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 ⅖-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

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	1.26	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
19A2	35.2	0.96	44.23	3.98	5.47	17.07	40.31
19A3	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.

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Antigenic and Electrophoretic Changes of α-Arachin after Heating *in Vitro*

The two major protein zones detected in the cold-insoluble fraction of the peanut by electrophoresis consist of subunits of α-arachin. After heating dry for 1 hr, α-arachin showed indistinct electrophoretic and immunologic properties above 145° and was nonantigenic after heating above 175°. Between 110 and 195°, decreased solubility of α-arachin followed an exponential-like curve. After heating the cold-insoluble fraction in solution for 1 hr, immunoprecipitation of α-arachin was partially identical to that of the control at 100° but was completely inhibited when heated above that temperature. These results suggested modifications in the steric arrangements of determinant groups in α-arachin.

The immunological characterization of some of the peanut proteins was reported by Daussant *et al.* (1969). From this study the major protein in the classic arachin fraction of Johns and Jones (1916) was named α-arachin. Analysis by acrylamide electrophoresis showed that the two major protein zones contained in the cold-insoluble fraction of the peanut proteins were protomers of α-arachin (Neucere, 1969, 1972). Previous studies (Neucere *et al.*, 1969; Thomas and Neucere, 1973) showed differential stability of general proteins and enzymes after applying wet and dry heat to whole seeds, and α-arachin was the most stable. The purpose of the present research was to investigate further the effects of wet and dry heat on "isolated" α-arachin as measured by electrophoresis, immunochemistry, and solubility changes.

PROCEDURE

Heat Treatments and Protein Extractions. Preparation of the cold-insoluble fraction from Virginia 56-R peanuts was reported previously (Neucere, 1969). The material used in the present study was the precipitate before ammonium sulfate fractionation (P_2 in above reference). Five samples (freeze dried) of 100 mg each were dissolved in 1.0 ml of phosphate buffer, pH 7.9, ionic strength 0.2, sealed in vials and were heated for 1 hr at 80, 90, 100, 110, 120, 130, 145, and 155°, respectively. Similar samples were dry-heated in open Petri dishes for 1 hr at 110, 120, 130, 145, 155, 175, and 195°, respectively. Each of these was extracted in 1.0 ml of the above buffer for analysis.

Analytical Methods. Protein contents were determined by the method of Lowry *et al.* (1951). Immunoelectrophoresis (IEA) and simple electrophoresis were carried out according to Grabar and Williams (1953) in 1.5% Ionagar gel (Oxoid, Ltd., London) and Agarose (simple electrophoresis). (It is not the policy of the Department to recommend the products of one company over those of any other engaged in the same business.) Electrophoresis was conducted in 0.025 *M* veronal buffer, pH 8.2, with a voltage gradient of 4 V/cm for 2 hr at room temperature. Antibody-in-gel electrophoresis was done according to Laurell (1966) in 1.5% Ionagar employing 10 V/cm for 16 hr at room temperature. Double diffusion was performed according to Ouchterlony (1949). Disk electrophoresis was conducted by the method of Davis (1964) using 7.5% acrylamide in the running gel and 3.0% in the stacking gel; the electrophoresis was performed at a constant current of 3 mA per tube for approximately 1 hr at 5°. All protein zones on Agarose slides, disk gels, and immunoprecipitates were stained with 0.1% Amido Black in 7.0% acetic acid and destained with 7.0% acetic acid.

RESULTS AND DISCUSSION

The immunoelectrophoretic analysis of the dry-heated samples is shown in Figure 1. Up to 130° (sample 4), essentially no precipitin variation from that of the control was observed. However, the minor antigenic protein or the so-called α-arachin contaminant (arrow 1) was inactivated at higher temperatures. At 155°, the precipitin line of α-arachin appeared diffused with a slight anodic shift. Analysis after heating at 175° showed a wide indistinct line that formed a double arc (arrow 2 in sample 7), but all antigenic activity was destroyed at 195°.

From the solubility changes induced by dry heat presented in Figure 2, it is evident that denaturation was not an "all" or "none" process. At 130°, for example, solubility was decreased to one-half, yet some antigenic species of α-arachin remained in solution. The relationship of solubilized protein and temperature is approximately exponential within the range indicated.

Analyses of samples containing equal protein concentrations heated in solution are shown in Figures 3 and 4. Part A of Figure 3 showed the relative changes in electrophoretic migration of the precipitin complexes. At 80°, a slight increase in migration of the conical peaks were observed for both α-arachin (a) and the arachin contaminant (ac). However, at 90° the reverse was observed for α-arachin and the contaminant was completely inactivated; only a trace of activity was observed at 100° and no reaction occurred at 110°. The increase in migration could be due to either an increase in charge of the antigens or a reduction of active determinant groups on the antigens. On