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APPLICATION OF GAS CHROMATOGRAPHY–MASS SPECTROMETRY IN ROUTINE AND RESEARCH IN CLINICAL CHEMISTRY

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SUMMARY

We have worked out a screening procedure which comprises at least four essential steps: the clinical selection of the patients with special problems and the pretreatment before the samples are taken; a gross evaluation of the selected patients with ordinary clinical chemical tests; analysis of samples, usually urine or serum, by the gas chromatographic–mass spectrometric screening system; and further biochemical studies of the abnormal metabolic conditions which may have been discovered.

The mass spectra are recorded by an on-line data system, which greatly facilitates the interpretations. The interpretation of the spectra is also helped by our off-line library matching of an unknown spectrum against 25,000 reference spectra stored in a central computer.

THE RESPONSIBILITY OF CLINICAL CHEMISTRY

Our generation has witnessed an explosive development in the biological sciences —biochemistry, molecular biology, genetics, pharmacology, biophysics, etc. It is to be regretted, however, that these laboratory developments are running far ahead of clinical medicine. The gap between what is possible to do for patients and what is actually being done is steadily increasing. There are several obvious reasons for this gap, for example lack of resources. Of particular significance is, however, the lag period between progress at the scientific frontier and its penetration and introduction into the medical curriculum and postgraduate training of medical personnel.

Clinical chemistry carries a great responsibility in this context. The basic education as well as the daily training of clinical chemists should make them particularly suited for bridging the gap between laboratory and bed-side medicine.

It is no secret, however, that many departments of clinical chemistry have serious difficulties in fulfilling such an obligation. The demands on these departments have increased enormously in the course of the last ten years. In hospitals of 500–1000 beds the clinicians yearly request hundreds of thousands, or even millions, of tests in a diversified spectrum of analyses. The clinical chemists have had to tackle a logarithmic increase in work load, despite lack of trained personnel at all levels.

It is obvious that such a strained and hectic working situation gives little time

and peace of mind for liaison between rapidly advancing research activities and the patient. Because of these difficulties for the clinical chemist, other colleagues at the hospital try to do the job as bridge-builders according to their special interests. This is the main reason why many hospitals in addition to a central laboratory also have a multitude of so-called special laboratories, with a corresponding multiplication of expensive equipment and disintegration of technical resources and milieu.

However, clinical chemistry must not be reduced to a technically advanced assembly line for efficient analysis. The increase in routine demands must level off and competent personnel must be trained. The clinical chemist must be given the opportunity to use his theoretical and instrumental expertise to help the clinicians resolve complicated clinical problems.

THE PLACE OF GAS CHROMATOGRAPHY–MASS SPECTROMETRY IN CLINICAL CHEMISTRY

Most of the work carried out by a clinical chemistry laboratory is performed according to routine procedures organized in detail with respect to speed, efficiency, reproducibility and back-up in case of break-downs. Tests and analyses requested by the clinician are fed into laboratory sections for assembly-line type of work, and the results are fed back to the clinician at the necessary speed.

Gas chromatography–mass spectrometry (GC–MS) in its present form does not fit easily into such a working routine. The equipment is expensive but still unreliable, with frequent instabilities and break-downs. The technique as practiced today is more an art than a standardized routine. A great number of tricks and pitfalls in extraction procedures, preparation of derivatives, packing of columns and running of GC systems are only gradually overcome after long training. The MS procedures with on-line and off-line data processing techniques of course require highly trained personnel. It seems obvious, however, that the overwhelming ability of GC–MS to analyse microamounts of a multitude of components in body fluids or urine samples from patients and its impressive sensitivity and accuracy in establishing chemical structures carries obvious potentialities in clinical chemistry.

GC–MS has been shown to give valuable information in the following areas of clinical chemistry:

(1) Identification of toxic or noxious compounds and determination of their concentration in serum and urine in intoxicated emergency cases. It seems reasonable that at least one GC–MS set-up should be available in emergency hospitals serving cities or areas of some size.

(2) The diagnosis of inborn errors of metabolism. As a great number of such diseases may be diagnosed by such a technique, it can easily be foreseen that the limited screening systems applied to neonates should be completed, or even replaced, by such a technique.

(3) The routine analyses of compounds occurring in minute amounts (*e.g.* hormones) could well be taken over by GC–MS technique.

(4) Pronounced metabolic disturbances might well be evaluated and aetiologically diagnosed by GC–MS analyses of abnormal metabolites. Such an application would have to await research evaluations of the type to be later quoted.

The prime prerequisite for the inclusion of GC–MS in clinical chemistry is

that these laboratories consider the above problems part of their responsibility. In addition to the assembly-line production of tests requested by the clinician, they should also accept the challenge of evaluating particular patients and problems in detail.

The second point is that the GC–MS equipment at present available should be considerably improved. Simpler and less expensive machinery would be required. When a clinical chemistry laboratory has taken on certain responsibilities, the important work of a hospital requires equipment with lesser break-down time than at present is the case. Of particular significance would be the development of mass spectrum libraries comprising compounds of biological interest. Possibly, specialized libraries should be developed comprising groups of compounds, such as, *e.g.*, drugs and drug metabolites, hormones, intermediary metabolites, etc. Also, standardized and stable techniques permitting analyses on a picogram scale would permit clinical chemical investigations on minute tissue biopsies or tissue cultures. The development of GC–MS systems using non-fragmenting ionization techniques (chemical ionization, field ionization and external ionization) would furthermore permit analyses to be performed also on mixtures of compounds.

OUR APPROACH IN THE USE OF GC–MS IN CLINICAL CHEMISTRY

We have been using GC–MS for nearly ten years at our department of clinical chemistry in Oslo. Gradually procedures were developed which to a large extent are determined by the type of work our hospital is doing in accordance with its university affiliation. Thus, as we have no emergency responsibilities, little work has been done in the area of toxic and noxious compounds. Instead, work has been concentrated on the diagnosis of known and unknown inborn errors of metabolism as well as on the evaluation and aetiological diagnosis of pronounced metabolic disturbances of other types.

At present our GC–MS system gives information of 500–800 metabolites in samples from serum or urine. This screening procedure will briefly be reviewed below, and some of our findings in the area of inborn errors of metabolism and other metabolic abnormalities will be mentioned. A lot of our work is currently dealing with the routine screening of inborn errors on samples from patients from all over Europe. Part of the work must, however, be judged as a research evaluation on novel inborn errors or on the possible routine application of GC–MS to other areas of clinical chemistry.

Our screening procedure comprises at least four essential steps, of which GC–MS is only one.

Selection and pretreatment of patients

In the search for inborn errors of metabolism one may either attempt a screening of, *e.g.*, all neonates, or one may attempt a closer examination of clinical cases. The former approach is being carried out to a large extent in many laboratories using simple methods, such as paper or thin-layer chromatography or chemical spot tests. In our experience the sensitive technique of GC–MS should be applied in the latter approach, *i.e.* to selected patients only.

In this work the investigation starts with an alert clinician who observes

particular signs or symptoms and at the same time is informed about our interests and activities. He should be particularly aware of peculiar smells from the body and body fluids of the patient, a hereditary history of similar diseases in the family, mental retardation, lasting acidosis or major deviations in the excretion of end-products of the various metabolic pathways.

We have attempted with our technique to also re-investigate a number of well-defined metabolic disorders such as renal failure, liver failure, untreated cases of pronounced endocrine disorders and various types of acidosis (*e.g.* respiratory, diabetic, renal, acetonaemic acidosis). In these studies, a close contact with a number of departments and a close cooperation with clinicians must obviously be maintained.

In too many cases exciting peaks in the gas chromatograms have turned out to be drugs or drug metabolites. Apart from a great number of well-known metabolites of well-known drugs, we have come across more unexpected urinary constituents such as phthalic acid, which could be traced back to cellulose phthalate in a drug capsule, trichloroacetic acid, from a rectal suppository containing chloral hydrate, N-acetyltryptophan, traced back to an intravenous albumin treatment where, for some reason, the manufacturer had added N-acetyltryptophan as stabilizing agent, and benzyl alcohol, traced back to the heparin solution in use.

Obviously, the minimum requirements for pretreatment of the patient before attempting a detailed study of his metabolic pattern should be: (1) no drug should have been used for the last week; (2) dietary excesses should be avoided for the last days; and (3) the urine and serum samples should be collected in the morning. It seems clear, however, that as the sensitivity and reproducibility of the analytical system is increased, one will be forced to put the patients on a standardized synthetic diet for at least days before serum or urine samples are collected.

Gross evaluation of patients using ordinary clinical chemical tests

The second step in our screening of a patient is to carry out a number of ordinary clinical chemical tests in order to determine in the urine the end-metabolites of the major metabolic pathways. The excretion of electrolytes, acid equivalents, ammonia, urea, creatinine and uric acid is determined. When possible we also determine the acid–base parameters of the blood and serum electrolytes, and determine cations and anions which may serve as a guideline as to the presence of abnormal compounds.

Some of our most interesting observations have been made at this stage, *i.e.* without the use of GC–MS. Thus, on the basis of ordinary tests we described four cases of a novel familial magnesium absorption deficiency in neonates[1], a diseased state where the babies may be saved by increased oral magnesium administration. Also at this stage the new inborn error of metabolism known as familial plasma lecithin:cholesterol acyltransferase (LCAT) deficiency[2] was described in our laboratory. This inborn defect in the transport mechanism of cholesterol has created considerable interest.

The GC–MS screening system

Having selected a patient from clinical and clinical chemical evaluations, the GC–MS system is brought into use. This screening system, which has gradually been developed in the course of several years, attempts to discover major variations in as

many metabolites as possible. The system, which has been described in detail elsewhere[3,4], permits up to eight different GC systems to be run and may give rise to as many as 600–800 GC peaks, depending upon the attenuation of the gas chromatograph. After deproteinization (in 80% ethanol) of the sample and removal of the ethanol in order to avoid transesterification at later stages, the sample is treated according to Fig. 1. Urine and spinal fluid can be analysed directly. The system is a collection of well established procedures and is not at all particularly advanced. We are working to the classical macroscale of 10^{-6} to 10^{-7} g and using flame ionization

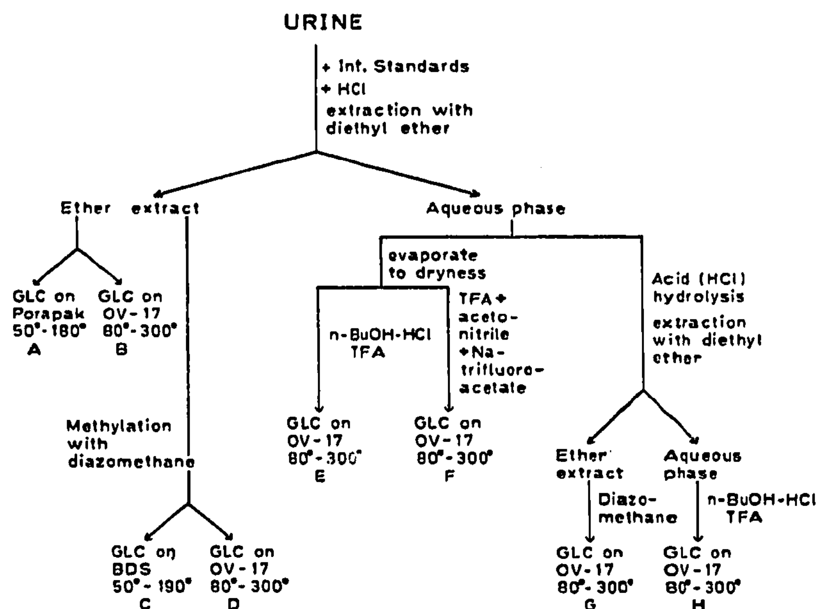

Fig. 1. Principles of the GC procedures.

detectors to visualize peaks in the GC. Reproducibility of the procedure is promoted by using a few well-trained technicians and by standardizing all the steps of the procedures. In particular, the GC columns are prepared to be as identical as possible. The columns are kept clean by packing the first and the last 10 cm with inert carrier material only, and this is frequently changed. A total of six GC set-ups with the desired type of column packing is available.

After the addition of internal standards (*n*-eicosane, trehalose and *α*-amino-octanoic acid) and acidification the sample is divided into an ether extract and an aqueous phase.

The ether extract is run on Porapak and OV-17 in order to separate very volatile and somewhat less volatile lower alcohols, acetone, acetaldehyde and fatty acids as well as hydrocarbons and certain barbiturates.

After methylation with diazomethane the ether extract is run on BDS and OV-17 to separate aliphatic and aromatic acids, certain phenols and di- and tri-carboxylic acids (when extracted).

Part of the aqueous phase is converted into *n*-butyl N-trifluoroacetyl (N-TFA) derivatives of amines and amino acids. In another part the carbohydrates are converted to O-TFA derivatives.

Some indications as to the presence of high-molecular-weight compounds and conjugates are obtained by acid hydrolysis of the aqueous phase followed by GC of a methylated sample or of *n*-butyl ester TFA derivatives.

The gas chromatograms are evaluated visually and all major or unexpected peaks are identified on a Varian Model CH 7 low-resolution mass spectrometer (Varian-Mat, Bremen, G.F.R.). Previously, the mass spectra were recorded manually. Recently, this difficult and time-consuming procedure was replaced by an on-line data system (Spectro-System 100 MS; Varian-Mat, Bremen, G.F.R.). This system permits repetitive scanning with storage of the recorded spectra on magnetic tape followed by a subsequent computer analysis which permits, *inter alia*, recording of the gas chromatograms (total ion current), mass chromatography, normalization of spectra, subtraction of background spectra and oscilloscope display of several spectra simultaneously for comparative purposes. (see Fig. 2).

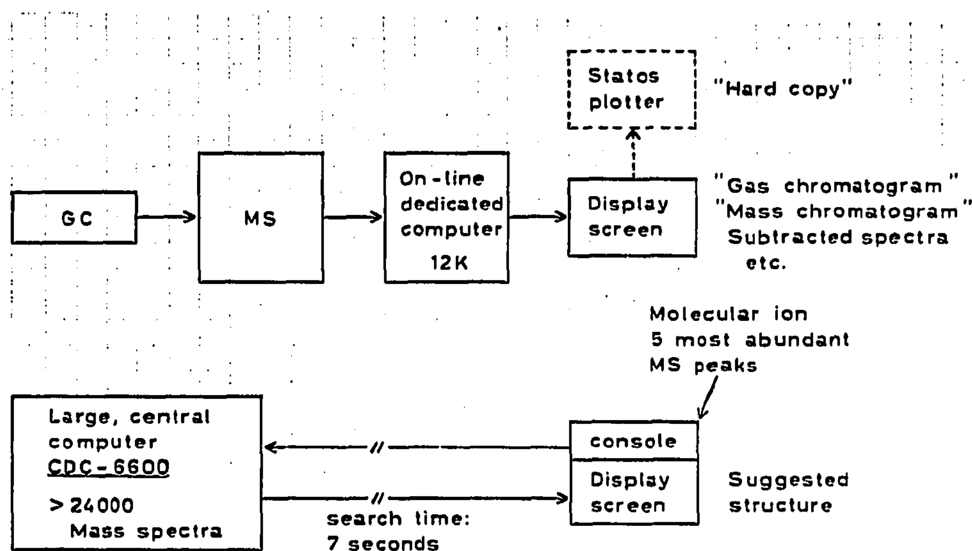

Fig. 2. The GC–MS–computer system.

Interpretation of the spectra is greatly helped by our off-line library matching of an unknown spectrum against a reference file of more than 25,000 abbreviated spectra stored in a fast and large central computer (CDC-6600). This file, which regrettably contains a lot of compounds of remote biological interest, originates from available computations[5–7]. About 500 spectra are our own recordings. Matching of an unknown mass spectrum against the reference file can be carried out by using the five strongest peaks either in order of decreasing intensity or at random. Concordances with all peaks, with four peaks or with only three peaks may be displayed. Also a

search for spectra with identical apparent M^+ can be carried out. This computer-matching is indicative only, and the final verification of the structure of an unknown compound is always based upon comparison with the spectrum of authentic compounds. Quite often one also has to carry out several chemical tests and chemical modifications of the original unknown molecule in order to elucidate its structure. High-resolution mass spectrometry is also available and has sometimes been used in our work.

The search time for the complete mass spectrum file of 25,000 requires about 7 sec. However, the complete GC–MS system as visualized in Fig. 1 permits a maximum of two to three patient samples to be run per day.

Further study of the abnormal metabolic conditions discovered

In many instances our system helps in confirming or excluding the diagnosis of well-known diseases or inborn errors of metabolism. In these cases our work with the patient usually stops after the interpretation of the GC–MS recordings.

In a number of instances, however, we have discovered new inborn errors of metabolism in our patients, or we have found unexpected metabolites in clinically well-known disturbances of metabolism. Such findings have precipitated medical and biochemical problems which have been further investigated in our laboratory by a variety of biochemical or physiological techniques carried out *in vivo* in man or mammals and *in vitro* on fibroblast cultures, tissue slices, cell subfractions or purified enzymes.

RESULTS OF OUR STUDIES ON INBORN ERRORS OF METABOLISM

About 1500 different enzymes have been characterized in mammals. As no regulator, repressor or operator gene or cistron could be imagined safe from mutation, it is generally felt that the number of inborn errors which may occur should be as large. The number actually described at present is approximately 150 diseases only. Probably a great number of enzyme defects will be incompatible with a complete pregnancy because they affect cofactors or structures essential for cell life (ATP production, DNA replication, cell division, membrane structure, etc.). Such defects should probably be sought as "unborn" errors of metabolism among early abortions. No doubt, the total number of inborn and unborn errors of metabolism could be expected to be far greater than the 150 diseases so far described.

The GC–MS screening system described above may detect about forty of the known inborn errors of metabolism (Table I). Several of these have been found by our screening procedure in the course of the last few years, usually as a verification of clinical diagnosis.

Particularly challenging and rewarding to us has been the fact that our screening system has disclosed several novel metabolic defects. Also the procedure of GC–MS has contributed considerably to our work on biochemical defects in previously described diseased states.

Refsum's disease

Klenk and Kahlke[8] described in 1963 the accumulation of phytanic acid —a branched hexadecanoic acid of isoprenoid structure— in a patient suffering from

TABLE I

INBORN ERRORS OF METABOLISM DETECTABLE BY THE SCREENING SYSTEM

Name of disease	*Compounds detectable by the GC–MS methods*	*Name of disease*	*Compounds detectable by the GC–MS methods*
Alcaptonuria	Homogentisic acid	Hypertryptophanaemia	Tryptophan
Carnosinaemia	Carnosine	Hypervalinaemia	Valine
Congenital lactacidosis	Lactic acid	Isovaleric acidaemia	Isovaleric acid, β-hydroxyisovaleric acid, isovalerylglycine
Cystathioninuria	Cystathionine		
Cystinuria	Cystine		
Diabetes mellitus	Glucose, β-hydroxybutyric acid, acetoacetic acid	Maple syrup urine disease	Valine, leucine, isoleucine, α-ketoisovaleric acid, α-ketoisocaproic acid, α-keto-β-methylvaleric acid
Essential fructosuria	Fructose		
Essential pentosuria	L-Xylose		
Galactosaemia	Galactose, amino acids	Methylmalonic acidaemia	Methylmalonic acid
L-Glyceric aciduria	L-Glyceric acid, oxalic acid	Non-ketotic hyperglycinaemia	Glycine
Hartnup's disease	Neutral amino acids		
Histidinaemia	Histidine, imidazoleacetic acid	Oast-House disease	α-Hydroxybutyric acid
		Ornithinaemia	Ornithine
Homocystinuria	Homocystine, methionine	Orotic aciduria	Orotic acid
β-Hydroxyisovaleric aciduria and β-methylcrotonylglycinuria	β-Hydroxyisovaleric acid, β-methylcrotonylglycine	Phenylketonuria	Phenylalanine, phenylpyruvic acid, phenyllactic acid, *o*-hydroxyphenylacetic acid
Hydroxylysinuria	Hydroxylysine		
Hydroxyprolinaemia	Hydroxyproline	Propionic acidaemia	Propionic acid
Hyper-β-alaninaemia	β-Alanine, β-aminoisobutyric acid, γ-aminobutyric acid	Pyroglutamic aciduria	Pyroglutamic acid (pyrrolidone-2-carboxylic acid)
Hyperlysinaemia	Lysine	Refsum's disease	Phytanic acid
Hypermethioninaemia	Methionine, α-keto-γ-methiolbutyric acid	Renal glycosuria	Glucose
		Short-chain fatty acidaemia	Butyric acid, caproic acid
Hyperoxaluria	Oxalic acid, glycolic acid, glyoxylic acid	Tyrosinosis	Tyrosine, *p*-hydroxyphenylpyruvic acid, *p*-hydroxyphenyllactic acid
Hyperprolinaemia	Proline		
Hypersarcosinaemia	Sarcosine		

Refsum's disease (heredopathia atactica polyneuritiformis). GC–MS played an essential role in our studies on two patients in Oslo suffering from this disease (Fig. 3). We found the accumulation of phytanic acid to be specific for Refsum's disease in a screening of more than 100 patients with various neurological disorders[9]. In studies where MS was used for the analysis of deuterium it was found, in cooperation with Steinberg's group in Bethesda, that enrichment of the total body water with $^{2}H_2O$ for six months resulted in incorporation of deuterium in cholesterol in a predictable way whereas only trace amounts accumulated in phytanic acid isolated from serum[10]. This experiment excluded endogenous synthesis of phytanate. Further studies showed that the phytol fragment of the chlorophyll molecule and phytanic acid in various foods are the sources of serum phytanic acid. By diet free from such constituents, phytanate disappeared from the serum of our two Oslo patients[11]. Since 1967 their serum and organ samples have shown only the trace amounts of phytanate normally present. As no relapses have occurred during the fifteen patient-years of observation in these two patients, and as some improvement particularly in nerve conduc-

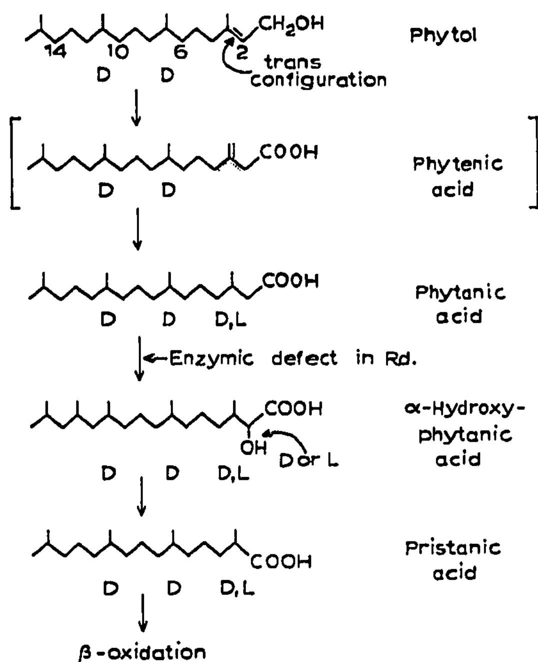

Fig. 3. Formation, degradation and stereochemistry of phytanic acid. R.d. = Refsum's disease.

tivity has been observed, it seems reasonable to conclude that most or all of the clinical symptoms of this disease are caused by phytanate accumulation.

Three research groups simultaneously showed that phytanate accumulation in these patients was due to an inborn enzyme defect in a hitherto not recognized α-oxidation mechanism existing in mammals[12–14]. Our approach in this work was to synthesize ^{14}C-labelled acids containing methyl branchings which prevented ω- and β-oxidations and to test their metabolism in the patients. The test compounds (^{14}C-labelled 3,6-dimethyloctanoic acid and 3,14,14-trimethylpentadecanoic acid) were shown by GC–MS to be degraded in man by a new, alternative metabolic pathway which was found to be an α-oxidation mechanism shortening the fatty acid carbon chain from the carboxyl end by CO_2 removal. This α-oxidation mechanism converts phytanic acid (C_{16}) into pristanic acid (C_{15}), which can be subjected to β-oxidation. Refsum's disease was shown to be an inborn error in this α-oxidation mechanism[14].

Methylmalonic acidaemia

In 1967, independently of Oberholzer *et al.*[15], we described methylmalonic acidaemia in a neonate who in the course of the first 48 h of life developed severe metabolic acidosis[16]. Based on the occurrence of large amounts of glycine in the serum of the child, we pointed out that patients previously diagnosed as having hyperglycinaemia actually may suffer from methylmalonic acidaemia[16,17].

Immediately on finding large amounts of methylmalonic acid in the serum and urine of the patient, we instituted cyanocobalamine treatment, but without apparent effect. A diet low in propionic acid precursors significantly reduced the

urinary excretion of methylmalonic acid and led to a considerable improvement in the clinical condition. However, the patient developed a severe infection and died of septicaemia.

After the death of another neonate we were asked to investigate a small amount of serum (1.5 ml) which was the only sample left of the patient[18]. Large amounts of methylmalonic acid made it likely that the patient had died from methylmalonic acidaemia. Upon closer examination of the serum by thin-layer chromatographic separation of the ether extract followed by GC–MS, we found in addition large amounts of propionic acid and a new metabolite, β-hydroxy-*n*-valeric acid. Moreover, the fatty acid pattern of the serum after hydrolysis showed an increased amount of odd-numbered acids (pentadecanoic and heptadecanoic). Probably, these, as well as β-hydroxy-*n*-valeric acid, stem from condensation reactions where propionyl-CoA may compete with acetyl-CoA. Accumulation of propionic and odd-numbered fatty acids has not previously been described in patients with methylmalonic acidaemia in which the enzyme defect is believed to be located in the methylmalonyl-CoA mutase step (Fig. 4).

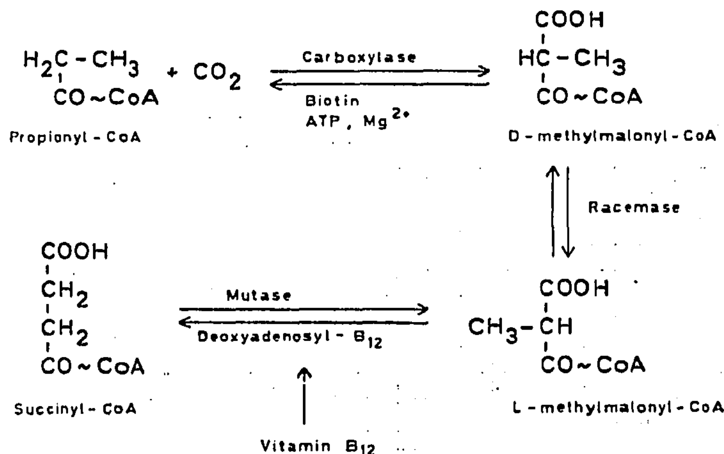

Fig. 4. Degradative pathway of propionyl-CoA. The patient with β-hydroxy-*n*-valeric acidaemia had a block at the racemase step.

β-Methylcrotonyl-CoA carboxylase deficiency

Three years ago we were presented with urine and serum samples from a girl, four months of age, with symptoms resembling those of Werdnig–Hoffmann's disease. The urine of the child had a peculiar smell, resembling that of cat's urine. We found that the child excreted large amounts of β-hydroxyisovaleric acid and β-methylcrotonylglycine[19]. It was concluded that she was suffering from a novel metabolic disorder with a deficiency at the β-methylcrotonyl-CoA carboxylase step of leucine degradation (Fig. 5). The administration of moderate amounts of biotin did not improve her status. On a diet containing the minimum requirement of leucine, the excretion of the abnormal metabolites rapidly dropped. However, the child died from pneumonia at the age of nine months. A child suffering from a similar metabolic disorder was later described by Gompertz *et al.*[20]. In this case large amounts of biotin apparently cured the child, eliminating the excretion of abnormal metabolites.

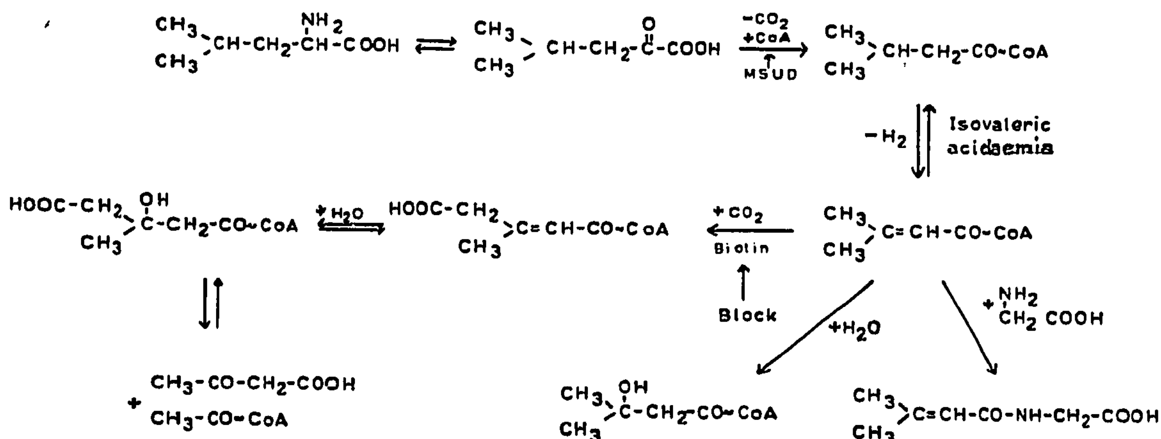

Fig. 5. Degradative pathway of leucine. The block in the patient with β-methylcrotonyl-CoA carboxylase deficiency and the defects in maple syrup urine disease (MSUD) and isovaleric acidaemia are indicated.

Pyroglutamic aciduria

In 1970 we found a novel metabolic error in a 19-year-old mentally retarded male suffering from retarded psychomotoric development, slight spastic tetraparesis and metabolic acidosis[21]. He was found to excrete in the urine 30–40 g of L-pyroglutamic acid (L-2-pyrrolidone-5-carboxylic acid) daily and his metabolic acidosis could be shown mainly to be due to the accumulation of L-pyroglutamic acid in extracellular fluid. By tracer experiments it could be shown that the patient in fact produced of the order of 60–80 g per 24 h of L-pyroglutamic acid, *i.e.* about two times more than the amount excreted in the urine. The 30–40 g per 24 h not excreted seems to have been degraded via glutamic acid (Fig. 6).

When a new metabolic error is discovered by the accumulation or excretion of abnormal metabolites, one can usually identify immediately the enzyme involved because of detailed knowledge of mammalian metabolism. This is not the case with

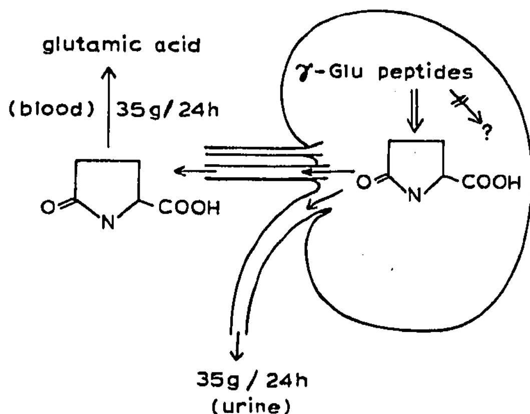

Fig. 6. The most likely explanation for the formation and metabolism of pyroglutamate in a patient with pyroglutamic aciduria.

pyroglutamic aciduria. Where in the mammalian metabolism do these large amounts of L-pyroglutamate originate? Are such amounts normally formed as intermediates in the metabolism of man? Our studies indicate that the inborn error in this patient is not related to urea formation or to ammonium ion formation by the kidneys. Of the known routes of formation of pyroglutamic acid in mammals, only γ-glutamyl substrates seem capable of producing the amounts seen in the patient. A defect could be envisaged in the metabolism of γ-glutamyl peptides, possibly related to an amino acid γ-glutamyl cycle[22,23].

It should be stressed that pyroglutamic acid is ninhydrin-negative and will therefore not be detected by the ordinary routine screenings of inborn errors related to amino acid metabolism. Several more cases may therefore have been overlooked. Recently, a second patient suffering from a similar disease was found in Sweden[24].

STUDIES ON CLINICALLY WELL-DEFINED DISTURBANCES OF METABOLISM

GC–MS techniques offer a rich opportunity to carefully re-investigate a number of well-defined metabolic disturbances such as renal failure, liver failure, metabolic acidosis and alkalosis, pronounced endocrine disorders, etc. Such investigations may open up new areas for the use of this technique in routine clinical chemistry.

Some of our results from such studies are given below.

In clinical terms acidosis or alkalosis are classified as respiratory or metabolic, and their degree measured by various parameters such as blood pH, pCO_2 and standard bicarbonate. When investigating serum and urine samples from such patients more carefully, using the GC–MS technique, it was found that all patients with ketotic acidosis excrete substantial amounts of adipic (C_6) and suberic (C_8) acids[25]. The daily excretion of these dicarboxylic acids parallelled the degree of ketosis and reached levels of 750 and 150 mg, respectively. In some cases succinic and glutaric acids were found in amounts of 1200 and 170 mg per day, respectively. In experiments with [1-^{14}C]- and [16-^{14}C]hexadecanoic acids strong indications were found that the dicarboxylic acids originate from long-chain monocarboxylic acids, which are first ω-oxidized to long-chain dicarboxylic acids and subsequently shortened by a mitochondrial β-oxidation. For some reason the dicarboxylic acids leak out of the mitochondria at chainlengths of six to eight carbon atoms.

In patients suffering from lactic acidosis another abnormal metabolite was excreted, *viz.* 2-hydroxybutyric acid[26]. The clinical significance and metabolic origin of this compound are at present under investigation. Possibly, the 2-hydroxybutyrate originates from 2-oxobutyrate, which may be a normal intermediate in the degradation of some amino acids.

Toxic products present in glucose–fructose mixtures used for intravenous nutrition[27]

Very recently we were confronted with a one-month-old baby suspected of having a metabolic disease. For several days the baby was given intravenous infusions of a glucose–fructose mixture supplemented with amino acids, electrolytes, vitamins, and lipids.

GC–MS analyses revealed large quantities of 5-hydroxymethyl-2-furoic and furan-2,5-dicarboxylic acids in the urine of the patient. Both these compounds have been shown to be present in small quantities in normal adult urine. Urine samples

collected a few days after stopping the intravenous nutrition did not contain the furan derivatives.

As it is well known that glucose and, in particular, fructose readily yield furan derivatives on heating at acid pH, it was thought that the furan derivatives in the urine of this patient might stem from the glucose–fructose mixture used for parenteral nutrition. GC–MS analysis of the sugar mixture showed indeed that 5-hydroxymethyl-2-furaldehyde, 2-(2′-hydroxyacetyl)-furan and laevulinic acid were all present in considerable quantities (120 mg/dl, 9 mg/dl, and 6 mg/dl, respectively) in the sterile glucose–fructose solution. These compounds, as well as a fourth contaminant that we were able to identify, 2-keto-3-deoxyglucose, are all formed when fructose is heated at acid pH, *e.g.*, as is the case during heat sterilization (Fig. 7).

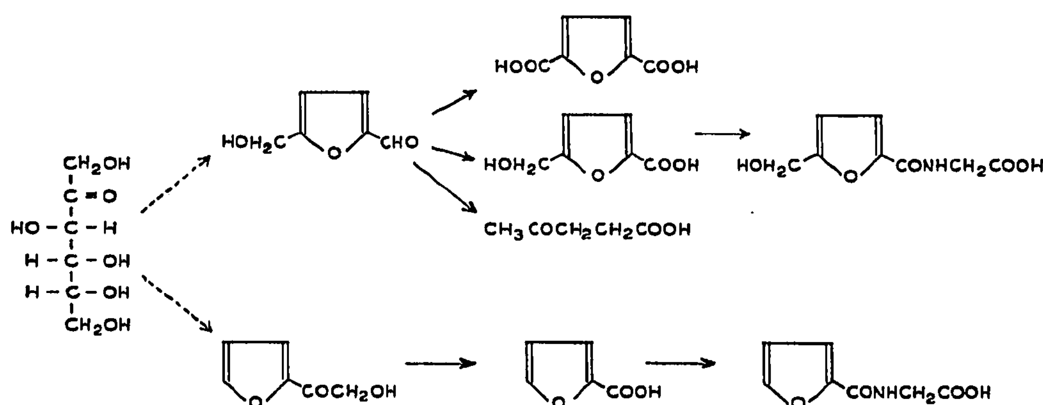

Fig. 7. Formation of furan derivatives from fructose by heating at acid pH.

Quantitative studies showed that about 50% of the infused 5-hydroxymethyl-2-furaldehyde had been metabolized in the body of our patient and was excreted in the urine in more oxidized forms, *viz.*, as 5-hydroxymethyl-2-furoic and furan-2,5-dicarboxylic acids. The glycine conjugates of both these compounds were absent from the urine. The remaining 50% of the infused furan derivatives was left in the body, probably bound to proteins.

It should be pointed out that aldehydes in general are reactive compounds capable of interacting with the thiol and amino groups of proteins. Because of these properties aldehydes actually may block SH-groups essential for cell division, and thus act as cytotoxic agents. Thus, it is clear that 5-hydroxymethyl-2-furaldehyde and 2-(2′-hydroxyacetyl)-furan are potentially toxic compounds one would like to avoid in mixtures used for intravenous nutrition. These compounds are inevitably formed in considerable quantities if a fructose-containing solution with a pH lower than 3.5 is heated to 110–130° for 1 or 2 h. Such conditions, which unfortunately seem to be employed by some pharmaceutical firms for sterilization of their sugar mixtures, should consequently be changed.

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