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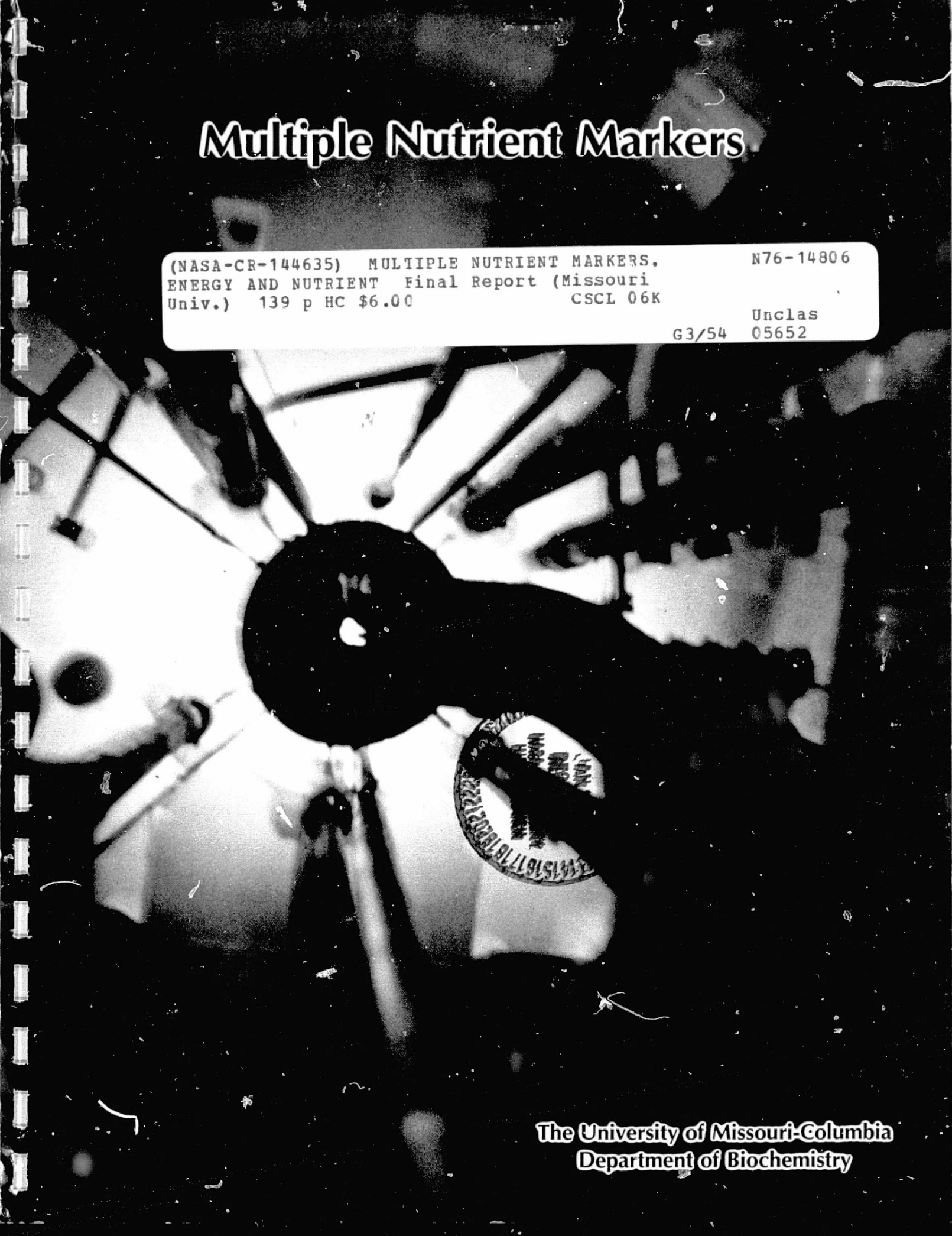
Multiple Nutrient Markers



The University of Missouri-Columbia
Department of Biochemistry

ERRATA SHEET
NAS9-12369, Univ. of Missouri, Columbia, MO FINAL REPORT
"ENERGY & NUTRIENT INTAKE MONITORING"

Proof Marks for Multiple Nutrient Markers

Page	Line	Change
Abs.	7	~~were~~ was
	11	manipulation
1	12	digestibility
9	1	~~,~~ last comma
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10	5	~~,~~ comma
21	18	lanthanous,
29	2	perform
	5	analysis,
	6	counsul
34	15	nonabsorbability
45	19	: → ;
58	9	earth
60	10	add of at end
67	1	Kotb
77	6	Rhesus
	10	preceeded
86	1	~~is~~ are
96	6 up	versatility
98	11	~~is~~ are
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104	11	occurence
	19	digesta;
	4 up	~~50%~~ 50%
106	3 up	marker
110	6	hesitancy
114	3 up	needed;
115	2	foods;
121	last	Luckey,

ENERGY AND NUTRIENT INTAKE MONITORING

FINAL REPORT

PREPARED FOR

NATIONAL AERONAUTICS AND SPACE ADMINISTRATION

L.B. JOHNSON MANNED SPACECRAFT CENTER

Houston, Texas 77058

NASA CONTRACT NAS9-12369

THE UNIVERSITY OF MISSOURI

COLUMBIA, MISSOURI

1975

RESEARCH REPORT

ENERGY AND NUTRIENT INTAKE MONITORING

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FORWARD

This is the final report of a study conducted at the University of Missouri, Columbia, Missouri (UMC). The work was done for the National Aeronautics and Space Administration, Johnson Manned Spacecraft Center at ~~Clearbrook~~ Houston, Texas, under contract NAS9-12369 during the period of November 1, 1971 to March 30, 1975. The program was technically monitored by Dr. Paul C. Rambaut and Dr. Malcolm Smith of the NASA Food and Nutrition Branch. Vita of the authors are given in Appendix A.

The authors acknowledge, with thanks, the essential contributions of Dr. Mike Kay, Dr. Jim Vogt and Mr. Don Gray of the University of Missouri Research Reactor; Dr. A. Kotb, formerly of the Biochemistry Department and presently with the Ministry of Health in Doha, Qatar; Dr. Ruth Baldwin, Department of Food Science and Nutrition, UMC; Dr. Boyd Terry, Department of Surgery, UMC; and Dr. Pat Manning and K.D. Cary of the School of Veterinary Medicine.

The work focused in the Department of Biochemistry of the Medical School. However, much work was done at the Sinclair Comparative Research Farm of the University by Dr. Hutcheson, who is also in the Department of Animal Husbandry.

The cover illustrates the core of the high density neutron reactor of the University of Missouri and the heart of the analytic system for multiple nutrient markers. This report was printed by the Technical Education Service of the University.

T. D. Luckey

T.D. Luckey

Professor of Biochemistry

May, 1975

ABSTRACT

This program on multiple nutrient markers was intended to develop a passive system to determine in-flight intake of nutrients. The only action required of the flight crew is to take one marker capsule with each meal. Analysis of the stools provides all the information needed.

Non-absorbed markers will be placed in all foods in proportion to the nutrients selected for study. Fecal analysis for each marker indicates how much of the nutrients marked were eaten. Fecal analysis for both marker and nutrient allows apparent digestibility to be calculated.

The markers chosen on the basis of non-absorbability and non-toxicity were the non-radioactive heavy metal oxides. These have the further advantage that they can be analyzed by neutron activation analysis without chemical munipulation.

Feasibility tests in rats indicated the diurnal variation of several markers, the transit time for markers in the alimentary tract, the recovery of several markers, and satisfactory use of selected markers to provide indirect measurement of apparent digestibility; the latter compared well with the classic direct chemical measurement.

Three successive generations of mice were fed 0, 1, 10, 100, and 1000 times the anticipated use level of 10 markers. Their growth, maturation, health, reproduction and lactation performance showed no ill effects from the markers. Carcass analysis showed no accumulation of the metals. Monkeys fed the same markers at 10 times the use level showed no residual metals in a variety of tissues. The balance study in monkeys showed good recovery of markers.

Detailed recommendations are provided for human feasibility studies. It is recommended that these be performed as soon as possible.

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I INTRODUCTION

Difficulties encountered in nutritional balance studies include obtaining objective evidence for the exact nutrient intake and collecting all excreta. These difficulties are compounded in large animal studies, clinical environments and even in metabolic wards. In the new monitoring system with multiple markers, all food available to a subject would have incorporated into it nonabsorbed markers placed into each food item at a predetermined ratio of marker to nutrient. A different marker is utilized for each nutrient under consideration. The recovery of each marker in the feces would indicate how much of each marked nutrient had been eaten; analysis of the fecal material for each nutrient would indicate how much was not absorbed; and the apparent digestability could be calculated. If an exact quantity of daily intake markers were taken by capsule, then fecal analysis for the intake marker would allow considerable information to be obtained without having to measure either the quantity of food intake or to make a complete collection of excreta.

There is a real need to accurately and objectively monitor in-flight nutrient intake of individual astronauts for the problems of energy, water, and calcium utilization. These problems intensify and it has been predicted that more nutritional problems will arise as time of flight increases or as a wide variety of relatively untrained personnel are flown. The evaluation of Apollo diets indicated that mineral deficiencies would predictably be the limiting physical factor on space flights of more than a few weeks duration at zero G. Improvements have been made but continuous objective evaluation of the human element is important as the program proceeds to longer manned space flights or to the use of less rigorously processed personnel.

Subjective recording of food consumption has been sporadic at best and quite unacceptable at worst. The increased use of open pantry makes this method more complex, more time consuming for in-flight records by astronauts and open to question from an objective viewpoint. Therefore an alternate objective method to record in-flight individual nutrient intake is being developed by the use of nutrient markers.

The objective of this project is to develop a passive, accurate method to record in-flight nutrient intake. The need for exact knowledge of selected nutrient intake is highlighted by the weight loss, negative calcium and nitrogen balances and possible water disturbance in most of the astronauts. The method is also needed for certain clinical work and will be useful generally in nutritional studies where several nutrients need to be monitored at one time.

A simple procedure by which the intake of one or more nutrients (i.e. protein, calories, calcium, etc.) can be determined with minimum subject participation would be to add nonabsorbed markers to each food in the pantry; each marker would be present in a specified ratio to the nutrient being marked. Data from fecal analyses would provide adequate information to determine not only intake but also apparent utilization of the nutrient. A system of single-treatment analyzed multiple markers is being developed for 5-10 different nutrients. This method will provide information needed to prevent or alleviate the metabolic losses routinely experiences by astronauts in space. The method being developed provides the desired information without the subject recording anything; this allows a check and a confirmation of other information.

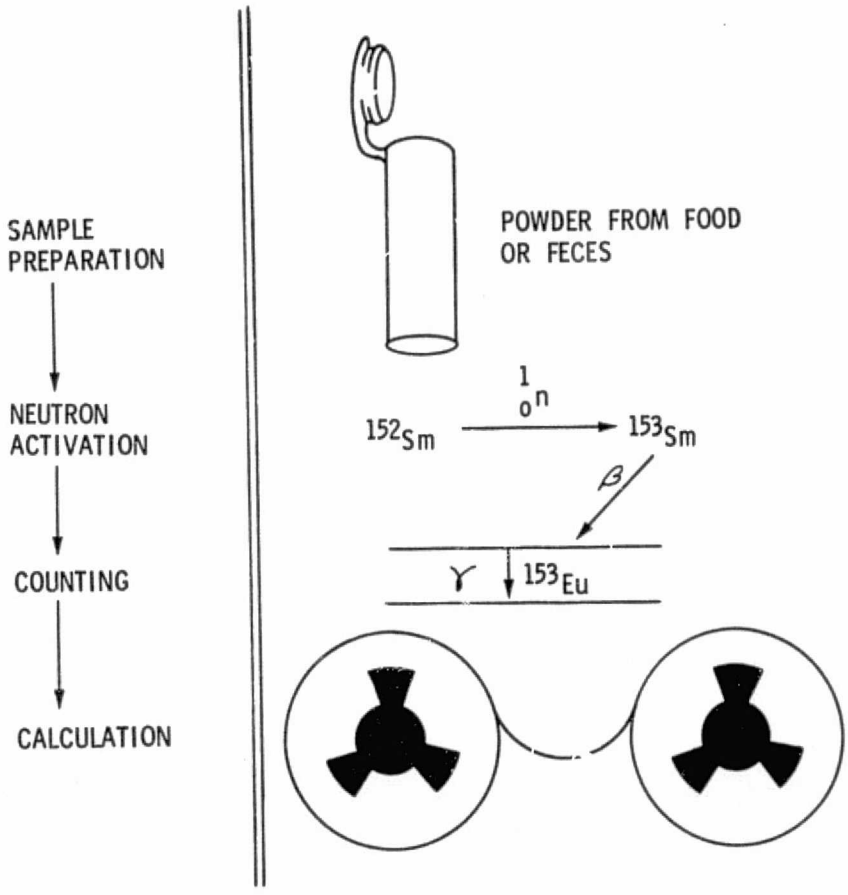
The series of animal studies summarized in this report established the feasibility of the method for humans and illustrate the general usefulness of the method. The rat studies involved transit times, recovery values,

diurnal variablitiy, examination of the daily marker concept, and an evaluation of multiple heavy metal markers to obtain indirect apparent digestibility of 4 nutrients using 3 different diets. The mice study established the inherent safety of selected markers fed at 1, 10, 100, and 1000 times the anticipated use level in 4 generations (3 complete generations). The monkey balance study and tissue analysis showed that no heavy metals could be found when 10 times the use level had been fed for 2 months. This was reinforced by carcass analysis of second generation mice fed 100 times the use level to maturity.

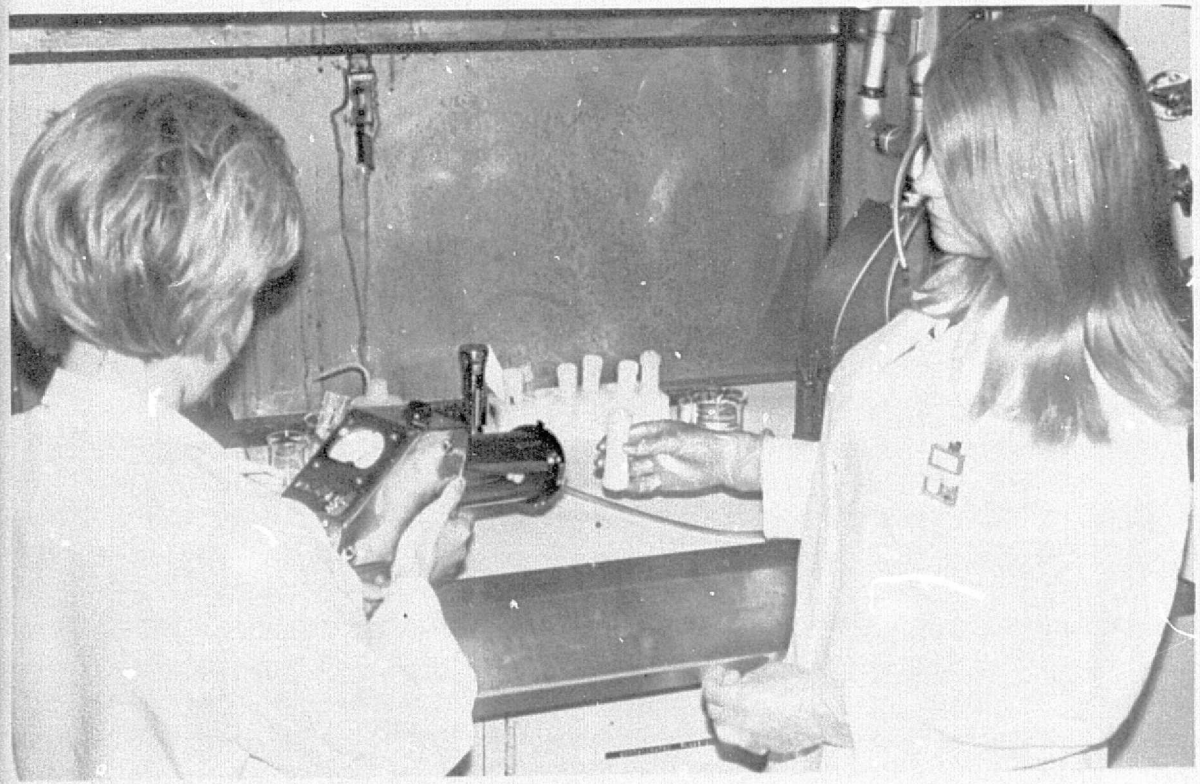
The work is introduced pictorially.

SAMPLE TREATMENT

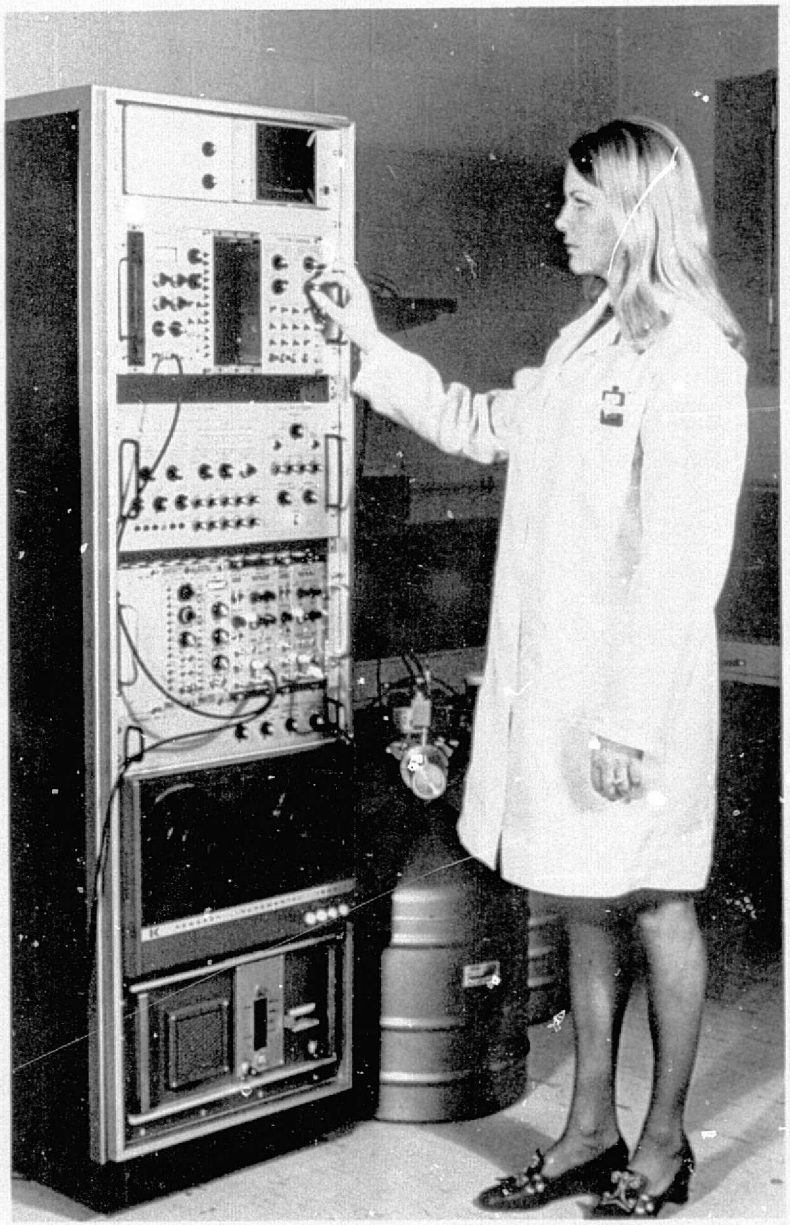
The samples are heat-sealed in plastic containers and the metals activated to radioactive states (see front cover for the core of the reactor). Following a suitable decay period to allow atoms, such as ^{22}Na, to decay, the samples are placed in a counter and the resultant data is evaluated by computer.



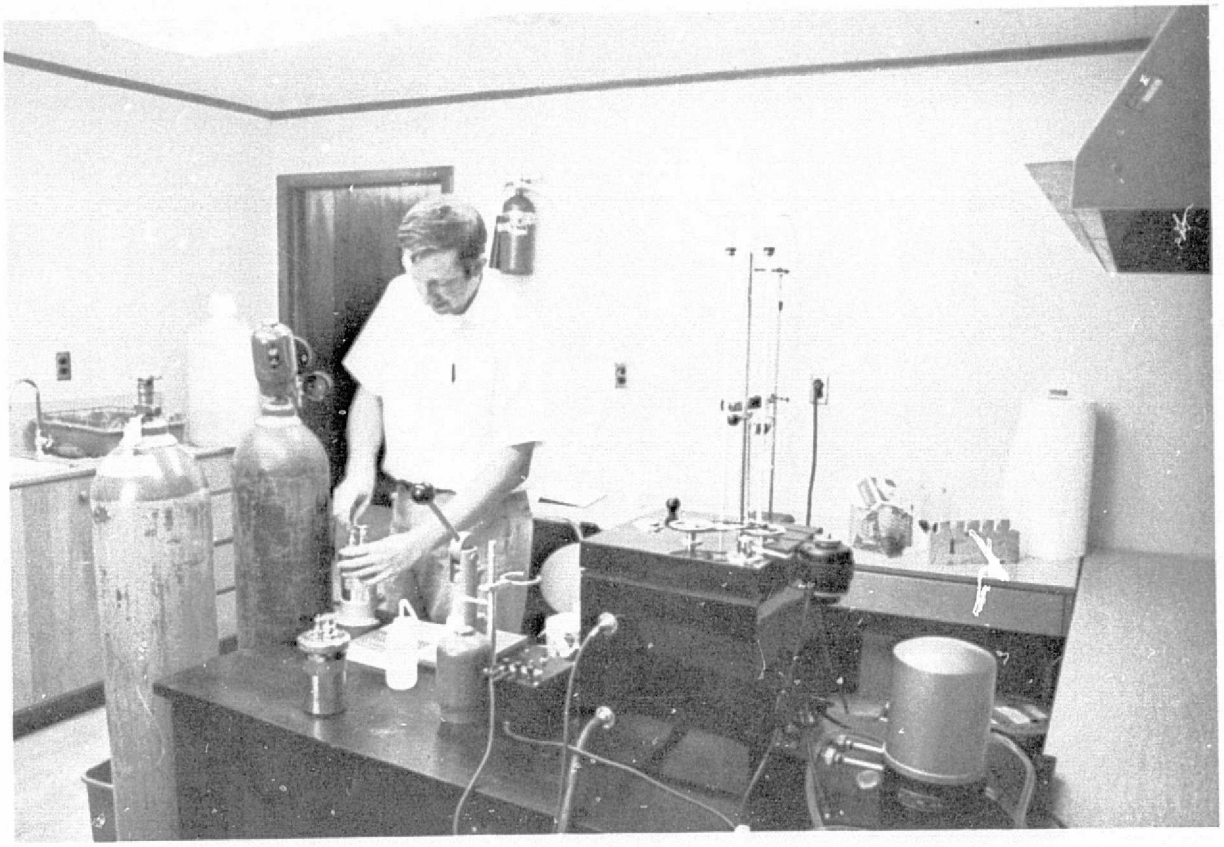
RADIATION SAFETY IS FIRST

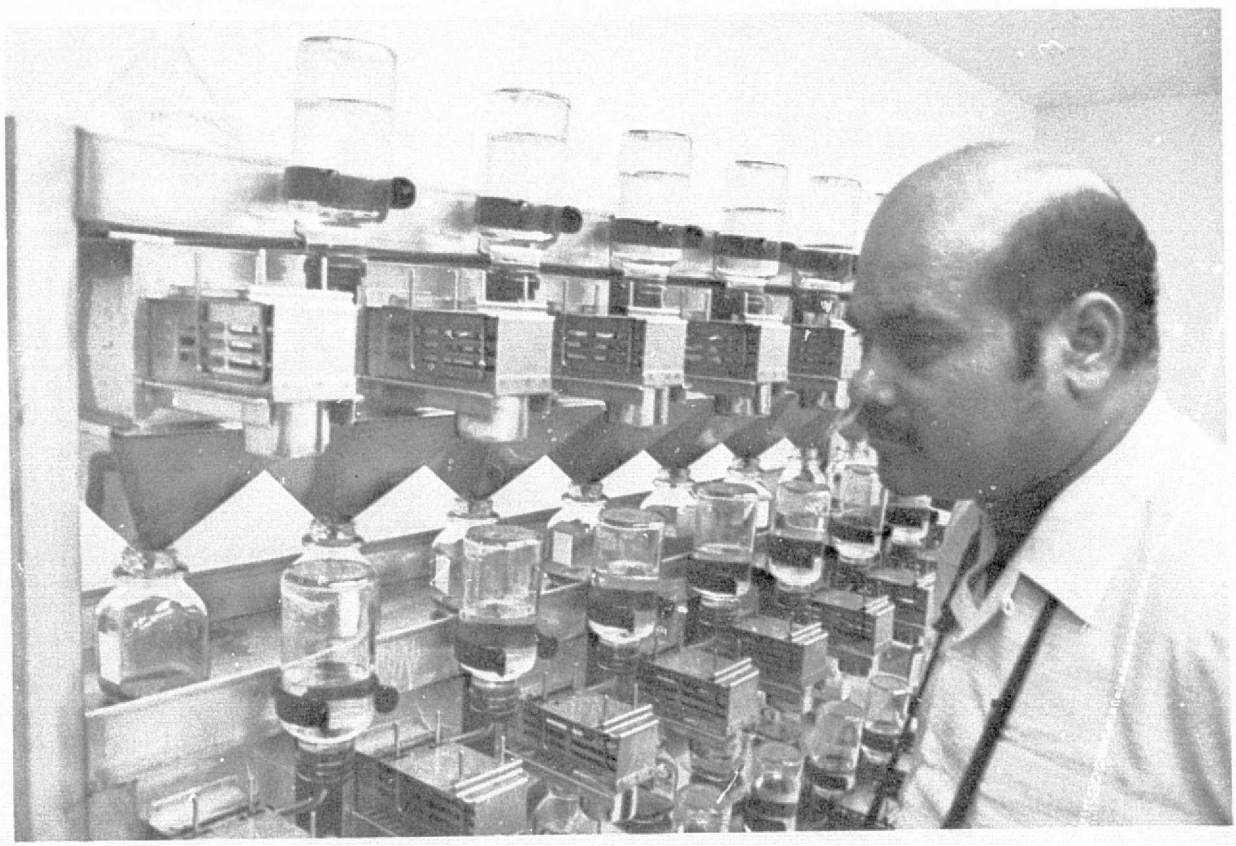


COUNTING THE RADIATED SAMPLE



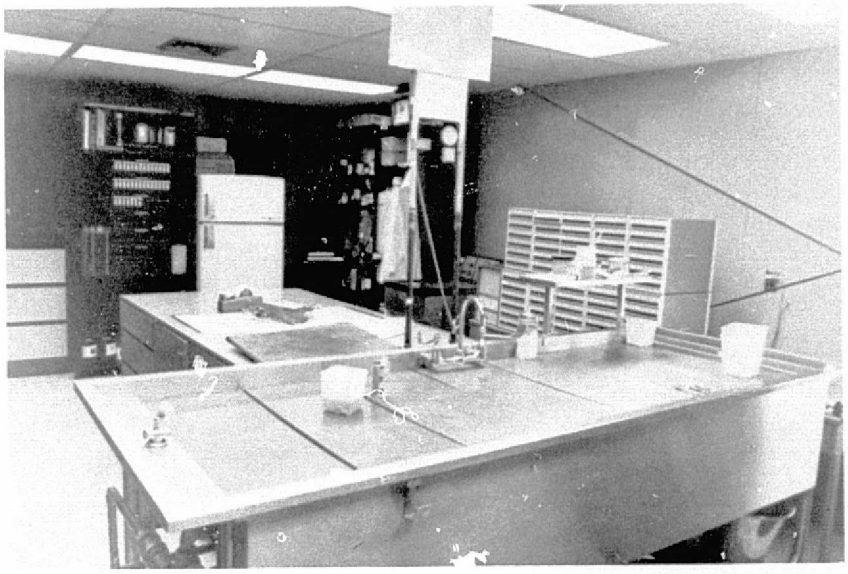
DR. HUTCHESON PREPARES THE BOMB CALORIMETER.



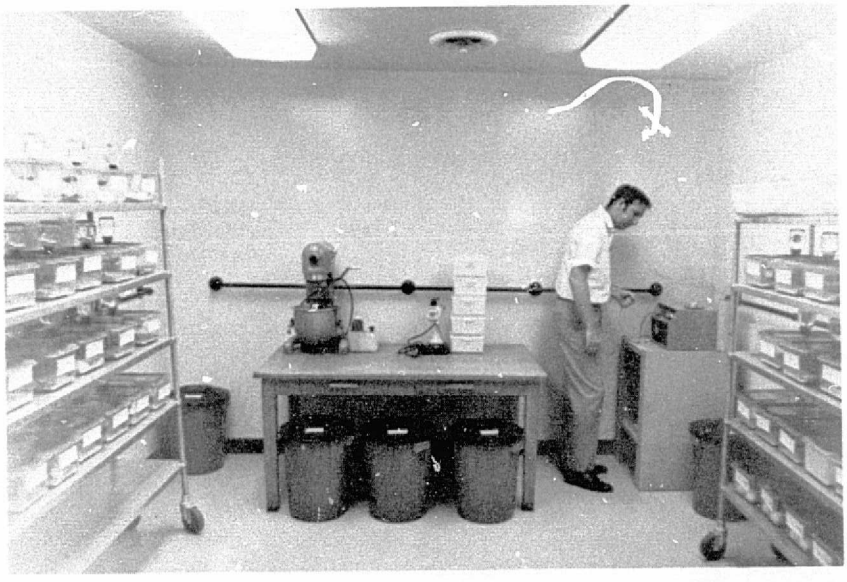


DR. VENUGOPAL CHECKS THE MOUSE METABOLISM CAGES.

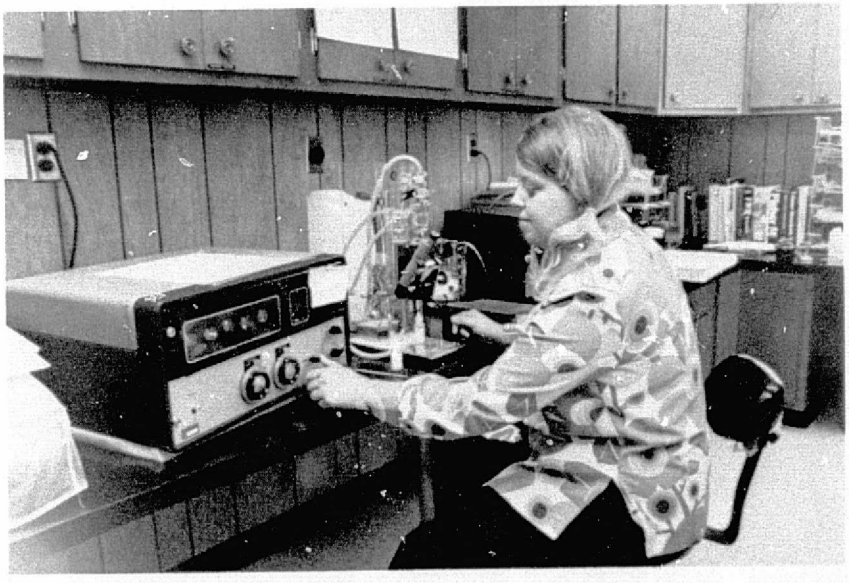
NECROPSY



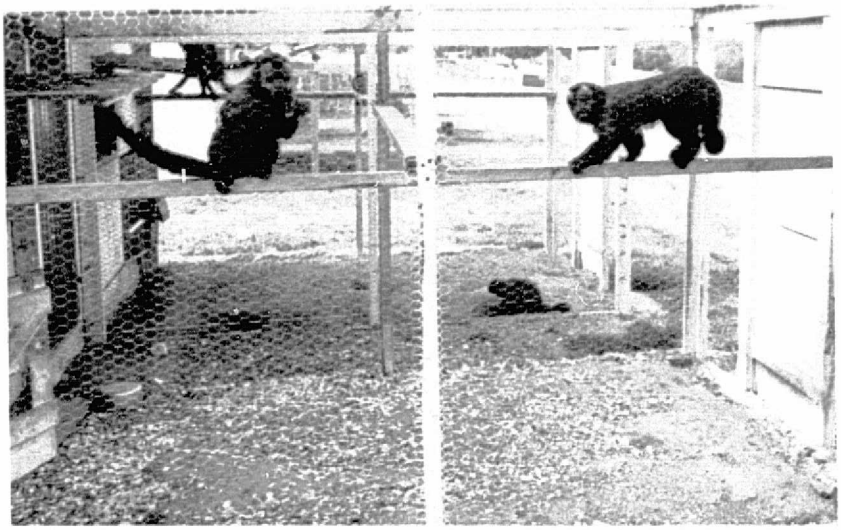
WEIGHING DIET



HEMATOLOGY

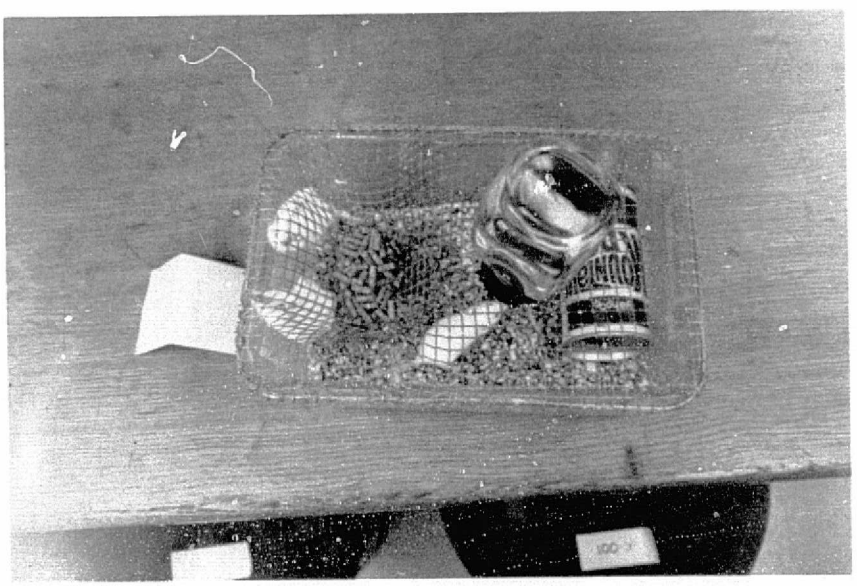


MONKEY EXERCISE



MOUSE SAFETY TEST

MOUSE HOUSE



II REVIEW OF HEAVY METAL MARKERS

A. MARKERS IN NUTRITION

"The Lord set a mark upon Cain". Genesis 4:15

Marker, indicator, tracer, reference substance, or index substance, are terms applied by workers in nutrition and physiology to a number of materials used in the qualitative or quantitative determination (usually indirect) of physiologic or nutritional phenomena. Since ancient times, markers have been used by man in ever increasing quantities and in ever more sophisticated ways. Anciently, counting black sheep in a white herd gave an estimate of the total number of sheep. However, it was not until the late nineteenth century that the use of an inert material in studies of food utilization was proposed. Colored glass beads were used early in the present century to study digestive tract function. Ingestion of radio opaque compounds was utilized shortly after X-rays were first utilized to observe the digestive tract. Since the late 1950's, the use of radioactive isotopes and techniques of radioactivation analysis has been increasingly helpful in solving many challenging problems in studies of physiology and nutrition.

The diversified use of markers in studies of digestive tract function and food utilization has accumulated much information which merits a comprehensive review of the subject. A variety of materials and procedures have been used repeatedly, but the authors have found no complete review of the topic. Our interest is limited to dietary markers, markers which would be put into the diet, which would occur in the food eaten by the animals or which would be taken orally at any time. Excluded from consideration are injected markers and most metabolites which originate within the animals. Nor have we considered dyes and radioactive materials used to trace animal movements in the wild and a great number of substances, radioactive and non-radioactive used only as qualitative or quantitative indicators in medicine in different diagnostic techniques; for example, the study of the function of some organs and the localization of certain tumors in the body. Also excluded are materials which have been used as indicators to indirectly determine body composition in vivo, e.g., tritium and deuterium have been used in the "dilution technique" to determine total body water. The determination of the body ^{40}K content has been used to estimate "lean body mass".

We have reviewed the use of markers in nutrition and some characteristics of individual markers with an evaluation of their usefulness in studies of food intake, food passage and food absorption in the gastrointestinal tract of man and animals. The techniques of feces sampling when the marker method is used, are also discussed. The extensive application of a number of indicators to study the rate of passage of food residues in the digestive tract is a major consideration. Consumption and digestibility of forage by grazing animals has been an area of interest and challenge to nutritionists; the indicator technique has contributed greatly to this area. Food utilization studies in space suggest interesting problems which could utilize new application of the indicator method.

Observation from nature or simple studies in the home or laboratory can provide much information about the nutrition of the animal being studied. Fecal pellets in the hunt offer a most valuable spoor to provide information about the animal being tracked. This might indicate the type of food, the size of the animal, something of its state of health, and how long it has been since the animal passed this location. Fecoliths, petrified fecal pellets, are useful in studying eating habits and ecology of prehistoric animals. Stomach contents (e.g., bits of undigested bone, feathers, sand, dirt, or stone from the stomach of a bird which had been eaten) tell the hunter much about his quarry. Such information provides classic information of the food habits and food chains of wild animals.

An early use of markers was the study of the rate of passage of food residues through the digestive tract of animals and man. The use of primitive markers has practically ceased; today, use of radioactive isotopes and radioactivation analysis adds convenience and precision to marker techniques. A variety of food utilization studies have taken advantage of the indicator technique. Studies of digestion, absorption, and retention of different food nutrients were found to be more convenient, less costly and sometimes more precise with the use of suitable indicators than the conventional method.

The markers used in food utilization or rate of food passage may be grouped in various ways; completeness and clarity suggest the classification given in Table I.

TABLE II-1

CLASSIFICATION OF MARKERS

A. Elements

1. Inert metals (heavy and rare earths)
2. Natural isotopes (^{40}K)
3. Artificial isotopes (^{144}Ce)

B. Compounds

1. Inorganic
   a. Metal oxides (Cr_2O_3, Fe_2O_3, TiO_2)
   b. Mineral salts ($BaSO_4$, CuSCN)
2. Organic
   a. Natural dyes (carmine, brilliant blue, chromogen)
   b. Synthetic dyes (methylene blue, crystal violet, basic fushsin, aniline blue, anthraquinone violet)
   c. Other (cellulose, lignin, plant sterols)

C. Particulates

1. Polymers (polyethylene glycol, polystyrene, glass, rubber)
2. Cells (yeast and bacteria)
3. Charcoal
4. Metal particles (sized aluminum particles)
5. Other particulates (seeds, cotton string)

A second grouping is useful from a physiologic viewpoint. Theoretically, any substance which can be classified in any of the following groups could be used as a marker in nutrition.

A. Absorbable

1. Completely absorbed from the alimentary tract and recovered in the urine. Substances that fit in this group may be referred to as "urinary indicators". Sweat and gaseous exchange are possible excretory paths for these compounds. A variable portion may be stored and some may be lost from skin functions.
2. Partly absorbed from the alimentary tract with a fixed percentage recovered unchanged in the urine.

B. Non-absorbable fecal markers

Substances in this category are absorbed minimally from the alimentary tract and may be virtually completely recovered unchanged in the feces. These substances are referred to as "fecal markers". The residue of compounds partially absorbed (A-2 above) could be useful as fecal markers. Most of the work with markers has involved the use of substances belonging to category B due to the availability of many substances that are known to fit in this group. Very limited examples are known of materials in category A that for practical reasons and under a variety of conditions may be accepted for quantitative use. Materials within category B may be characterized according to the following categorization:

1. External Markers
   a. Early markers
   b. Dyes
   c. Metal oxides
   d. Mineral salts
   e. Polyethylene glycol
   f. Radioactive markers ^{51}Cr, ^{131}Ba
   g. Rare earth elements ^{106}Ru, ^{91}Y, ^{140}La, ^{95}Zr, $^{46,47}Sc$, ^{144}Ce, Dy, Eu
   h. Microorganisms
   i. Sized particulates

2. Internal markers
   a. Silica
   b. Lignin
   c. Chromagen
   d. Fecal nitrogen
   e. The acid soluble fecal fraction
   f. Methoxyl and fiber

A variety of terms have been used in this field. A group of these are presented with definition and/or example to illustrate their meaning in this review.

Extent of passage: The point which undigested residue of the food comes to or has passed through the alimentary tract may be determined by the use of markers without otherwise disturbing the subject. Under certain conditions of digestive system malfunction or disorder, the food residue may be blocked at certain points along the digestive tract. The term "extent of passage" is used to describe how far a given ingested material was allowed to pass.

Passage of ingesta: Many terms have been used to describe the passage of ingested material through the alimentary tract. Most important of these are "transit time", "retention time", "rate of passage" and "rate of flow". These terms have been frequently used interchangeably; this has caused much confusion. To eliminate this confusion, we propose the following definitions to be compatible with the derivation of these terms.

1. Transit time - This is the time it takes the digesta of a given meal to pass through the alimentary tract or certain segments of it. This time also represents the retention time of this digesta in the tract or the particular segment. A simple way of calculating this time is by recording the time of first or last appearances of the marked residue of a meal. The "mean retention time" is a useful term calculated according to Castle (1956, for method of calculation see section on dyes).
2. Rate of flow - This term denotes the distance traveled by the digesta of a given meal through the alimentary tract (or certain segments of it) in a given amount of time.
3. Rate of passage - This refers to the quantity of digesta that passes a certain point along the alimentary tract in a given amount of time.

Digesta: Food and ingested material which is subjected to digestion within the digestive tract. Technically, it would include secretions, and excretions (mucosal cells) from different digestive organs.

External indicator: An indicator or a marker which is added to the diet or taken orally, e.g., chromic oxide.

Internal indicator: An indicator or a marker which occurs naturally in the diet, e.g., lignin.

Grab sampling: This is a technique for the sampling of feces where a sample is taken manually from the rectum. This method is mostly used with large animals.

Sward sampling: This is another technique for feces sampling specifically used with grazing animals. In this method, fecal samples are collected from the sward of grazed areas.

The criteria of nutrition markers for effective study were presented by Alvarez (1948). These requirements have been modified and augmented herein. For a given material to qualify as a marker in nutrition, it should:

a. have no toxic, physiologic or psychologic effects,
b. be neither absorbable nor metabolized (within the alimentary tract),
c. have no appreciable bulk,
d. mix intimately with the usual food and remain uniformly distributed in the digesta,
e. have no influence on alimentary secretion, digestion, absorption, normal motility of the digestive tract, or excretion.
f. have no discernible influence upon the microflora of the alimentary tract,
g. have qualities that allow ready, precise quantitative measurements and,
h. have physical-chemical properties which make it discernible throughout the digestive process.

A nutritional marker may be administered in one of the following ways depending on the nature of both the marker and the study:

a. it may be taken with the food after mixing with part or all of the diet, or drink
b. it may be taken in a pill or capsule when its color or taste is undesirable or when it is unchewable,
c. it may be prepared as a powder or solution to be taken orally or via suppository or fistula,

d. internal markers occur naturally in the diet and are therefore ingested as a part of the diet.

Conclusions

Dietary markers are useful tools in studies of food utilization including food and nutrient intake, passage of ingesta through the digestive tract, digestibility, and absorption of nutrients in man and animals. Studies of food consumption and utilization under grazing conditions call for special application of the marker method. The following conclusions are drawn regarding the suitability of markers for different studies:

1. The stained particle technique is widely used to measure the rate of food passage in ruminants. The counting of the stained particles recovered in feces is laborious and its validity is questioned. An alternative is to use a nonabsorbed dye that can be completely recovered and quantitatively measured in the feces; this has yet to be used in a wide variety of trials.
2. Several dyes have been used to color the feces of ruminants. Best coloring was with methylene blue, crystal violet, basic fuchsin or aniline blue. Carmine has been used to color human feces and those of monogastric animals to allow separation of experimental periods. A brilliant blue-methylcellulose mixture is recommended for the purpose in man.
3. Chromic oxide has been the most widely used fecal marker in human and animal studies. In ruminants, best results are obtained when the marker is given in sustained-release form by mixing with a suitable carrier. Availability of radioactive chromic oxide ($^{51}Cr_2O_3$) allows the use of minute amounts of marker and eliminates the time-consuming procedure of chemical estimation.
4. Titanium oxide has been used in studies of calcium and phosphorus absorption and protein digestibility. It can be estimated directly or in Kjeldahl digests of food and feces.
5. Barium sulphate has been used in man for extent and rate of passage studies and for chickens in food utilization studies. Radioactive $^{131}BaSO_4$ has been used to study the absorption of ^{59}Fe and ^{131}I-labelled nutrients. Cuprous thiocynanate has been successfully used in balance studies with man.

6. Several elements of the rare earths and other inert metals have been qualified as fecal markers and used in studies of passage and absorption of nutrients. Included in this group are the radioactive nuclides ^{144}Ce, ^{46}Sc, ^{47}Sc, ^{95}Zr, ^{140}La, ^{91}Y, ^{106}Ru ^{198}Au. Dysprosium, europium and gold are non-radioactive members of this group that can be estimated accurately by radioactivation analysis.
7. Soluble markers have been used in many physiological and nutritional studies in man and animals. Variation in results and the frequently reported failure to achieve complete recovery of polyethylene glycol (PEG) in the feces, especially in ruminants in some nutritional conditions, suggest re-examination of this material as a nutritional marker. It is suggested that further studies be conducted using a radioactive form of this material. Chromium-EDTA complex has been suggested as a substitute for PEG. Radioactive EDTA-^{51}Cr allows easy and precise measurement. Small quantities may be absorbed. Further work with this material, especially on its toxicology, is needed before it is recommended for wider use.
8. Microbiological techniques based on the use of thermophilic spores of bacteria such as B. subtilis to measure transit time in the digestive tract are interesting but have not been studied enough in regard to health and accuracy. The technique devised for ruminants should be evaluated in comparison with other techniques.
9. Internal markers used for food utilization studies with grazing animals include silica, lignin, methoxyl, fiber, chromogens, fecal nitrogen and the acid-soluble fecal fraction. Only chromogen and fecal nitrogen seem to be reliable indices to digestibility of forage. The use of these two indicators may continue until a better marker becomes available. Because chromogen is not absorbed from the digestive tract, it can be used in both the ratio technique and the fecal index method. Available data suggest that the latter is more reliable than the ratio technique in grazing studies.
10. It is recommended that the use of glass beads, small seeds, rubber, charcoal and metal particles in studies of the rate of passage, and iron oxide, silica, Anthraquinone Violet and Monastral Blue in digestibility

studies should cease. This conclusion is drawn on the basis of contradiction in results and repeated failure to achieve complete recovery. Plastic particles have been used in studies of digestibility and food passage. Colored plastic particles may be used to mark individual defecations when sward sampling is used in grazing studies. Further studies are needed on particle size and specific gravity of metal particulates as nutritional markers. This summary was abstracted from the definitive presentation of markers in nutrition by Kotb and Luckey (1972).

II REVIEW OF HEAVY METAL MARKERS

B. HEAVY METAL TOXICITY (From Venugopal and Luckey, 1975).

Man's increased effectiveness in industralization brings him into contact with rare minerals of the earth for which evolution provided no effective homeostatic mechanisms. At the same time man accentuates his environmental pollution from industrial and agribusiness wastes. While the essential and toxic roles of minerals in nutrition are well studied, a hitherto little explored role for minerals is their use as nutrient markers. A monitoring system is being developed in this laboratory using heavy metal oxides as multiple nutritional markers for a wide variety of studies including transit time, rate of flow, extent of passage and apparent digestibility. Kotb and Luckey (1972) reviewed the use of nutritional markers, which included both organic and inorganic compounds, and defined the prerequisites for suitable nutritional markers. Although some markers are not found to be entirely acceptable, heavy metals or their complexes appear to be most promising for acceptable markers (Kotb and Luckey, 1972). Neutron activation and atomic absorption analyses enable the quantification of these metals at exceedingly low concentrations.

Passive diffusion, facilitated diffusion and active transport characterize different methods for the passage of metal ions across the surface of intestinal and other biological membranes. The major sites of absorption in the alimentary tract are the duodenum, jejunum and ileum. The membrane pore size in the duodeunm is nine times greater than in the ileum. Passage across is not simple diffusion and there is no generally accepted theory on ion transport. Many factors such as concentration, electrochemical gradient energy from oxidative metabolism and the degree of hydration of the ions or small molecules are involved in ion transport.

A comprehensive summary of the toxicity of all metals is in preparation. The toxicity of metals is influenced by the mode of administration. Modes of entry include: (1) oral administration with variable amounts and rates of absorption from the gastrointestinal tract, (2) intravenous administration with fast distribution to tissues, (3) inhalation through the lung, (4) subcutaneous administration, (5) intraperitoneal administration, (6) intramuscular injection, and (7) absorption through the skin.

Criteria for metal toxicity in mammals are growth retardation, decreased fullness of health and intellectual capability, detrimental changes in reproductive cycle with mortality of offspring, increased morbidity, pathological changes, appearance of tumors and chronic disease symptoms and decreased longevity. Metal toxicity is the inherent capacity of the metal to affect adversely any biologic activity; the adverse effect could be an interaction of the metal with a protein or enzyme leading to changes in physiologic and metabolic processes or an interaction with DNA leading to mutation and change in behavior. Toxicity is also due to the antimetabolite activity of metal anions, formation of stable chelates or precipitates with essential metabolites, catalytic decomposition of essential metabolites, irreversible conformational changes in structure of macromolecules and disturbance of cell membrane permeability.

Toxic effects are dose dependent, the response of an organism to the toxin may be diphasic, a phase of positive biologic effects followed by a phase of pharmacotoxic action. Accumulation of excessive amounts of essential metals due to breakdown or inadequate functioning of the homeostatic excretory mechanism, or to excessive absorption from the diet may cause toxicity. Renal insufficiency and bilary obstruction may allow metal accumulation.

Mammalian systems detoxify metal ions by sequestering them in erythrocytes and leukocytes. The lysosomes of the macrophages may accumulate the insoluble and colloidal particles of the metal compounds and excrete them as residual vacuoles. When the body burden of the toxic metals increase, new proteins such as metallothionins and Cd-binding proteins are synthesized in the liver and kidney.

The mechanism of metal toxicity is complex and much is yet to be elucidated of toxicity at subcellular, cellular and organismic level. In general, light essential metals maintain the dynamic reversibility in biological processes; heavy metals fix biological structures and systems into irreversible and inflexible conformations and thereby prove toxic.

Rare earth metals or lanthanons comprise a group of fifteen metals, lanthanum (La), cerium (Ce), praseodymium (Pr), neodymium (Nd), promethium (Pm), samarium (Sm), europium (Eu), gadolinium (Gd), terbium (Tb), dysprosium (Dy), holmium (Ho), erbium (Er), thulium (Tm), ytterbium (Yb), and lutetium (Lu). These are the inner transitional elements. Although the atomic weights of these elements increase in small units with increase in atomic number, their chemical properties, metabolism, and involvement in biochemical systems are strikingly similar (Venugopal and Luckey, 1975).

Lanthanons, and yttrium form highly basic oxides of trivalent metals, but within these metals electropositivity and basicity differs. The addition of successive electrons into the rare earth 4 f orbitals increases the binding energy of the valence electrons. Ce and Gd have electrons in 5 d orbitals. The increase in binding energy decreases the atomic and ionic size and the basicity. Differences in basicity are dependent upon relative tendency towards electron loss or gain. The more basic the material the less will be the tendency to attract electrons. Lanthanum is most basic and

lutetium least basic. The divalent Ce, Nd, and terbium are much less basic than trivalent lanthanons.

The solubility of rare earth salts decreases with decreased basicity; the solubility products of lanthanon hydroxides are in the range 10^{-19} to 10^{-24}; the heavier lanthanons (from Gd to Lu) are less soluble than the lighter members. Poor solubility confers adsorptive capacity since absorption to some other material is inverse to solubility function, the less soluble the more the tendency to get adsorbed and vice versa. The adsorptive effects of lanthanons occur at concentrations less than the molar solubility of the hydroxides and at radiocolloidal concentration. At pH 7.4 to 8.1 the light lanthanons (La to Gd) precipitate and at pH 6.3 to 6.7 the leavy lanthanons precipitate.

The capacity of lanthanons to form insoluble complexes with phosphates enable them to function at low concentration as non-specific "phosphatases", cleaving off phosphate groups from essential metabolites such as ATP, glycerophosphates, nucleotides, and nucleic acids. Heavier lanthanons complex with protein more easily than do the light lanthanons and this ability to form stable plasma protein metal complexes may be involved in their coagulant action. EDTA-complexes with the rare earth metals are soluble and so stable that they are excreted in the same form. The poor gastrointestinal absorption of lanthanon compounds is due to their poor solubility, their basicity which results in the easy formation of hydroxides in precipitable, colloidal, or radiocolloidal forms at tissue pH level, and their tendency to adsorb to particulate food ingesta. The gastrointestinal intake of lanthanons is poor in all species. The great insolubility of the rare earth oxides makes their toxicity negligible. Toxicity of the metals and their individual salts following different routes of administration are

reviewed by Venugopal and Luckey (1975). The basic References are displayed in Appendix C:

a) Markers in Nutrition

b) Critical Review of Heavy Metal Toxicity in Mammals

c) Heavy Metal Toxicity, Safety and Hormology.

The data is given in Table II-B-1.

TABLE II-B-1

TOXICITY OF RARE EARTH METAL AND YTTRIUM COMPOUNDS
(Venugopal and Luckey, 1975)

Compound	Species	Mode of Administration	LD_{50} in mg of metal/Kg body weight
Lanthanum			
Oxide	rat	oral	>8500
Chloride	rat	oral	2370
	rat	ip	197
	mouse	ip	146
	mouse	sc	1373
	rabbit	iv	84
	guinea pig	ip	74
	frog	ip	393
Sulfate	rat	oral	>2450
	rat	ip	134
Nitrate	mouse	ip	132
Acetate	rat	oral	4400
	rat	ip	209
Amm. nitrate	rat	oral	830
	rat	ip	153
Citrate chloride	mouse	ip	44
	guinea pig	ip	34
Cerium			
Chloride	rat	oral	1200
		iv	50
	mouse	oral	3000
		ip	201
	frog	sc	120
Nitrate	rat	oral	1355
		ip	93
		iv	16
Europium			
Chloride	mouse	oral	2075
		ip	228
Nitrate	rat	oral	1704
		ip	72
	mouse	ip	109
Gadolinium			
Chloride	mouse	oral	> 850
		ip	232
Nitrate	rat	oral	1743
		ip	80

TABLE II-B-1 (cont.)

Compound	Species	Mode of Administration	LD_{50} in mg of metal/Kg body weight
Terbium			
Chloride	mouse	oral	2175
		ip	234
Nitrate	rat	oral	>1750
		ip	91
	mouse	ip	168
Dysprosium			
Chloride	mouse	oral	3290
		ip	251
Nitrate	rat	oral	1103
		ip	105
	mouse	ip	110
Holmium			
Chloride	mouse	oral	3140
		ip	243
Nitrate	rat	oral	1078
		ip	97
	mouse	ip	115
Erbium			
Chloride	mouse	oral	2700
		ip	233
Nitrate	rat	ip	84
		iv	13♂, 19♂
Praesodymium			
Chloride	rat	ip	750
	mouse	oral	1700
		ip	226
		sc	944
	guinea pig	ip	71
	frog	sc	500
	rabbit	sc	200
Nitrate	rat	oral	800
		ip	79
		iv	2.4, 12.5, 25
	mouse	ip	94
Neodymium			
Chloride	rat	ip	150
	mouse	oral	2125
		ip	240
		sc	160
	guinea pig	sc	140
		iv	70
	frog	sc	250
	rabbit	iv	80

TABLE II-B-1 (cont.)

Compound	Species	Mode of	LD_{50} in mg of metal/Kg body weight
Nitrate	rat	oral	905
		ip	89
		iv	2.1♀; 22♂
	mouse	ip	80
Samarium			
Chloride	mouse	oral	> 840
		ip	241
	guinea pig	sc	412
	frog	sc	150
Nitrate	rat	oral	957
		ip	96
		iv	3♀; 20♂
	mouse	ip	106
	guinea pig	sc	500
	frog	sc	660
Thulium			
Chloride	mouse	oral	2635
		ip	204
Nitrate	rat	ip	104
	mouse	ip	94
Ytterbium			
Chloride	mouse	oral	2995
		ip	186
Nitrate	rat	oral	1195
		ip	94
	mouse	oral	93
Lutetium			
Chloride	mouse	oral	4400
		ip	195
Nitrate	rat	ip	125
	mouse	ip	130♀; 108♂
Yttrium			
Oxide	rat	ip	395
Chloride	rat	ip	132
	mouse	ip	88
Nitrate	rat	ip	117
	mouse	ip	415
	rabbit	iv	125

III MARKERS ANTICIPATED AND USED

The analytical leveland the detection limits for metals are so low by neutron activation analyses that small doses of metal oxides could be used in these studies. One limitation in this new monitoring system would be the amount of these or interfering metals present in the food or environment. The metal oxides chosen for markers must be present in negligible quantities in food and feces, and must have no interference with the detection of another marker during the counting of radiation following neutron activation. Surprisingly, some Group III metals such as scandium and lanthanum are present in commercial laboratory chow at levels that could be used as internal markers. The porcelain feeding dish was found to cause uranium contamination in mouse feces. Astronaut feces are contaminated with the gold from their visors and life support tubes used for extra vehicular activities.

From the above consideration, a list of potential minerals for use as nutrient markers is presented in a later section (V-A). This list should be useful wherever multiple markers are needed. This list is cumulative in the sense that the first gives the best analytical sensitivity in the presence of each of the elements below, according to the method of Gray and Vogt (1974). Therefore, a selection of multiple metals for any use should proceed from the top.

The above information was developed from consideration of the physical-chemical character of each element, the individuality of spectral decay radiation of the activated species in the presence of other activated radionuclides, and the experience gained during this research. The markers presently recommended following the experiments are listed in Table III-1.

TABLE III-1

Recommended Markers

Element	Isotope	Half-life	Detection Limit (μg)**
*Sc	^{46}Sc	83.8 days	0.1
Ce	^{141}Ce	32.5 days	0.2
Ir	^{192}Ir	74.4 days	5×10^{-3}
Cr	^{51}Cr	27.8 days	0.2
*Eu	^{152}Eu	12.2 years	0.02
Sb	^{122}Sb	2.8 days	0.05
Au	^{198}Au	2.7 days	5×10^{-3}
*Tb	^{160}Tb	73 days	0.1
Lu	^{177}Lu	6.8 days	0.01
*Yb	^{175}Yb	4.2 days	0.04
*Sm	^{153}Sm	1.9 days	2×10^{-3}
*La	^{140}La	1.7 days	0.2

**These detection limits relate to the detection of the individual elements (a) not in company with any of the others shown, (b) in a matrix of diet or dried feces, and (c) following an approximate 20 minute irradiation and decay period of 4 to 7 days.

Table III-1 was compiled using a sort of rating system taking into account the major factors affecting the accurate determination of the actual peak areas used to calculate μg of the markers (or ppm). Those marked by an asterisk are the ones most recently used. The order shown in Table 1 is "best" at the top to "worst" at the bottom. It was assumed that combinations of these elements would probably be used and this assumption also went into the ordering of the table.

This table supplies a list of markers which can be analyzed from a single irradiation (∿ 20 minutes) and after a single decay period (4 to 7 days).

The metals used in each experiment to establish this recommended list are given in the description of each experiment.

IV RESEARCH

A. MULTIPLE ANALYSIS OF HEAVY METALS

A major advance in nutritional markers was offered by the potential to preform experiments with multiple markers which could be readily quantified in a single procedure which involved no chemical manipulation other than drying and sampling. Single markers had been studied using neutron activation analysis and radioactive metals are useful for some purposes as reviewed by Kotb and Luckey (1972). In consul with Dr. Mike Kay, procedures have been worked out (Gray and Vogt, 1974) which provide a quantitative method for the analysis of multiple heavy metal markers using non-radioactive metal oxides. The samples are processed with no wet chemistry which helps to reduce the cost of the procedure. Markers, diets and excreta may be handled without special procedures for radioactive material. The final sample in a heat sealed plastic vial is hazardous only following radiation. This presents obvious advantages for large animals, animals on pasture, and people in most situtations. This procedure adds a new dimension to the use of markers in nutrition.

A summary of the method (Gray and Vogt, 1974) is given as fig. IV-A-1. This system of nutrient intake monitoring has considerable flexibility in that several stable elements may be used to simultaneously monitor many different nutrients. The list of usable markers may be expanded and, by utilizing different irradiation and decay time schemes, may be applied to a number of different matrices. Of course, the selection of suitable markers must be a cooperative endeavor. The analytical chemist can make recommendations based on his knowledge of the capabilities and limitations of his equipment as well as analytical detection limits for the particular element. The biochemist must then eliminate those elements which do not

meet requirements for absorption and toxicity. Gray and Vogt suggest markers which, from a purely analytical standpoint, may prove useful in such a system of nutrient intake monitoring. Their tables were compiled from a consideration of published values of the nuclear parameters involved, such as cross-section, γ ray intensity, etc. The assumption has been made that counting would be done on a Ge(Li) detector. Note that the sensitivities listed are for practical analytic purposes. The detection sensitivity for separate, pure metals would require orders of magnitude less material. This method is simple, costly, reliable, and sensitive enough that only a few mg/day of some of the least active substances on earth are required for results with a 5% error. This was reported in March 1973 to NASA.

FIGURE IV-A-1. Publication of Gray and Vogt, 1974.

COMMUNICATIONS

Neutron Activation Analysis of Stable Heavy Metals as Multiple Markers in Nutritional Monitoring

A method is reported by which instrumental neutron activation analysis of various heavy metal elements has been applied to the monitoring of nutrient intake of laboratory animals. Some data are presented illustrating reproducibility. The scope of the method may be expanded by utilization of more or different markers. Tables are included, listing elements which may be useful as nutritional markers. It is hoped that ultimately this system may be applied to studies involving man.

The use of radioactive tracers has long been recognized as a valuable tool in the study of propagation of trace elements through a biological system. Recently a system of neutron activation analysis (NAA) was applied to a study of ration digestibility in cattle by Ellis and Huston (1968) and Olbrich *et al.* (1971). This study demonstrated the applicability of an inert marker element in ration intake experiments. By using inert markers, the problems associated with the handling of radioactive materials in a semi-uncontrolled situation may be avoided. Also, the samples collected may be stored for an indefinite period of time before analysis.

Particularly since solid state γ ray spectrometers came into widespread use, instrumental neutron activation analysis has been applied to a wide variety of sample types for the determination of a great many of the naturally occurring elements. While neutron activation analysis is often an expensive system, in those cases where a large number of samples are analyzed for several different elements, Ge(Li) detectors allow the analyst to minimize the "per element" cost. As a rapid and accurate system of multielement analysis, then, instrumental NAA should be especially well suited to a study of nutrient intake involving multiple stable markers.

One phase of an Apollo diet evaluation provided an opportunity to incorporate more than one marker in a nutrient intake study. Despite the closed and closely controlled environment which a space capsule represents, individual reporting, the counting of food packages, and the total food balance of the capsule do not provide indisputible evidence of the food and nutrient intake of any given astronaut.

Several studies have been completed in which suitable elements were used to monitor intake of multiple nutrients of laboratory animals (Luckey *et al.*, 1972). The markers were added to the animals' feed in amounts proportional to the content of the individual nutrient which each was intended to monitor. This paper describes the analytical techniques applied in those studies. The flexibility inherent in this system of analysis allows specific methodology to be modified as the list of markers is expanded or modified.

EXPERIMENTAL SECTION

Preparation and Irradiation. The samples consisted of about 0.9 g of diet, tissue, or feces, which were predried, weighed, and heat-sealed into ultrasonically cleaned ⅔-dram polyethylene irradiation vials. The sample vials were packaged in polyethylene "rabbits" and irradiated in the pneumatic tube facility of the University of Missouri's Research Reactor for 20 min at a thermal flux of approximately 5×10^{13} n cm^{-2} sec^{-1}. (The neutron flux was monitored with a piece of Al wire containing 0.058% Au.) Upon return from the reactor the samples were set aside for a period of about 1 week to allow for the decay of interfering shorter-lived isotopes, principally ^{24}Na.

Counting. After decay the samples, without transfer from the irradiation vials, were placed in 17 × 100 mm plastic counting tubes. Previous studies had shown that the vials themselves did not contribute any activity to the observed spectra. All counting was done on a 45 cm^3 Ge(Li) detector coupled to an 8192-channel pulse height analyzer. Each sample was counted for 1000 sec live-time and the accumulated spectrum was read onto 800 bpi computer-compatible magnetic tape. A spectrum analysis was then carried out on the tape by the University's IBM 360/65 computer using the program BARFF (Vogt, 1971).

Standards. Standards were prepared from solutions having accurately known concentrations of those elements being used as markers. A mixed standard was prepared from these solutions such that an aliquot of the mixed standard solution contained the marker elements in amounts which approximated those encountered in the sample analysis. Aliquots of the mixed standard solution were then evaporated into cleaned polyethylene vials which were sealed, irradiated, and counted in the manner described above.

Data Reduction. From a purely analytical standpoint, the choice of markers is based on several factors. One of the most important factors is the γ ray used in the analysis. As in any multielement survey by NAA, even with high-resolution Ge(Li) detectors, a major concern is interference from other γ rays in the sample at approximately the same energy. The markers used in these studies were chosen partially because each emitted a major γ ray free of such interferences. Computer analysis of the γ ray spectra yielded peak areas for each γ ray peak encountered in the spectra. Then for each of the markers to be determined in standards and samples, a single major interference-free γ ray was chosen. These peak areas were flux and decay corrected where necessary and the peak areas used to calculate ppm values for each marker.

RESULTS AND DISCUSSION

Table I presents typical data obtained. These data are presented solely to illustrate reproducibility.

The data in Table I resulted from six replicate analyses on each of two sample sets designated 17 and 19. Each set consisted of duplicate samples supplied to the reactor facility and designated A and B. The samples themselves were samples of dried mouse feces collected after a 24-hr balance study utilizing a diet containing the markers shown. The sets were obtained from two different groups of mice, both of which were fed the same marked diet under controlled conditions. The averages for each set appear, along with the values for one mean standard deviation.

From a practical standpoint, it is of interest to have

BAAB

Table I. Ppm Values for Markers Used in Nutrition Studies

	Sm	Eu	Tb	Yb	Sc	La	Dy
17A1	38.2	0.82	36.41	4.54	6.31	18.22	45.36
17A2	38.9	1.18	38.44	4.39	6.45	19.72	42.19
17A3	37.3	0.92	41.21	4.42	6.12	17.86	39.95
17B1	41.7	1.25	45.33	4.98	6.75	20.89	40.22
17B2	40.7	1.20	36.89	4.75	6.66	19.90	35.10
17B3	35.0	0.95	36.09	3.96	5.76	16.42	39.58
19A1	45.7	1.08	54.86	5.43	6.86	23.06	44.43
9A2	35.2	0.96	44.23	3.98	5.47	17.07	40.31
9A3	33.4	0.85	38.05	3.99	4.95	16.36	41.13
19B1	43.3	1.41	41.23	5.42	6.55	21.46	39.11
19B2	39.5	1.17	38.20	4.45	5.98	19.43	40.12
19B3	44.2	1.38	41.71	4.93	6.63	19.99	38.25
				Averages			
17	38.6 ± 1.0	1.06 ± 0.08	39.1 ± 1.5	4.51 ± 0.14	6.34 ± 0.15	18.8 ± 0.7	40.4 ± 1.4
Sample SD	2.41	0.19	3.60	0.35	0.37	1.63	3.37
19	40.2 ± 2.1	1.14 ± 0.09	43.0 ± 2.5	4.70 ± 0.27	6.08 ± 0.30	19.6 ± 1.0	40.6 ± 0.9
Sample SD	5.05	0.22	6.23	0.66	0.75	2.55	2.15

Table II. Proposed List of Markers to be Used in Nutrition Studies (Short Irradiation, Short Decay Time)[a]

Element	Isotope	Half-life	γ ray used in analysis, keV	Sensitivity, μg
Ga	^{72}Ga	14.3 hr	834.1	0.15
Sr	^{87m}Sr	2.8 hr	388.5	3×10^{-2}
Rh	^{104m}Rh	4.4 min	556.0	1×10^{-2}
In	^{116m}In	54 min	1293.4	5×10^{-3}
Re	^{188}Re	16.7 hr	155.1	3×10^{-3}
Nd	^{149}Nd	1.8 hr	269.6	6×10^{-2}
Dy	^{165}Dy	2.3 hr	94.6	2×10^{-4}
Er	^{171}Er	7.5 hr	308.1	3×10^{-2}
Pd	^{109m}Pd	4.8 min	188.9	0.1

[a] Irradiation, 1 to 3 min; decay, 5 min to 2 hr.

Table III. Proposed List of Markers to be Used in Nutrition Studies (Long Irradiation, Long Decay Time)[a]

Element	Isotope	Half-life	γ ray used in analysis, keV	Sensitivity, μg
Lu	^{177}Lu	6.8 days	208.4	9×10^{-3}
Ir	^{192}Ir	74.4 days	467.9	3×10^{-3}
Yb	^{175}Yb	4.2 days	396.1	3×10^{-2}
Tb	^{160}Tb	73 days	879.4	9×10^{-2}
Sm	^{153}Sm	1.9 days	103.2	1.5×10^{-4}
La	^{140}La	40.2 hr	1595.4	0.12
Sc	^{46}Sc	84 days	889.4	0.50
Eu	^{152}Eu	12.2 yr	1407.5	5×10^{-2}
Ho	^{166}Ho	27.3 hr	80.0	2×10^{-3}
Gd	^{159}Gd	18.5 hr	363.5	2×10^{-2}
Pr	^{142}Pr	19 hr	1575.0	0.60

[a] Irradiation, approximately 20 min; decay, approximately 1 week.

some idea of the confidence to be placed on a single determination, since an analytical program of this type often involves the analysis of several hundred samples and replicate analyses are not feasible. Therefore, Table I also includes values for the sample standard deviation, corresponding to the 67% confidence level of a single determination. These values appear immediately below the corresponding averages. With the exception of Eu, the values are less than 15% of the corresponding average.

This system of nutrient intake monitoring has considerable flexibility in that several stable elements may be used to simultaneously monitor many different nutrients. The list of usable markers may be expanded and, by utilizing different irradiation and decay time schemes, may be applied to a number of different matrices. Of course, the selection of suitable markers must be a cooperative endeavor. The analytical chemist can make recommendations based on his knowledge of the capabilities and limitations of his equipment as well as analytical detection limits for the particular element. The biochemist must then eliminate those elements which do not meet requirements for absorption and toxicity. A critical review on heavy metal toxicity is now in press (Venugopal and Luckey, 1973). Tables II and III present a proposed list of markers which, from a purely analytical standpoint, may prove useful in such a system of nutrient intake monitoring. The tables were compiled from a consideration of published values (Lederer *et al.*, 1968) of the nuclear parameters involved, such as cross-section, γ ray intensity, etc. The assumption has been made that counting would be done on a Ge(Li) detector.

It should be emphasized that these recommendations represent only a starting point in marker selection. Absorptivity or toxicity of the oxides of all these elements has not been considered. Several of the elements listed, namely Dy, Lu, Ir, Tb, Yb, Sm, La, Sc, and Eu, have already been used in nutrition studies with varying degrees of success (Hutcheson *et al.*, 1973; Luckey *et al.*, 1972). In these studies the oxides were used almost exclusively. The sensitivities shown are detection limits and are not intended to be recommendations as to the level which should be present for an accurate analysis in any given case. However, a general rule of thumb in selecting a marker or a series of markers from these tables is that those having the smallest value for sensitivity are most desirable in the analytical sense. Thus, the best would be Sm, the next best Dy, and so on.

Further studies are now underway in which nutritional monitoring of monkeys, applying this system of analysis, is being conducted. It is hoped that ultimately the system may be applied to human beings using as many as 20 markers.

ACKNOWLEDGMENT

The authors wish to express their gratitude to Drs. T. D. Luckey, Dave Hutcheson, and B. Venugopal for supplying both the samples and the opportunity to carry out this work. We also wish to thank Mrs. Vickie Spate and Miss Mary Mussachia for their valuable assistance in irradiation, counting, and data reduction.

IV RESEARCH

B. RAT FEASIBILITY STUDIES

Evaluation of markers in nutritional studies (1) indicated that few classic markers (index substances) should be used without serious reservations. However, good recoveries were reported for heavy metal oxides and salts. Radioactivation analysis of heavy metals (2) in appropriate samples provides further advantages for long-term experiments and for those conditions where radioactivity of untreated samples is undesirable, e.g. prolonged administration to humans and large animal feed lots. Pioneering work by Ellis (2) and Martz et al. (3) with a single marker measured by radio-activation analysis suggested the feasibility of using multiple markers. Cerium (Ce), terbium (Tb), ytterbium (Yb), lutetium (Lu), and iridium (Ir) were chosen for their negligible absorption and convenience of analysis by a single method, radioactivation analysis, to be utilized in a multiple marker feasibility study.

The concept examined experimentally was that recovery, rate of passage and/or apparent digestibility could be determined for several nutrients simultaneously by appropriate use of one marker for daily food intake plus one marker for each nutrient studied.

Following preliminary evaluations of the utility, sensitivity and compatibility of heavy metals in group analyses, the individual studies performed were: (a) recovery of selected heavy metals following single dose administration; (b) determination of the time needed for stabilization of the excretion curve of the markers used under conditions of constant intake; (c) comparison of the apparent digestibility of energy, calcium, potassium and iron by both classic and marker methods using different diets; and (d) determination of diurnal variation of excretion of the

markers. Satisfactory results from such studies would establish good feasibility for the use of multiple heavy metal markers in nutrition.

Methodology

Materials

Animals. Adult, male Sprague-Sawley rats were individually housed in stainless steel metabolism cages. The room was lighted from 8:00 a.m. to 10:00 p.m.

Water. The animals had continuously available distilled, ion exchange water in plastic waterers having plastic-covered stoppers and stainless steel nipples. The waterers were changed each week. Water taken from the waterers had approximately one million ohms resistance.

Diets. The stock diet was a single batch of Purina rat pellets ground with a cutting mill to pass a 20 mesh screen. Markers were added as needed by thoroughly mixing dilute nitric acid (0.01 N) solutions of four markers into the control diet. Corn oil was blended into the diet for the high energy diet (9:1, diet: oil, W/W). Alpha Cell was mixed into the diet for the low energy diet (4:1, diet: cellulose, W/W). All diets were stored under refrigeration. The estimated composition is given in Table IV-B-1.

Except in the recovery experiment, all rats ate one grain of puffed rice immediately prior to each feeding. The recovery experiment was performed with one grain of puffed rice for each marker given to each rat once, at the beginning of the experiment.

Food intake was carefully monitored in all experiments. Diet was fed *ad libitum* for two hours twice daily (8:30 - 10:30 a.m. and 8:00 - 10:00 p.m.) except in the recovery experiment where food was available continuously.

TABLE IV-B-1

Diet Composition (%)

	Lab Chow* Listed	Chow + Fat Calculated	Chow + Fiber Calculated
Dry Weight	91.0	92	93
Protein	23.4	21.1	18.7
Fat	4.5	14.1	3.6
Fiber	5.2	4.7	24.2
TDN	75	67 + 10	60
Gross Energy K Cal/gm	4.25	4.7	3.4
Ca	1.30	1.17	1.04
P	.86	.77	.67
K	.82	.74	.66
Na	.49	.44	.39
Fe (µg/gm)	198	178	158

* Purina Mills, St. Louis, Missouri

The rationale for the two hour ad libitum feeding twice daily was to allow a specific relationship of the intake marker to a known dietary pattern. The rats adapted readily to this regime which is not unlike their laboratory habits (a big meal after midnight and a small meal in the morning when fresh food and water are provided) according to the authors observations. The hunger of the rats encouraged them to readily eat the puffed rice prior tc each meal.

Samples of ground stock diet handled with plastic gloves and homogenized in a stainless steel mixer and equipment for collection of excreta were checked for natural occurrence of these elements; no marker concentrations were found which were significantly above minimum detection limits.

Markers. The markers used, the ratio of marker to one specific nutrient and information about each marker is given in Table IV-B-2. The quantity of markers in the diets as fed are presented in Table IV-B-3. Markers selected for nonabsorptibility in the digestive tract and compatible simultaneous determination by neutron activation analysis were cerium (Ce), ytterbium (Yb), iridium (Ir), lutetium (Lu), and terbium (Tb). The metals (at least 99.99% pure from Ventron) were weighed and dissolved in nitric acid. Stock solutions of the nitrate salts (Yb, 4.54 mg/ml; Tb, 5.03 mg/ml; Ir, 2.86 mg/ml; Lu, 4.74 mg/ml; Ce, 5.00 mg/ml) were used to prepare suitable dilutions in 0.01 N HNO_3. Since the desired dietary quantities were below analytical detection limits, the nutrient markers were mixed with the diet in fivefold concentrations for neutron activation analysis and then diluted with unmarked diet for feeding. Following ingestion, the markers became concentrated through bioincrassation due to absorption of nutrients; this provided the approximate fecal concentrations required for activation analysis.

TABLE IV-B-2

Nutrient Markers in Diets

Element	Cerium	Ytterbium	Terbium	Iridium	Lutetium
Isotope generated	^{141}Ce	^{175}Yb	^{160}Tb	^{192}Ir	^{177}Lu
Half life, days	32.5	4.2	73.0	74.4	6.8
γ Ray Energy, Kev	145.4	396.1	879.4	316.5	108.4
Nutrient Marked	Daily intake	K Cal	Ca	K	Fe
Balance Experiment					
Intended (μg Marker/unit nutrient)	50/day	0.060/K Cal	0.020/mg/ Ca	0.020/mg K	0.200/mg Fe
					0.7

TABLE IV-B-3

Multiple Marker Data (1)

Diet No.	Kind	Metal	Dietary Level (Mean ± SE)	Fecal Level (Mean ± SE)
I	Stock	Yb	0.046 ± 0.038	0.123 ± 0.064
		Tb	0	0.022 ± 0.012
		Ir	0	0
		Lu	0.037 ± 0.013	0.122 ± 0.013
		Ce	0.021 ± 0.021	1.78 ± 0.58
II	Stock plus Markers	Yb	0.250 ± 0.082	1.517 ± 0.045
		Tb	0.310 ± 0.021	1.408 ± 0.013
		Ir	0.172 ± 0.029	1.140 ± 0.093
		Lu	0.167 ± 0.023	0.894 ± 0.057
		Ce	0.399 ± 0.029	5.846 ± 0.040
III	High Energy	Yb	0.225 ± 0.016	1.577 ± 0.049
		Tb	0.186 ± 0.077	1.450 ± 0.034
		Ir	0.193 ± 0.090	0.982 ± 0.035
		Lu	0.148 ± 0.006	0.894 ± 0.057
		Ce	0.204 ± 0.005	5.796 ± 0.034
IV	High Fiber	Yb	0.134 ± 0.022	0.512 ± 0.077
		Tb	0.212 ± 0.038	0.656 ± 0.053
		Ir	0.177 ± 0.020	0.506 ± 0.026
		Lu	0.128 ± 0.039	0.371 ± 0.017
		Ce	0.118 ± 0.091	2.034 ± 0.003

(1) Corrected by substracting the appropriate quantity found for diet and feces of rats in Group I from diets and feces for groups II, III and IV.

Each marker was incorporated into the diets at the lowest concentration estimated to provide a 5% error of analysis with duplicate fecal samples. Lower concentrations were known to give less precise data due to the inherent background radiation through the counter. Higher concentrations or more replicates would provide increased analytical accuracy.

The data at the concentrations used generally showed a 5% error. This level was designated the use level. Except in the recovery and stabilization studies, markers were fed six or more days prior to the beginning of the experimental period.

Cerium, the intake marker, was given in a carrier. Puffed rice grains were infused with a known amount of the salt solution and left to air day on a plastic sheet (Table IV-B-4). In the stabilization experiment each rice particle was intended to contain 5µg cerium and in the digestibility study each rice particle was intended to contain 25 µg cerium.

Analyses. Individual consumption of diet and excretion of feces and urine were recorded daily. Individual fecal collections were dried at 70-75° for 24 hours. Dried samples were stored at room temperatures. Prior to analyses each was ground to a fine powder with porcelain mortar and pestle. Activation analysis was performed on duplicate and sometimes triplicate triturated aliquots in heat-sealed plastic containers using a method designed for multiple analyses (2). The samples were activated in a neutron flux and stored until the sodium decay allowed detection of minor peaks. A radiation spectrum was measured at preprogrammed energies and the decay rates of known standards were used to quantitate the amount of Ce, Ir, Lu, Tb and Yb on a µg/g basis.

TABLE IV-B-4

Markers in Puffed Rice

Marker	Rice Particle (µg/particle)		
	Quantity Intended	Quantity Found	
		Ave.	Duplicates*
Ce	50	39.940	44.185 35.732
Yb	25	22.916	23.817 22.015
Tb	25	22.905	23.379 22.431
Ir	25	23.913	25.158 22.668
Lu	25	19.100	19.619 18.581

*Duplicate particles containing one marker each were diluted by mixing into 1 g of diet (5 g for the Ce pellet) for activation analysis.

The bomb calorimeter was utilized for energy determinations. In order to determine Ca, and K, weighed aliquots were wet ashed in ternary acid, a mixture of 70% nitric acid, 70% perchloric acid, and 96% sulphuric acid in a 10:4:2 V/V ratio. Approximately one gram of diet or feces was accurately weighed and treated with 12.5 ml of the ternary acid and slowly digested. Within one hour a clear digest of the sample was obtained. The volume was decreased to 5 or 6 ml with consequent separation of white precipitate; upon the addition of HNO_3 (1.1 V/V concentrated HNO_3 and water) the precipitate dissolved and the solution was diluted with water to 25 ml for diet and 100 ml for fecal samples. K and Ca were determined by flame photometer using standard methods. Fe was estimated colorimetrically by the thiocyanate method in the 1-10 µg range. These methods were outlined by Oser (5).

Since each marker was incorporated in a known ratio to a given nutrient, analysis of feces for both marker and nutrient allowed calculation of the apparent digestibility of the nutrient:

$$\% \text{ Ap. Dig.} = \left[1 - \left(\frac{Nf}{Nd} \times \frac{Md}{Mf}\right)\right] 100.$$

% Ap. Dig. = percent apparent digestibility;

Nf = nutrient concentration in feces;

Nd = nutrient concentration in diet;

Md = marker concentration of diet;

Mf = marker concentration of feces.

Note that quantitative data for neither diet intake nor feces output is required; the concentrations alone provide the index of apparent digestibility. This concept was reviewed by Kotb and Luckey (1).

Experimental Designs

Recovery. The recovery experiment provided information about the rate of excretion of a single food particle and the percentage recovery of each marker. Fecal excretion of five heavy metal markers was determined for four rats (average weight = 255 g). Each was starved overnight and then each was fed 5 rice grains, one containing 50 µg of Ce and four each containing 25 µg of Tb, Yb, Lu and Ir. The rats were fed ad libitum thereafter. Complete collection of feces and urine by 24 hour periods was made for seven days.

Stabilization. The stabilization experiment was designed to indicate the initial pattern of excretion and to define the period required to reach uniform excretion of the markers. Four rats (mean body weight = 303 g) were fed ad libitum twice daily, for a total of 4 hours, a ground stock diet which contained four markers. Five µg of Ce were given twice daily in puffed rice. Complete collection of feces and urine by 24 hour periods was made for seven days.

Apparent Digestibility. The apparent digestibility experiment provided a comparison of direct and index methods using multiple markers. The digestibility experiment was six days duration following an adaptation period of six days. Twenty-four rats (mean body weight = 269 g) were divided into four groups which were randomly assigned to treatments. Group I served as the control and was fed unmarked, ground stock diet. Group II was fed ground stock diet with markers incorporated. Group III received a high energy diet and markers. Group IV received a low energy diet and markers. Prior to the morning feeding, 25 µg Ce was fed in puffed rice to each rat in Groups II, III, and IV. Rats in Group I were fed untreated rice. Rats were restricted to two feedings daily (8:30-10:30 a.m.; 8:00-10:00 p.m.).

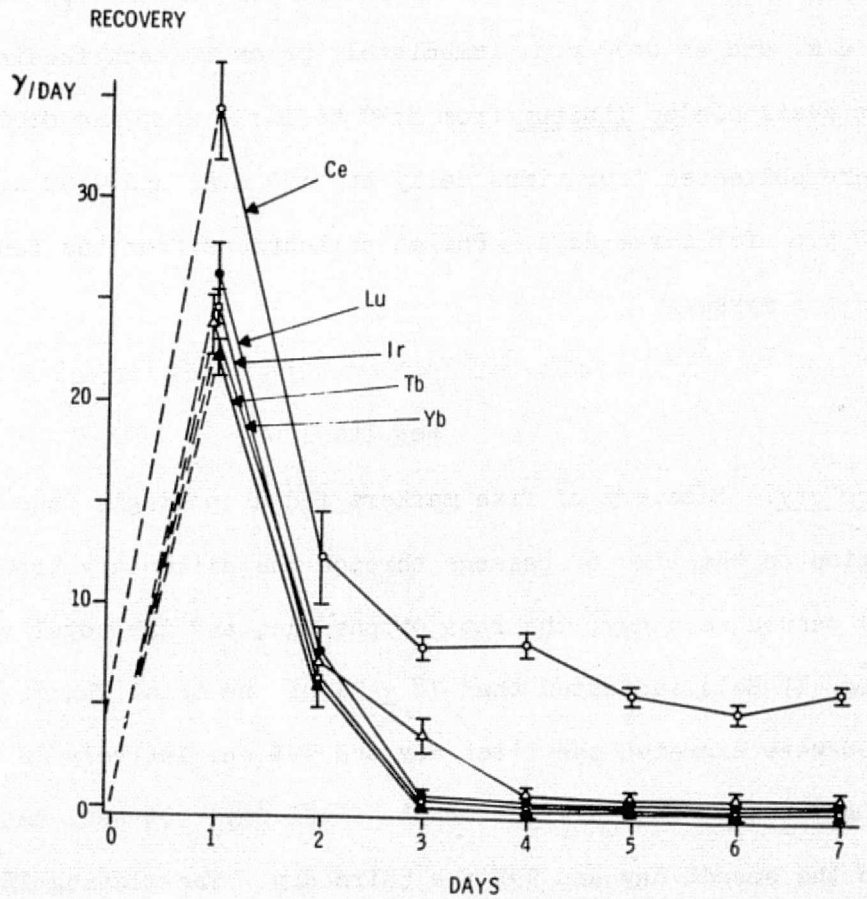
Complete 24 hour fecal samples were collected for six days from each rat, pooled for analyses and subsequent calculation of apparent utilization of Ca, K, Fe and energy.

Diurnal Variation. A diurnal variation experiment was carried out to study the inter- and intra-day variations in marker excretion following an adaptation period of two weeks. The four rats (mean body weight = 312 g) used in the apparent digestibility study were fed a ground stock diet with the markers incorporated. Ce was fed in puffed rice, 25 µg twice daily at 8:30 a.m. and at 8:00 p.m. immediately prior to each feeding period. Food was available ad libitum from 8:30 to 10:30 a.m. and 8:00 to 10:00 p.m. Feces were collected four times daily at 3:00 a.m. and 9:00 a.m. and 3:00 p.m. and 9:00 p.m. for three days. Pooled collections from the four rats were analyzed for markers.

Results

Recovery. Recovery of five markers fed as a single dose provided information on the time of passage through the alimentary tract, the quantity passed each day, the rate of passage, and the total recovery. The data (Fig. IV-B-1) indicated that 78 ± 1% of the total Tb, Ir, and Lu recovered were excreted the first day and 99% cumulatively in the first two days. Only 61% of Yb was excreted the first day, 90% was cumulatively excreted the second day and 99% the third day. The missing 1% was indistinguishable from the background at the analytical limits of sensitivity. When the background level of Ce was deducted from the Ce data, 88% of the total Ce recovered was excreted the first day and 94% was cumulatively excreted by the second day. Tb, Ir, and Lu are superior markers by this criterion. The data indicate that any one-day collection will have relatively large

IV-B-1. Fecal recovery of a single dose of five heavy metals from adult rats fed continuously. The mean value from four rats ± one standard error is displayed.



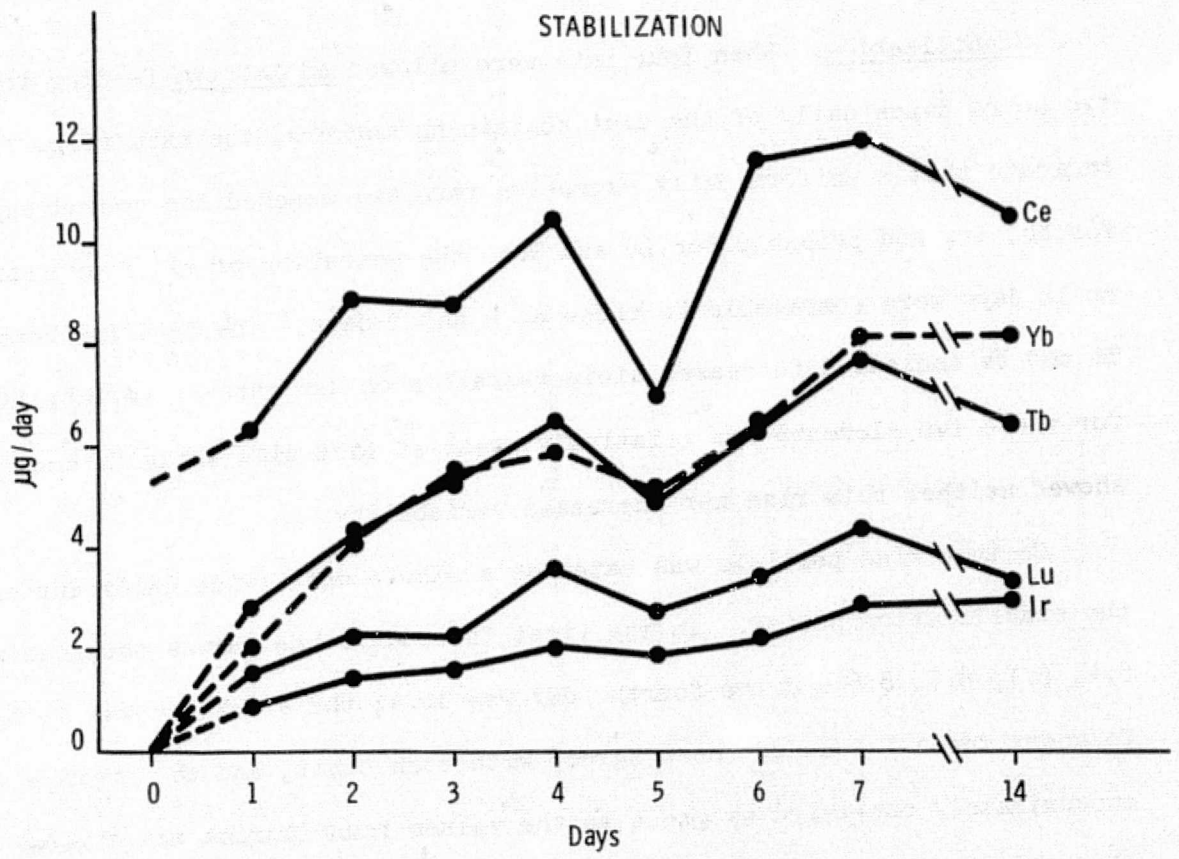
quantities of fecal material from the previous one or two days rations. During the last three days of the experiment the average fecal excretion of these metals was 5.41 µg Ce, 0.28 µg Lu, 0.19 µg of Yb, 0.07 µg of Tb and 0.01 µg Ir per rat per day. Accepting these as background levels at the analytical limit of the method, each nutrient marker was completely excreted within three days following administration of 25 or 50 microgram quantities.

Stabilization. When four rats were allowed ad libitum feeding for two hours twice daily of the diet containing markers, the data (Fig. IV-B-2) indicate that a uniform daily excretion rate was reached the second day for Yb, Ir, and probably for Lu and Tb: the excretion of all four metals at 14 days were comparable to those at 2 and 7 days. The data for both Tb and Yb indicated increased bioincrassation on day three. Variability for these two elements was relatively great at that time while Lu and Ir showed neither this rise nor increased variability.

Ce in a rice particle was eaten as a single dose twice daily during the stabilization period. In the first three days the values obtained were 6.4, 8.7, 8.7, 8.6 and the fourth day was 10.4; the sixth day was 11.6 µg Ce/g dry feces. None of these agreed with each other, and the average was approximately one-third as great as the values found during the six-day digestion study.

Apparent Digestibility. When the apparent digestibility (absorbability) was calculated for four different nutrients (Table IV-B-5), the direct method gave results with considerable variability in calcium and iron when diet I and II were compared. Both nutrients gave significantly ($p < 0.05$) less apparent digestibility in the presence of the marker compounds. The presence of marker entailed added mixing of the diet; this increased oxidation may

IV-B-2. Stabilization of fecal excretion of four metals incorporated intc the diet. Diet was fed ad libitum for 2 two-hour periods daily. The mean value from four rats and one standard error is displayed.



have caused the difference. The variability was higher for these nutrients than for energy and K, as shown by the standard errors. The utilization of calcium was decreased ($p = 0.05$) by the high fat diet (diet III), a predictable result when one considers the insoluble soaps which might be formed. The data on apparent digestibility of energy was relatively consistent between treatments: approximate energy digestibility was 83% for the control and high energy diets and a lower percentage for the high fiber diet. The apparent digestibility of potassium was 89 $\pm$ 3% for all of the diets.

Apparent digestibility for these same four nutrients was determined by markers and data from direct and indirect methods were compared (Table IV-B-6). There was no significant difference in K digestibility when direct and marker methods were compared. The values obtained by the indirect method were consistently higher than those obtained by the direct method for both energy and Fe. This difference was significant ($p < 0.05$) for energy digestibility in the high fiber diet. Calcium apparent digestibility was significantly higher ($p < 0.05$) by the indirect method for both the high and the low energy diet. Most Ca data had a high standard error: Fe data for diets III and IV showed a high standard error.

Diurnal Variation. Collection of feces every six hours provided information about the fecal excretion pattern of rats fed the marked diet during two hour periods twice daily. Since this experiment followed the six-day balance study, the animals were well adjusted to the routine and the excretion patterns were undoubtedly stable. Variability within each period was not available because the samples were pooled in order to obtain enough material for analysis. The data for the four nutrient markers (Fig. IV-B-3) indicate a relatively small variation when expressed as

TABLE IV-B-5

Six-Day Direct Apparent Digestibility Study[1]

Diet No	Kind	Nutrient	Dietary Level (Mean ± SE)	Fecal Level (Mean ± SE)	Direct Apparent Digestibility (Mean ± SE)
I	Stock	Avg g[2]	22.0 ± 0.40	4.16 ± 0.71	
		Kcal/g	4.07	3.72 ± 0.081	82.8 ± 2.18
		Ca mg/g	11.6	30.0 ± 0.83	51.1 ± 2.97
		K mg/g	25.0	10.8 ± 0.96	91.8 ± 8.01
		Fe mg/g	1.22	2.02 ± 0.188	68.7 ± 4.21
II	As I	Avg g[2]	22.1 ± 0.28	4.80 ± 0.11	
		Kcal/g	4.07	3.71 ± 0.058	80.2 ± 1.56
		Ca mg/g	11.6	35.4 ± 1.90	33.8 ± 5.37
		K mg/g	25.0	15.1 ± 0.80	86.9 ± 5.30
		Fe mg/g	1.10	2.45 ± 0.073	51.4 ± 2.63
III	High Energy	Avg g[2]	20.2 ± 0.56	3.95 ± 0.05	
		Kcal/g	4.78	3.91 ± 0.065	84.1 ± 1.66
		Ca mg/g	10.4	38.8 ± 0.81	27.1 ± 2.09
		K mg/g	23.0	11.6 ± 0.35	90.1 ± 3.02
		Fe mg/g	1.15	1.76 ± 0.087	70.1 ± 4.94
IV	High Fiber	Avg g[2]	24.1 ± 0.38	9.60 ± 0.18	
		Kcal/g	4.49	3.92 ± 0.036	65.2 ± 1.32
		Ca mg/g	9.20	14.8 ± 0.45	35.9 ± 3.04
		K mg/g	26.7	9.48 ± 0.41	85.8 ± 4.32
		Fe mg/g	1.10	1.24 ± 0.050	55.1 ± 4.31

(1) Average values for duplicate or triplicate individual dry samples from six rats with standard errors. All mean values are corrected to 3.

(2) Average intake on a dry basis of ground chow and feces: g/rat/d.

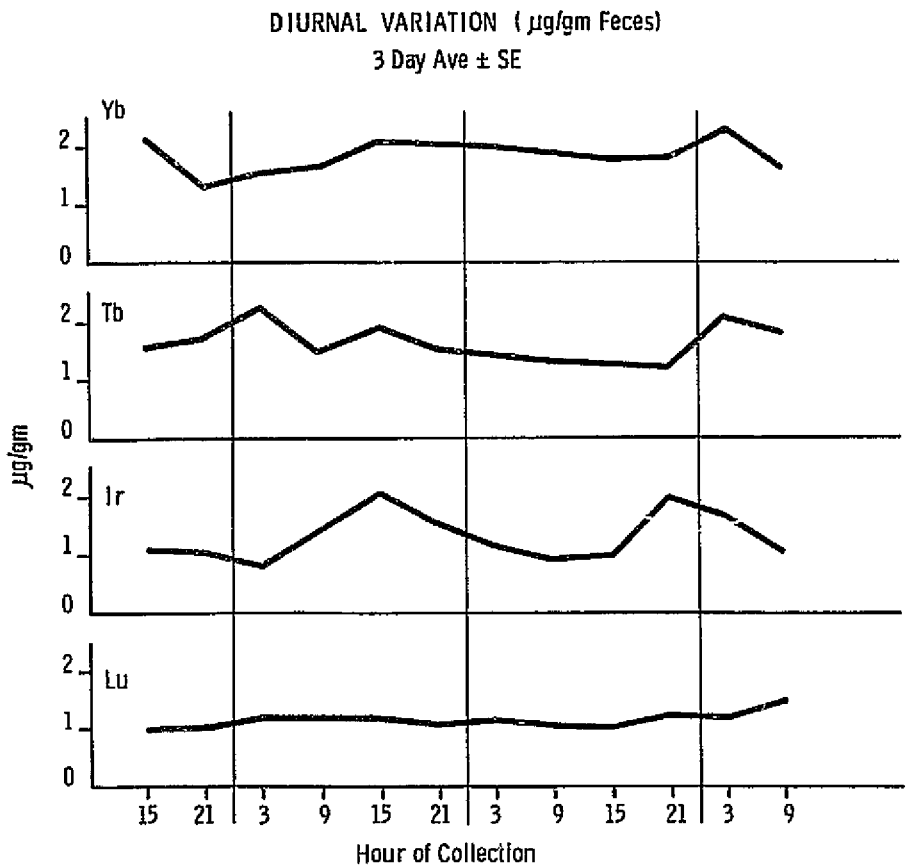
TABLE IV-B-6

Balance Study With Multiple Markers

Diet	Kind	Nutrient	Indirect apparent digestibility					% of Direct Apparent digest
			Tb	Yb	Lu	Ir	Mean	
			(Mean ± Se for each)					
	Stock	Kcal	80.0 ± 2.6	80.7 ± 3.0	83.0 ± 4.5	86.3 ± 5.2	82.5 ± 2.2	103(1)
		Ca	32.8 ± 5.1	49.7 ± 6.2	43.0 ± 7.1	53.9 ± 6.8	44.9 ± 4.3	106(1)
		K	86.7 ± 3.4	90.1 ± 3.9	88.7 ± 6.0	90.0 ± 5.6	89.1 ± 2.2	99.4(1)
		Fe	51.0 ± 2.1	63.3 ± 4.6	58.4 ± 1.2	66.4 ± 6.8	58.8 ± 2.9	99.5(1)
	High Energy	Kcal	90.8 ± 4.5	88.3 ± 3.1	86.5 ± 4.0	83.9 ± 3.8	87.4 ± 3.0	104
		Ca	52.1 ± 2.3	46.8 ± 6.8	38.3 ± 3.3	26.7 ± 4.0	41.0 ± 3.1	152
		K	93.5 ± 6.2	92.0 ± 4.2	91.7 ± 2.1	90.1 ± 3.56	91.9 ± 2.2	102
		Fe	80.4 ± 4.2	78.2 ± 3.9	74.7 ± 6.4	69.9 ± 5.5	75.8 ± 4.2	108
	High Fiber	Kcal	71.8 ± 3.9	77.2 ± 3.2	69.9 ± 4.2	69.5 ± 8.2	72.1 ± 3.7	111
		Ca	48.0 ± 8.1	57.9 ± 2.5	44.5 ± 7.2	43.7 ± 6.2	48.5 ± 3.9	135
		K	88.5 ± 6.3	90.7 ± 4.7	87.8 ± 6.1	87.6 ± 5.1	88.7 ± 4.2	103
		Fe	63.6 ± 5.4	70.5 ± 3.8	61.6 ± 4.6	60.6 ± 6.8	64.0 ± 4.7	116

(1) Based upon the average direct apparent digestibility for diets I and II (Kcal = 81.5%, Ca = 42.4%, K = 89.6% and Fe = 60.1%).

IV-B-3. Diurnal variation of four heavy metals in fecal excretion of rats fed ad libitum 2 hours in the morning and 2 hours at night. Their diet contained the metals mixed homogeneously. Each curve represents the results from four rats using pooled replicates.



concentration in the feces. Lutetium was remarkably constant. The metals were incorporated into the diet while Ce was given in puffed rice prior to each of the regular feeding times. The total Ce per collection period (top curve, Fig. IV-B-4) generally reflected the quantity of feces recovered for each period (center curve). The concentration of Ce in feces (bottom curve) showed one definite peak each day and generally was inversely related to the quantity of feces, and the quantity of each of the four markers excreted per collection period.

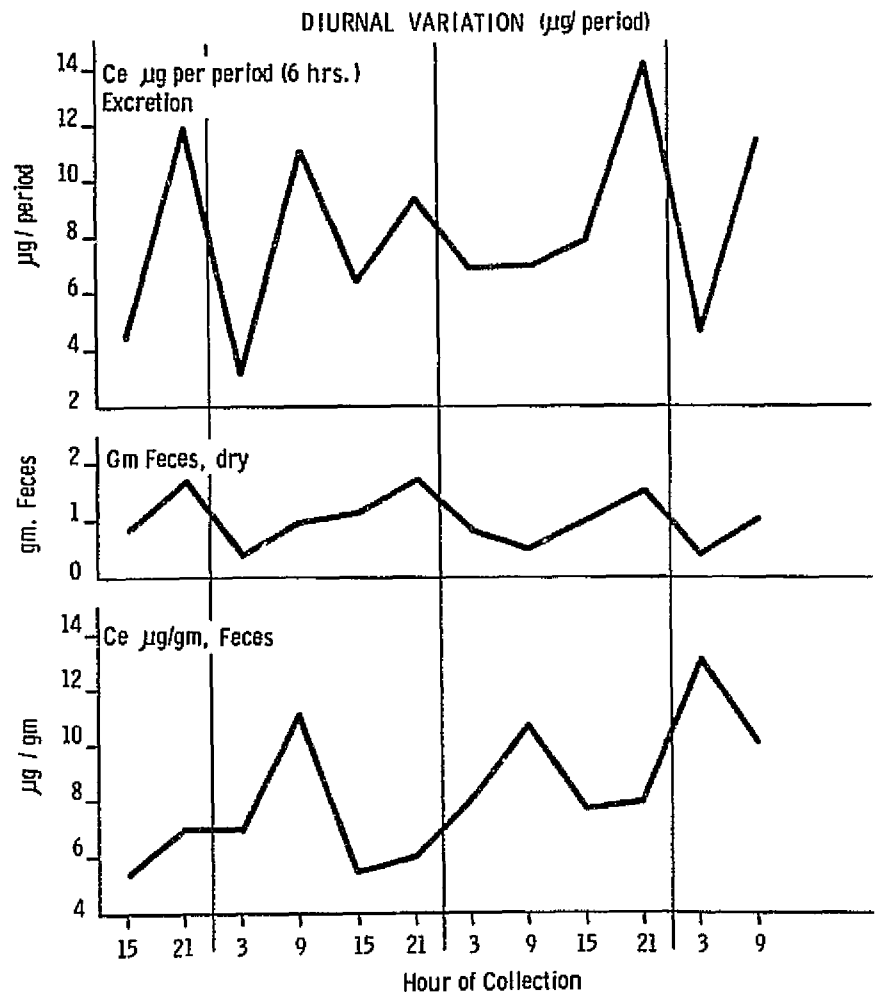
Discussion

In the recovery experiment, Ce was distinct from the other four metals in that the background was negligible before but not after the other four markers were added. Approximately 0.02 µg of Ce per g of stock diet was detected prior to addition of the other four heavy metals and almost 0.4 µg per g in the diet to which four other markers, but no Ce, was added. This suggests an analytical anomaly in which the addition of the other elements to this diet sensitized Ce detection in this method.

Excepting this discrepancy with Ce, the recovery experiment showed very clearly that over 90% of these heavy metals are recoverable within 48 hours of feeding. If the background quantity of approximately 6 µg of Ce per g feces were deducted from this experiment, then all of the Ce found above background data would have been voided in the first 48 hours. Recovery was greater than theory due to possible analytical errors.

In the stabilization experiment, 48 hours were needed to establish a steady state concentration of the four heavy metal salts incorporated into the diet in feces (Fig. IV-B-2). Ytterbium was the most variable of the four elements studied. It is interesting that the highest fecal level of

IV-B-4. Diurnal variation in fecal excretion of Ce given as a pulse at 8:30 a.m. and 8:00 p.m. daily. The four rats were fed as previously noted. Data for Ce was obtained from pooled samples.



Yb and Tb in this experiment was the average of that obtained in the digestion study in group II. Lutetium was about the same concentration in feces during stabilization period and during the digestion study. However, the concentration of Ir noted in the stabilization experiment was approximately one-half of that found in the six-day balance study. Since diets for the two experiments were mixed separately it is possible that this reflects a difference in the quantity of Ir present in different diets. Cerium was the most unreliable of the elements studied. Activation analysis of Ce in complex mixtures appears to be unsatisfactory and it is not presently in the multimarker list (IV-B-7). Unfortunately, the poor results with Ce prevented indirect estimate of food intake.

Apparent digestibility data could be extended by allowing each marker to represent any or all nutrients in a given diet. Therefore, the indirect apparent digestibility for each nutrient was calculated for the four nutrient markers (Table IV-B-6). Individual markers give consistent results for energy and K. Different markers gave less consistent results for Fe and considerable variation for Ca. When data for direct apparent digestibility of stock diets I and II and indirect apparent digestibility for all four markers were averaged, the two methods agreed well. This suggested the anomalous results obtained for Ca and Fe were partially due to analytical inconsistencies and could be compensated by more replicates; this was particularly noticeable for diet IV. A two-fold difference was obtained when Tb and Ir were utilized to determine Ca apparent digestibility in the high energy diet. Iridium gave excellent correlation with the direct method for the high energy diet and was the best marker for the high fiber diet. In general, the indirect method gave unexpectedly high values for the apparent digestibility of both Ca and Fe in adult rats.

Table IV-B-7

Analytical Levels

Element	Individual	Best 2	Best 3	Best 4	Best 5	All 6
Sc	0.5	1.0	1.5	1.5	1.5	1.5
Eu	0.1	0.1	0.4	0.8	0.8	0.8
Tb	0.5		1.0	2.5	2.5	2.5
Yb	0.2			1.5	1.5	1.5
Sm	0.01				0.2	0.2
La	1.0					5.0

The diurnal vatiation in fecal Lu excretion (Fig. IV-B-3) was virtually nil while that for Ir, Tb and Yb showed slight variations. The quantities of each excreted related directly to the amount of feces collected (middle graph, Fig IV-B-4) for each period. These minor differences in concentration suggest that some metals may have attached themselves to digesta or been sequestered in somewhat different manners than other metals. Such differences appeared to be negligible for the usual digestibility study. Lutetium appeared to be the least variable of the markers studied, while Ir, Yb and Tb were quite acceptable for studies such as those carried out herein. The complexities of multiple analyses are such that of the metals used here, Lu, Ir and Ce cannot be considered in the best six (Table IV-B-7). Singly each should be satisfactory.

The diurnal variation of Ce (Fig. IV-B-4) was much greater than that noted for the other four elements: this was anticipated since Ce was given as a single dose prior to each feeding. The data suggest that the quantity excreted per period was directly related to the time of passage of the single pulse fed. The peak concentrations of Ce in the feces indicate the single pulse does not spread evenly through all the digesta. This suggests the daily marker should be administered during or following each meal, 2-3 times daily, in order to provide better mixing with stomach digesta.

Our experience with multiple heavy metal markers for nutrition studies combined with consideration of decay rates which were most distinctive and sensitive (2) allows a listing of heavy metals (Table IV-B-7) which can be analyzed by neutron activation analysis using a single irradiation of 5×10^{13} n cm^{-1} sec^{-1} and a single decay period of 4 to 7 days. The values shown are micrograms of each element which corresponds to the "analytical level" for a 3% error in the analysis using triplicate 1/2 - 1 g samples.

The analytical levels are considerably greater than sensitivity levels; this is particularly true when precise quantitative data are required from a mixture of heavy metals. For example, Dy is detectable in very low concentrations by neutron activation analysis; but in a mixture the sensitivity of Dy detection is directly related to the quantity of Sm present due to the proximity of the Dy emission to the shoulder of Sm emission peak. Column 1 shows the analytical level for each element used individually and not in combination with any other element. The other columns show the analytical levels for combinations of the best 2, best 3, etc. Note that Ir, which gave the excellent conformity in the apparent digestibility study for diets III and IV,and Lu, which gave good results with diet II, are not on the list (Table IV-B-7) for desirability using activation analysis of multiple metals.

Radioactive heavy metals are easy to assay with good sensitivity: however in the low concentrations at which they are generally used, they may not exceed solubility limits and exist as ions and/or radiocolloids which could be absorbed. However, the same amount of radioactive metal diluted with small quantities of non-radioactive carrier was not absorbed. At concentrations slightly above solubility limits both non-radioactive and radioactive metals exist as radiocolloids, several ions clustered with a particle size much smaller than that of regular colloids; these radiocolloids may or may not be absorbed according to Kremers (6) and Schweitzer (7). Dilution of a radioactive marker with unlabelled carrier allowed Pfau and Abadir (8) to study gastrointestinal passage in bulls.

Another aspect to be considered is the safety of heavy metals in the diet. Hutcheson et al.(9) presented evidence which suggested that heavy metal oxides fed at 1000 times the anticipated use levels are safe for rats.

The toxicity of heavy metals has been reviewed by Venugopal and Luckey (10): the low quantities ingested in these experiments would not be expected to be nutritionally or physiologically evident in any studies with whole animals.

It is concluded from these experiments that some heavy metal salts may be used as multiple markers for a variety of different purposes (11). Cerium has not been satisfactory as a multiple marker with neutron activation analysis. Another marker should be utilized for the daily intake marker to increase the versatility of multiple markers. Application in human studies under metabolic ward or space flight conditions, using self selected diets from a variety of prepared foods, was anticipated. Nutrient markers would be put into all foods in a predetermined ratio to the designated nutrients. A daily food intake marker would indicate the proportion of the daily intake represented by any fecal sample. However, if incomplete sampling were practiced, the daily intake marker would need to be given in several doses in order to obtain a uniform distribution in feces. The fraction of daily intake marker and the quantity of any nutrient marker in a fecal sample would provide information for simple calculation of daily intake of the specific nutrient.

Summary

In this study, the nitrates of five metals (cerium, terbium, ytterbium, lutetium, and iridium) were fed to rats to determine the feasibility of their use as non-absorbed, multiple markers for recovery, passage and indirect apparent digestibility studies.

Fecal recovery of a single oral dose was completed within 72 hours. When the salts were mixed into the diet, 48-96 hours were required to

establish a steady state concentration of markers in feces. The diurnal variation of cerium in feces was found to be considerable when it was fed twice daily as a single dose prior to each feeding. When incorporated into the diet, negligible diurnal variation in fecal concentration was noted with Lu and small variation was seen with other metals. In nutrient apparent digestibility studies good agreement was generally found between direct and indirect multiple marker methods. The best marker was Ir. Experiments with a daily intake marker suggest that cerium was not satisfactory as a multiple marker in which neutron activiation analysis was the method of determination.

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IV RESEARCH

C. SAFETY OF HEAVY METAL MARKERS IN MICE

Heavy metals might provide the best approach for nutritional markers (1). These interests promoted a review and study of ten heavy metals. Seven of the ten metals considered are of the rare earth or lanthanoid series: Lanthanum (La), Samarium (Sm), Europium (Eu), Terbium (Tb), Dysprosium (Dy), Thurlium (Tm) and Ytterbium (Yb). Soluble salts of rare earth metals are hydrolyzed to insoluble hydroxides in the gastorintestinal tract and tend to absorb to particles of food ingested. The lanthanoid ions react with inorganic phosphate to form insoluble lanthanoid phosphates. The absorption of the rare earch metals is negligible; thus, they should be good markers. The gastrointestinal absorption of lanthanoids was poor in all species studied: rats and mice (2, 3), goats (4), cows (5) and calves (6).

Previously reported work has shown that (5ppm) scandium added in drinking water resulted in a slight but significant suppression of growth of mice at different intervals of age during the first six months but not at later intervals (7). Chronic ingestion of terbium, thulium and ytterbium chlorides (1000 ppm and 10,000 ppm in the diet) caused some suppression of growth in rats; but dysprosium and other rare earth metals chlorides did not (8). Studies of toxicity of the proposed minerals have been reviewed (9); no toxicity was noted from oral intake of the proposed metals to be studied. The stuqy described herein uses in combination the oxides of scandium (Sc), chromium (Cr), lanthanum (La), samarium (Sm), europium (Eu), terbium (Tb), dysprosium (Dy), thulium (Tm) and ytterbium (Yb) and barium sulfate. The use amounts of the elements were 0.12 ppm (Sc), 0.02 ppm (Cr), 0.40 ppm (La), 0.80 ppm (Sm), 0.036 ppm (Eu), 1.20 ppm (Tb), 1.20 ppm (Dy), 0.08 ppm (Tm), 0.12 ppm (Yb) and 0.008 (Ba).

Materials and Methods

Animals. Eighty females and 40 male weanling CF-1 mice were randomly assigned to 5 treatment groups. The groups consisted of animals fed no metal oxides and those fed metal oxides and barium sulfate added at 1, 10, 100 and 1000 times the proposed use amounts of the 10 heavy metals. The proposed use amounts of these markers in the diet was 1/5 of the concentration required for estimation by activation analysis with a 5% error (10). The concentration of these poorly absorbed dietary markers increased five fold in the feces by bioincrassation during the digestive process. Each dietary regime was continued through three successive generations. The experiment ended when the young from the third generation adults were weaned.

The sexes were housed in groups of 4 females or 2 males until initiation of breeding; then 2 males were housed with 4 females for 17 days for the breeding period. At the end of 17 days the females were placed in individual cages until young were born and weaned. The mice were individually weighed at three-week intervals until the breeding period. The litters were standarized at 2 days of age to 6 $\pm$ 1 pups for the lactation period. The dams were again weighed when the pups were weaned at 21 days. The second generation animals were randomly selected from offspring of the respective first generation groups and the third generation groups were selected from the offspring of the second generation. Each generation began at the same time to facilitate handling and record keeping. All generations were weighed and treated as described for the first generation. Animals were housed in standard polycarbonate mouse cages with Sanicell litter. The room was maintained with 12 hours of light and darkness each day. The temperature was maintained at 21 $\pm$ 3°. Five second generation adult mice from the control

and 100X groups killed and skinned. The alimentary tract was discarded and the remainder ground in a waring homogenizer and dried for whole carcass analysis. Five adult third generation mice were necropsied from the 100X group and the control group. Gross necropsy observations were made on the integument, cardiovascular, respiratory, alimentary, genital, urinary, endocrine, hemolytopoietic, nervous and musculoskeletal systems.

The basal diet was ground commercial[1] laboratory chow. Nine metal oxides and barium sulfate were added to the chow by thoroughly mixing the minerals in sequential dilutions of the basal diet and thorough mixing the appropriate series of concentrations desired. Each diet was then pelleted before analysis and feeding. Food and water was presented ad libitum. Table IV-C-1 illustrates the amount of metals added to the diet and the quantity determined by neutron activation analysis. The amount in the table corresponds to 10 times the proposed use level of metals in the diet. The 10X diet was analyzed in order that the quantity of the metals would be within analytical limits of neutron activation analysis. The 1X diet had only 1/10 of these quantities and the 100X and 1000X diets contained 100 and 1000 times these quantities, respectively. The elements Tm, Cr and Ba were not analyzed but were incorporated into the diet at the proposed level.

Blood Profiles. Five dams, 3 months of age, were selected from the control group and the 100X group and were bled via the tail for each generation. Erythrocyte and leukocyte counts were enumerated using an electronic cell counter, and the red cell size distributions were determined using a particle size distribution analyzer plotter[2]. Hemoglobin concentrations

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[1] Laboratory Chow, Ralston Purina, St. Louis, Missouri

[2] Model B; Coulter Electronics, Hialeah, Florida

were determined using a clinical hemoglobinometer and packed cell volumes were determined by the microhematocrit method. Serum protein, albumin, alpha-globulin, beta-globulin and gamma globulin were separated by microzone electrophoresis on cellulose polyacetate strips. The strips were stained with Ponceau S and the protein fractions were quantitated with a densitometer. Albumin/globulin ratios were calculated. Analysis of variance techniques were used to determine statistical differences.

Results

The general appearance of all mice in all groups was good. No morphologic anomalies were seen. Morbidity and mortality were less than 0.5% for the experiment. Necropsy examination of 5 random third generation adult mice fed the basal diet or 100X diet did not reveal any abnormalities. The necropsy protocol is indicated in Figure IV-C-1 for one of the sacrificed mice.

Weights of mice at different times during the experiment are presented in Table IV-C-2. The average daily gains of the mice in the 1000X and basal groups were significantly higher than other groups for the first generation, ($P < 0.01$). The average daily gains for the second generation were divided into three significantly different groups with 1X the highest, basal and 10X the intermediate and 100X and 1000X the lowest average daily gain, ($P < 0.01$). In the third generation the 100X had a significantly lower gain than the others ($P < 0.01$). The differences detected in average daily gains at different times were not consistent with levels of minerals added to the diet. There were no significant differences among the weights of the mice prior to mating nor the weight of dams at weaning.

The reproduction and lactation performance of the three generations

of mice is summarized in Table IV-C-3. No differences were detected in the number of females having litters within generations for the different groups. No significant differences were detected in average litter size or average weaning weight of the litters for any generation of treatment groups. The average litter size within generations was the highest for the second generation. Average weaning weight was not different for treatment groups within generations, however, the third generation had a significantly higher weaning weight for all treatment groups, ($P < 0.01$). The average weaning weight for three generations for all treatments were 7.42, 7.29 and 8.37 g, respectively. This difference in weaning weight may be attributed to adaptation of the mice to the management procedures. Less than 1.0% of the mice died from culling to weaning for the experiment.

Hematology and serum proteins for mice fed basal and 100X diets are summarized in Table IV-C-4. No differences for hematology variables and serum proteins were detected among animals in the same generation irrespective of whether they were fed the basal diet or the 100X diet. The leukocyte and erythrocyte counts, the pack cell volume, hemoglobulin, serum total protein and protein fractions were all within normal limits.

Carcasses of 100X mice and control mice were subjected to mineral analysis, Table IV-C-5. Sc and Sm were detected in both carcasses and skin and tail in the control and 100X mice. Tb and Yb were detected in both the control and 100X mice but only in the skin and tail. Eu was detected in the skin and tail of animals fed 100X diets. All levels detected were below analytical levels of the minerals; values noted in Table IV-C-5 are extrapolated values.

Discussion

The nutritional safety of these minerals as proposed markers is of

FIGURE IV-C-1

Necropsy Protocol, Sinclair Comparative Medicine Research Farm

Necropsy No. 72-100X

Experiment No. 72-044

Animal No.

D-L1 and D-R1

Animal Species: Mouse

Sex: Female Age: 4 Months

Investigator: Dr. Hutcheson

Dissector: Dr. Dee Carey

Post Mortem Interval: None

Date: 6-19-72

Gross Photographs: None

Photomicrographs: None

Clinical History and Clinical Diagnosis:

These two C-F1 female mice were fed a diet containing an enert heavy metal. These two mice had been on a diet containing 10 times the amount normally fed. These two animals had shown no untoward effects to the high amount of heavy metal contained in their diet.

The two animals were euthanatized by overdose of ether and a gross necropsy was performed.

Necropsy Diagnoses: None

Gross Necropsy Observations:

1. Integumentary System - No lesions seen.
2. Cardiovascular System - No lesions seen.
3. Respiratory System - No lesions seen.
4. Alimentary System -- Liver - No lesions seen.
   Pancreas - No lesions seen.
   GI tract - (stomach, small intestine, colon, large intestine) - No lesions seen.
5. Genital System -- Uterus - No lesions seen.
6. Urinary System -- Kidneys - No lesions seen.
7. Endocrine System -- Ovaries - No lesions seen.
   Adrenal - No lesions seen.
   Thyroids - not taken.

FIGURE IV-C-1 (cont.)

Pituitary - not taken.

Parathyroids - not taken.

8. Hemolytopoietic System -- Spleen - No lesions seen.

Lymph nodes - none appeared to be enlarged.

upmost interest for their use in a multiple marker system for accurate determination of nutrient intake and apparent utilization (4). These studies with mice indicate that the metal oxides of Sc, Cr, La, Sm, Eu, Tb, Dy, Tm, Yb and barium sulfate may be used safely as nutritional multiple markers. Three elements (Tm, Cr and Ba) were not determined. When these same diets were fed to monkeys for 12 weeks, no tissues were found with any metal present above analytical detection limits (12).

The gastrointestinal absorption of lanthanoid compounds is negligible due to their poor solubility at tissue pH levels; the basicity of their salts leads to the easy formation of hydroxides either as insoluble precipitates or colloidal forms (9). Poor solubility of lanthanoid oxides, hydroxides and salts at tissue pH levels enhances their tendency to absorb to particulate food ingesta (9). Although stable and water soluble chelates of lanthanoids such as EDTA-lanthanoids and citrate complexes are readily absorbed from the digestive tract, ingested oxides of Sc, La, Sm, Eu, Tb, Dy and Yb are poorly absorbed in mice as shown by tissue analyses. Negligible absorption from the digestive tract renders these oxides as useful markers (1). The slight growth depression from high levels of $TbCl_3$ (1000 ppm) (8) was not found in those studies with equally high levels of Tb_4O_7 (1440 ppm).

The results reported herein reenforce the data reported for monkeys (12) and other mammals (9) and support the conclusion that these heavy metal compounds are safe for use as nutritional markers. These same markers were not detectable in tissues of monkeys fed 10 times the use level for two months (12). A similar series of heavy metals, cerium, terbium, ytterbium, lutetium, and iridium, have been used as multiple nutrient markers (11).

Summary

Heavy metals have been proposed as nutrient markers to allow the accurate determinations of the time of passage, nutrient intake or apparent utilization of multiple nutrients. In order to evaluate possible toxic effects of scandium, chromium, lanthanum, samarium, europium, dysprosium, terbium, thulium, ytterbium, oxides and barium sulfate upon growth, general development, reproduction and lactation, mice were fed different levels of these compounds for three generations. The amount of elements fed were 0, 1, 10, 100, and 1000 times the use amount. The use amounts were 0.12 ppm Sc, 0.02 ppm Cr, 0.40 ppm La, 0.80 ppm Sm, 0.036 ppm Eu, 1.20 ppm Tb, 1.20 ppm Dy, 0.08 ppm Tm, 0.12 ppm Yb and 0.008 ppm Ba. The use amount was one-fifth of the concentration required for activation analysis. Mortality and morbidity were negligible. No consistent growth rate changes were observed; however, different groups showed different growth rates during different generations. The number of mice born showed no significant differences among treatment groups. Survival, growth rate, hematology, morphologic development, maturation, reproduction and lactational performance were comparable in mice fed the different levels of ten heavy metal oxides to those mice fed the basal diet. Carcass and skin analysis of mice fed 100 times the use level were comparable to those of control mice. All heavy metals detected were below analytic limits of analysis.

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TABLE IV-C-1

Elements added to the diet

(µg/g)

Element	Basal Diet	Addition Proposed	Final Analysis[1]
Europium	0.04 ± 0.02	0.36	0.32 ± 0.04
Samarium	0.33 ± 0.02	8.00	11.11 ± 3.56
Lanthanum	0.69 ± 0.02	4.00	6.08 ± 2.05
Dysprosium	0.25 ± 0.02	12.00	11.38 ± 1.62
Ytterbium	0.05 ± 0.02	1.20	1.12 ± 1.18
Scandium	0.12 ± 0.01	1.20	1.58 ± 1.70
Terbium	0.02 ± .01	12.00	11.02 ± 4.03
Thulium	NA[2]	.80	NA
Chromium	NA	.20	NA
Barium	NA	.08	NA

[1]Mean of 5 samples ± standard error of mean.
[2]NA = not analyzed.

TABLE IV-C-2

Average body weight of mice

(g)

Experimental Groups	Average Daily Gain 3 Wks To 6 Wks Of Age			Weight Prior To Mating			Weight Of Dams At Weaning		
	Generation			Generation			Generation		
	First	Second	Third	First	Second	Third	First	Second	Third
Basal	.200[a]	.296[b]	.258[a]	26.61	29.37	30.78	32.52	28.82	29.61
	(±.009)[1]	(±.013)	(±.012)	(±0.40)	(±2.23)	(±2.23)	(±0.72)	(±0.92)	(±0.72)
1 X	.106[b]	.360[a]	.286[a]	26.71	29.16	37.04	32.23	29.12	29.20
	(±.010)	(±.010)	(±.017)	(±0.43)	(±1.23)	(±3.22)	(±0.90)	(±1.22)	(±1.51)
10 X	.108[b]	.328[b]	.250[a]	26.61	29.94	28.81	31.61	29.34	26.68
	(±.012)	(±.017)	(±.011)	(±0.46)	(±0.77)	(±2.25)	(±0.87)	(±1.44)	(±1.28)
100 X	.134[b]	.207[c]	.133[b]	27.14	26.21	33.41	32.78	28.12	29.75
	(±.013)	(±.007)	(±.006)	(±0.67)	(±0.96)	(±3.34)	(±0.73)	(±0.83)	(±0.90)

cont'd

TABLE IV-C-2, cont'd

Experimental Groups	Average Daily Gain 3 Wks To 6 Wks Of Age			Weight Prior To Mating			Weight Of Dams At Weaning		
	Generation			Generation			Generation		
	First	Second	Third	First	Second	Third	First	Second	Third
1000 X	.230[a]	.211[c]	.280[a]	28.74	30.20	28.55	33.71	29.31	27.64
	(±.014)	(±.009)	(±.012)	(±0.55)	(±1.41)	(±1.77)	(±1.30)	(±1.24)	(±1.58)

Superscripts differ statistically significant ($P<0.01$) when groups within columns are compared.

[1]Standard error of the mean

TABLE IV-C-3

Reproduction and lactation performance in mice fed heavy metals

Experimental Groups	Females Having Litters, Generation			Average Litter Size, Generation			Average Weaning Weight, g, Generation		
	First	Second	Third	First	Second	Third	First	Second	Third
Basal	14/16	14/16	14/16	6.40 (±0.61)[1]	8.70 (±0.6)	7.00 (±0.3)	7.58 (±0.21)	7.31 (±0.31)	7.73 (±0.26)
1 X	13/16	16/16	12/16	7.60 (±0.7)	9.06 (±0.5)	6.03 (±0.9)	7.49 (±0.28)	7.00 (±0.28)	8.88 (±0.39)
10 X	12/16	16/16	12/16	6.60 (±0.6)	8.20 (±0.6)	8.67 (±1.0)	7.61 (±0.26)	7.14 (±0.21)	8.09 (±0.20)
100 X	14/16	14/16	14/16	8.10 (±0.7)	8.36 (±0.6)	8.17 (±0.7)	7.14 (±0.22)	7.80 (±0.22)	8.81 (±0.39)
1000 X	12/16	16/16	14/16	6.90 (±0.9)	10.45 (±0.5)	7.17 (±1.1)	7.33 (±0.26)	7.20 (±0.18)	8.36 (±0.29)

[1]Standard error of the mean.

TABLE IV-C-4

Hematology and serum proteins for control and 100X diets

	Control			100 X		
Generations	1st	2nd	3rd	1st	2nd	3rd
Leukocyte Count	7.37	7.95	7.60	8.59	7.62	8.00
$X10^3$, cells/mm^3	(±0.52)[1]	(±0.89)	(±0.72)	(±0.74)	(±0.92)	(±0.85)
Erythrocyte Count	6.90	6.42	6.82	6.87	6.51	6.81
$X10^6$, cells/mm^3	(±0.62)	(±0.62)	(±0.83)	(±0.54)	(±0.75)	(±0.71)
Packed Cell Volume %	46.70	45.20	45.80	42.60	46.30	46.10
	(±5.80)	(±8.20)	(±7.20)	(±6.20)	(±9.15)	(±7.50)
Hemoglobin	16.40	15.20	15.70	16.20	15.60	16.00
(g/100 ml)	(±1.20)	(±1.80)	(±2.20)	(±1.07)	(±2.10)	(±1.80)
Serum Total Protein	7.20	6.50	6.80	6.92	6.80	7.05
(g/100 ml)	(±0.65)	(±0.55)	(±0.82)	(±0.66)	(±0.56)	(±0.58)
Albumin	4.25	3.85	3.91	4.25	3.72	4.02
(g/100 ml)	(±0.92)	(±0.72)	(±0.56)	(±0.43)	(±0.40)	(±0.72)

cont'd

TABLE IV-C-4, cont'd

	Control			100 X		
Generations	1st	2nd	3rd	1st	2nd	3rd
αGlobulins	1.18	1.02	1.24	1.18	1.22	1.28
(g/100 ml)	(±0.24)	(±0.21)	(±0.23)	(±0.16)	(±0.18)	(±0.25)
βGlobulins	1.34	1.25	1.30	1.36	1.27	1.35
(g/100 ml)	(±0.15)	(±0.22)	(±0.31)	(±0.12)	(±0.21)	(±0.28)
γGlobulins	0.43	0.55	0.40	0.52	0.62	0.51
(g/100 ml)	(±0.06)	(±0.07)	(±0.05)	(±0.02)	(±0.10)	(±0.06)
Albumin/Globulin	1.18	1.38	1.34	1.39	1.16	1.28
	(±0.21)	(±0.25)	(±0.18)	(±0.16)	(±0.18)	(±0.22)

[1]Standard error of the mean is given in parentheses.

TABLE IV-C-5

Heavy metals (ppm) in carcass of second generation adult mice fed control and 100X diets

	CONTROL		100X	
	Carcass	Skin and Tail	Carcass	Skin and Tail
Scandium	0.015	0.015	0.02	0.02
Chromium	NA	NA	NA	NA
Lanthanum	--	--	0.05	--
Samarium	0.02	0.02	0.06	0.25
Europium	--	--	--	0.15
Dysprosium	--	--	--	--
Terbium	--	0.06	--	1.16
Thulium	NA	NA	NA	NA
Ytterbium	--	0.04	--	0.09
Barium	NA	NA	NA	NA

- was not detected. NA not analyzed.

IV RESEARCH

D. MONKEY BALANCE STUDY

Introduction

A review of markers in nutrition indicate that the use of heavy metal markers might provide the best method for exact determination of nutrient intake and/or apparent utilization. The use of radioactive metals for markers in nutrition may present certain problems such as the disposal of large quantities of radioactive waste; or minute quantities of radiomarkers may be absorbed from the gastrointestinal tract, since the markers could be in the form of absorbable ions or radiocolloids at these concentration levels. Therefore, the new and not wholly explored course is to use metals in the form of oxides or salts which can be neutron activated following the experiment to provide radioactive identification of the product in vitro. The interest in the use of heavy metal compounds as multiple markers in nutrition prompted this balance study of heavy metals.

The pilot study involved feeding monkeys Purina chow with the 10X level of the minerals. Three adult monkeys were used to determine the feasibility of using rare earth metals as nutritional markers. Two monkeys received ten rare earth metals in the diet for 56 days and at the end of this period a 7 day balance study was conducted while one monkey was the control.

Methods

Analysis. The basic method for analysis of multiple heavy metals in a single sample have been described (Gray and Vogt, 1974). Duplicate samples are dried, triturated, and replicate aliquotes are weighed and

subjected to neutron activation analysis. The results are presented as ppm on a dry weight basis. Budget restrictions allowed only 6 of the 10 metals to be determined and reported. The metals used are samarium (Sm), scandium (Sc), lanthanum (La), terbium (Tb), europium (Eu), dysprosium (Ds), ytterbium (Yb), thulium (Tm), chromium (Cr), and barium (Ba).

Animals. Three young adult male Rhesis monkeys were utilized for the experiments. Each was maintained in a metabolism cage throughout the entire period of 2 months. They were fed once daily with unrestricted intake for 2 hours. Tap water was available continuously. Necropsy was preceeded by nembutal anesthesia. Each animal was placed in a plastic bag after skinning in order to decrease possible contamination of internal organs with external particles. Food consumption and complete feces collections were made during the balance study.

Diet. Commercial laboratory chow was ground to 20 mesh, mixed with the appropriate minerals or nothing in a mixer with a universal type arm action and pelleted into 1 cm cubes. These were moistened and balled together prior to feeding. The composition of the chow is indicated in Table IV-D-1 Ten heavy metals were incorporated into the diet at a level 10 times the level; nine were oxides and Ba was incorporated as the sulfate. These metal oxides were at least 99.9% pure. The quantities intended and the quantities found by analysis are presented in Table IV-D-2. The control monkey was fed the repelleted chow and the other two were fed the marked chow throughout the acclimitization period of 56 days and the 7 day balance study.

Erbium was given as a daily marker: 25µg were administered daily in a capsule containing lactose diluent. This was balled into a small quantity of diet and fed prior to letting each monkey eat ad libitum.

TABLE IV-D-1

Purina Laboratory Chow

Approximate Chemical Composition*			
Nutrients**			
PROTEIN %	23.40	Chlorine %	.54
Arginine %	1.38	Fluorine, ppm	35.00
Cystine %	.32	Iron, ppm	198.00
Glycine %	1.20	Zinc, ppm	58.00
Histidine %	.60	Manganese, ppm	51.00
Isoleucine %	1.20	Copper, ppm	18.00
Leucine %	1.60	Cobalt, ppm	.40
Lysine %	1.41	Iodine, ppm	1.70
Methionine %	.43	VITAMINS	
Phenylalanine %	1.03	Carotene, ppm	6.50
Threonine %	.94	Menadione (added), ppm	--
Tryptophan %	.28	Thiamin, ppm	17.70
Valine %	1.24	Riboflavin, ppm	8.50
FAT %	4.50	Niacin, ppm	110.30
FIBER %	5.20	Pantothenic Acid, ppm	24.80
TDN %	75.00	Choline, ppm X 100	24.00
NFE (by difference) %	50.80	Folic Acid, ppm	5.90
Gross Energy, KCal/gm	4.25	Pyridoxine, ppm	3.80
ASH %	7.30	Biotin, ppm	.07
Calcium %	1.20	B-12, mcg/Kg	37.00
Phosphorus %	.86	Vitamin A, IU/gm	12.00
Potassium %	.92	Vitamin D. IU/bm	5.00
Magnesium %	.24	Alpha-tocopherol, IU/Kg.	74.00
Sodium %	.45	Ascorbic Acid, Mg/gm	--

*Based on latest ingredient analysis information. Since nutrient composition of natural ingredients varies analysis will differ accordingly.

**Nutrients expresses as per cent of ration except where otherwise indicated. Moisture content, though variable, is assumed to be 10.0% for the purposes of calculations.
Purina Laborato~ Chow, Ralston Purina Company, Checkerboard Square, St. Louis, Missouri, 6318[illegible], USA

TABLE IV-D-2

Elements Added To The Diet, 10 Times Use Level (ppm)

Element	Proposed	Analysis[1]
Europium	0.36	0.32 ± 0.04
Samarium	8.00	11.11 ± 3.56
Lanthanum	4.00	6.08 ± 2.05
Dysprosium	12.00	11.38 ± 1.62
Ytterbium	1.20	1.12 ± 1.18
Scandium	1.20	1.58 ± 1.70
Terbium	12.00	11.02 ± 4.03
Thulium	.80	NA[2]
Chromium	.20	NA
Barium	.08	NA

[1]Mean of 5 samples ± standard error of mean ppm dry basis.

[2]NA not analyzed.

Results

The general appearance and disposition of all monkeys was excellent throughout the entire experiment. There were no apparent differences in food and water intake between the control and the treatment monkeys. During the 56 day pre-balance period the control gained 121g while the treated monkeys gained 114 and 156g respectively. Necropsy revealed no abnormalities in either gross examination or hematology. A sample necropsy protocol is illustrated (Fig. IV-C-1).

The results from the 7 day balance study were divided into a 4- and a 3-day study in order to detect any trends (Table IV-D-3). In general, no trends were seen following the 56 day pre-balance period, and there was fair agreement between the two treatment animals. The balance data was incorporated into a single 7 day study and averaged to show the % accounted for each metal. When the 7 day data was expressed as balance (Table IV-D-4), most of the metals showed balances which seemed to be within limits of experimental variation. Four metals, Sm, Sc, Eu and Ds, were not accumulated. Lanthenum was questionable and Tb was possibly accumulated.

At the end of 63 days of continuous feeding the animals were sacrificed and their tissues analyzed for 8 rare earth metals (Table IV-D-5). No La, Dy, or Er was detected in any tissues. Although each of the other metals was detected in one or more tissues, in no case did the amount detected come within 0.1 of the limits for analytic certainty. Thus, all tissues contained no significant level of any of the metals.

Discussion

The general health exhibited by the monkeys fed 10 times the anticipated use level of 10 heavy metal markers is further evidence of the safety of this

TABLE IV-D-3

Balance Data for Monkeys for 7 Days

Metals	Trt.	4 Day Study Intake[1]	4 Day Study Output[1]	4 Day Study % Accounted	3 Day Study Intake[1]	3 Day Study Output[1]	3 Day Study % Accounted	7 Day Study Intake[1]	7 Day Study Output[1]	7 Day Study % Accounted	7 Day Average
Sm	1	1256	1396	111.1	1300	1420	109.1	2256	2816	110.2	107.0
	2	1704	1747	102.5	1659	1740	104.8	3363	3487	103.7	
Sc	1	209	214	102.0	217	215	98.9	426	429	100.7	99.6
	2	284	281	98.7	277	271	97.7	561	552	98.4	
La	1	580	552	95.1	601	599	99.7	1181	1151	97.5	95.7
	2	787	777	98.6	767	682	88.9	1554	1459	93.9	
Tb	1	1682	1407	83.6	1742	1507	86.4	3424	2914	85.1	87.8
	2	2283	2487	108.9	2223	1585	71.3	4506	4072	90.4	
Eu	1	37	39	104.8	38	42	109.6	75	81	108.0	105.0
	2	50	52	103.3	49	49	100.0	99	101	102.0	
Ds	1	1958	1995	101.9	2028	1988	98.0	3986	3983	99.9	102.2
	2	2657	2966	111.6	2587	2509	96.0	5244	5475	104.4	
										Average Total	99.6

[1] mg/day

TABLE IV-D-4

Heavy Metal Balance in Monkeys

Metal[1]	Monkey	7 Day Balance[2] %	Average
Sm	1	-10.2	-7.0
	2	- 3.7	
Sc	1	- 0.7	0.5
	2	1.6	
La	1	2.5	4.3
	2	6.1	
Tb	1	14.9	12.3
	2	9.6	
Eu	1	- 0.8	-1.4
	2	- 2.0	
Ds	1	0.1	-2.2
	2	- 4.4	

(1) Fed as the oxide.

(2) $\frac{\text{Intake - Output}}{\text{Intake}} \times 100$

TABLE IV-D-5

Tissue Analysis of Monkeys Fed Metals for 63 Days

Tissue		Metals, PPM (dry) Sm	Yb	Sc	La	Tb	Eu	Dy	Er
Bone	Control	—[1]	—	.02[2]	—	—	—	—	—
	Trt. 1	—	.05	—	—	—	—	—	—
	Trt. 2	—	—	—	—	—	—	—	—
Duodenum	Control	.01	.07	—	—	—	—	—	—
	Trt. 1	—	—	—	—	—	—	—	—
	Trt. 2	.01	.04	—	—	—	—	—	—
Skin & Hair	Control	—	—	—	—	—	—	—	—
	Trt. 1	—	.03	—	—	—	.02	—	—
	Trt. 2	.01	—	.01	—	—	—	—	—
Heart	Control	—	—	—	—	—	—	—	—
	Trt. 1	—	—	—	—	—	—	—	—
	Trt. 2	.01	.04	.01	—	.09	—	—	—
Liver	Control	—	—	—	—	—	—	—	—
	Trt. 1	—	.04	—	—	—	—	—	—
	Trt. 2	—	—	—	—	—	—	—	—
Kidney	Control	—	—	—	—	—	—	—	—
	Trt. 1	—	—	—	—	—	—	—	—
	Trt. 2	—	—	—	—	—	.02	—	—
Lung	Control	—	—	—	—	—	—	—	—
	Trt. 1	—	—	—	—	—	—	—	—
	Trt. 2	.02	.06	—	—	—	—	—	—
Fat	Control	—	—	—	—	—	—	—	—
	Trt. 1	—	—	—	—	—	—	—	—
	Trt. 2	—	—	—	—	—	—	—	—
Spleen	Control	—	—	—	—	—	—	—	—
	Trt. 1	—	—	—	—	—	—	—	—
	Trt. 2	—	—	—	—	—	—	—	—
Muscle	Control	—	—	—	—	—	—	—	—
	Trt. 1	—	—	—	—	—	—	—	—
	Trt. 2	—	—	—	—	—	—	—	—
Blood	Control	—	—	—	—	—	—	—	—
	Trt. 1	—	—	—	—	—	—	—	—
	Trt. 2	—	—	—	—	—	—	—	—
Analytic Limts		0.50	3.0	3.0	6.0	5.0	1.5	0.006	

[1] Represents no metal detection

[2] Average of duplicate samples

system. The lack of toxicity, the good balance found of most of the metals, and the detection of only background levels of any metal marker in monkey tissue following 2 months of feeding these high levels show the safety of these markers. This is longer than most balance or recovery experiments. It should be understood that the levels detected were simply blips above the background counting and were 10-100 times lower than the lower limit for analytical purposes, the levels needed for a reliability of 2-5% error. Since all metals were quantitatively accounted for (note the overall recovery of 99.6 in Table IV-D-3), the balance was satisfactory for a pilot study involving 2 treatment and 1 control monkeys. This result, added to the rat feasibiltiy study and the safety test of four generations of mice fed 10, 100 and 1000 times the anticipated use level, is quite adequate as a base to plan human experiments.

Excepting Tb, and possibly La, the balance study was good. One possible explanation for the positive Tb balance would be that bioincrassation increased the mineral milieu in which the Tb was reacting and a 5 fold increased concentration of Ca, Fe, or the other rare earth metals may have depressed the apparent radiaiton response by making the background higher.

The single pulse method for recovery gives extraordinarily high values during the next collection periods and the values then become too low for accurate measure within 3-4 days; overall, this is not a good approach. The balance method shows recovery of each marker better than does the method of giving a single dose and measuring fecal recovery over the next few days. The good recovery and the lack of tissue markers is evidence that the rare earth metals oxides are useful for multiple markers in nutrition.

Summary

This monkey study, in which 10 times the anticipated use level of heavy metal markers were fed, further illustrates the nutritional safety of the metals studied. The tissues analyzed generally illustrated that the metals were not detectable. The balance study indicated that all the metals could be accounted for and that there is no apparent absorption of these metals. It is concluded that these metal oxides can safely be used as nutritional multiple markers.

V RECOMMENDATIONS FOR HUMAN STUDIES

A. TASTE TEST

Taste test data is needed in order to learn whether or not the heavy metal oxides will impart objectionable odors or tastes during the processing of the foods. Selected foods from the Skylab list were purchased from the local supermarket and cooked as indicated:

a) Sausage - The frozen sample is thawed out fully. Small patties are made and wrapped in "Reveal" see thru wrap. Baked at 375 for 20 minutes and served hot. Both marked and unmarked samples.

b) Hamburger - same as above. Baked for 30 minutes. Marked and unmarked samples.

c) Peas in cream sauces - Cooked according to instructions on the carton; water used instead of milk; margarine instead of butter; margarine containing the marker oxides is used for the marked sample.

d) Creamed corn - contents from the can warmed to serving temperature after mixing one gram of lactose to the contents of the can. Lactose mixture containing the oxides is used for the marked sample.

e) Pudding - Cooked according to instructions on the carton using homogenized whole milk.

f) Tomato juice - mixed 100 gs. of tomato puree with 150 ml of water and used.

g) Apple sauce - Refrigerated sample was used as such.

h) Milk - Refrigerated sample used after efficient shaking and mixing.

i) Oleomargarine - The sample is to be smeared on a regular bread sample.

j) Bread, biscuits and cookies - frozen samples thawed and warmed to serving temperature.

Following the above processing, all foods to be used were analyzed for energy, fat, protein, Ca and Fe. The data are given in Table V-A-1; this table provides the foods to be eaten in a 4 day cycle as well as the nutrients per serving. Samples of the foods were treated with the appropriate

quantities of markers, cooked and analyzed.

The amount of each marker to be added was estimated from consideration of the anticipated amount of dry feces and the amount of each marker which would give acceptable quantity of metal for accurate analysis. The analytic limits, tha amount desired in 50g feces and 5 times the estimated desired quantity are listed in Table V-A-2. The amount expected per day (per 50 g dry feces) was allocated to each nutrient according to its anticipated daily intake (Table V-A-3). This calculation gave the ratio of marker to nutrient to use for the simple calculation of the quantity of each marker to add to each food (Table V-A-4). When the marked, cooked foods were prepared for serving, duplicate samples were dried and analysed. The marker content found is given in Table V-A-5. Food to which no marker had been added were found to have no measurable quantities of any of these markers. The daily intake marker, Tb, was found in none of the marked foods; it would be given by capsule orally 3 times daily.

These foods are then ready for a panel of taste experts who would be given marked and unmarked foods at appropriate intervals for a standard 1-2 comparison test. In a preliminary trial with students, the applesauce was judged to be tainted.

This work provides a practical guide for future completion of taste tests.

TABLE V-A-1

Menu, Weight of Serving and Composition of Foods

Diet 1 and 3

Food	Weight	Joules	Protein	Fat	CHO	Ca	P	Fe	Mg	K
	g		g	g	g	mg	mg	mg	mg	mg
°eggs, dried (1 tablespoon)	14	347	6.6	5.8	0.6	26	112	1.2	6	648
°sausage (pork lean)	40	586	12.7	11.2	-	5.2	110	1.6		330
°pancake ? (4" dia.)	90	870	6.4	6.4	30.6	90	126	1.2	22	110
honey (1 tablespoon)	21	268	0.1	-	16.4	4	3	0.2	-	-
butter (1 pat)	5	151	-	4.0	-	1	1	-	-	-
milk skimmed 2% fat	245	527	8.5	4.0	11.5	298	268	0.1	-	336
tomato juice (puree)	100	163	1.7	0.2	8.9	13	34	1.7	20	426
°coffee or tea										
°fish fillet (haddock)	100	690	19.6	6.4	5.8	40	247	1.2	-	348
°biscuits	70	837	5.3	10.5	28	73	90	1.0	-	-
butter (1 pat)	5	151	-	4.0	-	1	1	-	-	-
apple sauce	100	381	0.2	0.1	23.8	4	5	0.5	-	65
°coffee or tea										
°Prime rib roast, 3 slices fat and bone trimmed off	159	1686	42.6	10.0	-	12	325	5.2	36	670
°rolls 2	70	837	6.8	2.0	40	32	64	1.6	16	68
butter 2 pats	10	301	-	8.0	-	2	2	-	-	-
ˣpeas green cooked 2/3 cup	100	297	5.4	0.4	10.0	23	99	1.8		196
lettuce	50	41.8	-	-	2.0	20	-	-	-	-
salad dressing	15	251	0.3	4.5	2.5	-	-	-	-	-
pudding ready mixed cooked	100	518	3.4	3.0	22.8	102	95	0.3		136
°coffee or tea										
<u>snacks</u>										
°cookies oatmeal (2)	35	732	4.0	6.0	24	21	33	0.6	-	-
Tang (twice) 16.8 g each	32.6	527	-	0.1	32	99	42	-	1.7	6
TOTAL		10,163	123.6	86.6	258.9	866.2	1657	18.2		2756

°to be cooked

ˣto be warmed

Diet 2

Food	Weight g	Joules	Protein g	Fat g	CHO g	Ca mg	P mg	Fe mg	Mg mg	K mg
°egg dried (tablespoon)	14	347	6.6	5.8	0.6	26	112	1.2	6	64.8
°sausage (pork lean)	40	586	12.7	11.2	-	5.2	110	1.6	-	330
°biscuits	70	837	5.3	10.5	28.0	73	90	1.0	-	-
honey (tablespoon)	21	268	0.1	-	16.4	4	3	0.2	-	-
butter	5	151		4.0		1	1			
milk skimmed (2% fat)	245	527	8.5	4.0	11.5	298	268	0.1	-	336
tomato juice (puree)	100	163	1.7	0.2	8.9	13	34	1.7	20	426
coffee or tea										
xturkey 3 slices with	100	837	31	7.6	-	30	400.	5.1	-	398
gravy	24	377	-	1.0	20.0	-	-	-	-	-
°rolls	70	837	6.8	2.0	40.0	32	64	1.6	16	68
butter	5	151	-	4.0	-	1	1	-	-	-
peach slices in syrup	100	368	0.4	0.1	22.4	4	13	0.5	-	124
°coffee or tea										
°hamburger (lean)	112	1004	30.6	12.6	-	13.2	259	3.9	24	625
°bread (2 slices)	46	469	4.0	-	24	38	24	1.2	1.2	28
lettuce salad	50	41.8	-	-	1	20	-	-	-	-
salad dressing	15	251	0.3	4.5	2.5	-	-	-	-	-
xcorn (1/2 cup cooked)	80	335	2.2	0.7	16.4	4	41	0.4	17	81
butter 2 pats	10	301	-	8.0	-	2	2	-	-	-
pudding ready mix cooked	100	519	3.4	3.0	22.8	102	95	0.3	-	136
snacks										
°cookies oatmeal (3)	56	1067	6.0	910	32	21	33	0.6	-	-
Tang (2)	33	527	-	0.1	32	99	42	0.i	1.7	6
TOTAL		9920 (2371 Cal)	119.6	88.3	279	5782	92	19.5		2623

C. 2

Diet 4

Food	Weight g	Joules	Protein g	Fat g	CHO g	Ca mg	P mg	Fe mg	Mg mg	K mg
°egg dried (tablespoon)	14	347	6.6	5.8	0.6	26	112	1.2	6	64.8
°sausage (pork lean)	40	586	12.7	11.2	-	5.2	110	1.6	-	330
°biscuits	70	837	5.3	10.5	28.0	73.0	90	1.0	-	-
honey (tablespoon)	21	268	0.1	-	16.4	4	3	0.2		10
butter 1 pat	5	151	-	4.0	-	1	1	-	-	-
milk (2% fat) fresh	245	527	8.5	4.0	11.5	298	268	0.1		336
tomato juice (puree)	100	163	1.7	0.2	8.9	13	34	1.7	20	426
°coffee or tea										
°hamburger (extra lean)	112	1004	30.6	12.6	-	13.2	259	3.9	24	625
butter	5	151	-	4.0		1	1	-	-	-
xrolls 2	70	837	6.8	2.0	40.0	32	64	1.6	16	68
lettuce	50	41.8	-	-	2	20	-	-	-	-
peaches sliced in syrup	100	368	0.4	0.1	22.4	4	13	0.5		124
°coffee or tea										
xturkey roast 3 slices	100	837	31	7.6	-	30	400	5.1		398
with gravy	24	377	-	1.0	20	-	-	-	-	-
°bread (2 slices)	46	469	4	-	24	38	24	1.2		28
lettuce salad	50	41.8	-	-	2	20	-	-	-	-
salad dressing	15	251	0.3	4.5	2.5	-	-	-	-	-
xcorn cooked 1/2 cup	80	335	2.2	0.7	16.4	4	41	0.4	17	81
butter (2 pats)	10	301	-	8.0	-	2	2	-	-	-
pudding ready mix cooked	100	519	3.4	3.0	22.8	102	95	0.3	-	136
<u>snacks</u>										
°cookies oatmeal (3)	56	1067	6.0	9.0	32.0	21	33	0.6		
Tang (2)	33	527	-	0.1	32.0	99	42	0.1	1.7	6
TOTAL		10,000 (2391 Cal)	119.3	88.3	275	806.2	1592	19.5		2623

TABLE V-A-2

Estimated Quantity of Markers Desired

	Quantity of the Oxide Desired		
Metal	Analytic limit	Amount per 50g Feces	5 X Use Level for Analysis
	ppm	mg	mg
Sc	3.0	150	0.75
Eu	1.6	80	0.40
Tb	5.0	250	1.25
Sm	0.4	20	0.100
La	10.0	500	2.5
Yb	3.0	150	0.75

TABLE V-A-3

Allocation of Marker to Nutrient and the Ratio of Marker to Nutrient

Nutrient	Amount/day	Marker	µg/day	µg of marker/nutrient
iron	20 mg	La	2500	125/1 mg
calcium	880 mg	Yb	750	850/1 g
lipid	88 g	Sc	750	8.5/1 g
protein	120 g	Eu	400	3.33/1 g
energy	2500	Sm	100	0.2/Joule
daily marker	--	Tb	1200	400/capsule three capsules a day

TABLE V-A-4

Amount of Metal (mg) to be Added to Each Food (g)
to Provide the Proper Marker/Nutrient Ratio (1)

Marker		Eu	Sc	Yb	La	Sm
Food	g weight	Protein	Fat	Calcium	Iron	Energy
Pork Sausage	3000	2.537	5.712	0.265	15	0.336
		3.044	11.424	0.3047	17.55	0.398
Hamburger	6500	5.094	5.355	0.561	46.875	0.48
		6.1128	10.71	0.6451	54.46	0.569
Eggs	1400	2.097	4.76	2.391	13.65	0.339
		2.5164	9.52	2.7495	15.97	0.402
Tomato Puree	7500	0.503	0.1275	0.828	27.09	0.12
		0.6036	0.255	0.9522	31.69	0.1422
Butter	2000	0.0362	14.195	0.340	23.95	0.57
		0.0434	28.39	0.391	28.02	0.6754
Honey	2100	0.033	--	0.340	2.5	0.256
		0.0396		0.391	2.925	0.3033
Tang	3500	0.072	0.0298	8.806	35.63	0.484
		0.0864	.0596	10.1269	41.687	0.5735
Milk	1 gallon	0.436	0.527	3.935	0.193	0.078
		0.5232	1.054	4.5252	0.2258	0.0924
Salad dressing	1120	0.186	4.964	2.18	0.3	0.26
(blue cheese)		0.2232	9.928	2.507	0.351	0.3081
Turkey	4000					
Turkey gravy	80 g in 800	4.2	2.6	1.03	32.9	0.330
	ml of water	5.04	5.2	1.1845	38.493	0.391
Prime rib	8000					
Beef gravy	100 g in 800	7.166	4.275	0.531	35.35	0.822
	ml of water	8.6	8.55	0.6105	41.36	0.974
Fish	4000	2.65	0.611	1.496	7.0	0.426
Tartar Sauce	800	3.18	1.222	1.7204	8.19	0.5048

TABLE V-A-4 cont'd

Marker		Eu	Sc	Yb	La	Sm
Apple sauce	4000	0.0113	0.034	0.136	2.5	0.1456
		0.0135	0.068	0.1564	2.925	0.1725
Peaches	4000	0.0226	0.034	0.136	2.5	0.1408
whipping cream		0.0271	0.068	0.1564	2.925	0.1668
Corn in cans	3200	0.293	0.238	0.136	5.0	0.128
		0.3516	0.476	0.1564	5.85	0.151
Peas	3200	0.772	1.360	0.625	32.0	0.192
		0.9264	2.72	0.7187	37.44	0.2275
Bread Rolls	3850	1.60	2.72	0.75	50.0	0.592
	160	1.92	5.44	0.8625	58.5	0.7015
Biscuits	4000 solid	2.187	5.10	2.125	40.6	0.7
	+ water	2.6244	10.2	2.4437	47.5	0.83
Bread	3750	0.949	0.412	2.422	33.625	0.544
		1.1388	0.824	2.7853	39.34	0.545
Pancake	1800 powder	1.15	0.758	2.125	25.0	0.408
	+ water = 4500	1.38	1.516	2.4437	29.25	0.4834
Cookies	2800 +	0.452	5.414	1.19	28.12	0.405
	nuts etc.	0.5424	10.828	1.3685	32.94	0.48
Pudding	1950 dry +	1.0	3.187	9.69	29.0	0.54
	9 l. milk	1.2	6.374	11.1435	33.93	0.64

(1) The top value gives the mg metal and the lower value gives the mg of metal oxide to be added to the respective quantity of food.

TABLE V-A-5

Analysis of Prepared Foods for Markers

(data is ppm on a dry basis)

Nutrient Marked	Cal.	Fat	Daily	Ca	Fe	Prot.
Food Nutrient Marker	Sm	Sc	Tb	Yb	La	Eu
Corn (M)	0.14	0.64	--	--	6.58	0.49
Biscuits (M)	0.21	3.17	--	1.39	15.5	1.23
Bread (M)	0.26	1.82	--	0.76	18.7	--
Rib Roast (M)	0.21	2.38	--	--	11.4	2.25
Rolls (M)	0.16	1.71	--	--	20.4	0.96
Peaches & Cream (M)	--	--	--	--	--	--
Milk (M)	0.12	1.42	--	7.59	0.49	1.10
Apple Sauce (M)	0.24	0.45	--	--	5.06	--
Cookies (M)	0.14	2.46	--	0.55	9.56	0.14
Pancake (M)	0.25	1.71	--	1.72	20.1	1.01
Egg (M)	0.18	5.11	--	2.74	13.3	2.20
Pork Sausage (M)	0.34	8.07	--	--	14.6	2.33
Tang (M)	0.11	--	--	2.59	8.83	--
Peas (M)	0.22	2.33	--	0.26	38.9	1.07
Tomato Puree (M)	0.18	0.42	--	1.38	36.6	0.76
Hamburger (M)	0.21	3.82	--	0.59	25.9	3.03
Pudding (M)	0.19	2.23	--	3.64	9.75	0.43
Turkey with Gravy (M)	0.23	2.85	--	--	23.5	3.28

V RECOMMENDATIONS FOR HUMAN STUDIES

B. RECOVERY OF MULTIPLE HEAVY METAL MARKERS AND FOOD TRANSIT TIMES IN HUMANS

Introduction

In their review of markers in nutrition Kotb and Luckey (1972) define several terms applied to the passage of digesta through the alimentary tract:

> Transit time is the time it takes the digesta of a meal to pass through the alimentary tract or segments of it. This time also represents the retention time in the tract or the particular segment.
>
> Rate of passage refers to the quantity of digesta (as weight or proportion) that passes a point along the alimentary tract in a given time.
>
> Rate of flow denotes any quantity of digesta (as weight or proportion) that travels a distance in a given time (e.g., g/m h).
>
> Rate of transport denotes the distance (in length or proportion of length) travelled by the digesta of a meal through the alimentary tract or segments of it in a given time.

Quantitative determination of transit time has been estimated using dyes, stained straw and other particulates, radioactive metals and other standard markers. Most of these may be criticized; the most promising are the heavy metal oxides (Kotb and Luckey, 1972). Therefore, systematic development of the use of non-radioactive heavy metal oxides for multiple markers in nutrition studies was begun. Analytic problems and method were presented (Gray and Vogt, 1974). The feasibility and versatility of the use of multiple markers in rat transit times, diurnal variation, and apparent digestibility was established (Luckey et al. 1975). A literature review indicated no oral toxicity of multiple rare earth oxides as oral nutrient markers (Hutcheson et al. 1975). The safety was affirmed in monkeys fed 10 times the use level for 2 months in a study of balance,

retention and recovery (See Section IV-D). These animal tests indicated that multiple heavy metal markers could be used in humans. A preliminary experiment was performed to provide information on a comparison of different metals in the determination of recovery and transit time. This pilot study is presented as a basis for recommendations for a definitive study.

Methods

Subjects. Following the policies of the committee for research involving human subjects (Appendix D), 3 adult males were given a complete physical examination which included about 30 clinical tests immediately before and following the experiment. The physical character of the subjects is given in Table V-B-1.

TABLE V-B-1

Characteristics of Human Subjects

Subject	Height (cm)	Weight (K)	Description	Birth
BV	169	87.5	Stout	India
TL	177	86.4	Medium	USA
DH	183	79.5	Lean	USA

Each subject ate his usual food with the only restriction being that no highly spiced foods would be eaten immediately prior to or during the experiment. A single capsule containing multiple heavy metal markers (Table V-B-2) was taken with the noon meal. DH took his pill prior to the meal, TL took his pill at mid meal and BV took his pill following lunch. All feces were collected individually in plastic foil.* These were tied,

* Reveal Wrap

labeled, placed in freezer cartons and immediately frozen. Paper wipes were kept if they had any particulates on them, soiled wipes were not saved.

The frozen samples were weighed with correction being made for the plastic foil. The foil was opened and the samples air dried at 38° C to dryness (72 hours was found to be adequate). The dry specimens were triturated by mortar and pestle to a uniform powder, triplicate samples were taken by accepted sampling procedures (roll on a plastic 20 times at right angles and take representative portions from 5 areas), and the samples weighed into plastic, capped vials for neutron activation analysis. Control stools were collected one day prior to taking the markers.

Results

The excretion data from the 3 subjects is given in Table V-B-3. Sample results are displayed in graphic form. The average total recovery of a typical metal for all 3 subjects (Fig. V-B-1) indicates that about 1/3 of the marker was excreted the first day, another 1/3 was excreted the second day, about 1/4 the next 2 days and the remainder the 5th day. The amount found on subsequent collection is negligable. The same data is displayed in another form in Figure IV-B-2. This again shows that the amount recovered after the 5th day is negligible. The weight of each marker recovered for each subject for each metal analyzed is displayed in Figure V-B-3.

Dried urine samples showed no detectable markers.

The subjects had no signs of abnormality due to the metals. The clinical data and the physical examination following the experiment gave the same picture of health as that at the beginning.

TABLE V-B-2

Recovery Studies - Human Experiment

Ratio of Marker Oxides: Markers are to be used at 100X level

Markers	Analytical level: µg of metal/gm of dry substance 1X	Metal present in capsule at 100X level	Metal Oxides the capsule
Tb_4O_7	5.0	50	58.8
Sc_2O_3	3.0	39	60.0
La_2O_3	6.0	60	70.2
Eu_2O_3	1.5	15	18.0
Sm_2O_3	0.5	5	5.9
Dy_2O_3	0.006	0.06	0.07
Yb_2O_3	3.0	30	34.5
Tm_2O_3	0.1	10	12.8
Cr_2O_3	0.1	1.0	1.3
$Ba\ SO_4$	0.1	1.0	1.7
			263.27

Each capsule contains:

markers	263.27
methyl cellulose	36.73
	300.00

TABLE V-B-3

Excretion Data of 3 Subjects

Day	Subject BV		Subject TL		Subject DH	
	Urine L	Feces g dry	Urine L	Feces g dry	Urine L	Feces g dry
-1	1.33	54.5	2.65	23.0	.92	47.0
1	1.07	36.0	1.89	22.7	.86	49.3
2	1.22	47.3	1.63	16.0	1.77	37.8
3	1.08	40.9	1.70	16.4	1.88	19.3
4	--	43.4	--	9.4	--	44.1
5	--	34.4	--	48.3	--	30.1
6	--	34.2	--	10.3	--	25.6
7	--	26.3	--	24.1	--	29.4
8	--	50.0	--	19.0	--	26.3

Day -1 = Control samples taken one day before the metal markers.

Collections were not taken on day zero.

Urine was not collected following the third day post pill.

Figure V-B-1 The average recovery from all subjects from a single pulse at noon on day zero. Displayed are the mean values with one standard deviation from data of 3 subjects with duplicate analyses of 3 replicates from each triturated specimen.

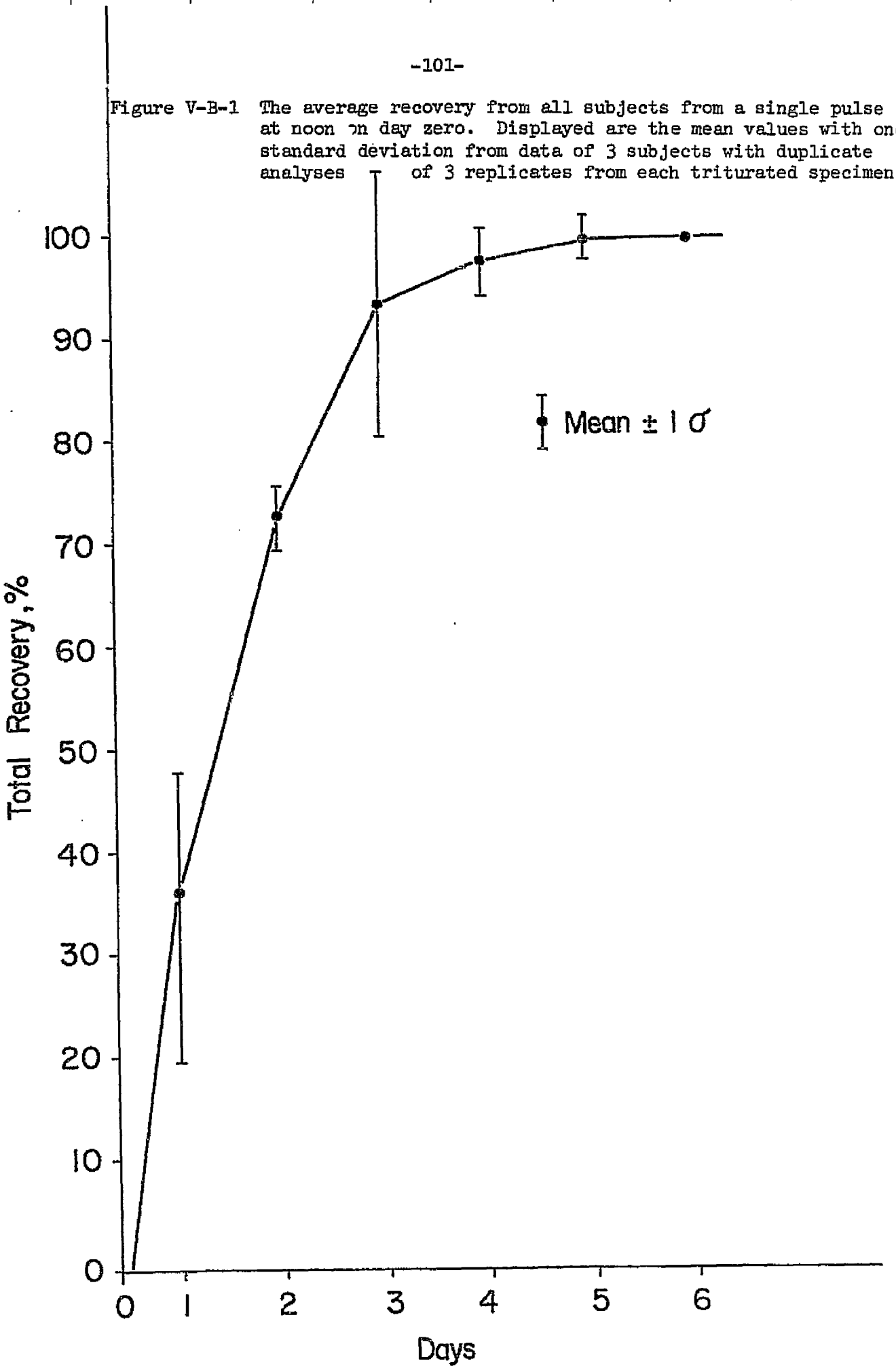


Figure V-B-2 The daily recovery from 3 subjects treated as in Fig. 1.

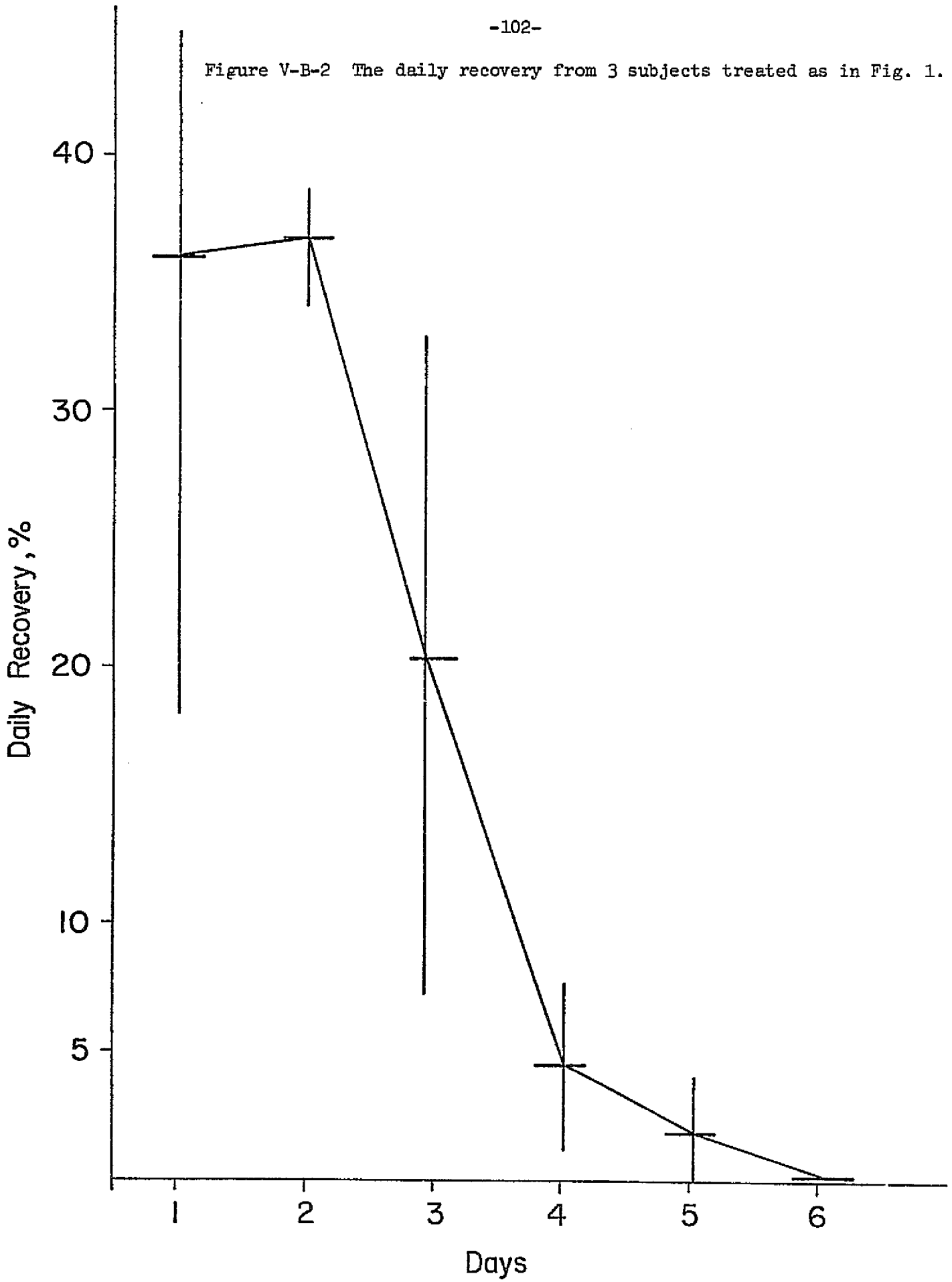


Figure V-B-3a Individual data for each subject showing the daily quantities recovered for: a) scandium; b) lanthanum; c) samarium; d) europium; and e) ytterbium.

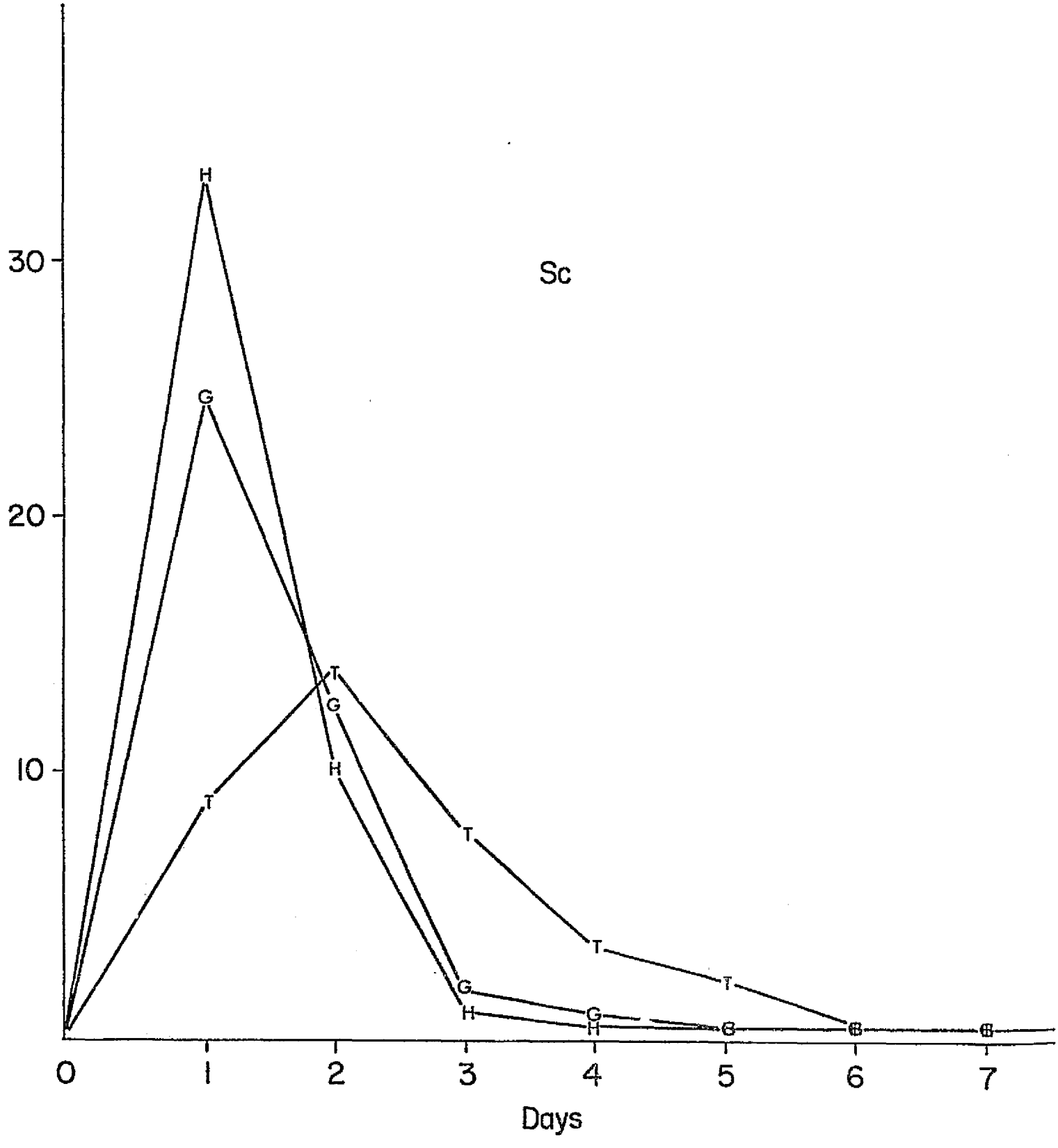


Figure V-B-3b. Lanthanum

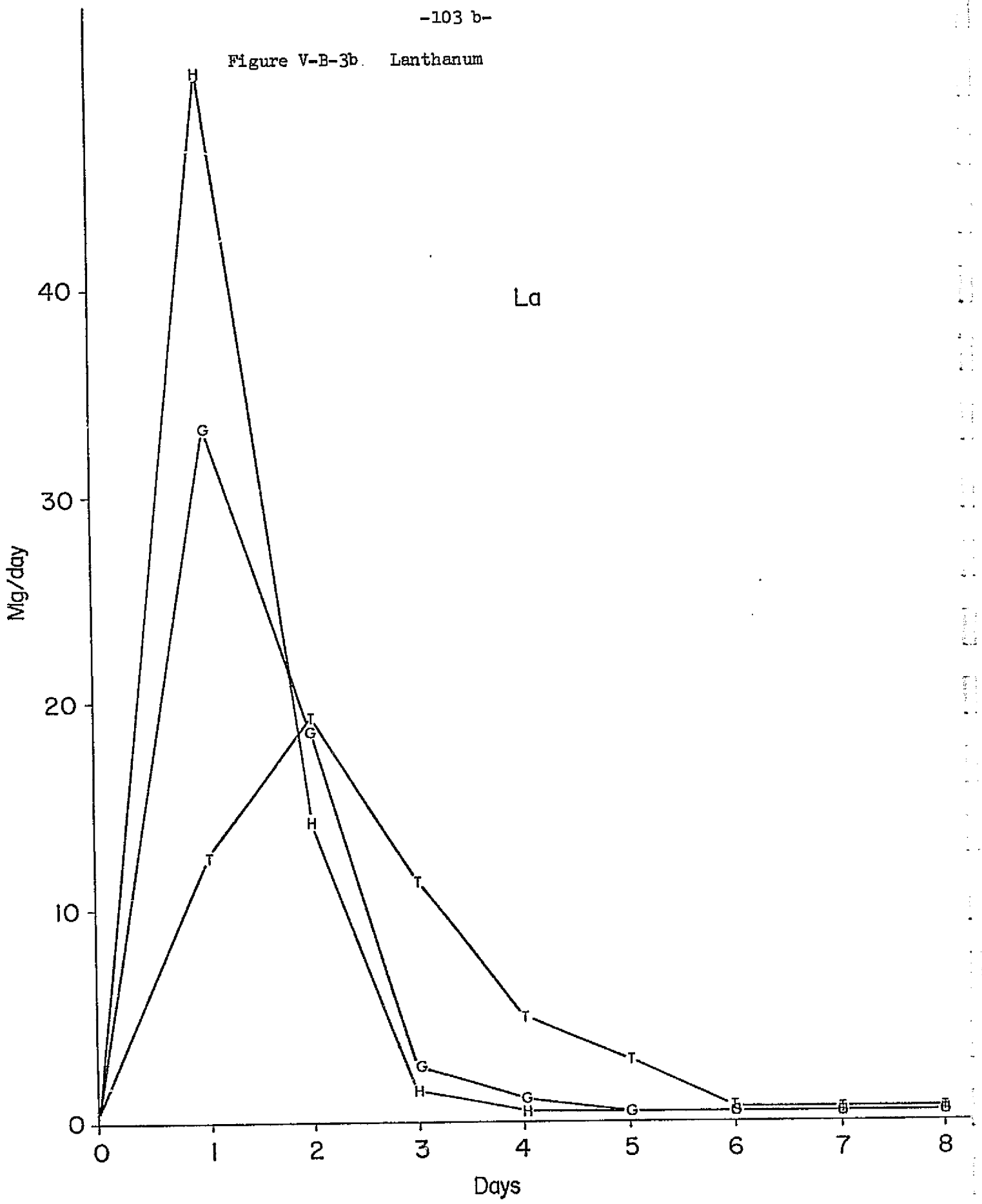


Figure V-B-3c Samarium

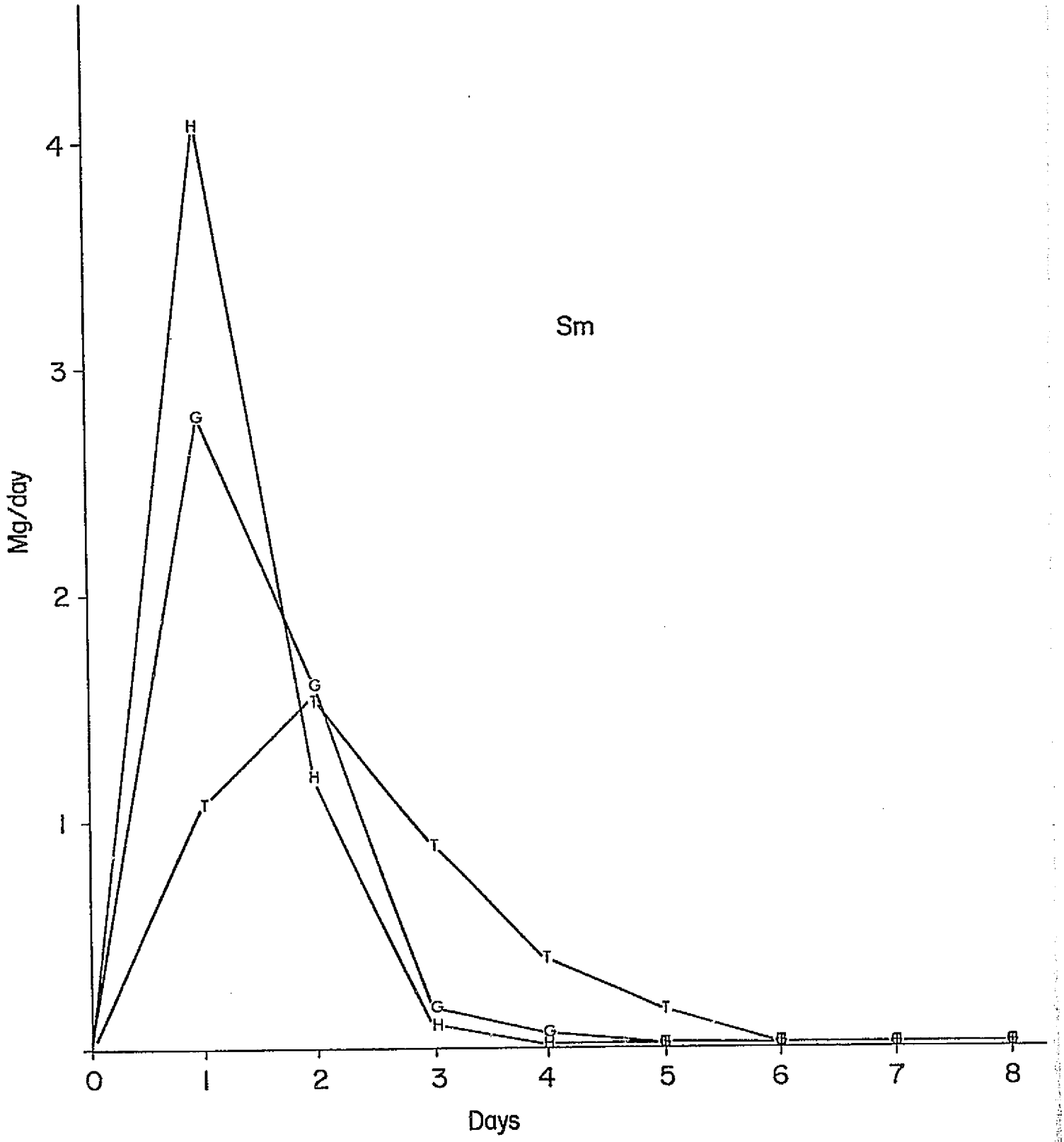


Figure V-B-3d Europium

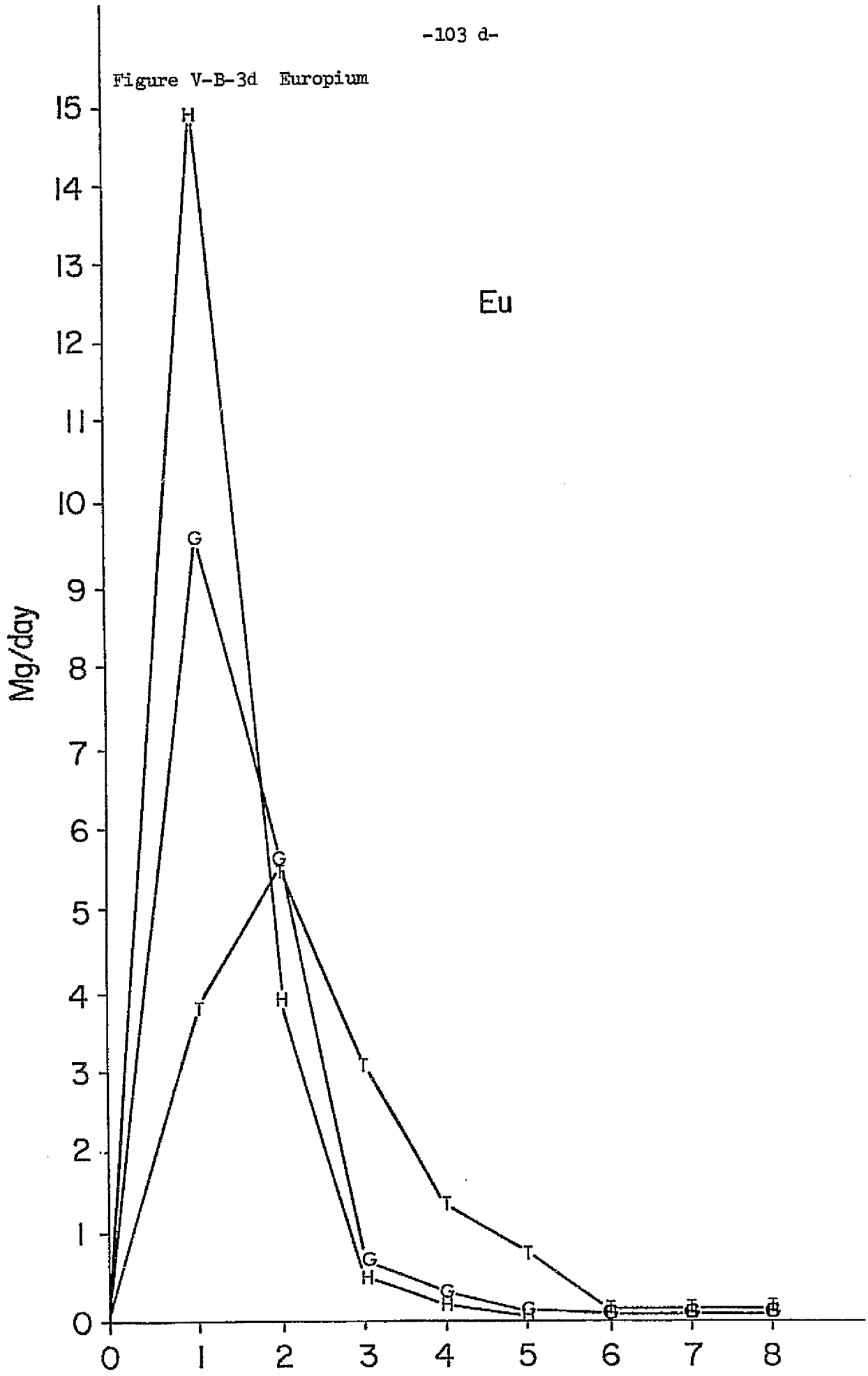
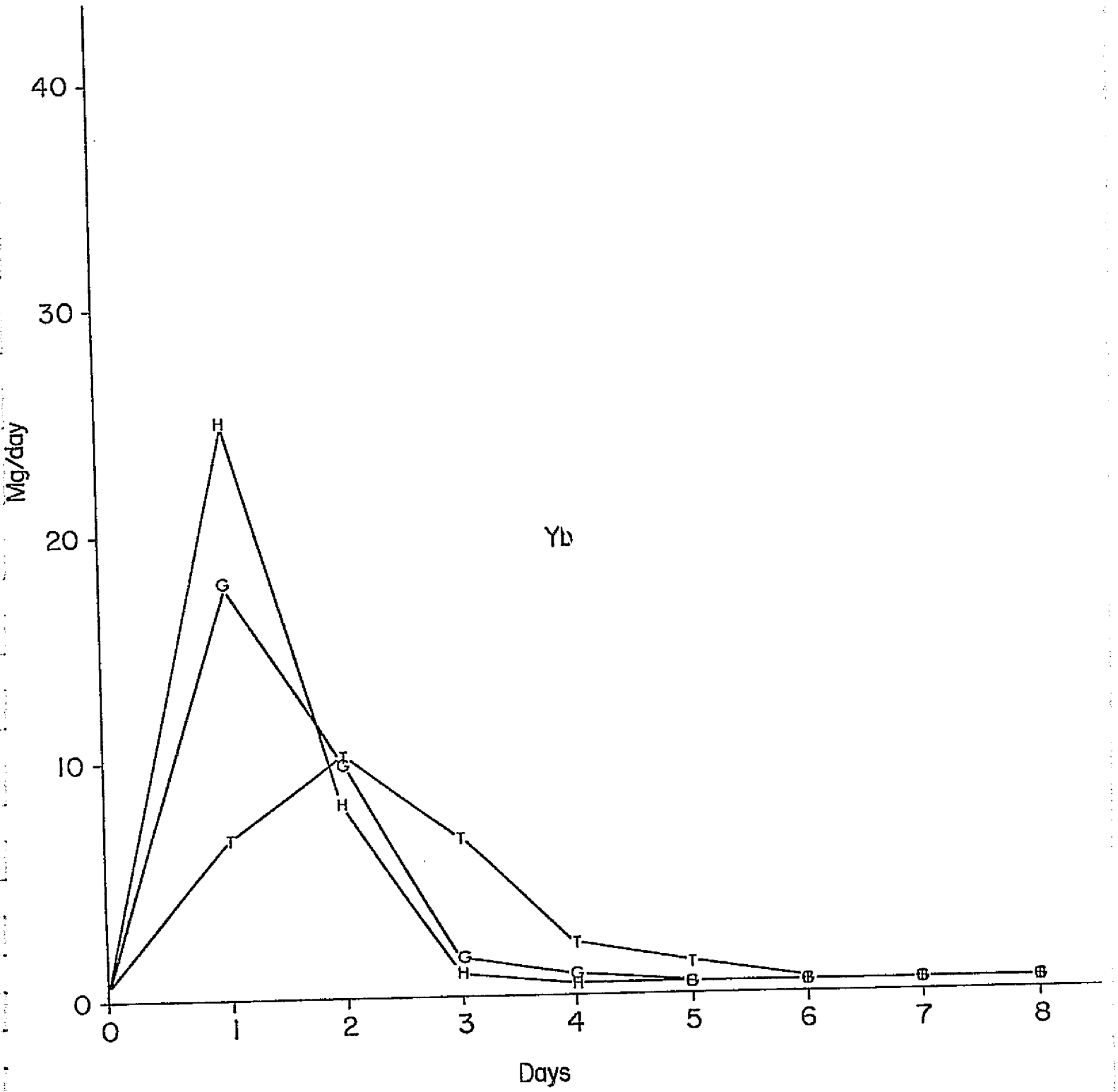


Figure V-B-3e Ytterbium



Discussion

The pattern of excretion of all metals is similar for each subject. Note that subject DH took his pill just prior to eating and subject BV took the markers following the meal. It seems possible that the patterns of excretion are related to the relative time of intake as much as to the individual characteristics of the subjects. This will be explored in future experiments. The similarity of the 5 markers would suggest that the other heavy metal markers would be similar. The raw data show the least variability for scandium; therefore, it might be the best single metal to study for the kinds of information sought. Scandium does occur in certain foods and feedstuffs; therefore, care must be taken to include background data. The natural occurance of scandium was not a problem in this preliminary work. It is of interest to note that no differences in excretion patterns were noted between scandium and ytterbium, although the atomic weights are about 4 fold different, 45 and 173 respectively.

Most determinations of transit time measure only the first appearance of a dye in feces after it has been administered. The times obtained would vary depending upon whether the dye were easily distinguished (as brilliant blue) or not (as carmine) from fecal color. Dyes may not flow through the alimentary tract at the same rate as food and digesta, these are among the reasons that Kotb and Luckey (1972) concluded that only heavy metal oxides would make good markers. Quantification of the metal oxides gives a proper index of the passage of food through the alimentary tract. The first 5%, 1/3, 507, 2/3 or 95% can readily be calculated, depending upon which conceptual base is desired. This has not been done for these data because the experiment was a preliminary work done to define problems.

The experiment clearly shows that the multiple marker system is

functional for humans. Multiple nutrient markers can be taken simultaneously, determined by a single discriminatory method, and provide comparable data. The final key will be to actually use a variety of rare earth metals to mark specific nutrients for humans, as was done in the rat feasibility tests. This is outlined in the next section (V-C).

The success of this experiment suggests a variety of other experiments which would provide useful information about the intestinal physiology of man. Normally, collections could stop at 5 days. Different markers could be used to mark different meals or different parts of a single meal. The difference in transit time between fat, thin and normal persons could be determined. The effects of fiber, mild cathartics and a variety of foods upon transit time could be determined. This experiment has opened the possibility to do a number of refined, quantitative experiments. More definitive tests should be carried out as soon as possible.

Summary

In a pilot study three human subjects were given 10 heavy metals in a single capsule at a noon meal and complete collections of feces was continued for 8 days. Urine was collected for 3 days. Preliminary results indicated that no markers were detectable in the dried urine. Recovery of 5 markers (the other 5 were not analyzed) showed that they all followed the same excretion pattern. About 1/3rd was excreted in the first 24 hours, another 1/3 was excreted the 2nd day, about 1/4th was excreted the third and fourth combined days, a small amount was found the fifth day and negligible quantities thereafter. This preliminary data opened the way for confirmatory work in the future.

V RECOMMENDATIONS FOR HUMAN STUDIES

C. BALANCE AND APPARENT DIGESTIBILITY

Introduction

Evaluation of markers in nutrition (Kotb, A.B. and T.D. Luckey, 1972, Markers in Nutrition) indicated that few classic markers should be used without serious reservations. However, good recoveries were reported for heavy metal oxides and salts. Radioactive heavy metals provide easy determination and good sensitivity. Neutron activation analysis of heavy metals provides further advantages for those conditions where radioactivity of untreated samples is not desirable, e.g. large animal feed lots, prolonged administration to humans. Radioactive heavy metals may usually be less desirable than inactive heavy metals for use of multiple markers.

The precept to be examined is that simple, precise balance and/or utilization of a variety of nutrients could result from the use of an intake marker plus one marker for each nutrient studied. Each nutrient marker would be incorporated in the food in a predetermined ratio to its designated nutrient. If the daily marker were ingested (one capsule with each of 3 meals), records and quantitative fecal collection could be eliminated. The intake marker provides an index of that fraction of the daily food intake which is represented by any portion of feces collected. The fecal concentration of nutrient marker is multiplied by the inverse fraction of the daily proportion of intake marker found to provide accurate estimation of the nutrient intake. Analyses for markers and nutrients in feces excreted completes the data needed to estimate utilization and/or balance of each marked.

The concept of multiple nutrient markers using heavy metal oxides which are analyzed by a single procedure has been developed in this laboratory

during the course of our NASA contract. The analytic method was reported by Gray et al. (1972) and Gray and Vogt (1974). A complete literature review of the toxicity of heavy metals has been completed (Critical Review of Heavy Metal Toxicity in Mammals, submitted by B. Venugopal and T.D. Luckey in 1974 for publication as a NASA Technical Report and Heavy Metal Toxicity, Safety and Hormology by T.D. Luckey, B. Venugopal and D.P. Hutcheson as Supplement Vol. 1 of Environmental Quality and Safety, Georg Thieme Publ. Stuttgart, 1975). Rat feasibility tests showed the utility of the system for measuring transit time, recovery, balance, diurnal variation, and apparent digestibility using 4 different metals to mark 4 different nutrients (Luckey et al. 1975). The safety of 10 metal markers was established by feeding groups of mice 1, 10, 100, and 1000 times the anticipated use level of 10 metal markers through 3 generations with no unusual effects noted for growth rate, maturity, general health and appearance, reproduction, lactation, necropsy examination and hematology (Hutcheson et al. 1975). A pilot study was made with monkeys to establish safety by feeding 10 times the use level to 2 monkeys for 2 months and a 1 week balance study of 5 metal markers; the absence of tissue retention in monkeys and the health and necropsy information gave proof that these metals were harmless under the conditions used. This work was summarized by Hutcheson et al. (1975 b), and is being written for separate publication. The last preliminary study was the transit time in humans reported in the above subsection (V-B).

The preliminary findings outlined above provide information for guidelines to be followed in depth as soon as possible. Approval for any such experiment would need to be obtained from the committee for Research Involving Human Subjects.

Methodology

The experimental diet is designed for three persons for a ten-day balance study. The marked diet will be the only material available to these subjects for six days prior to the balance study. Each subject is a self control with all food and drink weighed and recorded. Complete urine and fecal samples will be retained for analyses. Blood samples will be taken before, during and after the experiment. A complete physical examination should be administered before and following the study.

The food preparation will be very carefully supervised in order to obtain good representation of the exact quantities of mineral markers desired for each food, depending upon the nutrient represented by each marker. The minerals to be used, the nutrients represented by each and the ratios for each are given in Table V-A-3. Each subject will take the daily marker with methyl cellulose in three 100 mg capsules. One capsule is taken with the last few bites of each of three meals during the day.

Analyses of the feces and calculation of daily consumption of each nutrient will be made by using the markers. Chemical analyses for the nutrients being studied will be made in order to compare the efficiency of utilization of the nutrients being studied by both classic and marker methods. The nutrient analyses will be done be standard methods. The data will be processed and discussed from the viewpoint of feasibility and problems which may have arisen and suggestions for future use of the method.

The protocol was accepted by the UMC Human Experimentation Committee. The diet details are also given there. Possible nutrient-marker combinations are given in Table V-C-1. The formula for indirect determination of apparent digestibility is:

$$\% \text{ Apparent Digestibility} = 100 - 100 \left(\frac{\text{Marker in diet}}{\text{Marker in feces}} \times \frac{\text{nutrient in feces}}{\text{nutrient in diet}}\right).$$

TABLE V-C-1

Suggested Nutrients to be Marked and Markers

Nutrient	Marker
Calories	Yb-Ytterbium
Protein	Au-gold
Fat	Eu-Europium
Carbohydrate	Dy-Dysprosium
Ca	Tb-Terbium
P	Ho-Holmium
Mg	Er-Erbium
Fe	Lu-Lutetium
Cu	T-Titanium
Na	Sc-Scandium
K	Ir-Iridium
B_{12}	Gd-Gadolinium
Folate	La-Lanthanum
Cr	Zr-Zirconium
Collection	Ce-Cerium
H_2O (in food)	PEG-polyethylene glycol
Fecal	BB-Brilliant Blue
Others	Cr-Edta=Chromium EDTA
	La-Lanthanum
	Y-Yttrium
	Ru-Ruthinium
	Cr_2O_3-Chromic oxide
	ZrO-Zirconium oxide
	T_iO-Titanium oxide
	$BaSO_4$-Barium Sulfate

VI EVALUATION AND PROBLEMS

The concept of the use of multiple nutrient markers has been validated by feasibility studies in rate, safety studies involving 4 generations of mice, balance and retention studies in monkeys, and very preliminary studies in human subjects. An exhaustive literature search on the toxicity of non-radioactive, heavy metal oxides reveals that they are not toxic when taken orally. There should be no hesitency to use selected heavy metal oxides as nutritional markers in any species.

Recommendations and consultation were provided by numerous personal conferences and via telephone, letters and reports. All requests were properly followed to our best ability and to the apparent satisfaction of the NASA contract officer. It is anticipated that the good work and advice presented will contribute to a continuation of work in this area in the near future.

Problems

One problem which has repeatedly surfaced during this report is that the analytical system is as costly as the rest of the work combined. This is due to the computer time needed to determine the best of various radiation peaks for each metal under each condition. Increased replicates would reduce the error. The practical problems of learning the sensitivity of any given metal in the presence of 5-10 other metals occasionally resulted in the absence of data from metals fed due to the loss of analytical sensitivity of one metal in the presence of relatively large amounts of another.

The problem of particle size for the marker has not been explored. Powders (ca. 200 mesh) have been used; the validity for this being the best

has not been established. A study is needed to determine which type of nutrient the metal oxide powders follow closely, i.e. fat, carbohydrate, protein, fiber or mineral.

There are isolated reports of cancers or tumors being found following administration of some of the non-radioactive heavy metals. The validity and meaningfulness of these reports need to be evaluated.

VII NUTRIENT INTAKE MONITORING FOR FUTURE SPACE CREWS

Introduction

Literature review of markers in nutrition (Kotb and Luckey, 1972) and on heavy metal toxicity (Venugopal and Luckey, 1975) combined with the experimental studies in the previous sections of this report give ample evidence that selected non-radioactive, heavy metal oxides provide good markers for a system of multiple nutrient markers to indirectly record intakes and apparent digestibility of selected nutrients.

The methods for incorporation of these markers into foods and sample preparation are detailed in this section. Concrete examples are given. When different nutrients are selected and as more food types are needed, considerable ingenuity may be demanded. The basic methods are applicable to each nutrient and for a considerable number of markers. Suggestions for nutrients to be marked and markers available are given in Table VII-1. Recommended markers for different nutrients are listed in Table VII-2. Although other tables of recommended markers appear throughout this report, the use of markers not in Table VII-2 in the presence of a variety of other metals is complex and may require further refinement of the system.

The examples to be used are listed in Table VII-3.

Marker Concentrations and Marker-Nutrient Ratios

The reasoning process that leads to the estimation of the quantity of marker to be used and the marker-nutrient ratio is exemplified in Table VII-3. Information about which marker to use comes from a consideration of solubility, absorption, retention, toxicity and transit characteristics. The heavy metal oxides were chosen with these characteristics in mind. The estimation of quantity comes from a consideration of the sensitivity of the method used

in conjunction with the matrix being examined. The lowest level which provides acceptable accuracy is chosen. Cognizance is taken of the fact that the sensitivity of determination of any given metal varies with the presence of other metals with interferring characteristics. The remarkable similarity of the rare earth metals extends to the radiation character of their radionuclides. Any would show excellent sensitivity and have good analytic characteristics if used singly. The presence and concentration of other metals may cause any given peak of radiation to be useless when one metal is used with others. This explains the important changes seen in Table VII-2 and the complexity of adding each new element to this list. These complexities also are the reason that different combinations of metals have been used throughout the experiments reported. The model will be developed with the 6 recommended metals.

All data are reported as ppm element. Therefore, the atomic weights and the equivalent weights of the compounds being considered must be respected. These are given in columns 1 and 2, Table VII-3. Preliminary work establishes the analytic limit of sensitivity for the accuracy needed using triplicate samples, each run in duplicate (column 3). The size of sample is estimated -- about 50 to 100 g of dry feces per day indicate the quantity of each metal or oxide needed for the lowest sensitivity (column 4). In order to have a safety factor and to bring the accuracy above to lowest limit, 5-10 times the minimum level is recommended (column 5).

The nutrients to be followed are selected and methods for their quantification are perfected. The anticipated amount of each nutrient to be eaten each day is estimated (column 6). The above information allows direct calculation of marker-nutrient ratio to be calculated from columns 5 and 6. This is done (column 7) and conveniently rearranged (column 8) to provide

a quantity of each marker that will be added to each food for each of the nutrients designated. Thus, for each gram of protein, we would add 0.01 mg of scandium or 0.0153 mg of Sc_2O_3 to each food which contains protein. Similar procedure would follow for each marker-nutrient combination.

Daily Marker

Our experience indicates that the daily marker should be administered 3 times daily. Thus, the values given in column 5 should be divided into 3 equal parts and quantitatively inserted into capsules for ingestion at mid meal three times per day. Under usual conditions this marker will be excreted as indicated in Figure V-B-3. The fraction of this marker found in any stool sample indicates that proportion of the daily intake is represented in the sample.

A different daily marker for each person provides a laboratory check on the labels.

Marker Incorporation Into Food

Each batch of food prepared for eating must be analyzed for the nutrients to be marked. Food composition tables may be used for rough estimates, but not for experimental work. The content of nutrient determines the quantity of marker to be added to each food. Then the marker must be added to each food in a manner that assures any portion of that food eaten will provide the appropriate amount of markers. Table VII-4 gives explicit examples for a breakfast. The quantity cooking would be determined. Assume that 108 breakfasts were needed, three are needed for testing and about 10% more than is planned should be provided. Therefore, 120 times each of the quantities given in Table VII-4 should be carefully weighed out and

throughly mixed. The minerals can be readily mixed into about one half of the foods, these include powders (spray dried eggs, sugar, salt, pepper, dried puddings, and baked food ingredients), viscous liquids and sauces and purees. The metal oxides did not interfere with baking of bread, cookies and buns or with pancakes and fritters. Whole and chunky foods present problems which were generally solved by having the subjects eat complete servings, with plates scrapped clean. The combinations of vegetables with sauce, meat with gravy, sliced fruit with juice, salad with dressing, and fish with sauce are used because they provide a vehicle to carry measured amounts of the metal mix. Since all helpings are weighed and all food is eaten completely, this practice did not introduce significant error.

Analysis is performed on 3-5% of the items. The crew is free to eat any food available (as all are marked). Each must take one capsule at mid meal 3 times daily, and each must eat all of any food started. Individualized servings must be maintained for chunky foods. Baked foods may be shared. All fluids must be vigorously shaken for a few seconds to suspend the insoluble markers.

Stools should be kept daily with identification and date recorded on the container. These are best handled in the frozen state. They are dried (at 40° unless a nutrient is being studied which is heat sensitive), thoroughly ground and sampled. Three 2 g samples are provided for each analysis. Sample size for nutrient analysis will vary with the number and kind of studies being made, usually 2 samples of 4 g is adequate.

The nutrient analysis is done by standard methods. The neutron activation analysis is done according to the method outlined by Gray and Vogt (1974) which is given as Figure IV-A-1. The results are reported in ppm on a dry basis. Calculations for a variety of nutritional functions may be made by accepted formulae.

TABLE VII-1

Suggested Nutrients to be Marked and Markers*

Nutrient	Marker
Calories	Yb-Ytterbium
Protein	Au-gold
Fat	Eu-Europium
Carbohydrate	Dy-Dysprosium
Ca	Tb-Terbium
P	Ho-Holmium
Mg	Er-Erbium
Fe	Lu-Lutetium
Cu	T-Titanium
Na	Sc-Scandium
K	Ir-Iridium
B_{12}	Gd-Gadolinium
Folate	La-Lanthanum
Cr	Zr-Zirconium
Collection	Ce-Cerium
H_2O (in food)	PEG-polyethylene glycol
Fecal	BB-Brilliant Blue
Others	Cr-Edta=Chromium EDTA
	La-Lanthanum
	Y-Yttrium
	Ru-Ruthinium
	Cr_2O_3-Chromic oxide
	ZrO-Zirconium oxide
	T_iO-Titanium oxide
	$BaSO_4$-Barium Sulfate

* Any should be satisfactory for the daily marker.

TABLE VII-2

Analytical Levels

Element	Individual	Best 2	Best 3	Best 4	Best 5	All 6
Sc	0.5	1.0	1.5	1.5	1.5	1.5
Eu	0.1	0.1	0.4	0.8	0.8	0.8
Tb	0.5		1.0	2.5	2.5	2.5
Yb	0.2			1.5	1.5	1.5
Sm	0.01				0.2	0.2
La	1.0					5.0

TABLE VII-3

Determination of Marker Level and Marker-Nutrient Ratio

Column	1	2	3	4	5		6	7	8
			Analytic Level	Fecal Anticipated	Fecal/day	Nutrient	Approximate Amount/day	Ratio	Weight
Element	Weight	Equivalent Weight	ppm	mg/100g	µg		g	mg/g	mg/g
Sc	44.96	44.96	1.5	150	1000	Protein	100	1/100	0.01
Eu	152.0	152.0	0.8	80	500	Carbohydrate	250	1/500	0.002
Tb	158.9	158.9	2.5	250	1000	Fat	100	1/100	0.01
Yb	173.0	173.0	1.5	150	1000	Iron	0.3	1/.030	33
Sm	150.4	150.4	0.2	20	100	Vitamin C	0.1	1/1.0	1.0
La	138.9	138.9	5.0	500	3000	"Daily Marker"	(3 X 1mg)	--	--
Oxide									
Sc_2O_3	137.9	69.0	2.30	230	1532	Protein	100	1.53/100	.0153
Eu_2O_3	351.9	176.0	0.927	92.7	579	Carbohydrate	250	.232/100	.0232
Tb_2O_3	365.9	183.0	2.87	287	1148	Fat	100	1.15/100	.0115
Yb_2O_3	394.1	197.1	1.71	171	1140	Iron	0.3	1.14/0.3	3.76
Sm_2O_3	348.7	174.4	0.232	23.2	116	Vitamin C	0.1	1.16/1	1.16
La_2O_3	325.8	162.9	5.87	587	3522	"Daily Marker"	(3 X 1.17mg)		

TABLE VII-4

Examples of Markers to be Added to Different Foods

Food	Serving Weight g	Protein	Sc	Carbohydrate	Eu	Fat	Tb	Iron	Yb
Eggs, dried	14	6.6	0.0660	0.6	1.20	5.8	58	1.2	39.6
Sausage	40	12.7	0.127	0	0	11.2	112	1.6	52.8
Tang, dry	32.6	0	0	32.0	64.0	0.1	1	0	0
Pancake	90	6.4	0.064	30.6	61.2	6.4	64	1.2	39.6
Milk,2%	245	8.5	0.0850	11.5	23.0	4.0	40	0.01	0.330
Honey	21	0.1	0.001	16.4	32.8	0	0	0.2	6.60
Butter	5	0	0	0	0	4.0	40	0	0
Sugar	5	0	0	5.0	10.0	0	0	0	0
Coffee or tea		0	0	0	0	0	0	0	0

VIII OVERVIEW OF ASTRONAUT NUTRITION WORK BY LUCKEY

Previous work completed by T.D. Luckey includes research with M. Bengson on Long Term Biological Isolation of Primates and Mice during the period Jan. 1969 to Jan. 1970. This work is summarized in four volumes under contract NAS 9-9000. This work is reported in the scientific literature by T.D. Luckey, M.H. Bengson and M.C. Smith, Apollo Diet Evaluation: A Comparison of Biological and Analytical Methods Including Bioisolation of Mice and Gamma Radiation of Diet in Aerospace Med., 44: 888-901 (1973) and Effect of Bioisolation and the Intestinal Flora of Mice upon Evaluation of an Apollo Diet. J. Aerospace Med. 45: 509-518 (1974) by T.D. Luckey, M. H. Bengson and H.I. Kaplan. The next phase was Apollo Diet Evaluation during the period June 1970 to June 1971. The final report of this contract, NAS 9-10955, indicated serious deficiencies in particularly early Apollo diets. The inexactness of knowledge of food intake led to the need for a method to be developed which would provide the information with minimum astronaut participation.

The method proposed was to incorporate nutrient markers into all foods available; analysis of individual stools would provide an accurate index of the daily intake of the desired nutrients. Nutrients would be marked by adding proportionate quantities of nonabsorbed heavy metal oxides which could be analyzed in one operation using neutron activation analysis (Activation Analysis of Stable Elements Used as Nutritional Markers. Transactions of Amer. Nuclear Soc. 15: 149, 1972, D.H. Gray, M. Kay, J.R. Vogt and T.D. Luckey, and Neutron Activation Analysis of Stable Heavy Metals as Multiple Markers in Nutritional Monitoring. J. Agr. Food Chem. 22: 144-146, 1974, D.H. Gray and J.R. Vogt).

The problem was to devise a simple procedure by which the intake of one or more nutrients (i.e. protein, calories, calcium etc.) could be determined with minimum subject participation. This method provides information needed to prevent or alleviate the metabolic losses routinely experienced by astronauts in space. Astronaut weight loss may be due to excessive calorie, water and/or protein loss. Some astronauts provided accurate logs of food intake, others did not. The method being developed provides the desired information without the subject recording anything and allows a confirmation of other information.

The new method also will be useful in clinics, metabolic wards, limited population studies and in large animal efficiency studies. This method has been developed under contract NAS 9-12369; the principle investigator is Dr. T.D. Luckey, Professor of Biochemistry at the University of Missouri-Columbia in collaboration with Dr. D.P. Hutcheson, Associate Professor of Animal Husbandry and Dr. B. Venugopal, research associate.

Work completed to date includes (1) a literature survey of markers, (2) feasibility studies with rats (3) safety studies in mice and monkeys, (4) balance study with monkeys and (5) recommendations for human feasibility studies:

(1) A critical review of the literature on markers used in human and animal studies was made (A.R. Kotb and T.D. Luckey, Markers in Nutrition, Nutr. Abst. and Rev. 42: 813-845, 1972). This review clearly indicated that of all markers used, only the heavy metal (oxides) could satisfy stringent criteria of acceptability. Further literature review showed there was no toxicity known from ingestion of the non-radioactive heavy metal oxides chosen. The reviews are presented in Heavy Metal Toxicology, Safety and Hormology, in Environmental Quality and Safety. Ed. T.D. Luckey Georg Thieme

Publ. Stuttgart 1975, and Critical Review of Heavy Metal Toxicity in Mammals by B. Venugopal and T.D. Luckey in a NASA Technical Report submitted in 1974.

(2) Feasibility studies were performed with rats which showed (a) safety was not a critical problem in short-term experiments, (b) recovery of heavy metal oxides was acceptable, (c) diurnal variation was not a major problem, and (d) apparent digestibility of nutrients could be determined using the multiple marker method. This work is published: Heavy Metals as Nutrient Markers. IX International Congress of Nutr., Mexico Summaria, p. 56, 1972. T.D. Luckey, A. Kotb, and J. Vogt. Rat Feasibility Studies of Multiple Nutrient Markers. J. Nutr. 106: 1266-1276, T.D. Luckey, A.R. Kotb, J.R. Vogt, and D.P. Hutcheson.

(3) Safety studies showed that no detectable uptake of heavy metals occurred in monkeys fed excessive quantities of heavy metals for two months and that mice fed 1,000 times the anticipated use quantities showed no effects through three successive generations. This work has been published: Nutritional Safety of Heavy Metals Selected for Nutritional Markers. Fed. Proc. 32: 914 Abs., 1973, D.P. Hutcheson, B. Venugopal, T.D. Luckey, P.C. Rambaut and M. Smith; Safety of Heavy Metals as Nutritional Markers in Environmental Quality and Safety, Suppl. Vol 1, pp 74-80, 1975, D.P. Hutcheson, D.H. Gray, B. Venugopal, and T.D. Luckey; and Nutritional Safety of Heavy Metals in Mice. J. Nutr. 106: 670-675, 1975. D.P. Hutcheson, D.H. Gray, B. Venugopal and T.D. Luckey.

(4) The monkey safety and balance studies are in preparation for publication: Multiple Heavy Metal Marker Balance in Monkeys by D.P. Hutcheson, D.H. Gray, B. Venugopal and T.D. Luckey. The unqualified success of the animal work allowed human work to proceed. Preliminary work indicates the

recovery of markers is expected to be satisfactory and balance studies have been recommended. Evidence accumulated to date shows the markers are ot absorbed and are safe for man. (Rare Earth Metal Oxides as Nutritional Markers in Humans. 1974. Fed. Proc. Abstract 33: 703. B. Venugopal, D.P. Hutcheson, D.H. Gray and T.D. Luckey,) Continued research on man rated markers should be funded as soon as possible.

Contract NAS 9-12369 began in October 1971 and was extended through March 1975. Funds are needed to utilize this new methodology in the next space flights. This will allow accurate determination of the causes for astronaut weight losses and will begin a serious reevaluation of the energy, protein and mineral needs of man in space. The latter information will allow more accurate projection of the quantity of foods needed, the most efficient use of foods in manned space flight, and provide the most efficient planning for future space flights.

Utilization of the methodology would not only bring the previous year's work to fruition but would also dramatize NASA's new methodology as a useful tool for future nutrition research in the broad field of public health.

IX PUBLICATIONS

Gray, D., M. Kay, J.R. Vogt and T.D. Luckey, Activation Analysis of Stable Elements Used as Nutritional Markers. Transactions of Amer. Nuclear Soc. 15: 149, Abs. 1972.

Gray, D.H. and J.R. Vogt, Neutron Activation Analysis of Stable Heavy Metals as Multiple Markers in Nutrition. Agr. and Food Chem. 22: 144-146, 1974.

Hutcheson, D.P., B. Venugopal, T.D. Luckey, P.C. Rambaut and M. Smith, Nutritional Safety of Heavy Metals Selected for Nutritional Markers. Fed. Proc. 32. 914 Abs., 1973.

Hutcheson, D.P., D.H. Gray, B. Venugopal and T.D. Luckey, Nutritional Safety of Heavy Metals in Mice. J. Nutr., 105: 670-675.

Hutcheson, D.P., D.H. Gray, B. Venugopal, and T.D. Luckey, Safety of Heavy Metals as Nutritional Markers. Environmental Quality and Safety, Suppl. 1: 4-73, 1975.

Kotb, A. and T.D. Luckey, Markers in Nutrition. Nutr. Abstr. and Rev. 42: 813-845, 1972.

Luckey, T.D., A. Kotb, and J. Vogt, Heavy Metals as Nutrient Markers. IX International Congress of Nutr., Mexico Summaria p. 56, Abs. 1972.

Luckey, T.D., A.R. Kotb, J.R. Vogt and D.P. Hutcheson, Feasibility Studies in Rats Fed Heavy Metals as Multiple Nutrient Markers. J. Nutr., 105: 660-669.

Luckey, T.D., Introduction to Heavy Metal Toxicology, Safety and Hormology. Environmental Quality and Safety, Suppl. 1: 1-3, 1975.

Luckey, T.D., Hormology with Inorganic Compounds. Environmental Quality and Safety, Suppl 1: 81-103, 1975.

Venugopal, B., D.P. Hutcheson, D.H. Gray and T.D. Luckey, Rare Earth Metal Oxides as Nutritional Markers in Humans. 1974. Fed. Proc, Abstract 33: 703, 1974.

Venugopal, B. and T.D.Luckey, Critical Review of Heavy Metal Toxicity in Mammals. NASA Technical Report, 1975 Submitted.

Venugopal, B. and T.D. Luckey, Toxicology of Non-Radioactive Heavy Metals and Their Salts. Environmental Quality and Safety, Suppl. 1: 4-73, 1975.

X APPENDICES

A. Vita for a) Dr. T.D. Luckey, Principle Investigator
b) Dr. B. Venugopal, Research Associate
c) Dr. D.P. Hutcheson, Collaborator

B. Guidelines and Policy for Research Involving Human Subjects

C. Basic Publications:
a) Markers in Nutrition.
b) Critical Review of Heavy Metal Toxicity in Mammals.
c) Heavy Metal Toxicity Safety and Hormology.

Thomas Donnell Luckey - Resumé

PRECEDING PAGE BLANK NOT FILMED

PERSONAL

BIRTH: [redacted], [redacted].
MARRIED: Pauline Miller, 1943; 3 daughters - Jane, Mary and Donna.
HOBBIES: Photography and Building.

EDUCATION

Colorado A. and M., 1936-38, 1939-41, B.S. in Chemistry
University of Wyoming, 1938-39, and summers 1939 and 1940.
Texas A. and M., 1941-42
University of Wisconsin, 1942-44, M.S. in Biochemistry
University of Wisconsin, 1944-46, Ph.D. in Biochemistry

ACADEMIC

Texas A. and M., 1941-42, Teaching Assistant
University of Wisconsin, 1942-46, Research and Teaching Assistant
University of Notre Dame, 1946-52, Assistant Research Professor
University of Notre Dame, 1952-54, Associate Research Professor
University of Missouri, 1954-present: Professor of Biochemistry (Chairman, 1954-68), Department of Biochemistry and Chairman of Graduate Nutrition Area, 1970-72.

INDUSTRIAL

Board of Directors and Secretary, Mygrodol Products, Inc., South Bend, Indiana, 1951-53. Consultant: Mygrodol Products, Inc., 1948-53 and Whitehall Pharmacy, 1951-53. McDonnell Aircraft Corporation, 1965-66.

SOCIETIES

American Chemical Society, American Association for the Advancement of Science, Sigma Xi, The Institute of Nutrition, American Society for Microbiology, Phi Chi Medical Fraternity (Advisor), Society for Experimental Biology and Medicine, Association of American University Professors, Association for Gnotobiotics (Board of Directors, 1971-75), New York Academy of Science, and Missouri Academy of Science, Smithsonian Institute.

FELLOWSHIPS AND HONORS

Charter Chairman - West Central States Biochemistry Conference 1959, Conference Director, 1963-74. N.S.F. Travel Fellowship IV Inter. Cong. Nutrition, Paris, 1957; V. Inter. Cong. Microbiol., Stockholm, 1958; Symposium V on Germfree Animals at the VII Inter. Cong. Microbiol. Stockholm, 1958. Chairman, UMC Section of the ACS, 1959, Vice Chairman, 1957-58. Commonwealth Fellowship 1961-62 for study abroad. Member, Scientific Advisory Committee of Cancer Research Center, 1962-. American Institute of Nutrition Travel Fellowships, International Nutrition Congress at Edinborough (1963) and Prague (1969). Principal speaker at International Symposium on Microecology, 1964, Berlin. Organizer and Moderator of the Symposium on Gnotobiology at IX International Congress of Microbiology, Moscow, 1966. Keynote speaker oi the International Symposium on Germfree Life, Nagoya, 1966. Guest lecturer, Romanian Academy of Science, October, 1968. Visiting Scientist, Space Technology Center, Valley Forge, 1968-69. Organizer: 1st, 2nd and 3rd International Symposia on the Ecology of the Intestinal Flora, Columbia, 1970,'72,'74. Medical Advisory Board, Educational Films, Inc., Riverside Calif. 1970-. Special Lecturer, 2nd and 3rd International Symposia for Biological Medicine, Lausanne, 1972,'73. Subcommittee on Interaction of Nutrition and Infections, Food and Nutrition Board of U.S. Nat'l. Acad. Science, 1972-74.

AUTHOR

GERMFREE LIFE AND GNOTOBIOLOGY, 1963, and numerous articles and papers on germfree animals, nutrition, vitamins, antibiotics and hormones. EDITED: ADVANCES IN GERMFREE RESEARCH AND GNOTOBIOLOGY, 1968; INTESTINAL MICROFLORA, 1970; THYMIC HORMONES, 1972; INTESTINAL MICROECOLOGY, 1972.

PRODUCER-DIRECTOR - Educational Movies:

THE USE AND CARE OF THE ANALYTICAL BALANCE, McGraw-Hill Co., NITROGEN DETERMINATION.

X-A

BALAKRISHNA VENUGOPAL

1009 Locust
Columbia, Missouri
65201
314- 442-3900

Biochemistry
University of Missouri
Columbia, Mo, 65201.
314- 882-8795

Education:

Ph.D., Food Science and Nutrition Major, Biochemistry minor - University of Missouri - 1970.

M.S Biochemistry and Nutrition Majors - 1959

M.A. Chemistry Major, Physics minor - 1954

B.S. Chemistry major, Biology minor - 1947 (all from Madras University, India)

Ph.D Thesis Research:

Meat Tenderness - Physico-chemical properties of porcine leukocyte lysosomal hydrolases - their involvement in tenderization.

Employment History:

Assistant Professor Chemistry, Madras Veterinary College India 1949 -1952

Associate Professor and Chairman, Chemistry and Biochemistry
Madras Veterinary College, India 1954 - 1963
1970 - 1971

Research Assistant, Food Science Dept., Univ. of Missouri 1966 - 1970

Research Associate, Biochemistry Dept., Univ. of Missouri 1971 till date

Chief Biochemist and Quality control Officer, Veterinary Biological Products Co., Ranipet, India 1963 -1965

Professional Bodies and Honor Societies:

Member: American Association for Advancement of Science.
American Chemical Society
Institute of Food Technologists
American Meat Science Association
American Society of Animal Science

Associate: Royal Institute of Chemistry, London.

Fellow: Royal Chemical Society, London
Association of Food Scientists and Technologists, India.

Member: Sigma Xi and Gamma Sigma Delta.

Bio-data:

Born: [redacted] at [redacted].
Height: 5' 7½"
Weight: 186 lbs.
Status: Single.
Visa: Immigrant

X-A

CURRICULUM VITA

Name: David Paul Hutcheson

Social Security Number: [redacted]

Address: 2604 Violet Court

Home Phone Number: 445-7610

Born: [redacted]

Wife: Peggy Sue Hutcheson

Children: John Paul --- May 2, 1967
Sherry Lynn --- June 14, 1969

Degrees: Ph.D. --- University of Missouri --- 1970 --- Animal Nutrition
M.S. --- University of Missouri --- 1967 --- Animal Nutrition
B.S. --- Texas A & M --- 1963 --- Animal Science

Professional Organizations:

Sigma Xi
Gamma Sigma Delta
American Society of Animal Science
American Society of Veterinary Physiologist and Pharmacologist

Honors, Awards, Special Recognitions, Etc:

1972 --- Gamma Sigma Delta Junior Faculty Award of Merit

Previous Employment:

1969-1973; Biostatistician Sinclair Comparative Medicine Research Farm and Assistant Professor Veterinary Physiology and Pharmacology; University of Missouri

1963-1969; Research Assistant of Animal Husbandry; University of Missouri

Present Position:

1973-present; Biostatistician Sinclair Comparative Medicine Research Farm and Associate Professor of Veterinary Physiology and Pharmacology

Dissertations:

Stability and early physiological effects of diethylstilbestrol. M.S. Thesis, University of Missouri, Columbia, MO.

Stability of diethylstilbestrol and its effects on serum growth hormone, plasma urea nitrogen and performance in lambs. Ph.D. Thesis, University of Missouri, Columbia, MO.

Committees:

Interdisciplinary Nutrition (Ch 1971 to present) Columbia Campus
Radioisotope University Wide Safety Sub Committee (1971 to present)
Computer Committee (1970 to 1972) Veterinary School

X-B GUIDELINES AND POLICY

COMMITTEE FOR RESEARCH INVOLVING HUMAN SUBJECTS

January 30, 1975

The following is submitted to acquaint you and your faculty with the current guidelines and policy of the University of Missouri-Columbia Medical Center Committee for Research (Projects) Involving Human Subjects. (CRIHS) This committee functions in accordance with the policy of our parent institution, the University of Missouri, and the United States Department of Health, Education and Welfare.

University policy states "It shall be policy of the University that all research projects which involve human experimentation shall be subject to review and approval by an appropriate, officially appointed human experimentation committee prior to project initiation, and without respect to the source of funding or sponsorship. This policy shall include all faculty, staff and student research regardless of source of support."

University policy further states "human experimentation is defined as being any research which may put an individual at risk or who may be exposed to physical, psychological, sociological or other harm as a result of participation in a research project."

The Department of Health, Education and Welfare Rules and Regulations (Federal Register May 30, 1974) includes in their policy statement the reviewing committee shall determine whether the subject will be placed at risk and, if risk is involved, whether:

> "The risks to the subject are so outweighed by the sum of the benefit to the subject and the importance of the knowledge to be gained as to warrant a decision to allow the subject to accept these risks;

"the rights and welfare of any such subject will be adequately protected; legally effective informed consent will be obtained by adequate and appropriate methods."

"Subject at risk" is defined as any individual who may be exposed to the possibility of injury, including physical, psychological or social injury, as a result of participation as a subject.

DHEW further defines subject at risk as "any individual who might be exposed to the possibility of harm (physical, psychological, sociological or other) as a consequent of participation as a subject in any research, development or demonstration activity . . . which goes beyond the application of established and accepted methods necessary to meet his needs."

DHEW defines clinical research as "an investigation involving the biological, behavioral, or psychological study of a person, his body or his surroundings. This includes, but is not limited to any medical or surgical procedure, any withdrawal or removal of body tissue or fluid, any administration of a chemical substance, any deviation from normal diet or daily regimen, and any manipulation or observation of bodily processes, behavior or environment."

Clinical research comprises four categories of activity:

1. Studies which conform to established and accepted medical practice with respect to diagnosis or treatment of an illness.

2. Studies which represent a deviation from accepted practice, but which are specifically aimed at improved diagnosis, prevention or treatment of a specific illness in a patient.

3. Studies which are related to a patient's disease but from which he or she will not necessarily receive any direct benefit.

4. Investigative, non-therapeutic research in which there is no intent or expectation of treating an illness from which the patient is suffering, or in which the subject is a "normal control" who is not suffering from an illness but who volunteers to participate for the potential benefit of others.

The University of Missouri-Columbia Medical Center's Committee for Research Involving Human Subjects basically concerns itself to the assurance that the rights of (1) the subject, (2) the investigator and (3) the institution are, in fact, protected and not placed at undue risk. We do not evaluate protocols submitted to us for review for scientific merit. We perceive that to be the responsibility of the investigator, the departmental research committee, and the departmental chairman, as well as (when appropriate) the Clinical Research Center. If the protocol involves the use of radioisotopes, the protocol must be reviewed and approved by a Columbia Campus radiation safety committee prior to submission to the CRIHS.

N.B. This Committee and the University of Missouri, as well as the Department of Health, Education and Welfare consider children, prisoners and the mentally infirm vulnerable subjects because they have limited capacity to consent to their involvement in experiments. Protocols that would involve these individuals should be explicit as to the risks and/or benefits and the method of obtaining an informed consent, as the potential for abuse is higher for these groups.

X APPENDICES

C. BASIC PUBLICATIONS

1) :

MARKERS IN NUTRITION

A. R. KOTB* AND T. D. LUCKEY

DEPARTMENT OF BIOCHEMISTRY, SCHOOL OF MEDICINE, UNIVERSITY OF MISSOURI, COLUMBIA, MO. 65201, USA

CONTENTS

INTRODUCTION

Marker, indicator, tracer, reference substance and index substance are terms applied by workers in nutrition and physiology to a number of materials used in the qualitative or quantitative estimation (usually indirect) of physiological or nutritional phenomena. The diversified use of markers has accumulated much information which merits a thorough review of the subject. The scope of this review is limited to dietary markers, markers which would be put into the diet, which would occur in the food eaten by the animals or which would be taken orally at any time. Excluded from consideration are injected markers and most metabolites which originate within the animals, dyes and radioactive materials used to trace animal movements in the wild state and a great number of substances, radioactive and non-radioactive, used only as qualitative or quantitative indicators in different medical diagnostic techniques.

This review presents a summary of papers important for the development of concepts for the use of markers in nutrition and some characteristics of individual markers with an evaluation of their usefulness in studies of food intake, food passage and food absorption in man and animals. The extensive application of indicators to study the rate of passage of food residues in the digestive tract makes this a major consideration. Consumption and digestibility of forage by grazing animals has been an area of interest and challenge to nutritionists; the indicator technique has contributed greatly to this. Also discussed are techniques of faeces sampling when the marker method is used.

An early use of markers was the study of the rate of passage of food residues, glass beads and small seeds through the digestive tract of animals and man. Today, the use of radioactive nuclides and radioactivation analysis provides added convenience and precision to marker techniques. Studies of digestion, absorption and retention of nutrients were found to be more convenient, less costly and sometimes more precise with the use of suitable indicators than with the conventional method.

Classification of Markers

The markers used for food utilisation or rate of food passage may be grouped in various ways; completeness and clarity suggest the classification given in Table 1. A second grouping is useful from a physiological viewpoint. Theoretically, any substance which can be classified in any of the following groups could be used as a marker in nutrition.

*Present address: Animal Production Department, Ministry of Agriculture, Dokki, Cairo, Egypt, UAR

Reprinted from NUTRITION ABSTRACTS AND REVIEWS, Volume 42, No. 3, pp. 813–845, July 1972

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C. BASIC PUBLICATIONS

2):

CRITICAL REVIEW OF HEAVY METAL TOXICITY IN MAMMALS

by

B. VENUGOPAL and T.D. LUCKEY

NASA Technical Report

Submitted

July 22, 1974

X APPENDICES

C. BASIC PUBLICATIONS

3):

Heavy Metal Toxicity, Safety and Hormology

by
T. D. Luckey, B. Venugopal and D. Hutcheson

12 Figures, 44 Tables

1975
Georg Thieme Publishers, Stuttgart
Academic Press New York · San Francisco · London
A Subsidiary of Harcourt Brace Jovanovich, Publishers