A study of human muscle metabolism in relation to exercise, training and peripheral vascular disease

A thesis for the degree of M.D.,
University of Leicester

1987

by
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Abstract

The introduction to this thesis comprises two parts. A comprehensive review of the mechanisms and control of human skeletal muscle metabolism with emphasis on anaerobic metabolism and the effects of training, and a review of atherosclerosis and peripheral vascular disease with special mention of skeletal muscle metabolism in vascular disease together with an account of areas in which drugs may help in its treatment. There follows a detailed account of the technique for biopsying human skeletal muscle together with the analytical methods for the fluorimetric assay of high energy phosphates, glycogen, glyclolytic intermediates and succinic dehydrogenase activity. Three experimental chapters are then presented. The first describes the Anaerobic Work Test (AWT) and the metabolic changes that occurred in the quadriceps muscle of man after 6 and 30 s of supra-maximal exercise. This showed that within 6 s of exercise of this magnitude more than 50% of ATP was already being supplied by glycolysis. Over a 30 s sprint of the same intensity almost 70% of the energy was supplied by glycolysis and marked reduction in ATP occurred in the presence of significant amounts of PCr. The second experimental chapter examined the influence of high intensity training on the 30 s AWT and a trend for the resulting improvement in performance to be associated with an increased glycolytic capacity was revealed. The final experimental chapter combined a study of metabolic changes in the gastrocnemius muscle of patients with ischaemic rest pain together with a trial of intravenous naftidrofuryl in its treatment. Changes in muscle metabolism observed in patients with rest pain (low glycogen, ATP, PCr and SDH levels) were felt to represent disuse rather than a direct effect of muscle ischaemia and the drug naftidrofuryl conferred no obvious metabolic benefit although it did appear to possess analgesic properties. In the final discussion an hypothesis on fuel supply as a mechanism for fatigue is presented in the light of the metabolic changes reported.

The work for this thesis was carried out between 1981 and 1986 in the Department of Surgery, University of Leicester. Unless stated, all the work contained within was carried out entirely by the author and has not previously been submitted for another degree. Part of the work on which this thesis is based has been published in the following papers and the studies pertaining to them will be referred to in the text by the roman numerals.

I) Boobis LH, Wootton SA and Williams C (1983)
Human muscle metabolism during brief maximal exercise
J Physiol (Lond), 338, 21P

II) Boobis LH, Wootton SA and Williams C (1983)
Influence of sprint training on muscle metabolism during brief maximal exercise in man
J Physiol (Lond), 342, 36P-37P

III) Boobis LH and Bell PRF (1982)
Can drugs help patients with lower limb ischaemia?
Br J Surg, 69 (Suppl 1), S17-S23

IV) Boobis LH and Bell PRF (1984)
The place of drug therapy in peripheral arterial disease
In BIMR Surgery 4, Vascular Surgery (edited by PRF Bell and NL Tilney) pp 12-52. London: Butterworths

V) Boobis LH, Cheetham ME, Wootton SA and Williams C (1987)
Metabolic aspects of fatigue during sprinting
In Exercise - Benefits, Limits and Adaptations (edited by DAD Macleod, RJ Maughan, M Nimmo, T Reilly and C Williams)
London: Spon Limited (in the press)

Aknowledgements

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Much of the work in studies I and II was carried out in the Department of Sports Science, University of Loughborough with the collaboration of Professor Clyde Williams, who stimulated my continued interest in the fascinating field of exercise physiology. He has been a constant source of encouragement throughout and has sustained belief in my ability to complete this work when at times I had all but given up. My collaboration with him and his department has always been enjoyable and to Professor Williams I owe my eternal thanks.

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for

Caro, Nathalie and Sophie

"Veins which by the thickening of their tunicles in the old restrict the passage of the blood, and by this lack of nourishment destroy their life without any fever, the old coming to fail little by little in slow death."

Leonardo da Vinci (1452-1519), Dell' Anatomia, Fogli B

(translated by

Edward MacCurdy in The Notebooks of Leonardo da Vinci, Vol I, Ch III)

Index

Abstract I

Statement of Originality II

Acknowlegements III

Dedication IV

Index V

List of Tables IX

List of Plates and Figures XI

1 General Introduction 1

2 Literature review - Muscle metabolism 5

2.1 Anaerobic muscle metabolism - an historical review 5
2.1.1 The lactic acid era
2.1.2 The phosphogen era 6
2.2 The mechanism of muscular contraction 9
2.3 Energy provision and control during short term exercise 11
2.3.1 Adenosine triphosphatases 11
2.3.2 Regeneration of ATP 13
2.3.3 Control of glycogenolysis - phosphorylase 17
2.3.4 Control of glycolysis - phosphofructokinase 21
2.3.5 The creatine phosphokinase reaction 22
2.3.6 The adenylate kinase reaction 24
2.3.7 Adenylate deaminase 25
2.3.8 Amplification of control in energy supply 26
2.4 The importance of muscle fibre types in working human muscle 31
2.5 Fatigue 34
2.5.1 Central fatigue
2.5.2 Peripheral fatigue 35
2.6 The effect of training on high intensity exercise 45
2.6.1 Animal studies 46
2.6.2 Cross sectional studies in man 46
2.6.3 Prospective studies and high intensity training 52
2.6.4 Evidence for fibre type transformation 54

3 Atherosclerosis and peripheral vascular disease - literature review 55

3.1 Prevalence of peripheral vascular disease 55
3.2 Natural history of peripheral vascular disease 57
3.3 Aetiology of atherosclerosis 60
3.3.1 Platelets 61
3.3.2 Cholesterol 63
3.3.3 Thrombosis 64

3.4 Pathophysiological factors affecting outcome in occlusive arterial disease 65
3.4.1 Collateral circulation 65
3.4.2 Vascular spasm 66
3.4.3 Sympathetic innervation 68
3.4.4 Rheological abnormalities 70
3.5 Skeletal muscle metabolism and peripheral vascular disease 71
3.5.1 Metabolic changes within the muscle 71
3.5.2 The effect of training on intermittent claudication 77
3.6 Exercise and atherosclerosis 79
3.7 Areas in which drugs may help arterial disease 81
3.7.1 Antilipaemic drugs 85
3.7.2 Antiplatelet drugs and prostaglandins 87
3.7.3 Anticoagulants 101
3.7.4 Thrombolytic therapy 103
3.7.5 Vasodilators 105
3.7.6 Rheological agents 110
3.7.7 Metabolic enhancers 112
3.7.8 Other drugs 119
3.7.9 Beta blockers 120
3.7.10 Conclusions 121

4 Methodology 122

4.1 Muscle biopsies 122
4.1.1 Skin preparation 122
4.1.2 Biopsy procedure 124
4.1.3 Initial treatment of biopsies for metabolite assays 125
4.2 Sample preparation 126
4.2.1 Preparation for muscle metabolite assay 126
4.2.2 Preparation for muscle oxidative capacity 128
4.3 Metabolite assays 129
4.3.1 General principles 129
4.3.2 Fluorimetric assays for muscle metabolites 132
4.4 Succinate dehydrogenase assay 153
4.4.1 Assay of enzyme activity 154

5 Metabolic response to brief maximal exercise 158

5.1 Introduction 158
5.2 Protocol 159
5.3 Methods 159
5.3.1 The anaerobic work test (AWT) 159
5.3.2 Blood sampling 163
5.3.3 Muscle biopsies 163
5.4 Results 164
5.4.1 Power output 164
5.4.2 Changes in blood metabolites 164
5.4.3 Muscle metabolites 164
5.4.4 Muscle pH 171
5.4.5 ATP turnover 171
5.4.6 Glycogenolytic and glycolytic rates 173
5.5 Discussion 173
5.5.1 Peripheral fatigue - a protective mechanism 176
5.5.2 Muscle pH 176

5.5.3 *Glycogenolytic and glycolytic rates* 177
5.5.4 *ATP turnover and power output* 180
5.5.5 *Fall in ATP concentration* 181
5.5.6 *Aerobic contribution to high intensity exercise* 182
5.6 Conclusions 182

6 Influence of interval training on the 30 s AWT 184

6.1 Introduction 184
6.2 Protocol 185
6.3 Methods 185
6.3.1 *Maximum oxygen uptake* 185
6.3.2 *Anthropometric measurements* 187
6.3.3 *Training method* 187
6.3.4 *The anaerobic work test (AWT)* 188
6.4 Results 188
6.4.1 *Power output* 188
6.4.2 *Anthropometric characteristics* 188
6.4.3 *Functional capacity* 192
6.4.4 *Changes in blood metabolites* 192
6.4.5 *Muscle metabolites* 192
6.4.6 *Muscle pH* 201
6.4.7 *ATP turnover* 201
6.4.8 *Glycogenolytic and glycolytic rates* 201
6.5 Discussion 205
6.5.1 *Phosphogens and phosphogenolysis* 205
6.5.2 *Glycogen, glycogenolysis and glycolysis* 206
6.5.3 *Pyruvate* 208
6.5.4 *ATP turnover rates* 209
6.5.5 *Conclusions* 209

7 Metabolic changes in gastrocnemius muscle in patients with ischaemic rest pain and the influence of treatment with intravenous naftidrofuryl 211

7.1 Introduction 211
7.1.1 *Muscle metabolism in ischaemic rest pain* 211
7.1.2 *Naftidrofuryl* 212
7.2 Protocol 213
7.2.1 *Withdrawal and exclusions from the trial* 214
7.3 Methods 216
7.3.1 *Administration of the test substance* 216
7.3.2 *Clinical assessment* 217
7.3.3 *Physiological assessment* 220
7.3.4 *Blood sampling* 224
7.3.5 *Muscle biopsies* 224
7.4 Results 224
7.4.1 *Patient details* 224
7.4.2 *Changes in muscle metabolism with rest pain* 224
7.4.3 *Naftidrofuryl trial* 229
7.5 Discussion 240
7.5.1 *Changes in muscle metabolism with peripheral vascular disease* 240
7.5.2 *Naftidrofuryl trial* 244
7.5.3 *Conclusions* 248

8 Final discussion and Summary 249

8.1 Fatigue 249
8.1.1 Central fatigue 249
8.1.2 Peripheral fatigue 251
8.2 Metabolic response to brief maximal exercise 252
8.2.1 An hypothesis on fuel supply as a mechanism for fatigue 254
8.3 Influence of interval training on muscle metabolism with the 30 s Anaerobic Work Test (AWT) 257
8.4 Metabolic changes in muscle of patients with ischaemic rest pain 259
8.5 The use of naftidrofuryl in the treatment of ischaemic rest pain 260
8.6 Summary 262

Appendices

A1 Apparatus, chemicals, biochemicals and reagents 263

A2 Spectrophotometric assays for validation of standards 279

A3 Assays for blood metabolites 292

References 298

List of Tables

Table 2.1 Fuel storage and energy supply for human skeletal muscle 14

Table 2.2 Regulators of phosphofructokinase 27

Table 2.3 Muscle metabolite concentrations in man at rest and after various types of muscular work 29-30

Table 2.4 Differences in muscle fibre type characteristics in man 33

Table 2.5 ATP turnover rates and glycolytic and glycogenolytic rates for various types of muscular work 43

Table 2.6 Functional and biochemical changes as a result of physical training in man
2.6a Functional changes 49
2.6b Morphological and enzyme changes 50
2.6c Metabolite changes 51

Table 3.1 Prevalence of peripheral arterial disease of the lower limb 56

Table 3.2 Natural history of peripheral vascular disease of the lower limb 58

Table 3.3 Drugs used in peripheral vascular disease 83-84

Table 4.1 Coefficients of variation for muscle metabolite assays 133

Table 5.1 Performance characteristics of 6 s and 30 s AWT 165

Table 5.2 Blood lactate and glucose concentrations and a summary of performance characteristics in response to the 6 s and 30 s AWT 166

Table 5.3 Muscle metabolite results before and after the 6 s and 30 s AWT 167

Table 5.4 Statistical summary of metabolite changes after the 6 s and 30 s AWT 169

Table 5.5 Calculated values for muscle pH, glycogenolytic and glycolytic rates and anaerobic ATP turnover rates 175

Table 6.1 The influence of training on the performance characteristics of the 30 s AWT 189

Table 6.2 Training induced changes in anthropometric measurements and functional capacity 191

Table 6.3 Influence of training on blood lactate and glucose concentrations in response to 30 s AWT 193

Table 6.4 Influence of training on muscle metabolites in response to 30 s AWT 194

Table 6.5 Statistical summary of the influence of training on metabolite changes after 30 s AWT
6.5a Changes in muscle metabolites with the AWT and as a result of training 195
6.5b The effects of training, exercise and the interaction between training and exercise 196

Table 6.6 Influence of training on calculated values for muscle pH, glycogenolytic and glycolytic rates and anaerobic ATP turnover rates 203

Table 6.7 Anaerobic ATP turnover rates calculated from changes in PCr, lactate and pyruvate corrected for a constant total Cr concentration of 124.4 210

Table 7.1 Naftidrofuryl rest pain trial - patient details 225

Table 7.2 Metabolite values and succinic dehydrogenase activity of gastrocnemius muscle in rest pain patients and normal controls 227

Table 7.3 Metabolite values and succinic dehydrogenase activity of gastrocnemius muscle in diabetic and non-diabetic rest pain patients 228

Table 7.4 Amputation and reconstructive surgery rates for naftidrofuryl trial 230

Table 7.5 Linear analogue scores for subjective parameters
7.5a All patients 231
7.5b Patients who completed 10 days intravenous therapy 232

Table 7.6 Statistical summary of analogue scores of subjective parameters 234

Table 7.7 Analgesia requirements
7.7a All patients 237
7.7b Patients who completed 10 days intravenous therapy 238

Table 7.8 Haemodynamic measurements; plethysmographically measured segmental blood pressure and calf blood flow. 239

Table 7.9 Changes in blood metabolites in response to intravenous therapy in naftidrofuryl rest pain trial; patients who completed 10 days I.V. therapy. 241

Table 7.10 Changes in muscle metabolites in response to intravenous therapy during naftidrofuryl rest pain trial. 242

List of Plates and Figures

Plate 1	The various types of muscle biopsy needles.	123
Figure 1	Intramuscular anaerobic substrates as moles/kg wet weight and molar equivalents of high energy phosphate (as ATP).	15
Figure 2	Schematic representation of the length of time each anaerobic substrate would last at maximum recorded ATP turnover rate.	16
Figure 3	Outline of the Embden-Myerhof pathway.	18
Figure 4	Outline of the activation and control of glycogen phosphorylase.	19
Figure 5	A scheme for the development of fatigue.	36
Figure 6	Areas in which drugs might help lower limb ischaemia.	82
Figure 7	The platelet vessel wall prostaglandin inter-action and a scheme of thromboxane A_2 and prostacyclin synthesis.	88
Figure 8	Outline of the protocol in study I examining the metabolic response to brief maximal exercise.	160
Figure 9	Diagram of the fatigue profile obtained during the Anaerobic Work Test (AWT).	162
Figure 10	Three dimensional pathways chart illustrating the percentage change in muscle glycolytic intermediates after the 6 and 30 s AWT.	170
Figure 11	Derived total ATP turnover rates calculated from metabolite changes after 6 and 30 s AWT with the relative contributions of ATP, PCr and glycogen.	172
Figure 12	Glycogenolytic and glycolytic rates calculated from metabolite changes after 6 and 30 s AWT.	174
Figure 13	Plot of glucose 6-phosphate and lactate with best fit curve for data from studies I and II.	179
Figure 14	Outline of the protocol for study II examining the influence of interval training on the metabolic response to the 30 s AWT	186
Figure 15	Influence of training on the fatigue profile and power generated during the 30 s AWT.	190
Figure 16	Three dimensional pathways chart illustrating the influence of training on the percentage change in glycolytic intermediates after the 30 s AWT.	200

Figure 17 Derived total ATP turnover rates during the 30 s AWT before and after training. Values for study I included for comparison. 202

Figure 18 Glycogenolytic and glycolytic rates during the 30 s AWT before and after training. Values for study I included for comparison. 204

Figure 19 Summary of the protocol for the naftidrofuryl rest pain trial. 215

Figure 20a Linear analogue charts for Rest Pain, Paraesthesia and Coldness. 218

Figure 20b Linear analogue charts for General Wellbeing, Appetite and Sleep. 219

Figure 21a Line plot of scores for Pain, Paraesthesia and Coldness. 235

Figure 21b Line plot of scores for Wellbeing, Appetite and Sleep. 236

Figure 22 Pathways to fatigue. 250

Figure 23 Logarithmic plot of glycolytic ATP turnover against work load (power ouput) from data in table 2.5 and from studies I and II. 258

Chapter 1

1 General Introduction

Although there is no immediate, apparent relationship between healthy subjects capable of performing supramaximal exercise and patients with ischaemic rest pain due to peripheral arterial disease whose exercise tolerance is severely limited, there are similarities between the two groups.

The ability to perform high intensity exercise is dependent primarily on the ability of the exercising muscle to access and utilise energy in an almost instantaneous manner. Where the exercise is truly supramaximal provision of energy is almost exclusively anaerobic and fatigue, which can be considered to comprise of central and peripheral components, limits the amount of work that can be performed. Central fatigue can be influenced by motivation, pain, perceived severity of exercise, dyspnoea and the central effects of systemic metabolic changes. Peripheral fatigue can be considered to comprise three main parts; impaired activation/excitation of the motor units, impaired energy supply and impaired actin-myosin cross bridge coupling.

Patients with severe peripheral vascular disease can be considered analogous in their response to minimal exercise as normal subjects are to supramaximal exercise, in that both have a relative deficiency in oxygen supply for the demands of the working muscle. When the patient has pain at rest there may well be an oxygen lack even without exercise thus producing a similar metabolic situation in the resting state, and the presence of pain may enhance the analogous situation by contributing to the patients' central perception of fatigue. The study of skeletal muscle metabolism in response to high intensity exercise in normal subjects may therefore provide a realistic model to examine the

metabolic changes that may occur in occlusive arterial disease.

As energy for muscular work in both situations will be provided primarily by anaerobic means, an increase in performance or an improvement in pain will necessitate more energy being made available to the muscle cells either in absolute terms or by more complete utilisation of the available energy stores. An increase in high-energy phosphogens would provide the potential for this increased energy provision by the former means and some studies on high intensity training have reported this finding (e.g. Karlsson et al, 1972; Eriksson et al, 1973). More universally recorded has been an increase in the rate of glycolysis with training, and it has been suggested that training allows the muscle to adapt to the resultant increase in lactate by an improvement in its ability to buffer hydrogen ions (e.g. Sharp et al, 1983; Parkhouse and Mackenzie, 1984) thus allowing an increased rate of ATP resynthesis.

Patients with intermittent claudication have been reported as exhibiting similar changes in muscle enzyme activity as with physical training, namely an increased oxidative capacity (Holm et al, 1971; Holm, 1972) although more recent work has been contradictory and surprisingly claudicants do not have a high anaerobic capacity (Henriksson et al, 1980). There is little information on metabolite levels in this group of patients but ATP levels have been demonstrated as being depressed (Pernow et al, 1975). Physical training would appear to have no effect on glycolytic enzymes while oxidative capacity does seem to be improved (Saltin, 1981).

The drug naftidrofuryl is claimed to be of benefit to patients with intermittent claudication and ischaemic rest pain by virtue of its metabolic effects. It has been stated that it improves tissue oxidative potential by increasing succinate dehydrogenase activity and restoring

high energy phosphate levels (Maynaud et al, 1973; Elert et al, 1976). If this is the case then it would appear to have the potential of conferring on these patients a very favourable metabolic effect and confirmation of its value is thus required.

The aim of the studies reported in this thesis was firstly to examine the changes in muscle metabolites that occur during supramaximal dynamic exercise before and after high intensity sprint training, and secondly to compare these changes with those that may occur in patients with ischaemic rest pain due to occlusive lower limb arterial disease. This second aim was to be combined with a double blind trial of the drug naftidrofuryl in the treatment of these patients with ischaemic rest pain.

These studies are presented in four main experimental chapters. As a large part of the work was involved in setting up the analytical techniques for assaying the muscle biopsies, chapter 4 describes in some detail the methodology involved.

Chapter 5 outlines the design and nature of the experiment investigating the metabolic responses to brief maximal exercise using an all-out bicycle ergometer test (the Anaerobic Work Test) with which the subject exercises to exhaustion in 30 seconds. An attempt to identify the relative contribution to energy supply by the high energy phosphates and by glycolysis was made by biopsying the subjects at six seconds into the exercise period and at the termination of exercise (30 seconds).

Chapter 6 examines the effects of high intensity interval training on those metabolic changes that were reported after 30 seconds of exercise. The aim of the study was to determine whether a metabolic basis could be demonstrated for the increased ability to perform work which is seen with this type of training.

The final experimental chapter presents the metabolic changes that occurred in the gastrocnemius muscle of patients with rest pain. This study was combined with a double-blind trial of the administration of the drug naftidrofuryl to a group of these patients, the control group of patients providing the information for the first part of the study. Biopsies were obtained prior to treatment and again after five days and ten days of intravenous administration, and biochemical assessment was combined with measurement of blood flow. Placebo response was monitored by recording the subjective parameters of pain and related symptoms. The aim of this study was to determine whether or not the metabolic changes recorded mirrored those of the first study, and whether or not naftidrofuryl was of benefit metabolically or symptomatically to these patients.

Throughout all the studies involving subjects and patients the nature of the study or trial was explained and muscle biopsies were only taken after informed consent, the studies having had prior approval of the hospital or university ethical committee. At all times the most important priority was the welfare of the subjects and patients and although no experiment had to be halted because of undue distress to those participating, it was made clear to them that they could withdraw from the studies at any time.

Chapter 2

2 Literature Review - Muscle metabolism

2.1 Anaerobic muscle metabolism - an historical review

2.1.1 The lactic acid era

Lavoisier, in the years prior to the French Revolution was the first person to show that muscular work increased combustion within the body. This he attributed to the burning of a substance ("air fixé") in the lungs brought there by the blood (cited by Asmussen, 1971). It took a further half century before Magnus in 1837 concluded that combustion took place not only in the lungs but in the entire body (cited by Asmussen, 1971). By this time Berzelius, as reported by du Bois-Raymond (1877), was aware of the production of lactic acid by contracting muscle although its origins were uncertain. In 1867 Hermann reported that it arose from the explosive combination with inbuilt oxygen of a giant molecule called "inogen" (cited by Asmussen, 1971), but at the beginning of this century Fletcher and Hopkins (1907) showed that lactic acid was formed from glycogen stored within the muscle and oxygen utilization occurred in the recovery phase, there being no oxygen stored in an intra-molecular form. In 1922 Meyerhof and Hill shared the Nobel Prize in physiology for their theory which seemed to explain all the main changes in metabolism during exercise: as a direct result of muscular work there was a sudden production of lactic acid from glycogen; glycogen was resynthesised aerobically by combustion of part of the lactic acid formed. They postulated that a "steady state" could be reached as a result of a dynamic equilibrium between these two processes and that the "oxygen debt" paid after stopping exercise was caused by

the slow return to the resting state (Meyerhof, 1920; Hill, 1965). By combining this theory with his own thermodynamic studies, Hill further summarised anaerobic muscular contraction into five recognisable steps:

1). glycogen disappered

2). lactic acid appeared in equivalent amounts

3). CO_2 was driven off

4). heat was produced proportional to the lactic acid

5). hydrogen ion concentration rose

Even with this knowledge the primary biochemical reaction in muscular contraction was considered to be the release of lactic acid from glycogen, a large precursor molecule known as a "lactacidogen"; the liberated hydrogen ions were alleged to neutralize negative charges on the contractile protein filaments and so allow them to shorten. Doubt on this theory was already being cast by the discovery that the amount of lactic acid that could be produced by muscle was greatly increased by the addition of a buffer (Kondo, 1912) and by Embden's finding that much of the lactic acid was produced after the contraction was over (Embden, 1924). However it was not until the discovery of the phosphogens that Hill in 1932 was able to put forward his "revolution" in our understanding of muscle physiology and the "lactic acid era" would end and the "phosphogen era" begin (Hill, 1965).

2.1.2 The phosphogen era

In 1927 Eggleton and Eggleton, and Fiske and Subbarow published independently the finding that an organic phosphate (identified by the latter authors as phosphoryl creatine) decreased during muscular contraction, with a corresponding increase in the amount of inorganic phosphate, and increased with recovery (Eggleton and Eggleton, 1927;

Fiske and Subbarow, 1927). The lactic acid theory did not "lie down" however until Lundsgaard described his findings in 1930 that if muscle was poisoned with mono-iodoacetic acid anaerobic contractions could still take place without the production of lactate by the breakdown of phosphocreatine (Lundsgaard, 1930). It was presumed therefore that the basis for mechanical work was the splitting of phosphocreatine into inorganic phosphate and free creatine, lactic acid being formed as a secondary event to rebuild phosphocreatine.

During this "revolution" however, evidence for a more immediate energy source was accumulating. In 1929 Lohmann discovered adenosine triphosphate in muscle extracts, and in 1934 reported that the only way in which phosphocreatine could be utilised was by the rephosphorylation of adenosine diphosphate to ATP (Lohmann, 1934) and the hydrolysis of ATP and its rephosphorylation from PCr is known as the 'Lohmann Reaction'. It was rapidly realised that ATP was the immediate energy source for muscular contraction and that the primary reaction was the splitting of ATP into ADP and inorganic phosphate. Thus the breakdown of PCr and the accumulation of lactic acid were now relegated to a secondary recovery phase, although as no fall in ATP during muscular contraction had yet been demonstrated conclusive proof of the key role of ATP was a long time forthcoming. More indirect evidence was provided by Engelhardt and Ljubimowa (1939) who demonstrated that the myosin component of the muscle fibrils acted as an ATP cleaving enzyme (acto-myosin ATPase) thus affording a means of energy transfer during muscular contraction. Proof that ATP was indeed the primary energy source was finally provided with the demonstration by Cain et al (1962) that muscle in which creatine phosphokinase was inhibited with fluorodinitrobenzene (FDNB) could still contract by hydrolysis of ATP without utilisation of PCr.

In 1933 Margaria et al (1933) raised the concept of an "alactacid" oxygen debt as relating to the resynthesis of phosphogens (PCr and ATP). They had shown that after work of all intensities, there occurred an oxygen debt that was unrelated to the accumulation of blood lactate and that lactic acid increased after more intense exercise with a further increase in the oxygen debt. As a consequence of this Margaria postulated that the initial provision of the first few seconds of anaerobic energy was by PCr breakdown (Margaria et al, 1969). It was now possible to describe the apparent sequence of events that allowed muscular contractions and physical work to occur by utilisation of "fuels" within the muscle cell:

1) Energy for contraction was derived by hydrolysis of ATP to ADP directly by the muscle fibrils
2) Resynthesis of ATP occurred by rephosphorylation of ADP by PCr
3) Resynthesis of PCr took place
    a) anaerobically from glycolysis with the accumulation of lactate or
    b) aerobically by combustion of foodstuffs within the muscle cell

Our understanding of the exact biochemical sequence of events responsible for muscular contraction grew rapidly with the development of new scientific technology in the post-war years. Electron-microscopy allowed the ultrastructure of the muscle cell to be unravelled; the elucidation of the various biochemical pathways and the importance of the "high energy phosphate bond" was realised; the use of C^{14} labelled compounds and vascular catheterisation techniques allowed for the identification of muscular fuels and substrates and the determination of the fate of metabolites. However it was not until the reintroduction of

the muscle biopsy technique by Bergström in 1962 together with the microanalytical techniques of Lowry and Passonnau (Lowry et al 1964; Lowry and Passonneau, 1972) that direct examination of the changes occurring within the working muscle could be examined.

Using these methods, in 1971 Saltin et al (1971) were able to demonstrate the initiation of glycolysis within 10 seconds of exercise and surmised that it occurred the instant muscular contraction began, thus finally providing evidence to refute Margaria's alactacid interval, this work being confirmed by Jacobs et al (1983). Finally, in his excellent review, Brooks (1985) provides good evidence to refute the concept of an anaerobic threshold. He points out that the kinetics of lactic dehydrogenase are such that lactate is normally formed during aerobic work and that lactate appearance in the blood (and accumulation in the muscle) merely reflects a balance between lactate production and removal.

2.2 The mechanism of muscular contraction

The initiation of muscular contraction requires the transmission of a neuronal impulse along the peripheral nerve across the neuromuscular junction by release of acetylcholine. When sufficient transmitter has been released to change its internal potential to 40 mV above its resting potential of -90 mV an action potential is propogated in both directions along the muscle fibre (Hodgkin, 1951) and inwards along the transverse (T) tubule system (A.F. Huxley, 1974). These T-tubules are flanked by the lateral cysternae of the sarcoplasmic reticulum and transmission of the depolarization impulse to them results in calcium release (Fabiato and Fabiato, 1977). Calcium then diffuses into the muscle cytoplasm increasing its resting concentration from 10^{-7} mol/l to

10^{-5} mol/l and binds with the regulatory protein troponin C whose calcium binding sites are rapidly occupied (Kushmerick, 1983). This protein consists of two other components, whose interaction with tropomyosin, a fourth constituent of the thin filaments, results in a conformational change allowing actin to interact with the myosin head of the thick filament. The mechanism of the interaction which allows the thick and thin filaments to slide and cause muscle shortening was proposed by H.E. Huxley (1969) in which he postulated that force was developed by a rotation of the myosin head relative to the actin. A.F. Huxley and Simmons (1971) incorporated in this theory a mechanism for the stepwise shortening and elastic properties of contraction which have been shown to exist and it is likely that some variant of this mechanism occurs in nature. In this mechanism the myosin head oscillates between different stable positions dependent on the degree of shortening or stretch. During a single contraction each actin-myosin attachment (cross-bridge) is thought to act with repeated cycles of attachment, shortening and detachment and it is the process of detachment which is coupled to ATP hydrolysis by the myosin ATPase incorporated within the myosin head. Under conditions of stretch however, the position of the myosin head changes so that it can dissociate without utilisation of ATP. The exact mechanism whereby ATP hydrolysis is converted to mechanical work during each cross-bridge cycle is as yet unknown, but it is probable that some conformational change occurs in the myosin molecule when it binds to an ATP molecule. This may be in addition to the ATP required for its dissociation from actin so that two ATP molecules are utilised per cycle (Tonomura et al, 1961). Relaxation occurs when acetylcholine concentration falls as a result of cessation of neuronal impulses and calcium ions are actively transported back into the sarcoplasmic reticulum by an energy requiring process involving

calcium ATPase. The decrease in free calcium concentration allows dissociation of calcium ions from troponin and contraction stops (A.F. Huxley, 1974; Brooks and Fahey, 1984). Thus the electrical events leading to contraction and the chemical sequences producing it require energy which is only available from ATP hydrolysis; Na^+-K^+ ATPase maintains the Na^+/K^+ gradient across the cell, Ca^{2+} ATPase actively transports calcium back into the sarcoplasmic reticulum and actomyosin ATPase forms and breaks cross-bridges. Under extreme exercise this energy is provided exclusively by anaerobic sources.

2.3 Energy provision and control during short term exercise

The initiation of muscular contraction is therefore dependent on the release of Ca^{2+} and the energy for work is provided by the hydrolysis of ATP. For muscular work to continue the concentration of ATP must be maintained; therefore under steady-state non-fatiguing conditions of exercise, supply will equal demand. Under conditions of increasingly severe exercise maintainance of the rate of work is dependent on ATP resynthesis matching this increased demand (ATP turnover rate) and this will be determined by the rate at which ATP can be provided by its various regeneratory processes. Once the rate of ATP supply falls below that required then work rate must fall and fatigue occurs.

2.3.1 Adenosine triphosphatases

Adenosine triphosphate hydrolysis by actomyosin ATPase, Ca^{2+} ATPase and Na^+-K^+ ATPase for their various energy requiring steps can be summarised as follows:

$$ATP + H_2O \longrightarrow ADP + Pi + H^+ \qquad (2.1)$$

a) Actomyosin ATPase

In equation 2.1 actomyosin ATPase, which is involved in the formation and breaking of cross-bridges, has by far the highest activity. Margaria et al (1964) estimated that the muscle ATP turnover rate during 3 seconds of maximal stair climbing was in the order of 7 mmol ATP/kg d.m./s whereas with a standing high jump values between 25-30 mmol ATP/kg d.m./s have been described (CTM Davies, 1971; Di Prampero and Mogoni, 1981). McGilvery (1975) has calculated that this is equivalent to a maximum power delivery of 4-4.5 kilowatts and an ATP turnover rate of this magnitude would require a myofibrillar ATPase activity of 300-400 µmol/min/g wet weight.

b) Calcium ATPase

Calcium ATPase which is responsible for the active transport of Ca^{2+} back into the sarcoplasmic reticulum has been estimated to have a turnover rate of 0.4-0.8 mmol ATP/kg d.m./s (Kushmerick, 1983). It has been calculated that two Ca^{2+} are transported per ATP hydrolysed and that a continuous cycling of Ca^{2+} occurs during exercise to maintain the cytoplasmic calcium concentration at the level of 10^{-5} mol/l.

c) Sodium-potassium ATPase

Sodium-potassium ATPase activity during tetanic contraction has been estimated to be in the order of 0.2-0.6 mmol/kg d.m./s. It is thought that after each depolarisation to restore intracellular/extracellular Na^+/K^+ concentrations to their initial values 10 µmol of ATP are required (Kushmerick, 1983).

2.3.2 Regeneration of ATP

The regeneration of ATP during muscular contraction occurs by four mechanisms

$$PCr + ADP + H^+ \longrightarrow ATP + Cr \quad (2.2)$$

$$glycogen + 3ADP + 3Pi \longrightarrow 3ATP + 2La^- + 2H^+ \quad (2.3)$$

$$2ADP \longrightarrow ATP + AMP \quad (2.4)$$

$$acetyl\ CoA + O_2 + ADP \longrightarrow ATP + CO_2 + H_2O \quad (2.5)$$

The first three reactions occur anaerobically in the cytoplasm and the fourth aerobically in the mitochondria, acetyl CoA being derived from either pyruvate or fatty acids for subsequent oxidation in the Krebs cycle. A summary of the fuels available to the muscle and the amount and rate of energy supply are given in table 2.1 and illustrated for anaerobic conditions in figures 1 and 2.

Complete oxidation of glycogen requires that the electrons are removed from NADH for reduction of oxygen in the mitochondria. As the mitochondrial membrane is impermeable to the pyridine nucleotides (as the redox state [the NAD^+/NADH ratio] has to be maintained at a more oxidised state in the cytoplasm than the mitochondria [1000:1 against 10:1]) (Newsholme and Start, 1973) there must exist a means of transferring the electrons. This is achieved by different methods in white muscle and red muscle by means of the glycerol-3-phosphate shuttle (or glycerol-1-phosphate shuttle [Crabtree and Newsholme, 1972]) and the malate-aspartate shuttle respectively (McGilvery, 1975; Newsholme and Start, 1973). Under conditions of oxygen lack, the cell's redox state is maintained by reducing pyruvate to lactate by the conversion of NADH to NAD^+ but with the penalty to the cell of an accumulation of lactate and H^+.

Table 2.1 Fuel storage and energy supply for human skeletal muscle

	amount of substrate in moles	moles ~Pi (ATP) per mole of substrate	O_2	maximum ATP turnover (μmoles/g/s)	work time at maximum power (min)
anaerobic sources					
ATP → ADP	0.16	1	-	2.6	0.03
PCr → Cr	0.60	1	-	2.0	0.17
glycogen → lactate	2.50	3	-	1.2	1.2
glucose → lactate	0.02	2	-	0.8	0.01
aerobic sources					
glycogen → $CO_2 + H_2O$	2.50	37	6.17	0.5	90
glucose → $CO_2 + H_2O$	0.02	36	6.00	0.5	0.7
FFA → $CO_2 + H_2O$	60.00	138	5.63	0.25	10 days

Amount of substrate calculated for theoretical 70 kg man with a muscle mass of 30 kg, a muscle glycogen content of 70 mmoles/kg wet weight, a liver glycogen content of 500 mmoles and a total body fat content of 20%. Aerobic work time calculated for a VO_2max of 55ml/kg at a work rate of 70% VO_2max (maximum ATP turnover rates are expressed as μmoles/g/s wet weight and assume each substrate is the sole energy source, and for aerobic sources assume an unlimited oxygen supply).

(Data compiled from McGilvery, 1975; Hultman and Sjöholm, 1983; Sahlin, 1986)

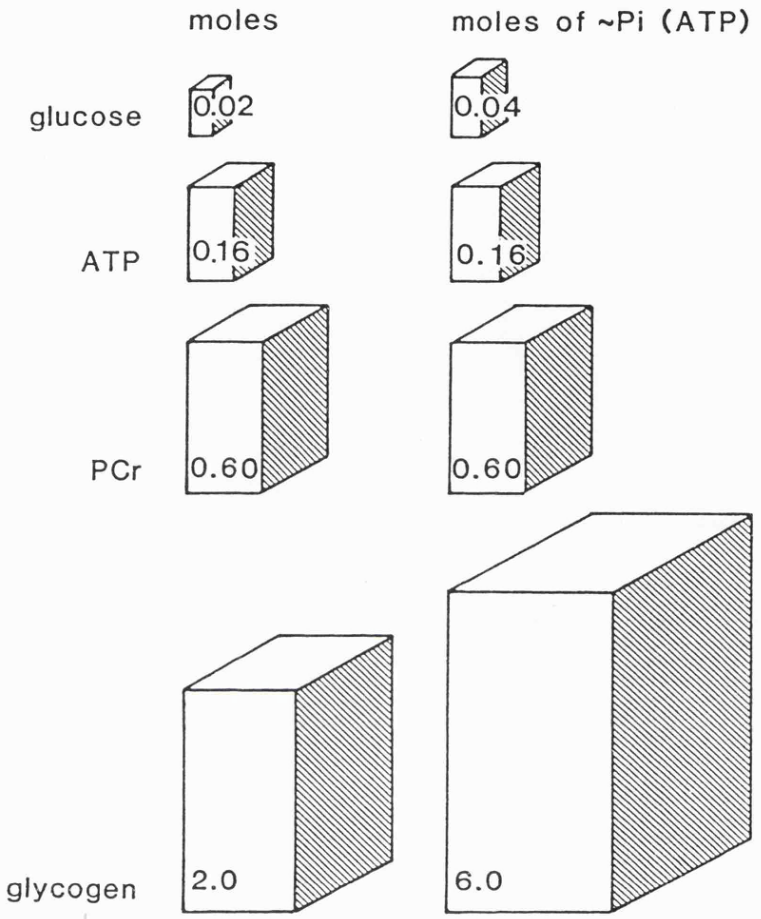


Figure 1 Diagramatic representation of the stores of intramuscular anaerobic substrates as moles/kg wet weight and molar equivalents of high energy phosphate (as ATP generated). As can be seen, glycogen is by far the most abundant fuel.

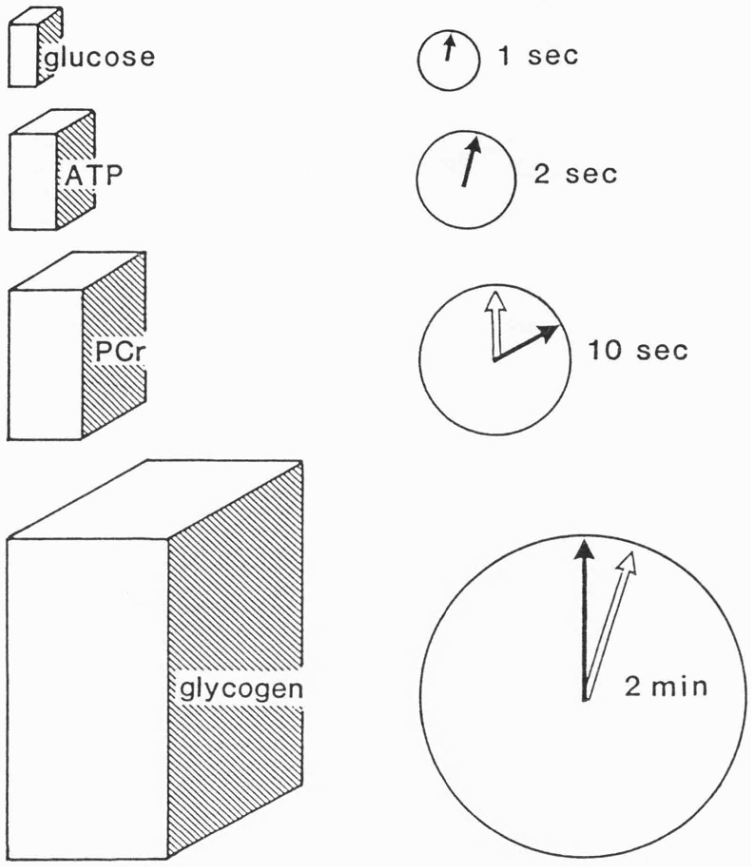


Figure 2 Schematic representation of the length of time each anaerobic fuel store would last at its maximum recorded ATP turnover rate.

2.3.3 Control of glycogenolysis - phosphorylase

Glycolysis is the conversion of glycogen to lactate through the Embden-Meyerhof pathway, whereas glycogenolysis is the entry of glycogen into the pathway by the phosphorylation of the glucosyl units to glucose 1-phosphate. As long as sufficient ATP can be regenerated by glycolysis, the adenine nucleotide pool will remain constant and the adenylate kinase reaction (equation 2.4) will be of secondary importance. An outline of the Embden-Myerhof pathway is illustrated in figure 3. For each 6-carbon glucosyl unit metabolised to two 3-carbon lactate molecules four molecules of ATP are generated and one is utilised in the phosphorylation of fructose 6-phosphate giving a net gain of three.

The entry of glycogen into the glycolytic pathway is initiated by phosphorylase which is considered to be the rate limiting enzyme of glycogen degradation. This enzyme exists in two forms and their interconversion is part of a complex system controlled by an enzyme cascade (figure 4). Phosphorylase a (phosphorylated), (the active form) is present in low concentrations (mole fraction of 22%) in the resting state when the b form (non-phosphorylated) is considered to be essentially inactive (Chasiotis, 1983). The b form is inhibited by ATP and glucose 6-phosphate and activated by AMP and IMP (Danforth, 1965; Aragon et al, 1980), unlike the a form which is unaffected by these inhibitors and is active even in the absence of AMP (Cori and Green, 1943).

Resynthesis of glycogen is catalysed by the enzyme glycogen synthetase which also exists in two interconvertible forms, I, the active non-phosphorylated form and D the inactive, phosphorylated form (Friedman and Larner, 1963). Although the inhibition of synthetase D by

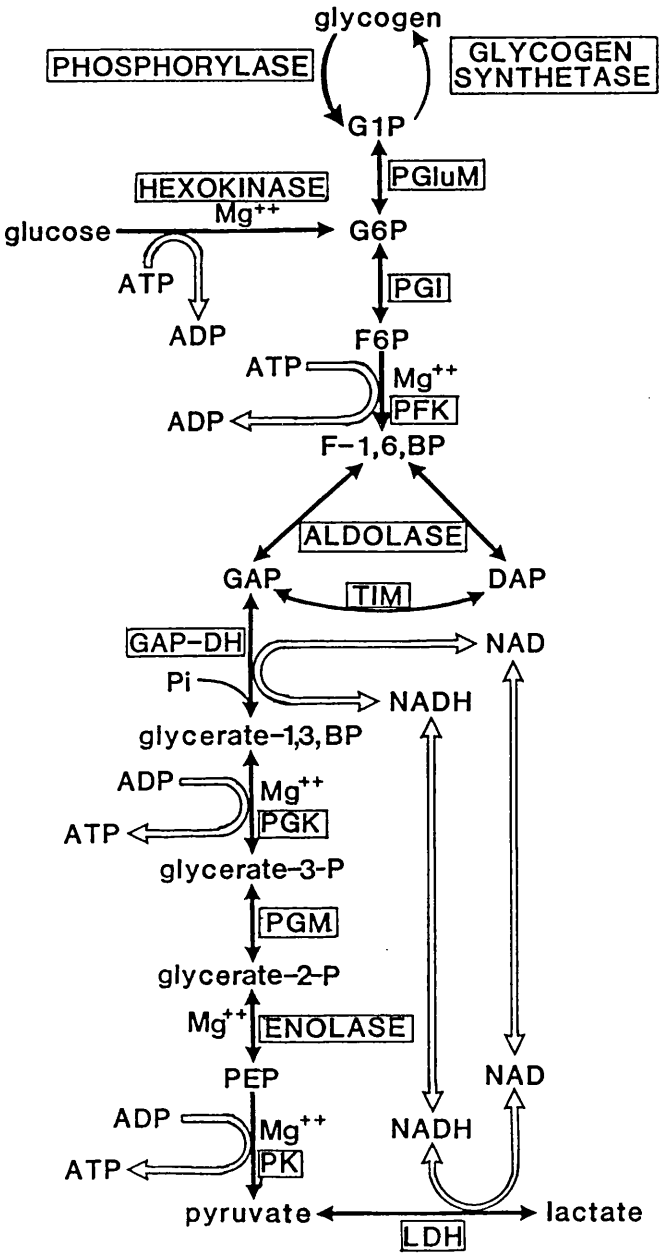


Figure 3 Outline of the Embden-Myerhof pathway. Each 6-carbon glucosyl unit requires one molecule of ATP before it is split into two 3-carbon residues each of which generates 2 moles of ATP, giving a net gain of 3 ATP. Glucose itself requires 2 molecules of ATP for its entry into the pathway making it a much less efficient fuel. The formation of lactate acts a as means of regenerating NAD^+ to maintain the redox potential of the cell in the absence of oxygen.

G1P=glucose 1-phosphate; G6P=glucose 6-phosphate; F6P=fructose 6-phosphate; F-1,6,BP=fructose 1,6-bisphosphate; GAP=glyceraldehyde 3-phosphate; DAP=dihydroxyacetone phosphate; PEP=phosphoenolpyruvate

PGluM=phosphoglucomutase; PGI=phosphoglucose isomerase; PFK=phosphofructokinase; GAP-DH=glyceraldehyde-3-phosphate dehydrogenase; PGK=3-phosphoglycerate kinase PGM=phosphoglycerate mutase; PK=pyruvate kinase; LDH=lactic dehydrogenase

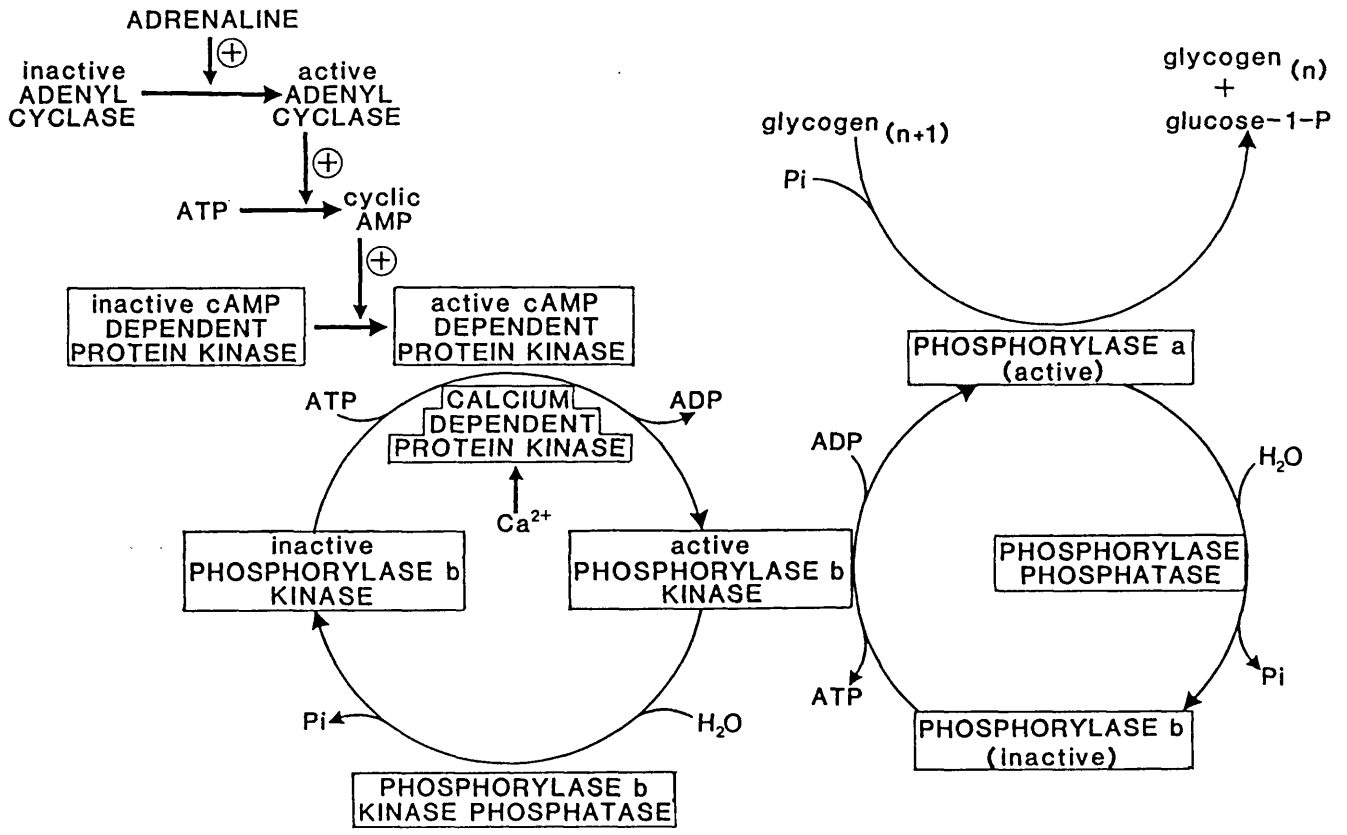


Figure 4 Outline of the activation and control of glycogen phosphorylase. Although ATP is required for the activation of the enzyme the amount is quantitatively very small in terms of the cellular ATP turnover. The activation of phosphorylase is initiated by a cascade system of reactions akin to that seen with blood clotting, so that once the reaction has been started, it rapidly accelerates.

ATP, ADP and Pi (Piras et al, 1968) can be overcome by high concentrations of glucose 6-P under most physiological conditions it is probably inactive (Newsholme and Start, 1973). Synthetase I is less susceptible to inhibitors and exhibits full activity even with low glucose 6-P levels (Chasiotis, 1983).

Phosphorylase interconversion is regulated at both the hormonal level by the activation of cyclic AMP by adrenaline and at the contractile level by the release of Ca^{2+} the resulting protein kinase activating phosphorylase kinase and promoting the formation of phosphorylase a (Chasiotis, 1983). On the initiation of muscular contraction an increase in cytoplasmic Ca^{2+} will activate phosphorylase a whose activity is related to Pi concentration, its substrate for glycogen phosphorylation. Chasiotis et al (1982) have shown that the K_m of phosphorylase a for Pi in the absence of AMP is 26.2 mmol/l Pi but with the increase in free AMP which is likely to occur with intense exercise the K_m falls to 6.8 mmol/l Pi. As free Pi at rest is in the region of 2-3 mmol/l (Ackerman et al, 1980; Meyer et al, 1985) this would explain the extremely low resting glycogenolytic activity. After 25 s of isometric contraction (fatigue in 60 s) Pi concentration rises to 18 mmol/l providing a saturating concentration for phosphorylase. This explains the finding that the average glycogenolytic rate is twice the phosphorylase a activity assayed at 11 mmol/l Pi. The alternative explanation (that both phosphorylase a and b are active) does not take into account the changes in Pi kinetics (Chasiotis, 1983). Thus during intense muscular activity the observed glycogenolytic rate is close to the V_{max} of the enzyme, estimated at 2.4 mmol glucosyl units/kg d.m./s. An individual's phosphorylase activity is dependent on the percentage fast twitch fibres which have been demonstrated as possessing 2-3 times the activity as slow twitch fibres (Harris et al, 1976). During

isometric contraction (knee extensors to fatigue in 60 s) and cycle ergometer exercise (fatigue in 6 min) there was an increase in the mole fraction of phosphorylase a to 53% and 34% at 25 s and 30 s respectively (Chasiotis et al, 1982) while at exhaustion activity had fallen to below resting levels (16% and 17%). This retransformation of a to b parallels the decline in force or work and may be due to a decreased calcium release from the sarcoplasmic reticulum (McLean, 1986).

2.3.4 Control of glycolysis - phosphofructokinase

The hexosemonophosphates thus formed are then metabolised to lactic acid and the rate limiting enzyme for this pathway is phosphofructokinase (PFK) which phosphorylates fructose-1-P to fructose 1,6-BP (Newsholme and Start, 1973). Various kinetic studies have identified a number of in vitro modulators of the enzyme. Activity of PFK is low in resting muscle and despite ATP being a substrate for its reaction physiological concentrations inhibit the enzyme (Uyeda, 1979). Citrate, Mg^{2+}, H^+, PCr, and a number of 3-carbon phosphorylated glycolytic intermediates all inhibit PFK either directly or by enhancing ATP inhibition (Lowry and Passonneau, 1966; Krzankowski and Matschinusky, 1969; Storey and Hochacha, 1974; Triverdi and Danforth, 1966; Uyeda, 1979). Similarly a number of activators acting either directly or by de-inhibition of ATP inhibition have been reported and these include the other reaction substrate fructose 6-P, AMP, ADP, Pi, cyclic AMP, NH_4^+, K^+, and a number of 6-carbon diphosphorylated intermediates (Breitner, 1979; Dobson et al, 1986; Hue et al, 1982; Lowry and Passonneau, 1966).

2.3.5 The creatine phosphokinase reaction

The reactants in equation 2.2 are considered to be in equilibrium, the creatine phosphokinase (CPK) equilibrium, but it is important to realise that each reactant exists as a number of ionic species, particularly magnesium chelates and undissociated acids. As a result of this, equilibrium is dependent not only on the relative concentrations of the main reactants but also on the concentrations of Mg^{2+} and H^{+} and McGilvery has pointed out the importance of this by showing that there can be a 10 fold change in the apparent equilibrium constant with quite small changes in pH and Mg^{2+} concentration (McGilvery, 1975). Creatine phosphokinase and its reactants are located in the muscle cytoplasm and are in close association with actomyosin ATPase. It is not surprising therefore that the activities of the two enzymes are closely linked or that Cr can only be rephosphorylated by ATP. As ATP is used the concentration of ADP will rise, and it has been suggested (McGilvery, 1975) that as the metabolism of fuels by the muscle is controlled primarily by the ADP concentration, this rise is prevented by the reactions in equations 2.2 and 2.3.

As the activity of CPK is greater than that of actomyosin ATPase (Newsholme et al, 1978) ATP concentrations are maintained at the expense of PCr and significant falls in ATP do not appear to occur until PCr falls to less than 60% of its resting value (Hultman et al, 1967). In Hultman's study (the first using the muscle biopsy technique in exercising human muscle) lactate was not measured and it was concluded that the changes in phosphogens represented an alactic interval. Jan Karlsson (1971) in his thesis reported a close relationship between muscle lactate and PCr concentrations, but felt that this was an expression of the O_2 deficit particularly as there was a linear

relationship between the relative workload (as a percentage of VO_2max) and phosphogen depletion. Sahlin et al (1975) in a study on 7 male subjects took biopsies from the quadriceps muscle before and after isometric contraction of the knee extensors (25 s or fatigue at 68% of MVC [maximum voluntary contraction]) under conditions of varying circulatory occlusion. They measured pH (by the homogenate technique) and determined muscle ATP, ADP, AMP, PCr and lactate concentration. With isometric contractions greater than 40% MVC (Edwards et al, 1972) the muscle is not perfused, thus under all their experimental conditions the muscle could be considered a closed system. Muscle pH was found to decrease from 7.09 at rest to 6.56 at fatigue and there was a linear correlation between pH and muscle lactate. It was concluded that the PCr content after exercise was a reflection of the equilibrium of the CPK reaction which itself was determined by the muscle pH. This was supported by a linear relationship between $\log K_{CPK}$ and pH, no recovery of PCr until the circulation was returned and a recovery of pH and PCr at the same rate. A small decrease in ATP with a reciprocal increase in ADP and AMP was found in two subjects who sustained contraction to 3 min. These findings were confirmed in a larger study using both dynamic (6 min cycle ergometer to exhaustion; n=24) and isometric exercise (40-95% MVC: n=30) (Harris et al, 1977) when a constant but non-linear relationship between lactate and PCr was also demonstrated.

In an attempt to determine the time-course of PCr resynthesis, Harris et al (1976a) repeated Sahlin's 1975 study with an additional group of 4 subjects who performed 300 W cycle ergometer exercise to exhaustion (6 min). They were able to show a biphasic curve to PCr regeneration with an initial fast phase ($t\frac{1}{2}$=21-22s) and then a slow phase ($t\frac{1}{2}$=170 s). The rapid phase recovery was 2-3 mmoles/kg d.m./s and in all cases recovery after isometric exercise was slower at both 2 and

4 min. Hexosemonophosphates after exhaustive isometric exercise were 25 mmoles/kg d.m. and the concentration had fallen to 10 mmoles at 4 min. This would generate 45 mmoles ATP by glycolysis, the amount by which PCr had recovered. No recovery of PCr occurred during circulatory occlusion and there was no significant decrease in ATP levels with either type of exercise. A mechanism for the two rates of PCr resynthesis was suggested by Sahlin et al (1979) after a study in which 11 subjects (6 with an occluded circulation) performed exhaustive exercise on a cycle ergometer (6-11 min at 300 W). In vitro incubation of some of the biopsies was undertaken in either 95% N_2 + 5% CO_2 or 95% O_2 + 5% CO_2 following which they were frozen and assayed. After exhaustive exercise, as had been previously demonstrated, resynthesis of PCr was inhibited by circulatory occlusion. Fifteen minutes incubation of the muscle in O_2 resulted in a 68% recovery of PCr without change in lactate (or by extrapolation pH); they speculated that the rapid phase of resynthesis was dependent on the availability of oxygen, and the slow phase on recovery in muscle pH. Additionally, in keeping with Newsholme's views (see discussion of Wilkie, 1981), they found no evidence on the basis of the mass action ratio of the adenylate kinase reaction to support the NMR finding that 90% of ADP within the cell is protein bound (McGilvery, 1975; Wilkie, 1981).

2.3.6 The adenylate kinase reaction

In their outstanding work on the chemistry of muscle contraction, Cain et al (1962) were able to demonstrate adenylate kinase activity in partially restoring ATP concentration after two stimulated contractions in isolated frog muscle in which CPK activity had been inhibited by FDNB. Adenosine diphosphate was decreased and AMP increased by the same

amount. It was concluded that this demonstration of the adenylate kinase reaction [despite being partially inhibited by FDNB (RE Davies, 1971)] operating during muscular contraction was important in the "cyclical regeneration of ATP from ADP" and maintaining low levels of ADP to ensure that the ATP/ADP ratio was kept high. This simple concept of the adenylate kinase reaction functioning merely to reduce ADP levels when its rate of rephosphorylation has been substantially reduced by a decline in reactions 2.2 and 2.3 has been challenged in favour of a role for it in the control of glycolysis (Newsholme and Start, 1973; McGilvery 1975) (vide infra).

2.3.7 Adenylate deaminase

Adenosine monophosphate concentrations in muscle are controlled by deamination of the pyridine to inosine monophosphate. The enzyme's activity is particularly high in fast glycolytic fibres and although it has a pH optimum of 6.5 (Setlow and Lowenstein, 1967) it is also active at normal pH (Dudley and Terjung, 1985).

$$\text{AMP} \longrightarrow \text{IMP} + NH_4 \qquad (2.6)$$

This reaction is irreversible but AMP can be reformed by two further reactions

$$\text{IMP} + \text{aspartate} + \text{GTP} \longrightarrow \text{adenylsuccinate} + \text{GDP} + \text{Pi} \qquad (2.7)$$

$$\text{adenylsuccinate} \longrightarrow \text{AMP} + \text{fumarate} \qquad (2.8)$$

Adenylate deaminase with its pH optimum of 6.5, a value which is widely reported as occurring at exhaustion (Sahlin, 1978; Costill et al, 1983; Sahlin and Henriksson, 1984) by its combined action with adenylate kinase will maintain the ATP/ADP ratio at the expense of depleting the

total adenine nucleotide pool (see table 2.3). The decrease in ATP seen during exhaustive work in man and the horse, has been shown to be stoicheiometrically related to an increase in IMP concentration (Harris and Hultman, 1985; Snow et al, 1985; table 2.3).

2.3.8 Amplification of control in energy supply

It has been estimated that to go from rest to maximum exercise over a period of seconds a 1000 fold increase in the rate of flux through the Embden-Meyerhof pathway is required (McGilvery, 1975). Such an enormous increase is unlikely to occur as a result of simple changes modulating a single enzyme. Newsholme and Start (1973) have proposed a mechanism of control for the regulatory enzyme PFK that allows the required acceleration of glycolysis to occur. The enzyme as has been stated is influenced by a number of activators and inhibitors as summarised in table 2.2.

a) Control by ATP

Since 1959 it has been known that PFK activity is very sensitive to a fall in pH. Although ATP is a substrate for the enzyme, the basis for this pH sensitivity is ATP-induced inhibition (Dobson et al 1986). This allows the concept of a simple feed back system for control of the enzyme; when ATP is used, its concentration falls and PFK is stimulated. Adenosine triphosphate concentration is however remarkably constant during exercise and although ATP depletion has been described during various types of dynamic and isometric exercise (table 2.3), even under the most extreme conditions (repeated electrical stimulation with an occluded blood flow) when lactate accumulation reached 170 mmoles/kg d.m. the maximum decrease in ATP recorded was 63% (Harris and Hultman, 1985). As it has been calculated that to change enzyme activity from

Table 2.2. Regulators of phosphofructokinase

activators	inhibitors
cyclic AMP	ATP
AMP	PCr
ADP	Mg^{2+}
NH_4^+	H^+
K^+	3-phosphoglycerate
fructose-1,6-BP	2-phosphoglycerate
fructose-2,6-BP	2,3-diphosphoglycerate
glucose-1,6-BP	phosphoenolpyruvate
fructose-6-P	citrate

10% to 90% would require at least a four fold change in regulator concentration it is obvious that changes in ATP itself are not sufficient to affect control on PFK.

b) Amplification of control by ATP

The adenylate kinase reaction is ideally placed to provide a means of amplifying small changes in ATP as the reaction is close to equilibrium and the concentrations of ATP, ADP and AMP are about one order of magnitude apart (Harris et al, 1974; table 2.3). Any small change in ATP will result in a much larger percentage increase in ADP and AMP (a 10% change in ATP would produce a four fold increase in AMP) and although this theoretical effect has been shown to occur in isolated frog muscle poisoned by DNFB (Cain et al, 1962) and in insect flight muscle (Newsholme and Start, 1973) there is lack of convincing evidence for its occurrence in man. There are, though, analytical difficulties in measuring AMP in needle biopsy specimens of muscle and of the few studies cited which report values for this metabolite, mention of this is made (see Harris et al, 1977). A summary of the metabolic changes occurring during various types of muscular work is given in table 2.3.

The amplification of control of PFK is therefore a complex interaction of inhibitors and de-inhibitors and activators. With high intensity exercise a fall in PCr (and to some extent ATP) by hydrolysis will increase Pi; deamination of AMP will result in an equimolar accumulation of IMP and NH_4^+; accumulation of fructose 6-P and fructose 1,6-BP occurs. All these postive modulators will tend to release PFK from ATP inhibition despite the progressive fall in pH with lactate accumulation and the maintainance of ATP levels.

Table 2.3 Muscle metabolite concentrations in man at rest and after various types of muscular work

Study	type of work	n	work rate (W)	work time (min)	PCr	TAN	ATP	ADP	AMP	IMP	GLY	G6P	F6P	FBP	PYR	LA
					metabolite (mmoles/kg dry muscle)											
Hultman	rest	45	--	-	68	-	24	-	-	-	-	-	-	-	-	-
et al (1967)	cycle	5	65	5	52	-	21	-	-	-	-	-	-	-	-	-
	cycle	8	130	5	26	-	22	-	-	-	-	-	-	-	-	-
	cycle	8	200	5	13	-	19	-	-	-	-	-	-	-	-	-
	cycle	12	300	*1.4	7	-	15	-	-	-	-	-	-	-	-	-
Karlsson	rest	3	--	-	68	-	17	-	-	-	350	4.6	-	-	-	5.5
and Saltin	cycle	3	265	6.3	19	-	13	-	-	-	-	4.8	-	-	-	41.3
(1971)	cycle	3	265	*16.5	16	-	13	-	-	-	-	7.8	-	-	-	52.2
	cycle	3	310	2.4	19	-	11	-	-	-	-	7.8	-	-	-	52.2
	cycle	3	310	*6.3	17	-	14	-	-	-	-	12.2	-	-	-	69.6
	cycle	3	380	*2.4	10	-	12	-	-	-	-	14.8	-	-	-	70.0
Karlsson	rest	28	--	-	69	-	18	-	-	-	324	-	-	-	-	7.7
et al (1971)	cycle	13	245	6	28	-	17	-	-	-	270	-	-	-	-	47.4
	cycle	13	425	*2-3	13	-	12	-	-	-	183	-	-	-	-	98.7
	cycle	15	145	10	43	-	15	-	-	-	248	-	-	-	-	28.7
	cycle	15	215	10	20	-	13	-	-	-	143	-	-	-	-	56.5
	cycle	15	260	*3-6	14	-	11	-	-	-	96	-	-	-	-	73.5
Harris	rest	81	--	-	76	27	24	3.2	0.1	-	349	1.7	0.3	0.3	0.4	5.1
et al (1974)																
Sahlin	rest	2	--	-	86	29	26	3.2	0.2	-	-	-	-	-	0.3	3.1
et al (1975)	rest (occ)	4	--	-	62	29	26	3.2	0.2	-	-	-	-	-	0.7	5.7
	isom (occ)	6	68%	0.4	27	29	24	3.5	0.2	-	-	-	-	-	1.0	51.2
	isom (occ)	8	68%	*0.8	7	25	21	3.5	0.2	-	-	-	-	-	1.6	91.3
Rehunen	rest	12	--	-	78	-	24	-	-	-	210	2.1	-	-	-	12.2
et al (1978)	†sprint	5	3x300m		20	-	17	-	-	-	16	8.6	-	-	-	47.8
	†sprint	4	3x40m		61	-	24	-	-	-	-	-	-	-	-	27.0
	†sprint	3	20+40+60m		37	-	24	-	-	-	-	-	-	-	-	37.5
Sahlin	rest	21	--	-	72	27	-	-	-	0.0	-	-	-	-	-	5.4
et al (1978)	cycle	10	?150	9	33	26	-	-	-	-	-	-	-	-	-	-
	cycle	11	?300	*7	10	21	-	-	-	3.7	-	-	-	-	-	100.5
Sahlin	cycle	5	300	*7.7	8	-	19	3.3	-	-	-	-	-	-	0.8	122.0
et al (1979)	isom (occ)	4	62%	*1.0	3	22	18	3.6	0.2	-	-	-	-	-	-	89.1
Sutton	rest	5	--	-	61	-	21	4.0	-	-	257	1.3	0.2	0.2	0.5	8.5
et al (1981)	cycle	5	195	20	32	-	12	3.5	-	-	43	2.9	0.3	0.5	0.7	39.9
	cycle	5	280	*4.56	22	-	12	3.8	-	-	23	1.6	0.3	0.4	0.8	63.8

Table 2.3 (continued)

Study	type of work	n	work rate (W)	work time (min)	PCr	TAN	ATP	ADP	AMP	IMP	GLY	G6P	F6P	FBP	PYR	LA
					metabolite (mmoles/kg dry muscle)											
¶Jacobs	rest	9	--	-	63	-	21	-	-	-	360	-	-	-	-	9.0
et al (1982)	cycle (AWT)	9	560	*0.5	25	-	14	-	-	-	278	-	-	-	-	60.5
Sjöholm	stim (occ)	6	71%	0.75	9	21	16	4.2	0.4	6.0	-	-	-	-	-	99.3
et al (1983)																
Sahlin and	rest	8	--	-	66	25	22	2.9	0.1	-	-	-	-	-	-	3.5
Henriksson	isom	8	60%	*0.84	7	24	21	3.7	0.1	-	-	-	-	-	-	83.9
(1984)																
¶Jones	rest	10	--	-	68	-	20	-	-	-	-	0.6	0.4	0.9	-	7.8
et al (1985)	isok (1.0)	2	770	0.17	20	-	16	-	-	-	-	12.0	1.3	3.6	-	62.0
	isok (2.3)	2	945	0.17	17	-	13	-	-	-	-	12.0	1.3	6.1	-	70.2
	isok (1.0)	5	770	*0.5	28	-	13	-	-	-	-	20.9	5.2	8.7	-	134.8
	isok (2.3)	5	945	*0.5	43	-	19	-	-	-	-	20.9	6.0	4.4	-	126.1
¶McCartney	rest	8	--	-	62	-	23	-	-	-	373	2.3	0.5	1.2	-	6.2
et al (1986)	isok (1.7)	8	990	*0.5	18	-	14	-	-	-	294	29.8	5.3	5.3	-	125.7
¶Cheetham	rest	8	--	-	88	-	28	2.7	-	-	280	1.4	0.4	0.1	0.2	2.7
et al (1986)	#sprint	8	535	*0.5	31	-	18	2.2	-	-	212	18.6	4.4	0.3	2.9	78.0
Spriet	rest	7	--	-	79	28	25	2.9	0.3	0.5	-	1.8	0.3	0.2	0.2	5.1
et al (1987)	stim (occ)	7	72%	0.43	16	26	22	3.6	0.4	2.4	-	10.4	2.1	0.4	2.3	65.6
	stim (occ)	7	72%	0.85	9	21	17	3.7	0.4	7.9	-	8.5	1.8	0.3	3.4	101.7
	stim (occ)	7	72%	1.28	5	19	15	3.9	0.4	10.6	-	8.6	1.7	0.3	2.8	135.2
	stim (occ)	7	72%	1.71	6	18	14	3.6	0.5	11.6	-	8.1	1.7	0.3	2.5	145.3

PCr=phosphocreatine; TAN=ATP+ADP+AMP; ATP=adenosine triphosphate; ADP=adenosine diphosphate; AMP=adenosine diphosphate; IMP=inosine monophosphate; GLY=glycogen; G6P=glucose 6-phosphate; F6P=fructose 6-phosphate; FBP=fructose 1,6-bisphosphate; PYR=pyruvate; LA=lactate

W=watts; min=minutes; m=meters

¶Studies with supramaximal work rate, work rate cited is maximum attained average power before decline
†track sprint, distances in meters; 3x300m, rest interval 3 min and 5 min; 3x40m, rest interval 5 min; 20+40+60m, rest interval 0.5 min
#treadmill sprint
*exercise test to exhaustion (subjects unable to continue)

x%=force as %MVC (maximum voluntary contraction)

cycle=cycle ergometer; isom=isometric knee extension; occ=occluded circulatiion; isok=isokinetic cycle ergometer (pedal revolution in Herz); stim=electrical stimulation; sprint=all out sprint

c) Substrate cycling as a means of accelerating glycolysis

As McGilvery (1975) has stated very large increases in the rate of glycolysis are required in going from rest to maximum exercise. Newsholme (1971) has proposed a substrate cycle between fructose 6-P and fructose 1,6-BP as a means of providing this acceleration. There exists in muscle the enzyme fructose 1,6-bisphosphatase (FBPase) which catalyses the conversion of FBP back to F6P, the reverse of the PFK reaction. It is far less active than PFK and the effect of AMP on the two enzymes is sigmoidal, in that small changes in AMP concentrations, by simultaneously inhibiting FBPase and de-inhibiting PFK, can result in a many hundred times increase in the rate of glycolysis (Newsholme and Start, 1973). Evidence has been presented (McGilvery, 1975) to show that the maximum activity of PFK occurs with an AMP concentration of 0.1-0.4 mmoles/kg d.m and a 50% inhibitory concentration of AMP for FBPase from 0.6-1.5 mmoles/kg d.m. (which does not take into account the effect of other possible inhibitors). From table 2.3 it can be seen that the values reported for AMP in man in part support Newsholme's hypothesis.

2.4 The importance of muscle fibre types in working human muscle

The preceding discussion is on the assumption that muscle behaves in a homogeneous manner during exercise but in fact this is not the case. In man, skeletal muscle consists of a mixture of fibres with different morphological and biochemical properties in contrast to most other animals where each muscle consists primarily of one fibre type (Karlsson, 1979). Based on the fibres' colour (related to myoglobin concentration), speed of contraction, staining characteristics for ATPase at different pH incubations and histochemical staining for oxida-

tive and glycolytic enzyme activities, it is possible to identify two main fibre groups: red, slow twitch (ST), type I, oxidative (SO) and white, fast twitch (FT), type II, glycolytic. White fibres can be further subgrouped into IIa and IIb based on their staining for myofibrillar ATPase (there is a third subgroup, IIc, which represents less than 1% of the total and is quantitatively unimportant in man). Type IIa share some of the characteristics of type I and are also known as FTa or Fast Oxidative Glycolytic (FOG) and type IIb are known as FTb or Fast Glycolytic (FG). The different characteristics of the three main fibre types are summarised in table 2.4

The characteristics of type II fibres are such that it is probable that during high intensity exercise there occur more profound metabolic changes than has been estimated by analysis of the heterogeneous mixture of fibre types obtained at muscle biopsy. During maximal dynamic exercise (exhaustion in 80 s) and isometric contractions at 50% MVC (exhaustion in 60 s) Essén and Häggmark (1975) found the variation in lactate and glycogen content of pooled type I and type II fibres so great that no conclusions could be drawn, but under both exercise conditions at exhaustion there was a tendency for more lactate to accumulate in the type II fibres. Tesch and Karlsson (1977) were unable to clarify matters with their study on isometric contraction at different work loads (25%, 50% and 75% MVC). They demonstrated in the three subjects studied that at all work loads, lactate accumulation was related to the fibre type that predominated. The following year, studying 10 subjects after isokinetic exercise to exhaustion, they were able to show that type II fibres were indeed better at forming lactate (Tesch et al, 1978). In a delightful study of 28 skiers biopsied on the slopes, unskilled skiers displayed selective glycogen depletion of type IIb fibres (Nygaard et al, 1978), a situation only seen after maximum

Table 2.4 Differences in muscle fibre type characterstics in man

	Type I (ST or SO)	Type IIa (FTa or FOG)	Type IIb (FTb or FG)	Reference
distribution (ATPase)	34-54%	32-43%	9-23%	1,2,15,16
pH lability	<3.9/>10.4	<4.9/>10.8	<4.5/>10.8	3
myoglobin	red	intermediate	white	18
contractility enzymes				
actomyosin ATPase	low	high	high	5,22
adenylate kinase	low	high	high	22
CPK	intermediate	intermediate	intermediate	9,22
AMP deaminase	low	high	high	19
glycolytic enzymes				
phosphorylase	low	intermediate	high	7,8,9
PFK	low	intermediate	high	5,7,9
FBPase	low	intermediate	high	9
LDH	low	high	high	9,20,23
mitochondrial density	high	intermediate	low	10
mitochondrial enzymes				
SDH	high	intermediate	low	5
ACDH	high	intermediate	low	9
fuel stores				
PCr	*intermediate-high	high-*very high		*16,17,21
ATP	*intermediate-high	high-*very high		*16,17,11
glycogen	high	high		5
triglycerides	low	high		5
fibre area	intermediate	large	small	12,18
capillary density	intermediate	intermediate	low	12,18
fibre/capillary ratio	high	intermediate	intermediate	18
resistance to fatigue				
aerobic exercise	high	low		4,6,13
anaerobic exercise	low	high		4,13,22
peak torque	low	high		12,14,22

Fibre characteristics are based largely on the finding in quadriceps muscle in untrained males age 16-35 years; *based on NMR studies on cat muscle

References:

1) Ball et al, 1983
2) Blomstrand et al, 1986
3) Brooke & Kaiser, 1970
4) Costill et al, 1976
5) Essén et al, 1975
6) Gollnick et al, 1972
7) Gollnick et al, 1974
8) Harris et al, 1976b
9) Hintz et al, 1980
10) Hoppeler, 1986
11) Jansson et al, 1985
12) Karlsson, 1979
13) Karlsson et al, 1981
14) Larsson & Karlsson, 1978
15) Larsson et al, 1978
16) Meyer et al, 1985
17) Rehunen & Härkönen, 1980
18) Saltin et al, 1977
19) Terjung et al, 1986
20) Tesch et al, 1978
21) Tesch et al, 1985
22) Thorstensson, 1976
23) Thorstensson et al, 1977

voluntary contractions (Secher and Nygaard, 1976), thus confirming that type II fibres exhibit a different metabolic behaviour to type I and that bad skiers expend a lot of effort in fighting the skis!

Snow et al (1985) has suggested that the ATP depletion seen with high intensity exercise may be restricted to type II fibres. This is supported by the finding that AMP deamination to IMP occurs readily only in FT fibres (Meyer and Terjung, 1980), AMP deaminase activity being twice as high as in ST fibres (Meyer et al, 1980), and at the 6th International Symposium on the Biochemistry of Exercise evidence was presented for a selective depletion of ATP and accumulation of IMP in type II fibres after exhaustive exercise in man (Jansson et al, 1985). At the same meeting Tesch et al (1985) reported that greater PCr breakdown occurred in FT fibres after exhaustive isokinetic exercise, these fibres possessing in addition a higher resting PCr concentration (90 against 81 mmoles/kg d.m.) than ST fibres.

Therefore if the selective behaviour of FT fibres is considered together with the information obtained in studying "whole muscle", there is considerable evidence to support the current theories on the control of energy supply during high intensity exercise.

2.5 Fatigue

Edwards (1983) has defined fatigue as the failure to generate or maintain the required or expected force or power output. In the early studies of muscular fatigue it was usual to attribute it to a failure of the central nervous system (Waller, 1891; Setchenov, 1903 [cited by Asmussen, 1971]; Mosso, 1915), and indeed Asmussen and Mazin (1978) quite recently supported the view that recovery from local or peripheral fatigue was influenced by some central nervous factor - the Setchenov

phenomenon. As early as 1914, however, Weber (1914) suggested that fatigue was local in origin, the brain simply modifying local events. Since then fatigue has been considered to comprise of peripheral and central components which Edwards (1978) has elegantly classified on the basis of failure of each of the various events leading from the volition of initiating a muscular contraction to the eventual force or power output. An adaptation of Edwards' scheme is illustrated in Figure 5.

2.5.1 Central fatigue

Central fatigue can be influenced by motivation, pain, perceived severity of exercise, dysnoea and the central effects of systemic metabolic changes. In addition psychological stress and sleep deprivation may change central awareness of fatigue. Despite these interactions, Merton et al (1981) demonstrated by direct stimulation of the motor cortex that in fatigued muscle the pathway conducts normally, and concluded that the muscle fibre fails and rather than the pathway. Bigland-Ritchie (1981) also showed that the ratio of stimulated to voluntary force remained constant as the muscle fatigued. Even in those cases where there was an apparent decline in voluntary force in excess of stimulated force, this constant relationship could be restored by encouraging the subject to make an extra effort.

2.5.2 Peripheral fatigue

The process by which a neuronal impulse results in muscular contraction is known as excitation-contraction coupling and peripheral fatigue can be considered to comprise of three main components on the basis of this process; impaired excitation/activation of the motor units, impaired actin-myosin cross bridge coupling and impaired energy supply.

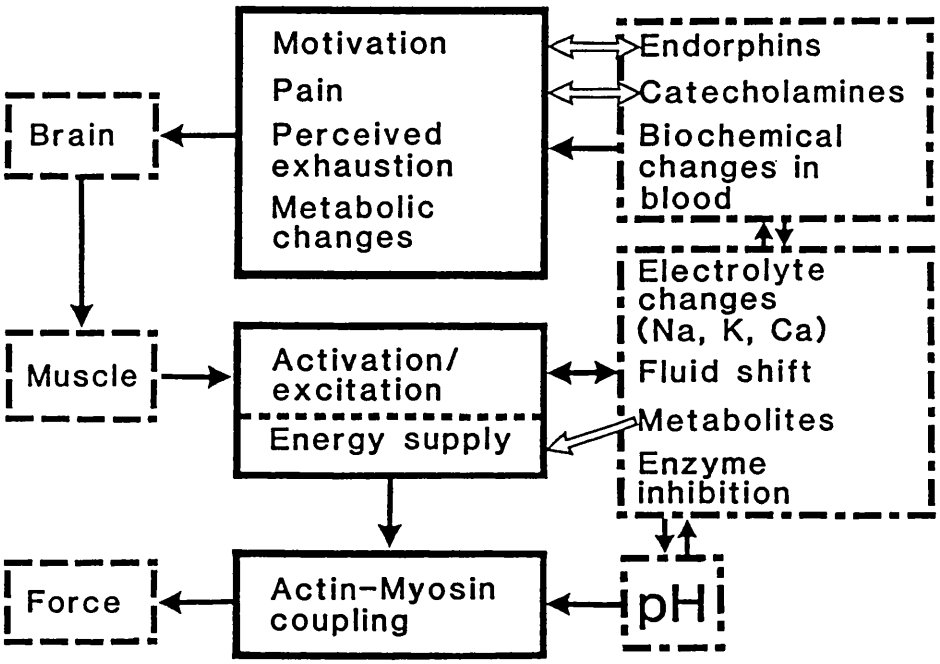


Figure 5 A scheme for the development of fatigue. Fatigue is a reduction in the required or expected force or power output. It is dependent on central (the brain) and peripheral components (muscle), the latter being dependent on activation/excitation, energy supply and cross-bridge coupling. As a result of exercise the biochemical changes shown on the left occur and these can influence both the central and peripheral components. Of considerable importance is the reduction in pH which can act both directly on the contractile properties of the muscle cell and by altering enzyme activity.

a) Activation/excitation coupling

Changes in excitation/activation have been suggested as a mechanism contributing to peripheral fatigue (Jones, 1981) possibly by a reduction in calcium accumulation by the sarcoplasmic reticulum and recent work by McLean et al (1986) supports this. Jones also postulated that an accumulation of interstitial potassium could slow membrane conduction and even lead to the failure of propogation of the action potential with a resultant loss of force. At the 6th Biochemistry of Exercise Symposium in Copenhagen, Hník et al (1986) presented work confirming contraction induced changes in muscle potassium. They demonstrated increases in interstitial K^+ concentrations of the order of 10-15 mmol/l and in addition stated that continued muscular contraction resulted in a loss of K^+ from the muscle with the result that ionic inbalance may result after prolonged work. This was clearly shown by Juel (1986) who demonstrated a reduction in intracellular K^+ from 165 mmol/l to 129 mmol/l after 6 min of dynamic knee extension at 100% VO_2max and it may be that this ionic change is due to an impairment of Na^+-K^+-ATPase (Sahlin, 1986). Jones and Bigland-Ritchie (1986) at the same conference felt that the T-tubule K^+ concentration was likely to be considerably higher and that this accumulation of extracellular K^+ could be minimised by the reduction in motor unit firing seen with sustained isometric contraction. Further evidence for the importance of K^+ as mediator of fatigue has been presented by Sjøgaard (1986). Under conditions of maximal and submaximal dynamic exercise the ratio of $[K^+_i][K^+_e]$ changed sufficiently (from 40:1 to 20:1) to decrease the resting muscle membrane potential from -90mV pre-exercise to -75mV at exhaustion, a value that could well impair membrane excitability and consequently muscle contraction.

b) Actin-myosin cross bridge coupling

After exhaustive high intensity exercise muscle lactate concentration is in the order of 100 mmoles/kg d.m. (table 2.3). As the increase in lactate is accompanied by an equimolar increase in $[H^+]$ this results in a fall in muscle pH of about 0.5 units to 6.5 (Hermansen and Osnes, 1972; Sahlin et al, 1976; Sahlin et al, 1978a; Costill et al, 1983; Sahlin and Henriksson, 1984). A more dramatic fall in pH is prevented by the buffering capacity of muscle which has been estimated to be about 60 Slykes (Sahlin and Henriksson, 1984). Cellular acidosis as a major component of fatigue was first suggested by Hill and Kupalov (1929) and since then force generation in animal muscle has been shown to be decreased by incubation in an acid medium (Hill, 1955; Sahlin et al, 1983; Mainwood and Renaud, 1985). Incubation of isolated rat extensor digitorum longus muscle in 30.8% CO_2 resulted in a 45% decrease in tetanic tension (which declined during the 1.28 s tetanus) and a marked prolongation of the relaxation time (Sahlin et al, 1983). The muscle pH under these conditions fell from a control value of 7.01 to 6.67, and muscle metabolite estimation revealed a significant increase in ADP and a 56% fall in PCr. These results were confirmed in isolated frog sartorius and mouse EDL both incubated with 30% CO_2, when the tetanic force was reduced by 30% and 35% respectively (Mainwood and Renaud, 1985). Force reduction when frog sartorius was stimulated to fatigue with the same proton load was 75% and this decline in tension could be maintained by incubation during recovery in an acid medium. This was contrary to the findings that rat EDL exhibited the same reduction in force whether the fall in pH was due to lactate accumulation (Sahlin et al, 1981) or CO_2 incubation (Sahlin et al, 1983). As a result of this, Mainwood and Renaud (1985) proposed two components to acidotic induced fatigue; a direct effect of intracellular acidosis and a change in the

state of one or more of the steps of excitation-contraction coupling.

The use of the "skinned fibre" technique has made it possible to study force generation of muscle fibres divorced from the effects of excitation-contraction coupling (Donaldson and Kerrick, 1975). A number of studies have shown that force generation of isolated skinned muscle fibres decreases over the pH range 7.0-6.5 (Fabiato and Fabiato, 1978; Robertson and Kerrick, 1979; Hermansen, 1981). Within species, FT fibres appeared to be more sensitive to a fall in pH than ST fibres and it has been suggested that this effect is due to a reduction in cross-bridge cycling by inhibition of actomyosin ATPase (Donaldson, 1983). This effect appears to be mediated by changes in Ca^{2+} sensitivity. When the pH falls, the sarcoplasmic reticulum binds more calcium (Nakamura and Schwartz, 1970); the amount of Ca^{2+} required to produce a given tension is increased with an increased $[H^+]$ (Robertson and Kerrick, 1976); there is a decrease in Ca^{2+} sensitivity to actomyosin ATPase precipitated by a reduction in calcium binding to troponin (Blanchard et al, 1984). In addition to the effects of pH, Cooke and Pate (1985) have demonstrated a decrease in force generation on incubating skinned muscle fibres in high concentrations of Pi. Over the range 1-2 to 15-20 mmol/l Pi (the range that is encountered during intense exercise) force was reduced up to 30% and this effect was in addition to the pH induced reduction in tension so that a combination of a [Pi] of 20 mmol/l and a pH of 6.5 resulted in a decline in force of 75%.

The importance of acidosis in exercise induced fatigue was elegantly demonstrated by Sutton et al (1981) who showed that endurance time with high intensity exhaustive exercise (95% VO_2max) was considerably shortened by induced acidosis (NH_4Cl ingestion) and improved by induced alkalosis ($NaHCO_3$ ingestion). With acidosis there was a

tendency for a reduced lactate efflux from muscle, and the accumulation of hexosemonophosphates together with a lower muscle lactate at exhaustion pointed to an inhibition of glycolysis at the level of PFK. However to complicate matters, maximal exercise (125% VO_2max) does not seem to be improved by induced alkalosis (Katz et al, 1984) and supra-maximal dynamic exercise (four bouts of 30 s on an isokinetic cycle ergometer at 100 rpm) achieving a maximum peak power of 1560 W was not affected by either alkalosis ($NaHCO_3$ ingestion) or acidosis (NH_4Cl ingestion and respiratory acidosis with 5% $FiCO_2$) (McCartney et al, 1983). Although it has been suggested that protons leave the cell at the same rate as lactate (Mainwood et al, 1972) or certainly no faster, (Hirche et al, 1975) the conclusions reached by Mainwood et al (1972) can be questioned. They based their statement on the finding that PCr and ATP recovery preceded lactate recovery and that tension which they considered to be a function of pH followed the latter. However from Sahlin et al's (1975) work PCr concentration is linearly related to $\log K_{CPK}$ and PCr recovery is dependent on pH. In addition Sahlin (1978) in his thesis was able to demonstrate in the first minute of recovery a H^+ ion efflux in excess of lactate. Subsequent to this it has been shown (Sejersted et al, 1984) that during high intensity exhaustive exercise, the release of protons, on a molar basis, from working muscle exceeds that of lactate. Therefore during submaximal exercise, extra-cellular factors by influencing the release of metabolites from the muscle cell may influence its activity. In maximal and supramaximal exercise however, the activity of the muscle seems less dependent on its extracellular environment and the rate limiting processes appear to be intracellular.

c) Fuel supply

During high intensity exercise fuel is supplied by the immediate energy source ATP and rephosphorylated by stores of PCr and glycogen. Wilkie (1981) suggested that muscular fatigue was indeed due to a chemical fuel shortage, particularly that of PCr, but in an appendix to the same paper produced evidence to show that this was unlikely to be the case. Although PCr levels drop to very low levels during extreme exercise they are never completely depleted and the PCr concentration may be more a reflection of intracellular pH (Sahlin et al, 1975) than a true depletion. The moderate changes in ATP concentrations seen are insufficient to influence the activity of acto-myosin ATPase the K_m for this enzyme for ATP being in the micromolar range (Edwards et al, 1975).

Glycogen depletion during brief high intensity exercise does not occur. However if complete glycogen depletion of both FT and ST fibres is induced then reduction in muscle strength and increased susceptibility to fatigue is seen (Jacobs et al, 1981). As a corollary to this an increased anaerobic capacity is not seen with an artificially high muscle glycogen induced by a glycogen-loading regimen (Maughan and Poole, 1981). Thus if fuel shortage is a major factor in fatigue it will be dependent on the ATP turnover rate. It is generally accepted that ATP from glycolysis and PCr are the suppliers of fuel during brief high intensity exercise and it is probable that they supply fuel concurrently. Bessman and Geiger (1981) have put forward an attractive hypothesis which integrates these two energy supplies. Their phosphocreatine shuttle hypothesis purports to show that the ADP bound to the myosin head can be rephosphorylated only by PCr and the creatine produced is converted back to PCr by mitochondrial ATP from glycolysis and oxidative phosphorylation. Although Newsholme et al (1978) have earlier presented an argument against the existence of a phosphocreatine

shuttle, the condition that they placed on the possibility of it existing (that phosphogen kinase activity should be at least twice the maximum rate of ATP utilisation of myofibrillar ATPase) is however met by their findings in skeletal muscle where in most cases it is 10-fold greater. Fuel supply (ATP turnover rate) to maximally working muscle may therefore be dependent on the rate of glycolysis, phosphocreatine acting only as a "battery to start the engine".

From table 2.3 it is possible to calculate rates of anaerobic ATP turnover and glycolysis and these are summarised in table 2.5.

$$\text{total ATP turnover} = -\Delta PCr - \Delta ATP + 1.5 \times \Delta lactate + 1.5 \times \Delta pyruvate \qquad (2.6)$$

$$\text{ATP glycolysis} = 1.5 \times \Delta lactate + 1.5 \times \Delta pyruvate \qquad (2.7)$$

As pyruvate concentration amounts to no more than 2% of lactate it can be ignored in the calculations.

Rates of glycogenolysis and glycolysis can be similarly calculated (Hultman and Sjöholm, 1983):

$$\text{glycogenolyis} = \Delta G6P + 0.33 \times \Delta G6P + \tfrac{1}{2}(\Delta lactate + 0.1 \times \Delta lactate) \qquad (2.8)$$

$$\text{glycolysis} = \tfrac{1}{2}(\Delta lactate + 0.1 \times \Delta lactate) \qquad (2.9)$$

It is obvious that during anything other than brief high intensity exercise there will be a contribution from aerobic energy sources which will increase with decreasing work load. However as the maximum rate of lactate efflux from the leg has been estimated to be about 4-5 mmol/min (Jorfeldt et al, 1978), and if we assume that during cycle ergometer exercise at sub-maximal work load half of the muscle mass of the lower limbs (20 kg) is working, then this will be equivalent to a loss of lactate from the muscle of 2 mmol/kg d.m./min. This corresponds to an underestimate of the glycolytic and glycogenolytic rates of 0.02

Table 2.5 ATP turnover rates and glycolytic and glycogenolytic rates for various types of muscular work

Study	type of work	work rate (W)	work time (m)	rates in mmols/kg d.m./s ATP turnover total	PCr	Glyco	glyco-lysis	glcogen-olysis
Karlsson and	cycle	265	6.3	0.28	0.13	0.14	0.05	0.05
Saltin (1970)	cycle	265	*16.5	0.12	0.05	0.07	0.03	0.03
	cycle	310	2.4	0.87	0.40	0.49	0.18	0.21
	cycle	310	*6.3	0.40	0.13	0.25	0.09	0.12
	cycle	380	*2.4	1.11	0.40	0.67	0.25	0.34
Karlsson	cycle	245	6	0.28	0.11	0.17	0.06	-
et al (1971)	cycle	425	*2.5	1.32	0.37	0.91	0.33	-
	cycle	145	10	0.10	0.04	0.05	0.02	-
	cycle	215	10	0.21	0.08	0.12	0.04	-
	cycle	260	*4.5	0.60	0.20	0.37	0.13	-
Sahlin	isom (occl)	68%	0.4	4.43	1.16	2.84	1.04	-
et al (1975)	isom (occl)	68%	*0.8	3.93	1.15	2.09	0.79	-
Sahlin	cycle	300	*7.7	0.53	0.14	0.38	0.14	-
et al (1978)	isom (occl)	62%	*1.0	3.34	1.15	2.09	0.79	-
Sutton et al (1981)	cycle	195	20	0.07	0.02	0.04	0.01	0.02
¶Jacobs et al (1982)	cycle (AWT)	450	*0.5	4.08	1.27	2.58	0.94	-
Sjöholm et al (1983)	stim (occl)	71%	0.75	4.70	1.49	3.14	1.15	-
Sahlin and Henriksson (1984)	isom	60*	*0.84	3.58	1.17	2.37	0.88	-
¶Jones	isok (1.0Hz)	750	0.17	13.07	4.70	7.97	2.92	4.41
et al (1985)	isok (2.3Hz)	820	0.17	14.86	5.00	9.18	3.36	4.85
	isok (1.0Hz)	670	*0.5	7.92	1.33	6.35	2.33	3.23
	isok (1.0Hz)	600	*0.5	6.78	0.83	5.92	2.17	3.07
¶McCartney et al (1986)	isok (1.7Hz)	700	*0.5	7.74	1.47	5.98	2.19	3.14
¶Cheetham et al (1986)	sprint	535	*0.5	6.00	1.90	3.77	1.38	2.14
Spriet et al (1987)	stim (occl)	72%	1.71	2.86	0.71	2.05	0.75	0.83

glyco=ATP from glycolysis; PCr=ATP from PCr breakdown

¶Studies with supramaximal work rate; work rate cited is mean power output over the duration of the test

Other details as for table 2.3

mmoles/kg d.m./s and to the glycolytic ATP turnover rate of 0.05 mmoles/kg d.m./s.

As can be seen in all cases, the ATP turnover from glycolysis exceeds that from PCr even when the duration of exercise is as brief as 10 seconds (Jones et al, 1985). Glycogenolysis appears to be able to accelerate sufficiently rapidly to meet the demands of the glycolytic pathway as in all cases it exceeds it. The maximum rate as calculated from the data of Jones et al (1985) of 4.85 mmol/kg d.m./s is twice the maximum activity recorded by Chasiotis in vitro for phosphorylase a + b after dynamic exercise (Chasiotis, 1983). In addition the highest ATP turnover rate from glycogen (9.2 mmol/kg d.m./s equivalent to 127 µmol/g wet weight/min) is about twice the maximum rate that has been previously reported (see table 2.3).

Electrically stimulated muscle with an occluded circulation enables metabolic changes to be examined under extreme conditions, and using this technique Hultman and Sjöholm (1983) have shown that within 10 seconds of stimulation glycolysis already contributed 40% of the total ATP turnover of 5.6 mmol/kg d.m./s and that the decline in force was proportional to the ATP turnover rate estimated from metabolic changes in serial biopsies taken after 5, 10, 20, 30, 40 and 50 s of stimulation. However after 50 s force production was still almost 80% of its initial value. During the first 5 s of contraction the rate of glycogenolysis exceeded glycolysis by 2.5 times and although it declined while glycolysis increased to its maximum rate at the end of the period of stimulation, it always exceeded it. In a separate study (Hultman et al, 1983) it was shown that even after 1.26 s of stimulation, that 20% of the energy supply of 11 mmol/kg d.m./s had been supplied by glycolysis. Spriet et al (1987) took this experiment with stimulated quadriceps muscle one stage further. Using impulses of 1.6 s stimulation

with 1.6 s rest the muscle was stimulated for 204.8 s corresponding to a contraction time of approximately 100 s. Biopsies were taken at 25, 50, 75 and 100 s. Force on this occasion declined to less than 20% of its initial value and there was again a corresponding decrease in ATP turnover. Glycolysis contributed 60% of the initial energy supply and declined during the test to 16% of its original rate of 1.26 mmol/kg d.m./s when it supplied all of the energy for contraction. The initial glycogenolytic rate of 1.68 mmol/kg d.m./s declined again more rapidly than glycolysis to a level equal to it in the last contraction interval. During this time muscle pH fell from 7.00 to 6.45. It was concluded that as ATP and PCr were still available and glycolysis continued despite the H^+ ion concentration, ATP production was not a limitation to force production. As the decrement in ATP turnover and in force at the end of the 100 s stimulation were the same it could also be argued that they were indeed cause and effect.

It is likely then that there is no single cause of fatigue; there is evidence to support failure of excitation/ activation coupling, actin-myosin cross bridge cycling and tension, and in the rate of energy supply. It is currently impossible to state whether or not the latter is due to an inability to supply ATP, primarily from glycolysis, or whether it is due to an impairment in utilisation caused by the former.

2.6 The effect of training on high intensity exercise

Physical training improves fitness and performance and in view of the differences in the muscle fibres types it will be apparent that different activities will stress different fibre groups. It has been demonstrated that marathon running depletes glycogen in ST fibres (Jacobs et al, 1981) whereas maximum isometric contractions depletes

FT fibres (Secher and Nygaard, 1976).

2.6.1 Animal studies

Although Saubert et al (1972) demonstrated an increase in FOG fibres at the expense of FG in white muscle of rats sprint trained for 11 weeks, this was not accompanied by any change in glycolytic or oxidative enzyme activity. The same year Staudte et al (1973) reported an increased activity of HK and CS in both red and white muscle. On this occasion the rats were sprint trained for 21 days only and red muscle (soleus) also demonstrated an increased GP and CPK activity. Using both sprint and endurance trained rats Hickson et al (1976) found no difference in metabolic activity between the two groups at both 8 and 16 weeks of training. There was again no change in glycolytic enzyme activity but oxidative capacity was improved in SO, FOG and FG muscle types. In a further study of sprint trained rats, Davies et al (1981) found no change in muscle oxidative capacity despite an increase in VO_2max and concluded that these two parameters were dissociated. There is therefore no clear pattern of the adaptive changes in muscle to high intensity training in the rat, and it is likely that activities that are considered entirely anaerobic or entirely aerobic will have considerable overlap so that most training schedules will combine both elements and this is presumably the case in man.

2.6.2 Cross sectional studies in man

In man, some insight into the adaptive changes with training for different intensities of exercise has been provided by cross sectional studies of populations with different levels of fitness. An early study by Johnson et al (1969) revealed that athletes appeared to be better at

fully oxidising fat than untrained individuals over a prolonged run. No mention of the training status of the athletes was however made. A large study of groups of varying physical activities (Gollnick et al, 1973) demonstrated that oxidative enzyme activity (SDH) was highest in those participating in endurance sports and was even higher in those muscle groups engaged extensively in endurance work (e.g. bicyclists). There was no difference in PFK activity in any of the groups but ST fibres were most numerous in the endurance athletes who had a greater oxidative capacity in both fibre types than untrained ST fibres. Muscle glycogen was much higher in trained than untrained subjects, with values of 600 against 350 mmol/kg d.m. for a subgroup of 6 trained and 10 untrained. These findings were confirmed by Costill et al (1976) who again looked at groups with differing athletic prowess. They found that the % ST fibres were greatest in distance runners and lowest in sprinters. Oxidative capacity was lowest in strength athletes (shot-putt and discus throwers) but glycolytic capacity highest in sprinters. These findings appeared to confirm Saltin's (1973) and Gollnick et al's (1973) view that muscle fibre distribution is by and large determined genetically whereas enzyme activity can be altered by training. Sahlin and Henriksson (1984) showed that trained athletes (top games players) demonstrated a higher muscle buffer capacity (β) (determined from pH change and lactate accumulation with an occluded circulation) than untrained (sedentary) subjects. Muscle oxidative capacity was higher in the trained group but there was no difference in muscle fibre morphology or glycolytic enzyme activity. Further evidence that athletes who undertake sports with high intensity exercise (sprinters and rowers) have a higher muscle buffering capacity was provided by Parkhouse et al (1985). Endurance athletes were found to have a buffering capacity (measured by titration on a deproteinised homogenate) similar to un-

trained controls and β was significantly correlated with muscle carnosine concentrations and the % FT fibres. The latter finding was contrary to that of Sahlin and Henriksson (1984) although they did find a relationship between FT fibres and post-exercise lactate.

Holloszy and Coyle (1984) summarised the adaptive changes in muscle to endurance training and concluded that endurance training causes an increase in the mitochondrial content and oxidative capacity; a tendency for FTb fibres to become FTa fibres; an increase in VO_2max; a greater reliance of fat as a fuel with less glycogen utilisation and consequently a lower lactate (blood and muscle) for the same sub-maximal activity. This latter point is at variance with Brooks' work (Donovan and Brooks, 1983; Brooks, 1985) which suggests that lactate turnover is directly correlated with the metabolic rate and that endurance training improves lactate clearance rather than decreasing production. This does not seem to be a function of blood flow, as muscle blood flow at rest and the same submaximal work rate is similar in endurance trained and untrained subjects (see Hudlická, 1977 for review) although higher maximum flows have been described (Clausen, 1973). Training does however increase the capillary density (Anderson and Henriksson, 1977) predominantly round highly oxidative fibres (Ingjer, 1979) and this arrangement may allow better muscle performance with the same overall blood flow.

Many of these studies cited suffer from the criticism that they look in a sense at a self-selected population and it difficult to deduce which changes are due to the effects of training and which are genetic. A better understanding of the induced changes can be obtained from studies on subjects before and after training. Table 2.6 summarises some of this work with an emphasis on high intensity training.

Table 2.6a Functional and biochemical changes as a result of physical training in man

Study	n(sex)	training model (work load W)	training frequency time/week	training duration months	change in VO_2max	improvement in performance
Varnauskas et al, 1970	5(m) 2(f)	Cycle ergometer (220-305W)	5min x 5 3 x week	6 weeks	+30%*	--
Karlsson et al, 1972	14(m)	endurance and interval	3 x week	7 months	+24%*	260-305W* (at VO_2max)
Gollnick et al, 1973	6(m)	cycle ergometer (85-95% VO_2max)	60min x 1 4 x week	5 months	+13%*	--
Eriksson et al, 1973	8(m)	calisthenics, running & sports	60min x 1 3 x week	4 months	+15%*	140-165W* (at VO_2max)
(boys 11-13)	5(m)	cycle ergometer	20min x 1 3 x week	6 weeks	+6%	-
Thorstensson et al, 1975	4(m)	interval sprint	4 x week	8 weeks	+3%	MVC +12%* Sargeant's jump +9%* Margaria test +4%* 25m sprint -0.03s*
MacDougall et al, 1977	9(m)	resistance (arms)	4 x week	5 months	-	MVC +28%*
Houston and Thomas, 1977	5(m)	interval sprint	4 x week	6 week	+4%*	60s run -14%* leg press load +35%*
Bylund et al, 1977	20(m)	calisthenics and endurance (80-90% VO_2max)	3 x week	7 months	+26%*	4km run -33%*
Weltman et al, 1978	13(f)	AWT - cycle ergometer	3 x week	6 weeks	+10.5%*	AWT +12%*
Costill et al, 1979	5(m)	isokinetic stength (1 leg)	6s bouts 4 x week	7 weeks	-	peak torque +4%*
	5(m)	isokinetic stength (1 leg)	30s bouts 4 x week	7 weeks	-	peak torque +4%*
Sharp et al, 1986	8(m)	AWT - cycle ergometer	8 x 30s 4 x week	8 weeks	+8%	peak torque +26%*

AWT = anaerobic work test; MVC = maximum voluntary contraction
* indicates a significant change

Table 2.6b (continued)

Study	n(sex)	% ST fibres pre	% ST fibres post	% change in fibre area ST	% change in fibre area FT	% change in various enzymes: glycolytic GP	glycolytic HK	glycolytic PFK	glycolytic LDH	oxidative SDH	oxidative CytOx	contractility CPK	contractility AK
Varnauskas et al, 1970	5(m) 2(f)	-	-	-	-	-	-	-	-	+44*	-	-	-
Gollnick et al, 1973	6(m)	32	36	+23*	-8	+117*	-	-	-	+94*	-	-	-
Eriksson et al, 1973 (boys 11-13)	5(m)	55	49	-	-	-	-	+83*	-	+30*	-	-	-
Thorstensson et al, 1975	4(m)	41	44	+3	+11	-	-	-	+6	-	-	+60	+16
Houston and Thomas, 1977	5(m)	42	41	-	-	-	-	-	-3	-	-	-	-
Bylund et al, 1977	20(m)	35	35	+25*	+20*	+29*	+26*	-4	-	-	+158*	-	-
Costill et al, 1979	5(m	45	42	-4	+5	-6	-	+6	-	+3	-	-1	0
	5(m)	48	47	+2	+10	+11*	-	+23*	-	+11*	-	+15*	+13*
Sharp et al, 1986	8(m)	-	-	-	-	-	-	+9	-	+45*	-	-	-

GP = glycogen phosphorylase; HK = hexokinase; PFK = phosphofructokinase;
LDH = lactic dehydrogenase; SDH = succinate dehydrogenase; CytOx = cytochrome oxidase
CPK = creatine(phospho)kinase; AK = adeylate kinase (myokinase)

* indicates a significant change

Table 2.6c (continued)

		changes in metabolites (mmoles/kg dry muscle)												
		ATP				PCr				glycogen		lactate		
		pre-exer		post-exer		pre-exer		post-exer		pre-exer		post-exer		other
Study	n(sex)	pre	post	pre	post	pre	post	pre	post	pre	post	pre	post	changes
Varnauskas et al, 1970	5(m) 2(f)	-	-	-	-	-	-	-	-	-	-	-	-	MBF +45%*
Karlsson et al, 1972	14(m)	17	21*	11	16	74	73	14	19	317	417*	74	84	
Gollnick et al, 1973	6(m)	-	-	-	-	-	-	-	-	313	791*	-	-	
Eriksson et al, 1973 (boys 11-13)	8(m)	19	21*	17	17	63	89*	19	26	235	310*	38	59*	
Thorstensson et al, 1975	4(m)	22	23	-	-	87	76	-	-	-	-	-	-	ATPase +29%*
MacDougall et al, 1977	9(m)	22	26*	-	-	74	78*	-	-	376	500*	-	-	arm +11%*
Houston and Thomas, 1977	5(m)	25	29	-	-	75	78	-	-	-	-	-	-	
Sharp et al, 1986	8(m)	-	-	-	-	56	66	16	24	-	-	91	111*	β +36%*

pre-exer = pre-exercise or resting samples; post-exer = immediately after exhaustive exercise
pre = pre-training; post = post training

MBF = maximal blood flow; ATPase = Mg^{2+} activated ATPase; arm = arm girth
β = muscle buffering capacity

* indicates a significant change

2.6.3 Prospective studies and high intensity training

The duration of training in these studies ranged from 6 weeks to 7 months and although in all the studies some objective improvement had been demonstrated, Saltin et al (1977) have estimated that although ST fibre cross sectional area may have increased maximally within two months, the 10-30% improvement seen in VO_2max over the first 8 weeks or so of training may improve to 50% over the next 9 months to 2 years. Similarly, mitochondrial enzyme activity continues to increase over 2 years of training as does capillarisation of the muscle fibres. Conversion of oxidative FTb to oxidative-glycolytic FTa fibres appears to be maximal at twelve months. The maximum rate of change of these adaptations is in the first 3 months of training and although these estimates were in relationship to endurance training it is likely that they can be applied to interval training. Despite the chronicity of training induced adaptation a period of training of more than 8 weeks would seem ideal.

An increase in VO_2max was invariably demonstrated even with apparently anaerobic training and a significant improvement in performance was recorded in all studies. As found with endurance trained athletes there was no change in the %FT/ST fibre distribution and MacDougall (1986) has confirmed the constancy of fibre types in his studies on bodybuilders and heavy resistance training. There was a tendency for ST fibre area to increase with endurance training and FT fibres to increase with sprint or resistance training in keeping with the findings expressed in the reviews by Saltin et al (1977) and Gollnick and Hodgson (1986). These changes in fibre morphology resulted in significant alterations in the percentage fibre area occupied by different fibre types. Gollnick et al (1973) found an increase in the

relative percentage area of ST fibres whereas Costill et al (1979) found a decrease in the percentage area of ST fibres and an increase in FTa in both legs trained at different relative work loads. As the number of fibres is thought not to change in man (MacDougall, 1986), any marked increase in fibre area would be as a result of fibre hypertrophy and would result in an increase in muscle mass as evident in MacDougall et al's (1977) finding of an increase in arm girth.

An improvement in muscle oxidative capacity in agreement with the changes in VO_2max was seen with both types of training as was an increase in the glycolytic potential. Two studies demonstrated an improvement in contractility enzymes (creatine kinase and adenylate kinase) with "anaerobic" training, but despite Eriksson et al (1973) and MacDougall et al's (1977) findings of an increased phosphogen concentration this was not invariably found and four studies found no change in PCr levels. Muscle glycogen was, as expected, uniformly increased and post exercise lactate appeared to increase with training consistent with an improved glycolytic capacity. Sharp et al (1986) reported an elevated muscle buffering capacity in keeping with the findings of Sahlin and Henriksson (1984) and Parkhouse et al (1984) in their cross sectional studies. One study demonstrated an increased maximum thigh blood flow measured by Xenon clearance (Varnauskas, 1970), a finding that Leinonen (1980) confirmed in endurance trained but not sprint trained subjects (international distance runners and sprinters).

The improved performance in high intensity training would appear to be as a result of an increased VO_2max and muscle oxidative capacity, an increase in FOG fibre types and FT fibre diameter resulting in reduced fatigability, and increased strength but no change in ST/FT fibre distribution. An increase in glycolytic enzyme activity is associated with an increased muscle glycogen and a higher muscle lactate at

exhaustion which may be a reflection of a higher glycolytic ATP turnover rate. There is no change in muscle ATP or PCr concentrations nor in the apparent ATP turnover from PCr. The improvement in muscle buffering capacity may be important in modifying the effects of the higher proton load that an increased glycolytic flux would produce.

2.6.4 Evidence for fibre type transformation

Although it is widely stated that changes in muscle fibre types do not occur as a result of training, Pette (1984) has produced convincing evidence to suggest that this is not the case. In a detailed review he states that chronic stimulation of small mammalian muscle (either electrically or by exercise) induces a fast to slow fibre transformation. This transformation is progressive with the quantity and duration of the stimulus and proceeds in an orderly sequence. Early enzyme changes result in a white to red metabolic transformation. Simultaneously cytosolic Ca^{2+} binding and sequestration is reduced by a decrease in parvalbumin and changes in the sarcoplasmic reticulum. Fast to slow transformation is completed by an exchange of fast with slow type myosin isoforms. He concludes that the phenotype of a muscle fibre is dynamic and is modified according to its functional demand and that transformation in the reverse direction, slow to fast, also occurs. Further evidence has been provided by Bouchard et al (1986) who in their detailed study of non-twin brothers, dizygotic and monozygotic twins felt that the genetic effect in muscle fibre distribution was quite low and that common environmental conditions contributed more to type I fibre distribution than genetic factors. The demonstration that it is possible to reconstruct damaged heart muscle with chronically stimulated latissimus dorsi muscle in man is evidence of the extreme adaptation of which skeletal muscle is capable (Carpentier and Chachques, 1985).

Chapter 3

3 Atherosclerosis and peripheral vascular disease - literature review

Although atherosclerotic changes have been described in the arteries of Egyptian mummies (Sandison, 1962), historical descriptions of the manifestations of the disease are sparse (Barnett and Fraser, 1955) and it was not until 1831 that Bouley (1831) made the first description of intermittent claudication due to vascular obstruction in the horse. It was another 15 years before the syndrome was reported in man, but it was not until 1858 that Charcot clearly defined and described the symptoms as we know them today (cited by Hansteen and Lorentsen, 1974). Since then, to have made any real progress in the treatment of atherosclerosis we should be able to refute John Wyckoff's statement of half a century ago: 'At present, no evidence exists that there is any specific mode of therapy which can either cure or affect the progress of atherosclerosis' (Wyckoff, 1933)

3.1 Prevalence of peripheral vascular disease

The prevalence of lower limb arterial disease in a population is difficult to ascertain, but from the studies summarised in Table 3.1, it can be seen that it increases with age and has been consistently found to be about 3% of males and 1% of females in the age group 60-64 years. Females appear to develop the disease about 10 years later than males. Despite the fact that only a small proportion of these patients require reconstructive surgery, 10,000 operations were performed upon arteries in England and Wales in 1978, with a male to female ratio of 3:1 (Department of Health and Social Security, 1981).

Table 3.1 Prevelance of peripheral arterial disease of the lower limb

Author	Population studied	Criteria for peripheral arterial disease	Males no studied	Males age group	Males prevalence (%)	Females no studied	Females age group	Females prevalence (%)
Widmer et al, 1964	Swiss pharmaceutical workers, Basle	History of IC Absence of foot pulses Occilography Arteriography	547 347 153	50-54 55-59 60-65	3.6 5.2 7.5	92 37 none	50-54 55-59 none	2.1 2.6
Reid et al, 1966	British postal workers	History of IC	676	50-59	2.4	none		
Kannel et al, 1970	Sample population Framingham, USA	History of IC	2573 2226 1619	50-54 55-59 60-64	1.0 1.8 3.2	3259 2892 2159	50-54 55-59 60-64	0.5 0.8 1.3
Hughson et al, 1978	General practitioners lists, Oxfordshire, UK	History of IC ABPI <75%	1544	45-69	2.2	1382	50-65	1.2
Schroll & Munck, 1981	Sample population of Glostrup, Denmark, born in 1914	History of IC Absence of foot pulses ABPI <90%	360	60	3.3	306	60	1.0

IC = intermittent claudication; ABPI = ankle/brachial pressure index

3.2 Natural history of peripheral vascular disease

This considerable problem is eased somewhat by the relatively benign course of the disease with regard to local problems. Juergens et al (1960) published the details of 520 patients who attended the Mayo Clinic from 1939-1948 with a diagnosis of atherosclerosis obliterans of the lower limb. They found that the overall amputation rate was 4.9% within 5 years, and even when patients with ischaemic ulceration or gangrene were considered, only 19.6% required amputation within this period. These findings on the natural history of the disease, together with those of Silbert and Zazeela (1958), the late Ken Bloor (1961) and subsequent large studies are summarised in Table 3.2. The overall good prognosis is confirmed, even in the presence of rest-pain, pre-gangrene or gangrene when amputation is not the inevitable outcome. Bloor (1961) found that 47% and Taylor and Calo (1962) only 24% of such patients lost a limb. The risk of death is increased to at least twice that of a similar population (Kannel et al, 1970) and this increased cardio-vascular mortality is indicative of intermittent claudication being a manifestation of a generalised disease. Silbert and Zazeela (1958) followed up 399 diabetics in their series and reported a 34.3% amputation rate compared with 8% for non-diabetics and a mortality at 10 years four times that of non-diabetics. Inflow (iliac artery) obstruction would appear to have a better prognosis than outflow (femoral artery). Juergens et al (1960) reported that 4.1% of aorto-iliac occlusions lost a limb compared with 5.5% of femoral artery occlusions, whereas Taylor and Calo (1962) listed the presence of aorto-iliac occlusion as a good prognostic factor. Jelnes et al (1986) in an excellent study from Copenhagen have drawn attention to some changes in the pattern of the disease over the last decade. Of the 257 patients

Table 3.2 Natural history of peripheral vascular disease of the lower limb

Author	no of patients	Female (%)	Follow-up (years)	overall mortality (%)	vascular deaths (%)	prognosis % stable or improved	amputation rate (%)	diabetics (%)
Silbert & Zazeela, 1958	799	14.8	3-15	33.5	64	65.5	8.0	0
Silbert & Zazeela, 1958	399	34.6	3-15	68.6	64	37.4	34.3	100
Juergens et al, 1960	520	8.1	3-10	47.0	‡75	no data	4.9	excluded
Bloor, 1961	1476	10.5	4-10	45.5	84	71.0	9.7	4.0
Taylor & Calo, 1962	412	8.0	3-12	21.0	51	82.7	10.0	4.5
Begg & Richards, 1962	198	7.0	5-12	46.5	86	68.9	7.1	4.0
Ulrich et al, 1973	304	23.0	0.6-6.4	17.4	no data	75.0	4.0	6.9
Imperato et al, 1975	104	no data	0.5-8	no data	no data	78.8	5.8	no data
Wilson et al, 1980*	53	excluded	5	9.0	100	45.0	2.0	excluded
Källerö et al, 1981†	193	25.0	8.5-11.5	48.7	61	#73.9	5.2	excluded
Jelnes et al, 1986	257	38.9	6-8	44.0	48	82.9	7.0	5.4
Lassila et al, 1986¶	312	19.0	8.8-11.8	60.3	68	69.2	no data	14.7

* Patients with femoro-popliteal occlusion only
† Study of 312 patients referred for venous occlusion plethysmography with intermittent claudication (IC): 119 patients with normal findings excluded from analysis
# Based on numbers who had reconstructive surgery and/or amputation
¶ Based on a retrospective study of 320 patients of whom 94% (312) were traced; prognosis calculated from those patients with IC who progressed to advanced ischaemia

studied, 100 were women, in contrast to the earlier studies. This they attributed to the increasing social liberation and change in smoking habits of women over the last 30 years. Over the 9 years of follow-up the disease progressed in 42 patients (16%) of whom 24 underwent reconstructive surgery and 18 had primary amputations. They found the most important index in predicting progression of the disease was the ankle or toe systolic pressure and no patient with an ankle systolic pressure greater than 70 mm Hg underwent amputation. Unlike earlier studies diabetes was not associated with an increased risk despite a similar incidence (5.4%) and the absence of the femoral pulse did not carry a more favourable prognosis. Mortality was 44% (twice the expected) of which 58% was vascular with male sex, age at presentation less than 60 years, hypertension and a low distal segmental limb pressure all positive risk factors. Diabetes was again not a risk factor in excess mortality which is contrary to what would be expected (Jonason and Ringqvist, 1985a) and it was suggested that many diabetics may present with advanced disease causing a bias in their apparent outcome in this study. Cronenwett et al (1984) had earlier pointed out the importance of a decline in ankle systolic pressure as an indicator of an increased likelihood of operation and Jonason and Ringqvist (1985b) found distal segmental pressure (as a variable dependent of multiple arterial stenosis) to be a significant risk factor. In a study of 224 non-diabetic claudicants (62 females) aged less than 75 years and followed up for five years, the variables they found which significantly correlated with the development of rest pain were the continuation of smoking and evidence of multiple arterial stenoses. A further paper by the same authors on 151 of these patients (27 females) (Jonason and Ringqvist, 1986), in agreement with other series, reported that 41% improved, 32% were stable and 27% had deteriorated of whom 18 underwent

reconstructive surgery or amputation.

3.3 Aetiology of atherosclerosis

Our understanding of the aetiology of atherosclerosis is based on several well defined risk factors together with a number of hypotheses reinforced by experimental evidence of its pathogenesis. Currently accepted major risk factors are ageing, the male sex, raised serum cholesterol, hypertension, ECG evidence of left ventricular hypertrophy, diabetes, tobacco smoking and physical inactivity (Kannel et al, 1970; Kannel et al, 1976; Schroll and Munck, 1981). For peripheral vascular disease, smoking habits, blood pressure, cholesterol and diabetes appear to be the strongest risk factors. Diabetes as a risk factor poses some problems as the other accepted risk indices are insufficient to account for the excess arterial disease in diabetics (Jarrett, 1981). Some light may have been thrown on the problem by the report of a possible genetic marker for atherosclerosis and its association with diabetes (Owerbach et al, 1982). The implication was that male sex and hyperglycaemia were by themselves important in the development of the disease although less important than the DNA sequences. Banga and Sixma (1986) however, in a detailed review of diabetes and vascular disease, pointed out that some of the alterations in platelet function, red blood cells and haemostasis and the lipoprotein disturbances seen in diabetics without vascular disease could be corrected by careful diabetic control. This suggested a causative rather than a passive role in the development of atherosclerosis, and non-enzymatic glycosylation of proteins, the central abnormality of diabetes, could be an important link between these various abnormalities and the disease. The recent decrease in the incidence of coronary heart disease in the United States in association

with dietary and life-style changes (decreased saturated fat and cholesterol intake and cessation of cigarette smoking) has shown that by altering risk factors it is possible to influence the outcome of the disease (Stern, 1979). It is likely though that in an individual, development of the disease is by combination of exposure to the local environment, general genetic predisposition, and interaction between the constituents of the blood and the vessel wall (Benditt and Gown, 1980).

3.3.1 Platelets

The most attractive hypothesis now being postulated as the major determinant of the cause of atherosclerosis is the interaction of platelets and the vascular endothelium. Platelet aggregation is brought about by a number of agonists including ADP, collagen, thrombin and thromboxane A_2 (Moncanda and Higgs, 1986) and adhesion of platelets to the vessel wall then gives rise to the release phenomena mediated through an increase in calcium concentration. In the normal physiological state, platelet-endothelial homeostasis is maintained by the opposing actions of vasoconstrictory and pro-aggregatory thromboxane A_2 and vasodilatory and anti-aggregatory prostacyclin (eprostenol) (Gryglewski, 1980). This maintenance of endothelial integrity by its production of the 'hormone' prostacyclin seems to be a crucial protective barrier between the blood vessel wall and its environment. A deficiency in prostacyclin production has been shown to occur in atherosclerotic arteries, diabetes and as a result of cigarette smoking. In addition it may be that prostacyclin has a regulatory role on arterial cholesterol metabolism (Moncanda and Higgs, 1986).

Endothelial injury can be produced by a variety of factors and may manifest itself in a spectrum of damage, from subtle changes in

permeability to complete desquamation (see Stemerman et al, 1984 for review). Among the many agents that have been shown experimentally to produce injury are mechanical damage, homocystinuria, immunological mechanisms, chronic hyerlipidaemia, endotoxins, viruses, certain products associated with tobacco smoking and haemodynamic forces (Schwartz and Mitchell, 1962; Caro et al, 1969; Stemerman and Ross, 1972; Fry, 1973; Schonfeld, 1975; Texon, 1976, Harker and Ross, 1979; Poston, 1979; Benditt and Gown, 1980; Thomas, 1981). Following endothelial injury, the platelets aggregate and release a number of chemical mediators as dense body granules and α-granule proteins together with a number of lysosomal enzymes and lipid derived mediators. Dense bodies contain serotonin and adenine nucleotides. The α-granule proteins comprise adhesive proteins, platelet factor 4, β-thromboglobulin and platelet derived growth factor (Chesterman and Berndt, 1986). The subendothelial environment appears to favour the accumulation of the cationic α-granule proteins (Chesterman and Berndt, 1986), and one of the most important appears to be the mitogenic platelet derived growth factor which has been identified and shown to cause medial myocyte migration and proliferation (Ross and Glomset, 1976; Ross, 1980). In man abnormality of platelet function has been described both in diabetics and in atherosclerosis (Horlick, 1961; Davis, 1973; Colwell et al, 1978) and hypercholesterolaemic patients have platelets which are abnormally sensitive to aggregating agents (Carvalho et al, 1974). The importance of platelets in the aetiology of atherosclerosis is supported by the fact that thrombocytopenic animals and pigs with von Willebrand's disease are protected from atherosclerosis (Schafer and Handin, 1979; Weksler and Nachman, 1981). However the finding of extensive atherosclerosis at autopsy in an

elderly man who had suffered from severe von Willebrand's disease adds to the controversy surrounding this problem (Kernoff et al, 1981).

Dyerberg and Bang (1982) added considerable weight to the hypothesis that the platelet/vessel-wall interaction is the key to development of atherosclerosis, by their study of Greenland Eskimos who have a nearly 10-fold lower incidence of ischaemic heart disease deaths than their countrymen living in Denmark. They found a diet-associated plasma lipid level and lipoprotein pattern accepted as low risk for ischaemic heart disease, together with an intake of n-3 marine fatty acids 5-fold higher in native Eskimos than in their Danish counterparts. Dietary intake of n-6 fatty acids were halved. There was a corresponding increase in plasma eicosapentaenoic acid (EPA) and a decrease in arachidonic acid, and the authors were able to demonstrate a shift in prostaglandin synthesis from arachidonic acid to EPA with a resultant new 'n-3' family of prostaglandins. The thromboxane A_2 analogue (thromboxane A_3) was of low biological activity, whereas the prostacyclin analogue (PGI_3) was biologically very potent, resulting in a decrease in platelet aggregability and a tendency to bleed. These findings were confirmed both in the population study and in volunteer studies by feeding experiments. This is further supported by the finding that a high EPA (fish) diet prolongs platelet survival in patients with heart disease (Hay et al, 1982).

3.3.2 Cholesterol

The complex role of lipoproteins as metabolically active carriers for cholesterol and triglycerides and their role in atherosclerosis has been well reviewed by Schonfeld (1975), Benditt and Gown (1980) and Lewis (1983). As a risk factor in developing atherosclerosis

cholesterol works in a straightforward way; the higher the blood cholesterol, the greater the risk. It is important to realise that absolute cholesterol levels are less meaningful than the levels of pro-atherogenic low density lipoproteins (LDL) and anti-atherogenic high density lipoproteins (HDL). This relevance is further emphasised by studies on patients with inborn errors of lipid metabolism; the familial hyperlipoproteinaemias are well recognised as being associated with a greatly increased risk of atherosclerosis, whereas families with genetically low levels of LDL or high levels of HDL are less at risk than normal (Schonfeld, 1975; Benditt and Gown, 1980). The exact mechanism of entry of lipids into the arterial wall is uncertain but there appears to be a non-specific transfer mechanism for lipoprotein macromolecules from the plasma into the arterial wall which involves albumin and is related to particle size with additional influx of free cholesterol (Stender, 1982). These local high concentrations of lipid could result in the formation of lipid peroxides, which inhibit prostacyclin synthesis with effective endothelial damage (Gryglewski, 1980).

3.3.3 Thrombosis

Although the importance of mural thrombosis as a final insult to vessel patency is self evident, its contribution to the development of the atherosclerotic lesion has remained controversial since Rokitansky's encrustation hypothesis of 1844 was so eloquently reintroduced by Duguid (1949). There is evidence that both increased levels of plasma fibrinogen and defective fibrinolysis are associated with occlusive arterial disease (Davidson and Walker, 1981; Lowe and Forbes, 1981), and Woolf (1978) has presented evidence to show that both fibrin and

platelet antigens can be demonstrated within sub-endothelial arterio-sclerotic plaques. This suggests that thrombi may contribute to the development of pre-existing plaques by being incorporated within the arterial wall and a recent review by Smith (1986) concludes that "there is extensive evidence that enhanced blood coagulation is a risk factor not only for thrombotic occlusion but also for atherogenesis".

The early lesion of atherosclerosis thus ensues and as a result of myocyte proliferation [perhaps of monoclonal origin (Benditt, 1977)], accumulation of extracellular lipid, cellular debris and thrombus, it develops into the complicated lesion. Progressive fibrosis, haemorrhage and calcification further narrow the artery until complete obstruction occurs, this final event being the result of further mural thrombosis (Ross and Glomset, 1976; Constantinides, 1981). The outcome for the limb then depends on a number of other pathophysiological factors.

3.4 Pathophysiological factors affecting outcome in occlusive arterial disease

3.4.1 Collateral circulation

The number of pre-existing collaterals and their degree of development are decisive factors in the course of the arterial disease. Collateral development can be divided into two phases; an acute phase which determines the initial outcome and a slow phase lasting several months which determines the eventual recovery, although this is never complete (Longland, 1953; John and Warren, 1961). Development of these vessels is dependent on three main factors; mechanical, metabolic and neuronal. Of all the factors, the pressure gradient across the occluded vessel would seem to be the most important, both in the initial acute

phase when there is an immediate drop and in the slow recovery phase when there are intermittent presssure gradients in response to exercise (Winblad et al, 1959; John and Warren, 1961). Metabolic factors in the form of hypoxia and 'vasodilatory metabolites' have been postulated but never convincingly demonstrated (Hansteen and Lorentsen, 1974). Perfusion of a limb with oxygenated blood cannot prevent the development of collaterals in the presence of a pressure gradient across the occluded vessel (Winblad et al, 1959; John and Warren, 1961), and conversely John and Warren (1961) demonstrated that perfusion with normotensive hypoxic blood resulted in the regression of collaterals immediately after their development in a manner identical to the removal of the arterial occlusion. The importance of neuronal factors is uncertain. From Longland's work (1953) it would appear that there is some sympathetic innervation of collateral vessels, but he was not able to demonstrate any lasting benefit to collateral circulation by sympathectomy either in an experimental animal or in man, and this corroborated the earlier study of Dornhorst and Sharpey-Schafer (1951).

3.4.2 Vascular spasm

Leary and Allen (1941) and Lindquist (1945) interpreted the decrease in calf oscillometric reading and the diminution or loss of the distal leg pulses on exercise in patients with intermittent claudication as due to vascular spasm. In 1950 Lindqvist (1950) was able to reconcile the apparent contradiction of findings (muscular vasodilatation above an arterial block with apparent distal vasoconstriction during exercise) by explaining it as due to haemodynamic alteration in the distribution of insufficient flow of blood for the limb's need - the 'borrowing-lending' phenomenon of DeBakey (DeBakey et al, 1947).

Hansteen and Lorentsen (1974) in their excellent review of the problem, further conclude that there is no evidence to support the 'vasospasm theory'. This is hardly surprising when one considers the mechanism of control of blood flow in skeletal muscle and the relative importance of neurogenic and metabolic determinants.

Resting blood flow of 2-4 ml/100 g/min is determined by the basal vascular smooth muscle tone which is dependent on myogenic and adrenergic sympathetic control. Although muscle contains both α and β receptors nervous control of muscle blood flow is only of importance for regulation during haemorrhage, the 'fight and flight' response and possibly at the beginning of exercise (Hudlická, 1985). Sympathetic blockade has been shown to decrease resting muscle blood flow both in normal subjects and patients with peripheral vascular disease (Hoffman and Jepson, 1968; Wright and Cousins, 1972) whereas, as a result of the metabolic demands of exercise or following timed ischaemia, flow can increase five to ten times or more in healthy subjects (Strandness and Sumner, 1975; Clausen, 1973). In skin, basal tone is primarily of sympathetic (α-adrenergic) origin but, despite this, there is also a reactive hyperaemia following circulatory arrest (Standness and Sumner, 1975). However, whereas in muscle the accumulation of vasoactive metabolites during ischaemia can abolish any sympathetic vasoconstrictor effect, it would appear that adrenergic stimulation will maintain vasoconstrictor tone in skin (Cooper et al, 1955). Andersen and Saltin (1985) have recently demonstrated the enormous potential exercising skeletal muscle possesses for increasing its blood flow. Using single leg dynamic exercise on a Krogh bicycle ergometer modified so that the fly wheel momentum returned the relaxed limb after knee extension, and with the calf muscle isolated by an occlusion cuff, quadriceps blood flow was determined by a thermal dilution technique. Blood flow

increased linearly with work rate (10 to 60 W), levelling off at the higher rates, and during maximum work was 250 ml/100 g/min. Therefore when a large percentage of the muscle mass is involved in exercise there must be some increase in vascular resistance to the exercising limbs. Although the main regulatory mechanism during exercise is the adaptation to metabolic demands, the important regulators appearing to be potassium ions (in mixed and glycolytic muscle) and inorganic phosphate and adenosine (in oxidative muscle) (Hudlická, 1985), Andersen and Saltin's results suggest the existence of a central regulatory mechanism.

3.4.3 Sympathetic innervation

Rutherford and Valenta (1971), in their elegant study on dogs, showed that although lumbar sympathectomy could increase resting and post-exercise muscle blood flow in the absence of inflow obstruction, with occlusion it resulted in a decrease in flow in keeping with the findings in man of Hoffman and Jepson (1968) and confirmed by Wright and Cousins (1972). As the natural history of the disease is so benign, it is hardly surprising that uncontrolled trials report favourable results in the use of lumbar sympathectomy in the treatment of intermittent claudication (Smithwick, 1957; Postlethwaite, 1973). Fife and Quinn (1975), however, in a controlled trial of 25 patients, were unable to demonstrate that phenol sympathectomy conferred any benefit in intermittent claudication and the mean improvement in almost 600 claudicants treated by lumbar sympathectomy (by a variety of methods) in nine other studies reviewed by Cotton and Cross (1985) was only 34% whereas 25% were worse. It should now be accepted in the light of available clinical and experimental evidence that lumbar sympathectomy has little part to play in the management of the claudicant, a sentiment shared by

Lindenauer and Cronenwett (1982).

In contrast, there is still a considerable body of opinion which feels that sympathectomy has a role in the treatment of patients with cutaneous ischaemia (Gillespie, 1973; Imparato, 1979). Rutherford and Valenta (1971) clearly demonstrated that sympathectomy increased blood flow, particularly in the more distal part of the extremity, but felt that part of the increase could have been caused by arteriovenous shunting. This was confirmed by Delaney and Scarpino (1973) who, again in a dog experiment, were able to show that about 20% of skin blood flow post sympathectomy was non-nutritive, being shunted across the arterio-venous anastamoses. Thulesius et al (1973) studied the effects of lumbar sympathetic blockade in 16 patients with rest pain or ischaemic ulcers and found the procedure only to be of benefit if the ankle blood pressure was above 60 mm Hg. The importance of ankle pressure in predicting outcome was confirmed by Yao and Bergan (1973) and Walker and Johnson (1980). Frøysaker (1973) was, however, unable to demonstrate any benefit to 32 patients with similar disease, although he did not record ankle pressures. In the studies reviewed by Cotton and Cross (1985) more than 600 limbs had sympathectomy for rest pain, skin ischaemia or gangrene, and success varied from a greater than 60% improvement to the procedure being detrimental to the patient. At present the available evidence would suggest that patients with critical ischaemia (rest pain with skin lesions or gangrene with an ankle systolic pressure less than 60 mmHg or without skin lesions or gangrene with a pressure less that 40 mmHg) (Bell et al, 1982) will not benefit from sympathectomy as skin vessels are already maximally dilated, but patients with 'less critical' cutaneous ischaemia may benefit from a redistribution of limb blood flow. Additionally, there appears to be no

extra benefit to the patient in an operative sympathectomy with its attendent risks and mortality, and until it can be shown unequivocally that the procedure has a definite place in the treatment of limb ischaemia, chemical ablation should be used in those patients in whom it is felt sympathectomy may help.

3.4.4 Rheological abnormalities

Apart from the finding of an elevated plasma fibrinogen level in atherosclerosis other rheological abnormalities have been reported; primarily an elevated whole blood viscosity and a reduced erythrocyte deformability (Dormandy et al, 1973; Störmer et al, 1974; Reid et al, 1976; Ehrly and Köhler, 1976; Dormandy et al, 1978), the latter being a measure of the internal viscosity, geometry and visco-elastic properties of the erythrocyte membrane (Chien, 1977). Whole blood viscosity is a measure of its individual components - haematocrit (of most importance), plasma viscosity, fibrinogen, and red cell aggregation and deformability (Begg and Hearns, 1966; Chien et al, 1967a, 1967b).

There is no reason to doubt the importance of the ability of red cells to deform in the microcirculation where they pass through capillaries significantly smaller than themselves, but the finding that this property is abnormal in atherosclerosis is difficult to explain, and it has not been shown that more rigid erythrocytes are associated with defective tissue perfusion in vivo. Since Weed et al (1969) described the importance of ATP and calcium in maintaining the ability of the red cell to deform in vitro, the explanation for the altered deformability has been postulated as an abnormally low red cell ATP level and this was "confirmed" by Buchanan and Moodley in 1977. However their conclusions were based on results obtained from only four athero-

sclerotic patients. Maughan et al (1984) in a detailed study, found no difference in erythrocyte ATP levels between 20 elderly patients with and 20 without peripheral arterial disease and 40 young normal controls (half of whom were smokers). There was an equal number of males and females in each group and it would appear that red cell deformability in vivo is not related to cellular ATP levels, and it is unlikely that red cell ATP content plays a part in the aetiology of atherosclerosis.

3.5 Skeletal muscle metabolism and peripheral vascular disease

3.5.1 Metabolic changes within the muscle

In the early 1960's Pentecost (1964) described a prolongation of the return to normal of post-exercise blood flow in patients with lower limb arterial disease, but there was no difference between the patients and normal subjects in external iliac vein oxygen saturation for the same work load (50 W). Two years later it was reported that oxygen extraction was significantly higher in patients with femoral artery occlusion who stopped working prematurely, than controls exercising at the same work rate (Hlavová et al, 1966). Pernow and Zetterqvist (1968) divided patients with intermittent claudication into four groups depending on the site of arteriographically determined arterial obstruction (8 iliac, 13 common or proximal superficial femoral, 18 distal superficial femoral artery and 7 popliteal and distal arteries). They showed at equivalent work loads (25, 50 and 75 W) that oxygen extraction was greatest the more proximal the obstruction and in all patients was greater than the controls. Arterial and venous lactate was also determined and they found that the net lactate release from the leg was greater the more proximal the block. This they considered to be a reflection of a greater mass of exercising ischaemic muscle when inflow

was reduced and they concluded that their results gave "virtual conclusive support to the concept that anaerobiosis is responsible for the muscle failure in intermittent claudication". Following reconstructive surgery (n=10) however, although oxygen extraction was reduced, it did not parallel the return of blood flow to normal (Bellman and Zetterqvist, 1966) and this was attributed to non-nutritive post-operative shunting of blood-flow. In an attempt to explain these findings Holm (Holm, 1972; Holm et al, 1972) studied the metabolic activity of gastrocnemius and quadriceps muscle in patients with claudication (n=33) and rest pain (n=11). Compared with a control group (n=18) there was no difference in succinate oxidase activity in quadriceps muscle but the enzyme was significantly more active in claudicant gastrocnemius. The rate of incorporation of U-^{14}C-glucose into glycogen, lipids and CO_2 was greater for both muscles in the claudicant group but no different for patients with rest pain. Lactate incorporation was only significantly different for the claudicant gastrocnemius. As an improved oxidative capacity and glycolytic potential is a feature of the adaptive response to endurance training (see chapter 2) it was postulated that some common mechanism was responsible for triggering the changes. An explanation for the increased oxygen extraction could thus be provided by the increase in oxidative enzymes. Holm quite rightly felt though, that it was not possible to say if the total metabolic capacity was increased or whether it was at the expense of other substrates, and the finding that FFA utilisation was much lower in patients (14 claudicants) than controls (Hagenfeldt et al, 1972) pointed to the latter. Pernow et al (1973 and 1975) reaffirmed the earlier findings on lactate release and arterio-femoral venous oxygen difference in 13 severe claudicants, and in

addition measured muscle ATP, PCr, glycogen and lactate concentrations at rest and after exhaustive bicycle ergometer work (10-100 W) prior to and following reconstructive arterial surgery. Resting ATP concentrations were 18.3 mmoles/kg dry muscle (d.m.), significantly less than the control values (23.4) but resting PCr, glycogen, glucose 6-P and lactate were similar at 76.5, 363, 1.3 and 4.8 mmol/kg d.m. and 82.2, 375, 1.7 and 6.1 mmol/kg d.m. for the patient and control groups respectively. At exhaustion the patients' ATP levels had fallen to 16.5, PCr was considerably depleted at 31.3 and lactate markedly elevated at 52.6 with glucose 6-P less so at 3.9 mmol/kg d.m. These changes were in contrast to the minimal changes seen in the control group working at similar loads but very similar to the changes they recorded after maximal work to exhaustion. Less glycogen was utilised by the patients for their equivalent rise in muscle lactate which was a reflection of their impaired lactate release. After reconstructive surgery the patients' metabolic response to exercise approximated to the control values, although again the leg arterio-venous oxygen difference remained elevated. It is probable that the changes reported are due to an impaired lactate and proton clearance secondary to the impaired blood supply. This would result in a fall in pH, an alteration in the creatine kinase equilibrium and a secondary fall in PCr and fatigue would then ensue (see chapter 2). Following operation an improved blood supply would restore the efflux of metabolites to normal and an improvment in work rate would follow accordingly in addition to any benefit of an increased aerobic capacity.

An early study on changes in enzyme activity in patients with peripheral vascular disease (Digiesi et al, 1975) reported depressed levels of glycolytic and citric acid cycle enzymes, the latter contrary to the findings of Holm et al (1972) and Falholt et al (1974). The

latter authors looked at enzymes involved in the Embden-Meyerhof pathway, the hexose-monophosphate shunt, the citric acid cycle and lipid metabolism on muscle obtained at operation for reconstructive surgery on eight patients with lower limb arterial disease. Their findings of an increased lipid metabolic activity over controls (based on the ratio of 3-hydroxyacyl-CoA dehydrogenase to glycerol-3-phosphate dehydrogenase) was not in agreement with Hagenfeldt et al's (1972) findings and this 'preference' for fats increased with increasing muscle ischaemia. Citric acid cycle and glycolytic activity were similar to control values but there was an increase in the hexose-monophosphate shunt. Their findings are difficult to explain in terms of economy of energy as this trend was associated with a reciprocal decline in citric acid enzyme activity, but may be a response to injury reaction as seen in ischaemic myocardium (Gudbjarnason et al, 1968) whereby the increase in HMP shunt and lipid activity are a precurser to lipid synthesis, and their finding of elevated intracellular free fatty acids would support this. These results were partly substantiated by Bylund et al (1976) who in an investigation of 39 patients (23 claudicants, 11 with rest pain and 5 with frank ischaemia) and 25 controls examined samples of gastrocnemius, retrieved at operation, for the activity of various mitochondrial and glycolytic enzymes. In agreement with their earlier work they found evidence for an increased oxidative capacity in the claudicant group; cytochrome-c-oxidase (CytCox) was 45%, citrate synthetase (CS) was 56% and 3-OH-acyl-CoA-dehydrogenase (ACDH) was 81% greater than control activity. Activity of CytCox had fallen to 18% greater and 13% less than the reference values in the rest pain group and severely ischaemic group respectively and although CS activity remained elevated, ACDH demonstrated a similar trend. Glycolytic enzyme activty (PFK) was above

normal in the patient group as a whole but depressed in the severely ischaemic patients in whom LDH activity was also low. The reverse was found with the control enzyme of glycogenolysis (glycogen phosphorylase) whose activity was depressed and activity increased with increasing ischaemia. Hexokinase activities showed no change but glucose 6-P dehydrogenase activity (reflecting the HMP shunt activity) was increased. The findings that oxidative capacity is depressed in most ischaemic tissue is to be expected but the finding that β-oxidation of fats and citric acid cycle enzyme activity is increased in these patients with only marginal elevation of glycolytic enzymes and depression of glycogen breakdown is more difficult to explain. Tappan (1957) in his study of guinea pigs living on the top (14,000 m) and bottom (sea level) of the Peruvian Andes has shown that a low oxygen tension induces increased oxidative and glycolytic capacity and by interpretation of their data an increase in mitochondria. This work was confirmed by Holm et al (1973b) with hypoxic rats. The low oxygen tension due to decreased blood flow and an improved oxygen extraction in claudicants may thus induce these adaptive changes. Glycogenolysis will be limited because of the reduced anaerobic capacity but glycolysis may have to increase to 'force' the formation of lactate and regeneration of ATP.

In an attempt to better understand these changes Henriksson et al (1980) and Hammarsten et al (1980) looked at muscle fibre types and capillarisation in 16 and 12 patients respectively with intermittent claudication. Both papers reported no difference in the distribution of ST and FT fibres between the patient and control goups but differed in their findings in the FTa and FTb subgroups. Henriksson found significantly more FTb fibres than the controls whereas Hammarsten found an increase in FTa fibres. They were however in agreement with the

finding that capillary density was most increased in FTa fibres and although this was true to a lesser extent for the other fibre types, it was only a significant change in the former authors' study who also found that SDH activity was significantly less in the patients. The improvement in capillarisation was in both studies a consequence of smaller fibres rather than any change in the total number of capillaries per fibre. Henriksson et al (1980) attributed their findings of a lower oxidative capacity in their patients than previous studies as perhaps being a function of fitness of the control group, but as these were comparable in Bylund et al (1976) and Holm et al's (1972) study, this is unlikely. Similar results were reported by Clyne et al (1982) with no change in the distribution of fibre type, an overall decrease in fibre area and an increased capillary fibre density consequent upon an unchanged capillary/fibre ratio. Their overall conclusions were that these results suggested a detraining or disuse effect rather than an adaptive response. Elander et al (1985) have not substantiated these conclusions. Using isolated mitochondria from 10 male patients and 12 matched controls they demonstrated an increased volume density of both subsarcolemmal and intermyofibral mitochondria, which displayed an oxidative capacity 48-64% greater than the controls. Thus it appears that patients with intermittent claudication do exhibit changes in muscle metabolism similar to those seen with endurance training.

Two recent papers (Christensen et al, 1986; Holdich et al, 1986) have demonstrated a useful application of the increased oxygen extraction seen during exercise in patients with lower limb arterial disease. Both papers show that patients with arterial disease can be separated from those with normal blood flow by monitoring transcutaneous oxygen tension during exercise allowing patients with false claudication

(Tait et al, 1985) to be identified.

3.5.2 The effect of training on intermittent claudication

a) Changes in functional capacity

The beneficial effects of exercise in intermittent claudication were first suggested by Erb in 1898 (cited by Saltin, 1981), but it was not until the late 1960's that this treatment was commonly recommended. Larsen and Lassen (1966) reported the results of a controlled trial of 14 patients with stable claudication treated by daily exercise (walking) and found that the exercise group increased their walking distance threefold while the control group was unchanged. The following year Skinner and Strandness (1967) confirmed this impovement in a group of five patients who underwent a similar training programme and demonstrated an increase in the maximum walking time, the time of onset of claudication pain and a reduction in the post exercise ankle systolic pressure drop. They interpreted this as representing an increase in collateral circulation. Another small controlled study of 13 patients (Ericsson et al 1970) also found that the walking distance increased only in the trained group and this was associated with an increase in peak blood flow following timed tourniquet ischaemia of 46% and this improvement carried over to the symptom-free leg. In a well conducted but uncontrolled trial, Zetterquist (1970) trained 9 male patients with stable claudication for 3-4 months by a combination of interval (short, repeated periods of cycle ergometer exercise) and endurance training (daily walking for at least one hour). He found that although the pain free walking distance increased from 190 to 330 meters, and the total walking distance from 320 to 400 meters there was no measurable change in the plethysmographically measured peak calf blood flow. A further

controlled trial (Dahllöf et al, 1974) in which 10 patients underwent 6 months of training and another uncontrolled trial (Sørlie and Meyer, 1978) of 10 patients undergoing a supervised training programme for 3-4 months confirmed Zetterquist's results. In both studies there was a significant improvement in the walking distance which Sørlie and Meyer additionally quantified as total work done on a bicycle ergometer but no change in peak calf blood flow. More recently Clifford et al (1980) have supported these findings by showing that there was a 75%-80% improvement in exercise tolerance in 18 out of 19 patients who completed a 6 months training schedule. Two patients out of the original 21 deteriorated and underwent arterial reconstruction but there was no failure to respond of those remaining patients even if they continued to smoke.

b) Metabolic changes

In his study, Zetterquist (1970) demonstrated a further reduction in venous oxygen saturation as a result of training but this was not associated with any measurable changes in blood lactate. Dahllöf et al (1974) in their controlled study examined biopsy specimens of gastrocnemius for metabolic analysis as detailed in their earlier paper (Holm et al, 1972, vide supra). They found that training increased succinic oxidase activity and the incorporation rate of U-^{14}C-glucose into glycogen, lipids, lactate and CO_2. Total muscle glycogen content was unchanged but there was an improved insulin sensitivity with training. These results were all changes in the same direction (other than the insulin) as the differences found between patients and controls (Holm et al, 1972) and suggest that the stimulus to the changes previously reported are indeed adaptive changes. Although Zetterquist (1970) found no changes in lactate in his study this was not the case with Sørlie and

Myhre (1978). As well as confirming that training increased O_2 extraction they found that not only was there a lower lactate release during exercise to exhaustion after training but this occurred at a higher work rate. Although it appears that training can increase the oxidative contribution to the provision of energy this does not seem to be true for the glycolytic enzymes (Saltin, 1981) and as blood flow is not improved this may again be a reflection of an impaired lactate and hydrogen ion clearance which is not improved by training. It is a paradoxical finding that there is an apparently greater reliance on FFA metabolism but this would reduce lactate production and allow more complete oxidation of fuels through the citric acid cycle (Newsholme, 1981). This is supported by the finding that the incorporation rate of glucose into lipids and CO_2 is increased. Saltin (1981) has reported a trend for an improvement in capillarisation with training and this may contribute to the widened arterio-venous oxygen difference. Thus the effects of training appear to be a summation of a number of small changes; an improved O_2 extraction with an improved capillarisation, a possible (marginal) improvement in blood flow, a switch from glycolysis to β-oxidation of fats and a concurrent improvement in the muscle oxidative capacity. This together with any increase in the efficiency in the patients' walking technique and a better pain tolerance could certainly account for the improvements in performance that undoubtedly occur in patients with intermittent claudication who undergo supervised training programmes.

3.6 Exercise and atherosclerosis

There is now substantial evidence to indicate that physical activity is protective against atherosclerosis and in particular

coronary artery disease. Morris et al (1953) first drew attention to this in their study on bus drivers and bus conductors and sedentary and active postal workers. Since then numerous other retrospective and prospective studies have confirmed this (see Schneider et al, 1986 for references). A very high level of physical fitness does however not prevent the disease, as evident from the finding that 50% of marathon runners who died suddenly had coronary atherosclerosis (Noakes and Opie, 1979) and Walker and Robert (1980) found that five runners who died running all had severe heart disease. Paffenbarger et al (1978) have shown that the relative risk of a fatal first heart attack is reduced by 50% irrespective of other risk factors, smoking included, with a weekly recreational physical energy expenditure of more than 2000 kcal. This decreased risk of myocardial infarction depended on the level of activity in adulthood and was not dependent on activity as a student. Schneider et al (1986) provide evidence to show that a number of atherosclerotic risk factors are potentially improved by exercise and these are; diabetes and glucose intolerance, hyperinsulinaemia, hyperlipidaemia, coagulation abnormalities, hypertension, hormonal abnormalities, and obesity. Despite the obvious potential benefits of regular exercise many people are put off the idea because of the fear of sudden death. This risk is very small and Thomson et al (1982) has estimated it to be one death per 396,000 man hours of jogging, equivalent to one death per year for every 7260 joggers. The commonest associated finding with sudden death was co-existing coronary atherosclerosis and Siscovick et al (1984) showed that the risk of cardiac arrest during exercise was 5-fold in those with the highest level of habitual activity and 56-fold in those who were habitually inactive (<20 min exercise per week). An increased risk of sudden death is not invariable and most studies have shown that training reduces the chance of a fatal heart attack during

exercise when compared with the incidence in the general population (Schneider et al, 1986). Excessive endurance exercise is however not neccessarily beneficial and may lead to hypertrophic cardiomyopathy (Maron et al, 1986) and amenorrhoea and demineralisation of bone (Prior, 1987). Similarly, very high intensity exercise (as in squash) may be associated with increased lipid peroxidation and production of free radicals with consequent detrimental effects (Viinikka et al, 1984). As a counsel of excellence all persons should indulge in regular moderate exercise from an early age as this will reduce the small extra risk of commencing an exercise programme with established heart disease and continue their level of activity into old age. If this policy were adopted the incidence of atherosclerotic disease would be substantially reduced over and above the beneficial effects of dietary changes and abolition of tobacco products.

3.7 Areas in which drugs may help in peripheral arterial disease

Any approach to drug therapy for lower limb ischaemia must take into account the natural history of the disease, making controlled trials of assessment essential. It is hardly surprising that in such a common disease with a wide variety of aetiological and pathophysiological variables, a multitude of drugs with varying actions have been used to try and alter or improve these factors. Figure 6 outlines the areas in which they might help and table 3.3, although by no means complete, lists most of the drugs advocated or advertised for the treatment of atherosclerosis in the U.K.

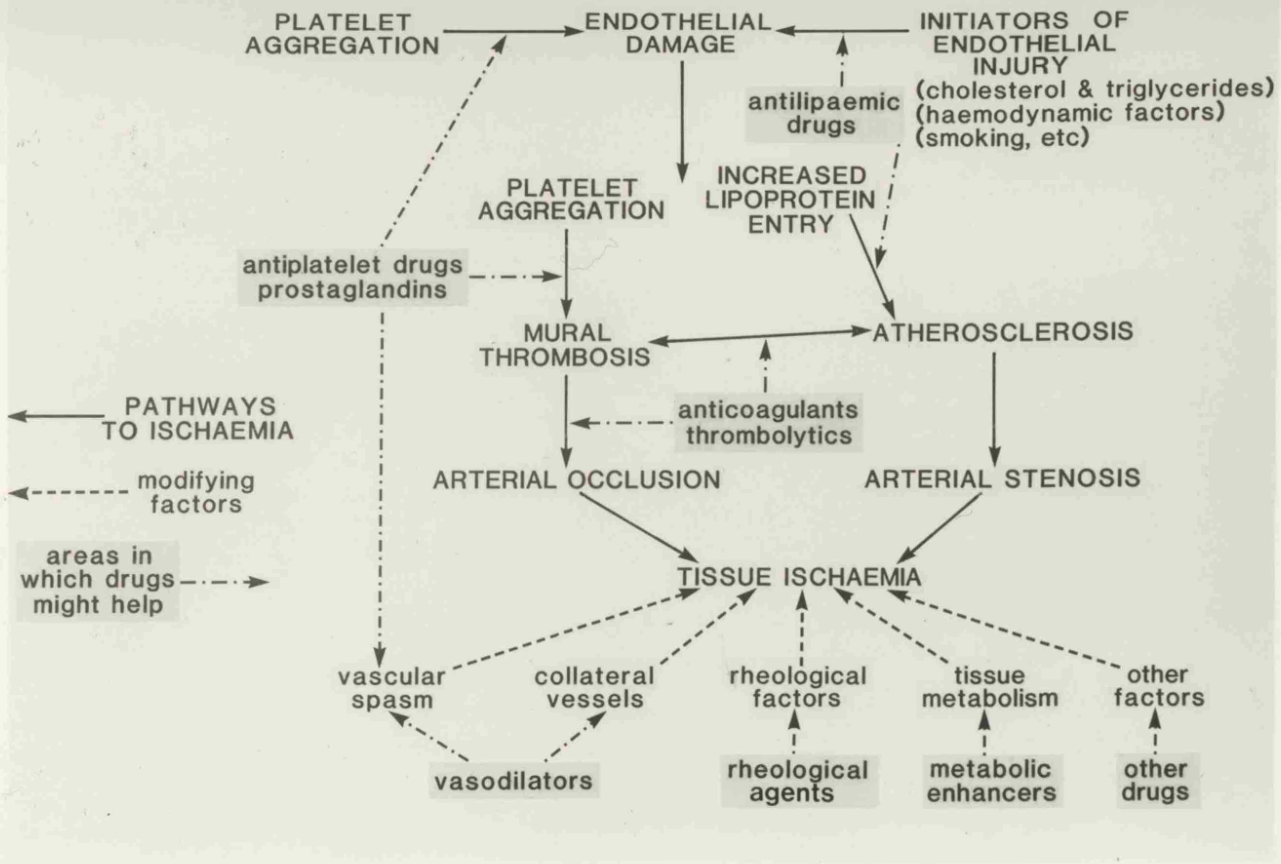


Figure 6 Areas in which drugs might help lower limb ischaemia. A scheme for the development of atherosclerotic ischaemia is shown together with factors that may modify the eventual outcome. Points at which drugs may help by either altering the pathoglogical pathways or by acting on these modifying factors are illustrated.

Table 3.3 Drugs used in peripheral vascular disease

Mode of action	Drug	Pharmacology	Other important information and action
Antilipaemic drug	Cholestipol	Binds intestinal bile acids; enhances bile acid sythesis of hepatic LDL receptors	Used in type IIa and IIb hyperlipo-proteinaemia
	Cholestyramine	As cholestipol	Type IIa and IIb
	Clofibrate	Enhances LDL activity; decreases plasma FFA; inhibits hepatic VLDL synthesis	Type III; increased rate of malignancy and gallstones
	Probucol	Inhibits hepatic cholesterol synthesis; enhances synthesis and excretion of bile acids	Type IIa and IIb
	Dextrothyroxine	Thyroid hormone analogue	Type IIa and IIb
	Nicotinic acid	Decreases hepatic cholesterol synthesis; decreases plasma FFA; enhances lipo-protein lipase activity	Type IIa, IIb, III, IV, V, and ?I
Antiplatelet drugs	Aspirin	Cyclo-oxygenase inhibitor	Selective platelet enzyme inhibition at low dose
	Dipyridamole	Phosphodiesterase inhibitor	Also direct acting vasodilator and antilipaemic
	Ketanserin	Inhibits serotonin induced platelet aggregation	Also reduces whole blood viscosity
	Sulphinpyrazone	Cyclo-oxygenase inhibitor	
	Ticlopidine	Inhibits fibrinogen and Von Willebrand factor binding to platelet glycoprotiens	May also stimulate adenyl cyclase
Prostaglandins	Prostaglandin E_1 (PGE_1)	Potent direct acting vasodilatory and antiplatelet effects	Destroyed on first pass through pulmonary circulation
	Prostacyclin	Similar to PGE_1	Naturally occurring endothelial hormone

Table 3.3 (continued)

Mode of action	Drug	Pharmacology	Other important information and action
Anticoagulants and thrombolytics	Nicoumalone		
	Phenindione	Vitamin K inhibitors	
	Warfarin		
	Streptokinase	Fibrinogen activator	Antigenic ++
Vasodilators	Bamethan	β-adrenergic agonist	Acts on muscle vessels only
	Cinnarizine	Direct smooth muscle relaxant; blocks calcium uptake into cells	Acts on skin and muscle; lowers blood viscosity, improves RBC deformability
	Cyclandelate	Papaverine-like smooth muscle relaxant	Acts on skin and muscle vessels; claimed to induce collateral
	Isoxsuprine	β-adrenergic agonist	Acts on muscle vessels only
	Nicotinic acid	Direct action on skin vessels	Also fibrinolytic (includes nicofuranose, xanthinol nicotinate, inositol nicotinate, nicotinyl alcohol)
	Phenoxybenzamine	α-adrenergic blocker	Acts on skin vessels only
	Thymoxamine	α-adrenergic blocker	Acts on skin vessels only
	Depropranex	Vasoactive pancreatic extract	Direct acting smooth muscle relaxant
Rheological agents	Dextrans	Haemodilutant	Also inhibit red cell aggregation
	Anabolic steroids	Fibrinolytic	Includes testosterone and stanozolol
	Ancrod	Potent fibrinolytic	Potent antigen, also anticoagulant
Metabolic Enhancers	Glucose-insulin	Direct effect on cellular metabolism	Decreases FFA metabolism and increases glycolysis
	Oxpentifylline	Claimed to improve red cell deformability by increasing ATP and cAMP	Also induces prostacyclin synthesis
	Naftidrofuryl	Claimed to improve tissue oxidative capacity in the ischaemic state	Also papaverine-like smooth muscle relaxant and local anaethetic properties
Other drugs	Mineralocorticoids	Induce sodium retention and hypertension	Given with excess sodium chloride

LDL=low density lipoproteins; FFA=free fatty acids; VLDL=very low density liproteins; I, IIa, IIb, III, IV, V refer to types of hyperlipoproteinaemia; ATP=adenosine triphosphate; cAMP=cyclic AMP (adenosine monophosphate)

3.7.1 Antilipaemic drugs

Of the many aetiological factors and initiators of endothelial damage, cholesterol and triglycerides are the only ones which can be directly influenced by drug therapy (as well as by diet and exercise). Patients with lower limb arterial disease have been found to have elevated blood cholesterol and triglyceride levels (Juergens et al, 1960; Schroll and Munck, 1981), but despite this the use of low fat diets and antilipaemic drugs has proved disappointing in the treatment of occlusive arterial disease (Kramsch et al, 1981), although epidemiological evidence has demonstrated that a diet induced reduction in cholesterol reduces the incidence of coronary heart disease (Hjermann et al, 1981; Lipid research clinics, 1984). This can in part be explained by the fact that, in patients with uncomplicated atherosclerosis, their moderate elevation of plasma lipids is only one of many risk factors involved in the development of their disease. Patients with proven hyperlipoproteinaemias, however have their lipid abnormality as the primary aetiological factor and animal experiments have shown that reversal of induced hyperlipidaemias can produce regression of the early atheromatous lesions produced (Constantinides, 1981). Can this be said of hyperlipidaemia associated atherosclerosis in man? Malinow (1981) reviewed the available literature on regression of human atherosclerosis and found 32 cases of regression reported by serial arteriography, 15 cases occurring in patients with femoral artery disease. Treatment was by a variety of methods; many of the studies were anecdotal and it is likely that a number of negative observations were unreported. Assessment of regression in man, however, is handicapped by several problems; we cannot examine the arteries histologically and chemically, we cannot subject patients to repeated exposure of their

vessels and examine them directly, and repeated angiography requires particular attention to technique to ensure that no variability of procedure is introduced. In addition we may mistake thrombi or even arterial spasm (Lörelius, 1980) for atheromatous lesions. Despite these problems, Duffield et al (1982) reported a controlled trial of plasma lipid reduction in peripheral atherosclerosis due to hyperlipoproteinaemia in which 24 patients with types IIa, IIb, III and IV hyperlipidaemia were equally divided into treatment and non-treatment groups. Therapy consisted of cholestyramine (with or without nicotinic acid) with clofibrate for type III patients. Regression or progression of atheromatous disease was assessed by serial angiography after an interval of 15-24 months. Although considerable reduction in plasma cholesterol and particularly pro-atherogenic LDL was obtained, the authors were unable to demonstrate regression of atheromatous plaques and the disease progressed in both groups, although less quickly in the treated group. This concurs well with Constantinides' (1981) view that early lesions (fatty streaks and myocyte proliferation) are capable of regression, but when the disease reaches the stage of clinical presentation regression will not occur, irrespective of treatment, and the best we can achieve is the prevention of progression of the disease and its more serious complications. Antilipaemic drugs, therefore, have no part to play in the treatment of atherosclerosis per se and should be reserved for patients with proven hyperlipidaemias when ideally therapy should be instituted before established atheromatous changes occur.

LaRosa (1982) has recently reviewed the mode of action of the lipid-lowering drugs and their indications and pharmacology are reviewed in table 3.3. Three new drugs have recently been introduced: compactin and mevinolin are competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase, the rate limiting enzyme of cholesterol synthesis

and both reduce LDL levels without influencing antiarthrogenic HDL levels. Gemfibrozil is related to clofibrate and has been shown in a recent study of 34 patients with type II and type IV hyperlipidaemias comparing the two drugs (Nash, 1982), to be as effective as clofibrate in lowering cholesterol and LDL levels but more effective in reducing triglyceride and increasing HDL concentrations.

3.7.2 Antiplatelet drugs and prostaglandins

The infectious enthusiasm with which authors such as Gryglewski (1980) have presented their hypothesis on the thromboxane/prostacyclin theory of the pathogenesis of atherosclerosis is reflected in the current wave of publications on this aspect of treatment of the disease. The aim of prostaglandin and antiplatelet therapy is to selectively inhibit thromboxane A_2 production or to increase endogenous prostacyclin (or administer exogenous prostacyclin or its analogue) to alter effectively the balance of power in favour of the vasodilatory anti-aggregatory prostacyclin, thus preventing platelet aggregation, endothelial damage and the development of atheroma. An outline of the platelet vessel wall interaction and the site of action of the anti-platelet drugs is illustrated in figure 7.

a) Antiplatelet drugs

A number of drugs have been shown to possess antiplatelet properties (Versraete and Kienast, 1986) but only three have been used to any extent as platelet suppressive therapy: aspirin, dipyridamole and sulfinpyrazone, although ketanserin has recently been added to this list (Bounameaux et al, 1985). These drugs have been the subject of considerable investigation with regard to their value in the secondary

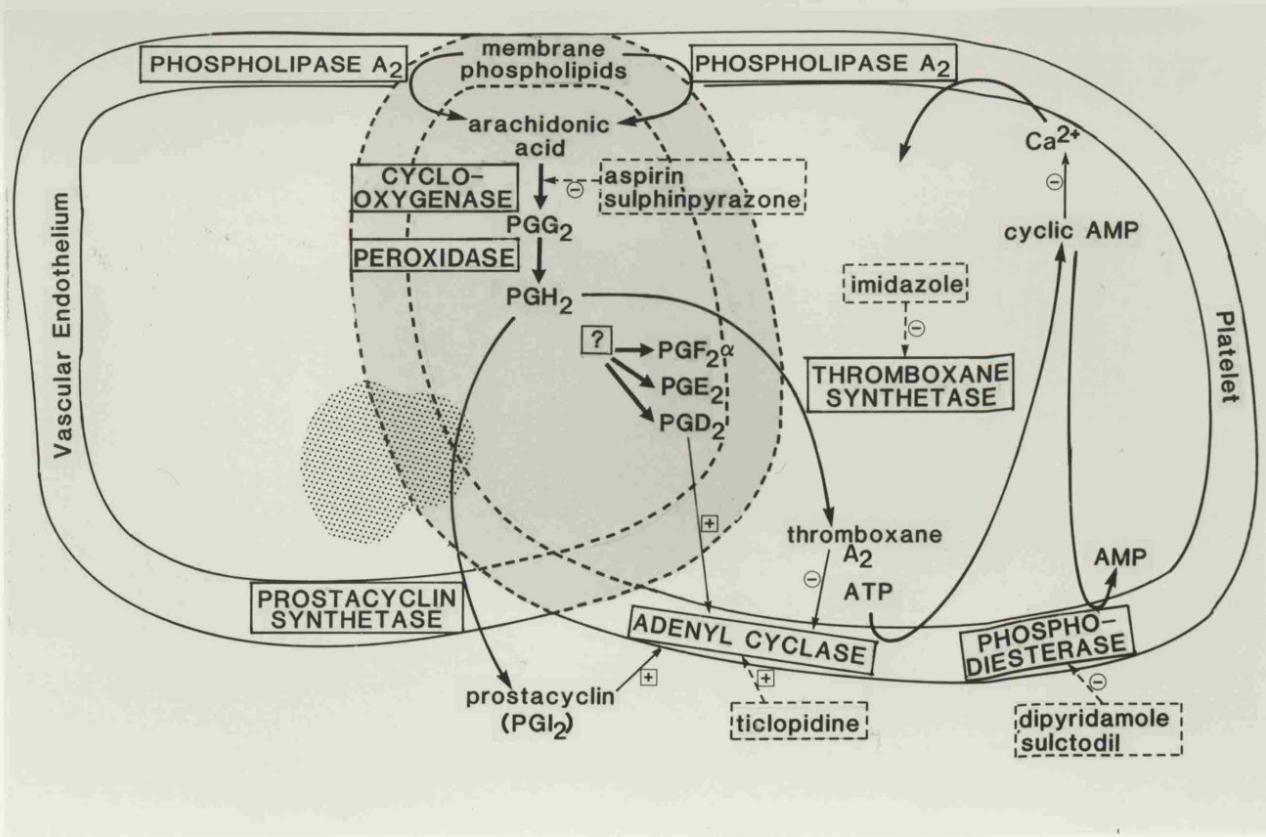


Figure 7 The platelet vessel wall prostaglandin inter-action and a scheme of thromboxane A_2 and prostacyclin (PGI_2) synthesis. Arachidonic acid is released from membrane phospholipids by phospholipase A_2 (activated by Ca^{2+} ions in the platelets). PGI_2 is produced only by vessel wall prostacyclin synthetase. Thromboxane A_2 is produced only by platelet thromboxane synthetase. Cyclic AMP inhibits the availablity of Ca^{2+} for platelet phospholipase A_2. Cyclic AMP production is positively regulated by PGI_2 and the intermediate prostaglandin PGD_2 and negatively by thromboxane A_2. The site of action of the antiplatelet drugs is indicated; platlelet cyclo-oxygenase is much more sensitive to inhibition by aspirin than the endothelial enzyme.

prevention of myocardial infarction, preventing coronary artery bypass graft occlusion and in the treatment of patients with stroke in evolution and thrombotic stroke. The results of the trials in this aspect of their use were presented at the Workshop on platelet-active drugs in the secondary prevention of cardiovascular events at the National Institute of Health, Bethesda, Maryland, 1980 (Proceedings of the workshop on platelet-active drugs in the secondary prevention of cardiovascular events, 1980). The post-myocardial infarction trials discussed were: Elwood et al; Coronary Drug Project Aspirin Study (CDPA); German-Austrian Multicentre Prospective Clinical Trial; Elwood and Wiliams; Aspirin Myocardial Infarction Study (AMIS); Elwood and Sweetnam; Persantine Aspirin Reinfarction Study (PARIS I); Anturane Reinfarction Trail (ART), and those in the treatment of transient ischaemic attacks (TIA), Aspirin in Transient Ischaemic Attacks (AITIA) and Recent Recurrent Presumed Cerebral Emboli (RRPCE). Genton (1980) in summarising the results of these trials, reached these conclusions: "Aspirin in the cerebrovascular trials dramatically reduced the incidence of death from stroke, especially in male patients, but there was no evidence of benefit from sulfinpyrazone. In contrast, aspirin in coronary artery studies showed a trend, and in two studies significantly reduced the incidence of non-fatal infarction but it consistently reduced mortality rates. Dipyridamole used in combination with aspirin did not produce conclusive differences. Sulfinpyrazone, when begun within one month after infarction, was reported to decrease mortality sharply, especially from sudden death and, to a lesser degree, to reduce the incidence of non-fatal recurrent infarction." May et al (1982) were much less enthusiastic. They felt that these trials had been unable to demonstrate any significant difference between intervention and control groups for total mortality and in these large trials involving a total

of 13,000 patients, no definite benefit of platelet active drugs had been shown. More recently these numerous trials have been extensively reviewed by Gallus (1986). After considering the additional data of the PARIS II, the Anturane Reinfarction Italian Study (ARIS) and the EPSIM research group findings he felt that sulphinpyrazone on its own conferred doubtful benefit, and when dipyridamole was used in combination with aspirin, the benefit was primarily due to the aspirin component. He concludes that the low cost, relative safety and trend towards a reduced mortality with aspirin alone justify its use. With regard to TIA's the most recent large controlled trial [American-Canadian Co-operative Study Group (1985)] has been unable to demonstrate any additional benefit of dipyridamole when used in combination with aspirin over aspirin alone. Following completed thrombotic stroke aspirin with or without dipyridamole has been shown to be of benefit (Bousser et al, 1983) as has fluribiprofen (another non-steroidal anti-inflammatory drug) but the newer antiplatelet agent, suloctodil, has not been demonstrated to be of use (Gent et al, 1985). The reduction in ischaemic complications by the treatment with aspirin of patients with symptomatic carotid artery disease must also be interpreted with some caution. Carson et al (1981) demonstrated that rapid progression of carotid artery atherosclerosis occurred in 27 patients receiving aspirin or aspirin and dipyridamole for 6-24 months prior to their urgent carotid endarterectomies. All these patients had initial resolution of their ischaemic attacks with antiplatelet therapy, but despite this their disease progressed to a greater than 90% stenosis in each case.

In all these trials with aspirin, the dose used has been between 300 and 1500 mg per day. Hanley et al (1982) have recently reported that the optimum dosage of aspirin appears to be 40 mg on alternate

days, this level producing suppression of the platelet thromboxane A_2 with minimal disturbance to the vessel wall prostacyclin. This finding of an optimum dose far less than used in published trials may partly explain the variability of results and trials using this 'ultra-low' dosage could be very enlightening.

The results of antiplatelet drugs in the prevention of coronary artery bypass graft obstruction are also contradictory; McEnamy et al (1976) studied 412 grafts in 216 patients, 220 grafts in 111 patients being followed-up from 6-40 months and found graft patency rates of 80% in the aspirin-treated group against 72% in the placebo group. Warfarin was given to a third group who exhibited patency of 82%. In 1978, Pantley et al (1978) reported the results of a smaller group of 50 patients who received no medication (24 patients) aspirin 975 mg/day and dipyridamole 325 mg/day, or warfarin (13 patients each), medication being commenced on the third post-operative day, and no difference in graft patency at 6 months was found (82%, 80% and 79% respectively). Sharma et al (1982) confirmed the failure of aspirin alone or in combination with dipyridamole to improve graft patency. Antiplatelet therapy was received by 98 patients with 42 no treatment controls and the overall patency was the same for the three groups studied: 83% for aspirin (975 mg/day) and dipyridamole (225 mg/day), 78% for aspirin alone (975 mg/day) and 80% for the controls, medication being again administered 3-5 days after surgery. However, Chesebro et al (1982) in the large Mayo Clinic double blind placebo-controlled prospective study of dipyridamole plus aspirin, did show a significant improvement in graft patency in the treatment group. A total of 407 patients receiving 1008 distal grafts were randomised into active drug or placebo groups and received dipyridamole 400 mg/day for 48 hours pre-operatively, dipyridamole 100 mg on the morning prior to surgery and 1 hour post-

operatively, followed by dipyridamole 75 mg and aspirin 325 mg 7 hours postoperatively and three times daily thereafter. At one month the overall graft patency was 97% for the treatment group and 90% for the control group, and at 6 months, 96% versus 85%, the difference being highly significant. The authors conclude that the reason for the failure of previous trials to confer benefit was institution of therapy in the postoperative period, after the thrombotic process had already begun. Since then other controlled studies have demonstrated similar benefits, including one with low dose aspirin [100 mg/day (Lorentz et al, 1984)] provided therapy was started before surgery, but no trial of antiplatelet therapy started more than 48 hours after operation has shown any benefit (see Gallus, 1986 for references).

In contrast, there is very much less published data on the use of antiplatelet drugs in patients with peripheral vascular disease, and there has been no primary prevention trial and only a few trials to prevent vessel occlusion in patients with symptomatic disease. Hess et al (1985) recently reported a double-blind, placebo-controlled, randomised trial in 199 patients with symptomatic peripheral vascular disease. During the two year period of study they measured the progression of atheroma by serial arteriography. They showed that the combination of aspirin (990 mg/day) and dipyridamole (225 mg/day) was better than aspirin alone (900 mg/day), both of which were significantly better than placebo in slowing the progression of the disease. Interestingly, both regimes were more effective in the presence of additional risk factors (smoking and hypertension) and less so when these were absent. Antiplatelet drugs have been more carefully evaluated in their use in improving the outcome after arterial reconstructive surgery. As there is excellent long term patency of

large vessel surgery regardless of drug therapy most trials have concentrated on patients with reconstruction below the inguinal ligament. Bollinger et al (1981) conducted a prospective study of 120 patients undergoing femoral-popliteal endarterectomy treated with aspirin 1 g/day, aspirin 1 g/day and dipyridamole 225 mg/day or coumadine. The study was not double blind and there were no controls, but aspirin alone or in combination appeared to improve the 2 year patency rate, 80% against 56% for coumadine. Three subsequent controlled trials involving 237 patients (Green et al, 1982; Kohler et al, 1984; Satiani, 1985) of whom 61 received aspirin 975 mg or 650 mg/day, 60 received aspirin 975 mg plus dipyridamole 225 mg/day and 116 were placebo controls have not been able to demonstrate any improvement in graft patency although Green et al (1982) found that a sub-group with above knee anastamoses faired significantly better than placebo. These findings are despite McCollum's (1981) study which demonstrated a reduced graft thrombogenicity in an acute randomised double-blind trial of 40 patients undergoing femoro-popliteal bypass grafting. Using indium labelled platelets they looked at the effects of aspirin 300 mg/day and dipyridamole 225 mg/day or placebo on graft thrombogenicity. At 1 week they were able to demonstrate a decreased platelet accumulation in the non-sapheous group, this being most marked in PTFE grafts, although no change was detectable in the very low native thrombogenicity of autologous vein grafts. In a further double-blind controlled trial, Goldman et al (1983) did report a significant improvement in graft patency in the 27 patients who received aspirin 900 mg/day and dipyridamole 225 mg/day. The effects of antiplatelet drugs on graft patency are superficially promising and further research in those patients who are considered to be at high risk of graft occlusion is warranted.

Smith and Warren (1981) studied the effects of 2 weeks treatment of dipyridamole 300 mg/day, nicotinic acid 150 mg/day or placebo administered in a randomised double-blind manner in six patients with intermittent claudication, and found that dipyridamole significantly improved exercise blood flow measured by radio-xenon clearance. In the latter part of the study the dipyridamole was given openly for an additional 2 months, resulting in a further apparent improvement in blood flow, but despite this no improvement in walking distance was reported. It is difficult to draw conclusions from such a small study and the authors do not discuss the likely mode of action of the drug, although it is very unlikely that any antiplatelet effects could manifest themselves in such a short period of time.

Three newer antiplatelet drugs, ticlopidine, suloctodil and ketanserin, have recently been reported as treatment for peripheral vascular disease. Ticlopidine, a potent inhibitor of platelet aggregation, is thought to potentiate endogenous prostacyclin by an effect on platelet adenyl cyclase. Hurlow et al (1981) in a randomised double-blind study of 54 male patients with intermittent claudication, treated with ticlopidine or placebo for a 6 month period, were able to demonstrate an improvement in peak blood flow but not walking distance in those patients under 60 years receiving the active drug, but Hamilton et al (1981a) in a similar trial of 23 patients were unable to confirm its beneficial effects. In a 6-week double-blind study of ticlopidine against placebo in 193 patients with ischaemic ulceration of the lower extremities, Katsumura et al (1982) showed that patients receiving the active drug exhibited greater healing of their ulcers, although the degree of pain was unaltered. However, the lack of objective measurements in the assessment of the patients casts doubts on the validity of

the results.

Suloctodil's antiplatelet effect is thought to be by inhibition of platelet phosphodiesterase and it possesses an additional vasodilatory property. Although there is very little clinical information on this drug two placebo-controlled trials in claudicants have been reported. Verhaeghe et al (1981) in a double-blind trial of 45 patients with a 3-month placebo run-in followed by 6 months' therapy with placebo or suloctodil, reported that an improvement in walking distance occurred paradoxically in the placebo-treated group. Removing patients whose walking distance was limited by factors other than claudication reversed these findings but changes in limb flow and pressures were consistently similar in both groups. Jones et al (1982) in a similar trial without a placebo run-in studied 40 patients, of whom 31 completed 6 months' therapy. Although they found an increased walking distance in both groups, this was only significant in the suloctodil group; 166 ± 37 m before and 207 ± 45 m at the end of the trial for placebo and 178 ± 18 m and 270 ± 52 m for the active drug. These differences are not in fact significant between the two groups, and like the Belgian study they found that limb blood flow and pressure changes were similar. As the drug was withdrawn from the market in 1985 because it was found to induce hepatitis, in some cases fatally (Laporte and Capella, 1986), further evaluation of the drug is no longer relevant.

Ketanserin is a serotonin inhibitor and although serotonin itself is only a weak stimulator of platelet aggregation it can act as an amplifier of aggregation induced by other agonists (Verstraete and Kienast, 1986). This drug has been shown to dramatically improve walking distance and haemodynamics in patients with intermittent claudication (De Cree et al, 1984), but an attempt to confirm this work by the original Belgian researchers (The Centre for Thrombosis and Vascular

Research, University of Leuven) and the vascular unit of St James' and St George's Hospitals, London produced some surprising results (Bounameaux et al, 1985). Eighteen patients received the active drug and 19 a placebo which they continued for 4 months after 1 month (or longer if required) placebo run-in period. All patients had a thigh/brachial systolic pressure index of less than 0.75 and after the 4 month treatment period, all patients took placebo for a further 2 months. During treatment with ketanserin, clear-cut inhibition of serotonin induced platelet aggregation was observed but despite this no changes in pain-free walking distance, total walking distance or any measured haemodynamic parameter was recorded. In contrast patients receiving placebo had increases in both pain-free and total walking distance. These results demonstrate not only the probable ineffectiveness of this new drug in peripheral vascular disease but also the importance of placebo controlled trials when assessing such products.

b) Prostaglandins

Are the results of treatment of peripheral vascular disease by the administration of prostaglandins, in particular the deficient 'hormone' prostacyclin more encouraging? As these substances are pharmacologically relatively unstable, they are suitable only for parenteral administration which confines their use to the acute treatment of end-stage peripheral vascular disease; rest-pain, ischaemic ulcers and pre-gangrene. Prostaglandin E_1 (PGE_1) was found to be a potent inhibitor of platelet aggregation (although 40 times less active than prostacyclin) and a powerful vasodilator. It is hardly surprising, therefore, that in view of the importance of prostaglandins in the platelet/vessel wall interaction, interest was expressed in PGE_1 as a therapeutic agent. The

initial reports of the drug's effectiveness were anecdotal and not uniformly encouraging, and as it is largely destroyed in its first pass through the lungs, it was initially given intra-arterially.

In 1973 Carlson and Eriksson (1973) first reported from the Karolinska Institute, Stockholm, the successful use of PGE_1 in the treatment of severe peripheral vascular disease in four patients and, in 1976, the same group (Carlsson and Olsson, 1976) having observed that during intra-arterial administration symptomatic relief was often observed in the contralateral leg, reported clinical improvement in six out of eight patients who had received PGE_1 by intravenous infusion at a dose of 2-4 μg/h for 3 days. No side effects were encountered and, in some patients, improvement was observed for up to 17 weeks. Nielsen et al (1976) reported a further uncontrolled study on the physiological changes of the intra-arterial administration of PGE_1 in six patients with occlusive arterial disease and three normal subjects. They found that, in keeping with other systemic vasodilators, PGE_1 decreased digital blood pressure and increased blood flow only proximal to an arterial occlusion, whereas in a normal limb blood flow increased uniformly. A further uncontrolled study of 100 patients with end-stage disease was reported in 1982 from West Germany (Gruss et al, 1982) in which PGE_1 was administered by continuous intra-arterial infusion over a period of 2-112 days at a dose of 0.1-0.2 ng/kg/min. There was little objective assessment of the patients recorded, and only 47 patients avoided major amputation, of whom an unstated number required more local debridement. Out of 29 diabetics in the study 22 required amputation and there were a number of complications associated with the long term arterial infusions. As the authors reported that only two limbs required subsequent amputation over a 6-month follow-up period following hospital discharge, they concluded that the results were long lasting.

However, these conclusions are not justified as the group who did not require initial amputation were 'self-selected' to form a less severely affected sub-group. In addition, they were maintained on antiplatelet therapy during PGE_1 treatment and after discharge (dipyridamole, aspirin 60 mg every 72 hours, and warfarin when possible). In a double-blind controlled trial of PGE_1 for severe lower limb ischaemia (14 patients and 16 controls) administered by the intravenous route at a dose of 10 ng/kg/min for 56 hours, Telles et al (1984) failed to demonstrate any benefit, 50% of the PGE_1 patients and 31% of the placebo patients undergoing amputation within 4 weeks of the study. Half of the patients were diabetic but as a sub-group there was no difference between them and the non-diabetics. More recently (Hirai and Nakayama, 1986) attention has been once more drawn to the mode of administration of PGE_1. In the study PGE_1 was administered to 12 patients with ischaemic ulceration or rest pain by both intravenous and intra-arterial routes and although the authors state that ankle/brachial pressure index was recorded this data is not given. They found that haemodynamic improvement was far greater with intra-arterial administration and that in three patients who received three 7-day courses of intravenous PGE_1 (dose 1-6 ng/kg/min) without benefit, ulcer healing occurred with intra-arterial PGE_1 (dose 0.1-0.3 ng/kg/min) which resulted in improvement in a total of 10 out of the 12 patients. However as the duration of intra-arterial therapy was 17-75 days it is apparent that the treatments were not comparable and the clinical outcome may not have been due entirely to the acute haemodynamic changes reported.

The availability of commercially produced prostacyclin (PGI_2), the metabolism of which is suppressed in atherosclerosis (Gryglewski, 1980; Moncanda and Higgs, 1986), allowed a more rational approach to treatment

by administration of the 'absent hormone'. Initial reports of treatment of advanced atherosclerosis with PGI_2 were again uncontrolled but encouraging. In 1979, Szczeklik et al (1979) published the first of such reports; five patients with chronic ischaemic ulcers of the feet were treated with intra-arterial PGI_2 by continuous infusion over 72 hours at a dose of 2-10 ng/kg/min. They found symptomatic and objective improvement in all patients, with complete ulcer healing in three and there was an associated increase in hyperaemic calf blood flow which persisted for up to 6 weeks after discharge. At the International Vascular Symposium, London, 1981, three further uncontrolled trials were presented. Negus et al (1981) treated 23 patients, of whom 20 were able to complete 72 hours of intravenous therapy, and symptomatic relief with improvement in pedal blood flow occurred in 16. However after 18 months follow-up only five patients had avoided amputation. Hamilton et al (1981b) reported the results of treating 13 patients by the intravenous route (5-10 ng/kg/min) for 72 hours. The two claudicants experienced no symptomatic improvement, although the 11 patients with lower limb ischaemia reported symptomatic relief during therapy but their symptoms largely reccurred after 7 days. Side-effects were common; facial and generalised flushing, headaches, and gastrointestinal disturbances, and after 12 months only three patients (out of 10 with rest pain) had not undergone reconstructive surgery or amputation. Another small open trial by Sprignall et al (1981) in which intravenous PGI_2 was given to eight patients (two diabetic) with a mean ankle/brachial systolic pressure index of 0.09 in the six non-diabetics, did not demonstrate any benefit. Belch et al (1983) reported the first double-blind, placebo-controlled trial of intravenous PGI_2 in 28 patients with ischaemic rest pain; 15 patients received PGI_2 2.5-10 ng/kg/min for 96 hours and 13 patients received placebo (the glycine buffer, pH 10). Although Doppler

ankle systolic pressures were measured, the mean value of 50 mm Hg suggests that the patients did not fall into the group of severe critical ischaemia, and the absence of any early operative intervention in either placebo or treatment group tends to confirm this. However, all patients receiving PGI_2 reported improvement in their symptoms and this was maintained in nine at 1 month and seven at 6 months. This compares with initial symptomatic improvement of only three in the control group which lasted 6 months in only one patient. Side effects were considerably less than Hamilton et al's (1981b) study and as the authors were unable to demonstrate any significant alteration in platelet function between the two groups, they suggested that the therapeutic effect of prostacyclin may have been vasodilatory. The most recent study of prostacyclin in severe peripheral ischaemia has, like the later studies on PGE_1 failed to confirm its early promise. Cronenwett et al (1986) in a well conducted placebo-controlled double-blind study of 27 patients with rest pain or ischaemic ulcers, of whom 13 received placebo, were unable to show any beneficial effect with the active drug. Eight patients in each group were diabetics, and the mean ankle/brachial and toe/brachial pressure index was 0.44 and 0.10 respectively. Prostacyclin (or the glycine buffer) was administered intravenously at a dose of 6 ng/kg/min for 72 hours and 54% of the patients receiving placebo and 31% the active drug were considered to be therapeutic successes.

Obviously we still require further well-controlled trials on the use of prostacyclin (and its synthetic analogues when these inevitably become available) although a response to treatment pattern does seem to be emerging; patients with severe or critical ischaemia (Bell et al, 1982) do badly, whereas those with less severe disease may exhibit long-

lasting benefit. It may be a long time before we accept, as Gryglewski (1980) suggested: "In advanced atherosclerosis, prostacyclin analogues may be considered to supplant the absent hormone and may be used as insulin is used in the treatment of diabetes".

3.7.3 Anticoagulants

It is hardly surprising, in view of the importance of mural thrombosis as a final insult to the patency of an artery diseased by atheroslerosis and the possible contribution of thrombi to the development of atherosclerotic plaques, that anticoagulant therapy has long been prescribed for atherosclerotic disease.

The only long-term anticoagulants used with any frequency have been the vitamin K antagonists, the coumarins. These drugs have had a chequered history in their use, both as short-term anticoagulants in acute myocardial infarction and as long-term treatment for secondary prophylaxis, and the 'trials and tribulations' of their use have been well reviewed by Mitchell (1981) and Gallus (1986). These reviews support the use of short term anticoagulants when there is a high risk of venous or arterial thromboembolism. With regard to long term anticoagulation, from the best controlled study to date (Sixty Plus Reinfarction Group, 1980), it would appear that oral anticoagulants do reduce the incidence of recurrent myocardial infarction with a small increase in haemorrhagic strokes, which is more than outweighed by a decrease in mortality from thrombotic strokes. Mitchell (1981), however, felt that the evidence to date failed to show that anticoagulants conferred any benefit in decreasing mortality after acute myocardial infarction.

The results of the use of anticoagulants as prophylaxis against postoperative occlusion following coronary artery bypass surgery are, as has been noted, inconclusive. McEnany et al (1976), in a randomised study of 320 grafts in 111 patients, found a graft patency rate of 84% in warfarin treated patients, against 72% for placebo, whereas Pantely et al (1978), in a similar study of 131 grafts in 50 patients, found no difference between the groups. Two subsequent randomised trials involving 630 grafts (Gohlke et al, 1981; McEnany et al, 1982) have demonstrated a 40% reduction in the graft occlusion rate when compared with placebo but the addition of aspirin and dipyridamole does not appear to further improve patency (Brooks et al, 1985).

Despite the possible benefit of anticoagulants to patients with coronary atherosclerosis, there is scant evidence to support their use in peripheral disease. Tillgren (1965) reported the results of a Swedish trial in which 202 patients received long term anticoagulants (more than 3 months) with 111 controls. The trial was neither double blind nor placebo-controlled and the control group were from a different hospital population. Despite this there were fewer thrombotic occlusions in the treatment group, although the propogation of existing clot did not seem to be affected, the information being obtained by serial arteriography. Additionally, there was no significant difference in the incidence of myocardial infarction or cerebrovascular incidents between the two groups. Richard and Begg (1967) confirmed Tillgren's findings in a similar study of 70 patients but De Smit et al (1981) in a report of two studies, a prospective randomised trial of 220 patients and a placebo-controlled, double-blind trial of 160 patients, were unable to demonstrate any beneficial effects of anticoagulant therapy. A recent Austrian study (Kretschmer et al, 1986) has reported promising results in the use of coumarine in improving saphenous vein graft

patency after bypass for femoropopliteal occlusive disease. In a random, controlled trial, 34 patients received coumarine and 37 acted as controls. Graft patency was significantly improved at 12 and 18 months in the anticoagulant group whether or not the graft was performed for limb salvage or claudication and irrespective of the position of the distal anastamosis with relation to the knee. Patency rates (for all patients) were 90% and 82% for the treatment group and 72% and 67% for the control group at 12 and 18 months respectively.

3.7.4 Thrombolytic therapy

Fibrinolytic therapy in the treatment of acute coronary artery occlusion is currently undergoing a revival of massive proportions and the benefits, problems and logistics have been the subject of a recent excellent review by J.R.A. Mitchell (Mitchell, 1986). He feels that it has not yet been established whether or not thrombolytic therapy will prove a viable mode of treatment for the 160,000 people who die each year from myocardial infarction. However, the results of the Gruppo Italiano per lo studio della streptocinaisi (GISSI) study which randomised 11,712 patients to receive either 1.5×10^6 units of streptokinase over 1 hour within 12 hours of infarction or routine coronary care treatment, has shown an 18% decrease in mortality in the thrombolytic group with maximum benefit in those patients treated within 1 hour and no improvement when streptokinase was delayed for more than 6 hours after the onset of pain. This does suggest that early post-infarct mortality can be reduced by high dose intravenous streptokinase provided treatment is initiated early enough.

This enthusiasm for streptokinase seems to have rekindled interest in this mode of treatment in acute thrombotic peripheral arterial

occlusion and this has coincided with the explosive expansion of interventional radiography (transluminal angioplasty) in the management of arterial occlusions. Although thrombolytic therapy by streptokinase has long been advocated for the treatment of both acute and chronic arterial occlusions there have been to date no controlled trials on this form of therapy. Verstraete et al (1966) reported restoration of arterial patency in 12 out of 15 patients with arterial occlusions of less than 72 hours' duration who received intra-arterial or intravenous streptokinase at a dose of 1.25×10^6 units over 30 min followed by 10^5 units/h for 3 days. In 1970 the same group (Amery et al, 1970) published the results of an extended study of 85 patients of whom 68 had arterial occlusions of less than 72 hours duration, where the overall success rate was only 31%, embolic occlusions responding more favourably than thrombotic ones. There was a very high complication rate and a 35% mortality in patients treated although in the majority of cases death could not be directly ascribed to streptokinase therapy. Patients with occlusions of more than 72 hours' duration (17) fared uniformly badly and this failure of chronic occlusions to respond to lysis by streptokinase was further highlighted by a third report (Verstraete et al, 1971) in which only 3 of 35 occlusions in 23 patients underwent complete lysis. This finding was at variance with those of Poliwoda et al (1969) and Hume et al (1970) who using the same initial reduced dosage schedule of 2.5×10^5 units in the first 30 minutes both reported more favourable results with long-standing occlusions; in the former study, 11 of 27 patients and in the latter, 4 of 15 patients responded favourably. In both reports, side effects were again common, but there were no associated mortalities. In the study by Verstraete et al (1971) they found that some patients with stenosis underwent occlusion during

streptokinase therapy and that there was a high incidence of post-treatment thrombo-embolic episodes.

Regional low dose therapy in which streptokinase is delivered at the site of occlusion by arterial catheter at a dose of 5000 units per hour has been advocated as a means of achieving clot lysis without the attendent haemorrhagic complications of systemic therapy (Dotter et al, 1974). Recent studies, however have shown that major complications are still common with this technique (Hamelink and Elliott, 1986) and that if low dose treatment is prolonged, streptokinase antibody development can be a serious problem (Sørentsen and Hegedüs, 1986). The hetrogeneous nature of these studies makes it difficult to comment on the efficacy of this form of treatment, the former authors reporting a success rate of 67% and the latter 37% although in both studies the numbers were small. Despite the introduction of low dose intra-arterial streptokinase the drug still has no part to play in the routine management of arterial occlusion by virtue of its antigenic, haemorrhagic and thromboembolic side effects. Its use should be confined to those patients with a recent peripheral artery occlusion who are poor candidates for surgery. Whether or not the new developments in fibrinolytic therapy (recombinant tissue plasminogen activator, acetylated streptokinase and prourokinase) will prove more effective remains to be seen, but the first clues to their efficacy will come from trials on myocardial infarction.

3.7.5 Vasodilators

The use of vasodilators in the treatment of peripheral arterial insufficiency rests on two hypotheses: that vasospasm occurs in ischaemic tissues and that these drugs may stimulate collateral vessel

development. The evidence for the former is lacking and for the latter is very scant indeed.

The nicotinic acid group of drugs which includes nicofuranose, xanthinol nicotinate, inosital nicotinate, nicotinyl alcohol and nicotinic acid itself, are direct acting skin vasodilators, although they have additional antilipaemic and fibrinolytic effects. As these drugs have no effect on skeletal muscle vasculature it is hardly surprising that there has been uniform failure to demonstrate any advantageous effect despite their other 'beneficial' actions. In 1952 Hamilton and Wilson (1952) investigated a number of drugs advocated in the treatment of intermittent claudication and studied the effects of treating 10 patients with nicotinic acid for 3-6 weeks. Symptomatic improvement occurred in none. Coffman and Mannick (1972) reported the results of plethysmographically measured resting calf and foot blood flow and calf blood flow determined by ^{131}I-sodium iodide disappearance-rate during exercise before and after oral dosing with nicotinyl alcohol in 16 patients with femoral-popliteal and aorto-iliac disease, and found the drug to have no effect in either group. In their detailed and excellent study of vasodilators in peripheral arterial disease, Hansteen and Lorentsen (1974) published the results of parenteral (30 patients) and oral (21 patients) therapy with nicotinic acid and xanthinol nicotinate on resting and peak blood flow (after exercise or reactive hyeraemia). They showed quite conclusively that intravenous (and to a lesser extent intramuscular) adminstration increased resting flow, but in all cases significantly reduced peak flow. Intra-arterial adminstration in three patients resulted in a fall in resting fore-foot blood flow which was associated with a fall in ankle systolic pressure. Oral therapy also resulted in a significant reduction in resting fore-foot blood flow, although this was less marked if the initial flow was

less than 4 ml/100 g/min. Mashiah et al (1978) in an objective assessment of 6 weeks oral nicofuranose in 13 patients with unilateral superficial femoral artery occlusion, found a significant reduction in plethysmographically measured peak calf flow in the asymptomatic leg, the symptomatic leg being unaltered, and in a double-blind trial of nicotinic acid and placebo, Smith and Warren (1981) confirmed these findings in six patients using xenon-133 measured anterior tibial blood flow.

In view of the therapeutic failure of nicotinic acid, it is predictable that the α-adrenergic blockers should share the same fate, as they have a similiar vasodilatory effect on skin vessels. These drugs, phenoxybenzamine, thymoxamine (which are relatively pure α-blockers acting on skin vessels only) and tolazoline (which has an additional direct effect on vascular smooth muscle) have been shown in several studies to be ineffective in peripheral vascular disease. Hamilton and Wilson (1952) studied 18 patients (10 in an open trial and eight in a double-blind placebo-controlled trial) who received oral tolazoline for 3-12 weeks and found that no patient improved his exercise tolerance during treatment. The following year Moser et al (1953) studied the effects of oral phenoxybenzamine and oral or intra-arterial tolazoline in 28 patients with arterial occlusion of the lower limb and were unable to demonstrate any beneficial effect, particularly in the 10 patients with atherosclerosis. In 1959 Gillespie (1959) showed that intravenous tolazoline, phenoxybenzamine or chlopromazine decreased plethysmographically measured blood flow more often than they increased it, while flow to the unaffected leg was uniformly increased. Thymoxamine was shown by Kane (1970) not to improve plethysmographically measured pedal blood flow in nine patients who received the drug intra-

venously as a single dose, while he demonstrated that it reduced foot blood flow in 14 patients with previous lumbar sympathectomies. Coffman and Mannick (1972) found that acute oral ingestion of tolazoline did not improve resting calf or foot blood flow in 15 patients, and the same drug administered intra-arterially by Hansteen and Lorentsen (1974) to seven patients (six with severe pedal ischaemia) did not produce any significant improvement in blood flow.

Cyclandelate, a direct acting, papaverine-like, smooth muscle relaxant, acting on both skin and muscle vasculature, is claimed to induce collateral vessel development largely on the basis of a study by van Hell (1974). He examined the effects of the drug on the collateral development in the hind limb of the rabbit after major vessel ligation. The conclusions of the study, that the drug accelerates the development of collateral vessels, are not borne out by the finding that after 4 and 5 weeks of treatment the rabbits receiving cyclandelate lagged behind the control group in their development of collaterals (three rabbits in each group). In addition, the second larger study had 8 of 22 rabbits excluded from evaluation, making the final analysis dubious. Initial clinical studies suggested that it benefited patients with intermittent claudication, although the studies of Fremont (1964) and Ross (1965) were uncontrolled. Reich (1977) in a double-blind, placebo-controlled cross-over study examined the effects of the drug in 39 patients, of whom seven were excluded from analyis. The author found that those patients receiving cyclandelate exhibited an improvement in skin temperature and calf blood flow measured by strain gauge plethysmograph both at rest and after exercise, but exercise tolerance increased during the second course of treatment whether or not the patients were receiving the active drug. It is hardly surprising, therefore, that Coffman (1979a), after finding that a single oral dose of 800 mg had no effect

on foot or calf blood flow felt that there was insufficient evidence to support its use.

Isoxuprine and bamethan are β-adrenergic agonists acting primarily on skeletal muscle blood vessels, but despite this favourable locus of action Strandness (1970) reported on the 'ineffectiveness of isoxuprine in intermittent claudication'. A total of 22 patients with lower limb arterial disease underwent a double-blind, placebo-controlled study of oral therapy for 4 weeks. No improvement in post-exercise ankle blood pressure response was detected and the drug conferred no benefit to the patient. In their study of vasodilators, Coffman and Mannick (1972) found no change in either resting foot or calf blood flow or exercise calf blood flow in 16 patients treated acutely with oral isoxuprine and Hansteen and Lorentsen (1974) treated five patients with the drug intravenously, none of whom exibited any change in either resting or reactive hyperaemic calf blood flow. There was however a significant reduction in systemic blood pressure and an increase in pulse rate after the injection.

Cinnarizine is an antihistamine with a number of 'beneficial actions'. It is claimed to be an anti-vasoconstrictor rather than a vasodilator, by blocking calcium ion entry into smooth muscle cells, and acts on both skin and muscle vessels (Emanuel and Will, 1977). Additionally, it is claimed to be a viscosity lowering agent by virtue of a similar effect on erythrocyte membrane, so improving red cell deformability (Di Perri et al, 1977). It has been shown in controlled studies to improve exercise tolerance in intermittent claudication. Staessen (1977) treated 57 patients in a double-blind placebo-controlled study and found a significant improvement in walking distance in those treated with cinnarizine. However, this was a muticentre trial

involving eight Belgian general practitioners and the limitations of this type of study with such small numbers must be borne in mind. Barber et al (1980) undertook a similar study of 51 patients with 13 general practitioners in Glasgow, 48 of whom completed the trial and also reported favourable results. In an open uncontrolled plethysmographic study of 24 patients receiving the drug for 12 weeks, de Cree et al (1977) reported an improvement in pre-treatment values of hyperaemic calf blood flow in 16, with a decrease in flow in six. These findings were at variance with those of Mashiah et al (1978) who, in an open study of 6 weeks' oral therapy in 13 patients measured peak calf flow in both the symptomatic and asymptomatic limbs and, in addition, failed to record any improvement in whole blood viscosity. An important paper by Laporte and Capella (1986) however, draws attention to serious extrapyramidal symptoms associated with the drug when used in elderly patients as a cerebral vasodilator. As its therapeutic effectiveness is in doubt it should not be used in the treatment of intermittent claudication.

3.7.6 Rheological agents

The rheological approach to the treatment of peripheral vascular disease was initiated by the use of dextrans as a means of improving the flow property of blood, and the use of low molecular weight dextrans as viscosity lowering agents has been well reviewed by Strandness and Sumner (1975). They concluded that any beneficial effects that accrued from such therapy was by virtue of haemodilution effectively increasing cardiac output and secondarily reducing haematocrit and blood viscosity. Improvement in femoral blood flow in patients with occlusive arterial disease tended to mirror changes in cardiac output, the effects of any

viscosity reduction being of less importance. These results are transitory as 70-80% of 40,000 molecular weight dextrans are excreted within 12 hours. Additionally, haemodilution as a means of increasing blood flow has been further questioned by the finding by Milligan et al (1982) that after venesection in 14 patients with polycythemia, plethysmographically measured calf blood flow failed to increase sufficiently to compensate for the reduction in muscle haemoglobin supply.

As fibrinogen is the most important determinant of blood viscosity, and defective fibrinolysis has been reported to be associated with the atherosclerotic state (see Smith, 1986 for review), an attempt to improve fibrinolysis as a therapeutic measure in the treatment of atherosclerosis has been advocated. Anabolic steroids were found to increase fibrinolysis (Fearnely and Chakrabarti, 1962) and testosterone has been administered therapeutically in the treatment of intermittent claudication. Hentzer and Cort Madsen (1964) studied the effect of a double-blind placebo-controlled trial of intramuscular testosterone in 36 patients with 62 diseased legs, treatment being continued for 6 months. No difference between the two groups could be detected, either in subjective improvement or in alterations in objective findings; pulse amplitude, venous filling time, walking distance, or xenon-133 measured anterior tibial blood flow.

The anabolic steroid stanozolol has been shown more recently to reduce plasma fibrinogen levels in normal subjects (Preston, 1981). Clinical trials with the drug have demonstrated its ability to achieve this effect in patients with atherosclerosis, although convincing evidence for any parallel objective improvement in the patients' vascular status is lacking. Walker (1981) concluded her review of the use of anabolic steroids in arterial disease by stating that there was

no good evidence to support their use, a view that was echoed by Hessel and Kluft (1986) five years later.

Acute fibrinolysis by ancrod has been claimed to produce dramatic improvements in patients both with intermittent claudication and with rest pain (Ehrly, 1976). Lowe et al (1982), however, reported the results of a double-blind placebo-controlled trial of ancrod in the treatment of 27 patients with ischaemic rest pain. Either saline or ancrod (280 units in 50 ml of saline administered intravenously over 16 hours on the first day followed by 140-1280 units given over 5 min daily to keep the plasma fibrinogen less than 1 g/l) was administered for 7 days, and despite a dramatic fall in the plasma fibrinogen with a resultant reduction in plasma and whole blood viscosity in the ancrod-treated patients, there were no clinical or haemodynamic differences between the two groups.

There is no evidence that chronic or acute reductions in plasma fibrinogen benefit patients with occlusive arterial disease, and the importance of viscosity in blood flow is further refuted by the observation that decreasing the viscosity of blood flowing through a stensosis can in some circumstances increase the flow resistance by increasing the flow turbulence (Coffman, 1979b).

3.7.7 Metabolic enhancers

Oxpentifylline is claimed to improve erythrocyte deformability by increasing the red cell ATP content (Buchanan and Moodley, 1977; Störmer et al, 1977; Schubotz and Mühlfellner, 1977) as well as having a fibrinolytic (Jarrett et al, 1977) and an anti-platelet effect (Stefanovich et al, 1977), the latter action appearing to be by stimulation of prostacyclin synthesis by the vessel wall (Matzky et al,

1982). By virtue of these actions it is asserted to be effective in the treatment of peripheral vascular disease and it is the only recent vasoactive drug to have been approved by the Food and Drug Administration of the United States (Spittell, 1985). This was largely as the result of a large double-blind placebo-controlled multicentre study involving some of the major vascular units in the United States (Porter et al, 1982). The investigators entered 158 patients into the study of whom 128 were accepted. Of these 25 were excluded from the active drug group and 21 from the placebo group so that only 52% of patients (82) were included for analysis. Both groups of patients increased both their pain free walking distance and total walking distance although this improvement was significantly greater in the oxpentifylline group. A recent report from Italy (Poggesi et al, 1985) presents haemodynamic evidence to support Porter et al's (1982) clinical findings. The Italian study demonstrated that the drug "significantly enhanced resting and post-ischaemic blood flow of the lower limb and simultaneously decreased peripheral vascular resistance both in healthy subjects and patients" and this was associated with an increased prostacyclin synthesis in vivo. No mention of the patients' exercise tolerance was made. Not all studies have supported these findings. Mashiah et al (1978) studied 13 patients who received oral oxpentifylline openly for 6 weeks and found that there was no change in resting blood flow, but demonstrated a marked reduction in post-exercise peak calf blood flow in both the symptomatic and asymptomatic legs. No effect on whole blood viscosity was found. Quinton et al (1981) in a double-blind, placebo-controlled trial treated 30 stable claudicants and were unable to demonstrate any significant improvements in exercise tolerance or differences between the two groups in erythrocyte

deformability, whole blood viscosity, plasma fibrinogen or changes in platelet reactivity. Maughan et al (1984) found that oral oxpentifylline administered for 7 days to 10 patients with severe peripheral vascular disease did not significantly alter erythrocyte ATP content which was, in itself, not significantly different from values found in normal healthy subjects. No change in the clinical status of the patients was noted. Although oxpentifylline seems free from side effects, its undisputed benefit is far from established and although it is unlikely to do harm to patients with peripheral vascular disease, before rushing for the prescription pad we should take heed of Laporte and Capella's (1986) warning that "useless drugs are not placebos".

A more fundamental approach to metabolic manipulation of ischaemic skeletal muscle has recently been reported (Falholt and Falholt, 1986). In order to suppress FFA concentrations and increase glycolysis in the ischaemic myocardium, treatment with glucose-insulin has been suggested, and shown to improve cardiac function (for references see Falholt and Falholt, 1986). As the changes in severely ischaemic muscle are similar to those described in the myocarium by Gudbjarnasson et al, (1968; vide supra), the authors administered 50% glucose with 60 units insulin and 40 mmoles potassium per litre at the rate of 1.3 ml/min through a central venous catheter to 6 patients who had consented to a mid-thigh amputation for severe lower limb ischaemia. Amputation was postponed for 4 days after informed consent by the patients and biopsies from the soleus muscle were obtained before commencing and after 3 days of the glucose-infusion. The muscle biopsy was assayed for a number of enzymes; triosephosphate dehydrogenase (GAPDH) as representative of total glycolysis, glucose 6-phosphate dehydrogenase (G-6-PDH) as representative of the hexose-monophosphate shunt, alphaglycerophosphate dehydrogenase (GDH) as a link between carbohydrate and lipid metabolism

and 3-hydroxyl-CoA dehydrogenase (HAD) representing fatty acid oxidation/lipid synthesis. Before the infusion was commenced there was a significant increase in the ratio of lipid to glycolytic enzymes when compared to healthy controls and there was evidence of an increased activity of the HMP shunt, these results being in keeping with their previous work (Falholt et al, 1974). After 3 days of glucose-insulin infusion there was a normalisation of the increased lipid/glycolytic activity and a marked reduction in the very high pre-treatment intra-celluar FFA content of the muscle. Clinically these changes seemed to be accompanied by a diminution or abolition of pain and the clinical improvement was also evident in the contralateral limb. The switch from FFA metabolism to glycolysis could have several beneficial effects on the cell. Free fatty accumulation within the cell may have an inhibitory effect on enzyme function and calcium soap production would have a detergent effect on the cell membrane function. Insulin could reverse these adverse effects by diminishing lipid synthesis and enhancing triglyceride formation and improving glucose entry into the cell. If these results are confirmed and accompanied by an equivalent clinical improvement this technique offers interesting possibilities in the management of the severely ischaemic limb.

The drug naftidrofuryl is advertised as being of benefit to patients with intermittent claudication and rest pain, emphasis being placed on its alleged metabolic effects. Pharmacologically it is a papaverine-like direct smooth muscle relaxant with claims of an additional ability to improve tissue oxidative potential in the ischaemic state. Meynauld et al (1973) showed from the manufacturer's research laboratories that, in mice, the drug improved cerebral ATP concentration, glucose uptake and succinate dehydrogenase activity. In

an in vivo experiment in five healthy male subjects, Shaw and Johnson (1975) studied exercise provoked metabolism before and after adminstration of the drug by measuring blood lactate and pyruvate. After dosing with naftidrofuryl they found the post-exercise lactate/pyruvate ratio was reduced and this was interpreted as indicating an improvement in the oxidative capacity following an anaerobic state. Although not significantly so, post-exercise blood lactate itself was higher after giving the drug, which is the opposite of what would be expected were their conclusions correct. Elert et al (1976) conducted an open trial of placebo (nine patients) or intravenous naftidrofuryl (18 patients) on the effects of gastrocnemius muscle metabolism in patients with severe claudication and rest pain. They reported that the drug increased muscle high energy phosphate levels and decreased lactate concentration, but as patients with more severe disease were all given the active drug, the base-line values in the two groups were not comparable. In 1981, Burns et al (1981), in a controlled-trial of 34 patients (15 receiving the active drug) reported that naftidrofuryl significantly decreased urinary nitrogen loss in the first 3 post-operative days after major surgery, and suggested that this protein sparing effect may have been due to stimulation of carbohydrate or fat metabolism.

Ruckely et al (1978) in a 3 month double-blind placebo-controlled trial (25 in each group) of naftidrofuryl in the treatment of intermittent claudication, found no significant difference between the two groups in any of the parameters recorded. Haemodynamic measurements included claudication distance, arm/ankle systolic pressure index, and gastrocnemius blood flow measured by ^{99m}technetium clearance. In a further double-blind. placebo-controlled trial of 128 patients (of whom 98 completed the study) Clyne et al (1980) administered naftidrofuryl or

placebo for a period of 6 months and recorded treadmill measured walking distance and ankle systolic pressure. Both groups significantly improved the distance they could walk before the onset of claudication, but in a sub-group of over the age of 60 this improvement was confined to those on the active drug who also reported subjective improvement, assessed by asking the patients whether they felt better or not. No significant differences, however, were recorded in total walking distance or ankle systolic pressure irrespective of age. A similar claudicant study was carried out by Walters et al (1980) on 69 patients of whom 56 completed 6 months' treatment of either naftidrofuryl or placebo. No haemodynamic differences were detected between the two groups who were objectively assessed by measurement of pre- and post-exercise ankle systolic pressure and treadmill walking distance. Subjective assessment consisted of assigning a score to 6 to asymptomatic legs and 3 to symptomatic legs at the commencement of the trial, and the values scored were significantly higher in those patients over 60 years who received the active drug, although the two groups as a whole were again similar. It is difficult, however, to ascribe this selective effect of naftidrofuryl in the over 60 age group to any obvious pharmacological effect. A muticentre double-blind controlled trial of naftidrofuryl and placebo from Germany (Trübstein et al, 1984) recruited 133 patients of whom 54 receiving the active drug and 50 placebo completed 12 weeks of treatment. Total walking distance improved to the same extent in both groups although pain free walking distance improved significantly more in the naftidrofuryl group but haemodynamic parameters were unaltered.

Wong and McBain (1980) in a study on the importance of personality measurement in the assessment of response to treatment for ischaemic

rest pain used 24 patients treated with 10 days intravenous naftidrofuryl as their model. Although there was no control group and the primary aim of their report was not a discussion of the use of the drug in the management of these patients there was a definite trend towards improvment. Greenhalgh (1981) reported on a placebo-controlled trial of intravenous naftidrofuryl in the treatment of ischaemic rest pain, in which 16 patients received a twice daily 2 hourly infusion of placebo for 7 days followed by 200 mg of active drug for a further 10 days, patients being continued on oral naftidrofuryl for a further 6 months. No differences in Doppler ankle systolic pressures nor in analgesic requirements were recorded during the two treatment periods. In 1982 Meehan et al (1982) published the results of a further trial of 7 days intravenous, followed by oral naftidrofuryl administered to 22 patients with a control group of 15 patients who received no therapy. The drug (200 mg) was administered twice daily over 2 hours in 500 ml of normal saline. Subjective observations only were assessed by means of linear analogue charts measuring five parameters relevant to ischaemic rest pain and five more general ones, and the results revealed a significant symptomatic improvement in those patients treated with naftidrofuryl, but the absence of a placebo control, especially in view of the method of administration should be noted. A further study by Raftery (1982) in which 30 patients with rest pain were entered into a randomised double-blind controlled trial showed that the number of patients in whom improvement was maintained was greater in the treatment group than the placebo group although the differences were not significant.

There is therefore some evidence to support the use of naftidrofuryl in the treatment of ischaemic rest pain although its role in the treatment of claudication is less definite.

3.7.8 Other drugs

Induced hypertension with mineralocorticoids and sodium chloride has been shown to be of benefit in the severely ischaemic limb, the aim being to increase mean arterial pressure by 15-20 mm Hg. Lassen et al (1968) treated five patients with ischaemic foot ulcers with fludrocortisone or desoxycortisone and sodium chloride over several months, and reported healing of ulcers in all patients. No side effects other than fluid retention were noted. Calf blood flow was measured during the course of treatment in one patient and revealed an increase in flow proportional to the increase in pressure. The same group (Larsen and Lassen, 1973) published the results of 69 patients with rest pain not amenable to reconstructive surgery treated with the same regime. A total of 57 patients were able to complete a course of therapy and satisfactory results were observed in 50. No deaths occurred that were ascribed to the treatment but patients with coronary insufficiency or hypertension were excluded. No record, however, was made of haemodynamic assessment in these patients. Hansteen and Lorentsen (1974), in their study of vasodilators in arterial insufficiency, administered intravenous metaraminol to five patients with intermittent claudication to increase systolic pressure by 50 mm Hg. Hypertensive therapy resulted in a significant decrease in plethysmographically measured resting calf blood flow, and a small but insignificant increase in hyperaemic peak calf flow. The absence of controlled trials and detailed haemodynamic assessment of this potentially hazardous mode of therapy dictates that further studies are required before acceptance of its efficacy.

3.7.9 Beta blockers

As intermittent claudication has been reported as a side-effect of non-selective β-blocker therapy, their use in patients with peripheral vascular disease has been generally considered to be contraindicated. Clement (1980) in his thesis on the circulation in human limbs, conducted a double-blind, cross-over trial of the effects of placebo, propranolol (80 mg twice daily) and metoprolol (100 mg twice daily) each given for 2 months on 10 male patients with stable intermittent claudication. Patients underwent a 4 week placebo run-in period and exercise tolerance was assessed before the onset of treatment and during each treatment period by treadmill walking tests. Ankle systolic pressures were also measured in seven patients. No difference in the time of onset of claudication, maximum walking distance or ankle pressure was found to occur as a result of β-blocker therapy. Lepäntalo et al (1981) confirmed these findings by studying the effects of β-blocker withdrawal on 28 claudicants who were receiving the treatment for hypertension or angina. The same group (Lepäntalo et al, 1985) have more recently provided further evidence demonstrating that no association between β-blockade and intermittent claudication exists. Hurst et al (1981) reported opposite findings in their study of eight hypertensive claudicants who underwent a randomised, blind, cross-over trial of 2 weeks' therapy of placebo, propranolol, pindolol or atenolol. Exercise calf blood flow was measured by xenon-133 clearance and the authors found that flow was reduced by about 30% during β-blocker therapy, there being no difference between non-selective and cardioselective ones. Studies on normal subjects have shown that although anaerobic endurance and strength are unaffected by β-blockers, aerobic endurance and power are both decreased, the effect being more pronounced with non-selective

blockers (Kaiser, 1984). On balance it would appear that β-blocker therapy is not detrimental to the exercise tolerance of patients with intermittent claudication but those in whom a β-blocker is indicated would be best advised to use a selective type.

3.7.10 Conclusions

The large number of drugs and the variety of therapies for the treatment of atherosclerosis of the lower limbs are testimony to their ineffectiveness. There is no single drug which has been shown unequivocally either to "cure or affect the progress of the disease" and Wykoff's dogma seems as true today as it was half a century ago. We have made numerous strides in our understanding of the pathogenesis of atherosclerosis over the last decade but in spite of this it seems unlikely that any therapy will ever be effective in reversing the considerable changes of the advanced disease. Our approach for the future must surely be to prevent atherosclerosis by eliminating risk factors where possible and searching for ways in preventing the occurrence of atherosclerotic lesions in the presence of unfavourable conditions. The recent work by Kramsch et al (1981) on the prevention of atherosclerosis in hypercholesterolaemic monkeys and the study by Dyerberg and Bang (1982) on the Greenland Eskimos, must surely be steps in the right direction. Until then the best we can offer is symptomatic relief and advice in exercise, diet and avoidance of tobacco. There may also be some future in attempting to halt the progress of the disease by inhibition of the major pathogenic pathway which seem to be the platelet-vessel wall interaction.

Chapter 4

4 Methodology

4.1 Muscle biopsies

Muscle biopsies were taken from the mid-portion of vastus lateralis muscle in the studies involving high intensity exercise on healthy volunteers, and from the medial head of gastrocnemius in the studies on patients with peripheral vascular disease. The quadriceps muscle was chosen for the exercise studies as this muscle is maximally stimulated during cycle ergometer sprinting (Bigland-Ritchie and Woods, 1974) and Karlsson (1971) had previously shown that despite its large size biopsies from the central portion of the muscle were representative of what was happening in the whole muscle. In addition Harris et al (1974) had published normal values on metabolite levels in this muscle in man. Gastrocnemius was chosen for the patient group as in severe peripheral vascular disease there is a progression of the disease distally and the most severely affected muscle group that it was felt could be safely biopsied was the calf, and it was in biopsies of gastrocnemius that Elert et al (1976) had demonstrated an improvement in metabolism with naftidrofuryl therapy. Biopsy samples were obtained using the University College Hospital or Northern Hospital Supplies modification of the Bergström-Stille needle (plate 1) (Young et al, 1978; Bergström, 1962).

4.1.1 Skin preparation

The skin was swabbed with an antiseptic solution of iodine in 70% isopropyl alcohol and then infiltrated intradermally using a 25 gauge needle with 1% plain lignocaine. The subcutaneous tissues and the

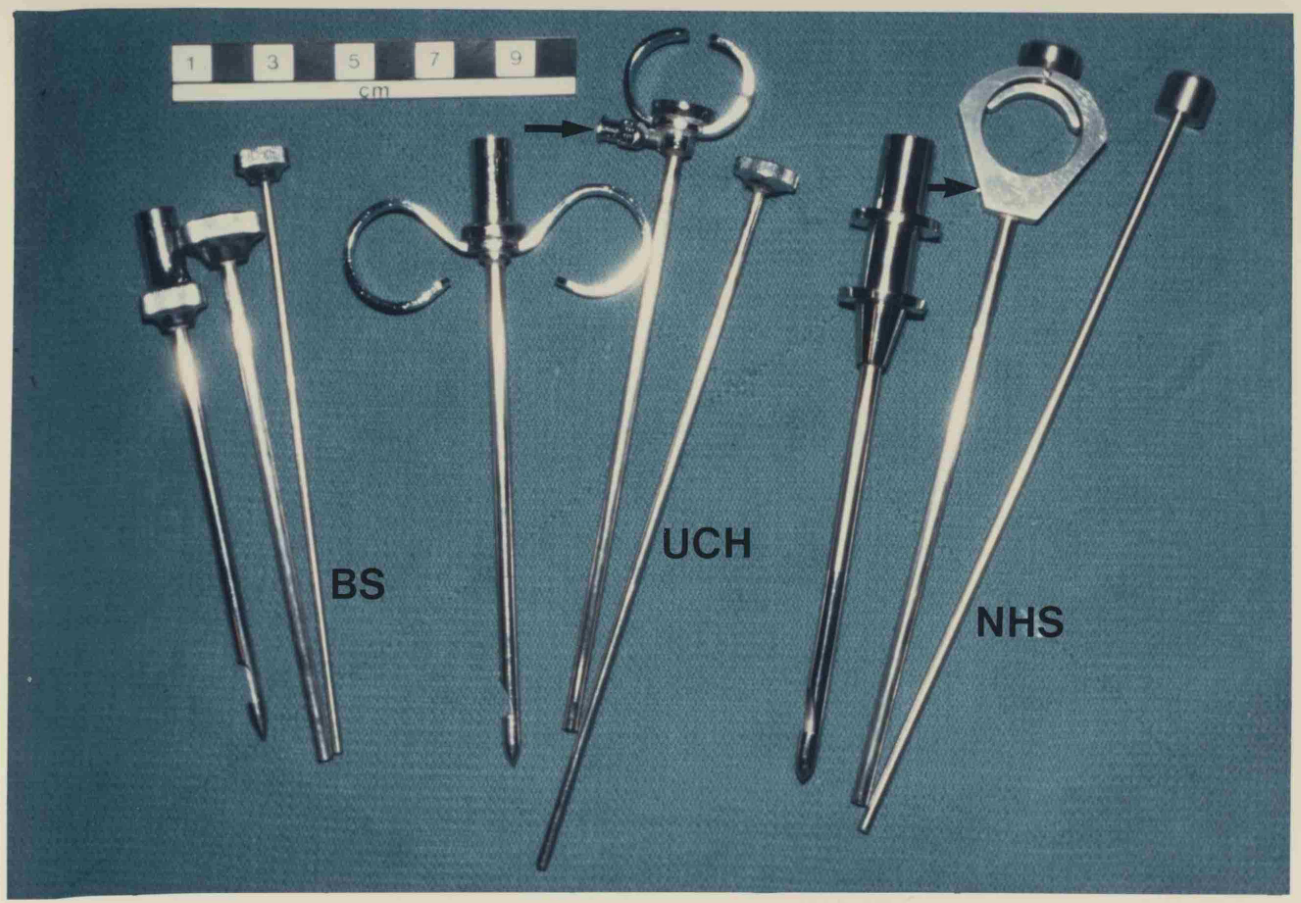


Plate 1 Illustration of the various types of muscle biopsy needles. The designer of the needle is given in parenthesis. The arrows indicate the suction port.

BS=Bergström-Stille (Bergström); UCH=University College Hospital (Jones); NHS=Northern Hospital Supplies (Maughan).

muscle fascia but not the muscle itself were then infiltrated with a 21 gauge needle using a total of 50 mg of anaesthetic solution (5 ml). For the studies on normal subjects in whom a postexercise biopsy was to be taken, this was repeated at a further site 2 cm from the first injection and after 3 or 4 min either one or two 5 mm skin incision was made through the anaesthetised skin and the muscle fascia with a number 11 scalpel blade (Swann-Morton). For the patient group, two biopsies were taken with different needles through the same incision. In the exercise studies, after the resting biopsy had been obtained both wounds were covered with a dry sterile gauze dressing held in place with microporous adhesive tape. The biopsy sites remained anaesthetised for up to 90 minutes following this procedure and this allowed the subjects to carry out their exercise test and then have a post-exercise muscle biopsy without further skin preparation or delay.

4.1.2 Biopsy procedure

For the quadriceps biopsy the needle was introduced into the body of the muscle in a lateral direction at an angle of about 20° to the vertical and perpendicular to the axis of the femur. The biopsy from gastrocnemius was taken by inserting the needle into the medial head of the muscle in a postero-inferior direction so that the axis of the needle pointed towards the heel. To regulate the depth from which the biopsy was taken the notch of the needle was positioned so that it lay just beneath the muscle fascia. Rapid suction was applied by a 60 ml disposable syringe via a luer-lock extension tube connected to the suction port of the needle and the biopsy retrieved by the guillotine effect of the inner cylindrical blade on the muscle prolapsing into the notch. Firm pressure applied with the hand against the muscle further

pushing it into the notch improved the size of the specimen (40-100 mg wet weight). In the exercise studies pre-exercise biopsies were carried out 5 mins after a warm-up with the subjects seated on the bicycle ergometer, having fasted from the night before. Post-exercise biopsies were taken with the subjects still seated on the bicycle ergometer to reduce the time between completing the exercise test and retrieving the sample and the delay was timed as being between 2 and 4 seconds. In the naftidrofuryl trial the resting biopsies were taken at the patients' bedside after an overnight fast. Of the two biopsies taken the first was for metabolite studies and was handled as below whereas the second biopsy was for estimation of the muscle oxidative capacity and on retrieval from the needle and placed in ice cold 150 mmol/l potassium chloride solution for subsequent processing. After both biopsies had been obtained the wound(s) were recleaned with alcoholic iodine and closed with sterile adhesive skin closures and a small first aid dressing (Band-Aid). The wound was then covered with a sterile gauze pad and adhesive strapping (Elastoplast), the latter being retained for about 4 hours. No restrictions were placed on activity after the biopsies and experience showed that the more active the person was afterwards the less discomfort was felt. There were no complications associated with any of the biopsies and the procedure was well tolerated, the subjects or patients varying in the discomfort experienced during the procedure. Most were unaware of the biopsy or at worst felt some deep visceral sensation and the only time it was painful was on the rare occasions that fascia was included with the specimen.

4.1.3 Initial treatment of biopsies for metabolites assays

Immediately on retrieval of the biopsy the needle was plunged into a container of Freon maintained at its freezing point with liquid

nitrogen. Although the temperature of the Freon (-150°C) was less than that of the liquid nitrogen (-186°C) its higher thermal conductivity ensured a more rapid freezing of the sample. The delay in removing the sample from the muscle to its immersion in Freon was between 400 - 600 ms. The needles were stored with the muscle biopsies in a Dewar flask containing liquid nitrogen until they were freeze dried. This was carried out in an Edwards Modulyo Freeze Drier at a temperature gradient of -50°C between the drying chamber temperature of -10°C and the freezer core at -60°C. The large specimen drying chamber was able to accommodate more than a dozen needles and after 12 hours was elevated to its upper position (chamber temperature 0°C) and drying completed. The biopsies were then easily ejected from the needles and were stored dessicated in screw top micro-reaction tubes (Sarstedt No. 72.694) in a -70°C freezer in sealed bags until removed for assay.

4.2 Sample Preparation

4.2.1 Preparation for muscle metabolite assay

a) Preparing the freeze dried muscle powder

Reagent

Petroleum ether

After removal from the freezer, the samples were allowed to warm to room temperature before opening the tubes. All connective tissue, surface blood and fat was carefully removed from the specimen and the muscle fibres teased apart with fine forceps following which the muscle was powdered in a miniature agate mortar and pestle. The powdered muscle was then transferred to a second micro-reaction tube (Sarstedt

No. 72.693) and defatted by agitation with 1 ml volumes of petroleum ether (Flear and Florence, 1961). Two or three changes of ether were used and this in addition enabled any blood admixed with the muscle powder to be separated from the fibres. Blood tended to remain in suspension in the ether longer than the muscle powder and could therefore be discarded with the supernatant. The remaining ether was allowed to evaporate in a fume cupboard over about 1 hour. Three to 5 mg aliquots of prepared powder were then accurately weighed into microreaction tubes (Sarstedt No. 72.692) using a 7-place electro-balance (Mettler).

b) Deproteinisation of sample and extraction of metabolites

Reagents

Potassium hydrogen carbonate 2.1 mol/l; Perchloric acid 0.5 mol/l, EDTA 1 mmol/l

Deproteinisation was carried out in a cold room at 0-4°C. The tube containing the sample was cooled by immersion in liquid nitrogen, being careful not to allow any to spill inside, and 100 μℓ of 0.5 mol/l perchloric acid per 1 mg of powder was added. This instantly froze in the tube which was then racked in a methanol-solid CO_2 mixture at -20°C. A batch of samples pertaining to one experiment was processed at one time and once perchloric acid had been added to all of the tubes they were tightly closed and mechanically agitated in the cold room until the perchloric acid began to thaw and mix with the muscle powder. The samples were agitated for a further 10 minutes to ensure complete extraction of all metabolites. The tubes were then spun at 0°C in an microcentrifuge (Eppendorf 5414) to precipitate the muscle pellet and a measured volume of extract removed with a volumetric pipette (Gilson) (usually 20 μℓ less than the volume of perchloric acid originally added)

and transferred to another micro-reaction tube. Neutralisation of the extract was carried out by the addition of 25μℓ of 2.1 mol/l $KHCO_3$ per 100μℓ of supernatant. After CO_2 evolution had ceased (helped by gentle agitation) the tubes were recentrifuged and the neutralised extract decanted and stored refrigerated for assay the same day or in a liquid nitrogen freezer for later assay. The precipitated muscle pellet was stored at -20°C for subsequent assay of acid insoluble glycogen.

4.2.2 Preparation for muscle oxidative capacity

Reagents

Potassium chloride 0.15 mol/l; Sodium hydroxide 2 mol/l; Chappell-Parry Medium

All procedures were carried out either over ice or at 0°C. The biopsy specimen of muscle was transferred from the cold KCl solution onto a watch glass. Fat, connective tissue and blood were carefully removed, the sample blotted to dry it, quickly weighed on a analytical balance (Sartorius), and transferred back into a 10 ml pyrex beaker containing fresh 150 mmol/l KCl. With fine iris scissors the muscle was finely chopped and then, using a polythene transfer pipette (the muscle tended to firmly adhere to a glass pipette), transferred to an disposable plastic centrifuge tube (LP4[P]) where the mince was thoroughly rinsed in several changes of KCl before being centrifuged at 1500 g for 2 min (MSE Chillspin). The supernatant was discarded and the pellet of minced muscle was placed in an all glass 1 ml mini-homogeniser (Uniform [Potter-Elvehjam design]) using a specially shaped stainless steel spoon (if the muscle sample weighed less than 10 mg a 0.1 ml homogeniser was used). Ten volumes by weight of Chappell-Parry medium were then added (Ernster and Nordenbrand, 1967) and the muscle

homoginised over ice for 2 min at 30 s intervals using a Citenco drive unit running at 1000 rpm connected to the homogeniser pestle by a rubber clutch. Further Chappell-Parry medium was then added to make the final 1:20 dilution and the diluted homogenate was then transferred into Sarstedt 1 ml screw-top micro-reaction tubes with a plastic transfer pipette with the tip drawn out (by holding it firmly in a pair of pliers and applying firm and steady traction) to fashion a long proboscis. The sample was then stored on ice for immediate, or in the liquid nitrogen freezer for subsequent assay.

4.3 Metabolite Assays

All assays were carried out using the principles as described by Lowry and Passonneau (1972) and as introduced to the author by Dr. R.J. Maughan, Department of Surgery, Aberdeen University. Many of the assays were modified in the light of laboratory experience and 'bugs' and the assays as described are the final versions which give predictable and highly reproducible results. Where assays are based on another particular analytical method reference is given.

4.3.1 General principles

All assays were based on the reduction or oxidation of pyridine nucleotides and concentrations of these substances were determined in a filter fluorimeter (Locarte) using 10 x 75 mm light wall rimless test-tubes (Pyrex) as fluorimeter tubes. Small portions of reagents were stored frozen in 1.5 ml microcentrifuge tubes (Eppendorf 3810). The concentration of the standard solutions was established spectrophotometrically using a molar extinction coefficent for NAD(P)H of 6.3 x 10^3

at 340 nm. Working at this wavelength disposable polystyrene cuvettes exhibited negligible absorbance thus obviating the need for expensive quartz cuvettes. Volumetric glass wear and reagent bottles were cleaned with a laboratory detergent (Decon 90) with a final rinse in double distilled water and were oven dried. All buffers were prepared in a laminar flow cupboard and filtered through a 0.2 micron filter before bottling to reduce micro-organism contamination. Lactate assays, which are prone to air borne contamination, were also carried out in the laminar flow cupboard. Experience showed that the Pyrex fluorimeter tubes were still best cleaned by the method of Lowry and Passonneau (1972). The tubes were placed in a 600 ml Pyrex beaker, covered with 50% nitric acid and boiled in a water bath for 5-10 min followed by rinsing twice in distilled water, boiling once more in distilled water, rinsing a further twice in distilled water and rinsing finally in double distilled water. To reduce blank fluorescence the tubes were air dried; the beakers were covered with 'cling film' which was perforated with numerous small holes and inverted and elevated over the bench allowing free circulation of air. They were then stored tightly covered with fresh 'cling film' until used. Assays involving fluorescent development of oxidised NADH were carried out using grade I NADH (100%) as this purity of cofactor was found to give the lowest blank values. This was also used for AMP assays as it was found that there was a significant amount of AMP in grade III NADH resulting in an unacceptably high blank. The spectrophotometric assays for validating the reactions and determining the concentration of the standard solutions are detailed in appendix 2. For the fluorimetric assays which were all carried out within the fluorimeter tube, the sample was first added and the reaction started with the reaction mixture containing all enzymes, and was in addition followed on a chart recorder using the highest concentration of

standard. This ensured that the reaction was working and allowed a check on the time course and end point. A reaction mixture volume of 0.2 ml was used throughout and on completion of the reaction 1 ml of sodium carbonate buffer, 20 mmol/l, pH 10 was added from a volumetric dispenser (Oxford Dispenser [fixed volume]) to enable fluorescence to be read. Whenever possible samples were assayed in duplicate except where a small sample size precluded this although it was found possible to estimate all the metabolites on as little as 1 mg of muscle powder. All the assays for a batch of samples were performed over a period of four days, the muscle extract being frozen in liquid nitrogen immediately after sufficient sample for the assay being carried out at that time had been removed. Repeated freeze-thaw cycles of the standard solutions had demonstrated that no loss of metabolites was encountered by this method. Prior to commencing a batch of fluorimetric assays, the concentration of the frozen reaction standard was rechecked photometrically. Batches of 10-12 samples were assayed at the one time with phosphocreatine, creatine and the adenine nucleotides being assayed on the day of extraction. The hexosemonophosphates, glucose, fructose-1,6-diphosphate and the triose phosphates were assayed on the following day and pyruvate, lactate, alanine and citrate and glycogen and the ketone bodies on the third and fourth days. The standard curves were always found to be linear and metabolite concentrations were calculated using a linear regression programme taking into account all neccessary blanks, tissue blanks, standards and dilutions on a programmable calculator (Casio FX-602P). As 0.125 ml of neutralised extract was equivalent to 1 mg of muscle powder the concentration of the metabolite in freeze dried muscle is as follows:

$$[\text{metabolite}]_{\text{muscle}}\ \text{mmol/kg} = 0.125 \times [\text{metabolite}]_{\text{extract}}\ \mu\text{mol/l}$$

The coefficient of variation (CV%) for the assays is summarised in table 4.1 and was calculated from 20 consecutive paired samples using the equation:

$$CV\% = \frac{\text{standard deviation between pairs}}{\text{mean of pairs} \times \sqrt{2}} \times 100$$

4.3.2 Fluorimetric assays for muscle metabolites

a) Adenosine monophosphate and adenosine diphosphate

$$AMP + ATP \xrightarrow{MK} 2ADP$$

$$ADP + PEP \xrightarrow{PK} ATP + Pyruvate$$

$$pyruvate + NADH + H^+ \xrightarrow{LDH} Lactate + NAD^+$$

Reagents

Buffer: Imidazole low fluorescence 50 mmol/l, pH 7.0

Cofactor: NADH (grade I) 1 mmol/l

Enzymes: MK 35 U/ml; PK 75 U/ml; LDH 40 U/ml

Additional reagents: ATP 0.5 mmol/l; PEP 2 mmol/l; $MgCl_2$ 0.1 mol/l; KCl 3 mol/l; EDTA 0.1 mol/l

Standard: ADP 2 mmol/l (stock solution): AMP 1 mmol/l

Working standards were prepared daily from the stock solution as follows:

stock solution (μℓ)	0	10	30	50
water (μℓ)	2000	1990	1970	1950
ADP concentration (μmol/l)	0	10	30	50
AMP concentration (μmol/l)				25

Table 4.1 Coefficient of variation for muscle metabolite assays

Metabolite	CV%
glycogen:	
total	1.79
acid insoluble	0.65
acid soluble	2.92
Phosphocreatine	1.48
Adenosine triphosphate	1.35
Adenosine diphosphate	2.58
Adenosine monophosphate	12.88
Glucose	3.40
Glucose 1-phosphate	8.32
Glucose 6-phosphate	1.01
Fructose 6-phosphate	8.52
Fructose 1,6-bisphosphate	4.64
Triose phosphates	5.14
Lactate	3.50
Pyruvate	1.14
Citrate	3.84
Alanine	1.09
β-hydroxybutyrate	5.26
Succinic dehydrogenase:	
complete assay	7.96
fumarate assay	2.33

$$CV\% = \frac{SD \times 100}{mean \times \sqrt{2}}$$

Immediately prior to analysis sufficient reaction mixture for 3 sets of duplicate blanks, standards and samples was prepared as follows:

Reaction mixture (final concentration)

Buffer 1 ml; $MgCl_2$ 20μℓ (2 mmol/l); KCl 25μℓ (75 mmol/l); ATP 10μℓ (5 μmol/l); NADH 6μℓ (6 μmol/l); PEP 10μℓ (20 μmol/l); EDTA 2μℓ (0.2 mmol/l); LDH 10μℓ (0.4 U/ml)

The mixture was divided into three portions and further additions made as follows:

1) as above (pyruvate reagent, sufficient for duplicate samples and water blanks)
2) PK 10μℓ/ml (0.75 U/ml) (ADP reagent, sufficient for duplicate samples, water blanks and standards)
3) PK 10μℓ/ml (0.75 U/ml) and MK 10μℓ/ml (0.35 U/ml) (AMP reagent, sufficient for duplicate samples plus 1 ml to run the AMP assay on the chart recorder)

Procedure

Three sets of duplicate 20μℓ of water (blank) and samples and 1 set of duplicate standards were pipetted into the fluorimeter tube. To each set of tubes was added one of the reaction mixtures starting with the AMP reagent and finishing with the pyruvate reagent, the ADP reagent being added to the standards. A further 1 ml of AMP reagent was added to 0.1 ml of AMP 25 μmol/l and the reaction followed in the fluorimeter to confirm that it was running and to check the time course. After incubating for 30 min at room temperature, carbonate buffer 1 ml was then added to each tube and fluorescence read after thorough mixing. The AMP reagent gives the sum of 2 x AMP, ADP and pyruvate, the ADP reagent the sum of ADP and pyruvate and the pyruvate reagent pyruvate

alone. Concentrations of 2 x AMP and ADP are determined by subtraction and from the standard curve. No attempt is made to calculate pyruvate with this assay, these values being used as a sample blank.

b) Adenosine triphosphate and phosphocreatine

$$\text{PCr} + \text{ADP} \xrightarrow{\text{CPK}} \text{Creatine} + \text{ATP}$$

$$\text{ATP} + \text{Glucose} \xrightarrow{\text{HK}} \text{ADP} + \text{G6P}$$

$$\text{G6P} + \text{NADP} \xrightarrow{\text{G6P-DH}} \text{6-P-Gluconolactone} + \text{NADPH} + \text{H}^+$$

$$\text{6-P-Gluconolactone} + \text{H}_2\text{O} \longrightarrow \text{6-P-Gluconate}$$

Reagents

Buffer: TRIS-HCl 50 mmol/l, BSA 0.02%, pH 8.1

Cofactor: NADP 5 mmol/l

Enzymes: CK 380 U/ml; HK 28 U/ml; G6P-DH 7 U/ml

Additional reagents: ADP 10 mmol/l; Glucose 10 mmol/l; Dithiothreitol 50 mmol/l; $MgCl_2$ 0.1 mol/l

Standard: ATP 2 mmol/l (stock solution); PCr 2 mmol/l

Working standards were prepared daily from the stock solution as follows:

stock solution (µℓ)	0	50	100	200
water (µℓ)	2000	1950	1900	1800
ATP concentration (µmol/l)	0	50	100	200
PCr concentration (µmol/l)				200

Immediately prior to analysis sufficient reaction mixture for 3 sets of duplicate blanks, standards and samples was prepared as follows:

Reaction mixture (final concentrations)

Buffer 1ml; NADP 10μℓ (50 μmol/l); ADP 10μℓ (0.1 mmol/l); Glucose 10μℓ (0.1 mmol/l); Dithiothreitol 10μℓ (0.5 mmol/l); $MgCl_2$ 50μℓ (5 mmol/l); G6P-DH 5μℓ (0.035 U/ml)

The mixture was divided in three portions and further additions made as follows:

1) as above (G6P reagent, sufficient for duplicate samples and water blanks)
2) HK 5μℓ/ml (0.14 U/ml) (ATP reagent, sufficient for duplicate samples, water blanks and standards)
3) HK 5μℓ/ml (0.14 U/ml) and CK 10μℓ/ml (0.9 U/ml) (PCr reagent, sufficient for duplicate samples, water blanks plus 1 ml to run the PCr assay on the chart recorder)

Procedure

10μℓ of extract was pipetted into a fluorimeter tube, diluted to 60μℓ with water and 10μℓ volumes of this transferred to 5 other tubes to give 3 sets of duplicate samples. Three sets of duplicate water blanks and 1 set of standards were also prepared using 10μℓ volumes. To each set of tubes was added 0.2 ml of one of the reaction mixtures starting with the PCr reagent and finishing with the G6P reagent, the ATP reagent being added to the standards. A further 1 ml of PCr reagent was added to 50μℓ of PCr 0.2 mmol/l and the reaction followed in the fluorimeter to confirm it was running and to check the time course. After incubating for 20 min at room temperature, carbonate buffer 1 ml was added to each tube, and after thorough mixing fluorescence measured. The PCr reagent gives the sum of PCr, ATP and G6P, the ATP reagent the sum of ATP and G6P and the G6P reagent G6P on its own. Concentrations of ATP and PCr are determined by subtraction and from the standard curve

and corrected for the 1:6 dilution. No attempt is made to calculate G6P which is used as a sample blank.

c) Creatine

$$\text{Creatine} + \text{ATP} \xrightarrow{\text{CPK}} \text{PCr} + \text{ADP}$$

$$\text{ADP} + \text{PEP} \xrightarrow{\text{PK}} \text{ATP} + \text{Pyruvate}$$

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{Lactate} + \text{NAD}^+$$

Reagents

Buffer: Imidazole low fluorescence, 50 mmol/l, pH 7.5

Cofactor: NADH 1 mmol/l

Enzymes: CK 1500 U/ml; PK 75 U/ml; LDH 40 U/ml

Additional Reagents: ATP 10 mmol/l; PEP 2 mmol/l; $MgCl_2$ 0.1 mol/l; KCl 3 mol/l; EDTA 0.1 mol/l

Standard: Creatine 2 mmol/l (stock solution)

Working standards were prepared daily from the stock solution as follows:

stock solution (µℓ)	0	50	100	150
water (µℓ)	2000	1950	1900	1850
creatine concentration (µmol/l)	0	50	100	150

Immediately prior to analysis sufficient reaction mixture for 2 sets of duplicate blanks, standards and samples was prepared as follows:

Reaction mixture (final concentration)

Buffer 1 ml; $MgCl_2$ 50µℓ (5 mmol/l); KCl 10µℓ (30 mmol/l); ATP 20µℓ (0.2 mmol/l); PEP 25µℓ (25 µmol/l); NADH 10µℓ (10 µmol/l); EDTA 1 µℓ (0.1 mmol/l); LDH 5µℓ (0.2 U/ml); PK 10µℓ (0.75 U/ml)

The mixture was divided into two portions and further additions made as follows:

1) as above (ADP reagent, sufficient for duplicate samples and water blanks)
2) CK 10μℓ (15 U/ml) (Creatine reagent, sufficient for duplicate samples, water blanks, standards and 1 ml to run on the chart recorder)

Procedure

As the concentration of creatine in the extract is between 0.3 and 1.0 mmol/l the extract is diluted 1:8 by pipetting 5μℓ of extract into a fluorimeter tube and adding 35μℓ of water. Two sets of duplicate samples, blanks and standards are then prepared using 10μℓ of diluted extract, water and standards. To each set of tubes was added 0.2 ml of one of the reaction mixtures commencing with the creatine reagent which was added to the standards, A further 1 ml of the creatine reagent was added to 50μℓ of the creatine standard 150 μmol/l and the reaction in this tube followed on the chart recorder. The tubes were incubated at room temperature for 20-30 min. Carbonate buffer 1 ml was then added, the tubes thoroughly mixed and fluorescence measured. The Cr reagent gives the sum of Cr, ADP and pyruvate and the ADP reagent the sum of ADP and pyruvate. Concentration of Cr is determined by subtraction and from the standard curve, correcting for the 1:8 dilution. The ADP plus pyruvate values are used as tissue blank.

d) Glucose 6-phosphate, glucose 1-phosphate, fructose 6-phosphate and Glucose

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{G6P} + \text{ADP}$$

$$\text{G1P} \xrightarrow{\text{PGluM}} \text{G6P}$$

$$\text{F6P} \xrightarrow{\text{PGI}} \text{G6P}$$

$$\text{G6P} + \text{NADP}^+ \xrightarrow{\text{G6P-DH}} \text{6-P-Gluconolactone} + \text{NADH} + \text{H}^+$$

Reagents

Buffer: TRIS-HCl 0.1 mol/l, 0.02% BSA, pH 8.1

Cofactor: NADP 50 mmol/l

Enzymes: HK 28 U/ml; PGluM 4 U/ml; PGI 35 U/ml; G6P-DH 7 U/ml

Additional reagents: G16DP 0.25 mmol/l; Dithiothreitol 50 mmol/l; EDTA 0.1 mmol/l; $MgCl_2$ 0.1 mol/l

Standard: G6P 2 mmol/l (stock solution); G1P 2 mmol/l

Working standards were prepared daily from the stock solution as follows:

stock solution (µℓ)	0	10	30	50
water (µℓ)	2000	1990	1970	1950
G6P concentration (µmol/l)	0	10	30	50
G1P concentration (µmol/l)				50

Immediately prior to analysis sufficient reaction mixture for 4 set of duplicate blanks, standards and samples was prepared as follows:

Reaction mixture (final concentration)

Buffer 1 ml; NADP 10µℓ (50 µmol/l); $MgCl_2$ 10µℓ (1 mmol/l); Dithiothreitol 10µℓ (0.5 mmol/l); EDTA 5µℓ (0.5 mmol/l); G16DP 2µℓ (0.5 µmol/l); G6P-DH 5µℓ (0.35 U/ml)

The mixture was divided into four portions and further additions made as follows:

1) as above (G6P reagent, sufficient for duplicate samples, water blanks and standards)
2) HK 5μℓ/ml (0.14 U/ml) (Glucose reagent, sufficient for duplicate samples and water blanks)
3) PGluM 10μℓ/ml (0.04 U/ml) (G1P reagent, sufficient for duplicate samples, water blanks and 1 ml to follow reaction on chart recorder)
4) PGI 10μℓ/ml (0.35 U/ml) (F6P reagent, sufficient for duplicate samples and water blanks)

Procedure

For resting samples 4 sets of duplicate 20μℓ of water (blank) and samples and 1 set of standards were pipetted into the fluorimeter tubes. After supramaximal exercise hexosemonophosphate concentrations can increase to greater than 30 mmol/kg d.m., equivalent to an extract concentration of 240 μmol/l. To simplify the assay and use one range of concentrations of standards, these samples were diluted 1:5 with water; to 32μℓ of extract was added 128μℓ of water and 20μℓ of the diluted extract was dispensed as above. To each set of tubes was pipetted 0.2 ml of one of the reaction mixtures starting with the glucose and F6P reagents and finishing with the G6P reagent which was added to the standards. A further 1 ml of the G1P reagent was added to 0.1 ml of G1P 50 μmol/l and the reaction in this tube followed on the chart recorder. The tubes were incubated for 20 min at room temperature and the reactions stopped by boiling for 2 min in a water bath. When cool 1 ml of carbonate buffer was added and after thorough mixing fluorescence was read. Glucose, G1P and F6P concentrations which are the sums of these

metabolites and G6P, were calculated from the standard curve and subtraction of G6P concentration and corrected for dilution in the post exercise samples.

e) Fructose 1,6-bisphosphate and triose phosphates

$$\text{F16BP} \xrightarrow{\text{aldolase}} \text{DAP} + \text{GAP}$$

$$\text{DAP} \xrightarrow{\text{TIM}} \text{GAP}$$

$$2\text{GAP} + 2\text{NAD}^+ \xrightarrow{\text{GAP-DH}} 2\text{glycerate-3-P} + 2\text{NADH} + 2\text{H}^+$$

Reagents

Buffer: Imidazole Low Fluorescence 50 mmol/l, pH 7.5

Cofactor: NAD 50 mmol/l

Enzymes: Aldolase 9 U/ml; TIM 240 U/ml; GAP-DH 180 U/ml

Additional Reagents: Sodium Arsenate 0.1 mol/l; Mercaptoethanol 1 mol/l; EDTA 0.1 mol/l

Standard: F16DP 1 mmol/l (stock solution)

Working standards were prepared daily from the stock solution as follows:

stock solution (μℓ)	0	5	10	20
water (μℓ)	2000	1995	1990	1980
F16DP concentration (μmol/l)	0	2.5	5	10
NADH equivalent (μmol/l)	0	5	10	20

Immediately prior to analysis sufficient reaction mixture for two sets of duplicate blanks, standards and samples was prepared as follows:

Reaction mixture (final concentration)

Buffer 1 ml; NAD 2μℓ (0.1 mmol/l); Sodium Arsenate 10μℓ (1 mmol/l); Mercaptoethanol 2μℓ (2 mmol/l); EDTA 10μℓ (1 mmol/l); GAD-PH 4μℓ (0.72 U/ml); TIM 10μℓ (24 U/ml)

The mixture was divided into two parts and further additions made as follows:

1) As above (Triose Phosphate reagent, sufficient for duplicate samples and water blanks)
2) Aldolase 2μℓ /ml (0.018 U/ml) (F16DP reagent, sufficient for duplicate samples, water blanks, standards and 1 ml to follow the reaction on the chart recorder)

Procedure

For resting samples two sets of duplicate 20μℓ of water (blank) and samples and one set of standards were pipetted into the fluorimeter tubes. After supramaximal exercise, the concentration of F16BP plus the Triose Phosphates can increase to 10 mmol/kg d.m. which is equivalent to an extract concentration of 80 μmol/l. To simplify the assay so that only one range of standards was required the postexercise samples were diluted 1:5 with water; 64μℓ of water was added to 16μℓ of sample and 20μℓ of the diluted extract dispensed as above. To each set of tubes was added 0.2 ml of one of the reagents, the F16BP reagent being added to the standards and a further 1 ml was added to 0.1 ml of F16BP 10 μmol/l and the reaction in this tube followed on the chart recorder. The tubes were incubated for 30 min at room temperature. Carbonate buffer 1 ml was then added and fluorescence read after thorough mixing. The triose phosphate reagent gives the sum of GAP and DAP whereas the F16BP reagent gives the sum of Triose Phosphates and 2 x F16BP (each mole of F16BP produces 2 moles NADH). Concentrations are determined by

subtraction and the standard curve and the F16BP result divided by two to give the true level. Postexercise values are corrected for dilution.

f) Pyruvate (by development of fluorescence in strong alkali)

$$\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{LDH}} \text{Lactate} + NAD^+$$

Reagents

Buffer: Imidazole low fluorescence, 50 mmoi/l, pH 7.0

Cofactor: NADH (grade 1) 1 mmol/l

Enzyme: LDH 10 U/ml

Additional Reagents: EDTA 0.1 mol/l; HCl 4 mol/l; NaOH 6 mol/l

Standard: Pyruvate 2 mmol/l (stock solution)

Working standards were prepared daily from the stock solution as follows;

stock solution (μℓ)	0	10	20	30
water (μℓ)	2000	1990	1980	1970
Pyruvate concentration (μmol/l)	0	10	20	30

Immediately prior to analysis sufficient reaction mixture for 2 sets of duplicate blanks, standards and samples was prepared as follows:

Reaction mixture (final concentration)

Buffer 1 ml; NADH 2μℓ (2 μmol/l); EDTA 10μℓ (1 mmol/l)

The mixture was divided into two portions and further additions made as follows:

1) as above ('NAD' reagent, sufficient for duplicate samples and water blanks)
2) LDH 10μℓ/ml (0.1 U/ml) (Pyruvate reagent, sufficient for duplicate

samples, water blanks, standards and 1 ml to follow reaction on chart recorder)

Procedure

Two sets of duplicate 10μℓ of water (blank) and samples and 1 set of standards were pipetted into the fluorimeter tubes. To each set of tubes was added 0.2 ml of one of the reaction mixtures, the pyruvate reagent being added to the standards. A further 1 ml of the pyruvate reagent was added to 50μℓ of pyruvate 10 μmol/l and the reaction in this tube followed on the chart recorder. Post-exercise pyruvate levels may increase more than ten fold to an extract concentration of 25 μmol/l. Although the range of standards used should cover this concentration the development of fluorescence results in an appreciable contribution from native NAD. This is taken into account by the tissue blanks ('NAD' reagent) but dilution of the postexercise samples with an equal volume of water ensures that all values lie within the standard range. After incubating the tubes for 5-10 min at room temperature, 20μℓ of 4 mol/l HCl was added to destroy any remaining NADH. The tubes were mixed and after a further 5 min 1 ml of 6 mol/l NaOH was carefully added and the tubes immediately and thoroughly mixed (this was done by three or four 15 s mixes on a vortex mixer because of the high viscosity of the NaOH). After 60 min incubation in the dark (covered in a drawer or cupboard) fluorescence was read exposing the tubes to a minimum of incident light to prevent decay in the fluorescence. Pyruvate concentration was determined by subtraction of the tissue blanks and from the standard curve, correcting the postexercise samples for dilution.

g) Citrate (by development of fluorescence in strong alkali)

$$\text{Citrate} \xrightarrow{CL} \text{Oxaloacetate} + \text{Acetate}$$

$$\text{Oxaloacetate} + \text{NADH} + H^+ \xrightarrow{MDH} \text{Malate} + NAD^+$$

Reagents

Buffer: TRIS-HCl 50 mmol/l, pH 7.6

Cofactor: NADH 1 mmol/l

Enzymes: CL 3.0 U/ml; MDH 35 U/ml

Additional Reagents: $MgCl_2$ 0.1 mol/l; EDTA 0.1 mol/l; HCl 4 mol/l; NaOH 6 mol/l

Standard: Citrate 2 mmol/l (stock solution)

Working standards were prepared daily from the stock solution as follows:

stock solution (µℓ)	0	10	20	30
water (µℓ)	2000	1990	1980	1970
citrate concentration (µmol/l)	0	10	20	30

Immediately prior to analysis sufficient reaction mixture for 2 sets of duplicate blanks, standards and samples was prepared as follows:

Reaction mixture (final concentration)

Buffer 1 ml; $MgCl_2$ 20µℓ (2 mmol/l); EDTA 10µℓ (1 mmol/l); NADH 2µℓ (2 µmol/l); MDH 10µℓ (0.35 U/ml)

The mixture was divided into 2 portions and further additions made as follows:

1) as above (Oxaloacetate reagent, sufficient for duplicate samples and water blanks)
2) CL 5µℓ/ml (0.015 U/ml) (Citrate reagent, sufficient for duplicate

samples, water blanks, standards and 1 ml to follow the reaction on the the chart recorder)

Procedure

Two sets of duplicate 10μℓ of water (blank) and samples and 1 set of standards were pipetted into the fluorimeter tubes. To each set of tubes was added one of the reaction mixtures, the citrate reagent being added to the standards. To a further 1 ml was added 50μℓ of citrate 30 μmol/l and this reaction followed on the chart recorder. The tubes were incubated for 15-20 min at room temperature and 20μℓ of HCl 4 mol/l added to destroy any remaining NADH. After a further 5 min incubation 1 ml of NaOH 6 mol/l was carefully added, the tubes thoroughly mixed (see pyruvate assay) and incubated in the dark for 60 min to develop NAD fluorescence which was then read with minimal exposure to incident light. Citrate concentration is calculated by subtraction of the oxaloacetate values and from the standard curve.

h) Lactate

$$\text{Lactate} + NAD^+ \xrightarrow{LDH} \text{Pyruvate} + NADH + H^+$$

$$\text{Pyruvate} + \text{Hydrazine} \longrightarrow \text{Pyruvate hydrazone}$$

Reagents

Buffer: Hydrazine 1.1 mol/l, EDTA 1 mmol/l, pH 9.9

Cofactor: NAD 50 mmol/l

Enzyme: LDH 1250 U/ml (undiluted)

Standard: Lactate 2 mmol/l (stock solution)

Working standards were prepared daily from the stock solution as follows:

stock solution (μℓ)	0	50	100	150
water (μℓ)	2000	1950	1900	1850
Lactate concentration (μmol/l)	0	50	100	150

Immediately prior to analysis sufficient reaction mixture for 1 set of duplicate samples, water blanks and standards plus 1 ml to follow the reaction on the chart recorder, was prepared as follows:

Reaction mixture (final concentration)

Buffer 1 ml; NAD 10μℓ (0.5 mmol/l); LDH 6μℓ (8 U/ml)

Procedure

Following supramaximal exercise muscle lactate can increase from a resting value of 2-4 mmol/kg d.m. to more than 100 mmol/kg d.m. equivalent to an extract concentration of 0.8 mmol/l. To simplify the reaction and use only one set of standards, postexercise samples were diluted 1:8 with water; to 5μℓ of extract was added 35μℓ of water. Into duplicate tubes was pipetted 10μℓ of samples (or diluted sample), water (blank) or standard and 0.2 ml of reaction mixture was added. To a further 1 ml of reaction mixture was added 50μℓ of lactate 0.15 mmol/l and the reaction in this tube followed on the chart recorder. After 30 min incubation 1 ml of carbonate buffer was added, the contents mixed and fluorescence read. Lactate concentration was calculated from the standard curve and corrected for dilution.

i) Glycogen

The assay for glycogen was carried out on both the acid precipitated muscle pellet (acid insoluble glycogen) and the neutralised extract (acid soluble glycogen) by analysis of glucosyl units obtained

after acid hydrolysis of glycogen by the method of Jansson (1981).

Acid Hydrolysis

Reagents: HCl 1 mol/l; NaOH 6 mol/l

Procedure

To the precipitated muscle pellet was added 0.1 ml of 1 mol/l HCl per 1 mg of muscle powder and to 10μℓ of neutralised extract was added 0.1 ml of 1 mol/l HCl. Both samples were incubated in tightly sealed screw top micro-reaction tubes in a boiling water bath for 2 hours after which the tubes were centrifuged for 1 min. For assay the supernatant from the muscle pellet was decanted and diluted with an equal volume of double distilled water and used un-neutralised whereas the 0.11 ml of the acid hydrolysed neutralised extract was neutralised with 15μℓ of 6 mol/l NaOH.

Acid insoluble glycogen was assayed spectrophotometrically using a Glucose Test Combination, GOD/Perid method and acid soluble glycogen was assayed by fluorimetric assay of the glucose produced.

Acid insoluble glycogen - Glucose Test Combination, GOD-Perid method

$$\text{glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} \text{gluconate} + H_2O_2$$

$$H_2O_2 + \text{ABTS} \xrightarrow{\text{POD}} \text{coloured complex} + H_2O$$

GOD = glucose oxidase

POD = horseradish peroxidase

ABTS = di-ammonium 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonate)

The intensity of the colour change is directly proportional to the concentration of glucose and using an appropriate standard the glucose concentration of a sample can be estimated.

Reagents

Buffer/enzymes/chromogen: made up as directed in kit (phosphate buffer 0.1 mol/l, pH 7.0; POD 0.8 U/ml; GOD 10 U/ml; ABTS 1.0 mg/ml). This was found to remain active for several months when stored protected from light at 0-4°C.

Standard: Glucose 0.505 mmol/l

Procedure

Duplicate blanks, standards and samples were prepared by adding 40µl of water, standard and the unneutralised diluted hydrolysed extract of the precipitated muscle pellet to 1 ml plastic semi-micro cuvettes into which was dispensed 1 ml of the GOD/Perid reagent from an Oxford dispenser. The samples were incubated at room temperature for 25 - 30 mins and were then read against the distilled water blank at 610 nm within 30 mins of the end of the incubation period.

Acid insoluble glycogen as glucosyl units mmol/kg d.m. is calculated from the equation

$$\text{glycogen} = \frac{2xE_{sample}}{E_{standard}} \times 0.505 \times 100$$

Acid soluble glycogen - fluorimetric glucose assay

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{G6P} + \text{ADP}$$

$$\text{G6P} + \text{NADP}^+ \xrightarrow{\text{G6P-DH}} \text{6-P-Gluconolactate} + \text{NADPH} + \text{H}^+$$

Reagents

Buffer: TRIS-HCl 0.1 mol/l, BSA 0.02%, pH 8.1

Cofactor: NADP 5 mmol/l

Enzymes: HK 28 U/ml; G6P-DH 7 U/ml

Additional Reagents: ATP 50 mmol/l; $MgCl_2$ 0.1 mol/l; EDTA 0.1 mol/l;

Dithiothrietol 50 mmol/l; HCl 1 mol/l

Standard: Glucose 0.505 mmol/l (stock solution)

Working standards were prepared daily from the stock solution as follows:

stock solution (μℓ)	0	20	50	100
water (μℓ)	505	485	455	405
glucose concentration (μmol/l)	0	20	50	100

Immediately prior to analysis sufficient reaction mixture for two sets of duplicate blanks, standards and samples was prepared as follows:

Reaction mixture (final concentration)

Buffer 1 ml: NADP 6μℓ (30 μmol/l); ATP 6μℓ (0.3 mmol/l); $MgCl_2$ 10μℓ (1 mmol/l); EDTA 5μℓ (0.5 mmol/l); Dithiothreitol 10μℓ (0.5 mmol/l); G6P-DH 3μℓ (0.02 U/ml)

The mixture was divided into two portions and further additions made as follows:

1) As above (G6P reagent, sufficient for duplicate samples and water blanks)
2) HK 5μℓ/ml (0.14 U/ml) (Glucose reagent, sufficient for duplicate samples, water blanks and standards plus 1 ml to allow reaction to be followed on chart recorder).

Procedure

Two sets of 20μℓ of samples and water (blanks) and 1 set of standards were pipetted into fluorimeter tubes. To each set was added 0.2 ml of one of the reaction mixtures, the glucose reagent being added to the standards. To a further 1 ml of this reagent was added 0.1 ml of glucose 0.1 mmol/l and this reaction followed on a chart recorder.

After 15-20 min incubation at room temperature 1 ml carbonate buffer was added to each tube and the fluorescence read after thorough mixing. Acid soluble glycogen concentration (as glucosyl units) is calculated by subtraction of the G6P values and from the standard curve and multiplied by 12.5 to correct for the dilution involved in the acid hydrolysis. As this value includes muscle glucose this must be subtracted from the result to give the true concentration.

j) Alanine

$$\text{Alanine} + NAD^+ \xrightarrow{ADH} \text{Pyruvate} + NADH + H^+$$

$$\text{Pyruvate} + \text{Hydrazine} \longrightarrow \text{Pyruvate hydrazone}$$

Reagents

Buffer: TRIS-Hydrazine 1 mol/l, EDTA 1 mmol/l, pH 10.0

Cofactor: NAD 50 mmol/l

Enzyme: Alanine dehydrogenase (ADH) 150 U/ml (undiluted)

Standard: L-Alanine 2 mmol/l (stock solution)

Working standard were prepared daily from the stock solution as follows:

stock solution (μℓ)	0	50	100	200
water (μℓ)	2000	1950	1900	1800
L-Alanine concentration (μmol/l)	0	50	100	200

Immediately prior to analysis sufficient reaction mixture for duplicate samples, water blanks and standards was prepared as follows:

Reaction Mixture (final concentration)

Buffer 1 ml; NAD 2.5μℓ (0.125 mmol/l); ADH 5μℓ (0.75 U/ml)

Procedure

To 10μℓ of samples, water and standards in a fluorimeter tube was added 0.2 ml of the reaction mixture, 50μℓ of L-Alanine 0.2 mol/l was added to a further 1 ml of the reagent and the reaction in this tube followed on the chart recorder. The tubes were incubated for 40-45 min at room temperature, 1 ml of carbonate buffer added and after mixing, fluorescence read. Alanine concentration was determined from the standard curve.

k) <u>β-Hydroxybutyrate</u>

$$\beta\text{-Hydroxybutyrate} + NAD^+ \xrightarrow{\text{3-HBDH}} \text{Acetoacetate} + NADH + H^+$$

$$\text{Acetoacetate} + \text{Hydrazine} \longrightarrow \text{Acetoacetate hydrazone}$$

Reagents

Buffer: Hydrazine 1.1 mol/l, EDTA 1 mmol/l, pH 9.0

Cofactor: NAD 50 mmol/l

Enzyme: 3-HBDH 15 U/ml (undiluted)

Standard: β-Hydroxybutyrate 2 mmol/l (stock solution)

Working standards were prepared daily from the stock solution as follows:

stock solution (μℓ)	0	5	10	20
water (μℓ)	2000	1995	1990	1980
β-Hydroxbutyrate (μmol/l)	0	5	10	20

Immediately prior to analysis sufficient reaction mixture for duplicate samples, blanks and standards was prepared as follows:

Reaction mixture (final concentration)

Buffer 1 ml; NAD 2.5μℓ (0.125 mmol/l); 3-HBDH 5μℓ (0.075 U/ml)

Procedure

Into duplicate tubes was pipetted 20μℓ of sample, water (blank) and standard and 0.2 ml of the reaction mixture was added. To a further 1 ml of reaction mixture was added 0.1 ml of β-hydroxybutyrate 20 μmol/l and the reaction in this tube followed on a chart recorder. After 60 min incubation at room temperature 1 ml of carbonate buffer was added, the contents mixed and fluorescence read. β-Hydroxybutyrate concentration was determined from the standard curve.

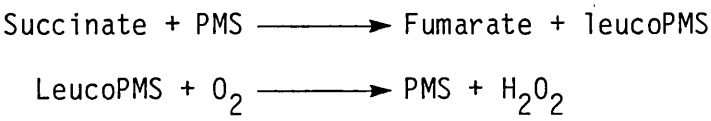
4.4 Succinate dehydrogenase assay

As the drug naftidrofuryl was purported to increase the activity of mitochondrial succinic dehydrogenase perhaps by increasing its affinity for its substrate (Meynaud et al, 1973), an assay of the activity of this component of the electron transfer chain was undertaken in the rest pain study. Ackerall and Singer (personal communication) recommended using the PMS/DCIP method as they felt that this would give the best sensitivity for the small size of sample obtained (see Singer et al, 1973; Singer, 1974 and Ackerall et al, 1978 for reviews). However despite their recommendations of dialysing and/or washing the homogenate by centrifugation a number of problems could not be overcome. These included a variable (and unknown) loss of part of the sample and rapid non-enzymatic reduction of the dyes. Purified mitochondria would have overcome these problems but the yield as prepared by the method of Ernster and Nordendrand (1967) was too small to provide sufficient sample for assay. It was therefore decided to adopt the PMS/fluorimetric assay as described by Essén et al (1975) and Henriksson et al (1980). As the homogenisation medium used in preparation of mitochondria appeared to give the best activity of the enzyme this was

retained for the initial homogenisation (vide supra) rather than the 0.3 mol/l phosphate buffer (Henriksson et al, 1980) and the initial 1:20 homogenate was then diluted to 1:100 with the phosphate buffer. The advantages of this assay over those based on artificial electron acceptors is that it overcomes the problems of non-enzymatic dye reduction by measuring the product of the reaction, fumarate rather than photometric changes in the dyes (or cytochrome C). Enzyme activity estimated by measurement of fumarate produced should therefore be equal to the activity obtained by manometric determination of the utilisation of oxygen without the problems associated with the Warburg apparatus (see Arrigoni and Singer, 1962). Although mitochondrial permeablity to PMS was theoretically limiting, disrupting the mitochondria with phospholipase and calcium did not affect the rate of fumarate production and it was concluded that PMS entry was optimal with the initial homogenisation alone. The rate of production of fumarate was directly proportional to the amount of homogenate used; doubling the amount doubled the rate and halving the amount halved it. Analysis of the fumarate produced was by fluorimetric assay as decribed by Lowry and Passonneau (1972).

4.4.1 Assay of enzyme activity

a) Incubation with PMS/Succinate



Reagents

Buffer: Potassium phosphate 0.3 mol/l, BSA 0.05%, pH 7.7

Cofactor: PMS 10 mmol/l

Enzyme: Muscle homogenate 1:20 in Chappell-Parry medium

Additional Reagents: Sodium Succinate 1.5 mol/l, pH 7.7; Antimycin A 0.1 mmol/l; NaOH 1 mol/l; Bromobenzene

Immediately prior to analysis sufficient reaction mixture for 2 duplicate pairs of samples was prepared on ice as follows:

Reaction mixture (final concetration)

Buffer 1 ml; PMS 50μℓ (10 mmol/l); Antimycin 1μℓ (0.1 μmol/l)

The mixture was divided into two portions and further additions made as follows:

1) as above (control reagent)
2) Sodium Succinate 0.1 ml/ml (0.15 mol/l) (succinic dehydrogenase reagent)

Procedure

The 1:20 muscle homogenate was diluted to 1:100 with cold phosphate buffer (20μℓ of homogenate and 180μℓ of buffer) and 20μℓ of this diluted enzyme preparation (equivalent to 0.2 mg of muscle wet weight) was added to 0.15 ml of each of the reaction mixtures in micro-reaction tubes with several small holes in the top (Eppendorf 3810). The tubes were transferred to a water shake bath at 37°C and incubated for 30 min. They were then transferred back onto ice and the reaction stopped with 0.3 ml of NaOH 1 mol/l followed by the addition of bromobenzene 0.15 ml to extract the remaining PMS. After mixing, the tubes were centrifuged for 1 min in an Eppendorf micro-centrifuge and the supernatant decanted for assay of the fumarate produced.

b) Fluorimetric assay of fumarate produced

$$\text{Fumarate} \xrightarrow{\text{fumarase}} \text{Malate}$$

$$\text{Malate} + \text{NAD}^+ \xrightarrow{\text{MDH}} \text{Oxaloacetate} + \text{NADH} + \text{H}^+$$

$$\text{Oxaloacetate} + \text{Glutamate} \xrightarrow{\text{GOT}} \text{Aspartate} + \alpha\text{-Ketoglutarate}$$

Reagents

Buffer: 2-amino-2-methyl-propan-1-ol 0.1 mol/l, pH 9.9

Cofactor: NAD 50 mmol/l

Enzymes: Fumarase 3500 U/ml (undiluted); GOT 90 U/ml; MDH 600 U/ml

Additional reagents: Glutamate 1 mol/l, pH 9.9; EDTA 0.1 mol/l

Standard: Fumarate 2 mmol/l (stock solution)

Working standards were prepared daily from the stock solution as follows:

stock solution (μℓ)	0	20	50	100
water (μℓ)	2000	1980	1950	1900
Fumarate concentration (μmol/l)	0	20	50	100

Immediately prior to assay sufficient reaction mixture for the duplicate pairs of control and succinic dehydrogenase tubes, 1 set of standards and 1 ml to follow the reaction on a chart recorder was prepared as follows:

Reaction mixture (final concentration)

Buffer 1 ml; NAD 4μℓ (0.2 mmol/l); Glutamate 10μℓ (10 mmol/l); EDTA 10μℓ (1 mmol/l); Fumarase 2μℓ (7 U/ml); MDH 6μℓ (3.6 U/ml); GOT 5μℓ (0.45 U/ml)

Procedure

Into paired duplicate fluorimeter tubes was pipetted 20μℓ of each supernatant, water and 1 set of standards. To each tube was added 0.2 ml of the reaction mixture. In addition 0.1 ml of fumarate 0.1 mmol/l was added to 1 ml of the reaction mixture and the reaction in this tube followed on a chart recorder. The tubes incubated for 20-30 min. After adding 1 ml of carbonate buffer fluorescence was read and the concentration of fumarate calculated from the standard curve. Enzyme activity was expressed as μmol/g/min and was determined by the following equation:

$$\text{SDH activity} = \frac{[C] \times D}{t}$$

where [C] = fumarate concentration in supernatant (μmol/l)

D = dilution factor (2.35 l/g muscle)

t = incubation time (30 min)

The coefficient of variation for the whole assay on two seperate samples of homogenate (from one biopsy specimen) was 7.96% and for the fluorimetric assay alone 2.33% calculated from 20 consecutive pairs.

Chapter 5

5 Metabolic response to brief maximal exercise

The purpose of this study was to quantify as accurately as possible the power output and fatigue profile generated during a maximal anaerobic work test (AWT), and relate the changes in performance to the accompanying metabolic changes as determined from needle biopsy specimens of muscle before the exercise test, after the period of maximal power output and at the end of the 30 s sprint,

5.1 Introduction

As discussed in chapter 2, there is now evidence to indicate that under conditions of supramaximal exercise, at least, energy for muscular contraction is provided simultaneously by PCr breakdown and glycolysis. To assess the relationship between the work done and the consequent metabolic changes it is necessary to employ a work test that allows reproducible measurement of power output, which ideally should be monitored continuously, and at the same time allow physiological measurements of the subject to be made. A number of such tests based on the bicycle ergometer have been described but the Wingate test as developed by Bar-Or (1980) has the advantage that it was designed to relate an individual's functional capacity to the metabolic response induced by the test and it has been estimated that the aerobic contribution to energy using this test is as little as 13%. Jacobs et al (1983), using the Wingate test, have shown the feasibility of obtaining post-exercise muscle biopsies after as little as 10 s of a bicycle ergometer sprint when they clearly demonstrated after this short interval the considerable contribution to energy provision by glyco-

lysis. A modification of this test was employed using a bicycle ergometer (Monark) from which power output was recorded by means of analogue to digital conversion of the current generated by a DC motor linked to the bicycle fly wheel and fed directly to a micro-computer (Lakomy, 1986). The validity of this method and the importance of a warm-up prior to undertaking the 30 s AWT has been discussed in detail by Wootton (1984). As power output using this test was always seen to decline after 6 s it was felt that a biopsy taken at this time, rather than the 10 s of Jacobs et al (1983), would be a more sensitive index of the metabolic changes in the period of maximal force generation.

5.2 Protocol

Four male subjects participated in this study, age 21-40 y, weight 71.2 ± 8.3 kg and height 1.73 ± 0.07 m. Each subject underwent either a 6 or 30 s AWT separated by a period of 7 days, the order of the test being randomly presented to the subjects. Muscle biopsies for metabolite studies were taken from the vastus lateralis muscle after the warm-up and after the exercise test with the subject still seated on the bicycle ergometer. Capillary blood samples were taken at the time of the muscle biopsies and 5 min after the sprint for determination of blood glucose and lactate. An outline of the protocol is illustrated in figure 8.

5.3 Methods

5.3.1 The anaerobic work test

The subjects rested at least 30 min prior to the test having fasted overnight and taken no prior exercise. While resting the muscle biopsy

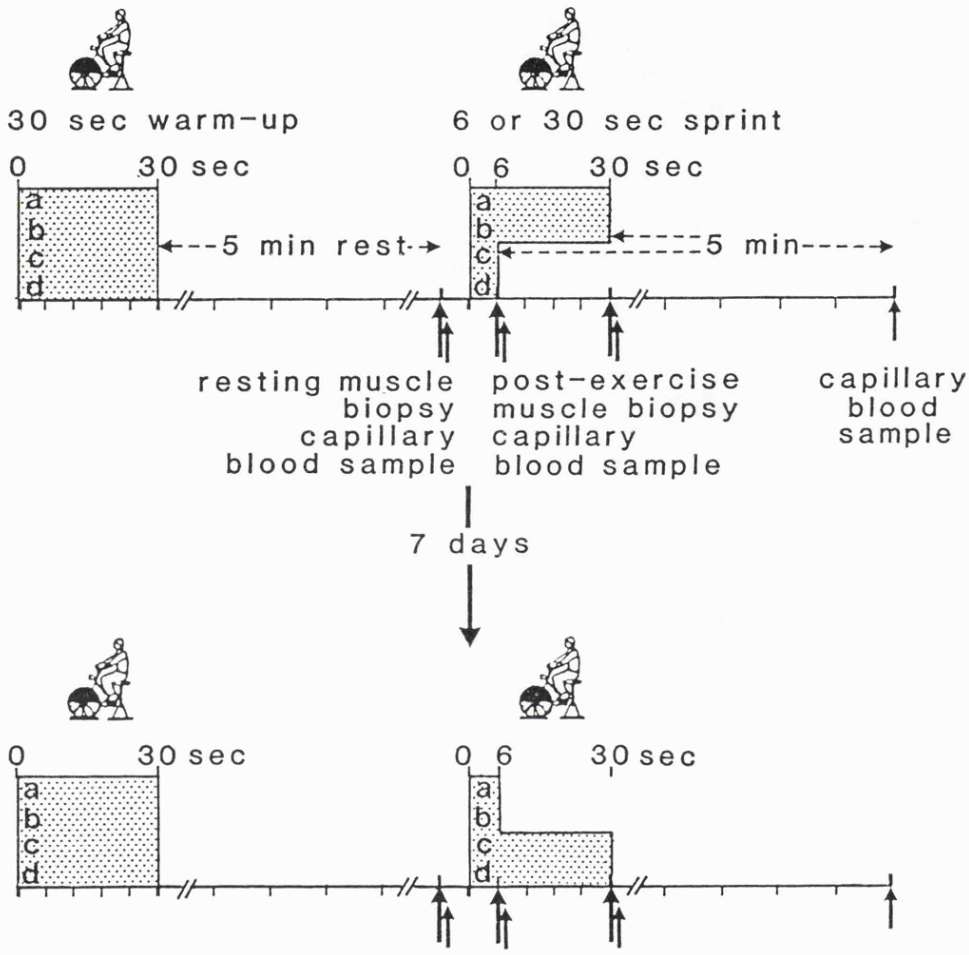


Figure 8 Outline of the protocol in study I examining the metabolic response to brief maximal exercise. Note that the "resting" biopsies were taken just prior to the sprint 5 minutes after a 30 second warm-up. The order in which the subjects performed the sprints (6 or 30 seconds) was randomly determined and reversed after 7 days.

sites were prepared following which the subjects transferred to the bicycle ergometer. Saddle height was adjusted so that the knee was maintained in a few degrees of flexion with the pedal at its lowest postion, toe clips being used to secure the position of the feet. Prior to undergoing the AWT the subjects performed two 30 s warm-ups, with 30 s recovery between each, the first at a pedal frequency of 85 rpm and the second at 115 rpm, with a load of 1.5 kp. The purpose of the warm-up was to familiarise the subjects with the exercise test and allow calibration of the DC motor-microcomputer system without inducing any feeling of exhaustion. Five minutes after completing the warm-up, when the pre-test biopsy had been obtained, the load was set to a predetermined level that ellicited the maximum power output for that individual while allowing a pedal frequency of 100-120 rpm and typically this was found to be 0.075 kp/kg. With the load supported the subject was instructed to maintain a pedal frequency of 25 rpm, the computer based data acquisition system was started and during a 3 s countdown the load was introduced. On the command of 'go' the subject accelerated as quickly as possible against the full load to their maximum pedal velocity and a timer was simultaneously started. During the AWT the subject remained seated on the bicycle and considerable verbal encouragement was given to entreat him to give maximum effort. After 15 s, a count down of the time remaining was given and immediately on finishing the sprint, the instruction 'stop' was issued and the load removed. The instant the pedals stopped, the postexercise biopsy and capillary blood samples were taken following which the subject was transferred to a couch for a period of passive recovery until after the last blood sample.

The characteristic fatigue profile obtained is illustrated in fig 9. From this four main values were derived: peak power output (PPO),

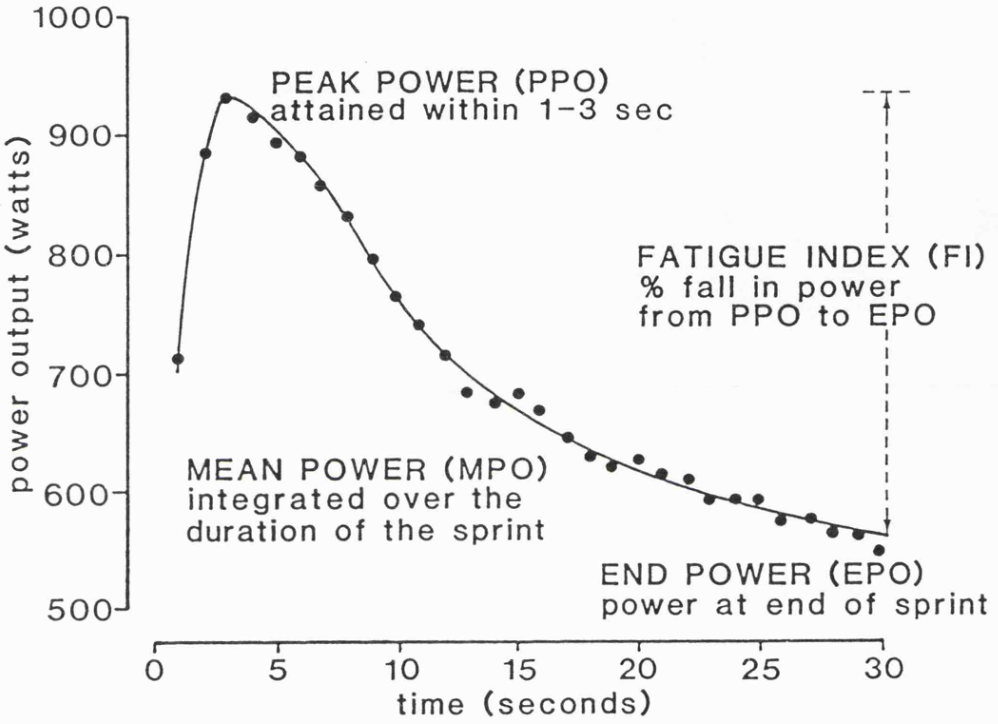


Figure 9 Diagram of the fatigue profile obtained during the Anaerobic Work Test (AWT). Note the measured values of PPO, MPO and EPO and the derived fatigue index (FI). Peak power was attained within 1-3 seconds and values greater than 1 kw were observed.

the maximum power attained during the sprint; end power output (EPO), the lowest power recorded towards the end of the sprint; mean power output (MPO), the average power sustained over the test; fatigue index (FI%), the percentage fall in power from PPO to EPO. The coefficient of variation for 5 tests (3 subjects) for PPO, EPO, MPO and FI% for the 30 s sprint were 3.5%, 3.8%, 2.1% and 4.6% respectively.

For the truncated 6 s AWT the same methods as the 30 s test were employed but after 6 s the subject was instructed to stop pedalling, the load removed and biopsy and blood tests obtained as described above. The coefficient of variation of the variables of power output for the shorter sprint was similar to that obtained for the 30 s test.

5.3.2 Blood sampling

Arterialised capillary blood samples from the pre-warmed hand were obtained by the finger-prick technique the blood being collected in duplicate 25μℓ disposable glass capillary tubes (Dade). The blood was immediately deproteinised in 0.25 ml of perchloric acid 0.25 mol/l to give a 1:11 dilution and centrifuged for 60 s in an micro-centrifuge (Eppendorf) to precipitate the denatured protein. Blood lactate and glucose was determined on the un-neutralised extract as detailed in appendix 3.

5.3.3 Muscle biopsies

Muscle biopsies were taken from the vastus lateralis component of the quadriceps muscle as described in chapter 4.

5.4 Results

5.4.1 Power output

Table 5.1 details the performance characteristics of the four subjects.

The PPO and MPO over the first 6 s of the exercise test was the same for the 30 s sprint and the truncated 6 s sprint confirming that the shortened sprint reflected an accurate sample of the initial 6 s of the test. Within this period 26% of the total work had been done.

5.4.2 Changes in blood metabolites

Blood lactate increased from 1.39 mmol/l at rest to 1.83 mmol/l at the end of the 6 s sprint, and after 5 min this had risen to 5.01 mmol/l. After the 30 s sprint the resting value of 1.27 mmol/l had risen to 4.49 mmol/l and reached 12.41 mmol/l at 5 min, a tenfold increase. Blood glucose concentrations were relatively unchanged at the end of both sprints but after 5 min increased by about 0.5 mmol/l to 6.11 mmol/l after the 6 s test and about 1.0 mmol/l to 6.52 mmol/l after the 30 s AWT. Blood metabolite results together with a summary of the performance characteristics are shown in table 5.2.

5.4.3 Muscle metabolites

Muscle metabolite results are summarised in table 5.3. Biopsy samples were not obtained in two subjects prior to the 6 s sprint and the mean for all the resting biopsies are given as the results compared favourably with published data, and there was no obvious difference between pre-exercise values for the two groups. Statistical analysis was by paired T-test between the individual resting values, using the

Table 5.1. Performance characteristics of 6 s and 30 s AWT

		Subject				
		CW	HKL	SAW	CM	Mean (±SD)
Body Wt (kg)	6 s	71.45	82.70	66.10	64.40	71.16 (±8.26)
	30 s	72.00	81.00	66.20	65.00	71.05 (±7.30)
Load (kp)		5.50	6.25	5.00	4.75	5.38 (±0.66)
30 s AWT	mean rpm	109.0	119.0	126.0	131.3	121.3 (±9.6)
	MPO (W)	599	743	631	624	649 (±64)
	PPO (W)	852	1086	879	869	922 (±110)
	EPO (W)	464	476	458	440	460 (±15)
	FI%	45.6	56.2	47.9	49.4	49.8 (±4.6)
	PPO_i (W)	877	1130	927	895	957 (±117)
	MPO_{0-6s}	767	991	796	779	833 (±106)
	EPO_{6s}	773	988	793	794	837 (±101)
MPO_{0-30s}/kg body wt		8.32	9.17	9.53	9.60	9.16 (±0.59)
MPO_{0-6s}/kg body wt		10.73	11.98	12.04	12.10	11.71 (±0.66)
6 s AWT	mean rpm	149.6	162.3	163.7	158.6	157.6 (±8.1)
	MPO (W)	803	1014	819	753	847 (±115)
	PPO (W)	872	1064	892	826	914 (±104)
	EPO (W)	777	962	790	776	826 (±91)
MPO_{0-6s}/kg body wt		11.24	12.26	12.39	11.69	11.90 (±0.53)

MPO=mean power output; PPO=peak power output; EPO=end power output;
FI%-fatigue index (% decrease in PPO); PPO_i=instantaneous PPO

Table 5.2 Blood lactate and glucose concentrations and a summary of performance characteristics in response to 6 s and 30 s AWT [mean (±SD)]

	6 s AWT	30 s AWT
Blood lactate (mmol/l)		
Pre-AWT	1.39 (±0.29)	1.27 (±0.25)
0 min post AWT	1.83 (±0.41)	4.49 (±0.25)
5 min post AWT	5.01 (±0.77)	12.41 (±1.21)
Blood glucose (mmol/l)		
Pre-AWT	5.61 (±0.46)	5.44 (±0.45)
0 min post AWT	5.71 (±0.31)	5.63 (±0.47)
5 min post AWT	6.11 (±0.76)	6.52 (±0.64)
Power output (watts)		
PPO	914 (±104)	922 (±110)
MPO_{0-6s}	847 (±115)	837 (±106)
EPO_{6s}	826 (± 91)	833 (±106)
MPO_{0-30s}	-	649 (±64)
EPO_{30s}	-	460 (±15)
FI%	9.5 (±2.4)	49.8 (±4.6)

Table 5.3 Muscle metabolite results before and after the 6 s and 30 s AWT in mmol/kg d.m. [mean (±SD)]

	post 6 s sprint (n=4)	pre-AWT (n=6)	post 30 s sprint (n=4)
glycogen - total	217.98 (±36.79)	261.21 (±21.78)	181.91 (±38.77)
acid insoluble	201.03 (±35.68)	245.19 (±18.36)	164.87 (±38.82)
acid soluble	16.95 (±3.10)	16.04 (±6.43)	17.04 (±5.45)
PCr	54.84 (±9.79)	84.25 (±3.72)	28.78 (±3.86)
ATP	22.17 (±0.95)	24.33 (±0.70)	13.67 (±2.54)
ADP	4.01 (±0.37)	3.79 (±0.21)	3.64 (±0.16)
AMP	0.23 (±0.05)	0.19 (±0.04)	0.15 (±0.05)
TAN	26.41 (±1.22)	28.31 (±0.80)	17.46 (±2.64)
Glucose	2.68 (±0.77)	1.86 (±0.67)	2.88 (±1.12)
G1P	0.54 (±0.16)	0.08 (±0.04)	0.69 (±0.22)
G6P	11.02 (±3.78)	1.33 (±0.35)	19.53 (±3.18)
F6P	1.98 (±0.67)	0.18 (±0.09)	3.33 (±0.89)
F16DP	0.53 (±0.19)	0.11 (±0.11)	3.13 (±3.97)
Triose phosphates	0.38 (±0.04)	0.32 (±0.06)	0.55 (±0.10)
Lactate	28.46 (±6.69)	8.51 (±1.71)	89.33 (±16.65)
Pyruvate	0.46 (±0.14)	0.17 (±0.04)	1.31 (±0.53)
Citrate	1.02 (±0.29)	0.91 (±0.16)	0.81 (±0.16)
Alanine	10.00 (±2.05)	8.41 (±1.47)	13.17 (±1.86)
β-hydroxybutyrate	0.36 (±0.05)	0.32 (±0.06)	0.35 (±0.05)

PCr=phosphocreatine; ATP=adenosine triphosphate; ADP=adenosine diphosphate; AMP=adenosine monophosphate; TAN=ATP+ADP+AMP (total adenine nucleotide pool); G1P=glucose 6-phosphate; G6P=glucose 6-phosphate; F6P=fructose 6-phosphate; F16DP=fructose 1,6-diphosphate

mean values where two samples were available, and the postexercise results. After 6 s there was as expected an appreciable fall in PCr (35%) whereas ATP, ADP and AMP levels remained more constant. Although there was no difference in TAN the small drop in ATP itself was significant at the 5% level. More dramatic however was the fall in glycogen (44 mmol/kg d.m. glucosyl units), both the total and acid insoluble fractions falling markedly, although the acid soluble component remained constant. There was a marked increase in hexose monophosphates (HMP) (850%), G1P, G6P and F6P all accumulating significantly, and in lactate (330%) with a much more modest increase in those metabolites below PFK in the Embden-Meyerhof pathway (210%). No changes in citrate or alanine were observed. Despite the extreme fatigue after the 30s sprint, PCr levels were still 34% of the resting values but there was now a 44% fall in ATP and a 38% drop in TAN concentration but no change in ADP or AMP. In addition HMP had increased to 15 times the pre-exercise levels (G1P 8.6 times, G6P 14.7 times and F6P 18.5 times) and lactate was now 10 times greater with a corresponding fall in glycogen of 79 mmol/kg d.m. (glucosyl units) equivalent to 30% of the resting concentration. Again the decrease was confined to the acid insoluble fraction. Fructose bisphosphate and the triose phosphates had now risen to 9 times their basal levels and alanine concentrations were now significantly greater than the pre-exercise values. A summary of the statistical results is given in table 5.4 and the changes are illustrated on the 3 dimensional pathways chart in figure 10. Interestingly, 85% of the glycogen utilised during the 30 s test could be accounted for by the accumulation of glycolytic intermediates and lactate, but during the 6 s sprint only 56% of the glycogen used could be accounted for in this way.

Table 5.4 Statistical summary of metabolite changes after 6 s and 30 s AWT. One tailed paired T-test, 3 degrees of freedom

metabolite	t-statistic pre-exercise v 6 s AWT	pre-exercise v 30 s AWT	6 s AWT v 30 s AWT
glycogen - total	4.60***	6.75****	4.72***
acid insoluble	5.60***	7.00****	7.62*****
acid soluble	0.24	0.15	0.03
PCr	6.99****	6.75****	4.72***
ATP	2.42*	5.99****	7.43****
ADP	-1.86	-0.70	2.53*
AMP	-0.98	1.56	1.52
TAN	1.79	6.10****	8.06*****
Glucose	-1.47	-2.10	-0.18
G1P	-4.37***	-4.64***	-1.91
G6P	-4.55***	-11.01*****	-3.54**
F6P	-4.65***	-6.43****	-1.95
F16DP	-3.15*	-1.31	-1.11
Triose phosphates	-0.85	-2.87*	-1.45
Lactate	-4.43***	-8.25*****	-5.69****
Pyruvate	-3.67**	-3.85**	-3.03*
Citrate	1.15	1.77	2.77*
Alanine	-0.83	-4.62***	-4.63***
β-hydroxybutyrate	-13.00*****	-1.60	0.14

Abbreviations as for table 5.3

level of significance

*p<0.05	t>2.35
**p<0.025	t>3.18
***p<0.01	t>4.54
****p<0.005	t>5.84
*****p<0.0025	t>7.45

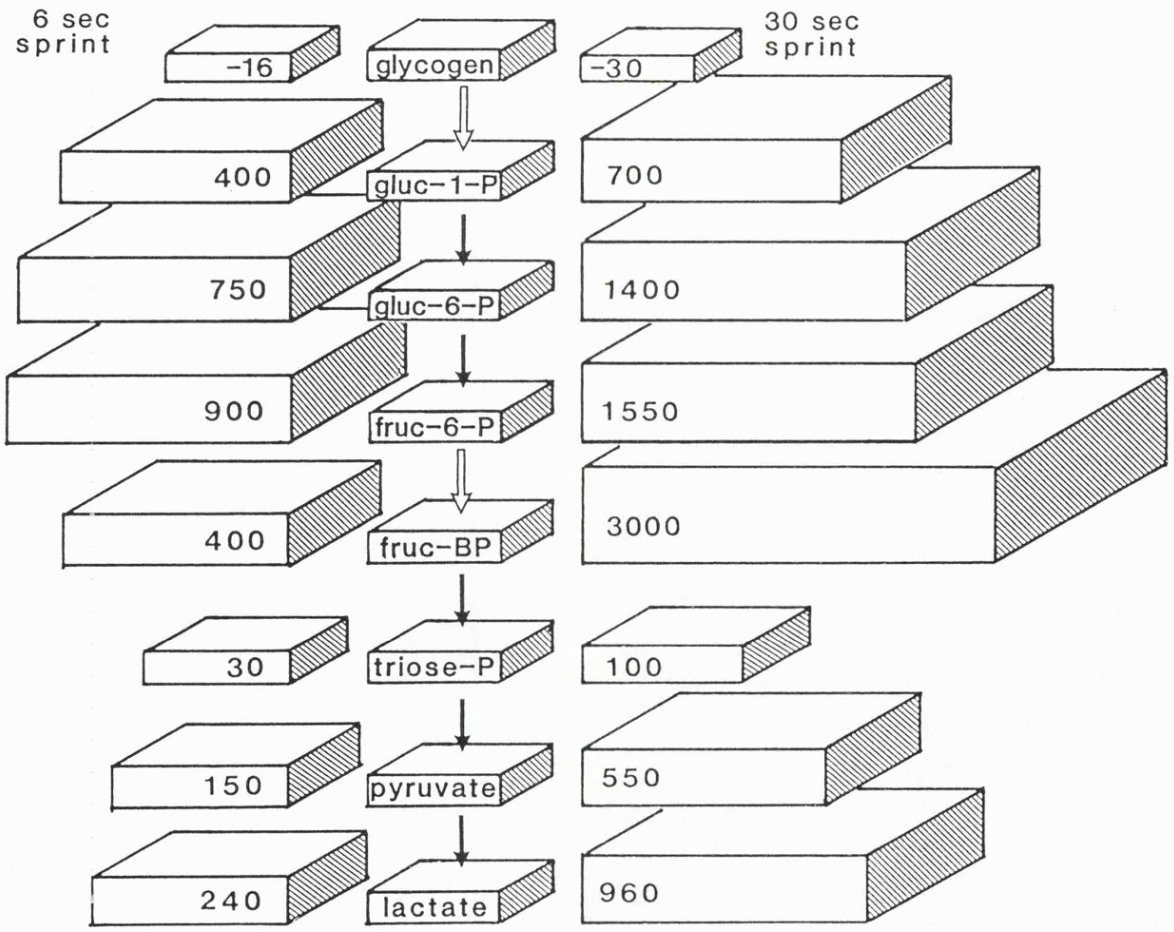


Figure 10 Three dimensional pathways chart illustrating the percentage change in muscle glycolytic intermediates after a 6 and 30 s AWT. The central boxes represent 100% by volume of the resting values and the two open arrows the control enzymes of glycogen phosphorylase and phosphofructokinase respectively. The percentage change after the sprint is illustrated by an equivalent change in the volume of the appropriate box.

5.4.4 Muscle pH

During dynamic exercise, Sahlin et al (1976) have demonstrated a linear relationship between blood lactate and muscle pH measured by the homogenate method:

$$pH = 7.06-0.00413(lactate+pyruvate) \quad (5.1)$$

Thus at rest the derived pH was 7.02 and this fell after 6 s to 6.94 and at the end of the sprint to 6.69.

5.4.5 ATP turnover

The total rate of ATP turnover can be derived from the decrease in PCr and ATP and the accumulation in lactate and pyruvate and the contribution from glycolysis alone by the increases in the latter two metabolites (Sahlin and Henrikssen, 1984).

$$\text{total ATP turnover} = -\Delta PCr-\Delta ATP+1.5\times\Delta lactate+1.5\times\Delta pyruvate \quad (5.2)$$

$$\text{glycolytic ATP turnover} = 1.5\times\Delta lactate+1.5\times\Delta pyruvate \quad (5.3)$$

Total ATP turnover from anaerobic sources during the 6 s sprint reached 12.5 mmol/kg d.m./s (range 7.0 - 12.5) to which glycolysis contributed 49% (5.0 mmol/kg d.m./s). Over the 30 s test total ATP turnover averaged 6.3 mmol/kg d.m./s (range 5.2 - 7.5) and by using the change in metabolite concentrations between the end of the 6 s and 30s tests ATP turnover between 6 and 30 s was calculated to have fallen to 5.4 mmol/kg d.m./s (range 3.3 - 6.7) of which glycolysis now provided 80%. These changes and the relative contributions from the different anaerobic sources are illustrated in figure 11.

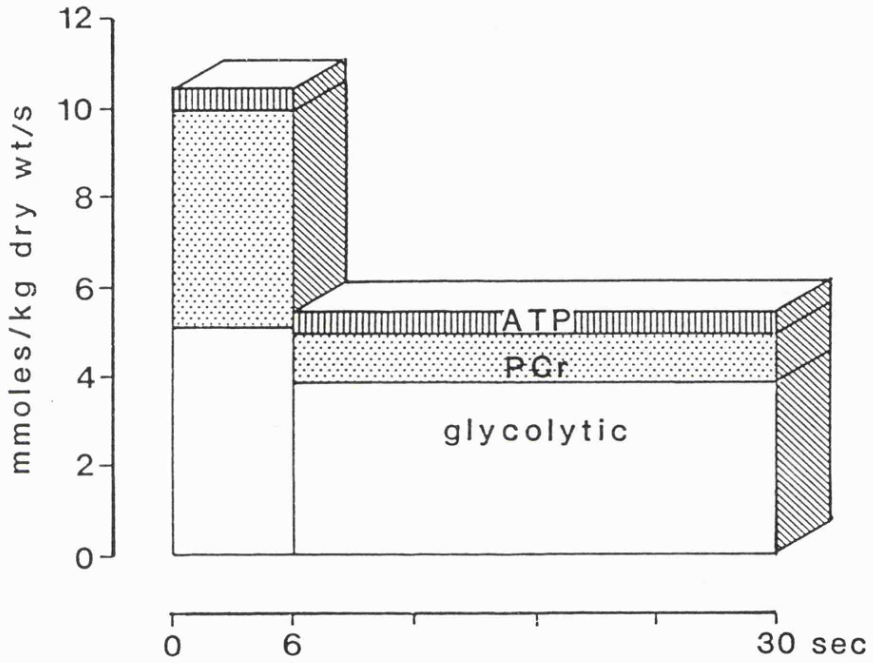


Figure 11 Derived total ATP turnover rates calculated from metabolite changes after a 6 and 30 s AWT with the relative contributions of ATP, PCr and glycogen. At 6 seconds there was an equal contribution to energy supply from glycolysis and PCr whereas after 30 seconds glycolysis provides most of the ATP.

5.4.6 Glycogenolytic and glycolytic rates

The rate of glycogenolysis can be calculated from the rate of accumulation of all the glycolytic intermediates above and below PFK in the pathway. Glycolysis can be determined from the increase in lactate and pyruvate. Estimates of these values can be derived from the formulae of Hultman and Sjöholm (1983).

$$\text{glycogenolysis} = \Delta G6P + 0.33 \times \Delta G6P + \tfrac{1}{2}(\Delta\text{lactate} + 0.1 \times \Delta\text{lactate}) \quad (5.4)$$

$$\text{glycolysis} = \tfrac{1}{2}(\Delta\text{lactate} + 0.1 \times \Delta\text{lactate}) \quad (5.5)$$

Within 6 s of exercise glycogenolysis had reached a rate of 4.0 mmol/kg d.m./s (range 3.2 - 6.6), exceeding glycolysis by more than 100%. After 30 s the rate of glycogen breakdown had fallen by 43% to 2.3 mmol/kg d.m./s whereas glycolysis had only fallen by 20% (from 1.8 to 1.5 mmol/kg d.m./s). Figure 12 illustrates these changes.

The derived values for pH and rates of ATP turnover, glycogenolysis and glycolysis are summarised in table 5.5.

5.5 Discussion

Our subjects studied comprised extremely well motivated individuals, a mixture of recreational games players and runners who usually had a vested interest in the study, so psychological drive and motivation was invariably high. As it is possible to account for almost all of the decrease in muscle glycogen by accumulation of the products of glycogenolysis and glycolysis (for the 30s test at least), it is felt that it is justified in considering this model as a "closed system", with there being very little interchange between the muscle cell and its immediate extracellular environment and the circulation. It is very

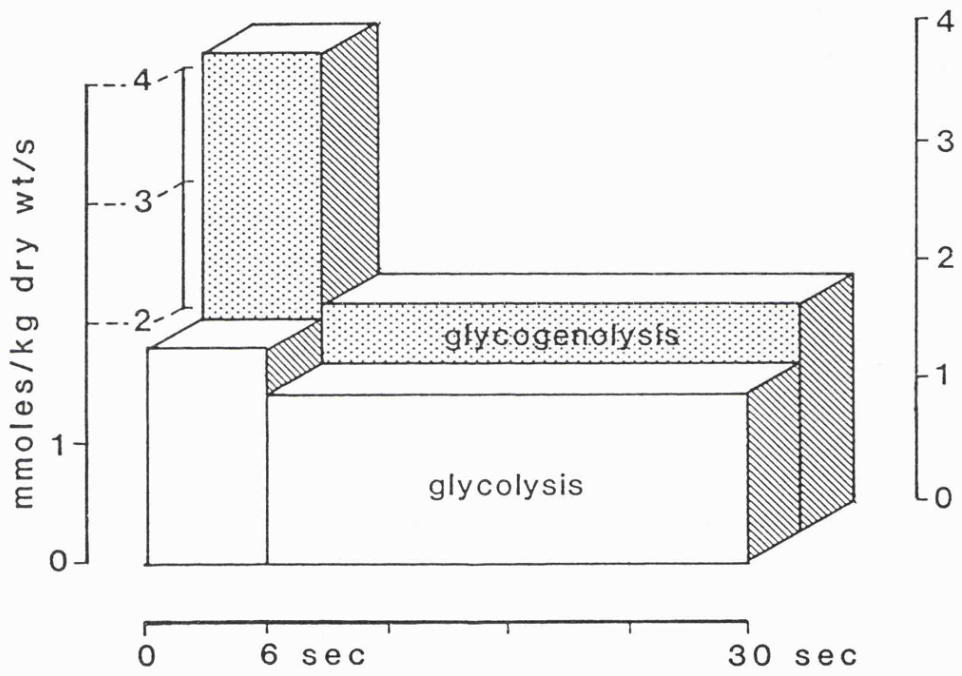


Figure 12 Glycogenolytic and glycolytic rates calculated from metabolite changes after a 6 and 30 s AWT. At 6 seconds the rate of entry of glycogen into the glycolytic pathway more than doubles its utilisation, whereas after 30 seconds the two rates have levelled off although there is always an excess of glycogen entering the pathway.

Table 5.5 Calculated values for muscle pH, glycogenolytic and glycolytic rates and anaerobic ATP turnover rates, total, glycolytic, from PCr and from ATP. Rates are given as mmol/kg d.m./s [mean (±SD)]

	6 s AWT (0-6 s)	30 s AWT (0-30 s)	30 s - 6 s AWT (6-30 s)
pH before sprint	7.02 (±0.01)	7.02 (±0.01)	---
pH after sprint	6.94 (±0.03)	6.69 (±0.08)	---
ΔpH	-0.08	-0.33	-0.25
glycogenolytic rate	3.99 (±1.76)	2.29 (±0.50)	1.87 (±0.75)
glycolytic rate	1.82 (±0.82)	1.48 (±0.36)	1.40 (±0.49)
total ATP turnover	10.27 (±2.44)	6.30 (±1.05)	5.30 (±1.59)
ATP glycolytic	5.04 (±2.27)	4.10 (±0.99)	3.86 (±1.36)
ATP from PCr	4.89 (±1.40)	1.85 (±0.21)	1.09 (±0.49)
ATP from ATP	0.35 (±0.03)	0.35 (±0.12)	0.35 (±0.01)

unlikely therefore that there occurred sufficient circulating biochemical changes from muscle metabolites in the course of the sprint to modulate central fatigue.

5.5.1 Peripheral fatigue - a protective mechanism

Although fatigue is undesirable in those wishing to maintain a high work load it is an important protective mechanism for the organism. Using the rate of glycolysis achieved by one subject in the 6 s test of 2.8 mmol/kg d.m./s and a glycogen content of 300 mmol/kg d.m., if the body's entire skeletal muscle mass were to produce lactate at that rate the muscle glycogen stores would be depleted in less than 2 min and death would ensue from profound acidosis (Newsholme and Leech, 1983a). Even with a buffering capacity of 60 Slykes (Sahlin and Henriksson, 1984) complete break-down of all glycogen would generate 140 mmol/kg wet muscle of hydrogen ions and cellular pH would fall to less than 5. It is likely therefore that there is some feedback mechanism initiated by the products of glycolysis that prevents damage to the cell occurring.

5.5.2 Muscle pH

Muscle pH has been shown to be linearly related to lactate concentration (Sahlin et al, 1976) and after the 30 s sprint pH falls to about 6.6, a value that is widely reported as occurring at exhaustion (Sahlin, 1978; Costill et al, 1983; Sahlin and Henriksson, 1984). Although it has been clearly demonstrated that a low pH can reduce force generation in isolated electrically stimulated muscle, a reduction in pH from 7.01 to 6.67 resulting in a 45% decrease in tetanic tension (Sahlin et al, 1983) and in "skinned fibres" (see Chapter 2, section 2.5.2) supramaximal dynamic exercise does not seem to be influenced by induced

acidosis (McCartney et al, 1983). The rate limiting processes for force generation during the AWT does not appear to be a simple function of an increased proton load and the importance of potassium ions has been discussed (Chapter 2, section 2.5.2).

5.5.3 Glycogenolytic and glycolytic rates

For glycolysis to proceed efficiently, it is important that it is exceeded by the rate of glycogenolysis and this condition was met during both the 6 s and 30 s tests. As 40% of glycogen mobilised occurred within the first 20% of the sprint it is likely that there is a very early and rapid increase in phosphorylase activity followed by an exponential fall. By 6 s, the breakdown of PCr has already resulted in sufficient Pi release to allow a saturating concentration for phosphorylase a and b. A glycogenolytic rate of 6.6 mmol/kg d.m./s (the highest value calculated of the 4 subjects) would require a total phosphorylase activity of 400 mmol/kg d.m./min, which is more than 2.5 times the estimated V_{max} for the enzyme reported by Chasiotis (1983), and it was only after 6 s of the test that the glycogenolytic rate fell to values in keeping with Chasiotis' measured activity. If the decrease in muscle glycogen is used to calculate phosphorylase activity instead, the estimated rates are greater still with mean results of 430 mmol/kg d.m./min within the first 6 s, falling to 90 mmol/kg d.m./min after 6 s. However if the mean glycogenolytic activity over the 30 s sprint is considered, values of 140 and 170 mmol/kg d.m./min are obtained by calculation from intermediates and from the decrease in glycogen respectively in close agreement with Chasiotis who measured activity after 25 s of isometric or 30 s of dynamic exercise. It may be therefore that the initial in vivo phosphorylase activity during supramaximal

exercise is much greater than has been previously described.

As summarised in table 2.5 these high glycogenolytic rates can be confirmed by calculation of the data presented by Jones et al (1985). Their study, using a constant velocity bicycle ergometer (isokinetic) at two pedal frequencies, 60 and 140 rpm, gave a computed activity of 190 and 180 mmol/kg d.m./min over a 30 s sprint and in the two subjects in whom they measured metabolites at 10 s of exercise the rates were 260 and 290 mmol/kg d.m./min at the slower and faster cycling speeds.

Phosphofructokinase is accepted as the control enzyme of glycolysis, as glycogen phosphorylase is the rate limiting enzyme for glycogen degradation (Newsholme and Start, 1973), and the "stacking-up" of the HMP above PFK certainly lends weight to the regulatory role of this enzyme. The initial glycolytic rate of 1.8 mmol/kg d.m./s fell to 1.4 after 6 s, this 22% fall being much less than the 53% fall in glycogenolysis. Despite this and in keeping with the studies in Table 2.5, the rate of glycogenolysis always exceeded the requirements by glycolysis. Unlike the findings of Hultman and Sjöholm (1983) with electrically stimulated muscle there was no evidence that glycolysis increased with time, a finding that was not confirmed by Spreit et al (1987) using a similar model. Plotting G6P concentration against lactate does not give such a clearly linear relationship as Hultman and Sjöholm (1983) found (figure 13). The graph appears to be curvilinear with an initial rapid rise in G6P and then plateauing at the highest concentrations, this being a reflection of the very high initial glycogenolytic rate declining much more rapidly than glycolysis.

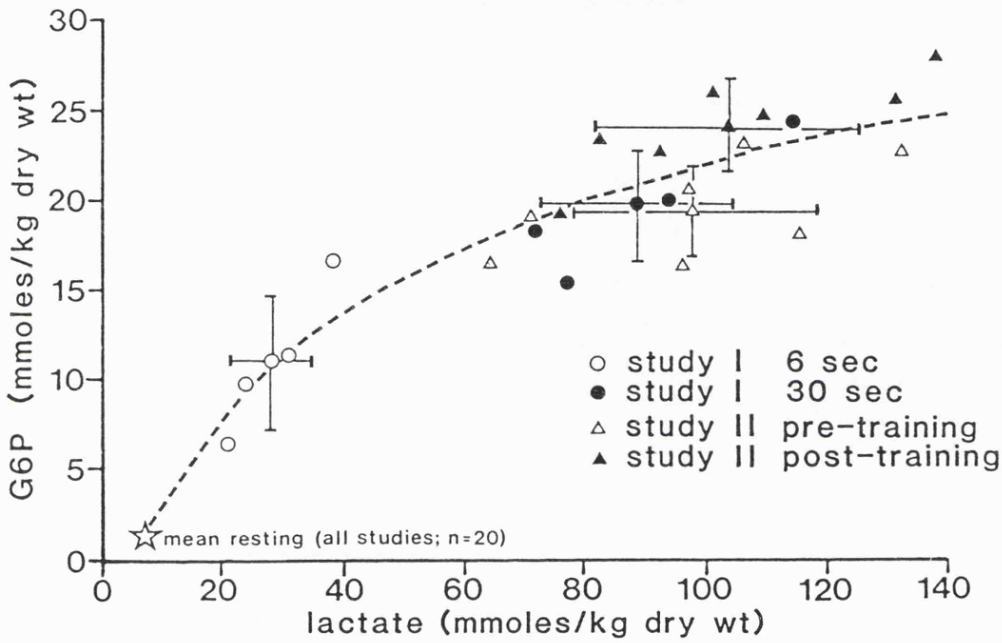


Figure 13 Plot of glucose 6-phosphate and lactate with best fit curve for data from studies I and II. Means and error bars (1 SD) are shown

5.5.4 ATP turnover and power output

There was an approximately equal contribution from PCr and glycolysis to the ATP turnover rate of 10.3 mmol/kg d.m./s in the initial 6 s of the test (PCr 47% and glycolysis 49%, ATP itself contributing to the remainder). The highest value obtained, 12.5 mmol/kg d.m./s, is close to the maximum rate calculated by McGilvery (1973) (11 to 17 mmol/kg d.m./s). This had fallen to 5.3 mmol/kg d.m./s after 6 s of which glycolysis now provided 73% and ATP itself 6.6%. Energy supply had therefore declined by 46% of the peak value at 6 s between 6 and 30 s due primarily to the 78% drop in its contribution from PCr, a fall from 4.9 to 1.1 mmol/kg d.m./s. Over this period glycolysis declined by just 23% and it would appear that despite their initial shared contribution to energy supply, ATP from PCr decays more than three times as rapidly as the rate of glycolysis. These changes have been shown graphically in figure 12.

Using the figure cited by Newsholme and Leech (1983b) of muscle being able to perform 24 kj of work per mole ATP, assuming a muscle water content of 77% (Karlsson, 1971) and a maximally exercising muscle mass of 50% of the body's total of 30 kg [upper leg muscle mass is approximately 10 kg (Wootton, 1984)] it is possible to calculate theoretical rates of ATP turnover for a given amount of work. An estimated value in the first 6 s of the sprint would be 10.3 mmol/kg d.m/s, over the 30 s sprint 7.8 mmol/kg d.m./s, these values proving remarkably close to those determined from changes in metabolites. It is extremely likely therefore that the biopsy technique gives an accurate picture of the profound biochemical changes occurring within the muscle. As the provision of energy during this type of exercise other than in the initiation of muscular activity is primarily from glycolysis, it

follows that the maintainance of the glycolytic rate is of prime importance in sustaining a required or desired rate of work. When ATP turnover falls the rate of mechanical work will decline, and fatigue will be experienced.

5.5.5 Fall in muscle ATP concentration

The dramatic reduction in ATP concentration found after the sprints is not seen with isometric exercise or electrical stimulation (Hultman and Sjöholm, 1983) but it has been demonstrated using a bicycle ergometer test of similar work intensitity (Jacobs et al, 1982; Jones et al, 1985; Table 2.3) and more recently during treadmill sprinting (Cheetham et al, 1986). Although this decrease in ATP concentration with intense dynamic exercise has been observed since Hultman et al's (1967) study of 20 years ago, it was not until 1978 that Sahlin et al (1978b) found that they could account for it by an increase in IMP. Harris and Hultman (1985) were able to reproduce this more pronounced fall in ATP by repeated electrical stimulation of quadriceps with an occluded circulation (they were able to produce a 63% fall in ATP with lactate concentrations reaching 170 mmol/kg d.m.), and found that the decrease in ATP was accompanied by a stocheiometric increase in IMP, findings which have been confirmed during dynamic exercise in horses (Snow et al, 1985). Hultman et al (1967) suggested that a reduction in ATP only occurred when PCr concentrations had been reduced by 60% but from the studies summarised in table 2.3 it can be seen that with extreme dynamic exercise a fall occurs before PCr supplies are exhausted, and after the 6 s sprint when PCr levels were still 65% of the resting concentration a small but significant decline in ATP was already evident. The significance of this will be further discussed in chapter 8.

5.5.6 Aerobic contribution to high intensity exercise

The contribution of aerobic metabolism in exercise of this nature is unknown. It is certain that some oxidative phosphorylation occurs and the finding that it is only possible to account for a little over half of the products of glycolysis (estimated from glycogen breakdown) after the 6 s sprint may reflect a greater percentage contribution from oxygen utilisation in the initial stages of exercise. It has recently been estimated (Cheetham et al, unpublished observations) that during treadmill sprinting of a similar intensity, the oxygen consumption is 16 ml/kg body weight. As 1 litre of O_2 is equivalent to 0.0447 moles and 6.17 moles of ATP are produced per mole of O_2 in the complete aerobic metabolism of glycogen (McGilvery, 1973), this is equivalent to an extra ATP turnover rate of 0.64 mmol/kg d.m. Even if this were supplied in the first 6 s of the test it is only 10% of the energy supply that would be provided if all the extra glycogen "lost" in the 6 s sprint were metabolised by oxidative phosphorylation and it is may be that the initial decrease in glycogen is an overestimate. This unaccounted glycogen may alternatively represent loss of lactate from the working muscle, and if the the intial part of the G6P/lactate curve is affected by loss of lactate from the muscle then taking this into account would restore linearity to the relationship reported by Hultman and Sjöholm (1983) which used an occluded circulation.

5.6 Conclusions

After 6 s of supramaximal exercise on a bicycle ergometer it was found that half of the muscle's energy supply was already being supplied by glycolysis. At exhaustion (after a 30 s anaerobic work test) glycolysis contributed to more than two thirds of the muscle's ATP turnover

and a marked decline in muscle ATP concentration was observed despite significant amounts of PCr remaining. A final discussion of the implications of these findings in the mechanism of fatigue will be undertaken in chapter 8.

Chapter 6

6 Influence of interval training on the 30 s AWT

This second study on the metabolic changes as a result of the 30s AWT was designed to confirm the findings reported in the first study and to examine the influence of interval training on the ability to perform the AWT and on the associated metabolic changes.

6.1 Introduction

As discussed in chapter 2, section 2.6, a period of 8 weeks training should be sufficient to induce adaptation to the training stimulus. Although the studies cited in table 2.6 reported an improved performance as a result of training the adaptive metabolic changes were variable. Despite the "anaerobic" type of exercise used an increase in VO_2max was invariable but changes in glycolytic activity were unpredictable. Karlsson et al (1972) found that with a 7 month training period there was no increased accummulation of muscle lactate after a maximal exercise test to exhaustion. However Eriksson et al (1973) found that young boys (11-13 years) trained on a bicycle ergometer for just 6 weeks demonstrated an almost doubling of PFK activity. This was associated with a 36% increase in lactate concentration at exhaustion, and in addition a significant increase in ATP and PCr levels as a consequence of training were described. By contrast, Sharp (1986) found no change in PFK activity with 8 weeks training using the bicycle ergometer AWT test as a training model. Despite this they reported an increase in post-exercise lactate after training with a concommitant increase in muscle buffering capacity. No change in PCr was reported but both the pre- and post-training values seemed very low (56 and 66

mmol/kg d.m.). A detailed study of the influence of training on the metabolic changes produced by supramaximal exercise has not been previously described.

6.2 Protocol

Seven male subjects participated in this study of the effects of high intensity interval training on performance and anaerobic muscle metabolism (age 21-31 y, weight 72.4 ± 12.2 kg and height 1.73 ± 0.08 m). Each subject performed the 30 s AWT before and after 8 weeks of intensive interval training. Although all of the subjects had indulged in regular recreational sports, none had undergone high intensity training. The post-training AWT was performed 6 days after completion of the training schedule to exclude any acute changes in muscle metabolism directly as a result of the training session. As before, muscle biopsies were taken with the subject seated at the bicycle ergometer after the warm-up and after the sprint. Capillary blood samples were taken at the time of the muscle biopsies for determination of blood glucose and lactate and again 5 min after the completion of the sprint. An outline of the protocol is illustrated in figure 14.

6.3 Methods

6.3.1 Maximum oxygen uptake

Maximum oxygen uptake was determined by the levelling off criteria of Åstrand and Rodahl (1977) with the expired air being collected into 150 l Douglas bags and the O_2 and CO_2 content measured by a mass spectrometer (Centronics RGA200).

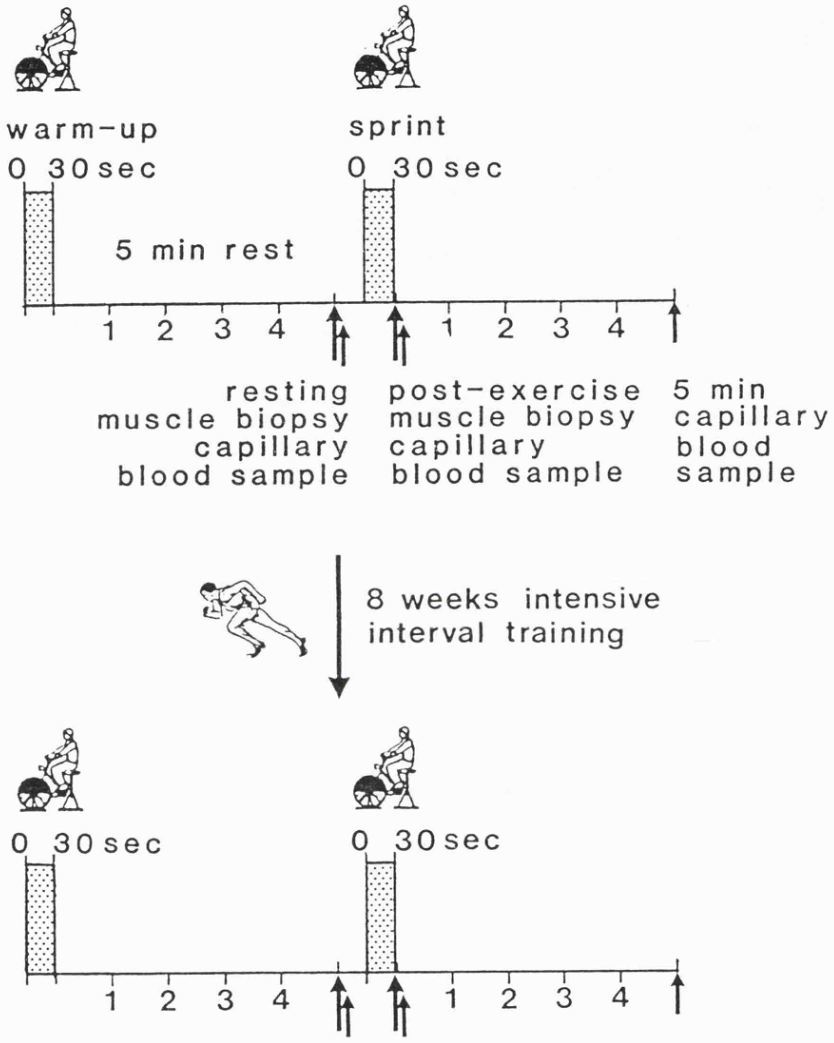


Figure 14 Outline of the protocol for study II examining the influence of interval training on the metabolic response to the 30 s AWT. As in study I, the "resting" biopsy was taken 5 minutes after a 30 second warm-up. The post-training sprint was performed 6 days after 8 weeks of intensive interval training to exclude any acute effect of training.

6.3.2 Anthropometric measurements

Body weight was measured to the nearest 50 g (Person Weigher, Avery Ltd.) and height, limb length and girth determined to the nearest mm using anthropometric tape and Harpenden anthropometers (Holtain Ltd.). Body fat was calculated from skinfold thickness measured with Harpenden skinfold calipers (Holtain Ltd.) by the method of Durnin and Rahaman (1967). Using the measured parameters limb volume and composition was estimated by the method of Jones and Pearson (1969).

6.3.3 Training method

Interval training was performed as a group for 8 weeks. Training sessions lasted from 1-2 h and were carried out 5-6 times per week. Sprint training consisted of high intensity interval running over distances ranging from 40-400 m with a recovery interval between sprints between 30 and 300 s, the longer periods of recovery being after the longer runs. In addition plyometric training (consisting of bounding, depth jumping, squat-thrusts etc) was carried out between sprint sessions. The training period was preceded by a warm-up and ended with a warm-down. Training was essentially the programme of the sprint athlete with the omission of weight training which was intentionally excluded to limit any increase in muscle mass, an occurrence which would make interpretation of results more difficult. During the period of training the subjects were to keep to their normal diet and life-styles. After 6 days of completion of the training schedule the subjects underwent their post-training 30 s AWT with muscle biopsies.

6.3.4 The anaerobic work test

This was carried out in the same fashion as the 30 s AWT in chapter 5 and muscle biopsies and blood sampling was undertaken as outlined therein.

6.4 Results

6.4.1 Power output

After 8 weeks of training there was a considerable improvement in the subjects' performance during the AWT. Peak power output, a measure of the maximum rate of work that could be achieved, increased significantly by 7.5% and although the maintainance of this improved work rate throughout the 30 s sprint was not sustained, EPO was still significantly higher than before training (5.5%). As a result of the enhanced ability to perform work during the first 10-15 s of the test MPO was further increased by 8.1%. Despite these improvements the percentage drop in power output (FI%) was unaltered (table 6.1). The changes in the fatigue profile generated are illustrated in figure 15.

6.4.2 Anthropometric characteristics

To exclude significant changes in muscle mass as a factor influencing training induced improvements in performance detailed anthropometric measurements were made. Although training resulted in a significant reduction in body fat content both fat free mass and lean upper leg volume (LULV) were unaltered (table 6.2). As can be seen, training resulted in only a small change (+1.8%) in thigh musculature as represented by LULV and correcting performance characteristics for this does not materially alter the improvements recorded (table 6.1).

Table 6.1 The influence of training on the performance characteristics of the 30 s AWT [mean (±SD)]

	Before Training	After Training	% change	F-Test Ratio
Body Wt (kg)	72.39 (±12.20)	72.66 (±11.70)	+ 0.4	0.26
LULV (l)	9.80 (±1.36)	9.96 (±1.38)	+ 1.8	5.94*
Load (kp)	5.43 (±0.59)	5.43 (±0.59)	---	---
mean rpm	125.1 (±7.6)	135.4 (±9.2)	+ 8.2**	49.66**
MPO (W)	682 (±109)	737 (±116)	+ 8.1**	46.78**
PPO (W)	931 (±130)	1001 (±131)	+ 7.5**	55.91**
EPO (W)	490 (±90)	517 (±102)	+ 5.5*	12.71*
FI%	47.7 (±3.8)	48.6 (±4.8)	+ 1.9	0.80
PPO_i (W)	969 (±138)	1024 (±129)	+ 5.7**	23.96**
MPO/kg body wt	9.4 (±1.5)	10.1 (±1.6)	+ 7.7**	48.85**
PPO/kg body wt	12.9 (±1.8)	13.8 (±1.8)	+ 7.1**	22.67**
EPO/kg body wt	6.8 (±1.2)	7.1 (±1.4)	+ 5.1**	21.87**
MPO/l LULV	69.5 (±2.6)	73.9 (±3.1)	+ 6.3**	23.37**
PPO/l LULV	95.1 (±4.1)	100.7 (±5.5)	+ 5.9**	14.24**
EPO/l LULV	49.8 (±2.9)	51.6 (±3.9)	+ 3.6*	11.32*

LULV=lean upper leg volume (both legs); MPO=mean power output; PPO=peak power output; EPO=end power output; FI%=fatigue index (% decrease in PPO); PPO_i=instantaneous PPO

Statistical analysis by 1-way ANOVA for correlated means

*$p<0.05$ $F>5.99$
**$p<0.01$ $F>13.75$

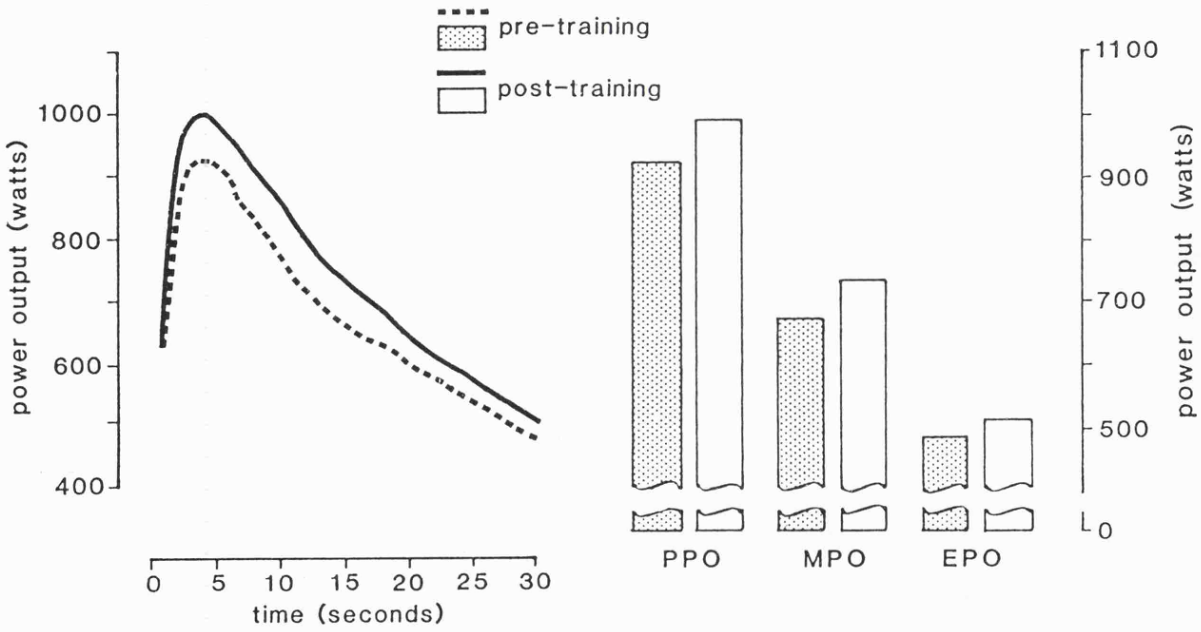


Figure 15 Influence of training on the fatigue profile and power generated during the 30 s AWT. There were significant improvements in PPO (Peak Power Output), MPO (Mean Power Output) and EPO (End Power Output) while the fatigue index was unaltered

Table 6.2 Training induced changes in anthropometric measurements and functional capacity [mean (±SD)]

	Before Training	After Training	% change	F-Test Ratio
Body Weight (kg)	72.39 (±12.20)	72.66 (±11.60)	+ 0.4	0.80
% Body Fat	8.70 (±2.07)	7.68 (±1.93)	- 11.7**	21.90**
Density	1.0793 (±0.0049)	1.0811 (±0.0046)	+ 0.2**	14.00**
Absolute Body Fat (kg)	6.29 (±1.71)	5.59 (±1.70)	- 11.1**	21.24**
Fat Free Mass (kg)	66.10 (±11.30)	67.05 (±10.70)	+ 1.4	4.45
Total Thigh Volume (l)	5.89 (±0.76)	5.88 (±0.75)	- 0.2	0.06
Lean Thigh Volume (l)	4.90 (±0.68)	4.98 (±0.69)	+ 1.8	0.05
Fat Thigh Volume (l)	1.00 (±0.25)	0.90 (±0.21)	- 9.9	1.15
Total Calf Volume (l)	2.70 (±0.41)	2.67 (±0.44)	- 1.0	0.36
Lean Calf Volume (l)	2.37 (±0.35)	2.36 (±0.37)	- 0.5	0.51
Total Leg Volume (l)	8.59 (±1.17)	8.55 (±1.17)	- 0.5	0.36
Total Leg Fat (%)	15.42 (±2.89)	14.18 (±2.59)	- 8.0**	15.52**
VO_2max test				
duration (min)	7.23 (±1.09)	7.71 (±1.01)	+ 6.6*	7.15*
work load (watts)	294 (±30)	300 (±29)	+ 2.0	2.12
VO_2max (l/min)	3.85 (±0.41)	3.90 (±0.47)	+ 1.3	0.57

Limb volumes are for the left leg only

Statistical analysis by 1-way ANOVA for correlated means

*$p<0.05$ $F> 5.99$
**$p<0.01$ $F>13.75$

6.4.3 Functional capacity

Although training resulted in small increases both in the VO_2max and the corresponding work load, these were not significant. The duration that the work could be maintained was however significantly greater, and it may have been that the failure to demonstrate a training induced increase in VO_2max was related to the sensitivity and reproducibility of the test as three subjects demonstrated a small increase, three a decrease and one remained the same (table 6.2).

6.4.4 Changes in blood metabolites

Blood lactate and glucose exhibited similar changes in concentration in response to the 30 s AWT as recorded in the first study. Glucose levels were unchanged immediately after the sprint but consistently rose after 5 min ($p<0.001$), whereas lactate concentrations had already risen more than two-fold at 0 min and reached a concentration of more than 12 mmol/l at 5 min. Although there was a tendency for post-training increases in response to the test to be lower, these changes were not significant and no change in post-exercise glucose concentrations with training was noted (table 6.3).

6.4.5 Muscle metabolites

Changes in muscle metabolites are summarised in table 6.4. Statistical analysis was by Student's Paired t-Test for the effect of exercise and training on changes in muscle metabolites and by a 2 way ANOVA to test within subject variation by exercise, training and the interaction between training and exercise on changes in metabolites. Tables 6.5a and 6.5b summarise these statistical results.

Table 6.3 Influence of training on blood lactate and glucose concentrations in response to 30 s AWT [mean (±SD)]

	Before Training	After Training	% change
Blood Lactate (mmol/l)			
Pre-AWT	1.59 (±0.51)	1.61 (±0.32)	+ 1.3 (ns)
0 min post AWT	*4.40 (±1.02)	*3.95 (±0.92)	- 1.3 (ns)
5 min post AWT	*13.30 (±1.36)	*12.56 (±1.43)	- 5.6 (ns)
Pre - 0 min post AWT	2.82 (±1.17)	2.34 (±0.76)	- 17.0 (ns)
Pre - 5 min post AWT	11.72 (±1.39)	10.95 (±1.39)	- 6.6 (ns)
0 - 5 min post AWT	8.90 (±0.82)	8.62 (±1.05)	- 3.1 (ns)
Blood Glucose (mmol/l)			
Pre-AWT	5.37 (±0.61)	5.52 (±0.42)	+ 2.8 (ns)
0 min post AWT	5.43 (±0.51)	5.58 (±0.52)	+ 2.8 (ns)
5 min post AWT	*6.38 (±0.57)	*6.58 (±0.67)	+ 3.1 (ns)

*$p<0.001$; One-tailed Student's Paired t-Test, pre-AWT v 0 min and 5 min post AWT
ns=not significant

effect of training tested by 1 way ANOVA for correlated means

Table 6.4 Influence of training on muscle metabolites in response to 30 s AWT in mmol/kg d.m. [mean (±SD)]

	before training		after training	
	pre AWT	after AWT	pre AWT	after AWT
glycogen - total	264.34 (±34.80)	190.36 (±39.70)	361.07 (±55.77)	250.63 (±43.22)
acid insoluble	216.89 (±14.00)	157.77 (±25.61)	321.13 (±45.15)	236.26 (±34.83)
acid soluble	47.46 (±24.46)	32.58 (±17.20)	39.87 (±19.34)	26.08 (±10.63)
Cr	27.89 (±7.64)	85.87 (±9.11)	19.42 (±6.18)	88.81 (±7.88)
total Cr	123.89 (±9.54)	119.00 (±6.49)	105.80 (±3.64)	117.62 (±5.93)
PCr	94.42 (±6.72)	33.13 (±6.62)	86.43 (±5.62)	28.81 (±3.31)
ATP	21.73 (±1.88)	12.18 (±2.94)	20.47 (±2.01)	11.10 (±2.44)
ADP	3.17 (±0.39)	2.99 (±0.39)	3.08 (±0.17)	3.52 (±0.22)
AMP	0.25 (±0.05)	0.20 (±0.06)	0.18 (±0.06)	0.23 (±0.11)
TAN	25.15 (±2.24)	15.38 (±3.22)	23.73 (±2.00)	14.84 (±2.51)
Glucose	2.24 (±0.45)	3.71 (±0.50)	2.65 (±0.63)	3.62 (±0.74)
G1P	0.12 (±0.04)	1.02 (±0.24)	0.14 (±0.07)	1.97 (±0.37)
G6P	1.24 (±0.17)	19.38 (±2.62)	1.14 (±0.26)	24.20 (±2.67)
F6P	0.17 (±0.07)	3.77 (±0.87)	0.15 (±0.09)	6.42 (±1.04)
F16BP	0.12 (±0.06)	1.44 (±1.30)	0.05 (±0.01)	1.45 (±1.30)
Triose phosphates	0.17 (±0.07)	0.61 (±0.28)	0.36 (±0.05)	0.57 (±0.23)
Lactate	8.91 (±3.73)	97.56 (±21.93)	8.31 (±3.54)	104.15 (±22.12)
Pyruvate	0.69 (±0.41)	3.26 (±1.26)	0.21 (±0.16)	0.96 (±0.46)
Citrate	1.17 (±0.24)	0.83 (±0.24)	1.25 (±0.13)	0.67 (±0.13)
Alanine	9.05 (±0.91)	11.02 (±2.18)	10.22 (±1.10)	12.47 (±1.21)
β-hydroxybutyrate	0.38 (±0.10)	0.38 (±0.08)	0.32 (±0.08)	0.32 (±0.04)

Cr=free creatine; total Cr=total creatine; PCr=phosphocreatine; ATP=adenosine triphosphate; ADP=adenosine diphosphate; AMP=adenosine monophosphate; TAN=ATP+ADP+AMP (total adenine nucleotide pool); G1P=glucose 6-phosphate; G6P=glucose 6-phosphate; F6P=fructose 6-phosphate; F16BP=fructose 1,6-bisphosphate

Table 6.5a Statistical summary of the influence of training on metabolite changes after 30 s AWT

	untrained pre AWT v post AWT	trained pre AWT v post AWT	pre AWT trained v untrained	post AWT trained v untrained
glycogen - total	t=13.43****	t=10.30****	t=-3.70*	t=-2.82*
acid insoluble	6.46****	13.36****	-4.79***	-4.66***
acid soluble	2.99*	2.80*	-0.67	-0.75
Cr	-17.45****	-17.72****	4.37***	-0.78
total Cr	1.45	-3.53***	6.44***	-0.37
PCr	17.96****	26.25****	3.34*	2.39*
ATP	8.05****	14.69****	1.14	-0.69
ADP	-0.48	-3.00*	0.55	-0.17
AMP	2.29*	-0.87	1.78	-0.50
TAN	8.01****	14.27****	1.19	-0.32
Glucose	-6.00****	-3.58***	-1.72	-0.22
G1P	-9.25****	-11.19****	-0.92	-5.68***
G6P	-17.19****	-21.98****	1.09	-5.01***
F6P	-10.62****	-14.91****	0.38	-4.44***
F16BP	-2.48*	-2.64*	2.39**	-0.08
Triose phosphates	-3.03*	-2.79*	4.29***	-1.34
Lactate	-11.21****	-11.90****	0.35	-0.67
Pyruvate	-6.69****	-4.66***	4.16***	5.01***
Citrate	2.90*	8.00****	-0.77	-1.46
Alanine	-2.25*	-7.14****	-2.04	-1.72
β-hydroxybutyrate	-0.51	-0.34	0.89	-1.60

Abbreviations as for table 6.4

Significance of changes in muscle metabolites with AWT, tested by one-tailed Student's paired t-test. Changes as a result of training tested with a two-tailed Student's paired t-test.

levels of significance

one-tailed		two-tailed
t>1.90	p<0.05*	t>2.37
t>3.00	p<0.01**	t>3.40
t>3.40	p<0.005***	t>4.03
t>5.41	p<0.0005****	

Table 6.5b Statistical summary of the influence of training on metabolite changes after 30 s AWT

	within subject variation		
	training	30 s AWT	interaction training/AWT
glycogen - total	F= 11.79*	F=350.38**	F= 6.96*
acid insoluble	25.87**	217.17**	8.76*
acid soluble	0.55	14.96**	0.02
Cr	1.00	339.96**	25.85**
total Cr	9.96*	1.20	54.00**
PCr	13.71*	544.63**	1.93
ATP	1.01	210.59**	0.02
ADP	0.00	2.19	0.10
AMP	0.62	0.00	1.78
TAN	0.60	198.05**	0.39
Glucose	0.41	142.29**	1.13
G1P	44.38**	207.85**	23.69**
G6P	19.42**	424.47**	32.76**
F6P	18.38**	398.57**	20.95**
F16BP	0.25	6.63*	0.54
Triose phosphates	2.19	8.72*	9.81
Lactate	0.28	188.45**	0.70
Pyruvate	26.18**	56.44**	21.70**
Citrate	0.32	41.12**	3.32
Alanine	5.04	18.77**	0.17
β-hydroxybutyrate	0.16	0.00	0.58

Abbreviations as for table 6.4

The effect of training, exercise and the interaction between training and exercise determined by 2-way ANOVA (repeated measures).

level of significance

$p<0.05$* $F> 5.99$
$p<0.01$** $F>13.75$

a) Pre-AWT metabolites

Muscle glycogen increased by 37% as a result of training, a value similar to that reported by other studies (see table 2.6). This was confined to the acid insoluble fraction and although Jansson (1981) has suggested that the acid soluble glycogen may be the more metabolically active this fraction remained constant. Both free and total creatine concentrations were lower after training and this was associated with a small but significant decrease in PCr and a small but non-significant decrease in ATP. Whether this represents a true fall in phosphogen concentration or not is difficult to say. Certainly because of the variability of values due to contamination with blood and small fragments of connective tissue, Harris et al (1976) have suggested that all metabolites should be corrected for a constant total creatine concentration of 124.4 mmol/kg d.m. It is possible that due to an increase in active capillaries as a result of training (see chapter 2, section 2.6.2), more red blood cells was included in the post training biopsy specimen which was taken after a period of warm-up exercise. As the extraction method used was designed to eliminate such errors as much as possible, no attempt was made to correct for a constant Cr level to compensate for this change in the reference base but it will be assumed that this represents a sampling error. Lower resting F16BP levels were measured after training and this may indicate a favourable change in the substrate cycling of F6P to F16BP (see Newsholme and Leech, 1983b). It is difficult to account for the changes seen in pyruvate concentrations. The levels found after training are similar to those in the first study whereas the pre-training levels are much higher, and they are considerably higher than the reference range for normal metabolites that has been established. It is possible that these high pyruvate values represent metabolic changes to the warm-up which were abolished by the

adaptation to training rather than true changes in resting concentrations (see discussion).

b) Post-AWT metabolites

After the sprint the metabolic changes were very close to those reported in the previous study. Total glycogen fell 28% (74 mmol/kg d.m. glucosyl units) and this was largely confined to the acid insoluble fraction although a small decrease in the soluble glycogen was also seen. There was no change in total Cr, demonstrating the efficiency of the extraction method for the metabolites, and PCr and free Cr changed reciprocally there being a 65% fall in the former (58 mmol/kg d.m.) and a 208% rise in the latter (58 mmol/kg d.m.). As before there was a 44% fall in ATP and a corresponding 39% decrease in TAN. Hexose monophosphates demonstrated a dramatic increase (16-fold) with G1P increasing 750%, G6P 1460% and F6P 2100% and there was a small but significant increase in glucose (66%). Confirming the importance of the regulatory role of PFK there was a more modest 6-fold increase in F16DP and triose phosphates. Lactate demonstrated an 11-fold increase to a level of almost 100 mmol/kg d.m. (similar to the first study) but the higher pre-AWT pyruvate levels were reflected in a much higher post-exercise concentration. There was again a small but significant increase in alanine but there was now in addition, a significant fall in muscle citrate concentration (29%).

After training 111 mmol/kg d.m. of glycogen were utilised representing a 31% drop from, it should be noted, the much higher initial concentration and this rate of glycogen utilisation was significantly increased by the training effect. Interestingly post-sprint total Cr levels were the same before and after training and were close to the reference value suggested by Harris et al (1976) of 124.4

mmol/kg d.m. This resulted in there being a significant interaction between training and the AWT on Cr and total Cr. As exercise per se is not going to alter muscle Cr this lends further support to the argument that the after training pre-AWT values were artifactually low. Applying a 17% correction factor to the pre-AWT values to take this into account did not alter the relationships between the other metabolites with the exception that the significant difference between the pre-AWT pyruvate levels was abolished. Additionally a small but significant difference in muscle glucose now existed. Despite this it was felt that no correction should be applied and the raw data was used throughout. Phosphocreatine and ATP decreased by the same amounts as before training (67% and 46% respectively) and there was a small increase in ADP, not seen prior to training. The most obvious effects of training were the considerable increase in the HMP as would be expected by the increased mobilisation of glycogen. Total HMP were now 23-fold more than the pre-AWT values with corresponding increases in G1P (1300%), G6P (2000%) and F6P (3650%) but there was no absolute increase in the concentrations of F16DP and the triose phosphates when compared with the values before training. Although muscle lactate had now increased 12.5-fold this did not represent a significant training induced increase and training had no effect on the changes in citrate and alanine concentrations. The changes in the glycolytic intermediates are illustrated in the 3-dimensional pathways chart in figure 16. Before training 97% of the glucosyl units from glycogen could be accounted for but this fell to 74% after training.

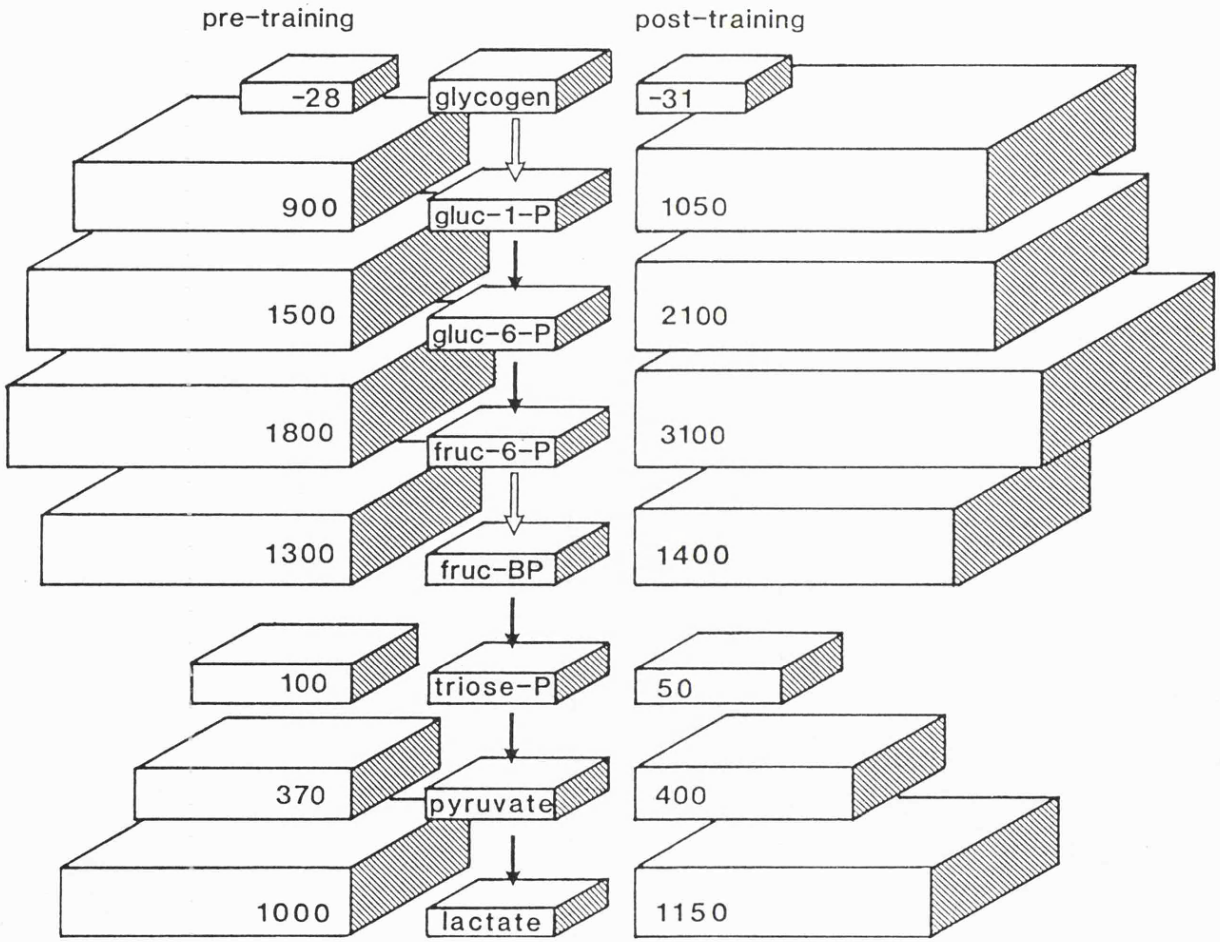


Figure 16 Three dimensional pathways chart illustrating the influence of training on the percentage change in glycolytic intermediates after the 30 s AWT. Details as for Figure 10. The backing-up of glycolytic intermediates above PFK (the open arrow between fruc-6-P and fruc-BP) is very evident.

6.4.6 Muscle pH

Both pre-exercise and post-exercise muscle pH as derived from the changes in lactate and pyruvate (equation 5.1) were the same as the first study and no training effect was observed, the post sprint pH being 6.64 and 6.63 before and after training respectively.

6.4.7 ATP turnover

Pre-training total ATP turnover and the relative contributions from glycolysis, PCr and ATP were similar to the first study, the rate and contributions being 6.92 mmol/kg d.m./s and 66%, 29% and 5% respectively. There was a small increase in total ATP turnover after training to 7.06 mmol/kg d.m./s and the proportions altered a little, glycolysis now supplying 68%, PCr 27% and ATP 4%, but none of these trends were significant (figure 17). Rates of ATP turnover together with derived values for pH, glycogenolysis and glycolysis are summarised in table 6.6.

6.4.8 Glycogenolytic and glycolytic rates

Glycogenolysis and glycolysis were calculated as previously described (equations 5.4 and 5.5). Before training values were very simliar to those obtained in the first study with rates of 2.43 and 1.63 mmol/kg d.m./s for glycogenolysis and glycolysis respectively. With training glycogen breakdown increased to 2.73 mmol/kg d.m./s and the corresponding increase of glycolysis to 1.76 mmol/kg d.m./s resulted in the ratio of glycogenolysis to glycolysis remaining constant at 1.5:1 but these increases did not reach statistical significance (figure 18).

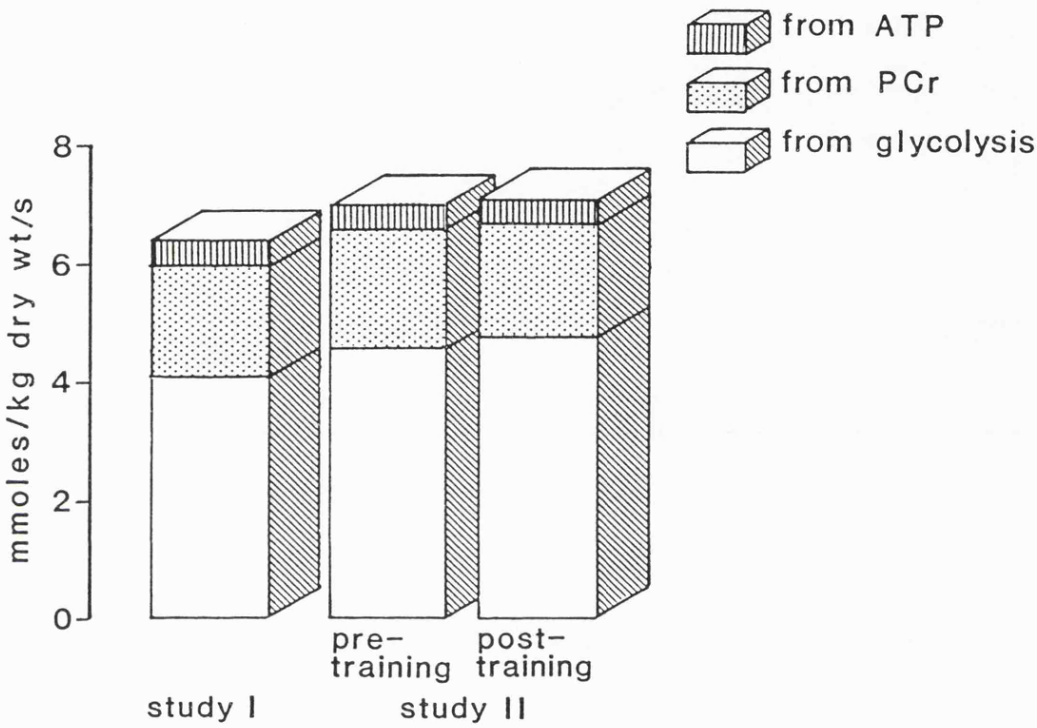


Figure 17 Derived total ATP turnover rates during the 30 s AWT before and after training. Values for study I included for comparison. There were no significant differences between the relative contribution to ATP supply from the different anaerobic sources comparing Study I and pre- and post-training Study II.

Table 6.6 Influence of training on calculated values for muscle pH, glycogenolytic and glycolytic rates and anaerobic ATP turnover rates (total, glycolytic, from PCr and from ATP). Rates are given as mmol/kg d.m. [mean (±SD)]

	before training	after training	% change
pH before sprint	7.02 (±0.02)	7.02 (±0.02)	0.0 (ns)
pH after sprint	6.64 (±0.10)	6.63 (±0.10)	- 0.15 (ns)
ΔpH	-0.38	-0.39	+ 2.6 (ns)
glycogenolytic rate	2.43 (±0.47)	2.73 (±0.43)	+12.3 (ns)
glycolytic rate	1.63 (±0.38)	1.76 (±0.39)	+ 8.0 (ns)
total ATP turnover	6.92 (±1.37)	7.06 (±1.25)	+ 2.0 (ns)
ATP glycolytic	4.56 (±1.08)	4.83 (±1.08)	+ 5.9 (ns)
ATP from PCr	2.04 (±0.30)	1.92 (±0.19)	- 5.9 (ns)
ATP from ATP	0.32 (±0.10)	0.31 (±0.06)	- 3.1 (ns)

ns=not saignificant, tested by two-tailed Student's paired t-test and one-way ANOVA for correlated means

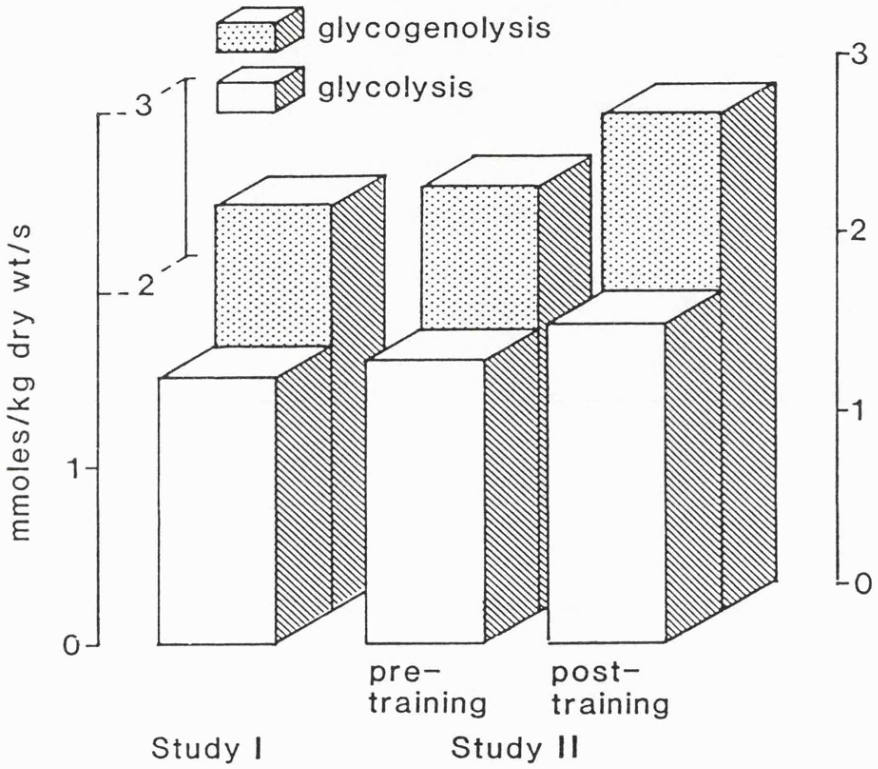


Figure 18 Glycogenolytic and glycolytic rates during the 30 s AWT before and after training. Values for study I included for comparison. Although the rate of glycogenolysis increased after training this was not a significant gain. Glycolysis was largely unchanged.

6.5 Discussion

The improvements in performance with training are similar to those reported in other studies employing a supra-maximal bicycle ergometer test (Weltman et al, 1978; Campbell et al, 1979; Belcastro et al, 1981) although it is notable that the previous studies have used female subjects. Similar increases in different aspects of performance have also been reported using different exercise and training models (see table 2.6). Metabolic adaptations of muscle to high intensity interval training have been variably reported. An increase in VO_2max has been an invariable finding even with apparently anaerobic training (table 2.6) and this has been associated with an increase in muscle oxidative capacity. Although the ratio of %FT/%ST fibres does not appear to change, there is a tendency for the FT fibre area to increase with sprint or resistance training (Thorstensson et al, 1975; Bylund et al, 1977; Costill et al, 1979).

6.5.1 Phosphogens and phosphogenolysis

As it has been suggested that PCr and ATP stores may be higher in FT fibres (see table 2.4) such a change could lead to an increased muscle phosphogen composition. This has been demonstrated by a number of workers for both PCr (Eriksson et al, 1973; MacDougall et al, 1977) and ATP (Karlsson et al, 1972; Eriksson et al, 1973; MacDougall et al, 1977) but has not been found to be an invariable metabolic adaptation (see table 2.6). Evidence to support the importance of an improved contribution to energy supply from phosphogenolysis is provided by the finding by some workers (Thorntensson et al, 1975; Costill et al, 1979) that contractility enzymes (CPK and ATPase) are increased by training. However the findings reported in this chapter do not entirely support

the theory that phosphogen contribution to anaerobic energy supply is increased by training. There was a small but significant (although probably artifactual) decrease in pre-AWT PCr concentration while ATP levels were unchanged. The decrement in PCr before training was 61.3 mmol/kg d.m. over the 30 s sprint compared to 57.6 mmol/kg d.m. after training (a significant difference). Correcting all values, though, for a constant total Cr concentration of 124.4 mmol/kg d.m. resulted in a 18% increase in the post-training utilisation of PCr, values which were still significantly different but reversed in direction. Consequently anaerobic energy contribution from PCr was altered by an equivalent and significant amount and although total ATP turnover was increased this change was not significant. No such change was found when similarly correcting ATP concentrations. It remains debatable therefore whether or not training increases muscle phosphogen concentrations and the contribution of PCr to anaerobic energy supply. Using the raw data no change is found but when corrected for a constant total Cr a significant interaction between exercise and training is revealed.

6.5.2 Glycogen, glycogenolysis and glycolysis

Muscle glycogen is uniformly found to increase with training and a value of the order of 30% has been frequently reported (see table 2.6). Although the rate of glycogenolysis and lactate accumulation is decreased when glycogen falls below about 50% of its initial value (150 mmol/kg d.w.) there is no evidence to support an increased glycolytic activity with an elevated glycogen (Jacobs, 1981). An increase in muscle glycogen by itself is unlikely to result in an improved rate of glycolysis. Training induced improvements in glycogenolytic and glycolytic enzyme activity have been reported. Gollnick et al (1973) found a

more than doubling of phosphorylase activity after 5 months bicycle ergometer training and an increase of 29% was reported by Bylund et al (1977) after 7 months training. After a much shorter training interval (7 weeks) Costill et al (1979) still found a significant increase (11%) with an associated improvement in PFK activity of 23% and Eriksson et al (1973) found an 83% increase in PFK after just 6 weeks of bicycle ergometer training in 11-13 year old boys. Although the utilisation of glycogen as determined from a fall in total glycogen was considerably increased by training, from 74 to 110 mmol/kg d.m. over the 30 s sprint, this was not associated with a corresponding increase in glycolytic intermediates and lactate of the same magnitude. Before training 72 mmol/kg d.m. of intermediates equivalent to 97% of the loss of glycogen could be accounted for but after training although the accumulation of intermediates increased to 83 mmol/kg d.m. this was only 75% of the fall in glycogen. As there was no associated increase in the blood lactate immediately after the sprint or at 5 min, this loss is unlikely to represent a marked increase in lactate clearance. Additionally, as discussed in chapter 5, it is extremely unlikely that this amount of glycogen can have been subject to complete aerobic breakdown. One possibility is that as glycogen is relatively fixed within the fibre matrix there can be no diffusion effect whereas lactate has been shown to diffuse between fibres (Essén and Häggmark, 1975) and this coupled with the variability of the analytical methods could account for this finding. Therefore although an increased glycogenolytic activity occurs using changes in glycogen, the smaller increase calculated from intermediates (2.43 to 2.73 mmol/kg d.m./s) is not significant. Consequently the similarly derived glycolytic rates are not significantly different before and after training (table 6.6). Although the higher post-AWT

muscle lactate seen after training was not statistically significant, with the trend reported by Eriksson et al (1973) and Sharp et al (1986), it may as the latter workers suggest reflect an improvement in muscle buffering capacity. Whether this occurs by change in physico-chemical buffering or improved hydrogen ion clearance (transient buffering) remains to be answered.

6.5.3 Pyruvate

Training resulted in a decrease in muscle pyruvate levels both pre-AWT and after the sprint without further changes in citrate or alanine concentrations. Although this could reflect a training induced increase in LDH activity this enzyme does not seem to be induced by training (Thorstensson et al, 1975; Houston and Thomas, 1977). Additionally Brooks (1985), as discussed in chapter 2, section 2.1, pointed out that the kinetics of LDH are such that lactate is normally produced without pyruvate accumulation. Although training did not significantly increase muscle alanine concentrations it did increase by about the same amount as the decrease in pyruvate. Felig (1977) has reviewed the importance of alanine as a means of transferring an oxidisable fuel to the liver while at the same time utilising the ammonia produced by AMP deamination and although under normal circumstances the alanine-pyruvate cycle is of little quantitative importance to the exercising muscle (Newsholme and Leech, 1983b) it is possible that under extreme conditions the pathway may be useful in decreasing muscle NH_4 concentration which itself has been implicated in fatigue (see Mutch and Banister, 1983 for review). However as the formation of 2-oxoglutarate by Kreb cycle activity is dependent on the availability of oxygen, this mechanism, if it occurs, would by necessity need to be very short lived in supramaximal

(anaerobic) exercise.

6.5.4 ATP turnover rates

As most of the energy supply during the sprint is derived from glycolysis it is not surprising that anaerobic ATP turnover rates are not influenced by training, using the values derived from changes in phosphogen concentration and from accumulation of glycolytic intermediates (table 6.6). Recalculation of these values with metabolite concentrations corrected for a total creatine concentration of 124.4 is shown in table 6.7. Despite the resultant marked increase in total ATP turnover and the greater glycolytic ATP turnover with these new derived values these changes were still not significant. As discussed above ATP from PCr was now greater, but the relative percentage contribution to energy supply from each source was not materially altered.

6.5.5 Conclusions

Training does not therefore appear to alter the relative contribution of the various components of anaerobic energy supply. There is a trend towards a greater provision to energy from glycolysis if the accumulation in HMP and lactate and the marked fall in glycogen are considered together but these changes are not significant. These findings are in keeping with the conclusions of Parkhouse and McKenzie (1984) who in their excellent review on anaerobic performance surmised that with sprint-training the resultant increases in power output are greater than the enhanced ability to produce ATP.

Table 6.7 Anaerobic ATP turnover rates calculated from changes in PCr, ATP, lacatate and pyruvate corrected for a constant total Cr concentration of 124.4 in mmol/kg d.m./s [mean (±SD)]

	before training	after training	% change	
total ATP turnover	7.10 (±1.53)	7.84 (±1.31)	+10.4	(ns)
ATP glycolytic	4.79 (±1.13)	5.06 (±1.13)	+ 6.8	(ns)
ATP from PCr	2.01 (±0.31)	2.37 (±0.22)	+17.9	$p<0.01$
ATP from ATP	0.30 (±0.11)	0.41 (±0.06)	+36.7	(ns)

	uncorrected values		corrected values	
	before	after	before	after
% total ATP turnover				
ATP glycolytic	65.9	68.4	67.5	64.5
ATP from PCr	29.5	27.2	28.3	30.2
ATP from ATP	4.5	4.4	4.2	5.2

Tested by two-tailed Student's paired t-test and one-way ANOVA for correlated means.

ns=not significant

before=before training; after=after training

Chapter 7

7 Metabolic changes in gastrocnemius muscle in patients with ischaemic rest pain and the influence of treatment with intravenous naftidrofuryl.

The purpose of this study was to examine any changes in muscle metabolism that occurred in patients with ischaemic rest pain due to occlusive arterial disease. This was combined with a trial of the assessment of intravenous naftidrofuryl examining three important areas of the drug's actions; clinical, physiological and biochemical.

7.1 Introduction

7.1.1 Muscle metabolism in ischaemic rest pain

Changes in skeletal muscle metabolism in patients with peripheral vascular disease have been discussed in chapter 3, section 3.5. Although there has been a considerable amount of work on the enzyme adaptations to ischaemia, there is far less information on metabolite changes. Pernow et al (1975) have reported a lower than normal ATP concentration in patients with intermittent claudication, but PCr, glycogen, G6P and lactate levels were similar to control values. These samples were however taken from the quadriceps muscle. In ischaemic rest pain, there is insufficient blood circulating for the aerobic needs of the limb even at rest, and this deficit is more marked distally. It was felt that under these circumstances the most severely affected muscle that could be safely biopsied was the calf muscle. Although there is not the same ammount of data on normal values for metabolites in the gastrocnemius there is no evidence that resting levels of

glycogen, phosphogens and metabolic intermediates are any different to the quadriceps (Maughan, personal communication). Elert et al (1976) published values for patients with severe claudication and rest pain. They found a considerably depressed PCr concentration (mean 51 mmol/kg d.m.) and surprisingly very high ATP levels (mean 27.6 mmol/kg d.m.), with those patients with more severe disease (rest pain) having more depressed concentrations (37 and 21 mmol/kg d.m. respectively). Adenosine monophosphate levels seemed high (1.2 mmol/kg d.m.) and muscle lactate was in the order of 20 mmol/kg d.m. Despite the variability of the results, if the trend reported is true then there should be some value in studying muscle metabolites in the severely ischaemic limb. Additionally, although muscle oxidative capacity seems to be increased in patients with intermittent claudication (Elander et al, 1985) there is some evidence to suggest that it is depressed in patients with more severe ischaemia (Bylund et al, 1976).

7.1.2 Naftidrofuryl

The drug naftidrofuryl (Praxilene, Lipha) was developed at the Lipha-Observal Research Centre, Lyon (Szarvasi et al, 1966). Initial pharmacology revealed it to have a wide range of actions. It is a powerful local anaesthetic (four times more effective than lignocaine), has antiserotonin and antibradykinin effects and is a potent vasodilator (Fontaine et al, 1968). Additionally, the drug has been shown to have a beneficial effect on tissue glucose utilisation, particularly when it is hypoxic (Meynaud et al, 1973 and 1975; Shaw and Johnson, 1975) and it is thought that one of the drug's actions is somehow mediated through its ability to increase succinic dehydrogenase activity with a subsequent improvement of oxidative phosphorylation (Meynaud et al, 1973).

Despite its wide range of activity, naftidrofuryl exhibits remarkably few side effects. It is not associated with a fall in blood pressure or with the "borrowing-lending" phenomenon and the only problem that has received any attention has been that of peripheral thrombophlebitis at the site of infusion, although this was somewhat controversial (Fontaine et al, 1968; Woodhouse and Eadie, 1977; Heidrich, 1978; Heidrich and Shartl, 1978). Naftidrofuryl has had wide use in France and Germany prior to its introduction into this country in 1972, and numerous studies have shown it to be effective in the treatment of patients with peripheral vascular disease of the legs, even when associated with rest pain (Di Maria, 1968; Eichhorn, 1969; Becker, 1970; Böttinger and Raithel, 1971; Hauch and Lévai, 1973; Ratschow, 1976; Pohle et al, 1979; see Chapter 3, section 3.7.7). In spite of this there had not been a well controlled double-blind study of the drug, investigating all important areas of action.

7.2 Protocol

The trial was organised by the author, recruiting patients of Professor P.R.F Bell and Mr. W.W. Barrie at the Leicester Royal Infirmary and Leicester General Hospital respectively. It was structured as a double-blind controlled trial with two main groups, diabetics and non-diabetics. Patients were eligible for the trial if they had ischaemic rest pain due to superfical femoral artery occlusion which did not abate after 48 hours bed rest and analgesia, and in whom the ankle systolic pressure was less than 40 mm Hg if no skin lesions and less than 60 mm Hg if ulcers or skin lesions (Bell et al, 1982). Patients received the test-substance intravenously for 10 days followed by oral therapy for a period of 3 months during which time they were

followed-up monthly. The test-substances (intravenous and tablet preparations) were supplied by Lipha Pharmaceuticals in an already randomised allocation of active drug and placebo. The code for the trial was kept by Mr. T. Brown, Technical Director of Lipha and (in sealed envelopes) by the pharmacies of the two hospitals.

On admission patients had their relevant data recorded on a proforma sheet prepared by the author and had a chest X-ray, ECG, full blood count, platelet count, plasma viscosity, prothrombin time, biochemical profile (SMAC) and blood sugar, together with an arteriogram before any treatment was commenced. Minor gangrene of the toes was dealt with at the discretion of the consultant in charge, and any concomitant disease treated as necessary. Mobility of the patients was at the discretion of the ward sister.

Clinical assessment was by use of daily linear analogue rating scales, and daily inspection of the affected limb. Physiological assessment was by measurement of resting and hyperaemic calf blood flow together with an estimate of ankle and calf systolic blood pressure by strain gauge plethysmography on days 0, 4 and 9 of intravenous therapy. Biochemical assessment was carried out on days 0, 5 and 10 on needle biopsy specimens of gastrocnemius muscle, and on a concomitant sample of venous blood. Clinical assessment was repeated at monthly follow-up for 3 months, compliance of treatment being checked by tablet count at each follow-up visit. After this, patients were discharged from the trial. A summary of the protocol is illustrated in figure 19.

7.2.1 Withdrawal and exclusions from the trial

a) Withdrawal

All patients in whom there was a clinical deterioration or obvious

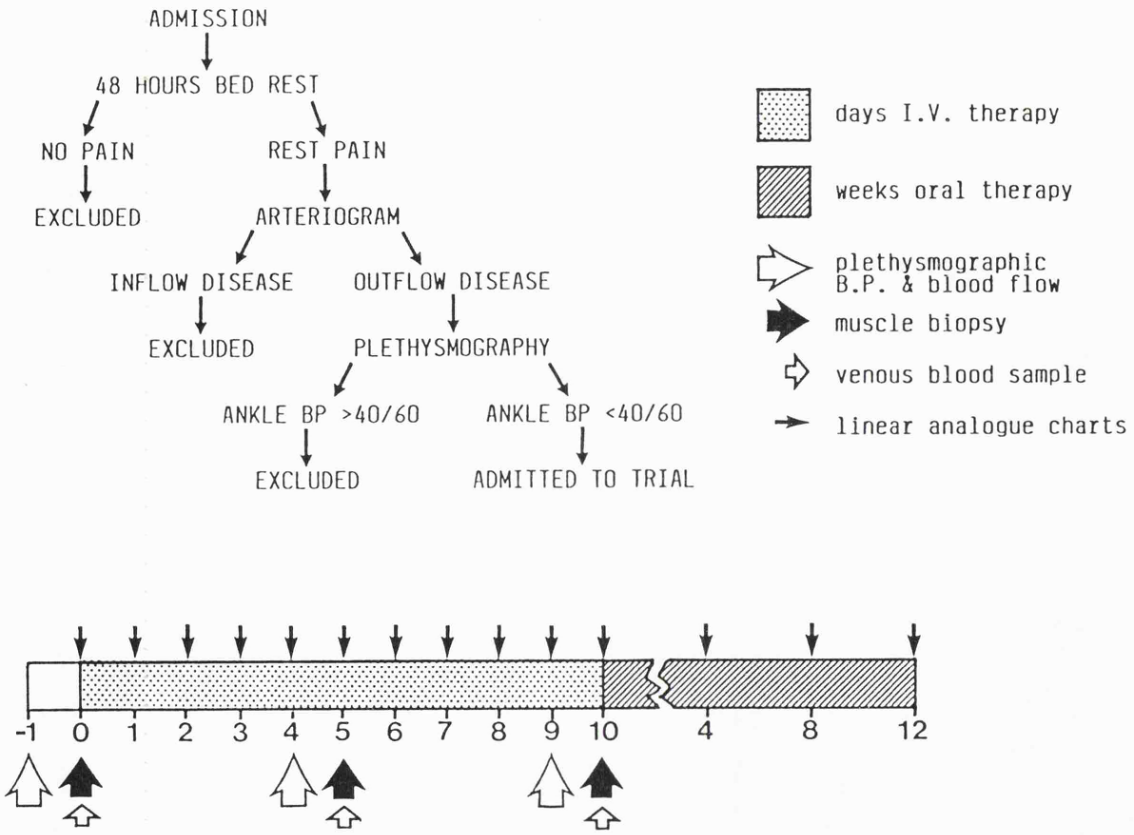


Figure 19 Summary of the protocol for the naftidrofuryl rest pain trial. A flow chart as to the selection of patients is illustrated together with an outline of the timing of the various measurements and tests.

Ankle BP 40/60 = Ankle sytolic pressure of 40 mmHg without skin lesions and 60 mmHg with skin lesions.

failure to relieve disturbing rest pain were withdrawn, the decision being made by the consultant in charge, and alternate treatment undertaken.

b) Exclusions

i) All patients with absent femoral pulses; these patients were treated by reconstructive surgery.

ii) All patients who obviously required amputation at initial assessment

iii) All patients who had an atrio-ventricular conduction defect.

7.3 Methods

7.3.1 Administration of the test substance

Having entered the trial, patients were allocated a trial number which corresponded to the test substance (drug or placebo). This was given by continuous intravenous infusion into a central venous catheter (inserted by the author by the percutaneous infraclavicular route) by an peristaltic infusion pump (IVAC model 531) at a dose of 1200 mg in 1 litre of 0.9% saline administered over a period of 24 h. Although there had been considerable discussion as to whether or not naftidrofuryl caused peripheral thrombophlebitis (Woodhouse and Eadie, 1977 and subsequent correspondance) it has been suggested that this side effect was most likely to occur when a high concentration was used at a low flow rate (Charlesworth, 1977). As these criteria were met by the chosen method of administration, the volume of infusate being kept deliberately low to prevent acute haemodynamic changes, a central route of administration was used. The dosage was higher than normally given in this country but common on the continent and it was chosen because it

was felt that in view of the drug's effects and short half-life (40 min) a continuous infusion at that concentration should be given to ensure a constant therapeutic blood level of 500 ngm/ml (Ratschow, 1976; Lartigue-Mattei et al, 1980). The trial substance was supplied already added to the perfusate, the bags having been prepared under strict aseptic conditions by the Sterile Products Unit at the Leicester Royal Infirmary Pharmacy under the control of Mrs. Tessa Hamp. After 10 days patients were converted to oral therapy at a dose of 200 mg 8 hourly.

7.3.2 Clinical assessement

a) Subjective sensations

Assessment of pain and related symptoms was performed by the use of linear analogue charts (figures 20a and 20b). Ratio scaling, in which a patient is asked to assign a number, usually from 0 to 10, proportional to the relative magnitude of pain between the extremes of no pain and unbearable pain has been shown to be a reproducible method (Clarke and Spear, 1964). Substitution of the numbers by line length gives a visual analogue scale, the two extremes of pain being separated by a clearly defined line of fixed length (usually 10 cm) with clear cut-off points at each end, and in addition this can be used for the measurement of other feelings (Aitken, 1969). The patient marks on the line the point at which he or she feels his or her pain falls, and the distance from the "no pain" end is measured in mm and scored. Advantages include infinite variability, the obviation of bias towards favoured numbers and the ability to statistically analyse the results (Bond and Pilowski, 1966). Linear analogue scales have been shown to be accurate and reproducible (Revill et al, 1976; Downie et al, 1978) and the technique has been successfully used in assessing the response to treatment with

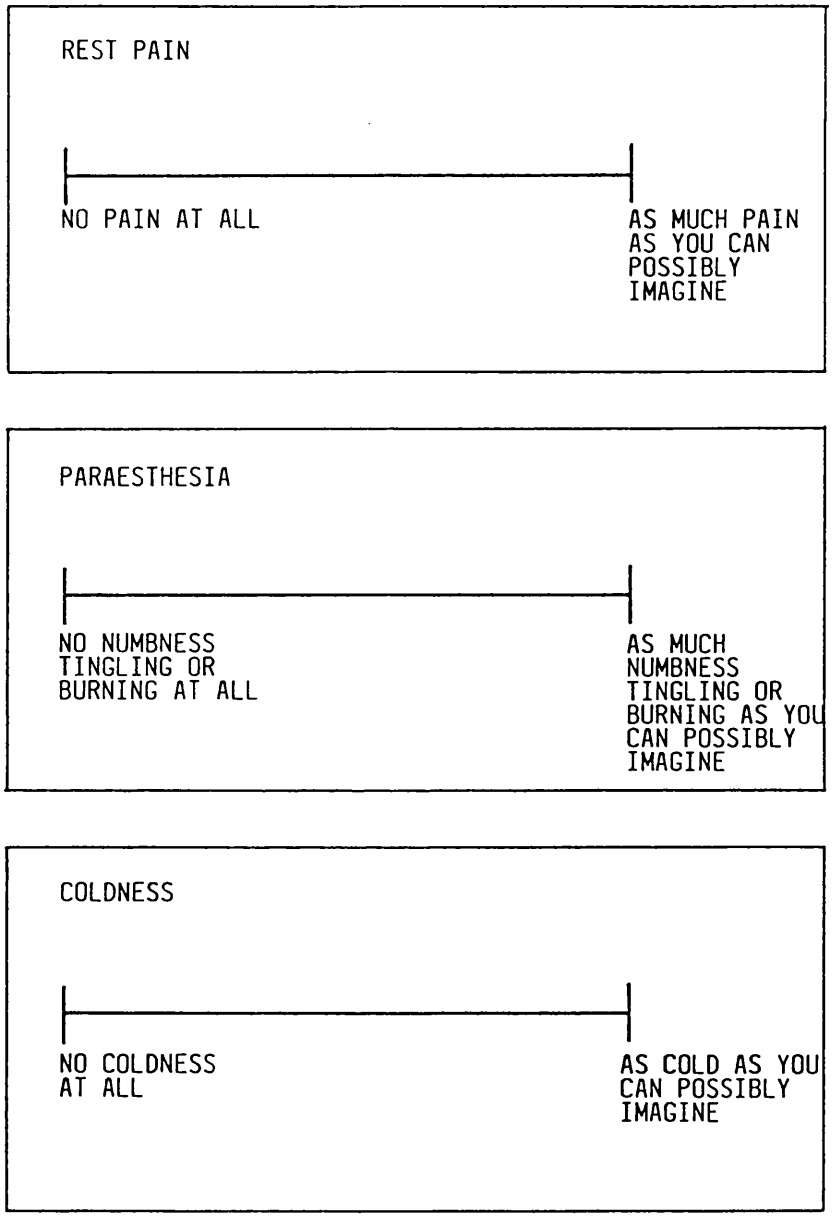
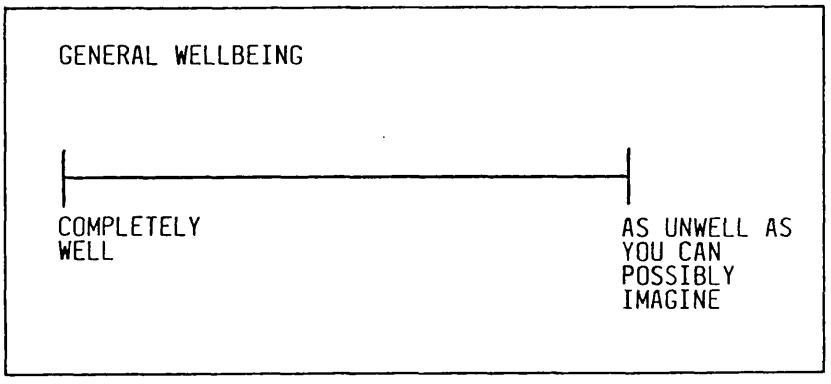
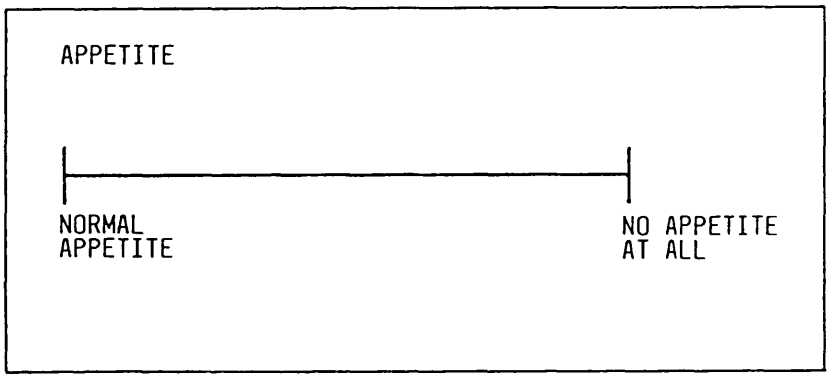


Figure 20a Linear analogue charts for Rest Pain, Paraesthesia and Coldness. The lines measured 100 mm and each parameter was printed on a seperate form.





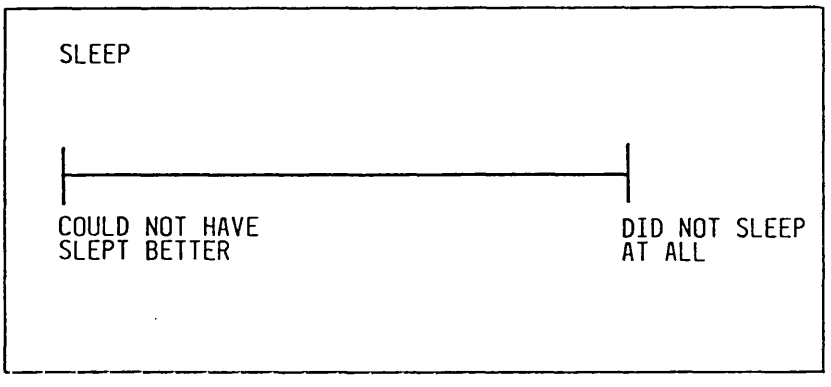


Figure 20b Linear analogue charts for General Wellbeing, Appetite and Sleep. See Figure 20a.

intravenous naftidrofuryl (Wong and McBain, 1980; Meehan et al, 1982).

Six charts each containing an individual parameter (pain, paraesthesia, coldness, general wellbeing, appetite and sleep) were handed to the patients at 9.00 a.m. each morning, or 4 h after the last administration of analgesia, if the patient had recently received this. After initial familiarisation before starting the trial, in which assistance and explanation in their use was given, the patient was encouraged to fill in the charts on his or her own. All patients adapted very well to their concept and no problems were encountered in their use.

b) Analgesia requirements

Analgesia was standardised to paracetamol tablets 0.5 g, dihydrocodeine tablets 30 mg and morphine sulphate injections 10 mg. These were given on demand 4 hourly, if required, at doses of 0.5-1 g, 30-60 mg and 10-20 mg respectively. Because it was felt likely that the more severe the patients' pain the stronger the analgesia that they would be given, a scoring system for analgesia consumption was adopted; each 0.5 g tablet of paracetamol scored 1, 30 mg of DF118 scored 2 and 10 mg of morphine scored 4. This allowed statistical analysis to be made on the total consumption using non-parametric tests.

7.3.3 Physiological assessment

a) Arteriography

As all patients being admitted to the trial were being considered for limb salvage surgery, arteriography was a prior requisite for inclusion. This was carried out by the radiology departments at the two hospitals where patients were based, using standard radiological techniques. To quantify the angiographic appearances, a scoring system

was again adopted. This was based on the appearance of the lower limb arteries at three sites; above the inguinal ligament, the upper leg and below the knee. A score of 0 was given if the artery was apparently disease free, 1 if atheromatous but not more than 70% stenosed, 2 if more than 70% stenosed but not occluded and 3 if occluded. These scores were given to the inflow vessels (common iliac, external iliac or common femoral, whichever was most severely diseased), the superficial femoral or popliteal segment, the profunda femoris and each of the three tibial vessels. Thus a patient with a normal arterial tree would score 0 and if all vessels were completely blocked he would score 18.

b) Measurement of leg blood flow and segmental blood pressure

This was carried out at initial assessment of the patient (day "0"), when if the ankle pressure measurements did not fulfil the criteria of the trial the patient was excluded, and again after 4 and 9 days of intravenous treatment. The procedure was carried out at the patients' bed side on the ward which offered several advantages: it was less trouble to the patients as they did not have to be moved to another department; the patients were well rested; as the wards were at the relatively constant temperature of 24°C ambient temperature as a variable affecting blood flow was excluded; the problem of transporting patients attached to an infusion pump was overcome. Measurement of resting flow was obtained using the average value of 20 consecutive meaurements taken at 12 s intervals, using a venous occlusion pressure of 30 mm Hg with an 8 s inflation time and a 4 s deflation time. Peak flow was measured as the maximum hyperaemic response to 3 min tourniquet ischaemia. An arterial cuff pressure of 250 mm Hg was used for this and the patients tolerated the 3 min ischaemia very well. Sampling intervals of 12 s were used to record the reactive hyperaemia with the

same venous occlusion pressure and ratio of inflation to deflation time as for resting flow. Ankle and calf systolic pressure were determined as the pressure at which an increasing volume change was noted with progressive deflation of calf and thigh cuffs respectively.

Both blood flow and ankle pressures were measured with mercury in silastic strain gauges using a Medimatic SP2 dual channel plethysmograph with an automatic balancing strain gauge bridge system and a Flowmatic automatic inflation/ deflation unit (Medimatic, Copenhagen). Whitney (1953) has validated the principle of mercury in rubber strain gauge plethysmography, and its use in evaluating patients with peripheral vascular disease was the subject of an excellent paper by Myers (1964) who drew attention to the importance of a delay in the time to peak flow as indicative of disease proximal to the strain gauge. Since then a number of modifications of the device (employed in the Medimatic SP2) have been made, the most significant of which has been an automatic electrical calibration (Hallböök et al, 1970). The validity of the procedure has further been demonstrated by Englund et al (1972) who were able to show a linear relationship between blood flow measured by direct perfusion of the leg and plethysmographically. Dahn (1965) has provided evidence to suggest that the optimum period of arterial occlusion to induce the most reproducible hyperaemic response is 3 min, while Lorentsen et al (1970) in a very thorough study have drawn attention to the importance of the venous occlusion pressure. Although this is not critical in normal subjects, a collecting pressure below diastolic pressure being adequate, the fall in blood pressure seen in patients with vascular disease after exercise or in response to tourniquet hyperaemia makes the collecting cuff pressure more critical. For the study, as Lorentsen et al (1970) reported, a pressure of 30 mm Hg was

found to give reproducible results, this pressure being sufficiently low not to occlude the thigh circulation yet high enough to effectively occlude the venous system. In addition to the peak blood flow and time to peak flow, the duration of the hyperaemic response was measured (Van De Water et al, 1980). The technique and validity of strain gauge plethysmography in the measurement of segmental systolic blood pressure has been the subject of an excellent thesis by Gundersen (1972) and the principles as descibed by him were used.

The coefficient of variation for resting flow measured serially over a period of 4 min (20 consecutive measurements) was 2.5% and a single measurement repeated over a period of 3 days was 11.5%. For peak blood flow the coefficient of variation for 3 consecutive measurements was 8.4% and three measurements made over 3 days 13.6%. For time to peak flow the variations were 7.5% and 12.4% (time being measured at 12 s intervals) and for the duration of the hyperaemic response the values were 11.5% and 17.6%. Segmental blood pressure measurements were more reproducible, the coefficient of variation for 3 consecutive measurements being 2.4% and when made on different days 7.0%, this value falling to 4.6% when corrected for changes in the systemic blood pressure.

7.3.4 Blood sampling

Venous blood samples were taken just prior to the muscle biopsies on the day that the infusion was to start and after 5 and 10 days of treatment. Blood and serum were deproteinised as described in appendix 3 and deproteinised blood, deproteinised serum and serum were analysed for glucose, lactate, pyruvate, alanine, acetoacetate, β-hydroxybutyrate and citrate, glycerol and free fatty acids respectively (appendix 3).

7.3.5 Muscle biopsies

Two biopsies were taken from the medial head of gastrocnemius under local anaesthetic as described in chapter 4 prior to starting treatment and after 5 and 10 days of infusion. One sample was "snap-frozen" in Freon at its freezing point for freeze-drying and subsequent analysis of metabolites (glycogen, PCr, ATP, ADP, AMP, glucose, G1P, G6P, F6P, F16BP, triose phosphates, lactate, pyruvate, alanine, citrate and β-hydroxybutyrate) and the second sample collected in ice cold KCl 0.15 mmol/l for assay of succinic dehydrogenase activity (chapter 4).

7.4 Results

7.4.1 Patient details

Patient details are summarised in table 7.1. As can be seen the four groups were well matched for age, sex, number of diabetics, the presence or absence of systemic atherosclerosis and cigarette smoking habits. As expected less diabetics were smokers but there were no other differences between diabetics and non-diabetics or between the treatment or control groups (Fisher exact test and two tailed Student's t-test for independent samples). The one exception was the duration of oral therapy for the placebo group diabetics and this was because all these patients underwent early amputation. However the incidence of diabetic and non-diabetic amputations was not statistically different.

7.4.2 Changes in muscle metabolism with rest pain

Eight patients with significant vascular impairment of the non-rest pain leg agreed to undergo pre-treatment biopsies of that leg, repeated at days 5 and 10 of the trial. The values for the metabolites and SDH

Table 7.1 Naftidrofuryl rest pain trial - patient details (values are given ±SEM)

	active drug (n=13)		placebo (n=15)	
parameter	diabetics (n=6)	non-diabetics (n=7)	diabetics (n=6)	non-diabetics (n=9)
males	3	6	4	6
females	3	1	2	3
age in years	69 (±0.4)	67 (±4.9)	63 (± 5.3)	73 (±3.7)
cigarette smokers	3	6	2	8
history of IHD	4	3	3	5
history of CVD	3	3	1	4
weeks of IC	4.8 (±1.5)	10.0 (±5.9)	24.4 (±14.0)	3.2 (±5.7)
weeks of rest pain	3.1 (±0.7)	2.6 (±0.7)	4.6 (± 1.6)	5.9 (±1.6)
digital ulcers	5	4	6	4
digital infection	1	2	4	1
digital gangrene	2	1	5	1
days IV infusion	7.7 (±1.3)	8.7 (±0.9)	10.0 (± 0)	8.7 (±0.7)
weeks oral therapy	5.3 (±2.0)	6.6 (±2.0)	0 (± 0)	7.6 (±1.4)
major amputation	3	3	6	1

IHD=ischaemic heart disease; CVD=cerebro-vascular disease; IC=intermittent claudication; IV=intra-venous

activity in this less severely affected limb were identical to the more painful leg and this data was included for subsequent analysis. Values from 10 healthy controls are included for comparison with the patients' data. These normals comprised 7 males and 3 females aged 22-40 who were undergoing biopsies in the course of another experiment for which metabolite values were determined. In addition they agreed to a second resting biopsy for SDH assay. They were recreationally fit but could not be described as highly trained. Several differences between the normal controls and the patients were noted; muscle glycogen was significantly higher in the control group and similary PCr and ATP concentrations were depressed in the rest pain group. No differences in hexosemonophosphates were found, but muscle glucose was much higher in the patients who in addition demonstrated a higher muscle lactate concentration. The most noticeable difference between the two groups however was the muscle oxidative capacity as estimated by SDH activity, this enzyme being almost twice as active in the normal controls. The results together with a statistical summary on the differences are listed in table 7.2.

As more than 40% of the patients were diabetic it was important to determine whether there were any major metabolic differences in the muscle of diabetics and non-diabetics and table 7.3 lists the values for these two groups seperately. As can be seen, other than a higher muscle glucose there was no statistical difference between them, and as the rest pain group had a higher muscle glucose than the normals it was considered justified to combine the data for subsequent analysis.

Table 7.2 Metabolite values and succinic dehydrogenase (SDH) activity in rest pain patients and normal controls. Metabolite concentrations in mmol/kg dry weight and SDH activity in µmol/g wet weight/min [(means (±SD)]

	rest pain (n=28)	control (n=8)	normal (n=10)	rest pain v control	rest pain v normal
glycogen	265.9 (81.2)	262.9 (90.1)	331.1 (51.5)	t= 0.07	t= 1.96*
PCr	65.3 (15.0)	62.9 (10.8)	88.8 (9.8)	0.43	4.65***
ATP	19.3 (4.2)	19.5 (5.5)	24.1 (1.2)	-0.13	2.50**
ADP	3.07 (0.49)	2.93 (0.61)	3.11 (0.27)	0.63	0.14
AMP	0.19 (0.10)	0.19 0.08)	0.16 (0.04)	0.02	-0.49
glucose	7.13 (3.83)	6.21 (3.72)	1.71 (0.59)	0.60	-3.79***
G1P	0.10 (0.12)	0.07 (0.08)	0.08 (0.02)	-0.45	-0.90
G6P	1.36 (1.07)	1.00 (0.89)	1.25 (0.47)	0.86	0.06
F6P	0.16 (0.17)	0.16 (0.10)	0.14 (0.08)	0.00	-0.21
F16BP	0.38 (0.42)	0.35 (0.22)	0.19 (0.11)	0.14	-0.39
triose phosphates	0.27 (0.12)	0.26 (0.14)	0.27 (0.08)	0.21	0.13
lactate	12.25 (6.78)	9.29 (4.89)	5.99 (2.10)	1.14	-1.97*
pyruvate	0.27 (0.16)	0.21 (0.07)	0.16 (0.04)	1.14	-1.64
citrate	0.72 (0.50)	0.79 (0.28)	0.56 (0.09)	-0.35	-1.63
alanine	7.58 (2.58)	6.20 (1.83)	5.74 (1.75)	1.40	-1.50
β-hydroxbutyrate	0.38 (0.22)	0.43 (0.33)	0.36 (0.10)	0.37	0.17
SDH	2.89 (0.95)	2.47 (0.65)	5.59 (1.34)	0.73	5.81***

PCr=phosphocreatine; ATP=adenosine triphosphate; ADP=adenosine diphosphate:
AMP=adensosine monophosphate; G1P=glucose 1-phosphate; G6P=glucose 6-phosphate;
F6P=fructose 6-phosphate; F16BP=fructose 1,6-bisphosphate

control=samples taken from opposite leg to rest pain leg, but with significant vascular impairment
normal =healthy normal controls (7 males, 3 females)

Significance of differences between rest pain and control samples and rest pain and normal samples tested with Student's t-test for independent samples (one tailed as null hypothesis assumed that normal and control biopsies were both less anaerobic than rest pain biopsies)

level of significance

$t>1.71$ $p<0.05$*
$t>2.49$ $p<0.01$**
$t>3.75$ $p<0.0005$***

Table 7.3 Metabolite values and succinic dehydrogenase (SDH) activity in rest pain patients. Differences between diabetics and non-diabetics on day 0 of infusion.

Metabolite concentrations in mmol/kg dry weight and SDH activity in µmol/g wet weight/min [(means (±SD)]

	non-diabetic (n=21)	diabetic (n=16)	t-statistic
glycogen	274.0 (83.3)	243.4 (83.3)	0.82
PCr	67.1 (13.0)	61.2 (15.2)	1.23
ATP	20.4 (4.6)	18.9 (3.9)	1.69
ADP	3.09 (0.39)	3.00 (0.67)	0.71
AMP	0.17 (0.08)	0.20 0.11)	-0.82
glucose	5.46 (3.07)	9.10 (3.75)	-3.01*
G1P	0.12 (0.13)	0.05 (0.05)	1.81
G6P	1.24 (0.51)	0.97 (0.86)	1.16
F6P	0.15 (0.10)	0.16 (0.13)	-0.21
F16BP	0.21 (0.15)	0.65 (0.51)	0.74
triose phosphates	0.27 (0.13)	0.28 (0.10)	-0.31
lactate	10.08 (6.35)	13.71 (6.22)	-1.70
pyruvate	0.22 (0.12)	0.31 (0.17)	-1.78
citrate	0.83 (0.53)	0.60 (0.26)	1.79
alanine	7.06 (1.76)	7.57 (2.52)	-0.60
SDH	3.05 (0.77)	2.37 (1.09)	0.44

Abbreviations as for Table 7.2

Significance between muscle metabolites of diabetics and non-diabetics tested with Student's t-test for independent samples (two-tailed).

levels of significance

$t>2.11$ $p<0.05$
$t>2.90$ $p<0.01$*

7.4.3 Naftidrofuryl trial

a) Clinical outcome

Twenty one patients completed 10 days intravenous treatment, 9 receiving the active drug and 12 placebo, all withdrawals being for uncontrolled rest pain. All subsequently underwent major amputation, three undergoing prior limb salvage reconstruction. Of the remainder, of those receiving naftidrofuryl, four underwent limb salvage operations of whom one and one other required a major amputation. Of the placebo group, four also underwent limb salvage surgery of whom one and three others required major amputation. There was no significant difference in the numbers (diabetics, non-diabetics or both) requiring amputation nor in the time they started the trial to the time of eventual surgery (table 7.4).

b) Linear analogue scores of subjective parameters

There was no difference in the initial scores of pain or any of the other parameters measured (two tailed Student's t-test for independent samples). In addition, the mean scores of all patients and those receiving a full 10 days intravenous therapy were similar. For this reason, full analysis of variance was carried out on the "complete treatment" groups. A summary of the patients' scores is shown in tables 7.5a and 7.5b. Statistical analysis revealed significant improvements in pain for those receiving the active drug for the parameters of pain, coldness, wellbeing , appetite and sleep when compared over the full 10 day period. Patients receiving placebo nevertheless also scored significantly less for pain and paraesthesia over the period of intravenous treatment. When the duration of therapy was broken down into two 5 day periods (0-5 and 5-10 days) however, for those patients receiving naftidrofuryl, significance was only maintained for pain (over the

Table 7.4 Amputation and reconstructive surgery rate for naftidrofuryl trial

	non-diabetics		diabetics		total	
	placebo	active	placebo	active	placebo	active
	(n=9)	(n=7)	(n=6)	(n=6)	(n=15)	(n=13)
reconstructive surgery	2 (22%)	3 (43%)	2 (33%)	2 (33%)	4 (27%)	5 (38%)
time to operation (weeks)	6.0	10.1	0.2	10.2	3.1	8.5
amputation	1 (11%)	3 (43%)	6 (100%)	3 (50%)	7 (47%)	6 (46%)
time to amputation (weeks)	18.0	2.4	3.9	8.4	5.9	5.4

Statistical analysis by Fisher's exact test (for numbers in groups) and Mann-Whitney U-test (for time to surgery)

Table 7.5a Linear analogue scores (mm) for subjective parameters, all patients

Score on day number [mean(±SEM)]

active drug	0 (n=13)	1 (n=13)	2 (n=13)	3 (n=11)	4 (n=11)	5 (n=11)	6 (n=10)	7 (n=10)	8 (n=9)	9 (n=9)	10 (n=9)
Pain	69 (7)	64 (8)	50 (8)	48 (9)	39 (10)	37 (10)	31 (9)	32 (10)	21 (7)	21 (10)	18 (8)
Paraesthesia	45 (10)	41 (10)	37 (10)	31 (10)	29 (9)	24 (8)	25 (8)	29 (9)	20 (7)	17 (7)	16 (6)
Coldness	52 (11)	25 (8)	37 (10)	26 (9)	21 (7)	21 (7)	17 (6)	19 (7)	17 (6)	15 (7)	15 (7)
Wellbeing	42 (8)	48 (9)	37 (9)	30 (8)	34 (7)	27 (9)	28 (7)	25 (9)	17 (5)	14 (6)	15 (5)
Appetite	30 (10)	31 (10)	27 (9)	26 (8)	19 (6)	21 (8)	20 (8)	21 (9)	10 (5)	11 (5)	9 (4)
Sleep	73 (8)	51 (8)	57 (8)	32 (9)	30 (5)	30 (7)	34 (7)	27 (7)	21 (8)	16 (6)	15 (7)

placebo	0 (n=15)	1 (n=15)	2 (n=15)	3 (n=15)	4 (n=15)	5 (n=14)	6 (n=14)	7 (n=14)	8 (n=13)	9 (n=13)	10 (n=13)
Pain	72 (6)	50 (8)	57 (8)	55 (8)	54 (8)	45 (9)	40 (8)	44 (9)	48 (9)	48 (9)	46 (9)
Paraesthesia	58 (9)	43 (8)	53 (8)	48 (9)	41 (8)	41 (9)	34 (9)	36 (9)	31 (8)	40 (9)	42 (10)
Coldness	45 (10)	27 (8)	25 (8)	24 (8)	29 (8)	29 (8)	24 (7)	36 (10)	30 (9)	27 (8)	24 (8)
Wellbeing	43 (8)	43 (8)	38 (8)	36 (8)	41 (9)	39 (9)	37 (9)	28 (6)	34 (7)	41 (8)	41 (9)
Appetite	24 (8)	37 (8)	26 (8)	28 (8)	24 (7)	27 (8)	31 (8)	28 (8)	31 (9)	40 (9)	40 (9)
Sleep	67 (8)	53 (8)	50 (8)	50 (8)	43 (6)	48 (8)	49 (8)	40 (6)	47 (8)	49 (7)	44 (6)

Table 7.5b Linear analogue score for subjective parameters, patients who completed 10 days intravenous therapy

Score on Day Number [Means (±SEM)]

active drug (n=9)	0	1	2	3	4	5	6	7	8	9	10
Pain	62 (8)	53 (7)	37 (6)	38 (7)	28 (9)	28 (9)	25 (7)	25 (8)	21 (7)	21 (10)	18 (8)
Paraesthesia	27 (9)	23 (7)	18 (7)	18 (6)	23 (8)	17 (7)	19 (6)	23 (7)	20 (7)	17 (7)	16 (6)
Coldness	35 (12)	22 (8)	22 (8)	26 (11)	22 (8)	21 (8)	19 (7)	20 (8)	18 (6)	16 (7)	16 (6)
Wellbeing	37 (9)	37 (10)	23 (8)	23 (7)	32 (9)	20 (7)	24 (7)	19 (7)	17 (5)	14 (6)	15 (5)
Appetite	25 (11)	23 (10)	24 (9)	19 (8)	16 (7)	12 (6)	14 (6)	14 (6)	10 (5)	11 (5)	9 (4)
Sleep	64 (10)	45 (8)	51 (10)	26 (10)	30 (7)	26 (8)	31 (7)	23 (8)	21 (8)	15 (6)	15 (7)

placebo (n=13)	0	1	2	3	4	5	6	7	8	9	10
Pain	72 (7)	53 (9)	58 (9)	55 (9)	52 (8)	41 (8)	41 (8)	44 10)	48 (9)	48 (9)	46 (9)
Paraesthesia	54 (10)	42 (9)	50 (9)	45 (10)	37 (8)	38 (8)	31 (9)	38 (8)	31 (8)	40 (9)	39 (9)
Coldness	43 (10)	27 (9)	23 (9)	21 (8)	26 (7)	24 (7)	21 (7)	33 (10)	30 (9)	27 (8)	24 (8)
Wellbeing	38 (8)	43 (9)	37 (9)	34 (8)	43 (10)	40 (10)	39 (9)	30 (7)	34 (7)	41 (8)	41 (9)
Appetite	27 (9)	37 (9)	28 (9)	30 (9)	24 (8)	28 (9)	33 (9)	30 (9)	32 (9)	39 (9)	26 (7)
Sleep	56 (9)	53 (8)	48 (8)	52 (9)	45 (7)	51 (8)	52 (8)	42 (6)	47 (8)	49 (7)	44 (6)

initial 5 days) and sleep (over both periods) while the placebo group retained significance for pain in the first period. A statistical summary is shown on table 7.6 and the results are shown graphically in figures 21a and 21b.

c) Analgesia requirements

No difference in analgesia requirments was demonstrated between the two groups and there was no change in analgesia consumption as a response to treatment with placebo or active drug (Friedman two-way analysis of variance). The results for the complete treatment groups and those who completed 10 days of intravenous therapy are listed in tables 7.7a and 7.7b.

d) Changes in haemodynamic measurements

No differences were encountered in haemodynamic measurements for diabetics and non-diabetics, and the values determined for all patients were similar to those for the patients who completed 10 days treatment. In addition the values of the non-rest pain legs were similar for the placebo and active drug groups. The values recorded in those patients who completed 10 days treatment are summarised in table 7.8. As can be seen administration of the test substance had no significant effect on systemic or segmental blood pressure, blood flow, either resting or peak, or any of the measurements derived from the hyperaemic response [statistical analysis by two-tailed Student's t-test for independent samples (across groups) and one-way ANOVA (repeated measures)].

e) Blood metabolites

Blood metabolites, including blood glucose, were the same for the diabetic and non-diabetic patients, and for the treatment group as a whole the values were the same as blood samples assayed for the same

Table 7.6 Statistical summary of analogue scores of subjective parameters

	rest pain group (n=9)			placebo group (n=13)		
	days 0-10	days 0-5	days 5-10	days 0-10	days 0-5	days 5-10
Pain	F= 9.24**	F= 8.73**	F= 0.79	F= 3.41**	F= 4.74**	F= 0.65
Paraesthesia	0.73	-	-	2.12**	1.61	0.64
Coldness	2.90**	2.16	1.69	1.48	-	-
Wellbeing	3.12**	1.88	2.16	1.45	-	-
Appetite	2.74**	2.41	2.26	0.48	-	-
Sleep	8.05**	5.89**	2.89*	0.91	-	-

Statistical analysis by 1-way ANOVA for repeated measures. Where significance revealed over 10 day period, days 0-5 and 5-10 tested seperately. Complete treatment groups only

levels of significance

days 0-10		days 0-5 & days 5-10
F>1.95	p<0.05*	F>2.45
F>2.55	p<0.01**	F>3.51

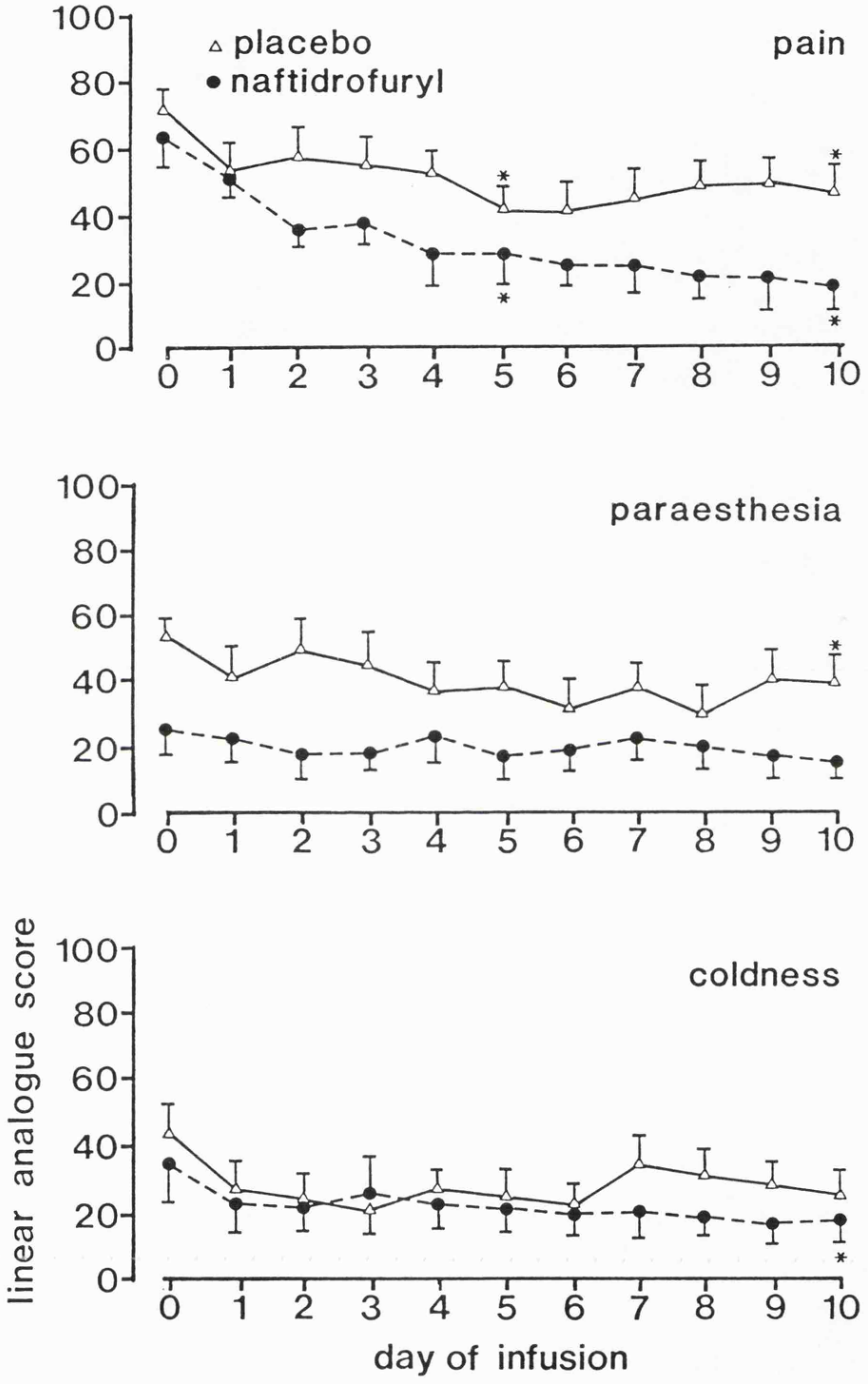


Figure 21a Line plot of scores for Pain, Paraesthesia and Coldness. Means and error bars (1 SEM) are shown. Significant differences are indicated by an asterisk

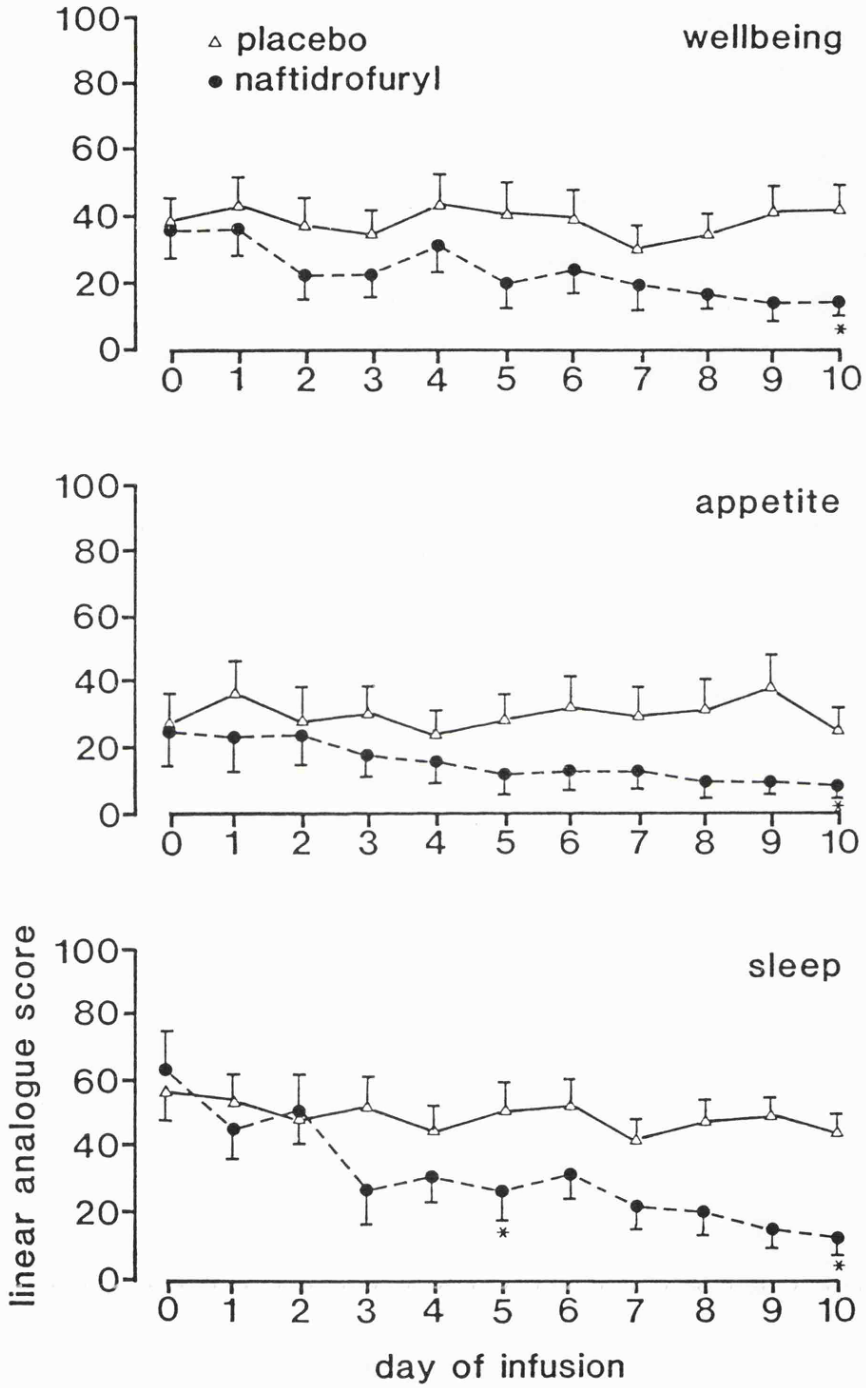


Figure 21b Line plot of scores for Wellbeing, Appetite and Sleep. See Figure 21a

Table 7.7a Analgesia requirements, all patients

number of doses on day number [mean (±SEM)] (n=number of patients)

active drug	0 (n=13)	1 (n=13)	2 (n=13)	3 (n=11)	4 (n=11)	5 (n=11)	6 (n=10)	7 (n=10)	8 (n=9)	9 (n=9)	10 (n=9)
Morphine 10mg IM	0.9(0.5)	1.1(0.5)	1.2(0.7)	1.5(0.8)	1.5(0.9)	1.4(1.0)	1.7(1.3)	0.4(0.4)	0.4(0.4)	0.4(0.4)	0.4(0.4)
DF118 30mg PO	2.3(0.7)	1.8(0.6)	1.3(0.6)	1.5(0.8)	1.5(0.7)	1.2(0.5)	1.5(0.8)	1.2(0.6)	2.0(0.9)	1.6(0.7)	1.0(0.7)
Paracetamol 500mg PO	0.5(0.2)	0.6(0.3)	0.6(0.3)	0.4(0.4)	0.5(0.4)	0.7(0.3)	0.2(0.2)	0.8(0.4)	0.4(0.3)	0	0
placebo	**0 (n=15)**	**1 (n=15)**	**2 (n=15)**	**3 (n=15)**	**4 (n=15)**	**5 (n=14)**	**6 (n=14)**	**7 (n=14)**	**8 (n=13)**	**9 (n=13)**	**10 (n=13)**
Morphine 10mg IM	0.8(0.4)	0.9(0.5)	1.4(0.7)	0.9(0.6)	1.1(0.6)	1.1(0.8)	1.0(0.7)	0.7(0.5)	0	0.3(0.3)	0.5(0.5)
DF118 30mg PO	1.7(0.5)	1.7(0.5)	1.9(0.6)	2.0(0.7)	1.2(0.5)	1.4(0.5)	1.5(0.5)	1.6(0.6)	1.8(0.7)	1.8(0.6)	1.6(0.7)
Paracetamol 500mg PO	0.7(0.5)	0.5(0.3)	0.7(0.3)	0.4(0.3)	1.6(0.6)	1.1(0.6)	1.0(0.5)	1.1(0.6)	0.5(0.4)	1.0(0.6)	0.5(0.3)

DF118=dihydrocodeine; IM=intramuscular; PO=oral

Table 7.7b Analgesia requirements, patients who completed 10 days intravenous therapy

number of doses on day number [mean (±SEM)]

active drug (n=9)	0	1	2	3	4	5	6	7	8	9	10
Morphine 10mg IM	0.4(0.4)	0.4(0.4)	0.4(0.4)	0.6(0.4)	0.4(0.4)	0.4(0.4)	0.4(0.4)	0.4(0.4)	0.4(0.4)	0.4(0.4)	0.4(0.4)
DF118 30mgs PO	1.2(0.7)	1.2(0.5)	1.1(0.7)	1.0(0.7)	1.2(0.7)	1.4(0.6)	1.7(0.9)	1.3(0.7)	2.0(0.9)	2.0(0.9)	1.6(0.7)
Paracetamol 500mg PO	0.6(0.3)	0.9(0.5)	0.9(0.4)	0.4(0.4)	0.7(0.5)	0.9(0.4)	0.2(0.2)	0.9(0.5)	0.4(0.3)	0	0
Analgesia summed	4.8(1.8)	5.1(1.5)	4.9(1.8)	4.7(2.2)	4.9(1.8)	5.6(1.7)	5.3(2.1)	5.3(1.8)	6.2(2.0)	4.9(2.0)	3.8(2.1)

placebo (n=13)	0	1	2	3	4	5	6	7	8	9	10
Morphine 10mg IM	0.7(0.5)	1.1(0.6)	1.2(0.7)	1.1(0.7)	1.0(0.6)	1.2(0.8)	1.1(0.7)	0.7(0.5)	0	0.3(0.3)	0.5(0.5)
DF118 30mg PO	1.7(0.6)	1.7(0.6)	1.7(0.6)	1.5(0.6)	0.9(0.5)	1.2(0.5)	1.5(0.6)	1.7(0.6)	1.8(0.7)	1.8(0.6)	1.6(0.7)
Paracetamol 500mg PO	0.8(0.5)	0.8(0.4)	0.6(0.3)	0.5(0.3)	1.7(0.6)	1.2(0.7)	1.1(0.6)	1.2(0.7)	0.5(0.4)	1.0(0.6)	0.5(0.3)
Analgesia summed	6.9(1.8)	8.5(2.2)	8.9(2.7)	7.8(2.5)	7.5(2.4)	8.6(3.0)	8.3(2.7)	7.7(1.9)	4.2(1.2)	5.8(1.4)	5.5(2.1)

DF118=dihydrocodeine; IM=intramuscular; PO=oral

Analgesia summed = 4xMorphine 10mg + 2xDF118 30mg + Paracetamol 500mg

Table 7.8 Haemodynamic measurements in patients who completed 10 days I.V. therapy: blood pressure in mm Hg; blood flow in ml/100ml muscle/min; time in seconds [mean (±SD)]

rest pain limb	active drug (n=9)			placebo (n=12)		
measurement	day 0	day 5	day 10	day 0	day 5	day 10
systolic BP	172 (35)	171 (35)	171 (35)	164 (36)	164 (36)	164 (36)
diastolic BP	80 (8)	80 (8)	80 (8)	83 (12)	83 (12)	83 (12)
ankle BP	43 (11)	42 (10)	50 (13)	41 (12)	40 (8)	42 (11)
calf BP	90 (26)	100 (25)	102 (23)	95 (23)	91 (10)	94 (15)
ankle/brachial pressure index	0.25 (0.07)	0.25 (0.08)	0.29 (0.12)	0.25 (0.09)	0.24 (0.06)	0.26 (0.11)
calf/brachial pressure index	0.52 (0.13)	0.58 (0.06)	0.60 (0.13)	0.58 (0.10)	0.55 (0.11)	0.57 (0.10)
resting flow	2.64 (0.92)	3.02 (1.67)	2.50 (0.82)	3.18 (1.96)	2.89 (1.76)	3.51 (2.30)
peak flow	5.68 (4.81)	5.73 (4.11)	6.77 (5.80)	5.35 (2.64)	5.21 (2.31)	6.21 (3.87)
time to peak flow	37 (20)	42 (29)	49 (38)	38 (24)	49 (44)	45 (20)
time to return to resting flow	146 (73)	170 (72)	216 (92)	257 (129)	221 (159)	193 (152)

control limb	active drug (n=7)			placebo (n=10)		
measurement	day 0	day 5	day 10	day 0	day 5	day 10
ankle BP	88 (18)	98 (29)	85 (26)	85 (20)	82 (19)	93 (18)
calf BP	125 (15)	122 (18)	120 (223	115 (32)	110 (28)	123 (15)
ankle/brachial pressure index	0.51 (0.11)	0.57 (0.16)	0.49 (0.10)	0.52 (0.12)	0.50 (0.14)	0.57 (0.14)
calf/brachial pressure index	0.73 (0.16)	0.71 (0.07)	0.70 (0.06)	0.70 (0.14)	0.67 (0.18)	0.75 (0.13)
resting flow	2.50 (0.85)	2.49 (0.85)	2.40 (0.79)	3.11 (1.84)	2.75 (1.68)	3.49 (2.73)
peak flow	7.49 (3.29)	7.14 (3.29)	7.01 (2.38)	9.01 (4.08)	7.64 (4.04)	7.94 (4.39)
time to peak flow	30 (20)	28 (20)	18 (17)	41 (42)	33 (21)	48 (66)
time to return to resting flow	188 (92)	162 (65)	189 (86)	191 (52)	187 (105)	182 (90)

series of metabolites taken from the 10 normal controls who provided muscle biopsies. As can be clearly seen no changes were found as a response to treatment over the 10 days of therapy and there was no difference between the placebo and active drug groups. The results for those patients who completed 10 days intravenous therapy are summarised in table 7.9.

f) Muscle metabolites

As no substantial difference between diabetics and non-diabetics and those who had an additional biopsy from the non-rest pain leg was found, values for all biopsies in the active drug and placebo groups are considered together. No change in metabolites as a response to treatment with naftidrofuryl was noted. In particular there was no increase in high energy phosphates (ATP and PCr) levels which were depressed when compared with normal subjects. Neither was there any improvement in the activity of succinate dehydrogenase which was again much lower in the patients with rest pain. The only significant change was a reduction in muscle glycogen which occurred within the first 5 days for the patients receiving the active drug and over the 10 days in those receiving the placebo. Table 7.10 summarises the muscle metabolite results.

7.5 Discussion

7.5.1 Changes in muscle metabolism with peripheral vascular disease

a) Muscle metabolites

As the reference values for the normal metabolites were from healthy active subjects of a much younger age than the patients studied it could be argued that the differences recorded could be the effects of age rather than vascular disease. Möller (1981) in his thesis on

Table 7.9 Changes in blood metabolites in response to intravenous therapy in naftidrofuryl rest pain trial (patients who completed 10 days I.V. therapy)

mmoles/l [mean (±SD)]

	active drug (n=9)			placebo (n=13)		
metabolite	day 0	day 5	day 10	day 0	day 5	day 10
glucose	6.53 (2.67)	6.15 (2.66)	6.54 (2.84)	4.80 (0.82)	4.56 (0.83)	4.41 (1.13)
lactate	1.31 (0.47)	1.58 (0.56)	1.23 (0.43)	0.91 (0.32)	0.97 (0.32)	0.95 (0.43)
pyruvate	0.057 (0.038)	0.063 (0.028)	0.059 (0.033)	0.064 (0.048)	0.059 (0.030)	0.066 (0.041)
alanine	0.394 (0.142)	0.404 (0.176)	0.412 (0.140)	0.377 (0.163)	0.393 (0.167)	0.372 (0.167)
citrate	0.061 (0.016)	0.053 (0.017)	0.072 (0.049)	0.067 (0.033)	0.053 (0.023)	0.057 (0.024)
β-hydroxybutyrate	0.139 (0.047)	0.127 (0.095)	0.128 (0.054)	0.121 (0.057)	0.082 (0.082)	0.083 (0.049)
acetoacetate	0.023 (0.021)	0.029 (0.024)	0.030 (0.011)	0.029 (0.033)	0.022 (0.031)	0.017 (0.025)
glycerol	0.111 (0.031)	0.092 (0.024)	0.100 (0.029)	0.108 (0.031)	0.099 (0.032)	0.103 (0.013)
free fatty acids	0.400 (0.148)	0.328 (0.153)	0.350 (0.086)	0.422 (0.189)	0.285 (0.167)	0.354 (0.142)

Table 7.10 Changes in muscle metabolites in response to intravenous therapy during naftidrofuryl rest pain trial

Muscle metabolites in mmol/kg dry weight [mean (±SD)]
Succinic dehydrogense activity in μmol/g wet weight/min [mean (±SD)]

	active drug						placebo					
	day 0 (n=18)		day 5 (n=13)		day 10 (n=12)		day 0 (n=18)		day 5 (n=16)		day 10 (n=14)	
glycogen	284.6	(94.4)	208.4*	(42.1)	198.8*	(62.0)	247.7	(69.4)	226.7	(53.3)	193.9*	(51.8)
PCr	65.9	(12.9)	62.8	(9.5)	60.1	(10.9)	63.5	(15.4)	60.2	(11.0)	59.8	(10.0)
ATP	19.9	(5.1)	20.1	(4.7)	19.0	(5.2)	18.8	(3.6)	18.6	(3.2)	18.2	(3.2)
ADP	3.12	(0.53)	3.02	(0.58)	3.08	(0.37)	2.94	(0.50)	3.01	(0.70)	2.69	(0.72)
AMP	0.18	(0.10)	0.22	(0.11)	0.23	(0.09)	0.19	(0.09)	0.24	(0.17)	0.20	(0.12)
glucose	6.39	(3.56)	7.67	(3.13)	7.79	(3.66)	7.48	(4.02)	7.36	(3.06)	7.41	(3.34)
G1P	0.09	(0.11)	0.23	(0.27)	0.13	(0.16)	0.09	(0.11)	0.10	(0.06)	0.13	(0.11)
G6P	1.39	(1.27)	1.86	(0.85)	1.96	(1.64)	1.14	(0.71)	1.01	(0.44)	1.09	(1.09)
F6P	0.14	(0.15)	0.23	(0.19)	0.24	(0.29)	0.17	(0.16)	0.13	(0.10)	0.16	(0.22)
F16BP	0.41	(0.51)	0.24	(0.22)	0.24	(0.28)	0.33	(0.19)	0.25	(0.18)	0.31	(0.30)
triose phosphates	0.32	(0.13)	0.30	(0.11)	0.31	(0.13)	0.22	(0.09)	0.25	(0.12)	0.26	(0.17)
lactate	11.02	(5.48)	13.39	(6.86)	13.90	(8.57)	12.17	(7.44)	12.48	(5.54)	14.74	(8.76)
pyruvate	0.27	(0.16)	0.31	(0.14)	0.25	(0.15)	0.25	(0.14)	0.21	(0.12)	0.25	(0.17)
citrate	0.83	(0.57)	0.90	(0.49)	0.82	(0.29)	0.64	(0.27)	0.75	(0.27)	0.75	(0.29)
alanine	7.27	(2.16)	7.47	(2.05)	7.30	(1.97)	7.25	(2.84)	6.94	(2.31)	6.44	(1.90)
β-hydroxybutyrate	0.31	(0.15)	0.38	(0.19)	0.34	(0.15)	0.51	(0.38)	0.43	(0.20)	0.32	(0.15)
succinic dehydrogenase	3.15	(0.83)	3.41	(0.52)	3.50	(0.70)	2.51	(0.92)	2.68	(1.02)	2.66	(0.87)

* significantly different from day 0 values; significance over three measurements tested by 1-way ANOVA with repeated measures; between measurement tested by Student's t-test for correlated means (one-tailed).

for ACTIVE DRUG $F=4.54$, $p<0.05$; 0 v 5 days $t=2.46$, $p<0.025$; 0 v 10 days $t=2.46$, $p<0.025$; 5 v 10 days ns
for PLACEBO $F=9.93$, $p<0.01$; 0 v 5 days $t=0.80$, ns; 0 v 10 days $t=1.89$, $p<0.05$; 5 v 10 days $t=1.31$, ns

skeletal muscle adaptation to ageing, studied 22 healthy elderly subjects without intercurrent disease and reported phosphogen values which although lower than the younger subjects were much higher than the patients. These elderly subjects had a mean age of 67.8 and the PCr, ATP and ADP levels were 72.7, 23.2 and 2.84 mmol/kg d.m. respectively, values which are similar to those of Harris et al (1974). In 8 patients with chronic obstructive lung disease he found significantly lower PCr (62.3 mmol/kg d.m.) and ATP (19.9 mmol/kg d.m.) values and as ADP and AMP concentration appeared stable, this effectively reduced the energy charge potential of the muscle cell. A similar reduction in muscle PCr and ATP was demonstrated by MacDougall et al (1977) after a period of immobilisation in normal healthy volunteers, ADP concentrations again remaining unchanged. As the phosphogen concentrations found in the rest pain patients revealed a similar pattern to these two studies it is possible that forced inactivity brought about by the patients' disability was the prime stimulus for these changes, rather than the vascular disease itself. The lower ATP/ADP ratio and higher lactate (and by inference a lower muscle pH) will reduce the free energy from ATP hydrolysis (Newsholme and Start, 1973; Sahlin et al, 1978b) with the consequence that the muscle will have to expend more energy for a given amount of work. This coupled with the lower muscle glycogen (Jacobs, 1981), which may again be a function of disuse, would induce an earlier onset of muscular fatigue. Muscle glucose is a mean of the intra-cellular and extracellular values and there are problems in making deductions regarding the relative concentrations in the two compartments (see Newsholme and Start, 1973). One could however hypothesise that as diabetics had a significantly higher glucose than the non-diabetics who in turn had higher values than the normal controls, the extracellular rather than intracellular glucose was elevated. However although an

impaired glucose tolerance in patients with peripheral vascular disease has been described, this has not been an invariable finding (Holm et al, 1973) despite the association between diabetes and atherosclerosis (Owerbach et al, 1982)

b) Muscle oxidative capacity

The normal value obtained for SDH activity was less than but similar in magnitude to that reported by Essén et al (1975) and Henriksson et al (1980) who described values of 9.7 and 8.7 µmol/g wet weight/min respectively. The latter authors reported SDH activity in patients with intermittent claudication 40% lower than their control values, contrary to Bylund et al (1976) who demonstrated an increased activity of muscle oxidative capacity (reflected in cytochrome oxidase activity) in claudicants although patients with severe ischaemia had lower (but not significantly so) values than normal. Holm et al (1972) did find a significantly lower succinic oxidase activty in the gastrocnemius muscle of patients with rest pain or gangrene, and the enzyme's activity was also much lower than normals in the less severely ischaemic quadriceps muscle. The finding of a much lower SDH activity in the patient group further supports inactivity as an important factor in the metabolic changes described, as muscle oxidative capacity can be substantially improved by training both in normal subjects and in those with peripheral vascular disease (see Chapter 2, section 2.6 and Chapter 3, section 3.5.2).

7.5.2 Naftidrofuryl trial

a) Clinical outcome

A combined amputation and reconstructive surgery rate of 70%

confirms the severity of the disease in these patients and must be contrasted with the results of other studies purporting to involve patients with "critical ischaemia". In a study of 28 patients assessing the effectiveness of PGI_2 in the treatment of rest pain, none required early operative intervention (Belch et al, 1983) and although in the controlled study by Greenhalgh (1981) of naftidrofuryl for ischaemic rest pain all but 3 of the 16 patients underwent reconstructive surgery, only 1 had an initial ankle systolic pressure of less than 50 mm Hg. Raftery (1982) described an amputation rate of only 20% but did not report objective measurements of pedal perfusion. Statistical analysis of haemodynamic variables as predictors of amputation (Point biserial coefficient, amputation as the dichotomous variable) revealed significant correlations between loss of the limb and the following parameters; time to peak flow ($p<0.05$), time to return to resting flow ($p<0.025$), severity of inflow atheroma ($p<0.01$), severity of outflow atheroma ($p<0.025$). Surprisingly ankle systolic pressure was a poor predictor (see Jelnes et al, 1986) as was the peak hyperaemic flow and this was particularly so as there was good correlation between distal systolic pressure and measurements of flow. The close correlation between the delay and duration of the hyperaemic response and the arteriographic findings are in keeping with the work of Myers (1964) and Van De Water et al (1980).

b) Measurement of subjective parameters and analgesic requirments

The significant placebo effect on the relief of pain highlights the need for controlled trials particularly when a drug is being assessed for its pain relief. Wong and McBain's (1980) study revealed a similar biphasic response to pain relief, the greatest improvement occurring in the first half of the treatment period. Although Meehan et al (1982)

employed analogue scales recording a number of parameters similar to the ones described above, in their controlled study, they did not find a marked placebo response although it should be noted that measurements were made only prior to and after 7 days of intravenous treatment and a trend in improvement may have been missed. Unlike Raftery (1982) no effect on analgesia requirements was noted. Naftidrofuryl treated patients did report an increased overall level of subjective improvement, most marked within the first 5 days of treatment.

c) Haemodynamic measurements

Measurement of limb blood flow and pressure was undertaken to exclude changes in flow or pedal peripheral resistance as factors influencing the response to treatment. Although strain gauge plethysmography proved reliable and reproducible, it was found to be a time consuming method. Each series of measurements took the author more than 1 hour, and although an attempt was made to develop a direct computerised differentiation and smoothing of the strain gauge signal to simplify interpretation of the traces this proved impossible to achieve. Movement artifact, muscle fasciculation, and superimposed pulse wave forms all occured at about the same frequency making it impossible to remove noise from the signal. It was found that the only reliable way to calculate flows was by manually drawing a tangent to each of the 100 curves obtained in the recordings for one leg and bypassing artifact by "eye-balling". Significant correlations were found between ankle systolic pressures, calf systolic pressures, peak blood flow and time to peak flow. Despite its vasodilatory properties no changes in blood flow or pressure were observed in either the control limb or the rest pain limb during naftidrofuryl administration confirming Greenhalgh's (1981) findings.

d) Metabolic changes

No changes were observed in any of the blood or muscle metabolites measured which could be attributed to the effects of the active drug. The only positive finding over the course of the period of treatment was a significant fall in glycogen in both the active drug and placebo groups. This was almost certainly the effect of immobility (MacDougall et al, 1977) as despite the fact that no restriction was placed on activity the presence of a continuous intravenous infusion did restrict the patients. In addition, the majority felt more comfortable in bed.

The initial paper on the metabolic effects of naftidrofuryl examined the in vitro effects of the orally administered drug on mouse brain (Meynaud et al, 1973). In the light of current knowledge on the control of metabolism, the authors' proposal that an increase in cerebral oxygenation would lead to an increase in ATP concentration above the normal levels needs to be treated with some caution. Similarly their claims of an increased succinic oxidase activity seemed to have been due to it influencing the binding of malonate at mmolar concentrations. Their second paper (Meynaud et al, 1975), failed to confirm any effect of the drug on mouse brain ATP concentration 15 min after dosing but purported to show significant decreases in lactate and glucose concentrations after this short interval. Two hours after administration, ATP and in addition PCr concentrations were found to be again elevated and this effect seemed to be dose related, being present at 50 and 150 mg/kg but not at 15 mg/kg. They also claimed to show a partial protective effect of the drug in reducing the fall in ATP levels seen with delayed freezing of the brain while at the same time preventing as much rise in lactate. It is possible that these effects if they occur, are due to enzyme inhibition, and if that is the case

then it is extremely unlikely that any beneficial effects will accrue in vivo. Elert et al's (1976) paper suffers from a number of anomalies. Pre-treatment concentrations of ATP and PCr were very much higher in the control group, and the naftidrofuryl treated group did not even reach these values after 5-6 days of treatment. As discussed earlier, the metabolite values in their study are not in agreement with the generally accepted normal range and despite their claims of a profound metabolic effect no mention of the clinical outcome is made. The protein sparing effect of naftidrofuryl reported by Burns et al (1981) is interesting but difficult to explain and it is of note that it has not been possible to repeat their findings.

7.5.3 Conclusions

The changes in muscle metabolism observed in patients with ischaemic rest pain, namely a low glycogen, ATP and PCr concentration and a depressed level of SDH activity, appear to represent the effects of inactivity rather than, as had been postulated, the direct effects of muscle ischaemia. Thus the response to chronic ischaemia is not comparable with those changes seen after high intensity exercise.

Substantive evidence for a significant metabolic effect of naftidrofuryl either in vivo or in vitro is lacking and the failure to demonstrate any clinical, haemodynamic or metabolic effect is striking. Although the drug does appear to confer some benefit over placebo in relief of pain and associated symptoms, this may be more a reflection of its local anaesthetic property than anything else. There is no evidence to support its use in the treatment of the critically ischaemic limb, and its use in the treatment of these patients must be in doubt.

Chapter 8

8 Final discussion and Summary

8.1 Fatigue

8.1.1 Central fatigue

Common to both the athlete striving for supramaximal performance and the patient with peripheral vascular disease, unable to walk more than a few score yards, is fatigue. This can be defined as the failure to maintain a required or expected power output (or in the case of isometric contraction, force) (Edwards, 1983) a phenomenon experienced during both these extreme examples. In chapter 2, section 2.5 the concept of fatigue and its various components was discussed and figure 22 summarises the factors that can influence it.

a) Subjective factors

Although central fatigue is not usually considered to be a limiting factor in well motivated sportsmen, with the more adverse cortical feedback of poor motivation and pain in patients with lower limb arterial disease, this may indeed limit muscle function.

b) Catecholamines

Cheetham et al (1986) have demonstrated a 4-fold increase in catecholamine concentration (both adrenaline and noradrenaline) 3 min after a 30 s treadmill sprint of the same intensity as the AWT. A significant correlation was found between both the rate of glycolysis and blood glucose levels, and as this was true both for adrenaline and noradrenaline the increase in catcholamines could not have been entirely due to peripheral spill-over. Peak catecholamine concentrations have

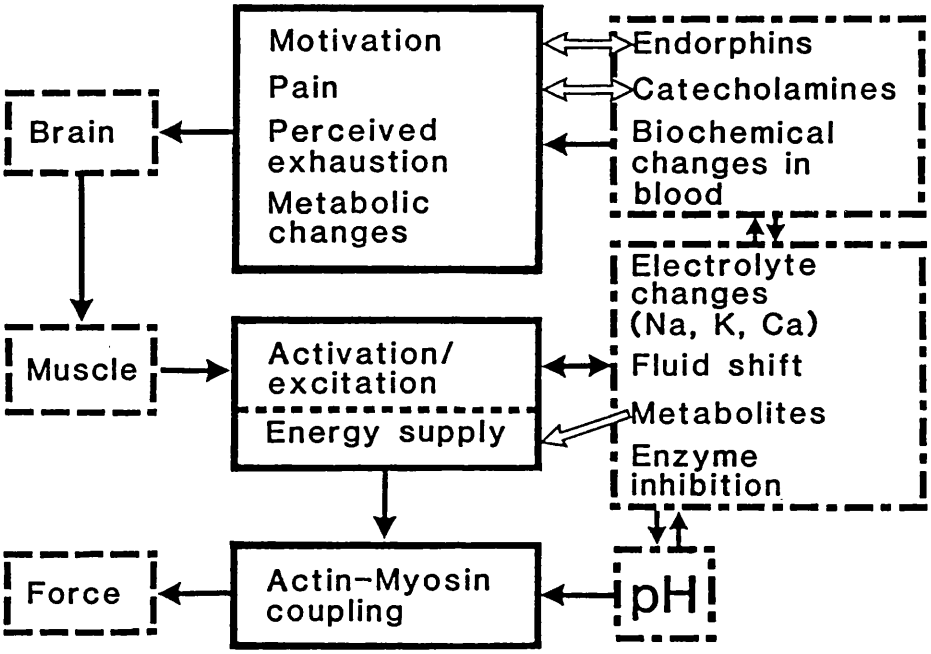


Figure 22 Pathways to fatigue. The basic step in force production is the instruction to the muscle by the brain to contract (right hand boxes). Some of the components of this "command chain" are shown in the central boxes (solid lines) and the factors modifying these factors are shown on the left. These peripheral changes not only influence the muscle's immediate environment but also influence central effects. A reduction in muscle pH seens to be a key factor in the development of peripheral fatigue.

been shown to occur immediately on cessation of a maximal anaerobic work test (MacDonald et al, 1983) whereas peak blood glucose and lactate concentrations are not reached for 5 min. It is unlikely then that even if adrenaline and noradrenaline increase hepatic glucose production either by a direct effect on liver glycogen or indirectly by stimulating pancreatic glucagon production (Näveri et al, 1985) the exercising muscle will benefit from this late availability of blood glucose. The exact mechanism for this early rise is unclear but it is probable that the initial control at least is peripheral (see Galbo, 1983 for review). Therefore the likely benefit from this rapid rise in catecholamines would be to initiate the transformation of phosphorylase b to a in readiness for rapid mobilisation of glycogen (Chasiotis et al, 1983).

c) Influence of β-endorphins on fatigue

Little information is available on the changes in β-endorphins in anaerobic exercise, but Brooks et al (1986) have demonstrated a rise, of the same order of magnitude as the catecholamines, after a 30 s bicycle ergometer sprint. It would be interesting to conjecture that this rise in endogenous opioids could alter cortical appreciation of pain and decrease central fatigue in addition to any effect that they have at the neuromuscular junction (Haynes et al, 1983).

8.1.2 Peripheral fatigue

The complex mechanisms of peripheral fatigue have been discussed in depth in chapter 2. As no measurements of ionic fluxes, particularly K^+, were made in the AWT studies of chapters 5 and 6 it is not possible to comment on the contribution of failure of activation/excitation coupling to fatigue. However there is increasing evidence to implicate changes in K^+ as being important in its development. Although muscle pH

was not directly measured, as it had been shown to be linearly related to lactate (Sahlin et al, 1976) derived pH values were discussed. However it has recently been shown (Cheetham and Boobis, unpublished observations) that after high intensity training the measured muscle pH was much greater than that which should be expected from the increase in lactate. This was not associated with any change in the physico-chemical buffering capacity of the muscle and implies an increase in transient buffering from transmembrane proton flux. In view of these findings it is neccessary to treat with caution derived pH values after dynamic exercise.

8.2 Metabolic response to brief maximal exercise

There was a surprising contribution to energy supply by glycolysis after as little as 6 s, when more than 50% of the ATP turnover was already being provided by the Embden-Meyerhof pathway. The only other study in which it is possible to calculate the rates of ATP turnover and the relative contribution from glycogen after very brief supramaximal dynamic exercise (10 s) revealed even more striking results (Jones et al, 1985). Of a total ATP turnover of 15 mmol/kg d.m./s, glycolysis provided more than 60%.

If PCr were the primary anaerobic fuel during the initial few seconds of maximal work then it would be expected to provide the greater proportion of energy. In addition, PCr stores were still very well maintained at more than 65% of the resting concentration at a time when muscular fatigue was already evident. As a result of this initial high glycolytic rate there was already significant lactate accumulation with a corresponding increase in hydrogen ions and decrease in pH. After 6 s a significant accumulation of a number of activators of PFK had

accrued (see table 2.2) and those measured included F16BP and F6P. The concentration of ATP was already beginning to show signs of falling and it is possible that additional changes in ADP and AMP occurred at levels below the sensitivity of the analytical methods. After the full 30 s sprint the metabolic changes in studies I and II, were the same, although the increase in glucose seen in the first study was not significant. Despite the extreme fatigue and obvious intensity of the sprint, almost 40% of the resting PCr was still available but by now 44% of the ATP had been used up. This is contrary to Hultman et al's (1967) conclusions that decreases in ATP are not seen until at least 60% of the PCr has been utilized, but from the studies summarised in table 2.3 it is evident that this reduction in ATP has been widely observed. Although it has been demonstrated that this is associated with an equimolar increase in IMP concentration (Harris and Hultman, 1985; Snow et al, 1985; table 2.3) a satisfactory explanation for this phenomenon has not been given. Karlsson and Saltin (1970) although observing the fact, chose to proffer no comments and a year later Karlsson (1971) failed to provide any insight into the occurence. The study of Jones et al (1985) cited above also recorded a marked reduction of ATP with preservation of PCr levels but again in their discussion they ascribed the initial few seconds of exercise as being responsible for the fall in PCr, its subsequent levels being maintained by glycolysis and at the expense of ATP. As Sahlin et al (1975) have shown a linear relationship between $\log K_{CPK}$ and pH (and by inference the production of lactate), it is clearly not possible for PCr levels to be maintained by glycolysis with its inevitable accumulation of lactate and fall in pH. The work by Spriet et al (1987) may provide some clues to the mechanism behind these apparently paradoxical changes. They demonstrated, by serial biopsies

of electrically stimulated muscle with an occluded circulation, a progressive decline in ATP turnover, as well as glycolysis and force production which were all reduced to less than 20% of their initial values at the end of 100 s of stimulation. As ATP concentration did not fall below 14 mmol/kg d.m. and PCr was still present in a significant concentration they concluded that as phosphogens were still present while force production was severely depressed, ATP supply could not be the limiting factor in the continuation of muscular contraction. These findings seem to contradict those seen in dynamic exercise when dramatic declines in ATP are seen despite high relative power output still being maintained. A recent study by Katz et al (1986) demonstrated a linear correlation between %FT fibres and the ATP turnover rate during isometric contraction to fatigue. They reported similar changes in metabolites to those found after the 30 s AWT although PCr was much more dramatically reduced, in keeping with other reports after isometric exercise. A significant correlation between the rate of ATP turnover from glycolysis and PCr and the contraction duration was described. It was considered likely that the rates reflected changes in metabolites responsible for or associated with fatigue rather than causing fatigue.

8.2.1 An hypothesis on fuel supply as a mechanism for fatigue

The phosphocreatine shuttle as eloquently presented by Bessman and Geiger (1981) has recently been the subject of an excellent review by Gollnick (1986). As discussed in chapter 2, section 2.5.2, the existence of this mechanism allows integration of the energy supply by PCr and glycolysis. An hypothesis involving this mechanism in the development of fatigue which may explain the different levels of PCr and ATP utilization seen with dynamic and isometric exercise is presented by

the author.

The shuttle hypothesis assumes that the ATP bound to the myosin head, which provides energy for cross-bridge formation and which is hydrolysed by acto-myosin ATPase, can only be rephosphorylated by PCr. As the ATP used in providing fuel for the other energy requiring processes of muscular contraction (the excitation/contraction process by Ca^{2+} and Na^{+}-K^{+} transport, hydrolysed by Ca^{2+} ATPase and Na^{+}-K^{+} ATPase respectively) is not bound, it can therefore be rephosphorylated directly by glycolysis or oxidative phosphorylation.

Kushmerick (1983) has shown that during dynamic exercise a relatively small percentage of ATP is used for the excitation process whereas during isometric contraction this is very high and has been estimated at 30% of the total. During dynamic exercise hydrogen ion flux exceeds lactate (Sejersted et al, 1984) so that there is a higher muscle pH for a given lactate; during isometric contraction above 40% MVC no ionic flux occurs (Edwards et al, 1972) and pH is linearly related to lactate (Sahlin et al, 1976). If PCr were the primary energy source in initiating extreme muscular activity then it could be expected that it would be possible to demonstrate depletion of this fuel. From the results summarised in Table 2.3 and the findings of Study I it would appear that the more intense the exercise and the briefer its duration the less PCr is used. This is quite the opposite to what would be expected and it is postulated that other than the priming effect of PCr in starting contraction all the energy during anaerobic exercise is supplied by glycolysis via the PCr shuttle.

As there is a linear relationship between the rate of energy supply via glycolysis and work rates at all intensities (vide infra) it would appear that when the glycolytic rates is insufficient to maintain the

required ATP turnover rate for that work load, fatigue will occur even if PCr remains. Sahlin et al (1975) have shown that its concentration is determined by the intracellular pH via the CPK equilibrium reaction, being linearly related to pH and dependent on pH for its recovery. During supramaximal dynamic exercise extremely high ATP turnover rates exist (Studies I and II; Table 2.5). Glycolysis can only maintain energy supply at this rate for a few seconds before metabolic inhibition occurs. If during this time, the PCr shuttle is unable to rephosphorylate ADP at the very high rates required, the adenylate kinase reaction may reduce the ADP bound to the myosin head to maintain a high ATP/ADP ratio with a resultant fall in ATP concentration (see Chapter 2, section 2.3). The decline in power output will then match the decline in glycolysis but the higher terminal pH (protons being lost in excess of lactate) will result in a higher PCr at exhaustion.

During isometric exercise a much lower maximal ATP turnover rate occurs (see Table 2.5). A significant amount of ATP (provided by glycolysis) will be "stolen" for the excitation processes (Kushmerick, 1983). The result of this will be that less ATP will be available for cross-bridge formation and the rate of ATP turnover by acto-myosin ATPase will acordingly be diminished. The PCr shuttle may now be able to rephosphorylate the ADP formed without the need for the adenylate kinase reaction so a fall in ATP does not occur. As H^+ efflux does not occur, there will be a lower pH at exhaustion than during dynamic exercise and this is reflected by a lower PCr concentration (see Table 2.3). With electrically stimulated muscle, metabolic changes are similar to those during isometric contraction (see Table 2.3) but when the muscle is "driven" abnormally (Spriet et al, 1987) cross-bridge formation occurs at a higher rate than would normally occur with the result that the decaying energy supply from glycolysis via the PCr

shuttle can no longer meet the muscle's requirements. This may again result in an accumulation of ADP so that the adenylate kinase reaction results in a fall in ATP.

This hypothesis still allows for the metabolic control of muscular contraction as discussed in chapter 2. In addition it may help explain the finding that a reduction in ATP appears to be more marked in FT fibres which are capable of a much faster rate of contraction and hence require a much higher rate of energy supply.

8.3 Influence of interval training on muscle metabolism with the 30 s anaerobic work test

If the hypothesis outlined above has any validity it would be necessary to demonstrate that the increase in power output associated with training is accompanied by a higher ATP provision by glycolysis. Although the study demonstrated a trend in this direction the changes were not significant. However a recently completed study in which 8 subjects underwent a similar training schedule using a supramaximal treadmill sprint as the exercise model has revealed a significant improvement in glycolytic ATP turnover to accompany the improvement in performance (Cheetham and Boobis, unpublished observations). Additionally, if a logarithmic plot of glycolytic ATP turnover against work load is carried out using the data from table 2.5 and correcting for lactate efflux using an estimated value of 0.05 mmoles/kg d.m./s (from Jorfeldt et al, 1978), a linear relationship is obtained (figure 23). This clear relationship between the energy supply from the Embden-Meyerhof pathway and power output at all work rates further supports the key role of glycolysis in muscular contraction at all levels of activity.

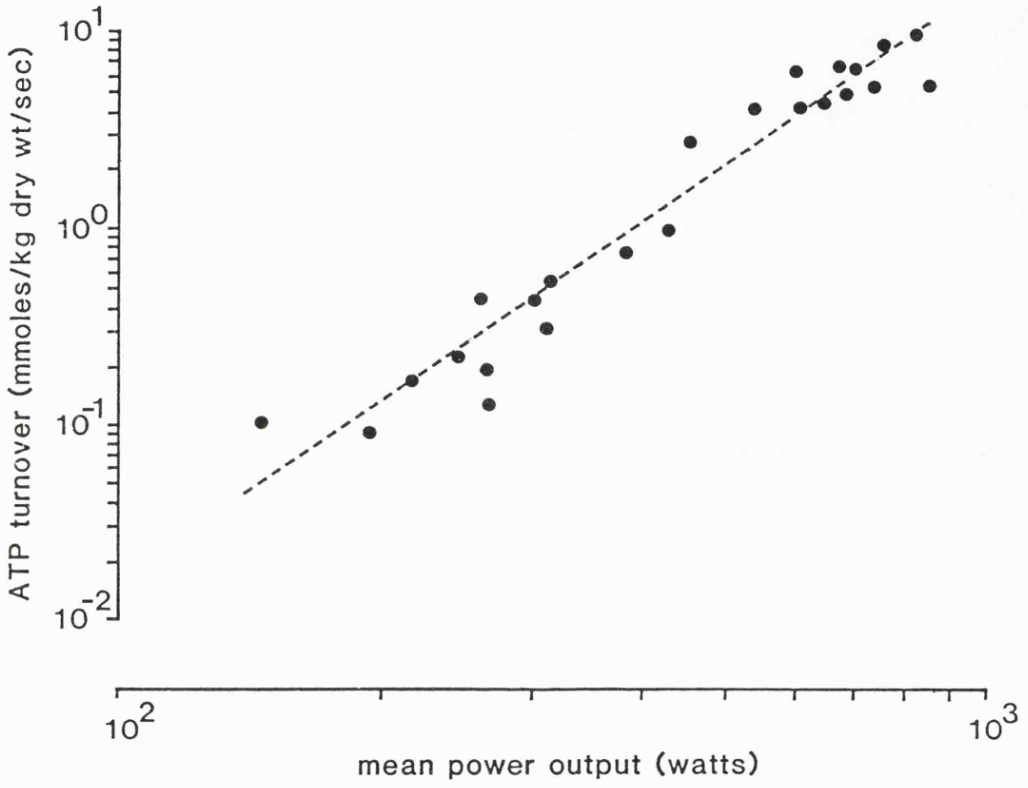


Figure 23 Logarithmic plot of glycolytic ATP turnover against work load (power ouput) from data in table 2.5 and from studies I and II. Values are corrected for lactate efflux of 0.05 mmol/kg d.m./s. (n=24; r=0.97)

8.4 Metabolic changes in muscle of patients with ischaemic rest-pain

Despite the invasive nature of the biopsies, they were very well tolerated and no complications from them were encountered. The evidence presented in the discussion in chapter 7 would suggest that the changes in muscle metabolism found were more a reflection of inactivity or disuse than of ischaemic changes within the muscle. If a more distal muscle had been biopsied such as in the foot, then the direct effects of ischaemia may have been more apparent but this could not be considered realistic because of the risks involved. A criticism of interpretation of the metabolite changes is the failure to have measured total creatine as a reference to metabolite concentrations. This was because the patient samples were processed before the creatine assay had been validated but Möller (1980) has shown that the creatine content in the muscle of elderly patients with and without disease is the same as reported by Harris et al (1975) in normal controls. As the normal range of metabolites reported in chapters 5 and 6 (including creatine) are in close agreement with other studies there is no reason however to doubt the validity of the results. A second criticism could be the examination of resting metabolite levels; if the patients had undergone some sort of exercise test prior to biopsy a better picture of the metabolic processes would have been obtained. Although this in theory could have been done, the patients' pain and immobility prevented in many cases even gentle foot movement and such a study would have effectively excluded those with the most severe disease. Examination of muscle removed at amputation would have been informative and would have provided additional important information.

The importance of exercise in peripheral vascular disease has been discussed in Chapter 3, and the finding that patients with severe lower

limb atherosclerosis can be considered to be "de-trained" lends support to the argument that training can be beneficial. Rauramaa (1986) has recently drawn attention to the beneficial effects of exercise in stimulating prostacyclin production, which has been likened in atherosclerosis to the deficient hormone insulin in diabetes (Gryglewski, 1980) and Grimby (1986) has demonstrated both the feasibility of and the adaptive changes that occur in training elderly patients (70-81 years). Thus even patients with severe ischaemia may benefit from some form of exercise if it can be tolerated.

8.5 The use of naftidrofuryl in the treatment of ischaemic rest pain

The failure to demonstrate any therapeutic benefit as a response to naftidrofuryl is similar to what has been found in the use of prostacyclin (PGF_2) in the treatment of rest pain. Cronewett (1986) studied a comparable group of 27 patients with rest pain or ischaemic ulcers with PGF_2 or placebo and was unable to show that the active drug conferred any benefit. A similar response to lumbar sympathectomy is seen to occur, with those patients with most severe ischaemia deriving least benefit. There is no evidence to support the claims that naftidrofuryl has any beneficial metabolic effect on resting muscle but again it may have been informative to have tested the response to exercise. Although the drug appeared to result in a lower analogue score for subjective parameters including pain, there was a signifcant placebo response and more patients were withdrawn for uncontrolled pain who were receiving the active drug than placebo (4 and 2 respectively) but this difference was not significant. Evidence to support its widespread use in the treatment of severe pedal ischaemia or in buying time prior to constructive surgery is not supported. Metabolic manipulation

using a glucose-insulin infusion (Falholt and Falholt, 1986) has been demonstrated to induce favourable metabolic changes with observed clinical improvements. This is as yet uncontrolled and as the early uncontrolled trials of PGE_1 and PGF_2 failed to live up to their promise, caution must be exhibited before unwarranted claims for the use of this method are made.

8.6 Summary

1) Supramaximal exercise for 6 s resulted in more than 50% of the ATP being supplied by glycolysis.

2) Over a 30 s sprint of the same intensity almost 70% of the energy was supplied by glycolysis and marked reductions in ATP occurred in the presence of significant amounts of PCr. A hypothesis for these findings is presented.

3) Training revealed a trend for the resulting improvement in performance to be associated with an increased glycolytic capacity and although not statistically significant, this trend has been confirmed in a subsequent study.

4) The changes in muscle metabolism observed in patients with rest pain (low glycogen, ATP, PCr and SDH levels) represented disuse rather than a direct effect of muscle ischaemia as had been postulated and were thus not comparable with those seen after high intensity exercise.

5) The drug naftidrofuryl when used in the treatment of ischaemic rest pain confers no obvious clinical or metabolic benefit.

Appendix 1

A1 Apparatus, chemicals, biochemicals and reagents

A1.1 Apparatus

-70°C Freezer: Le Rose -70°C freezer (New Brunswick Scientific (U.K.) Ltd., Watford)

Analytical Balance: Sartorius Series 200 (R.W. Jennings & Co. Ltd., Scientech House, East Bridgeford, Nottingham)

Centrifuge: MSE Chilspin (MSE Scientific Instruments, Crawley)

Centrifuge tubes: LP/3P and LP/4P tubes (Luckham Ltd., Burgess Hill, Sussex)

Cuvettes: Sarstedt 1ml disposable semi-micro cuvettes, No. 67.742 (Sarstedt Ltd., Beaumont Leys, Leicester)

Flask Shaker: Gallenkamp flask shaker (Gallenkamp, Belton Road West, Loughborough)

Fluorimeter: Locarte Filter Fluorimeter LFM8-9 with mercury arc lamp and quartz optics; primary filter: Corning No. 5860, and secondary filters: Corning No. 4303 and 3387 (Lowry and Passonneau, 1972) (Locarte Co., Wendell Road, London)

Fluorimeter tubes: Pyrex 10x75 mm light wall rimless test-tubes, No. 1622/02 (Fisons Scientific Apparatus, Bishop Meadow Raod, Loughborough)

Freeze Dryer: Edwards EF4 Modulyo Freeeze Dryer with Specimen Dryer Accessory No. 10-F029-10-100; Edwards E2M5 Series Rotary Vacuum Pump (Edwards High Vacuum, Manor Road, Crawly, West Sussex)

Homogenisers: Uniform mini homogeniser; 0.1 ml No. H103/33-34/94 and 1ml No. H103/32-35/94 (Jencons (Scientific) Ltd., Leighton Buzzard, Beds.)

Homogeniser Drive Unit: Citenco Varilab Stirrer (Scientific

Industries International Inc. (U.K.) Ltd., Loughborough)

Liquid Nitrogen Freezer: Union Carbide LR-40 (Jencons (Scientific) Ltd., Leighton Buzzard, Beds.)

Microbalance: Mettler ME22/BE22/BA25 (Gallenkamp, Belton Road West, Loughborough)

Micro-capillary tubes: Dade 25 μℓ Volupette disposable glass capillary tubes (Scientific Industries International Inc. (U.K.) Ltd., Loughborough)

Microcentrifuge: Eppendorf 5414 (Anderman & CO. Ltd, Central Avenue, East Mosley)

Microreaction tubes: Eppendorf 3810 1.5 ml microcentrifuge tubes (Anderman & Co. Ltd., Central Avenue, East Mosley): Sarstedt 1.5 ml screw-top microtubes No. 72.692; Sarstedt 2 ml screw-top microtubes No. 72.693; Sarstedt 2 ml screen printed screw-top microtubes No. 72.694 (Sarstedt Ltd., Beaumont Leys, Leicester)

Pipettes: Gilson Pipetman and Gilson Microman adjustable pipettes (Anachem Ltd., Luton, Beds.)

Spectrophotometer: Pye Unicam SP1800 ultraviolet spectrophotometer; AR 25 series recorder (Pye Unicam Ltd., Cambridge)

Universal 30 ml bottles: Sterilin No. 128A (Sterilin, Teddington, Middlesex)

Volumetric Dispensers: Oxford Dispenser 1 ml (fixed volume), 500 ml; Oxford Pipettor Model SA, 1.2 l; Oxford Pipettor Model R, 500 ml (BCL The Boehringer Corporation (London) Ltd., Bell Lane, Lewes)

Vortex mixer: Vortex Genie (Scientific Industries International Inc. (U.K.) Ltd., Loughborough)

Water bath: Grant, J/GE10 (R.W. Jennings & Co. Ltd., Cientech House, Eastbridgeford, Nottingham)

Water bath, shaking: Grant SS40 (R.W. Jennings & Co. Ltd.)

A1.2 Chemicals

Ammonium sulphate, specially low in heavy metals for enzyme work: Chloroform, grade Aristar: Cupric nitrate trihydrate, grade Analar: Ethanol absolute, grade Aristar: Ethylenediaminotera-actic acid (EDTA), grade Analar: Hydrochloric acid, grade Analar: Magnesium chloride hexahydrate, grade Analar: Magnesium sulphate heptahydrate, grade Analar: Methanol, grade Aristar: n-Heptane, grade Analar: Nitric Acid (fuming) (95%), grade Analar: Perchloric acid (72%), grade Analar: Petroleum ether (petroleum spirit) 30-40°C, grade Analar: Potassium carbonate (anhydrous), grade Analar: Potassium chloride, grade Analar: Potassium dihydrogen orthophosphate, grade Analar: Potassium hydrogen carbonate, grade Analar: di-Potassium hydrogen orthophospate trihydrate, grade Analar: Potassium hydroxide, grade Aristar: Sodium carbonate (anhydrous), grade Analar: Sodium chloride, grade Analar: tri-Sodium citrate, grade Analar: Sodium hydrogen carbonate, grade Analar: Sodium hydroxide, grade Analar: Zinc chloride, grade Analar

BDH Chemicals Ltd., Broom Road, Parkstone, Poole BH12 4NN

2-Amino-2-methylpropan-1-ol, free base: 2-Amino-2-methylpropan-1-ol hydrochloride, crystalline: Hydrazine hydrate 100% (64% hydrazine): Hydrazine sulphate, crystalline: Imidazole, grade I crystalline: Imidazole, grade III crystalline, fluorimetric grade: Triethanolamine hydrochloride, 99%, crystalline: 2-(2-Thiozyl[azo])-p-cresol, crystalline (TAC): TRIS [tris(hyroxymethyl)aminomethane] base, reagent grade: TRIS hydrochloride, reagent grade

Sigma London Chemical Co. Ltd., Fancy Road, Poole, BH17 7NH

A1.3 Biochemicals, cofactors, reactants and standards

Acetoacetic acid, lithium salt

Adenosine 5'-diphosphate (ADP), di(cyclohexylammonium) salt, grade V: 98-100%, crystalline from equine muscle

Adenosine 5'-monophosphate (AMP), sodium salt, type III: 99-100%, crystalline from equine muscle

Adenosine 5'-triphosphate (ATP), disodium salt, grade II, crystalline (for Chappell-Parry medium only)

Adenosine 5'-triphosphate (ATP), disodium salt 99-100% (vanadium free), crystalline from equine muscle

L-Alanine, crystalline

Albumin, Bovine crystallised and lyophilised (BSA)

Albumin, Bovine crystallised and lyophilised, fatty acid free

Antimycin A from Streptomyces kitazawaenis, crystalline

Bromobenzene (bromobenzol)

D-Fructose 1,6-bisphosphate (F16BP, FBP), trisodium salt, 98-100%, crystalline

α-D-Glucose 1,6-diphosphate (G16DP), tetra(cyclohexylammonium) salt hydrate, 95-100%, crystalline

D-Glucose-6-phosphate (G6P), monosodium salt, crystalline

L-Glutamic acid, monosodium salt, 99-100%, crystalline

Glycerol, 99%

DL-β-Hydroxybutyric acid, sodium salt, crystalline

L(+)-Lactic acid, free acid, grade L-I, 98-100%, crystalline

2-Mercaptoethaol, 1.0 mol/l in 0.02 mol/l KOH

β-Nicotinamide-adenine dinucleotide (NAD), free acid, grade III, 98%

β-Nicotinamide-adenine dinucleotide, reduced (NADH), disodium salt, grade III, 98%

β-Nicotinamide-adenine dinucleotide phosphate (NADP), sodium salt, 98-100%

Palmitic acid, free acid, 99%, crystalline

Phenazine methosulphate (PMS)

Phosphocreatine (PCr) disodium salt hydrate, 98-100%, crystalline

Phospho(enol)pyruvate (PEP) Tri(cyclohexylammonium) salt, crystalline

Pyruvic acid, sodium salt, type II, 99+%, crystalline

Succinic acid, disodium salt hexahydrate, crystalline

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Glucose Test-Combination, GOD-Perid method

β-Nicotinamide-adenine dinucleotide, reduced (NADH) disodium salt, grade I, 100%

The Boehringer Corporation (London) Ltd., Bell Lane, Lewes, East Sussex BN7 1LG

Citric acid, grade Analar

Dithiothreitol (Clelands reagent)

D-Glucose, grade Analar

Sodium arsenate heptahydrate, grade Analar

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A1.4 Enzymes

L-Alanine dehydrogenase from bacillus subtilis, 30 U/mg, 5 mg/ml, suspension in ammonium sulphate solution, 2.4 mol/l (L-Alanine:NAD oxidoreductase (deaminating), EC 1.4.1.1)

Aldolase from rabbit muscle, 9 U/mg, 10 mg/ml, crystalline suspension

in ammonium sulphate solution 3.2 mol/l (D-Fructose-1,6-bisphosphate D-glyceraldehyde-3-phosphate lyase, EC 4.1.2.13

Citrate lyase (CL) from aerobacter aerogenes, 0.25 U/mg, lyophilisate, stabilised with BSA, sucrose, $MgSO_4$ and EDTA (Citrate oxaloacetate-lyase, EC 4.1.3.6)

Creatine kinase (Creatine phosphokinase) (CK) from rabbit muscle, 380 U/mg, lyophilised (ATP:creatine N-phosphotransferase, EC 2.7.3.2)

Fumarase from pig heart, 350 U/mg, 10 mg/ml, crystalline suspension in ammonium sulphate solution, 3.2 mol/l (L-Malate hydro-lyase, EC 4.2.1.2)

Glucose-6-phosphate dehydrogenase (G6P-DH) from yeast, grade I, 350 U/mg, 1 mg/ml, suspension in ammonium sulphate solution, 3.2 mol/l (D-glucose-6-phosphate:NADP 1-oxidoreductase, EC 1.1.1.49)

Glutamate-oxaloacetate transaminase (GOT) from pig heart, 200 U/mg, 2 mg/ml, suspension in ammonium sulphate solution, 3.2 mol/l (L-Aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1)

Glyceraldehyde-3-phosphate dehydrogenase (GAP-DH) from rabbit muscle, 80 U/mg, 10 mg/ml, crystalline suspension in ammonium sulphate solution, 3.2.mol/l; EDTA 0.1 mmol/l (D- Glyceraldehyde-3-phosphate:NAD oxido-reductase (phosphorylating), EC 1.2.1.12)

Glycerol dehydrogenase from Enterobacter aerogenes, 25 U/mg, 20 mg/ml, suspension in ammonium sulphate solution, 3.2 mol/l (Glycerol:NAD^+ 2-oxidoreductase, EC 1.1.1.6)

Hexokinase (HK) from yeast, 140 U/mg, 2 mg/ml, suspension in ammonium sulphate solution, 3.2. mol/l (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1)

3-Hydroxybuturate dehydrogenase (3-HBDH), from Rhodopseudomonas spheroides, grade II, 5 mg/ml, 3 U/mg, suspension in ammonium sulphate solution, 3.2 mol/l (D-3-Hydroxybutyrate:NAD oxidoreductase, EC

1.1.1.30)

Lactic dehydrogenase (LDH) from beef heart, 250 U/mg, 5 mg/ml, crystalline suspension in ammonium sulphate solution, 3.2 mol/l (L-Lactate:NAD oxidoreductase, EC 1.1.1.27)

Malate dehydrogenase (MDH) from pig heart (mitochondrial), 1200 U/mg, 5 mg/ml, suspension in ammonium sulphate suspension (L-Malate:NAD oxido-reductase, EC 1.1.1.37)

Myokinase (Adenylate kinase) (MK), from rabbit muscle, 360 U/mg, 2mg/ml, suspension in ammonium sulphate solution, 3.2 mol/l (ATP:AMP phosphotransferase, EC 2.7.4.3)

Phosphoglucomutase (PGluM), from rabbit muscle, 200 U/mg, 2 mg/ml, suspension in ammonium sulphate, 3.2 mol/l,(α-D-Glucose-1,6-biphosphate:α-D-glucose-1-phosphate phosphotransferase, EC 2.7.5.1)

Phosphoglucose isomerase (PGI), from yeast, 350 U/mg, 2 mg/ml, suspension in ammonium sulphate, 3.2 mol/l, (D-Glucose-6-phosphate ketol-isomerase, EC 5.3.1.9)

Pyruvate kinase (PK), from rabbit muscle, 200 U/mg, 2 mg/ml, crystalline suspension in ammonium sulphate solution, 3.2 mol/l (ATP:pyruvate 2-O-phosphotransferase, EC 2.7.1.40)

Triose phosphate isomerase (TIM) from rabbit muscle, 500 U/mg, 2 mg/ml, crystalline suspension in ammonium sulphate solution, 3.2 mol/l (D-Glyceraldehyde-3-phosphate ketol-isomerase, EC 5.3.1.1)

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A1.5 Reagents

A1.5.1 Buffers

2-Amino-2-methyl-propan-1-ol 0.5 mol/l (pH 9.9); 3.14 g 2-amino-2-methylpropan-1-ol HCl and 2.23 g 2-amino-2-methylpropan-1-ol free base dissolved in water, pH adjusted if required and diluted to 100 ml.

2-Amino-2-methyl-propan-1-ol 0.10 mol/l, hydrazine 0.20 mol/l, EDTA 1 mmol/l (pH 9.9); 891.4 mg 2-amino-2-methyl propan-1-ol, 1 ml hydrazine hydrate and 37.2 mg EDTA.Na_2.$2H_2O$ dissolved in water, pH adjusted with HCl to 9.9 and diluted to 100 ml.

Chappell-Parry Buffer (pH 7.4) (50 mmol/l TRIS-HCl buffer, pH 7.4; 100 mmol/l KCl; 5 mmol/l $MgSO_4$; 1 mmol/l EDTA): 0.505 g TRIS base, 0.131 g TRIS HCl, 0.746 g KCl, 0.123 g $MgSO_4.7H_2O$, 37.2 mg EDTA.$2H_2O$ dissolved in water, pH adjusted to 7.4 if required, and diluted to 100 ml.

Chappell-Parry Medium (Buffer with 1 mmol/l ATP) prepared by adding 20 ml of Chappell-Parry Buffer to 0.2 ml of 0.1 mol/l ATP (grade II)

Hydrazine 1.1 mol/l, EDTA 1 mmol/l (pH 9.0); 1.3 g hydrazine sulphate, 5.0 ml hydrazine hydrate and 37.2 mg EDTA dissolved in water, pH adjusted to 9.0 and diluted to 100 ml.

Imidazole 50 mmol/l (pH 7.0); 0.341 g of imidazole base dissolved in water, add 3 ml of 1.0 mol/l HCl, pH adjusted if required and diluted to 100 ml

Imidazole Low Fluorescence 50 mmol/l (pH 7.0); 0.341 g of low fluorescence imidazole base dissolved in water, add 3 ml of 1.0 mol/l HCl, pH adjusted if required and dilute to 100 ml

Imidazole Low Fluorescence 50 mmol/l, Zinc Chloride 0.3 mmol/l (pH 7.0); 0.409 mg $ZnCl_2$ dissolved in 10 ml of low fluorescence imidazole buffer 50 mmol/l, pH 7.0.

Imidazole 50 mmol/l (pH 7.5); 0.341 g of imidazole base dissolved in water, add 1.6 ml of 1.0 mol/l HCl, pH adjusted if required and diluted to 100 ml.

Imidazole Low Fluorescence 50 mmol/l (pH 7.5); 0.341 g of low fluorescence imidazole base dissolved in water, add 1.6 ml of 1.0 mol/l HCl, pH adjusted if required and diluted to 100 ml.

Potassium phosphate 1 mol/l, EDTA 1 mmol/l (pH 7.0); 15.98 g $K_2HPO_3.3H_2O$, 4.08 g KH_2PO_4, 37.2 mg EDTA dissolved in water, pH adjusted to 7.0 and diluted to 100 ml.

Potassium phosphate 50 mmol/l, magnesium chloride 5 mmol/l, BSA 0.05% (pH 7.5); 0.046 g $K_2HPO_4.3H_2O$, 0.116 g KH_2PO_4, 0.102 g $MgCl_2.6H_2O$ and dissolved in water and diluted to 100 ml, 50 mg BSA added to surface and allowed to dissolve on its own.

Potassium phosphate 0.3 mol/l, BSA 0.05% (pH 7.7); 0.45 g KH_2PO_4 and 6.09 g K_2HPO_4 dissolved in water and diluted to 100 ml, 50mg BSA added to surface and allowed to dissolve on its own.

Sodium carbonate 20 mmol/l (pH 10.0); 0.84 g $NaHCO_3$ and 1.06 g Na_2CO_3 EDTA dissolved in water, pH adjusted to 10.0 and diluted to 1 l.

Sodium carbonate 0.1 mol/l (pH 10.6); 0.17 g $NaHCO_3$ and 0.85 g Na_2CO_3 dissolved in water, pH adjusted if required and diluted to 100 ml

TRIS HCl 20 mmol/l, BSA 0.02% (pH 8.1); 0.158 g TRIS HCl, 0.121 g TRIS base dissolved in water, pH adjusted if required and diluted to 100 ml, 20 mg BSA added to surface and allowed to dissolve on its own.

TRIS HCl 50 mmol/l, BSA 0.02% (pH 8.1); 0.788 g TRIS HCl, 0.606 g TRIS base dissolved in water, pH adjusted if required and diluted to 100 ml, 20 mg BSA added to surface and allowed to dissolve on its own.

TRIS HCl 0.1 mol/l, BSA 0.02% (pH 8.1); 1.576 g TRIS HCl, 1.211 g TRIS base dissolved in water, pH adjusted if required and diluted to 100 ml, 20 mg BSA added to surface and allowed to dissolve on its own.

TRIS HCl 50 mmol/l (pH 7.6); 0.591 g TRIS HCl and 0.151 g TRIS base dissolved in water, pH adjusted if required and diluted to 100 ml.

TRIS-Hydrazine 1.0 mol/l, EDTA 1 mmol/l (pH 10.0); 0.484 g TRIS base, 5 ml hydrazine hydrate and 37.2 mg EDTA dissolved in water, adjust to pH 10.0 with 1 mol/l HCl and diluted to 100 ml.

Stability of buffers: all buffers were stable at 0-4°C for at least 6 months but were changed if micro-organisim contamination occured.

A1.5.2 Enzymes

a) Dilutant

TRIS-HCl 20 mmol/l, BSA 0.02% (pH 8.1) except for Citrate Lyase [Low Fluorescence Imidazole 50 mmol/l, Zinc Chloride 0.3 mmol/l (pH 7.0)] and LDH [Ammonium sulphate 2.1 mol/l, BSA 0.2%]

b) Working solutions

Alanine dehydrogenase 150 U/ml (undiluted)

Aldolase 9 U/ml, 10μℓ stock suspension in 90μℓ dilutant

CK 380 U/ml, 0.1 mg solid dissolved in 0.1 ml of dilutant

CK 1500 U/ml, 0.4 mg solid dissolved in 0.1 ml of dilutant

CL 3 U/ml, 1.2 mg solid dissolved in 0.1 ml of imidazole dilutant

Fumarase 3500 U/ml (undiluted)

GAP-DH 180 U/ml, 20μℓ stock suspension in 65μℓ dilutant

GDH 500 U/ml (undiluted)

GOT 90 U/ml, 20μℓ stock suspension in 70μℓ dilutant

G6P-DH 7 U/ml, 20μℓ stock supension in 90μℓ dilutant

3-HBDH 15 U/ml (undiluted)

MDH 600 U/ml, 10μℓ stock suspension in 90μℓ dilutant

MDH 35 U/ml, 5μℓ stock suspension in 0.85 ml dilutant

HK 28 U/ml, 10μℓ stock suspension in 90μℓ dilutant

MK 35 U/ml, 5μℓ stock suspension in 0.1 ml dilutant

LDH 1250 U/ml (undiluted)

LDH 40 U/ml 5μℓ stock suspension in 0.15 ml ammonium sulphate dilutant

LDH 10 U/ml 5μℓ stock suspension in 0.62 ml ammonium sulphate dilutant

PGluM 4 U/ml, 5μℓ stock suspension in 0.495 ml dilutant

PGI 35 U/ml, 5μℓ stock suspension in 95μℓ dilutant

PK 75 U/ml, 25μℓ stock suspension in 80μℓ dilutant

TIM 240 U/ml, 5μℓ stock suspension in 0.195 ml dilutant

Stability of enzymes: all stock suspensions and lyophylised solids are stable for at least a year at 0-4°C. The diluted enzymes were prepared fresh daily with the exception of citrate lysase which was prepared weekly, and LDH which was stable for several weeks.

A1.5.3 Cofactors

ADP 50 mmol/l; 33.76 mg di(cyclohexylammonium) salt dissolved in 0.975 ml water, neutralised with 25μℓ 2 mol/l NaOH and 0.2 ml stored as 50 aliquots at -20°C (stable indefinitely)

ADP 10 mmol/l; 0.8 ml of ADP 50 mmol/l diluted to 4.0 ml with water and stored as 1 ml aliquots at -20°C (stable indefinitely)

Arsenate 0.1 mol/l; 3.12 g sodium salt dissolved in water and made up to 100 ml. Stored at 0-4°C (stable for months)

ATP 50 mmol/l; 30.1 mg of disodium salt (99-100%) dissolved in 0.95 ml water, neutralised with 0.05 ml of 2 mol/l NaOH, and stored as 0.1 ml aliquots at -20°C (stable indefinitely)

ATP 10 mmol/l; 6.02 mg of disodium salt (99-100%) dissolved in 0.99 ml water, neutralised with 0.01 ml 2 mol/l NaOH, and stored as 0.1 ml

aliquots at -20°C (stable indefinitely).

ATP 0.5 mmol/l; 1.9 ml water added to 0.1 ml ATP 10 mmol/l and stored as 1 ml aliquots at -20°C (stable indefinitely)

ATP 0.1 mol/l (for Chappell-Parry medium); 61.4 mg disodium salt (grade II) dissolved in 0.9 ml water, neutralised with 0.1 ml of 2 mol/l NaOH and stored as 0.2 ml aliquots in 30 ml Sterilin screw-top polystyrene tubes at -20°C.

Glucose 0.1 mol/l; 36.03 mg D-Glucose dissolved in 2 ml of water and 0.5 ml stored as 0.1 ml aliquots at -20°C (stable).

Glucose 10 mmol/l; 1.5 ml of glucose 0.1 mol/l diluted to 15 ml and stored as 1 ml aliquots at -20°C (stable indefinitely)

Glutamic acid 1.0 mol/l (pH 9.9); 1.87 g monosodium salt dissolved in water, pH adjusted to 9.9 and diluted to 10 ml, Stored as 1 ml aliquots at -20°C (stable indefinitely).

G16DP 0.25 mmol/l; 1.538 mg tetra(cyclohexylammonium) salt dissolved in 2 ml of water and stored as 0.2 ml aliquots at -20°C (stable indefinitely).

NAD 50 mmol/l stock solution; 36.03 mg free acid dissolved in 1 ml water and stored as 0.5 ml aliquots at -20°C (stable indefinitely).

NADH 5 mmol/l stock solution (grade III); 4 mg disodium salt (grade III) dissolved in 1 ml of carbonate buffer 0.1 mol/l, pH 10.6 and stored at -70°C (stable indefinitely).

NADH 1 mmol/l (grade III); 0.4 ml carbonate buffer 0.1 mol/l, pH 10.6 added to 0.1 ml NADH 5 mmol/l and stored at 0-4°C (stable for 2-3 weeks) or -70°C (stable indefinitely)

NADH 1 mmol/l (grade I); 0.75 mg disodium salt (grade I) dissolved in 1 ml of carbonate buffer 0.1 mol/l, pH 10.6 and stored at -70°C (stable indefinitely)

NADP 50 mmol/l; 42.3 mg sodium salt dissolved in 1 ml water and stored

as 0.2 ml aliquots at -20°C (stable indefinitely)

NADP 5 mmol/l; 0.2 ml of NADP 50 mmol/l diluted to 2 ml with water and stored as 0.5 ml aliquots at -20°C (stable indefinitely).

PEP 30 mmol/l; 15 mg tri(cyclohexylammonium) salt dissolved in 1 ml of water and stored as 0.1 ml aliquots at -70°C (stable indefinitely).

PEP 2 mmol/l; 1.4 ml water added to 0.1 ml PEP 30 mmol/l and stored as 0.1 ml aliqots at -70°C (stable indefinitely)

PMS 10 mmol/l; 15.32 mg dissolved in water and diluted to 5 ml. Stored protected from light at 0-4°C (stable for several weeks).

Pyruvate 0.1 mol/l; 2.2 mg sodium salt dissolved in 2 ml of water and stored as 0.1 ml aliquots at -70°C (stable indefinitely).

Succinate 1.5 mol/l (pH 7.7); 4.05 g disodium salt hexahydrate dissolved in water, pH adjusted to 7.7 and diluted to 10 ml. Stored as 1 ml aliquots at -20°C (stable indefintely).

Stablity of cofactors: all cofactors were stable as indicated; for fluorimetric assays the concentration of NADH was checked spectrophotometrically prior to commencing a batch of assays.

A1.5.4 Standards

All standards with the exception of glucose, glycerol and palmitic acid were made up by dissolving the stated weight in 8 ml of water, checking the concentration spectrophotometrically and diluting to the concentration indicated. ADP and ATP were neutralised with 1 and 2 equivalents of NaOH respectively. Aliquots of 1 ml were then stored at -20°C (unless otherwise stated) and were stable indefinitely.

Acetoacetate 2 mmol/l; 2.2 mg of lithium salt: ADP 2 mmol/l; 13.5 mg of di(cyclohexylammonium) salt: L-Alanine 2 mmol/l; 1.8 mg of free

acid: AMP 1 mmol/l; 3.6 mg of free acid: ATP 2 mmol/l; 12 mg disodium salt: Citrate 2 mmol/l; 5.9 mg of trisodium salt: F6P 2 mmol/l; 7.5 mg disodium salt: F16BP 1 mmol/l; 4.9 mg of trisodium salt: Fumarate 2 mmol/l; 3.2 mg sodium salt: G1P 2 mmol/l; 7.7 mg of dipotassium salt: G6P 2 mmol/l; 5.6 mg of sodium salt: Glucose 0.505 mmol/l; Boehringer standard from glucose test-combination, stored at 0-4°C (stable several months): Glycerol 2 mmol/l; 18.42 mg of glycerol dissolved in 100 ml of water and concentration checked spectrophotometrically, stored as 1 ml aliquots at -20°C: β-Hydroxybutyrate 2 mmol/l; 4.2 mg of sodium salt: Lactate 2 mmol/l; 1.8 mg free acid: Lactate 10 mmol/l; 9.01 mg of free acid: Palmitate 1 mmol/l, BSA 3%; 25.64 mg palmitic acid dissolved in acetone in a 100 ml volumetric flask and 5 ml of KOH 50 mmol/l added, made up to 100 ml with 3% fatty acid free BSA and stored at 0-4°C (stable for several weeks): PCr 2mmol/l; 6.9 mg of disodium salt: Pyruvate 2 mmol/l; 2.2mg of sodium salt, stored at -70°C (stable for many months)

A1.5.5 Other Reagents

Ammonium sulphate 2.1 mol/l, BSA 0.2%; 5.55 g $(NH_4)2SO_4$ dissolved in water and diluted to 20 ml, 40 mg BSA added to surface of solution and allowed to dissolve on its own.

Antimycin A 0.1 mmol/l; 0.55 mg antimycin A dissolved in 10 ml of 100% ethanol. Stored at -70°C.

BSA 3%, fatty acid free; 3 g crystallised and lyophilised bovine albumin, fatty acid free, carefully added to about 75 ml of water, allowed to dissolve on its own, pH adjusted to 7.0 and made up to 100 ml. Stored at 0-4°C, stable for 2-3 weeks.

CHM extraction solution for FFA assay; chloroform 280 ml, n-heptane 210 ml and methanol 10 ml. Stored at room temperature in Oxford Pipettor Model SA.

Copper reagent, stable, for FFA assay (pH 8.0-8.2); triethanolamine HCl 0.75 g, copper nitrate 3.25 g, sodium chloride 25 g dissolved in water, pH adjusted if required and made up to 100 ml.

Dithiothreitol 50 mmol/l; 77.13 mg dissolved in 10 ml of water

EDTA 0.1 mol/l; 3.72 g $EDTA.Na_2.2H_2O$ dissolved in water and diluted to 100 ml.

Hydrochloric acid 1.0 mol/l; 10.3 g (8.7 ml) 35.4% HCl, SG 1.18 diluted to 100 ml with water.

Hydrochloric acid 4.0 mol/l; 41.2 g (34.9 ml) 35.4% HCl, SG 1.18 diluted to 100 ml with water.

Magnesium chloride 0.1 mol/l; 2.03 g $MgCl_2.6H_2O$ dissolved in water and diluted to 100 ml.

Mercaptoethanol 1 mol/l; stock solution in 0.02 mol/l KCl

Nitric Acid 50%; 500 ml water added to 300 ml of fuming HNO_3 (95%), SG 1.50

Perchloric acid 0.25 mol/l; 2.04 ml 72% $HClO_4$, SG 1.70 diluted to 100 ml with water

Perchloric acid 0.5 mol/l, EDTA 1 mmol/l: 37.2 mg EDTA dissolved in water with 4.08 ml 72% $HClO_4$, SG 1.70 and diluted to 100 ml.

Potassium chloride 3.0 mol/l; 22.37 g KCl dissolved in water and made up to 100 ml.

Potassium hydrogen carbonate 2.1 mol/l: 21.03 g $KHCO_3$ dissolved in water and diluted to 100 ml.

Potassium hydroxide 50 mmol/l; 0.281 g KOH dissolved in water and diluted to 100 ml.

Sodium hydroxide 2 mol/l; 8 g NaOH dissolved in water and diluted to

100 ml. Stored at room temperature.

Sodium hydroxide 6 mol/l; 120 g NaOH dissolved in water and diluted to 500 ml (for fluorescent development assays, allowed to stand in sunlight for several hours before use). Stored at room temperature.

TAC 0.01% (colour reagent for FFA assay); 10 mg TAC dissolved in 100 ml ethanol. Stored in Oxford Pipettor Model R at 0-4°C (stable for several months).

All reagents were stored at 0-4°C and were indefinitely stable unless otherwise stated.

Appendix 2

A2 Spectrophotometric assays for validation of standards

A2.1 General points

All assays were carried out in a 1 ml semi-micro disposable polystyrene cuvette at 340 nm, reading against air, and were followed to completion on a chart recorder. The concentration of the standard was calculated from the following equation:

$$[C] = \frac{Vc \times E}{6.3 \times Va} \qquad (A2.1)$$

where [C] = the concentration of the standard in mmol/l

Vc = the final cuvette volume in ml

Va = the volume of standard added in ml

$\Delta E = E_2 - E_1$

E_1 = the initial absorbance

E_2 = the final absorbance

6.3 = the mmolar extinction coefficient for NADH/NADPH at 340 nm

Where assays involved the oxidation of NADH, the concentration of NADH was checked beforehand to ensure that there was about 50% excess for the reaction. Unless otherwise indicated 20$\mu\ell$ of an approximately 2 mmol/l standard was added to 1 ml of reaction mixture after reading E_1, E_2 being read after a constant end point had been reached. The time course of the reaction is given in minutes.

A2.1.1 Acetoacetate

$$\text{Acetoacetate} + \text{NADH} + H^+ \xrightarrow{\text{3-HBDH}} \beta\text{-OH butyrate} + NAD^+$$

Reagents

Buffer: Potassium phosphate 1 mol/l, EDTA 1 mmol/l, pH 7.0

Cofactor: NADH 5 mmol/l

Enzyme: 3-HBDH (undiluted)

Standard: Acetoacetate 2 mmol/l

Reaction mixture (final concentration)

Buffer 1ml; NADH 12μℓ (0.06 mmol/l); 3-HBDH 10μℓ (0.15 U/ml)

Reaction complete in 30-40 min.

A2.1.2 Adenosine diphosphate (ADP)

$$\text{ADP} + \text{PEP} \xrightarrow{\text{PK}} 2\text{ATP} + \text{Pyruvate}$$

$$\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{LDH}} \text{Lactate} + NAD^+$$

Reagents

Buffer: Imidazole 50 mmol/l, pH 7.0

Cofactor: NADH 5 mmol/l

Enzymes: PK 75 U/ml; LDH 40 U/ml

Other reagents: PEP 30 mmol/l; $MgCl_2$ 0.1 mol/l; KCl 3 mol/l; EDTA 0.1 mol/l

Standard: ADP 2 mmol/l

Reaction mixture (final concentration)

Buffer 1 ml; $MgCl_2$ 20μℓ (2 mmol/l); KCl 25μℓ (75 mmol/l); NADH 15μℓ (0.075 mmol/l): PEP 10μℓ (0.3 mmol/l): LDH 10μℓ (0.4 U/ml)

To 20μℓ of standard was added 1 ml of reaction mix and E_1 read. PK 4μℓ (0.3 U/ml) was then added, mixed thoroughly and the reaction followed to completion (2-4 min) and E_2 read. A further 4μℓ of PK was added to obtain the absorbance of the enzyme (usually insignificant) and the concentration of the standard calculated ($\Delta E = E_1 - E_2 - E_{PK}$)

A2.1.3 Alanine

$$\text{Alanine} + NAD^+ \xrightarrow{ADH} \text{Pyruvate} + NADH + H^+$$

$$\text{Pyruvate} + \text{Hydrazine} \longrightarrow \text{Pyruvate hydrazone}$$

Reagents

Buffer: TRIS-Hydrazine 1 mol/l, EDTA 1 mmol/l, pH 10.0

Cofactor: NAD 50 mmol/l

Enzyme: Alanine dehydrogenase (ADH) 150 U/ml (undiluted)

Standard: Alanine 2 mmol/l

Reaction mixture (final concentration)

Buffer 1 ml; NAD 10μℓ (0.5 mmol/l); ADH 5μℓ (0.75 U/ml)

Reaction complete in 20-30 min

A2.1.4 Adenosine monophosphate (AMP)

$$AMP + ATP \xrightarrow{MK} 2ADP$$

$$ADP + PEP \xrightarrow{PK} ATP + \text{Pyruvate}$$

$$\text{Pyruvate} + NADH + H^+ \xrightarrow{LDH} \text{Lactate} + NAD^+$$

Reagents as for ADP with the addition of

Enzymes: MK 35 U/ml

Other reagents: ATP 10 mmol/l

Standard: AMP 1 mmol/l

Reaction mixture as for ADP with the addition of ATP 10μℓ/ml (0.1 mmol/l) and the incorporation of PK 4μℓ/ml (0.3 U/ml). Reaction mixture added to the standard as in ADP assay and the reaction started with the addition of MK 10μℓ (0.35 U/ml) and followed to completion (10-15 min). A futher 10μℓ MK then added to obtain the absorbance of the enzyme. Calculations as for ADP noting that as 2 moles of NADH are produced per mole of AMP then the AMP concentration is the calculated result divided by 2. For this reason the AMP standard was made up to 1 mmol/l.

A2.1.5 Adenosine triphosphate (ATP)

$$\text{ATP} + \text{Glucose} \xrightarrow{\text{HK}} \text{ADP} + \text{G6P}$$

$$\text{G6P} + \text{NADP}^+ \xrightarrow{\text{G6P-DH}} \text{6-P-Glucolactone} + \text{NADH} + \text{H}^+$$

Reagents

Buffer: TRIS-HCl 50 mmol/l, BSA 0.02%, pH 8.1

Cofactor: NADP 50 mmol/l

Enzymes: HK 28 U/ml; G6P-DH 7 U/ml

Other reagents: Glucose 0.1 mol/l; $MgCl_2$ 0.1 mol/l

Standard: ATP 2 mmol/l

Reaction mixture (final concentration)

Buffer 1 ml; NADP 50μℓ (0.5 mmol/l); $MgCl_2$ 50μℓ (5 mmol/l); Glucose 10μℓ (1 mmol/l); G6P-DH 10μℓ (0.07 U/ml)

To 20μℓ of 2 mmol/l standard of ATP 1 ml of reaction mixture added

and E_1 read. HK 10μℓ added, the reaction followed to completion (5 min) and E_2 read. A further 10μℓ of HK then added to determine the absorbance of the enzyme and concentration of standard calculated using $\Delta E = E_2 - E_1 - E_{HK}$.

A2.1.6 Citrate

$$\text{Citrate} \xrightarrow{CL} \text{Oxaloacetate} + \text{acetate}$$

$$\text{Oxaloacetate} + \text{NADH} + H^+ \xrightarrow{MDH} \text{Malate} + NAD^+$$

Reagents

Buffer: TRIS-HCl 50 mmol/l, pH 7.6

Cofactor: NADH 5 mmol/l

Enzymes: CL 3 U/ml; MDH 35 U/ml

Other reagents: $MgCl_2$ 0.1 mol/l; EDTA 0.1 mol/l

Standard: Citrate 2 mmol/l

Reaction mixture (final concentration)

Buffer 1 ml; NADH 12μℓ (0.06 mmol/l); $MgCl_2$ 20μℓ (2 mmol/l); EDTA 10μℓ (1 mmol/l); CL 20μℓ (0.06 U/ml); MDH 10μℓ (0.35 U/ml)

Reaction started with standard and completion in less than 10 min.

A2.1.7 Creatine

$$\text{Creatine} + \text{ATP} \xrightarrow{CK} \text{PCr} + \text{ADP}$$

$$\text{ADP} + \text{PEP} \xrightarrow{PK} \text{ATP} + \text{Pyruvate}$$

$$\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow{LDH} \text{Lactate} + NAD^+$$

Reagents

Buffer: Imidazole 50 mmol/l, pH 7.5

Cofactor: NADH 5 mmol/l

Enzymes: CK 1500 U/ml; PK 75 U/ml; LDH 60 U/ml

Additional reagents: ATP 10 mmol/l; PEP 30 mmol/l; $MgCl_2$ 0.1 mol/l; KCl 3 mol/l

Standard: Creatine 2 mmol/l

Reaction mixture (final concentration)

Buffer 1 ml; $MgCl_2$ 50μℓ (5 mmol/l); KCl 10μℓ (75 mmol/l); NADH 15μℓ (0.075 mmol/l); ATP 10μℓ (1 mmol/l); PEP 6.5μℓ (0.2 mmol/l); PK 10μℓ (0.75 U/ml); LDH 4μℓ (0.2 U/ml)

To 20μℓ of standard was added 1 ml of the reaction mix and E_1 recorded. CK 10μℓ (15 U/ml) was then added and the reaction folowed to completion (10-20 min) and E_2 read. The absorbance of the CK was then determined and the concentration of the standard calculated.

A2.1.8 Fructose 1,6-bisphosphate (F16BP)

$$\text{F16BP} \xrightarrow{\text{aldolase}} \text{DAP} + \text{GAP}$$

$$\text{DAP} \xrightarrow{\text{TIM}} \text{GAP}$$

$$2\text{GAP} + \text{NAD}^+ \xrightarrow{\text{GAD-DH}} 2\ \text{Glycerate-3-phosphate} + \text{NADH} + \text{H}^+$$

Reagents

Buffer: Imidazole 50 mmol/l, pH 7.5

Cofactor: NAD 50 mmol/l

Enzymes: Aldolase 9 U/ml; TIM 240 U/ml; GAP-DH 180 U/ml

Additional reagents: Sodium arsenate 0.1 mol/l; EDTA 0.1 mol/l;

Mercaptoethanol 1 mol/l

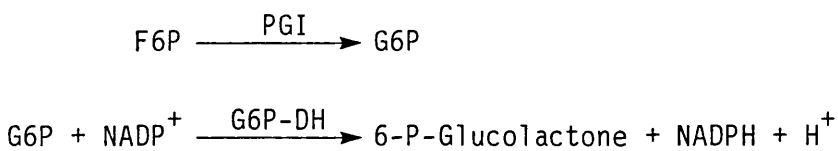
Standard: F16BP 1 mmol/l

Reaction mixture (final concentration)

Buffer 1 ml; NAD 20μℓ (1 mmol/l); EDTA 10μℓ (1 mmol/l); Sodium arsenate 10μℓ (1 mmol/l); Mercaptoethanol 2μℓ (2 mmol/l); GAPDH 10μℓ (1.8 U/ml); TIM 10μℓ (2.4 U/ml); Aldolase 10μℓ (0.09 U/ml)

Reaction complete in about 20 min. As 2 moles of NAD are reduced per mole of F16BP the actual concentration of the standard is half the concentration calculated from the change in absorbance.

A2.1.9 Fructose 6-phosphate (F6P)



Reagents as for G6P with the addition of:

Enzyme: PGI 35 U/ml

Standard: F6P 2 mmol/l

Reaction mixture as for G6P with the incorporation of the enzymes in the mixture; PGI 10μℓ/ml (0.35 U/ml) and G6P-DH 10μℓ/ml (0.07 U/ml). Reaction started with the standard and takes 10 min to go to completion.

A2.1.10 Fumarate

$$\text{Fumarate} \xrightarrow{\text{fumarase}} \text{Malate}$$

$$\text{Malate} + \text{NAD}^+ \xrightarrow{\text{MDH}} \text{Oxaloacetate} + \text{NADH} + \text{H}^+$$

$$\text{Oxaloacetate} + \text{glutamate} \xrightarrow{\text{GOT}} \text{Aspartate} + \alpha\text{-ketoglutarate}$$

Reagents

Buffer: 2-amino-2-methyl-propan-1-ol 0.1 mol/l, pH 9.9

Cofactor: NAD 50 mmol/l

Enzymes: Fumarase 3500 U/ml; MDH 600 U/ml; GOT 90 U/ml

Additional reagents: Glutamate 1 mol/l, pH 9.9; EDTA 0.1 mol/l

Reaction mixture (final concentration)

Buffer 1 ml; NAD 40μℓ (2 mmol/l); Glutamate 40μℓ (40 mmol/l); Fumarase 4μℓ (17.5 U/ml); MDH 10μℓ (6 U/ml); GOT 10μℓ (0.9 U/ml)

Reaction to completion in 15-20 min

A2.1.11 Glucose

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{G6P} + \text{ADP}$$

$$\text{G6P} + \text{NADP}^+ \xrightarrow{\text{G6P-DH}} \text{6-P-Gluconolactone} + \text{NADPH} + \text{H}^+$$

Reagents as for G6P with the addition of

Enzyme: HK 28 U/ml

Additional reagents: $MgCl_2$ 0.1 mol/l

Standard: Glucose 0.505 mmol/l (Boehringer test standard)

Reaction mixture as for G6P with the addition of ATP 10μℓ/ml (0.5 mmol/l) and the incorporation of the enzymes into the mixture; HK

10μℓ/ml (0.28 U/ml) and G6P-DH 10μℓ/ml (0.07 U/ml). On this occasion reaction started with a standard of known concentration and assay used to validate the reaction (completion in 4-6 min).

A2.1.12 Glycerol

$$\text{Glycerol} + \text{NAD}^+ \xrightarrow{\text{GDH}} \text{Dihydroxyacetone} + \text{NADH} + \text{H}^+$$

$$\text{Dihyroxyacetone} + \text{Hydrazine} \longrightarrow \text{Dihydroxyacetone-hydrazone}$$

Reagents

Buffer: 2-amino-2-methyl-propan-1-ol 0.1 mol/l, hydrazine 0.2 mol/l, EDTA 1 mmol/l, pH 10.0

Cofactor: NAD 50 mmol/l

Enzyme: GDH 500 U/ml (undiluted)

Standard: Glycerol 2 mmol/l

Reaction mixture

Buffer 1 ml; NAD 40μℓ (2 mmol/l); GDH 5μℓ (2.5 U/ml)

Reaction complete in 45-50 min

A2.1.13 Glucose 1-phosphate (G1P)

$$\text{G1P} \xrightarrow{\text{PGluM}} \text{G6P}$$

$$\text{G6P} + \text{NADP}^+ \xrightarrow{\text{G6P-DH}} \text{6-P-Gluconolactone} + \text{NADPH} + \text{H}^+$$

Reagents as for G6P with the addition of

Enzyme: PGluM 4 U/ml

Additional reagent: G16DP 0.25 mmol/l

Standard: G1P 2 mmol/l

Reaction mixture as for G6P with the addition of G16DP 2μℓ/ml (0.5 μmol/l) and the incorporation of the enzymes into the mixture; PCluM 10μℓ/ml (0.04 U/ml) and G6P-DH 10μℓ/ml (0.07 U/ml). Reaction started with the standard and completed in 4-6 min.

A2.1.14 Glucose 6-phosphate (G6P)

$$G6P + NADP^+ \xrightarrow{G6P\text{-}DH} 6\text{-}P\text{-}Gluconolactone + NADPH + H^+$$

Reagents

Buffer: TRIS-HCl 0.1 mol/l, BSA 0.02%, pH 8.1

Cofactor: NADP 50 mmol/l

Enzyme: G6P-DH 7 U/ml

Other reagents: EDTA 0.1 mol/l

Standard: G6P 2 mmol/l

Reaction mixture (final concentration)

Buffer 1 ml; NADP 10μℓ (0.5 mmol/l); EDTA 5μℓ (0.5 mmol/l)

Reaction started with 10μℓ G6P-DH (0.07 U/ml) and after completion (3-5 min) absorbance of enzyme determined.

A2.1.15 β-Hydroxybutyrate

$$\beta\text{-}Hydroxybutyrate + NAD^+ \xrightarrow{3\text{-}HBDH} Acetoacetate + NADH + H^+$$

$$Aetoacetate + Hydrazine \longrightarrow Acetoacetate\ hydrazone$$

Reagents

Buffer: Hydrazine 1.1 mol/l, EDTA 1 mmol/l, pH 9.9

Cofactor: NAD 50 mmol/l

Enzyme: 3-HBDH (undiluted)

Standard: β-Hydroxbutyrate 2 mmol/l

Reaction mixture (final concentration)

Buffer 1 ml; NAD 10μℓ (0.5 mmol/l); 3-HBDH 10μℓ (0.15 U/ml)

Completion in about 30-40 min.

A2.1.16 Lactate

$$\text{Lactate} + NAD^+ \xrightarrow{LDH} \text{Pyruvate} + NADH + H^+$$

$$\text{Pyruvate} + \text{Hydrazine} \longrightarrow \text{Pyruvate hydrozone}$$

Reagents

Buffer: Hydrazine 1.1 mol/l, EDTA 1 mmol/l, pH 9.0

Cofactor: NAD 50 mmol/l

Enzyme: LDH 1250 U/ml (undiluted)

Standard: Lactate 2 mmol/l

Reaction mixture (final concentration)

Buffer 1 ml; NAD 40μℓ (2 mmol/l); LDH 15μℓ (20 U/ml)

Reaction complete in 15-20 min.

A2.1.17 β-Nicotinamide adenine dinucleotide (reduced) (NADH)

$$\text{Pyruvate} + NADH + H^+ \xrightarrow{LDH} \text{Lactate} + NAD^+$$

Reagents

Buffer: Imidazole 50 mmol/l, pH 7.0

Cofactors: NADH '5' mmol/l; NADH '1' mmol/l

Enzyme: LDH 60 U/ml

Additional reagent: Pyruvate 0.1 mol/l

Reaction mixture (final concentration)

Buffer 1 ml; Pyruvate 10μℓ (1 mmol/l)

To 1 ml of reaction mixture was added either 10μℓ NADH '5 mmol/l' or 40μℓ NADH '1 mmol/l', E_1 read and 1μℓ of LDH then added and reaction followed to completion (1-2 min) and NADH concentration calculated from equation A2.1

A2.1.18 Phosphocreatine (PCr)

$$\text{PCr} + \text{ADP} \xrightarrow{\text{CPK}} \text{Creatine} + \text{ATP}$$

$$\text{ATP} + \text{Glucose} \xrightarrow{\text{HK}} \text{ADP} + \text{G6P}$$

$$\text{G6P} + \text{NADP+} \xrightarrow{\text{G6P-DH}} \text{6-P-Gluconolactone} + \text{NADPH} + \text{H}^+$$

Reagents as for ATP with the addition of

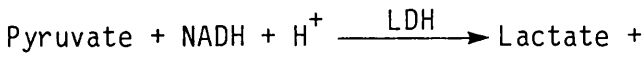
Enzyme: CK 380 U/ml

Additional reagents: ADP 50 mmol/l

Standard: PCr 2 mmol/l

Reaction mixture as for ATP with the addition of ADP 10μℓ/ml (0.5 mmol/l) and the incorporation of HK 10μℓ/ml (0.28 U/ml) in the mixture. After reading E_1 10μℓ CK were added and the reaction followed to completion (10 min) and E_2 read. The absorbance of the enzyme was determined by adding a further 10μℓ and the concentration of the standard calculated as for ATP.

A2.1.19 Pyruvate



Reagents

Buffer: potassium phosphate 1 mol/l, EDTA 1 mmol/l, pH 7.0

Cofactor: NADH 5 mmol/l

Enzyme: LDH 10 U/ml

Standard: Pyruvate 2 mmol/l

Reaction mixture (final concentration)

Buffer 1 ml; NADH 12μℓ (0.06 mmol/l); LDH 10μℓ (0.01 U/ml)

Reaction started with the standard and complete in 2-3 min. If slow drift of end point experienced with the phosphate buffer, use imidazole 50 mmol/l, pH 7.0.

Appendix 3

A3 Assays of blood metabolites

A3.1 Fluorimetric assays

These were essentially the same as the equivalent assays for muscle metabolites. Deproteinisation of whole blood was carried out at two dilutions; 0.25 ml of blood was added immediately on withdrawal to 0.5 ml of ice cold 0.6 mol/l perchloric acid and 25μℓ of blood was was added to 0.25 ml of 0.25 mol/l perchloric acid. Both samples were spun for 30 s in an Eppendorf micro-centrifuge to precipitate the protein sediment. The supernatant of the former was neutralised with 70μℓ of $KHCO_3$, 2.1 mol/l per 0.3 ml and was used for the determination of acetoacetate, citrate and pyruvate while the latter was used un-neutralised for assay of alanine, β-hydroxybutyrate, glucose and lactate. Both extracts were stored at -70°C. In addition, 25μℓ of the serum from 1 ml of blood was deproteinised with 0.25 ml of 0.25 mol/l perchloric acid for determination of glycerol and with the remainder (for assay of FFA) stored at -20°C.

A3.1.1 Acetoacetate

This ketone body is not measurable in muscle and the fluorimetric assay for blood is described.

$$\text{acetoacetate} + \text{NADH} + \text{H} \xrightarrow{\text{3-HBDH}} \beta\text{-Hydroxybutyrate} + \text{NAD}^+$$

Reagents

Buffer: Potassium phosphate 1 mol/l, EDTA 1 mmol/l, pH 7.0

Cofactor: NADH 1 mmol/l

Enzyme: 3-HBDH 15 U/ml (undiluted)

Additional Reagents: Sodium carbonate buffer 20 mmol/l, pH 10.0

Standard: Acetoacetate 2 mmol/l (stock solution)

Working standards were prepared daily from the stock solution as follows:

stock solution (μℓ)	0	10	20	40
water (μℓ)	2000	1990	1980	1960
acetoacetate concentration (μmol/l)	0	10	20	40

Immediately prior to analysis sufficient reaction mixture for duplicate samples and standards plus 1 ml to follow the reaction on a chart recorder was prepared as follows:

Reaction Mixture

Buffer 1 ml; NADH 5μℓ (5 μmol/l); 3-HBDH 5μℓ (0.075 U/ml)

Into duplicate fluorimeter was pipetted 25μℓ of sample and standard and 0.2 ml of reaction mixture was added. A further 0.125 ml of acetoacetate 40 μmol/l was added to 1 ml of reaction mixture and this reaction followed on a chart recorder. The tubes were incubated for about 30 min at room temperature, carbonate buffer 1 ml added and fluorescence read. Acetoacetate concentration was calculated from the standard curve and corrected for dilution (1:3). The between pair coefficient of variation for 20 consecutive duplicate samples was 8.21%.

A3.1.2 Glycerol

The assay for glycerol was carried out as had been recently described using glycerol dehydrogenase (Boobis and Maughan, 1983)

Reagents:

Buffer: 2-amino-2-methyl-propan-1-ol 0.1 mol/l, hydrazine 0.2 mol/l, EDTA 1 mmol/l, pH 9.9

Cofactor: NAD 50 mmol/l

Enzyme: GDH 500 U/ml (undiluted)

Additional Reagents: Perchloric acid 0.25 mol/l

Standard: Glycerol 2 mmol/l

Working standards were prepared daily from the stock solution as follows:

stock solution (μℓ)	0	20	30	50
perchloric acid 0.25 mol/l (μℓ)	2000	1980	1970	1950
glycerol concentration (μmol/l)	0	20	30	50

Immediately prior to analysis sufficient reaction mixture for duplicate samples, blanks and standards plus 1 ml to follow the reaction on a chart recorder was prepared as follows:

Reaction Mixture (final concentration)

Buffer: 1 ml; NAD 20μℓ (1 mmol/l); GDH 5μℓ (2.5 U/ml)

To duplicate 50μℓ of samples (un-neutralised), blanks and standards in fluorimeter tubes was added 0.2 ml of the reaction mixture. A further 0.25 ml of glycerol 50 μmol/l was added to 1 ml of the mixture and the reaction in this tube followed on a chart recorder. The tubes were incubated for 60-90 min at room temperature, 1 ml carbonate buffer was added and fluorescence read. Glycerol concentration was calculated from the standard curve and corrected for dilution. The between pair coefficient of variation for 20 consecutive duplicate samples was 3.63%.

A3.1.4 Differences between assays for blood and muscle metabolites

The remaining fluorimetric assays for blood metabolites were similar to those used for the same metabolite measured in blood with the exception of the differences outlined below.

a) β-Hydroxybutyrate

Standard range 10-50 μmol/l and NAD increased to 0.25 mmol/l (final concentration). 20μℓ samples assayed. The between pair coefficient of variation for 20 consecutive duplicate samples was 2.89%.

b) Pyruvate

Standard range 10-50 μmol/l, NADH 7 μmol/l and LDH 0.05 U/ml (final concentration). Readings made on native fluorescence of NADH on 20μℓ samples after addition of 1 ml of carbonate buffer (10-15 min incubation) and only single set of duplicate tubes used. The between pair coefficient of variation for 20 duplicate samples was 7.33%.

c) Citrate

Same standards used but NADH increased to 6 μmol/l (final concentration) as sample volume increased to 20μℓ per tube. Readings made on native fluorescence of NADH after addition of carbonate buffer 1ml and citrate concentration calculated from standard curve and corrected for oxaloacetate concentration and dilution. The between pair coefficient of variation for 20 consecutive duplicate samples was 4.21%

d) Alanine

Same standards used but perchloric acid 0.25 mol/l used as dilutant. Sample size increased to 20μℓ and NAD to 0.25 mmol/l (final concentration). The between pair coefficient of variation for 20 consecutive duplicate samples was 1.81%.

e) Lactate

Standard range 0.10-0.50 mmol/l with perchloric acid 0.25 mol/l as dilutant; NAD increased to 1 mmol/l and LDH to 15 U/ml (final concentrations). Sample size was increased to 20μℓ per tube and the between pair coefficient of variation for 20 duplicate samples was 1.99%

A3.2 Spectrophotometric assays

A3.2.1 Glucose

This was measured spectrophotometrically using the glucose test combination (GOD-Perid method) as described in the determination of acid insoluble glycogen. Using an un-neutralised perchloric acid extract instead of uranyl acetate, as recommended, did not adversely affect the assay. The sample size was 40μℓ and a single standard (0.505 mol/l) was used, the between pair coefficient of vartiation for 20 duplicate samples being 1.38%.

A3.2.2 Free Fatty Acid (FFA) assay

A colorimetric assay based on the method of Noma et al (1973) was used. However, a number of problems were experienced in trying to scale down the assay and use disposable micro-reaction tubes rather than glass stoppered test tubes. Different batches of tubes and those from different manufacturers gave very variable and often unacceptably high blank values. This was thought to be due to lipids either within the plastic or as releasing agent for the moulds. It was eventually found that Eppendorf 1.5 ml microcentrifuge tubes (ref. 3810) gave a low and reproducible blank result. All glassware was cleaned as for the fluorimeter tubes.

Reagents

CHM extraction solution; Stable Copper Reagent, pH 8.1; TAC 0.01% (colour reagent); BSA (FFA free) 3%

Standard: Palmitic acid 1 mmol/l in 3% FFA free BSA (stock solution)

Working standards were prepared daily for the stock solution as follows:

stock solution (ml)	0	0.50	1.00	2.00
water (ml)	2.00	1.50	1.00	0
Palmitic acid concentration (mmol/l)	0	0.25	0.50	1.00

To 1.5 ml Eppendorf microreaction tubes was added 40μℓ of serum or standards, 0.2 ml of copper reagent and 1.2 ml of CHM. The tubes were tightly closed and shaken mechanically in a specially constructed rack for 4 min following which they were centrifuged for 1 min in an Eppendorf microcentrifuge. One ml of the upper phase was carefully transferred to a glass test tube containing 0.25 ml of TAC. A green colour linearly proportional to the FFA concentration developed immediately and aborbance was read at 610 nm against a water blank in a 1 ml semi-micro cuvette (glass or quartz). Concentration of FFA was calculated from the the standard curve and the between pair coefficient of variation for 20 consecutive duplicate samples was 3.29%

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