Isolation and Some Properties of Human Metallothionein*

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ABSTRACT: The most highly purified preparations of metallothionein isolated from human renal cortex obtained so far contain 4.2% cadmium, 2.6% zinc, 0.5% mercury, and 0.3% copper, maximally a total of 8.9 g-atoms/molecular weight of 10,500 ± 1050, thus being quite similar to the equine protein [Kägi, J. H. R., and Vallee, B. L. (1961), *J. Biol. Chem. 236*, 2435]. The presence of mercury in human metallothionein was traced to the use of organic mercurials during therapy of patients whose kidneys served for isolation of the protein. For each metal atom of human metallothionein three mercapto groups appear to be available for binding, quite similar to the equine protein. The total metal-binding capacity is accounted for by 26 silver-titratable sulfhydryl residues/mole, and in accord with this, preliminary amino acid analyses indicate that cysteinyl residues represent a minimum sulfur content of 8.1%. Human and equine metallothionein both exhibit a similar absorption band at 250 mμ, characteristic of cadmium mercaptide chromophores. Both proteins also exhibit closely similar rotatory dispersion. There is a large positive Cotton effect centered at 254 mμ, due to the asymmetric binding of cadmium to multiple sulfur ligands.

Metallothionein was first isolated from equine renal cortex by Margoshes and Vallee (1957). Further purification showed it to contain 5.9% cadmium and 2.2% zinc. The metal-free protein, thionein, contains 16.3% nitrogen and 9.3% sulfur (Kägi and Vallee, 1961). A very similar metalloprotein has now been isolated from the renal cortex of the human kidney through the application of an improved isolation procedure in which gel filtration substitutes for salt fractionation, used previously (Kägi and Vallee, 1960).

While cadmium and zinc constitute most of the metal content of the equine protein, some preparations of human metallothionein also contain substantial quantities of mercury. The occurrence of this metal was traced to the therapeutic use of mercurial diuretics in the patients from whom kidneys were obtained at autopsy. In the best preparations obtained thus far, the total metal content of human metallothionein amounts to 8.9 g-atoms/10,500 mol wt. There are three silver-reactive mercapto groups/g-atom of metal bound. Both values are close to those previously found in the equine protein.

Materials and Methods

Reagents and Glassware. Analytical grade chemicals and metal-free water were used throughout. The preparation of metal-free water, metal-free buffers, and the necessary precautions to avoid metal contamination have been described (Thiers, 1957). Solutions were stored in polyethylene containers at 4°. All dialyses were performed in cellulose casings (Visking-Nojax, 27/32), precleaned, and treated as previously described (Kägi and Vallee, 1960).

pH measurements were made potentiometrically at 23 ± 2° either with a Beckman Model G or with a Radiometer (Copenhagen) Model 25 pH meter.

Conductivity determinations were performed with a Radiometer conductivity meter at 23 ± 2°.

Protein concentrations were determined gravimetrically after precipitation with trichloracetic acid and drying at 104° (Hoch and Vallee, 1953). The absorbance at 250 mμ served as alternate method for estimating the concentration of metallothionein (Kägi and Vallee, 1961). The absorptivity was determined to be 6.8 ml mg^{-1} cm^{-1} for a preparation of highly purified human protein. A Beckman DU spectrophotometer was used throughout.

Metal analyses were carried out by emission spectrography (Vallee, 1955). Cadmium, zinc, and copper were also determined by atomic absorption spectrophotometry after dilution of chromatographic fractions in water (Fuwa and Vallee, 1963; Fuwa *et al.*, 1964; Pulido *et al.*, 1966). Mercury was measured by atomic absorption spectrophotometry (Cobain, 1965; K. Fuwa, W. A. Cobain, and B. L. Vallee, unpublished data).

Reactive mercapto groups were determined by amperometric titration with Ag^+ (Benesch *et al.*, 1955) in a supporting electrolyte solution (Tris, KCl, HNO_3) at pH 7.5 (Hoch and Vallee, 1960).

Gel Filtration Chromatography. Gels were prepared by swelling in metal-free distilled water or dilute Tris buffer for at least 10 days. The following gels were employed: Sephadex (Pharmacia, Uppsala, Sweden),

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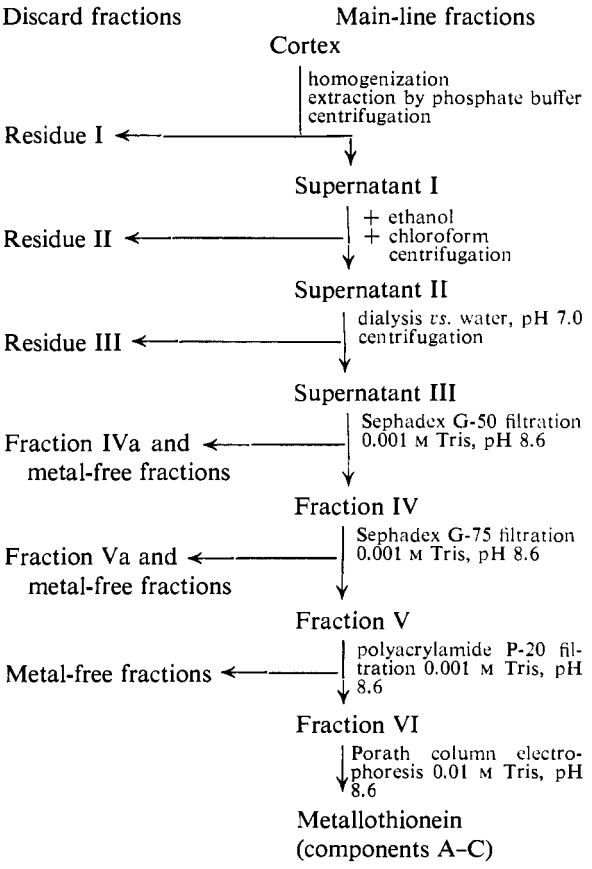
Figure 1: Purification of Human Kidney Metallothionein.



G-25 (Lot 4370) fine beads; G-50 (Lot 4120) fine beads; G-75 (Lot 5225); and polyacrylamide gel, P-20 (Bio-Rad, Lot 2703). Fine particles were eliminated by decantation and the absorbent beds were packed at room temperature by pouring a thin slurry of gel particles in buffer solution into glass columns, partially filled with buffer. Slurry was added until the desired bed height was obtained. A flow rate between 15 and 25 ml/hr was maintained.

Preparative column electrophoresis was performed in a Porath column, 2.9 × 30 cm (LKB Model 3340 C), packed with ethanolized cellulose and equilibrated with 0.01 M Tris–HCl, pH 8.6. The column was prepared and the sample applied as described by Porath and Hjertén (1962). Runs (19 hr) using 525 v, 6 ma were performed with the column cooled to 10 ± 1°.

Disk gel electrophoresis was performed on samples containing 0.1–0.2 mg of protein (Davis, 1964).

Molecular weights were estimated by gel filtration (Andrews, 1965), utilizing a 148 × 1 cm Sephadex G-75 column, equilibrated with 0.04 M Tris–HCl, pH 8.1, at 23 ± 2°. Protein samples, 1–3 mg in 1 ml, were applied to the column and their elution volumes were estimated by spectrophotometric measurement on 2-ml eluate fractions. The void volume, V_0, was determined after every second run with Blue Dextran

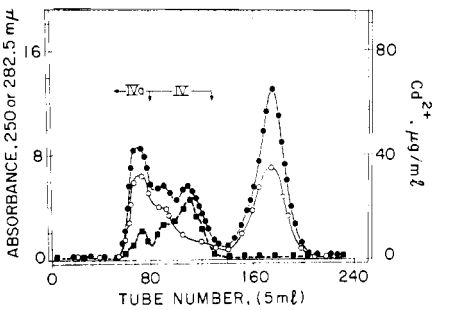


FIGURE 2: Chromatography of human kidney metallothionein on Sephadex G-50. Supernatant III (3.1 g), in 47 ml of 0.001 M Tris, pH 8.6, was chromatographed on a 56 × 4 cm (540 ml) bed of Sephadex G-50 fine beads at a flow rate of 25 ml/hr, 4°. Cadmium (■) absorbances at 250 (●) and 282.5 mμ (○) were measured. Fractions (5 ml) were collected. As indicated by the arrows, the cadmium-rich fraction IV was pooled and lyophilized. The balance was discarded.

(Pharmacia, Uppsala, Sweden), a polysaccharide with an average molecular weight of 2×10^6.

Absorption spectra were determined with a recording spectrophotometer (Cary Model 15) using 1-cm light-path quartz cells.

Optical rotation was measured with a Cary Model 60 recording spectropolarimeter over the range 220–500 mμ at 23 ± 2°; cells with fused quartz end plates and 2–20-mm path length were used. The slit width of the instrument was programmed to yield constant energy over the entire spectral range. Measurements were performed with protein concentrations varying from 0.5 to 0.05 mg/ml to eliminate the possibility of spurious Cotton effects (Urnes and Doty, 1961). Base lines were recorded in the same cells containing buffer only. Molar rotation with respect to cadmium was calculated from the equation $[M]_\lambda^T = [\alpha]_\lambda^T MW/100$ where $[\alpha]_\lambda^T$ = specific rotation and MW = minimum molecular weight calculated for 1 g-atom of cadmium present in the protein.

Isolation of Human Metallothionein

Human kidneys were obtained from patients of both sexes whose ages ranged from 18 to 71 years and who had died without exhibiting primary renal disease. The organs were shown to be free of pathological changes both by gross and histological features. The kidneys were frozen as soon as possible and stored at −20°. Unless otherwise stated all operations were carried out at 4°. For each preparation 12–14 kidneys are thawed overnight, rinsed with water, the capsule removed, and the cortex separated from the medulla. The kidney cortex is cut in small strips and homogenized in an electric blendor for 20 sec. The procedure is an extension of that previously employed for equine metallothionein (Kägi and Vallee, 1960) (Figure 1). The homogenate of kidney cortex is extracted with an equal volume of 0.05 M sodium phosphate buffer,

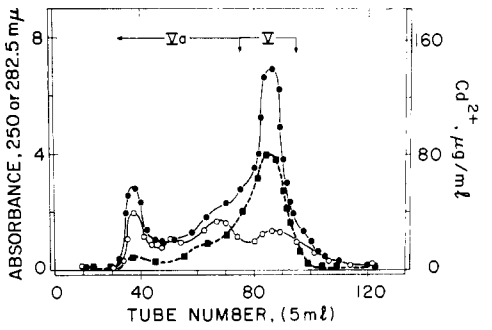


FIGURE 3: Chromatography of human kidney metallothionein on Sephadex G-75. Fraction IV (616 mg) in 15 ml of 0.001 M Tris, pH 8.6, was chromatographed on a 127 × 2.2 cm (490 ml) bed of Sephadex G-75, at a flow rate of 18 ml/hour, 4°. Cadmium (■) absorbances at 250 (●), and 282.5 mμ (○) were measured. As indicated by the arrows, the 5-ml fractions were pooled to yield cadmium-rich fraction V which was lyophilized. Fraction V-a was discarded.

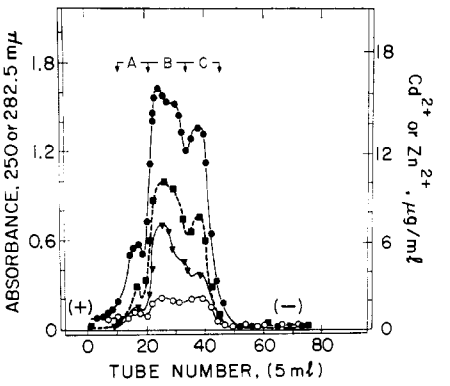


FIGURE 4: Electrophoresis of human kidney metallothionein in Porath column. Fraction VI (115 mg) in 14 ml of 0.01 M Tris–HCl, pH 8.6, was separated in a 2.9 × 30 cm cellulose bed (285 ml) at 10°, 525 v, 6 ma. After 19 hr the material was displaced with buffer and collected in 5-ml fractions at a flow rate of 0.2 ml/min. Cadmium (■) and zinc (▼) absorbances at 250 (●) and 282.5 mμ (○) were measured. Fractions were pooled as indicated by the arrows to yield components A–C.

pH 7.0, for 2 hr with continuous stirring. To remove heavy particles the slurry is centrifuged in an International refrigerated centrifuge at 1000*g* for 60 min to yield supernatant I.

With constant stirring 1.2 parts of 95% ethanol, followed by 0.095 part of chloroform, prechilled to −20°, are added dropwise to the turbid supernatant I. The massive reddish and rubbery precipitate, residue II, is separated by centrifugation at 1000*g* for 1 hr. The resultant clear yellow supernatant II is freed from organic solvents and salt by overnight dialysis *vs.* two changes of water, adjusted, and maintained at pH 7.0 by the addition of 0.01 M sodium hydroxide. The fine precipitate formed on dialysis, residue III, is removed by centrifugation at 32,600*g* in a Spinco Model L preparative ultracentrifuge to yield supernatant III (approximately 1500 ml) which is lyophilized.

Fractionation is continued by chromatography on a series of gels having varying exclusion limits. The elution of metallothionein is monitored by measuring both the cadmium content and the absorbance, at 250 and 282.5 mμ of the fractions. Tris is added to supernatant III to yield a final concentration of 0.001 M, pH 8.6, and the resultant solution is passed through a 56 × 4 cm Sephadex G-50 column at a flow rate of 25 ml/hr. The molecular weight exclusion limit of the gel was 10,000 as measured with dextran (Figure 2). The major fraction of the total cadmium content is eluted just after the first major absorbance peak, and occurs in the position of partially excluded material. The material showing close correspondence between the cadmium content and the absorbance at 250 mμ is pooled and lyophilized (fraction IV). Both fraction IVa, containing a minor amount of cadmium associated with higher molecular weight material, and the second major absorbance peak, containing low molecular weight material, are discarded.

Fraction IV dissolved in 0.001 M Tris, pH 8.6, is rechromatographed on a 127 × 2.2 cm Sephadex G-75 column, with an exclusion limit of 50,000 mol wt, at a flow rate of 18 ml/hr (Figure 3). Approximately 75% of the total cadmium applied to the column is recovered in a single peak in the position of nonexcluded, lighter material. This cadmium-rich fraction is pooled and lyophilized (fraction V), while the heavier and excluded material (fraction Va) containing a relatively small amount of cadmium is discarded. Fraction V is purified further by passage through a 127 × 2.2 cm column of polyacrylamide gel (P-20), with an exclusion limit of 20,000, equilibrated with 0.001 M Tris, pH 8.6, at a flow rate of 15 ml/hr. The resultant cadmium-containing peak is well separated from two peaks of heavier and lighter impurities. The combined eluate for the cadmium-containing peak is lyophilized to constitute fraction VI or crude metallothionein.

Further purification of fraction VI is performed by column electrophoresis. After the buffer has been exchanged for 0.01 M Tris–HCl, pH 8.6, on a 50 × 1 cm Sephadex G-25 column, fraction VI is applied to the Porath column. After 19 hr of electrophoresis, 5-ml fractions are eluted at a flow rate of 0.2 ml/min. Figure 4 shows the resultant elution diagram in which one minor and two major components are observed moving toward the anode. These are designated at components A–C of metallothionein. These components were also demonstrated by disk gel electrophoresis. Further studies were confined to components B and C which accounted for more than 90% of the cadmium applied to the column.

TABLE I: Metal Content of Fractions Attending Purification of Metallothionein.[a]

Fraction	(a) Cd[b]	(b) Cd	(c) Zn	(d) Hg[b]	(e) Cu[b]	(f) Ca	(g) Mg	(h) Sr	(i) Cr	(j) Al	(k) Fe	(l) Mn	(m) Pb
Cortex	140	130	350	...[c]	...	400	520	0.8	3.4	25	380	2.2	nd[d]
Buffer extract	130	<250	410	...	...	110	>950	1.3	6.1	22	>800	120	>23
Supernatant II	2,510	2,300	1,610	...	...	840	7,520	nd	41	28	360	nd	nd
Fraction IV	5,650	6,120	...	...	...	420	1,470	3.7	<24	23	nd	nd	26
Fraction V	10,100	10,500	5,300	...	...	2,880	1,160	15	<63	<44	nd	nd	<290
Fraction VI	29,500	28,400	17,200	3,750	1,710	1,120	nd	nd	99	170	430	110	nd
Metallothionein component B	42,200	...	26,700[b]	5,380	2,540	...	...	...	...	...	...	...	...
Metallothionein component C	38,500	...	19,900[b]	4,760	4,600	...	...	...	...	...	...	...	...

[a] Metal content is given in micrograms per grams of dry weight of material precipitated by 10% trichloroacetic acid, except for the cortex (line 1) where it is based on dry weight of the whole tissue. [b] Atomic absorption analysis. Balance of determinations performed by emission spectrography. [c] (...) = not determined. [d] nd = not detected.

TABLE II: Accumulation of Mercury in Human Kidney Metallothionein; Metal Content (micrograms per gram) of Fractions Attending Purification.

Fraction	Preparation 1[a]				Preparation 2[b]			
	Cd	Zn	Fe	Hg[c]	Cd	Zn	Fe	Hg[c]
Supernatant I	130	790	1,180	...[d]	...	...	...	...
Supernatant III	7,590	5,520	130	7,320	10,500	6,410	120	680
Fraction IV	11,600	6,670	...	18,300	13,000	7,560	280	710

[a] Kidneys from patients known to have been treated with organic mercurials. [b] Kidneys from individuals not known to have been exposed to organic mercurials. [c] Atomic absorption analysis. Balance of determinations performed by emission spectrography. [d] (...) not determined.

Results

The metal content of the fractions obtained during the isolation procedure, in μg/g of protein, is shown in Table I. Cadmium determined by atomic absorption spectrophotometry (column a) and by emission spectrography (column b) are in good agreement. The cadmium content rises from 130 to 140 μg/g of cortex to 42,200 and 38,500 μg/g in the purest fractions of components B and C of metallothionein, respectively. This corresponds to 3.9 and 3.7 g-atoms/10,500 mol wt (*vide infra*). The limited amounts of components B and C obtained precluded their analysis by emission spectrography.

The 300-fold enrichment of the material with respect to cadmium is accompanied by an increase in the concentration of zinc (column c), from 350 μg/g in the cortex to 26,700 and 19,900 in components B and C, a 75- and 50-fold enrichment, respectively, and corresponding to 4.3 and 3.2 g-atoms of zinc/10,500 mol wt. In addition to cadmium and zinc, purified fractions of metallothionein contain up to 4600 μg/g of copper and 5380 μg/g of mercury, or 0.7 and 0.3 g-atom/mole. Iron represents an insignificant fraction of the total metal content of fraction VI. Magnesium, strontium, lead, and other metals are removed during fractionation while the concentrations of calcium, aluminum, chromium, and manganese remain virtually unaltered.

The molar sum of cadmium, zinc, copper, and mercury ranged from 7.9 to 8.9 g-atoms/10,500 g of protein, though at comparable stages of purification in different preparations the amount of mercury varied. Since neither procedural nor analytical factors were found to account for this variation, the possibility was examined that the mercury content of the protein might be the result of the medical history of the patients whose kidneys were employed for the isolation of metallothionein. It developed that some of the patients who served as donors of the starting material, indeed, had been treated previously with mercurials. Fraction IV of metallothionein was therefore isolated from

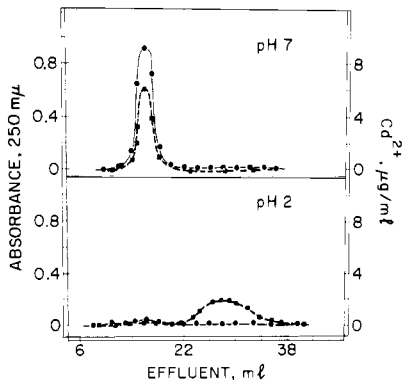


FIGURE 5: Removal of cadmium from human metallothionein. Human metallothionein, 0.4 mg in 2 ml of water, was adjusted to pH 2 with 0.1 N HCl and applied to a Sephadex column, G-25, 50 × 1 cm, fine beads, equilibrated with 0.01 N HCl. The control experiment was performed with gel equilibrated with 0.001 M Tris, at pH 7.0. Absorbances at 250 mμ (●) and cadmium (■) were measured in each of the 2-ml fractions. The apoprotein, thionein, formed on exposure to pH 2 has no absorbance at 250 mμ. Ionic cadmium appears in the late fractions of the chromatogram.

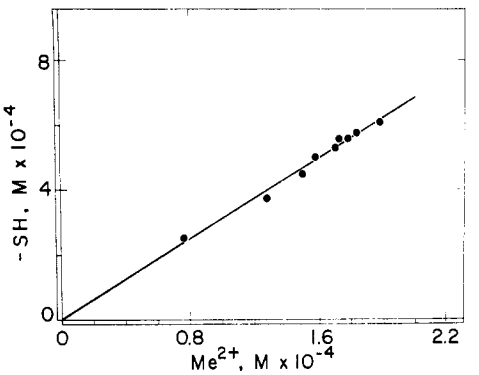


FIGURE 6: Correlation of silver-titratable mercapto groups with the sum of cadmium, zinc, and copper present in human kidney metallothionein. Fractions of component B after Porath column electrophoresis; correlation coefficient, r, = 0.9459.

TABLE III: Representative Yields in Isolation of Human Metallothionein.

Fraction	Cd (%)	Zn (%)	TCA Ppt (%)
Cortex	100	100	100
Buffer extract	41	45	39
(II) Supernatant of ethanol + $CHCl_3$ precipitation	40	10.4	2.28
(III) Supernatant after dialysis *vs.* H_2O	25	6.2	0.80
(IV) Metal-containing fraction in Sephadex G-50 chromatography	24	2.8	0.60
(V) Metal-containing fraction on Sephadex G-75 chromatography	11	2.2	0.15
(VI) Metal-containing fraction in polyacrylamide chromatography	8	1.7	0.05
Metallothionein, components B-C of Porath electrophoresis	7	1.6	0.03

eight kidneys of patients who had received at least 80,000 μg of mercury in the form of mercurial diuretics[1] during the 2 months prior to their demise (Table II, preparation 1). This fraction was also isolated from six kidneys of patients whose medical history showed no known exposure to mercury (Table II, preparation 2). Preparation 1 contained 18,300 μg/g of mercury, almost equimolar to the amount of cadmium present. The mercury content of preparation 2, however, was quite low, *i.e.*, 710 μg/g (Table II).

The total final yield of material measured by trichloracetic acid precipitation of the protein represents approximately 0.03% of the initial dry weight of the kidney cortex. It contained 7% of the total cadmium and 1.6% of the zinc present in the original dry tissue (Table III).

While metals remain firmly bound to metallothionein under the conditions employed for its purification, they can be removed at low pH. Human metallothionein, component B, 3×10^{-5} M, is adjusted to pH 2.0 by the addition of 0.1 N HCl and passed through a Sephadex column, G-25, 50 × 1 cm, equilibrated with 0.01 M HCl. While cadmium, zinc, and copper are removed quantitatively from the protein and recovered in the fractions containing nonexcluded material, as shown for cadmium in Figure 5, mercury remains bound under these conditions. The removal of cadmium causes the disappearance of the characteristic absorbance at 250 mμ (*vide infra*). Recombination of the metal-containing fractions with the apoprotein, thionein, and readjustment of the pH to 7.0 or addition of excess Cd^{2+} at this pH restores the absorption characteristics of metallothionein. Similar results were obtained with the equine protein.

Amperometric titration with Ag^+ of the peak fractions of components B and C demonstrates 26.5 and 25.2 reactive mercapto groups/10,500 g of human metallothionein, respectively. Concomitantly, cadmium, zinc, and copper, but not mercury, are displaced from the

[1] Each milliliter of organic sodium meralluride (mercuhydrin), Lakeside Laboratories, Inc., Milwaukee, Wis., the mercurial diuretic most commonly used in these patients contained the equivalent of 40,000 μg of mercury.

TABLE IV: Estimation of Molecular Weight of Human Metallothionein by Gel Filtration.

Protein[a]	Mol Wt	Method Employed	Reference	Wavelength Employed for Estn (mμ)	V/V_0[b]
Metallothionein (equine kidney)	10,000	SD[c]	Kägi and Vallee (1961)	250	2.14
Metallothionein components B and C (human kidney)	10,500		This study	250	2.11
Cytochrome *c* (horse heart)	12,400	AAA[d]	Margoliash (1962)	410	2.07
Ribonuclease (bovine pancreas)	13,700	AAA	Hirs *et al.* (1956)	280	2.02
α-Chymotrypsin (bovine pancreas)	25,000	AAA	Hartley (1962)	280	1.85
Carboxypeptidase A (bovine pancreas)	34,600	AAA	Smith and Stockell (1954); Bargetzi *et al.* (1963)	280	1.71

[a] Cytochrome *c* was obtained from Sigma Biochemical Co., Lot 114B-7150; ribonuclease, chymotrypsin, and carboxypeptidase A were obtained from Worthington Biochemical Co., Lot No. 6089–90, 60668–9, and COA 707–11, respectively; metallothionein from horse kidney was prepared by the method of Kägi and Vallee (1961). [b] Average of three determinations. [c] SD = sedimentation and diffusion. [d] AAA = amino acid analysis.

protein and can be removed by passage through Sephadex. Moreover, when measured in successive fractions of eluate after column electrophoresis the ratio of titratable mercapto groups to the sum of the metal atoms which can be displaced is constant. As gauged from the slope of the correlation plot, close to three sulfhydryl groups are titrated per metal atom bound to the protein (Figure 6). While the content of titratable sulfhydryl groups and the metal content changes as purification of the protein progresses, the ratio of three mercapto groups per metal atom is attained in relatively impure fractions and remains constant thereafter. Amino acid analyses, to be reported in detail elsewhere, demonstrate that cysteine accounts for 25–27% of the amino acid residues of both components B and C of human metallothionein, similar to the equine protein (Kägi and Vallee, 1961).

The molecular weights of components B and C were estimated by Sephadex G-75 gel filtration. Using the ratio of the elution volume, V, to void volume, V_0, as a measure of molecular weight, human metallothionein was compared with a number of proteins of known molecular dimensions. The proteins selected as markers, their molecular weights, and the spectral characteristics employed for their detection in the eluate fractions are shown in Table IV together with the experimental values of V/V_0.

Although differences in mobility were observed on disk gel and Porath column electrophoresis, components B and C of metallothionein behaved identically with respect to gel filtration: on Sephadex G-75 each of these components is eluted, as a single symmetrical peak, in the same position. These peaks, with a ratio $V/V_0 = 2.11$, are eluted after horse heart cytochrome *c* ($V/V_0 = 2.07$) and bovine ribonuclease ($V/V_0 = 2.02$), and slightly before equine metallothionein ($V/V_0 = 2.14$). The plot of the V/V_0 *vs.* the logarithm of the known molecular weight of the proteins used as calibration standards is linear (Figure 7). On this basis the molecular weight of components B and C is estimated to be 10,500 ± 1050.

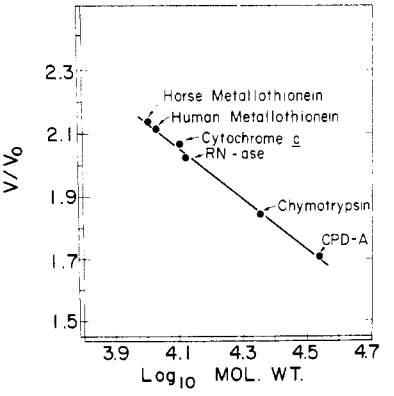


FIGURE 7: Determination of the molecular weight of human metallothionein by gel filtration. Relationship between elution volume, V/V_0, and molecular weight of standard proteins. Sephadex G-75, 148 × 1 cm, 0.04 M Tris-HCl, pH 8.1, 23 ± 2°; flow rate: 18 ml/hr. Procedure is as described in the text and Table IV.

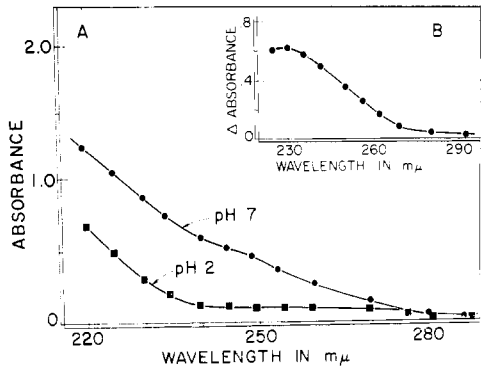


FIGURE 8: Absorption spectra of human metallothionein and thionein. (A) Absorbances of metallothionein (●) and of thionein (■), 0.05 mg/ml, dissolved in 0.001 M Tris, pH 7.0, are plotted as a function of wavelength. The characteristic absorbance shoulder at 270 mμ is abolished upon removal of the metals by exposure to pH 2.0, yielding the spectrum of the apoprotein, thionein. (B) Difference spectrum of metallothionein *vs.* thionein (●).

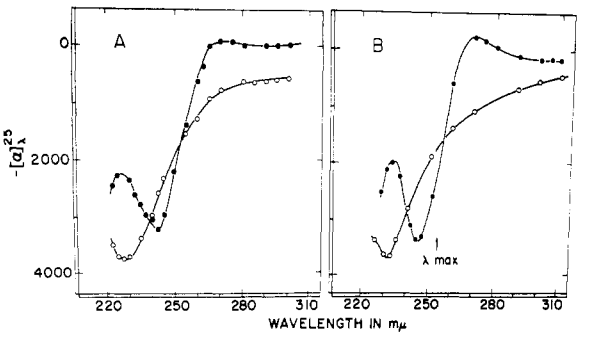


FIGURE 9: Optical rotatory dispersion of human (A) and equine (B) metallothionein and thionein. Metallothionein (●) and the apoprotein, thionein (○), 0.5–0.05 mg/ml, are prepared as described in the text. The specific rotation, $[\alpha]^{23}$, is plotted as a function of the wavelength. Metallothionein exhibits a positive Cotton effect, centered at 254 mμ, with a peak at 268 mμ and a trough at 245 mμ. A second peak is observed at 230 mμ. Removal of cadmium results in the plain negative rotatory dispersion of thionein.

The ultraviolet absorption spectrum of human metallothionein exhibits a broad shoulder at 250 mμ with an absorptivity of 6.8 ml mg^{-1} cm^{-1} for the purest fractions (Figure 8A) near that previously reported for the equine protein (Kägi and Vallee, 1961). Cadmium is removed on exposure of the native protein to pH 2.0 and correspondingly this shoulder disappears, yielding the structureless spectrum of the apoprotein, thionein. Subsequent readjustment to neutral pH restores the spectrum of the native protein. The difference spectrum of thionein and metallothionein reveals a broad absorption band, extending from 250 to 220 mμ, the lower limit of the spectral region examined (Figure 8B). From the difference spectrum, a molar absorptivity ε of the cadmium chromophore at 250 mμ of 17,200 cm^{-1} $mole^{-1}$ l. can be calculated.

The optical rotatory dispersion characteristics of human and equine metallothionein are unusual and closely similar to one another (Figures 9A and B). The equine protein was prepared by the procedure previously described (Kägi and Vallee, 1961). The large Cotton effect of equine metallothionein between 310 and 225 mμ, described before (Ulmer *et al.*, 1962), is centered at about 254 mμ near the characteristic absorption shoulder. The maximum of this Cotton effect is at 268 mμ and the minimum is at 245 mμ. Removal of cadmium by acidification to pH 2.0 and passage through Sephadex G-25 makes it apparent that this Cotton effect is superimposed upon the plain dispersion of the apoprotein, thionein. It is restored by addition of cadmium to thionein at pH 7.0. There are indications of a second, probably positive Cotton effect below 230 mμ.

The magnitude of the 254 mμ Cotton effect of human and equine metallothionein is a function of the cadmium content. The sum of the difference rotations, Δ[α], of thionein and of metallothionein at 268 and 245 mμ indicate a total amplitude of the Cotton effect of 1570° for the human and 2250° for the equine protein, in direct proportion to the cadmium content of the two proteins. Hence, based on cadmium, in both proteins the Cotton effect has a total molar amplitude, $[M]^T$, of 42,500°.

Discussion

The presence of substantial amounts of cadmium in the kidney of various species including the human (Klein and Wichmann, 1945; Voinar, 1950; Malyuga, 1941; Vallee, 1960), led to the isolation of metallothionein, a cadmium- and zinc-containing protein from equine renal cortex (Margoshes and Vallee, 1957; Kägi and Vallee, 1960, 1961). Horse kidneys were chosen for the initial studies because of their relatively high cadmium content (Vallee, 1960).

The present data confirm the existence of a similar protein in the human renal cortex. In both instances the selective accumulation of cadmium served as the primary means for the identification and isolation of a specific macromolecule of unknown biological function, which could not have been identified readily by other means. The demonstration of this protein in the human and its similarity to that of the horse lends support to the hypothesis that analogous proteins will be found in yet other species, most likely accounting for the preferential occurrence of cadmium in the kidney cortex of different animal species, *e.g.*, the murine (Berlin and Ulberg, 1963; Gunn and Gould, 1957), the bovine (Klein and Wichmann, 1945) and porcine (Forney *et al.*, 1955), and the canine, among other mammalian kidneys (Vallee, 1960). The occurrence of similar or identical proteins in other organs is under study.

The procedure described for the isolation of equine metallothionein (Kägi and Vallee, 1960) was adapted to the purification of the human protein. Fractionation with ethanol and chloroform was retained as an effective procedure for early removal of hemoglobin and other components. Precipitation with ammonium sulfate was eliminated by substituting gel filtration employing a series of chromatographic steps resulting in progressive purification of the protein as a function of molecular size (Figures 2 and 3).

The elimination of salt fractionation not only removes potential hazards of denaturation but also markedly reduces losses of material during long periods of dialysis and avoids potential sources of metal contamination. The high absorbance at 250 mμ, characteristic of cadmium binding to equine thionein, is also typical of human metallothionein. It provides a simple and convenient means with which to follow cadmium-containing thionein (Kägi and Vallee, 1961). At advanced stages of purification of human metallothionein absorbance at 250 mμ and cadmium content, measured independently, were highly correlated, quite analogous to measurements on the equine protein. The correlation coefficient, r, for these two parameters was 0.98 for successive fractions of component B. It should be remembered, however, that employment of absorbance at 250 mμ, typical of cadmium–thionein, as a criterion of purification not only excludes thionein itself from the final isolated material but also complexes of thionein with other metals.

The ability to measure cadmium, zinc, and mercury accurately and rapidly by atomic absorption spectrophotometry throughout the purification procedure constitutes significant progress in the analytical chemical procedures required for the identification and isolation of such systems (Fuwa and Vallee, 1963; Fuwa *et al.*, 1964; Pulido *et al.*, 1966). Atomic absorption spectrophotometry proved particularly useful for the work with the human protein since both the total content of cadmium and its concentration in the human kidney are substantially lower than in the equine renal tissue.

Human metallothionein, which has a single peak on gel filtration, was purified further by preparative electrophoresis resulting in the appearance of three components which differ but slightly in metal content and absorbance characteristics (Figure 4). Since component A constitutes but a small fraction of the total, only components B and C were studied here. Efforts to achieve their complete separation are in progress.

In order of magnitude the metal content of components B and C of human metallothionein is comparable to that of equine metallothionein. Component B of human metallothionein contained 4.2% cadmium, 2.6% zinc, 0.5% mercury, and 0.25% copper, corresponding to 3.9, 4.3, 0.3, and 0.4 g-atoms of metal/mole, respectively, a total of 8.9 g-atoms of metal/10,500 mol wt.

The zinc content of component C, 1.9% or 3.3 g-atoms/mole, was lower and the copper content, 0.46% or 0.76 g-atom/mole, was higher than that of component B. Together with 3.8% or 3.7 g-atoms of cadmium and 0.47% or 0.25 g-atom/mole of mercury there is a total of 7.9 g-atoms/10,500 mol wt. It has been suggested previously that the binding of each atom of cadmium or zinc to metallothionein might induce a negative charge (Kägi and Vallee, 1961). Aside from possible variations in amino acid composition, the difference of about 1 g of metal between components B and C, perhaps due to loss of metal during purification, might readily account for the differences in electrophoretic mobility.

In the course of this work equine metallothionein, prepared by the method of Kägi and Vallee (1960, 1961), was also purified further by preparative electrophoresis as described here for the human protein. After electrophoresis a single, symmetrical peak with high absorbance at 250 mμ was eluted from the Porath column, well separated from minor components containing contaminating material with high absorbance at 280 mμ. The electrophoretic homogeneity of this material was verified also by starch gel electrophoresis. It contained 59,500 μg/g of cadmium and 16,900 μg/g of zinc, 310 μg/g of iron, and 1500 μg/g of copper, a total of 8.4 g-atoms/10,000 mol wt. This is in close agreement with the metal content previously reported (Kägi and Vallee, 1961). This material was employed for comparison of molecular weights (Table IV, Figure 7).

The occurrence of mercury in the human protein can be attributed at least in part to the therapeutic use of mercurials as shown by the high mercury content of partially purified metallothionein isolated from kidneys of patients who had been exposed to such drugs (Table II). Clearly, the chemical basis for the accumulation of mercury requires further investigation. But in view of the characteristic affinity of mercury for sulfur ligands (Stricks *et al.*, 1954; Schwarzenbach *et al.*, 1955; Gurd and Wilcox, 1956; Lenz and Martell, 1964) and since the element is eliminated slowly by the human kidney (Storlazzi and Elkins, 1941; Goldwater and Nicolau, 1966), it is not surprising to find mercury associated with metallothionein, a renal protein characterized by a high content of mercapto groups. Kidneys of rats and dogs receiving various mercurials also accumulate mercury up to 61.5 μg/g of wet tissue (Swensson *et al.*, 1959), perhaps for analogous reasons. Based on the occurrence of metallothionein in kidney, physiological studies have, in fact, endeavored to implicate metallothionein in renal function through studies of the effects of cadmium, zinc, and mercury on tubular sodium reabsorption (Vander, 1963). The diuretic effect of mercurials, leading to their widespread use for this purpose, may or may not be pertinent to these findings, though the potential causal relationships deserve further study.

For both components B and C of human metallothionein, the estimated molecular weight was 10,500 ± 1050, very near that of the equine protein, 10,000 ± 260 measured by sedimentation and diffusion (Kägi and Vallee, 1961). The molecular weight of human metallothionein was estimated by comparing its be-

havior on gel filtration with that of proteins of known molecular weight, using equine metallothionein, the molecular weight of which has been established independently, as one of these. The equine protein, together with the globular proteins used as calibration standards, lie on one line, allowing an estimate for the molecular weight of the human protein to be made. Such molecular weight estimates may be considered to be accurate to 10% (Andrews, 1964, 1965). Horse metallothionein and, presumably, the human protein have a low partial specific volume, *i.e.*, 0.65 g^{-1} ml (Kägi and Vallee, 1961), due to the high metal and cysteine content. Therefore, the molecular weight estimated by gel filtration would tend to be at the lower limit. A revision of the exact value may ultimately be required when more material becomes available for detailed studies.

Exposure of the protein to low pH, followed by gel filtration, separates metals from human metallothionein. Cadmium and zinc dissociate from the equine protein over the pH range 7–2, cadmium being bound more firmly than zinc, in accord with the known stability constants of their complexes with sulfur ligands (Kägi and Vallee, 1961; Gurd and Wilcox, 1956). Mercury–sulfur complexes are much firmer and, as expected, the mercury–thionein complex does not dissociate even at pH 2. This finding is consistent with previous experiments demonstrating that at neutral pH, dialysis of equine metallothionein *vs. p*-mercuribenzoate displaces cadmium and zinc (Kägi and Vallee, 1960). Mercury is also bound more firmly than silver and thus, unlike cadmium, zinc, and copper, it is not removed during Ag^+ titration.

The preponderance of free sulfhydryl groups of thionein due to the large proportion of cysteinyl residues constitutes an unusual feature of this protein. Components B and C of the human protein contain 26.5 and 25.2 mercapto groups/10,500 mol wt, respectively, compared to the 26/mole of equine metallothionein (Kägi and Vallee, 1961). Based on the number of mercapto groups of metallothionein and preliminary amino acid analyses, the protein has a minimal sulfur content of about 8.1%, closely similar to that of equine metallothionein (Kägi and Vallee, 1961). A protein from wool containing 6.7% sulfur is the only one known to approach such high values (Gillespie, 1962). Here, however, most of the sulfur is in the form of cystine; in the native protein free no mercapto groups were found.

Although their sum remains nearly constant, the mole fraction of different metals in the equine and human proteins differ; similarly, they vary in metallothionein isolated from different kidneys (Table II). These observations support earlier suggestions that at least cadmium and zinc replace each other isomorphically, by competing for identical binding sites (Margoshes and Vallee, 1957).

The 0.3 g-atom of mercury, a small fraction of the total metal content, does not reduce the total number of titratable mercapto groups significantly. The ratio of the sum of the number of gram atoms of cadmium, zinc, copper, and mercury to the total number of titratable mercapto groups in various preparations of human metallothionein suggests that three mercapto groups are available to bind 1-g atom of metal.

The characteristic ultraviolet absorption spectrum, which is analogous to that of equine metallothionein, provides additional evidence for the stoichiometry of this interaction (Figure 8). Thus, the location and order of magnitude of the molar coefficient of absorptivity of the cadmium-dependent absorption band may be attributed to a charge-transfer transition similar to that of complexes of cadmium with two or more mercaptide ligands, such as 2-mercaptoethanol and 2,3-dimercaptopropanol (Kägi and Vallee, 1961). The absorption band of the zinc mercaptide chromophore (Kägi and Vallee, 1961), with a maximum at 215 mμ, most likely also contributes to the difference absorption spectrum (Figure 8B). While the spectra of mercury or copper–thionein have not been characterized as yet, preliminary data indicate that mercury induces an absorption band in equine metallothionein with a shoulder at 270 mμ. Since mercury is not removed at pH 2.0, it may account in part for the residual absorption of the human protein observed in this region (Figure 8A).

The almost identical rotatory dispersion characteristics of both metallothioneins give support to a unique, sterically limited asymmetric orientation of the multidentate metal ligand sites. The large positive Cotton effects of both proteins, centered at 254 mμ and directly dependent on the cadmium content, fully confirms the results of earlier studies of optical rotatory dispersion of equine metallothionein (Ulmer *et al.*, 1962). They are abolished by removal and restored on readdition of cadmium. Their molar amplitude, *i.e.*, 42,500°, calculated per g-atom of cadmium, is identical in both proteins. Thus, the amplitudes of the Cotton effects reflect the cadmium contents. They are typical examples of extrinsic Cotton effects due to the interaction of a chromogenic group, not part of the peptide chain, with asymmetric loci of these proteins (Ulmer and Vallee, 1965).

The biological activity of human metallothionein has not been studied as yet, though the pertinent considerations have been discussed (Kägi and Vallee, 1960, 1961). The physical characteristics of the two proteins are consistent with their role in a wide range of biochemical and physiological mechanisms such as catalysis, transport, storage, immune phenomena, or heavy metal detoxication. This latter suggestion gains support from the isolation of a metallothionein-like protein from livers of rabbits exposed to cadmium for long periods of time (Piscator, 1963). The potential participation of metallothionein in the pharmacological action of mercurial diuretics, suggested by the present data, may represent a new avenue of approach to the establishment of a functional role.

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