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Isolation of a Zinc α_2-Macroglobulin from Human Serum*

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ABSTRACT: An α_2-macroglobulin constitutes the principal zinc metalloprotein of human serum. The material, separated successively by DEAE-cellulose chromatography and gel filtration, accounts for 30 to 40% of the total zinc content of serum. It is homogeneous in the ultracentrifuge and exhibits a sedimentation constant consistent with that known for α_2-macroglobulin. Disc gel and immuno electrophoresis show one major and two faint minor bands. Immunochemical properties, trypsin–protein esterase activity, and the failure of soybean trypsin inhibitor to inactivate the trypsin–protein complex—under conditions where trypsin itself is inhibited—all serve to establish the identity of this zinc protein as α_2-macroglobulin. During purification, trypsin–protein esterase activity of fractions increases in parallel with zinc content which varied from 320 to 770 μg of zinc/g in a number of α_2-macroglobulin preparations. A direct role for zinc in the esterase activity of the tryspin–α_2-macroglobulin complex is not known thus far.

Zinc in human serum is almost entirely bound to protein. Its concentration is maintained within well-defined limits in normal individuals and is depressed in several metabolic and neoplastic disorders (Vikbladh, 1951; Vallee, 1959; *Lancet*, 1968). During the past 15 years, the identification of a number of zinc metalloenzymes has clarified many general aspects of the metabolic role of zinc, but the zinc content of the known zinc enzymes accounts for very little of the element found in human serum (Himmelhoch *et al.*, 1966). Hence, the nature and significance of all the circulating zinc proteins in man has remained unknown.

Recently atomic absorption and spark emission spectroscopy have been employed to identify zinc and other metals in discrete protein fractions of human serum resolved by DEAE-cellulose chromatography (Himmelhoch *et al.*, 1966). The present communication extends these investigations and demonstrates that the zinc present in normal human serum is composed of two principal species: one fraction, which is firmly bound to α_2-macroglobulin, and a second fraction, more loosely associated with albumin.

Materials

Materials were chosen with a view toward minimizing metal contamination during experimental procedures, as described previously (Himmelhoch *et al.*, 1966). Stock solutions of Tris (Trizma Base, Sigma Chemical Co.) were demineralized by passage through columns of Chelex 100 (Bio-Rad Laboratories) by the method of Himmelhoch *et al.* (1966), except that the resin was not prewashed with EDTA. Succinic acid (Fisher Scientific Co.) and HCl (Aristar, British Drug Houses, Ltd.) were found satisfactory for use without further demineralization.

Chromatographic Media. DEAE-cellulose (DE-32, Whatman Co.) was cycled as outlined previously (Peterson and Chiazze, 1962). After washing with 0.5 M HCl, it was brought to the pH and conductivity of the starting buffer and packed at 15 psi under nitrogen, without exposure to EDTA.

Sephadex G-100 (Pharmacia Fine Chemicals, Inc.) and agarose (Bio-Rad Laboratories, Agarose A 0.5 m) were washed with 0.1 M Tris·HCl, pH 8.0, until the zinc concentration of the effluent was comparable with that of the applied buffer, as verified by atomic absorption spectrometry (Fuwa and Vallee, 1963; Fuwa *et al.*, 1964; Iida *et al.*, 1967).

Methods

Serum samples were obtained as previously noted (Himmelhoch *et al.*, 1966). Plasma was processed from material stored in commercially available plastic transfer packs (Fenwal Co.).

Prior to all chromatographic procedures the starting material was dialyzed against two 20-fold volumes of starting buffer at 5°. The buffer was changed at 18 hr, and then dialysis was stopped after 6 hr. Scanty precipitates were removed by centrifugation and discarded.

In each of four pilot studies, 10 ml of serum was chro-

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Step	Fraction	Discard
	Serum or plasma	
1a. Chromatography: DEAE-cellulose		Material not containing zinc and fraction B
	Fraction A	
1b. Gel filtration: Agarose A 0.5 m or Sephadex G-100		Fraction AII
	Fraction AI	
1c. Chromatography: DEAE-cellulose		Remaining material not containing zinc
	α_2-Macroglobulin	

FIGURE 1: Scheme for isolation of α_2-macroglobulin from human serum.

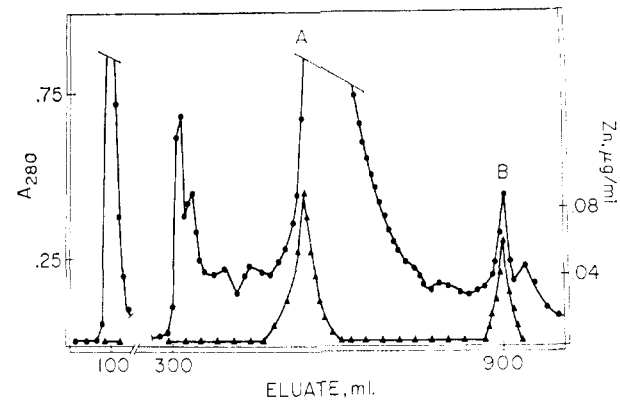

FIGURE 2: DEAE-cellulose chromatography of whole serum. Serum (10 ml) was dialyzed for 24 hr against two changes of 20 volumes each of 0.04 M Tris, 0.005 M succinic acid and applied to a 2 × 30 cm column of DEAE-cellulose prepared as outlined under Methods. Elution was carried out with the described 1080-ml gradient using a Varigrad mixer. Absorbance at 280 mμ (●—●—●) and zinc concentration (▲—▲—▲) are plotted.

matographed on a 2 × 30 cm column of DEAE-cellulose using 1080 ml of a complex gradient of Tris buffer and succinic acid, constituted as previously described (Himmelhoch *et al.*, 1966). Zinc was measured in the eluate by atomic absorption spectrometry and protein was detected by measuring absorbance at 280 mμ using a Zeiss PMQ II spectrophotometer. A conductivity meter (Radiometer, Copenhagen) with a CDC 114 electrode, and a pH meter (Radiometer, Copenhagen), with a 6-K 2021 C glass electrode, were used to determine conductivity and pH, respectively. The zinc-rich fractions were concentrated by pressure dialysis using PM-30 membranes in a Diaflo concentration cell (Amicon Corp.) and further resolved on a 2 × 50 cm column of Sephadex G-100 equilibrated with 0.1 M Tris·HCl, pH 8.6. In two of these separations, carrier-free ^{65}Zn (New England Nuclear Corp.) was added immediately prior to dialysis and chromatography in order to determine the distribution of exogenous zinc within the system. Radioactivity was measured with a γ-ray spectrometer (Tracerlab, Inc.). At each step, the components of the zinc-rich fractions were examined by disc gel electrophoresis under conditions suggested by the manufacturer (Canalco Corp.)[1] and by immunoelectrophoresis with mono- and polyvalent antisera (Scheidegger, 1955).

Larger quantities of α_2-macroglobulin, adequate for purposes of physical-chemical characterization, were prepared employing the general procedures outlined in Figure 1. Serum or plasma (200–400 ml), pooled from two or more donors, was used as the starting material. This was dialyzed against three changes of 20–30 volumes of 0.05 M Tris–0.02 M succinic acid, pH 7.6, and then chromatographed on a (4 × 80 cm) column of DEAE-cellulose (Figure 1, step 1a). The starting buffer was 0.05 M Tris and 0.02 M succinic acid, pH 7.6, and the limit buffer was 0.12 M Tris and 0.05 M succinic acid, pH 7.6. A linear gradient consisting of 2.0 l. each of starting buffer and limit buffer was used. The zinc-rich fraction A (see Figure 1, step 1a) was further resolved on a 4 × 75 cm column of Agarose A, 0.5 m, equilibrated with 0.1 M Tris·Cl, pH 8.0 (Figure 1, step 1b). The agarose-excluded material was rechromatographed on a 2 × 30 cm column of DEAE-cellulose using 100 ml each of the starting and limit buffers described above (Figure 1, step 1c), yielding highly purified protein.

Trypsin–protein esterase activity (hereafter referred to as the esterase activity) among the serum proteins, a function unique to α_2-macroglobulin (Schultze and Heremans, 1966), was measured titrimetrically using 0.01 M tosyl-DL-arginine methyl ester (TAME, Sigma Chemical Co.) in 0.001 M Tris-Cl, pH 7.5, as substrate, twice-recrystallized bovine trypsin (Worthington Biochemicals), 0.08 mg, and soybean trypsin inhibitor (Worthington Biochemicals), 0.10 mg (Haverback *et al.*, 1962; Mehl *et al.*, 1964). The order and volumes of additions were: trypsin (0.20 ml); sample to be tested for the esterase activity or buffer (0.20 ml); and soybean inhibitor (0.20 ml) in a total volume of 0.60 ml. After 5 min of incubation at 25°, 0.30 ml of this mixture was added to the substrate (2.70 ml) to start the reaction. The assay was performed at 25°, pH 7.5, titrating with 0.10 N NaOH in a pH-Stat (Radiometer, Copenhagen) with an automatic recorder (Ole Dich, Copenhagen).

The activities of known zinc metalloenzymes, carboxypeptidase, alcohol dehydrogenase, and alkaline phosphatase, as well as that of lactic dehydrogenase were tested in selected fractions, using standard assay procedures (Wacker *et al.*, 1956; Coleman and Vallee, 1962; Blair and Vallee, 1966; Plocke *et al.*, 1962).

Protein dry weight was determined by the trichloroacetic acid precipitation method (Hoch and Vallee, 1953). In all samples zinc was measured by atomic absorption (Fuwa and Vallee, 1963; Fuwa *et al.*, 1964) and in two preparations magnesium, manganese, iron, copper, chromium, and nickel were also measured by this procedure. Metal content was also established by emission spectrography (Vallee, 1955). Sedimentation velocity was measured at 22° in the Spinco Model E analytical ultracentrifuge equipped with an RTIC unit and a phase plate as Schlieren diaphragm.

Results

Chromatography of whole serum on DEAE-cellulose, using the salt and pH gradient previously described (Himmelhoch *et al.*, 1966), results in a broad separation of the major proteins present (Figure 2). Two zinc peaks, labeled A and B, in accord with the scheme of Figure 1, are identified.

[1] The standard Tris-glycine pH 9.5 system.

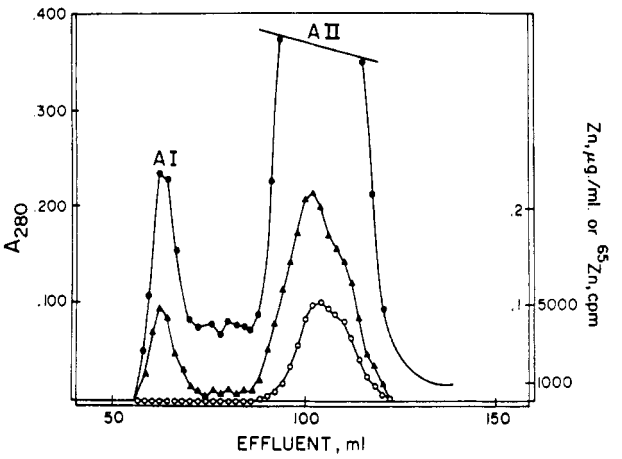

FIGURE 3: Gel filtration of fraction A. Fraction A, obtained from DEAE-cellulose chromatography of whole serum, was concentrated to 2 ml by pressure dialysis and applied to a 2 × 50 cm column of Sephadex G-100 equilibrated with 0.10 M Tris·Cl, pH 8.6. Carrier-free ^{65}Zn (20 μg) was added to serum prior to chromatography. Absorbance at 280 mμ (●—●—●), total zinc measured by atomic absorption (▲—▲—▲), and ^{65}Zn distribution (○—○—○) are plotted. The two peaks in order of elution are termed AI and AII, respectively. Similar results were obtained using Agarose A 0.5 m.

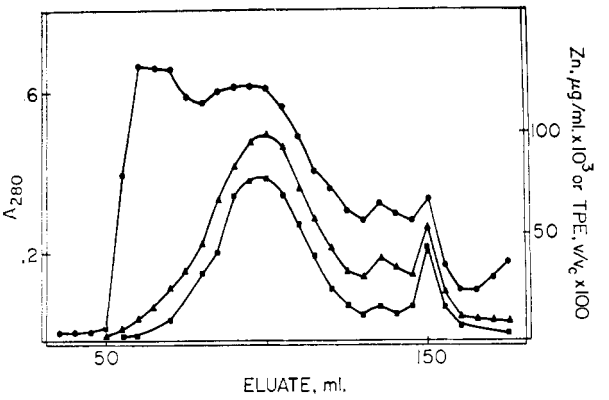

FIGURE 4: DEAE-cellulose chromatography of AI. Material constituting fraction AI obtained from Agarose gel filtration was dialyzed for 24 hr against two changes of 20 volumes each of 0.05 M Tris, 0.02 M succinic acid and applied to a 2 × 30 cm column of DEAE-cellulose as described in Methods. Gradient elution was carried out with 100 ml each of starting and limit buffer at 4°. Absorbance at 280 mμ (●—●—●), zinc content (▲—▲—▲), and the esterase activity (■—■—■) are plotted.

Peak A elutes at pH 7.5 and a conductivity of 1.0 mmho on the rising shoulder of the large albumin peak. Peak B elutes at pH 6.6, but its zinc content is lost on dialysis and, hence, has not been examined further. Further resolution of peak A is achieved by gel filtration on Sephadex G-100 (Figure 3). This procedure separates two major protein fractions, AI and AII, each containing significant amounts of zinc. AI appears in the excluded volume while AII emerges at a volume corresponding to an approximate molecular weight of 60,000, the latter based on comparison with the behavior of bovine hemoglobin and human albumin on the same column.

In order to examine the results of potential zinc contamination, carrier-free ^{65}Zn was added to serum in twofold excess over its native zinc content. When dialyzed immediately thereafter and chromatographed on DEAE-cellulose, 80% of the radioactivity is recovered in peak A (Figure 2), while the remainder is associated with peak B. When A is resolved by gel filtration, ^{65}Zn associates solely with AII (Figure 3). Under the conditions employed, the isotope neither binds nor exchanges with the zinc in AI. These findings indicate that fraction AI binds its native zinc very firmly and likely does not accumulate exogenous zinc during isolation.

The proteins constituting fractions AI and II have been analyzed by immunoelectrophoresis with antihuman serum. AII contains solely albumin while α_2-macroglobulin is the dominant immunoreactive species in AI (Figure 5, *q.v. infra*).

The identity of AI as α_2-macroglobulin and its functional integrity are confirmed by measuring its characteristic biological activity, trypsin–protein esterase. This activity is based on the ability of α_2-macroglobulin to bind trypsin, forming an enzymatically active complex. However, in the complex the esterase and peptidase activities of trypsin are protected from inactivation by soybean trypsin inhibitor. The amount of complex formed, and hence the degree of protection of trypsin activity, is proportional to the amount of α_2-macroglobulin present (Haverback *et al.*, 1962; Mehl *et al.*, 1964).

The esterase activity parallels the zinc concentration in fraction A from the initial DEAE-cellulose chromatogram and in fraction AI after gel filtration. Moreover, when AI is rechromatographed on DEAE-cellulose (Figure 1, step 1c) three zinc-containing protein peaks appear, each of which has the esterase activity closely related to its zinc content (Figure 4). The presence of multiple chromatographic peaks, each with the esterase activity, is consistent with the reported microheterogeneity of α_2-macroglobulin (Ganrot and Laurell, 1966).

The content of zinc and its relationship to biological activity have been determined at each stage of purification of the protein in accord with procedures commonly employed in the characterization of zinc metalloenzymes (Vallee and Wacker, 1970). Table I shows a typical preparation of α_2-macroglobulin in which both zinc and protein concentrations as well as the esterase activity are measured in the pooled

TABLE I: Zinc Content and Trypsin–Protein Esterase Activity of Fractions during Purification.[a]

	Zn (μg/g of protein)	Esterase Activity (units/g of protein)	Esterase Activity/Zn (units/μg)
Plasma	46	31	0.7
Fraction A	120	920	7.6
Fraction AI	234	2960	12.7
α_2-Macroglobulin	343	6060	17.7

[a] The measurement of trypsin–protein esterase activity is described in Methods. One unit equals 2.0 μmoles of H^+ released per minute in the presence of an otherwise completely inhibitory amount of soybean trypsin inhibitor.

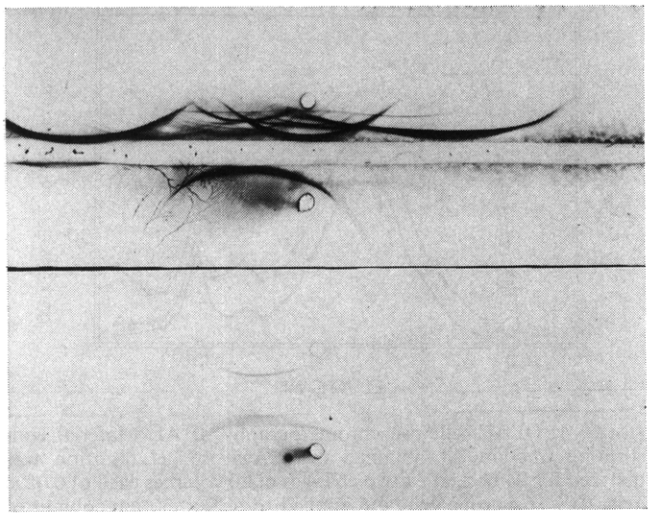

FIGURE 5: Immunoelectrophoresis demonstrating α_2-macroglobulin. Material from the major zinc-containing peak (AI) after rechromatography on DEAE-cellulose was pooled and then concentrated by pressure dialysis to a protein concentration of 50 mg/ml. Top: upper well, whole serum; lower well, zinc-containing α_2-macroglobulin. Antibody is rabbit antihuman serum. Bottom: upper well, whole serum; lower well, zinc-containing α_2-macroglobulin. Antibody is rabbit antihuman-α_2-macroglobulin.

TABLE II: Metal Content of Preparations of α_2-Macroglobulin.

Preparation	Starting Material	Zn (μg/g)
A.	Zinc Content by Atomic Absorption Spectrometry	
1	Individual serum[a]	390
2	Individual serum[a]	380
3	Individual serum[a,b]	320
4	Individual serum[a,b]	450
5	Pooled serum	770
6	Pooled plasma	560
7	Pooled plasma	340

B. Metal Content[c] by Emission Spectroscopy

	Zn	Ba	Fe	Ca	Cr	Mn	Mg
5	710	10	290	270	Tr	35	250
7	325	*	240	65	30	*	Tr

[a] Obtained from same donor. [b] These separations gave no signal for the following elements by atomic absorption spectroscopy: Co; Cr; Fe; Mg; Mn; and Ni. [c] Measured in micrograms per gram of protein: *, not detected; Tr, trace.

peak material comprising the α_2-macroglobulin-rich fractions. With progressive purification, the zinc content per gram of protein and the specific esterase activity of α_2-macroglobulin increase concomitantly. The ratio of catalytic activity to zinc concentration also increases, as expected of a system in which the activity measured is associated with one zinc protein, while inactive, zinc-containing moieties are eliminated. Notably, the most highly purified material does not exhibit any of the activities of known zinc enzymes potentially or actually present in serum, such as alkaline phosphatase, carboxypeptidase, or alcohol dehydrogenase.

Table II summarizes the zinc content of the most highly purified material in each of seven preparations studied so far, as measured by atomic absorption spectrometry (Table IIA). Preparation numbers 3 and 4 were also examined for Mg, Mn, Fe, Co, Cr, and Ni by means of this method though none of these elements could be found.

The metal content of two preparations (5 and 7, Table IIA) was also determined by emission spectrography (Table IIB). There was close agreement for the values of the zinc content in the two samples as determined by both methods, *i.e.*, 770 and 710 as well as 340 and 325 μg/g, respectively.

In Table IIA preparations 1–4, samples all prepared from the same starting material, vary least in zinc content, as might be expected. In contrast, preparations 5 to 7 were prepared from sera of different individuals. When analyzed by emission spectrography, preparations 5 and 7 contained significant quantities of iron and perhaps calcium (Table IIB).

The zinc-containing fraction AI was characterized further by immuno- and physicochemical procedures. Figure 4 shows the results of immunoelectrophoresis of a 5% solution of pooled material from the first zinc peak after DEAE rechromatography of peak AI. (Figure 5). The analysis employs *anti*human serum (top) and anti-α_2-macroglobulin (bottom). This pooled material precipitates with anti-α_2-macroglobulin, and only trace amounts of extraneous material are present, as demonstrated by the presence of two faint non-α_2-macroglobulin arcs on analysis with antihuman serum.

The sedimentation of rechromatographed fraction AI, in concentrations of 4 and 8 mg/ml, yields s values of 18.6 and 15.5, respectively, confirming its large molecular size. Figure 6 shows the sedimentation of an 8 mg/ml solution at a speed of 36,500 rpm. A single species is seen at 10 (left) and 40 (right) min after rotor speed is attained. The same material (40 μg) has been examined by disc electrophoresis in 4 and 7% polyacrylamide gels at pH 8.2 (Figure 7). One major and two faint minor bands are discernible, indicating a high degree of purification.

Discussion

α_2-Macroglobulin has herein been identified as a zinc metalloprotein of human serum. The close association of zinc with this macroglobulin during purification conforms to criteria proposed for identification of metalloproteins (Vallee, 1955; Vallee and Wacker, 1970). During isolation, the esterase activity, characteristic of α_2-macroglobulin, parallels zinc concentration, and both increase concomitantly during successive stages of purification. The activity:zinc ratio also increases, probably due to the elimination of zinc in albumin, and, possibly, small amounts in other protein moieties (Table I). Zinc in α_2-macroglobulin does not exchange with ^{65}Zn during isolation, suggesting that the metal is bound with a high stability constant, a feature also characteristic of other metalloproteins (Vallee and Wacker, 1970). Zinc is present in all preparations of α_2-macroglobulin, obtained as outlined herein, whether serum or plasma serves as starting material. The amounts of zinc reported, *i.e.*,

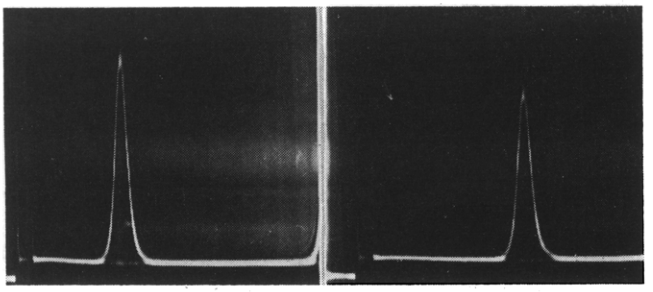

FIGURE 6: Ultracentrifugation of zinc-containing α_2-macroglobulin. The photographs are taken at 10 and 40 min after attaining speed (35,600 rpm) at a phase-plate angle of 70°. The material examined is pooled from the major zinc-containing peak after DEAE-cellulose rechromatography (Figure 5). The protein concentration was 8 mg/ml in 0.05 M Tris·Cl, 0.1 M NaCl (pH 8.0). The direction of sedimentation is from left to right.

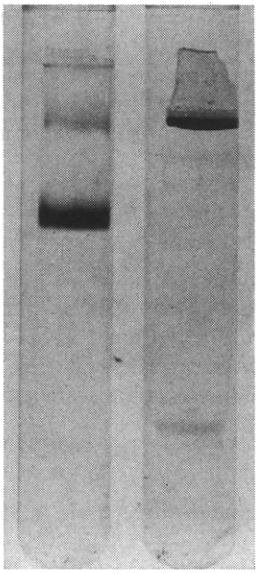

FIGURE 7: Disc gel electrophoresis of zinc-containing α_2-macroglobulin. The material (40 μg) pooled from the major zinc-containing peak after DEAE-cellulose rechromatography was submitted to electrophoresis in 4% (left) and 7% polyacrylamide gels (right). Electrophoresis was at 100 V for 1 hr. The anode is at bottom. The buffer was 0.025 M Tris-glycine, pH 8.2.

320–770 μg/g of protein (Table II), correspond to 3.3–7.9 g-atoms of Zn/mole of α_2-macroglobulin (molecular weight approximately 840,000) (Schonenberger *et al.*, 1968). While these amounts of zinc are stoichiometrically significant, caution must be exercised in establishing exact numerical relationships. The problems encountered in assigning precise molar stoichiometry to the metal content of multichain zinc proteins (Adelstein and Vallee, 1958; Simpson *et al.*, 1968; Drum *et al.*, 1969) have been detailed recently (Drum *et al.*, 1969).

While the α_2-macroglobulin here isolated exhibits a biological activity unique among the serum proteins, it must be emphasized that it is not known to catalyze any biological reactions. The characteristic assay for this α_2-macroglobulin depends on the ability of this protein to form a trypsin complex which exhibits esterolytic in the presence of otherwise completely inhibitory concentrations of soybean trypsin inhibitor. Thus, it must be kept in mind that under the conditions present, α_2-macroglobulin itself does not hydrolyze the trypsin substrate, tosylarginine methyl ester (Haverback *et al.*, 1962; Mehl *et al.*, 1964); the esterolytic activity of trypsin is maintained fully on binding to α_2-macroglobulin when soybean trypsin inhibitor would completely abolish tryptic activity in its absence.

Zinc (and other) metalloenzymes are inhibited when the active site metal is removed or complexed by chelating agents (Vallee and Wacker, 1970). Preliminary investigations suggest that chelating agents which remove zinc from α_2-macroglobulin neither abolish the enzymatic activity nor the protective characteristic of the trypsin–α_2-macroglobulin complex. The complex owes its enzymatic activity to trypsin which, of course, is not a metalloenzyme and, hence, would not be expected to be inhibited by chelating agents. In what manner, if any, the zinc atoms of α_2-macroglobulin might play a role in trypsin binding and/or the functions and structures of α_2-macroglobulin in protecting the activity of trypsin from inhibition is not clear. While it is conceivable that zinc might be a component of another species bound strongly to α_2-macroglobulin, there is no current evidence to support this hypothesis.

The zinc in α_2-macroglobulin represents a substantial fraction of the total serum zinc, estimated to be 30–40%, based on Agarose gel filtration. Most of the remaining zinc is apparently bound to albumin (Vikbladh, 1951; Vessel and Bearn, 1957). While albumin readily binds ^{65}Zn, as shown earlier by Gurd and Goodman (1952), fraction AI does not take up ^{65}Zn or exchange easily with it (Figure 3). Thus the characteristics of α_2-macroglobulin are in accord with those of metalloproteins, while the looser association of zinc with albumin resembles more closely a "metal–protein complex" (Vallee, 1955; Vallee and Wacker, 1970).

Iron and possibly calcium are present in significant amounts in the preparations analyzed by emission spectrography (Table IIB). However, in two other preparations (no. 3 and 4, Table IIA), iron was not detected by atomic absorption spectroscopy. Since iron and calcium have been detected in significant quantities in zinc metalloproteins derived from other sources (Vallee *et al.*, 1959; Simpson *et al.*, 1968), the potential significance of these observations requires further evaluation.

The detection of zinc in α_2-macroglobulin offers further opportunity for the exploration of the biological role of a macromolecule whose physiologic function is not completely understood. Since α_2-macroglobulin also binds other proteins of biologic importance, *e.g.*, thrombin and plasmin (Schultze *et al.*, 1963), the relationship of the metal to the binding of each of these proteins can now be examined. Moreover, the implications of the presence of zinc in α_2-macroglobulin assume greater potential significance when it is recalled that several human diseases are accompanied by marked depression of serum zinc concentrations (Vallee, 1959; Parisi and Vallee, 1969). Therefore, the significance of low concentrations of serum zinc may now be explored through studies of the partition of the element between the α_2-macroglobulin and albumin fractions in such disease states. The relationship of copper to ceruloplasmin in sera from patients with Wilson's disease and the state of saturation of transferrin in iron deficiency or iron

overload may be cited as precedents in which the pursuit of metal–protein interactions in human biochemistry has ultimately aided the elucidation of disease processes. Investigations utilizing both normal and pathologic sera are currently in progress in order to enlarge upon the role of zinc in α_2-macroglobulin.

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