Acknowledgments

We are very thankful to Dr. E. Miller for details of the procedure for separating CNBr peptides from tropocollagen, and to Silvia Padilla for her excellent technical assistance.

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Isolation of a Nickel α_2-Macroglobulin from Rabbit Serum*

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ABSTRACT: Measurements of nickel were performed by atomic absorption spectrometry upon serum protein fractions which were separated successively by ultrafiltration and by column chromatography on Sephadex G-200 and on DEAE-cellulose. Rabbit serum was used as the starting material, since measurements showed that nickel concentrations in serums from rabbits (mean = 9.3 μg/l.; std dev $\pm$ 2.2; range 6.5–14.0; N = 24) were higher than in serums from man or ten other mammalian species. Nickel in rabbit serum was found to exist in three forms: (a) ultrafiltrable nickel (16% of total Ni), (b) albumin-bound nickel (40% of total Ni), and (c) in a nickel–metalloportein which was named "nickeloplasmin" (44% of total Ni). Disc gel and immunoelectrophoresis showed that purified nickeloplasmin migrates as a single protein band in the α_2-globulin region. Sedimentation equilibrium studies indicated that nickeloplasmin is a macroglobulin, with estimated molecular weight of 7.0×10^5. Following dialysis of rabbit serum against buffer containing Ni(II), the albumin-bound nickel became increased, but nickel content of the nickeloplasmin fraction was not affected.

Himmelhoch *et al.* (1966) separated human serum proteins by gradient chromatography on DEAE-cellulose columns, and measured the trace metal content of the eluted protein fractions by means of emission spectrography. Their data suggested the presence in serum of a metalloprotein which is rich in nickel, and which does not contain any detectable calcium, magnesium, strontium, barium, iron, zinc, manganese, aluminum, chromium, lead, cadmium, molybdenum, or tin. The present study confirms the existence of a serum nickel metalloprotein (which we have named "*nickeloplasmin*"), and furnishes additional information regarding its chemical properties. Rabbit serum was selected as the starting material for isolation of serum nickeloplasmin, since pre-

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liminary measurements indicated that concentrations of nickel in rabbit serums were greater than in serums from man or ten other mammalian species.

Materials

Precautions. Concentrated acid and base reagents were of Ultra Pure grade (E. Merck Co., Darmstadt, West Germany). Water was deionized and distilled in an all-glass still. Glassware was washed with nitric acid (200 g/l.) and rinsed with copious water immediately before use. Dialysis tubing, ultrafiltration membranes, chromatographic media, and buffer reagents were demineralized as described by Himmelhoch *et al.* (1966) and by Parisi and Vallee (1970).

Collection of Blood. In order to determine nickel concentrations in serums from healthy adult animals, blood was collected by venepuncture from 4 Jersey cattle (2♂, 2♀), 4 American domestic horses (2♀, 2♂), 3 British Alpine goats (1♀, 2♂), 7 Yorkshire white pigs (4♀, 3♂), 4 Shropshire sheep (2♀, 2♂), 4 beagle dogs (2♀, 2♂), 3 American domestic cats (2♀, 1♂), 24 New Zealand white rabbits (12♀, 12♂), 3 Dutch black rabbits (1♀, 2♂), and 3 American brown rabbits (1♀, 2♂). Blood was collected by cardiac puncture from 3 guinea pigs (2♀, 1♂), 3 Syrian golden hamsters (1♀, 2♂), 11 Fischer white rats (6♀, 5♂), 4 New Hampshire red chickens (2♀, 2♂), and 4 Maine lobsters (2♀, 2♂). The guinea pigs, hamsters, and rats were anesthetized with ether. A dental drill was used to remove a disk (1-cm diameter) from the carapace of each lobster in order to facilitate cardiac puncture. Precautions to avoid nickel contamination of the blood samples were observed, as previously described (Nomoto and Sunderman, 1970). After the blood had clotted at room temperature, the serum was removed and was stored frozen at −15° until analysis. As the starting material for purification of nickeloplasmin, pooled serum (6 l.) from approximately 150 New Zealand white rabbits was prepared by Pel-Freez Biologicals, Inc., Rogers, Ark., with precautions to avoid nickel contamination. The pooled rabbit serum was shipped frozen, and was received in the laboratory within 4 days after the blood was collected.

Methods

Nickel analyses in serums and chromatographic fractions were performed by the atomic absorption method of Nomoto and Sunderman (1970), with the following modification. Samples (5 ml) of serums or fractions were transferred to centrifuge tubes, and 6 ml of trichloroacetic acid solution (150 g/l.) was slowly added to each tube, with constant mixing. The tubes were kept at 25° for 30 min and then were centrifuged at 900*g* for 15 min. The protein-free supernatant fluids were decanted into a second set of centrifuge tubes. Trichloroacetic acid solution (4 ml) was added to each of the original tubes, and the precipitated proteins were resuspended by means of a Vortex mixing apparatus. The tubes were again centrifuged at 900*g* for 15 min, and the protein-free washings were combined with the original supernatant fluids. Analysis was continued as described by Nomoto and Sunderman (1970) beginning at step 4.

Zinc analyses were performed by the atomic absorption technique of Prasad *et al.* (1965).

Ceruloplasmin analyses were performed by the *p*-phenylenediamine oxidase technique of Sunderman and Nomoto (1970).

Protein analyses were performed by the biuret technique of Savory *et al.* (1970).

Cellulose acetate electrophoresis was performed by the Microzone technique of Savory *et al.* (1970).

Disc electrophoresis in acrylamide gel (4%) was performed with a Canalco gel electrophoresis apparatus (Canal Instrument Corp., Bethesda, Md.). The buffer was Tris–glycine (0.025 mole/l., pH 8.6); the current was 100 V and the duration of electrophoresis was 1 hr. Protein staining with coomassie blue was performed by the technique of Weber and Osborne (1969).

Immunoelectrophoresis in agar gel was performed by the Ouchterlony method (1968), using horse anti-rabbit antiserum (Kallstadt Laboratories, Inc., Minneapolis, Minn.).

Sedimentation equilibirum measurements were performed at 22° in a Spinco Model E analytical ultracentrifuge, as described by Yphantis (1964), except that photoelectric scanning was used instead of interference optics. For estimations of the molecular weight of nickeloplasmin, a partial specific volume of 0.73 ml/g was assumed (Schachman, 1957).

Ultrafiltration. A Diaflo pressure dialysis apparatus (Model TCF-10 with PM-10 membrane (Amicon Corp., Lexington, Mass.)) was used for ultrafiltration of pooled rabbit serum. The specific gravities of the serum, ultrafiltrates and residues were determined at 20° with a micropyknometer, so that the concentrations of nickel per liter of solution could be converted to concentrations per kilogram of H_2O. Computations of ultrafiltrable nickel were performed as described by Sunderman (1966).

Column chromatography on Dextran Gel. Sephadex G-200 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) was equilibrated at 25° with Tris-HCl buffer (pH 8.6, 0.1 mole/l.), and was introduced into a chromatography column (2.5 × 45 cm) equipped for ascending migration. The packed column of Sephadex G-200 was washed with Tris-HCl buffer for 48 hr before the first fractionation, and for 24 hr before each subsequent fractionation. The void volume of the column was 50 ml, and the flow rate averaged 20 ml/hr. The absorbance of the effluent buffer was monitored at 280 nm. Pooled rabbit serum (150 ml) was concentrated to 37.5 ml by ultrafiltration and then was diluted to 75 ml with Tris-HCl buffer. Ten milliliters of the resultant solution was taken for each chromatographic fractionation. Effluent buffer from the chromatographic column was collected in 5.5- or 11-ml fractions. Five or ten milliliters of each fraction was used for nickel analysis, and 0.5 or 1 ml was used for measurements of total protein and electrophoretic fractionations.

Column chromatography on DEAE-cellulose was performed at 25° by descending migration in a 2.5 × 45 cm column. The DEAE-cellulose (Sigma Chemical Co., St. Louis, Mo.) was packed to a height of 20 cm, and was washed with "starting" buffer (Tris-HCl, pH 8.6, 0.25 mole/l.) for 48 hr before the first fractionation. Before each subsequent fractionation, the column was washed for 18 hr with "final" buffer (Tris-HCl, pH 8.6, 1.0 mole/l.), and then for 6 hr with starting buffer. The flow rate averaged 40 ml/hr. The starting material for chromatography on DEAE-cellulose was pooled fractions A + B, which were obtained by chromatography on Sephadex G-200 (see Results). Fifteen milliliters of pooled fractions A + B was concentrated to 5 ml by centrifugal ultrafiltration using a Centriflow CF-50A membrane (Amicon Corp.), and was layered on the top of the DEAE-cellulose column. Elution was performed with a linear concentration gradient (300 ml) of Tris-HCl buffer (pH 8.6) ranging from 0.25 to 1.0 mole/l. The absorbance of the effluent was monitored at 280 nm, and 8- or 10-ml fractions were collected for nickel and zinc analyses and for electrophoretic fractionations. Protein concentra-

TABLE I: Nickel Concentrations in Serums from Healthy Adults of Several Species.

Species	No. of Subjects	Nickel Concn (μg/l.) Mean ± Std Dev	Range
Man (*Homo sapiens*)	40	2.6 ± 0.8	1.1–4.6
Jersey cattle (*Bos taurus*)	4	2.6	1.7–4.4
American domestic horses (*Equus caballus*)	4	2.0	1.3–2.5
British alpine goats (*Capra linius*)	3	3.5	2.7–4.4
Yorkshire white pigs (*Sus scrofa domestica*)	7	5.3 ± 1.9	3.5–8.3
Shropshire sheep (*Ovis aries*)	4	3.3	2.9–4.2
Beagle dogs (*Canis familiaris*)	4	2.7	1.8–4.2
American domestic cats (*Felis catus*)	3	3.7	1.5–6.4
New Zealand white rabbits (*Oryctolagus cuniculus*)	24	9.3 ± 2.2	6.5–14.0
Dutch black rabbits (*Oryctolagus cuniculus*)	3	7.2	6.6–7.9
American brown rabbits (*Oryctolagus cuniculus*)	3	8.7	8.6–8.9
Guinea pigs (*Cavia porcellus*)	3	4.1	2.4–7.1
Syrian golden hamsters (*Mesocricetus auratus*)	3	5.0	4.2–5.6
Fischer white rats (*Rattus* sp.)	11	2.7 ± 1.0	0.9–4.1
New Hampshire red chickens (*Gallus domesticus*)	4	3.6	3.3–3.8
Maine lobsters (*Homarus americanus*)	4	12.4	8.3–20.1

tions in these fractions were estimated on the basis of their absorbances at 280 nm, calibrated by reference to absorbances of dilutions of starting material (pooled fractions A + B) which had been analyzed for protein content by the biuret technique.

Dialysis Experiments. Pooled rabbit serum (50 ml) was placed in cellophane dialysis tubing and dialyzed for 24 hr at 6° in a rotating dialysis apparatus (Oxford Laboratories, Redwood City, Calif.). The dialysis fluid consisted of 4 l. of NH_4Cl solution (0.15 mole/l., pH 7.4), containing $NiCl_2$ (0.12 mg of Ni/l.).

Results

The concentrations of nickel which were found in serums from several species of animals are compared in Table I to the nickel concentrations in serums from 40 healthy human volunteers, as reported by Nomoto and Sunderman (1970). There were no significant differences between the nickel concentrations in serums from males and females, in any of the species. The highest mean concentration of nickel was present in lobster serum. Of the mammalian species which were studied, the highest concentrations of nickel were found in rabbit serums. Since the nickel content of rabbit serum averaged 3.5 times that of human serum, rabbit serum was chosen as the starting material for purification of nickeloplasmin. The nickel concentration in pooled rabbit serum which was prepared by Pel-Freez Biologicals, Inc. (9.0 μg/l.), did not differ significantly from the mean concentration of nickel in 24 individual serums from New Zealand white rabbits which were collected in the authors' laboratory (9.3 μg/l.).

TABLE II: Partition of Nickel in Pooled Rabbit Serum.

Fraction	Proportion of Total Serum Ni[a] (%)	Ni Concn (μg/l. of Serum)
Pooled rabbit serum	100	9.0
Ultrafiltrable nickel	16 (15–17)	1.4
Albumin-bound nickel	40 (31–52)	3.6
Nickeloplasmin	44 (39–53)	4.0

[a] Mean (and range), based upon three separate experiments.

Ultrafiltrable nickel in pooled rabbit serum averaged 16% of the total serum nickel (Table II). A typical chromatographic fractionation of serum proteins and nickel on Sephadex G-200 is illustrated in Figure 1. The starting material was 10 ml of the residue obtained after ultrafiltration of rabbit serum. The recovery of nickel in the sum of the collected fractions was 101% of the nickel which was present in the starting material. Virtually identical chromatographic fractionations were obtained in three separate experiments. Chromatography on Sephadex G-200 separated serum protein-bound nickel into two distinct moieties: (a) *nickeloplasmin*, which was present in fractions A and B; and (b) *albumin-bound nickel*, which was present in fractions E–K. Ceruloplasmin was not detected in fractions A and B, but was present in fractions C and D. Electrophoresis on cellulose acetate showed that fractions A–F contained serum globulins, and fractions E–K contained serum albumin. As indicated in Table II, an average of 40% of total nickel in rabbit serum was present in the albumin fractions (E–K) and 44% was associated with nickeloplasmin (fractions A and B).

The starting material for column chromatography on DEAE-cellulose was 15 ml of combined fractions A and B

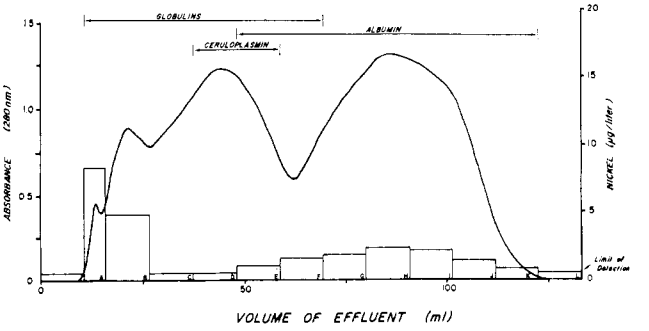

FIGURE 1: Chromatography of rabbit serum proteins on dextran gel (Sephadex G-200), as described in Methods. Absorbance at 280 nm is plotted as a solid line, and nickel concentrations are shown by bar graphs.

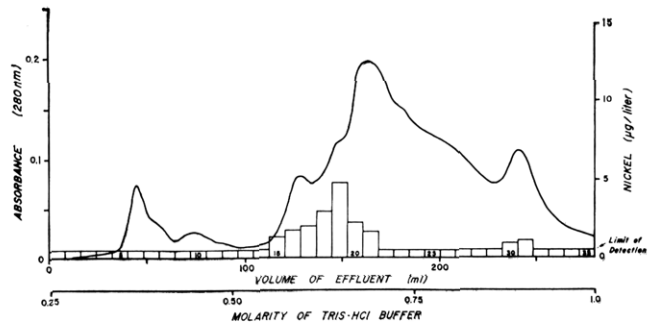

FIGURE 2: DEAE-cellulose chromatography of combined fractions A + B obtained by chromatography on Sephadex G-200 (see Methods). Absorbance at 280 nm is plotted as a solid line, and nickel concentrations are shown by bar graphs.

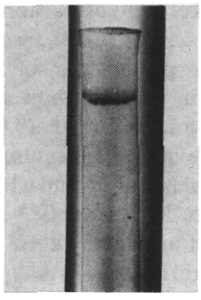

FIGURE 3: Disc electrophoresis of rabbit serum nickeloplasmin. Material (40 μg of protein) in fraction 19 obtained by DEAE-cellulose chromatography was submitted to electrophoresis in 4% acrylamide gel at 100 V for 1 hr (see Methods). The anode is at the bottom. Protein was stained with coomassie blue.

obtained by chromatography on Sephadex G-200. A typical chromatographic fractionation of proteins and nickel on DEAE-cellulose is illustrated in Figure 2. The recovery of nickel in the sum of the collected fractions was 109% of the nickel which was present in the starting material. Qualitatively identical chromatographic fractionations were obtained in three separate experiments. A nickel-containing protein (nickeloplasmin) was eluted from the DEAE-cellulose column in a peak which was distributed in fractions 15–21. Fraction 19, which was eluted at a buffer concentration of 0.65 mole/l., consistently contained the highest relative concentration of nickel. In the three experiments, the concentrations of nickel in fraction 19 were 2.3, 4.7, and 4.8 μg/l., respectively.

A zinc-containing protein was eluted from the DEAE-cellulose column in a peak which was distributed in fractions 9–12, corresponding to buffer concentrations ranging from 0.40 to 0.53 mg per l. There was no detectable zinc in any of the other chromatographic fractions. The zinc–protein peak was completely separated from the nickeloplasmin peak. Electrophoresis on cellulose acetate showed that the protein in fraction 19 obtained in each of the three experiments migrated as a narrow band in the α_2-globulin region of the serum electrophoretic pattern. Disc electrophoresis on acrylamide gel showed that the fraction 19 obtained in the third experiment contained a single protein peak (Figure 3). Immunoelectrophoresis of this fraction 19 revealed a single precipitin arc in the α_2-macroglobulin region (Figure 4). Sedimentation equilibrium studies indicated that the protein in fraction 19 was a macroglobulin with an approximate molecular weight of 7.0×10^5.

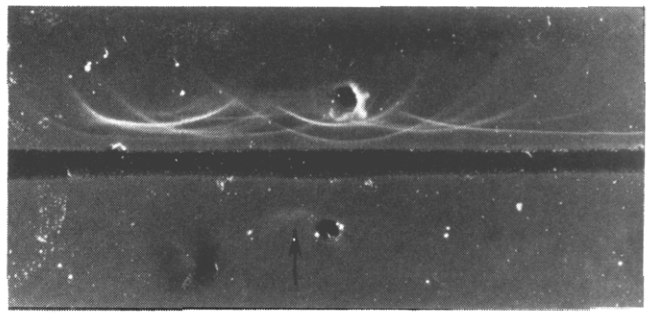

FIGURE 4: Immunoelectrophoresis of rabbit serum nickeloplasmin. Antibody is horse antirabbit serum. The anode is at the left. Top well: whole rabbit serum. Bottom well: fraction 19 obtained by DEAE-cellulose chromatography (protein 70 μg/ml). Nickeloplasmin is seen as a single precipitin arc (arrow) in the α_2-macroglobulin region.

TABLE III: Nickel:Protein Ratios during Purification of Serum Nickeloplasmin.

Purification Step	Protein Concn (g/l.)	Ni Concn (μg/l.)	Ni: Protein Ratio (μg of Ni/g of Protein)
Pooled rabbit serum	60	9.0	0.15
Residues after ultrafiltration	146	23.5	0.13
Fraction B (chromatography on Sephadex G-200)	3.3	4.9	1.5
Fraction A (chromatography on Sephadex G-200)	1.0	8.3	8.3
Fraction 19 (chromatography on DEAE-cellulose)	0.070	4.8	68

The concentrations of protein and nickel which were present in certain fractions which were obtained by ultrafiltration and chromatography are listed in Table III. It may be seen that the nickel/protein ratios (μg of Ni/g of protein) increased from 0.13 in the residue after ultrafiltration, to 8.3 in fraction A obtained by chromatography on Sephadex G-200, and to 68 in fraction 19 obtained by chromatography on DEAE-cellulose. Based upon an estimated molecular weight of 7.0×10^5, purified nickeloplasmin contained approximately 0.82 g-atom of nickel/mole of protein.[1]

When pooled rabbit serum was dialyzed for 24 hr *vs.* 80 volumes of NH_4Cl buffer which contained Ni(II) in a concentration of 120 μg/l., the concentration of nickel in the serum increased from 9.0 to 530 μg per l. The dialyzed serum was concentrated by ultrafiltration and then subjected to chromatography on Sephadex G-200. The concentration of nickel in pooled fractions A + B was the same in a control

[1] Caution should be exercised in interpreting such estimates of molar stoichiometry of the metal content of metalloproteins, as emphasized by Parisi and Vallee (1970). If computations are based upon a reported molecular weight of 8.2×10^5 for serum α_2-macroglobulin (Schultze and Heremans, 1966), the purified nickeloplasmin contains approximately 0.95 g-atom of nickel/mole of protein.

sample which was not dialyzed against Ni(II). Massive increases in the concentrations of nickel were found in fractions E–K, which contained serum albumin. From this experiment, it appears that Ni(II) becomes bound to serum albumin *in vitro*, and that it does not become bound to serum nickeloplasmin.

Discussion

Himmelhoch and associates (1966) suggested that a nickel–metalloprotein might be present in serum, based upon: (a) the repeated demonstration of nickel in a single chromatographic fraction of proteins obtained from dialyzed human serum, and (b) the fact that the nickel-containing protein fraction did not contain any other detectable trace metals. For chromatographic fractionations on DEAE-cellulose, Himmelhoch *et al.* (1966) employed a Tris-succinate buffer system, with a concentration gradient ranging from 0.045 to 0.6 mole per l. They found that the nickel–metalloprotein was eluted in the limit buffer (0.6 mole/l.). This concentration corresponds approximately to the molarity of Tris-HCl buffer (0.65 mole/l.) at which rabbit serum nickeloplasmin was eluted from DEAE-cellulose in the present study. From our observations, it appears that nickel is firmly bound to nickeloplasmin, so that it is not removed during successive purification procedures. The sequential increases in the ratios of nickel to protein which were observed during stepwise purification of nickeloplasmin conform to one of the proposed criteria for identification of metalloproteins (Wacker, 1970; Vallee and Wacker, 1970). The failure of the nickeloplasmin fraction to bind Ni(II) after equilibrium dialysis is also in accordance with the properties of metalloproteins. The present observation that serum nickeloplasmin is an α_2-macroglobulin is strikingly similar to the finding by Parisi and Vallee (1970) that the principal zinc metalloprotein of human serum is also an α_2-macroglobulin. Parisi and Vallee did *not* detect any nickel in purified preparations of serum zinc α_2-macroglobulin, nor did the present authors detect any zinc in chromatographic fractions containing nickeloplasmin.

In vitro binding of Ni(II) to serum albumin, which was observed in the present study, is in agreement with earlier reports (Coddington and Perkins, 1961; Rao, 1962). Based upon optical rotatory dispersion and circular dichroism spectra of Ni(II) complexes with synthetic peptides and bovine serum albumin, the amino, carboxyl, and imidazole ligands probably constitute the principal binding-sites for Ni(II) (Bryce and Gurd, 1966; Tsangaris *et al.*, 1969). The occurrence of nickel in preparations of human serum albumin has been reported by Russanov and Balevska (1964) and Malvano *et al.* (1967).

Clinical interest in serum nickel-containing proteins has been stimulated by the finding that serum nickel concentrations are frequently increased following myocardial infarction. D'Alonzo and Pell (1963) observed increased nickel concentrations in 19 of 20 serums which were obtained from patients with acute myocardial infarction within 24 hr after admission to the hospital. Sunderman *et al.* (1970) reported increased nickel concentrations in 21 of 29 serums which were collected from patients with acute myocardial infarction during the period from 12 to 36 hr after onset of symptoms. Determinations of the concentrations of nickel in albumin and globulin fractions of serum proteins from healthy human volunteers and from patients with myocardial infarction are currently in progress in our laboratory. Attempts are also being made (a) to isolate nickeloplasmin from human as well as rabbit serum, (b) to perform spectrographic analyses of purified nickeloplasmin for possible content of other trace metals, (c) to measure the *in vivo* incorporation of ^{63}Ni into rabbit serum nickeloplasmin, and (d) to determine whether nickeloplasmin possesses the trypsin–protein esterase activity which is characteristic of serum α_2-macroglobulins (Parisi and Vallee, 1970).

Acknowledgments

We thank Mrs. Maria Nechay and Mr. Richard Morang for skillful technical assistance; Dr. William Daniels and Mr. Andrew Brennan for assistance in obtaining blood from several species of animals; Dr. Robert Votaw and Mr. Robert S. Munson for protein fractionations by disc gel electrophoresis; Dr. Irwin H. Lepow and Mr. David Y. Conlin for immunoelectrophoretic studies; and Dr. Eugene Wampler, Dr. Maria Decsy, and Miss Florence Woodiel for estimations of molecular weight by high-speed sedimentation equilibrium.

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