0163-4984/93/3701–0027 $02.40

Essential Trace Elements in Humans

Serum Arsenic Concentrations in Hemodialysis Patients in Comparison to Healthy Controls

DANIEL R. MAYER,*[,1] WALTER KOSMUS,[1] HELMUT POGGLITSCH,[2] DAVID MAYER,[3] AND WOLFGANG BEYER[4]

[1]Institute for Analytical Chemistry, Karl-Franzens-University Graz, Universitätsplatz 1, 8010 Graz; [2]Department of Nephrology, Karl-Franzens-University Graz, Medical Center, Auenbruggerplatz 11, 8036 Graz; [3]Merkur Sanatorium, Eckerstraße 98, 8020 Graz; and [4]Institute for Medical Chemistry and Pregl Laboratory, Karl-Franzens-University Graz, Harrachgasse 21, 8010 Graz, Austria

Received August 5, 1992; Accepted August 21, 1992

ABSTRACT

Serum arsenic concentrations of persons suffering from renal failure and undergoing hemodialysis treatment (n = 85) and of healthy controls (n = 25) were determined by hydride-generation AAS technique after microwave digestion. The results were evaluated by comparing the values of both groups, considering physiological factors and individual data, as well as comorbid conditions of the hemodialysis (HD) patients. Serum arsenic levels were diminished in the patient group compared with controls (mean values 8.5 ± 1.8 ng/mL vs 10.6 ± 1.3 ng/mL). Furthermore, additional diseases within the hemodialysis group, particularly injuries of the central nervous system (CNS), vascular diseases, and cancer, were correlated to occasionally markedly decreased serum arsenic concentrations. It was concluded that arsenic homeostasis is disturbed by HD treatment and certain additional diseases. Desirable arsenic concentrations in the body seem to be reasonable. This consideration results in the conclusion that arsenic could play an essential role in human health. Thus,

*Author to whom all correspondence and reprint requests should be addressed.

reference arsenic concentrations in different human tissues and body fluids should be established in order to recognize not only arsenic intoxication, but also arsenic deficiency. Perhaps arsenic deficiency contributes to the increased death risk of HD patients, and therefore, arsenic supplementations for patients with extremely low serum arsenic concentrations should be taken into account.

Index Entries: Arsenic; hemodialysis; serum; microwave digestion; hydride-generation atomic absorption spectrometry; essential trace elements.

INTRODUCTION

Biological and medical trace element research has progressed constantly in recent years, yielding an enormous abundance of data (*1*,*2*). To clear up the influence of environment and nutrition on the human body, and for clinical diagnosis of trace element intoxication or deficiency, all kinds of human tissues and body fluids have been analyzed. "Normal values" have been estimated for each essential trace element. However, it is rarely discussed whether these values are desirable or are only the result of the element occurrence in the environment or food—which may be the same sometimes! Nevertheless, "normal" or "reference" values, which we define as desirable or recommended trace element concentrations in certain tissues of healthy individuals, are necessary to verify variations in elemental concentrations, resulting from physiological or pathological changes and environmental influences. In fact, various diseases, like cancer, may lead to or arise from trace element disorders. On the other side, abnormalities in the metabolism of certain trace elements are involved in the pathogenesis of human diseases, like Menke's (steely hair syndrome) and Wilson's (hepatolenticular degeneration). However, in most cases, it cannot be stated whether trace element imbalances are the cause of a certain disease or vice versa.

This brings us to an important consideration in the discussion of what an essential trace element is: If the body status of an element is disturbed as a consequence of a disease, it seems reasonable that there exists a need for this element in the organism in order to preserve its health. Consequently, the deficiency of a certain element should allow to infer that this element is essential for life. We suggest that this criterion be added to the definition of "essentiality" (*3*). In the following, we will proceed according to this consideration in order to answer the question of a potential essentiality of the ultratrace element arsenic for humans.

Risks of Hemodialysis Treatment

One group that is very strongly affected by trace element alterations and additionally suffering from numerous diseases is patients with chronic renal failure, undergoing hemodialysis treatment (*4*–*7*). Hayashi

(*4*) found decreased concentrations of Fe, Ni, Co, Cd, and Pb in hair of hemodialyzed persons, whereas the levels of Se, Sn, Mo, and Cr were increased, and only Na, K, Ca, P, and Mg values were adequate compared to healthy controls. Other authors observed decreased serum concentrations of Se (*8,9*), Zn, and Mg (*9*), and elevated Cu levels in patients with chronic renal failure (*9*). Marumo et al. (*5*) found As, V, Al, Zn, Mn, Cu, Mg, Ca, and Br concentrations in hair, plasma Al levels, and As, Mn, and Cu concentrations in fingernails to be higher in hemodialyzed persons compared with healthy individuals, and significantly lowered plasma Zn values in the HD group. In addition to other factors, for instance, comorbid conditions, like atherosclerotic heart disease, high blood pressure, or diabetes mellitus, trace element alterations are an important factor in the increased death rate of HD patients (*10–15*). Thus, a considerable number of reports discuss the reasons for the trace element alterations and the influence of hemodialysis treatment on the trace element status (*8,9,16–18*). The possible causes of trace element disorders of uremic patients may be failure of renal excretion as a consequence of the kidney disease, loss of certain elements across the dialysis membrane, and contamination of dialyzates with trace elements.

Most of the publications dealing with the clinical aspects of trace element alterations in HD patients and the effects on human health caused by these alterations are related to a few elements, namely Cu, Zn, Mn, Pb, Al, Pt, Cr, Cd, and Se (*19*). However, important data about others, particularly arsenic, are still missing, although some literature data suggest that the body store of arsenic may be changed in persons with chronic renal failure (*10*).

Arsenic, an Essential Trace Element?

Arsenic is primarily branded as highly toxic for humans. So far, only allowable concentrations in drinking water, food, or soil have been established by several agencies. However, since experiments of Anke et al. (*20*), demonstrating on goats and minipigs that there exist arsenic requirements for animals, evidence has been accumulating that arsenic could play an essential role in life processes. Furthermore, as early as 1966 (*41*) and even before, effects of arsenic on the healthy appearance of skin and hair of mice, rats, and horses were well known. It was also discussed then that arsenic may aid hemoglobin production, promote growth in tissue cultures, and stimulate the growth of plants. Recent data indicate that it may be essential for the organism, and therefore a deficiency syndrome may exist (*21,22*). A requirement of 50 ng of arsenic/g of diet for animals has been proposed (*23*). Arsenic is also famous for its interactions with selenium (*24,25*), for instance, sodium arsenite proved to be protective against toxic levels of certain forms of inorganic selenium (*26,27*). On the other hand, Obermeyer et al. reported enhanced toxicity of relatively untoxic forms of selenium, when dispensed together with arsenite (*28*).

The beneficial properties of arsenic become even more evident when we take a closer look at its role in biological cycles: It catalyzes glutathione biosynthesis (*29*), is involved in the arginine, membrane phospholipid, and zinc metabolism (*30*), and stimulates the bile excretion. So, since arsenic undoubtedly is important for living organisms, it has become a proposed essential element (*3*). Therefore, it seems reasonable that a certain quantity of arsenic in the human body, supplied with daily food intake, could be desirable, and recommended arsenic concentrations in human tissues and body fluids should be established. Mertz (*31*) extrapolated the requirements for animals to humans and found 25–30 μg to be a minimum daily requirement. Normal serum arsenic concentrations presented in literature show a remarkable diversity between 1 ng/mL and 20 ng/mL (*1,32–34*). In order to contribute to the elucidation of its role in the human body by providing additional data about arsenic, the serum arsenic concentrations of healthy adults and hemodialyzed patients of Styria, a federal state of Austria, were determined. The serum arsenic concentrations were determined and compared with individual data and physiological factors, like sex and weight, as well as comorbid conditions, like diabetes mellitus or cardiomyopathy. Additionally, since the biological functions of arsenic and selenium are connected very closely, for instance, via glutathione and glutathione peroxidase (*24,29*) or their antagonistic interactions, we will complete our studies in a subsequent publication by determining serum selenium concentrations of the same personal groups and comparing them with the results presented in this article.

MATERIALS AND METHODS

Subjects

One hundred and nine individuals participated in this study and were divided into two main groups: group 1: 25 healthy volunteers (13 females and 12 males), and group 2: 84 patients (41 females and 43 males) having been dialyzed between 1 and 18 yr, two or three times a week with treatment durations of 4–7 h. No distinction was made between hemofiltration and hemodialysis treatment.

Both groups were subdivided according to sex, weight, and smoking habits. Furthermore, group 2 was subdivided according to accompanying diseases (*see* Tables 1–4).

Sample Collection

Blood samples were collected by venipuncture with disposable steel needles connected to plastic tubes (Type BL 376 C 00554501, No. 89101680, Bellco P.O., Mirandola, Italy). After coagulation at room temperature, the serum was immediately separated by centrifugation at 3000

Table 1
Serum Arsenic Levels (ng/mL) of Healthy Individuals (Group 1) and HD Patients (Group 2), Expressed as Median M and Lower–Upper Quartile L-U Quartile

Group No.	N^a	M	L-U quartile
I	25	10.6	9.6–11.8
II	84	9.1	7.1–10.1

[a]*N* = Number of Individuals.

Table 2
Sex-Specific Serum Arsenic Concentrations (ng/mL) of Healthy Controls (Group 1) and HD Patients (Group 2)

	Males			Females		
Group No.	*N*	M	L-U quartile	*N*	M	L-U quartile
I	13	11.0	9.2–11.8	12	10.4	9.6–11.3
II	43	9.1	6.9– 9.8	41	9.0	7.6–10.1

rpm for 15 min, lyophilized, and wet-ashed by microwave dissolution as described below.

Equipment and Reagents

Serum arsenic concentrations (ng/mL) were determined after serum dissolution with a modified household microwave oven by hydride-generation atomic absorption spectrometry with a model Hitachi Z-6100, equipped with a lab-constructed hydride system and a hollow cathode arsenic lamp from Cathodeon with recommended conditions of the manufacturer (*35*). Highest-quality analytical reagents (Merck Suprapur) were used throughout the whole experimentation. Working curves were established with arsenic(III) standard (MerckTitrisol). All labware was cleaned by carefully soaking with HNO_3 (subboiled distilled) for approx 10 h, followed by rinsing twice with HNO_3, rinsing three times with deionized H_2O, and drying on a rubber mat.

Digestion Procedure

Freeze-dried serum samples (3 mL) were mineralized by microwave digestion as described previously (*35*). Quality control was carried out by processing five samples of NIST bovine liver (1577a) SRM with the serum analysis and simultaneously performing recovery studies by spiking five samples with 5, 10, 20, 30, and 40 μg arsenic(III). Results are listed below.

Determination of Serum Arsenic Concentrations

Digestion aliquots of 1 mL in 14 mL HCl 3% were used for arsenic determination with the hydride technique as outlined previously (*35*).

Table 3
Serum Arsenic Concentrations (ng/mL) in Relation to Smoking Habits of Healthy Individuals (Group 1) and HD Patients (Group 2)

	Nonsmokers			Smokers		
Group No.	*N*	M	L-U quartile	*N*	M	L-U quartile
I	13	10.4	9.9–11.6	12	10.3	9.1–11.6
II	69	9.1	7.5–10.1	15	8.1	4.3– 9.2

Table 4
Serum Arsenic Concentrations (ng/mL) of HD Patients According to Additional Diseases

Additional disease or diet	*N*	M	L-U quartile
Diabetes mellitus			
Yes	12	8.9	8.7– 9.7
No	72	9.1	6.9–10.1
Hypertension			
Yes	19	8.8	6.6– 9.6
No	65	9.1	7.0–10.1
Liver			
Yes	11	9.1	6.3–10.6
No	73	9.0	7.0–10.1
Cardiomyopathy			
Yes	17	9.0	7.2–10.3
No	67	9.1	6.6– 9.8
Cancer			
Yes	10	7.0	3.3–12.3
No	74	9.1	7.1– 9.9
Vascular diseases			
Yes	11	6.3	3.9– 9.6
No	73	9.1	7.7–10.1
CNS diseases			
Yes	8	4.7	4.4– 5.6
No	76	9.1	7.6–10.1
Diet			
Yes	61	9.0	6.6–10.0
No	23	9.1	7.4–10.0

Three determinations were performed for each sample. Reagent blanks were processed with the samples. The detection limit of the method (3 × standard deviation of the blank) was found at 0.02 ppb. No reduction from the pentavalent to the trivalent state of arsenic was required prior to the determinations, since reduction procedures (*36*) showed no effects on the signal heights. Analytical results were obtained by calculating the mean values of three blank corrected determinations of each sample. The concentrations for NIST bovine liver were found between 43.5 and 50.3 ng/g with certified values of 47 ± ng/g. Recoveries were found within a range of 100 ± 8%. These data demonstrate that the applied digestion

and analysis methods afforded the most reproducible, precise, and reliable results.

Statistical Analysis

Exploratory data analysis was used to exhibit the data graphically. The results are presented as box and whisker plots. Precise values are outlined numerically in tables as medians with lower and upper quartiles. The distributions of serum arsenic concentrations of HD patients and controls are examined as frequency histograms.

RESULTS AND DISCUSSION

Figures 1 and 2 show the distributions of the serum As levels for groups 1 and 2. The numerical statistical data median, lower and upper quartile for controls, HD patients, and subgroups are reported in Tables 1–4. The results are charted as box and whisker plots in Figs. 3 and 4.

Generally, it can be stated that the values of dialysis patients disperse in a markedly larger extent than those of controls, as can be seen from box plots 1 and 2 in Fig. 3. Table 1 reveals that the serum arsenic concentration is decreased by a factor of 20% in the HD group compared with healthy controls. The causes for this effect may be, as described before, failure of renal excretion or loss of arsenic across the dialysis membrane. Furthermore, the results indicate that not only arsenic accumulation (5), but also arsenic deficiency may arise in the body of HD patients. Within both groups, no significant sex-specific variations in serum arsenic concentrations can be observed. Only healthy females appear to have somewhat lower values than healthy males (Table 2 and boxes 3–6 in Fig. 3).

The results concerning the smoking habits are somewhat contrary (Table 3 and boxes 7–10 in Fig. 3). Serum arsenic concentrations of healthy smokers and healthy nonsmokers are quite similar. However, all statistical parameters indicate that among that HD group, nonsmokers appear to have slightly higher serum arsenic concentrations than smokers.

The subclasses according to additional diseases within the HD group demonstrate a wide range of serum arsenic concentrations (Table 4 and Fig. 4). No significant variation can be pointed out in connection with the comorbid conditions diabetes mellitus, hypertension, liver diseases, and cardiomyopathy (boxes 1–8 in Fig. 4). All of them show no detectable effects on the serum arsenic levels, which are quite comparable to those observed for reference individuals within the HD group. Furthermore, the values of HD patients on diet are quite comparable to the others, indicating that no inadequate dietary intake of arsenic seems to occur (boxes 15 and 16 in Fig. 4).

Dramatically decreased serum arsenic concentrations can be observed for subjects suffering from cancer, vascular diseases, and diseases

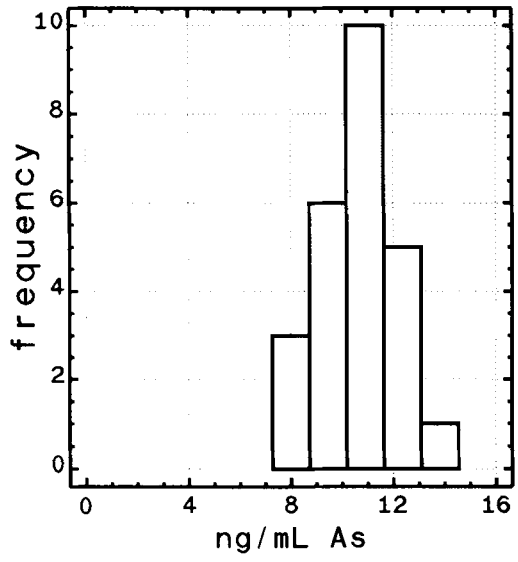

Fig. 1. Serum arsenic value distribution observed in control group (group 1).

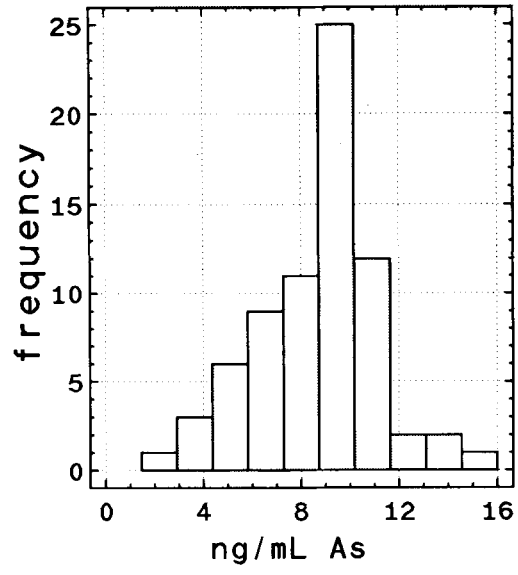

Fig. 2. Serum arsenic value distribution observed in the HD group (group 2).

of the CNS (Table 4 and boxes 9–14 in Figure 4). In spite of the relative low number of individuals being examined, the tendency toward very low serum arsenic concentrations is obviously and, thus, may be universally valid for these collectives of patients. The results suggest that the arsenic body turnover could be affected by some kinds of cancer as it is known from other elements, like selenium (*38–40*). The influence of

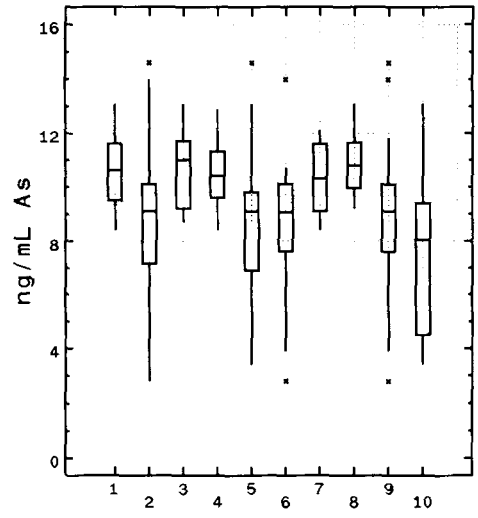

Fig. 3. Box and whisker plots of serum arsenic concentrations of healthy controls and HD patients. Legends to box numbers:
1–group 1, 2–group 2, 3–males group 1, 4–females group 1, 5–males group 2, 6–females group 2, 7–nonsmokers group 1, 8–smokers group 1, 9–nonsmokers group 2, 10–smokers group 2.

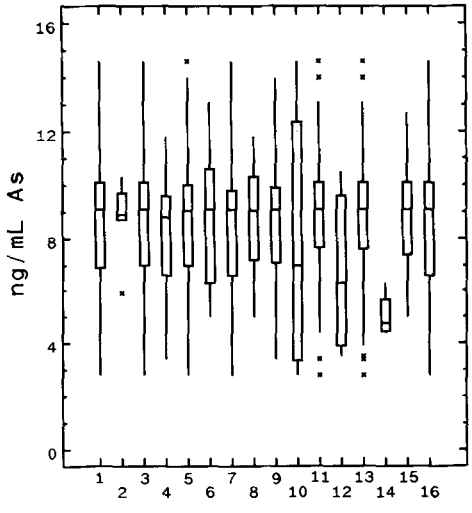

Fig. 4. Box and whisker plots serum arsenic concentrations of HD patients according to additional diseases. Legends to box numbers:
1/2–diabetes no/yes, 3/4–hypertension no/yes, 5/6–liver diseases no/yes, 7/8–cardiomyopathy no/yes, 9/10–cancer no/yes, 11/12–vascular diseases no/yes, 13/14–CNS-diseases no/yes, 15/16–diet no/yes.

vascular diseases on the arsenic status is not explainable for us. The reduced serum arsenic concentrations in HD patients with CNS diseases indicate that arsenic may be involved in the neural and brain metabolism, perhaps acting as cofactor in certain enzymes. In order to verify this hypothesis, much has to be established regarding possible neurochemical functions of arsenic. For instance, the determination of arsenic concentrations in different brain regions, primarily in association with neurological disorders, like Parkinson's disease, or investigations concerning the etiology of neurological disorders relative to arsenic, may help to confirm an essential function of arsenic in the CNS.

CONCLUSIONS

Our results suggest that for the purpose of a complete clinical diagnosis, the trace element status of HD patients should be established. Additionally, we recommend examining the trace element status in the course of preventive medical checkups. This can be achieved by analyzing certain tissues or body fluids, like blood serum. Analytical techniques meeting highest requirements, like Inductively Coupled Plasma Mass Specrometry (ICP-MS), Graphite Furnace Atomic Absorption Spectrometry (GFAAS), Neutron Activation Analysis (NAA), or Hydride-Generation Atomic Absorption Spectrometry (HGAAS), are available for this purpose. These most sophisticated automated methods offer low detection limits and present only small measurement standard deviations. Furthermore, with the exception of HGAAS, they allow simultaneous determinations of numerous elements. Thus, they are excellently suitable to estimate even the smallest variations in body trace element concentrations, which already may affect human health.

Although it is well known that several physiological factors, diseases, and individual habits influence the serum concentrations of several trace elements, the scientific literature is extremely poor concerning information on variations of arsenic concentrations in the human body, relating to these parameters. In our study, we could clearly demonstrate that serum arsenic concentrations are reduced in HD patients and additionally correlate to several comorbid conditions, like injury of the CNS or vascular diseases. As a consequence of the fact that certain diseases may reduce serum arsenic concentrations, we arrive at the conclusion that a homeostatic regulation of the arsenic body turnover seems to be most likely. A homeostatic regulation, however, that keeps the concentration of an element in the body at a constant level and being disturbed in the described way is only conceivable for elements that are essential or at least desirable for the proper function of the human organism. Thus, our results suggest that a minimum requirement of arsenic for humans can be stated.

In consequence of our study we postulate that arsenic should be considered or may be defined to be essential for human life processes.

Desirable normal or reference thresholds for arsenic should be established with accurate and precise methods to determine not only arsenic intoxication, but also arsenic deficiency. For instance, Morisi and Patriarca (*37*) elaborated an excellent concept that allows the estimation of reference values and the confirmation that specific biological parameters, like trace element concentrations, are subjected to variations in ill conditions. In cases where arsenic values are below previously defined recommended concentrations and therefore deficiencies may exist, arsenic supplementations should be taken into account. The required quantities may be calculated on the basis of Mertz's former suggestions (*31*).

Additionally, our results propose that arsenic deficiency could contribute to the increased death risk of people undergoing a hemodialysis treatment, and supplementation of small quantities may help to prolong the life of patients belonging to this most endangered group of humans. Further research work has to be done in order to investigate the influences of physiological and pathological sources on serum arsenic levels. First, they may be of great scientific and clinical importance and, second, they may help to outline and to understand the biological functions of this fascinating element. Finally, these investigations may result in the conclusive evidence that the trace element arsenic has essential importance for humans.

REFERENCES

1. J. Versieck and R. Cornelis, *Trace Elements in Human Plasma or Serum*, CRC Press, FL, 1989.
2. G. V. Iyengar, W. E. Kollmer, and H. J. M. Bowen, *The Elemental Composition of Human Tissues and Body Fluids*, Verlag Chemie, Weinheim, FRG, 1978.
3. E. Frieden, *J. of Chem. Education* **62,** 917 (1985).
4. M. Hayashi, *Tokushima, J. Exp. Med.* **37,** 103 (1990).
5. F. Marumo, Y. Tsukamoto, S. Iwanami, T. Kishimoto, and S. Yamagami, *Nephron* **38,** 267 (1984).
6. N. M. Thomas, B. J. Stevens, T. J. Humphrey, and R. C. Atkins, *Kidney Int.* **33,** 9 (1983).
7. Y. Tsukamato, S. Iwanami, and F. Marumo, *Nephron* **26,** 174 (1980).
8. B. Dworkin, S. Wesely, W. S. Rosenthal, E. M. Schwartz, and L. Weiss, *Am. J. of Med. Sci.* **293/1,** 6 (1987).
9. D. Tsekova, K. Tsachev, I. Gruev, and S. Pina, *Vutr. Boles.* **29/3,** 69 (1990).
10. P. W. Eggers, *Am. J. Kidney Dis.* **15/5,** 414 (1990).
11. T. B.Wiegmann and M. D. Jameson, *Med. Clin. North. Am.* **74/4,** 945 (1990).
12. E. G. Lowrie and N. L. Lew, *Am. J. Kidney Dis.* **15/5,** 458 (1990).
13. A. J. Collins, G. Hanson, and A. Umen, *Am. J. Kidney Dis.* **15/5,** 422 (1990).
14. M. Odaka, *Am. J. Kidney Dis.* **15/5,** 410 (1990).
15. A. Hull, *Nephrol. News Issues* **4/4,** 28 (1990).
16. A. C. Alfrey and W. R. Smythe, *Ann. Contractors Conf. Artif. Kidney Program Natn. Arthritis, Metabol. Digest. Dis.*, National Institute of Health, Bethesda, 1981, p. 110.
17. A. Salvadeo, C. Minoia, S. Segagni, and G. Villa, *Int. J. Artif. Organs* **2,** 17 (1979).

18. A. C. Alfrey and W. R. Smythe, *Proc. 8th Congr. Nephrol.*, Athens, 1982, p. 1007.
19. C. J. Menendez-Botet and M. K. Schwartz, *Anal. Chem.* **63,** 194 (1991).
20. M. Anke, A. Henning, M. Grün, M. Partschefeld, B. Groppel, and H. Lüdke, *Arch. Tierernähr.* **26,** 742 (1976).
21. W. Mertz, *Science* **213,** 1332 (1981).
22. *Arsenic. Environmental Health Criteria* 18. World Health Organization, Geneva, 1981.
23. M. Anke, M. Grün, M. Partschefeld, B. Groppel, and A. Hennig, in *Trace Element Metabolism in Man and Animals,* M. Kirchgessner, ed., Freising-Weihenstephan, 1976, p. 248.
24. M. El-Bagiarmi, H. Ganther, and M. Sunde, *Rev. Toxicol. Environm. Sci.* **8,** 585 (1980).
25. O. A. Levander, *Environ. Health Perspect.* **19,** 159 (1977).
26. I. S. Palmer and C. W. Bonhorst, *Agric. Food Chem.* **5,** 928 (1957).
27. A. L. Moxon and M. A. Rhian, *Physiol. Rev.* **23,** 305 (1943).
28. B. D. Obermeyer, I. S. Palmer, O. E. Olson, and A. W. Halverson, *Toxicol. and Appl. Pharmacol.* **20,** 135 (1971).
29. D. V. Frost, in *Proceedings des 3. Spurenelement Symposiums: Arsen*, M. Anke, H. J. Schneider, and Ch. Brückner, eds., Jena, 1980.
30. F. H. Nielsen and E. O. Uthus, in *Biochemistry of the Essential Trace Elements,* E. Frieden, ed., Plenum, New York, 1984, p. 319.
31. W. Mertz, in *Proceedings des 3. Spurenelement Symposiums: Arsen*, M. Anke, H. J. Schneider, and Ch. Brückner, eds., 1980, p. 14.
32. O. Plantin, *Acta Neurol. Scand.* **70,** 95 (1984).
33. V. Blekstad, J. Jonsen, E. Steinness, and K. Helgeland, *Acta Med. Scand.* **216,** 25 (1984).
34. G. V. Iyengar and J. Woittiez, *Clin. Chem.* **34/3,** 474 (1988).
35. D. Mayer, S. Haubenwallner, W. Kosmus, and W. Beyer, *Anal. Chim. Acta.* **268/2,** 315 (1992).
36. B. Welz and M. Melcher, *Vom Wasser* **59,** 407 (1982).
37. G. Morisi and M. Patriarca, *Acta Chim. Hung.* **128,** 581 (1991).
38. G. Kallistratos, A. Evangelou, K. Seferiadis, P. Vezyraki, and K. Barboutis, *Nephron* **41,** 217 (1985).
39. F. Kiefer, *Ars Medici* **2,** 60 (1987).
40. J. M. D. Malvy and D. Amèdèe-Manesmes, in *Selenium in Medicine and Biology,* J. Néve and A. Favier, eds., Walter de Gruyter, Berlin, 1989, p. 267.
41. H. A. Schroeder and J. J. Balassa, *J. Chron. Dis.*, **19,** 85 (1966).