The analysis of biological fluids for the antenatal and perinatal diagnosis of metabolic disease

Christopher John Pullin
University of Wollongong

UNIVERSITY OF WOLLONGONG
COPYRIGHT WARNING

You may print or download ONE copy of this document for the purpose of your own research or study. The University does not authorise you to copy, communicate or otherwise make available electronically to any other person any copyright material contained on this site. You are reminded of the following:

This work is copyright. Apart from any use permitted under the Copyright Act 1968, no part of this work may be reproduced by any process, nor may any other exclusive right be exercised, without the permission of the author.

Copyright owners are entitled to take legal action against persons who infringe their copyright. A reproduction of material that is protected by copyright may be a copyright infringement. A court may impose penalties and award damages in relation to offences and infringements relating to copyright material. Higher penalties may apply, and higher damages may be awarded, for offences and infringements involving the conversion of material into digital or electronic form.

 Unless otherwise indicated, the views expressed in this thesis are those of the author and do not necessarily represent the views of the University of Wollongong.

Recommended Citation
THE ANALYSIS OF BIOLOGICAL FLUIDS FOR THE ANTENATAL AND PERINATAL DIAGNOSIS OF METABOLIC DISEASE

A thesis submitted in fulfilment of the requirements for the award of the degree of

DOCTOR OF PHILOSOPHY

from

THE UNIVERSITY OF WOLLONGONG

by

CHRISTOPHER JOHN PULLIN, B.Sc., M.Sc., Dip.Ed.
This Thesis is dedicated to my wife

Hendy Elizabeth

and to my children

Andrew, David and Deborah

"Διδω, εφ' ου κύριος και θεός ἡμῶν,"  
Αποκαλύφθη εἰς ἡμᾶς 4,12  
(Revelation of John 4:12)
TABLE OF CONTENTS

SYNOPSIS 3

INTRODUCTION 6

RESULTS AND DISCUSSION

PRENATAL DIAGNOSIS OF INHERITED DISEASE
BY METABOLIC PROFILING

a. Development of methods for the analysis of metabolites in amniotic fluid 60

b. Prenatal Diagnosis of methylmalonic acidemia 84

c. Prenatal Diagnosis of glutaric aciduria 97

PERINATAL DIAGNOSIS OF INHERITED DISEASE
BY METABOLIC PROFILING

a. Dicarboxylic aciduria: A suspected defect in fatty acid oxidation 110

b. Studies of a patient with propionic acidemia: The identification of 3-keto-2-methylvaleric acid and 3-hydroxy-2-methylvaleric acid 140

EXPERIMENTAL 169

APPENDIX A: Correction formula for intensity measurement 196

APPENDIX B: Elution temperatures and characteristic mass spectral ions 201

APPENDIX C: Computer sorting of National Institute of Health MS Library 209

APPENDIX D: Clinical summaries of patients studied in this work 250

BIBLIOGRAPHY 260

ACKNOWLEDGEMENTS 283

PUBLICATIONS
SYNOPSIS
Gas chromatography/mass spectrometry profiling methods for the identification and accurate quantitation of organic acid metabolites in urine, amniotic fluid and serum have been developed. These methods allow quantitation of organic acids in body fluid samples with a lower limit of detection of 0.3 μg/ml and a precision of 0.1 μg/ml, with less than 4% variation between duplicate samples. An isotope dilution method was developed to validate the profiling method for methylmalonic acid.

The methods were applied to prenatal diagnosis of genetic diseases. Three pregnancies at risk for methylmalonic acidemia were monitored. In all three the normal amniotic fluid levels of MMA allowed prediction of normal foetuses, a result confirmed by enzyme measurements on cell cultures and by perinatal studies of the infants. Results obtained during one pregnancy showed raised levels of maternal MMA excretion which are at variance with literature reports that the genotype of the foetus rather than the mother determines maternal MMA excretion during pregnancy.

In a pregnancy at risk for glutaric aciduria, amniotic fluid studies showed an increase in glutaric acid concentration of fifteen times normal at fifteen weeks gestation. As a result an affected foetus was predicted and the prediction was confirmed by enzyme studies on cultured cells. At termination the AF glutaric acid level had risen to forty times normal. These results suggest that the quantitation of relevant metabolites in amniotic fluids in appropriate cases is a reliable method of prenatal diagnosis, in those pregnancies where the foetus is at risk for an organic acidemia.

Gas chromatographic/mass spectrometric profiling and quantitation of metabolites has also been applied to the perinatal diagnosis of genetic diseases. Urines of three children who presented with an episode of a disease resembling Reye's syndrome were found to contain large quantities of the di-
carboxylic acids, adipic and suberic acid as well as the glycine conjugate of suberic acid, suberylglycine. A variety of other dicarboxylic acids, both saturated and unsaturated, were also found in the urine during the acute clinical stage. The glycine conjugate of hexanoic acid, hexanoylglycine, was also elevated when the children were sick. The excretion of these metabolites could be markedly increased by fasting the patients for periods of greater than ten hours. These results indicate that the children may have a defect in fatty acid \( \beta \)-oxidation which becomes clinically significant during periods of prolonged fasting when fatty acid oxidation becomes the major source of energy.

Two abnormal metabolites, 3-keto-2-methylvaleric acid and 3-hydroxy-2-methylvaleric acid, have been identified and quantitated in the urine of a child with propionic acidemia. These metabolites may have been produced as a result of self-condensation of propionyl-CoA. Data is presented to show that the unusual ketone 3-pentanone, which has been observed previously in the urine of patients with propionic acidemia, is produced as a result of the decarboxylation of 3-keto-2-methylvaleric acid. The identification of these and other characteristic metabolites enabled the post-mortem diagnosis of propionic acidemia in a child who had died following a disease characterised by acidotic episodes.
INTRODUCTION
Individual variation, health and disease are controlled in large part by genetic mechanisms. This touchstone of modern human biology and medicine has its roots in the medical studies of the eighteenth and nineteenth centuries but has assumed its present position of eminence only during the last thirty years. From studies of molecular biology and of causes and courses of disease have begun to emerge a scientific basis of diagnosis and treatment.

The study of human genetics has been responsible for and dependent on three related subdisciplines:

(a) Population genetics in which studies are made of the frequency, distribution and effects of specific genetic defects.

(b) Cytogenetics which studies the location and organisation of genes on the forty six human chromosomes.

(c) Biochemical genetics which studies the chemical identity of the gene and its products, the mechanisms of control of cellular metabolism by genes and the disturbances of cellular metabolism caused by lack of expression of genes.

The term "inborn error of metabolism", introduced by Garrod at the turn of the century applies to the group of diseases studied by biochemical genetics in which we can define the inborn errors as genetically determined biochemical disorders due to specific congenital defects in the structure or function of protein molecules. Many of the inborn errors have clinical and pathological consequences which are a direct result of the specific biochemical disorder. However the importance of these conditions go far beyond their clinical impact. The treatment of an individual patient must always remain the prime concern of medicine but studies of patients with specific inborn metabolic errors have been instrumental in elucidating normal biochemical pathways, in establishing the structure and function of various species of macromolecules and in illuminating genetic concepts and mechanisms. Thus the definition of the enzymic defect in phenylketonuria, albinism and alkaptonuria provided important information about the pathway of aromatic amino acid metabolism. Similarly studies of haemoglobin variants provided insight into the structures and functions of the
normal haemoglobin molecule and much of our current thinking on carbohydrate metabolism is based on studies of galactosemia, the glycogenoses and pentosuria.

GOALS AND LIMITATIONS OF STUDY OF GENETIC DISEASES

Investigations of inborn errors of metabolism have two independent goals. The first is to define the diagnostic, therapeutic and prognostic implications of the disease for the affected subject and for others suffering from a similar defect. The second is to investigate the biochemical and genetic events which are responsible for the clinical disturbance and to relate these to the normal patterns of metabolism. The closer we come to this second goal the more rational we become about the first. Thus the identification that the enzymic defect in isovaleric acidemia is on the path of leucine catabolism, gives rise to the premise that the isovaleric acid is solely (or at least predominantly) derived from dietary leucine and restriction of this amino acid will decrease isovaleric acid accumulation. Treatment of the patient with low leucine diets lead to normal physical and mental development. Similarly the identification of the enzymatic defect in patients with galactosemia provided the chemical basis for the accumulation of galactose-1-phosphate in body tissue. This fact provided not only the rationale for dietary treatment of the condition which prevents and reverses cerebral symptoms but also has provided a method of detection of carriers of the mutant gene.

This situation does not exist for all genetic diseases. In some diseases such as sickle cell anaemia we have a reasonable amount of information about the biochemical defect but this gives little basis for treatment. Conversely for some diseases such as organic acidurias we have effective dietary therapy but have little information on the structural defects in the enzymes involved. In Tay-Sachs disease we know that certain glycolipids accumulate in cells owing to a specific catabolic enzyme deficiency but we have no effective means of reversing the condition. Finally for many diseases such as Huntington's Chorea, family studies and morphologic changes suggest a specific inborn metabolic disturbance but we have no "biochemical handle" with which to
advances which one may expect in the future. They also suggest the need to think of inborn errors of metabolism as signposts on the road to an understanding of the ways in which genetic variation and subsequent protein structure influence our individual variation and normal physiology. Thus the study of genetic diseases is of major importance in understanding the biochemical reactions in health and disease and not a study of rare defects which only affect a few individuals.

**GENETIC MUTATION AND MACROMOLECULES**

Genetic diseases are caused by a variation in the nucleotide sequence of the chromosomes in the nucleus of the cell. This variation, known as a mutation, may be expressed as a change in the structure or function of macromolecules such as enzymes in a cell. Such mutations may be expressed as a clinical or chemical disturbance, or they may remain unknown either because they cause no significant functional disturbance or because they are incompatible with life. Such genetically controlled structural variation is found for all proteins studied and may be responsible for the variant forms of a given inborn error which exhibits biochemical and genetic heterogeneity e.g. phenylketonuria and cystinuria.

**FUNCTIONAL DERANGEMENT DUE TO GENETIC VARIATION**

Although Garrod stressed enzymatic defects which produce a block in an anabolic or catabolic pathway, inborn errors have been described for all types of protein. Mutations have been described affecting function of cell membranes in the intestine, kidney and other organs which lead to diminuation in the transport of sugars, amino acids, phosphates, vitamins, cations and water.

These mutations may reflect the malfunction, absence or alteration of specific membrane carrier protein or an abnormality in the membrane receptor sites for hormones or other mediators. Other mutations involve serum proteins such as albumin, lipoprotein, immunoglobulins or transferrins and may have important clinical consequences or none at all.
or absence of an intracellular enzyme function. The functional consequences of this loss of enzyme activity are of greatest interest and concern. The loss of functional activity may be produced by complete cessation of enzyme synthesis or by production of a mutant protein which has lost the ability to carry out its usual function. It is often difficult to distinguish between these two possibilities but immunological and enzymatic studies may allow the distinction between lack of synthesis and production of an antigenically similar but functionless protein to be made. Such studies have been carried out for a small number of inborn errors of metabolism such as galactosemia, Lesch-Nyhan syndrome and muscle phosphorylase deficiency but many diseases remain to be studied in this fashion.

Other disorders are characterised by only a partial loss of enzymic activity, which may be expressed in several ways. Firstly the mutant enzyme may have a markedly reduced affinity for its substrate. This defect has been demonstrated in citrullinemia and some defects of glucose-6-phosphate dehydrogenase activity. Secondly the mutant enzyme may have increased thermo-lability which may explain the reduced activity. Similarly instability or increased rate of destruction of a mutant enzyme may give lowered activity. In some cases the mutant enzyme binds essential cofactors abnormally. Thus in cystathioninuria patients the cystathioninase activity of an in vitro liver homogenate is markedly increased when pyridoxyl phosphate is added to the incubation medium and nearly normal activity can be restored by the presence of large amounts of this co-factor. This alteration of activity is paralleled in vivo by a reduction in cystathionine excretion in these patients when treated with large amounts of pyridoxine. Similarly in one variant of methylmalonic acidemia, Baumgartner, Bachmann and Wick have demonstrated that activity of methylmalonyl-CoA mutase enzyme can be restored to normal activity in vitro and in vivo by the addition of large amounts of 5'-deoxyadenosylcobalamin which is the specific co-factor for the mutase apoenzyme. Other vitamin dependent inborn errors have been described for several vitamins including vitamin B12. Studies of patients with B12 responsive methylmalonic aciduria have shown that the defect may be in the pathway of B12 metabolism which produces the active coenzyme as well as
A, B, C, D Substrate and products of major reaction pathway.

F, G Products of minor reaction pathway.

T_A Transport system for A

E_{AB}, E_{BC}, E_{CD}, E_{AF}, E_{FG} Enzymes catalysing conversion of A to B,
B to C, C to D, A to F and F to G.

Cell Membrane

FIGURE 1. Schematic representation of biochemical sequence in a cell.
Two other mechanisms of reduced enzymatic activity are of importance. Mutations could affect polypeptide sub-units in such a way that when these are assembled into functional enzymes the activity is impaired. A parallel situation exists where the globin chain of haemoglobin M interferes with the redox state of iron in the haemoglobin complex causing methaemoglobinemia. Such a defect has not yet been described for an enzymic or membrane protein. Finally we must consider the case in which the same reaction is catalysed by more than one enzyme in the one cell. Total deficiency of one enzyme could lead to partial reduction of net activity. This situation has been described in Fabry's disease and for Tay-Sachs disease and may be much more common than previously recognised as an explanation of genetic heterogeneity.

Although the known inborn errors of metabolism are usually caused by a decrease or loss of activity the converse may also apply. In acute intermittent porphyria hepatic aminolevulinic acid synthetase activity is increased several fold and increased enzyme activity is characteristic of several other diseases.

CONSEQUENCES OF ENZYME DEFECTS

The intracellular effect of a genetic alteration of a protein will depend on factors such as its function and the severity of the defect. However it is possible to make generalisations of the consequences of the enzyme defects. If we consider the sequence of reactions in Fig. I we can define five possible consequences.

(a) **Precursor Deficiency**

If the specific transport system $T_A$ for a substance $A$ across a membrane is defective the intracellular concentration of $A$ may be too low to saturate the enzyme $E_{AB}$. This can then slow the entire sequence and lead to a deficiency of all products $B$, $C$ and $D$. This mechanism is evident in Hartnup disease where tryptophan transport is defective and leads to an intracellular deficiency of nicotinamide. It is also evident in pernicious anaemia where transport of vitamin $B_12$ across the intestinal wall is defective, leading to a
deficiency of the cofactor for methylmalonyl-CoA mutase.

(b) Precursor Accumulation
If the enzyme $E_{CD}$ is defective then the precursors A, B, C may accumulate in the cell. This has been shown for methylmalonic aciduria where a defect in the methylmalonyl-CoA racemase or mutase causes the intracellular accumulation of methylmalonyl-CoA and the excretion of large amounts of methylmalonic acid.

(c) Alternative Pathway Utilisation.
If the conversion of substance A to substance B is impaired by a defect in the enzyme $E_{AB}$ an accessory pathway to F and G may become prominent. Thus in phenylketonuria the lack of phenylalanine hydroxylase activity leads to overproduction and excretion of phenylpyruvic, phenyllactic and phenylacetic acids.

(d) Product Deficit.
If product D is the physiologically active product of the reaction sequence, a block in any of the steps leading to its formation may result in inadequate production of D. This is illustrated in vitamin B$_{12}$ responsive methylmalonic acidemia where a defect in B$_{12}$ metabolism causes a deficit of co-factor for the methylmalonyl-CoA mutase enzyme and thus accumulation of methylmalonic acid. Similarly in albinism melanin is not formed because of lack of tyrosinase activity.

(e) Lack of Feedback Control
The end product of the reaction sequence D may regulate the activity of the first enzyme in the pathway $E_{AB}$. This control regulates the flux of material through the pathway. This process has been extensively studied in microbial systems and is of importance in man. Thus in the Lesch-Nyhan syndrome the enzyme defect in hypoxanthine-guanine phosphoribosyl transferase results in the failure to regulate synthesis
of uric acid and leads to marked over production of the end product.

**CLINICAL MANIFESTATION OF ENZYME DEFECTS**

The clinical expression of enzyme defects may vary tremendously. Some defects such as pentosuria, iminoglycinuria and β-aminoisobutyric aciduria are harmless; others such as branched chain ketoaciduria propionic aciduria and phenylketonuria lead to severe metabolic disturbances early in infancy which may result in early death or profound mental and physical handicaps. Although the mutations responsible for the inborn errors are already in the gamete at conception they may not be expressed until many years after birth. For example acute intermittent porphyria rarely manifests autonomic nervous system involvement before puberty and Huntington's Chorea rarely shows clinical symptoms before the age of forty.

Sex may also play a large role in clinical expression of disease. Some inborn errors are sex linked (generally only expressed in the male) and may be inherited as dominant or recessive traits. In other diseases the role of sex is more subtle e.g. the unusual occurrence of gouty arthritis in the pre-menopausal female.

Organ involvement may relate directly to the tissue in which the defect is expressed, but in many disorders this is not true. In cystinuria the defective resorption of cystine leads to the formation of cystine calculi and in hepatic glycogenoses the enzymatic liver defects are responsible for the accumulation of liver glycogen. In phenylketonuria and acute intermittent porphyria however specific enzymatic liver defects lead to manifestation in the central or autonomic nervous system, possibly due to the accumulation in the circulation of toxic metabolites.

**DIAGNOSIS OF METABOLIC DEFECTS**

The earliest recognition of the concept of inborn errors of metabolism by Garrod grew out of his interest in and studies of alkaptonuria, albinism, cystinuria and pentosuria. He noted that in alkaptonuria, patients excreted large amounts of homogentisic acid in their urine almost from birth and this excretion could be increased by administration of protein or the amino acids phenylalanine and tyrosine.
HEREDITARY AMINOACIDOPATHIES WITH DETECTABLE EXCESS OF SOLUBLE METABOLITES (Cumulative Number)

FIGURE 2. Growth of knowledge about inborn errors of amino acid metabolism.

The open circles indicate findings made in the pre-partition chromatography era; filled circles reflect findings derived from the use of partition chromatography on filter paper and the ninhydrin stain to locate amino acids in biological fluids; the squares represent at least a dozen diseases identified through the application of GC-MS methods to biological fluids.
These observations, together with his studies of the families of the affected patients lead to his postulation that alkaptonuria resulted from the deficiency of a normal enzyme responsible for the catabolism of homogentisic acid, such deficiency being inherited as a recessive trait. The identification of the homogentisic acid depended on the qualitative chemical tests available and on the preparation of derivatives which could be characterised by physical or chemical tests.

This pattern of identification of accumulated or missing metabolites is fundamental to the diagnosis of inborn errors of metabolism. For the first half of this century the identification of specific metabolites depended exclusively on either a few qualitative chemical tests or on the preparation of derivatives which could be characterised by physico-chemical means. Included in these tests were the ferric chloride test for the detection of phenols, the cyanide-nitroprusside reagent for the detection of disulphides and the use of phenylhydrazine for the detection of keto acids. The limits of detection and the limited range of tests accounted for the few known inborn errors of metabolism.

In 1946 Dent published his papers on partition chromatography of amino acids in urine and serum. This technique, coupled with the sensitive and specific ninhydrin stain for amino acids was then applied to the investigation of human disease. Two decades later, the exponential growth of knowledge fostered by this technology had given birth to some fifty "ninhydrin positive" characterised diseases. (See Fig. 2, after Scriver and Rosenberg). The sensitivity and effectiveness of Dent's techniques compared to the other techniques in use at that time however produced the false impression that most errors of metabolism were due to aberrant metabolism of amino acids. By 1970 the rate of increase of new discoveries had begun to decline because most ninhydrin positive compounds which accumulate in genetic diseases had been discovered.

The first paper on gas chromatography appeared in the literature in 1952 and the method was quickly put to use in the petroleum industry where it mushroomed into a major analytical tool in the 1950's. As the technique developed and commercial equipment became available the technique was soon applied in other fields such as biology and chemistry.
TABLE 1
SOME ENZYME DEFECTS LEADING TO ORGANIC ACIDURIAS

<table>
<thead>
<tr>
<th>Defective Pathway</th>
<th>Major Organic Acids</th>
<th>Metabolic Pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Methylacetoacetyl CoA-β-ketothiolase</td>
<td>α-Methylacetoacetic</td>
<td>Isoeucine catabolism</td>
</tr>
<tr>
<td>Isovaleryl CoA dehydrogenase</td>
<td>α-Methyl-β-hydroxylutyrlic</td>
<td>Leucine catabolism</td>
</tr>
<tr>
<td>β-Methylcrotonyl CoA carboxylase</td>
<td>Isovaleric</td>
<td></td>
</tr>
<tr>
<td>3-hydroxy-3-methylglutaryl CoA lyase</td>
<td>β-methylcrotonic</td>
<td></td>
</tr>
<tr>
<td>Branched chain amino acid decarboxylase</td>
<td>3-hydroxy-3-methylglutaric</td>
<td></td>
</tr>
<tr>
<td>Glutaryl-CoA dehydrogenase</td>
<td>α-ketoisocaproic</td>
<td>Leucine, isoleucine and valine catabolism</td>
</tr>
<tr>
<td>Not known</td>
<td>α-keto-β-methyl valeric</td>
<td></td>
</tr>
<tr>
<td>α-Ketoacidipic dehydrogenase</td>
<td>α-keto-isovaleric</td>
<td></td>
</tr>
<tr>
<td>Glutathione synthetase</td>
<td>Glutaric</td>
<td>Lysine catabolism</td>
</tr>
<tr>
<td>Propionyl CoA carboxylase</td>
<td>Glutaric and other acids</td>
<td>&quot;Acyl-CoA&quot; metabolism</td>
</tr>
<tr>
<td>Methylmalonyl racemase</td>
<td>α-ketoacidipic</td>
<td>Lysine and tryptophan catabolism</td>
</tr>
<tr>
<td>Methylmalonyl CoA mutase</td>
<td>Pyroglutamic</td>
<td>Glutathione</td>
</tr>
<tr>
<td>Terminal enzyme for Adenosyl B12 synthesis</td>
<td>Propionic</td>
<td>Propionate catabolism</td>
</tr>
<tr>
<td>Proximal enzyme for Adenosyl B12 synthesis</td>
<td>Methylmalonic</td>
<td>Methylylmalonate catabolism</td>
</tr>
<tr>
<td>Block in Methyl B12 and Adenosyl B12 synthesis</td>
<td>Methylmalonic</td>
<td>Methylylmalonate catabolism</td>
</tr>
<tr>
<td>Pyruvate decarboxylase</td>
<td>Methylmalonic</td>
<td>Methylylmalonate catabolism</td>
</tr>
<tr>
<td>Pyruvate dehydrogenase</td>
<td>Pyruvic</td>
<td>Methylylmalonate catabolism</td>
</tr>
<tr>
<td>Succinyl-CoA: 3-ketoacid-CoA transferase</td>
<td>Pyruvic</td>
<td>Methylylmalonate catabolism</td>
</tr>
<tr>
<td></td>
<td>Succinic</td>
<td>&quot;Ketone Body metabolism&quot;</td>
</tr>
</tbody>
</table>
The discovery of the basic principles and the initial development of the mass spectrometer were largely due to physicists and in 1913 J.J. Thompson carried out his classic studies on the isotopes of the elements. By the 1950's this area of instrumentation had advanced to the stage where a commercial system became readily available. During the early 1960's the gas chromatograph and mass spectrometer were coupled to give a very powerful analytical tool which was capable of separating and uniquely identifying a wide range of organic compounds. The application of this technique to the diagnosis and study of inborn errors of metabolism allowed the examination of non-ninhydrin positive compounds with a sensitivity comparable to that of the ninhydrin technique for amino acids. This has lead to an increase in the pace of discovery of new inborn errors.

**ORGANIC ACIDEMIAS**

Several inborn errors of amino acids or carbohydrate metabolism have now been described in which the major metabolites accumulating in the blood and urine are organic acids or organic acid derivatives (see Table 1). All have symptoms of central nervous system involvement and if untreated lead to severe mental retardation or death. For most of these a biochemical aetiology has been established by enzyme studies, although an understanding of the exact structural defects is often incomplete. Similarly our knowledge of the complete clinical spectrum or range of metabolic derangement is limited at present but as further studies are made and results of treatment assessed our understanding of the group of diseases will be widened. The mode of inheritance of most of these diseases seems to be by autosomal recessive genes.

This group of diseases have presented many problems in diagnosis. Acidoses and central nervous system dysfunction are characteristic of organic acidemias. Since most severe cases present in the neonatal period, central nervous system dysfunction may be difficult to assess owing to the limited range of responses available to the neonate. In addition some of the symptoms are also characteristic of such common neonatal conditions as anoxia during birth, cardiorespiratory disease,
renal disease or infection.

Three common presentations have been observed:

1. A previously well newborn or young infant becomes rapidly ill, usually with persistent vomiting. There may be a rapid progression to coma, apneic spells and death. In milder situations clearing of symptoms is likely but further attacks may be triggered by protein feeding or mild infection.

2. An infant or child fails to achieve normal growth and development potential. These children often have a history of periods of lethargy, failure to feed, irritability and mild vomiting and may have neurological defects.

3. An infant may present with acute neonatal central nervous system signs such as local or generalised seizures, staring spells or myoclonus. Their electroencephalographic patterns may include a variety of abnormalities.

Biochemical studies show a metabolic acidosis, often with an accompanying lactic acidosis. Ketosis may or may not be present. Hyperglycinemia, hyperammonemia and hypoglycemia are other laboratory findings frequently present. Plasma and urine amino acids are usually normal or manifest non specific elevations.

Treatment rationale is similar in all these diseases and is based on restriction of the substrate to the affected enzyme. Initial treatment begins by stopping the accumulation of the toxic metabolites by removing the precursors, control of the acidosis and fluid balance, and provision of an energy source such as glucose until the accumulated toxins are excreted. Attempts are then made to enhance the enzyme activity by supplying large amounts of cofactors.

Successful treatment of a large number of cases has shown that early diagnosis of infants with organic acidurias is essential if permanent neurological damage is to be prevented. Long term management emphasizes the limitation of toxic metabolites by the use of low protein or artificial diets and this usually controls the acute attacks. Since amino
acids are needed for growth and metabolism a careful balance must be maintained between toxic accumulation of metabolites and an adequate supply of amino acids. Thus accurate analytical methods are required for quantitation of acid excretion and other biochemical parameters to ensure the balance between restricting intake and adequate supply of amino acids for growth and development.

**DIAGNOSIS OF ORGANIC ACIDEMIAS**

The diagnosis of organic acidemias and their genetic aetiology depends on the definition of the enzyme defects responsible for the accumulation of organic acids. However, the identification of the acids, together with a knowledge of normal metabolic pathways, in most cases allows one to postulate the location of the defect. The diagnostic problem then becomes the identification and quantitation of the organic acid accumulating in the cells. The ultimate proof of the diagnosis however relies on enzyme studies in isolated tissue or cultures. The buildup of acids within the tissues usually leads to an accumulation of the metabolites in blood and then in the urine. Thus the diagnostic problem becomes the identification of the organic acids or their derivatives in serum or urine.

The identification of the acids of intermediary metabolism however presents considerable analytical difficulties since the physico-chemical differences between them are often small. Consequently the analytical techniques used have been based on chromatography since small quantities of similar or isomeric compounds can be separated. Techniques which have been applied successfully include electrophoresis, paper, thin layer, liquid partition and gas chromatography. Whilst each method has certain advantages the final choice often depends on the interests of the investigator, the technical capabilities of the laboratory and the particular compounds being investigated.

Since the diagnosis of metabolic errors might require the identification of many classes of organic acids such as ketoacids, hydroxyacids, mono and poly-carboxylic acids and glycine conjugates the analytical method of choice must be able to detect all these classes of compounds. The most useful techniques which have been found to be suitable are liquid partition chromatography, gas chromatography (GC) and gas
chromatography-mass spectrometry (GC-MS). Most workers have favoured the use of GC-MS for urinary organic acid analysis. However GC-MS has one major limitation: it can only handle volatile constituents or compounds which can be readily converted into volatile derivatives. It has been estimated that because of this limitation, analytical methods based on GC detect only about 20% of the total number of substances present in a complex biological mixture.\(^48,49\)

Techniques for examination of highly polar molecules, thermally unstable and high molecular weight compounds have provided the impetus for the development of alternative techniques to complement GC-MS. The development and application of high pressure liquid chromatography (HPLC) in clinical chemistry\(^50,51\) and the recent development of micro HPLC-MS\(^52\) interfaces to the analysis of organic acids may well lead to the detection of a new group of inborn errors of metabolism.

At present it is clear that GC and GC-MS are the major techniques of multicomponent analyses of body fluids in use today and in this thesis only the application of GC-MS for organic acid profiling will be considered. A number of excellent reviews of this area have recently appeared.\(^53-58\)

The use of high resolution MS has not become widespread in biochemical profiling but it may be used to advantage if it is available.\(^59\) Advances in column technology\(^57,60-62\) and the more common use of high resolution capillary columns\(^63-66\) together with the wider use of alternative modes of MS ionisation\(^67-69\) (especially chemical ionisation\(^70-72\) (CI)) have increased both the sensitivity and the number of components which can be identified in an analysis of a body fluid. The application of computer technology\(^73-75\) to GC-MS has enabled not only the collection, storage, manipulation and display of large amounts of data from a single experiment but also the concurrent computer control of the GC-MS system, the ability to identify a component by comparison of its low resolution mass spectrum to library spectra (either during or after the data collection) and the measurement of relative concentration of each component.\(^76-78\)
FIGURE 3. CI and EI mass spectra of the TMS derivative of MMA and $^2\text{H}_3$-MMA.

Note the minor fragmentation of the molecular ion in CI-MS and the low intensities of the ions at m/c 250 and 221 which retain the label in the EI-MS.
METHODS OF QUANTITATION OF METABOLITES

The methods of quantitation of metabolites by GC have been known for many years but the use of GC-MS computer systems has allowed new techniques to be developed. The simplest of these techniques is to use the total ion current plot of the MS alone or in conjunction with an FID trace produced by sample splitting.

The commercial availability of compounds labelled with stable isotopes of hydrogen, carbon, nitrogen, oxygen, sulphur etc. has increased the use of such compounds as internal standards, with the increasing demand causing a fall in prices. These standards are ideal for quantitative MS investigations, since they are clearly distinguishable by MS from the unlabelled compound but are chemically practically identical with it. The use of stable isotopic labelled internal standards also enhances the extraction efficiency as the standard effectively increases the total concentration of material in the biological fluid. Compounds containing \(^{13}\text{C}\) are the preferred standards as there are no detectable isotope effects in the GC separations, absorption losses, extraction losses, rates of derivative formation or production of ions in the MS source. The use of deuterium labelled compounds usually offers a cheaper and more easily synthesised alternative but deuterium labelled compounds may show alterations in GC retention and the label may also be chemically exchangeable during the analysis. The use of such standards should be checked using calibration curves.

A major increase in sensitivity may be achieved by the use of chemical ionisation MS (CI-MS). The appropriate choice of reagent gas (usually isobutane or methane) and source conditions leads to the production of very few product ions, the major one being the protonated molecular ion \((\text{MH}^+)\). This ion retains any isotopic label which may be present which is not necessarily true for the fragment ions in the EI spectrum (see Fig. 3). The increased sensitivity of negative ion CI-MS will give further advances in this area.

The choice of derivatives for quantitative work is governed by absorption behaviour and stability but these may be modified for MS considerations. Thus trimethylsilyl (TMS) derivatives of acids are easily
a. Addition of internal standard.
b. Hydrolysis of conjugates of acids.
c. Chemical modification of acids e.g. formation of keto acid oximes.
d. Extraction of basic and neutral compounds.
e. Acidification and extraction of organic acids.
f. Further purification.
g. Drying of extracts
h. Concentration of extracts
i. Derivatisation
j. Separation of components (G-C)
k. Analysis and identification of components of interest


Note that not all steps may be used in a particular analysis.
hydrolysed but are very helpful for identification and quantitation as they usually give intense characteristic M-15 ions in the mass spectrum.

The general method of workup of samples (see Fig. 4) includes the addition of internal standards, possible hydrolysis of conjugates, extraction and concentration of the extract. Frequently a number of purification, separation and derivatisation steps are needed before measurement by the MS and consequently an internal standard method is the method of choice. A suitably labelled internal standard may not be available and an analog or homolog may then be chosen. The assumption made in this method is that the ratio of internal standard to analyte is not changed during any chemical or physical operations and thus the yields of each sample treatment or extraction step need not be determined. A single measurement of the concentration ratio of the analyte to the internal standard is all that is necessary. The usefulness of the method can be checked by investigation of calibration mixtures. In some instances the use of labelled internal standards allows procedures to be simplified. For example it may be adequate to carry out only one solvent extraction of a biological fluid since further extractions may increase the amount of material and of interfering substances but the ratio of labelled to unlabelled material will not change. Purification of extracts may however be needed since some impurities may contribute to the MS measurements.

Three major methods of MS measurement have emerged. First the repetitive scanning of a preselected mass range during the gas chromatographic run gives the optimum in the versatility of mass spectral information collection. This method allows the processing of data to generate individual ion current profiles referred to as Mass Chromatograms which can be used for comparison of unknown and internal standard. This technique is statistically inefficient for the quantitation of ion intensity since much of the time is spent measuring masses of no particular value to the analysis as well as the time spent in regions of the scan between masses. The method does however accommodate complex samples, as all eluting peaks will have representative mass spectra recorded, and the presence of a compound need not be known prior to its elution from the GC. Improvements to the accuracy and sensitivity
may be obtained by limiting the range scanned, high speed scanning, threshold sensitivities and mathematical treatment of data.

The technique of detection of a limited number of ions (multiple ion selection - MIS) has been found to extend the sensitivity and the accuracy of analyses. The focus of the mass spectrometer must be alternated between two or more $m/e$ values in a time sequence. This is easily accomplished in a quadrupole instrument by altering the DC voltages. In a magnetic instrument the magnetic field cannot respond fast enough and the accelerating voltage is usually switched.

The best sensitivity is obtained when the whole time is spent monitoring a single ion (SIM). Instrumentally this is usually implemented by the use of closed loop feed back control to ensure the instrument is always on the top of the peak. An extension of this technique is the provision of two detectors to allow for instrumental variations. This specialised hardware requirement is not usually available with GC-MS but is used for specific applications requiring the highest accuracy. The major disadvantages of MIS and SIM include the limited number of compounds that can be analysed per GC experiment, the difficulties in verifying peak identity (especially at low levels), the need for appropriate labelled internal standards, the inability to demonstrate the presence of unexpected compounds and the instrumental difficulties associated with the implementation of such an analysis especially on magnetic instruments.

**DIRECT CI-MS**

Most results available at this time have been obtained by GC or GC-MS studies but a recent report by Issacher and Yinon has taken advantage of the lower fragmentation obtainable with CI-MS. They have examined crude urine extracts using solid probe CI-MS and have identified the acids present by their protonated molecular ions. Patterns are obtained at different probe temperatures to examine both low and high volatility carboxylic acids. The method does not give quantitative results but diagnostic patterns were obtained with some gross metabolic defects. This technique may possibly be developed into a screening procedure for gross abnormalities but needs much more work to enable the identification of more subtle changes in excretion patterns.
FIGURE 5. Early demonstration of biochemical individuality by metabolic profiling (after Williams)

The metabolic patterns of 4 subjects were obtained by measurement of the taste threshold (Numbers 1-17) and urinary metabolites (Numbers 18-31). The lengths of the lines are indicative of the amounts of compounds measured.
Following the development of GC methods for amino acids Summons et al and others developed methods for analysis of amino acids which eliminated the need for complete chromatographic resolution of mixture by monitoring the characteristic ions in the mass spectrum of the GC column effluent. Halpern and others have developed this idea using a combination of specific extractions of classes of compounds, labelled internal standards, derivatisation, differences in volatility and the increased intensity of the protonated molecular ion in CI-MS to enable quantitative measurements of metabolites without prior chromatographic separations. This method has been shown to be applicable to a variety of classes of compounds including amino acids, fatty acids, steroids, drugs and free and bound cholesterol. The methods can be used to analyse mixtures in a fast reproducible manner with minimum pretreatment. Good agreement with other methods is obtained and the method is applicable to microsamples collected on filter paper. However analyses using these techniques must be checked for the possibilities of interferences in MS and for specificity and sensitivity.

METABOLIC PROFILING

The concept that individuals might have a "metabolic pattern" that would be reflected in the constituents of their biological fluids was first developed and tested by Williams et al in the late 1940's and early 1950's.

Utilising data from 200,000 paper chromatograms he was able to show that the excretion patterns for a variety of substances varied greatly from individual to individual, but that these patterns were relatively constant for a given individual. (see Fig. 5 after Gates and Sweeley). Williams then studied samples from a variety of groups including alcoholics, schizophrenics and mental hospital patients and produced suggestive evidence for characteristic metabolic patterns associated with each group. This work was not repeated by others due to the technical complexity of the programme and the ideas of the utility of this approach lay dormant until the late 1960's when advances in gas and liquid chromatography enabled similar studies to be carried out.
The term "metabolic profile" was introduced by the Hörnings in 1971 who originally defined it to mean "multicomponent GC analyses that define or describe metabolic patterns for a group of metabolically or analytically related metabolites". This definition has been modified and broadened so that quantitative information is often included as well as results obtained by techniques other than GC.

Initially the metabolic profiles obtained were qualitative in nature i.e. the identity of the metabolites was the major consideration, but in the last few years the emphasis has been on the development of quantitative methods for the analysis of components of biological fluids.

TREATMENT OF SAMPLES PRIOR TO SEPARATION AND IDENTIFICATION

A. Collection, Storage and Transport.

Most clinical laboratories do not have direct access to GC-MS-computer systems and thus the initial organic acid screening of samples is usually carried out by GC alone. In the case of a positive screening result the samples may then be transported to a specialised centre for further investigations. Collection, transport and storage of specimens is rarely under the control of the specialised laboratory and changes may occur in specimens before receipt by the specialised laboratory. Fasting blood and the first morning urine specimens are the preferred samples and these should be stored at low temperatures to prevent decomposition of metabolites. Information about dietary habits and drug intakes by the patient is of value and help eliminate false positive results. Further samples may be requested or studies of twenty-four hour excretion of metabolites may be required. In some cases follow up studies on other samples from the same patient may be required to confirm the presence of an unusual metabolite.

B. Isolation of Organic Acids

Three principal methods for the isolation of acidic constituents of urine prior to derivatisation and GC are currently in use. These methods and their relevant merits have been reviewed and their efficiencies have been compared. The first is based on solvent
extraction using a variety of solvents, such as ether and ethyl acetate. The extraction efficiencies may be increased by saturating the urine with sodium chloride. A recent variation of this technique uses the absorption of the urine onto a solid phase cellulose resin and elution of the hydrophobic acids and neutrals with organic solvents. This variation yields better results in terms of recovery and precision. Fitch et al have shown that small aliphatic molecules and aromatic acids are satisfactorily quantitated using solvent extraction. Whilst tricarboxylic acids are also isolated the reproducibility and their recoveries are poor.

The second method is based on ion exchange chromatography. This was first suggested by Horning and intensively studied by Chalmers and Watts. There have been conflicting reports of the merits of this approach, and problems have been encountered with the high concentration of sulphate and phosphate which are present in urine. This interference may be overcome by the use of a barium hydroxide preprecipitation step but this may lead to the possibility of co-precipitation or even decomposition of some organic acids. DEAE-Sephadex is the most widely used ion exchange resin but others such as Dowex-3 have also been used. Following absorption of the acids on the resin and washing to separate neutral molecules the acids are eluted with aqueous pyridinium acetate and the solution lyophilised. To counter losses of the more volatile acids during the lyophilisation step some workers use an elution with hydrochloric acid followed by neutralisation of the eluate with sodium bicarbonate. The ion exchange method gives reproducible results with the majority of organic acids, and is the only technique available that isolates polyhydroxy acids such as glyceric acid but poor recoveries are obtained for the tricarboxylic acids which are co-precipitated by the initial barium hydroxide treatment. Further complications are caused by the partial lactonisation of aldonic acids. The third method is limited to the isolation of more volatile acids and makes use of either steam distillation or vacuum distillation.

The choice of which extraction method is to be used will be based on the requirements of the analysis. The ion exchange methods are more laborious and time consuming but are the best for studies of very polar or highly water soluble molecules. In most metabolic diseases
however the deviations from normality are usually so large that the use of ion exchange methods are not justified as the use of solvent extraction methods together with recovery studies can give accurate fast results both for qualitative and quantitative studies.

The use of other chromatographic pre-treatments for class separation of compounds such as alumina or charcoal absorption, XAD-2 hydrophobic polystyrene resin, or silica gel which have been of great use in drug, catecholamine, oestrogen and lipid analysis have not been particularly useful in the isolation of organic acids and have not been used.

Urine is the only body fluid which is normally devoid of protein. All other fluids contain small (amniotic fluid, spinal fluid) or large amounts of protein (serum, seminal fluid, synovial fluid). It has been suggested that spinal fluid and amniotic fluid can be treated in the same way as urine but no recovery studies have been reported to test this assumption. Other physiological fluids have to be deproteinised to avoid interference by protein. If non destructive methods such as gel filtration, dialysis or membrane filtration are used, some compounds which are tightly bound to protein and water insoluble low molecular weight substances such as long chain fatty acids may be lost. This is not a problem with other classes of metabolites such as carbohydrates and amino acids. Protein precipitation agents such as sulphosalicylic acid followed by extraction or ion exchange have been recommended, but it is known that some acids may be co-precipitated with the protein. Direct extraction of serum with ion-solvent pairs such as ammonium carbonate-ethyl acetate have been widely used for drug extractions but this method does not extract organic acids.

Mamer has suggested that precipitation of protein using ethanol after which the ethanol is removed in vacuo and the extract is treated in the same way as urine. Other authors have reported success using variations of this method and acetone and methanol have also been used. Recovery studies have not been reported for these methods.

A number of the above techniques have been adapted to the measurement of methylmalonic acid in serum and amniotic fluid. For measurement of this metabolite Wang et al and other groups have ignored
TABLE 2

PRODUCTS FORMED BY SILYLATION OF FUNCTIONAL GROUPS

<table>
<thead>
<tr>
<th>REACTANT GROUP</th>
<th>INTERMEDIATE PRODUCT</th>
<th>REACTION PRODUCT</th>
</tr>
</thead>
<tbody>
<tr>
<td>$R_1$CH-OH</td>
<td></td>
<td>$R_1$CH-O-Si(CH$_3$)$_3$</td>
</tr>
<tr>
<td>$R_1$C=O</td>
<td>$R_1$C=NOH</td>
<td>$R_1$C=N-O-Si(CH$_3$)$_3$</td>
</tr>
<tr>
<td>$R_1$SH</td>
<td></td>
<td>$R_1$S-Si(CH$_3$)$_3$</td>
</tr>
<tr>
<td>$R_1$NH</td>
<td></td>
<td>$R_1$NH-Si(CH$_3$)$_3$</td>
</tr>
<tr>
<td>$R_1$C=NH</td>
<td></td>
<td>$R_1$C=N-Si(CH$_3$)$_3$</td>
</tr>
</tbody>
</table>
protein effects and measured serum levels with the same methods as are
commonly used for urine methylmalonic acid (MMA). Oberholzer and
other co-workers developed a method in which the protein of diluted
acidified serum was coagulated by heat before the colourimetric estima-
tion of MMA was done. The scarcity of results reported for serum
levels of metabolites and the wide variety of methods reported indicate
the difficulties of reproducible extraction methods for organic acids from
protein containing fluids.

The difficulties of extraction of fatty acids and other lipids from
protein containing fluids have generally been overcome by the development
of special methods such as the Folch or Dade extraction.

C. Derivatisation of the Organic Acids

The acids obtained by extraction or ion exchange are in general too
involatile for direct GC analysis. The aim of derivatisation is to en-
hance volatility and suppress the absorption and decomposition of the
acids on the surface of the chromatographic packing. To be of value the
derivatisation step must be chemically simple and it must proceed
quantitatively.

The urinary acid fraction contains a complex mixture of mono and
poly-carboxylic acids, hydroxy and keto-acids, phenols, phenolic acids
and conjugates of these compounds with glycine and glucuronic acids.
The two most useful reactions which have been used for derivatisation are
silylation and esterification (especially methylation).

Trimethylsilyl (TMS) ethers and esters are the most popular deriv-
atives for the GC of organic acids since they are easy to prepare
quantitatively and conveniently, are safe to handle and have good
chromatographic properties. (Table 2). The silyl derivatives are partic-
ularly suitable for GC-MS and commercially available deuterium labelled
TMS reagents may be used to obtain derivatives with different molecular
weights. The silyl derivatives give characteristic fragmentation patterns
in EIMS, and usually have a strong \( \{M-15\}^+ \) ion in the spectrum to indicate
the molecular weight. Reference spectra of a large number of organic acid
silyl derivatives are also readily available.

The reagent of choice is \( N_{2}O\)-bis(trimethylsilyl)trifluoroacetamide
(BSTFA)\textsuperscript{170} which is one of the most potent silylating reagents available. It has the added advantage that the reaction by-products are highly volatile and elute with the solvent and any excess reagent. The reagent is often used in conjunction with catalytic amounts of trimethylchlorosilane or pyridine.

Sodium salts of acids are generally more difficult to silylate than free acids, but recent investigations\textsuperscript{171} have shown that bis(trimethylsilyl)acetamide (BSA) in the presence of trimethylchlorosilane and/or hydroxylamine converts these salts directly into volatile TMS derivatives. The TMS derivatives of organic acids are highly water sensitive, in particular the di-TMS derivatives of dicarboxylic acids\textsuperscript{172} are readily hydrolysed by even traces of water. However by strict exclusion of moisture during preparation and the use of molecular sieve and septum sealed containers the derivatives may be kept for several months without decomposition. The silylation of N-acylglycines may give rise to multiple GC peaks\textsuperscript{173} from the presence of incompletely derivatised material. However, when longer silylating times and stronger reagents,\textsuperscript{173-175} or methyl esters are chosen as derivatives\textsuperscript{176} a single GC peak is obtained. The two structural isomers of the TMS derivative of keto acids may also have different GC elution times and these acids are usually determined when the keto group is first derivatised with hydroxylamine\textsuperscript{164,174,177} (or a substituted hydroxylamine\textsuperscript{108,120-122,178}) before silylation. Combined silylation and methylation yields derivatives with good chromatographic properties\textsuperscript{108,179} and combined ethylation and silylation has been preferred for some SIM studies.\textsuperscript{180}

The methyl esters of organic acids have excellent chromatographic properties and can be prepared with diazomethane. The mass spectra of methyl esters are usually simple and easier to interpret than those of the corresponding silyl compounds. Despite the hazards associated with diazomethane, its use gives esters in quantitative yield in a simple smooth and rapid procedure. The method may also lead to certain by-products, artifacts and unexpected results,\textsuperscript{181,182} but it is still the method of choice for esterfication. Other diazo alkanes have been used to prepare higher molecular weight esters.\textsuperscript{166}

Many alternative methods of esterification have been used. These
<table>
<thead>
<tr>
<th>Acid</th>
<th>Disease</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propionic Acid</td>
<td>Propionic Acidemia</td>
<td>134</td>
</tr>
<tr>
<td></td>
<td>Methylmalonic Acidemia</td>
<td>189</td>
</tr>
<tr>
<td>Butyric Acid</td>
<td>Glutaric Aciduria Type 2</td>
<td>190</td>
</tr>
<tr>
<td>2-Methylpropionic Acid (Isobutyric)</td>
<td>Glutaric Aciduria Type 2</td>
<td>190</td>
</tr>
<tr>
<td>Hexanoic Acid</td>
<td>Glutaric Aciduria Type 2</td>
<td>190</td>
</tr>
<tr>
<td>Tiglic Acid</td>
<td>Propionic Acidemia</td>
<td>191</td>
</tr>
<tr>
<td>2-Methylbutyric Acid</td>
<td>Jamaican Vomiting Sickness</td>
<td>192</td>
</tr>
<tr>
<td>3-Methylcrotonic Acid</td>
<td>3-Methylcrotonyl glycinuria</td>
<td>193</td>
</tr>
<tr>
<td>Isovaleric Acid</td>
<td>Isovaleric Acidemia</td>
<td>133</td>
</tr>
<tr>
<td></td>
<td>Jamaican Vomiting Sickness</td>
<td>192</td>
</tr>
<tr>
<td></td>
<td>Glutaric Aciduria Type 2</td>
<td>190</td>
</tr>
<tr>
<td>Propionylglycine</td>
<td>Propionic Acidemia</td>
<td>194</td>
</tr>
<tr>
<td>2-Methylbutyryl-glycine</td>
<td>Propionic Acidemia</td>
<td>195</td>
</tr>
<tr>
<td>3-Methylcrotonyl glycinine</td>
<td>3-Methylcrotonyl Glycinuria</td>
<td>193</td>
</tr>
<tr>
<td></td>
<td>3-Methylcrotonyl Glycinuria</td>
<td>196</td>
</tr>
<tr>
<td></td>
<td>Biotin Responsive</td>
<td></td>
</tr>
<tr>
<td>Tiglylglycine</td>
<td>Propionic Acidemia</td>
<td>197</td>
</tr>
<tr>
<td></td>
<td>3-Methylcrotonyl Glycinuria</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Biotin Responsive</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2-Methyl Acetoacetyl CoA</td>
<td>196</td>
</tr>
<tr>
<td></td>
<td>Thiolase Deficiency</td>
<td>198</td>
</tr>
<tr>
<td>Isovalerylglycine</td>
<td>Isovaleric Acidemia</td>
<td>188</td>
</tr>
<tr>
<td></td>
<td>Jamaican Vomiting Sickness</td>
<td>192</td>
</tr>
<tr>
<td>Hexanoylglycine</td>
<td>Glutaric Aciduria Type 2</td>
<td>190</td>
</tr>
</tbody>
</table>
include an acid/alcohol combination such as methanol/hydrochloric acid, methanol/boron trifluoride, methanol/thionyl chloride, the reaction of silver salts with alkyl halides, the use of crown ethers or other dehydration catalysts, and the injection port pyrolysis of tetraalkylammonium salts of acids. 

There have been a wide variety of derivatives and methods developed for specialised separations and specific combinations of derivatising agents employed for poly-functional compounds. These specialised methods are not generally applicable to the profiling of organic acids.

D. Special Methods required for Short Chain Acids

Short chain (C₁₂ - C₈) monocarboxylic acids are volatile and therefore require no derivatisation for GC. These acids as well as their glycine conjugates have some value for the diagnosis of human metabolic diseases (See Table 3). The presence of these acids may sometimes be detected by smell, but the proper diagnosis of disease depends on quantitative measurement of the acid. Methods for sample preparation have included steam distillation, vacuum distillation and solvent extraction. In some cases, minimum sample treatment may even include injection of raw urine, ethanol deproteinised serum or urine from which the metal ions have been removed by ion exchange resins. GC separations on such phases as Porapak, FFAP (Carbowax 20M-nitroterephthalic acid), Chromosorb 105, SP 1200/orthophosphoric acid on Chromosorb W and many others have been used. One recommended method uses carrier gas saturated with formic acid and gives analyses with a relative standard deviation of less than 4%.

Alternative approaches to short chain fatty acid analyses have included derivatisation to improve the gas chromatographic properties. These have included formation of benzyl esters, p-bromophenacyl esters and p-phenylphenacyl esters and the use of TMS esters prepared via trimethylsilylimidazole. The derivatisation techniques are not popular and despite the problems associated with the GC of free acids the latter approach is generally used.
TABLE 4
SOME ARTEFACT METABOLITES DETECTED BY GAS LIQUID
CHROMATOGRAPHY OF ORGANIC ACID EXTRACTS

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Probable Source</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adipic acid</td>
<td>Medium chain triglyceride feed</td>
<td>213</td>
</tr>
<tr>
<td>N. Acetytryptophan</td>
<td>Protein solution stabiliser</td>
<td>213</td>
</tr>
<tr>
<td>Benozoic acid</td>
<td>Bacterial metabolite</td>
<td>214</td>
</tr>
<tr>
<td>Benzyl alcohol</td>
<td>Soft drinks, berries, jams, coffee</td>
<td>215</td>
</tr>
<tr>
<td>Chloral gluconride</td>
<td>I.V. Phenobart</td>
<td>215</td>
</tr>
<tr>
<td>Chlorocresol</td>
<td>Neparin</td>
<td>215</td>
</tr>
<tr>
<td>Citric acid homologue</td>
<td>I.V. Phenobarb</td>
<td>213</td>
</tr>
<tr>
<td>Citric acid</td>
<td>&quot;Lemon&quot; drinks</td>
<td>213</td>
</tr>
<tr>
<td>p-Cresol</td>
<td>&quot;Lemon&quot; drinks</td>
<td>213</td>
</tr>
<tr>
<td>2,6-Diethylaniline metabolites</td>
<td>Anticoagulant</td>
<td>213</td>
</tr>
<tr>
<td>4-Ethylphenol</td>
<td>Gut flora</td>
<td>214</td>
</tr>
<tr>
<td>2-5-Furandicarboxylic acid</td>
<td>Soft drinks, berries, jams, coffee</td>
<td>215</td>
</tr>
<tr>
<td>2-Furoylglycine</td>
<td>I.V. Phenobarb</td>
<td>213</td>
</tr>
<tr>
<td>Nippuric acid</td>
<td>Sterilised I.V. fructose</td>
<td>219</td>
</tr>
<tr>
<td>4-Hydroxyphenyl acetic acid</td>
<td>Cooked food</td>
<td>219</td>
</tr>
<tr>
<td>5-Hydroxycoumaran</td>
<td>Sterilised I.V. fructose</td>
<td>219</td>
</tr>
<tr>
<td>4-Hydroxyphenyl hydroacrylic acid</td>
<td>Antipyretic lotion</td>
<td>219</td>
</tr>
<tr>
<td>5-Hydroxymethyl-2-furoic acid</td>
<td>Berries, jams, soft drinks</td>
<td>215</td>
</tr>
<tr>
<td>Levalinic acid</td>
<td>I.V. Phenobarb</td>
<td>213</td>
</tr>
<tr>
<td>N-methylpyruvic acid</td>
<td>Bactrep. nassalis overgrowth</td>
<td>215</td>
</tr>
<tr>
<td>2-Mercaptobenzothiazide</td>
<td>Anticoagulant</td>
<td>215</td>
</tr>
<tr>
<td>3,4-Methylcnehexanedioic acid</td>
<td>Phenylglycine overgrowth</td>
<td>215</td>
</tr>
<tr>
<td>Oxalic acid</td>
<td>Phenylacetic acid</td>
<td>219</td>
</tr>
<tr>
<td>3-Oxodipropylacetic acid</td>
<td>Phenylacetic acid</td>
<td>219</td>
</tr>
<tr>
<td>Pantothenic acid</td>
<td>Phenylacetic acid</td>
<td>219</td>
</tr>
<tr>
<td>Phenol*</td>
<td>Phenylacetic acid</td>
<td>219</td>
</tr>
<tr>
<td>Phenylpropionic acid</td>
<td>Phenylacetic acid</td>
<td>219</td>
</tr>
<tr>
<td>Phenoxycetic acid</td>
<td>Phenylacetic acid</td>
<td>219</td>
</tr>
<tr>
<td>Phenylisobutyric acid</td>
<td>Phenylacetic acid</td>
<td>219</td>
</tr>
<tr>
<td>Phthalic acid</td>
<td>Phenylacetic acid</td>
<td>219</td>
</tr>
<tr>
<td>Propylene glycol</td>
<td>Phenylacetic acid</td>
<td>219</td>
</tr>
<tr>
<td>Pyruvotonic acid</td>
<td>Phenylacetic acid</td>
<td>219</td>
</tr>
<tr>
<td>Phenylethylmalonamide metabolites</td>
<td>Phenylacetic acid</td>
<td>219</td>
</tr>
<tr>
<td>2-N-propylglutaric acid</td>
<td>Phenylacetic acid</td>
<td>219</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>Epilim metabolite</td>
<td>221</td>
</tr>
<tr>
<td>Salicyluric acid</td>
<td>Epilim metabolite</td>
<td>221</td>
</tr>
<tr>
<td>Sebacic acid</td>
<td>Epilim metabolite</td>
<td>221</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>Epilim metabolite</td>
<td>221</td>
</tr>
<tr>
<td>Tributylphosphosphate</td>
<td>Epilim metabolite</td>
<td>221</td>
</tr>
<tr>
<td>Trichloracetic acid</td>
<td>Epilim metabolite</td>
<td>221</td>
</tr>
<tr>
<td>Thymol</td>
<td>Epilim metabolite</td>
<td>221</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>Epilim metabolite</td>
<td>221</td>
</tr>
<tr>
<td>Crotonic acid</td>
<td>Epilim metabolite</td>
<td>221</td>
</tr>
<tr>
<td>Dipropylacetic acid</td>
<td>Epilim metabolite</td>
<td>221</td>
</tr>
<tr>
<td>Isovaleric acid</td>
<td>Epilim metabolite</td>
<td>221</td>
</tr>
<tr>
<td>3-Butyrolactic acid</td>
<td>Epilim metabolite</td>
<td>221</td>
</tr>
<tr>
<td>Trans-3-methyl-2-hexenoic acid</td>
<td>Epilim metabolite</td>
<td>221</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>Epilim metabolite</td>
<td>221</td>
</tr>
<tr>
<td>3-Methylcrotonic acid</td>
<td>Epilim metabolite</td>
<td>221</td>
</tr>
<tr>
<td>3-Hydroxyisovaleric acid</td>
<td>Epilim metabolite</td>
<td>221</td>
</tr>
<tr>
<td>3-Hydroxyisovaleric acid</td>
<td>Epilim metabolite</td>
<td>221</td>
</tr>
<tr>
<td>Polyethylene bags</td>
<td>Epilim metabolite</td>
<td>221</td>
</tr>
<tr>
<td>Decarboxylation of methylmalonic acid</td>
<td>Epilim metabolite</td>
<td>206</td>
</tr>
<tr>
<td>Bacterial metabolite</td>
<td>Epilim metabolite</td>
<td>221</td>
</tr>
</tbody>
</table>
ARTIFACTS AND PITFALLS

A variety of artifacts and pitfalls may occur during the profiling of physiological fluids (See Table 4 after Hammond). The presence of a particular component in a fluid extract may easily be misinterpreted as an unusual metabolite which bears a casual relationship to the patient's state of health although the origin of the component may be due to contamination. Conversely the absence of a component, the presence of which is considered diagnostic, may cause the diagnosis of a condition to be missed.

Instrumental problems such as loss of resolution and sensitivity which may go unnoticed for periods of time without adequate quality control may cause metabolites to be missed. This is especially true of intermittent faults in equipment. The problem of ghost peaks may also arise especially in short chain fatty acid analysis.

Chemical contamination of the extract is common. It may be aggravated by the concentration effect of solvent removal and may arise from sample containers, anticoagulants, solvents, reagents or plasticisers.

Bacterial contamination especially of urine specimens may give rise to serious problems. Hammond has shown that about 45% of samples received showed moderate or profuse bacterial growth on arrival in the laboratory and Hansen et al have shown that many common bacteria can produce organic acids in short time spans. Bacteria or faulty storage of sample may also lead to the disappearance of metabolites from the samples.

Numerous problems may be introduced during sample work up and derivatisation. Artifacts may be formed by processes such as the dehydration of hydroxy acids, decarboxylation, trans-esterification or acid or base catalysed decomposition of compounds such as glucose. Similarly GC may be responsible for the unexpected formation of compounds such as 5-hydroxycoumarin from homogentisic acid.

Perhaps the most serious problems arise from the ingestion of dietary
TABLE 5
METABOLITES IDENTIFIED IN AMNIOTIC FLUID

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Ketoisovaleric acid</td>
<td>139</td>
</tr>
<tr>
<td>2-Ketoisocaproic acid</td>
<td>257, 139</td>
</tr>
<tr>
<td>Maleic acid</td>
<td>139, 475</td>
</tr>
<tr>
<td>p-Hydroxybenzoic acid</td>
<td></td>
</tr>
<tr>
<td>Lauric acid</td>
<td>139</td>
</tr>
<tr>
<td>Myristic acid</td>
<td>139</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>257, 139</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>257, 139</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>257, 139</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>139</td>
</tr>
<tr>
<td>Glycolic acid</td>
<td>257</td>
</tr>
<tr>
<td>3-Hydroxyisobutyric acid</td>
<td>257</td>
</tr>
<tr>
<td>3-Hydroxyisovaleric acid</td>
<td>257</td>
</tr>
<tr>
<td>Urea</td>
<td>257</td>
</tr>
<tr>
<td>2-Ketocaproic acid</td>
<td>257</td>
</tr>
<tr>
<td>Glycerol</td>
<td>257</td>
</tr>
<tr>
<td>Phosphoric acid</td>
<td>257</td>
</tr>
<tr>
<td>Glyceric acid</td>
<td>257</td>
</tr>
<tr>
<td>2-Methyl-3-hydroxybutyric acid</td>
<td>257</td>
</tr>
<tr>
<td>2-Hydroxybenzoic acid</td>
<td>257</td>
</tr>
<tr>
<td>2-Hydroxyglutaric acid</td>
<td>257</td>
</tr>
<tr>
<td>3-Hydroxy-3-methylglutaric acid</td>
<td>257</td>
</tr>
<tr>
<td>4-Hydroxybenzoic acid</td>
<td>257</td>
</tr>
<tr>
<td>3-Hydroxyphenylacetic acid</td>
<td>257</td>
</tr>
<tr>
<td>Gentisic acid</td>
<td>257</td>
</tr>
<tr>
<td>Homovanillic acid</td>
<td>257</td>
</tr>
<tr>
<td>4-Hydroxyphenyllactic acid</td>
<td>257</td>
</tr>
<tr>
<td>Hexadecanol</td>
<td>257</td>
</tr>
<tr>
<td>Pentose alcohol</td>
<td>257</td>
</tr>
<tr>
<td>Heptadecanoic acid</td>
<td>257</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>257</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>257</td>
</tr>
</tbody>
</table>
factors and drugs by the patients. Many patients may be on a wide variety of drugs as well as intravenous alimentation. Particular attention must be paid to acidic drug metabolites which could be mistaken for endogenous metabolites. All classes of drugs must be considered as Jellum et al have shown that even skin ointments containing salicylic acid can lead to interferences in urine profiles. Similarly a restricted diet may cause distorted excretion patterns of metabolites, especially in very sick patients who may be receiving little or no protein in their alimentation and so produce only small amounts of diagnostic metabolites.

ORGANIC ACID PROFILE STUDIES

Since Markey et al published their table of urinary organic acids in 1974 many new metabolites have been discovered by GC-MS methods. Later listings have included urinary studies of normal adults and newborns and have covered diet and individual variation on organic acid excretion.

Most papers have dealt with urine and very little information exists on the organic composition of other body fluids. Some quantitative results on serum from patients with organic acidurias have been reported. Since no recoveries have been reported for these studies the results must remain suspect. Cerebrospinal fluid has been examined especially for the presence of acidic catecholamine metabolites. Variations of profiles due to neurological disease have been found but further investigation is required to determine if this can be used as a diagnostic criteria. Saliva, seminal fluid and synovial fluid have been examined for organic acids as has dialysis fluid from nephrectomised patients. Amniotic fluid is of interest because of the possibility of antenatal diagnosis, however only a few papers have dealt with the organic acid content and concentration (Tables 5,6) in contrast to the number reported for lipid profiles which are of clinical value in assessing foetal lung maturation.

There has been a report on the organic acid profile of tissue homogenates where both differences and similarities between organs are shown. Sensitivity was such that samples obtained by needle biopsy could be examined but the usefulness of such studies remain to be established.

In all studies of other fluids the profiles obtained are similar to
## TABLE 6

**METABOLITES IDENTIFIED AND QUANTITATED IN AMNIOTIC FLUID**

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Concentration mM/l (mean ± SD)</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Range</td>
</tr>
<tr>
<td>Lactate</td>
<td>6.7/2.9</td>
<td>4.1-14.2</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.13/0.04</td>
<td>0.06-0.20</td>
</tr>
<tr>
<td></td>
<td>*8.2/4.0</td>
<td>0.013-0.097</td>
</tr>
<tr>
<td></td>
<td>0.046/0.021</td>
<td>0.06-0.24</td>
</tr>
<tr>
<td>3-hydroxybutyrate</td>
<td>0.114 /0.0159</td>
<td></td>
</tr>
<tr>
<td>2-hydroxybutyrate</td>
<td>0.05</td>
<td>0.03-0.10</td>
</tr>
<tr>
<td>Succinate</td>
<td>0.009/0.007</td>
<td>0.004-0.027</td>
</tr>
<tr>
<td>2-ketoglutyrate</td>
<td>*7.4/3.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.013/0.008</td>
<td>0.004-0.035</td>
</tr>
<tr>
<td>Citrate</td>
<td>*55.5/12.7</td>
<td>4.6-7.72</td>
</tr>
</tbody>
</table>

* Values are in mg/L

ϕ Values are in mg/100 ml
those obtained from urine although individual components may differ in concentration. The levels reported in serum are generally very much lower than those present in urine. The definition of the normal organic acid patterns in body and in other fluids provides the basis for the identification of abnormal patterns which may be present in the disease state.

**COMPUTERS IN THE IDENTIFICATION OF COMPOUNDS**

The widespread use of GC-MS for the examination of biological fluids has brought with it a number of problems. The most notable of these is the amount of data generated in a typical experiment where several thousand mass spectra may be recorded. Apart from pattern recognition techniques which have been of great value in analysis of volatiles and in the pyrolysis GC-MS of bacteria and cells most of the work on GC-MS profiling of body fluids ultimately ends up with the problem of identifying certain peaks on the gas chromatogram from their corresponding mass spectra. Manual interpretation of the relevant mass spectra is often difficult and time consuming. The visual comparison of the unknown spectrum with a catalogue of known mass spectra often gives valuable clues with regard to structure. A number of such collections of spectra have been published, containing up to 40,000 spectra and details of these and other data bases are available.

The development of computing facilities has allowed this process of matching and interpreting spectra to be automated. Three general approaches have been made to computer applications.

(a) The development of methods in which a file or library of encoded reference spectra is searched to find the best match with a similarly encoded unknown spectrum.

(b) The use of a pattern recognition approach in which the features determined from known mass spectra are used to provide classification of unknown spectra of compounds which are not in the data base used to determine the features.

(c) The use of data systems to elucidate structures of unknowns using fragmentation rules and known fragmentation patterns. This approach attempts to automate that taken by an organic chemist when he manually attempts to interpret a spectrum.
The aim of the first approach is to retrieve an identical spectrum to the reference spectrum. However any practical system should be able to retrieve spectra of the same compound even when distorted by instrumental variation. Many library systems will retrieve the spectra of structurally similar compounds if no matching spectrum is in the library. This approach has received the widest attention because of its innate simplicity, generality and the availability of large spectral libraries.

In file searching the spectra in the library are coded, typically by extracting from the complete spectrum some subset of the total data. This selection generally transforms the spectrum into a code directly suitable for computer searching thereby reducing both computer storage and search time requirements. The similarly coded unknown spectrum is then compared with either the complete set of library codes or some subset of these to find known spectra which "best fit" the unknown. A wide variety of algorithms have been used to determine matching of library and unknown spectra. The algorithm used will depend on the encoding method used and usually gives an output of the M best matching compounds with some measure of similarity.

The pattern recognition approach addresses a general problem: can an obscure property of a collection of objects (in this case compounds) be predicted and/or detected using indirect measurements (in this case mass spectra) made on the object, which are known to be related to the property via some unknown relationship? The assumption is made in this approach that if measurements are made of this obscure property then objects having properties in common will have similar measurements. In most applications to mass spectra interpretation non parametric statistical methods are used, in which no assumptions are made about underlying statistical relationships. This type of mass spectral interpretation is useful for identifying certain features in a mass spectrum even if the compound is not in the library and has demonstrated high reliability in answering suitable problems. It also has advantages of speed in execution once the relevant factors are determined. However most systems based on pattern recognition are generally inferior for the unique identification of unknowns, may require large amounts of computational effort to determine the factors to be recognised, and may be very sensitive to the
libraries used to determine the factors. Factor analysis has been used to determine the relationships used to identify the spectrum of the unknown. The best use of pattern recognition appears to be in the prediction of the mass spectrum where predictive abilities of 70 - 90% have been obtained.

The application of our knowledge of fragmentation patterns appropriate to various compound types and sub-structures gives the basis of the third approach to the interpretation of mass spectrum. Such knowledge, although imperfect, is considerable (e.g. refs. 295-297). However, it cannot always be formulated into rules for ready use in computer programmes. It is likely that with the application of computer techniques to large libraries to find these associations of known structural and spectral features, this problem will be decreased.

These techniques have been applied by many workers to analyse simple monofunctional molecules, to suggest molecular weights or for the sequencing of peptides are often used in conjunction with high resolution MS. The best known application of mass spectral theory to mass spectral interpretation is the application of heuristic programming approach by the Stanford group. This programme recognises features in the mass spectrum of the unknown, generates all possible structures and their predicted mass spectra and tests the generated spectra against that of the unknown. The programme has been applied to a wide range of compounds and mixtures and can make use of metastable ion data and high resolution MS if available. The programme has some interactive intervention during execution to save time by discarding some intermediate structures. The programme is especially useful for isomeric and structurally related compounds. The artificial intelligence approach suffers from the large computer storage and execution time requirements, as well as the difficulty of programming and has not been generally applied or developed.

The simple search techniques offer a very useful treatment of a large data base and together with the use of specialised libraries can give many clues to the identification of unknown compounds. Many of these library researching techniques are being applied to small specialised libraries of mass spectra which can be searched quickly, often using the micro or mini computer that controls the GC-MS system.
### Table 7

**NEW METABOLIC DISORDERS DISCOVERED BY GC/MS PROFILING**

<table>
<thead>
<tr>
<th>Disease</th>
<th>Metabolites Detected</th>
<th>Year</th>
<th>Ref*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Refsum’s disease** (phytanic acid storage disease)</td>
<td>Phytanic acid</td>
<td>1963</td>
<td>333</td>
</tr>
<tr>
<td></td>
<td>Isovaleric acid, isovalerylglycine, 3-hydroxyisovaleric acid</td>
<td>1965</td>
<td>367</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1965</td>
<td>368</td>
</tr>
<tr>
<td></td>
<td>Isovaleric acidemia</td>
<td>1966</td>
<td>133</td>
</tr>
<tr>
<td></td>
<td>Methylymalonic aciduria</td>
<td>1967</td>
<td>161</td>
</tr>
<tr>
<td>Type I</td>
<td></td>
<td>1967</td>
<td>152</td>
</tr>
<tr>
<td>Type II</td>
<td></td>
<td>1970</td>
<td>369</td>
</tr>
<tr>
<td>Type III</td>
<td></td>
<td>1972</td>
<td>370</td>
</tr>
<tr>
<td>Type IV</td>
<td></td>
<td>1973</td>
<td>371</td>
</tr>
<tr>
<td>Type IV? Methylmalonic and 3-hydroxy-n-valeric acidemia</td>
<td>Methylymalonic, propionic, 3-hydroxy-n-valeric and odd-chain fatty acids</td>
<td>1970</td>
<td>134</td>
</tr>
<tr>
<td>Type I: biotin-unresponsive</td>
<td>Propionic, 3-hydroxypropionic, methylcitric, 3-hydroxy-3-methylglutaric and 3-hydroxybutyric acids and propionylglycine</td>
<td>1970</td>
<td>372</td>
</tr>
<tr>
<td>Type II: biotin-responsive</td>
<td></td>
<td>1970</td>
<td>373</td>
</tr>
<tr>
<td>3-Methylcrotonyl-CoA carboxylase deficiency</td>
<td>3-Methylcrotonylglycine, 3-hydroxyisovaleric acid</td>
<td>1970</td>
<td>374</td>
</tr>
<tr>
<td>Type I: biotin-unresponsive</td>
<td>3-Methylcrotonylglycine, tiglylglycine, 3-hydroxyisovaleric, 3-methylcrotonic, methylcitric and 3-hydroxypropionic acids and 3-hydroxy-3-methylglutaric and 3-hydroxybutyric acids, not 3-methylcrotonylglycine</td>
<td>1971</td>
<td>193</td>
</tr>
<tr>
<td>Type II: biotin-responsive***</td>
<td></td>
<td>1974</td>
<td>374</td>
</tr>
<tr>
<td>Type III?:</td>
<td></td>
<td>1976</td>
<td>376</td>
</tr>
<tr>
<td>Pyroglutamatic aciduria (3-Oxoprolinuria, glutathione, synthetase deficiency)</td>
<td>Pyroglutamatic acid (2-oxoproline)</td>
<td>1970</td>
<td>377</td>
</tr>
<tr>
<td>Acyl CoA-dehydrogenase deficiency? (congenital dicarboxylic aciduria)</td>
<td></td>
<td>1974</td>
<td>378</td>
</tr>
<tr>
<td>2-Methylacetoacetic and 2-methyl-3-hydroxybutyric acidemia</td>
<td>Saturated C₄-C₈ dicarboxylic acids, C₆-C₉ cis-5-monounsaturated dicarboxylic acids</td>
<td>1973</td>
<td>379</td>
</tr>
<tr>
<td></td>
<td>2-Methylacetoacetic, 2-methyl-3-hydroxybutyric acids and tiglylglycine</td>
<td>1976</td>
<td>380</td>
</tr>
<tr>
<td>D-Glyceric acidemia</td>
<td>D-Glyceric acid and glycine</td>
<td>1974</td>
<td>381</td>
</tr>
<tr>
<td>Type I</td>
<td></td>
<td>1976</td>
<td>382</td>
</tr>
<tr>
<td>Type II</td>
<td></td>
<td>1976</td>
<td>383</td>
</tr>
<tr>
<td>2-Keto adipic aciduria</td>
<td>2-Keto adipic, 2-hydroxyadipic and 2-amino adipic acids</td>
<td>1975</td>
<td>384</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1975</td>
<td>385</td>
</tr>
<tr>
<td>Glutaric aciduria</td>
<td>Glutaric, 3-hydroxyglutaric and glutaconic acids</td>
<td>1975</td>
<td>386</td>
</tr>
<tr>
<td>Type I</td>
<td>Glutaric, (no glutaconic), lactic, isobutyric, isovaleric, propionic, 2-methylbutyric, ethylmalonic and 3-hydroxybutyric acids and several metabolites associated with lactic- and keto-acidoses</td>
<td>1977</td>
<td>387</td>
</tr>
<tr>
<td>Type II</td>
<td>1976</td>
<td>388</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1976</td>
<td>389</td>
<td></td>
</tr>
<tr>
<td>Carnitine deficiency</td>
<td>Adipic, pimelic and suberic acids</td>
<td>1975</td>
<td>388</td>
</tr>
<tr>
<td></td>
<td>Adipic, suberic and sebacic acids and suberylglycine</td>
<td>1976</td>
<td>347</td>
</tr>
<tr>
<td>Non-ketotic dicarboxylic aciduria (carnitine deficiency?)</td>
<td>3-Hydroxy-3-methylglutaric, 3-methylglutaric, 3-hydroxyisovaleric and 3-methylglutaric acids (not 3-methylcrotonic acid)</td>
<td>1976</td>
<td>355</td>
</tr>
<tr>
<td>3-Hydroxy-3-methylglutaric aciduria</td>
<td></td>
<td>1976</td>
<td>209</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1976</td>
<td>225</td>
</tr>
<tr>
<td>Glyceroluria</td>
<td>Glycerol</td>
<td>1977</td>
<td>358</td>
</tr>
</tbody>
</table>

* Only the first published references on the first patients are cited.
** Classified and described clinically by Refsum (334) many years before GC and MS methods were used to discover the accumulated metabolite.
*** A recent investigation (379) indicates that some of the patients with biotin-responsive propionic acidemia and 3-methylcrotonyl-CoA carboxylase deficiency may suffer from a combined carboxylase deficiency due to a defect in the transport and/or metabolism of biotin rather than the synthesis of the apoenzymes.
RESULTS OF GC-MS PROFILING

Since Klenk and Kahlke used GC-MS methods to separate and identify an unknown metabolite (phytanic acid) in Refsum's disease, gas phase analytical methods have become increasingly important in the diagnosis and study of metabolic diseases. Since that time about 25 new diseases (Table 7, after Jellum) have been discovered and about 50 to 60 previously described inborn errors of metabolism can also be diagnosed and studied using this technique. In general these diseases are recognised because of the occurrence of new or pathological metabolites or large increases in normal metabolites, identifiable by means of GC-MS. Subsequent biochemical work, such as enzyme studies on cells grown in tissue culture and metabolic studies using stable and/or radio isotopes and dietary loading studies are needed to confirm the diagnosis and to monitor treatment.

The major problem is to find appropriate cases for GC-MS study by defining the clinical criteria used in the initial screening of cases. Nyhan discussed some of the problems of selecting patients for screening and points out a number of cases where a misdiagnosis of pyloric stenosis was made and an operation was carried out before genetic disease was properly diagnosed.

Organic acid profiling has also been used to study other disease states such as ketoacidosis, lactic acidosis, mental retardation, stroke, congenital dicarboxylic aciduria, Jamaican vomiting sickness, gastro intestinal disorders, disease related to catecholamine metabolism, respiratory distress syndrome, bacterial and viral infections and Reyes Syndrome.

PRENATAL DIAGNOSIS OF GENETIC DISEASE

Examination of the newborn population shows that two to four per cent have some type of birth defect which can be considered genetic in origin. The term "birth defect" is used in a general sense to encompass all types of structural, metabolic and other abnormalities which derive from genetic or other prenatal causes. The birth defects detected postnatally represent only a small fraction of all abnormalities that result from genetic aberrations because most result in early spontaneous
<table>
<thead>
<tr>
<th>Disorder</th>
<th>Metabolites Detectable by GC/MS</th>
<th>Abnormal Amino Acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaptonuria</td>
<td>Homogentisic acid</td>
<td>No</td>
</tr>
<tr>
<td>Argininosuccinic aciduria</td>
<td>Argininosuccinic acid</td>
<td>Yes</td>
</tr>
<tr>
<td>Alaninuria</td>
<td>Lactic, pyruvic acids</td>
<td>No</td>
</tr>
<tr>
<td>Carnitine deficiency</td>
<td>Adipic, pimelic, suberic acids</td>
<td>Yes</td>
</tr>
<tr>
<td>Dicarboxylic aciduria</td>
<td>Lactic, adipic, suberic, suberylglycine, sesamic, C_{12}-3-unsaturated dicarboxylic acid</td>
<td>No</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>Glucose, 8-hydroxybutyric acid, acetoacetic acid</td>
<td>No</td>
</tr>
<tr>
<td>Essential fructosuria</td>
<td>Fructose</td>
<td>No</td>
</tr>
<tr>
<td>Essential pentosuria</td>
<td>L-Xylulose</td>
<td>Yes</td>
</tr>
<tr>
<td>Fructose 1-6 diphosphatase</td>
<td>Lactic, pyruvic, 2-oxoglutaric acids</td>
<td>Yes</td>
</tr>
<tr>
<td>Galactosemia</td>
<td>Galactose, amino acids</td>
<td>No</td>
</tr>
<tr>
<td>Glucaric aciduria</td>
<td>Glucaric, gluconic acids, glucaric-1, 4-lactic acid</td>
<td>Yes</td>
</tr>
<tr>
<td>Glyceric acidemia (Type 1)</td>
<td>Glyceric acid</td>
<td>No</td>
</tr>
<tr>
<td>Glyceric acidemia (Type 2)</td>
<td>Glyceric acid</td>
<td>Yes</td>
</tr>
<tr>
<td>Glucose-6-phosphatase deficiency</td>
<td>Lactic, pyruvic acids</td>
<td>No</td>
</tr>
<tr>
<td>Glutaric acidemia</td>
<td>Glutaric, glutaric, 3-hydroxyglutaric acids</td>
<td>No</td>
</tr>
<tr>
<td>Glutaric acidemia Type 2</td>
<td>Butyric, ethylmalonic, glutaric acids</td>
<td>No</td>
</tr>
<tr>
<td>Glutathione synthetase deficiency</td>
<td>Pyroglutamic acid</td>
<td>No</td>
</tr>
<tr>
<td>Glycogen storage disease Type I</td>
<td>Lactic, zoxoglutaric acids</td>
<td>No</td>
</tr>
<tr>
<td>Glycogen storage disease Type II</td>
<td>2-oxoglutaric, acetoacetic, acetoacetic, sebacic acids</td>
<td>No</td>
</tr>
<tr>
<td>Glycogen synthetase deficiency</td>
<td>Lactic acid</td>
<td>No</td>
</tr>
<tr>
<td>Glyceroluria</td>
<td>Glycorol</td>
<td>Yes</td>
</tr>
<tr>
<td>Hartnup disease</td>
<td>Neutral amino acids</td>
<td>Yes</td>
</tr>
<tr>
<td>Hawkinsuria</td>
<td>4-hydroxyxyclohexylactic acid</td>
<td>Yes</td>
</tr>
<tr>
<td>Histidinemia</td>
<td>Imidazole lactic, imidazole pyruvic, imidazole lactic acid</td>
<td>Yes</td>
</tr>
<tr>
<td>Homocystinuria with methyl-</td>
<td>3-hydroxy-3-methylglutaric acid, 3-methylglutaric acid, 3-hydroxyisovaleric acid</td>
<td>No</td>
</tr>
<tr>
<td>malonic aciduria</td>
<td>Xanthuronic</td>
<td>No</td>
</tr>
<tr>
<td>Hydroxymethyl-CoA lyase deficiency</td>
<td>2-oxo-4-methylbutyric acid</td>
<td>Yes</td>
</tr>
<tr>
<td>Hydroxykynureninuria</td>
<td>Oxalic, glycolic, glyoxylic acids</td>
<td>No</td>
</tr>
<tr>
<td>Hypermethiozemia</td>
<td>Oxalic, 1-glutaric acid</td>
<td>No</td>
</tr>
<tr>
<td>Hyperoxaluria Type 1</td>
<td>Oxalic, glycolic, glyoxylic acids</td>
<td>No</td>
</tr>
<tr>
<td>Hyperoxaluria Type 2</td>
<td>Pipolectic (piperidine-2-carboxylic) acid</td>
<td>Yes</td>
</tr>
<tr>
<td>Hyper-pipocelotamia</td>
<td>1-pyrolidine-5-carboxylic acid</td>
<td>Yes</td>
</tr>
<tr>
<td>Hyper-prolominia</td>
<td>Indolylacrylglycin                              Isovalerylglycine, 3-hydroxyisovaleric, isovaleric, hexanoyl glycine, isovaleryl glycine</td>
<td>No</td>
</tr>
<tr>
<td>Isovaleric acidimia</td>
<td>Isovalerylglycine, 3-hydroxyisovaleric, isovaleric, hexanoyl glycine, isovaleryl glycine</td>
<td>No</td>
</tr>
<tr>
<td>Jamaican vomiting sickness</td>
<td>Adipic, C_{12}-3-unsaturated, ethylmalonic glutaric, 3-hydroxyisovaleric, isovaleric,</td>
<td>No</td>
</tr>
<tr>
<td>(Hypoglycin A toxicity)</td>
<td>2-methyl succinic methylene cyclopentylacetic, 2-methylbutyric, sebacic, suberic acids,</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>hexanoyl glycine, isovaleryl glycine</td>
<td>No</td>
</tr>
<tr>
<td>Condition</td>
<td>Acids</td>
<td>Response</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
<td>------------------------------------------------------------------------</td>
<td>----------</td>
</tr>
<tr>
<td>Xetoacidosis</td>
<td>Acetoacetic, 3-hydroxybutyric, 3-hydroxy-isobutyric, 3-hydroxyisovaleric, 2-methyl-3-hydroxybutyric, adipic, sebacic, suberic acids</td>
<td>No</td>
</tr>
<tr>
<td>2-ketoaciduria</td>
<td>2-ketoacid, 2-hydroxyacid, 2-amino adipic</td>
<td>Yes</td>
</tr>
<tr>
<td>Lactic acidosis</td>
<td>Lactic, 2-hydroxybutyric acids</td>
<td>No</td>
</tr>
<tr>
<td>Lesch Nyhan syndrome</td>
<td>Uric acid</td>
<td>No</td>
</tr>
<tr>
<td>2-methylcrotonyl CoA carboxylase deficiency Type I (biotin unresponsive)</td>
<td>3-methylcrotonylglycine 3-hydroxyisovaleric acid</td>
<td>No</td>
</tr>
<tr>
<td>3-methylcrotonyl CoA carboxylase deficiency Type II (biotin responsive)</td>
<td>2-ethylhydroxyacrylic acid, 3-hydroxyisovaleric, 3-hydroxy-3-methylglutaric, 3-hydroxypropionic, methylcitric, 3-methylcrotonic acid, 3-methylcrotonyl glycine, tiglylglycine</td>
<td>No</td>
</tr>
<tr>
<td>2-ketoadipic aciduria</td>
<td>2-ketoadipic, 2-hydroxyacid, 2-amino adipic</td>
<td>Yes</td>
</tr>
<tr>
<td>Lactic acidosis</td>
<td>Lactic, 2-hydroxybutyric acids</td>
<td>No</td>
</tr>
<tr>
<td>Lesch Nyhan syndrome</td>
<td>Uric acid</td>
<td>No</td>
</tr>
<tr>
<td>2-ketoadipic aciduria</td>
<td>2-ketoadipic, 2-hydroxyacid, 2-amino adipic</td>
<td>Yes</td>
</tr>
<tr>
<td>Lactic acidosis</td>
<td>Lactic, 2-hydroxybutyric acids</td>
<td>No</td>
</tr>
<tr>
<td>3-ketoisovaleric aciduria</td>
<td>Lactic, 2-hydroxybutyric acids</td>
<td>No</td>
</tr>
<tr>
<td>3-hydroxyisovaleric aciduria</td>
<td>3-hydroxyisovaleric acid</td>
<td>No</td>
</tr>
<tr>
<td>2-methyl-3-hydroxybutyric aciduria</td>
<td>3-methylcrotonylglycine 3-hydroxyisovaleric acid</td>
<td>No</td>
</tr>
<tr>
<td>Maple syrup urine disease</td>
<td>2-ethylhydroxyacrylic acid, 3-hydroxyisovaleric, 3-hydroxy-3-methylglutaric, 3-hydroxypropionic, methylcitric, 3-methylcrotonic acid, 3-methylcrotonyl glycine, tiglylglycine</td>
<td>No</td>
</tr>
<tr>
<td>Methylmalonic aciduria (Types I-IV)</td>
<td>Methylnalonic acid, proionic, 3-hydroxybutyric acid, 3-methylcrotonic acid, 3-hydroxy-3-methylglutaric, 3-hydroxybutyric, 3-methylvaleric, 2-hydroxyisovaleric, acetoacetic acid, 3-hydroxyisovaleric acid, pyruvic acid</td>
<td>No</td>
</tr>
<tr>
<td>Neuroblastoma</td>
<td>Methylnalonic, proionic, 3-hydroxybutyric acid, 3-methylcrotonic acid, 3-hydroxy-3-methylglutaric, 3-hydroxybutyric, 3-methylvaleric, 2-hydroxyisovaleric, acetoacetic acid, 3-hydroxyisovaleric acid, pyruvic acid</td>
<td>No</td>
</tr>
<tr>
<td>Oat house disease</td>
<td>Orotic acid</td>
<td>No</td>
</tr>
<tr>
<td>Orotic aciduria</td>
<td>Orotic acid</td>
<td>Yes</td>
</tr>
<tr>
<td>Phenylketonuria</td>
<td>Phenylpyruvic, phenyl-lactic, 2-hydroxyphenylactic, mandelic, 4-hydroxyphenylpyruvic, 4-hydroxyphenyl-lactic acids, Lactic, pyruvic acids</td>
<td>Yes</td>
</tr>
<tr>
<td>Phosphatase dehydrogenase deficiency</td>
<td>Propionic, 3-hydroxypropionic, methylcitric 3-hydroxy-3-methylglutaric, 3-hydroxybutyric, acetoacetic, 3-ketovaleric, tiglic acids, propionyl glycine, tigly glycine, lactic, pyruvic acids</td>
<td>Yes</td>
</tr>
<tr>
<td>Propionic acidemia</td>
<td>Propionic, 3-hydroxypropionic, methylcitric 3-hydroxy-3-methylglutaric, 3-hydroxybutyric, acetoacetic, 3-ketovaleric, tiglic acids, propionyl glycine, tigly glycine, lactic, pyruvic acids</td>
<td>Yes</td>
</tr>
<tr>
<td>Pyruvic dehydrogenase deficiency (various forms)</td>
<td>3-hydroxy-4-hydroxymandelic acid, catecholamines and metabolites, Phtylic acid</td>
<td>No</td>
</tr>
<tr>
<td>Pheochromocytoma</td>
<td>Glucose</td>
<td>No</td>
</tr>
<tr>
<td>Refsum's disease</td>
<td>Saccharopine</td>
<td>Yes</td>
</tr>
<tr>
<td>Renal glycosuria</td>
<td>Orotic acid</td>
<td>No</td>
</tr>
<tr>
<td>Saccharopinuria</td>
<td>Phytic acid</td>
<td>No</td>
</tr>
<tr>
<td>Short chain fatty acidemia</td>
<td>Glucose</td>
<td>No</td>
</tr>
<tr>
<td>Succinyl CoA-3-ketoacid CoA transferase deficiency</td>
<td>Saccharopine</td>
<td>Yes</td>
</tr>
<tr>
<td>Tryptophan malabsorption (blue dieter syndrome)</td>
<td>Butyric and caproic acids</td>
<td>No</td>
</tr>
<tr>
<td>Tyrosinorin (Kodes)</td>
<td>3-hydroxybutyric, acetoacetic acids</td>
<td>No</td>
</tr>
<tr>
<td>Tyrosinemia</td>
<td>Kynurenic, xanthurenic and indole-3-acetic acid</td>
<td>No</td>
</tr>
<tr>
<td>Tyrosine amino transferase deficiency</td>
<td>4-hydroxyphenyl pyruvic, 4-hydroxyphenylacetic, 4-hydroxyphenyllactic acids</td>
<td>Yes</td>
</tr>
<tr>
<td>Urocanic aciduria</td>
<td>4-hydroxyphenyl pyruvic, 4-hydroxyphenylacetic, 4-hydroxyphenyllactic acids</td>
<td>Yes</td>
</tr>
<tr>
<td>Xanthurenic aciduria</td>
<td>4-hydroxyphenyl pyruvic, 4-hydroxyphenylacetic, 4-hydroxyphenyllactic acids</td>
<td>Yes</td>
</tr>
<tr>
<td>Xanthurenic aciduria</td>
<td>4-hydroxyphenyl pyruvic, 4-hydroxyphenylacetic, 4-hydroxyphenyllactic acids</td>
<td>Yes</td>
</tr>
<tr>
<td>Disorders</td>
<td>Percent</td>
<td></td>
</tr>
<tr>
<td>--------------------------------------------------------------------------</td>
<td>---------</td>
<td></td>
</tr>
<tr>
<td><strong>CHROMOSOME DISORDERS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Autosomal disorders, e.g. trisomy 21, trisomy 18, etc.</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>Sex chromosome disorders, e.g. XO and X deletions, XXX, XXY, etc.</td>
<td>0.35</td>
<td></td>
</tr>
<tr>
<td><strong>TOTAL CHROMOSOMAL DISORDERS</strong></td>
<td>0.55%</td>
<td></td>
</tr>
<tr>
<td><strong>MONOGENIC DISORDERS. MENDELIAN INHERITANCE</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Autosomal recessive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Severe mental retardation</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>Deafness, blindness</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>Cystic fibrosis</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Adrenogenital syndrome</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>Albinism</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>Amino, organic acidurias</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>Mucopolysaccharidoses</td>
<td>0.0005</td>
<td></td>
</tr>
<tr>
<td>Tay Sachs disease</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Galactosemia</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td>**X-linked, e.g. Duchenne muscular dystrophy, haemophiliases, etc.</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Autosomal dominant, e.g. Marfan syndrome, neurofibromatosis, myotonic dystrophy</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td><strong>TOTAL MONOGENIC DISORDERS</strong></td>
<td>0.36%</td>
<td></td>
</tr>
<tr>
<td><strong>MULTIFACTORIAL DISORDERS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Congenital malformations, e.g. spina bifida, anencephaly, congenital heart, pyloric stenosis, etc.</td>
<td>1.65</td>
<td></td>
</tr>
<tr>
<td>Common diseases, e.g. diabetes, malignancy, allergies</td>
<td>?</td>
<td></td>
</tr>
<tr>
<td><strong>TOTAL MULTIFACTORIAL DISORDERS</strong></td>
<td>&gt;1.65%</td>
<td></td>
</tr>
<tr>
<td><strong>TOTAL FREQUENCY OF GENETIC DISORDERS</strong></td>
<td>&gt;2.56%</td>
<td></td>
</tr>
</tbody>
</table>
abortions.

These birth defects may be divided into three general categories or classes (Table 9, after Epstein and Golbus). The first category comprises non-lethal chromosome disorders which affect about 0.6 per cent of the newborn population. The second is the group of Mendelian disorders which result from abnormalities of single genes. It is estimated that about 0.4 per cent of the newborn population are affected by such conditions and this figure does not include diseases which have a high incidence in specific racial or other groups. The organic acidemias fall into this group. The third group are less well defined and include conditions which are influenced by environmental factors. They include congenital malformations which affect about 1 per cent of the population and multifactorial diseases such as diabetes and schizophrenia which are known to have a genetic component. The first two categories of genetic diseases are the ones most amenable to current techniques of prenatal diagnosis.

Although techniques of prenatal diagnosis are too expensive at present to be used as a general screen for all pregnant women it is possible to define groups in which the incidence of disease is expected to be high. Thus the incidence of Down's syndrome in the general population is about 1 per 1000 live births, but when maternal age reaches forty the incidence is about 1 in 17 and when the mother reaches forty-five years the incidence is about 1 in 12. Similarly the chances of a child with an autosomal recessive condition such as methylmalonic aciduria being born is much less than 1 in 10,000 but if a couple have already had one affected child the risk rises to 1 in 4 for each subsequent pregnancy.

There are a number of advantages in prenatal diagnosis of affected foetuses. The opportunity to have non-affected children by selective abortion of affected foetuses can have a profound effect on the mental health of a couple and on their economic and social circumstances. In the absence of an abortion decision on moral or ethical grounds, the immediate treatment of children at, or even before birth may prevent the mental retardation which accompanies these diseases. The definition and screening of high risk groups may also have significant economic advantages for the
community despite the moral, legal and ethical problems raised. Figures collected for Downs Syndrome patients indicate that to screen all pregnant women over forty for this abnormality would cost about 20% of the amount required to pay for lifetime institutional care if the detected affected foetuses were born. To screen all women over thirty-five the cost is about equal to that of caring for the affected children and if women over thirty were screened the cost would be about five times the cost of care. Similar results have been demonstrated for Tay-Sachs disease when Ashkenazi Jews were screened, using mass screening programmes to detect heterozygotes. In this case a definite cost benefit could be shown, as the incidence in this population was shown to be as high as 1 in 15.

Intrauterine diagnosis during pregnancy was a matter of interest even to the ancients and ancient texts from Egypt (about 1350 B.C.) and Greece suggest methods for determining the sex of the foetus. In this century major advances in prenatal diagnosis have been made by the examination of maternal body fluids using the knowledge, techniques and technology of the disciplines of chemistry, biology and medicine. In 1930 a report of amniocentesis and amniography in late pregnancy appeared and in 1950 Bevis suggested the link between amniotic fluid, bilirubin levels and severity of haemolytic disease of the newborn. In 1963 Liley published the first treatment of disease in utero when he described the technique of interuterine transfusion.

The major impetus to the development of prenatal diagnosis of genetic disorders derives from three simultaneous technical advances in the late 1950's; the technique for obtaining samples of amniotic fluid early in gestation and the willingness to use it; the development of methods for the culture of human cells in vitro; and the development of better techniques of cytogenetic analysis. In 1966 several reports of karotyping of human amniotic cells appeared, in 1967 the diagnosis of a chromosome translocation was reported and in 1968 Nadler reported the diagnosis and its confirmation after abortion of a case of Downs syndrome as well as confirmed diagnoses of a number of biochemical abnormalities.
### TABLE 10

**INBORN ERRORS OF METABOLISM THEORETICALLY DIAGNOSABLE BEFORE BIRTH (AFTER MILUNSKY)**

#### LIPIDOSSES

<table>
<thead>
<tr>
<th>Condition</th>
<th>Diagnosis Made</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol-ester storage disease</td>
<td></td>
</tr>
<tr>
<td>Fabry disease*</td>
<td></td>
</tr>
<tr>
<td>Faber disease</td>
<td></td>
</tr>
<tr>
<td>Gaucher disease*</td>
<td></td>
</tr>
<tr>
<td>Generalized gangliosidosis (GM1 gangliosidosis Type 1)*</td>
<td></td>
</tr>
<tr>
<td>Juvenile GM2 gangliosidosis (GM2 gangliosidosis Type 2)*</td>
<td></td>
</tr>
<tr>
<td>Tay-Sachs disease (GM2 gangliosidosis Type 1)*</td>
<td></td>
</tr>
<tr>
<td>Sandhoff's disease (GM2 gangliosidosis Type 2)*</td>
<td></td>
</tr>
</tbody>
</table>

#### MUCOPOLYSACCHARIDOSES

<table>
<thead>
<tr>
<th>Condition</th>
<th>Diagnosis Made</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPS I-Hurler*</td>
<td></td>
</tr>
<tr>
<td>MPS I-Scheie</td>
<td></td>
</tr>
<tr>
<td>MPS II A - Hunter*</td>
<td></td>
</tr>
<tr>
<td>MPS II B - Hunter*</td>
<td></td>
</tr>
<tr>
<td>MPS III A - Sanfilippo*</td>
<td></td>
</tr>
<tr>
<td>MPS III B - Sanfilippo*</td>
<td></td>
</tr>
</tbody>
</table>

#### AMINO ACID AND RELATED DISORDERS

<table>
<thead>
<tr>
<th>Condition</th>
<th>Diagnosis Made</th>
</tr>
</thead>
<tbody>
<tr>
<td>Argininosuccinic aciduria*</td>
<td></td>
</tr>
<tr>
<td>Aspartylglucosaminuria</td>
<td></td>
</tr>
<tr>
<td>Citrullinemia*</td>
<td></td>
</tr>
<tr>
<td>Congenital hyperammonemia*</td>
<td></td>
</tr>
<tr>
<td>Histidinemia*</td>
<td></td>
</tr>
<tr>
<td>Hypervalinemia</td>
<td></td>
</tr>
<tr>
<td>Immobulinuria</td>
<td></td>
</tr>
<tr>
<td>Isoeucine metabolism disorder</td>
<td></td>
</tr>
<tr>
<td>Maple-syrup-urine disease:</td>
<td></td>
</tr>
<tr>
<td>Severe infantile</td>
<td></td>
</tr>
<tr>
<td>Intermittent</td>
<td></td>
</tr>
<tr>
<td>Methylnalonic aciduria</td>
<td></td>
</tr>
<tr>
<td>Unresponsive to vitamin B12*</td>
<td></td>
</tr>
<tr>
<td>Responsive to vitamin B12*</td>
<td></td>
</tr>
</tbody>
</table>

#### DISORDERS OF CARBOHYDRATE METABOLISM

<table>
<thead>
<tr>
<th>Condition</th>
<th>Diagnosis Made</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fucosidosis</td>
<td></td>
</tr>
<tr>
<td>Galactokinase deficiency</td>
<td></td>
</tr>
<tr>
<td>Galactosaemia*</td>
<td></td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase deficiency*</td>
<td></td>
</tr>
<tr>
<td>Glycogen-storage disease (Type II)</td>
<td></td>
</tr>
<tr>
<td>Glycogen-storage disease (Type III)</td>
<td></td>
</tr>
</tbody>
</table>

#### MISCELLANEOUS HEREDITARY DISORDERS

<table>
<thead>
<tr>
<th>Condition</th>
<th>Diagnosis Made</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acatalasemia</td>
<td></td>
</tr>
<tr>
<td>Acute intermittent porphyria</td>
<td></td>
</tr>
<tr>
<td>Adenosine deaminase deficiency*</td>
<td></td>
</tr>
<tr>
<td>Chediak-Higashi syndrome</td>
<td></td>
</tr>
<tr>
<td>Congenital erythropoietic porphyria</td>
<td></td>
</tr>
<tr>
<td>Congenital nephrosis*</td>
<td></td>
</tr>
<tr>
<td>Lysosomal acid phosphatase deficiency*</td>
<td></td>
</tr>
<tr>
<td>Lysyl-ornithine-protein hydroxylase deficiency</td>
<td></td>
</tr>
<tr>
<td>Myoclonic muscular dystrophy</td>
<td></td>
</tr>
<tr>
<td>McKusick-patella syndrome</td>
<td></td>
</tr>
<tr>
<td>Ornate aciduria*</td>
<td></td>
</tr>
<tr>
<td>Haemophilia*</td>
<td></td>
</tr>
<tr>
<td>Cystinosis*</td>
<td></td>
</tr>
<tr>
<td>Familial hypercholesterolemia</td>
<td></td>
</tr>
<tr>
<td>Glutathionuria</td>
<td></td>
</tr>
<tr>
<td>Hypophosphatasia*</td>
<td></td>
</tr>
<tr>
<td>3-cell disease</td>
<td></td>
</tr>
<tr>
<td>Leigh's encephalopathy</td>
<td></td>
</tr>
<tr>
<td>Lesch-Nyhan syndrome*</td>
<td></td>
</tr>
<tr>
<td>Protoporphyria</td>
<td></td>
</tr>
<tr>
<td>Saccharopinuria*</td>
<td></td>
</tr>
<tr>
<td>Sickle cell anemia</td>
<td></td>
</tr>
<tr>
<td>Tentacular feminization</td>
<td></td>
</tr>
<tr>
<td>Tellerian pigmentosa</td>
<td></td>
</tr>
</tbody>
</table>

* Diagnosis made
During the last decade the scope of possible prenatal diagnosis has been widened from chromosomal aberration to include about fifty metabolic diseases (Table 10, after Milunsky) and a number of multifactorial congenital malformations.

The most direct technique of examining the foetus is by visual examination and instruments have been developed for this purpose. Techniques of radiological and radioisotope visualisation of the uterine cavity, foetus and placenta have been developed but the most useful method of foetal visualisation is that of ultrasonic scanning. This technique is not useful for prenatal diagnosis of genetic disease but is essential in safe sampling of amniotic fluid or foetal tissue. Techniques have been developed for sampling the placenta, foetal blood and tissue but at present these are not safe and reliable and are restricted to studies of diseases such as haemoglobinopathies which cannot be studied by other techniques.

For most types of prenatal information we must use indirect sampling of the foetus through the analysis of the amniotic fluid and its constituents. The ability to obtain amniotic fluid for analysis referred to as amniocentesis is basically the mechanical problem of having an amniotic cavity large enough for the safe insertion of a needle to withdraw fluid for study. The amount of fluid increases during gestation and at 13-14 weeks it is possible to safely withdraw samples of 15 to 25 ml. with little risk to the foetus or mother using ultrasound to locate the pools of fluid.

The criteria for diagnostic amniocentesis include the possibility of diagnosing the disease by the study of the fluid, the cells in the fluid or in cells cultured from the fluid; the disease must be severe enough to warrant the attempt; the lack of effective treatment of the disease of in utero or neonatal treatment; and in general the desire to consent to termination if the disease is diagnosed. To balance the advantage of prenatal diagnosis there are a number of problems and risks including the failure to obtain fluid, or contamination of the fluid with maternal blood giving cells that are not representative of the foetus, the low risks of damage to the foetus or
inducing abortions, the sampling of only one of a twin pregnancy, Rh isoimmunisation of the mother and moral, legal and ethical consequences. There may also be problems with the cell culture as only some cells may grow giving cell clones which may not be representative of the population of foetal cells, or no viable cells may grow or the cells' characteristics may change during subculturing. Appropriate control cell lines may assist in delineating these problem areas.

Some diseases such as phenylketonuria are not expressed in the fibroblast like cell cultures from amniotic fluid and are not amenable to this type of study. One problem is the large amounts of cells needed for enzymic analysis. This may mean four to eight weeks of culture before sufficient numbers of cells are available and even using micro methods two to four weeks are still required. There is only a relatively short period between the earliest time an amniocentesis can be performed and the latest time at which termination should be performed. Longer culture periods can also lead to psychological and other difficulties for the patient and doctor. Thus there is need for fast and reliable tests for metabolic diseases using amniotic fluid or cells from the fluid.

ORIGIN AND COMPOSITION OF AMNIOTIC FLUID

Amniotic fluid was considered as a stagnant pool until a few years ago, but is now seen as a dynamic system which continually interchanges with the amniotic sac, the maternal and the foetal circulations. The contents of the uterus during pregnancy are ultimately derived from the mother but the metabolism of all cells inside the uterus is under the genetic control of the foetus. Current evidence indicates that the fluid originates as a transudate from maternal and foetal serum across the placenta, foetal membranes and umbilical cord. The foetus also secretes fluid into the amniotic fluid from the renal tract, respiratory tract, skin and appendages.

There are two basic types of process which are responsible for the production and composition of amniotic fluid. These are referred to as exchange and net transfer. The first involves bidirectional diffusion across membranes in equal or nearly equal amounts in each direction. It is extensive in terms of the numbers of molecules involved (e.g. water exchanges at rates of 4-500 ml. per hour) but results in a neatly balanced system. It is operative throughout the whole of pregnancy and
during early pregnancy appears to be the only functional mechanism. Membranes such as the chorioamnion, foetal skin, foetal surface of the placenta and umbilical cord are involved.

The second mechanism, that of net transfer, is of lesser importance in terms of the number of molecules involved which are insignificant compared to exchange. In terms of the final composition of the fluid this process is the most important. The foetal skin keratinises and becomes impermeable at about the eighteenth week and from about the fourteenth week the foetal kidneys begin to function. Especially in the later stages of pregnancy foetal urination becomes a major source of fluid and foetal breathing movements point to a possible excretory function of the lungs. Foetal swallowing is the major source of removal of fluid and solutes during this transfer process.

The fluid consists of water with a large array of solutes. These include electrolytes, uric acid and creatinine, cholesterol, carbohydrates, organic acids, amino acids, hormones, enzymes (Table 11) and other proteins and lipids. In the fluid a number of viable intact cells as well as cell debris, vermix, non viable cells and organelles from disrupted cells are suspended. There are relatively few cells (2-3000/ml) before the twelfth week of gestation, but by the fourteenth week this has increased to about 30,000 cells/ml and this figure rises until the end of pregnancy. These cells are desquamated from the foetal skin, amnion, tracheobronchial tree, gut and urogenital tract and about 50% are viable at fourteen weeks. These viable cells are undergoing anaerobic metabolism and this may account for the high levels of lactic acid and related acids in the fluid (Table 6).

Although water, electrolytes and other diffusible compounds may originate from the mother, the amniotic cavity is a foetal compartment surrounded as it is by placenta and membranes all of which result from the products of conception. The amniotic fluid constituents therefore reflect the foetal condition despite the constant interchange across the placenta from the foetal to maternal circulation.
### TABLE 11
ENZYMES WHICH HAVE BEEN DETECTED IN AMNIOTIC FLUID,
UNCULTIVATED AMNIOTIC FLUID CELLS AND CULTIVATED
AMNIOTIC FLUID CELLS

<table>
<thead>
<tr>
<th>Amniotic Fluid</th>
<th>Uncultivated Amniotic Fluid Cells</th>
<th>Cultivated Amniotic Fluid Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid phosphatase</td>
<td>Acid phosphatase</td>
<td>Acid ceramidase</td>
</tr>
<tr>
<td>Aldolase</td>
<td>Aldolase</td>
<td>Acid lipase</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>Alkaline phosphatase</td>
<td>Acid phosphatase</td>
</tr>
<tr>
<td>a-Arabinosidase</td>
<td>a-Arabinosidase</td>
<td>Adenosine deaminase</td>
</tr>
<tr>
<td>a-Arabinosidase</td>
<td>a-Arabinosidase</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>a-Galactosidase</td>
<td>a-Galactosidase</td>
<td>a-Arabinosidase</td>
</tr>
<tr>
<td>a-1,4-Glucosidase</td>
<td>a-1,4-Glucosidase</td>
<td>a-Fucosidase</td>
</tr>
<tr>
<td>a-Mannosidase</td>
<td>a-Mannosidase</td>
<td>a-Galactosidase</td>
</tr>
<tr>
<td>a-Hexosaminidase</td>
<td>a-Hexosaminidase</td>
<td>a-1,4-Glucosidase</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>Acid phosphatase</td>
<td>a-Ketoisocaproate deacetylase*</td>
</tr>
<tr>
<td>a-Galactosidase</td>
<td>a-Galactosidase</td>
<td>a-L-iduronidase</td>
</tr>
<tr>
<td>a-Hexosaminidase</td>
<td>a-Hexosaminidase</td>
<td>a-Hexosaminidase</td>
</tr>
<tr>
<td>a-Galactosidase</td>
<td>a-Galactosidase</td>
<td>a-Hexosaminidase</td>
</tr>
<tr>
<td>a-1,4-Glucosidase</td>
<td>a-1,4-Glucosidase</td>
<td>a-Hexosaminidase</td>
</tr>
<tr>
<td>a-Fucosidase</td>
<td>a-Fucosidase</td>
<td>a-Hexosaminidase</td>
</tr>
<tr>
<td>a-Galactosidase</td>
<td>a-Galactosidase</td>
<td>a-Hexosaminidase</td>
</tr>
<tr>
<td>a-1,4-Glucosidase</td>
<td>a-1,4-Glucosidase</td>
<td>a-Hexosaminidase</td>
</tr>
</tbody>
</table>

* Enzymes associated with amino acidurias or organic acidurias.
Prenatal diagnosis using amniotic fluid

Amniotic fluid obtained by amniocentesis during early pregnancy offers three possible sources for diagnostic analysis: uncultured cells, cultured cells and amniotic fluid. The majority of prenatal diagnoses have been made using cultured cells. These have been used for cytogenetic studies, for the detection of chromosome abnormalities and for sex determination in the diagnosis of sex linked disorders. Cultured amniotic fluid cells have recently been used a great deal in the study of biochemical inborn errors known to be characterised by an enzyme deficiency or some other specific biochemical abnormality. Prenatal diagnosis of biochemical defects depends on the demonstration of the lack of the required enzyme or accumulation of the metabolites in the cells. The enzymes that have been found in cultivated amniotic fluid cells obtained during the mid trimester of pregnancy are outlined in Table 11.

The determination of normal ranges of enzyme activity in amniotic fluid cells is often difficult. Results may depend on factors such as substrate specificity, low levels of enzyme present, contamination with maternal cell lines, assay methods used, stability of the enzyme, cell cultivation and medium and length of time of cultivation. The cultured cells may also show different enzyme levels in different types of cell lines and the use of mixed cell cultures may give false results. Despite the delay and technical difficulties involved, cultivated amniotic fluid cells are the most reliable techniques for prenatal diagnosis of inborn errors of metabolism at present.

A second source of material for enzyme and metabolite analysis is uncultured amniotic fluid cells, but biochemical analysis of such cells has not been very successful. The problems of enzyme determination are similar to those in cultured cells with the further complication that a large but unknown percentage of cells are non viable and have no measurable enzyme activity. The other major problem is the low overall activity thus making microenzyme determination almost obligatory. Disorders such as Pompe's disease and Tay-Sachs disease have been diagnosed using uncultured cells and some intracellular storage diseases have been diagnosed by electron microscopy of such cells.

Prenatal diagnosis of biochemical metabolic defects using these
**TABLE 12**

**BIOCHEMICAL DISORDERS THAT HAVE BEEN DIAGNOSED PRENATALLY**

*(AFTER EPSTEIN AND GOLBUS)*

<table>
<thead>
<tr>
<th>DISORDER</th>
<th>METABOLIC DEFECT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid phosphatase deficiency</td>
<td>Lysosomal acid phosphatase deficiency</td>
</tr>
<tr>
<td>Adenosine deaminase deficiency (combined immunodeficiency)</td>
<td>Adenosine deaminase deficiency</td>
</tr>
<tr>
<td>Adrenogenital syndrome</td>
<td>C-11 or C-21 steroid hydroxylase deficiency</td>
</tr>
<tr>
<td>Argininosuccinic aciduria†</td>
<td>Argininosuccinic acid synthetase deficiency</td>
</tr>
<tr>
<td>Citrullinemia†</td>
<td>Argininosuccinic acid synthetase deficiency</td>
</tr>
<tr>
<td>Cystinosis</td>
<td>Cystine accumulation</td>
</tr>
<tr>
<td>Fabry disease</td>
<td>Ceramidetrihexoside α-galactosidase deficiency</td>
</tr>
<tr>
<td>Fucosidosis</td>
<td>α-Fucosidase deficiency</td>
</tr>
<tr>
<td>Galactokinase deficiency</td>
<td>Galactokinase deficiency</td>
</tr>
<tr>
<td>Galactosemia</td>
<td>Galactose-1-phosphate uridylyltransferase deficiency</td>
</tr>
<tr>
<td>Generalized gangliosidosis (GM1 gangliosidosis, Type I)</td>
<td>5-Galactosidase deficiency</td>
</tr>
<tr>
<td>Juvenile gangliosidosis (GM1 gangliosidosis, Type II)</td>
<td>5-Galactosidase deficiency</td>
</tr>
<tr>
<td>Gaucher disease</td>
<td>Glucocerebrosidase deficiency</td>
</tr>
<tr>
<td>Glycogen storage disease, Type II (Pompe disease)</td>
<td>α-1,4-Glucosidase deficiency</td>
</tr>
<tr>
<td>Hemoglobinopathy (sickle cell)</td>
<td>Synthesis of hemoglobin S</td>
</tr>
<tr>
<td>Hunter syndrome</td>
<td>α-L-iduronidase deficiency</td>
</tr>
<tr>
<td>Hurler syndrome</td>
<td>β-Galactosidase deficiency</td>
</tr>
<tr>
<td>Hypophosphatemia (some types)</td>
<td>Alkaline phosphatase deficiency</td>
</tr>
<tr>
<td>I-cell disease</td>
<td>Multiple lysosomal enzyme deficiencies</td>
</tr>
<tr>
<td>Isovaleric acidemia</td>
<td>Isovaleryl CoA dehydrogenase deficiency</td>
</tr>
<tr>
<td>Ketotic hyperglycinemia</td>
<td>Propionyl CoA carboxylase deficiency</td>
</tr>
<tr>
<td>Krabbe disease</td>
<td>Galactocerebrosidase β-galactosidase deficiency</td>
</tr>
<tr>
<td>Lesch-Nyhan syndrome</td>
<td>Hypoxanthine guanine phosphoribosyltransferase deficiency</td>
</tr>
<tr>
<td>Maple syrup urine disease†</td>
<td>Branched chain ketoacid dehydrogenase deficiency</td>
</tr>
<tr>
<td>Maroteaux-Lamy syndrome</td>
<td>Arylsulfatase A deficiency</td>
</tr>
<tr>
<td>Menkes disease</td>
<td>Copper accumulation</td>
</tr>
<tr>
<td>Metachromatic leukodystrophy</td>
<td>Methylmalonic aciduria</td>
</tr>
<tr>
<td>Niemann-Pick disease</td>
<td>Methylmalonic CoA mutase deficiency</td>
</tr>
<tr>
<td>Placental sulfatase deficiency</td>
<td>Sphingomyelinase deficiency</td>
</tr>
<tr>
<td>Porphyria - acute intermittent type</td>
<td>Placental sulfatase deficiency</td>
</tr>
<tr>
<td>Pyruvate decarboxylase deficiency</td>
<td>Uroporphyrinogen I synthetase deficiency</td>
</tr>
<tr>
<td>Sandhoff disease</td>
<td>Pyruvate dehydrogenase deficiency</td>
</tr>
<tr>
<td>Sanfilippo syndrome, Type A</td>
<td>Hexosaminidase A and B deficiency</td>
</tr>
<tr>
<td>Sanfilippo syndrome, Type B</td>
<td>N-acetyl-α-glucosaminidase deficiency</td>
</tr>
<tr>
<td>Tay-Sachs disease</td>
<td>Hexosaminidase A deficiency</td>
</tr>
<tr>
<td>α-Thalassemia</td>
<td>Decreased synthesis of α chain of hemoglobin</td>
</tr>
<tr>
<td>β-Thalassemia</td>
<td>Decreased synthesis of β chain of hemoglobin</td>
</tr>
<tr>
<td>Wolman disease</td>
<td>Acid lipase deficiency</td>
</tr>
<tr>
<td>Xerodermia pigmentosa</td>
<td>UV endonuclease deficiency</td>
</tr>
</tbody>
</table>

* Organic acidemias
† Amino acidurias
methods have been practised for the last ten years and some one
thousand cases have been studied \(^{389,418,443}\) (Table 12 after
Epstein and Golbus \(^{389}\)).

Cell free amniotic fluid has also been examined and used for
prenatal diagnosis. A number of metabolic diseases such as
Hunters syndrome and Tay-Sachs disease have been diagnosed on the
basis of enzyme deficiency in the cell free fluid but the origin and
characteristics of the enzymes present (see Table 11) are largely
unknown.

The soluble components of amniotic fluid have also been subjected
to various types of analysis. \(^{456,437}\) Amniotic fluid contains one
tenfold to one twentieth of the protein content of serum and some attempts
have been made to study protein disorders. The study of alpha-fetoprotein
for the diagnosis of neural tube defects is a notable exception
and screening methods are available using maternal serum levels to
indicate a high risk group in which amniocentesis should be performed.

The screening for metabolites in amniotic fluid has not proved very
useful, however, few representative studies are available. The
increasing foetal contribution to the fluid during pregnancy should
cause the concentration of the metabolites to increase in the fluid.
This has been demonstrated for the concentration of methylmalonic
acid in a foetus affected with methylmalonic aciduria \(^{446}\) and for steroids
in a case of adrenogenital syndrome. \(^{447,448}\) It should be noted that
no abnormality was shown in amniotic fluid branched chain amino acid
levels in one foetus affected with maple syrup urine disease. \(^{449}\)
Morrow \(^{450}\) has suggested that the reason for this is that the maternal
circulation can clear the foetal circulation of the particular
metabolite, especially if the metabolite can be used by maternal cells.
In the case of methylmalonic aciduria however the active substrate is
methylmalonyl coenzyme-A and this must be hydrolysed before it can
diffuse into the foetal extracellular space. Thus MMA appears in the
amniotic fluid and is carried via the maternal circulation to appear in
the maternal urine. This process should occur for other organic acids
thus making metabolite measurement suitable for prenatal organic
acidemia diagnosis. Thus examination of amniotic fluid and maternal urine could be a useful adjunct to cell culture in prenatal diagnosis of organic acidemias. Up to now experience has been limited to methylmalonic aciduria and further developments in this field must await suitable techniques and application to other diseases.

AIMS OF THIS WORK

The use of GC-MS profiling of metabolites in amniotic fluid offers the possibility of fast accurate prenatal and perinatal diagnosis of metabolic disease. The aim of the work reported in this thesis is the study of metabolic disease of genetic origin using techniques of GC and GC-MS with special emphasis on the prenatal diagnosis of such disease in cases where the risk of an affected foetus is known to be high. The low levels of acidic metabolites present in amniotic fluid reported in the profiling studies (Table 6) and the presence of relatively high levels of protein in the fluid suggested the need for quantitative recovery studies and methods suitable for accurate precise analysis.
RESULTS AND DISCUSSION
PRENATAL DIAGNOSIS OF INHERITED DISEASE
BY METABOLIC PROFILING

a. DEVELOPMENT OF METHODS FOR THE ANALYSIS OF METABOLITES IN AMNIOTIC FLUID

The diagnosis of organic acidurias and acidemias depends on the identification and quantitation of organic acids present in biological fluids. In order to examine the use of metabolite concentrations in amniotic fluid for the prenatal diagnosis of metabolic diseases accurate methods of quantitation of these metabolites must be available. This analytical problem may be divided into two parts, namely the separation of the acids from the matrix and the derivatisation and analysis of the organic acids. Very little has been reported on the isolation and quantitation of organic acids from amniotic fluid. Hagenfeldt and Hagenfeldt quantitated six acids in amniotic fluid and identified nine other acids by using a perchloric acid protein precipitation method that they developed for serum. Nichols et al. did not pretreat the fluid before extraction but used a preliminary basic extraction to remove neutral and basic substances which interfered with the GC profile. Other early work in the quantitation of specific acids in amniotic fluid relied on colourimetric or enzymatic methods of analysis using perchloric deproteinised amniotic fluid.

Results for the quantitation of methylmalonic acid (MMA) in amniotic fluid have been published. Morrow et al. used automatic column chromatography on silicic acid for separation and quantitation of MMA (I) using untreated amniotic fluid. The same group has recently reported a method in which amniotic fluid is saturated with sodium chloride, acidified and extracted in similar fashion to a urine specimen. Rosenberg and co-workers have reported the preliminary separation of MMA (I) using an ion exchange column followed by extraction of the column eluate.

Despite this variation in reported methods Jellum's review states that due to low protein levels in amniotic fluid acidic metabolites may be extracted from it using the same methods as those developed for urine.
### TABLE 13
RECOVERY OF MMA (I) AND ADIPIC ACID (II) FROM AMNIOTIC FLUID AND URINE USING SOLVENT EXTRACTION AND ION EXCHANGE METHODS

<table>
<thead>
<tr>
<th>SPECIMEN</th>
<th>TOTAL PROTEIN</th>
<th>ACID</th>
<th>RECOVERY(^{a})(%)</th>
<th>Solvent Extr. Method (157)</th>
<th>Ion Exchange Methods</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MMA (I)</td>
<td></td>
<td>Solvent Extr. Method (157)</td>
<td>Ion Exchange Methods</td>
</tr>
<tr>
<td>Amniotic Fluid Pool 1 (14-17 weeks)</td>
<td>5.6 g/l</td>
<td></td>
<td></td>
<td>Solvent Extr. Method (157)</td>
<td>Ion Exchange Methods</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Solvent Extr. Method (157)</td>
<td>Ion Exchange Methods</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MMA (I)</td>
<td></td>
<td>Solvent Extr. Method (157)</td>
<td>Ion Exchange Methods</td>
</tr>
<tr>
<td>Amniotic Fluid Pool 2 (35 weeks)</td>
<td>2.9 g/l</td>
<td>MMA (I)</td>
<td></td>
<td>Solvent Extr. Method (157)</td>
<td>Ion Exchange Methods</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adipic (II)</td>
<td></td>
<td>Solvent Extr. Method (157)</td>
<td>Ion Exchange Methods</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MMA (I)</td>
<td></td>
<td>Solvent Extr. Method (157)</td>
<td>Ion Exchange Methods</td>
</tr>
<tr>
<td>Urine</td>
<td></td>
<td>MMA (I)</td>
<td></td>
<td>Solvent Extr. Method (157)</td>
<td>Ion Exchange Methods</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adipic (II)</td>
<td></td>
<td>Solvent Extr. Method (157)</td>
<td>Ion Exchange Methods</td>
</tr>
</tbody>
</table>

\(^{a}\) Results are expressed as mean % recovery ± 2 x SD and were obtained by analysing at least 3 samples by GC of the TMS derivatives on Apiezon-N.
For our initial investigation one solvent extraction and two ion exchange methods were selected for detailed examination. The solvent extraction method was modified to use ethyl acetate rather than diethyl ether as extracting solvent as the latter caused stable gel formation with some samples of amniotic fluid. Initial experiments were based on extracting acidified amniotic fluid directly but a large number of extraneous substances were co-extracted which interfered with the GC analysis of some acids. This problem was overcome by pre-extraction of the interfering components at pH 7-9. This method gave a recovery of aqueous 96% when standard solutions of acids were used.

The two ion exchange methods examined were Giorgio and Plout's procedure which uses Dowex 3x4 resin for absorption of organic acids, elution with dilute HCl followed by neutralisation and lyophilisation and Thompson and Markey's method which uses barium hydroxide precipitation of sulphate and phosphate followed by absorption of the acids on DEAE-Sephadex followed by elution with pyridinium acetate and lyophilisation. Both methods gave measured recoveries of greater than 95% when standard solutions of acids were used. Both methods were tried on samples of pooled amniotic fluid. One pool was derived from fluid obtained at 14-17 weeks gestation and the other pool was derived from fluid obtained after 35 weeks gestation. For comparison the methods were also tried on a urine sample. MMA (I) and adipic acid (II) were added to the samples and the samples were worked up as described above and the acids were quantitated on the GC as the TMS derivatives.

The results obtained for the 2 amniotic fluids and urine samples are shown in Table 13. These results show low recoveries of acids by all methods and wide variations in recovery between the two acids.

The lower recoveries of MMA (I) may be due to the ready decarboxylation of the acid in strongly basic or acidic solution but this loss was not seen when standard solutions or urine were used. This loss then must be due to the sample matrix. The major difference between the urine and amniotic fluid samples is the presence of protein.
TABLE 14

RECOVERY OF MMA (I) AND ADIPIC ACID (II)
FROM DILUTED SERUM\textsuperscript{a} USING SOLVENT EXTRACTION

<table>
<thead>
<tr>
<th>DILUTION OF SERUM\textsuperscript{a} (Parts H\textsubscript{2}O added)</th>
<th>APPROXIMATE PROTEIN CONCENTRATION\textsuperscript{b}</th>
<th>% RECOVERY\textsuperscript{c}</th>
<th>MMA (I)</th>
<th>Adipic Acid (II)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nil</td>
<td>g/l</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>80</td>
<td>2-14</td>
<td>64-77</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>40</td>
<td>6-19</td>
<td>70-82</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>26</td>
<td>10-24</td>
<td>76-85</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>72-79</td>
<td>92-98</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} Pool obtained from 100 healthy blood donors

\textsuperscript{b} Measured colourimetrically on Technicon Autoanalyser.

\textsuperscript{c} Results are range of mean % recovery ± 2 SD for 3 samples by GC of the TMS derivatives on Apiezon-N.
The amniotic fluid protein content however varies with the gestation period from 0.7-8.2 g/l (mean 3.85 g/l) at 13 weeks to a peak of 4.0-8.0 g/l (mean 6.28 g/l) at 25-28 weeks and gradually falls to about 1.5-7.0 g/l (mean 2.96 g/l) at term. The recovery of MMA (I) from the amniotic fluid samples is less for those samples with a higher protein content and this suggests that the variable protein content is responsible for the low recoveries. When Giorgio and Plout's method was used interfering peaks due to phosphate and sulphate TMS derivatives made the quantitation of some organic acids difficult. This result was unexpected as the reported concentration of phosphate and sulphate in amniotic fluid are comparable to those in serum which are 10-20 times less than those found in urine. However much of the phosphate (~85%) and sulphate (~50%) in serum and amniotic fluid are combined as esters and the free ions may be released on hydrolysis. The levels of organic acids present however is much lower than those in urine and the increased sensitivity required for the analysis increases the effect of the presence of sulphate and phosphate. Thompson and Markey suggested a precipitation of sulphate and phosphate ions with barium hydroxide to remove this interference but since this step may lead to the removal of organic acids as insoluble barium salts, the ion exchange approach was abandoned.

In order to test the suggestion that the increased protein content could interfere with the extraction of organic acids, standard amounts of MMA (I) and adipic acid (II) were added to diluted pool serum and the samples were worked up and quantitated as described above. The protein content of the solutions varied from 8-80 g/l. The results obtained in these experiments are shown in Table 14. These results show that as the protein content of the solution is increased the recovery of both acids falls and this effect is more pronounced for MMA (I). The reason for the lower recovery of MMA is not clear but similar results were also obtained when other substituted malonic acids such as ethyl malonic acid (III) or propylmalonic acid (IV) were extracted from serum or amniotic fluid. The results also highlight the variability in the extraction of the acid even when identical matrices were used and that the reproducibility of the extraction increases as the protein content falls.
<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>TOTAL PROTEIN</th>
<th>ACID</th>
<th>RECOVERY&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2 µg/ml added acids</td>
<td>5 µg/ml added acids</td>
</tr>
<tr>
<td>Amniotic Fluid Pool 1</td>
<td>5.6 g/l</td>
<td>MMA (I)</td>
<td>29 ± 7</td>
</tr>
<tr>
<td>(14-17 weeks)</td>
<td></td>
<td>Adipic</td>
<td>79 ± 6</td>
</tr>
<tr>
<td>Amniotic Fluid Pool 2</td>
<td>2.9 g/l</td>
<td>MMA (I)</td>
<td>39 ± 8</td>
</tr>
<tr>
<td>(35 weeks)</td>
<td></td>
<td>Adipic (II)</td>
<td>85 ± 7</td>
</tr>
<tr>
<td>Serum</td>
<td>80 g/l</td>
<td>MMA (I)</td>
<td>3 ± 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adipic (II)</td>
<td>62 ± 7</td>
</tr>
<tr>
<td>Serum diluted with 2 parts H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>26 g/l</td>
<td>MMA (I)</td>
<td>15 ± 8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adipic (II)</td>
<td>64 ± 7</td>
</tr>
<tr>
<td>Standard Solution</td>
<td></td>
<td>MMA (I)</td>
<td>94 ± 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adipic (II)</td>
<td>97 ± 2</td>
</tr>
</tbody>
</table>

<sup>a</sup> Results are expressed as mean % recovered ± 2 SD for 3 samples and were obtained by GC analysis of the TMS derivatives on Apiezon-N.
<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>TOTAL PROTEIN BEFORE ULTRAFILTRATION</th>
<th>ACID</th>
<th>RECOVERY&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>2 µg/ml</td>
</tr>
<tr>
<td>Amniotic Fluid</td>
<td>5.6 g/1</td>
<td>MMA (I)</td>
<td>26 ± 5</td>
</tr>
<tr>
<td>Pool 1 (14-17 weeks)</td>
<td></td>
<td>Adipic (II)</td>
<td>74 ± 8</td>
</tr>
<tr>
<td>Amniotic Fluid</td>
<td>2.9 g/1</td>
<td>MMA (I)</td>
<td>37 ± 4</td>
</tr>
<tr>
<td>Pool 2 (35 weeks)</td>
<td></td>
<td>Adipic (II)</td>
<td>83 ± 9</td>
</tr>
<tr>
<td>Serum</td>
<td>80 g/1</td>
<td>MMA (I)</td>
<td>6 ± 6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adipic (II)</td>
<td>60 ± 5</td>
</tr>
<tr>
<td>Serum Diluted with</td>
<td>20 g/1</td>
<td>MMA (I)</td>
<td>13 ± 7</td>
</tr>
<tr>
<td>3 parts H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td></td>
<td>Adipic (II)</td>
<td>81 ± 5</td>
</tr>
<tr>
<td>Standard Acids</td>
<td></td>
<td>MMA (I)</td>
<td>91 ± 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adipic (II)</td>
<td>94 ± 3</td>
</tr>
</tbody>
</table>

<sup>a</sup> Results are expressed as mean % recovery ± 2 SD for 3 samples and were obtained by GC analysis of the TMS derivatives on Apiezon-N.
These results suggest that the protein content of the amniotic fluid is responsible for the organic acid analysis problems and alternative methods of removing protein from the samples should be examined.

In order to determine whether the acids could be retained in the water fraction of the fluids an attempt was made to separate the protein and the MMA (I) and adipic acids (II) using gel filtration. Molecular sieve chromatography using Sephadex G-10 resin was used to separate the high and low molecular weight fractions of serum and amniotic fluid which contained the added MMA (I) and adipic acids (II). The results of these experiments are shown in Table 15. The recovery of a standard solution of acids from the low molecular weight fraction is virtually quantitative but that from the various protein containing fluids is low and variable.

The possibility of separating the acids from the protein by washing was also examined. An ultrafiltrate of fluids was obtained by centrifugation in a size selective semi-permeable membrane cone which retained compounds with a molecular weight of greater than 25,000. The sample was ultrafiltered and the residue made up to the original volume of the sample and further centrifuged. A sample of standard acids gave recovery of 85% in the first centrifugation. The protein content of the ultrafiltrates as measured by trichloracetic acid turbidimetry was less than 100 mg/l. The results of these experiments (Table 16) show that the acids could not be washed from the protein and again show the lower recoveries of added acids from solutions with higher protein concentration. These results suggested that the assumption that the protein content of amniotic fluid is too low to cause interference is incorrect and that the presence of protein was causing the low recoveries of the organic acids.

The usual method of removing such interference in a clinical chemistry method is by precipitation of the protein. Precipitation involves the changing of protein structure to lower the solubility of the molecules. Proteins carry both positive and negative charges and the precipitation process depends on factors such as pH of the solution and dielectric properties of the solvent. There have been a large number of methods developed to achieve this, most of which leave small amounts of protein in the solution. However, such techniques must be studied to ensure that
### TABLE 17

**RECOVERY OF MMA (I) AND ADIPIC ACIDS (II)**

FROM AMNIOTIC FLUID USING PROTEIN PRECIPITATION METHODS

<table>
<thead>
<tr>
<th>REAGENT</th>
<th>% RECOVERY&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% RECOVERY&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SAMPLE</td>
<td>17 weeks</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Amniotic Fluid Pool 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(14-17 weeks)</td>
</tr>
<tr>
<td>ACID</td>
<td></td>
<td>MMA (I)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adipic (II)</td>
</tr>
<tr>
<td>Ethanol (371)</td>
<td>34-47</td>
<td>86-93</td>
</tr>
<tr>
<td>Methanol (152)</td>
<td>32-46</td>
<td>84-94</td>
</tr>
<tr>
<td>Heat &amp; Acetic Acid (161)</td>
<td>46-53</td>
<td>92-96</td>
</tr>
<tr>
<td>Perchloric Acid (235)</td>
<td>10-24</td>
<td>29-47</td>
</tr>
<tr>
<td>Sulphosalicylic Acid (455)</td>
<td>9-21</td>
<td>31-53</td>
</tr>
<tr>
<td>Tungstic Acid (142)</td>
<td>11-30</td>
<td>32-50</td>
</tr>
<tr>
<td>Zinc Sulphate + Sodium Hydroxide (142)</td>
<td>31-46</td>
<td>80-92</td>
</tr>
<tr>
<td>Zinc Sulphate + Barium Hydroxide (Somogyi) (142)</td>
<td>32-45</td>
<td>80-94</td>
</tr>
</tbody>
</table>

<sup>a</sup> Results are expressed as the range of recovery of the acids obtained from 2 samples which were analysed by GC of the TMS derivatives on Apiezon-N.
<table>
<thead>
<tr>
<th>REAGENT</th>
<th>REF.</th>
<th>SAMPLE</th>
<th>Serum</th>
<th>Serum Diluted with 3 parts H₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>MMA (I)</td>
<td>Adipic (II)</td>
</tr>
<tr>
<td>Ethanol</td>
<td>371</td>
<td>3-17</td>
<td>62-80</td>
<td>6-22</td>
</tr>
<tr>
<td>Methanol</td>
<td>152</td>
<td>4-20</td>
<td>64-80</td>
<td>6-26</td>
</tr>
<tr>
<td>Heat + Acetic Acid</td>
<td>161</td>
<td>25-46</td>
<td>66-86</td>
<td>28-50</td>
</tr>
<tr>
<td>Perchloric Acid</td>
<td>235</td>
<td>0-12</td>
<td>30-55</td>
<td>2-18</td>
</tr>
<tr>
<td>Sulphosalicylic Acid</td>
<td>455</td>
<td>2-16</td>
<td>49-65</td>
<td>0-14</td>
</tr>
<tr>
<td>Tungstic Acid</td>
<td>142</td>
<td>3-17</td>
<td>37-62</td>
<td>7-24</td>
</tr>
<tr>
<td>Zinc Sulphate + Sodium Hydroxide</td>
<td>142</td>
<td>12-26</td>
<td>82-94</td>
<td>16-29</td>
</tr>
<tr>
<td>Zinc Sulphate + Barium Hydroxide (Somogyi)</td>
<td>142</td>
<td>14-32</td>
<td>81-97</td>
<td>16-35</td>
</tr>
</tbody>
</table>

a Results are expressed as the range of recoveries of the acid obtained from 2 samples which were analysed by GC of the TMS derivative on Apiezon-N.
the analyte does not precipitate with the protein, that there is no interference from reagents and that there is no decomposition of the analyte or any other methodological bias in the result.

Proteins may be precipitated by either physical or chemical means. Physical methods include ultrafiltration, microdiffusion and heat denaturation. Chemical techniques include antigen-antibody reactions, protein dehydration by use of organic solvents and insoluble salt formation with reagents such as perchloric acid, sulphasalicylic acid or tungstic acid. The general nature of these chemical precipitation reactions appears fairly certain although some details remain obscure.

A number of reports of the application of these methods to organic acids in serum have appeared in the literature. The most often reported method for the measurement of MMA (I) in serum is that developed by Oberholzer et al\(^1\) in which serum is diluted thirty times in dilute acetic acid and the protein is denatured by heating. Methods using organic solvents to dehydrate protein molecules and to lower the dielectric content of the solution have been used for the examination of organic acids in serum, disrupted cells and other protein containing fluids. Acetone,\(^1\) methanol\(^131,152,153\) and ethanol\(^144-146,371\) have all been used in proportions varying from one to ten volumes of solvent for each volume of serum. Precipitation methods using perchloric acid\(^235\) and sulphasalicylic\(^455\) acid have also been used to examine MMA (I) and other organic acids in serum. Unfortunately in all these reported methods for organic acids no recovery experiments were reported.

We have now carried out recovery studies using Oberholzer et al's\(^1\) method, alcohol precipitation\(^152,371\) and a variety of anionic\(^142,235,455\) and cationic\(^142\) precipitating agents with both amniotic fluid and serum. (Tables 17, 18) In each case the use of standard solutions of acids gave recoveries of greater than 90%. The highest recoveries in the experiments were obtained using the more gentle physical precipitation method of Oberholzer et al.\(^1\) The anionic precipitating agents such as perchloric and sulphasalicylic acids gave the lowest recoveries suggesting that the free acids are precipitated with the protein. The cationic precipitating
FIGURE 6. Recovery of MMA and adipic acid from amniotic fluid at various dilutions. 5ug/ml acid added.

FIGURE 7. Recovery of MMA and adipic acids from serum at various dilutions. 5ug/ml acid added.
FIGURE 8. Recovery of MMA and adipic acids from amniotic fluid after samples allowed to stand at pH 10 before acidification and extraction of organic acids.

FIGURE 9. Recovery of MMA and adipic acids from serum after samples allowed to stand at pH 10 before acidification and extraction of organic acids.
### TABLE 19

**RECOVERY OF MMA (I) AND ADIPIC ACIDS (II)**

FROM AMNIOTIC FLUID AND SERUM USING SODIUM DODECYL SULPHATE

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>ACID</th>
<th>% RECOVERY^d</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No SDS Present</td>
</tr>
<tr>
<td>Amniotic Fluid Pool 1\textsuperscript{a}</td>
<td>MMA (I)</td>
<td>78 ± 5</td>
</tr>
<tr>
<td>(14-17 weeks)</td>
<td>Adipic Acid (II)</td>
<td>93 ± 3</td>
</tr>
<tr>
<td>Amniotic Fluid Pool 2\textsuperscript{a}</td>
<td>MMA (I)</td>
<td>79 ± 4</td>
</tr>
<tr>
<td>(35 weeks)</td>
<td>Adipic Acid (II)</td>
<td>93 ± 3</td>
</tr>
<tr>
<td>Serum\textsuperscript{b}</td>
<td>MMA (I)</td>
<td>78 ± 4</td>
</tr>
<tr>
<td></td>
<td>Adipic Acid (II)</td>
<td>95 ± 3</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Amniotic fluid samples were diluted with water containing SDS (2 ml) and allowed to stand at pH9-11 for 20 mins. before extraction.

\textsuperscript{b} Serum was diluted with water (9 ml) containing SDS and allowed to stand at pH9-11 for 20 mins. before extraction.

\textsuperscript{c} Final concentration in the solution 0.5%.

\textsuperscript{d} Results were reported as mean % recovery ± 2 SD and were obtained by analysing 5 samples by GC of the TMS derivatives on Apiezon-N.
agents such as zinc sulphate and the Somogyi procedure give higher but variable levels of recoveries as do the alcohol precipitation methods. The use of alcohol as precipitating agent gave some problems in the GC analysis due to the presence of a number of extra peaks which interfere with the organic acid determinations. These were presumably due to the presence of lipid materials and other small molecules.

The higher recovery obtainable from the slow precipitation in Oberholzer's method and in the diluted serum specimens suggested that the effect of diluting the protein containing fluids as well as the time required for the dissociation of the acids from the protein be investigated. Warner and Vahouny have already shown that standing in alkaline solutions is important for the dissociation of chemically bound MMA (I) and succinic acids (IX) in tissue homogenates.

A series of experiments were carried out to find the optimum dilution for serum and amniotic fluid to obtain maximum recoveries of the acids. (Figs. 6 and 7) These results were obtained by adding varying amounts of water to the fluid before extraction from alkali to remove neutral compounds, acidification and extraction of the acids. The results show that the addition of 2-3ml water/ml fluid gives maximum recovery of the two acids from amniotic fluid and the addition of 8-10ml of water is required per ml of serum.

Experiments showed that the time of standing in acid solution before extraction of the acids had no measurable effect but that when the solutions were allowed to stand at pH 9-11 the recovery of the acids was improved. (Figs. 8 and 9). For both the amniotic fluid and serum the optimum time of standing at alkaline pH is 15 to 20 minutes. This time presumably represents the time required to dissociate the tightly bound acids from the protein.

The addition of sodium dodecylsulphate (SDS) was also investigated and was shown to improve the recoveries of the acids. (Table 19). These results show that the recovery of the acids was improved by about 5% when SDS was used. The use of SDS resulted in a number of additional peaks on
<table>
<thead>
<tr>
<th>ACID</th>
<th>LEVEL ADDED</th>
<th>RECOVERIES&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Amniotic Fluid Pool 1 (14-17 weeks)</th>
<th>Amniotic Fluid Pool 2 (35 weeks)</th>
<th>Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Absolute</td>
<td>Relative</td>
<td>Absolute</td>
<td>Relative</td>
</tr>
<tr>
<td>Adipic</td>
<td>5 µg</td>
<td>95 ± 3</td>
<td>95 ± 4</td>
<td>95 ± 4</td>
<td>97 ± 4</td>
</tr>
<tr>
<td></td>
<td>2 µg</td>
<td>96 ± 4</td>
<td>95 ± 3</td>
<td>95 ± 3</td>
<td>97 ± 4</td>
</tr>
<tr>
<td>Succinic</td>
<td>5 µg</td>
<td>84 ± 3</td>
<td>87 ± 3</td>
<td>85 ± 4</td>
<td>89 ± 4</td>
</tr>
<tr>
<td></td>
<td>2 µg</td>
<td>85 ± 3</td>
<td>86 ± 3</td>
<td>85 ± 4</td>
<td>88 ± 4</td>
</tr>
<tr>
<td>Glutaric</td>
<td>5 µg</td>
<td>82 ± 3</td>
<td>86 ± 3</td>
<td>84 ± 3</td>
<td>87 ± 3</td>
</tr>
<tr>
<td></td>
<td>2 µg</td>
<td>83 ± 4</td>
<td>87 ± 4</td>
<td>83 ± 3</td>
<td>87 ± 3</td>
</tr>
<tr>
<td>Levulinic</td>
<td>5 µg</td>
<td>71 ± 6</td>
<td>74 ± 7</td>
<td>74 ± 5</td>
<td>77 ± 5</td>
</tr>
<tr>
<td></td>
<td>2 µg</td>
<td>69 ± 4</td>
<td>73 ± 4</td>
<td>70 ± 4</td>
<td>74 ± 4</td>
</tr>
<tr>
<td>2-Hydroxyisocaproic</td>
<td>5 µg</td>
<td>70 ± 5</td>
<td>73 ± 5</td>
<td>73 ± 3</td>
<td>77 ± 5</td>
</tr>
<tr>
<td></td>
<td>2 µg</td>
<td>71 ± 5</td>
<td>75 ± 4</td>
<td>73 ± 4</td>
<td>76 ± 4</td>
</tr>
<tr>
<td>Phenylacetic</td>
<td>5 µg</td>
<td>86 ± 7</td>
<td>90 ± 7</td>
<td>87 ± 3</td>
<td>91 ± 3</td>
</tr>
<tr>
<td></td>
<td>2 µg</td>
<td>86 ± 4</td>
<td>91 ± 4</td>
<td>86 ± 4</td>
<td>90 ± 4</td>
</tr>
<tr>
<td>p-Hydroxyphenylacetic</td>
<td>5 µg</td>
<td>93 ± 5</td>
<td>97 ± 5</td>
<td>94 ± 3</td>
<td>98 ± 3</td>
</tr>
<tr>
<td></td>
<td>2 µg</td>
<td>89 ± 3</td>
<td>94 ± 3</td>
<td>94 ± 3</td>
<td>99 ± 4</td>
</tr>
<tr>
<td>Myristic</td>
<td>5 µg</td>
<td>99 ± 4</td>
<td>103 ± 4</td>
<td>98 ± 5</td>
<td>102 ± 5</td>
</tr>
<tr>
<td></td>
<td>2 µg</td>
<td>100 ± 3</td>
<td>105 ± 3</td>
<td>98 ± 4</td>
<td>103 ± 4</td>
</tr>
</tbody>
</table>

<sup>a</sup> Results are reported as mean % recovery ± 2 SD and were obtained by analysing 3 samples by GC as the TMS derivatives on Apiezon-N. Pimelic acid was used as external standard and Adipic acid (II) used as internal standard.
FIGURE 10. GC profile from SE-30 packed column of the silylated organic acid extract from normal amniotic fluid.

The peaks were identified as: 1, lactic acid; 2, 2-hydroxybutyric acid; 3, 3-hydroxybutyric acid; 4, urea; 5, glycerol; 6, succinic acid; 7, phosphoric acid; 8, adipic acid (internal standard); 9, malic acid; 10, citric acid; 11, di-n-butyl phthalate; 12, palmitic acid; 13, linoleic acid; 14, stearic acid.
The peaks were identified as: 1, 3-hydroxybutyric acid; 2, 2-hydroxy-3-methylbutyric acid; 3, 2-hydroxybutyric acid; 4, succinic acid; 5, phosphoric acid; 6, glutaric acid; 7, adipic acid; 8, dodocyl-1-ol; 9, pimelic acid (internal standard); 10, dodocane; 11, dodocysulphuric acid; 12, citric acid. SS = solvent suppression.
FIGURE 12. GC profile from the SE-30 packed column of the silylated organic acid extract from normal serum.

FIGURE 13. GC profile from SE-30 packed column of the silylated extract from amniotic fluid at alkaline pH.

Temperature programmed at 10°C per minute.
the GC after derivatisation. These were shown to be due to the presence of dodecylsulphuric acid-\text{\textregistered}MS, dodecyl-1-ol-\text{\textregistered}MS and the related homologous compounds. These compounds were present in the reagent, and the alcohol and acid components increased when the reagent was kept for some time. The presence of the acids and alcohol peaks could be minimised by the use of a freshly prepared solution of SDS. The presence of these peaks in the GC profile did not interfere with the measurement of any of the acids studied but where this possibility exists the lower recoveries of acids obtainable without SDS must be tolerated.

The increased recoveries in the presence of SDS may be explained by its mode of action. This chemical interacts with the water layers associated with the surface of protein molecules and alters the tertiary structure of the molecules to give globulin like molecules. It is a gentle method of denaturing protein which gives molecules which are usually insoluble under acid conditions but soluble in base. This explains the precipitation of the protein when the specimens were acidified to pH 1.

The extraction of a series of acids of different types was measured using the above method. Known amounts of the acids were added to samples of amniotic fluid and serum pools and the recovery of each measured. The results of this study are presented in Table 20. These results show that the added acids were all extracted with reasonable recoveries but only the non polar fatty acid was extracted quantitatively. The reproducibility of extraction is high and even when lower recoveries are obtained this allows accurate quantitation of the acids in amniotic fluid and serum. The use of this solvent extraction method thus allows fast accurate quantitation of the metabolite in amniotic fluid or serum samples.

The normal profiles of amniotic fluid and serum obtained using this method are shown in Figs. 10 to 12 and the profile of the alkaline extract of serum is shown in Fig. 13. This alkaline extract contains a large number of components which were not identified but included phenols, aldehydes, ketones and alcohols.
MALONIC ACID DIETHYL ESTER
TRIDEOUTEROMETHYL MALONIC ACID DIETHYL ESTER (V)
TRIDEOUTEROMETHYL MALONIC ACID (VII)

FIGURE 14. Synthesis of $^2H_3$-MMA.

FIGURE 15. CI-MIS calibration curve for MMA.
The use of this method allowed the quantitation of organic acids in amniotic fluid samples with a lower limit of detection of approximately 0.3 μg/ml and a precision of 0.1 μg/ml. Variations of MMA (I) concentration in duplicate samples of less than 4% were obtained using this method.

To overcome the inherent difficulties of measurement of small amounts of organic acids in amniotic fluid and serum, an isotope dilution method was considered to validate the analysis of MMA (I). Trideuteromethylmalonic acid diethyl ester (V) was prepared by the malonic ester synthesis using trideuteromethyl iodide (VI) (Fig. 14). The ester was hydrolysed with potassium hydroxide and the extracted solid recrystallised to constant melting point. The compound was found to contain about 6% hexadeuterodimethylmalonic acid (VII). The trideuteromethylmalonic acid (2H3-MMA) (VIII) had an isotopic purity of greater than 99%. The TMS derivative of 2H3-MMA (VIII) had a fractionally shorter retention time than the unlabelled MMA (I) but the two compounds could not be separated by GC and MIS was used for measurement of the ratio of 2H3-MMA (VIII) to MMA (I). Using 2H3-MMA (VIII) as an internal standard, a calibration curve for MMA (I) was then prepared over a range of 0-25.0 μg of MMA (I).

The ion pairs at m/e 218.3, 221.3 and 247.3, 250.3 were chosen for El-MIS as the ions at 221.3 and 250.3 retained the deuterium label. The ratios were measured using El-MIS as was the ratio of the protonated molecular ions at m/e 263.3, 266.3 using Cl-MIS. These peak heights ratios were corrected for forward contribution of the MMA (I) to the internal standard (VIII) and back contribution of the internal standard (2H3-MMA) (VIII) to the MMA (I) using a calculator programme (see Appendix A). The corrected peak height ratios gave straight line calibration curves with correlation coefficients of greater than 0.99 in each case. The Cl-MIS calibration curve obtained using uncorrected and corrected peak height ratios (Fig. 15) shows that use of the correction extends the linear region of the calibration curve and gives a straight line curve which passes through the origin. As expected the Cl-MIS mode gave intensities which were much higher than those obtained when the fragment ions pairs were used. As we have shown the use of either Cl or El-MIS gives comparable results, the use of Cl-MIS was chosen to give greater sensitivity and precision. Injection of aliquots containing 10 ng of MMA (I) gave reproducible analysis with variations between duplicate samples of
TABLE 21
COMPARISON OF LEVELS OF MMA (I) IN AMNIOTIC FLUID
OBTAINED USING GC PROFILING AND ISOTOPE DILUTION

<table>
<thead>
<tr>
<th>SAMPLE NO.</th>
<th>CONCENTRATION OF MMA (I)(e) ((\mu g/\text{ml fluid}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GC Profiling</td>
</tr>
<tr>
<td></td>
<td>FID Detector(c)</td>
</tr>
<tr>
<td>1</td>
<td>0.32</td>
</tr>
<tr>
<td>2</td>
<td>0.30</td>
</tr>
<tr>
<td>3</td>
<td>1.06</td>
</tr>
<tr>
<td>4</td>
<td>0.25</td>
</tr>
</tbody>
</table>

\(a\) Samples are from fluid collected 14-17 weeks and organic acids were analysed as TMS derivatives.

\(b\) Adipic acid internal standard.

\(c\) 3% SE 30 packed column.

\(d\) SE54 WCOT capillary column with open coupling to Varian MAT 44 MS.

\(e\) Results are mean of duplicate determinations.
less than 2% for amniotic fluid samples. Using the capillary column for separation of the MMA (I) the lower limit of detection was approximately 0.05 µg/ml of fluid and analyses were made to a precision of 0.02 µg/ml.

Table 21 shows the results obtained when four samples were analysed for MMA (I) using a packed column GC method with FID, a capillary column GC method using the total ion current plot of the mass spectrometer and the isotope dilution method. These results show that the isotope dilution gives slightly higher values for the amount of methylmalonic acid present but the results obtained using the GC method with the packed column and FID are sufficiently accurate for clinical diagnosis.
b. PRENATAL DIAGNOSIS OF METHYLMALONIC ACIDEMIA

In 1962 Cox and White demonstrated an increase in urinary excretion of MMA (I) in patients with vitamin $B_{12}$ deficiency. Inherited methylmalonic aciduria was first described in 1967 by two groups of investigators in retarded infants who suffered from recurrent vomiting, life threatening attacks of ketoacidosis and intolerance to high protein feedings. Since the original description of the disease a number of distinct variants have been described. The enzyme defect responsible for the disease variant may be either in the propionate to succinate pathway or in the pathway of vitamin $B_{12}$ metabolism in which 5-deoxyadenosylcobalamin, the co-factor for $L$-methylmalonyl-CoA carbonyl mutase is produced (Fig. 16).

Clinically patients with increased MMA (I) excretion are characterised by protein intolerance, bouts of ketoacidosis and inhibited growth and development. Patients with deficiencies in the racemase or mutase activity generally die at an early age. In patients with defects of vitamin $B_{12}$ metabolism treatment with doses of the vitamin often produce clinical and biochemical improvement and can lead to a lowering of the excretion of MMA (I). The frequency and size of the dose required varies with individual patients and may vary in periods of metabolic stress such as infection. The dose is not related to the measured serum levels of the vitamin or metabolic precursors but may be assessed by measurement of the daily excretion of MMA (I).

The defects can be demonstrated in cultured fibroblasts and amniotic fluid cells and hence prenatal diagnosis is possible. However several features of this inborn error of metabolism make it possible to evaluate the foetus by means other than cultured cells and so avoid the time required for cell culture.

Morrow suggested that diagnosis may be made by analysing mutase activity in non cultured cells. In an affected pregnancy both amniotic fluid and maternal urine contain excess MMA (I), since MMA (I) is not readily metabolised and consequently accumulates in both foetal and maternal fluids.
**TABLE 22**

ENZYME STUDIES OF CELLS CULTURED FROM AMNIOTIC FLUID OBTAINED AT 15 WEEKS GESTATION FROM MRS V.T. (3RD PREGNANCY) AND FROM SKIN BIOPSY CELLS OF AFFECTED CHILD

### A. STUDIES OF VITAMIN B12 METABOLISM

#### 1. CO ENZYME SYNTHESIS BY INTACT CELLS

<table>
<thead>
<tr>
<th></th>
<th>V.T. Amniotic fluid cells</th>
<th>Control Amniotic fluid cells</th>
<th>Affected child fibroblast cells</th>
<th>Control fibroblast cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-Deoxyadenosine Co VIII cobalamin pg cobalamin/ mg cells</td>
<td>0.48</td>
<td>0.54</td>
<td>0.23</td>
<td>0.16</td>
</tr>
<tr>
<td>Methylcobalamin pg cobalamin/ mg cells</td>
<td>0.10</td>
<td>0.18</td>
<td>0.92</td>
<td>0.12</td>
</tr>
</tbody>
</table>

**Total Cobalamin Uptake pg cobalamin/ mg cells**
- V.T. Amniotic fluid cells: 1.2
- Control Amniotic fluid cells: 2.1
- Affected child fibroblast cells: 6.3
- Control fibroblast cells: 1.1

#### 2. 5-DEOXYADENOSINECOBALAMIN SYNTHESISING ACTIVITY IN CELL EXTRACTS

<table>
<thead>
<tr>
<th></th>
<th>V.T. Amniotic fluid cells</th>
<th>Control Amniotic fluid cells</th>
<th>Affected child fibroblast cells</th>
<th>Control fibroblast cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-Deoxyadenosine Co VIII cobalamin pg cobalamin/ mg protein/30 min.</td>
<td>14.1</td>
<td>123</td>
<td>17.6</td>
<td>25.9</td>
</tr>
</tbody>
</table>

### B. STUDIES OF PROPIONATE-SUCCINATE PATHWAY

#### 1. METHYLMALONYL COA MUTASE ACTIVITY*(HOLONENZYME IN CELL EXTRACT)

<table>
<thead>
<tr>
<th></th>
<th>V.T. Amniotic fluid cells</th>
<th>Control Amniotic fluid cells</th>
<th>Affected child fibroblast cells</th>
<th>Control fibroblast cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>173 pmoles succinate/mg protein/min.</td>
<td>173</td>
<td>123</td>
<td>&lt; 1</td>
<td>224</td>
</tr>
</tbody>
</table>

#### 2. 1-PROPIONATE FIXATION IN INTACT CELLS DURING 10 HOURS

<table>
<thead>
<tr>
<th></th>
<th>V.T. Amniotic fluid cells</th>
<th>Control Amniotic fluid cells</th>
<th>Affected child fibroblast cells</th>
<th>Control fibroblast cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.77 natoms 14C/mg protein</td>
<td>3.77</td>
<td>4.61</td>
<td>9.93</td>
<td>7.50</td>
</tr>
<tr>
<td>4.88 natoms 14C/mg protein</td>
<td>4.88</td>
<td>6.61</td>
<td>10.00</td>
<td>8.75</td>
</tr>
</tbody>
</table>

**Measured using a racemic mixture of D and L methylmalonyl CoA. Defect in racemase enzyme is indicated by half activity of tested cell and must then be further investigated. Normal cells convert over 80% of substrate to succiny1-CoA.**
The major reason for developing the present analytical method was to accurately measure small amounts of organic acids in amniotic fluid in order to distinguish the pregnant heterozygote carrying a foetus affected with an organic acidemia from one carrying an unaffected foetus. During the course of this work two pregnant heterozygotes at risk for methylmalonic aciduria were studied during three pregnancies.

Mrs. V.T. had two children suffering from methylmalonic acidemia but one has since died. These children were diagnosed to have a defect in methylmalonyl-CoA mutase apoenzyme and had normal vitamin $B_{12}$ metabolism.

Amniocentesis was performed at 15 weeks gestation and successful cell cultures were begun. Cells were 46 XY karotype indicating a male foetus. The MMA (I) concentration of the fluid was measured and a value of 0.92 µg/ml was obtained using GC profiling. The identity of the peak assigned to MMA (I) in the profile was confirmed by GC/MS and no extraneous ions were present in the MS. When the isotope dilution method became available a comparable result was obtained by isotope dilution. When 15 normal amniotic fluids of 14-18 weeks gestation were examined using the technique a mean value of 0.72 µg/ml with a standard deviation of 0.42 µg/ml (Normal range less than 1.56 µg/ml) was obtained for MMA (I).

The value obtained for the MMA (I) concentration for Mrs. V.T. indicated that the foetus would be unaffected and so the pregnancy was allowed to continue. This prediction was supported by the enzyme studies performed by Dr. M.J. Mahoney. (Table 22)

The studies of vitamin $B_{12}$ metabolism show that both the cultured amniotic fluid cells and cultured fibroblasts from Mrs. V.T.'s affected surviving child have normal uptake of radioactive vitamin $B_{12}$ and normal formation of $\text{Adenosylcobalamin}$ and methylcobalamin in both intact cells and cell extracts. The metabolism of propionate to succinate was examined in two parts. A mixture of D & L-methylmalonyl-CoA was used as substrate by cell extracts to measure methylmalonyl-CoA mutase haloenzyme and the results obtained show a clear distinction between the cultured fibroblasts from the affected child and cells grown from the amniotic fluid, which are within the normal range. Similarly the fixation of $^{14}$C-propionate shows a clear distinction between the amniotic fluid cells and controls, and
**TABLE 23**

**RECOVERY OF STANDARD ACIDS ADDED TO URINE**

<table>
<thead>
<tr>
<th>ACID</th>
<th>ACID ADDED:</th>
<th>5 µg/ml</th>
<th>50 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a% RECOVERY:</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Absolute</td>
<td>Relative to Adipic Acid</td>
<td>Absolute</td>
</tr>
<tr>
<td>Adipic Acid (II)</td>
<td>93 ± 3.8</td>
<td>100%</td>
<td>94 ± 2.7</td>
</tr>
<tr>
<td>Methylmalonic Acid (I)</td>
<td>79 ± 3.2</td>
<td>85 ± 3.4</td>
<td>82 ± 2.7</td>
</tr>
<tr>
<td>γ-Ketovaleric Acid</td>
<td>67 ± 5.7</td>
<td>72 ± 6.0</td>
<td>72 ± 5.9</td>
</tr>
<tr>
<td>2-Hydroxyisocaproic Acid</td>
<td>65 ± 5.9</td>
<td>70 ± 6.3</td>
<td>67 ± 4.2</td>
</tr>
<tr>
<td>Succinic Acid</td>
<td>83 ± 3.2</td>
<td>89 ± 3.4</td>
<td>84 ± 3.0</td>
</tr>
<tr>
<td>Phenylacetic Acid</td>
<td>85 ± 6.2</td>
<td>91 ± 6.7</td>
<td>86 ± 3.7</td>
</tr>
<tr>
<td>Glutaric Acid (X)</td>
<td>86 ± 2.5</td>
<td>92 ± 2.7</td>
<td>86 ± 3.0</td>
</tr>
<tr>
<td>p-Hydroxyphenylacetic Acid</td>
<td>92 ± 2.3</td>
<td>99 ± 2.5</td>
<td>94 ± 3.1</td>
</tr>
<tr>
<td>Lauric Acid</td>
<td>94 ± 3.5</td>
<td>101 ± 3.8</td>
<td>95 ± 3.3</td>
</tr>
</tbody>
</table>

*a Each value is the mean % recovery ± 2 SD obtained from at least 3 experiments and were obtained by GC of the TMS derivatives on Apiezon-N.*
those of the affected child. This data also illustrates an enhancement of activity in the affected child's cells in the presence of a gross excess of hydroxycobalamin.

In order to study the urinary excretion of MMA (I) during pregnancy, methods were required to accurately measure normal excretion levels of MMA in urine. The solvent extraction method used for screening urinary metabolites was adapted to this purpose using adipic acid (II) (10 μg) as internal standard. A GC calibration curve for MMA (I) was prepared using the ratios of the peak height for MMA (I) to the peak height of an internal standard over a range of acid concentrations (0-100 μg). The curve obtained was a straight line with a correlation coefficient of 0.99.

Initial experiments were done by extracting acidified urine directly with ethyl acetate but in some samples extraneous substances such as thymol and cresol were present which interfered with the measurement of some acids, particularly MMA (I). The interfering substances were preservatives and some appeared to be related to diet as they were not present in all specimens collected from the same person. The problem was overcome by saturating the urines with sodium bicarbonate and extracting the basic and neutral compounds at pH 7-8 before acidifying the urine to pH 1 and re-extracting the organic acids with ethyl acetate. Recoveries of acids from biological fluids however may not be quantitative and for accurate quantitation of metabolites recovery studies are also necessary. Such studies were carried out for MMA (I) and other representative biologically important acids. This was done by adding known amounts of the acids and the internal standard to a "normal" urine sample and extracting the urine, followed by addition of an external standard to the ethyl acetate. The results of these studies are shown in Table 23. The acids were not extracted quantitatively but re-extraction of the urines failed to improve the yields significantly. MMA (I) could be measured by this method with a lower limit of detection of 0.4 μg/ml and a precision of 0.1 μg/ml.

When the isotope dilution method for MMA (I) became available calibration curves were constructed using TMS derivatives both by E1-MIS using the ion pairs at m/e 218/221 and 247/250, and by CI-MIS using the protonated molecular ions at m/e 263.3 and 266.3. These calibration
**TABLE 24**

**COMPARISON OF RESULTS OBTAINED FOR URINARY MMA (I) USING GC PROFILING AND ISOTOPE DILUTION METHODS**

<table>
<thead>
<tr>
<th>SAMPLE NO.</th>
<th>METHOD</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GC PROFILING</td>
<td>CI/MIS(^a)</td>
<td>EI/MIS(^b)</td>
</tr>
<tr>
<td></td>
<td>µg MMA/ml</td>
<td>µg MMA/ml</td>
<td>µg MMA/ml</td>
</tr>
<tr>
<td>1</td>
<td>8.1</td>
<td>8.2</td>
<td>8.6</td>
</tr>
<tr>
<td>2</td>
<td>3.6</td>
<td>3.3</td>
<td>3.6</td>
</tr>
<tr>
<td>3</td>
<td>4.7</td>
<td>4.4</td>
<td>5.3</td>
</tr>
<tr>
<td>4</td>
<td>1.7</td>
<td>1.9</td>
<td>1.8</td>
</tr>
<tr>
<td>5</td>
<td>1.1</td>
<td>0.9</td>
<td>1.0</td>
</tr>
</tbody>
</table>

\(^a\) Results obtained using protonated molecular ions at m/e 263.3, 266.3

\(^b\) Results obtained using fragment ions at m/e 247, 250.

\(^c\) Results obtained using fragment ions at m/e 218, 221.
<table>
<thead>
<tr>
<th>WEEKS GESTATION</th>
<th>MMA (I) CONCENTRATION&lt;sup&gt;c&lt;/sup&gt;</th>
<th>MMA (I) EXCRETION&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/ml</td>
<td>µmol/l</td>
</tr>
<tr>
<td>MRS. V.T.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>3.8</td>
<td>32</td>
</tr>
<tr>
<td>14</td>
<td>3.3</td>
<td>28</td>
</tr>
<tr>
<td>15</td>
<td>10.9</td>
<td>92</td>
</tr>
<tr>
<td>19 weeks</td>
<td>8.1</td>
<td>68</td>
</tr>
<tr>
<td>33 weeks Day 1</td>
<td>11.1</td>
<td>100</td>
</tr>
<tr>
<td>33 weeks Day 2</td>
<td>5.3</td>
<td>58</td>
</tr>
<tr>
<td>6 days P.P.</td>
<td>3.6</td>
<td>30</td>
</tr>
<tr>
<td>10 weeks P.P.*</td>
<td>2.5</td>
<td>22</td>
</tr>
<tr>
<td>Normal Controls b</td>
<td>1.8 ± 1.1</td>
<td>1.7 ± 1.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values obtained by GC profiling of TMS derivatives on SE30. Comparable values were obtained using the isotope dilution procedure.

<sup>b</sup> Values are expressed as mean ± SD of 19 samples obtained from 11 pregnant and 8 non-pregnant females.

<sup>c</sup> All results have been corrected for recovery.

* P.P. = post partum
curves were linear over the range of 0-250 μg MMA (I)/ml urine and had

correlation coefficients (obtained by the least squares method) of
greater than 0.99. The MMA (I) concentration of a number of urine
samples were measured using GC profiling with adipic acid (II) as internal
standard and isotope dilution and the results are shown in Table 24.
These results show good agreement between the two methods and between
and EI-MIS the CI-MIS methods. The higher ion currents obtained in CI-MIS gave
better precision of analysis and this method was used for all further
isotope dilution studies. The majority of urine results reported in this
work have been measured by the GC profiling method but some have been
checked by the isotope dilution method.

A number of urine specimens were obtained from Mrs. V.T. during the
course of the pregnancy. The first specimens obtained were spot specimens
and excretion levels were related to urinary creatinine. All other
specimens were pooled 24 hour collections. Examination of 24 hour urine
collections from 8 normal subjects and 11 pregnant patients showed no
significant difference between the excretion of MMA in the two groups.
The excretion obtained in these patients was 1.7 ± 1.1 μmol/μmol creat­
inine (mean ±SD)(normal range <3.9 μmol/μmol creatinine) and all patients
excreted less than 4 mg/day of MMA (1.6 ± 1.0 mg/day (mean ±SD)). The
values obtained for Mrs. V.T. are shown in Table 25. The MMA (I) level
of the random urine specimen collected at 13 weeks is within the normal
range but after this time the patient excreted increased amounts of
MMA (I). The two specimens collected during week 33 show the daily
variation in excretion of MMA (I) but all specimens were above the upper
limits of the normal range and had not returned to normal by six days
after parturition. By 10 weeks after parturition however the excretion
had returned to normal and examination of further random samples has shown
no other abnormal levels of MMA (I). During the pregnancy normal levels
of vitamin B12 were demonstrated in this patient and no signs of vitamin
B12 deficiency were observed.

At delivery a sample of amniotic fluid was obtained and analysed for
MMA. This sample showed a concentration of 2.46 μg/ml by the GC
profiling method. The identity of the peak assigned to MMA (I) in the
profile was confirmed by GC-MS and no extraneous ions were present in the MS. Examination of 8 specimens of amniotic fluid of greater than 35 weeks gestation showed a mean value of 1.35 µg/ml with a standard deviation of 0.65 µg/ml (normal range < 2.65 µg/ml). The value obtained for the amniotic fluid from Mrs. V.T. was within the normal range.

Urine was obtained from the newborn at 4 days of age when on a normal diet and showed MMA (I) concentration of 0.75 µg/ml (6.3 µmol/l), 3.5µmol MMA (I)/µmol creatinine. The total excretion of MMA was 0.16 mg/day. These values are all within the measured normal range. Since that time the child has remained healthy except for an episode of haemolytic-uremic syndrome.

Mrs. V.T. undertook a further pregnancy and amniocentesis was again performed at 15 weeks. Amniotic fluid MMA (I) was 0.34 µg/ml in this specimen and this result was confirmed by isotope dilution. Examination of cultured cells showed no abnormality of methylmalonyl-CoA metabolism and a normal foetus was predicted. The pregnancy was allowed to continue and an unaffected child was born at term. Urine samples from this child collected in the first week of life were within the normal range. Unfortunately no urine studies were carried out during the fourth pregnancy.

Mrs. G.P. was a woman whose second child was affected with methylmalonic acidemia caused by a defect in methylmalonyl-CoA mutase enzyme. During her third pregnancy amniocentesis was performed at 15 weeks gestation and the amniotic fluid contained0.60µg/ml MMA (I) which was within the measured normal range (less than 1.56µg/ml). A normal foetus was predicted and this was supported by finding normal levels of methylmalonyl-CoA mutase activity in cultured amniotic fluid cells. The pregnancy was allowed to continue and a healthy child was delivered at term. Analysis of specimens of urine obtained from the child in the first weeks of life contained normal levels of MMA (I) confirming the in-utero prediction. No urine studies were carried out during this pregnancy.
# TABLE 26

**AMNIOTIC FLUID MMA (I) CONCENTRATION**

<table>
<thead>
<tr>
<th>PATIENT</th>
<th>WEEKS GESTATION</th>
<th>MMA CONCENTRATION µg/ml fluid</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>GC Profiling</td>
<td>Isotope Dilution</td>
<td></td>
</tr>
<tr>
<td>V.T. Preg. 1</td>
<td>15</td>
<td>0.92</td>
<td>0.96</td>
<td></td>
</tr>
<tr>
<td></td>
<td>38</td>
<td>2.46</td>
<td>2.57</td>
<td></td>
</tr>
<tr>
<td>V.T. Preg. 2</td>
<td>15</td>
<td>0.34</td>
<td>0.48</td>
<td></td>
</tr>
<tr>
<td>G.P.</td>
<td>15</td>
<td>0.60</td>
<td>0.65</td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>14-18</td>
<td>0.72 ± 0.42</td>
<td>0.72±0.37</td>
<td></td>
</tr>
<tr>
<td>(mean ± SD)b</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>35+</td>
<td>1.35 ± 0.65</td>
<td>1.43±0.62</td>
<td></td>
</tr>
<tr>
<td>(mean ± SD)c</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**a** Values obtained by GC profiling of TMS derivatives on SE30 and are corrected for recoveries. The identity of the methylmalonic acid was confirmed by GC/MS. Comparable values were obtained by isotope dilution method.

**b** Results from 15 AF specimens obtained at 14-18 weeks.

**c** Results from 8 AF specimens obtained after 35 weeks for fetal lung maturity studies.
The results obtained in this work show that in the pregnancies monitored, the use of amniotic fluid levels of MMA (I) are as good in the prediction of the clinical status of the foetus as the enzyme studies. The metabolite measurements have the added advantage that the results are available within one working day of receipt of the specimen rather than the 4-8 weeks required for growth and analysis of cultured amniotic fluid cells. Morrow\textsuperscript{157} has also suggested that the absence of MMA (I) in amniotic fluid, particularly if confirmed by another amniocentesis is very strong evidence that the foetus is unaffected. Thus the use of amniotic fluid MMA (I) level is a valuable aid to prenatal diagnosis of this condition, particularly if there is only a short time available between amniocentesis and the latest time of therapeutic abortion or in cases where cell lines are not cultured successfully. The metabolite is also stable in the fluid when stored frozen and this alleviates problems of sample transport which may occur with cell culture.

Few reports have been published on MMA (I) excretion in heterozygotes \textsuperscript{156,157,159,160,446,467} and especially during pregnancy. Heterozygotes usually excrete levels of MMA (I) comparable to the levels obtained in normal controls.\textsuperscript{155,158,160,161,456} Thus the normal range obtained in these investigations using pregnant and non pregnant patients is a valid comparison for heterozygotes MMA (I) excretion.

The exception to this is shown in the report by Netzloff et al\textsuperscript{468} in which a heterozygote who excreted increased amounts of MMA (I) post partum was studied but no levels of acid excretion were given.

A number of studies have been carried out in pregnant heterozygotes. These studies have suggested that the genotype of the foetus rather than that of the mother determines maternal excretion during pregnancy. If the foetus is unaffected the maternal excretion of MMA (I) is normal during pregnancy.\textsuperscript{158,160} If however the foetus is affected the urinary MMA (I) excretion rises. The time at which this increased excretion occurs however appears to be variable. Morrow et al\textsuperscript{446} demonstrated normal MMA (I) levels in maternal urine in a heterozygote with an affected foetus at 20 weeks gestation but showed a large increase (approximately six times normal) by week 31. Gompertz et al\textsuperscript{467} did not measure MMA (I) excretion before week
36 when they found a four times normal increase. Nakamura et al.\textsuperscript{131} found a rise in MMA (I) excretion comparable to that shown in our patient by 23 weeks and Bakker et al.\textsuperscript{158} also reported a similar rise by week 22. Mahoney et al.\textsuperscript{156} however reported levels that were distinctly raised to 2.5 times normal by week 16 and dropped into the normal range within 2 days after the foetus was aborted. This data has given rise to the assumption that the maternal urinary MMA (I) excretion remains normal if the foetus is not affected and that monitoring of maternal urinary MMA (I) affords promise for prenatal diagnosis of affected foetuses, particularly if the mother is unwilling to undergo amniocentesis. Our studies of Mrs. V.T. show that this assumption is not correct as this heterozygote excreted abnormal amounts of MMA (I) when the amniotic fluid and cell cultures indicate a normal foetus and this prediction was proven by perinatal studies.

The excretion levels in Mrs. V.T. rose to nearly three times normal which is nearly as high as those in patients where the foetus was affected. This abnormal excretion began as early as week 14 and did not return to normal until more than six days after delivery. This slow decline of MMA (I) excretion is not characteristic of other reported pregnancies\textsuperscript{156, 158, 446, 467} where raised urinary MMA (I) was obtained when the foetus was affected and normal excretion was found within 48 hours of delivery or termination. The only other case where raised urinary MMA (I) excretion has been reported in a pregnant heterozygote with an unaffected foetus is that of Netzlof et al.\textsuperscript{468} but in his patient abnormal excretion of MMA (I) occurred even when the patient was not pregnant and this atypical behaviour of a heterozygote may be due to vitamin $B_{12}$ deficiency. This possible cause was excluded in our patient by serum $B_{12}$ and haematological studies.

The daily variation in excretion during this time is shown by the variation of the two specimens obtained during week 33. Mrs. V.T. was not on any controlled diet during the collection but her diet is usually low in protein and high in carbohydrates and fats. The variation in MMA excretion during these two days may thus reflect protein intake as well as variability of metabolism.
FIGURE 17. Pathways of lysine, 5-hydroxylysine and tryptophane degradation through glutaryl-CoA.

Glutaryl CoA has not been detected as a free intermediate. Glutaric acid, glutaric acid and 3-hydroxyglutaric acid are excreted by patients with a block at position 1 but are not usually present in normal patients.
c. PERNATL DIAGNOSIS OF GLUTARIC ACIDEMIA

Large amounts of glutaric acid (X) have been detected in the urine of patients with two clearly different syndromes. One syndrome, biochemically dominated by glutaric aciduria and lactic aciduria was reported by Przyrembel et al in a neonate who died at 70 hours after birth. Besides glutaric (X) and lactic acids (XI), a number of mono and dicarboxylic acids, as well as several amino acids were found in elevated concentrations in blood and urine. Defective oxidation of the branched chain amino and keto acids was demonstrated in fibroblasts in this patient. This syndrome has been named glutaric aciduria type II.

The other syndrome, glutaric aciduria type I is an autosomal recessive inherited inborn error in the catabolism of lysine (XII), tryptophane (XIII), and hydroxylysine (XIV) (Fig. 17). The condition was first described by Goodman et al in 1975 and seven patients have been reported. The disease is characterised clinically by an intermittently progressive dyskinetic syndrome of choreoathetosis with hyperkinesia and dysarthria and may or may not involve mental retardation. The disease usually runs a progressive course and death in a Reye syndrome-like state has been observed. As well as glutaric acid (X) all patients are reported to excrete increased amounts of 3-hydroxyglutaric acid (XV) and glutaconic acid (XVI). Some may excrete increased amounts of 2-amino adipic acid (XVII) and saccharopine (XVIII) together with 2-keto adipic acid (XIX). The histological changes in the putamen and caudate nuclei in this disease resemble those seen in Huntington's Chorea and Parkinson's disease. During the course of this work studies were made of a pregnant heterozygote for glutaric acidemia Type I where the foetus was at risk for the disease.

Mrs. V.M. has a previous child who had been diagnosed as having glutaric acidemia when the mother was about 8 weeks pregnant. The affected child was eighteen months old and appeared to be normal until 2 weeks before, when, following an upper respiratory tract infection she developed an acute dyskinetic reaction and lost the ability to stand, sit or crawl. Organic acid chromatography showed the presence of glutaric acid (X)
TABLE 27
GLUTARYL COA DEHYDROGENASE ACTIVITY IN CULTURED FIBROBLASTS FROM AFFECTED CHILD, PARENTS AND NORMAL CONTROLS

<table>
<thead>
<tr>
<th></th>
<th>Glutaryl CoA Dehydrogenase Activity&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nanomoles CO&lt;sub&gt;2&lt;/sub&gt;/hr/mg protein</td>
</tr>
<tr>
<td>R.M. (Affected child)</td>
<td>0</td>
</tr>
<tr>
<td>V.M. (mother)</td>
<td>1.31</td>
</tr>
<tr>
<td>J.M. (father)</td>
<td>1.69</td>
</tr>
<tr>
<td>Control</td>
<td>4.23</td>
</tr>
<tr>
<td>Normal Controls</td>
<td>2.54 ± 0.26&lt;sup&gt;b&lt;/sup&gt; (N = 6)</td>
</tr>
<tr>
<td>Heterozygotes</td>
<td>1.19 ± 0.25&lt;sup&gt;c&lt;/sup&gt; (N = 13)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values obtained by S.I. Goodman, University of Colorado.

<sup>b</sup> Values obtained by D.T. Whelan et al. (473).

<sup>c</sup> Values obtained by E. Christensen & N.J. Brandt (477).
## TABLE 28

CONCENTRATION OF GLUTARIC ACID FOUND IN AMNIOTIC FLUID EXTRACTS OF PATIENT V.M. AND CONTROL SUBJECTS

<table>
<thead>
<tr>
<th></th>
<th>DATE COLLECTED</th>
<th>GESTATION (WEEKS)</th>
<th>GLUTARIC ACID $^a$ (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V.M.</td>
<td>19/10/78</td>
<td>15.5</td>
<td>4.35</td>
</tr>
<tr>
<td>V.M.$^b$</td>
<td>11/12/78</td>
<td>22.5</td>
<td>11.7</td>
</tr>
<tr>
<td>Control 1</td>
<td>19/10/78</td>
<td>15</td>
<td>0.38</td>
</tr>
<tr>
<td>Control 2</td>
<td>19/10/78</td>
<td>16</td>
<td>0.14</td>
</tr>
<tr>
<td>Control 3$^c$</td>
<td>1-10/5/78</td>
<td>14-20</td>
<td>0.22</td>
</tr>
</tbody>
</table>

$^a$ All values are corrected for recovery.

$^b$ Specimen obtained at termination.

$^c$ This sample was obtained from a pool of 15 amniocentesis specimens.
TABLE 29
GLUTARYL-CoA METABOLISM IN CULTURED AMNIOTIC FLUID CELLS FROM V.M., PREGNANT HETEROZYGOTE WITH NORMAL FOETUS AND NORMAL CONTROLS\textsuperscript{a}

<table>
<thead>
<tr>
<th></th>
<th>Glutaryl CoA Metabolised Nanomoles CO\textsubscript{2}/hr/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>V.M.</td>
<td>Not Detected</td>
</tr>
<tr>
<td>Pregnant Heterozygote</td>
<td>2.44</td>
</tr>
<tr>
<td>Control I</td>
<td>1.03</td>
</tr>
<tr>
<td>Control II</td>
<td>4.94</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Results obtained by S.I. Goodman, University of Colorado.
(19.4 μmol/μmol creatinine) but 3-hydroxyglutaric acid (XV) and glutaconic acid (XVI) were absent. The glutaryl-CoA dehydrogenase levels obtained in fibroblast culture from the child and the parents were measured and no activity of the enzyme could be shown in fibroblasts or leucocytes from the child (Table 27). The parents also showed reduced levels of the enzyme which are comparable to those shown by the heterozygotes reported by Whelan et al. and by Christensen and Brandt.

Amniocentesis was performed at 15 weeks gestation and successful cell cultures were begun. The concentration of glutaric acid (X) in the amniotic fluid was measured and a value of 4.35 μg/ml obtained using the GC method (Table 28). The GC-MS of the peak assigned to glutaric acid contained no extraneous ions. A careful search was made but no 3-hydroxyglutaric acid (XV), glutaconic acid (XVI) or 2-ketoadipic acid (XIX) could be found in the fluid. The amino acid levels in this specimen were all within the normal range and no α-amino adipic acid (XVII) or saccharopine (XVIII) were found.

The recovery of glutaric acid (X) added to pooled amniotic fluid showed a recover of $95 \pm 2\%$ (n=6) relative to the adipic acid (II) internal standard. The average recovery of the internal standard was $91 \pm 6\%$ (n=20) relative to pimelic acid (XX).

When twenty four control specimens of amniotic fluid obtained from patients with estimated gestational ages of 14-20 weeks were examined the mean concentration of glutaric acid (X) was 0.28 μg/ml with a standard deviation of 0.11 μg/ml.

The presence of a large increase of glutaric acid (X) in the amniotic fluid indicated the foetus was affected and further evidence for this was obtained when the enzyme results became available. No glutaryl-CoA dehydrogenase activity was detectable in cells cultured from the amniotic fluid (Table 29) whereas the cells cultured from amniotic fluid from another pregnant heterozygote who was later shown to have a normal child, contained normal levels of the enzyme.

These results suggested that the foetus was homozygous for the defect and it was decided that the pregnancy should be terminated. A further
### TABLE 30

**TISSUE GLUTARIC ACID LEVELS AND ENZYME STUDIES ON ABORTUS FROM PATIENT V.M. AND NORMAL CONTROLS**

<table>
<thead>
<tr>
<th>Glutaric Acid (µg/g wet weight)</th>
<th>Foetus A</th>
<th>Glutaric Acidemia (476)</th>
<th>Control Foetuses (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutaric acid (µg/g wet weight)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>17.0</td>
<td>330</td>
<td>96</td>
</tr>
<tr>
<td>Kidney</td>
<td>70.2</td>
<td>1000</td>
<td>1330</td>
</tr>
<tr>
<td>Brain</td>
<td>112.0</td>
<td>109</td>
<td>88\textsuperscript{a}, 165\textsuperscript{b}</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutaryl-CoA dehydrogenase (nmoles CO\textsubscript{2}/hr/mg protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>0.15</td>
<td>0\textsuperscript{c}</td>
<td>0</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.03</td>
<td>0\textsuperscript{c}</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamate dehydrogenase (µmoles NADH oxid/min/mg prot)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>0.38</td>
<td>1.44\textsuperscript{c}</td>
<td>0.45</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Frontal cortex  
\textsuperscript{b} Basal ganglion  
\textsuperscript{c} Isolated mitochondria
FIGURE 18. The total ion current profile from the SE-54 capillary column of the silylated organic acid extract of amniotic fluid obtained from patient V.M. at 15 weeks gestation (A) and a pool of fluid from 15 normal patients 14-20 weeks gestation (B).

The peaks were identified as: 1. lactic acid; 2. 3-hydroxybutyric acid; 3. succinic acid; 4. glutaric acid; 5. adipic acid (internal standard); 6. duodecanol; 7. pinellic acid (external standard).
specimen of amniotic fluid was obtained at termination and the concentration of glutaric acid (X) in it measured. The level of 11.7 μg/ml (Table 28) obtained indicates a large increase in the concentration of the metabolite in the intervening seven weeks and is about forty times the normal level of glutaric acid. A total ion current profile of the 15 week specimen is shown in Fig. 18 compared to a specimen from a pool of 15 normal controls.

Studies of glutaric acid (X) content and enzyme activities were carried out on tissue homogenates from the abortus (Table 30). These results are compared to values obtained on two patients with glutaric acidemia at autopsy and on three control human foetuses ranging in gestational age from 16 to 24 weeks. The liver, kidney and brain of the abortus at risk showed markedly increased amounts of glutaric acid (X) and almost no glutaryl-CoA dehydrogenase activity, a pattern which is observed in tissues from glutaric acidemia patients but not in controls. The glutamate dehydrogenase activity is normal showing that the tissue was still viable at the time of the assay.

The cerebral hemispheres and cerebellum of the foetus were normal to both gross and microscopic examination, but there were some slight alterations in the size distribution of the nuclei in the striatum, which contained many prominent and darkly staining nuclei.

The absence of urinary glutaconic (XVI) and 3-hydroxyglutaric acid (XV) in the affected child is of interest as it is at variance with all other reported cases of this disease and would suggest a different enzyme mutant present in this family. The precise location of the enzyme defect for glutaric aciduria type 1 has not been determined although a number of mutants have been demonstrated. Two possible defects have been suggested to explain the observed urinary products of the reported patients.

The accumulation of glutaryl-CoA and the excretion of glutaric acid (X) are consistent with a defect in glutaryl-CoA dehydrogenase. The appearance of 3-hydroxyglutaric acid (XV) and glutaconic acid (XVI) may then be explained as being due to an alternative pathway for the oxidation of glutaric acid (X) similar to the one postulated for the conversion.
of propionic acid (XXI) to 3-hydroxypropionic acid (XXII) in patients with propionic or methylmalonic acidemia. This defect could also explain why the leucocytes of patients can metabolise free glutaric acid. In contrast to this postulated defect Stokke et al. have suggested that the reaction proceeds via two enzymes, glutaryl-CoA dehydrogenase and glutaryl-CoA dehydrogenase and that the defect is in glutaryl-CoA dehydrogenase. The presence of 3-hydroxyglutaric acid (XV) is then explained by hydration of abnormally high intercellular concentrations of glutaryl-CoA followed by decylation. Similar hydration has been shown to occur normally in the synthesis of 3-hydroxymethylglutaryl-CoA from 3-methylglutaconyl-CoA and has been suggested as the basis for excretion of β-hydroxisovaleric acid (XXIII) in patients with β-methylcrotonyl-CoA carboxylase deficiency. However, although the main degradative pathway of glutaryl-CoA in mammalian cells is well established, it has to date been impossible to isolate glutaryl-CoA as an intermediate or to separate glutaryl-CoA dehydrogenase activity from that of decarboxylation. The method of enzyme analysis of this sequence of reactions is by measurement of $^{14}$CO$_2$ produced and does not differentiate between the two possibilities as the production of carbon dioxide from glutaryl-CoA could take place by either mechanism.

Examination of the metabolite excretion pattern of this child however suggests that no alternative pathway exists for the metabolism of glutaric acid (X) which produces glutaric (XVI) and 3-hydroxyglutaric (XV) acids. The two enzyme reaction sequence of Stokke appears to be correct and the parents of this child are thus heterozygous for a defect in glutaryl-CoA dehydrogenase with the other reported patients having a defect of glutaryl-CoA dehydrogenase.

The nature of the movement disorder and its similarity to that found in Huntington's Chorea and Parkinson's disease suggests that the biochemical cause of the movement disorders may be the toxic effect of the high acid levels on enzymes in the basal ganglia. It is thought that proper balance between acetylcholine, dopamine and γ-aminobutyric acid-containing fibres in the basal ganglia is necessary for normal movement. Stokke et al. have shown that glutarate (X) glutarate (XVI) and 3-hydroxyglutarate (XV) are all potent inhibitors of glutamate decarboxylase (γ-aminobutyric acid synthetase) which produces γ-aminobutyric acid. Since
the highest concentrations of γ-aminobutyric acid and γ-aminobutyric acid synthetase are found in the basal ganglia the high concentration of glutaric acid (X) found in the brain of the affected children and the abortus would thus inhibit the synthesis of γ-aminobutyric acid in basal ganglia and cause the clinical symptoms.

The treatment of glutaric aciduria by dietotherapy, vitamin (riboflavin) supplementation and drugs (Lioresal® a γ-aminobutyric acid replacement and sodium valproate) has been only variably effective, perhaps because irreversible damage has been done to the basal ganglia by the time the diagnosis is usually established. The observation that glutaric acid (X) is significantly accumulated in the brain even during foetal life in this disorder raises the possibility that neuronal damage may be done even in utero. The mild striatal changes which were noted in the affected foetus may reflect such damage or may be due only to terminal anoxia. Careful study of the histology of the striatum in normal and affected abortuses will be necessary in the future if this issue is to be resolved.

This work describes the first instances in which pregnancies at risk for glutaric acidemia have been monitored for this disease, and shows that affected and unaffected foetuses can be distinguished with comparative ease by measuring the glutaryl-CoA dehydrogenase activity in cultured amniotic fluid cells or the concentration of glutaric acid (X) in the amniotic fluid. The foetus predicted to be affected had the in utero diagnosis confirmed by determination of glutaric acid (X) and glutaryl-CoA dehydrogenase in tissues while a foetus predicted to be unaffected had normal glutaryl-CoA dehydrogenase activity in umbilical cord fibroblasts and did not show glutaric aciduria on two occasions during the first week of life.

Glutaric acid may accumulate in the amniotic fluid of the affected foetus because, like many other non-amino organic acids it is poorly reabsorbed from the glomerular filtrate and appears in the foetal urine and thus in the amniotic fluid. A similar mechanism has been suggested as the basis of the accumulation of abnormal metabolites in the amniotic
fluid of foetuses with methylmalonic acidemia, argininosuccinic acidemia and propionic acidemia. An alternative mechanism of accumulation may be diffusion through the foetal skin, umbilical cord and placenta of the large amounts of acid stored in the foetal cells.

Although it is technically easier to measure the concentration of glutaric acid (X) in amniotic fluid than to culture cells to measure glutaryl-CoA dehydrogenase, both investigations should be made for in utero diagnosis of glutaric acidemia. Further investigations are also required to determine the amniotic fluid levels of glutaric acid (X) in the case of a foetus heterozygous for the mutant allele.
Mrs. V.M. is undertaking a further monitored pregnancy. Amnio­centesis was performed at 15 weeks and analysis of the amniotic fluid showed a concentration of 0.62 µg/ml of glutaric acid, the presence of which was confirmed by GC-MS. The normal range obtained using the above method with a packed column has a mean of 0.72 µg/ml with a standard deviation of 0.15 µg/ml (normal range 0.5-1.1 µg/ml, 24 specimens).

A 24 hour urine specimen collected at the same time had a glutaric acid concentration of 0.86 µg/ml (0.9 µmol/mmol creatinine). The specimen had a total volume of 1570 ml (creatinine 7200 µmol/l) and this contained 1.35 mg of glutaric acid. The normal values obtained for 16 specimens of non-pregnant patients has a mean of 1.8 mg/day and a standard deviation of 1.2 mg/day (normal range < 5 mg/day).

Measurement of glutaryl-CoA dehydrogenase activity in cultured amniotic fluid cells shows normal levels and no abnormalities of glutaryl-CoA metabolism.

These results indicate that the foetus is not affected with glutaric acidemia and the pregnancy is continuing.
Experience has shown that relatively few inborn errors of metabolism can be diagnosed by clinical criteria alone and that detection generally depends upon screening those patients with suspicious symptoms for a wide range of metabolites. The application of GC and GC/MS for the profiling of classes of metabolites has lead to the identification of many new disorders, particularly the organic acidemias.

The organic acids which accumulate in organic acidemias are water soluble, ninhydrin negative acids most of which originate from the intermediary metabolism of amino acids. However some organic acids, such as propionic (XXI) and pyruvic (XXIV) may also be produced from sources such as lipids or carbohydrates. Very few genetic diseases have been described in which a defect in fatty acid oxidation is the primary biochemical lesion responsible for an organic acidemia. These include a defect in green acyl-CoA dehydrogenase and glutaric aciduria type II. Refsum's disease, in which the defective metabolism of branched chain fatty acids leads to an accumulation of phytanic acid (XXV), is usually classed as a lipid storage disease, since phytanic acid (XXV) is not water soluble and only small amounts of free acid are excreted.

The organic acidemias have been discovered and chemically characterised primarily by GC and GC/MS. The metabolites identified and detected in the urine in organic acidemias may not be the unutilised substrate of the defective enzyme system but may be the product (s) of the substrate modified by alternate pathways. Alternatively the unutilised substrate may or may not accumulate in body fluids. For example, alternate pathways found in several organic acidemias are glycine conjugation and conversion to the hydroxy analogue of aliphatic monocarboxylic acids. These alternate pathways produce the major metabolites seen in isovaleric acidemia during remission when little free isovaleric acid (XXVI) is excreted.
Some biochemical changes not directly related to the primary enzyme defect have been observed in several organic acidemias. These may include hyperglycinemia, hypoglycemia, hyperammonemia, long chain ketonuria, ketosis and lactic acidosis. These accompanying biochemical changes are highly significant because of their importance for diagnosis but may be misleading if too much importance is placed on them because the primary biochemical defect may be overlooked.

Clinically all of the organic acidemias are accompanied by either severe ketoacidosis with lethargy and coma or by some neurological manifestations. These acute clinical expressions of the disease may be episodic, reflecting the ability of alternate pathways to detoxify unutilised substrate of the defective enzymes except under severe metabolic stress. The disease may or may not be accompanied by mental deficiency and may demonstrate genetic heterogeneity. These clinical variations are not sufficient for diagnosis on clinical grounds alone and diagnosis rests on the chemical analysis of metabolites in serum or urine and enzymatic assays.

 Organic acidurias are not only secondary to genetic enzyme defects but may be inducible by nutritional and toxic factors. Examples of induced organic aciduria are the methylmalonic aciduria of vitamin $B_{12}$ deficiency and Jamaican vomiting sickness.

The major problem in the diagnosis of the organic acidemias is the need for rapid identification of the organic acids, enabling early diagnosis and life saving treatment of the patients. The preliminary screening for organic acids may be by paper or thin layer chromatography, chemical tests or GC urine screens, but the unequivocal identification of the abnormal acids relies on GC-MS. Our preliminary screening system has been described.

There have been a large number of systems described for the isolation of organic acids from urine. In this work urine samples were saturated with sodium bicarbonate and interfering substances extracted at pH 7-8 before acidifying the urine to pH 1 and extraction of the organic acids with ethyl acetate. When large amounts of acetoacetic acid (XXVII) or other keto acids were present in the urine these compounds were converted to the oximes or methoximes by the method of Lancaster et al, before
extraction. The oximes and methoximes have characteristic EI fragmentation patterns and GC retention times.

The preferred acid derivatives for GC and GC-MS are the methyl or trimethylsilyl esters. Silyl derivatives were chosen for our studies, as the Wollongong laboratory has a lot of experience with these derivatives. A mass spectral library (the Markey library of 1913 compounds of biological interest) was also available which contained many mass spectra of TMS derivatives. Methyl esters were also used at various stages of this investigation.

Most of our early work was done with Apiezon N stationary phase. This phase was chosen initially because of good resolution characteristics for low molecular weight acids and its high thermal stability. The use of this phase caused some problems as the TMS derivatives of dicarboxylic acids and some keto acids hydrolysed or decomposed during chromatography. In view of this, later work was done with an SE-30 packed column. Some of the later GC work was also done on an SE-54 wall coated capillary column. GC elution temperatures on the three columns used and the diagnostic mass spectral ions used for the identification of metabolites seen in this study have been listed in Appendix B.

To assist in the MS identification of unknown compounds, a series of specialist mass spectral libraries were sorted by computer from the NIH collection of 20,000 mass spectra. These specialist libraries were sorted according to the presence of elements such as silicon, nitrogen, halogens or oxygen in the molecule and molecular weights and then tables of frequency for the biggest fifteen peaks in each spectrum were generated. This work is described in Appendix C.

Fatty acids play an important role as an energy source. Higher plants and animals can store large amounts of neutral fat as a fuel reserve in a highly concentrated form (~ 9 kcal/g). The oxidation of fatty acids usually provides at least half the oxidative energy in the liver, kidneys, heart muscle and resting skeletal muscle. During periods of fasting fatty acid oxidation becomes virtually the sole source of energy with gluconeogenesis providing the glucose requirements of brain cells.
FIGURE 19. Activation of fatty acids for β-oxidation.

ATP = adenosine triphosphate, AMP = adenosine monophosphate
FIGURE 20. β-Oxidation of fatty acids.

One acetyl CoA is removed during each pass through the sequence. One molecule of palmitoyl CoA yields 8 molecules of acetyl CoA. FAD = flavine adenine dinucleotide, FADH₂ = reduced FAD, NAD = nicotinamide adenine dinucleotide, NADH₂ = reduced NAD, CoA = coenzyme A.
The major endogenous source of fatty acids for fuel is storage fat in the form of fat droplets in the cytoplasm which consists largely of triacylglycerols. Since fatty acids must be in the free form before they can undergo activation and oxidation, the triacylglycerols must first undergo hydrolysis by intracellular lipases to yield free fatty acids and glycerol. Another source of fatty acids is the metabolic turnover of membrane phospholipids.

Fatty acid oxidation is an intra-mitochondrial process and may be divided into two major areas: the activation and transfer of the fatty acid to the intramitochondrial space and the oxidative degradation of the activated fatty acid (Fig. 19).

Long chain fatty acids are first activated by an energy-requiring step in which the fatty acid ester of Coenzyme-A (CoA) is formed enzymatically at the expense of adenosine triphosphate (ATP). There are three enzymes responsible for this reaction each of which is specific for a given range of fatty acid chain lengths and will activate saturated, unsaturated, 2- or 3-hydroxy fatty acids.

Fatty acids have only a limited ability to cross the inner membrane as CoA esters and an enzyme fatty acid CoA: carnitine fatty acid transferase catalyses transfer of the fatty acyl group from its thioester linkage with CoA to an oxygen-ester linkage with the hydroxyl group of carnitine (XXVIII). The 0-fatty acyl carnitine so formed readily passes through the inner membrane. In the last part of the activation-transfer process the fatty acid acyl group is transferred from carnitine (XXVIII) to intramitochondrial CoA by the action of intramitochondrial carnitine fatty acid transferase. The fatty acyl CoA now is used as substrate by the fatty acid oxidation cycle, which occurs in the inner matrix compartment (Fig. 20).

The fatty acyl-CoA ester first undergoes enzymatic dehydrogenation at the α and β carbon atoms to form a Δ^Δ\text{2,3} - unsaturated fatty acyl-CoA as product. There are four different enzymes for this reaction, each of which is specific for a given range of fatty acid chain lengths.
FIGURE 21. \( \omega \)-Oxidation of fatty acids.

\( \text{NADP}=\text{nicotinamide adenine dinucleotide phosphate, NADPH}_2=\text{reduced NADP} \)
The $\Delta^2,3^2$ bond of the $\Delta^2,3^2$-enoyl-CoA ester is reversibly hydrated to give an L-3-hydroxyacyl-CoA and this reaction is catalysed by a stereospecific enzyme, 3-hydroxyacyl-CoA hydrolyase. In the next step of the fatty acid cycle, the L-3-hydroxy-fatty-acyl-CoA is dehydrogenated to form 3-ketofatty-acyl-CoA by L-3-hydroxy fatty acyl-CoA dehydrogenase. This enzyme is relatively nonspecific for fatty acid chain length but absolutely stereospecific for the L-stereoisomer.

In the last step of the fatty acid oxidation sequence which is catalysed by $\beta$-ketothiolase, the 3-ketofatty-acyl-CoA undergoes cleavage by interaction with a molecule of free CoA to yield a carboxylterminal two-carbon fragment as free acetyl-CoA and the CoA ester of the fatty acid shortened by two carbon atoms. This thiolytic cleavage reaction has a high negative free energy change and is energetically favoured. There appear to be two or three forms of the enzyme each specific for different chain lengths.

The fatty acyl-CoA, now two carbon atoms shorter, then re-enters the sequence of reactions at the dehydrogenation reaction and this cycle continues until the acid is completely oxidised.

This process is known as $\beta$ oxidation and normally accounts for over 95% of fatty acid oxidation. However an alternative pathway for oxidation of fatty acids exists, known as $\omega$ oxidation (Fig. 21). In this process the carbon atom which is furthest from the acid group is hydroxylated and then oxidised to another acid group. This oxidation probably occurs on the endoplasmic reticulum. The dicarboxylic acid formed can then be transported into the mitochondria and undergo $\beta$-oxidation to shorten the carbon chain in two unit steps. The appearance of dicarboxylic acids in the urine is indicative of increased $\omega$-oxidation.

There have been a number of clinical conditions reported where the urinary excretion of adipic acid (II) and other dicarboxylic acids is increased. These include Glycogen Storage disease, Reyes syndrome, Congenital Lactic Acidosis and Carnitine deficiency. An increased excretion of adipic (II) and suberic (XXIX) acids has also been observed in ketosis where the excretion of these dicarboxylic acids appear to
FIGURE 22. GC profile from Apiezon-N packed column of the silylated organic acid extract from urine obtained from patient M.E. at the time of the attack.

The peaks were identified as: 1, 3-hydroxybutyric acid; 2, urea; 3, glutaric acid; 4, adipic acid; 5, suberic acid.
parallel the degree of ketosis. However, in all these conditions the amount of adipic (II) and other dicarboxylic acids excreted is small and is associated with the excretion of large quantities of other organic acids such as lactic acid (XI) or 3-hydroxybutyric acid (XXX).

The investigations reported here have been carried out on 3 unrelated male children each of non-consanguinous parents and aged 6-14 months, who presented in a semi-comatose state. A history of two to four days mild gastro-enteritis prior to admission was given and physical examination showed gross hepatomegaly with mild dehydration. The children all showed a mild metabolic acidosis with low serum glucose. Serum transaminases were normal or mildly raised to about twice normal and plasma ammonia and amino acids were both normal. One patient was reported to have fat globules in his urine.

The patients improved on treatment with intravenous fluids and glucose and after one to three days were clinically well. The hepatomegaly gradually resolved over some weeks and, although the patients have shown susceptibility to infections, prompt treatment of these have prevented further acute attacks.

Our first patient (M.E.) was the seventh child of unrelated Australian parents, born at term following a normal pregnancy. Two days prior to admission the patient became febrile and began to have diarrhoea. Persistent vomiting continued for the next forty-eight hours and the patient became increasingly drowsy. On admission the semi-comatose, peripherally cyanosed child was mildly hypothermic (axillary temperature 35.2°C) with normal pulse and blood pressure and 8 cm of firm hepatomegaly.

A GC profile of the silylated extract of a urine sample taken from the patient close to the time of admission (Fig. 22) was dominated by one large peak which was identified by mass spectrometry as the disilyl derivative of adipic acid (II). Smaller amounts of suberic (XXIX) and sebacic (XXXI) acids were also detected in the urinary extract. Lactic (XI) and 3-hydroxybutyric (XXX) acids, which elute at the positions indicated on the profile, were present only at low levels. Estimation of the amount of adipic acid present in the urine at the time of admission showed that up to 4000 mmole of adipic acid were excreted/mole creatinine.
FIGURE 23. GC profile from the SE-30 packed column of the methylated organic acid extract from the urine obtained from patient M.E. at the time of the attack.

The peaks were identified as follows: 1, 3-hydroxybutyric acid; 2, adipic acid; 3, 3-methyladipic acid; 4, pimelic acid; 5, octenedioic acid; 6, sebacic acid; 7, hexanoylglycine + p-hydroxyphenylacetic acid; 8, azeleic acid; 9, decenedioic acid; 10, sebacic acid; 11, hippuric acid; 12, unknown; 13, suberylglycine.
**FIGURE 24. Synthesis of suberylglycine.**

```
HOOC-(CH₂)₆-COOH
SUBERIC ACID (XXIX)

(i) SOCl₂

(ii) CH₃OH (1 mole)

CH₃OOCC-(CH₂)₆-COCl

\[ \text{H₂N-CH₂-COOCH₃ HCl} \]
\[ + \text{N(CH₂ - CH₃)₃} \]

CH₃OOCC-(CH₂)₆-CO-NH-CH₂-COOCH₃

K OH

HOOC-(CH₂)₆-CO-NH-CH₂-COOH
SUBERYLGLYCINE (XXXIII)
```
The methylated extract of a urine sample taken during the attack was also examined by GC-MS. The GC trace of this sample is shown in Fig. 23. In addition to the large peaks of adipic (II) and suberic (XXIX) acids a major peak (peak 13) was observed which eluted at 217 °C. This peak was identified as the methylester (XLII) of suberylglutamic (XXXII). The mass spectrum of this compound matched with that of a sample of suberylglutamic (XXXII) synthesised by Gregersen et al. and was identical with that of a sample of suberylglutamic (XXXII) synthesised by a modification of the anhydrous Schotten-Baumann procedure developed by Sweetman et al. for the synthesis of tiglylglycine (Fig. 24). This is only the second time that the excretion of this compound has been reported. The glycine conjugates (XXXIII-XXXVI) of succinic (IX), glutaric (X), adipic (II) and sebacic (XXXI) acid were also synthesised but were not detected in the urinary extract of this patient. The glycine conjugate (XXXVII) of the monocarboxylic acid, hexanoic acid (XL), was present in significant amounts. The presence of this compound was confirmed by GC-MS by comparison with a sample synthesised as above. The glycine conjugates (XXXVIII, XXXIX) of butanoic (XLII) and octanoic (XLIII) acid were also synthesised but were not detected in the urine sample.

Other unusual compounds detected as their methyl ester derivatives were the monounsaturated dicarboxylic acids, octenedioic acid (XLIV) and decenedioic acid (XLV) and the unsaturated form of decenedioic acid (XLVI). These compounds were identified using mass spectrometry by reference to library spectra and those published by Tanaka and Lindstedt et al. The positions of the double bonds could not be determined from the mass spectra and were not determined by other methods.

The patient was resuscitated with intravenous glucose and his conscious state returned almost to normal within twenty four hours. Normal feeds were reintroduced at this stage and the child discharged ten days after admission with 6 cm firm hepatomegaly below the costal margin.

The appearance of the child's metabolic profile changed considerably
TABLE 31

CONCENTRATIONS OF METABOLITES DETECTED IN THE URINE OF M.E. AT THE TIME OF THE ATTACK AND IN A CLINICALLY UNAFFECTED PERIOD

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>CONCENTRATION (mmole/mole creatinine)</th>
<th>M.E. Attack</th>
<th>M.E. Unaffected</th>
<th>Normal Children (n=5, mean and range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adipic Acid</td>
<td></td>
<td>1747</td>
<td>6 - 8</td>
<td>6 (2 - 11)</td>
</tr>
<tr>
<td>Suberic Acid</td>
<td></td>
<td>764</td>
<td>7 - 10</td>
<td>9 (4 - 20)</td>
</tr>
<tr>
<td>Suberylglycine</td>
<td></td>
<td>429</td>
<td>&lt;9</td>
<td>N.D.</td>
</tr>
<tr>
<td>Hexanoic Acid*</td>
<td></td>
<td>182</td>
<td>166</td>
<td>N.D.</td>
</tr>
<tr>
<td>Octanoic Acid*</td>
<td></td>
<td>216</td>
<td>5</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

* Determined after acid hydrolysis

FIGURE 25. Family pedigree of patient M.E.
with his clinical condition, so that the recovery from the attack and
the regression of the hepatomegaly were associated with a drop in the
level of the urinary adipic acid (II) to the levels seen in normal
children. The other unusual metabolites detected at the time of the
attack also either fell to normal levels or disappeared from the urine
although significant amounts of hexanoylglycine (XXXVII) remained.

The amounts of some of these unusual metabolites detected in a urine
sample taken at the time of the attack and also in a clinically unaffected
period are shown in Table 31. It can be seen that although the
excretion of adipic (II) and suberic (XXIX) acids is within the normal
range during the clinically unaffected period, there is a very marked
increase in the excretion of these dicarboxylic acids at the onset of the
attack. At this time the quantity of suberic acid (XXIX) excreted as
the glycine conjugate (XXXII) accounted for approximately 35% of the
total suberic acid (XXIX) present in the urine.

This is considerably lower than that found in the patient described
by Gregersen et al., where the percentage of total suberic acid (XXIX)
present as suberylglycine (XXXII) ranged from 65% to 75%.

As well as the marked increase in the dicarboxylic acids, the mono­
carboxylic acids hexanoic (XL) and octanoic (XLIII) were measured after
acid hydrolysis and these were also found to increase during the attack.
However, the increase in the excretion of hexanoic acid (XL) was only
slight.

Since all of the unusual metabolites detected in the urine of the
patient appeared to be derived from fatty acids and especially from
increased ω-oxidation, we considered that this patient could have a
defect in β-oxidation of fatty acids. The possibility that this was
a genetic defect was strongly suggested by consideration of the family
pedigree (Fig. 25). Two other siblings a boy aged two years and a girl
aged 10 months had died suddenly during episodes of a gastro-enteritis­
like illness, when, after two days of diarrhoea and vomiting, they had
become drowsy for a few hours and abruptly died. To test this hypothesis
a fasting study was performed to determine whether the excretion of these
unusual acids could be induced as a result of an increase in endogenous
fat metabolism. During this study there was no clinical change observed
FIGURE 26. The excretion of adipic acid, suberic acid and suberylglycine in the urine of M.E. (age 8 months) as a function of the time of fasting.
FIGURE 27. The excretion of adipic acid and suberic acid in the urine of M.E. (age 2 years) as a function of the time of fasting.
in the child. The acid excretion during the fasting study (Fig. 26) when the child was eight months old shows that there is a marked increase in the urinary excretion of adipic (II) and suberic (XXIX) acids after 10 hours fasting. After 14 hours of fasting, the urinary adipic acid (II) concentration had increased to more than thirty times the normal level and the suberic acid (XXIX) to more than twelve times the normal level. The level of suberylglycine (XXXII) at this stage was also significantly increased above normal.

Although adipic acid (II), suberic acid (XXIX) and suberylglycine (XXXII) were the only metabolites to be monitored quantitatively during the fast, all of the unusual metabolites present in the urine at the time of the attack were observed in the urine sample obtained at the end of the fast.

Five normal children (aged from nine to sixty months) and one child diagnosed as having Reyes syndrome (aged two years nine months) were fasted under similar conditions and, in each case, no increase in the excretion of dicarboxylic acids was observed. Two of these children were fasted for more than thirty hours without raising the excretion of either adipic (II) or suberic acid (XXIX). In all control studies no hexanoic acid (XL) or hexanoylglycine (XXXVII) was observed in any urine.

In order to test the effect of an exogenous fat load M.E. was given a load of dairy fat (39 g) at the age of ten months. A twenty four hour urine collected after the load showed an increase of two to three times the levels of adipic (II) and suberic (XXIX) acids with suberylglycine (XXXII) and hexanoylglycine (XXXVII) also being detected.

Sixteen months after the initial fasting study, the patient was again fasted and once again the urinary levels of dicarboxylic acids were markedly raised (Fig. 27). On this occasion however, the increase in excretion did not commence until the child had fasted for seventeen hours.

These results suggest that the attack which caused the admission of
this child to hospital may have resulted from an inability to fast for lengthy periods of time and we have therefore been reluctant to subject the patient to fasts of prolonged duration in order to observe clinical symptoms.

Our second patient (R.Z.) was the second child of unrelated parents born at 35 weeks gestation. His neo-natal course and development were satisfactory. At fourteen months he contracted a middle ear and throat infection and gastro-enteritis. On day four his condition suddenly deteriorated with signs of peripheral shutdown necessitating respiratory support. He had a mild metabolic acidosis and a Dextrostick test was negative for sugar (serum glucose <20 mg/100 ml). Treatment was begun with glucose and general supportive therapy. During day six the child began to fit and EEG indicated a severe metabolic encephalopathy. A GC profile of the silylated extract of a urine specimen obtained on day five showed the presence of a large peak which was identified by mass spectrometry as the disilyl derivative of adipic acid (II). Smaller amounts of suberic acid (XXIX) were also detected in the urine. This specimen also showed only low levels of lactic (XI) and 3-hydroxy-butyric (XXX) acids.

A liver biopsy was done on day seven of the illness. This showed considerable intracellular lipid accumulation in a non specific macro-globular pattern which is characteristic of sick children. The characteristics of microvesicularity and universal small droplet fat infiltration without inflammation under light microscopy and enlargement of mitochondria, decrease in matrix density and loss of mitochondrial dense bodies under electron microscopy which indicate Reyes syndrome were all absent.

By day eight the child was conscious and eating normally, although irritable and improved rapidly until discharged.

At twenty months of age the child was re-admitted for a fat load study. The normal total daily intake of milk fat was given at breakfast, after a fifteen hour fast. The patient became very ill after twenty minutes and regurgitated the majority of the fat load. He became ketotic over the next few hours and required intravenous glucose for
FIGURE 28 The excretion of adipic acid and suberic acid in the urine of R.Z. (age 2½ years) as a function of the time of fasting.
FIGURE 29. GC profile from the SE-30 packed column of the methylated organic acid extract from patient R.Z. at the end of the fasting study (age 2 years 3 months).

The peaks were identified as: 1. glutaric acid; 2. adipic acid; 3. thymol; 4. pimelic acid; 5. 3-hydroxyadipic acid (?); 6. octenedioic acid; 7. suberic acid; 8. p-hydroxyphenylacetic acid; * hexanoylglycine; 9. 3-hydroxysuberlic acid (?); 10. decenedioic acid; 11. hippuric acid; 12. unknown M.Wt 204; 13. 3-hydroxysebacic acid (?); 14. 3-hydroxybenzoylglycine; 15. unknown M.Wt 270; 16. 3-hydroxydodecanoic acid (?); 17. suberylglycine.
FIGURE 30. GC profile from the SE-30 packed column of the methylated organic acid extract from the urine obtained from patient A.M. at the end of the fasting study (age 16 months).

The peaks were identified as: 1, adipic acid; 2, 3-methyladipic acid (?); 3, unknown Wt. 186; 4, pimelic acid; 5, octenedioic acid; 6, suberic acid; 7, citric acid; 8, unknown; 9, azelaic acid; 10, heptanoylglycine (?); 11, N-methylaspartic acid; 12, decenedioic acid; 13, sebacic acid; 14, hippuric acid; 15, 3-hydroxysebacic acid (?); 16, palmitic acid; 17, euberylglycine.
### TABLE 32

**PLASMA CARNITINE LEVELS FOR PATIENT R.Z.**

**MEASURED DURING FASTING STUDY**

<table>
<thead>
<tr>
<th>Hours of Fasting</th>
<th>Carnitine (nmole/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>30.0</td>
</tr>
<tr>
<td>6</td>
<td>18.4</td>
</tr>
<tr>
<td>12</td>
<td>44.5</td>
</tr>
<tr>
<td>18</td>
<td>37.1</td>
</tr>
</tbody>
</table>

Normal controls (498) 16-101 (mean 49)
resuscitation. Examination of two subsequent twenty-four hour urine collections showed that adipic acid (II) was increased in both specimens. The first collection showed six times normal levels and the second showed three times normal levels.

At two and a quarter years of age a diagnostic fast of fifteen hours duration was performed on R.Z. During this time he became drowsy and ketotic and began to vomit after fourteen hours. The amounts of adipic acid (II) and suberic acid (XXIX) found during this fasting study (Fig. 28) show that the excretion of adipic acid (II) is markedly increased after eleven hours of fasting and had risen to more than ten times normal. A GC profile of the methylated extract of the final urine sample obtained during this fast is shown in Fig. 29. This profile reveals that suberylglycine (XXXII) is also present but accounts for only about 10% of the total suberic acid. Hexanoylglycine (XXXVII) is also present in small amounts as are the unsaturated dicarboxylic octenedioic (XLIV) and decenedioic (XLV) acids. The hydroxy acids (XLVII-XLIX) corresponding to adipic acid (II), suberic acid (XXIX), and sebacic acid (XXXI) have also been tentatively identified by comparison with library spectra. These metabolites were not present in samples obtained before the fasting experiment was begun.

In order to test the possibility that carnitine (XXVIII) deficiency was the cause of the increased ω-oxidation, total acid soluble carnitine (XXVIII) was measured in plasma samples obtained during this study (Table 32). These results show some variation during the study but are all within the normal range of 16-101 nmole/ml (mean 49 nmole/ml) reported by Ikeakor and Lake.

A urine sample was also obtained from this child during a mild intercurrent infection and the acid profile examined by GC-MS. The abnormal metabolites were again detected. The adipic acid (II) level was found to be raised to about ten times normal and that of suberic acid (XXIX) to twice normal.

Our third patient (A.M.) was the second child of unrelated Australian parents. He was born at term and had some neonatal feeding difficulties with CNS involvement and no acidosis, but these resolved and he had
developed normally until eleven months of age. Four days prior to admission he developed mild vomiting and diarrhoea and two days before admission he became lethargic. He did not respond to treatment with intravenous fluids, developed 4 cm of firm hepatomegaly and became clinically dehydrated. A low blood sugar and mild metabolic acidosis were abnormal laboratory findings. The child responded to intravenous glucose immediately and over the next twenty-four hours he became less drowsy with a gradual return to normal activity and conscious state with the hepatomegaly gradually resolving over two weeks.

Examination of a urine sample obtained during this period showed the presence of ketonuria and fat globules. GC-MS of the TMS derivatives of an extract of the urine specimen showed the presence of greatly increased amounts of adipic acid (II), with raised levels of suberic (XXIX) and sebacic (XXXI) acids.

At thirteen months of age the child was re-admitted and after a fifteen hour fast received a fat load. He became mildly unwell, began vomiting about two hours after the fat load and became ketonuric. His blood glucose fell and he was given intravenous glucose and fluids. A urine specimen collected after the fat load showed that the adipic acid (II) level had risen to about fifty times normal and that the suberic acid (XXIX) level had risen to five times normal.

When a fasting study was performed on A.M. low normal blood glucose levels were maintained by the use of intravenous glucose. At the end of seventeen hours the serum triglycerides and free fatty acids had risen above normal and the patient was clinically well. Mild ketonuria developed at the end of fifteen hours fasting. After six hours from the start of the fast the levels of suberic acid (XXIX) began to rise and after eight hours the levels of adipic acid (II) also began to rise. At fifteen hours the adipic acid (II) level was twenty five times normal and the suberic acid (XXIX) level was twelve times normal. A GC profile of the methylated urinary extract of the sample obtained at fifteen hours (Fig. 30) showed that the suberylglycine (XXXII) peak accounted for about 65% of the suberic acid (XXIX). Pimelic acid (XX), sebacic acid (XXXI), octenedioic (XLIV) and decenedioic (XLV) acid were confirmed by GC-MS to be present in this sample and the hydroxy acid (XLIX) of
Sebacic acid (XXXI) was tentatively identified. Hexanoylglycine (XXXVII) was also present in significant amounts.

Although the presence of saturated and unsaturated dicarboxylic acids, suberylglycine (XXXII) and hexanoylglycine (XXXVII) characterise the metabolic profiles of these children, there was considerable variation in the relative amounts of the various compounds detected in the urines of the three children as well as in urine samples taken at different times from the same child.

The biosynthesis of dicarboxylic acids via the ω-oxidation of fatty acids was first studied by Verkade et al. and later by several groups of researchers. Their results suggest that ω-oxidation provides an alternative pathway for the oxidation of fatty acids. The end products of ω-oxidation are short chain dicarboxylic acids, since the long chain dicarboxylic acids initially formed by ω-oxidation on the microsomes can be further degraded by the mitochondrial β-oxidation system. It seems likely that the dicarboxylic acids observed in these three patients have arisen from a combination of ω and β-oxidation.

The relative importance of the ω and β-oxidation pathways to the overall metabolism of fatty acids under a variety of conditions is not known. The results obtained by Antony and Landau suggest that in rat liver, β-oxidation is the predominant pathway under normal conditions. However, experiments using branched chain fatty acids or enzyme inhibitors such as methylene cyclopropylacetyl-CoA have shown that ω-oxidation may assume a major role when the β-oxidation pathway is hindered. Thus there are a number of conditions known where an increased excretion of dicarboxylic acids in the urine is associated with a disturbance in β-oxidation.

Recently a number of reports have appeared in the literature concerning children who have been found to excrete large quantities of dicarboxylic acids as the major urinary organic acids. Przyrembel et al. have described a child with glutaric aciduria type II whose urine contained large amounts of C₆, C₈ and C₁₀ dicarboxylic acids as well as greatly elevated levels of lactic (XI) (10.3 mole/mole creatinine) and glutaric acid (X) (9.6 mole/mole creatinine). The presence of branched-chain amino acid metabolites in the urine indicated a generalised defect
in the activity of acyl-CoA dehydrogenases. Glutaric acid (X) was often observed as a minor component in the urinary GC profile of M.E. and the other two children, however the amounts present were well below the levels reported for glutaric aciduria type II.

Carnitine (XXVIII) deficiency is known to cause an increased excretion of adipic (II), pimelic (XX) and suberic (XXIX) acids as well as unsaturated dicarboxylic acids. In most cases the clinical symptoms described have been mainly in relation to muscle dysfunction although often effects are seen in liver function as well.\(^3\)\(^8\)\(^8\) There are three distinct types of syndromes with respect to carnitine (XXVIII) and lipid metabolism. The first is a deficiency\(^5\)\(^0\)\(^7\) or partial deficiency\(^5\)\(^0\)\(^8\) of carnitine palmitoyl transferase in muscle and possibly liver. The second is where muscle carnitine (XXVIII) levels are low but plasma levels are normal suggesting some defect in carnitine (XXVIII) transport into muscle cells. The third is a deficiency of carnitine (XXVIII) in muscle, plasma and liver described by Kaparti et al\(^3\)\(^8\)\(^8\) which suggests a defect in carnitine (XXVIII) biosynthesis which occurs in the liver. In the last syndrome an increase in dicarboxylic acid excretion was noted due to increased \(\omega\)-oxidation and this should occur in the other deficiency syndromes also although it has not been specifically reported. Our patients had significant differences in dicarboxylic acid excretion pattern to that described by Kaparti et al\(^3\)\(^8\)\(^8\) and this fact, together with the normal levels of plasma carnitine (XXVIII) found in R.Z. during the fasting experiment and the presence of hexanoylglycine (XXXVII) suggests that carnitine (XXVIII) deficiency is not the cause of the attacks in our patients.

The clinical pictures and the organic acid profiles observed in the three boys at the time of the attacks closely resemble those reported in patients suffering from Jamaican Vomiting Sickness, a disease produced by the ingestion of unripe akee fruit.\(^1\)\(^9\)\(^2\) This fruit contains a toxic amino acid derivative, hypoglycin A. A metabolite of this amino acid, methylenecyclopropylacetic acid, inhibits the transport of fatty acids into mitochondria\(^5\)\(^1\)\(^0\) and also inhibits dehydrogenation of several acyl CoA derivatives\(^5\)\(^1\)\(^1\) leading to the disruption of fatty acid \(\beta\)-oxidation and a massive urinary excretion of adipic acid (II) and other dicarboxylic acids. Hexanoylglycine (XXXVII) was also
excreted in large amounts in this disease.

A similar clinical case to that of our patients is described by Chalmers et al in identical twin boys following an adenovirus infection. One boy died before admission to hospital but the other recovered rapidly after treatment with intravenous glucose. Urine samples taken at the time of the attack showed elevated levels of adipic and suberic acids, unsaturated dicarboxylic acids and octanoic acid. The similarities between this disease and Jamaican vomiting sickness were noted by the authors who suggested that the attacks may have resulted from the ingestion of a toxic analogue of hypoglycin A. Further investigation by Chalmers and Watts has shown that unripe sloe berries may have been the causitive agent in these cases. In the case of the three boys described in this report, the results obtained from the fasting experiments indicate that the disease in these children is caused by a deficiency in endogenous metabolism rather than the ingestion of a toxic compound.

Tanaka et al have reported a patient suspected of having a deficiency of green acyl-CoA dehydrogenase, the enzyme responsible for catalysing the dehydrogenation of C₄-C₈ CoA esters. This child excreted large amounts of adipic acid (II), hexanoylglycine (XXXVII) and 2-ethylmalonic acid (III). In our patients adipic acid (II) and hexanoylglycine (XXXVII) were elevated but 2-ethylmalonic acid (III) was not detectable.

A patient who appears to have most features in common with the three children presented in this report, is the boy described by Gregersen et al. This child was prone to episodes of lethargy and unconsciousness but responded rapidly to intravenous glucose. At the times of these attacks, the urine was found to contain large quantities of dicarboxylic acids as well as suberylglycine (XXXII), the metabolite present in the urine of our patients.

The results of the fasting studies carried out on our patients indicate an inability to adequately cope with the flux of fatty acids through the β-oxidation pathway at times when the body is dependent on fat as the source of energy, and it would appear that periods of prolonged fasting may have induced the attacks which were responsible for the
admission of these children to hospital. In support of this view it is of interest to note that, the patient described by Gregersen et al. died suddenly following a 39 hour fast for diagnostic purposes.

The reason for the acute clinical state of the patients is unclear. Although low concentrations of glutaric acid (X) are nephrotoxic over long periods of time and high concentrations cause permanent neurological damage, adipic acid (II) and other dicarboxylic acids are relatively non-toxic and non-neurotoxic. Thus the relatively high concentration of dicarboxylic acids is probably not the cause of the clinical state.

Short chain fatty acids are however, very neurotoxic and are known to cause coma when given to experimental animals. This neurotoxicity explains the observation that in isovaleric acidemia serum acid concentration parallels the levels of consciousness during acute attacks. Short chain fatty acids also cause electro-encephalographic changes such as slow waves and spindles by causing depression of the activating system of the reticular formation in midbrain neocortex and by interfering with neuronal membrane function. The symptoms may thus be caused by the intracellular build up of monocarboxylic acids affecting the brain cells in combination with hypoglycemia caused by inhibition of gluconeogenesis.

The results from these patients suggest that the patients have a defect in one of the enzymes of the $\beta$-oxidation pathway. The variability of the metabolites excreted may indicate a different defect in $\beta$-oxidation or may reflect genetic heterogeneity of a single defect. This question cannot be settled until the individual enzymes for $\beta$-oxidation are assayed in each patient. The evidence obtained here suggests that the defect is in one of the chain length specific enzymes of the $\beta$-oxidation pathway and is thus probably in the dehydrogenase or thiolase enzymes. This defect then causes the utilisation of the alternate $\omega$-oxidation pathway to produce energy from the fatty acids.

The appearance of an almost normal GC profile under normal dietary conditions may be explained by the 'fat-sparing' effect of carbohydrates since, when adequate carbohydrate is available, only minute quantities of fatty acids are oxidised to provide energy. Thus a deficiency in
β-oxidation may not become significant until the body becomes reliant on fat for its energy requirements. The time at which this occurs will depend mainly on the amount of glycogen stored in the body. Analysis of the respiratory quotients suggests that approximately 8-10 hours after a meal glycogen stores become depleted and the body becomes dependent on fat as the source of energy. This time period coincides with the increased urinary excretion of dicarboxylic acids observed in the three children during fasting studies.
b. STUDIES OF PATIENTS WITH PROPIONIC ACIDEMIA: THE IDENTIFICATION OF 3-KETO-2-METHYLVALERIC ACID AND 3-HYDROXY-2-METHYLVALERIC ACID

In 1961, Childs et al described a male infant with episodic metabolic ketoacidosis, protein intolerance and remarkably elevated plasma glycine concentration. Extensive studies of glycine metabolism failed to reveal a definitive abnormality and investigation of the protein intolerance showed that five amino acids were ketogenic in the patient. These were leucine (L), isoleucine (L1), valine (LII), threonine (LIII) and methionine (LIV). During the next seven years the hyperglycinemia syndrome was differentiated into non-ketotic and ketogenic forms and some of the cases of the latter were shown to be cases of methylmalonic acidemia. Investigation of the initial case showed that it was not methylmalonic acidemia and further investigation showed the presence of butanone (LV), pentanone (VI) and hexanone (LVII) as well as acetone (LVIII). The butanone (LV) was postulated to arise from the spontaneous decarboxylation of 2-methylacetoacetic acid (LIX) an intermediate of isoleucine (LI) metabolism. In 1968 Hommes et al reported a patient whose neonatal death from severe metabolic acidosis was associated with extremely high concentrations of propionic acid (XXI) in the blood (>100 x normal) together with high liver concentration of unusual C15 and C17 fatty acids but no hyperglycinemia was found. Hsai et al used enzyme studies to demonstrate that a sibling of the original patient had an almost complete inability to metabolise propionate (XXI) and that in another patient no activity of propionyl-CoA carboxylase (EC 6.4.1.3) could be shown. The parents of one patient showed a partial deficiency of enzyme activity. Since that time a number of other patients have been described with a defect of this enzyme and prenatal diagnosis has been carried out by enzyme measurements of cultured cells and by measurement of secondary metabolites in amniotic fluid. An asymptomatic patient with a partial enzyme deficiency has recently been described.

The propionyl-CoA carboxylase enzyme has been crystallised from pig heart and studied extensively. It is a mitochondrial enzyme with a molecular weight of about 700,000 with 4 molecules of biotin as prosthetic group for the apoenzyme. The biotin is bound through an amide linkage in which the terminal carboxyl group of biotin reacts with
FIGURE 31. Metabolic pathways leading to the production of propionyl CoA.
The carboxylation reaction occurs in two stages in which bicarbonate is first bound to the biotin-apoenzyme complex through the biotin and the complex then reacts with propionyl-CoA to form D-methylmalonyl-CoA. The first stage of the reaction requires ATP and magnesium ions. The biotin cofactor requirement of the enzyme has been reflected in the discovery of some patients with a biotin responsive variant of the disease.

This lack of activity of propionyl CoA carboxylase causes a build up of propionyl-CoA. Propionyl-CoA is a key intermediate in the metabolism of many precursors (Fig. 31). It is an obligate metabolite of isoleucine (LI) which is found in large amounts in food protein. Threonine (LIII) and methionine (LIV) may be metabolised to propionyl-CoA through α-ketobutyryl-CoA and Hsai et al have shown that this is an almost quantitative pathway in methylmalonic acidemia. Valine (LI) may also be metabolised to propionate though β-hydroxyisobutyrate (LX), Odd chain fatty acids with 15 or 17 carbons which are minor constituents of body and dietary lipids, comprise 2-3% of fatty acids and produce 1 mole of propionate (XXI) per mole of fatty acid oxidised. Large dietary intake of fat may make this a major source of propionate (XXI). A minor but significant source of propionate (XXI) is the side chain cleavage of cholesterol to bile acids and propionic acid (XXI). A source of propionic acid (XXI) of major importance in ruminants is bacterial fermentation in the gut but this source has not been evaluated in man. Studies by Snyderman et al using antibiotics to sterilise the alimentary canal suggest that this may be a significant source of propionate (XXI) in man.

The next step in the metabolism of propionyl-CoA is the carboxylation to D-methylmalonyl-CoA and thence to succinyl-CoA for use in the citric acid cycle. Many alternative minor pathways for metabolism of propionyl-CoA became important when this sequence is blocked and lead to the synthesis of the characteristic metabolites found in propionic acidemia. These pathways include conjugation with glycine to give propionylglycine.
FIGURE 32. Metabolic pathways for propionyl CoA

The normal pathway is through methylmalonyl-CoA and succinyl-CoA to the citric acid cycle but if this pathway is blocked urinary excretion of products from the alternate pathways is observed.
FIGURE 33. The total ion current profile from the SE-54 capillary column of the silylated organic acid extract of urine obtained from patient Z.K. at the time of admission when he was severely ketotic.

The peaks were identified as: 1, lactic acid; 2, 3-hydroxypropionic acid; 3, 3-hydroxybutyric acid; 4, 3-hydroxy-2-methylbutyric acid; 5, acetoacetic acid (isomer 1); 6, 3-hydroxyvaleric acid; 7, acetoacetic acid (isomer 2); 8, 3-ketovaleric acid; 9, 3-keto-2-methylvaleric acid; 10, 3-hydroxy-3-methylglutaric acid; 11, 3-keto-3-methylvaleric acid; 12, 3-hydroxy-3-methylglutaric acid; 13, methylcitric acid; 14, 4-hydroxyphenyllactic acid. SS = solvent suppression
ω-oxidation to give 3-hydroxypropionic acid (XXII), condensation with oxalacetate (LXII) to give methylocitric acid (LXIII) and condensation with acetyl-CoA to give 3-keto-n-valerate (LXIV) (Fig. 32).

The most prominent biochemical feature of the defect in propionyl-CoA carboxylase is the accumulation of propionyl-CoA, which is the metabolite immediately preceding the enzyme block. This metabolite is hydrolysed and excreted as propionic acid (XXI). Compounds occurring earlier in the pathway are also excreted as well as secondary metabolites of propionic acid (XXI). Metabolites such as 2-butanone (LV), 3-pentanone (LVI), 3-hexanone (LVII), methyl citric acid (LXIII), tiglic acid (LXV), propionylglycine (LXI), tiglylglycine (LXVI), 3-hydroxypropionate (XXII), 3-keto-2-butyrate (LIX), 3-hydroxy-2-methyl-butyrate (LXVIII), 3-hydroxy-n-valerate (LXIX) and 3-keto-n-valerate (LXIV) have been identified in these patients. The presence of such secondary metabolites may give the initial indication of the diagnosis, as propionic acid (XXI) cannot be seen on the usual urinary screen for organic acids and specialised methods are required to detect it.

Our patient (ZK) was healthy at birth after a normal pregnancy, labour and delivery, but developed a severe metabolic acidosis at 48 hours. The perinatal deaths of 2 siblings suggested the possibility of an inborn error of metabolism as the basis for the acidosis. The organic acid profile of the child's urine when first admitted with ketosis (60U?) (Fig. 33) was dominated by a large peak of 3-hydroxybutyric acid (XXX) and smaller peaks of lactic acid (XI) and acetoacetic acid (XXVII) which are characteristic of ketoacidosis. In addition there were a number of peaks of compounds which have been described as being elevated in propionic acidemia. Duran suggests that methylcitric acid (LXIII) and 3-hydroxypropionic acid (XXII) were considered to be diagnostic of propionyl-CoA carboxylase deficiency and these compounds were present though only as minor components. The five carbon acids 3-keto-n-valeric acid (LXIV), 3-hydroxy-n-valeric acid (LXIX) and 3-hydroxy-2-methyl-butyric acid (LXVIII) were major constituents of the specimen and Duran suggests these may be frequently found in urine from patients with propionyl-CoA carboxylase deficiency when they are ketotic. These compounds were identified by comparison of the mass spectra of the TMS derivatives with those in our library as well as those published by Sweetman et al and Ando et al. A urinary keto acids and ketone screen showed the
FIGURE 34. The total ion current profile from the SE-54 capillary column of the silylated organic acid extract of urine obtained from patient Z.K. after the ketosis had been controlled.

The peaks were identified as: 1, lactic acid; 2, 3-hydroxypropionic acid; 3, 3-hydroxybutyric acid; 4, 3-hydroxy-2-methylbutyric acid; 5, 3-hydroxyisovaleric acid; 6, 3-hydroxyvaleric acid; 7, acetoacetic acid; 8, 3-hydroxy-2-methylvaleric acid; 9, 3-keto-2-methylvaleric acid (isomer 1); 10, 3-keto-2-methylvaleric acid (isomer 2); 11, 3-keto-2-methylvaleric acid (isomer 3); 12, propionylglycine; 13, glutaric acid; 14, adipic acid; 15, fumar-2-hydroxy-5-methyl-5-carboxylic acid; 16, 2-hydroxyglutaric acid; 17, 3-hydroxy-3-methylglutaric acid; 18, 4-hydroxyphenylacetic acid; 19, methyl citric acid; 20, 4-hydroxyphenyllactic acid; 21, palmitic acid. SS = solvent suppression.
FIGURE 35. Synthesis of propionylglycine (LXI).
presence of acetone (LVIII), butanone (LV) and 2-pentanone (LVI) all of which have been reported in propionic acidemia. The presence of these metabolites suggested a diagnosis of propionic acidemia and serum propionate (XXI) was measured. The result obtained was 3.5 mM/l (normal = 1.29 ± 0.52 µM/l and serum glycine (LXX) was 356 µM/l (normal range of 92-392 µM/l. The identity of the propionic acid (XXI) was confirmed by GC-MS of the butyl ester formed on pyrolysis of the tetrabutylammonium salt in the injection port of the gas chromatograph.

The diagnosis of a *propionyl-CoA carboxylase* defect causing propionic acidemia was confirmed by the enzyme studies which were not available until after the patient's death. The activity of *propionyl-CoA carboxylase* in cultured skin fibroblast extracts was 30 pmol/min/mg protein. A series of normal patients had activities of 860± 200 pmol/min/mg protein. Thus Z.K. had about 3.5% normal activity of the enzyme. The fibroblasts complemented into the pcc BC group of Gravel et al. No other patients in this complementation group have been biotin responsive suggesting that an initial impression of biotin responsiveness may have been due to some other factor in the vigorous initial treatment. Management of the patient was based on control of the ketoacidosis by fluid therapy, peritoneal dialysis and exchange transfusions to remove accumulated organic acids, as well as protein restrictions.

The extract of a urine sample obtained the following day when ketosis was under control is shown in Fig. 34. At this time the serum propionate (XXI) concentration was 2 mM/l and glycine concentration was 382 µM/l. This profile reveals large peaks of 3-hydroxypropionic acid (XXII), 3-hydroxy-2-methylbutyric acid (LVIII) and methylcitric acid (LXIII). 3-keto-n-valeric acid (LXIV) and 3-hydroxy-n-valeric acid (LXIX) which were components of the first sample were barely detectable in this sample. Two metabolites, 3 hydroxyisovaleric acid (XXIII) and propionylglycine (LXI) appeared which were not observed in the previous sample. The presence of these metabolites was confirmed by GC-MS by comparison with authentic samples. Propionylglycine (LXI) was synthesised (Fig. 35) by a modification of the anhydrous Schotten-Baumann procedure developed by Sweetman et al. for the synthesis of tiglylglycine (LXVI). In this method an acid chloride is reacted with glycine methyl ester hydrochloride (LXXI) in the
FIGURE 36. The preparation of 3-keto-2-methylvaleric acid (LXVII) via oxidation of propionaldol and the Claisen condensation of ethyl propionate.
presence of base under anhydrous conditions at room temperature and
the resulting acylglycine methyl ester hydrolysed in methanolic sodium
hydroxide to obtain the free acylglycine. This method has a number of
advantages over the usual methods of synthesis of acylglycines
in which the acid chloride or anhydride is reacted with glycine in
basic aqueous media which gives a product which is often contaminated
with free acid. The advantages of Sweetman et al's method include
the production of the methyl ester as intermediate which may be
purified by distillation. The method gave yields of 85% for the
condensation and 80% for the hydrolysis. The preparation of the acid
chloride and glycine methyl ester hydrochloride proceeded in
quantitative yield.

Two other prominent peaks were present in the GC trace (peaks 8 and 13,
Fig. 34) which could not be identified. CI-MS of peak 13 indicated the
compound had a molecular weight of 274 as the TMS derivative and that
peak 11 had an identical molecular weight. Further examination showed
peak 11 had an identical EIMS. The peak at m/e 245 corresponding to a loss
of 29 amu from the molecular ion suggested the presence of an ethyl group
within the molecule. Comparison of the mass spectrum with that of 3-keto-
n-valeric acid (LXIV) showed that the major fragment ions of the un-
known were m/e values 14 amu higher in the unknown than in 3-keto-n-
valeric acid (LXIV) suggesting the presence of an extra methylene unit.
A possible structure of 3-keto-2-methylvaleric acid (LXVII) was post-
ulated and the synthesis of this compound was undertaken.

Two different syntheses of this compound were carried out. The first
approach (Fig. 36A) was via the oxidation of propionaldol (LXXII). The
preparation of propionaldol (LXXII) has been described from propanal
(LXXIII) by the Aldol reaction. The required aldehyde is readily obtained
by the oxidation of propanal (LXXIV). Propionaldol (LXXII) was prepared in low yield using sodium hydroxide catalyst but oxidation
of the product gave no 3-keto-2-methylvaleric acid (LXXVII).
This may have been due to the acid in the Jones reagent reversing the
condensation. When Spath et al's modification (Fig. 36B) was
attempted the intermediate 2,4-diethyl-5-methyl-6-hydroxy-1,3-dioxane
(LXXV) was isolated in 51% yield, but distillation from adipic acid (II)
regenerated propanal (LXXIII) and gave no propionaldol (LXXII). This
FIGURE 37. (a) The EI mass spectrum of peak 13 from the GC shown in Fig. 34. (b) The EI mass spectrum of the silylated derivative authentic 3-keto-2-methylvaleric acid.

\[
\text{CH}_3-\text{CH}_2-C-\text{CH}-\text{COOC}_2\text{H}_5
\]

3-KETO-2-METHYLVALERIC ACID ETHYL ESTER (LXXVII)

\[
\text{CH}_3-\text{CH}_2-\text{CH}-\text{CH}-\text{COOC}_2\text{H}_5
\]

3-HYDROXY-2-METHYLVALERIC ACID ETHYL ESTER

\[
\text{CH}_3-\text{CH}_2-C\text{-CH}-\text{COOH}
\]

3-HYDROXY-2-METHYLVALERIC ACID (LXXIX)

FIGURE 39. (a) The EI mass spectrum of peak 8 from the GC profile shown in Fig. 34. (b) The EI mass spectrum of the silylated derivative of authentic 3-hydroxy-2-methylvaleric acid.
approach was abandoned in favour of the Claisen condensation\(^5\) (Fig. 36C). Self condensation of ethyl propionate (LXXVI) in the presence of sodium ethoxide gave 3-keto-2-methylvaleric acid ethyl ester (LXXVII) which may be hydrolysed to the required acid. Condensation was affected in reasonable yield and the product was hydrolysed at 4\(^\circ\) to minimise decarboxylation. Acidification of the sodium salt was followed by immediate extraction of the acid. This hydrolysis procedure has been used by Krüger\(^5\) for the isolation of acetoacetic acid. The yield of ketoacid (LXXVII) from the ester was over 80\%. The dried acid was derivatised with BSTFA and GC-MS analysis of the product showed it to be identical with that of the unknown urinary component (Fig. 37). Two peaks were obtained in the GC profile of both the authentic and urinary 3-keto-2-methylvaleric acid (LXXVII). These silylation products are the cis and trans-enol derivatives. By increasing the derivatisation time the majority of the earlier eluting peak was converted to the second product which we believe is the thermodynamically more stable trans-enol derivative.

The compound present in peak 8 of the silylated urinary acid profile (Fig. 34) was shown to have a molecular weight of 276 by EI-MS. Further examination showed that peak 9 in the profile also had a molecular weight of 276. Both compounds had very similar fragmentation patterns by EI-MS. On the basis of their EI-MS the compounds were tentatively identified as 3-hydroxy-2-methylvaleric acid (LXXIX). A sample of authentic hydroxy acid was synthesised by sodium borohydride reduction of the keto acid ethyl ester (LXXVII) followed by base hydrolysis (Fig. 38). The two silyl derivatives were shown to have the same GC retention times and mass spectra as peak 8 and 9 in the profile (Fig. 39). The reason for the presence of the two silyl derivatives is due to the fact that the hydroxy acid contains two asymmetric carbon atoms (Fig. 38) and thus can give rise to two diastereoisomers and the two peaks correspond to the disilyl derivatives of these two diastereoisomers of 3-hydroxy-2-methylvaleric acid (LXXIX). A similar phenomenon was observed for methylcitric acid (LXIII) where the silyl derivative was resolved into two peaks on the capillary column (Fig. 34 - peaks 21 and 22).
FIGURE 40. (a) The serum concentration of propionic acid (■—■) compared with (b) the urinary concentrations of 3-hydroxypropionic acid (○—○) and 3-keto-2-methylvaleric acid (●—●) in patient Z.K. during the course of his treatment.
The absolute configuration of the two diastereoisomers of methylcitric acid (LXIII) excreted by a patient with propionic acidemia have been determined previously.

Both 3-keto-2-methylvaleric acid (LXVII) and 3-hydroxy-2-methylvaleric acid (LXXIX) were found consistently in urine samples taken from this patient during his illness. The most likely origin of 3-keto-2-methylvaleric acid (LXVII) appeared to be the thiolase-catalysed self condensation of propionyl-CoA and this suggested that the urinary excretion of the keto acid (LXVII) may be a useful indicator of intracellular and serum propionic acid levels.

The urinary levels of 3-keto-2-methylvaleric acid (LXVII) and serum levels of propionate (XXI) were monitored together to investigate a possible correlation and the results obtained show (Fig. 40) a good correlation between 3-keto-2-methylvaleric acid (LXVII) and another propionate metabolite, 3-hydroxypropionic acid (XXII). There seemed to be a reasonable correlation between the urinary excretion of these two metabolites and serum propionate (XXI) concentration after 90 hours but there was a marked deviation from this relationship in the sample at the time of admission when the child was severely keto-acidotic. This may be due to the intensive treatment of the child or may be due to a lag in the production of metabolites of propionic acid (XXI).

Another possible explanation for the presence of 3-keto-2-methylvaleric acid (LXVII) and 3-hydroxypropionate (XXII) is that they are formed in the urine after excretion by the kidney. For example they could be formed in the bladder or during storage.

In order to test whether 3-keto-2-methylvaleric acid (LXVII) or 3-hydroxy-2-methylvaleric acid (LXXIX) had arisen artificially from the self condensation of propionic acid on storage, propionic acid was added to normal urine to give a final concentration of 5 mM and the solution was incubated at 37°C for 22 hours. Extraction and derivatisation of the organic acids failed to show the presence of either 3-keto-2-methylvaleric acid (LXVII) or 3-hydroxy-2-methylvaleric acid (LXXIX).
The unusual ketone, 3-pentanone (LVI) has been shown to be present in the urines of patients with propionic acidemia and methylmalonic acidemia by means of the test although the mechanism of its formation has not been discussed. Since 3-keto-2-methylvaleric acid (LXVII) is a β-keto acid, it is possible that it could be responsible for the formation of 3-pentanone (LVI) by a decarboxylation mechanism. The identification of 3-keto-2-methylvaleric acid (LXVII) in the urine of our patient prompted us to explore the possibility that 3-pentanone (LVI) could be derived from this β-keto acid.

There are two ways in which 3-pentanone (LVI) could be produced from 3-keto-2-methylvaleric acid (LXVII): the spontaneous decarboxylation of the acid in vivo or the artefactual production of the ketone in vitro due to the high concentration of acid in the 2,4-DNP reagent used. Support for the latter mechanism as the source of the pentanone is given by a study of the decarboxylation reaction of acetoacetic acid. The first order rate constant for the decomposition of acetoacetic acid is $4 \times 10^{-3} \text{sec}^{-1}$ in 0.2 M acid (a half life of 5.8 mins) and increases with acid concentration whereas that for the anion is $8 \times 10^{-6} \text{sec}^{-1}$ (half life 24 hours).

In order to test these possibilities methods of analysis of free ketones in urine and for ketone-DNP derivatives had to be developed. The extraction of free ketones from urine without extracting the keto acids had been reported by Liebich and Huesgen. These authors extracted heptanones from basic urine with cyclohexane and analysed the dried organic extract by mass fragmentography. Our approach was to use their solvent extraction method with pentane and analyse for the presence of ketones by GC using a suitable derivative. The oximes of acetone (LXXX), butanone (LXXXI), 3-pentanone (LXXXII) and 3-heptanone (LXXXIII) were prepared but attempts to separate them from the solvent on the available columns were unsuccessful. Similarly the methoximes of acetone (LXXXIV), butanone (LXXXV) 3-pentanone (LXXXVI) and 3-heptanone (LXXXVII) were prepared but were found to co-elute with the solvent. Derivatisation of the oximes with BSTFA gave some separation from the solvent but interference was found from the reagent impurities and by-
products. Bachmann et al reported the separation of the dinitrophenyl-
hydrazones of ketones on lightly loaded silicone columns and the 2,4-
dinitrophenylhydrazones of acetone (LXXXVIII), butanone (LXXXIX), 3-
pentanone (XC) and 3-heptanone (XCI) were prepared. The Dexil-300
packing material used by these workers was not available and the use of
SE30 gave poor resolution of the derivatives. However use of an OV-17
capillary column gave adequate separation of the free ketones. The
method gave recoveries of greater than 97% for added 3-pentanone (LVI)
and the standard curve using 3-heptanone (XCI) as an internal standard
was a straight line with correlation coefficient of 0.99 over the
range 0-1500 μg 3-pentanone (LVI).

The 2,4-dinitrophenylhydrazones were also analysed by TLC in two
solvent systems. One system, that of Seakins et al, separated
2,4-DNP derivatives and the other, that of Daum et al, was used to
separate neutral derivatives. The identity of the - - - A derivatives was
confirmed by solid probe EI and CI mass spectrometry.

In an initial experiment, several microlitres of 3-keto-2-methyl-
valeric acid (LXVII) were added to the 2,4-DNP reagent. An immediate
precipitate was formed and TLC and MS analysis showed that this was
the 2,4-DNP derivative (XC) of 3-pentanone (LVI). A precipitate of 3-
pentanone- (XC) was also obtained when urine containing 5mM 3-keto-
2-methylvaleric acid (LXVII) was treated with the reagent under the
conditions used for urine screening. Since this urine sample had been
thoroughly extracted with pentane to remove 3-pentanone (LVI) prior
to the addition of the dinitrophenylhydrazine reagent, it seemed that
the 3-pentanone- (XC) must have been produced as a result of the
decarboxylation of the keto acid or the ketoacid- in acid solutions.
When 5mM solutions of 3-keto-2-ethylvaleric acid (XCVIII) or 3-keto-2-
methylcaproic acid (XCV) in urine were similarly treated with 2,4-DNP
reagent a precipitate of 3-hexanone (XC) was obtained.

Treatment of 5 mM solution of 2-methylacetoacetic acid (LIX) in urine
with the reagent gave a precipitate which contained about equal amounts
of butanone (LXXXIX) and 2-methylacetoacetic acid (XCVI) while
treatment of a 5mM solution of acetoacetic acid (XXVII) gave less than 10%
FIGURE 41. The urinary concentrations of 3-pentanone and 3-keto-2-methylvaleric acid as a function of time.

The lines drawn represent lines of best fit determined by linear regression analysis. The high initial level of 3-pentanone resulted from the fact that the 3-keto-2-methylvaleric acid added to urine contained a significant amount of this ketone as degradation product. 3-Keto-2-methylvaleric acid (---), 3-pentanone (-----).
of acetone-^2,4-DNP (LXXXVIII) the major product being acetoacetic acid-2,4-DNP (XCVII). Smith has also described the formation of acetone-2,4-DNP (LXXXVIII) from solutions of acetoacetic acid (XXVII). When 5 mM aqueous solutions of keto acids were treated with 2,4-DNP reagent similar results were obtained. In each case the ketone must have been produced as a result of the decarboxylation of the keto-acid or the keto acid in acid solution.

To determine whether 3-pentanone (LVI) could be formed by decarboxylation of urinary 3-keto-2-methylvaleric acid (LXVII) within the body, the keto acid was added to normal urine at a concentration of approximately 5 mM and the solution incubated at 37°C. Samples were removed at various time intervals for the measurement of 3-pentanone (LVI) and the residual keto acid (LXVII). These results are summarised in Fig. 41 and show an increase in pentanone (LVI) and a decrease in keto acid (LXVII) concentrations. This suggests that the keto acid is readily decarboxylated at body temperatures.

Using the above technique, urine samples of Z.K. which had been shown to contain large amounts of 3-keto-2-methylvaleric acid (LXVII) were shown to contain large amounts of 3-pentanone (LVI) (0.35 mole/mole creatinine in the 2nd urine sample, Fig. 34). The identity of this ketone as 3-pentanone (LVI) was confirmed by GC-MS of the free ketone and TLC and MS of its 2,4-DNP derivative. In urine samples taken after treatment of the patient 3-keto-2-methylvaleric acid (LXVII) concentrations were low, and 3-pentanone (LVI) could not be detected.

Our second patient (E.B.) was a child who died at age 22 months after an episodic illness characterised by drowsiness, hypotonia and myoclonic jerking. In earlier episodes this was accompanied by peripheral circulatory failure and hypothermia. Later the major feature of his illness was the presence of severe acidosis. No diagnosis was made while he was alive but the amino acid chromatograms showed elevations of urinary and serum glycine. He had elevated serum ammonia and neutropenia but studies of glycine metabolism in E.B. failed to provide a reason for the elevated serum glycine. The child also had sugar intolerance and malabsorption and his protein restricted diet contained medium chain triglycerides.
FIGURE 42. Total ion current profile from the SE-54 capillary column
the silylated organic acid extracts from the urine of E.B.
when medium chain triglycerides were the major calorie
source.

The peaks were identified as: 1, lactic acid; 2, 3-hydroxypropionic acid; 3, 3-hydroxybutyric acid; 4, 3-hydroxy-2-methylbutyric acid; 5, 3-hydroxyisovaleric acid; 6, 3-hydroxyvaleric acid; 7, 3-hydroxy-2-methylvaleric acid; 8, succinic acid; 9, 3-keto-2-methylvaleric acid; 10, propionylglycine; 11, glutaric acid; 12, adipic acid; 13, unknown; M.W. 236; 14, suberic acid; 15, 16, methylcitric acid; 17, o-bactic acid.
as the major source of calories. At the age of seven months episodes of keto acidosis began. Urinary studies at this time gave equivocal results, one group suggesting methylmalonic acid (I) as the major metabolite and another claiming the metabolite was in fact acetoacetic acid (XXVII).

At the age of twelve months he entered a period of relative stability and growth when on a restricted protein diet. Episodes of ketoacidosis continued and at 22 months the child died following a viral infection. Enzyme studies were not available for this patient as attempts to culture cells were unsuccessful.

A sample of urine from E.B. had been collected when he was 13½ months old when he in relatively good health. This sample showed slightly increased glycine concentration and was negative for ketones and ketoacids. The metabolic profile of this urine sample is shown in Fig. 42. The profile is dominated by large peaks due to adipic (II) and succinic acids (IX).

The presence of the dicarboxylic acid peaks due to glutaric acid (X), pimelic acid (XX) suberic acid (XXI) and sebacic acid (XXXI) may be explained by the use of the medium chain triglyceride as calorie source causing saturation of the β-oxidation pathway and consequent ω-oxidation of the fatty acids. The presence of lactic (XI) acid and 3-hydroxybutyric (XXX) acid may be explained by mild acidosis.

The large peak due to 3-hydroxypropionic (XXII) acid suggests the diagnosis of propionic acidemia and other metabolites which were identified to support this diagnosis were 3-hydroxy-2-methylbutyric acid (LXVIII), propionylglycine (LXI) and methylcitric acid (LXIII). 3-Keto-2-methylvaleric acid (LXVII) and 3-hydroxy-2-methylvaleric acid (LXXIX) were also present but in small amounts.

One further prominent peak in the GC trace (peak 13 Fig. 42) could not be identified. Cl-MS of the peak indicated the compound had a molecular weight of 288 as the silyl derivative. Examination of the MS suggested a number of similarities to that of 3-keto-2-methylvaleric acid (LXVII) and two possible structures were proposed: that of 3-keto-2-methylcaproic acid (XCIV) or 3-keto-2-ethylvaleric acid (XCI) with
FIGURE 43. Mixed Claisen condensation of ethyl propionate and ethyl butyrate.
FIGURE 44. Preparation of 3-keto-2-methylvaleric acid (XCIV) and 3-keto-2-ethylcaproic acid (XCIII) via the Reformatsky reaction.
the former being the most likely structure. The ethyl esters of these compounds were synthesised using the mixed Claisen condensation between ethyl propionate (LXXVI) and ethyl butyrate (XCVIII) (Fig.43). These compounds could not be separated by fractional distillation but were separable on a capillary GC column. A portion of the mixture was hydrolysed and the resulting acids silylated to obtain their GC-MS. Unfortunately unequivocal identification of the acids could not be made and it was decided to synthesise the individual keto acids. This was done according to Fig. 44 by a Reformat$ky reaction. 3-Hydroxy-2-methylcaproic acid ethyl ester (CIV?) was prepared from 2-bromopropionic acid ethyl ester (XCIX), butanal (C) and zinc. In a similar fashion 3-hydroxy-2-ethylvaleric acid ethyl ester (CIII) was prepared from propanal (CI) and 2-bromobutyric acid ethyl ester (CII). The keto acids were prepared by oxidation and hydrolysis of the hydroxy acids.

Comparison of the GC retention times and MS of the unknown and the synthesised 3-keto-2-methylcaproic acid (XCV), 3-keto-2-ethylvaleric acid (XCI) showed that the unknown was not identical to either compound and further attempts to identify it were abandoned.

The diagnosis of propionic acidemia is well supported however in this patient both on clinical and biochemical grounds although no enzyme studies are available. Treatment with high levels of biotin gave no improvement in E.B. although in Z.K. a mild response was seen. However neither patient could be classed as biotin responsive.

The two cases described here show a large number of unusual metabolites which have been described as being elevated in patients with propionic acidemia. There appears to be considerable variation in the excretion pattern of these secondary metabolites in these patients and in those described in the literature. The origin of many of these metabolites may be explained by examination of the precursors and metabolites of propionylCoA (Figs. 31 and 32). The reason for the raised levels of glycine shown by our patients and many of those in the literature remains obscure but is still a valuable indication to the condition.
The variability of the excretion pattern of metabolites can cause difficulties with the diagnosis of this disease even in laboratories which have well established GC screening procedures. Under conditions such as these, the identification of 3-keto-2-methylvaleric acid (LXVII) and 3-hydroxy-2-methylvaleric acid (LXXIX) as major urinary components in our patients may be a valuable aid in the future diagnosis of propionic acidemia on the basis of secondary metabolites. These two compounds do not appear to have been reported previously as components of human metabolism although Przyrembel et al speculate that 3-keto-2-methylvaleric acid (LXVII) may be the structure of an unknown compound eluting with fumaric acid in their studies of a patient with propionic acidemia. The evidence these authors present however is not conclusive.

Both 3-keto-2-methylvaleric acid (LXVII) and 3-hydroxyvaleric acid (LXXIX) have been detected in urine samples from the two patients we have encountered with propionic acidemia. In addition, we have also detected low levels of these acids in some patients with methylmalonic acidemia. However, neither the hydroxy acid nor the keto acid have been found in urine samples taken from normal controls.

It seems likely that 3-keto-2-methylvaleric acid (LXVII) arises by an enzyme catalysed self condensation of propionyl CoA in a reaction analogous to the formation of acetoacetate from acetyl CoA. Several 3-ketoacyl-CoA thiolases are known which catalyse the general reaction involving the condensation of two molecules of thioesters to produce a 3-ketothioester with the release of a thio alcohol. Since 3-ketoacyl-CoA thiolases have been described in mammalian systems with broad substrate specificites, it is conceivable that, under conditions where propionyl CoA is greatly elevated, one or more of these enzymes could form 3-keto-2-methylvaleryl CoA by the condensation of two moles of propionyl CoA. Enzymic reduction of the 3-keto-2-methylvaleryl-CoA, catalysed by 3-hydroxy-acyl-CoA dehydrogenase (E.C. 1.1.1.35) could then yield 3-hydroxy-2-methylvaleryl CoA. Hydrolysis of the CoA esters of these two compounds would then produce 3-keto-2-methylvaleric acid (LXVII) and 3-hydroxy-2-methylvaleric acid (LXXIX).
The proposed modes of formation of 3-hydroxy-2-methylvaleric acid (LXVII) and 3-keto-2-methylvaleric acid (LXXIX) in this patient could best be tested by administration of stable isotope labelled propionic acid (XXI) or isoleucine (LI), followed by an examination of the labelling patterns in the two urinary acids. However these loading studies have not been possible due to the death of the patients. The increase in the suggested keto acid in Przyrembel et al's patient following an isoleucine (LI) load however supports this mode of formation.

The excretion of urinary ketones in a patient with ketotic hyperglycenaemia and patients with ketosis due to starvation, diabetes or a high fat intake has been investigated by Menkes. The patient with hyperglycenaemia was found to excrete elevated amounts of two unusual ketones, 2-butanone (LV) and 3-pentanone (LVI). Tsao and Pfeiffer proposed that 2-butanone (LV) was formed through the spontaneous decarboxylation of 2-methylacetooacetic acid (LIX). The origin of the 3-pentanone (LVI) excreted in the urine of patients with propionic acidemia and methylmalonic acidemia however has remained obscure, although Menkes postulated that this ketone could arise via the condensation of propionyl CoA and methylmalonyl CoA. In this work we have demonstrated that the 3-pentanone (LVI) observed in the urine of patients with propionic acidemia and methylmalonic acidemia can arise from the decarboxylation of 3-keto-2-methylvaleric acid (LXVII). This decarboxylation was shown to occur when the keto acid was incubated in normal urine at 37°C and would presumably be faster in urines of lower pH from acidotic patients. It is possible that some 3-pentanone may be produced enzymically from 3-keto-2-methylvaleric acid (LXVII) in a reaction analogous to the formation of acetone (LVIII) from acetoacetic acid (XXVII), catalysed by acetoacetate decarboxylase (E.C.4.1.1.4.).

The screening procedure for the presence of unusual ketones usually involves the addition of 2,4-dinitrophenyldrazine in 2 M HCl to urine, and it was also demonstrated that 3-pentanone (XC) could arise directly from the breakdown of urinary 3-keto-2-methylvaleric acid (LXVII) during the course of the reaction. This decarboxylation should be taken into account when studying or quantitating urinary ketones as 2,4-DNP derivatives.
An elevated concentration of glycine in plasma and urine has been described as being characteristic of propionic acidemia, but in our first patient (Z.K.) the serum and urinary levels of glycine at the time of admission were within the normal range. At that time the serum propionic acid (XXI) concentration was 3.5 mM. Analysis of the urinary amino acids at that time did however show increased amounts of cystine and lysine. These amino acids were also found to be elevated in the urines of three of eight patients with propionic acidemia investigated by Duran et al. Two of these three patients did not show hyperglycinuria. Although neither hyperglycinuria nor hyperglycinaemia was present during the acute illness period in our patient, once the serum levels of propionic acid were controlled by treatment, both hyperglyceinemia and hyperglycinuria appeared.

From an analysis of the gas chromatography profiles and the results of the quantitation of urinary metabolites in Z.K. it would seem that under conditions where excess acetyl CoA was available there appeared to be a preferential condensation between propionyl CoA and acetyl CoA. Thus, the major propionate-derived metabolites appearing in the urine when the child was ketotic were 3-ketovaleric acid (LXIV) and 3-hydroxyvaleric acid (LXIX). However when the ketosis had been controlled, self-condensation to form 3-keto-2-methylvaleric acid (LXVII), condensation with oxaloacetate (LXII) to form methylcitric acid (LXIII) as well as oxidation to produce 3-hydroxypropionic acid (XXII) and condensation with glycine (LXX), seemed to be the major routes of propionyl CoA metabolism.

These observations may have important implications for screening laboratories since, under conditions where a child is ketotic, the major secondary metabolites to look for in cases of propionic acidemia may be 3-ketovaleric acid (LXIV) and 3-hydroxyvaleric acid (LXIX) whereas in non-ketotic patients the diagnostic metabolites appear to be 3-hydroxypropionic acid (XXII) and methylcitric acid (LXIII). A negative relationship between the excretion of 3-hydroxybutyric acid (XXX) and methylcitric acid (LXIII) was noted by Duran et al. in their study of patients with propionic acidemia.
It is interesting to speculate on the reason why 3-keto-2-methylvaleric acid (LXVII) and 3-hydroxy-2-methylvaleric acid (LXXIX) have not been observed previously in other patients with propionic acidemia, but were major urinary metabolites in patient Z.K. The reason for this may be related to the elevated serum levels of propionic acid (XXI) seen in this child, even after control of the ketosis. This observation could perhaps indicate that these two metabolites, which apparently arise from the condensation of two propionyl CoA molecules, may be relatively more prominent in patients who have very high serum levels of propionic acid.

**NOTE ADDED IN PROOF**

The excretion of 2-methyl-3-keto-valeric (LXVII) acid has also been reported in a publication by Lennert, W., Schumann, L., Urbanek, R., Niederhoff, H., Bohm, N., *Eur J. Pediatr.*, 128, 197 (1978). These authors also suggest that the compound may be formed by self-condensation of propionyl-CoA.
EXPERIMENTAL
**GENERAL**

Gas chromatographic (GC) separations were carried out with a Becker Packard model 419 chromatograph (Delft, The Netherlands) fitted with flame ionisation detectors. The columns employed were a 1.8mx6mm glass column packed with 3% Apiezon-N on acid washed dimethyldichlorosilane (DMCS) treated Chromasorb-W (80-100 mesh) and a 1.8mx6mm DMCS treated glass column packed with 3% SE-30 on DMCS treated Gas Chrom-Q (80-100 mesh). Helium was used as carrier gas at a flow rate of 30 ml min$^{-1}$. The injection ports and detectors were maintained at 260° C. Programmed separations were carried out at 6° C min$^{-1}$ from 70° or 80° with an initial isothermal delay ,using a linear temperature programmer.

The identification of urinary metabolites was confirmed by combined gas chromatography-mass spectrometry (GC-MS) in which a Varian Aerograph GC (model 2700) was interfaced, via a jet separator to a Dupont 491B mass spectrometer fitted with a dual electron impact/chemical ionisation source. The GC was fitted with a 1.8mx6mm glass column packed with either 6% Apiezon-N or 3% SE-30 on DMCS treated Chromasorb-W (80-100 mesh). A helium flow rate of 25 ml min$^{-1}$ was used, and during analysis the temperature was programmed from 80° C to 250° C at a rate of 6° C min$^{-1}$.

Electron impact (EI) mass spectra were recorded at a source temperature of 240° C and an ionising voltage of 70 eV with a block voltage and repeller voltage of 1400V. Chemical ionisation (Cl) mass spectra were recorded using isobutane at 0.5 to 1 Torr with an electron energy of 70 eV, and a block voltage and repeller voltage of 1400VDC. The MS was interfaced with a Nova 1220 computer (Data General Corporation, U.S.A.).

GC-MS and quantitative multiple ion selection (MIS) were carried out using a Varian MAT44 GC-MS system in which a 25m SE-54 (Jaeggi) wall coated capillary column was directly coupled to the ion source of the mass spectrometer. For GC a helium flow rate of 2 ml min$^{-1}$ was used, and during analyses the temperature was programmed from 70° C to 220° C at 4° C min$^{-1}$. 
EI mass spectra were obtained using an ionising voltage of 70 eV and an emission current of 0.8 mA. The ion source was maintained at 200°C, the transfer lines at 250°C and the injection port of the gas chromatograph at 250°C. The total ion current was measured from mass 50 to 500 and was recorded with 320mV full scale.

Cl mass spectra were obtained using isobutane at 45-50Pa with an ionising voltage of 160 eV and an emission current of 0.2 mA.

The concentration of amino acids were determined by ion exchange chromatography using a Technicon TSM amino acid analyser.

Infrared spectra (IR) were recorded on a Perkin Elmer Model 237 infra red spectrophotometer and were calibrated using polystyrene film.

All melting points were measured in open capillaries using a thermometer calibrated with standard substances. All boiling points were uncorrected.

p-Tolylsulphonylmethylnitrosamide, propionic acid, propionyl chloride, butanoic acid, butanoic anhydride, 2-bromobutyric acid ethyl ester, 2-bromobutyric acid, α-methylacetoacetic acid ethyl ester, trideuteromethyl iodide, methylmalonic acid, ethylmalonic acid and succinic acid were obtained from Fluka AG chemische Fabrick Buchs, Switzerland. Propanol, butanol, acetone, ethanol, methanol, ethyl propionate, ethyl acetoacetate pentane and silver oxide were obtained from Ajax Chemical Ltd., Sydney, Australia.

Adipic acid, glycine, glutaric acid, diethyl malonate and tetrabutylammonium bromide were purchased from Merck Chemical Co., Darmstadt, Germany.

Thionyl chloride, chromic oxide, butanone, pentanone, heptanone, 2,4-dinitrophenylhydrazine, sodium borohydride, sodium, molecular sieve (5A and 3A) and zinc wool were supplied by BDH Chemicals Limited, Poole, England. Suberic acid, sebacic acid, hexanoic acid and octanoic acid were supplied by Sigma Chemical Company, Missouri, U.S.A.. Succinic anhydride and adipic acid monomethyl ester monochloride were obtained from
Solvents used for extraction of organic acids were analytical reagent grade and were obtained from Ajax Chemical Co., Sydney, Australia. Each batch was tested for the presence of organic acids before use.

Benzene was dried by azeotropic distillation and distilled before use.

Dry ethanol and dry methanol were prepared by distillation of the alcohol from magnesium ethoxide or methoxide.

The BSTFA used for formation of TMS derivatives contained 1% TMCS as catalyst and was obtained from Regis Chemical Co., Chicago, U.S.A. in 1 ml or 10 ml ampoules stored under nitrogen. Reaction vials (1 ml) fitted with rubber caps and tapered reaction vials fitted with teflon lined rubber septa were supplied by Regis Chemical Co., and were cleaned with CrO\textsubscript{3} - H\textsubscript{2}SO\textsubscript{4}.

Standard solutions of organic acids were made up in ethanol and were stored in a deep freeze for periods of up to one month. A range of concentrations of the individual organic acids were prepared by pipetting standard solutions of an organic acid and internal standard into reaction vials and, after derivatisation, calibration curves were determined by comparing the GC peak height of the organic acid with the peak height of the internal standard.

Quantitation of individual organic acids was achieved by measurement of the peak height of each acid relative to the peak height of the internal standard and interpolating from a standard curve. The recovery of individual acids was determined by the method of standard additions using a normal urine, serum or amniotic fluid. All reported results for patient samples in this thesis have been corrected for recovery.
Preparation of Diazomethane

*p-Tolylsulphonylmethyl-nitrosamide* (2.24g, 10 mmol) was dissolved in ether (30 ml) and the solution was cooled in ice. A solution of potassium hydroxide (0.4g) in ethanol (10 ml; 95%) was added and if a precipitate formed more ethanol was added to dissolve it. After 5 mins the ethereal solution of diazomethane was distilled into an ice/salt bath cooled receiver, the outlet of which was connected to a trap containing cold ether (30 ml). The solution from both receivers was combined and stored over potassium hydroxide pellets in a freezer for periods of up to five days.

Preparation of Tetrabutylammonium Hydroxide (~0.4 M) in Methanol

Tetrabutylammonium bromide (3.22g, 10 mmol), silver oxide (1.75g, 7.5 mmol) and anhydrous methanol (20 ml) were stirred together for two hours. The solids were removed by filtration and the solution diluted to 25 ml. Aliquots of the solution were titrated to phenolphthalein end point with \( \text{H}_2\text{SO}_4 \) (0.100 M) to check the concentration and the solution stored over molecular sieve below 0° C.

Preparation of Jones Reagent

Chromic oxide (26.7g, 0.27 mol) was dissolved in water (40 ml), sulphuric acid (21.3 ml) was added and the solution was diluted to 100 ml. This solution is 8N in chromic acid and contains the calculated amount of sulphuric acid.

Preparation of 2,4-Dinitrophenylhydrazine Reagent

This reagent was prepared according to Seakins et al. and was a saturated solution of 2,4-dinitrophenylhydrazine (~0.5%) in hydrochloric acid (2 M).

Screening Procedure for Organic Acids in Urine

Urine (1 ml) was saturated with sodium bicarbonate and then extracted with ethyl acetate (3 x 2 ml). This extract was discarded. The aqueous layer was then adjusted to pH 1 with hydrochloric acid, saturated with sodium chloride and extracted with ethyl acetate (3 x 3 ml). The organic
layers were dried (MgSO₄), filtered and evaporated to dryness under a stream of nitrogen at room temperature. The dried extract was silylated with BSTFA (50μl; 60°C; 30 min) and an aliquot injected into the gas chromatograph.

**Extraction and Quantitation of Organic Acids in Amniotic Fluid**

Internal standard (5μg; usually adipic acid (II)), water (2 ml) and sodium dodecylsulphate (0.3 ml of 5% solution) were added to amniotic fluid (1 ml). The solution was saturated with salt and adjusted to pH 9-11 with sodium hydroxide (1 M). The solution was left to stand at room temperature for 20 mins and extracted with ethyl acetate (3 x 3 ml) and these extracts were discarded. The solution was acidified to pH 1 with hydrochloric acid (5 M) and extracted with ethyl acetate (3 x 3 ml). External standard (5μg; usually pimelic acid (XX)) was added to the combined extracts which were dried (MgSO₄) and evaporated under a stream of nitrogen. The residue was dissolved in a few drops of ethyl acetate and transferred to a tapered reaction vial. The dried residue was derivatised for GC analysis with BSTFA (30μl; 65°C; 1 h). An aliquot (0.5-1μl) of the solution was injected into the chromatograph. Quantitation of acids was achieved by measurement of the peak heights of the unknown, relative to the peak height of the internal standard and interpolating from a standard calibration curve. The external standard was used to check the recovery of internal standard. For the preparation of methyl esters the dried residues were derivatised by treatment with diazomethane.

**Extraction and Quantitation of Organic Acids in Serum or Plasma**

Internal standard (5μg; usually adipic acid (II)), water (8 ml), sodium dodecylsulphate (1 ml; 5% solution) were added to serum (1 ml). The solution was saturated with salt and adjusted to pH 9-11 with sodium hydroxide (1 M). The solution was left to stand at room temperature for 20 mins and extracted with ethyl acetate (3 x 10ml) and these extracts were discarded. The solution was acidified to pH 1 with hydrochloric acid (5 M) and extracted with ethyl acetate (3 x 30 ml). External standard (5μg; usually pimelic acid (XX)) was added to the combined extracts which were dried (MgSO₄) and evaporated under a stream of nitrogen.
nitrogen. The residue was dissolved in a few drops of ethyl acetate and transferred to a tapered reaction vial. The dried residue was derivatised for GC analyses with BSTFA (30µl, 65°C, 1h) and an aliquot injected into the chromatograph. For the preparation of methyl esters the dried residue was treated with diazomethane and evaporated to about 30µl and an aliquot injected into the chromatograph.

Quantitation of Urinary Organic Acids

To urine (1 ml) or diluted urine (1 ml) was added an internal standard (usually adipic acid (II); 50 µl of 1mg/ml solution in ethanol) and sufficient sodium bicarbonate to obtain a saturated solution. The solution was extracted with ethyl acetate (3 x 3 ml) and these extracts were discarded. The urine was acidified to pH 1 with hydrochloric acid and then extracted with ethyl acetate (3 x 3 ml). The organic extracts were dried (MgSO₄) and the solvent removed under a stream of nitrogen at room temperature. The residue was transferred to a small vial using ethyl acetate and again evaporated to dryness. The dried residue was derivatised for GC analysis with BSTFA (50µl; 65°C; 30 min) and an aliquot (usually 1µl) was taken for injection.

Preparation of Oxime-TMS Derivatives of Urinary Keto Acids

Internal standard (50µg; usually adipic acid (II)) was added to urine (1 ml) and the solution adjusted to pH 10 with sodium hydroxide. Freshly prepared hydroxylamine hydrochloride solution (200µl; 100 mg in 1 ml) was added and the solution incubated at 65°C for 30 mins. The cooled solution was saturated with salt, the pH adjusted back to 10 if necessary and then extracted with ethyl acetate (3 x 2 ml). These extracts were discarded. The aqueous layer was acidified to pH 2 with hydrochloric acid (1 M) and extracted with ethyl acetate (3 x 2 ml). The ethyl acetate was dried (MgSO₄) and evaporated under a stream of nitrogen. The residue was dissolved in a few drops of ethyl acetate and transferred to a reaction vial and again dried under nitrogen. The residue was derivatised for GC analysis with BSTFA (50µl; 65°C; 30 mins) and an aliquot injected into the chromatograph.
Hydrolysis and Quantitation of Urinary Acylglycines

To a volume of urine containing 1 mmol creatinine, was added hydrochloric acid (3 ml; 6 M) and the solution heated at 110° for 3 hours. The internal standard (adipic acid (II)) was added to the cooled solution and the acids extracted with ether (3 x 3 ml). The ether extracts were dried (MgSO₄) and evaporated at room temperature under a stream of nitrogen to final volume of about 100μl. BSTFA (50μl) was added and the extract heated (60°; 3 min). GC analyses were performed on 3% SE30 using an initial temperature of 55°C.

Measurement of Propionic Acid (XXI) in Urine

Propionic acid was measured by a modification of the sample pretreatment method of Chalmers et al combined with the GC method of Otterstein and Bartley. Urine (200μl) and internal standard (isobutyric acid (CV) (5mg/ml; 100μg) were mixed with a portion of damp neutral Dowex 50W x 12 resin (H⁺ Form; Biorad Ltd.). The tube was mixed by inversion and allowed to stand for ten minutes and centrifuged. An aliquot (5μl) was injected into the GC.

The separation was carried out on a 1.8 M x 2 mm column packed with 100% SP 1200/1% H₃PO₄ at a column temperature of 125° in a Hewlett Packard model 402 chromatograph with flame ionisation detector and using nitrogen as carrier gas. The injector temperature was 140° and the detector temperature 150°. The column was flushed with injections of phosphoric acid or water between samples.

Measurement of Propionic (XXI) Acid in Serum

Propionic acid was measured by a modification of the sample treatment method of Remésy and Demigne combined with the GC method of Otterstein and Bartley. Serum (200μl), internal standard (isobutyric acid; 5 mg/ml; 20μl) and ethanol (1 ml) were mixed together in a glass stoppered centrifuge tube, allowed to stand for 15 minutes and centrifuged. The supernatant was decanted, neutralised with sodium hydroxide
(20μl; 0.2 M) and evaporated to dryness under a stream of nitrogen. The residue was reconstructed with distilled water (15μl) and acidified with phosphoric acid (5μl) just prior to injection. An aliquot (0.2-2μl) was injected into the GC.

**Test for Production of 3-Keto-2-methylvaleric Acid (LXVII) and 3-Hydroxy-Propionic Acid (XXII) in Urine**

To freshly voided urine (100 ml; pH 5.5) from a healthy male subject was added propionic acid (XXI) (37 mg, 0.5 mmol) and the urine warmed at 37° C for 24 hours. An acidified aliquot (3 ml) was saturated with sodium chloride, adipic acid (II) (50μg) was added and the solution extracted with ether (3 x 10 ml). The extracts were dried (MgSO₄), the solvent removed and the silylated (BSTFA; 50μl; 65°; 30 mins) residue examined by GC. No trace of 3-keto-2-methylvaleric acid (LXVII) or 3-hydroxypropionic acid (XXII) were found.

**Measurement of Urinary 3-Pentanone (LVI)**

To urine (4 ml) were added internal standard (100μl of 1 mg/ml of 3-heptanone (XCII) in water) and saturated sodium carbonate solution (100μl). The pH was checked and if it was below 9 more sodium carbonate was added. The solution was immediately extracted with pentane (0.5 ml), the organic phase separated and an aliquot of this solution was injected onto a 60 m OV-17 SCOT column (Scientific Glass Engineering). After injection, the GC oven temperature was maintained at 50° C for four minutes and then increased at 3° C min⁻¹. Quantitation of 3-pentanone was achieved by measurement of the peak height of 3-pentanone (LVI) relative to the peak height of 3-heptanone (XCII) and interpolating from a standard curve.

**Reaction of Urinary Acetoacetic Acid (XXVII) with 2,4- Dinitrophenyl-hydrazine Reagent**

Freshly voided urine (100 ml) was spiked with freshly prepared acetoacetic acid (XXVII) (51 mg; 0.5 mmol). A portion of the solution was basified (NaOH; 1M) and then extracted with ether. The ether extracts showed no acetone (LVII)) by GC or 2,4-dinitrophenylhydrazine reagent.
Spiked urine (8 ml) and 2,4-dinitrophenylhydrazine reagent (8 ml) were left to stand at room temperature (3 hours). A slight cloudiness developed in the solution and when this was recovered by filtration, washed and dried weighed < 1 mg. The precipitate was examined by solid probe CIMS and TLC (silica gel, chloroform:acetic acid (100 : 7); hexane:chloroform (1:1) and was shown to be the 2,4-dinitrophenylhydrazone of acetoacetic acid (XCVII) with a trace of the 2,4-dinitrophenylhydrazone of acetone (LXXXVIII). On standing for a further 48 hours a further amount of precipitate formed which had the same composition.

Reaction of Urinary 2-Methylacetoacetic Acid with 2,4-Dinitrophenylhydrazine Reagent

Freshly voided urine (100 ml) was spiked with freshly prepared 2-methylacetoacetic acid (LIX) (58mg; 0.5 mmol). A portion of the urine was basified (NaOH; 1M) and extracted with ether. The ether extracts showed no butanone (LV) by GC or 2,4-dinitrophenylhydrazine reagent.

Spiked urine (8 ml) and 2,4-dinitrophenylhydrazine reagent were left to stand at room temperature. Cloudiness developed after 10 minutes and a large amount of precipitate had developed after 3 hours. The precipitate was recovered by filtration, washed and dried (yield 9 mg). The precipitate was examined by solid probe CIMS and TLC (silica gel, chloroform:acetic acid (100; 7) and hexane:chloroform (1:1) and was shown to be a mixture of the 2,4-dinitrophenylhydrazones of 2-methylacetoacetic acid (XCVI) and butanone (LXXXIX) in the ratio of 3:2.

Reaction of Urinary 2-Methyl-3-oxovaleric Acid (LXVII) with 2,4-Dinitrophenylhydrazine Reagent

Freshly voided urine (100 ml) was spiked with freshly prepared 3-Keto-2-methylvaleric acid (LXVII) (65 mg, 0.5 mmol). A portion of the solution was basified (NaOH; 1M) and extracted with ether (3 x 2 ml). The dried (Mg SO₄) ether extracts showed no pentanone (LVI) by GC or 2,4-dinitrophenylhydrazine reagent.

Spiked urine (8 ml) and 2,4-dinitrophenylhydrazine reagent were mixed together at room temperature. A small amount of gas was produced and
a precipitate began to form immediately. After ten minutes the solution was filtered and the precipitate was washed and dried. No further precipitate formed in the urine on standing. The precipitate (11 mg.) was examined by solid probe CIMS and TLC (silica gel, chloroform:acetic acid (100:7) and hexane:chloroform (1:1) and was shown to be the 2,4-dinitrophenylhydrazone of pentanone (XC). No trace of the acid derivative was found.

Reaction of Urinary 3-Keto-2-methylcaproic Acid (XCIV) with 2,4-Dinitrophenylhydrazine Reagent

When urine (100 ml) spiked with freshly prepared 3-keto-2-methylcaproic acid (XCIV) (72 mg; 0.5 mmol) was treated with 2,4-dinitrophenylhydrazine reagent as described above a quantitative yield of the 2,4-dinitrophenylhydrazone of 3-hexanone (CVI) was obtained in 10 minutes.

Reaction of Urinary 3-Keto-2-ethylvaleric Acid (XCIII) with 2,4-Dinitrophenylhydrazine reagent

When urine (100 ml) spiked with freshly prepared 3-keto-2-ethylvaleric acid (XCIII) (72 mg; 0.5 mmol) was treated with 2,4-dinitrophenylhydrazine reagent as described above a quantitative yield of 3-heptanone-2,4-dinitrophenylhydrazone (CVI) was obtained in 10 minutes.

Preparation of Diethyl Trideuteromethylmalonic Acid (V)

Sodium (1.6g, 0.07 mol) was dissolved in dry absolute ethanol (30 ml) in a 100 ml flask equipped with a reflux condenser and drying tube (CaCl₂). Redistilled diethyl malonate (11.1g, 0.07 mol) was added to the stirred solution. After 5 mins trideuteromethyl iodide (VI) (10g, 0.07 mol) was added slowly during one hour while the flask was gently heated. When all the trideuteromethyl iodide (VI) had been added the mixture was heated under reflux for a further half hour and then neutralised with acetic acid (5 ml). The majority of the alcohol was removed by distillation and dilute hydrochloric acid (10 ml; 0.3M) was added. The aqueous lower layer was separated from the ester and extracted with ether (3 x 20 ml). The combined organic layers were dried by shaking quickly with calcium
chloride, filtered, and the solvent removed. The crude ester was shaken for exactly one min with a cold solution of sodium hydroxide (1 ml; 30%) and then with dilute hydrochloric acid and dried (MgSO$_4$). The crude ester was distilled to give pure trideuteromethylmalonic acid diethyl ester (V) bp 88-90°/16 mm (lit. for unlabelled ester 82-3°/12 mm), yield 5.6g (46%), Cl-MS [MH]$^+$ 177.

**Preparation of Trideuteromethylmalonic Acid (VIII)**

Trideuteromethylmalonic acid diethyl ester (V) (3.5g, 20 mmol), was added dropwise to a stirred solution of potassium hydroxide (7 ml; 50%) under reflux during 1h. The solution was heated under reflux for a further 2h and then distilled until the vapour temperature reached 100°. The cooled alkaline solution was washed with ether (2 x 10 ml) and acidified with cold hydrochloric acid (50%) to pH 2. The crude acid was extracted with ethyl acetate (5 x 20 ml), the extracts dried (MgSO$_4$) and the solvent removed. The residue was recrystallised from benzene-ether then three times from ethyl acetate-light petroleum to give pure trideuteromethylmalonic acid (VIII) m p 125-6° (lit. for unlabelled acid 126-7°) yield 2.0g (83%).

**Preparation of Glycine Methyl Ester Hydrochloride (LXXI)**

To anhydrous methanol (35 ml) cooled in dry ice acetone was added freshly distilled thionyl chloride (3.5 ml) drop wise. Glycine (LXX) (5g, 66 mmol) was then added and the mixture refluxed for 45 min. After removal of the solvent the crystalline residue was dissolved in methanol and the solution evaporated to dryness. The residue was finally recrystallised from methanol-ether to give pure glycine methylester hydrochloride (LXXI), long needles, m.p. 176° (lit. mp 177°) yield 7.9g (94%).
Preparation of Propionylglycine (LXI)

Propionyl chloride (2.3g, 25 mmol) and glycine methyl ester hydrochloride (LXXI) (3.8g, 30 mmol) were stirred together in dichloromethane (30 ml). Triethylamine (6.1g, 60 mmol) in dichloromethane (15 ml) was added dropwise and the solution stirred at room temperature for 16 hours. Dilute hydrochloric acid (20 ml; 20% was added and the layers separated. The dichloromethane layer was washed (H₂O), dried (MgSO₄) and the solvent removed. The residue was distilled under reduced pressure to give pure propionylglycine methyl ester bp 130/2.5 mm, yield 3.1g, (85%), Cl-MS (MH)⁺ 146. The compound had a retention time of 7.5 min (elution temperature 125°) on 3% SE30 and the El mass spectrum contained ions at m/e 146 (MH)⁺, 145 (M)⁺, 113 (M-CH₃OH)⁺, 86 (M-COOC₂H₅)⁺ and 67 (M-NHCH₂COOCH₃)⁺.

A portion of the methyl ester (725 mg, 5mmol) was refluxed in sodium hydroxide (15 ml; 1.25 M in 50% ethanol) for 1 hour. Water (10 ml) was added and the solution was extracted with ether (3 x 20 ml). These extracts were discarded and the solution acidified (6 M HCl) and extracted with ether (3 x 20 ml). The ether extracts were dried (MgSO₄) and the solvent removed. The residue was recrystallised from acetone to give pure propionylglycine (LXI), mp 126° (lit mp 126-7°), yield 520 mg (80%). Treatment of a sample of this compound with BSTFA gave the disilyl derivative which had an elution time of 11.3 mins (elution temperature 148°) on 3% SE 30, Cl-MS (MH)⁺ 276, and the El spectrum contained ions at m/e 275 (M)⁺, 260 (M-CH₃)⁺, 232 (M-CH₃ + CO)⁺, 158 (M-CO₂MS)⁺ and 102 (TMS-(NH-CH₂))⁺. A small amount of monosilyl derivative was also formed which had an elution time of 11.2 min (elution temperature 147°) on 3% SE30, Cl-MS (MH)⁺ and the El spectrum contained ions at m/e 203 (M)⁺, 188 (M-CH₃)⁺, 159 (M-CO₂)⁺, 144 (M-(CO₂ + CH₃))⁺, and 131 (M-(TMS + H))⁺.

Preparation of Butyrylglycine (XXXVIII)

Butanoic acid (2.2g, 25 mmol) was refluxed with thionyl chloride (15 ml) for 4 hours. The excess thionyl chloride was removed under vacuum and benzene (2 x 10 ml) added and removed under vacuum. The acid chloride was treated as above to give butanoylglycine methyl ester bp 126°/2 mm (lit 118/0.9 mm), yield 2.8 g (72%) Cl-MS (MH)⁺ 160. The compound had
a retention time of 9.2 min (elution temperature 135°) on 3% SE 30 and the El mass spectrum contained ions at \( m/e \) 160 {MH}⁺, 159 {M}⁺, 131 \( \text{CH}_2 = \text{C} (\text{OH}) \text{NHCH}_2 \text{COOCH}_3 \)� (McLafferty rearrangement), 127 {M-CH\(_3\)OH}⁺ 100 {M-\( \text{COOCH}_3 \)}⁺ and 71 {M- (NH-CH\(_2\)-COOCH\(_3\))}⁺. Hydrolysis of a portion of the ester (800 mg, 5 mmol) and recrystallisation of the residue from ether gave pure butyrylglycine (XXXVIII) mp 69° (lit mp 68-70°) yield 410 mg (56%). Treatment of a sample of this compound with BSTFA gave the disilyl derivative which had a retention time of 12.7 mins (elution temperature 156°) on 3% SE 30, Cl-MS {MH}⁺ 290 and the El mass spectrum contained ions at \( m/e \) 289 \( \text{M} \)⁺, 274 \{M-CH\(_3\)\}, 246 \{M(CH+CH\(_3\))\}, 218 \{M-(TMS+2H)\}, 200 \{M-0TMS\}⁺, 172 \{M CO\(_2\)TMS\}⁺, 102 \{TMSNHCH\(_2\)\}⁺.

**Preparation of Hexanoylglycine(XXXVII)**

Hexanoic acid (XL) (0.2g, 25 mmol) was treated as above to give hexanoylglycine methyl ester, bp 133-5°/3mm (lit 51° bp 127-30°/2.5mm), yield 2.6g (56%), CI-MS \{MH\}⁺ 188. The compound had a retention time of 13.2 min (elution temperature 159°) on 3% SE-30 and the El mass spectrum contained ions at \( m/e \) 188 \{MH\}, 187 \{M\}⁺, 158 \{M-C\(_2\)H\(_5\)\}, 156 \{M-CH\(_3\)\}, 144 \{M-C\(_3\)H\(_7\)\}, 131 \{CH\(_2\) = \text{C} (\text{OH}) - \text{NH-CH}_2 - \text{COOCH}_3 \} (\text{McLafferty rearrangement}) 128 \{M-\text{COOCH}_3\}, 99 \{M-(NHCH\(_2\)-COOCH\(_3\))\}, 98 \{M-(NH\(_2\)-CHCOOCH\(_3\))\}⁺ and 71 \{C\(_5\)H\(_1\)\}⁺. Hydrolysis of the ester (930mg, 5 mmol) and recrystallisation of the residue from ethyl acetate/hexane gave pure hexanoylglycine (XXXVII) mp 94° (lit 174° mp 93-4°) yield 420 mg (48%). Treatment of a sample of this compound with BSTFA gave the disilyl derivative which had a retention time of 16.5 minutes (elution temperature 179°) on 3% SE 30, Cl-MS \{MH\}⁺ 318 and the El mass spectrum contained ions at \( m/e \) 317 \{M\}⁺, 230 \{M-(TMS + CH\(_3\))\}⁺, 201 \{M-CO\(_2\)TMS + H\}⁺, 102 \{TMSNH = CH\(_2\)\}, 99 \{C\(_5\)H\(_1\) C = 0\}⁺.

**Preparation of Octanoylglycine**

Octanoic acid (3.6g, 25 mmol) was treated as above to give octanoylglycine methyl ester bp 143/2.5, yield 3.9 g (72%), Cl-MS \{MH\} 216. The compound had a retention time of 17.5 min (elution temperature 185° C) on 3% SE 30 and the El mass spectrum contained ions at 216 \{MH\}⁺,
215 \{M\}^+, 184 \{M-CH_3\}^+, 156 \{M-COOCH_3\}^+, 144 \{M-C_5H_11\}^+, 131 \{CH_2 = C(OH)NHCH_2COOCH_3\}^+ \text{(Mclafferty rearrangement)}, 127 \{M - (NH_2CH_2COOCH_3)\}^+ and 99 \{C_7H_15\}^+. Hydrolysis of the ester (1.15g, 5 mmol) and recrystallisation of the product from ethyl acetate/hexane gave pure octamethylglycine mp 101.5-2.5°C, yield 560 mg (56%). Treatment of a sample of this compound with BSTFA gave the disilyl derivative which had a retention time of 20.2 mins (elution temperature 201°C) on 3% SE 30 Cl-MS \{MH\}^+ 345 and the El mass spectrum contained ions at m/e 345 \{M\}^+, 248 \{M-(TMS + CH_3)\}^+, 127 \{C_7H_15CO\}^+, 102 \{TMSNH = CH_2\}^+.

Preparation of Adipylglycine (XXXV)

Adipic acid monomethyl ester monochloride (4.5 g, 25 mmol) was treated as above to give adipylglycine dimethyl ester bp 198-202°/2.5 mm, yield 2.3 g (40%), Cl-MS \{MH\}^+ 232. The compound had a retention time of 19.7 mins. (elution temperature 198°C) on 3% SE 30 and the El mass spectrum contained ions at m/e 232 \{MH\}^+, 231 \{M\}^+, 200 \{M-CH_3\}^+, 172 \{M-COOCH_3\}^+, 158 \{M-(NHCH_2COOCH_3)\}^+, 131 \{CH_2 = C(OH)NHCH_2COOCH_3\}^+ \text{(Mclafferty rearrangement)}, 115 \{(CH_2)4COOCH_3\}^+, 111 \{M-(CH_3OH + (NHCH_2COOCH_3))\}^+, 89 \{NH_2CH_2COOCH_3\}^+ and 73 \{CH_2COOCH_3\}^+. Hydrolysis of the ester (1.15g; 5 mmol) and recrystallisation of the residue from ethylacetate/hexane gave pure adipylglycine (XXXV) mp 143° (lit575 mp 139-46°) yield 0.67g (60%).

Preparation of Suberylglycine (XXXII)

Suberic acid (XXIX) (4.3g, 25 mmol) was refluxed with freshly distilled thionyl chloride (15 ml) for 4 hours. The excess thionyl chloride was removed under vacuum and the last traces of reagent removed by treating the residue with dry benzene (2x10ml) and removing the solvent under vacuum. The acid chloride was dissolved in benzene (20 ml) and methanol (0.8 g, 25 mmol) in benzene (5 ml) added dropwise to the cooled stirred solution during 10 min. The mixture was stirred for 1 hour and the solvent removed under vacuum. The crude suberic acid monomethyl ester monochloride was treated as above to give suberylglycine dimethyl ester bp 214°/0.3mm, yield 1.9 g (30%) Cl-MS \{MH\}^+ 260. The compound had a retention time of 22.8 mins. (elution temperature 217°C) on 3% SE 30 and the El mass spectrum contained ions at m/e 260 \{MH\}^+, 259 \{M\}^+. 
228 \{M-CH_3O\}^+, 200 \{M-COOCH_3\}^+, 186 \{M-(CH_2COOCH_3)\}^+, 171 \{M-NHCH_2COOCH_3\}^+, 139 \{M-(CH_3OH + NHCH_2COOCH_3)\}^+, 131 \{CH_2=C(OH)NHCH_2COOCH_3\} (McLafferty rearrangement). Hydrolysis of the ester (1.3 g, 5 mmol) and recrystallisation of the residue from the ethyl acetate/hexane gave pure suberyl-glycine (XXXII) mp 126° (lit 575 125-6°) yield 0.52g (45%)

Preparation of Sebacylglycine (XXXVI)

Sebacic acid (XXXI) (2.0g, 10mmol) was treated as above to give sebacylglycine dimethyl ester bp 235°/0.2mm, yield 1.73g (60%), CI-MS \{MH\}^+ 288. The compound had a retention time of 26.6 min (elution temperature 241°) on 3% SE 30 and the EI mass spectrum contained ions at m/e 288 \{MH\}^+, 287 \{M\}^+, 256 \{M-CH_3O\}^+, 228 \{M-COOCH_3\}^+, 199 \{M-NHCH_2COOCH_3\}^+, 131 \{CH_2-COHNHCH_2COOCH_3\}^+ (McLafferty rearrangement). Hydrolysis of the ester (0.85g, 3 mmol) and recrystallisation of the residue from ethyl acetate/hexane gave pure sebacylglycine (XXXVI) mp 120° (lit 575 mp 123-126°) yield 0.28g (37%).

Preparation of Glutarylglucose (XXXIV)

Glutaric acid (1.6g, 12.5 mmol) was treated as above. Only a small amount of glutarylglucose dimethyl ester was formed and most of the glutaric acid was recovered as mono methyl glutarate. GC of the diazomethane treated product on 3% SE 30 gave 2 peaks with retention times 5.7 mins. (elution temperature 114°) and 16.3 mins (elution temperature 178°). GC-MS showed the first peak to be dimethyl glutarate by comparison with an authentic sample. The EI mass spectrum of the second peak contained ions at m/e 218 \{MH\}^+, 217 \{M\}^+, 185 \{M-CH_2OH\}^+, 158 \{M-COOCH_3\}^+, 129 \{M-(NHCH_2COOCH_3)\}^+ and 101 \{M-(CONHCH_2COOCH_3)\}^+ and was identified as glutarylglucose dimethyl ester by comparison with the published mass spectrum.

Preparation of Succinylglycine (XXXIII)

Succinic anhydride (3g, 30 mmol) was heated under reflux with methanol (8 ml) for 1.5 hours. The solvent was removed and the residue treated with benzene (2 x 10 ml) and evaporated to dryness. The residue was treated at room temperature with thionyl chloride (5 ml) for 3 hours
and then heated at 70° for 1 hour. The excess thionyl chloride was removed under vacuum and the residue treated with benzene (2 x 10 ml) which was removed under vacuum. The crude chloroform was treated as above to give succinylglycine dimethyl ester bp 184°/3 mm, yield 2.3 g (34%) Cl-MS \( \{\text{MH}\}^+ \) 204. The compound had a retention time of 13.8 mins. (elution temperature 163°) on 3% SE 30 and the EI mass spectrum contained ions at \( m/e \) 204 \( \{\text{MH}\}^+ \), 203 \( \{M\}^+ \), 172 \( \{M-\text{CH}_3\}^+ \), 171 \( \{M-\text{CH}_2\text{OH}\}^+ \), 144 \( \{M-\text{COOCH}_3\}^+ \), 115 \( \{M-(\text{NHCH}_2\text{COOCH}_3)\}^+ \), 112 \( \{M-(\text{COOCH}_3+\text{CH}_2\text{OH})\}^+ \), 87 \( \{\text{CH}_2\text{COOCH}_3\}^+ \). Hydrolysis of the ester (1.0 g ; 5 mmol) and recrystallisation of the residue from ethyl acetate/hexane gave pure succinylglycine mp 145° (lit 145.5-146° C) yield 0.46 g (53%).

Preparation of Propanal (LXIII)

A solution of potassium dichromate (108 g, 0.36 mol) and sulphuric acid (80 ml) in water (660 ml) was added dropwise with stirring to boiling propanol (66 g, 1.1 mol) in a two litre flask fitted with a fractionating column. The addition was made at such a rate that the temperature at the top of the column did not rise above 75° and this took 20-30 minutes. The mixture was then heated for a further 15 minutes until the temperature at the top of the column rose above 75°. The crude product (32 g) was collected in an ice-cooled flask and dried with magnesium sulphate. Slow distillation through a short fractionating column gave pure propanal (LXIII), bp 48° (lit 47-50°), yield 21 g (33%).

Preparation of Butanal (c)

Butanal (C) was prepared using the above method and substituting butanol (Cl) (82 g, 1.1 mol) for propanol. The product had bp 73-2° (lit 74.5°), yield 30 g (38%).

Attempted Preparation of 3-Keto-2-methylvaleric Acid via Aldol Condensation using a Sodium Hydroxide Catalyst

Sodium hydroxide (0.11 ml, 2.5M) was added to cooled (4°) stirred propanol (LXIII) (5.8 g, 0.1 mol) during thirty minutes. The reaction mixture was stirred for a further 90 minutes and tartaric acid (421 mg, 0.1 mol) was added. The acid solution was filtered and the filtrate distilled
(water bath, $70^\circ$) until no more passed over. The residue weighed 0.68g (12% yield).

A portion of the residue (0.116g) was dissolved in acetone and cooled in an ice bath. Jones' reagent was added dropwise to the stirred solution until a permanent orange colour was obtained. This required 0.27ml, instead of the theoretical usage of 0.25 ml.

The solution was stirred for a further 10 minutes, water (20 ml) was added and the solution was saturated with salt and extracted with ether (3 x 25 ml). The combined ether extracts were washed (satd. NaCl) dried ($\text{Na}_2\text{SO}_4$) and the ether removed. The residue (0.85g) was examined by GC-MS but no evidence of the desired product was found.

The reaction was repeated at room temperature and the intermediate condensation product distilled (bp $84^\circ/11$ mm; lit $578^\circ$ bp of propionaldol (LXII) $84-6^\circ/11$ mm), yield 1.6g (14%). MS examination of this product indicated a Cl molecular weight of 116. The intermediate (400mg) in acetone (40 ml) was oxidised with Jones' reagent until no further decolourisation of the reagent occurred (0.9 ml). The solution was saturated with NaCl and extracted with ether (3 x 50 ml) and the ether extracts were back extracted with sodium hydroxide (2 x 10 ml; 1 M). The neutralised ($\text{HCl}$) aqueous layer was again extracted with ether (3 x 20 ml) and the dried ($\text{MgSO}_4$) extract examined by GC-MS. No 3-keto-2-methylvaleric acid (LVII) was found in this extract.

**Attempted Preparation of Propanaldol (LXII) using a Potassium Hydroxide Catalyst**

Propanal (26g, 0.45 mol) and ethanol (5g, 0.11 mol) were stirred at room temperature and potassium hydroxide (1.2ml; 0.7 M) was added dropwise so that the temperature did not exceed $35^\circ$. Stirring was continued for two hours and acetic acid (0.66 ml) was added. The excess propanal was removed on a Rotovap at room temperature but no propanaldol (LXII) was obtained.
Attempted Preparation of Propanaldol (LXIII) using a Potassium Carbonate Catalyst

Saturated potassium carbonate (20 ml) was added dropwise to propanal (16.4g, 0.28 mol) and the mixture stirred at 8°C for 4 hours. The solution was filtered and 2,4-diethyl-5-methyl-6-hydroxy-1,3-dioxane (8.4g) was obtained by vacuum distillation of the filtrate bp 84°/1 mm, (yield 51%). Distillation of the compound from adipic acid in vacuo gave no propanaldol (LXII) but regenerated propanal (LXIII).

Preparation of 3-Keto-2-methylvaleric Acid Ethyl Ester (LXXVII)

Finely divided sodium (4.6g, 0.2 mol) was prepared by melting sodium beneath xylene and then cooling the mixture with vigorous magnetic stirring, in a three necked flask equipped with a reflux condenser and a thermometer. The powdered sodium was washed with ether (2 x 50 ml) by decantation and suspended in ether (50 ml). Absolute ethanol (11.7 ml, 0.2 mol) was added, the mixture stirred until the sodium had dissolved and the solvent was removed by distillation. Ethyl propionate (LXVI) (122g, 1.2 mol) was added and the mixture heated and stirred under reflux for sixty hours keeping the pot temperature at 95°C. At intervals of about 12 hours the ethanol formed was removed by fractional distillation. The reaction mixture was cooled and acetic acid (36g, 1M) was added to the stirred mixture at such a rate that the temperature did not rise above 20°C. The layers were separated and the aqueous layer washed with ether (4 x 50 ml). The combined organic layers were dried (Na2SO4) and the solvent removed. The crude product was fractionally distilled under reduced pressure to give pure 3-keto-2-methylvaleric acid ethyl ester (LXXVII) bp 108-10/22mm (lit556 88-90°/12mm), yield 10.1g (32%); pyrazolone mp 108-10°C (lit556 108-10°C). IR (CHCl3) 1741 cm⁻¹ (ester stretch); 1717 cm⁻¹ (keto group); 1191 cm⁻¹ (conjugated ester stretch); Cl-MS \( [\text{MH}]^+ \) 159.

The TMS derivative of the ester showed two peaks on GC with elution time of 7.7 and 8.4 minutes (elution temperature 100.4, 103.4°C) on SE54. Both had \( \text{Cl-MS(MH)}^+ \) 231 and both El mass spectra contain ions at \( m/e \) 230 [M]⁺, 215 [M-C,H₃]⁺, 201 [M-C₂H₅]⁺, 187 [M-(C₅H₅)]⁺, 185 [M-C₂H₅O]⁺, 184 [M-C₂H₅OH]⁺, 157 [M-TMS]⁺, 113, 112, 111, 83 [M-(TMS +...
**Preparation of 3-Keto-2-Methylvaleric Acid (LXVII)**

The ethyl ester (LXXVII) (100 mg, 0.6 mmol) was hydrolysed with potassium hydroxide (2.5 ml; 1.8 M in 50% ETOH) at 4°C for 24 hours. The unreacted ester was extracted from the basic solution with ether (3 x 10 ml). The solution was cooled in an ice bath and the pH carefully adjusted to 1 with hydrochloric acid (2 M). The acid was immediately extracted with ether and the extracts were washed and dried (MgSO₄). Pure 3-keto-2-methylvaleric acid (LXVII) was obtained by removal of the solvent under a stream of nitrogen as an oil (68 mg, 83%). Cl-MS (MH)⁺ 130. The oil could be stored in a freezer (-20°C) for periods of up to two weeks without significant decomposition.

GC of a sample of the acid derivatized with BSTFA gave three peaks with retention times 4.0 mins, 8.5 mins and 9.6 mins. (elution temperatures 85.8, 104.1 and 108.4°C on SE 54. On further heating with the reagent the first peak disappeared and this was shown to be the mono-silyl derivative of the keto acid Cl-MS (MH)⁺ 203 with ions in the E1 spectrum at m/e 202 (M)⁺, 187 (M-CH₃)⁺, 173 (M-C₂H₅)⁺, 146 ((CH₃)₃SiO C(OH)-CH-CH₃)⁺ (McLafferty rearrangement), 143 (M-(CH₃)₂SiH)⁺, 130 (M-TMS + H)⁺, 129 (M-TMS)⁺, 115, 86 (C₂H₅-C(OH) = CH-CH₃)⁺ (McLafferty rearrangement of m/e 130), 75 ((CH₃)₂SiOH)⁺, 73 (TMS) and 57 (C₂H₅CO)⁺.

The other two peaks (retention time 8.5 and 9.6 minutes) had Cl-MS) (MH)⁺ 275 and identical E1 mass spectra which contained ions at m/e 274 (M)⁺, 259 (M-CH₃)⁺, 245 (M-C₂H₅)⁺, 185 (M-OTMS)⁺, 184 (M-HOTMS)⁺, 169 (M-(CH₃ + HOTMS)⁺, 157 (M-COOTMS)⁺, 147 ((CH₃)₂Si=OTMS)⁺, 75 ((CH₃)₂SiOH)⁺ and 73 (TMS)⁺ and were assigned to the cis/trans isomers of the disilyl derivative of the enol CH₃CH₂ C(OH)=CH(CH₃)COOH.
Preparation of 3-hydroxy-2-methylvaleric acid (LXXIX)

3-Keto-2-methylvaleric acid ethyl ester (LXXVII) (200mg, 1.26 mmol) and the sodium borohydride reagent (2.5 ml; 6mg dissolved in 1 ml of 0.25M ethanolic NaOH) were warmed together in a screw-capped tube at 65°C for three hours. Acetic acid (200 ml) and water (2 ml) were added and the solution was extracted with ether (3 x 10 ml). The ether layers were dried (MgSO_4) and distilled to give pure 3-hydroxy-2-methylvaleric acid ethyl ester (192mg, yield 95%). Free 3-hydroxy-2-methylvaleric acid (LXXIX) was obtained by hydrolysing the ester (100mg, 0.6 mmol) with potassium hydroxide (2.5 ml, 10% KOH in 50% EtOH) at 65°C for two hours. After extracting the unreacted ester from the basic solution with ether and acidification to pH1 with hydrochloric acid the solution was extracted with ether (3 x 5 ml), the ether extracts were dried (MgSO_4) and the solvent removed. Pure 3-hydroxy-2-methylvaleric acid (LXXIX) was obtained as an oil, yield 68mg (83%) CI-MS (MH)^+ 133.

G C of a sample of the acid derivatised with BSTFA gave the disilyl derivative which gave two peaks due to the 2 diastereoisomers of retention times 7.3 minutes and 7.6 minutes (elution temperature 99.1°C and 100°C) on SE 54. Both had a molecular weight of 276, CI-MS (MH) 277 and an El mass spectra which contained ions at m/e 261 (M-CH_3)^+, 247 (M-C_2H_5)^+, 218 (M-(CO_2 + CH_3) + H)^+, 203 (M-TMS)^+, 147 ((CH_3)_2Si=OTMS)^+, 131 (CH_3CH_2CHO-TMS)^+, 117 (COOTMS)^+, 75 ((CH_3)_2SiOH)^+ and 73 (TMS)^+.

G C of a sample of the acid treated with diazomethane followed by BSTFA derivatisation gave the methyl ester silyl derivative which also gave two peaks with retention times 5.9 and 6.2 minutes on SE 54 (elution temperatures 93.5°C and 94.4°C). Both had molecular weights of 218, CI-MS (MH)^+ 219 and El mass spectra which contained ions at m/e 218 (M)^+, 203 (M-CH_3)^+ , 189 (M-CH_3O)^+, 174 (M-(C_2H_5 + CH_3)COO)^+, 143 (M-(COOCCH_3 + CH_4))^+, 133 (M-(TMS + CH_4))^+, (CH_2=OTMS)^+, 75 ((CH_3)_2SiOH)^+, 73 (TMS)^+.

Preparation of Acetone Oxime (LXXX)

Acetone (0.5 ml) was added to a solution of hydroxylamine hydrochloride (1g) and sodium acetate trihydrate (2g) in water (10 ml) and the
mixture shaken. A few drops of methanol were added to obtain a clear solution and the flask was heated on a water bath for 10 mins. The solution was cooled and the precipitate recovered by filtration. The product was recrystallised from methanol/water to give pure acetone oxime (LXXX) mp 59° (lit 577 mp 59°), Cl-MS [MH]+ 74.

Preparation of Butanone Oxime (LXXXI)

Butanone was treated as above to give pure butanone oxime (LXXXI) bp 152°C (lit 580 bp 152°C), Cl-MS [MH]+ 88.

Preparation of 3-Pentanone Oxime (LXXXII)

3-Pentanone was treated as above to give 3-pentanone oxime (LXXXII) bp 165° (lit 580 bp 165°), Cl-MS [MH]+ 102.

Preparation of 3-Heptanone Oxime (LXXXIII)

3-Heptanone was treated as above to give 3-heptanone oxime (LXXXIII) bp 193° (lit 580 bp 193° C), Cl-MS [MH]+ 130.

Preparation of Acetone Methoxime (LXXXIV)

This compound was prepared by a modification of the above method. Acetone (1.0 ml) was added to a solution of methoxylamine hydrochloride (2g) and sodium acetate trihydrate (g) in water (20 ml) and the mixture shaken. A few drops of methanol were added to obtain a clear solution and the flask was heated on a water bath for 10 mins. The cooled mixture was extracted with ether (3 x 10 ml), and the extracts were dried and the solvent removed.

Distillation of the residue gave pure acetone methoxime (LXXXIV) bp 72° (lit 581 bp 72-72.5), Cl-MS [MH]+ 88.
Preparation of Butanone Methoxime (LXXXV)

Butanone was treated as above to give pure butanone methoxime (LXXXV) bp 91° (lit bp 91.5°), CI-MS {MH}⁺ 102.

Preparation of 3-Pentanone Methoxime (LXXXVI)

3-Pentanone was treated as above to give pure 3-pentanone methoxime (LXXXVI) bp 116 (lit bp 116-117°) CI-MS {MH}⁺ 116.

Preparation of 3-Heptanone Methoxime (LXXXVII)

3-Heptanone was treated as above to give pure 3-heptanone methoxime (LXXXVII) bp 142°, CI-MS {MH}⁺ 143.

Preparation of Acetone 2,4-Dinitrophenylhydrazone (LXXXVIII)

2,4-Dinitrophenylhydrazine was suspended in methanol (5 ml) and sulphuric acid (0.5 ml) was added. To the filtered solution was added acetone (0.2 ml) and the mixture was shaken and then left to stand for 10 mins. The crude product was recovered by filtration and recrystallised from ethanol to give pure acetone 2,4-dinitrophenylhydrazone (LXXXVIII) mp 128° (lit mp 128°), CI-MS {MH}⁺ 239.

Preparation of Butanone 2,4-Dinitrophenylhydrazone (LXXXIX)

Butanone was treated as above to give butanone 2,4-dinitrophenylhydrazone mp 115° (lit mp 115°), CI-MS {MH}⁺ 253.

Preparation of 3-Pentanone 2,4-Dinitrophenylhydrazone (XC)

3-Pentanone was treated as above to give pentanone 2,4-dinitrophenylhydrazone mp 156° (lit mp 156°) CI-MS {MH}⁺ 267.

Preparation of 3-Heptanone 2,4 Dinitrophenylhydrazone (XCI)

3-Heptanone was treated as above to give 3-heptanone 2,4-dinitrophenyl-
Preparation of Ethyl Butyrate (XCVIII)

Butyric anhydride (79g, 0.5 mol), ethanol (138g, 3 mol) and sulphuric acid (ml) were heated under reflux for four hours. Most of the alcohol (\(\approx 90 \text{ ml}\)) was removed by distillation. The residue was washed with water (50 ml), potassium carbonate (50 ml; saturated solution) and sodium chloride (50 ml; saturated solution) and dried (\(\text{MgSO}_4\)). On distillation the residue gave pure ethyl butyrate (XCVIII) bp 121-2\(^0\) (lit bp 121.6\(^0\)), yield 92.1g (79%), CI-MS \{MH\}\(^+\) 117.

Preparation of Mixed Condensation of Ethyl Propionate (LXXVI) and Ethyl Butyrate (XCVIII)

Dry sodium ethoxide (0.2 mol) was prepared in a three necked flask equipped with a reflux condenser, a thermometer and a magnetic stirrer by dissolving sodium (4.6g, 0.2 mmol) in excess ethanol (50 ml) and the solvent by distillation. Ethyl propionate (LXXVI) (51g, 0.5 mol) and ethyl butyrate (XCVIII) (58g, 0.5 mol) were added and the mixture stirred and heated under reflux for forty hours with a pot temperature of 95\(^0\). The ethanol and some ethyl propionate (total volume 60 ml) was removed by fractional distillation every twelve hours. The reaction mixture was cooled and acetic acid (36g, 6M) was added to the stirred mixture at such a rate that the temperature did not rise above 20\(^0\).

The layers were separated and the aqueous layer was washed with ether (4 x 50 ml). The combined organic layers were dried (\(\text{Na}_2\text{SO}_4\)) and the solvent removed. The residue was fractionally distilled under vacuum and two fractions with bp range of 102-120\(^0\) and 124-144 were collected. The esters could be easily separated by capillary GC but further fractional distillation did not improve the separation.

The first fraction bp 102-120\(^0\) (mainly 110\(^0\) C)/25 mm weighed 2.6g and contained 3-keto-2-methylvaleric acid ethyl ester (LXXVII) (12.5%), 3-keto-2-ethylvaleric acid ethyl ester (44.7%) 3-keto-2-methylcaproic acid ethyl ester (31.3%) and 3-keto-2-ethylcaproic acid ethyl ester (11.6%). The second fraction bp 122-144\(^0\) (mainly 139-141\(^0\) C)/25 mm weighed 3.4g
and contained 3-keto-2-methylvaleric acid (4.4%), 3-keto-2-ethylvaleric acid ethyl ester (16%), 3-keto-2-methylcaproic acid ethyl ester (47.9%) and 3-keto-2-ethylcaproic acid ethyl ester (31.6%). A small portion of each fraction (100mg) was hydrolysed as described for 3-keto-2-methylvaleric acid ethyl ester and the residue obtained (70mg) was silylated and the GC-MS was run on SE 54.

The GC of the silylated residue of fraction A showed the presence of 6 peaks. Peak 1 had retention time of 8.7 minutes, (elution temperature 104.6°C), CI-MS {MH}+ 274, with identical mass spectrum to that obtained for the TMS derivative of 3-keto-2-methylvaleric acid. Peak 2 had a retention time of 10.0 mins (elution temperature 110.2°C) CI-MS {MH}+ 289 and had an EI mass spectrum which contained ions at 288 {M}+, 273 {M-CH3}+, 259 {M-C2H5}+, 245 {M-CO2 + H}+, 199 {M - O-TMS}+, 198 {M-(H + O-TMS)}+ 185, 184, 171 {M-COOTMS}+, 147 ((CH3)2 Si=O=TMS)+ and this spectrum was in agreement with 2-ethyl-3-ketovaleric acid (XCIII) diTMS.

Peak 3 had a retention time of 10.8 mins (elution temperature 112.9°C) CI-MS {MH}+ 289. The EI mass spectrum contained ions which suggested the presence of a mixture of 2-ethyl-3-ketovaleric acid (XCIII) diTMS and 3-keto-2-methylcaproic acid (XCIV) diTMS.

Peak 4 had a retention time of 11.7 minutes (elution temperature 116.7°C), CI-MS {MH}+ 289, and had an EI mass spectrum which contained ions at m/e 288 {M}+, 273 {M-15}+, 245 {M-CO2 + H}+, 218 {M-(C2H7 + CO)-H}+, 199 {M-O-TMS}+, 198 {M-TMSOH}, 183 {M-(CH + TMSOH)}+, 170, 147 ((CH3)2 Si=O=TMS)+ and 131 and this was assigned to 3-keto-2-methylcaproic acid (XCIV) diTMS.

Peaks 5 and 6 had retention times of 11.9 and 12.5 (elution temperature 117.2°C and 119.4°C), CI-MS {MH}+ 303 and had identical mass spectrum containing ions at 302 {M}+, 287 {M-CH3}+, 231, 217, 213 {M-TMSO}+, 212 {M-TMSOH}, 197 {M-CH3 + TMSOH}+, 185 {M-COOTMS}+, 184 {M-HCOOTMS}+, 171, 147 ((CH3)2 Si=O=TMS)+ and these were assigned to 3-keto-2-ethylvaleric acid.
GC of the silylated residue from fraction B showed the presence of the same 6 peaks with identical retention times, elution temperatures and mass spectra.

Preparation of 2-Bromopropionic Acid Ethyl Ester (CII)

2-Bromopropionic acid (30.6 g, 0.2 mol) and thionyl chloride (30g, 0.25 mol) were heated under reflux for 1 hour. The excess thionyl chloride was distilled off and the residue added dropwise with stirring to cold ethanol (10g, 0.22 mol). After stirring at room temperature for 1 hour the solution was distilled to give pure 2-bromopropionic acid ethyl ester (CII) bp 76°/25mm (lit 570 bp 63°/14 mm) yield 30.5 g (85%), CI-MS \{MH\}^+ 181, 183.

Preparation of 3-Hydroxy-2-methylcaproic Acid Ethyl Ester (CIV)

To zinc (9.6g, 0.15 mol) was added a small portion of a mixture of 2-bromopropionic acid ethyl ester (20g 0.11 mol) and butanal (9g, 0.125 mol) in benzene (60 ml). A crystal of iodine was added, the mixture warmed to initiate the reaction and the remainder of the mixture added. The mixture was heated under reflux for three hours and sulphuric acid (3.5M; 120 ml) was then added to the cooled mixture. The organic layer was separated and the aqueous layer extracted with ether (3 x 30 ml). The combined organic layers were dried (MgSO_4) and the solvents removed. The residue was distilled in vacuo to give pure 3-hydroxy-2-methylcaproic acid ethyl ester bp 107-10^0/25 mm (lit 584 bp 115-21^0/38 mm), yield 3.5g (19%) CI-MS \{MH\}^+ 175.

GC of the silyl derivative of the ester gave 2 peaks with elution times 7.8 minutes and 8.1 minutes on SE 54 (elution temperature 100.6° and 102.2°) with identical molecular weights, CI-MS \{MH\}^+ 247 and El mass spectra which contained ions at m/e 231 \{M-CH_3\}^+, 203 \{M-C_3H_7\}^+, 174 \{M-(C_3H_7+C_2H_5)\}^+, 157 \{M-OMS\}^+, 145 \{CH(CH_2CH_2CHOMS)\}^+, 130, 117 \{COOTMS\}^+, 103 \{CH_2=O=OMS\}^+, 75 \{(CH_3)_2SiOH\}^+, 73 \{TMS\}^+. 
Preparation of 2-Ethyl-3-Hydroxyvaleric Acid Ethyl Ester (CII)

A portion of a mixture of 2-bromobutyric acid ethyl ester (CII) (25g, 0.13 mol), propanal (9.7 g, 0.17 mol) and benzene (66 ml) was added slowly to zinc (13.7g, 0.21 mol) containing a crystal of iodine and the mixture was gently warmed until the reaction began. The remainder of the reaction mixture was then added and the solution heated under reflux for 3 hours. Dilute sulphuric acid (3.5M; 120 ml) was added to the cooled solution and after vigorous stirring the mixture was separated and the organic layer was dried (MgSO₄) and the solvent removed. The residue was distilled to give pure 2-ethyl-3-hydroxyvaleric acid ethyl ester bp 123-4/25 mm (lit° bo 95-7°/12mm), yield 5.4 g (25%) Cl-MS \{MH\}⁺ 175.

GC of the silyl derivative of the ester gave 2 peaks with elution times 7.3 and 7.6 minutes (elution temperatures 98.2° and 99.6° with identical molecular weights, Cl-MS \{MH\}⁺ 247. The E1 mass spectra contained ions at 231 \{M-CH₃\}⁺, 217 \{M-C₅H₅\}, 173 \{M-COOC₂H₅\}, 157 \{M-OTMS\}⁺ 131 \{M-CH(C₂H₅)COOTMS\}, 117 \{COOTMS\}⁺, 75 \{(CH₃)₂SiOH\}⁺ 73 \{TMS\}⁺.

Oxidation of 2-Ethyl-3-Hydroxyvaleric Acid Ethyl Ester (CIII)

2-Ethyl-3-hydroxyvaleric acid ethyl ester (CIII) (350mg, 2 mmol) was dissolved in acetone (35 ml) and the solution was cooled to 5°. The acid was titrated with Jones' reagent until a permanent orange colour persisted (0.6 ml). The stirring was continued for 30 minutes and water (30 ml) was then added and the solution was extracted with ether (4 x 50 ml). The ether extracts were washed with saturated sodium chloride (2 x 25 ml) and dried (MgSO₄). The ether was evaporated to give 2-ethyl-3-ketovaleric acid ethyl ester in quantitative yield. This ester was shown to be pure by GC and TLC (silica gel, benzene/chloroform; 95:5). A small portion was hydrolysed with NaOH and the resulting 2-ethyl-3-ketovaleric acid (XCIII) was silylated. The retention times and mass spectrum of the trimethylsilyl derivative was identical to that obtained for 2-ethyl-3-ketovaleric acid (XCIV) made via the mixed Claisen condensation.
Oxidation of 3-Hydroxy-2-Methylcaproic Acid Ethyl Ester (CIV)

3-Hydroxy-2-methylcaproic acid ethyl ester (CIV) (350mg, 2 mmol) was dissolved in acetone and cooled in ice to 5°C with stirring. The solution was titrated with Jones' reagent until a permanent orange colour was obtained (0.7 ml). The stirring was continued for 30 minutes and water (30 ml) was then added and the solution saturated with salt before extraction with ether (3 x 50 ml). The ether extracts were washed (satd NaCl; 2 x 20 ml), dried (MgSO₄) and the solvent removed. 3-Keto-2-methylcaproic acid ethyl ester was obtained in quantitative yield and its purity demonstrated by GC and TLC (silica gel; benzene chloroform (95:5)). A small portion was hydrolysed with NaOH and the resulting 3-keto-2-methylcaproic acid (XCIV) was silylated. The retention times and mass spectra of the two diastereoisomers were identical to those obtained for 3-keto-2-methylcaproic acid made via the mixed Claisen condensation.
APPENDIX A

CORRECTION FORMULA FOR INTENSITY MEASUREMENTS
During the course of this work it was found that the use of isotopically labelled internal standards which were only moderately enriched isotopically gave calibration curves which were not linear over extended regions. To overcome this problem a formula was developed which, when applied to the uncorrected peak height ratio, gives the absolute ratio between internal standard and unknown.

For a pure material A with an ion at \( m/e \, M \) let the ratio of the intensity of the \( M + n \) peak to the \( M \) peak be \( R \). For another pure material B with an ion of \( m/e \, M^* \) let the ratio of the \( M^* - n \) peak to the \( M^* \) peak be \( S \).

Diagramatically this may be represented as follows:

**FOR PURE MATERIAL A**

\[
\frac{I_{M+n}}{I_M} \quad \frac{x}{x} = R
\]

**FOR PURE MATERIAL B**

\[
\frac{I_{M^* - n}}{I_{M^*}} \quad \frac{y}{y} = S
\]
When we examine a mixture of the two materials A and B where the $M + n$ peak due to compound A is coincident with the $M^*$ peak of compound B we have the case where the measured intensity of $M$ is due to a contribution $x$ from compound A and $y$ from compound B and the intensity at $M^*$ is similarly due to a contribution $x$ from compound A and $y$ from compound B and this may be represented as follows:

\[ X \]

\[ Y \]

\[ X \]

\[ Y \]

\[ M \]

\[ M^* \]

\[ m/e \]

Where $I_M$ = observed intensity of peak at mass $M$

$I_{M^*}$ = observed intensity of peak at mass $M^*$

$x$ = contribution of compound A to $I_M$

$y$ = contribution of compound B to $I_M$

$x$ = contribution of compound A to $I_{M^*}$

$y$ = contribution of compound B to $I_{M^*}$

We can see that $x = RX$

$y = SY$

Let

\[ I_M \]

\[ I_{M^*} \]

\[ = J \]

$J$ is the observed ratio between the intensities at masses $M$ and $M^*$

We can write $I_M = X + y = X + SY$ \hspace{1cm} -(1)$

$I_{M^*} = y + x = Y + RX$ \hspace{1cm} -(2)$
On multiplying Eqn. (1) by R we obtain

\[ R \cdot IM = R \cdot X + R \cdot S \cdot Y \quad -(3) \]

Subtracting Eqn (2) from Eqn (3)

\[ IM^* - R \cdot IM = Y - R \cdot S \cdot Y \quad -(4) \]

This may be rearranged to give:

\[ Y = IM^* - R \cdot IM \]

\[ 1 - R \cdot S \]

Similarly we see that

\[ X = IM - S \cdot IM^* \]

\[ 1 - R \cdot S \]

The actual ratio of compound A to B is given by -

\[ X = \frac{IM - S \cdot IM^*}{1 - R \cdot S} \]

\[ Y = \frac{IM^* - R \cdot IM}{1 - R \cdot IM/IM^*} \]

by substituting J for IM

\[ IM^* = J (1 - S/J) \]

\[ 1 - RJ \]

\[ = J - S \]

\[ 1 - RJ \]

Thus by measuring S and R in separate experiments we may correct the observed ratio to give the true ratio of the amounts of A and B present.
FIGURE A-1. CI-MS calibration curve for lecithin using 1-(\(^{13}\)C-palmitoyl)-2-palmitoyllecithin internal standard and measuring the intensity of the protonated molecular ions of the palmitic acid obtained by hydrolysis.

\[ \text{ratio} \left( \frac{^6\text{C}}{^{13}\text{C}} \right) \]
The use of this correction may be illustrated by the analysis of lecithin by hydrolysis and measurement as palmitic acid. The lecithin internal standard available contained 50% of $^{13}$C labelled palmitic acid and the samples after hydrolysis were analysed by measurement of the 257/258 peaks of the protonated molecular ions of palmitic acid in the CI isobutane mass spectrum. The contribution from natural isotopic labelling in the unknown to the intensity of the 258 peak was 18.5% of the intensity of the 257 peak, (R) and in the pure internal standard the ratio of the 257 peak to the 258 was 93.8% (S). The curves obtained using 10µg of internal standard are shown in Fig. A-1. These curves show that use of the correction substantially improves the range of linearity of the curve.

The formula was used for correction of the experimental mass spectral intensity ratios in all isotope dilution results, reported in this thesis and a programme was written for use with a portable calculator to obtain corrected intensities.
APPENDIX B

ELUTION TEMPERATURES
AND
CHARACTERISTIC MASS SPECTRAL IONS
### APPENDIX B

**TABLE B-1**

**ELUTION TEMPERATURES AND CHARACTERISTIC MASS SPECTRUM IONS**

**FOR TMS DERIVATIVES OF SOME METABOLITES SEEN IN THIS STUDY**

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>ELUTION TEMPERATURE (°C)</th>
<th>CHARACTERISTIC IONS IN MASS SPECTRUM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>APIEZON</td>
<td>SE30</td>
</tr>
<tr>
<td>Glyoxylic acid methoxime</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propylene glycol</td>
<td>101</td>
<td>104</td>
</tr>
<tr>
<td>Phenol</td>
<td>114</td>
<td>106</td>
</tr>
<tr>
<td>Pyruvic acid methoxime</td>
<td>108-9,</td>
<td>113</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>106</td>
<td>109</td>
</tr>
<tr>
<td>Hexanoic acid</td>
<td>111</td>
<td>111</td>
</tr>
<tr>
<td>Glycolic acid</td>
<td>111</td>
<td>112</td>
</tr>
<tr>
<td>2-Ketoisovaleric methoxime</td>
<td>113, 119</td>
<td></td>
</tr>
<tr>
<td>2-Ketobutyric acid methoxime</td>
<td>114</td>
<td></td>
</tr>
<tr>
<td>α-Hydroxy-2-methylbutyric acid</td>
<td>119</td>
<td>115</td>
</tr>
<tr>
<td>Glyoxylic acid oxime</td>
<td>115</td>
<td></td>
</tr>
<tr>
<td>Compound</td>
<td>134</td>
<td>117</td>
</tr>
<tr>
<td>--------------------------------------------------</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>Oxalic acid</td>
<td>113</td>
<td>117</td>
</tr>
<tr>
<td>γ-Ketovaleric acid oxime</td>
<td></td>
<td>117</td>
</tr>
<tr>
<td>Ketomalonic acid methoxime</td>
<td>117, 151</td>
<td></td>
</tr>
<tr>
<td>2-Hydroxy-3-methylbutyric acid</td>
<td>121</td>
<td>118</td>
</tr>
<tr>
<td>Pyruvic acid oxime</td>
<td>118</td>
<td></td>
</tr>
<tr>
<td>Acetoacetic acid methoxime</td>
<td>119</td>
<td></td>
</tr>
<tr>
<td>3-Hydroxybutyric acid</td>
<td>122</td>
<td>120</td>
</tr>
<tr>
<td>Levulinic acid</td>
<td>120</td>
<td></td>
</tr>
<tr>
<td>3-Hydroxypropionic acid</td>
<td>116</td>
<td>121</td>
</tr>
<tr>
<td>3-Hydroxyisobutyric acid</td>
<td>118</td>
<td>122</td>
</tr>
<tr>
<td>α-Ketobutyric acid oxime</td>
<td>122</td>
<td></td>
</tr>
<tr>
<td>2-Keto-3-methylvaleric acid methoxime</td>
<td>123, 128</td>
<td></td>
</tr>
<tr>
<td>Methylmalonic acid</td>
<td>128</td>
<td>126</td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>144</td>
<td>126</td>
</tr>
<tr>
<td>2-Methyl-3-hydroxybutyric acid</td>
<td>125</td>
<td>127</td>
</tr>
<tr>
<td>2-Hydroxyisocaproic acid di TMS</td>
<td>128</td>
<td></td>
</tr>
<tr>
<td>2-Hydroxy-3-methylvaleric acid</td>
<td>134</td>
<td>128</td>
</tr>
</tbody>
</table>
180, 165, 149, 137=135, 109, 107, 105, 91.
234, 219≈190, 147.
275, 260, 186, 170, 158, 147, 133, 129, 103.

262, 247, 219, 190, 147<145.

247, 232, 204, 158, 147, 130, 114, 100, 89.
203, 188, 147, 144, 116, 89.
248, 233, 191, 143, 130.
188, 173, 155, 145, 131, 129, 99, 98, 75, 73.
248, 233, 218, 177, 147, 143, 103.
261, 246, 218, 172, 163, 147, 144, 133, 131, 128.
231, 216, 203, 200, 189, 172, 156, 147, 114, 113, 89.
262, 247≈218.
194, 179, 135, 105.
262, 247, 218, 191, 88.

276, 261, 233, 219, 217, 190, 159, 147, 143, 133, 117, 103, 69.
<table>
<thead>
<tr>
<th>Chemical Name</th>
<th>Column 1</th>
<th>Column 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-Hydroxy-3-methylbutyric acid</td>
<td>131</td>
<td>129</td>
</tr>
<tr>
<td>Urea</td>
<td>134</td>
<td>129</td>
</tr>
<tr>
<td>2-Ketoisocaproic acid methoxime</td>
<td></td>
<td>130</td>
</tr>
<tr>
<td>Acetoacetic acid</td>
<td>137</td>
<td>131</td>
</tr>
<tr>
<td>Phosphoric acid tri TMS</td>
<td>135</td>
<td>132</td>
</tr>
<tr>
<td>3-Hydroxyvaleric acid</td>
<td></td>
<td>132</td>
</tr>
<tr>
<td>O-Cresol</td>
<td>133</td>
<td></td>
</tr>
<tr>
<td>Octanoic acid</td>
<td>143</td>
<td>134</td>
</tr>
<tr>
<td>2-Methyl-3-hydroxyvaleric acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenylacetic acid</td>
<td>145</td>
<td>134</td>
</tr>
<tr>
<td>2-Keto-3-methyl-n-valeric acid oxime</td>
<td></td>
<td>134</td>
</tr>
<tr>
<td>Maleic acid</td>
<td>140</td>
<td>135</td>
</tr>
<tr>
<td>Di-ethylene glycol di TMS</td>
<td></td>
<td>135</td>
</tr>
<tr>
<td>2-Ketoisovaleric acid oxime</td>
<td></td>
<td>135</td>
</tr>
<tr>
<td>Levulinic acid methoxime</td>
<td></td>
<td>135</td>
</tr>
<tr>
<td>3-Ketovaleric acid</td>
<td></td>
<td>137</td>
</tr>
<tr>
<td>Thymol</td>
<td>157</td>
<td>138</td>
</tr>
<tr>
<td>Succinic acid</td>
<td>141</td>
<td>138</td>
</tr>
<tr>
<td>2-Ketoisocaproic acid oxime</td>
<td></td>
<td>138</td>
</tr>
<tr>
<td>B-2 (cont.)</td>
<td>262, 247, 205, 131, 115, 95.</td>
<td></td>
</tr>
<tr>
<td>-------------</td>
<td>-------------------------------</td>
<td></td>
</tr>
<tr>
<td>92.8</td>
<td>204&lt;189, 171, 147, 114, 100.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>231, 216≈200≈189, 157, 110, 99, 89.</td>
<td></td>
</tr>
<tr>
<td>92.14; 97.93</td>
<td>246, 231, 232=157.</td>
<td></td>
</tr>
<tr>
<td>96.0</td>
<td>314, 299, 283, 211.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>247, 233, 205, 189, 147, 144, 133, 131.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>180≈165, 149, 135, 91.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>216, 201, 132, 117.</td>
<td></td>
</tr>
<tr>
<td>98.9</td>
<td>261, 247, 218, 207, 205, 203, 191, 171, 157, 147, 144, 133, 131, 115.</td>
<td></td>
</tr>
<tr>
<td>99.7</td>
<td>208, 193, ≈164.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>289, 274, 261, 247, 200, 172, 147.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>250, 235, 207, 205, 191, 161, 147, 145, 117, 103, 101</td>
<td></td>
</tr>
<tr>
<td></td>
<td>275, 260, 232, 186, 158, 147.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>217, 202, 186, 127, 119, 100, 89.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>260, 245, 231, 171, 170, 163, 155.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>222, 207, 165, 149, 105.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>289, 274, 247, 200, 172, 147, 133, 129, 110, 89, 82.</td>
<td></td>
</tr>
<tr>
<td>Compound</td>
<td>Page</td>
<td></td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>3-Keto-2-methylvaleric acid oxime</td>
<td>139</td>
<td></td>
</tr>
<tr>
<td>Acetoacetic acid oxime</td>
<td>142</td>
<td></td>
</tr>
<tr>
<td>2-Methylacetoascetic acid</td>
<td>142</td>
<td></td>
</tr>
<tr>
<td>Fumaric acid</td>
<td>151</td>
<td></td>
</tr>
<tr>
<td>Fumaric acid</td>
<td>143</td>
<td></td>
</tr>
<tr>
<td>Glyceral acid tri TIMS</td>
<td>145</td>
<td></td>
</tr>
<tr>
<td>3-Ketovaleric acid oxime</td>
<td>146</td>
<td></td>
</tr>
<tr>
<td>2-Methyl-3-ketovaleric acid</td>
<td>146</td>
<td></td>
</tr>
<tr>
<td>Glycerol tri TIMS</td>
<td>139</td>
<td></td>
</tr>
<tr>
<td>N-Propionyl glycine mono TMS</td>
<td>147</td>
<td></td>
</tr>
<tr>
<td>N-Acetylglycine di TMS</td>
<td>147</td>
<td></td>
</tr>
<tr>
<td>Glutaric acid</td>
<td>157</td>
<td></td>
</tr>
<tr>
<td>3-Methylglutaric acid</td>
<td>160</td>
<td></td>
</tr>
<tr>
<td>3-Methylglutaconic acid</td>
<td>164, 171</td>
<td></td>
</tr>
<tr>
<td>3-Methylglutaconic acid</td>
<td>155, 160</td>
<td></td>
</tr>
<tr>
<td>Butanoyl glycine di TMS</td>
<td>156</td>
<td></td>
</tr>
<tr>
<td>Butanoyl glycine mono TMS</td>
<td>157</td>
<td></td>
</tr>
<tr>
<td>Ketomalonic acid oxime</td>
<td>157</td>
<td></td>
</tr>
</tbody>
</table>
289, 274, 259, 200, 184.
261, 247, 201.

105.3
260, 245, 217, 171, 157, 155, 147, 143, 133.
322, 307, 292, 205, 189, 147, 133, 130, 117, 103.
275, 260, 232, 186, 185, 170, 158, 147, 129.

104.0
259, 247, 218, 174, 163, 157, 147, 133, 131.
130, 117, 103.
308, 293, 218, 205, 147, 133, 129, 117, 103.

108.6
203, 188, 159, 144, 132, 131, 104, 102, 88, 86.
261, 246, 218, 190, 176, 174, 172, 171, 147, 145=144, 132, 130, 104, 102.

111.8
276, 261, 233, 204, 187>186, 158, 147, 117, 116.
290, 275, 247, 204, 201, 172, 159, 147.
288, 273, 244, 229, 198, 183, 170, 109, 82.
289, 218, 202, 200, 189, 173, 158, 145, 132, 131, 130, 116, 104, 102, 100.
145, 132, 131, 130, 116, 104, 102, 100.
349, 334, 262, 172, 155, 147, 133, 131, 99, 84.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention Time 1</th>
<th>Retention Time 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adipic acid</td>
<td>171</td>
<td>163</td>
</tr>
<tr>
<td>Furan-2-hydroxymethyl-5-carboxylic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Hydroxyphenylacetic acid</td>
<td>186</td>
<td>172</td>
</tr>
<tr>
<td>Pimelic acid</td>
<td>185</td>
<td>173</td>
</tr>
<tr>
<td>2-Hydroxyglutaric acid</td>
<td>185</td>
<td>174</td>
</tr>
<tr>
<td>3-Hydroxy-3-methylglutaric acid</td>
<td>185</td>
<td>174</td>
</tr>
<tr>
<td>Phenyllactic acid</td>
<td>186</td>
<td>176</td>
</tr>
<tr>
<td>2-Ketoglutaric acid tri-TMS</td>
<td>184</td>
<td>177</td>
</tr>
<tr>
<td>3-Hydroxyphenylacetic acid</td>
<td>189</td>
<td>177</td>
</tr>
<tr>
<td>Hexanoylglycine mono TMS</td>
<td></td>
<td>178</td>
</tr>
<tr>
<td>Glutaconic acid</td>
<td>189</td>
<td>178</td>
</tr>
<tr>
<td>2-Ketoglutaric acid methoxime</td>
<td></td>
<td>178</td>
</tr>
<tr>
<td>Hexanoylglycine di TMS</td>
<td></td>
<td>179</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>179</td>
<td>179</td>
</tr>
<tr>
<td>4-Hydroxyphenylacetic acid</td>
<td>193</td>
<td>180</td>
</tr>
<tr>
<td>2-Ketoglutaric acid oxime</td>
<td></td>
<td>184</td>
</tr>
<tr>
<td>Tartaric acid</td>
<td>184</td>
<td></td>
</tr>
<tr>
<td>3-Hydroxy-3-methyl tartaric acid</td>
<td>185</td>
<td></td>
</tr>
<tr>
<td>4-Hydroxy-cyclohexylacetic acid</td>
<td>188, 193</td>
<td></td>
</tr>
</tbody>
</table>
| 3-2 (cont.) | 128.6 | 290, 275, 145, 111.  
   |       | 136.8 | 271, 197, 169, 147, 123.  
   |       |       | 296, 281, 252, 164, 149, 147.  
   |       | 146.4 | 304, 289, 217, 204, 199, 186, 173, 155, 147, 125, 117.  
   |       | 151.5 | 247, 203, 157, 147, 129, 85.  
   |       |       | 310, 267, 220, 193.  
   |       |       | 362, 347, 318, 291.  
   |       |       | 296, 281, 252, 164, 149.  
   |       |       | 274, 259, 95.  
   |       |       | 319, 304, 288, 244, 229, 202, 198, 186, 156, 147, 89.  
   |       |       | 282, 267, 209, 195, 193, 147, 135.  
   |       | 157.1 | 296, 281, 252, 179, 164.  
   |       |       | 377, 362, 260, 244, 170, 156, 147.  
   |       |       | 378, 363, 273, 231, 247, 199.  
<p>|       |       | 287, 212, 204, 197, 171, 170, 147.  |</p>
<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention Time 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suberic acid</td>
<td>200</td>
</tr>
<tr>
<td>Phenylpyruvic acid di TMS</td>
<td>207</td>
</tr>
<tr>
<td>Homovanillic acid</td>
<td>194</td>
</tr>
<tr>
<td>Phenylpyruvic acid methoxime</td>
<td>191</td>
</tr>
<tr>
<td>2-Ketoadipic acid methoxime</td>
<td>191</td>
</tr>
<tr>
<td>Phenylpyruvic acid oxime</td>
<td>192</td>
</tr>
<tr>
<td>2-Ketoadipic acid oxime</td>
<td></td>
</tr>
<tr>
<td>Hippuric acid</td>
<td>224</td>
</tr>
<tr>
<td>Octanoylglycine mono TMS</td>
<td>200</td>
</tr>
<tr>
<td>Octanoylglycine di TMS</td>
<td></td>
</tr>
<tr>
<td>Citric acid</td>
<td>205</td>
</tr>
<tr>
<td>4-Hydroxy-3-methoxy mandelic acid</td>
<td>208</td>
</tr>
<tr>
<td>Methyl citrate</td>
<td></td>
</tr>
<tr>
<td>Sebacic acid</td>
<td>225</td>
</tr>
<tr>
<td>4-Hydroxyphenyllactic acid</td>
<td>224</td>
</tr>
<tr>
<td>4-Hydroxyphenylacetic acid</td>
<td>224</td>
</tr>
<tr>
<td>Di-n-butylphthalate</td>
<td>228</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>249</td>
</tr>
<tr>
<td>p-Hydroxyphenylpyruvic oxime</td>
<td></td>
</tr>
<tr>
<td>p-Hydroxyphenylpyruvic methoxime</td>
<td></td>
</tr>
</tbody>
</table>

Retention Times:
- 1: 186, 189
- 2: 191
- 3: 192
- 4: 197
- 5: 199
- 6: 200
- 7: 201
- 8: 203
- 9: 204
- 10: 208
- 11: 208
- 12: 211
- 13: 210
- 14: 230
- 15: 227
| 318, 303, 187=169, 129, 217, 204. |
| 308, 293, 265, 237, 190, 147, 118, 90. |
| 326, 311, 267, 209, 179. |
| 265=250=189, 147, 116. |
| 333, 318, 302, 258, 170, 147, 89. |
| 323, 308, 280, 206, 189, 147, 118, 117, 91, 90, 89. |
| 391, 376, 302, 258, 184, 170, 147. |
| 251, 236, 206=105. |
| 414, 399, 371, 297. |

172.2
| 346, 331, 217=215, 204, 166, 149, 147, 129, 117. |

177.3
| 398, 308, 179. |
| 326, 311, 267. |
| 278, 223, 205, 149. |

191.1
<p>| 328, 313, 145, 132. |
| 411, 396, 277, 206, 205, 190, 179, 147, 116, 105. |
| 353, 338, 278, 277, 205, 190, 116, 105. |</p>
<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>ELUTION TEMPERATURE (°C)</th>
<th>CHARACTERISTIC IONS IN MASS SPECTRUM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Apiezon</td>
<td>SE30</td>
</tr>
<tr>
<td>Glutaric acid</td>
<td>114</td>
<td>160, 129, 128, 112, 100, 101</td>
</tr>
<tr>
<td>3-Hydroxybutyric</td>
<td>115</td>
<td>145, 113, 90, 88, 86, 84, 74, 70, 69.</td>
</tr>
<tr>
<td>Propionylglycine</td>
<td>125</td>
<td>174, 143, 114, 111, 101, 73, 74.</td>
</tr>
<tr>
<td>Adipic acid</td>
<td>127</td>
<td>159, 144, 142, 131, 128, 127, 103, 100, 90, 88, 71.</td>
</tr>
<tr>
<td>Butanoylglycine</td>
<td>135</td>
<td>150, 135, 115, 107, 105, 91.</td>
</tr>
<tr>
<td>3-Ketoadipic</td>
<td>139</td>
<td>188, 157, 128, 125, 115, 101, 97, 96, 87, 83, 74, 73, 69.</td>
</tr>
<tr>
<td>Octenediolic acid</td>
<td>151</td>
<td>118.2</td>
</tr>
<tr>
<td>Substance</td>
<td>Value</td>
<td></td>
</tr>
<tr>
<td>---------------------------------</td>
<td>-------</td>
<td></td>
</tr>
<tr>
<td>Suberic acid</td>
<td>154</td>
<td></td>
</tr>
<tr>
<td>Citric acid</td>
<td>155</td>
<td></td>
</tr>
<tr>
<td>Hexanoylglycine</td>
<td>159</td>
<td></td>
</tr>
<tr>
<td>p-Hydroxyphenylacetic acid</td>
<td>159</td>
<td></td>
</tr>
<tr>
<td>Decenedioic acid</td>
<td>175</td>
<td></td>
</tr>
<tr>
<td>Glutarylglycine</td>
<td>178</td>
<td></td>
</tr>
<tr>
<td>Hippuric acid</td>
<td>183</td>
<td></td>
</tr>
<tr>
<td>Octanoylglycine</td>
<td>185</td>
<td></td>
</tr>
<tr>
<td>Adipylglycine</td>
<td>198</td>
<td></td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>211</td>
<td></td>
</tr>
<tr>
<td>Suberylglycine</td>
<td>217</td>
<td></td>
</tr>
<tr>
<td></td>
<td>141.1</td>
<td></td>
</tr>
</tbody>
</table>
234, 143≈101.
187, 158, 156, 144, 131, 128, 103, 99, 90, 89, 88, 71.
166, 151, 107, 77.

228, 196, 165, 164, 137, 136, 123, 122, 119, 95, 94, 81≈80≈74, 67.
218, 217, 185, 158, 129, 101.

193, 161, 134, 105, 77, 74.
215, 184, 156, 144, 131, 127, 103, 99, 90.

231, 200, 172, 168, 158, 143, 140, 131, 115, 111, 90, 83, 73.
259, 228, 200, 186, 171, 139, 131, 111, 103, 99, 90, 83, 60.
APPENDIX C

COMPUTER SORTING OF NATIONAL INSTITUTE OF HEALTH MS LIBRARY
The large amount of data generated in a typical GC-MS profiling experiment leads to the problem of identification of the peaks obtained on the GC from their recorded mass spectra. Manual interpretation of the relevant mass spectra is often difficult and time consuming and identification may be greatly assisted by reference to mass spectral libraries. The simplest method of identification of the unknown compounds is to match the mass spectrum of the unknown with that of a known compound. The problem of identification is then changed to that of searching a library to find an identical mass spectrum to that of the unknown or a structurally similar compound.

The National Institute of Health Library of 20,000 mass spectra was available in our laboratory. The library was in the form of three computer files, one of which contained information about each compound such as the formula, molecular weight, identification number as well as information about the mass spectra recording conditions (CHEM10) The other two files contained identification numbers and mass intensity data of the ions present in the mass spectrum for each compound (CHEM11 and CHEM12). These files were not in readily accessible form and contained a number of errors. The aim of this work was to facilitate ready access to the library to help in the identification of unknowns.

The initial section of the work was the development of a computer programme to print out the complete spectra. The file of information was processed to form a new file (NIHNAMES) which contained a single line of information about each compound. This line contained the identification number, the molecular weight, the formula, the name and the original library in which the mass spectrum was recorded for each compound. A programme was developed, written in Fortran, to read the name file (NIHNAMES) and the MS data file (CHEM11 and CHEM12) for the name, information and MS of each compound. This information and the complete mass spectrum of each compound was printed out in a standard format with 12 mass intensity pairs per line (Programme PRINT, Fig. C-1, C-2). The first section of the programme (start to 2 Fig. C-1, lines 1-62, 74, 76, Fig. C-2) reads a line from the compound information,
(NIHNames) searches the MS data file (CHEM 11) to find the data for the relevant compound, checks for and corrects errors in the data and reads the mass and intensity of the ions into arrays. The second part of the programme (2 to end Fig. C-1 lines 63-73 Fig. C-2) calculates the number of lines of output, prints the identification number, molecular weight, formula, name and library source of the compound and then prints out the mass and intensity information (Fig. C-3).

The process of searching such a large library for matching spectra is still time consuming and there have been many approaches to simplifying the search task (see Ref. 73, Chapter 5).

Since the organic acid samples used in this work were all TMS derivatives, the compounds with matching spectra must all contain silicon. The creation of a sublibrary of silicon containing compounds thus eliminates a large number of compounds from consideration. The use of specialised libraries has been reported by other authors \(^{57, 90, 587, 187}\) of whom have used only spectra generated in their laboratories. The generation of a sublibrary of all silicon containing compounds was carried out using the conversational time sharing processor (CTS) text editor available on the Univac 1106 computer to search the molecular formula in each line of NIHNames for the presence of silicon. If silicon was present the line of information about the compound was moved and a new file (TMSComps) (Fig. C-4) created containing only those compounds which contained silicon in the molecular formula. This file was used as input for the encoding programme.

One of the problems in constructing a successful library matching system is the selection of a suitable coding scheme which will compress data sufficiently to allow a large data base but still retain as much information as possible. The compression of data bases has a number of advantages since the retaining of the full spectrum presents an inordinate amount of data for storage and searching except for small specialised libraries.\(^ {591}\) Abbreviation of the spectra also improves the performance of the search by minimising the variability of recorded spectra. For example the use of a minimum intensity threshold ensures that low intensity peaks of doubtful reproducibility are not used in the
mactching procedure. Fortunately the majority of spectra contain much more information that is required for a unique identification of the compounds. Thus Mathews and Morrison showed that the matching of six to eight major peaks in terpene spectra gave results comparable to use of the full spectra.

The choice of an encoding system can be made in terms of three basic systems:

(a) Recording of the intensity of a limited number of peaks where the number is usually between five and ten. This method is simple and direct both in encoding and subsequent matching and allows a surprisingly unique description of each component. The method is most applicable to compounds of lower molecular weights and especially where libraries of limited size are employed. A natural development of this approach is the encoding of N peaks every M mass units which improves the discrimination between higher mass compounds. Mathews and Morrison have shown that encoding the 6 or 8 most intense peaks in each spectrum gave the best matching of the very similar mass spectra of terpenes.

(b) The transformation of the spectrum into features such as the presence of ion series, low medium and high mass characteristic ions, small and large neutral losses, secondary neutral losses and fingerprint ions.

(c) Encoding as a mathematical function in which a single valued representation of the spectrum is calculated to be stored in the data bank.

The problems with the latter two included the large volume of calculation needed for treatment of the library and the difficulties of finding suitable functions or features.

The use of intensity information in encoding spectra necessitates an increase in the storage space required. However, the addition of some intensity data enables better matching results.

The encoding scheme chosen was the use of the mass and intensity values of the 15 most intense peaks in the spectrum above \( m/e 30 \) (Big 15). Although Mathews and Morrison showed that 6-8 peaks gave
maximum discrimination for terpenes experience with a library system using the 10 most intense peaks showed that, since TMS compounds have mass spectrum a number of intense ions in their A which are related to the TMS group, use of more than 10 peaks was required to obtain a unique representation of the MS of structurally similar TMS compounds. The intensity values were normalised on the most intense peak (range 0-99). (Fig. C-5, C-6) A FORTRAN programme was developed (BIG15, Fig. C-5, C-6) to read the name file (TMSCOMPS) and the MS data file (CHEM11 and CHEM12) for the required information and compute and output the 15 most intense peaks with normalised intensities. The input of data to this programme was the same as for PRINT with the added step of clearing the intensity array before the data input for each compound (lines 1-62, 67-69). A subroutine (BIG, Fig. C7, C8) was called to find the 15 most intense peaks and write them into new arrays (A-B Fig. C-7, Lines 1-13 Fig. C-8). These arrays were resorted into order of decreasing mass using a ripple sort (B-C Fig. C-7 lines 14-34 Fig. C-8) and an array combining the mass and intensity data passed back to the main programme (C-END Fig. C-7 and lines 35-40, Fig C-8). The main programme then prints out the compound identification and the 15 most intense peaks in order of decreasing mass (Fig. C-9) (File BIG15).

To facilitate the matching of spectra of unknown compounds whose molecular weights were known by CI-MS it was considered desirable to sort the output of programme BIG 15 into order of ascending molecular weights. In the FORTRAN programme written to do this (FILESORT, Fig. C-10, C-11) the file to be sorted was copied into a direct access file (also called a random access file) and the number of compounds calculated. The line numbers of the lines containing the identification number and molecular weight were calculated, and these values were read from the direct access file into arrays (A to B Fig. C-10 Fig. C-11 lines 1-28). The mass array was then sorted using a monkey-puzzle sort (called a list processing sort or tree sort) (B-C Fig. C-10, lines 31-57 Fig. C-11). This efficient sorting algorithm uses two associated vectors to point to the items that are to the left and right of any item. It suffers from the fact that a large amount of workspace is needed but was chosen because the items to be sorted do not need to be moved. The latter section of the programme (C to D, Fig. C-10 lines 52-74 Fig. C-11) is used to read the data for each compound from the direct access file and
write it on the output file (SORTBIG15) in order of increasing molecular weight.

In order to further facilitate searching the library and to prepare it for computer searching a programme was written to generate tables of frequency of molecular weight, frequency of occurrence of ions of a given m/e in the BIG15 and to list all spectra which contained an ion of a given m/e (MFTABLE, Fig. C-13, C-14). The amount of space required for the information to generate the spectra list table (400K words) was greater than the available user core in the Univac 1106 (64K words) and an unformatted direct access file was used for this table. The maximum number of spectra whose individual identification numbers will be recorded if a peak of given m/e is present is specified as 130. The direct access file is structured as an array of 3000 lines of 132 words. The first two words in any line contain the m/e value for the ion and the frequency of occurrence of the ion and the remainder of the line is an array of numbers to identify the compounds containing that ion among the 15 most intense ions. Lines 1-1000 contain arrays using the NIH identification numbers, lines 1001-2000 contain arrays using identification numbers based on the molecular weight and lines 2001-3000 have arrays containing the line number of the name, identification data and BIG15 MS in a second direct access file. These files have been retained for adaption to an already available MS library search on the Univac 1106 (QUADNOVA*RCHEM).

The data input section of the programme (A-I Fig. C-13, lines 1-65) reads in the compound information and the 15 most intense peaks in the mass spectrum (the output of FILESORT). A new identification number is calculated containing the molecular weight and the molecular weight frequency array and the number of spectra are updated. For each mass (in a BIG15 MS) the relevant lines are read from the direct access file into core, the identification arrays are updated and then rewritten to the direct access file (I-B Fig. C-13, lines 68-111 Fig. C-14). The identification number, name, formula, molecular weight and BIG15 MS are written to the other direct access file and to the output file. The programme then returns for further data input (B-C Fig. C-13 lines 111-125 Fig. C-14).

When all data has been processed the programme sorts out the number of
spectra, the molecular weight frequency table, and the frequency of occurrence of each m/e value from 31 to 1000 (C-D Fig. C-13, lines 126-160 Fig. C-14). The programme next prints out the lists of identification numbers of the mass spectra containing ions of each m/e value. Both the NIH identification number and the identification number based on the molecular weight are printed out (D-E Fig. C-13, lines 161-216 Fig. C-14). The molecular weight frequency array and the ion occurrence frequency array were then written to the first 20 lines of the direct access file.
START

1. Read first 6 lines CHEM11 and discard

2. Read from NIHNAMEs the information about compound

3. Read mass, intensity pair from CHEM11 into array

   Is identification same as from NIHNAMEs

      YES

      2. Is mass = molecular weight?

         YES

         Write out compound identification + error message

      NO

      NO

      Write out compound identification + error message

1
YES

Is molecular wt. correct

NO
Correct molecular weight

YES

Error in CHEM11 data

YES
Read next 100 lines CHEM11

NO

Define IDUMMY - counter to read in correct number of mass/intensity pairs from CHEM11

Is IDUMMY correct for this compound

NO
Change counter IDUMMY

3
FIGURE C-1. Flowchart for programme PRINT.
C PROGRAM PRINT

1 C FILE UNIT 14 WHICH CONTAINS THE IDENTIFICATION NUMBER, THE
2 MOL. WEIGHT, THE FORMULA AND THE NAME OF THE COMPOUND.
3 MASS VALUES, THE MASS AND THE INTENSITY OF THE MASS SPECTRAL PEAK.
4 SPECIAL FILE CHEM11.
5 C THE INTENSITY VALUES ARE READ INTO 2 ARRAYS OF MASS AND INT SLP LONG.
6 C THE TWO ARRAYS CONTAIN MASS INTENSITY PAIRS.
7 C VARIABLES USED
8 C ING = IDENTIFICATION NO. READ FROM UNIT 14, NAMES.
9 C MOLWT = MOLECULAR WEIGHT READ FROM UNIT 14.
10 C FORM = FORMULA READ FROM UNIT 14.
11 C NAME = NAME OF COMPOUND READ FROM UNIT 14.
12 C SOURCE = SOURCE TO FIND FULL SPECTRUM.
13 C ID = IDENTIFICATION NO. READ FROM UNIT 16.
14 C MW = NUMBER OF MASS VALUES READ FROM UNIT 16.
15 C INT = NUMBER OF INTENSITY VALUES READ FROM UNIT 16.
16 C FILES ARE THEN PRINTED IN A COMPRESSED FORM.

20 DIMENSION MASS(500), INT(500), JUNK(125)
21 DIMENSION FORM(5), NAME(5), SOURCE(21)

26 COMMON MASS, INT, FORM, NAME, SOURCE.

27 THE FIRST 6 LINES OF CHEM11 ARE FILLED WITH JUNK AND ARE READ IN AND DISCARDED.

31  DO 1, 1210
32  I = 1, 1210

33  IF (ING .LT. 161) JUNK
34  IF (ING .LT. 161) JUNK
35  IF (ING .LT. 161) JUNK
36  IF (ING .LT. 161) JUNK
37  IF (ING .LT. 161) JUNK

40  IF (ING = 297) GO TO 41
41  IF (ING = 423) GO TO 42
42  IF (ING = 111) MOLWT = 1114
43  IF (ING = 751) MOLWT = 1674
44  IF (ING = 1114) GO TO 45
45  IF (ING = 1115) GO TO 97

50  IDUMMY=0
51  IF (ING = 208) IDUMMY=32
52  IF (ING = 208) IDUMMY=32
53  IF (ING = 208) IDUMMY=32
54  IF (ING = 208) IDUMMY=32
55  IF (ING = 208) IDUMMY=32
56  IF (ING = 208) IDUMMY=32
57  IF (ING = 208) IDUMMY=32
58  IF (ING = 208) IDUMMY=32
59  IF (ING = 208) IDUMMY=32
60  IF (ING = 208) IDUMMY=32
61  IF (ING = 208) IDUMMY=32
62  IF (ING = 208) IDUMMY=32
63  IF (ING = 208) IDUMMY=32
64  IF (ING = 208) IDUMMY=32
65  IF (ING = 208) IDUMMY=32
66  IF (ING = 208) IDUMMY=32
67  IF (ING = 208) IDUMMY=32
68  IF (ING = 208) IDUMMY=32
69  IF (ING = 208) IDUMMY=32
70  IF (ING = 208) IDUMMY=32
71  IF (ING = 208) IDUMMY=32
72  IF (ING = 208) IDUMMY=32
73  IF (ING = 208) IDUMMY=32
74  IF (ING = 208) IDUMMY=32
75  IF (ING = 208) IDUMMY=32
76  IF (ING = 208) IDUMMY=32
77  IF (ING = 208) IDUMMY=32
78  IF (ING = 208) IDUMMY=32
79  IF (ING = 208) IDUMMY=32
80  IF (ING = 208) IDUMMY=32
81  IF (ING = 208) IDUMMY=32
82  IF (ING = 208) IDUMMY=32
83  IF (ING = 208) IDUMMY=32
84  IF (ING = 208) IDUMMY=32
85  IF (ING = 208) IDUMMY=32
86  IF (ING = 208) IDUMMY=32
87  IF (ING = 208) IDUMMY=32
88  IF (ING = 208) IDUMMY=32
89  IF (ING = 208) IDUMMY=32
90  IF (ING = 208) IDUMMY=32
91  IF (ING = 208) IDUMMY=32
92  IF (ING = 208) IDUMMY=32
93  IF (ING = 208) IDUMMY=32
94  IF (ING = 208) IDUMMY=32
95  IF (ING = 208) IDUMMY=32
96  IF (ING = 208) IDUMMY=32
97  IDUMMY=0
98  IDUMMY=0
99  IDUMMY=0
100  IDUMMY=0
101  IDUMMY=0
IF (IONO.FO.701) IDUMMY=189

DO 60 J=2,TIDUMMY

READ (16,200,ERR=130,FIND=15D) IP,NHV,MASS(J),INT(J)

IF (MASS(J) .GT. 40) SIGX=SIGX+TNT(J)

IF (MASS(J) .GT. MOLWT) GO TO 70

STOP

CONTINUE

70 LINE=2(J,J/12)+1

WRITE (15,11) TEQ,MOLAT,FORM,NAME,SOURCE

LE=1

WRITE (15,1) LE

IF (LE .LT. MALS(T)) GO TO 69

CONTINUE

80 CONTINUE

GO TO 20

90 READ (16,20) IDN,NVM,MSS,INT

GO TO 50

95 WRITE (6,210) IDNO,MOLAT,FORM,NAMF,SOURCE

GO TO 20

100 WRITE (6,220) IDNO,MOLAT,FORM,NAMF,SOURCE

GO TO 20

110 WRITE (6,230) IDNO,MOLAT,FORM,NAMF,SOURCE

GO TO 20

120 WRITE (6,240) IDNO,MOLAT,FORM,NAMF,SOURCE

GO TO 20

130 WRITE (6,250) IDNO,MOLAT,FORM,NAMF,SOURCE

GO TO 20

140 WRITE (6,260) IDNO,MOLAT,FORM,NAMF,SOURCE

GO TO 20

150 WRITE (6,170)

STOP

FORMAT (12(I4,1H,14,1H,1X))

FORMAT (*) PROGRAM HANGED END OF FILE * 

FORMAT (20.24)

FORMAT (I6,14,1X,52A4,2,A4,1Y,2A4)

FORMAT ()

FORMAT (///,1Y,11,1Y,54A7,2A4,1Y,2A4,///)

FORMAT (*) DYN FROM CHEM1 IS NOT EQUAL TO THAT FROM THSCOMP1

FORMAT (*) IDNO FROM CHEM1 =,1S)

FORMAT (*) ERROR IN CHEM1 14 

FORMAT (///,1Y,11,1Y,54A7,2A4,1Y,2A4,///)

END
FIGURE C-3. Output from programme PRINT.
<table>
<thead>
<tr>
<th>MSC 699R</th>
</tr>
</thead>
<tbody>
<tr>
<td>54.22</td>
</tr>
<tr>
<td>93.210</td>
</tr>
<tr>
<td>137.265</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>MSC 748R</th>
</tr>
</thead>
<tbody>
<tr>
<td>42.250</td>
</tr>
<tr>
<td>65.590</td>
</tr>
<tr>
<td>85.170</td>
</tr>
<tr>
<td>105.433</td>
</tr>
<tr>
<td>122.170</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>MSC 7C1R</th>
</tr>
</thead>
<tbody>
<tr>
<td>67.103</td>
</tr>
<tr>
<td>94.160</td>
</tr>
<tr>
<td>137.550</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>MSC 7C3R</th>
</tr>
</thead>
<tbody>
<tr>
<td>53.109</td>
</tr>
<tr>
<td>76.340</td>
</tr>
<tr>
<td>107.160</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>MSC 7C4R</th>
</tr>
</thead>
<tbody>
<tr>
<td>56.250</td>
</tr>
<tr>
<td>81.920</td>
</tr>
<tr>
<td>98.100</td>
</tr>
<tr>
<td>123.180</td>
</tr>
<tr>
<td>Line</td>
</tr>
<tr>
<td>------</td>
</tr>
<tr>
<td>82</td>
</tr>
<tr>
<td>83</td>
</tr>
<tr>
<td>84</td>
</tr>
</tbody>
</table>

FIGURE C-4. A part of TMSCOMPS generated by the CTS text editor on the Univac 1106 for preparation of the sublibrary of silicon containing compounds.
Read first 6 lines CHEM11 and discard
Zero intensity array
Read from NIHAMES THE information about compound
Read line from CHEM11
Is identification number same as NIHAMES
YES
Is molecular wt. correct
YES
Error in CHEM11 data
YES
Define IDUMMY-counter to read in correct number of mass/intensity pairs from CHEM11

Is IDUMMY correct for this compound
YES

Correct molecular weight
Read next 100 lines CHEM11

Change counter IDUMMY
NO

YES
FIGURE C-5. Flowchart for programme BIG15.

1. Read mass/intensity pair from CHEM11 into array

2. Is identification same as NIH NAMES
   - YES: Go to subroutine BIG to find Big15
   - NO: Go to next step

3. Is mass = molecular weight
   - YES: Write out heading for compound-identification no., formula, name, library, source.
   - NO: Write BIG15 array

4. Any more compounds
   - YES: Go back to step 1
   - NO: END
C PROGRAM BIG15

C THIS PROGRAM IS DESIGNED TO USE THE INPUT FILES:

C A FILE UNIT IN WHICH CONTAINS THE IDENTIFICATION NUMBER, THE MOLECULAR
C WEIGHT, THE FORMULA AND THE NAME OF THE COMPOUND EG GUANIDINE-MS

C A SPECIAL FILE QUADGRAV.CHM1

C THE INTENSITY VALUES ARE READ INTO AN ARRAY OF INTENSITIES 100 TO LONG.

C WHICH THE POSITION IN THE ARRAY INDICATES THE MASS OF THAT PEAK & A PEAK OF

C MASS 245 AND INTENSITY 999 WOULD BE IN POSITION INT245)

C VARIABLES USED

C A JUNK = RUBBISH IN FIRST LINES CHEM1II

C B IDNO = IDENTIFICATION NO READ FROM UNIT 14 IE. TMSCOMPOUNDS

C C MOLWT = MOLECULAR WEIGHT READ FROM UNIT 14

C D FORM = FORMULA READ FROM UNIT 14

C E NAME = NAME OF COMPOUND READ FROM UNIT 14

C F SOURCE = SOURCE READ TO FIND FULL SPECTRUM

C G ID = IDENTIFICATION NO READ FROM UNIT 16

C H M NW = NUMBER OF MASS VALUES READ FROM UNIT 16

C I MASS = MASS VALUE OF MASS-INT PAIR READ FROM UNIT 16

C J INTEN = INTENSITY USED WHEN CHECKING IDNC1 READ FROM UNIT 16

C K DROMU = DROMY INDEX CALCULATED IF SUBROUTINE

C DIMENSION INT(200), MOLWT(300), JUNK(120), FORM(5)

C DIMENSION NAME(20), SOURCE(15)

C THE FIRST 6 LINES OF CHEM1II ARE FILLED WITH JUNK AND ARE READ IN AND

C READ (16,160) JUNK

C CLEAR THE INTENSITY ARRAY

C DO 10 J=1,200

C 10 READ (16,160) JUNK

C READ FROM LIST OF COMPOUNDS IDNO,MOLWT,FORM,NAME,SOURCE

C 10 READ (16,190) IDNO,MOLWT,FORM,NAME,SOURCE

C READ DOWN CHEM1II TO FIND THE START OF THE INFORMATION FOR THE REQUIRE

C NO READ (16,200,ERR=1200) IDNO,MOLWT,FORM,NAME,SOURCE

C IF (IDNO.EQ.IDNO) GO TO 51

C GO TO 46

C THIS SECTION SORTS OUT THE ERRORS IN CHEM1II

C 50 IF (IDNO.EQ.97) GO TO 90

C IF (IDNO.EQ.114) GO TO 90

C IF (IDNO.EQ.111) GO TO 90

C IF (IDNO.EQ.473) GO TO 90

C IF (IDNO.EQ.393) MOLWT=115

C IF (IDNO.EQ.673) MOLWT=167

C IF (IDNO.EQ.351) IDNUM=46

C IF (IDNO.EQ.354) IDNUM=33
FIGURE C-6 Listing of programme BIG15
A

START

I

Set max intensity(I) = intensity of mass 31

J

31, molecular weight

Is Max Int(I) > Int(J)?

NO

YES

Set new Max Int(I) = Int(J)
Set new Mass(I) = MASS(J)

J

Set intensity Mass(I) = 0

I

B

L

1.15

2

Set a limit on the no. of passes through the arrays(JJ) and a flag to indicate if an exchange has occurred

3
FIGURE C-7, Flowchart for subroutine BIG
SUBROUTINE BIG(MOLWT, INT, MBIG15)
DIMENSION INT(2000), MBIG15(3U), MINT(15), MAXMAS(15)
DO 20 I = 1, 15
MINT(I) = INT(31)
MAXMAS(I) = 31
DO 10 J = 32, MOLWT
IF (INT(J) .LE. MINT(I)) GO TO 10
MINT(I) = INT(J)
MAXMAS(I) = J
CONTINUE
NUM = MAXMAS(I)
INT(NUM) = G
CONTINUE
GO TO 50
L = 1, 15
C SET A LIMIT ON THE PASS THROUGH THE DATA
JJ = L
C SET A FLAG TO INDICATE NO EXCHANGES HAVE OCCURRED
MARK = G
DO 99 K = 1, JJ
KP1 = K + 1
IF (MAXMAS(K) .GE. MAXMAS(KP1)) GO TO 99
C EXCHANGE THE TWO MASS AND INTENSITY ARRAYS AND SET FLAG
JEXCH = MAXMAS(KP1)
JEXCH1 = MINT(KP1)
MAXMAS(KP1) = MAXMAS(K)
MINT(KP1) = MINT(K)
MAXMAS(K) = JEXCH
MINT(K) = JEXCH1
MARK = 1
CONTINUE
99 CONTINUE
C TEST TO SEE IF ANY EXCHANGES HAVE TAKEN PLACE
IF(MARK .EQ. 0) GO TO 30
CONTINUE
DO 30 U = 1, 15
M9IG15(U) = MAXMAS(M)
30 CONTINUE
MBIG15(N) = MAXMAS(M)
RETURN
END

FIGURE C-8. Listing of subroutine BIG.
FIGURE C-9. Output from programme BIG15.
<table>
<thead>
<tr>
<th>Time</th>
<th>74.7</th>
<th>73.82</th>
<th>59.5</th>
<th>58.3</th>
<th>45.17</th>
<th>44.4</th>
<th>43.7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time</td>
<td>56.9</td>
<td>55.5</td>
<td>47.2</td>
<td>45.4</td>
<td>44.3</td>
<td>43.2</td>
<td>41.2</td>
</tr>
<tr>
<td>Time</td>
<td>75.3</td>
<td>74.4</td>
<td>73.46</td>
<td>59.5</td>
<td>58.1</td>
<td>45.6</td>
<td>43.2</td>
</tr>
<tr>
<td>Time</td>
<td>73.99</td>
<td>59.6</td>
<td>56.10</td>
<td>55.6</td>
<td>47.6</td>
<td>45.19</td>
<td>43.6</td>
</tr>
<tr>
<td>Time</td>
<td>61.5</td>
<td>59.3</td>
<td>47.5</td>
<td>45.16</td>
<td>44.1</td>
<td>43.13</td>
<td>42.2</td>
</tr>
<tr>
<td>Time</td>
<td>85.11</td>
<td>75.25</td>
<td>74.9</td>
<td>73.99</td>
<td>57.19</td>
<td>45.20</td>
<td>41.13</td>
</tr>
<tr>
<td>Time</td>
<td>95.48</td>
<td>81.10</td>
<td>75.20</td>
<td>74.9</td>
<td>73.99</td>
<td>45.19</td>
<td>39.10</td>
</tr>
</tbody>
</table>
A

START

M

1,1000000

Read input line from BIG15

Last line of input

YES

Calculate number of compounds

NO

Write to direct temporary Direct access File (DA)

Write last line to DA file

1,Number of Compounds

M

N

Calculate line number of lines which contain Identification number (IDNO) & Molecular Wt.

Read Identification number and molecular weight from calculated line.

N

Write no of compounds on line printer

B

Zero pointers used in sort

2
Set elements in pointer arrays to zero

Select mass of compound 1, i.e., J = 1

Is Mass(I) > Mass(J)?

NO

Is left pointer = 0 = ILB(J)?

NO

Set J = left pointer ILB(J)

YES

Set right pointer = -J = IRB(I)

left pointer vector J = I

Set IRB(I) = IRB(J)

YES

Set new J = IRB(J)

NO

Is right pointer J < 0

Reset J = 1

J = ILB(J)

Is left pointer(J) (J > 0, ILB(J) > 0)

YES

C

2

1

4

3
FIGURE C-10. Flowchart for programme FILESORT.
COMPILE 1 (X+2)

C PROGRAM FILE SORT

C THIS PROGRAM TREATS FILES CREATED BY PROGRAM SORT AND SOPTS THE

C COMPOUNDS INTO ASCENDING MOLECULAR WEIGHT

C THE PROGRAM THEN REWRITES THE FILES IN ORDER OF INCREASING MOLECULAR

C WEIGHT

C THE FILE TO BE SORTED IS ASSIGNED TO FILE 14 AND IS DECLARED A

C RANDOM ACCESS FILE

C THE ING AND MOLECULAR WEIGHT ARE READ INTO 2 ARRAYS AND THE

C MOLECULAR WEIGHT SORTED BY A MONKEY SORT - SEE DAY, AC, COMPUTER

C TECHNIQUES - CAMBRIDGE UNIV PRESS 1972 -DL1.4624/32 PRO

C THE OFFICE FILE STATEMENT CONTAINS 4 FIELDS

C A = THE NUMBER OF RECORDS IN THE FILE - IN THIS CASE THE NUMBER OF LINE

C B = NUMBER OF CHARACTERS PER RECORD

C C = A LETTER DEFINING THE TYPE OF RECORD - F MEANS FORMATTED CHARACTERS

C D = AN INTEGER VARIABLE WHICH DEFINES A LINE AFTER IT IS READ

C ILF AND IRB ARE VECTORS WHICH DEFINES THE SORTED ARRAY.

DEFINE FILE 14{15D, 12F1.PT)

COMMON MASS (200), IDNO (200), ILF (200), IRB (2000)

DO 10 M = 1, L 10

READ (13, 20L, END = 20J) COPY

WRITE (14, * ) COPY

READ (14, IX, 20L, END = 20J) COPY

SET THE POINTERS TO ZERO

ILB (I) = 0

IPB (I) = 0

THE FOLLOWING LOOP MUST BE DONE FOR EACH RECORD.

DO 99 I = 2, NC0MP

ILB (I) = 0

IPB (I) = 0

IF (MASS(I).GT.MASS(J)) GO TO 70

IF (ILB(J) .EQ. 0) GO TO 60

J = ILB (J)

GO TO 50

60 IRB (I) = J

70 IF (IRB (J).LE.0) GO TO 80

J = IRB (J)

80 WRITE (13, * ) COPY

99 CONTINUE

END
GO TO 5L
IRB(I)=IRB(J)
CONTINUE
C THE FOLLOWING STATEMENTS READ THE ITEMS AND WRITE THEM INTO A NEW FILE
J=1
GO TO 1L
100 IRB(J)+1 GO TO ILEC
110 IF (FILE(J).GT.0) GO TO 1LJC
120 JPT=+1(J-1)*6
READ (14,JPT,233) IDN,MOLWT,FORM,NAME,SOURCE
140 IF (IDN+NE,F1ON(J)) GO TO 190
150 WRITE (16,JPT) IDN,FORM,NAME,SOURCE
160 IF (I10(J)) 160,170,150
170 J=IRB(J)
GO TO 1L
180 IF (J=IRB(J)) 160,170,150
GO TO 1L
190 WRITE (6,270)
WRITE (6,220) N,IX
STOP
200 WRITE (6,220) N,IX
STOP
210 WRITE (6,220) N,IX
GO TO 1L
220 FORMAT (33A4)
230 FORMAT (16, I4,5A4,2A4,I4, A4)
240 FORMAT (5A4)
250 FORMAT (33A4)
260 FORMAT (16, A4)
270 FORMAT (' ERROR- IGN0 NOT EQUAL TO ION')
END

FIGURE C-11. Listing of programme FILESORT
<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular Weight (amu)</th>
<th>Molar Mass (g/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PALMITIC ACID-METHYL ESTER</td>
<td>360 270 C17.H34.O2</td>
<td>MGH 512</td>
</tr>
</tbody>
</table>

FIGURE C-12. Output from programme FILESORT.
START

Define files, dimension and zero arrays, spectrum count. Set number of spectra for recording identification numbers (NSPEC)

Read compound information + Big15

Is Mol. Wt < 30 or > 1000

YES

Reset Mol. Wt.

NO

Calculate new index number = Mol wt x 1000 + Frequency

Update Mol. Wt. Frequency Array

Update Spectra Counter

1, 15

1

Is Mass > 1000

YES

Set mass = 999

10 4 1
Is mass < 31?

Work out line numbers in DA File
Read Frequency of peak from Array.

Peak Freq. > NSPEC?

Peak Freq. = 0?

Read from DA File mass, freq., identification numbers of MS which contain peak

Update frequency array, counter from DA file.

Updated Freq. > NSPEC?

Write updated frequency back to DA file.
Update Spectrum no.array,index no.array,IDNO array

Write Spectrum no.array,index no.array,IDNO array back to DA file

Write information, compound identification and BIG15 to second DA File

Any more input?

Write out number of spectra

Write headings for Mol.Wt. table

Calculate line numbers + counters

Write-out line of Mol. wt.freqs.
Write headings for ion freq. table

Calculate line numbers + counters

Write out line of ion frequencies

Write headings for table of mass spectrum numbers.

Freq = 0 ?

Freq < NSPEC

Will no. of lines fit page?

YES

Take new page + print headings

NO

YES

NO

11

5

1.50

MI

31,1000

MJ

6

7

8
Write peak frequency + Mass

Take new page + print headings

Calculate lines required

Will no. of lines fit page?

YES

Write peak frequency + Mass

Calculate nos. + counters

Write lists of identification nos. + IDNOs.

NO

Take new page + print headings

Will no. of lines fit page?

YES

Write peak freq. = 0 + Mass

Calculate line nos. in DA file

Write Mass + zero freq. to DA file
Calculate counters + line nos.

Write molecular wt. array to first 10 lines DA file, ion frequency array to lines 11-20

END

FIGURE C-13. Flowchart for programme MFTABLE.
C PROGRAM MFTABLT

PROGRAM TO SORT DIG 15 SPECTRA OF MASS SPECTRA AND
PRODUCE A TABLE OF MASSES, THE FREQUENCY OF EACH MASS
AND A LIST OF SPECTRA WHICH CONTAIN THE PEAK

THE PROGRAM PRODUCES TWO DIRECT ACCESS FILES:
1. A FILE ATTACHED TO LOGICAL UNIT 14 WHICH CONTAINS
   THE MASS, THE FREQUENCY OF THAT MASS, AND THE ID NUMBER
   OF THE SPECTRA CONTAINING THE PEAK AS WELL AS THE POSITION
   OF THE SPECTRUM IN ANOTHER DIRECT ACCESS FILE.
2. A FILE ATTACHED TO LOGICAL UNIT 15 WHICH CONTAINS
   THE INFORMATION CHEMICAL NAME BASED ON MOLECULAR WEIGHT,
   THE SPECTRA AND THE DIGEST IS PEAKS TOGETHER WITH THEIR
   INTENSITY

THE PROGRAM ALSO PRODUCES A PRINTOUT OF THE DIG 15 SPECTRA
WHICH ARE IN THE SAME ORDER AS THE INPUT SPECTRA BUT WHICH
HAVE THE ID NUMBER CHANGED TO THAT CALCULATED FROM MOLECULAR
WEIGHT TIMES FRACTION A NUMBER TO UNIQUELY IDENTIFY EACH SPECTRUM.

VARIABLES USED:

1. IDNC = IDENTIFICATION NUMBER FROM DIG 15 FILE
2. MWTFR = MOLECULAR WEIGHT
3. FORM = FORMULA
4. NAME = NAME
5. SOURCE = SOURCE OF SPECTRA IN ORIGINA COLLECTION
6. MASS = MASS OF SPECTRUM
7. INT = INTENSITY OF PEAK
8. MWTFR = MOLECULAR WEIGHT FREQUENCY - NO OF COMPOUNDS
9. WITH MOLECULAR WEIGHT
10. IPKFP = FREQUENCY OF A GIVEN PEAK MASS M
11. ICOUNT = POSITION OF IDENTIFICATION NUMBERS IN ARRAY

DIMENSION MWTFR(1U6), MASS(1S), INT(1S), IPKFP(1U6)
DIMENSION NAME(2), SOURCE(2), FORM(5)
DEFINE FILE 15(1LC, F.TD,V, IJL), 14(1LC,132, V, IJL)
DIMENSION LIST(132), LIST1(132), IPKFP(132), MH(27)
DATA IPKFP /11,0,0,0, MWTFR /132=0/
READ (12,160,END=50) IDNO, MOLWT, FORMNAME, SOURCE
READ (12,170,END=50) (MASS(I),INT(I),1351,15)
IF (MOLWT.GT.1000) MOLWT=1000
IF (MOLWT.LT.3) MOLWT=3
C
TO CALCULATE THE INDEX NUMBER
MOLWT=INT(MOLWT)+MTFR(MOLWT)
IF (MOLWT.GT.1000) MOLWT=999
IF (MOLWT.LT.3) MOLWT=3
SET THE COUNTER
ICOUNT=ICOUNT+1
TO CALCULATE: THE INDEX NUMBER
MWTFR(MOLWT)=MWTFR(MOLWT)+1
SET THE COUNTERS TO THE RELEVANT MASS
IF (MASS(I).GE.9999) MASS(I)=999
IF (MASS(I).LE.9.99) GO TO 4
KON=MASS(I)
KON1=KON+1.0
KON2=KON1+1000
NUM=IPFR(KUN)
READ FROM THE DIRECT ACCESS FILE
IF (NUM.LE.1) GO TO 20
IF (NUM.GT.NSPEC) GO TO 20
READ (14*KON) KUN,IPFR(LIST(I),I,I,J).
READ (14*KON2) KUN2,IPFR2(LIST(I),I,I,J).
IF (KUN2.LT.KUN) GO TO 22
IPFR1(KUN)=IPFR(KON)
READ (14*KUNZ) KUNZ,IPFR3(LIST(I),I,I,J).
IF (KUNZ.LT.KUN) GO TO 22
IPFR1(KUN)=IPFR(KON)
NUM=NUM+1
IF (NUM.LE.1) GO TO 20
IF (NUM.GT.NSPEC) GO TO 20
END
WRITE RESULTS TO DA FILE

WRITE (14'KON1) MASS(I),NUM,(LIST(IJ1),IJ1=1,NUM)
WRITE (14'KON1) MASS(I),NUM,(LIST(IJ2),IJ2=1,NUM)
WRITE (14'KON2) MASS(I),NUM,(ISPIR(IJ3),IJ3=1,NUM)

GO TO 47
WRITE (14'KON1) MASS(I),NUM
WRITE (14'KON1) MASS(I),NUM
WRITE (14'KON2) MASS(I),NUM

WRITE THE SPECTRUM TO THE OTHER DA FILE
WRITE (15'COUNT) ION,MOL,MNC,FORM,NAME,SOURCE,(MASS(I),INT(I)),
WRITE (16,'KON2) MASS(I),INT(I),I=1,15)
READ IN NEXT SPECTRUM
GO TO 15
WRITE OUT THE NUMBER OF SPECTRA.
WRITE (18,15C) I COUNT
WRITE OUT THE FREQUENCY OF EACH MOLECULAR WT.
WRITE (16,21C)
DO 7 S 1 N I = 1,149
CALCULATE MASSES.
DO 8 M(I)=I,J=1,120
SET COUNTERS
K1=N1*2+1
K7=K1+19
C WRITE OUT ARRAY OF MASS FREQUENCIES

WRITE (18,200) (MF(J),J=1,20),(*WTPF(M),M=K1,K2)

C WRITE OUT TABLE OF MASS/FREQUENCIES
WRITE (18,22) 
DO 9, K1=1,49
9 CONTINUE
WRITE (18,200) (MF(J),J=1,20),(*WTPF(H),MT=K1,K2)

C WRITE OUT THE MASS/FREQUENCY ARRAY
WRITE (18,200) (MF(J),J=1,20),(*WTPF(H),MT=K1,K2)

C WRITE OUT THE MASS SPECTRUM NO TABLE
WRITE (18,200) (MF(J),J=1,20),(*WTPF(H),MT=K1,K2)

DO 130 I=1,NL1X
  IF (IPFR(I) .LE.R) GO TO 110
  IF (IPFR(I) .LT.NSPEC) GO TO 110
  LINEN=NL1X
  IF (LINEN.GE.56) NPAGE=NPAGE+1
  WRITE (13,250) NPAGE
  WRITE (18,200) I,IPFR(I)
  NLINF-LINFN
  IF (LINEN.GE.56) NLINF=LINEN+5
  WRITE (18,200) I,JKLB
  WRITE (18,250) I,JKLB
  WRITE (18,200) I,JKLB
  GO TO 130

THIS SECTION WRITES OUT THE IDNO'S OF THE SPECTRA WHICH CONTAIN THE PEAKS.
C

HEAD (14*J, IFR, LIST(IJ), IJ=1,J)

IF (IJ+1 M J)

READ (14*J, IFR, LIST(IJ), IJ=1,J)

L=

LINES=(IFR/15+1)

C RESET LINE COUNTERS, TAKE NEW PAGE IF NECESSARY.

LINES=LINES+(LINES=1)

IF (LINES(1)<56) WRITE (19,24) NPAGE

IF (LINES(2)<56) WRITE (1I,270) (LIST(M), M=15*L)

IF (LINES(3)<56) WRITE (11,270) (LIST(M), M=15*L)

C CONTINUE

IF (LINES(4)<56) WRITE (18,250) I.IFR(I) 

DO 12 K=1,LINES

IF (IFR.LT. L) L=IFR

WRITE (10,254) (LIST(M), M:IS,L)

WRITE (11,270) (LIST(M), M=IS,L)

CONTINUE

MWTFR (1) = COUNT

DC 14(JJ)=1,J)

J1=JJ=10*J1

J2=J1+1

J3=J1+1

C

WRITE (14,JJ) (MWTFR(I), I=J1,J2)

WRITE (14,J1) (I.IFR(I), I=J1,J2)

STOP

C

FORMAT (***,'NUMBER OF SPECTRA =','I7)

FORMAT (///,'TABLE OF OCCURRENCE OF MOLECULAR WEIGHT ***)

FORMAT (///,'TABLE OF OCCURRENCE OF MASS SPECTRAL PEAKS ***)

FORMAT (///,'TABLE OF PEAKS/SPECTRUM NUMBERS ***)

FORMAT (///,'TABLE OF PEAKS/SPECTRUM NUMBERS ***)

FORMAT (///,'TABLE OF PEAKS/SPECTRUM NUMBERS ***)

FORMAT (///,'IDENT NO ***)

END

FIGURE C-14. Listing of programme MFTABLE.
SPECTRA OF TRIMETHYLSILYL DERIVATIVES

272004 C15.H32.02.SI LAURIC ACID-MONOTMS
258.10 257.51 145.16 132.39 129.25 117.51 104.13 75.02 74.11 73.99 57.9 55.24 45.12 43.42 41.35 36

273000 C12.H27.N.02.SI2 BISTRIMETHYLSILYL PIPEROLIC ACID
230. 3 158. 4 157.14 156.99 147. 4 84. 5 75. 5 74. 4 73.49 59. 5 45.11 44.2 43.3 41.35

273001 C11.H23.N.03.SI2 PYROGLUTAMIC ACID-OTMS
258. 5 258.16 157.20 156.99 93. 5 75.40 73.70 47. 5 45.20 44. 5 43.5 31. 0 31. 0 31. 0 31. 0

273002 C11.H23.N.03.SI2 D-TMS-2-PYRROLIDONE-5-CARBOXYLATE
273. 5 258.10 157.20 156.99 93. 5 75.40 73.70 47. 5 45.20 44. 5 43.5 31. 0 31. 0 31. 0 31. 0

273003 C11.H23.N.03.SI2 PYROGLUTAMIC ACID-OTMS
258. 6 230. 8 158. 4 157.14 156.99 148. 2 147.19 75. 9 74. 7 73.78 59. 3 58. 2 45.12 44. 3 43. 3

273004 C12.H27.N.02.SI2 CYCLOLEUCINE-DITMS
230. 4 168. 3 158. 4 157.15 156.99 147. 8 84. 5 75. 7 74. 6 73.58 59. 5 45.11 44. 2 43. 2 41. 2

273005 C11.H23.N.03.SI2 PYROGLUTAMIC ACID-OTMS
258. 6 230. 6 221. 3 158. 4 157.13 156.99 147.15 75.10 74. 7 73.82 59. 5 58. 3 45.17 44. 4 43. 7

274000 C12.H26.03.SI2 BISTRIMETHYLSILYL 2-KETOISOCAPROIC ACID
274. 6 259.19 215. 8 157.6 156.10 149.11 148.18 147.99 141. 7 133. 7 75.16 74. 7 73.83 69.15 45.23

274001 C11.H22.04.SI2 BISTRIMETHYLSILYL ITACONIC ACID
259.13 230. 6 215. 7 149.10 148.16 147.99 133. 5 75.17 74. 8 73.90 67. 5 47. 5 45.17 43. 4 40. 6

274002 C11.H23.05.8.SI2 A-FUCOPYRANOSIDE-1-METHYL-3,4-METHYLBORONATE-2-TMS
157.43 146.29 143.15 131.33 130.96 115.40 89.32 75.65 74.20 73.99 59.24 45.32 44.36 43.26 41.16

274003 C11.H22.04.SI2 ITACONIC ACID-OTMS
259.15 230. 7 201. 6 149. 8 148.14 147.93 83.12 75.25 74. 9 73.99 67. 5 47. 5 45.19 43. 6 40. 7

FIGURE C-15.Big15 mass spectra produced by programme MFTABLE.
TABLE OF OCCURRENCE OF MOLECULAR WEIGHT

<table>
<thead>
<tr>
<th>MASS</th>
<th>21</th>
<th>22</th>
<th>23</th>
<th>24</th>
<th>25</th>
<th>26</th>
<th>27</th>
<th>28</th>
<th>29</th>
<th>30</th>
<th>31</th>
<th>32</th>
<th>33</th>
<th>34</th>
<th>35</th>
<th>36</th>
<th>37</th>
<th>38</th>
<th>39</th>
<th>40</th>
</tr>
</thead>
<tbody>
<tr>
<td>FREQ</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>41</td>
<td>42</td>
<td>43</td>
<td>44</td>
<td>45</td>
<td>46</td>
<td>47</td>
<td>48</td>
<td>49</td>
<td>50</td>
<td>51</td>
<td>52</td>
<td>53</td>
<td>54</td>
<td>55</td>
<td>56</td>
<td>57</td>
<td>58</td>
<td>59</td>
<td>60</td>
<td>61</td>
</tr>
<tr>
<td>FREQ</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>81</td>
<td>82</td>
<td>83</td>
<td>84</td>
<td>85</td>
<td>86</td>
<td>87</td>
<td>88</td>
<td>89</td>
<td>90</td>
<td>91</td>
<td>92</td>
<td>93</td>
<td>94</td>
<td>95</td>
<td>96</td>
<td>97</td>
<td>98</td>
<td>99</td>
<td>100</td>
<td>101</td>
</tr>
<tr>
<td>FREQ</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>101</td>
<td>102</td>
<td>103</td>
<td>104</td>
<td>105</td>
<td>106</td>
<td>107</td>
<td>108</td>
<td>109</td>
<td>110</td>
<td>111</td>
<td>112</td>
<td>113</td>
<td>114</td>
<td>115</td>
<td>116</td>
<td>117</td>
<td>118</td>
<td>119</td>
<td>120</td>
<td>121</td>
</tr>
<tr>
<td>FREQ</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>121</td>
<td>122</td>
<td>123</td>
<td>124</td>
<td>125</td>
<td>126</td>
<td>127</td>
<td>128</td>
<td>129</td>
<td>130</td>
<td>131</td>
<td>132</td>
<td>133</td>
<td>134</td>
<td>135</td>
<td>136</td>
<td>137</td>
<td>138</td>
<td>139</td>
<td>140</td>
<td>141</td>
</tr>
<tr>
<td>FREQ</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>141</td>
<td>142</td>
<td>143</td>
<td>144</td>
<td>145</td>
<td>146</td>
<td>147</td>
<td>148</td>
<td>149</td>
<td>150</td>
<td>151</td>
<td>152</td>
<td>153</td>
<td>154</td>
<td>155</td>
<td>156</td>
<td>157</td>
<td>158</td>
<td>159</td>
<td>160</td>
<td>161</td>
</tr>
<tr>
<td>FREQ</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>161</td>
<td>162</td>
<td>163</td>
<td>164</td>
<td>165</td>
<td>166</td>
<td>167</td>
<td>168</td>
<td>169</td>
<td>170</td>
<td>171</td>
<td>172</td>
<td>173</td>
<td>174</td>
<td>175</td>
<td>176</td>
<td>177</td>
<td>178</td>
<td>179</td>
<td>180</td>
<td>181</td>
</tr>
<tr>
<td>FREQ</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>4</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>11</td>
<td>0</td>
<td>8</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

FIGURE C-16. Table of frequency of occurrence of molecular weights produced by programme MFTABLE.
### TABLE OF OCCURRENCE OF MASS SPECTRAL PEAKS

<table>
<thead>
<tr>
<th>MASS</th>
<th>FREQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>21</td>
<td>0</td>
</tr>
<tr>
<td>22</td>
<td>0</td>
</tr>
<tr>
<td>23</td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>26</td>
<td>0</td>
</tr>
<tr>
<td>27</td>
<td>0</td>
</tr>
<tr>
<td>28</td>
<td>0</td>
</tr>
<tr>
<td>29</td>
<td>0</td>
</tr>
<tr>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td>31</td>
<td>12</td>
</tr>
<tr>
<td>32</td>
<td>8</td>
</tr>
<tr>
<td>33</td>
<td>10</td>
</tr>
<tr>
<td>34</td>
<td>4</td>
</tr>
<tr>
<td>35</td>
<td>5</td>
</tr>
<tr>
<td>36</td>
<td>9</td>
</tr>
<tr>
<td>37</td>
<td>0</td>
</tr>
<tr>
<td>38</td>
<td>0</td>
</tr>
<tr>
<td>39</td>
<td>0</td>
</tr>
<tr>
<td>40</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>MASS</th>
<th>FREQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>41</td>
<td>366</td>
</tr>
<tr>
<td>42</td>
<td>70</td>
</tr>
<tr>
<td>43</td>
<td>172</td>
</tr>
<tr>
<td>44</td>
<td>12</td>
</tr>
<tr>
<td>45</td>
<td>58</td>
</tr>
<tr>
<td>46</td>
<td>23</td>
</tr>
<tr>
<td>47</td>
<td>36</td>
</tr>
<tr>
<td>48</td>
<td>23</td>
</tr>
<tr>
<td>49</td>
<td>7</td>
</tr>
<tr>
<td>50</td>
<td>82</td>
</tr>
<tr>
<td>51</td>
<td>1985</td>
</tr>
<tr>
<td>52</td>
<td>99</td>
</tr>
<tr>
<td>53</td>
<td>1776</td>
</tr>
<tr>
<td>54</td>
<td>99</td>
</tr>
<tr>
<td>55</td>
<td>264</td>
</tr>
<tr>
<td>56</td>
<td>35</td>
</tr>
<tr>
<td>57</td>
<td>165</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>MASS</th>
<th>FREQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>58</td>
<td>5</td>
</tr>
<tr>
<td>59</td>
<td>102</td>
</tr>
<tr>
<td>60</td>
<td>412</td>
</tr>
<tr>
<td>61</td>
<td>208</td>
</tr>
<tr>
<td>62</td>
<td>53</td>
</tr>
<tr>
<td>63</td>
<td>46</td>
</tr>
<tr>
<td>64</td>
<td>199</td>
</tr>
<tr>
<td>65</td>
<td>207</td>
</tr>
<tr>
<td>66</td>
<td>79</td>
</tr>
<tr>
<td>67</td>
<td>411</td>
</tr>
<tr>
<td>68</td>
<td>87</td>
</tr>
<tr>
<td>69</td>
<td>77</td>
</tr>
<tr>
<td>70</td>
<td>208</td>
</tr>
<tr>
<td>71</td>
<td>24</td>
</tr>
<tr>
<td>72</td>
<td>22</td>
</tr>
<tr>
<td>73</td>
<td>17</td>
</tr>
<tr>
<td>74</td>
<td>36</td>
</tr>
<tr>
<td>75</td>
<td>29</td>
</tr>
<tr>
<td>76</td>
<td>572</td>
</tr>
<tr>
<td>77</td>
<td>90</td>
</tr>
<tr>
<td>78</td>
<td>63</td>
</tr>
<tr>
<td>79</td>
<td>36</td>
</tr>
<tr>
<td>80</td>
<td>172</td>
</tr>
<tr>
<td>81</td>
<td>28</td>
</tr>
<tr>
<td>82</td>
<td>50</td>
</tr>
<tr>
<td>83</td>
<td>80</td>
</tr>
<tr>
<td>84</td>
<td>253</td>
</tr>
<tr>
<td>85</td>
<td>72</td>
</tr>
<tr>
<td>86</td>
<td>120</td>
</tr>
<tr>
<td>87</td>
<td>70</td>
</tr>
<tr>
<td>88</td>
<td>80</td>
</tr>
<tr>
<td>89</td>
<td>63</td>
</tr>
<tr>
<td>90</td>
<td>36</td>
</tr>
<tr>
<td>91</td>
<td>217</td>
</tr>
<tr>
<td>92</td>
<td>285</td>
</tr>
<tr>
<td>93</td>
<td>44</td>
</tr>
<tr>
<td>94</td>
<td>249</td>
</tr>
<tr>
<td>95</td>
<td>22</td>
</tr>
<tr>
<td>96</td>
<td>143</td>
</tr>
<tr>
<td>97</td>
<td>30</td>
</tr>
<tr>
<td>98</td>
<td>59</td>
</tr>
<tr>
<td>99</td>
<td>39</td>
</tr>
<tr>
<td>100</td>
<td>59</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>MASS</th>
<th>FREQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>101</td>
<td>79</td>
</tr>
<tr>
<td>102</td>
<td>87</td>
</tr>
<tr>
<td>103</td>
<td>445</td>
</tr>
<tr>
<td>104</td>
<td>83</td>
</tr>
<tr>
<td>105</td>
<td>290</td>
</tr>
<tr>
<td>106</td>
<td>53</td>
</tr>
<tr>
<td>107</td>
<td>208</td>
</tr>
<tr>
<td>108</td>
<td>24</td>
</tr>
<tr>
<td>109</td>
<td>36</td>
</tr>
<tr>
<td>110</td>
<td>17</td>
</tr>
<tr>
<td>111</td>
<td>19</td>
</tr>
<tr>
<td>112</td>
<td>49</td>
</tr>
<tr>
<td>113</td>
<td>79</td>
</tr>
<tr>
<td>114</td>
<td>150</td>
</tr>
<tr>
<td>115</td>
<td>411</td>
</tr>
<tr>
<td>116</td>
<td>77</td>
</tr>
<tr>
<td>117</td>
<td>106</td>
</tr>
<tr>
<td>118</td>
<td>45</td>
</tr>
<tr>
<td>119</td>
<td>120</td>
</tr>
<tr>
<td>120</td>
<td>106</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>MASS</th>
<th>FREQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>121</td>
<td>110</td>
</tr>
<tr>
<td>122</td>
<td>8</td>
</tr>
<tr>
<td>123</td>
<td>20</td>
</tr>
<tr>
<td>124</td>
<td>7</td>
</tr>
<tr>
<td>125</td>
<td>41</td>
</tr>
<tr>
<td>126</td>
<td>35</td>
</tr>
<tr>
<td>127</td>
<td>27</td>
</tr>
<tr>
<td>128</td>
<td>48</td>
</tr>
<tr>
<td>129</td>
<td>572</td>
</tr>
<tr>
<td>130</td>
<td>175</td>
</tr>
<tr>
<td>131</td>
<td>279</td>
</tr>
<tr>
<td>132</td>
<td>103</td>
</tr>
<tr>
<td>133</td>
<td>468</td>
</tr>
<tr>
<td>134</td>
<td>24</td>
</tr>
<tr>
<td>135</td>
<td>197</td>
</tr>
<tr>
<td>136</td>
<td>30</td>
</tr>
<tr>
<td>137</td>
<td>33</td>
</tr>
<tr>
<td>138</td>
<td>33</td>
</tr>
<tr>
<td>139</td>
<td>39</td>
</tr>
<tr>
<td>140</td>
<td>39</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>MASS</th>
<th>FREQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>141</td>
<td>39</td>
</tr>
<tr>
<td>142</td>
<td>48</td>
</tr>
<tr>
<td>143</td>
<td>211</td>
</tr>
<tr>
<td>144</td>
<td>58</td>
</tr>
<tr>
<td>145</td>
<td>175</td>
</tr>
<tr>
<td>146</td>
<td>102</td>
</tr>
<tr>
<td>147</td>
<td>1193</td>
</tr>
<tr>
<td>148</td>
<td>258</td>
</tr>
<tr>
<td>149</td>
<td>285</td>
</tr>
<tr>
<td>150</td>
<td>27</td>
</tr>
<tr>
<td>151</td>
<td>46</td>
</tr>
<tr>
<td>152</td>
<td>30</td>
</tr>
<tr>
<td>153</td>
<td>29</td>
</tr>
<tr>
<td>154</td>
<td>53</td>
</tr>
<tr>
<td>155</td>
<td>71</td>
</tr>
<tr>
<td>156</td>
<td>86</td>
</tr>
<tr>
<td>157</td>
<td>106</td>
</tr>
<tr>
<td>158</td>
<td>56</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>MASS</th>
<th>FREQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>159</td>
<td>160</td>
</tr>
<tr>
<td>160</td>
<td>160</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>MASS</th>
<th>FREQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>161</td>
<td>63</td>
</tr>
<tr>
<td>162</td>
<td>24</td>
</tr>
<tr>
<td>163</td>
<td>52</td>
</tr>
<tr>
<td>164</td>
<td>34</td>
</tr>
<tr>
<td>165</td>
<td>49</td>
</tr>
<tr>
<td>166</td>
<td>36</td>
</tr>
<tr>
<td>167</td>
<td>22</td>
</tr>
<tr>
<td>168</td>
<td>22</td>
</tr>
<tr>
<td>169</td>
<td>97</td>
</tr>
<tr>
<td>170</td>
<td>80</td>
</tr>
<tr>
<td>171</td>
<td>75</td>
</tr>
<tr>
<td>172</td>
<td>82</td>
</tr>
<tr>
<td>173</td>
<td>155</td>
</tr>
<tr>
<td>174</td>
<td>106</td>
</tr>
<tr>
<td>175</td>
<td>89</td>
</tr>
<tr>
<td>176</td>
<td>62</td>
</tr>
<tr>
<td>177</td>
<td>51</td>
</tr>
<tr>
<td>178</td>
<td>32</td>
</tr>
<tr>
<td>179</td>
<td>162</td>
</tr>
</tbody>
</table>

**FIGURE C-17.** Table of frequency of occurrence of mass spectral ions produced by programme MFTABLE.
<table>
<thead>
<tr>
<th>MASS</th>
<th>FREQUENCY</th>
<th>NIH ID. NUM</th>
<th>IDENT NO</th>
</tr>
</thead>
<tbody>
<tr>
<td>51</td>
<td>50</td>
<td>4344</td>
<td>123000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>36906</td>
<td>162001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40358</td>
<td>166000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6488</td>
<td>166001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>70</td>
<td>166003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2439</td>
<td>179001</td>
</tr>
<tr>
<td>52</td>
<td>36</td>
<td>4344</td>
<td>123000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>38107</td>
<td>162000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>36906</td>
<td>162001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>189</td>
<td>172007</td>
</tr>
<tr>
<td></td>
<td></td>
<td>183</td>
<td>195000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>198002</td>
<td></td>
</tr>
<tr>
<td>53</td>
<td>35</td>
<td>2392</td>
<td>74000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4739</td>
<td>86000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4755</td>
<td>88000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5987</td>
<td>100000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5812</td>
<td>108000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2389</td>
<td>108001</td>
</tr>
<tr>
<td>54</td>
<td>34</td>
<td>5995</td>
<td>164000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2437</td>
<td>165000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6532</td>
<td>172002</td>
</tr>
<tr>
<td></td>
<td></td>
<td>169</td>
<td>172007</td>
</tr>
<tr>
<td></td>
<td></td>
<td>53</td>
<td>172008</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2057</td>
<td>180001</td>
</tr>
<tr>
<td>55</td>
<td>33</td>
<td>2570</td>
<td>248009</td>
</tr>
<tr>
<td></td>
<td></td>
<td>43487</td>
<td>256000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>244</td>
<td>288001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>188</td>
<td>301001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>35849</td>
<td>418002</td>
</tr>
</tbody>
</table>

**TABLE OF PEAKS/SPECTRUM NUMBERS**

**FIGURE C-18. Listing of identification numbers**
of Big15 MS which contain an ion of stated m/e.
APPENDIX D

CLINICAL SUMMARIES OF PATIENTS STUDIED IN THIS WORK
Prenatal Diagnosis - V.T. - Methylmalonic Acidemia

Amniocentesis was performed at 15 weeks gestation on a woman whose two previous children were affected with methylmalonic acidemia which was not diagnosed till they were over two years of age. A report on these children has appeared. One child died at the age of 3 years 2 months, The other is still living and excretes up to 3g MMA(I) per day. The enzyme defect was shown to be at the methylmalonyl CoA mutase apoenzyme with normal cobalamin metabolism. The children were not B₁₂ responsive although a mild response was shown to very large amounts of added hydroxycovalamin in cultured cells. The surviving child is well and developing on a self selected low protein diet and has slightly below average mental development (IQ is in the low normal range.)

Enzyme studies and amniotic fluid MMA(I) levels predicted that the foetus was not affected and the pregnancy was allowed to proceed to delivery which occurred at term. Urine samples from the baby collected during the first week showed no increase in MMA(I) and thus confirmed the diagnosis. The baby is well and normal but did suffer from an episode of haemolytic-uremic syndrome at 1 year of age.

A further pregnancy undertaken by this couple has been monitored using amniotic fluid MMA(I) levels and enzyme assays. These both predicted an unaffected foetus and the pregnancy was allowed to proceed to delivery. MMA(I) was present in normal amounts in samples collected during the neonatal period confirming the in utero diagnosis. The baby has been healthy and well since birth.

Prenatal Diagnosis - G.P. - Methylmalonic Acidemia

Amniocentesis was performed at 16 weeks gestation on a woman whose second child was affected with methylmalonic acidemia. This was diagnosed at 7 months of age as a defect in the methylmalonyl CoA mutase enzyme following investigations of the child for failure to thrive.

Analysis of the amniotic fluid showed a normal level of methylmalonic acid present. Examination of the cultured amniotic fluid cells showed that they also had normal levels of methylmalonyl CoA mutase activity. The foetus was predicted to be unaffected and pregnancy was allowed to proceed
to delivery which occurred at term. Urine samples from the baby collected during the first weeks of life showed no increase in methylmalonic acid and thus confirmed the in utero prediction.

PRENATAL DIAGNOSIS - V.M. - GLUTARIC ACIDURIA

Amniocentesis was performed at 15 weeks gestation on a woman whose only other child had been diagnosed as having glutaric aciduria seven weeks previously at the age of 18 months. The child had appeared to be normal until two weeks before when, following an upper respiratory tract infection and some vomiting, she developed an acute dyskinetic reaction with dystonia and tremor and lost the ability to stand, sit and crawl. Recovery from the encephalopathy was slow and incomplete, leaving residual dystonia and athetosis. Organic acid chromatography of the urine showed glutaric aciduria (22mg/mg creatinine) without 3-hydroxyglutaric aciduria or glutaric aciduria and glutaryl-CoA dehydrogenase activity was deficient in both peripheral leucocytes and cultured fibroblasts.

The diagnosis of an affected foetus was predicted seven weeks after amniocentesis and the pregnancy was terminated at 22½ weeks by intra-amniotic injection of prostaglandins with the foetus being born dead 20 hours after induction. Autopsy was performed 4 hours after delivery; half the brain was fixed in buffered formalin for histological examination and the liver, kidney and remainder of the brain were frozen at -80° and maintained at that temperature until enzyme analysis was performed.

PERINATAL DIAGNOSIS -M.E. - DICARBOXYLIC ACIDURIA

M.E. is the seventh child of unrelated Australian parents. Of his six siblings, two had died suddenly during episodes of gastroenteritis, when following two days of diarrhoea and vomiting with apparently little systemic effect, they had become very drowsy for a few hours and abruptly died.

M.E. is the second of non-identical twins born at term following a normal pregnancy. There were no neonatal problems and he had developed normally. His weight at six months was at the 75th percentile and his
height was at the 97th percentile. Two days prior to admission the patient became febrile and began to have mild diarrhoea. Persistent vomiting continued for the next forty-eight hours and a few hours before admission the child had become increasingly drowsy. On admission the patient was hypothermic (axillary temperature 35.2°C) and was noted to have 8 cm of firm hepatomegaly below the costal margin. He had normal blood pressure and pulse but was semi-conscious and peripherally cyanosed but was not clinically dehydrated. He was slightly hypertonic but no other CNS or general clinical abnormality was noted. On admission the following laboratory results were obtained: blood glucose 1.4 mM\textsuperscript{-1}, blood pH 7.386; pO\textsubscript{2} 108.4 mm Hg; pCO\textsubscript{2} 25.4 mm Hg; base excess - 8.6 mM\textsuperscript{-1}, bicarbonate 17.3 mM\textsuperscript{-1}; sodium 129 mM\textsuperscript{-1}, potassium 4.4 mM\textsuperscript{-1}, urea 17 mM\textsuperscript{-1}, serum aspartate aminotransferase 68 U/l (Normal < 20 U/l); serum alanine aminotransferase 31 U/l (normal < 20 U/l). Haematological studies were normal and blood, urine and CSF cultures were negative.

Urinalysis showed mild ketonuria but was otherwise negative. Urinary amino acids were within the normal range at the time of the attack.

The child was resuscitated with intravenous glucose and his conscious state returned almost to normal within twenty-four hours. Mild irritability continued for the next few days. Normal feeds were reintroduced after twenty-four hours and the child was discharged after ten days with 6 cm of firm hepatomegaly persisting below the costal margin and no changes in the serum transaminases. The hepatomegaly and raised transaminases resolved during the next six weeks and the child has developed normally since that time.

At eight months of age a fasting study showed early development of dicarboxylic aciduria with no clinical effects and at ten months a fat load study also had no clinical effects. The fasting study was repeated at two years of age and despite the absence of clinical effects gross dicarboxylic aciduria recurred.

**PERINATAL DIAGNOSIS - R.Z. - DICARBOXYLIC ACIDURIA**

R.Z. is the second child of unrelated parents. He was born at 35
weeks to a mother with a previous bad obstetric history. The breech birth was rapid and the child showed no respiratory distress but developed mild neonatal jaundice (74 µM⁻¹) which resolved without treatment. He developed normally and at eight months his milestones were satisfactory and his weight was on the 75th percentile.

At fourteen months he contracted a bilateral otitis media and infected throat and began to vomit. He was treated with antibiotics but was unable to retain fluids and continued vomiting for two days when treatment with Stemitil failed to control the vomiting. He continued to vomit and his condition deteriorated and he was admitted on the fourth day. Examination of the child showed that he was mildly dehydrated, with no diarrhoea or raised temperature, normal heart, blood pressure and urinalysis but having an enlarged liver (5cm below right costal margin). Chest x-ray revealed a coin wedged in the oesophagus which had been present for a number of days and was later removed.

The child's condition suddenly deteriorated very rapidly. He lapsed into semi-consciousness, had obvious signs of peripheral shutdown and dextrosticks test was negative for sugar. Blood gas analysis showed a pH of 7.25, pO₂ of 105 mm Hg and pCO₂ of 11 mm Hg. After the onset of coma the pH was 7.15, pO₂ 60 mm Hg, and pCO₂ 15 mm Hg with a base excess of -18. Treatment was begun with intravenous dextrose but because of rapidly deepening coma and marked restlessness, paralysis of the respiratory muscles with curare and intubation with assisted ventilation was begun.

The patient's condition remained comatosed and acidotic for 5 days with the pH varying between 7.24 and 7.27 despite treatment with base. An electroencephalogram taken at that time showed a deep sleep record. Treatment was supplemented with lasix, antacid and dexamethasone but the serum calcium dropped dramatically during day five. On day six despite pethidine given to control restlessness the child began to fit in short generalised convulsions. The electroencephalogram showed mixed variable patterns and indicated severe metabolic encephalopathy. The metabolic acidosis was over-corrected and the pH rose to 7.48 and pCO₂ to 35 mm Hg with a base excess of +10. The blood calcium was still low but towards the end of the day a few purposeful movements occurred and the pH began to return to normal despite intermittent fits.
Fitting had ceased by day seven and normal consciousness began to return. A liver biopsy done at this time showed considerable lipid accumulation in a non-specific macroglobular pattern commonly seen in sick children but failed to show the microvesicularity, mitochondrial and other histological changes seen in Reye's syndrome.\textsuperscript{496,497} By day eight the child was able to breathe by himself and began to open his eyes and from that time the improvement was rapid until discharge on day sixteen.

Since that time the child has remained well except for some minor illnesses. At 20 months of age the child was admitted for a fat load study. The normal daily intake of milk fat was given at the end of a fifteen hour fast. The patient became ill and vomited after twenty minutes. He became very ketotic over the next few hours. Intravenous glucose was required for resuscitation. At twenty-seven months of age the child was fasted for fifteen hours. During this time he became drowsy and ketogenic and began to vomit after 14 hours.

**PERINATAL DIAGNOSIS - AM - DICARBOXYLIC ACIDURIA**

A.M. was born at term following a normal pregnancy with a birth weight of 3.70 Kg. Lethargy was noted from twenty-four hours of age following attempts at breast feeding, and the child was tube fed with expressed breast milk. Persistently poor feeding, repeated hypertonic posturing and vomiting led to transfer to a pediatric hospital for further investigation. On admission he had lost weight (weight was 3.25 Kg) and was noted to be lethargic, with poor Moro reflex and no other CNS findings. There were no other clinical findings. Blood gases, serum calcium, and serum magnesium were normal and blood sugar was 2.7 mmol\textsuperscript{-1}.CSF, urine and blood culture proved sterile and haematological investigations were normal. The problems continued during the next week but when normal feeding began the child was discharged apparently normal. A.M. subsequently thrived with normal developmental milestones on a normal diet until eleven months of age.

Four days prior to admission A.M. developed mild vomiting and diarrhoea. Two days before admission he became lethargic and was admitted to a peripheral hospital. The child initially improved with intravenous fluids but over the next forty-eight hours became increasingly unresponsive, and
developed 4 cm of hepatomegaly. On transfer to Royal Alexandra Hospital for Children he was unresponsive to most stimuli and hypotonic. He had no other CNS abnormalities but the firm liver edge was 4 cm below the costal margin and clinically he was markedly dehydrated.

Biochemical investigation showed a blood sugar of 1.1 mMl\(^{-1}\). Blood gases were pH 7.396, base excess -9.2 mMl\(^{-1}\), standard bicarbonate 16.5 mMl\(^{-1}\) and pCO\(_2\) 25.1 mm Hg. Plasma sodium was 143 mMl\(^{-1}\), plasma potassium 4.2 mMl\(^{-1}\), plasma urea 14.0 mMl\(^{-1}\) and haemoglobin 10.8 g/100 ml. The child responded immediately to intravenous glucose given on arrival, becoming more alert and active. However he remained drowsy during the next twenty-four hours with a gradual return to normal activity and normal conscious state. During this time he was maintained on intravenous glucose and other fluids for rehydration. Serum transaminases were initially raised but ammonia and lactate were both normal. The electrolytes remained normal and urea gradually returned to normal on rehydration. Blood, CSF and urine culture proved sterile. Urinary investigations showed fat globules, ketonuria and high levels of adipic acid on admission but these resolved during the first twenty-four hours. Following the initial recovery the child remained clinically well but hepatomegaly (4 to 6 cms) and mildly raised serum transaminases persisted, but slowly resolved during the next two weeks. Serum cholesterol was normal with mildly raised serum triglycerides (1.3 mMl\(^{-1}\)). Serum alpha-1-antitrypsin, copper and ceruloplasmin were normal and the child was discharged after two weeks.

At thirteen months of age the child was readmitted for investigation. He was at the 75\(^{th}\) percentile for height and weight and was clinically well. After a fifteen hour fast the child received a fat load and became mildly unwell and drowsy with repeated vomiting. His blood glucose fell to 2.7 mMl\(^{-1}\) and he revived after a few hours of intravenous glucose and fluids.

The child continued to thrive physically on a normal diet and he has remained developmentally normal. At sixteen months of age he was re-assessed and subjected to a twenty-four hour fast during which normal glycemia was maintained and he was clinically well. Mild ketonuria developed at fifteen hours of fasting and adipic and other dicarboxylic acids were grossly raised.
Since that time he has continued to thrive and his development remains at about the 75th percentile.

PERINATAL DIAGNOSIS - Z.K. - PROPIONIC ACIDEMIA

The patient (Z.K.) was a newborn boy of half first cousin Turkish parents. Two sisters had died aged 2 days and 2 months in Turkey. A 7 year old sister and a 4 year old brother are normal.

Z.K. was born at term after a normal pregnancy, labour and delivery. Birth weight was 2.820kg. The infant was initially fed a milk formula containing 2.8g protein per 100 ml, but he was noted to be unwell at 48 hours with depressed conscious state and acidotic breathing, and was said to smell ketotic. Mild jaundice was present. Laboratory investigation revealed a marked metabolic acidosis (pH 7.06, bicarbonate 4 mMl$^{-1}$ pCO$_2$ 14 mm Hg and base excess -25) and the nitroprusside test for urine for ketones was strongly positive. Intravenous sodium bicarbonate was given. The infant was transferred to Royal Children's Hospital, Melbourne, at 60 hours of life.

Initial investigations confirmed the metabolic acidosis and demonstrated a plasma ammonia of 569μM ml$^{-1}$ (Reference range < 100μM ml$^{-1}$). High voltage electrophoresis showed elevated lysine and cystine in urine and elevated lysine and decreased alanine in serum. Thin layer chromatography of urinary keto acids, as the DNP derivatives showed increased acetone, butanone and pentanone. Other serum results were as follows: sodium 152 mM$^{-1}$, potassium 4.4 mM$^{-1}$, chloride 110 mM$^{-1}$, calcium 1.65 mM$^{-1}$, magnesium 1.0 mM$^{-1}$, urea 11.4 mM$^{-1}$, glucose 4.3 mM$^{-1}$, pO$_2$ 74 mm Hg, and total bilirubin 140 μMl$^{-1}$. The initial haematology was normal but later leucopenia (3.2 x 10$^3$ mm$^{-3}$) and thrombocytopenia (15 x 10$^3$ mm$^{-3}$) appeared. A urine screen for short chain fatty acids was positive for propionic acid and screening for urinary organic acids showed elevated lactic, 3-hydroxybutyric,acetoacetic and 4-hydroxyphenyllactic acids plus many other elevated unidentified acids.

Management of Z.K. consisted of intravenous 10% dextrose, peritoneal
dialysis from 65 hours of life, exchange transfusions at 90 and 100 hours of life and megavitamin therapy including biotin (100 mg) orally immediately after the second exchange transfusion. Rapid improvement in the infant's clinical condition followed. Dietary protein was introduced and increased to 1.5 gm/Kg/day on the eleventh day of life. Biotin was continued at a dose of 10 mg/day orally. The impression of biotin responsiveness gained during the initial illness was to have been formally tested later, however, a further metabolic crisis developed after two months of relatively good progress on biotin and protein restriction. Hyperammonemia (1700 μM·l⁻¹) and propionic acidemia proved uncontrollable and he died at the age of three months.

PERINATAL DIAGNOSIS - E.B. - PROPIONIC ACIDEMIA

E.B. was born at term to a healthy 18 year old unmarried mother and there was no family history available. Labour and delivery were uneventful and birth weight was 4.280 kg. He made very satisfactory progress in the first week of life and was discharged to a children's home.

He was admitted to Royal Children's Hospital, Melbourne, at the age of fourteen days because of drowsiness and hypotonia. These features were accompanied by myclonic jerking and some vomiting and diarrhoea. Examination showed no apparent physical abnormalities with some minor liver enlargement (2 cm below RCM). During the next month there were several episodes of gram negative septicemia with peripheral circulatory failure and deterioration of conscious state. Vomiting and diarrhoea were fairly constant problems. During this phase electrolyte estimations never showed more than a mild acidosis and the pH did not drop below 7.26 with a base excess of -6. Myclonic twitches occurred whenever the child was ill. The episodes of septicemia were treated with antibiotics. Biochemical investigations showed massive amounts of urinary glycine and raised serum glycine (4.1mg/100ml, 40 x normal) with raised blood ammonia (262μg/100 ml, 2.5 x normal). Neutropenia was a striking feature and a diagnosis of primary hyperglycinaemia was made but the absence of significant acidosis suggested significant differences to the type described by Nyhan and Childs. A number of studies of glycine metabolism were performed but no further information was found.
Maintenance of the child based on this diagnosis consisted of very low protein feeding with maximum calories from other sources. The patient showed severe sugar intolerance and malabsorption and medium chain triglycerides were used as the major calorie source with a gradual increase in glucose content. Initial protein levels were restricted to 0.5mg/Kg/day but after some time this was increased to 1g/kg/day.

Over the next several weeks there were wide variations in the clinical state and the declines had no apparent reason. Serum and urine glycine levels had returned to the normal range when low protein feeding was instituted and did not ever rise above the normal range again. During this period the blood ammonia was continually rising.

At the age of seven months an episode leading to a severe decline occurred, precipitated by a viral infection. During this episode the blood ammonia failed to rise but the child became severely acidic. This pattern of severe episodes of acidosis with massive ketonuria marked each decline in condition of the child for the rest of his life. Identification of the urinary acids gave equivocal results. Chemical tests suggested that methylmalonic acid was present but gas chromatography suggested the major compound present was acetoacetic acid and not methylmalonic acid. This conflict of results remain unresolved.

At the age of eight months a particularly severe illness occurred which was only controlled with IV fluids and massive amounts of bicarbonate. After this time he entered a fairly even period of good health and developed to about 60% of normal.

At sixteen months of age an attempt was made to feed him a normal diet. This produced initially only a very slight metabolic acidosis which was compensated. For three weeks he remained in good health but then became acutely acidotic and after restabilisation the normal diet was withdrawn.

His final illness began with a viral infection which led to massive acidosis which was difficult to control. He never regained physical strength and remained floppy and lethargic till his death. Aspiration of milk led to a persistent chest infection which was never satisfactorily
cleared. Massive amounts of vitamins and trace elements were given but no improvement in clinical condition occurred. He died at age twenty two months.

During this prolonged illness it was clear that protein overload either by exogenous protein or indigenous protein released during acute infections resulted in acute intoxication. During the first 6-7 months this state of cerebral intoxication was not accompanied by any acidosis but hyperglycinaemia and hyperammonemia were constant features. From 7 months until death each of the many episodes of deterioration were accompanied by severe ketoacidosis and the absence of significant hyperammonemia or hyperglycinaemia. All episodes of ketoacidosis could be explained by protein overload, endogenous or exogenous. The most probable diagnosis is a defect in the pathway of some organic acid.
Garrod, A.E., *Lancet* 2, 1, 73, 142, 214 (1908)


27 Frimpter, G.W., Science, 149, 1095 (1965)
40 Shih, V.E., Laboratory Techniques for the Detection of Hereditary Metabolic Disorders, CRC Press Pty. Ltd., Cleveland, 1973
41 Dent, C.E. Lancet, 2, 637 (1946)
42 Dent, C.E., Biochem J., 43 169 (1948)
44 James, A.T. and Martin, A.J.P., Analyst, 77, 915 (1952)
45 Wien, W. Ann. Phys. Chem. 65 440 (1898)
46 Thompson, J.J., Rays of Positive Electricity, Longmans Green, New York, 1913


90 Budde, W.L. and Eichelberger, J.W. J. Chromatogr, 134, 147 (1977)
93 Issachar, D. and Yincn, J. Biomed Mass Spectrom, 6, 47 (1979)
94 Husek, P., and Macek, K., J. Chromatogr, 113, 139 (1975)
103 Williams, R.J. et al. Biochemical Institute Studies IV, Individual Metabolic Patterns and Human Disease: An Exploratory Study Utilising Predominantly Paper Chromatographic Methods, U. of Texas Publication No. 5109, U. Texas, Austin, 1951
104 Williams, R.J., Biochemical Individuality: The Basis for the Genetotrophic Concept, U. Texas Press, Austin, 1956
114 Liebich, H.M., J. Chromatogr 112, 551 (1975)
124 Reid, E., Analyst (Lond) 101, 1, (1976)
163 Kuksis, A., J. Chromatogr., 143, 3 (1977)
167 Drozd, J., J. Chromatogr., 113, 303 (1975)
169 Vouros, P. and Harvey, D.J., Anal. Chem., 45, 7 (1973)


Gloor, R. and Leidner, H., Chromatographia, 2, 618 (1976)


Rasmussen, K., Ando, T., Nyham, W.L., Hull, D., Cottom, D., Kilroy, A.W. and Wadlington, W., J. Pediat., 81, 970 (1972)


212 Umeh, E.O., J. Chromatogr., 56, 29 (1971)
215 Perry, T.L. and Hansen, S. in Application of Gas Chromatography-Mass Spectrometry to the Investigation of Human Disease, McGill University-Montreal Children's Hospital, Montreal, 1974, p.89
220 Armstrong, M.O. and Shaw, K.N.E., J. Biol. Chem., 225, 265 (1975)
228 Vessman, J., J. Chromatogr., 100, 153 (1974)


Räihä, N.C.R., Pediatrics, 32, 1025 (1963)


Kim, Y.J. and Felig P., Metabolism, 21, 507 (1972)


Biemann, K. and McMurray, W., Tetrahedron Lett., 647 (1965)
Biemann, K. and Fennessey, P.V., Chimia, 21, 226 (1967)
Nyhan, W.L. (ed), Heritable Disorders of Amino Acid Metabolism: Patterns of Clinical Expression and Genetic Variation, John Wiley and Sons, New York, 1974
Pettersen, J.E., Diabetes, 23, 16 (1974)
Tanaka, K., J. Biol. Chem., 247, 7465 (1972)


Barnes, N.D., Hull, D., Balgobin, L. and Gompertz, D., Lancet, 2, 244 (1970)


Gregersen, N., Brandt, N.J., Christensen, E., Grøn, I., Rasmussen, K. and Brandt, S., J. Pediat., 90, 740 (1977)


Epstein, C.J. and Golbus, M.S., Amer. Scientist, 65, 703 (1977)

Culliton, B.J., Science, 190, 537 (1975)


398 Bevis, D.C.A., Lancet, 2, 443 (1950)
401 Carrel, A. in Nobel Lectures: Physiology or Medicine, 1942-62, Elsevier, Amsterdam, 1964
404 Hsai, D.Y.Y., Metabolism, 19, 308 (1970)
405 Millman, W.J., Genet., 2, 259 (1971)
408 Nadler, H.L., Pediat., 42, 912 (1968)
409 Nadler, H.L., J. Pediat., 74, 132 (1969)
410 Valenti, C., Schutta, E.J. and Kehaty, T., Lancet, 2, 220 (1968)
448 Nichols, J., Lancet, 1, 83 (1970)
460 Cox, E.V. and White, A.M., Lancet, 2, 853 (1962)


Kyllerman, M. and Steen, G., Neuropediatric, 8, 397 (1977)


Ando, T., Rasmussen, K., Nyham, W.L. and Hull, D., Proc. Nat. Acad. USA, 69, 2807 (1972)


Stokke, O., Eldjarn, L., Jellum, E., Pande, H. and Waaler, P.E., Pediatrics, 49, 726 (1972)


Brandt, N.J., Gregersen, N., Christensen, E., Grøn, I.H. and Rasmussen, K., J. Pediat., 94, 669 (1979)

Goodman, S.I., personal communication


499 Verkade, P.E. and Van Der Lee, J., Biochem. J., 28, 31 (1934)


502 Verkade, P.E., Chem. Ind. Lond., 57, 704 (1938)

503 Preiss, B. and Bloch, K., J. Biol. Chem., 239, 85 (1964)


505 Björkhem, I., J. Biol. Chem., 251, 5259 (1976)


514 Watts, R.W.E., personal communication

515 Rose, W.C., J. Pharmacol. Exp. Ther., 42, 147 (1924)


521 Holmqquist, B. and Ingvar, D.H., Experientia, 13, 331 (1957)


526 Childs, B. and Nyhan, W.L., Pediatrics, 33, 403, (1964)
527 Gerritsen, T., Kaveggia, E. and Waisman, H.A., Pediatrics, 36, 882 (1965)
528 Menkes, J.H., J. Pediat., 69, 413 (1966)
529 Hsiè, Y.E., Scully, K.J. and Rosenberg, L.E., Lancet, 1, 757 (1969)
533 Wolf, B., Paulsen, E.P. and Hsiè, Y.E., J. Pediat., 95, 563 (1979)
542 Snyderman, S.E., Sansarico, C., Norton, P., Phansalkar, S.V., Pediatrics, 50, 925 (1972)
547 Fu, S-C.J. and Mak, D.H.S., J. Chromatogr., 54, 205 (1971)
548 Bondi, S. and Eissler, F., Biochem. Z., 23, 499 (1910)
551 Neilson, A.J. and Houlihan, W.J., Org. Reactions, 16, 80
556 Hauser, C.R. and Hudson, B.E., Org. React., 1, 267 (1942)
557 Krüger, R.C., J. Amer. Chem. Soc., 74, 5536 (1952)
564 Shriner, R.L., Org. React., 1, 1 (1942)
570 Handbook of Chemistry and Physics, 70th Edn., Chemical Rubber Publishing Co., Cleveland, 1975
573 Beilstein, Bd 4, Suppl.3, 1155
574 Beilstein, Bd 4, Suppl.3, 1159
578 Beilstein, Bd III, Suppl.1, 423
580 CRC Handbook of Tables for Organic Compound Identification, 3rd Edn., CRC Press Ltd., Cleveland, 1967

581 Beilstein, Bd 1, 650
582 Beilstein, Bd 1, 669
583 Beilstein, Bd 1, Suppl.III, 2811
584 Beilstein, Bd 3, Suppl.II, 440
585 Beilstein, Bd 3, 701
ACKNOWLEDGEMENTS
The author wishes to thank his supervisor, Professor Berthold Halpern for his continual guidance and encouragement throughout this work and for the privilege of working with him. The author is also indebted to Dr. Roger Truscott who collaborated with him in some of the work. Thanks are also due to Associate Professor Peter Bolton who acted as supervisor for six months and as a consultant for the computer treatment of the mass spectral libraries.

The author would also like to thank John Korth and Larry Hick for recording the numerous mass spectra and for maintenance of the gas chromatographs. Thanks are also due to the laboratory and workshop staff for their help with supplies and equipment. The author is also indebted to Quentin Addison and the Computer Centre staff for their cooperation and assistance.

The author would like to thank Dr. Allan Snoswell (Waite Agricultural Research Institute, University of Adelaide) for carnitine measurements, Dr. Jerry Mahoney (Department of Human Genetics, Yale University, U.S.A.) for enzyme studies on patients with methylmalonic acidemia and Dr. S.I. Goodman for his collaboration in the prenatal diagnosis of glutaric aciduria.

The author is deeply indebted to Dr. Bridget Wilcken from the Oliver Latham Laboratories, Sydney who was responsible for initiating the prenatal studies of the patients under her care and was associated with the studies of the dicarboxylic aciduria patients. Thanks are also due to Dr. Grahame Wise (Prince of Wales Hospital, Randwick) for his cooperation in the provision of samples for the prenatal diagnosis of glutaric aciduria in his patient and to Dr. F. Gunseit (Prince of Wales Hospital, Randwick) and Drs. Martin Silink and Hillary Griffiths (Royal Alexandra Hospital for Children, Camperdown) for their part in sample collection and fasting studies of the dicarboxylic aciduria patients under their care. The author would also like to thank Professor David Danks (Genetics Research Unit, Royal Melbourne Children's Hospital, Melbourne) for initiating the studies of the patients with propionic acidemia and to thank Judy Hammond and staff for their part in collecting samples, preliminary screening and measurement of serum propionate.
The author wishes to thank his fellow research students Albert, Anne, Danny, Gary, Chris, Peter, Wayne, Tony, John, Tim and Eric together with his many friends for their encouragement and friendship throughout this work.

Thanks are due to Marjorie Hall for typing the drafts of this thesis and the author would specially thank Jean Maxwell-Wright for typing the final copy and her assistance in all aspects of producing this thesis.

The author gratefully acknowledges the receipt of the Ampol Postgraduate Award and a Commonwealth Postgraduate Award during the tenure of which this work was carried out.

Finally, the author gratefully acknowledges the loving support, patience, understanding and forebearance of his wife Wendy, and his children during the various stages of this project.
PUBLICATIONS
Antenatal Diagnosis of Glutaric Acidemia

Stephen I. Goodman and Delfido A. Gallegos

Department of Pediatrics, University of Colorado Health Sciences Center, Denver, Colorado

Christopher J. Pullin, Berthold Halpern and Roger J.W. Truscott

Department of Chemistry, The University of Wollongong, Wollongong, New South Wales, Australia

Grahame Wise

The Prince of Wales Children's Hospital, Randwick, New South Wales, Australia

Bridget Wilcken

Oliver Latham Laboratories, North Ryde, New South Wales, Australia

Eamonn D. Ryan and Donald T. Whalen

Departments of Pediatrics and Pathology, McMaster University Medical Center, Hamilton, Ontario, Canada

Supported in part by Maternal and Child Health Special Project No. 252 and National Institutes of Health Grant HD-04024
Antenatal Diagnosis of Glutaric Acidemia

Introduction

Glutaric acidemia is an inborn error of amino acid metabolism which is characterized clinically by an intermittently progressive dyskinetic syndrome in childhood, chemically by the excretion of large amounts of glutaric and (usually) 3-hydroxyglutaric acids in urine, and pathologically by chronic neuronal loss in the putamen and caudate nuclei (1-8). The disorder is due to deficiency of glutaryl-CoA dehydrogenase, an enzyme in the pathway of lysine, hydroxylysine and tryptophan oxidation, and is inherited as an autosomal recessive trait (5,9).

The purpose of this communication is to document in-utero diagnosis of this disorder, which can be established by demonstrating deficiency of glutaryl-CoA dehydrogenase in cultured amniotic cells and (probably) accumulation of glutaric acid in amniotic fluid.
Case Reports

Pregnancy A

Amniocentesis was performed at 15 weeks gestation on a woman whose only previous child had been diagnosed as having glutaric acidemia some seven weeks earlier at the age of 18 months. The child had appeared to be normal until two weeks before when, following an upper respiratory tract infection and some vomiting, she developed an acute dyskinetic reaction with dystonia and tremor and lost the ability to stand, sit and crawl. Recovery from the encephalopathy was slow and incomplete, leaving residual dystonia and athetosis. Organic acid chromatography of urine showed glutaric aciduria (22.6 mg/mg creatinine) without 3-hydroxyglutaric and glutaconic aciduria, and glutaryl-CoA dehydrogenase activity was deficient in both peripheral leukocytes and cultured fibroblasts.

The diagnosis of an affected fetus was predicted seven weeks after amniocentesis and the pregnancy was terminated at 22-1/2 weeks by intra-amniotic injection of prostaglandins, with the fetus being born dead 20 hours after induction. Autopsy was performed 4 hours after delivery; half of the brain was fixed in buffered formalin for histological examination and the liver, kidney and remainder of the brain were frozen at -80°C and maintained at that temperature until enzyme analysis was performed about 5 weeks later.

Pregnancy B

Amniocentesis was performed at 15 weeks gestation on the mother of a child with glutaric acidemia who has already been reported (5); the fetus was predicted to be unaffected and pregnancy was allowed to proceed to delivery, which occurred at 41 weeks. Glutaric acid was not detected in either of two urine samples collected during the first week of life, and the activity of glutaryl-CoA dehydrogenase in fibroblasts cultured from the umbilical cord was 0.76 nmoles CO₂/hr/mg protein (Hamilton laboratory normal = 0.54 ± 0.04), confirming the in-utero
prediction. The baby, a male, did however have congenital heart disease (transposition of the great vessels, coarctation of the aorta, and patent ductus arteriosus) which required surgical correction.

Methods

The activities of glutaryl-CoA dehydrogenase in cultured amniotic cells and in tissue sonicates, and of glutamate dehydrogenase in tissue sonicates, were measured as previously described (1,10). The method used to measure tissue content of glutaric acid has also been described (7).

In the Denver and Hamilton laboratories, amniotic fluid content of glutaric acid was measured by gas chromatography using the technique of internal standardization (11); internal standards used were phenylacetic acid (Denver) and eicosane (Hamilton). Organic acids in 1 ml amniotic fluid were extracted and silylated as previously described (12) using 10 mc1 BSTFA and 2 mc1 TMCS, and 2 mc1 of the derivatized extract was chromatographed on a 10 foot column of OV-22 on 90/100 mesh Supelcoport (Supelco Inc., Bellefonte, Pa.). The carrier gas was nitrogen (30 ml/min) and the column was kept at 90° for 4 minutes before the oven temperature was raised to 280° at the rate of 8°/minute. The identity of organic acids in the extract was established by combined gas chromatography-mass spectrometry using an AEI MS-12 medium resolution mass spectrometer.

In the Wollongong (Australia) laboratory, amniotic fluid content of glutaric acid was measured as follows. Adipic acid (5 mcg), water (2 ml) and 5% sodium dodecylsulfate (0.3 ml) were added to 1 ml amniotic fluid and the solution was saturated with NaCl and adjusted to pH 9-11 with 1M NaOH. After standing at room temperature for 20 minutes the solution was extracted three times with 3 ml ethyl acetate, and the extracts were discarded. The solution was then acidified to
pH 1 with 5 M HCl and the organic acids were extracted into ethyl acetate. Pimelic acid (5 mcg) was added as an external standard and the extract was dried with MgSO₄ and evaporated under nitrogen. The dried residue was derivatized using 30 mc1 BSTFA (1 hour at 65°) and approximately 0.5 mc1 of the silylated extract was injected onto a 20m x 0.2mm (i.d.) capillary column which was wall-coated with SE-54. The inlet split was 10:1. Carrier gas was helium (2 ml/min) and temperature was programmed to rise fro 80° to 250° at 6°/minute. All peaks were identified by feeding the gc effluent through an open coupling into a Varian-MAT44 quadrupole mass spectrometer, and the concentration of glutaric acid was measured by the technique of internal standardization (internal standard = adipic acid).

Results

The results of the studies performed on the two fluids obtained by amniocentesis are shown in Table I. Both glutaric acid concentration in the fluid and activity of glutaryl-CoA dehydrogenase in cultured amniotic cells were abnormal in pregnancy A and it was decided that the pregnancy should be terminated. The concentration of glutaric acid in amniotic fluid at termination of pregnancy, also shown in Table I, had increased considerably in the intervening 7 weeks.

Table II shows the results of studies performed on the abortus and compares them to values obtained on two patients with glutaric acidemia at autopsy and on three control human fetuses ranging in gestational age from 16 to 24 weeks. The liver, kidney and brain of the abortus at-risk contained markedly increased amounts of glutaric acid and almost no glutaryl-CoA dehydrogenase activity, a pattern which is observed in tissues in glutaric acidemia but not in controls.

The cerebral hemispheres and cerebellum of the fetus were normal to both gross and microscopic examination, but there were some slight alteration in the size distribution of nuclei in the striatum, which contained many prominent and small darkly-staining nuclei.
Discussion

This report describes the first instances in which pregnancies at risk for glutaric acidemia have been monitored for this disease, and shows that affected and unaffected fetuses can be distinguished with comparative ease by measuring glutaryl-CoA dehydrogenase activity in cultured amniotic cells and the concentration of glutaric acid in the amniotic fluid. Two pregnancies were monitored; the fetus predicted to be affected had the in-utero diagnosis confirmed by determinations of glutaric acid and glutaryl-CoA dehydrogenase in tissues, while the fetus predicted to be unaffected had normal glutaryl-CoA dehydrogenase activity in umbilical cord fibroblasts and did not have glutaric aciduria on two occasions during the first week of life.

Glutaric acid may accumulate in the amniotic fluid of the affected fetus because, like many other non-amino organic acids, it is poorly reabsorbed from the glomerular filtrate and appears in fetal urine and thus in the amniotic fluid (13); a similar mechanism has been suggested as the basis of the accumulation of abnormal metabolites in the amniotic fluid of fetuses with methylmalonic acidemia (14-16), arginosuccinic acidemia (17,18) and propionic acidemia (19). Although it is technically easier to measure the concentration of glutaric acid in amniotic fluid than it is the activity of glutaryl-CoA dehydrogenase in cultured amniotic cells, in-utero diagnosis of glutaric acidemia should probably rest on the latter determination until it can be shown that glutaric acid does not accumulate in the amniotic fluid of the fetus heterozygous for the mutant allele.

The treatment of glutaric acidemia by dietotherapy, vitamin (riboflavin) supplementation, and drugs (LioresalR and valproic acid) has been only variably effective (1,3,5,8,20,21), perhaps because irreversible damage has been done to
the basal ganglia by the time the diagnosis is usually established. The observation that glutaric acid is significantly accumulated in the brain even during fetal life in this disorder raises the possibility that neuronal damage may be done even in-utero. The mild striatal changes which were noted in the affected fetus may reflect such damage or may be due only to terminal anoxia, and careful histopathological examination of the striatum in normal and affected abortuses will be necessary in the future if this issue is to be resolved.
| Table I |
|--------------------------|--------------------------|
| **Amniotic fluid** | **Amniotic cell** |
| Glutaric acid | Glutaryl-CoA dehydrogenase |
| - mcg/ml - | - nanomoles CO₂/hr/mg protein - |

**Pregnancy A**

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Glutaric acid</th>
<th>Glutaryl-CoA dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amniocentesis (15 wk)</td>
<td>4.35¹</td>
<td>not detected²</td>
</tr>
<tr>
<td>Termination (22 wk)</td>
<td>11.7¹,13.3²</td>
<td></td>
</tr>
</tbody>
</table>

**Pregnancy B**

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Glutaric acid</th>
<th>Glutaryl-CoA dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amniocentesis (15 wk)</td>
<td>not detected²,³</td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>0.28±0.11(m±sd)¹</td>
<td>1.03, 4.94²</td>
</tr>
</tbody>
</table>

¹Wollongong laboratory
²Denver laboratory
³Hamilton laboratory
Glutaric acid
(mcg/g wet weight)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>17.0</td>
</tr>
<tr>
<td>Kidney</td>
<td>70.2</td>
</tr>
<tr>
<td>Brain</td>
<td>112.0</td>
</tr>
</tbody>
</table>

Glutaryl-CoA dehydrogenase
(nmoles CO₂/hr/mg prot)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>0.15</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Glutamate dehydrogenase
(mcmoles NADH oxid/min/mg prot)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>0.38</td>
</tr>
</tbody>
</table>

1. Frontal cortex
2. Basal ganglion
3. Isolated mitochondria
Table II

<table>
<thead>
<tr>
<th>Glutaric acidemia (7,8)</th>
<th>Control Fetuses (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>330</td>
<td>96</td>
</tr>
<tr>
<td>1000</td>
<td>1330</td>
</tr>
<tr>
<td>109</td>
<td>88&lt;sup&gt;1&lt;/sup&gt;, 165&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>0&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td>0&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td>1.44&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0.45</td>
</tr>
</tbody>
</table>
Glutaric aciduria: A 'new' disorder of amino acid metabolism.

2. Gregersen, N., Brandt, N.J., Christensen, E., Grønn, I., Rasmussen, K.
and Brandt, A. Glutaric aciduria: Clinical and laboratory findings


4. Brandt, N.J., Brandt, S., Christensen, E., Gregersen, N. and Rasmussen, K.
Glutaric aciduria in progressive choreo-athetosis. Clinical Gen. 13: 77-80,
1978.

5. Whelan, D.T., Hill, R., Ryan, E.D. and Spate, M. L- Glutaric aciduria:

6. Stokke, O., Goodman, S.I., Thompson, J.A. and Miles, B.S. Glutaric
aciduria: Presence of glutaconic and β-hydroxyglutaric acids in urine.

7. Goodman, S.I., Norenberg, M.D., Shikes, R.H., Breslich, D.J. and Moe, P.G.
Glutaric aciduria: Biochemical and morphologic considerations. J. Pediat.


acyl-CoA dehydrogenase deficiency (Glutaric aciduria type II) with transient
hypersarcosinemia and sarcosinuria; possible inherited deficiency of an

Aerograph , Walnut Creek, California, 1969, pp 150-151.


fectivity. The converse is often true for patients with acute Type B hepatitis. Similarly, fibrinogen and prothrombin complex are highly infectious and yet generally HBsAg-negative. The occurrence of overt hepatitis B in human beings after receipt of only highly infectious and yet generally HBsAg-negative blood is not uncommon. Lastly, plasma was still infectious when diluted far beyond the range of any current radioimmunoassay and far beyond the dilution achieved by freezing and deglycerolization. Thus, the fact that our starting inoculum was HBsAg-positive by radioimmunoassay is not the relevant point. Of note was the initial infectivity titer. The initial 1-ml inoculum containing 10^6 infectious units per milliliter was diluted in 500 ml of whole blood before freezing and deglycerolization. This procedure reduced the concentration of virus to approximately 2 × 10^3 infectious units per milliliter, a level well below that previously observed in chronic carriers of hepatitis B virus and one that should have subjected the freeze-wash process to a fair test.

Because of the disparity between hepatitis B antigenicity and infectivity and because a low dose of virus is not necessarily equated with development of mild disease, we did not consider it justifiable to commit an additional large number of this scarce and expensive animal resource to establish the concentration of hepatitis B virus that might be rendered noninfectious by freezing and deglycerolization. The question raised by Dr. Goldfinger can only be answered by a large-scale, controlled, prospective study in human beings. In the interim, we believe that our chimpanzee studies subjected freezing and deglycerolization to a valid test and that these studies do not currently support the use of frozen erythrocytes to reduce the incidence of Type B post-transfusion hepatitis.

We omitted the controls suggested by Dr. Smith because the inoculum had previously been "pedigreed" in other chimpanzees so that its infectivity titer and clinical sequelae were known. One can only use the chimpanzee as a model for infectivity, not for clinical illness, since overt disease almost never occurs in them regardless of the infectivity titer of the inoculum. In terms of chronicity, one would anticipate the opposite of what Dr. Smith suggests. In both human and chimpanzee studies the tendency toward chronicity has been highest in subjects receiving the most dilute inoculum. Thus, in theory at least, frozen red cells that did not prevent infection, but reduced the titer of virus, would be more, rather than less, likely to result in a chronic carrier state.

Harvey J. Alter, M.D.
Paul V. Holland, M.D.
Robert J. Gerety, M.D.
National Institutes of Health
Bethesda, MD 20014


ZOLLINGER-ELLISON SYNDROME WITH MULTIPLE ENDOCRINE ADENOMATOSIS TYPE II

To the Editor: We report here an unusual association of Zollinger-Ellison syndrome with multiple endocrine adenomatosis Type II in a young man.

This 28-year-old man was admitted to the Yale-New Haven Hospital because of epigastric pain. There was no recent history of vomiting, melena or salicylate abuse. He was a member of a local kindred with multiple endocrine adenomatosis Type II (Sipple's syndrome). Five years before, medullary carcinoma of the thyroid was treated by total thyroidectomy; follow-up serum calcitonin levels and a liver biopsy revealed metastatic disease. One year previously, he had entered the Beth Israel Clinical Research Center for evaluation of diarrhea and weight loss; statorrhoea (excretion of 16.9 g of stool fat per day) and an elevated serum calcium were noted, and a barium-meal examination showed only a nonspecifically abnormal small bowel. Small-bowel biopsy and tests for urinary 5-hydroxyindoleacetic acid and phenylpyruvic acid were normal. The serum gastrin was 150 pg per milliliter (normal, less than 200). The diarrhea was thought to be a result of the high serum calcium level or of some other tumor product and was brought under control with diphtheria toxin and sodium thiocyanate. This improvement persisted, but at the time of discharge some degree of diarrhea was still present. The patient continued to feel well, with no evidence of recurrent diarrhea. His serum calcitonin level remained elevated.

This patient shares several features common to other patients with multiple endocrine adenomatosis Type II. The diarrhea may have been due to multiple endocrine adenomatosis Type II, which has been associated with increased pancreatic secretin. Alternatively, the diarrhea may have been due to gastrin-secreting tumor. This possibility is supported by the lack of weight loss and the presence of elevated serum gastrin levels. The diagnosis of multiple endocrine adenomatosis Type II in this patient has been confirmed by the finding of an elevated serum calcitonin level and by the presence of multiple endocrine adenomatosis Type II in his relatives.

Harvey J. Alter, M.D.
Paul V. Holland, M.D.
Robert J. Gerety, M.D.
National Institutes of Health
Bethesda, MD 20014

DICARBOXYLIC ACIDURIA: THE RESPONSE TO FASTING

R.J.W. TRUSCOTT a,*, L. HICK a, C. PULLIN a, B. HALPERN a, B. WILCKEN b, H. GRIFFITHS c, M. SILINK c, H. KILHAM c and F. GRUNSEIT d

a Department of Chemistry, University of Wollongong, P.O. Box 1144, Wollongong, N.S.W. 2500 (Australia), b Oliver Latham Laboratory, Sydney, N.S.W. (Australia), c Royal Alexandra Hospital, Sydney, N.S.W. (Australia) and d Prince of Wales Children’s Hospital, Sydney, N.S.W. (Australia)

(Received October 23rd, 1978)

Summary

The urine of a child who presented with an episode of a disease resembling Reye's syndrome was found to contain large quantities of the dicarboxylic acids adipic and suberic acids, as well as the glycine conjugate of suberic acid, suberyl glycine. A variety of other dicarboxylic acids, both saturated and unsaturated, were also found in the urine at the time of the attack. It was found that the excretion of these unusual metabolites could be markedly increased by fasting for periods of greater than 10 h.

These results indicate that the patient may have a defect in fatty acid oxidation which becomes clinically significant during periods of prolonged fasting.

Introduction

There have been a number of clinical conditions reported where the excretion of adipic acid in urine is increased. These include glycogen storage disease [1], Reye’s syndrome [2] and congenital lactic acidosis [3]. An increased excretion of adipic and suberic acid has also been observed in ketosis [4], where the excretion of these dicarboxylic acids appears to parallel the degree of ketosis. However in all these conditions the amount of adipic acid excreted is small, and the increased excretion of this dicarboxylic acid is associated with the excretion of large quantities of other organic acids such as lactic acid or 3-hydroxybutyric acid.

We now describe a patient who was found to excrete considerable quantities of adipic acid and other dicarboxylic acids without having significantly elevated urinary levels of either lactic or 3-hydroxybutyric acid. A preliminary report on this work has been presented [5].

* To whom correspondence should be addressed.
Materials and methods

Case report

The male patient (M.E.) was the seventh child of unrelated Australian parents, born at term following a normal pregnancy. Two of his six siblings, a boy aged 2 years and a girl aged 10 months, died suddenly during episodes of a gastroenteritis-like illness, when, after two days of diarrhoea and vomiting, they had become very drowsy for a few hours, upon which they abruptly died.

Two days prior to admission the patient became febrile and began to have mild diarrhoea. Persistent vomiting continued for the next 48 h and a few hours before admission the child became increasingly drowsy.

On admission the patient was hypothermic (axillary temperature 35.2°C), semi-conscious and was noted to have 8 cm of firm hepatomegaly below the costal margin. At the time of the attack the following laboratory results were obtained: blood glucose, 1.4 mmol/l; blood pH 7.38; blood bicarbonate, 17.3 mmol/l; serum aspartate aminotransferase, 68 U/l; serum alanine aminotransferase 31 U/l. Urinary amino acids at the time of admission were within the normal range.

The patient was resuscitated with intravenous glucose and his state of consciousness returned almost to normal within 24 h. Normal feeds were reintroduced at this stage and the child was discharged ten days after admission with 6 cm firm hepatomegaly below the costal margin.

Materials

Chemicals were obtained as follows: Succinic acid (1,4-butanedioic acid) from May and Baker Ltd., Dagenham, U.K.; hexanoic acid, octanoic acid, adipic acid (1,6-hexanedioic acid) and suberic acid (1,8-octanedioic acid) from Sigma Chemical Co., St. Louis, MO, U.S.A.; and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) from Regis Chemical Co. Morton Grove, IL, U.S.A.

Identification of compounds

The identification of urinary metabolites was confirmed by combined gas chromatography-mass spectrometry (GC/MS) in which a Varian Aerograph (2700)GC was interfaced, via a jet separator, to a Dupont 491B mass spectrometer fitted with a dual electron impact/chemical ionisation source. The GC was fitted with a 6 ft × ½ inch glass column packed with either 6% Apiezon N or 3% SE-30 on 80–100 mesh dimethyldichlorosilane-treated Chromosorb W. A helium flow rate of 25 ml/min was used, and during the analyses the temperature was programmed from 80°C to 250°C at a rate of 6°C/min.

Extraction and quantitation of urinary acids

Succinic acid (10 μg) was added as an internal standard to 1 ml of urine and the solution was acidified to pH 1 with HCl (0.1 ml, 6 mol/l). Saturated aqueous NaCl (1 ml) was then added and the mixture extracted with ethyl acetate (3 × 2 ml). The organic extracts were pooled, evaporated to dryness with a stream of dry nitrogen, and the dried residue derivatised for GC analysis with BSTFA (50 μl, 60°C, 30 min). Quantitation of individual acids was achieved by measurement of the peak heights of each acid relative to the peak height of the
internal standard and interpolating from a standard calibration curve. The recoveries of adipic acid and suberic acid using the above method were determined by extracting 1-ml samples of water spiked with 20 μg of each of the compounds. The efficiency of extraction was calculated to be 94% for adipic acid and 96% for suberic acid.

For the examination of organic acids and acyl glycines as the methyl ester derivatives, urine samples were extracted with diethyl ether/ethyl acetate and the extract methylated at room temperature with an ethereal solution of diazomethane.

Hydrolysis of acylglycines

Urine samples containing 1 mmol creatinine were hydrolysed in 3 ml of 6 M HCl (110°C, 3 h). The internal standard was added to the hydrolysate and the organic acids were extracted with diethyl ether (3 × 3 ml). The pooled ether extract was dried over MgSO₄ and evaporated at room temperature to a final volume of approximately 100 μl. BSTFA (50 μl) was then added and the extract silylated (60°C, 30 min). GC analyses were performed on 3% SE-30 as described previously, using an initial temperature of 55°C.

Results

A GC profile of the silylated extract of a urine sample taken from the patient close to the time of admission is shown in Fig. 1. The profile was dominated by one large peak which was identified by mass spectrometry as the disilyl derivative of adipic acid. Smaller amounts of suberic and sebacic acids were also detected in the urinary extract. Lactic acid and 3-hydroxybutyric acid which elute at the positions indicated on the profile were present only at low levels. Estimation of the amount of adipic acid present in the urine at the time of admission showed up to 4 mol adipic acid per mol creatinine.

The methylated extract of a urine sample taken during the attack was also examined by GC/MS. The result is shown in Fig. 2. In addition to the large peaks of adipic and suberic acids a major peak (peak 13) was observed which eluted at 217°C. This peak was identified as the methyl ester of suberyl glycine. The mass spectrum of this compound matched that of suberyl glycine shown by Gregersen et al. [6] and was identical with that of a sample of the reference compound synthesized in our laboratory. This is only the second time that the excretion of this compound has been reported. The glycine conjugates of adipic and sebacic acid were not detected in the urinary extract of this patient although the glycine conjugate of the monocarboxylic acid, hexanoic acid, was present in significant amounts.

Other unusual compounds detected as their methyl ester derivatives were the mono-unsaturated dicarboxylic acids, octenedioic and decenedioic, and the di-unsaturated form of decenedioic acid. These compounds were identified using mass spectrometry by reference to the spectra published by Tanaka [7] and Lindstedt et al. [3]. The positions of the double bonds could not be determined from the mass spectra and were not determined by other methods. The appearance of the child’s metabolic profile changed considerably with his clinical condition, so that the recovery from the attack and the regression of
Fig. 1. Gas chromatogram of the silylated urinary organic acids from M.E. at the time of the attack. Peaks were identified as follows: 1, 3-hydroxybutyric acid; 2, urea; 3, glutaric acid; 4, adipic acid; 5, suberic acid.

Fig. 2. Gas chromatogram of the methylated urinary organic acids from M.E. at the time of the attack. Peaks were identified as follows: 1, 3-hydroxybutyric acid; 2, adipic acid; 3, 3-methyladipic acid; 4, pimelic acid; 5, octenedioic acid; 6, suberic acid; 7, hexanoyl glycine + p-hydroxyphenylacetic acid; 8, azelaic acid; 9, decenedioic acid; 10, sebacic acid; 11, hippuric acid; 12, unknown; 13, suberyl glycine.
the hepatogemaly were associated with a drop in the level of urinary adipic acid to the levels seen in normal children of approximately 10 mmol/mol creatinine. The other unusual metabolites detected at the time of the attack also either fell to normal levels or disappeared from the urine although significant amounts of hexanoyl glycine remained.

The amounts of some of these unusual metabolites detected in a urine sample taken at the time of the attack and also in a clinically unaffected period are shown in Table I. It can be seen that, although the excretion of adipic and suberic acids is within the normal range during the clinically unaffected period, there is a very marked increase in the excretion of these dicarboxylic acids with the onset of the attack. At this time, the quantity of suberic acid excreted as the glycine conjugate accounted for approximately 35% of the total suberic acid present in the urine. This is considerably lower than that found in the patient described by Gregersen et al. [6] where the percentage of total suberic acid present as suberyl glycine ranged from 65 to 75%.

As well as the marked increase in the excretion of the dicarboxylic acids, the monocarboxylic acids hexanoic and octanoic, when measured after acid hydrolysis, were also found to increase during the attack. However, the increase in the excretion of hexanoic acid was only slight.

Since all of the unusual metabolites detected in the urine of this patient appeared to be derived from fatty acids, we considered that this patient could have a defect in fatty acid metabolism. To test this hypothesis, a fasting study was performed to determine whether the excretion of these unusual acids could be induced as a result of an increase in endogenous fat metabolism. The results of this fasting study are shown in Fig. 3. At the time of this fast the child was 8 months of age. The graphs show that there is a marked increase in the urinary excretion of adipic and suberic acids after approx. 10 h fasting. After approx. 14 h of fasting, the urinary adipic acid concentration had increased to more than 30 times the normal level and the suberic acid concentration to 12 times the normal level. The excretion of suberyl glycine at this stage was also significantly increased above normal.

Although adipic acid, suberic acid and suberyl glycine were the only metab-

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (mmol/mol creatinine)</th>
<th>Normal children (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M.E.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Attack</td>
<td>Unaffected</td>
</tr>
<tr>
<td>Adipic acid</td>
<td>1747</td>
<td>6—8</td>
</tr>
<tr>
<td>Suberic acid</td>
<td>764</td>
<td>7—10</td>
</tr>
<tr>
<td>Suberyl glycine</td>
<td>429</td>
<td>&lt;9</td>
</tr>
<tr>
<td>Hexanoic acid *</td>
<td>182</td>
<td>166</td>
</tr>
<tr>
<td>Octanoic acid *</td>
<td>216</td>
<td>5</td>
</tr>
</tbody>
</table>

* Determined after acid hydrolysis.
Fig. 3. The excretion of adipic acid, suberic acid and suberyl glycine in the urine of M.E. as a function of the time of fasting.

olites to be monitored quantitatively during the fast, all of the unusual metabolites present in the urine at the time of the attack were observed in the urine sample taken at the end of the fast.

Five normal children (aged from 9 to 60 months) and one child diagnosed as having Reye’s syndrome (aged 45 months) were fasted under similar conditions and in each case, no increase in the excretion of dicarboxylic acids was observed. Two of these children (aged 9 and 36 months) were fasted for more than 30 h without raising the excretion of either adipic or suberic acid above the normal level.

Sixteen months after the initial fasting study, the patient was again fasted and once again the urinary levels of dicarboxylic acids were markedly raised. On this occasion however, the increase in excretion did not commence until he had fasted for 17 h. This delay may reflect a decreased rate of lipolysis with age. These results suggest that the attack which caused the admission of this child to hospital may have resulted from an inability to fast for lengthy periods of time, and we have therefore been reluctant to subject the patient to fasts of prolonged duration.
Since the discovery of the first patient with dicarboxylic aciduria, we have examined two other unrelated children with similar clinical histories whose urines at the time of admission were found to contain large amounts of adipic acid as well as the other unusual dicarboxylic acids found in the urine of the first child. Both of these children (aged 17 and 27 months) were found to excrete greatly increased amounts of adipic acid, and suberic acid on fasting.

Although the presence of saturated and unsaturated dicarboxylic acids and suberyl glycine characterise the metabolic profiles of these children, there was considerable variation in the relative amounts of the various compounds detected in the urines of the three children as well as in urine samples taken at different times from the same child.

Discussion

The biosynthesis of dicarboxylic acids via the $\omega$-oxidation of fatty acids was first studied by Verkade et al. [8] and later by several groups of researchers [9–11]. Their results suggest that $\omega$-oxidation provides an alternative pathway for the oxidation of fatty acids. The end products of $\omega$-oxidation are short chain dicarboxylic acids, since the long chain dicarboxylic acids formed by $\omega$-oxidation on the microsomes can be further degraded by the mitochondrial $\beta$-oxidation system [12]. It seems likely therefore that the dicarboxylic acids observed in the urine of M.E. and the other two boys have arisen from fatty acids by a combination of $\omega$- and $\beta$-oxidation.

The relative importance of the $\omega$- and $\beta$-oxidation pathways to the overall metabolism of fatty acids under a variety of conditions is not known. The results obtained by Antony et al. [13] suggest that in rat liver, $\beta$-oxidation is the predominant pathway under normal conditions, however, experiments using branched-chain fatty acids [14] or enzyme inhibitors such as methylene cyclopropylacetyl-CoA, [7] have shown that $\omega$-oxidation may assume a major role when the $\beta$-oxidation pathway is hindered. Thus there are a number of conditions known where an increased excretion of dicarboxylic acids in the urine is associated with a disturbance in $\beta$-oxidation [15,16].

Recently a number of reports have appeared in the literature concerning children who have been found to excrete large quantities of dicarboxylic acids as the major urinary organic acids. Przyrembel et al. [17] have described a child with glutaric aciduria type II whose urine contained large amounts of $C_6$, $C_8$ and $C_{10}$ dicarboxylic acids as well as greatly elevated levels of lactic (10.3 mol/mol creatinine) and glutaric acid (9.6 mol/mol creatinine). The presence of branched-chain amino acid metabolites in the urine indicated a generalised defect in the activity of acyl-CoA dehydrogenases. Glutaric acid was often observed as a minor component in the urinary GC profile of M.E. and the other two children, however the amounts present were well below the levels reported for glutaric aciduria type II.

Carnitine deficiency is known to cause an increased excretion of adipic, pimelic and suberic acids as well as unsaturated dicarboxylic acids. Although there were significant differences between the metabolic profiles observed under normal dietary conditions for the three children and the patient with systemic carnitine deficiency described by Karpati et al. [15], carnitine
deficiency cannot be excluded as the cause of the disease seen in these children.

The clinical pictures and the organic acid profiles observed in the three boys at the time of the attacks closely resemble those reported in patients suffering from Jamaican vomiting sickness, a disease produced by the ingestion of unripe akee fruit [16]. This fruit contains a toxic amino acid derivative, hypoglycin A. A metabolite of this amino acid, methylene cyclopropyl acetic acid inhibits the transport of fatty acids into mitochondria [18] and also inhibits the dehydrogenation of several acyl-CoA derivatives [19] leading to the disruption of fatty acid β-oxidation and a massive urinary excretion of adipic acid and other dicarboxylic acids.

A similar clinical case to that of M.E. has been described by Chalmers et al. [20] in identical twin boys following an adenovirus infection. One boy died before admission to hospital but the other recovered rapidly after treatment with intravenous glucose. Urine samples taken at the time of the attack showed elevated levels of adipic and suberic acids, unsaturated dicarboxylic acids and octanoic acid. The similarities between this disease and Jamaican vomiting sickness were noted by the authors who suggested that the attacks may have resulted from the ingestion of a toxic analogue of hypoglycin A. In the case of the three boys described in this report, the results obtained from the fasting experiments indicate that the disease in these children is caused by a deficiency in endogenous metabolism rather than the ingestion of a toxic compound.

Tanaka et al. [21] have reported a patient suspected of having a deficiency of green acyl-CoA dehydrogenase, the enzyme responsible for catalysing the dehydrogenation of C4—C8-CoA esters. This child excreted large amounts of adipic acid, hexanoyl glycine and 2-ethylmalonic acid. In our patients adipic acid and hexanoyl glycine were elevated but 2-ethylmalonic was not detectable.

A patient who appears to have most features in common with the three children presented in this report, is the boy described by Gregersen et al. [6]. This child was prone to episodes of lethargy and unconsciousness but responded rapidly to intravenous glucose. At the times of these attacks, the urine was found to contain large quantities of dicarboxylic acids as well as suberyl glycine, the metabolite present in the urine of M.E.

The results of the fasting studies carried out on our patients indicate an inability to cope adequately with the flux of fatty acids through the β-oxidation pathway at times when the body is dependent on fat as the source of energy, and it would appear that periods of prolonged fasting may have induced the attacks which were responsible for the admission of these children to hospital. In support of this view it is of interest to note that the patient described by Gregersen et al. died suddenly following a 39-h fast for diagnostic purposes.

If these children do have a defect at the level of fatty acid oxidation, the appearance of an almost normal GC profile under normal dietary conditions may be explained by the ‘fat-sparing’ effect of carbohydrates since, when adequate carbohydrate is available, only minute quantities of fatty acids are oxidised to provide energy. Thus a deficiency in β-oxidation may not become significant until the body becomes reliant on fat for its energy requirements. Analysis of the respiratory quotients [22] suggests that approx. 8—10 h after a meal, the body becomes dependent on fat as the source of energy. This time
period coincides with the increased urinary excretion of dicarboxylic acids observed in the three children during fasting studies.

Acknowledgements

This work was supported by a grant from the National Health and Medical Research Council of Australia.

References

7 Tanaka, K. (1972) J. Biol. Chem. 247, 7465—7478
10 Kusunose, M., Kusunose, E. and Coon, M.J. (1964) J. Biol. Chem. 239, 1374—1380
13 Antony, G.J. and Landau, B.R. (1968) J. Lipid Res. 9, 267—269
The Identification of 3-Keto-2-methylvaleric Acid and 3-Hydroxy-2-methylvaleric Acid in a Patient with Propionic Acidemia‡

Roger J. W. Truscott, Christopher J. Pullin, and Berthold Halpern
Department of Chemistry, The University of Wollongong, Wollongong, NSW 2500, Australia

Judy Hammond, Eric Haan and David M. Danks
Departments of Biochemistry and Pediatrics, Royal Children's Hospital, Parkville, Victoria 3052, Australia

Two abnormal metabolites, 3-keto-2-methylvaleric acid and 3-hydroxy-2-methylvaleric acid, have been identified and quantitated in the urine of a child with propionic acidemia. These metabolites may be produced as a result of the self-condensation of propionyl-CoA. Data are presented to show that the unusual ketone, 3-pentanone, which has been observed previously in the urine of patients with propionic acidemia, is produced as a result of the decarboxylation of 3-keto-2-methylvaleric acid.

INTRODUCTION

The investigation of children suspected of having an inborn error of metabolism, often involves a screening procedure for urinary organic acids, since the identification of abnormal organic acids can often provide valuable information about the site of an enzyme defect. ¹

Propionic acidemia is an inborn error of metabolism in which there is a defect in the activity of propionyl-CoA carboxylase (E.C.6.4.1.3). ² This defect in activity may result from a mutant propionyl-CoA carboxylase holoenzyme or from a deficiency in the utilization of biotin, the coenzyme involved in carboxylation. This disease is difficult to detect by the usual urinary screening procedures since the pattern of metabolites excreted by patients with propionic acidemia varies considerably. It appears, however, that a diagnosis can be made on the basis of the secondary metabolites, 3-hydroxypropionic acid and methylcitric acid, which appear in the urine even at times when the excretion of propionic acid is not greatly elevated. ³

We now describe the identification of two previously unreported metabolites, 3-keto-2-methylvaleric acid and 3-hydroxy-2-methylvaleric acid, in the urine of a child with propionic acidemia. These two metabolites, both of which appear to result from the condensation of two propionyl-CoA molecules, may provide an additional means for the identification of propionic acidemia on the basis of secondary metabolites.

EXPERIMENTAL

Case history

The patient (Z.K.) was a newborn boy of half first cousin Turkish parents. Two sisters had died of unknown causes aged two days and two months in Turkey. A seven year old sister and four year old brother are normal.

Z.K. was born at term after a normal pregnancy, labour and delivery. Birth weight was 2.820 kg. The infant was initially fed a milk formula containing 2.8 g protein per 100 ml but he was noted to be unwell at 48 h, with depressed conscious state and acidic breathing, and was said to smell ketotic. Mild jaundice was present. Laboratory investigation revealed a marked metabolic acidosis (pH 7.06, bicarbonate 4 mM L⁻¹, pCO₂ 14 mmHg and base excess −25) and the nitroprusside test of urine for ketones was strongly positive. Intravenous sodium bicarbonate was given.

The infant was transferred to the Royal Children's Hospital at 60 h of life. Initial investigation confirmed the metabolic acidosis and demonstrated a plasma ammonia of 569 μM L⁻¹. High voltage electrophoresis showed elevated lysine and cystine in urine and elevated lysine and decreased alanine in serum. Thin layer chromatography (TLC) of urinary ketones, as the DNP derivatives, showed increased acetone, butanone and pentanone. The initial full blood count was normal, but later leucopenia (3.2 x 10³ mm⁻³) and thrombocytopenia (15 x 10³ mm⁻³) appeared. Fibroblastic cell cultures are being established for assay of propionyl-CoA carboxylase activity.

Management consisted of intravenous 10% dextrose, peritoneal dialysis from 65 h of life, exchange transfusions at 90 and 110 h of life, and megavitamin therapy including biotin 100 mg orally immediately after the second exchange transfusion. Rapid improvement in the infant’s clinical condition began around this time. Dietary protein was introduced and increased to 1.5 g kg⁻¹ day⁻¹ on the eleventh day of life. Biotin was continued at a dose of 10 mg daily. The impression of biotin responsiveness gained during the initial illness was to have been formally tested later, however a further metabolic crisis developed after two months of relatively good progress on biotin and protein restriction. Hyperammonemia (1700 μM L⁻¹) and propionic acidemia proved uncontrollable and he died.

Abbreviation: DNP = dinitrophenyl hydrazone.
† Author to whom correspondence should be addressed.
The concentrations of amino acids in serum were determined by ion-exchange chromatography using a Technicon TSM amino acid analyser. Serum propionic acid was measured by gas chromatography (GC) according to the method of Remesy and Demigne.

In the routine screening procedure for urinary organic acids, NaCl (0.5 g) was added to 1 ml of urine, the pH adjusted to 10 with NaOH and the solution extracted with ethyl acetate (3 x 2 ml). This extract was discarded. The aqueous layer was then adjusted to pH 1 with HCl, and extracted twice with ethyl acetate (2 x 2 ml) and once with diethyl ether (1 x 2 ml). The pooled organic phases were evaporated to dryness with nitrogen at room temperature. The dried urinary extract was silylated with 125 µl of BSTFA containing 1% TMCS (60 °C, 30 min) and an aliquot corresponding to 40 nmol creatinine injected onto the GC column.

The separation and identification of urinary metabolites was performed by gas chromatography mass spectrometry (GCMS) using a Varian MAT44 GCMS system in which a 25 m SE-54 (Jaeggi) wall-coated capillary column was directly coupled to the ion source of the mass spectrometer. For GC, a helium flow rate of 2 ml min⁻¹ was used, and during the analyses the temperature was programmed from 70-220 °C at 4 °C min⁻¹.

Electron impact (EI) mass spectra were obtained using an ionizing voltage of 70 eV and an emission current of 0.8 mA. The ion source was maintained at 200 °C, the transfer lines at 250 °C and the injection port of the gas chromatograph at 250 °C. The total ion current was measured from mass 50 to 500 and was recorded with 320 mV full scale.

Chemical ionization (CI) mass spectra were obtained using isobutane at 45-50 Pa with an ionizing voltage of 160 eV and an emission current of 0.2 mA.

For the quantitation of urinary metabolites, adipic acid (50 µg) was added to a urine sample containing approximately 1 µmol creatinine and the solution made up to 1 ml with H₂O. This solution was acidified to pH 1 with 6 M HCl, saturated NaCl solution (1 ml) added, and the mixture extracted with diethyl ether (3 x 3 ml). The pooled ether extract was dried over MgSO₄, filtered, then evaporated at room temperature to a final volume of approximately 100 µl. N,O-bis-(trimethylsilyl)-trifluoracetamide (BSTFA) (50 µl) was then added and the extract silylated (60 °C, 30 min). The derivatives were analysed by GC on a 6 ft x 1 mm column of 3% SE-54 on 80/100 mesh Gas Chrom Q (Applied Science Labs). Quantification of individual acids was achieved by measurement of the peak heights of each acid relative to the peak height of 4-heptanone and interpolating from a calibration curve.

RESULTS

The organic acid profile of the child when first admitted with ketosis is shown in Fig. 1. At the time of admission the concentration of propionic acid in the serum was 3.5 mM (normal = 1.29 ± 0.52 µM) and the serum glycine level was 356 µM which is within the normal range of 92-392 µM. The urinary organic acid profile was obtained by chromatographing the silylated acids on an SE-54 capillary column which we have previously shown to give excellent separation of these derivatives.

The organic acid profile was dominated by a large peak of 3-hydroxybutyric acid and smaller peaks of lactic acid and acetoacetic acid. In addition to these metabolites, which are characteristic of ketosis, there were a number of other compounds present in the urinary extract which have been described as being elevated in propionic acidemia. Methylocetic acid and 3-hydroxypropionic acid, metabolites which have been considered to be diagnostic of propionyl-CoA carboxylase deficiency, were present as only minor components in this first urine sample, but the five-carbon acids 3-ketovaleric, 3-hydroxyvaleric and 3-hydroxy-2-methylbutyric acid were major constituents of the chromatogram. These compounds were identified on the
Figure 1. The total ion current profile from the SE-54 capillary column of the silylated urinary extract from patient Z.K. This urine sample was taken at the time of admission, when the child was severely ketotic. The numbered GC peaks were identified by MS as follows: 1, lactic acid; 2, 3-hydroxypropionic acid; 3, 3-hydroxybutyric acid; 4, 3-hydroxy-2-methylbutyric acid; 5, acetoacetic acid (isomer 1); 6, 3-hydroxyvaleric acid; 7, acetoacetic acid (isomer 2); 8 and 9, 3-ketovaleric acid; 10, 2-methylacetoacetic acid; 11, 3-keto-2-methylvaleric acid; 12, 3-hydroxy-3-methylglutaric acid; 13 and 14, methylcitric acid; 15, 4-hydroxyphenyllactic acid. SS = solvent suppression.

The extract of a urine sample taken on the following day when the ketosis had been controlled produced a very different GC trace (Fig. 2). At this time, the serum propionic acid concentration was 2 mM and the serum glycine 382 μM.

The urinary profile on this occasion revealed large peaks of 3-hydroxypropionic acid, 3-hydroxy-2-methylbutyric and methylcitric acid. 3-Ketovaleric and 3-hydroxyvaleric which were major components of the first urinary extract were barely detectable in the second urine. In contrast, two metabolites, 3-hydroxyisovaleric acid and propionyl glycine appeared which were not observed in the previous urinary extract. The glycine conjugate of propionic acid, which has been described previously in a patient with propionic acidemia by Rasmussen et al.,11 was identical on the basis of retention time and mass spectrum with a sample of the authentic compound synthesized in our laboratory.

In addition to these metabolites, two prominent peaks were present in the GC trace (peaks 8 and 13, Fig. 2) which could not be identified. A CI mass spectrum showed that the compound in peak 13 had a molecular weight of 274 as the silyl derivative, and the EI mass spectrum displayed a prominent ion at m/z 245 corresponding to a loss of 29 amu from the molecular ion, suggesting the presence of an ethyl group within the molecule. Comparison of the EI mass spectrum with that of 3-ketovaleric acid showed that there was an increase of 14 amu in all of the major high mass fragments. This information allowed us to postulate that the unknown compound was 3-keto-2-methylvaleric acid.

The authentic compound was synthesized via a Claisen condensation reaction using ethyl propionate, and the silyl derivative was shown to be identical with that of the unknown urinary component on the basis of GC retention time and mass spectrum (Fig. 3). As expected, two peaks were obtained in the GC profile of the silyl derivatives of both the authentic and urinary 3-keto-2-methylvaleric acid (peaks 11 and 13). Presumably these peaks are the disilyl cis- and trans-enol derivatives. With an increased time of derivatization, the earlier eluting peak was converted to the later peak which may correspond to the thermodynamically more stable trans-enol derivative.
3-KETO- AND 3-HYDROXY-2-METHYLVALERC ACID

Figure 2. The total ion current profile from the SE-54 capillary column of the silylated urinary extract from patient Z.K. This urine sample was taken after the ketosis had been controlled. The numbered GC peaks were identified by MS as follows: 1, lactic acid; 2, 3-hydroxypropionic acid; 3, 3-hydroxybutyric acid; 4, 3-hydroxy-2-methylbutyric acid; 5, 3-hydroxyisovaleric acid; 6, 3-hydroxycyclohexanecarboxylic acid; 7, acetoacetic acid; 8 and 9, 3-hydroxy-2-methylvaleric acid; 10, 3-ketovaleric acid; 11, 3-keto-2-methylvaleric acid (isomer 1); 12, 2-methylacetoacetic acid; 13, 3-keto-2-methylvaleric acid (isomer 2); 14, propionylglycine; 15, glutaric acid; 16, adipic acid; 17, furan-2-hydroxymethyl-5-carboxylic acid; 18, 2-hydroxyglutaric acid; 19, 3-hydroxy-3-methylglutaric acid; 20, 4-hydroxyphenylacetic acid; 21 and 22, methylcitric acid; 23, 4-hydroxyphenyllactic acid; 24, palmitic acid.

The compound present in peak 8 of the urinary profile (Fig. 2) was shown to be 3-hydroxy-2-methylvaleric acid, the corresponding hydroxy acid of 3-keto-2-methylvaleric acid. This compound was synthesized by borohydride reduction of the authentic 3-keto-2-methylvaleric acid, and the silyl derivative was shown to have the same GC retention time and mass spectrum as the urinary component (Fig. 4). Once again, two GC peaks having almost identical mass spectra were obtained for both the authentic and the urinary 3-hydroxy-2-methylvaleric acid (peaks 8 and 9, Fig. 2). These two peaks presumably correspond to the disilyl derivatives of the two diastereoisomers of 3-hydroxy-2-methylvaleric acid. A similar phenomenon was observed for methylcitrate where the silyl derivative was resolved into two peaks on the capillary column (peaks 21 and 22). The absolute configurations of the two diastereoisomers of methylcitrate excreted in a patient with propionic acidemia have been determined previously.12

3-Keto-2-methylvaleric acid was found consistently in urine samples taken from the patient during his illness. Since the most likely origin of 3-keto-2-methylvaleric acid appeared to be a thiolase catalysed self-condensation of propionyl-CoA, the urinary levels of 3-keto-2-methylvaleric acid were monitored together with the serum levels of propionic acid to determine if the urinary excretion of this organic acid could be used as a useful indicator of the serum propionic acid concentration.

The results of this study are shown in Fig. 5. It can be seen that the urinary excretion of 3-keto-2-methylvaleric acid correlates well with the excretion of another propionate-derived metabolite, 3-hydroxypropionic acid. There seemed also to be a reasonable correlation between the excretion of these two urinary metabolites and the serum concentration of propionic acid from 90 h onwards; however, there was a marked deviation from this relationship in the samples taken at the time of admission. At this time, the child was severely ketotic.

In order to test whether 3-keto-2-methylvaleric acid or 3-hydroxy-2-methylvaleric acid had arisen artefactually from the self-condensation of propionic acid in
urine, propionic acid was added to normal urine to give a final concentration of 5 mM and the solution incubated at 37 °C for 22 h. Extraction and derivatization of the organic acids as described in the Experimental section failed to show the presence of either 3-keto-2-methylvaleric acid or 3-hydroxy-2-methylvaleric acid.

The unusual ketone, 3-pentanone, has been identified as the DNP derivative in the urines of patients with propionic acidemia and methylmalonic acidemia, although the mechanism of its formation has been unknown. The identification of 3-keto-2-methylvaleric acid in the urines of patients with these diseases prompted us to explore the possibility that 3-pentanone could be derived from this \( \beta \)-keto acid by decarboxylation.

In an initial experiment, several microlitres of 3-keto-2-methylvaleric acid was added to 2 ml of a saturated solution of 2,4-dinitrophenylhydrazine in 2 M HCl. The precipitate which formed was analysed by TLC, and was shown to be composed predominantly of a compound which co-chromatographed with the DNP derivative of authentic 3-pentanone. The structure of this compound, as 3-pentanone-DNP, was confirmed by direct insertion mass spectrometry. A precipitate of 3-pentanone-DNP was also obtained when 1 ml of a saturated solution of dinitrophenylhydrazine in 2 M HCl was added to an equal volume of urine containing 5 mM 3-keto-2-methylvaleric acid. Since this urine sample had been thoroughly extracted with pentane to remove 3-pentanone prior to the addition of the dinitrophenylhydrazine, it seemed that the 3-pentanone-DNP must have been produced as a result of the decarboxylation of the keto acid or the keto acid-DNP derivative in acid.
solution. A similar phenomenon was observed for urinary 2-methylacetoacetic acid, where a precipitate of 2-butanone-DNP was obtained. The formation of acetone-DNP from solutions of acetoacetic acid has been described by Smith.\textsuperscript{15}

To determine whether 3-pentanone could be formed by decarboxylation of urinary 3-keto-2-methylvaleric acid within the body, the keto acid was added to normal urine at a concentration of approximately 5 mM and the solution incubated at 37 °C. Samples were removed at various time intervals for the measurement of 3-pentanone and residual keto acid. The results are shown in Fig. 6 and demonstrate that 3-keto-2-methylvaleric acid is readily decarboxylated by standing in urine at 37 °C.

Using solvent extraction with pentane, urine samples of Z.K. which had been shown to contain large amounts of 3-keto-2-methylvaleric acid were also found to contain corresponding large amounts of 3-pentanone (0.35 mol mol\textsuperscript{-1} creatinine in the second urine sample, Fig. 5). The identity of this ketone as 3-pentanone and not 2-pentanone, a ketone which has also been described as being elevated in this disease,\textsuperscript{13} was confirmed by GCMS. In urine samples taken after treatment, when the concentrations of 3-keto-2-methylvaleric acid were low, 3-pentanone could not be detected by our method.

**DISCUSSION**

Although a large number of unusual metabolites have been described as being elevated in patients with propionic acidemia, there appears to be considerable variability in the excretion pattern of these secondary metabolites. This factor can cause difficulties with the diagnosis of this disease even in those laboratories which have well established gas chromatographic screening procedures. Under conditions such as these, the identification of 3-keto-2-methylvaleric acid and 3-hydroxy-2-methylvaleric acid as major urinary components in our patient, may be a valuable aid in the future diagnosis of propionic acidemia on the basis of secondary metabolites. These two compounds do not appear to have been reported previously as components of human metabolism.

Both 3-keto-2-methylvaleric acid and 3-hydroxy-2-methylvaleric acid have been detected in urine samples from the two patients with propionic acidemia which we have encountered. In addition, we have also detected low levels of these acids in patients with methylmalonic acidemia; however, neither the hydroxy acid nor the keto acid could be found in urine samples taken from normal controls.

It seems likely that 3-keto-2-methylvaleric acid arises by an enzyme catalysed self-condensation of propionyl-CoA in a reaction analogous to the formation of acetoacetate from acetyl-CoA. Several 3-ketoacyl-CoA thiolases are known which catalyse the general reaction involving the condensation of two molecules of thioesters to produce a 3-ketothioester with the release of a thioalcohol.\textsuperscript{16} Since 3-ketoacyl-CoA thiolases have been described in mammalian systems with broad substrate specificities,\textsuperscript{17} it is conceivable that, under conditions where propionyl-CoA is greatly elevated, one or more of these enzymes could form 3-keto-2-methylvaleryl-CoA by the condensation of two molecules of propionyl-CoA. Enzymic reduction of the 3-keto-2-methylvaleryl-CoA, catalysed by 3-hydroxyacetyl-CoA dehydrogenase (E.C.1.1.1.35) could then yield 3-hydroxy-2-methylvaleryl-CoA. Hydrolysis of the CoA esters of these two compounds would then produce 3-keto-2-methylvaleric acid and 3-hydroxy-2-methylvaleric acid.

The proposed modes of formation of 3-hydroxy-2-methylvaleric acid and 3-keto-2-methylvaleric acid in this patient could best be tested by administration of stable isotope labelled propionic acid or isoleucine followed by an examination of the labelling patterns in the two urinary acids. However, these loading studies were not achieved before the child died.

The excretion of urinary ketones in a patient with ketotic hyperglycaemia and patients with ketosis due to starvation, diabetes or a high fat intake has been investigated by Menkes.\textsuperscript{13} The patient with hyperglycaemia was found to excrete elevated amounts of two unusual ketones, 2-butanone and 3-pentanone. Tsao and Pfeiffer\textsuperscript{18} proposed that 2-butanone was formed through the spontaneous decarboxylation of 2-methylacetoacetic acid. The origin of the 3-pentanone excreted in the urine of patients with propionic acidemia and methylmalonic acidemia has remained obscure, however, although Menkes postulated that this ketone could arise via the condensation of propionyl-CoA and methylmalonyl-CoA.\textsuperscript{13} In this paper we have demonstrated that the 3-pentanone observed in the urine of patients with propionic acidemia and methylmalonic acidemia can arise from the decarboxylation of 3-keto-2-methylvaleric acid. This decarboxylation was shown
to occur when the keto acid was incubated in normal urine at 37 °C and would presumably be faster in urines of lower pH. It is possible that some 3-pentanone may be produced enzymically from 3-keto-2-methylvaleric acid in a reaction analogous to the formation of acetone from acetoacetic acid, catalysed by acetoacetate decarboxylase (E.C.4.1.1.4). 19

The screening procedure for the presence of unusual ketones usually involves the addition of 2,4-dinitrophenylhydrazine in 2 M HCl to urine, and it was also demonstrated that 3-pentanone-DNP could arise directly from the breakdown of urinary 3-keto-2-methylvaleric acid during the course of the reaction. This decarboxylation should be taken into account when studying or quantitating urinary ketones as DNP derivatives.

An elevated concentration of glycine in plasma and urine has been described as being characteristic of propionic acidemia, 1 but in our patient the serum and urinary levels of glycine at the time of admission were within the normal range. At this time the serum propionic acid concentration was 3.5 mM. Analysis of the urinary amino acids at this time did show increased amounts of cystine and lysine, however. These amino acids were also found to be elevated in the urines of three of the eight patients with propionic acidemia investigated by Duran et al. 2 Two of these three patients did not show hyperglycinuria. Although neither hyperglycinuria nor hyperglycinemia was present during the acute illness period in our patient, once the serum levels of propionic acid were controlled by treatment, both hyperglycinemia and hyperglycinuria appeared.

From an analysis of the GC profiles and the results of the quantitation of urinary metabolites in this patient, it would seem that under conditions where excess acetyl-CoA was available, there appeared to be a preferential condensation between propionyl-CoA and acetyl-CoA. Thus, the major propionate-derived metabolites appearing in the urine when the child was ketotic were 3-ketovaleric acid and 3-hydroxyvaleric acid. However, when the ketosis had been controlled, self-condensation to form 3-keto-2-methylvaleric acid, condensation with oxaloacetate to form methylicitric acid as well as oxidation to produce 3-hydroxypropionic acid and condensation with glycine, seemed to be the major routes of propionyl-CoA metabolism.

These observations may have important implications for screening laboratories since, under conditions where a child is ketotic, the major secondary metabolites to look for in cases of propionic acidemia may be 3-ketovaleric acid and 3-hydroxyvaleric acid, whereas in non-ketotic patients the diagnostic metabolites appear to be 3-hydroxypropionic acid and methylicitric acid. A negative relationship between the excretion of 3-hydroxybutyric acid and methylicitric acid was noted by Duran et al. in their study of patients with propionic acidemia.

It is interesting to speculate on the reasons why 3-keto-2-methylvaleric acid and 3-hydroxy-2-methylvaleric acid have not been observed previously in other patients with propionic acidemia, but were major urinary metabolites in this patient. The reason for this may be related to the elevated serum levels of propionic acid seen in this child, even after control of the ketosis. This observation could perhaps indicate that these two metabolites, which apparently arise from the condensation of two propionyl-CoA molecules, may be relatively more prominent in patients who have very high serum levels of propionic acid.

Acknowledgements
This work was supported by a grant from the National Health and Medical Research Council of Australia.

REFERENCES

Application Note No. 33

Measurement of Urinary Methylmalonic Acid Using Quantitative Multiple Ion Selection

Authors:
J. Korth, C. J. Pullin, R. J. W. Truscott, B. Halpern

The University of Wollongong
Department of Chemistry
Wollongong, N.S.W. 2500, Australia
Measurement of Urinary Methylmalonic Acid Using Quantitative Multiple Ion Selection

by J. Korth, C. J. Pullin, R. J. W. Truscott and B. Halpern, the University of Wollongong, Department of Chemistry, Wollongong, N.S.W. 2500 Australia

Methylmalonic acid (MMA) is a metabolite in the catabolic pathway of branched chain amino acids and of fatty acids with an odd number of carbon atoms (Fig. 1). Normal adults excrete less than 5 mg/day of this acid in the urine. Excessive excretion of MMA in urine (levels of up to 5000 mg/day) can arise as a result of either vitamin B12 deficiency (Pernicious Anaemia) or a defect in one of the enzymes involved in the degradative pathway of MMA. Four inborn errors of metabolism leading to increased excretion of MMA are known: a deficiency of the enzymes racemase or isomerase and two abnormalities of vitamin B12 metabolism.\textsuperscript{1} Clinically, patients with increased MMA excretion present with protein intolerance, intermittent keto acidosis and show inhibited growth and development. Patients with deficiencies in the racemase or isomerase enzyme activity generally die at an early age, but patients with defects of vitamin B12 metabolism may respond to vitamin B12 therapy. The frequency and size of the dose required for treatment varies with individual patients and may need adjustment in periods of metabolic stress. Since the therapeutic dose is not related to the measured serum concentration,\textsuperscript{2} the effectiveness of the treatment is best assessed by measurement of the daily urinary excretion of MMA.

Published methods for the quantitative determination of urinary MMA rely on the use of thin layer\textsuperscript{3} or gas chromatography.\textsuperscript{4} However, the presence of other urine constituents with similar retention behaviour makes the analysis of small amounts of MMA both difficult and unreliable.

To overcome these inherent difficulties we now describe an isotope dilution method using GC/MS with $[^2H]_3$-MMA as an internal standard. Quantitative multiple-ion selection (MIS: QUANTITATIVE) was selected since this mode of operation allows precise intensity measurements of the specific ions involved and thus provides a sensitive method for determining the levels of excreted MMA within the range of interest. Since the method is applicable to a large number of compounds, we describe it in some detail, by way of example.

![Fig. 1 Major pathway of production and catabolism of methylmalonic acid](image)
In general, quantitative MIS requires the preparation of a suitable derivative of the compound of interest as well as that of an appropriate internal standard. The validity of the method in a specific case is established by constructing a calibration curve using the intensities of relevant ions involved. For monitoring complex mixtures quantitative MIS requires accurate and reproducible retention times. To meet these requirements isothermal oven conditions are desirable as this eliminates the need for time consuming column stabilization prior to each injection (Fig. 2).

For the TMS derivatives of MMA we found an oven temperature of 95 °C to be satisfactory, since this gave good separation from the solvent and a relatively short retention time (Fig. 3). As part of the process of establishing these parameters, we obtained EI spectra of both \([\text{H}_2\text{H}]_3\) MMA and MMA which determined those fragments that retain the deuterium label (Fig. 4 and Fig. 4a).

### Table: GC Parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCIB</td>
<td>684 μb</td>
</tr>
<tr>
<td>PSOU</td>
<td>11 μb</td>
</tr>
<tr>
<td>VELE</td>
<td>71.5 V</td>
</tr>
<tr>
<td>CEMI</td>
<td>0.00 mA</td>
</tr>
<tr>
<td>VSEM</td>
<td>20.1 V</td>
</tr>
<tr>
<td>TGCI</td>
<td>248 °C</td>
</tr>
<tr>
<td>TGCO</td>
<td>95.3 °C</td>
</tr>
<tr>
<td>TGCS</td>
<td>249 °C</td>
</tr>
<tr>
<td>TGCL</td>
<td>256 °C</td>
</tr>
<tr>
<td>TSOU</td>
<td>199 °C</td>
</tr>
</tbody>
</table>

**Fig. 2** GC parameters entered in the MIS: QUANTITATIVE programme to obtain isothermal conditions (95 °C) for a total period of 4 minutes
Fig. 3 Total ion-current profile of synthesized [2H]$_3$ - MMA. Peak 1 was tentatively identified as dimethylmalonic acid

Fig. 4 El spectrum of the TMS-derivative of [2H]$_3$ - MMA. Comparison with Fig. 4a shows retention of the deuterium label in fragments m/e = 250 and 221

Fig. 4a El spectrum of the TMS - derivative of unlabelled MMA.
After determining the retention time, the measuring time window defining parameters START and DURATION (DUR) were entered to just cover the on-column time of MMA. The short duration time and the high resolving power of the WCOT column ensured that no other interfering peaks were encountered within the measurement window. The same ions were also monitored prior to the elution of MMA to allow adjustments for background noise contributions where necessary.

After entering the accurate mass values and GC parameters shown in Fig. 5, the programme was started by pressing "/" on the keyboard and waiting till the message "READY FOR START" appeared on the visual display unit. A calibration sample was then injected and the space bar depressed on the keyboard. The solvent was diverted for 2 minutes, after which all of the eluent was taken into the mass spectrometer by activating the appropriate switch. Several EI runs were also carried out for both labelled and unlabelled MMA injected separately, to establish percentage cross contribution due to natural isotope abundances, and to calculate the purity of the synthesized internal standard. Results of a typical run are shown in Fig. 6.

![Fig. 5 Parameters entered for EI quantitative MIS](image)

![Fig. 6 Results for EI quantitative MIS of \([2\text{H}]_3 - \text{MMA}\). By averaging the results of five consecutive injections the following results for \([2\text{H}]_3 - \text{MMA}\) were obtained.](image)
Optimum CI conditions (i.e. PCIB = 468 µb) were then obtained for MMA and \([^2\text{H}]_3\) - MMA using iso-butane as reagent gas (Fig. 7). Typical results are shown in Fig. 8. The values found were used in making appropriate adjustments in establishing a calibration curve for CI conditions (Fig. 9).

Patient samples were run under the same conditions as those used for calibration purposes, with the exception that, following the elution of MMA, other higher boiling components were removed from the column by a suitable setting of the oven temperature. Prior to the next run the programme was re-initialized automatically by typing "RN" and pressing the "RETURN" key on the keyboard. A typical set of results for two patients' samples are shown in Fig. 10 to Fig. 13. The results show the close correspondence between values obtained by either CI or EI quantitative MIS and allows double checking of values if the question of interfering ions arises.

---

**Fig. 7** Table of values entered for CI (isobutane) quantitative MIS

<table>
<thead>
<tr>
<th>PCIB</th>
<th>TGCI</th>
<th>PSOU</th>
<th>VELE</th>
<th>CEMI</th>
<th>VSEM</th>
<th>TIME INTENSITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>466 µb</td>
<td>250 °C</td>
<td>0.0 nA</td>
<td>158 V</td>
<td>0.00 mA</td>
<td>2.09 V</td>
<td>100.0</td>
</tr>
</tbody>
</table>

**Fig. 8** Data obtained for unlabelled MMA run in the CI quantitative MIS mode. The result shows that the contribution to m/e = 266 from unlabelled MMA must be taken into account particularly when monitoring high levels of MMA.

**Fig. 9** Calibration curve for methylmalonic acid. Plot of peak height ratios of MMA against constant amount of internal standard (50 µg \([^2\text{H}]_3\) - MMA)

Key:
- □ = m/e 218 versus 221
- ○ = m/e 247 versus 250
- △ = m/e 263 versus 266
**Fig. 10**  Real time visual display (in the Cl quantitative MIS mode), of the ion-currents corresponding to indicated mass values.

**Fig. 11** A typical set of Cl results obtained for different urine extracts. Since the internal standard (m/e = 266.3) is equivalent to 50 µg, the above percentage values give an immediate indication of the relative amount of MMA present in the volume of urine extracted. The upper line shows that the background intensities prior to elution of MMA are negligible.

**Fig. 12** Real-time visual display record in the El quantitative MIS mode of the silylated urine extract 2.
Results and Discussion

The calibration curves obtained by EI/MIS and CI/MIS were straight lines with correlation coefficients obtained by the least-squares method of > 0.99 in each case (Fig. 9). The urinary excretion of MMA as determined by MIS from a methylmalonic aciduria patient on vitamin B12 therapy is presented in Table 1. On the basis of the decrease in the daily MMA excretion, patient 1 was diagnosed as being responsive to vitamin B12. The results obtained for patient 2 indicate a near normal MMA excretion rate.

<table>
<thead>
<tr>
<th>CI/MIS Using 263/266</th>
<th>EI/MIS Using 247/250</th>
<th>EI/MIS Using 218/221</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Patient 1.</strong></td>
<td><strong>µg MMA/ml</strong></td>
<td><strong>mg/day</strong></td>
</tr>
<tr>
<td>Before B12 injection</td>
<td>894</td>
<td>500</td>
</tr>
<tr>
<td>Day 10 after 1st injection of B12</td>
<td>428</td>
<td>152</td>
</tr>
<tr>
<td><strong>Patient 2</strong></td>
<td><strong>4.4</strong></td>
<td><strong>5.7</strong></td>
</tr>
</tbody>
</table>

Table 1 MMA excretion of 2 MMA patients on Vitamin B12 therapy
Instrument: MAT 44 digital GC/MS system

GC conditions: Open-coupled 25 m, SE-54 (Jaeggi) WCOT column, 0.3 mm ID

He flow rate: 2 ml/min

Injection split ratio: 1:10

Column temperature:

(i) Calibration samples: 95 °C Isothermal

(ii) Urine extracts: 95 °C Isothermal for 4 minutes, then 230 °C for 4 minutes

Paper speed: 1 cm/min

MS conditions:

Source: 200 °C

Emission: 0.8 mA (El)

0.2 mA (Cl)

e Energy: 70 eV (El)

150 eV (Cl)

Sample preparation:

A solution of [2H]3-MMA in acetone (50μl, 1 mg/ml) was added to urine (50-1000 μl). The solution was saturated with sodium bicarbonate, extracted twice with ethyl acetate (2 ml) and the organic phase discarded. The aqueous solution was then acidified to pH1 extracted three times with ethyl acetate (2 ml) and the combined extracts dried over Na2SO4. The ethyl acetate solution was then evaporated to dryness and the dry residue heated with bis (trimethylsilyl)trifluoroacetamide (BSTFA) (50 μl) at 65 °C for 30 min.

References


METHYLMALONIC ACIDAEMIA: RAISED URINARY LEVELS
DURING PREGNANCY IN AN OBLIGATE HETEROZYGOTE WITH NORMAL FOETUS

B. Wilcken and C. Pullin*

Oliver Latham Laboratory,
N.S.W. Health Commission, Sydney

*Department of Chemistry,
University of Wollongong, N.S.W.

Methylmalonic acidaemia can readily be diagnosed prenatally. It has been suggested that the finding of raised urinary and/or amniotic fluid methylmalonic acid (MMA) levels in a pregnancy at risk for methylmalonic acidaemia could be used as an indication for therapeutic abortion, without waiting for enzyme studies.

Methylmalonic acidaemia due to methylmalonyl Coenzyme A apomutase deficiency was diagnosed in sisters aged 3 and 4½ years. The parents undertook a further pregnancy and maternal urinary MMA levels were monitored. Amniocentesis was performed at 15 weeks.

Urinary MMA was 3.25 mg/G creatinine at 13 weeks (normal), and ranged up to 11.8 mg/day, 12.7 mg/G creatinine at 33 weeks of pregnancy.

The levels in control pregnant women were under 4 mg/day.

Amniotic fluid studies performed by Dr. M.J. Mahoney indicated normal propionate and vitamin B12 metabolism in the foetus. Further studies performed post-natally confirmed this. Maternal urinary MMA levels was 8.6 mg/day at day 6, and 3.5 mg/day at 10 weeks postpartum.

In this obligate heterozygote for MMA due to apomutase deficiency, increased urinary MMA levels occurred during pregnancy with a normal foetus.
ADIPIC ACIDURIA:
A POSSIBLE NEW INBORN ERROR OF FATTY ACID METABOLISM

R. Truscott, C. Pullin, B. Halpern
Wollongong University, N.S.W.

B. Wilcken
Oliver Latham Laboratory, Sydney, N.S.W.

M. Silink, H. Griffiths, H. Kilham
Royal Alexandra Hospital, Sydney, N.S.W.

and

F. Grunseit
Prince of Wales Children's Hospital, Sydney, N.S.W.

Adipic acid and other short chain dicarboxylic acids occur in normal urine in tiny quantities. Larger amounts are found during moderate to severe ketosis.

Three unrelated boys were admitted to hospital aged 6 - 14 months. All had a history of 2 - 4 days vomiting and were semi-comatose. There was gross hepatomegaly. Serum transaminases were moderately elevated; blood ammonia level was normal in two, and moderately raised in one. In all there was easily correctable hypoglycaemia and metabolic acidosis; urine and plasma amino acids were normal. Gas liquid chromatography of urine confirmed by mass spectrometry revealed no ketones, but massive adipic aciduria, with up to 6000 μg adipic acid/mg creatinine. In all cases recovery was rapid, hepatomegaly regressed, and there was no interval adipic aciduria.

Fasting for sixteen hours reproduced a marked adipic aciduria in two cases, adipic acid being excreted in increasing amounts from eleven hours, but had no effect in two control children.

Adipic aciduria may be caused by an inborn error of fatty acid metabolism in these patients, in one of whom there is a history of death in infancy of two siblings, from unknown cause.
DICARBOXYLIC ACIDURIA: A POSSIBLE DEFECT IN FATTY ACID METABOLISM

C.J. Pullin, R.J.W. Truscott, and B. Halpern
University of Wollongong, N.S.W. 2500

B. Wilcken
Oliver Latham Laboratory, SYDNEY, N.S.W. 2000

M. Silink, H. Griffiths and H. Kilham
Royal Alexandra Hospital, SYDNEY, N.S.W. 2000

F. Grunseit
Prince of Wales Children's Hospital, SYDNEY, N.S.W. 2000

Investigations will be described on three unrelated males aged 6 - 14 months who presented in a semi-comatose state with a history of 2 - 4 days vomiting and diarrhoea. The children were characterized by an easily correctable hypoglycaemia, mild metabolic acidosis and gross hepatomegaly with normal serum transaminases and amino acids.

GC/MS of urinary acid extracts revealed large amounts of adipic, suberic and sebacic acids together with suberyl glycine. Only small amounts of lactic and β-hydroxybutyric acid were present. The above diagnostic GC pattern was not present when the children were well but could be provoked by fasting.

A genetic defect is suggested by the infantile death of two siblings of one patient following a similar pattern of illness. We suggest that these patients may have a defect in short chain acyl CoA decarboxylase which when fat is the major energy source, leads to increased ω-oxidation of fatty acids and a consequent excretion of dicarboxylic acids.