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CHIRALITY OF URINARY METABOLITES IN INHERITED METABOLIC DISORDERS

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An important group of inherited metabolic disorders in man produce abnormal excretion of organic acids in the urine. Diagnosis is usually based on identification of the abnormal metabolites, working back from there to characterise the defect at enzymatic level. The chirality of these metabolites may be significant in that the different enantiomers of a substance usually have different metabolic origins. Thus, knowledge of the chirality of a metabolite aids in the understanding of the mechanism of the disorder.

The chirality of a number of urinary metabolites in inherited metabolic disease was examined using gas chromatography-mass spectrometry, most of the analyses being performed using single ion monitoring. This analytical method requires the use of chiral reference compounds of known configuration. Chiral lactic and glyceric acids are available commercially, but a range of chiral 3-hydroxy acids were prepared by the reduction of the corresponding 3-ketocarboxylic acid esters with fermenting baker's yeast. After hydrolysis of the esters, separation of the enantiomers is based on their reaction with a suitable chiral reagent to form a volatile mixture of the diastereoisomers which are resolved by gas-liquid chromatography using a capillary column with a non-chiral stationary phase. Information from the analysis of the chiral standards was used to assign the absolute configuration of the following urinary metabolites; lactic acid in an unusual case of lactic aciduria, glyceric acid in the glyceric acidurias, 3-hydroxybutyrate in ketonuria, 3-hydroxyvalerate in propionic acidaemia, 2-methyl 3-hydroxybutyrate in β-ketothiolase deficiency and 3-hydroxyadipic acid in hydroxydicarboxylic aciduria. The biochemical significance of the chirality of each of these metabolites is discussed.

The assigning of configuration to urinary 3-hydroxyvalerate in propionic acidaemia, 2-methyl 3-hydroxybutyrate in β-ketothiolase deficiency and 3-hydroxyadipic acid in hydroxydicarboxylic aciduria represents original work and has helped to elucidate the metabolic origins of these compounds.

CONTENTS. 1

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1. INTRODUCTION.

1.1 Inherited Metabolic Disease.

The common feature of all inherited metabolic disorders is a qualitative or quantitative change in a protein which has some function in the body. Genes in the nuclei of cells code for amino acids and their arrangement into polypeptides and more complex proteins. This vast number of proteins form and control single cells, their structure into tissues and organs and the metabolism of the body as a whole. Alteration of a single base in a gene can lead to substitution of an amino acid in a polypeptide chain which ultimately changes the nature of the protein product. This may involve synthesis of a protein with an abnormal structure which renders it incapable of performing its normal function. Alternatively, if the change is in the regulatory section of the gene, it may lead to the production of reduced or increased quantities of that protein or a total lack of protein synthesis. A person who inherits the abnormal gene will therefore have a defect associated with the mechanism of a fundamental body protein. This protein may be an enzyme, in which case there could be profound effects upon its structure and catalytic function.

Most of the documented metabolic disorders are the result of an enzyme defect, but some inborn errors of metabolism are known in which the abnormality is in some other mechanism. An example is cystine-lysinuria, leading to the formation of cystine stones in the kidney and bladder, which is due to a defect in the renal transport system of cystine and related amino acids, including lysine.

Most of the inherited metabolic disorders are determined by genes on the autosomes, that is, a chromosome which is not a sex chromosome. Many of the disorders are known to have an autosomal recessive mode of inheritance and this can be explained in simple Mendelian terms (Figure 1.1). Both the mother and father are heterozygous for the variant gene. This means that, given random association of genes at conception, there is a 1 in 4 chance that they will produce an affected child.

1.1.1 Enzyme defects.

All of the inherited disorders discussed in this dissertation are the result of enzyme defects in organic acid metabolism and it is useful to examine in general terms the metabolic consequences of such a defect. Under normal circumstances a particular enzyme reaction is part of a metabolic pathway such as that illustrated in figure 1.2a showing the catabolism of W to Z. In the event of a deficiency of the enzyme (E_3), which catalyses the conversion of Y to Z, there may be a number of outcomes. There may simply be a failure to form the end product of the pathway (Figure 1.2b), an accumulation of substances prior to the block (Figure 1.2c) and the formation of secondary metabolites from the accumulated precursors (Figure 1.2d).

The accumulation of substances prior to the metabolic block and the production of secondary metabolites from these precursors are major factors in the presentation and diagnosis of a disorder.

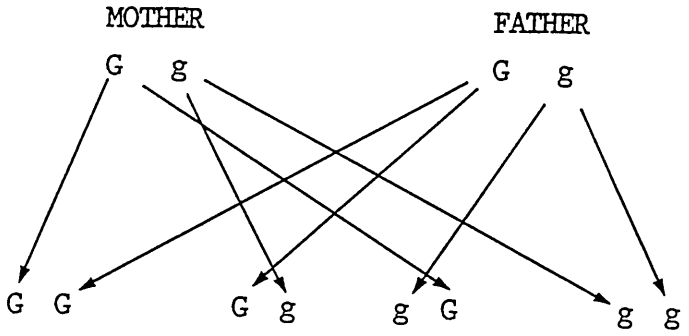


Figure 1.1 Diagram to show the inheritance pattern for a gene G and a mutant form g. GG = homozygous normal Gg/gG = heterozygous carriers gg = homozygous recessive = affected.

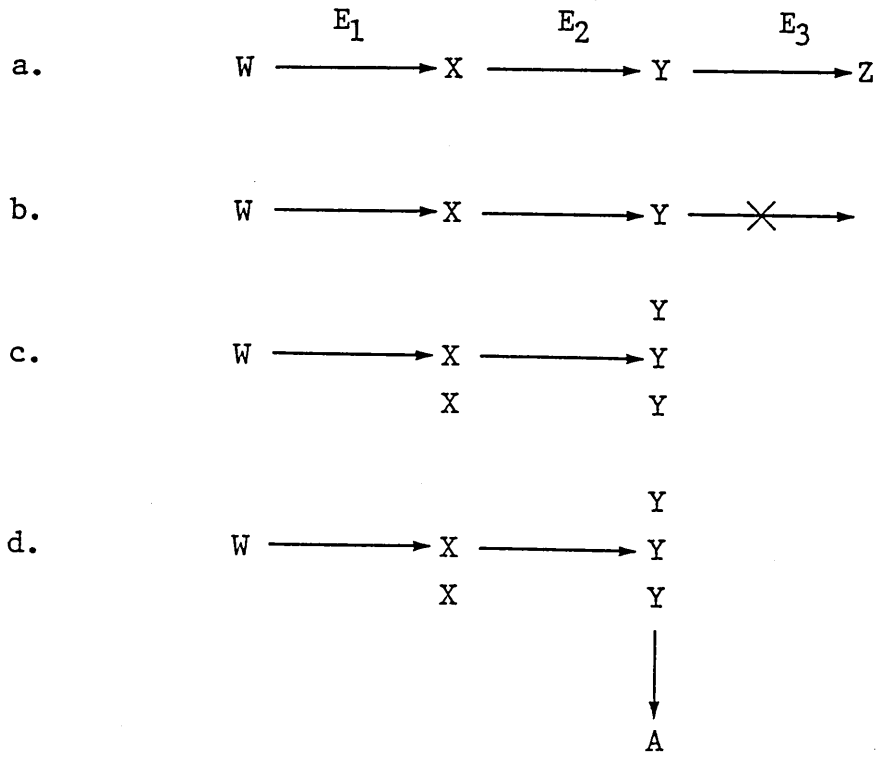


Figure 1.2 The effect of an enzyme deficiency (E_3) in the hypothetical metabolic pathway from W to Z.

1.2 Chirality.

It is appropriate to discuss the meaning of chirality before examining its significance to the organic acidurias.

A chiral molecule is one which is not superimposable upon its mirror image, that is, it has no reflectional symmetry. A carbon atom which has four different atoms or groups directly bonded to it is said to be asymmetric or chiral. An example is carbon 2 in lactic acid (2-hydroxypropionate) (Figure 1.3). The two forms of the acid are optical isomers and are called enantiomers. The enantiomers will rotate a plane of polarised light in opposite directions but to an equal degree. The one which rotates polarised light to the left is the laevrorotatory (l) enantiomer and the one which rotates it to the right the dextrorotatory (d) enantiomer. An equimolar mixture of the two enantiomers has no effect upon the light and is known as a racemic mixture. Apart from this property, enantiomers are chemically and physically identical.

A more complex molecule than lactic acid may have many chiral carbons. Such molecules are known as diastereoisomers (Figure 1.4). Diastereoisomers are not enantiomers, but they are stereoisomers. Unlike enantiomers, which differ only in their response to polarised light, diastereoisomers have completely different chemical and physical properties.

The term prochiral refers to a trigonal centre which would become chiral on the introduction of an appropriate fourth substituent. It also refers to a tetrahedral centre with two similar groups which would become chiral on the substitution of one of the identical groups for a fourth different group.

Figure 1.3 The enantiomers of lactic acid.

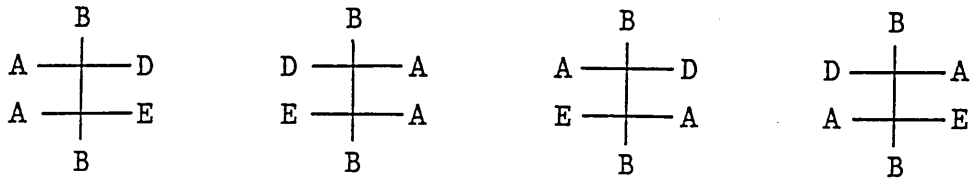


Figure 1.4 Plane diagrams to illustrate diastereoismerism. Molecules 1 and 2 are enantiomers, as are 3 and 4. Molecules 1 and 3 are stereoisomers, but they are not enantiomers. They are diastereoisomers, as are 2 and 4. Molecules 1 and 2 have configurations such that identical substituents can eclipse each other and are known as the erythro forms. Molecules 3 and 4 are the threo forms; identical substituents are not able to eclipse each other.

1.2.1 The designation of absolute chemical configuration.

One convention for the designation of chemical configuration to a molecule is to relate its structure to the configuration of glyceraldehyde where the designation is D or L. This method has no correlation with the d/l system outlined above.

The accepted method for the unambiguous designation of absolute configuration was developed by Prelog et al. (1966). In this the four substituents attached to the carbon atom are ranked in order of decreasing atomic weight. The asymmetric carbon is then viewed from the substituent opposite the group of lowest atomic weight and the orientation of the remaining substituents around the carbon atom in order of decreasing atomic weight determined. If the orientation is clockwise the chiral carbon is said to have the R configuration and if anti-clockwise, the S configuration. (Figure 1.5).

1.2.2 The stereospecificity of enzymes.

The stereospecificity of enzyme-catalysed reactions is one of their major properties and there are numerous examples in nature of the biological activity of chiral compounds. In man, carboxylation of propionyl CoA via the action of propionyl CoA carboxylase (EC 6.4.1.3) produces R(L) methylmalonyl CoA. This is then converted to the S(D) enantiomer via a racemase which moves the alpha-hydrogen of the R-methylmalonyl CoA. The R enantiomer is then converted to succinyl CoA via the action of a specific B_{12}-dependant mutase (EC 5.1.99.1) (Figure 1.6) (Harper's Biochemistry 1988). The production of cinnamic acid from L-phenylalanine via the enzyme phenylalanine ammonia lyase (EC 4.3.1.5),

Order of priority OH > COOH > CH_3 >H

S R

Figure 1.5 Diagram to illustrate the absolute configuration of the enantiomers of lactic acid.

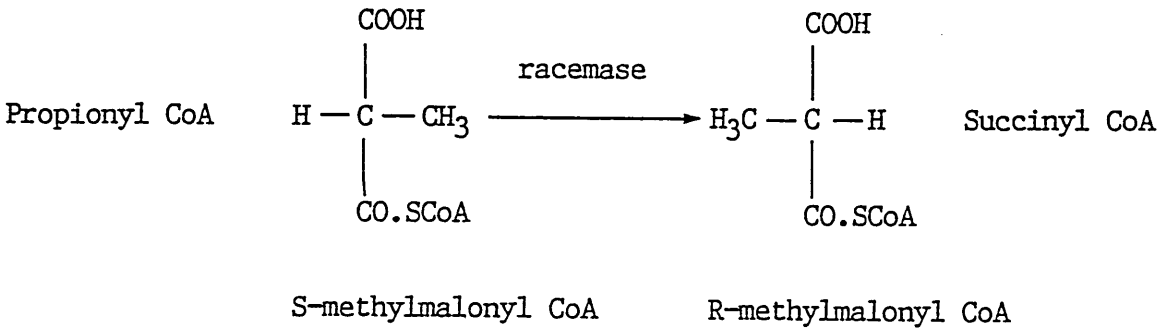


Figure 1.6 The inversion of configuration of methylmalonyl CoA via methylmalonyl CoA racemase.

for example, is part of the process by which lignin is formed in higher plants. The enzyme will act upon the L enantiomer of phenylalanine only (Bartlett and Battersby 1974).

A further example is that of the boring insect Gnathotricus sulcatus which appears to respond to the S enantiomer of its pheromone sulcatol in laboratory assay and this response appears to be inhibited by the R enantiomer (Mori 1981).

1.2.2.1 The mechanism of enantioselection.

Enantioselection, the ability of enzymes to distinguish between enantiomers, can be explained in terms of the differences in interactions between the enantiomer and a chiral active site. The preference of an enzyme for one substrate enantiomer from a racemic mixture can range from total to very slight depending on the structure of the substrate. A few enzymes are known which can act equally on both enantiomers, but this is rare. An example is the hydrogenation of chiral allenes by the bacterium Clostridium kluyveri; each enantiomer is reduced equally to give cis and trans products (Rambeck et al. 1974).

Some enzymes can also make prochiral distinctions; something which is illustrated by the formation of chiral alcohols from acyclic ketones by yeast oxidoreductases (Sih and Chen 1984). This property can be explained by the formation of two diastereoisomeric transition complexes between the enzyme and the prochiral substrate. For enantioselection to occur one of the transition complexes must have a lower free energy (ΔG) so that this is favoured over the other. The degree of enantiomeric excess (ee) is determined by the difference in free energy. Where this difference is ≥ 3 kcal $mole^{-1}$ an optically pure product would result. An

would result. An enzyme will therefore be highly enantioselective if it can distinguish between enantiotopic faces in a substrate molecule. If there is no discrimination the result is a racemic mixture. For example; 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30) from Rhodopseudomonas spheroides catalyses the reaction:-

$$\text{D-3-Hydroxybutyrate} + NAD^+ \longrightarrow \text{Acetoacetate} + NADH + H^+$$

The enzyme does not react with L-3-hydroxybutyrate even when it is present in high concentrations. (Krebs et al. 1969). In contrast, 3-hydroxybutyrate produced by the reduction of acetoacetate with an aqueous solution of potassium borohydride consists of a racemic mixture.

1.2.2.2 The significance of enzyme stereospecificity to inherited metabolic disease.

The stereospecificity of enzyme reactions means that the configuration of urinary metabolites in the organic acidurias may be of diagnostic significance in that different enantiomers of the same substance can originate from different metabolic pathways. Lactic acid produced normally in man has the L configuration, but cases of children with short-gut syndrome have been reported who excrete D-lactic acid of bacterial origin (Perlmutter et al. 1983 and Haan et al. 1985). Chalmers et al. (1980) reported a case of D-2-hydroxyglutaric aciduria and proposed that this disorder was due to a defect in the degradation of 5-aminolaevulinic acid. 2-Hydroxyglutarate is also excreted in glutaric aciduria type II and has been shown to consist mainly of the D enantiomer (Goodman et al. 1982). D-2-Hydroxyglutarate is normally

oxidised to 2-ketoglutarate in man via D-2-hydroxyglutarate dehydrogenase (EC 1.1.99.2) and it is this enzyme which Chalmers et al. 1980 suggest is deficient in the case of D-2-hydroxyglutaric aciduria. L-2-Hydroxyglutaric aciduria has also been reported, but the biochemical basis is unknown (Duran et al. 1980). Two or more glyceric acidurias are known. In one there is the excretion of D-glyceric acid (Wadman et al. 1976, Kolvraa et al. 1976 and Duran et al. 1987) and in the other L-glyceric acid is produced. (Williams and Smith 1968 and Mistry et al. 1988).

Knowledge of the configuration of a urinary metabolite can also help to determine its origin by comparison with the configuration of compounds from systems known to operate in cells. Kamerling et al. (1982) determined that urinary 5-hydroxyhexanoate produced by some patients with impaired fatty acid oxidation systems consisted mainly of the L-enantiomer and concluded that it was a by-product of extra-mitochondrial (ω-1)-oxidation of fatty acids. This conclusion was partially determined by comparison with the work of Hamberg and Bjorkhem (1971) who found that when decanoic acid was incubated with rat liver microsomes the resulting 9-hydroxydecanoic acid was mainly of the L configuration.

Following initial study by Pollitt et al. (1985) of a new disorder in which there is a defect in malonic semialdehyde dehydrogenase (EC 1.2.1.15), Manning and Pollitt (1985) examined the chirality of some metabolites of R and S-methylmalonyl semialdehydes in this disorder affecting valine and thymine metabolism. Examination of the configuration of some urinary metabolites after ingestion of deuterated isobutyric acid suggested that the R enantiomer of methylmalonic

semialdehyde is the substrate of methylmalonic semialdehyde dehydrogenase.

1.3 The Organic Acidurias.

The organic acidurias/acidaemias (the presence of organic acids in the urine and blood) cover defects in many areas of body biochemistry including fatty acid oxidation, amino acid metabolism and pyruvate/lactate metabolism. The major feature of the organic acidurias is the accumulation of abnormal amounts of organic acids in the body fluids and their excretion into urine. The presence of organic acids in body fluids affects the body's acid/base buffering system causing lowering of blood pH and, consequently, a metabolic acidosis. It is this, combined with the presence of toxic amounts of precursors and their conjugates, which account for the illness.

The clinical symptoms associated with organic acidurias share many features. Patients often present early in life with acidosis, vomiting, drowsiness, convulsions and possibly coma. The illness is potentially fatal in young babies and those who do survive may be mentally handicapped. Some patients present later in childhood having had either prolonged failure to thrive or a sudden acute attack which may be associated with infection.

It is extremely important that these disorders be diagnosed quickly since therapy can often improve the patient's prognosis. Diagnosis is based on organic acid analysis of a body fluid, usually urine. This is done most effectively by gas chromatography (GC) or gas chromatography-mass spectrometry (GC-MS) in the first instance, followed by further biochemical investigation. For GC analysis, the organic acids are extracted from the urine and a stable derivative formed which enables their separation by gas chromatography. Tentative identification of the acids may then be made on the basis of retention time; that is, the time

taken for a particular compound to elute from the chromatography column expressed relative to an homologous series of hydrocarbons. GC-MS, in which a mass spectrum of each compound eluting from the chromatography column is obtained, is necessary for unequivocal identification (Figure 1.7).

Once the organic acids in a urine sample have been identified, diagnosis can be made by examining the overall pattern of excretion. It is important to have an understanding of the normal urinary organic acid excretion pattern in man and to appreciate that the urinary organic acids of a patient with a metabolic disorder may appear normal when not in crisis (Appendix I Chromatogram 1).

Following GC-MS analysis, further biochemical investigation such as enzyme assay of cultured skin fibroblasts or tissue biopsy is normally carried out for confirmation.

The following is an outline of the inborn errors of metabolism and related conditions which were investigated. In each case, the enzyme deficiency, the general clinical presentation and the abnormal urinary metabolites are described. The abnormal urinary metabolites described were detected at this laboratory as part of the routine analysis of samples. Most of this work was not performed by the author.

1.3.1 Lactic aciduria.

Figure 1.8 shows the formation of lactic acid from pyruvic acid. Lactic aciduria can result from many different uninherited conditions such as severe anaemia. It can also be due to the disturbance of pyruvate or lactate metabolism secondary to another organic aciduria. A

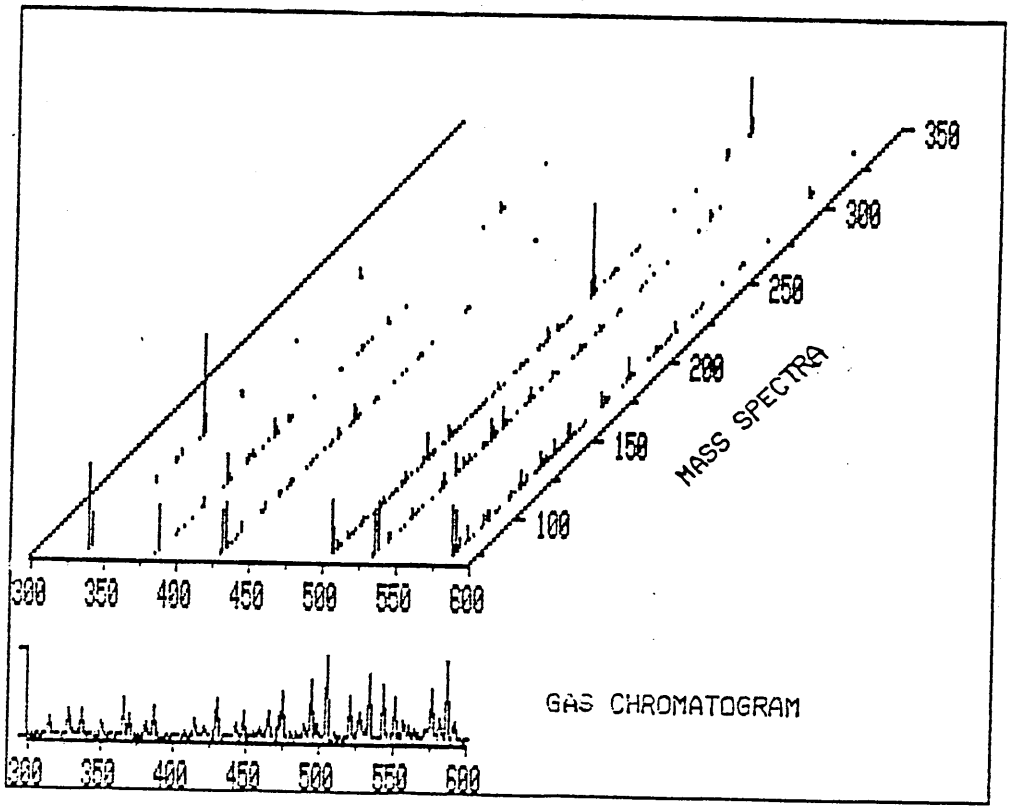
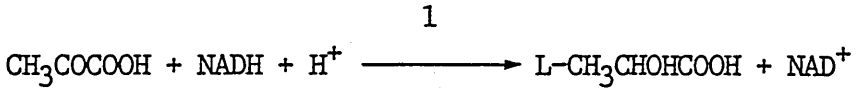


Figure 1.7 Diagram showing the mass spectra of selected peaks from the gas chromatogram.



1 = Lactate dehydrogenase (EC 1.1.1.27).

Figure 1.8 The formation of L-lactic acid from pyruvate.

concentration of 2.5mmol/L or above of lactic acid in the blood of a child would constitute a lactic acidosis but, due to renal tubule reabsorption of lactate, a lactic aciduria would not be noticed until the blood lactate concentration reached about 7mmol/L.

The child examined at this laboratory was a boy who was born at 27 weeks gestation and who suffered from a number of severe problems associated with prematurity. At 4 months of age he died due to pneumonia and septicaemia and subsequent post-mortem examination showed brain, liver and kidney abnormalities. A number of urine samples had been collected by the medical staff when the child was between 10 and 17 weeks of age and GC-MS analysis showed significant excretion of 3-hydroxypropionic acid, 1,3-propandiol and lactic acid (Pollitt et al. 1987). 3-Hydroxypropionic acid excretion is a feature of propionic acidaemia, but fibroblast assay of propionyl CoA carboxylase ruled out the disorder in this case. A further 2 children, who also died in infancy, showed the same profiles. In another patient with a similar illness, the 3-hydroxypropionic acid and 1,3-propandiol disappeared on the administration of the antibiotic neomycin and so were probably caused by the activity of gut bacteria.

Lactic acid produced normally in mammals has the L configuration, but some cases are known in which lactic acid with the D configuration is excreted. This D-lactic acid is of bacterial origin. Therefore, the configuration of the lactate produced by this child was determined to discover whether this too was a product of bacterial metabolism.

1.3.2 The glyceric acidurias.

The configuration of glyceric acid excreted by any patient must be assigned for diagnostic purposes.

1.3.2.1 D(R)-Glyceric aciduria.

D-Glyceric aciduria is a rare inborn error of metabolism characterised by high concentrations of D-glyceric acid in the blood and urine. Two quite different presentations of glyceric aciduria have been described in the literature consisting of five patients in all. One patient suffering from mild metabolic acidosis and developmental delay (Wadman et al.1976), one patient described by Van Schaftingen (1988) with developmental delay and hypotonia and three with mental retardation, siezures and hypotonia (Brandt et al. 1976, Grandgeorge et al. 1980 and Duran et al. 1987). D-Glycerate kinase deficiency (EC 2.7.1.31) (Figure 1.9A) has been demonstrated in Van Schaftingen's patient and it is postulated that Wadman's patient also suffered from the same defect. Loading experiments with fructose lead to the theory that Duran's (1987) patient suffered from a deficiency of triokinase (EC 5.3.1.1) and similar results with Brandt's (1976) patient suggested that this deficiency could account for the more severe presentation of this form of glyceric aciduria. Triokinase catalyses the phosphorylation of dihydroxyacetone to dihydroxyacetone phosphate and of glyceraldehyde to glyceraldehyde-3-phosphate.

We have examined urine from five patients at our laboratory. Patient 1 presented with neurological abnormalities including unusual eye movements in the immediate peri-natal period. Urinary organic acids were

examined by GC-MS and showed a large peak of glyceric acid, but no other abnormality. In another instance (patient 2), glyceric acid was detected in the urine of a child under investigation for speech delay and microcephaly. Three siblings of this child (patients 3 to 5) also excreted glyceric acid, but were asymptomatic (Appendix I Chromatogram 2).

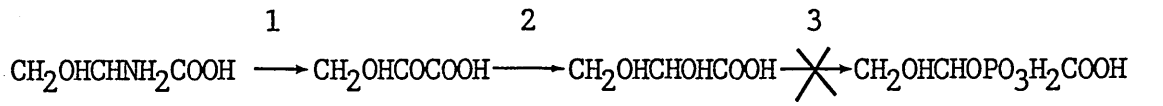
1.3.2.2 L(S)-Glyceric aciduria.

The human metabolic disorder primary hyperoxaluria type 2 is characterised by recurrent renal calculi, hyperoxaluria and L-glyceric aciduria. It has been recently demonstrated in liver that this is due to a deficiency of the enzymes glyoxylate reductase (EC 1.1.1.26) and D-glycerate dehydrogenase (EC 1.1.1.29) (Figure 1.9B) (Mistry et al.1988).

Generally, the disorder presents during the first decade of life with the patients suffering from oxalate renal calculi of which surgical removal is sometimes necessary. In more severe cases the stones recur and nephrectomy was ultimately carried out. The disorder usually leads to early death due to kidney failure or heart block caused by damage to the nerves in heart muscle by oxalate crystals. Analysis of the urinary organic acids from a patient with this disorder showed excretion of large amounts of glyceric acid together with smaller amounts of oxalic acid (Appendix I Chromatogram 3).

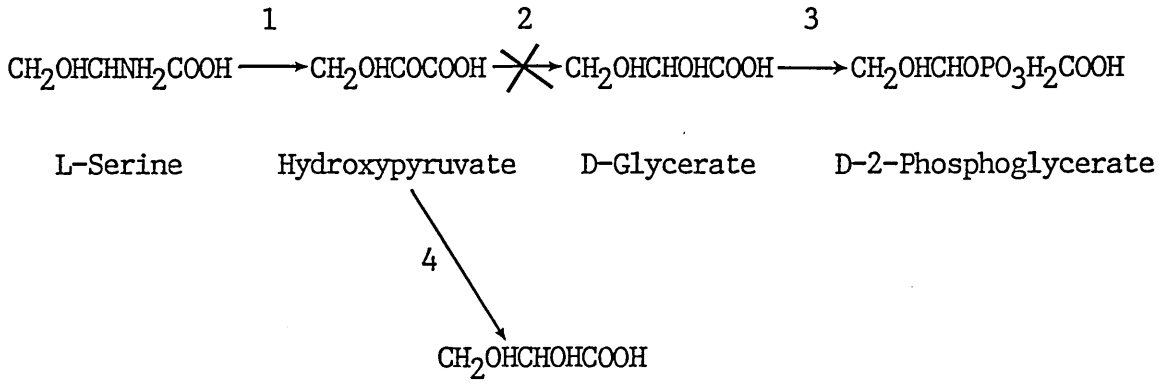
This disorder was also demonstrated coincidentally in a closed colony of cats when certain animals died with renal failure (Blakemore et al.1988). The affected animals showed muscle weakness beginning between 5-9 months of age. This eventually became profound and was accompanied by renal failure and death. Muscle biopsy carried out by the

A.



L-Serine Hydroxypyruvate D-Glycerate D-2-Phosphoglycerate

B.



L-Serine Hydroxypyruvate D-Glycerate D-2-Phosphoglycerate

L-Glycerate

1 = L-serine-pyruvate aminotransferase (EC 2.6.1.51)
2 = D-glycerate dehydrogenase (EC 1.1.1.29)
3 = D-glycerate kinase (EC 2.7.1.31)
4 = Lactate dehydrogenase (EC 1.1.1.27)

Figure 1.9 The enzyme defects associated with the glyceric acidurias.
A. D-glyceric aciduria as a result of D-glycerate kinase deficiency leading to the accumulation of D-glycerate.
B. L-glyceric aciduria as a result of D-glycerate dehydrogenase deficiency leading to the accumulation of hydroxypyruvate which is reduced to L-glycerate.

veterinary surgeons during the stage of profound muscle weakness showed denervation atrophy, although biopsy taken before the onset of crisis showed no abnormality.

Subsequent post-mortem examination carried out by the veterinary surgeons found swollen kidneys with oxalate crystals in the renal tubules. Also, the proximal regions of motor and sensory axons were distended with neurofilaments.

Urinary organic acid analysis showed excretion of glyceric acid and oxalic acid similar to the human pattern (Appendix I Chromatogram 4).

1.3.3 Ketosis.

Ketosis is a condition which occurs when fat stores are the body's main energy source such as during a fast or when nutrient intake is low due to vomiting. At these times the fuel for metabolism is shifted from glucose to fatty acids and ketone bodies (acetoacetate and 3-hydroxybutyrate). The heart and brain especially rely on the ketone bodies as their main energy source during starvation. This change in fuel usage is known as ketosis and ketonuria occurs when acetoacetate and 3-hydroxybutyrate are found in the urine.

A child was investigated at this hospital after presenting with Reye-like episodes. Reye's syndrome is an illness, which may have a number of causes, characterised by vomiting, convulsions, lethargy, hypoglycaemia and enlarged liver which can progress to coma and death if not treated. There is often a mild illness such as a childhood infection before the onset of the more severe symptoms of the syndrome (Reye 1963). There appears to be uncoupling of oxidative phosphorylation in Reye's syndrome which causes inhibition of many mitochondrial enzymes

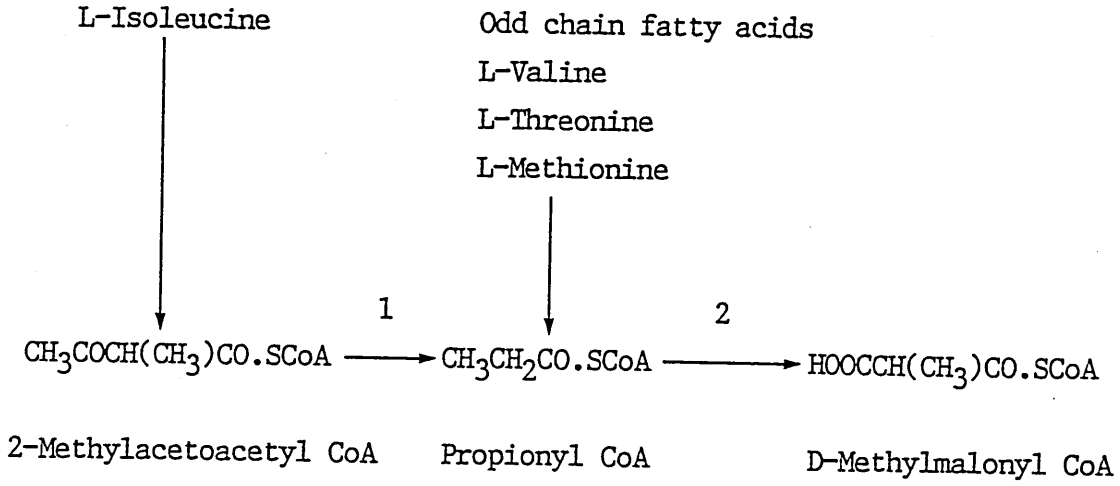
(Tracey et al. 1987). This acute type of presentation is associated with disorders of fatty acid metabolism such as medium chain acyl CoA dehydrogenase deficiency, an enzyme involved in mitochondrial β-oxidation. Patients with this disorder are unable to catabolise fats to produce ketone bodies during a fast (see also section 1.3.6). Therefore, urine was collected by the medical staff during a 24 hour fast to test for fatty acid oxidation defects and the organic acids were examined. This showed a large ketonuria, but no other abnormality (Appendix I Chromatogram 5).

The 3-hydroxybutyric acid excreted during ketosis is known to have the D(R) configuration and the configuration of the 3-hydroxybutyrate excreted by the above patient is assigned for confirmation only.

1.3.4 Propionic acidaemia.

Propionic acidaemia is a human inherited metabolic condition caused by a deficiency of the mitochondrial, biotin-dependent enzyme propionyl CoA carboxylase (Hsia et al. 1969). This enzyme catalyses the carboxylation of propionyl CoA to D-methylmalonyl CoA on the catalytic pathway of isoleucine, valine, threonine and methionine (Figure 1.10). The disorder has an autosomal recessive mode of inheritance.

Typically, patients present dramatically during the first few days of life with rapid respiration and refusal of feeds and often vomiting and convulsions. The illness progresses with dehydration and drowsiness and eventually, coma. Mental retardation and early death usually occur, but in those children who do survive, acute ketotic crises are provoked by infection and protein meals. (Wolf et al. 1981).



1 = β-Ketothiolase (EC 2.3.1.9)

2 = Propionyl CoA carboxylase (EC 6.4.1.3)

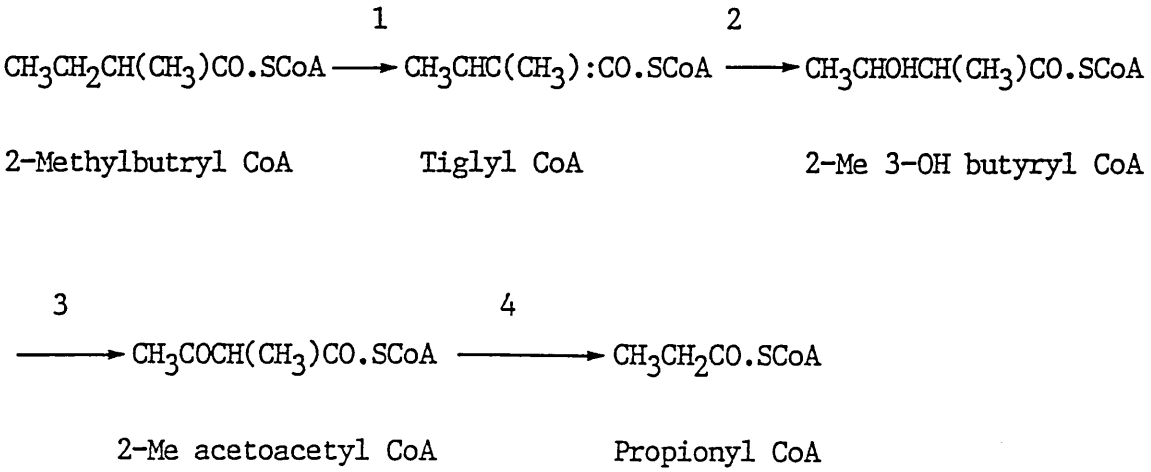
Figure 1.10 The site of the defect in propionic acidaemia.

Examination of the urinary organic acids from a child suffering from propionic acidaemia shows a number of metabolic products formed due to the accumulation and secondary metabolism of propionyl CoA, some of these formed when it substitutes for acetyl CoA in other metabolic pathways such as ketogenesis. There is large excretion of 3-hydroxypropionic acid, propionylglycine and methylcitric acid together with smaller amounts of 3-oxo and 3-hydroxyvaleric acids, 2-methyl 3-oxovaleric acid and 2-methyl acetoacetic acid (Appendix I Chromatogram 7).

The 3-hydroxyvaleric acid produced in propionic acidaemia is thought to be formed in an analagous manner to 3-hydroxybutyrate in ketosis and its configuration has not been previously investigated. It was therefore determined in two patients suffering from propionic acidaemia.

1.3.5 2-Methylacetoacetyl CoA thiolase deficiency (β-ketothiolase deficiency).

This inherited disorder is due to the deficiency of the thiolase which catalyses the cleavage of 2-methylacetoacetyl CoA to acetyl CoA and propionyl CoA in the isoleucine catalytic pathway. There are three different β-ketothiolases in mammalian tissue, one cytoplasmic and two mitochondrial. One of the mitochondrial enzymes is specific for acetoacetyl CoA and 2-methylacetoacetyl CoA while the other enzyme has a wider substrate specificity. It has been demonstrated that it is the acetoacetyl CoA and 2-methylacetoacetyl CoA specific thiolase which is deficient in this disorder (EC 2.3.1.9) (Middleton and Bartlett 1983) (Figure 1.11).



1 = 2-Methyl branched chain dehydrogenase (EC 1.2.4.4)
2 = Enoyl CoA hydratase (crotonase) (EC 4.2.1.17)
3 = 3-Hydroxyacyl CoA dehydrogenase (EC 1.1.1.35)
4 = Methylacetoacetyl CoA thiolase (EC 2.3.1.9)

Figure 1.11 The enzyme defect in 2-methylacetoacetyl CoA thiolase deficiency.

The clinical presentations of this disorder appear to differ widely, but conform to 3 distinct types and have common features. The first type consists of a child who had persistant vomiting in the first few days of life and who later developed cardiomyopathy (Keating et al. 1972 and Henry et al. 1981). In the second group no symptoms were seen during the first year of life, but acidosis and vomiting and in some cases lethargy and coma following infections developed in the second or third year (Daum et al. 1973 and Robinson et al. 1979). In the third group there are no symptoms until after the fourth year when the illness usually presents with frequent recurrent headaches (Gompertz et al. 1982).

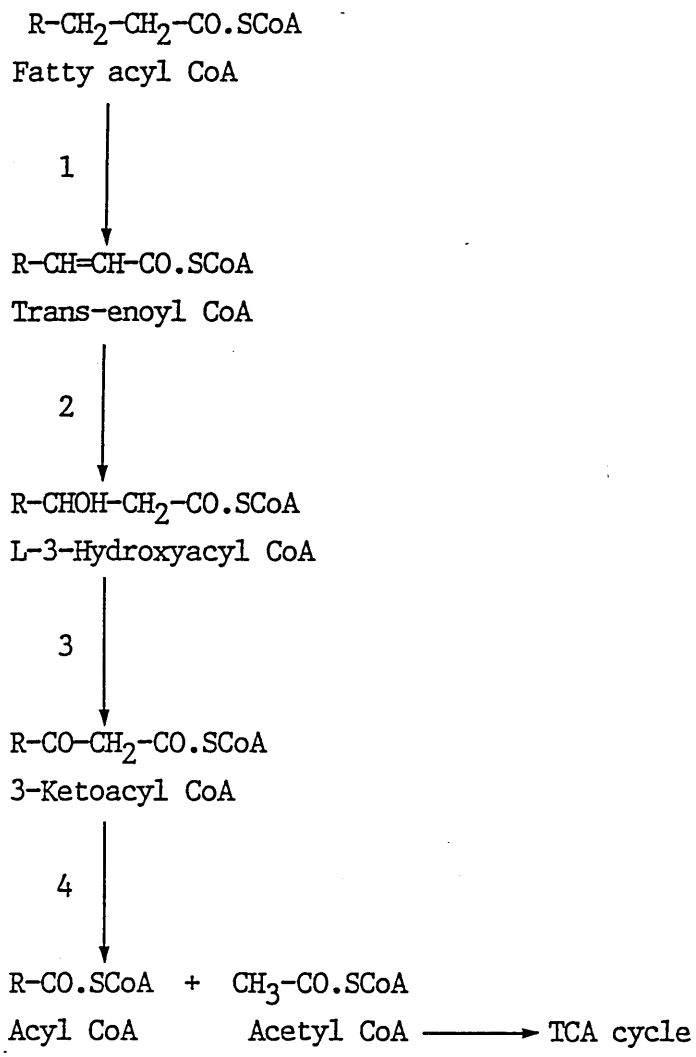
Urinary organic acids from patients with this disorder show excretion of large amounts of the ketone bodies and of threo- and erythro-2-methyl 3-hydroxybutyric acid, 2-methyl 3-ketobutyric acid and tiglylglycine (Appendix I Chromatogram 6).

Although Liebich and Forst (1984) observed that in ketoacidosis the 2-methyl 3-hydroxybutyrate excreted consisted mainly of the threo form, the absolute configuration of urinary 2-methyl 3-hydroxybutyric acid excreted by patients with this disorder has not previously been investigated. Configuration was therefore assigned in one patient with 2-methylacetoacetyl CoA thiolase deficiency who presented during the first week of life.

1.3.6 3-Hydroxydicarboxylic aciduria.

3-Hydroxydicarboxylic aciduria is a comparatively recently discovered disorder of fatty acid oxidation in which there is urinary excretion of even-chain dicarboxylic acids and of large amounts of 3-hydroxydicarboxylic acids combined with an absence of ketonuria. The actual enzyme defect is still under investigation but is thought to lie on the β-oxidation spiral with either L-3-hydroxyacyl CoA dehydrogenase (EC 1.1.1.35) or 3-oxoacyl CoA thiolase (EC 2.3.1.16) (Figure 1.12).

There appears to be two distinct presentations of 3-hydroxy-dicarboxylic aciduria. The first, which includes the majority of the patients characterised so far, present with an illness similar to Reye's syndrome within the first year of life; that is, vomiting, lethargy and convulsions progressing to coma and possibly death. One patient had been classified as a case of sudden infant death as had two siblings of two other patients in this group. The majority of patients suffering from this presentation of 3-hydroxydicarboxylic aciduria have died. The second, smaller group of patients have a chronic presentation consisting of progressive liver disease with fatty infiltration and cirrhosis and also non-ketotic hypoglycaemia on fasting. Non-ketotic hypoglycaemia on fasting means that the patient has low blood sugar, but the normal change from glycolytic to ketotic metabolism by using fat supplies does not take place. Hence, no ketone bodies are produced and this is one of the main features of a disorder of fatty acid oxidation. Inability to β-oxidise fatty acids means that acetyl CoA available for ketogenesis becomes depleted so that these patients have very high concentrations of free fatty acids in their plasma and low 3-hydroxybutyrate. Continuation



1 = Acyl CoA dehydrogenase (EC 1.3.99.3)
2 = Enoyl CoA hydratase (EC 4.2.1.17)
3 = L-3-Hydroxyacyl CoA dehydrogenase (EC 1.1.1.35)
4 = 3-Ketoacylthiolase (EC 2.3.1.16)

Figure 1.12 The mitochondrial β-oxidation cycle.

of fasting in these patients would lead to collapse of mitochondrial function and death.

Urinary organic acid profiles of both groups of patients shows major peaks of adipic, suberic and sebacic acids together with 3-hydroxy-adipic, 3-hydroxysuberic, 3-hydroxysebacic and 3-hydroxydecanedioic acids. Smaller amounts of unsaturated dicarboxylic acids and some odd chain dicarboxylic acids are seen. 4-Hydroxyphenyllactic and 4-hydroxyphenylpyruvic acids are also major features (Appendix I Chromatogram 8).

The 3-hydroxyadipic acid excreted by a subject ingesting dodecanedioic acid has been shown to have the L configuration (Svendsen et al. 1985). It is also known that D-3-hydroxyacyl ACP's (acyl carrier protein) are formed as part of the fatty acid synthetase system. However, the configuration of 3-hydroxyadipic acid excreted by patients with hydroxydicarboxylic aciduria has not previously been assigned. Its configuration was therefore determined in two patients with this disorder; one from each of the two types of clinical presentation.

1.4 Resolution of Asymmetric Compounds by Gas Chromatography.

Two methods exist for the resolution of asymmetric metabolites by GC. One involves the reaction of the enantiomers with a chiral reagent to create mixtures of diastereoisomers which are resolved on a non-chiral chromatography column. Use of GC to separate diastereoisomers is an extension of the classical resolution procedure in which their adsorption and partition differences are exploited. The other involves direct resolution of the enantiomers by stereoselective sorption on a chiral column. All the analyses in this dissertation make use of the first of the two methods and therefore resolution of enantiomers on a chiral column will not be discussed further.

1.4.1 Derivatisation.

The first step in resolution of metabolites by GC is to select a suitable chiral derivative. This must conserve the optical purity of the asymmetric carbon and be thermally and sterically stable at the high temperatures of the chromatographic process. It is also important that the reaction of the derivative with the chiral metabolite be largely quantitative, especially in analyses where only small amounts of compounds are present. It should be noted that resolution on a non-chiral column (an optically inactive stationary phase) is not able to distinguish between enantiomers, that is, ll can not be separated from dd nor ld from dl. Enantiomers can only be distinguished by the use of an optically pure resolving agent.

The resolution process itself depends upon the differential interactions between the diastereoisomers and the stationary phase of

the GC column. Many factors can influence this and there are some general rules which apply to the selection of a suitable derivitising reagent which help to optimise this process. Feibush and Spialter (1971) stated that the distance between the chiral carbons of the compound and the derivitising reagent should be kept as small as possible, ideally less than three atoms. The presence of an aromatic group or acyl groups in the resolving reagent is desirable as they increase the number of interactions between the diastereoisomer and the stationary phase via hydrogen bonding or π-orbital overlap. It has also been shown that a large difference in the size of the groups attached to the derivatising reagent's chiral carbon can be effective (Blau and King 1977).

1.4.2 Designation of absolute configuration by gas chromatography.

Determination of the configuration of an asymmetric compound by gas chromatography is based on the order of elution from the column of the diastereoisomers formed from the chiral compound and a chiral derivative. Co-chromatography of a sample of the single enantiomer, optically-pure derivative with a sample containing a mixture of diastereoismers allows identification of the enantiomers. This means that a standard compound of known configuration derivatised with the optically-pure chiral reagent is necessary.

The main text discusses the production of such standard compounds and their role in determining the configuration of various urinary metabolites in metabolic disease. The biochemical significance of the results is also discussed.

2. EXPERIMENTAL.

2.1 Materials.

The following materials were obtained from Sigma Chemical Company, Dorset, U.K.:-
Bistrimethylsilyl trifluoroacetamide with 1% chloromethylsilane.
Coenzyme A (lithium salt).
Crotonase (EC 4.2.1.17)
L-Cysteine (free base).
Flavin adenine dinucleotide
β-Hydroxybutyrate dehydrogenase (EC 1.1.1.30) (Type V lyophilised powder from Rhodopseudomonas spheroides).
Glutaryl CoA.
D,L- and D-Glyceric acid (2,3-dihydroxypropionate).
N-methyl-N-nitro-N-nitrosoguanidine.
Nicotinamide adenine dinucleotide (Grade III from yeast).
Octanoyl CoA
Phenazine methosulphate
Trifluoracetic anhydride

The following materials were obtained from Aldrich Chemical Company, Dorset, U.K.:-
Cyanoacetic acid (99%+ Gold Label).
Diethyl 3-oxoadipic acid.
Dodecanedioic acid (1,10-decanedicarboxylic acid).
Ethyl 2-methyl acetoacetate (ethyl 2-methyl 3-ketobutyrate).
Ethyl propionylacetate (ethyl 3-ketopentanoate).
3-Oxoadipic acid.
R-2-Phenylpropionic acid
Sodium thiosulphate pentahydrate.
Tetrabutylammonium dihydrogen phosphate (97%).

The following materials were obtained from BDH Limited, Dorset, U.K. All are'AnalaR' grade except where stated:-
Absolute alcohol.
Acetic anhydride.

Adipic acid (GPR) (Hexanedioic acid).
D,L- and L-Alanine.
Ammonium chloride.
Benzene.
Dichloromethane.
Diethyl ether.
1,4-Dioxan.
Di-potassium hydrogen phosphate.
Ethyl acetate.
Ferric chloride.
Hydrochloric acid (Aristar)
Hydroxyammonium chloride.
D,L- and L-Lactic acid.
Lithium aluminium hydride.
l-Menthol.
Methanol.
Methyl acetoacetate.
Potassium borohydride.
Potassium dihydrogen phosphate.
Pyridine.
Sulphuric acid (Aristar).
Sodium chloride.
Sodium hydrogen carbonate.
Sodium hydroxide.
Sodium nitroprusside.
Sucrose.
Trichloroacetic acid.

Some materials were obtained from other suppliers:-
Thionyl chloride - May and Baker Limited, Manchester, U.K.
D,L-2-Phenylpropionic acid - Koch-Light Limited, Haverhill, U.K .
Ethyl buytrylacetate - Fluka Chemical Ltd., Glossop, U.K.
1-Octanol - Fisons Laboratory Reagents Limited, Loughborough, U.K.
M^{C}Dougalls fast-action dried yeast.

2.1.1 Equipment.

The MSE minor 's' bench-top centrifuge was purchased from Fisons PLC, Crawley, Sussex.
The CE212 Variable Wavelength UV Monitor was obtained from Cecil Instruments, Cambridge, U.K.
The Varian gas chromatograph was obtained from Varian Associates Limited, Walton on Thames, Surrey, U.K.
The VG 12-250 mass spectrometer was purchased from VG Masslab Limited, Altringham, Cheshire, U.K.
The HPLC equipment consisting of a Gilson 302 HPLC pump and Gilson 116 detector was obtained from Anachem Biochemistry and Chromatography, Luton, U.K.. The HPLC gradient maker was a Kontron model 425 fitted with a Kontron 420 gradient pump. These were obtained from Kontron Instruments, Watford, U.K..

2.2 Extraction of Urinary Organic Acids.

Urine (fresh or stored frozen at -20°C, 2ml) was saturated with sodium chloride and the pH adjusted to 1 with dilute hydrochloric acid. If required, internal standard (0.5mg/ml heptadecanoic acid in ethyl acetate, 0.5ml) was added at this point.

The urine was extracted by vigorous shaking with approximatly an equal volume of ethyl acetate. After allowing the aqueous and inorganic phases to separate, the organic layer was decanted and dried over anhydrous sodium sulphate. This process was repeated once with ethyl acetate and twice with diethyl ether. The pooled organic extract was reduced using a rotary evaporator and water bath at 37°C leaving an oily residue of organic acids. The residue was then be derivatised using one of the following procedures.

2.3 Derivatisation Procedures.

2.3.1 Trimethylsilylation.

The trimethylsilyl (TMS) derivative was made by the addition of bistrimethylsilyl trifluoroacetamide with 1% chlorotrimethylsilane (100μl) and redistilled pyridine (50μl). The sample was then heated at 80°C for about 20 minutes or left at room temperature overnight. The derivative is very stable and may be stored for months with no ill effect.

This derivative was used for the initial examination of the standard compounds and for the urinary organic acid analysis of patients with inherited metabolic disease.

2.3.2 Methylation.

2.3.2.1 Methylation with diazomethane.

Diazomethane was generated in a MNNG diazomethane apparatus purchased from Aldrich Chemical Company using the method of Fales et al.(1973). N-Methyl-N-nitro-N-nitrosoguanidine (MNNG) (133mg) was placed in the inner tube along with water (0.3ml). The top was sealed with a screw cap with a rubber septum in the centre. Diethyl ether (3ml) was placed in the outer tube. The two tubes were then assembled with an O-ring and pinch clamp and the lower part immersed in an ice-bath. Sodium hydroxide (5M 0.6ml) was injected dropwise through the septum of the inner tube. This reacted with the N-methyl-N-nitro-N-nitrosoguanidine releasing gaseous diazomethane which dissolved in the ether. The reaction was complete after about 40 minutes.

The fresh ethereal solution of diazomethane was added dropwise to the sample until the pale yellow colour persisted, showing complete methylation.

2.3.2.2 Methylation with methanol and thionyl chloride.

The sample was dissolved in a few millilitres of cold methanol and a few drops of thionyl chloride added to this. The solution was refluxed for 30 minutes. After cooling, the methanol and thionyl chloride were removed with a stream of nitrogen (Blau and King 1977).

This method has been found by the author to cause racemisation of some chiral 3-hydroxydicarboxylic acids (see section 4.2.5) and is

therefore not recommended for samples which are subsequently to be derivatised for diastereoisomer resolution.

2.3.3 2-Phenylpropionyl derivatives.

2.3.3.1 Preparation of 2-phenylpropionyl chloride.

Dry benzene was obtained by storing benzene over sodium metal. Pyridine was dried by storing over potassium hydroxide pellets.

2-Phenylpropionyl chloride was prepared according to the method of Hammerstrom et al.(1973). 2-Phenylpropionic acid (200mg) and thionyl chloride (240μl) were mixed at 0°C then heated at 70°C for 30 minutes. Dry benzene (0.5ml) was added and the mixture evaporated to dryness with nitrogen. A further aliquot of dry benzene was added and the solution re-evaporated to dryness. The residue was dissolved in dry benzene (2.4ml) and stored at 4°C.

2.3.3.2 Preparation of the derivative.

2-Phenylpropionyl derivatives were prepared according to the method of Kamerling et al.(1982).

After methylation of the sample with an ethereal solution of diazomethane, the excess of diazomethane and ether were removed with a stream of nitrogen. The residue was treated with dry pyridine (50μl), dry benzene (200μl) and 2-phenylpropionyl chloride solution (100μl). The mixture was kept for 2 hours at room temperature.

Dry benzene (2ml) was added and the organic phase washed twice with aqueous sodium hydrogen carbonate solution (1M 1ml) and twice with water

(1ml). The benzene solution was dried over anhydrous sodium sulphate and evaporated to dryness with nitrogen. The residue was dissolved in a few drops of ethyl acetate. The sample may be stored at 4°C for some months.

This derivative was used to examine the enantiomers of standard 3-hydroxybutyric acid (Figure 2.1), 3-hydroxyvaleric acid (Figure 2.2), 3-hydroxyoctanoic acid (Figure 2.3) and 3-hydroxyadipic acid (Figure 2.4). It was also used in the analysis of the following urinary metabolites:- 3-hydroxybutyric acid in ketosis, 3-hydroxyvaleric acid in propionic acidaemia and 3-hydroxyadipic acid in hydroxydicarboxylic aciduria.

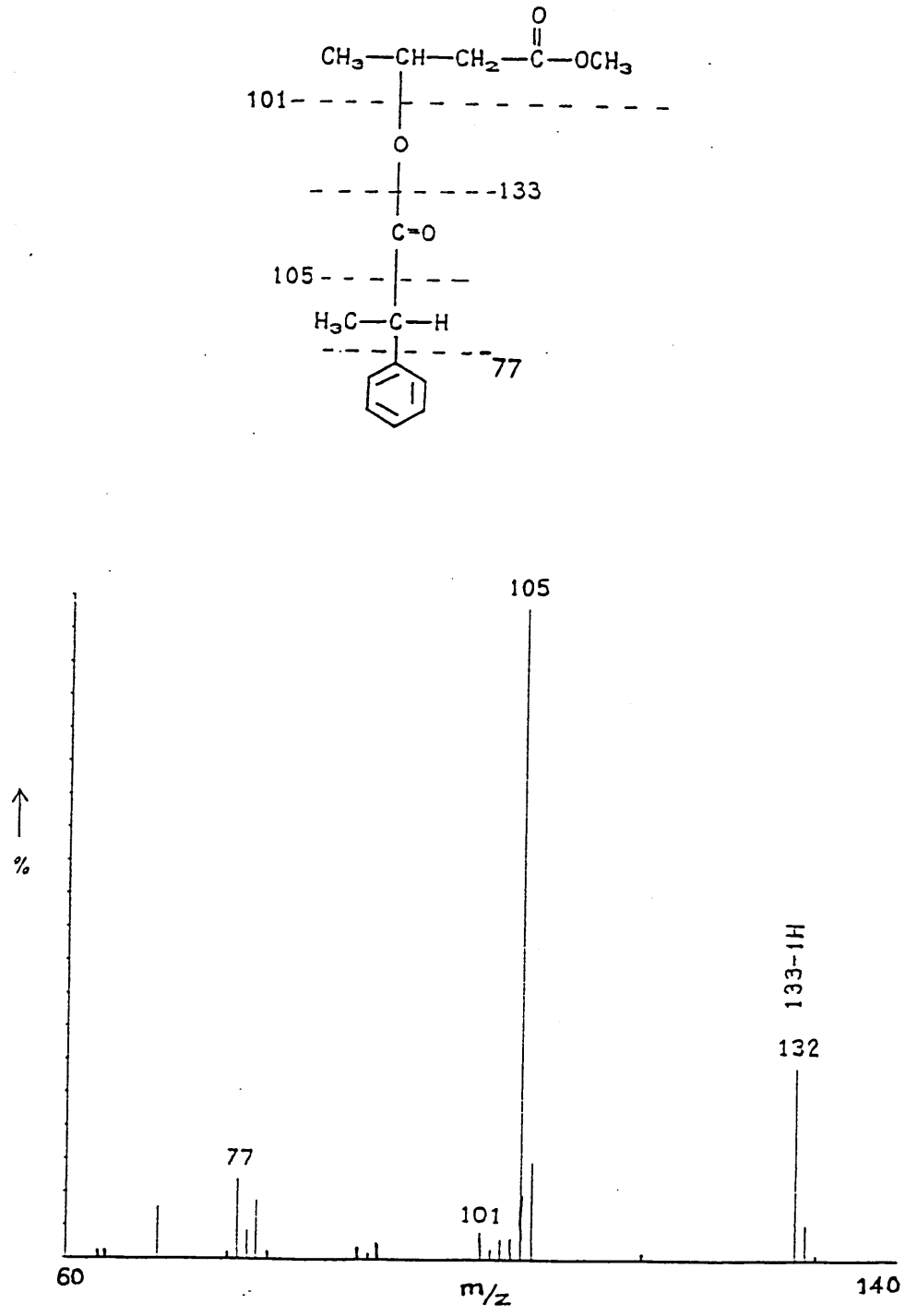
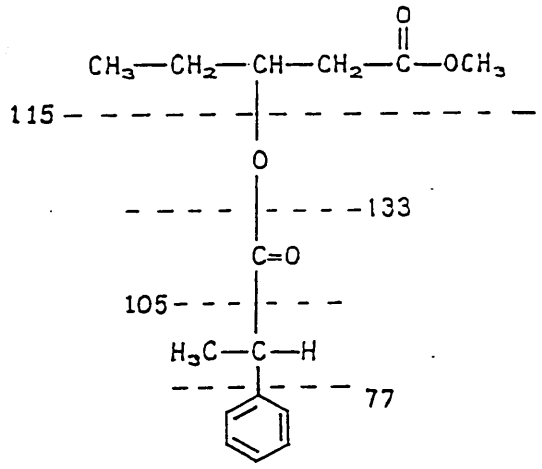


Figure 2.1 The electron impact mass spectrum of the methyl ester R-2-phenylpropionyl derivative of R, S 3-hydroxybutyrate.



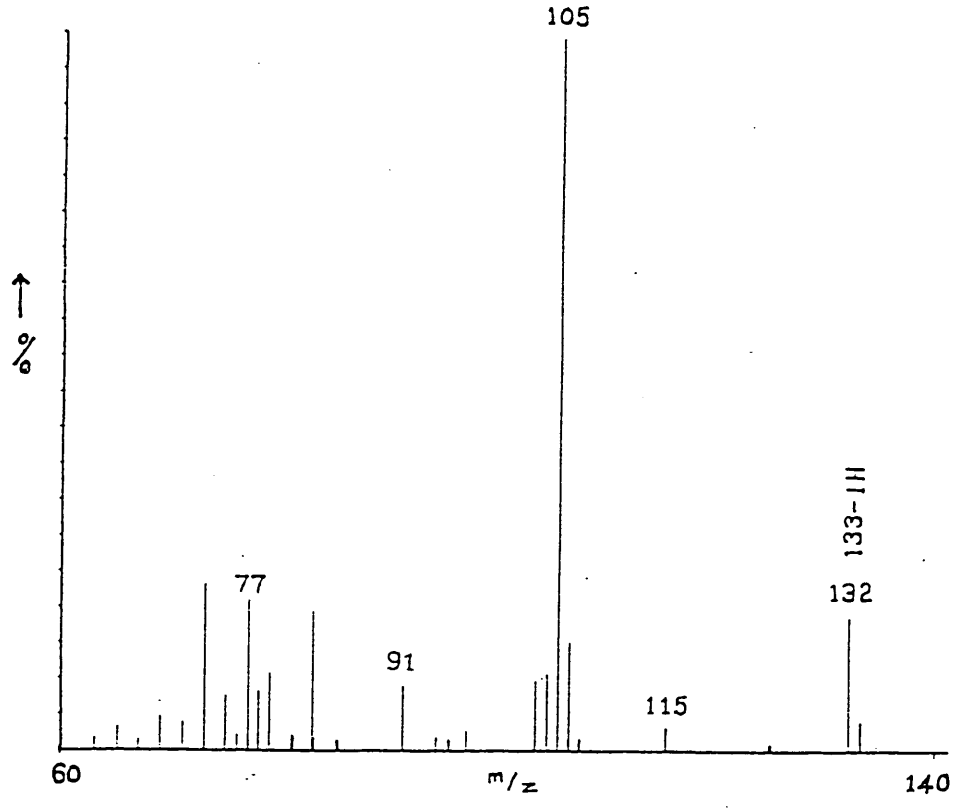
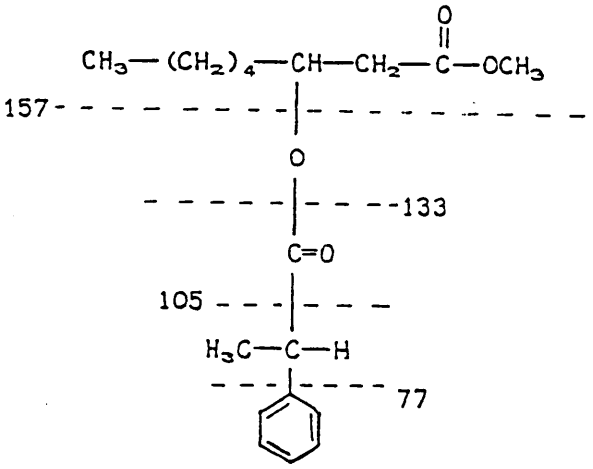


Figure 2.2 The electron impact mass spectrum of the methyl ester R-2-phenylpropionyl derivative of R,S 3-hydroxyvaleric acid.



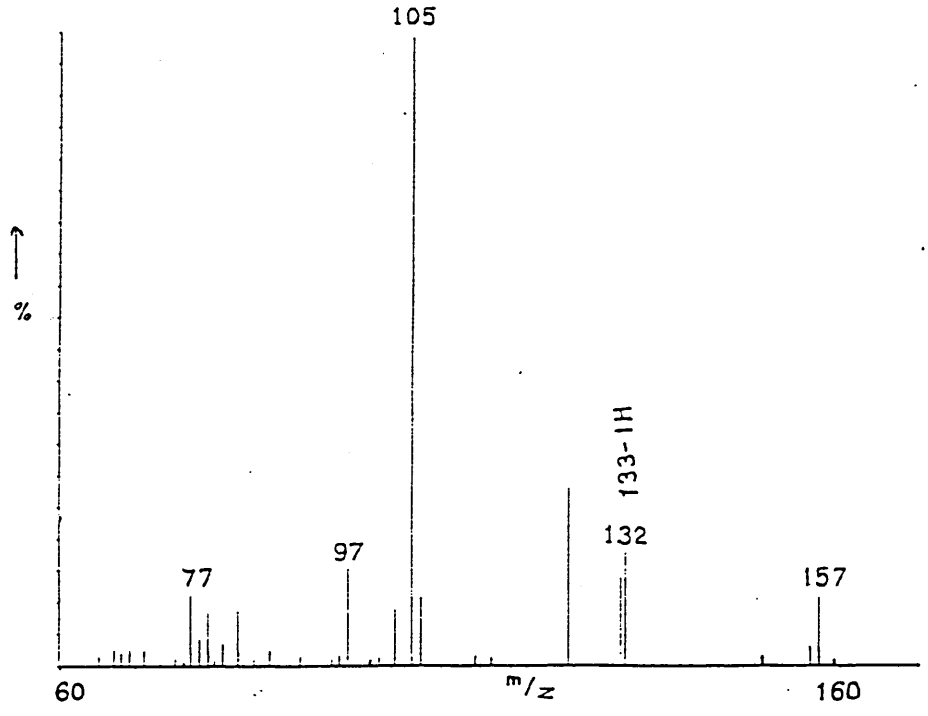


Figure 2.3 The electron impact mass spectrum of the methyl ester R-2-phenylpropionyl derivative of D, L 3-hydroxyoctanoic acid.

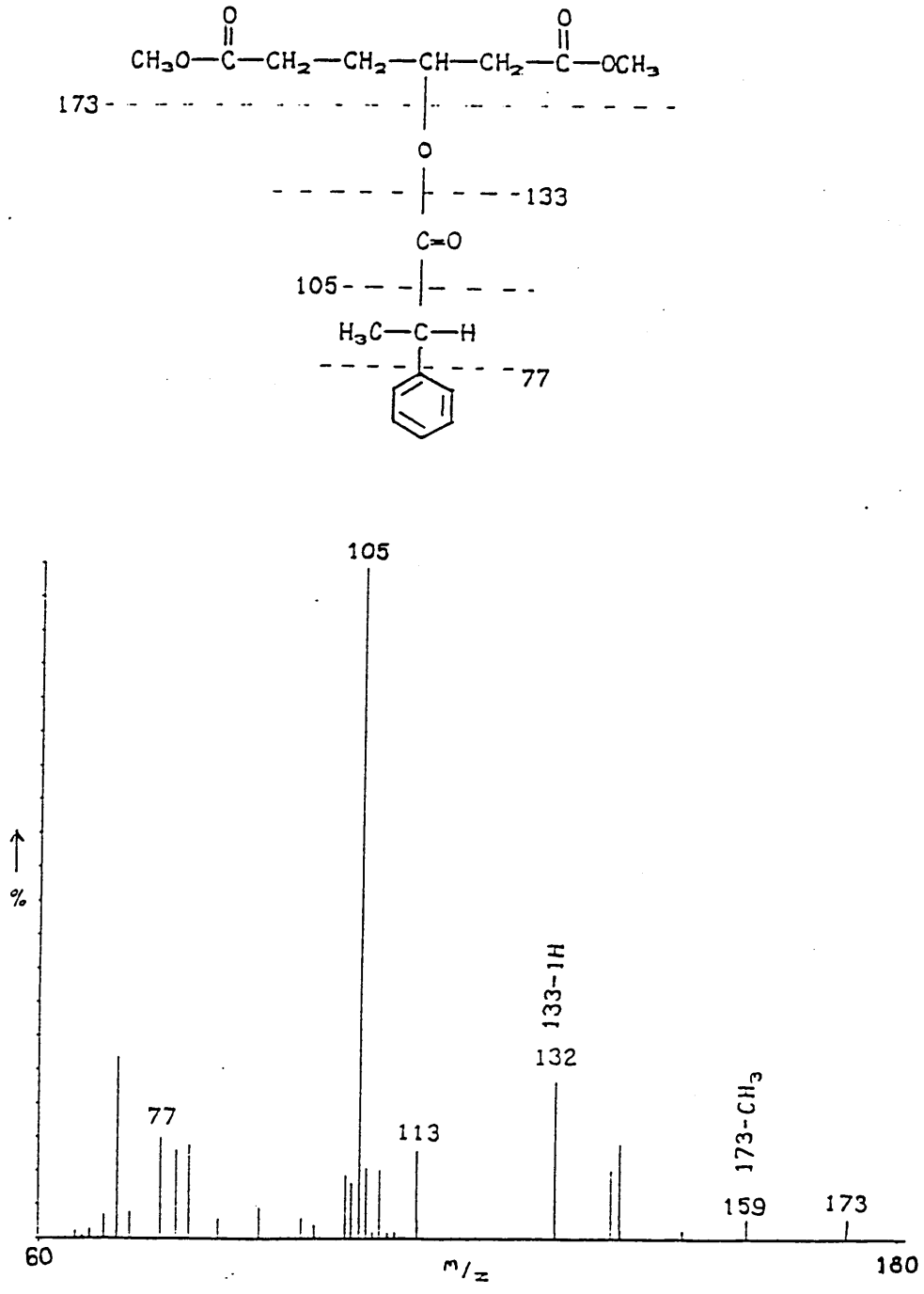


Figure 2.4 The electron impact mass spectrum of the dimethyl ester R-2-phenylpropionyl derivative of R,S 3-hydroxyadipic acid.

2.3.4 N-Trifluoroacetyl-L-alanyl derivatives.

2.3.4.1 Preparation of N-trifluoroacetyl-L-alanyl chloride.

N-Trifluoroacetyl-L-alanyl chloride was prepared according to the method of Souter et al.(1975) on a one tenth scale.

L-Alanine (100mg) was placed in a flask and cooled in an ice-bath. Trifluoroacetic anhydride (TFA) (1ml) was added and the mixture shaken until the alanine dissolved. Excess of TFA was removed by a stream of nitrogen. Chilled thionyl chloride (0.5ml) was added and the mixture kept at room temperature for 15 minutes. The excess of thionyl chloride was then evaporated under nitrogen and the residue dissolved in dichloromethane (10ml).

The reagent was stored at 4°C.

2.3.4.2 Preparation of the derivative.

Method (i).

This is based on the method of Svendsen et al.(1985) for urinary organic acid analysis. N-TFA-L-alanyl chloride (0.5ml) was added to the compound to be derivatised (2mg) and the solution kept at room temperature with occasional shaking for 3 days.

Unreacted reagent was removed by hydrolysing with water (1ml) and the solution extracted with diethyl ether (3 x 2ml).The pooled ether was dried over anhydrous sodium sulphate and evaporated to dryness with nitrogen. The residue was dissolved in a small amount of methanol and methylated with diazomethane.

This method was used to prepare the derivative of methyl 3-hydroxyoctanoic acid.

Method (ii)

The sample was methylated and the excess of diazomethane and ether removed with a stream of nitrogen. N-TFA-L-alanyl chloride (0.5ml) and dry pyridine (50μl) were added and the solution kept at room temperature for 3 days. The extraction of the sample was carried out as for preparation (i). These derivatives were analysed immediately.

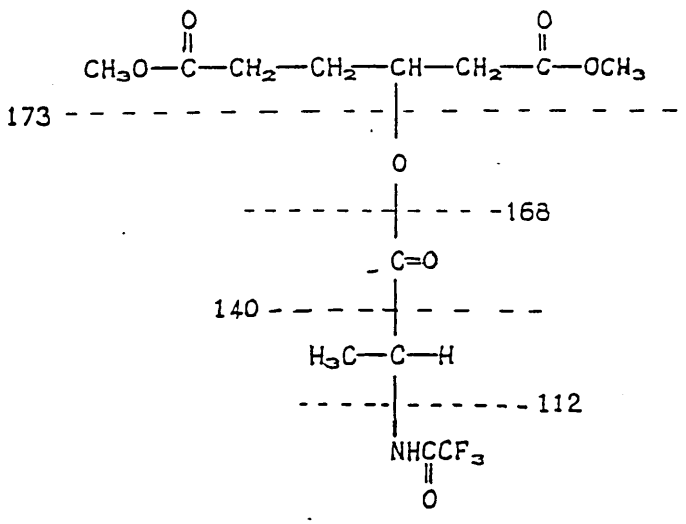
This method was used to prepare the dimethyl ester N-TFA-L-alanyl derivative of 3-hydroxyadipic acid (Figure 2.5) (see section 2.7).

2.3.5 Preparation of O-acetylated-l-menthyl esters.

O-Acetylated-l-menthyl esters were prepared according to the method of Wadman et al.(1976).

l-Menthol (300mg) was added to the standard compound (2mg) and dry hydrogen chloride gas was bubbled through the solution at 110°C for 2 hours. Hydrogen chloride gas was produced by dropping concentrated sulphuric acid on to sodium chloride. Excess of hydrogen chloride and menthol were then removed by a stream of nitrogen. The ester was acetylated with pyridine-acetic anhydride (1:1 1ml) at 100°C for 30 minutes. The reagents were evaporated with absolute ethanol by a stream of nitrogen and the residue dissolved in chloroform. These derivatives may be stored at 4°C for months with no ill effect.

The derivative was used to examine the enantiomers of standard glyceric acid (Figure 2.6) and 2-methyl 3-hydroxybutyrate (Figure 2.7).



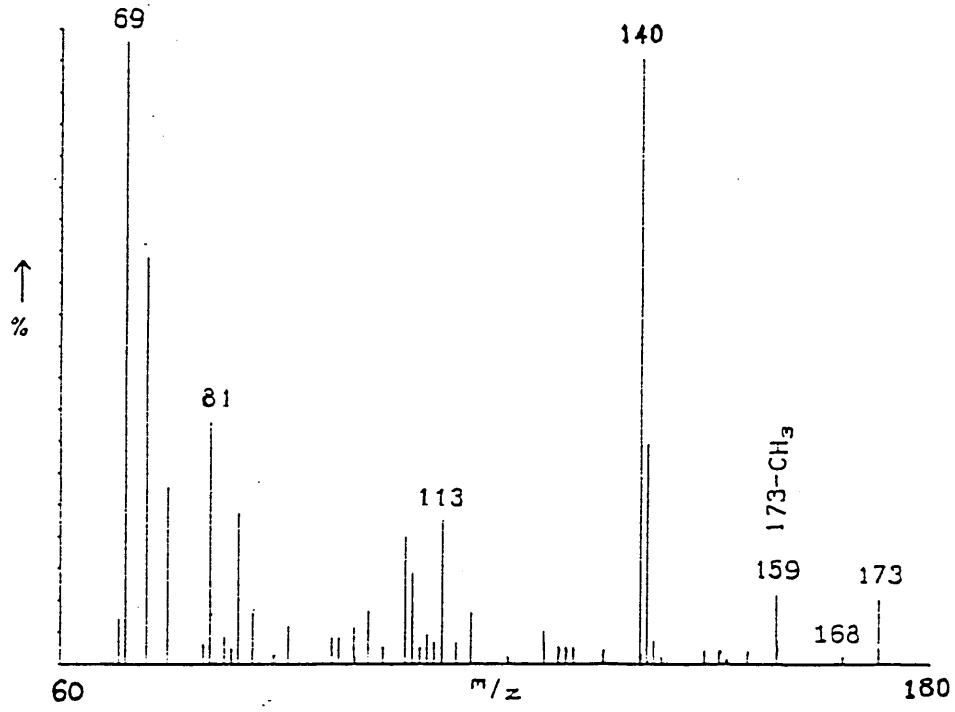
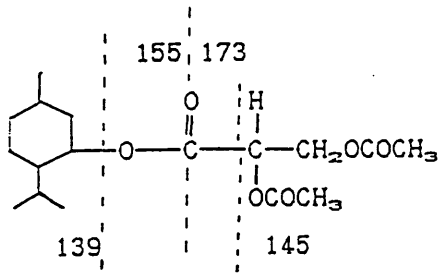


Figure 2.5 The electron impact mass spectrum of the dimethyl ester *N*-TFA-L-alanyl derivative of R,S 3-hydroxyadipic acid.



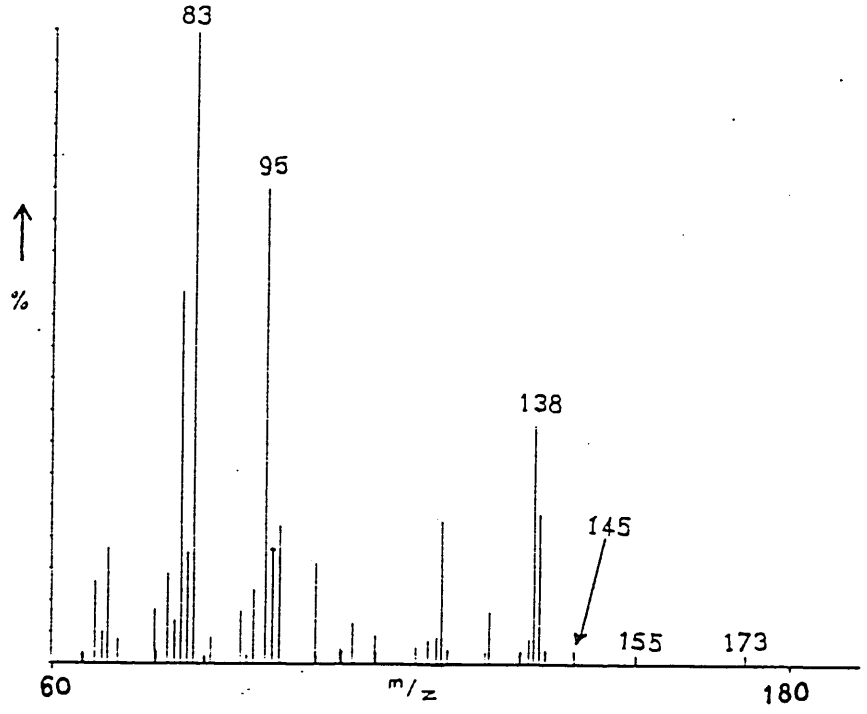
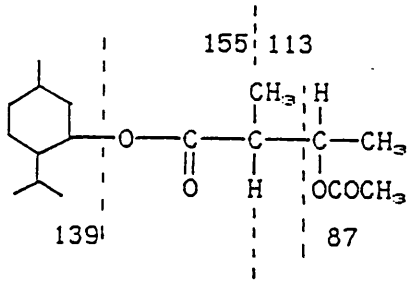


Figure 2.6 The electron impact mass spectrum of the *O*-acetylated-l-menthyl derivative of D,L glyceric acid.



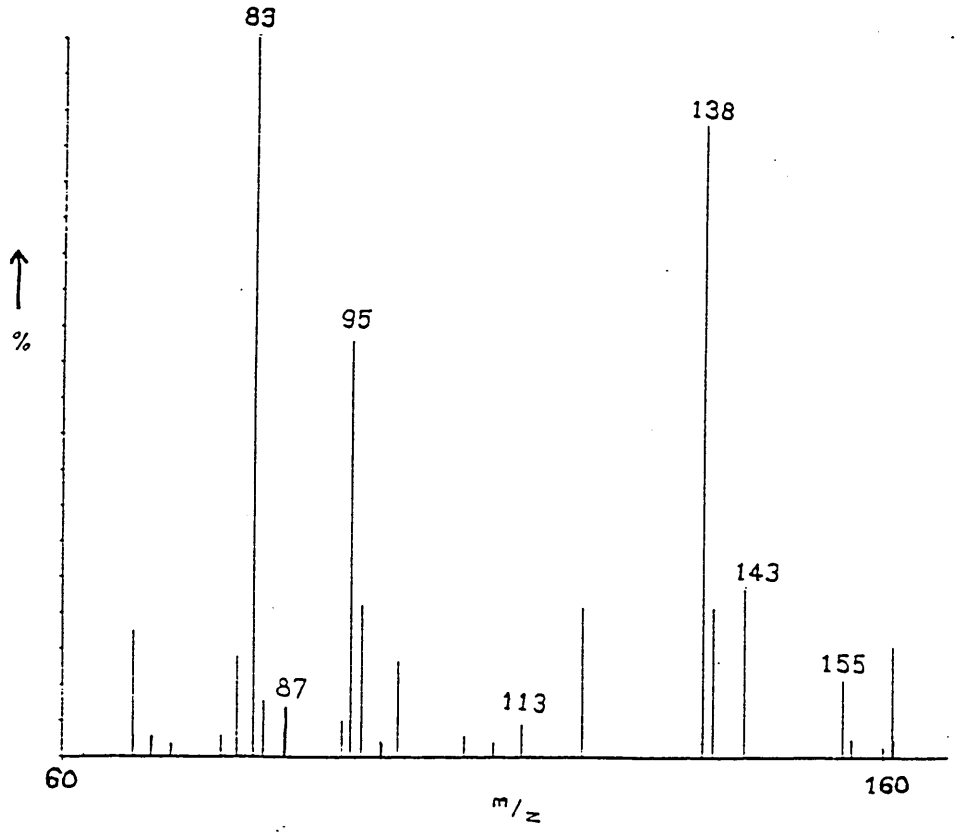


Figure 2.7 The electron impact mass spectrum of the *O*-acetylated-l-menthyl derivative of R,S 2-methyl 3-hydroxybutyric acid.

It was also used in the analysis of urinary glyceric acid in D-glyceric acidaemia and primary hyperoxaluria type II and of 2-methyl 3-hydroxybutyric acid in β-ketothiolase deficiency.

2.3.6 Preparation of O-trifluoroacetylated-l-menthyl esters.

O-Trifluoroacetylated-l-menthyl esters were prepared according to the method of van Gennip et al.(1981).

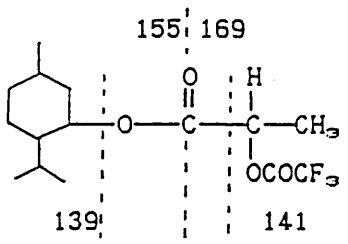
Menthylation was carried out as shown in section 2.3.5. The l-menthyl ester was treated with TFA (1ml) at room temperature for 30 minutes. The excess of TFA was removed by nitrogen and the residue dissolved in ethyl acetate. These derivatives may be stored at 4°C for months without deterioration.

The derivative was used to examine standard lactic acid (Figure 2.8) and the corresponding urinary metabolite in lactic aciduria.

2.4 Racemic Standards.

D,L-3-Hydroxyoctanoic acid was prepared by the Reformatsky reaction (Vogel 1957).

RS-3-hydroxyadipate was prepared adding potassium borohydride to an aqueous solution of 3-oxoadipate. The solution was kept at room temperature for about 2 hours then extracted under acidic conditions.



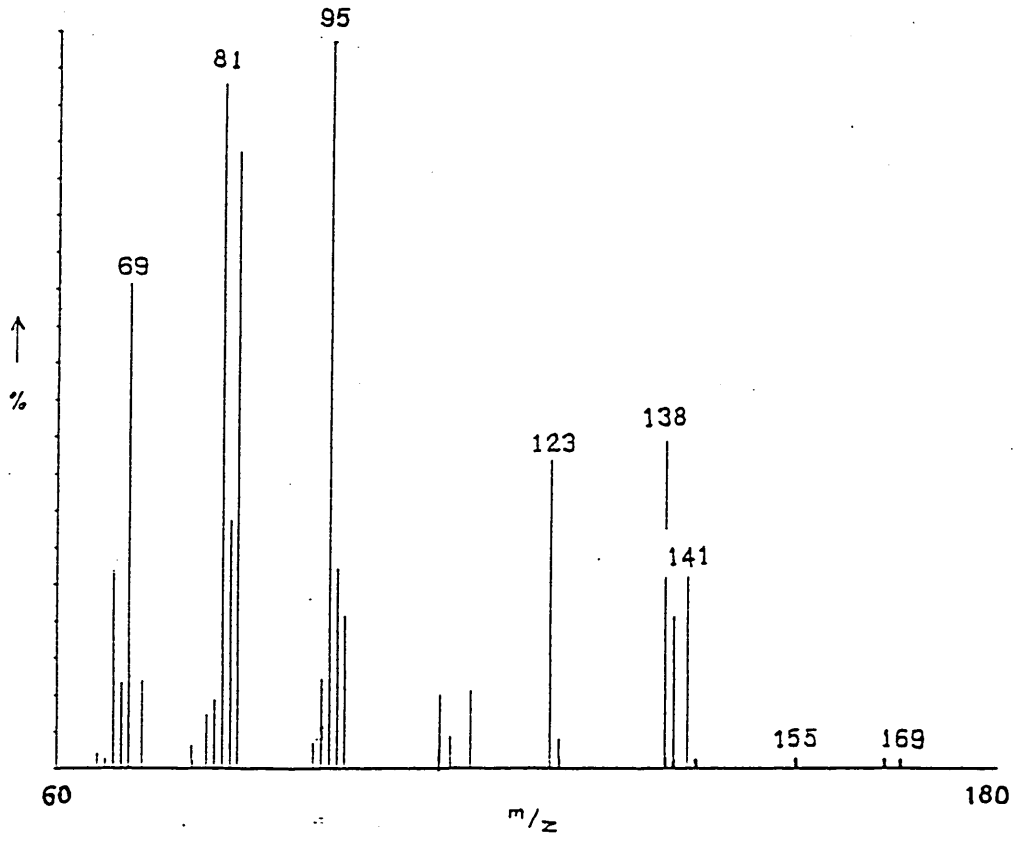


Figure 2.8 The electron impact mass spectrum of the *N*-TFA-l-menthyl derivative of D,L lactic acid.

2.5 Preparation of Standards of Known Configuration.

2.5.1 Yeast method.

Many of the chiral compounds necessary to determine the absolute configuration of the urinary metabolites were not available commercially. Therefore, standards of known configuration were prepared by the action of yeast oxidoreductases on various β-keto carboxylic acid esters. The method is based on that of Mori and Tanida (1981) for the production of ethyl S-3-hydroxybutyric acid from ethyl acetoacetate (Figure 2.9), but the extraction procedure was modified.

Dried yeast (1.8g) was dispersed in tap water (30ml) at 33°C and sucrose (4.8g) was added to it. The flask was shaken for a few minutes during which active fermentation began. The β-keto carboxylic acid ester (0.3g) was added and the flask kept at 33°C for 4 hours with occasional shaking.

The culture was cooled to room temperature and a portion of the solution extracted with an equal volume of diethyl ether. To separate the aqueous and organic layers and to remove the yeast, the solution was centrifuged in a MSE minor 's' bench-top centrifuge at speed 5 for 10 minutes. The ether layer was decanted and dried over anhydrous sodium sulphate. The solvent was then removed in vacuo leaving an oily deposit of the β-hydroxy carboxylic acid ester.

This method was used to prepare methyl S-3-hydroxybutyrate from methyl acetoacetate, ethyl 2-methyl 3-hydroxybutyrate (2S,3S:2R,3S 9:1) from ethyl 2-methylacetoacetate, ethyl 3-hydroxypentanoate from ethyl propionylacetate, octyl S-3-hydroxypentanoate from octyl 3-ketopentanoate, ethyl 3-hydroxyhexanoate from ethyl butyrylacetate and

diethyl 3-hydroxyadipate from diethyl 3-ketoadipate.

The alkyl groups were removed from the carboxylic acid ester by boiling with potassium hydroxide (0.5M) in a test-tube for about a minute. After cooling, the solution was acidified and the hydroxycarboxylic acid extracted with diethyl ether.

2.5.2 Preparation of methyl S-3-hydroxypentanoate via the yeast reduction of octyl 3-ketopentanoate.

The product of the action of yeast oxidoreductases on ethyl propionylacetate was a mixture of the R and S enantiomers of ethyl 3-hydroxypentanoate. Therefore the octyl ester was made and reacted with the yeast, and the methyl ester made subsequently as follows.

Methyl S-3-hydroxypentanoate was prepared according to the method of Mori et al.(1985).

2.5.2.1 Preparation of octyl cyanoacetate.

A mixture of cyanoacetic acid (9g), 1-octanol (12g), benzene (50ml) and concentrated sulphuric acid (0.15ml) was heated under reflux with azeotropic removal of water for 3 hours. After cooling, ether was added to the mixture to dilute. This was washed with water, saturated sodium hydrogen carbonate solution and sodium chloride solution (5%) and then dried over anhydrous sodium sulphate. The solvent was removed in vacuo.

2.5.2.2 Preparation of octyl 3-ketopentanoate.

A solution of ethyl magnesium bromide in dry ether (dried by storing

over sodium metal) (3M 100ml) was placed in round-bottomed flask. It was cooled and kept under nitrogen. Octyl cyanoacetate in 40ml dry ether was added dropwise with stirring over a period of 20 minutes. Stirring was continued for 15 minutes after the addition of the reagent and the mixture was left at room temperature overnight.

The mixture was then poured into an ice-cooled, saturated ammonium chloride solution (35ml) and the flask rinsed with saturated ammonium chloride solution and dilute hydrochloric acid. The pH of the resulting two-phase mixture was adjusted to 1 with concentrated hydrochloric acid. The mixture was then stirred vigorously at room temperature for 3 hours followed by extraction with ether. The ether extract was washed with sodium thiosulphate solution (5%), saturated sodium hydrogen carbonate solution and sodium chloride solution (5%), dried over anhydrous sodium sulphate and concentrated in vacuo.

2.5.2.3 Preparation of methyl S-3-hydroxypentanoate.

Octyl S-3-hydroxypentanoate was made from octyl 3-ketopentanoate by the method outlined in section 2.5.1 (Figure 2.10). Aqueous potassium hydroxide (1.1% 0.6ml) was added to a solution of octyl S-3-hydroxypentanoate (20mg) in ethanol (1ml). The mixture was kept at room temperature with occasional shaking for 1.5 hours. It was then concentrated in vacuo to remove the ethanol and extracted with ether to remove the octanol. The aqueous layer was acidified to pH1 with dilute hydrochloric acid (1M) and extracted with ethyl acetate. The extract was dried over anhydrous sodium sulphate and concentrated in vacuo. Methylation was performed with diazomethane.

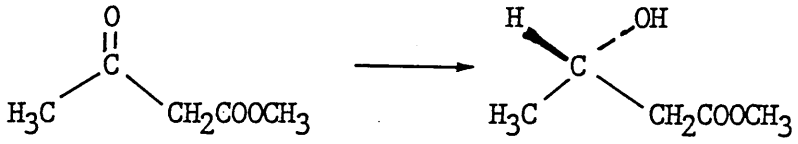


Figure 2.9 The production of methyl S-3-hydroxybutyric acid from methyl acetoacetate via the action of yeast oxidoreductases.

Figure 2.10 The production of octyl S-3-hydroxypentanoic acid from octyl 3-ketopentanoate via the action of yeast oxidoreductases.

2.6 Other Enzymatic Preparations.

2.6.1 Preparation of L-3-hydroxyoctanoic acid.

This method is based on an assay for medium chain acyl CoA dehydrogenase by Kolvraa et al.(1982). Octanoyl-CoA was incubated with acyl-CoA dehydrogenase in rat liver homogenate which converts it to octenoyl CoA. This reaction uses flavine adenine dinucleotide (FAD) as coenzyme with phenazine methosulphate (PMS) as electron acceptor. The presence of excess purified crotonase converts the unsaturated CoA ester to L-3-hydroxyoctanoyl CoA as shown in figure 2.11.

The following were placed in a test-tube :-

Potassium dihydrogen phosphate buffer pH 7.5 (200mM 350μl)

Cysteine (0.6mg/ml 50μl)

FAD (0.83mg/ml 50μl)

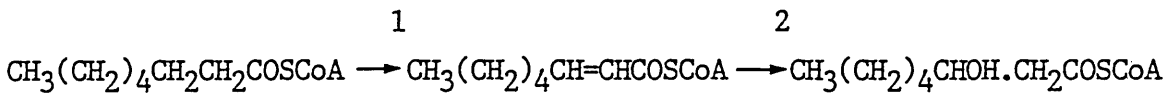
PMS (22mg/ml 50μl)

Octanoyl CoA (0.5mg 50μl)

Crotonase (0.8mg solid diluted to 0.8ml. Further diluted 1 in 50. (25μl))

Rat liver homogenate (rat liver (0.5g) homogenised with potassium dihydrogen phosphate buffer pH 7.5 (5mM 10ml), (125μl))

The mixture was incubated at 37°C for 45 minutes. Sodium hydroxide (0.1M 0.5ml) was added and the solution heated at 60°C for 30 minutes to hydrolyse the CoA ester. After cooling, the solution was acidified to about pH 1 with dilute hydrochloric acid and extracted with ether and ethyl acetate. The extract was dried over anhydrous sodium sulphate and the solvent removed in vacuo.



1 = Acyl CoA dehydrogenase.

2 = Crotonase.

Figure 2.11 The production of 3-hydroxyoctanoyl CoA using purified crotonase.

2.6.2 Preparation of L-3-hydroxyadipate from adipyl CoA.

The preparation of L-3-hydroxyadipate from adipyl CoA was carried out in a similar manner to the preparation of L-3-hydroxyoctanoate from octanoyl CoA shown above.

2.6.2.1 Preparation of adipyl chloride.

Adipyl CoA was prepared according to the method of Kolvraa and Gregersen (1986) as follows.

Dioxan was distilled over lithium aluminium hydride before use. Adipic acid (2.3g) was treated with thionyl chloride (2g) in dioxan (25ml) and the mixture refluxed for 5 hours. After cooling, a small sample of the mixture was evaporated to dryness with nitrogen and cold methanol added. The mixture was kept at room temperature for 30 minutes after which the solvent was removed and the presence of the adipyl chloride was checked by GC-MS.

2.6.2.2 Nitroprusside reaction for free coenzyme A (Stadtman, 1957).

Sodium nitroprusside (1.5g) was dissolved in sulphuric acid (1M 5ml), absolute methanol (95ml) and concentrated ammonia solution (10ml).The white precipitate was filtered off leaving a clear red solution.

The test involved placing a drop of the test solution onto a strip of filter paper and dipping it into the nitroprusside reagent. Appearance of a pink colour indicated the presence of free coenzyme A.

2.6.2.3 Hydroxamic Acid Test for CoA Esters (Lipmann and Tuttle, 1945).

The term "%" refers to grammes of compound per 100ml.

Neutralised hydroxylamine reagent:-
Aqueous hydroxyammonium chloride (28%) and aqueous sodium hydroxide (14%) were mixed in equal parts.
Ferric chloride reagent:-
Aqueous ferric chloride (5% in hydrochloric acid (0.1M)), aqueous trichloroacetic acid (12%) and hydrochloric acid (3M) were mixed in equal parts.
Hydroxylamine reagent (1ml) was added to test solution (2ml) and the solution kept at room temperature for 10 minutes. Ferric chloride reagent (3ml) was added. The immediate appearance of a purple/brown colour indicated the presence of CoA esters.

2.6.2.4 Preparation of adipyl CoA.

Co-enzyme A (0.03mmol) and sodium hydrogen carbonate (83mg) were dissolved in degassed water (3ml) and adipyl chloride (0.5mmol) in dioxan (6ml) was added slowly. The pH was kept at 8 by the addition of sodium hydrogen carbonate and the atmosphere above the reaction kept free of oxygen by a stream of nitrogen. The reaction was monitored by the nitroprusside test for free coenzyme A. After 1.5 hours further adipyl chloride was added and the reaction allowed to continue for a further 30 minutes.

The pH of the solution was adjusted to 6 with dilute hydrochloric acid and the dioxan removed <u>in vacuo</u>. Water was added until the solution

became clear and the excess of free adipic acid extracted with diethyl ether. The presence of CoA esters was checked using the hydroxamic acid test and was positive.

The purity of the adipyl CoA was also checked by high performance liquid chromatography (HPLC) according to the method of Dugan et al. (1987). Chromatography was carried out using a C18 column. The mobile phase, containing tetrabutylammonium phosphate (0.025M), consisted of a linear gradient of 1-50% methanol which began immediately after injection and reached final conditions after 20 minutes. The flow rate was 2ml/minute and the absorbance measured at 254nm. A standard of glutaryl CoA (3nmol/20μl) was examined along with the adipyl CoA preparation. A peak was detected after 16.8 minutes in both samples which was attributed to coenzyme A itself. Glutaryl CoA was detected as a single peak eluting at 23.6 minutes, whereas the adipyl CoA preparation contained two peaks which eluted at 23.2 and 23.8 minutes. The two peaks were attributed to mono and di-sustituted adipyl CoA (Figure 2.12).

2.6.2.5 Preparation of L-3-hydroxyadipate.

Adipyl CoA was substituted for octanoyl CoA in the experiment shown in section 2.6.1. A small amount of the product was examined by GC-MS, but was found to consist of adipic acid only. This indicated that the adipyl CoA had not been metabolised at all; the adipic acid resulting from alkaline hydrolysis of the adipyl CoA.

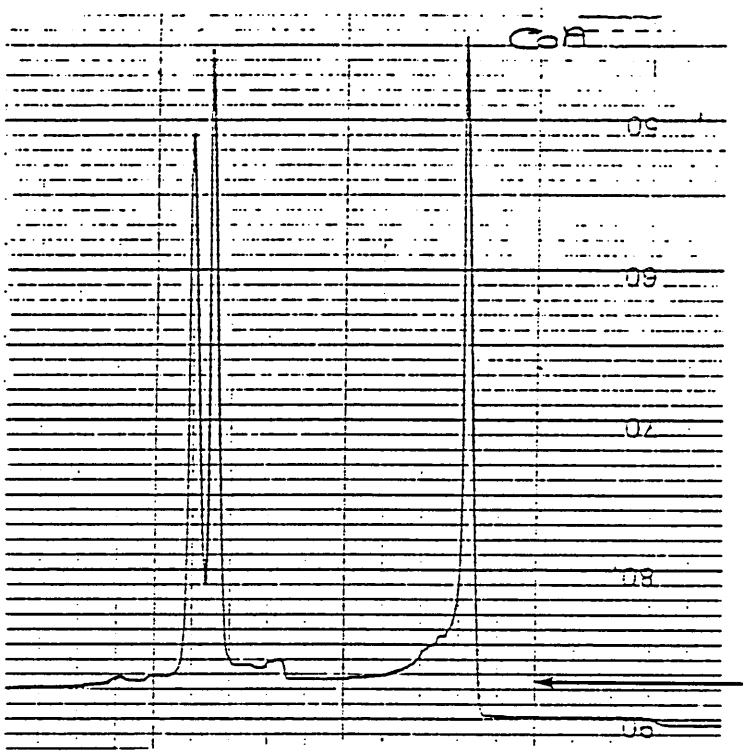


Figure 2.12 The HPLC trace of the adipyl CoA preparation. The single peak is attributed to coenzyme A itself and the two peaks to mono- and di-substituted adipyl CoA.

2.6.3 Isolation of L-3-hydroxyadipate from human urine.

Svendsen et al.(1985) isolated S-3-hydroxyadipate from human urine where a subject had ingested dodecanedioic acid (20mM). Therefore, dodecanedioic acid (13mM, mixed in a fruit yoghurt) was taken by mouth and 5 samples of urine collected over 5.5 hours. A sample (2ml) of each aliquot of urine was extracted and examined by GC-MS, but none of the samples contained 3-hydroxyadipate.

2.6.4 Examination of the action of Rhodopseudomonas spheroides on 3-oxoadipate.

The action of β-hydroxybutyrate dehydrogenase from Rhodopseudomonas spheroides on 3-oxoadipate was examined. The assay follows the decrease in absorbance at 340nm as nicotinamide adenine dinucleotide (NADH) is oxidised to NAD and was initially set up with acetoacetate as substrate as shown.

The following reagents were prepared:-

Phosphate buffer pH 6.8 (0.1M):-

Potassium dihydrogen phosphate (1.36g) was dissolved in water (100ml).

Dipotassium hydrogen phosphate (1.74g) was dissolved in water (100ml).

The solutions were mixed 39 to 61 parts respectively and the adjusted to 6.8.

Phosphate buffer pH 6.8 (10mM):-

Potassium dihydrogen phosphate (0.68g) was dissolved in water (100ml).

Dipotassium hydrogen phosphate (0.87g) was dissolved in water (100ml).

The solutions were mixed 19 to 81 parts respectively and 100ml of the resulting solution was diluted to 500ml with water. The pH was adjusted

to 6.8.

NADH in water (10mM).

Substrate in water (0.1M).

The commercial preparation of β-hydroxybutyrate dehydrogenase (Sigma) consisted of 5.6 units in ammonium sulphate suspension (3.2M 500 l).

The following were placed in a silica cuvette:-

Phosphate buffer pH 6.8 (0.1M 1ml)

NADH (10mM 100μl)

Acetoacetate (0.1M 100μl)

Water to 3ml

The solution was stabilised at 25°C and β-hydroxybutyrate dehydrogenase suspension (10μl) was added to start the reaction. The decrease in absorbance at 340nm was measured with a Cecil UV spectrophotometer connected to a chart recorder.

The reaction proceeded rapidly so was repeated using 3-oxoadipate as substrate under the same conditions. In this case, there was evidence of a very slight decrease in absorbance at a much reduced rate relative to the first experiment. However, it was decided to scale up the reaction to determine whether enough 3-hydroxyadipate could be produced to be used as a standard.

The following were placed in a conical flask:-

Phosphate buffer pH 6.8 (0.1M 15ml)

NADH (10mM 1.5ml)

3-oxoadipate (0.1M 1.5ml)

Water (27ml)

β-hydroxybutyrate dehydrogenase (3 units in cold phosphate buffer pH 6.8 (10mM 200 l))

The flask was kept at 26°C overnight for the reaction to proceed.

An aliquot (10ml) was extracted and the extract was examined by GC-MS, but there had been no reduction of the 3-oxoadipate.

2.7 Preparation of the Dimethyl Ester 3-Hydroxyadipate N-TFA-L-Alanyl Derivative.

The action of yeast oxidoreductases on diethyl 3-ketoadipate was to produce mainly one enantiomer of diethyl 3-hydroxyadipate, but its absolute configuration could not be assigned using R-2-phenylpropionyl derivatives directly because it had not been possible to prepare a sample of 3-hydroxyadipate of known configuration by any of the methods outlined in sections 2.6.2, 2.6.3 and 2.6.4. It was important therefore, to prepare the corresponding N-TFA-L-alanyl derivative of 3-hydroxyadipate and to relate this to the unequivocal synthesis of dimethyl L-3-hydroxyadipate N-TFA-L-alanyl ester produced by the potassium permanganate oxidation of L-1,7-octadien-4-ol N-TFA-L-alanyl ester (Svendsen et al. (1985)). However, all attempts to produce dimethyl 3-hydroxyadipate N-TFA-L-alanyl derivatives by method (i) (the method used by Svendsen for urinary analysis) in section 2.3.4.2 were unsuccessful. It was possible though, to produce the derivative of methyl 3-hydroxyoctanoic acid by this method.

3-Hydroxyadipate is known to lactonise readily under acidic conditions. This consists of the formation of an ester bond between the hydroxy group and the carboxyl group at the far end of the molecule producing a five-membered ring (Figure 2.13). Therefore method (i) was repeated but 3-hydroxyadipate was methylated first to protect the carboxyl groups and prevent lactonisation. This method was also unsuccessful, though it was possible to prepare diethyl 3-hydroxyadipate

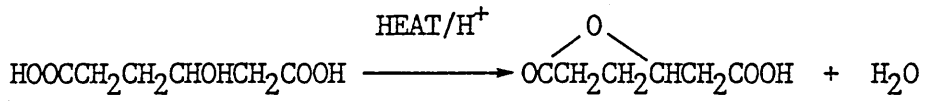


Figure 2.13 The formation of 3-hydroxyadipate lactone.

N-TFA-L-alanyl ester directly using this method.

It was postulated that the presence of a base in the reaction mixture could stop the formation of the 3-hydroxyadipate lactone by neutralising the acidic conditions. Therefore, dry pyridine (50μl) was added along with the N-TFA-L-alanyl chloride to the reaction mixture according to preparation (i). This method was also unsuccessful. However, when this was repeated with 3-hydroxyadipate that had been methylated first according to method (ii) this was successful: GC-MS analysis revealed a peak with the characteristic spectrum illustrated in Svendsen's paper (1985) (Figure 2.5). Method (ii) was therefore repeated with R,S 3-hydroxyadipate.

2.8 Gas Chromatography.

Analyses were carried out on a Varian gas chromatograph model 3700 with a flame ionisation detector. The instrument was fitted with a 25m BP1 (OV101 equivalent) fused silica capillary column using helium as carrier gas. The injector temperature was 250°C and the split ratio was 20:1. Samples were injected at 80°C . The oven was programmed to hold at 80°C for 5 minutes then rise to 260°C at a rate of 10°C per minute. The temperature was held at 260°C for 5 minutes before cooling.

Glyceric acid acetylated-l-menthyl esters were analysed using gas chromatography only. For these analyses the sample was injected at 80°C. The temperature was increased rapidly to 180°C and held for 20 minutes. The temperature was then increased to 260°C in the same way as above.

2.9 Gas Chromatography-Mass Spectrometry.

GC-MS analyses were carried out on a VG Masslab 12-250 quadrupole mass spectrometer linked to a Hewlett-Packard 5790A gas chromatograph. The gas chromatograph was fitted with a 12.5m BP1 fused silica capillary column coated with a crosslinked dimethyl silicone liquid phase. The injector was set at 250°C and the split ratio was 15:1. The carrier gas was helium. Electron impact mass spectra were acquired at an ionising potential of 70eV with the source temperature set at 200°C. Qualitative analysis involved scanning continuously over the mass range $^{m}/_{z}$ 60-600 with the gas chromatograph programmed from 80-260°C so that a mass spectrum of each compound eluted from the column is obtained. Where a more accurate or quantitative analysis was required single ion monitoring (SIM) was carried out. In this operation, the instrument focussed on ions characteristic of the particular compound under analysis over a pre-set period of time based on information taken from a scanning acquisition run.

The N-TFA-L-alanyl derivatives of methyl RS-3-hydroxyadipate were analysed using a 25m vitreous silica capillary column with a BP20 (Carbowax 20M equivalent) liquid phase with the oven programmed from 80-260°C at a rate of 10°C per minute. There was an isothermal section for a duration of 10 minutes at the end of the run.

2.10 The Designation of Configuration to the Standard Compound Derivatives.

Glyceric acid di-*O*-acetylated-l-menthyl esters were not examined by GC-MS, but by GC only.

The R,S derivatives of the standard compounds were analysed using a scanning acquisition run with the GC oven programmed linearly from 80-260°C and using a BP1 (OV101 equivalent) column. This allowed checking of the spectrum against a library file or calculated fragments and determined whether or not the diastereoisomers could be resolved. If there was poor resolution, the rate of oven temperature increase was decreased or an isothermal section was set into the temperature programme. Once the temperature programme was optimised, a SIM programme was then set up for each compound.

Each standard R,S derivative was then co-chromatographed with the corresponding optically-pure standard derivative to determine the order of elution of the enantiomers from the column.

2.10.1 The absolute configuration of the standard 3-hydroxyadipate.

The diastereoisomers of the *N*-TFA-L-alanyl derivative of dimethyl 3-hydroxyadipate did not separate using a BP1 column even under isothermal conditions. Analyses were therefore carried out using a 25m BP20 (Carbowax equivalent) capillary column as used by Svendsen *et al.*(1985). The GC oven was programmed linearly from 80-260°C with the derivative eluting during the 260°C isothermal section at the end of the run. A SIM programme was set up scanning for ions 159 and 173 (Figure 2.5). The results were cross-referenced with those obtained with the R-2-

phenylpropionyl derivatives. This involved noting which enantiomer was produced by the yeast method using N-TFA-L-alanyl derivatives, the information being available from Svendsen's work (1985). This information was used to determine the order of elution of the diastereoisomers from the same preparation using R-2-phenylpropionyl derivatives. All further analysis of 3-hydroxyadipate was performed using the R-2-phenylpropionyl derivative.

2.11 Designation of the Absolute Configuration of some Urinary Metabolites Excreted in Inherited Metabolic Disease.

The absolute configuration of the metabolites produced in various inborn errors of metabolism described in the introduction were determined. This was done by co-chromatographing the urine derivative with the R,S standard derivative. In each case the analyses were performed using the same chromatographic conditions and single ion monitoring programme as the corresponding standard.

3.1 Results.

The resolution of each of the chiral standard derivatives by gas chromatography and the order of elution of the enantiomers are shown in figures 3.1 to 3.8.

Chiral lactic and glyceric acids are available commercially and methods for the resolution of their enantiomers are well known (Figures 3.1 and 3.2) (Wadman et al. 1976 and van Gennip et al. 1981). Figure 3.3 shows the separation of the diastereoisomers of the O-acetylated-l-menthyl esters of 2-methyl 3-hydroxybutyrate.

A range of 3-hydroxymonocarboxylic acids and one 3-hydroxydicarboxylic acid were prepared by yeast reduction of their corresponding 3-ketocarboxylic acid esters. These chiral 3-hydroxy compunds were analysed using R-2-phenylpropionyl derivatives (Figures 3.4 to 3.6). Figure 3.7 shows the resolution of the enantiomers of 3-hydroxyoctanoate using this derivative. 2-Phenylpropionyl derivatives have been used to resolve the diastereoisomers of methyl 2-, 3-, 14-, 15-, 16- and 17-hydroxyoctadecanoates and methyl 3-hydroxydecanoate. Attempts to resolve methyl octadecanoates with the hydroxyl group on other carbons were unsuccessful (Hammarstrom and Hamberg 1973). The derivative has also been used to determine the absolute configuration of urinary 5-hydroxyhexanoic acid in patients with dicarboxylic aciduria (Kamerling et al. 1982).

Figure 3.8 shows the resolution of the dimethyl 3-hydroxyadipate N-TFA-L-alanyl derivative which was used to assign the configuration of

diethyl 3-hydroxyadipate produced by the baker's yeast reduction of diethyl 3-ketoadipate. This derivative was used by Svendsen et al. (1985) to determine the configuration of urinary 3-hydroxyadipate excreted by patients with hydroxydicarboxylic aciduria. It has also been used in the gas chromatographic resolution of some amphetamines and related amines (Souter et al. 1975).

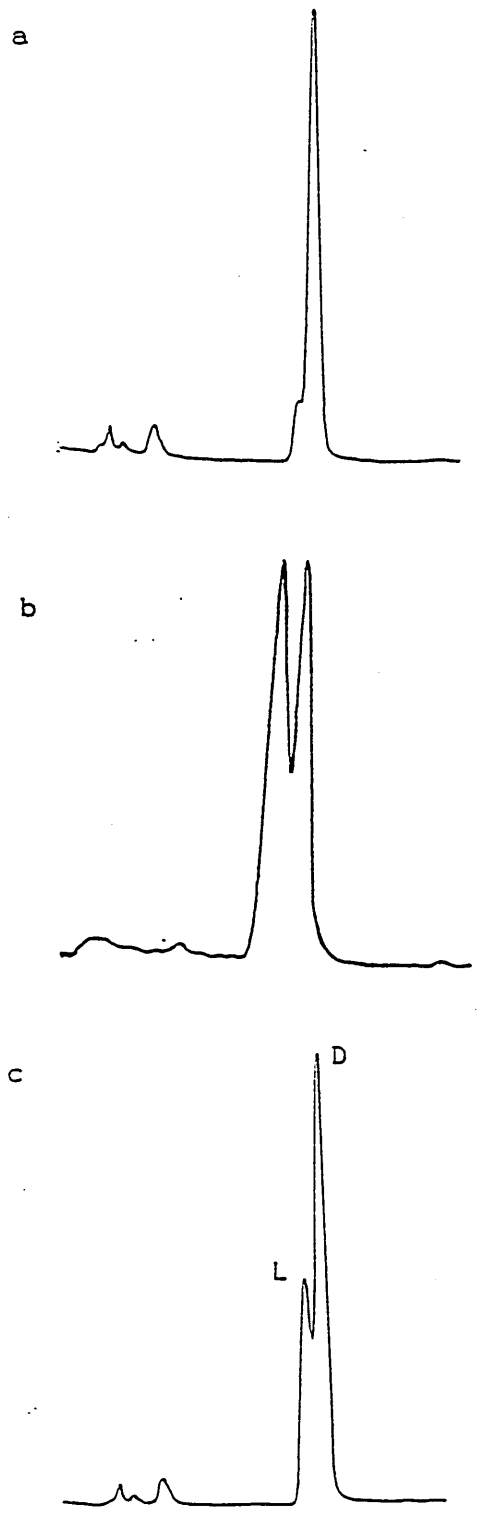


Figure 3.1 Capillary gas chromatograms of the *O*-acetylated-l-menthyl esters of a) D-glyceric acid b) D,L-glyceric acid c) co-chromatography of a) and b) to show the order of elution of the enantiomers.

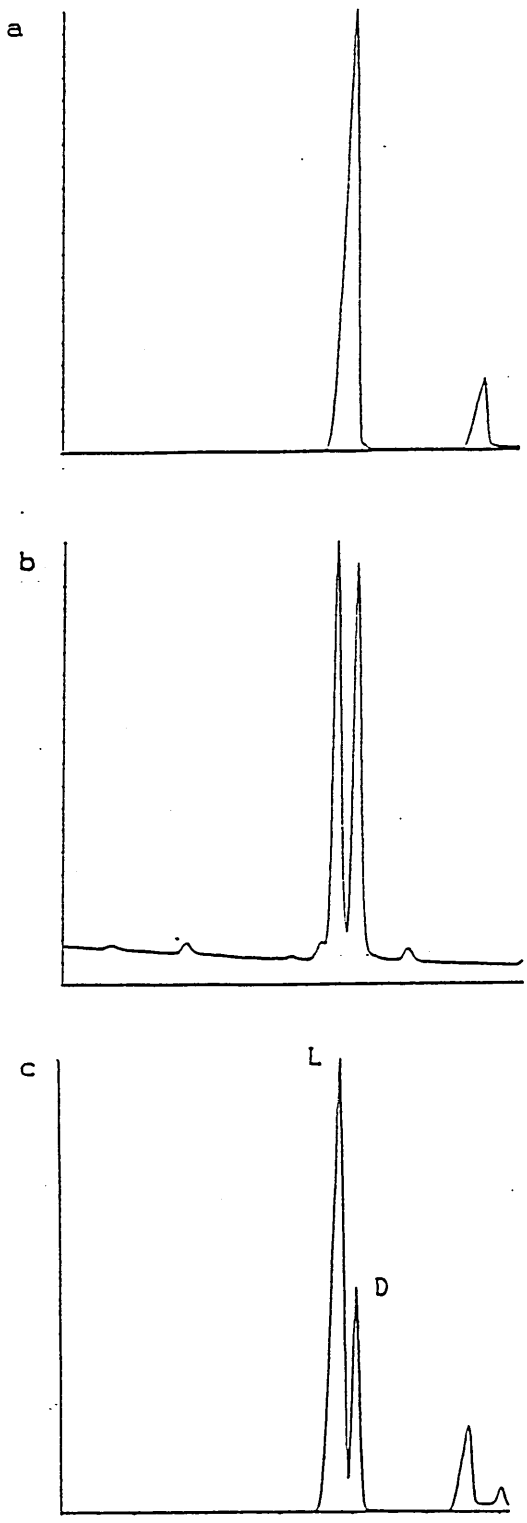


Figure 3.2 Single ion gas chromatograms $^{m}/_{z}$ 143 of the *N*-TFA-l-menthyl esters of a) L-lactic acid b) D,L-lactic acid c) co-chromatography of a) and b) to show the order of elution of the enantiomers

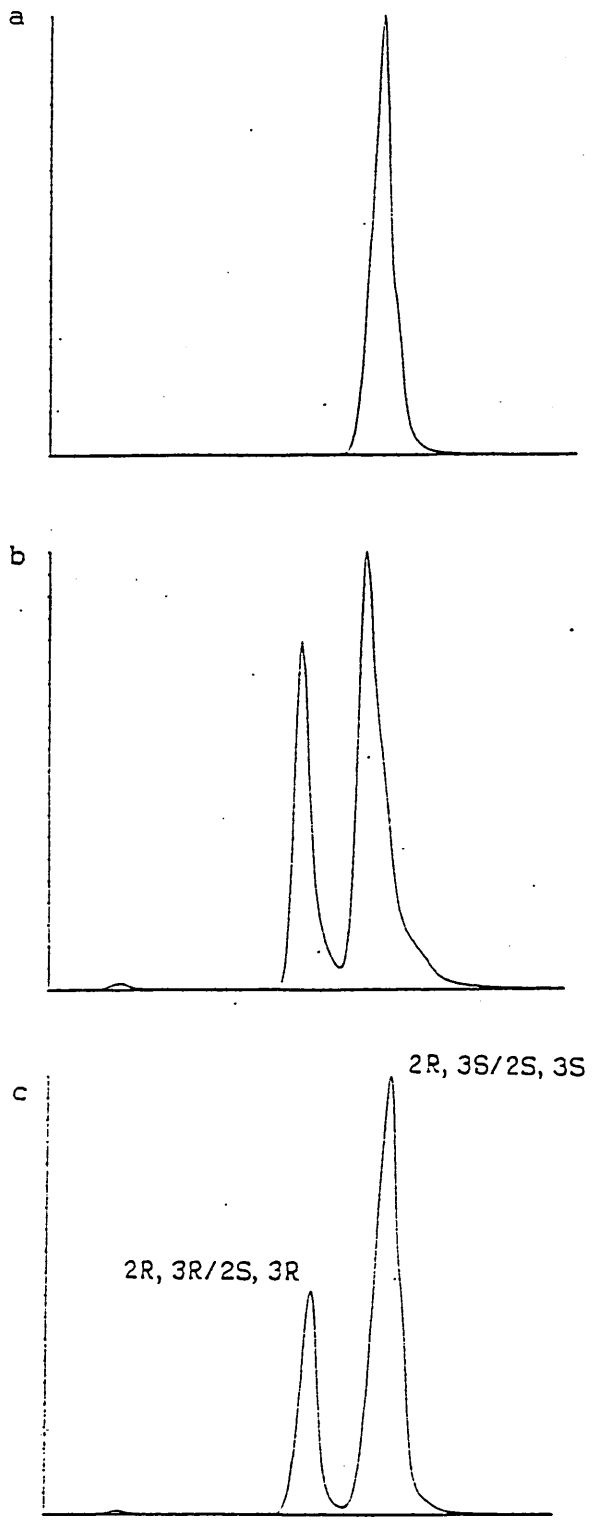


Figure 3.3 Single ion gas chromatograms $^{m}/_{z}$ 143 of a) *O*-acetylated-l-menthyl esters of 2R, 3S: 2S, 3S (1: 9) 2-methyl 3-hydroxybutyric acid b) O-acetylated-d, l-menthyl esters of 2R, 3S: 2S, 3S (1: 9) 2-methyl 3-hydroxybutyric acid c) co-chromatography of a) and b) to show the order of elution of the diastereoisomers.

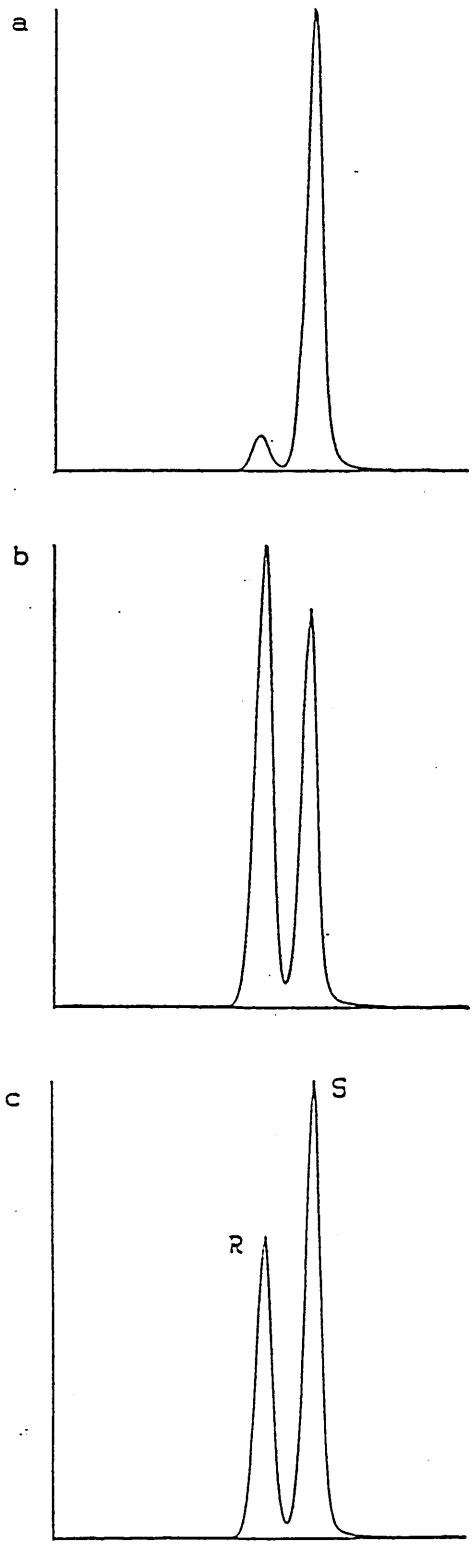


Figure 3.4 Single ion gas chromatograms $^{m}/_{z}$ 101 of the dimethyl R-2-phenylpropionyl esters of a) S-3-hydroxybutyric acid b) R,S-3-hydroxybutyric acid c) co-chromatography of a) and b) to show the order of elution of the enantiomers.

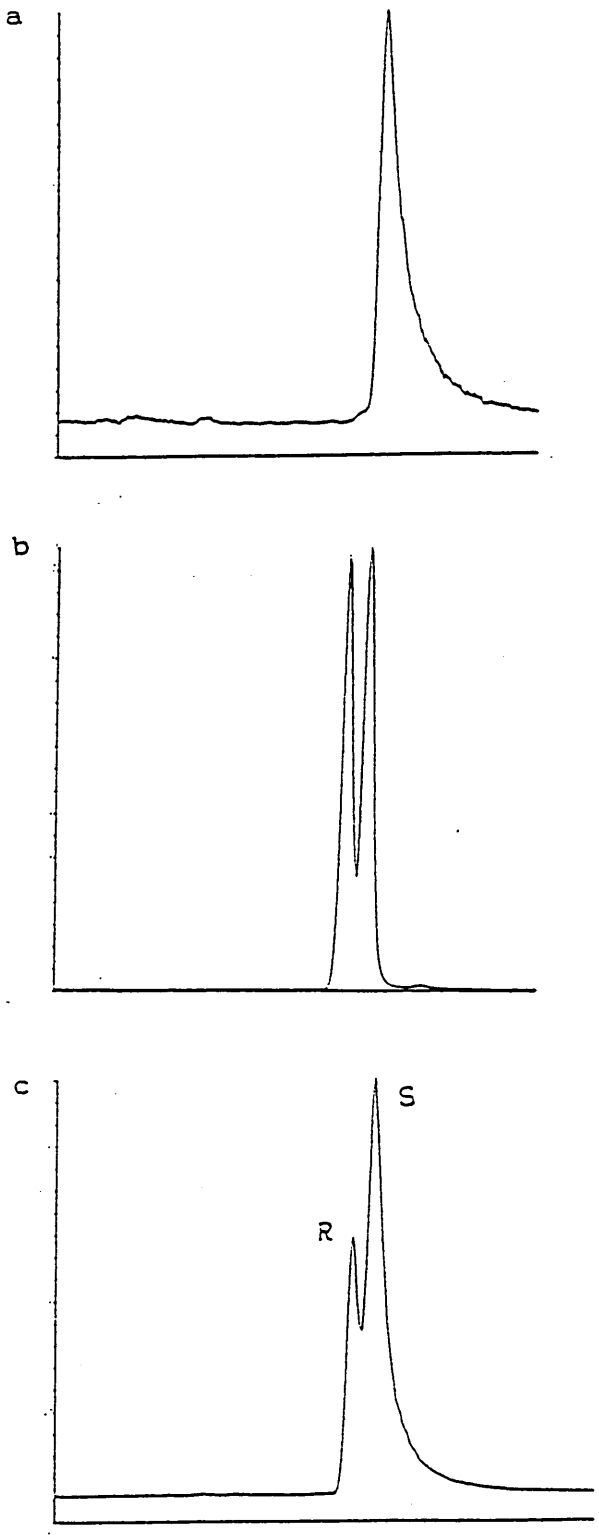


Figure 3.5 Single ion gas chromatograms $^{m}/_{z}$ 115 of the R-2-phenylpropionyl esters of a) S-3-hydroxyvaleric acid b) R,S-3-hydroxyvaleric acid c) co-chromatography of a) and b) to show the order of elution of the enantiomers

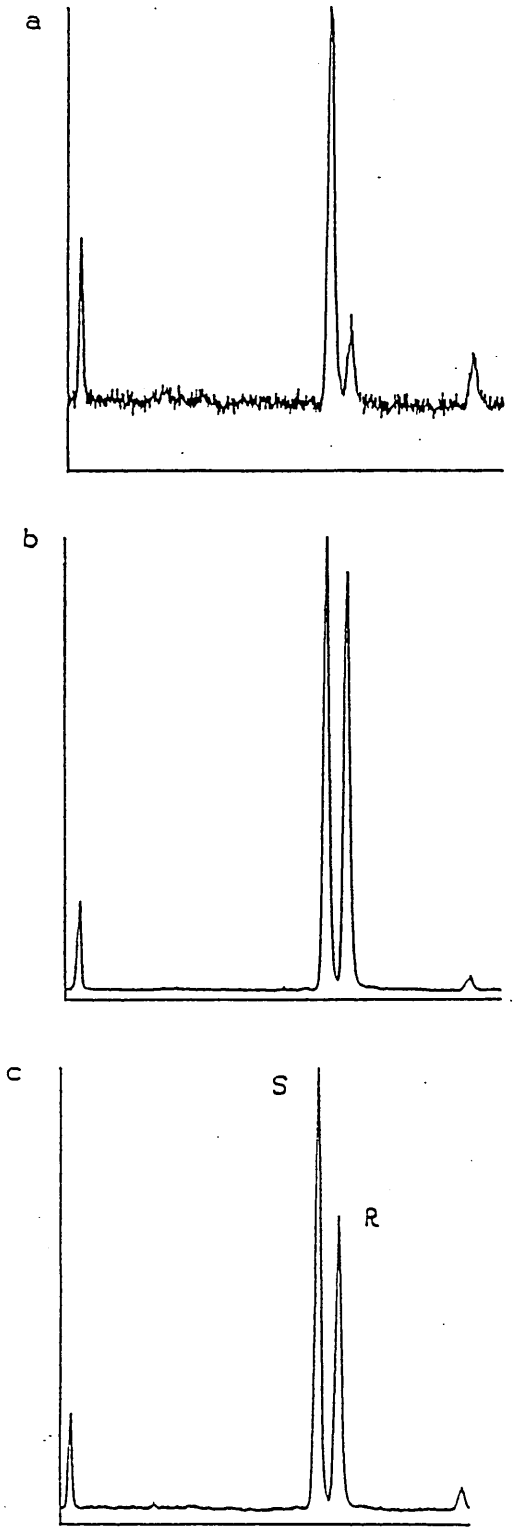


Figure 3.6 Single ion gas chromatograms $^{m}/_{z}$ 173 of the dimethyl R-2-phenylpropionyl esters of a) 3-hydroxyadipic acid from the yeast reduction of dimethyl 3-ketoadipic acid b) R,S-3-hydroxyadipic acid c) co-chromatography of a) and b) to show the order of elution of the enantiomers.

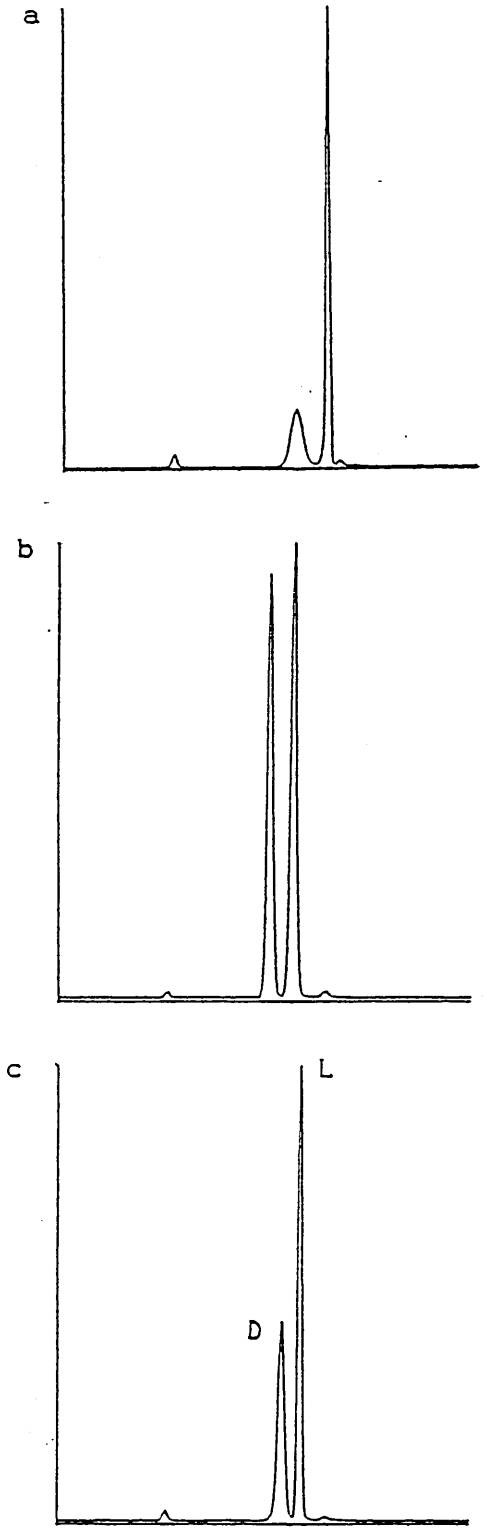


Figure 3.7 Single ion gas chromatograms $^{m}/_{z}$ 157 of the dimethyl R-2-phenylpropionyl esters of a) L-3-hydroxyoctanoate b) D,L-3-hydroxyoctanoate c) co-chromatography of a) and b) to show the order of elution of the enantiomers.

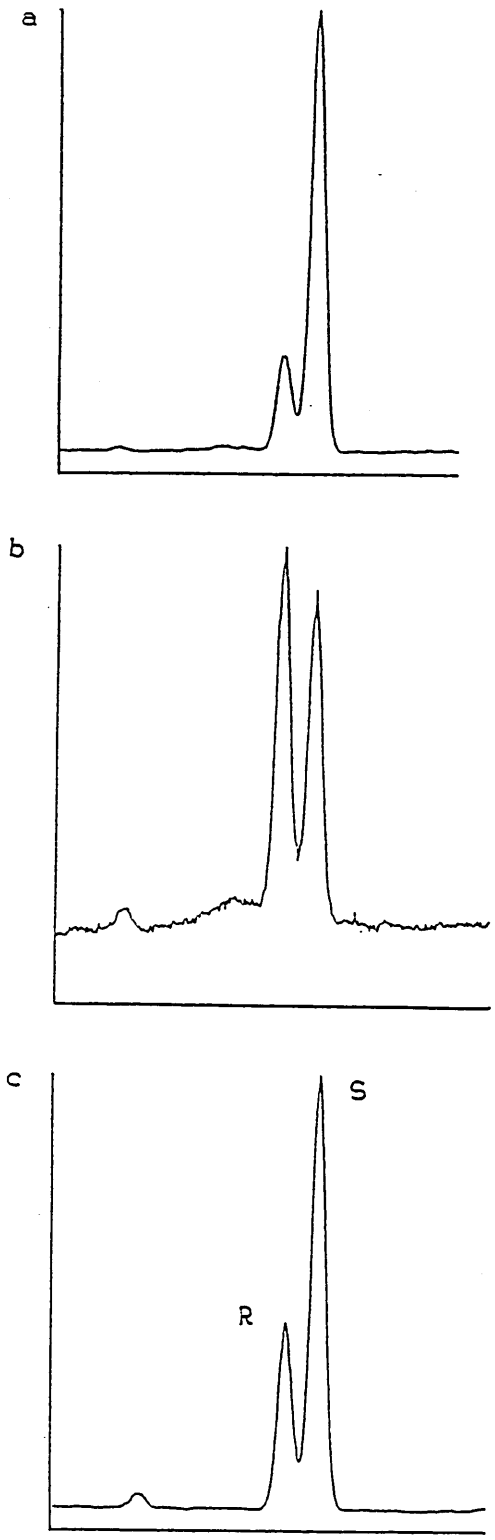


Figure 3.8 Single ion gas chromatograms $^{m}/_{z}$ 173 of the dimethyl *N*-TFA-L-alanyl esters of a) 3-hydroxyadipic acid from the yeast reduction of dimethyl 3-ketoadipic acid b) R,S-3-hydroxyadipic acid c) co-chromatography of a) and b) to determine the order of elution of the enantiomers.

3.2 Discussion.

3.2.1 Enantiospecific Reductions using Baker's Yeast.

The ability of micro-organisms to accomplish stereoselective reactions is well recognised in organic chemistry. The first recorded use was by Le Bel in 1881 when he used Bacterium termo to selectively destroy one enantiomer of propane-1,2-diol. There are a number of advantages to the use of microbial reactions in organic synthesis. Firstly, enzyme reactions generally take place under mild conditions with the necessary co-factors already present in the whole-cell system. Secondly, micro-organisms possess a number of enzymes which can be induced to quite high levels, the inducer normally being the substrate itself. Often modification of only one of a number of similar substituents within a substrate molecule can be achieved or even a sequence of reactions. Many microbial enzymes have relaxed specificities which means that they can catalyse reactions on unnatural compounds to give products of high optical purity.

3.2.2 Asymmetric reduction of carbonyl compounds.

The asymmetric reduction of carbonyl compounds by bakers yeast (Saccharomyces cerevisiae) to yield chiral alcohols is a convenient method due to the ease of the reaction and because bakers yeast is inexpensive and available in large quantities. Chemical catalysts such as organometallic componds have not been widely applied to this type of reaction because of the problems associated with the design. The asymmetric reduction of ethyl acetoacetate to give S-3-hydroxybutyrate

is a recognised process and provides a useful chiral building block for more complex molecules (Mori 1981). Indeed the applications of bakers yeast reactions are widespread. It has been used to prepare optically-active bicycloheptenols necessary for the preparation of prostaglandin $F_{2\alpha}$ (Roberts 1985). It has also been employed in the preparation of - unsaturated aromatic aldehydes which are subsequently used in the production of L-daunosamine (vitamin E) (Fuganti and Grasselli 1985).

3.2.3 The yeast oxidoreductases.

The production of S-3-hydroxybutyrate by bakers yeast yields a product of high, but variable, optical purity. This means that if there is only one yeast oxidoreductase it is able to interact with both faces of the carbonyl group. Wipf et al. (1983) observed that the optical purity of 3-hydroxybutyrate could be improved by continuous, slow feeding of the fermenting yeast mixture to achieve low substrate concentration. It has also been demonstrated that the optical purity of various esters changes with concentration (Zhou et al. 1983) which implies that there must be more than one oxidoreductase which generate alcohols of opposite configuration at different rates. The most recent work on these -keto reductases has succeeded in purifying and examining the kinetics of three enzymes from yeast cytosol. These are a fatty acid synthetase complex with a stereochemical preference for D alcohols, a further D enzyme and an L enzyme. Experiments with various mutants of Saccharomyces cerevisiae devoid of certain components of the fatty acid synthetase complex confirm that the -keto reductase component is a major competing enzyme in the reduction of 3-keto carboxylic acid esters (Sih et al. 1985 and 1986). The observation of Wipf can be explained in

that the L enzyme has a higher V_{max}/K_M ratio than the D enzyme in relation to acetoacetate.

3.2.4 The Prelog rule.

Whilst the yeast production of 3-hydroxybutyrate yields a product of high optical purity, similar reduction of ethyl 3-ketopentanoate afforded a mixture of the R and S enantiomers of 3-hydroxypentanoate (Frater 1979). These results can be explained by reference to the Prelog rule (Prelog 1964). This states that higher stereoselectivity in the microbial reduction of ketones can be achieved if the carbonyl moiety is flanked by a bulky and a small group. If there is little difference in the size of the flanking groups the product is unlikely to be of high optical purity. This general rule has been useful in predicting the stereochemical course of reactions involving acyclic ketones (e.g. Bucciarelli et al. 1983). A number of reports of achieving the desired enantiomer of various alcohols are known in which the size of the substituents flanking the carbonyl group have been altered according to the Prelog rule. In this way Mori et al. 1985 experimented with the production of both enantiomers of methyl-3-hydroxypentanoate using various derivatives. The R enantiomer of methyl 5-phenylthio-R-3-hydroxypentanoate was produced by yeast reduction of methyl 5-phenylthio-3-oxopentanoate. Methyl S-3-hydroxypentanoate was prepared by yeast reduction of octyl 3-oxopentanoate followed by alkaline hydrolysis of the product and methylation with diazomethane. Similarly, after studying the effect of the size of the ester grouping on the chirality of the products after exposure to yeast, Zhou et al. (1983) produced octyl R-4-chloro-3-hydroxybutyrate by the yeast reduction of octyl 4-

chloro-acetoacetate and hydrolysis of the product. This was subsequently used in the production of L-carnitine, a factor associated with the metabolism of fatty acids in man. Hirama et al. 1983 have also experimented with the production of a range of trifunctional 3-hydroxy esters utilising the bakers yeast reduction of hydrolysed 3-ketocarboxylates. These observations all lead to the general conclusion that the enzyme affording S(L) alcohols prefer large, hydrophobic substituents at the carboxy end of the 3-ketocarboxylic acid, whereas the R(D) enzymes prefer the substituents at the hydrocarbon end (Sih and Chen 1984).

3.2.5 Preparation of chiral standards by yeast asymmetric reduction.

The asymmetric reduction of β-ketocarboxylic acid esters was utilised to prepare a range of chiral β-hydroxycarboxylic acid esters as standards for the urinary analysis.

3.2.5.1 S-3-Hydroxybutyric acid.

Methyl acetoacetate was reduced by yeast to give methyl S-3-hydroxybutyric acid (Figure 3.4). This is slightly different from the method of Mori (1981) in that he used the ethyl ester of acetoacetate in his preparations. However, Lemieux and Giguere (1951) produced L(S)-3-hydroxybutyric acid by the yeast reduction of free acetoacetate. Since the yeast reduction of both the ethyl ester of acetoacetate and the free acid both yield the S enantiomer of 3-hydroxybutyrate, it was assumed that similar reduction of the methyl ester would give the same product. This was subsequently confirmed in the analysis of ketosis where the

D(R) enantiomer of 3-hydroxybutyrate is known to be excreted (Section 1.3.3 and 4.2.3).

3.2.5.2 S-3-Hydroxypentanoic acid.

The yeast reduction of ethyl 3-ketopentanoate was found to yield an almost equal mixture of the two enantiomers of 3-hydroxypentanoate. This observation was also recorded by Frater in 1979. This suggests that both the R and S reductases in yeast are acting upon the substrate yielding hydroxy compounds of opposite configuration (Zhou et al. 1983). S-3-Hydroxypentanoate was therefore produced by the method Mori et al. (1985) by the yeast reduction of octyl ester of 3-ketopentanoate. This increase in the size of the ester grouping attached to the carboxyl group favours the S(L) reductase and hence the product is exclusively the S enantiomer of 3-hydroxypentanoate (Figure 3.5).

3.2.5.3 3-Hydroxyhexanoic acid.

Ethyl 3-ketohexanoate was reduced by baker's yeast to yield mainly one enantiomer of 3-hydroxyhexanoate, the chirality of which was not known. The only other reference to a related reaction known to the author is that of Lemieux and Giguere (1951) who produced R-3-hydroxyhexanoic acid by the yeast reduction of free 3-ketohexanoate. Application of the Prelog rule to this problem suggested that the yeast reduction of ethyl 3-ketohexanoate would also produce R-3-hydroxyhexanoate and this is explained as follows. The yeast reduction of ethyl acetoacetate yields S-3-hydroxybutyrate, but similar reduction of ethyl 3-ketopentanoate gives a mixture of the two enantiomers of 3-hydroxypentanoate. It would

therefore be expected that further lengthening of the carboxylic acid molecule by an additional two carbons would favour the R(D)-enzyme still more and yield a product with mainly the R configuration (see section 3.2.4).

However, examination of chiral 3-hydroxyhexanoate by gas chromatography as the corresponding methyl ester R-2-phenylpropionyl derivative produced a different conclusion. It was observed that the single enantiomer of 3-hydroxyhexanoate corresponded to the second peak of the R,S mixture to be eluted from the column, that is, the one with the longer retention time. Examination of figures 3.4, 3.5 and 3.7 shows that for similar derivatives of 3-hydroxybutyrate, 3-hydroxypentanoate and 3-hydroxyoctanoate the S enantiomer has a longer retention time than the R enantiomer. Hammarstrom and Hamberg (1973) showed that the diastereoisomers of the D-2-phenylpropionyl derivative of methyl 3-hydroxydecanoate had a similar order of elution, that is, the L enantiomer had the longer retention time. However, they used a gas chromatography column with a QF-1 stationary phase for their analyses which is more polar than the OV101 phase used for the above work and this means that the results can not be compared directly. It is suggested that, in keeping with the order of elution of related methyl 3-hydroxycarboxlic acid derivatives, the single enantiomer of methyl 3-hydroxyhexanoate examined had the S configuration and, therefore, that the yeast reduction of ethyl 3-ketopentanoate yields ethyl S-3-hydroxyhexanoate.

This result is obviously at variance with the result predicted by the Prelog rule and the reason for this is not known to the author.

3.2.5.4 3-Hydroxyadipic acid.

The application of bakers yeast to the reduction of 3-keto dicarboxylic acids has not been previously examined as far as is known. In addition, no information on the relationship of the Prelog rule to 3-keto dicarboxylic acid esters is known to the author.

Gas chromatography of the dimethyl ester R-2-phenylpropionyl derivative of 3-hydroxyadipic acid produced by yeast reduction showed that it consists of mainly one enantiomer (Figure 3.6a), but the chirality was not known. The order of elution trend of monocarboxylic acid derivatives which was used to assign the configuration of 3-hydroxyhexanoate described above could not be applied to this problem because it can not be assumed that the same pattern will follow for dicarboxylic acid derivatives.

It was therefore necessary to compare the configuration of 3-hydroxyadipate produced by the yeast with some of known configuration. Repeated unsucessful attempts were made to prepare dimethyl 3-hydroxyadipate N-TFA-L-alanyl derivative using 3-hydroxyadipate from the yeast reaction in the method used by Svendsen et al. (1985) for urine analysis (section 2.3.4 method(i)). However, the same procedure was successful when 3-hydroxyoctanoic acid was substituted for 3-hydroxyadipate which indicated that the problem did not involve the reagent itself.

It is noteworthy that Svensen et al. used a chemical method to prepare their dimethyl L-3-hydroxyadipate N-TFA-L-alanyl derivative. This involved the preparation of L-1,7-octadien-4-ol from L-malic acid (Bartlett et al. 1984) which was derivatised with N-TFA-L-alanyl chloride directly and subsequently oxidised with potassium permanganate

to the 3-hydroxyadipate derivative. The derivative was then methylated with diazomethane. A molecular model of the L-3-hydroxyadipate used by Svensen and his co-workers was constructed from the diagram in their paper and this was shown to be the S configuration.

Synthesis of the dimethyl 3-hydroxyadipic acid N-TFA-L-alanyl derivative was only possible after some manipulation of Svendsen's method (section 2.7 and 2.3.4.2 method (ii)), and then the yield was small. The fact that the derivative could be produced when the carboxyl groups of the 3-hydroxyadipate were esterified and in the presence of a base to neutralise the acidic conditions supports the idea that lactonisation was the reason for the failure of the original method. This is obviously something that Svendsen and his co-workers would not have come across by using the standard chemical preparation from L-1,7-octadien-4-ol outlined above. However, it is difficult to understand how they did not encounter this problem using the direct method of derivatisation in their analysis of urinary 3-hydroxyadipate.

Once dimethyl 3-hydroxyadipate N-TFA-L-alanyl derivative was synthesised it was used as a cross reference to assign the configuration of the 3-hydroxyadipate prepared by yeast reduction (Figure 3.6 and 3.8). Examination of these figures shows that the action of yeast oxidoreductases on diethyl 3-ketoadipate is to produce diethyl S-3-hydroxyadipate. This situation could be similar to that of 3-hydroxyhexanoate in that application of the Prelog rule would lead to the expectation that the product would have the R configuration. The reason why the product actually has the S configuration is not known.

All subsequent analysis of 3-hydroxyadipate was carried out using 2-phenylpropionyl derivatives because of the much shorter sample preparation time (2 hours as against 3 days for N-TFA-L-alanyl

derivatives) with much greater yields. In addition, 2-phenylpropionyl derivatives can be analysed using an OV101 capillary column which is in everyday use in the laboratory and do not necessitate the use of a delicate Carbowax polar column.

3.2.6. Other chiral 3-hydroxyadipic acid preparations.

Because of the problems associated with the N-TFA-L-alanyl derivatives, various attempts were made to prepare 3-hydroxyadipate of known configuration in order to assign the configuration of the yeast 3-hydroxyadipate with 2-phenylpropionyl derivatives directly. These were unsuccessful and are discussed below.

3.2.6.1 The preparation of 3-hydroxyadipic acid from adipyl CoA using purified crotonase and liver homogenate.

The attempt to produce S-3-hydroxyadipate from adipyl CoA in an analogous method to that used for the preparation of S-3-hydroxyoctanoate from octanoyl CoA (Figure 3.7) was not successful. Kolvraa et al. (1986) used rat liver mitochondrial and peroxisomal preparations in their experiments to investigate the β-oxidation of dicarboxylic acids in rat liver rather than a crude homogenate. It may be that the acyl CoA dehydrogenase was rendered inactive in the simple system used in this experiment or that there was significant thiolase activity in the homogenate which acted upon the adipyl CoA.

3.2.6.2 The action of β-hydroxybutyrate dehydrogenase on 3-oxoadipate.

The action of β-hydroxybutyrate dehydrogenase from Rhodopseudomonas spheroides on acetoacetate in the presence of NADH yields D-3-hydroxybutyrate. The organism is known to produce D-3-hydroxypentanoate and D-3-hydroxyhexanoate in a similar way from the appropriate substrates at about a twentieth of the rate (Krebs et al. 1969). Whether D-3-hydroxyadipate could be produced in a similar way from 3-ketoadipate was investigated. The experiment was unsuccessful. It is envisaged that the presence of the carboxyl group at the end of the 3-ketoadipate molecule in place of a hydrocarbon chain in the monocarboxylic acid substrates interfered with the binding of the enzyme and hence no reaction took place.

3.2.6.3 The excretion of 3-hydroxyadipate following ingestion of dodecanedioic acid.

Adipic, suberic and sebacic acids together with the corresponding 3-hydroxydicarboxylic acids have been detected in the urine of volunteers ingesting dicarboxylic acids (Svendsen et al. 1985). It is accepted that the 3-hydroxycarboxylic acids are formed as intermediates in the β-oxidation of mono and dicarboxylic acids in man. Svendsen used urine from a subject who had previously ingested dodecandioate to examine the chirality of the excreted 3-hydroxyadipate. It was hoped that by repeating this experiment enough 3-hydroxyadipate could be produced to act as a standard in identifying the configuration of the enantiomer produced in the yeast reduction. This experiment was also unsuccessful. Although there was a significant dicarboxylic aciduria and large amounts

of unmetabolised dodecanedioic acid in the urine, there did not appear to be any 3-hydroxyadipic acid present. The presence of adipate, suberate and sebacate indicated that the dodecanedioate was β-oxidised, but the relative intermediates were not seen.

3.2.7 The asymmetric reduction of α-methyl β-keto esters.

A number of authors have examined the asymmetric reduction of α-methyl β-keto esters because this structure appears in many natural compounds. A variety of micro-organisms have been studied and it seems that all four possible stereoisomers of an α-methyl β-keto ester can be obtained by selecting the appropriate organism (Akita et al. 1982). Frater (1979b) reported that the bakers yeast reduction of ethyl 2-methylacetoacetate afforded ethyl 2-methyl 3-hydroxybutyrate of 9:91 2R,3S:2S,3S. Similar yeast reduction in our laboratory has confirmed this result (Figure 3.3).

Examination of molecular models of 2R,3S and 2S,3S 2-methyl 3-hydroxybutyrate show that the 2S,3S molecule has a threo configuration, that is, identical groups can not eclipse each other. The yeast reduction of ethyl 2-methylacetoacetate therefore yields a product mainly of the threo configuration.

3.3 Summary of the Configurations of the 3-Hydroxy Compounds prepared by the Yeast Method.

Chiral standard from the yeast reduction of the corresponding 3-keto compound.	Configuration
Methyl 3-hydroxybutyric acid	S
Ethyl 3-hydroxypentanoic acid	R,S
Octyl 3-hydroxypentanoic acid	S
Ethyl 3-hydroxyhexanoic acid	S
Diethyl 3-hydroxyadipic acid	S
Ethyl 2-methyl 3-hydroxybutyric acid	2R,3S:2S,3S 1:9

4. INHERITED METABOLIC DISORDERS - RESULTS AND DISCUSSION.

4.1 Results.

The results of the urinary organic acid analyses are shown in figures 4.1 to 4.8., but are summarised below.

Chiral urinary metabolite	Disorder	Configuration
Lactic acid	Lactic aciduria	S
D-Glyceric acid	D-Glyceric aciduria	R
L-Glyceric acid	Primary hyperoxaluria (II) (Human and feline)	S
3-Hydroxybutyric acid	Ketosis	R
3-Hydroxypentanoic acid	Propionic acidaemia	R
2-Methyl 3-hydroxybutyric acid	β-Ketothiolase deficiency	2S,3S/ 2R,3S
3-Hydroxyadipic acid	Hydroxydicarboxylic aciduria	S

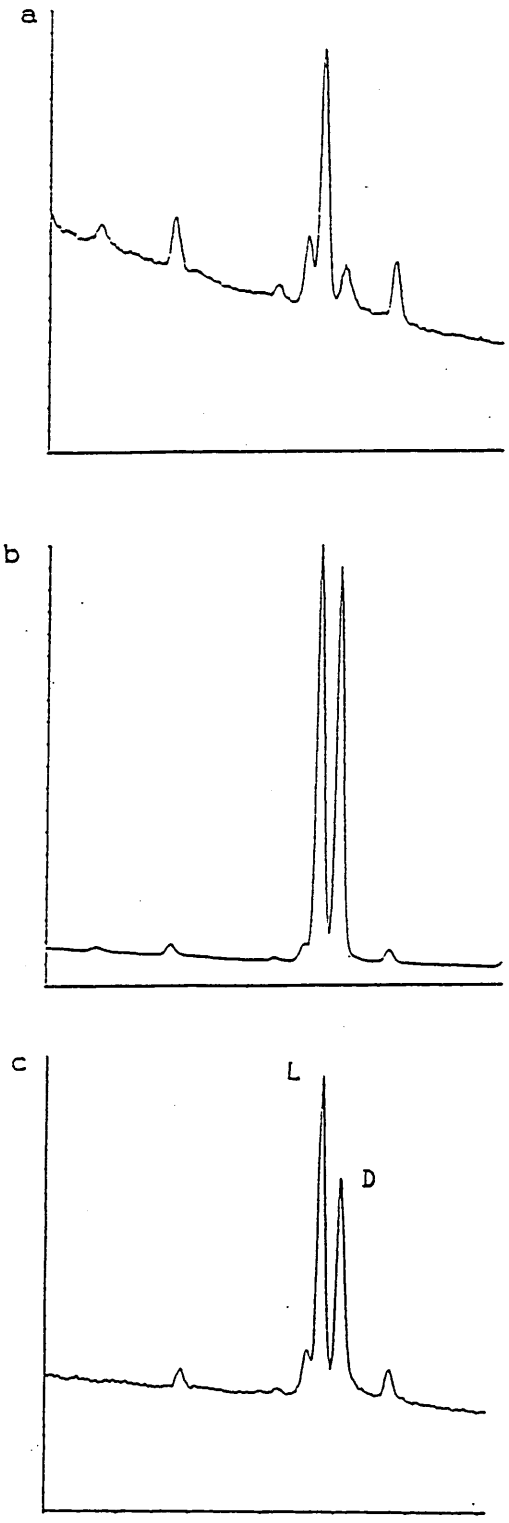


Figure 4.1 Single ion gas chromatograms $^{m}/_{z}$ 143 of the *N*-TFA-l-menthyl esters of a) urine extract from a child with lactic aciduria b) D,L-lactic acid c) co-chromatography of a) and b) to determine the configuration of the excreted lactic acid.

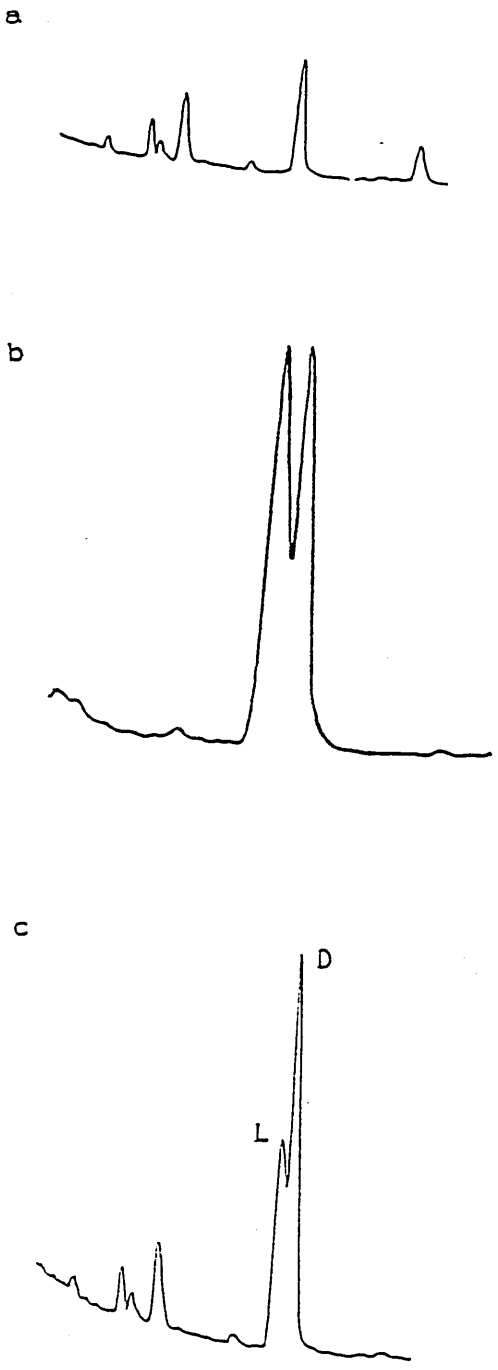


Figure 4.2 Capillary gas chromatograms of the *O*-acetylated-l-menthyl esters of a) urine extract from a child with glyceric aciduria b) D,L-glyceric acid c) co-chromatography of a) and b) to determine the configuration of the excreted glyceric acid.

Figure 4.3 Capillary gas chromatograms of the *O*-acetylated-l-menthyl esters of a) urine extract from a cat with hyperoxaluria type II b) D,L-glyceric acid c) co-chromatography of a) and b) to determine the configuration of the excreted glyceric acid.

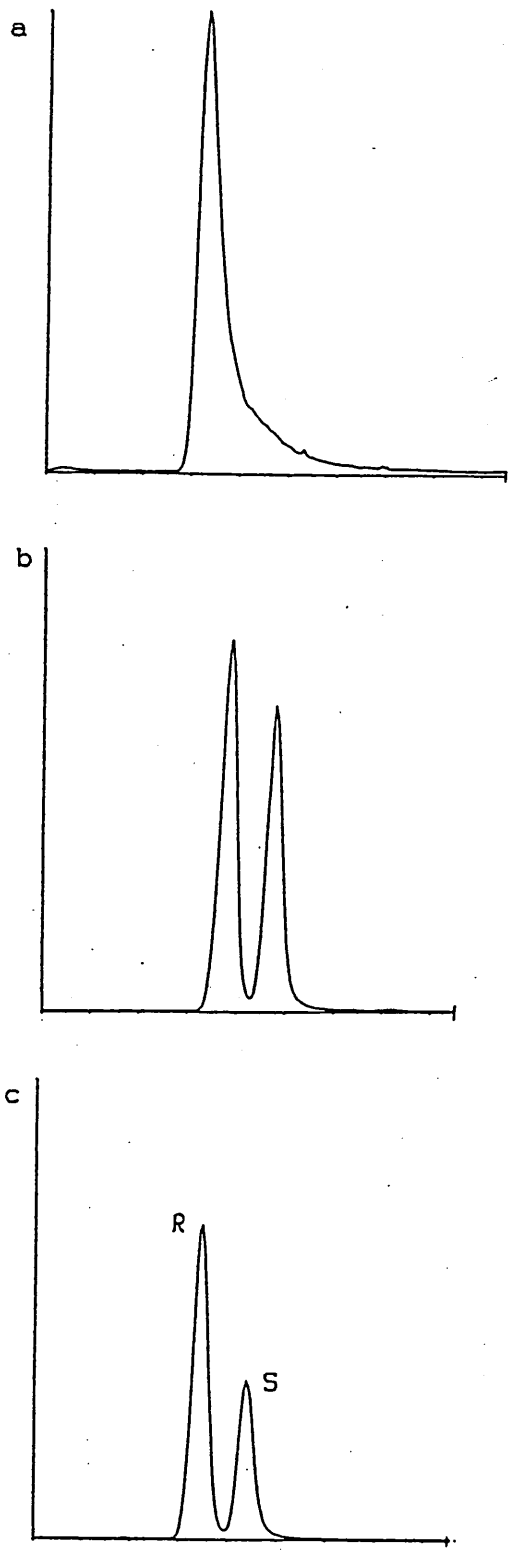


Figure 4.4 Single ion gas chromatograms $^{m}/_{z}$ 101 of the dimethyl R-2-phenylpropionyl esters of a) urine extract from a child with ketosis b) R, S-3-hydroxybutyric acid c) co-chromatography of a) and b) to show the configuration of the excreted 3-hydroxybutyric acid.

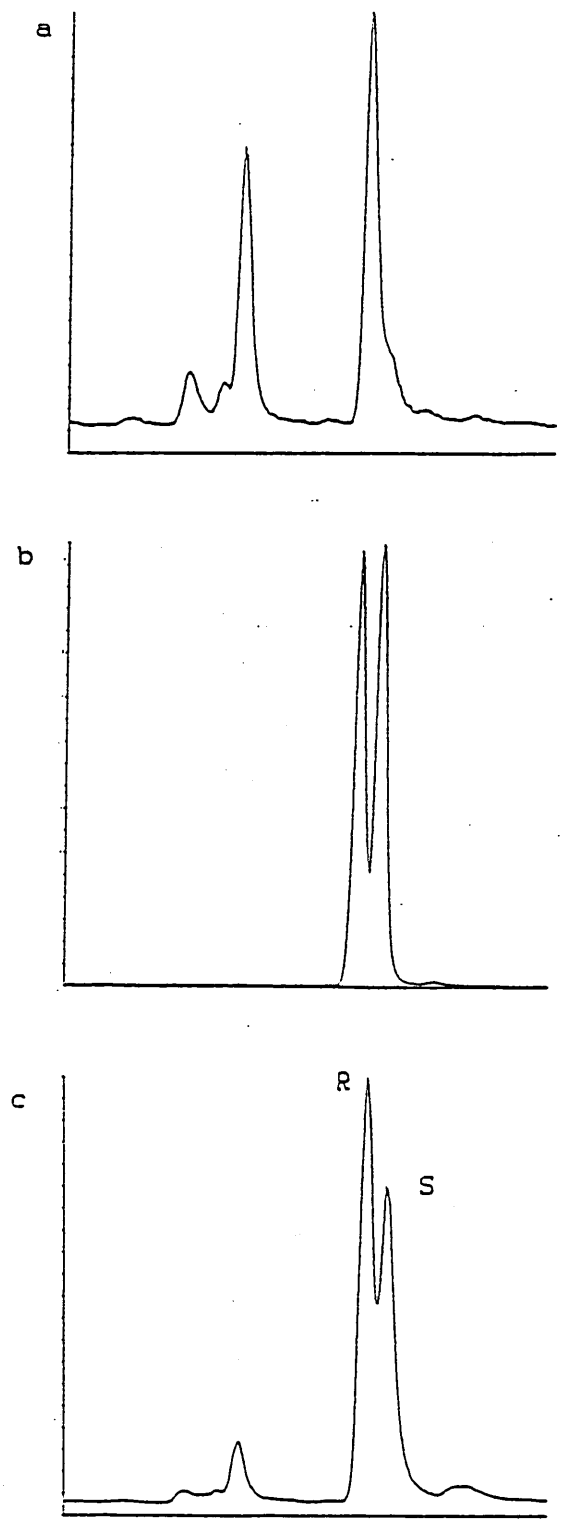


Figure 4.5 Single ion gas chromatograms $^{m}/_{z}$ 115 of the methyl R-2-phenylpropionyl esters of a) urine extract from a patient with propionic acidaemia b) R,S-3-hydroxyvaleric acid c) co-chromatography of a) and b) to determine the configuration of the excreted 3-hydroxyvaleric acid.

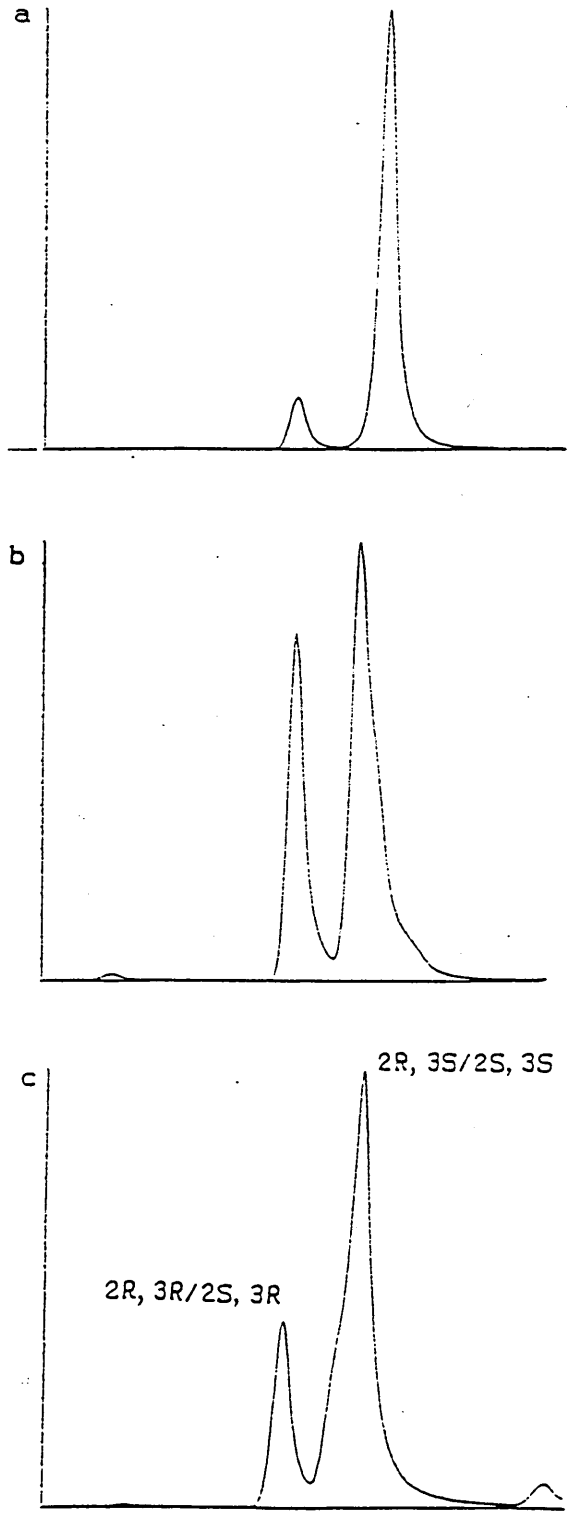


Figure 4.6 Single ion gas chromatograms $^{m}/_{z}$ 143 of a) O-acetylated-l-menthyl esters of a urine extract from a patient with β-ketothiolase deficiency b) O-acetylated-d, l-menthyl esters of 2R, 3S: 2S, 3S (1:9) 2-methyl 3-hydroxybutyric acid c) co-chromatography of a) and b) to determine the configuration of the excreted 2-methyl 3-hydroxybutyric acid.

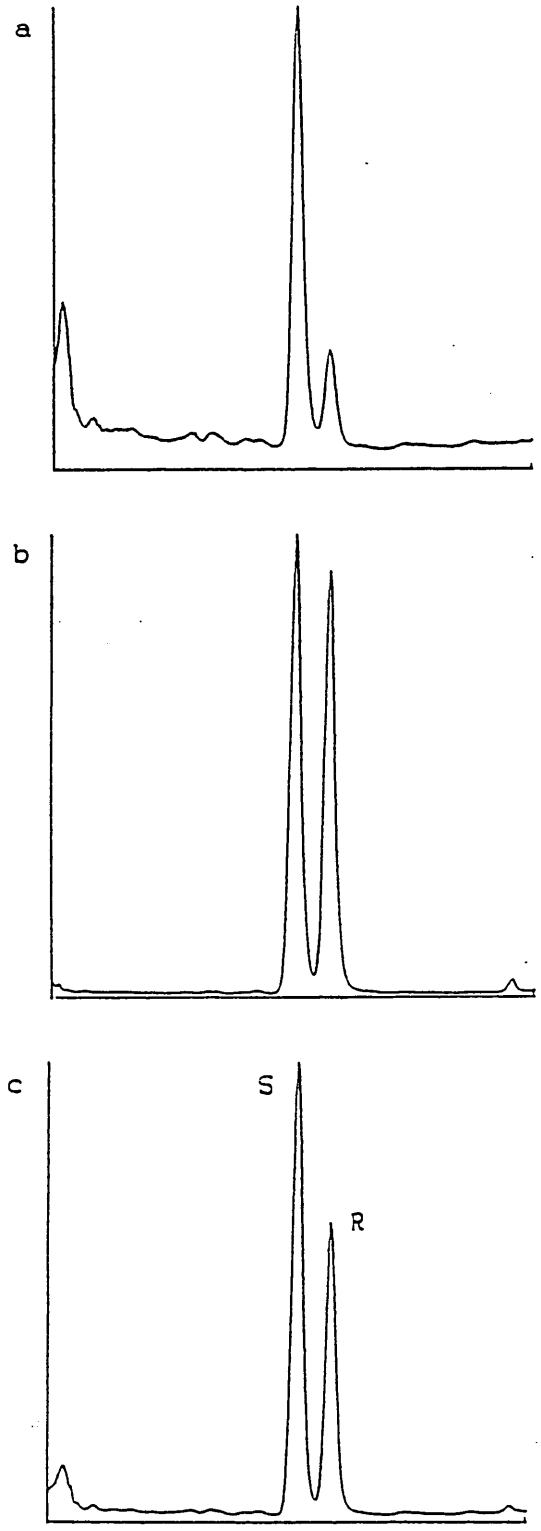


Figure 4.7 Single ion gas chromatograms $^{m}/_{z}$ 173 of the dimethyl R-2-phenylpropionyl esters of a) urine extract from a patient with hydroxydicarboxylic aciduria b) R,S-3-hydroxyadipic acid c) co-chromatography of a) and b) to show the order of elution of the enantiomers.

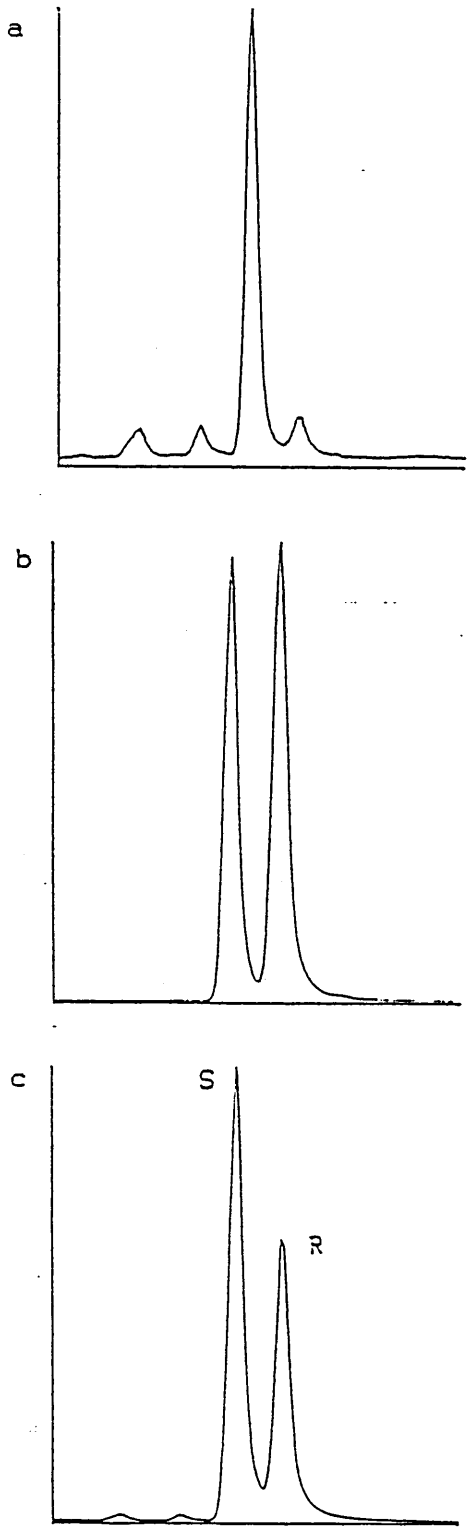


Figure 4.8 Single ion gas chromatograms $^{m}/_{z}$ 173 of the dimethyl R-2-phenylpropionyl esters of a) urine extract from a second patient with hydroxydicarboxylic aciduria b) R,S-3-hydroxyadipic acid c) co-chromatography of a) and b) to show the order of elution of the enantiomers.

4.2 Discussion.

4.2.1 Lactic Aciduria

The child investigated for the cause of the abnormal excretion of 3-hydroxypropionic acid, 1,3-propandiol and lactic acid was shown to excrete the L enantiomer of lactic acid (Figure 4.1). Lactic acid produced normally in mammals as a result of pyruvate metabolism has the L configuration, but two children have been reported with short bowel syndrome who excreted large amounts of D-lactic acid thought to be due to the overgrowth of Lactobacillus acidophilus (Haan et al. 1985 and Perlmutter et al. 1983). Another patient was described by Duran et al.in 1977 who excreted D-lactic acid of unknown origin.

It is usual to find some lactic acid in urinary organic acid extracts from normal subjects and from those with metabolic disorders. Since the lactic acid excreted by this child had the L configuration it is assumed that, unlike the other abnormal metabolites, it was probably not of bacterial origin and that this type of microbial infection is different from those reported previously in short bowel syndrome.

4.2.2 The Glyceric Acidurias.

4.2.2.1 D(R)-Glyceric aciduria.

D-Glyceric aciduria resulting from D-glycerate kinase deficiency has been proven in one patient (A) (Van Schaftingham 1988) and its mechanism is easily understood (Figure 1.9A). Both this patient (A) and Wadman's patient (B) (1976) showed a response to serine loading consisting of an

increase in urinary D-glycerate which suggests that Wadman's patient (B) also suffered from the same enzymatic defect.

As a result of loading experiments with fructose and dihydroxyacetone phosphate which caused increased urinary D-glycerate, Duran et al.(1988) suggested that the D-glyceric aciduria in their patient (C) could be the result of triokinase deficiency. This enzyme catalyses the phosphorylation of glyceraldehyde to glyceraldehyde-3-phosphate on the fructose catabolic pathway. It also catalyses the phosphorylation of dihydroxyacetone to dihydroxyacetone phosphate which then enters the glycolytic pathway. Glyceraldehyde can alternatively be oxidised to D-glycerate by the enzyme aldehyde dehydrogenase. The hypothesis is that a deficiency of triokinase would promote the conversion of glyceraldehyde to D-glycerate. It is likely that the ability of the serine degradation pathway would be limited, with the concommitent accumulation of D-glycerate. Furthermore, in the absence of triokinase, dihydroxyacetone could be oxidised to hydroxypyruvate by aldehyde dehydrogenase (EC 1.2.1.3/4/5) and then converted to D-glycerate by D-glycerate dehydrogenase. This would also overload the capacity of the pathway with the same result as before.

Duran was not able to demonstrate increased D-glycerate excretion after serine loading (patient C) as had Van Schaftingen (A) and Wadman (B), but Bonham et al. (in press) suggest that this does not necessarily exclude D-glycerate kinase deficiency because the levels of serine administered were not high enough to challenge the pathway (Duran's patient (C) had a plasma serine level of 467 mol/L compared with 750 mol/L for Wadman's patient (B)). They confirmed this by giving a serine load to one of their patients (patient 2 in section 1.3.2.1) which resulted in a plasma serine level of 417 mol/L, but no increase in D-

glycerate excretion. An increased load resulting in a plasma serine level of 717 mol/L showed marked increase urinary of D-glycerate.

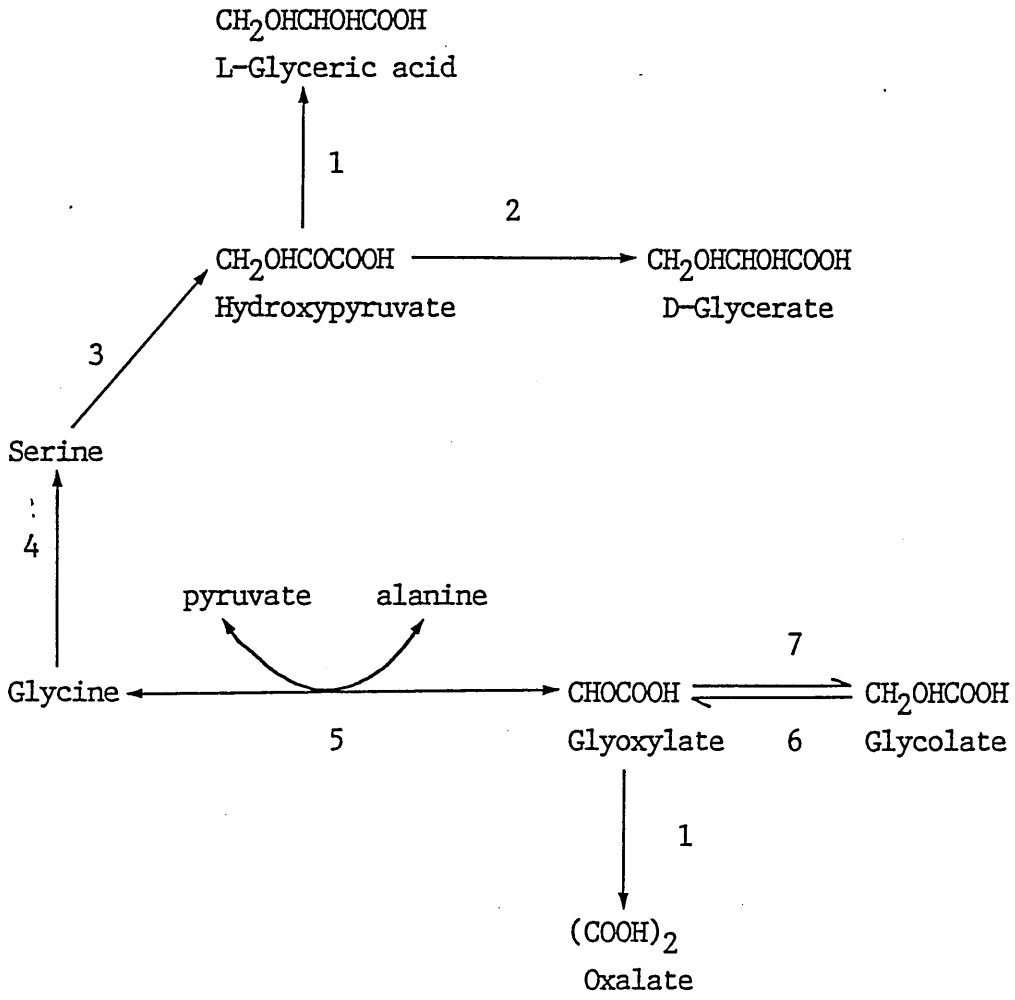
The child presenting with neurological abnormalities and who excreted D-glycerate (patient 1 in section 1.3.2.1 and Figure 4.2) has not been further investigated, but is believed to be progressing normally. Two of the four siblings (patients 3 to 5) whose organic acids showed D-glycerate excretion were the subjects of serine and fructose loading experiments by Bonham et al. The plasma serine levels reached 934 and 717 mol/L and were accompanied by an increase in D-glycerate excretion. There was also an increase in urinary D-glycerate following the fructose load. They conclude that the defect in these patients is consistant with a deficiency of D-glycerate kinase as described by Van Schaftingen. It is worth noting that triokinase levels were normal in Van Schaftingen's patient (A), but they were not measured in any of the other patients. Kolvraa et al. (1976) has found significantly lower activity of D-glycerate dehydrogenase in the leucocytes of a patient (D) with D-glyceric aciduria, but since this enzyme is the proven defect in primary hyperoxaluria type II (L-glyceric aciduria) (Mistry et al. 1988) it is difficult to understand how it could result in D-glyceric aciduria.

Although a deficiency of D-glycerate kinase seems to be the most likely cause of the excretion of D-glyceric acid there does not appear to be any explanation as yet for the range and severity of the symptoms accompanying this disorder.

4.2.2.2 Human primary Hyperoxaluria type II (L(S)-glyceric aciduria)

Primary hyperoxaluria type II is due to a deficiency of the enzymes D-glycerate dehydrogenase and glyoxylate reductase. D-Glycerate dehydrogenase catalyses the reduction of hydroxypyruvate to D-glyceric acid on the serine degradation pathway (Snell 1986). The characteristic L-glyceric aciduria is caused by the oxidation of the accumulated hydroxypyruvate by L-lactate dehydrogenase in the cytosol to L-glyceric acid (Williams and Smith 1968). Hydroxypyruvate itself has not been found in the urine of any person with the disorder.

The origin of the oxalosis has been the subject of a number of hypotheses (William and Smith 1968, Chalmers and Lawson 1982). However, glyoxylate is known to be the main precursor of oxalate and it is thought that the inability to reduce glyoxylate to glycolate due to a deficiency of glyoxylate reductase could result in its oxidation to oxalate by cytoplasmic L-lactate dehydrogenase (Mistry et al. 1988). D-Glycerate dehydrogenase also has glyoxylate reductase activity and there is speculation that they are in fact the same enzyme (Williams and Smith 1968, Dawkins and Dickens 1965 and Chalmers and Lawson 1982).
This situation would be similar to the oxalosis in the disorder primary hyperoxaluria type I in which there is a deficiency of the enzyme alanine-glyoxylate aminotransferase (EC 2.6.1.44) (Figure 4.9). The disease is characterised by hyperoxaluria and glycolic aciduria, but no glyceric aciduria. The mechanism of the oxalosis is explained by the passage of the accumulated glyoxylate from the peroxisome to the cytosol where it is reduced to oxalate by lactate dehydrogenase (Danpure et al. 1986).



1 = L-Lactate dehydrogenase (EC 1.1.1.27)
2 = D-Glycerate dehydrogenase (EC 1.1.1.29)
3 = Serine-pyruvate aminotransferase (EC 2.6.1.51)
4 = Serine-hydroxymethyltransferase (EC 2.1.2.2)
5 = Alanine-glyoxylate aminotransferase (EC 2.6.1.44)
6 = Glycolate oxidase (EC 1.1.3.15)
7 = Glyoxlate reductase (EC 1.1.1.26)

Figure 4.9 The origin of the L-glyceric aciduria and oxalosis in primary hyperoxaluria type 2.

4.2.2.3 Feline primary hyperoxaluria type II (L(S)-glyceric aciduria).

Figure 4.3 shows the L-glyceric acid excreted by a cat with primary hyperoxaluria type II.

It is extremely rare that an animal model for a human genetic disease be found. Danpure et al. 1989 has stated that the enzymes D-glycerate dehydrogenase and glyoxylate reductase, which are deficient in the human condition, are also deficient in affected cats. Blakemore et al. (1988) have also stated that the feline disorder is inherited in an autosomal recessive mode.

Comparison of the clinical manifestations of the disorder in man and cats show a number of differences. The peripheral neuropathy seen in cats is not a symptom of the disease in man. In addition, the renal problems associated with the feline disorder are much more acute. Danpure et al. (1989) have suggested that the more severe presentation of the disorder in cats is due to a difference in the intracellular site of glyoxylate metabolism. This is related to the observation that feline alanine-glyoxylate aminotransferase is located in the mitochondria rather than the peroxisome as in man (Noguchi et al. 1978). If cytosolic glyoxylate reductase has a greater role in the feline metabolism of glyoxylate then the consequences of a deficiency of the enzyme would be greater.

4.2.3 Ketosis and propionic acidaemia.

4.2.3.1 Ketosis.

The observation that the 3-hydroxybutyrate produced by the child during a fast had the R(D) configuration (Figure 4.4) is the expected result and is explained as follows.

Under high rates of fatty acid oxidation the liver produces large quantities of acetyl CoA, two molecules of which can condense to form acetoacetyl CoA. This is a reversal of the acetoacetyl CoA thiolase reaction whereby acetoacetyl CoA reacts with another CoA molecule to produce two molecules of acetyl CoA. The acetoacetyl CoA condenses with another molecule of acetyl CoA to form 3-hydroxy 3-methyl glutaryl CoA (3-HMG CoA) via the enzyme 3-HMG CoA synthase. 3-HMG CoA is acted upon by the enzyme HMG CoA lyase which produces free acetoacetate and acetyl CoA. Acetoacetate spontaneously forms acetone or, via the action of the mitochondrial enzyme D-3-hydroxybutyrate dehydrogenase, D-3-hydroxybutyrate (Figure 4.10).

4.2.3.2 Propionic Acidaemia

The clinical presentation of propionic acidaemia is so non-specific that detailed laboratory investigation is necessary. GC-MS analysis of the urinary organic acids shows a number of characteristic metabolites which are formed by secondary metabolism of the accumulated propionic acid. The major diagnostic metabolites are 3-hydroxypropionate and methylcitrate.

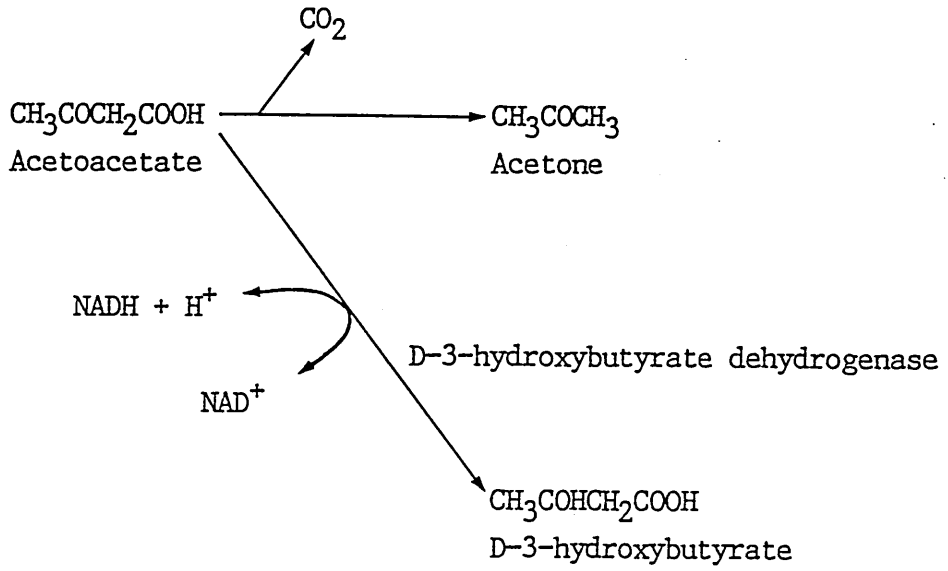


Figure 4.10 The formation of the ketone bodies.

3-Hydroxypropionate is thought to be formed when propionic acid is β-oxidised producing acrylyl CoA which can subsequently be hydrated to the 3-hydroxy compound (Ando et al. 1972). Methylcitrate is produced when a molecule of propionyl CoA substitutes for a molecule of acetyl CoA in reacting with oxaloacetate by the action of citrate synthetase in the tricarboxylic acid (TCA) cycle. It is interesting in that two isomeric forms of the molecule are formed. Brandage et al. (1977) have distinguished the diastereoisomers using trimethyl esters as the 2R,3S and the 2S,3S isomers in a ratio of 1:2. Weidman and Drysdale (1979) have found this ratio to vary between patients and be temperature dependant. From experiments to study the mechanism of the enzyme reaction, they concluded that the competitive inhibition of citrate synthesis by methylcitrate produces an overall slowing of the rate of the TCA cycle. This is responsible for many of the severe symptoms of the disorder.

Propionylglycine and tiglylglycine may be present in the urine of propionic acidaemia patients formed via the action of glycine N-acylase. Tiglylglycine is thought to be formed when tiglyl CoA accumulates due to competition for crotonase by acrylyl CoA which, in turn, is formed from propionate. Glycine N-acylase has a low affinity for propionyl CoA, but the suggestion is that the accumulation of the substrate might have an inductive effect on the enzyme and hence glycine synthesis (Chalmers and Lawson 1982).

3-Oxo and 3-hydroxyvalerate are thought to be formed when propionyl CoA substitutes for acetyl CoA in the formation of ketone bodies via the 3-hydroxy 3-methylglutaryl CoA pathway, the mechanism of which is outlined above. This origin is confirmed by the analytical work which shows that it is the R(D) enantiomer of 3-hydroxyvalerate which is

produced in propionic acidaemia (Figure 4.5) in a similar way to the production of D(R)-3-hydroxybutyrate via the action of D-3-hydroxybutyrate dehydrogenase. Further evidence for this includes the observation made in at least one patient examined in this laboratory who excreted a small amount of 3-hydroxy 3-ethylglutarate in a similar manner to 3-hydroxy 3-methylglutarate in ketosis. 2-Methyl 3-hydroxyvalerate can also result from the condensation of two molecules of propionyl CoA and 2-methyl 3-hydroxybutyrate from the analogous situation between acetyl CoA and propionyl CoA. The 2-methyl 3-hydroxybutyrate in the patient examined here consisted mainly of the erythro diastereoismer accompanied by a small amount of the threo diastereoisomer. This is similar to the case of 2-methylacetoacetyl CoA thiolase deficiency described below. Menkes (1966) identified a number of abnormal ketones including 2-butanone, 2-pentanone and 3-pentanone which are thought to be the products of the spontaneous decarboxylation of the appropriate oxo acids in a similar way to the production of acetone from acetoacetate.

The competition of propionyl CoA with acetyl CoA in various pathways concerning fatty acids synthesis also results in the production of abnormal lipids and triglycerides found in this disorder.

4.2.4 2-Methylacetoacetyl CoA Thiolase Deficiency.

Middleton and Day (1988) have recently confirmed the metabolic role of acetoacetyl CoA thiolase in human fibroblasts. They have shown that the absence of the same enzyme which causes 2-methyl 3-hydroxybutyric aciduria also causes greatly reduced ability to oxidise acetoacetate in vitro. This factor is expressed in patients with the disorder by their

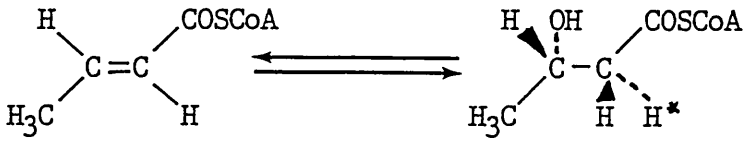
severe ketoacidosis.

The results of the GC-MS analysis of the O-acetylated-L-menthyl derivative of the urinary organic acids showed that the excreted 2-methyl 3-hydroxybutyric acid had either the 2R,3S or the 2S,3S configuration (Figure 4.6).

2-Methyl 3-hydroxybutyryl CoA is produced by the action of crotonase on tiglyl CoA (Robinson et al. 1956) (Figure 1.11). 2-Methyl 3-hydroxybutyrate excreted in urine of patients with this disorder may be formed by hydrolysis of the accumulated CoA ester or by the reduction of 2-methylacetoacetyl CoA and hydrolysis of the ester. In their work on the stereochemistry of the crotonase (enoyl CoA hydratase) reaction Willardson and Eggerer (1975) determined that hydration of crotonyl CoA to 3-hydroxy[2^3H]butyrate proceeded by the syn addition of water to give a product with the 2R,3S configuration as shown in figure 4.11.

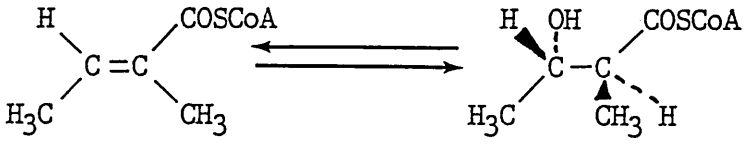
Therefore,it is predicted that, when tiglyl CoA is syn hydrated addition of the hydroxyl group to carbon 3 is exactly the same giving the S configuration. However, the situation at carbon 2 is slightly different in that the presence of a methyl group in place of a hydrogen changes the order of priority of the substituents around the carbon. This gives carbon 2 an S configuration as shown in figure 4.11. Thus, 2-methyl 3-hydroxybutyrate produced by crotonase hydration should have the 2S,3S configuration.

The baker's yeast reduction of ethyl 2-methylacetoacetate gives 2-methyl 3-hydroxybutyrate of mainly the 2S,3S diastereoisomer (Frater 1979b), that is, the threo configuration. This corresponds to the second peak of the two eluted from a OV101 chromatography column using trimethylsilyl esters. In urine from a patient with 2-methylacetoacetyl CoA thiolase deficiency the major peak of 2-methyl 3-hydroxybutyrate



Crotonyl CoA

3-Hydroxy[2^{3}H]butyrate
(2R,3S)



Tiglyl CoA

3-Hydroxy 2-methylbutyrate
(2S,3S)

Figure 4.11 The stereochemistry of the crotonase reaction.

corresponds to that with the lesser retention time and is therefore the erythro diastereoisomer (Appendix 1 Chromatogram 6). This must consist of the 2R,3S configuration and is at variance with the predicted result.

The oxidation of 2-methyl 3-hydroxybutyryl CoA to 2-methyl-acetoacetyl CoA by 3-hydroxyacyl CoA dehydrogenase is a reversible reaction (Middleton and Bartlett 1983). It could be that, due to the accumulation of 2-methylacetoacetyl CoA, the reverse reaction would take place to a greater extent. If the 2R,3S form of 2-methyl 3-hydroxy-butyryl CoA is more stable than the 2S,3S form then the 2R,3S configuration would be produced preferentially via the reverse reaction and would account for the observed result. It would also follow that the small peak of threo (2S,3S) 2-methyl 3-hydroxybutyrate is produced by the action of crotonase on tiglyl CoA directly and hydrolysis of the product further back up the pathway.

The observation that carbon 3 of the 2-methyl 3-hydroxybutyrate molecule has the S configuration regardless of the configuration of carbon 2 is significant. It means that it is unlikely that 2-methylacetoacetate is reduced by β-hydroxybutyrate dehydrogenase since this would result in the D(R) configuration at carbon 3 which is the situation in ketosis and for 3-hydroxyvalerate in propionic acidaemia.

4.2.5 3-Hydroxydicarboxylic Aciduria

The L(S) configuration of urinary 3-hydroxyadipic acid excreted by patients with hydroxydicarboxylic aciduria is the same as that excreted by normal subjects who have previously ingested dicarboxylic acids (Figure 4.7 and 4.8).

The range of even and odd-chain dicarboxylic acids, their 3-hydroxy

analogues and unsaturated dicarboxylic acids in the urine of these patients (Appendix I Chromatogram 8) shows that the fatty acid -oxidation spiral is compromised. The presence of high concentrations of 4-hydroxyphenyllactic and pyruvic acids is indicative of damage to liver enzymes. Pollitt et al. (1987) have suggested that the urinary organic acid excretion pattern is consistent with a defect of the 3-hydroxyacyl CoA dehydrogenase or the oxoacyl CoA thiolase. This would lead to an accumulation of medium chain 3-hydroxymonocarboxylic acids in the mitochondria. β-Oxidation of 3-hydroxydodecanoate would produce 3-hydroxydodecanedioic acid which, if ω-oxidised from the other end of the molecule, would give a range of even chain dicarboxylic acids and their 3-hydroxy analogues. This process would probably take place in the peroxisomes.

However, knowledge of the configuration of the 3-hydroxyadipate excreted in this disorder does not mean that the exact nature of the defect can be located because the L(S) enantiomer would result if the deficiency lay with L-3-hydroxyacyl CoA dehydrogenase or through 'backing-up' of metabolites from the 3-oxoacyl CoA thiolase step. It does, however, show that it is unlikely that the 3-oxoadipyl CoA is being reduced by some other dehydrogenase enzyme to give D(R)-3-hydroxyadipyl CoA in a similar way to the production of D(R)-3-hydroxybutyrate from acetoacetate.

The absolute configuration of the other 3-hydroxydicarboxylic acids excreted in this disorder has not been directly assigned as part of this dissertation. However, in one of the urinary analyses the organic acid extract was methylated using methanol and thionyl chloride in place of diazomethane. When the sample was subsequently examined as the R-2-phenylpropionyl derivative it was found that the 3-hydroxyadipate and 3-

hydroxysebacate had racemised to some extent. This was assumed to be due to the methylation process. Comparison of this sample with another from the same patient where methylation was performed with diazomethane showed that the dimethyl 3-hydroxyadipate and dimethyl 3-hydroxysebacate (3-hydroxydecanedioic acid) corresponded to the diastereoisomer with the shorter retention time. Figure 3.6 shows that for dimethyl 3-hydroxyadipate R-2-phenylpropionyl derivative the S enantiomer has the shorter retention time. It is therefore assumed that 3-hydroxysebacate excreted by patients with hydroxydicarboxylic aciduria also has the S(L) configuration. This is to be expected since the 3-hydroxydicarboxylic acids excreted in this disorder are thought to be formed by the same process. 3-Hydroxysuberic acid (3-hydroxyoctanedioic acid) was not identified in the derivative preparation.

The reason for the two types of clinical presentation and the exact nature of the defect in this disorder are still under investigation.

5. CONCLUSIONS.

This dissertation has examined two of the main aspects of the determination of configuration of chiral metabolites by GC-MS: the production of chiral reference compounds where they are not available commercially and their use in determining the absolute configuration of urinary metabolites in inherited metabolic disease.

The baker's yeast reduction of β-keto carboxylic and α-methyl β-keto carboxylic acid esters to produce chiral reference compounds has been discussed. The ease of the practical aspects of this method has great advantage over the often complicated and protracted procedures involved in purely chemical methods. It has been shown that production of a particular chiral intermediate can be achieved by manipulation of the size of the ester grouping on the precursor according to the Prelog rule. It has also been shown however, that the Prelog rule has limitations and that configuration of chiral standards produced with reference to this rule may need to be checked by biochemical methods. In the case of 3-hydroxyhexanoate for example, the configuration could have been checked by preparing S(L)-3-hydroxyhexanoate from hexanoyl CoA in a similar manner to the preparation of S(L)-3-hydroxyoctanoate from octanoyl CoA. The work could also be developed to examine the asymmetric yeast reduction of β-keto dicarboxylic acid esters and the application of the Prelog rule to this.

The configurations of a number of chiral metabolites in various inherited metabolic disease have been assigned. This has been performed for diagnostic purposes such as in the glyceric acidurias and some types of lactic aciduria or to provide information on the biochemical mechanisms of the enzyme defect such as the work on propionic acidaemia

and 3-hydroxydicarboxylic aciduria.

The determination of the absolute configuration of urinary 3-hydroxyadipate excreted in hydroxydicarboxylic aciduria is valuable, but further analysis concentrating on the measurement of appropriate enzymes in the liver of persons with this disorder is necessary to confirm the exact defect.

The analysis of 3-hydroxypentanoic acid in propionic acidaemia has confirmed the hypothesis that this metabolite is produced when propionyl CoA substitutes for acetyl CoA in ketogenesis.

The examination of the absolute configuration of urinary 2-methyl 3-hydroxybutyric acid is not conclusive, but has presented the idea that this metabolite is produced mainly by the reverse reaction of the oxidation of 2-methyl 3-hydroxybutyryl CoA to 2-methylacetoacetyl CoA rather than directly from hydrolysis of 2-methyl 3-hydroxybutyryl CoA itself.

In general terms, the dissertation has aided in the overall understanding of the biochemical mechanisms at work in some of the organic acidurias.

The use of GC-MS and single ion monitoring as an analytical tool in this work has been invaluable. It has enabled chiral standards and metabolites to be examined on the basis of calculated characteristic ions and the results expressed clearly. This is relevant where there are many compounds in a sample such as in urinary analysis and where the concentration of a particular analyte is very small. It is doubtful whether the analysis of 3-hydroxyadipate N-TFA-L-alanyl esters especially could have been accomplished using GC alone.

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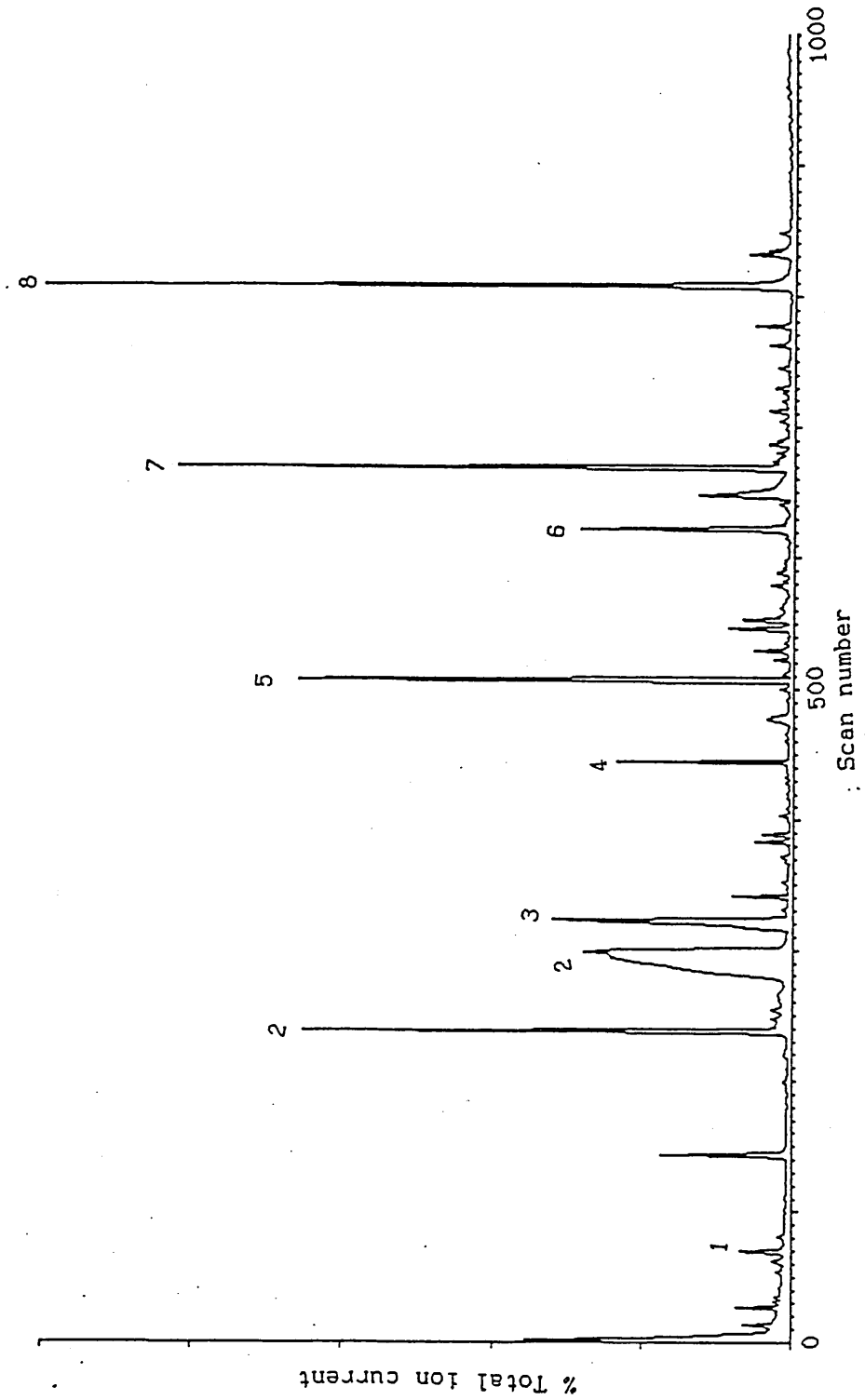
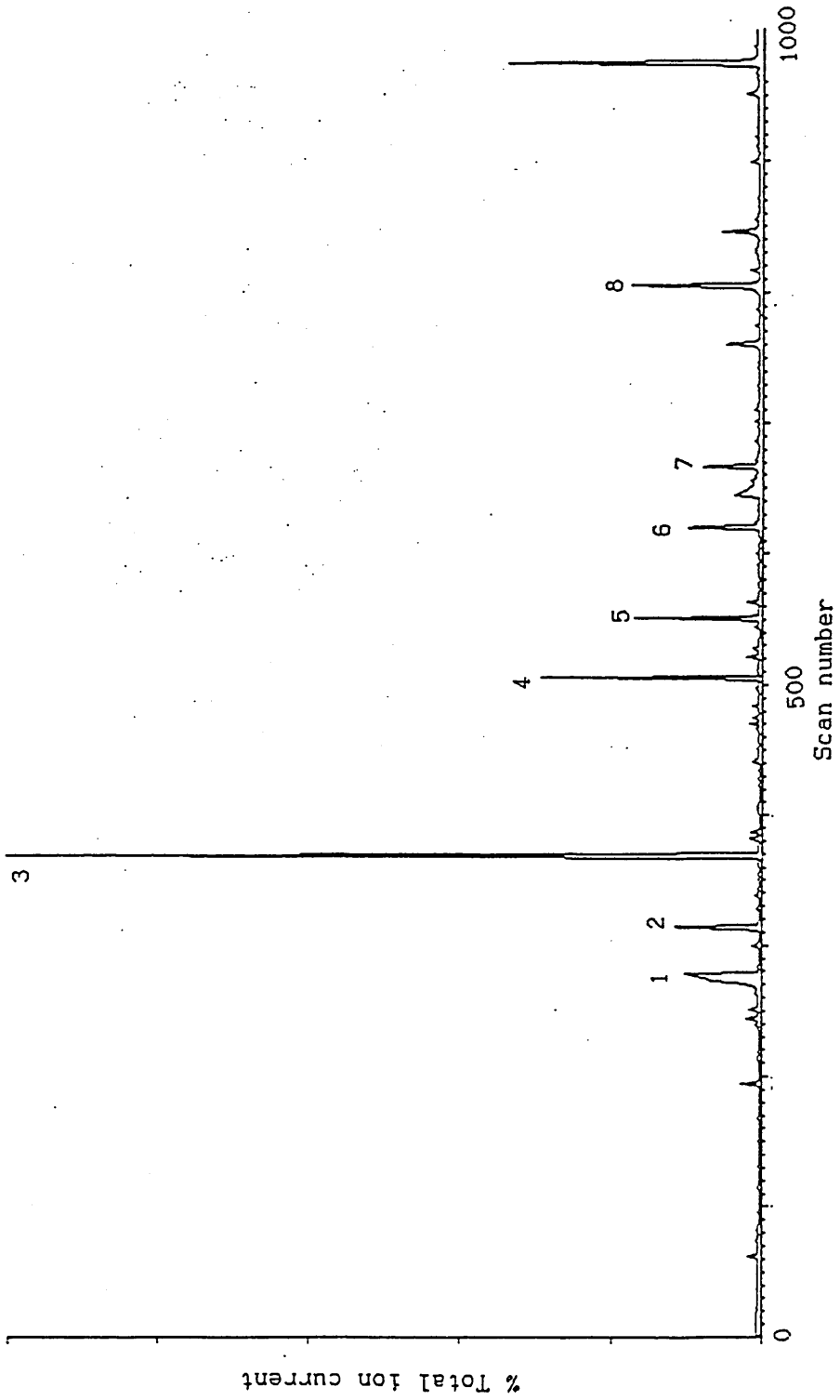
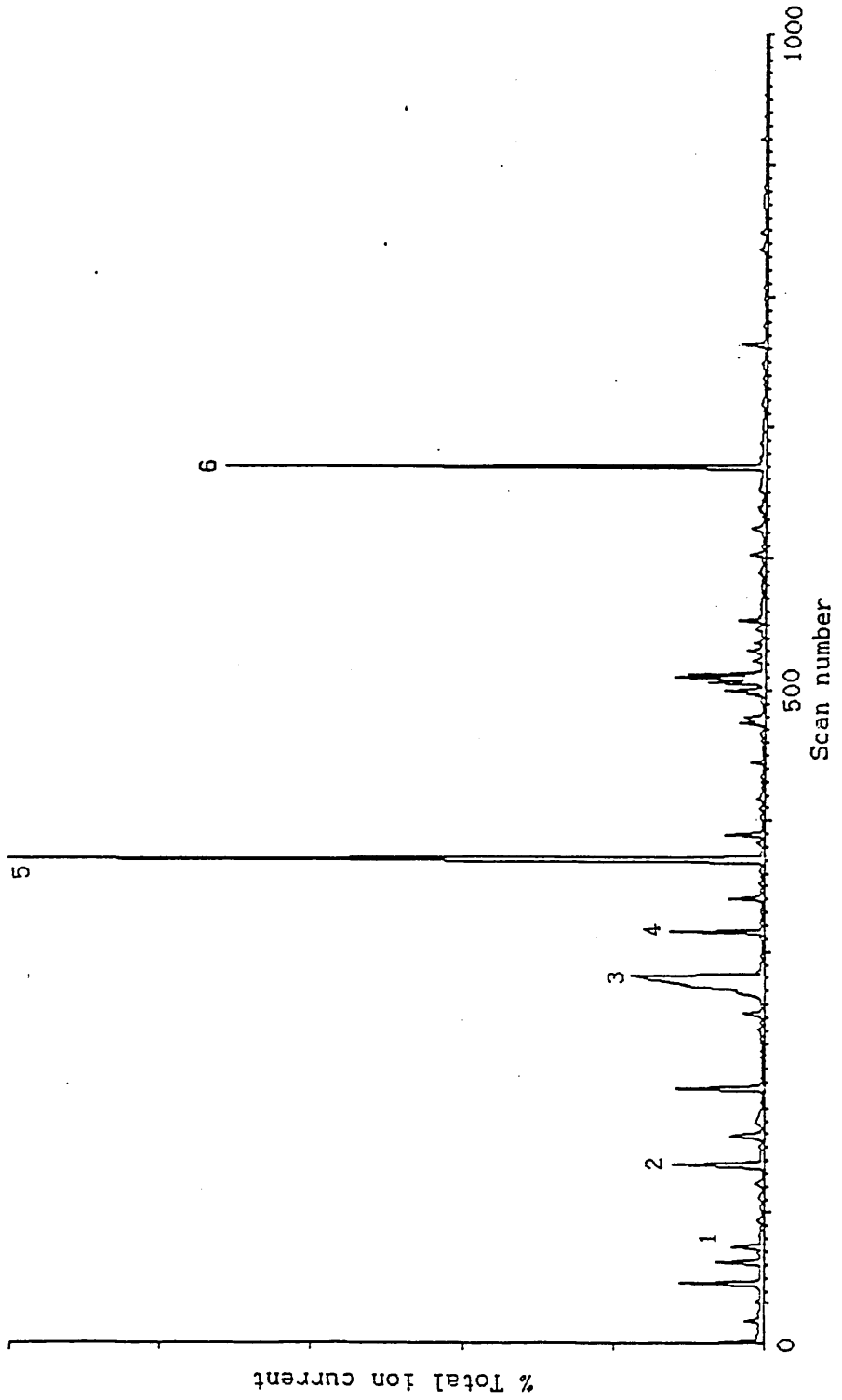


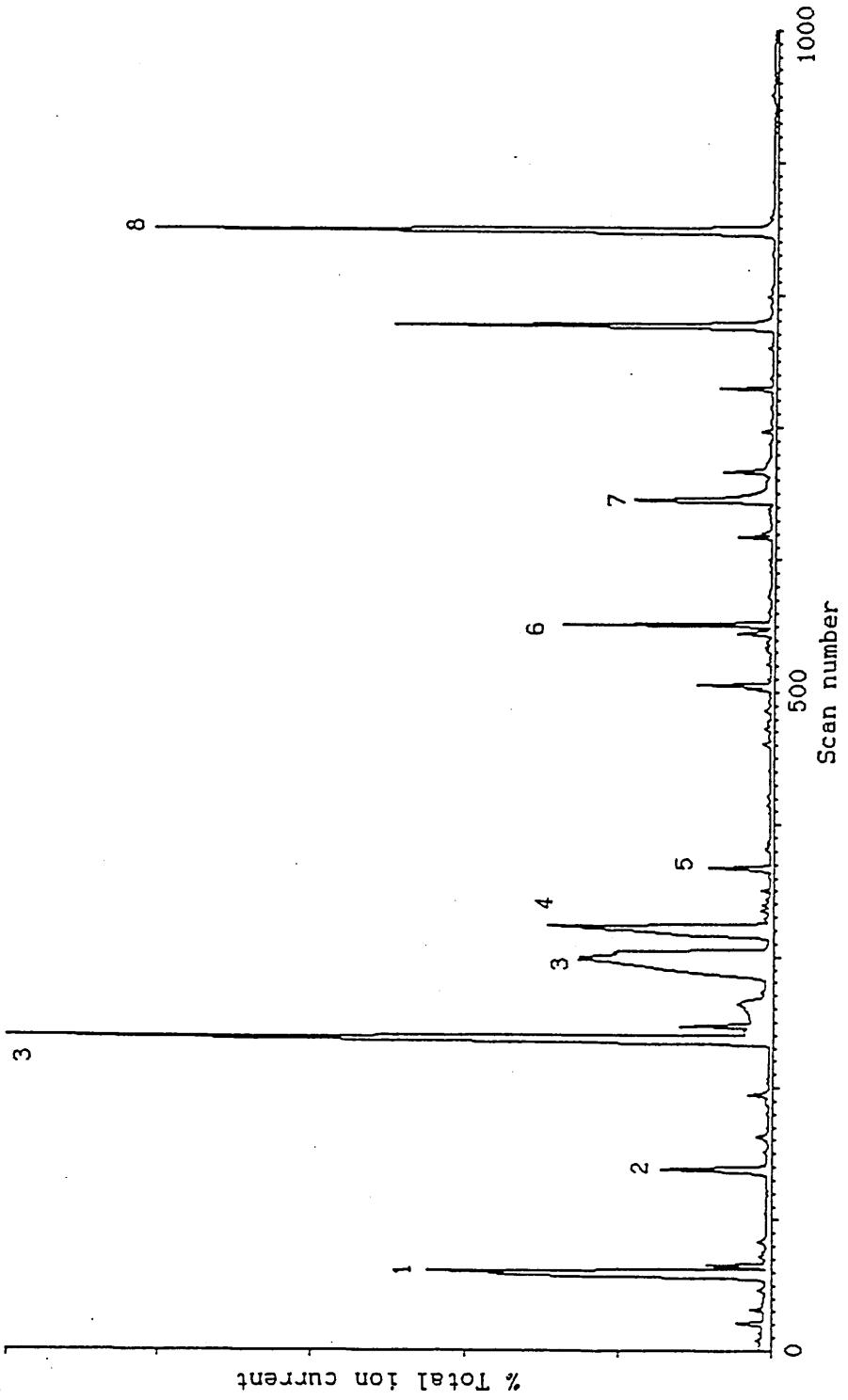
1. Total ion current chromatogram of urine organic acids from a normal child. 1. Lactic acid 2. Urea 3. Phosphate 4. 2-Deoxytetronic acid 5. Pyrogallol 6. Aconitic acid 7. Citric acid 8. Internal standard (margaric acid).



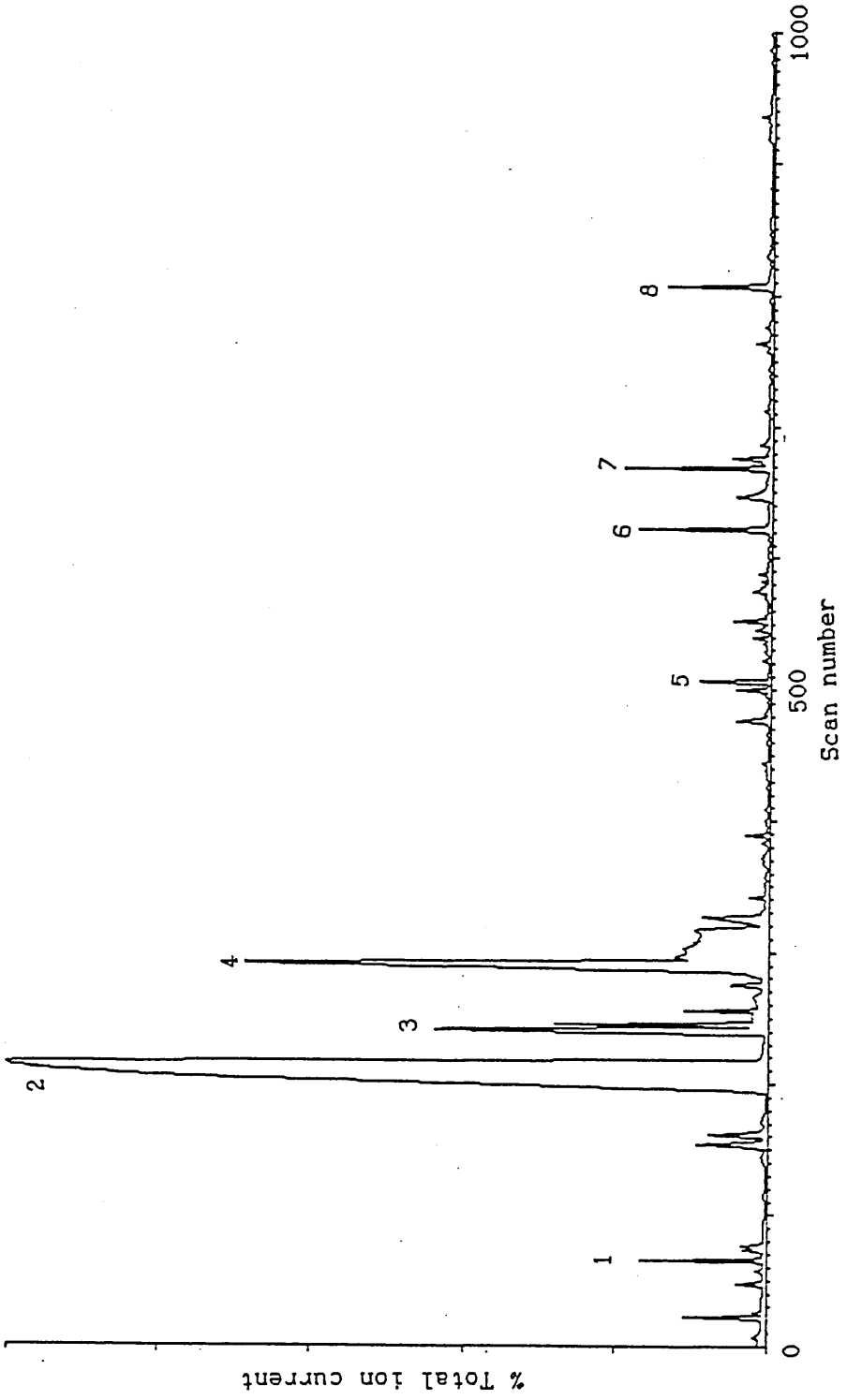
2. Total ion chromatogram of urine organic acids from a child with D-glyceric acidaemia. 1. Urea 2. Phosphate 3. Glyceric acid 4. Pyrogallol 5. 4-hydroxyphenylacetic acid 6. Aconitic acid 7. Citric acid 8. Internal standard.



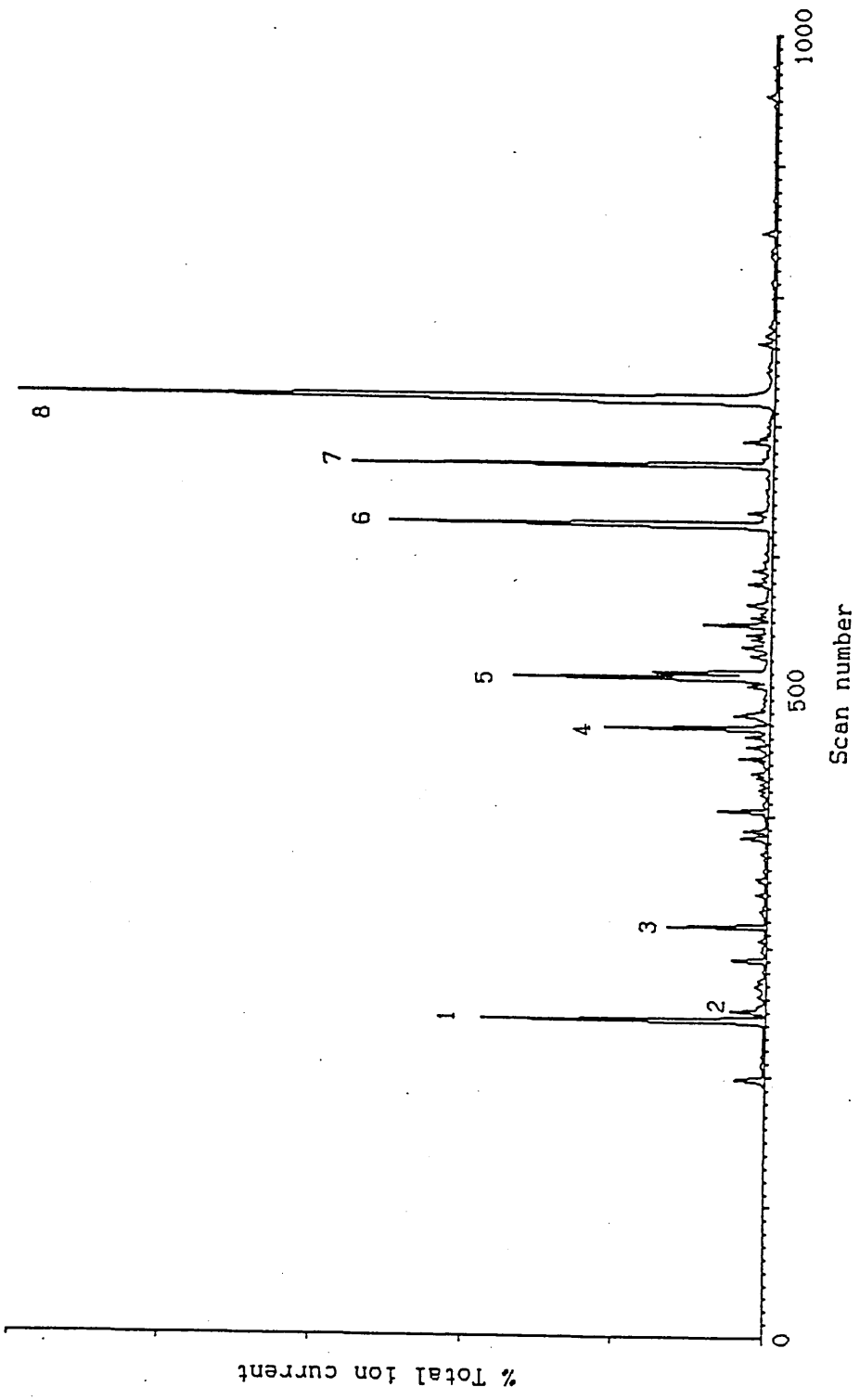
3. Total ion chromatogram of urine organic acids from a child with L-glyceric aciduria (hyperoxaluria type II). 1. Lactic acid 2. Oxalic acid 3. Urea 4. Phosphate 5. Glyceric acid 6. Hippuric acid.



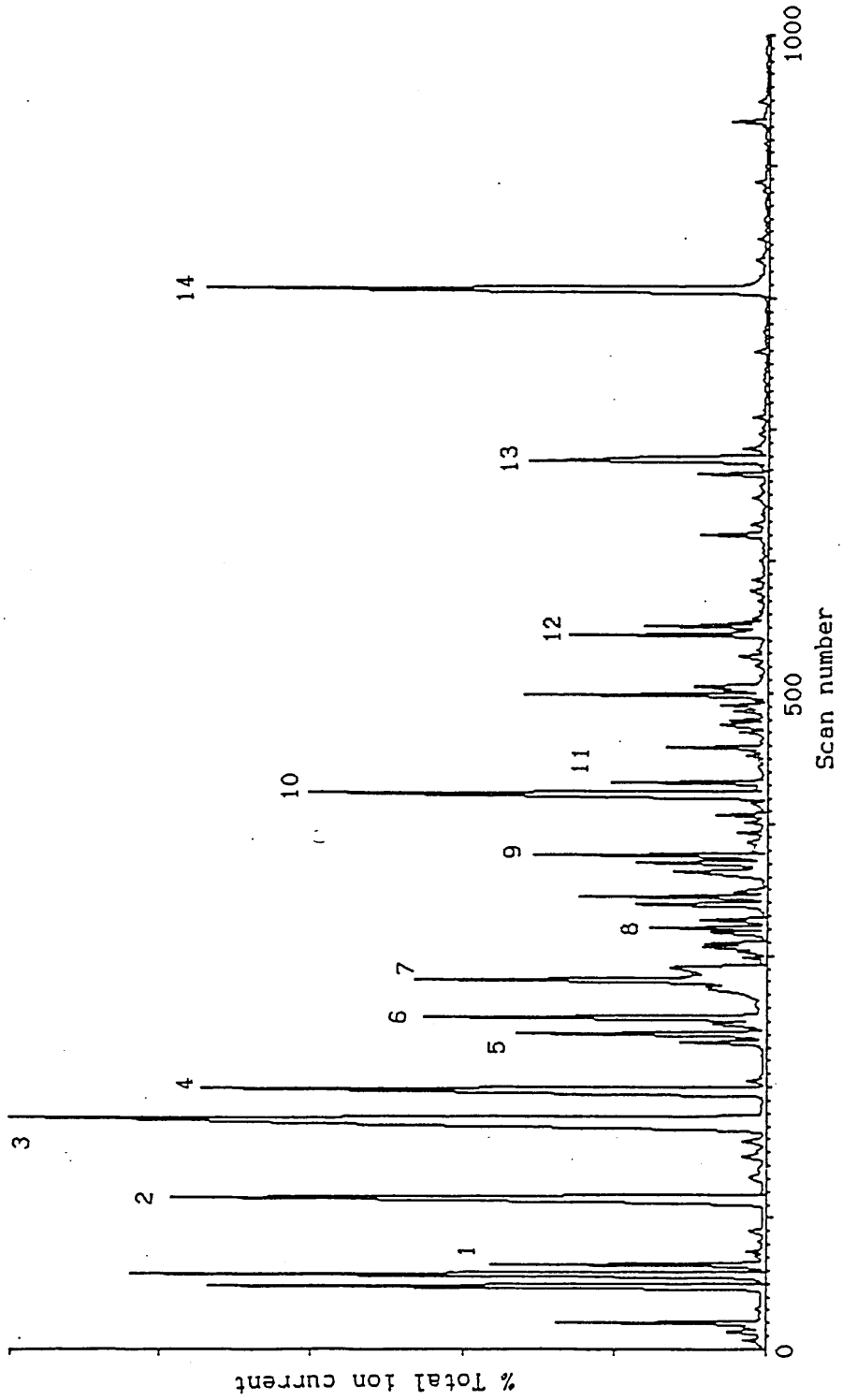
4. Total ion chromatogram of urine organic acids from a cat with feline hyperoxaluria type II. 1. Lactic acid 2. Oxalic acid 3. Urea 4. phosphate 5. Glyceric acid 6. 4-Hydroxyphenylacetic acid 7. Hippuric acid 8. Internal standard.



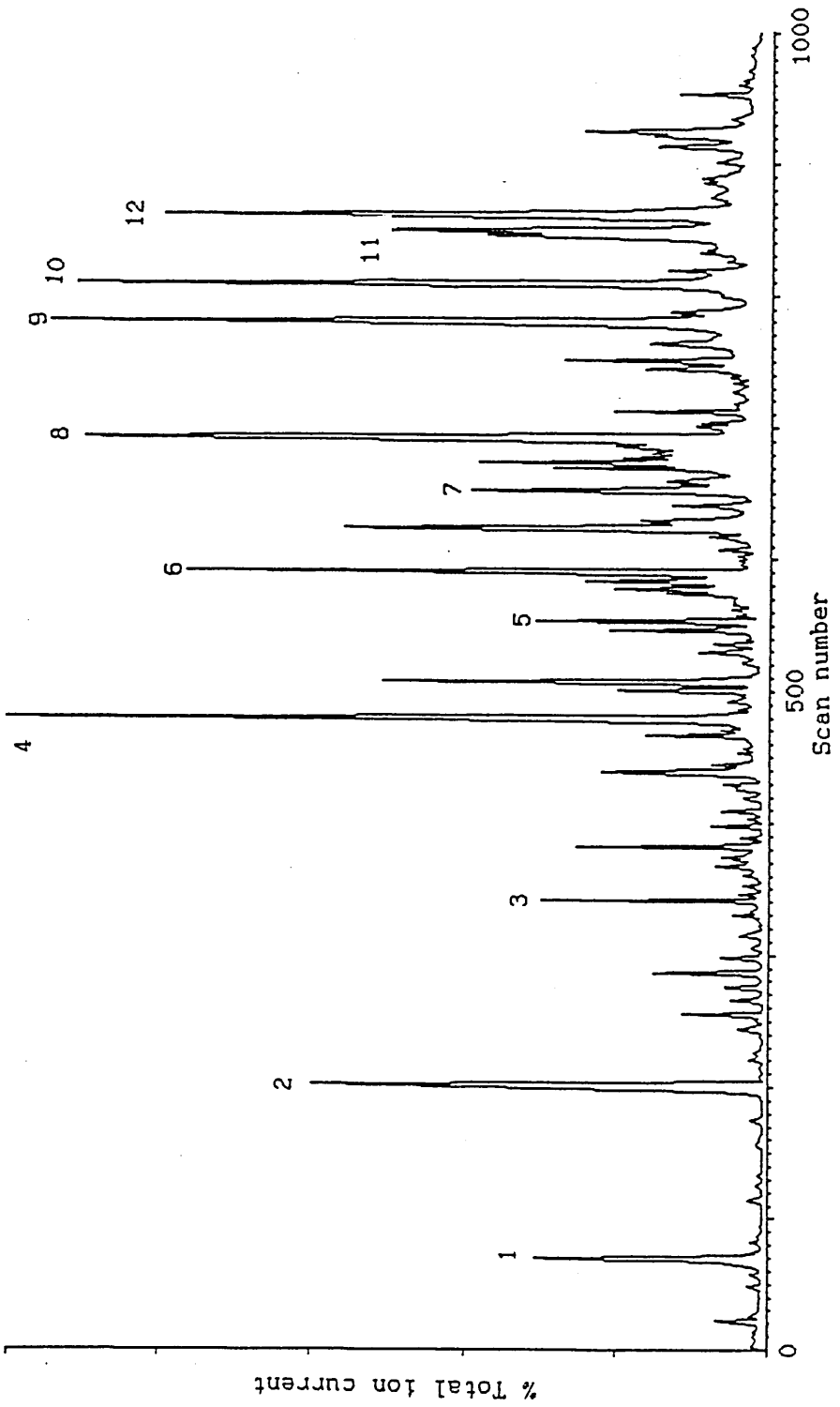
5. Total ion chromatogram of urine organic acids from a child with ketonuria. 1. Lactic acid 2. 3-Hydroxybutyric acid 3. Urea 4. Acetoacetic acid 5. Pyrogallol 6. Aconitic acid 7. Citric acid 8. Internal standard.



6. Total ion chromatogram of urine organic acids from a child with β-ketothiolase deficiency. 1.erythro2-methyl 3-hydroxybutyric acid 2. threo 2-methyl 3-hydroxybutyric acid 3. Ethylmalonic acid 4. 2-Methylglutaconic acid 5. Tiglylglycine 6. Aconitic acid 7. Citric acid 8. Internal standard.



7. Total ion chromatogram of urine organic acids from a child with propionic acidaemia. 1. Lactic acid 2. Butanediol 3. 3-Hydroxypropionic acid 4. 3-hydroxybutyric acid 5. 2-Methyl 3-hydroxybutyric acid 6. 3-Hydroxyisovaleric acid 7. 3-Hydroxyvaleric acid 8. 3-Oxovaleric acid 9. 3-Oxo 2-methylvaleric acid 10. Propionylglycine 11. 3-Methylglutaconic acid 12. 3-Hydroxy 3-methylglutaric acid 13. Methylcitric acid 14. Internal standard.



8. Total ion chromatogram of urine organic acids from a child with hydroxydicarboxylic aciduria. 1. Lactic acid 2. 3-Hydroxybutyric acid 3. Succinic acid 4. Adipic acid 5. 4-Hydroxyphenylacetic acid 6. Suberic acid and 3-hydroxyadipic acid 7. Hippuric acid 8. Sebacic acid and 3-hydroxysuberic acid 9. 3-Hydroxysebacic acid 10. Internal standard. 11. 3-Hydroxydodecendioic acid 12. 3-Hydroxydodecandioic acid.

Studies and lectures undertaken in fulfillment of the requirements for the degree of M.Phil.

General seminars and lectures.

Attendence at the seminars held in the Department of Chemical Pathology, Sheffield Children's Hospital 1986-89. This includes one seminar presented by myself entitled "L-Glyceric Aciduria in the Cat".

Attendence at lectures and meetings held by the Sheffield Institute of Medical Laboratory Sciences Discussion group 1987.

Attendence at lectures held for the Fellow of the Institute of Medical Laboratory Sciences examination at Sheffield City Polytechnic 1987-88.

Attendence at various post-graduate lectures held by the Department of Paediatrics, Sheffield Children's Hospital 1987-89.

Attendence at some meetings of the University of Sheffield Biochemical Society 1988.

Attendence at some relevant undergraduate lectures held in the Department of Chemistry and the Department of Biochemistry 1988.

Specific Seminars and Conferences.

The Sir Ernest Finch Memorial Lectures chaired by Professor June Lloyd entitled "Recent Advances into Lipid Inborn Errors of Metabolism." March 1987. Held at Sheffield Children's Hospital.

Day seminar entitled "Fatty Acid Oxidation Disorders" held at the Queen Elizabeth Hospital, Birmingham. May 1987.

The SSIEM (Society for the Study of Inborn Errors of Metabolism) Annual Symposium on lipoprotein disorders held at Sheffield University in September 1987.

Working party on "Acute Life Threatening Events and Cot Death related to Inborn Errors of Metabolism". Held at Whirlow Grange Conference Centre, Sheffield. September 1987.

Day seminar entitled "Biochemical Disorders in Children" held at St. James Hospital, Leeds in November 1988.

Attendence at a seminar on the use of enzymes and whole cells for the preparation of chiral intermediates in organic synthesis presented by Professor S. Roberts of the University of Exeter. Held by the University of Sheffield Organic Chemistry Group in March 1989.

Related Reading.

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Published Material.

Some of the work presented in this dissertation has been published elsewhere and a copy of each of the following is enclosed.

Primary hyperoxaluria and L-glyceric aciduria in the cat.
W.F. Blakemore, M.F. Heath, M.J. Bennett, C.H. Cromby and R.J. Pollitt.
Poster presentation at the 1988 conference of the Society for the Study of Inborn Errors of Metabolism (S.S.I.E.M.).

Primary hyperoxaluria and L-glyceric aciduria in the cat.
W.F. Blakemore, M.F. Heath, M.J. Bennett, C.H. Cromby and R.J. Pollitt.
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D-Glyceric acidaemia: possibly a harmless biochemical anomaly.
J.R. Bonham, R.J. Pollitt, C.H. Cromby, K.H. Carpenter, E. Worthy and T.C. Stephenson.
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J.R. Bonham, R.J. Pollitt, C.H. Marsland, K.H. Carpenter, E. Worthy and T.C. Stephenson.
Poster presentation at the Association of Clinical Biochemists 1989 meeting in Birmingham.

PRIMARY HYPEROXALURIA AND L-GLYCERIC ACIDURIA IN THE CAT

W.F. Blakemore and M.F. Heath, Department of Clinical Veterinary Medicine, University of Cambridge, M.J. Bennett, Department of Chemical Pathology, Children's Hospital, Sheffield, and C. H. Cromby and R.J. Pollitt, Neonatal Screening Laboratory, Middlewood Hospital, Sheffield.

A number of cats in a closed colony developed peripheral neuropathy and died in renal failure between 5 and 9 months of age. The neurological signs, which become fully manifest when the animals are in renal failure, are those of profound muscle weakness. Pathologically, the proximal regions of motor and sensory axons are distended with neurofilaments. Muscles also show evidence of denervation. The kidneys of all affected animals contain severe intratubular depositions of calcium oxalate crystals. Analysis of urine from control and affected animals by an enzymic assay for oxalate indicated hyperoxaluria in some affected cats. The urines were further analysed for organic acids by extraction and GC-MS. L-glycerate was found in the urines of 6 cats, all of which had peripheral neuropathy and/or renal failure, and showed hyperoxaluria at some stage. No cats without urinary L-glycerate showed clinical signs or significant hyperoxaluria. The affected animals were related, and the condition appears to be inherited as an autosomal recessive. As yet, carrier animals have not been identified biochemically. The combination of primary hyperoxaluria and L-glyceric aciduria is equivalent to a human metabolic disorder, hyperoxaluria type II. In the human disorder a deficiency of D-glycerate dehydrogenase has been identified in leukocytes. This deficiency could cause the accumulation of hydroxypyruvate, which would then be reduced to L-glycerate by lactate dehydrogenase and excreted in the urine. The cause of the increased urinary oxalate is not clear at present, but may represent another route for the catabolism of hydroxypyruvate. A feline model of hyperoxaluria type II will be useful in the understanding of the human disorder, and may have relevance to sporadic cases of oxalosis in the cat.

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Author(s) : J.R. Bonham, R.J. Pollitt, C.H. Cromby, K.H. Carpenter, E. Worthy and T.C. Stephenson

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Title : **D-glyceric aciduria: possibly a harmless biochemical anomaly**

There are very few reports of D-glyceric aciduria and most associate the condition with serious disease, particularly developmental delay and neurological problems. The degree of glyceric aciduria reported is variable. One case additionally showed hyperglycinaemia.

We have investigated a family of 5 siblings, four of whom showed D-glyceric aciduria, verified by GC-MS of *O*-acetylated l-menthyl esters. D-Glycerate was excreted in the range 3-6 mmol/mmol creatinine. There was no hyperglycinaemia. The index case, aged 5 years, showed speech delay and microcephaly. The remaining children, the oldest 14 years, are clinically completely normal. A further unrelated case showed acidosis and unusual eye-movements during the neonatal period but has since developed normally. A sib had previously died neonatally, apparently of septicaemia.

It is tempting to conclude that isolated D-glyceric aciduria is completely harmless but it remains possible that, like medium chain acyl-CoA dehydrogenase deficiency, for example, its adverse effects are expressed only in particular circumstances.

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D[+] Glyceric Acidaemia — Is It a Disease?

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D[+] glyceric acidaemia is an extremely rare condition which has been described on only three occasions. It has been associated with neonatal metabolic acidosis, convulsions and mental retardation at 2 years of age and protein intolerance in a child who died aged 1 year. In each case it was implied that the metabolic disturbance gave raise to the symptomotology.

We describe a family of 5 siblings in whom 4 excreted large amounts of D[+] glycerate verified by GCMS of o-acetylated methylesters. The index case shows speech delay and microcephaly. The remaining family members of whom the eldest is 13 years old are all entirely developmentally normal.

This unique family strongly suggests that D[+] glyceric acidaemia is a benign condition. It is an important reminder that biochemical individuality need not necessarily imply disease.

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Multivariate Screening: A Computerised Method

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Screening for clinical disorders is an important growth area for clinical chemistry laboratories. Measurement of maternal serum AFP (mAFP) is well established as a screen for neural tube defect. Recently mAFP has also been shown to be useful in screening for the risk of Down's syndrome, either alone or with greatly improved precision, when in combination with maternal serum HCG and oestriol.

The statistical method used for screening with 3 variables is not commonly known since most statistics text books do not contain the formula for multivariant normal distribution. However, only uni-and bi-variant functions can conveniently be written as expanded formulae. For distributions with more than 2 parameters there are too many variables to handle manually, and a computer capable of matrix algebra is necessary.

I present an explanation of the multivariate normal distribution and a BBC-Basic program which has general application and can perform the necessary calculations for combinations of up to 10 analytes.

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Bias Changes Induced in Two-Site Immunoradiometric Assays for LH by Using Different Monoclonal Antibodies

DENISE BULOCK, JOHN KANE and *IAN LAING
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Large bias differences have been noticed in external quality assessment schemes for Luteinising Hormone (LH). Two-site immunoradiometric (IRMA) assays employing two monoclonal antibodies show a negative bias to radioimmunoassays (RIA) or mixed monoclonal/polyclonal IRMA's. We have developed two IRMA's to LH using polyclonal anti LH as solid phase antibody and different ^{125}I labelled monoclonal antibodies to intact LH. All other reagents were the same for both assays. The two assays had very similar within and between batch precision profiles and recovered exogenous LH (IRP 68/40) quantitatively. However, large differences were noticed when analysing patient samples, with results using one monoclonal antibody up to 50% less than those with the other ($Y = 1.821X - 0.72$ $r = 0.92$). The higher results agreed well with those obtained in RIA (RIA = 0.80 × IRMA + 2.1 $r = 0.98$). The results were almost certainly due to subtle differences in the two monoclonal antibodies recognising different species of LH in plasma and the pituitary IRP 68/40 standard.

J. Inher. Metab. Dis. 11 Suppl. 2 (1988) 215–217

Short Communication

Primary Hyperoxaluria and L-Glyceric Aciduria in the Cat

W. F. BLAKEMORE[1], M. F. HEATH[1], M. J. BENNETT[2], C. H. CROMBY[3] and R. J. POLLITT[3]*

[1]*Department of Clinical Veterinary Medicine, University of Cambridge;* [2]*Department of Chemical Pathology, Sheffield Children's Hospital;* [3]*Neonatal Screening Laboratory, P.O. Box 134, Middlewood Hospital, Sheffield S6 1TP, UK*

L-Glyceric aciduria (primary hyperoxaluria type 2; McKusick 26000) is a rare inherited disorder characterized in man by recurrent calcium oxalate nephrolithiasis, chronic renal failure and early death from uraemia. To date only eight cases have been described in the literature (Williams and Smith, 1968; Chalmers *et al.*, 1984). A defect in D-glycerate dehydrogenase has been demonstrated in human leukocytes (Williams and Smith, 1968). Deficiency of this enzyme results in the accumulation of hydroxypyruvate which is then reduced to L-glycerate by lactate dehydrogenase. The cause of the increased urinary oxalate is unclear but may represent an alternative route of hydroxypyruvate metabolism.

We present here a number of cats with L-glyceric aciduria and hyperoxaluria who may represent the first animal model of this severe condition.

CLINICAL SIGNS AND PATHOLOGICAL FINDINGS IN AFFECTED CATS

The affected cats develop acute renal failure between 5–9 months of age. At this time they show signs of weakness, which progresses to profound weakness over a period of a few days, and muscle biopsy shows evidence of denervation atrophy. At post mortem examination such animals show prominent accumulation of neurofilaments in the proximal axons of ventral horn cells and dorsal root ganglion cells of the spinal cord. The kidneys are usually swollen and the renal tubules contain oxalate crystals.

Prior to the onset of renal failure it is difficult to demonstrate specific neurological deficits although affected cats are noticed to be weaker than their littermates. Muscle biopsy at this early stage has so far failed to show abnormalities of muscle. However, examination of one animal prior to the onset of renal failure has shown that the swelling of proximal axons is present in animals not in renal failure.

The condition has been seen in cats of both sexes and all affected cats are related; present evidence indicates that the mode of inheritance is an autosomal recessive.

* Corresponding Author

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BIOCHEMICAL INVESTIGATIONS

Oxalate was determined using a commercial kit (Crider and Curran, 1984). Organic acids were extracted from urine by ethyl acetate and ether solvent extractions, converted to their trimethysilyl derivatives using bis(trimethylsilyl)-trifluoroacetamide containing 1% chlorotrimethylsilane, and examined by gas chromatography–mass spectrometry. Glyceric acid was quantitated by capillary gas chromatography as the trimethylsilyl derivative against a standard of DL-glyceric acid extracted under similar conditions. The configuration of the abnormal feline glyceric acid peak was determined by capillary gas chromatography of the *O*-acetylated l-menthyl esters (Kamerling *et al.*, 1977).

Examination of the urine of affected animals indicated L-glyceric aciduria and intermittent hyperoxaluria. These features are the hallmarks of the human disorder primary hyperoxaluria type 2. Data for random cystocentesis samples from affected cats, related but unaffected animals, and unrelated controls are shown in Table 1; for comparison data are shown from human cases of primary hyperoxaluria type 2 and their relatives.

Table 1 Urine oxalate and glycerate concentrations in L-glyceric aciduria

	Oxalate	*Glycerate*
Cats:		
affected ($n=6$)	17–523	120–11 100
related ($n=11$)	2–66	0
controls ($n=6$)	1–2	0
Humans[a]:		
cases ($n=4$)	44–136	290–1350
related ($n=10$)	14–86	0

Values are mmol(mol creatinine)$^{-1}$
[a] Data of Chalmers *et al.* (1984)

DISCUSSION

Peripheral neuropathy of the type present in these cats is not a feature of primary hyperoxaluria type 2 in man. Accumulations of neurofilaments in the proximal portion of motor and sensory axons of the spinal cord are a feature of β,β'-iminodipropionitrile intoxication and of a dominantly inherited condition in the dog (canine spinal muscular atrophy in Brittany spaniels). The renal lesions are more severe in the cats than those seen in primary hyperoxaluria type 2 in man. These differences indicate either that D-glycerate dehydrogenase deficiency manifests differently in the cat or that we are dealing with a different condition. Nevertheless this may be a useful animal model in which to study the mechanism of hyperoxaluria as a consequence of L-glyceric aciduria.

ACKNOWLEDGEMENT

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