Comparison of Copper Binding Components in Dog Serum with Those in Other Species

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Abstract. **The copper content of dog serum and its distribution to copper binding proteins was compared with that of rat and mouse. Total serum Cu concentrations of dogs and mice were one third those of the rat. Plasma ceruloplasmin, determined by azide-inhibitable oxidase activity with two substrates, was 8-fold less in the dog and 9- to 20-fold less in the mouse than in the rat, and, in both dogs and mice, there was 70–75% less ceruloplasmin Cu, determined by atomic absorption after gel filtration. In the dog, the largest proportion of total and exchangeable serum Cu was with the transcuprein fraction. Only one third as much Cu was with albumin in the dog (and mouse) versus the rat, and this was released much more readily through dialysis. In dogs and mice, the exchangeable (nonceruloplasmin) serum copper pool was half the size of that in rats and humans. Especially in the mouse (but also in rats and dogs), a small proportion of the exchangeable pool appeared bound to ferroxidase II. We conclude that the dog may rely more on transcuprein and low molecular weight complexes and less on albumin and ceruloplasmin for transport of copper to cells.** [P.S.E.B.M. 1992, Vol 200]

Man and the rat have been the main objects of research on copper distribution and metabolism in the mammal. From work with these species, it is apparent that the liver and kidney are the organs richest in copper (1). Indeed, the liver is a central organ of copper metabolism, being (with kidney) the initial recipient of most incoming dietary or injected copper (2); the source of nondialyzable serum copper (3, 4) in the form of ceruloplasmin (5), into which newly absorbed copper is rapidly incorporated, and which comprises about 65% of the copper in this fluid (6, 7); and a major source (and route) for copper excretion from the body, via the bile (8, 9). Copper homeostasis appears to be maintained primarily, but not exclusively, by varying excretion (10, 11), so that copper concentrations in liver (and other organs and tissues) stay rather constant, even with large differences in copper intake (12) or injection (10).

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Upon absorption, copper is initially carried to the liver from the intestine by the blood plasma, attached to albumin and transcuprein (2, 13). Albumin is the most abundant plasma protein. Transcuprein is much less abundant and less well characterized, with an M_r of 270,000–300,000 (2, 14). A high affinity binding site for copper on rat and human albumin involves the three N-terminal amino acids, including a histidine in the third position (15, 16). It has a dissociation constant of 10^{-17} alone, and 10^{-22} M in the presence of histidine, which forms a ternary albumin-Cu-his complex (17). The affinity of transcuprein for copper must be even greater, because it preferentially binds added copper in the presence of large amounts of albumin (2, 13, 14). Transcuprein and albumin rapidly exchange copper with each other (as determined with ^{67}Cu and ^{64}Cu isotopes), and it is possible that transcuprein is the actual donor of the metal to hepatocytes, since albumin tends to slow uptake of Cu(II) when added to cultured hepatocytes (18) and other cells (19).

In hepatocytes, incoming copper is used in at least three ways: some of it goes directly into the bile, some is used for internal enzymes (such as cytochrome *c* oxidase and superoxide dismutase), and a significant portion is incorporated into ceruloplasmin for export

into the blood plasma. Ceruloplasmin, in the circulation, becomes a source of copper for peripheral cells and tissues (2, 10, 20), where it is absorbed by a process involving specific cell surface receptors (11, 21–23).

While concentrations of copper in blood (and blood plasma) are not as high as those in liver, kidney, and some other tissues, copper in human blood is a quantitatively significant part of the whole body copper pool (1, 11). In rats and humans, the total plasma concentration is normally just over 1 μg/ml (very similar to concentrations of Fe and Zn). When plasma or serum is fractionated by gel chromatography on Sephadex G-150 (2, 6, 7, 11), the main copper binding components are partially separated, with the immediate elution of transcuprein (M_r = 270,000–300,000), followed by ceruloplasmin (132,000), albumin (68,000), and some low molecular weight components of unknown nature (6). Based on gel profiles of a fair number of samples (determined by furnace atomic absorption), the percentage of plasma copper attributable to these components in human plasma (or serum) is about 8%, 65%, 18%, and 8%, respectively (2, 7, 11). With regard to albumin and transcuprein, this means about 80–150 ng of Cu/ml associated with each fraction, although the albumin concentration alone (about 40 mg/ml) would allow a total binding of more than 40 μg of Cu/ml. (The albumin sites are, thus, highly unsaturated.) The distribution of copper among components of rat plasma is quite similar (2; this study).

While reviewing work on the binding of copper to albumin, we became aware that the dog presents an aberration of the "normal" picture of plasma copper distribution and metabolism described. Dog albumin is missing the crucial histidine in the third position from the N terminus (24, 25). Thus, it has a much lower (and perhaps rather nonspecific) affinity for copper. This suggests that albumin might not play the same role in copper transport in the dog as it does in other species and that transcuprein might be even more important. The dog also has the propensity to accumulate high levels of copper in the liver (26–28), the extreme being the Bedlington terrier, which routinely dies of liver copper toxicosis (29–31). The Bedlington terrier has thus been proposed as an animal model for Wilson's disease, which presents a similar picture for the human. However, dogs in general have the same tendencies, even normal dogs having liver copper concentrations more than 10 times those for the rat or human (about 40 μg/g or more) (30). These phenomena might be linked to differences in serum copper carriers, although another factor is reduced biliary excretion (32).

Apart from albumin, the copper components of dog serum have not been well characterized, nor has the actual distribution of copper among components of the serum been examined. The present work demonstrates that this distribution differs in several different ways from that of other species, but that albumin is still a carrier of some exchangeable copper in the plasma.

Materials and Methods

Serum Samples. Samples (1–5 ml) of fresh, leftover dog serum (less than 8 hr old) were obtained courtesy of Dr. Richard Glassberg, our local veterinarian, at Sunnycrest Animal Care Center (Fullerton, CA). None of the dogs used as sources had any serious health problems. Some (5–10 ml) samples of fresh blood were also obtained from 10 healthy dogs at the local dog pound. The dogs were of a variety of breeds, mature (weighing 20 kg or more), and of both sexes. Serum was collected by centrifugation. Rat serum was obtained from normal, adult, female Sprague-Dawley rats, as described previously (2). Human serum from adult women was volunteered by several laboratory personnel and the blood was drawn at the University Medical Center. Mouse serum was obtained from adult females of the Swiss and BALB/c strains, as described for the rat. All serum samples were either analyzed fresh or (more frequently) stored at −20°C until use. Several episodes of freezing and thawing had no affect on the enzyme activities assayed in the sample, although samples were frozen and thawed no more than twice.

Ceruloplasmin and Ferroxidase II Assays. The activity of both enzymes was assayed by following the oxidation of synthetic substrates spectrophotometrically in the absence (ceruloplasmin plus ferroxidase II activity) and presence (ferroxidase II activity only) of N_3^- (3 m*M*). Oxidase activity was measured with *p*-phenylenediamine by continuous monitoring of absorbance at 540 nm (at 37°C), as described previously (33), and with *o*-dianisidine by comparing the absorbance at 10 min and 40 min at 30°C, after acid precipitation (34).

Copper Analysis. The copper content of serum and purified fractions was determined by furnace atomic absorption spectroscopy using a model 457 spectrometer from Instrumentation Laboratories (Wilmington, DE). Serum samples were diluted 5-fold with 20 m*M* K phosphate buffer (pH 7.0) prior to analysis.

Binding of Copper to the Exchangeable Serum/Copper Pool. The binding of copper to components of the exchangeable (nonceruloplasmin) copper pool of serum was monitored by *in vitro* addition of tracer radioactive copper (2–50 ng of Cu) to 1.0-ml portions of serum, followed by fractionation on 50-ml columns of Sephadex G-150, equilibrated with a 20 m*M* K phosphate buffer (pH 7.0) as described previously (2). Aliquots (10 ng or less) of radioactive copper as ^{67}Cu(II) (carrier free, from the reactor at the University of Missouri; courtesy of Dr. Kurt Zinn), all as the 1:1 (mole:mole) nitrilotriacetic acid (NTA) complex, were added to serum 30 min to 18 hr before samples were fractionated on gel columns. One-milliliter fractions

were collected and analyzed for radioactivity using an automatic, multichannel gamma counter (Gamma Trac 1191; Tracor Analytic, Mountain View, IL). Radioactivity was corrected for decay to a specified zero time.

Purification of Copper Binding Proteins. Partial purification of radioactively labeled copper binding proteins was achieved with ammonium sulfate fractionation and gel chromatography. Previously frozen serum was brought to 50% saturation with solid ammonium sulfate and incubated for 1 hr at 4°C. Aliquots (1.0 ml) of supernatants or precipitates (suspended in 20 m*M* K phosphate buffer [pH 7.0]) were applied to 50-ml columns of Sephadex G-150 (Pharmacia, Piscataway, NJ) or Ultrogel AcA34 (LKB, Gaithersburg, MD), equilibrated, and eluted with the same phosphate buffer. Collected fractions were counted for ^{67}Cu activity and monitored for absorbance at 280 nm (as well as ceruloplasmin oxidase activity or albumin content). Pseudoaffinity separation was performed on some samples with Affigel Blue, which has a high affinity for albumin. The gel was obtained from Bio-Rad (Richmond, CA) and small portions were added to protein samples as a suspension equilibrated with 20 m*M* K phosphate (pH 7.0). Separation of bound and free proteins was accomplished by centrifugation and repeated washing (with the same buffer).

Albumin Assay. Albumin concentrations in serum and purified fractions were assayed as the capacity to bind bromcresol green, described in Tietz (35).

Results

The total serum copper concentrations of samples from dogs, rats, and mice were analyzed by furnace atomic absorption spectroscopy. The results (Table I) show that sera from dogs and mice had much lower copper concentrations than those of rats. Since ceruloplasmin accounts for about 65% of the total serum copper in rats (and humans) (6), it was conceivable that a reduced ceruloplasmin concentration might be the reason for the reduced total serum copper concentrations (in dog and mouse sera). The initial step to assess this possibility was to measure ceruloplasmin oxidase activity with *p*-phenylenediamine (pPD). Table I shows that total pPD oxidase activity in dog and mouse sera was indeed much lower than that in rat (and human) serum.

In rats and humans at least, this oxidase activity is dependent primarily upon ceruloplasmin, but a much larger copper protein, ferroxidase II (36), also contributes some activity. The activity due to ceruloplasmin but not that of ferroxidase II is inhibited by azide ions. Therefore, pPD oxidation was also measured in the presence of azide. As expected, ferroxidase II activity (measured in the presence of azide) was only a very small portion of the total oxidase activity in rat serum. In the dog, the net azide independent activity was similar to that of the rat and was thus, a larger portion of total oxidase activity. The ferroxidase II activity in mouse serum was 2- to 3-fold higher and, in this species, was about half of the total oxidase activity with pPD. Net ceruloplasmin oxidase activity (Table I) was calculated by substracting pPD oxidase activity in the presence of azide from total activity. On this basis, net ceruloplasmin activity was 6-fold lower in the dog than in the rat and 9-fold lower in the mouse. Since total serum copper was only 3-fold lower, this implies either that there are differences in the copper content of the ceruloplasmin in these species (affecting specific enzyme activity) or that there are differences in the abundance of copper associated with *other* serum components, or both.

The differences in oxidase activity of dog and other sera were confirmed using another substrate for ceruloplasmin, *o*-dianisidine (Table II). Instead of a 5-fold difference in total oxidase activity between the dog and the rat (observed with pPD), the difference was more than 10-fold with this substrate. Total oxidase activity in mouse serum appeared to be half that of the dog, with *o*-dianisidine, whereas with pPD the activities were the same (Table I). (In all cases, very little activity remained in the presence of azide, indicating that *o*-dianisidine was not a good substrate for ferroxidase II.) There also were small, but not statistically significant, differences in the total albumin concentrations in the three species (Table II), based on the binding of bromcresol green and using bovine albumin as a standard.

Further evidence that ceruloplasmin was less abun-

Table I. Serum Copper, Ceruloplasmin Oxidase, and Ferroxidase II Activities in the Dog, Rat, and Mouse[a]

Species	Serum Cu (ng)	pPD Oxidase activity (10^{-2} nmol/min/ml)		
		Total activity		Net ceruloplasmin oxidase
		$-N_3^-$	$+N_3^-$ (ferroxidase II)	
Rat	976 ± 225 (4)	19 ± 6 (4)	0.8 ± 0.6 (6)	18 ± 6 (4)
Dog	322 ± 139 (11)	3.8 ± 0.4 (13)	0.6 ± 0.2 (14)	3.2 ± 0.5 (13)
Mouse	392 ± 142 (3)	3.9 ± 0.3 (9)	1.8 ± 0.3 (9)	2.0 ± 0.4 (9)

[a] Data are expressed as the mean ± SD (*n*).

Table II. Concentrations of Albumin and *o*-Dianisidine Oxidase Activity in Dog, Rat, and Mouse Sera[a]

	o-Dianisidine oxidase activity (10^{-2} μmol/min/ml)		Albumin (mg/ml)
	$-N_3^-$	$+N_3^-$	
Dog	8.4 ± 1.8 (8)	0.2, 0.0 (2)	33 ± 3 (4)
Rat	93 ± 11 (6)	3 ± 3 (4)	28 ± 1 (3)
Mouse	4.1 ± 1.1 (4)	0.5 (1)	25, 23 (2)

[a] Data are expressed as the mean ± SD (*n*).

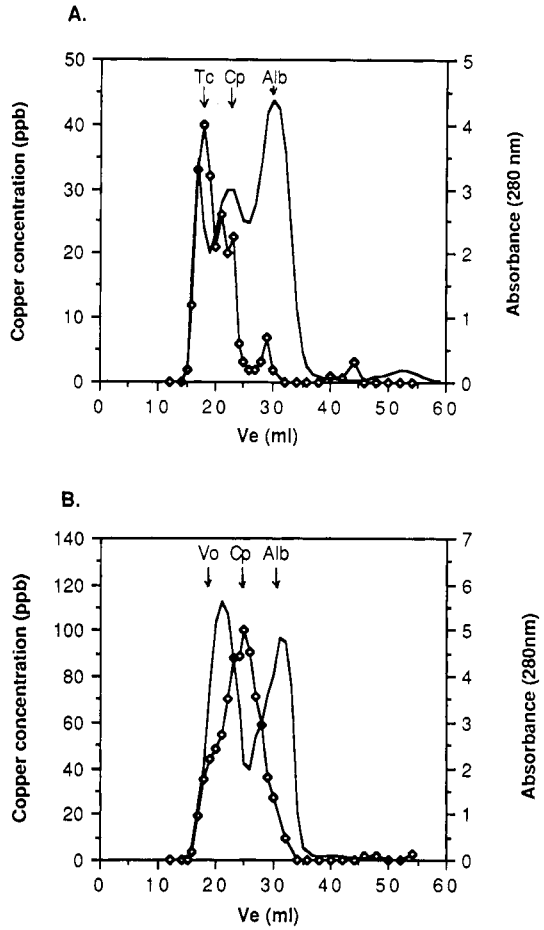

Figure 1. Fractionation of serum copper (◇) and 280-nm absorbing material (–) on columns of Sephadex G-150. Aliquots (1.0 ml) of (A) pooled dog serum or (B) rat serum were applied to 50-ml columns of gel. Fractions were assayed for copper (ppb) by furnace atomic absorption spectroscopy and for protein by absorbance at 280 nm. Arrows indicate void volume (Vo) or transcuprein (Tc) elution, and the elution of ceruloplasmin (Cp; determined by oxidase activity) and albumin (determined with the pure bovine standard). Copper contained in 15–19 ml was taken as that with transcuprein, in 20–27 ml as that with ceruloplasmin, and in 28–33 ml as that with albumin.

dant in canine than in rat serum was obtained by fractionation on columns of Sephadex G-150. Figure 1 shows the elution of 280-nm absorbing material and copper (determined by furnace atomic absorption) from 1.0-ml samples of serum applied to 50-ml gel columns. The elution positions of ceruloplasmin and albumin (determined by pPD oxidase activity and with pure albumin, respectively) are indicated by arrows. It is evident that in the case of the dog (Fig. 1A), most of the copper eluted not with ceruloplasmin but in the void volume. The void volume is where transcuprein and ferroxidase II would both elute, since their apparent mol wt are 270,000–300,000 (2, 14) and about 800,000, respectively (36). Based on determinations on two different pooled serum samples, the void volume peak contained about 47% of the total copper (44% and 50% in two determinations) or a total of about 200 Cu ng/ml of serum, whereas the ceruloplasmin peak contained about 42% (or about 180 ng/ml) (see Legend to Fig. 1 for details). A small amount of copper also eluted in the position of albumin (about 9%, or 45 ng/ml) and with low molecular weight ligands eluting between 40 and 50 ml (2%; 6 ng/ml). Thus, most of the copper in dog serum appeared to be associated with the void volume/transcuprein fraction, much less with ceruloplasmin, and almost none with the albumin fraction. Figure 1B shows the contrast with rat serum, which clearly had most of its copper in the ceruloplasmin fraction (66%) and much less in the transcuprein (18%) and albumin (15%) fractions (which had about equal amounts). A single pooled mouse serum sample (not shown) gave intermediate results: with 33% in the void volume, 53% with ceruloplasmin, 13% with the albumin fraction, and 1% with components of low molecular weight. Protein elution profiles (absorbance at 280 nm) were quite similar for all three species.

Additional evidence that canine transcuprein was binding more copper, and that canine albumin was binding less copper, than these proteins in the rat was obtained by examining the distribution of tracer ^{67}Cu(II) after its *in vitro* addition to serum to label the exchangeable serum copper pool (Figs. 2 and 3). (Ceru-

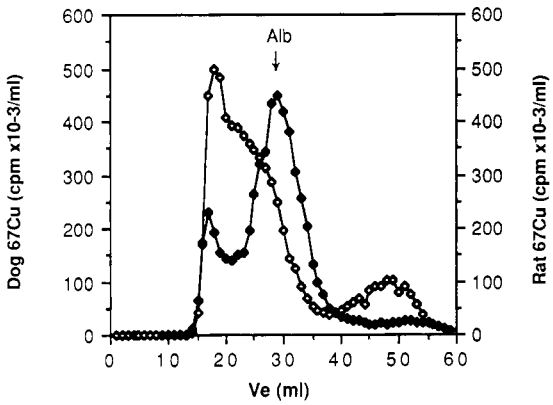

Figure 2. Elution of radioactive copper from serum samples applied to Sephadex G-150 (as in Fig. 1). Elution positions of transcuprein (Tc), ceruloplasmin (Cp), and albumin (Alb) are as described in Figure 1. Serum samples (1.0 ml) from rat (♦) and dog (◇) were incubated with ^{67}Cu-NTA prior to chromatography. The elution of albumin (arrow) was determined by binding of bromcresol green (see Materials and Methods).

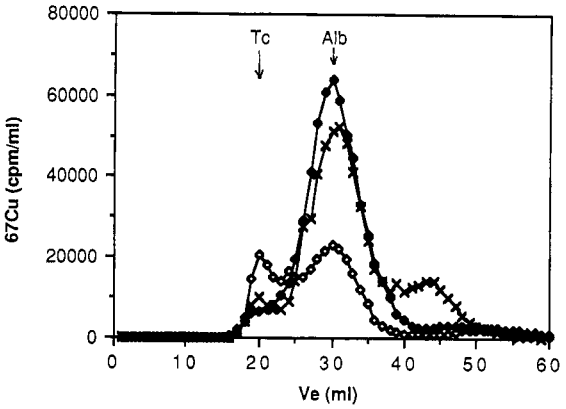

Figure 3. Fractionation of 50% saturation ammonium sulfate supernatants of ^{67}Cu-labeled sera, from dogs (◇), rats (◆), and mice (x), on Sephadex G-150 (as in Figs. 1 and 2).

loplasmin copper in not exchangeable and so is not part of this pool.) Samples of serum (1.0 ml) from the rat and the dog were incubated with ng quantities of ^{67}Cu-NTA for 1 to 18 hr prior to fractionation on 50-ml gel columns (see Materials and Methods). Timing made no difference. In the case of the rat, there were two peaks of radioactivity (Fig. 2), the first being the void volume/transcuprein fraction and the second being the albumin fraction (2, 23). The proportion of radioactivity associated with the void volume (transcuprein) peak versus that in albumin was about 1:4. The distribution of radioactivity in dog serum was quite different (Fig. 2). A much larger proportion of radioactivity was associated with the void volume/transcuprein fraction. A larger proportion was also with components of low molecular weight (or NTA) (between 45 and 50 ml), and there was no clear-cut peak coinciding with the elution of albumin. Thus, in the dog, the proportion of exchangeable copper held by transcuprein and low molecular weight ligands (like the actual copper) was greater than in the rat, and less of the exchangeable copper was attached to albumin.

The degree of participation of transcuprein, albumin, and other proteins in the exchangeable copper pool of dog and rat sera was also assessed by other methods, beginning with ammonium sulfate fractionation. Samples of pooled, fresh-frozen serum from both species, labeled with ^{67}Cu *in vitro*, were brought to 50% saturation with solid, granular ammonium sulfate at 4°C. This procedure partially separates globulins (which tend to precipitate) and albumin (which tends to stay in solution). The serum data in Table III show that for the rat, about two thirds of the radioactivity remained in the supernatant. This roughly corresponds to the proportion of radioactivity eluting with albumin on gel columns (Fig. 2). The opposite was true in the case of dog serum (Table III), in which less of the radioactive copper eluted in the albumin region of the gel column (Fig. 2). (The mouse appeared to be like the rat.)

Table III. Ammonium Sulfate Fractionation of Serum and Serum Albumin Fractions[a]

Source	Total ^{67}Cu at start (10^{-3} cpm)	^{67}Cu in fractions after treatment with 50% saturation ammonium sulfate	
		Supernatant (10^{-3} cpm) (%)	Precipitate (10^{-3} cpm) (%)
Serum			
Rat	3688	2532 (69)	1157 (31)
Dog	3576	1468 (41)	2108 (59)
Mouse	2549	1769 (69)	780 (31)
Albumin fraction			
Dog	392	317 (81)	73 (19)
Rat	341	291 (85)	50 (15)

[a] Serum treated with ^{67}Cu-NTA to label albumin and transcuprein, or the serum albumin fraction obtained by chromatography of such serum on Sephadex G-150 (Fig. 2, 27–34 ml), were treated with ammonium sulfate (50% saturation) for 1 hr (4°C) prior to centrifugation. Aliquots of precipitate (dissolved in phosphate buffer) were counted for ^{67}Cu and compared with those for the original ^{67}Cu-treated material.

"Albumin" fractions from gel columns (obtained by separating ^{67}Cu-labeled serum on Sephadex G-150) were also pooled and treated with 50% saturation ammonium sulfate (Table III). Specifically, fractions for elution volumes from 27 to 34 ml (Fig. 2) were pooled, corresponding to an M_r from about 50,000–100,000 K. In the case of sera from both rats and dogs, all but 15–20% of the radioactivity remained soluble. This strongly suggests that even in dog serum, most of the exchangeable copper not with transcuprein is actually with albumin. This was further supported by the findings for Sephadex gel chromatography of the *supernatants* from ^{67}Cu(II)-treated whole serum, 50% saturated with ammonium sulfate (Fig. 3). Most of the radioactivity in these albumin-rich supernatants was, indeed, with the albumin. A significant portion of the ^{67}Cu, however, was also in the void volume where transcuprein would elute, since albumins are only partially separated from globulins with 50% saturation ammonium sulfate; some ^{67}Cu was with low molecular weight components. For the dog, 33% and 9% of the radioactivity, respectively, were with the transcuprein and low molecular weight fractions. Percentages were 19% and 5%, respectively, for the rat, and 14% and 28% for the mouse. (It is to be noted that the overall lower radioactivity in the dog versus mouse and rat ammonium sulfate supernatants reflects the greater proportion of "exchangeable" ^{67}Cu that had been removed by precipitation of the globulins.)

For both rat and dog serum, the radioactive globulin material (that *precipitated* with ammonium sulfate) was also fractionated further, in this case on large-pore gels (Fig. 4). (Precipitates would contain transcuprein,

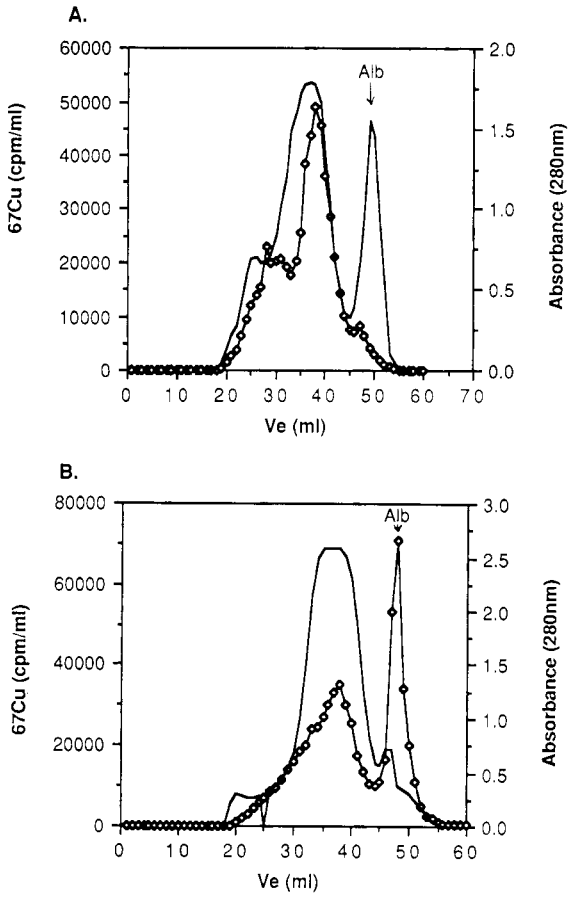

Figure 4. Elution of radioactive copper-binding components, in ammonium sulfate *precipitates* of serum, on large-pore gels. Aliquots of ammonium sulfate precipitates (50% saturation) obtained from ^{67}Cu-NTA-treated, pooled serum from (A) rats and (B) dogs were applied to 50-ml columns of Ultrogel AcA 34. Data show elution of radioactivity (◇) and absorbance at 280 nm (–). The peak elution position of albumin standard is indicated by the arrow.

ferroxidase II, and other globulins, and much less albumin.) The objective was to determine whether most of globulin radioactivity was associated with transcuprein. Transcuprein has an apparent mol wt of 270,000–300,000 (2, 14) and would elute with a peak in the region of 38 ml in this column. As shown for the rat in Figure 4A, most of the radioactivity (about 65%) in this fraction did indeed elute in the position of transcuprein. However, a significant portion (about 25%) was also associated with much larger proteins eluting closer to the void volume, and a small portion was with albumin. Ferroxidase II (M_r = 800,000) would elute in the void volume. The results for the mouse (not shown) were like those for the rat. The major radioactive component in the dog globulin fraction (ammonium sulfate precipitate) was again with transcuprein (Fig. 4B) (about 45%), but a considerable portion of the radioactivity (about 30%) eluted exactly in the position of the albumin that had remained with the globulins. This suggests that canine dog albumin precipitates more readily than rat albumin with ammonium sulfate. (Again, about 25% of the globulin radioactivity was with low molecular weight ligands.) Taken together with the data in Table III and from Figure 3, it can be calculated that the overall distribution of exchangeable ^{67}Cu(II) to transcuprein and albumin was as follows: 33% to transcuprein and 60% to albumin in the case of rat serum (with the rest going almost equally to larger and much smaller serum components), and 41% to transcuprein and 42% to albumin in the case of the dog (with a higher proportion of the remainder going to larger proteins).

Further evidence that in the dog ^{67}Cu(II) added to serum was still binding to albumin was obtained with the pseudoaffinity gel, Affigel Blue, which has a high affinity for this protein. ^{67}Cu-Labeled albumin fractions from Sephadex G-150 chromatography (after ammonium sulfate fractionation of serum) were treated with Affigel Blue to remove albumin and the ^{67}Cu that bound to it. Irrespective of the species, 72–88% of the radioactivity in samples from dogs, rats, and mice rapidly adsorbed to the gel. No statistically significant differences were apparent.

The relative affinities of the different albumins for copper were also examined by dialyzing ammonium sulfate supernatants (obtained from ^{67}Cu-labeled serum) against 10 μM NTA in phosphate-buffered saline (pH 7.0) over several days and monitoring release of radioactivity. (NTA forms a chelate with Cu(II) [37].) As shown in Figure 5, the affinity of canine albumin was much lower than that of rat or murine albumin; the dog albumin fraction released more than 60% of its copper after 5 days, and the rat and mouse albumin fractions released only 10% during the same period.

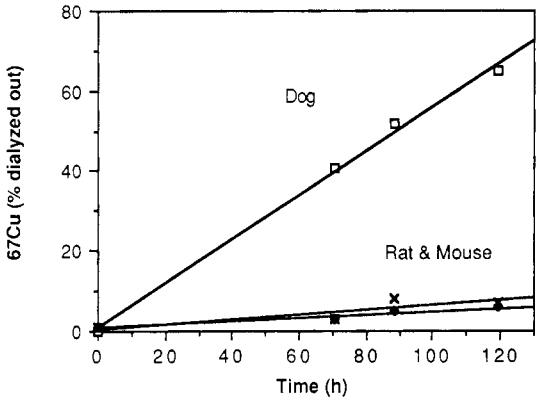

Figure 5. Release of radioactive copper from albumin fractions during dialysis against NTA. Supernatants from dog (□), rat (♦), and mouse (x) serum samples, incubated overnight with ^{67}Cu-NTA and then fractionated with 50% saturation ammonium sulfate, were dialyzed against 10 μM NTA in 0.9% NaCl buffered with 10 mM K phosphate (pH 7.0) at 4°C. Aliquots of dialysates were counted for radioactivity at various times after the start of dialysis. Released radioactivity was compared with the total in the original sample.

Discussion

We have shown that the blood plasma of the dog differs considerably from that of the rat, mouse, and human with regard to the distribution of copper among its binding components, especially ceruloplasmin, transcuprein, and albumin. The estimated contributions of these components to plasma copper in the dog, rat, mouse, and human are summarized in Table IV, based on the present studies and on earlier work with human samples. In the case of the rat and human, and presumably also in other species, ceruloplasmin is the main copper-carrying component. It is not part of the exchangeable plasma copper pool and appears to deliver its copper to cells through an interaction with specific membrane receptors (11, 13). Ceruloplasmin has at least two major functions, one being the transport and delivery of copper from the liver to nonhepatic tissues (other than the kidney) (2, 11, 20, 38) and the other the protection of the interstitial space and cell membranes against oxygen radicals (11, 39).

In the case of the dog, much less copper seems to be associated with ceruloplasmin than in the rat or human. This conclusion is based on our findings of a much lower oxidase activity and the presence of much less copper in the ceruloplasmin fractions. Thus, it seems likely that dog serum also contains less ceruloplasmin protein. A larger proportion of serum copper in this species appears to be associated with the transcuprein fraction, and total serum copper is less than half that in the rat (or human). The mouse appears to have about the same amount of total serum copper and ceruloplasmin copper as the dog, while having about half the net oxidase activity and more ferroxidase II (Table IV).

Our data for total serum copper in the dog are close to those reported by others who (like us) used the sensitive furnace atomic absorption techniques (30, 40, 41) and lower than those from some earlier studies in which less sensitive colorimetric (42) or flame atomic absorption (43) techniques were employed (and gave values close to 0.75 μg/ml). The only previous report comparing rat and canine serum oxidase activity (using a pPD in the absence of azide) gave results in general agreement with our findings (42). (The units cannot be compared, but the difference was 6-fold, as compared with 5-fold in our studies.)

Apart from ceruloplasmin, most (or even all) of the nonceruloplasmin copper in plasma may be part of the exchangeable plasma copper pool. This pool is a much larger proportion (about 60%) of total serum copper in the case of the dog (Table IV) and a much smaller proportion in the case of the rat and the other species. On an absolute basis, however, not just total serum copper but also the non-ceruloplasmin (exchangeable) copper pool is smaller in the dog and mouse than in the rat and human, since the total copper of serum is so much less. As a consequence, there is also less copper associated with albumin in the blood of both the dog and the mouse. However, canine serum has about the same amount of copper in the transcuprein fraction as the rat (Table IV), making this the most prominent copper-containing fraction in the blood plasma (even more than ceruloplasmin). This was not so for the mouse.

Canine albumin has a much lower affinity for copper (24, 44) than that of the human and probably most other species, which one would expect would reduce the amount of copper associated with this protein in canine serum. The lower copper affinity of canine albumin is ascribable to a lack of histidine as the third residue from the N terminus. Bovine, rat, and human albumins have the sequence Asp-(or Glu)-Ala-(or Thr)-His-Lys-Ser (25), whereas dog albumin has the sequence Glu-Ala-Tyr-Lys-Ser. (Mouse albumin has the sequence Leu-His-Asn (45), which, thus, also includes a histidine.) Human albumin-Cu has a dissociation constant of 10^{-17} M (in the absence of histidine) (17). We have shown that the dissociation constant for the rat and mouse must also be low (13; Fig. 4). In our dialysis studies with 10 μM NTA, only 10% of the radioactivity on rat and mouse albumin was released over 5 days. In previous studies (13), we found that dialysis of ^{67}Cu-labeled rat albumin against phosphate-

Table IV. Summary of Copper Associated with Serum Components[a]

Species	Average total Cu (ng/ml)	Ceruloplasmin (ng/ml)	Total exchangeable pool (ng/ml) (%)	Transcuprein fraction (ng/ml) (%)	Albumin fraction	Low mol wt fraction
Dog	322	135–180 (3.0)[b]	187 (58)[c]	150–200 (15)[d]	29	6
Mouse[e]	392	200 (2.0)	184 (47)	130 (30)	51	4
Rat	976	644 (18)	332 (34)	176 (35)	146	10
Human[f]	1030	600 (17?)	430 (42)	120	170	110

[a] Average total serum Cu and percentages of copper recovered in different components upon fractionation on Sephadex G-150.
[b] Net ceruloplasmin oxidase activity with pPD (Table I).
[c] Percentage of total serum copper.
[d] Percentage in nontranscuprein components of greater molecular weight (Fig. 4).
[e] Based on analysis of one sample of pooled serum.
[f] From Wirth and Linder (40).

buffered saline (pH 7.0) with no added chelator released ^{67}Cu at about half this rate, whereas 100 μM L-histidine (K_d for Cu-His_2 10^{-17} M) released most of the copper within 2 days. Clearly, the affinity of dog albumin for copper was much less; the rate of release of copper from albumin was about 12 times faster for the dog than for the rat and mouse. Nevertheless, it still took days for the copper to be released to the chelating agent, NTA.

Thus, despite its lower affinity for copper, canine albumin still appeared to be an important component of the exchangeable serum copper pool. Considerably less copper was associated with the albumin fraction and much more with transcuprein in the case of the dog than in the rat (Fig. 1, A versus B). However, as in the other species tested, about 75% of the copper in the albumin fraction (Table III, Fig. 3) was indeed with albumin per se. Thus, even in the absence of the usual high affinity site, canine albumin is an important carrier of exchangeable copper, albeit in a smaller plasma copper pool. Since the dog also has much less ceruloplasmin as a potential circulating source of copper for cells, this leaves transcuprein as the most prominent copper carrier in canine blood. Whether this circumstance could be related to the propensity of dogs for liver copper accumulation remains to be explored.

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