INTERNATIONAL ATOMIC ENERGY AGENCY

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SUMMARY REPORT

ADVISORY GROUP MEETING

on

STABLE ISOTOPE LABELLED COMPOUNDS IN BIOMEDICAL STUDIES

International Atomic Energy Agency
Headquarters Vienna, Austria
26 to 29 November 1985

Edited by: H. Vera Ruiz and
R.M. Parr

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I. INTRODUCTION

Deuterium (^{2}H), ^{13}C, ^{15}N and ^{18}O are naturally-occurring, non radioactive, heavier forms of the atoms that comprise more than 90% of all living tissue. Their discovery in the 1930's and application as tracers laid the foundations for modern biochemical concepts, but their use was overshadowed by the emergence of tritium and carbon 14 usage after 1945. In recent years, ethical constraints on human exposure to radioactivity have grown, especially in studies of infants, children and pregnant or lactating women. The safety of stable isotopes, their applicability to studies of protected populations as well as to surveys of nutritional status, and the absence of storage or disposal problems has led to a resurgence in their application. Their use provides essential quantitative information on nutritional requirements needed for on-going global food policy deliberations.

During the last decade the use of stable isotope labelled compounds in biomedical and nutritional studies has expanded rapidly, particularly for studies on energy expenditure and metabolic processes involving proteins, carbohydrates, fat and other important nutrients.

Several of these procedures, using compounds labelled with isotopes of biologically important elements, i.e., ^{2}H, ^{13}C, ^{15}N, ^{18}O and others, are now used routinely for clinical studies in many advanced countries. In developing countries, however, largely due to the high costs of both instrumentation and labelled compounds, these techniques have not yet been widely used despite, in some cases a high prevalence of important medical problems to which they could be usefully applied such as malnutrition, malabsorption and diseases associated with these.

The Agency organized the present Advisory Group Meeting to assess and review recent progress in the use of stable isotopes in biomedical and related studies, and to recommend specific actions by it to facilitate information exchange on this subject. This meeting followed two previous IAEA meetings: a Technical Committee Meeting (1977) and a Consultants' Meeting (1981).

The present Meeting was attended by 12 experts representing 8 Member States of the IAEA. The names of the experts are listed in Annex I. Its purpose as described in the Information Sheet was (1) to discuss the current status of stable isotope techniques and applications in nutritional and biomedical studies, 2) to assess the applicability of these techniques to some important nutritionally related problems prevalent in developing countries, 3) to advise the Agency on future programmes, 4) to define the purpose and scope of a new co-ordinated research programme on this topic which the Agency plans to initiate in 1986, and to prepare a protocol for it, and 5) to draw up a tentative programme and participants' list for a scientific Seminar which is due to take place at the Agency's Headquarters in November 1986.

The programme of the meeting was restricted to topics involving applications of stable isotopes of the lighter elements (H, C, N, O). Heavier elements such as Ca, Fe, Zn and Se were not considered. The following report summarises the views expressed at the Meeting.

II. ^{15}N STUDIES

Unlike methods for measuring body energy expenditure, those for studying the kinetics of metabolic processes are obliged to use isotopes since no other technique can give any information on reaction rates. Studies of nitrogen metabolism are further restricted to using the stable isotopes of nitrogen (^{15}N) because the radioactive form of this element (^{13}N) has a half life too short for any practicable purpose. This restriction to a stable isotope, unfortunately, makes measurement of the isotope technically and practically more difficult. At present there are two basic methods of analysis: isotope-ratio mass-spectrometry (IRMS) and ion-emission spectroscopy (IES). Relative to IES, IRMS is more expensive in terms of capital investment and in operational costs but is more precise and sensitive.

Applications of ^{15}N in kinetic studies are numerous and include amino acid and protein turnover, urea metabolism, intestinal absorption, purine and pyrimidine metabolism, body function tests and body composition.

There are several methods currently available for measuring the rate of whole-body protein turnover in man but most are research techniques which are complex in practice and cannot be applied to large populations. Recent development of the stochastic end-product approach of Waterlow and co-workers have made it possible to measure protein turnover over a relatively short period of nine hours in many subjects simultaneously. The practical aspects of this technique are also very simple and it would appear an ideal technique for use in field conditions or in developing countries where services and facilities may be restrictive.

The amino acid and protein requirements for normal health in a range of physiological states, and in pathological states are only understood to a limited extent. The conception that nitrogen moves with relative ease between amino acids is not entirely correct. There is increasing evidence that the movement of nitrogen through metabolic pathways is controlled. When the demand for nitrogen increases relative to the available supply, adaptive responses lead to the more efficient utilisation of nitrogen. Importantly, the flux of nitrogen through the bowel changes, dependant upon an interaction with the resident bacterial flora. There is provisional evidence indicating that essential and non-essential amino acids may be made available to the host through the metabolic activity of the bacteria. The dietary intake of non-digestible carbohydrate, serving as a source of energy, exerts a major influence on the growth and activity of the bacteria. As the habitual diets taken in the developing countries are relatively rich in non-digestible carbohydrate, the possible implications for amino acid requirements can be assessed using ^{15}N techniques.

In ^{15}N studies on diagnosis of metabolic functions it has been shown that malfunction of the liver, which is an important organ for the regulation of protein synthesis in the body, can be characterized by several new tests (i.e. ^{15}N-ammonium-test, ^{15}N-hippurate-test, ^{15}N-methacetin-test). Further tests using ^{15}N for the examination of malabsorption, maldigestion or hormone deficiency have been developed. Kinetic data on parameters of protein metabolism are

obtainable only by the use of stable isotope techniques. Standardized routine methods are now available. Metabolic data such as N-flux, protein synthesis, utilization or pool size are calculated on the basis of relatively simple metabolic models and analytical techniques (emission spectrometry for ^{15}N analysis).

It seems that the most appropriate methodologies for developing countries are those which only require ^{15}N analysis of simple urinary end products (e.g. total nitrogen, ammonia, urea, hippurate or amino acids).

III. ^{13}C BREATH TESTS

^{13}C breath tests are non-invasive probes of enzyme function and organ status in which the appearance of $^{13}CO_2$ in the breath reflects metabolism of the tracer molecule after oral ingestion. Technology for collection, storage, automatic purification and isotopic analysis of respiratory CO_2 is well established and studies can be carried out at sites remote from the analysis laboratory. A variety of nutrient probes has been developed. These include naturally enriched substrates such as corn glucose, corn cereal and corn oil as well as specifically labelled compounds such as trioctanoin-1-^{13}C, triolein, cholyl glycine-1-^{13}C, dimethyl aminoantipyrine and methacetin. The substrates are applied parenterally or, preferentially, orally to different population groups, including newborn babies, pregnant women and patients at risk for or suffering from functional disorders or diseases of the gastrointestinal tract, the pancreas, the liver, or depleted of nutritional factors essential for normal organ function, growth and development. ^{13}C urea has been shown to be of test value as a probe of gastric disorders. The ability to collect stools excreted after a test probe and to determine the excess ^{13}C by combustion of the organic matter to CO_2 extends the utility of ^{13}C measurements to balance studies and the estimation of substrate/nutrient malabsorption.

IV. THE NON-INVASIVE MEASUREMENT OF TOTAL ENERGY EXPENDITURE (TEE) USING $^{2}H_{2}{}^{18}O$ LABELLED H_2O

Clinicians and nutritionists in developing and developed countries require precise estimates of energy expenditure in free living human subjects. At the population level, estimates will provide information of the type required by WHO/FAO expert committees for scientifically based recommendations. For individuals, information may be needed in the dietary management of obesity, or indeed any disorder in which there is a failure to maintain energy balance.

Previously available methodologies have either proved to have low sensitivity (eg. food intake measurements coupled with balance studies) or impractical for field studies (eg. whole body calorimetry).

Sufficient validations are now available for us to be reasonably confident that this method will be valuable provided that the investigators are fully aware of the need to have a reasonable knowledge of the environmental conditions and the physiology of the subjects under investigation. Naive application of the method could lead to gross inaccuracies and an inability to make comparisons between different populations or identifiably different groups (eg. lean and obese) within a single community. The full potential of this methodology requires precise estimates of changes in body composition, especially loss or gain of body fat.

Our brief leads us to suppose that the major thrust of the IAEA's efforts will be towards the measurement of energy expenditure in the developing world, and standard protocols will need to be designed with this in mind.

Important points to consider in such protocols are:

1. The measurement of resting metabolic rate (RMR). This is essential because TEE/RMR ratios form the basis of current estimates of energy requirements.

2. The measurement of dietary intake. Information about the composition of the diet is desirable and a study which included measurements of dietary status would have considerable advantages over one that did not.

3. Sample numbers analyzed will be compatible with the generation of observed errors of 0.5-1.0% on estimates of the slopes and intercepts of isotope disappearance curves of urinary isotope concentrations. Errors of this magnitude will lead to estimates of energy expenditure that are precise to the level of ±5%.

V. SAFETY AND BIOLOGICAL CONSEQUENCES OF ENRICHED STABLE ISOTOPE USAGE

Unlike radioactive isotopes, stable isotopes present no inherent toxicological hazard in their use. The introduction of ^{2}H, ^{13}C, ^{15}N or ^{18}O tracer molecules adds to the pre-existing levels of these isotopes already present in tissue. These additions are usually smaller than the amounts being consumed daily in food, water and air. Changes in tissue concentrations of stable isotopes in such studies are comparable to the natural variations arising from dietary and water isotope sources. These observations lead to the conclusion that within the levels of currently accepted usage there is no evidence whatsoever to indicate any health hazards resulting from the use of stable isotopes tracers in human nutritional and medical studies, nor are there any theoretical reasons for expecting such hazards.

The biological effects of stable isotopic substitution in enzymatic, cellular, or physiological processes can be subdivided into two categories: those that involve deuterium and those that involve all other elements found in an organism. Because of the large mass difference between deuterium and hydrogen, there is a corresponding effect on the chemical bond reactivity. This same difference aids in the concentration and enrichment of deuterium in virtually limitless quantities. These quantities have enabled extensive investigation of the enrichment level required for toxicological manifestations.

The mass differences for isotopes of higher elements, however, are much smaller; their physical properties are more similar, and thus the enrichment of such isotopes becomes more and more expensive. Moreover, the quantities necessary to investigate high levels of exposure become prohibitive in cost. Toxicity studies of the stable isotopes of biological interest, therefore, were undertaken in inverse order of their discovery (^{18}O, discovered 1929, toxicity determined 1975; ^{13}C, discovered 1929, toxicity determined 1973, ^{2}H, discovered 1932, toxicity determined 1933). Thus, it was within the same year that deuterium was first discovered, that the first studies of its biological effects were initiated.

Between 1934 and 1939, 216 papers were published on the biological effects of deuterium. Most of these papers appeared within the first three years; the last 7 were published in 1939. Thus, after the classic studies were completed, it was not until the price of deuterium dropped from $20 to $0.20 per gram that interest was rekindled in this area.

The depth of study is much more attenuated for ^{13}C, and is represented by several publications which stem from the Los Alamos Scientific Laboratory. No evidence of toxicity was found at the highest enrichments attained (60%) in two mice. To date, there has been no report of an investigation of whole organism response to ^{15}N enrichment levels.

The physical chemistry of ^{18}O has been reported excellently in the literature. Only because of the substantial resources of the Stable Isotope Department of the Weizmann Institute, however, was it possible for Samuels and his co-workers to raise three generations of mice in an atmosphere of 90% $^{18}O_2$ and to provide their drinking water as 90% $H_2{}^{18}O$. No physiological or biochemical effects were noted and the mice reproduced normally through each generation with no increase in infant mortality.

The margin of safety in the application of stable isotopes is large. The enrichment of total body water with deuterium may be as high as 1% to 2%, and is virtually limitless in the cases of ^{13}C, ^{15}N, or ^{18}O (where the sheer economic cost of substantial tissue isotope replacement

is prohibitive). For this reason, and for the more important feature, absence of radiation, the use of these tracers in protected populations has been strongly favoured by human investigation review committees. This perspective, however, must be provided in an accurate manner to the subject or guardian from whom informed consent is required. The information in Table 1 may be used for this purpose. Listed is the natural abundance for each isotope, calculated as mg/kg/body weight, compared with normal daily intake, and with the amount used in most foreseeable studies. These values demonstrate the absence of any perturbation in body composition when stable isotopes are used.

Table 1. Natural stable isotope content of the human body, daily consumption of stable isotopes, and quantities of stable isotopes used in conventional tracer studies

	^{2}H (mg/kg)	^{13}C (mg/kg)	^{15}N (mg/kg)	^{18}O (mg/kg)
Body content	15	1980	111	130
Intake as:				
Food	0.23	99.9	0.15	20.8
Water	6.7			40.0
Air	--	--	--	66.4
Tracer dose	5	15	10	60

The following is a list of some research groups currently using or developing the $^{2}H_{2}$ ^{18}O technique:

P. Klein	(Houston)
W.A. Coward	(Cambridge)
D.A. Schoeller	(Chicago)
K. Westerterp	(Maastricht, Netherlands)
W.P.T. James	(Rowett Research Institute, Aberdeen, UK)
E. Forsum	(Huddinge, Sweden)
Prof. Noack	(Institute for Nutrition, Academy of Sciences, Potsdam, GDR)
A. Ferro-Luzzi	(National Institute of Nutrition, Rome, Italy)

VI. NUTRITIONAL PROBLEMS IN DEVELOPING COUNTRIES POSSIBILITIES FOR STABLE ISOTOPE STUDIES

The main nutritional problems in developing countries are related to deficiency states due to insufficient or inadequate intake of food or specific nutrients, impaired absorption or bioavailability of nutrients, excessive losses, increased metabolic demands, or combinations of all these. Other influences are dietary factors such as the presence of substances that enhance or interfere with a nutrient's availability, host factors such as metabolic responses to chronic low intakes, pathologic conditions such as diarrhoeal and chronic diseases, environmental factors such as cyclic climate changes, and social and behavioral circumstances such as work demands and cultural constraints. Stable isotopes can be used to study the impact of these factors on nutritional status and their metabolic, physiological and pathological consequences. Such studies include the absorption and bioavailability of nutrients from local diets, measurement of total energy expenditure, assessment of body composition, protein and amino acid metabolism, intestinal malabsorption, influence of infections and malnutrition on dietary requirements, functional consequences of malnutrition, adaptation to low dietary intakes, and pharmacodynamics in malnourished individuals. These aspects must be investigated under the dietary, sanitary, ecological and social conditions prevailing in developing countries.

The factors that must be considered to decide the priorities for such investigations are: a) whether stable isotopes provide the best tool for the intended research; b) the relevance of the problem to be studied; c) the quality of the work that can be done; and d) the technical and financial feasibility of doing the research.

The main limitations and constraints for the use of stable isotopes in developing countries are the cost and availability of the isotopes and labelled compounds, the costs and complexities of several analytical techniques and instruments, and the training of the necessary personnel. There are several options to overcome these limitations, namely:

a) making the isotopes and instruments available to scientists in developing countries, providing technical support to solve problems of methodology and instrumentation, and training local technical personnel;

b) establishing support laboratories or making arrangements with existing facilities to do the analytical work required by the developing country scientists;

c) conducting international collaborative projects with the participation of scientists from developing and more developed countries.

VII. AVAILABILITY OF STABLE ISOTOPES AND PRODUCTION OF LABELLED COMPOUNDS

Despite occasional fluctuations in production levels, the stable isotopes of carbon (^{13}C), nitrogen (^{15}N), and oxygen (^{18}O) continue to be produced in multi-kilogram quantities per year at facilities throughout the world. These levels are adequate for projected levels of usage, though some users have expressed concern that this may not be true for ^{18}O labelled water. Particularly significant is the increasing pace of activities at universities, research institutes, and commercial enterprises to develop efficient methods for the incorporation of these isotopes into appropriate labelled substrates. This has resulted in the availability of increased quantities of a wider variety of compounds for biomedical studies.

Biotechnological processes for the preparation of stable isotope labelled compounds for biomedical studies are being used. Such syntheses, which were previously tedious, inefficient, and costly, can now be made conveniently and economically by the use of microorganisms. In this way ^{15}N labelled amino acids, antibiotics, and other metabolites have been prepared.

The need to incorporate the label imposes restrictions on conventional microbial processes. The a priori limiting quantity of isotopic

precursor, the danger of label dilution from external and internal pools, and the necessity of recovering residual starting materials for reuse, dominate the process design.

Examples drawn from immobilized bacterial preparations of L-[^{15}N]alanine, L-[^{15}N]aspartate, L-[^{15}N]tyrosine and L-[^{15}N]DOPA demonstrate how these preparations have been modified to conserve ^{15}N.

Fermentative production of L-[^{15}N]glutamic and L-[^{15}N]lysine has also been achieved under conditions in which only 10% of the initial $^{15}NH_4Cl$ remained unused. Compared to ^{13}C, ^{15}N is superior for labelling. Because of the limited number of nitrogenous groups in biomolecules the label is localized, while ^{13}C must be introduced to specific sites. Therefore immobilized biocatalysts are preferred to fermentative preparations for ^{13}C labelling. Furthermore, in the latter, losses from carbohydrate catabolism are prohibitive and must be overcome.

VIII. MODERN INSTRUMENTATION

The application of stable isotopes in biomedical studies continues to rely heavily on the mass-spectrometric determination of isotope ratios ($^{2}H/^{1}H$, $^{13}C/^{12}C$, $^{15}N/^{14}N$ and $^{18}O/^{16}O$) in simple gases like hydrogen, carbon dioxide and nitrogen derived as metabolic end-products or from chemical conversion of metabolites isolated from tissue or body fluids. Biomedical studies generate a large number of samples and there is a need for improvements in the speed and cost effectiveness of gas/mass-spectrometric methods and for alternate inexpensive methods of rapid isotope analysis, eliminating where possible the steps of isolation, purification and chemical conversion of metabolites. Several promising developments to these ends were discussed at the Meeting.

One working paper detailed a method developed for combining and automating both the sample preparation and the isotope-ratio mass

spectrometric measurements to enable rapid determination of the $^{15}N/^{14}N$ ratio in N_2 derived from a variety of biological specimens. The method allows the analysis of samples as small as 5 µg with a total conversion and analysis time of 5 minutes. The system should also be capable of the analysis of $^{13}C/^{12}C$ ratios of CO_2 from the same combusted sample.

Another paper detailed the development and performance evaluation of a nondispersive infrared heterodyne ratiometer for the determination of the $^{13}C/^{12}C$ ratios in CO_2. The performance of the instrument, which should be less expensive to construct than an isotope-ratio mass spectrometer, is equal to that of a geochemical isotope-ratio mass spectrometer, equipped with an automatic sample purification system, with respect to precision and accuracy. The instrument offers the advantages of much higher sample throughput and lack of requirement for sample purification. Having no high vacuum and high voltage components, the instrument is rugged and can be operated and maintained by non-specialists. Other instruments that measure isotope abundance without mass spectrometry include ^{15}N emission spectrometry and the measurement of high-enrichment deuterium by IR absorption. These are especially applicable for use in developing countries.

The Meeting recommended that more needs to be done to stimulate the use of instruments that are cheaper and simpler to handle and maintain, such as infrared spectroscopy for $^{2}H_2O$ analysis and ion-emission spectroscopy for ^{15}N. It should be recognized that these instruments are less sensitive and precise than mass spectrometry and that their application may be limited to certain purposes (e.g., assessment of total body water), but their cost, overall handling features and related sample preparation might make them more suitable for specific uses in developing countries.

In other papers presented at the Meeting, the possibility of using nuclear magnetic resonance (NMR) spectroscopy for analysis of isotopically labelled compounds was discussed. Although the application of NMR to biology and medicine is a rather recent development, the

technique has been used in analytical and structural chemistry for over three decades and is a widely dispersed methodology. Recent advances in techniques for acquiring data on present instruments allow the determination of ^{13}C content by proton NMR spectroscopy of crude urine, serum, fecal, and tissue samples in measurement times as short as 5 min. There is also a high probability that the ^{15}N content can be measured in certain samples by proton NMR. The method requires large sample sizes (0.5 ml or more) but eliminates or reduces chemical processing and has a wide potential applicability in the analysis of untreated (or minimally treated) biological samples.

IX. CO-ORDINATED RESEARCH PROGRAMME AND SEMINAR

The AGM on Stable Isotopes in Biomedical Studies recommends the establishment by the Agency of a coordinated research programme on stable isotope applications to nutritional problems in developing countries. Some of the suggested topics are:

- The study of dietary energy requirements by measurement of energy expenditure through the use of $^{2}H_{2}^{18}O$ tracer techniques. Direct assessment of actual energy expenditure in chronically malnourished populations, as well as under various work regimens, has a major policy implication in setting standards of adequate nutrition. This work can be expected to be of importance to other international organizations such as the World Health Organization (WHO), the Food and Agriculture Organization (FAO), and the United Nations University (UNU).

- Body composition measurements employing ^{2}H and/or ^{18}O labelled water to measure total body water and thereby lean body mass and percent body fat, are essential in the evaluation of body changes during starvation, catch-up growth and maturation as well as during pregnancy and lactation. These are also used to validate indirect measures in specific group populations.

- ^{15}N amino acid and protein metabolism and the exploration of the roles of non-protein nitrogen sources and the colonic flora in meeting essential amino acid requirements are important areas of study in understanding adaptation to low protein intakes.

- Organ function in malnutrition, studied by ^{13}C-labelled substrates and evaluated by $^{13}CO_2$ excretion in breath or by the excretion of ^{15}N labelled substrates in urine, followed by end-product analysis, will for example, pinpoint origins of steatorrhea, bile-salt losing enteropathies, lactose maldigestion and carbohydrate intolerance.

- Absorption and bioavailability of dietary nutrients, naturally enriched with ^{13}C or specifically enriched with ^{15}N, can be used to guide refeeding of infants with locally available foods, and the dietary management of individuals who are recovering from chronic diarrhoea.

- Drug metabolism is often altered in malnourished individuals, altering the efficacy of treatment. Liver function tests with ^{13}C aminopyrine or ^{15}N methacetin can provide important information on the remaining capacity of the organ to dispose of drugs.

Regarding the proposed IAEA Seminar on Applications of Stable Isotopes in Human Nutritional and Medical Studies, which is expected to take place in November 1986, it was recommended that this should cover the same subject area as the present Advisory Group Meeting, but with emphasis on the topics listed above.

X. OTHER RECOMMENDATIONS

Analytical Services

Access to analytical measurements of stable isotopic contents of samples collected by investigators in the coordinated research programme must be explored by the Agency. The consideration of sample sizes, numbers and isotopic enrichment as well as the cost of contract measurements will be important in resource allocations. While research agreements with laboratories in developed countries will be helpful in quality control, the anticipated work load is unlikely to be accommodated within the latter facilities.

Reference Materials

There is a need for reference materials with enriched levels of isotopes.

Several participants offered to supply these to the Agency free of charge. The following is a priority list.

Unit Size	Isotope	Form	Enrichment level required
1 gm	^{2}H	Water	500 and 1000°/oo vs SMOW
100 mg	^{13}C	$NaHCO_3$(solid)	100 and 500°/oo vs PDB
100 mg	^{13}C	UL-glucose(solid)	100 and 500°/oo vs PDB
100 mg	^{15}N	$(NH)_2SO_4$(solid)	50 and 250°/oo
100 mg	$^{15}N_2$	$^{15}N_2$-urea(solid)	50 and 250°/oo
10 gm	^{18}O	Water	250 and 500°/oo vs SMOW.

The recommended number of units of each is 1000. They should be labelled, stored and distributed by the Agency. Intercomparisons should be organized to establish recommended values of the enrichment factors, and participating laboratories should share the analytical values obtained.

Technical Assistance

There is a compelling need for assistance under the Technical Cooperation programme to provide equipment and training. Fellowships are needed for periods up to 3 months and courses of up to 14 days as well as consultation by experts at the sites of the CRP contractors. The efficient and accurate use of stable isotope tracers requires familiarity with appropriate tracer administration, sample collection and storage, and preparation for eventual isotopic analysis as well as conversion of analytical results into physiological parameters.

It is important to consider that a commitment must be made by the developing country (or institution) and the organization(s) that support it, such that the essential supplier and infrastructure to prepare samples and run the instruments will be available and that the personnel that was trained to do the analyses are assigned for that purpose over a period of time that will permit adequate utilization of these resources.

ANNEX I

International Atomic Energy Agency
Advisory Group Meeting
on
"Stable Isotope Labelled Compounds in Biomedical Studies"

IAEA Headquarters, Vienna, Austria
26 to 29 November 1985
Meeting Room A-0742

LIST OF PARTICIPANTS

Dr. L. Thun (*)
Dept. of Pediatrics
(Kinderklinik)
University of Vienna
Währinger Gürtel 74-76
A-1090 Vienna
Austria

Dr. H. Helge
Kinderklinik und Poliklinik (WE 16)
Freie Universität Berlin
Heubnerweg 6
D-1000 Berlin 19
FGR

Dr. H. Faust
Akademie der Wissenschaften der DDR
Zentralinstitut für Isotopen- und
Strahlenforschung
Permoserstrasse 15
DDR-7050 Leipzig
German Democratic Republic

Dr. B. Torun
INCAP
Carretera Roosevelt
Zona 11
Apartado Postal 1188
Ciudad de Guatemala
Guatemala

Dr. A. Lapidot
Isotope Research
The Weizmann Institute of Science
Rehovot
Israel

Dr. A. A. Jackson
University of Southampton
Dept. of Nutrition
Boldrewood
Bassett Crescent East
Southampton
United Kingdom

(*) observer

Dr. W.A. Coward
University of Cambridge and
Medical Research Council
MRC Dunn Nutrition Unit
Milton Road
Cambridge CB4 1XJ
United Kingdom

Dr. D. Halliday
Clinical Research Centre
Division of Clinical Sciences
Watford Road
Harrow, Middlesex HA1 3UJ
United Kingdom

Dr. G. Overend
VG Isogas Limited
Isotopic Mass Spectrometry
Aston Way
Middlewich
Cheshire CW10OHT
United Kingdom

Dr. P.D. Klein
Texas Children's Hospital
6608 Fannin, Suite 601
Houston, Texas 77030
USA

Dr. N.A. Matwiyoff
Centre for Non Invasive Diagnosis
University of New Mexico
School of Medicine
900 Camino de Salud, N.E.
Albuquerque, New Mexico 87131
USA

Dr. Elisabet Forsum
Department of Medical Nutrition
Karolinska Institute
Huddinge Hospital F 69
S-141 86 Huddinge
Sweden

Dr. E. Fern
Nestlé
Research Department
Avenue Nestlé 55
CH-1800 Vevey
Switzerland

IAEA Staff Members

Prof. V. Ferronsky
Dr. M. Nofal
Dr. R. Parr, Scientific Secretary
Dr. H. Vera Ruiz, Scientific Secretary