and tissues. In Cell surface and malignancy fugarty International Center proceedings, No. 24, Sept. 1974. Ed. P. T. Mora.
DHEN Pub. pp.5-16

NEVILLE, D. M. Jr. 1975

Isolation of cell surface membrane fractions from mammalian cells and organs.

In Membrane biology Vol. 3, Ed. E. Korn, pp.97-145. Plenum Publishing Corp. New York.

NEWMAN, H. A. I., GORDESKY, S. E., HOPPEL, S. E. AND COOPER, C. 1968

The action of digifonin on rat liver mitochondria. Phospholipid content.

Biochem.J. 107:381-385

NEWSHOLME, E. A. AND START, C. (1973)

Regulation in metabolism, Wiley - Interscience, London.

NICKSON, J. K. AND JONES, M. N. (1977)

Reconstitution of the monosaccharide transport system of the human erythrocyte membrane.

Biochem.Soc.Trans. 5:147-149

NIELSEN, N. A. (1933)

Skand.Arch.Physiol. 66:1

cited by L. L. Miller 1973

NIKKILA, E. A., AND OJALA, K. (1965)

Induction of hyperglyceridaemia by fructose in the rat.

Life Sci. 4:937-943

NIXON, D. A. 1963

The transplacental passage of fructose, urea and mesomositol in the direction from foetus to mother, as demonstrated by perfusion studies in the sheep.

J.Physiol.(Lond.) 166:441-444

NOVIKOFF, A. B. (1959)

In Subcellular particles. Ed. T. Hayashi. Ronald Press. New York. 1959 p.11.

O'BRIEN, J. S. (1970)

Molecular Anatomy of a membrane. In Membranes: structure and function. Eds. J. R. Villanueva and F. Ponz. Acad. Press London and New York. FEBS symposium Vol. 20, 1970, pp.33-38

OLDENDORF, W. H. (1971)

Brain uptake of radiolabelled amino acids, amines, and hexoses after arterial injections.

Amer.J.Physiol. 221(6):1629-1639

ORGEL, L. E. (1959)

"The hydrogen bond", in Biophysical Science. Ed. Oncley, J. L. Wiley Pub. New York. 1959

OSTERHOUT, W. J. V. 1930

The accumulation of electrolytes. II Suggestions as to the nature of accumulation in Valonia.

J.Gen.Physiol. 14:285-300

OVERATH, P., SCHAIRER, H. U., AND STEFFEL, W. (1970)

Correlation of in vivo and in vitro phase transitions of membrane lipids in Escherichra coli.

Proc.Nat.Acad.Sci.U.S. 67:606

OVERATH, P. AND TRAUBLE, H. (1973)

Phase transitions in cells, membranes and lipids of Escherichia coli. Detection by fluorescent probes, light scattering and dilatometry.

Biochem. 12:2625-2634

OVERTON, E. (1899) cited by Wilbrandt and Rosenberg, 1961.

Uber die allgemeinen osmotischen eigenschaften der zelle, ihre vermutlischen ursachen und ihre bedeutung fur die physiologie

Vjschr.Naturf.Ges.44:88-135

PAPPER, S. SAXON, L., PROUT, T. E. AND ALPERT, H. C. 1956

The effects of cortisone on the fructose and glucose tolerance tests of normal men.

J.Lab.Clin.Med. 48:13-19

PARDEE, A. B. (1966)

Purification and properties of a sulphate-binding protein from salmonella typhimurium.

J.Biol.Chem. 241:5886-5892

PARDEE, A. B. (1967)

Crystallization of a sulphate-binding protein (permease) from salmonella typhimurium.

Science, N.Y. 156:1627-1628

PARDEE, A. B. (1968)

Membrane transport proteins. Proteins that appear to be parts of membrane transport systems are being isolated and characterized.

Science, N.Y. 162:632-637

PARDEE, A. B. AND PRESTIDGE, L. S. (1966)

Cell-free activity of a sulphate binding site involved in active transport.

Proc.Natn.Acad.Sci.U.S.A. 55:189-191

PARDEE, A. B., PRESTIDGE, L. S., WHIPPLE, M. D. AND DREYFUSS, J. (1966)

A binding site for sulphate and its relation to sulphate transport into salmonella typhimurium.

J.Biol.Chem. 241:3962-3969

PARDRIDGE, W. M., JEFFERSON, L. S. (1975)

Liver uptake of amino acids and carbohydrates during a single circulatory passage.

Amer.J.Physiol. 228(4):1155-1161

PARISI, R. (1969)

Management of chronic renal failure by isolated jejunal loop perfusion.

Brit.J.Urol. 41:603-604

PARK, C. R., BORNSTEIN, J. AND POST, R. L. (1955)

Effect of insulin on free glucose content in rat diaphragm in vitro.

Am.J.Physiol. 182:12-16

PARK, C. R., CROFFORD, O. B., AND KONO, T. (1968)

Mediated (non-active) transport of glucose in mammalian cells and its regulation.

J.Gen.Physiol. 2965-3185

PARK, C. R. AND JOHNSON, L. H. (1955)

Effect of insulin on transport of glucose and galactose into cells of rat muscle and brain.

Am.J.Physiol. 182:17-23

PARK, C. R., MORGAN, H. E., HENDERSON, M. J., REGEN, D. M. CADEMAS, E., AND POST, R. L. (1961)

Hormones and organic metabolism. V. the regulation

of glucose uptake in muscle as studied in the perfused rat heart.
Recent.Progr.Hormone Res. 17:493-538

PAULING, L. (1960)
The nature of the chemical bond. Cornell Univ. Press, Ithaca, New York, 1960

PEREIRA, J. N. AND JANGAARD, N. O. (1971)
Different rates of glucose and fructose metabolism in rat liver tissue in vitro.
Metabolism, 20:392-400

PHUTRAKUL, S. AND JONES, M. N. (1979)
The permeability of bilayer lipid membranes on the incorporation of erythrocyte membrane extracts and the identification of the monosaccharide transport proteins.
Biochim.Biophys.Acta M67(2):188-200

PIMENTEL, G. C. AND MCCLELLAN, A. L. (1960)
The hydrogen bond. Reinhold Pub. New York, 1960

PONDER, E. (1955)
Red cell structure and its breakdown "Protoplasmatologia", Vol. 10, Part 2, Springer, Vienna.

POWIS, G. (1970)
Perfusion of rat's liver with blood: transmitter overflows and gluconeogenesis.
Proc.Roy.Soc. B 174:503-515

RANDLE, P. J. (1961)
Monosaccharide transport in muscle and its regulation.

In: Membrane transport and metabolism. Eds. A. Kleinzeller and A. Kotyk. Symposia Csav. 1961 pp.431-433

RANDLE, P. J. AND SMITH, G. H. (1958).
Regulation of glucose uptake by muscle. 1. The effects of insulin, anaerobiosis and cell poisons on the uptake of glucose and release of potassium by isolated rat diaphragm.
Biochem.J. 70:490-500

RAZIN, S., ARGAMAN, M. AND AVIGAN, J. 1963
Chemical composition of mycoplasma cells and membranes
J.Gen.Microbiol. 33:477-487

REGEN, D. M. AND MORGAN, H. E. 1964
Studies of the glucose-transport system in the rabbit erythrocyte.
Biochim.Biophys.Acta 79:151-166

RENKIN, E. M. (1955)
Effects of blood flow on diffusion kinetics in isolated, perfused hindlegs of cats; double circulation hypethesis.
Am.J.Physiol. 183:125-136

RENKIN, E. M. (1959)
Transport of potassium-42 from blood to tissue in isolated mammalian skeletal muscles
Am.J.Physiol. 197:1205-1210

RICE, P. A., ROURKE, J. E., NESBITT, EL L. (1976)
In vitro perfusion studies of the human placenta.

IV. Some characteristics of the glucose transport system in the human placenta.
Gynecol.Invest. 7:213-221

RIFKIND, B. M., LAWSON, D. H. AND GALE, M. (1966)
Effect of short-term sucrose restriction on serum-lipid levels
Lancet 2:1379-1381

RIGGS, T. R., WALKER, L. M., CHRISTENSEN, H. N. 1958
Potassium migration and amino acid transport
J.Biol.Chem. 233:1497-1484

RITCHIE, H. D. AND HARDCASTLE, J. D. (1973)
Isolated organ perfusion.
Crosby Lockwood, London, 1973

ROBERTSON, J. D. (1960)
The molecular structure and contact relationship of cell membranes
Prog.Biophys. 10:343-347

ROBERTSON, J. D. 1964
Unit membranes: A review with recent new studies of experimental alterations and a new subunit structure in synaptic membranes.
In: "Cellular membranes in development" (M. Locke ed.), pp.1-81. Acad. Press New York

ROSECAN, M., DAUGHADAY, W. H. 1954
Comparison of insulin treatment with and without added carbohydrate in human diabetic ketosis
J.Clin.Invest. 1954 33:49-56

ROSENBAUM, J. L. (1972)

The use of resin hemoperfusion in the treatment of acute intoxication

Clin.Toxicol. 5:331-335

ROSENBERG, T. AND WILBRANDT, W. 1957

Uphill transport induced by counterflow

J.Gen.Physiol. 41:289-296

ROSS, B. D. 1972

Perfusion techniques in biochemistry -a laboratory manual in the use of isolated perfused organs in biochemical experimentation.

Clarendon Press, Oxford. 1972

ROSS, B. D., HEMS, R., FREEDLAND, R. A., KREBS, H. A. 1967

Carbohydrate metabolism of the perfused rat liver

Biochem.J. 105:869-875

ROTHFIELD, L. I. AND ROMEO, D. (1971)

In: Structure and Function of Biological Membranes. Rothfield, L. I. ed. New York, N.Y., Acad. Press, p.251

ROUILLER, C. (1969)

The liver - morphology, biochemistry, physiology. Vol. 2, Acad. Press, New York and London, 1969

SABATINI, D. D., BENSCH, K., AND BANNETT, R. J. (1963)

Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation.

J.Cell.Bio. 17: 19-58

SALTON, M. R. J. AND FREER, J. H. 1965

Composition of the membranes isolated from several gram-positive bacteria.

Biochim.Biophys.Acta. 107:531-538

SCHIMASSEK, H. 1962

Perfusion of isolated rat liver with a semisynthetic medium and control of liver function

Life Sci. 11:629-634, 1962

SACKS, J. AND BAKSHY, S. (1957)

Insulin and tissue distribution of pentose in nephrectomized cats.

Am.J.Physiol. 189:339-342

SCHIMASSEK, H. 1962

Regulation of lactate and pyruvate levels in perfusion medium by the isolated rat liver

Life Sci. 11:635-638

SCHMITT, F. O., BEAR, R. S. AND PALMER, K. J. (1941)

X-ray diffraction studies on structure of nerve myelin sheath

J.Cell.Comp.Physiol. 18:31-42

SCHMITT, F. O., BEAR, R. S. AND PONDER, E. 1936

Optical properties of the red cell membrane

J.Cell.Comp.Physiol. 9: 89-92

SCHRODER, H. LEICHTWEISS, H. P. AND MADEE, W. 1975

The transport of D-glucose, L-glucose and D-mannose across the isolated guinea pig placenta

Pfluegers Arch. 356:267-275

SCHULTZ, S. G. AND CURRAN, P. F. 1970

Coupled transport of socium and organic solutes.

Physiol.Rev. 1970 50:637-718

SELWYN, M. J. AND DAWSON, A. P. (1977)
Model membranes and transport systems
Biochem.Soc.Trans. 5: 1621-1629

SEN, A. K. AND WIDDAS, W. F. (1962a)
Determination of the temp. and pH dependence of glucose transfer across the human erythrocyte membrane measured by glucose exit
J.Physiol. 160:392-403

SEN, A. K. AND WIDDAS, W. F. (1962b)
Variations of the parameters of glucose transfer across the human erythrocyte membrane in the presence of inhibitors of transfer
J.Physiol. 160:404-416

SERROU, B. C., SOLASSOL, C., MEISS, L., GELIS, C, AND ROMIEU, C. 1973
Lysosomal enzymes in liver preservation.
In: Isolated liver perfusion and its applications.
Eds. I. Bartosek, A. Guaitani and L. L. Miller.
Raven Press, New York, 1973, pp.127-134

SESTOFT, L. 1974
Regulation of fructose metabolism in the perfused rat liver. Interrelation with inorganic phosphate, glucose, ketone body and ethan of metabolism
Biochim. Biophys. Acta 343: 1-16

SESTOFT, L. AND FLERON, P. 1974
Determination of the kinetic constants of fructose transport and phosphorylation in the perfused rat liver.

Biochim.Biophys.Acta 345:27-38

SHANNON, J. A. AND FISHER, S. 1939

The renal tubular reabsorption of glucose in the normal dog

Am.J.Physiol. 122:765-771

SHELLEY, H. J. (1979)

Transfer of carbohydrates. In Placental transfer, eds. Chamberlain, G. V. P. and Wilkinson, A. W. Pitman Medical, London --.118-141

SHORT, A. H. (1980)

Personal communication

SIMONDS, J. P. AND BRANDES, W. W. (1929)

The effect of peptone on the hepatic veins in the dog

J.Pharmacol. 35:165-169

SINGER, S. J. (1971)

A fluid lipid-globular protein mosaic model of membrane structure

Ann.N.Y.Acad.Sci. 195:16-23

SINGER, S. J. AND NICOLSON, G. L. (1972)

The fluid mosaic model of the structure of cell membranes

Science 175:720-731

SINGER, S. J. (1974)

The molecular organization of membranes

Ann.Rev.Biochem. 43:805-833

SJOSTRAND, F. S. (1967)

Electron microscopy of cells and tissues.

Acad.Press, New York and London.

SOSKIN, S., ESSEX, H. E. HERRICK, J. P. AND MANN, F. C. (1938)

The mechanism of regulation of the blood sugar by the liver.

Am.J.Physiol. 124:558-567

SOSKIN, S. AND LEVINE, R. (1946)

Carbohydrate metabolism

Chicago Univ. Press, Chicago, 1946

STECK, T. L. (1972)

In: Membrane Research. Ed. C. F. Fox, Acad. Press New York, pp.71-93

STECK, T. L. (1974)

The organization of proteins in the human red blood cell membrane. A review.

J.Cell.Biol. 62:1-19

STEIN, W. D. 1956

The permeability of erythrocyte ghosts

Exptl.Cell.Res. 11:232-234

STEIN, W. D. (1964)

A procedure which labels the active centre of the glucose transport system of the human erythrocyte.

IN: The Structure and activity of enzymes. Ed. T. W. Goodwin, B. S. Hartley, and J. I. Harris.

pp.133-137. Acad. Press, New York

STEIN, W. D. 1967

The movement of molecules across cell membranes.

Acad. Press, New York, pp. 126-176

J.Physiol. 100:432-458

TSUKAGOSHI, N. AND FOX, C. F. (1973)

Transport system assembly and the mobility of membrane lipids in Escherichia coli

Biochem 12:2816-2822

TYGSTRUP, N. AND WINKLER, K. (1954)

Kinetics of galactose elimination

Acta Physiol. Scand. 32:354-362

TYGSTRUP, N. AND K. WINKLER 1958

Galactose blood clearance as a measure of hepatic blood flow

Clin.Sci. 17:1-9

ULRICH (1934)

Planta 23: 146

cited by Davson, H. and Danielli, J. F. (1952)

VANDENHEUVEL, F. A. (1965)

Structural studies of biological membranes. The structure of myelin

Ann.N.Y.Acad.Sci. 122:57

VERZAR, F. AND MCDOUGAL, E. J. (1936)

Absorption from the intestine

Longmans, London, 1936

VOGEL, G., LAUTERBACH, F. AND KROGER, W. 1965

Die bedeutung des natriums fur die renalen transporte von glucose und para amino hippuraaure

Arch.Ged.Physiol. 283:151-159

VON FREY M., AND GRUBER, M. (1885)

Arch.Physiol. 9:519

cited by L. L. Miller 1973

WALKER, D. G. (1960)

The transmission of sugars across the goat placenta

J.Biochem. 74:287

WALLACH, D. F. H. 1968

Cellular membranes and tumour behaviour: a new hypothesis

Proc.Natl.Acad.Sci. (U.S.A.) 61:868-874

WALLACH, D. F. H. 1972

The plasma membrane: Dynamic perspectives, genetics and pathology. Springer-Verlag New York. The English Universities Press Ltd. London

WALLACH, D. F. H. AND ZAHLER, P. H. (1966)

Protein conformations in cellular membranes.

Proc. Nat.Acad.Sci. 56:1552-1559

WATKINS, W. M. 1966

Blood group substances

Science, 152:172-181

WEBER, M., BIRCHER, J., STREBEL, H., HAECKI, W., SCHOLL, E. AND PRESIG, R. (1973)

Herterologons and homologous pig liver perfusion: a functional comparison. In:Isolated liver perfusion and its applications. Eds. I. Bartosek, A. Guaitani, L. L. Miller. Raven Press, New York, 1973, pp.95-99

WEICHSELBAUM, T. E., MARGRAF, H. W. AND ELMAN, R. (1953)
Metabolism of intreavenously infused fructose in man
Metabolism 2:434-449

WHITTAM, R. (1964)
Transport and diffusion in red blood cells. Monographs of the Physiological Society No. 13. Edward Arnold Ltd., London, p.152

WIDDAS, W. F. 1952
Inability of diffusion to account for placental glucose transfer in the sheep and consideration of the kinetics of a possible carrier transfer.
J.Physiol.(Lond.) 118:23-39

WIDDAS, W. F. 1954
Facilitated transfer of hexoses across the human erythrocyte membrane.
J.Physiol.(Lond.) 125:63-180

WIDDAS, W. F. 1955
Hexose permeability of foetal erythrocytes
J.Physiol. 127:318-327

WILBRANDT, W. 1950
Permeabilitatsprobleme
Arch.Exp.Pathol.Pharmakol. 212:9-31
cited by Lefevre 1972

WILBRANDT, W., FREI, S. AND ROSENBERG, T. 1959
The kinetics of glucose transport through the human red cell membrane
Exper.Cell.Res. 11:69-64

WILBRANDT, W. AND ROSENBERG, T. 1961

The concept of carrier transport and its corollaries in pharmacology

Pharmacol.Rev. 13(2), 109-183

WILFONG, R. F., NEVILLE, D. N. Jr. 1970

The isolation of a brush border membrane fraction from rat kidney

J.Biol.Chem. 245:6106-6112

WILLIAMS, T. F., EXTON, J. H., PARK, C. R., AND REGEN, D. M. 1968

Stereospecific transport of glucose in the perfused rat liver

Am.J.Physiol. 215:1200-1209

WILSON, R. H. 1932

Effect of phlorizin on the rate of absorption from the gastrointestinal tract of white rat

J.Biol.Chem. 97:597-502

WILSON, G., ROSE, S. P., FOX, C. F. (1970)

The effect of membrane lipid unsaturation on glycoside transport.

Brochem.Biophys.Res.Commun. 38:617-623

WILSON, T. H. AND VINCENT, T. N. (1955)

Absorption of sugars in vitro by the intestine of the golden hamster

J.Biol.Chem. 216:851-866

WOODS, H. F. AND ALBERTI, K. G. M. M. (1972)

Dangers of intravenous fructose

Lancet 1972, 2:1354-1357

WOODS, H. F., EGGLESTON, L. V., KREBS, H. A. (1970)
The cause of hepatic accumulation of fructose-1-phosphate on fructose loading
Biochem.J.119:501-510

YUDILEVICH, D. L. AND DEROSE, N. (1971)
Blood-brain transfer of glucose and other molecules measured by rapid indicator dilution
Am.J.Physiol. 220:841-846

YUDILEVICH, D. L., RENKIN, E. M., ALVAREZ, O. A. AND BRAVO, I. (1968)
Liver distribution and uptake of molecules studied by rapid indicator-dilution technique
Circ.Res. 23:325-336

YUDKIN, J. (1964)
Dietary fat and dietary sugar in relation to ischaemic heart disease and diabetes.
Lancet 2:4-5

YUDKIN, J. AND MORLAND, J. (1967)
Sugar intake and myocardinal infarction
Amer.J.Clin.Nutr. 20:503-505

YUDKIN, J. AND RODDY, J. (1964)
Levels of dietary sucrose in patients with occlusive attrerosclerotic disease
Lancet 2:6-7

ZIERLER, K. L. 1959
Effect of insulin on membrane potential and potassium content of the rat muscle

Am.J.Physiol. 197:515-523

ZIERLER, K. L. (1961)
A model of a poorly-permeable membrane as an alternative to the carrier hypothesis of cell membrane penetration
Bull.Johns Hopkins Hosp. 109:35-48

ZIERLER, K. L. (1962)
Theoretical basis for indicator dilution methods for measuring flow and volume
Circulation Res. 10:393-407

ZIERLER, K. L. (1965)
Equations for measuring blood flow by external monitoring of radioisotopes
Circ.Res. 16: 309-321

ZIMMERMAN, G., TENNYSON, C. AND DRAPANAS, T. (1971)
Studies of preservation of the liver and pancreas by freezing techniques.
Transplantation Proc. 3:657-664

ZIMMERMANN, G., TENNYSON, C. BOECKL, O. DRAPANAS, T. (1973)
Isolated liver perfusion following long-term freezing and storage. Studies of metabolic activity and function.
In: Isolated liver perfusion and its applications. Eds. I. Bartosek, A. Guaitani and L. L. Miller. pp.87-93. Raven Press, New York.