INDICES OF FATTY ACID METABOLISM

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ABBREVIATIONS

%CE	-	Percentage esterified carnitine
3HB	-	3-Hydroxybutyrate
ATP	-	Adenosine triphosphate
AMP	-	Adenosine monophosphate
BSTFA	-	bis-(trimethylsilyl)- trifluoroacetamide with 1% trimethyl-chlorosilane added
CC	-	Correlation coefficient
CoA	-	Coenzyme A
CoQ	-	Coenzyme Q
CPT	-	Carnitine palmitoyl transferase
CV	-	Coefficient of variation
DCA	-	Dicarboxylic aciduria
DMSO	-	40% v/v dimethyl sulphoxide
ETF	-	Electron transfer flavoprotein
FAD	-	Flavin adenine dinucleotide (oxidised form)
$FADH_2$	-	Flavin adenine dinucleotide (reduced form)
FFA	-	Free fatty acids
GC	-	Gas chromatography
HG	-	Hexanoylglycine
HPLC	-	High performance liquid chromatography
IS	-	Internal standard
LCAD	-	Long chain acyl-coenzyme A dehydrogenase
LCHAD	-	Long chain hydroxyacyl-coenzyme A dehydrogenase
MCAD	-	Medium chain acyl-coenzyme A dehydrogenase
MS	-	Mass spectrometry
NAD	-	Nicotinamide adenine dinucleotide (oxidised form)
NADH	-	Nicotinamide adenine dinucleotide (reduced form)
NADP	-	Nicotinamide adenine dinucleotide phosphate (oxidised form)
NADPH	-	Nicotinamide adenine dinucleotide phosphate (reduced form)
PPG	-	Phenyl-propionylglycine
SCAD	-	Short chain acyl-coenzyme A dehydrogenase
SD	-	Standard deviation
SG	-	Suberylglycine
SIDS	-	Sudden infant death syndrome
TMS	-	Trimethylsilyl derivative

CONTENTS

SUMMARY

During the fed state energy requirements are met by glycolysis of carbohydrates. When the stores of carbohydrates are diminished, for example during prolonged fasting, metabolism switches to that of fatty acids. Fatty acids are broken down by ß-oxidation within the mitochondrial matrix. Prolonged fasting results in the production of ketone bodies. These can also be used as an energy source by the brain.

In defects of fatty acid metabolism where individual steps are inhibited or blocked, such as medium chain acyl-CoA dehydrogenase deficiency, an abnormal accumulation of the metabolites that lead up to the block, or their breakdown products, is often seen. Non-compensatory levels of metabolites following the site of the defect also occur.

In the fed state, when flux through the defective fatty acid pathway is minimal, metabolic profiles can appear completely normal. It is therefore often necessary to induce metabolic stress before a full laboratory investigation can proceed.

Interpretation of individual metabolite quantitations can often be difficult and a variation of 'normal values' according to metabolic state can lead to misinterpretation. Comparison between the concentrations of related metabolites along the fatty acid metabolic pathway may diminish the need for exact knowledge of the metabolic state and by correlation plotting could clearly identify abnormal relationships.

This thesis describes an investigation into the efficacy of paired metabolite

correlation plots in preliminary detection of defects in fatty acid metabolism.

In certain inborn errors of fatty acid metabolism where the ß-oxidation cycle is affected, abnormal urine metabolite patterns have been used as diagnostic markers. Similar patterns have been reported in the urine of healthy newborns and termed generalised neonatal dicarboxylic aciduria[177].

This report documents an investigation of the connections between generalised neonatal dicarboxylic aciduria and a number of overlying factors (*vis* type of feed, gender, sibling history of sudden infant death syndrome and urine carnitine levels).
Also discussed is the development of two laboratory assays. A radio-enzymatic method was developed and used to determine the levels of total, free and acyl carnitine in urine or blood. Suberyl, hexanoyl, and phenylpropionyl glycine in urine can be quantitated by use of stable isotope internal standards and gas chromatography / electron impact mode mass spectrometry. Synthesis and calibration of such internal standards is described.

Finally, methods used to culture and store skin fibroblasts from biopsy samples are included as an appendix. These fibroblasts can then be used in various diagnostic tests such as carbon dioxide release and electron transfer flavoprotein enzyme analysis. The costs encountered during tissue culture could be avoided by medium term storage of the biopsy material prior to culture to await sufficient clinical evidence to merit such analyses. Preliminary results of extended cryogenic storage and viability of recovered specimens are also included.

CHAPTER 1

INTRODUCTION

1.1 A BRIEF HISTORY

Fatty acids have two major roles throughout the mammalian kingdom; as integral components of membrane lipids and as the body's main energy store in the form of triacylglycerols.

Under energy stress, when carbohydrate supply is low, triacylglycerols are oxidised to yield relatively large amounts of energy. Mole for mole, the oxidation of fatty acids such as oleic acid yields more than three times the amount of ATP than does the oxidation of glucose.

The theory of fatty acid oxidation has existed since the beginning of the century. Geelmugden found ketones to be produced from fatty acids in 1897. In 1904 Knoop was revolutionary in his use of synthetically labelled fatty acids. He fed dogs odd and even, straight chain fatty acids with phenyl groups attached to the ω-carbon and deduced that fatty acids were shortened by 2 carbon units at a time starting at the carbonyl end. He thus devised the theory of ß-oxidation. It was not until the 1950's that the organisation of such a pathway began to unfold. The mid 1950's saw the discovery of co-enzyme A, CoA, and its role as carrier molecule for fatty acids. Tissue fractionation was also developed around this time and paved the way to the discovery not only of subcellular particles such as mitochondria but also the acylation of carnitine. Work involving the acylation of carnitine and its action as cofactor during fatty acid oxidation then led to the elucidation of its role as carrier for fatty acyl groups as they cross the inner mitochondrial membrane.

1.2 OVERVIEW OF OXIDATIVE METABOLISM

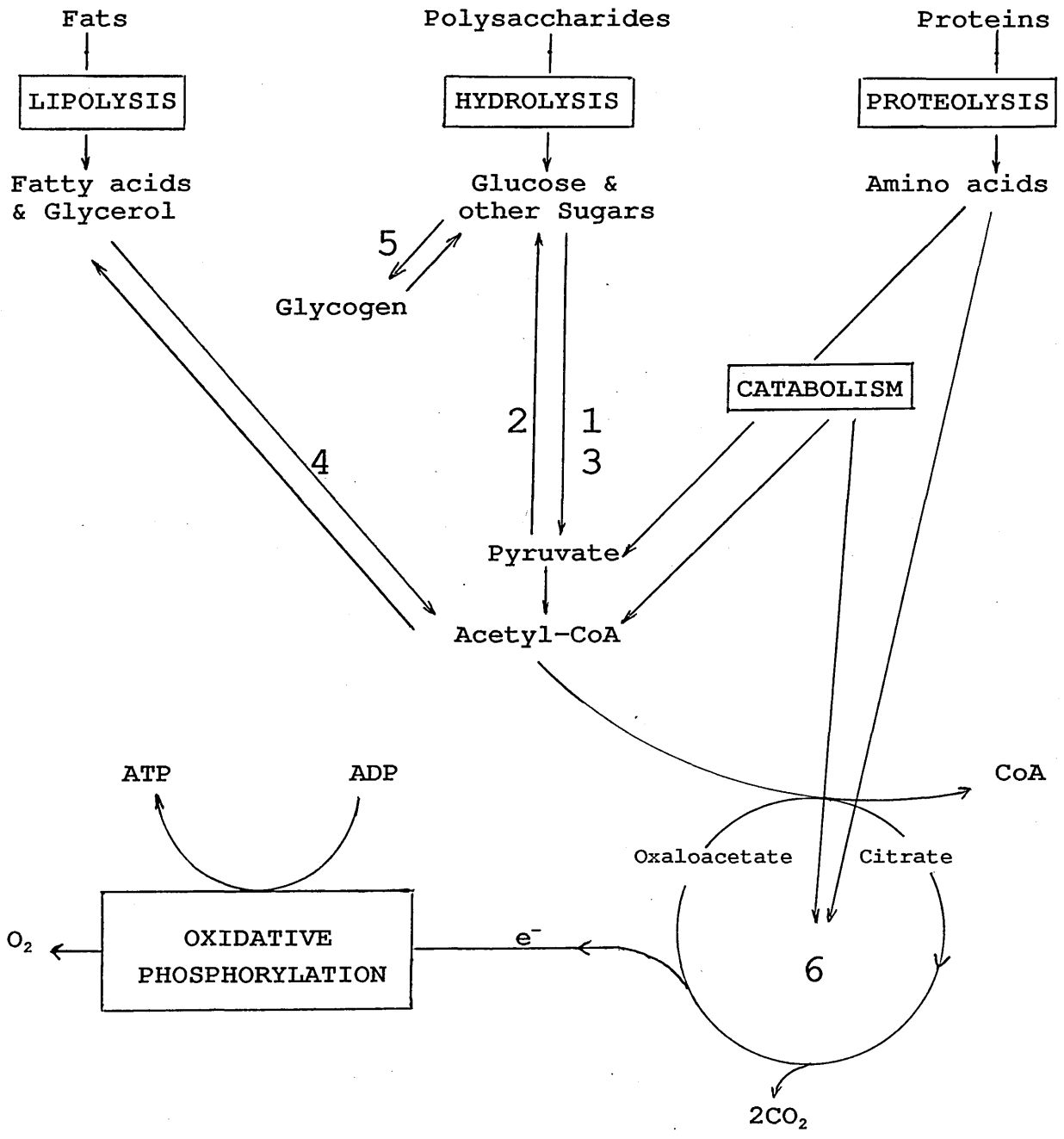


Fig. 1.01. The processes involved in oxidative metabolism.

On average a well-fed 70kg man has a calorie distribution as shown in table 1.01. Depending on his activity his body energy requirements can range from 1600kcal to 6000kcal per 24 hours. The carbohydrate pool could be exhausted by just one day's fast.

The production of energy and synthetic material is accomplished essentially by the six metabolic pathways.

1. Glycolysis
2. Gluconeogenesis
3. Pentose phosphate pathway
4. Fatty acid synthesis / ß-oxidation
5. Glycogen synthesis / degradation
6. Citric acid cycle

Table 1.01. Calorific distribution of energy in an average 70kg man[2].

ORGAN	AVAILABLE ENERGY (kcal)		
	Glucose or Glycogen	Triglycerides	Mobilizable Proteins
Blood	60	45	0
Liver	400	450	400
Brain	8	0	0
Muscle	1,200	450	24,000
Adipose tissue	80	135,000	40

The brain's predominant fuel source is glucose. It is therefore important to maintain tolerable blood glucose levels (greater than 3 mmol/l) at all times. This is achieved during times of low blood glucose by the body producing glucose from glycogen breakdown and gluconeogenesis. At the same time maximum supply to the brain is ensured by a switch of the major peripheral organs from glycolysis to fatty acid oxidation. Muscle protein breakdown may also occur supplying gluconeogenesis with pyruvate, lactate and alanine. However, as this would be detrimental to the activity of the body, and therefore its capability to find food or defend itself, proteolysis is limited until starvation is severe.

1.2.1 METABOLIC FLOW DURING THE WELL FED STATE

During well fed states glycolytic breakdown of glucose produces biosynthetic precursors (pyruvate and NADH) and energy (ATP).

$$C_6H_{12}O_6 + NAD^+ \longrightarrow CH_3{-}\overset{\overset{O}{\|}}{C}{-}COO^- + 2ATP + 2NADH$$

These compete with ß-oxidation for NAD and electron transport chain access, minimising fatty acid oxidation. Work with isolated mitochondria has shown fatty acids, once in the matrix, to be oxidised in preference to pyruvate and citrate. Pyruvate and most other citric acid cycle intermediates only slightly effect ß-oxidation whilst fatty acids can almost halt pyruvate and citrate oxidation[3,4].

Insulin secreted from the pancreas stimulates glycogen synthesis and hepatic glycolysis. Uptake of glucose by muscle and liver is enhanced along with uptake by muscle of the branched amino acids valine, leucine and isoleucine to supply protein synthesis. General protein synthesis is increased and proteolysis inhibited. Acetyl-CoA carboxylation in the liver is stimulated increasing malonyl-CoA levels and inhibiting ß-oxidation of longer chain fatty acids (see section 1.6.2). Glycerol phosphate acyl transferase is the enzyme responsible for the conversion of glycerol-3-

phosphate to phosphatidate which is subsequently converted to triacylglycerol. Its activity in the mitochondria is enhanced by insulin thereby further increasing lipogenic rates. The synthesised fatty acids travel to adipose tissue in the form of very low density lipoprotein, VLDL, where their abundance, along with glucose, leads to triacylglycerol formation and storage. Lipolysis is inhibited.

Vasopressin with Ca^{2+} ions inhibit fatty acid oxidation[5] by increasing glycolysis, lactate formation[6], and acetyl-CoA carboxylation.

The effect of the adrenal cortex hormones on fatty acid oxidation is unclear. Mineralocorticoids, in general, suppress ketone levels by inhibiting ß-oxidation probably via the malonyl-CoA mechanism[7,8] (see section 1.6.2). However, the glucocorticoids stimulate adipose fatty acid release[9] which increases the supply to ß-oxidation. They also promote gluconeogenesis and a primary deficiency in glucocorticoids is often associated with an elevated rate of ketogenesis. This is thought to be a result of an increased reliance on ketone bodies for energy due to a decreased supply from glycolysis.

The sex hormones have opposite effects on the formation of the fatty acid binding protein that transports fatty acids to the endoplasmic reticulum for esterification. Oestradiol elevates the proteins formation whilst testosterone suppresses. Therefore, females are more prone to the esterification pathway whereas males favour ß-oxidation.

1.2.2 METABOLIC FLOW DURING FASTING

Upon fasting, glucagon along with catecholamine hormones act in a very similar fashion opposing the action of insulin. Glucagon targets the liver whereas the catecholamines act mainly on muscle. Glycogenolysis, fatty acid oxidation, gluconeogenesis, muscle proteolysis and brown adipose cell lipolysis are all enhanced. Glycogen synthesis, fatty acid synthesis, insulin production and uptake of

glucose by muscle are inhibited.

Thyroid hormones stimulate triglyceride release from adipose tissue[10] and regulate ß-oxidation in the liver. The triglycerides are hydrolysed to free fatty acids and travel to the muscle and liver to undergo ß-oxidised.

Such a change in fuel supply to the muscle prevents pyruvate conversion to acetyl-CoA. Instead pyruvate with lactate and alanine travel to the liver to be incorporated into glucose production. By the same pathway glycerol produced during triacylglycerol breakdown is also used to produce glucose. Hepatic fatty acid oxidation elevates citrate and acetyl-CoA levels leading to the switching off of glycolysis.

As fatty acid oxidation continues acetyl-CoA levels rise. High gluconeogenic rates deplete oxaloacetate concentrations. Under these conditions the tri-carboxylic acid cycle cannot complete the oxidation of the acetyl groups rapidly enough. The ketone bodies acetoacetate and 3-hydroxybutyrate are then produced. Their build up increases as the fast progresses. Brain is capable of using such metabolites as a fuel source and after 3 days a third of its total fuel supply consists of ketone bodies. After several weeks this proportion rises to dominate the supply. Heart and muscle can also utilise these ketone bodies, in fact in vitro acetoacetate is metabolised in preference to glucose. (see section 1.8.1 for further discussion of ketogenesis).

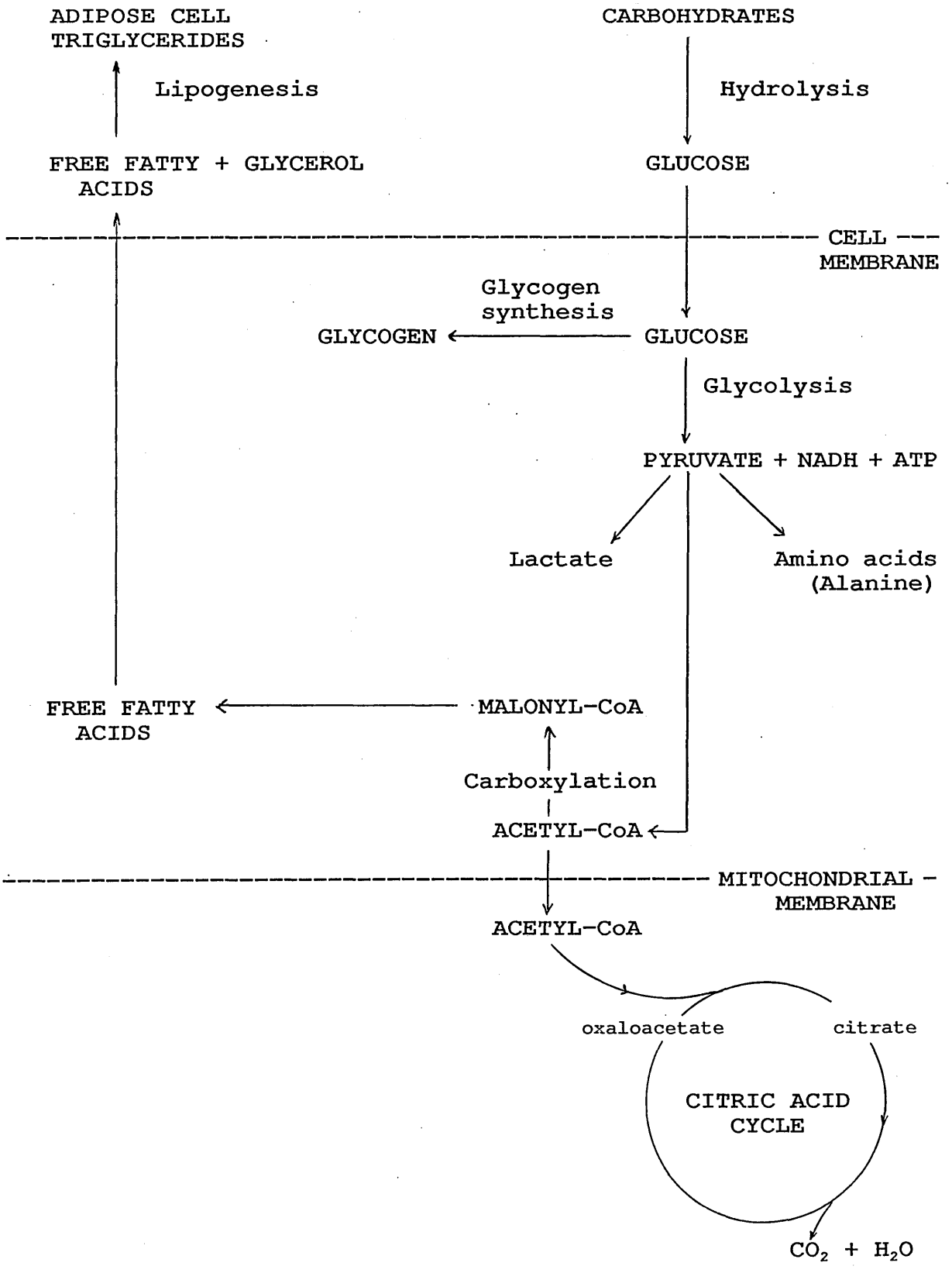


Fig. 1.02. Metabolic flow in muscle and liver during high carbohydrate states.

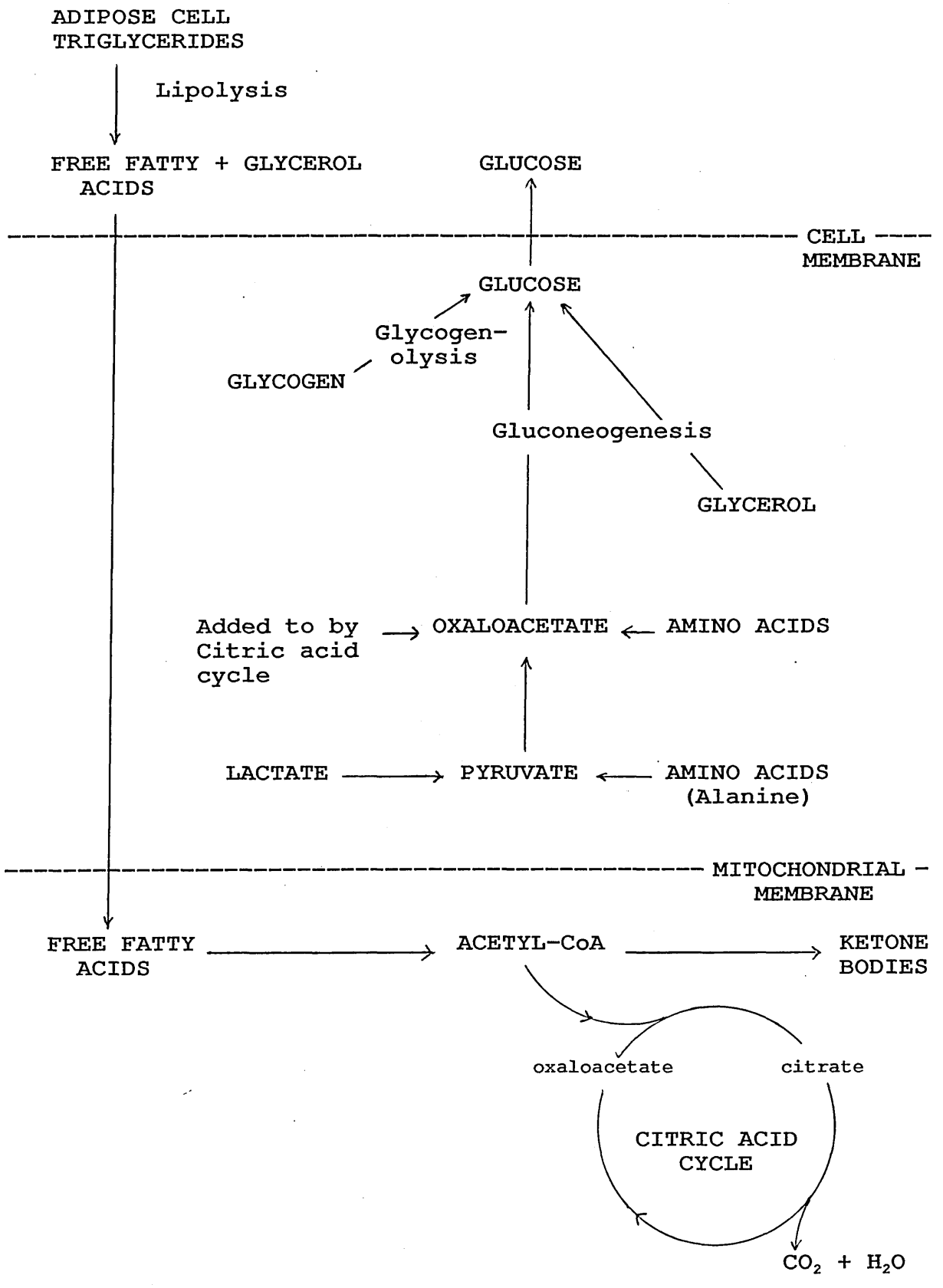


Fig. 1.03. Metabolic flow in muscle and liver tissue during low carbohydrate states

1.3 DEVELOPMENT AND CONTROL OF ß-OXIDATION DURING THE PERINATAL PERIOD

Long chain fatty acids are essential during embryonic and fetal development. They are used both for structural esterification as well as serving as a major energy source postnatally.

Prenatal supply is either from fetal tissue or from the mother. Studies on rodents show synthetic rates to be high in a variety of tissues, in particular liver, lung and brain. Synthesis is mostly by the de novo pathway in the cytosol but also occurs via chain elongation in mitochondria[11] and in microsomes[12]. Such synthesis decreases at birth with an increase in fat intake and again increases upon weaning[13,14] as a less fatty diet is adopted.

The sudden removal of maternal blood nutrient supply and the abrupt change in environment at birth creates significant metabolic stress. As a result there is a switch from glucose metabolism to fatty acid metabolism. A preparation for this switch can be seen in the latter stages of gestation. Fat depots increase and mitochondria are seen to associate with myofibrils in readiness for a more efficient energy production.

At birth a sudden increase in free fatty acids precedes an increase in serum ketone levels a few hours later. A drop in the respiratory quotient from 1 to 0.8 over the next few days, highlights an increase in fatty acid oxidation[15,16]. Hepatic glycogen depletion indicates the demand for glucose in maintaining metabolic homeostasis.

Main regulatory factors affecting such a switch in metabolism from glycolysis to ß-oxidation are :-

1. Fatty acid availability - suggested by the increase in serum fatty acids prior to fatty acid oxidation increase and also a drop in fatty acid and

ketone oxidation upon weaning when fat intake is decreased.

2. Rates of fatty acid transfer, including levels of intracellular fatty acid binding proteins.

3. Activity of the key enzymes acyl-CoA synthase and carnitine acyl transferase. These enzymes show an increase in activity correlating with increase fatty acid oxidation[17].

4. Oxygen supply - emergence into the more aerobic postnatal environment enhances tissue oxygen supply and facilitates the aerobic oxidation of fatty acids.

5. Carnitine supply.

A correlation has been found between ß-oxidation rates and the amount of carnitine in feed. Breast milk, richer in carnitine than artificial feeds is accompanied by higher plasma carnitine and ketone levels as early as 42 hours after birth[18,19]. This, in turn, leads to a higher rate of increase of ß-oxidation during metabolic stress.

Hepatic fatty acid de novo synthesis varies over birth, suckling and weaning, whereas that in lung[20] and brain[21] remains relatively constantly high. This is thought to be a result of brain and lung requirements for myelin and surfactant respectively for which fatty acids are precursors.

1.4 COMPARTMENTATION OF THE B-OXIDATION PATHWAY

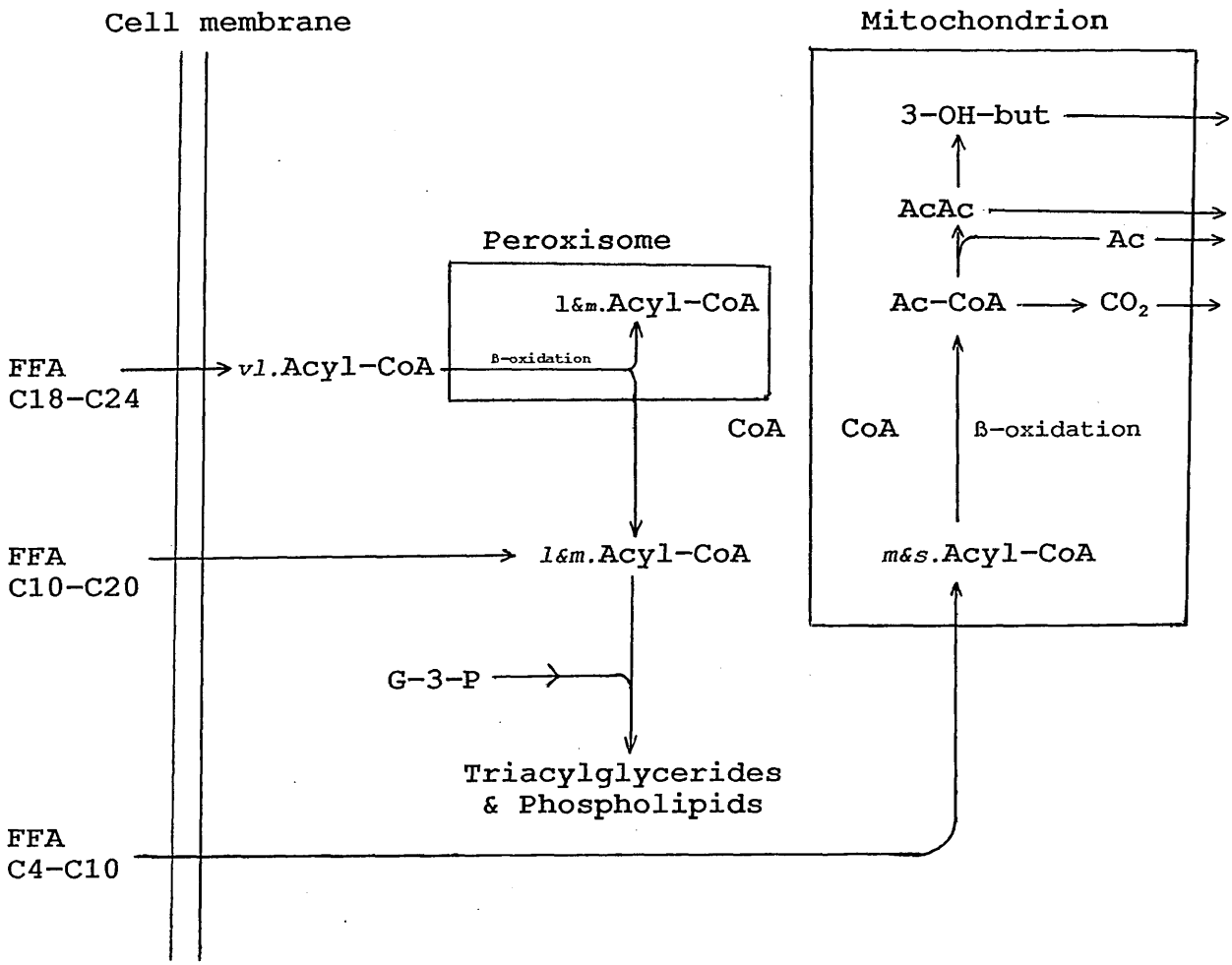


Fig. 1.04. Distribution of ß-oxidation within the cell. Abbreviations: FFA, free fatty acid; Ac-CoA, acetyl- CoA; Ac, acetate; G-3-P, glycerol-3-phosphate; *s*, short; *m*, medium; *l*, long; *vl*, very long chain length.

There is an amount of uncertainty in the short, medium, long and very long nomenclature used to describe the carbon chain lengths of fatty acids. As a result in referencing current literature these categories may be seen to overlap slightly.

Fatty acid oxidation operates in three main subcellular compartments.

1. Extramitochondrial compartment.
2. Peroxisomes.
3. Mitochondrial matrix.

1.4.1 EXTRA - MITOCHONDRIAL COMPARTMENT

This includes the endoplasmic reticulum, cytosol and outer mitochondrial membrane. Here long chain (C12-26) fatty acids passing into the cell are converted to acyl-CoA esters by the action of acyl-CoA synthases.

FA + ATP + CoA = Acyl-CoA + AMP + PPi

These esters can then be either incorporated into phospholipids and triacylglycerides or oxidised by the ß-oxidation pathway.

Shorter chain free fatty acids (C4-10) can pass directly into the mitochondrion prior to CoA esterification.

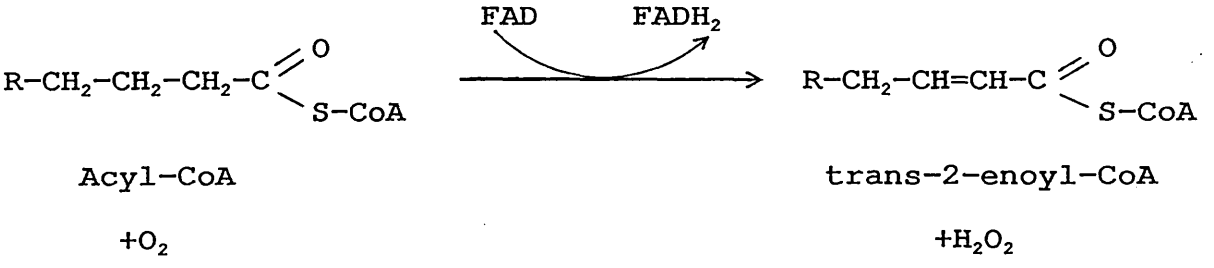
1.4.2 PEROXISOMAL ß-OXIDATION

In the peroxisomes partial ß-oxidation has a chain shortening effect, particularly important for the longer fatty acids (C22-C26)[22-24].

Although the steps involved in this ß-oxidation are the same as those of the mitochondrial pathway, the enzymes involved differ. Carnitine, carnitine-acyl transferase and carnitine translocase are not required for transport of long acyl-CoA's into the organelle. However, medium-chain carnitine acyltransferase and carnitine acetyl transferase proteins have been isolated[25,26]. These are thought to play a role in transfer of partial oxidation products to the tri-carboxylic acid cycle

and electron transport chain of the mitochondria.

Acyl-CoA oxidase catalyses the first step in the pathway. Fatty acyl-CoA is converted to trans-2-enoyl-CoA with concomitant reduction of the enzyme's prosthetic group FAD to $FADH_2$. The reduced prosthetic group is then re-oxidised by direct interaction with oxygen.



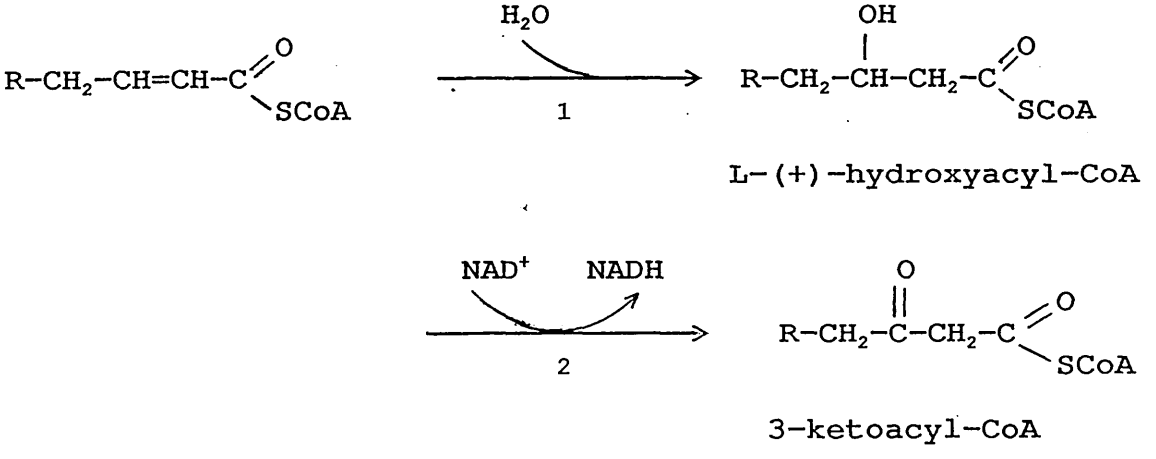
Acyl-CoA

$+O_2$

trans-2-enoyl-CoA

$+H_2O_2$

The enoyl-CoA is acted upon by a bifunctional protein. Possessing both 2-enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activity this protein hydrates enoyl-CoA to L-(+)-hydroxyacyl-CoA and converts this to 3-ketoacyl-CoA with simultaneous reduction of NAD^+ to NADH.

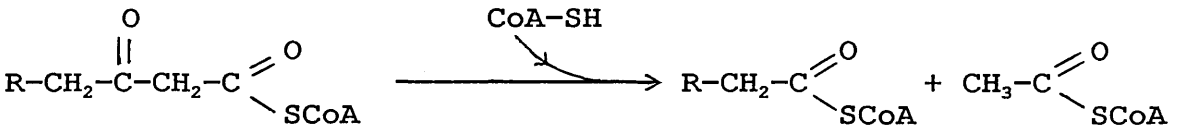


L-(+)-hydroxyacyl-CoA

3-ketoacyl-CoA

1 - 2-enoyl-CoA hydratase activity
2 - 3-hydroxyacyl-CoA dehydrogenase activity

Finally, acetyl-CoA is produced by 3-ketoacyl-CoA thiolase.



This enzyme has been seen to have maximal activity for long chain length substrates indicating peroxisomal ß-oxidation's preference for long chain fatty acids. Best substrates for peroxisomal ß-oxidation are saturated C12-C26 and unsaturated C18:1, C18:2 and C22:1 chain length fatty acids. C8 and C10 chain lengths are oxidised to a lesser extent. C6 and C4 oxidation is inappreciable[27]. Affinity for dicarboxylic acids is even greater than that for the corresponding fatty acids (monocarboxylic acids)[28-30].

An important difference between the two ß-oxidation pathways is in the first step. The mitochondrial pathway is connected to the electron transport chain via an acyl-CoA dehydrogenase flavoprotein. In the peroxisome oxidation of the $FADH_2$ is directly by oxygen.

Defective peroxisomal ß-oxidation results in the accumulation of the longer chain fatty acids. Adrenoleukodystrophy, an 'X'-linked disorder resulting from peroxisomal very long chain acyl-CoA synthetase deficiency, causes an accumulation of C24 and C26 fatty acids in the adrenals and central nervous system[31].

1.4.3 MITOCHONDRIAL ß-OXIDATION

Of main interest in this study is mitochondrial ß-oxidation. It is here where acyl-CoA esters undergo complete ß-oxidation ultimately producing acetyl-CoA. This pathway shall now be discussed.

1.5 ACYL-CoA PRODUCTION FOR ß-OXIDATION

1.5.1 THE SYNTHASES

Fatty acids enter the cell by simple diffusion after binding to a lipoprotein receptor on the cell membrane.

Once in the cytosol two classes of enzymes are involved in their conversion to acyl-CoA esters. The acyl-CoA synthases catalyse their formation whilst acyl-CoA hydrolases facilitate the reverse reaction ensuring a constant supply of CoA for other metabolic processes.

The enzymes within each class differ in their carbon chain length specificities for free fatty acids. This, along with a wide distribution throughout many body tissues, ensures the activation of a wide range of free fatty acid lengths in a wide variety of tissues.

The synthases involved in the supply of fatty acyl-CoA esters for ß-oxidation are :-

1. Butyryl-CoA synthase (EC 6.2.1.?)
2. Medium chain acyl-CoA synthase (EC 6.2.1.2)
3. Long chain acyl-CoA synthase (EC 6.2.1.3)

1.5.1.1 Butyryl-CoA synthase

This enzyme exists solely within the mitochondrial matrix of extrahepatic tissues such as heart[36]. It has not been isolated in liver and has only limited importance in ß-oxidation.

1.5.1.2 Medium chain acyl-CoA synthase

Heptanoate and octanoate are the preferred substrates for medium chain acyl-CoA synthase however activity throughout carbon chain lengths C4 -C12 is also exhibited. A second enzyme present within the mitochondrial matrix acts upon medium chain fatty acids. Its specificity for benzoate and also activity towards salicylate indicates an involvement in the production of glycine conjugates of aromatic carboxylic acids[37].

1.5.1.3 Long chain acyl-CoA synthase

Long chain acyl-CoA synthase activity has been found in the microsome[38] and peroxisomes[39,40] of the liver and in the outer mitochondrial membrane[41,42]. Such a distribution gives each organelle its own capacity to activate long chain free fatty acids. However it is not known for sure whether separate acyl-CoA pools exist. In the liver activity is concentrated in the peroxisomes with the remainder spread uniformly between the mitochondria and endoplasmic reticulum.

Regulation is mainly via substrate availability and acyl-CoA product inhibition.

1.6 THE ROLE OF CARNITINE IN ß-OXIDATION

1.6.1 TRANSPORT ACROSS THE INNER MITOCHONDRIAL MEMBRANE

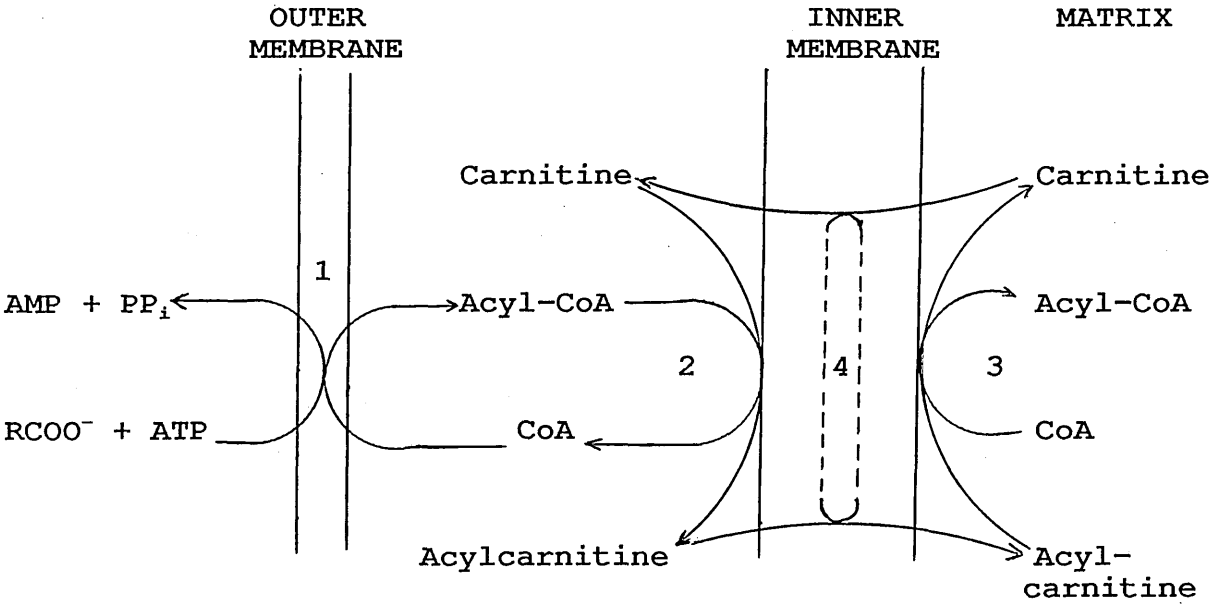


Fig. 1.05. Carnitine dependant transport of long chain fatty acids into the mitochondrial matrix.
1 - Long chain acyl-CoA synthase
2 - Carnitine-palmitoyl transferase I
3 - Carnitine-palmitoyl transferase II
4 - Carnitine translocase

The inner mitochondrial membrane is impermeable to acyl-CoA's of lengths in excess of C16 which make up a large proportion of available fatty acids. Such esters are carried across this barrier by carnitine, carnitine acyltransferase and acyl-carnitine translocase interaction.

Firstly the CoA ester is converted to acyl-carnitine by a carnitine-palmitoyl

transferase I bound to the outer surface of the inner mitochondrial membrane[43,44]. Acyl-carnitine translocase then carries the carnitine ester across the membrane. Once on the matrix side acyl-CoA is regenerated by carnitine-palmitoyl transferase II and the carnitine recycled back across the membrane on a 1:1 basis with the influx of acyl-carnitine[45-47]. There also appears to be a slow, unidirectional, inward flow of carnitine which may be involved in preventing intra-mitochondrial acyl-CoA build up. During times of low tissue acylcarnitine concentrations the translocase process may become rate limiting to ß-oxidation. This inward flow of carnitine would then become more significant and may help to prevent an excessive build up of intra-mitochondrial acyl-CoA concentrations by decreasing the inner acylcarnitine /carnitine ratio relative to that outside the mitochondria.

Carnitine uptake by the liver during times of fast is stimulated by glucagon[45,48]. However, a correlation between carnitine concentration and ß-oxidation is difficult to distinguish because of interference from other regulatory factors.

Tissue carnitine depletion results in lipidosis (accumulation of triacylglycerol). Lipidosis in heart muscle caused by the diphtheria toxin can be decreased by carnitine therapy[48].

1.6.2 CARNITINE-PALMITOYL TRANSFERASE

There are thought to be three carnitine acyltransferases involved in ß-oxidation[49]. The roles of carnitine-octanoyl transferase, found in the peroxisomes[49], and carnitine-acetyl transferase, found in both peroxisomes and mitochondria[50], are unclear. Of greatest importance is the involvement of carnitine-palmitoyl transferase.

There are thought to be two separate isoenzymic forms of carnitine-palmitoyl transferase. Coded for on separate gene loci, they are carnitine palmitoyl

transferase I, CPT I, and carnitine-palmitoyl transferase II, CPT II. They are localised on the outer and inner surface respectively, of the inner mitochondrial membrane[51].

Both long chain acyl-CoA and long chain acyl-carnitine are amphipathic, capable of binding to protein and membrane surfaces therefore the enzyme kinetics vary with surrounding environment and membrane constitutions[52,53]. This may be one source of discrepancy between results from different workers in this area[54,55].

An interesting observation with carnitine-palmitoyl transferase (EC 2.3.1.21) is that one substrate, palmitoyl-CoA competitively inhibits the attachment of the second substrate, carnitine. This effect may be used to prevent flooding of the matrix with long chain acyl-CoA under rapid fatty acid intake. It has also been noticed that the outer transferase shows lowest and limiting activity.

During high carbohydrate states elevated acetyl-CoA carboxylation results in an increase in malonyl-CoA levels. Malonyl-CoA inhibits CPT I[56] and is a key regulatory factor in fatty acid oxidation. The effect is most prominent in muscle and adipose tissue[57], areas of high ß-oxidation capability. It has been shown that in rat mitochondria prolonged fasting results in a drop not only in the level of malonyl-CoA, as one would expect, but also in its inhibitory power on ß-oxidation[58,59,8]. This is due to both a decrease in sensitivity of CPT I to malonyl-CoA and a shift of activity from CPT I to CPT II (total CPT activity remaining relatively constant).

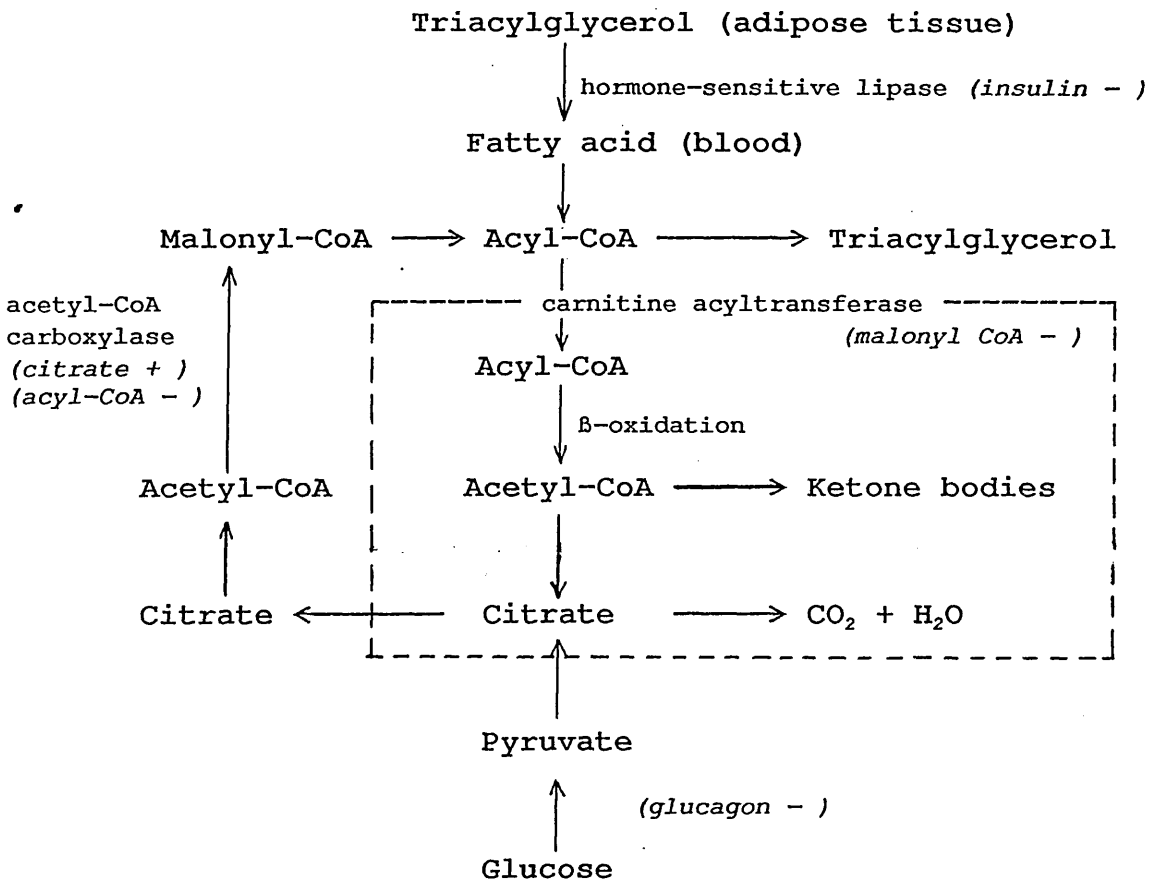
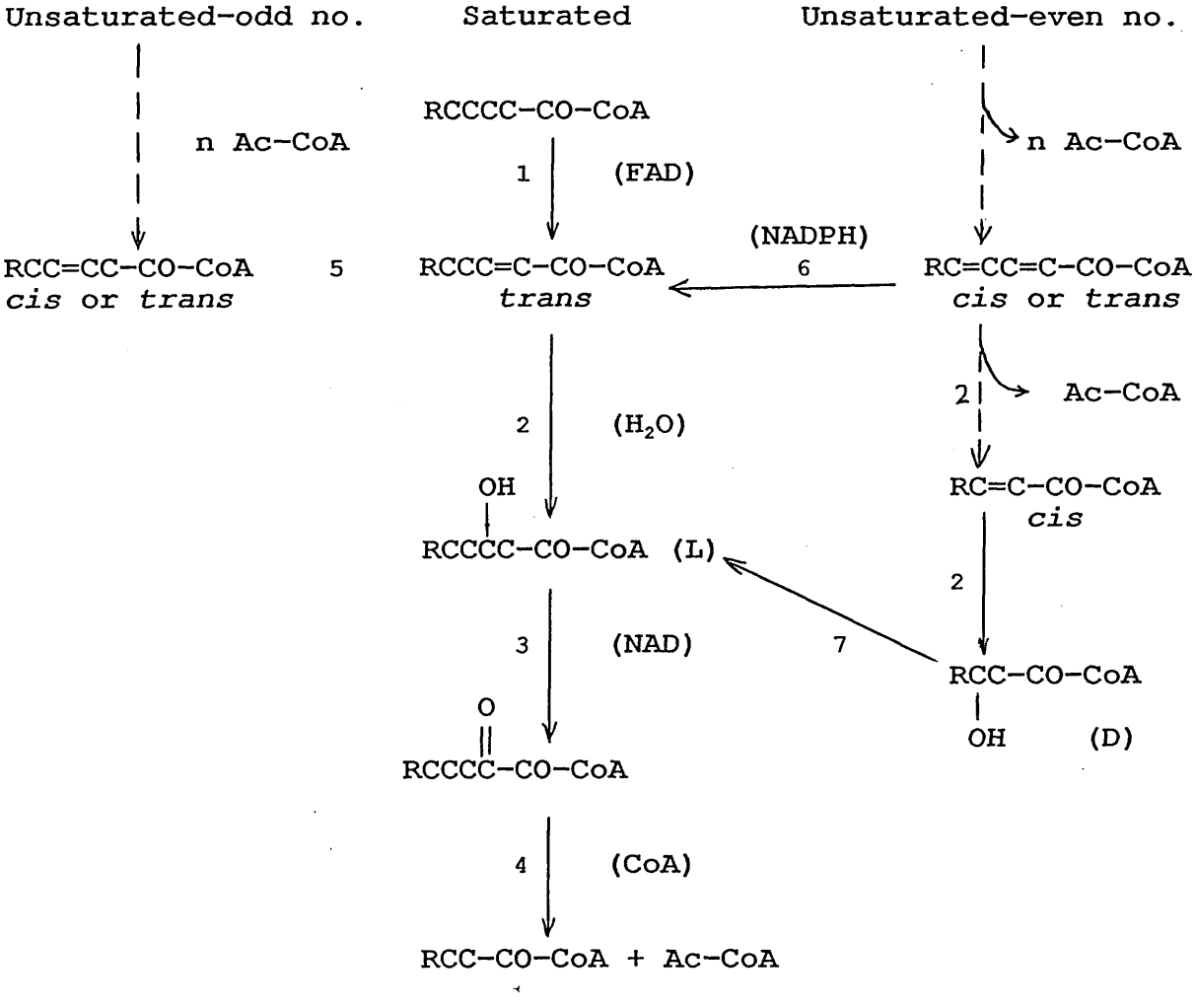


Fig. 1.06. The action (- inhibitory; + stimulatory) of key metabolites on fatty acid oxidation[60].

Glycerol phosphate acyl transferase is the principal enzyme involved in the synthesis of phosphoglyceride and triacylglycerol from glycerol phosphate. It exists in the endoplasmic reticulum and outer mitochondrial membrane[61]. During well fed states its activity increases as that of CPT I falls. Having a lower Km for palmitoyl-CoA, glycerol phosphate acyl transferase competes with CPT I and inhibits ß-oxidation[62,63].

1.7 THE MAJOR ENZYMES OF THE ß-OXIDATION PATHWAY

1.7.1 MITOCHONDRIAL ß-OXIDATION OF STRAIGHT CHAIN SATURATED FATTY ACIDS



1 - Acyl-CoA dehydrogenases.
2 - Enoyl-CoA hydratases (crotonases).
3 - L-(+)-3-Hydroxyacyl-CoA dehydrogenases.
4 - Acetyl-CoA acyltransferases (thiolases).
5 - Enoyl-CoA isomerase.
6 - 2,4-dienoyl-CoA 4-reductase.
7 - 3-hydroxyacyl-CoA epimerase.

Fig. 1.07. The enzymes involved in the ß-Oxidation of straight chain saturated and unsaturated fatty acids.

The extreme low levels of ß-oxidation intermediates is extraordinary even when considering the ability of the enzymes involved to suppress such build up. These intermediates have been seen not to act as true intermediates, that is they have insufficient turnover and no precursor product relationships (as shown by isotope trace experiments[64]). The probable explanation for such low levels would be the existence of the ß-oxidation enzymes within a complex. A complex that would be loosely constructed so as to fall apart upon mitochondrial disruption, thus evading detection. The detectable intermediates would represent true intermediates that have temporarily escaped the complex. Acetyl-CoA would possess the ability to escape such a complex permanently.

The enzymes involved in mitochondrial ß-oxidation of saturated, straight chain acyl-CoA esters can be grouped into four main classes.

1) Acyl-CoA dehydrogenases.
2) Enoyl-CoA hydratases (crotonases).
3) L-(+)-3-Hydroxyacyl-CoA dehydrogenases.
4) Acetyl-CoA acyltransferases (thiolases).

1.7.1.1 Acyl-CoA dehydrogenases

Three different acyl-CoA dehydrogenases are involved in mammalian mitochondrial ß-oxidation of straight chain fatty acids. They have over-lapping specificities[65-68] and similar structures[67]. Short chain acyl-CoA dehydrogenase (SCAD), medium chain acyl-CoA dehydrogenase (MCAD) and long chain acyl-CoA dehydrogenase (LCAD) show significant activity for acyl-CoA's with carbon chain lengths 4 to 6, 6 to 12, and 12 to 20 respectively. They act sequentially on acyl-CoA esters that are continuously being shortened by ß-oxidation[69]. All possess an FAD prosthetic group that is reduced as trans 2-enoyl-CoA is produced. $FADH_2$ is then re-oxidised by the action of electron transfer flavoprotein (ETF), and electron transfer flavoprotein dehydrogenase (ETFD)[70] also known as ETF: ubiquinone oxidoreductase (ETF:QO). These feed the electrons

into the mitochondrial electron transport pathway (respiratory chain) at the coenzyme Q - cytochrome b level.

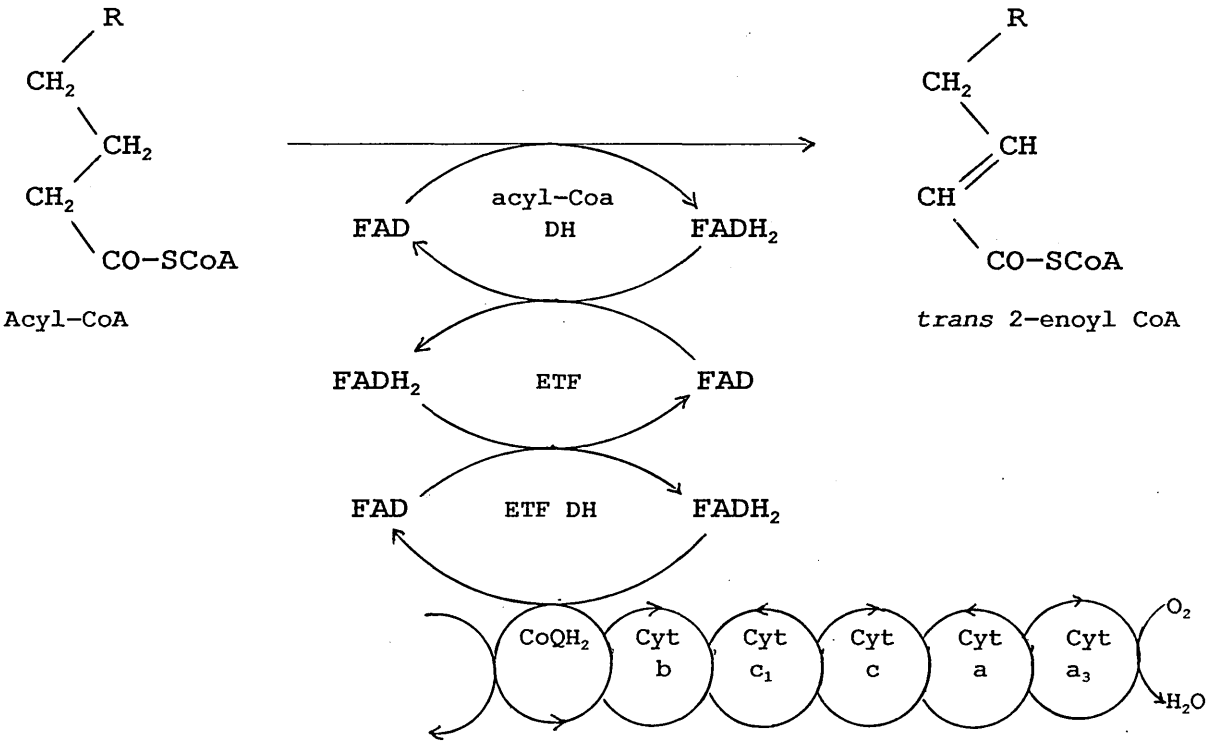


Fig.1.08. Interaction of the acyl-CoA dehydrogenases with the electron transport chain. (cyt = cytochrome, DH = dehydrogenase, $CoQH_2$ = coenzyme Q - reduced form).

The three ß-oxidation acyl-CoA dehydrogenases are strongly inhibited by a product further down the ß-oxidation pathway 3-ketoacyl-CoA[68]. In this way this class of enzymes supply a certain amount of regulation to the pathway.

1.7.1.2 Enoyl-CoA hydratases

The hydratases facilitate the addition of water to the *trans* carbon double bond of trans-2-enoyl-CoA producing L-(+)-3-hydroxyacyl-CoA. They also show a lower

activity towards *cis* double bonds often contained in unsaturated fatty acids such as linoleic acid where D-(-)-3-hydroxyacyl-CoA is formed (see section 1.7.2).

Two enoyl-CoA hydratases have been isolated one with short, the other with long chain length specificity[71,72,75]. The former reacts only poorly with 2,4 dienoyl-CoA esters[73,74], a product of oxidation of some unsaturated fatty acids. It also shows activity towards branched enoyl-CoA esters, evidence of its involvement in the breakdown of branched chain amino acids and other compounds.

1.7.1.3 L-(+)-3-hydroxyacyl-CoA dehydrogenases

Two L-(+)-3-hydroxyacyl-CoA dehydrogenases have been isolated with overlapping specificities. Once again one short chain and one long chain specific form.

They catalyse the oxidation of L-(+)-3-hydroxyacyl-CoA with the simultaneous reduction of NAD, whilst showing no activity for the D-(-)-hydroxyacyl-CoA product of *cis*-enoyl-CoA hydration.

1.7.1.4 Acetyl-CoA acyltransferases (thiolases)

Finally at least two thiolases[76,77] of similar structure catalyse the thiolytic cleavage of 3-ketoacyl-CoA via an acetylated enzyme intermediate[78,79]. Acetyl-CoA is produced along with an acyl-CoA two carbon units shorter than its pre-ß-oxidation cycle parent molecule.

Whilst one thiolase shows broad chain length specificity the other is specific for acetoacetyl-CoA[76] as is a third cytosolic enzyme[80] involved in cholesterol biosynthesis[76].

A heavy displacement of the general thiolase reaction towards acetyl-CoA production along with an inverse relationship between Km and substrate carbon chain length helps to prevent build up of ß-oxidation intermediates[76,81,82].

Whilst the acetyl-CoA/CoA ratio within the mitochondrial matrix increases with ß-oxidation that within the cytosol does not[83]. This is important as acetyl-CoA inhibits pantothenate kinase within the cytosol, the rate limiting enzyme of CoA production. Otherwise, as ß-oxidation progressed, CoA levels would drop which would be counter productive towards acyl-CoA production and hence ß-oxidation.

1.7.2 MITOCHONDRIAL ß-OXIDATION OF UNSATURATED FATTY ACIDS

Unsaturated fatty acids such as linoleic acid possess *cis* carbon double bonds (figure 1.09.). ß-Oxidation of these fatty acids eventually results in the production of two particular esters δ3-*cis*-enoyl-CoA and δ2-*trans*-δ4-*cis*-dienoyl-CoA. The hydratases cannot react with the first and only poorly with the second, probably due to resonance between the two double bonds creating a significant amount of δ3 carbon double bond character. In addition hydratase action on δ2-cis-dienoyl-CoA leads the pathway down another fruitless road in the production of D-(-)-3-hydroxyacyl-CoA inactive towards hydroxyacyl-CoA dehydrogenase.

ß-oxidation is continued pass these stumbling blocks by three additional enzymes (figure 1.07),

1. Enoyl-CoA isomerase,
2. Dienoyl-CoA reductase,
3. Hydroxyacyl-CoA epimerase.

1.7.2.1 Enoyl-CoA isomerase

This isomerase (EC.5.3.3) shows a preference for *cis* but can convert both *cis* and *trans* δ3 carbon double bonds to δ2 *trans*[84-87].

1.7.2.2 Dienoyl-CoA reductase

Two dienoyl-CoA reductases are found in mitochondria 2-enoyl reductase (EC.1.3.1.8.) and 2,4-dienoyl-CoA 4-reductase (EC 1.3.1.-)[88]. The reason for the former is unsure however, the latter, also found in peroxisomes reduces both *cis* and *trans* δ4 carbon double bonds. Both use NADPH in their action which is regenerated mainly by the energy dependant NADH:NADP *trans*-hydrogenase

(EC.1.6.1.1.). This explains why ß-oxidation of δ4 unsaturated fatty acids is strongly suppressed in uncoupled mitochondria, where NADPH levels will be depleted.

1.7.2.3 Hydroxyacyl-CoA epimerase

The 3-hydroxyacyl-CoA epimerase (EC.5.1.2.-) converts non-oxidisable D-(-)-3-hydroxyacyl-CoA to the dehydrogenase substrate L-(+)-3-hydroxyacyl-CoA. The importance of such an epimerase is diminished by two factors. Firstly, enoyl-CoA hydratase has a low affinity for 2,4-dienoyl-CoA and therefore production of D-(-)-3-hydroxyacyl-CoA is limited. Secondly, 2,4-dienoyl-CoA is more readily converted to 2-enoyl-CoA by 2,4-Dienoyl-CoA 4-reductase.

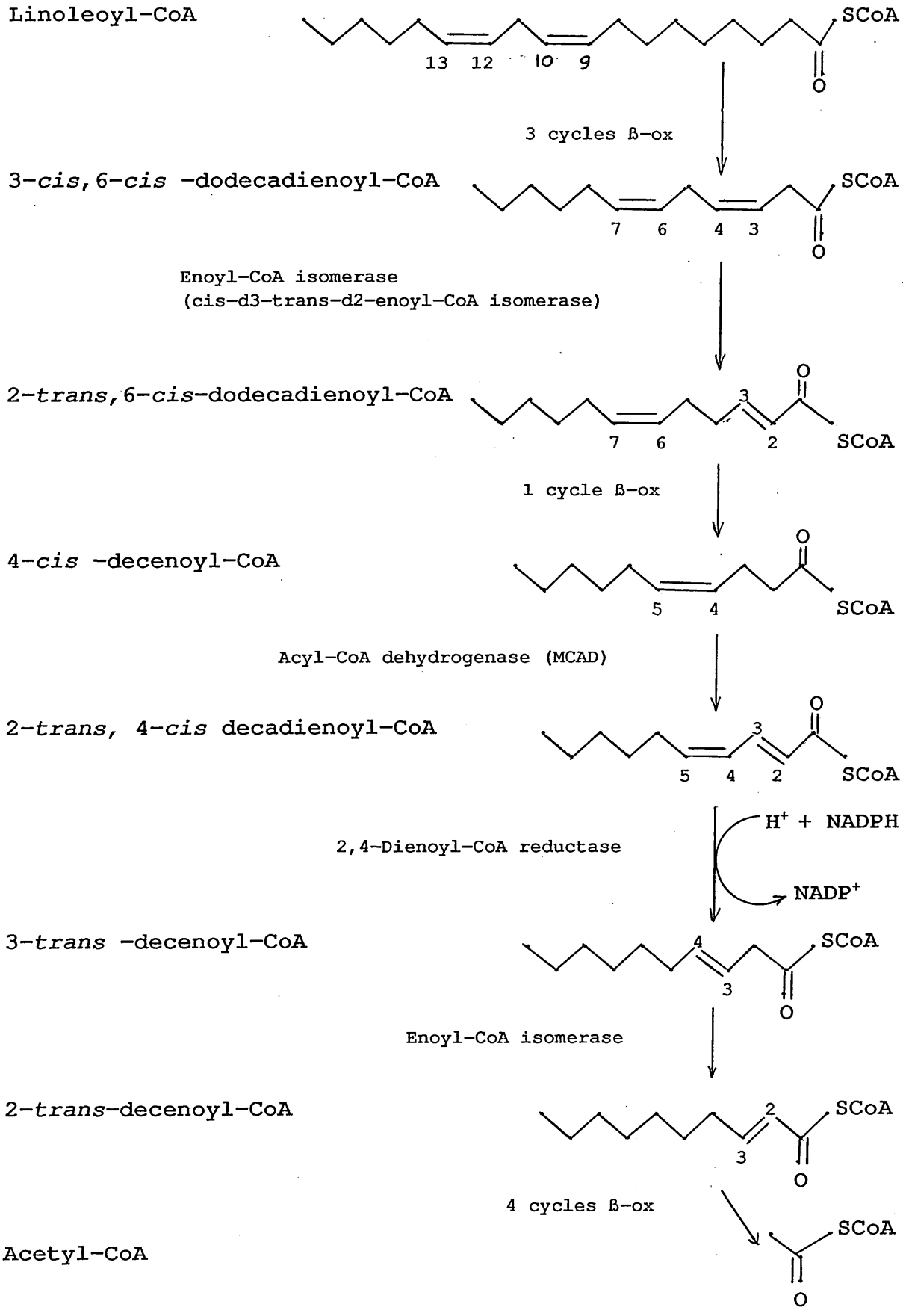


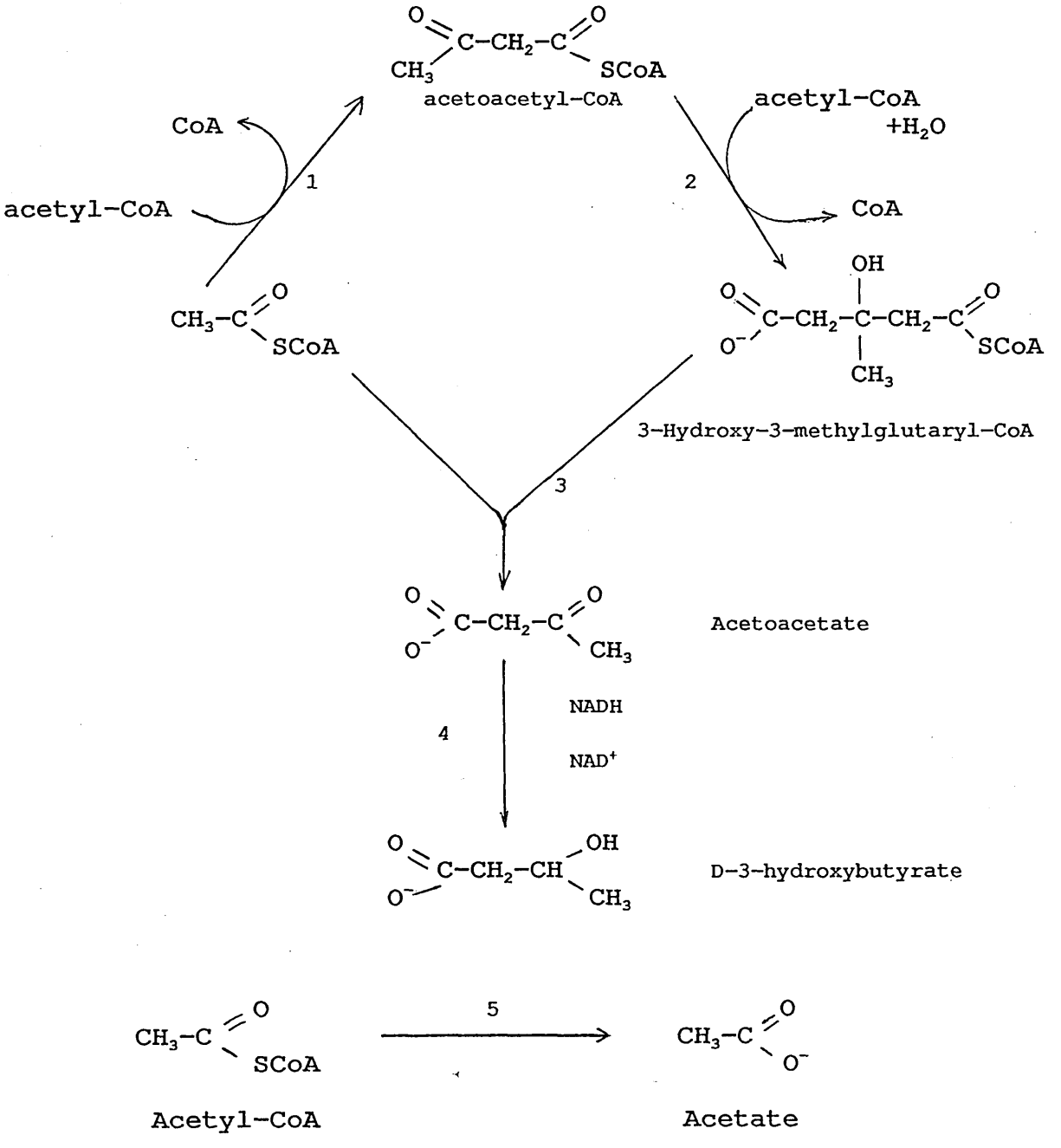
Fig.1.09. Mitochondrial ß-Oxidation of Linoleoyl-CoA[100].

1.7.3 INDUCTION OF THE ENZYMES INVOLVED IN ß-OXIDATION

ß-Oxidation is induced by prolonged carbohydrate depletion. That is to say, during fasting fatty acid oxidation rates per mitochondrial mass are higher (an increase of 50% in some cases[89]) in animals that have already fasted for some time than in those previously well fed[90-92]. Mitochondrial CPT[93], acyl-CoA dehydrogenase[94], and 2,4-dienoyl-CoA 4-reductase[95] and ETF[96] have all been seen to be induced in this way. Total mitochondrial mass can also increase.

Similarly, diets high in long chain *trans* fatty acids and C22 fatty acids, poorly oxidised in the mitochondria, induce peroxisomal ß-oxidation[97-99].

1.8 KETONE PRODUCTION VIA THE HYDROXYMETHYLGLUTARYL-CoA CYCLE



1 = 3-Keto thiolase
2 = 3-Hydroxy-methylglutaryl-CoA synthase
3 = 3-Hydroxy-methylglutaryl-CoA lyase
4 = D-(-)-3-hydroxybutyrate dehydrogenase
5 = Acetyl-CoA hydrolase

Fig. 1.10. The production of ketone bodies via hydroxymethylglutaryl-CoA.

In the liver and kidneys the main ß-oxidation product, acetyl-CoA, has three fates. It can be completely oxidised to carbon dioxide and water by the tri-carboxylic acid cycle, form a substrate for lipogenesis (significant only during well fed conditions) or be converted to the ketone bodies, 3-hydroxybutyrate, acetoacetate and the hydrolysis product acetate.

1.8.1 MECHANISM OF KETOGENESIS

During prolonged fast acetyl-CoA levels rise due to increased ß-oxidation rates and the oxaloacetate levels fall due to utilisation by gluconeogenesis. In addition, the electron transport chain will be further reduced by high ß-oxidation rates and the NADH/NAD ratio will increase. The steps of the citric acid that are dependant on the NAD concentration, such as the oxidation of isocitrate, α-keto-glutarate and malate, will therefore be suppressed. The net result is that the rate of production of acetyl-CoA will exceed the capability of the citric acid cycle to complete oxidation. When this occurs, acetyl-CoA is utilised by ketogenesis at an increasing rate.

Initial assumptions were that ketones were an incidental by product of elevated ß-oxidation. However, current thinking tends to regard ketone production as a deliberate means to compensate for the lack of ability to metabolise fatty acids at an appreciable rate.

Acetoacetate is formed from the condensation of acetyl-CoA and acetoacetyl-CoA via 3-hydroxy-3-methylglutaryl-CoA. The rate limiting step of the condensation is catalysed by 3-hydroxy-methylglutaryl-CoA synthase (EC.4.1.3.5). 3-Hydroxy-methylglutaryl-CoA lyase (EC.4.1.3.4), in the presence of Mg^{2+} [101], then facilitates the cleavage of hydroxy-methylglutaryl-CoA to form acetoacetate and acetyl-CoA[102]. This enzyme is localised exclusively in the mitochondria of organs with high ketogenic potential, such as the liver and kidneys[103]. Hydrolase action on acetoacetyl-CoA would also produce acetoacetate, however, such an enzyme has

not been isolated.

D-(-)-3-hydroxybutyrate is formed by D-(-)-3-hydroxybutyrate dehydrogenase (EC .1.1.1.30) action on acetoacetate with NADH. This enzyme is present in all tissues and is bound firmly to the inner membrane of the mitochondria facing towards the matrix. This position suggests that 3-hydroxybutyrate/acetoacetate in the liver is in equilibrium with NADH/NAD in the mitochondrial matrix[104]. Phosphatidyl choline is also required for activity[105,106].

Acetate production in the mitochondria is facilitated by acetyl-CoA hydrolase[107]. It has also been found in the liver cytosol[108]. In rat brown adipose tissue this enzyme is strongly inhibited by CoA therefore limiting acetate formation to conditions of high acetyl-CoA/CoA ratios[109,110].

These ketone bodies can then travel to other tissues where -

1) 3-hydroxybutyrate is converted to acetoacetate by 3-hydroxybutyrate dehydrogenase,
2) acetoacetate is converted to acetoacetyl-CoA by succinyl-CoA:acetoacetyl-CoA transferase[111,112] and subsequently to acetyl-CoA by thiolase,
3) acetate is converted to acetyl-CoA by acetyl-CoA synthase.

Oxidation is then completed by the entrance of the acetyl-CoA into the tri-carboxylic acid cycle at these sites.

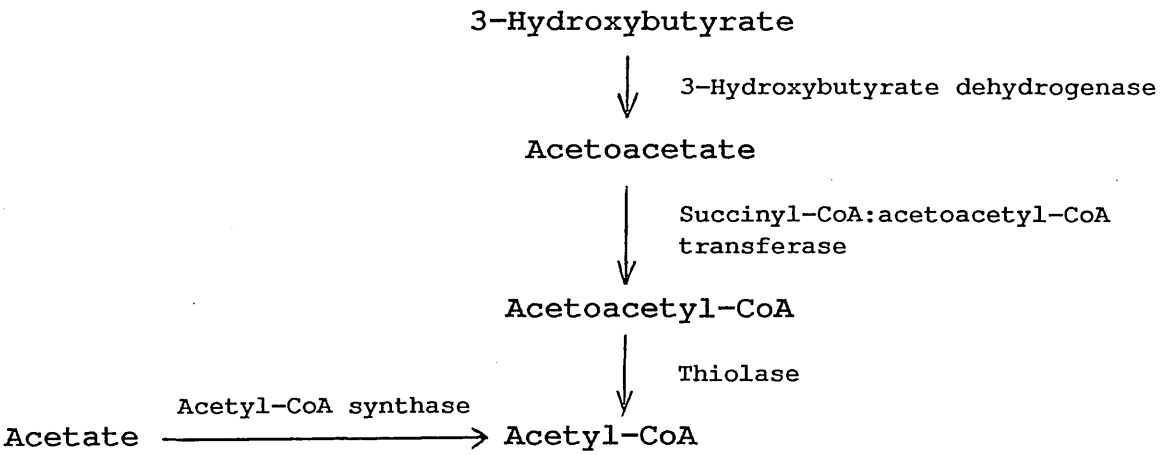


Fig. 1.11. The reconversion of ketone bodies to acetyl-CoA in extrahepatic tissue.

1.9 ω-OXIDATION OF FATTY ACIDS

ω-Oxidation, first demonstrated in 1934[113] occurs within the microsomes (ie the endoplasmic reticulum) in organs such as liver and kidney. The initial step, the hydroxylation of the last (ω) or the next to last (ω-1) carbon of the fatty acid, is catalysed by a mixed function oxidase system within the endoplasmic reticulum. Cytochrome P450 and cytochrome P450 reductase are involved and the presence of NADPH and oxygen is necessary for activity. ω-Hydroxy fatty acids are formed along with a small amount of (ω-1)-hydroxy fatty acid. ω-Hydroxy fatty acids are oxidised to dicarboxylic acids by alcohol and acetaldehyde dehydrogenases in the endoplasmic reticulum or cytosol[117] and the longer dicarboxylic acids converted to dicarboxylic mono-CoA esters by long chain acyl-CoA synthase in the endoplasmic reticulum or mitochondria[118,119]. A certain amount of chain shortening by peroxisomal ß-oxidation may also occur. Monocarboxyl esters of carnitine can be produced[119] but are less well oxidised in the mitochondria.

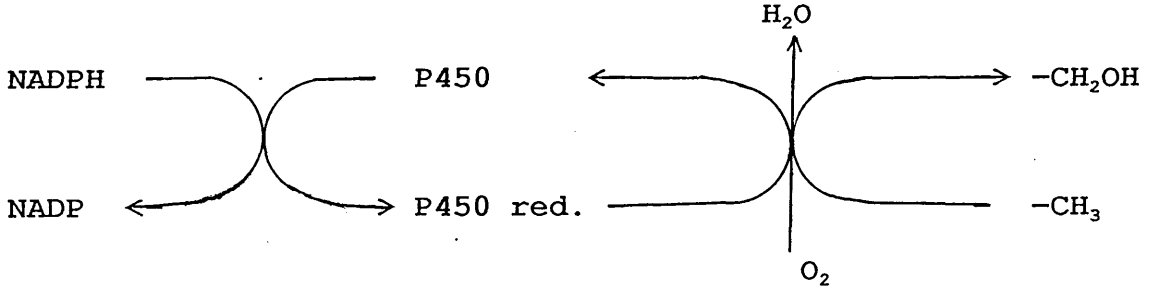


Fig. 1.13. The mixed function oxidase system of ω-oxidation.

Although it is conceivable that ω-oxidation can produce succinate and therefore glucose, this effect is thought to be gluconeogenicaly insignificant.

Under normal conditions only 4-5% of straight chain fatty acids (monocarboxylic acids) undergo ω-oxidation with a preference for medium chain lengths C10-C14. The pathway plays a significant role in the oxidation of branched and substituted fatty acids[114,115] which are poor substrates for ß-oxidation such as 2,2-dimethylstearate. The dicarboxylic acids that are produced are then ß-oxidised[116] forming either shorter dicarboxylic acids or acetyl-CoA. During times of high fatty acid oxidation flux these dicarboxylic acids build up and along with the concomitant ketone bodies are excreted in the urine to create a ketotic dicarboxylic aciduria. When there is a block in ß-oxidation the production of acetyl-CoA and therefore ketogenesis is diminished. A build up in fatty acids accelerates ω-oxidation increasing production of dicarboxylic acids. Depending on the position of the block in ß-oxidation pathway subsequent dicarboxylic acid shortening will be constrained. For example in MCAD deficiency ß-oxidation will halt at C6 chain lengths. This along with an absence of ketones results in the characteristic hypoketotic C6, C8 and C10 (adipate, suberate, sebacate) dicarboxylic aciduria profile associated with this disease.

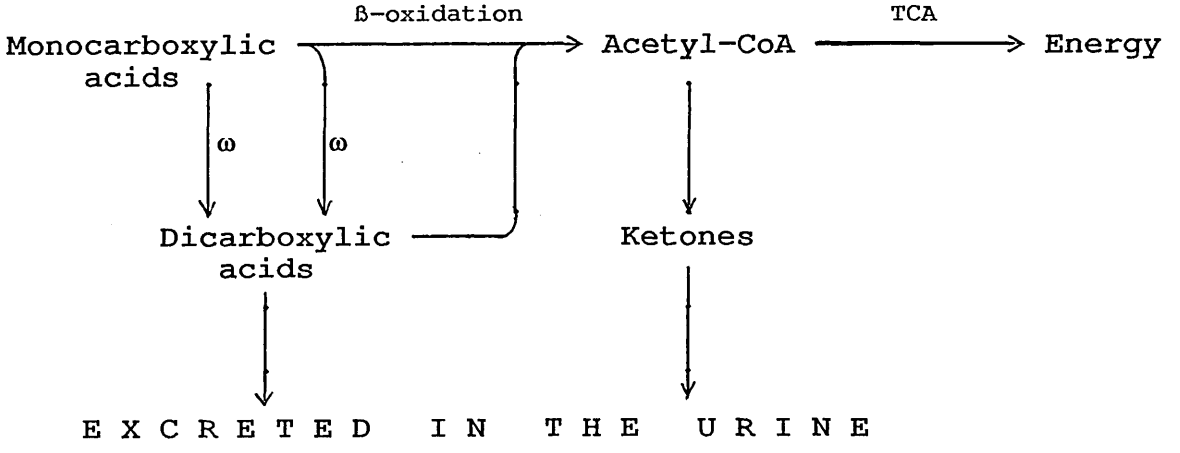


Fig. 1.12. The production of a ketotic dicarboxylic aciduria. (TCA = tri-carboxylic acid cycle)

1.10 CLINICAL FEATURES OF DEFECTS IN FATTY ACID OXIDATION

To date (1991) there are 9 documented errors in fatty acid metabolism (table 1.02). When one considers the number of enzymic steps involved in the pathway there may be a possible ten or eleven yet to be reported. The first six defects, deficiencies in LCAD, MCAD, SCAD, LCHAD, ETF and ETFD of which MCAD deficiency is the most common have all been implicated in sudden infant death syndrome, SIDS. All disorders are inherited in an autosomal recessive way with a 1 in 4 chance of inheritance.

1.10.1 GENERAL PRESENTATION

Most of the disorders in fatty acid metabolism show acute decompensation and Reye like symptoms of hypoketotic hypoglycaemia, elevated free fatty acids, hyperammonaemia and hepatic fat accumulation, during times when fatty acid oxidation is needed. Symptoms are of three basic types:

1. Symptomatic hypoglycaemia[121,122];
2. Hepatic encephalopathy and Reyes like illness[123-128];
3. Sudden unexpected death[129-137].

They result from either fatty acid build up, fatty acid metabolic intermediate build up or hypoglycaemia and as such show fasting intolerance. The two CPT deficiencies[138] and carnitine transport defect show slightly different patterns of presentation (see section 1.10.8)

Presentation is often preceded by an independent illness often too mild to merit hospitalisation. An increase in body energy requirement during illness, concomitant loss of appetite, vomiting and diarrhoea all diminish carbohydrate

stores and place emphasis on fatty acid metabolism. Any fault in this pathway will then be magnified and, if untreated, could result in coma and death.

It is often the case that laboratory profiles appear normal whilst the patient is metabolically balanced. This limits the detection of any disorder to times when the particular defective step of the pathway is required. However, it is now evident that measurement of glycine and carnitine conjugates may provide a method of detection of such defects during the asymptomatic period (see section 1.11.2 and 1.11.3).

Table 1.02. Clinical features and acute sample organic acid profiles of fatty acid oxidation defects.

DEFECT	CLINICAL FEATURES	ORGANIC ACIDS
LCAD	Fasting intolerance, SIDS, cardiomyopathy	Adipic, suberic, sebacic, C12-,C14-dicarboxylic acids
MCAD	Fasting intolerance, SIDS	Adipic, suberic, sebacic (saturated and unsaturated), hexanoyl-, suberyl- and 3-phenylpropionylglycine, octanoylcarnitine, 5-hydroxyhexanoic, 7-hydroxyoctanoic acid
SCAD	Fasting intolerance, neurological seizures, acidaemia, myopathy	Ethylmalonic, methylsuccinic butyrylglycine, butyrylcarnitine
LCHAD	Fasting intolerance progressive liver disease	Adipic, suberic, sebacic (saturated and unsaturated) 3-hydroxy C8, C10, C12, C14 dicarboxylic acids
ETF& ETFD	(a) Severe form: Hypoglycaemia, acidaemia, neonatal death (b) Mild form: Fasting intolerance, muscle weakness	Same as LCAD, MCAD, SCAD plus glutaric, 2-hydroxyglutaric, isovalerylglycine, isobutyrylglycine, isovalerylcarnitine, glutarylcarnitine
CPTliver	Fasting intolerance	None
CPTmuscle	Exercise induced	None (myoglobinuria)
Carnitine transport defect	Progressive skeletal & cardiac muscle weakness (endocardial fibroelastosis), fasting intolerance	None

1.10.2 GENERAL MANAGEMENT

On identification management is usually straight forward. Carbohydrate supply should be ensured and periods of fast minimised thus avoiding the need for fatty acid metabolism. Special diets can be used to avoid the step in the pathway which is defective. For example, medium chain triglyceride oil alleviates the need for LCAD and is a useful addition to glucose therapy in deficiency of this enzyme.

1.10.3 LONG CHAIN ACYL-CoA DEHYDROGENASE DEFICIENCY

Initially described in 1985[127] to date fourteen cases, nine females and five males, have been documented eleven of which have been positively identified by ETF assay. A deficiency in this enzyme essentially blocks long chain acyl-CoA production and hence ß-oxidation of C16 to C20 fatty acids. LCAD deficiency seems to follow one of 3 clinical pathways.

1. Onset in the first six months with high mortality rate.
2. Onset between six months and one year with acute decompensation similar to MCAD deficiency.
3. Onset approximately one year of age with stress induced rhabdomyolisis similar to muscular CPT II deficiency[139].

One case presented at 8 years of age[140].

Most patients have had reported hepatomegaly and were detected to be hypoglycaemic (glucose level of 1.2 mmol/l[140]) with inappropriately low urine ketone levels. Elevated ammonia and transaminase levels have been reported in several but not all patients. Low serum carnitine and increased percent esterified carnitine have been reported[140] (table 1.03) with acetyl-carnitine the predominant acyl-carnitine. Liver biopsy and necropsy investigations have shown fatty infiltrations. Organic acid profiles during balanced metabolic states appear normal. During presentation C6, C8, C10, C12 and C14 dicarboxylic are present.

Suberyl, hexanoyl and phenylpropionyl-glycines are not detected and no carnitine conjugates have been seen.

Table 1.03. Plasma total (TOT) and percentage esterified carnitine levels in LCAD deficiency[140].

	MEAN	RANGE	NORM.
TOT (μmol/l)	16	10–28	46±10
% Esterified	80	48–100	<25

Direct enzyme assay remains the only reliable means of diagnosis of this defect.

1.10.4 MEDIUM CHAIN ACYL-CoA DEHYDROGENASE DEFICIENCY

The most common of the disorders of ß-oxidation is medium chain acyl-CoA dehydrogenase deficiency with an incidence of 1:5000 to 1:10,000. Initially described in 1976[123] the age at onset usually ranges from 2-47 months[141] although cases have been reported presenting as early as two days and as late as eight years of age.

Urine organic acid analysis, during onset, has detected the medium chain dicarboxylic acids, adipic, suberic, and sebacic acids, the carnitine conjugates octanoyl-, hexanoyl-, and *cis*-4-decenoylcarnitine, and the glycine conjugates hexanoyl- and suberylglycine. The presence of phenylpropionyl glycine, a product of intestinal 3-phenylpropionic acid has been proposed as a pathagnomic marker[142-145] although it may be absent in the newborn because of insufficient gut colonisation[146]. In addition, the presence of 5-hydroxyhexanoic and 7-hydroxyoctanoic acids, products of ω-1 oxidation, may also prove useful. In some cases, plasma GC/MS organic acid analysis has also shown increased octanoic, decanoic and *cis*-4-decenoic acids[73,74], even during the asymptomatic period .

High esterified and low free carnitine values occur. Muscle, liver, and plasma total carnitine is usually approximately 25% of normal[148]. The mechanism for this secondary carnitine deficiency, often seen in many of the ß-oxidation disorders, is unclear but is probably due to impaired renal handling of free and esterified carnitine[149,161]. Carnitine treatment does not prevent illness during fasting[148].

1.10.5 SYSTEMIC SHORT CHAIN ACYL-CoA DEHYDROGENASE DEFICIENCY

There have been three documented cases of systemic short chain acyl-CoA dehydrogenase, SCAD, deficiency. Initial presentation is within the first few days of life and in one case has proved fatal. There is also a second myopathic form of the disease, detected in 2 patients over 30 years of age.

The three cases of systemic SCAD deficiency presented with abnormal organic aciduria but with varying profiles. Major metabolites lactate, 3-hydroxybutyrate, 2-hydroxybutyrate, butyrate, ethylmalonate, methylsuccinate, adipate, 3-hydroxyisovalerate were also detected. Similar organic acid profiles have been seen in two patients shown not to be SCAD deficient by enzyme analysis[50].

As with MCAD deficiency low free and high esterified plasma carnitines have been noticed.

Tritium and carbon-14 release assays on skin fibroblast indicate impaired butyrate oxidation but normal octanoate and palmitate metabolism[151,152].

ETF linked assay shows only partially defective butyrate oxidation due to overlapping MCAD activity. In one patient octanoate oxidation was also diminished indicating MCAD deficiency.

Difficulties in the diagnosis of such a rare and symptomatically variable disorder are exaggerated by interference from MCAD activity. Direct enzyme analysis remains the only reliable diagnostic assay.

1.10.6 LONG CHAIN HYDROXYACYL-CoA DEHYDROGENASE DEFICIENCY

Long chain hydroxyacyl-CoA dehydrogenase (LCHAD) deficiency is very similar to LCAD and MCAD deficiencies in presentation. Routine laboratory analysis provides little indication of the disease but fasting produces marked hypoglycaemia with inappropriately low ketosis and elevated lactate, pyruvate and urate levels. Serum free carnitine levels can be suppressed to 60% whilst long chain acyl carnitine increases (similarly in liver and muscle). Organic acid profiles at presentation show 3-hydroxydicarboxylic acids (C6, C8, C10, C12, C14), and medium chain saturated and mono unsaturated dicarboxylic acids. As the assay for this enzyme becomes more widely available the number of diagnoses will no doubt increase. Other metabolic blocks after the acyl-CoA dehydrogenase step have been seen to produce 3-hydroxydicarboxylic acidurias. For example thiolase defects can result in the excretion of 3-hydroxycarboxylic acids resulting from an accumulation of unstable 3-keto compounds.

1.10.7 ELECTRON TRANSFER FLAVOPROTEIN AND ELECTRON TRANSFER FLAVOPROTEIN DEHYDROGENASE DEFICIENCY (glutaric aciduria type II and ethylmalonic adipic aciduria)

1.10.7.1 Glutaric aciduria type II

First documented in 1976[153], this disease presents soon after birth in either a mild or severe, often fatal, form. The defect is at the electron transfer flavoprotein or electron transfer flavoprotein - ubiquinone oxidoreductase level[154]. Because of the involvement of ETF and ETFD in the acyl-CoA dehydrogenase reactions, presentation is often suggestive of a multiple acyl-CoA dehydrogenase deficiency. Of particular importance is the presence of glutaric acid in the urine. As a result this deficiency is often referred to as glutaric aciduria type II. During presentation glutarate appears as a substantial peak on a gas-liquid chromatography organic acid profile and also occurs in smaller amounts during the asymptomatic period. The metabolism of branched chain amino acids and lysine is also effected.

Studies on skin fibroblasts show diminished oxidation of butyrate octanoate and palmitate.

1.10.7.2 Ethylmalonic adipic aciduria

This related disorder has been detected in many patients[126,155] where ethylmalonate is the predominant peak during the asymptomatic period. The position of the block within fatty acid metabolism is still unsure and therefore no direct enzyme assay is available.

Often patients suffering from ethylmalonic adipic aciduria respond well to riboflavin supplementation.

1.10.8 SYSTEMIC CARNITINE DEFICIENCY AND CARNITINE TRANSPORT DEFECT

The three possible carnitine related disorders affecting the transfer of long chain fatty acids across the mitochondrial membrane are:

1) Carnitine transport defect.
2) Carnitine-palmitoyl transferase deficiency (CPT I and II).
3) Systemic carnitine deficiency.

Carnitine transport defect presents usually within the first year of life. Symptoms include cardiomyopathy (thought to be the cause of death in some cases), skeletal muscle weakness[156], marked hypoglycaemia with inappropriately low ketosis, hyperammonaemia and raised transaminases with total plasma carnitine levels that can be as low as 10% of normal. In 50% of cases encephalopathy has been reported.

The few cases of reported CPT I deficiency have a very similar presentation to carnitine transport defect all have shown encephalopathy. Slightly more cases of muscular CPT II than CPT I have been documented. Onset is generally much later than CPT I deficiency, between 6 years to late adulthood. There appears to be two clinical forms of CPT II deficiency[189]. Both show muscular symptoms such as skeletal muscle weakness and pain on exercise, but in the second there is also hepatic and cardiac dysfunction with rhabdomyolisis myoglobinuria sometimes leading to renal failure. It has been suggested[189] that CPT II activity is insufficiently low, in the more common muscle form, to impair long chain fatty acid oxidation in liver and heart. Residual activity in fibroblasts of 25% for the muscular form and 10% for the hepato-cardio-muscular form have been recently quoted along with the observation that palmitate oxidation is not significantly effected until such a residual activity falls below 20%.

Systemic carnitine deficiency, as suggested by subnormal non-crisis plasma free

carnitine levels, has often proved to be a secondary effect of an enzyme defect such as MCAD deficiency[157,158] or carnitine transport defect[156,159]. Further, low free carnitine may be a reflection of an elevated acyl-carnitine level and not a result of carnitine deficiency (paired correlation plots would be of use here - see sections 3.2.1 and 4.1). There is therefore conjecture as to the existence of any definite systemic carnitine defect.

1.11 LABORATORY EVALUATIONS OF ERRORS IN FATTY ACID METABOLISM

1.11.1 CLINICAL LABORATORY ASSAYS

Clinical laboratory assays useful during assessment of fatty acid oxidation defects are :-

BLOOD ANALYSIS

Acid-base studies.
Glucose quantitation.
Ammonia and liver enzyme measurements.
Plasma carnitine evaluations.
- Total plasma carnitine.
- Free plasma carnitine.
- Esterified / total - *This often provides a more reliable indicator than just TOTAL or ESTERIFIED and can act as a diagnostic indicator during the well period. (See later chapters)*

Free fatty acid quantitation.
3-Hydroxybutyrate measurements.
- FFA / 3HB - *Once again a ratio of values is more diagnostically useful. High ratios, double normal in some cases, indicate metabolic defect. Normal ratios are approximately unity (see later).*

URINE ANALYSIS

Ketone evaluation. - *It is possible that crisis urine ketones levels can be of sufficient abundance to register positive on spot tests contradicting the hypoketotic presentation of the fatty acid oxidation defects.*

Organic acid analysis. - *Qualitatively by GC. A growing number of centres are now using more sensitive GC/MS methods.*

One limitation of urinary organic acid analysis during acute crisis is that a fatty acid metabolic defect lying prior to mitochondrial ß-oxidation would not produce a build up of oxidation metabolites and therefore no such abnormal organic acid pattern would occur. Defects in fatty acid transport into the mitochondria (acyl-CoA activation, CPT deficiency or carnitine transport defect) do not produce abnormal urinary organic acid patterns (see section 1.10.8).

1.11.2 GLYCINE CONJUGATE QUANTITATION

The glycine conjugates hexanoyl-, suberyl-, and 3-phenylpropionyl-glycine occurring in the acyl-CoA dehydrogenase deficiencies may be present, but in very small amounts, during the asymptomatic period.

Use of stable isotopic internal standards provides a means of quantifying glycine conjugates making early diagnosis of deficiencies such as that of MCAD possible. Such assays involving advanced chemical ionisation mode GC/MS have been used with very high success rates[160]. However, use of single ion monitoring, electron impact mode GC/MS provides a more widely available means of glycine conjugate quantitation. (see section 2.4).

The basic idea behind using an internal standard in GC quantitation assays is to use a standard with chromatographic properties as similar as possible to those of the compound to be quantitated (the quantitate), whilst maintaining a detectable difference in elution times between the two. A deuterated form of the quantitate has almost identical properties but is eluted slightly earlier. Total ion GC/MS methods lack the sensitivity to distinguish compounds with such similar retention times. However, with the MS running in selective ion monitoring mode the difference between similar ion fragments from both molecules can be distinguished. Ions from the IS will be a number of AMU heavier (depending on the number of deuteriums on the molecule) than those from the quantitate.

1.11.3 CARNITINE CONJUGATE QUANTITATION

Sophisticated assays for carnitine conjugate quantitation are available involving fast atom bombardment mass spectrometry (FAB/MS). Even greater sensitivity is gained by use of FAB MS/MS (two MS used in tandem)[161-163]. Acyl carnitines can be detected by TLC[164] although this method lacks sufficient sensitivity to detect such low levels as are present outside crisis.

Other methods have been investigated to replace GC/MS such as high performance liquid chromatography (HPLC)[165,166], HPLC/MS[167], thin layer chromatography and nuclear magnetic resonance spectroscopy[168]. However, to date all lack sufficient sensitivity to be of any great use for carnitine quantitation in fatty acid oxidation defect diagnosis. My own experience with HPLC, GC and GC/MS upholds this statement.

Radio-isotope exchange HPLC has been used to determine octanoyl carnitine levels but this method is still under development.

1.11.4 USE OF METABOLIC STRESS INDUCTION IN THE EVALUATION OF METABOLIC DEFECTS

It is not always possible to obtain laboratory samples during spontaneous presentation. However, crisis can be forced whilst the patient is available for testing by subjecting them to metabolic stress. There are three principle methods used to induce metabolic stress. They are;

1. Controlled fasting,
2. High fat dieting,
3. Metabolite loading tests.

Fasting or subjecting a patient with a fatty acid metabolic defect to a high fat diet must be rigorously supervised and has proved fatal[169]. Loading tests involve the administration of high concentrations of fatty acid metabolic pathway intermediates, that precede the suspected block, to magnify any abnormal metabolic trait. Carnitine loading elevates the levels of diagnostic acylcarnitine in MCAD deficiency[134]. 3-Phenyl-propionic acid, PPA, loading increases urinary 3-phenyl-propionyl glycine concentrations detected by HPLC or GC/MS[170]. The PPA loading test is used as a specific test for MCAD deficiency where phenylpropionyl glycine is excreted in the urine as well as hippuric acid the normal metabolite of PPA. The test may prove to be useful in other metabolic defects. For example, an LCHAD deficient patient undergoing PPA loading tests has been reported to produce small amounts of 3'-hydroxyphenylpropionyl glycine[171].

1.11.5 FATTY ACID OXIDATION DEFECT IDENTIFICATION BY USE OF FIBROBLAST CULTURE

Work with skin fibroblasts provides a relatively non-invasive technique to evaluate fatty acid metabolic defects in the laboratory without the need for metabolically stressing the patient.

Fatty acids labelled with either tritium or carbon-14 are oxidised to produce $^{3}H_20$ or $^{14}C0_2$ respectively. Use of such radio labelling can be employed in the elucidation of the position of a metabolic block[172]. However, such assays cannot identify specific enzyme defects and false negative results can occur. These may be connected with the presence of non-mitochondrial ß-oxidation or overlapping chain length specificities of related enzymes.

Because of the expense and time required for fibroblast culture it is an advantage to be able to store the biopsy material without immediate culture (see appendix 1). In this way the investigator can await sufficient evidence of fatty acid metabolic defect to merit further investigation. Cryoscopic storage techniques published by K.J. Fowler[173] have been further developed and evaluated by the author and are discussed in appendix 1.

1.11.6 DIRECT ENZYME ANALYSIS OF DEFECTS IN FATTY ACID METABOLISM

Work in this area concentrates around the acyl-CoA dehydrogenase defects. Necessary for enzyme activity is the presence of ETF a natural electron acceptor for the dehydrogenase reaction as acyl-CoA is dehydrogenated to enoyl-CoA. The absence of a commercial supply of ETF adds to the complexity of an already time consuming and technically demanding assay. Development is still under way of an assay involving a synthetic electron acceptor and incorporating a colour end point[174].

As well as the three acyl-CoA dehydrogenases, direct enzyme assays have been used to measure CPT, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase activities. Carnitine transport protein can also be measured by indirect assay. However, none of these assays are, by any means, routine.

CHAPTER 2

METHODS AND MATERIALS

2.1 ROUTINE CLINICAL LABORATORY ASSAYS

2.1.1 BLOOD SAMPLING PROCEDURE FOR LABORATORY ANALYSIS

The following assays were all carried out on the Cobas Bio spectrometric autoanalyser. The quality control used, unless otherwise stated, was the standard procedure well established within the laboratory.

Blood samples were collected by thumb prick from young children presenting at the Sheffield Childrens Hospital with febrile illness, and a control group of children awaiting unrelated surgical procedure.

The plasma or serum was separated by centrifugation at approximately 3500 revs/min for 5 minutes on a Heraeus Christ Minifuge2 centrifuge (Heraeus Equipment Ltd) and stored at -20°C for a maximum of two weeks prior to assay.

2.1.2 PLASMA GLUCOSE QUANTITATION

Samples were collected into glass containers pretreated with sodium fluoride preservative. A minimum volume of 50 µl serum was used in the assay.

This assay incorporated (+)Glucose GDH single shot assay kit marketed by Roche MA (Kit no. 0715182). The procedure relied on the conversion of 3-D-glucose to D-gluconate in the presence of NAD by glucose dehydrogenase (GDH).

$$\text{3-D-Glucose} + \text{NAD} + H_2O \xrightarrow{\text{GDH}} \text{D-Gluconate} + \text{NADH}$$

Mutarotase was added to accelerate the transition from α to ß isomeric forms of

D-glucose. The decrease in absorbance at 340nm was directly proportional to the original glucose concentration.

2.1.3 PLASMA LACTATE QUANTITATION

Collection and storage of samples was identical to the procedure adopted for serum plasma glucose quantitation. A minimum assay sample size of 50 µl plasma was used.

Lactate levels were measured using the (+)lactate two shot assay kit marketed by Boehringer Manheim GmbH, W.Germany (kit no. 149993). Lactate 5mmol/l (BCL) stored at 4°C was used as standard. The principle of the assay relied on lactate dehydrogenase (LDH) action in converting lactate to pyruvate with concomitant reduction of NAD. The pyruvate was then reacted with glutamate in the presence of glutamate-pyruvate transaminase (GPT) producing alanine and oxoglutarate. An increase in absorbance at 340 nm was directly proportional to the original lactate concentration.

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

$$\text{Pyruvate} + \text{Glutamate} \xrightarrow{\text{GPT}} \text{Alanine} + \text{Oxoglutarate}$$

2.1.4 PLASMA 3-HYDROXYBUTYRATE QUANTITATION

Collection and storage of samples was identical to the procedure adopted for plasma glucose quantitation.

(+)3-Hydroxybutyrate dehydrogenase, HBDH (Sigma, type 2; stored at 4°C) action on 3-hydroxybutyrate in the presence of NAD (BDH Ltd.) produced acetoacetate.

There was a concomitant rise in absorbance at 340 nm that was directly proportional to the original level of 3-hydroxybutyrate. Tris-(hydroxymethyl)-methylamine-[2-amino-2-(hydroxymethyl)propane-1, 3-diol], (BDH Ltd) was used to maintain an optimal working pH 8.5 (1.2g Tris, 74mg EDTA in 100ml deionised water, adjusted to pH8.5 with 8M sodium hydroxide, stored at 4°C) and to this 0.25g oxalic acid (Sigma) was added to prevent interference from lactate. 3-Hydroxybutyrate (Sigma) calibration concentrations of 0.96, 2.4 and 7.2 mmol/l were used.

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

2.1.5 PLASMA FREE FATTY ACID QUANTITATION

Heparin stimulates lipoprotein lipase which releases free (non-esterified) fatty acids from triglycerides. Hence, blood samples collected in heparinised containers were not suitable for assay.

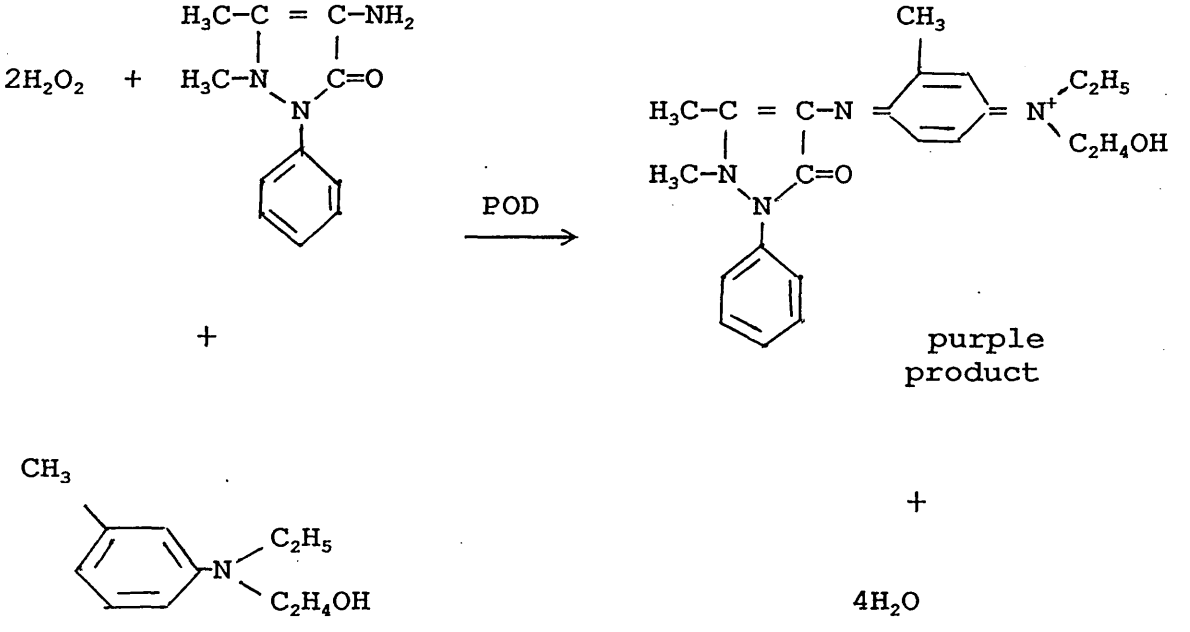
The method used the (+)Free fatty acids two shot kit marketed by WAKO Chemicals (kit no. 994-75409). Oleic acid, 1 mmol/l, was used as standard. In the presence of ATP, Mg^{2+}, CoA and acyl-CoA synthase (ACS) free fatty acids were converted to acyl-CoA.

$$\underset{\text{(FFA)}}{\text{RCOOH}} + \text{ATP} + \text{CoA} \xrightarrow{\text{ACS}} \text{Acyl-CoA} + \text{AMP} + PP_i$$

These thiol esters were then oxidised in the presence of acyl-CoA oxidase (ACO) producing hydrogen peroxide.

$$\text{Acyl-CoA} + O_2 \xrightarrow{\text{ACO}} \text{2,3-}\textit{trans}\text{-enoyl-CoA} + H_2O_2$$

Oxidative condensation of 3-methyl-N-ethyl-N-(3-hydroxyethyl)-aniline and 4-aminoantipyrine in the presence of hydrogen peroxide and peroxidase (POD) produced a purple colour complex with an absorption maximum of 550nm.



The density of this colour provided a direct indication of the original concentration of free fatty acid.

Interference from vitamin C 's antioxidant qualities and its ability to react with hydrogen peroxide is eliminated by the addition of ascorbate oxidase at the outset of the reaction.

2.2 CARNITINE QUANTITATION BY RADIO-ENZYMATIC ASSAY

2.2.1 OVERVIEW

This method was developed from those of Cederblad & Lindstedt[175] and Jalaludin Bhuiyan *et al*[176].

Free carnitine in urine or plasma was converted to [C^{14}] labelled acetylcarnitine by the addition of [C^{14}] labelled acetyl-CoA in the presence of carnitine-acetyl transferase (CAT). N-Ethylmaleimide was used to capture the acetyl-carnitine and prevent the reverse reaction thus avoiding falsely lowered results.

$$\text{Free carnitine} + [C14]\text{Acetyl-CoA} \xrightarrow{\text{CAT}} [C14]\text{Acetylcarnitine}$$

[C^{14}]Acetylcarnitine was then separated from unreacted [C^{14}]acetyl-CoA on a Dowex anion exchange resin column. Total carnitine content was measured as free carnitine after alkaline hydrolysis. The amount of esterified carnitine (long and short) was calculated as the difference between total and free concentrations.

2.2.2 PREPARATION

The assay was run in duplicate and a separate standard curve set up with concentrations of 0, 10, 20, 40μM carnitine (Sigma) every assay run. Initially, a 60μM concentration was also used, but as the intra assay variation became less this point was exclude.

The standards were subjected to the same conditions as the samples undergoing free carnitine quantitation. That is, not including the hydrolysis step. Similar standards subjected to the hydrolysis did not give any significantly different results. Therefore it was not thought necessary to run independent standard curves for free and acylated carnitine evaluation.

Dowex standard grade columns were prepared from Dowex 1-X8(Cl) particle size 0.075-0.150mm (100-200 mesh), BDH Ltd. This was purified and fined by mixing with an equal volume of 1M sodium hydroxide for approximately 1 hour and bringing back to neutrality by repeated washing with physiological saline. This slurry was then used immediately or stored, submersed in saline, at 4°C until required. Standard glass pasteur pipettes cut at their construction and plugged with silanised glass wool were filled to a depth of 4cm with the Dowex slurry and washed through with saline prior to use.

[C^{14}]Acetyl-CoA 100μM(0.2μCi/ml) was prepared from 10μCi [C^{14}]acetyl-CoA (Amersham CFA390) mixed with 2.64mg unlabelled acetyl-CoA (Sigma) made up to 50ml with deionised water and stored at -20°C in batch size aliquots.

The carnitine-acetyl transferase was stored undiluted at 4°C.

2.2.3 PROCEDURE

Hydrolysis of the sample (50µl) was carried out by incubation at 37°C for 1 hour with 1M potassium hydroxide (100µl), subsequently returning to neutrality with 1M hydrochloric acid (100µl).

To 20µl of 40mM N-ethylmaleimide (Sigma) and 50µl 1M Hepes buffer (Sigma) either 50µl of hydrolysed sample or 25µl of both neat sample (or standard) and deionised water was added.

100µl Of 100µM [C^{14}]acetyl-CoA was then added and the reaction initiated by the addition of 30µl dilute (1 part enzyme to 10 parts deionised water) carnitine-acetyl transferase. After incubation at 37°C for 1 hour the reaction mixture was passed onto the Dowex column and washed through with two lots of 0.75ml deionised water. Any unreacted [C^{14}]acetyl-CoA was retained. The eluent, containing [C^{14}]acetylcarnitine, was collected in miniature polyethylene reactavials (Cannberra Packard) and mixed with 10ml scintillation fluid (Packard Picofluor40 Universal liquid scintillation solution - aqueous capacity 5-6cm^3 aq in 10ml). The ß activity was then counted for two minutes on a scintillation counter (Miniaxi Tri-carb 4000 series) and used as an indication of the isotope content of the solution. A standard curve was set up to evaluate the carnitine content in the original sample.

2.2.4 ASSAY OF LONG AND SHORT CHAIN ACYLCARNITINES

Although not used during this assay, it is also possible to differentiate between long chain acylcarnitines (LCAC) or acid insoluble acylcarnitines and short chain acylcarnitines (SCAC) or acid soluble acylcarnitines[176,185,188]

The protein and LCAC are precipitated from the plasma sample by perchloric acid. The supernatant is then neutralised with potassium hydroxide and centrifuged to remove potassium chlorate. The difference between total and free carnitine results (measured by the same methods as already described) represents the SCAC content of the original sample. The pellet is hydrolysed by potassium hydroxide at 60°C for 1 hour to release all LCAC as free carnitine. The protein is then precipitated with further perchloric acid and the supernatant neutralised with potassium hydroxide and assayed as normal. The result is a measurement of the original LCAC in the plasma sample.

2.3 ORGANIC ACID ANALYSIS BY GAS CHROMATOGRAPHY / MASS SPECTROMETRY

This procedure involved two steps, an initial solvent extraction and trimethylsilyl derivatisation, followed by injection onto a gas chromatograph or gas chromatograph - mass spectrometer.

2.3.1 SOLVENT EXTRACTION AND DERIVATISATION

Either of three sample volumes were used depending on the creatinine level of the urine. These were 2.0, 1.0 or 0.5ml according to creatinine values of less than 0.5, 0.5 to 2.0 and greater than 2.0 mmol/l respectively. In the latter two cases the volume was made up to 2ml with deionised water. In the event of insufficient urine availability creatinine quantitation was omitted and the maximum volume possible was used in the extraction. In this case no water was added if the urine looked particularly dilute.

1 ml Of heptadecanoic acid (15mg to 100ml with water) internal standard was added to the urine solution which was acidified with 5N hydrochloric acid and extracted in the presence of sodium chloride with two 5ml aliquots of ethyl acetate and two 5ml diethyl ether. Water was removed from the pooled organic layer over anhydrous granular sodium sulphate (BDH Ltd.). After drying under nitrogen the extract was derivatised by redissolving in two drops pyridine and mixing with six drops bis-(trimethylsilyl)- trifluoroacetamide with 1% trimethylchlorosilane added (BSTFA) (Sigma). This was then left for at least 1 hour at room temperature. At least 0.5µl of derivasate was used for analysis.

2.3.2 ANALYSIS BY GAS CHROMATOGRAPHY

Initial analysis was by flame ionisation gas chromatography on a Philips PU4500 chromatograph fitted with an SGE bonded phase fused silica BP10 column (moderately polar OV1701 equivalent) 25m x 0.22mm (internal diameter) x 0.33mm (outer diameter) x 0.25µM (film thickness). Elution was carried out under a programmed temperature gradient of 80°C for 5 minutes followed by a rise of 8°C per minute to a final temperature of 260°C and held for a further 5 minutes. The injector temperature was set to 250°C and that of the detector to 300°C. A column pressure of approximately 5 PSI and a flow rate of 1ml/min through the column was established with helium carrier gas. An insert split ratio of 20:1 was used.

2.3.3 ANALYSIS BY GAS CHROMATOGRAPHY / MASS SPECTROMETRY

On the occurrence of abnormal spectra the sample was analysed by electron impact mode gas chromatography / mass spectrometry using a Hewlett Packard 5890 series II gas chromatograph fitted with a Hewlett Packard HP.1 crossed linked methyl silicone gum column 12m x 0.2mm(internal diameter) x 0.33µM (film thickness) with a Hewlett Packard 5971A mass selective detector. The temperatures and flow rates were identical to those used for the preliminary gas chromatography (section 2.3.2). A scan rate of 1 per second with an inter-scan delay of 0.03 seconds was used. The mass spectrum was viewed and investigated through Hewlett Packard G1030A MS Chemstation (DOS series) software in a Microsoft Windows3 environment installed on a Hewlitt Packard Vectra QS/165 personal computer.

2.4 PREPARATION OF ISOTOPE INTERNAL STANDARDS FOR THE QUANTITATION OF URINARY GLYCINE CONJUGATES BY GAS CHROMATOGRAPHY / MASS SPECTROMETRY

The initial intention was to synthesise deuterated suberylglycine (SG), hexanoylglycine (HG), and phenylpropionylglycine (PPG), not available commercially, and use these to quantitate any non-deuterated conjugates in urine of children (see section 1.11.2). In order to quantitate the amount of deuterated glycine conjugate in each internal standard (IS) it was necessary to synthesise non-deuterated forms of each compound also commercially unavailable. Unfortunately, time constraints prevented any full analysis of the synthesised IS. Further, although initial calibration results seemed acceptable (see section 3.5), difficulty was experienced in reproducing the data. The main problem seemed to lie with the sensitivity of the chromatography. Running the assay in splitless mode to concentrate the amount of sample passing onto the column did not alleviate the problem.

The procedures used were adaptations of those described by Kolvraa and Gregersen[30].

Choice of solvent combinations for extraction and recrystallisation procedures resulted from lengthy assessments of the effectiveness of a variety of different arrangements including types and volumes of solvents to be used and recrystallisation procedures. Analysis procedures to confirm the presence of the chloride derivatives and glycine conjugates were similar to those used for regular urinary organic acid analysis (see section 2.3.3) but with the mass spectrometer running in single ion monitoring mode. The ion fragments monitored for the acylglycines are shown in results table 3.21.

It was decided to aim for significant synthesis of deuterated glycine conjugates carrying 4 or more deuterium groups. In this way identical natural isotopic abundance in urine samples would be insignificant and similar ion fragments from quantitate and IS would be nicely separated by the chromatography (see section 1.11.2).

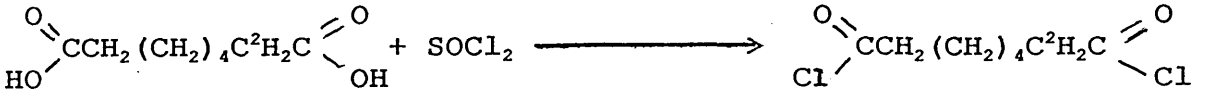
Resultant deuterated acylglycine product mixtures contain several isotopic compounds deuterated to various degrees (see results table 3.20.). In choosing the isotopic compound to be used as IS abundance was also taken into account. For each of the three conjugates the final isotopes chosen were those possessing four deuterium groups.

2.4.1 DEUTERATED SUBERYLGLYCINE INTERNAL STANDARD PREPARATION

2.4.1.1 Deuterated suberylchloride synthesis

Because of thionyl chloride's explosive reaction with water all apparatus was oven dried.

To 0.2g previously prepared (2-$^2H_2$)-suberic acid, 5ml thionyl chloride (stored at 4°C) was added drop wise. The mixture was refluxed for 1 hour, and left to cool. Excess thionyl chloride was removed under nitrogen leaving a dark yellow oil (approximately 100µl). Presence of a number of deuterated suberylchloride isotopes was detected by GC/MS, the most abundant being (2-$^2H_2$)-suberyl-dichloride.



(2-$^2H_2$)-suberic acid | thionyl chloride | (2-$^2H_2$)-suberyl--dichloride

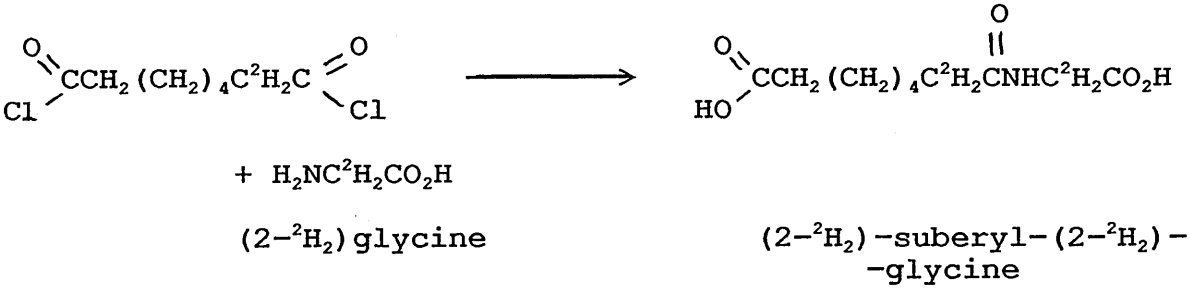
2.4.1.2 Deuterated glycine solution preparation

A solution of $^2H_2$-glycine was prepared at 4°C by dissolving 500mg $^2H_5$-glycine (Sigma) in 5ml 1N sodium hydroxide.

2.4.1.3 Deuterated suberylglycine synthesis

At room temperature the suberate / suberyl chloride reflux mixture was added to the deuterated glycine / sodium hydroxide solution and mixed for 1½ hours over vortex. After centrifugation at 3000 revs/min for 5 minutes any precipitate was removed.

The supernatant was then acidified to pH1 by 6N hydrochloric acid, and extracted into three volumes of 30ml ethylacetate and three volumes of 30ml diethyl ether. The pooled organic layer was dried over sodium sulphate and evaporated to dryness under nitrogen. The resultant solid contained (2-$^2H_2$)-suberyl-(2-$^2H_2$)-glycine.



2.4.1.4 Calibration solution synthesis

A similar procedure to that used for deuterated SG synthesis was used for the production of the non-deuterated conjugate.

0.5ml Suberoyldichloride (Aldrich Chemical Co. Ltd.) was added to previously prepared glycine solution (500mg glycine, Sigma, to 5ml sodium hydroxide). After mixing by vortex for 1½ hours the mixture was centrifuged and the supernatant extracted into three 30ml volumes of each ethylacetate and diethyl ether. Evaporation left a white residue containing both suberate and SG.

2.4.1.5 Calibration solution purification

It was only possible to purify the mixture to a 1:4 ratio of suberate to SG by extraction with hexane and petroleum ether. Much more success was achieved through recrystallisation from an ethylacetate, hexane, petroleum ether mixture.

The suberate / SG powder was dissolved in a minimal volume of ethylacetate hexane and petroleum ether (approximately 1:1:1). This solution was then evaporated to approximately 50% volume in a water bath and left overnight at 4°C. White granular crystals separated out of solution. GC/MS confirmed these to be SG with the absence of any detectable suberate.

2.4.2 DEUTERATED HEXANOYLGLYCINE INTERNAL STANDARD PREPARATION

2.4.2.1 Deuteration of hexanoic acid

2ml Hexanoic acid (1.84g) was added to 2ml sodium deuteroxide. The salt that formed was then dissolved in 4ml deuterium oxide (^2H_2O). The solution was placed in a 20ml tissue digestion vessel with teflon liner (Northern Media) and left at 140°C for 5 days. The resultant solid was acidified with concentrated hydrochloric acid, to convert from the sodium salt to the acid, and extracted into three volumes of 30ml ethylacetate and three volumes 30 ml diethyl ether. After evaporation of the organic layer under nitrogen the presence of (2-$^2H_2$)-hexanoic acid was confirmed by GC/MS.

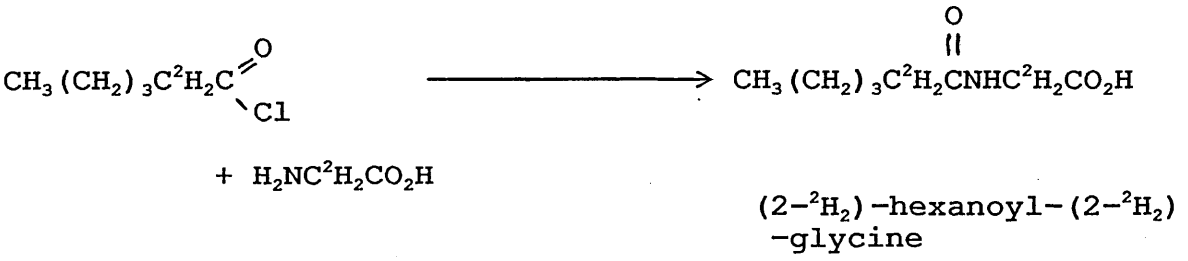
2.4.2.2 Deuterated hexanoylchloride synthesis

The process was a repeat of the thionyl chloride reflux used to produce deuterated suberyl chloride. After reflux the apparatus containing the reaction mixture was left overnight under anhydrous conditions before removal of excess thionyl chloride under nitrogen. GC/MS confirmed the presence of a number of deuterated hexanoylchloride isotopic compounds. The most abundant being (2-$^2H_2$)-hexanoylchloride.

(2-$^2H_2$)-hexanoic acid (2-$^2H_2$)-hexanoylchloride

2.4.2.3 Deuterated hexanoylglycine synthesis

The mixture containing (2-$^2H_2$)-hexanoyl chloride was added to 5ml 1M sodium hydroxide. This solution was then mixed by vortex with deuterated glycine / sodium hydroxide solution. The mixture was left overnight at room temperature, centrifuged and the supernatant rotary-evaporated to a powder.



The powder was then acidified with 1N hydrochloric acid, extracted into three 30ml volumes of each ethylacetate and diethyl ether, dried over sodium sulphate, evaporated to dryness and stored at -20°C. The presence of (2-$^2H_2$)-hexanoyl (2-$^2H_2$)-glycine was confirmed by GC/MS.

2.4.2.4 Calibration solution

Previously prepared and purified HG was used.

2.4.3 DEUTERATED PHENYLPROPIONYLGLYCINE INTERNAL STANDARD PREPARATION

2.4.3.1 Deuteration of phenylpropionic acid

The method incorporated for the deuteration of hexanoic acid was used replacing the hexanoic acid with 0.5g of phenylpropionic acid. On cooling a white solid formed. This was acidified and extracted into three 30ml volumes of each ethylacetate and diethyl ether, and evaporated to a white solid. The presence of (2′-$^2H_2$)-phenylpropionic acid was confirmed by GC/MS.

2.4.3.2 Deuterated phenylpropionylchloride synthesis

Again a similar method as for deuterated suberyl chloride was used, with the (2′-$^2H_2$)-phenylpropionic acid and thionyl chloride, to produce a white powder containing (2′-$^2H_2$)-phenylpropionyl chloride.

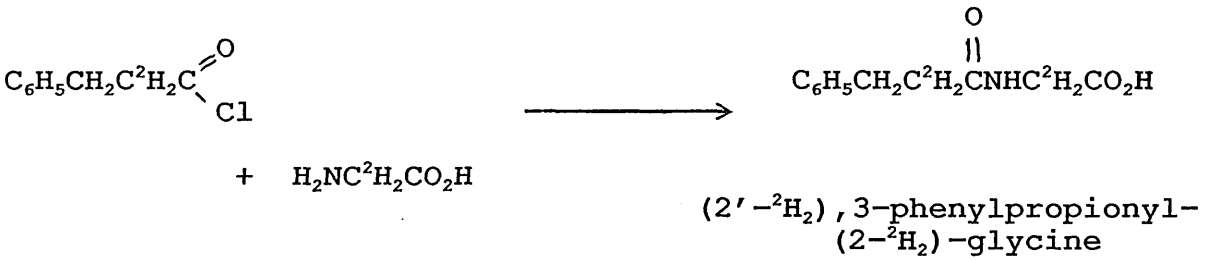
(2′-$^2H_2$)-phenyl-propionic acid

(2′-$^2H_2$)-phenyl--propionyl chloride

2.4.3.3 Deuterated phenylpropionylglycine synthesis

The powder was dissolved in 6-7ml 1N sodium hydroxide and added to previously prepared deuterated glycine / sodium hydroxide solution and mixed for 1½ hours over vortex. The solution was then acidified with hydrochloric acid,

extracted into three volumes of 30ml ethylacetate and three volumes of 30ml diethyl ether, dried over sodium sulphate and evaporated to dryness. The presence of (2'-$^2H_2$),3-phenylpropionyl-(2-$^2H_2$)-glycine was confirmed by GC/MS.



2.4.3.4 Calibration solution synthesis

1ml Commercial phenylpropionyl chloride (Hydrocinnamoyl chloride 98%, Aldrich Chemical Co. Ltd.) was added to 1g glycine in 7ml sodium hydroxide and mixed for 1½ hours by vortex. The whole reactant mixture (no supernatant was obtainable) was acidified to pH1 with 1N hydrochloric acid and extracted into three volumes of 15ml ethylacetate and three volumes of 15ml diethyl ether. The extract, containing PPG, was evaporated to an oil and stored at 4°C.

2.4.3.5 Calibration solution purification

The glycine conjugate / carboxylic acid mixture was dissolved in hexane and ethylacetate (5-10ml) and evaporated to 50% volume. Storing at 4°C for 2 hours resulted in the separation of white crystals subsequently shown to be PPG with the absence of any detectable phenylpropionic acid.

2.4.4 CALIBRATION OF DEUTERATED CONJUGATES

2.4.4.1 Initial concentration approximations

Approximately half of each deuterated HG and PPG and all of the SG (see section 3.5.2.1) were dissolved in 100ml volumes of methanol. For each non-deuterated conjugate approximately 2.5mg (the exact weight was recorded) was dissolved in a 100 ml volume of methanol. The concentration of each solution was calculated (see section 3.5.2).

1ml Of each non-deuterated standard and 100µl of each deuterated solution were mixed, acidified with 1N hydrochloric acid and extracted in the presence of sodium chloride into two 5ml volumes of ethylacetate and two 5ml volumes of diethyl ether. The organic layer was dried over sodium sulphate and evaporated to dryness under nitrogen. The solid was TMS derivatised with 2 drops of pyridine and 8 drops of BSTFA, as used for standard organic acid analysis (see section 2.3.1). The derivasate was analyzed by GC/MS under operating conditions previously described (see section 2.3.3).

Selective ion monitoring was used to detect ion fragments for each of the TMS derivatives TMS1 and TMS2 for the deuterated and non-deuterated conjugates (see section 3.5.2). A dwell time of 0.08 seconds with an inter-scan delay of 0.02 seconds was used. The abundances of these ion fragments, as indicated by the peak areas, were calculated by integration. For each compound the areas from both derivatives (TMS1 and TMS2) were summed and the ratio of deuterated to non-deuterated conjugate areas evaluated. These values were multiplied by the known concentration of non-deuterated compound to calculate that of the deuterated conjugate. This was then extrapolated back, accounting for the 1:10 mix of deuterated to non-deuterated conjugate, to determine the concentration of the original stock (see section 3.5.2.1).

The volume of stock solution required in 100ml with methanol to provide the

required working concentration (see section 3.5.2.1) was then calculated for deuterated HG and PPG. For deuterated SG the working concentration obtained by dissolving the total yield in 100ml of methanol was calculated (see section 3.5.2.1).

It was intended that once calibrated equal volumes of the three deuterated conjugates would be mixed and a 100µl aliquot of this cocktail added to 1ml of the urine sample to be extracted. Therefore, it was necessary to account for a dilution factor of 30 fold when extrapolating from working to stock concentrations.

2.4.4.2 Accurate calibrations

A cocktail of equal volumes of the three deuterated conjugates was mixed and 100µl aliquots of this was used in five assays with varying concentrations of non-deuterated standards. The conditions for these assays were identical to those of section 2.4.4.1 and each was run in quadruplicate. Calibration curves were drawn for deuterated to non-deuterated conjugate peak area ratios (see section 3.5.3).

2.5 INTERMEDIARY METABOLITES INVESTIGATION

2.5.1 AIMS OF THE INVESTIGATION

To determine the quality of any correlations between fasting values of various metabolites involved in fatty acid oxidation. The efficacy of such correlations as diagnostic tools for errors in fatty acid oxidation was then investigated by the inclusion of data from patients with known or suspected defects. The effect of length of fast on the metabolite levels was also examined by means of correlation plotting. Finally, the use of these fasting profiles in conjunction with the paired metabolite correlations for fatty acid oxidation defect elucidation was discussed.

2.5.2 SAMPLE COLLECTION, STORAGE AND ANALYSES

Approximately 5ml venous samples were collected by venepuncture from children either before feeding and admitted to the Sheffield Childrens Hospital with febrile illness or having been fasted overnight (4 hours minimum) either to await surgical procedure or in order to investigate suspected metabolic defect. The metabolic stress required for the investigation was a result of either the febrile illness or the fast. The specimens were separated and the plasma stored at -4°C. The following analyses were performed;

Glucose

Lactate

3-Hydroxybutyrate

Free fatty acids

Total, free and esterified carnitine.

2.5.3 CHOICE OF SAMPLE

Any deviations from normal intermediary metabolite levels caused by errors in fatty acid metabolism would be expected to increase with length of fast as demand on the ß-oxidation pathway rises. It was therefore decided where a series of samples were taken from a patient as the fast progressed the last collected was to be used for the following investigation. In this way length of fast would be maximised within the constraints of the study and any deviations from normality would be emphasised. It was hoped that any abnormal trends as a result of defective fatty acid metabolism would therefore be clearer and more defined.

2.5.4 PAIRED METABOLITE CORRELATIONS

Correlations between pairs of metabolites were investigated using patients with no diagnosed or suspected metabolic defect (ie. a zero value for the variable DIAGNOSE - see appendix 2). The efficacy of the use of these plots as indicators in the diagnosis of fatty acid metabolic defects was then investigated. To do this data from a number of patients having diagnosed metabolic defects were incorporated into the plots and their positions in relation to the non diagnosed points discussed. In addition data from several children suspected of having a fatty acid metabolic defect were incorporated into the study to provide examples of the use of paired metabolite correlation to indicate more precisely the site of such defects.

2.5.5 RESPONSE TO FAST

The response of these metabolites to fasting was then studied. An evaluation of the usefulness of such fasting profiles in conjunction with paired metabolite correlations as indicators of fatty acid metabolic defect was severely limited by a difficulty in obtaining exact fasting times (see Discussion).

2.6 GENERALISED DICARBOXYLIC ACIDURIA INVESTIGATION

2.6.1 AIMS OF THE INVESTIGATION

This investigation formed part of a larger study to evaluate the use of GC and GC/MS urine organic acid analysis in the neonatal diagnosis of errors in fatty acid oxidation.

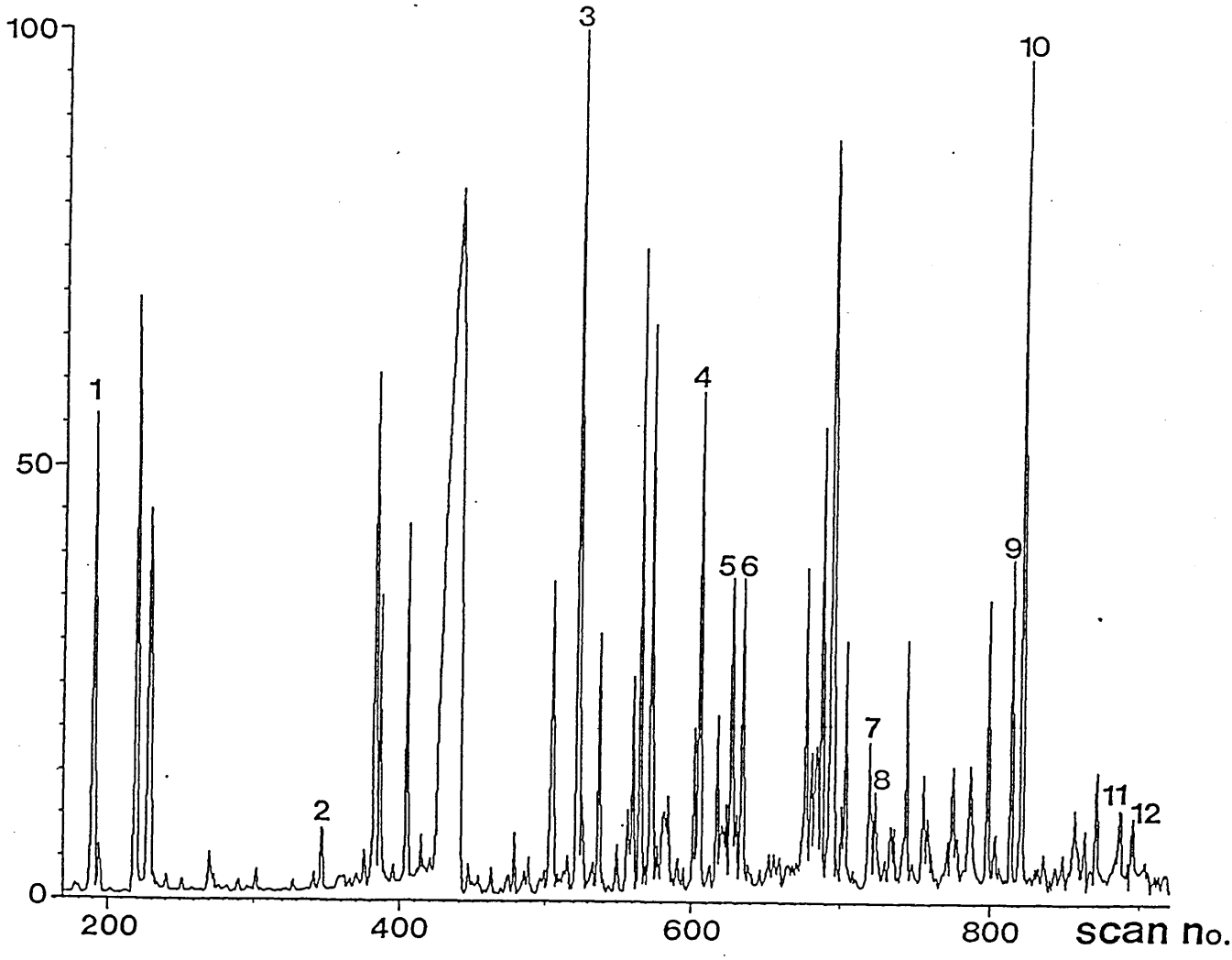


Fig.2.01 GC/MS trace showing generalised DCA in a one day old child. The peaks are: 1, 3-hydroxybutyric acid; 2, ethylmalonic acid; 3, adipic acid; 4, lactone of 3-hydroxy adipic acid; 5, octenedioic acid; 6, suberic acid; 7, 3-hydroxyoctenedioic acid; 8, 3-hydroxysuberic acid; 9, 3-hydroxysebacic acid; 10, internal standard; 11, 3-hydroxdodecenedioic acid;12 3-hydroxydodecanedioic acid.[177]

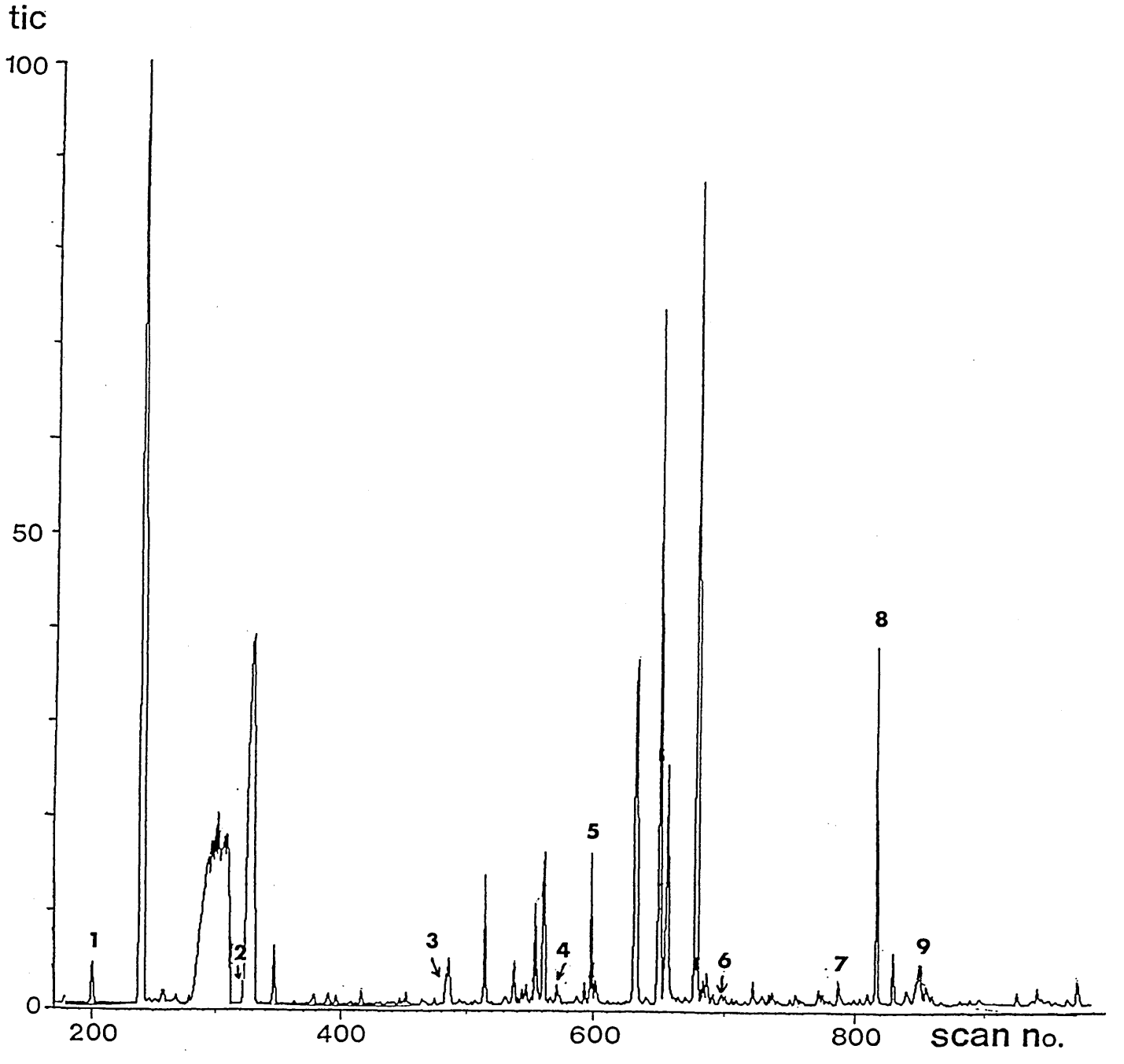


Fig.2.02 GC/MS trace showing classic urine metabolite trace in a child presenting with MCAD deficiency. The peaks are: 1, 3-hydroxybutyric acid; 2, ethylmalonic acid; 3, adipic acid; 4, hexanoyl glycine; 5, suberic acid; 6, sebacic acid; 7, 3-hydroxysebacic acid; 8, internal standard; 9, suberyl glycine.[177]

During the course of this larger study a urinary organic acid pattern containing low levels of ethylmalonic, adipic, suberic and octenedioic acids as well as 3-hydroxy-adipic, -suberic and -sebacic acids (see figure 2.01) was often seen[177] in both siblings of SIDS patients and control urines. This pattern, whilst being quantitatively much lower, was similar to that considered to be a diagnostic finding in crisis samples from patients with fatty acid oxidation defects such as MCAD deficiency (see figure 2.02.). The cause of this finding, referred to as generalised neonatal dicarboxylic aciduria (generalised DCA), is investigated further in this thesis as detailed below.

2.6.2 SAMPLE COLLECTION, STORAGE AND ANALYSES

Random urine samples were collected onto cotton wool balls during the first five days of life, preferably days 1, 3 and 5, from siblings of sudden infant death syndrome patients. Matched control urine samples were also collected from babies born on the same day in the same unit.

The urine was stored at -20°C as soon as possible, within 24 hours of collection, until required for investigation. The analyses performed were organic acid analysis by either gas chromatography or gas chromatography mass spectrometry and total, free and esterified carnitine quantitations.

2.6.3 CHOICE OF SAMPLE

Where samples were taken on successive days of life the results obtained from the earliest sample were used. In this way the period of greatest metabolic stress, ie that closest to the time of birth, was analysed. Also, it has been shown that the occurrence of generalised DCA is most prominent on the first day of life[177].

2.6.4 GENERALISED DICARBOXYLIC ACIDURIA RELATIONSHIPS

The relationship between generalised dicarboxylic aciduria and a number of overlying factors was investigated. These factors were; sibling history of sudden infant death syndrome, gender, whether the baby was bottle or breast fed and the number of days elapsed between birth and sample collection.

2.6.5 CARNITINE EVALUATIONS

Measurements of carnitine values (total, free, esterified) for all patients allowed reference range calculations for urinary carnitine levels in newborns, and an investigation of their reliance on the above mentioned factors. Separate reference ranges were calculated for those subjects exhibiting, not exhibiting, or regardless of generalised DCA.

Variations in urine concentrations were corrected for by dividing by the level of creatinine.

Because of a skewed distribution, reference ranges were calculated as the 2.5th to 97.5th inter percentile range. Appendix 4 shows normal ranges (mean ± 2 SD) calculated based on normal distributions of raw data. The differences between these and the inter-percentile ranges indicate the amount of skew in the sample distributions. Also included, for reference, are normal ranges calculated from logarithms of the total and free carnitine values and inverse of the logarithm of the percent esterified values. Some of the distributions still showed slight skew after logarithmic conversion and therefore the percentile ranges were used in preference.

2.6.6 STATISTICAL ANALYSIS

2.6.6.1 Two Tailed Fisher's Exact Method

This test was used to test the null hypotheses that there is no association between the presence of generalised dicarboxylic aciduria and;

1. sibling history of sudden infant death syndrome,
2. method of feed - breast or artificial, and
3. gender.

The p value for each frequency distribution is calculated as;

$$p = \frac{r_1!\ r_2!\ s_1!\ s_2!}{N!\ \ a!\ b!\ c!\ d!}$$

where r_1 = The frequency of generalised DCA.
r_2 = The frequency of no generalised DCA.
s_1 = The frequency of one of the values for the possibly associated factor (one of the three listed above).
s_2 = The frequency of the alternative value for the possibly associated factor.
N = Total sample size for the test.
a,b,c,d = The frequencies of the four possible combinations of the groups used for r_1, r_2, s_1 and s_2.

The two tailed Fisher's probability represents the probability of obtaining the observed or a more extreme distribution given that there is no association between the presence of generalised DCA and the three possibly associated factors being tested. This is achieved by calculating the probabilities for all possible distributions that would produce equivalent r_1, r_2, s_1 and s_2 values to those observed. All p values less than or equal to that of the observed distribution are then summed. The result is the two tailed Fisher's probability. Alternatively the more widely known chi-squared test could have been used. However, this is only an approximation to Fisher's exact test and should not be used with expected frequencies of less than five as was the case here.

2.6.6.2 The Wilcoxon Rank Sum Test

The Wilcoxon rank sum test was used to distinguish any significant differences between the medians of total, free and esterified carnitine data in relation to gender, feed (artificial or breast) presence of generalised dicarboxylic aciduria and whether the patient was a sibling of a sudden infant death syndrome victim.

Significance testing was carried out on the null hypothesis that the medians of each sample were equal. Calculation of the level of significance of this hypothesis was based on the formula;

$$\phi(Z) = \int_{-\infty}^{z} \frac{1}{\sqrt{2\pi}} e^{-\frac{1}{2}t^2} dt$$

where the Wilcoxon rank sum statistic Z was calculated as;

$$= \frac{\text{(smallest sum of ranks) - (expected sum of ranks)}}{\text{(standard deviation of ranks)}}$$

2.7 MISCELLANEOUS

2.7.1 DATA ANALYSIS COMPUTER SOFTWARE

Data manipulation was aided by use of Statistical Analysis System version 6.03 software (SAS Institute Inc. USA), run on various IBM compatible personal computers.

CHAPTER 3

RESULTS

3.1 ROUTINE LABORATORY ASSAY QUALITY CONTROL DATA

When measuring plasma free fatty acid, lactate, 3-hydroxybutyrate and glucose for the intermediary metabolites investigation samples were incorporated into routine laboratory batches. Table 3.01 shows coefficients of variation (CV%) and a description of the two quality control materials (QCI and QCII) used in theses batches.

Table. 3.01. Quality control results for routine intermediate metabolite assays.

Assay	Year	CV%	QCI	QCII
Free fatty acids	1988	4.2	Nycomed lo bovine serum	Ciba Corning bovine 3
	1989	4.4		
	1990	4.0		
Lactate	1988	5.2	Sigma metabolite control	Sigma metabolite control
	1989	4.9		
	1990	3.6		
3-Hydroxy-butyrate	1988	9.7	Sigma metabolite control	Sigma metabolite control
	1989	9.6		
	1990	9.0		

3.2 INTERMEDIARY METABOLITES INVESTIGATION

Exact fasting times were not recorded in many cases. Therefore it would be unwise to attempt any reference range evaluations on such a non homogeneous population. Never the less summary statistics for data collected are very comparable to those quoted by JP Bonnefont[178], JI Wolfsdorf[179] and KJB Lamers[180]. These workers quoted exact fasting times between 15 and 24 hours and it was seen that the change in metabolite levels within this range was small.

Table 3.02 shows sample size, median, minimum and maximum for the intermediary metabolites from patients with no diagnosed disorder of fatty acid metabolism (ie variable DIAGNOSE = 0, see appendix table A2.2).

Table 3.02. Basic statistical analysis.

	Glucose (mmol/l)	Lactate (mmol/l)	Free fatty acids (mmol/l)	Total Carnitine (µmol/l)
Sample size	106	113	113	31
Median	4.4	1.4	0.9	35.0
Minimum	1.0	0.6	0.1	15.0
Maximum	10.4	6.2	3.3	88.0

All values were taken from plasma samples

Table 3.02. continued

	Free Carnitine (µmol/l)	Esterified Carnitine (%)	3-Hydroxy-butyrate (mmol/l)	FFA / 3 -HB
Sample size	32	31	112	109
Median	23.5	35.4	0.7	1.3
Minimum	10.0	0	0	∞
Maximum	45.0	73.3	4.6	16.5

All values were taken from plasma samples

3.2.1 PAIRED METABOLITE CORRELATION PLOTS

The following plots and statistics show the correlation between plasma intermediary metabolite levels for patients having no diagnosed irregularity in fatty acid metabolism. Superimposed on these are data from infants with various errors of fatty acid metabolism. All points for the non-diagnosed patients are represented by a cross whilst those for the diagnosed children are shown by the value of the variable DIAGNOSE (see appendix table A2.2).

All lactate plots (figures 3.01. and 3.07 to 3.11.) showed poor correlations. This was probably an indication of the weak link between lactate levels and the other intermediary metabolite levels due to factors extraneous to fatty acid oxidation (see section 4.1.1.4).

All plots against total carnitine (figures 3.03., 3.08., 3.12. and 3.16.) showed poor correlation, so too did the plot of free carnitine against glucose (figure 3.04.). Infact, all plots between the three carnitine values (total, free and esterified) were thought to be of little use in the diagnosis of errors in fat metabolism and were not included in these results. Similarly, plots against FFA/3HB were also excluded on the basis that any note worthy points would also be exhibited in the plots of the single metabolites.

A very good correlation was seen between 3HB and %CE (figure 3.18., cc = 0.8). Further, all of the plots against %CE (excluding those involving total and free carnitine and lactate) possessed correlation coefficients with modulus values above 0.6 (figures 3.05 and 3.14 and table 3.03). %CE values were chosen in preference to straight forward acylcarnitine values during the early stages of this study as it became apparent that stronger correlations would be exhibited using the percentage values.

The remaining plots all showed recognisable correlations with correlation coefficient modulus values above 0.4.

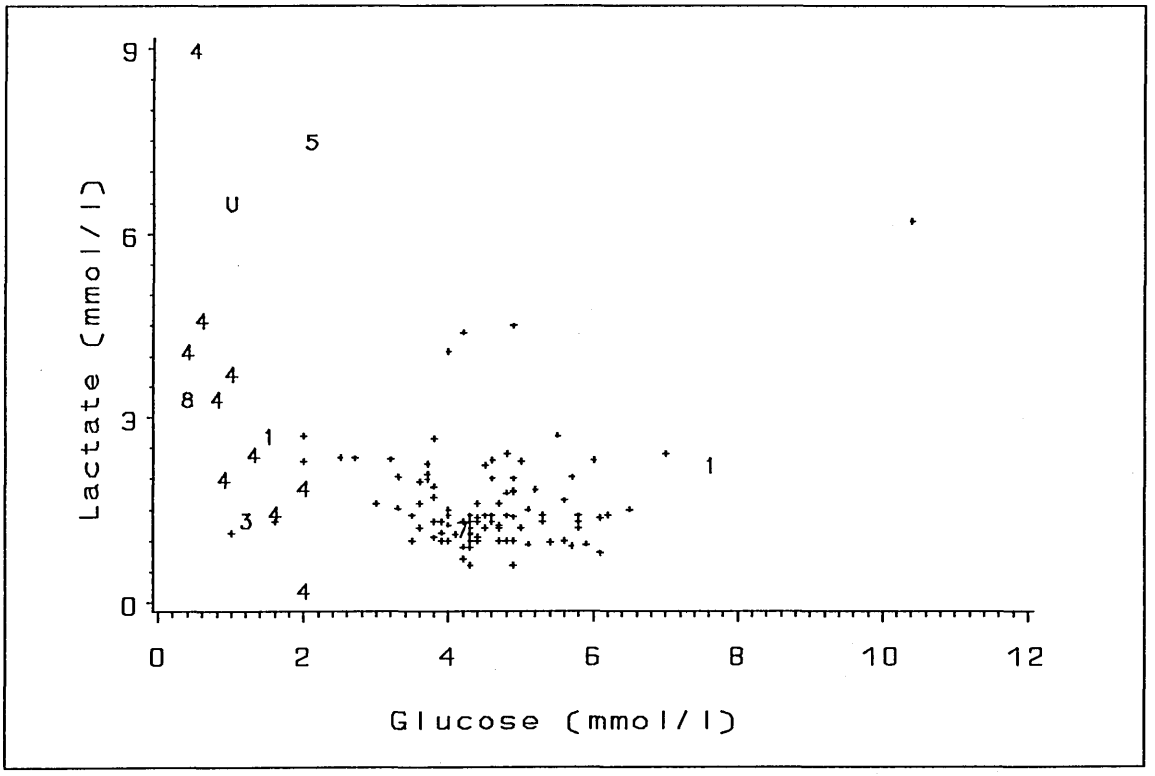


Fig.3.01. Plot of lactate against glucose.

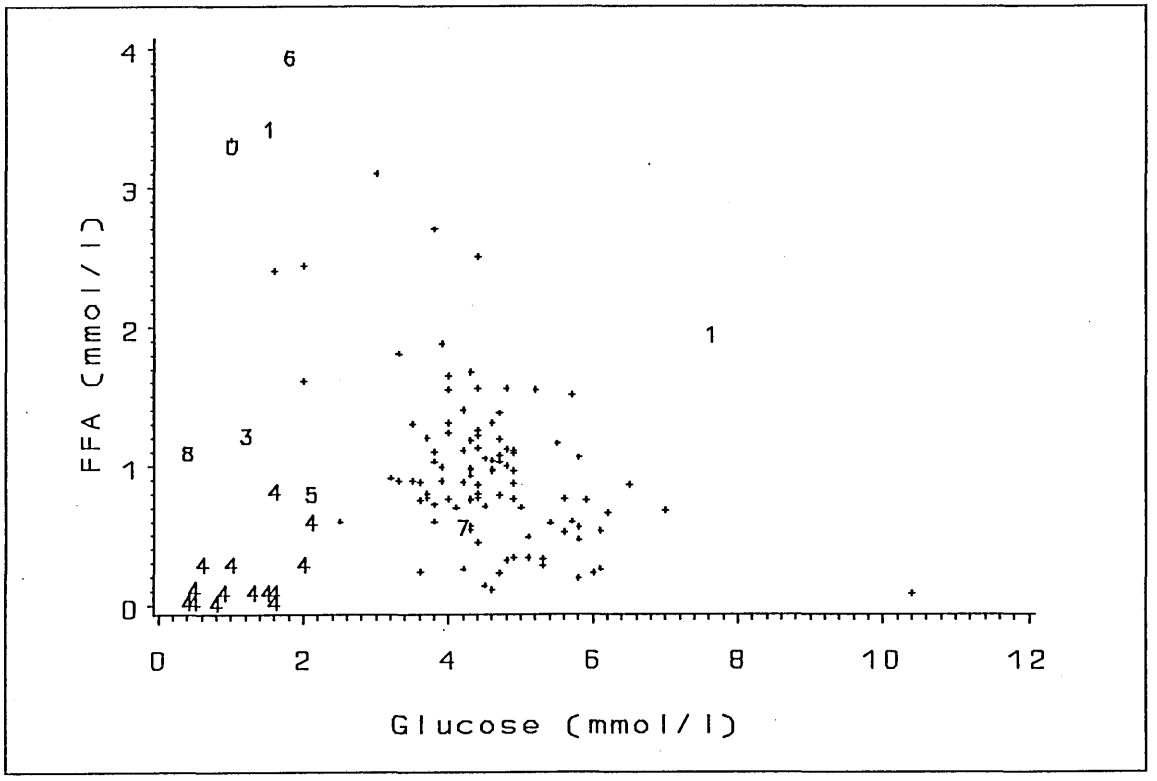


Fig.3.02. Plot of free fatty acids against glucose.

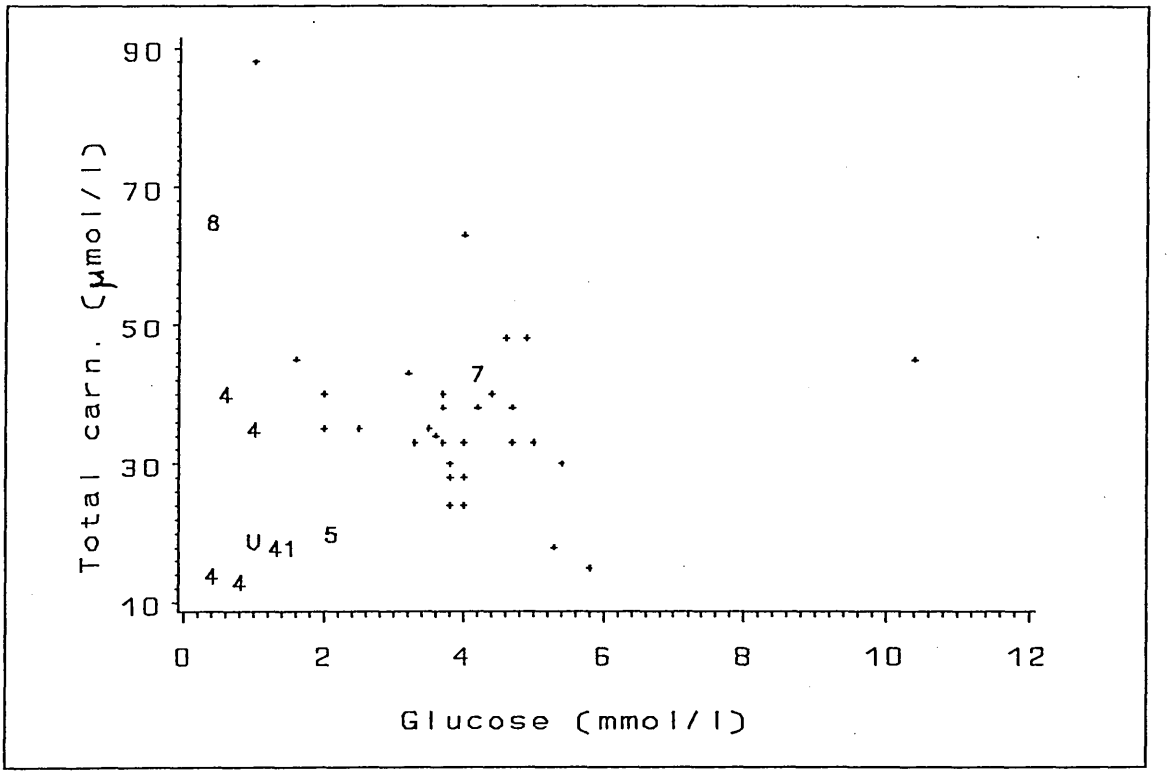


Fig.3.03. Plot of total carnitine against glucose.

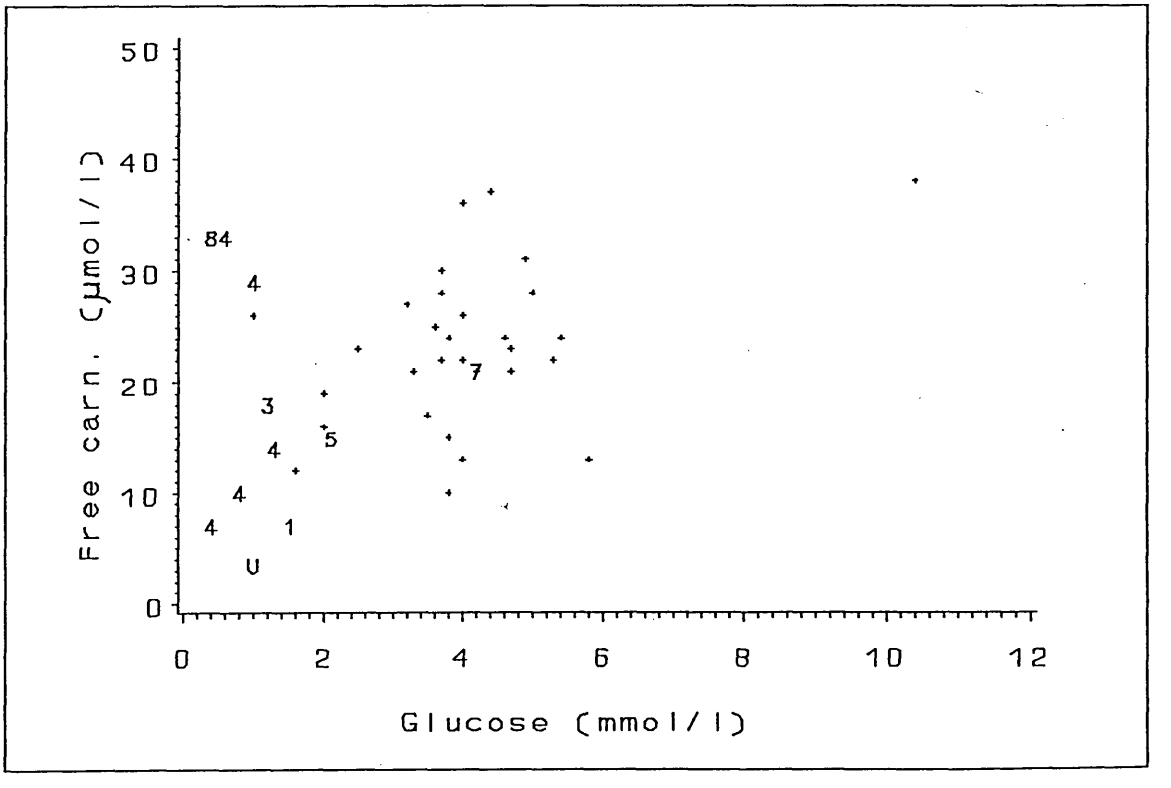


Fig.3.04. Plot of free carnitine against glucose.

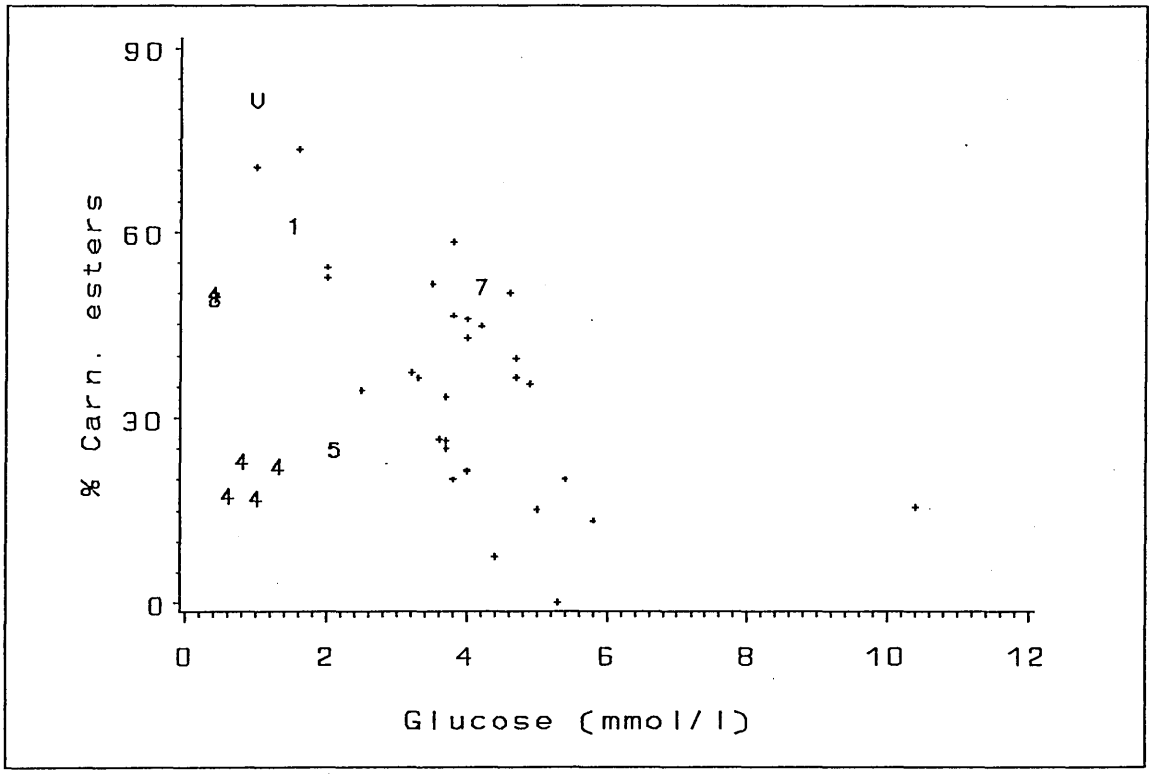


Fig.3.05. Plot of % esterified carnitine against glucose.

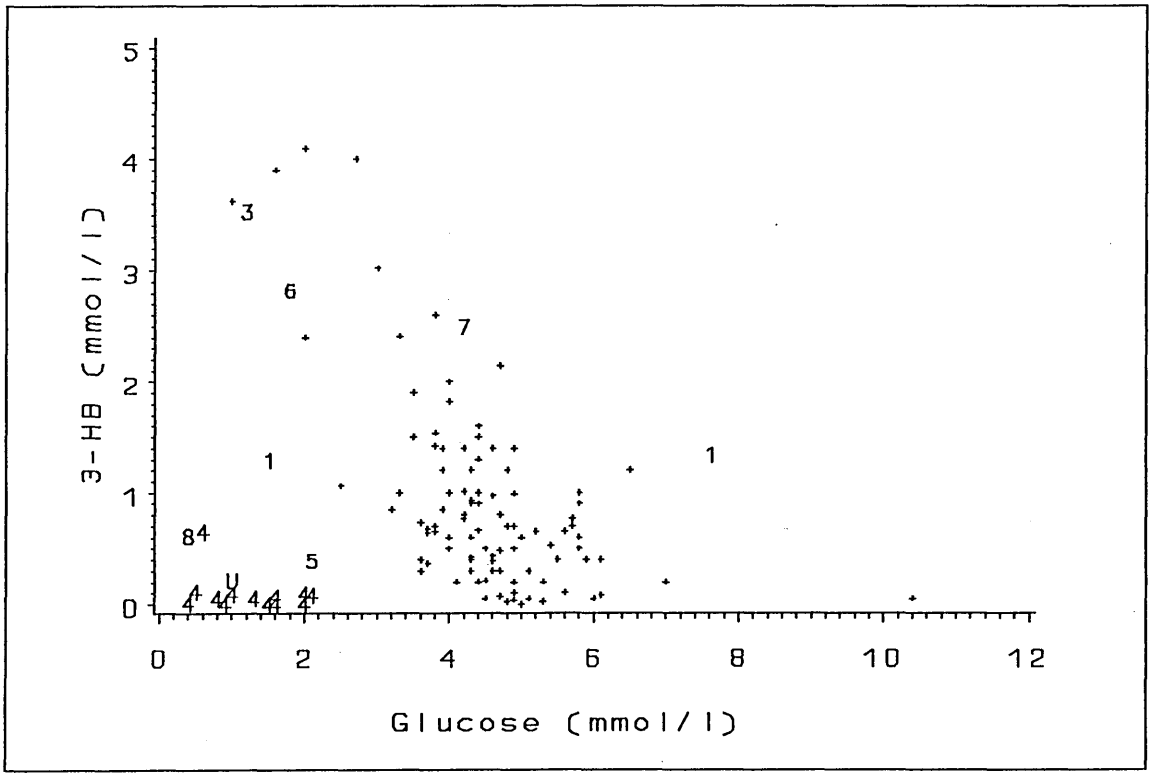


Fig.3.06. Plot of 3-hydroxybutyrate against glucose.

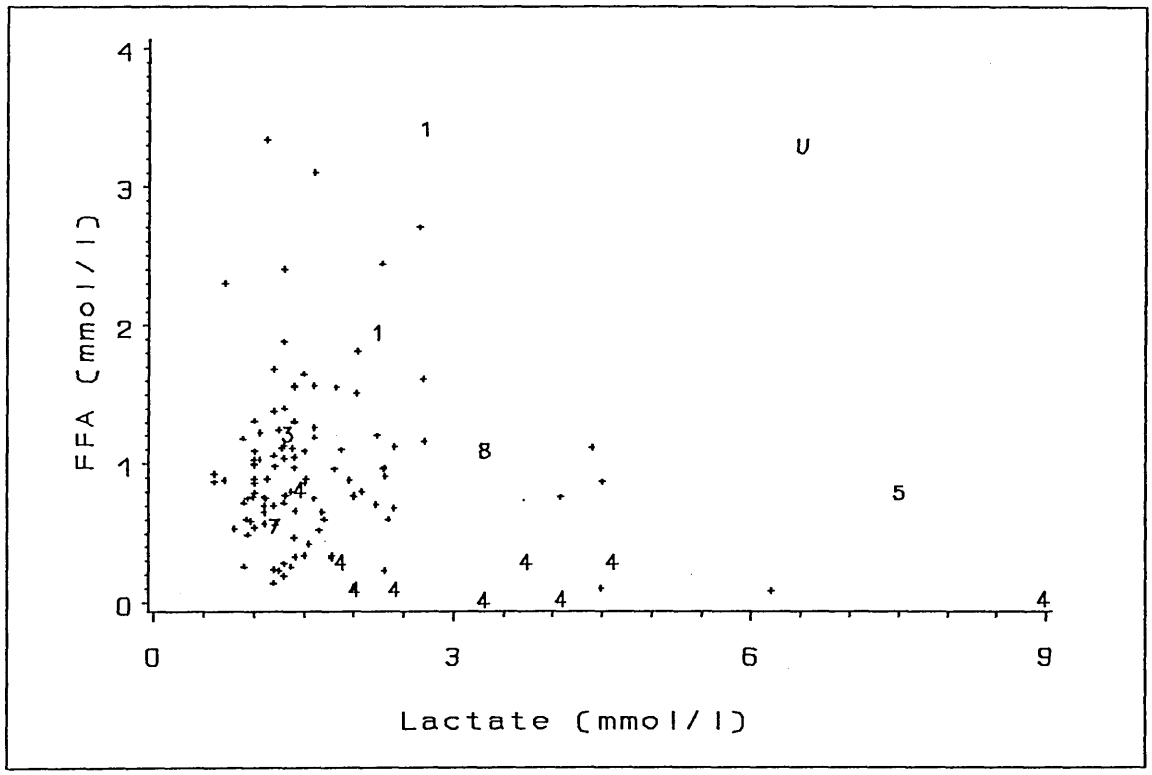


Fig.3.07. Plot of free fatty acids against lactate.

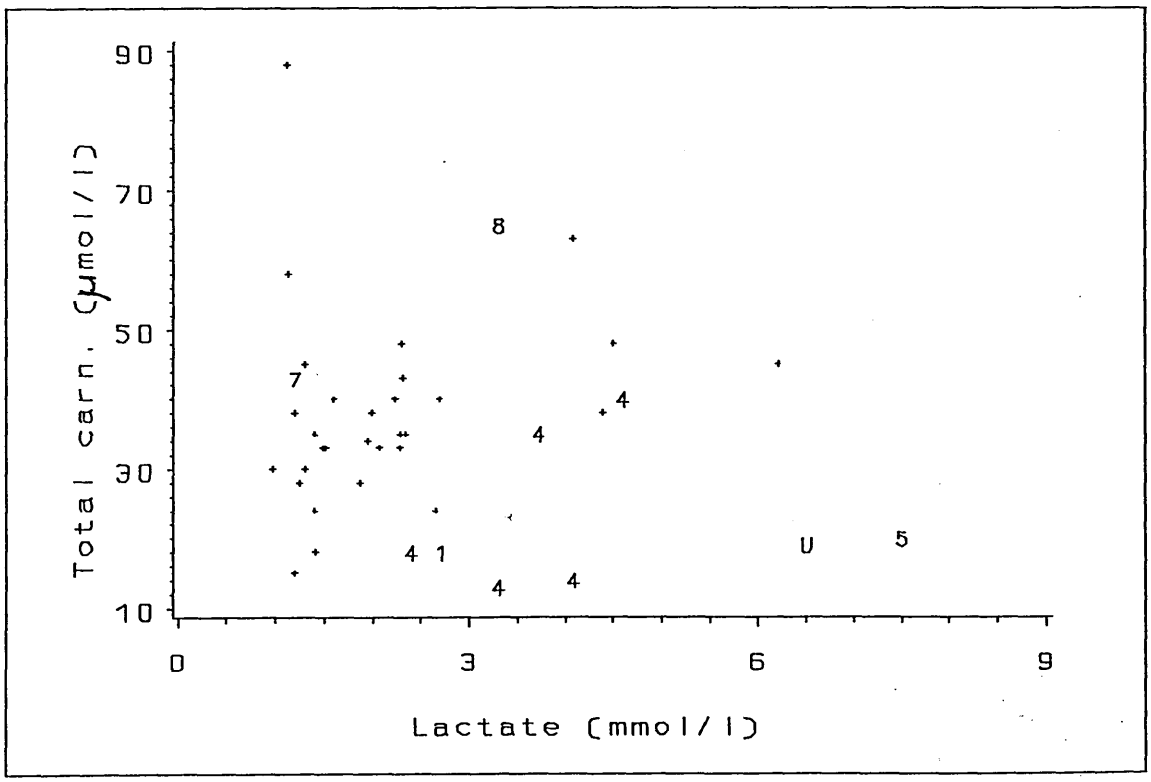


Fig.3.08. Plot of total carnitine against lactate.

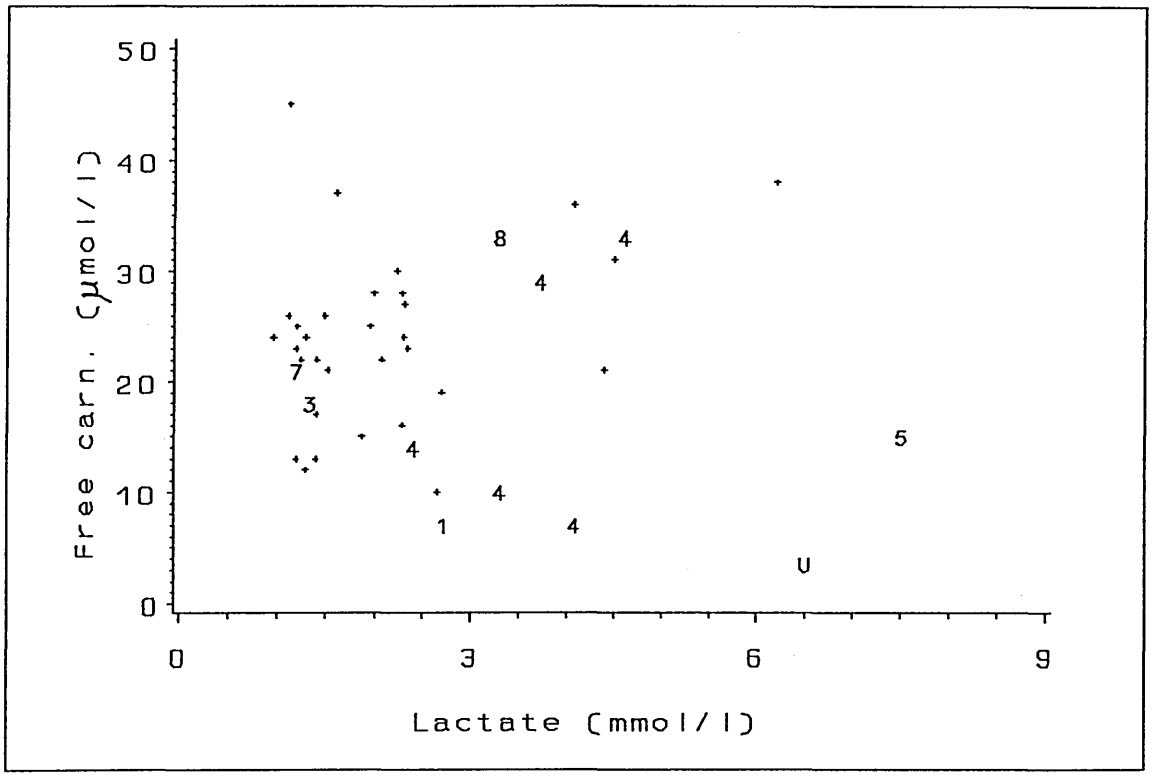


Fig.3.09. Plot of free carnitine against lactate.

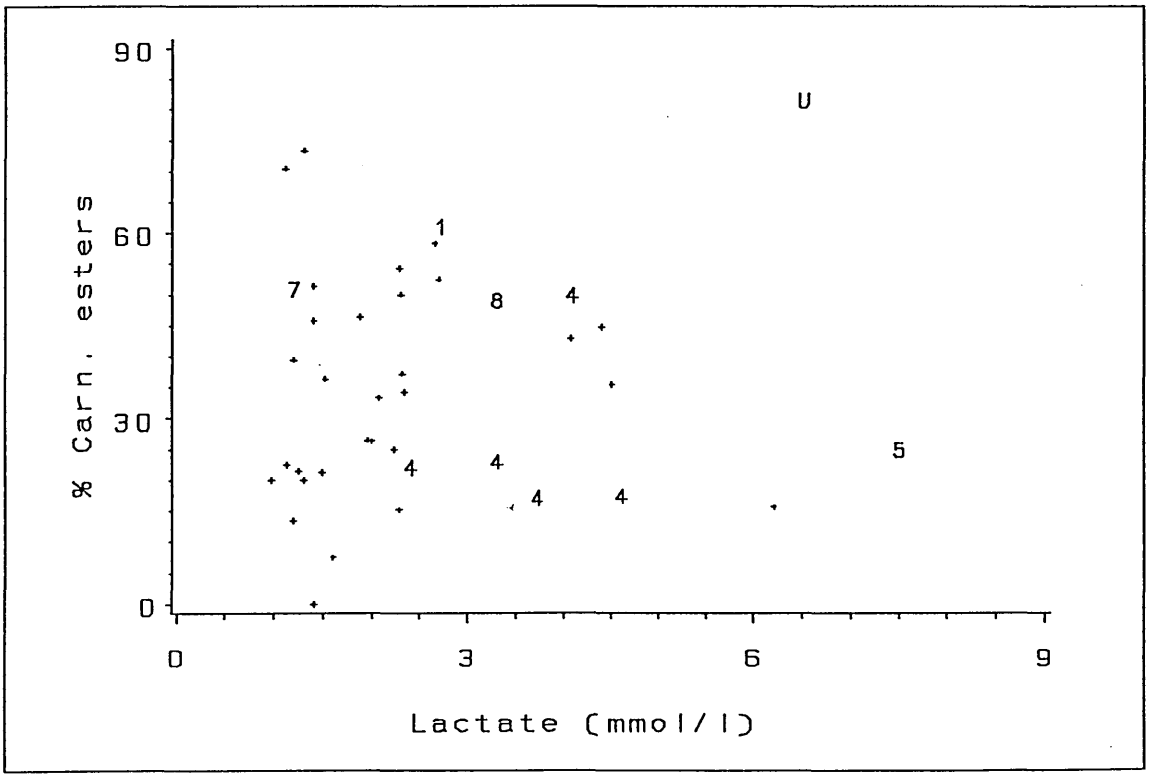


Fig.3.10. Plot of % esterified carnitine against lactate.

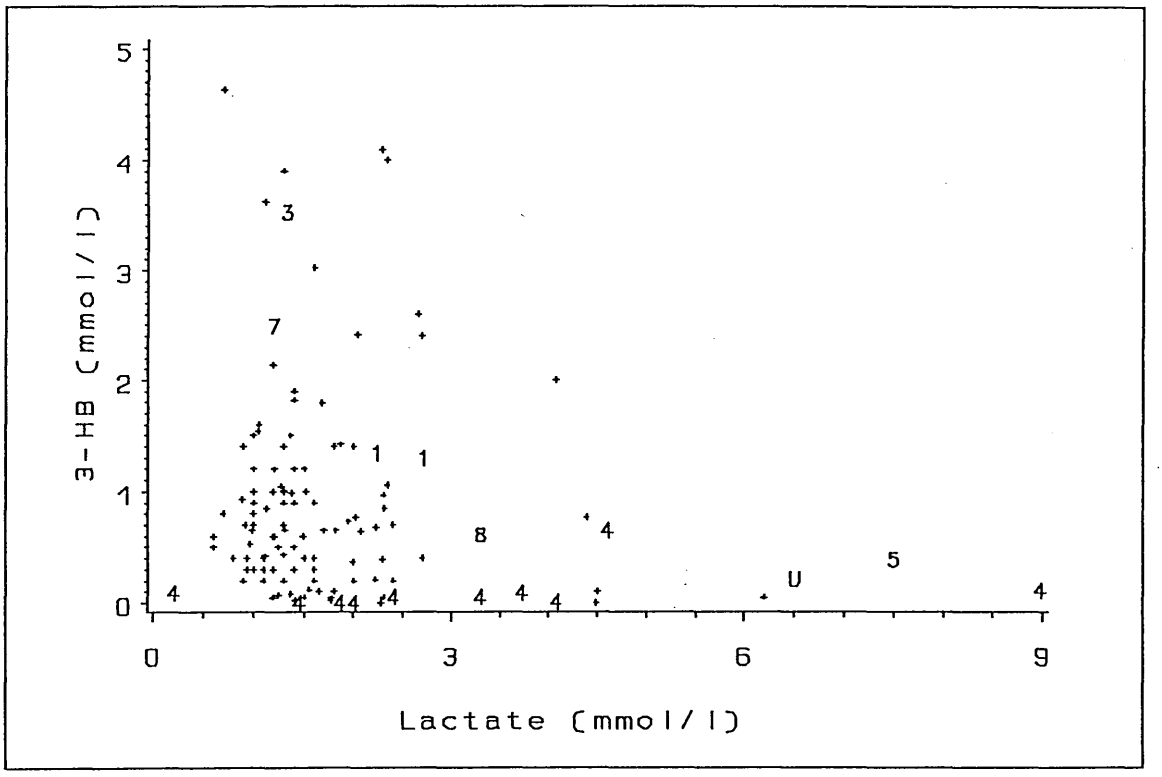


Fig.3.11. Plot of 3-hydroxybutyrate against lactate.

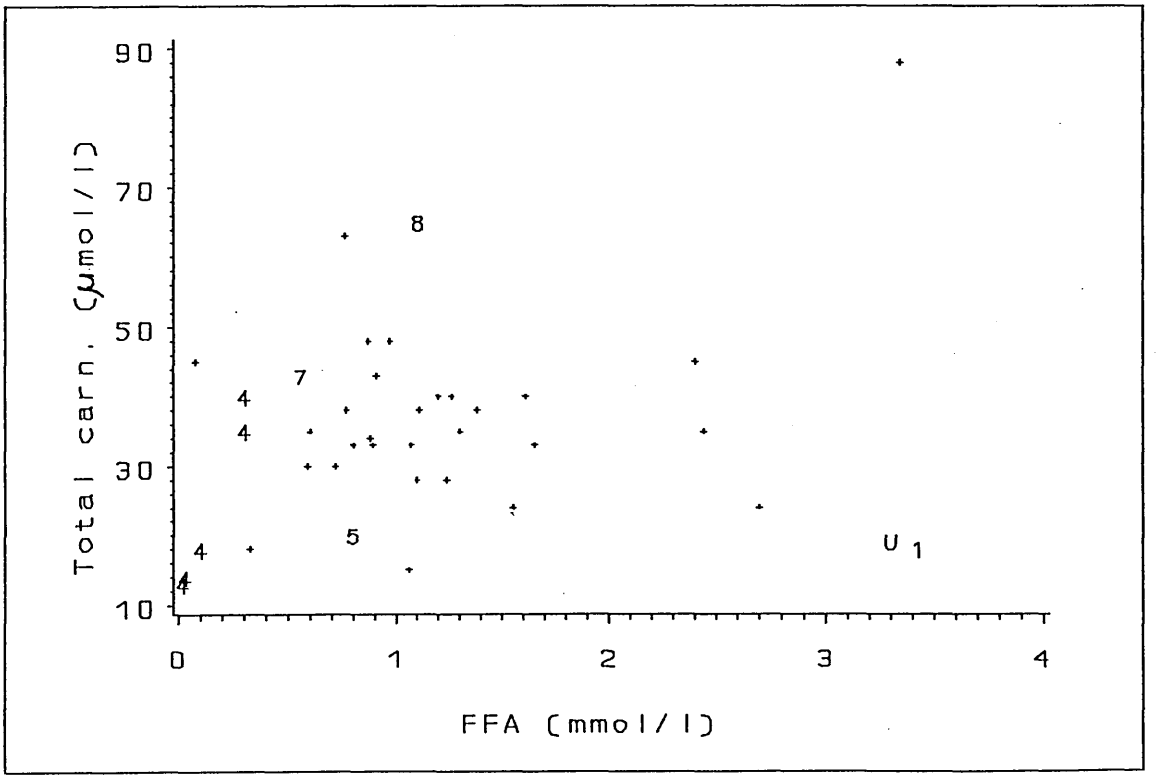


Fig.3.12. Plot of total carnitine against free fatty acids.

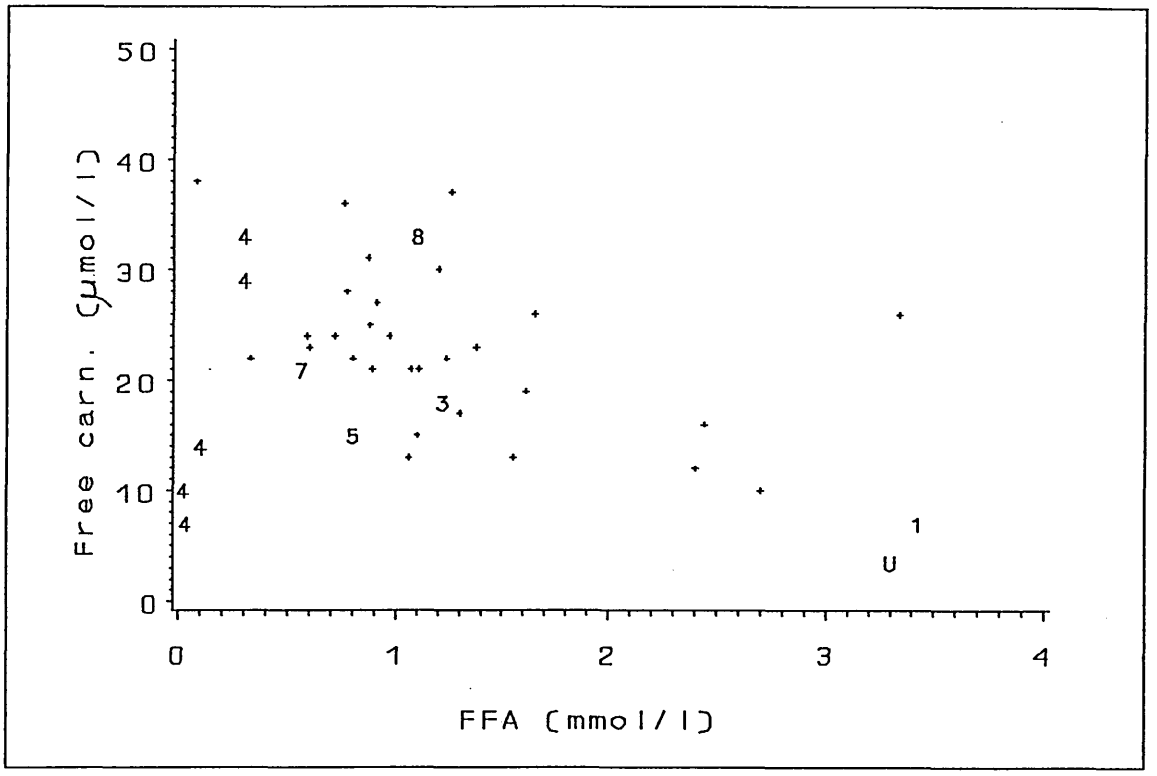


Fig.3.13. Plot of free carnitine against free fatty acids.

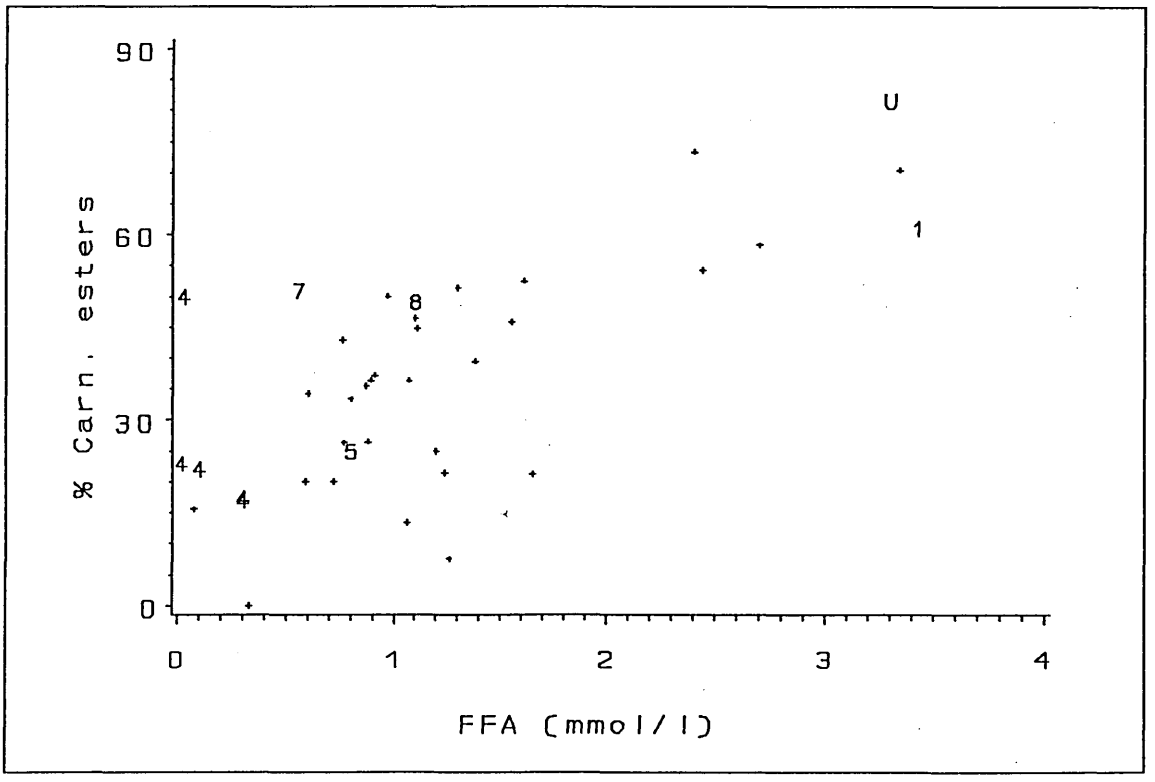


Fig.3.14. Plot of % carnitine esters against free fatty acids.

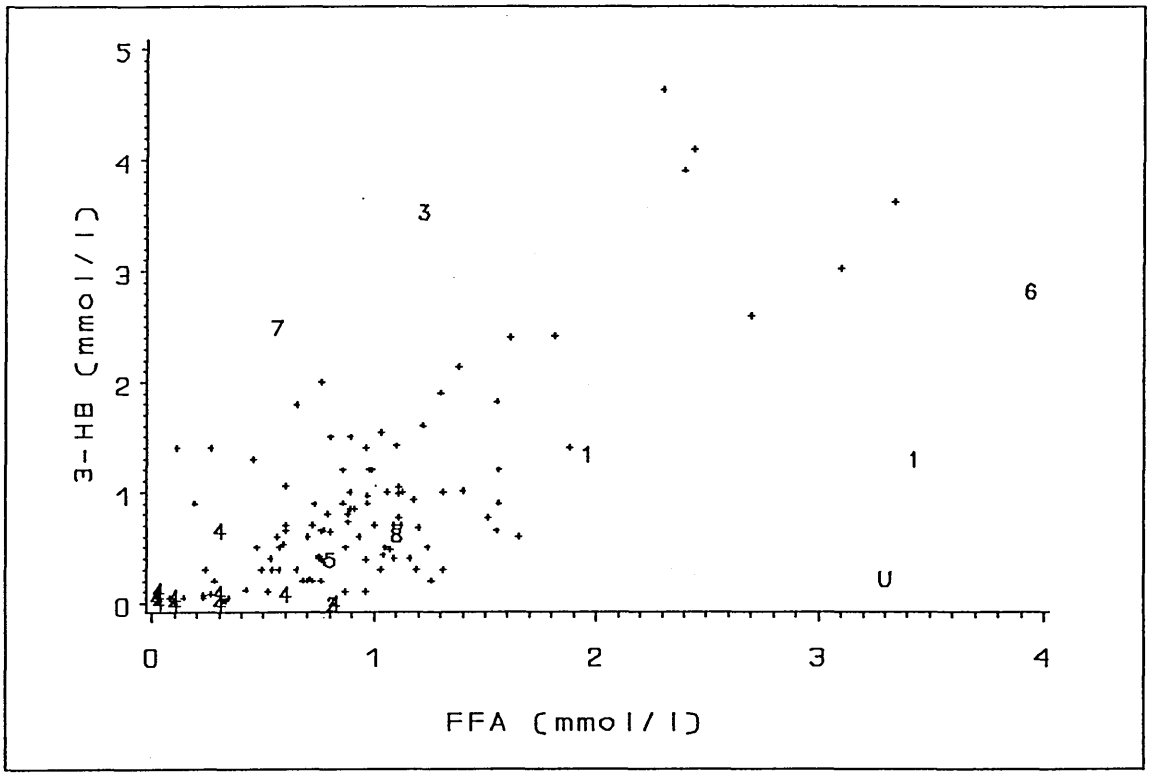


Fig.3.15. Plot of 3-hydroxybutyrate against free fatty acids.

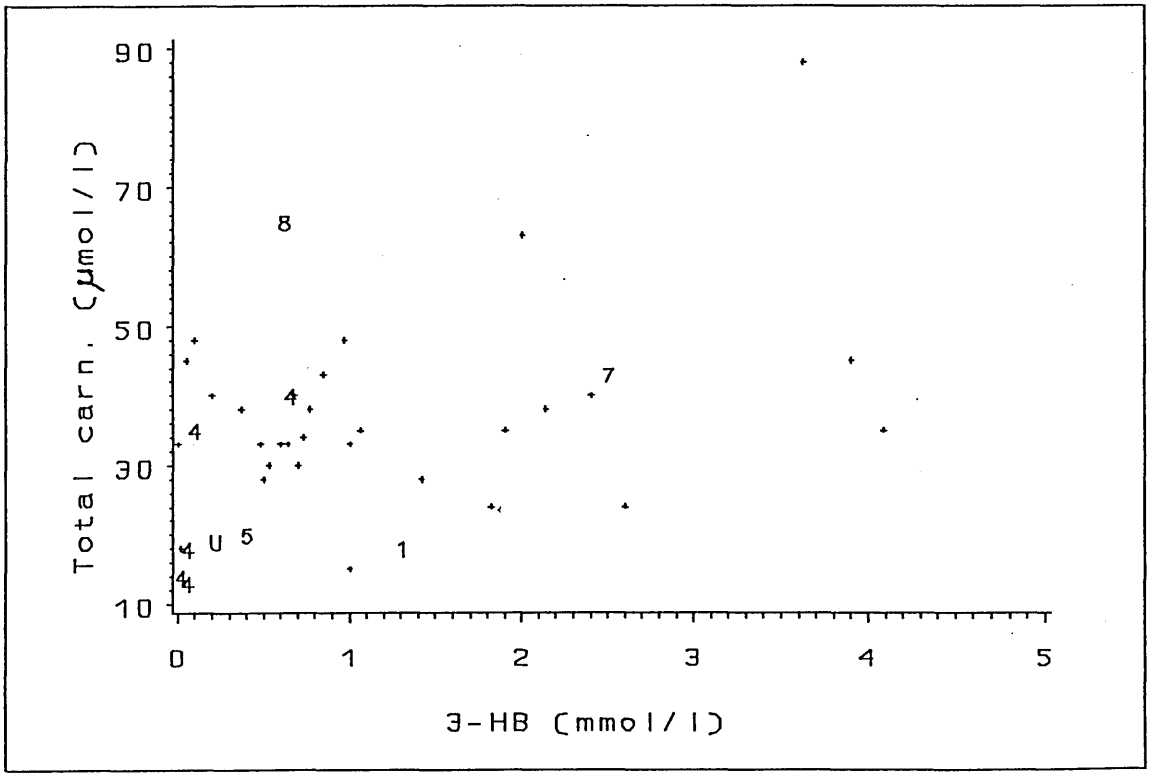


Fig.3.16. Plot of total carnitine against 3-hydroxybutyrate.

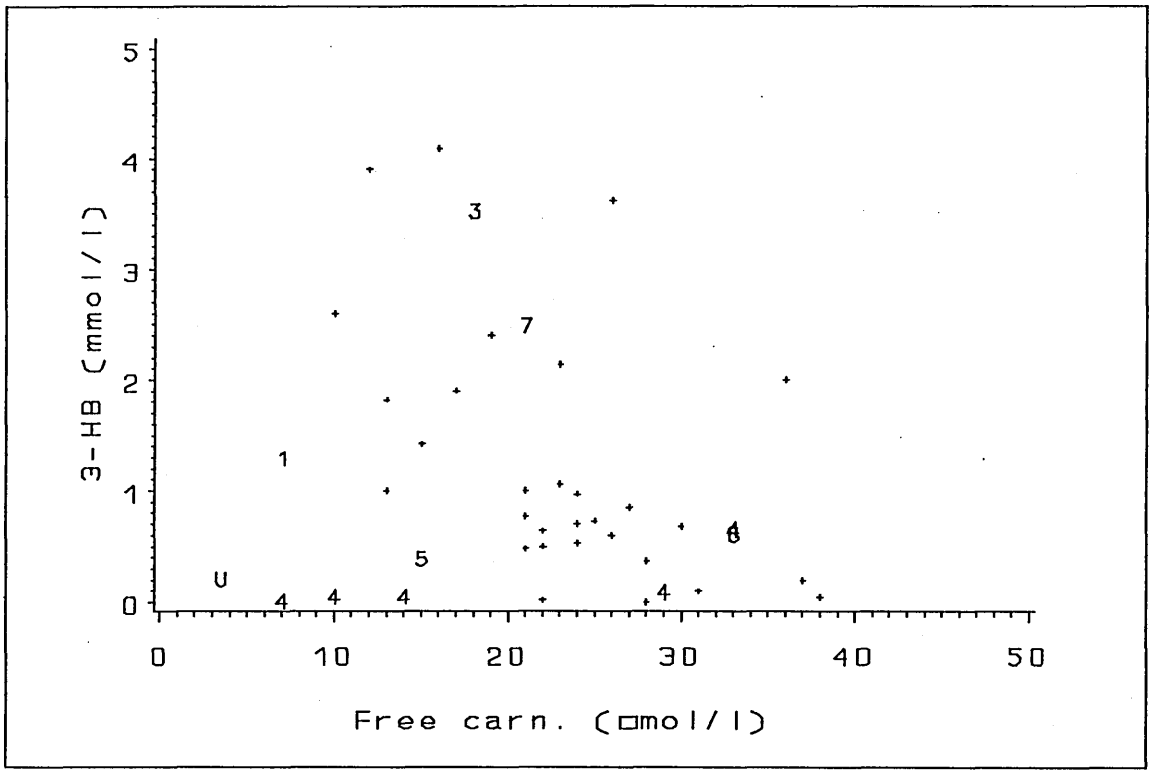


Fig.3.17. Plot of 3-hydroxybutyrate against free carnitine.

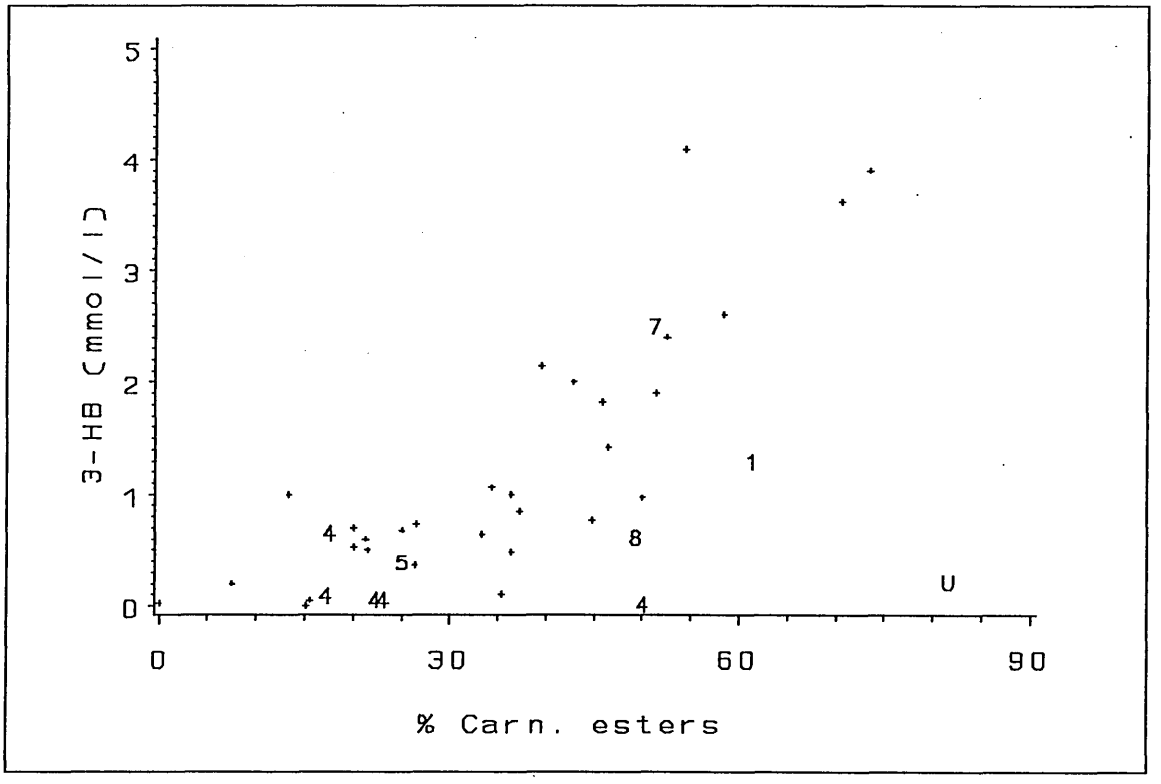


Fig.3.18. Plot of 3-hydroxybutyrate against % esterified carnitine.

Table 3.03 summarises the correlation coefficients, p values of no correlation and sample sizes for all possible correlations between the metabolite values collected from subjects with no diagnosed fatty acid oxidation defect (ie variable DIAGNOSE = 0 - see appendix table A2.2). Spearman correlation coefficients were used in preference to the more standard Pearson values. In dealing with the rank values of the variables as opposed to the numeric the Spearman correlation coefficients are less affected by outliers than would be the Pearson counterparts. It was seen however, that conclusions drawn during this study were still evident if Pearson calculations were used.

Table 3.03 Correlation coefficients, p value of no correlation, and sample sizes for data collected from subjects with no diagnosed fatty acid oxidation defect.

	Spearman Corr. Coef. / Prob > |R| under Ho: Rho=0 / Number of Observations						
	HB	CARNT	CARNF	CARNE	FFA3HB	FFA	LACT
GLUC Glucose	-0.53096 0.0001 101	-0.26620 0.1551 30	0.22576 0.2220 31	-0.61140 0.0003 30	0.38865 0.0001 99	-0.42110 0.0001 102	-0.07606 0.4451 103
LACT Lactate	-0.06270 0.5191 108	0.29468 0.1139 30	0.14765 0.4280 31	0.17351 0.3592 30	0.13169 0.1805 105	0.05730 0.5558 108	
FFA Free fatty acids	0.58376 0.0001 110	0.05585 0.7735 29	-0.44738 0.0150 29	0.62528 0.0003 29	-0.14151 0.1422 109		
FFA3HB FFA / 3HB	-0.86067 0.0001 109	-0.15569 0.4200 29	0.41209 0.0263 29	-0.66371 0.0001 29			
CARNE % Esterified carnitine	0.82953 0.0001 30	0.30139 0.0994 31	-0.49667 0.0045 31				
CARNF Free carnitine	-0.57316 0.0009 30	0.57981 0.0006 31					
CARNT Total carnitine	0.14790 0.4354 30						

3.2.2 FASTING PROFILES

Data from patients with no diagnosed error of fatty acid metabolism and for which exact fasting times were recorded were used to provide the following plots (figures 3.19. to 3.24.) These, along with the accompanying statistics, show the effect of fasting on intermediary metabolite levels. Also included are data taken from patients with defects in fatty acid metabolism. Unfortunately, because of a lack of exact fasting times the patients included here were limited to those diagnosed as hypoglycaemic hyperinsulinaemic and patient 69 with a suspected defect in glycogen storage or mobilisation. All points for the non-diagnosed patients are represented by a cross whilst those for the diagnosed children are shown by the value of the variable DIAGNOSE (see appendix table A2.2).

The lactate and total carnitine profiles showed poor correlations and therefore are not shown. Notably, although the correlation coefficient for the FFA/3HB profile (figure 3.24) was only -0.3, visually there did appear to be a more clear correlation. The poor coefficient appeared to be a result of a wide spread of values between fast times of 5.5 and 8.5 hours. All other plots showed good correlations with modulus correlation coefficients between 0.49 and 0.63.

Table 3.04. summarises the Spearman correlation coefficients, p value of no correlation and sample size for the effect of fast on metabolite values measured in subjects with no diagnosed defects in fatty acid oxidation.

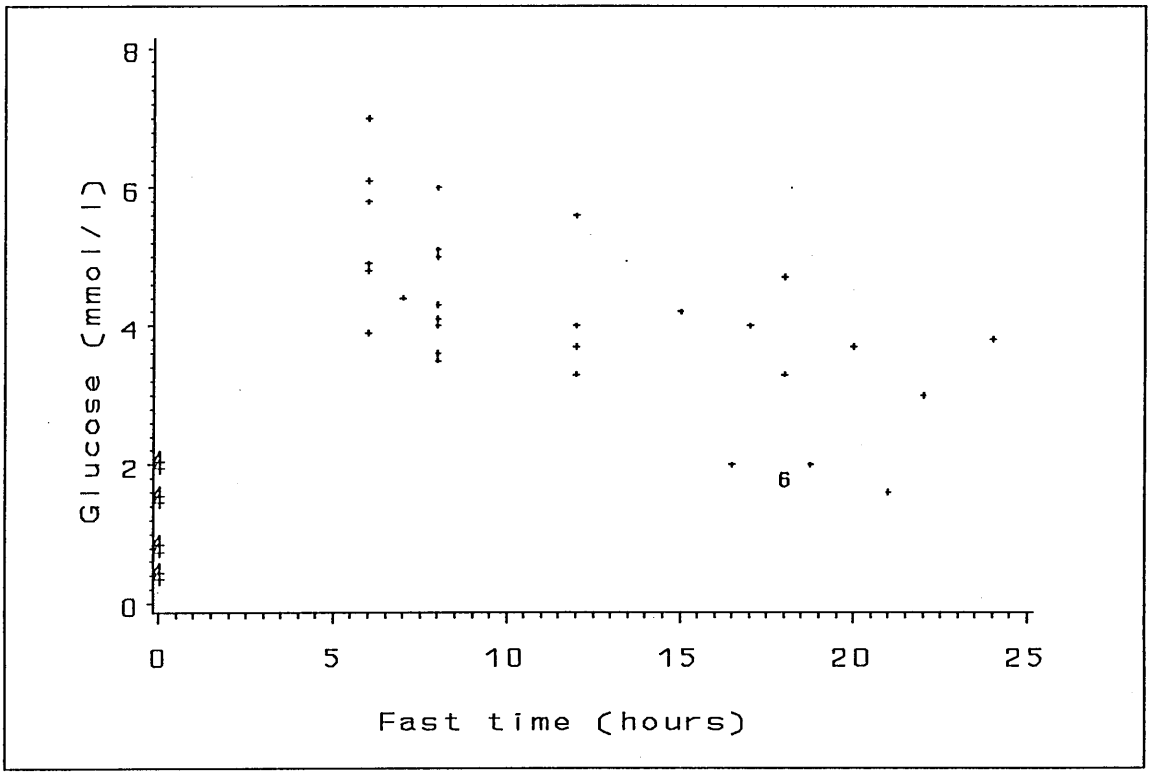


Fig.3.19. Plot of glucose against length of fast.

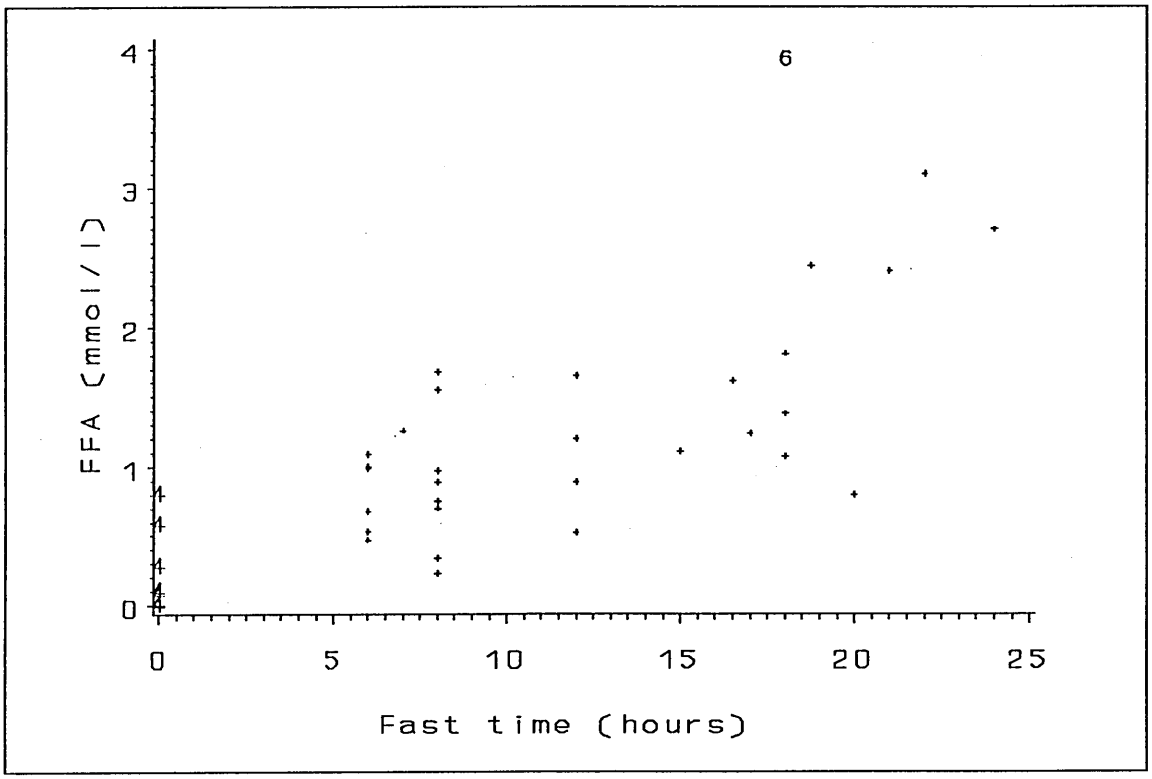


Fig.3.20. Plot of free fatty acids against length of fast.

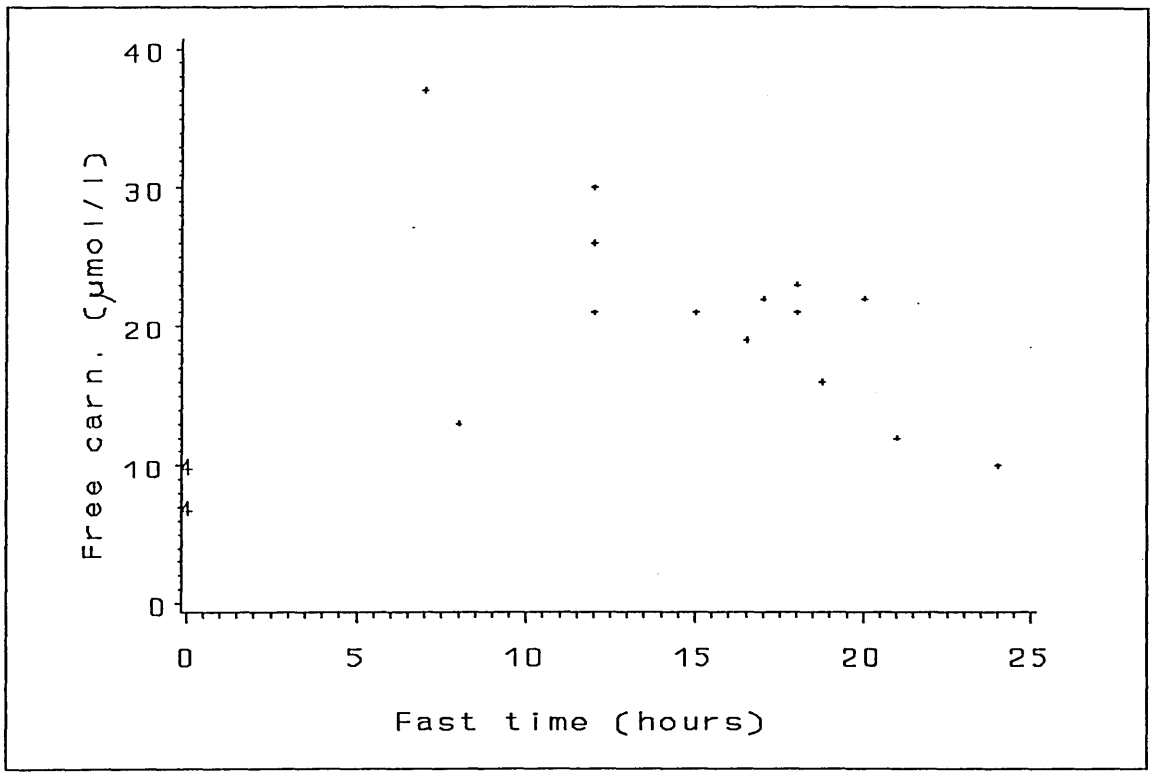


Fig.3.21. Plot of free carnitine against length of fast.

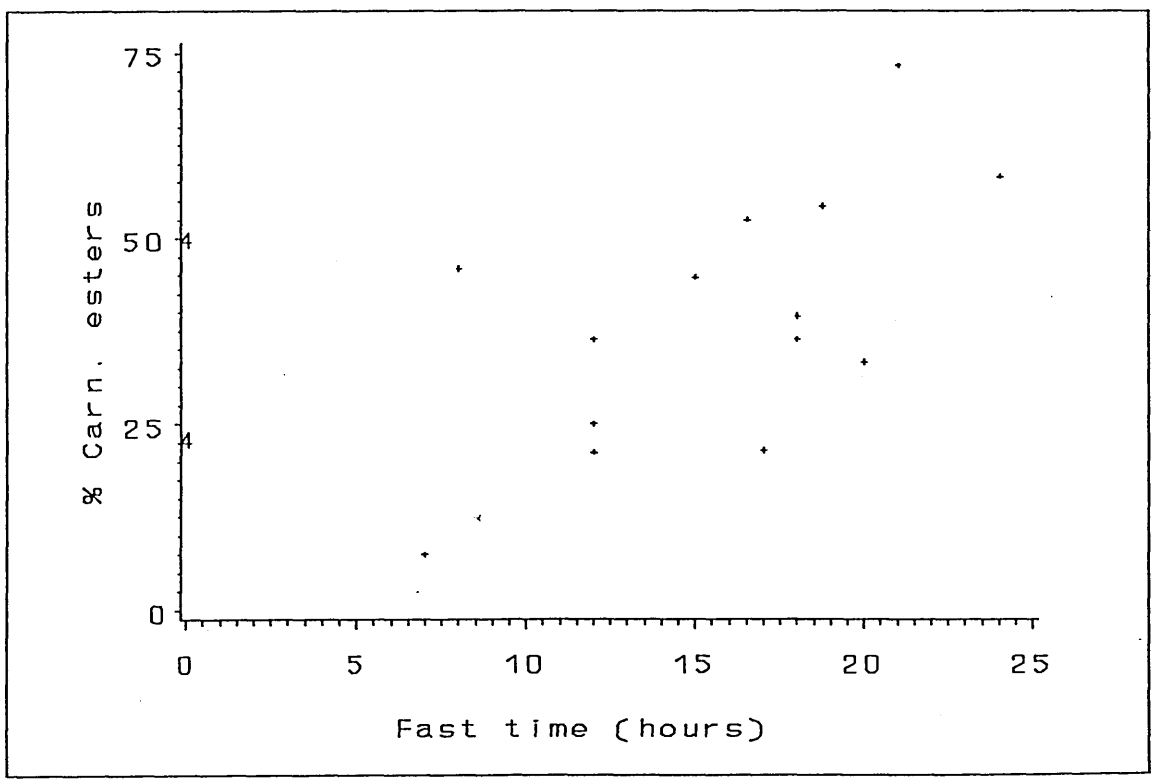


Fig.3.22. Plot of esterified carnitine against length of fast.

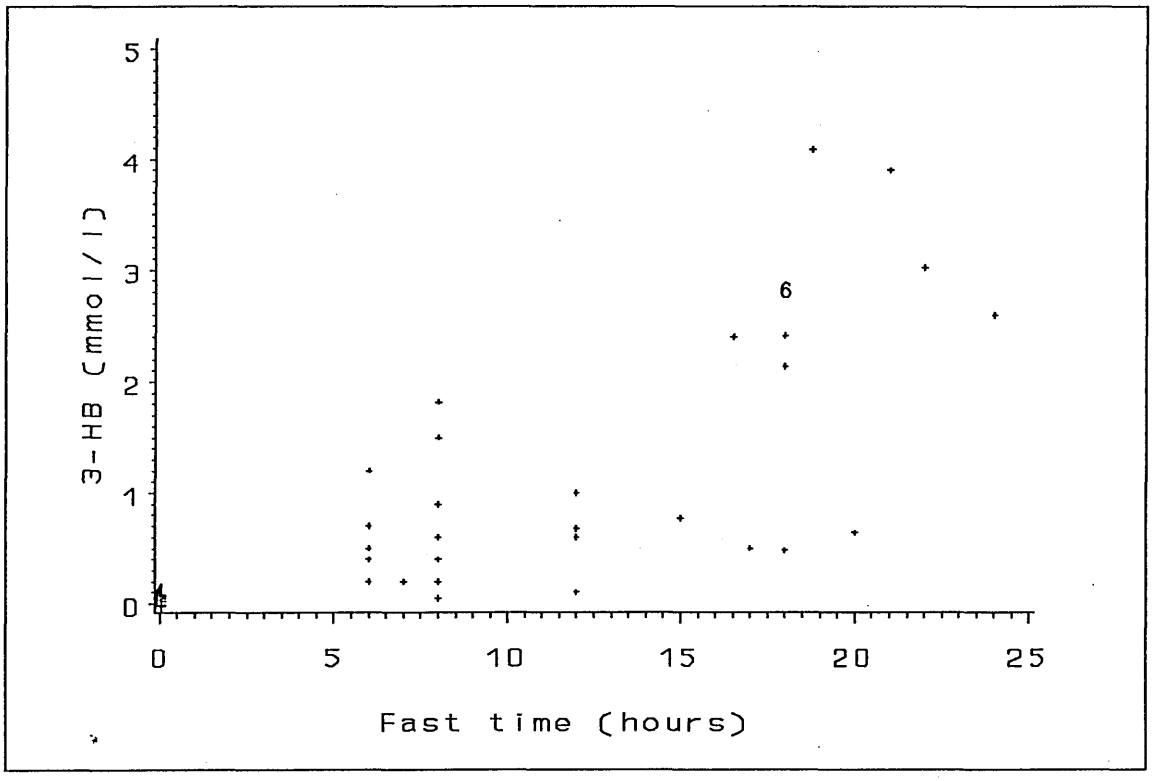


Fig.3.23. **Plot of 3-hydroxybutyrate against length of fast.**

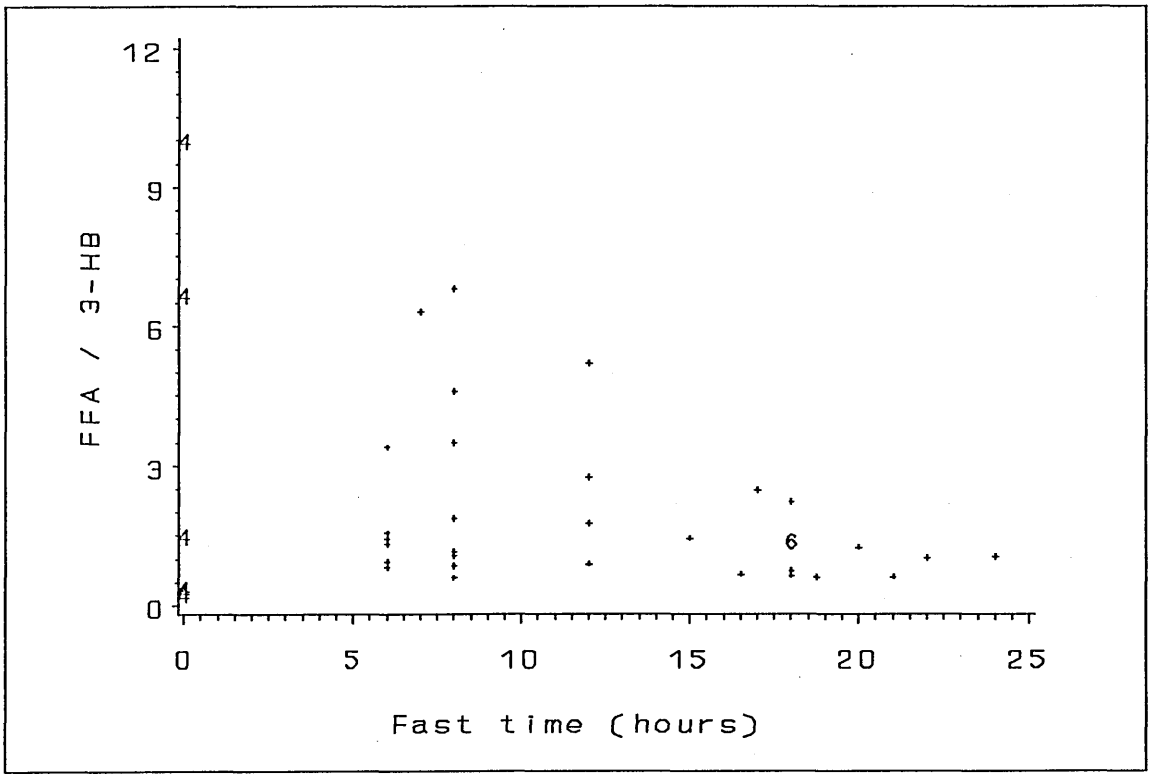


Fig.3.24. **Plot of free fatty acids / 3-hydroxybutyrate against length of fast.**

Table 3.04. Spearman correlation coefficients (C), p value of no correlation (P), and number of observations (N) for the effect of length of fast on intermediary metabolites

	C	P	N
GLUC Glucose	-0.62747	0.0002	31
LACT Lactate	0.49234	0.0057	30
FFA Free fatty acids	0.59656	0.0004	31
FFA3HB Free fatty acids / 3-hb	-0.31120	0.0941	30
CARNE % Esterified carnitine	0.58518	0.0279	14
CARNF Free carnitine	-0.52222	0.0554	14
CARNT Total carnitine	-0.07200	0.8068	14
HB 3-Hydroxybutyrate	0.52571	0.0028	30

All correlations were against length of fast

3.3 GENERALISED DICARBOXYLIC ACIDURIA INVESTIGATION

3.3.1 SUBSET DEPENDENCY

Fisher's two tailed exact method was used to test the hypothesis that generalised DCA was not associated to; 1. Sibling history of sudden infant death (SIB of SIDS); 2. Method of feeding - breast or artificial; and 3. Gender. The resultant p values are shown in table 3.05. High values for 'SIB of SIDS' and 'Gender' indicate no connection between these parameters and the occurrence of generalised DCA, whereas, the value for 'Feed' is much lower.

In addition, table 3.06. shows the distribution of subjects within the subsets set up by the possible combinations of the three parameters mentioned above against presence and absence of generalised DCA. The percentage of all breast fed neonates that showed generalised DCA is more then twice the percentage of all bottle fed neonates that showed similar patterns, whilst, the percentage of breast feeders that showed no DCA is less than half that for bottle feeders.

These points indicate a connection between generalised DCA and method of feeding.

Table 3.05. Table of p values based on non-association of generalised dicarboxylic aciduria and; 1. Sibling history of sudden infant death; 2. Method of feeding; 3. Gender.

	CLASS		
	SIB of SIDS	Feed	Gender
Gen. DCA or non gen. DCA	0.77	0.08	0.73

Table 3.06 Distribution of patients exhibiting generalised dicarboxylic aciduria (Gen. DCA) and those with no detectable urinary dicarboxylic acids (Non DCA) according to sibling history of SIDS, type of feed, and gender.

Class	N	Distribution of patents			
		Gen. DCA		Non DCA	
		Fqy	%	Fqy	%
Sibling of SIDS	55	28	50.9	27	49.1
No history of SIDS	14	8	57.1	6	42.9
Breast feed	13	9	69.2	4	30.8
Artificial feed	9	2	22.2	7	77.8
Male	16	11	68.8	5	31.3
Female	18	11	61.1	7	38.9

N = number in class, Fqy = frequency of patients within subclass, % = percentage of class within subclass.

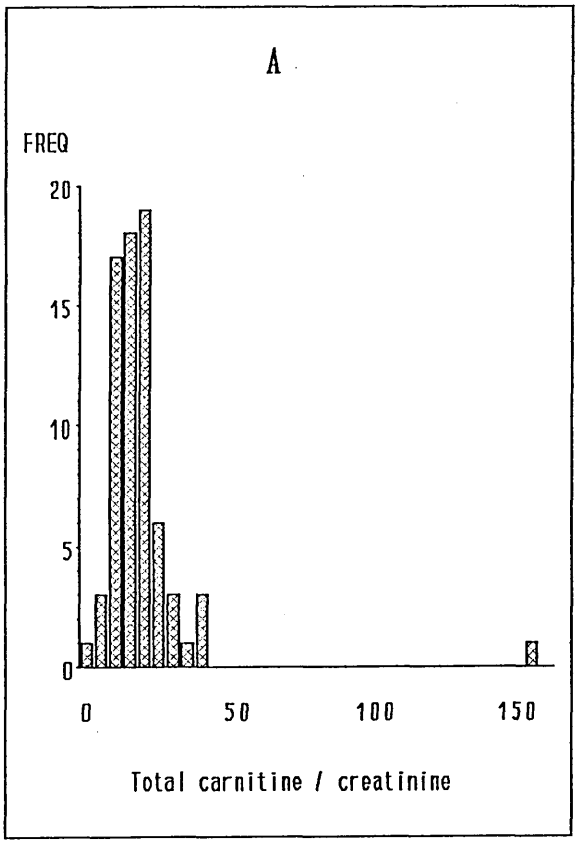
3.3.2 STATISTICAL ANALYSIS AND REFERENCE RANGE EVALUATIONS FOR NEONATAL URINARY CARNITINE LEVELS

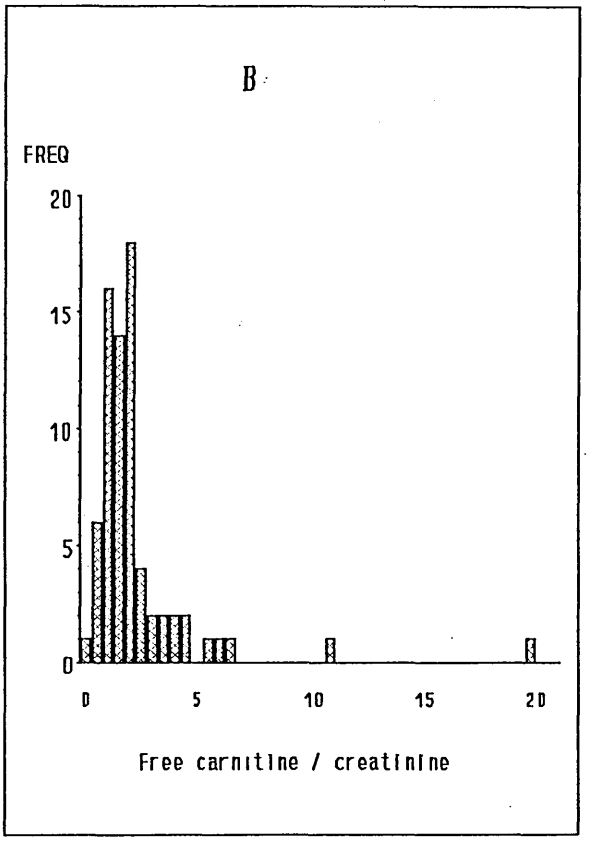
3.3.2.1 Frequency plots and statistics

Because of an apparent association between carnitine values and generalised DCA (see later) separate statistical investigation and reference range evaluations were also carried out on two subsets of the study group, one contained babies showing generalised DCA and the other contained babies that do not.

The following frequency plots, figures 3.25., 3.26. and 3.27. and summary statistics tables 3.07, 3.08. and 3.09 show the distributions of carnitine values with, without and regardless of presence of generalised DCA.

All total and free carnitine values are given in µmol/mmol creatinine.





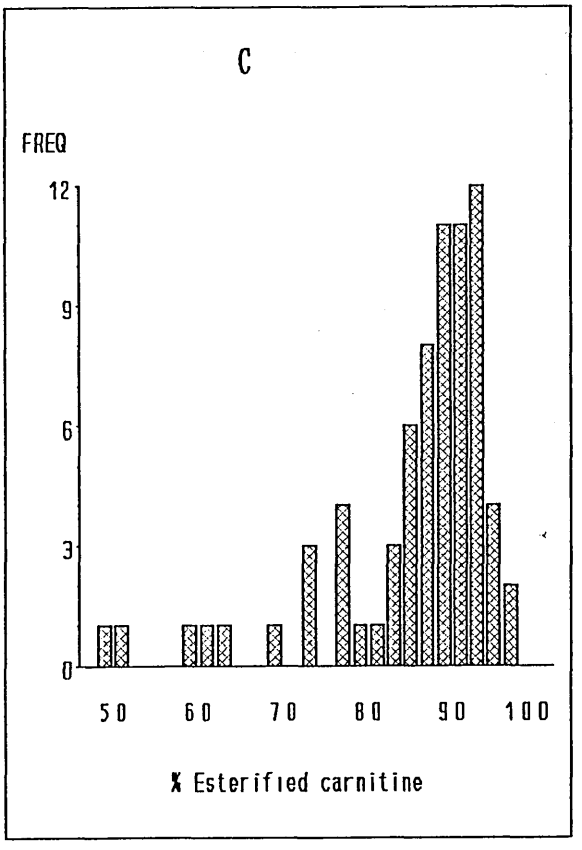
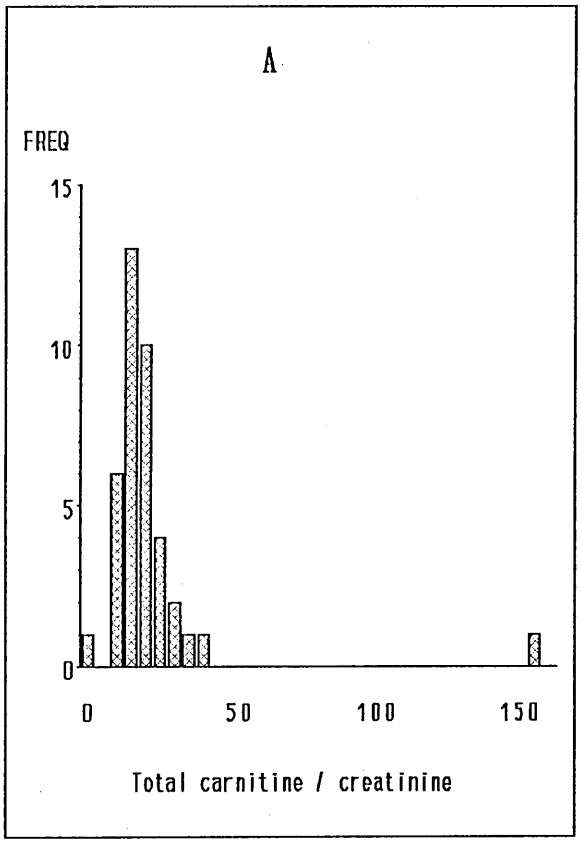


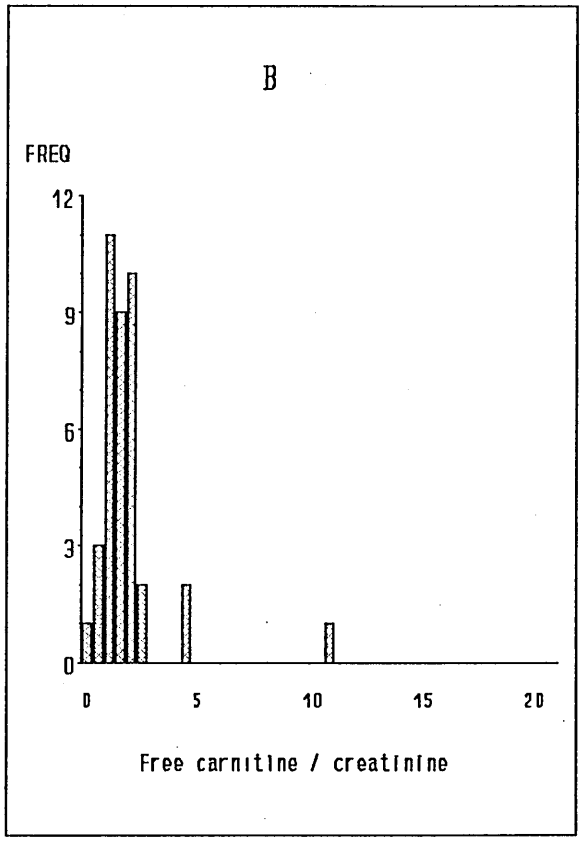
Fig.3.25.

Frequency plots of carnitine data (total, free and esterified) for all patients involved in the generalised DCA investigation.

Table 3.07. Basic statistical analysis of carnitine data (total, free and esterified) for all patients involved in the generalised DCA investigation.

	URINE CARNITINE		
	Total	Free	Esters
Sample size	72	72	72
Mean	19.1	2.3	86.7
Standard deviation	18.0	2.7	10.1
Median	16.2	1.7	89.8
Upper quartile	20.5	2.2	93.1
Lower quartile	11.5	1.0	85.1
Minimum	1.1	0.2	50.0
Maximum	153.9	20.0	97.7
Coef. of variation	94.5	118.0	11.6





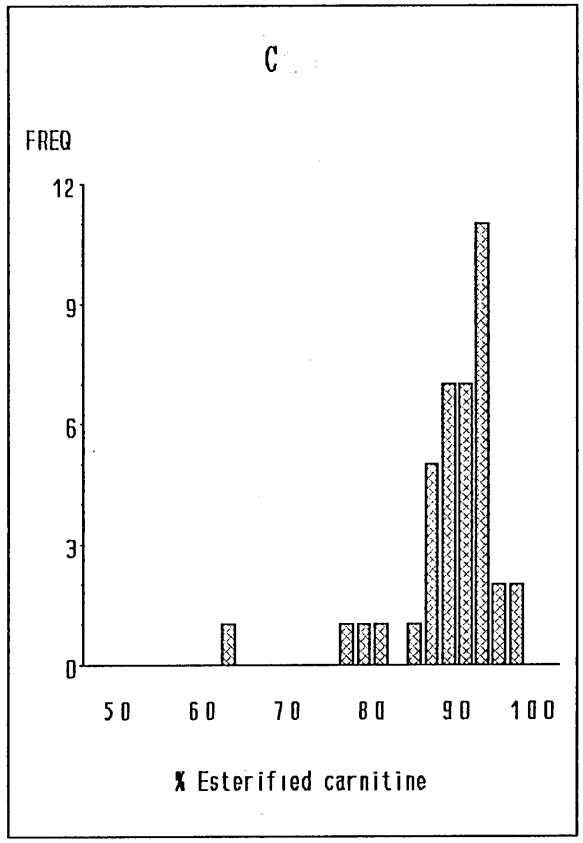
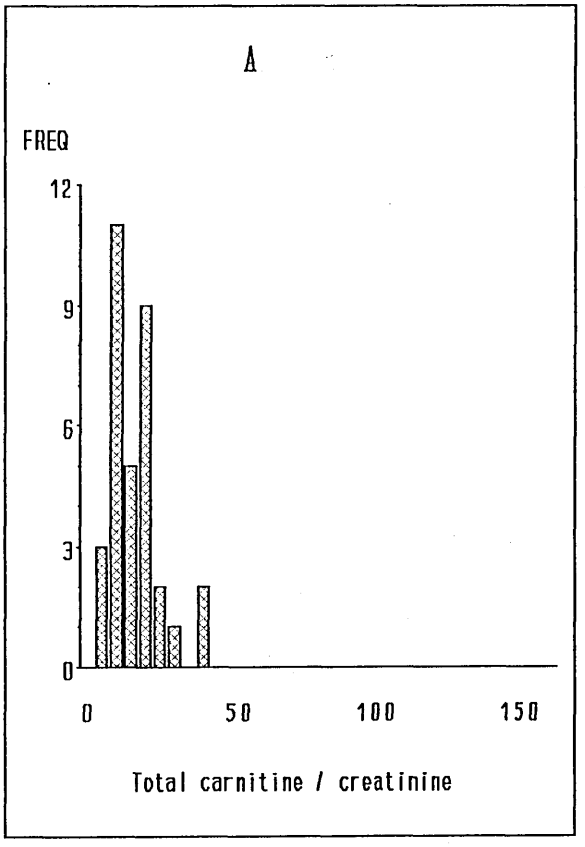


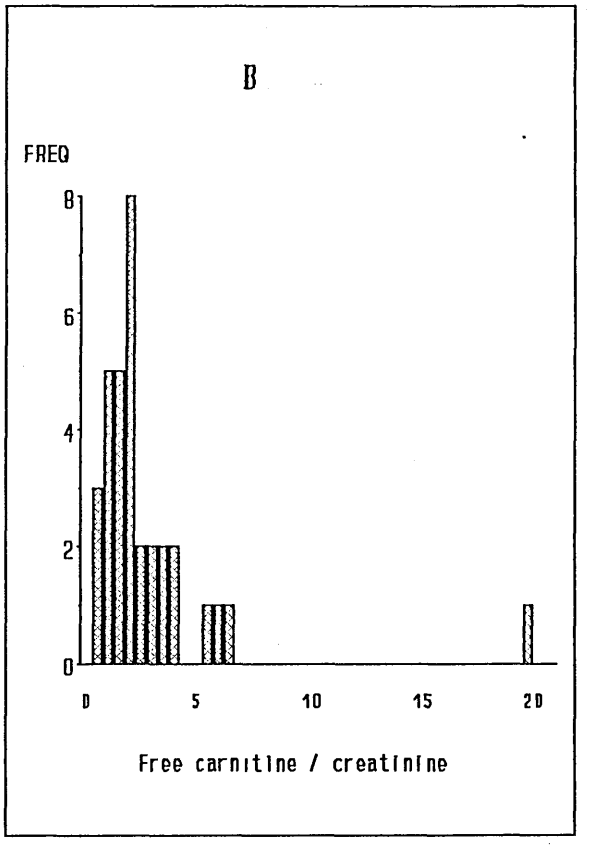
Fig.3.26.

Frequency plots of carnitine data (A; total, B; free and C; esterified) for children exhibiting a generalised DCA.

Table 3.08. Basic statistical analysis of carnitine data (total, free and esterified) for children exhibiting a generalised DCA.

	URINE CARNITINE		
	Total	Free	Esters
Sample size	39	39	39
Mean	21.6	1.8	91.0
Standard deviation	22.9	1.7	6.2
Median	17.4	1.3	92.2
Upper quartile	22.3	1.9	94.2
Lower quartile	13.2	0.9	88.9
Minimum	1.1	0.2	63.6
Maximum	153.8	10.8	97.6
Coef. of variation	106.4	96.4	6.9





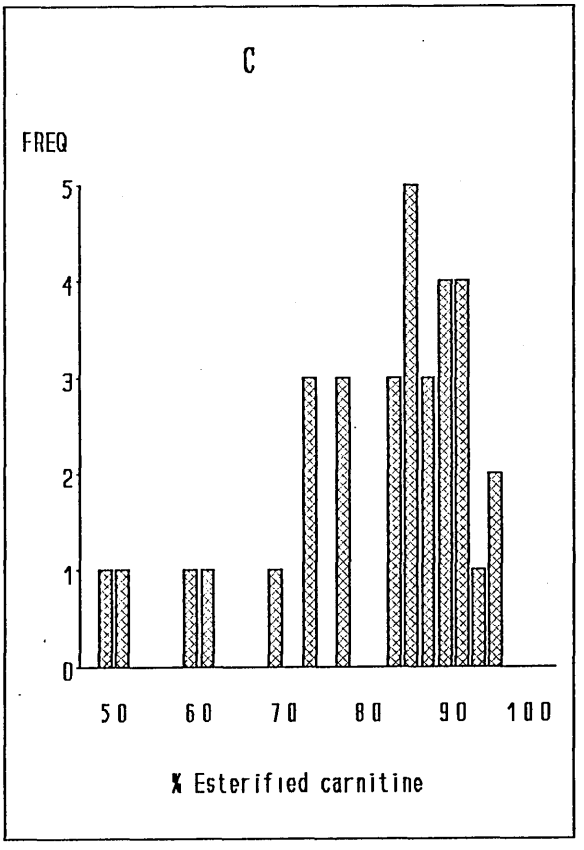


Fig.3.27.

Frequency plots of carnitine data (total, free and esterified) for children having no detectable DCA.

Table 3.09. Basic statistical analysis of carnitine data (total, free and esterified) for children having no detectable DCA.

	URINE CARNITINE		
	Total	Free	Esters
Sample size	33	33	33
Mean	16.1	2.9	82.4
Standard deviation	8.9	3.4	12.0
Median	13.5	2.0	85.7
Upper quartile	20.0	2.9	90.3
Lower quartile	9.4	1.3	77.4
Minimum	4.3	0.6	50.0
Maximum	42.0	20.0	95.9
Coef. of variation	55.4	120.4	14.6

3.3.2.2 Urinary carnitine reference ranges

All carnitine values showed a skewed distribution (see previous section). The reference ranges were therefore approximated. The representation used was the 2.5th to 97.5th inter percentile range. The 2.5th percentile is that value below which 2.5% of all data lie whilst the 97.5th percentile is that value above which 2.5% of all data lie. Below is an example calculation of the 2.5th to 97.5th percentile range using urinary total carnitine data for all patients in the study. Table 3.10. summarises the ranges calculated for the subsets mentioned previously.

Calculation of 2.5th percentile

The nth value corresponding to the 2.5th percentile

$$= \frac{N_{max}}{100} \times 2.5 = \frac{72}{100} \times 2.5 = 1.8 \text{ th}$$

$$N_{1.8} = N_1 + 8\,\frac{[\,N_2 - N_1\,]}{10} = 1.14 + 8\,\frac{[\,4.34 - 1.14\,]}{10}$$

$$= 3.70$$

Calculation of 97.5th percentile

The nth value corresponding to the 97.5th percentile

$$= \frac{N_{max}}{100} \times 97.5 = \frac{72}{100} \times 97.5 = 70.2 \text{ th}$$

$$N_{70.2} = N_{70} + \frac{2\,[N_{71} - N_{70}\,]}{10} = 40.00 + \frac{2\,[42.00 - 40.00]}{10}$$

$$= 40.40$$

Table 3.10. Table showing the 2.5th and 97.5th percentiles calculated; 1. regardless of presence of urinary DCA (ALL); 2. for patients exhibiting generalised DCA (DCA); and 3. for patients with no detectable DCA (NO DCA).

Group	Nth value 2.5%	Nth value 97.5%	Carnitine value	2.5th percentile	97.5th percentile
ALL	1.8	70.2	total	3.7	40.4
			free	0.5	7.5
			% esterified	51.9	96.6
DCA	1.0	38.0	total	1.1	39.7
			free	0.2	4.6
			% esterified	63.6	97.1
NO DCA	0.8	32.2	total	3.6	40.4
			free	0.5	9.3
			% esterified	41.3	95.7

Median values were calculated for total, free and esterified carnitine for each condition of the four parameters - presence of generalised DCA, sib. of SIDS, feed and gender. Null hypotheses stating that these medians did not change with the condition of each parameter was tested using the Wilcoxon rank sum test. The medians and resultant p values are tabulated in tables 3.11. to 3.14..

Low p values of no association for free and particularly esterified carnitine in table 3.11. indicates a high likelihood of an association between these values with generalised DCA.

Table 3.11. Table of medians and p values for the null hypothesis that the carnitine values (total, free and esterified) do not differ with the presence or absence of generalised DCA.

Carnitine value	MEDIAN VALUE		p value of no association
	Generalised DCA present	Generalised DCA absent	
Total	17.4	13.5	0.0723
Free	1.3	2.0	0.0292
% Esterified	92.2	85.7	0.0002

There does not seem to be a connection between any carnitine values and sibling history of SIDS, table 3.12.

Table 3.12. Table of medians and p values for the null hypothesis that the carnitine values (total, free and esterified) do not differ with the presence or absence of sibling history of sudden infant death.

Carnitine value	MEDIAN VALUE		p value of no association
	Sibling of SIDS patient	No sibling history	
Total	15.4	18.7	0.1652
Free	1.7	2.0	0.3666
% Esterified	89.8	88.2	0.8991

Low p values for total carnitine against type of feed, table 3.13., and gender, table 3.14., suggests higher total carnitine for breast fed neonates over bottle feeders and for females in comparison to males.

Table 3.13. Table of medians and p values for the null hypothesis that the carnitine values (total, free and esterified) do not differ with the method of feeding - breast or artificial.

Carnitine value	MEDIAN VALUE Breast feed	MEDIAN VALUE Artificial feed	p value of no association
Total	19.8	10.8	0.0275
Free	2.1	1.8	0.6163
% Esterified	89.8	81.3	0.0825

Table 3.14. Table of medians and p values for the null hypothesis that the carnitine values (total, free and esterified) do not differ with gender.

Carnitine value	MEDIAN VALUE Male	MEDIAN VALUE Female	p value of no association
Total	13.8	18.3	0.0310
Free	1.8	1.5	0.6662
% Esterified	90.0	91.5	0.2477

3.3.3 RELATIONSHIP OF CARNITINE VALUES TO AGE WITHIN THE FIRST FIVE DAYS OF LIFE

Urinary total, free and esterified carnitine (µmmol/mmol creatinine) were plotted against age for all infants within the first week of life. These plots are shown in figures 3.28. to 3.30., and the associated correlation coefficients, p values of no correlation and sample sizes are shown in table 3.15.. Because of the possible relationship between carnitine values and generalised DCA separate correlation coefficients and p values of no correlation for those subjects showing generalised DCA and those not showing any DCA were also examined. These again are included in table 3.15. and provide evidence that there is no change in carnitine values, with or without generalised DCA, over the first five days of life.

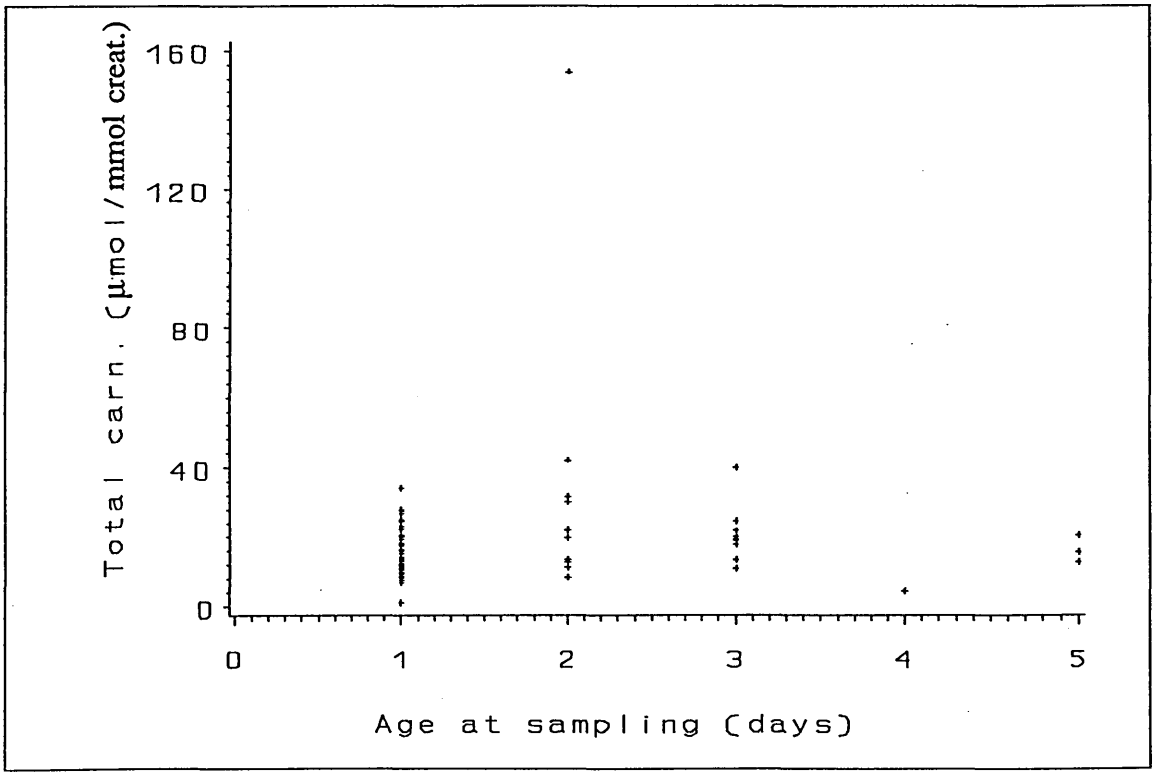


Fig.3.28. A plot of total urine carnitine against age.

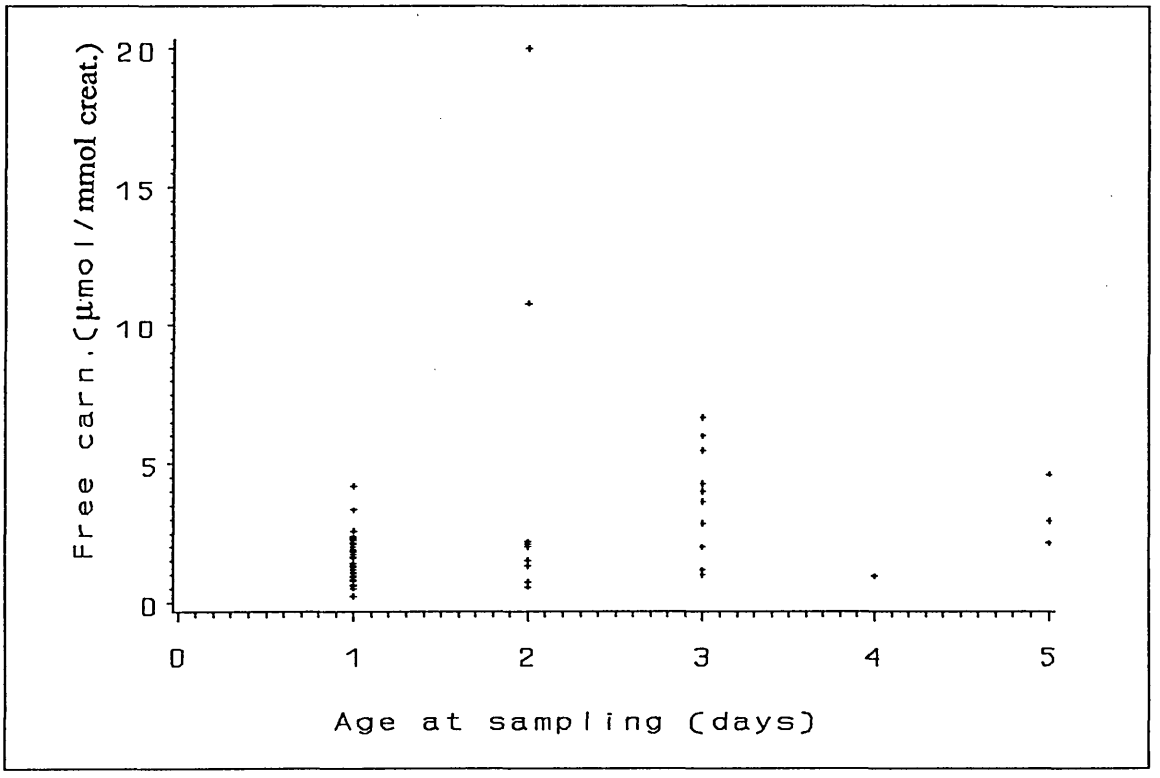


Fig.3.29. A plot of free urine carnitine against age.

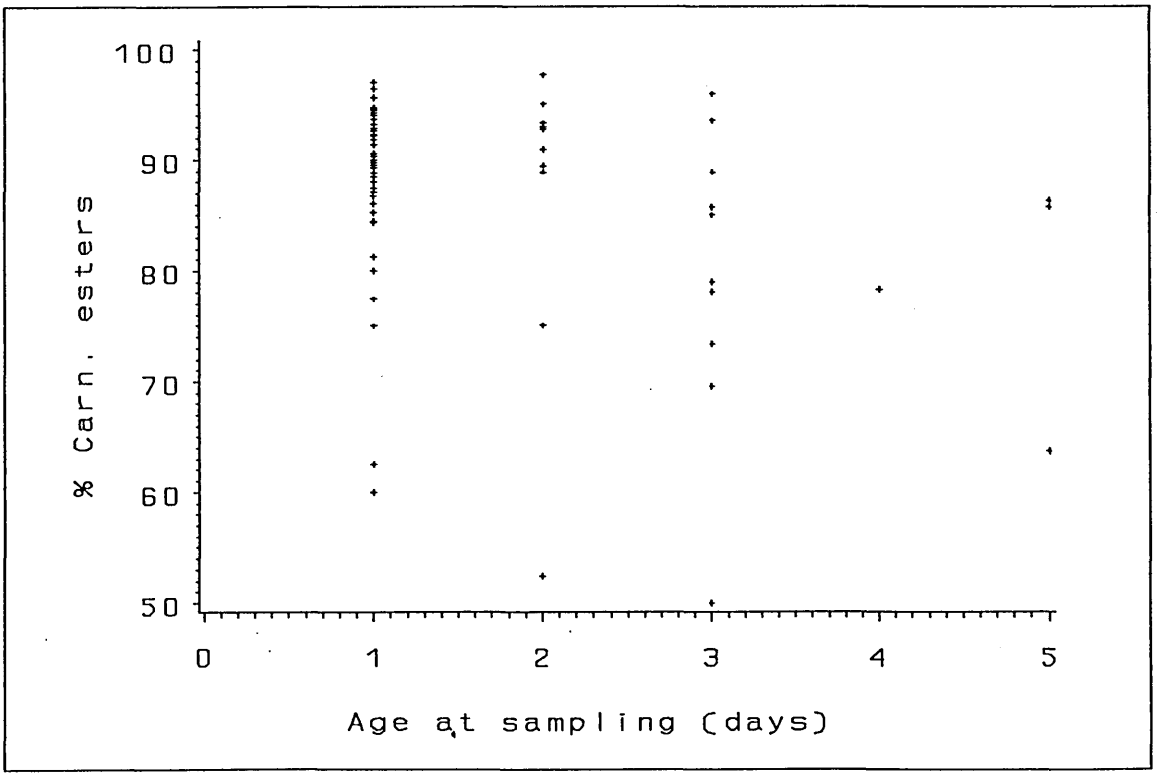


Fig.3.30. A plot of percentage esterified urine carnitine against age.

Table 3.15. Correlation coefficients (C), p value of no correlation (P), and number of observations (N) for the plots of total (TC), free (FC), and esterified carnitine (AC) against age for data collected from; 1. all neonates regardless of generalised DCA; 2. those exhibiting generalised DCA; 3. those not exhibiting DCA.

	Spearman Correl. Coef. / Prob > |R| under Ho: Rho=0 / N		
	TC	FC	AC
DAY	0.23571	0.39886	-0.27663
Age at sampling (days) for neonates	0.0512	0.0007	0.0214
regardless of generalised DCA	69	69	69
DAY	0.15265	0.29451	-0.17779
Age at sampling (days) for neonates	0.3741	0.0812	0.2995
exhibiting generalised DCA	36	36	36
DAY	0.38587	0.36371	-0.17905
Age at sampling (days) for neonates	0.0266	0.0375	0.3188
not exhibiting any DCA	33	33	33

3.4 DEVELOPMENT OF A RADIO-ENZYMATIC ASSAY TO QUANTITATE CARNITINE IN URINE AND PLASMA

Total carnitine values where multiplied by 2.5 to correct for the dilution factor. It was found that values above 70μmol/l gave rise to unacceptable duplicate values. Therefore, samples recording levels in excess of 60μmol/l were diluted accordingly and re-assayed.

3.4.1 PRECISION

The intra-batch variation was calculated by assaying five aliquots from the same randomly collected plasma sample. Table 3.16. shows the results of the assay.

Table 3.16. Results of the radio-enzymatic assay to investigate precision and recovery

Aliquot	Carnitine level (μmol/l)		Spiked 1:1 with 20μmol/l carnitine	
	Free	Total	Free	Total
1	52	56	46	43
2	53	58	44	45
3	54	59	43	44
4	52	57	45	46
5	54	59	45	42
MEAN	53.0	57.8	44.6	44.0
STANDARD DEVIATION	0.9	1.2	1.0	1.4
COEF. OF VARIATION	1.6	2.1	2.2	3.1

3.4.2 RECOVERY

An aliquot of the plasma used for the above intra batch precision was spiked 1:1 with 20 µmol/l carnitine, split into five and assayed simultaneously with the unspiked batch. The results are shown in table 3.16. The mean free carnitine concentration of the plasma that was spiked was 53µ mol/l. The expected overall free carnitine concentration was therefore,

$$\frac{53 + 20}{2} = 36.5 \text{ µmol/l}$$

Observed overall free carnitine concentration was,

44.6 µmol/l

Percentage recovery was therefore,

$$\frac{44.6}{36.5} \times 100 = 122\%$$

The mean total carnitine concentration of the plasma that was spiked was 57.8 µmol/l. The expected overall total carnitine concentration was therefore,

$$\frac{57.8 + 20}{2} = 38.9 \text{ µmol/l}$$

Observed overall total carnitine concentration was,

44 µmol/l

Percentage recovery was therefore,

$$\frac{44.0}{38.9} \times 100 = 113\%$$

In summary, the precision of this assay at approximately 50 to 60 µmol/l has a standard deviation of 0.9 µmol/l (CV 1.6) for free plasma carnitine and 1.2 µmol/l (CV 2.1) for total plasma carnitine. The recoveries for free and total carnitine were 122% and 113% respectively.

3.4.3 QUALITY ASSURANCE AND CONTROL

The quality control material used for the plasma carnitine quantitation was the investigators own plasma collected after 12 hr fast. Standard deviation and coefficient of variation results for total carnitine, table 3.17., are somewhat higher than those for free during both 1988 and 1989. This was assumed to be a reflection of an increase in experimental error due to the extra hydrolysis step and inherent dilutions involved in the total carnitine analysis. As one can see from figure 3.31. the quality control results could be somewhat erratic resulting in a number of batch rejections due to values outside the mean ± 2 SD range. Such a variation, whilst acceptable, is thought to be a reflection of the problematic nature of this assay. Overall CV's were all reasonable for a non-automated assay of this sort.

Table 3.17. Quality control results for radio-enzymatic assay of plasma carnitine during the period of the intermediate metabolites investigation.

	Plasma carnitine					
Year	Free			Total		
	Mean	SD	CV%	Mean	SD	CV%
1988	47.9	2.3	4.8	52.2	7.6	7.3
1989	24.2	1.2	5.0	65.4	5.7	8.7

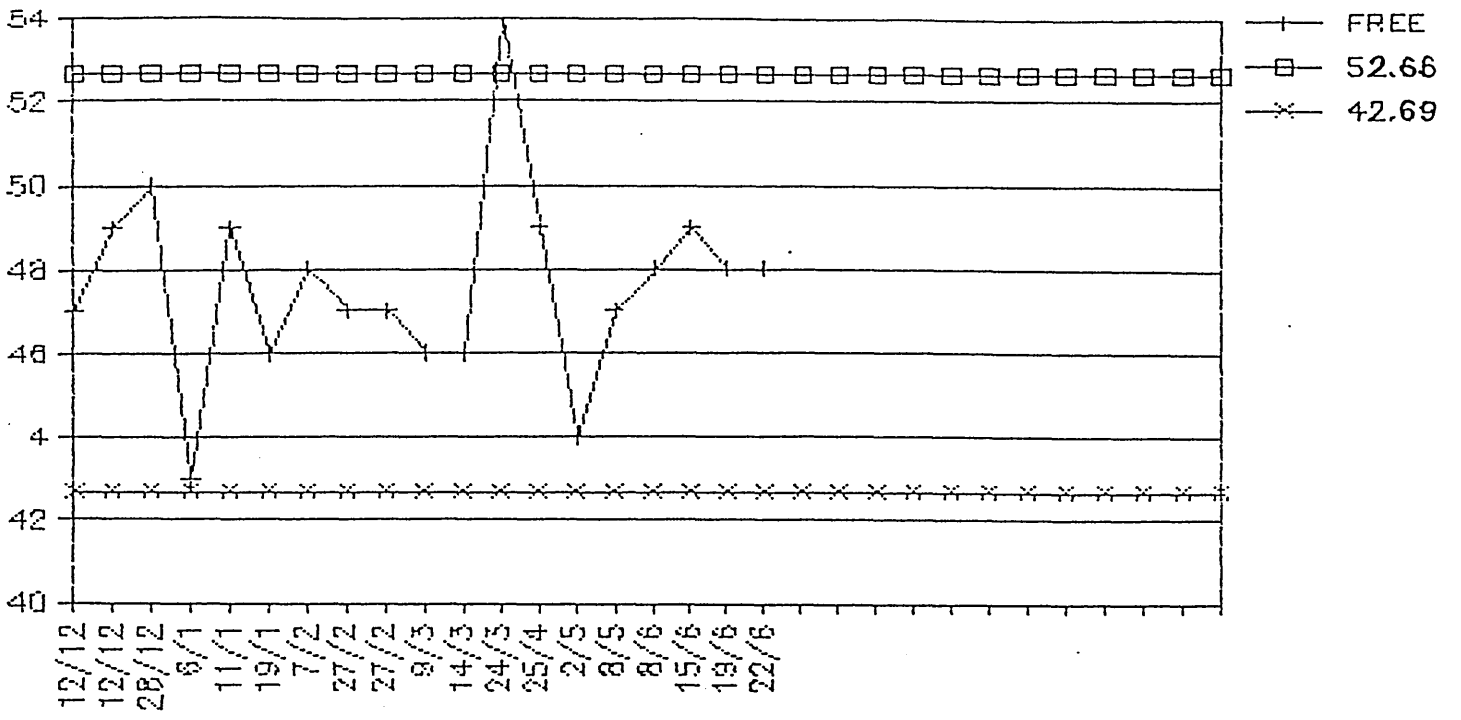


Fig.3.31. Example of quality control chart (1987-88) for Free Plasma Carnitine showing the ± 2SD limits.

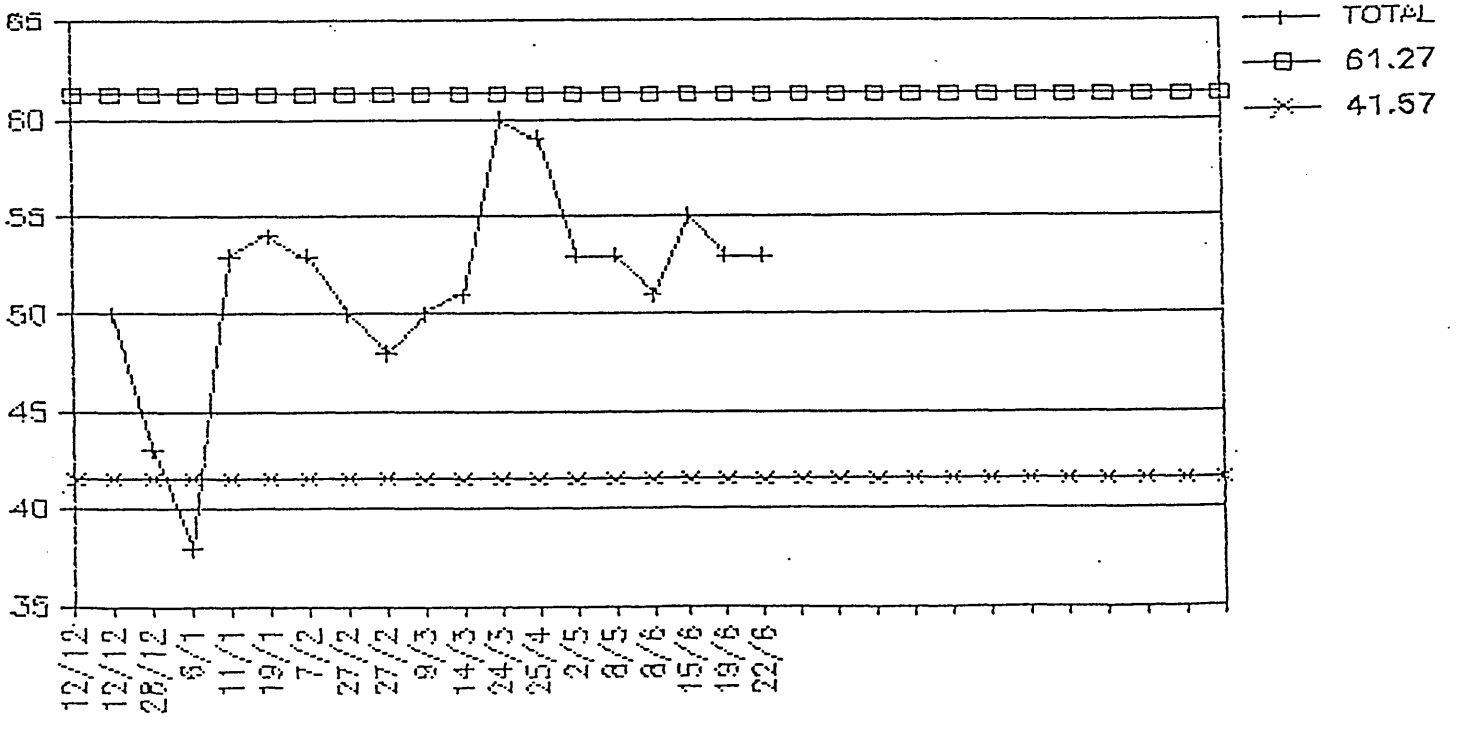


Fig.3.32. Example of quality control chart (1987-88) for Total Plasma Carnitine showing the ± 2SD limits.

The standard curve used during the assay was seen to vary a surprising amount between assays (figure 3.33. and table 3.18.). As a result the standard curve was recalculated for each assay.

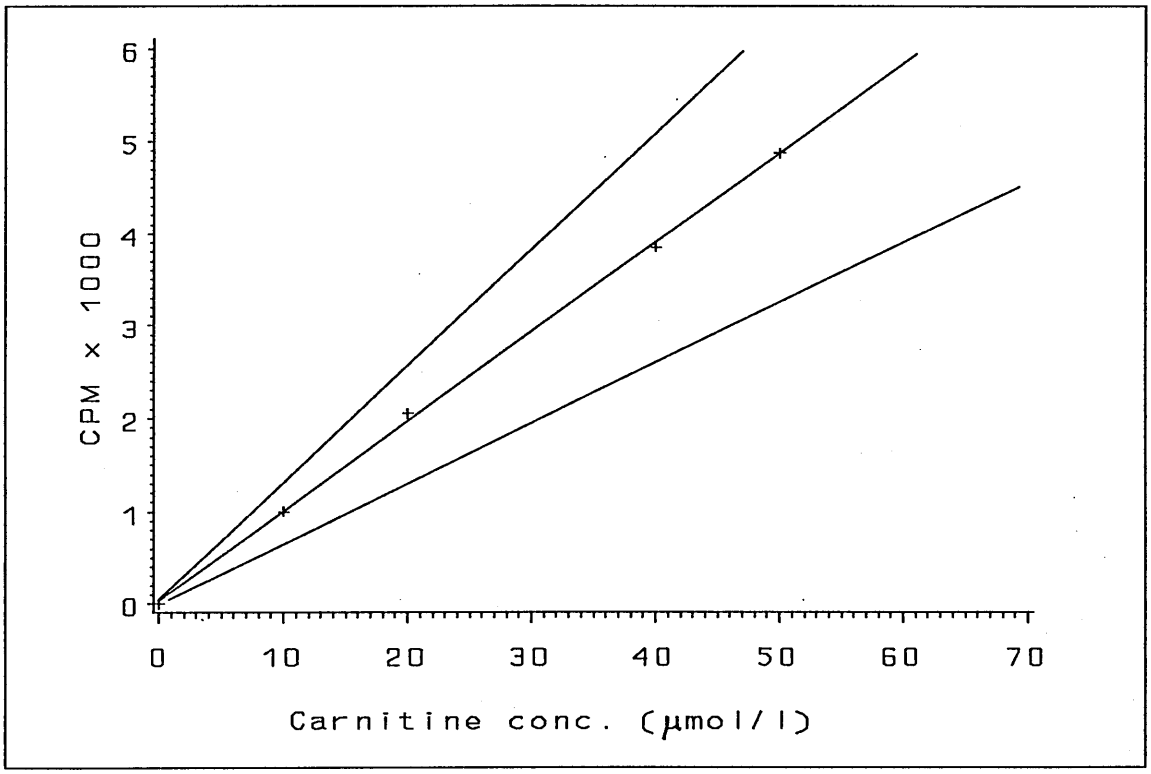


Fig.3.33. **An example of the standard curve calculated during the radio-enzymatic evaluation of plasma carnitine. Also shown are the upper and lower limits for the variation of standard curve during the period July 1989 and February 1990.**

Table 3.18. Mean counts per minute values for four standards 10, 20, 40, and 60 µmol/l free carnitine along with standard deviations and coefficients of variation for values collected over the period July 1989 to February 1990.

Standard conc.	Counts per minute Mean	S.D.	C.V.%
10	1027	80	7.8
20	1929	80	4.1
40	3866	192	5.0
50	5682	120	2.1

Table 3.19. shows an example of results taken from a typical assay run.

Table 3.19. Example of typical duplicate counts per minute results.

Sample	CPM	Mean CPM	Mean CPM - Blank	E.C.C. (µmol/l)	Multi. Factor	A.C.C. (µmol/l)
0 standard (blank)	262 263	263	-	0		0
10 µM std	1128 1125	1127	864	10		10
20 µM std	2001 2042	2022	1759	20		20
40 µM std	4040 4362	4201	3938	40		40
patient 1 (free)	3559 3436	3498	3235	35		35
patient 1 (total)	5174 5189	5182	4919	53.5	2.5	134

CPM = Counts per minute; E.C.C. = Equivalent carnitine concentration read from standard curve; A.C.C. = Actual carnitine concentration in original sample.

3.5 PREPARATION OF ISOTOPE INTERNAL STANDARDS FOR THE QUANTITATION OF URINARY GLYCINE CONJUGATES BY GAS CHROMATOGRAPHY / MASS SPECTROMETRY

3.5.1 PREPARATION RESULTS

Table 3.20. shows the relative abundances of the six isotopic forms of the deuterated hexanoic acid and deuterated hexanoyl glycine (HG) following their respective syntheses. The tetra-deuterated HG was selected to be used as internal standard, IS. Similarly, the tetra-deuterated phenylpropionyl glycine (PPG), with a relative abundance of 31%, and tetra-deuterated suberyl glycine (SG), with a relative abundance of 43%, were selected as the IS isotopic compounds.

The ion fragments that were selectively scanned and used for quantitation are shown in table 3.21. and table 3.23.. Their retention times were seen to vary slightly. Table 3.21. shows an example of typical retention times and the retention time periods over which scanning was carried out. Also shown are published methylene unit values for both TMS derivatives of the non-deuterated conjugates.

Table 3.20. Abundances of hexanoic acid and hexanoyl glycine following their respective syntheses.

No. of deuterated hydrogen ions on molecule	Percentage of total abundance	
	Hexanoic acid	Hexanoylglycine
0	0.19	1.07
1	5.40	0.85
2	77.01	2.51
3	12.88	9.97
4	4.31	73.47
5	0.39	12.12

Table 3.21. Typical retention times for the ion fragments scanned and the windows over which scanning was carried out for the detection of deuterated and non-deuterated glycine conjugates.

Parent compound	TMS deriv.	D or N	Meth. units (AMU)	Ion fragment	Retention time (minutes)	Scan window (minutes)
SG	1	D	22.34	364	23.17	22.5 - 24.0
		N		360	23.20	
	2	D	22.37	436	23.50	
		N		432	23.55	
HG	1	D	16.11	162	15.21	14.5 - 16.5
		N		158	15.25	
	2	D	16.48	204	15.78	
		N		200	15.81	
PPG	1	D	19.83	268	20.09	19.5 - 21.0
		N		264	20.11	
	2	D	19.96	355	20.22	
		N		351	20.24	

D = deuterated conjugate, N = non-deuterated conjugate

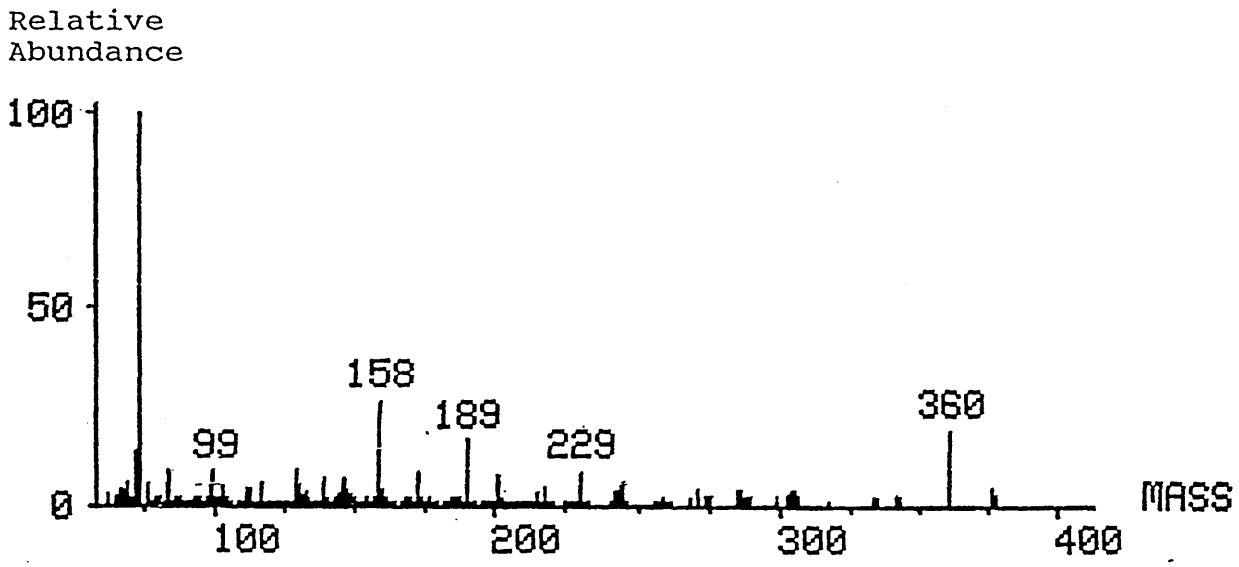


Fig.3.34. GC/MS spectrum (relative abundance against mass) for the TMS1 derivative of non-deuterated SG,

TMS1 and TMS2 derivatives were detected immediately following synthesis of both SG and deuterated SG. However, at the time of calibration each compound was present solely in the TMS1 form.

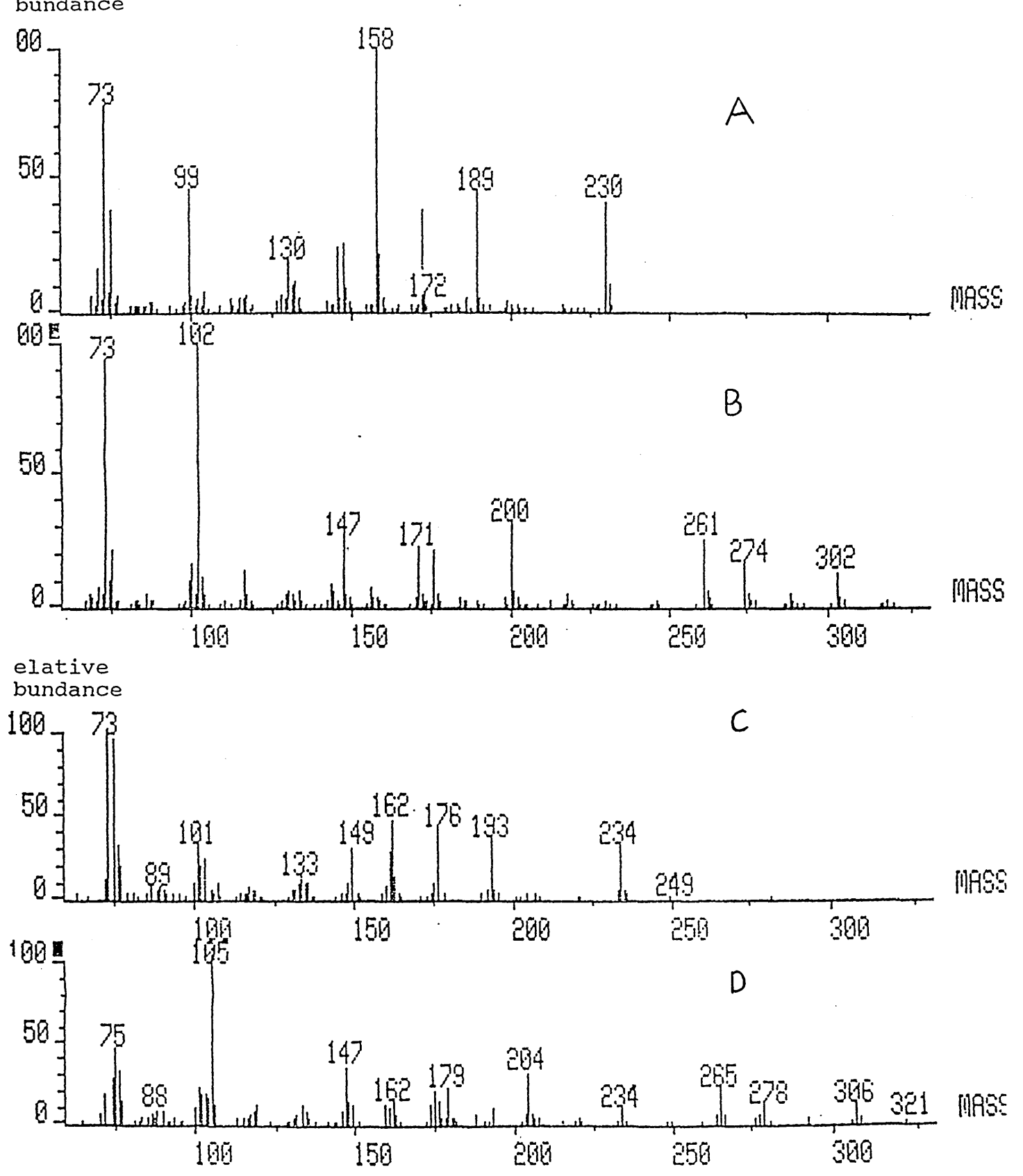


Fig.3.35. GC/MS spectra (relative abundance against mass) for;
A: TMS1 derivative of non-deuterated HG,
B: TMS2 derivative of non-deuterated HG,
C: TMS1 derivative of ($^2H_4$)-HG,
D: TMS2 derivative of ($^2H_4$)-HG.

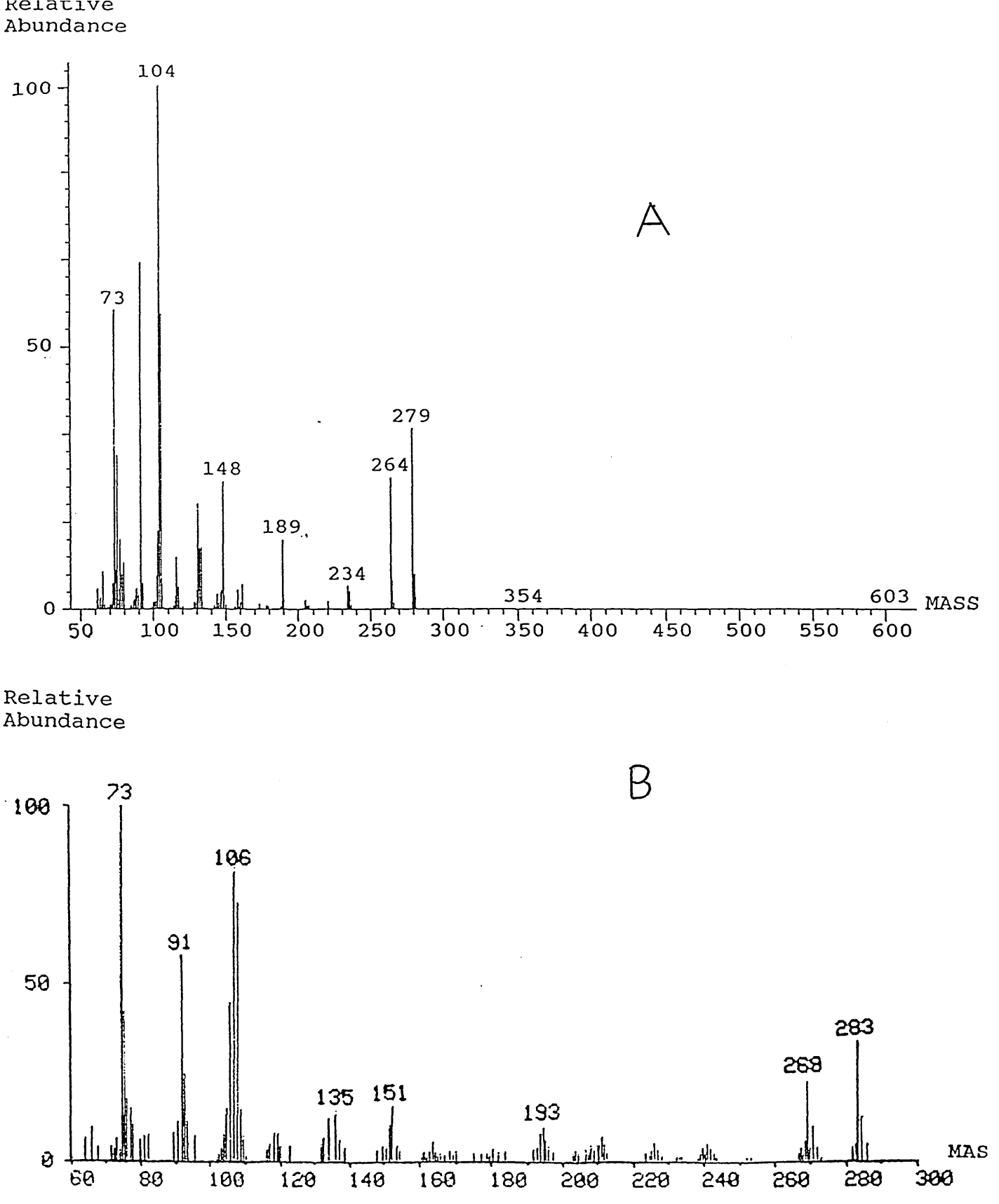


Fig.3.36. GC/MS specta (relative abundance against mass) for;
A: TMS1 derivative of non-deuterated PPG,
B: TMS1 derivative of $(^2H_4)$-PPG.

3.5.2 CONCENTRATION EVALUATIONS

Table 3.22. shows the weights and calculated concentrations of the non-deuterated conjugates used to evaluate the concentrations of the deuterated conjugates.

Table 3.22. Calculations of non-deuterated glycine conjugate solution concentrations.

Glycine conjugate	Molecular weight (AMU)	Weight in 100ml Methanol (mg)	Conc. (µmol/l)
SG	303	2.54	83.8
HG	173	2.65	153.2
PPG	207	2.61	126.1

Table 3.23. summarises which ion fragments were used to detect each TMS derivative of each glycine conjugate deuterated or non-deuterated; resultant peak areas from a single ion monitoring GC/MS analysis; calculated deuterated/non-deuterated peak area ratios for each glycine conjugate; concentrations of the non-deuterated conjugates used (from table 3.22.);and resultant concentrations of the deuterated compounds in the assay.

Table 3.23. Preliminary evaluations of deuterated glycine conjugate concentration.

Glycine conjugate	TMS deriv.	D or N	Scan ion	Peak area	Ratio D/N	Conc. N	Conc. D
SG	1	D	364	428981	0.165	83.8	138
		N	360	2606297			
	No TMS2 present at time of calibration.						
HG	1	D	162	405011	0.318	153	487
		N	158	1843545			
	2	D	204	1563831			
		N	200	4338218			
PPG	1	D	268	20977302	10.321	126	13004
		N	264	1907429			
	2	D	355	25518423			
		N	351	2597491			

D = deuterated conjugate, N = non-deuterated conjugate. All concentrations in µmol/l. A dilution factor of x10 (see section 2.4.4.1) was taken into account when calculating the final column.

3.5.2.1 Choice of working concentration

It was important to supply deuterated IS working concentrations that were both close to the normal urinary glycine conjugate levels (expected to be seen in the vast majority of cases) and yet not too far removed from any abnormally elevated levels, such as those detected in medium chain acyl-CoA dehydrogenase deficiency. For this reason, it was decided to use working deuterated HG and PPG solutions equivalent to the upper limit of the normal control results published by Rinaldo *et al*[143] (table 3.24). Table 3.25 shows the data used to calculated the volumes required to produce such concentrations assuming a creatinine level of 1mmol/l. Yields of deuterated SG proved insufficient, at such high concentrations, to supply a significant volume to cater for an adequate

number of urine assays. It became apparent that in order to produce 100ml of working IS the total SG yield would produce a concentration of 4.6 µmol/l (table 3.26.). Such a concentration, whilst not as high as one would have liked, was acceptable.

Table 3.24. Ranges of normal control values for glycine conjugates found in the urine of children ranging from 1 week to 9 months of age.

GLYCINE CONJUGATE	MOLECULAR WEIGHT	RANGE OF NORMAL CONTROL VALUES	
		*µg/mg creat.	µmol/mmol creat.
SG	303	0 - 95	0 - 35.4
HG	173	0.21 - 1.9	0.14 - 1.24
PPG	207	0 - 1.1	0 - 0.6

***Ranges taken from Rinaldo *et al*[143]. Unit conversions based on creatinine molecular weight of 113 AMU. Abbreviations: creat. = creatinine.**

Table 3.25. Evaluation of the volume of stock deuterated HG and PPG required in 100ml with methanol in order to create the concentrations recorded in table 3.24.

Glycine conjugate	Stock conc.	*Required working conc.	Required stock conc.	Required vol.in 100ml
HG	487	1.24	37.2	7.64
PPG	13005	0.6	18.0	0.14

All concentrations in µmol/l. *Upper limit of control values[143] assuming creatinine of 1 mmol/l. A factor of 30 was used in calculating the required stock concentration (sec. 2.4.4.1.)

Table 3.26. Evaluation of the working concentration of deuterated suberylglycine obtained by using a stock solution of the total yield of conjugate in 100ml methanol.

Glycine conjugate	Stock concentration	Dilution factor	Working concentration
SG	138	30	4.6

All concentrations in μmol/l.

3.5.3 CALIBRATION

Preliminary results for the accurate calibration of the deuterated glycine conjugates are listed in table 3.27.. The corresponding calibration curves are shown in figures 3.37. to 3.39.. Whilst these preliminary results seemed acceptable they proved difficult to reproduce and time constraints prevented any further analyses.

Table 3.27. Calibration results for the accurate calibration of deuterated SG, HG and PPG.

Glycine conjugate	STD conc. µmol/l	Quadruplicate 1	2	N/D peak area ratios 3	4
SG	0	0.035	0.057	0.072	0.088
	1	0.175	0.155	0.181	0.169
	2	0.312	0.299	0.295	0.315
	3	0.495	0.482	0.475	0.491
	4	0.645		0.648	
	5		0.751	0.735	0.725
HG	0.00	0	0	0	0
	0.25	0.275	0.243		0.253
	0.50	0.389	0.421	0.410	0.378
	0.75	0.606	0.556	0.691	0.589
	1.00	0.812	0.855	0.805	0.942
	1.25	1.031	1.015	1.025	
PPG	0.0	0	0	0	0
	0.1	0.221			0.277
	0.2	0.385	0.354	0.244	0.405
	0.3	0.437	0.523	0.481	0.444
	0.4	0.584	0.568	0.608	0.564
	0.6	0.865	0.888	0.935	0.955

N = non-deuterated conjugate; D = deuterated conjugate.

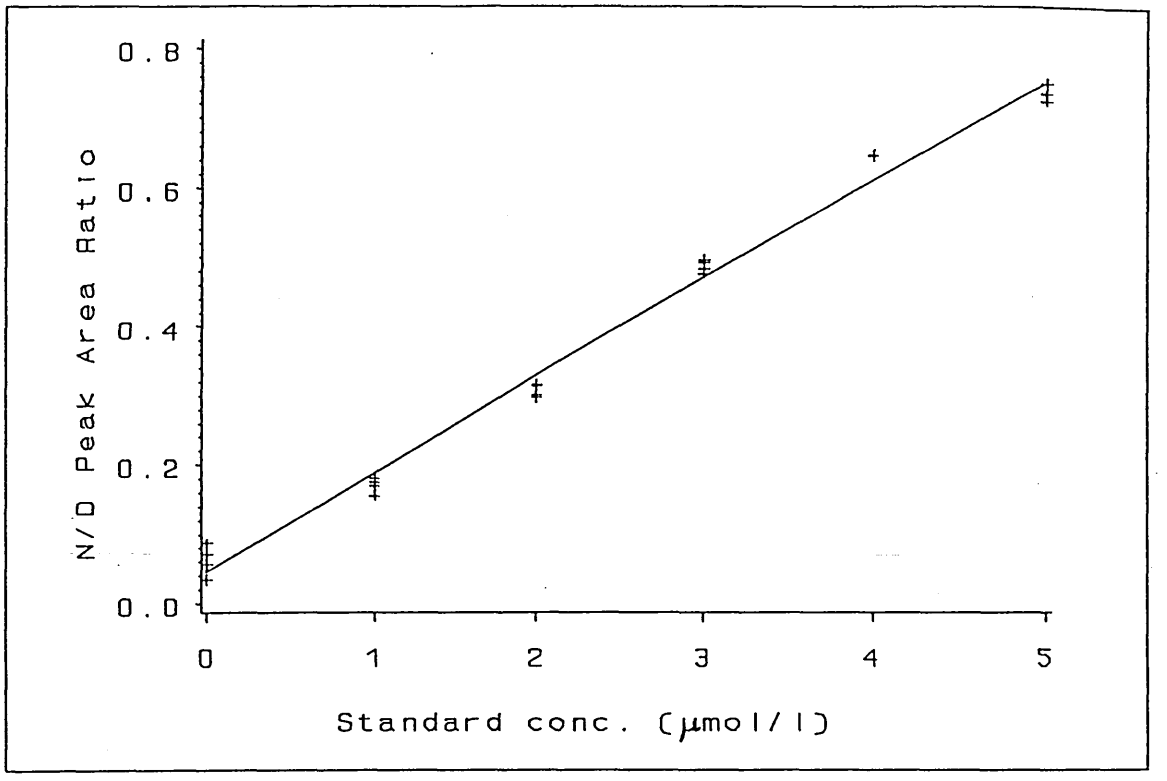


Fig.3.37. Calibration curve for deuterated SG by linear regression.

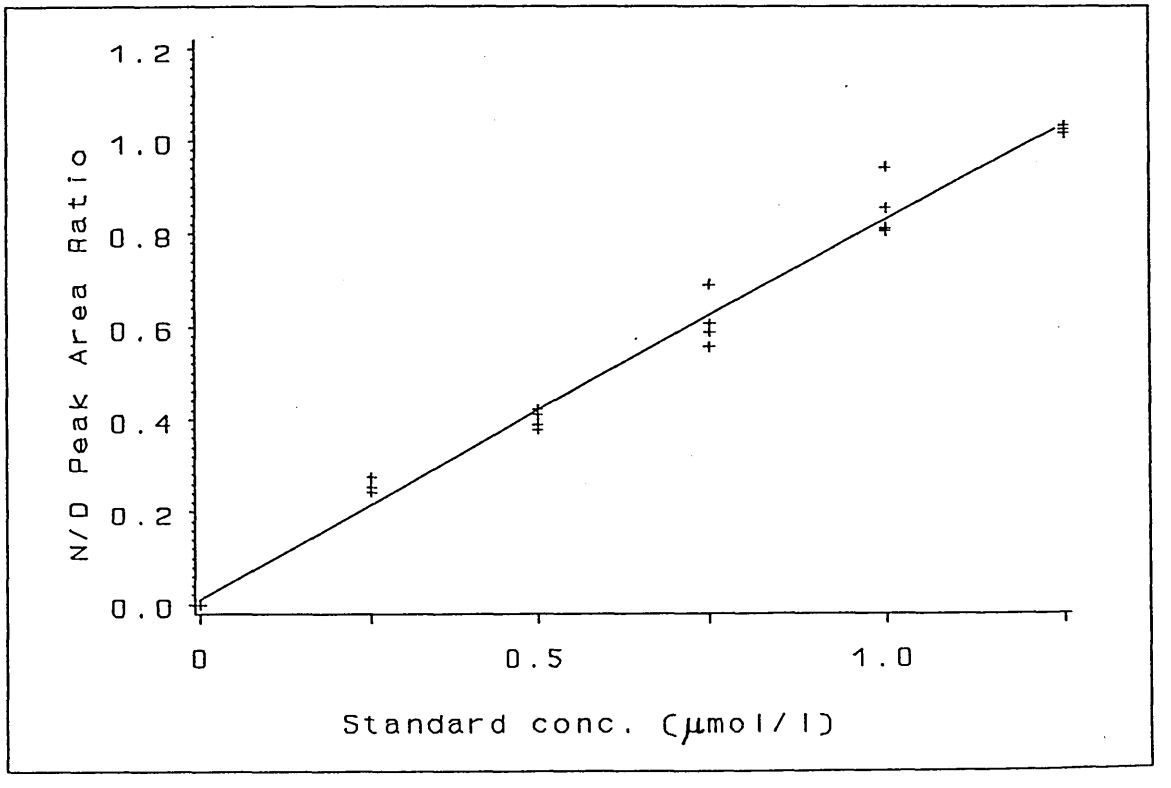


Fig.3.38. Calibration curve for deuterated HG by linear regression.

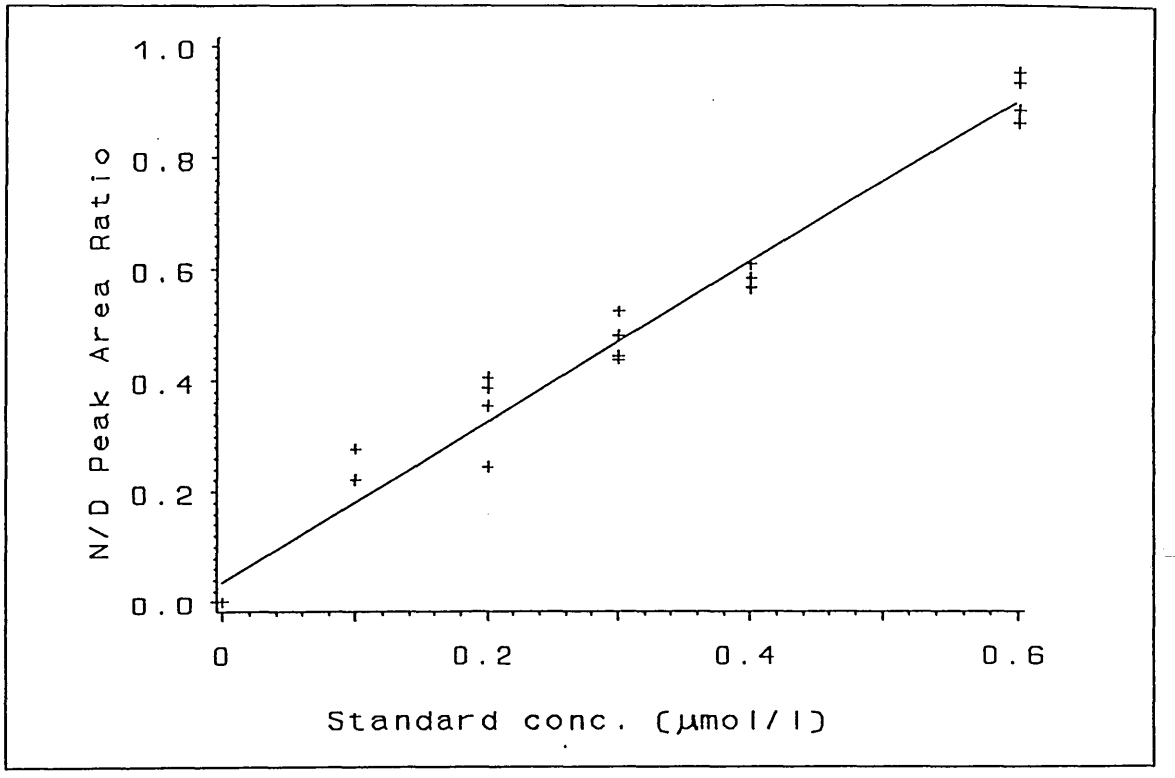


Fig.3.39. Calibration curve for deuterated PPG by linear regression.

CHAPTER 4

DISCUSSION

4.1 INTERMEDIARY METABOLITES INVESTIGATION

4.1.1 PAIRED METABOLITE CORRELATION PLOTS AND FASTING PROFILES (NORMALS)

In considering paired metabolite correlation plots involving control patients one must expect variation as a result of differing glucose homeostasis, hormonal and metabolic adaptation[178] and glycogen reserves. All of these factors may change with age[178] (see section 4.1.4) as well as the general state of health of the subject.

4.1.1.1 Supply of free fatty acids to the mitochondria

As free fatty acids are release from adipose tissue in response to low blood carbohydrates one would expect a negative correlation between free fatty acids,FFA, and glucose values, figure 3.02.. A poor correlation (cc=-0.4 table 3.03) may be partly caused by unevenly distributed glucose values but may also be a sign of a successful homeostatic mechanism. This is now explained.

In response to low blood glucose levels, fat metabolism is increasingly used as a fuel source. If one were to plot activities of glycolysis and lipolysis against blood carbohydrate levels an initial relationship similar to that of region A in figure 4.01. would be seen.

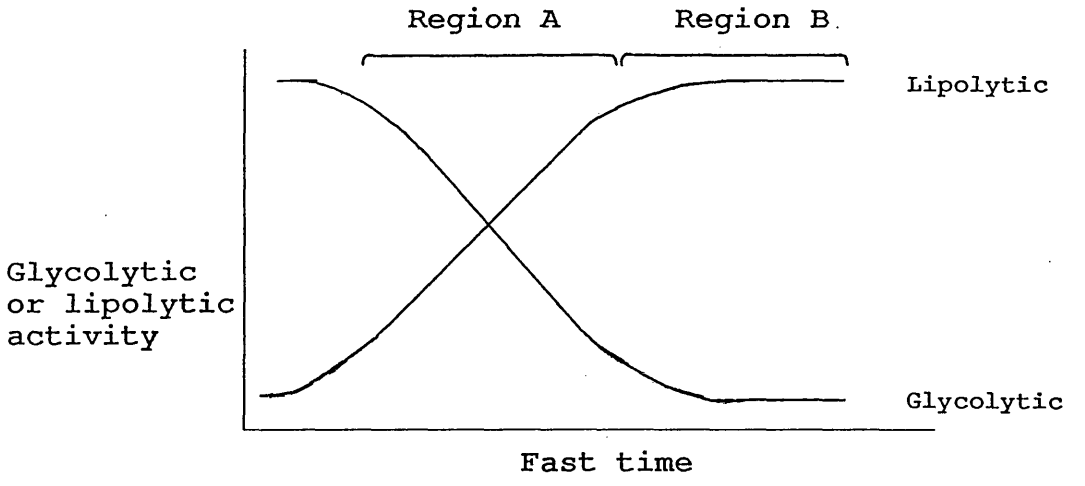


Fig.4.01. Theoretical plot of activities of glycolysis and lipolysis against blood carbohydrate levels.

However, as glucose levels fall lower, glucose conservation techniques (see section 1.2.2) become more pronounced. These have the effect of decreasing the rates of both the decline in glucose levels and the increase in fat metabolism (region B figure 4.01.).

In a healthy patient the level of FFA can be taken as an indicator of the rate of fat metabolism. Therefore, if the population of samples in this study extends into region B then the correlation of FFA(and to a lesser extent the %carnitine esters and 3-hydroxybutyrate) against glucose will be diminished. Any such effect would be expected to show on fasting profiles for glucose and FFA. It is difficult to see any indicative pattern in Figures 3.19. and 3.20. although it should be noted that the patients used for these plots were only a proportion of all patients in the study. Therefore, it may be the case that some samples were taken outside region A of figure 4.01.

Finally, the p value of no correlation for FFA against glucose controls is very low ($p=0.0001$, table 3.03) and, even when considering such a large sample size, is quite significant providing further evidence of correlation.

4.1.1.2 Transport into the mitochondrial matrix

As the CoA esters of the FFA reach the mitochondria they are converted to acyl carnitine to be transported across the inner mitochondrial membrane. Thus the level of esterified carnitine would be expected to increase with increasing FFA. As FFA increase in response to low blood glucose, carnitine esters should also be seen to increase. This is reflected in the plots of esterified carnitine against glucose (figure 3.05) and FFA (figure 3.14) which show very good linear correlations (CC=-0.6 P=0.0003, and CC=0.6 P=0.0003 respectively, table 3.03).

The overall poor appearance of the other carnitine correlation plots is thought to be partially attributable to a smaller sample size. One negative result for esterified carnitine was recorded (Patient 103). Here the total and free values were very similar 18 µmol/l and 22 µmol/l respectively. A difference of 4 µmol/l at approximately the 20 µmol/l level lay within the limits of the variation of the assay (±2SD = ±2.4 - Quality control results 1989) and on this basis the negative carnitine result was assumed to be zero in the correlation plots. This value remains the lowest carnitine value whether it is negative or zero. Therefore, the correlation coefficients, being calculated on the ranks of the values, will remain unaffected.

Expressing carnitine esters as a percentage of the total carnitine value was seen to produce better correlations with the other metabolites than did the straight forward esterified values. The reason for this is not clear but it may indicate some relationship between total and esterified values. For example, a diminished supply of carnitine would limit esterification. However, this would not be expected in normal healthy individuals. Further, if total carnitine was limiting to esterification the amount of free carnitine would be minimal. Low free carnitine values were not evident in these results.

4.1.1.3 Production of ketone bodies

Production of 3-hydroxybutyrate, 3HB, one of the ketone bodies, in association with increasing ß-oxidation rates is shown in correlations of this metabolite against glucose, FFA and percent carnitine esters, %CE (figures 3.06, 3.15 and 3.18 respectively).

Correlation of 3HB to %CE is extremely good (cc=0.8) possessing the highest correlation coefficients of all the plots. Interestingly the correlation coefficient for plots with metabolites involved in early stages of fatty acid metabolism decrease in accordance with how far one has travelled back along the pathway (see figure 4.03.). For example the correlation of 3HB against %CE is very high whilst that for 3HB against FFA is lower with 3HB against glucose lower still.

4.1.1.4 Correlation of intermediary metabolite values with lactate

There is no apparent correlation of lactate with any of the intermediary metabolites (figures 3.01. and 3.07. to 3.11.). KJB Lamers only reported a very slight correlation with glucose (cc=0.34) and JP Bonnefont[178] reported no significant difference between 15 and 24 hour fasting lactate values from children between the age of 1 and 12 months.

Lactate is both utilised by gluconeogenesis and produced by active skeletal muscle as described by the Cori cycle (figure 4.02.). The activity of the subjects in this study was not controlled and therefore, neither was their production of lactate. Further, gluconeogenesis is not solely reliant on the levels of lactate. Glycerol and amino acids also feed this pathway. Therefore, it is difficult to predict whether lactate will increase or decrease with length of fast. It maybe that lactate is too separated from fatty acid metabolism to show any clear relationships in plots against the other intermediary metabolites.

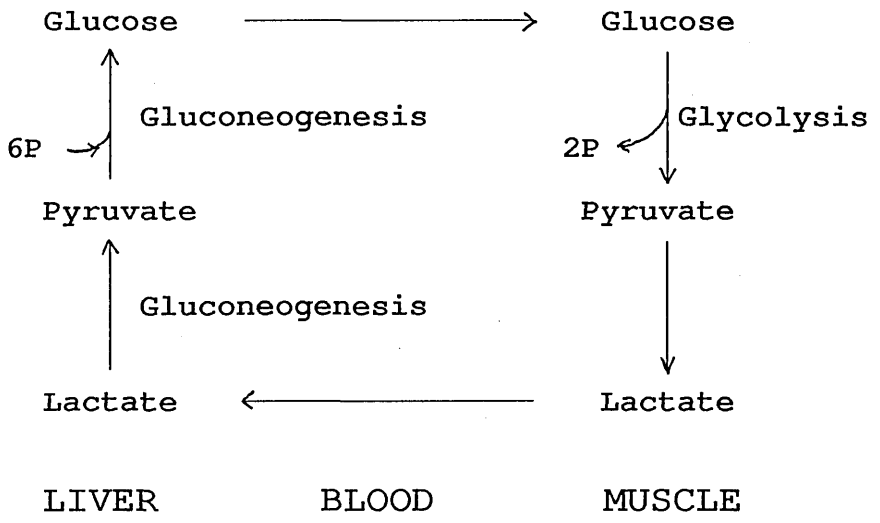


Fig.4.02. The Cori cycle (P = ATP equivalents).

4.1.1.5 Effects of length of fast on intermediary metabolite levels

The negative correlation between glucose and length of fast (figure 3.19.) was as expected. The lactate profile is again poor probably for the same reasons discussed earlier. The other intermediary metabolites FFA, %CE and 3HB all increase with fast (figures 3.20., 3.22., 3.23. respectively), as a result of rising demand for energy from non-glycolytic pathways. Total carnitine showed no change in response to fast and therefore because of the increase in %CE there was a necessary decrease in free carnitine (figure 3.21.). Interestingly, FFA/3HB decreased as the length of fast increased (figure 3.24.). This indicates a growing rate of 3HB production relative to that for FFA. From figure 3.23. the relationship between 3HB and fasting time may be non-linear suggesting an increase in the rate of 3HB production with fast, within the first 25 hours at least. Such a response may be connected to an increasing use of ketones as an energy source. From figure 3.20, the rate of FFA increase may also rise during fast but to a lesser extent.

4.1.2 PAIRED METABOLITE CORRELATION PLOTS AND FASTING PROFILES WITH DEFECTS OF FATTY ACID OXIDATION

4.1.2.1 The basic principles

The basic principle involved in the use of paired metabolite correlations as indicators of fatty acid oxidation defects is to use the normality/abnormality of each plot as an indicator of the states of the pathway/s involved between the two metabolites in question. Obviously, the closer these metabolites are along the fatty acid metabolic pathway (as regards the number of basic stages in between) the fewer the number of metabolic processes involved. For example, a direct conversion of one metabolite into a second would normally only involve one basic process (although this may involve several chemical stages). A paired correlation of these two metabolites would then provide a direct indication of the state of this process. Similarly, comparison of results obtained from a plot involving say three successive metabolic processes and a paired plot involving just the latter two of these processes would deductively provide and indication of the state of the first. By such deductive reasoning and direct investigations each stage, from one metabolite to the next, along the metabolic pathway can be tested.

In short, for a section of the fatty acid oxidation pathway to be tested in this way the initial and final metabolites involved within that section must be measured and included in appropriate paired metabolite correlations.

4.1.2.2 Medium chain acyl-CoA dehydrogenase deficiency

Appropriate samples from two diagnosed medium chain acyl-CoA dehydrogenase (MCAD) deficient patients, patients 40 and 94 (both shown by point 1 on the correlation plots), were available to the study.

In this disorder the metabolic block lies within the ß-oxidation cycle in the mitochondrial matrix (see section 1.10.4). Free fatty acid response to low carbohydrate levels is therefore unaffected. This is reflected in the normal position of patient 40 on the FFA against Glucose plot (figure 3.02.). Unfortunately, patient 94 was treated with glucose prior to sampling. This resulted in her apparent non-fasted state (glucose = 7.6 mmol/l).

The increase in carnitine esters during fasting, as acyl groups are transferred across the inner mitochondrial membrane, is also unaffected by MCAD deficiency. This is indicated by the normal position of the values measured for patient 40 on the free and esterified carnitine against glucose plots (figures 3.04. and 3.05.). An apparent depletion of total carnitine for patient 40, noticed on the total carnitine against glucose plot (figure 3.03.), may be an indication of the secondary, systemic carnitine deficiency often associated with MCAD deficiency. Carnitine values for patient 94 were unavailable

Hypoketotic hypoglycaemia is evident for patient 40 on the plot of 3HB against glucose (figure 3.06.). Once again patient 94 did not appear to be sufficiently stressed to require ß-oxidation for energy thus no indication of fatty acid oxidation defect is seen. In fact, it has been documented that a blood glucose level of below 3 mmol/l is necessary to clearly discriminate fatty acid oxidation defects by correlations of 3HB against glucose[178] (see section 4.1.4).

The normality of the lactate levels for both patients is indicative of an unaffected gluconeogenesis.

4.1.2.3 Glycogen storage and mobilisation defects

Patient 69, represented by point 6, shows a defect in the storage or mobilisation of glycogen. This results in depressed glycogenolysis and therefore abnormally low glucose during fasting. However, although glucose values are slightly low on the fasting profile (figure 3.19.), the point is not clearly distinguishable from the control group probably due to homeostatic conservation of glucose as previously explained. However, the high value on the fasting profile for FFA (figure 3.20.) does suggest an increased reliance on fatty acids as a fuel source. FFA and 3HB levels in relation to glucose appear normal (figures 3.02 and 3.06). Although data for lactate and carnitine were not available one would also expect these to follow the normal trend of the control values. Carnitine values after twelve hours of fast were available and showed normal levels. These were not included in the plots due to the sample selection procedure (see section 2.5.3).

A similar normal pattern is suggested for patient 95 (point 2) diagnosed as suffering from glycogen storage disease type 1A. In this disease a deficiency of microsomal glucose-6-phosphatase severely suppresses glucose-6-phosphate conversion to glucose. Therefore, again glycogenolysis is diminished. Very high lactate levels are also usually exhibited. Unfortunately data on this patient is limited to just 3HB and FFA. From the plot between these two metabolites (figure 3.15.) it appears that this patient was not fasted as long as patient 69 that is to say FFA and 3HB are much lower. As such the demand on glycogenolysis for glucose was not as great and therefore any defect would not be as prominent. This would explain the closeness of this point to the control values. The importance of sufficient fasting times in order to distinguish such defects on paired correlation plots is discussed further in section 4.1.4.1.

The position of this defect is not encompassed by the metabolites measured in the study and therefore, the correlation plots cannot be used to distinguish the position. However, even in these circumstances, correlation results can still supply evidence of a defect.

4.1.2.4 Isolated glucocorticoid deficiency

As mentioned in section 1.2.1, the glucocorticoids facilitate FFA release from adipose tissue[9]. Further, adrenalectomy in rats has been reported to cause an increase in ketone body levels[187] which is probably due to the use of ketone bodies as an alternative fuel source. A deficiency of these hormones will therefore result in insufficient FFA levels at times of metabolic stress but elevated 3HB in relation to the FFA that are released. These points are reflected in patient 96 (point 3). An inappropriate FFA level with respect to that of glucose is shown in figure 3.02. The normality of the stages between FFA reaching the liver and their acyl-CoA esters being converted to acylcarnitine is reflected in a normal free carnitine against FFA plot, figure 3.13.. Total carnitine and therefore %CE values were not available. Finally, elevated 3HB in relation to FFA levels is indicated in figure 3.15. reflecting the rise in ketone levels in the absence of the adrenal cortex hormones.

Both free carnitine against glucose (figure 3.04) and 3HB against free carnitine (figure 3.17) plots are on the upper limit of normal but there is no distinct departure from the normal values. Usually, one would expect inappropriate FFA compared with glucose and raised 3HB production to result in raised values for free carnitine against glucose and 3HB against free carnitine respectively. However, the way in which the corticoids suppress ketone levels is by inhibition of ß-oxidation. The site of this inhibition is thought to be that of carnitine palmitoyl transferase I and the method by an elevation in the malonyl-CoA level. Therefore, the absence of elevated 3HB against free carnitine may be a reflection of an inhibited acyl carnitine production maintaining a high free carnitine level. An inhibited carnitine esterification should further increase free carnitine with respect to glucose. Surprisingly, this is not seen in figure 3.04 and the reason for this is unclear. An increase in the rate of gluconeogenesis, in an attempt to supply the energy that a suppressed ß-oxidation cannot, may enhance glucose levels counteracting any elevated free carnitine value. However, such a glucose enhancement is not evident in figure 3.02 and would presumably result in a further increase in free carnitine.

4.1.2.5 Hyperinsulinaemic hypoglycaemia

A number of patients, clinically consistent with the diagnosis of hyperinsulinaemic hypoglycaemia, have been included into the study (point 4's). None were fasted but the nature of the disease supplied the required hypoglycaemia.

In this disease there is an abnormally high level of insulin, a pancreatic hormone that plays a key role in the control of the direction of flow through the metabolic pathways. It increases synthesis of glycogen, fatty acids and proteins and promotes glycolysis. In this way the blood glucose level is decreased and FFA release is inhibited.

A primary indication of this disease is very low plasma FFA and glucose. This is clearly shown in FFA against glucose correlation (figure 3.02.). Naturally, if FFA in comparison to glucose is low so too will be the levels (in relation to glucose) of the related metabolites further along the fatty acid metabolic pathway, namely carnitine esters and 3HB whilst free carnitine will be elevated. Again this can be seen in the relevant correlations against glucose (figures 3.04., 3.05. and 3.06.), although, three points show almost normal free carnitine against glucose plots. This is discussed later in this section.

From the plots of lactate against glucose (figure 3.01.) all the points, whilst exhibiting a distinct shift because of the extremely low glucose levels, span the whole of the control range of lactate levels with some showing elevated values. A particularly high lactate is seen for (patient 133). Insulin does decrease gluconeogenesis by lowering the activities of some of the enzymes involved such as pyruvate carboxylase and fructose-6-diphosphatase. Therefore, a high lactate level in respect to low glucose may occur. However, as discussed in section 4.1.1.4., it is difficult to predict the type of correlation between lactate and the other metabolites.

Carnitine values were available for five of the patients, 115, 116, 122, 132 and 134 . All showed inappropriate %CE in relation to their glucose levels (figure 3.05) as mentioned above. Three showed low total carnitine values which seemed to counteract the depressed %CE against glucose to produce normal free carnitine against glucose (figure 3.04.). They also either showed normal (patients 132 and 114) or high (patient 134) %CE in relation to FFA and 3HB (figures 3.14. and 3.18 respectively). It is likely that this, along with the low total carnitine level, is the reason for the low free carnitine in relation to FFA and 3HB (figures 3.13. and 3.17.). Such values for the other two of the five patients were normal.

The reasons for an apparent carnitine deficiency in three of the hyperinsulinaemic hypoglycaemic patients, and also for the elevated %CE in relation to FFA and 3HB for patient 134 are unclear. These patients tended to be very young at sampling and much younger than most of the control children. Therefore, any comparison against the controls should be treated with caution.

All patients showed normal 3HB against FFA plots (figure 3.15.).

4.1.3 PAIRED METABOLITE CORRELATION PLOTS AND FASTING PROFILES WITH UNKNOWN DEFECTS OF FATTY ACID OXIDATION

Four patients suspected of having a defect in energy production during times of low blood glucose levels were included in the study. They provided examples of the use of paired metabolite correlation in diagnosing such defects and the way in which an overall picture such as that in figure 4.03. can be formed. Figure 4.03. is a simplified diagrammatic representation of the way in which the seven metabolites, measured in the study, are linked. It should be noted that it is not a representation of the reactions of the metabolic pathway.

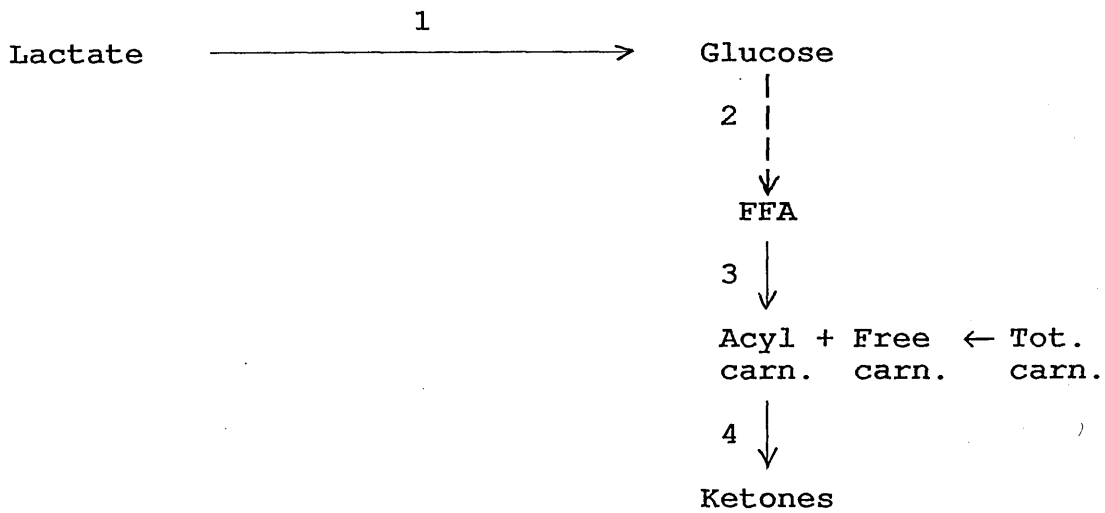


Fig.4.03. An example of the type of overall picture that can be built up from evidence provided by paired metabolite correlation studies. (KEY: 1 = gluconeogenesis; 2 = production of FFA in response to plasma glucose levels; 3 = passage of FFA into the cell and their incorporation into acyl carnitine; 4 = the transport of acyl carnitine into the mitochondria, the incorporation of the acyl groups into ketogenesis and the production of 3-hydroxybutyrate.

4.1.3.1 Defect in gluconeogenesis

For patient 9 (point 5) two steps on figure 4.03. appear abnormal. High lactate and low glucose levels suggest a defect in gluconeogenesis (step 1) whilst an inappropriately low FFA value in comparison to glucose (figure 3.02) indicates a defect in FFA production or mobilisation in response to hypoglycaemia (step 2).

Free and esterified carnitine values in relation to FFA and 3HB appear normal (figures 3.13, 3.14, 3.17 and 3.18). This indicates an unaffected transfer of acyl groups across the mitochondrial membrane and ketone production (steps 3 and 4 respectively).

It is possible that there exists a threshold rate of free fatty acid release from adipose tissue. If glucose levels were to fall below that which would lead to this maximum rate of free fatty acid release (as may be the case with defective gluconeogenesis) then it may appear that there was a defect in FFA response to hypoglycaemia. This is exactly what is observed for this patient. However, the apparent capability of some patients to liberate much more free fatty acids in response to equivalent or lower glucose levels seems to contradict this postulate. Alternatively a real second defect may be present in this patient.

Therefore, patient 9 results are consistent with a defect in gluconeogenesis with the possibility of a second defect in FFA production or mobilisation.

4.1.3.2 Defect in acetoacetate utilisation

Separation of patient 43, represented by point 7, from the controls is not so clear.

Lactate and glucose values are normal suggesting a normal step 1 on figure 4.03. The FFA against glucose plot (figures 3.02.) appears normal reflecting appropriate FFA release in relation to glucose (step 2 on figure 4.03). The 3HB against glucose plot (figure 3.06.) lies on the limit of the control group but there is no definite separation. Whilst %CE is slightly elevated in relation to FFA (figure 3.14) all other carnitine plots against FFA and glucose (figures 3.03 to 3.05, 3.12, 3.13) show borderline-normal positions. This indicates that acyl transfer into the mitochondria (step 3 on figure 4.03) is probably normal. 3HB against esterified carnitine (figure 3.18.) appears borderline-normal reflecting an appropriate ketone production (step 4 on figure 4.03). Most noticeable is the 3HB against FFA plots (figure 3.15.). Here the point is elevated.

A suppressed ketone body utilisation will result in a concomitant build up of both acetoacetate and 3HB. During early stages of fast as ß-oxidation exists as the major energy source a defect in ketone utilisation will pass largely unnoticed. As the fast progresses into the second and third day an increased reliance on ketones as fuel sources will magnify any inherent defect. Upper borderline 3HB levels are evident in the intermediary metabolite plots but because of a limited fast time (less than 16 hours) the contribution of ketogenesis to the patients overall energy supply is insufficient to create any outstanding effect.

These results indicate a normal fatty acid metabolism with all steps on figure 4.03. appearing normal. High 3HB in respect to FFA may be a result of defective ketone body utilisation although this does not explain the apparent normality of the other 3HB plots or the elevated %CE against FFA plot. This disorder has subsequently been associated, by alternative methods, with a defect in acetoacetate utilisation.

4.1.3.3 Spontaneous hypoglycaemia

Patient 119, point 8, was entered into the study as a control. Eight days later she suddenly developed spontaneous hypoglycaemia, without fast. Extremely low glucose levels are apparent in all glucose plots indicating the hypoglycaemia. Lactate levels appear borderline agreeing with a normal gluconeogenic utilisation (step 1 on figure 4.03).

The FFA against glucose plot (figure 3.02.) is suppressed suggesting a metabolic defect lying within the release of FFA during times of low blood glucose (step 2 figure 4.03).

Total carnitine levels are high but esterified carnitine production in accordance to the glucose level (figure 3.05.) is low despite an abundance of free carnitine. This reinforces the idea of defective free fatty acid generation (step 2). %CE increase with increasing FFA appears normal (figure 3.14) indicating normal acyl-CoA conversion into carnitine esters (step 3 figure 4.03).

3HB production compared to the level of %CE is normal (figure 3.18), evidence for good ß-oxidation-ketone production interplay (step 4 figure 4.03.) and 3HB against FFA (figure 3.15.) are quite normal again echoing the normality of fatty acid metabolism following free fatty acid arrival at the mitochondria.

In summary, paired metabolite correlation plots for patient 119 provide evidence of a fatty acid metabolic defect lying in liberation of free fatty acids from adipose tissue in response to low carbohydrate levels in the blood.

4.1.3.4 Defects in gluconeogenesis and 3-hydroxybutyrate production

For patient 121, represented by the point U, the glucose level is very low and that of lactate elevated. This suggests a possible gluconeogenic defect (step 1 on figure 4.03.). The FFA and free and esterified carnitine levels in relation to glucose all appear normal as does that of %CE (figures 3.02, 3.04, 3.05) and %CE against FFA (figure 3.14). This indicates a normal fatty acid release and acyl group transport into the mitochondrial matrix (point 2 and 3 figure 4.03). 3HB levels are very much suppressed in relation to glucose (figure 3.06.), free fatty acids (figure 3.15) and %CE (figure 3.18) indicating a suppressed ketogenesis (point 4 figure 4.03).

Further evidence for the gluconeogenic defect is given by the similarities between points for this patient and those of patient 9 represented by point 5 (see section 4.1.3.1).

Paired metabolite plots therefore provide evidence for the presence of two defects in fatty acid metabolism. One lies within the gluconeogenic incorporation of lactate. The second lies in the production of ketones in conjunction with high ß-oxidation flux. Such a duel block would explain the difficulty in diagnosis of this child's defects up to her inclusion into the study.

4.1.4 POSSIBLE FUTURE DEVELOPMENTS

4.1.4.1 Exact fasting times

Although, exact fast times would be particularly useful on the fasting profiles, one can see that they are not essential for paired metabolite correlation studies. However, it is important that the length of fast is sufficient to exert enough pressure on fat metabolism and ketosis to elicit any defect. This period would seem to be typically more than six hours or, more specifically, until glycaemia levels fall below 3mmol/l. Although most of the abnormal samples were below this limit, the vast majority of normal patients exhibited glucose values between 3 and 5mmol/l. Normal data collected over a wider range of fast would increase definition of the correlations and therefore would be a possible refinement for further studies.

4.1.4.2 Necessary correlations

It should also be noted, as suggested in section 4.1.2.1, that not all the correlation plots used in this study are necessary for an assessment of the defect of a patient. Usually if all metabolite levels are known only the correlations between adjacent metabolites on figure 4.03. are necessary with the remainder serving only to reinforce any conclusions. Also paired metabolite correlations can prove useful even if the metabolites involved do not encompass the position of the defect as shown in section 4.1.2.3.

4.1.4.3 Dependence on age

JP Bonnefont and co-workers[178] have shown that levels of oxidation metabolites during fast differ with age depending on the quality of glucose homeostasis. They

state that children older than 7 years show better glucose conservation and later onset of fatty acid metabolism as a result of starvation. In this study, although all patients were young, records were too sparse to collect an 'age' variable. Perhaps this should be considered in further studies.

4.1.4.4 Increase in sample size

If paired metabolite plots were set up within the clinical laboratory, data could be continuously added to build up a substantial sample size. Correlations would no doubt become much clearer against which data from suspected fatty acid oxidation defects could be easily compared. The biochemist would therefore have a quick, reliable and easy to use indicator as to the metabolic step or region presenting abnormal. Relevant treatment and further testing could then be carried out. Also, the inclusion of more data from diagnosed patients would strengthen the reliability of these procedures as well as highlight their limitations, a very important factor.

A number of samples may be collected from a subject and in this way fasting profiles could be set up for single individuals and compared with the fasting profiles calculated from multi-patient populations included in this study. Unfortunately, although in some cases such multiple samples from individual patients were collected the data was too sparse to permit any sensible investigation in this area.

As sample size increases and the difference between normal and abnormal becomes more defined computerisation of the whole process may be an option. This on-line facility could then refer back to the clinician any patients producing anything other than completely normal plots.

4.2 GENERALISED DICARBOXYLIC ACIDURIA INVESTIGATION

4.2.1 THE ASSOCIATION WITH THREE POSSIBLY RELATED FACTORS

From the Fisher's two tailed analysis of the connection between generalised DCA and sibling history of SIDS, feed and gender (table 3.05.) one can see high p values for the association of sibling history of SIDS (0.77) and gender (0.73) with generalised DCA. This suggests no association, whilst that for the method of feeding (0.08) is appreciably lower providing evidence of a possible relationship, if only at the 10% level of significance. Also, from table 3.06. there are similar percentages of males and females and similar percentages of siblings and non-siblings of SIDS within each of the two classes 'presence' and 'absence of generalised DCA'. The percentage of breast fed neonates exhibiting generalised DCA is more than three times that for bottle feeders and the percentage of bottle fed neonates that show no such urinary pattern is more than twice that for breast feeders.

These findings indicate that sibling history of SIDS and gender are not associated with generalised DCA but there is evidence that the method of feeding is connected to the phenomenon. It should be noted that sample sizes are low and whilst Fisher's exact test is an accepted test to use on such small numbers any statistical conclusions should be treated with caution.

4.2.2 URINARY CARNITINE REFERENCE RANGE EVALUATIONS

The frequency diagrams and basic statistics (figures 3.25. to 3.27. and tables 3.07. to 3.09.) show skewed distribution for all carnitine evaluations. In a normal healthy baby, as the time difference between birth and sampling increases, the remaining metabolic stress produced by birth will diminish. Hence, any abnormal carnitine values resulting from this stress will return to normal as time progresses. Maebashi et al[181] reported a return to normal carnitine excretion within 5 days following a several fold elevation due to a 5 day fast. A variation of sampling age may therefore create skewed distributions. However, figures 3.28. to 3.30. do not indicate any significant relationship between carnitine values and age. It is likely that such a short time period following birth is not sufficient to create any detectable change in carnitine levels. Perhaps larger sample size would have supplied a more normal distribution.

Reference ranges obtained from the subset containing both generalised DCA and non DCA patients are in good agreement with published data for children 3 months and upwards[182] (see table 4.01.). Variations in normal range representations coupled with differences in the units of the reported data often created complications in such comparisons. In table 4.01. unit conversion was based on a creatinine molecular weight of 113 AMU and %CE values were calculated from the limits of the normal ranges quoted for total and acylcarnitine. It should also be noted that Chalmers quoted his normal ranges as mean ± standard deviation rather than mean ± 2(standard deviation) which is statistically more comparable to the 2.5th to 97.5th percentile range approximations used in this study.

Table 4.01. Normal ranges quoted by Chalmers *et al*[182].

Carnitine value	Normal range
Total	5.6 - 22.6 µmol/mmol creat
Free	1.3 - 10.3 µmol/mmol creat
Esters	54.4 - 76.8 %

4.2.3 THE CONNECTION BETWEEN CARNITINE VALUES AND GENERALISED DICARBOXYLIC ACIDURIA

Data from table 3.11 show that the levels of urinary free and percent esterified carnitine may be related to the presence/absence of generalised DCA at the 5 percent level of significance. Indeed, the probability of being incorrect in assuming a difference in percent esterified results is very low ($p = 0.0002$). Therefore, there is significant evidence, at the 5% level, that the percent esterified proportion of urinary carnitine is greater in neonates showing generalised DCA than those showing no DCA within the first five days of life.

A logistic regression analysis of the data was carried out. This test, whilst outside the statistical scope of this thesis, is specifically designed to analyse the interdependence of a number of variables within a population. The results can be found in table 4.02..

Table 4.02. Logistic regression analysis results on data from the generalised DCA investigation.

Variable	Interdependence with Generalised DCA
Sibling history of S.I.D.S.	No
Bottle or artificial feed	Yes
Gender	No
Total carnitine	No
Free carnitine	Possible
% Esterified carnitine	Yes
Number of days after birth	Possible

A possible link between type of feed and generalised DCA is shown and supports theories discussed later. A connection between %CE and generalised DCA is also indicated and supports previous conclusions. A possible dependence of DCA on the number of days after sampling, agrees well with previously published observations[177].

The p values from table 3.12. suggest that there is no difference in carnitine values dependant on sibling history of SIDS.

A p value of approximately 0.03 (table 3.13.) for total carnitine with type of feed suggest a significantly higher urinary total carnitine value in breast fed neonates than in those bottle fed (at the 5% level of statistical significance). Artificial feeds have been shown in some cases to be deficient in carnitine. This may result in higher carnitine excretion by breast fed neonates in comparison to those bottle fed as the renal threshold is surpassed by oral intake. However, this is not to say that all extra carnitine in breast milk is excreted. There does not appear to be any difference in free carnitine ($p=0.62$) suggesting that the extra total carnitine is incorporated into acyl esters. However, a p value of 0.08 for association between %CE and feed supports this theory at the 10% level of significance but not at the 5% level.

In the case of the sex of the infant (table 3.14.) it appears, at the 5% level, that urine total carnitine values are higher for female newborns than males. There is no apparent difference in free and %CE. Published data for unstressed subjects between 16 and 60 years[181,183,184] agree with a difference in carnitine excreted across the sex's but show males to possess the highest values. Unfortunately these values were quoted in mg/day and therefore could not be compared quantitatively to the results of this study.

4.2.4 POSSIBLE FUTURE DEVELOPMENTS

The significance of the generalised DCA is as yet unknown. It is thought to be a harmless anomaly reflecting an immaturity of the fatty acid metabolic pathway. However, it would seem natural to assume that as the pattern is very similar to the more enhanced DCA accepted as part of an indicative trait in medium chain acyl-CoA dehydrogenase (see section 1.10.4), it would be preferable to avoid such a situation. Inclusion of L-carnitine supplement in artificial feed may help to alleviate the problem. Further studies with the inclusion of carnitine supplements in the feed of babies presenting with generalised DCA and measurement of their plasma as well as urine carnitine values would provide further information about the connection between carnitine and generalised DCA.

The presence of generalised DCA is not thought to be a problem in the organic acid diagnoses of acyl-CoA dehydrogenase deficiencies where similar patterns are much more exaggerated.

4.3 RADIO-ENZYMATIC ASSAY TO QUANTITATE CARNITINE

4.3.1 CHOICE OF ASSAY MATERIALS

In order to achieve acceptable results for this assay it was necessary to investigate the effects of many conditions. 15ml Reactavials were used because of the small volumes of liquid encountered during the assay. Such volumes were kept to a minimum in order to concentrate the label and stabilise resultant scintillation counts. In order to maintain small volumes Picofluor 40 scintillation fluid was chosen because of its high aqueous volume capacity (5 - 6 cm^3 aqueous in 10 ml scintillation fluid). Consideration was also given to the material used for the elution column container. Glass columns (Pasteur pipette) were selected over plastic (1ml syringe) to avoid the possibility of any retention of the label by the plastic. Length of column and amount of washing were carefully investigated before the 4cm depth and two 0.75ml washes were chosen based on intra-batch variation and recovery values.

The actual column material was chosen to be Dowex 1-X8 (chloride form) as used by Jalaludin Bhuiyan *et al*[185]. It was found essential that this Dowex was pretreated with sodium hydroxide before use and brought back to neutrality with saline. Otherwise, scintillation counts became spurious providing poor duplicates and unreliable results. The hydroxide washing had the effect of cleaning and fining the dowex. As a result of an excess of hydroxyl ions some of the chloride ions on the resin particles may have been displaced. It was these chloride groups that were exchanged for the acetyl-CoA in order to retain the unreacted [^{14}C]acetyl-CoA during the elution stage of the assay. Such a displacement by hydroxide ions would be limited by the resins stronger affinity for chloride ions. However, to ensure the resins former chloride state neutrality was returned by washing with physiological saline rather than deionised water.

4.3.2 POSSIBLE FUTURE DEVELOPMENTS

As this assay becomes more routinely used there are a number of areas that could become more 'automated'. For example, the use of multi-pipettes or a premixed cocktail of most of the reagents (excluding the enzyme) to reduce the number of pipette actions per sample. This would help to standardise the assay more thus decreasing intra- and inter-batch variations and increasing reliability and accuracy.

4.4 PREPARATION OF ISOTOPE INTERNAL STANDARDS FOR THE QUANTITATION OF URINARY GLYCINE CONJUGATES BY GAS CHROMATOGRAPHY / MASS SPECTROMETRY

4.4.1 RESPONSE TO DEUTERATION METHODS

For SG the abundance of 43% for the $^2H_4$ compound relative to the total abundance for the 6 isotopic compounds $^2H_0$ to $^2H_5$ was good.

Table 3.20. shows the results of a single ion monitoring GC/MS analysis of both deuterated hexanoic acid and the final deuterated HG produced. Di-deuterated hexanoic acid was seen to be the most abundant isotope and therefore, not surprisingly, the $^2H_4$-glycine conjugate dominated in the final product. A relative abundance of 73% was extremely good. The greater success of the deuteration of this molecule in comparison with that of the SG would be partially attributable to HG's shorter carbon chain length. The greater the chain length the more stable the molecule and the less prone are the hydrogen groups to replacement by deuteriums. Even though a small amount of non-deuterated compound still remained at the end of the synthesis (1.07% of the total HG) this was accepted as insignificant and infact was undetectable during calibration (table 3.27 and figure 3.38)

A lower relative abundance for $^2H_4$-PPG of 31%, again was probably a reflection of the larger carbon chain length responding poorly to such forced exchange synthesis.

4.4.2 DEUTERATED STANDARD CALIBRATION

As already mentioned calibration results for all three deuterated compounds produced good linearity (table 3.27 and figures 3.37 to 3.39) but unfortunately poor reproducibility. The non zero peak area ratio for the zero standard on figure 3.37 indicates the presence of non-deuterated SG in the deuterated sample. The regression lines for HG and PPG (figures 3.38 and 3.39) also do not pass through zero but in considering the inter-assay variation, suggested by the scatter of the plots, this does not necessarily indicate presence of non-deuterated HG or PPG in the deuterated sample. Further, actual zero results were recorded for the zero standards for HG and PPG.

However, in using such calibration curves the presence of background levels of non-deuterated conjugates in the deuterated standard would not effect quantitation results.

4.4.3 FUTURE DEVELOPMENT

More success has subsequently been achieved with the same stock isotopes by use of methyl ester derivatisation[190], as used by Ramsdell and Tanaka[191], rather than trimethylsilyl derivatisation, initially chosen as this was the method already in place for regular urine organic acid analysis.

CLOSING SUMMARY

The intermediary metabolites investigation shows that paired metabolite correlation plotting can provide a clear indication of abnormal relationships and defect positions. In many cases a more evident picture than would be gained from interpretation of the individual laboratory results. This, along with the reduced need for exact knowledge of metabolic status, suggests that such plots can be useful in the diagnosis of fatty acid oxidation defects prior to more specific tests such as enzyme analyses.

Reference data correlations between the plasma intermediary metabolites glucose, free fatty acids, percent esterified carnitine and 3-hydroxybutyrate were good indicating significant inter-relationships during fasting. Plots involving more distantly related metabolites, such as lactate, provide weaker and therefore less useful correlations. Control of external factors that may affect metabolic states, such as exercise and age, may enhance correlations.

Separation of abnormal values resulting from errors in fatty acid oxidation was sufficiently clear to allow confident predictions of the general positions of the majority of the defects investigated. One requirement for a position to be clearly identified is that the metabolites either side of the position must be included in appropriate correlation plots. However, it has been shown that, in some cases, evidence of defect positions not encompassed by the metabolites measured can also be provided. A primary necessity in using paired metabolite correlations is the induction of sufficient metabolic stress, in this case carefully supervised fasting, to ensure the manifestation of any abnormality. Results may appear completely normal during times of minimal fatty acid oxidation flux.

The general dicarboxylic acid investigation provides evidence that the occurrence of neonatal generalised DCA is not associated with sibling history of SIDS or gender. However, it is associated with breast feeding, as opposed to feeding with artificial milk compounds. Also, urine total and percent esterified carnitine values, on the whole, seem to be higher in urine exhibiting generalised DCA. Additional investigations involving carnitine supplementation and analysis of the association between carnitine values and types of feed would provide more information.

The occurrence of generalised DCA may be related to a depletion of tissue carnitine limiting ß-oxidation. Dicarboxylic acids would then be produced via ω-oxidation. The pattern diminishes within a few days after birth and current thinking regards it
as a harmless result of an immaturity in the fatty acid oxidation pathway.

The radio-enzymatic assay of carnitine proved to be a reliable and cost effective means of measuring urine and plasma carnitine levels with the possibility of extension to differentiate between long and short chain length acylcarnitines.

The preparation of glycine conjugate isotope internal standards was very problematic. Difficulties were encountered in reproducing calibration results using trimethylsilyl derivatives. More success has since been achieved using methyl ester derivatisation.

APPENDIX 1

CRYOGENIC STORAGE AND FIBROBLAST TISSUE CULTURE OF SKIN BIOPSIES

1.1 SAMPLE SELECTION

Skin biopsies (approx. 2mm in diameter if possible) were routinely collected post mortem from children at Sheffield Childrens Hospital and transported to the laboratory in sterile culture media (see appendix section 1.3). The biopsy was selected for culture if subsequent liver microscopy revealed evidence of fatty infiltration. In a number of cases the biopsy was cryogenically preserved until clinical evidence merited culture (see appendix section 1.6).

The explant was set up for culture or cryogenic storage within a few hours of collection (typically within one hour).

1.2 ENVIRONMENTAL CONTROL

Aseptic conditions were facilitated by the use of a laminar flow cabinet (MDH Intermed).

1.3 MEDIA PREPARATION

Growth media was prepared by mixing 100ml culture media (Hams F10 nutrient mixture with 2mM hepes buffer and L-Glutamine, Flow laboratories) with 30ml sterile foetal bovine serum (Flow laboratories; stored at -20°C) and 1ml penicillin / streptomycin mix (50µg/ml streptomycin sulphate : 100 U/ml benzylpenicillin, Evans Medical Ltd, in PBS). The mixture was then filtered through cellulose acetate filters (Flowpore D26, pore size 0.2µm, pyrogen free; Flow laboratories) and stored at 4°C until required. A sample was incubated at 37°C for a few days before use to ensure the absence of visible bacterial growth.

Trypsinisation solution. 0.2g Ethylenediamine tetra-acetic acid disodium salt (EDTA, BDH) was added to 400ml phosphate buffered saline (PBS, 1 tab to 100ml deionised

water) and autoclaved at 115°C for 20 min on a Dent & Hellyer labclave (Intermed MDH). 2.5% Sterile lyophilised trypsin (GIBCO) was first dissolved in 20ml of the EDTA solution and then mixed with the rest. The solution was then stored in batch size aliquots at -20°C.

Cryogenic storage solution. An initial 40% v/v dimethyl sulphoxide (DMSO, Sigma) in PBS solution was diluted 1 in 5 with growth media to create a 10% v/v solution.

1.4 FIBROBLAST INITIALISATION

The biopsy was cut into small pieces. 3-5 pieces were placed into each of three nunc tubes (Nunclon delta Intermed) and a flamed, cooled cover slip laid on top to prevent their movement. 2ml growth media was then added to cover the biopsy sections and the arrangement incubated at 37°C. Three times a week the tubes were examined under the microscope (CK Olympus Tokyo, magnification anomalous) for signs of fibroblastic growth and the growth media replaced with fresh. The time taken to initialise fibroblastic growth varied but was typically 1 - 2 weeks.

1.5 TRANSFER OF FIBROBLASTS - TRYPSINISATION

When fibroblastic growth was sufficient to cover the whole of the flat surface of the nunc tube, the fibroblasts were then transferred into a culture vessel(50ml wide canted neck with 25cm^2 growth area and plug sealed screw cap, Falcon Labware; supplied by Fahrenheit laboratory supplies) as follows.

The growth media was replaced with 3ml trypsinisation solution and left for approximately 1 minute until the cells were seen to be beginning to loose their elongated shape and lift off the surface of the tube. The majority of the enzyme solution was then removed and the cells left in the remnants of the solution for 5 to 10 minutes at 37°C to totally break their adhesion to the surface. 5ml Of growth

media was then added washing the fibroblasts off the sides of the tube. This solution was poured into a culture vessel and feeding continued as before. When growth was confluent over the surface of the vessel the cells were retrypsinised and either split into two such vessels or transferred into a larger vessel.

1.6 CRYOGENIC STORAGE OF FIBROBLAST CELLS

After 3 or 4 passages (sufficient to establish normal growth but to avoid excessive multiplication of anomalies caused by culturing) the cells were trypsinised and resuspended in 2ml cryogenic storage solution. The fibroblast solution was placed in a high chemical resistant polypropylene cryogenic vile (Bibby) and gently frozen, initially to -80°C before placing in liquid nitrogen vapour.

1.7 RECOVERY OF FROZEN CELLS

The thawing of fibroblasts was carried out quickly to avoid hypotonic shock. The cryotube was placed at 37°C and the cell suspension transferred to a nunc tube with 10ml PBS. After centrifugation at 1000 rev/min for 5 minutes the supernatant was removed and the cells washed again with a further 10ml PBS, centrifuged and the supernatant removed. The pellet was resuspended in growth media and after 24 hours incubation, when the cells had adhered to the surface of the tube, the growth media was renewed to remove any remaining trace of DMSO. The cells were then cultured as previously described.

1.8 TESTING FOR MYCOPLASMA INFESTATION

Prior to cryogenic storage or before use for assay the fibroblast culture was tested to ensure the absence of mycoplasma. These contaminating microorganisms metabolise substrates such as butyric and octanoic acid (but not palmitate[192]). Any extraneous

metabolic activity would result in falsely elevated enzyme activity results from assays such as CO_2 release.

The method used was a modification of that described by Russel *et al*[193]. A coverslip was placed in nunc tube containing a suspension of the cells to be tested. After a few days incubation the cells settled and adhered to the cover slip. Incubation continued until adequate growth was seen i.e. approximately a monolayer covering the majority of the cover slip. Any more and the density of the cells would swamp the detection procedure, any less and the cells would be too sparse to provide confident results.

The growth media was then replaced with a 3:1 ethanol: glacial acetic acid fixative, and left at room temperature for at least twenty minutes. The fixative was removed and the cells washed twice with 1-2ml Gurrs buffer (BDH). To 5 ml Gurrs buffer in each tube 0.05ml of 5×10^{-5} g/ml Hoescht 33258 stain (bis-benzimide, Sigma) was added. After incubation at 37°C for 30 minutes the cover slip was removed, placed inverted on a microscope slide and examined under a fluorescence microscope (Vickers Fluorescence Microscope M17, magnification x100, with very low fluorescence submersion oil, blue filter exciter 2, FICT 5 +GG455 [3mm] OG515 [3mm]).

The stain binds to the DNA of both cell nuclei and mycoplasma (largely DNA) and fluoresces on excitation with U.V light.

A brilliant mottled effect within the cytoplasm is indicative of mycoplasma infestation.

1.9 TREATMENT FOR MYCOPLASMA INFESTATION

On detection of mycoplasma the appropriate sanitation procedures of all materials used were carried out with careful quarantining of the infected cell lines. The treatment kit B.M. Cyclin marketed by Boehringer Manhiem was used as directed by

the manufacturers. This treatment has been seen to temporarily suppress ß-oxidation by up to 70% but full activity is regained after a few days away from treatment[186].

1.10 CRYOGENIC STORAGE OF SKIN BIOPSIES

This method was adapted from that of K.J. Fowler[173]. Skin biopsies arriving at the laboratory were immediately placed in a 10% v/v solution of culture medium and DMSO. After approximately 1 hour to allow penetration of DMSO into the cells the arrangement was frozen at -80°C.

1.11 RECOVERY OF CRYOGENICALLY STORED BIOPSY

As with cultured cell recovery, thawing was carried out quickly to avoid cellular damage. The frozen medium and biopsy were placed at 37°C and the biopsy removed as soon as it was free from the frozen solution. The biopsy was placed in growth media to wash and prepared for culture whilst submerged in fresh medium. After 24 hours incubation the growth medium was carefully replaced to remove any further trace of DMSO. Culturing procedures then continued as normal.

1.13 RESULTS AND DISCUSSION OF CRYOGENIC STORAGE OF BIOPSIES

Ten skin biopsy samples were cryogenically stored and recovered. Storage times ranged from 1 to 12 months. Two of the biopsies that had been stored for 12 months failed to grow after thawing whilst a third showed quite normal viability for culture in all three nunc tubes. All other biopsies were cultured successfully although in one case cultures in only two of the three tubes survived whilst for another biopsy growth in one tube was significantly suppressed.

Although these results are very preliminary and are not a thorough viability investigation they do show that it is possible to store skin biopsies in this way for much longer than the initial 23 days published by Fowler.

Storage of biopsy material in liquid nitrogen vapour as are skin fibroblast cells may result in extending possible storage times without losing the capacity for culture. This would then provide much more scope for retrospective studies using assays requiring tissue culture and again limit the costs involved in culture that is subsequently deemed unnecessary (as mention in section 1.11.5).

APPENDIX 2

DATA OBTAINED DURING THE INTERMEDIARY METABOLITES INVESTIGATION

Table A2.1. A description of the variable names used in the intermediary metabolite investigation.

Variable name	Description
PATIENT	Patient number
DIAGNOSE	Diagnosis of patient
SAMPLE	Sample number
FASTCOM	Comment associated with length of fast
FAST	Length of fast (hours)
GLUC	Glucose (mmol/l)
LACT	Lactate (mmol/l)
HB	3-Hydroxybutyrate (mmol/l)
FFA	Free fatty acids (mmol/l)
FFA3HB	Free fatty acids / 3-hydroxybutyrate
CARNF	Free carnitine (μmol/l)
CARNT	Total carnitine (μmol/l)
CARNE	% Esterified carnitine

Table A2.2. A list of all values for the variable DIAGNOSE and the corresponding diagnosis decode.

DIAGNOSE Value	Representative disease
0	No diagnosed disorder of fatty acid metabolism
1	Medium chain acyl-CoA dehydrogenase deficiency
2	Glycogen storage disease type 1A
3	Isolated glutacorticoid deficiency
4	Hyperinsulinaemic hypoglycaemia
5	Defect in gluconeogenesis
6	Limited glycogen stores or mobility
7	Defective acetoacetate utilisation
8	Spontaneous hypoglycaemia
U	Duel defect

Table A2.3. A list of all values for the variable FASTCOM and the corresponding decode.

FASTCOM value	Description
*	Unconfirmed fast time
>	Fast time greater than value of variable FAST
6 to	Fast time between 6 and value of variable FAST
6 or	Fast time either 6 or value of variable FAST
approx	Fast time only approximately equal to the value of variable FAST

OBS	PATIENT	DIAGNOSE	SAMPLE	FASTCOM	FAST	GLUC	LACT	HB	FFA	FFA3HB	CARNT	CARNF	CARNE
1	1	0	1	*	18	5.8	1.2	1.00	1.06	1.06000	15	13	13.3333
2	2	0	2		.	3.8	1.3	0.70	0.72	1.02857	30	24	20.0000
3	3	0	3	* >	6	4.3	1.0	0.30	0.54	1.80000	.	.	.
4	4	0	4		7	4.4	1.6	0.20	1.26	6.30000	40	37	7.5000
5	5	0	5	*	5	4.5	1.4	0.50	1.05	2.10000	.	.	.
6	6	0	6		8	4.3	1.4	0.90	0.97	1.07778	.	.	.
7	7	0	7	*	6	4.6	1.4	0.30	1.31	4.36667	.	.	.
8	8	0	8	>	6	4.0	1.0	1.00	1.31	1.31000	.	.	.
9	9	5	109	*	18	2.1	7.5	0.40	0.80	2.00000	20	15	25.0000
10	10	0	10	>	7	4.9	1.8	1.40	0.96	0.68571	.	.	.
11	11	0	11	*	8	4.3	1.3	.	.	.	.	.	.
12	12	0	12		8	4.3	1.2	.	1.68	.	.	.	.
13	13	0	13	*	4	10.4	6.2	0.05	0.08	1.60000	45	38	15.5556
14	14	0	14		8	3.5	1.0	1.50	0.89	0.59333	.	.	.
15	15	0	15		8	4.1	1.1	0.20	0.70	3.50000	.	.	.
16	16	0	16		6	3.9	1.0	1.20	0.99	0.82500	.	.	.
17	17	0	17	>	6	3.6	1.2	0.30	0.24	0.80000	.	.	.
18	18	0	18	*	6	4.6	2.0	1.40	0.11	0.07857	.	.	.
19	19	0	19		6	4.8	1.0	0.70	1.00	1.42857	.	.	.
20	20	0	20		6	5.8	1.4	0.50	0.47	0.94000	.	.	.
21	21	0	21	*	8	4.3	0.6	0.60	0.93	1.55000	.	.	.
22	22	0	22		8	5.0	1.2	0.60	0.70	1.16667	.	.	.
23	23	0	23	*	8	4.4	1.6	0.90	1.56	1.73333	.	.	.
24	24	0	24	*	6	4.9	2.0	0.20	0.76	3.80000	.	.	.
25	25	0	25		.	3.9	1.3	1.40	1.88	1.34286	.	.	.
26	26	0	26	*	6	5.3	1.3	0.20	0.28	1.40000	.	.	.
27	27	0	27	* >	6	5.8	1.3	0.90	0.19	0.21111	.	.	.
28	28	0	28		6	7.0	2.4	0.20	0.68	3.40000	.	.	.
29	29	0	29		6	4.9	1.0	0.70	1.09	1.55714	.	.	.
30	30	0	30		.	4.4	1.3	1.00	1.13	1.13000	.	.	.
31	31	0	31		6	6.1	0.8	0.40	0.53	1.32500	.	.	.
32	32	0	32	*	8	4.7	1.6	0.30	1.19	3.96667	.	.	.
33	33	0	33	6 to	8	4.7	1.0	0.80	0.79	0.98750	.	.	.
34	34	0	34		8	3.6	1.6	0.40	0.75	1.87500	.	.	.
35	35	0	35	* >	8	4.3	1.1	0.30	0.57	1.90000	.	.	.
36	36	0	74		8	6.0	2.3	0.05	0.23	4.60000	.	.	.
37	37	0	37		8	5.1	1.5	0.05	0.34	6.80000	.	.	.
38	38	0	38	* >	8	4.4	1.0	0.90	0.86	0.95556	.	.	.
39	39	0	39	* >	8	4.9	0.6	0.50	0.87	1.74000	.	.	.
40	40	1	40		.	1.5	2.7	1.30	3.42	2.63077	18	7	61.1111
41	41	0	41	* >	6	5.5	2.7	0.40	1.16	2.90000	.	.	.
42	42	0	42	* >	8	4.3	1.1	0.40	0.76	1.90000	.	.	.
43	43	7	43	* 6 or	16	4.2	1.2	2.50	0.56	0.22400	43	21	51.1628
44	44	0	44		.	4.9	4.5	0.10	0.87	8.70000	48	31	35.4167
45	45	0	61	* >	8	.	0.9	0.20	0.72	3.60000	.	.	.
46	46	0	46		.	4.2	0.9	1.40	0.26	0.18571	.	.	.
47	47	0	47		.	4.2	0.7	0.80	0.88	1.10000	.	.	.
48	48	0	48		.	4.5	1.2	0.05	0.14	2.80000	.	.	.
49	49	0	49		.	4.8	2.4	0.70	1.12	1.60000	.	.	.
50	50	0	50		.	4.7	1.0	0.30	1.03	3.43333	.	.	.
51	51	0	58		.	.	1.8	0.10	0.96	9.60000	.	.	.
52	52	0	52		.	6.5	1.5	1.20	0.86	0.71667	.	.	.
53	53	0	53		.	4.8	1.4	1.20	1.56	1.30000	.	.	.
54	54	0	104		.	.	.	0.50	0.57	1.14000	.	.	.
55	55	0	55		.	.	1.1	0.30	0.65	2.16667	.	.	.
56	56	0	56		.	.	1.5	0.40	1.09	2.72500	.	.	.

OBS	PATIENT	DIAGNOSE	SAMPLE	FASTCOM	FAST	GLUC	LACT	HB	FFA	FFA3HB	CARNT	CARNF	CARNE
57	57	0	57.0		.	4.4	.	1.30	0.45	0.3462	.	.	.
58	58	0	58.0		.	5.9	0.94	0.40	0.75	1.8750	.	.	.
59	59	0	59.0	* >	12.00	5.7	0.92	0.70	0.60	0.8571	.	.	.
60	60	0	60.0		.	4.4	1.06	1.60	1.22	0.7625	.	.	.
61	61	0	61.0		.	5.1	0.94	0.30	0.49	1.6333	.	.	.
62	62	0	79.0		.	5.8	1.21	0.60	0.56	0.9333	.	.	.
63	63	0	63.0		.	5.7	2.02	0.77	1.51	1.9610	.	.	.
64	64	0	64.0	*	6.00	4.4	1.31	0.66	0.77	1.1667	.	.	.
65	65	0	65.0		.	4.3	1.21	1.20	0.98	0.8167	.	.	.
66	66	0	66.0		12.00	5.6	1.65	0.10	0.52	5.2000	.	.	.
67	67	0	67.0	approx	12.00	4.4	1.37	1.50	0.80	0.5333	.	.	.
68	68	0	68.0		.	3.9	1.13	0.85	0.89	1.0471	.	.	.
69	69	6	69.1		12.00	3.5	.	2.70	2.68	0.9926	33	13	60.6061
70	69	6	69.2		18.00	1.8	.	2.83	3.94	1.3922	.	.	.
71	70	0	70.1		12.00	6.2	.	0.16	0.89	5.5625	33	23	30.3030
72	70	0	70.2		18.00	4.7	.	0.48	1.07	2.2292	33	21	36.3636
73	71	0	71.0		.	4.6	1.30	0.43	1.04	2.4186	.	.	.
74	72	0	72.0		.	4.9	1.38	0.99	1.11	1.1212	.	.	.
75	73	0	73.0		.	4.2	1.30	1.01	1.40	1.3861	.	.	.
76	74	0	74.0		.	6.1	1.37	0.08	0.26	3.2500	.	.	.
77	75	0	75.0		.	4.7	1.25	0.07	0.23	3.2857	.	.	.
78	76	0	76.0		.	4.9	1.78	0.04	0.34	8.5000	.	.	.
79	77	0	77.0		.	.	1.27	1.05	1.11	1.0571	.	.	.
80	78	0	78.0		.	.	1.68	1.79	0.65	0.3631	.	.	.
81	79	0	79.0		.	4.3	1.12	0.42	0.75	1.7857	.	.	.
82	80	0	80.0		.	.	1.55	0.12	0.42	3.5000	.	.	.
83	81	0	81.0		.	6.2	1.41	.	0.66	.	.	.	.
84	82	0	82.0		.	4.4	.	.	2.50	.	.	.	.
85	83	0	83.0		.	4.5	2.22	0.21	0.71	3.3810	.	.	.
86	84	0	84.0		.	5.6	0.99	0.65	0.76	1.1692	.	.	.
87	85	0	85.0		.	4.8	1.77	0.02	0.32	16.0000	.	.	.
88	86	0	86.0		.	4.3	0.89	0.93	1.18	1.2688	.	.	.
89	87	0	87.0		.	4.6	2.29	0.39	0.96	2.4615	.	.	.
90	88	0	88.0		.	5.2	1.82	0.65	1.55	2.3846	.	.	.
91	89	0	89.0		.	3.8	1.05	1.54	1.03	0.6688	.	.	.
92	90	0	90.0		.	.	1.13	.	.	.	58	45	22.4138
93	91	0	91.0		.	.	0.71	4.63	2.30	0.4968	.	.	.
94	92	0	92.0		.	.	.	0.89	0.73	0.8202	.	.	.
95	93	0	93.0		.	.	4.49	0.00	0.10	.	.	.	.
96	94	1	94.0		.	7.6	2.22	1.35	1.95	1.4444	.	.	.
97	95	2	95.1		.	.	.	0.03	0.81	27.0000	.	.	.
98	95	2	95.2		.	.	.	0.00	0.71	.	.	.	.
99	95	2	95.2		.	.	.	0.00	0.81	.	.	.	.
100	96	3	96.0		.	1.2	1.33	3.53	1.22	0.3456	.	18	.
101	97	0	97.0		.	2.7	2.33	4.00	.	.	.	.	.
102	98	0	98.0		.	5.0	2.28	0.00	.	.	33	28	15.1515
103	99	0	99.1		11.00	4.1	1.96	0.51	0.57	1.1176	40	34	15.0000
104	99	0	99.2		14.00	3.9	1.98	1.46	1.17	0.8014	45	30	33.3333
105	99	0	99.3		18.00	3.3	2.03	2.41	1.81	0.7510	.	.	.
106	100	0	100.1		13.00	4.3	1.21	0.48	0.85	1.7708	.	.	.
107	100	0	100.2		15.50	3.9	1.23	1.92	1.15	0.5990	30	22	26.6667
108	100	0	100.3		18.75	2.0	2.28	4.09	2.44	0.5966	35	16	54.2857
109	101	0	101.0		.	3.6	1.21	.	.	.	.	25	.
110	102	0	102.0		.	5.4	0.97	0.53	0.59	1.1132	30	24	20.0000
111	103	0	103.0		.	5.3	1.41	0.02	0.33	16.5000	18	22	-22.2222
112	104	0	104.1		16.00	4.2	1.30	0.40	2.10	5.2500	.	.	.

OBS	PATIENT	DIAGNOSE	SAMPLE	FASTCOM	FAST	GLUC	LACT	HB	FFA	FFA3HB	CARNT	CARNF	CARNE
113	104	0	104.2		22.0	3.0	1.60	3.02	3.10	1.0265	.	.	.
114	105	0	105.1		15.0	4.5	1.50	0.85	1.25	1.4706	26	15.0	42.3077
115	105	0	105.2		8.0	4.0	1.40	1.82	1.55	0.8516	24	13.0	45.8333
116	106	0	106.1		15.0	4.8	1.30	1.39	1.23	0.8849	40	30.0	25.0000
117	106	0	106.2		18.0	4.7	1.20	2.14	1.38	0.6449	38	23.0	39.4737
118	107	0	107.1		.	.	1.64	2.00	0.45	0.2250	55	32.0	41.8182
119	107	0	107.2		.	4.0	4.07	2.00	0.76	0.3800	63	36.0	42.8571
120	108	0	108.1		12.0	4.4	3.37	0.60	0.88	1.4667	34	23.0	32.3529
121	108	0	108.2		15.0	4.2	4.39	0.77	1.11	1.4416	38	21.0	44.7368
122	109	0	109.0		.	3.7	1.99	0.37	0.77	2.0811	38	28.0	26.3158
123	110	0	110.1		12.5	3.1	2.68	1.20	1.02	0.8500	44	24.0	45.4545
124	110	0	110.2		16.5	2.0	2.69	2.40	1.61	0.6708	40	19.0	52.5000
125	111	0	111.1		8.0	4.5	1.16	0.10	0.70	7.0000	30	25.0	16.6667
126	111	0	111.2		12.0	4.0	1.49	0.60	1.65	2.7500	33	26.0	21.2121
127	112	0	112.1		8.0	3.7	2.20	0.05	0.28	5.6000	35	31.0	11.4286
128	112	0	112.2		12.0	3.3	1.52	1.00	0.89	0.8900	33	21.0	36.3636
129	113	0	113.1		17.0	3.3	1.02	1.53	1.40	0.9150	24	11.0	54.1667
130	113	0	113.2		24.0	3.8	2.65	2.60	2.70	1.0385	24	10.0	58.3333
131	114	4	114.0		.	1.3	2.40	0.06	0.10	1.6667	18	14.0	22.2222
132	115	4	115.0		.	1.0	3.72	0.10	0.30	3.0000	35	29.0	17.1429
133	116	0	116.1		9.0	4.2	1.43	0.48	1.20	2.5000	36	30.0	16.6667
134	116	0	116.2		12.0	3.7	2.23	0.68	1.20	1.7647	40	30.0	25.0000
135	117	0	117.1		17.0	3.9	2.71	0.45	0.90	2.0000	30	25.0	16.6667
136	117	0	117.2		20.0	3.7	2.07	0.64	0.80	1.2500	33	22.0	33.3333
137	118	0	118.0		.	3.8	1.70	0.65	0.60	0.9231	.	.	.
138	119	0	119.1		21.0	1.6	1.30	3.90	2.40	0.6154	45	12.0	73.3333
139	119	8	119.0		.	0.4	3.30	0.62	1.10	1.7742	65	33.0	49.2308
140	120	0	120.0		.	3.5	1.40	1.90	1.30	0.6842	35	17.0	51.4286
141	121	U	121.0		.	1.0	6.50	0.22	3.30	15.0000	19	3.5	81.5789
142	122	4	122.0		.	0.6	4.60	0.66	0.30	0.4545	40	33.0	17.5000
143	123	0	123.1		.	2.5	5.04	0.30	0.60	2.0000	38	25.0	34.2105
144	123	0	123.2		.	2.5	2.34	1.06	0.60	0.5660	35	23.0	34.2857
145	124	0	124.1		.	4.2	1.70	0.34	0.90	2.6471	30	22.0	26.6667
146	124	0	124.2		.	3.8	1.87	1.42	1.10	0.7746	28	15.0	46.4286
147	125	0	125.1		12.0	4.2	1.02	0.21	0.60	2.8571	33	24.0	27.2727
148	125	0	125.2		17.0	4.0	1.25	0.50	1.24	2.4800	28	22.0	21.4286
149	126	0	126.0		.	3.2	2.31	0.85	0.91	1.0706	43	27.0	37.2093
150	127	0	127.0		.	1.0	1.12	3.62	3.34	0.9227	88	26.0	70.4545
151	128	0	128.0		.	4.6	2.30	0.97	0.97	1.0000	48	24.0	50.0000
152	129	0	129.0		.	3.6	1.95	0.73	0.88	1.2055	34	25.0	26.4706
153	130	4	.		0.0	1.6	.	0.08	0.03	0.3750	.	.	.
154	131	4	.		0.0	1.5	.	0.01	0.10	10.0000	.	.	.
155	132	4	.		0.0	0.8	3.30	0.06	0.02	0.3333	13	10.0	23.0769
156	133	4	.		0.0	0.5	8.97	0.12	0.03	0.2500	.	.	.
157	134	4	.		0.0	0.4	4.08	0.02	0.03	1.5000	14	7.0	50.0000
158	135	4	.		0.0	1.6	.	.	0.10	.	.	.	.
159	136	4	.		0.0	0.5	.	.	0.12	.	.	.	.
160	137	4	.		0.0	2.1	.	0.09	0.60	6.6667	.	.	.
161	138	4	.		0.0	2.0	1.86	0.01	0.30	30.0000	.	.	.
162	139	4	.		0.0	1.6	1.45	0.01	0.82	82.0000	.	.	.
163	140	4	.		0.0	0.9	2.00	0.01	0.10	10.0000	.	.	.
164	141	4	.		0.0	2.0	0.20	0.10	.	.	.	.	.

APPENDIX 3

DATA OBTAINED DURING THE GENERALISED DICARBOXYLIC ACIDURIA INVESTIGATION

Table A3.1. A description of the variable names used in the generalised dicarboxylic aciduria investigation.

Variable name	Description
PATIENT	Patient number
MF	Male(1) or female(0)
SC	Sibling(1) or control(0) (C=cousin of SIDS)
BA	Breast(1) or artificial(0) feed (B=both)
DCANON	Generalised DCA present(1) or absent(0)
DAY	Age at sampling (days)
CREAT	Urine creatinine
T	Urine total carnitine
TC	Total carnitine / creatinine
LOGTC	Log (Total carnitine / creatinine)
F	Urine free carnitine
FC	Free carnitine / creatinine
LOGFC	Log (Free carnitine / creatinine)
AC	% Esterified carnitine
LOGAC	Log (% Esterified carnitine)

OBS	PATIENT	MF	SC	BA	DCANON	DAY	CREAT	T	TC	LOGTC	F	FC	LOGFC	AC	LOGAC
1	1	1	1	.	1	3	17.0	308.0	18.118	1.25810	20.0	1.1765	0.07058	93.5065	1.97084
2	2	.	1	.	1	1	7.4	78.0	10.541	1.02286	6.0	0.8108	-0.09108	92.3077	1.96524
3	3	.	1	.	1	1	7.7	134.0	17.403	1.24061	15.0	1.9481	0.28960	88.8060	1.94844
4	3	.	1	.	1	4	9.3	145.0	15.591	1.19289	10.0	1.0753	0.03152	93.1034	1.96897
5	4	.	1	.	1	1	11.4	150.0	13.158	1.11919	9.0	0.7895	-0.10266	94.0000	1.97313
6	4	.	1	.	1	2	1.2	18.0	15.000	1.17609	3.0	2.5000	0.39794	83.3333	1.92082
7	5	1	1	.	1	1	9.0	103.0	11.444	1.05859	10.0	1.1111	0.04576	90.2913	1.95565
8	6	.	1	.	1	1	5.5	138.0	25.091	1.39952	13.0	2.3636	0.37358	90.5797	1.95703
9	6	.	1	.	1	3	1.4	40.0	28.571	1.45593	10.0	7.1429	0.85387	75.0000	1.87506
10	7	1	1	.	1	1	5.1	140.0	27.451	1.43856	11.0	2.1569	0.33382	92.1429	1.96446
11	8	0	1	.	1	1	2.4	39.0	16.250	1.21085	4.0	1.6667	0.22185	89.7436	1.95300
12	8	0	1	.	1	3	3.8	74.0	19.474	1.28945	4.0	1.0526	0.02228	94.5946	1.97587
13	8	0	1	.	1	5	1.0	20.0	20.000	1.30103	2.0	2.0000	0.30103	90.0000	1.95424
14	9	.	1	.	1	1	3.8	48.0	12.632	1.10146	5.0	1.3158	0.11919	89.5833	1.95223
15	9	.	1	.	1	3	3.5	55.0	15.714	1.19629	3.0	0.8571	-0.06695	94.5455	1.97564
16	9	.	1	.	1	5	1.0	15.0	15.000	1.17609	2.0	2.0000	0.30103	86.6667	1.93785
17	10	0	1	.	1	1	14.4	233.0	16.181	1.20899	13.0	0.9028	-0.04442	94.4206	1.97507
18	11	1	1	.	1	1	6.4	70.0	10.938	1.03892	5.0	0.7812	-0.10721	92.8571	1.96782
19	11	1	1	.	1	3	2.8	48.0	17.143	1.23408	3.0	1.0714	0.02996	93.7500	1.97197
20	11	1	1	.	1	5	1.6	20.0	12.500	1.09691	3.0	1.8750	0.27300	85.0000	1.92942
21	12	1	0	.	1	1	8.7	121.0	13.908	1.14327	7.0	0.8046	-0.09442	94.2149	1.97412
22	13	0	0	.	1	1	5.5	110.0	20.000	1.30103	7.0	1.2727	0.10474	93.6364	1.97144
23	13	0	0	.	1	3	1.1	28.0	25.455	1.40577	3.0	2.7273	0.43573	89.2857	1.95078
24	14	0	1	.	1	1	11.5	206.0	17.913	1.25317	11.0	0.9565	-0.01931	94.6602	1.97617
25	14	0	1	.	1	3	0.7	180.0	257.143	2.41017	10.0	14.2857	1.15490	94.4444	1.97518
26	14	0	1	.	1	5	0.9	41.0	45.556	1.65854	38.0	42.2222	1.62554	7.3171	0.86434
27	15	0	0	.	1	1	3.3	113.0	34.242	1.53456	4.0	1.2121	0.08355	96.4602	1.98435
28	15	0	0	.	1	3	10.3	236.0	22.913	1.36007	14.0	1.3592	0.13329	94.0678	1.97344
29	15	0	0	.	1	5	1.6	34.0	21.250	1.32736	4.0	2.5000	0.39794	88.2353	1.94564
30	16	0	1	.	1	1	5.2	95.0	18.269	1.26172	7.0	1.3462	0.12909	92.6316	1.96676
31	16	0	1	.	1	3	10.3	290.0	28.155	1.44956	13.0	1.2621	0.10111	95.5172	1.98008
32	16	0	1	.	1	5	1.3	25.0	19.231	1.28400	2.0	1.5385	0.18709	92.0000	1.96379
33	17	.	1	.	1	1	2.0	49.0	24.500	1.38917	4.0	2.0000	0.30103	91.8367	1.96302
34	17	.	1	.	1	2	2.2	168.0	76.364	1.88289	48.0	21.8182	1.33882	71.4286	1.85387
35	18	1	1	.	1	1	6.0	92.5	15.417	1.18799	5.0	0.8333	-0.07918	94.5946	1.97587
36	19	0	0	1	1	1	4.2	70.0	16.667	1.22185	9.0	2.1429	0.33099	87.1429	1.94023
37	20	.	1	.	1	1	2.9	81.0	27.931	1.44609	5.5	1.8966	0.27796	93.2099	1.96946
38	20	.	1	.	1	3	1.0	25.0	25.000	1.39794	3.0	3.0000	0.47712	88.0000	1.94448
39	20	.	1	.	1	5	1.3	58.0	44.615	1.64948	20.0	15.3846	1.18709	65.5172	1.81636
40	21	1	1	.	1	2	2.7	85.0	31.481	1.49806	2.0	0.7407	-0.13033	97.6471	1.98966
41	21	1	1	.	1	3	0.7	18.0	25.714	1.41017	1.0	1.4286	0.15490	94.4444	1.97518
42	22	.	1	1	1	1	5.4	78.0	14.444	1.15970	10.0	1.8519	0.26761	87.1795	1.94041
43	22	.	1	1	1	3	7.5	133.0	17.733	1.24879	7.0	0.9333	-0.02996	94.7368	1.97652
44	23	0	1	.	1	.	6.5	131.0	20.154	1.30436	7.0	1.0769	0.03218	94.6565	1.97615
45	24	.	C	.	1	1	4.2	64.0	15.238	1.18293	8.0	1.9048	0.27984	87.5000	1.94201
46	25	1	0	1	1	1	5.5	128.0	23.273	1.36685	10.0	1.8182	0.25964	92.1875	1.96467
47	26	.	1	.	1	5	2.6	33.0	12.692	1.10354	12.0	4.6154	0.66421	63.6364	1.80371
48	27	.	1	.	1	2	3.3	45.0	13.636	1.13470	5.0	1.5152	0.18046	88.8889	1.94885
49	28	.	1	.	1	1	6.6	7.5	1.136	0.05552	1.5	0.2273	-0.64345	80.0000	1.90309
50	28	.	1	.	1	3	2.0	30.0	15.000	1.17609	3.0	1.5000	0.17609	90.0000	1.95424
51	28	.	1	.	1	5	0.9	14.0	15.556	1.19189	2.0	2.2222	0.34679	85.7143	1.93305
52	29	.	0	1	1	1	2.5	51.0	20.400	1.30963	1.5	0.6000	-0.22185	97.0588	1.98704
53	30	.	1	1	1	2	6.0	119.0	19.833	1.29740	8.0	1.3333	0.12494	93.2773	1.96978
54	30	.	1	1	1	3	2.6	45.0	17.308	1.23824	4.5	1.7308	0.23824	90.0000	1.95424
55	30	.	1	1	1	5	1.0	18.0	18.000	1.25527	2.0	2.0000	0.30103	88.8889	1.94885
56	31	.	0	0	1	1	2.3	28.0	12.174	1.08543	3.0	1.3043	0.11539	89.2857	1.95078
57	31	.	0	0	1	5	2.1	36.0	17.143	1.23408	2.5	1.1905	0.07572	93.0556	1.96874

OBS	PATIENT	MF	SC	BA	DCANON	DAY	CREAT	T	TC	LOGTC	F	FC	LOGFC	AC	LOGAC
58	32	0	1	1	1	2	1.3	200	153.846	2.18709	14.0	10.7692	1.03218	93.0000	1.96848
59	32	0	1	1	1	4	10.2	20	1.961	0.29243	6.0	0.5882	-0.23045	70.0000	1.84510
60	33	0	0	1	1	3	2.1	41	19.524	1.29056	9.0	4.2857	0.63202	78.0488	1.89237
61	34	1	1	0	1	1	1.7	16	9.412	0.97367	3.0	1.7647	0.24667	81.2500	1.90982
62	34	1	1	0	1	3	2.3	36	15.652	1.19457	5.0	2.1739	0.33724	86.1111	1.93506
63	34	1	1	0	1	5	1.0	23	23.000	1.36173	5.0	5.0000	0.69897	78.2609	1.89354
64	34	.	0	1	1	1	7.6	91	11.974	1.07823	10.0	1.3158	0.11919	89.0110	1.94944
65	35	0	1	.	1	1	7.6	76	10.000	1.00000	4.0	0.5263	-0.27875	94.7368	1.97652
66	36	1	1	1	1	1	4.4	98	22.273	1.34777	10.0	2.2727	0.35655	89.7959	1.95326
67	37	1	1	1	1	5	0.7	11	15.714	1.19629	1.5	2.1429	0.33099	86.3636	1.93633
68	38	.	1	.	0	1	3.1	36	11.613	1.06494	5.0	1.6129	0.20761	86.1111	1.93506
69	39	.	1	.	0	1	7.6	58	7.632	0.88261	5.0	0.6579	-0.18184	91.3793	1.96085
70	40	.	1	.	0	1	5.8	40	6.897	0.83863	15.0	2.5862	0.41266	62.5000	1.79588
71	40	.	1	.	0	3	5.6	70	12.500	1.09691	8.0	1.4286	0.15490	88.5714	1.94729
72	40	.	1	.	0	5	0.9	19	21.111	1.32451	8.0	8.8889	0.94885	57.8947	1.76264
73	41	.	1	.	0	1	6.9	58	8.406	0.92458	9.0	1.3043	0.11539	84.4828	1.92677
74	42	.	1	.	0	1	3.3	31	9.394	0.97285	7.0	2.1212	0.32658	77.4194	1.88885
75	42	.	1	.	0	2	2.8	35	12.500	1.09691	7.0	2.5000	0.39794	80.0000	1.90309
76	42	.	1	.	0	4	1.1	15	13.636	1.13470	3.0	2.7273	0.43573	80.0000	1.90309
77	43	1	1	.	0	1	2.8	25	8.929	0.95078	3.0	1.0714	0.02996	88.0000	1.94448
78	44	.	1	.	0	2	1.5	33	22.000	1.34242	3.0	2.0000	0.30103	90.9091	1.95861
79	45	.	1	.	0	1	4.0	78	19.500	1.29003	9.0	2.2500	0.35218	88.4615	1.94675
80	45	.	1	.	0	3	2.2	38	17.273	1.23736	4.0	1.8182	0.25964	89.4737	1.95170
81	45	.	1	.	0	5	1.3	26	20.000	1.30103	3.5	2.6923	0.43012	86.5385	1.93721
82	46	.	1	.	0	1	1.4	20	14.286	1.15490	2.0	1.4286	0.15490	90.0000	1.95424
83	47	.	1	.	0	1	2.3	16	6.957	0.84239	4.0	1.7391	0.24033	75.0000	1.87506
84	48	.	0	.	0	3	2.2	24	10.909	1.03779	12.0	5.4545	0.73676	50.0000	1.69897
85	48	.	0	.	0	5	1.3	19	14.615	1.16481	10.0	7.6923	0.88606	47.3684	1.67549
86	49	0	1	1	0	3	2.0	49	24.500	1.38917	2.0	1.0000	0.00000	95.9184	1.98190
87	49	0	1	1	0	5	0.4	13	32.500	1.51188	1.5	3.7500	0.57403	88.4615	1.94675
88	50	0	1	.	0	1	1.7	31	18.235	1.26091	3.0	1.7647	0.24667	90.3226	1.95580
89	51	.	0	.	0	1	0.6	16	26.667	1.42597	2.5	4.1667	0.61979	84.3750	1.92621
90	52	0	1	B	0	3	0.5	9	18.000	1.25527	1.0	2.0000	0.30103	88.8889	1.94885
91	53	1	1	.	0	1	2.7	34	12.593	1.10012	5.0	1.8519	0.26761	85.2941	1.93092
92	54	.	1	1	0	1	1.1	13	11.818	1.07255	1.0	0.9091	-0.04139	92.3077	1.96524
93	55	.	0	.	0	3	2.1	46	21.905	1.34054	14.0	6.6667	0.82391	69.5652	1.84239
94	55	.	0	.	0	5	1.2	66	55.000	1.74036	41.0	34.1667	1.53360	37.8788	1.57840
95	56	0	1	0	0	2	1.5	19	12.667	1.10266	2.0	1.3333	0.12494	89.4737	1.95170
96	56	0	1	0	0	3	1.4	20	14.286	1.15490	1.5	1.0714	0.02996	92.5000	1.96614
97	56	0	1	0	0	4	1.3	18	13.846	1.14133	4.5	3.4615	0.53927	75.0000	1.87506
98	57	.	1	0	0	2	1.6	48	30.000	1.47712	3.5	2.1875	0.33995	92.7083	1.96712
99	58	0	1	.	0	5	1.7	35	20.588	1.31362	5.0	2.9412	0.46852	85.7143	1.93305
100	59	1	0	B	0	3	1.1	15	13.636	1.13470	4.0	3.6364	0.56067	73.3333	1.86530
101	59	1	0	B	0	5	0.8	14	17.500	1.24304	1.0	1.2500	0.09691	92.8571	1.96782
102	60	.	1	1	0	3	1.0	40	40.000	1.60206	6.0	6.0000	0.77815	85.0000	1.92942
103	60	.	1	1	0	5	1.0	20	20.000	1.30103	5.0	5.0000	0.69897	75.0000	1.87506
104	61	0	1	.	0	3	1.0	19	19.000	1.27875	4.0	4.0000	0.60206	78.9474	1.89734
105	62	.	1	.	0	3	0.7	14	20.000	1.30103	2.0	2.8571	0.45593	85.7143	1.93305
106	62	.	1	.	0	5	1.0	31	31.000	1.49136	15.0	15.0000	1.17609	51.6129	1.71276
107	63	.	1	0	0	1	1.3	14	10.769	1.03218	1.0	0.7692	-0.11394	92.8571	1.96782
108	64	.	0	.	0	1	1.7	23	13.529	1.13128	1.0	0.5882	-0.23045	95.6522	1.98069
109	64	.	0	.	0	3	0.8	16	20.000	1.30103	6.0	7.5000	0.87506	62.5000	1.79588
110	64	.	0	.	0	5	0.5	26	52.000	1.71600	18.0	36.0000	1.55630	30.7692	1.48812
111	65	.	1	0	0	2	0.5	21	42.000	1.62325	10.0	20.0000	1.30103	52.3810	1.71917
112	65	.	1	0	0	5	1.2	86	71.667	1.85532	58.0	48.3333	1.68425	32.5581	1.51266
113	66	1	1	0	0	2	1.9	16	8.421	0.92537	4.0	2.1053	0.32331	75.0000	1.87506
114	67	.	1	0	0	4	5.3	23	4.340	0.63745	5.0	0.9434	-0.02531	78.2609	1.89354

OBS	PATIENT	MF	SC	BA	DCANON	DAY	CREAT	T	TC	LOGTC	F	FC	LOGFC	AC	LOGAC
115	67	.	1	0	0	5	0.7	18	25.7143	1.41017	9.0	12.8571	1.10914	50.0000	1.69897
116	68	1	1	0	0	1	1.2	10	8.3333	0.92082	4.0	3.3333	0.52288	60.0000	1.77815
117	68	1	1	0	0	3	1.0	13	13.0000	1.11394	1.0	1.0000	0.00000	92.3077	1.96524
118	68	1	1	0	0	4	0.4	6	15.0000	1.17609	1.0	2.5000	0.39794	83.3333	1.92082
119	69	.	1	.	0	2	3.5	40	11.4286	1.05799	2.0	0.5714	-0.24304	95.0000	1.97772
120	69	.	1	.	0	3	1.5	16	10.6667	1.02803	1.5	1.0000	0.00000	90.6250	1.95725
121	69	.	1	.	0	5	0.6	6	10.0000	1.00000	1.0	1.6667	0.22185	83.3333	1.92082
122	70	0	0	1	0	1	3.8	68	17.8947	1.25273	9.0	2.3684	0.37446	86.7647	1.93834
123	71	.	.	.	1	.	10.2	405	39.7059	1.59885	15.0	1.4706	0.16749	96.2963	1.98361
124	72	.	.	.	1	.	5.4	96	17.7778	1.24988	9.0	1.6667	0.22185	90.6250	1.95725

APPENDIX 4

URINARY CARNITINE NORMAL RANGES FOR THE GENERALISED DICARBOXYLIC ACIDURIA INVESTIGATION BASED ON NORMAL DISTRIBUTIONS AND NORMAL LOGARITHMIC DISTRIBUTIONS

See section 2.6.4 for an explanation of the ranges and calculations shown here.

Table A4.1 Normal ranges based on normal distributions of the raw data

	Carnitine Value		
	Total	Free	% Ester
DCA & Non DCA	19.1 ± 36.1	2.3 ± 5.4	86.8 ± 20.2
Generalised DCA	21.6 ± 45.8	1.8 ± 3.4	90.5 ± 12.4
Non DCA	16.1 ± 18.0	2.9 ± 6.9	82.4 ± 24.0

Normal ranges calculated as; (mean) ± 2(standard deviations).

Table A4.2 Normal ranges based on normal distributions of the logarithmic results.

	Carnitine Value		
	*Log (Total)	*Log (Free)	*Log (100 − %Ester)
DCA & Non DCA	1.2 ± 0.5	0.2 ± 0.6	1.0 ± 0.6
Generalised DCA	1.2 ± 0.6	0.1 ± 0.6	0.9 ± 0.5
Non DCA	1.1 ± 0.4	0.3 ± 0.7	1.2 ± 0.6
	¥Total	¥Free	¥% Ester
DCA & Non DCA	5.0 to 50.1	0.4 to 6.3	60.2 to 97.5
Generalised DCA	4.0 to 63.1	0.3 to 5.0	74.9 to 97.5
Non DCA	5.0 to 31.6	2.5 to 10.0	36.9 to 96.0

***Normal logarithmic ranges calculated as; (mean) ± 2(standard deviations).**
¥Ranges converted back from logarithmic values to non-logarithmic values.

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