

Department of Physiology,
Anatomy and Genetics

The interaction between ketone bodies and carbohydrate in human metabolism

Doctor of Philosophy Thesis

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Thesis Abstract: The human brain cannot oxidise fat, so ketone bodies (KBs) are produced in the liver from fat to provide energy in starvation, the brain having a high capacity to oxidise KBs. KB levels rise as insulin falls, with reduced glucose availability, thereby providing a signal to conserve carbohydrate and to switch energy use to fat. **Methods**: Here, studies were conducted into the relationship between KB and carbohydrate metabolism using exercise and controlled diets to alter substrate availability. A ketone ester (KE) drink was used to raise D-β-hydroxybutyrate (βHB, $\pm^{13}$C-label) levels, at rest and during exercise. Glucose disposal, insulin and other markers of carbohydrate and fat metabolism, KB oxidation and glycogen synthesis rates were investigated by blood sampling, glucose clamp studies, ^{13}C breath analysis and muscle biopsies. **Results**: βHB oxidation accounted for ≥ 28% of resting VO_2, and increased 3-fold with exercise, but was 24% lower when exercising after a low-carb diet. After glycogen-depleting exercise, endogenous insulin levels rose two-fold, glucose disposal increased 32% and glycogen synthesis increased 50% during a 2-hour hyperglycaemic (10 mM glucose) clamp following the KE drink. In overnight fasted healthy controls and type-2 diabetic participants (T2DM), KE increased insulin and lowered glucose concentrations vs. control (zero calorie) drink. There was an increase in glucose disposal during euglycaemic (5 mM glucose) clamping in healthy controls, but no difference in T2DM patients. KE lowered circulating free fatty acids and peripheral lipolysis in T2DM patients. **Conclusion**: βHB can replace glucose as a fuel, increasing glucose disposal and glycogen synthesis, and increase insulin in health and disease. When carbohydrate is low, muscle βHB oxidation is downregulated to spare KBs for the brain, effects directly or indirectly conserving carbohydrate and protecting the brain. This work is the first to show the metabolic effects of an exogenous (dietary) ketone ester in human, particularly on carbohydrate metabolism, and the effect of substrate availability on ketone oxidation.

Declaration

The work presented in this thesis is my own including:

Study design

Ethics Applications

Participant recruitment

Experimental studies

Data analysis

Data presentation

Unless acknowledged below:

Dr Pete Cox assisted with study design of the work presented in chapters 3 and 4 and the tracer development and experimental studies presented in chapter 3.

Mr Huw Stradling (laboratory assistant) assisted with the conduct of studies presented in chapter 4.

Miss Catriona McNeil conducted the ^{13}C mass spectrometry analysis presented in chapter 3.

Dr Matthew Frise assisted with the experimental studies presented in chapter 5, as the blinded investigator adjusting the euglycaemic clamp infusions.

Mrs Sandy Humphreys conducted the analysis of substrates for the work presented in chapter 5.

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Table of Contents

1 Introduction 1

1.1 Meeting the energy requirements of the human brain 1

1.2 Discovery of ketone bodies 5

1.3 The metabolic characteristics of ketone bodies 6

1.3.1 Ketone bodies 6

1.3.2 Synthesis 9

1.3.3 Oxidation 12

1.3.4 Energetics and efficiency 13

1.3 Potential experimental and therapeutic uses of ketone bodies 18

1.3.1 Fuel substrate 18

1.3.2 Carbohydrate sparing 19

1.3.3 Diabetes, insulin resistance and the metabolic effects of ketone bodies 21

1.4 Ketone ester 24

1.5 Outline programme of work 26

2 General Methods 28

2.1 Ethics 28

2.1.1 Ethical approval 28

2.1.2 Consent 28

2.2 Participants and screening 28

2.2.1 Inclusion criteria – studies 1 and 2 29

2.2.2 Exclusion criteria – studies 1 and 2 29

2.2.3 Inclusion criteria – study 3 29

2.2.4 Exclusion criteria – study 3 30

2.3 Anthropometry 30

2.4 Cardiopulmonary exercise testing 30

2.4.1 Indirect calorimetry 30

2.4.2 $\dot{V}O_2$max test protocol 31

2.4.3 Calibration of indirect calorimetry methods 31

2.4.4 Determination of peak power output (PPO) and maximal oxygen uptake ($\dot{V}O_{2max}$) 31

2.5 Exercise interval protocol to deplete glycogen 32

2.6 Fasting and dietary manipulation 33

2.6.1 High-carbohydrate diet 33

2.6.1 Low-carbohydrate diet 33

2.7 Study drinks 34

2.7.1 Ketone ester drinks 34

2.7.2 Control drinks 34

2.8 Blood handling and analysis 34

2.8.1 Blood sampling 34

2.8.2 Blood sample storage. 35

2.8.3 Analysis of blood samples 35

2.9 Muscle biopsies and storage 36

2.9.1 Muscle biopsy 36

2.9.2 Muscle glycogen analysis 37

2.10 Intravenous infusions 37

2.11 Glucose clamp procedures 38

2.11.1 Hyperglycaemic clamp 38

2.11.2 Euglycaemic clamp 39

3 Ketone oxidation 40

Abstract 40

3.1 Introduction 41

3.1.1 Ketone oxidation during exercise 41

3.1.2 Ketone oxidation and exercise intensity 44

3.1.3 Ketone oxidation in competition with other energy substrates 44

3.2 Methods 46

3.2.1 Participants 46

3.2.2 Baseline visit 46

3.2.3 Collection of gas samples 46

3.2.4 Drink preparation and ^{13}C-labelling 47

3.2.5 Blood sampling and analysis 48

3.2.6 Glycogen depletion 48

3.2.7 Controlled diet 48

3.2.8 Lipid infusion 49

3.2.9 ^{13}C label calculations 49

3.2.10 Mass spectrometry analysis 50

3.2.11 Statistics 51

3.2.12 Study design 51

3.3 Results 52

3.3.1 Diet composition 52

3.3.2 Exercise 53

3.3.3 Blood metabolites 55

3.3.4 Ketone concentration 58

3.3.5 ^{13}C tracer analysis 60

3.4 Discussion 62

3.4.1 Diets 62

3.4.2 Exercise 63

3.4.3 Blood metabolites 63

3.4.4 Ketone kinetics 64

3.4.5 Exercise intensity and ketone oxidation 67

3.4.6 Carbohydrate availability and ketone oxidation 69

3.5 Conclusion 73

4 Ketones and glycogen recovery following exercise 74

Abstract 74

4.1 Introduction 75

4.1.1 Glycogen and Ketones 75

4.1.2 Carbohydrate delivery 76

4.2 Methods 78

4.2.1 Participants 78

4.2.2 Baseline visit 78

4.2.3 Glycogen depletion protocol 79

4.2.4 Ketone and control drinks 79

4.2.5 Blood sampling and analysis 79

4.2.6 Hyperglycaemic clamp and sham clamp 79

4.2.7 Muscle biopsy 81

4.2.8 Glycogen analysis 81

4.2.9 Statistics 81

4.2.10 Study design 81

4.3 Results 82

4.3.1 Glycogen depletion protocol 82

4.3.2 Clamp conditions 84

4.3.3 Lactate 85

4.3.4 Insulin 86

4.3.5 Glucose disposal 86

4.3.6 Glycogen 87

4.4 Discussion 90

4.4.1 Experimental protocol 90

4.4.2 Glucose disposal and endogenous insulin 91

4.4.3 Ketones and insulin release – adaptive significance 94

4.4.4 Changes in other metabolites 95

4.4.5 Ketone bodies and glycogen synthesis 96

4.5 Conclusion 99

5 Ketones and glucose disposal in type 2 diabetes 101

Abstract 101

5.1 Introduction 102

5.2 Methods 103

5.2.1 Participants 103

5.2.2 Ketone and control drinks 104

5.2.3 Blood sampling and analysis 105

5.2.4 Euglycaemic clamping 105

5.2.5 Statistics 106

5.2.6 Study design 107

5.3 Results 108

5.3.1 Clamp conditions 108

5.3.2 Metabolite concentrations 109

5.3.3 Initial effects of ketones on insulin and glucose 110

5.3.4 Insulin and glucose disposal 111

5.3.5 Lactate handling 112

5.3.6 Insulin sensitivity 112

5.4 Discussion 113

5.4.1. Metabolite effects 113

5.4.2 Insulin concentration and glucose disposal 115

5.4.3 Early effects of acute ketosis 116

5.5 Conclusion 117

6. General Discussion 118

6.1 Summary of key novel findings: 119

6.2 The brain and carbohydrate 120

6.3 Ketones are an alternative fuel for the brain and skeletal muscle 120

6.4 Progressive starvation alters tissue oxidation of ketone bodies to protect the brain 124

6.5 Ketones are a signal to preserve carbohydrate 125

6.6 Therapeutic implications of ketone bodies in diabetes 126

6.7 Conclusion 128

References 129

1 Introduction

1.1 Meeting the energy requirements of the human brain

The fundamental importance of ketone bodies is to supply energy to the brain during starvation. The brain has a large and continuous energy demand and is not able to oxidise fat to any significant extent. The brain is an organ vital to vertebrate survival. Vertebrates face the constant challenge of an uncertain energy supply, driving the need to store energy. In the face of this selection pressure, ketone bodies have emerged as an adaptation to link the body's vast energy store and the brain's unceasing demand for energy.

The human brain demands a large and continuous supply of energy. In humans approximately 20% of oxygen consumption and 25% of glucose oxidation (100-150 g/day) is accounted for by the brain, which represents just 2% of the body mass (1). This high, and sustained, requirement for glucose is particularly striking in the context of the limited body stores of carbohydrate, in the form of the polymer glycogen. The body stores approximately 500 times more energy as fat than as carbohydrate (Figure 1.1) (2).

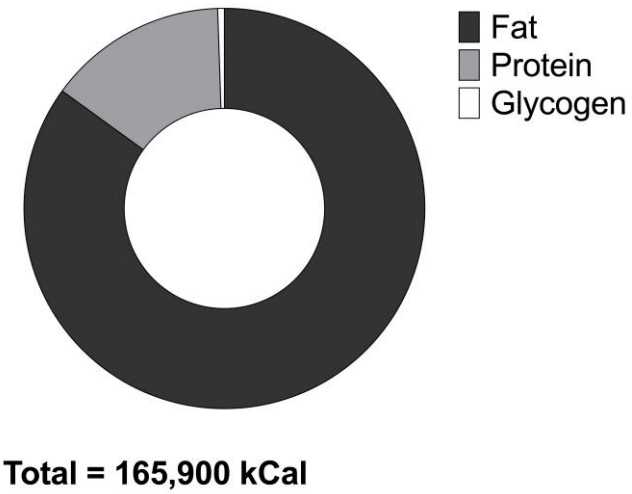


Figure 1.1 – Absolute energy storage (70 kg man - from Cahill (2))

The reason for the overwhelming predominance of fat as the human energy store is its high energy density. The maximum theoretical energy density of fat is 9.3 kcal/g while that of carbohydrate is 4.0 kcal/g (3). Furthermore, glycogen is stored with 1-2 g of water, in a concentrated aqueous solution (4), whereas fat is stored anhydrously, further exacerbating the difference in efficiency of storage by weight. Consequently, across many phyla, mobile organisms (land vertebrates, migratory birds and insects), or parts of organisms (e.g. seeds, nuts and pollen) store energy as fat, in contrast to sessile organisms (e.g. trees), which store energy as carbohydrate.

Of the relatively limited human stores of carbohydrate, in the form of glycogen, approximately 75% is within skeletal muscle. Muscle lacks the enzyme glucose-6 phosphatase. Glucose-6-phosphatase converts glucose-6-phosphate, which cannot cross the cell membrane, into glucose, which can. Therefore, once deposited as glycogen, the glucose stored in skeletal muscle is confined to this tissue. The remaining 25% of stored glucose is accounted for by the approximately 100-150 g (400 – 600 kcal) of liver glycogen that may be mobilised for immediate glucose requirements throughout the body. This quantity of carbohydrate could supply the requirements of the adult brain for approximately 24 hours (5, 6). Indeed Cahill demonstrated in the 1970s that the liver effectively makes no net contribution to glucose supply beyond 2-3 days (7).

It is therefore evident that the brain needs an alternative energy substrate to glucose (or lactate during high-intensity exercise (8)). When glucose reserves are exhausted, *new* glucose must be synthesised from protein. On initial fasting, this requires 75 g of protein/day. With increasing duration of fasting, insulin levels fall causing increased peripheral lipolysis (9). Plentiful availability of non-esterified fatty acids and suppressed

insulin levels lead to increased synthesis of ketone bodies in the liver (*ketogenesis*) (10). As ketogenesis increases, ketone bodies progressively replace glucose as an energy substrate for the brain, and the amount of protein required for *de novo* synthesis of glucose (*gluconeogenesis*) falls to 20 g/day (2, 3). This adaptation *extends* the survivable duration of human starvation from 10 to 57-73 days (2, 11, 12).

Ketone bodies provide the link between fat and the brain's ceaseless energy demands. The liver acts as a *transducer*, converting inaccessible energy, in circulating fats, into the useable substrates: ketone bodies. Indeed in prolonged fasting, ketone oxidation accounts for 60 % of the brain's oxygen consumption (13).

"One can speculate that keto acids exist solely as nature's way of providing a water-soluble derivative of fat that penetrates the blood-brain barrier". Owen et al., 1967 (13).

Ketone oxidation reduces the body's daily glucose requirement. Beta-oxidation of long chain fatty acids, yields the 2-carbon moieties that are used to synthesise ketone bodies, but the liver also harnesses bond energy from beta-oxidation to drive all those hepatic processes, collectively termed *gluconeogenesis*, which convert lactate, alanine and glycerol to glucose (3, 7). This further reduces the amount of 'new glucose' that must be synthesised. Indeed it has been shown that, as the brain adapts to use ketones, the level of hepatic gluconeogenesis falls and >75% of gluconeogenesis is accounted for by lactate, pyruvate and glycerol (14). That is to say, there is a significant decrease in the dependence upon gluconeogenic precursors derived from protein (Figure 1.2).

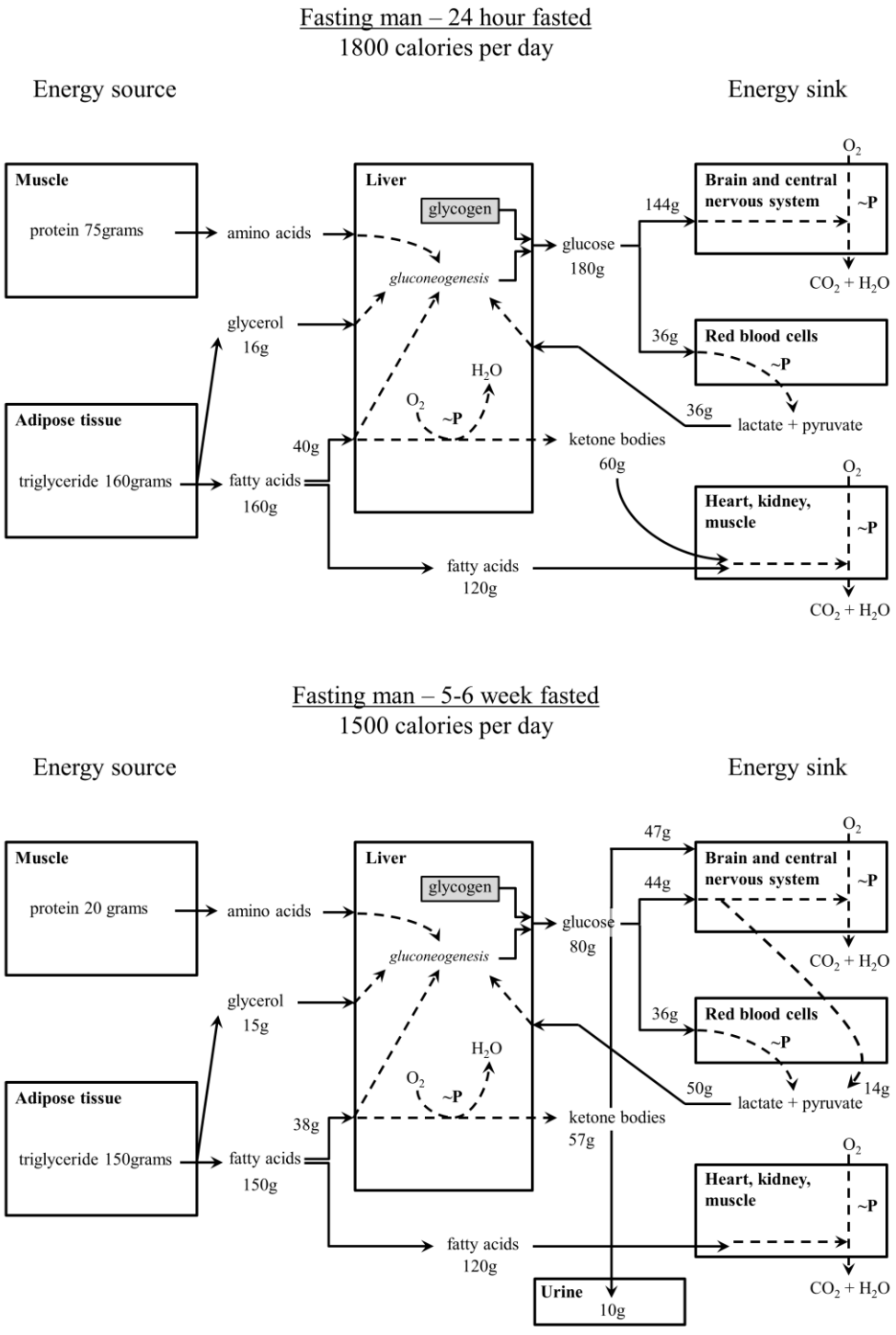


Figure 1.2 – Diagram representing the adaptations to prolonged starvation. Increasing consumption of ketone bodies by the central nervous system reduces the requirement to consume the body's protein for gluconeogenesis. After Cahill (1970) (2).

A further survival advantage of this preservation of body protein concerns the reduction of nitrogen excretion. The provision of ketone bodies, as an oxidative fuel to brain, skeletal muscle, heart and kidneys greatly reduces the catabolism of protein that would otherwise be essential to provide gluconeogenic substrate. The consequent reduction in urea excretion, the major osmolyte in urine, reduces the obligate urine requirement to only 200 ml per day, thus also conserving body water (2).

Ketone bodies are the metabolic adaptation to link the brain with the body's energy depot, adipose tissue, in times of dietary carbohydrate restriction. They are synthesised constitutively in the liver, when insulin is low, peripheral lipolysis is active and circulating non-esterified fatty acids abundant. Ketone bodies are therefore also ideally placed to signal *carbohydrate deficit*, orchestrating a series of metabolic shifts that downregulate glucose oxidation and upregulate glucose storage and oxidation of fat. The net effect of ketones is to displace glucose in supplying the brain's rapacious energy requirements and to reduce whole-body glucose consumption, shifting energy use to the more abundant fat stores. The consequent reduction in requirement for gluconeogenic precursors from muscle greatly prolongs the time before body protein is fatally denuded. Ketone bodies have evolved to mitigate the harm of prolonged carbohydrate restriction; therefore, their metabolism is intimately enmeshed with that of glucose.

1.2 Discovery of ketone bodies

Through identification in the urine of patients in diabetic coma (15, 16), acetoacetate's role in human pathophysiology was recognised first in 1889. Ketone bodies, in concentrations exceeding 20 mM, were soon known to be responsible for the diabetic ketoacidosis, associated with insulin deficiency (17). In 1906, the German pathologist Bernhard Naunyn suggested that ketone bodies were a by-product of incomplete combustion of fat. After this

inauspicious beginning, 50 years passed before it was shown that ketone bodies were synthesised by the liver as an adaptation to fasting, to be oxidised in a variety of extrahepatic tissues (18, 19). Benedict, in his studies of a Maltese man ('Mr L') fasting for 31 days, observed a progressive rise in D-β-hydroxybutyrate to plateau at ~6 mM (20). Mr L was a member of the Metaphysical Society of Malta (21), whose habit was to periodically fast for many weeks at a time. Then, in 1970, George Cahill described the essential function of ketone bodies in conserving muscle protein and extending survival five-fold during total starvation (2).

1.3 The metabolic characteristics of ketone bodies.

1.3.1 Ketone bodies

The term 'ketone bodies' is used to describe acetoacetate, β-hydroxybutyrate and acetone (Figure 1.3). In fact, β-hydroxybutyrate is a carboxylic acid rather than a true ketone and acetone, produced by the spontaneous decarboxylation of acetoacetate, is rapidly volatilised and excreted in the lung and is therefore of little biological significance. In this thesis the collective term ketone bodies will therefore be used to refer to acetoacetate and β-hydroxybutyrate. Acetoacetate is non-chiral, while β-hydroxybutyrate exists in two enantiomers, a D- and an L- form. Only D-β-hydroxybutyrate is used as an energy substrate and further reference to β-hydroxybutyrate (βHB) will only be to the D- form.

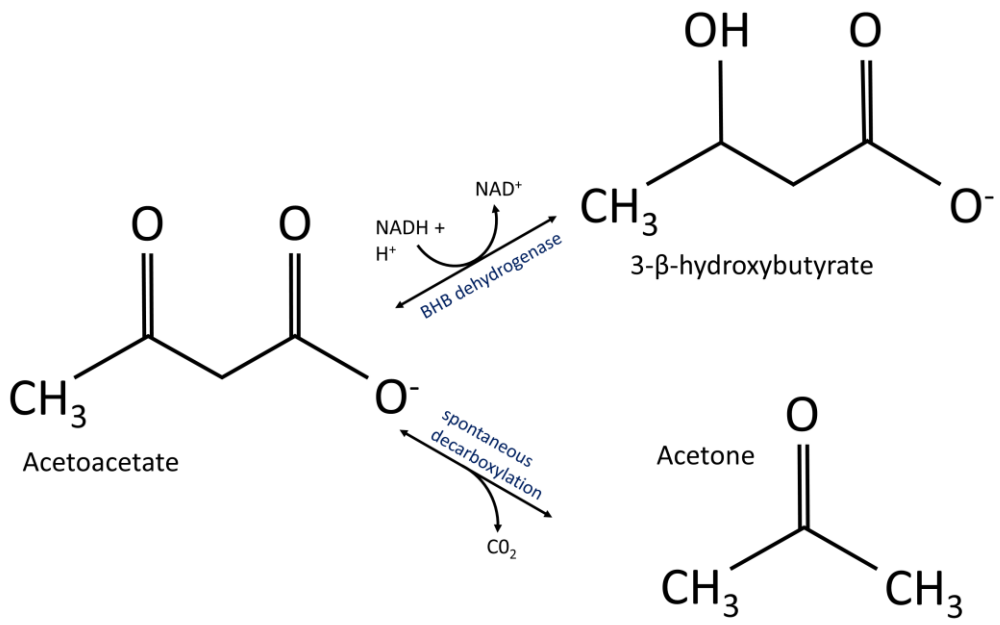


Figure 1.3 – Interrelationships between the ketone bodies.

Ketone bodies are small, water-soluble 4-carbon fuel substrates that are transported across cell membranes via the ubiquitous monocarboxylate transporters (MCTs), which are fundamental to the transport of lactate and pyruvate as well as the ketone bodies (22, 23).

The typical concentration of ketone bodies in fed humans is 0.05-0.1 mM (24), but levels increase rapidly (Figure 1.4) with fasting (2, 25), carbohydrate restriction (26), increased peripheral lipolysis, for example in the neonate (27) or following prolonged exercise (28), and if there is a pathological inability to access abundant plasma glucose, as in Type 1 diabetes, (29, 30) or deficiency of 3-oxoacid-CoA transferase (para. 1.2.3) (31). Chronic alcoholism, combined with malnutrition and decreased glycogen stores may lead to a fall in insulin/glucagon ratio and increase in NADH/NAD^+ ratio secondary to the metabolism of ethanol via alcohol dehydrogenase (Figure 1.5). High-levels of circulating non-esterified fatty acids, carbohydrate depletion and low insulin levels associated with alcoholic malnutrition all contribute to a rise in βHB and acetoacetate (32). The ratios of

βHB/acetoacetate and lactate/pyruvate are also raised secondary to their coupling to NADH/NAD^+ (33-35).

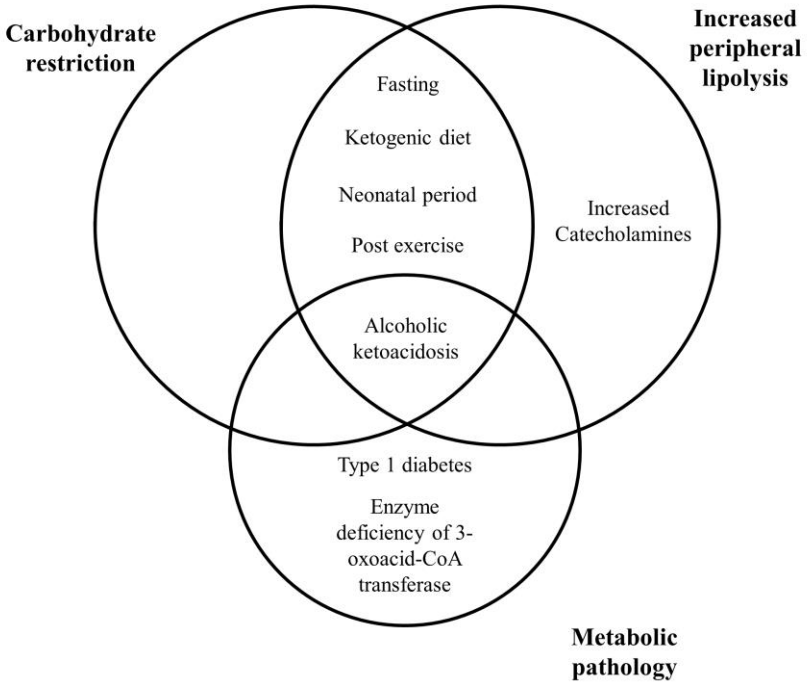


Figure 1.4 – Physiological and pathological causes of ketosis

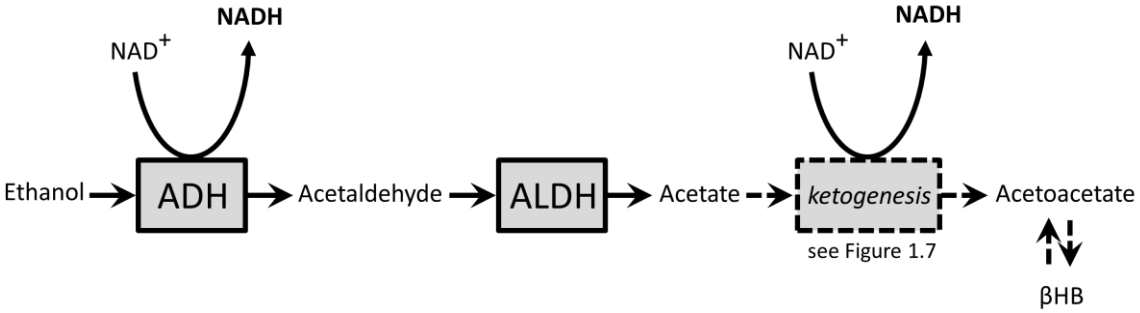


Figure 1.5 – The metabolism of ethanol, illustrating how excess ethanol leads to an increase in the NADH/NAD^+ couple. ADH = alcohol dehydrogenase; ALDH = acetaldehyde dehydrogenase.

1.3.2 Synthesis

The synthesis of ketone bodies, termed *ketogenesis*, is inversely linked to the availability of carbohydrate. This relationship underscores the metabolic *niche* occupied by ketone bodies as a replacement fuel for the central nervous system under conditions of starvation and depletion of carbohydrate reserves. The reciprocal regulation with carbohydrate can be divided into *extra-hepatic* and *intra-hepatic* mechanisms.

Extra-hepatic regulation is based upon the insulin/glucagon ratio. When carbohydrate availability is low, the insulin/glucagon ratio falls leading to increased peripheral lipolysis and a rise in circulating non-esterified fatty acids, the substrate for ketogenesis.

Intra-hepatic regulation is based on the liver's capacity for both synthesis and beta-oxidation of fatty acids. There are a number of other metabolic pathways operating within the hepatocyte, which represent opposite directions of carbon flux e.g. glycolysis and gluconeogenesis or glycogenolysis and glycogen synthesis. In such cases there are distinct *control* enzymes in each process (i.e. that occur only in the metabolic pathway of one direction of the carbon flux) which are under opposite, reciprocal control. The result is that futile *cycling* in which flux occurs simultaneously in both directions cannot occur. In the case of reciprocal regulation of fatty acid synthesis and ketogenesis the key regulatory enzyme is carnitine palmitoyl transferase I (CPT I), an enzyme, located in the outer mitochondrial membrane, involved in the commitment of fatty acyl-CoA to beta-*oxidation* within the mitochondrion. The critical regulator of CPT I is malonyl-CoA, a molecule which is the first commitment step in hepatic fatty acid *synthesis*. Malonyl-CoA represents a carbohydrate *sensor* which, by inhibiting CPT I, downregulates beta-oxidation of fat and ketogenesis (36). In isolated hepatocytes, the processes of lipogenesis and ketogenesis occur reciprocally (Figure 1.6) (37).

Abundant hepatic glucose leads, via glycolysis, to plentiful pyruvate and, through the anaplerotic enzyme pyruvate carboxylase, to the generation of excess oxaloacetate within the mitochondrion. As oxaloacetate (OAA) and acetyl-CoA can form citrate, mitochondrial citrate levels rise and citrate is shuttled into the cytosol. Cytosolic citrate is hydrolysed back into acetyl-CoA and OAA. In the cytosol, acetyl-CoA is carboxylated to malonyl-CoA, committing this molecule to fatty acid synthesis and inhibiting beta-oxidation and ketogenesis (38). Conversely, in times of carbohydrate scarcity and abundant circulating fat, fatty acyl-CoA is bound to carnitine and shuttled across the mitochondrial membrane where it undergoes beta-oxidation to acetyl-CoA. When glucose is scarce, there is no excess in OAA and, rather than forming citrate for oxidation, acetyl-CoA units are combined via the thiolase reaction, to form acetoacetyl-CoA. Acetoacetyl-CoA combines with another acetyl-CoA molecule (HMG-CoA synthase reaction) to form β-hydroxy-3-methylglutaryl-CoA (HMG-CoA). HMG-CoA can be split by HMG-CoA lyase to yield acetoacetate and acetyl-CoA (39). Acetoacetate can be reduced to D-β-hydroxybutyrate. The pathways of ketogenesis are shown in Figure 1.7. The ratio D-β-hydroxybutyrate/acetoacetate is set by the redox potential, and thus is in equilibrium with the $NADH/NAD^+$ ratio.

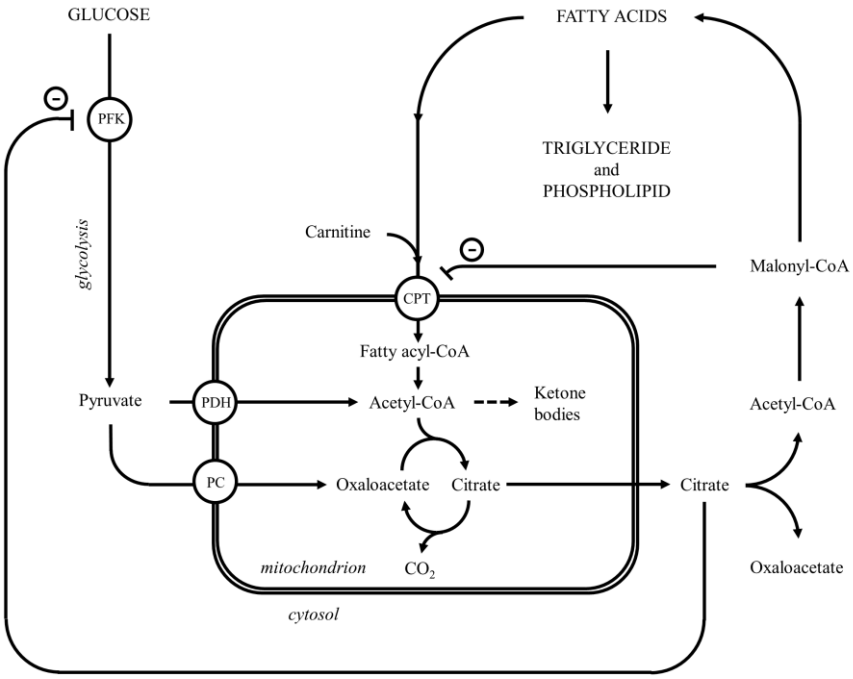


Figure 1.6 – Reciprocal control of lipogenesis and ketogenesis. Abundant glucose availability leads to an increase in acetyl-CoA and the shuttling of citrate into the cytosol. In the cytosol citrate is cleaved and acetyl-CoA is converted to malonyl-CoA, which inhibits mitochondrial uptake of fatty acids. Abundant fatty acids cause increased transport to the mitochondrion and increase production of citrate which is shuttled to the cytosol. As cytosolic fatty acid concentrations are also high, citrate is not cleaved to yield acetyl-CoA and instead accumulates, downregulating glycolysis via inhibition of phosphofructokinase. CPT – carnitine palmitoyl transferase; PDH – pyruvate dehydrogenase; PC – pyruvate carboxylase; CoA-coenzyme A; PFK - phosphofructokinase.

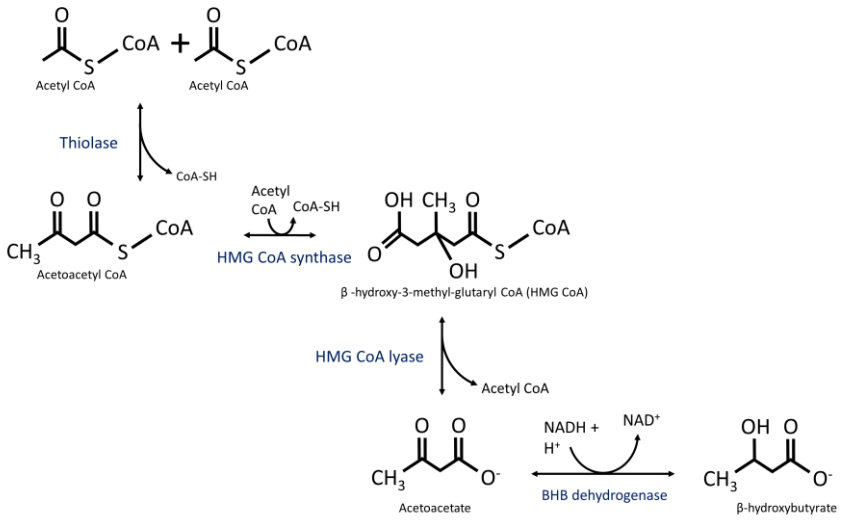


Figure 1.7 – Synthesis of ketone bodies. CoA – coenzyme A; HMG – β-hydroxy-3-methylglutaryl; BHB – β-hydroxybutyrate.

1.3.3 Oxidation

As long ago as 1943, it was suggested that the principal function of ketone bodies was as oxidative substrates in the peripheral tissues (40). A large proportion (35-62%) of ^{14}C in a ^{14}C-labelled infusion of ketone bodies is recovered as respiratory CO_2, which represents 30-50% of the total substrate oxidation in rats and dogs at rest (41, 42). There is, however, also evidence for the incorporation of ketone bodies into fat, as part of the rapid process of lipogenesis in both rodent lactating mammary gland (43) and the developing central nervous system of suckling rats (44-46). The incorporation of ketone bodies into lipid occurs in the cytosol and requires the enzyme *acetoacetyl-CoA synthetase*:

$$\textit{Acetoacetate} + \textit{ATP} + \textit{CoA} \rightarrow \textit{acetoacetyl-CoA} + \textit{AMP} + \textit{pyrophosphate}$$

Oxidation of ketone bodies occurs in the mitochondria (Figure 1.8). βHB is first oxidised to acetoacetate via *β-hydroxybutyrate dehydrogenase*:

$$\beta\textit{HB} + \textit{NAD}^{+} \leftrightarrow \textit{acetoacetate} + \textit{NADH}$$

Acetoacetate is activated by the transfer of a coenzyme A group from succinyl-CoA in the first span of the Krebs cycle (*also termed* the tricarboxylic acid (TCA) *or* citric acid cycle). This reaction is catalysed by *3-oxoacid CoA-transferase*, also called *succinyl-CoA transferase* (SCOT). Acetoacetyl-CoA is then cleaved by the enzyme *thiolase* to produce 2 molecules of acetyl-CoA, the common substrate for terminal oxidation to CO_2 in the Krebs cycle:

$$\textit{Acetoacetate} + \textit{Succinyl-CoA} \rightarrow \textit{Acetoacetyl-CoA} + \textit{succinate}$$

$$\textit{Acetoacetyl-CoA} + \textit{R-CoA} \leftrightarrow \textit{2 acetyl-CoA} + \textit{R}$$

Acetoacetyl-CoA synthetase and succinyl-CoA transferase (SCOT) are present in a variety of extrahepatic tissues. The activity of SCOT is typically 10-fold that of acetoacetyl-CoA synthetase.

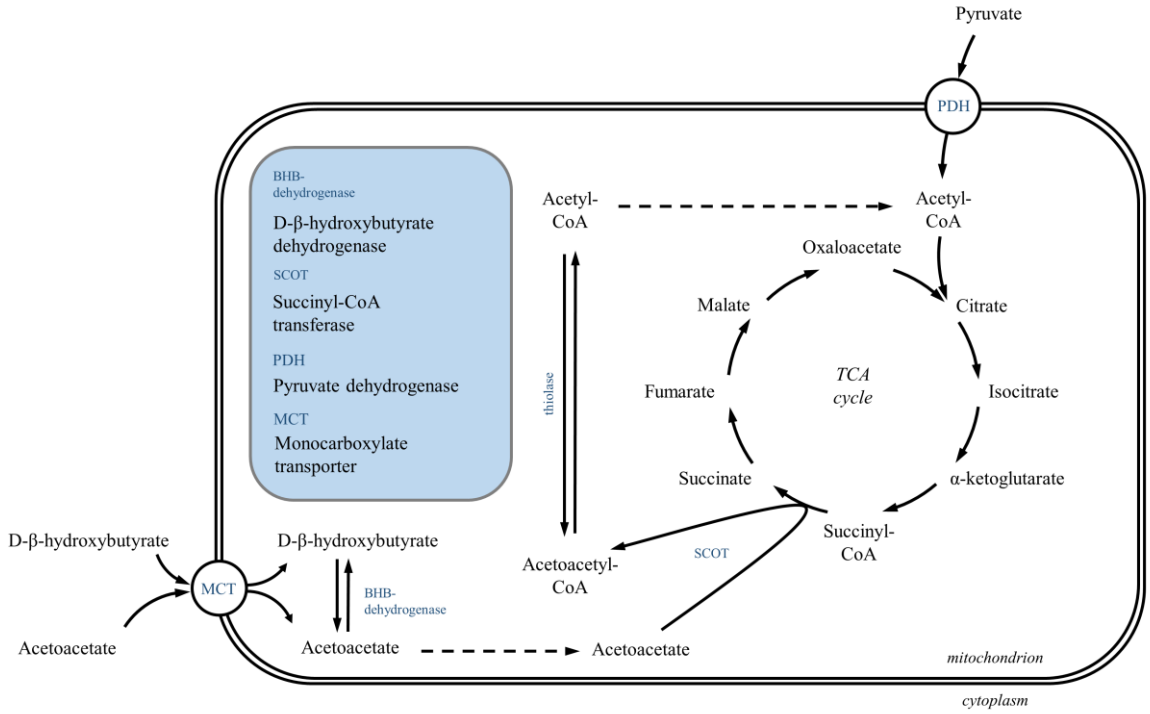


Figure 1.8 – Oxidation of ketone bodies in the mitochondrion.

Succinyl-CoA-3-oxoacid transferase is not present in the hepatocyte and therefore ketone body oxidation, and a futile cycle of synthesis and oxidation, cannot occur.

1.3.4 Energetics and efficiency

The *efficiency* of a metabolic fuel substrate can be measured by a number of parameters (Table 1.1).

E*nergy density* is a measure of the calorific content of a substrate per unit mass. This measure is further complicated by the fact that some substrates can only be stored in association with water, thus increasing the *effective* mass of the stored fuel and decreasing energy density. In

the case of glucose, stored as glycogen, each gram of carbohydrate is stored in association with 1-2 grams of water (4). Fat, on the other hand, is stored anhydrously and so it, and ketone bodies, which are derived from it, do not suffer this loss of efficiency by weight.

Oxygen efficiency is a measure of the calories released per unit quantity of oxygen, when a substrate is fully oxidised. Oxygen efficiency is proportional to the respiratory quotient (RQ).

$$RQ = \text{production of carbon dioxide } (\dot{V}CO_2 \text{ in litres per min}) / \text{uptake of oxygen } (\dot{V}O_2 \text{ in litres per min})$$

This is of interest, because it contributes towards changes in fuel selection during states in which oxygen delivery is limited: exposure to high altitude (47); high-intensity exercise (48, 49); tissue infarction (50) and heart failure (51). It is also reasonable to hypothesise that substrate selection may also be influenced by reduced oxygen availability in pulmonary ventilation-perfusion mismatch and severe anaemia.

The RQ for any substrate can be calculated based on its stoichiometry and its complete oxidation to carbon dioxide (CO_2) and water (52). For a substrate $C_xH_yO_z$ the formula for complete oxidation is:

$$C_xH_yO_z + (x + y/4 - z/2)\ O_2 \rightarrow x\ CO_2 + (y/2)\ H_2O$$

thus oxidation of this substrate gives an RQ:

$$x/(x + y/4 - z/2)$$

Some authors have considered the energy efficiency of substrates in terms of their calorific equivalents for the production of CO_2 (53). This raises intriguing questions when we consider the fact that ventilation is entrained to the excretion of CO_2 (54). One can therefore

consider the *ventilatory efficiency* of the substrate. The oxidation of substrates with a lower RQ may be associated with a reduced work of breathing and even of reduced subjective breathlessness.

Energy substrates differ in terms of their *specific enthalpy* – a term derived from the Greek for the heat (*thalpein*) within (*en*-) describing the amount of heat (*heat of combustion*) within a given amount (mass) of a substance. The SI units are joules per kilogram. The complete oxidation of a substrate can therefore also be described in terms of the change in enthalpy (ΔH) occurring with complete oxidation under equivalent conditions of temperature and pressure (Table 1.1).

This fundamental difference in the *thermodynamic* characteristics of a substrate sets an absolute limit to the quantity of energy that may be captured as adenosine triphosphate (ATP) the universal *currency* of energy metabolism. However, the efficiency of the conversion of energy in covalent bonds in an energy substrate to the high-energy phosphate bonds of ATP or phosphocreatine (PCr) may be limited further by the different pathways active in the complete oxidation of different substrates. The pathways of particular interest, with regard to energetically efficient oxidation are those linking the Krebs cycle and the respiratory electron transport chain (ETC), and those of the ETC itself.

The absolute limit to the energy availability of the ETC is determined by the REDOX span between the NAD couple (at complex I) and the oxygen couple for the reduction of oxygen to form water:

$$NADH_{mito} + 2H^{+} + \tfrac{1}{2}O_2 \rightarrow H_2O + NAD^{+}_{mito}$$

The maximum available energy (ΔG) is determined by the equation:

$$\Delta G' = -nF\Delta E$$

Where n = number of electrons (n=2 as the equation is completed by a pair of electrons); F = 96.485 kJ/mol/V and ΔE is the electrical potential (in volts) between the negative NAD couple and the positive O_2 couple.

The energy flow of the passage of sequential pairs of electrons, carried to the ETC by the reducing *equivalents* NADH and FADH is captured in the *pumping* of protons from the mitochondrial interior to the intermembrane space. The return of these protons down an electrochemical gradient, where it occurs via the F_1ATP_{ase}, leads to the phosphorylation of ADP to regenerate ATP:

$$\boldsymbol{ADP + P_i \rightarrow ATP}$$

On the basis of the equations above, and the regeneration of three molecules of ATP per pair of electrons, the theoretical maximum $\Delta G'_{ATP}$ for the hydrolysis of cytosolic ATP is -59.2 kJ/mol (55). Given that the measured $\Delta G'_{ATP}$ for heart, liver and red cells ranges from -54 to -58 kJ/mol it is evident that oxidative phosphorylation is highly efficient (56).

Ketone bodies have a high heat of combustion (Table 1.1 - normalised to 2C units). In fact, βHB is so reduced (high hydrogen to carbon ratio) that its heat of combustion is 31% higher than that of pyruvate, the product of glycolysis. Within the ETC this additional energy is captured through the further reduction (increased electrical negativity) of the NAD couple at complex I and the further oxidation of the Q/QH_2 couple at complex III. This increase in REDOX span ($\Delta E_{Q/NADH}$) is associated with a higher energy of hydrolysis of ATP.

This is believed to be the explanation for the 25% increase in *hydraulic work*, with no increase in oxygen uptake ($\dot{V}O_2$) of an isolated perfused rat heart when a physiological ratio of 4 mM βHB : 1 mM acetoacetate was added to the perfusate in addition to the control perfusion with 10 mM glucose (55). Further evidence that switching to ketone bodies as an oxidative substrate may enhance efficiency comes from studies of AV difference across the

brain in prolonged fasting of obese patients. Owen and colleagues (1967) found that the total brain blood flow was 45 ml/min and the oxygen uptake 2.7 ml/min (13). These values are 21% and 25% lower than the normal values in human brains on a *normal* western diet (57).

Table 1.1 – Energetic content and efficiencies of βHB and other substrates

Substrate		Formula	RQ	Energy/ mass kcal/g	Energy / L O_2 kcal/l	Energy / L CO_2 kcal/l	Energy/ mole ΔH kcal/mol	Energy per C_2 unit ΔH kcal/C_2
Ketone	BHB	$C_4H_8O_3$	0.89	4.69	4.8	5.4	-487	-243
Carbohydrate	Glucose	$C_6H_{12}O_6$	1	3.72	5	5	-670	-224
	Pyruvate	$C_3H_4O_3$	1.2	3.17	4.9	4.1	-279	-186
Fat	Palmitate	$C_{16}H_{32}O_2$	0.7	9.31	4.6	6.7	-2385	-298

The RQ and *efficiency* parameters for βHB ($C_4H_8O_3$) and pyruvate ($C_3H_4O_3$) in the context of the *standard* energy substrates glucose ($C_6H_{12}O_6$), the archetypal carbohydrate, and palmitate ($C_{16}H_{32}O_2$), as an example of fat. Energy per unit gas volume is calculated using the equation for complete oxidation and the molar volume of gas at standard temperature and pressure. The energy/mole is divided by the molar quantity of gas and the volume of that gas at STP (22.4 dm^3) to yield energy per litre of gas at STP. Adapted from Veech et al 2004 (56).

1.3.5 Ketone bodies as metabolic signals.

βHB and acetoacetate were first identified as energy substrates used to link the central nervous system with the body's principal energy stores in adipose tissue. However, it is emerging that βHB also has an important role as a signalling molecule.

βHB is a ligand for two known G-protein coupled receptors. The PUMA-G receptor (also known as the hydroxycarboxylic acid receptor 2 [HCAR2] or as Gpr109) was first identified as the receptor for nicotinic acid (vitamin B3) (58), which activates the receptor only in supraphysiological concentrations of 500-3,000 mg daily (59). We now know that βHB binds and activates PUMA-G at physiological concentrations (60) inhibiting lipolysis in an apparent negative feedback mechanism to restrict the supply of fatty acids to the liver and

to prevent metabolic acidosis from the unrestricted synthesis of ketone bodies. βHB also binds to and antagonises the free fatty acid receptor FFR3 (also known as Gpr41) present in the autonomic sympathetic ganglia and, in mice, βHB acts via FFR3 to reduce both sympathetic drive and metabolic rate (61).

Post-translational modification via inhibition of the ubiquitous histone deacetylases (HDACs) was first observed for the short-chain fatty acid butyrate (62). Butyrate differs from βHB by a single hydroxyl group and indeed inhibition of class I HDACs by βHB has also been described (63). βHB has been shown in a mouse model to cause global hyperacetylation, including hyperacetylation of the promoter and upregulation of transcription of FOXOA3 (the vertebrate homologue of the stress-response transcription factor in worms DAF16, which regulates lifespan). One biological effect was to increase resistance to oxidative stress (64).

It has even been suggested that, as ketone bodies can increase the mitochondrial concentration of acetyl-CoA by 9-18 fold (55), this may in itself enhance intracellular acetylation, even if requiring citrate shuttling to increase cytosolic and nuclear acetyl-CoA concentrations (65).

1.3 Potential experimental and therapeutic uses of ketone bodies

1.3.1 Fuel substrate

In the 1930s, it became apparent that animal tissues have the capacity to take up large quantities of circulating ketone bodies (18, 19, 66). In 1941, using an anaesthetised rabbit model, Wick and Drury reported that up to 85% of resting oxygen consumption could be accounted for by the uptake and complete oxidation of a sodium β-hydroxybutyrate salt, infused at 0.13 mmol/kg/min (67). It is known that the healthy adult liver can produce up to

185 g of ketone bodies each day and that ketone bodies provide up to 6% of energy following the overnight fast and 30-40% of energy after a 3 day fast (29).

In 1974, Reichard and colleagues demonstrated significant ketone body oxidation in eight obese patients who had been admitted to hospital for *therapeutic fasting* at rest. Ketone body synthesis and complete oxidation to CO_2 was determined using a 3-^{14}C-labelled acetoacetate tracer. This study revealed that, with prolonged (2-24 days) starvation, the total ketone body concentrations rose to 7-10 mM, and ketone body production climbed steadily to plateau at about 2 weeks. The peak ketone production was 150 g per day (approximately 700 kcal equivalent converted to ketone bodies from endogenous fat) and the peak ketone oxidation was 130 g per day (the remainder being lost in urine or used in non-oxidative metabolism), which is equivalent to approximately 600 kcal *burned* (68). In a study of the same participants, using a specific AV difference study across the forearm muscle compartment they found that, with prolonged fasting (24 days), the ketone body concentrations continued to rise, but the ketone body uptake, particularly of βHB actually fell slightly from the peak levels at around 2 weeks (69).

1.3.2 Carbohydrate sparing

In their first study of more prolonged exercise as a potential stimulus to the uptake of ketone bodies by muscle, Féry and Balasse also looked at ketone rate of disposal (R_d) in the 60 mins following a 2-hour exercise bout of moderate intensity (50% $\dot{V}O_{2max}$). They observed that during this period, the skeletal muscle uptake of ketone bodies was 0.012 mmol/kg/min, a 3-fold increase in muscle ketone body disposal vs. resting oxidation (70). They were then the first to suggest the *utility* of post-exercise ketosis, a phenomenon that had first been reported by Courtice and Douglas in 1936 (71). It was already known that human skeletal muscle would resynthesise glycogen following exercise, even in a fasted state (72). Féry and

Balasse noted the finding, in rat skeletal muscle, that ketone bodies inhibit glycolysis and increase glycogen synthesis (73), and proposed that post-exercise ketosis may be part of an adaptation to ensure glycogen replenishment. Wahren and colleagues had already demonstrated that, following exercise, skeletal muscle glycogen is preferentially restored in preference to hepatic glycogen (72). The first recognition that ketone bodies might inhibit glucose utilisation was made in 1952 by Drury and Wick (74), who showed that an infusion of β-hydroxybutyrate decreased glucose oxidation in the rabbit. Acetoacetate or β-hydroxybutyrate have now been demonstrated to reduce glucose, or pyruvate, oxidation in heart (75), diaphragm (76), soleus muscle (73) and kidney (77). All of these tissues share the characteristics that they are capable of a high rate of glycolytic flux, and all can oxidise ketone bodies.

This well-conserved effect of the ketone bodies has been interpreted as a means to redirect tissue substrate use away from carbohydrate oxidation and towards oxidation of fat.

> *The net result of this interaction between glucose and ketone bodies in the peripheral tissues is a sparing of carbohydrate, either as glucose itself or as lactate and pyruvate that can be reconverted to glucose via the Cori cycle.* Robinson and Williamson, 1980 (24)

The mechanism by which ketone bodies inhibit glycolysis in favour of the oxidation of alternative substrates is now widely accepted. It occurs by 2 processes. First, the rapid increase in available acetoacetate within the mitochondrion leads to a many-fold increase in acetyl-CoA concentration. An increase in [acetyl-CoA]/[CoA] ratio leads to an increase in all intermediates of span 1 of the Krebs cycle (78). The rapid increase in mitochondrial citrate leads to shuttling of citrate into the cytosol where it exerts a powerful inhibition on

the enzyme of proximal glycolysis phosphofructokinase (Figure 1.6)(79). Second, the increase in [acetyl-CoA]/[CoA] and [NADH]/[NAD+] ratio (55) also inhibits the activity of the pyruvate dehydrogenase (PDH) complex, via activation of pyruvate dehydrogenase kinase (PDK), which phosphorylates PDH at any of 3 serine residues, all of which inactivate the enzyme (75, 80). The overall effect of an abundance of ketone bodies is to inhibit carbohydrate oxidation, in favour of the oxidation of fat and some ketones. In driving this shift in fuel preference, the ketone bodies preserve precious carbohydrate reserves for the brain and minimise the need for protein catabolism to provide gluconeogenic precursors.

1.3.3 Diabetes, insulin resistance and the metabolic effects of ketone bodies

There are fascinating implications for the use of ketone bodies in diabetes, metabolic syndrome and in temporary states of insulin resistance (e.g. severe infection, traumatic brain injury).

1.3.3.1 Ketones as an insulin-mimetic factor

Insulin increases cell membrane glucose flux through increased incorporation of the *inducible* glucose transporter channels (GLUT-4) into the sarcolemma (81) and increases the [acetyl-CoA]/[CoA] through upregulation of the activity of PDH (82, 83). Perfusion of an isolated rat heart with a physiological ratio of ketone bodies (βHB:acetoacetate, 4:1) showed how ketone bodies can mimic the key effects of insulin. Ketone bodies raise intracellular glucose concentration through the provision of an alternative substrate (75) and inhibiting glucose oxidation in the heart (75), diaphragm (76), and kidney (77) of rats. Like insulin, ketone bodies also raise the mitochondrial [acetyl-CoA]/[CoA]. In the case of ketone bodies, this is not achieved by upregulating decarboxylation of pyruvate, but by providing an alternative supply of acetyl-CoA. Acetoacetate is *activated* by the addition of a -CoA moiety via succinyl-CoA-3-oxoacid transferase (SCOT). Acetoacetyl-CoA is then cleaved

by thiolase to yield 2 molecules of acetyl-CoA (55). Both insulin and ketone bodies therefore result in the same upregulation of the metabolites of span 1 of the Krebs cycle and both cause an increase in the *hydraulic efficiency* (a measure of the external work performed per unit of oxygen consumption) of the isolated perfused rat heart by about 25% (84). Given that diabetes is a disease of *energy crisis* and insulin resistance, the potential for ketone bodies to replicate the mitochondrial conditions seen with insulin stimulation and to enhance the capacity for work performed per unit oxygen is interesting. Furthermore, we know that during prolonged fasting, circulating insulin is low and ketone bodies take on the functions that it would normally serve. There would appear, therefore, to be potential for a state of ketosis to ameliorate the harm of insulin resistant states.

1.3.3.2 Ketones as an oxidative substrate in diabetes

Diabetic dogs, goats and rabbits have the same capacity to take up ketone bodies (measured by AV difference) as do non-diabetic controls (85, 86). The fraction of oxygen uptake accounted for by the oxidation of acetoacetate was 44% (85). This underlines the possibility that ketones may *bypass* the pathological limitation of access to substrates seen in diabetes. In a rare form of *Leigh syndrome*, afflicting a young boy with deficiency of the PDH complex, symptoms, and cerebral pathology (assessed by interval neuroimaging) improved markedly with a ketogenic diet (87).

1.3.3.3 Ketones as a stimulus for endogenous insulin release

In addition to the potential of ketone bodies to bypass the limitations of both glucose uptake and PDH flux seen in states of insulin resistance, there is also evidence that ketone bodies have the potential to augment endogenous insulin release. The first report of ketone bodies causing endogenous insulin release was in 1964 in dogs (88). Early studies in man frequently failed to demonstrate any increase in insulin secretion in response to ketone bodies (89-91). Then in 1970, using catheters placed in the hepatic portal vein (for the purposes of hepatic

imaging in two colorectal cancer patients), Balasse and colleagues (1970) identified an increase in hepatic portal vein insulin in response to acetoacetate infusion (92). However, this insulinotropic effect was not detectable in the peripheral circulation. Given that the liver can clear 45% of insulin from the hepatic portal system (93), this is perhaps unsurprising, and may explain the negative findings of those who reported no insulinotropic effect of ketone bodies after sampling the peripheral circulation. Owen and colleagues *did* demonstrate an insulinotropic effect in normal, obese and diabetic subjects in 1973 (94). Using a rapid acetoacetate infusion, achieving acetoacetate concentrations of approximately 4 mM and total ketone bodies approaching 5.5 mM, they saw a brief doubling of endogenous insulin in ONF subjects from 25 to 50 mU/l.

1.3.3.4 Ketogenic diets and carbohydrate restriction in diabetes

The first therapeutic use of a ketogenic diet was in the treatment of intractable seizure episodes in epilepsy, as early as 1921, (95, 96). In 1924, the effective use of this treatment was presented by clinicians at the Mayo clinic (97). Though the exact mechanism of the anticonvulsant effect has never been confirmed, it is believed to result from the hyperketonaemia induced by the very low carbohydrate content. De Vivo et al. concluded that the increase in electroconvulsive threshold in rats on a hyperketogenic diet was an increased *energy charge*. They demonstrated a rise in the [ATP]/[ADP] ratio in the brains of acutely starved rats versus the control-fed group (98). In an elegant experiment in 1999, Pan and colleagues demonstrated that initiation of a ketogenic diet raised the content of high energy phosphates in the brains of sufferers of intractable epileptic seizures using ^{31}P magnetic resonance spectroscopy (99).

However, dietary advice for the treatment of type 2 diabetes, from Europe and North America, recommends against a low-carbohydrate diet (defined conservatively by the American Diabetes Association as <130 g/day carbohydrate) (100, 101). In spite of this,

there is evidence that in a population with type 2 diabetes a low-carbohydrate diet improves glycaemic control (102-104); reduces, or eliminates, the need for medication (105); is at least as effective as a low-fat diet for weight loss (106-109) and improves the cardinal features of the metabolic syndrome (increased high density lipoprotein (HDL) and low density lipoprotein (LDL) diameter, reduction in triglyceride (TG) and reduction in apo B/apo A1 ratio) (108, 110). Investigators in the field have pointed out that these benefits typically occur at a *tipping point* when whole body metabolism shifts from deposition of energy as adipose fat to mobilisation of fat and fat oxidation. This *tipping point* invariably coincides with the onset of *ketonuria* (111).

Ketonuria has been used as a marker of fat mobilisation in low-carbohydrate diets, rather than implicated mechanistically in the benefits of the diet *per se*. However, it is intriguing to consider that, given their recognised effects as signalling molecules in themselves (65), ketone bodies may play a role in the beneficial metabolic modulation in carbohydrate restriction.

1.4 Ketone ester

Therapeutic use of ketosis has always depended upon either carbohydrate restriction (< 20 g of total oral carbohydrate per day) or, in the case of intractable paediatric seizures, a more extreme diet (≤ 1 g of protein and 10-15 g of carbohydrate) introduced by Peterman et al at the Mayo clinic (97). The Peterman diet is sometimes referred to as the 4:1 diet, as it contains 4-fold the amount of fat, by weight, as protein and carbohydrate combined. This diet is generally recognised to be unpalatable (112) and adherence has proved difficult (56). Furthermore, it is known that excessively high circulating levels of fat can reduce energy availability to the heart and risk cardiomyopathy (51). Indeed there have been reports of cardiomyopathy in children on a ketogenic diet (113).

The vast majority of experimental work conducted on the metabolic consequences of ketosis in man and animals has been conducted either using fasting/carbohydrate restriction or the infusion or dietary feeding of ketone body salts (for review see Robinson and Williamson (24)). While ketone salts are effective at raising circulating ketone body concentrations they deliver a salt load which, quite apart from being harmful in the longer term, confounds *normal* physiology by inducing considerable fluid shifts and renally-mediated changes in salt and water handling, associated with changes in the renin-angiotensin-aldosterone axis.

Recently, an orally-available, safe, well-tolerated ester molecule: R-3-hydroxybutyl-R-3-hydroxybutyrate has been developed for human use (114, 115) (Figure 1.9). The ester bond is cleaved by ubiquitous carboxylesterases and esterases, within the stomach and small intestine, and the products: 3-hydroxybutyrate and 1,3-butanediol absorbed. The hepatic portal vein carries both constituent molecules to the liver where 1,3-butanediol is metabolised by alcohol dehydrogenase and aldehyde dehydrogenase into 3-hydroxybutyrate (116, 117). Consequently, each molecule of ester yields 2 molecules of D-β-hydroxybutyrate. Following ingestion of the ester, peripheral levels of βHB rise within 15-30 mins and are maintained (at rest) for at least 2 hours (118).

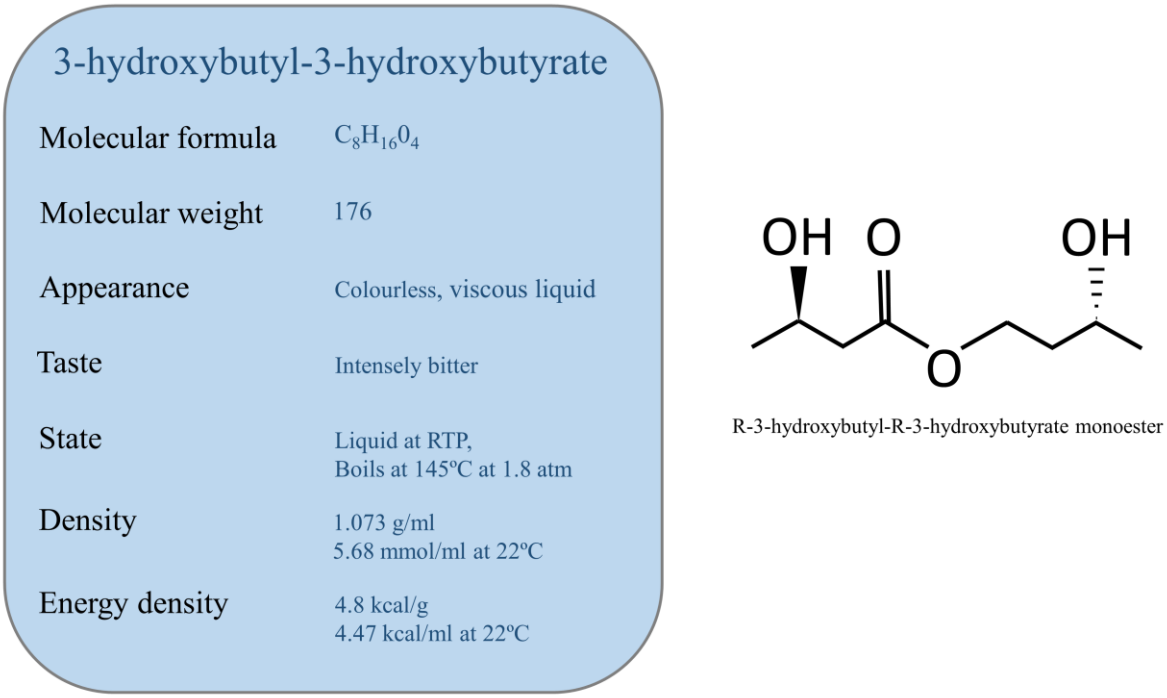


Figure 1.9 – The ketone ester: R-3-hydroxybutyl-R-3-hydroxybutyrate

This novel ketone ester makes it possible to investigate the actions of ketone bodies without the need to cause a potentially harmful increase in the level of circulating fat, or the introduction of a confounding, and also potentially harmful, salt-load.

More specifically, for the first time, this ketone ester allows investigation of the effects of the co-administration of both ketone bodies and plentiful carbohydrate – a novel condition, not seen in nature.

1.5 Outline programme of work

The work described in this thesis is focused on the effects of ketone bodies on carbohydrate metabolism.

Ketone bodies are an *alternative* substrate and are used in states of carbohydrate deprivation to spare, and ultimately to largely replace, glucose to meet the energetic requirements of the

central nervous system. They spare carbohydrate not only by providing the only significant alternative substrate for the brain, but also by acting as a metabolic *signal* to shift the body's energetic requirements away from carbohydrate to those derived directly, and indirectly (via ketones themselves), from fat.

This work explores three questions:

- To what extent can ketone bodies be *burned* as a fuel in skeletal muscle, and what are the influences of exercise intensity and other fuel substrates on ketone oxidation?
- Can ketone bodies increase skeletal muscle glycogen synthesis following glycogen-depleting exercise?
- Can ketone bodies alter carbohydrate and fat metabolism in diabetes, and do any such alterations offer therapeutic potential?

2 General Methods

2.1 Ethics

2.1.1 Ethical approval

All the studies presented in this thesis were considered, and received favourable opinion, by approved National Health Service (NHS) or Ministry of Defence (MOD) research ethics committees.

- Study 1: The effects of diet and exercise on ketone body metabolism – 14/EE/0063
- Study 2: Oral ketones in exercise endurance and recovery – 508/MODREC/14
- Study 3: Exogenous oral ketones and insulin sensitivity in type 2 diabetes – 15/SW/0198

All studies were conducted in accordance with the principles and guidance on ethical research practice, stated in the declaration of Helsinki (119).

2.1.2 Consent

Study participants were fully briefed about all aspects of the protocol and were provided with a participant information sheet (PIS), approved by the research ethics committee, at least 24 hours prior to providing written, informed consent. They were all made aware that their consent was voluntary and could be withdrawn at any time, without the need for explanation, and with no harm to their medical care or professional status.

2.2 Participants and screening

Participants in studies 1 and 2 were well-trained athletes who all completed at least 4 – 6 hours of aerobic exercise per week, in at least 3 exercise sessions. Participants in study 3

were either participants with type 2 diabetes, who did not require treatment with insulin, or were healthy individuals with no history of metabolic illness.

2.2.1 Inclusion criteria – studies 1 and 2

- Willing and able to give informed consent
- Male, or female taking the combined oral contraceptive pill, aged 18-45 years
- $\dot{V}O_{2max}$ >3.8 l/min

2.2.2 Exclusion criteria – studies 1 and 2

- Inability to perform bicycle ergometer exercise
- Insufficient $\dot{V}O_{2max}$
- Any history of cardiovascular or metabolic disease
- Current or recent chest infection
- Current or recent musculoskeletal injury
- Pregnancy or current breastfeeding
- Allergy to egg, soya or peanut [study 1 only – relative contraindication to Intralipid®]

2.2.3 Inclusion criteria – study 3

- Participant is willing and able to give informed consent for participation in the study
- Male or Female, aged 18 to 75 years
- EITHER type 2 diabetes (controlled with diet and/or metformin) not requiring insulin or sulphonylurea OR healthy individual with no history of cardiovascular or metabolic disease
- Alcohol intake within the nationally recommended weekly limit (< 15 units for women, < 22 units for men)

2.2.4 Exclusion criteria – study 3

- Taking insulin or sulphonylurea drugs (eg gliclazide and glibencamide)
- Poorly controlled diabetes
- Pregnancy or current breastfeeding

2.3 Anthropometry

All participants had height and weight measurements recorded using a wall-mounted scale and calibrated Seca 813 balance (Seca Ltd., Birmingham, UK). Body mass index (BMI kg/m^2) was recorded and body surface area was calculated according to the Mosteller equation (120).

2.4 Cardiopulmonary exercise testing

2.4.1 Indirect calorimetry

Simultaneous measurements of oxygen uptake and elimination of carbon dioxide were made on a *breath by breath* basis using a proprietary system (Metalyzer® 3B, Cortex, Leipzig, Germany), which continuously samples mixed gases at the mouth, via an aspiration line connected to a mask sealed over the participants nose and mouth (Hans Rudolph V2 7450 series mask, Cranlea, Birmingham, UK). The Metalyzer® contains an oxygen electrode for direct measurement of oxygen content and to calculate the carbon dioxide content. Results were saved to a dedicated study computer and displayed continuously using the linked software package (MetaSoft® version 7.9.1, Cortex, Leipzig, Germany). Data were analysed, after each visit, by applying a 30 second average to determine $\dot{V}O_2$ and $\dot{V}CO_2$, and other derived parameters (such as respiratory exchange ratio), at each time point.

2.4.2 $\dot{V}O_2$max test protocol

The $\dot{V}O_2$max test protocol chosen was a progressive increase in workload commencing with 3 minutes at 100W and increasing to volitional fatigue in a series of 25W increments every 3 minutes. The reason that this step-protocol was chosen, in preference to a progressive ramp, was to allow the collection of data at 'pseudo-steady-state' at submaximal intensities.

2.4.3 Calibration of indirect calorimetry methods

Calibration of oxygen and carbon dioxide concentrations and volume measurements was performed before and after each test. Gas concentrations were calibrated using the '2-point method' with room air and a control gas of known oxygen and carbon dioxide concentration (17.14% O_2, 5.03% CO_2 control gas, Cranlea, Birmingham, UK). Calibration of the turbine was performed using a standard 3 l syringe (Hans Rudolph 3 litre calibration syringe, Cranlea, Birmingham, UK). The Metalyzer® has an in-built thermometer and the exact local barometric pressure was inputted, at each visit, using a barometer within the laboratory, checked against the measurement at the local Royal Air Force base.

2.4.4 Determination of peak power output (PPO) and maximal oxygen uptake ($\dot{V}O_{2max}$)

Tests of maximum workload (PPO) and maximal $\dot{V}O_2$ uptake were performed in order to prescribe work intensities corresponding to a known proportion of $\dot{V}O_{2max}$ and for the prescription of work intensity intervals used to deplete muscle glycogen (see 2.5).

Maximum effort tests were conducted on an Ergoselect 100 stationary cycle ergometer (Ergoline, Bitz, Germany). This electrically braked ergometer was set for a fixed external workload, independent of the cadence (revolutions per min of the crank). All tests were conducted following an overnight fast, with water taken *ad libitum*. All tests were started at

a low workload of 100 watts. After each period of 3 mins, the workload was increased by 25 watts until volitional fatigue. Work max was defined according to Currell et al (121):

PPO = Power output at step n-1 +

((seconds completed in step n /180s) × workload increment per step [watts])

The test was recorded as maximal if one of the following criteria was satisfied:

- The oxygen uptake reached a *plateau* phase in which the increase in uptake was less than 0.2 L/min
- The respiratory exchange ratio exceeded 1.1

2.5 Exercise interval protocol to deplete glycogen

Participants attended the laboratory following a 12-hour overnight fast. Glycogen was depleted as described by Taylor and colleagues (122). Following a 10-min warm-up at 50% $\dot{V}O_2$ max, participants commenced intermittent intensity, 2-min intervals alternating 90% PPO efforts with 50% PPO recovery. When fatigued at this intensity, defined as the inability to turn the crank at 60 revolutions per min (RPM), the upper interval workload was decreased to 80% PPO before resumption of intervals. When fatigue occurred at 80% PPO, the upper workload was decreased further to 70% PPO. When work could not be maintained at 70% PPO at a cadence of at least 60 RPM, the upper workload was reduced to 60% PPO. Exhaustion, and protocol completion, was defined by the inability to complete two mins at 60% PPO. The participant's heart rate was monitored throughout (Polar H7 heart rate monitor, Polar, Kempele, Finland) and water was consumed *ad libitum*.

2.6 Fasting and dietary manipulation

All study visits commenced at 0700-0800 hours and followed a 10 – 12 hour overnight fast. During this fast, only water was permitted. Participants were requested not to perform exercise, other than that prescribed in the protocols, for 48 hours prior to each visit and were requested to abstain from alcohol for 24 hours.

Study 1 required participants to observe either a high-carbohydrate or a low-carbohydrate diet for 36 hours prior to each study visit. Ingredients for all diets were provided to participants either directly, by hand, at their baseline visit, or via supermarket delivery using a major UK supermarket delivery service. The diet composition was not blinded.

2.6.1 High-carbohydrate diet

The high-carbohydrate diet comprised a variety of drinks and foods with a high-carbohydrate content. The diet was formulated to achieve a carbohydrate intake of 6-10 g/kg/day in line with the American College of Sports Medicine guidance on carbohydrate consumption for athletes pursuing a ‘High endurance’ program of physical activity in ‘Nutrition and Physical Performance’ (123).

2.6.1 Low-carbohydrate diet

The low-carbohydrate diet comprised a variety of foods with a very low-carbohydrate content. The diet was formulated to match the calorie intake of the high carbohydrate diet. A very low-carbohydrate diet is usually defined as fewer than 20 grams of carbohydrate per day (124). This study used a slightly more moderate target of 20-50 g/day, in view of the increased difficulty of restricting carbohydrate when simultaneously needing to consume a high total calorific content – in order to match the high-carbohydrate diet. The low-

carbohydrate diet was also prescribed so as to provide <10% of total daily calories from carbohydrate.

2.7 Study drinks

2.7.1 Ketone ester drinks

The ketone ester dose in Studies 1 and 2 was 0.573 ml/kg of participant bodyweight. This is equal to 615 mg/kg of ester. In Study 3 the ketone ester dose was 0.4 ml/kg (430 mg/kg).

In all studies, the total volume of ester was diluted by the addition of water (7 parts water:1 part ketone ester). Each ketone drink had the bitter taste partially masked by the addition of 3g of proprietary aspartame sweetener and 2 g each of a commercial orange (product number 10814226/4) and lemon/lime (product number 10866736/2) flavour additive (Symrise, Holzminden, Germany).

2.7.2 Control drinks

Control drinks were composed of a volume of water equal to the total volume of the ketone drinks. They contained the same quantity of aspartame sweetener and commercial citrus flavour additives as the ketone drinks. The control drinks were taste-matched to the ketone drinks by the addition of 0.045 ml/kg of commercial bitter agent (product number 648352, Symrise, Holzminden, Germany).

2.8 Blood handling and analysis

2.8.1 Blood sampling

Blood samples were drawn from 22 gauge peripheral venous cannulas (Venflon®, Becton Dickinson, Plymouth, UK) inserted into a vein in the participant's antecubital fossa or forearm. Where possible, cannulas were placed in the non-dominant arm. Blood samples

were saved directly into sealed, vacuum tubes (Vacutainer®, ®, Becton Dickinson, Plymouth, UK). Ethylenediaminetetraacetic acid (EDTA) tubes were used for plasma samples. Tubes with a clot activator and gel were used to collect serum samples. Sealed tubes containing samples were transferred immediately to be stored in iced water (at 4ºC).

2.8.2 Blood sample storage.

Within four hours of blood being drawn, chilled samples in vacutainers were centrifuged at 4ºC at 3,600 rpm in a Beckman Avanti 30 centrifuge (Beckman-Coulter, Brea, CA, USA) using a F0850 rotor (mean radius 6.35cm).

Given that:

$$g = (1.118 \times 10^{-5}).\ R.\ S^2$$

where R = rotor radius (cm) and S = rotor speed (revolutions per min)

The mean centrifugal separation force was 900g. The supernatant of centrifuged samples was pipetted into Eppendorf tubes and stored at -80ºC until analysis.

2.8.3 Analysis of blood samples

Samples were thawed on the benchtop for 40 mins prior to analysis.

All samples were analysed in duplicate for glucose, lactate, non-esterified fatty acids (NEFA), triacylglycerol and βHB using a commercial benchtop automated spectrophotometric analyser (ABX Pentra 400, Montpellier, France). The machine was calibrated prior to each batch of samples and all samples were compared with internal control standards that were freshly prepared each day of analysis.

Acetoacetate assays were performed according to the spectrophotometric enzymic method described by Williamson et al (125), based upon the following reaction, catalysed by β-hydroxybutyrate dehydrogenase:

$$\textit{Acetoacetate} + \textit{NADH} \leftrightarrow \textit{β-hydroxybutyrate} + \textit{NAD}^{+}$$

Absorbance at 340 nm was used as a measure of NADH concentration and was measured before and after the addition of β-hydroxybutyrate dehydrogenase. The change in absorption was compared with that from a range of known acetoacetate standards in order to determine acetoacetate concentrations within the samples.

Insulins were analysed using a commercial ELISA kit (product number 10-1113-10 Mercodia, Uppsala, Sweden).

Blood substrate (but not insulin) analyses for study 3 were conducted in the laboratory of the Oxford Centre for Diabetes, Endocrinology and Metabolism (OCDEM) under the supervision of the laboratory manager Ms Sandy Humphreys. Serum samples were analysed for acetoacetate and D-β-hydroxybutyrate, lactate, glucose, NEFA, triglyceride and glycerol.

2.9 Muscle biopsies and storage

2.9.1 Muscle biopsy

Muscle biopsies were taken under local anaesthetic (2% lidocaine hydrochloride) injected subcutaneously and infiltrated up to 3 cm deep into underlying muscle. Individual samples were taken from lateral incisions (approximately 8 mm in length) 2 -3 cm apart, over the distal third of the *vastus lateralis*. Sampling was performed using a Bard Monopty™ Core Biopsy Instrument, 12 French gauge, 10 cm long (Bard Biopsy Systems, Tempe, AZ, USA). Four passes, up to 2 cm deep, were made with the biopsy instrument yielding approximately 100 mg

of tissue per biopsy. Samples were immediately frozen, in labelled Eppendorf tubes, in liquid nitrogen. They were then stored at -80 ºC for later analysis.

2.9.2 Muscle glycogen analysis

Muscle glycogen analysis was conducted according to the method of Van Loon et al (126). Samples were freeze dried under negative pressure in an Edwards Modulyo apparatus (Edwards, Crawley, UK) at approximately 2 millibar and -50ºC for 48 hours. Approximately 5 mg of freeze dried muscle was powdered, dissected free of all collagen, fat and other non-muscle tissue, and the powder hydrolyzed by incubation in 500 µl of 1 M HCl for 3 h at 100°C in a boiling water bath. Samples were allowed to cool to room temperature and then neutralized with 250 µl 0.12 mol/l Tris-2.1 mol/l KOH saturated with KCl. After centrifugation, 150 µl of supernatant was analysed in duplicate for glucose concentration using a commercial benchtop automated spectrophotometric analyser (ABX Pentra 400, Montpellier, France).

Glycogen concentration was calculated from the glucose concentration in the recovered supernatant and expressed as mmol glycosyl units/kg dry weight of muscle:

$$\textit{Glycogen} = (0.75 \times (\textit{glucose mM}) \times 1000) / \textit{dry muscle sample weight}$$

2.10 Intravenous infusions

Intravenous infusions of 20% dextrose and Intralipid® (both supplied by Baxter Healthcare, Newbury, UK) were given through 20 gauge peripheral venous cannulas (Venflon®, Becton Dickinson, Plymouth, UK) inserted into a vein in the participant's antecubital fossa. The infusion pumps were Carefusion Alaris^{TM} GP devices, used in conjunction with compatible *single-use* giving sets.

2.11 Glucose clamp procedures

All clamps were conducted in accordance with the original description of de Fronzo and colleagues (127) who devised both hyperglycaemic and euglycaemic clamp techniques. All clamp studies required the insertion of 2 peripheral cannulas: a 22 gauge peripheral venous cannula in the dorsum of the hand and a 20 gauge peripheral venous cannula in the antecubital fossa of the contralateral arm. The 22 gauge cannula was used for sampling blood at 5 min intervals to allow frequent monitoring of blood glucose concentrations. The hand and forearm around the sampling cannula were heated to 40-44 ºC using two therapeutic heat pads (HK35 Beurer, Ulm, Germany) wrapped around the limb to cause maximal vasodilatation and so generate a 'pseudoarterialized' blood sample. The very small samples drawn from this cannula were analysed for glucose concentration in a *near-patient*, validated benchtop analyser (HemoCue 201^{+}, Radiometer, Copenhagen, Denmark). The larger diameter 20 gauge cannula was used for the infusion of 20% dextrose and (in the euglycaemic clamp only) both 20% dextrose and insulin.

2.11.1 Hyperglycaemic clamp

The aim of the hyperglycaemic clamp was to raise the circulating blood glucose concentration of a fasted individual to 10 mM as rapidly as possible and then to *clamp* the glucose at 10 mM for the remainder of a 2 hour period. The original purpose of this clamp was to investigate beta-cell function by analysing endogenous insulin response to this *supraphysiological* glucose stimulus.

A priming dose of 240 mg/kg of glucose was infused over the first 15 mins and then the rate of a 20% dextrose infusion was adjusted, over the remaining 2 hours, based on blood glucose measurements taken every 5 mins to maintain the whole blood glucose at 10 mM.

This method of providing carbohydrate following exhaustive exercise was chosen for three reasons. First, the technique provided a robust means to standardise the glucose availability to skeletal muscle across different visits for the same participant, and between different individuals. Second, by delivering glucose intravenously, it was possible to avoid the variations in time and magnitude of nutrient delivery that complicate oral ingestion and enteral absorption. Finally, it was possible to ensure that glucose delivery to the muscle was at least as high as would be provided by recommended optimum post-exercise carbohydrate feeding of 1.0 – 1.2 g/kg/h over 4-6 hours (128). This meant that any increase in glucose disposal and/or glycogen synthesis that occurred with the ketone intervention was more likely to be biologically relevant.

2.11.2 Euglycaemic clamp

The aim of the euglycaemic clamp was to maintain the fasting blood glucose (i.e. 4 mM glucose) for the duration of the 2 hour clamp, while simultaneously giving a fixed dose of exogenous insulin (e.g. 40 mU/m^2/min). The *clamp* at fasting glucose concentration was achieved through a variable rate infusion of 20% dextrose. The theoretical basis of the euglycaemic clamp was to *standardise* insulin concentration and to *prevent* endogenous hepatic glucose output. In this state, the amount of glucose infused was equal to the amount of glucose taken up by the tissues. The more *sensitive* the tissues are to insulin, the more 20% dextrose will need to be infused to maintain *euglycaemia*.

3 Ketone oxidation

Abstract

Background: The work presented in this chapter sought to demonstrate ketone body oxidation during exercise in humans. It also aimed to determine the effects on ketone oxidation rate of both exercise intensity and the availability of other substrates.

Methods: This study was of a 4-arm, randomised, crossover design. Prior to study visits all participants consumed a regulated high or low-carbohydrate diet for 36 hours followed by an overnight fast. Study visits involved consuming a ^{13}C-labelled ketone ester drink and completing one hour of exercise. During this exercise, blood samples were collected for metabolite measurements and breath samples were collected for later analysis of ^{13}C enrichment by mass spectroscopy.

The conditions studied were:

- A prime – High-carbohydrate diet + light intensity exercise 40% $\dot{V}O_{2max}$.
- A – High-carbohydrate diet + high intensity exercise 75% $\dot{V}O_{2max}$.
- B – High-carbohydrate diet, IV fat infusion + high intensity exercise 75% $\dot{V}O_{2max}$.
- C – Low-carbohydrate diet, IV fat infusion + high intensity exercise 75% $\dot{V}O_{2max}$.

Results: Ketone bodies were oxidised at rest and during exercise. Increasing exercise intensity increased the absolute quantity of ester oxidised (20, 53 and 68 mmol/h at rest, 40% and 75% $\dot{V}O_{2max}$ respectively) and reduced the relative contribution to oxidation (28%, 11% and 8% $\dot{V}O_2$ at rest, 40% and 75% $\dot{V}O_{2max}$ respectively). Less ^{13}C-label was recovered following a low-carbohydrate diet than a high-carbohydrate diet (1.42 mmol vs. 1.76 mmol $p < 0.05$).

Conclusion: Ketone bodies are oxidised as a fuel substrate, with the greatest contribution to total oxidation occurring at rest. The reduced oxidation following carbohydrate depletion may result from decreased anaplerosis and impaired regeneration of oxaloacetate via pyruvate carboxylase thus reducing the concentration of Krebs cycle intermediates so limiting Krebs cycle flux.

3.1 Introduction

In the anaesthetised rabbit, ketone oxidation may account for 85% of resting oxygen uptake (67). Human results indicate a more modest contribution of ketone oxidation. Published studies report that ketone oxidation provides 3-6% of the energy requirement following an overnight fast, rising to 30-40% after a 3 day fast (29, 70, 129). Following prolonged fasting of up to 24 days, the human liver may produce 150 g of ketone bodies per day (68). Peak ketone oxidation at this point is about 130 g/day of ketone bodies, equivalent to 600 kcal of energy. The balance of ketone bodies are lost in the urine or undergo non-oxidative metabolism.

3.1.1 Ketone oxidation during exercise

The results of studies concerning ketone oxidation during exercise are more variable (Table 3.1). Wahren and colleagues were the first to quantify ketone oxidation in integrated human exercise studies of healthy controls and in type 1 diabetic patients who had abstained from insulin treatment for 24 hours (130). They determined that there was no significant oxidation of ketone bodies in healthy controls (using AV difference across the leg), at rest or during exercise (40 mins, 60% $\dot{V}O_{2max}$), and that in the type 1 diabetic group the oxidation of ketone bodies could account for only 4% of total oxygen uptake ($\dot{V}O_2$). They obtained similar results when the study was repeated at a lower exercise intensity (40 mins, 45% $\dot{V}O_{2max}$) (131). Rennie et al. not only found that there was no significant uptake of ketone bodies during 30 mins exercise at 60% $\dot{V}O_{2max}$ but also that there was a net *appearance* of ketone bodies during the exercise period (132). This demonstration of ketone appearance during exercise is not an isolated finding (133, 134). With the large body of work from Féry and Balasse on ketone oxidation (Table 3.1 studies 3, 4, 6 & 7) it emerged that the duration of fasting prior to ketone oxidation studies was inversely proportional to the ketone oxidation

rate during subsequent exercise (70). Indeed in this study Féry and Balasse showed, consistent with the findings of Rennie et al. (132), that after 3-5 days fasting rather than ketone bodies being *disposed* of during exercise there was a net *release* (by rate of appearance calculation) during exercise. Intriguingly, this result contradicted an earlier study by the same group in which they had demonstrated ketone uptake by exercising muscle in individuals fasted for 3 days exercising for 30 mins at 60% $\dot{V}O_{2max}$. A possible explanation for this contradiction is discussed at the end of the chapter (3.4.6).

Table 3.1 – Studies of ketone uptake and ketone oxidation during exercise in man.

Study	Participants	Pre-study preparation	Resting total ketone (mM)	Resting βHB (mM)	Exercise	Rate of disappearance of ketones (various units)	Ketone oxidation as $\%\dot{V}O_2$ or $\%\dot{V}CO_2$	Reference
1.	8 healthy male	ONF	0.16	0.1	40 mins 55-60% $\dot{V}O_{2max}$	*not reported*	0% $\dot{V}O_2$	Wahren, Hagenfeldt and Felig. 1975(130)
	8 Type 1 DM	ONF and 24h off insulin	2.05	1.51	40 mins 55-60% $\dot{V}O_{2max}$	*not reported*	4% $\dot{V}O_2$	
2.	5 male	ONF	0.075	--	30 mins at 60% $\dot{V}O_{2max}$	-0.034 mmol/min	*not reported*	Rennie et al. 1976(132)
3.	7 male	3 day fasted	4.8	3.8	30 mins at 60% $\dot{V}O_{2max}$	1.3 mmol/min	10% $\dot{V}CO_2$	Balasse, Féry, Neef. 1978(129)
4.	6 male	ONF	0.2	0.14	2 h 45% $\dot{V}O_{2max}$	0.004 mmol/kg/min	*not reported*	Féry and Balasse. 1983(70)
	6 male	ONF	0.31	0.22	2 h REST	0 mmol/kg/min	*not reported*	
	6 male*	3-5 day fasted	5.67	4.52	2 h 50% $\dot{V}O_{2max}$	-0.003 mmol/kg/min	*not reported*	
	7 male	ONF	0.33	0.26	2 h 44% $\dot{V}O_{2max}$	0.012 mmol/kg/min	*not reported*	
5.	6 healthy male	ONF	0.08	0.038	40 mins 45% $\dot{V}O_{2max}$	0.03 mmol/min	1% $\dot{V}O_2$	Wahren et al. 1984(131)
	9 Type 1 DM	ONF and 24hr off insulin	1.1	0.81	40 mins 45% $\dot{V}O_{2max}$	0.28 mmol/min	5% $\dot{V}O_2$	
6.	10 male	18 hr fasted	0.31	0.21	2 h 50-55% $\dot{V}O_{2max}$	0.4 (rest) to 0.7 (exercise) mmol/min	*not reported*	Féry and Balasse. 1986(135)
	5 male	62 hour fasted	3.21	2.52	2 h 50-55% $\dot{V}O_{2max}$	1.2 (rest) to 1.2 (exercise) mmol/min	*not reported*	
	6 male*	112 hour fasted	5.67	4.52	2 h 50% $\dot{V}O_{2max}$	1.6 (rest) to 1.4 (exercise) mmol/min	*not reported*	
7.	7 male + 1 female	ONF + acetoacetate infusion	6.0	--	2 h 50% $\dot{V}O_{2max}$	2.0 (rest) to 2.2 (exercise) mmol/min	*not reported*	Féry and Balasse. 1988(136)
	6 male*	112 hour fasted	5.67	4.52	2 h 50% $\dot{V}O_{2max}$	1.6 (rest) to 1.4 (exercise) mmol/min	*not reported*	

ONF = overnight fasted, R_d=rate of disappearance

3.1.2 Ketone oxidation and exercise intensity

Given the considerable discrepancies between the findings of Wahren and colleagues and the work of Féry and Balasse regarding the contribution of ketone bodies to substrate oxidation during exercise, it is clear that further work is warranted. No data have been published, from healthy participants, on the effect of exercise intensity on ketone oxidation.

3.1.3 Ketone oxidation in competition with other energy substrates

In view of the critical role of ketone bodies in preserving carbohydrate stores during starvation, it is interesting to consider whether the local availability of carbohydrate may influence muscle ketone oxidation. It seems reasonable to speculate that when whole body carbohydrate, and so also muscle glycogen, levels fall to a critically low level that this may signal a need to *spare* ketone bodies for oxidation within the central nervous system. We know, from the pioneering work of Randle and colleagues, that there is a form of substrate 'competition' between the utilisation of free fatty acids and glucose in cell oxidation (137). This glucose-fatty acid ('Randle') cycle describes the observation that relative substrate availability in skeletal muscle and adipocytes determines what ratio of fuels is oxidised and/or converted into stored fat in the form of triglyceride. A mechanism for this *competition* between glucose and fatty acids, seen in liver, was first suggested by McGarry et al (36, 38) in 1977. In summary, abundant availability of glucose leads to plentiful oxidation of pyruvate to yield acetyl-CoA within the mitochondrion. Excess acetyl-CoA can be *shuttled*, via citrate, to the cytosol where it is available for fatty acid synthesis. The first committed step in fatty acid synthesis is the conversion of acetyl-CoA to malonyl-CoA. Malonyl CoA inhibits the transport of cytosolic fatty acids into the mitochondrion by inhibiting carnitine palmitoyl transferase 1 (CPT1) (38). Thus, when glucose is plentiful, fatty acid oxidation is inhibited and synthesis increases. Conversely when fatty acid concentrations are high, (e.g.

during fasting), and acetyl CoA is provided in excess by beta oxidation of fat, the same citrate shuttling from the mitochondrion to the cytosol results in inhibition of proximal glycolysis by reducing the activity of phosphofructokinase (79). In addition, the increases in acetyl-CoA/CoA and $NADH/NAD^+$ inhibit the activity of the pyruvate dehydrogenase complex (as described in 1.3.2) thus reducing terminal glycolysis by end-product inhibition (75, 80). This in-series inhibition of proximal and terminal glycolysis shifts energy requirements away from carbohydrate and toward fat.

The availability of an oral ketone ester, which can rapidly raise circulating levels of ketone bodies without a salt load or concomitant elevation of free fatty acids makes it possible to investigate the novel suggestion that there is a ‘glucose-ketone cycle’.

The work described in this chapter sought to confirm ketone oxidation in exercising healthy human participants, given an oral ketone ester, and to determine the effects of both exercise intensity and substrate availability on ketone oxidation rate.

3.2 Methods

3.2.1 Participants

Six well-trained male athletes were recruited to the study (General Methods 2.2). All performed 4-6 hours per week of endurance training and had a $\dot{V}O_{2max}$ > 3.8 l/min (Table 3.2). All participants provided written informed consent.

Table 3.2 – Baseline characteristics of study participants

Characteristic	Mean ± SEM
Age (years)	35.5 ± 2.6
Height (m)	1.84 ± 0.05
Weight (kg)	81.9 ± 6.9
BMI	23.9 ± 1.0
% body fat	15.5 ±1.4
$\dot{V}O_{2max}$ (l/min)	4.7 ± 0.3
$\dot{V}O_{2max}$ (ml/kg/min)	58.6 ± 2.6
Peak power output (W)	349 ± 18

3.2.2 Baseline visit

Each participant attended a baseline visit to complete a cardiopulmonary exercise test to volitional fatigue (General Methods 2.4). This allowed the measurement of maximal oxygen uptake ($\dot{V}O_{2max}$) and peak power output (PPO). All participants also received a ketone drink, followed by blood and exhaled breath sampling (3.2.3 and 3.2.4) to establish the contribution of ketone bodies to oxidation at rest.

3.2.3 Collection of gas samples

At predefined time points, participants breathed through a one-way valve, low-resistance gas collection circuit. Fifty litres of exhaled breath were collected in a Douglas bag (138). Mixed samples of the breath were aspirated via a sealed port, into 12 ml sealed, evacuated

glass Exetainer® tubes (Labco, Lampeter, Wales, UK) and stored for later ^{13}C and ^{12}C CO_2 analysis by mass spectroscopy.

3.2.4 Drink preparation and ^{13}C-labelling

Ketone drinks were prepared as previously described (General Methods 2.7). In addition to the standard drink, a small volume of ^{13}C-labelled monoester was mixed in with the drinks. Two labels were used (Figure 3.1) in place of a universally labelled ester molecule. In one label, the 4 carbons in the hydroxybutyrate moiety were labelled and in the other, 4 carbons of the hydroxybutyl moiety were labelled. The dose of each of the 2 labelled esters was 0.0025 ml/kg. This is equivalent to a dose of 0.0142 mmol of universally labelled ester and 0.1136 mmol of ^{13}C atoms.

The labelled esters were produced by Shasun Pharmaceuticals Limited (Shasun Pharmaceuticals Ltd., Northumberland, UK) under good manufacturing practice (GMP) conditions. Conversion of ^{13}C-labelled butanediol was carried out using the standard stoichiometry of 1.4 equivalents of ethyl-3-hydroxybutyrate. The reaction was carried out at 20-25 °C for 5 hours, whilst distilling off ethanol at reduced pressure (~3 mbar) to drive the reaction. After removing the enzyme by filtration, short-path distillation using Kugelrohr apparatus was performed.

Conversion of ^{13}C-labelled ethyl-3-hydroxybutyrate was carried out with butanediol charged in excess (1.4 equivalents). The reaction was carried out at 20-25 °C for 4 hours at ~5 mbar and then 3.5 hours at ~2 mbar. After removing the enzyme by filtration, short-path distillation using Kugelrohr apparatus was performed.

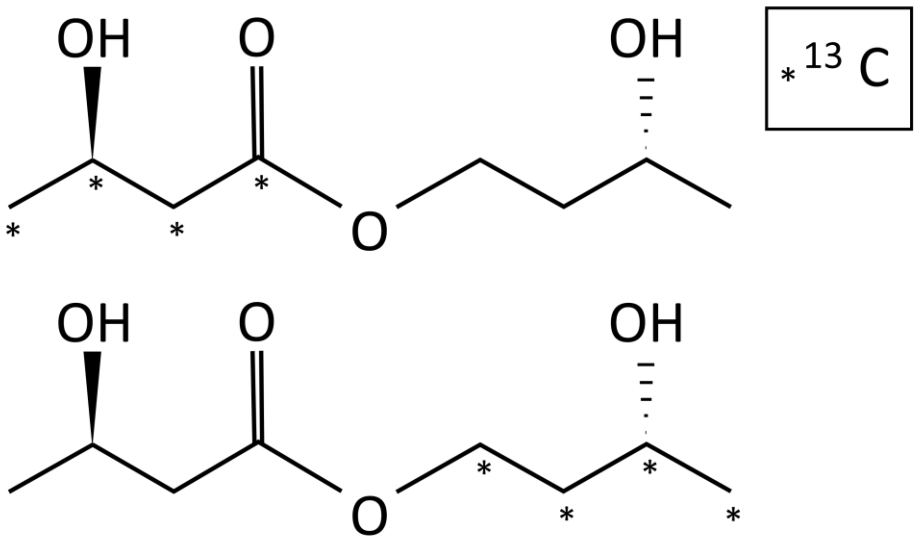


Figure 3.1 – ^{13}C-labelled ketone monoester

3.2.5 Blood sampling and analysis

Blood sampling and analysis was conducted as previously described (General Methods 2.8).

3.2.6 Glycogen depletion

Thirty six hours prior to each study visit participants completed a standardised high-intensity interval exercise protocol to the point of physical fatigue (General Methods 2.5). This protocol has been demonstrated to reliably deplete skeletal muscle glycogen (8, 122).

3.2.7 Controlled diet

Immediately following each glycogen depletion, participants consumed a prescribed high-carbohydrate or low-carbohydrate diet (General Methods 2.6) up to, and including, the evening meal before the overnight fast preceding the study visit. This dietary manipulation was used to set the substrate conditions for each study visit.

3.2.8 Lipid infusion

Enhanced availability of non-esterified fatty acids was achieved by an infusion of 20% Intralipid® (Baxter Healthcare, Staines, UK) at 60 ml/h. Intralipid® is a clinical-grade lipid emulsion for human intravenous infusion. It is derived from soy bean oil and egg-yolk phospholipids. Intralipid® contains a mixture of triglycerides (majority unsaturated fatty acids) and non-esterified fatty acids (linoleic acid (44-62%); oleic acid (19-30%); palmitic acid (7-14%); α-linoleic acid (4-11%) and stearic acid (1.4-5.5%)). The maximum infusion rate in adults is 1 ml/min, with the first dose not to exceed 500ml.

3.2.9 ^{13}C label calculations

Substrate oxidation calculations were based upon the recovery of ^{13}C-labelled ketone monoester carbon atoms as $^{13}CO_2$ in exhaled breath.

The original dose of ^{13}C in ketone drinks was calculated:

TTR of drink* x *number of mmol of carbon atoms in drink

TTR = tracer (^{13}C)/tracee (^{12}C)

mmol of carbon atoms in drink = mmol of ester administered* x *8

The quantity of ^{13}C recovered was calculated by measuring the mmol/min of $^{13}CO_2$ exhaled at the end of the exercise when a *plateau* had been achieved. Plateau = *steady state* $^{13}CO_2$ release. An *area under the curve* (AUC) for mmol $^{13}CO_2$ vs. time was calculated (mmol/min $^{13}CO_2$.min = mmol $^{13}CO_2$) yielding the mmol of $^{13}CO_2$ recovered in one hour at steady state. The AUC was based upon a 60 min period at the *steady state* rate of production.

^{13}C recovery (%) = ^{13}C in drink dose / $^{13}CO_2$ recovered in breath

A number of studies have demonstrated that, at one hour of steady state exercise, the spurious loss into, or recovery from, the bicarbonate pool of ^{13}C-label becomes negligible

(139-141). The same investigations have also demonstrated that one hour of steady-state exercise also obviates the need for an acetoacetate correction to allow for loss of ^{13}C label into intermediates of the Krebs cycle or its cataplerotic pathways. Van Hall (1999) concluded that, under the condition of steady state exercise lasting at least one hour, that bicarbonate and acetoacetate corrections were not required (142).

3.2.10 Mass spectrometry analysis

Gas chromatography – mass spectrometry (GC-MS) was conducted at the Oxford Centre for Diabetes, Endocrinology and Metabolism (OCDEM).

Drink samples were analysed for ^{13}C enrichment according to the following validated method (143). Samples were pipetted into 10 ml glass tubes and acidified to pH 1 with 1 M-HCl. The 3-OHB in drink samples was extracted into 4·5 ml ethyl acetate–diethyl ether (1:1, v/v) in 10 ml glass tubes for mixing by hand for 2 min and then by rotary mixing for 1 h. The aqueous and organic phases were separated by centrifugation, and the upper organic phase was evaporated to dryness under N2 at room temperature. t-Butyl dimethylsilyl derivatives of 3-OHB were prepared by the addition of 20 µl pyridine and 20 µl N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide + 1% t-butyl dimethylchlorosilane.

Samples of exhaled breath were analysed according to a validated method (144). Carbon dioxide was separated from the presence of other gases using a capillary column (CPPoraPLOTQ; Varian Ltd, Oxford, United Kingdom) with dimensions of 27.5 m x 0.32 mm x 10 µm on the GC-combustion isotope ratio MS. Splitless injection mode, an injection volume of 40 µl, and an injector temperature of 110 °C were used. The oven temperature was kept constant at 35 °C, and the total run time was 10 min. The column flow was held constant at 1.2 ml/min. The results were not accepted if background enrichment was not within the range of natural enrichment. The ^{13}C enrichment results (from ketone ester drinks

and breath samples) expressed as $\delta^{13}C‰$ were converted to the tracer-to-tracee ratio (TTR) using the following equation:

$$TTR\ (^{13}C{:}^{12}C) = [(\delta^{13}C‰/1000) + 1] \times 0.0112372$$

The TTR of a baseline measurement (before administration of the stable-isotope tracer) was subtracted from each sample TTR to account for natural abundance.

3.2.11 Statistics

One way ANOVA was used to analyse the energy content and composition of the diets. Two-way ANOVA was used to compare ketone oxidation and metabolite levels. A p value of < 0.05 was taken to indicate statistical significance. Unless otherwise stated, the p values reported are those of post-hoc Tukey tests following significant findings of ANOVA analyses.

3.2.12 Study design

The study was a 4-arm, randomised crossover design (Figure 3.2).

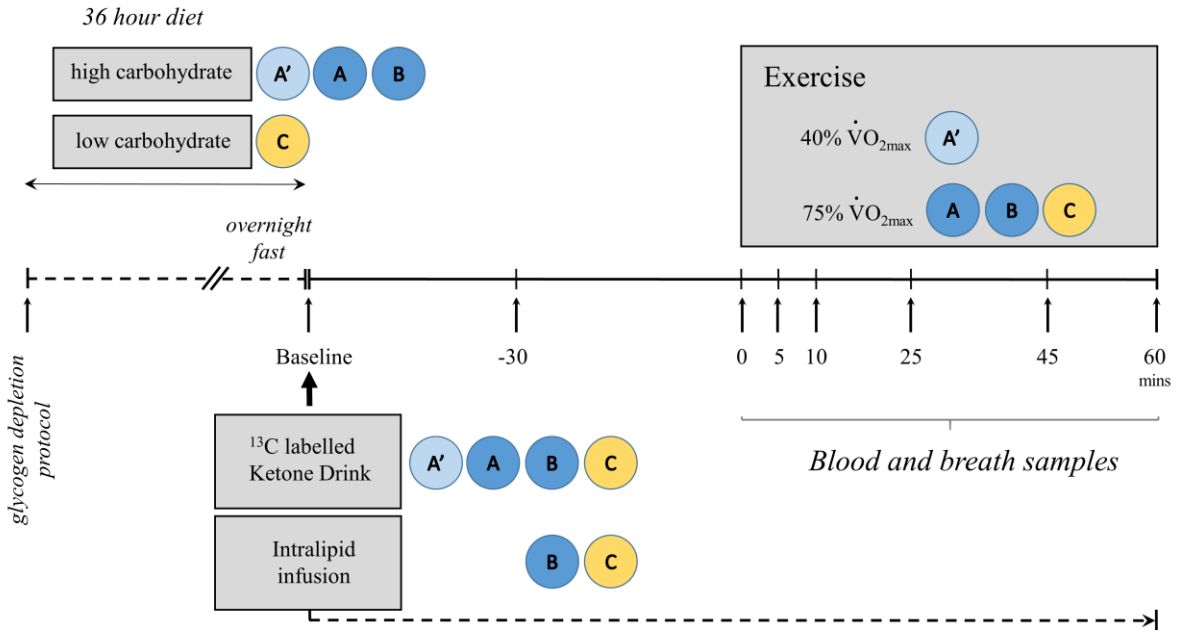


Figure 3.2 – Schematic of study visit

All visits occurred 36 hours after a glycogen depletion protocol followed by a controlled high or low-carbohydrate diet and an overnight fast. At each visit, participants consumed a ^{13}C-labelled ketone drink followed by a 60 min rest period. An intravenous infusion of intralipid was given at 2 of the study visits to enhance substrate availability of non-esterified fatty acids. After the rest period, participants completed a 60 min bout of exercise at a constant exercise intensity. The four study conditions were:

- Condition A' - Carbohydrate *replete*, exercise at 40% $\dot{V}O_{2max}$
- Condition A - Carbohydrate *replete*, exercise at 75% $\dot{V}O_{2max}$
- Condition B - Carbohydrate *replete*, IV fat infusion, exercise at 75% $\dot{V}O_{2max}$
- Condition C – Carbohydrate *deplete*, IV fat infusion, exercise at 75% $\dot{V}O_{2max}$

Breath and blood samples were collected at the start of each visit and then at pre-determined intervals during the study (Figure 3.2). Trials were conducted at 16-18ºC in the exercise physiology laboratory in the Department of Physiology, Anatomy and Genetics at the University of Oxford (DPAG).

3.3 Results

3.3.1 Diet composition

The calorie intake and carbohydrate consumption across all diets are shown in Table 3.3 and Figure 3.3. There was no significant difference in calorie intake between any of the diets.

Twenty-fold less carbohydrate was consumed in condition C vs. all other conditions ($p < 0.01$).

Table 3.3 – Diet composition: calories and carbohydrate consumption

Study condition	kcal/24h	CHO/24h	CHO/kg/24h
A prime - 40% $\dot{V}O_{2max}$ CHO diet	3855 ± 639	658 ± 74**	7.3 ± 0.4**
A - 75% $\dot{V}O_{2max}$ CHO diet	3566 ± 331	608 ± 40**	7.4 ± 0.8**
B - 75% $\dot{V}O_{2max}$ CHO diet, iv fat	3918 ± 501	723 ± 110**	7.9 ± 0.5**
C - 75% $\dot{V}O_{2max}$ LOW CHO diet, iv fat	3720 ± 373	29 ± 4	0.4 ± 0.1

Values are mean ± SEM; CHO = carbohydrate; ** = $p < 0.01$ vs. C

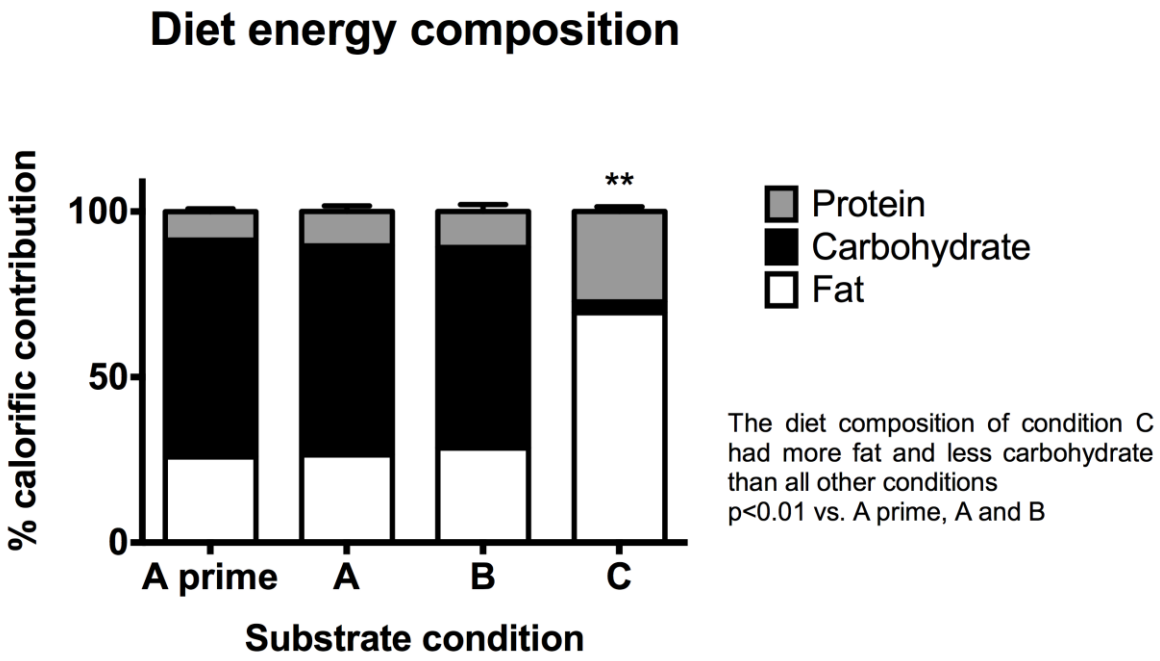


Figure 3.3 – Composition of controlled diets across study conditions

3.3.2 Exercise

Heart rate and the Borg scale, which is a rating of perceived exertion (RPE) are shown in Figure 3.4. The heart rate was 51 bpm at rest, 112 bpm at 40% $\dot{V}O_{2max}$ (A prime) and 157 bpm at 75% $\dot{V}O_{2max}$ (A, B and C), with no difference between the 3 conditions at 75% $\dot{V}O_{2max}$. The RPE, on completion of exercise under high-carbohydrate diet conditions was 15.8 at 75% $\dot{V}O_{2max}$ (A and B) and 10.2 at 40% $\dot{V}O_{2max}$ (A prime). However, under low-

carbohydrate diet conditions, exercise at 75% $\dot{V}O_{2max}$ (C) was associated with a 17% increase in RPE to 18.5, $p < 0.0001$.

$\dot{V}E$, $\dot{V}O_2$, and RERs (Figure 3.5) were lower at 40% $\dot{V}O_{2max}$ (A prime), and lower still at rest, than under all 75% $\dot{V}O_{2max}$ conditions (A, B and C).

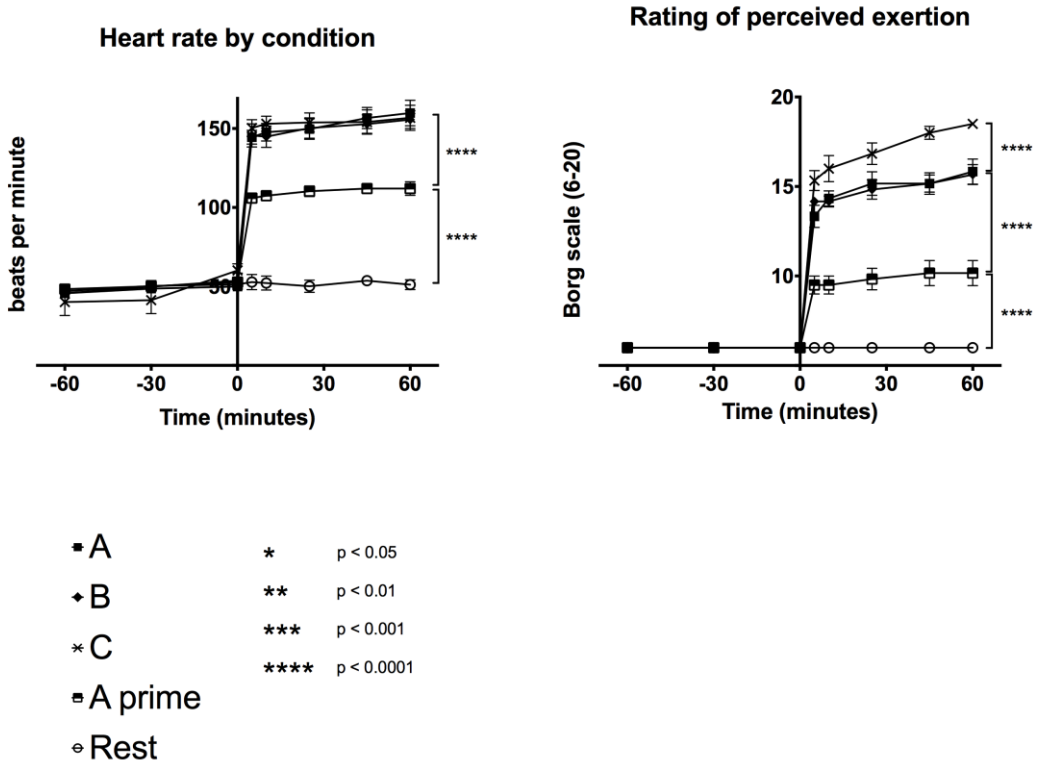


Figure 3.4 – Heart rate and rating of perceived exertion (Borg scale 6-20) during one hour at rest, following the ^{13}C-labelled drink, and the subsequent one hour of exercise.

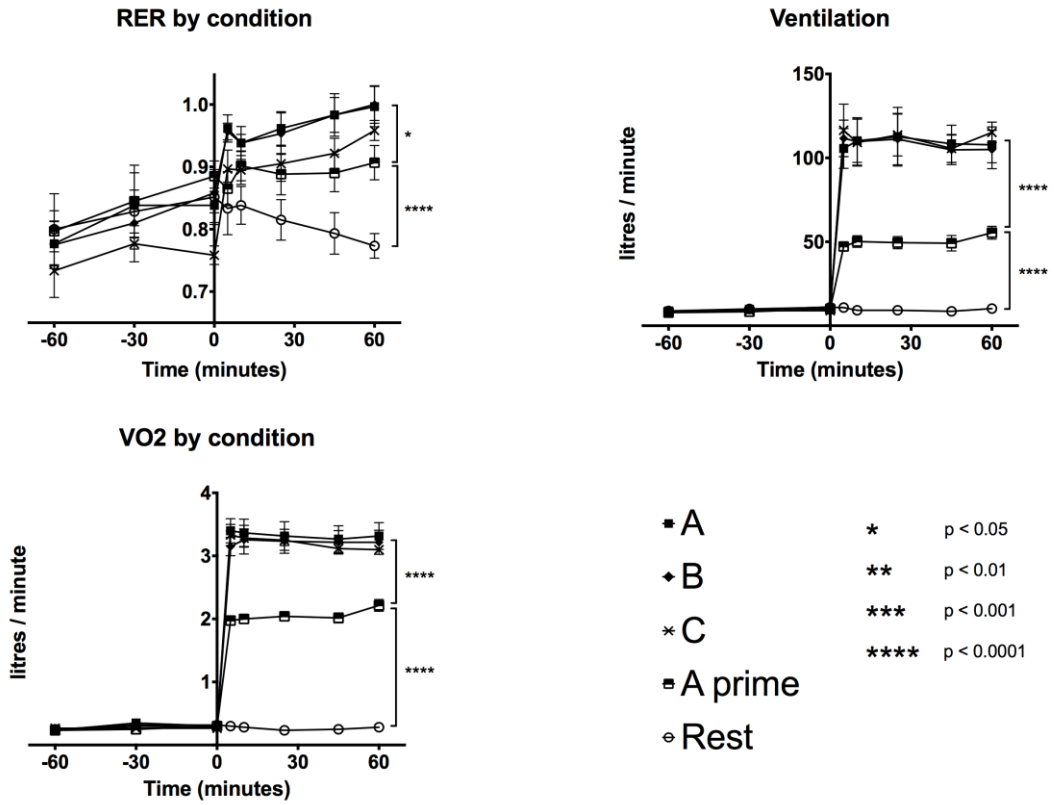


Figure 3.5 – Ventilation, $\dot{V}O_2$ and respiratory exchange ratio (RER) during one hour at rest, following the ^{13}C-labelled drink, and the subsequent one hour of exercise.

3.3.3 Blood metabolites

Resting plasma glucose (Figure 3.6) fell by ~1.2 mM during one hour of rest following ketone ingestion from 5.0 mM to 3.8 mM ($p < 0.0001$). At the completion of exercise, plasma glucose was significantly higher following the high-carbohydrate diet and exercise at 40% $\dot{V}O_{2max}$: 4.0 mM or 75% $\dot{V}O_{2max}$: 3.8 mM than following a low-carbohydrate diet and exercise at 75% $\dot{V}O_{2max}$: glucose 3.0 mM.

Plasma lactate (Figure 3.6) did not change during the rest period following ketone ester ingestion. There were significant increases in lactate in the first 30 mins of exercise in all conditions at 75% $\dot{V}O_{2max}$. Lactate was significantly higher following the high-carbohydrate diet.

NEFA levels (Figure 3.6) were elevated on arrival following the low-carbohydrate diet (C) 0.58 mM vs. all other conditions, and remained 0.3-0.5 mM higher than all other conditions throughout the visit ($p < 0.0001$). Although NEFA levels were not elevated to the same degree by an infusion of Intralipd® alone following the high-carbohydrate diet (B), there was significant elevation of NEFA levels in comparison to both of the conditions, which did not include Intralipid® infusion, 40% $\dot{V}O_{2max}$ (A prime) and 75% $\dot{V}O_{2max}$ (A).

Plasma TAG (Figure 3.6) rose rapidly from the start of the study visit in conditions B and C. Both of these conditions included a 2-hour infusion of Intralipid®, starting at the beginning of the visit ($t = -60$ to $t = 60$). By the first plasma measurement at $t = -30$ there was a highly significant, approximately twofold, increase in TAG concentration in condition B and C vs. all other conditions. By the end of the 60 min exercise period the TAG level had risen to threefold that of the other conditions.

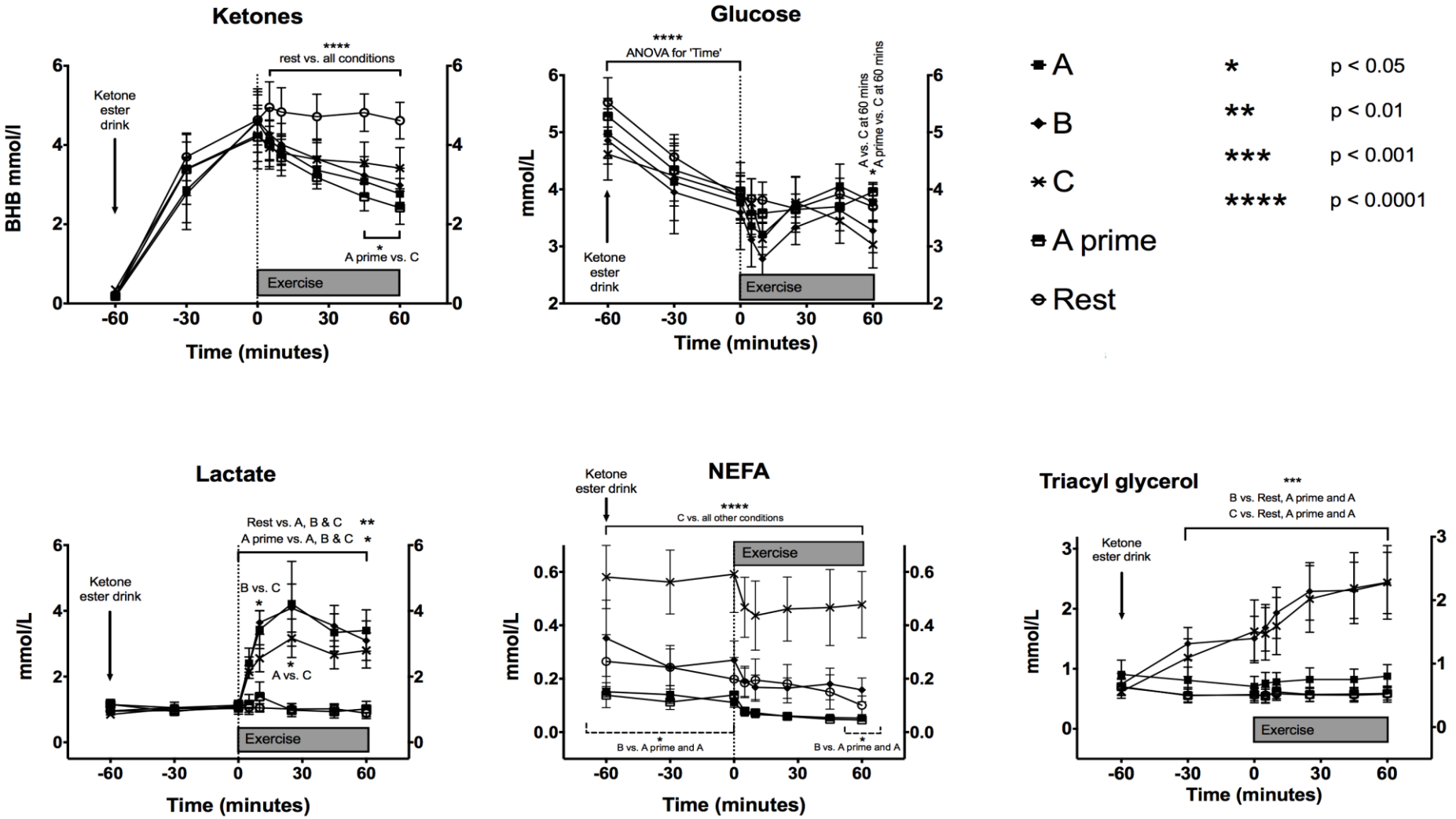


Figure 3.6 – Blood metabolites during one hour at rest following ketone ester ingestion and over the subsequent hour either at rest or exercising at 40% $\dot{V}O_{2max}$ or 75% $\dot{V}O_{2max}$.

3.3.4 Ketone concentration

The fasted βHB concentration at the start of each visit was higher following the low-carbohydrate diet (C) 0.35 mM than following the high-carbohydrate diet conditions (A prime, A and B) 0.20 mM. This difference was significant vs. condition B ($p < 0.05$). In the resting condition, the βHB peaked at 5.0 mM after 65 mins. Under all exercise conditions, the βHB peak occurred at the end of the one hour rest prior to exercise, and had fallen from this peak by 5 mins of exercise in all cases.

From min 10 of exercise the βHB was lower in all exercise conditions than at rest ($p < 0.0001$). During the last 15 mins of exercise the ketone concentration was significantly lower following high-carbohydrate diet and 40% $\dot{V}O_{2max}$ (A prime) vs. low-carbohydrate fed and 75% $\dot{V}O_{2max}$ (C). At the completion of exercise, βHB was 2.4 mM in A prime vs. 3.4 mM in C ($p < 0.05$).

Figure 3.7 shows the difference between the area under the curve (AUC) at rest and during the exercise conditions during the final 15 mins of exercise:

$$\Delta AUC_{45\text{-}60} = AUC_{REST\ 45\text{-}60} - AUC_{CONDITION\ 45\text{-}60}$$

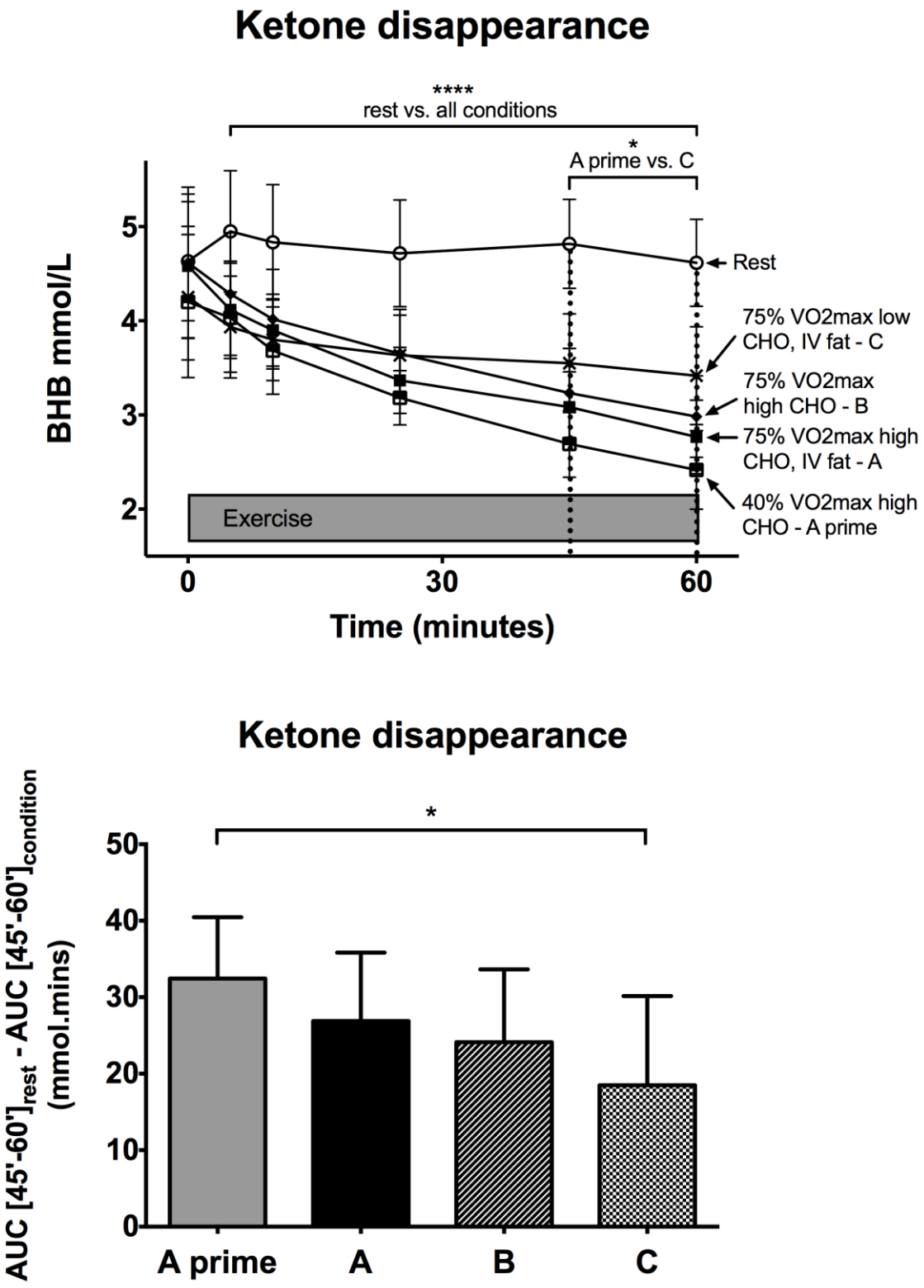


Figure 3.7 – βHB kinetics. Upper panel - one hour either at rest or exercising at 40% $\dot{V}O_{2max}$ (A prime) or 75% $\dot{V}O_{2max}$, following either a high-carbohydrate (A and B) or a low-carbohydrate (C) diet. The vertical dotted lines at 45' and 60' demonstrate the region of interest for calculating $AUC_{45\text{-}60}$. Lower panel - βHB kinetics displayed as $\Delta AUC_{45\text{-}60}$. * = $p < 0.05$; **** = $p < 0.0001$.

3.3.5 ^{13}C tracer analysis

Carbon dioxide at rest was 12.5 mmol/min. Percentage recovery of ^{13}C from a labelled ester drink is a surrogate measure of oxidation (142). Figure 3.8 shows the quantity of exhaled carbon dioxide ($\dot{V}CO_2$, upper left panel), the ^{13}C enrichment of the breath (TTR $^{13}C/^{12}C$, upper right panel) and the product of these measurements and is the rate of production of $^{13}CO_2$ in breath (lower left panel).

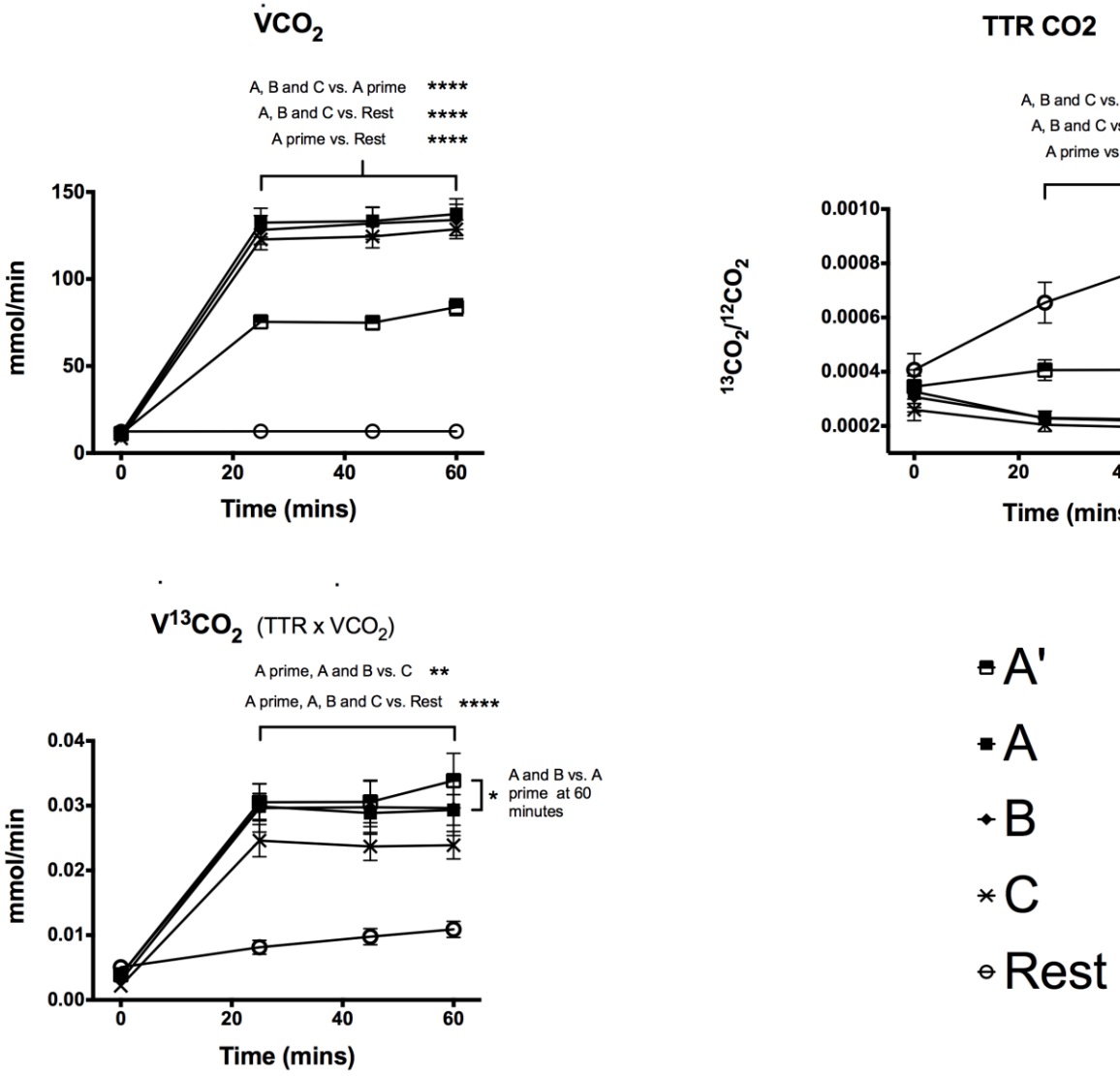


Figure 3.8 – ^{13}C analysis. Upper left panel: CO2 output in mmol/l/min ($\dot{V}CO2$). Upper right panel: 13C enrichment of exhaled breath samples: tracer to tracee ratio (TTR) = 13C/12C. Lower left panel: $^{13}CO2$ in mmol/l/min (= product of TTR.$\dot{V}CO_2$).

During exercise, the carbon dioxide production rose rapidly to a plateau at 25 mins. Enrichment of evolved CO_2 at rest (Figure 3.8, upper right panel) was approximately twofold greater than enrichment at 40% $\dot{V}O_{2max}$ ($p < 0.0001$) which was twofold the enrichment of all three conditions at 75% $\dot{V}O_{2max}$ ($p < 0.0001$).

Recovery of labelled carbon dioxide (Figure 3.8, lower left panel) was approximately threefold higher during exercise than at rest throughout the exercise period ($p < 0.0001$). Significantly less label was recovered under the 75% $\dot{V}O_{2max}$ condition following a low-carbohydrate diet (C) than in all conditions which followed a high-carbohydrate diet. At the end of the 60 min exercise period the amount of label recovered at 40% $\dot{V}O_{2max}$ slightly exceeded that from the two carbohydrate fed conditions at 75% $\dot{V}O_{2max}$.

Table 3.4 shows the absolute and percentage recovery of ^{13}C and the calculated contribution of ketone ester to energy expenditure and oxygen uptake.

Table 3.4 Ketone oxidation by substrate and exercise condition

Condition	Recovery of ^{13}C mmol	Recovery of ^{13}C (%) $^{13}C_{BREATH}/^{13}C_{DRINK}$	Ester oxidation mmol/h	Energy from ester kcal/hour	Fraction of oxidation (%) $\dot{V}O_2$ester/ $\dot{V}O_2$total
Rest	0.65 ± 0.07	8 ± 1	20 ± 2	16 ± 1	28 ± 3
A prime - 40% $\dot{V}O_{2max}$ CHO diet	1.84 ± 0.18*†	20 ± 2*†	53 ± 2*†	42 ± 1*†	11 ± 1*†
A - 75% $\dot{V}O_{2max}$ CHO diet	1.73 ± 0.11*†	24 ± 4*	68 ± 17*	54 ± 14*	8.0 ± 1.4*
B - 75% $\dot{V}O_{2max}$ CHO diet, iv fat	1.79 ± 0.23*†	21 ± 3*	56 ± 7*	44 ± 5*	7.0 ± 0.8*
C - 75% $\dot{V}O_{2max}$ LOW CHO diet, iv fat	1.42 ± 0.12*	17 ± 3*	44 ± 5*	35 ± 4*	5.5 ± 0.7*

Values are mean ± SEM; ∗ = $p < 0.05$ vs. resting condition; † = $p < 0.05$ vs. condition C

3.4 Discussion

This study demonstrated that βHB was oxidised at rest and during both low and high intensity exercise. Increasing exercise intensity led to an increase in the absolute quantity of ester oxidised (20, 53 and 68 mmol/h at rest, at 40% $\dot{V}O_{2max}$ and at 75% $\dot{V}O_{2max}$ respectively) and to a fall in the relative contribution to total oxidation (28%, 11% and 8% of $\dot{V}O_2$ at rest, at 40% $\dot{V}O_{2max}$ and at 75% $\dot{V}O_{2max}$ respectively). Significantly more ^{13}C-label was recovered during high-intensity exercise (75% $\dot{V}O_{2max}$) following a high-carbohydrate diet than following low-carbohydrate diet (1.76 mmol vs. 1.42 mmol).

3.4.1 Diets

The controlled diets were calorie matched across conditions and there was a 20-fold increase in carbohydrate intake between high-carbohydrate (663 g/day across A prime, A and B) and low-carbohydrate (29 g/day: condition C). This amount was striking, given the very high calorific intake of the daily diets (3,760 kcal/day). The high calorie intake was to achieve

the high-carbohydrate doses required to maximise glycogen synthesis and to differentiate the carbohydrate availability between substrate conditions.

3.4.2 Exercise

The subjective rating of perceived exertion (RPE), albeit in un-blinded participants, was significantly higher following the low-carbohydrate diet. This increased subjective sensation of exertion is likely also to reflect skeletal muscle glycogen depletion, which has long been known to signal profound fatigue (145).

3.4.3 Blood metabolites

There was a striking 1.2 mM fall in plasma glucose during the first hour following ketone ester administration, common to all conditions. When resting, the fall in blood glucose continued until 85 mins post ester drink to 3.7 mM, a 1.8 mM fall in blood glucose. This hypoglycaemic effect of ketone bodies has been well described (89, 146-154). Although there is no agreement on the reason that ketone bodies cause blood glucose levels to fall. The fact that ketone infusions do not always increase peripheral insulin in intact animals (152, 155) suggests that hypoglycaemia may be caused by increased glucose disposal independent of insulin, i.e. increased insulin sensitivity (24). It is possible, however, that while endogenous insulin from the pancreas may not reach the peripheral circulation, there may be a rise in the hepatic portal circulation sufficient to restrict hepatic glucose output, and studies have confirmed an increase in hepatic portal vein insulin caused by exogenous ketone infusion (88, 92, 151). Furthermore, the liver clears ~43% of hepatic portal vein insulin at the first pass (93). It is therefore conceivable that a small quantity of insulin may be released into the portal circulation, sufficient to reduce hepatic glucose output but insufficient, after first-pass hepatic clearance, to be detectable in peripheral blood.

Lactate levels did not differ at rest. At the onset of exercise, lactate levels rose to 4.2 mM in condition A and 4.1 mM condition B. Reflecting lower skeletal muscle glycogen, following a low-carbohydrate diet, the peak in condition C was lower. This difference at peak was significant for A vs. C, and it is likely that the difference was not significant vs. condition B due to the simultaneous IV fat infusion. Increased NEFA leads to inhibition of glycolysis (137), which would account for a reduction in lactate.

NEFA levels were approximately twofold lower during exercise at 40% $\dot{V}O_{2max}$ and at 75% $\dot{V}O_{2max}$ following high-carbohydrate diet than under resting conditions. Given that the insulin suppression and catecholamine release of exercise cause increased lipolysis, the fall in circulating fatty acids probably represented an increased rate of disposal in skeletal muscle rather than a decrease in production. The effect of an Intralipid® infusion for one hour before, and then during, exercise at 75% $\dot{V}O_{2max}$ following a high-carbohydrate diet (B) was sufficient to abolish the effect of exercise on NEFA. The effect of glycogen depletion, 36 hours prior to the study visit, followed by the low-carbohydrate diet caused marked elevation of NEFA levels, being fourfold higher at rest, and remaining higher throughout exercise.

3.4.4 Ketone kinetics

The high cost of producing a tailored ^{13}C-labelled substrate constrained the study design to use a single-bolus delivery of labelled substrate, rather than continuous iv infusion to achieve a steady state, prior to commencing the exercise intervention. Nevertheless, the ^{13}C-labelled ketone ester allowed quantification of the oxidation of exogenous ketones. Even before considering the ^{13}C data, there was evidence from peripheral blood ketone kinetics to suggest not only the effectiveness of the ketone ester drink to rapidly and consistently elevate βHB levels, but also that ketones were consumed as an oxidative substrate. At rest, the peak

ketosis occurred at 65 mins post-drink administration. Under all exercise conditions, this peak was not reached, and circulating βHB levels fell rapidly from the onset of exercise. Figure 3.7 demonstrates that during the first 15 mins of exercise, regardless of intensity or substrate preparation, the rate of ketone disappearance was similar. βHB disappearance was greatest at 40%$\dot{V}O_{2max}$ (A prime) and least at 75%$\dot{V}O_{2max}$ following a low-carbohydrate diet (C).

The ^{13}C data confirm that βHB was oxidised as a fuel. ^{13}C recovery in breath underscores differences in the rate of ketone disappearance/oxidation: which was greatest at 40% $\dot{V}O_{2max}$ and was least at 75% $\dot{V}O_{2max}$ under conditions of carbohydrate restriction and fat infusion. The oxidation rate calculated from ^{13}C enrichment of breath correlated closely with the blood ketone surrogate ΔAUC during the final 15 mins of exercise (Figure 3.9).

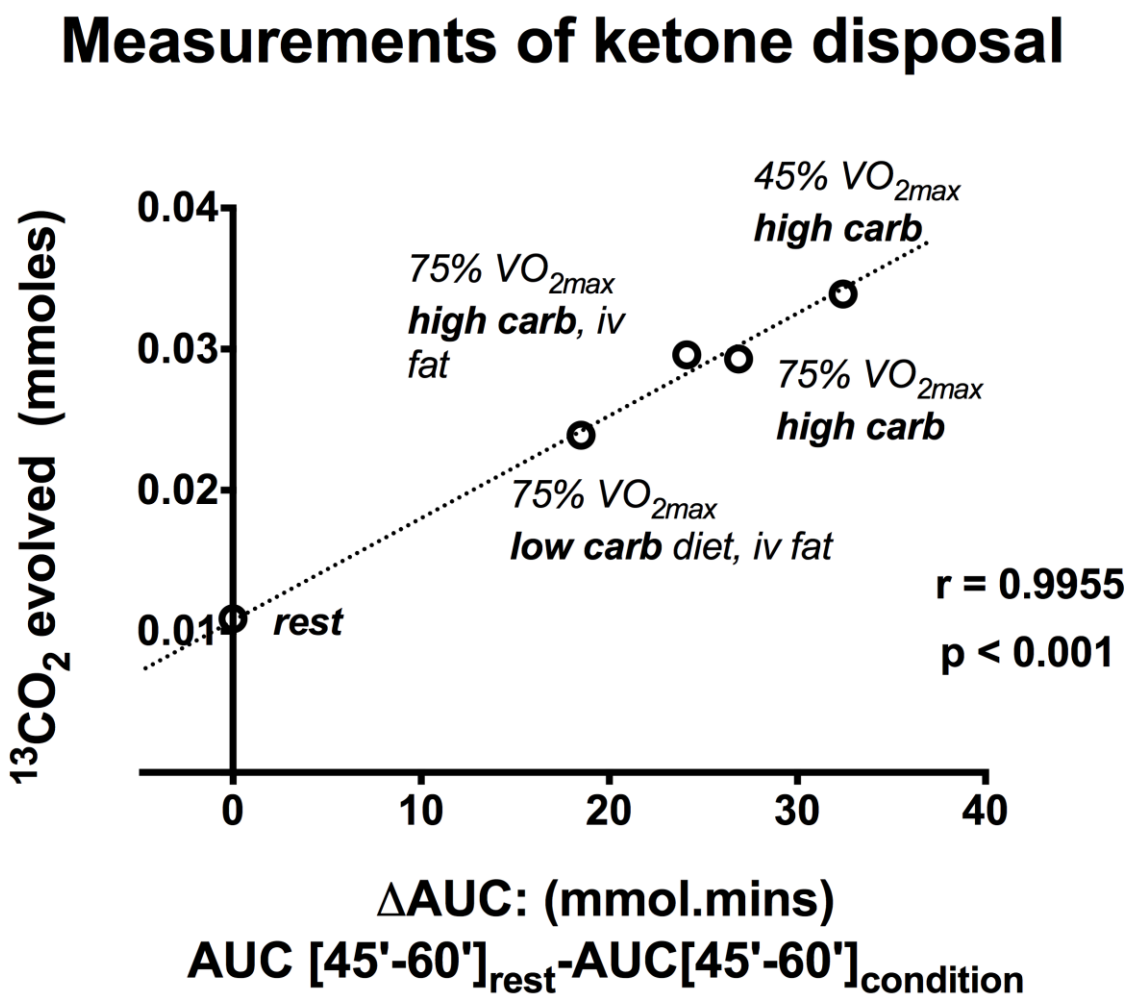


Figure 3.9 – Correlation of ^{13}C recovery with Δ $AUC_{45\text{-}60}$ during the final 15 mins of exercise. Data points represent mean values of all participants.

This correlation suggests that the use of ketone disappearance from peripheral blood was a good measure of ketone oxidation, at least during exercise.

Steady state exercise over one hour at both 40% and 75% $\dot{V}O_{2max}$ provided ample time to reach a steady state of ^{13}C enrichment in breath. At rest, by contrast, the TTR and TTR.VCO_2 were both still rising 2 hours after the ester drink. This meant that a key assumption made in calculating oxidation: that the TTR.VCO_2 represents a maximal, steady state value, is

unsafe and that ketone oxidation at rest (28% of $\dot{V}O_2$ – Table 3.4) was significantly underestimated.

3.4.5 Exercise intensity and ketone oxidation

Higher intensity exercise (75% $\dot{V}O_2$), when there was plentiful glycogen in skeletal muscle (conditions A and B), caused the maximum rate of βHB oxidation. Oxidation rates were lower during IV fat infusion, which raises the possibility that, as another donor of acetyl-CoA moieties, NEFA may *compete* with ketone bodies for oxidation.

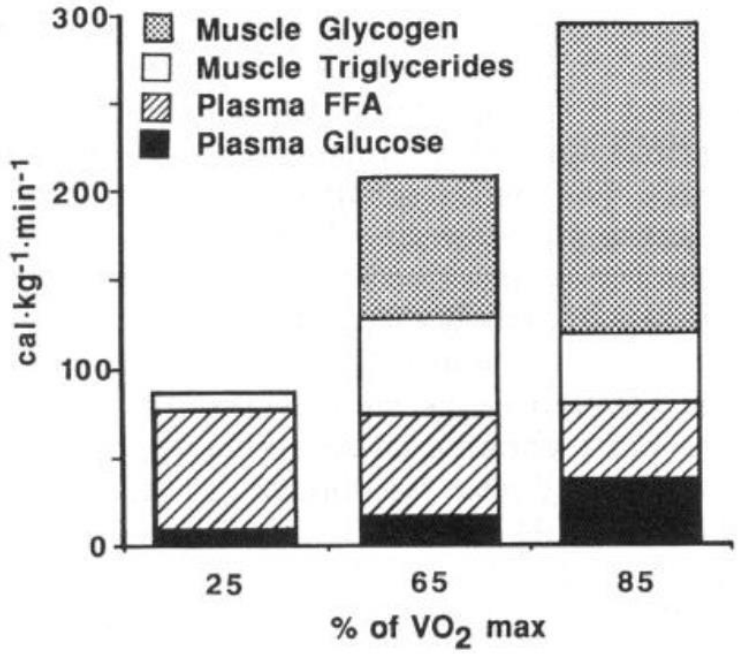
The smallest absolute ester oxidation was seen at rest: 20 mmol/h. However, when the proportion of oxygen uptake attributable to ester ($\dot{V}O_2$ester) is calculated; based on the requirement of 10 molecules of oxygen to completely oxidise one molecule of ester:

$$C_8H_{16}O_4 + 10\ O_2 \leftrightarrow 8\ CO_2 + 8H_20$$

it is clear that the ranked *relative* importance of ketones to total oxygen uptake was reversed. At least 28% of oxygen uptake at rest was accounted for by βHB oxidation. This is much less than the 85% of oxygen uptake attributed to ketone oxidation in anaesthetised rabbits (67), but is higher than in published studies of humans fasted for between 3 and 5 days (70, 129) in whom the resting βHB concentration was 4-5 mM. Two hours after the ketone drink, the rate of ^{13}C recovery was still increasing at rest. 28% is likely to be a significant underestimate of the βHB contribution to oxidation. The most likely explanation for the relatively larger contribution of ketone oxidation in our participants at rest, in comparison to studies using starvation to achieve a similar degree of ketosis, is that our participants had not been subjected to all the other effects of 3-5 days carbohydrate restriction (70). Fasting lowers levels of stored and circulating carbohydrate, suppresses insulin and upregulates peripheral lipolysis raising circulating NEFA levels (1). The use of an oral ketone ester makes it possible to dissect out the effects of starvation that are attributable to ketosis alone.

For this reason Féry and Balasse eventually compared the ketone oxidation rate caused by 3-5 days fasting with an infusion of acetoacetate to *clamp* the ketone body concentrations at the same level in overnight fasted subjects (136). They found that the ketone body disposal rate during 2-hours of exercise (50% $\dot{V}O_{2max}$) increased by 57% when equimolar ketone bodies were supplied by exogenous infusion rather than endogenously, secondary to fasting.

At 40% $\dot{V}O_{2max}$, the relative contribution of exogenous ketone oxidation to total oxygen uptake was lower than at rest, but still accounted for 11% of $\dot{V}O_2$. Although an increase to 75% $\dot{V}O_{2max}$ increased the absolute quantity of ketone oxidation, the relative contribution of ketones to $\dot{V}O_2$ fell as total $\dot{V}O_2$ increased proportionately faster than ketone oxidation. At 75% $\dot{V}O_{2max}$, ketone ester oxidation accounted for 8% of oxygen uptake ($\dot{V}O_2$). These data are reminiscent of the reduction of the contribution of fat to whole body oxidation, with increasing exercise intensity, as the proportion of carbohydrate oxidation increases (48, 49) - Figure 3.10.



3.10 – Figure from Romijn et al. (48) demonstrating the reducing contribution of fat, and increasing contribution of carbohydrate to total energy supply with increased exercise intensity.

Fatty acids and ketone bodies undergo oxidative metabolism in the same final pathway. They are dependent upon a sufficient supply of oxygen, continued flux through the Krebs cycle and uninterrupted flow through the electron transport chain. It is therefore unsurprising that βHB behaves like fatty acids with regard to contribution to oxidation with changing exercise intensity.

3.4.6 Carbohydrate availability and ketone oxidation

The most striking finding of this study was the marked effect of carbohydrate restriction on the capacity of skeletal muscle to oxidise βHB. The restriction of dietary carbohydrate for 36 hours following a glycogen depleting exercise session, resulted in a 30% reduction in ketone oxidation (measured by $\dot{V}O_2$ester) from 8% to 5.5%.

It is important to distinguish whether the differences seen in ketone oxidation rate between carbohydrate replete exercise at 75% $\dot{V}O_{2max}$ and carbohydrate deplete exercise at 75% $\dot{V}O_{2max}$ were due to the elevated NEFA levels or carbohydrate depletion. The inclusion of condition B in the protocol, which combined carbohydrate replete exercise at 75% $\dot{V}O_{2max}$ combined with an Intralipid® infusion to elevate NEFA, allowed the effects of NEFA elevation and carbohydrate depletion to be considered independently. The striking similarity of ^{13}C recovery from carbohydrate replete exercise without (A) and with (B) elevated NEFA supports the conclusion that the reduced ketone oxidation seen following glycogen depleting exercise and a 36-hour low-carbohydrate diet (C) is attributable to carbohydrate depletion rather than to elevation of circulating fatty acids.

There is an aphorism among exercise physiologists that “Fat burns in the flame of carbohydrate”. This whimsical phrase is intended to suggest that carbohydrate is one of the principle sources of carbon to replenish, or increase, the intermediates of the Krebs cycle (156) allowing the oxidation of any fuel to proceed to completion. Anaplerosis is a term,

coined by Kornberg in 1966, that refers to any metabolic pathway that supplements a metabolic cycle (157). The reverse process is cataplerosis. In the case of the Krebs cycle anaplerosis and cataplerosis are in balance. If cataplerosis exceeds anaplerosis for a time, then flux through the cycle is reduced, as the oxaloacetate, required to condense with acetyl-CoA to regenerate citrate, is exhausted. Likewise, when abundant acetyl-CoA is available, from the beta oxidation of fatty acids within the mitochondrion, then, in the absence of any anaplerotic *resupply*, this will rapidly exhaust the resting supply of oxaloacetate. During exercise, the density of Krebs cycle intermediates may actually increase by up to fourfold, with an associated increase in Krebs cycle flux of 70-fold (158). This anaplerotic effect increases the capacity of skeletal muscle to oxidise fat (158). When skeletal muscle glycogen is depleted, the capacity to burn fat, as well as aerobic oxidation of carbohydrate, are impaired (159). This is referred to colloquially as ‘hitting the wall’ among runners, or ‘bonking’ among cyclists.

The findings of this study raise the possibility that ketones behave similarly to fat, not only in terms of their relationship to exercise intensity, but also to carbohydrate availability. Both these substrates are subject to the same restrictions in Krebs cycle flux capacity that result from an increase in the cataplerosis/anaplerosis ratio (Figure 3.11).

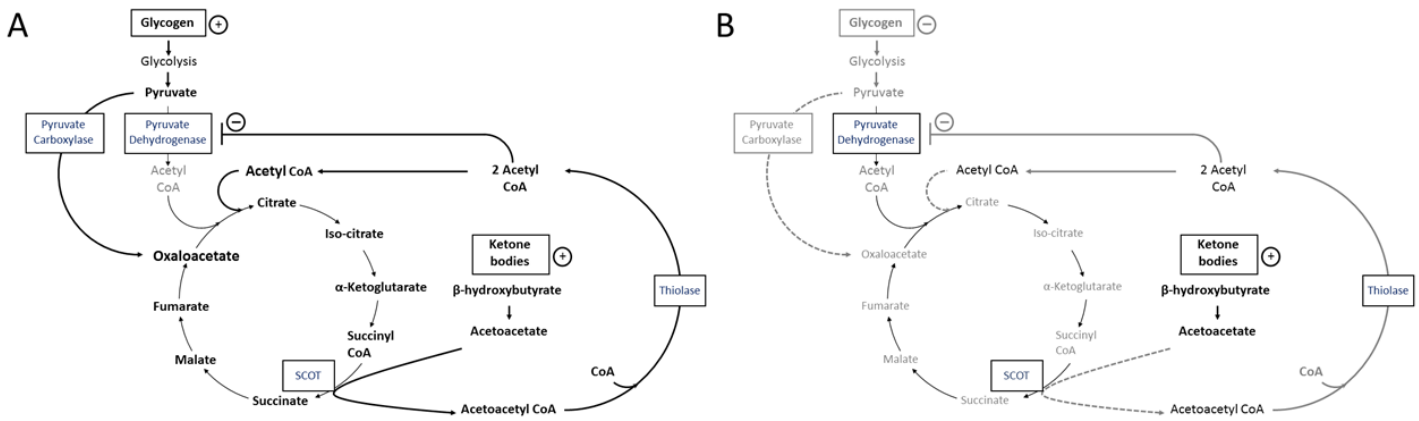


Figure 3.11 – suggested mechanism to explain the importance of carbohydrate availability to ketone oxidation. In the carbohydrate replete state (A) there is anaplerotic supply of oxaloacetate, allowing expansion of Krebs cycle intermediates and enhanced Krebs cycle flux. When carbohydrate is depleted (B) there is inadequate availability of oxaloacetate to condense with acetyl-CoA, which is abundant, reducing the capacity for oxidation. CoA = coenzyme A; SCOT = succinyl-CoA-3-oxoacid transferase.

This same hypothesis would account for the contradictory findings of Féry and Balasse, who described ketone net *uptake* by skeletal muscle in 3-day fasted individuals exercising at 60% $\dot{V}O_2$max for 30 mins (129) but net *release* in 3-day fasted individuals exercising at 50% $\dot{V}O_2$max for 2 hours. The exercise intensity is similar in the two studies but the duration differs substantially. Given the hypothesis proposed above (Figure 3.11), it is plausible that net uptake, seen in short duration exercise, is reversed when the muscle glycogen stores are exhausted. This duration has been described to be 60-90 mins which lies in between the durations of these contrasting studies (145, 160). Indeed, if we examine the time course of βHB during the overnight fasted, 2-hour exercise study of Féry and Balasse (Figure 3.12) we do indeed see a fall in βHB and acetoacetate during the first 60 mins, followed by a subsequent rise during the final 60-120 mins. The data from this study, suggest that muscle glycogen concentration is an important determinant of ketone oxidation rate.

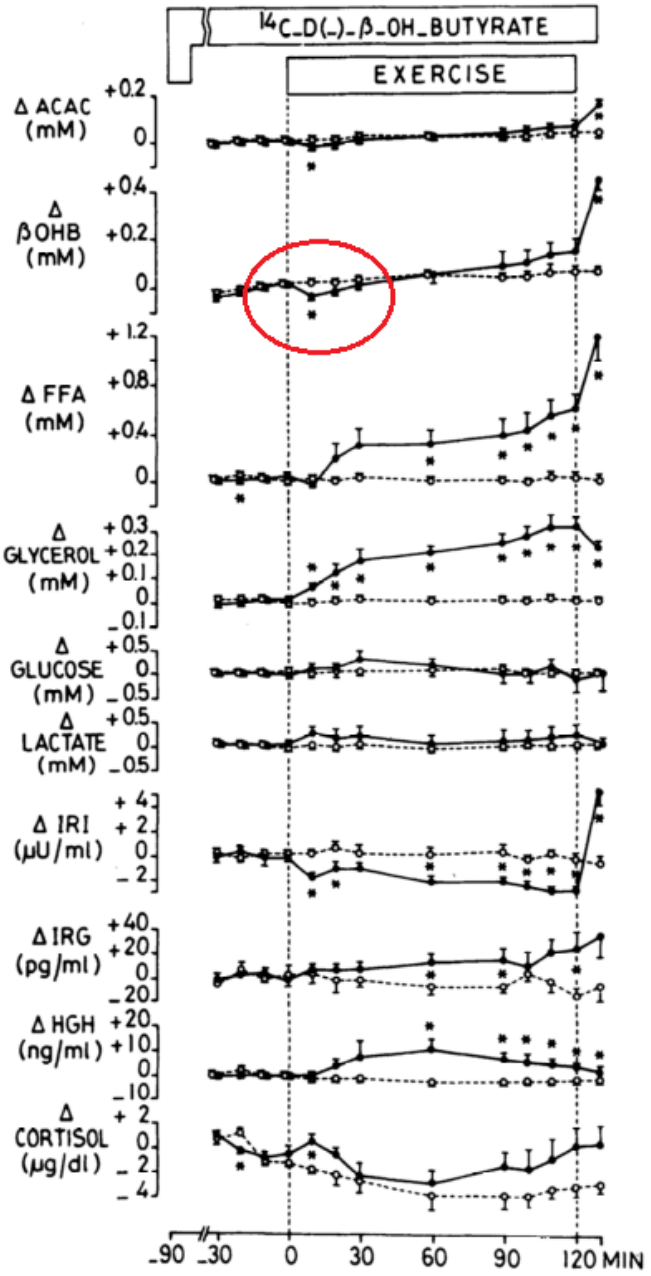


Figure 3.12 – excerpt from Féry and Balasse (1983) (70). The fall in circulating βHB (circled in red) is reversed from approximately 60 min, which would coincide with glycogen depletion in these overnight fasted, untrained participants.

Conclusion

Ketones were oxidised at rest, and during light and high intensity exercise, in man. Total ketone body oxidation increased with exercise intensity. However, as oxygen uptake increased, the relative contribution of ketone bodies to total oxidation fell, reminiscent of the behaviour of fatty acids as a fuel. These findings strongly support the novel hypothesis that skeletal muscle glycogen is necessary to permit ketone oxidation. Glycogen's permissive function may act through its putative role as an anaplerotic donor.

Following the conclusion that carbohydrate availability influences βHB metabolism, the work described in the next chapter addresses the reciprocal question of whether βHB availability may influence carbohydrate metabolism.

4 Ketones and glycogen recovery following exercise

Abstract

Background: The work presented in this chapter sought to determine, for the first time in humans, whether exogenous ketones, given as a ketone ester drink, can increase glucose disposal beyond the levels achieved by carbohydrate alone.

Methods: This study was of a 3-arm, randomised, blinded crossover design. Following glycogen-depleting exercise, participants recovered for 2 hours, during which they were given, in randomised visit order:

- control drink, then saline infusion – *saline control*
- control drink, then glucose infusion *clamped* at 10 mM – *glucose control*
- ketone drink then glucose infusion *clamped* at 10 mM – *glucose plus ketone*

A biopsy was taken before and after each of the 2 hour recovery periods for the measurement of muscle glycogen content.

Results: Following ingestion of the ketone ester, βHB levels rose rapidly to a peak of 5.3 mM at 40 mins, and remained ≥ 3.3 mM throughout the clamp. During 2 h recovery, glucose disposal was 32% faster for *glucose plus ketone* than *glucose control* (0.83 vs. 0.63 g/kg/h, $p < 0.0001$). Faster glucose disposal was associated with 50% higher post-recovery glycogen (246 vs. 164 mmol/kg dry weight muscle, $p < 0.05$) and a 2-fold greater endogenous insulin release (31 vs. 16 mU/l, $p < 0.001$) by the end of the 2 h glucose clamp.

Conclusion: In humans, a ketone ester drink increased glucose uptake and enhanced muscle glycogen synthesis beyond levels achieved by hyperglycaemia alone. A possible mechanism for this is increased uptake of glucose, mediated by higher insulin levels, combined with ketone-mediated inhibition of glycolysis, both acting to divert glucose-6-phosphate away from oxidation and towards glycogen synthesis.

4.1 Introduction

4.1.1 Glycogen and Ketones

In 1966, Jonas Bergström and Eric Hultman demonstrated that muscle glycogen is the key determinant of endurance exercise capacity in man. Following the publication of their findings in *Nature*, a seminal series of experiments confirmed that endurance correlates with initial muscle glycogen stores, and that glycogen exhaustion critically impairs the capacity for external muscular work (145, 160, 161). These discoveries prompted decades of investigation into the optimal dose and formulation of carbohydrate, and supplemental insulinotropic amino acids, to enhance muscle glycogen recovery (*see below* 4.1.2). During exercise, regular consumption of soluble carbohydrate has a beneficial effect on endurance capacity (162). However, exogenous carbohydrates are unable to provide more than 15-25% of ongoing energy requirements and glycogen falls inexorably with ongoing exercise (163, 164). The issue of how to maximise the storage of skeletal muscle glycogen therefore remains central to exercise physiology.

In addition to providing a key alternative to glucose for brain, skeletal muscle and cardiac metabolism (2), ketone bodies act as a powerful signal to conserve precious stores of carbohydrate and switch to the alternative energy source, fat (65). In rodent and canine models, ketone body supplementation, in addition to high-dose carbohydrate, can increase the deposition of glycogen within both skeletal and cardiac muscle (73, 84, 165). The same effect has never been demonstrated in man. Recently, it has been demonstrated in man that ketone bodies can alter fuel preference during exercise, away from carbohydrate and towards fat oxidation (166), recapitulating earlier findings in healthy, anaesthetised dogs (147). It is reasonable therefore to test whether ketones may also influence fuel handling in the post exercise period.

4.1.2 Carbohydrate delivery

Given the importance of glycogen to the performance of both short-duration, high-intensity exercise and long bouts of low-intensity endurance efforts, there has been considerable interest in how to maximise carbohydrate delivery by optimising the diet following exercise (167).

There is evidence to suggest that carbohydrate replenishment should be initiated early. Ivy et al found that there was 45% lower glycogen recovery if carbohydrate refeeding was delayed by 2 hours compared to starting immediately post-exercise (168). Several studies have examined the effects of carbohydrate on glycogen recovery post exercise (169, 170). In these studies, Blom and Ivy concluded independently that glycogen synthesis could not be enhanced by delivering more than 0.7-0.75 g/kg/h carbohydrate. In contrast to these findings, van Loon et al (2000) found that giving doses of 1.0-1.2 g/kg/h *did* increase glycogen synthesis post exercise (126). The reason for these contradictory findings may have been the *frequency* of carbohydrate intake of the total dose. In the studies of Blom and Ivy, drinks were taken at 2-hourly intervals, whereas in van Loon's study , boluses were given every 30 mins. Some studies have reported higher glycogen synthesis rates resulting from even higher carbohydrate doses of 1.0 -1.85 g/kg/h at frequent intervals (15-60 min) (171-174).

It is known that at sub-maximal carbohydrate replenishment doses, the addition of protein (e.g. wheat protein hydrolysate) or specific amino acids (arginine, glutamine, leucine, phenylalanine) can increase glycogen synthesis rates (presumed to be through insulinotropic mechanisms: substances that stimulate insulin release) (8). However, the insulinotropic amino acid, leucine, does not enhance glycogen synthesis over and above that associated with 1.2 g/kg/h carbohydrate alone (174).

An important consideration of the ideal diet to optimise glycogen synthesis is the precise composition of the carbohydrates used. Kiens et al (1990) have demonstrated that, in an isocaloric, equal carbohydrate dose, comparison between a high glycaemic index and low glycaemic index, the high glycaemic index formulation resulted in ~61% faster glycogen synthesis during the first 6 hours of recovery compared to the low glycaemic index (175).

Fructose requires conversion into glucose in the liver before it is available for glycogen synthesis (176). This is likely to be the reason that isocaloric consumption of fructose, compared to glucose, is associated with lower rates of glycogen synthesis (170, 177). However, the rate of intestinal uptake of glucose is limited by its dependence on the sodium –glucose cotransporter (SGLT1). Saturation of SGLT1 limits gut uptake of glucose to rates ≤ 1g/min (178). Fructose (a 'multiple transportable carbohydrate' (121)) is transported across gut epithelium by GLUT-5, rather than SGLT1, and so provides an additional, albeit slower, route of uptake for carbohydrate. For this reason, the oral delivery of a mixture of glucose and fructose enhanced exogenous carbohydrate oxidation during exercise by 13-65% (179, 180).

When taking into account the amassed evidence for optimising glycogen recovery following exercise, no oral, post-exercise regimen in man has been shown to be superior to 1.0-1.2 g/kg/h carbohydrate over 4-6 hours (128, 159).

The aim of the study in this chapter was to determine if the provision of ketone bodies, in the form of a ketone ester supplement, could enhance glycogen synthesis beyond that achievable by supraphysiological levels of carbohydrate alone.

4.2 Methods

4.2.1 Participants

Twelve well-trained male British servicemen were recruited to the study (General Methods 2.2). All performed 4-6 hours per week of endurance training and had a $\dot{V}O_{2max}$ > 3.8 l/min. All participants provided written informed consent.

Table 4.1 – Baseline characteristics of study participants

Characteristic	Mean ± SEM
Age (years)	32.8 ± 0.5
Height (m)	1.77 ± 0.01
Weight (kg)	75.8 ± 0.4
BMI	24.3 ± 0.1
Fasting glucose	4.30 ± 0.03
$\dot{V}O_{2max}$ (l/min)	4.30 ± 0.04
$\dot{V}O_{2max}$ (ml/kg/min)	57.0 ± 0.4
Peak power output (W)	316 ± 3

4.2.2 Baseline visit

Each participant attended a baseline visit to complete a cardiopulmonary exercise test to volitional fatigue (General Methods 2.4) to allow the measurement of maximal oxygen uptake ($\dot{V}O_{2max}$) and peak power output (PPO). PPO was used to prescribe the workloads for the glycogen depletion protocol.

4.2.3 Glycogen depletion protocol

Participants attended each study visit following an overnight fast and started the visit by completing a standardised high-intensity interval exercise protocol to the point of complete physical fatigue (General Methods 2.5). This protocol has been demonstrated to reliably deplete skeletal muscle glycogen (8, 122).

4.2.4 Ketone and control drinks

After completion of glycogen depletion, participants consumed either a ketone drink, or an acaloric taste and appearance-matched control drink of equal volume. The ketone drink contained 0.573 ml/kg [615 mg/kg] of the ketone ester (R)–3–hydroxybutyl (R)–3 hydroxybutyrate. The natural bitter taste was partially masked with a citrus flavouring (Symrise, Holzminden, Germany) and proprietary sweetener. The control drink contained the same citrus and sweetener components as the ketone drink. The bitter taste was matched with the addition of a weight-dosed commercial bitter agent (Symrise, Holzminden, Germany). The drink was given immediately following the post-glycogen-depletion muscle biopsy and the clamp was started 30 mins following ingestion.

4.2.5 Blood sampling and analysis

Blood sampling and analysis was conducted as previously described (General Methods 2.8).

In addition, blood samples taken at 5 min intervals during the clamp were measured for whole blood glucose with a near-patient benchtop device (HemoCue 201^{+}, Radiometer, Copenhagen, Denmark).

4.2.6 Hyperglycaemic clamp and sham clamp

The hyperglycaemic clamp was conducted according to the original method described by de Fronzo and colleagues (127). This method of providing carbohydrate following exhaustive

exercise was chosen for three reasons. First, the technique provides a robust means to standardise the glucose availability to skeletal muscle across different visits for the same participant, and between different individuals. Second, by delivering glucose intravenously, it was possible to avoid the variations in time and magnitude of nutrient delivery that confound oral ingestion and enteral absorption methods. Finally, it was possible to ensure that glucose delivery to the muscle was at least as high as would be provided by recommended optimum post-exercise carbohydrate feeding of 1.0–1.2 g/kg/h over 4-6 hours (128). This meant that any difference in glucose disposal and/or glycogen synthesis observed with the ketone intervention was more likely to be biologically relevant.

Participants had two IV cannulae: one in the antecubital fossa (20 gauge), for infusion of 20% glucose (20% dextrose, Baxter Corporation, Staines, UK) and one in the dorsum of the contralateral hand (22 gauge) for sampling whole blood glucose at five min intervals. The limb containing the sampling cannula was heated to 40-44 ºC using two therapeutic heat pads (HK35 Beurer, Ulm, Germany) wrapped around the hand and forearm to cause maximal vasodilatation and generate a 'pseudoarterialized' blood sample. A priming dose of 240 mg/kg glucose was given over the first 15 mins of the clamp, after which the glucose infusion rate was adjusted to maintain a whole blood glucose of 10 mM, measured from the pseudoarterialized sample using a validated benchtop analyser (HemoCue 201^{+}, Radiometer, Copenhagen, Denmark). At the end of the 2-hour clamp, a second muscle biopsy was taken. The participant was then given a moderate glycaemic index meal and the dextrose infusion rate was gradually reduced to zero. Participants were observed until euglycaemia had been maintained for a period of 30 mins after cessation of glucose infusion. All outward appearances and measurements associated with the saline *sham* clamp were identical; the fluid infused was 0.9% NaCl solution rather than 20% glucose.

4.2.7 Muscle biopsy

Muscle biopsies were all conducted as previously described (General Methods 2.9). Biopsies were taken immediately following completion of the glycogen depletion protocol and then immediately following the 2-hour recovery period.

4.2.8 Glycogen analysis

Muscle glycogen analysis was conducted according to the protocol described by van Loon et al (126) as described above (General Methods 2.9.2).

4.2.9 Statistics

A two-way ANOVA was used to compare metabolite and insulin levels and experimental conditions across the 3-arms of the study. Glucose disposal and comparisons of muscle glycogen between the *glucose control* and the *glucose plus ketone* condition were analysed using Student's t-tests. A p value of < 0.05 was taken to indicate statistical significance.

4.2.10 Study design

The study was of a randomised, blinded, crossover design, comprising three arms. Participants attended a baseline visit for familiarisation with experimental conditions and to complete an incremental maximum exercise test, measuring maximum oxygen uptake ($\dot{V}O_{2max}$) and peak power output (PPO), which allowed individualised prescription of the glycogen depletion protocol. Each study visit followed an overnight fast and commenced with a standardised, validated exercise-interval protocol to deplete muscle glycogen (122). Participants ingested a taste- and appearance-matched ketone or control drink prior to *either* a two hour hyperglycaemic clamp with IV 20% glucose infusion to *clamp* whole blood glucose at 10 mM, *or* a *sham* clamp using normal saline (0.9% NaCl solution). The three recovery conditions used were:

- Control drink then saline *sham* clamp - *saline control*
- Control drink then hyperglycaemic clamp – *glucose control*: 'gold standard'
- Ketone ester drink then hyperglycaemic clamp – *glucose plus ketone*

Muscle samples were obtained from the *vastus lateralis* straight after glycogen depletion and then immediately following the two-hour recovery period. Venous blood was sampled at five min intervals for whole blood glucose and at regular intervals to measure energy substrate and insulin concentrations (Figure 4.1).

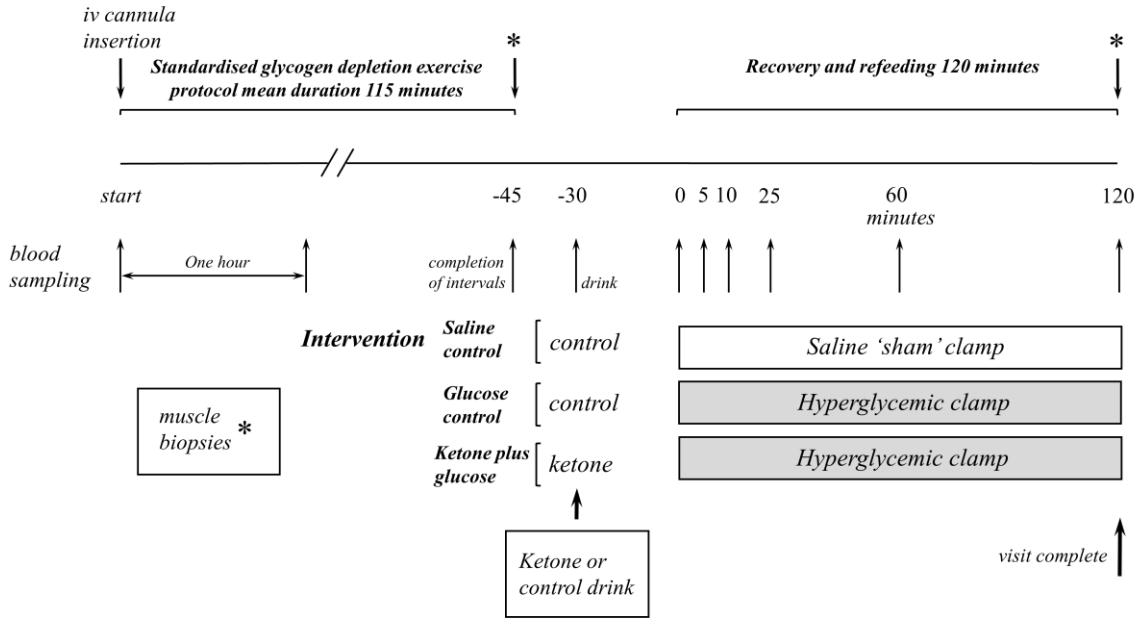


Figure 4.1 – Schematic of study visit.

4.3 Results

4.3.1 Glycogen depletion protocol

In total, 36 glycogen depletion protocols were completed for the three conditions. The characteristics of these glycogen depletion episodes are shown in Table 4.2 and heart rates during glycogen depletion are shown in Figure 4.2.

Table 4.2 – Characteristics of glycogen depletion protocol

Characteristic	Mean ± SEM
Number of ntervals	58 ± 1
Number of intervals at 90% PPO	27 ± 1
Duration of depletion (mins)	115 ± 2
Total external work done (megajoules)	1.70 ± 0.03
Total external work done (kilocalories)	400 ± 7
Whole blood glucose at completion (mM)	3.30 ± 0.04

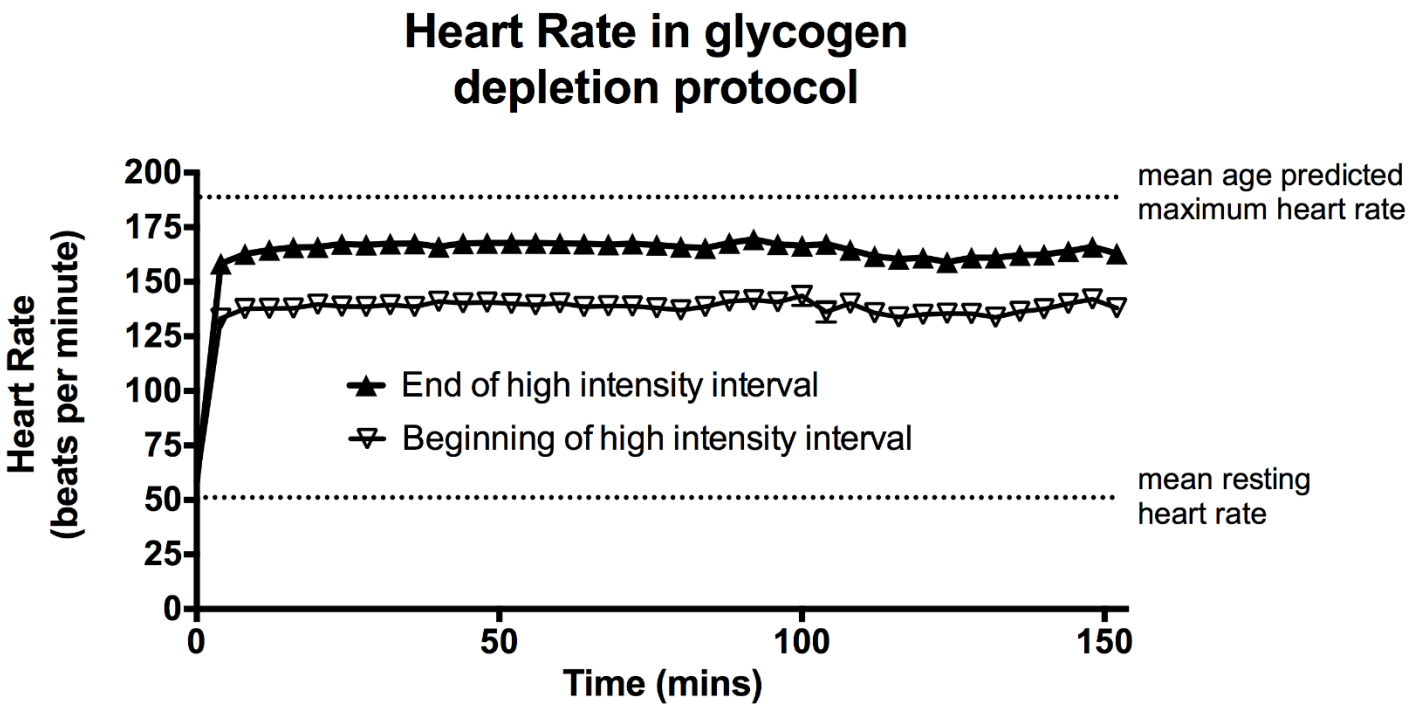


Figure 4.2 – Heart rate during glycogen depletion protocols

4.3.2 Clamp conditions

4.3.2.1 Ketone concentration

In both the glucose control and the saline control groups, βHB levels (Figure 4.3) increased from 0.20 to 0.75 mM, $p < 0.0001$ in the 45 mins following cessation of exercise. In the *glucose control,* post-exercise ketosis was abolished during the hyperglycaemic clamp, with βHB falling to 0.2 mM by the end of the clamp. Following ingestion of ketone ester, βHB levels rose rapidly to peak at 5.3 mM at 40 mins after ingestion with βHB falling to 3.3 mM by the end of the clamp. There was a difference between *glucose plus ketone* and the other two conditions throughout the clamp ($p < 0.0001$). In the *saline control* (with no exogenous glucose or ketone bodies) βHB continued to rise throughout the clamp to a peak of 1.6 mM, differing from the *glucose control* for the final hour of the clamp ($p < 0.05$).

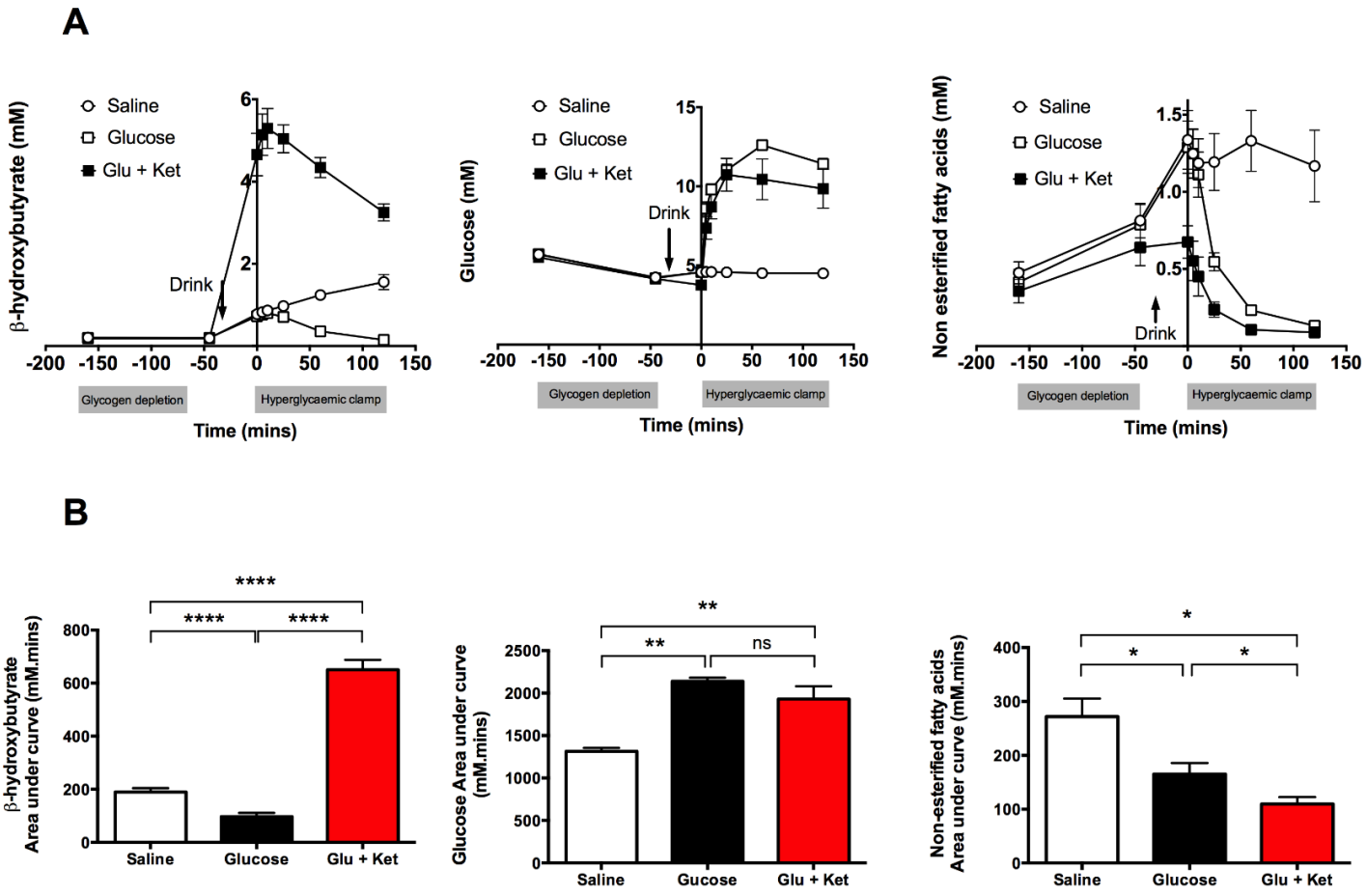


Figure 4.3 – D-β-hydroxybutyrate, glucose and non-esterified fatty acid (NEFA) concentrations during each study visit (Panel A). Area under the curve (AUC) measures of βHB, glucose and NEFA during the 2-hour recovery clamp (Panel B).

4.3.2.2 Glucose concentration

Plasma glucose (Figure 4.3) at the start of each visit, following an overnight fast, was 5.8 mM, but fell by 30% to 4.1 mM ($p < 0.0001$) following glycogen depletion. In the *saline control*, plasma glucose was 4.5 mM during the clamp, lower than both *glucose control* (10.7 mM) and *glucose plus ketone* (9.4 mM) throughout ($p < 0.001$). Plasma glucose did not differ significantly between *glucose control* and *glucose plus ketone*.

4.3.2.3 Non-esterified fatty acid concentration

Following the overnight fast, the mean NEFA concentration was 0.40 mM (Figure 4.2). After glycogen depletion exercise it rose to 0.75 mM ($p < 0.0001$). In both the *glucose control* and *saline control,* NEFA levels continued to rise after the drink to 1.3 mM ($p < 0.0001$). In *glucose plus ketone,* the rise after the drink was abolished (peak 0.68 mM) and NEFA was lower than both control conditions from 0-10 mins of the clamp ($p < 0.0001$). At the onset of the hyperglycaemic clamp, NEFA levels fell in the *glucose control*. In *saline control* NEFA remained at ≥ 1.2 mM throughout the clamp, and was higher than the other two conditions ($p < 0.0001$).

4.3.3 Lactate

Plasma lactate (Figure 4.4) was 1.5 mM at rest and rose to 5.5 mM following glycogen depletion ($p < 0.0001$). After 45 mins of rest the lactate fell to 1.8 mM and 1.7 mM in *glucose control* and *saline control* respectively. In *glucose plus ketone,* lactate fell significantly less, to 2.1 mM, higher than either control condition ($p < 0.0001$). The lactate level remained elevated in *glucose plus ketone*, between 0.4 – 0.6 mM above both control conditions ($p < 0.0001$).

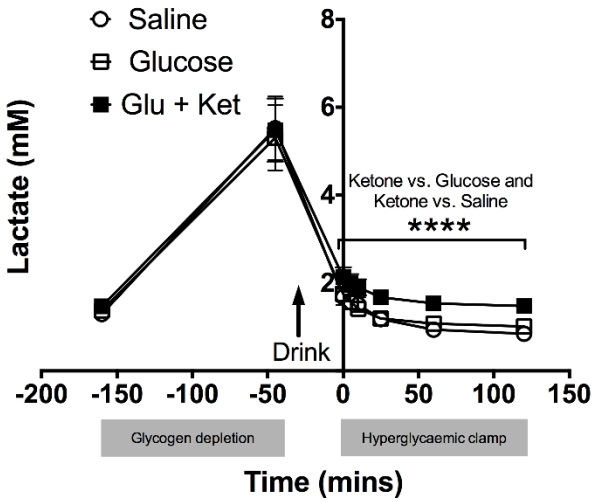


Figure 4.4 – Plasma lactate during study visits.

4.3.4 Insulin

Following an overnight fast, plasma insulin was 3.7 mU/l (Figure 4.5). After glycogen depletion, it fell to 1.0 mU/l ($p < 0.0001$). In the *saline control*, in which no exogenous glucose was administered the insulin remained below 2.2 mU/l for the remainder of the clamp, lower than throughout *glucose control* and *glucose plus ketone* ($p < 0.05$). At completion of the hyperglycaemic clamp, insulin levels rose to 16.4 mU/l in *glucose control* and to 31.1 mU/l in *glucose plus ketone* ($p < 0.001$).

4.3.5 Glucose disposal

Glucose disposal (Figure 4.5) during the clamp was 22% higher in *glucose plus ketone* than *glucose control* (0.83 g/kg/h vs. 0.63 g/kg/h, p 0.0001). The difference in glucose disposal was significant from 45 mins to the end of the 2-hour clamp ($p < 0.01$).

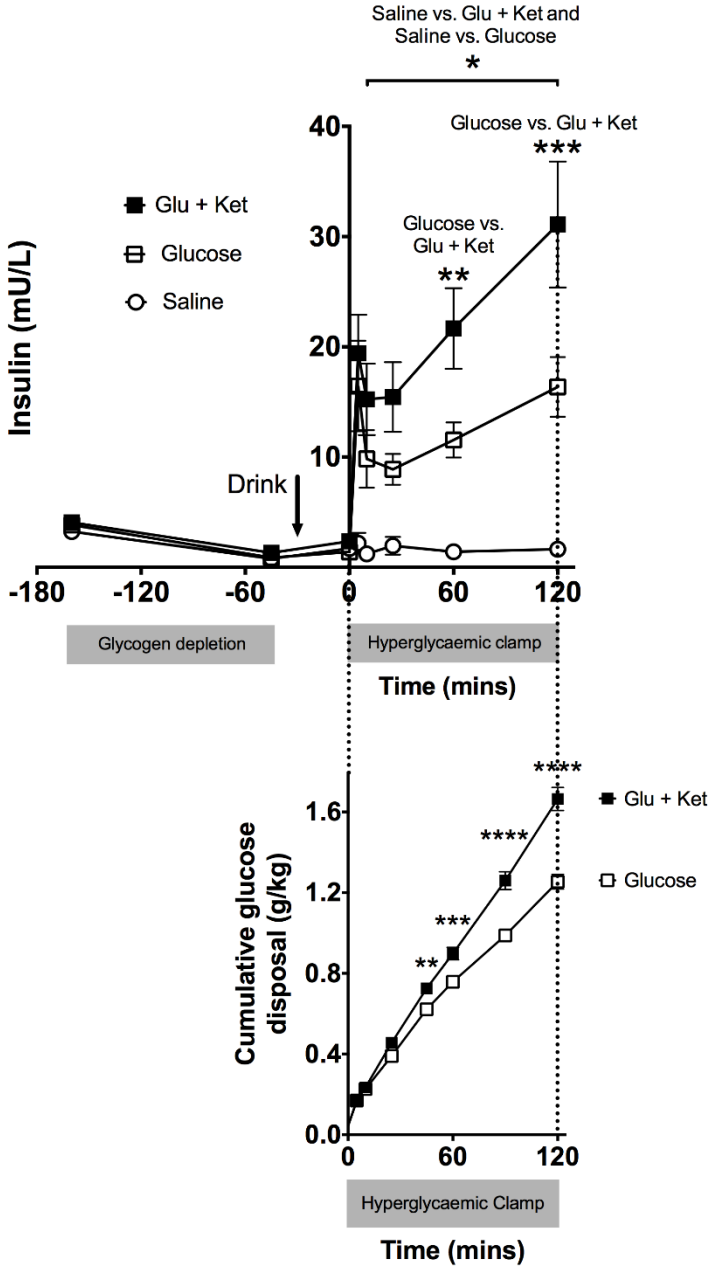


Figure 4.5 – Insulin concentration throughout the study visits and cumulative glucose disposal during the 2-hour recovery clamp.

4.3.6 Glycogen

Following depletion, glycogen (mean of all conditions) was 115 mmol/kg dry weight of muscle (mmol/kg $_{DW}$). The quantity of glycogen in skeletal muscle increased under all conditions (Table 4.3, Figure 4.6) but following recovery was higher in *glucose plus ketone* than *glucose control*. The increase in glycogen over 2-hours of recovery was higher in

glucose plus ketone vs. *glucose control* and higher in *glucose control* vs. *saline* control (Table 4.3).

Table 4.3 Glycogen concentrations pre and post recovery

Condition	Pre-recovery glycogen mmol/kg$_{DW}$	Post-recovery glycogen mmol/kg$_{DW}$	Increase in glycogen mmol/kg $_{DW}$/h
Saline control	134 ± 28	167 ± 34	35 ± 23
Glucose control	94 ± 15	164 ± 13	70 ± 13‡
Glucose plus ketone	132 ± 20	246 ± 32*†	114 ± 23*††

Values are mean ± SEM

∗ = p < 0.05 for *glucose plus ketone* vs. *glucose control;* † = p < 0.05 for *glucose plus ketone* vs. *saline control;* †† = p < 0.01 for *glucose plus ketone* vs. *saline control;* ‡ = p < 0.05 for *glucose control vs. saline.*

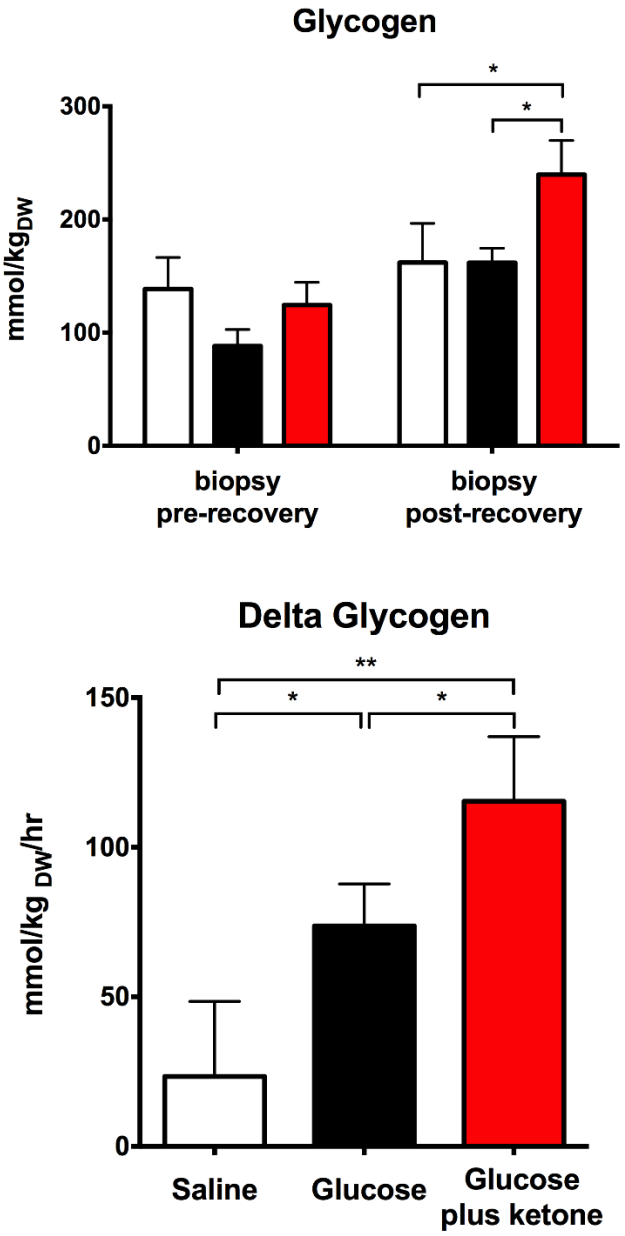


Figure 4.6 – Skeletal muscle glycogen pre- and post- recovery period (Panel A). The increase in glycogen during 2 hours recovery (Panel B).

4.4 Discussion

The novel findings of this study were that the supply of exogenous ketones, in addition to a *gold standard* dose of carbohydrate, increased whole-body glucose disposal (32% increase, $p < 0.0001$) and endogenous insulin release (doubling of insulin in the second hour of recovery, $p < 0.01$) in comparison to a *gold standard* dose of carbohydrate alone. These changes were associated with an increase of more than 50% in the post-recovery muscle glycogen. None of these findings have previously been reported in man.

4.4.1 Experimental protocol

High doses of oral carbohydrates can cause gastric irritation and stomach pain (123). Furthermore, there is considerable inter-individual variation in the rates of uptake of orally-delivered carbohydrate into the bloodstream. So, in a controlled study using the oral route of carbohydrate delivery, it is difficult to determine how much glucose has reached the skeletal muscle and other tissues. As a further confounder, it is not known what interaction may occur between oral ketone bodies and oral carbohydrate that may interfere with either the rates of carbohydrate or ketone body absorption. John Ivy's group have demonstrated, at rates very similar to those used in this study, that the iv infusion of glucose *per se* does not alter either endogenous insulin release, or glycogen synthesis rates compared to the same glucose solution delivered orally (126).

For these reasons it was decided to study the effects of exogenous ketone bodies delivered orally as a ketone monoester whilst providing carbohydrate intravenously. The use of an intravenous hyperglycaemic clamp, as described originally by de Fronzo et al (127), allowed the exact circulating concentration of glucose to be adjusted and maintained, and therefore rigid standardisation of the glucose availability between different individuals, and within individuals, across study visits. Another advantage of this method, in addition to allowing

robust *matching* of glucose availability, was that it permitted precise and accurate measurement of the rate of glucose disposal throughout the clamp period.

4.4.2 Glucose disposal and endogenous insulin

The consensus within the exercise physiology community is that the optimum oral dose of carbohydrate for recovery of muscle glycogen following exercise is 1.0 – 1.2 g/kg/h of multiple transportable carbohydrates over 4-6 hours (159, 181). It is interesting that in the current study, the maximum rate of glucose disposal with a supraphysiological availability of glucose was only 0.63 g/kg/h, even following arduous, glycogen-depleting exercise. This suggests that at least part of an oral dose of carbohydrate may not be rapidly assimilated into muscle, but instead supplies a gastric, vascular and/or hepatic *reservoir* of carbohydrate, which then allows muscle glycogen synthesis over a protracted period.

The striking finding of this study has that the addition of ketones to a *supraphysiological* concentration of glucose led to a 32% increase in glucose disposal, over and above that caused by 10 mM circulating glucose alone. The increased glucose intake may have been explained by the *doubling* of endogenous insulin release in response to the simultaneous presence of glucose (10 mM) and βHB (3-5 mM).

There is an initial, rapid and relatively 'insulin insensitive' period of glucose uptake and glycogen synthesis immediately following the cessation of exercise, lasting approximately 30-60 mins (167, 182) and believed to be due to physical-contraction-dependent mechanisms that increase the incorporation of the glucose transporters, GLUT4, into the sarcolemma of exercising muscle (183-185) and a contraction-mediated increase in glycogen synthase activity (186, 187). As the mechanically-coupled effects subside, glucose supply to skeletal muscle more closely approximates the resting insulin-dependent state, but with *enhanced* insulin-sensitivity (188). This biphasic response, and the initial *insulin*

insensitive period, may explain some findings of the study (Figure 4.3). The hyperglycaemic clamp commenced 45 mins after completion of glycogen-depleting exercise. In spite of an early and clear separation in endogenous insulin release between the two hyperglycaemic conditions, the maximal differences in glucose disposal were not seen until approximately 1½ hours post exercise (Figure 4.5). A possible explanation for this is that, for the first 60-90 mins post exercise the muscle was still completely, or relatively, insensitive to insulin because GLUT-4 incorporation at the sarcolemma and glycogen synthase activity were already at a maximum, subsequent to contraction (167, 182). After 90 mins, when there is no residual mechanically-mediated enhancement of glucose uptake, the effects of an increase in endogenous insulin release are easier to discern.

On further analysis of the rates of glucose disposal, there was a strong, significant negative correlation between body mass index (BMI) and the rate of glucose disposal (Figure 4.7) in both the *glucose control* and in the *glucose plus ketone* conditions. The fact that no correlation existed for glucose disposal and insulin sensitivity, assessed by baseline homeostatic model assessment of insulin resistance (HOMA) scores (189), suggests that BMI may be a surrogate for adaptation to endurance exercise and capacity to recover carbohydrate stores. BMI may be a better predictor of the capacity for glucose uptake post-exercise than resting insulin sensitivity.

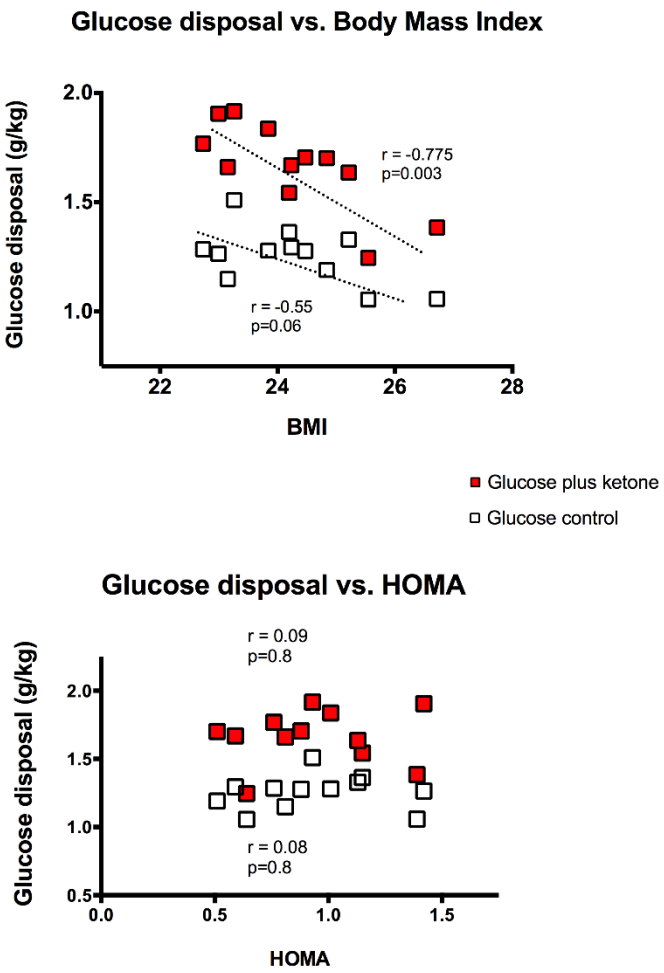


Figure 4.7 – Glucose disposal with BMI and HOMA. Upper panel: glucose disposal vs. Body Mass Index (BMI), Lower panel: glucose disposal vs. homeostatic model assessment of insulin resistance (HOMA) score.

The near-patient, benchtop-analyser *whole blood* glucose measurement was 3.3 mM following glycogen depletion, contrasting with 4.1 mM *plasma* glucose. It is accepted that whole blood glucose is 10-15% lower than plasma glucose, based on the difference between plasma and whole blood volume accounted for by the red cell volume (190). Exercise has the potential to cause dehydration, but is also known to raise haematocrit by a combination of vascular exudation of fluid and the *autologous* transfusion of red cells into the circulation from the spleen (191). The resulting increase in haematocrit by any of these mechanisms

effectively *dilutes* the plasma with cells and is therefore likely to account for the 20% discrepancy that we found between whole blood and plasma glucose.

4.4.3 Ketones and insulin release – adaptive significance

It is notable that endogenous insulin was unaffected by the ketone drink 30 mins after ingestion. The mean glucose concentration at this time was 4.1 mM. Within five mins of starting the glucose infusion however, a larger increase in insulin release in the *glucose plus ketone* vs. the *glucose control* became evident. It is known from work performed on isolated rat beta cells in culture that ketone bodies promote insulin release only in the presence of glucose (5 mM), with no insulinotropic response to ketone bodies in the absence of glucose (192). The same investigators demonstrated that, in rats, there is a threshold of 1 mM βHB for any insulin release and the potentiation of insulin release increases with βHB concentration to a peak at 10 mM with no further increase at 15 mM. The order of efficacy of ketone bodies in potentiation of insulin release was D-βHB > racemic mixture of βHB > acetoacetate [efficacy of D-β-hydroxybutyrate approximately twofold that of acetoacetate] (192). Physiological situations in which βHB and glucose may simultaneously be elevated to levels that cause an insulin response include the transition from fasting to fed, and feeding during post-exercise ketosis. In both these situations one might speculate that an increase in endogenous insulin into the portal circulation would be useful to signal an end to hepatic glucose output, in order to conserve precious liver glycogen.

The doubling of endogenous insulin release has biological implications beyond enhanced glucose disposal, glycogen synthesis and glycogen preservation. Insulin has an anabolic action on skeletal muscle, inhibiting muscle catabolism that normally follows exercise (193, 194). In the situation of adequate carbohydrate provision during post-exercise ketosis,

insulin may protect the skeletal muscle from inappropriate breakdown, such as that demonstrated to occur during high-intensity physical activity on military operations (195).

4.4.4 Changes in other metabolites

Figure 4.3 demonstrates the reduction in circulating NEFA resulting from elevated βHB, an effect first described by Björntorp et al in 1966 (196). The mechanism for this effect is the negative feedback of βHB at the PUMA-G receptor in adipose tissue to restrict peripheral lipolysis (58, 60). This mechanism is essential both to prevent an uncontrolled ketoacidosis and to enable efficient use of fat stores in situations of low insulin/glucagon ratio, to prevent wasteful *excess* lipolysis. In the absence of ketone-mediated negative feedback, the NEFA levels in the *saline control* continued to rise steadily following cessation of exercise, reflecting appropriate increases in peripheral lipolysis under conditions of low glucose availability, and low insulin concentration. This physiological lipolysis represents an appropriate shift from carbohydrate to fat in supplying the body's energy requirements (28).

Lactate was elevated in the *glucose plus ketone* condition, compared to the controls, throughout the clamp. During exercise, ketone bodies *lower* the level of circulating lactate (166, 197), due to the suppression of glycolysis, both from product inhibition of the pyruvate dehydrogenase complex (caused by increased acetyl-CoA/CoA and increased NADH/NAD^+ ratios (78) and by inhibition of phosphofructokinase by increased citrate shuttling from mitochondrion to cytosol (79). The effect of ketone supplementation is to elevate both the βHB/acetoacetate ratio and acetyl-CoA levels (114, 166) leading to high acetyl-CoA/CoA and NADH/NAD^+ ratios which inhibit PDH flux and elevate pyruvate levels (75, 80). These effects, seen in exercise, are exacerbated at rest by the relatively decreased consumption of NADH (158). The elevated redox state of the mitochondrion can increase the redox state of the cytosol by *shuttling* of redox equivalents. Furthermore, the direct effect of a high

βHB/acetoacetate ratio in the cytosol, secondary to ketone ester administration will also serve to elevate the cytosolic NADH/NAD^+ ratio. The lactate dehydrogenase reaction is linked to the NADH/NAD^+ redox couple and an elevated NADH/NAD^+ ratio drives the reaction from pyruvate to lactate (Figure 4.8).

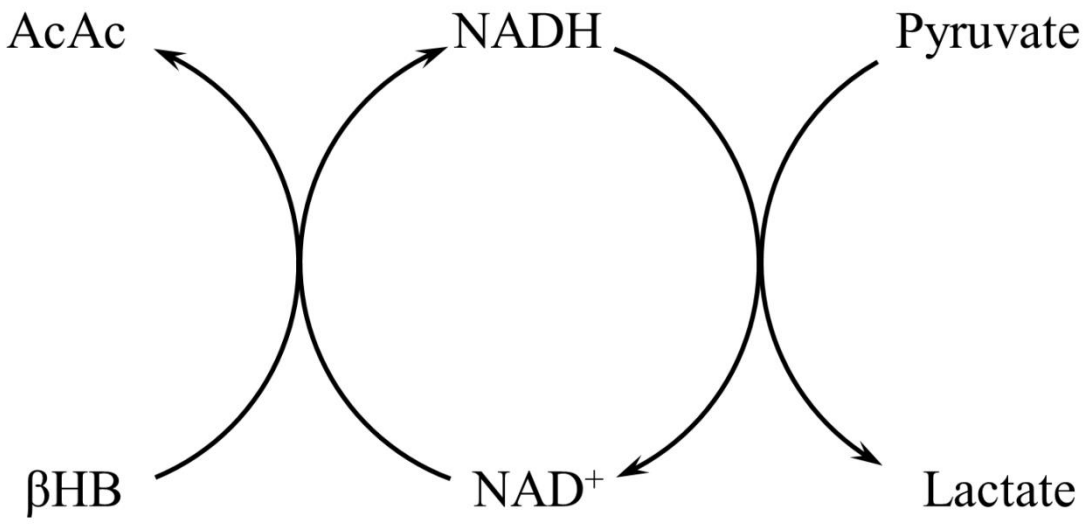


Figure 4.8 – Redox coupling of D-β-hydroxybutyrate and lactate (AcAc = acetoacetate)

It is likely therefore, that the increased *rate* of consumption of reducing equivalents during exercise will modulate the effects of ketone body inhibition on glycolysis and interconversion of pyruvate and lactate. This provides a potential explanation for D-β-hydroxybutyrate *increasing* lactate at rest and *decreasing* lactate during exercise.

4.4.5 Ketone bodies and glycogen synthesis

The same ketone body mediated mechanisms that inhibit both proximal (inhibition of *phosphofructokinase*) and terminal glycolysis (inhibition of *pyruvate dehydrogenase* kinase) are known to reduce glucose oxidation in the heart (75), diaphragm (76), and kidney (77) of rats and to both decrease glucose oxidation *and* increase glycogen synthesis in rat soleus muscle (73) and dog heart (165). The current study attempted to investigate if the same carbohydrate *sparing* and glycogen synthesis *acceleration* effects could be demonstrated in

man. It is interesting to compare the degree of glycogen depletion and subsequent rates of glycogen synthesis in this study (Table 4.3) with previous similar studies (Table 4.4).

Table 4.4 – Comparative list of human skeletal muscle glycogen studies

Study	Participants	Glycogen depletion protocol	CHO intake (g/kg)	CHO intake (g/kg/h)	Post exercise glycogen ($mmol/kg_{DW}$)	Time period (h)	Rate of glycogen synthesis ($mmol/kg_{DW}/h$)	Reference
1.	8 male	Cycling 2-min intervals at 90% and 50% VO_{2max} to exhaustion	84	1.2	106 176	0-3	40 25	Jentjens et al. (174)
2.	8 male	Cycling 2-min intervals at 90% and 50% VO_{2max} to exhaustion	55	0.75	72	0-5	25	Keizer et al. (198)
3.	11 male	Cycling 2h intervals at 70-100%max	77	1	116	0-2.5	37	McCoy et al. (199)
4.	5 male	Cycling 2-min intervals at 90% and 50% VO_{2max} to exhaustion	88	1.2	90	0-4	41	Van Hall et al. (8)
5.	8 male	Cycling 2-min intervals at 90% and 50% VO_{2max} to exhaustion	56	0.8	190	0-5	17	Van Loon et al. (126)
6.	8 male	Cycling 2h intervals at 60-65% and 70-75% VO_{2max}	56 **iv**	0.75	119 105 131	0-4	22 23.5 24	Reed et al. (200)
7.	5 male	Cylcing to exhaustion at 70% VO_{2max}	0	0	93	0-2.5	7	Maehlum & Hermansen (72)
Mean	*(excluding Study 7)*		69 ± 6	0.95 ± 0.09	123 ± 13		28 ± 3	

Values are mean ± SEM
All the studies referenced here quoted glycogen concentration in mmol glycosyl units per kg dry weight of muscle. This obviated the requirement to make a correction from mmol per kg wet weight to mmol per kg dry weight (x 4.35, based on an assumption of 77% water content by mass).

Glycogen depletion by high-intensity interval protocols in published studies achieved a mean *depleted* glycogen concentration of 123 $mmol/kg_{DW}$ (range 72-176) (8, 72, 126, 174,

198-200) in comparison to the current study's 115 mmol/kg $_{DW}$. The hourly rate of glycogen synthesis in a study that provided no fuel during 2.5 hours recovery was 7 mmol/kg $_{DW}$ (72), which is similar to the rate observed in the *saline control* 16 mmol/kg $_{DW}$/h. The mean rate of glycogen synthesis reported across a variety of carbohydrate doses during recovery is 28 mmol/kg $_{DW}$/h (range 17-41). The rates are, unsurprisingly, higher when the measurement is made following a dose at the optimal recommendation of 1.0-1.2 g/kg/h or the recovery period is short, because glycogen synthesis is faster in the early stages of recovery (167). This is reflected in the current study finding of a rate of 35 mmol/kg $_{DW}$/h in the *glucose control*. This context underscores the effect of adding βHB during recovery. In *glucose plus ketone*, the rate was 57 mmol/kg $_{DW}$/h, which is 40% higher than the highest value from any similar study in the literature (8, 126, 167, 198-200) and represents the first demonstration of a dietary intervention that enhances the recovery of glycogen following exhaustive exercise, beyond that achieved by the recommended 1.0-1.2 g/kg/h of multiple transportable carbohydrate alone (201).

Such a large effect to increase glycogen synthesis post-exercise demands an explanation. Naturally, a doubling of endogenous insulin release will cause a marked increase in glucose uptake into the muscle, at least once any post-exercise, insulin-independent phase of recovery has elapsed. When glucose enters the muscle cell it is *trapped* in irreversible phosphorylation, by the enzyme hexokinase, as glucose-6-phosphate. At this point, it has two main potential fates. First, the glucose can be oxidised to pyruvate/lactate in glycolysis and second, glucose-6-phosphate can be incorporated into glycogen.

Ketone bodies augment the supply of acetyl-CoA, inhibit distal glycolysis through inhibition of the pyruvate dehydrogenase complex and inhibit proximal glycolysis through inhibition of phosphofructokinase by citrate, mediated by *shuttling* of citrate from mitochondrion to cytosol. These mechanisms have been fully described above (1.3.2) (75, 80, 202). By

inhibiting glycolysis, ketone bodies favour the incorporation of glucose-6-phosphate into muscle glycogen (73, 165).

There is, however, another potential mechanism by which ketones may accelerate glycogen synthesis. It is possible that, in addition to increasing the circulating levels of insulin, βHB may also signal increased insulin *sensitivity* through an AMPK-dependent mechanism. AMPK activity has been implicated in the increased insulin sensitivity post-exercise (203) and ketone bodies have been demonstrated to increase AMPK activity in muscle in a rodent model (204). AMPK activity correlates inversely with glycogen concentration and glycogen is known to inhibit AMPK by binding to the carbohydrate-binding domain on its β-subunit (205). These findings have led Richter and Jensen to speculate that increasing levels of AMPK released from glycogen during exercise may explain the increased insulin sensitivity (206). If βHB can upregulate AMPK activity, it is plausible that, in addition to stimulating glucose uptake and storage by increasing insulin release from the pancreatic beta cell, it can also augment the rate of glycogen synthesis at any given insulin concentration. In short, ketone bodies may promote glycogen synthesis by enhancing both insulin *release* and insulin *sensitivity*, and by inhibiting glucose oxidation, thus favouring its incorporation into glycogen.

4.5 Conclusion

Following exhaustive exercise, the provision of exogenous βHB, in addition to high-dose carbohydrate, doubles endogenous insulin release, increases glucose disposal by 32% and increases glycogen content by 50% during the first two hours of recovery. Given that reduction of muscle glycogen stores impairs both moderate and high intensity exercise (207), and that the exhaustion of glycogen reserves during exercise leads to a critical failure

of power output (145, 160), any intervention that enhances glycogen synthesis is of great potential benefit to exercise performance.

The release of endogenous insulin and the disposal of glucose into body tissues are both essential to metabolism in the healthy population, but the importance of these fundamental mechanisms is highlighted in metabolic disease. The work described in the next chapter sought to determine whether D-β-hydroxybutyrate could influence endogenous insulin release, insulin sensitivity and glucose disposal in patients with type 2 diabetes.

5 Ketones and glucose disposal in type 2 diabetes

Abstract

Background: The work presented in this chapter sought to determine, for the first time in humans, whether βHB, can augment insulin release, increase insulin sensitivity and/or increase glucose disposal in participants with type 2 diabetes and healthy controls.

Methods: This study was of a 2-arm, randomised, controlled, double-blinded crossover design. Nine patients with type 2 diabetes and 9 healthy controls were recruited. After overnight fasting, participants ingested a ketone ester drink and undertook a two hour euglycaemic clamp (IV insulin at 40 mU/m^2/min & IV 20% glucose infusion) to *clamp* whole blood glucose at 5 mM.

- Intervention arm - ketone ester drink - 0.4 ml/kg (430 mg/kg)
- Control arm - taste and appearance-matched control drink

Results: The ketone drink caused βHB to peak at 4.6 mM (diabetics) and 4.2 mM (healthy controls), remaining ≥ 2.0 mM throughout the protocol. At 30 min post drink, insulin was elevated by 28% in the diabetic patients and by 64% in the healthy controls vs. control drink (Diabetic: 49 vs. 38 mU/l, $p < 0.05$. Healthy control: 24 vs. 14 mU/l, $p < 0.05$). At the same time, glucose was 7% (diabetes) and 15% (healthy) lower vs. control (Diabetic: 5.6 vs. 6.0 mM, $p < 0.05$. Healthy control: 5.1 vs. 6.0 mM, $p < 0.05$). From 0-45 mins of the euglycaemic clamp, glucose disposal was 80% higher in healthy controls following ketone vs. control (18 vs. 10 g, $p < 0.01$), with no difference in glucose disposal in the diabetic patients. There was significant suppression of fasting lipolysis in both groups.

Conclusion: In patients with type 2 diabetics and healthy subjects a ketone ester drink augmented insulin and suppressed glucose levels. The glucose lowering effect appeared to be mediated by enhanced beta-cell release of insulin, with no change in insulin sensitivity. Neither augmented insulin release nor increased glucose disposal were demonstrated during the euglycaemic clamp in the diabetic group, which may represent beta-cell failure, as part of diabetic pathophysiology, or loss of the insulin signal through a *diluting* effect of the infused exogenous insulin.

5.1 Introduction

In addition to the empirical evidence of improved metabolic health in obese and frankly diabetic patients on a ketogenic diet (102, 103, 108, 111, 124, 208, 209), there is direct molecular and metabolic evidence for a potential benefit of ketone bodies in insulin resistance. Adipose tissue has a key role in the development of insulin resistance (210), first demonstrated in the *ob/ob* mouse which has a homozygous mutation in the *leptin* gene. Deficient in leptin, the *ob/ob* mouse exhibited hyperphagia, hyperlipidaemia and insulin resistance (211, 212). Leptin is the first of a range of peptide hormones, *adipokines*, found to originate in adipose tissue. Leptin and adiponectin are 'anti-diabetic' adipokines that increase beta-oxidation of fat and stimulate insulin sensitivity in liver and skeletal muscle (213), this anti-diabetic effect is at least partly explained by activation of 5'-AMP-activated protein kinase (AMPK). AMPK is an 'energy gauge' activated by a reduction in ATP and a rise in AMP, and is upregulated by leptin and adiponectin (214). A potential effect of ketones in reducing insulin resistance may be through their action to upregulate AMPK activity, seen in ketogenic diets (215-217) and, in C57BL/6J mice, fed the ketone monoester R-3-hydroxybutyl-R-3-hydroxybutyrate (204). In this mouse model, the ketone ester upregulated AMPK and overcame insulin resistance. Ketone bodies also suppress peripheral lipolysis and reduce circulating fat levels (196, 218), thereby enhancing insulin sensitivity by reducing the substrate competition that elevated NEFA levels exert against oxidation of glucose (79, 137, 202) and by reducing the interference that lipids exert on insulin signalling (219, 220).

In the previous chapter, it was demonstrated in normal healthy male subjects, following glycogen-depleting exercise, that D-β-hydroxybutyrate increased endogenous insulin release and whole-body glucose disposal. The purpose of the work described in this chapter

was to determine whether βHB could increase endogenous insulin release, enhance insulin sensitivity and/or increase glucose disposal in both healthy controls and in patients with type 2 diabetes.

5.2 Methods

5.2.1 Participants

Nine participants with type 2 diabetes, not requiring treatment with insulin/sulphonylurea, and 9 healthy, age-matched controls were recruited to the study (General methods 2.2.3 - 2.2.4). The groups were well matched for age, height and sex. The diabetic participants had significantly higher body weights, fasting glucose and insulin concentrations, homeostatic model assessment of insulin resistance (HOMA(189)) and proportion of glycated haemoglobin (HbA1c %) (Table 5.1).

Table 5.1 - Baseline characteristics of study participants

Characteristic	Healthy controls Mean ± SEM	Type 2 diabetic participants Mean ± SEM
	n = 9	n = 9
Male/Female	6/3	5/4
Age (years)	52.4 ± 3.3	55.1 ± 2.7
Height (m)	1.75 ± 0.04	1.75 ± 0.03
Weight (kg)	80.9 ± 2.7	93.1 ± 6.8 *
BMI (kg/m^2)	26.4 ± 1.0	30.3 ± 2.2
Fasting glucose (mM)	4.8 ± 0.1	6.7 ± 0.3 **
Fasting insulin (mU/l)	4.6 ± 0.9	9.4 ± 1.8 *
HOMA score	1.0 ± 0.2	3.1 ± 0.7 *
HbA1c (%)	5.3 ± 0.1	6.4 ± 0.3 **

* $p < 0.05$ diabetes participants vs. healthy controls; ** $p < 0.01$ diabetes participants vs. healthy controls. HOMA – homeostatic model assessment of insulin resistance

For each study visit, participants reported to the metabolic ward of the Oxford Centre for Diabetes, Endocrinology and Metabolism (OCDEM) at 0800 hours, following an overnight fast. Participants with diabetes were also instructed to omit their oral diabetes medication on the morning of the visit.

5.2.2 Ketone and control drinks

Following a baseline blood test, participants consumed either a ketone drink, or a taste and appearance-matched control drink of equal volume (General Methods 2.7). The ketone drink contained 0.4 ml/kg [430 mg/kg] of the ketone ester (R) – 3 – hydroxybutyl (R) – 3 hydroxybutyrate. The natural bitter taste was partially masked with a citrus flavouring

(Symrise, Holzminden, Germany) and 3 g of proprietary sweetener, containing 0.6 g of sugars. The control drink contained the same citrus and sweetener components as the ketone drink. The bitter taste was matched with the addition of a weight-dosed commercial bitter agent (Symrise, Holzminden, Germany).

5.2.3 Blood sampling and analysis

Blood sampling and analysis was conducted as previously described (General Methods 2.8).

Blood samples, taken at 5 min intervals during the euglycaemic clamp, were measured for whole blood glucose with a near-patient benchtop device (HemoCue 201^{+}, Radiometer, Copenhagen, Denmark) to guide the dextrose infusion rate. A *fasted* sample of blood, collected (in an ethylene-diamine-tetraacetic acid [EDTA] tube) at the first visit, was sent to the laboratory of the John Radcliffe Hospital, Oxford, UK, for measurement of the percentage glycation of haemoglobin (HbA1c [%]).

5.2.4 Euglycaemic clamping

The euglycaemic clamp was conducted according to the method described by de Fronzo and colleagues (127). Participants had two IV cannulae sited: one in the antecubital fossa (20 gauge), for infusion of both insulin and glucose, and one in the dorsum of the contralateral hand (22 gauge) for sampling whole blood glucose at five min intervals. Insulin (Actrapid® 40 U/ml, Novo Nordisk®, Gatwick, UK) was infused at a rate of 40 mU/m^2/min and 20% glucose (20% dextrose, Baxter Corporation, Staines, UK) was infused at the rate required to maintain blood glucose at 5 mM.

The limb containing the sampling cannula was heated to 40-44 ºC using two therapeutic heat pads (HK35 Beurer, Ulm, Germany) wrapped around the hand and forearm to cause maximal vasodilatation and generate a ‘pseudoarterialized’ blood sample. At the end of the clamp, the participant was given a moderate glycaemic index meal and the dextrose infusion

rate was gradually reduced to zero. Participants were observed until euglycaemia had been maintained for a period of 30 min after cessation of glucose infusion. During the clamp, the infusion rate for the 20% dextrose required to maintain a whole blood glucose of 5 mM was recorded, and blood was sampled at regular intervals for metabolite and insulin concentrations.

The theoretical value of a euglycaemic clamp is that, by providing a fixed dose of insulin at a level higher than normally circulating in the overnight fasted state, hepatic glucose output is prevented, whilst simultaneously standardising the dose of insulin. Such conditions mean that the quantity of glucose infused is theoretically equal to the quantity of glucose being taken up by body tissues. As such, the glucose infusion rate provides an accurate, sensitive and reproducible measure of insulin sensitivity.

When the fasting glucose exceeded 6.5 mM, participants were given a *run in* clamp, which comprised a 40 $mU/m^2/min$ infusion of insulin with regular whole blood glucose measurements, until the blood glucose was less than 5 mM. At this point, the appropriate study drink was administered. The timing and nature of all further elements of the study was identical. When a *run in* clamp was used at the first visit, it was also administered at the second visit, to standardise the study conditions.

5.2.5 Statistics

A two-way ANOVA was used to compare metabolite and insulin levels and the rate of glucose disposal. Area under the curve (AUC) values were determined for selected metabolites and compared using a Student's t-test. A p value of < 0.05 was taken to indicate statistical significance.

5.2.6 Study design

The study (Figure 5.1) was of a randomised, double-blinded crossover design, comprising two arms. Each study visit followed an overnight fast and commenced with a fasting blood test. Participants ingested a study drink 30 mins prior to a two hour euglycaemic clamp that used exogenous IV insulin at 40 mU/m^2/min and IV 20% glucose infusion to *clamp* whole blood glucose at 5 mM. The drinks, given in randomised order, were either:

- A ketone ester drink - 0.4 ml/kg (430 mg/kg)
- A taste and appearance-matched control drink

Venous blood was sampled at intervals to measure substrates and insulin concentrations. The glucose infusion rate was adjusted by a blinded investigator, based on the previous whole blood glucose measurement.

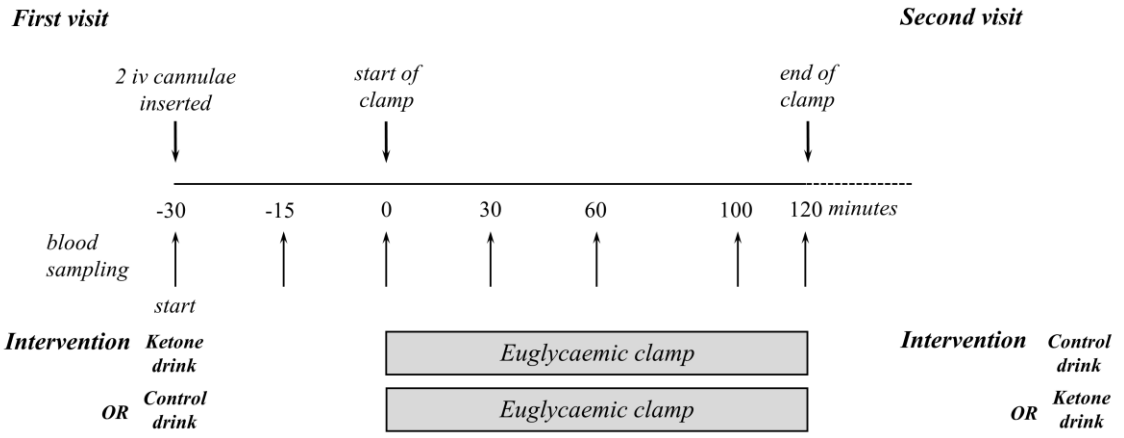


Figure 5.1 - Schematic of study visit. For each of the two visits, participants arrived following an overnight, had 2 venous cannulae sited and a baseline blood sample drawn. Following baseline measures, a drink was administered, followed by two further blood tests over 30 min. 30 min after the drink, the 2-h euglycaemic clamp was started. Blood samples were drawn at intervals during, and on completion of, the clamp.

5.3 Results

5.3.1 Clamp conditions

There were no significant baseline differences in the fasting concentrations of βHB and acetoacetate between groups or interventions. The ketone ester drink caused a rapid rise in ketone body concentration, and in βHB/acetoacetate ratio, which remained significantly elevated throughout the clamp in both diabetic participants and healthy controls (Table 5.2 and Figures 5.2 & 5.6 [lower right panel]).

Table 5.2 – Ketone body concentrations during study visits

Group	Intervention	D-β-hydroxybutyrate (mM)				Acetoacetate (mM)			
		Fasted	Start of clamp	Peak	End of clamp	Fasted	Start of clamp	Peak	End of clamp
Diabetes	Control	0.10 ± 0.04	0.05 ± 0.02	0.04 ± 0.01	0.02 ± 0.00	0.09 ± 0.02	0.06 ± 0.01	0.05 ± 0.01	0.04 ± 0.01
	Ketone	0.10 ± 0.03	4.33 ± 0.23 ****	4.64 ± 0.24 ****	2.95 ± 0.24 ††*****	0.10 ± 0.02	1.04 ± 0.07 ****	1.28 ± 0.09 ††††****	1.21 ± 0.08 ††††****
Healthy	Control	0.10 ± 0.01	0.06 ± 0.01	0.04 ± 0.01	0.01 ± 0.00	0.07 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	0.03 ± 0.01
	Ketone	0.10 ± 0.02	4.33 ± 0.23 ****	4.12 ± 0.30 ****	2.05 ± 0.27 ****	0.06 ± 0.02	0.86 ± 0.06 ****	0.97 ± 0.06 ****	0.87 ± 0.08 ****

Values = mean ± SEM
† $p < 0.05$, †† $p < 0.01$, †††† $p < 0.0001$ for diabetes (ketone) vs. healthy (ketone); **** $p < 0.0001$ ketone vs. control condition within group

The mean whole blood glucose concentration (Figure 5.2) during the 2-hour clamp did not differ between the diabetes group (5.0 ± 0.1) and controls (5.0 ± 0.0). A *run-in* clamp was necessary, at both visits, for four of the nine diabetic participants.

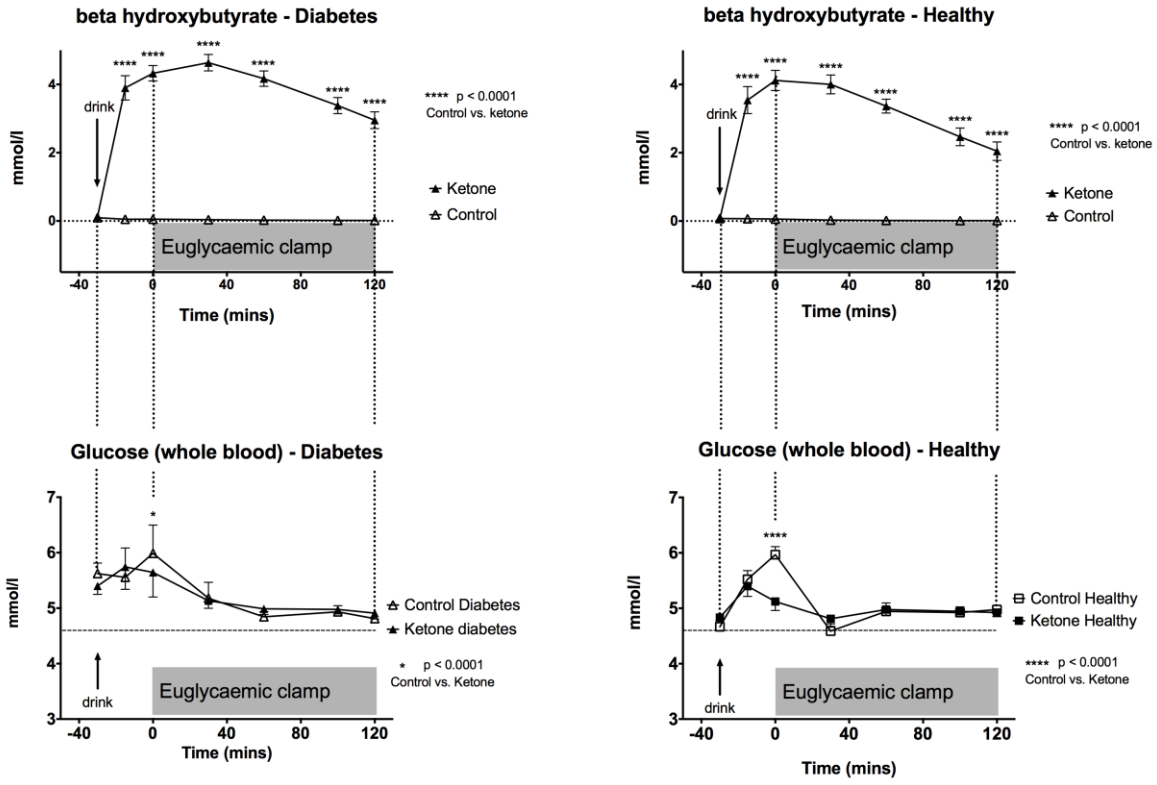


Figure 5.2 – D-β-hydroxybutyrate and glucose concentrations before and during the clamp

5.3.2 Metabolite concentrations

The levels of glycerol, non-esterified fatty acids and lactate are shown in Figure 5.3. Glycerol and NEFA did not differ between groups at rest. Following the ketone drink the levels of glycerol and NEFA fell in both groups vs. control. The significantly lower glycerol continued to the end of the clamp in the diabetic group, but not in healthy controls. NEFA fell to a barely detectable level in both groups by the end of the euglycaemic clamp. Lactate was significantly elevated at rest in the diabetic group vs. controls (mean difference 0.7 mM, $p < 0.0001$), which was maintained throughout the clamp in the control conditions. The effect of the ketone drink was to significantly elevate lactate levels throughout the 2nd hour of the clamp in both groups.

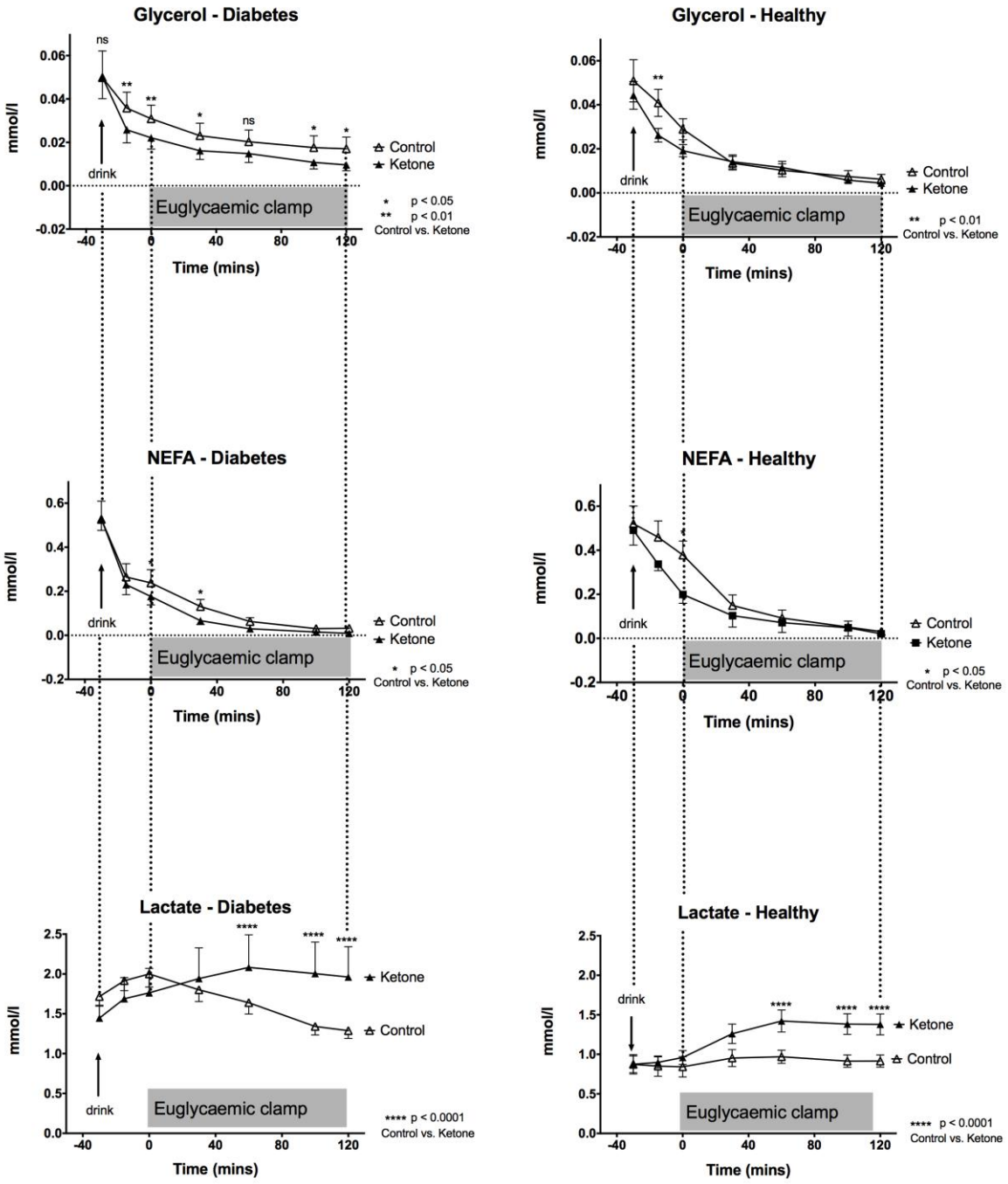


Figure 5.3 – Glycerol, NEFA and lactate concentrations before and during the clamp

5.3.3 Initial effects of ketones on insulin and glucose

During the 30 mins following drink consumption, prior to starting the clamp, there were differences in both glucose and insulin concentrations (Figure 5.4) between diabetic participants vs. controls. Insulin was elevated after 15 min in the control group and after 30 min in the diabetic group following the ketone vs. the control drink. Insulin levels were

elevated in the diabetes group partly due to the use of a *run in* clamp in four of the nine participants. Following the ketone drink, glucose was lower in both the diabetic and control groups at 30 minutes.

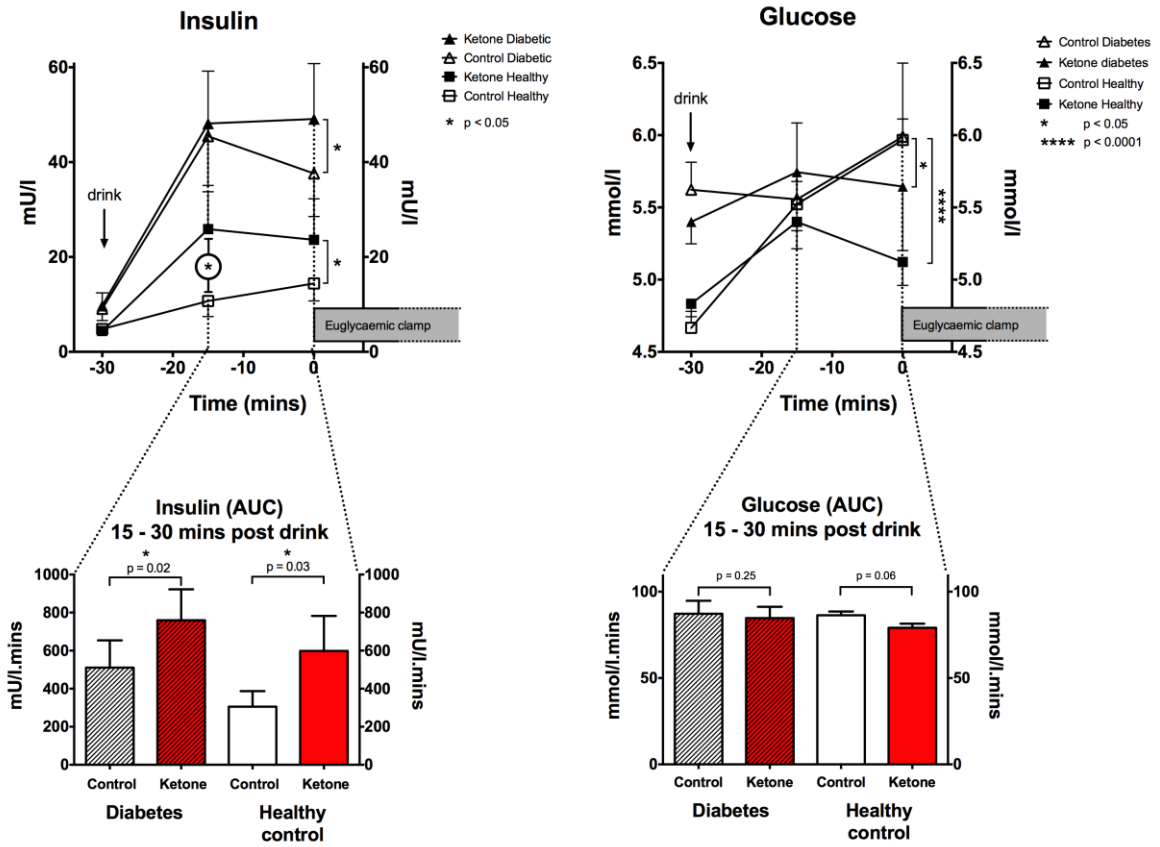


Figure 5.4 – Insulin and glucose concentrations following ketone and control drink ingestion

5.3.4 Insulin and glucose disposal

In the control group, ketones caused an increase in insulin concentration and glucose disposal throughout the first 45 minutes of the euglycaemic clamp (Figure 5.5), but this difference was not seen in the diabetic group.

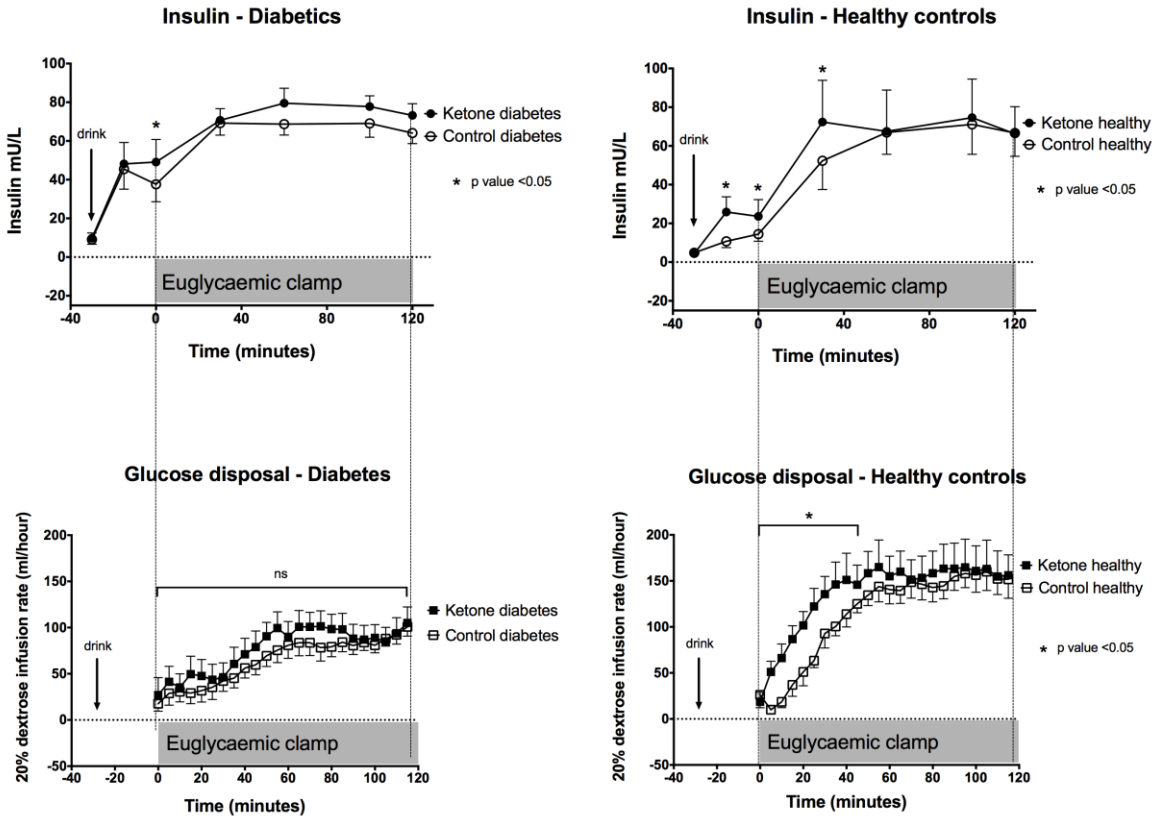


Figure 5.5 – Insulin concentration and glucose disposal during the euglycaemic clamp

5.3.5 Lactate handling

Prior to any intervention, the resting lactate levels were 0.7 mM higher in the diabetic group than the healthy controls ($p < 0.0001$) (Figure 5.6, upper left panel), and in this group there was a correlation ($r = 0.3$) between fasting lactate and fasting glucose (Figure 5.6, lower left panel).

5.3.6 Insulin sensitivity

There was an increase in glucose disposal in the control group but not in the diabetic group (Figure 5.5). Glucose disposal per unit insulin was calculated in the control group (Figure 5.6, upper right panel), as a measure of insulin sensitivity. There was no difference between the ketone intervention and control.

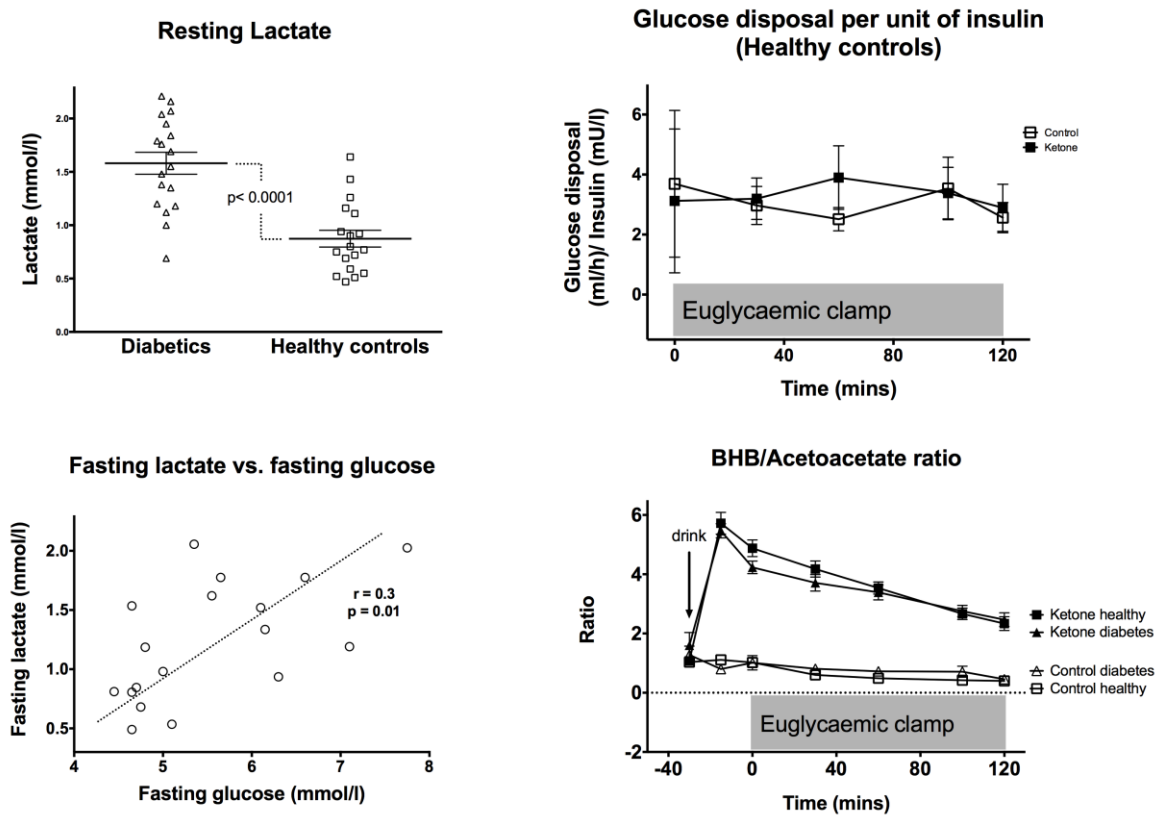


Figure 5.6 – Measurements of fasting lactate, fasting lactate vs. fasting glucose and of insulin sensitivity and βHB/acetoacetate ratio during the clamp.

5.4 Discussion

The key findings of this study were that consumption of a ketone ester drink caused an increase in endogenous insulin release, and a decrease in whole blood glucose in both healthy controls and patients with type 2 diabetes. In the healthy controls, this effect extended into the euglycaemic clamp period, with increased insulin during the first 30 mins and increased glucose disposal for the first 45 mins of the clamp vs. control. No significant effect on insulin, or glucose disposal, persisted during the clamp in the diabetic participants.

5.4.1. Metabolite effects

5.4.1.1 Suppression of lipolysis

This study demonstrated that circulating NEFA and glycerol, a surrogate for lipolysis, are lowered by ketone bodies in all participants vs. control. The effect was more marked in the

diabetic group, presumably because their lipaemia is more resistant to insulin than in healthy controls (221). In this context, the combination of ketones and insulin infusion was more effective at suppressing the lipaemia than insulin infusion alone. Ketone bodies were first reported to suppress peripheral lipolysis by Björntorp in 1966 (196). Initially, there was speculation as to the degree to which this effect was mediated by the increase in insulin release caused by ketone bodies (222, 223), particularly given that inhibition of peripheral lipolysis had been demonstrated to occur at insulin concentrations so low that their effect on carbohydrate metabolism was negligible (224). The finding that ketone bodies could suppress lipolysis when the insulin concentrations were controlled, convinced metabolic investigators that ketone bodies must inhibit lipolysis *independent* of insulin. The evidence included not only insulin treated rats, rendered diabetic by streptozotocin (225), and panreatectomised dogs (218), but also children with type 1 diabetes (226). The importance of derangements in lipid metabolism, rather than glucose metabolism, in the aetiology of diabetes is increasingly being recognised (221, 227). Lipid oversupply can reduce glucose uptake both by substrate competition (137) and by interference in cell-signalling pathways (219, 220). Thus the anti-lipolytic effect of ketone bodies is just as interesting as the potentiation effect on insulin release from pancreatic beta-cells.

5.4.1.2 Lactate handling

Resting lactate is elevated in diabetes (228), due to the impaired ability of diabetic tissues to oxidise both glucose and lactate (229, 230), largely due to a reduction in PDH flux (231, 232). Thus an increase in glucose will cause an increase in lactate by increased flux through lactate dehydrogenase. Here, there was a correlation between glucose and lactate concentrations in the 18 study visits of diabetic participants supporting the findings of Crawford et al. (228).

The elevated lactate during the clamp, in all subjects, may be explained by the redox coupling effect of an acute elevation of βHB, and thereby the βHB/acetoacetate, which may increase the mitochondrial NADH/NAD^+ ratio and the production of lactate from pyruvate (Figure 4.8). In both groups, the ketone drink produced a fivefold increase in βHB/acetoacetate that remained more than double the resting value throughout the clamp.

5.4.2 Insulin concentration and glucose disposal

Insulin concentrations were higher in both groups at the start of the clamp, 30 mins following ingestion of a ketone ester drink vs. control drink. This effect included those diabetic participants who received a standardised *run-in* clamp to achieve euglycaemia. Though this elevated insulin level continued in the early part of the clamp in healthy controls, there was no significant elevation during the clamp in diabetic participants. There was also no increase in glucose disposal in the diabetic participants, which may have resulted from an absence of augmented endogenous insulin release. In fact, insulin and glucose disposal were the same for the diabetic control and diabetic ketone intervention arms, suggesting that, contrary to the reported ketone-mediated increase in AMPK causing increased insulin sensitivity (204), there was no demonstrable change in whole-body insulin *sensitivity* in the diabetic group.

The aim of a euglycaemic clamp is to determine whole body insulin sensitivity, which is achieved by fixing the insulin concentration and inhibiting hepatic glucose output so that glucose infused to maintain euglycaemia is equal to glucose taken up by tissues. This provides a sensitive, accurate and repeatable measure of insulin sensitivity (127). As the ketone drink caused endogenous insulin release, during clamping in healthy controls, the insulin concentration was *not* rigidly standardised between the control and ketone intervention. Therefore glucose disposal per unit of circulating insulin was used as a surrogate for insulin *sensitivity*. This parameter was unchanged by the ketone intervention,

suggesting that the increase in glucose disposal during the early part of the clamp resulted from increased endogenous insulin *release*, rather than an increase in insulin *sensitivity*.

5.4.3 Early effects of acute ketosis

The most significant finding of this study was that in healthy fasted controls and fasted diabetic participants insulin was elevated, and glucose lowered, within 30 mins of consuming a ketone drink compared to a control drink. In the previous study, a similar level of ketosis following exercise did not cause a rise in endogenous insulin. It is possible therefore that there is, as previously suggested, a *threshold* glucose concentration below which the *potentiating* effect of ketones on beta cell insulin release does not operate. Indeed in the previous study, the mean plasma glucose following exhaustive exercise was 4.1 mM. In this study, the mean plasma glucose in healthy, overnight fasted participants was 5.3 mM. As stated above, in isolated beta-cells of rats, 5mM appears to be the threshold for ketone potentiation of insulin release in culture (192). However, it may also be that some specific feature of the post-exercise condition inhibits the beta-cell response to ketones. For example, during exercise insulin levels are suppressed by α-adrenergic stimulation of the beta-cell (233, 234). This effect may contribute to the lack of insulin potentiation by ketones in the 30 mins following exhaustive exercise.

Given that we know that beta-cell dysfunction is a part of the pathophysiology of type 2 diabetes (210), the absence of an increase in endogenous insulin during clamping may reflect metabolic pathology. It is also possible that an increase in portal insulin concentration may occur, but that hepatic insulin clearance renders this change undetectable in the peripheral circulation. This phenomenon has been described previously (92). Finally, it is quite plausible that the small, but physiologically potent, signal of increased peripheral insulin, which was detectable during the 30 mins between ketone ester ingestion and the start of the

clamp, was lost during the clamp because of a *dilution* effect during insulin infusion. This study may therefore represent a false-negative for the effect of ketone bodies on beta-cell insulin release in Type 2 diabetes and it may be that different methodologies may be able to address this question with a higher degree of sensitivity.

5.5 Conclusion

In healthy controls and participants with type 2 diabetes, following an overnight fast, a ketone ester drink increased endogenous insulin and decreased circulating glucose concentrations. During the euglycaemic clamp, an increase in endogenous insulin and glucose disposal continued in the initial stages of a euglycaemic clamp, following ketone intervention vs. control in healthy participants but not in participants with type 2 diabetes. Increased glucose disposal appeared to be mediated through increased insulin release and no change in insulin *sensitivity* was observed.

In addition, in both groups, ketones suppressed of peripheral lipolysis; the effect being more marked in diabetic participants than healthy controls.

Augmentation of insulin release and suppression of lipolysis, with reduction in circulating fats, are therapeutic goals in diabetes. Given that these two effects are seen following the D-β-hydroxybutyrate ester, ketone bodies may be the effectors of similar benefits in type 2 diabetics on low-carbohydrate diets (102-109). It is also possible that the elevation of circulating ketones by exogenous supplementation may have therapeutic potential in metabolic disease.

6. General Discussion

The aim of the work described in this thesis was to answer the following questions:

- Can βHB be used as a fuel in skeletal muscle, and how do exercise intensity and other substrates alter ketone oxidation?
- Can the provision of βHB, with abundant carbohydrate, increase skeletal muscle glycogen synthesis following glycogen-depleting exercise?
- Can βHB alter carbohydrate and fat metabolism in diabetes, and do any alterations offer therapeutic potential?

6.1 Summary of key novel findings:

- βHB is a fuel substrate, oxidised by the body.
- βHB oxidation is increased 3-fold during exercise.
- The relative contribution of βHB oxidation to total oxygen uptake ($\dot{V}O_2$) is greatest at rest and diminishes with increasing exercise intensity.
- βHB oxidation is reduced following glycogen-depleting exercise followed by a low-carbohydrate diet.
- βHB *increases* insulin release in healthy controls when plasma glucose is over ~5 mM.
- βHB *increases* insulin release in participants with type 2 diabetes following an overnight fast.
- βHB, with a circulating whole blood glucose of 10 mM, increases whole-body glucose disposal by 32% compared to control.
- With a circulating whole blood glucose of 10 mM, βHB increases muscle glycogen synthesis by 50% compared to control.
- βHB lowers circulating fat levels in all subjects, whether healthy controls or those with T2DM.

6.2 The brain and carbohydrate

The findings described in this thesis can be explained in the context of three selection pressures, which have structured hominid metabolism:

- In the absence of cellulase, access to dietary carbohydrate was scarce
- The brain grew large, but was unable to oxidise significant quantities of fat
- Body protein served a committed purpose and was not available as a *store* of energy

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"Primitive man faced several unique problems. Survival required the capacity to withstand prolonged periods of deprivation and yet the sparing of as much body protein as possible in order to be able to hunt successfully at the first available opportunity, or, on the other hand, to escape if preyed upon." George Cahill, 1968 (21)

Ultimately, the adaptations that have evolved in response to these pressures can explain the reciprocal relationships of ketone bodies and carbohydrate.

6.3 Ketones are an alternative fuel for the brain and skeletal muscle

An essential means by which ketone bodies spare carbohydrate in times of scarcity is to decrease carbohydrate consumption as a fuel, through ketone oxidation displacing the energy requirements normally met by carbohydrate.

The human brain can oxidise large quantities of ketone bodies, which supplant glucose by supplying up to 60% of the energy required during prolonged (5-6 weeks) starvation (Figure 6.1) (13). After 5-6 weeks fasting, ketone oxidation reaches 0.9 mmol/kg/h (approximately 85% is βHB and 15% acetoacetate).

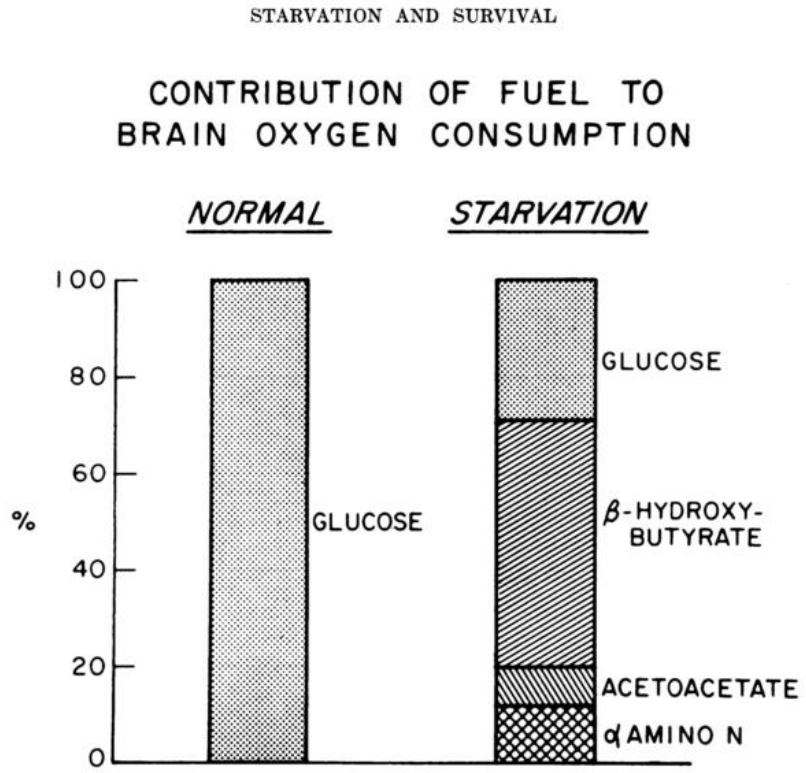


Figure 6.1 – The fuel use of the brain in the fed state, 'Normal', and after 5-6 weeks starvation, 'Starvation'(from Owen et al. 1967) (13).

The data presented in Chapter 3 showed, in healthy, overnight fasted male participants, that *whole-body* βHB oxidation was at least 40 mmol/h, or 0.5 mmol/kg/h, and accounted for at least 28% of resting oxygen uptake ($\dot{V}O_2$). Given that ^{13}C TTR was still rising at the end of the study period, this was likely to have been an underestimate (142). These values were higher than a similar study of *whole-body* ketone oxidation, using a primed infusion of [3-^{14}C] acetoacetate, which found a ketone oxidation rate of 26 mmol/h (129). The methodological differences were that in that study, the participants had been fasted for 3

days prior to the measurement. Furthermore, an isotopic label at position 3 (C-3) is more likely to be incorporated into the Krebs cycle, increasing the underestimation of oxidation, due to increased incorporation of the C-3 label into metabolic intermediates, thus preventing its collection as labelled CO_2 in breath (142). Other investigators suggest that, following a 3 day fast, with total ketone bodies at comparable levels to those achieved in the study here, ketones may account for 30-40% of the body's energy needs (29). With the onset of exercise, βHB oxidation, determined by ^{13}C recovery, increased 2.5-fold and 3-fold at 40% and 75% $\dot{V}O_{2max}$, respectively. No studies of ketone oxidation during high intensity (75% $\dot{V}O_{2max}$) exercise have been published, and reports of ketone oxidation at 45% $\dot{V}O_{2max}$ (70) attributed 3% of $\dot{V}O_2$ to ketone oxidation (compared to the current finding:11% of $\dot{V}O_2$ at 40% $\dot{V}O_{2max}$). The 3% contribution may have resulted from a much lower (only 0.6-0.8 mM) total ketone body concentration during exercise. Féry and Balasse have dominated publications on *whole-body* ketone oxidation during exercise (70, 129, 135). In 1988, Féry and Balasse extended their work with an elegant study comparing ketone oxidation during exercise (2 h at 50% $\dot{V}O_{2max}$) in participants with total ketones of 6-7 mM, either from fasting or from ^{14}C-labelled acetoacetate infusion (136). They demonstrated a significant exercise-related increase in the rate of disposal (R_d) and metabolic clearance rate (MCR) of ketone bodies (Figure 6.2), in the overnight fasted group on acetoacetate infusion, and no increase in the fasted group, before concluding "Exercise did not stimulate the capacity of muscle to extract ketones, as indicated by the lack of changes in MCR" (136).

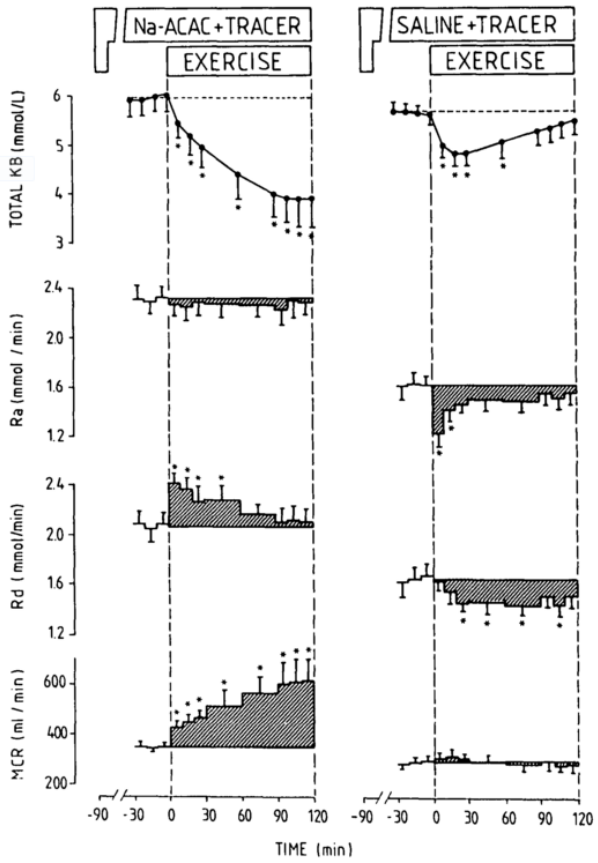


Figure 6.2 – Ketone body metabolism before and during 2-hours exercise at 50% $\dot{V}O_{2max}$. Ketone concentration; rate of appearance (R_a); rate of disappearance (R_d); and metabolic clearance rate (MCR) are shown in a group given a ^{14}C-acetoacetate tracer to elevate ketone body levels (left of figure) and another group in whom the same ketosis was achieved by fasting (right of figure). * = $p < 0.05$ in difference from resting condition. Taken from Fery and Balasse 1988 (136).

Their clear demonstration of an exercise-induced increase in ketone oxidation, followed by the rejection of this finding is perplexing.

In chapter 3, the finding that relative βHB contribution to total oxidation fell slightly with increased exercise intensity recapitulates the exercise intensity-oxidation rate seen for fat (48). The respiratory quotient for carbohydrate (1.0) is higher than that of fat (0.7) and βHB (0.89), reflecting a higher *oxygen-efficiency*. Furthermore, in the relative hypoxia of high-intensity exercise, ATP is increasingly generated by substrate-level phosphorylation from the anaerobic glycolysis of glucose, derived from the circulation or from glycogenolysis (49). However, the absolute contribution of ketone bodies to total substrate oxidation

increased with increasing exercise intensity. Indirect evidence for the oxidation of ketone bodies by exercising muscle comes from studies that demonstrate increased capacity for ketone oxidation and/or increased succinyl-CoA-3-oxoacid transferase (SCOT, the rate-limiting enzyme of ketone oxidation) activity in response to endurance training (71, 235-237). Whilst whole-body measurement of βHB oxidation are essential to explore the role of ketone bodies in integrated metabolism, an arteriovenous measurement approach, including arterial, femoral venous and hepatic portal catheterisation, with paired samples of arteriovenous or porto-systemic metabolite differences would allow more focussed analysis of ketone oxidation in the skeletal muscle of resting and exercising leg muscle and the endogenous output of ketone bodies from the liver. The other major advantage of this approach is that it allows for the measurement of the disposal/appearance of other metabolites, such as fat, glucose and amino-acids without the need for complex, multiple isotope labels.

6.4 Progressive starvation alters tissue oxidation of ketone bodies to protect the brain

Less ^{13}C-labelled CO_2 was recovered during exercise following a low carbohydrate diet (Chapter 3). The capacity for muscle to oxidise ketones falls with increased duration of fasting (69, 153), in contrast to brain (13). Furthermore, provision of dietary glucose can increase ketone oxidation (166). Laffel has suggested (29) that the decreased capacity of muscle to oxidise ketone bodies in fasting is caused by downregulation of SCOT (238). This hypothesis was rejected by van Itallie (2003) on the basis that no measured change in enzyme activity, has been of sufficient magnitude to account for the observed reduction in oxidation (11). An alternative hypothesis, to account for the differences due to diet in the current study, is that glycolysis from muscle glycogen is necessary for ketone oxidation.

Muscle glycogen is consumed slowly during fasting at rest, and consumed rapidly by high-intensity or prolonged exercise (207). Glycolytic flux can maintain Krebs cycle flux via pyruvate carboxylase, regenerating oxaloacetate from pyruvate, (156). Anaplerosis, of which the pyruvate carboxylase reaction is a major example, is necessary for the continued oxidation of any fuel that 'burns' in the Krebs cycle (239). This hypothesis would also explain the apparent fall in ketone oxidation in the overnight fasted state, between 60-90 minutes into a 2-hour exercise bout at 50% $\dot{V}O_{2max}$ (Figure 6.2) (136). In overnight fasted individuals, muscle glycogen depletion occurs at between 60-90 minutes of exercise at this intensity (145, 160). The teleological argument for this adaptation is that, as carbohydrate depletion progresses, the energy threat to the brain increases, making it advantageous for muscle to *spare* ketone bodies for the brain and to switch to consumption of fat, which the brain cannot oxidise. A downregulation of muscle ketone oxidation in starvation, another link between carbohydrate and ketone metabolism, underscores the pre-eminence of the brain and the need to conserve protein, by eking out precious carbohydrate.

6.5 Ketones are a signal to preserve carbohydrate

In Chapter 4, it was found that βHB, given with abundant glucose, caused a 2-fold increase in endogenous insulin release, increased whole body glucose disposal and increased glycogen synthesis following exercise. It would now be interesting to explore, with functional testing, whether this increase in the recovery of muscle glycogen translates into an improvement in physical endurance during subsequent exercise. The carbohydrate-conserving effect was mediated through increased insulin release, an effect that has been reported in cell-culture and animal studies (147, 192, 240, 241), and without a change in insulin sensitivity. However, other cell culture (241) and rodent (242) studies *have* reported increased insulin sensitivity with βHB. Increased insulin will not only conserve

carbohydrate in muscle glycogen, but will conserve hepatic glycogen through the restriction of hepatic glucose output, as demonstrated by Balasse and colleagues (92). Mikkelsen (2014) reported both hypoglycaemia and decreased glucose production, during ketone body infusions, with no evidence of increased insulin in the peripheral circulation, and concluded that these changes were directly caused by ketone bodies, rather than secondary to insulin release. It is interesting that Balasse et al (1970), also found no increase in peripheral insulin, but *were* able to detect a small increase in portal insulin, by placing a catheter in the hepatic portal vein. They concluded that this small increase in portal insulin was sufficient to account for the decreased hepatic output (92). The liver clears approximately 50% of insulin at first pass (93) making small changes in portal concentration undetectable in the peripheral circulation. In addition to the insulinotropic effect of βHB found here, ketone bodies also increase incorporation of glucose into adipose tissue (243) and reduce glucose oxidation in the heart (75), diaphragm (76), and kidney (77) of rats, supporting the central role of ketone bodies in the conservation of carbohydrate.

6.6 Therapeutic implications of ketone bodies in diabetes

Type 2 diabetes mellitus is a metabolic disorder characterised by hyperglycaemia, hyperlipidaemia and an inability to access the potential energy stored in fuel substrates. The global prevalence of diabetes is predicted to rise from 177 million in 2000 to 366 million in 2030 (244). The Cambridge MRC Epidemiology Unit estimates that 35-40% of Europeans will develop diabetes during their lives (245). Diabetes is associated with a reduced life expectancy (246) and confers an increased incidence of both large artery (macrovascular) atherosclerotic disease, including premature coronary artery disease and stroke, and small artery (microvascular) disease leading to retinopathy, nephropathy and neuropathy (247, 248). Up to 70% of patients will die of a cardiovascular cause, compared to 40% of the non-

diabetic population (249); the risk of developing heart failure is raised 2-fold in men and 5-fold in women (250) and the incidence of myocardial infarction is increased up to 5-fold (251).

The work presented in this thesis has consistently demonstrated the suppression of circulating free fatty acids, a phenomenon first described in 1966 (196). An insulinotropic effect of βHB has also been demonstrated in both healthy controls and participants with type 2 diabetes. This is an insulin *potentiating* effect, in that it appears to require plasma glucose in excess of ~5 mM.

Aretaeus of Cappadocia was the first to accurately describe diabetes in the second century AD (252). In the centuries since, the pathophysiological focus has been on glycosuria, hyperglycaemia and abnormal insulin metabolism. More recently, endocrinologists have suggested that disordered lipid, rather than glucose, metabolism may be the cause of this disease (221, 227). Lipid oversupply reduces glucose uptake by substrate competition (137) and by interfering in cell-signalling pathways (219, 220). This hypothesis for the aetiology of diabetes suggests that dyslipidaemia causes insulin insensitivity, and that hyperglycaemia is a secondary consequence. Thus, it may be the lipid-lowering effect of ketone bodies, rather than their potentiation of insulin release, which offers the most interesting therapeutic potential in treating diabetes.

6.7 Conclusion

Ketone bodies evolved to extend survival when dietary carbohydrate is unavailable. Consequently, their metabolism is inextricably linked with that of carbohydrate. In providing an alternative fuel to carbohydrate, and a powerful sign of its scarcity, they shift the body's metabolism away from glucose consumption towards the oxidation of fat. The possibility of providing ketone bodies rapidly and safely in an oral preparation, offers the potential to restore access to appropriate energy substrates in metabolic disease.

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