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# Spectrographic and Chromatographic Resolution of Metalloproteins in Human Serum*

S. Ralph Himmelhoch, Herbert A. Sober, Bert L. Vallee, Elbert A. Peterson, and Keiichiro Fuwa

ABSTRACT: Fractions of dialyzed human serum obtained by gradient chromatography on DEAE-cellulose columns were analyzed for metals by spark emission and atomic absorption spectroscopy. Such measurements required the prior development of a noninterfering eluting buffer and elimination of contaminating metals from the chromatographic system.

Iron, zinc, manganese, and nickel were found reproducibly in specific fractions, presumably as constituents of metalloproteins. Calcium, magnesium, and strontium were also localized, but their association with proteins seems more tenuous, since they are more loosely bound. Zinc is found in three distinct fractions from one of which a protein containing a stoichiometrically significant amount of zinc was partially purified. All three zinc fractions were separated from the activities of serum enzymes previously known to contain zinc.

Most metals are present in serum in such minute concentrations that, until recently, available analytical techniques were not adequate for their study. Furthermore, methods of protein separation used prior to the last decade involved conditions disruptive to protein–metal interactions. It has long been apparent, however, that metals present in blood serum function in conjunction with proteins, and whenever it has been possible to examine this association as, *e.g.*, for siderophilin and ceruloplasmin, areas of major biochemical importance have been uncovered (Putnam, 1960).

With advances in spark, flame emission, and atomic absorption spectroscopy (Vallee, 1960) and with the development of gentle protein separation methods of high resolution such as ion-exchange and molecular sieve chromatography (see review by Sober *et al.*, 1965) these problems have been overcome in principle. However, major technical obstacles remained before these methods could be combined to yield meaningful data.

In the present study, general procedures were developed which permit emission and atomic absorption spectroscopy of proteins obtained by means of chromatographic and electrophoretic protein fractionation methods. This required elimination of significant contamination and development of noninterfering buffer systems.

These general procedures were then applied to the study of dialyzed human serum: first, to determine the distribution of metals in fractions obtained by ion-exchange chromatography on DEAE-cellulose by spark emission spectrography; second, to study in greater detail the distribution of zinc and magnesium among such fractions utilizing the sensitivity of atomic absorption analysis; and finally, to achieve a partial purification of one of the three serum zinc proteins thus detected. Preliminary reports of this work have been given (Himmelhoch *et al.*, 1964).

* From the Biophysics Research Laboratory, Department of Biological Chemistry, Harvard Medical School, and the Division of Medical Biology, Peter Bent Brigham Hospital, Boston, Massachusetts, and the Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Public Health Service, Department of Health, Education and Welfare, Bethesda, Maryland 20014. *Received April 29, 1966.* This work was supported in part by Grant-in-Aid HE-07297 from the National Institutes of Health of the Department of Health, Education and Welfare and by the Nutrition Foundation.

## Methods and Materials

*Adsorbent.* The anion exchanger, DEAE-cellulose (Whatman Floc, DE-50, nominal capacity 1 mequiv/g, 100–230 mesh, lot no. 75-81/49-54 and 105-110) was washed as previously described (Peterson and Sober, 1962) and further freed of adsorbed metals by soaking in 0.01 M tetrasodium EDTA for at least 1 week. Experiments were performed with a serum sample volume of either 30 ("small scale") or 100 ml ("large scale"). For small-scale experiments, 18.0 g of DEAE-cellulose was suspended in 0.01 M sodium EDTA, pH 8.6, and packed into a 2.0 × 25 cm column with increasing air pressure (Peterson and Sober, 1962). For large-scale experiments a suspension of 95 g of DEAE-cellulose in 0.01 M sodium EDTA, pH 8.6, was pumped into a 4.5 × 50 cm column at a constant flow rate of 5 l./hr with a "Rollflex" pump (Cole-Parmer Instrument and Equipment Co., Chicago, Ill).

Once packed, the columns were placed in the cold room and washed with six column volumes of starting buffer. The conductivity and pH of the effluent were monitored after every column volume of buffer had passed through to ensure complete equilibration. Metal contamination was detected by shaking a 100-ml aliquot of the effluent with dithizone (Dithizone, Fisher certified reagent) in $CCl_4$.

*Solutions and Apparatus.* Special precautions were taken to avoid and eliminate metal contamination of serum and serum fractions during every stage of the experiment (Thiers, 1957). Aluminum needles (Monoject, Rohr Products, Inc., Deland, Fla.), polyethylene tubes and pump tubing, and "Lucite" columns and gradient mixers (Varigrads; Peterson and Sober, 1956) were used. Ashing was performed and the equipment was cleaned as described (Thiers, 1957).

The Tris–succinate chromatographic buffer system (Peterson and Chiazze, 1962) was prepared from "metal-free" stock solutions of Tris (Fisher, reagent grade) and succinic acid. Stock solutions were demineralized with columns of Chelex 100 (Bio-Rad, analytical grade chelating resin with a capacity of 0.33 mmole of $Cu(NH_3)_4^{2+}$/ml of resin bed, 100–230 mesh, control no. 5836-10 B1569). These columns were prepared by cycling the resin three times with 1 M NaOH and 1 M HCl and then packing from a suspension in 0.01 M tetrasodium EDTA. The packed column was washed free of EDTA by passage of several column volumes of distilled water until the conductivity was that of deionized water and the effluent pH was less than 9. A 1.8 × 25 cm bed containing about 9 g of Chelex resin was sufficient for at least 2 l. of 2.4 M Tris or 0.6 M disodium succinate stock solution. Since succinic acid stock solutions were too acid to be purified directly on Chelex resin, 0.6 M disodium succinate was cleaned on such a column and then converted to succinic acid by passage through a bed of Dowex-50 (Bio-Rad analytical grade cation exchanger AG50W-X12 with a capacity of 5.0 mequiv/dry g, 100–230 mesh, lot. no. 500704, control no. 4799-41 B-1092) in the hydrogen form. Prior to use, dialysis membranes prepared by boiling in $Na_2CO_3$ (Peterson and Chiazze, 1962) were soaked in 0.01 M EDTA, pH 8.6, for 1 week at room temperature.

## Experimental Procedure

*Samples.* Blood samples were drawn directly from individual donors into polyethylene bottles and allowed to clot at room temperature. The serum was removed by centrifugation and dialyzed on a rotating dialyzer against two 20-fold volumes of starting buffer at 5°. The buffer was changed after 18 hr, and then dialysis was stopped after 6 hr. When present, a scanty precipitate was removed by centrifugation and discarded.

*Chromatography.* After the sample had been applied and washed in, the column was connected to a peristaltic pump already adjusted to the appropriate flow rate. Fractions were collected in polyethylene tubes or bottles with time-indexed fraction collectors.

For small-scale experiments, a flow rate of 120 ml/hr was employed. The gradient volume was 2100 ml and the elution schedule was 168 ml of starting buffer, followed by the gradient (Peterson and Chiazze, 1962).

For large-scale experiments, a flow rate of 600 ml/hr was used. The gradient volume was 10,800 ml and the elution schedule was 864 ml of starting buffer, followed by the gradient (Peterson and Chiazze, 1962). Starting buffer was 0.005 M in succinic acid and 0.04 M in Tris. Limit buffer was 0.3 M in both Tris and succinic acid. The gradient composition (per cent limit buffer in each chamber) was 0, 2, 2, 10, 10, 10, 10, 10, and 100.

### *Analysis of Fractions*

*Preparation.* The absorbance at 280 mμ, pH, and conductivity of each fraction were determined and groups of tubes were pooled on the basis of the protein concentration profile to yield nine pooled fractions lettered A–H. The effluent volumes represented by each fraction were as follows: A, 0–3%; B, 3–36%; C, 36–40%; D, 40–53%; E, 53–64%; F, 64–73%; G, 73–85%; H, 85–100%. These were lyophilized and quantitatively transferred to platinum dishes for ashing.

Preashing in platinum evaporating dishes was carried out as described (Thiers, 1957). Ashing was performed under borosilicate glass dust covers in a muffle furnace (Electric Hot Pack Co., Model 7074), controlled electrically to maintain a temperature of 450°. If after 24 hr in the muffle furnace a carbon-free ash was not obtained, 2 drops of concentrated nitric acid was added to the crucible and evaporated on a hot plate at low temperature. The crucible was then returned to the muffle furnace for an additional 2 hr with the temperature now raised to 500°. In this manner, a white, easily soluble ash was invariably obtained.

The *spectrographic* procedure was essentially that reported previously (Vallee, 1955). The prepared graphite porous cup electrodes, 0.4-ml capacity, 2.5-cm length, 3-mm i.d., 6-mm o.d. (United Carbon Co.,

Bayside, Wis.) were handled with platinum-tipped forceps. The copper counter electrodes (Johnson and Matthey, Inc., London, "spec pure") were 6 mm in diameter and 5 cm long. Johnson and Matthey "spec pure" rods, metal sponge, flakes, or salts were used for preparation of standard solutions. "Metal-free" hydrochloric acid was prepared as described (Thiers, 1957). Eastman Kodak spectrographic plates, emulsion no. 103-0, were used throughout. Processing solutions were those recommended by the manufacturer.

A 3.4-m, Wadsworth mounting grating spectrograph (Jarrell-Ash Co.) employing a grating of 15,000 lines/in. and a Varisource R unit were used. The acid-resistant resin-coated sparking chamber was equipped with a platinum-coated electrode holder, a 2-mm gap divider, and an exhaust system which neutralized and filtered the escaping acid fumes. An Applied Research Laboratories microdensitometer with a slit width at 10 $\mu$ served to measure the intensities of the emission lines.

*Preparation of Standard Solutions*

Standard solutions containing iron, cobalt, nickel, cadmium, zinc, manganese, chromium, tin, strontium, calcium, copper, molybdenum, lead, barium, magnesium, and aluminum, and with vanadium added to each solution as an internal standard, were prepared in 6 N hydrochloric acid to obtain calibration curves over a range varying from 0.01 to 300 $\mu$g/ml for the different elements.

Vanadium (7 $\mu$g/ml) and 1.0 ml of "metal-free" hydrochloric acid were added to the ash of each sample which was then dissolved using gentle heat, if necessary. The contents of each crucible was transferred to acid-cleaned polyethylene test tubes. Sample solutions were sparked in duplicate and standard solutions in triplicate.

The emission lines of interest were identified and their intensities and that of the corresponding background were measured densitometrically. The ratios of the background-corrected intensities of the lines of the unknown elements to that of the internal standard were obtained. From these a log–log plot of metal concentration *vs.* relative intensity was constructed from which the metal contents of the fractions could be calculated. Zinc and magnesium were also measured by atomic absorption spectroscopy (Fuwa *et al.*, 1964; Wacker *et al.*, 1965).

*Test for Contamination in the Complete System.* The reagents employed were analyzed by spark emission spectrography both before and after purification to allow a direct assessment of the degree of metal contamination and its elimination. In addition, a "small-scale" chromatogram was performed without a serum sample and the total effluent ashed and analyzed to establish the final "blank" level.

*Precision of Spectrographic Analyses and Reproducibility of Dialysis.* A pooled serum sample from six normal donors was divided into 12 5-ml aliquots of which six were ashed and analyzed directly. The remaining six were dialyzed individually against starting buffer and then ashed and analyzed. Zinc was determined by atomic absorption (Fuwa *et al.*, 1964) since interference by the serum matrix would have required major modifications for its determination by the emission spectrographic method.

*Partial Purification of One Zinc Component.* Dry ammonium sulfate (120 g) was added slowly with continual stirring to 400 ml of serum. The mixture was allowed to stand for 30 min at 0° and then centrifuged at 10,000*g* in a Spinco Model L ultracentrifuge. The precipitate was dissolved in about 100 ml of 0.04 M Tris–HCl buffer, pH 8.6 (0.04 M in Tris and 0.01 M in HCl), and dialyzed against two 20-fold portions of the same buffer at 4°.

The redissolved, dialyzed precipitate was separated by electrophoresis on a bed of ethanolyzed cellulose (Munktell Cellulos-pulver, Grycksboro, Sweden) in a Porath column electrophoresis apparatus (LKB Model 5801-A) for 22 hr at 650 v. The cooling jackets were maintained at 2°. The column was equilibrated with 0.04 M Tris–HCl buffer, pH 8.6. Elution was performed at 600 ml/hr and 20-ml fractions were collected. The zinc-containing fractions were pumped directly onto a 4.5 × 50 cm bed of DEAE-cellulose and chromatography was carried out exactly as described for a "large-scale" serum chromatogram (*vide supra*).

The fractions containing the zinc component of interest were pooled, diluted by a factor of 1.3 with water, readjusted to pH 8.6 with 0.4 M Tris, and adsorbed to a 6-g bed of DEAE-cellulose equilibrated with starting buffer. Elution was effected with a step of 0.2 M succinic acid–0.44 M Tris buffer, pH 7.2.

The zinc component, thus concentrated to a volume of 15 ml, was applied to a 2.5 × 135 cm column of Sephadex G-200 (Pharmacia, Lot TO 1038) equilibrated with starting buffer. Elution was carried out at a flow rate of 10 ml/hr maintained with a Mariotte flask. Fractions (5 ml) were collected. Lactic acid dehydrogenase, glutamic acid dehydrogenase, carboxypeptidase, and alkaline phosphatase activities were measured by standard assay procedures (Wacker *et al.*, 1956; Adelstein and Vallee, 1958; Coleman and Vallee, 1962; Plocke *et al.*, 1962).

## Results

Table I presents the metal content of the chromatographic effluent without a serum sample. The first column gives the values obtained prior to purification of reagents and the second those obtained subsequent to the purification procedures described above. Unpurified reagents contained prohibitively high quantities of iron, zinc, copper, lead, and manganese. Substantial amounts of calcium, magnesium, and barium were also present. Purification reduced this contamination to acceptable levels for all of these metals, in fact, lowering the levels of barium, zinc, and manganese below the limit of detection of the method.

The metal content of serum samples prior to and after dialysis is recorded in Table II. The mean and coefficient of variation were derived from duplicate

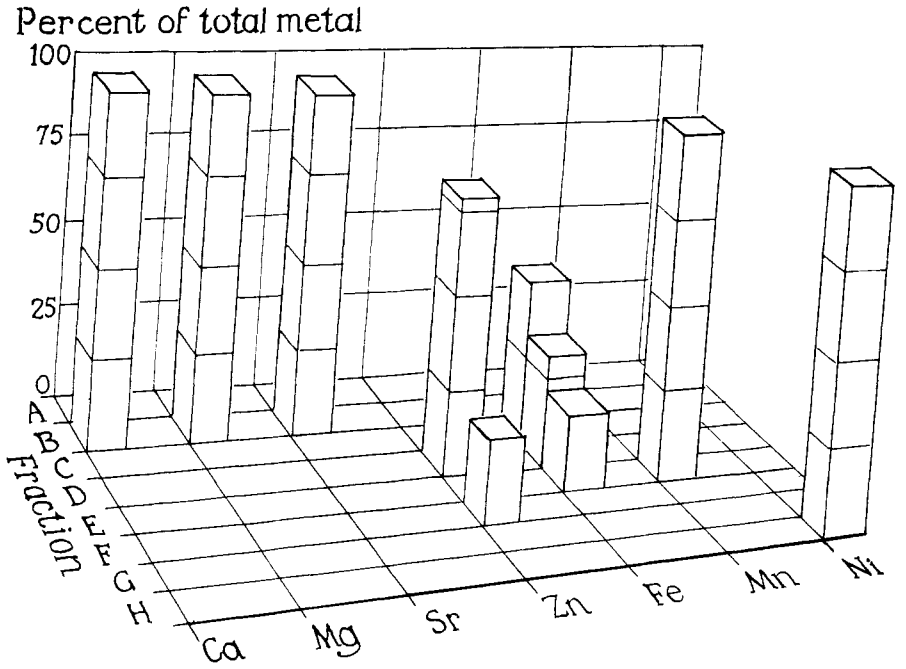


FIGURE 1: Distribution of metals in chromatographic fractions. The heights of the bars represent the percentage of each metal contained in each of the chromatographic fractions. The mean of eight experiments is shown.

TABLE I: Metal Content of Effluent before and after Purification of Reagents.[a]

| Metal | Metal Content of Effluent (parts per billion) Before Purification | After Purification |
|---|---|---|
| Calcium | 260 | 10 |
| Strontium | *b* | *b* |
| Magnesium | 50 | 4 |
| Barium | 10 | *b* |
| Iron | 60 | 3 |
| Nickel | 6 | 1 |
| Cobalt | *b* | *b* |
| Zinc | 48 | *b* |
| Cadmium | *b* | *b* |
| Copper | 40 | 3 |
| Manganese | 5 | *b* |
| Molybdenum | *b* | *b* |
| Lead | 6 | *b* |

[a] The total buffer solution employed in a conventional experiment was collected as a single sample after passage through a 2 × 2.5 cm column containing 18 g of DEAE-cellulose. The solution was evaporated to dryness under an infrared lamp (Thiers, 1957) and then ashed and analyzed as under Methods. Its metal content was compared with that of "purified" buffer passed through the column and analyzed in a similar fashion (see text). [b] Below limit of detection.

TABLE II: The Effect of Dialysis on the Metal Content of Serum.[a]

| Metal | Metal Concn (μg/100 ml) Serum (%) | Dialyzed Serum (%) |
|---|---|---|
| Calcium | 9760 ± 10 | 2920 ± 20 |
| Magnesium | 2090 ± 8 | 287 ± 25 |
| Iron | 119 ± 20 | 106 ± 40 |
| Zinc[b] | 115 ± 8 | 91 ± 17 |

[a] Six aliquots each of serum and individually dialyzed serum samples from the same stock were ashed separately and analyzed in duplicate. The data given are the arithmetic mean ± the coefficient of variation of the 12 analytical values obtained for the undialyzed and dialyzed samples. [b] Atomic absorption spectrophotometry (see, text).

sparkings of six aliquots ashed separately either with or without prior dialysis. The data obtained on whole serum are close to those generally reported in the literature for these metals, and the coefficients of variation similar to those cited for this method by Thiers and Vallee (1957). Except for iron, the values obtained after dialysis reflect substantial removal of apparently loosely bound metal. Dialysis, moreover, increased the coefficients of variation markedly.

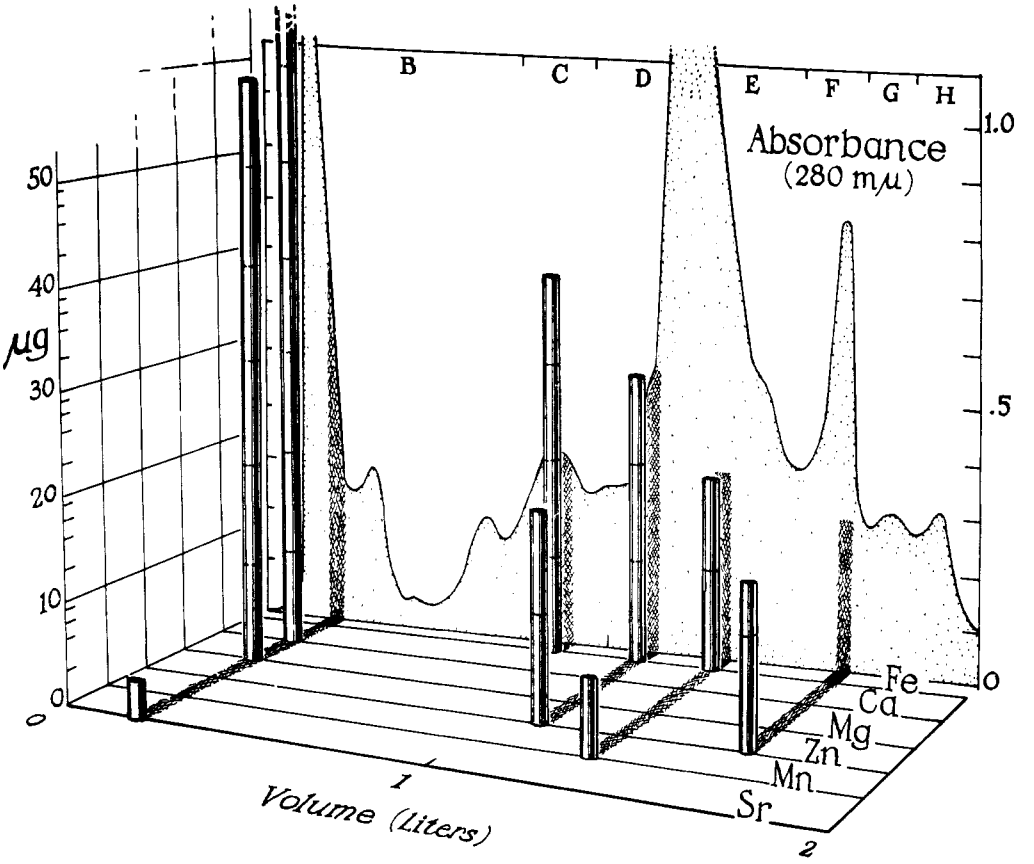


FIGURE 2: Metal distribution in relation to the protein concentration profile of a serum chromatogram. The bars represent the total micrograms of metal recovered in each fraction, designated in accord with Figure 1.

The concentration of metals in micrograms per fraction derived from 100 ml of serum are shown in Table III. Figure 1 permits direct comparisons on a relative scale by setting the total of each metal found in all fractions of Table III equal to 100%. The height of the bars then reflects the percentage of each metal found in each fraction. Calcium, magnesium, and strontium were consistently present in fraction B. Barium was the only alkaline earth which was variously detected in fractions B, C, and E. Iron was found in the contiguous fractions C–E suggesting the existence of multiple iron proteins. The finding of zinc in fractions D and F, separated by the zinc-poor fraction E, indicates the existence of at least two chromatographically distinct zinc proteins. Manganese was detected in only four out of eight experiments, a consequence of its low concentration in serum. When present it always appeared in fraction E, a situation quite analogous to that of nickel, which appeared only in fraction H.

TABLE III: Metal Content of Chromatographic Fractions of Serum (μg/Fraction per 100 ml of Serum).[a]

| | Fraction | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Metal | A | B | C | D | E | F | G | H |
| Calcium | | 1450 | | | | | | |
| Magnesium | | 380 | | | | | | |
| Strontium | | 1 | | | | | | |
| Barium | | 0.4 | 0.2 | | 0.7 | | | |
| Iron | | | 43 | 29 | 19 | | | |
| Zinc | | | | 45 | | 14 | | |
| Manganese | | | | | 4 | | | |
| Nickel | | | | | | | | 8 |

[a] In all instances where numerical values are not given the spectral lines of the elements in question were below the limits of detection. All values are the means of eight separate experiments. Each serum sample was fractionated by gradient chromatography on DEAE-cellulose as described in Methods.

Aluminum, chromium, and lead were encountered sporadically and adjudged to be contaminants. Cadmium, molybdenum, and tin were not detected in any experiments. The copper counter electrode employed in the spark precluded the analysis of this element (Vallee, 1955).

The combination of protein fractionation with spectrography resulted in good recovery of metals in the dialyzed serum employed as the starting material. When the contents of Ca, Mg, Zn, and Fe present in

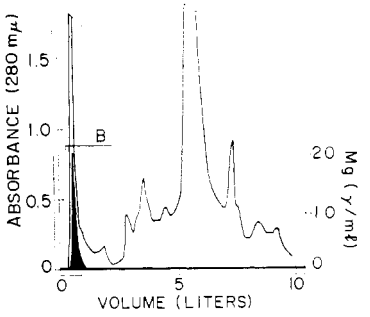


FIGURE 3: Magnesium distribution in a serum chromatogram (atomic absorption spectroscopy). The magnesium concentration, the shaded area, is shown beneath the effluent protein concentration as determined by absorbance at 280 mμ. The position of fraction B is indicated for reference.

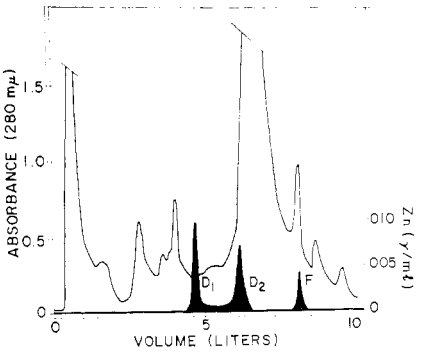


FIGURE 4: Zinc distribution in a serum chromatogram (atomic absorption spectroscopy). The zinc concentration, the shaded areas, is shown beneath the effluent protein concentration as determined by absorbance at 280 mμ. Three fractions containing zinc, $D_1$, $D_2$, and F, can be discerned.

all fractions were totaled, the recoveries were 79, 97, 94, and 86%, respectively.

The relationship of the metals detected to the known protein components of human serum can be derived from the position of each metal with respect to the concentration profile of the effluent protein. In Figure 2, presenting a single experiment, bars representing total metal content in micrograms are projected onto the protein concentration profile shown as the effluent absorbance at 280 mμ.

By disc electrophoresis the alkaline earth rich fraction, fraction B, contains only 7-S γ-globulins. Fractions C–E, the iron-rich fractions, contain most of the effluent with absorbance at 460 mμ, largely owing to their content of siderophilin and hemoglobin–haptoglobin. The sensitivity of the atomic absorption methods for zinc and magnesium permitted the determination of these elements in each of the 180 fractions collected.

The results obtained with magnesium are shown in Figure 3 where the effluent concentration of magnesium in micrograms per milliliter and the effluent profile of protein concentration are given. These data confirm the emission spectrographic results by demonstrating that magnesium emerges in a position corresponding to fraction B (Figures 1 and 2). Exhaustive dialysis of these fractions results in the loss of substantially all of the magnesium.

The atomic absorption method also confirmed the results on zinc by emission spectrography, but further resolved the data. The chromatographic position of zinc (Figure 4) corresponded to fractions D and F of previous experiments (Figure 1), but two distinct zinc components ($D_1$ and $D_2$) were clearly resolved within fraction D. Dialysis of each of these zinc components against starting buffer showed their zinc content to be nondialyzable.

By a multistage procedure described in Table IV 21 mg of purified component $D_1$ containing 11.5 μg of zinc (550 μg/g) was obtained from 400 ml of serum containing 436 μg of nondialyzable zinc and 28.6 g of protein (15 μg of zinc/g of protein, Table IV). Since analytical chromatography of a small aliquot of this serum prior to other treatment showed that 92 μg of zinc was associated with component $D_1$ and the remainder with components $D_2$ and F, the over-all yield was 13%. With the multistage purification procedure or with several modifications, 80–94% yield was always obtained until 10-fold purification was achieved. Be-

TABLE IV: Purification of Zinc-Containing Component $D_1$[a] of Serum.[b]

| Purification Step | Total Protein Fraction (g) | Nondialyzable Zinc/Fraction (μg) | Zinc/Protein (μg/g) |
|---|---|---|---|
| 1. Serum | 28.6 | 436 | 15 |
| 2. $(NH_4)_2SO_4$ precipitate | 13.2 | 305 | 23 |
| 3. Electrophoretic albumin fraction | 2.6 | 296 | 114 |
| 4. Fraction $D_1$ (DEAE-cellulose chromatography) | 0.2 | 28 | 140 |
| 5. Fraction $D_1$ (Sephadex G-200 chromatography) | 0.02 | 12 | 550 |

[a] For nomenclature see Results. [b] Serum (400 ml) was fractionated by means of the five steps shown. Analyses of the main-line fractions of the purification are shown. Protein weight was determined by TCA precipitation and zinc by atomic absorption spectroscopy as described under Methods.

yond this point the product became unstable and a large proportion of the zinc became dialyzable.

However, the zinc component $D_1$ was separated from the fractions of serum containing carboxypeptidase, lactic acid dehydrogenase, alkaline phosphatase, and glutamic dehydrogenase activities as early as the ammonium sulfate precipitation (step 2, Table IV). These activities were recovered in yields ranging from 75 to 92% in the dialyzed, ammonium sulfate supernatant solution.

## Discussion

The limitations of existent methods have precluded detection and measurement of metals in the minute concentrations in which they occur in serum. Only in the special cases of the colored proteins hemoglobin–haptoglobin, siderophilin, and ceruloplasmin have these methodological difficulties been circumvented. The development of a spark emission spectrographic method (Vallee, 1955) capable of simultaneous measurement of many metals with good accuracy in a single sample coupled with the development of techniques of ion-exchange chromatography of serum on columns of DEAE-cellulose (Peterson and Sober, 1956; Sober *et al.*, 1956), which can provide high resolution of proteins at essentially any required load, provided an avenue of approach to this problem. It is, in fact, analogous to that previously employed with subcellular particles (Thiers and Vallee, 1957).

However, the large quantities of buffer salts and adsorbent utilized in gradient chromatography, as well as the variation in buffer composition from fraction to fraction, posed major obstacles to the combination of these two methods. The problems of metal contamination increase in direct proportion to the quantity of buffer salts employed, and the variation in buffer composition makes assignment of an appropriate blank metal content difficult. Furthermore, previous chromatographic buffer systems uniformly contained phosphate ion (Sober *et al.*, 1956) known to interfere with spectrographic analysis (Ahrens and Taylor, 1961).

The methods described in this study and a previous one (Peterson and Chiazze, 1962), therefore, were designed to achieve a chromatographically effective buffer system free of metals to such a degree that correction for metal content would not be necessary.

The analytical demonstration of the absence of significant metal contamination (Table I), the details of the elution patterns obtained, and the recovery data document the achievement of these goals. The convenient extension of the same methods to systems using ammonium sulfate precipitation, preparative electrophoresis, and molecular sieve chromatography under metal-free conditions (Table IV) demonstrates their general usefulness.

The repeated occurrence of a metal in the same chromatographic position constitutes presumptive evidence of its association with a protein in that fraction, since prior dialysis removes both loosely bound or free metal ions. Thus, the present data indicate the existence in human blood serum of specific metalloproteins containing zinc, iron, manganese, and nickel.

The fact that manganese and nickel occur each in a single separate fraction suggests the possibility that they are associated with specific proteins. Further work is required to establish the significance of these observations.

Iron must be associated with multiple chromatographic components, since it was found in three distinct fractions. Each of these contains a band on disc electrophoresis, corresponding to siderophilin, in accord with the heterogeneity of this protein in other systems (Smithies, 1958). The possibility cannot be eliminated, however, that one or more of these fractions contains an additional iron-binding protein.

Calcium, magnesium, strontium, and barium are known to bind relatively weakly to proteins (Vallee, 1960). The atomic absorption data (Figure 4) show that dialysis can remove magnesium almost completely from the fractions in which it occurs. The specificity of the association between these alkaline earths and the proteins with which they emerge from the column cannot be settled by the present data. Their emergence in the first fraction after the column wash, the position which would be occupied by any metal which becomes dissociated from proteins during adsorption to the ion-exchange column, adds further uncertainty in this regard.

Spectrographic and atomic absorption analyses demonstrate the chromatographic resolution of at least three serum zinc proteins. Since a number of zinc enzymes are known to occur in serum, the enzymatic characteristics of these fractions were examined. The chromatographic activity profiles of lactic acid dehydrogenase, alkaline phosphatase, carboxypeptidase, and glutamic dehydrogenase overlap but are not identical with the zinc profile of fractions $D_1$ and $D_2$. The zinc profile of fraction F does not coincide with any of these activities. Furthermore, the purification procedure recorded in Table IV separates zinc proteins $D_1$ and $D_2$ completely from these enzyme activities. It would appear that these three moieties represent hitherto unrecognized proteins. As might be predicted from the magnitude of the activities observed, the known zinc enzymes of serum are present in such low concentration that the zinc associated with them is undetectable by the present analytical procedures.

The data given in Table IV document the existence of Zn–protein $D_1$ and define certain of its properties. The final ratio of zinc to protein achieved (550 $\mu$g/g) corresponds to 0.7 g-atom/mole of 120,000 mol wt, the approximate size indicated by chromatography on G-200 Sephadex. Since disc electrophoresis demonstrated the presence of one major and two minor bands, tentatively presumed to be impurities, the zinc content seems to be stoichiometrically significant.

In view of the reduced serum zinc concentration which has been observed in cases of Laennec's cirrhosis (Vallee *et al.*, 1956) the existence of these three zinc proteins is of special interest. Preliminary studies indi-

cate that the lowered serum zinc concentration in this disease is a reflection of a change in the amount of one of these firmly bound zinc moieties. Studies are in progress to complete the isolation of these proteins and to discern their functional role.

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