Identification of Inorganic Elements in Proteins in Human Serum and in DNA Fragments by Size Exclusion Chromatography and Inductively Coupled Plasma Mass Spectrometry with a Magnetic Sector Mass Spectrometer

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Abstract: A general method to identify trace elements in biological molecules is described. These measurements are made in only a few minutes without preliminary isolation and preconcentration steps. Many unusual or difficult elements, such as Cr, Se, Cd, Th, and U, can be observed at ambient levels bound to proteins in human serum. A new protein or family of proteins, that either contain Se or that bind Se molecules, is found with molecular weight ~ 760 kDa. Binding of metal cations to DNA restriction fragments can be studied by similar procedures both for essential elements such as Mn and Fe and toxic ones such as Cd and Pb. In particular, trace Pb, Cd, and Co are completely bound to DNA fragments. Reduction of chromate to a cation (probably Cr^{3+}) and subsequent binding of the Cr cation to DNA is also demonstrated.

Inorganic elements play key roles in the function of many biological molecules.[1] Metal ions such as Zn and Cu contribute to the structure and function of many enzymes, for example. The toxic activity of some trace metals such as Cd and Pb is thought to relate to their ability to compete with essential elements in proteins or to bind to DNA. There has been considerable recent interest in dietary selenium to prevent cancer[2] and delay the onset of AIDS symptoms.[3] Multielement analytical procedures are needed to study interactions between different trace elements. For example, occupational exposure to certain pairs of metals (e.g., Pb and Cu, Pb and Fe, or Fe and Cu) causes a much higher incidence of Parkinson's disease than exposure to any of the individual metals studied,[4] and selenium plays a role in the metabolism of iodine in thyroid hormones.[5]

With modern instrumentation for atomic spectroscopy, the total amount of ultratrace elements in biological specimens can generally be determined on a multielement basis. Preparation of the sample and minimizing contamination, rather than the performance of the instrument per se, are often the limiting steps in the accuracy of the measured concentrations. This is particularly the case with inductively coupled plasma−mass spectrometry (ICP-MS) with a magnetic sector mass analyzer, which has very high sensitivity and sufficient spectral resolution to separate atomic analyte ions from polyatomic ions. For example, detection limits for most elements are 1 ppt or better, and the major isotopes $^{52}Cr^+$ and $^{56}Fe^+$ can readily be separated from the interferences ArC^+ and ArO^+.[6]

This paper describes methodology that combines these recent advances in ICP-MS with chromatographic separations for the study of inorganic ions directly in biological samples. Size exclusion chromatography (SEC) is chosen for these initial experiments because it operates near physiological pH and does not require organic solvents that might denature the biological molecules or otherwise remove the metals of interest. The SEC column is robust and can tolerate repeated injections of a difficult sample such as serum. The ICP-MS measurement then identifies the element(s) present in particular chromatographic peaks. Such combinations of chromatography and ICP-MS are presently considered the preferred general method for the measurement of the molecular forms of trace elements.

SEC has been used previously for ICP-MS with low-resolution mass analyzers.[7] The main new aspects of the present work include (a) improved sensitivity and spectral resolution provided by the magnetic sector MS and (b) further improvements in sensitivity and reduction of spectral interferences via the use of microscale nebulization and solvent removal for

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introducing virtually all of the column effluent into the ICP. The type of information provided by these capabilities is then illustrated for several elements of current interest in proteins and DNA.

Instrumentation. The major instrumental components are (a) the SEC column,[8] (b) the microconcentric nebulizer and desolvation system,[9] (c) the ICP,[10] and (e) the magnetic sector MS.[11] The components and key operating conditions are identified in the appropriate footnotes. The compounds of interest are separated by SEC and injected on-line as discrete bands into the nebulizer. This particular nebulizer is designed for use at low liquid flow rates (typically 30−150 μL min^{-1}); it produces a mist of fine droplets that are efficiently transported out of the spray chamber. These droplets are then dried, and most of the solvent is removed by a condenser. Desolvation has been shown by others to improve ion transmission dramatically for this type of ICP-MS device compared to the sensitivity obtained when the sample is introduced as wet aerosol droplets.[12] The dry aerosol particles from the sample then enter the hot argon ICP where they are converted into atomic ions. These ions are extracted through a molecular-beam sampling system and analyzed by a reverse geometry double focusing mass analyzer.

Two resolution settings were used for the mass analyzer. At low resolution, the peaks are flat-topped with a width of approximately 0.2 mass unit at the base of the peak. The nominal value of $m/\Delta m$ is 300, where Δm is the peak width. At medium resolution ($m/\Delta m = 3000$), the narrower slits and the scanning process reduce the signal to about 10% of that obtainable at low resolution, so medium resolution is used only for elements such as Fe and Cr that suffer from spectral interference from polyatomic ions.[13]

SEC Separations. The retention behavior of the SEC column is calibrated by analyzing synthetic samples of pure proteins known to contain particular elements. Large proteins of high molecular weight are not retained and elute first, followed by smaller proteins; compounds with low molecular weights elute last. The calibration proteins, their molecular weights, and the elements monitored are thyroglobulin (670 000 g/mol or 670 kDa, I), apoferritin (440 kDa, Fe, Cu, and Zn), β-amylase (200 kDa, Cu), alcohol dehydrogenase (150 kDa, Zn (150 kDa, Zn), and carbonic anhydrase (29 kDa, Cu and Zn).

These pure proteins yield individual peaks with a width of 30−40 s at the base for a chromatographic resolution of ∼1.0. Thus, the column and sample introduction system are capable of reasonable chromatographic resolution if a relatively simple sample is injected. In particular, the extra dead volume added by desolvating the aerosol does not compromise the chromatographic peak shapes, at least when the bands from the column are already fairly wide (30−40 s). Heating the aerosol in the desolvation system can cause either memory or loss of volatile elements such as Hg and Os, however.

The observations that (a) these calibration mixtures yield single, sharp chromatographic peaks and (b) the retention times for the various calibration proteins fall on the same straight line when plotted vs molecular weight indicate that the SEC separation did not remove the inorganic elements from the proteins. We also injected blanks containing EDTA at ∼1 mM several times to see if inorganic elements are retained by the column. Such elements could then be removed from the column by proteins in subsequent injections and appear falsely to be bound to proteins in the original sample. Iron was the only element that gave a substantial chromatographic peak from the EDTA injections, and even that Fe peak was at a lower level than those seen from the serum or DNA samples. Thus, we believe the peaks shown below actually represent binding of the elements of interest to proteins or DNA in the original samples and are not merely due to artifacts of the SEC separation. Some elements (notably Pb) do produce an elevated, continuous background, however, as discussed below.

Samples. A human serum standard reference material was obtained as a freeze-dried solid;[14] 1.00 g was dissolved in 0.1 M tris−HCl buffer in deionized water, as directed by NIST. The pH of this reconstituted serum solution was approximately 7. Although this standard reference material was not intended for use as either a protein or a trace metal standard, it was used for the present work because it is readily available and can be handled with minimal precautions.

DNA restriction fragments were obtained commercially.[15] The DNA solution contained a range of fragments from 8 (molecular weight (MW) ∼5 kDa) to 587 base pairs (MW ∼387 kDa). It was diluted 1/50 in 25 mM tris buffer to a final concentration of 5 ppm DNA. This solution also contained 20 μM EDTA that had been added to the original DNA sample. The molecular weight calibrations derived for the proteins were assumed to be at least approximately valid for these DNA fragments.

The ICP produces primarily singly charged atomic ions, regardless of the original species in the sample solution. In the subsequent discussion, the ions observed by the mass spectrometer are denoted by citing the isotope monitored, which distinguishes the ions formed in the ICP from those present in the sample solution. For example, $^{208}Pb^+$ is detected with the mass spectrometer from either free Pb^{2+} ions in solution or Pb bound to proteins in the sample.

Metal Ions in Proteins from Human Serum. Element-selective chromatograms for Zn, Cu, and Pb are shown in Figure 1A. The concentrations corresponding to the largest peaks are

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(8) SEC conditions for protein separations (Figures 1−4): GPC 300 column (SynChrom Inc., 2 mm i.d. × 25 cm long), eluent aqueous tris−HCl buffer (0.1 M) at 100 μL/min, pH 6.9. Conditions for DNA separations (Figures 5 and 6): packing from GPC 300 column removed and packed into PEEK column by Keystone Scientific, Inc. (2 mm i.d. × 25 cm long), same eluent with tris buffer at 0.025 M and 1 mM EDTA. The injection volume was 3 μL.

(9) MCN and desolvation conditions: MCN (Transgenomic CETAC Technologies) with a single pass conical spray chamber, aerosol gas flow rate 0.7 L min^{-1}, makeup gas flow rate 0.3 L min^{-1}, heater temperature 140 °C, condenser temperature 0 °C.

(10) ICP conditions: outer gas flow rate 14 L min^{-1}, auxiliary gas flow 0.8 L min^{-1}, forward power 1.25 kW, sampling position 10 mm from load coil, on center.

(11) MS conditions: along with the ICP conditions, ion lens voltages etc. were adjusted to maximize the signal for analyte ions from standard solutions injected postcolumn before the chromatographic experiments. The accelerating voltage was nominally 4095 V.

(12) Hutton, R. C., personal communication, 1996.

(13) The m/z value transmitted during a chromatographic peak can be readily changed in the low resolution mode by changing the accelerating voltage with very little dead time between such hops. In the present work, this electrostatic peak switching procedure is generally done over a limited m/z range at a fixed magnetic field setting, although both the accelerating voltage and magnetic field strength can be switched in tandem. In medium resolution, the magnetic field is kept at a preset value corresponding to the middle of the mass region of interest. The accelerating voltage is then scanned and the resulting chromatogram is reconstructed for the ions of interest.

(14) Standard reference material 909a, Freeze-Dried Human Serum, National Institute of Standards and Technology (NIST).

(15) PBR322 HaeIII digest, Boehringer Mannheim.

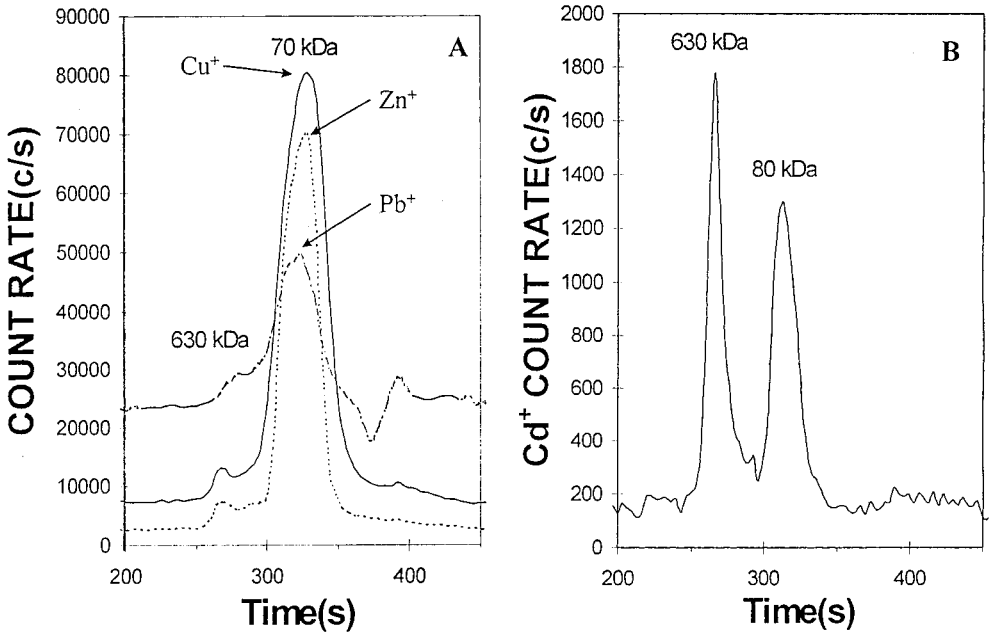

Figure 1. Element-selective chromatograms for $^{64}Zn^+$, $^{63}Cu^+$, and $^{208}Pb^+$ (a) and $^{114}Cd^+$ (b) on proteins from a single injection of NIST 909a human serum. Approximate molecular weights of the measured protein fractions are indicated on the figures. Concentrations for the largest chromatographic peaks are estimated to be ~3 ppb for Zn, 1 ppb for Cu, 0.2 ppb for Pb, and 90 ppt for Cd. Spectral resolution = 300. The GPC column[8] was used for Figures 1−4.

estimated in the captions to the figures.[16] The initial parts of these chromatograms show flat baselines, which have been deleted to conserve space. The major portions of these elements are bound to proteins that elute in a retention window from 300 to 360 s, which corresponds to a molecular weight range of 200 to 20 kDa. The maximum of the SEC profile for these elements is at 70 to 80 kDa, which is roughly the molecular weight of albumins, the most abundant proteins in serum. Copper and zinc also produce a small peak from large protein(s) at ~600 kDa.

There is a substantial background for $^{208}Pb^+$, which is actually Pb^+ ions from the column, buffer, and/or tubing. Despite the dip in the $^{208}Pb^+$ background and signal at 380 s, there is a hint of a small peak for "free" Pb, perhaps Pb^{2+}, at 400 s. Alternatively, this latter Pb chromatographic "peak" could represent a small (<10 kDa) Pb-binding protein found previously in erythrocytes.[17] There is significant environmental and toxicological interest in determining what fraction of the Pb in blood and serum is free or is bound to cells or proteins.

These measurements show that nearly all the Cu and Zn and most of the Pb in serum are bound to proteins. In these and subsequent chromatograms, the SEC peaks shown from serum are generally wider than those seen from pure proteins, probably because each SEC peak from serum represents a number of proteins. The chromatographic resolution provided by this SEC column is not capable of complete separations for such a complex sample as human serum. Also, the relative peak heights for different elements in the chromatograms do not directly reflect the relative concentrations.

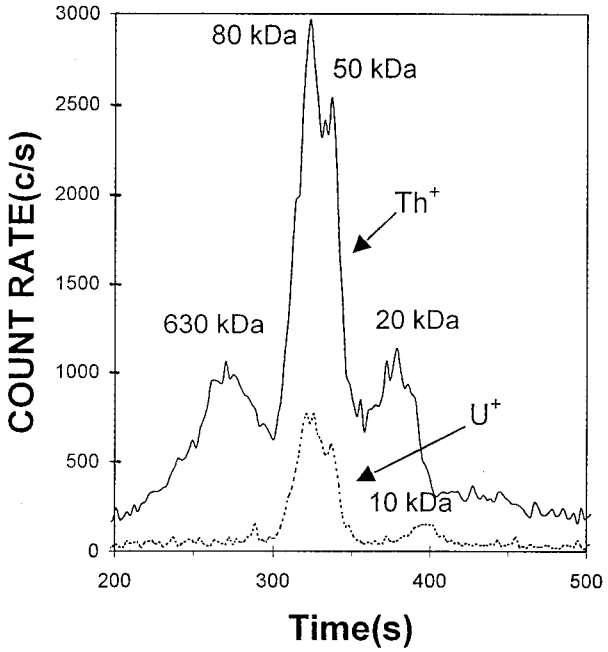

Figure 2. Chromatograms for $^{232}Th^+$ and $^{238}U^+$ on proteins in NIST 909a human serum. Concentrations for the largest peaks are ~3 ppt for Th and 1 ppt for U. Spectral resolution = 300.

The Cd chromatogram shown in Figure 1B was obtained during the same injection and elution cycle as that in Figure 1A. The two $^{114}Cd^+$ chromatographic peaks correspond to Cd bound to proteins at ~600 and 80 kDa. There is little or no "free" Cd. Traditionally, Cd, Cu, and Zn have been thought to be stored in metallothioneins.[18] However, parts A and B in Figure 1 show that chromatographic peaks in the appropriate molecular weight range for metallothioneins (~10 kDa) are

(16) These estimates are based on the typical sensitivity of the instrument for the isotopes monitored. They have not been derived from rigorous calibrations and are provided primarily for the general information of the reader. These estimates are not meant to represent accurate, quantitative measurements of concentration.

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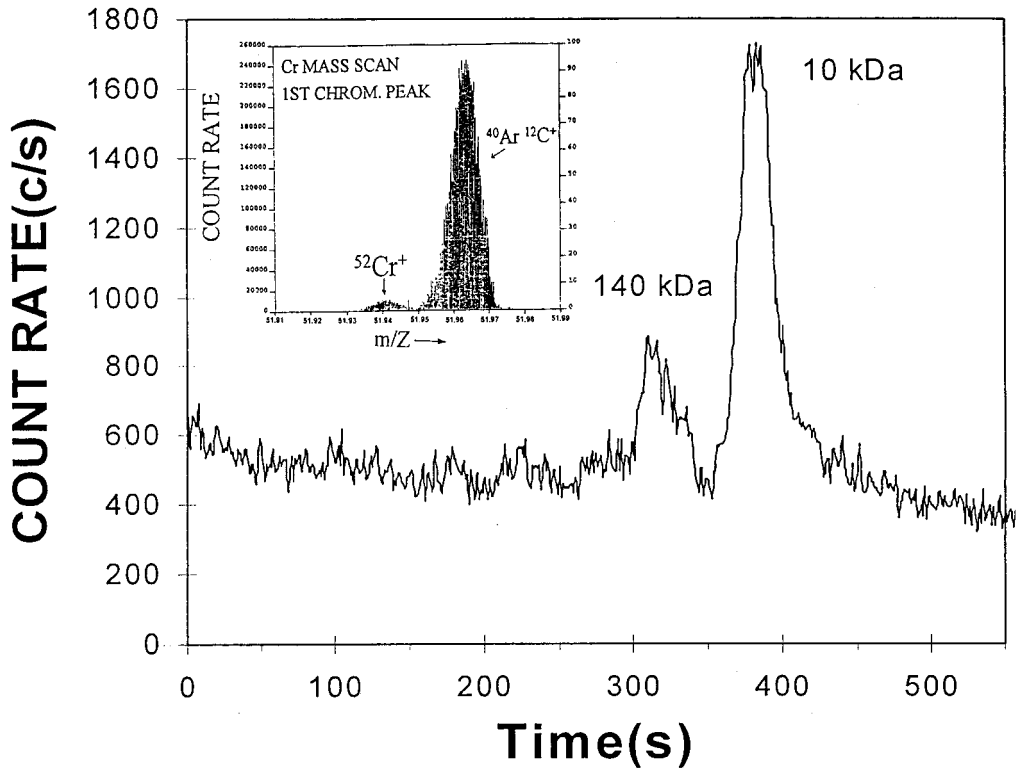

Figure 3. Chromatogram for $^{52}Cr^+$ in proteins from NIST 909a human serum, spectral resolution = 3000. The inset shows the mass spectral separation between $^{52}Cr^+$ and $^{40}Ar^{12}C^+$. The Cr concentration in the largest peak is ~0.1 ppb.

certainly not prominent and may not be present at all. Either these elements are not stored in metallothioneins in serum, or the metallothioneins are bound to other, larger proteins.

Another chromatogram for U and Th obtained from a different injection of serum is presented in Figure 2. Three distinct fractions containing Th are observed at the molecular weights shown. The major Th fraction is at ~80 kDa, as is the case for U. There is some U bound to either small proteins or other small molecules as well.

For the elements shown previously, spectral interferences are not severe, so low resolution (i.e., wide slits in the MS) is used to provide maximum signal. Chromium is a different matter because of spectral overlap with $^{40}Ar^{12}C^+$, as indicated in Figure 3. A mass scan done at medium spectral resolution during elution of the first Cr chromatographic peak is depicted in the inset to Figure 3. Spectral resolution of 3000 is sufficient to separate $^{52}Cr^+$ from $^{40}Ar^{12}C^+$. Not only is the signal from ArC^+ much larger than that from $^{52}Cr^+$, but the ArC^+ signal increases when proteins elute because they put more carbon into the plasma than the eluent alone. Thus, measurements at low spectral resolution would not suffice for the measurement of $^{52}Cr^+$ unless some other means was employed to remove ArC^+. Figure 3 also presents the reconstructed chromatogram for $^{52}Cr^+$, which shows Cr in two protein fractions at ~140 and 10 kDa.

The reader should note that none of these elements have been spiked into these serum samples. The signals shown represent the ambient levels of these elements in the samples as analyzed. It is possible that these reference serum samples were contaminated, either in production, during storage, or by us when they were redissolved. If so, the chromatograms still show that the extra trace metals added as contaminants bind to proteins. We have done some confirmatory experiments with fresh serum and found chromatographic binding patterns similar to those described in Figures 1−3, with lower concentrations for some elements, notably Cr and Th.

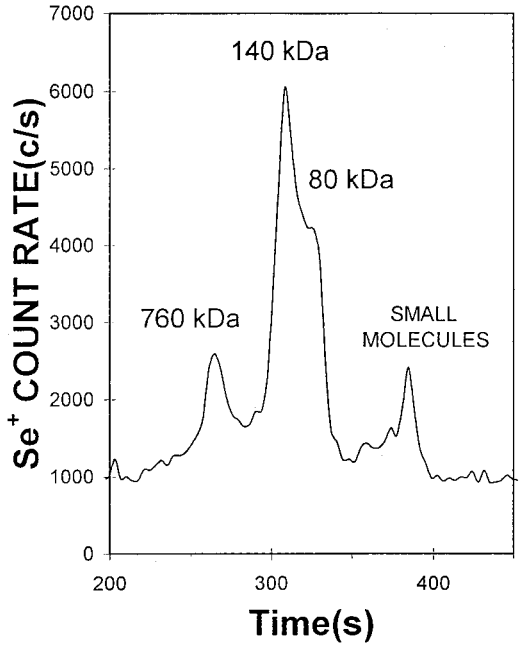

Figure 4. Chromatogram for $^{82}Se^+$ in proteins from NIST 909a human serum, spectral resolution = 300. The Se concentration in the largest peak is approximately 40 ppb.

Selenium in Proteins from Human Serum. Selenium is one of the most difficult elements for ICP-MS in that the ionization efficiency is low and the most abundant isotopes suffer from spectral overlap with the background ion Ar_2^+. We therefore use $^{82}Se^+$ at low spectral resolution despite possible overlap with $^{82}Kr^+$, a common contaminant of the argon used to operate the plasma. Fortunately, the total Se concentration in serum is fairly high, typically 90 ppb or more.[19]

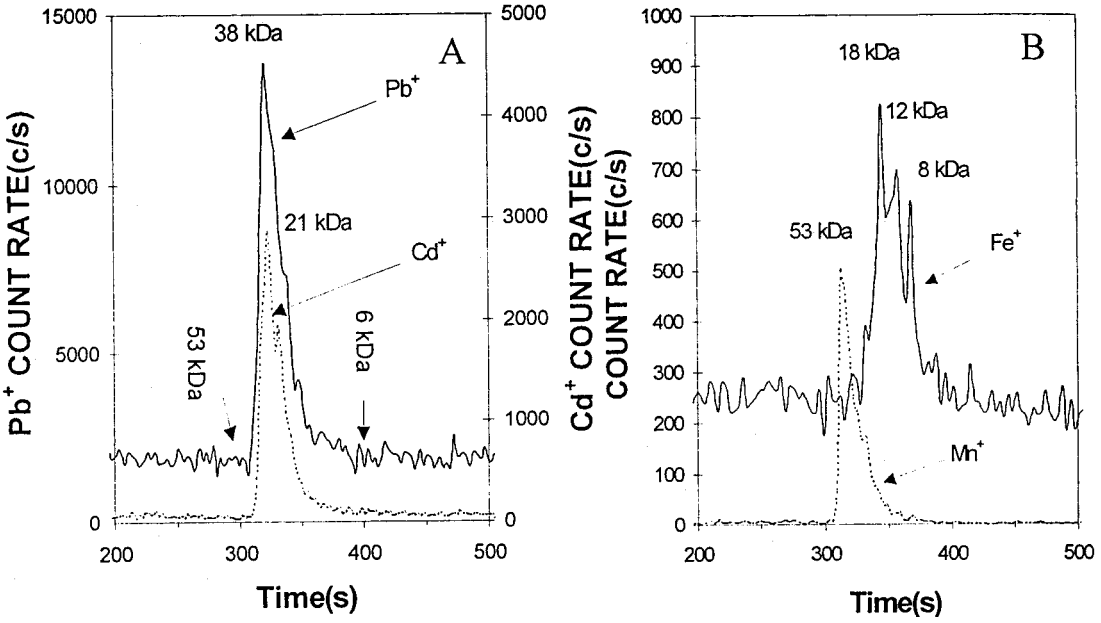

Figure 5. Chromatograms for $^{114}Cd^+$, $^{208}Pb^+$, $^{56}Fe^+$ and $^{55}Mn^+$ bound to DNA restriction fragments. For Cd and Pb, spectral resolution = 300; for Fe and Mn, resolution = 3000. These metal ions are impurities in the samples or solvents, not spikes. Concentrations are roughly 12 ppb each for Fe, Mn and Pb and 50 ppb for Cd. The homemade PEEK column[8] was used for the results shown in this and the final figure.

A selenium chromatogram obtained in this fashion is shown in Figure 4. Most of the Se is bound to three distinct protein fractions in the molecular weight ranges 760, 140, and 80 kDa. Some Se in small molecules also elutes after the proteins at a retention time of ~390 s.

The main selenoproteins that have been characterized are glutathione peroxidase (85 kDa), extracellular glutathione peroxidase (92 kDa), and selenoprotein P (three forms, 74, 61, or 57 kDa).[20] We see a chromatographic peak at ~80 kDa that could correspond to these species, in addition to peaks from two other, larger protein fractions that either contain selenium or bind selenium molecules.

With this system, calcium in serum elutes as a single chromatographic peak at about 320 s. Thus, $^{40}Ar^{42}Ca^+$ could contribute slightly to the chromatographic peaks labeled 140 and 80 kDa in Figure 4. None of the other chromatographic peaks in Figure 4 are caused by this polyatomic ion.

Binding of Metal Ions to DNA Fragments. Element-selective chromatograms are shown for four elements in a solution of DNA restriction fragments in Figure 5. In the first frame (Figure 5A), chromatographic peaks for Pb and Cd occur at retention times that correspond to the elution of DNA fragments in the molecular weight ranges 7−41 kDa. These peaks represent the ambient concentrations of these elements in the DNA, solvent, and buffers; none of these elements have been spiked into the samples. The chromatograms illustrate that these toxic elements are completely bound to DNA, at least at the trace levels encountered here.

The other frame of Figure 5 shows that Fe and Mn also readily bind to DNA; Mn binds to larger fragments than does Fe. Three or four different size fragments containing Fe are partially separated by the SEC column. Medium spectral resolution is necessary here to separate $^{56}Fe^+$ from $^{40}Ar^{16}O^+$ and is helpful for the measurement of Mn. We also found Co at substantial levels (~10 ppb) in these DNA solutions, much more so than in proteins from human serum. All the Co was bound to DNA. This latter observation is of interest because of other studies that show that cobalt ions bind readily to phosphodiesters similar to DNA.[21]

Reactions and Binding of Cr(VI) and Cr(III) with DNA. Three chromatograms for Cr at medium spectral resolution are shown in Figure 6. The DNA solution itself does not have appreciable levels of Cr, as shown by the dotted baseline in Figure 6A. One aliquot of the DNA solution is then spiked with CrO_4^{2-}, i.e., the probable form of Cr(VI) at pH 7, which is the pH of the sample. A second aliquot is spiked with Cr^{3+}. The two spikes contain equal numbers of Cr atoms. Chromium from the CrO_4^{2-} spike is observed bound to DNA (Figure 6A). Most of the Cr from the Cr^{3+} spike also binds to DNA (Figure 6B), with a small subsequent peak for Cr bound to small molecules.

Other experiments show that the oxoanion MoO_4^{2-} shows little tendency to bind to these DNA fragments, as expected if the main metal binding sites are the negatively charged phosphate groups. Thus, the original CrO_4^{2-} in the spike (Figure 6a) has been converted into a cation, otherwise the Cr from it would not bind to DNA.[22] One possible half reaction would be

$$CrO_4^{2-} + 8H^+ + 3e^- \rightarrow Cr^{3+} + 4H_2O$$

If CrO_4^{2-} has been reduced, some other component of the solution has been oxidized, most likely the DNA. *Oxidation of DNA in this fashion is one possible process responsible for carcinogenesis by Cr(VI).*[23] Rather than oxidize DNA directly,

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(20) Burk, R. F., Ed. *Selenium in Biology and Human Health*; Springer-Verlag: New York, 1994.

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(22) Hneihen, A. S.; Standeven, A. M.; Wetterhahn, K. E. *Carcinogenesis* **1993**, *14*, 1795.

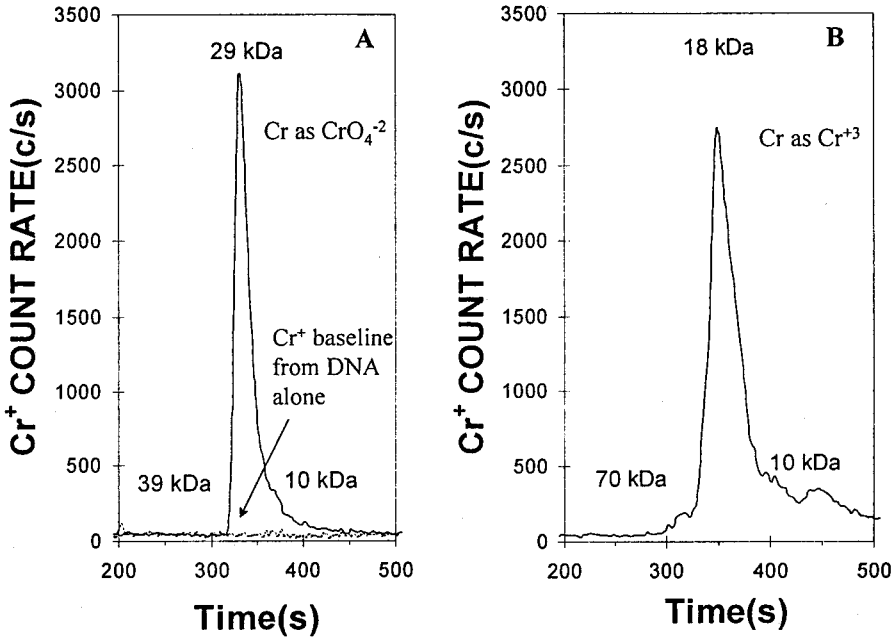

Figure 6. Chromatograms for $^{52}Cr^+$ from unspiked DNA fragments (baseline at bottom of frame a), DNA fragments spiked with CrO_4^{2-} at 33 ppb (a) and DNA fragments spiked with Cr^{3+} at 66 ppb (b). Spectral resolution = 3000.

chromate in actual biological systems could alternatively oxidize other compounds to produce reactive species such as O_2^- or $OH^•$, which then oxidize DNA.[23,24] The present work shows that the Cr cation formed (probably but not necessarily Cr^{3+}) can bind to DNA during or after the oxidation of DNA.

Conclusion

The main results of this study can be summarized as follows:

1. Even though the chromatographic separation dilutes the sample, ICP-MS with a magnetic sector instrument can observe many elements at ambient levels in human serum and provides the spectral resolution necessary to measure Cr and Fe using their major isotopes.

2. Most of the metals studied remain bound to the biological molecules during the chromatographic separation. Thus, SEC provides a "soft" chemical separation that does not remove the metals from the proteins or DNA fragments. Eventually, better chromatographic separations (probably by an additional, different separation mechanism such as ion exchange, affinity chromatography, or electrophoresis) or an additional spectroscopic measurement (such as electrospray MS) will be necessary to identify individual proteins in samples of this complexity. The authors expect that SEC will continue to be valuable as a preliminary fractionation and desalting step, followed by a different separation procedure that has been fine-tuned to optimize chromatographic resolution for a particular set of compounds.

3. In human serum, alkali metals (Cs, Rb, Li, and also Tl) are observed as free metal ions, while the alkaline earths Ba and Sr are mostly free ions with some bound to proteins.[25] Most other metals are observed bound to proteins, even those elements normally considered toxic. For most elements, the main protein fraction containing metals is around 80 kDa (e.g., Figure 1A), which probably corresponds to serum albumins.

4. Trace metal cations readily bind to DNA fragments. The Cr from a spike of CrO_4^{2-} also binds to DNA fragments, which indicates that the Cr(VI) has been reduced while the DNA has probably been oxidized. Such measurements are straightforward at Cr levels of ~50 ppb and could probably be performed at concentrations down near the physiological and present regulatory levels of Cr(VI) of ~1 ppb.

5. In principle, the ICP-MS device can measure several elements in the same molecules. This capability would be useful for identifying enzymes that contain ions from different elements.

6. The purity of the blanks remains a problem at these concentrations, especially for Pb. Completely metal-free chromatographic systems are advisable, i.e., no stainless steel columns. However, even if the samples are contaminated with additional metals at modest levels, the proteins and DNA fragments present therein readily bind the excess metals.

A number of valuable measurements should be possible based on the high selectivity and sensitivity of this general analytical method. These include the following: (a) estimation of the strength of binding of the inorganic elements by addition of complexing reagents with known binding constants, followed by chromatographic separation of the products, (b) comparison of binding patterns for metals in proteins that have had the disulfide bonds broken or have been denatured, and (c) spike experiments in which a tracer in a particular oxidation state (e.g., Mn^{2+}) can be followed into particular biomolecules. Some such studies should be possible more or less directly on biological liquids or on extracts from tissues without the laborious isolation procedures normally employed, which may themselves contaminate or alter the metal binding characteristics of interest. Alternatively, the isolation procedures already developed can now be applied to very small samples. These types of experiments are currently underway in our laboratory.

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