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Applications of Neutron Activation Analysis to the Study of Age-Related Neurological Diseases

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ABSTRACT

Although the etiology and pathogenesis of Alzheimer's disease, Pick's disease, and amyotrophic lateral sclerosis are still unknown, it has been suggested that perturbations in element metabolism may play a role. Even if not causative factors, these imbalances may prove to be markers that could aid in diagnosis. We have employed a sequential neutron activation analysis (NAA) procedure to determine elemental concentrations in brain, hair, fingernails, blood, and cerebrospinal fluid (CSF) of these patients and age-matched controls. Samples are first irradiated with accelerator-produced 14-MeV neutrons for determination of nitrogen and phosphorus, then with reactor thermal neutrons for the instrumental determination of 16–18 minor and trace elements, and, finally, reactor-irradiated again, followed by a rapid radiochemical separation procedure (RNAA) to determine four additional elements. Major advantages of NAA are: (1) its simultaneous multielement capability; (2) the relative freedom from reagent and laboratory contamination; (3) the absence of major matrix effects; and (4) an adequate sensitivity for most elements of interest. Ranges of concentrations by INAA and RNAA in selected control tissues and interelement correlations in control brain are presented to illustrate results obtained by the procedure. Longitudinal studies of tissues from Alzheimer's disease (AD) and amyotrophic lateral sclerosis (ALS) patients are still in progress.

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Index Entries: Trace elements; neutron activation analysis; brain; hair; fingernails; blood; Alzheimer's disease; Pick's disease; amyotrophic lateral sclerosis.

INTRODUCTION

The etiology and pathogenesis of age-related neurological diseases, such as AD, Pick's disease (PD), and ALS, are still not known. Among the hypotheses suggested is a possible role of trace element imbalance or toxicity. Unfortunately, only fragmentary data are available for many trace element concentrations in specific brain regions of patients with these and related neurological diseases. For some elements, values for even bulk control brain concentrations are lacking, and correlation of brain levels with other measures of extracerebral trace-metal status [e.g., analyses of blood, cerebrospinal fluid (CSF), hair, and fingernails] are virtually nonexistant.

Historically, elements implicated in AD have included aluminum (*1–4*), lead (*5–6*), manganese (*7*), silicon (*8–9*), and zinc (*10*). Recent studies, however, have failed to detect abnormal aluminum (*11–14*), manganese (*15*), or zinc (*16*) concentrations in various tissues and/or fluids from AD patients when compared to carefully age-matched controls. Altered brain calcium metabolism has been proposed as a factor in AD, based on observations of an increase in both aluminum and calcium in the cytoplasm of hippocampal neurons bearing neurofibrillary-tangles, in Guamanian patients with parkinsonism-dementia complex (*17*). However, formal study of AD patients has not revealed differences for either calcium or several additional elements in serum and hair, as compared to the patients' healthy spouses (*18*).

Zinc has been implicated in PD (*19,20*), but more recent work in our laboratory failed to disclose any significant differences in brain zinc levels between patients with advanced PD and age-matched controls (*21*). We did observe significant elevations of chlorine, iron, manganese, phosphorus, and sodium, together with significant decreases of cesium, chromium, rubidium, and selenium in PD brains (*21*). Because only 11 brain samples from 2 PD patients were available for study, firm conclusions can not be drawn relative to the importance of these imbalances in the pathogenesis of the disease.

A clinical syndrome resembling ALS has been observed from chronic mercury or lead intoxication (*22–25*). Studies of occupational histories and other epidemiological factors in ALS patients indicate that long-term heavy-metal exposure is more common in these patients compared to hospitalized control patients or normal subjects (*26–27*). In subsequent studies, analyses of spinal cord and brain postmortem have suggested that the levels of aluminum, calcium, lead, and manganese may be increased in ALS, but essential trace-element levels (e.g., copper and zinc) appear unchanged (*28–29*). Evidence has also been reported that

aluminum, calcium, and manganese may have an altered distribution in ALS and be concentrated in the vicinity of blood vessels in the spinal cord (*30–32*).

Many trace-element studies relating to neurological diseases have been restricted to only one, or at best, several elements. In some cases, adequate care has not been taken to prevent trace element contamination, especially in the sample collection process. Most studies have employed analytical techniques (e.g., atomic absorption and inductively coupled plasma emission spectrometry) that are based on atomic properties and require serious attention to matrix effects and interferences.

In this paper, a sequential neutron activation analysis procedure is described for the determination of more than 20 major, minor, and trace elements in brain, hair, fingernails, blood, and CSF. Application of this nuclear technique to the analysis of biological tissues offers the following major advantages: (1) a simultaneous multielement capability, which permits interelement correlation studies on the same tissue sample; (2) relative freedom from reagent and laboratory contamination and blank correction, which are inherent for analytical techniques requiring extensive sample processing or dissolution; (3) the absence of significant matrix effects, which permits simultaneous analyses of materials of different types (e.g., brain, hair, blood, bone); and (4) a sensitivity adequate to determine more than 80% of the known essential trace elements and many toxic elements common in the environment. The majority of these determinations are purely instrumental and rapid, which implies a low unit cost when done in quantity.

The principal disadvantages of the method are that no information is obtained on the chemical state of the elements, and it is a bulk analysis technique that does not provide information on cellular or subcellular localization. The NAA is essentially a batch technique, well-suited to simultaneous analyses of many samples. The technique is not often used in clinical applications, in which limited data on only a few samples are required on a short time scale. On-site accessibility to a nuclear reactor is certainly an advantage, but is not a necessity for long-term studies.

The major intrinsic limitations in the use of purely instrumental NAA (INAA) are: (1) primary interference reactions in which a second element produces the same indicator radionuclide as the element of interest; and (2) gamma-ray spectral interferences caused by close-lying lines of two or more radionuclides that cannot be resolved by the detector. Fortunately, primary interference reactions affect relatively few elemental determinations in biological matrices (e.g., a nuclear reaction on phosphorus produces an interference to aluminum determinations in brain). Spectral interferences can often be circumvented by: (1) the use of variable decay times prior to data acquisition in order to take advantage of differences between radionuclide half lives; and (2) a peak ratio correction method. The latter technique is employed when the interfering radionuclide emits several gamma rays with different energies, and a

peak area correction for the energy region of interest can be based on the interference-free peak.

EXPERIMENTAL

Sampling and Preirradiation Treatment

Brain Samples

Brain specimens are taken at the time of autopsy, typically within 2–24 h after death. For comparison, a few biopsy samples were also available in the early stages of our study. Specimens are normally taken from defined anatomic regions: the anterior and middle frontal lobes, anterior and middle temporal lobes, hippocampus, amygdala, superior parietal lobule, posterior occipital lobe, putamen, caudate, globus pallidus, thalamus, substantia nigra, cerebellar vermis, and cerebellar hemisphere. Approximately 75% of all brain specimens analyzed in our earlier published studies (*12,15,16,33,34*) were from the cerebral cortex. For these studies, cerebral and cerebellar cortical sections were selected to contain, by visual estimate, 50 ± 10% proportions of gray and white matter. More recently, separated gray and white matter samples have been analyzed.

After removal of the leptomeninges with Teflon instruments, brain samples are taken with high-purity, synthetic, quartz knives. The sections are pressed gently between sheets of Whatman 541 hardened, ashless filter paper to remove any surface fluids and placed in preweighed Ziploc polyethylene bags. Roughly, adjacent sections are taken for histologic evaluation. The specimens are maintained at −70°C until they are prepared for analysis.

The brain samples are diced several times with a quartz knife during freeze-drying to speed attainment of constant weight. The freeze-dried to wet-weight ratios (FD/WET) are determined for each individual sample and used to convert all our data on freeze-dried samples to a wet (fresh) weight basis. The FD/WET ratios have been determined to be 0.211 ± 0.002 for adult controls, 0.201 ± 0.003 for AD patients, 0.190 for PD patients, 0.232 ± 0.040 for ALS patients, and 0.124 ± 0.005 for infants. Freeze-dried brain sample sizes taken for INAA are typically 10–15 mg for the long irradiations and 100–250 mg for the short irradiations.

Hair and Fingernail Samples

Hair is initially cut close to the scalp at the nape of the neck and stored in virgin polyethylene vials. Prior to washing, the hair samples are cut into short pieces with surgical stainless-steel scissors used only for this purpose. Fingernails from both hands are also cut with surgical scissors and stored in similar vials.

Both hair and nail samples are washed after collection according to the International Atomic Energy Agency (IAEA) recommended proce-

dure (35). The samples are washed in the same vials in which they are stored. Fifteen milliliters of Distilled-in-Glass acetone are added to each vial, and the vials are agitated for 10 min on a mechanical shaker. This wash is discarded, and 15 mL of deionized–distilled water are added to the samples, which are again agitated for 10 min. This process is repeated for two more water washes and one final acetone wash. After washing, the hair and fingernails are placed in a dust-free enclosure and allowed to air-dry for 24–48 h. Nails are treated in a manner similar to hair, except that they are scraped with a quartz knife before washing to remove external dirt.

We have tested a series of alternate washing procedures and find no advantage over the IAEA procedure. It should be realized that the IAEA procedure does deplete alkali metals and halogens, especially in hair, but most protein-bound trace-element concentrations are little altered. Rivlin (36) has given a good review of experimental problems associated with hair analysis and in interpretation of trace element data on hair. These problems have been addressed in the development of our procedures.

Blood

Blood is allowed to flow through an in-dwelling, intravenous Teflon catheter into acid-washed polyethylene vials. The first 5 mL of blood are not collected to minimize any contamination that might be introduced during catheter insertion. Specimens of the blood are allowed to clot and are then centrifuged to separate the formed elements of blood ("cells") from the serum. Samples of serum and clot are freeze-dried separately for analysis. The FD/WET ratios for blood cells and serum average 0.295 and 0.095, respectively.

Cerebrospinal Fluid

Lumbar CSF is sampled through a plastic catheter inserted with a Touhy needle, which is then withdrawn. The initial 5 mL of CSF is discarded. Normally, 5–10 mL are collected for this work and immediately frozen and stored at −70°C. The samples are freeze-dried for 24 h prior to analysis. The FD/WET ratios have been determined to be approximately 0.011.

All of the above samples are handled in dust-free enclosures whenever possible, using talc-free polyethylene gloves. All containers and implements coming in contact with the samples are constructed of virgin polyethylene or quartz and are prewashed with Ultrex nitric acid (J. T. Baker), deionized–distilled water and Distilled-in-Glass methyl alcohol (Burdick & Jackson).

Irradiation and INAA Counting Procedures

The 14-MeV INAA determinations of phosphorus and nitrogen make use of the Kaman A-711 high-yield neutron generator at the University of Kentucky. The analytical nuclear reactions are $^{31}P(n,alpha)^{28}A1$

and $^{14}N(n,2n)^{13}N$. Counting is done with two 12.7 × 12.7 cm NaI(T1) scintillation detectors arranged at 180°.

Following the 14-MeV INAA determinations, the polyethylene-encapsulated samples are subjected to short, thermal neutron irradiations (5–15 s) using a nuclear reactor rabbit facility. Reactor irradiations are usually done at the Missouri Universities Research Reactor (MURR), Columbia, MO. Quantitation is based on simultaneous irradiation of National Bureau of Standards biological standard reference materials (SRMs) and comparison of the gamma-ray intensities of the irradiation-induced indicator radionuclides in the samples and standards. The short-lived radionuclides produced are counted at the reactor site using a 15% efficiency Ge(Li) gamma-ray detector. Elements normally determined in the short irradiations include aluminum, chlorine, iodine, manganese, sulfur, and vanadium. Other elements, such as calcium, copper, potassium, and sodium, may also be determined in some matrices at this time or later, following the long irradiation.

For the long thermal neutron reactor irradiations, aliquants of all types of samples are taken from the short irradiation vials, or the original sample containers, and heat-sealed in preweighed Suprasil synthetic quartz vials. Typically, 10–30 mg of sample are used. Samples and SRM comparator standards are irradiated at a thermal neutron flux density of 1.3–3.5 × 10^{13} neutrons/cm^2/s for periods up to 40 h. Samples are returned to our laboratory for counting with a 14% efficiency intrinsic Ge detector, which has full width at half maximum (FWHM) of 1.8 keV at ^{60}Co and is coupled to a Nuclear Data ND 680 pulse-height analyzer with peak search and data resolution software. The elements antimony, arsenic, bromine, calcium, cesium, chromium, cobalt, gold, iron, mercury, potassium, rubidium, scandium, selenium, silver, sodium, and zinc are normally determined in this irradiation. Not all the elements listed above for either the short or long irradiations are detected in each type of tissue.

Radiochemical Separations

Following the 14-MeV and reactor thermal neutron INAA determinations, arsenic cadmium, copper, and molybdenum are sequentially determined in the same samples by use of a second, long (30–40 h) reactor thermal neutron irradiation and a simple radiochemical separation procedure (*37*). This RNAA procedure involves dissolution of the reirradiated samples in a mixture of concentrated HNO_3 and H_2SO_4, with addition of carriers for each element to be determined. Quantitative extraction of ^{64}Cu, the ^{115}Cd—^{115m}In equilibrium pair and ^{99}Mo from the 0.5*M* HCl medium is accomplished with 0.048*M* diethyldithiocarbamate (DDTC) in chloroform. Arsenic is absorbed quantitatively from the residual aqueous phase (adjusted with 1.6*M* HCl–0.3*M* HF) on a tin dioxide ion exchange column. The separated phases are counted with an intrinsic Ge detector coupled to a multichannel analyzer. Decontamination factors from major

spectral interference elements (antimony, bromine, phosphorus, potassium, and sodium) are generally greater than 1000×.

Interferences

A classic example of a primary interference reaction is the $^{31}P(n, alpha)^{28}Al$ reaction, which interferes with the determination of aluminum in brain and other tissues by the $^{27}Al(n, gamma)^{28}Al$ reaction. This problem was addressed by first determining the phosphorus content of each sample by 14-Mev INAA, and then determining the ^{28}Al contribution from phosphorus by irradiating phosphorus standards along with the samples for reactor aluminum analyses (*12,38*). The phosphorus corrections in brain can be as high as 30–50% of the gross ^{28}Al activity observed. For INAA aluminum determinations in biological tissues to be practical, short, high flux density irradiations, a high-efficiency counting system, and precise determinations of phosphorus in the same sample are required.

The only other significant interference encountered in this study is a selenium spectral interference to the analytical peak used for the determination of mercury. The only useful photopeak of ^{203}Hg at 279 keV suffers interference from the 280-keV gamma ray emitted by ^{75}Se. Fortunately, the selenium indicator radionuclide has several intense gamma-rays (265 and 280 keV) and the interference can be easily resolved by irradiation of a pure selenium standard and application of the peak ratio method. The counts recorded in the 265-keV selenium peak are multiplied by the experimental peak ratio factor of 0.406 for selenium and then subtracted from the counts in the composite peak at 279–280 keV to yield the net ^{203}Hg counts. Cornelis et al. (*39*) have reviewed other potential interferences in the NAA of biological materials [e.g., the $^{54}Fe(n,alpha)^{51}Cr$ interference to the determination of chromium via the $^{50}Cr(n,gamma)^{51}Cr$ reaction]. Other interferences reported by Cornelis et al. do not exceed 2%, relative, for the data reported in this study.

RESULTS

In general, the precision of our INAA analyses is in the range of ±5% at the μg/g level and ±5–10% at the ng/g level, based on analyses of replicate samples. Agreement of our INAA data for international biological standards with literature or certified values is excellent (*33*).

Ranges of elemental abundances we have obtained using the sequential INAA–RNAA procedure are given in Figs. 1–3 for adult (age > 18 yr) control brain, hair, fingernail and blood samples, respectively. For some elements (e.g., iodine, sulfur, vanadium) only a few data are currently available, and no ranges are presented here. All values are ex-

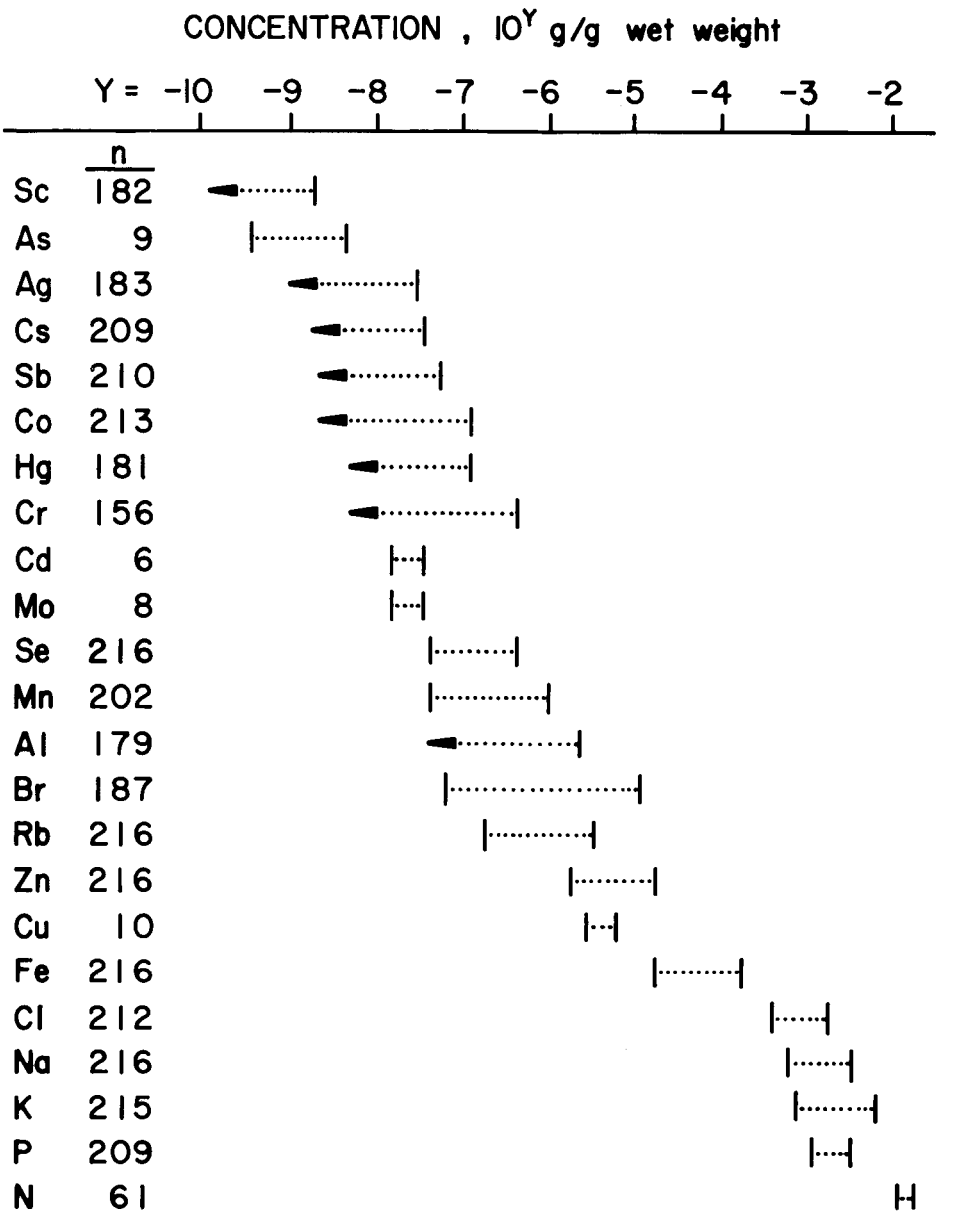

Fig. 1. Ranges of brain trace-element concentrations observed for adult controls using sequential INAA and RNAA (<--- = detection limit, *n* = samples analyzed, wet weight basis).

pressed on a "fresh," or wet-weight basis. Conversions to a freeze-dried weight basis may be made by use of the FD/WET ratios listed previously.

Our data for aluminum, manganese, and zinc in AD and age-matched control brains have been recently published (*12,15,16*). No significant differences between AD and age-matched control brain concentrations for these elements were observed. It should be remembered that NAA is a bulk sample technique, and cellular or highly localized imbalances would be difficult to detect in larger samples. Our results do

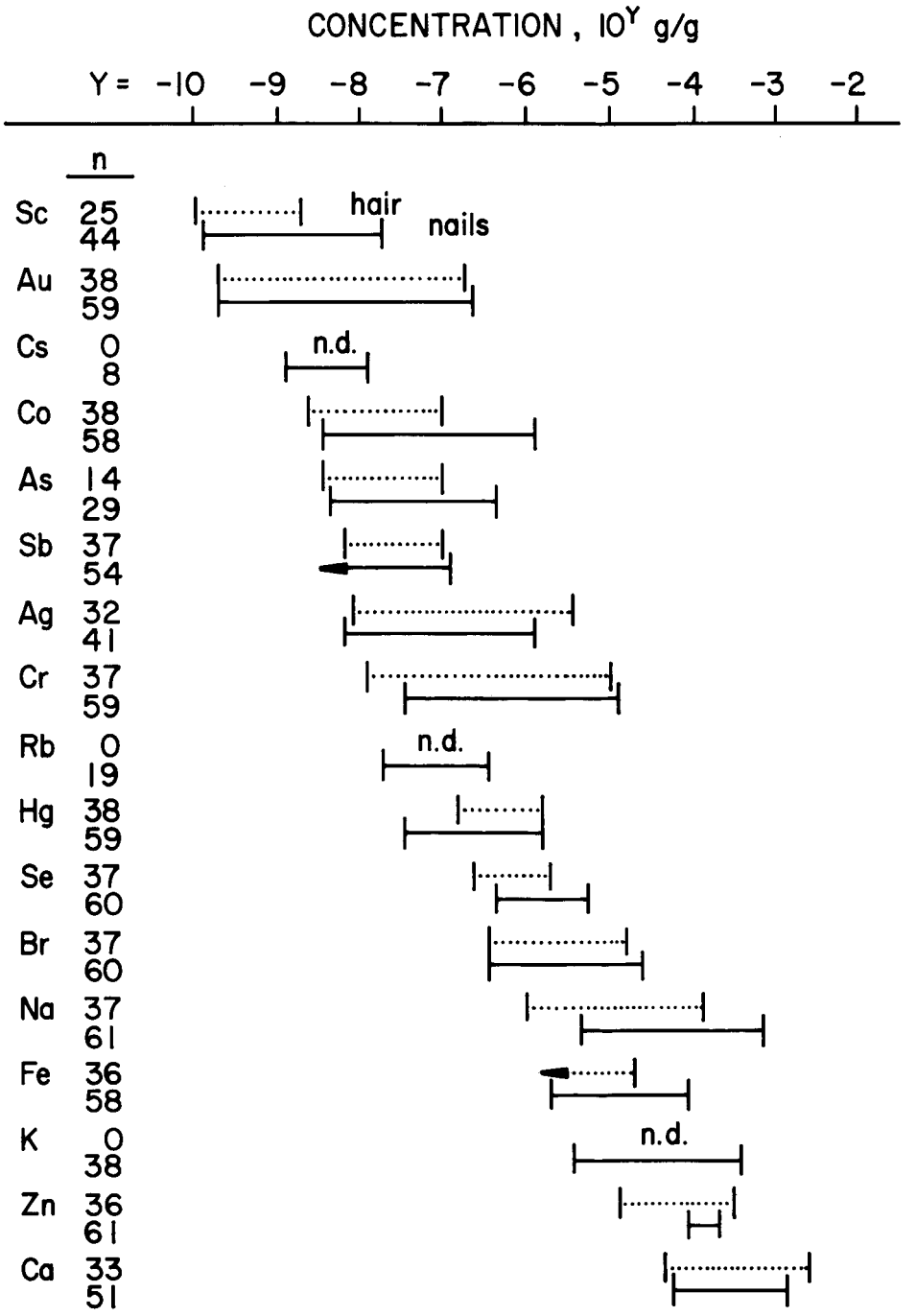

Fig. 2. Ranges of hair and fingernail trace element concentrations for carefully selected adult controls after washing according to the IAEA recommended procedure.

indicate that no significant imbalances in AD bulk brain burdens exist for these elements. We have observed, however, other significant disease–control brain trace-element imbalances in both AD and ALS. These results will be published elsewhere. As noted previously, we have also ob-

served significant imbalances for cesium, chlorine, chromium, iron, manganese, phosphorus, rubidium, selenium, and sodium in PD brains as compared to age-matched controls (*21*).

Analyses of various body tissues and fluids from ALS patients and hair and fingernails from AD patients are currently in progress. Both the AD and ALS studies are longitudinal. Samples of hair and fingernails (and in the case of ALS, various body fluids) are obtained several times each year from patients and their spouses who participate in support groups at the Sanders Brown Research Center on Aging at the University of Kentucky. Whenever possible, these generalized, body-level, trace-element determinations are followed by analyses of autopsy material (e.g., brain, spinal cord, CSF).

DISCUSSION

The concentration ranges in Figs. 1–3 illustrate the sensitivity and applicability of our NAA approach for a number of biologically important elements in several matrices that we have analyzed extensively, to date.

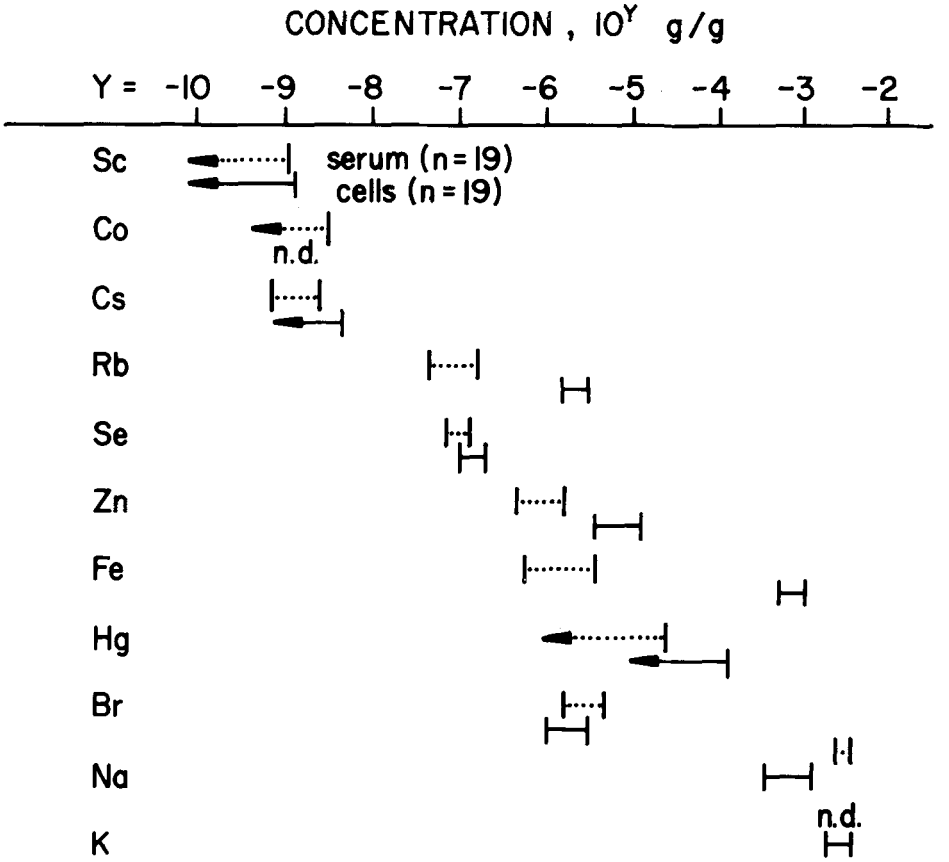

Fig. 3. Ranges of blood clot (cells) and serum trace-element concentrations for adult controls (wet weight basis). Data for additional elements are still being collected.

Only fragmentary data for a number of other elements have been obtained to date and their ranges are not presented here. Concentration ranges we observe in diseased tissues generally do not differ appreciably from those for aged-matched controls. It is only when analysis of variance tests for differences between means are applied to large data sets that significant differences in control and diseased tissues are observed.

Data we have obtained from hair and fingernail samples confirm observations by others that a consistent preanalysis washing procedure must be followed, since the wash conditions may have a great influence on trace element levels. The history of the sample must be carefully examined, since we have observed that commercial hair treatments can radically alter trace element levels (e.g., use of selenium-containing shampoos). We have observed significant differences in hair trace-element levels between age-matched control males and females and between young and old individuals of the same sex. Similar observations have been reported by Takeuchi et al. (*40*). Other factors reported to affect hair trace-element levels include environmental exposure (*41*), pregnancy (*42*), and disease state (*43*). As may be noted in Fig. 2, interpretation of hair and fingernail analyses is already difficult because of the wide ranges of concentrations observed in carefully selected age-matched controls. Careful sample selection and close attention to sample pretreatment are required to obtain meaningful data.

An important advantage of our simultaneous multielement NAA approach is well illustrated by its potential to provide interelement correlation information without sampling bias. Correlations for adult control brains are illustrated in Table 1. Positive correlations observed in diseased tissues, but not in controls, might aid in identification of environmental sources affecting tissue levels in the diseased patient. Negative correlations, such as that indicated for mercury and chromium in Table 1, may suggest an antagonistic relation. Although relatively few interelement correlation studies in brain have been reported in the literature, it should be noted that the positive selenium–iron and rubidium–zinc correlations observed in this work have also been observed by Hock et al. (*44*). Significant variation of trace element concentrations with age in controls, such as we have observed for brain aluminum levels (*12*), reinforce the need for careful age-matching in disease–control comparisons.

As mentioned previously, one potential deficiency of NAA is that it is essentially a "bulk sample" analytical technique. To minimize irradiation-induced pressure buildup in the sealed quartz vials used in the long irradiations, it is necessary to remove moisture by freeze-drying the samples prior to irradiation. Hence, the fresh weight of the analytical sample may be quite large. Ordinarily, samples of at least several milligrams are used in the irradiations, and the comparator standards are selected to have a similar matrix and size. Many of the multielement biological SRMs available from the National Bureau of Standards and other international standards are not certified to be homogeneous at the <20-mg sample-size level. This problem is resolved by including many

TABLE 1
Interelement Pearson Correlations in Adult Control Brains

Primary variable	Secondary variables: Significant correlations ($p < 0.05$)[a]
Ag	Co(+), Fe(+), Hg(+), Na(−), Sb(−), Sc(+), Zn(+), Age(−), FD/WET(+)
Al	Mn(+), Age(+)
Br	Sb(+)
Cl	Cr(−), Fe(−), Na(+), Rb(−), Age(+)
Co	Ag(+), P(+)
Cr	Cl(−), Hg(−), Na(−), Rb(+), Sb(+), Zn(−)
Cs	Sc(−)
Fe	Ag(+), Cl(−), Mn(+), Rb(+), Sc(+), Se(+), FD/WET(+)
Hg	Ag(+), Cr(−), P(+)
K	Rb(+), Zn(+), Age(−)
Mn	Al(+), Fe(+), Sc(+), Zn(+)
Na	Ag(−), Cl(+), Cr(−), Rb(−), Age(+), FD/WET(−)
P	Co(+), Hg(+), Sc(+), Se(+), Age(−), FD/WET(+)
Rb	Cl(−), Cr(+), Fe(+), K(+), Na(−), Zn(+), Age(−), FD/WET(+)
Sb	Ag(−), Br(+), Cr(+)
Sc	Ag(+), Cs(−), Fe(+), Mn(+), P(+)
Se	Fe(+), P(+), FD/WET(+)
Zn	Ag(+), Cr(−), K(+), Mn(+), Rb(+)

[a](+) = direct; (−) = inverse.

small standards in each irradiation unit and using their mean specific activity in computation of the unknown sample concentrations.

The size of the analytical sample is not important in studies of body fluids, but sample size can be a problem in tissue analyses. The use of relatively large tissue samples, especially in the short INAA irradiations, as contrasted with smaller samples used in other techniques, may explain some of the conflicting reports in the literature on trace element relationships to AD. Localized trace-element imbalances resulting from elemental redistributions or sporadic variations at the cellular or subcellular level might not be observed at the bulk sample level. Larger analytical samples, such as those used in NAA, are more appropriate for studies relating to generalized trace-element body burdens and for identifying elements of interest for the application of different methodologies. Clearly, the complementary application of both bulk sample and regional or microprobe techniques will be required to firmly establish any relation that may exist between trace elements and neurological diseases.

SUMMARY

A sequential-irradiation neutron activation analysis procedure has been developed, which permits the determination of more than 20 elements in biological materials. The procedure has been applied with success to the analysis of brain, hair, fingernails, blood cells (clots), blood serum, and CSF in longitudinal studies relating to the possible role of trace element deficiency/toxicity in the etiology and pathogenesis of several age-related neurological diseases.

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