STUDIES ON GLUCOSE AND 3-HYDROXYBUTYRATE METABOLISM IN THE PERFUSED HEART

by

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DECLARATION OF ORIGINALITY

I declare that this thesis was composed by myself and that the research which is described was mine.

A.M.N. Sultan.

CONTENTS

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ABSTRACT

Although the isolated perfused rat heart is often used in studies of metabolic interrelationships and control, there are large discrepancies in reported rates of metabolism measured in closed-circuit systems of perfusion. In this work the metabolism of the rat heart has been studied using a system of perfusion in which fresh perfusate is infused steadily into circulating perfusate whose volume is kept constant by continuous withdrawal at a rate equal to that of infusion. This system has several potential advantages. Substrates or products that are used or produced at a steady rate must reach constant concentration in the circulating perfusate. This has been shown in this work to happen with glucose as the sole substrate and with glucose and 3-hydroxybutyrate as competing substrates.

A further advantage is that constancy can be reached at low concentrations as readily as at high concentrations. This has enabled the kinetics of glucose utilization to be studied using a wide range of concentrations. The inhibition of glucose utilization by 3-hydroxybutyrate was also investigated using a wide range of concentration of the ketone body.

The system of perfusion also permits changes in metabolic rates to be followed. Glucose utilization by hearts from fed animals when perfused without insulin fell during the first half-hour of perfusion. In testing the possibility that this fall reflects the diminishing effect of endogenous insulin, the sensitivity of the isolated heart to insulin was found to be much greater than has been reported previously. This may be the result of the continuous infusion of insulin into the circulating perfusate or of the use of paper filters which were found to be associated with low rates of glucose utilization in the absence of insulin.

These attributes of the perfusion system have been used to investigate several aspects of glucose and 3-hydroxybutyrate metabolism with these substrates present singly or in competition. The main conclusion to be drawn from these studies was that 3-hydroxybutyrate is a poor substrate for the isolated heart but a potent inhibitor of glucose utilization. In the absence of insulin the ketone body at all concentrations inhibited glucose utilization and, when the hearts were taken from fasted animals, the inhibition was so great that glucose utilization was undetectable. Insulin at all concentrations opposed the inhibition but only completely restored glucose utilization when the concentration of 3-hydroxybutyrate was low.

Although the perfusion system is not suited to the measurement of low rates of utilization at high concentration of substrate, its merits investigated in the work reported in this thesis are sufficient to justify the analytical demands that it makes.

LIST OF FIGURES

LIST OF TABLES

GENERAL INTRODUCTION

The isolated perfused heart has been a popular preparation for biochemical study since Langendorff (1895) introduced the technique for perfusion of the coronary circulation from a cannula inserted into the aorta. One attraction is that the technique is comparatively easy. It allows substrates, hormones and oxygen to be delivered to the cells through the capillary circulation so that concentration gradients that are likely to be large in tissue slices are restricted. It also maintains the compartmentation of the organ into vascular, interstitial, and intracellular spaces so that regulation and limitation of metabolism through the rate of transfer between the compartments can be studied.

The rat has a heart of convenient size and metabolic activity. Its coronary flow is sufficient for the demands for oxygen to be met by oxygen in solution in simple saline medium. This independence from blood or haemoglobin gives the important advantage that the content of substrates and hormones in the perfusate can be closely controlled. Consequently the isolated perfused rat heart has been widely studied mainly for the information it can provide about cardiac metabolism and its regulation but also as a model for muscle in general.

The physiological relevance of the properties of the isolated rat heart perfused by the Langendorff technique can be questioned. It is denervated and the interrelation of nervous effects on hormonal regulation is often neglected. It performs no external work in that it does not pump fluid against an external resistance but only develops tension. Neely, Liebermeister, Battersby and Morgan (1967a) developed a system of perfusion that results in the heart doing external work.

Perfusate introduced through the left atrium is pumped from the left ventricle against a resistance that that also provides the pressure for the perfusion of the coronary arteries. This working preparation has been held to differ only quantitatively from the Langendorff or non-working preparation (Ross, 1972) so that only the advantage of being able to assess the constancy or otherwise in the aortic output justified the extra complexity and technical difficulties. However Taegtmeyer, Hems and Krebs (1980) concluded that the workload imposed on the isolated heart has important qualitative effects on cardiac metabolism so that the Langendorff preparation is of limited relevance to the situation in vivo.

The work presented in this thesis is concerned entirely with the Langendorff preparation but one of its objectives is to assess the usefulness of the preparation for metabolic investigations. However limited the physiological relevance of the preparation may be, confidence in its application for example to problems of metabolic regulation depends on the consistency of its properties in control conditions. The rate of glucose utilization by hearts isolated from fed rats and perfused with 5.5mM-glucose in the absence of insulin was found by Meuli and Froesch (1975) to be 30μmoles.g dry wt.$^{-1}$h^{-1} and by Wieland, Funcke and Löffler (1971) to be 420μmoles.g dry wt.$^{-1}$h^{-1}. Further examples of the variability of observed rates of glucose utilization by isolated hearts are given in Chapter 5.

In almost all cases the utilization of glucose and of other substrates by the perfused rat heart has been calculated from the fall in the amount of the substrate in a known volume of perfusate that is recirculated through the heart for a measured time. This technique of perfusion for making metabolic studies will be referred to as closed-circuit perfusion. In this system of perfusion change

in the concentration of both substrates and products in the perfusate is inevitable and necessary for the rates of metabolic process to be measured. This has some disadvantages. If the rate of utilization of a substrate is concentration-dependent then at low concentrations the rate must fall during the period of measurement. This is usually compensated for by making measurements over a short period or by using a large volume of perfusate. If the rate of utilization is not concentration-dependent a long period of measurement or a small volume of perfusate is favoured. When the inter-relationships of two competing substrates are studied the range of conditions is restricted if the utilization of each is to be measured without large changes in the concentration of the other. If changes in utilization with time are to be determined frequent estimates are required and small volumes of recirculated perfusate must be used especially if high concentrations of substrate are employed so that the rate of utilization is independent of substrate concentration (England and Randle, 1967).

A particular disadvantage of the closed-circuit system of perfusion is that the concentration of any constituent of a heart at the end of an experiment cannot be exactly related to the conditions of the perfusate that determine an observed rate of utilization of a substrate. Again this is particularly inconvenient if the rate is falling with the concentration of the substrate in the course of experiment. To overcome this disadvantage Fisher and O'Brien (1972) modified the system of closed-circuit perfusion so that fresh perfusate could be infused continuously into the system while keeping the volume of recirculated perfusate constant by withdrawing perfusate at a rate equal to that of infusion. In this system of perfusion with balanced infusion and withdrawal the concentration of a component of the perfusate that is used or produced by the heart at a constant rate must tend

to become constant. The rate of utilization or production can be measured from the difference between the amounts added to or withdrawn from the system. By calculating in addition the rate of change of the amount of substrate or product in the perfusate it is also possible with this system to determine the time-course of utilization or production.

When Fisher and O'Brien followed the time-course of glucose utilization in the presence of insulin they found it to be constant from the start of the experiment. In the absence of insulin the rate fell by at least 20% during the first 30 to 40 minutes of perfusion to a constant rate that was maintained for a further 30 minutes or more. This observation conflicts with claims that the rate of glucose utilization in the absence of insulin is constant in a closed-circuit system (Morgan, Henderson, Regen and Park, 1961; Randle, Newsholme and Garland, 1964; England and Randle, 1967).

Many studies of metabolism in the isolated rat heart have been made during the first 10, 15 or 20 minutes of perfusion (Morgan et al., 1961; Randle et al., 1964 and others, see Tables 17 and 18). However properties of hearts other than glucose utilization have been found to change in the early period of perfusion. Zachariah (1961) observed that the permeability of the cardiac cells of the rat to L-arabinose fell during the first 30 minutes of perfusion. When anti-insulin serum was given to the rat in vivo the permeability was relatively constant from the start of perfusion. Fisher and Williamson (1961a) reported that the consumption of oxygen, the rate of contraction and coronary flow rate all fell during the 20 minutes from the start of perfusion. These changes are attributed to the recovery of the heart from the trauma of isolation and to reflect in particular the diminishing effects of endogenous catecholamines. It has become common

for hearts to be perfused for up to 20 minutes before metabolic observations are made.

The objective of the work presented in this thesis was to test the usefulness of the system of perfusion of Fisher and O'Brien in metabolic studies. The system has advantages and limitations that in some respects complement those of closed-circuit systems. Its main advantage lies in being able to measure the rate of utilization or production at a constant concentration of substrate or product in the perfusate. The establishment of constant concentrations indicates constant rates of metabolism. However the time taken before a constant concentration in the perfusate is reached depends on the rate of infusion, the volume of recirculated perfusate and when the rate of consumption or production of substance becomes constant. In addition the recognition of a period of constancy is made with greater confidence the longer it can be followed. Consequently it was decided that whenever time permitted hearts would be perfused for 150 minutes. This would be adequate in the unfavourable circumstances of a rate of metabolism becoming constant after an hour when the half-time for the approach to a steady-state is no less than 15 minutes and ample when the rate is constant from the start of the experiment and the half-time is approximately 7 minutes. A disadvantage of this decision to make routinely prolonged experiments is that in some conditions such as the absence of insulin the results of Fisher and O'Brien indicate that constant metabolic rates would not be expected to be maintained to the end of the experiment. In that case the state of the heart at the end of the experiments is not representative of its state in the period of metabolic stability. By giving priority to the recognition of a state of metabolic stability and of its duration no attempt was made to analyse the constituents of the heart in that state.

A limitation on the application of the system of perfusion with balanced infusion and withdrawal is that numerous analyses must be made of the perfusate withdrawn from the system in order to establish the time-course of changes in the composition of the perfusate whereas in closed-circuit perfusion analysis is made of the perfusate at the beginning and end of the period of study. In this work an automated system for glucose analysis was available and was further developed for precision and economy so that the time-course of glucose concentration could be routinely determined. Otherwise analyses are time-consuming or expensive and restricted the number of investigations. However the time-course of change in the composition of the perfusate gives the time-course of utilization or production of a substance and not just the rate in conditions of constancy. In closed-circuit systems the determination of a time-course of a metabolic process also requires frequent sampling and analysis.

Fisher and O'Brien (1972) were able to study glucose utilization at low concentrations when the rate is concentration-dependent. A constant concentration was as readily established at low as at high concentrations. The system should also be well suited for studying the competition between two or more substrates. The rate of consumption of one could be determined at a defined concentration of itself and of the competing substrate if both are metabolized at a constant rate. A particular advantage is that the interaction should be able to be studied over a wide range of concentrations of both substrates. In this work the competition between DL-3-hydroxybutyrate and glucose has been studied because this ketone body has previously only been included in perfusate of closed-circuit system at concentrations (5mM to 10mM) that are at the upper end of its physiological range and because the competition has only been studied in experiments of

20 minutes duration. This investigation is presented in Part III of this thesis where Chapter 13 gives a more detailed introduction to the outstanding issues in this field.

The study of glucose utilization when it is the only exogenous substrate provided to the heart is described in Part II of this thesis. Apart from establishing a basis for comparison with results obtained with a second substrate the experiments in this part were intended to relate the properties of the isolated rat heart perfused with balanced infusion and withdrawal to results that have been obtained in closed-circuit system. In particular the claim of Fisher and O'Brien (1972) that glucose utilization by heart from fed rats perfused without insulin declines during the first 40 minutes of perfusion was tested because no corroborating reports have been made.

In the introductory chapter to Part II, the variability of estimates of glucose utilization obtained with the isolated perfused rat heart is discussed. The experiments reported in Part II and Part III also contribute to an understanding of the variability and to an analysis of the suitability not only of the particular perfusion system used but of the isolated non-working rat heart as an experimental preparation for metabolic studies. Details of the method of perfusion and its characteristics are included in Part I.

PART I

MATERIALS AND METHODS

CHAPTER 1

Animals, Materials and Media

1.1 Animals

Hearts were taken from male albino rats of Wistar stock weighing between 200-300g. Before being sacrificed the rats were kept in the departmental animal house for one week at least under a cycle of 12h of darkness and 12h of artificial light and at a constant temperature of 22°C. The rats were bred at the Centre for Laboratory Animals, The Bush, Milton Bridge, Penicuik where they were also kept under conditions of constant lighting and temperature. This precaution avoids seasonal variation in the sensitivity of the rat heart to insulin which has been demonstrated by Young (1965). Unless stated otherwise the animals had free access to food consisting of a standard laboratory diet (Oxoid 86, Oxoid Ltd., Basingstock, Hants.) and to water until the time of the experiment. When the rats were fasted food was withheld either overnight (18-20h) or for 48h, but water was freely available.

1.2 Materials

1.2.1 Biochemicals

DL-3-Hydroxybutyrate (sodium salt), nicotinamide-adenine dinucleotide (oxidized form, free acid), nicotinamide-adenine dinucleotide (reduced form, disodium salt), L-Lactate (1M-solution) were obtained from The Boehringer Corporation (London) Ltd., Lewes, East Sussex.

Acetoacetate (lithium salt), bovine albumin (fraction V, essentially fatty acid free) and triethanolamine hydrochloride were obtained from Sigma London Chemical Company Ltd., Poole, Dorset.

Sodium acetate (anhydrous powder) was purchased from Koch-Light Laboratories Ltd., Colnbrook, Buckinghamshire.

Bovine insulin (crystalline), D-glucose, glycine and all other chemicals were of the highest available quality and were obtained from BDH Chemicals Ltd., Poole, Dorset.

1.2.2 Enzymes

Glucose oxidase (EC 1.1.3.4) as Fermcozyme 653M and peroxidase (EC 1.11.1.7) were purchased from Hughes and Hughes Ltd., Harold Wood, Essex.

D-3-Hydroxybutyrate dehydrogenase (EC 1.1.1.30) and L-lactate dehydrogenase (EC 1.1.1.27) were obtained from The Boehringer Corporation (London) Ltd., Lewes, East Sussex.

1.2.3 Miscellaneous Materials

Guaiacum (Guaiac resin) was obtained from Hughes and Hughes Ltd., Harold Wood, Essex. Triton X-100 was obtained from BDH Chemicals Ltd., Poole, Dorset. Silicone MS Antifoam A was supplied by Hopkin and Williams Ltd., Chadwell Heath, Essex. Millipore filters were purchased from the Millipore Corporation, Bedford, Massachusetts, U.S.A. and paper filters from Whatman Ltd., Maidstone, Kent. Double-distilled water was used for all media and solutions.

1.3 Media

1.3.1 Modified Krebs Henseleit Medium

All perfusions in this work were carried out with a modification of the medium of Krebs and Henseleit (1932). The modification involves halving the concentrations of calcium and magnesium to allow for the binding of those ions by plasma proteins (Greene and Power, 1931). This Modified Krebs Henseleit Medium which will be referred to as MKHM throughout this thesis has the composition given in Table 1. When it is equilibrated at 37^{o}C with a gas mixture of 5% carbon dioxide in

Table 1

The composition of the perfusate (MKHM)

Component	Concentration (mM)	Osmotic Coefficient	Active Osmolarity (m.Osmoles)
NaCl	118.48	0.93	220.373
$NaHCO_3$	24.876	0.96	47.762
KCl	4.739	0.92	8.720
KH_2PO_4	1.186	0.87	2.064
$CaCl_2 \cdot 6H_2O$	1.270	0.86	3.277
$MgSO_4 \cdot 7H_2O$	0.593	0.58	0.6888
		Total	= 282.884

oxygen it has a pH of 7.4. In preparing the MKHM calcium chloride was always added last when all other constituents were almost at their final dilution. This avoids precipitation of calcium phosphate. Substrates, hormones and albumin ($0.5mg.ml^{-1}$) were included in the perfusate as indicated in the text without altering the composition of the electrolytes. Stock solutions of $liu.ml^{-1}$ insulin in 0.94%(w/v) sodium chloride containing 0.01M-hydrochloric acid were stored in a deep-freeze at $-20^{o}C$.

1.3.2 Saline A

After they were cut from the animals, hearts were immediately placed in a cold saline solution designated Saline A. This solution contains 142.2mM-sodium chloride and 0.5mM-sodium bicarbonate and has $285.4milliosmoles.L^{-1}$. When it is equilibrated with atmospheric carbon dioxide at $0^{o}C$ it has a pH of 7.4.

CHAPTER 2

Analytical Methods

2.1 The Estimation of Glucose

2.1.1 Introduction

β-D-glucose has been estimated throughout this work by an automated colorimetric method using β-D-glucose oxidase (EC 1.1.3.4) and peroxidase (EC 1.11.1.7) with an extract of gum guaiacum resin as the chromogen.

$$C_6H_{12}O_6 + H_2O + O_2 \xrightarrow[\text{Oxidase}]{\text{Glucose}} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{Chromogen} \xrightarrow{\text{Peroxidase}} H_2O + \text{Coloured product}$$

Many methods of glucose estimation have been based on the oxidation of glucose in the presence of glucose oxidase to gluconic acid with the formation of hydrogen peroxidase. Keilin and Hartree (1948) and Keilin and Hartree (1952) used catalase to decompose the hydrogen peroxide and measured manometrically the oxygen released while Froesch and Renold (1956) measured the decrease in the reducing power that accompanied the oxidation of glucose. However Keston (1956) used peroxidase to promote the oxidation of O-tolidine by hydrogen peroxidase and so introduced the basis of many methods of glucose estimation that differ mainly in the nature of the chromogen. Teller (1956) and Huggett and Nixon (1957) used O-dianisidine which has the advantage over O-tolidine in a manual method of giving a colour that is stable. Both O-tolidine and O-dianisidine are no longer used because they have been identified as carcinogens.

For this work a modification of the methods of Hill and Kessler (1961) and Hill (1965) for the automated estimation of glucose using gum guaiacum as chromogen has been used. This method has the advantage that the reagent is very stable and can be prepared in large quantities

and is extremely economical. Although the colour generated in the reaction with hydrogen peroxidase is unstable, this is a very minor inconvenience in an automated method. Since the experimental procedure used in this work required the analysis of up to 50 samples of perfusate and standard solutions an automated method was essential. Fisher and O'Brien (1972) used this method successfully and further development described here improved its economy and made it easily adaptable for the analysis of a wide range of glucose concentrations with little preparation of the sample being required.

2.1.2 Preparation of the Stock Gum Guaiacum Solution

(i) Extract 7.5g of powdered gum guaiacum with 125ml ethanol. It is sufficient to leave the mixture for 3h or overnight with occasional shaking.

(ii) Dissolve 450g for anhydrous sodium acetate in 6litres of distilled water and add 31ml of glacial acetate acid and 200ml of Triton X-100 with continuous mixing. This solution has a pH of 5.6.

(iii) Filter the gum guaiacum extract into the acetic buffer with mixing and make the volume up to 10litres with distilled water.

This stock solution of gum guaiacum should be left for 10 to 15 days when it darkens to a deep amber colour. This solution can be stored at room temperature indefinitely. Addition of the enzymes to a freshly prepared stock commonly but not always produces a blue colour. This phenomenon very rarely occurs when the stock solution has been left to stand.

2.1.3 Stock Solution of Peroxidase

A stock solution of peroxidase is prepared by dissolving 5mg of salt free lyophilised peroxidase powder in 5ml of distilled water. This solution is stable for 15 days when it is stored at $4^{o}C$.

2.1.4 Preparation of the Working Reagent

The working reagent is prepared by taking 67ml of the stock gum guaiacum solution and adding 33ml of distilled water and volumes of glucose oxidase (Fermcozyme) and of the stock peroxidase that are appropriate to the concentration of glucose to be estimated and are given in Table 2. The variation of the amount of the enzyme in the reagent is a modification in the presented work which is a departure from the practice of Fisher and O'Brien (1972) who used 1ml of Fermcozyme and 1mg of peroxidase per 100ml of reagent and diluted their samples to give an appropriate final concentration of glucose. By introducing this change considerable economy in the method has been achieved. The working reagent is stable for 3 weeks at $4^{o}C$.

2.1.5 System for Automated Analysis

Samples were aspirated from a Technicon Sampler Model II at the rate of 30 per hour with a distilled water wash between samples. Air ($0.4ml.min^{-1}$), sample ($2ml.min^{-1}$) and reagent ($3.4ml.min^{-1}$) were brought together by a Technicon proportioning pump (Fig. 1). The segmented stream of the reaction mixture passed through mixing coils for 5 minutes at $37^{o}C$ and then through a flow cell in a Perkin-Elmer Spectrophotometer Model 6/20. The percent transmission at 625nm was recorded on a Servoscribe 1s potentiometric recorder.

2.1.6 Application of the Method

Some precautions were followed throughout the work to ensure maximal accuracy:

(i) Mutarotation of the standard glucose solutions

The stock solution of glucose from which standards were prepared was always heated either for 20 minutes in a hot water bath ($60-80^{o}C$)

Table 2

Composition of working reagents

Glucose concentration in sample ($\mu g.ml^{-1}$)	Fermcozyme ($ml.100ml^{-1}$)	Stock Peroxidase ($ml.100ml^{-1}$)
5 to 35	0.05	0.17
20 to 140	0.025	0.025
40 to 100	0.025	0.05
100 to 700	0.005	0.025
500 to 1000	0.002	0.03

The volumes of Fermcozyme and stock peroxidase solutions in 100ml of the working reagents are given for the appropriate range of glucose concentrations.

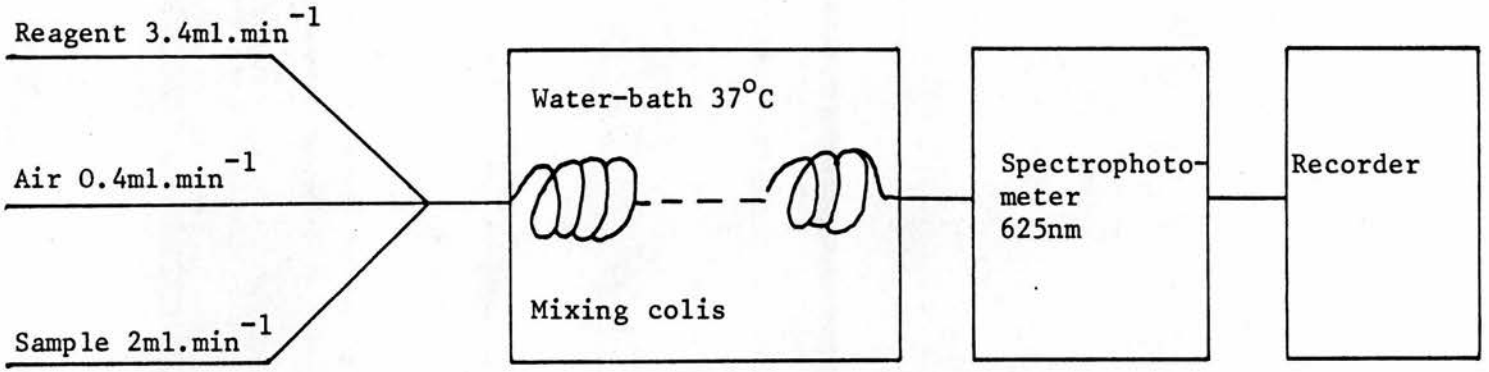

Fig. 1. Manifold for the automated analysis of glucose

or for 3 hours at 37°C. This promotes the equilibration of α and β anomers of glucose in a solution prepared from the solid α-D-glucose. In a method that involves a short reaction and does not require that the oxidation of glucose should be complete, it is important that the standards and unknowns solutions are similar in the composition of the α and β anomers.

(ii) Reagent temperature

The reagent was warmed to 37°C before use. This precaution is also helpful in a method which does not necessarily allow full colour development to occur.

(iii) Running order of standards and unknown samples

The practice of Fisher and Gilbert (1970a) was followed. Standard (S) and unknown (U) solutions were analysed in the following sequence;

$S_1S_2S_3S_4U_1U_2U_3U_4U_4U_3U_2U_1S_4S_3S_2S_1$

The average absorbance of each pair therefore refers to the same moment in the period over which the series is analysed. Consequently any regular changes in the performance in the spectrophotometer, or of the recorder or in the properties of the reagent are compensated for. The spectrophotometer and recorder were turned on at least 2 hours before use so that any changes in their performance should be regular ones.

2.1.7 Preparation of Unknown Samples

Samples of the perfusate were usually diluted but in some cases undiluted samples were used for analysis.

(i) Diluted sample

In experiments where hearts were perfused with 2.7mM-or 5.5mM-glucose, the samples of the perfusate and the infusate were diluted 5 and 10 times respectively to give an appropriate final glucose

concentration by taking 1ml or 0.5ml of the perfusate and making up to 5ml with distilled water. In these investigations the concentration of glucose in the diluted samples fell between 60 to 100μg.ml^{-1}.

(ii) Undiluted sample

In hearts perfused at low concentrations of glucose the perfusate samples were analysed without any dilution or treatment. The concentration of glucose in these samples varied between 4μg.ml^{-1} and 40μg.ml^{-1}.

2.1.8 The Linearity of the Method of Glucose Estimation

Table 2 shows the volumes of Fermcozyme and stock peroxidase solution in 100ml of the working reagent and the range of glucose concentrations for which each reagent was used. At low concentrations of glucose more of the enzymes were used to give optimum colour development. At high concentrations of glucose less enzyme was needed but the rate of colour development was still linearly related to glucose concentration as shown in Figs. 2 and 3.

The intercept of the y-axis is variable and reflects the variations in the properties of different preparations of stock gum guaiacum.

2.1.9 The Precision of the Estimation of Glucose

Fig. 4 shows the reproducibility of the traces when samples of standard solutions of glucose are analysed repeatedly. The slight tendency of the peak heights to increase is compensated for by running the series in mirror-image and taking the average of estimates equi-spaced about the mid-point.

Table 3 shows the mean of the estimated concentration of solutions of glucose in water varying from 10μg.ml^{-1} to 1000μg.ml^{-1} and the standard deviation (S.D.) of the estimates. The standard deviation is 1% or less of the mean regardless of the concentration.

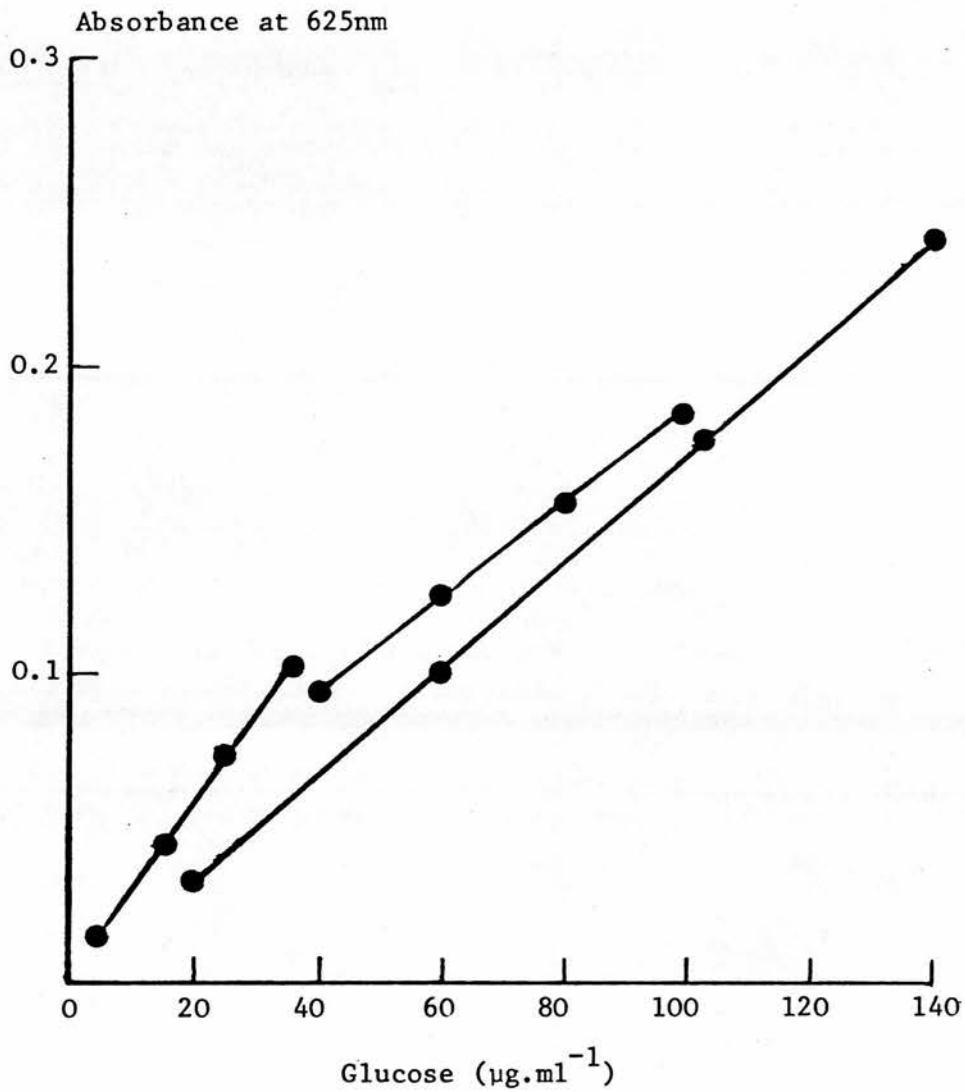

Fig. 2. Relationship between glucose concentration and absorbance at 625nm

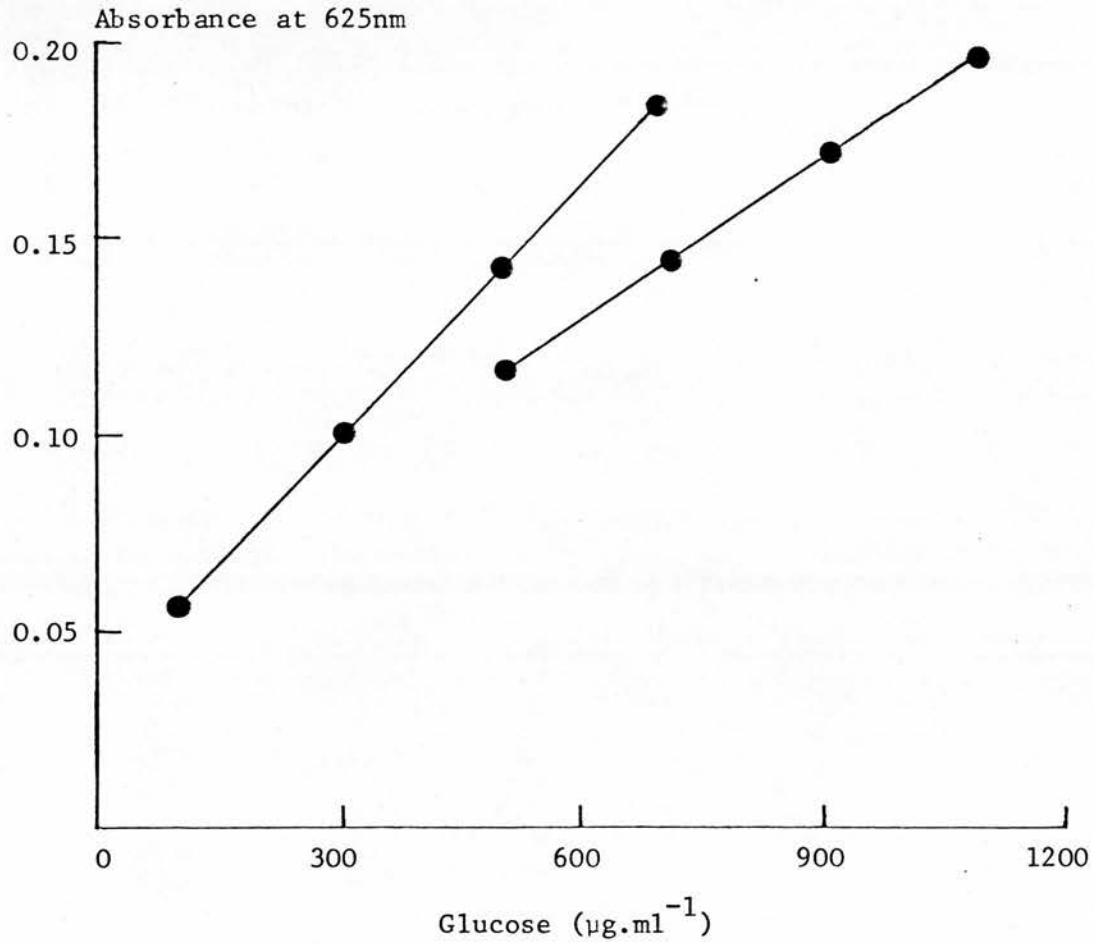

Fig. 3. Relationship between glucose concentration and absorbance at 625nm

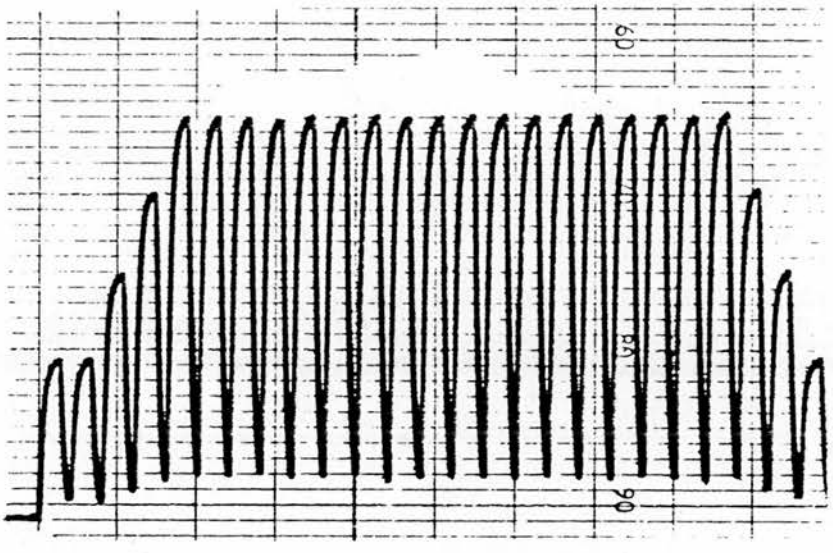

Fig. 4. The reproducibility of traces when samples of standard solutions of glucose are analysed repeatedly

Table 3

The precision of the estimate of the concentration of glucose in aqueous solution

Estimated Concentration ($\mu g.ml^{-1}$)	Standard Deviation	Percentage Error
9.944 (9)	0.057	0.57
20.091 (9)	0.072	0.36
69.300 (8)	0.590	1.0
100.050 (10)	0.660	0.65
492.200 (9)	2.490	0.50
992.300 (9)	8.700	0.87

Values are means and standard deviations of the numbers of observations given in parentheses.

2.1.10 The Accuracy of Glucose Estimation

(i) The effect of perfusate (MKHM) on glucose estimation

The content of electrolytes in samples of perfusate when analysed for glucose varied depending on whether the sample was diluted or not. To test the effect of electrolytes on glucose estimation five-fold dilutions of three samples of a solution of glucose containing 500$\mu g.ml^{-1}$ were made with MKHM and the resultant solutions analysed. The mean, standard deviation and recoveries of glucose from those solutions are given in Table 4 in comparison with a sample treated similarly with water. No effect of MKHM is evident.

(ii) The effect of albumin on glucose estimation

In some experiments the perfusate contained albumin at 0.5$mg.ml^{-1}$. The effect of the presence of albumin on the method for glucose estimation was tested by preparing solutions at two concentrations of glucose (100$\mu g.ml^{-1}$ and 60$\mu g.ml^{-1}$) and containing bovine serum albumin at up to 50$mg.100ml^{-1}$. Analysis of these solutions indicates (Table 5) that albumin at the tested concentrations has no effect on glucose estimation. In spite of this finding, in all experiments where albumin was used albumin was added to the standards.

(iii) The effect of DL-3-hydroxybutyrate on glucose estimation

Solutions of glucose were prepared with distilled water at two different concentrations (80$\mu g.ml^{-1}$ and 60$\mu g.ml^{-1}$) and containing DL-3-hydroxybutyrate at different concentrations as indicated in Table 6. The results show that there is no effect of DL-3-hydroxybutyrate on the method of glucose estimation. The concentrations of DL-3-hydroxybutyrate used in this test cover those of the perfusates used in this work.

(iv) The effect of acetoacetate on glucose estimation

The effect of acetoacetate on glucose estimation was tested by

Table 4

Effect of electrolytes on the estimation of glucose

	Estimated Glucose ($\mu g.ml^{-1}$)	Percentage Recovery
A	100.05 ± 0.65 (10)	100.05
B	100.50 ± 0.68 (8)	100.50
C	99.04 ± 0.42 (6)	99.00
D	100.06 ± 0.31 (6)	99.94

Values are means $\pm$ S.D. with the numbers of observations in parentheses. Samples A, B and C are of an aqueous solution of glucose diluted five-fold with MKHM. Sample D was treated in the same way with distilled water. The mean recovery of A, B and C is 99.85%.

Table 5

The effect of albumin on glucose estimation

Albumin ($mg.100ml^{-1}$)	Estimated Glucose ($\mu g.ml^{-1}$)	Per cent Recovery
0.0	99.53 ± 0.12 (4)	-
	60.30 ± 0.54 (8)	-
5	99.93 ± 0.12 (4)	100.4
	60.71 ± 0.00 (4)	100.7
10	100.16 ± 0.00 (4)	100.6
	60.82 ± 0.11 (4)	100.8
15	99.67 ± 0.58 (4)	100.1
	60.30 ± 0.00 (4)	100.0
20	100.11 ± 0.00 (4)	100.6
	60.53 ± 0.28 (4)	100.4
30	99.84 ± 0.00 (4)	100.31
	60.01 ± 0.13 (4)	99.5
40	100.01 ± 0.20 (4)	100.5
	59.73 ± 0.11 (4)	99.1
50	99.89 ± 0.14 (4)	100.4
	59.59 ± 0.11 (4)	98.8

Values are the mean $\pm$ S.D. of the estimated concentration of solutions of glucose containing different concentrations of albumin. The numbers of observations are in parentheses.

Table 6

The effect of DL-3-hydroxybutyrate on glucose estimation

DL-3-Hydroxybutyrate (mM)	Estimated Glucose ($\mu g.ml^{-1}$)	Per cent Recovery
0.0	79.48 ± 0.70 (8)	-
	61.11 ± 0.15 (8)	-
0.5	80.45 ± 0.09 (4)	101.3
	61.07 ± 0.00 (4)	99.9
1.0	86.43 ± 0.00 (4)	101.2
	62.27 ± 0.00 (4)	101.9
2.0	79.73 ± 0.09 (4)	100.3
	61.08 ± 0.11 (4)	100.0
6.0	80.67 ± 0.08 (4)	101.5
	62.21 ± 0.08 (4)	101.8
12.0	79.43 ± 0.13 (4)	99.9
	62.41 ± 0.00 (4)	102.1

Values are the mean $\pm$ S.D. of the estimated concentration of solutions of glucose containing different concentrations of DL-3-hydroxybutyrate. The numbers of observations are in parentheses.

preparing solutions containing glucose ($62\mu g.ml^{-1}$) and acetoacetate at a range of concentrations that include those met in perfusates analysed in this work. Analysis of these solutions shows (Table 7) that acetoacetate does not affect the estimates of glucose concentration.

2.2 The Estimation of D-(-)-3-Hydroxybutyrate

2.2.1 Introduction

The method of Williamson and Mellanby (1974) was used to estimate D-3-hydroxybutyrate. The method depends on the increase in the absorption at 340nm due to the formation of NADH when D-3-hydroxybutyrate is oxidized to a acetoacetate in the presence of D-3-hydroxybutyrate dehydrogenase (EC 1.1.1.30).

$$\text{D-(-)-3-Hydroxybutyrate} + NAD^+ \rightleftharpoons \text{Acetoacetate} + NADH + H^+$$

Samples of perfusate and infusate were diluted 50-fold without deproteinization. Aliquots of 2ml were then analysed without modification of the published method.

2.2.2 The Linearity of the Method

Figure 5 is a plot of the increase in absorbance at 340nm when aliquots were taken of solutions of DL-3-hydroxybutyrate diluted to have nominal concentrations of 40μM, 100μM, 160μM and 220μM. The relationship between nominal concentration and increase in absorbance is linear.

2.2.3 The Precision of the Method

When solutions of DL-3-hydroxybutyrate with nominal concentrations of 40μM, 100μM, 160μM and 220μM were analysed, the concentration of D-3-hydroxybutyrate in those solutions calculated from the molar extinction coefficient of NADH were as shown in Table 8. The error of the estimates varies between 2% and 8% and the proportion of D-3-hydroxybutyrate varies from 44.7% to 48.0%. This compares with a typical enzymatic analysis by the suppliers of 41%.

Table 7

The effect of acetoacetate on glucose estimation

Acetoacetate (mM)	Estimated Glucose ($\mu g.ml^{-1}$)	Per cent Recovery
0.0	62.07 ± 0.0 (4)	-
0.25	62.11 ± 0.0 (4)	100.1
0.50	62.54 ± 0.0 (4)	100.8
1.00	61.81 ± 0.19 (4)	99.6
2.00	61.85 ± 0.0 (4)	99.7

Values are the mean $\pm$ S.D. of the estimated concentration of solutions of glucose containing different concentrations of acetoacetate. The numbers of observations are in parentheses.

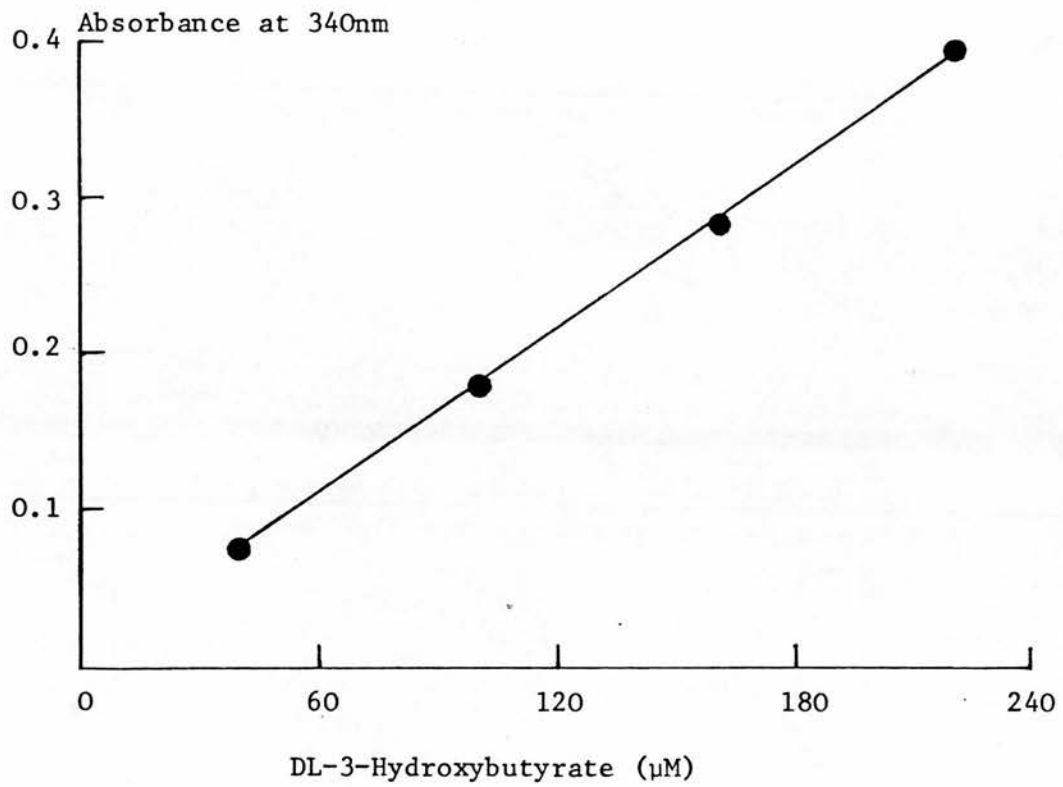

Fig. 5. Relationship between DL-3-hydroxybutyrate concentration and absorbance at 340nm

Table 8

Reproducibility of the estimation of and recovery of D-3-hydroxybutyrate

Nominal DL-3-Hydroxybutyrate (μM)	D-3-Hydroxybutyrate (μM)	Per cent Error	Recovery (%)
40	19.22±1.46 (6)	8	48.0
100	45.24±2.03 (6)	5	45.3
160	72.00±1.22 (6)	2	45.0
220	98.35±1.58 (6)	2	44.7

Values are the mean ± S.D. with the numbers of observations in parentheses. The recovery is calculated as the percentage of D-3-hydroxybutyrate in solutions of DL-3-hydroxybutyrate with the nominal concentration shown.

2.2.4 The Effect of Acetoacetate on the Estimation of D-3-Hydroxybutyrate

The effect of acetoacetate on the estimation of D-3-hydroxybutyrate was investigated by analysing solutions of varying content of acetoacetate and D-3-hydroxybutyrate. The results are given in Table 9. No significant effect of acetoacetate on D-3-hydroxybutyrate estimation is indicated.

2.3 The Estimation of Acetoacetate

2.3.1 Introduction

The method of Mellanby and Williamson (1974) was used to estimate acetoacetate. This method depends on the decrease in the absorption at 340nm due to the oxidation of NADH to NAD when acetoacetate is reduced to D-3-hydroxybutyrate in the presence of D-3-hydroxybutyrate dehydrogenase.

$$\text{D-(-)-3-Hydroxybutyrate} + NAD^+ \rightleftharpoons \text{Acetoacetate} + NADH + H^+$$

2.3.2 The Linearity of the Method

Fig. 6 is a plot of the decrease in the absorbance at 340nm when aliquots were taken of a solution of acetoacetate diluted to have concentrations of 10μM, 50μM, 90μM and 130μM. The relationship between acetoacetate concentration and the decrease in the absorbance is linear.

2.3.3 The Precision of the Method

When solutions of acetoacetate at nominal concentrations of 10μM, 50μM, 90μM and 130μM were analysed the concentration of acetoacetate in those solutions calculated from the molar extinction coefficient of NADH were as shown in Table 10. The coefficient of variation of the estimates varies between 1.7% and 5%, and the mean estimated concentration of acetoacetate varies from 90.3% to 99.6% of the nominal concentration.

Table 9

The effect of acetoacetate on the percent recovery of D-3-hydroxybutyrate

DL-3-Hydroxybutyrate (μM)	Acetoacetate (μM)				
	40	60	80	100	120
40	46.7	46.7	48.7	47.9	45.7
100	46.5	44.8	45.2	44.4	45.7
160	44.1	45.1	45.1	43.5	43.8
220	44.3	44.3	45.1	44.3	44.7

The values are the estimated percent content of D-3-hydroxybutyrate in solutions containing the nominal concentration of DL-3-hydroxybutyrate and acetoacetate shown.

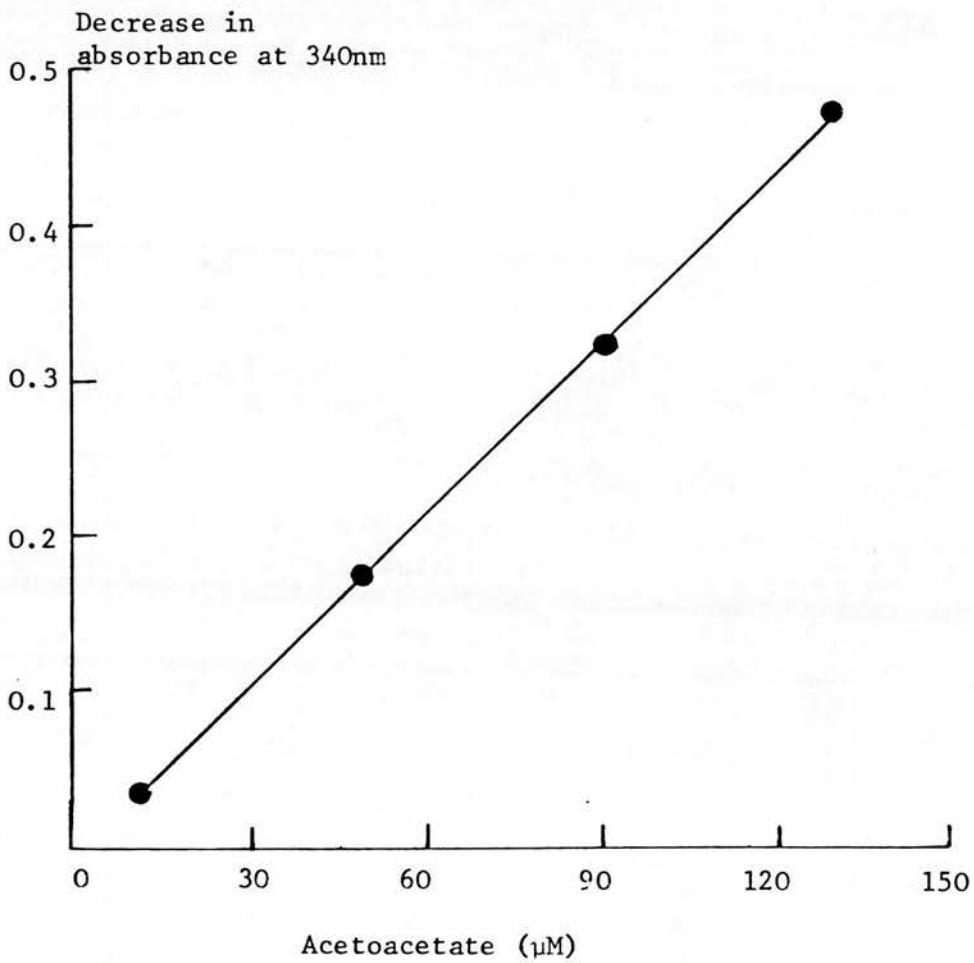

Fig. 6. Relationship between acetoacetate concentration and the decrease in absorbance at 340nm

Table 10

Precision of the estimation of acetoacetate

	Nominal Acetoacetate Concentration (μM)			
	10	50	90	130
Estimated conc .(μM)	9.79	49.8	87.98	117.45
Standard deviation	1.65	2.7	1.44	3.31
Coefficient of variation	1.7	5	2	3
No. of observations	6	6	3	6
Estimated/Nominal x 100	97.9	99.6	97.7	90.3

2.3.4 The Effect of DL-3-Hydroxybutyrate on the Estimation of Acetoacetate

The effect of DL-3-hydroxybutyrate on acetoacetate estimation was investigated by determining the apparent content of acetoacetate in solutions containing between 10μM and 130μM-acetoacetate and between 0.1mM and 8.0mM-DL-3-hydroxybutyrate. The results, shown in Table 11, indicate that DL-3-hydroxybutyrate at less than 2mM has no appreciable effect on the estimation of acetoacetate. However at concentrations above 4mM DL-3-hydroxybutyrate could interfere with acetoacetate estimation. In this work the perfusate samples for acetoacetate estimation were subject to four-fold dilutions so that the concentration of DL-3-hydroxybutyrate in the sample was always less than 3mM.

2.4 The Estimation of L-(+)-Lactate

L-Lactate was estimated by the method of Gutmann and Wahlefeld (1974), which depends on the increase in the absorption at 340nm when NAD^+ is reduced to NADH and lactate is oxidized to pyruvate in the presence of lactate dehydrogenase (EC 1.1.1.27).

$$\text{L-(+)-Lactate} + NAD^+ \rightleftharpoons \text{Pyruvate} + NADH + H^+$$

In Fig. 7 the relationship between the concentration of lactate and the increase in absorbance measured on two separate occasions is shown. The linearity and reproducibility of the method is evident.

2.5 The Estimation of Pyruvate

Few attempts were made to measure the concentration of pyruvate in the perfusate of hearts. When this was attempted the method of Passonneau and Lowry (1974) was used. This method is based on the decrease in absorption by NADH at 340nm when pyruvate is reduced to lactate in the presence of lactate dehydrogenase.

$$\text{Pyruvate} + NADH + H^+ \rightleftharpoons \text{L-Lactate} + NAD^+$$

Table 11

The effect of DL-3-hydroxybutyrate on the percent recovery of acetoacetate

Acetoacetate (μM)	DL-3-Hydroxybutyrate (mM)								
	0.10	0.20	0.40	0.80	1.20	1.6	4.0	6.0	8.0
10	112	103	94	100	100	100	101	100	100
50	88	90	91	91	88	88	93	86	86
90	87	90	89	88	85	83	89	89	84
130	91	92	91	89	89	88	-	-	-

The values are the estimated percent content of acetoacetate in solutions containing the nominal concentrations of acetoacetate and DL-3-hydroxybutyrate given.

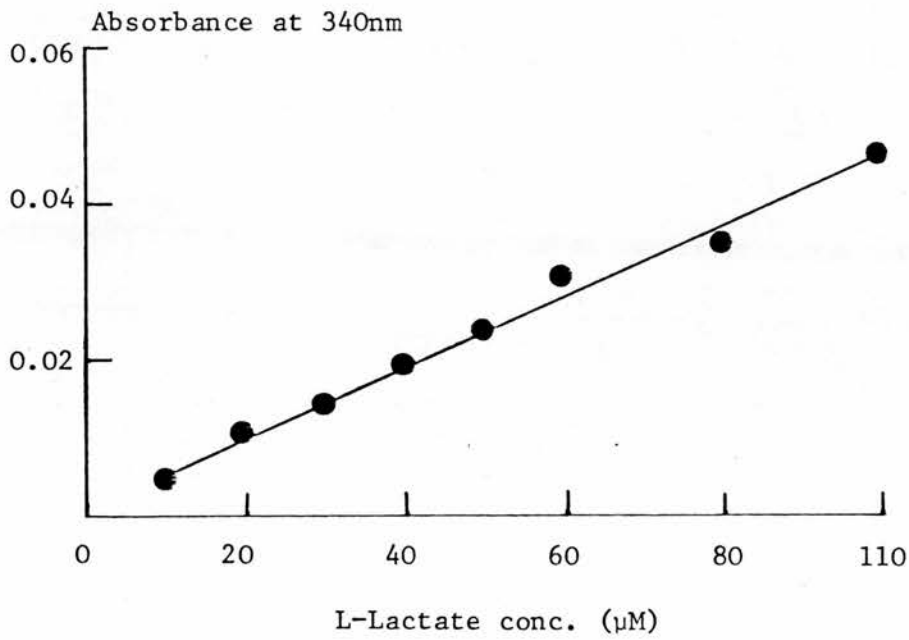

Fig. 7. Relationship between L-lactate concentration and absorbance at 340nm

Although this method is capable of measuring pyruvate at 0.1μM no pyruvate was detected in the perfusate of those hearts studied.

CHAPTER 3

Technique of Perfusion

3.1 Introduction

Langendorff (1895) established the technique of retrograde perfusion of the isolated heart. The basis of this technique is to introduce the medium to the heart through a cannula inserted into the aorta. The entry of the medium into the coronary circulation depends on the competence of the aortic valve. When the aortic valve is closed perfusate enters the coronary circulation.

The great majority (about 95%) of the coronary effluent leaves the heart via the coronary sinus and right atrium. Since the two venae cavae are cut the effluent is released against a minimal resistance. A small fraction of the effluent probably 5%,enters the left ventricle through the Thebesian veins and must be pumped from the chamber against the established perfusion pressure. Drainage of the left ventricle through the apex of the heart does not significantly affect the oxygen consumption (Arnold and Lochner, 1965) so that the beating heart perfused according to the principle of Langendorff has a basal requirement for energy. However the oxygen consumption of the Langendorff preparation does increase with increase in the aortic pressure (Neely et al., 1967a). In the work presented here as low a perfusion pressure was used as is consistent with maintaining adequate coronary flow. The Langendorff preparation is said to be non-working in so far as it does the minimum amount of external work.

Many different ways have been devised for delivering a warmed, oxygenated, and filtered medium to the cannula of the heart at an appropriate pressure. For studies of the rate of removal of substrates from the perfusate, recirculation of the perfusate through the heart is almost always necessary.

Some systems of perfusion have been especially widely used. For example the system of Bleehen and Fisher (1954) had the advantage of recirculation through a Soxhlet thimble filter but because perfusion pressure depended simply on hydrostatic pressure the apparatus was bulky and required at least 25ml of perfusate. Recirculation of perfusate was achieved through a gas-lift. By introducing roller pumps to provide both the force for perfusion and recirculation among other modifications, Morgan et al. (1961) developed a compact apparatus that needs as little as 5ml of perfusate. These and other less widely used systems for cardiac perfusion that follow the Langendorff principle are all closed systems whether large or small volumes of perfusate are recirculated. The activity of the heart continuously alters the composition of the perfusate and these alterations can be followed in studying the activity. However the conditions of perfusion are necessarily inconstant as was discussed in the General Introduction.

Fisher and O'Brien (1972) developed a new system of perfusion using the Langendorff principle that has the particular advantage that it permits the establishment of steady state conditions in a small volume of recirculated perfusate while still allowing the rates of metabolic activities to be measured. In this system fresh perfusate is continuously infused into the recirculated perfusate whose volume of about 7ml is kept constant by withdrawal at a rate equal to that of infusion. A substrate included in the perfusate must reach a constant concentration, in the recirculated perfusate provided that the heart is using it at a constant rate.

3.2 The Perfusion Apparatus

3.2.1 Outline

The system of Fisher and O'Brien (1972) is illustrated in Fig. 8.

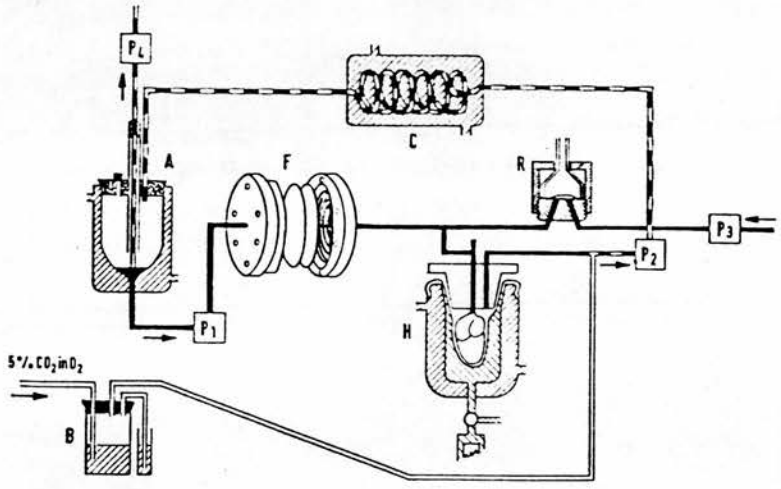

Fig. 8. Diagram of the perfusion system.

A: perfusate reservoir; B: buffer bottle;

C: coil for gas exchange; F: filter;

H: heart chamber; R: resistance; P_{1-4}: pumps.

Perfusate is pumped (P_1) from a reservoir (A) through a filter (F) at a rate (15-20ml.min^{-1}) in excess of the coronary flow rate. It may then pass through a resistance (R) or be presented to the heart (H) at a pressure determined by the resistance. The coronary effluent is collected by a second peristaltic pump (P_2) whose capacity is about eight times greater than the coronary flow rate and which can also draw in a 5% CO_2 in O_2 gas mixture which has passed through water in a buffer bottle (B). P_1 and P_2 were driven by the same motor. The mixture of gas and perfusate is combined with perfusate that has by-passed the heart and also receives infusate from a third pump (P_3) at a rate between 0.20ml.min^{-1} and 1.0ml.min^{-1}. The whole mixture of perfusate and gas returns to the reservoir via a coil (C) which increases the time available for gaseous exchange. This coil was omitted in one apparatus used in the later stages of this work in which perfusate was made to run down the walls of a longer reservoir. The alteration did not affect the properties of the hearts. The level of perfusate in the reservoir is kept constant at the bottom of a glass tube by pumping (P_4) through the tube at a rate in excess of that infusion. By keeping constant the level of perfusate in the reservoir the volume of recirculated perfusate is also kept constant. Perfusate withdrawn from the system is delivered to a fraction collector. Infusate is pumped from a second reservoir where it is warmed by water-jacketing and oxygenated by circulation through a gas-lift.

In this work the peristaltic pumps P_1 and P_2 were of variable speed and were constructed in the Department of Biochemistry, University of Edinburgh by Mr. A. Purdie and Mr. W. Tait. Pumps P_2 and P_4 were fixed speed and supplied by Schuco International Ltd., Woodhouse Road, London. The fraction collectors were Microfractionators supplied by Gilson, Villiers-le-bel, France.

The construction of the components of the apparatus was described by Fisher and O'Brien (1972) and in detail by O'Brien (1969). However the apparatus has been used with some minor modifications in the work presented here so for ease of reference the key components are described below. Their design is strongly influenced by the need to keep the volume of circulating perfusate as small as possible to that steady-state conditions may be established rapidly.

3.2.2 Heart chamber (Fig. 9)

A polythene stopper through which passed the metal cannula on which the heart was mounted was made to fit the mouth of the heart chamber. The heart on its mounting was introduced into an expanded finger from a polyvinyl disposable glove. Perfusate leaving the heart had to fill the finger and leave the chamber through a channel in the stopper. In this way the heart was immersed in the perfusate and good thermal contact maintained. The finger could be collapsed around the heart so that the volume of the perfusate held in the finger was minimized. The finger was stretched and collapsed by withdrawing water from or returning it to a compartment closed by the finger at one end and a syringe at the other. This compartment was itself water-jacketed and a three-way tap permitted the syringe to be filled with warm water when the finger was to be collapsed. In the design of Fisher and O'Brien (1972) the water of the compartment could be made continuous through the tap with a column of water that rose to no more than 3cm above the heart. Omission of this feature had no obvious effect on the properties of the hearts.

3.2.3 Filter (Fig. 10)

In this work filtration depended on a combination of Millipore and filter paper or on filter paper alone. In most of the experiments

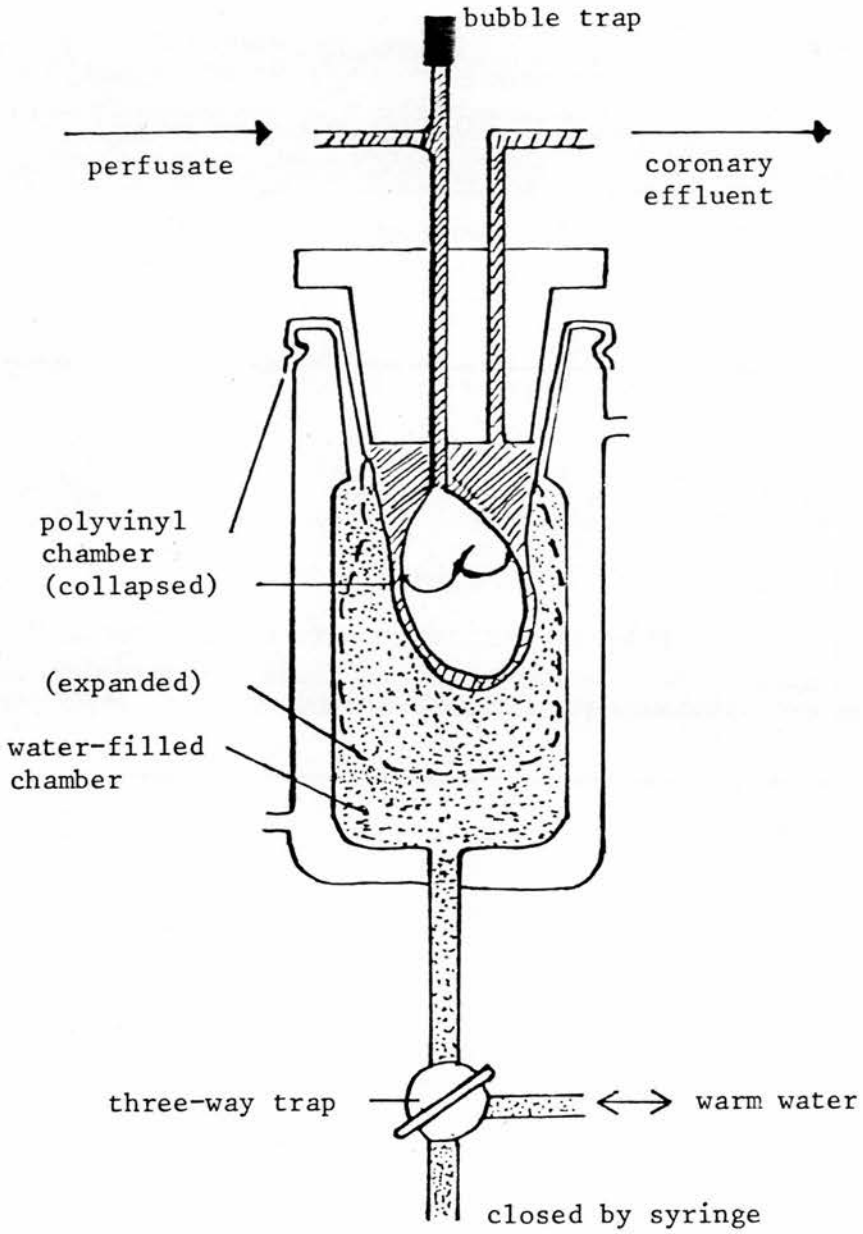

Fig. 9. Heart chamber

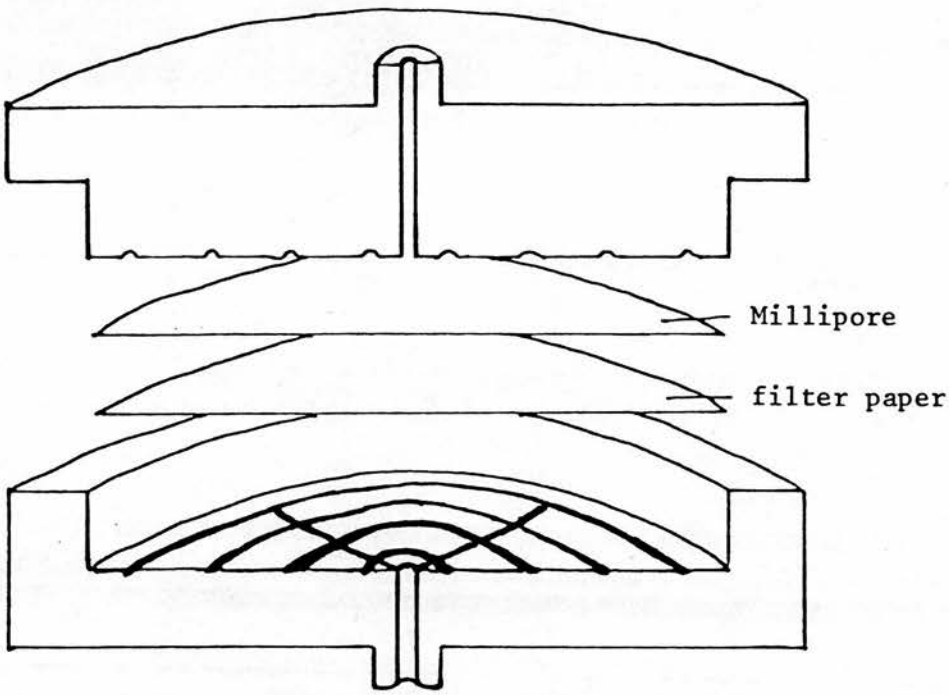

Fig. 10. Filter

reported here a Millipore filter 47mm in diameter and pore size 0.4μm was supported by a circle of hardened filter paper (Whatman No. 54). These two filters were held between the opposable surfaces of two interlocking circular Perspex discs into which eight concentric channels and four radial channels were cut to a depth of 0.5mm. Perfusate entered and left the device through channels drilled through the centre of each disc. A rubber ring made a seal when the two Perspex discs were bolted together.

Perfusate containing albumin ($0.5mg.ml^{-1}$) was found to block the Millipore filter after less than 30 minutes of perfusion. Using two discs of hardened filter paper (Whatman No. 54) prolonged perfusion was possible without any obvious harmful effect on the heart. However a filter consisting of two discs of Whatman No. 50 also blocked with perfusate containing albumin. In the experiments of Fisher and O'Brien (1972) the Millipore filter had a pore size of 3μm and was supported by Whatman No. 50 filter paper.

3.2.4 Resistance (Fig. 11)

The pressure of perfusate in the aorta was determined by the resistance to the passage of perfusate between a thin sheet of polyvinyl chloride and a Perspex surface. The resistance was varied by altering the air pressure over the sheet. Perfusate entered and left the resistance through channels drilled through a cylinder of Perspex. The polyvinyl chloride sheet covered an end surface of the cylinder and was held in place by a hollow Perspex cone which was clamped onto the cylinder. The air pressure in the cone could be varied and was measured by a Accoson sphygmomanometer which was calibrated against a column of water attached to the cannula in its heart chamber. Hearts were perfused at a pressure of 40mmHg.

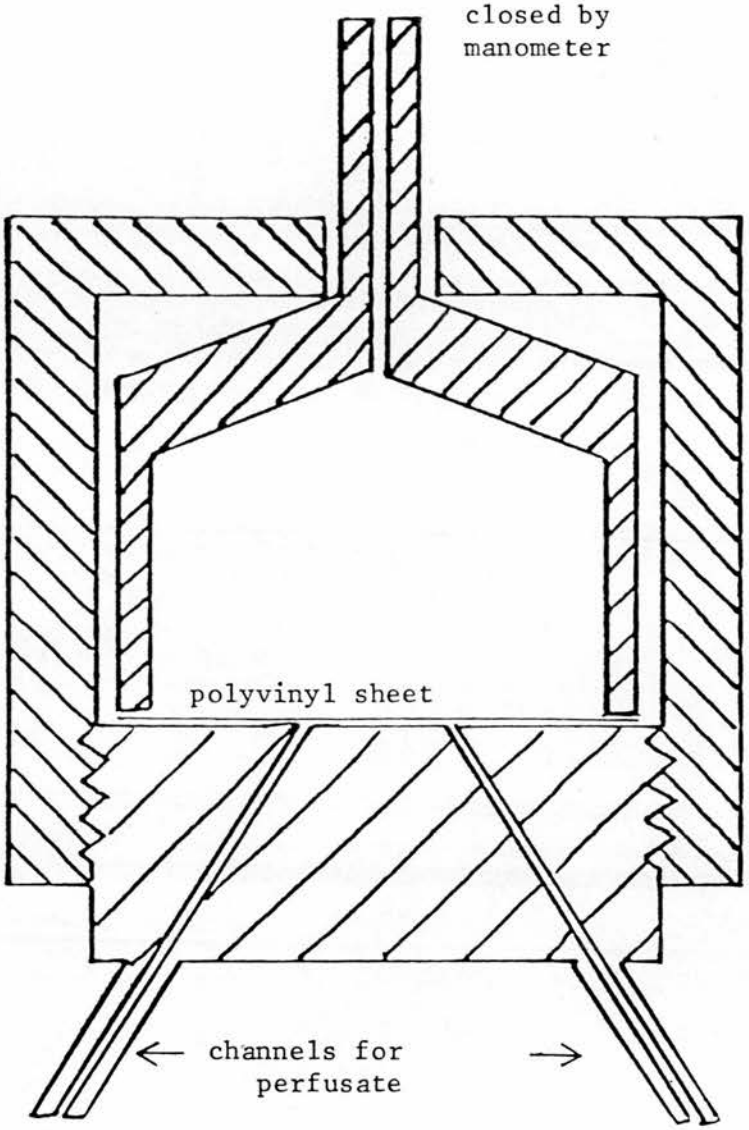

Fig. 11. Resistance.

3.2.5 Oxygenation

A 5% CO_2 in O_2 gas mixture was bubbled through water into a buffer bottle (Fig. 8, B) from which gas was drawn at approximately 40ml.min^{-1} to be mixed with the coronary effluent. That excess gas was always escaping from the buffer bottle could be seen by making it bubble through a test-tube of water. The gas which separates from the perfusate in the perfusate reservoir (Fig. 8, A) was allowed to escape and was not returned to the buffer bottle as was the practice of Fisher and O'Brien (1972). This avoids the need to make the reservoir air tight and should if anything improve the oxygenation. However the original system was capable of restoring the gas composition of the coronary effluent in one circuit of the apparatus as measured by oxygen electrodes (Personal communication Dr. J.A. O'Brien).

3.2.6 Temperature Control

Where possible the components of the apparatus were water-jacketed. The perfusate and infusate reservoirs, the heart chamber and when used the oxygenation coil were water-jacketed. When water at 38°C was circulated through the jacketing the temperature of the perfusate in the aortic cannula was 37.5°C. Strong lighting directed at the heart chamber also contributed by raising the local air temperature.

3.3 Use of the Apparatus

Before the heart was cannulated about 20ml of medium was recirculated for 15 to 20 minutes for warming, oxygenation and filtration. The tubing to the cannula and the cannula itself was filled with medium but further flow stopped with a screw clip so that all the perfusate circulated through the resistance. Similarly the tubing from the reservoir of infusate was filled but both infusion and withdrawal pumps were turned off.

On cannulation but before the heart was put into its chamber release of the screw clip immediately established perfusion which was continued for 15-20 seconds or until the blood had been washed from the heart. The heart was then placed in the heart chamber, the tubing to remove the coronary effluent attached and the chamber collapsed gently around the heart. The infusion and withdrawal pumps were started which would automatically establish the minimum circulating volume within 1 minute though this process was usually accelerated by withdrawing excess perfusate from the reservoir into a syringe.

The time when the perfusate withdrawn from the reservoir immediately after the minimum volume had been established reached the fraction collector was taken to be zero time for the start of the experiment. Samples were collected over five minute periods throughout an experiment.

The infusion rate was measured immediately before and immediately after the experiment by determining the time taken to suck 1ml of perfusate from a plastic container. The liquid did not separate into drops in this container and the end-point of the aspiration was easily seen when air entered the narrow-bore tubing that was used. In eighty randomly selected experiments the difference between the two estimates averaged 2.6%.

3.4 Isolation and Treatment of the Heart

The rat was anaesthetised with diethyl ether and the thoracic cavity was opened. Then the heart was removed and placed in cold Saline A (Section 1.3.2). While the heart was in the Saline A, attached tissues were removed and the aorta prepared for cannulation. The heart was then cannulated and washed free of blood with perfusate (Section 1.3.1). When the effluent of the heart was clear the heart

was introduced into the heart chamber. About 2 minutes passed between opening the chest and starting perfusion after cannulation.

This procedure differs from that of Fisher and O'Brien (1972) in which immediately after cannulation the heart was perfused at room temperature for 1 to 2 minutes with an oxygenated saline B solution. When equilibrated at room temperature with 5% CO_2 in O_2 gas mixture this solution (105mM-NaCl and 40mM-$NaHCO_3$) has a pH of 7.4 and physiological osmolarity. The heart was therefore exposed to an unphysiological pattern of electrolytes.

This preliminary perfusion at room temperature was used to make it easier to wash out the blood from the coronary circulation without causing clots to form. In this work washing out the blood with warm perfusate did not result in a noticeable increase in the proportion of the hearts becoming blocked. Moreover omitting the pre-perfusion avoids the risk of bubbles of gas entering the heart when it is transferred with its cannula and cannula holder from the pre-perfusion apparatus of the heart chamber. At the end of the experiment the heart was cut from the cannula and the aorta removed. The heart was blotted, weighed and dried at 110°C for 20-40h. The dry heart was covered while it cooled and then weighed to the fourth decimal place. No further loss of weight was measured when hearts which had been dried for 24h were dried for a further period of 24h. The error associated with the dry weight of the hearts was less than 1%.

3.5 Criteria of Successful Perfusion

The following criteria were checked for every heart perfused.

3.5.1 Mechanical Performance

The rate, force and rhythm of the heart were noted periodically. The rate of contraction was measurable by stopwatch only with difficulty

unless it fell below 200beats.min^{-1}. These slowly beating hearts were ignored because in studies in the same system adapted to measure pressure changes with a pressure transducer the rate of contraction was found to be normally between 250 and 350beats.min^{-1} (Personal communication by Dr. J.A. O'Brien).

3.5.2 Metabolic Stability

When experience had established that metabolic stability is normal (for instance a constant rate of glucose utilization), departure from a steady state was a criterion for rejection. Establishment of a steady state under any circumstance suggested a successful perfusion because failure would be expected to lead to inconstancy. In some experiments the rate of lactate production could give evidence of hypoxic metabolism.

3.5.3 Appearance

The softness and colour of the heart was checked at the end of each experiment. Absence of ischaemic patches was taken to be evidence of successful perfusion. The presence of ischaemic patches which might have formed late in the experiment were taken to indicate failure only if metabolic and mechanical criteria were also unfavourable.

3.5.4 Coronary Flow

At the end of each experiment a timed collection of the coronary effluent was made in a measuring cylinder. Hearts with low coronary flows (less than 25ml.g dry wt.$^{-1}$min^{-1}) were suspected of being blocked. They were accepted only if their appearance, vigour, and rate of glucose utilization suggested normal aerobic perfusion. Since the coronary flow could only be measured at the end of the experiment the results were not necessarily indicative of the state of the heart in

the middle of the experiments consequently most weight was put on the metabolic and mechanical stability of the heart.

CHAPTER 4

Characteristics of the Perfusion System

4.1 Time-course of Substrate and Product Concentrations

In the system of perfusion with balanced infusion and withdrawal the concentration of a substrate that is used at a steady rate or of a product that is produced at steady rate should tend to become constant. Examples of this are given here and in later chapters (6 and 18) and demonstrate that hearts in this perfusion system are or become metabolically stable. Analysis of the time-courses of substrate and product concentration in the perfusate allows the time-courses of the rates of utilization and production to be calculated. This is discussed in Section 4.2 of this chapter.

4.1.1 Perfusion with Glucose as Sole Substrate

Fig. 12 shows the time-courses of glucose and lactate concentration in the perfusate during an experiment of 150 minutes with a heart from a fed animal. The initial concentration of glucose of the perfusate and of the infusate was 5.5mM. No insulin was added to the perfusate. The concentration of perfusate glucose becomes steady after 60 minutes and remains so for a further 90 minutes. As will be shown in Chapter 6 a fall in the perfusate glucose concentration in the last 30 minutes is common. While the glucose concentration is steady the rate of glucose utilization must also be steady but earlier in the experiment the glucose concentration passed through a minimum after about 30 minutes. Since the rate of infusion and the composition of the infusate were unchanged throughout the experiment this pattern can only be explained as reflecting a decrease in glucose utilization to a steady rate.

The concentration of lactate in the perfusate decreases from 0.2mM after 12.5 minutes, is constant at 0.04mM from 72.5 minutes to

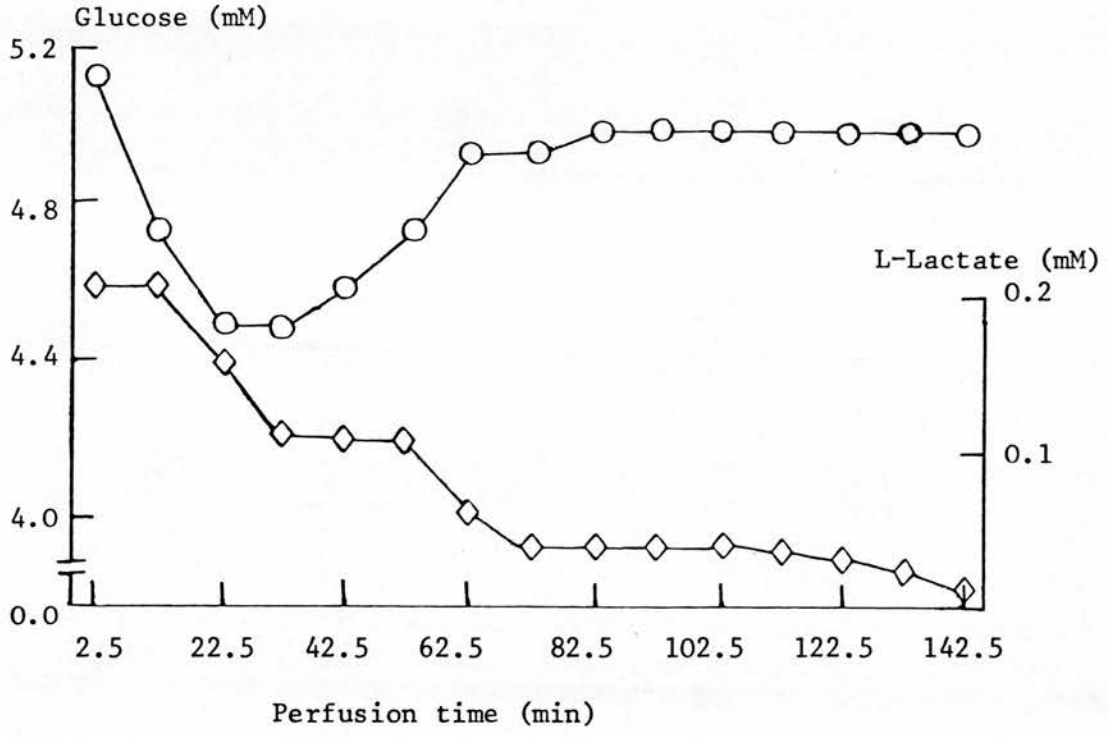

Fig. 12. Time-courses of perfusate glucose (circles) and lactate (diamonds) concentrations

A heart from a fed animal was perfused with MKHM containing initially 5.5mM-glucose.

122.5 minutes and finally falls until it is unmeasurable after approximately 140 minutes. The early fall in concentration does not necessarily indicate that the production of lactate decreases during this period. It could simply reflect the wash-out of lactate that had been produced by the heart during the period of hypoxia associated with its preparation for perfusion.

4.1.2 Perfusion with Glucose and D-3-Hydroxybutyrate as Substrates

Fig. 13 shows the time-courses of the concentrations of glucose, lactate, D-3-hydroxybutyrate and acetoacetate in the perfusate when a heart from a fed rat was perfused for 150 minutes. The infusate contained 5.5mM-glucose and 10mM-DL-3-hydroxybutyrate as did the perfusate at the start of the experiment. No insulin was included. The concentrations of the two substrates and the two products become constant within the limits of the methods of analysis after 50 minutes of perfusion. No pyruvate was detected in the perfusate. In the cases of D-3-hydroxybutyrate and acetoacetate the time-courses of concentration are consistent with a simple exponential approach to a steady state. The falling concentration of lactate suggests that either its rate of production fell initially or again that lactate formed during the isolation of the heart was washed out of the system. However the steady state concentration of lactate must indicate continuing lactate production at a lower but constant rate. The time-course of glucose concentration suggests an early minimum. In this experiment the infusion rate of 24ml.h^{-1} and the circulating volume of 7.5ml require that the half-time for the approach to a constant concentration of a substance produced or used at a steady state is 13 minutes. The relative flatness of the time-course of glucose concentration from as early as 2.5 minutes despite a fall in concentration

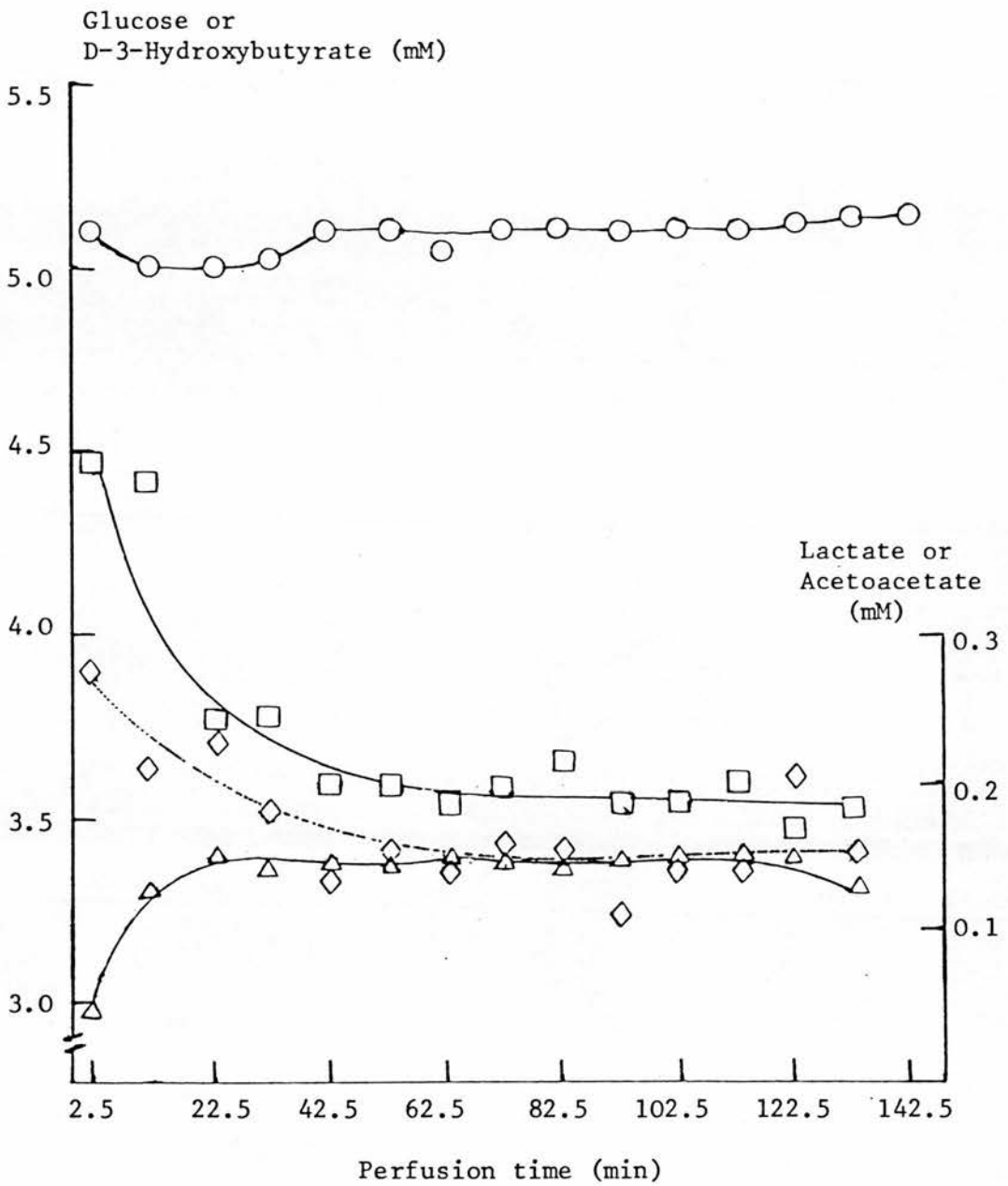

Fig. 13. Time-courses of perfusate glucose (circles), D-3-hydroxybutyrate (squares), acetoacetate (triangles) and L-lactate (diamonds) concentrations. The lines are the best fit lines drawn by eye.

A heart from a fed animal was perfused with MKHM containing initially 5.5mM-glucose and 10mM-DL-3-hydroxybutyrate.

from 5.5mM also indicates an initial fall in the rate of glucose utilization.

All four constituents of the perfusate attain clearly defined concentrations so that competing substrates can be studied each at defined concentrations of the other and without interference from the effects of the progressive accumulation of their products.

4.2 Precision of the Estimates of Utilization and Production

4.2.1 Estimates Made at Constant Perfusate Concentration

When the concentration of a substrate or product in the perfusate is constant the rate of utilization or production of the substrate is given by the equation (1)

$$v = i\,(a-x)/w \quad \ldots\ldots \quad (1)$$

where

v is the rate of utilization or production

i is the infusion rate

a is the infusate concentration

x is the perfusate concentration

w is the dry weight of the heart.

The precision of an estimate of utilization or production is not limited by the error in the determination of dry weight because the weights of hearts measured after periods of 12, 24 and 48h of drying was reproducible to within 0.5%. The results were expressed in terms of dry weight. Nor is the limiting error likely to be associated with measuring the infusion rate (Section 3.3) which remained constant during the course of an experiment to within 2.6%.

The main source of error is in the measurement of the difference between concentrations of the substrate or product in the infusate and the perfusate. In the case of product formation this amounts to

the error in the measurements of the concentration of product in the perfusate since in this work neither of the released products, acetoacetic acid or lactic acid have been included in the infusate. Therefore the probable precision of the estimation of utilization will be less than that of production so the remainder of this section will concentrate on the precision of the estimates of utilization.

It follows from equation (1) that the slower is the rate of infusion the greater will be the difference between the concentration of substrate in the perfusate and in the infusate at any particular rate of substrate utilization. However the rate of infusion also influences the time taken to reach a steady state. The half-time ($t_{\frac{1}{2}}$) for the approach to a steady state is given by

$$t_{\frac{1}{2}} = 0.693 \times P/i$$

where

0.693 is a constant (ln 2)

P is the volume of recirculated perfusate

i is the rate of infusion.

A short half-time is favoured by a high rate of infusion which reduces the difference in concentration between infusate and perfusate. This conflict between the usefulness of a large difference and a short half-time is minimized by the use of a small volume of recirculated perfusate. A volume of 6.5ml is as small as is practicable in the apparatus used but 7.0-7.5ml is more convenient and allows the polyvinyl finger in the heart chamber to sit loosely round the heart.

The range of experimental conditions that can be used is therefore restricted by the need to have a measurable difference in concentrations and a half-time for the approach to a steady state that is sufficiently short for an experiment to be complete while the heart remains

metabolically stable. The restrictions become greater at high concentrations of a substrate when its rate of utilization is independent of concentration because the difference between the infusate and perfusate concentration then becomes relatively smaller.

This effect is illustrated in Table 12 where examples are given of hearts perfused at low and high rates of infusion at 2.7mM or 5.5mM glucose with and without added insulin. In these examples the steady state concentration was determined as the mean of the concentrations of those sections which by inspection of a graph of the time-course of concentration appeared to be in a steady state. In Table 12 the standard deviation of the difference between infusate and perfusate concentration is calculated and expressed as a percentage of the difference. The mean and standard deviation of the concentrations of infusate and perfusate glucose are also given in the Table. The error in the estimate of the difference represents the error which in most circumstances would determine the precision of the estimate of glucose utilization. Although the error is acceptable under all circumstances, it is clear that low rates of infusion are preferable at physiological concentration of glucose especially in the absence of insulin. The use of higher concentrations of glucose such as 11mM or 33mM in the absence of insulin is not possible because it would require such a low rate of infusion that the half-time for the approach to a steady state would be 30 minutes or more.

In experiments with glucose and D-3-hydroxybutyrate as competing substrates in the absence of insulin the lower rate of infusion ($25ml.h^{-1}$) was used when the glucose concentration was 2.7mM or 5.5mM. Measurement of the rate of utilization of D-3-hydroxybutyrate was made easier by the relatively large differences in concentration between infusate (5mM) and perfusate (approximately 3.5mM) caused in

Table 12

The precision of the estimation of glucose utilization (see text for details)

Insulin ($2mU.ml^{-1}$)	Infusion Rate ($ml.h^{-1}$)	Infusate Conc. (mM)	Perfusate Conc. (mM)	Difference (mM)	Percent Error	Glucose Utilization ($\mu moles.g\ dry\ wt.^{-1}h^{-1}$)
-	25.9	2.74 ± 0.00 (4)	2.45 ± 0.04 (7)	0.296 ± 0.030	10	50
-	25.2	2.72 ± 0.01 (4)	2.30 ± 0.01 (7)	0.429 ± 0.180	4	81
-	60.5	2.74 ± 0.01 (3)	2.61 ± 0.01 (4)	0.140 ± 0.014	10	87
-	49.0	2.80 ± 0.00 (2)	2.55 ± 0.03 (5)	0.237 ± 0.024	10	85
-	29.5	5.51 ± 0.01 (2)	4.99 ± 0.03 (7)	0.517 ± 0.22	4	106
-	24.7	5.49 ± 0.03 (3)	4.92 ± 0.02 (8)	0.563 ± 0.025	4	112
-	56.3	5.52 ± 0.03 (3)	5.24 ± 0.03 (7)	0.28 ± 0.027	10	110
-	56.3	5.61 ± 0.01 (3)	5.47 ± 0.02 (4)	0.143 ± 0.014	10	57
+	24.7	2.75 ± 0.05 (3)	1.00 ± 0.02 (8)	1.749 ± 0.033	2	318
+	24.7	2.79 ± 0.02 (4)	1.40 ± 0.02 (6)	1.389 ± 0.02	1	243
+	55.4	2.70 ± 0.02 (2)	2.00 ± 0.01 (6)	0.696 ± 0.013	2	285
+	55.4	2.68 ± 0.02 (4)	2.00 ± 0.01 (9)	0.679 ± 0.015	2	321
+	23.1	5.78 ± 0.01 (2)	3.57 ± 0.00 (5)	2.205 ± 0.006	0.3	388
+	21.9	5.48 ± 0.05 (4)	3.18 ± 0.01 (4)	2.297 ± 0.032	1	335
+	56.3	5.46 ± 0.04 (4)	4.72 ± 0.02 (8)	0.737 ± 0.28	4	368
+	61.5	5.41 ± 0.08 (4)	4.69 ± 0.03 (13)	0.717 ± 0.046	7	309

part by the high proportion that was converted to acetoacetate.

In the rest of this thesis unless otherwise stated the calculation of utilization or production at constant concentration was based on fractions collected between 60 and 90 minutes although in some conditions metabolic stability was maintained to the end of a 150 minute experiment.

4.2.2 Estimates Made at Inconstant Perfusate Concentrations

When the concentration of a substance in the perfusate changes with time it is still possible to calculate the rate of formation or utilization of the substance. The rate of change of the amount of the substance in the recirculating perfusate must be estimated in addition to the contribution made by infusing and withdrawing the substance. The rate of utilization or production at any time is given by the equation (2)

$$v = i\,(a-x)/w - P(dx/dt)/w \quad \ldots\ldots \quad (2)$$

where

v is the rate of utilization or production

i is the rate of infusion

a is the infusate concentration

x is the perfusate concentration

P is the volume of circulated perfusate

t is the time.

A full description of the time-course of utilization or production is possible if the volume of circulated perfusate and the rate of change of concentration in the perfusate are known. The volume of circulated perfusate varied from one experiment to another mainly because it was impossible to collapse the polyvinyl finger round the heart in exactly the same way in each experiment. A minimum value

for the volume can be determined by switching off the infusion and withdrawal pumps at the end of an experiment and measuring the volume of the perfusate that can be pumped from the system. This procedure gave values in the range 6.5ml to 6.9ml. If about 10% of the perfusate remains in the apparatus on the surfaces and filter paper, 7.5ml is a reasonable estimate of the volume of recirculated perfusate. The volume cannot be as little as 6ml nor can be as large as 9ml because the introduction of so large a volume into the apparatus results in there being noticeably more perfusate in the reservoir. Table 13 shows that the effect of calculating rates of utilization assuming the volume to be 6 or 9ml rather than 7.5ml is small even when the term A that includes the volume of circulated perfusate contributes a large part of the estimate of the rate. Since the error in the value for the volume is unlikely to exceed 0.5ml the effect of this error on the estimate of utilization is unlikely to be greater than 5% in the worst case.

Two methods for the calculation of the rate of change of concentration were tested. In one, the coefficients of a fourth or higher order polynomial that best described the time-course of concentration were calculated and the rate of change of concentration was estimated from the first differential of the polynomial. This method worked adequately when the time-course of concentration was simple but if the concentration passed through an early minimum in a 150 minute experiment a fourth-order equation did not fit well to the minimum although the fit was good in the later stages. A higher-order polynomial usually gave a better fit to the minimum but not to the later period of relative constancy. In almost all cases the fit to the first and last points of the time-course of concentration was poor and this often lead to poor estimates of the rate of change of concentration

Table 13

Glucose utilization calculated for different volumes of perfusate

Time (min)	Perfusate Glucose (mM)	Term A with P at			Term B	A+B		
		6ml	7.5ml	9ml		6ml	7.5ml	9ml
2.5	5.27		-	-	-	-	-	-
12.5	4.53	167	208	250	138	305	346	388
22.5	3.88	131	163	196	233	364	396	429
32.5	3.44	62	77	93	297	359	374	390
42.5	3.36	15	19	23	309	324	327	332
52.5	3.31	12	15	18	316	328	331	334
62.5	3.26	17	21	25	322	340	344	348

The data for the perfusate glucose concentration are from a heart perfused with 5.5mM-glucose and 2mU.ml^{-1} insulin and with an infusion rate 21.88ml.h^{-1}.

Term A = $P \frac{dx}{dt} \cdot \frac{1}{w}$ and Term B = $i(a-x)/w$

A+B = rate of utilization μmoles.g dry wt.$^{-1}$h^{-1}.

at the second and penultimate points. These difficulties might have been avoided if more frequent estimates of concentration had been made at the early and late stages of the experiments, but to do this would have required that shorter or fewer experiments were done in order to keep within the capacity for analysis.

The second method of calculating the rate of change of concentration at a particular point in the time-course was based on the assumption that it approximated to the slope of the straight line joining the two neighbouring points. This procedure has the advantage of simplicity and also is applied directly to the experimental data rather than to idealized values derived from an equation fitted to the experimental data. Table 14 shows the contribution of the term A ($P.dx/dt.wt^{-1}$) that contains the rate of change of concentration, to the estimate of utilization at ten minute intervals throughout an experiment in which a heart perfused with insulin and glucose at an initial concentration of 5.5mM. The greatest contribution of term A to the estimate of utilization is 60% after 12.5 minutes of perfusion. The rate of change of concentration at 12.5 minutes is unlikely to be less than the slope of the line joining that point to its predecessor (2.5 minutes) or more than the slope of the line that joining the point to its successor (22.5 minutes). The rates of utilization of glucose at 12.5 minutes calculated using these extreme values for the rate of change of concentration are respectively 360 and 333μmoles.g dry wt.$^{-1}h^{-1}$ compared with the estimate of 346μmoles.g dry wt.$^{-1}h^{-1}$. Consequently even in the cases where the term A makes the greatest contribution the uncertainty in the estimate of the rate of concentration does not result in an unacceptable error in the estimate of utilization. Hearts which were perfused at lower concentrations of glucose and whose perfusate glucose followed a simple pattern had values for term A that

Table 14

Time-course of glucose utilization in the presence of insulin

Time (min)	Perfusate Glucose (mM)	Term A	Term B	A+B
2.5	5.27	-	-	-
12.5	4.53	208	138	346
22.5	3.88	163	233	396
32.5	3.44	77	297	374
42.5	3.36	19	308	327
52.5	3.31	15	316	331
62.5	3.26	21	323	344
72.5	3.17	11	336	347
82.5	3.18	-1	334	333
92.5	3.18	2	334	336
102.5	3.17	6	336	342
112.5	3.14	6	340	346
122.5	3.13	15	341	356
132.5	3.04	8	354	362
142.5	3.08	-	-	-

The data for the perfusate glucose concentration are from a heart perfused with 5.5mM-glucose and 2mU.ml^{-1} insulin and with an infusion rate 21.88ml.h^{-1}.

Term A = $P.\frac{dx}{dt} \cdot \frac{1}{w}$ and Term B = $i(a-x)/w$

A+B = The rate of utilization μmoles.g dry wt.$^{-1}$h^{-1}.

were a lower proportion of the estimate of utilization than in the example in Table 14.

In at least 80% of all experiments the precision with which the time-course of utilization was calculated was comparable to that illustrated in Table 14. However in some experiments when the time-course of glucose concentration passed through an early minimum or when the rate of change of concentration itself fell rapidly the estimation of the rate of change was in some instances more difficult. These patterns for the time-course of concentration establish in themselves that the rate of glucose utilization is falling during the early stages of the experiment. Table 15 illustrates the time-course of glucose utilization by a heart perfused without insulin and with, initially, 5.5mM-glucose. This experiment is typical in that the earliest estimate of glucose utilization is close to the mean for this condition and so is the constant rate in the middle of the experiment. The earliest estimate at 12.5 minutes has the highest contribution from the term involving the rate of change of concentration and the extreme values for the rate of change calculated as described above give acceptable limits to the rate of glucose utilization at 12.5 minutes of 124 and 177μmoles.g dry wt.$^{-1}$h^{-1} compared with estimate of 148μmoles.g dry wt.$^{-1}$h^{-1}. In this experiment the infusion rate was 22ml.h^{-1}. At a higher infusion rate the approach to a steady state would have been quicker once the rate of utilization had stabilized and the steady state concentration would have been higher in which case the time-course of concentration would probably have passed through a minimum as in Fig. 19, A. The rate of change of concentration at a point near the minimum calculated by the routine method was regarded as acceptable if inspection of the time-course of concentration indicated that it would provide a reasonable estimate because in this circumstance

Table 15

Time-course of glucose utilization in the absence of insulin

Time (min)	Perfusate Glucose (mM)	Term A	Term B	A+B
2.5	5.31	-	-	-
12.5	5.02	72	76	148
22.5	4.89	29	96	125
32.5	4.85	10	104	114
42.5	4.84	2	106	108
52.5	4.84	0	106	106
62.5	4.84	5	106	111
72.5	4.81	5	110	115
82.5	4.81	0	110	110
92.5	4.81	0	110	110
102.5	4.81	0	110	110
112.5	4.81	9	110	119
122.5	4.76	13	119	132
132.5	4.73	8	123	131
142.5	4.70	-	-	-

The data for the perfusate glucose concentration are from a heart perfused with 5.5mM-glucose and with an infusion rate 21.88ml.h^{-1}.

Term = $P.\frac{dx}{dt} \cdot \frac{1}{w}$ and Term B = $i(a-x)/w$.

A+B = The rate of utilization μmoles.g dry wt.$^{-1}h^{-1}$.

the calculated extreme values for the rate of change were necessarily too different to provide a useful test. This check which was applied routinely to all experiments indicated that the slope had always been given a reasonable value. This in turn suggests that although the rate of change of concentration was calculated from the concentration of a fraction whose mid-points of collection are separated by twenty minutes the frequency of sampling was adequate to describe changes in the conditions of perfusion.

Two other calculations of rates of utilization were made when the time-course of utilization showed that changes in the rate occurred early in the experiment. In one, rates of utilization were calculated for the times 7.5 minutes and 17.5 minutes by determining a rate of change of concentration from the data at 2.5 minutes and 12.5 minutes and 12.5 minutes and 22.5 minutes and assuming the concentration at 7.5 minutes and 17.5 minutes to be the average of the concentration at the neighbouring times. This gives an indication of the initial rate of glucose utilization and its subsequent change.

The second estimate is that of the overall rate of utilization in the period 2.5 minutes to 22.5 minutes. This can be calculated from the composition and volume of perfusate at the beginning and end of the period, the amount of glucose infused during this period and the amount of glucose withdrawn. The last term was calculated on the assumption that the concentration of glucose withdrawn during the first ten minutes was the average of the concentration at 2.5 minutes and 12.5 minutes and that during the second ten minutes it was the average of the concentration at 12.5 minutes and 22.5 minutes. This calculation is useful because it is equivalent to the estimate of utilization over the same period in a closed circuit system and helps comparison between this work and that of others.

4.3 The Effect of Infusion Rate on Glucose Utilization

Apart from affecting the precision of an estimate of utilization changes in infusion rate might affect the magnitude of the estimate. For instance the rate of lactic acid production might increase as the rate of infusion is increased if accumulation of lactic acid in the perfusate suppresses its own formation. The steady state concentration of lactic acid would tend to be lower at higher rates of infusion and so its rate of formation could tend to rise. This in turn might be reflected in the rate of glucose utilization. Alternatively any factor that influences glucose utilization and that can leave the heart would be expected to do so to a greater extent at higher rates of infusion. This is analogous to supposing that in a closed system of perfusion the volume of perfusate influences the rate of utilization or production of metabolites.

Table 16 shows the mean rates of glucose utilization in steady state conditions by groups of hearts perfused at different glucose concentrations, with or without insulin and at different infusion rates. There are no significant differences in the rates of glucose utilization by comparable groups that can be attributed to the rate of infusion, with the possible exception of hearts perfused with 2.7mM glucose and insulin. In the latter case the steady state concentrations differed considerably at the two rates of infusion and these differences may themselves have affected the rate of a concentration dependent process. In this thesis unless otherwise stated the results of experiments performed at 5.5mM-glucose have been aggregated regardless of infusion rate and similarly at 2.7mM-glucose in the absence of insulin but at 2.7mM-glucose in the presence of insulin the results obtained at different infusion rates have been treated separately.

Table 16

Effect of infusion rate on glucose utilization

No.	Infusion Rate ($ml.h^{-1}$)	Steady State Glucose Conc. (mM)	Glucose Utilization ($\mu moles.g\ dry\ wt.^{-1}h^{-1}$)
1	25.2 ± 0.5 (8)	2.3 ± 0.05	84 ± 6
2	51.8 ± 0.4 (4)	2.56 ± 0.04	74 ± 14
3	26.8 ± 1.4 (16)	4.85 ± 0.07	118 ± 11
4	56.25 (2)	5.33	107
5	24.8 ± 0.37 (7)	1.30 ± 0.07	261 ± 16
6	59.5 ± 1.5 (7)	1.98 ± 0.04	320 ± 21
7	26.7 ± 2.1 (8)	3.84 ± 0.13	302 ± 16
8	58.0 ± 0.9 (6)	4.64 ± 0.05	329 ± 9

Groups 1,2,5,6 received 2.7mM-glucose and groups 3,4,7,8 received 5.5mM-glucose at the infusion rates shown. Groups 5,6,7,8 were exposed to insulin at $2mU.ml^{-1}$. The numbers of observations are given in parentheses. All differences are insignificant except Group 5 vs Group 6 for which $0.05>P>0.02$. Group 4 shows the average of two observations, otherwise values are mean $\pm$ S.E.M. The mean rate of utilization and the mean glucose concentration for each heart was calculated from the values estimated at 62.5, 72.5, 82.5 and 92.5 minutes of perfusion.

4.4 Evidence of Aerobic Metabolism

4.4.1 Adequacy of Coronary Flow

To measure the coronary flow, the heart, while still being perfused, was taken from its chamber at the end of each experiment and the coronary effluent was collected in a measuring cylinder for one minute. This required that the volume of perfusate available to the heart was increased. Warmed oxygenated perfusate was taken from the infusate reservoir and added to the perfusate reservoir. No attempt was made to measure the coronary flow at the beginning of the experiment when priority was given to establishing as soon as possible a minimum volume of recirculating perfusate.

In 18 hearts perfused with 5.5mM-glucose the mean coronary flow rate and its S.E.M. was 49.8±3.2ml.g dry wt.$^{-1}$min^{-1} with a range from 29.9 to 89.2ml.g dry wt.$^{-1}$min^{-1}. Similarly in 12 hearts perfused with 2.7mM-glucose, the mean rate was 46.9±4.6ml.g dry wt.$^{-1}$min^{-1} with a range from 21.1 to 84.9ml.g dry wt.$^{-1}$min^{-1}. These values were obtained with a perfusion pressure of 40 to 44mm.Hg and were measured at the end of experiments lasting 150 minutes. Under the same conditions in 13 hearts perfused with 5.5mM-glucose and 2mU.ml^{-1} insulin the mean rate of coronary flow was 52.03±3.8ml.g dry wt.$^{-1}$min^{-1} with range from 17.61 to 72.9ml.g dry wt.$^{-1}$min^{-1}. Similarly in 14 hearts perfused with 2.7mM-glucose and 2mU.ml^{-1} insulin, the mean rate of coronary flow was 43.06±3.8ml.g dry wt.$^{-1}$min^{-1} with a range from 27.3 to 77.04ml.g dry wt.$^{-1}$min^{-1}. There is no statistically significant difference in the rate of coronary flow between hearts perfused with glucose and hearts perfused with glucose and insulin at either glucose concentration.

The possibility that large differences in coronary flow rate might cause or reflect limitations in supply of oxygen to the heart was examined.

The correlation between glucose utilization and coronary flow rate was tested for the four groups of hearts perfused with 2.7mM and 5.5mM-glucose with and without insulin at 2mU.ml^{-1}. The correlation coefficients varied from -0.51 to +0.17 and none were significantly different from zero. In these tests the rate of utilization in a period of steady state was compared with its coronary flow at the end of the experiment. As shown in Section 6.6 the glucose utilization tends finally to increase in hearts perfused without insulin for 150 minutes. So the comparison is not necessarily valid. However hearts perfused with insulin usually maintain their steady state to the end of the perfusion.

Opie (1965) measured glucose utilization in hearts perfused at high and low rates of coronary flow which he kept constant by adjusting the perfusion pressure. Although the pressure was increased by 39% and 62% in 20 minutes in maintaining flows of 34±2 and 105±9ml.g dry wt.$^{-1}$min^{-1} respectively the rates of glucose utilization in the two groups of hearts were similar being 168±21 and 196±22μmoles.g dry wt.$^{-1}$h^{-1} respectively.

4.4.2 Lactate Production

Fisher and O'Brien (1972) using the system of perfusion with infusion and withdrawal found that the rate of lactate production was less than 10μmoles.g dry wt.$^{-1}$h^{-1} in 42% of hearts perfused without insulin and at glucose concentrations between 0.25mM and 15mM and was less than 50μmoles.g dry wt.$^{-1}$h^{-1} in 78% of these experiments. As will be discussed (Section 5.1) the reported rates of lactate production by hearts perfused in closed circuit systems without insulin and with 5mM-to 11mM-glucose are very variable ranging from zero to 232μmoles.g dry wt.$^{-1}$h^{-1} (Table 17).

For this work three hearts were perfused for 150 minutes without insulin and with an initial glucose concentration of 5.5mM. One of these experiments was illustrated in Fig. 12 and discussed in Section 4.1.1. The other two experiments were similar. The rate of lactate production fell to steady low rate after 60 minutes and thereafter averaged 6μmoles.g dry wt.$^{-1}h^{-1}$ when the average rate of glucose utilization was 74μmoles.g dry wt.$^{-1}h^{-1}$. This low proportion of glucose converted to lactate indicates that the hearts were adequately supplied with oxygen.

PART II

UTILIZATION OF GLUCOSE AS THE SOLE SUBSTRATE

CHAPTER 5

Introduction

5.1 Variability of Estimates of Glucose Utilization

In the general introduction to this thesis the usefulness of the isolated perfused rat heart in making metabolic studies was suggested to depend on the stability and reproducibility of its properties. Measurements of glucose utilization by the isolated rat heart have been reported for many years and they differ considerably. In Table 17 examples are given of published estimates of glucose utilization by hearts taken from fed rats and perfused without insulin. The choice of examples was limited to work published in the last twenty years because the methods of perfusion used are still used nowadays and most of them are derived from systems described by Bleehen and Fisher (1954) and Morgan et al. (1961). The values quoted in Table 17 range from 30 to 420μmoles.g dry wt.$^{-1}$h^{-1}.

Such large variations in the rates observed by different authors are commented upon (e.g. Randle et al., 1964; Meuli and Froesch, 1975) but only one report has been found in the literature of very variable results in a single investigation. Bleehen and Fisher (1954) observed low, medium, and high rates of glucose utilization in the absence of insulin. The rates in these groups were 59, 120 and 252μmoles.g dry wt.$^{-1}$h^{-1} respectively. Cheung, Conover, Regen, Whitfield and Morgan (1978) using fasted rats apparently obtained rates of glucose utilization of 93 and 270μmoles.g dry wt.$^{-1}$h^{-1} when the concentrations of glucose were 11.0 and 9.4mM respectively, but did not comment on this difference. In contrast Randle et al. (1964) in three separate experiments measured the rate of glucose utilization as 239, 255 and 294μmoles.g dry wt.$^{-1}$h^{-1}. The production of lactate was not always measured in studies of glucose

Table 17

Glucose utilization and lactate production by hearts from fed rats

Initial Glucose conc. (mM)	Preperfusion Period (min)	Period of Estimation (min)	Glucose[A] Utilization (μmoles.g dry wt.$^{-1}$h^{-1})	Lactate[B] Production (μmoles.g dry wt.$^{-1}$h^{-1})	$\frac{B}{2A}$ X 100	Reference
5	15	15-75	72	0.0		Williamson and Krebs (1961)
5	5	5-35	299*	206	34	Opie et al. (1962)
5	15	15-45	293*	-		
5	5	5-35	345*	232	34	Opie et al. (1963)
5.5	0	0-15	239*	-		Randle et al. (1964)
5.5	0	0-15	255*	-		
5.5	0	0-15	294*	-		
5	10	10-40	406*	-		Shipp et al. (1964)
5	10	10-40	391*	184	23	Shipp (1964)
5	15	15-45	102	24	12	Williamson (1964)
5.5	10	10-40	62	14	11	Morgan et al. (1965)
5	15	15-75	77	14	9	Williamson (1965)
11.1	15	15-45	222	100	23	Chain et al. (1969)
5	15	15-45	238	92	19	
10	15	15-45	280	114	20	

Table 17 contd.

Initial Glucose conc. (mM)	Preperfusion Period (min)	Period of Estimation (min)	Glucose[A] Utilization (μmoles.g dry wt.$^{-1}$h^{-1})	Lactate[B] Production (μmoles.g dry wt.$^{-1}$h^{-1})	$\frac{B}{2A}$ X 100	Reference
5	10	10-60	129	58	22	Crass et al. (1969)
11	15	15-45	154*	70	23	Opie et al. (1971)
11	15	15-45	221*	116	26	
5	0	0-20	420*	64	8	Wieland et al. (1971)
7.7	0	70-80	86	-		Fisher and O'Brien (1972)
5	15	15-60	30	10	17	Meuli and Froesch (1975)
5	12	12-42	258	90	17	Orme et al. (1977)

Hearts from fed rats perfused with glucose.

*Units converted from wet weight taking the ratio of wet to dry weight to be 5:1.

utilization. However the available data are given in Table 17 and plotted in Fig. 14. There is a close correlation between the rates of lactate production and of glucose utilization with the exception of the data from Wieland *et al.* (1971).

5.2 Effect of Anti-Insulin Serum

The cause of the inconsistency in the measured rates of glucose utilization has been said to be differences in the amount of endogenous insulin remaining in the heart during the period of measurement. Mansford (1967) found that the administration of anti-insulin serum one hour before removal of the heart reduced the subsequent rate of glucose utilization by 58%. Addition of anti-insulin serum to the perfusate is without effect on the rate (O'Brien, 1969) but there is no reason to suppose that this treatment will increase the rate at which the insulin content of the isolated heart falls. It is also possible that the effect of anti-insulin serum administered *in vivo* comes about indirectly by altering the levels of other hormones as well as insulin and the availability of substrates other than glucose. Whether the effect is direct or indirect it is likely to involve a change in the permeability of myocardial cells because Zachariah (1961) showed that *in vivo* administration of anti-insulin serum reduced the initially high permeability to non-metabolized sugars that was found in the isolated heart.

5.3 Effect of Pre-perfusion and Extended Perfusion

Randle *et al.* (1964) when they compared their results with those of Williamson and Krebs (1961) also explained the three to four fold difference in terms of the effect of endogenous insulin. They suggested that the practice of Williamson and Krebs of perfusing the heart for 15 minutes before measuring glucose utilization during the next hour

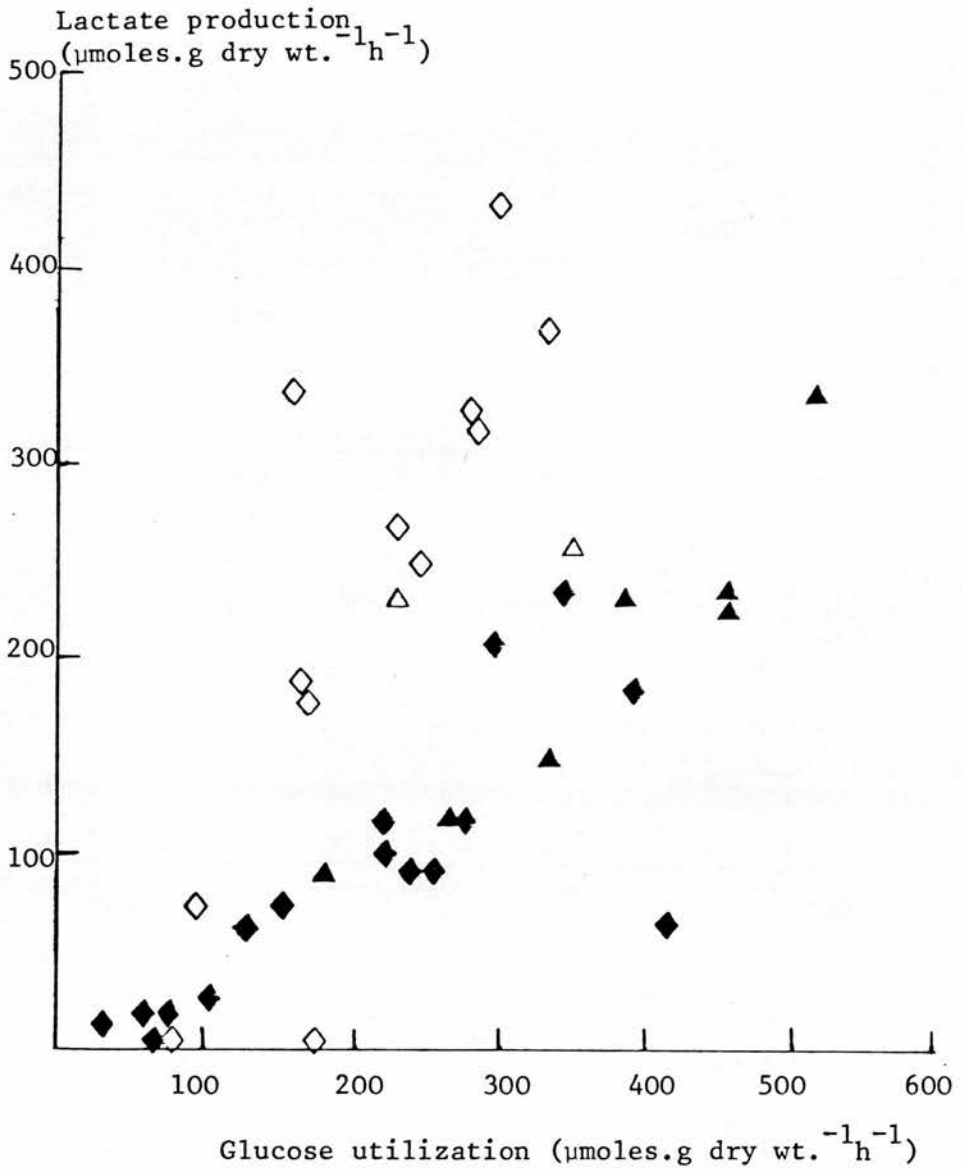

Fig. 14. The relationship between glucose utilization and lactate production. The data are taken from Tables 17 to 21. Open symbols indicate hearts from fasted animals, filled symbols indicate hearts from fed animals, diamonds indicate the absence of insulin, triangles indicate the presence of insulin.

of perfusion reduced the level and effect of endogenous insulin in comparison with their own procedure of measuring utilization as soon as practicable. They washed the heart through with 10 to 15ml of medium taking perhaps 2 minutes and then measured glucose utilization during 15 minutes of recirculated perfusion. Williamson and Krebs waited 15 minutes before making observations to allow a period of falling oxygen consumption and heart rate to pass (Fisher and Williamson, 1961a,b). This period before measurements are made has become common practice and is often known as the pre-perfusion period. Its duration varies from worker to worker and it may involve once-through or recirculated perfusion. The estimates of glucose utilization would be expected to vary with the duration of the preperfusion period if Randle et al. (1964) and Mansford (1967) are correct in believing that the level of endogenous insulin is the cause of the differences in the estimates.

Examination of Table 17 shows that the duration of the preperfusion has no obvious effect on the size of subsequent estimates of glucose utilization. Opie, Shipp, Evans and Leboeuf (1962) reported similar results (299 and 293μmoles.g dry wt.$^{-1}h^{-1}$) whether the preperfusion lasted 5 or 15 minutes respectively. Shipp, Matos, Kinzley and Crevasse (1964) using a preperfusion period of 10 minutes and making their estimate of utilization over the next 30 minutes obtained a value of 406μmoles.g dry wt.$^{-1}h^{-1}$.

There are few reports of investigations of the effect of the duration of perfusion on the estimate of glucose utilization. If endogenous insulin was having a diminishing effect as perfusion was prolonged its rate of utilization would be expected to fall with time. Randle et al. (1964) state that they found no difference in the glucose utilization by hearts from fed animals when measured during the first

15 minutes or the first 30 minutes of perfusion. In contrast to this are the results of Fisher and O'Brien (1972) who followed the time-course of glucose utilization during the approach to a steady state of glucose concentration and found that when the concentration exceeded 1mM the rate in the absence of added insulin declined during the first 40 minutes of perfusion and then became relatively constant. As was indicated in Section 4.1.1 and will be substantiated in Section 6 this behaviour has been confirmed in this work. The earliest estimate of utilization reported by Fisher and O'Brien was made at 10 minutes without preperfusion other than 3 to 4 minutes to wash away the blood and to establish a fixed volume of recirculating perfusate. They made few estimates at any one concentration of glucose but their published data indicate a rate of 86μmoles.g dry wt.$^{-1}$h^{-1} at around 7.7mM after 45 minutes of perfusion in comparison with the rate of between 239 and 294μmoles.g dry wt.$^{-1}$h^{-1} found by Randle et al. (1964) for the first minutes of perfusion following wash-out of blood. The overall rate of utilization found by Fisher and O'Brien (1972) between 15 and 45 minutes of perfusion was 155μmoles.g dry wt.$^{-1}$h^{-1} which in comparison with an equivalent period in the work of others is similar to the rate found by Opie, Mansford and Owen (1971), half that found by Opie et al. (1962) and 50% greater than that reported by Williamson (1964). Fisher and O'Brien (1972) argued that their results supported the concept of a washout of endogenous insulin influencing the rate of glucose utilization by the isolated heart. However although their results and those of Mansford (1967) and Zachariah (1961) are consistent with this idea there is considerable variation between results obtained under conditions when washout of insulin should be similar.

When the period of perfusion is extended the rate of glucose utilization in the absence of insulin appears to increase. In an

experiment lasting 200 minutes Fisher and O'Brien (1972) found a period of metabolic stability between 90 minutes and 120 minutes that was followed by a progressive increase in the rate of utilization. Neely, Libermeister and Morgan (1967b) reported that the rate of utilization were 77μmoles.g dry wt.$^{-1}$h^{-1} between 15 minutes and 75 minutes and 173μmoles.g dry wt.$^{-1}$h^{-1} between 85 minutes and 145 minutes.

5.4 Effect of Fasting

If endogenous insulin is the main cause of the large variation in reported estimates of glucose utilization, more consistent results should be obtained with hearts from fasted animals whose concentration of plasma insulin is low. Table 18 gives examples of estimates of glucose utilization by hearts taken from fasted rats and perfused without added insulin. Where possible the examples are taken from publications appearing during the same period and having the same origins as those in Table 17. In some instances the glucose utilization by hearts from fed and from fasted animals, were reported in the same publication and these results are brought together in Table 19. All workers observed a reduction of glucose utilization in hearts from fasted animals, although Shipp (1964) found only a small reduction. However Shipp *et al.* (1964) did find a marked reduction, using the same experimental procedure. Yet they found similar rates with hearts from fed animals while the rates reported by Shipp *et al.* (1964) for hearts from fasted rats were lower than those of Shipp (1964) by some 30%.

Inspection of Table 18 suggests that fasting reduces the scatter of results (73 to 335μmoles.g dry wt.h^{-1}) while still leaving a four-fold variation. However this reduction largely disappears if the unusually low rate found by Meuli and Froesch (1975) with fed animals

Table 18

Glucose utilization and lactate production by hearts from fasted rats

Duration of Fast (h)	Initial Glucose (mM)	Preperfusion Period (min)	Period of Estimation (min)	Glucose Utilization (μmoles.g dry wt.$^{-1}$h^{-1})	Lactate Production (μmoles.g dry wt.$^{-1}$h^{-1})	Reference
18	5	1	0-15	136	-	Morgan et al. (1959)
18	5	1	15-30	119		
18	5	1	0-15	150	-	Morgan et al. (1961)
18	5	1	15-30	142	-	
overnight	5	5	5-35	298	430	Shipp et al. (1961)
overnight	5	5	5-35	168	174	Opie et al. (1962)
overnight	5	5	5-35	230	268	Opie et al. (1963)
overnight	20	5	5-35	163	188	
96	5	5	5-35	157	334	
18	5.5	2	0-15	161	-	Randle et al. (1964)
18	5.5	2	0-15	114	-	
18	5.5	2	15-30	150	-	
40	5.5	2	0-15	89	-	
overnight	5	10	10-40	276	328	Shipp et al. (1964)
overnight	5	10	10-70	245	246	
overnight	5	5	5-35	306	-	Shipp (1964)
overnight	5	10	10-40	285	310	
overnight	5	10	10-40	335	366	

Table 18 contd.

Duration of Fast (h)	Initial Glucose (mM)	Preperfusion Period (min)	Period of Estimation (min)	Glucose Utilization (μmoles.g dry wt.$^{-1}$h^{-1})	Lactate Production (μmoles.g dry wt.$^{-1}$h^{-1})	Reference
18	4.7	15	15-75	90	70	Neely et al. (1967b)
18	5	15	15-75	77	3	
	-	-	85-145	173	0.5	
not given	9.4	10	10-70	270		Cheung et al. (1978)
	11	35	35-50	93	-	
	20	35	35-50	73	-	

All units except for the data of Neely et al. and Cheung et al. are converted from the wet to dry weight taking the ratio of wet to dry weight to be 5:1.

Table 19

The effect of fasting on glucose utilization

Glucose Utilization (μmoles.g dry wt.$^{-1}$ h^{-1})		Ratio $\frac{\text{Fasted}}{\text{Fed}}$	Reference
Fed	Fasted		
293	168	0.57	Opie et al. (1962)
345	230	0.66	Opie et al. (1963)
345	163	0.47	
294-239	161	0.54-0.67	Randle et al. (1964)
	114	0.38-0.47	
	89*	0.3 -0.37	
391	306,285,335	0.78,0.73,0.86	Shipp (1964)
406	276	0.68	Shipp et al. (1964)

The data are for rats fasted overnight except for (*) when the period of fasting was 40h. When data were corrected from wet to dry weight a ratio of 5:1 was assumed.

is discounted. Again there is a close correlation between the rate of lactate production and glucose utilization (Fig. 14). Fasting increases the proportion of the glucose consumed that is converted to lactate. A comparison of Tables 17 and 18 suggests from work done in one laboratory over a period of years that fasting of animals apparently increases the rate of utilization by the isolated heart. Morgan, Neely, Wood, Liebecq, Liebermeister and Park (1965) reported lower rates of utilizatio n by heart from fed animals than were found by Morgan et al. (1961), and attributed the difference to an improvement in technique; 'improved stability of the preparation has been accompanied by much lower rates of glucose uptake in aerobic non-working hearts than those found earlier with less satisfactory perfusion technique'.

Further improvements on the technique of Morgan et al. (1965) were claimed by Neely et al. (1967b) to explain their lower control values, and low rates of lactate formation. Morgan et al. (1965) used a 10 minute preperfusion and a thirty minute period of measurement whereas Morgan et al. (1961) did not preperfuse and usually used a 15 minute period of measurement but found no difference in rates of utilization in the first and second fifteen minute periods of perfusion. Other differences were that Morgan et al. (1965) included heparin in the perfusate during the perfusion and calcium-ethylene diamine tetra-acetic acid (Ca.EDTA) throughout the experiment. However the exclusion of CA-EDTA was without effect on glucose utilization in the absence of insulin and Orme, Kelly, Brendel and Bressler (1977) using the same procedure as Morgan et al. (1965) observed rates of 258umoles.g dry wt.$^{-1}$h^{-1} in comparison with 62 μmoles.g dry wt.h^{-1} by Morgan et al. (1965).

5.5 Variability of Glucose Utilization in the Presence of Insulin

Although the common finding, that glucose utilization is lower in hearts from fasting animals than in hearts from fed animals, suggests that the lower plasma insulin concentrations of the animals may have direct or indirect effects on the isolated hearts, large variations in the reported rates remain unexplained. When isolated hearts are perfused with insulin the variability of the results is reduced. Tables 20 and 21 give estimates of glucose utilization in the presence of insulin measured with hearts from fed and fasted animals respectively. In Table 20 the estimates range from 180 to 520μmoles.g dry wt.$^{-1}$h^{-1}. If the lowest value is ignored the range becomes 266 to 520μmoles.g dry wt.$^{-1}$h^{-1}. This lowest value and also the lowest value in the absence of insulin (Table 17) were found by Meuli and Froesch (1975). In both cases the estimates are the lowest by a wide margin but the technique used differs from that of most workers only in the inclusion of albumin in the perfusate. However Randle *et al.* (1964) found no effect of albumin on glucose utilization. In Table 21 the range is from 261 to 436μmoles.g dry wt.$^{-1}$h^{-1} which is small in comparison with the spread seen in the absence insulin.

The correlation (Fig. 14) between lactate production and glucose utilization by hearts from fed animals is again good.

5.6 Effect of Methodological Differences

From this analysis of the literature on glucose utilization by the isolated perfused rat heart it appears that what agreement there is between the results of different workers is mostly qualitative. It is always found that insulin stimulates glucose utilization and that when the point is specifically investigated (Table 19) the heart from a fasted animal uses less glucose in the absence of insulin than

Table 20

Glucose utilization by hearts from fed rats in the presence of insulin

Initial Glucose (mM)	Insulin ($mU.ml^{-1}$)	Preperfusion Period (min)	Period of Estimation (min)	Glucose Utilization ($\mu moles.g\ dry\ wt.^{-1}h^{-1}$)	Lactate Production ($\mu moles.g\ dry\ wt.^{-1}h^{-1}$)	$\frac{B}{2A} \times 100$	Reference
5	2	15	15-75	266	114	21	Williamson and Krebs (1961)
5.5	100	0	0-15	385*	230	30	Garland et al. (1962)
20	100	5	5-35	520*	334	32	Opie et al. (1963)
5.5	100	0	0-15	394*	-	-	Randle et al. (1964)
5.5	12	10	10-40	298		-	Morgan et al. (1965)
5	2	15	15-75	334	148	22	Williamson (1965)
11	2	15	15-45	456	230	25	Chain et al. (1969)
6	100	0	45-90	311	-	-	O'Brien (1969)
11		15	15-45	456*	226	25	Opie et al. (1971)
5	2	15	15-60	180	86	24	Meuli and Foresch (1975)

*Units are converted from wet to dry weight, taking the ratio of wet to dry weight to be 5:1.

Table 21

Glucose utilization by hearts from fasted rats and in the presence of insulin

Initial Glucose (mM)	Insulin ($mU.ml^{-1}$)	Preperfusion Period (min)	Period of Estimation (min)	Glucose Utilization ($\mu moles.g\ dry\ wt.^{-1}h^{-1}$)	Lactate Production ($\mu moles.g\ dry\ wt.^{-1}h^{-1}$)	Reference
5	100	0	0-15	277*	-	Morgan et al. (1959)
5	100	0	0-15	297*	-	Morgan et al. (1961)
5.5	100	0	0-15	23o*	230	Garland et al. (1962)
20	100	5	5-35	352*	256	Opie et al. (1963)
5.5	100	0	0-15	261*	-	Randle et al. (1964)
10	25	10	10-60	436	-	Cheung et al. (1978)

*Units are converted from wet to dry weight taking the ratio of wet to dry weight to be 5:1.

The period of fast was overnight or 18h except Randle et al. (40 hr), and Garland et al. (40-48 hr), and was not given by Cheung et al.

does a heart from a fed animal. Most disagreement is found in the amount of glucose used by hearts from fed animals when perfused without insulin. The disagreement cannot apparently be fully explained in terms of the effects of endogenous insulin persisting in the isolated heart.

If the source of the inconsistencies in the reported values of glucose utilization is methodological it should become apparent from the descriptions of the techniques employed by the authors of the papers referred to in Tables 17 to 21. No such explanation was found in a search that included the following features.

5.6.1 State and Nature of the Animals

Not all authors give full details of strain, sex and weight of the rats used and few refer to the conditions of heat and light in which they were kept before the experiment. Rats of the Wistar and Sprague Dawley strains have been used most commonly. Opie, Evans and Shipp (1963) used both strains and reported no effect of strain. Williamson and Krebs (1961), Williamson (1964) and Williamson (1965) have used different strains on different occasions with the same results while different workers e.g. Randle et al. (1964) and Williamson and Krebs (1961) have used the same strain with very different results. Only Meuli and Froesch appear to have used the Osborne Mendel strain so it is impossible to know whether this choice explains the low rates of glucose utilization that they observed.

The animals were male when the sex was described and weighed between 200 and 350g aged 42 to 72 days. The animals were therefore adult. When the animals were fed up to the time of use the composition of diet, if described, is usually said to be standard which implies a moderately high carbohydrate diet. The lack of information on the

temperature and condition of lighting in which the animals were kept is unfortunate because Young (1965) found that isolated hearts vary in their sensitivity to insulin according to the length of the day-light. He was able to reproduce the seasonal change by altering the exposure of rats to artificial light. Differences in sensitivity to insulin could explain some of the variability in glucose utilization because although it is probable that the animals were kept under constant conditions, and authors took such a precaution for granted, the period of lighting might easily vary between laboratories by two hours or more per day.

5.6.2 Anaesthetics and Method of Sacrifice

There are several differences in the ways the animals were treated before the removal of the heart. The rat may be anaesthetised with one of several anaesthetics. If so heparin may or may not be injected to reduce the likelihood of clots forming in the coronary circulation. Alternatively the rat may be decapitated and again heparin may be injected before this. No rates of glucose utilization less than 100μmoles.g dry wt.$^{-1}$h^{-1} have been reported when animals were decapitated but the use of anaesthetics does not necessarily lead to low rates, for example the extreme values in Table 17 were obtained with hearts from anaesthetised animals. The anaesthetic used is not always identified but Nembutal for example has been used with heparin by Randle et al. (1964) and Morgan et al. (1965) with very different rates of utilization being found. Those who have used ether anaesthesia (Bleehen and Fisher, 1954; Williamson and Krebs, 1961; Fisher and O'Brien, 1972) observed relatively low rates of utilization in the absence of insulin. In this work ether has been used because it is very volatile and should not persist long in the

isolated heart and because one purpose was to confirm the observations of Fisher and O'Brien. For the latter reason heparin was also avoided. Since the concentration of insulin in blood is influenced by the use of anaesthetics (Aynsley-Green, Biebuyck and Alberti, 1973) it is unfortunate that the nature and period of exposure to an anaesthetic are not routinely specified.

5.6.3 Preliminary Handling of the Heart

After they excised the heart almost all workers placed it in an ice-cold medium which results in the heart ceasing to beat, slows down all metabolic processes including that of blood coagulation and so enables the aorta to be prepared for cannulation. Some workers used a physiological saline solution but several used a bicarbonate buffer (Krebs and Henseleit, 1932). Such a medium has a pH of 7.4 when equilibrated with carbon dioxide at a partial pressure of 40mmHg and at 37°C. At 0°C to 4°C equilibrated with atmospheric carbon dioxide the pH is raised. In this work, following the practice of Fisher and O'Brien (1972) an isomolar saline solution (Saline A, Section 1.3.2) was used that has a pH of 7.4 when equilibrated with air at 0°C. Only Bleehen and Fisher (1954) and Meuli and Froesch (1975) prepared the heart for cannulation in warmer solutions. There is no detectable relation between the procedure used and the observed rate of glucose utilization.

5.6.4 Pre-perfusion Procedures

This aspect of perfusion technique was described earlier and it was argued that the duration of the period has no consistent effect on subsequent estimates of glucose utilization in the absence of added insulin.

5.6.5 Volume and Composition of the Perfusate

All perfusates are based on the medium of Krebs and Henseleit (1932). The original medium and modifications in which the concentrations of calcium or magnesium or both are halved to allow for the binding of these cations to plasma proteins (Greene and Power, 1931) have been used equally often. High and low rates of glucose utilization in the absence of insulin have been reported with both the standard and modified perfusate.

There are two ways in which the volume of recirculated perfusate might affect the estimate of glucose utilization. The first is an extension of the idea that endogenous insulin influences the rate of glucose consumption by the isolated heart. Dilution of the insulin through a large volume might increase the rate of loss of insulin from the heart. It was noted earlier that pre-perfusion without recirculation did not result in lower rates of utilization in the subsequent period of measurement than followed preperfusion with recirculation. However Opie et al. (1971) using 40ml and 15ml and 15 minutes of preperfusion reported value of 154μmoles.g dry wt.$^{-1}$h^{-1} while Williamson and Krebs (1961) by using 17-25ml and 15 minutes of preperfusion found 72μmoles.g dry wt.$^{-1}$h^{-1}. The second effect of volume that might be proposed would suggest that rates of glucose utilization should increase with volume. There is inevitable loss from the heart into the perfusate of those substances to which the cell membrane are permeable. Lactate and pyruvate are such substances and the amount of them that is lost and the amount of glucose that may be converted to them should increase with the volume of perfusate. Any such effect of volume is equivalent in the system used here to an effect of infusion rate. This was investigated and no effect found (Section 4.3). Most of the results in Table 17 were obtained with

hearts perfused with 15-30ml of perfusate. No effect of the volume or composition of perfusate on glucose utilization is therefore evident.

5.6.6 Perfusion Pressure

The oxygen consumption of the isolated heart increases with the pressure at which the perfusate is delivered to the heart (Opie et al., 1971; Neely et al., 1967a; Neely, Bowman and Morgan, 1969). Glucose consumption in the absence of insulin is thought to be limited by the rate of glucose permeation into the cells so would only be expected to vary with perfusion pressure if membrane permeability was also altering. Increased work by the heart and hypoxia do increase permeability to glucose (Opie et al., 1971; Neely, Whitmer and Rovetto, 1975). Neely et al. (1967a) found that glucose utilization varied with perfusion pressure but was relatively consistent between 40 and 60mmHg. Most of the authors quoted in Table 17 used perfusion pressures of 40 to 60mmHg and large differences in glucose consumption were found at the same pressure e.g. Shipp (1964): 391μmoles.g dry wt.$^{-1}$h^{-1} and Crass, McCaskill and Shipp (1969): 129μmoles.g dry wt.$^{-1}$h^{-1} both used 60mmHg.

5.6.7 Temperature

In most work the hearts were perfused at 37°C. Some authors (Opie et al., 1962; Meuli and Froesch, 1975) used perfusate at 38°C, others worked at 36°C (Neely et al., 1967a; Cheung et al., 1978) at 35°C. No systematic studies appear to have been made of the effects of such differences in temperature. It is unlikely to explain the variability of results because use of perfusate at 37°C is associated with a high degree of variability.

5.6.8 Filtration

Details of the method of filtration of perfusates are not always given. In Chapter 7 experiments are described that were made late in this project and showed that the nature of the filter affects the rate of glucose utilization at constant concentrations of glucose in the absence of insulin. This may be related to the observation of Zachariah (1961) who showed that the use of large sintered glass filters resulted in increased permeability to non-metabolized sugars when the perfusate did not contain protein.

5.6.9 Conclusion

To summarise there is no single variable in the technique of cardiac perfusion as reported in the literature of the subject that can explain the inconsistency of the published estimates of glucose utilization in the absence of insulin. This should mean that there is no reason for accepting any one estimate rather than another. However the higher estimates are the more dubious simply because their association with high rates of lactate formation suggests hypoxic metabolism. If the higher estimates in Table 17 are erroneous the error must have occurred regularly because the standard error of the mean of those estimates is not proportionally larger than the SEM for lower estimates. There are two possible sources of damage to the isolated heart which can not be deduced to have occurred from the description of the methodology. First faulty cannulation can give high rates of glucose consumption. Bleehen and Fisher (1954) showed that if the cannula is inserted so deep into the aorta that it affects the competence of the aortic valves the glucose utilization increases markedly. The left ventricle does more work in attempting to expel the perfusate that enters it. The objection to this as an explanation is that it is

unlikely to be a regular error. The second possibility concerns the time taken between excising the heart and establishing perfusion. Recovery from the trauma of isolation may be less complete the more the onset of perfusion is delayed. When the time taken is extended beyond 1 minute there is a much greater chance that the heart becomes unstable metabolically (Personal communication by J.A. O'Brien). Details on this aspect of technique are rarely if ever given. However Ross (1972) emphasises the need to minimize the period of anoxia during the isolation of the heart. The effect of glass filters observed by Zachariah (1961) was only evident after 40 minutes and does not explain differences in the early rates of glucose utilization.

5.7 Experimental Investigation

When there is so much uncertainty about the physiological relevance of estimates of glucose utilization that have been made in the past, it is justifiable to apply a different approach to the problem. The perfusion system used here has the advantages that the time course of utilization can be measured and that the stability of the metabolic performance by the heart can be followed. Several aspects of glucose utilization have been studied. The observations of Fisher and O'Brien (1972) on glucose utilization by hearts from fed rats have been extended according to a different experimental design. The effect of fasting has been investigated for the first time with the novel system of perfusion. In addition the sensitivity of the heart to insulin has been studied. The hypothesis that endogenous insulin modifies the rate of glucose utilization by the isolated heart implies that the heart is sensitive to physiological concentrations of insulin. Finally the particular facility of perfusion with infusion and withdrawal for using low concentrations of glucose has been used to extend to low concentrations the observations of O'Brien (1969) and to study the kinetics of glucose

utilization for evidence of the limiting factor. This touches on the wide subject of the effect of insulin on the kinetics of glucose permeation in heart muscle.

CHAPTER 6

Glucose Utilization by Hearts from Fed Animals

6.1 Introduction

The time-course of glucose utilization by hearts perfused in closed circuit systems has received little attention. What evidence there is on the glucose utilization by hearts from fed rats (Randle et al., 1964; England and Randle, 1967) suggests that the rate is constant with time at least during the first 30 minutes of perfusion but finally rises if insulin is absent (Neely et al., 1967b). Fisher and O'Brien (1972) using a system of perfusion with balanced infusion and withdrawal found that the pattern of the time-course varied with the presence or absence of insulin. Hearts perfused with insulin at $100mU.ml^{-1}$ showed constant rates of glucose utilization throughout the experiments. Without added insulin the rate decreased over a period of 30 to 40 minutes becoming then relatively constant. This behaviour was usual when the perfusate glucose concentration was greater than 1mM. At lower concentrations of glucose the rate of utilization was more commonly constant throughout the experiment. In that investigation a wide range of glucose concentrations was used and usually only two hearts were studied at any one concentration. Most experiments were done at lower concentrations than are usually used in closed-circuit systems. Therefore in this work the time-course of glucose utilization was reinvestigated with groups of hearts perfused under more restricted conditions. Also, in some experiments, the time-course of lactate production was studied so that changes in glucose utilization could be related to any variation in the rate of anaerobic metabolism.

Hearts were perfused with initial concentration of glucose in the perfusate being either 5.5mM or 2.7mM. Fresh perfusate also

containing glucose at 5.5mM or 2.7mM was infused into the recirculating perfusate at either a high rate about 50ml.h^{-1} or a low rate 20ml.h^{-1}. Insulin when used was present at 2mU.ml^{-1}. The collection and analysis of fractions and the calculation of results followed the standard procedure described in Sections 3.3 and 4.2. Two concentrations of glucose and two infusion rates were used to provide a variety of control conditions for hearts perfused with both glucose and another energy source which might inhibit glucose utilization. As was shown in Section 4.2 the precision of an estimate of utilization is less at high substrate concentration and at high infusion rates. When the rate of utilization is low as would be expected if glucose consumption in the absence of insulin is further reduced by inhibition, the range of usable concentrations and infusion rates is reduced. A glucose concentration of 2.7mM was chosen because O'Brien (1969) found that the rate of utilization at that concentration was not greatly reduced from the maximum so that more precise estimates can be achieved at rates close to those seen at physiological concentrations. Insulin was used at 2mU.ml^{-1} because more studies on glucose consumption have been made with this concentration than with any other and because rates of utilization are maximally stimulated at this concentration (Bleehen and Fisher, 1954).

6.2 The Time-Course of Perfusate Glucose Concentration in the Presence of Insulin

Hearts from fed animals were perfused with insulin at 2mU.ml^{-1} and glucose at 2.7mM or 5.5mM was infused at a high or low rate. In Fig. 15 examples of the time-course of perfusate glucose concentration are given for the four conditions. Fractions were collected for 5 minutes, alternate fractions were analysed and their concentrations plotted at the mid-point of the period. In all four cases the

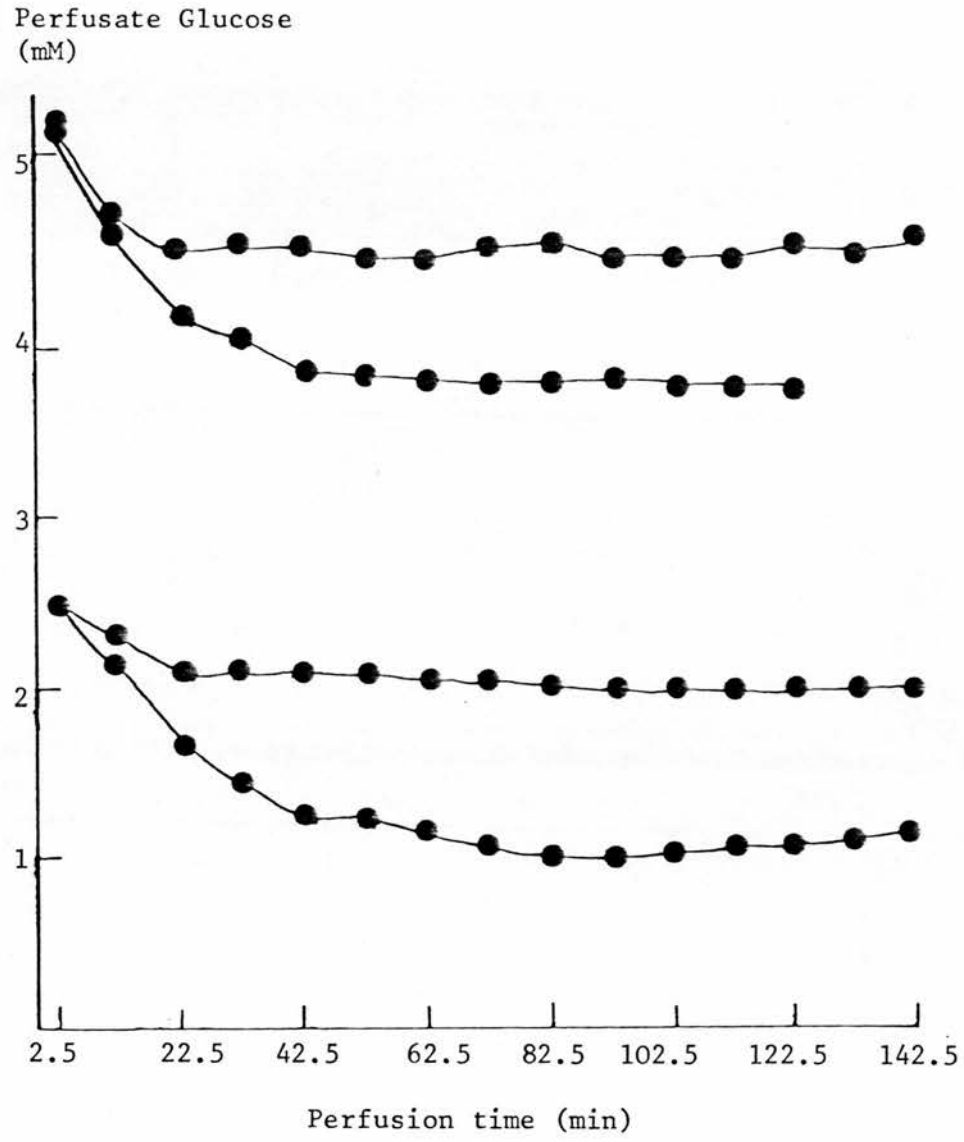

Fig. 15. Time-courses of perfusate glucose concentration in the presence of insulin

Hearts from fed animals were perfused with MKHM containing 5.5mM- or 2.7mM-glucose and 2mU.ml^{-1} insulin at high and low infusion rates. A and B 5.5mM-glucose, infusion rates 55 and 21ml.h^{-1} respectively C and D 2.7mM-glucose, infusion rates 55 and 25ml.h^{-1} respectively.

perfusate glucose concentration tends to become constant at a level that is usually maintained to the end of experiments lasting 150 minutes. The effect of changing the infusion rate is as would be expected. Reduction of infusion rate delays the attainment of a steady state and increases the difference in concentration of infusate and perfusate glucose.

6.3 The Time Course of Glucose Utilization in the Presence of Insulin

Fig. 16 shows the time-course of the mean rates of glucose utilization by 14 hearts perfused with 5.5mM-glucose and insulin at $2mU.ml^{-1}$. The results obtained from experiments using high and low infusion rates have been combined because the rate of infusion has no significant effect on the rates of utilization in these conditions (Section 4.3). The number of estimates that contributes to the mean decreases to 13 after 82.5 minutes and to 12 after 122.5 minutes because in two experiments leakage of perfusate from the apparatus caused the experiments to be stopped early. The rate of glucose utilization does not change significantly throughout the experiments and averages 317 ± 4 (S.E.M.) $\mu moles.g\ dry\ wt.^{-1} h^{-1}$ during the whole experimental period of 150 minutes and 313 ± 11 (S.E.M.) $\mu moles.g\ dry\ wt.^{-1} h^{-1}$ from 60 to 90 minutes when the concentration of glucose in the perfusate is constant.

Fig. 17 shows the time-course of the mean rates of glucose utilization by hearts perfused with 2.7mM-glucose and $2mU.ml^{-1}$ insulin at high and low infusion rates. In these experiments the rates of utilization are affected by the rate of infusion (Section 4.3) and the 7 hearts studied at each rate have been considered separately. In both sets of experiments the utilization of glucose is constant for most of the period of perfusion. At the higher infusion rate, although the pattern suggests that the rate initially increases to

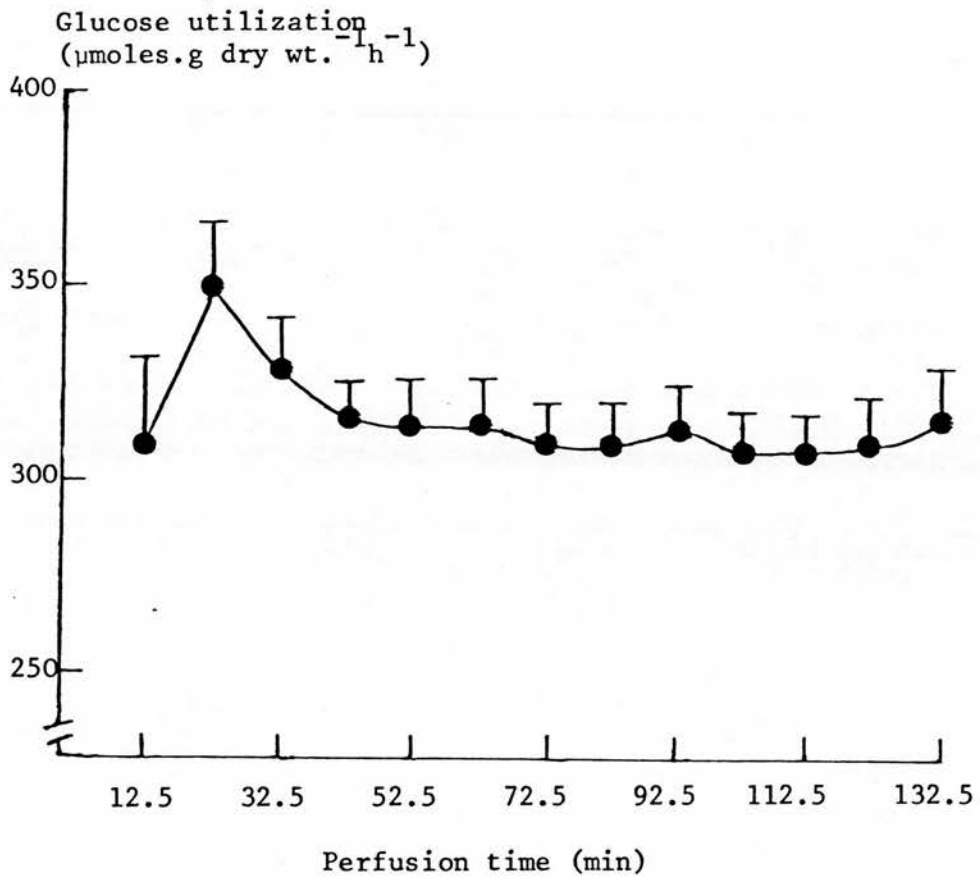

Fig. 16. The time-course of glucose utilization in the presence of insulin

14 hearts from fed animals were perfused with 5.5mM-glucose and 2mU.ml^{-1} insulin. Points are the mean $\pm$ S.E.M.

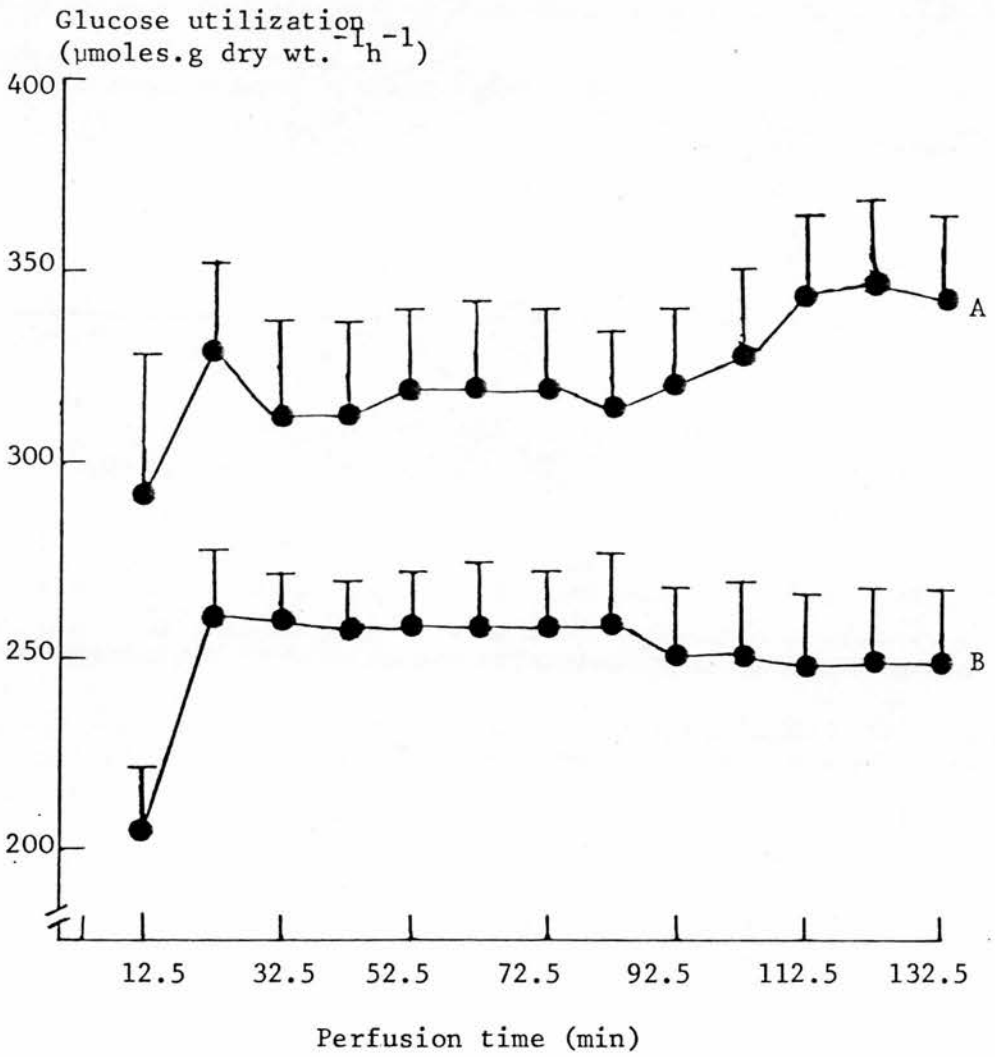

Fig. 17. Time-courses of glucose utilization in the presence of insulin

Hearts from fed animals were perfused with 2.7mM-glucose and 2mU.ml^{-1} insulin at high and low infusion rates A and B respectively.

Points are the mean $\pm$ S.E.M. of 7 experiments in each group.

a constant value that is maintained until, in the last half-hour of the perfusion, the rate rises again, these changes are not statistically significant. The rate of utilization during the period 60 to 90 minutes is 320 ± 21 (S.E.M.) umoles.g dry wt.$^{-1}h^{-1}$.

At the lower rate of infusion the rate of utilization at 12.5 minutes (208 ± 16 μmoles.g dry wt.$^{-1}h^{-1}$) is less than the rates during the period 60 to 90 minutes which average 261 ± 16 μmoles.g dry wt.$^{-1}h^{-1}$. This difference is significant at between the 2% and 5% level of probability. Because the estimation of the rate of glucose utilization at 12.5 minutes, when the concentration of perfusate glucose is inconstant is subject to greater error than in steady state conditions (Section 4.2.2), the possibility was tested that the rate of change of concentration at 12.5 minutes had been systematically underestimated. Table 22 shows that the estimated rates of utilization at 7.5 minutes, 17.5 minutes and throughout the period 2.5 to 22.5 minutes calculated as described in Section 4.2.2 are consistent with the rate at 12.5 minutes being lower than subsequent estimates.

6.4 The Time-Course of Lactate Production by Hearts Perfused with 5.5mM-Glucose and 2mU.ml^{-1} Insulin

The time-course of lactate production was investigated in three of the experiments in which hearts from fed rats were perfused with 5.5mM-glucose and 2mU.ml^{-1} insulin. Fig. 18 shows the time-course of the mean rate of lactate production in these experiments. There are no significant changes in the rate throughout the period of perfusion. However in the three experiments the rate of lactate production was reduced after 32.5 minutes in comparison with the rate at 22.5 minutes by 27%, 29% and 52% so that despite the variation in the rate of production the results suggest a fall in the rate in the first half-hour of perfusion. The rate of lactate production between 60 minutes

Table 22

Changes in glucose utilization in the early period of perfusion

Initial Glucose conc. (mM)	Insulin ($2mU.ml^{-1}$)	Glucose Utilization ($\mu moles.g\ dry\ wt.^{-1}h^{-1}$) at (min)				
		7.5	12.5	17.5	22.5	Overall
2.7 (12)	-	136 ± 11	126 ± 13	82 ± 10	81 ± 9	110 ± 11
5.5 (18)	-	165 ± 9	157 ± 9	124 ± 10	119 ± 9	143 ± 8
2.7* (7)	+	170 ± 15	208 ± 16	250 ± 20	262 ± 17	210 ± 16
2.7** (7)	+	231 ± 35	294 ± 35	316 ± 26	330 ± 25	291 ± 32
5.5 (14)	+	268 ± 24	305 ± 22	357 ± 26	344 ± 19	310 ± 20

Groups of hearts from fed animals were perfused with glucose and with or without insulin as shown in the Table.

(*) Low infusion rate; (**) High infusion rate, other results combined regardless the infusion rate. Values are mean $\pm$ S.E.M. and the numbers of observations are in parentheses. Overall utilization is the rate between 2.5 minutes and 22.5 minutes.

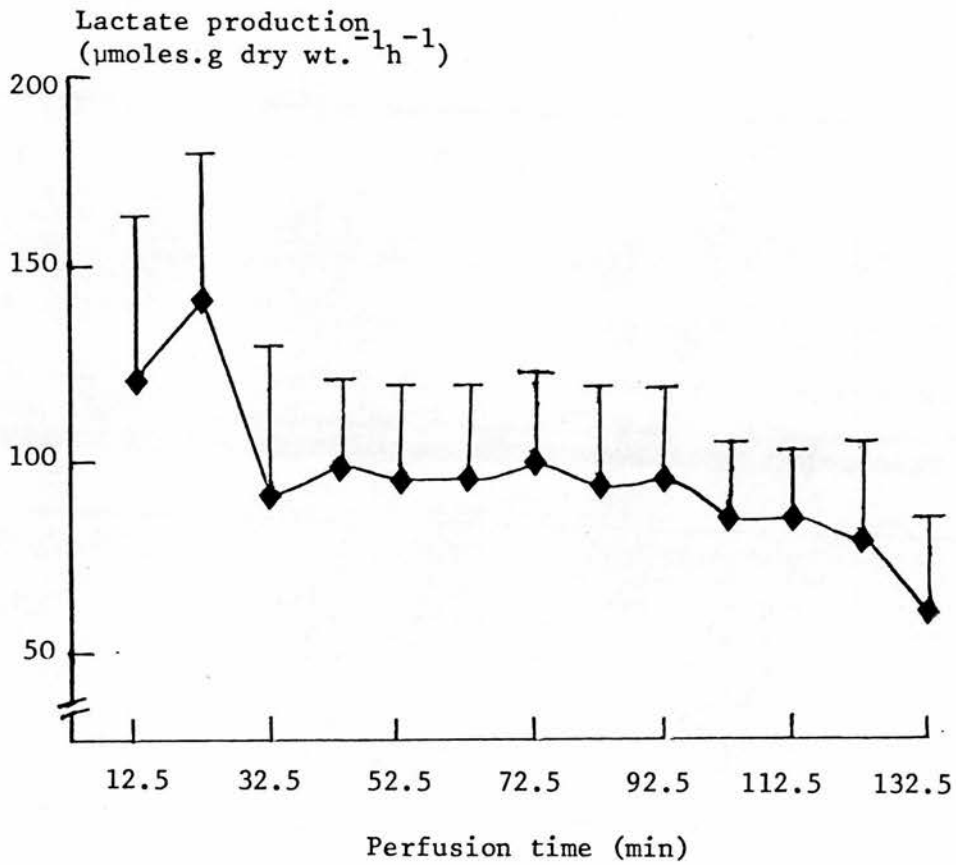

Fig. 18. The time-course of lactate production in the presence of insulin

Hearts from fed animals were perfused with 5.5mM-glucose and 2mU.ml^{-1} insulin. Points are the mean $\pm$ S.E.M. of 3 experiments.

and 120 minutes was 97μmoles.g dry wt.$^{-1}$h^{-1}. In this period of constancy in glucose and lactate production, between 10% and 29% (mean 18%) of the glucose was converted to lactate.

6.5 The Time-Course of Perfusate Glucose Concentration Without Added Insulin

Hearts from fed animals were perfused without added insulin and glucose at 2.7mM or 5.5mM was infused at a high or low rate. In Fig. 19 examples of the time-course of perfusate glucose concentration are given for the four conditions. In all four cases the concentration of glucose passes through an early minimum before rising to become effectively constant for periods of 30 minutes or more. Finally the concentration again falls but the time of the onset of the fall is variable.

6.6 The Time-Course of Glucose Utilization Without Added Insulin

The glucose utilization of groups of hearts whose perfusate received 2.7mM-glucose at high and low rates is not significantly different during the period 60 to 90 minutes (Section 4.3). Combination of all results with 2.7mM-glucose gives a time-course of the mean rate of utilization that is shown in Fig. 20.

The rate of glucose utilization with 5.5mM-glucose is also independent of the infusion rate (Section 4.3) and the time-course of the mean rate of utilization is shown in Fig. 20. In this case the number of observations that contribute to the calculations of the mean rates is reduced at later times of estimation. The reasons for the reductions were that three experiments were stopped after 100 minutes and, in two, analytical difficulties caused the loss of samples. In none of the five experiments were there reasons to disregard the remaining data.

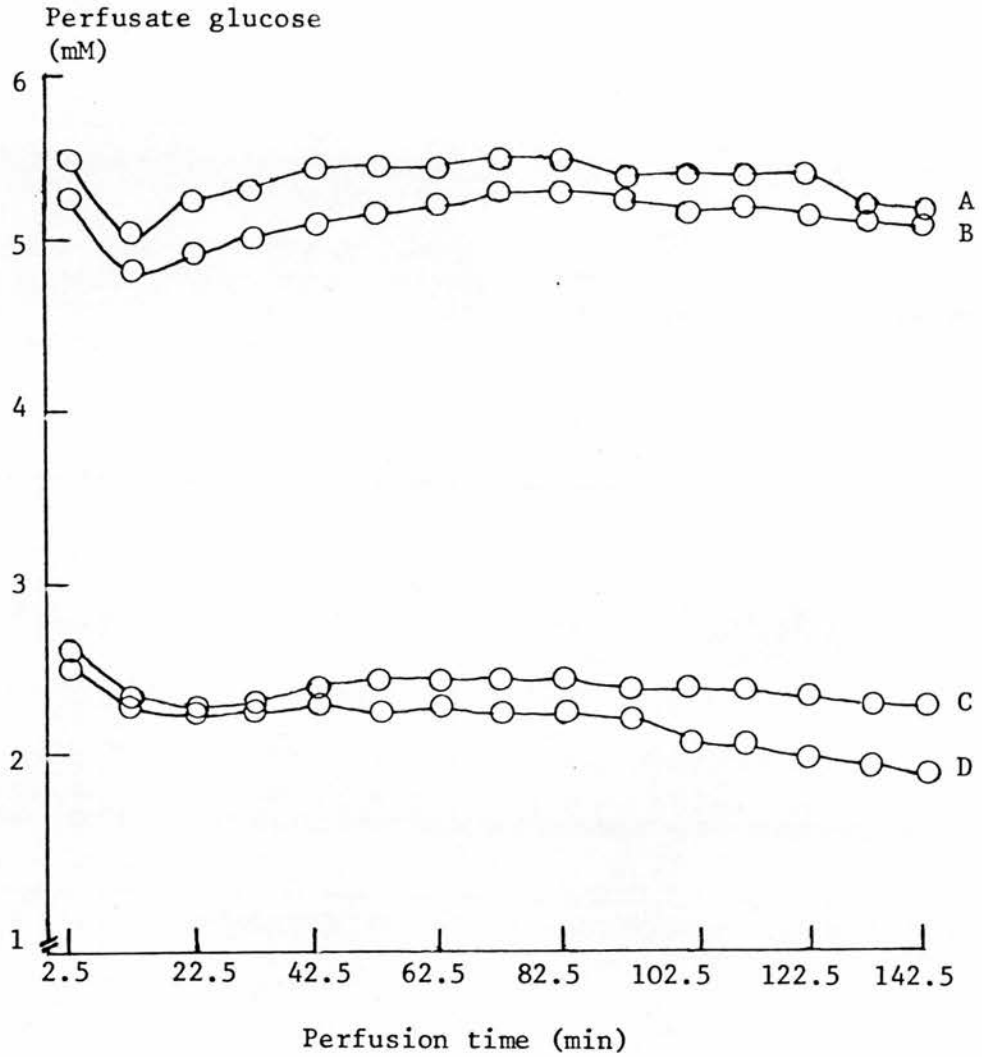

Fig. 19. Time-courses of perfusate glucose concentrations in the absence of insulin

Hearts from fed animals were perfused with 5.5mM- or 2.7mM-glucose at high and low infusion rates.

A and B 5.5mM-glucose, infusion rates 56 and 28ml.h^{-1} respectively

C and D 2.7mM-glucose, infusion rates 50 and 25ml.h^{-1} respectively.

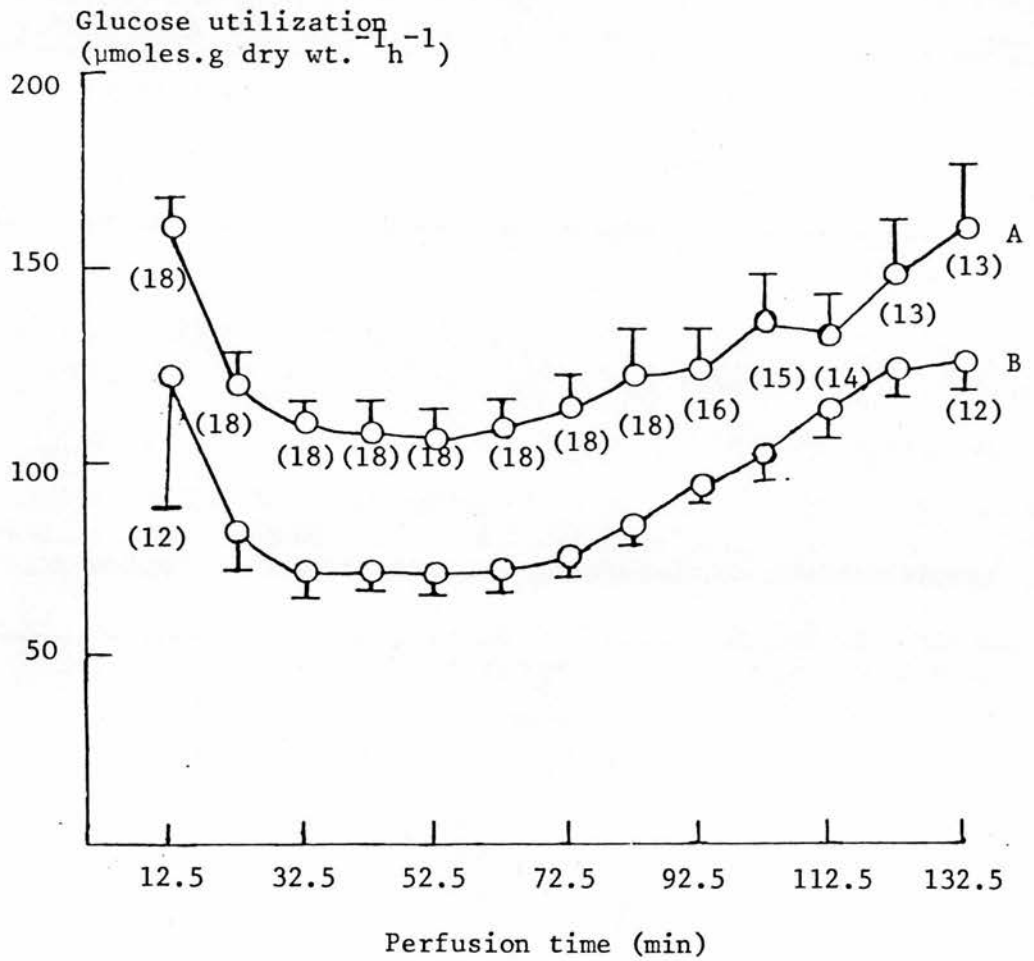

Fig. 20. Time-courses of glucose utilization in the absence of insulin

18 hearts from fed rats were perfused with 5.5mM-glucose (series A) and 12 hearts from fed rats were perfused with 2.7mM-glucose (series B). Points are means $\pm$ S.E.M.

With both 5.5mM-glucose and 2.7mM-glucose the time-course of utilization shows an early fall in the rate that is greatest between 12.5 minutes and 22.5 minutes. Table 22 shows the rates calculated for the times 7.5, 12.5, 17.5 and 22.5 minutes and for the period 2.5 to 22.5 minutes. These estimates also indicate that the rate of glucose utilization decreases early in the perfusion without insulin. Such a decrease is also suggested by the time-course of glucose concentration in these experiments. The rate falls during the first 30 minutes of perfusion, is then essentially constant for a further 30 or 40 minutes and finally increases during the last 60 minutes of the experiment.

6.7 The Time-Course of Lactate Production by Hearts Perfused with 5.5mM-Glucose

In three experiments the time-course of lactate production was investigated with hearts perfused with 5.5mM-glucose. The time-course of lactate concentration in one of these experiments was shown in Fig. 12 and the results from all three were given in outline in Section 4.4.2.

The time-course of the mean rates of lactate production is shown in Fig. 21. The rate is initially high and variable but falls during the first 50 minutes of perfusion and is thereafter no greater than 5μmoles.g dry wt.$^{-1}$h^{-1} and has an average of 3μmoles.g dry wt.$^{-1}$h^{-1} when the glucose utilization rate was lowest at 60 minutes. With the method of assay used the lowest measurable rate of lactate production was approximately 2μmoles.g dry wt.$^{-1}$h^{-1}. In the first half-hour of perfusion the lactate production accounted for approximately 11% of the glucose consumption and thereafter less than 5%.

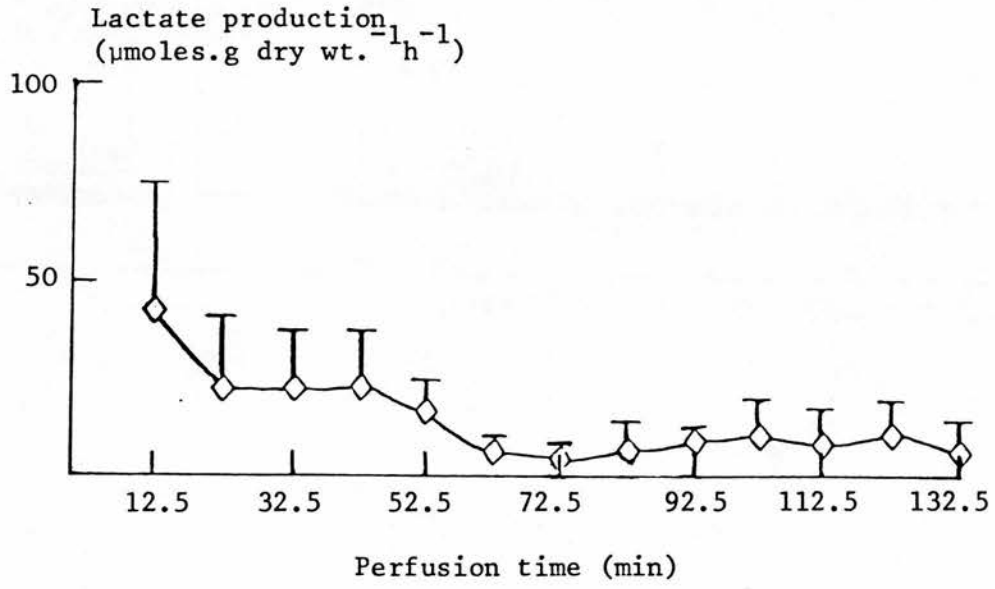

Fig. 21. The time-course of lactate production in the absence of insulin

Hearts from fed animals were perfused with 5.5mM-glucose.

Points are the mean $\pm$ S.E.M. of 3 experiments.

6.8 Discussion

6.8.1 Evidence of Metabolic Stability

In all the experiments reported in this chapter there were periods when the rate of glucose utilization was constant regardless of the concentration of glucose or the presence or absence of insulin. The shortest and most variable periods occurred in the absence of insulin while hearts perfused with 5.5mM-glucose and 2mU.ml^{-1} insulin normally used glucose at a constant rate for 150 minutes. This observation agrees with that of Fisher and O'Brien (1972). A constant rate of utilization suggests that the heart is in a stable metabolic condition but this condition may be normal and reflect properties of physiological relevance or abnormal if the heart undergoes metabolic changes during its isolation that are not reversed when perfusion is established.

The periods of metabolic stability were associated with relatively low rates of lactate production. When steady concentrations of glucose and lactate existed 18% of the glucose used in the presence of insulin could be accounted for by lactate production and, in the absence of insulin, lactate production was negligible. In comparison with published estimates of glucose utilization and lactate production (Tables 17 and 20, Fig. 14) the proportion of anaerobic metabolism observed in this work is low.

In Section 3.5 criteria were given for judging successful perfusion. Hearts which failed to satisfy the criteria of regular force and rate of contraction were rejected. Analysis of the perfusate glucose concentration showed that a steady state was not established. These hearts amounted to less than 10% of total so that selecting for mechanical or metabolic stability does not make an unrepresentative collection. No systematic study of lactate production by failing hearts

was made in this work but in an investigation in which lactate measurements were made routinely regardless of the condition of the heart there was a close association of mechanical and metabolic instability with high rates of glucose utilization and of lactate and glycerol phosphate release (unpublished observations by Dr. J.A. O'Brien). It seems probable that periods of metabolic stability with low rates of anaerobic activity have more relevance to the physiological situation than periods of unstable metabolic activity. At the least they provide a clear basis from which to make comparisons.

6.8.2 Glucose Utilization in the Presence of Insulin

The rate of glucose utilization at 5.5mM-glucose in the presence of $2mU.ml^{-1}$ insulin was $313 \pm 11 \mu moles.g\ dry\ wt.^{-1}h^{-1}$. This may be compared with other estimates in Table 20 in which most values are higher. The relatively low rate is probably the result in part of the associated low rate of lactate production and in part of the use of as low a perfusion pressure as will give an adequate coronary flow. Higher perfusion pressures should raise the rate of utilization by 77% for a doubling of perfusion pressure (Neely et al., 1967b).

The oxygen consumption of hearts at a perfusion pressure of 40mmHg as was used in this work was measured by Fisher and Williamson (1961a) and by O'Brien (1972). They found the rate of oxygen consumption to be between 1500 and $1700 \mu moles.g\ dry\ wt.^{-1}h^{-1}$ and Fisher and Williamson found that the oxygen consumption did not vary with the nature of the substrate in the perfusate. If glucose was the only substrate used this range of oxygen consumption would account for between 250 and $280 \mu moles.glucose.g\ dry\ wt.^{-1}h^{-1}$ which, allowing for approximately 20% conversion to lactate, accounts well for the observed rate of utilization. However another study of oxygen consumption does not agree that it is unaffected by the nature of the

substrate (Willebrands and Van Der Veen, 1967), but the conditions of perfusion were not comparable with those of this work. Fates of glucose other than carbon dioxide and lactate have not been quantified in hearts undergoing prolonged perfusion. If their rates change with time this does not affect the estimate of total glucose utilization and although the glycogen content of hearts perfused with glucose and insulin increases (e.g. Williamson and Krebs, 1961) this can not continue indefinitely. It seems reasonable to suppose that an isolated non-working rat heart can obtain at least 75% of its energy from perfusate glucose at near physiological concentrations.

In one respect the pattern of glucose utilization at 2.7mM-glucose in the presence of insulin differed from that seen with 5.5mM-glucose. When the infusion rate was low the rate of glucose utilization after 12.5 minutes was 20% less than the constant rate established afterwards. This difference was not the result of systematic underestimation of the rate of change of glucose concentration and was significant at a level of probability between 5% and 2%. An early low rate of utilization was not seen with 5.5mM-glucose nor with 2.7mM-glucose when the infusion rate was high but possibly occurs at lower concentrations (Chapter 10). A possible explanation of the effect was sought in the fact that hearts perfused without glucose or with very low concentrations of glucose were found to release glucose into the perfusate. The most likely source of this glucose is the glucose that was in the interstitial water when the heart was excised but it could conceivably arise intracellularly through the action of debranching enzyme (amylo-α-,1,6-glucosidase) (EC 3.2.1.33) on glycogen. However in experiments with hearts perfused without substrate all the glucose was released in the first five minutes of perfusion and if the equilibration of endogenous glucose with perfusate glucose is

complete in five minutes it cannot affect the estimate of utilization made at 12.5 minutes. In addition each heart would have to release approximately 1.5μmoles of glucose into the perfusate during the first 15 minutes of perfusion in order to cause a 20% underestimate of utilization. In substrate-free perfusions only 0.2μmoles of glucose was released in the first five minutes and none thereafter.

A possible explanation of low rate of utilization at 12.5 minutes when 2.7mM-glucose and 2mU.ml^{-1} insulin were infused slowly can be based on the fact that the only obvious difference in the conditions of perfusion is the concentration of glucose. At low concentrations of glucose there appears to be an inhibition of glucose utilization that is not evident at higher concentrations. Newsholme and Randle (1961) found an accumulation of glycolytic intermediates in hearts before perfusion was started and Randle et al. (1964) gave evidence that these intermediates could contribute to glycogen synthesis if glucose utilization was inhibited by pyruvate or DL-3-hydroxybutyrate. It is conceivable that if the clearance of these accumulated intermediates is inhibited they would themselves lead to the inhibition of the phosphorylation of glucose. This might happen if glycogen synthesis is relatively inhibited as might happen at low concentration of glucose since glucose is an activator of glycogen synthesis through its effect on phosphorylase a and phosphorylase phosphatase (Stalmans, Laloux and Hers, 1974). This mechanism is thought to be important in the regulation of glycogen metabolism in the liver. This tentative suggestion apparently requires that the response to the concentration of glucose is very sensitive because the concentration of the perfusate during the first 30 minutes differs by little whether 2.7mM-glucose is infused rapidly or slowly. However although at the higher rate of infusion the mean rates of glucose utilization at

12.5 minutes and 22.5 minutes did not differ significantly, in five out of seven experiments the rate increased and the percentage increase ranged from 7% to 70% with an average of 28%. This compares with a range of 15% to 47% with an average of 27% for the seven experiments conducted at the lower infusion rate.

6.8.3 Glucose Utilization in the Absence of Insulin

When no insulin was added to the perfusate the time-course of glucose utilization was similar with both 2.7mM and 5.5mM-glucose and agreed with the pattern reported by Fisher and O'Brien (1972) to be typical of experiments with glucose at concentrations greater than 1mM. The rate decreased for 30 to 40 minutes and then became relatively constant. Later the rate tended to increase again but the time when the rate started to rise varied considerably. Some hearts maintained a constant rate for 30 minutes but others maintained it for 80 minutes. With 5.5mM-glucose the mean rate of glucose utilization in the period of stability was 106μmoles.g dry wt.$^{-1}$h^{-1} and in three such hearts the lactate production in this period was no more than 5μmoles.g dry wt.$^{-1}$h^{-1}. If the rate of oxygen consumption was approximately 1500 to 1700μmoles.g dry wt.$^{-1}$h^{-1} and if all the glucose consumed was oxidized it could account for 37% to 42% of the oxygen consumption. This indicates that the endogenous reserves of glycogen and triglyceride met most of the energy requirements of the heart.

The tendency for the rate of utilization to rise towards the end of the experiments was not associated with any change in the rate of lactate production or with any consistent change in the mechanical performance of the heart. Neely et al. (1967b) compared the rate of glucose utilization and lactate production in the periods 15-75 minutes and 85-145 minutes in a closed-circuit system of perfusion and reported an increase of glucose utilization and a fall in lactate

production in the latter period. Because of the dependence on endogenous reserves when insulin is absent, the rise in glucose utilization might be linked to the depletion of the reserves. Experiments designed to examine this possibility are reported in Chapter 9.

The early fall in the rate of utilization amounts to 31% between 12.5 and 22.5 minutes with 5.5mM-glucose and 44% with 2.7mM-glucose. Fisher and O'Brien (1972) observed falls greater than 20% in the first hour of perfusion and attributed them to the diminishing effect of endogenous insulin. This hypothesis assumes that glucose utilization is sensitive to low concentrations of insulin. Experiments to test this are reported in Chapter 7. Randle et al. (1964) also assumed that endogenous insulin affected their results but found no difference in the rate of utilization in two successive 15 minute periods of perfusion with 5.5mM-glucose in the absence of added insulin. This indicates a rate of utilization of approximately 263μmoles.g dry wt.$^{-1}$h^{-1} throughout the first 30 minutes of perfusion whereas in this work the rate after 7.5 minutes was approximately 161μmoles.g dry wt.$^{-1}$h^{-1}. As was discussed in Chapter 5 there is no simple explanation to the variation in the reported rates of glucose utilization by hearts from fed rats perfused without insulin. The difference in rates is reflected in the rates of lactate production but it is not clear why lactate production should vary so much.

The rate of glucose utilization of 106μmoles.g dry wt.$^{-1}$h^{-1} during the period of metabolic stability is reasonably consistent with the rates published by other workers (Table 17) who also observed low rates of lactate production.

A comparison of the rates of glucose utilization measured during the periods of stability in the presence and absence of insulin (313 and 106μmoles.g dry wt.$^{-1}$h^{-1} respectively) shows that insulin causes

a three-fold stimulation of glucose utilization. However the stimulation is only two-fold after 12.5 minutes and at the end of experiments of 150 minutes duration. A three-fold stimulation is in the middle of the range of the results set out in Table 20.

In summary the results presented in this chapter show that the utilization of glucose by the isolated rat heart perfused with balanced infusion and withdrawal can be studied at constant concentrations of substrate in the perfusate. Whether insulin is present or not the rate of glucose utilization is stable for periods long enough to be useful in comparative studies and is characterised by a low degree of anaerobic metabolism.

CHAPTER 7

The Sensitivity of Glucose Utilization to Insulin

7.1 Introduction

Although endogenous insulin has been proposed to effect the utilization of glucose by the isolated hearts little work has been done on the sensitivity of this preparation to insulin. Bleehen and Fisher (1954) found that insulin at high concentration ($2mU.ml^{-1}$) caused a 3.7 fold stimulation of glucose utilization by hearts from fed animals. Insulin at $20\mu U.ml^{-1}$ had no effect and at $60\mu U.ml^{-1}$ doubled the rate of glucose utilization which was therefore increased to 55% of the maximum rate. At $200\mu U.ml^{-1}$ the rate increased to 73% of the maximum rate. Meuli and Froesch (1975) who reported unusually low rates of glucose utilization both in the presence and absence of added insulin obtained a six-fold stimulation with insulin at $2mU.ml^{-1}$, a five-fold stimulation at $200\mu U.ml^{-1}$, and a four-fold stimulation at $100\mu U.ml^{-1}$. No other systematic investigation of the sensitivity of the isolated heart to insulin has been found in the literature.

The significance attributed to sub-maximal stimulations of glucose utilization depends on what is taken to be the concentration of insulin in the plasma of the fed rat. Since Hales and Randle (1963) reported the range of insulin concentration in the fed rat to be 120 to $140\mu U.ml^{-1}$ improvements in the radioimmunoassay of insulin concentrations have led to lower values being found. However there is considerable variation in the observed concentration of insulin in the blood of the fed rat. For instance Hawkins, Alberti, Houghton, Williamson and Krebs (1971) found $30\mu U.ml^{-1}$ while Aynsley-Green *et al.* (1973) found $84\mu U.ml$. The results of Hawkins *et al.* together with the insensitivity of skeletal muscle to insulin (Zierler and Rabinowitz, 1964) in comparison with the response shown by adipose tissue

(Fain, Kovacev and Scow, 1966), has led Newsholme (1976) to propose that for skeletal muscle at least the direct effect of insulin on glucose utilization is usually unimportant in comparison with the indirect effect through the release of fatty acids from adipose tissue. The fatty acids released when the concentration of insulin is low would suppress the utilization of glucose by muscle. From the results of Bleehen and Fisher (1954) and of Meuli and Froesch (1975) it would seem that this hypothesis should include cardiac muscle if the insulin concentration of the blood of fed rat is only $30\mu U.ml^{-1}$ or even $84\mu U.ml^{-1}$. This would also indicate that the wash-out of endogenous insulin should be without effect on glucose utilization by isolated heart.

However the results presented in Section 6.6 show that isolated hearts are unable in the absence of insulin to satisfy their energy when glucose is the only substrate. Therefore the absence of fatty acids alone is not enough to allow cardiac muscle to use glucose at the maximum rate. If insulin does have important direct effects the sensitivity of cardiac muscle to insulin must be greater than has been reported for isolated hearts. However if cardiac muscle is so insensitive to insulin that changes in the concentration of the hormone do not effect glucose utilization *in vivo* an increase in glucose extraction that does occur in man as the concentration of fatty acid falls *in vivo* (Lassers, Wahlqvist, Kaijser and Carlson, 1971) must mean that the cells are permeable to glucose regardless of the lack of an effect of insulin. This would support the claim (Taegtmeyer *et al.*, 1980) that the properties of the non-working isolated heart are not representative of the heart *in vivo* and underline the importance of the increased permeability of hearts that do external work.

In this chapter an investigation into the sensitivity to insulin of glucose utilization by the isolated perfused rat heart is reported. In addition a study was made of the time-course of glucose utilization in hearts perfused without insulin after a period of perfusion with insulin at concentrations that had maximal or sub-maximal effects on utilization. The time-course in these experiments might be expected to be similar to the decrease in the rate of utilization by hearts perfused without added insulin if the decrease is caused by the loss of endogenous insulin.

7.2 Experimental Procedure

The study of the effect of a low concentration of insulin required some changes in experimental procedure. As is now commonly recognised the binding of insulin on glass (Ferrebee, Johnson, Mithoefer and Gardella, 1951), paper (Newerly and Berson, 1957) and plastic surfaces (Hill, 1959; Petty and Cunningham, 1974) can seriously reduce the concentration of insulin in solution. This effect can be avoided by the inclusion of albumin or other protein in the solution (Ferrebee et al., 1951; Wiseman and Baltz, 1961). However with protein in the perfusate the Millipore filters became blocked within half-an-hour. Instead hearts were perfused successfully when the Millipore filter was replaced by a second disc of hardened filter paper.

The adsorption of insulin-I^{131} on glass can be prevented by the inclusion of 0.5mg.ml^{-1} albumin in the medium (Wiseman and Baltz, 1961). This concentration of essentially fatty acid-free bovine serum albumin (Fraction V) was routinely used in the experiments reported in this chapter. Doubling or trebling the concentration of albumin was without obvious effect on the glucose utilization at 20μU.ml^{-1} or 60μU.ml^{-1}. Albumin in the perfusate binds calcium and magnesium ions but the use of albumin that had been dialysed for 48 hours against four changes

of perfusate did not affect the observed rate of glucose utilization. At the concentration used the albumin must have insigifnicant effect on the concentration of electrolytes.

The effects reported here are not specific to albumin, gelatin was also effective but a higher concentration ($1.25 mg.ml^{-1}$) was required.

7.3 Effects of Filter-Type and Albumin on Glucose Utilization

Five hearts from fed rats were perfused with MKHM containing 5.5mM-glucose and filtered through two layers of Whatman No. 54. The time-course of the concentration of perfusate glucose was similar to those shown in Section 6.5 for hearts perfused using Millipore filters. In all five experiments the perfusate glucose concentration passed through a minimum, indicating that the rate of glucose utilization was decreasing, and then became constant. The mean rate of glucose utilization measured when the perfusate glucose concentration was constant was $52 \pm 8 \mu moles.g\ dry\ wt.^{-1} h^{-1}$, whereas the rate found using Millipore filters was $117 \pm 10 \mu moles.g\ dry\ wt.^{-1} h^{-1}$. This marked and significant difference ($0.01 > P > 0.002$) in the rate of utilization was not associated with any obvious changes in the mechanical performance of the hearts.

It was not possible to study the effect of albumin on glucose utilization using Millipore filters because they blocked early in the experiments. In eight experiments hearts from fed rats were perfused with MKHM containing 5.5mM-glucose, $0.5 mg.ml^{-1}$ albumin and filtered by Whatman No. 54. The mean rate of glucose utilization measured during the period of constancy in perfusate glucose utilization was $75 \pm 11 \mu moles.g\ dry\ wt.^{-1} h^{-1}$. This does not differ significantly from the rate found with paper filters and without albumin but is significantly different ($0.02 > P > 0.01$) from the rate of utilization by hearts perfused using Millipore filters. Use of paper filters in these

experiments was associated with a reduction in glucose utilization. But albumin was without effect.

7.4 Effects of Low Concentrations of Insulin on Glucose Utilization

Table 23 shows the rates of glucose utilization measured during the period of constant perfusate glucose concentration when hearts were perfused with 5.5mM-glucose and insulin in the range 20 to 2000 $\mu U.ml^{-1}$ and with or without albumin at $0.5mg.ml^{-1}$. The perfusate was filtered with Millipore when albumin was excluded and with paper filters when albumin was present. Insulin at all concentrations increased the rate of glucose utilization in the presence of albumin. The rate of utilization at $60\mu U.ml^{-1}$ insulin was not significantly different from that at higher concentrations of the hormone. At less than $60\mu U.ml^{-1}$ insulin the rate of utilization depended on the concentration of insulin. In the experiments with Millipore filters and without albumin insulin did not affect the rate of glucose utilization when used at concentration of $160\mu U.ml^{-1}$ or less. With $2mU.ml^{-1}$ insulin the rate of glucose utilization was indistinguishable despite the difference in filter and the presence of albumin.

7.5 Effect of Perfusion with Insulin on Subsequent Glucose Utilization in the Absence of Added Insulin

Hearts from fed rats were perfused with MKHM containing 5.5mM-glucose, $0.5mg.ml^{-1}$ albumin and insulin at $2mU.ml^{-1}$, $80\mu U.ml^{-1}$ or $40\mu U.ml^{-1}$ for 30 minutes using paper filters. They were then transferred to a second apparatus where, after one minute of non-recirculated perfusion to mimic the period for the wash-out of blood from a freshly excised hearts, they were perfused for a further 90 or 100 minutes with MKHM containing only 5.5mM-glucose using a Millipore filter.

Table 23

The effect of insulin on glucose utilization

Insulin Concentration ($\mu U.ml^{-1}$)	Glucose Utilization ($\mu moles.g\ dry\ wt.^{-1}h^{-1}$)	
	Without Albumin	With Albumin ($0.5mg.ml^{-1}$)
0	117 ± 10 (18)	75 ± 11 (8)
20	-	136 ± 31 (4)
40	-	226 ± 17 (6)
60	119 ± 20 (7)	294 ± 30 (4)
80	114 ± 12 (7)	272 ± 12 (12)
160	106 ± 20 (5)	284 ± 40 (4)
250	-	324 ± 32 (4)
500	223 ± 17 (4)	318 ± 14 (4)
2000	313 ± 11 (11)	305 ± 19 (6)

Hearts were perfused with MKHM containing 5.5mM-glucose and albumin and insulin as indicated. Values are means $\pm$ S.E.M., and the numbers of observations are in parentheses.

The time-courses of the mean rate of glucose utilization in these experiments are shown in Fig. 22. The rates shown for hearts preperfused with 2mU.ml^{-1} insulin are the average of two experiments, and decrease over a period of 50 minutes from values characteristic of hearts exposed to high concentrations of insulin to an effectively constant rate of approximately 240μmoles.g dry wt.$^{-1}$h^{-1} that is approximately twice the rate typical of comparable insulin-free hearts.

Five hearts were perfused with insulin at 40μU.ml^{-1} for 30 minutes before being transferred to the insulin-free system. There were no significant changes in the rate of glucose utilization during the following 100 minutes of perfusion and the rate was indistinguishable from that of hearts perfused without insulin and with Millipore filters from the start of the experiment. Of these five experiments only one gave a time-course of perfusate glucose concentration in which there was an obvious minimum.

In six experiments insulin at 80μU.ml^{-1} was included in the perfusate for 30 minutes. In the subsequent two hours of perfusion without insulin the hearts behaved in two distinct ways. Three hearts established a constant concentration of perfusate glucose after 40 to 50 minutes of the second period of perfusion without it passing through a minimum and three showed the pattern of falling concentration followed by a rise to constancy. The mean rates of utilization of the two groups are shown in Fig. 22. The first estimate of utilization after the preperfusion with insulin was similar in both groups and to the rates seen in freshly excised hearts when perfused without insulin (Section 6.6). Thereafter the rate in one group fell to approximately half that found in hearts perfused with Millipore filters and without insulin from the start of the experiment. In the other group the rate remained approximately constant. In none of the six

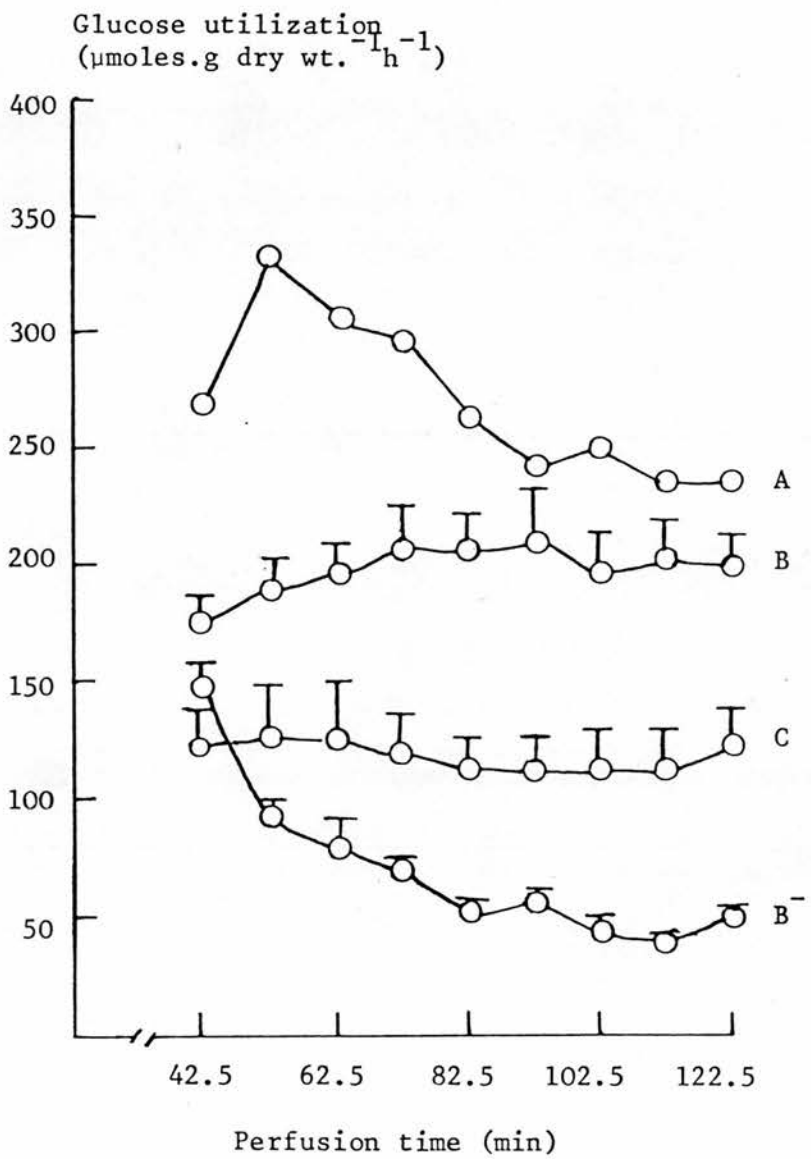

Fig. 22. The time-course of glucose utilization after preperfusion with insulin

Hearts from fed animals were perfused for 30 minutes with 5.5mM-glucose and insulin at A: 2mU.ml^{-1}, B and B^{-}: 80μU.ml^{-1}, C: 40μU.ml^{-1}). Points are the mean $\pm$ S.E.M. of the subsequent glucose utilization in the absence of insulin for 3 hearts (B, and B^{-}) and 5 hearts (C).

Values in series A are the averages from two experiments.

experiments were there any obvious mechanical or physical differences associated with the two groups.

7.6 Discussion

The results presented in Section 7.4 suggest that glucose utilization by the isolated perfused rat heart is more sensitive to insulin than appears to have been found before. The cause of this improvement is not certain. In comparison with Bleehen and Fisher (1954) the improvement could be explained by the inclusion of albumin in the perfusate but they found a two-fold stimulation by insulin at $60\mu U.ml^{-1}$ whereas in the absence of albumin in this work $60\mu U.ml^{-1}$ and $160\mu U.ml^{-1}$ insulin were both ineffective. In addition Meuli and Froesch (1975) included protein in their perfusate at $2.5mg.ml^{-1}$ but obtained a sub-maximal stimulation by $200\mu U.ml^{-1}$ insulin.

It is possible that the explanation lies in a difference in the system of perfusion. Meuli and Froesch (1975) using a fat-pad bioassay found that the activity of insulin originally at $220\mu U.ml^{-1}$ fell by 20% during closed-circuit perfusion for 60 minutes. The fall might be relatively greater at lower concentrations of insulin. Posner, Sotman and Antoniades (1968) also reported a fall in the insulin content of the perfusate of hearts despite the presence of albumin. Continuous infusion of insulin as in the experiments reported here would tend to maintain the concentration of insulin in the perfusate closer to that at the start of the experiment. However using $40\mu U.ml^{-1}$ insulin that causes a sub-maximal stimulation on glucose utilization, the effect was examined of doubling the infusion rate after an hour's perfusion but no change in glucose utilization was detected.

Another explanation may lie in the effect of the type of filter used. As was shown in Chapter 6 the use of Millipore filters following the practice of Fisher and O'Brien (1972) gives results which are in

reasonable agreement with what were taken to be the best in the literature. The rates of glucose utilization were low and associated with little lactate production. With paper filters the rate of utilization was reduced by 56% and with paper filters and albumin in the perfusate the reduction was 36%. This effect is unlikely to be an inhibition of glucose utilization attributable in some way to the paper filter because the same grade of paper filter was present in all experiments acting as a support to the Millipore filter. A reduction in glucose utilization when Millipore is replaced suggests that Millipore in some way tends to raise glucose utilization. The increase in utilization can not be linked to significantly increased production of lactate and when glucose utilization is maximally stimulated by insulin the rates with the two types of filters are indistinguishable. This suggests that the effect of Millipore might be to increase the permeability of the cardiac cells to glucose. If such an effect occurs and if the effect of insulin is not additive with it, the apparent sensitivity to insulin would be affected. This might explain how Bleehen and Fisher (1954) using a Soxhlet thimble filter found 60μU.ml^{-1} insulin to be effective in the absence of albumin while in this work using a Millipore filter that concentration of insulin was ineffective. Use of sintered glass filters are associated with an increase in the permeability of isolated rat hearts to non-metabolised sugars if protein is excluded from the perfusate (Zachariah, 1961) whereas there is no increase in permeability when paper filters are used regardless of the presence or absence of protein (Fisher and Gilbert, 1970a).

The implications of the sensitivity to insulin of glucose utilization by isolated perfused rat heart depends on what is taken to be the range of concentrations of insulin in the blood of the fed rat and the extent to which the response to insulin is altered when the work

of the heart is increased (Neely et al., 1967b; Taegtmeyer et al., 1980). If the concentration of insulin in the fed rat is about $30\mu U.ml^{-1}$ as found by Hawkins et al. (1971) this would be sufficient to cause a 2.5-fold increase in glucose utilization if insulin was the only regulator but the rate would still be 50% of the maximum. However the values reported by Hawkinset al. (1971) were found in rats kept in restraining cages. In these circumstances glucose tolerance has been shown to be reduced (Shah, Wongsurawat, Aran, Motto and Bowser, 1977), perhaps because of a relative inhibition of insulin secretion. In another study the concentration of plasma insulin in the fed rat was found to be $84\mu U.ml^{-1}$ (Aynsley-Green et al., 1973). At these concentration insulin alone would fully activate glucose utilization by cardiac muscle. However the response of the heart to insulin is likely to be complicated by the work required of the organ. Taegtmeyer et al. (1980) who used Millipore filters and $10mU.ml^{-1}$ insulin found no response of glucose utilization to insulin in hearts perfused under conditions of high work load and with glucose as the only substrate. At low work loads and at high work loads when a choice of substrates was available, insulin stimulated glucose utilization. Neely et al. (1967b) found that insulin could further stimulate the permeation of L-arabinose into heart muscle whose permeability had already been increased by a work-load. In fact the sensitivity of permeation to insulin was greater in the working than in the Langendorff preparation.

If the concentration of insulin in the blood of the fed rat is $40\mu U.ml^{-1}$ or more the sensitivity of the isolated heart to insulin is sufficient to suggest that the decline in utilization early in the perfusion of hearts in the absence of added insulin could be due to the wash-out of endogenous insulin. However the experiments designed

to test this possibility were inconclusive. Hearts exposed to 2mU.ml^{-1} insulin have high rates of glucose utilization (330μmoles.g dry wt.$^{-1}$h^{-1}) when subsequently perfused without insulin. These rates may be compared with that in Table 22 where the rate of utilization after 12.5 minutes of perfusion without insulin was 157μmoles.g dry wt.$^{-1}$h^{-1}. The average rate for all six hearts that had been exposed to 80μU.ml^{-1} insulin was 164μmoles.g dry wt.$^{-1}$h^{-1} after 12.5 minutes perfusion without insulin but the time-courses of utilization thereafter deviate so much that it is not justifiable to claim that this first estimate reflects the carry-over of insulin. In the case of hearts exposed to 40μU.ml^{-1} the constancy of the rate of utilization need not indicate any lack of effect of endogenous insulin when the concentration of plasma-insulin in vivo is uncertain.

Bleehen and Fisher (1954) also performed similar experiments. Hearts were exposed to insulin at 2mU.ml^{-1} and 0.2mU.ml^{-1} for 15 minutes and then perfused without insulin for a further 30 minutes. Only the higher concentration caused higher rates of utilization in the second period. Both studies show that the effects of insulin at high concentration do persist during subsequent perfusion without insulin but provide no good evidence for this happening at physiological concentration of insulin. Possibly hearts that have been perfused for 30 minutes and have an increased volume of interstitial fluid should not be compared with freshly excised hearts, but it is on the whole likely that the initial high rate of glucose utilization of hearts from fed animals perfused without insulin only indirectly reflects the elevated concentration of insulin in the animal.

CHAPTER 8

Glucose Utilization by Hearts from Fasted Rats

8.1 Introduction

Several investigations whose results are shown in Table 18 (Section 5.4) have concluded that glucose utilization in the absence of insulin by hearts isolated from fasted rats is less than that by hearts from fed animals. However in these investigations the estimates of the rate of glucose utilization were made during periods when the results presented in Chapter 6 suggest that the hearts from the fed rats were likely for at least part of the time to have been using glucose at a declining rate. If this decline reflects the disappearance of an effect of endogenous insulin, the early rate of glucose utilization by hearts from fasted rats might reasonably be expected to be lower, because the plasma insulin concentration of fasted rats is well established to be lower than that in fed rats (Hales and Randle, 1963; Aynsley-Green et al., 1973). However observed differences between the fed and fasted state need not simply be differences in the effect of insulin because the possibility exists of other effects resulting from the changed hormonal status and energy sources in the fasted rat. The time-course of glucose utilization by hearts from fasted animals therefore deserves investigation, not only to clarify the effect of any early changes in rate on the comparisons that might be made with hearts from fed animals but also to permit comparison of rates after perfusion for an hour or more. After prolonged perfusion the hearts may be reasonably supposed to be free of insulin and any differences in glucose utilization attributable to more lasting effects of fasting. In this respect the duration of fasting is also relevant for although it would not be expected to affect the insulin content of hearts perfused for more than one hour, lasting effects of fasting might

intensify with the duration of the fast. There is evidence of lasting effects of fasting in the observation that the rate of glucose utilization in the presence of added insulin by hearts from fasted animals is lower than that by hearts from fed animals (Opie et al., 1963; Randle et al., 1964 see Tables 20 and 21).

For these reasons the time-course of glucose utilization was studied in hearts from animals fasted for varying periods and perfused with and without added insulin.

8.2 The Time-Course of Glucose Utilization in the Absence of Added Insulin

Rats were fasted overnight or for 48 hr and their hearts were perfused with 5.5mM-glucose for 150 minutes or 120 minutes respectively. Regardless of the duration of fasting the concentration of glucose in the perfusate became constant without passing through a minimum as was the case with hearts from fed animals perfused without added insulin. Later the glucose concentration fell as the utilization increased. In this respect the behaviour of the hearts from fed and fasted animals is the same.

The time course of the average rate of glucose utilization by these hearts is shown in Fig. 23 together with the results obtained with hearts from fed rats. Whether the animals were fasted overnight or for 48 hr the early rate of glucose utilization by the isolated perfused hearts was less than that seen in hearts from fed animals. However the time-course of utilization in hearts from fasted rats was similar during two hours of perfusion regardless of the duration of the fast. Increasing the duration of the fast had the effect, if any, of increasing the rate of glucose utilization rather than lowering it further. Data from all experiments with fasted animals have been combined regardless of the duration of the fast and the mean of the rates are given in Table 24. The reduction in the rate of glucose

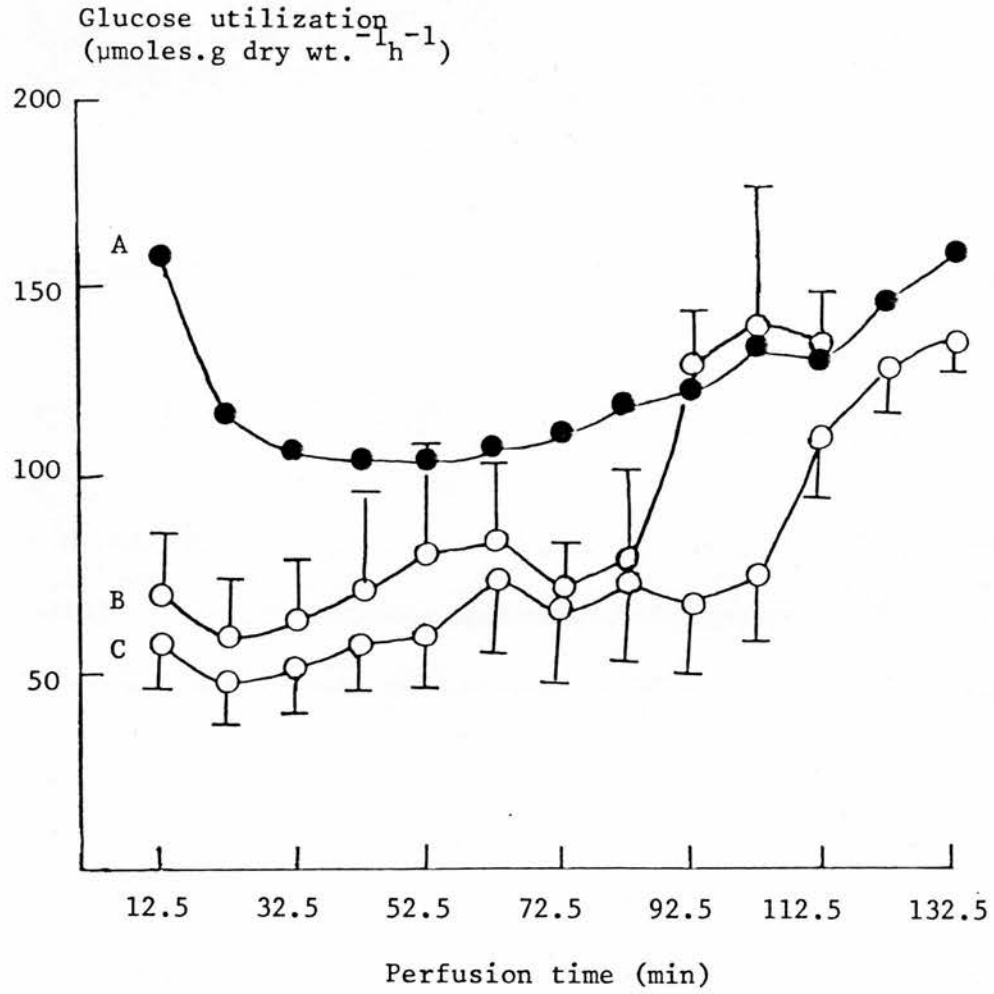

Fig. 23. Time-courses of glucose utilization by hearts from fasted animals

5 rats were fasted overnight (C) and 3 rats for 48h (B). Points are the mean $\pm$ S.E.M. Series A: results with hearts from fed rats (Fig. 20).

Table 24

Glucose utilization by isolated hearts from fed and fasted animals in the absence of insulin

	Time (minutes)												
	12.5	22.5	32.5	42.5	52.5	62.5	72.5	82.5	92.5	102.5	112.5	122.5	132.5
A Fed	157	118	108	103	106	112	121	121	123	134	130	147	158
B Fasted (Overnight)	50	41	45	49	49	68	66	75	98	123	137	146	174
C Fasted (48 hours)	70	61	64	72	79	84	72	78	131	135	131	-	-
D = (B+C)	58	49	52	58	60	74	68	76	111	128	135	156	174
A vs B	a	a	a	a	a	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
A vs D	a	a	a	a	a	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s

t - test;

a = 0.01>P>0.001

n.s = not significant

Hearts were perfused with 5.5mM-glucose. Values are the mean rate of glucose utilization expressed as μmoles.g dry wt.$^{-1}$h^{-1} at 10 minute intervals, for 18-13 hearts from fed rats, 5 hearts from overnight fasted rats and 3 hearts from rats fasted for 48 hours.

utilization by the hearts from fasted rats is initially 63% and falls to 30% after 60 minutes. Statistically the reduction in the rate of glucose utilization is not significant after 90 minutes of perfusion. The rate of glucose utilization ultimately increases in all experiments but the increase occurs earlier and is greater in the hearts from fasted animals. The rate of utilization at 90 minutes differs significantly ($0.05>P>0.02$) from that at 30 minutes in hearts from rats fasted overnight but not in hearts from fed rats.

8.3 The Time-Course of Glucose Utilization in the Presence of Insulin and $2mU.ml^{-1}$

Hearts from rats fasted overnight or for 48 hours were perfused with MKHM containing 5.5mM-glucose and $2mU.ml^{-1}$ insulin for 150 minutes or 120 minutes respectively. The concentration of glucose in the perfusate fell with time and became constant after 60 minutes. The time-courses of the mean rates of glucose utilization by hearts from rats fasted overnight and 48 hours are shown in Fig. 24 together with time-course found when hearts from fed animals were perfused with 5.5mM-glucose and $2mU.ml^{-1}$ insulin. The pattern of the time-course of glucose utilization by hearts from fasted animals during the first 40-50 minutes of perfusion period is different from that found with hearts from fed rats. There is an initial progressive increase in the rate that is very obvious in the hearts from rats fasted for 48 hours whose initial rate is significantly different from that seen in the fed state. In the hearts from rats fasted overnight it took about 30 to 40 minutes to reach a constant rate of glucose utilization which a run analysis suggests is significantly greater than the rate of utilization in hearts from fed rats. On the other hand with hearts from rats fasted for 48 hours, the rate of glucose utilization progressively increases during the first 50 minutes. Thereafter there

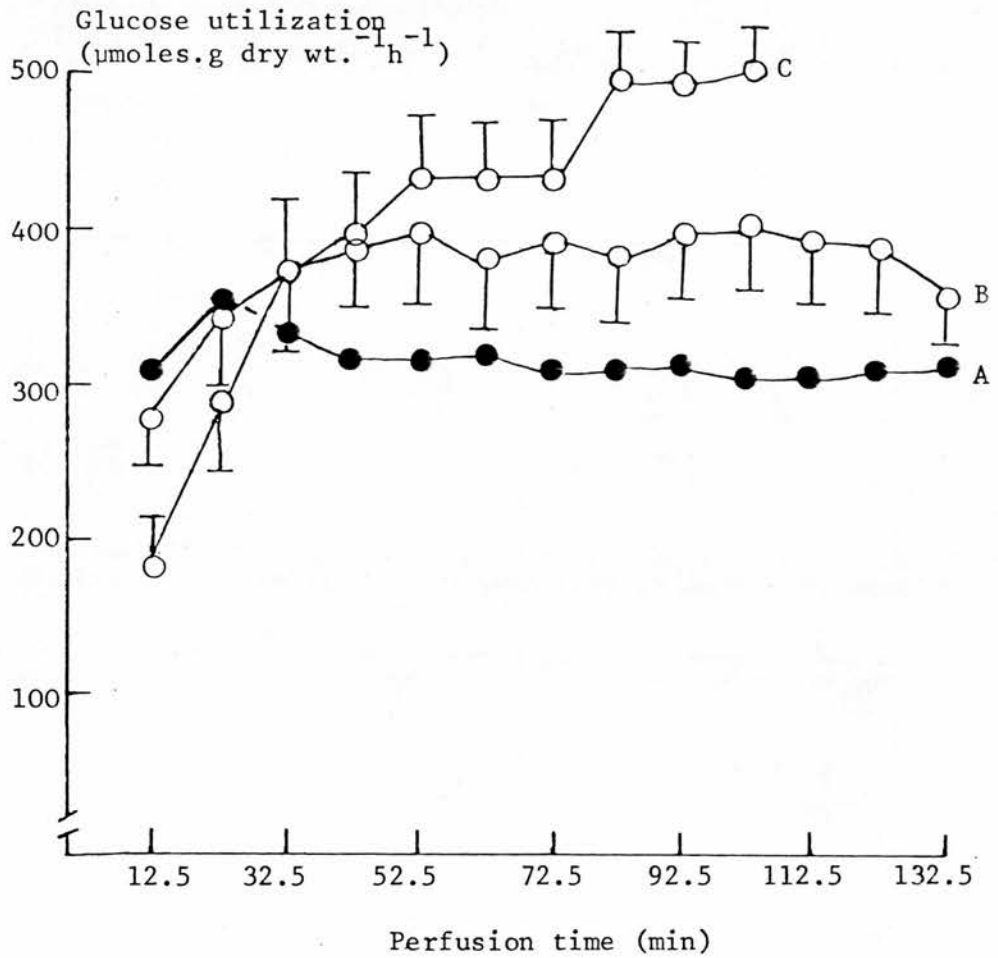

Fig. 24. Time-courses of glucose utilization in the presence of insulin by hearts from fasted animals

Hearts from rats fasted for overnight or 48h, B and C respectively and from fed rats A were perfused with 5.5mM-glucose and insulin 2mU.ml^{-1}.

3 rats were fasted overnight (B) and 4 rats for 48h (C). Points are the mean $\pm$ S.E.M. Series A: results with hearts from fed rats (Fig. 16).

is a period of relative constancy before the increase in utilization resumed. However the pattern does not differ significantly from one of continuous increase throughout the experiment. After 50 minutes of perfusion, glucose utilization in hearts from animals fasted for 48 hours is significantly higher than in the fed state (Table 25). Fasting and its duration affect the pattern of the time-course of glucose utilization and the rate of glucose utilizatio n by the isolated perfused rat heart in the presence of added insulin.

8.4 The Time-Course of Glucose Utilization in the Presence of Insulin 80μU.ml^{-1}

Hearts from animals fasted for 48 hours were perfused with MKHM containing 5.5mM-glucose, 0.5mg.ml^{-1} albumin and 80μU.ml^{-1} insulin for 120 minutes. Fig. 25 shows the time-course of the mean rate of glucose utilization in these experiments. As with the experiments at a higher concentration of insulin the rate of utilization by hearts from fasted animals was initially low but increased steadily until after 60 minutes it exceeded the rate achieved in hearts from fed animals (Table 26). Because of the effect of Millipore filters which was discussed in Chapter 7 a simple comparison with the experiments in which a higher concentration of insulin was used is not possible. When paper filters, albumin and a lower concentration of insulin are used the time-course of utilization in hearts from fasted rats is similar in pattern to that seen with Millipore filter and 2mU.ml^{-1} insulin but differ in that at all times the rate are lower at the low concentration of insulin.

8.5 Discussion

In the absence of insulin glucose utilization by hearts from fasted rats was lower during the first ninety minutes of perfusion than was found in hearts from fed animals. The degree of reduction ranged from

Table 25

Glucose utilization by hearts from fed and fasted animals in the presence of high concentration of insulin

	Time (minutes)												
	12.5	22.5	32.5	42.5	52.5	62.5	72.5	82.5	92.5	102.5	112.5	122.5	132.5
A Fed	309	351	328	316	315	317	310	310	314	307	306	311	317
B Fasted (Overnight)	277	342	372	386	394	379	390	382	389	398	390	386	359
C Fasted (48 hours)	186	285	371	396	428	428	432	494	489	531	-	-	-
A vs B	n.s	n.s	n.s	a	a	n.s	b	a	a	b	b	a	-
A vs C	b	n.s	n.s	b	c	c	d	d	d	d	-	-	-

a = 0.05 P 0.02

b = 0.02 P 0.01

c = 0.01 P 0.001

d = 0.001 P

n.s = not significant

Hearts were perfused with 5.5mM-glucose and 2mU.ml^{-1} insulin. Values are the mean rate of glucose utilization expressed as µmoles.g dry wt.$^{-1}$h^{-1} measured at 10 minutes intervals, 14-12 hearts from fed rats and for 3 and 4 hearts from rats fasted for overnight and 48 hours respectively.

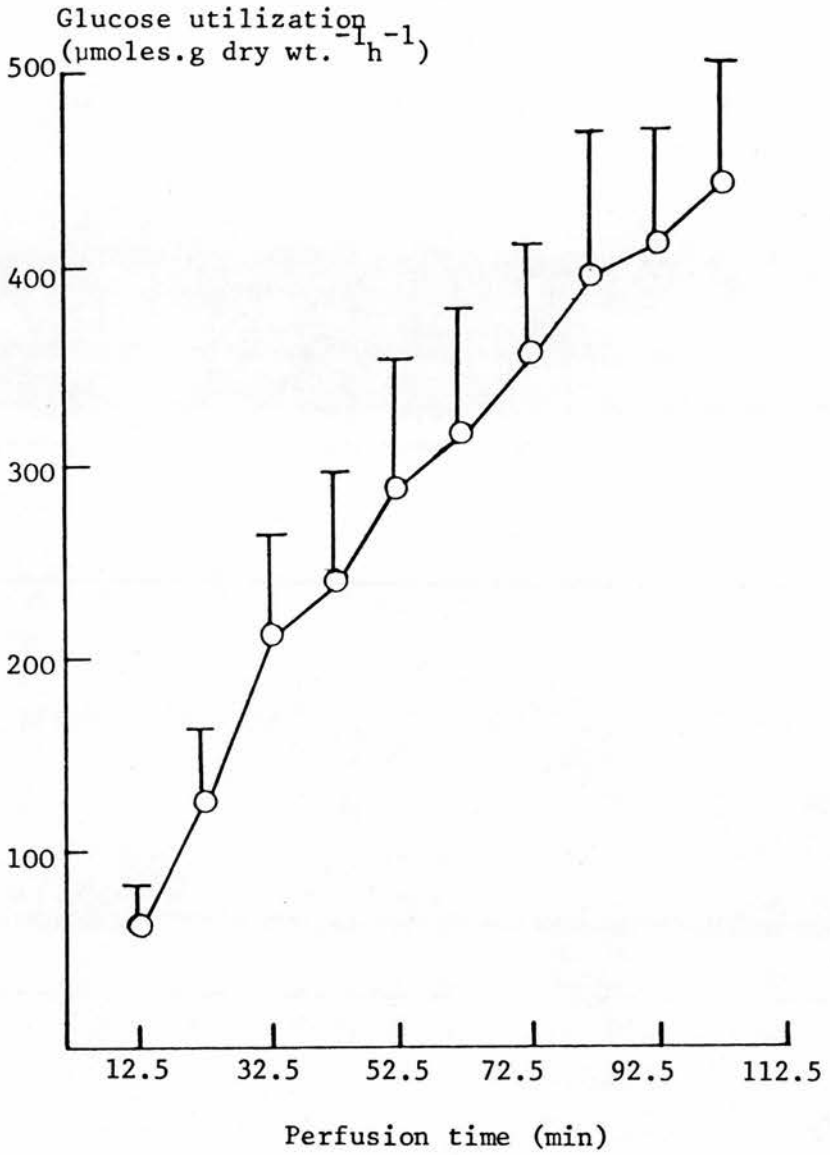

Fig. 25. The time-course of glucose utilization at low insulin concentration by hearts from fasted rats

5 rats were fasted for 48h and their hearts were perfused with MKHM containing 5.5mM-glucose, 80μU.ml^{-1} insulin and 0.5mg.ml^{-1} albumin. Points are the mean $\pm$ S.E.M.

Table 26

Glucose utilization at low insulin concentration

	Time (minutes)									
	12.5	22.5	32.5	42.5	52.5	62.5	72.5	82.5	92.5	102.5
Fed	237	243	237	257	266	262	272	278	284	289
Fasted (48 hours)	61	125	208	237	286	315	350	391	408	439

Hearts were perfused with 5.5mM-glucose, 0.5mg.ml^{-1} albumin and 80μU.ml insulin. Values are the mean rate of glucose utilization expressed as μmoles.g dry wt.$^{-1}$h^{-1} measured at 10 minute intervals, for 12 hearts from fed rats and 5 hearts from fasted rats.

63% after 12 minutes to zero after 90 minutes. Whereas the rate in hearts from fed animals initially falls and then becomes constant, and tends to rise again during the last stage of perfusion, in hearts from fasted rats the rate is initially constant, and tends to rise again during the last stage of perfusion.

The difference in the early part of the time-courses is consistent with the idea that the falling utilization by hearts from fed rats reflects the loss of endogenous insulin or of its effect because hearts from fasted rats would be expected to contain less insulin. However the constant rate of glucose utilization by hearts from fed rats that is established after 30 to 40 minutes of perfusion is greater than the constant rate in hearts from fasted animals that is maintained for the first 40 minutes of perfusion. Fasting appears to reduce glucose utilization by 45-50% when there is no reason to suppose that endogenous insulin is exerting any short-term effect. The duration of fasting has no effect on the size of this reduction in glucose utilization in the absence of added insulin. It appears that hearts from rats fasted overnight have a reduced ability to use glucose that cannot be attributed directly to a lack of endogenous insulin. Consequently fasting cannot be regarded as a convenient way of producing hearts free of insulin but otherwise identical to those of fed animals.

The rise in the rate of glucose utilization by hearts from fasted rats that occurs after 50 minutes of perfusion without insulin might reflect the release of an inhibition or the development of an activation of glucose metabolism or progressive failure of the preparation with associated changes in permeability to glucose. Earlier failure of hearts with lower initial rates of glucose utilization might occur because these hearts must make greater demands on their endogenous

reserves. In hearts from fasted rats the contribution of perfusate glucose to the energy needs of the heart is around half the contribution made to hearts from fed rats. However no mechanical deterioration was seen to accompany the increase in glucose utilization and fasting approximately doubles the amount of glycogen found in freshly excised rat hearts (Randle et al., 1964). Attempts to examine the effect of depletion of endogenous reserves on glucose utilization are described in the next chapter.

The release of an inhibition of glucose utilization is an attractive hypothesis because in the fasting state alternative substrates to glucose such as fatty acids or the ketone bodies are thought to inhibit the metabolism of glucose (Randle, Garland, Hales and Newsholme, 1963). In experiments of the length used here the effect of extracellular substrates that accompany the heart during its isolation can be ignored. It is also unlikely that any release of inhibition involves the washout of an inhibitor from the heart when the increase in utilization is delayed for 40 minutes after the start of the experiment. Conceivably regulation might be exerted by endogenous fatty acids themselves or a derivative of them that falls in concentration as it or its source is consumed.

Table 27 shows the effect of fasting on the rate of glucose utilization by hearts perfused without insulin reported from several laboratories and in this work. All agree that fasting reduces the rate of utilization and Randle et al. (1964) also agreed with the finding of this work that the duration of the fast did not significantly affect the rate of glucose utilization by hearts from fasted rats. Since it has been shown here that the early rate of utilization is constant in hearts from fasted animals these differences in the ratio found for those hearts cannot be attributed to variations in any

Table 27

Glucose utilization by isolated perfused hearts from fasted rats

Insulin	Glucose Utilization (μmoles.g dry wt.$^{-1}$h^{-1}) Fed	Fasted	Fasted:Fed	Period of Perfusion (minutes)	Reference
-	293	82	0.38	5-35	Opie et al. (1962)
-	345	230	0.66	5-35	Opie et al. (1963)
-		163	0.47	5-35	Opie et al. (1963)
-	249-239	161	0.54-0.67	0-15	Randle et al. (1964)
-		114	0.38-0.47		"
-		89*	0.3-0.37		"
-	391	306	0.78	5-35	Shipp (1964)
-		285	0.98	10-40	"
-		335	0.91	10-40	"
-	406	245	0.6	10-70	Shipp et al. (1964)
-	157	50	0.32	10-15	This work
-	107	53	0.49	30-60	
+	520	352	0.68	5-35	Opie et al. (1963)
+	394	261*	0.66	0-15	Randle et al. (1964)
+	309	243	0.79	10-15	This work
+		185**	0.60	10-15	"
+	313	368	1.2	50-70	"
+		429**	1.37	50-70	"

The hearts from fed and fasted animals were perfused with 5 to 5.5mM-glucose with 2mU.ml^{-1} in the work presented in this text and 100mU.ml^{-1} by others or without insulin. All rats were fasted for overnight except * for 40 hours and ** for 48 hours.

period of pre-perfusion. The size of the reduction reported is affected by the inclusion in the experimental period of the early phase of high rates of utilization by hearts from fed rats. The earliest estimate of 157μmoles.g dry wt.$^{-1}$h^{-1} made in this work at 12.5 minutes was found to be reduced by about 63% while in the periods of metabolic stability the reduction in rate was 45%.

Insulin at 2mU.ml^{-1} did not immediately restore the rate of glucose utilization to the levels found for hearts from fed rats. In hearts from rats fasted overnight the early rate was lower but not significantly so. However after an hour the hearts were using glucose at higher rates than did hearts from fed rats. In hearts from animals fasted for 48 hours, the rate of glucose utilization was significantly lower during the first 20 minutes of perfusion. Moreover the rate of glucose utilization progressively increases during the first 50 minutes of perfusion, possibly steadying between 50 and 75 minutes before increasing again. The rates reached towards the end of the experiments would if fully oxidized require 3000μmoles.oxygen.dry wt.$^{-1}$h^{-1} which is almost double that measured by O'Brien (1972) in hearts from fed animals. Repetition of these experiments measuring the rate of lactate production would help to explain the fate of the glucose. Although the results of these experiments with hearts from rats fasted for 48 hours differ particularly in their late stages from those obtained with hearts fasted overnight, they are qualitatively very similar to the results obtained when hearts from animals fasted for 48 hours were perfused with 80μU.ml^{-1} insulin. In both the rates are initially significantly less than those obtained with fed animals and are finally significantly greater. However at 80μU.ml^{-1} insulin the rate of utilization after 12.5 minutes is reduced by 70% while at 2mU.ml^{-1} the reduction is 39%. This difference may be explained in part or wholly by the effect of

Millipore filters on utilization when insulin has less than maximal effects and need not be taken to indicate an effect of increasing concentration of insulin. Nevertheless the resistance to the action of insulin at $80\mu U.ml^{-1}$ is considerable.

Opie et al. (1963) and Randle et al. (1964) found that $100mU.ml^{-1}$ insulin cannot fully restore glucose utilization by hearts from rats fasted for 18h and 40h respectively. Table 27 shows the ratio of glucose utilization in the presence of insulin in fasted:fed state. These other workers studied the rate of utilization in at most the first 35 minutes of perfusion using a closed-circuit systems of perfusion. An underlying increase in rate during this period would not be detectable. No comparable experiments appear to have been made in which glucose utilization by hearts from fasted rats was measured after prolonged perfusion.

CHAPTER 9

Glucose Utilization by Hearts After Perfusion Without Substrate

9.1 Introduction

Since perfusate glucose can provide the isolated heart with 80-100% of its energy needs in the presence of insulin but only 30-40% in the absence of insulin, the endogenous reserves of glycogen and triglyceride are likely to be consumed in meeting its demand for energy. It is conceivable that the depletion of these reserves is associated with the tendency for glucose utilization by hearts perfused without insulin to increase after 90 to 120 minutes of perfusion. Protein probably makes an insignificant contribution to the energy supply. Net proteolysis in the non-working perfusate rat heart has been estimated to be 15μmoles.g dry wt.$^{-1}$h^{-1} (Takala, Hiltunen and Hassinen, 1980). This may have an important anaplerotic role but could only make a minor contribution as a source of energy. Consequently glycogen and triglyceride can be regarded as the only endogenous sources of energy.

The glycogen content expressed in glucose equivalents of the freshly excised heart taken from fed rats has been found to lie in the range 45 to 91μmoles.g dry wt.$^{-1}$ (Fisher and Williamson, 1961a; Opie et al., 1962; Opie et al., 1963; Dhalla, Matoushek, Sun and Olson, 1973; Gartner and Vahouny, 1973). When hearts are perfused without substrate, the reported rates of fall in the glycogen content are variable. After 30 minutes of such perfusion the glycogen content may have fallen by 61% (Fisher and Williamson, 1961a), or 35% (Dhalla, Yates and Olson, 1972), while after 60 minutes the store is depleted by 79% (Fisher and Williamson, 1961a) or 70% (Dhalla et al., 1972). Fisher and Williamson (1961a) found that insulin in the absence of glucose had no effect on the rate of depletion of the store.

The amount of triglyceride in the freshly excised heart taken from fed rats has been found to lie in the range 13 to 19μmoles.g dry wt.$^{-1}$ (Denton and Randle, 1965; Denton and Randle, 1967; Olson and Hoescher, 1967; Dhalla et al., 1973). When hearts are perfused without substrate the observed rate of decrease of the store is variable as was the case with the glycogen store. However there is agreement that the decrease is slow at first. Dhalla et al. (1972) found no change in the triglyceride content after 45 minutes of perfusion without substrate and thereafter it fell by 40%, 55% and 75% after 60, 90 and 120 minutes perfusion respectively. Similarly Olson and Hoescher (1967) reported a negligible fall after 30 minutes of perfusion but a fall of 45% after 60 minutes and 68% after 90 minutes.

In this chapter the results of experiments to test the effect of a period of substrate-free perfusion on the subsequent rate of glucose utilization are described. A period of 45 minutes perfusion without substrate was chosen because this would be expected to reduce the glycogen store by approximately 50% with little effect on the triglyceride store. To achieve a similar reduction in the triglyceride store would have required substrate-free perfusion for 90 minutes by which time many hearts perfused with glucose in the absence of insulin are tending to increase their rate of glucose utilization. Substrate-free perfusion for longer than 45 minutes but less than 90 minutes appears to offer no particular advantage.

9.2 The Effect of Substrate-Free Perfusion on the Subsequent Glucose Utilization in the Absence of Insulin

Three hearts were perfused with MKHM containing no substrate for 45 minutes, and then the infusate was changed to one containing 5.5mM-glucose and perfusion continued for a further 105 minutes.

During the period of perfusion without substrate the hearts were strong and vigorous for approximately 30 minutes, but became slower and weaker in the following 15 minutes. On the infusion of glucose at 25ml.h^{-1} the hearts regained their rate and force of contraction and the concentration of perfusate glucose became constant after a further 40 to 50 minutes.

Another set of three hearts from fed rats were perfused with MKHM containing no substrate for 45 minutes, and were transferred to a second perfusion system, where they were perfused with the MKHM containing 5.5mM-glucose at an infusion rate of 26ml.h^{-1} for a further 120 minutes. The perfusate glucose concentration fell slowly during the rest of the experiments indicating a steady increase in the rate of utilization. However the time-course of the mean rate of glucose utilization for the two groups was similar.

If the mean rate of all six experiments at any time (Fig. 26) is compared with the corresponding rate in hearts provided with glucose throughout the experiment there are no significant differences. Run analysis indicates that the rate of glucose utilization is significantly reduced in the hearts perfused without substrate. However the data in the series are not independent.

9.3 The Effect of Depletion on Glucose Utilization in the Presence of Insulin

A group of six hearts were perfused with MKHM containing 2mU.ml^{-1} insulin, but no substrate for 45 minutes, and then MKHM containing 5.5mM-glucose and 2mU.ml^{-1} insulin was infused for a further 105 minutes. The mean rate of glucose utilization at 328 ± 11μmoles.g dry wt.$^{-1}h^{-1}$ was constant throughout the period of perfusion with glucose. This may be compared with the overall rate of glucose utilization of

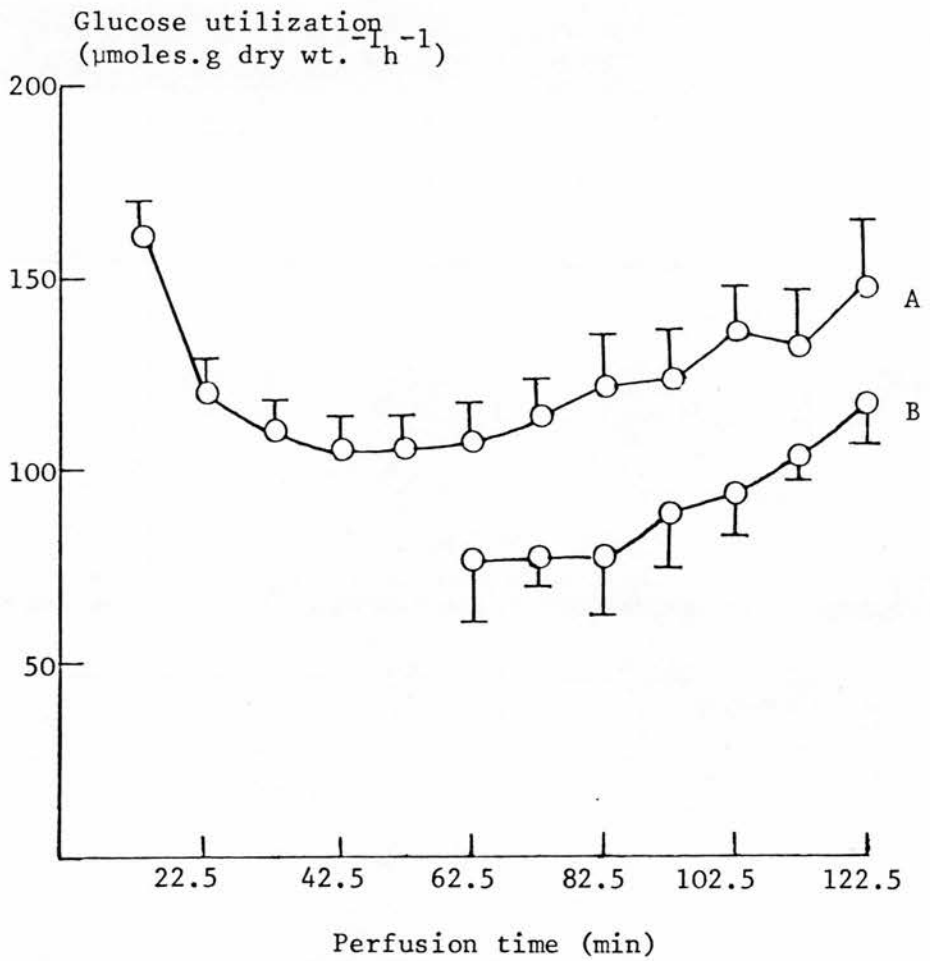

Fig. 26. Effect of preperfusion without substrate on subsequent glucose utilization

A: Hearts from fed animals perfused with 5.5mM-glucose from the beginning of the experiment. Data from Fig. 20.

B: 6 hearts from fed animals preperfused without substrate for 45 minutes and then perfused with 5.5mM-glucose as described in the text.

Points are the mean $\pm$ S.E.M.

329 ± 3 μmoles.g dry wt.$^{-1}$ h^{-1} by hearts perfused with 5.5mM-glucose and 2mU.ml^{-1} insulin from the start of the experiment.

9.4 Discussion

The rate of glucose utilization by hearts after 45 minutes of perfusion without substrate is not increased as would be expected if the depletion of endogenous reserves was related to the tendency for glucose utilization to increase in hearts subject to prolonged perfusion with glucose in the absence of insulin. Although perfusion without substrate had no detectable effect on subsequent glucose utilization in the presence of insulin, in the absence of the hormone the rate tended to be reduced.

If the consumption of endogenous reserves does influence the pattern of glucose utilization it seems unlikely that the depletion of the glycogen store is involved. It is possible that glucose utilization responds to the reduction of the triglyceride reserves or to the condition that starts their mobilization. The deterioration in the mechanical performance of the hearts suggests that the endogenous reserves cannot be mobilized sufficiently rapidly to satisfy the energy requirements so that a low concentration of free fatty acids in the heart or of their derivatives might permit an increased rate of glucose utilization.

The pattern of the time-course of glucose utilization in the absence of insulin shows no effect of the period of perfusion without substrate. It is particularly noticeable that there is no decline in the rate in the 30 minutes following the introduction of glucose, which suggests that the decline seen on the initiation of perfusion does depend on the condition of the freshly-excised heart.

CHAPTER 10

Glucose Utilization at Low Glucose Concentrations

10.1 Introduction

When the concentration of glucose in the perfusate is low enough for the rate of utilization to vary with the concentration there are disadvantages in using a closed-circuit system of perfusion as was discussed in the General Introduction. In contrast perfusion with balanced infusion and withdrawal should be particularly suitable for studying glucose utilization in these conditions because it should allow measurements to be made at constant concentrations of perfusate glucose with good precision since the differences in glucose concentration between the infusate and perfusate should be relatively large. The lowest concentration that can usefully be used is determined partly by the sensitivity of the analytical methods and partly by the difference in concentration of glucose entering and leaving the heart which may become so large that the heart is not uniformly perfused.

Apart from investigating the relationship between glucose utilization the concentration, experiments with low concentrations could help to explain the differences in the time-course of glucose utilization that were found at higher concentrations (Chapter 6). However rates of utilization may vary both with time and concentration before constant concentrations become established and this can be anticipated to complicate an analysis of the time-course at low concentrations of glucose. There are also important implications in the response of the rate of utilization to insulin at low concentrations of glucose. The sensitivity to low concentrations of insulin is relevant to the issue of the significance of the loss of endogenous insulin to the time-course of glucose utilization and also, together with the response to high insulin concentrations, to the mechanism of insulin action

on glucose permeation. Fisher and Gilbert (1970c) concluded that insulin increased the half-saturation constant for the permeation of pentoses and that the effect on the maximum rate of permeation increased with the concentration of insulin. Since at low concentrations of glucose an increase in the half-saturation constant of permeation might markedly offset the effect of insulin on the rate of permeation particularly if the insulin concentration is also low, the effect of insulin in these circumstances deserves investigation. The lowest concentrations of glucose that has been used in the perfusate of hearts from fasted rats were 0.83mM and 0.6mM in work by Morgan et al. (1961) and Cheung et al. (1978) respectively. These concentrations were found at the end of experiments in closed-circuit systems. Fisher and O'Brien (1972) measured glucose utilization by hearts from fed rats at a constant glucose concentration of 0.22mM. All these workers found insulin to stimulate glucose utilization at these or similar concentrations.

10.2 Experimental Conditions

Hearts from fed rats were perfused with MKHM containing initially 0.33mM, 0.11mM, 0.06mM or 0.03mM-glucose. Insulin when present was at 25mU.ml^{-1}, 2mU.ml^{-1}, 80μU.ml^{-1}, 60μU.ml^{-1} or 40μU.ml^{-1}. Albumin at 0.5mg.ml^{-1} was included when insulin was at a concentration of less than 2mU.ml^{-1} and in the appropriate control experiments.

10.3 Results and Discussion

10.3.1 Lower Practicable Limit of Glucose Concentration

When hearts were perfused with glucose at an initial concentration of 0.03mM and with insulin, the concentration of perfusate glucose between 60 and 90 minutes averaged 0.016mM. This concentration is at the limit of the sensitivity of the method used to estimate glucose

and the error associated with its measurement made it uncertain when a constant concentration was established in the perfusate. If it is assumed that the concentrations were constant and their average value between 60 and 90 minutes is taken the mean rate of utilization in five experiments was 4.9μmoles.g dry wt.$^{-1}$h^{-1}. In the light of this uncertainty, 0.06mM was adopted as the lowest initial concentration.

10.3.2 Glucose Utilization in the Absence of Insulin

Fig. 27 shows the time-course of the mean rate of glucose utilization by hearts perfused at initial glucose concentrations of 0.33mM, 0.11mM and 0.06mM using Millipore filters and at 0.33mM-glucose with 0.5mg.ml^{-1} albumin and paper filters. Whereas at high concentrations of glucose the use of paper filters was associated with a large reduction in the rate of glucose utilization (Section 7.3) at 0.33mM-glucose the reduction at any one time in the experiments is insignificant.

In both sets of experiments with an initial glucose concentration of 0.33mM the rate of utilization appears to be constant during the first 40 minutes of perfusion and to increase thereafter but only the increase of 43% between 37.5 minutes and 97.5 minutes in hearts perfused using Millipore filters is marginally significant ($0.05>P>0.02$). In the experiments conducted at 0.11mM- and 0.06mM-glucose the rate again appears to increase by 23% and 25% respectively after an early period of constancy but the increases are also only marginally significant. However the interpretation of the time-courses of utilization is complicated by the effect of the change in glucose concentration during the experiments. A high rate of infusion (50ml.h^{-1} to 60ml.h^{-1}) was used in all these experiments because the relatively large difference between the infusate and perfusate concentrations allowed utilization to be measured with good precision. The half-time for the approach to a steady state was therefore short at approximately

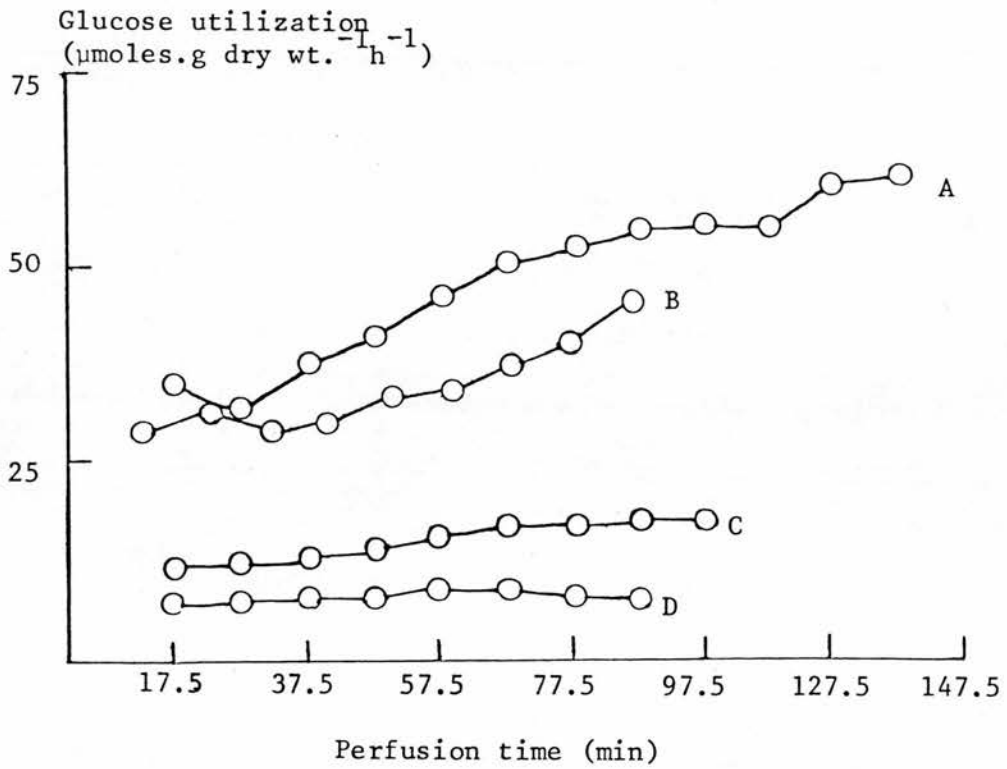

Fig. 27. Time-courses of glucose utilization at low glucose concentrations in the absence of insulin

Hearts from fed animals were perfused as follows: A, 6 experiments with initially 0.33mM-glucose; C, 5 experiments with initially 0.11mM-glucose; D, 6 experiments with initially 0.06mM-glucose and all in A, C, D with Millipore filters. Series B consists of 4 hearts perfused with MKHM containing 0.5mg.ml^{-1} albumin and 0.33mM-glucose using paper filters. Points are the means of the estimates of utilization.

5 minutes and consequently the perfusate concentration was effectively constant within 20 minutes of the start of the experiment and fell to a new constant value when the rate of utilization increased. It is clear from Fig. 27 and Table 28 that the rate of glucose utilization is dependent on the glucose concentration both in the early and later periods of constancy. The fall in the glucose concentration in the perfusate when the rate of utilization tended to increase presumably limited the increase relative to the rate measured at the early higher concentration. It is probable that the tendency for utilization to increase by hearts perfused without insulin at low concentrations of glucose has been underestimated in these experiments.

Fisher and O'Brien (1972) reported that when the concentration of infused glucose was less than 0.5mM, hearts perfused in the absence of insulin showed no change in the rate of glucose utilization in experiments lasting 90 minutes. However in only two experiments at the lowest concentrations of glucose were the conditions comparable with those of this work. In most experiments Fisher and O'Brien perfused hearts with anti-insulin serum in closed-circuit for five to seven minutes before establishing balanced infusion and withdrawal. In the two experiments in which there was no preperfusion with anti-insulin serum, the rate was constant throughout in one and rose initially in the other (Personal communication by Dr. J.A. O'Brien).

The cause of the possible increase in glucose utilization is largely a matter of speculation. If it reflects the wash-out of endogenous insulin, then at physiological concentration insulin must inhibit glucose utilization when glucose concentration is low. This is theoretically possible and is examined in the next section. Another possibility is the mechanism discussed in Section 6.8 in which

Table 28

Glucose utilization at low concentration of glucose

Infusate Glucose (mM)	Perfusate Glucose (mM)		Glucose Utilization (μmoles.g dry wt.$^{-1}$ h^{-1})	Period of Estimation (minutes)
0.06	0.040$\pm$0.001	(6)	7$\pm$0.5	27.5-37.5
	0.036$\pm$0.001	(6)	8$\pm$0.5	57.5-87.5
0.11	0.089$\pm$0.004	(5)	14$\pm$2.0	27.5-37.5
	0.080$\pm$0.004	(5)	17$\pm$1.0	67.5-97.5
0.33	0.258$\pm$0.009	(6)	33$\pm$7.0	27.5-37.5
	0.225$\pm$0.011	(6)	52$\pm$6.0	67.5-97.5
0.3	*0.276$\pm$0.016	(4)	31$\pm$9.0	22.5-32.5
	0.247$\pm$0.011	(4)	38$\pm$5.0	62.5-92.5

Hearts from fed rats were perfused with MKHM containing glucose as shown in the Table.

*These hearts perfused with MKHM containing glucose and 0.5mg.ml^{-1} albumin and using paper filters.

Values are mean $\pm$ S.E.M., and the numbers of observations are in parentheses.

accumulated glycolytic intermediates were proposed to inhibit glucose utilization when the glucose concentration is too low to promote their conversion to glycogen. That mechanism may be relevant to changes in the first twenty minutes of perfusion with insulin but it seems unlikely that it should apply to hearts after an hour perfusion without insulin. Finally the increase may be related to the consumption of endogenous stores but the experiments in which hearts were perfused without substrate did not indicate any effect on the depletion of reserves on the subsequent utilization of glucose. Hearts perfused at these low concentrations of glucose did noticeably weaken after approximately 90 minutes whereas no such change was seen at higher concentrations. The increase preceded the onset of weakening in the group as a whole and did not in any individual heart correlate with the change in mechanical performance.

10.3.3 Glucose Utilization in the Presence of Insulin

The effect of insulin on glucose utilization at low glucose concentration was studied at several concentrations of insulin and of glucose. Fig. 28 shows some representative examples of the time-course of the mean rate of glucose utilization in these experiments. Insulin at $2mU.ml^{-1}$ stimulated glucose utilization at all concentrations of glucose and the time-course of utilization showed no tendency for the rate to increase. Table 29 gives the concentration of glucose in the period of metabolic stability and the rate of glucose utilization at that concentration. The rate of glucose utilization is clearly dependent on the concentration of glucose.

With insulin at $25_{m}U.ml^{-1}$ the rate of glucose utilization was not significantly different from the rate when insulin was present at $2mU.ml^{-1}$. If as reported by Fisher and Gilbert (1970c) the maximum rate of transport of pentoses increases as the concentration of insulin

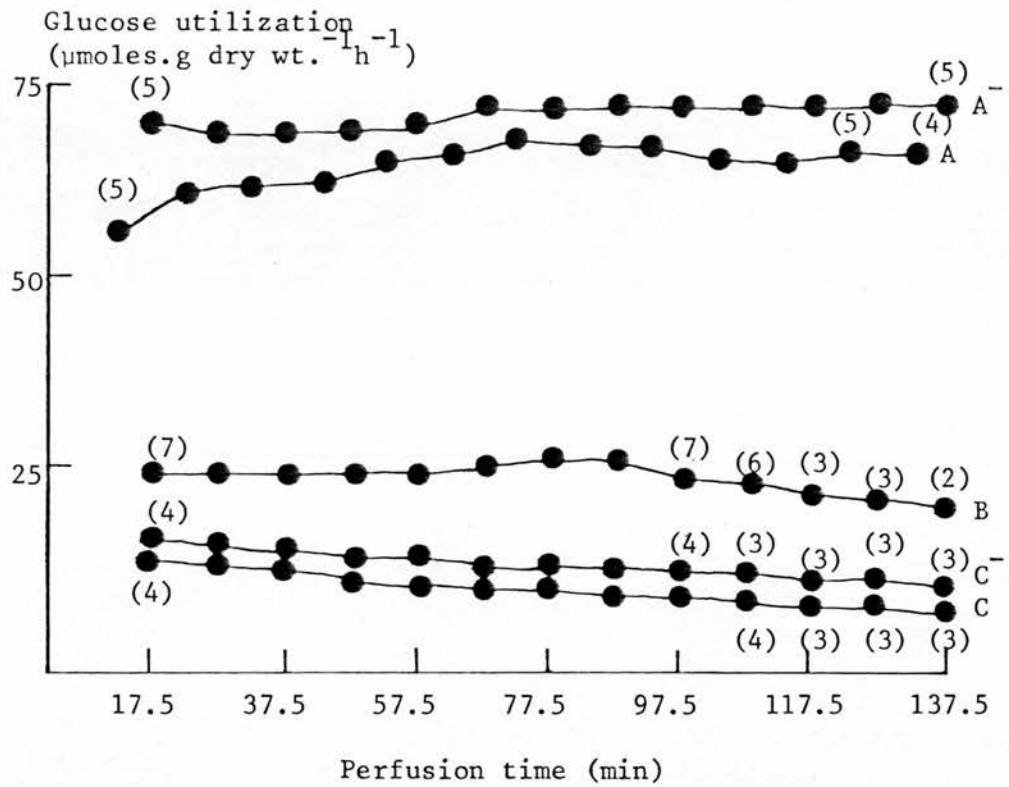

Fig. 28. Time-courses of glucose utilization at low glucose concentrations in the presence of insulin

Hearts from fed rats were perfused with 0.33mM; 0.11 and 0.06mM-glucose and 2mU.ml^{-1} insulin (A, B and C respectively), and with 0.33 and 0.06mM-glucose and 25mU.ml^{-1} insulin (A^- and C^-).

Points are the mean rates for the number of observations are in parentheses which change either because of the use of short experiments or because of technical problems.

Table 29

Glucose utilization at low concentration of glucose in the presence of insulin

Insulin ($mU.ml^{-1}$)	Infusate Glucose (mM)	Perfusate Glucose (mM)	Glucose Utilization ($\mu moles.g\ dry\ wt.^{-1}h^{-1}$)	Period of Estimation (minutes)
25	0.33	0.161 ± 0.04 (5)	72 ± 2.0	67.5-97.5
2		0.184 ± 0.04 (5)	68 ± 3.0	62.5-92.5
*0.08		0.204 ± 0.009 (4)	58 ± 4.0	62.5-92.5
*0.06		0.217 ± 0.006 (4)	68 ± 4.0	62.5-92.5
*0.04		0.211 ± 0.018 (5)	64 ± 4.0	62.5-92.5
2	0.11	0.058 ± 0.003 (7)	26 ± 2.0	67.5-97.5
25	0.06	0.029 ± 0.002 (4)	12 ± 0.1	67.5-97.5
2		0.035 ± 0.001 (4(	10 ± 0.1	67.5-97.5

Hearts from fed rats were perfused with MKHM containing glucose and insulin as shown in the Table.

*These hearts perfused with MKHM containing glucose, insulin and $0.5mg.ml^{-1}$ albumin and using paper filters.

Values are mean $\pm$ S.E.M., and the numbers of observations are in the parentheses.

is raised this is not reflected in the rate of glucose utilization at least by hearts perfused using Millipore filters. When paper filters were used and albumin at 0.5mg.ml^{-1} included in the perfusate, insulin at 80μU.ml^{-1} also stimulated glucose utilization and the rate was not significantly different from those found at the higher concentration of insulin (Figs. 28 and 29 , Table 29). At this and all other concentrations there was no evidence that insulin inhibited glucose utilization. However insulin at 40μU.ml^{-1} and 60μU.ml^{-1} did not immediately stimulate glucose utilization to the extent seen at the higher concentration of the hormone. The time-course of glucose utilization was indistinguishable in the two sets of experiments, both initially showing a rate intermediate between that seen with insulin at 80μU.ml^{-1} and that in the absence of insulin and both ultimately giving a rate comparable or greater than that seen at higher insulin concentrations. Conceivably in all experiments a tendency for glucose utilization to rise is underestimated because in reaching a steady state the glucose concentration in the perfusate fell by approximately 50% which even with a half-time of 5 minutes would be expected to influence the earliest estimate of glucose utilization.

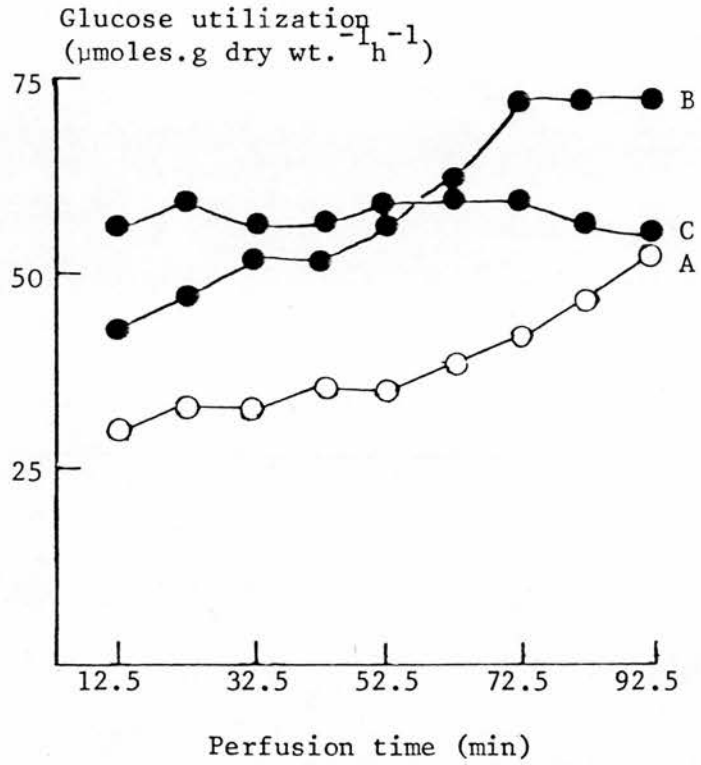

Fig. 29. Time-courses of glucose utilization at low glucose concentration and low insulin concentrations

Hearts from fed rats were perfused with 0.33mM-glucose, 0.5mg.ml^{-1} albumin and without insulin (A) or with insulin 60 and 80μU.ml^{-1} (B and C respectively).

Points are the mean of 4 experiments in each group.

CHAPTER 11

The Kinetics of Glucose Utilization

11.1 Introduction

The analysis of the kinetics of glucose utilization by the perfused heart is useful for what it can reveal about the overall process, the rate-limiting steps in the process and the effects of insulin on them. A particular advantage of the perfusion system used in this work is that glucose utilization can be measured when the concentration of perfusate glucose is constant. A rate can be associated with a clearly defined concentration rather than with a range of concentrations and without any uncertainties about changes in the rate of utilization independent of changes in glucose concentration. This is especially important in conditions where the rate of utilization varies with the concentration of the substrate. An analysis of the kinetics of glucose utilization measured in this work might therefore be a useful test of conclusions drawn from experiments made in closed-circuit systems of perfusion which restrict the lower limit of concentration that can usefully be used and in which the rate of utilization in the absence of added insulin may be measured when it is changing independently of glucose concentration.

11.2 Results

Data have been collected together from the experiments using hearts from fed animals perfused using Millipore filters that have been described in Chapters 6 and 10. Experiments with paper filters were made at too few concentrations of glucose to be used. The kinetic analysis has been applied to the results obtained in the period between 60 minutes and 90 minutes of perfusion when regardless of the concentration of glucose or the presence or absence of insulin both

the rate of glucose utilization and the concentration of perfusate glucose tend to be stable. Table 30 gives the mean perfusate glucose concentration and rate of glucose utilization for groups of these results obtained in the presence and absence of insulin ($2mU.ml^{-1}$). The tendency of the rate of utilization to reach a maximum regardless of increasing concentration of glucose is clear (Fig. 30). Consequently the kinetics of utilization were analysed on the assumption that they approximated to simple Michaelis-Menten kinetics. In Table 31 the apparent half-saturation constant (Ku) and maximum rates of utilization (Vu) in the presence and absence of insulin are given. These have been calculated by fitting three linear transformations of the Michaelis-Menten equation to the experimental data both in the groups shown in Table 31 and also with each experiment treated as an individual point. Examples are given in Fig. 31. In the absence of insulin Ku ranges from 0.3mM to 0.59mM and Vu ranges from 105 to 141 $\mu moles.g\ dry\ wt.^{-1}h^{-1}$. In the presence of insulin, Ku varies from 0.97mM to 3.8mM and Vu from 386 to 1189$\mu moles.g\ dry\ wt.^{-1}h^{-1}$. The variations in the estimates of the kinetic constants may result from the differences in statistical weight that the methods give to the experimental data and its associated error (Dowd and Riggs, 1965). However the intracellular utilization of perfusate glucose involves several physical processes so that the process may only approximate to a single Michaelis-Menten mechanism. If the fit is better at higher or lower concentration of glucose this will be reflected in estimates of Ku and Vu through the linear transformation that give greater or least weight to the relevant data.

11.3 Discussion

The consumption of glucose by the isolated heart involves several processes some of which may influence the relationship between the concentration of glucose in the perfusate and the rate of the

Table 30

Glucose utilization at different glucose concentration in the presence and absence of insulin

Infusate Glucose (mM)	Perfusate Glucose (mM)	Glucose Utilization (μmoles.g dry wt.$^{-1}$ h^{-1})
0.06	0.036$\pm$0.001 (6)	8$\pm$0.5
0.11	0.080$\pm$0.004 (5)	17$\pm$1.0
0.33	0.225$\pm$0.011 (6)	52$\pm$6.0
2.7	2.390$\pm$0.050 (12)	80$\pm$6.0
5.5	4.900$\pm$0.070 (18)	117$\pm$10
0.06*	0.035$\pm$0.001 (4)	10$\pm$0.1
0.11*	0.058$\pm$0.003 (7)	26$\pm$2.0
0.33*	0.184$\pm$0.004 (5)	68$\pm$3.0
2.7*	1.300$\pm$0.070 (7)	261$\pm$16.0
2.7*	1.980$\pm$0.040 (7)	320$\pm$21.0
5.5*	4.180$\pm$0.130 (14)	313$\pm$11.0

Hearts from fed rats were perfused with MKHM containing glucose as shown in the Table.

*These hearts were perfused with glucose and 2mU.ml^{-1} insulin.

Values are mean $\pm$ S.E.M., and the numbers of observations are in parentheses. The rate of glucose utilization was estimated between 60-90 minutes of perfusion.

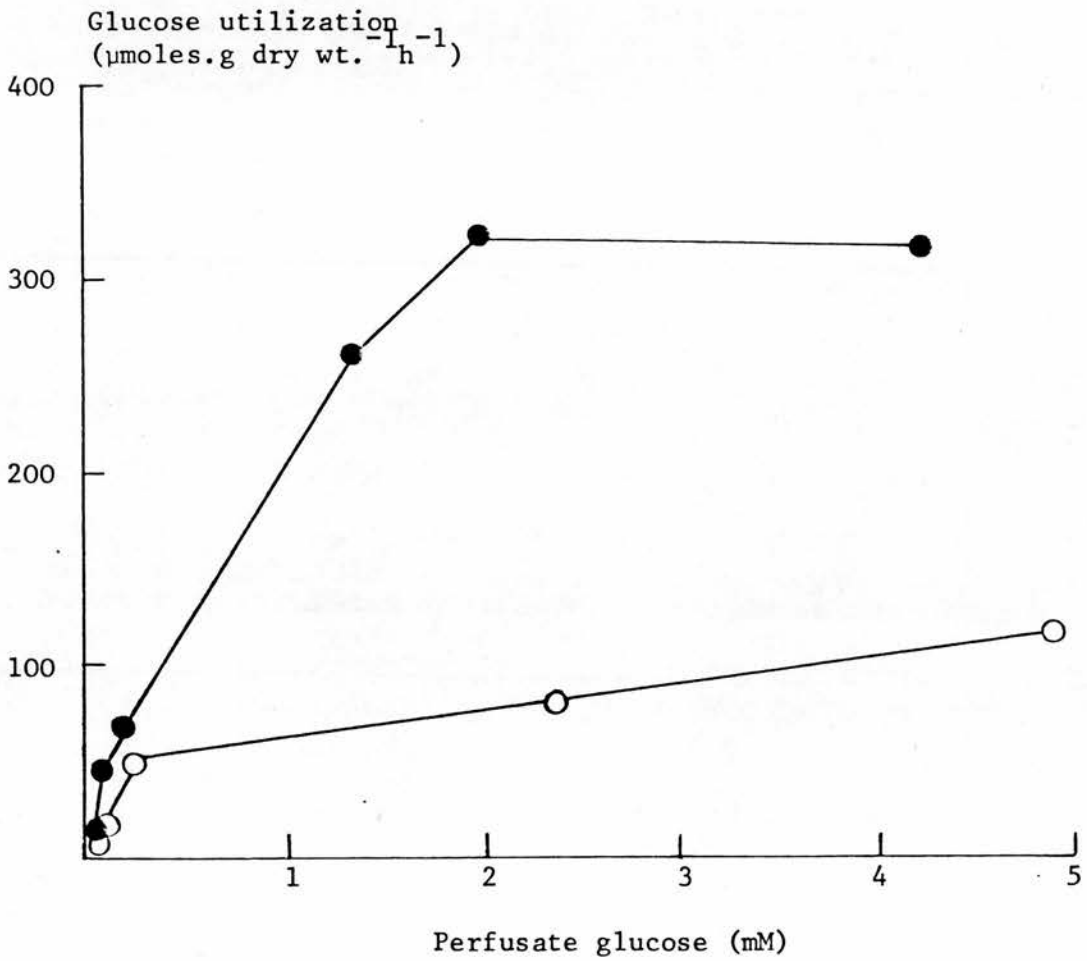

Fig. 30. The relationship between glucose utilization and perfusate glucose concentration in the presence and absence of insulin

Hearts from fed rats were perfused with ● and without ○ insulin 2mU.ml^{-1}.

Data are taken from Table 30.

Table 31

The apparent half-saturation constants (Ku) and maximum rates of utilization (Vu) in the presence and absence of insulin

Plot	Individuals		Groups		
	Ku (mM)	Vu (μmoles.g dry wt.$^{-1}$ h^{-1})	Ku	Vu	Insulin
$\frac{1}{v}$ vs $\frac{1}{s}$	0.45	109	0.59	141	-
$\frac{s}{v}$ vs s	0.48	105	0.59	123	-
v vs $\frac{v}{s}$	0.30	105	0.37	108	-
$\frac{1}{v}$ vs $\frac{1}{s}$	2.37	803	3.8	1189	+
$\frac{s}{v}$ vs s	0.97	386	1.04	424	+
v vs $\frac{v}{s}$	1.16	401	1.46	387	+

Data for s and v are taken from hearts from fed animals perfused with glucose at different glucose concentrations with or without insulin (2mU.ml^{-1}).

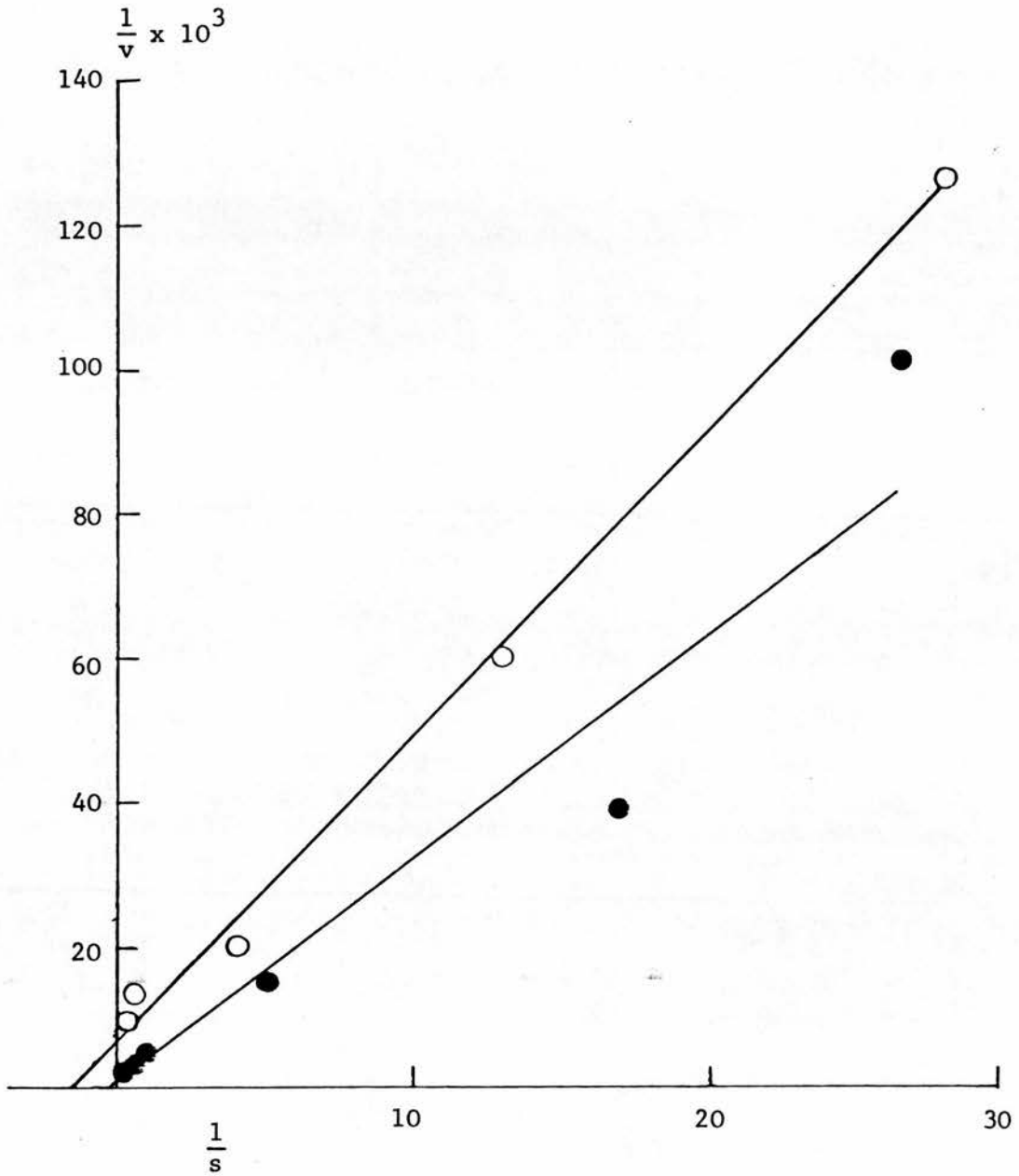

Fig. 31. Lineweaver-Burk plot of the relationship between glucose utilization and glucose concentration

Open circles indicate the absence of insulin, filled circles the presence of insulin. Data are taken from Table 30.

intracellular metabolism of glucose. Glucose is delivered to the heart in the flow of perfusate through the coronary vessels. It must then cross the capillary walls and pass through the interstitial fluid to the myocardial cells. Next, it must pass through the cell membrane and finally it is transformed in an enzyme-catalysed reaction.

Of these processes two can be shown to have no significant influence on the overall kinetics of glucose utilization in the conditions of the experiments. First the rate of supply of glucose to the heart is the product of the coronary flow rate and the concentration of glucose in the perfusate. At the two lowest steady state concentrations of glucose in the presence of insulin the mean rates of utilization of 10μmoles.g dry wt.$^{-1}$h^{-1} and 26μmoles.g dry wt.$^{-1}$h^{-1} would cause a fall in concentration of 16% across the heart at the average coronary flow rate of 44.6ml.g dry wt.$^{-1}$min^{-1}. The average concentration of glucose in the coronary circulation would be 8% less than the concentration in the perfusate. In these two worst cases the concentration gradient is still sufficiently small in comparison with biological variation for the heart to be treated as being uniformly exposed to glucose and the delivery of glucose to the heart is certainly not limiting the rate of utilization.

The second process that is unlikely to influence the overall kinetics of glucose utilization is the diffusion of glucose through the interstitial fluid after crossing the capillary wall. Heart muscle is well provided with capillaries which are on average separated by 16μm (Berne and Rubio, 1979). If all the capillaries are open the farthest glucose need diffuse is less than half this distance. From the diffusion coefficient of glucose in water and assuming that diffusion takes place through an area similar to that of the capillary walls the decrease in concentration across 8μm when glucose is being

used at 10μmoles.g dry wt.$^{-1}$h^{-1} would be 0.003mM. Since this calculation assumes that the interstitial water is unstirred whereas in the contracting isolated heart considerable exchange of water occurs between the vascular and interstitial compartment (Young, 1968), it is likely to overestimate the fall in concentration.

The remaining processes that influence or determine the kinetics of glucose utilization are the passages of glucose through the capillary wall and the cell membrane and the intracellular transformation of glucose. It has been assumed (Post, Morgan and Park, 1961) that in the absence of insulin the kinetics of utilization approximate to those of permeation through the cell membrane. There is good evidence for this assumption. In the absence of insulin, intracellular glucose is difficult to detect. When the extracellular glucose is at physiological concentrations no intracellular glucose has apparently been measurable (Morgan et al., 1961; Cheung et al., 1978) although at high concentrations of extracellular glucose (30mM) there are conflicting claims (England and Randle, 1967; Cheung et al., 1978). In the physiological range of glucose concentrations insulin not only increases the rate of glucose utilization but also increases the concentration of intracellular glucose (Morgan et al., 1961; Cheung et al., 1978). This suggests that a restriction of the entry of glucose into the cell is removed and this idea is supported by the fact that insulin increases the rate at which the extracellular and intracellular concentrations of non-metabolised sugars approach equilibrium. The properties of the permeation of some monosaccharides including glucose into muscle cells are consistent with the mechanism of facilitated diffusion proposed by Widdas (1952). The process is saturable, shows stereospecificity, is subject to competition between sugars and permits the phenomenon of counter-transport (Park, Crofford

and Kono, 1968). Consequently the saturation kinetics that characterise the restricted rate of glucose utilization are probably determined by the properties of permeation into the myocardial cells. If the measured rates of utilization can be taken to approximate to the rate of inward permeation with no significant outward transport, Ku, the apparent half-saturation of glucose utilization in the absence of insulin, and Vu, the maximum rate of glucose utilization, approximate to K_T, the half-saturation constant of glucose permeation, and V_T, the maximum rate of permeation. The failure of attempts to detect intracellular glucose do not wholly justify this approximation when the detection depends on the total amount of glucose that can be extracted from the heart being greater than the total amount of extracellular glucose calculated on the assumption that all extracellular water is at the same glucose concentration as of the perfusate (Morgan et al., 1961). Failure may indicate that there is a significant concentration difference across the capillary wall. When intracellular glucose is not detected despite attempts to allow for a transcapillary gradient (Cheung et al., 1978) the failure may still point to the difficulties of measuring the gradient, or simply underline how difficult it is to inhibit rapidly glucose metabolism at the end of an experiment. However the K_m of the isoenzymes of hexokinase that predominate in rat heart muscle have been estimated in vitro to be approximately 0.05mM. Since the V_{max} of phosphorylation in vivo is likely to be determined mainly by the extent of the inhibition of the enzyme by glucose-6-phosphate (England and Randle, 1967) its value in the absence of insulin must be at least as great as the highest rate of 320μmoles.g dry wt.$^{-1}$h^{-1} found in the presence of insulin. Rates of glucose utilization ranging from 8 to 117μmoles.g dry wt.$^{-1}$h^{-1} would require the intracellular glucose concentration to range from

0.0013mM to 0.017mM in an irreversible reaction catalysed by an enzyme with the above values for K_m and V_{max}. These concentrations are 3.5% and 0.35% respectively of the perfusate glucose concentration associated with these rates of utilization and suggest that measurement of intracellular glucose is impractical. They also suggest that there is no significant outward permeation of glucose from the cardiac cells.

Vu is therefore acceptable as a reasonable estimate of V_T, the rate of permeation in the absence of insulin. However Ku is not necessarily a good estimate of K_T because it must be an overestimate whose error depends on the size of the concentration gradient that must exist across the capillary wall. Since in this work the lowest glucose concentrations that have been used are about ten times more dilute than the lowest that appear to have been used in closed-circuit systems of perfusion and are lower than Ku, any extracellular gradient will have a greater effect in this work. It is unfortunate that there is considerable uncertainty about the permeability of the capillary wall to glucose in the isolated perfused rat heart. Studies *in vivo* cannot be applied to the isolated heart because the process of isolating the heart increases the permeability of the capillaries sufficiently for Evans Blue-albumin conjugate, which is restricted to the vascular space *in vivo*, to leave the capillaries *in vitro* (Sutherland and Young, 1966). The increase in permeability to the conjugate occurs immediately on excision of the heart and persists throughout experiments lasting 30 minutes. Extracellular markers equilibrate with or are washed out from the heart at rates that depend on their molecular size. It is reasonable to suppose that water soluble substances cross the capillary wall by diffusion as appears to be the case *in vivo* (Landis and Pappenheimer, 1963). Few measurements of the permeability constant (PS) of the capillaries of the isolated rat heart to glucose or

molecules of similar size have been made. Analysis of the wash-out of ^{14}C-glucose from the perfused rat heart gave a value for PS which suggested that the maximum rate of glucose utilization that could be sustained at a perfusate glucose concentration of 4mM would be 58μmoles.g dry wt.$^{-1}$h^{-1} (Kammermeier and Kammermeier, 1976). Since rates of glucose utilization greater than 300μmoles.g dry wt.$^{-1}$h^{-1} are commonly measured it seems that this approach to the measurement of PS is inadequate. Attempts to follow the time-course of the equilibriation of the extracellular water with sorbitol have produced limiting values for PS (Morgan et al., 1961) or an estimate of PS of 740hr.gml^{-1} (Cheung et al., 1978) that are consistent with reported rates of glucose utilization.

The use of very low concentrations of glucose in this work allows an indirect estimate of the capillary permeability constant to be made and also some indication about the effects of insulin on the kinetics of glucose permeation into the cardiac cells. Inspection of Table 30 shows that both in the presence and absence of insulin there is a linear relationship between glucose utilization and perfusate glucose concentration at the lowest concentration. Glucose utilization is directly proportional to glucose concentration but is stimulated by insulin which suggests that passage through the capillary wall is not the rate limiting step at least in the absence of insulin unless this process is sensitive to insulin. First order kinetics is a saturable process indicate that the concentration of substrate is negligible in comparison with the half-saturation constant. Therefore

$$v = \frac{V_T}{K_T} x \quad \text{and} \quad v_i = \frac{V_{T_i}}{K_{T_i}} x_i$$

where x and x_i are the concentrations of glucose in the interstitial space with the subscript i indicating the presence of insulin and are related to s and s_i the respective concentration of perfusate glucose thus:

$$v = PS\ (s-x) \qquad \text{and } v_i = PS\ (s_i - x_i)$$

$$\text{since } x = \frac{K_T}{V_T} v \qquad \text{and } x_i = \frac{K_{T_i} . v_i}{V_{T_i}}$$

$$\text{Then } v = PS\ \left(s - \frac{K_T V}{V_T}\right) \text{ and } v_i = PS\ \left(s_i - \frac{K_{T_i} . v_i}{V_{T_i}}\right)$$

Therefore

$$\frac{1}{PS} = \frac{s}{v} - \frac{K_T}{V_T} \text{ and } \frac{1}{PS} = \frac{s_i}{v_i} - \frac{K_{T_i}}{V_{T_i}}$$

Assuming that insulin does not alter capillary permeability it follows that

$$\frac{s}{v} - \frac{s_i}{v_i} = \frac{K_T}{V_T} - \frac{K_{T_i}}{V_{T_i}}$$

From Table 30 the mean values for s/v and s_i/v_i at the three lowest concentrations can be calculated

$$\frac{K_T}{V_T} - \frac{K_{T_i}}{V_{T_i}} = 0.0045 - 0.0035 = 0.001$$

K_T/V_T must have a true value no greater than 0.0045 when there would be no extracellular concentration gradient and no less than 0.001 if $\frac{K_{T_i}}{V_{T_i}}$ is to have a positive value. Equating V_T with Vu ranging from 105 to 141μmoles.g dry wt.$^{-1}$h^{-1}, K_T lies in the range 0.63mM to 0.18mM and is close to the estimate of Ku. The overall kinetics of glucose

utilization do therefore approximate to those of glucose permeation. The values for PS found by Cheung et al. entails a transcapillary gradient such that $\frac{K_T}{V_T}$ equals 0.003 and K_T is then between 0.32 and 0.42mM. The lower limiting value for K_T/V_T indicates that the lowest possible value for PS is 350hr.g.ml^{-1}.

The effect of insulin on the parameters of permeation can also be derived from the equation $K_T/V_T - K_{T_i}/V_{T_i} = 0.001$ but the conclusions are more speculative because of the lack of any independent estimate of K_{T_i} or V_{T_i}. However if for example K_T = 0.35mM and V_T=105μmoles.g dry wt.$^{-1}$h^{-1} K_{T_i}/V_{T_i} must equal 0.0011. Values for K_{T_i} and V_{T_i} that agree with this ratio can then be tested for their consistency with the observed rates of glucose utilization when glucose can be detected intracellularly. In Table 30 the net rate of glucose permeation, which in steady state conditions must equal the rate of glucose metabolism, has been calculated from the equation

$$v = \frac{V_i\ s}{K_i + s} - \frac{V_i\ y}{K_i + y}$$

where s is the perfusate glucose concentration and y is the intracellular glucose concentration. A value of 4mM was chosen for s because at that concentration utilization is near saturation and extracellular gradients are negligible. Also at around this physiological concentration intracellular glucose is measurable. Thus Cheung et al. found in hearts from fasted rats that when the perfusate glucose was 2.1mM the intracellular glucose concentration was 0.82mM and at 9.3mM extracellular glucose the intracellular glucose was 5.9mM. Other estimates of intracellular glucose by Morgan et al. (1961) are in general agreement. Consequently y was taken to be 1, 2 or 3mM when the extracellular concentration was 4mM.

K_{T_i} was chosen initially on the assumption that K_T is not altered by insulin. Three values for K_T 0.25, 0.35, and 0.4mM were taken representing the extremes and centre of the range of possible values. V_{T_i} was then calculated setting K_{T_i} equal to K_T and V_T equal to 105μmoles.g dry wt.$^{-1}h^{-1}$. Table 32 shows that the net rate of permeation is lower than the observed rate of utilization at 320μmoles.g dry wt.$^{-1}h^{-1}$ whenever K_{T_i} is assumed to equal K_T. When K_{T_i} and V_{T_i} are increased in proportion the net rate of permeation increases. This analysis suggests that the effect of insulin on glucose utilization is best explained by 10- to 25-fold increase in the maximum rate of permeation and 4-fold increase in the half-saturation constant.

The conclusion that insulin increases K_T is supported by the observation that the stimulation of glucose utiliz ation is less or no greater at low concentrations of perfusate glucose than at high concentrations. If no allowance is made for any extracellular gradient the stimulation of the lowest concentration is only about 50% compared with 300% at the highest concentration. If Cheung's estimate of capillary permeability is applied to the data of Table 32 the stimulation at the lowest comparable concentrations of interstitial glucose is restored to 300%. However at the low concentrations of glucose when phosphorylation is far from saturated the intracellular glucose concentration is probably 10 to 15% of the interstitial concentration if the K_m of hexokinase is 0.05mM. At the high concentrations of perfusate glucose the intracellular concentration in the presence of insulin is about 50% of the extracellular concentration. If the rate of outward permeation from the heart is a much smaller fraction of the inward permeation at low glucose concentrations an increase in V_T with no change in K_T should result in a greater stimulation of utilization by insulin in those conditions. The fact

Table 32

Effect of values of parameters of permeation on estimates of glucose utilization

	K_{Ti} (mM)	V_{Ti} (μmoles.g dry wt.$^{-1}h^{-1}$)	Glucose Utilization (μmoles.g dry wt.$^{-1}h^{-1}$) when Intracellular Glucose Concentration (y) is 1mM	2mM	3mM
	0.25	650	92	34	12
K_T = 0.25mM	0.50	1300	289	116	41
V_T = 105	1.00	2600	780	347	130
	2.00	5250	1750	875	350
	0.35	260	46	18	6
K_T = 0.35	0.70	525	138	58	21
V_T = 105	1.40	1050	340	160	62
	2.80	2100	1197	875	664
	0.40	220	43	17	6
K_T = 0.4	0.80	440	123	53	20
V_T = 105	1.60	880	290	140	55
	3.20	1760	559	301	126

The concentration of perfusate glucose was assumed to be 4mM.

Details are given in the text.

that this does not occur is consistent with an effect of insulin to raise K_T.

The significance of any effect of insulin on K_T depends on the relative values for K_T and the physiological concentration of extracellular glucose. When K_T is much less than the physiological concentration a reduction in K_T by insulin does not add much to a rise in inward permeation brought about by an increase in V_T. But as can be seen from Table 32 an increase in K_T, without necessarily markedly offsetting the effect of an increase in V_T on inward permeation, can reduce the competition between the processes of outward permeation and phosphorylation. In effect inhibition of outward permeation permits the saturation of hexokinase. However if K_T is greater than the physiological concentration of extracellular glucose an increase in K_T must inhibit both inward and outward permeation.

Other investigations into the effect of insulin on the permeation of sugars into muscle tissues have all agreed that V_T is increased but disagree as to whether K_T is increased or not. Decreases of K_T in response to insulin has only been claimed for adipose tissue (Iliano and Cuatrecasas, 1971). Fisher and Zachariah (1961) and Fisher and Gilbert (1970c) concluded that insulin increased K_T for the permeation of non-metabolised sugars into perfused hearts from fed rats. The increases varied from 27 fold to 31 fold with different sugars. However Cheung et al. (1978) found insulin had no effect on the K_T for the permeation of 3-O-methylglucose into hearts from fasted rats but increased V_T by between 9- and 36-fold. In a study also with hearts from fasted rats that was stressed to be semiquantitative (Post et al., 1961) insulin increased the estimate of K_T for the permeation of glucose from 9mM to 27mM. From the same laboratory,

the estimate of K_T for the permeation of glucose into hearts from fed rats was reported to be 1mM (Morgan et al., 1965) but no estimate of the effect of insulin was made. The above analysis of the kinetics of glucose utilization measured in this work suggests that for glucose permeation into hearts from fed rats K_T is increased by insulin from between 0.25mM and 0.45mM to between 1mM and 2mM while V_T is increased from 105μmoles.g dry wt.$^{-1}h^{-1}$ to between 1000 and 2500μmoles.g dry wt.$^{-1}h^{-1}$.

This conclusion is based on experiments with hearts perfused using Millipore filters. It was suggested earlier (Section 7.3) that the reduced rate of glucose utilization observed with hearts perfused in the absence of insulin using paper filters might be due to a reduced permeability to glucose. If this is the case V_T would be lower in these hearts. At 0.27mM-glucose paper filters did not significantly reduce glucose utilization which suggests a lower value of K_T (Section 10.3.2). In the presence of insulin the use of paper filters did not affect glucose utilization. This suggests that the general conclusion about the nature of the effect of insulin on the parameters of glucose permeation is applicable to these experiments regardless of the choice of filters.

CHAPTER 12

Summary

12.1 Stability of Glucose Utilization

At physiological concentrations of glucose the rate of glucose utilization by the isolated perfused rat heart becomes constant for at least 30 minutes regardless of the presence or absence of insulin. Glucose utilization is inconstant during the first 30 to 40 minutes of perfusion particularly in hearts from fed animals perfused without insulin.

12.2 Effect of Insulin on Glucose Utilization

Insulin stimulates glucose utilization at all concentrations of glucose in the range 0.035mM to 4.2mM. The stimulation is greater at the higher concentrations particularly in hearts perfused using paper filters since their rate of glucose utilization is low in the absence of the hormone. Analysis of the relationship between glucose utilization and glucose concentration in the perfusate suggests that insulin increases both the maximum rate and the half-saturation constant for glucose permeation.

12.3 Sensitivity of Glucose Utilization to Insulin

The rate of glucose utilization by hearts perfused using paper filters and albumin responds to insulin at $20\mu U.ml^{-1}$ and maximally at a concentration of $80\mu U.ml^{-1}$.

12.4 Effect of Fasting on Glucose Utilization

In the absence of added insulin hearts from fasted animals use glucose at a constant rate from the start of the experiment.
In comparison with hearts from fed rats fasting reduces the rate of glucose utilization after 60 minutes perfusion by 30%. Insulin does

not immediately restore the rate of glucose utilization but after an hour's perfusion the rate of glucose utilization in hearts from fasted animals is greater than in those from fed rats.

12.5 Effect of Depletion of Endogenous Reserves

Glucose utilization by heart that have been subjected to 45 minutes of perfusion without substrate is unaffected. This indicates that the state of the glycogen stores is not of major importance in determining the rate of glucose utilization in prolonged experiments.

PART III

INTERRELATIONSHIP BETWEEN GLUCOSE AND DL-3-HYDROXYBUTYRATE METABOLISM

CHAPTER 13

13.1 Introduction

Glucose in the presence of insulin can meet most of the energy requirements of the isolated perfused rat heart (Chapter 6). However *in vivo* glucose is not the only substrate available to the heart. Free fatty acids, for example, are always available and, of the ketone bodies, acetoacetate and D-3-hydroxybutyrate are also potential substrates in fasting animals.

These alternative sources of energy have been suggested to be important in the reduction of glucose oxidation by heart and other muscles in fasting or exercise (Randle *et al.*, 1963; Newsholme, 1976, 1981). Since the possible demand for glucose is large in comparison with the reserves of glycogen, particularly of the liver, a reduction in glucose utilization by muscle would tend to conserve what glucose is available for those tissues that depend on it and to minimize the need for gluconeogesis which is mainly satisfied at the expense of body protein.

The hypothesis that ketone bodies alter the metabolism of glucose in muscle is based mainly on experiments with the isolated heart of the rat, but there is no doubt that early in a fast the consumption of glucose by skeletal muscle of man and rats falls *in vivo* while that of fatty acid and ketone bodies rises (Owen and Reichard, 1971; Berger, Hagg and Ruderman, 1975). However the evidence for an effect of ketone bodies on glucose metabolism in skeletal muscle is inconclusive. In the isolated perfused rat hindquarter, ketone bodies are used at a rate determined by their concentration in the medium (Ruderman and Goodman, 1973) but they do not inhibit glucose uptake or glycolysis whether the muscle is resting or contracting or whether

insulin is present or not (Berger, Hagg, Goodman and Ruderman, 1976). Nevertheless lactate oxidation is inhibited by ketone bodies in resting and contracting muscle. This may reflect the effect of the mixture of the types of muscle fibre in the rat hind quarter. The muscle mass is quoted to be 55% white muscle (Maizels, Ruderman, Goodman and Lau, 1977). Only the remaining red muscle would be expected to metabolise the ketone bodies and to oxidise lactate but both red and white would contribute to the estimate of glucose uptake and of the glycolytic rate. When the soleus muscle preparation, which consists mainly of red fibres and has a much greater glycolytic rate than the hindquarter preparation, is incubated glucose uptake and glycolysis is inhibited by ketone bodies (Maizels et al., 1977), whereas no such effect is obtained in a predominantly white muscle, the extensor digitorum longus.

A further complication to the study of the regulation in skeletal muscle of glucose utilization by ketone bodies is the effect of physical training on the capacity to use ketone bodies. Training rats by exercise on a treadmill can increase the levels of 3-hydroxybutyrate dehydrogenase, 3-ketoacid CoA-transferase and acetoacetyl-CoA-transferase by between 40% and 470% with associated increases in the capacity to oxidize pyruvate and fatty acids (Holloszy, Winder, Fitts and Rennie, 1978). No such effect are observed in the heart in which even after the training period these enzymes have activities at least double those in skeletal muscle. Most investigations have been made on untrained animals in which any effect of ketone bodies on glucose utilization might be expected to be reduced.

The applicability to skeletal muscle of the concept of the regulation of glucose utilization by ketone bodies has not been established except in red muscle fibres showing high glycolytic rates. However the validity of the concept for cardiac metabolism is more

strongly based. Williamson and Krebs (1961) found that acetoacetate reduced both glucose utilization and oxidation in isolated rat hearts perfused with insulin. In the absence of insulin glucose utilization was not affected but glucose oxidation was inhibited. Minton and Raben (1962) obtained similar results. Hall (1961) also reported that acetoacetate inhibited the oxidation of glucose by hearts perfused in the absence of insulin. He based this conclusion on changes in $^{14}CO_2$ production when acetoacetate was introduced after 45 minutes of perfusion with glucose alone. Williamson and Krebs studied the period of one hour following an initial preperfusion of 15 minutes. In contrast Randle et al. (1964) made almost all of their observations during the first 15 minutes of perfusion. They used DL-3-hydroxybutyrate in most of these experiments and found it effective in inhibiting glucose utilization and oxidation both in the presence and absence of insulin. They explained the differences between their observations and those of Williamson and Krebs as the result of the effect of endogenous insulin. However they also showed that 3-hydroxybutyrate inhibited glucose utilization by hearts from fasted rats in which there should be less endogenous insulin. The inhibition of glucose utilization in the presence of insulin was associated with an increase in the concentration of intracellular glucose but in the absence of insulin this effect was only significant in hearts from fasted animals. In addition to this evidence of the inhibition of the phosphorylation of glucose an inhibition of the permeation of L-arabinose occurred in hearts from fed animals in the absence of insulin and in the presence of insulin at $0.5 mU.ml^{-1}$ but not with insulin at $1.0 mU.ml^{-1}$. With hearts from fasted animals, the ketone body only inhibited permeation in the presence of insulin at $0.5 mU.ml^{-1}$. Since endogenous insulin was assumed to be effective in the hearts

from fed rats it was suggested tentatively that D-3-hydroxybutyrate decreased the effect of insulin on transport at low but not at high concentrations of the hormone.

The results of experiments presented in the previous section on the time-course of glucose utilization at physiological concentrations cast doubt on the validity of conclusions based on the behaviour of hearts in the first 30 minutes of perfusion in the absence of insulin. It is when insulin concentrations are low that the effect of ketone bodies on glucose utilization are especially relevant to *in vivo* conditions although the response of diabetics with ketoacidosis to the administration of insulin has obvious importance. No work appears to have been done using longer periods of perfusion with which the results of Randle *et al.* (1964) can be compared. England and Randle (1967) again found inhibition of glucose utilization in the presence of insulin by DL-3-hydroxybutyrate in experiments lasting 25 minutes but made no observations without insulin. Wieland *et al.* (1971) found that 3-hydroxybutyrate and acetoacetate increased lactate production and diminished glucose uptake in the absence of insulin but they too perfused hearts from fed rats for 20 minutes without preperfusion and assumed that their results were influenced by endogenous insulin. They made no experiments with added insulin. Neely *et al.* (1969) give support to there being an effect of 3-hydroxybutyrate on glucose permeation. They found that this ketone body inhibited the permeation of 3-O-methylglucose into hearts from fasted rats whose glucose utilization had been stimulated by perfusion at high pressure (100mmHg). No studies were made with 3-hydroxybutyrate at lower pressures but palmitate inhibited permeation at low and high perfusion pressures. No other investigations of the effect of 3-hydroxybutyrate on glucose utilization by the perfused rat heart have been found in the literature.

The regulatory effect on glucose utilization ascribed to 3-hydroxybutyrate deserves to be investigated using hearts subject to prolonged perfusion when effects of endogenous insulin can be disregarded. The system of perfusion with balanced infusion and withdrawal is especially suited to this purpose when both substrates results in the formation of products that can accumulate in the perfusate. In Chapter 4 when describing the characteristics of the preparation, evidence was presented (Fig. 13) that glucose, lactate, 3-hydroxybutyrate and acetoacetate reach steady concentrations in the perfusate. In contrast in a closed circuit system the concentrations of all substrates and products must change continuously.

A second advantage of the perfusion system used in this work is that it allows substrates to be used at low concentrations as has been shown in the study of glucose utilization at low concentrations in Chapter 10. In the case of ketone bodies their concentration in vivo are usually much lower than those used in closed circuit systems of perfusion which require competing substrates to be at similar concentrations. In the fed rat the combined concentrations of acetoacetate and D-3-hydroxybutyrate are in the range 0.1mM to 0.3mM according to Robinson and Williamson (1980) with whom Hawkins et al. (1971) agreed giving 0.15mM, 0.22mM and 0.65mM as the concentrations in decapitated, anaesthetised and conscious, though restrained and catheterised, fed rats respectively. The concentration of total ketone bodies rises to 2.8mM, 3.0mM and 3.4mM in rats starved for two, three and four days respectively (Newsholme, 1976). In Chapters 14 and 15 the results are presented of a study of the effects in the presence and absence of insulin of 3-hydroxybutyrate over a wide range of concentrations on glucose utilization by hearts from fed rats. The investigation was extended to include the effect

of fasting and of different concentrations of insulin (Chapters 17 and 14). In addition since the initial study showed that the intensity of the inhibition of glucose metabolism by 3-hydroxybutyrate increases during the early period of the experiments the effect of the addition of the 3-hydroxybutyrate into the perfusate after preperfusion with glucose alone was also studied (Chapter 16).

In these investigations only the concentration of glucose in the perfusate was measured. Although it would have been desirable to have measured the time-course of the concentrations of glucose, lactate, 3-hydroxybutyrate and acetoacetate in all experiments this was not possible partly because of the time needed to make all these analyses and partly because of the expense of doing so. However the utilization of 3-hydroxybutyrate was studied in a more restricted series of experiments (Chapter 18).

The purpose of these experiments was to determine the extent to which 3-hydroxybutyrate can satisfy the energy requirements of the isolated heart whether as the only substrate or in competition with glucose and can affect the proportion of glucose that is converted to lactate. Although the effects of 3-hydroxybutyrate on glucose metabolism have received some attention as described above very little work appears to have been done on the utilization of 3-hydroxybutyrate by the perfused rat heart. Williamson and Krebs (1961) in their study of acetoacetate utilization refer to a single experiment in which a heart was perfused with 2mM D-3-hydroxybutyrate. Wieland et al. (1971) measured the consumption of D-3-hydroxybutyrate and glucose and the production of acetoacetate and lactate by six hearts perfused for 20 minutes with a perfusate containing initially 5mM-glucose and 10mM-DL-3-hydroxybutyrate. No other studies of 3-hydroxybutyrate metabolism by the isolated rat heart have been found in the literature.

The utilization of 3-hydroxybutyrate and the production of acetoacetate were therefore measured both in the presence and in absence of insulin when 3-hydroxybutyrate was the only substrate and in competition with glucose. These experiments were made with 10mM-DL-3-hydroxybutyrate and 5.5mM-glucose for comparability with the work of Wieland et al. (1971) and because these conditions are similar to those used by Randle et al. (1964) and others in studying the effect of the ketone body on glucose utilization.

CHAPTER 14

Glucose Utilization in the Presence of Insulin and of DL-3-Hydroxybutyrate

14.1 Introduction

In the presence of insulin the utilization of glucose by the perfused rat heart is inhibited by acetoacetate (Williamson and Krebs, 1961; Randle et al., 1964) and DL-3-hydroxybutyrate (Randle et al., 1964; England and Randle, 1967). The effect of DL-3-hydroxybutyrate was studied in a limited set of conditions. Randle et al. perfused hearts from fed rats with 5.5mM-glucose, 5.5mM-DL-3-hydroxybutyrate and 100mU.ml^{-1} insulin for 15 minutes. England and Randle also used hearts from fed rats and perfused with 14mM-glucose and 50mU.ml^{-1} insulin for 8 minutes before introducing 5.5mM-DL-3-hydroxybutyrate and perfusing for a further 20 minutes. In this chapter results are presented of experiments lasting for 150 minutes in which the perfusate initially contained 2.7mM- or 5.5mM-glucose, DL-3-hydroxybutyrate at one of six concentrations in the range 0.2mM to 10mM and insulin at 80μU.ml^{-1}, 2mU.ml^{-1} or 100mU.ml^{-1}. Although the time-course of D-3-hydroxybutyrate concentration was not determined in these experiments it had been established (Section 4.1.2 and Chapter 18) that with 10mM-DL-3-hydroxybutyrate steady-state conditions are established in the perfusate. It is likely that when the perfusate glucose becomes constant and indicates a constant rate of glucose utilization, the rate of D-3-hydroxybutyrate metabolism is also constant. As with low concentrations of glucose (Chapter 10) low concentrations of D-3-hydroxybutyrate should become constant in the course of the experiments.

The effect of varying the concentration of insulin was studied because in their experiments into the effect of DL-3-hydroxybutyrate on the permeability to L-arabinose of the perfused heart from fed and fasted rats Randle et al. (1964) concluded that the effect might depend on whether the concentration of insulin was low ($0.5mU.ml^{-1}$) or high ($100mU.ml^{-1}$). A comparison was therefore made of the effects of DL-3-hydroxybutyrate on glucose utilization over a wide range of concentrations of insulin (0.08, 2 and $100mU.ml^{-1}$) all of which cause maximum stimulation of glucose utilization in the system used.

14.2 Glucose Utilization by Hearts from Fed Rats Perfused with MKHM Containing 5.5mM-Glucose, DL-3-Hydroxybutyrate at 0.2mM, 1.0mM, 2.5mM, 5mM or 10mM and $2mU.ml^{-1}$ Insulin

Fig. 32 shows the time-course of the concentration of perfusate glucose in experiments in which the initial concentration of DL-3-hydroxybutyrate was 5mM or 10mM. In both case the concentration falls and passes through a minimum before rising to a constant value. This pattern indicates an initial decrease in the rate of glucose utilization and contrasts with that shown in Chapter 6 Fig. 16 when glucose is the only substrate in the presence of insulin and its utiliz ation is steady from the start of the experiment.

In Fig. 33 time-course are shown of the mean rate of glucose utilization by hearts exposed to DL-3-hydroxybutyrate at initial concentrations of 0.2mM, 1.0mM, 2.5mM, 5mM and 10mM. At the highest concentration of the ketone body the rate of glucose utilization falls during the first hour of perfusion and then becomes constant at a rate that is 58% of the initial rate. The degree of inhibition calculated at 10 minute intervals during these experiment is shown in Table 33. With 10mm-DL-3-hydroxybutyrate the earliest estimate

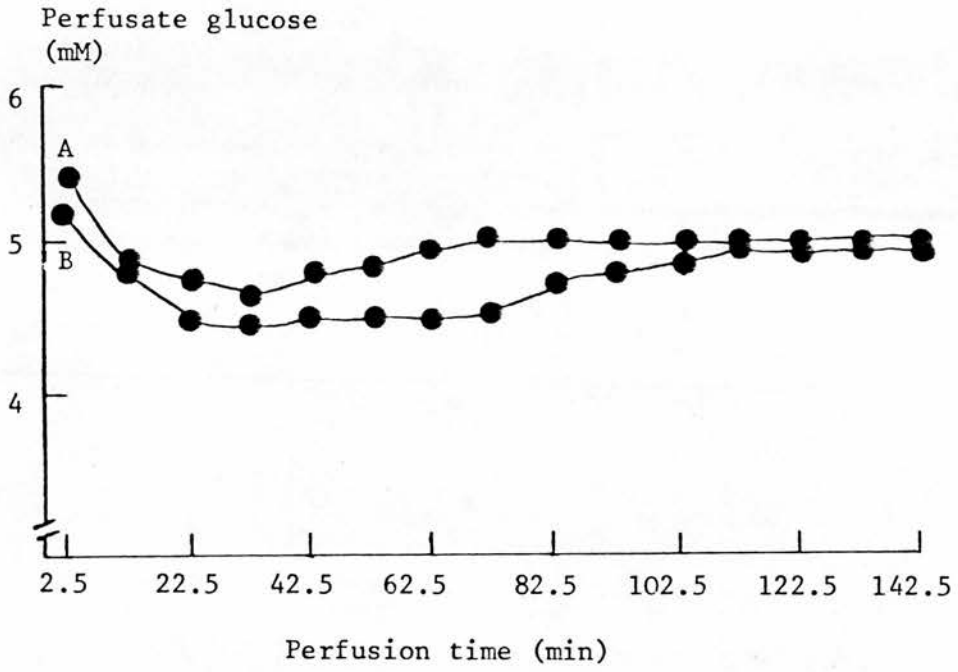

Fig. 32. Time-courses of perfusate glucose concentration in the presence of DL-3-hydroxybutyrate and insulin

Hearts from fed rats were perfused with 5.5mM-glucose, insulin $2mU.ml^{-1}$ and 5mM- or 10mM-DL-3-hydroxybutyrate (A and B respectively).

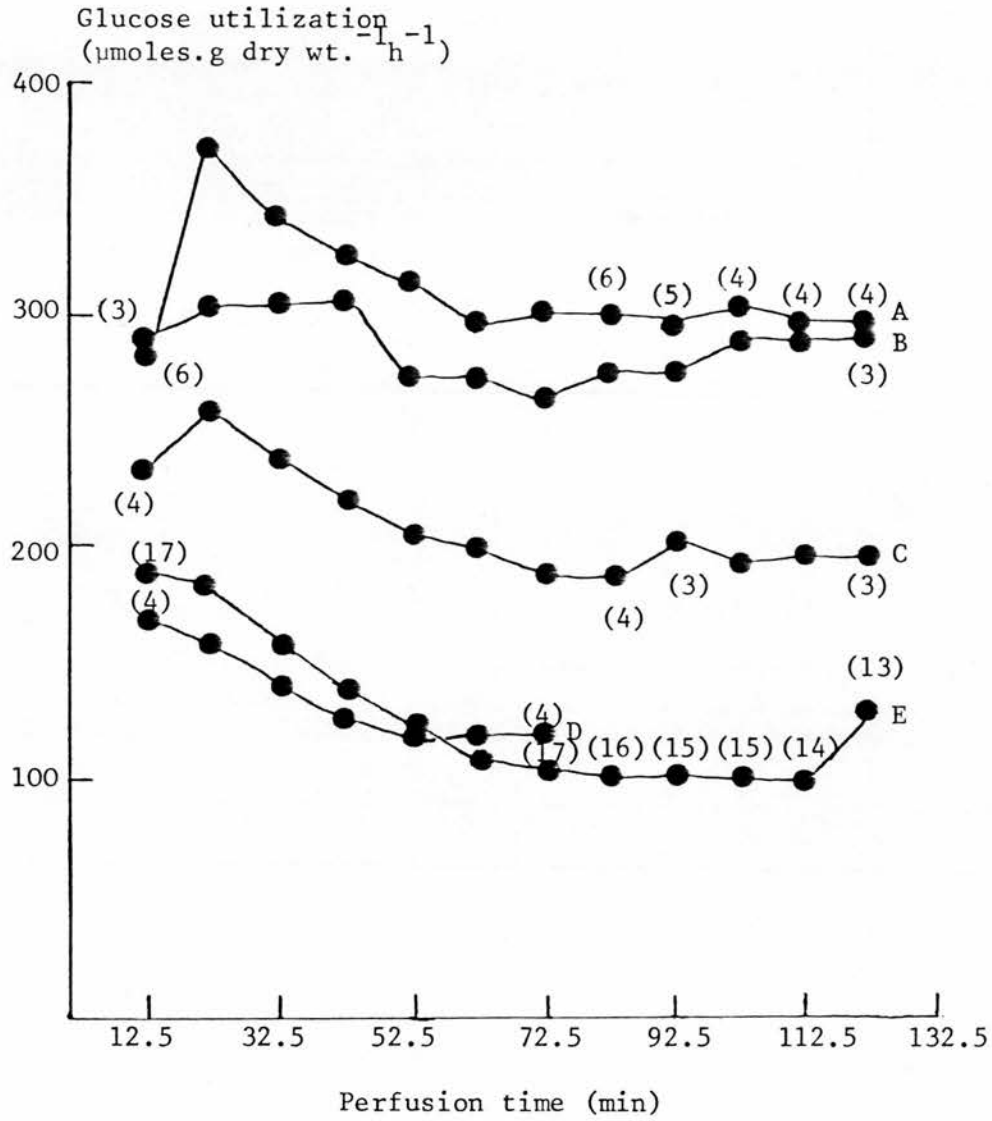

Fig. 33. Effect of concentration of DL-3-hydroxybutyrate on the time-course of glucose utilization at 5.5mM-glucose and 2mU.ml insulin

Hearts from fed rats were perfused with 5.5mM-glucose, 2mU.ml^{-1} insulin and DL-3-hydroxybutyrate at 0.2, 1, 2.5, 5 and 10mM for A, B, C, D, and E respectively.

Points are the mean of the number of observations are in parenthesis.

Table 33

The effect of DL-3-hydroxybutyrate concentrations on glucose utilization at 5.5mM in the presence of insulin ($2mU.ml^{-1}$)

Initial Conc. of DL-3-HB (mM)	% Inhibition of Glucose Utilization Time (minutes)										
	12.5	22.5	32.5	42.5	52.5	62.5	72.5	82.5	92.5	102.5	112.5
0.2	8	-6	-5	-3	0.0	7	3	4	6	-1	9
1.0	6	13	7	1	13	16	15	10	11	5	10
2.5	25	26	27	30	36	38	38	37	-	-	-
5.0	45	54	56	60	60	61	62	55	47	45	40
10.0	39	48	52	56	61	65	66	67	68	66	67

Hearts from fed rats were perfused with MKHM containing 5.5mM-glucose, $2mU.ml^{-1}$ insulin and DL-3-hydroxybutyrate (DL-3-HB) as shown in the Table.

of glucose utilization is reduced by 39% and the inhibition increases until it is 65% after an hour of perfusion. The rate is then similar to that seen with 5mM-glucose as the sole substrate in the absence of insulin and can be measured with a similar precision (Section 4.2).

When 2.5mM-DL-3-hydroxybutyrate was infused with 5.5mM glucose and $2mU.ml^{-1}$ insulin the time-course of glucose utilization (Fig. 33) followed a similar course but at all times the inhibition was less severe than with 10mM-DL-3-hydroxybutyrate. At 2.5mM and lower concentrations of the ketone body the earliest estimate of utilization was lower than the second. Since the time-course of glucose concentration in all these experiments makes the precision of the estimate of the rate of change of concentration uncertain the trend of the changes in glucose utilization was explored by calculating the rates at 7.5 minutes, 17.5 minutes and throughout the period 2.5 to 22.5 minutes (Table 34). The results of these calculations agree with that indicated by Fig. 33 namely that at the three lowest concentrations of DL-3-hydroxybutyrate the early rate of glucose utilization is low and that the rate tends afterwards to rise before falling to a constant value. With 0.2mM and 1.0mM-DL-3-hydroxybutyrate the steady rates of glucose utilization between 60 and 90 minutes of perfusion were not significantly inhibited although in both cases the rate were less than that observed in the absence of DL-3-hydroxybutyrate.

Mixed results were seen with 5mM-DL-3-hydroxybutyrate. Four hearts were studied and in all glucose utilization was inhibited progressively during the first hour of the experiments to an extent indistinguishable from that associated with 10mM-DL-3-hydroxybutyrate. Later in two experiments the rate rose again but remained relatively inhibited. In a third the rate stayed constant for a further 70 minutes and in the fourth the rate also stayed constant but technical

Table 34

Glucose utilization in the presence of DL-3-hydroxybutyrate and insulin during the early stage of perfusion

Initial DL-3-HB conc. (mM)	Glucose Utilization (μmoles.g dry wt.$^{-1}$h^{-1}) at Time (minutes) 7.5	12.5	17.5	22.5	Overall (2.5-22.5 minutes)
0.2	230$\pm$23	284$\pm$15	263$\pm$21	371$\pm$18	293$\pm$7 (6)
1.0	263$\pm$50	291$\pm$48	301$\pm$45	304$\pm$50	284$\pm$44 (3)
2.5	200$\pm$28	233$\pm$24	262$\pm$17	261$\pm$14	231$\pm$21 (4)
5.0	198$\pm$24	193$\pm$16	162$\pm$12	162$\pm$14	179$\pm$12 (4)
10.0	182$\pm$12	192$\pm$11	189$\pm$9	185$\pm$8	185$\pm$10 (17)

Hearts from fed rats were perfused with MKHM containing 5.5mM-glucose, 2mU.ml^{-1} insulin and DL-3-hydroxybutyrate as shown in the Table. Values are the mean $\pm$ S.E.M., and the numbers of observations are in parentheses.

difficulties caused the experiment to be stopped after a total of 90 minutes. Without particular reason for regarding any of these experiments as unrepresentative only the data obtained in the first 90 minutes have been combined.

These experiments indicate that when DL-3-hydroxybutyrate inhibits glucose utilization in the presence of insulin the inhibition increases during the first 40 to 60 minutes of exposure to the ketone body. The intensity of the inhibition is similar with 5mM or 10mM-DL-3-hydroxybutyrate, is less severe with 2.5mM and small or zero with 1.0mM and 0.2mM-DL-3-hydroxybutyrate. The concentration of D-3-hydroxybutyrate at the start of these experiments was found by enzymatic determination to be 45% of that of the mixture (Section 2.2.3). Since metabolism must lower the concentration of D-3-hydroxybutyrate, concentrations of less than 1mM can inhibit glucose utilization in the presence of insulin.

14.3 Glucose Utilization by Hearts from Fed Rats Perfused with MKHM Containing 2.7mM-Glucose, DL-3-Hydroxybutyrate at 1.25mM, 2.5mM, 5.5mM or 10mM and 2mU.ml^{-1} Insulin

The pattern of the inhibition of glucose utilization by DL-3-hydroxybutyrate at 2.7mM-glucose is similar in general to that at the higher concentration presented above. Fig. 34 shows the time-course of the mean rate of glucose utilization in the presence of 2mU.ml^{-1} insulin with glucose alone and with four different concentrations of DL-3-hydroxybutyrate. The rate of glucose utilization at 5.5mM-DL-3-hydroxybutyrate falls during the first 70 minutes of perfusion to 40% of the initial rate and the subsequent steady rate of 83μmoles.g dry wt.$^{-1}$h^{-1} involves an inhibition of 58% in the corresponding rate of utilization when glucose is the only substrate.

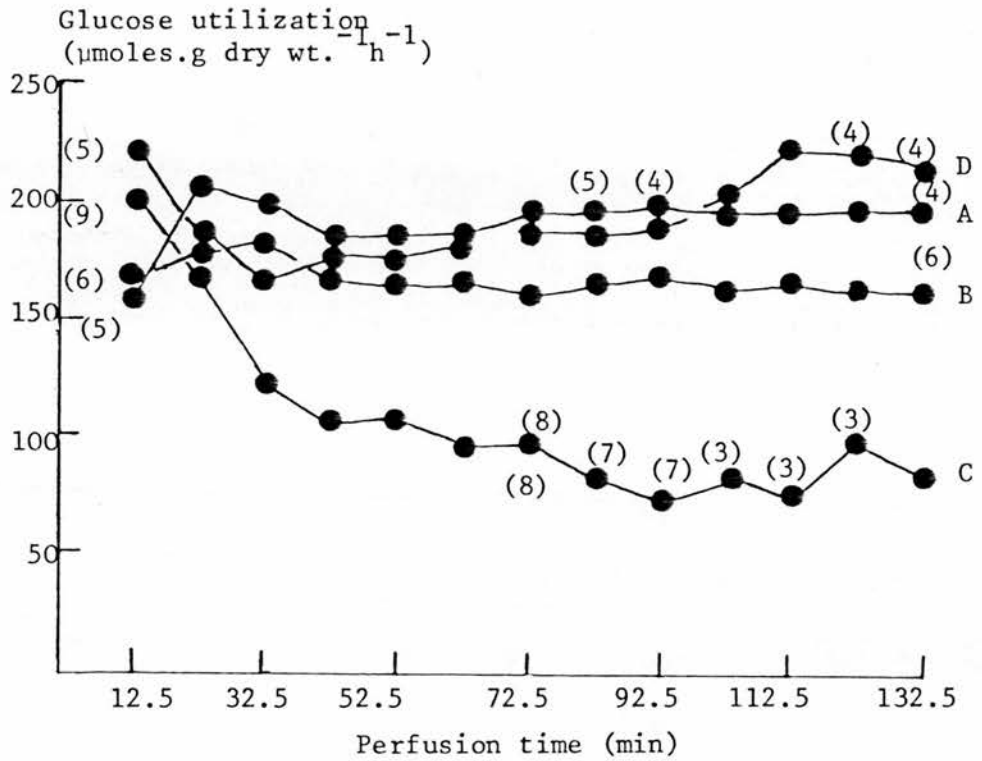

Fig. 34. Effect of concentration of DL-3-hydroxybutyrate on the glucose at 2.7mM-glucose and insulin 2mU.ml^{-1}

Hearts from fed rats were perfused with 2.7mM-glucose, 2mU.ml^{-1} insulin and DL-3-hydroxybutyrate at 1.25, 2.5, 5.5 and 10mM for A, B, C and D respectively.

Points are the mean of the numbers of observations are in parentheses.

There is no significant inhibition of utilization at the earliest estimate (Table 35) with 10mM or any other concentration of DL-3-hydroxybutyrate because the rate at this time is depressed with glucose alone (Section 6.3).

With 10mM-DL-3-hydroxybutyrate the rate of glucose utilization only falls 24% in the first half-hour of perfusion and is constant during the following hour with a value of 185μmoles.g dry wt.$^{-1}$h^{-1} which indicates an inhibition of 30%. Thus the inhibition of glucose utilization was always less and did not intensify to the same extent as with 5mM-DL-3-hydroxybutyrate and the steady state rates of these two concentrations of ketone body are significantly different ($P<0.001$). At the lowest concentrations of DL-3-hydroxybutyrate (1.25mM and 2.5mM) no intensification of the inhibition of utilization occurred after 22.5 minutes but the steady rates after an hour's perfusion represent an inhibition of 26% and 36% respectively.

14.4 Glucose Utilization by Hearts from Fed Rats Perfused with MKHM Containing 5.5mM-Glucose, 5mM-10mM-DL-3-Hydroxybutyrate and 100mU.ml^{-1} Insulin

Fig. 35 shows the time-course of the mean rate of glucose utilization by hearts perfused with 5.5mM-glucose, 5mM- or 10mM-DL-3-hydroxybutyrate and 100mU.ml^{-1} insulin. The rates of glucose utilization at the two concentrations of ketone body are indistinguishable at any time throughout the experiments (Table 36). Comparison with Fig. 33 and inspection of Table 36 shows that the rates of utilization in the period 50 to 90 minutes are significantly different from the rates found with insulin at 2mU.ml^{-1}. However the rates early in the experiments do differ at the two concentrations of insulin and at 12.5 minutes when 5mM-DL-3-hydroxybutyrate is used the

Table 35

The effect of DL-3-hydroxybutyrate on glucose utilization at 2.7mM in the presence of insulin (2mU.ml)

Initial Conc. of DL-3-HB (mM)	% Inhibition of Glucose Utilization at Perfusion Time (minutes)										
	12.5	22.5	32.5	42.5	52.5	62.5	72.5	82.5	92.5	102.5	112.5
1.25	17	15	18	24	27	26	21	16	13	9	8
2.5	19	29	31	33	34	36	39	37	34	36	35
5.5	31	49	59	66	66	70	75	77	73	77	75
10.0	-6	29	37	30	31	30	27	30	25	19	12

Hearts from fed rats were perfused with MKHM containing 2.7mM-glucose, 2mU.ml^{-1} insulin and DL-3-hydroxybutyrate as shown in the Table.

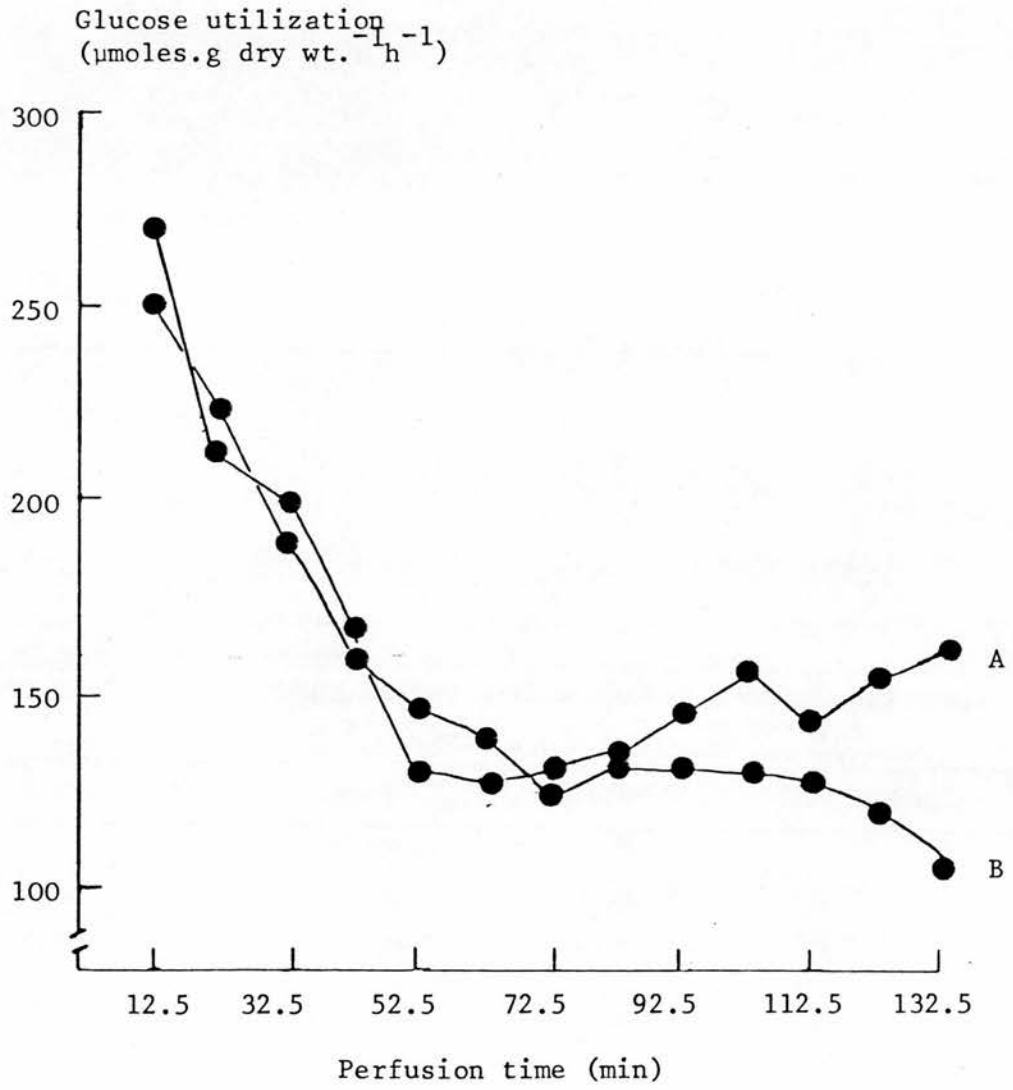

Fig. 35. Time-courses of glucose utilization at 5.5mM-glucose, 100mU.ml^{-1} insulin and DL-3-hydroxybutyrate at (A) 5mM and (B) 10mM

Points are the means of the results of 6 experiments (A) and 4 experiments (B) using hearts from fed rats.

Table 36

The effect of DL-3-hydroxybutyrate concentration on glucose utilization (5.5mM) at different concentrations of insulin

Initial DL-3-HB conc. (mM)	Insulin conc. ($mU.ml^{-1}$)	Glucose Utilization ($\mu moles.g\ dry\ wt.^{-1}h^{-1}$) at Perfusion Time (Minutes)										
		12.5	22.5	32.5	42.5	52.5	62.5	72.5	82.5	92.5	102.5	112.5
5	100	270**	215	199	167	131	127	133	135	147	158	144
10	100	251	225	190	159	146	140	123	133	131	130	128
5	2	170**	162	143	127	125	124	119	140	165	168	185
10	2	190	183	158	139	124	111	105	102	100	103	101
10*	0.08	203	189	148	127	135	128	108	132	104	171	-

Hearts from fed rats were perfused with MKHM containing 5.5mM-glucose, DL-3-hydroxybutyrate (DL-3-HB) and insulin as shown in the Table.

*In this group of hearts 0.5mg.ml^{-1} albumin was included in the perfusate and filter papers were used.

**These values are significantly different at between the 2% and 5% levels of probability.

Composition of the values at any one time show no other significant differences.

difference is significant at the 5% level of probability. The rate at 12.5 minutes in the presence of insulin at 100mU.ml^{-1} is not significantly inhibited by the presence of DL-3-hydroxybutyrate. At both concentrations of insulin the rate during the first 80 minutes of perfusion is not affected by the difference in concentration of DL-3-hydroxybutyrate.

14.5 Glucose Utilization by Hearts from Fed Rats Perfused with MKHM Containing 5.5mM-Glucose, 10mM-DL-3-Hydroxybutyrate, 0.5mg.ml^{-1} Albumin and 80μU.ml^{-1} Insulin

Fig. 36 shows the time-course of the mean rate of glucose utilization when insulin at 80μU.ml^{-1} is included in the perfusate together with 5.5mM-glucose and 10mM-DL-3-hydroxybutyrate. As was discussed in Section 7.4, insulin at this lower concentration is only effective if albumin is included in the perfusate presumably to prevent the adsorption of the insulin onto the surfaces of the perfusion apparatus. It was also necessary to use paper filters but the rates of glucose utilization in the presence of insulin at 80μU.ml^{-1} or at higher concentrations are not affected by the nature of the filter. The results obtained with 80μU.ml^{-1} insulin and DL-3-hydroxybutyrate should therefore be comparable with those presented in Table 23 (Section 7.4) where the rate of glucose utilization was steady throughout the experiment and indistinguishable from the rates found with 2mU.ml^{-1} insulin in the absence of albumin. The comparison again indicates that DL-3-hydroxybutyrate inhibits glucose utilization progressively. However this pattern may be misleading because the standard error of the mean of six experiments with 80μU.ml^{-1} insulin was unusually large for that number of observations and the differences between the rates of utilization at

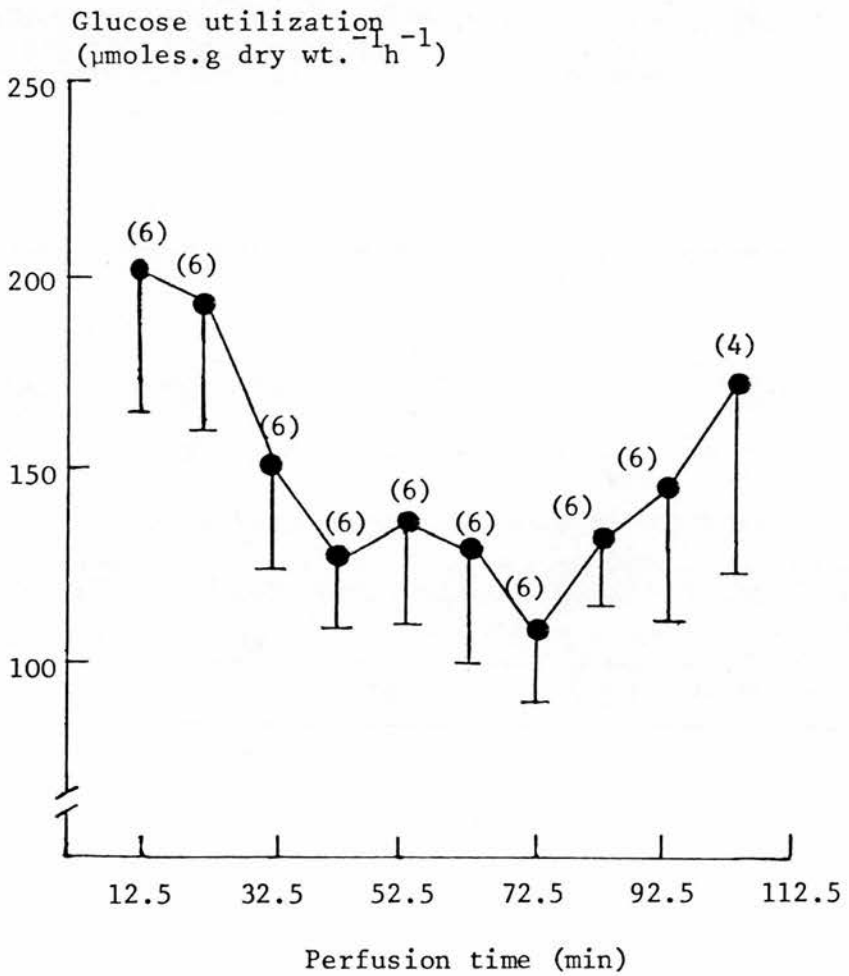

Fig. 36. The time-course of glucose utilization at 5.5mM-glucose, 10mM-DL-3-hydroxybutyrate and 80μU.ml^{-1} insulin

Points are the mean ± S.E.M. of 6 experiments using hearts from fed rats. The perfusate contained 0.5mg.ml^{-1} albumin and was filtered using paper filters.

12.5 minutes and 62.5 minutes are not significant. The rate of glucose utilization because 40 and 80 minutes is 126μmoles.g dry wt.$^{-1}h^{-1}$. This is significantly less than the rate of 274μmoles.g dry wt.$^{-1}h^{-1}$ found in the absence of the ketone body at this insulin concentration and significantly greater than the rate of glucose utilization of 75μmoles.g dry wt.$^{-1}h^{-1}$ found with 0.5mg.ml^{-1} albumin in the absence of insulin. The utilization is 54% inhibited in the steady state. Although the comparison is not strictly valid the pattern of inhibition of glucose with utilization shown in Fig. 36 is indistinguishable from that found with 2mU.ml^{-1} insulin and shown in Fig. 33.

14.6 Glucose Utilization by Hearts from Fed Rats Perfused with MKHM Containing 2.7mM-Glucose, DL-3-Hydroxybutyrate at 2.5mM, 5.0mM or 10mM and 100mU.ml^{-1} Insulin

Fig. 37 shows the time-course of the mean rate of glucose utilization when insulin at 100mU.ml^{-1} is present together with 2.7mM-glucose and DL-3-hydroxybutyrate at 2.5mM, 5.0mM or 10mM. Only two experiments were made with 10mM-DL-3-hydroxybutyrate and in this case the points are the average of the two estimates. Comparison with Fig. 34 and inspection of Table 37 shows that for most of the duration of the experiments the rate of glucose utilization in the presence of 2.5mM-DL-3-hydroxybutyrate is unaffected by the concentration of insulin. However the rate at 12.5 minutes is higher when 100mU.ml^{-1} is used. This rate is also higher than that found at 2.7mM-glucose and 2mU.ml^{-1} insulin in the absence of DL-3-hydroxybutyrate but no experiments were made with 100mU.ml^{-1} insulin in this circumstance. With 5mM and 10mM-DL-3-hydroxybutyrate the rates of utilization in the first half hour of perfusion were indistinguishable from one another and from the rates measured at the lower insulin concentration.

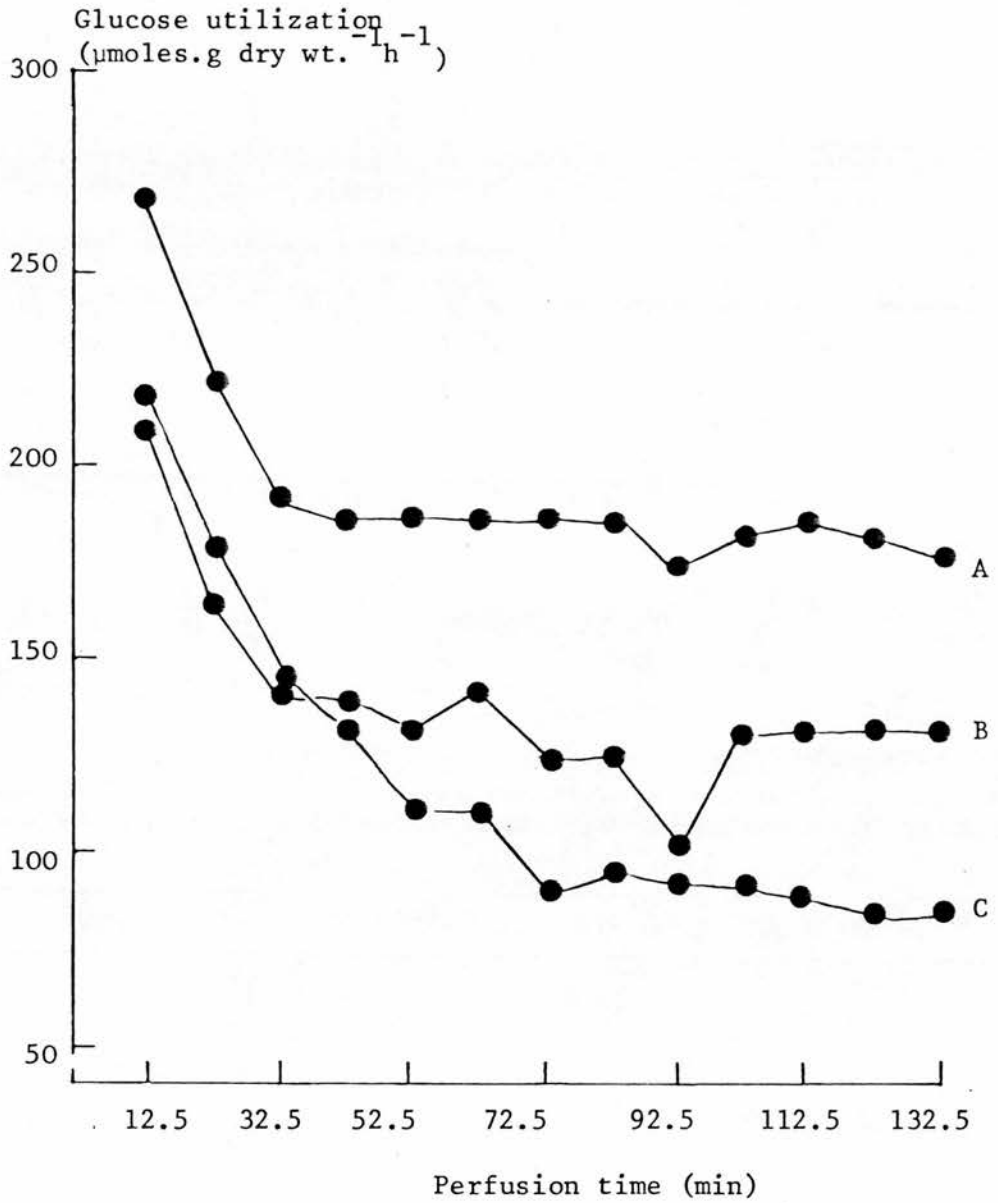

Fig. 37. The effect of concentration of DL-3-hydroxybutyrate on the time-course of glucose utilization at 2.7mM-glucose and 100mU.ml^{-1} insulin

Hearts from fed rats were perfused with 2.7mM-glucose, 100mU.ml^{-1} insulin and 2.5mM-, 5.5mM- and 10mM-DL-3-hydroxybutyrate (A, B and C respectively).

Points are the average of 3, 3 and 2 experiments for A, B and C respectively.

Table 37

The effect of DL-3-hydroxybutyrate concentration on glucose utilization at 2.7mM-glucose at different concentrations of insulin

Initial DL-3-HB conc. (mM)	Insulin conc. ($mU.ml^{-1}$)	Glucose Utilization ($\mu moles.g\ dry\ wt.^{-1}h^{-1}$) at Perfusion Time (Minutes)										
		12.5	22.5	32.5	42.5	52.5	62.5	72.5	82.5	92.5	102.5	112.5
2.5	100 (3)	267	219	190	184	184	185	183	181	174	180	183
5.0	100 (3)	207	163	139	138	131	142	121	125	98	128	130
10.0	100 (2)	217	167	142	129	111	110	87	94	89	86	89
2.5	2 (6)	168	185	181	172	171	169	158	165	167	163	163
5.5	2 (9)	204	167	128	108	108	96*	81	72	85	77	86
10.0	2 (5)	222	186	167	180	180	184	189	183	191	205	221

Hearts from fed rats were perfused with MKHM containing 2.7mM-glucose, DL-3-hydroxybutyrate (DL-3-HB), and insulin as shown in the Table. Values the average of the number of observations given in parentheses.

*The number of observations decreases from 9 to 3 at this point because a series of shorter experiments were made.

Later in the experiments the rates can be distinguished but whereas with 2mU.ml^{-1} the rates were lower with 5.5mM-DL-3-hydroxybutyrate the opposite is the case with 100mU.ml^{-1} insulin.

14.7 Discussion

These experiments indicate that DL-3-hydroxybutyrate inhibits glucose utilization in the presence of insulin to an extent that depends on the duration of the experiment, and on the concentration of the ketone body but, at least after the first hour of perfusion, is independent of the concentration of insulin. A full discussion of these results is given in Chapter 18 where experiments are described in which lactate production, D-3-hydroxybutyrate consumption and acetoacetate production are measured in comparable conditions. In addition more experiments into the development and release of inhibition of glucose utilization by DL-3-hydroxybutyrate are presented in Chapter 16. However it is useful now to compare the results with those of other workers.

Randle *et al.* (1964) reported that at 139μmoles.g dry wt.$^{-1}$h^{-1} glucose utilization was inhibited by 65% during the first 15 minutes of perfusion of hearts from fed rats with 5mM-glucose, 5mM-DL-3-hydroxybutyrate and 100mU.ml^{-1} insulin. The overall rate of utilization between 2.5 and 22.5 minutes measured in this work at the same concentrations of substrates and insulin was 241μmoles.g dry wt.$^{-1}$h^{-1} suggesting an inhibition of 22%. When the insulin concentration was 2mU.ml^{-1} the glucose utilization between 2.5 and 22.5 minutes at the same substrate concentrations was 179μmoles.g dry wt.$^{-1}$h^{-1}, which is an inhibition of 42%. However in the period of constancy after approximately an hour's perfusion the rates of glucose utilization in the presence of 5mM- or 10mM-DL-3-hydroxybutyrate

and at all concentrations of insulin were between 108 and 136μmoles.g dry wt.$^{-1}h^{-1}$ with the inhibition varying from 57% to 65%. Comparison of the percentage inhibition is complicated by the differences in rate found with glucose as the only substrate. The main discrepancy between this work and that of Randle et al. is in the development of the inhibition of utilization. Randle et al. reported that the rate of glucose utilization in their experiments did not change in a further period of perfusion of 15 minutes, but the rate they found compares well with the steady-state rate estimated here. In contrast, England and Randle (1967) who perfused rat hearts for 8 minutes with 14mM-glucose and 50mU.ml^{-1} insulin before adding DL-3-hydroxybutyrate to a final concentration of 5.5mM observed that the inhibition of glucose utilization increased during the following 15 minutes. The rate of glucose utilization when the ketone body was introduced was high at 498μmoles.g dry wt.$^{-1}h^{-1}$ and had been inhibited by 27% after 5 minutes, 42% after 10 minutes and by 46% after 15 minutes. The increase in inhibition was associated with an increase in the intracellular concentration of glucose-6-phosphate. These results support those presented here in that they show an intensification of inhibition but not over as long a period.

No comparable experiments have been found in the literature on the effect of the concentration of DL-3-hydroxybutyrate on the degree of inhibition of glucose utilization but Newsholme and Randle (1964) reported increasing effects of DL-3-hydroxybutyrate between 0.4mM and 5.5mM in raising the concentration of glucose-6-phosphate in perfused rat hearts. Studies on the effect of acetoacetate on glucose utilization (Williamson and Krebs, 1961) were made only with 5mM-acetoacetate, 5mM-glucose and 2mU.ml^{-1} insulin. The rates in the period 15 to 75 minutes in the absence and presence of acetoacetate

were 266 and 129μmoles.g dry wt.$^{-1}$h^{-1} respectively which is an inhibition of 52%. These are close to the values found in this work with 5.5mM-glucose and 10mM-DL-3-hydroxybutyrate, and 2mU.ml^{-1} insulin, but Williamson and Krebs did not report any change in the rate of utilization of glucose during the period of measurement.

In summary, most of the work described in this chapter was done in conditions that have not been previously examined. Where comparisons can be made some support for this work can be found although the agreement is not complete.

CHAPTER 15

Glucose Utilization in the Presence of DL-3-Hydroxybutyrate

15.1 Introduction

In the absence of added insulin glucose utilization by perfused hearts isolated from fed rats falls during the early period of perfusion. This pattern was also seen when hearts were perfused with insulin and high concentrations of DL-3-hydroxybutyrate and is readily distinguished from the relative constancy of glucose utilization when glucose is the sole substrate in the presence of insulin. Inhibition by ketone bodies of glucose utilization as well as that of glucose oxidation has not been observed in the absence of added insulin (Williamson and Krebs, 1961) except when conditions suggested the possible presence of endogenous insulin (Randle et al., 1964; Wieland et al., 1971). These claims suggest that in the absence of added insulin DL-3-hydroxybutyrate should depress the early rate of glucose utilization but have no effect on the rate during the period of metabolic stability established after 40 to 60 minutes of perfusion. The rate might therefore be low and constant throughout the experiment. To test these possibilities the effect was studied of DL-3-hydroxybutyrate of concentrations between 0.2mM and 10mM on glucose utilization at glucose concentrations of 2.7mM and 5.5mM in the absence of insulin.

15.2 Glucose Utilization by Hearts from Fed Rats Perfused with MKHM Containing 5.5mM-Glucose and 0.2mM-, 1.0mM-, 2.5mM-, 5mM-, or 10mM-DL-3-Hydroxybutyrate

Fig. 38 shows two examples of the time-course of the concentration of perfusate glucose that are representative of this series of experiments. Usually the concentration passed through a minimum

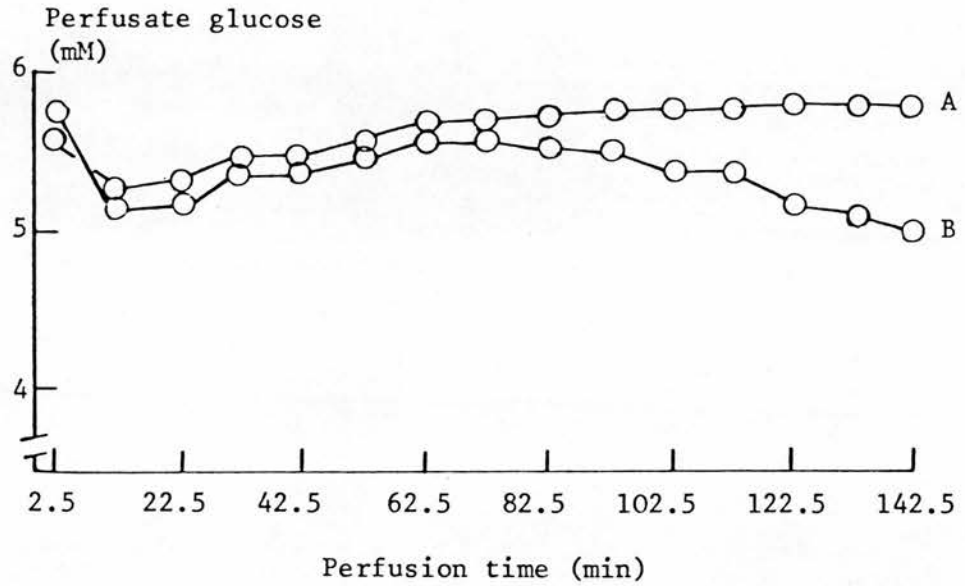

Fig. 38. Time-course of perfusate glucose concentration at 5.5mM-glucose and 5.5mM- and 10mM-DL-3-hydroxybutyrate

The data are the results from single experiments in which hearts from fed rats were perfused with 5.5mM-glucose and 5.5mM- or 10mM-DL-3-hydroxybutyrate A and B respectively.

before becoming constant or, less often, became constant rapidly before two half-times had passed for the theoretical approach to a steady state at a constant rate of utilization. Both patterns indicate an initial fall in the rate of glucose utilization to a constant rate which was maintained in some cases to the end of an experiment of 150 minutes. In other cases the glucose concentration tended to fall in the late stages of the experiment.

The time-course of the mean rate of glucose utilization at the different concentrations of DL-3-hydroxybutyrate are shown in Fig. 39. At all concentrations of the ketone body the rate of utilization decreases to constancy after 30 to 50 minutes, remains stable for a further hour and, at the three lowest concentrations finally increases. The experiments with 5.5mM-DL-3-hydroxybutyrate lasted for 60 minutes. Table 38 gives the mean and standard error of the mean for the rates of glucose utilization during the period of constancy in comparison with the rate measured over the equivalent period with glucose as the sole substrate. At all concentrations of DL-3-hydroxybutyrate the rates of glucose utilization are significantly reduced except at 5.5mM-DL-3-hydroxybutyrate compared with the rate in the absence of the ketone body but none are significantly different from the others. The similarity in the degree of inhibition after 40 minutes of perfusion regardless of the concentration of DL-3-hydroxybutyrate is also evident in Table 39 where are shown the percentage inhibitions at 10 minute intervals during the first 90 minutes of the experiments. Table 39 also shows that there is no such similarity in the early part of the experiments when in most cases there appears to be no initial inhibition while with 2.5mM-DL-3-hydroxybutyrate there is a constant degree of inhibition from the start and with 1mM-DL-3-hydroxybutyrate there is apparently no inhibition in the first 40 minutes of perfusion.

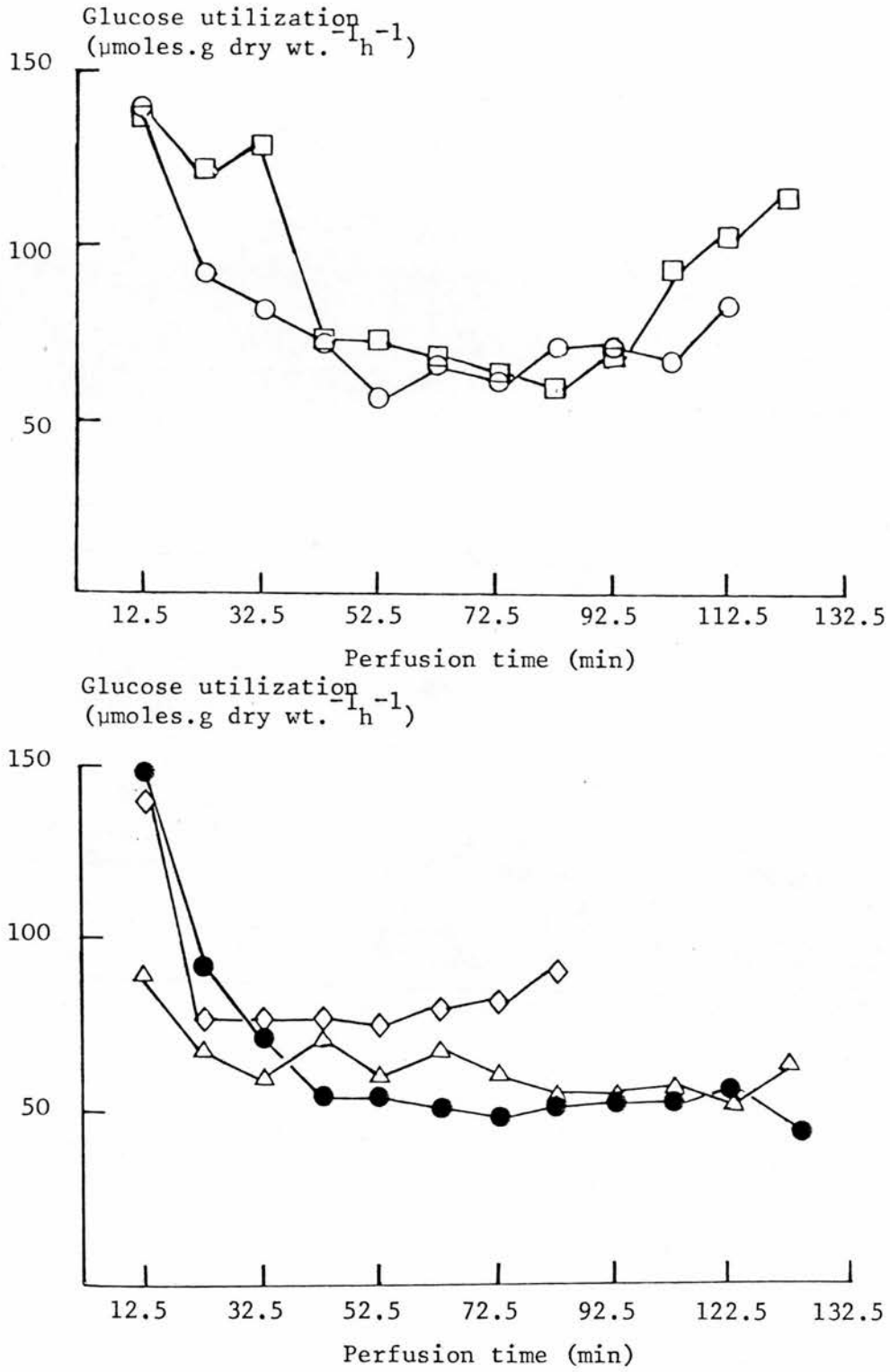

Fig. 39. Time-courses of glucose utilization at 5.5mM-glucose and at different concentrations of DL-3-hydroxybutyrate

Hearts from fed rats were perfused with 5.5mM-glucose and DL-3-hydroxybutyrate at different concentrations:- open circles 0.2mM (5), open squares 1mM (5), open triangles 2.5mM (3), open diamond 5mM (4) and close circles 10mM (10). Numbers of experiments are shown in parentheses.

Table 38

The effect of DL-3-hydroxybutyrate on glucose utilization at 5.5mM-glucose in the absence of insulin

DL-3-HB Conc. (mM)	Glucose Utilization (μmoles.g dry wt.$^{-1}$ h^{-1})	
0.0	117 ± 10 (18)	
0.2	65 ± 13 (5)	a
1.0	64 ± 16 (5)	a
2.5	60 ± 20 (3)	b
5.5	86 ± 11 (4)	n.s
10.0	51 ± 11 (10)	c

Hearts from fed rats were perfused with MKHM containing 5.5mM-glucose and DL-3-hydroxybutyrate (DL-3-HB) at different concentration as indicated in the Table.

Values are mean $\pm$ S.E.M., and the numbers of observations are in parentheses. Probability of difference from control being significant:

a = $0.02>P>0.001$

b = $0.05>P>0.02$

c = $P<0.001$

n.s = not significant

Table 39

The effect of DL-3-hydroxybutyrate on glucose utilization at 5.5mM-glucose in the absence of insulin

Initial DL-3-HB conc. (mM)	% Inhibition of Glucose Utilization at Time (Minutes)								
	12.5	22.5	32.5	42.5	52.5	62.5	72.5	82.5	92.5
0.2	10	21	24	32	55	38	41	45	42
1	13	5	-9	32	24	38	45	54	45
2.5	43	42	44	33	40	37	44	57	55
5.5	11	36	31	30	30	25	27	31	-28
10.0	3	21	36	47	44	51	57	59	57

Hearts from fed rats were perfused with MKHM containing 5.5mM-glucose and DL-3-hydroxybutyrate as shown in the Table.

15.3 Glucose Utilization by Hearts from Fed Rats Perfused with MKHM Containing 2.7mM-Glucose and 5.5mM- or 10mM-DL-3-hydroxybutyrate

Fig. 40 shows the time-course of the mean rate of glucose utilization by hearts perfused with 2.7mM-glucose alone or together with either 5.5mM- or 10mM-DL-3-hydroxybutyrate. At the lower concentration of ketone body two hearts were perfused for 150 minutes and three others for 90 minutes. The data for the first 90 minutes in all experimental conditions are also given in Table 40. At both concentrations of DL-3-hydroxybutyrate the rate of glucose utilization decreases at first and becomes constant after 40 minutes. With 5.5mM-DL-3-hydroxybutyrate the constant rate is significantly lower than in the absence of the ketone body. Since only three experiments were carried out with 10mM-DL-3-hydroxybutyrate the rate of utilization at any one time cannot be tested for significance. However a run analysis of the time-course of utilization in the presence and absence of DL-3-hydroxybutyrate suggests that there is a significant inhibition and that the inhibition increases with the concentration of the ketone body.

15.4 Discussion

These experiments indicate that DL-3-hydroxybutyrate inhibits the utilization of glucose in the absence of added insulin and when the isolated heart may be supposed to be free of endogenous insulin. However they provide no evidence of any significant inhibition of glucose utilization after 12.5 minutes of perfusion. These results conflict with those of Randle *et al.* (1964) who reported inhibition of glucose utilization by 5mM-DL-3-hydroxybutyrate during a 15 minute period of perfusion, when the rate of glucose utilization was 75μmoles.g dry wt.$^{-1}$h^{-1} compared with 255μmoles.g dry wt.$^{-1}$h^{-1}.

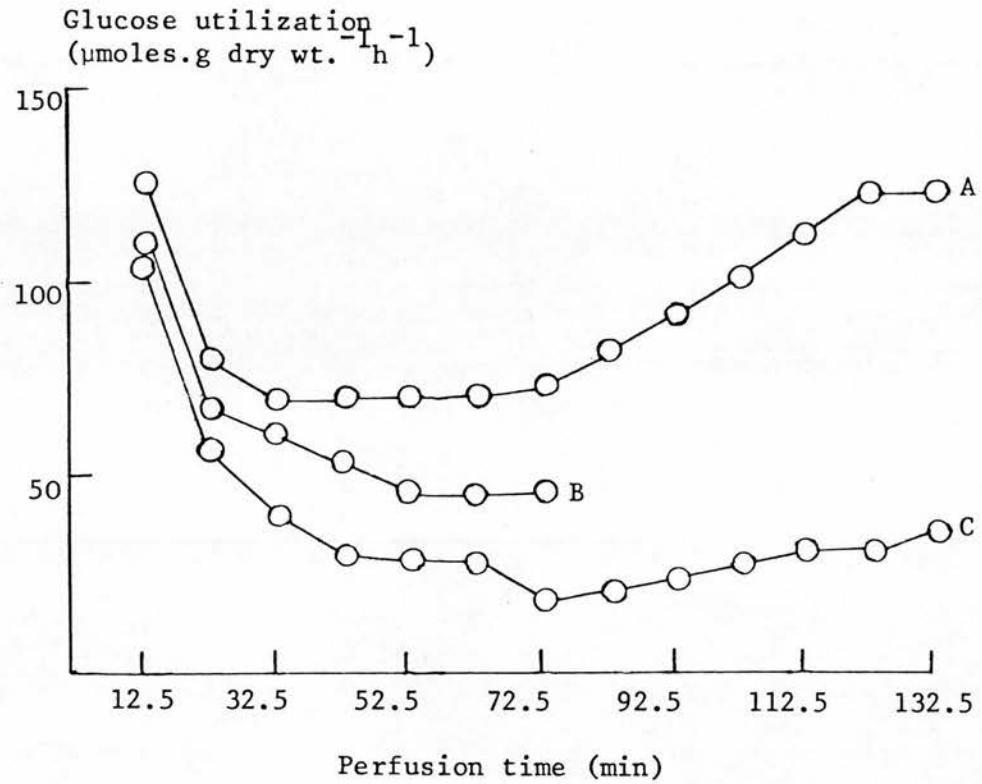

Fig. 40. Time-courses of glucose utilization at 2.7mM-glucose and 5.5mM- or 10mM-DL-3-hydroxybutyrate

Hearts from fed rats were perfused with 2.7mM-glucose alone (A) and with 5.5mM- or 10mM-DL-3-hydroxybutyrate B and C respectively.

Points are the average of 12, 5 and 3 experiments for A, B and C respectively.

Table 40

The effect of DL-3-hydroxybutyrate on glucose utilization at 2.7mM-glucose in the absence of insulin

Initial DL-3-HB (mM)	Glucose Utilization (μmoles.g dry wt.$^{-1}$h^{-1}) at Perfusion Time (Minutes)								
	12.5	22.5	32.5	42.5	52.5	62.5	72.5	82.5	92.5
0.0	126	81	71	69	71	70	74	83	93
5.5	109	67	62	53	45	46	47	-	-
10.0	106	57	41	29	27	27	17	19	23
5.5	13	17	13	23	37	34	36	-	-
10.0	16	30	42	58	62	61	77	77	75

Hearts from fed rats were perfused with MKHM containing 2.7mM-glucose and DL-3-hydroxybutyrate as indicated in the Table. Values are the average of 5 and 3 observations at 5.5mM- and 10mM-DL-3-hydroxybutyrate (DL-3-HB) respectively.

The conflict may reflect mainly the high rate of utilization found by Randle et al. with glucose as the only substrate in the absence of insulin because in the work reported here the rate of glucose utilization in the stable period averaged 65μmoles.g dry wt.$^{-1}$h^{-1} at all concentrations of the 3-hydroxybutyrate. Similarly the fact that Williamson and Krebs (1961) found no effect of acetoacetate on glucose utilization in the absence of insulin may also depend on the relatively low rate of 72μmoles.g dry wt.$^{-1}$h^{-1} they found with glucose as the only substrate. These differences emphasise the difficulties in interpretation when the properties of isolated rat hearts in an important reference state are very variable.

With the perfusion system used in this work, low rates of utilization at relatively high concentrations of substrate result in small differences in concentration between the infusate and perfusate which limits the precision of the estimate of utilization in steady state conditions (Section 4.2.1). With 5mM-glucose the precision is adequate to establish that DL-3-hydroxybutyrate significantly inhibits glucose utilization but no effect of the concentration of the ketone body on the degree of inhibition is detectable. At 2.7mM-glucose when the precision of the estimation of utilization is increased there is a significantly greater inhibition of glucose utilization with 10mM- than with 5mM-DL-3-hydroxybutyrate.

The effect of DL-3-hydroxybutyrate on glucose utilization in the presence and absence of insulin is compared in Fig. 41. Even when the concentration of D-3-hydroxybutyrate was initially no more than 0.09mM and should be reduced thereafter by metabolism the inhibition of glucose utilization in the absence of insulin was approximately 44%. Fig. 41 suggests that insulin could oppose the effects of low concentrations of D-3-hydroxybutyrate and cause a

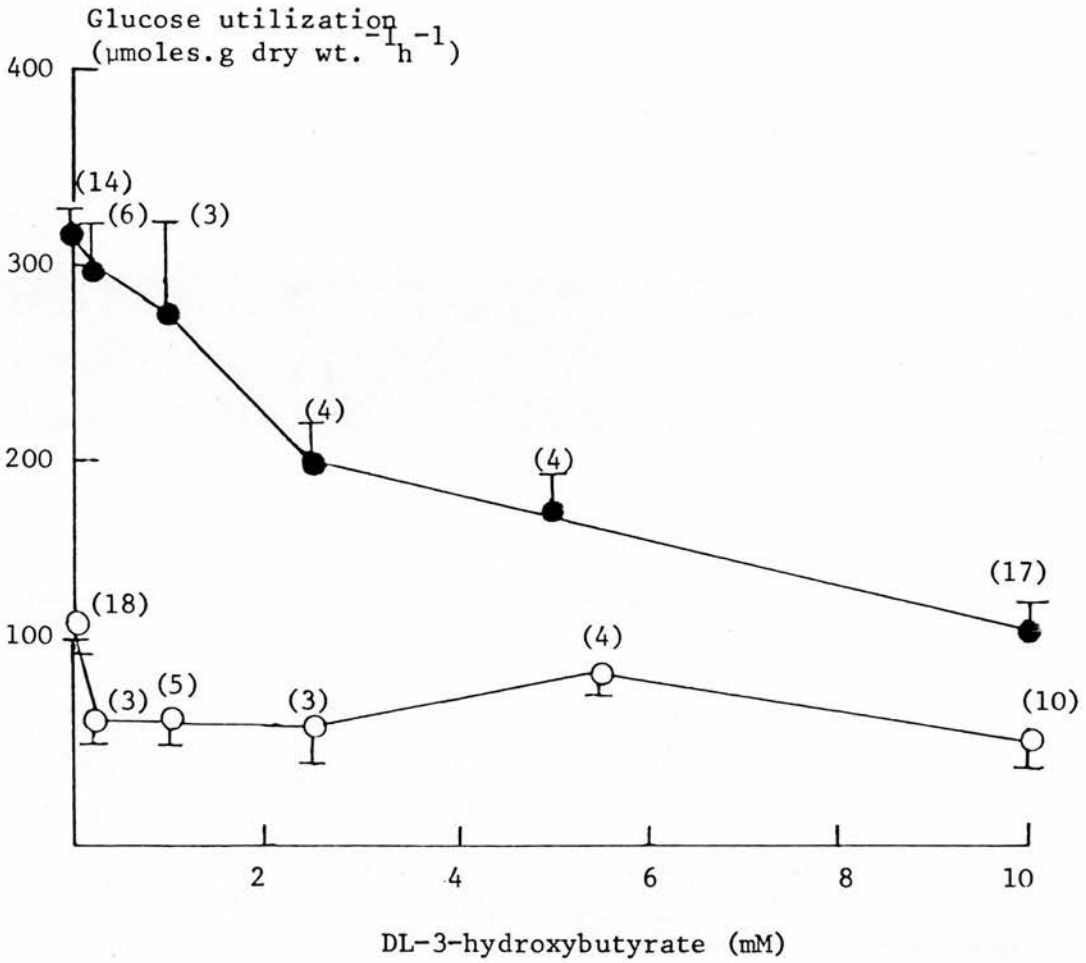

Fig. 41. The effect of DL-3-hydroxybutyrate on the utilization of glucose at 5.5mM-glucose in the presence and absence of insulin

Hearts from fed rats were perfused with 5.5mM-glucose with (closed circles) without (open circles) 2mU.ml^{-1} insulin and initial concentrations of DL-3-hydroxybutyrate as shown in the figure.

Points are the mean $\pm$ S.E.M. of the rates of utilization between 60 and 90 minutes and the numbers of observations are in parentheses.

4.5-fold increase in glucose utilization but would not be able to completely overcome the inhibitory effects of high concentrations. The confidence with which this impression can be accepted depends on the accuracy of the estimate of glucose utilization when glucose is the only substrate in the absence of insulin. The time-courses shown in Figs. 39 and 40 indicate that the metabolic stability is prolonged longer when both substrates are available especially with 10mM-DL-3-hydroxybutyrate. However if from all the experiments with 5mM-glucose as the sole substrate those are selected in which a constant rate was maintained until 100 minutes of perfusion their average rate of 104μmoles.g dry wt.$^{-1}h^{-1}$ is still significantly higher than that in the presence of DL-3-hydroxybutyrate.

CHAPTER 16

The Development and Release of the Inhibition by DL-3-Hydroxybutyrate of Glucose Utilization

16.1 Introduction

The experiments presented in Chapter 14 showed that when DL-3-hydroxybutyrate inhibits glucose utilization, the inhibition increases during the first hour of perfusion in the presence of insulin. An increase in the inhibition may also occur in the absence of insulin (Chapter 15) but detection of such an effect is complicated by the fact that glucose utilization falls during this period of perfusion when glucose is the only substrate in the absence of insulin. However both in the presence and absence of insulin the rate of glucose utilization is constant in hearts perfused for an hour with glucose but no ketone body. It seemed likely that analysis of the development of inhibition of glucose utilization would be helped if DL-3-hydroxybutyrate were to be introduced into the perfusate of hearts that had already reached a constant rate of utilization and especially if the perfusate glucose had also reached a constant concentration. Departure from a steady-state should be more easily detected than change in the rate of change of concentration. In addition each heart in these conditions can act as its own control. However if a steady-state is to be reached within an hour of perfusion without insulin, a high infusion rate must be used. Consequently all experiments of this sort were made with 2.7mM-glucose so that the rates of utilization could be measured with adequate precision.

In Chapter 9 the effect on glucose utilization of depletion of the endogenous reserves of the isolated heart was discussed. Although no effect of depletion was found when glucose was the only substrate,

the possibility was investigated that this might influence the time-course of the development of the inhibition of glucose utilization by hearts perfused with glucose, DL-3-hydroxybutyrate and insulin after 45 mins. of perfusion without substrate.

In addition the recovery of glucose utilization from inhibition by DL-3-hydroxybutyrate was studied by stopping the infusion of the ketone body after different periods of perfusion. The object of these experiments was to determine whether the time-course of the recovery of glucose utilization varied with the initial intensity of the inhibition.

All the experiments reported in this chapter are relevant to the wider issue of the normality of the properties of the isolated heart when perfusion is started.

16.2 Effect of 5.5mM-DL-3-Hydroxybutyrate on Glucose Utilization after 60 minutes Perfusion with 2.7mM-Glucose

Hearts from fed rats were perfused with MKHM containing 2.7mM-glucose for 60 minutes. The infusate was then changed to one of MKHM containing 2.7mM-glucose and 5.5mM-DL-3-hydroxybutyrate. At the same time the withdrawal pump was stopped and 0.1ml of MKHM containing sufficient DL-3-hydroxybutyrate to raise the concentration of 7.5ml of recirculating perfusate to 5.5mM was added to the perfusate reservoir. Recirculation without withdrawal was continued for one minute to allow complete mixing of the perfusate and withdrawal was then restarted. The normal circulating volume was re-established during the 5 minute period of collection of the first fraction in the new conditions of perfusion.

In seven experiments the rate of glucose utilization measured at 62.5 minutes was not significantly different from that observed

at 52.5 minutes. The rate after 72.5 minutes was reduced in five experiments and the mean per cent reduction in comparison with the rate over the period 52.5 minutes to 62.5 minutes was 40%. Table 41 shows the per cent reduction in rate at ten minute intervals from the introduction of DL-3-hydroxybutyrate. The inhibition does not increase after the first ten minutes of exposure, but decreases as the rate of utilization tended to increase towards the end of the experiment. The intensity of the inhibition is 46% at its greatest which is approximately 70% that reached when DL-hydroxybutyrate was present from the start of perfusion.

16.3 Effect of 5.5mM-DL-3-Hydroxybutyrate on Glucose Utilization after 60 Minutes Perfusion with 2.7mM-Glucose and 2mU.ml^{-1} Insulin

The procedure in these experiments was identical to that described above in Section 16.2. The per cent reductions in the rate at 10 minute intervals following the introduction of DL-3-hydroxybutyrate are given in Table 41. Again the inhibition does not increase after the first ten minutes of exposure and the intensity of the inhibition which remains constant at about 22% is less than that seen in equivalent experiments in which 5.5mM-DL-3-hydroxybutyrate was present in the perfusate from the start of the experiment.

16.4 Release of the Inhibition of Glucose Utilization by DL-3-Hydroxybutyrate

Hearts from fed rats were perfused with MKHM containing 2.7mM-glucose, 5.5mM-DL-3-hydroxybutyrate and 2mU.ml^{-1} insulin for 5, 30 or 60 minutes and then the infusate was changed to one lacking DL-3-hydroxybutyrate but otherwise of identical composition. Perfusion was continued so that the total duration of all experiments was 150 minutes. The infusion rate in these experiments averaged 0.51ml.min^{-1}

Table 41

Development of the inhibition by DL-3-hydroxybutyrate of glucose utilization

	% Inhibition of Glucose Utilization at Perfusion Time (Minutes)							
	62.5	72.5	82.5	92.5	102.5	112.5	122.5	132.5
A (5)	5	40	45	36	30	27	28	0.0
B (4)	15	26	19	21	19	19	18	13

Hearts from fed rats were perfused with MKHM containing 2.7mM-glucose and A without or B with insulin ($2mU.ml^{-1}$) for 60 minutes. The infusate was changed to one of MKHM containing 2.7mM-glucose, and 5.5mM-DL-3-hydroxybutyrate and, in series B, insulin ($2mU.ml^{-1}$). Numbers of observations are in parentheses.

so that the half-time for the removal of a non-metabolized substance from the perfusate was 10 minutes. Consumption of D-3-hydroxybutyrate by the heart would have increased the rate at which its concentration fell.

Fig. 42 shows the time-course of the mean rate of glucose utilization in these experiments and that found in the complete absence of DL-3-hydroxybutyrate. In all experiments in which hearts were exposed to DL-3-hydroxybutyrate, the rate of glucose utilization at 12.5 minutes was similar and inhibited by approximately 29%. In the experiments in which the exposure continued for 30 or 60 minutes the inhibition increased and reached 67%. After infusion of the ketone body had been stopped the rate of glucose utilization had increased within 2 to 3 half-times when the concentration of D-3-hydroxybutyrate could not be greater than 0.6-0.3mM. Inhibition was still evident after 4-5 half-times when D-3-hydroxybutyrate was no more than 0.15-0.075mM. Recovery of glucose utilization was slowest in the hearts exposed to DL-3-hydroxybutyrate for the shortest time but followed a similar pattern in the other two cases. At the end of the experiments there was no significant inhibition of glucose utilization but the small number of experiments made with the shortest exposure to DL-3-hydroxybutyrate do not permit statistical comparison.

16.5 Effect of the Depletion of Reserves on Inhibition of Glucose Utilization by DL-3-Hydroxybutyrate

Four hearts from fed rats were perfused for 45 minutes with MKHM containing 2mU.ml^{-1} insulin and no substrate. They were then transferred to an apparatus in which the perfusate contained 5.5mM-glucose, 10mM-DL-3-hydroxybutyrate and 2mU.ml^{-1} insulin and were perfused for a further 90 minutes.

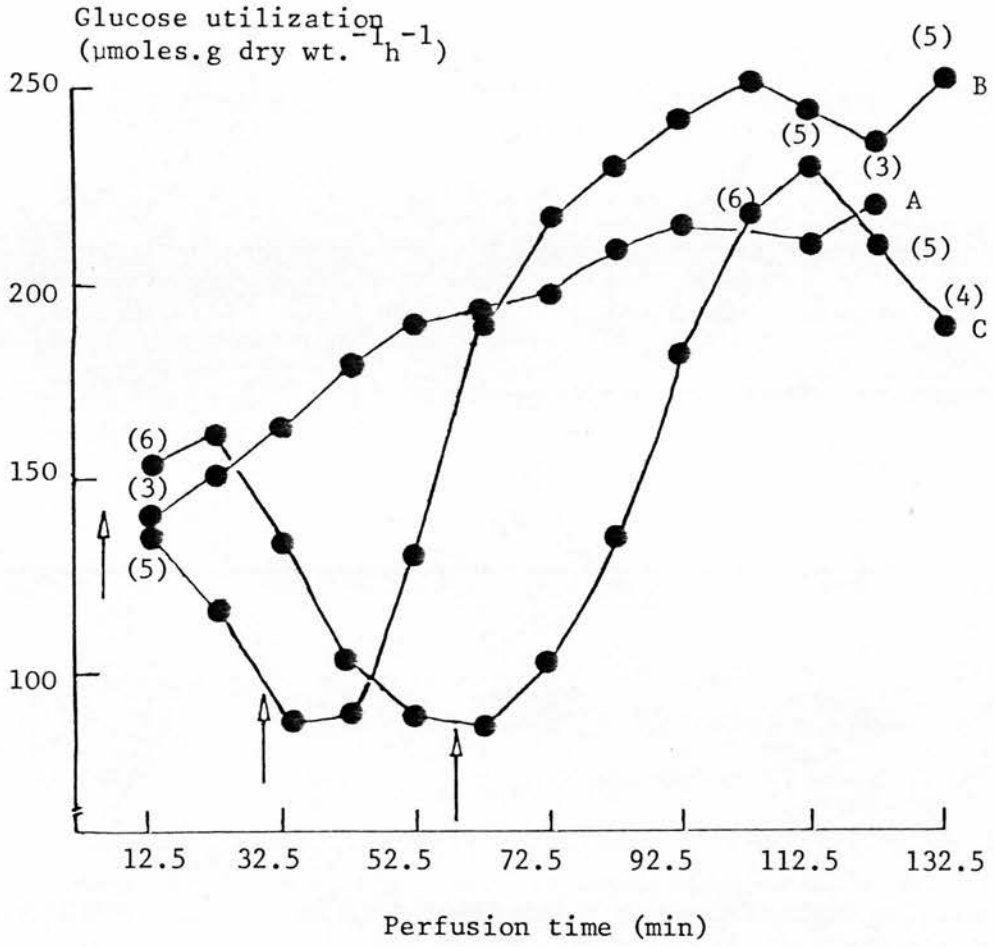

Fig. 42. Recovery of glucose utilization in the presence of insulin from inhibition by DL-3-hydroxybutyrate

Hearts from fed rats were perfused with 2.7mM-glucose, 2mU.ml^{-1} insulin and 5.5mM-DL-3-hydroxybutyrate for 5, 30 or 60 minutes (A, B and C respectively) when the infusate was changed to one containing 2.7mM-glucose and 2mU.ml^{-1} insulin as shown by arrows.

Points are the average of 3, 5 and 6 experiments (A, B, C respectively).

Fig. 43 shows the time-course of the mean rate of glucose utilization in these experiments. The inhibition of glucose utilization when it was first measured is similar to that seen at 12.5 minutes in experiments without the period of depletion (Fig. 33). The inhibition intensifies over a period of time that is also similar to the experiments without depletion but becomes greater than in any of them. In comparison with the rate of glucose utilization by hearts depleted of reserves but perfused only with glucose and insulin (Section 9.3) the rate is inhibited by 85%.

16.6 Discussion

Introduction of DL-3-hydroxybutyrate to the circulated perfusate after 60 minutes of perfusion with glucose alone is less effective in inhibiting glucose utilization, both in the presence and absence of insulin, than is its inclusion in the perfusate from the start of the experiments. With insulin the inhibition is reduced from 69% to 13% and without it from 40% to 23%. In addition there is no evidence of an intensification of the inhibition when DL-3-hydroxybutyrate is introduced after an hour's perfusion. However perfusion of hearts for 45 minutes without substrate before exposure to both glucose and the ketone body results in a pattern of falling utilization of glucose.

These experiments can be interpreted tentatively as suggesting that glucose utilization is progressively inhibited by DL-3-hydroxybutyrate in conditions associated with replenishment of glycogen stores. The glycogen content of the perfused rat heart increases when the perfusate contains glucose, insulin and a ketone body (Williamson and Krebs, 1961; Randle et al., 1964). But ketone bodies have little effect on the glycogen content in the absence of insulin.

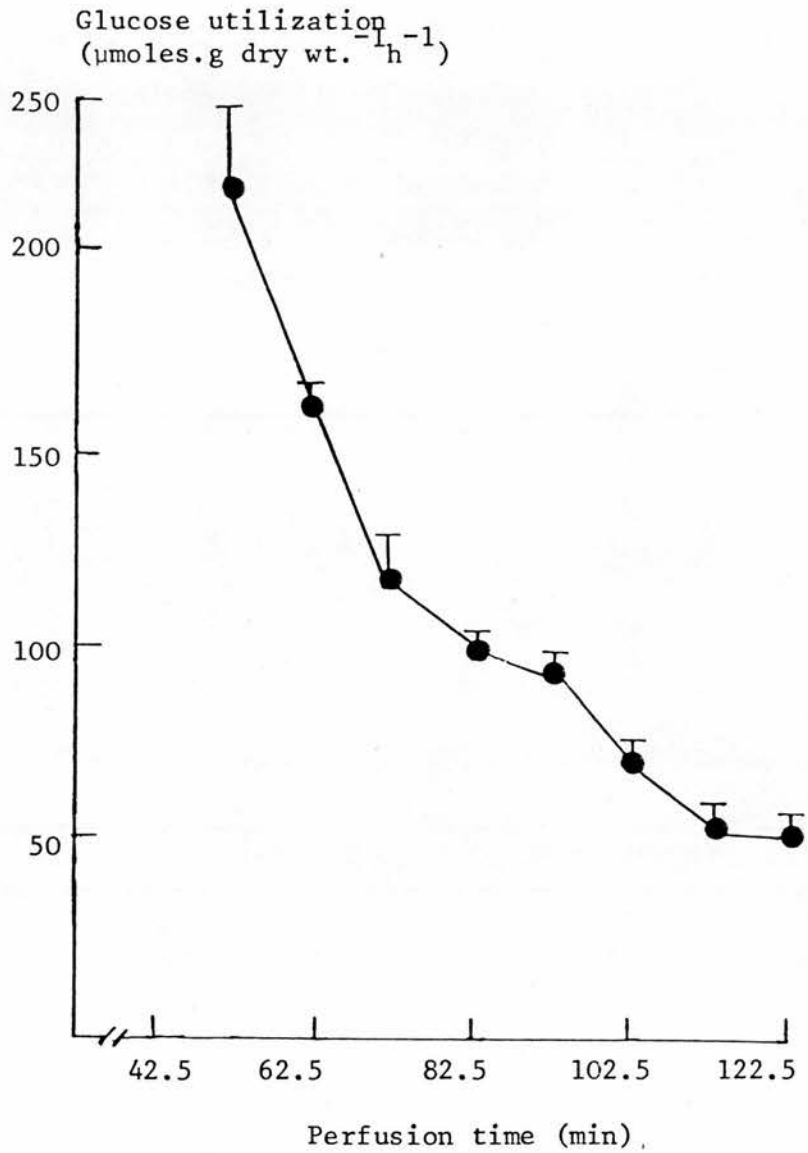

Fig. 43. The effect of DL-3-hydroxybutyrate on the time-course of glucose utilization in the presence of insulin after a period of substrate-free perfusion

4 hearts from fed animals were perfused without substrate in the presence of 2mU.ml^{-1} insulin for 45 minutes and then transferred to another apparatus in which the perfusate contained 5.5mM-glucose, 10mM-DL-3-hydroxybutyrate and 2mU.ml^{-1} insulin.

Points are the mean $\pm$ S.E.M.

Hearts perfused with glucose and insulin for one hour might have a maximum amount of glycogen whereas hearts that are recovering from the procedure of isolation or that have been perfused without substrate for 45 minutes would be able to increase their store of glycogen and in doing so might restrict the rise in the concentration of glucose-6-phosphate that accompanies the inhibition of glucose utilization by DL-3-hydroxybutyrate (England and Randle, 1967). While the synthesis of glycogen or the lack of it might determine whether the inhibition of glucose utilization by DL-3-hydroxybutyrate is or is not progressive, it does not explain the difference in the intensity of the inhibition that depends on whether or not the heart is preperfused with glucose alone.

An explanation for the observation that recovery of glucose utilization from inhibition was slowest when the exposure to DL-3-hydroxybutyrate was stopped early in the experiment when the intensity of the inhibition was least is a matter for speculation. However inhibition continued after all periods of exposure beyond the time when the concentration of the ketone body had fallen below the concentrations that are inhibitory in the presence of insulin (Section 14.2). This implies that the reversal of the changes that lead to the inhibition of glucose utilization may follow a similar time-course as their development.

CHAPTER 17

The Effect of Fasting on Glucose Utilization in the Presence of DL-3-Hydroxybutyrate

17.1 Introduction

Glucose utilization in the absence of added insulin was shown to be reduced in hearts from fasted animals in comparison with hearts from fed rats (Section 8.2). Whether DL-3-hydroxybutyrate can further reduce the rate is particularly important because fasting is the most common normal condition that is accompanied by an increase in the concentration of ketone bodies. Randle et al. (1964) reported that 5.5mM-DL-3-hydroxybutyrate halved the rate of glucose utilization in the absence of insulin by hearts from fasted animals to a value indistinguishable from that found with hearts from fed animals. They did not investigate the ability of insulin to oppose the inhibition.

When hearts from fasted animals were perfused with 5.5mM-glucose and $2mU.ml^{-1}$ insulin (Section 8.3) the rate of glucose utilization was at first low but rose to values higher than those reached by hearts from fed animals. The effect of DL-3-hydroxybutyrate on this time-course of glucose utilization was investigated and the results are presented in this chapter together with those obtained when hearts from fasted rats were perfused with glucose, DL-3-hydroxybutyrate and no insulin.

17.2 The Effect of DL-3-Hydroxybutyrate in the Absence of Insulin

In three experiments hearts from rats fasted for 48 hours were perfused with MKHM containing 5.5mM-glucose and 10mM-DL-3-hydroxybutyrate. In none of the experiments was it possible to detect a difference between the concentration of glucose in the infusate and that in any of the samples of perfusate. The rate of glucose utilization

was therefore so low as to unmeasurable. This indicates a very low rate because the method of estimation of glucose applied to twelve samples of perfusate collected over two hours would allow a difference of 1% in concentration from that of the infusate glucose to be detected although a rate of utilization could not then be determined with useful provision. In the conditions of the three experiments the rate of glucose utilization could not have exceeded 9 to 10μmoles.g dry wt.$^{-1}$h^{-1}.

17.3 The Effect of DL-3-Hydroxybutyrate in the Presence of Insulin

In four experiments hearts from rats fasted for 48 hours were perfused with MKHM containing 5.5mM-glucose, 10mM-DL-3-hydroxybutyrate and 2mU.ml^{-1} insulin. Fig. 44 shows the time-course of the mean rate of glucose utilization in these experiments. The rates measured at 22.5 and 42.5 minutes were significantly different from one another ($0.05>P>0.02$) but this is insufficient to establish that the rate of utilization fell during the early period of perfusion. From 32.5 minutes to the end of the experiment the rate of utilization averaged 84μmoles.g dry wt.$^{-1}$h^{-1}.

17.4 Discussion

The impossibility of measuring the rate of glucose utilization by hearts from fasted rats when perfused with glucose and DL-3-hydroxybutyrate is in itself an indication of the extent of the inhibition of glucose utilization. The inhibition can not be less than 90% in comparison with hearts from fed rats perfused with 5.5mM glucose in the same conditions. The only similar experiments that have been found in the literature are those of Randle et al. (1964) who measured glucose utilization in two successive 15 minute periods by hearts which were taken from rats fasted for 18 hours and which

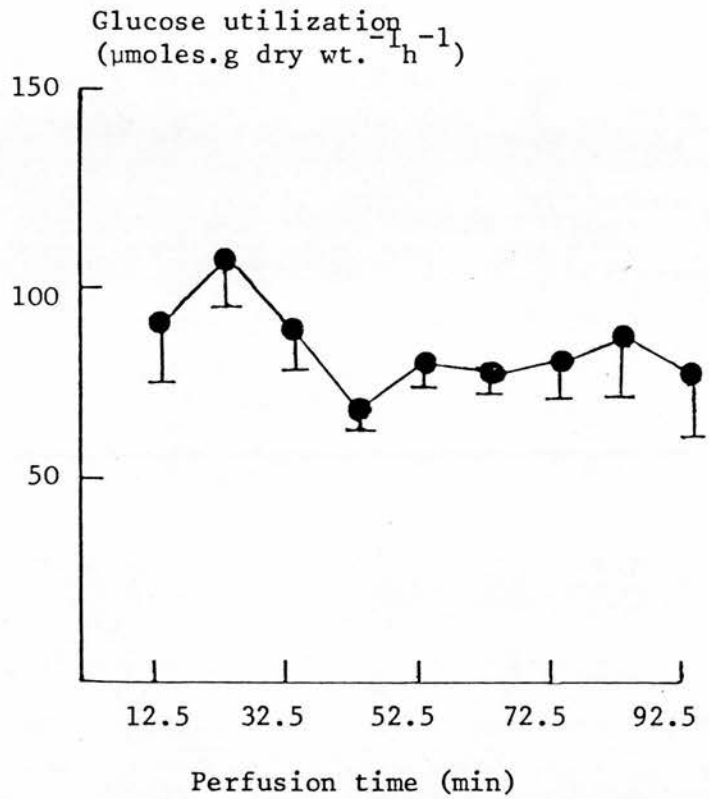

Fig. 44. The effect of DL-3-hydroxybutyrate on the time-course of glucose utilization in the presence of insulin by hearts from fasted rats

Hearts from rats fasted for 48h, were perfused with 5.5mM-glucose, 10mM-DL-3-hydroxybutyrate and 2mU.ml^{-1} insulin.

Points are the mean $\pm$ S.E.M. of 4 experiments.

were perfused with 5.5mM-glucose and 5.5mM-DL-3-hydroxybutyrate. In these experiments the rates of utilization in the two periods were equivalent to 80 and 75μmoles.g dry wt.$^{-1}$h^{-1} which are similar or greater that the rates found by Randle et al. and in this work when hearts from fed animals were perfused with the two substrates. This suggests that increasing the period of fasting increases the susceptibility of glucose utilization to inhibition by DL-3-hydroxybutyrate although with glucose as the only substrate the effect of fasting was as great in animals fasted overnight as in animals fasted for 48 hours (Section 8.2).

The rate of glucose utilization by hearts from fasted animals perfused with insulin, glucose and DL-3-hydroxybutyrate has not apparently been previously studied. The results are compared in Table 42 with the rates of glucose utilization in experiments that differed either in the use of fed rats or in the use of glucose as the only substrate. In hearts from fasted rats perfused with glucose and insulin, the rates increased from values indicative of inhibition relative to the fed state until they were the highest found in this work so that when DL-3-hydroxybutyrate was also present the constant rate of utilization throughout most of the experiment indicates a degree of inhibition rising from 50% to 84%. In contrast a comparison with the time-course of glucose utilization by hearts from fed rats perfused with insulin and the two substrates shows that fasting has a little effect on the degree of inhibition of glucose utilization. The apparent increase in the suceptibility of glucose utilization in the absence of insulin to inhibition by DL-3-hydroxybutyrate as fasting is prolonged is not evident when insulin is present.

Table 42

The effect of fasting on glucose utilization in the presence of DL-3-hydroxybutyrate and insulin

Initial DL-3-HB (mM)	Glucose Utilization (μmoles.g dry wt.$^{-1}$h^{-1}) at Perfusion Time (Minutes)												
	12.5	22.5	32.5	42.5	52.5	62.5	72.5	82.5	92.5	102.5	112.5	122.5	132.5
0.0*	186	285	371	396	428	428	432	494	489	-	-	-	-
10.0*	92	108	89	71	83	78	82	88	80	-	-	-	-
0.0	309	351	328	318	315	317	310	310	314	307	306	311	317
10.0	190	183	158	129	124	111	105	102	100	103	101	134	132
	% Inhibition of Glucose Utilization												
Fasted	50	62	76	82	81	82	81	82	84	-	-	-	-
Fed	39	48	52	56	61	65	66	67	68	66	67	57	58

Hearts from fed and fasted rats were perfused with MKHM containing 5.5mM-glucose, 2mU.ml^{-1} insulin, and with or without 10.0mM-DL-3-hydroxybutyrate.

*Heart from rats fasted for 48 hours.

CHAPTER 18

Studies of DL-3-Hydroxybutyrate Utilization

18.1 Introduction

In the introductory chapter to this part of the thesis the apparent lack of studies of the metabolism of DL-3-hydroxybutyrate by the non-working perfused rat heart was pointed out. Only one experiment appears to have been made in which D-3-hydroxybutyrate utilization was measured in the absence of insulin and of competing substrates (Williamson and Krebs, 1961) and a series of six experiments DL-3-hydroxybutyrate utilization was measured with glucose as a competing substrate (Wieland et al., 1971). In this chapter the results are presented and discussed of studies of D-3-hydroxybutyrate utilization and acetoacetate production with or without glucose and in the presence and absence of insulin. Lactate production was also measured whether glucose was present or not.

18.2 D-3-Hydroxybutyrate Utilization and Acetoacetate Production in the Absence of Insulin

Hearts from fed rats were perfused for 150 minutes with MKHM containing 10mM-DL-3-hydroxybutyrate. The hearts were vigorous and beat regularly throughout the experiments. An example of the time-courses of the concentrations of D-3-hydroxybutyrate, acetoacetate and lactate measured in alternate fractions collected over 5 minutes periods is shown in Fig. 45. The time-course of the D-3-hydroxybutyrate utilization and of acetoacetate and lactate production in four experiments is shown in Fig. 46 which includes the time-course of D-3-hydroxybutyrate oxidation calculated on the assumption that it is the difference between D-3-hydroxybutyrate utilization and acetoacetate production.

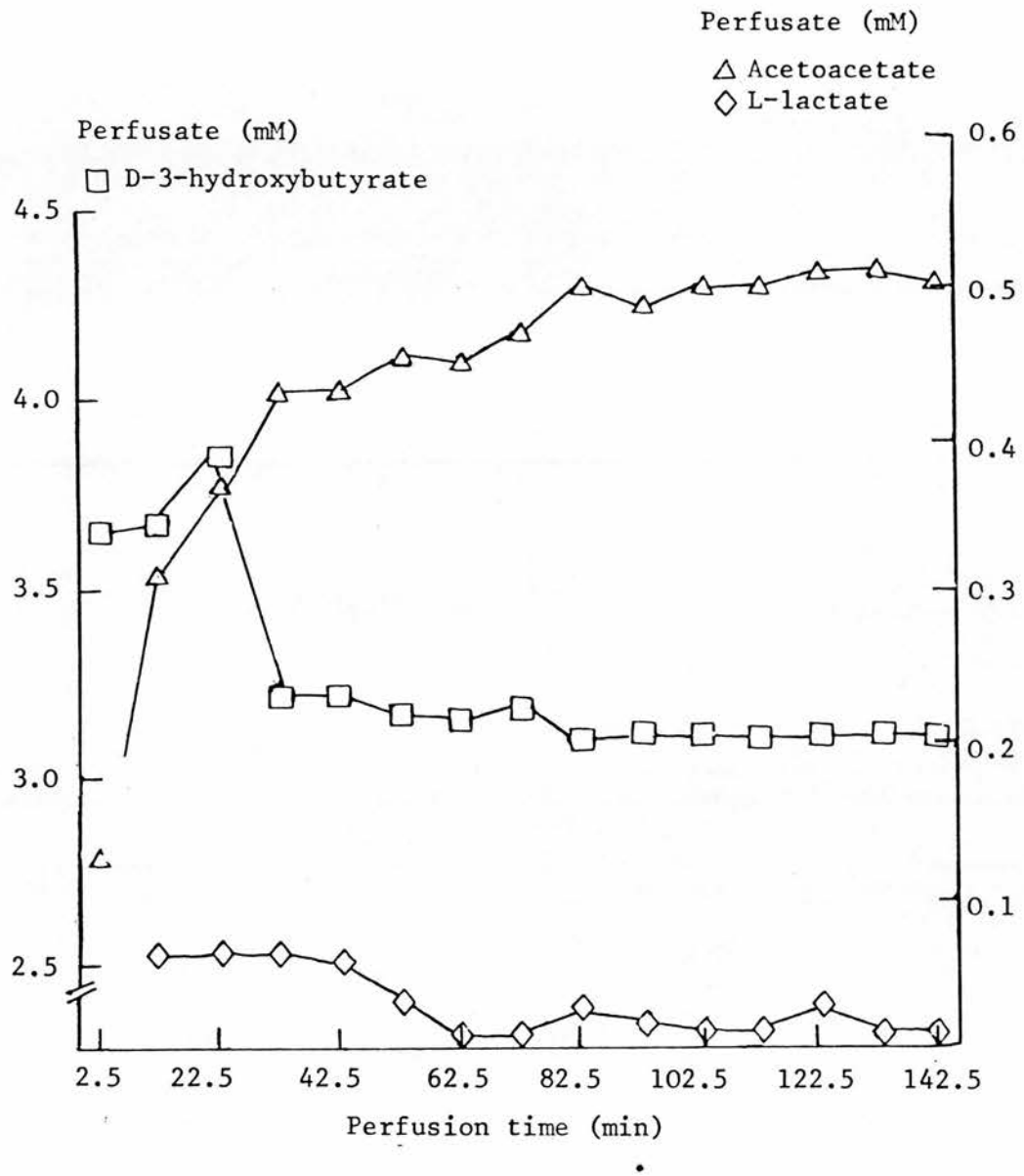

Fig. 45. Time-courses of the concentrations of D-3-hydroxybutyrate, acetoacetate and L-lactate

A heart from a fed animal was perfused with 10mM-DL-3-hydroxybutyrate.

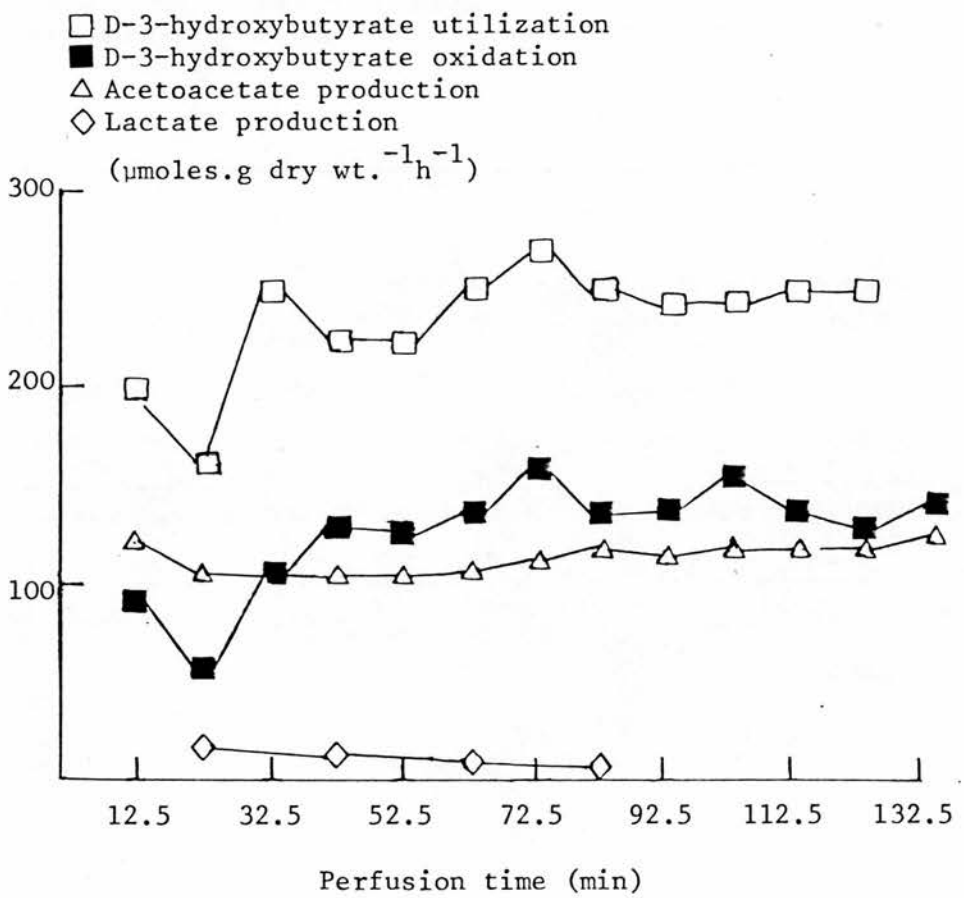

Fig. 46. Time-courses of D-3-hydroxybutyrate utilization and oxidation and acetoacetate and lactate production

Hearts from fed rats were perfused with 10mM-DL-3-hydroxybutyrate. Points are the mean of 4 experiments.

The concentration of lactate in the perfusate fell too low to measure but the fall does not reflect simply the wash-out of lactate but also a decreasing rate of lactate production that is not measurable after an hour's perfusion. The decreasing rate might indicate a developing inhibition of glycolysis or exhaustion of its source which might be for instance glycogen or the glycolytic intermediates that accumulate during the isolation of the heart (Randle et al., 1964).

The concentration of D-3-hydroxybutyrate and acetoacetate become constant within the limits of reproducibility of the methods of analysis for approximately the last hour of the experiments indicating constant rates of metabolism. In this period the rate of utilization of D-3-hydroxybutyrate was 255 ± 9 μmoles.g dry wt.$^{-1}$h^{-1} of which 116 ± 4 μmoles.g dry wt.$^{-1}$h^{-1} or 45% could be accounted for in acetoacetate production and 144 ± 6 μmoles.g dry wt.$^{-1}$h^{-1} were assumed to be completely oxidised. The rate of utilization in these experiments was estimated with good precision because the difference in concentration between infusate and perfusate D-3-hydroxybutyrate in the steady state was 25% of the infusate concentration and readily distinguished by the assay procedure (Section 2.2.3). The precision of the estimates of acetoacetate and lactate production was determined by the precision of the estimate of the concentration in the sample of perfusate (Sections 2.3.3 and 2.4).

In all of the four experiments the early changes in concentration of D-3-hydroxybutyrate shown in Fig. 45 suggest a falling rate of utilization that is also to be seen in Fig. 46. This fall in rate is reversed within half-an-hour of perfusion but the changes are not statistically significant and are not accompanied by any significant change in the production of acetoacetate.

18.3 D-3-Hydroxybutyrate Utilization and Acetoacetate Production in the Presence of Insulin

Four hearts from fed rats were perfused for 150 minutes with MKHM containing 10mM-DL-3-hydroxybutyrate and 2mU.ml^{-1} insulin, and three were vigorous and regular throughout the experiment. The fourth heart was distinctly weak and data from it have been ignored. The time-course of D-3-hydroxybutyrate, acetoacetate and lactate in one of three experiments are shown in Fig. 47 and time-course of the mean rate of D-3-hydroxybutyrate and of acetoacetate and lactate production are shown in Fig. 48.

As in the absence of insulin lactate is produced at a diminishing rate until after an hour it could not be measured precisely. Acetoacetate and D-3-hydroxybutyrate both reach concentrations that are constant within the limits of the experimental error from 50 minutes until the end of the experiment. In this period of constancy the rate of D-3-hydroxybutyrate utilization is 213$\pm$16μmoles.g dry wt.$^{-1}$h^{-1} and that of acetoacetate production is 90$\pm$5μmoles.g dry wt.$^{-1}$h^{-1} so that the mean rate of D-3-hydroxybutyrate oxidation is 120$\pm$8μmoles.g dry wt.$^{-1}$h^{-1}. These rates are similar to those found in the absence of insulin.

The pattern of the time-courses of the concentrations of D-3-hydroxybutyrate at the beginning of the experiments is different from that seen in the absence of insulin, in that they indicate an initially high rate of D-3-hydroxybutyrate utilization that decline to constancy during the first hour of perfusion. These changes are not statistically significant but are accompanied by a early low rate of acetoacetate production. The combination of the effects results in the rate of the oxidation of D-3-hydroxybutyrate in the presence of insulin during the first 30 minutes of perfusion being twice that found in the absence of insulin.

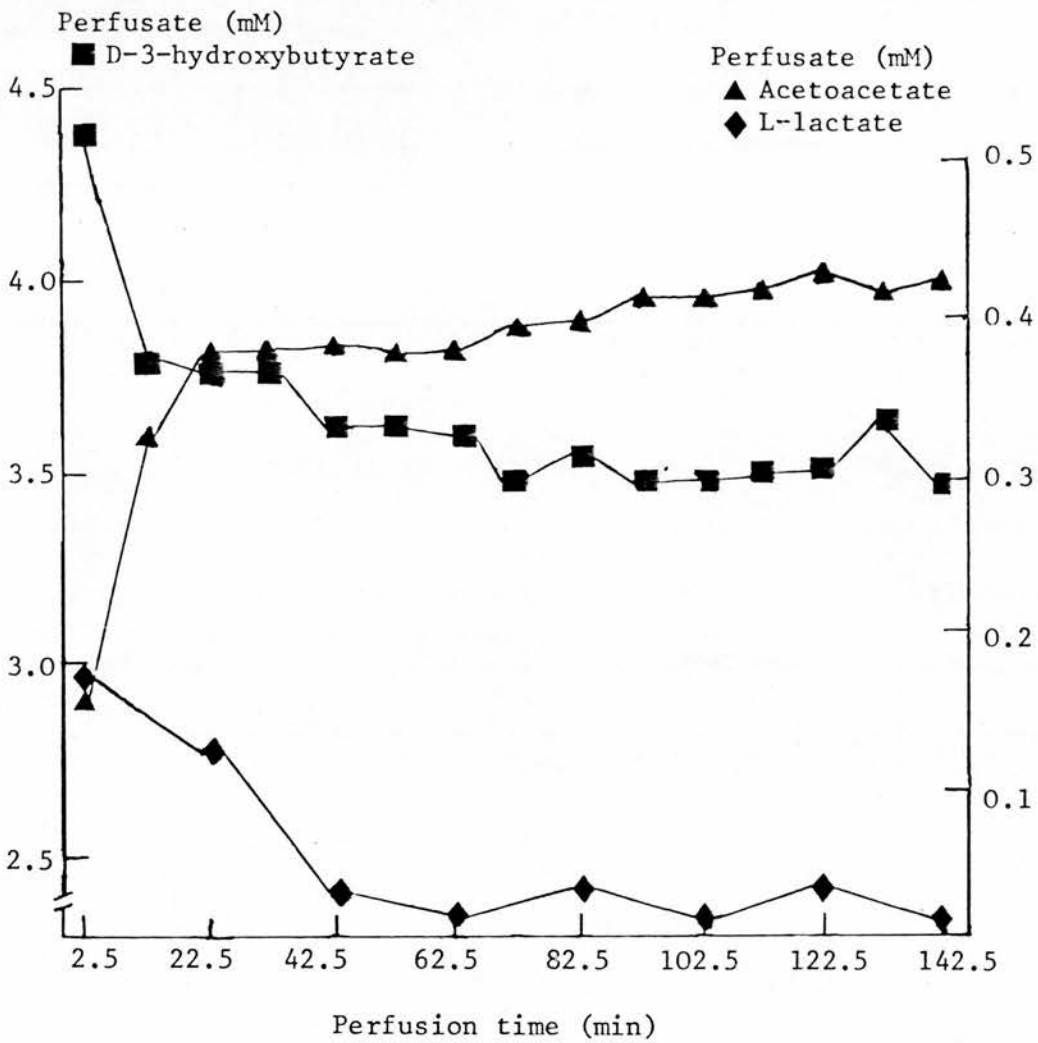

Fig. 47. Time-courses of the perfusate concentrations of D-3-hydroxybutyrate, acetoacetate and lactate in the presence of insulin

A heart from a fed animal was perfused with 10mM-DL-3-hydroxybutyrate and 2mU.ml^{-1} insulin.

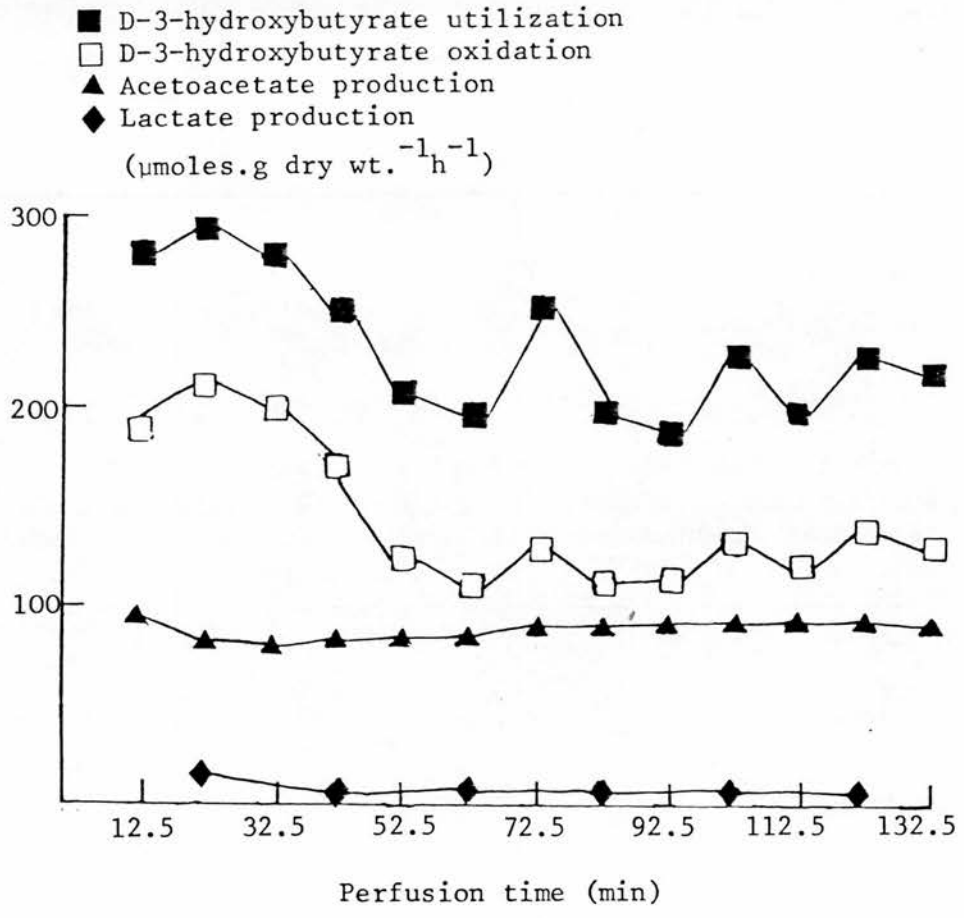

Fig. 48. Time-courses of D-3-hydroxybutyrate utilization and oxidation and acetoacetate and lactate production in the presence of insulin

Hearts from fed rats were perfused with 10mM-DL-3-hydroxybutyrate and 2mU.ml^{-1} insulin. Points are the mean of the three experiments.

18.4 Glucose and D-3-Hydroxybutyrate Metabolism in the Absence of Insulin

Hearts from fed rats were perfused for 150 minutes with MKHM containing 5.5mM-glucose and 10mM-DL-3-hydroxybutyrate. The concentrations of D-3-hydroxybutyrate, glucose, acetoacetate and lactate in the perfusate at ten minute intervals in one of these experiments are shown in Fig. 49 and the time-course of the mean rates of glucose and D-3-hydroxybutyrate utilization, and of acetoacetate and lactate production are shown in Fig. 50. In three experiments made in these conditions the concentrations of the four substances became constant within the first hour of perfusion and did not alter detectably until after 90 minutes of perfusion when in two of the experiments the concentration of D-3-hydroxybutyrate fell slowly and the concentration of lactate tended to rise.

These changes are reflected in Fig. 50 where the mean rate of D-3-hydroxybutyrate of utilization of 178 ± 11µmoles.g dry wt.$^{-1}$h^{-1} that is effectively constant from the start of the experiment rises after 90 minutes of perfusion. This increase is not accompanied by any change in the rate of acetoacetate production at 29 ± 3umoles.g dry wt.$^{-1}$h^{-1} and therefore suggests an increase in D-3-hydroxybutyrate oxidation for 147 ± 15µmoles.g dry wt.$^{-1}$h^{-1}. Acetoacetate production remained lower than in the absence of glucose.

Lactate production which fell to a constant rate after half-an-hour also increased after 90 minutes perfusion, whereas the rate of glucose utilization after an early fall did not alter significantly thereafter. However the rates of glucose utilization after the first hour of perfusion were lower than the 51 ± 11µmoles.g dry wt.$^{-1}$h^{-1} found after 60 minutes in the same condition when only glucose utilization was measured (Section 15.2). Of the three experiments reported here,

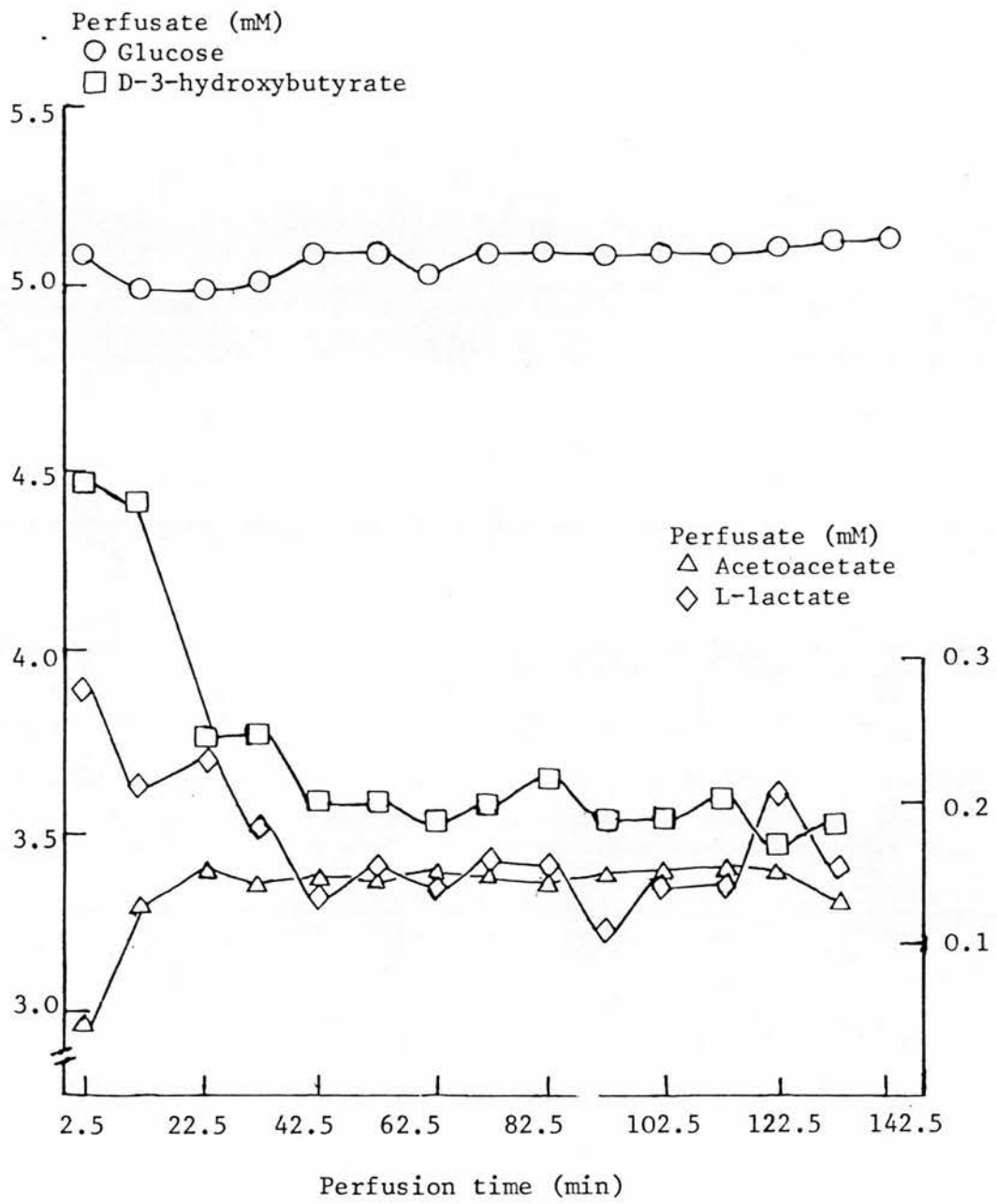

Fig. 49. Time-courses of perfusate concentrations of glucose, D-3-hydroxybutyrate, acetoacetate and L-lactate

A heart from a fed animal was perfused with 5.5mM-glucose and 10mM-DL-3-hydroxybutyrate.

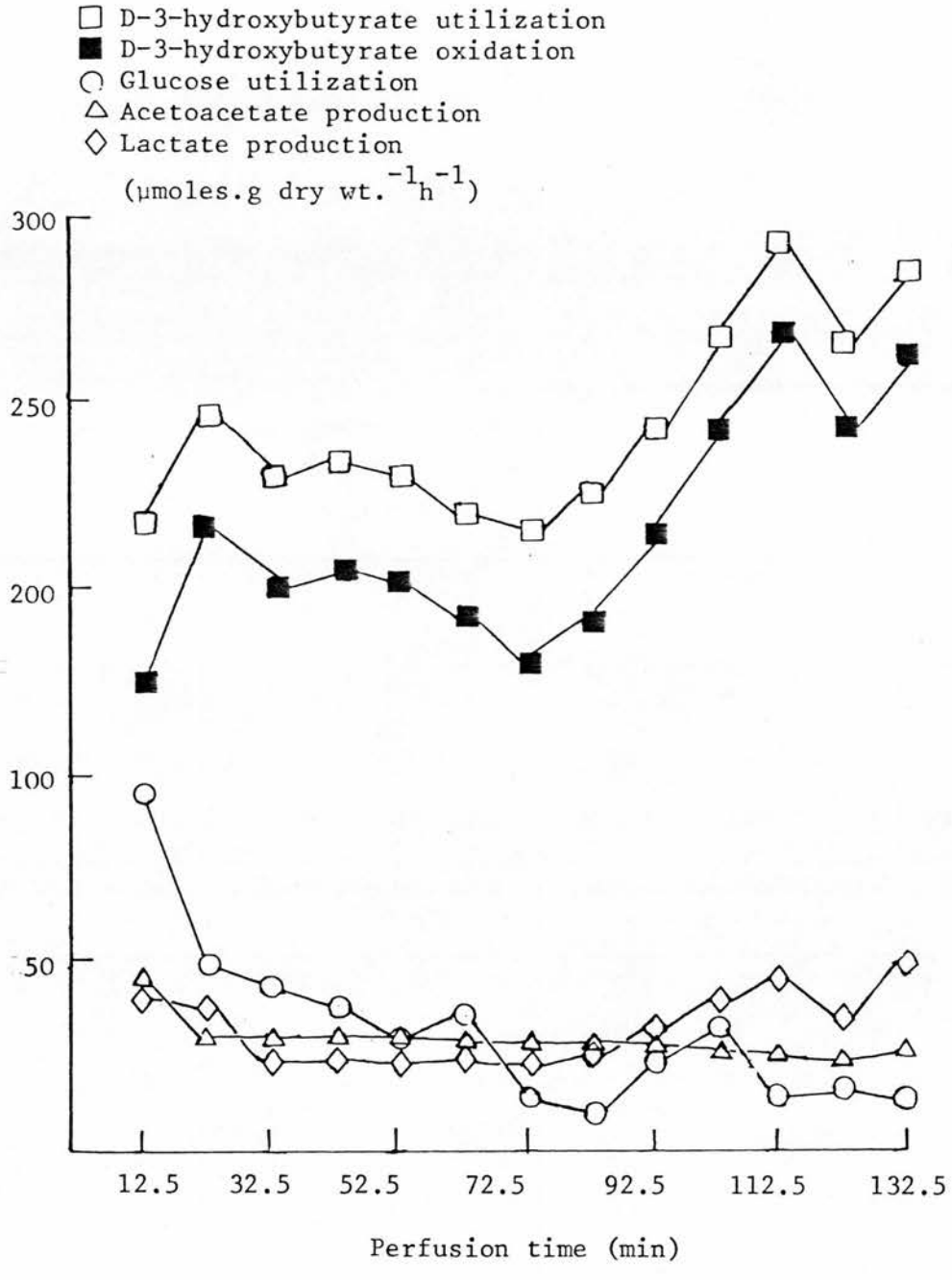

Fig. 50. Time-courses of glucose utilization and D-3-hydroxybutyrate and oxidation and acetoacetate and lactate production

Hearts from fed animals were perfused with 5.5mM-glucose and 10mM-DL-3-hydroxybutyrate. Points are the mean of three observations.

in one the rate of glucose utilization after 60 minutes was between 20 and 50μmoles.g dry wt.$^{-1}h^{-1}$ while in the other two although detectable it fell in the range of 3 to 20μmoles.g dry wt.$^{-1}h^{-1}$. However the rate of lactate production was similar in all three experiments and in the two cases in which glucose utilization was especially low the lactate produced was too great to be accounted for by the glucose used.

18.5 Glucose and D-3-Hydroxybutyrate Metabolism in the Presence of Insulin

Hearts from fed rats were perfused for 150 minutes with MKHM containing 5.5mM-glucose, 10mM-DL-3-hydroxybutyrate and 2mU.ml^{-1} insulin. The concentrations of glucose, D-3-hydroxybutyrate, acetoacetate and lactate at 10 minute intervals in one of the experiments are shown in Fig. 51 and the time-courses of the mean rate of utilization or production of these substrates are shown in Fig. 52.

In all four experiments the time-course of concentration of perfusate glucose and of glucose utilization was similar to that described in Section 14.2. When only glucose utilization was measured in hearts perfused in the same conditions. The fall in the rate of glucose utilization was not, after the first 20 minutes of perfusion, accompanied by a fall in lactate production in these experiments. In the fourth, analytical problems caused the data to be lost. The lactate production in Fig. 52 rises after approximately 70 minutes but this is the result of a 5-fold increase in one experiment which was accompanied by an increase of only 30% in glucose utilization.

The utilization of D-3-hydroxybutyrate and the production of acetoacetate and consequently the oxidation of D-3-hydroxybutyrate did not alter significantly during the course of the experiments.

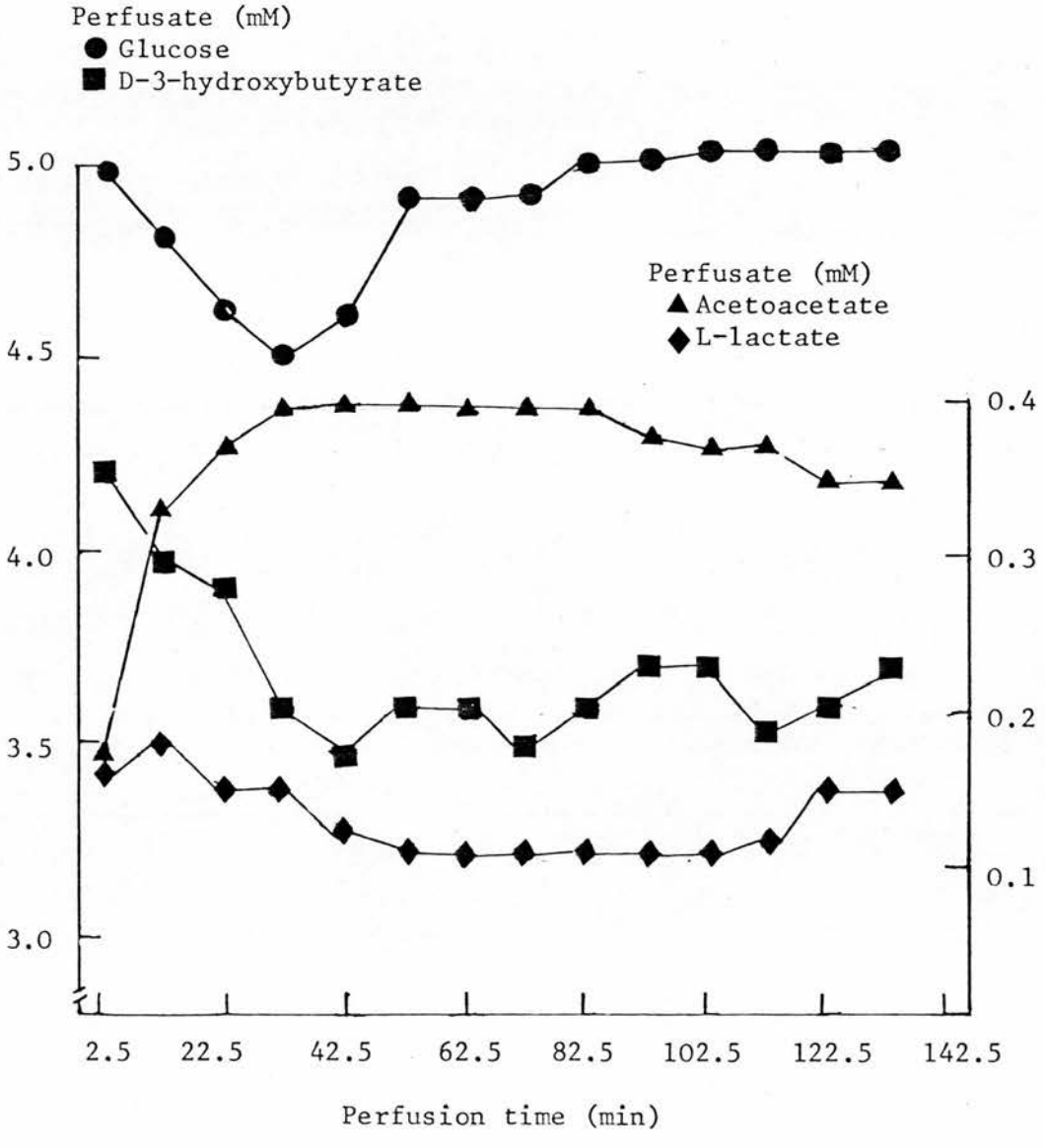

Fig. 51. Time-courses of perfusate concentrations of glucose, D-3-hydroxybutyrate, acetoacetate and lactate in the presence of insulin

A heart from a fed animal was perfused with 5.5mM-glucose, 10mM-DL-3-hydroxybutyrate and 2mU.ml^{-1} insulin.

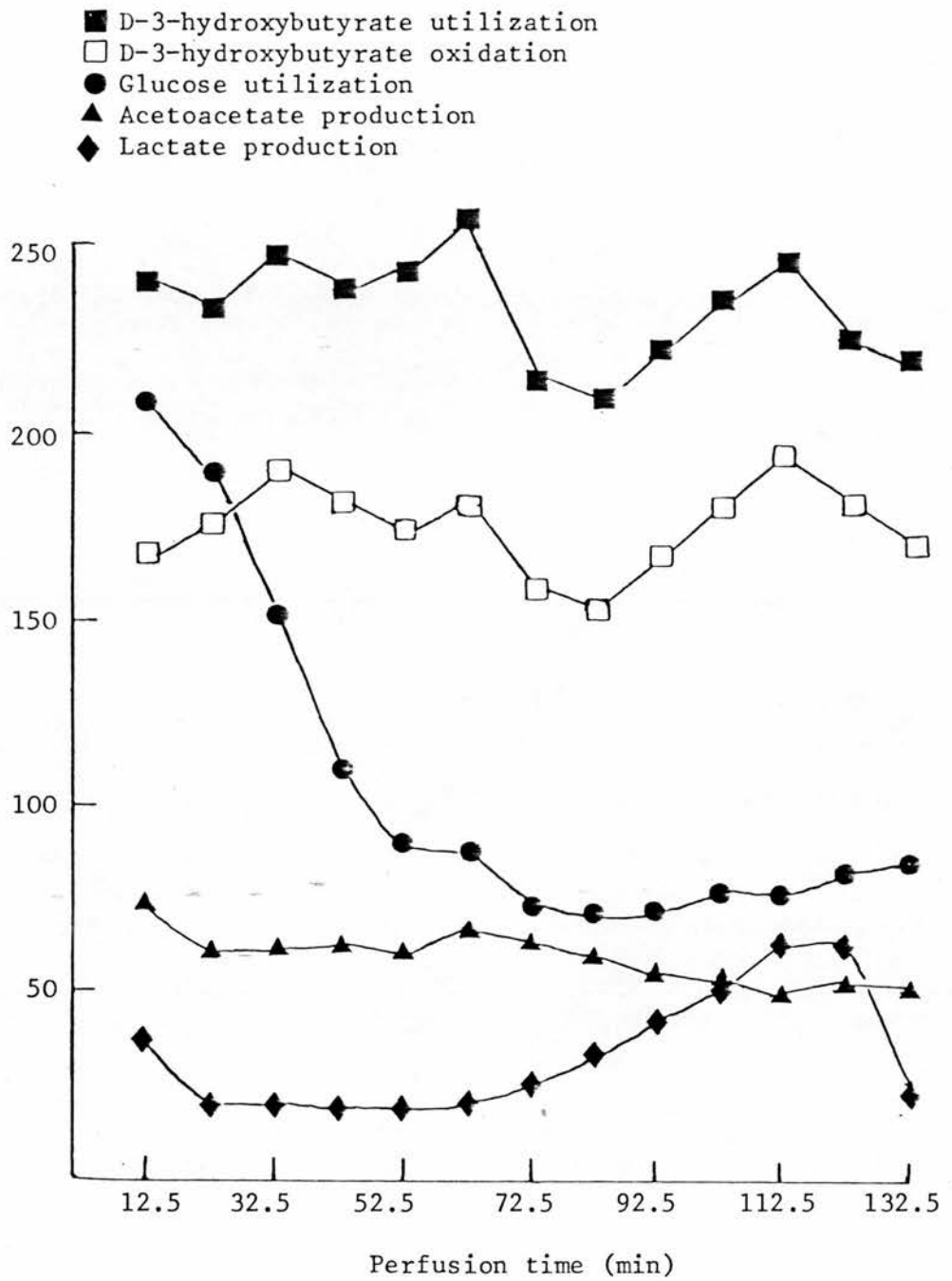

Fig. 52. Time-courses of glucose utilization and D-3-hydroxybutyrate utilization and oxidation and acetoacetate and lactate production in the presence of insulin

Hearts from fed animals were perfused with 5.5mM-glucose, 10mM-DL-3-hydroxybutyrate and 2mU.ml^{-1} insulin.

Points are the mean of 4 experiments.

The rates measured between 50 and 90 minutes were 219±20μmoles.g dry wt.$^{-1}$h^{-1} for D-3-hydroxybutyrate utilization, 61±8μmoles.g dry wt.$^{-1}$h^{-1} for acetoacetate production and 166±19umoles.g dry wt.$^{-1}$h^{-1} for D-3-hydroxybutyrate oxidation.

18.6 Discussion

The results presented in Section 18.4 and 18.5 of this chapter agree with evidence presented earlier (Chapters 14 and 15) of an inhibitory effect of DL-3-hydroxybutyrate on glucose utilization both in the presence and absence of insulin. The intensity of the inhibition increased with the time of perfusion in the presence of inulsin but in the absence of insulin not only was the intensity of the inhibition greater than shown earlier but in 2 out of 3 experiments the intensity increased with time. The proportion of the glucose consumed during the course of these experiments that can be accounted for by lactate production is shown in Table 43 together with the proportions found with hearts perfused with glucose as the only substrate. When DL-3-hydroxybutyrate was excluded from the perfusate the proportion of glucose consumed that might be converted to lactate fell or remained constant as the experiments progressed. In the presence of DL-3-hydroxybutyrate and glucose the rate of lactate production did not vary significantly with time whether insulin was present or not so that the change in the proportion of glucose utilization accountable by lactate formation must mainly reflect the fall in glucose utilization.

In the presence of insulin both the rate of lactate production and the proportion of glucose consumed that is accountable by lactate production during the early period of perfusion was lower when DL-3-hydroxybutyrate was included in the perfusate. This suggests that the early inhibition of glucose utilization is not mainly due to the

Table 43

Lactate production in the presence and absence of D-3-hydroxybutyrate

Initial DL-3-HB Conc. (mM)	% of Glucose Recovered as Lactate at Perfusion Time (Minutes)												
	12.5	22.5	32.5	42.5	52.5	62.5	72.5	82.5	92.5	102.5	112.5	122.5	132.5
0.0	17	21	13	18	9	2.5	2	2	7.5	2	6	3.5	-
10.0	21	37	27	32	40	36	88	156	67	61	173	109	192

Hearts from fed rats were perfused with MKHM containing 5.5mM-glucose and DL-3-hydroxybutyrate (DL-3-HB) as indicated in the Table.

inhibition of pyruvate oxidation but rather to inhibition of permeation or the conversion of glucose to pyruvate or both. This agrees with the conclusion of Randle et al. (1964) that DL-3-hydroxybutyrate reduced the rate of glycolysis in the presence of insulin but these workers did not measure lactate production. There appears to be no comparable studies of the effect of DL-3-hydroxybutyrate on glucose metabolism during prolonged perfusion. However Williamson and Krebs (1961) found from measurements after 15 and 75 minutes of perfusion that acetoacetate in the presence of insulin reduced glucose utilization and lactate production but in doing so doubled the proportion of glucose consumed that could be accounted for by lactate production. This procedure can take no account of changes in rate with time but the results are in reasonable agreement with the data presented here although in their experiments the reduction in lactate output was small.

In the absence of insulin, Tables 43 and 44 show that the presence of DL-3-hydroxybutyrate increased the rate of lactate production and the amount of glucose that may be converted to lactate. This suggests that inhibition of pyruvate oxidation has a greater effect in these conditions than when insulin is present. Williamson and Krebs (1961) also found acetoacetate to increase lactate production but in contrast to this work they found no change in the rate of glucose utilization after 15 minutes perfusion. Randle et al. (1964) found DL-3-hydroxybutyrate to inhibit the high rates of glucose utilization that they observed in experiments over 20 minutes and that they attributed to the effects of endogenous insulin. They did not measure lactate production. However Wieland et al. (1971) reported an inhibition in the presence of DL-3-hydroxybutyrate of glucose utilization, which they also thought was affected by endogenous insulin, a stimulation of lactate production and a halving of the amount of the active form

Table 44

D-3-Hydroxybutyrate and glucose utilization, D-3-hydroxybutyrate oxidation, and acetoacetate and lactate production

Initial Glucose (mM)	Initial DL-3-HB (mM)	Insulin ($2mU.ml^{-1}$)	D-3-Hydroxybutyrate Utilization	D-3-Hydroxybutyrate Oxidation	Acetoacetate Production	Lactate Production	Glucose Utilization	
-	10	-	255 ± 9	144 ± 6	116 ± 4	8 ± 2*		(4)
-	10	+	213 ± 16	120 ± 8	90 ± 5	5 ± 2		(3)
5.5	10	-	178 ± 11	147 ± 15	29 ± 3	31 ± 1	15 ± 7	(3)
5.5	10	+	219 ± 20	166 ± 19	61 ± 8	27 ± 6*	78 ± 10	(4)

Hearts from fed rats were perfused with MKHM containing glucose, DL-3-hydroxybutyrate (DL-3-HB), and insulin as shown in the Table. Values are mean $\pm$ S.E.M. μmoles.g dry wt.$^{-1}$ h^{-1} and the numbers of observations are in parentheses.

*Three observations only.

of pyruvate dehydrogenase. Garland, Newsholme and Randle (1964) observed that DL-3-hydroxybutyrate stimulated lactate production in the absence of insulin and glucose and inhibited the metabolism of pyruvate by pathways other than the conversion to lactate. The source of the pyruvate and lactate need not be glucose or glycogen. Ottaway and Sarkar (1958) found that the amount of lactate produced by hearts perfused with acetoacetate was greater than could be accounted for by changes in the glycogen content. Although it is possible (Seaman, 1957; Davies, 1958) that acetoacetate or D-3-hydroxybutyrate could be the source of the lactate as proposed by Ottaway and Sarkar there is also the possibility that the lactate is derived from amino-acids released by proteolysis. This could explain how after an hours perfusion the lactate production was found in two experiments in this work to be greater than could be expected from the rate of glucose utilization.

The rates of utilization and oxidation of D-3-hydroxybutyrate rate and of the production of acetoacetate in the conditions of the experiments described in this chapter are summarised in Table 44. It is notable that after 60 minutes the rates of oxidation of D-3-hydroxybutyrate are similar regardless of the presence or absence of glucose or insulin and the rate of utilization of the ketone body is unaffected by insulin or the combined presence of glucose and insulin but is reduced when glucose alone is present. The reduction in utilization but not of the oxidation of D-3-hydroxybutyrate is explained by the relatively larger fall in production of acetoacetate which is also reduced in the presence of glucose and insulin but not sufficiently to have a significant effect on the rate of D-3-hydroxybutyrate oxidation.

If it is assumed that the oxygen consumption of the non-working perfused rat heart is not affected by the nature of the substrates

that are used and is approximately 1600μmoles.g dry wt.$^{-1}h^{-1}$, the metabolism of 5mM-D-3-hydroxybutyrate could account for 40% of the oxygen consumption when it is the only substrate and a lower percentage in the presence of glucose. In the absence of insulin the inhibition of glucose utilization and of pyruvate oxidation is such that when the two substrates are both present the metabolism of the ketone body can barely substitute for the reduced supply of energy from the metabolism of glucose. In the presence of insulin the two substrates together provide less energy than does glucose when it is the only substrate. Although these conclusions are based on the assumption of a constant energy requirement, they do suggest that D-3-hydroxybutyrate is a poor substrate for the non-working perfused heart.

Williamson and Krebs (1961) in one experiment using 2mM-D-3-hydroxybutyrate concluded that the ketone body met 73% of the energy requirements of the heart in the first 15 minutes of measurement but only 28% an hour later when the concentration of D-3-hydroxybutyrate had fallen to 0.7mM. Although comparisons cannot fairly be made with the results of one experiment the possibility is suggested that L-3-hydroxybutyrate might affect the utilization of D-3-hydroxybutyrate or the metabolism of glucose. Wieland et al. (1971) found that L-3-hydroxybutyrate did not affect glucose utilization. Wieland et al. who measured the utilization of D-3-hydroxybutyrate and the production of acetoacetate in hearts perfused with glucose insulin reported rates approximately double those found in this here. Their results are equivalent to a utilization of D-3-hydroxybutyrate of 357μmoles.g dry wt.$^{-1}h^{-1}$ and a production of acetoacetate of 82μmoles.g dry wt.$^{-1}h^{-1}$ or a conversion of 23% of the D-3-hydroxybutyrate consumed to acetoacetate compared with 16% in this work. If the oxygen consumption of the hearts in the work of Wieland et al. (1971) is assumed to have

been 1600μmoles.g dry wt.$^{-1}h^{-1}$ the metabolism of D-3-hydroxybutyrate in their experiments would account for 80% of it and so indicate that the ketone body is a good energy-source. However there is some evidence that the oxygen consumption in these experiments was unusually high. When glucose was the only substrate the rate of glucose utilization in the absence of insulin during 20 minutes of perfusion was equivalent to 420μmoles.g dry wt.$^{-1}h^{-1}$ of which 64μmoles.g dry wt.$^{-1}h^{-1}$ could be accounted for by the production of lactate which is a relatively very low proportion (Fig. 14). Since no change in glycogen content would be expected (Randle et al., 1964), it appears that sufficient glucose is oxidised to account for 2272μmoles.g dry wt.$^{-1}h^{-1}$ and because insulin was absent it might be assumed that glucose alone was insufficient to meet the energy requirements or oxygen consumption. When 3-hydroxybutyrate and glucose were both present, their metabolism according to Wieland et al. could account for a potential rate of oxygen consumption of 2314μmoles.g dry wt.$^{-1}h^{-1}$. No experiments were made without glucose or with insulin so that although Wieland et al. agree with this work about the proportion of D-3-hydroxybutyrate utilization that is accounted for by acetoacetate production there are no independent tests of the observation that the proportion is higher particularly in the absence of glucose.

The reasons for the variations in the rate of acetoacetate production are a matter for speculation. Although the concentrations of D-3-hydroxybutyrate and acetoacetate become constant in the perfusate, neither the concentrations or their ratio are necessarily the same as those in the mitochondria because their concentration gradients between the perfusate and the mitochondria might easily differ. However it is reasonable to suppose that differences in the ratio of concentrations in the perfusate might reflect differences

in the ratio within the mitochondria and therefore in the ratio of the concentrations of NAD^+ and NADH. If so, a relative low concentration of acetoacetate in the perfusate when D-3-hydroxybutyrate oxidation is unchanged could indicate a more reduced state of mitochondria. However it is not clear why the presence of glucose should make the mitochondria more reduced particularly when the effect appears to be greatest when the rate of glucose oxidation is almost zero. Ottaway and McMinn (1979) on the basis of computer simulation studies have proposed that the tricarboxylic acid cycle is unable to operate using acetoacetate alone. Provision of a second source of acetylCoA would permit the utilization of acetoacetate. The proposal that is extended by Ottaway, McClelan and Saunderson (1981) does not agree with the observations described in this chapter when acetoacetate is derived from D-3-hydroxybutyrate.

There is some support for the conclusion that while it is a strong inhibitor of glucose utilization D-3-hydroxybutyrate is a poor source of energy. Acetoacetate is used in vivo by peripheral tissue more readily than D-3-hydroxybutyrate (Ruderman, Houghton and Hems, 1971; Owen and Reichard, 1971) and in prolonged fasting in man skeletal muscle releases D-3-hydroxybutyrate that is produced by reduction of acetoacetate. With a working heart Taegtmeyer et al. (1980) found that neither D-3-hydroxybutyrate nor acetoacetate could alone or together support the mechanical activity of the heart but could do so in the presence of glucose. They concluded that the rate of acetoacetate utilization found by Williamson and Krebs (1961) which could account for 73% of the oxygen consumption was the maximum rate attainable by rat cardiac muscle. Their work and that reported here suggests that the idea that ketone bodies are good or preferred sources of energy is questionable.

CHAPTER 19

Summary

19.1 Effect of DL-3-Hydroxybutyrate on Glucose Utilization in Hearts from Fed Animals

DL-3-Hydroxybutyrate inhibits glucose utilization in hearts from fed animals whether insulin is present or not. In the absence of insulin the inhibition increases with the concentration of DL-3-hydroxybutyrate up to 5mM but 10mM-DL-3-hydroxybutyrate does not consistently increase the inhibition. Insulin can oppose the inhibition at low concentrations (0.2 and 1mM) of the ketone body but not at high (2.5, 5 and 10mM). In the presence of insulin the inhibition intensifies during the first 60 minutes of perfusion and is constant thereafter. The intensification is not seen when DL-3-hydroxybutyrate is introduced to the perfusate after 60 minutes perfusion with glucose and insulin alone.

19.2 Effect of DL-3-Hydroxybutyrate on Glucose Utilization in Hearts from Fasted Animals

DL-3-Hydroxybutyrate (10mM) completely inhibits glucose utilization by hearts from fasted animals perfused without insulin but when insulin is present the inhibition of glucose utilization by the ketone body is similar to that seen in hearts from fed animals.

19.3 D-3-Hydroxybutyrate and Glucose Metabolism in Hearts from Fed Animals

In the presence of 10mM-DL-3-hydroxybutyrate a lower proportion of glucose utilized by hearts perfused with insulin is converted to lactate then when glucose is the only substrate, whereas in the absence of insulin a greater proportion of the glucose consumed can

be accounted for by lactate production. D-3-Hydroxybutyrate oxidation is unaffected by the presence of glucose or insulin but acetoacetate production is reduced in the presence of glucose. D-3-Hydroxybutyrate alone cannot meet the energy needs of the heart and in the presence of insulin scarcely substitutes for the energy lost through the inhibition of glucose utilization.

GENERAL DISCUSSION

The work that is presented in this thesis had general and specific objectives. The more general issues were those of the usefulness of the isolated rat heart as an experimental preparation and of the system of perfusion with balanced infusion and withdrawal for studying it. In Chapter 5 the variability in the reported rates of glucose utilization by the non-working perfused rat heart was discussed in details. Some of this variation may reflect changes in utilization with the time of perfusion but some must also reflect differences in experimental technique.

At the start of this project it was assumed that perfused hearts in the absence of insulin used glucose with low rates of lactate production. In general as was shown in Fig. 14 high rates of glucose utilization are associated with high rates of lactate production and reductions in these rates have been attributed to improvement in techniques (Neely et al., 1967b). The results obtained using Millipore filters seemed satisfactory (Chapter 8). However when later in the project it proved necessary to use paper filters for experiments in which the perfusate contained albumin, the reduction in the rate of glucose utilization showed the inadequacy of the original assumption. This experience shows the extent to which the performance of the isolated heart can be affected by the conditions of perfusion. It is unfortunate that the nature of the filter is rarely referred to in publications. However there is some evidence that paper filters are not associated with the increased permeability of cardiac cell that occurs in hearts perfused without protein using glass filter (Fisher and Gilbert,1970a). If permeability is affected it would be reasonable to expect more consistent results from measurements made when the rate of permeation is not a limiting factor which would explain why

the choice of filter did not affect the rate of glucose utilization in the presence of insulin. It seems likely that the general replacement of the Soxhlet filter used by Bleehen and Fisher (1954) with sintered glass filters was mistaken and the more recent use of Millipore filters has not been a complete remedy. While the perfused rat heart continues to be a popular experimental preparation a study of the effects of filters on the properties of preparation and of their causes is needed. As shown earlier there are many differences in the techniques used to perfuse hearts but no systematic investigation of their effects has been made.

Although the demonstration of an effect of technique on the properties of the perfused rat heart casts some doubt on its usefulness, more encouraging evidence was also obtained. Following the time-course of metabolism in long experiments has established that under most conditions studied there are periods when the rates of metabolism at least of glucose and D-3-hydroxybutyrate are constant. When rates were inconstant it was usually early in the experiment so that the use of periods of preperfusion is important when closed-circuit system are used. Short experiments without preperfusion are least trustworthy. It seems reasonable to assume that constant metabolic rates indicate a state of stability and that metabolic interrelationships can be best investigated in that state.

The system of perfusion with balanced infusion and withdrawal showed several advantages over the closed-circuit system. It is useful in enabling a rate of utilization or production to be associated with a well-defined concentration of a metabolite. This is a particular advantage when the rate of the process is concentration-dependent. The abilities to allow multiple steady-states to be established and to allow competing substrates to be used at very different concentrations

are also clear advantages compared with closed-circuit system. The technique also allows rates of metabolism to be followed when the concentration of the metabolite is changing but while this is useful when the rates are independent of the changes in concentration it is less helpful in conditions where the rates are likely to vary with changes in concentration.

The disadvantages of the technique as it was used in this work are mainly in the number of analyses that it requires. However where experience has established that steady states are established analysis could be limited to fractions collected in that period. But if the time-course of metabolism is of particular interest it would be helpful to make more frequent analyses when the composition of the perfusate is changing than was done in this work. The large number of analyses reflects the prolonged perfusions which allows periods of metabolic stability to be more easily detected but prevents any useful analysis of the heart itself from being made. With experience of the conditions when steady-states exist the heart could be taken for analysis in that period. Probably the greatest disadvantage of the technique is in measuring low rates of utilization when the concentrations of the substrate in the infusate and perfusate are high and similar. This was seen most clearly in the inability to detect glucose utilization by hearts from fasted animals perfused with glucose and DL-3-hydroxybutyrate in the absence of insulin. This difficulty could only be avoided by using lower infusion rates to increase the difference in concentration between infusate and perfusate. This would increase the half-time for the approach to a steady-state and in this work the longest half-times of between 15 and 20 minutes could not be much increased and the volume of circulated perfusate could not be much reduced to shorten the half-time.

It is possible that the rate and therefore the concentration difference to be measured would be bigger under any conditions of perfusion if the heart was required to do external work. The apparatus is easy to adapt for perfusion of the working heart and would not need the large volumes of the perfusate used for example by Taegtmeyer et al. (1980). However low rates of utilization at high concentrations of substrate are difficult to measure in any system.

A working heart system with balanced infusion and withdrawal would be useful to test the observations of Taegtmeyer et al. (1980) which lead them to suggest that the non-working heart has little physiological relevance. They based this suggestion on the comparison of their results with those of Williamson and Krebs (1961) who concluded that acetoacetate was a good substrate for the non-working heart. Since in this work D-3-hydroxybutyrate was found to be a poor substrate the question is not settled. It would be useful to extend the experiments reported here to the study of acetoacetate and particularly of combinations of acetoacetate and D-3-hydroxybutyrate as substrates for the non-working and for the working heart perfused in steady-state conditions.

Another observation of Taegtmeyer et al. (1980) was that insulin only promoted glucose utilization by the working heart in a limited set of conditions. They used Millipore filters so their conclusions that insulin is relatively unimportant in stimulating glucose utilization in comparison with the effect of work itself needs to be re-examined. Applied to the non-working heart the technique of perfusion with balanced infusion and withdrawal has proved useful in giving new knowledge of insulin sensitivity as well as of the regulation of glucose metabolism by a competing substrate. Applied to the working heart its potential could be developed further.

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