Mass spectrometric assay of stable isotopic enrichment for the estimation of protein turnover in man

By D. HALLIDAY and W. W. C. READ, *Nutrition Section, Clinical Research Centre, Watford Road, Harrow Middlesex HA1 3UJ*

The object of this communication is to focus attention on the design and analytical capabilities of isotope ratio and organic mass spectrometers and their potential areas of application to the study of amino acid and protein metabolism in man using stable isotopes.

The isotope ratio mass spectrometer

Dual inlet double collector gas isotope ratio mass spectrometers by inherent design, possess a low resolving power but extremely high sensitivity. These specialized instruments are capable of precise estimates of the heavy isotopic content of pure, low molecular weight permanent gases such as H_2, CO_2, N_2 and SO_2. For present purposes, consideration will be given only to the heavy non-radioactive isotopes of carbon and nitrogen.

The low resolution is due primarily to the very short analyser tube (and thus the ion beam path length), the width of the final resolving slits and the low strength of the permanent magnet. The high sensitivity is achieved by directly comparing the isotope ratio of the sample gas to that of a reference gas. The complete gas inlet system, situated in front of the analyser tube, is characteristically composed of variable volume bellows (reservoirs), metal capillaries and an automatic all metal changeover valve. The bellows permit equalization of pressures with respect to sample and reference gas as the ratio ultimately obtained is pressure dependant; the capillaries, crimped at their distal end, convert viscous to molecular gas flow such that the isotopic content of the gas entering the analyser is identical to that in the reservoirs. Finally, the changeover valve serves two purposes; it permits alternate entry of sample and reference gas into the analyser on a strict time basis and simultaneously permits the gas not entering the analyser to be bled away at an equal rate, thus maintaining the status quo with respect to pressure between the reference and sample gases.

The mass spectrometer itself consists of the analyser (flight tube) that houses an ion source, ion collectors and a deflection region (shown in Fig. 1). If one takes nitrogen gas as an example, the objective of isotopic analysis is to separate the m/e 28 (major) and 29 (minor) ion species (N retains its diatomic molecular structure) such that they can be collected and measured separately and a ratio of their intensities obtained. On entering the ion source gas molecules are ionized in a stream of electrons, extracted from the source under the influence of an accelerating voltage as a mixed ion beam, which is segregated on passage through the magnetic field into component ion beams of fixed mass:charge value. The

0029–6651/81/4033–5702 $01.00

segregated ion beams are focused into individual deep Faraday buckets, having passed through major and minor beam resolving slits and supressor electrodes which act both to minimize ion drift between the collectors and to reject any secondary electrons formed by ion collisions. The impact of the major and minor ion beams in their respective collector buckets produces a current. This original signal is amplified at two stages, transformed into a usable voltage and thence fed into voltage to frequency converters whose output pulse rate is linearly proportional to the input voltage. These pulses are fed into separate major and minor beam counters and the desired ratio obtained.

Translated into practical 'black box' terms the above type of system provides the basis for comparing the isotopic ratio of reference and sample gases under as nearly identical conditions as possible. Under optimal conditions an isotope ratio mass spectrometer can detect one ^{13}C or ^{15}N atom in 10^5 unlabelled atoms difference in the isotopic content of two gases. This available sensitivity can only be fully exploited if sufficient attention is given to the preparative stages involved in producing gas samples for isotopic analysis.

Sample preparation and isotopic fractionation

Despite extensive use of ^{15}N-labelled compounds in clinical investigations sample preparation techniques have undergone few significant developments in the

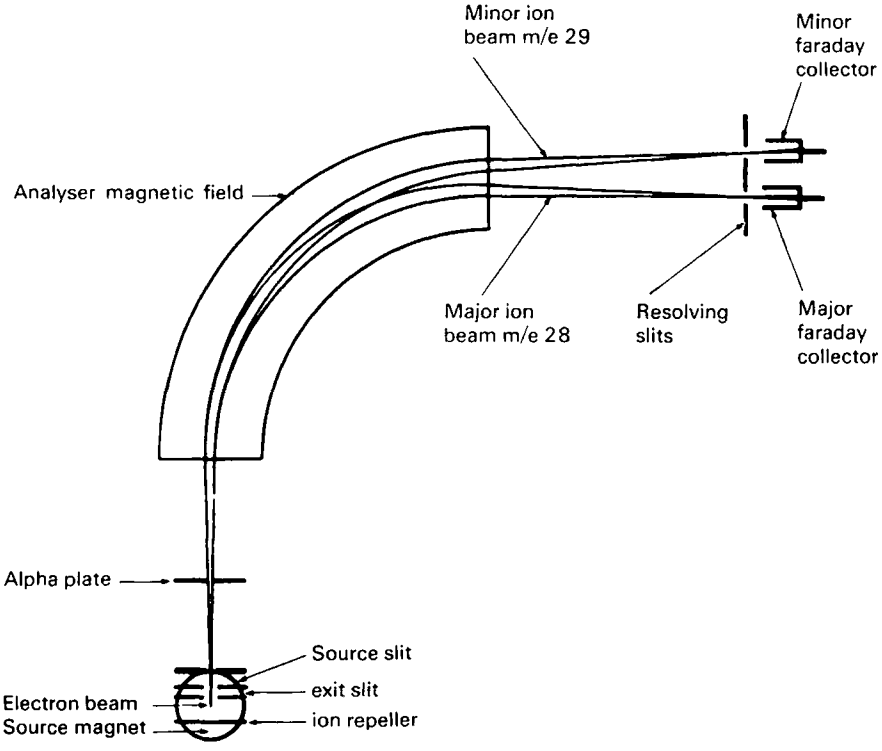

Fig. 1. Isotope ratio mass spectrometer analyser tube depicting the positional relationship of source, collectors and focusing plates.

https://doi.org/10.1079/PNS19810048 Published online by Cambridge University Press

last few decades. Essentially the molecular N_2 required for isotopic analysis is obtained by either Dumas combustion or the Kjeldahl-Rittenberg techniques (Rittenberg *et al.* 1948). In the latter case the ammonium salt resulting from the Kjeldahl digestion is recovered and oxidized to molecular N_2 by the action of sodium or lithium hypobromite. Investigation of N metabolism in man using ^{15}N-labelled amino acids ultimately requires measurement of the isotopic content of urinary ammonia, urea or α-amino N, and often the corresponding plasma components. The classical methods for the isolation or purification or both, of these components are at best laborious and at worst may result in an unacceptable level of isotopic fractionation. In our hands the aeration method for extracting NH_3 from urine requires 3 h to displace in excess of 90% of the NH_3 whilst the often used xanthydrol derivative of urea is difficult to recrystallize from solution. The use of urease to produce NH_3 from urea must be followed by an aeration or distillation process which again may not result in full recovery of the product. Whilst ion exchange resins have frequently been used to remove ammonia from urine as a clean-up procedure, the use of a resin which specifically combines with NH_3 has not been used in isotope work, to our knowledge. Hutchinson & Laby (1962) reported the use of the $^{+}Na/^{+}K$ form of Dowex 50 to extract ammonia from whole blood and thence treated the resin directly with Nessler's reagent to quantitate blood ammonia. We have developed and extended this principle to bind ammonia to the $^{+}Na/^{+}K$ form of Bio-Rad AG50-X8. When treated with sodium or lithium hypobromite the NH_3 is liberated quantitatively from the resin. Similarly, NH_3 liberated from urea by the action of urease or from α-amino acids following ninhydrin treatment may be handled in a similar fashion using a modification of a method described by Kennedy (1965). The appeal of this resin system lies in the stability of the resin-ammonia complex, its specificity, and the ease of batch processing samples. To date only methylamine, rarely present in urine, has been found to interfere with isotopic measurements. A generalized scheme of application of this resin system is given in Fig. 2.

The oxidation rate of ^{13}C-labelled amino-acids can be quantitated with a knowledge of the $^{13}CO_2$ enrichment in expired air and of total carbon dioxide production rate. In this instance sample preparation is a simple procedure as the 'combustion' has occurred in vivo and the required CO_2 may be differentially cryogenically trapped from expired air.

It is pertinent at this juncture to strike a cautionary note in relation to any protocol that requires the measurement of the isotopic content of $^{13}CO_2$ in expired air. This measurement is performed against a natural ^{13}C abundance of about 1·1%. Dietary constituents derived directly or indirectly from plant material can exhibit relatively large fluctuations in natural abundance (1·06–1·12%) which is a reflection of the enrichment or depletion of ^{13}C relative to ^{12}C as a result of isotopic fractionation. In higher plants isotopic fractionation occurs during the photosynthetic fixation of carbon dioxide with the ultimate formation of carbohydrate, which may occur by one of two synthetic pathways. The Calvin-Benson pathway results in the formation of a 3-carbon intermediate (C_3) whilst

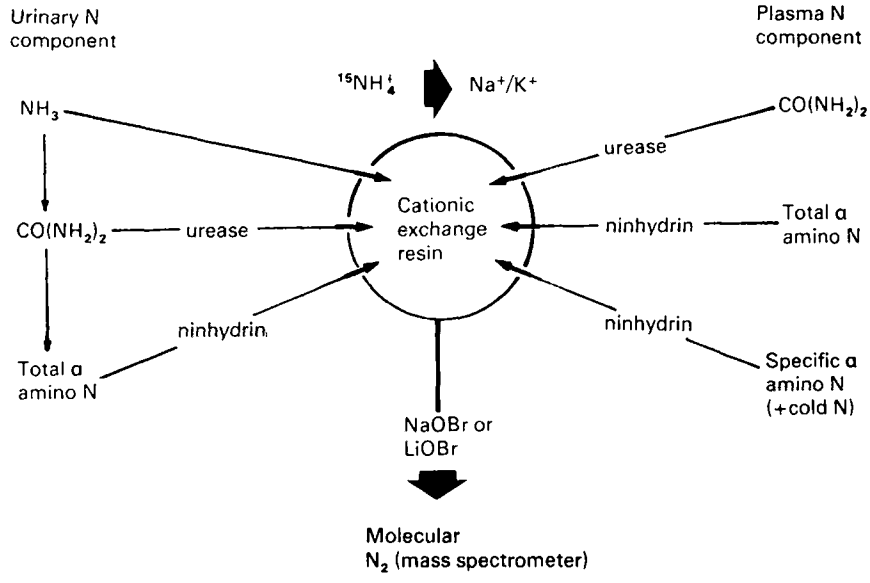

Fig. 2. Some areas of application using a resin ammonia complex with direct hypobromite reaction for the batch preparation of molecular N_2.

the alternative (C_4) or Hatch-Slack pathway proceeds through a 4-carbon intermediate. The carbohydrate formed by these two pathways differs markedly in ^{13}C content; C_3 plants (potato, sugar beet) having a lower enrichment than C_4 plants (sorghum, sugar cane, maize). Further discrimination against ^{13}C occurs in the conversion of carbohydrate to amino acids and fatty acids with the result that within a given system, lipid has a lower ^{13}C content than protein which itself is lower than that of carbohydrate. The natural $^{13}CO_2$ of expired air therefore will represent the mean of ^{13}C content of substrates being oxidized and it is necessary to ascertain this level prior to administration of any labelled compound. For human studies in the fasted state knowledge of likely baseline $^{13}CO_2$ fluctuations have been reported (Schoeller *et al.* 1977); in the fed state it is preferable to select, as far as possible, an isotopically neutral food.

This concept has general application in all isotope studies involving the measurement of enrichment in end products. Human tissues will reflect the isotopic ratios of their major food sources (Gaffney *et al.* 1978), suggesting that isotopic fractionation resulting from difference in rates of reaction and diffusion and equilibrium constants are minimal within the body. The available analytical instrumentation can detect these effects which therefore require evaluation in terms of 'zero' or 'baseline' samples when measuring small increments of enrichment in tissue samples or metabolic end products following tracer administration. This is equally important irrespective of whether ^{13}C- or ^{15}N-labelled compounds are the investigational tool.

https://doi.org/10.1079/PNS19810048 Published online by Cambridge University Press

Expression of isotopic enrichment, correction factors and sample size

Isotopic enrichment may be expressed in either of two forms; atom % excess which is widely used by biologists and clinicians, or alternatively in the del per ml nomenclature (δ^0/oo), favoured from the outset by environmentalists whose work is committed to detecting small naturally occurring isotopic differences. Calculation of these units for N is given below:

$$\text{atom \% excess} = \frac{R_S}{2+(R_S \times 10^{-2})} - \frac{R_R}{2+(R_R \times 10^{-2})}$$

$$\delta^0/oo = \frac{R_S - R_R}{R_R} \times 10^3$$

where R_S, R_R represent the m/e:$^{29}/_{28}$ ion intensity ratio of sample and reference gas respectively.

The only realistic instrumental correction of mass spectrometric data is for changeover valve 'crosstalk' between sample and reference gas (Mook & Grootes, 1973). The reference gas may be an international standard or more usually a convenient secondary laboratory standard. The use of PTFE greaseless taps and 'O' ring joints in vacuum preparation lines reduces the risk of atmospheric contamination (and thus the need to apply an argon correction) which may only become significant when preparing N samples in the 10–50 μg range. When high ^{15}N enrichments are measured (>1·0 atom % excess) the m/e 28, 29 and 30 intensities should be monitored as the relative amounts of these species are in a binomial distribution, $a^2:2ab:b^2$, where a and b represent the relative abundances of the two lighter isotopes. For precise $^{13}CO_2$ measurements a correction factor for oxygen isotopes in the sample may be applied (Craig, 1953).

The ability to determine isotopic enrichment with high sensitivity and precision permits the use of tracer levels of labelled amino acids in the knowledge that specific aspects of their metabolism can be monitored over considerable periods. It thus follows that investigations can become more cost effective as ^{13}C- and ^{15}N-labelled amino acids in general are not insignificantly priced. A final bonus arises from the fact that the mass spectrometer can be used as an accurate gas meter. That is to say, after calibration, it may be used to quantitate the original compound from which the analysed gas originated. Set against these obvious advantages, the limiting factor in areas of biological application of this instrumentation, is one of sample size. Realistically 0·1–1 mg of CO_2 or N_2 gas is required for optimum isotopic analysis on a routine basis. With some loss of accuracy and precision it is possible to reduce this sample size to 10 μg of the gas by invoking the use of cryogenic trapping agents or molecular sieves to confine the sample in a smaller initial volume, or ultimately exclude the entire inlet system and inject the sample directly into the analyser source. These alternatives however in no way constitute routine analysis for large numbers of samples. The limits of application of an isotope ratio mass spectrometer in relation to amino acid and protein metabolism studies are therefore imposed by the advantages and limitations outlined above.

Area of instrument—protocol application

[^{15}N]glycine is the amino acid that has been most widely employed to study dynamic aspects of whole body protein turnover in man since the pioneering work of Schoenheimer and Rittenberg some 40 years ago. Tracer administration of this ^{15}N-label has been as a single oral or intravenous pulse dose, constant intravenous infusion or by repetative oral feeding. Theoretical considerations of all these methods aimed at estimating whole body protein turnover in a variety of physiological and diseased states have been comprehensively reviewed (Waterlow *et al.* 1978). Without exception these methods depend on measurement of isotope excreted in an end product in the urine (urea or ammonia). Whether these estimates are calculated from the rate of change in end product labelling with time (compartmental analysis) or cumulative excretion of isotope (stochastic analysis) the enrichment measured will inevitably be low as a result of vast dilution within relevant body pools. Similar quantitative considerations apply when expired $^{13}CO_2$ is used to calculate amino acid oxidation rate, and indirectly protein turnover, following the administration of a ^{13}C-labelled amino acid. The isotope ratio mass spectrometer is thus ideally suited for these types of investigation where sample size is not a limiting factor but sample isotopic enrichment is low and requires accurate measurement.

An investigational protocol involving a constant infusion of a ^{13}C-carboxy and/or ^{15}N-amino labelled amino acid, if of sufficient duration, may permit detection of the enrichment in that amino acid incorporated into a specific protein or group of proteins. For example, it is possible, from approximately 10 mg of muscle protein (percutaneous biopsy) or plasma albumin, to purify the required fraction, hydrolyse the protein and isolate the amino acid of interest. The $^{13}CO_2$ can be liberated with ninhydrin and trapped cryogenically and the $^{15}NH_3$ subsequently distilled out and picked up on the $^{+}Na/^{+}K$, as prescribed previously. If the isotopic enrichment of the precursor pool from which synthesis occurs is independently measured then fractional protein synthetic rates of the above proteins can be calculated.

The possibility exists of performing isotopic enrichment assays on single plasma derived amino acids with the methodology outlined above (Halliday & McKeran, 1975). The sample preparation techniques involved and blood sample requirements, however, render this over-all protocol totally inappropriate for routine clinical studies. Blood sample size can be markedly reduced if the plasma amino acid of interest is initially isolated, quantitated and diluted with 'cold' acid ultimately to provide sufficient gas for isotope ratio mass spectrometric analysis. However, the procedures involved are protracted and one has incurred the additional errors of quantitation and dilution. They have been recently superseded by the development of sophisticated GC–MS–DS techniques for estimating the ^{13}C or ^{15}N-enrichment or both of individual plasma amino acids from less than 0·5 ml blood (Bier & Christopherson, 1979; Matthews *et al.* 1980).

An intermediate position along the spectrum of blood sample size requirements for mass spectrometric isotopic measurements of amino acids would be the use of a

uniformly ^{13}C- or ^{15}N-labelled whole protein. In this instance less than 5 ml of plasma would be sufficient for the estimation of total ^{13}C-carboxyl or total α-amino ^{15}N-labelling. We have recently employed constant nasogastric infusions of ^{15}N-yeast protein to investigate the possibility of calculating protein flux from the expected total α-amino ^{15}N plasma plateau under steady state conditions. Interest centred around the reproducibility of the method when absorption characteristics were monitored in the same subject, under similar dietary conditions (data rarely presented by authors), the possibility of reducing the time to achieve total α-amino ^{15}N plasma plateau with a priming dose of ^{15}N-yeast protein and finally to attempt to prime the urea pool such that ultimately all estimations could be performed on urinary urea. Previously, investigators using [^{15}N]glycine administered orally or intravenously, but without urea pool priming, have required some 48–72 h to produce an isotopic steady state in urinary urea in adult subjects (Steffee *et al.* 1976).

The results from our initial investigations on two male subjects, one normal and one CVA brainstem, are presented in Table 1. The procedures involved were approved by the Northwick Park Hospital Ethical Committee. Isotopic plateau with respect to total α-amino ^{15}N was attained in approximately 16 h and the infusions continued to 22 h. Five or six plasma samples analysed from each study at plateau produced a CV of less than 3%.

This section has been devoted to the capabilities and potential applications of isotope ratio mass spectrometry to various aspects of amino acid and protein metabolism in man using ^{13}C- and ^{15}N-labelled compounds.

An alternative method of $^{13}CO_2$ analysis is the use of infrared spectroscopy (Hirano *et al.* 1979). Recent general improvements and the potential of incorporating a tuneable laser source into this type of instrumentation may well

Table 1. *Quantitative aspects of protein turnover in two male subjects calculated from total α-amino ^{15}N plasma plateau levels resulting from nasogastric infusions of ^{15}N-yeast protein*

(Results are means with their standard deviations for five or six measurements of total plasma α-amino ^{15}N-enrichment between 16–22 h into the infusion when isotopic plateau had been achieved)

Subject	Diet (g protein/d)	Infusion no.	Flux (g/d)	Protein metabolism: Synthesis (g/kg per d) Mean	Synthesis SD	Catabolism (g/kg per d) Mean	Catabolism SD
1. Normal male	72	1	398	5·35	0·23	5·22	0·24
	72	2	411	5·46	0·29	5·43	0·32
	30	3	350	4·74	0·23	5·12	0·27
2. Brainstem CVA-male	40	1	405	6·74	0·22	6·73	0·29
	40	2	394	6·56	0·25	6·30	0·24

https://doi.org/10.1079/PNS19810048 Published online by Cambridge University Press

provide sufficient sensitivity, accuracy and precision for $^{13}CO_2$ breath analysis used in amino acid oxidation studies. The use of optical emission spectroscopy for ^{15}N analysis is dealt with elsewhere in this Workshop.

The organic mass spectrometer

The organic mass spectrometer is essentially an instrument for qualitative analysis and has been used in this way for many years. It is probably the most sensitive analytical instrument currently available, and can give structural details of a compound not to be determined by any other method.

Electron impact ionization

When a molecule is admitted to the instrument and hit by an energetic electron, an electron is normally removed, resulting in a positively charged ion, whose behaviour is easily influenced by variations in the electrostatic and magnetic fields through which it moves. Since deflection of an ion carrying unit charge, moving through constant electrical and magnetic fields is determined purely by the mass of the ion, it is relatively simple to separate ions of different masses. The most sophisticated instruments, which are always of the magnetic sector type are capable of separating ion beams of less than 10^{-3} mass units apart and therefore are easily able to discriminate between two such molecules as N_2 (mass 28.01504) and carbon monoxide (mass 28.0038). Under conditions of very low energy ionization by electrons, the main ion would be of the same m/e as molecular weight of the compound examined and its exact molecular weight determined; a value of greatest importance to the chemist. Under normal operating conditions of a mass spectrometer, the energy of ionizing electrons used is considerably in excess of that needed for ionization and the excess energy is transferred to the molecule. This results in instability which causes fragmentation of the ion into several smaller ions. The extent to which this occurs depends on the structure of the compound and frequently results in there being no ions of the molecular weight of the compound being present. However, under constant conditions, a given compound will give a constant spectrum of fragments, with the same relative intensities between the different fragments. If the instrument is made to scan through the m/e spectrum, and record the exact m/e and intensity of the fragments produced by a compound, a highly characteristic mass spectrum can be produced, which can be used for identification of a compound introduced into the instrument. The exact mass of each of the fragments, shown by the instrument is used by the chemist to determine the structure of each fragment, which gives a very considerable amount of information about the structure of the parent compound.

Almost all naturally occurring compounds contain a small percentage of molecules which contain an atom of a heavy isotope, resulting in a molecular ion of an m/e of one or two mass units greater than the majority of the ions from the compound. For example, carbon monoxide will in addition to an ion of 28.0038, produce an ion of 29.00747, and one of 30.01132. The ion of mass 29, which occurs to about 1% of the mass 28 intensity is due to the presence of ^{13}C in the

compound, and the mass 30 ion (about 0·2%) is due to ^{18}O. The ion of mass 29 does in fact contain a small amount of ^{17}O (about 0·037% of the main peak). If the isotope is randomly distributed throughout the molecule it will occur in equal abundance in all the fragments which contain the element, but if it is concentrated in a particular group of the molecule, it will occur mainly in fragments which contain that group.

In recent years, the ability of this type of mass spectrometer to quantitate the relative intensities of the ion beams due to the presence of various isotopes has been used as the basis of methods for assays of various compounds.

For these types of measurements, the very high resolving power is not necessary, and the simpler, less expensive quadrapole instrument is frequently used. In addition to its lower price, its requirement of less skilled personnel to operate it and its lower demand on floor space, it has the advantage that it is possible to switch between different m/e beams at a faster rate than the magnetic instrument.

Chemical ionization

In cases where a low intensity or absence of the ion from the whole molecule of the compound of interest would be unacceptable, a more gentle form of ionization is available. In this mode, known as chemical ionization, the ionizing electron beam interacts with a simple gaseous compound such as methane or NH_3 to give positively charged ions, which in turn ionize the compound of interest, usually by protonation. Excess energy transferred to the latter compound is usually far lower than in electron impact ionization, resulting in far less fragmentation. The extent to which fragmentation does occur may be predetermined by selection of the appropriate reagent gas, e.g. in terms of decreasing fragmentation $He<CH_4<(CH_3)_3CH<NH_3$. The main disadvantage of using a CI method, is that considerably greater contamination of the MS occurs than with EI, resulting in increased down time of the instrument.

Isotope abundance measurements

For measurement of the isotopic abundances in a compound, comparison of the molecular ion with that of the same mass number +1 or +2 units or both will give information about the total number of molecules containing one, or two isotopic tracers. If a medium resolution instrument is used (e.g. a quadrapole instrument) it is unable to differentiate between abundances of different isotopes (e.g. ^{13}C and ^{15}N) in the same molecule. If an amino acid has been labelled with a single ^{13}C and a single ^{15}N, a peak of molecular mass +1 will indicate that one of the isotopes has been lost in the compound, but be unable to indicate which one. By selection of a fragment of the molecule occurring in the spectrum, which contains only one of the labelled groups and comparing its isotopic ratios with that of the mass of the ion +1, it is possible to determine the abundance of a specific labelling atom. Modern instruments fitted with a sophisticated data system are capable of carrying out such measurements on several fragments with a fair degree of accuracy (CV<3%) on a single gas chormatographic peak of a duration of a few seconds and thus give

several pieces of required information from a single analysis. This type of multiple measurement has been given over twenty different names in the scientific literature (Watson *et al.* 1974) but we choose to call it Selected Ion monitoring.

Before this type of measurement can be used, it is necessary that fragments of reasonable intensity should be chosen, since the accuracy of any measurement in the mass spectrometer is a function, at least in part of the signal/noise ratio of the ion beam measured. It is moreover essential that the chemical structure of the fragment be positively identified. The identification given in the literature may not necessarily be correct. In an attempt to measure the total isotopic abundance in heptafluoroproprionyl leucine iso butyl ester labelled with ^{15}N, and with ^{13}C in the carboxyl group, we chose a fragment described as being the molecular ion less C_3F_7. This was found to contain a single isotope, instead of both, and examination by Dr Lawson (CRC, Clinical Chemistry) of the ion on a high resolution instrument showed that it was of entirely different structure containing only the isotopic N (unpublished observation).

Analyses as described presuppose that the compound of interest is introduced into the instrument in a pure form. The extreme sensitivity of the mass spectrometer—a great advantage when dealing with compounds only available in micro quantities—has the disadvantage that very small quantities of impurities can seriously interfere with mass spectra and measurements carried out thereon. It is therefore necessary to use a device which will separate the required compound from what is usually a complex mixture of like and unlike materials. When dealing with biological materials, it is usually possible to carry out an initial cleanup of the sample to remove most of the 'unlike' substances, e.g. removal of protein by precipitation. The 'like' materials may then be separated into their individual components by more sophisticated methods.

Gas chromatography

For the type of work in which we are interested, i.e. measurement of the isotopic content of amino acids, ketoacids and sugars, the method of choice for separation is gas–liquid chromatography. The general principles of the technique are too well known to need further description. When used in conjunction with a mass spectrometer, certain special factors have to be considered. When used in the conventional manner, column bleed normally results in an elevation of baseline, which although it may be annoying, can usually be compensated for in any calculation. When used with a mass spectrometer, the material bleeding from the column into the instrument produces its own spectrum, which may confuse identification of the sample, or when quantitative measurements are carried out may give erroneous results. Background spectra of this kind may often limit the useful sensitivity of the instrument. In addition, the continuous feed of liquid phase into the source of the mass spectrometer gradually contaminates the critical components of the instrument. Another source of extraneous peaks is residues from derivatizing agents, which have to be used to produce sufficiently volatile

compounds from all of the compounds that we are likely to investigate in biological studies. Use of plastic apparatus in preparations will frequently lead to interfering fragments.

The type of column used is usually left to the choice of the worker. There are basically three types of column available: the packed column, the so-called SCOT (Surface Coated Open Tubular) and the WCOT (Wall Coated Open Tubular) column. Each type has its own advantages and disadvantages. The packed column is normally limited to an over-all length of a few metres resulting in a limited degree of separation on a column. It is able however to deal with a considerably greater loading of material than the other two types. It is robust and comparatively easy to pack in the laboratory. The main disadvantage is that it needs a considerable flow of carrier gas to operate efficiently. This gas flow is far too great to admit directly into the mass spectrometer and a device which will remove most of the gas has to be placed between the outlet of the column and the inlet of the analyser tube. Several different types of separators have been described, but in all cases some of the emerging compound is lost as well as the carrier gas. Another disadvantage of the packed column is that the bleed rate is rather high.

Capillary columns offer several advantages over the packed column. The length of the column is nearly unlimited. Twenty-five and 30 meter columns are in standard usage, with resulting increase in separation ability. Column bleed is relatively low and the very small gas flow through them enables the effluent to be admitted directly into the mass spectrometer. Two of the former disadvantages; the great fragility of the column and the possibility of certain compounds reacting with the glass surface, have been virtually eliminated by use of silica columns. The main disadvantages still remaining are the very small samples that can be used; the columns are slower than packed columns, and the cost high. Preparation of one's own capillary columns in the laboratory is really not practicable. SCOT columns which were developed to avoid reactance of compounds with the capillary walls offer few advantages.

The stationary phase used in the columns depends on the type of compounds being separated. Several hundred of these are now available and there is no space to discuss them. The most generally useful column now available for biological work is probably a 25 m capillary column coated with OV101 or its equivalent.

Methods for protein turnover studies

We now turn to the application of the techniques to the kind of measurements needed for studies in protein turnover. These are usually limited to measurements of the isotopic abundance of ^{13}C and ^{15}N in blood amino acids and ^{13}C in ketoacids. It may also be necessary to investigate blood urea. Less than 1 ml of blood is used for these determinations.

None of these compounds will move on a column and have first to be derivatized to form a compound with a reasonably high vapour pressure at the temperatures used in the GC process. This temperature is limited by the nature of the liquid phase in the column.

When plasma amino acids are to be analysed it is always necessary to carry out a preliminary clean-up of the sample. Collection of the blood should be carried out in the absence of plastic apparatus and after removing the erythrocytes by centrifugation, the amino acids are removed from the plasma by an ion exchange resin. Dowex 50W X8, 100–200 mesh in the hydrogen form is suitable. Some workers prefer to deproteinize the plasma before the resin treatment, but we have not found it necessary. After thoroughly washing the resin, the amino acids are eluted with 3 M-ammonium hydroxide and the solution then thoroughly dried. We choose to use a rotary evaporator for this purpose. The residue is now ready for derivatization.

To produce a derivative which is suitable for the GC, it is necessary to acylate the amino groups (and possibly the hydroxyls) and to esterify the carboxyl group. The choice of reagents available for this purpose is extremely wide and an extensive review on the gas chromatography of amino acids with 415 references has been published (Husek & Macek, 1975). The choice of reagents has been extended since then. In theory, any of these derivatives can be used for the GC–MS analysis, but practical considerations limit the number that are in fact used. It is of course necessary that the derivative chosen should allow good separation on the GC column chosen and the compounds of interest should emerge from the column in a reasonable time. Where more than one amino acid is to be investigated, derivatization should be equally efficient on all amino acids. It is also desirable that a single derivative should be formed from each amino acid, and that it should take place in a reasonable time. During the course of derivatization, no structural change should take place in the amino acid other than the addition of the required groups. It is also necessary to choose reagents and solvents, residues from which will not damage the column or produce confusing fragments in the mass spectrometer. Above all, the derivative must produce usable ions in the instrument. To date, no derivatization procedure fits all the requirements listed. With one exception (Wolfensberger *et al.* 1979), all procedures deamidate glutamine and asparagine and many reagents oxidize cysteine to cystine. Trimethylsilylation tends to form multiple derivatives, particularly from glycine and is notorious for producing extraneous mass spectral peaks. Histidine is extremely difficult to derivatize completely unless an acid anhydride is co-injected on to the column, which can damage the column, or reduce its efficiency.

With these difficulties it is not really surprising that very few publications have appeared which use the GC–MS system for measuring isotopic abundances in amino acids from biological material.

In 1970, Vandenheuvel & Cohen published a report on the ^{13}C content of fourteen amino acids from algal protein grown in a $^{13}CO_2$ medium. They used the trimethylsilyl derivatives of the acids, run on SE30 or OV–17 packed columns. The paper gave good evidence for lack of isotopic fractionation in the methods used. Since this paper there have been a limited number of papers on the GC–MS analysis of amino acids. The derivative most widely used has been the *N*-trifluoracetyl *n*-butyl ester, run on a packed column and analysed by CI technique

(Pereira *et al.* 1973; Frick *et al.* 1977; Kingston & Duffield, 1978; Finlayson *et al.* 1980). The methyl ester (Zagalak *et al.* 1977) has also been used.

The *N*-heptafluoro *n*-propyl ester has been used with a packed column and CI (Robinson *et al.* 1978) and the *n*-butyl ester on a capillary column with EI (Farmer *et al.* 1980). Matthews *et al.* (1979) use the *N*-acetyl *n*-propyl ester on a packed column with CI.

A derivatization method has been described which does not deamidate glutamine and is suitable for GC–MS use has been described (Wolfensberger *et al.* 1979) but does not appear to have been widely used. The use of the dimethyl amino methylene methyl ester derivative has also been described to study plasma taurine enriched with [1,2-^{13}C]taurine in infants (Irving & Klein, 1980).

Most of these methods have been described with specific amino acids in view.

It is obvious that the technique of measuring isotope abundance in amino acids is in its infancy. Use of the capillary column with its inherently greater separation capability will almost certainly increase now that the silica column is available commercially. The range of acylating agents is increasing rapidly and it is unlikely that the best derivative of the amino acids has yet been developed.

Keto acid analyses

There is little in the literature which deals with the derivatization of α keto acids derived from amino acids. These acids can be gas chromatographed as their O-trimethylsilyl quinoxalinol derivatives. Most of the compounds were separated on a 1·2 m packed column of 2% OV101 (Langenbeck *et al.* 1975) and the work was extended to describe their single-ion detection in a quadrapole mass spectrometer after separating on a 3% Dexsill 300, or 3% OVI column (Langenbeck *et al.* 1977). Improved separation of the compounds could be effected by using a 2 m column packed with a mixed stationary phase (0·3% Carbowax 20M, 0·3% Silar 5 and 0·06% Lexan) on Chromosorb WAW (Schwarz *et al.* 1980). Other derivatives described have been trimethyl-silylated oximes run on a 6 column of 3% OVI or on 3% OV-17. Mass spectra are included in the paper (Lancaster *et al.* 1973). Methyl esters of the 2:4 dinitrophenyl hydrazones have also been used. The column was 4% SE30 but separation was not complete (Kallio & Linko, 1973). No publications have so far appeared which describe evaluation of isotopic content of keto acids by GC–MS. As with amino acids, much developmental work remains to be carried out in this field.

This presentation has indicated that although much developmental work remains to be carried out, the combination of the isotope ratio and organic mass spectrometers, with the associated techniques, gives us a tool of immense power to investigate almost any aspect of amino acid and protein metabolism in animals and man.

REFERENCES

Bier, D. M. & Christopherson, H. L. (1979). *Analyt. Biochem.* **94**, 242.
Craig, H. (1953). *Geochim. Cosmochim. Acta.* **3**, 53.
Farmer, P. B., Bailey, E., Lamb, J. H. & Connors, T. A. (1980). *Biomed. Mass Spectrom.* **7**, 41.

Finlayson, P. J., Christopher, R. K. & Duffield, A. M. (1980). *Biomed. Mass Spectrom.* **7,** 450.
Frick, W., Chang, D., Folkers, K. & Daves, G. D. (1977). *Analyt. Chem.* **49,** 1241.
Gaffney, J. S., Irsa, A. P., Friedman, L. & Slatkin, D. N. (1978). *Biomed. Mass Spectrom.* **5,** 495.
Halliday, D. & McKeran, R. O. (1975). *Clin. Sci. Mol. Med.* **49,** 581.
Hirano, S., Kanamatsu, T., Takagi, Y. & Akei, T. (1979). *Analyt. Biochem.* **96,** 64.
Husek, P. & Macek, K. (1975). *J. Chromat.* **113,** 139.
Hutchinson, J. H. & Laby, D. H. (1962). *J. Lab. clin. Med.* **60,** 170.
Irving, C. S. & Klein, P. D. (1980). *Analyt. Biochem.* **107,** 251.
Kallio, H. & Linko, R. R. (1973). *J. Chromat.* **76,** 229.
Kennedy, I. R. (1965). *Analyt. Biochem.* **11,** 105.
Kingston, E. E. & Duffield, A. M. (1978). *Biomed. Mass Spectrom.* **5,** 621.
Lancaster, G., Lamm, P., Sriver, C. R., Tjoa, S. S. & Mamer, O. A. (1973). *Clin. Chim. Acta.* **48,** 279.
Langenbeck, U., Hoinowski, A., Mantel, K. & Möhring, H-U. (1977). *J. Chromat.* **143,** 39.
Langenbeck, U., Möhring, H-U. & Dieckmann, K-P. (1975). *J. Chromat.* **115,** 75.
Matthews, D. E., Ben-Galim, E. & Bier, D. M. (1979). *Analyt. Chem.* **51,** 80.
Matthews, D. E., Motil, K. J., Rohrbaugh, D. K., Burke, J. F., Young, V. R. & Bier, D. M. (1980). *Am. J. Physiol.* **238,** E473.
Mook, W. G. & Grootes, P. M. (1973). *Int. J. Mass Spectr. Ion Phys.* **12,** 273.
Pereira, W. E., Hoyano, Y., Reynolds, W. E., Siemmous, R. E. & Duffield, A. M. (1973). *Analyt. Biochem.* **55,** 236.
Rittenberg, D., Nier, A. O. C. & Reimann, S. (1948). *Preparation and measurement of isotopic tracers* [J. W. Edwards, editor]. Ann Arbor: University of Michigan Press.
Robinson, J. R., Starratt, A. N. & Schlahetka, E. E. (1978). *Biomed. Mass Spectrom.* **5,** 648.
Schoeller, D. A., Schneider, J. F., Solomons, N. W., Watkins, J. B. & Klein, P. D. (1977). *J. Lab. clin. Med.* **30,** 412.
Schwarz, H. P., Karl, I. E. & Bier, D. M. (1980). *Analyt. Biochem.* **108,** 360.
Steffee, W. P., Goldsmith, R. S., Pencharz, P. B., Scrimshaw, N. S. & Young, V. R. (1976). *Metabolism* **25,** 281.
Vandenheuvel, W. J. A. & Cohen, J. S. (1970). *Biochim. biophys. Acta.* **208,** 251.
Waterlow, J. C., Garlick, P. J. & Millward, D. J. (1978). *Protein turnover in mammalian tissues and in the whole body.* Amsterdam: North-Holland Publishing Company.
Watson, J. F., Falkner, F. C. & Sweetman, B. J. (1974). *Biomed. Mass Spectrom.* **1,** 156.
Wolfensberger, M., Redweik, U. & Curtins, H-Ch. (1979). *J. Chromat.* **172,** 471.
Young, V. R. & Bier, D. M. (1980). *Am. J. Physiol.* **238,** E473.
Zagalak, M-J., Curtins, H-Ch., Leimbacher, W. & Redweik, U. (1977). *J. Chromat.* **142,** 523.

Printed in Great Britain