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Characterization of isoprotein patterns in tissue extracts and isolated samples of metallothioneins by reverse-phase high-pressure liquid chromatography

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Reverse-phase high-pressure ('performance') liquid chromatography was used to characterize the isometallothioneins in preparations isolated from tissues of a variety of animals by 'conventional' chromatographic methods. The resolution was such that isoproteins differing by a single serine→leucine difference in 61 residues could be easily separated. Yields from the reverse-phase support were typically 60–70% for the isoproteins. Comparisons of isometallothionein patterns after Cd^{2+}-induction in rabbits indicated that total metallothionein concentrations were about 4-fold higher in liver than kidney extracts from the same animal. In the extracts a minimum of four and six isometallothionein peaks were detected in kidney and liver respectively. Under acidic conditions, where the metals are removed from the protein, the chromatographic properties, i.e. hydrophobicities, of the isoproteins from kidney were identical with those of four of those found in liver. Although the same peaks appeared in tissue extracts from individual animals, concentration differences were apparent. Remarkably, no differences were observed between the isoprotein patterns of liver or kidney as a consequence of either Cd^{2+}- or Zn^{2+}-induction. Chromatography of the metal-containing forms at neutral pH in Tris buffer indicated that the relative ratios of the complexed metal ions in the isoproteins were found to be effectively identical, not only before and after chromatography, but also within the separated forms from a single tissue source.

Metallothioneins are small proteins that are easily identifiable by a number of physical characteristics (Kägi & Nordberg, 1979): (*a*) molecular weights in the region of 6500; (*b*) high content of either Zn and/or Cd or Cu; (*c*) amino acid compositions characterized by approx. 30% cysteine and the absence of aromatic amino acids; (*d*) optical properties typical of thiolate complexes. At physiological pH the metal complexes are quite stable, with dissociation constants estimated to range from 10^{-15} to 10^{-11} M for Cd and Zn respectively (Andersen *et al.*, 1979). Co-ordination is thought to be tetrahedral (Vašák, 1980), and an arrangement of the metal in clusters has been detected by n.m.r. and e.s.r. measurements of various derivatives (Otvos & Armitage, 1980; Vašák & Kägi, 1981).

Proteins possessing some or all of the above characteristics have been isolated from tissues of numerous species (Kägi & Nordberg, 1979), and their organ distribution, as well as quantitative determination of their concentration, has been most thoroughly documented for the mammals, where, in all likelihood, such proteins are ubiquitously distributed, albeit often at very low concentrations. Since their relative concentrations are highest in the kidney and liver of animals pretreated with injections of Cd^{2+} or Zn^{2+} salts, most investigations have been directed to such sources.

Within a particular organ a number of isometallothioneins can usually be detected. For example, liver preparations from horse and rabbit have indicated the presence respectively of two and three isoproteins, which have been shown to have slightly different primary structures. The most highly conserved positions within metallothioneins or similar proteins are those of the cysteine residues, which are all directly involved in metal binding. In mouse tissues two genes coding for metallothioneins-I and -II have been detected, and the nucleotide sequence of that for the isometallothionein-I has been determined (Glanville *et al.*, 1981). Direct measurement

Abbreviation used: h.p.l.c., high-pressure ('performance') liquid chromatography.

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of metallothionein-I mRNA concentrations before and after metal-ion induction have indicated that regulation occurs at the level of transcription and that the kinetics of accumulation and degradation differ (Durnam & Palmiter, 1981).

The presence of isometallothioneins in the various tissues has complicated their isolation, characterization and quantitative determination. Perhaps the most extreme example to date is that of the human liver, where two metallothionein-containing fractions can be separated by a combination of gel- and ion-exchange-chromatographic procedures (Bühler & Kägi, 1974; Kissling, 1979). Primary sequencing of the material designated metallothionein-2 indicated the presence of a single polypeptide chain (Kissling & Kägi, 1977). The situation was different for the metallothionein-1 pool, where at five positions in the primary sequence two, and in one position even three, different amino acid residues were found in approximately equal concentrations (Kissling & Kägi, 1979). Thus the insufficient resolving powers of 'conventional' separation techniques has thus far prevented the unambiguous determination of the primary structures of the components of this pool.

Recent developments in the application of reverse-phase h.p.l.c. both to peptide (Wilson *et al.*, 1981*a*,*b*) and to protein (Wilson *et al.*, 1982*a*,*b*; Berchtold *et al.*, 1982) isolations suggested that the technique might be a useful means to further the characterization of isometallothioneins, i.e. with respect to cellular concentrations, number, relative ratios and tissue differences. In addition to investigating each of these points, the present study illustrates the versatility of the method for the detection and isolation of the multiple forms of metallothioneins from samples prepared by using the well-established 'conventional' chromatographic techniques.

Materials and methods

Buffers and reagents

The compositions of the buffers used for h.p.l.c. were as follows: System I, buffer *A*, 0.1% trifluoroacetic acid; buffer *B*, 0.1% trifluoroacetic acid containing 60% (v/v) acetonitrile; System II, buffer *A*, 50mM-Tris/HCl buffer, pH 7.5; buffer *B*, 50mM-Tris/HCl buffer, pH 7.5, containing 60% (v/v) acetonitrile.

Water was twice distilled from quartz apparatus, and h.p.l.c.-quality acetonitrile was from J. T. Baker Chemicals (Phillipsburg, NJ, U.S.A.). Tris was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and trifluoroacetic acid ('zur Sequenzanalyse') from Fluka (Buchs, Switzerland). All other chemicals were of analytical grade and were obtained from either Fluka or Merck (Darmstadt, West Germany).

Plaice liver metallothionein was kindly supplied by Dr. J. Overnell and had been prepared as described by Overnell & Coombs (1979). Induction and isolation of rabbit liver metallothioneins-1 and -2 were performed as described by Kimura *et al.* (1979). Horse kidney metallothioneins-A and -B were prepared as described by Kägi *et al.* (1974) and Kojima *et al.* (1979). Rat liver metallothioneins-1 and -2 were isolated as described by Andersen *et al.* (1978). Chicken liver metallothionein was obtained by the method described by Bühler & Kägi (1974) for the isolation of human liver metallothioneins.

Apparatus

The h.p.l.c. instrument consisted of two Altex model 110A pumps, a Rheodyne model 7125 injector with either 10μl or 600μl sample loop, a Kontron model 200 microprocessor and a Uvikon 725 spectrophotometer equipped with an 8μl flow-through cell. Chromatography was performed on 4.6mm × 250mm Aquapore RP-300 columns (RP-8; 10μm particle size; 30nm-pore-size support) from Brownlee Laboratories (Santa Clara, CA 95050, U.S.A.). Column effluents were manually collected and dried by rotatory evaporation.

A Durrum D-500 analyser was used for amino acid analysis after sample hydrolysis in 6M-HCl *in vacuo* at 110°C for 22h. Metal analyses were performed on a Varian atomic-absorption spectrophotometer (model AA-975) equipped with a graphite tube atomizer (model GTA-95) and an automatic sampling unit. Standard metal solutions for instrument calibration were supplemented with apo-metallothionein (0.1mg/ml), since it was observed that sulphide formation occurred during sample heating and atomization, which led to inaccurate determination of Cd amounts.

Protein modifications

Cysteine residues were oxidized with performic acid (Hirs, 1967) before acid hydrolysis. Protein concentrations were determined either by amino acid analysis or by quantitative determination of titratable thiol groups. To this end the protein was diluted into a mixture of 900μl of 6M-guanidinium chloride/50mM-EDTA, pH 8.0, and 100μl of 5mM-5,5'-dithiobis-(2-nitrobenzoic acid) in 50mM-sodium phosphate buffer, pH 7.5. Absorption differences at 412nm were measured in a Zeiss spectrophotometer (model PMQ3) and concentrations were calculated by assuming a molar absorption coefficient $\varepsilon_{412} = 12\,600\,M^{-1}\cdot cm^{-1}$ for 5-thio-2-nitrobenzoate (Ellman, 1959).

Protein isolation

Tissue extractions were performed by using the procedure described by Bühler & Kägi (1974), as modified by Kimura *et al.* (1979). After homo-

genization in 20mM-Tris/HCl buffer, pH8.6, at 0–1°C with a weight/buffer volume ratio of 1:1, ethanol and chloroform (both at −30°C) were added to the homogenate (1.05:0.08:1.00, by vol.) and the precipitate was recovered by centrifugation at 27000*g* for 15min at −20°C (Sorvall centrifuge, SS-34 head). The soluble proteins were precipitated overnight at −30°C by the addition of 3vol. of ethanol (−30°C), and the precipitate was collected by centrifugation (as above). The pellet was dissolved in water, freeze-dried and redissolved in the specific buffer *A* for h.p.l.c. At this point, conventional isolation procedures for metallothioneins would continue with gel and ion-exchange chromatography (Bühler & Kägi, 1974).

Results and discussion

The availability of metallothionein preparations from rabbit, rat, chicken and plaice liver, as well as from horse kidney, which had been isolated by conventional methods (see above), provided the means of testing the suitability of reverse-phase h.p.l.c. for separation/isolation of isoproteins from a starting mixture containing only the closely related components. Preliminary h.p.l.c. experiments (results not shown) indicated little difference between using either the 0.1% trifluoroacetic acid or 0.1% H_3PO_4/10mM-$NaClO_4$ (see Wilson *et al.*, 1981*b*) buffer systems. Since the former is sufficiently volatile to allow for its removal by evaporation, thus circumventing the problem of salt formation, chromatography was performed in 0.1% trifluoroacetic acid and elution with a gradient of acetonitrile.

Analysis by h.p.l.c. of conventionally isolated horse kidney metallothioneins-A and -B (Fig. 1) suggested either significant amounts of cross-contamination or the presence of additional isoproteins. Preparative isolations of the designated peaks, and subsequent amino acid analysis (Table 1), indicated that peaks A-2 and A-3 from the metallothionein-A pool are different proteins, as shown simply by comparing the respective leucine values. As found by analysis of primary structure (Kojima *et al.*, 1979), two forms should be expected that have been shown to differ at a single position, one form having a serine residue and the other a leucine residue at position 54. The observed elution orders are those expected from hydrophobicity differences arising from serine/leucine substitutions, i.e. stronger interaction of the leucine derivative with the C-8 packing, with this in turn requiring a higher concentration of the organic eluent to effect elution. The compositions of the two pooled fractions indicate the expected serine/leucine differences, and perhaps another, involving a threonine residue, that has not been observed hitherto.

H.p.l.c. of the horse metallothionein-B material indicated the presence of minimally three isoproteins in the approximate proportions 14:1.6:1 (Fig. 1*b*). The amino acid composition of the third peak was determined (Table 1) and found to agree quite well with the expected values from the primary sequence (Kojima *et al.*, 1979). Although the amounts of material found in peaks 1 and 2 (Fig. 1*b*) were very small, the relative proportions of cysteic acid (after oxidation; see the Materials and methods section), lysine and aspartate were representative of metallothioneins. The elution points of the designated h.p.l.c. peaks of horse kidney metallothioneins-A and -B (Figs. 1*a* and 1*b*) are sufficiently different, especially when the original chromatograms are compared, to suggest the presence of five or six isoproteins. It should be noted that the values listed under 'B' in Table 1 for each metallothionein analysed represent the amino acid compositions of the preparations before h.p.l.c. As observed for the horse metallothionein fractions, as well as for most of the isoproteins chromatographed, the contaminating background is significantly decreased after the h.p.l.c. (compare isoleucine and/or leucine values before and after h.p.l.c.).

Rabbit liver metallothionein fractions, designated metallothioneins-1 and -2, displayed even greater heterogeneity when analysed (Figs. 1*c* and 1*d*). On the basis of similar elution points, the peaks designated 1-1 and 2-1 appear to represent cross-contamination due to incomplete separation during the final DEAE-Sephadex chromatographic step. However, the ion-exchange column resolves the two pools so well (see Fig. 2 in Kimura *et al.*, 1979) that it is presently thought that these peaks might well represent additional isoproteins. The double peaks observed in the peak 2-1 region suggest more than a single component in peak 1-1; the non-integral values for leucine in the amino acid composition support this conclusion. Peaks 1-2 and 1-3 differ primarily in the ratios of serine, leucine and arginine relative to lysine. The sequencing efforts of Kimura *et al.* (1979) have detected a serine→leucine substitution that, although not specifically identified (see footnotes to Table 1 in Kimura *et al.*, 1979), is probably located at position 54. This would account, at least partially, for the observed compositional differences. The analysis data for peak 2-2 agree with what is expected from the known primary sequence of rabbit liver metallothionein-B (Kimura *et al.*, 1979).

Preparative h.p.l.c. of plaice liver metallothionein yielded two predominant peaks (Fig. 2*a*) with slightly differing amino acid compositions (results not given). Since no heterogeneity has thus far been detected in the primary sequence (approx. 50% completed; see Overnell *et al.*, 1981), the significance of these differences remains unclear.

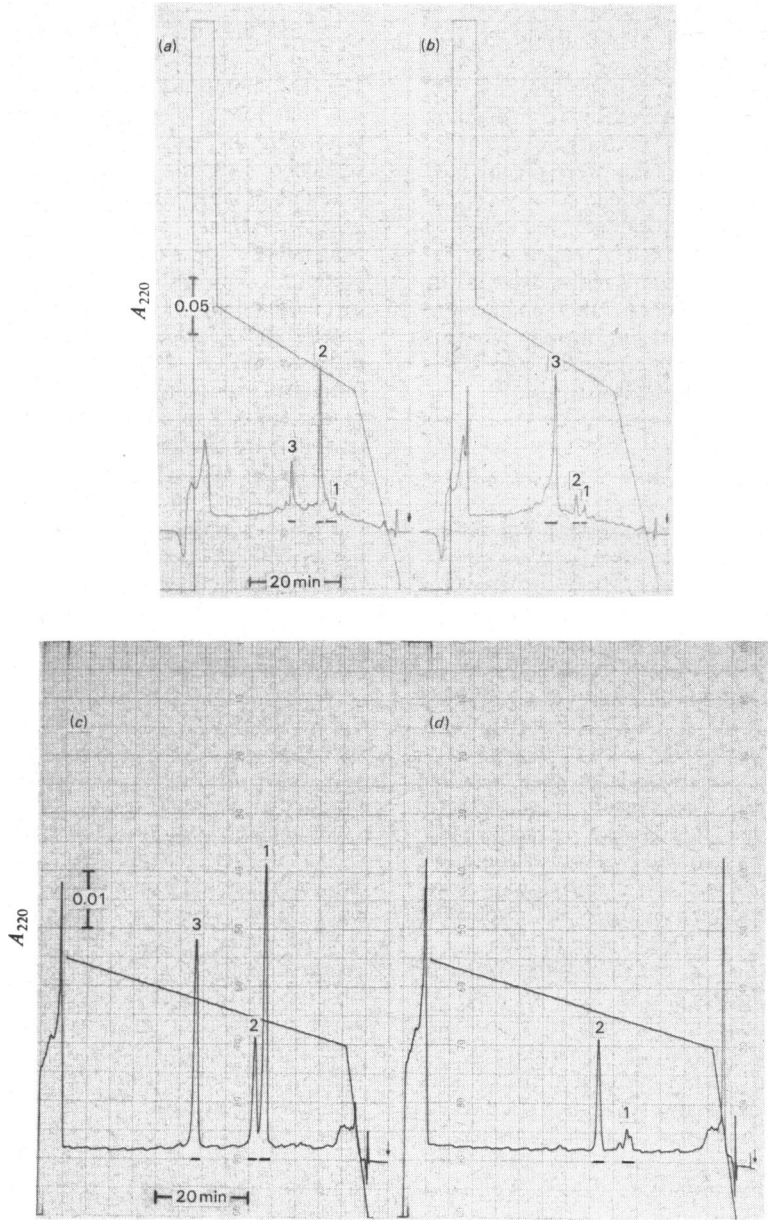

Fig. 1. *H.p.l.c. of isolated horse kidney metallothioneins-A (a) and -B (b) and rabbit liver metallothioneins-1 (c) and -2 (d) in trifluoroacetic acid buffers*

Amounts (μg) injected were 150, 150, 179 and 60 respectively. Chromatography was performed at 1 ml/min on an RP-300 column (46 mm × 250 mm) with a gradient (straight line) formed between buffer *A* (0.1% trifluoroacetic acid) and buffer *B* (0.1% trifluoroacetic acid containing 60% acetonitrile). Effluent was pooled as indicated by the horizontal bars; arrows indicate points of sample injection.

Chromatography of the chicken liver metallothionein fraction (fig. 2*b*) indicated the presence of a single component with elution properties characteristic of metallothioneins. Amino acid analysis supported this identification. The composition of the chicken liver metallothionein, as isolated in the

Table 1. *Amino acid compositions of peaks derived from the h.p.l.c. of horse kidney and rabbit liver metallothioneins*
For experimental details see the text. See Fig. 1 for the representative chromatograms. Compositions before h.p.l.c. are designated under 'B', those obtained after h.p.l.c. with the isometallothionein designation followed by the peak number. Collected samples were oxidized before hydrolysis and analysis; cysteine was determined as cysteic acid. Compositions are given relative to lysine content.

	Amino acid composition (residues/molecule)														
	Horse (kidney) metallothionein A				Horse (kidney) metallothionein B			Rabbit (liver) metallothionein-1					Rabbit liver metallothionein-2		
	Present work			Kojima *et al.*	Present work		Kojima *et al.*	Present work				Kimura *et al.*			Kimura *et al.*
Amino acid	B	A-2	A-3	(1979)	B	B-3	(1979)	B	1-1	1-2	1-3	(1979)	B	2-2	(1979)
Asp	3.3	2.7	2.8	3	3.3	2.8	3	3.9	4.2	4.0	3.9	4	5.1	4.8	4
Thr	3.2	2.8	1.8	3	1.5	1.0	1	3.4	4.0	3.2	2.8	3	3.0	2.8	3
Ser	7.7	7.4	5.2	8/7	8.1	7.3	8	7.8	8.3	8.3	7.2	8	7.3	7.7	9
Glu	3.2	2.2	2.1	2	4.1	3.0	3	2.3	2.4	2.3	2.4	2	2.4	1.0	1
Pro	3.5	3.0	3.1	3	2.7	2.0	2	2.1	2.3	1.9	2.0	2	2.7	2.3	2
Gly	7.8	6.4	6.3	7	6.1	4.9	5	3.6	4.0	3.5	3.4	4	4.5	3.8	4
Ala	5.4	4.8	4.7	5	7.4	6.9	7	6.4	6.5	6.5	7.1	7	8.2	8.3	8
Cys	21.6	18.8	19.6	20	23.8	19.1	20	20.3	20.0	20.9	20.2	20	19.2	20.4	20
Val	1.3	1.0	0.8	1	3.0	2.7	3	—	—	—	—	—	—	—	—
Met	1.0	0.9	0.9	1	0.9	1.0	1	1.0	1.0	1.0	1.0	1	1.0	0.8	1
Ile	0.3	—	—	—	0.3	—	—	1.0	1.2	1.0	1.1	1	1.1	0.8	1
Leu	0.6	—	0.8	—/1	0.5	—	—	0.6	0.4	0.3	1.1	1	0.5	—	—
Lys	6.0	6.0	6.0	6	7.0	7.0	7	8.0	8.0	8.0	8.0	8	8.0	8.0	8
Arg	1.8	1.7	1.5	2	1.2	0.9	1	0.5	—	0.6	0.9	1	—	—	—
Yield (%)	...	72.0				69.7				64.0				70.0	

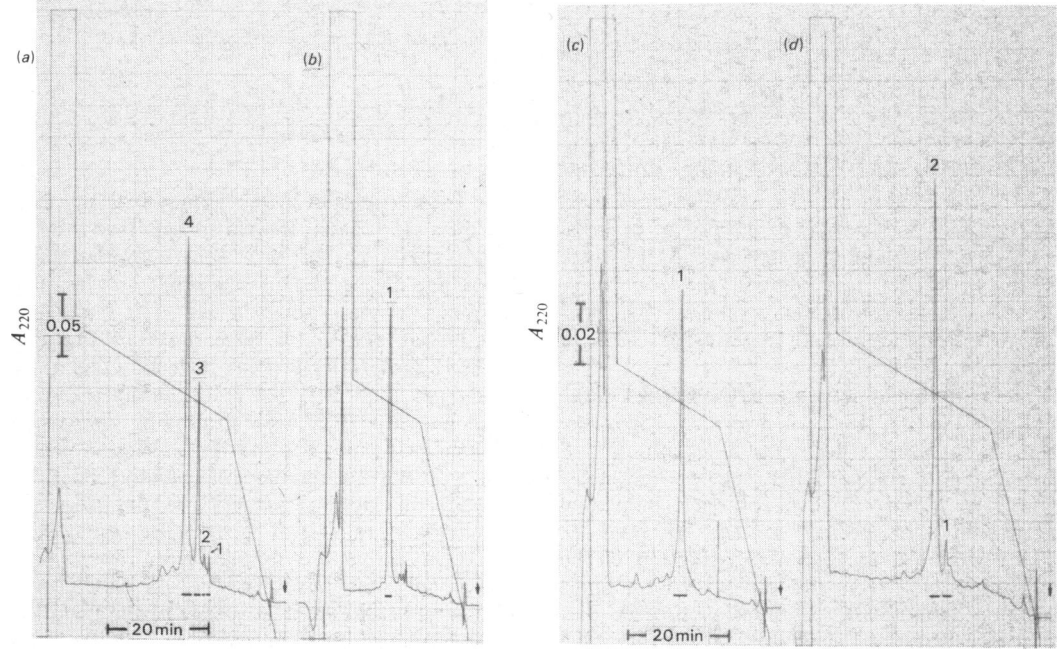

Fig. 2. *H.p.l.c. of isolated plaice liver metallothionein (a), chicken liver metallothionein (b) and rat liver metallothioneins-1 (c) and -2 (d) in trifluoroacetic acid buffers*
Amounts (μg) injected were 117, 104, 130 and 143 respectively. All other conditions were as given in Fig. 1 legend.

present work, is noteworthy in that it is the first that has been shown to contain integral amounts of the amino acid histidine. Figs. 2(*c*) and 2(*d*) illustrate the homogeneity of the isoprotein preparations from rat liver. Although each appears to consist of predominantly a single protein species, their amino acid compositions indicated increased homogeneity after h.p.l.c.

For reverse-phase h.p.l.c. to be useful for qualitative and quantitative detection of metallothioneins in tissue extracts, the yields and resolution must be compared with those obtained with conventionally isolated preparations. The average yield for those isoproteins chromatographed by h.p.l.c. was 65.2% (Table 1 and the values determined for the indicated peaks in Fig. 2). A variation in yield of ±5% between injections was established by performing repeated isolations of the isoproteins found in rabbit liver metallothionein-1 preparations (see Fig. 1*c*) and determining recoveries by amino acid analysis (results not given). On the basis of these results it would appear the recoveries are reproducible and that the average yields from the Aquapore RP-300 support are at least 65%. The resolution obtained was illustrated in Figs. 1(*a*) and 1(*c*) by the separation of the two isoproteins differing solely in a serine/leucine substitution within 61 amino acids.

The chromatography of a 'crude' extract from Zn^{2+}-induced rabbit kidney material is shown in Fig. 3. Those peaks with elution characteristics similar to those of the isolated isoproteins [37–45% of buffer *B* on the indicated gradient; see Figs. 1(*c*) and 1(*d*)] were collected, oxidized and their amino acid compositions determined (results not given). The compositions of peaks 2–5 were in agreement with the expected values (see Table 1), and clearly illustrated that the only proteins eluted within this area of the chromatogram are isometallothioneins. Within fractions 2–5 a total of 32.5 μg of material was recovered. Assuming a 65% recovery from reverse-phase h.p.l.c. (see above), the concentrations of the isometallothioneins would be 50 μg per 1300 μg of chromatographed material or 3.8% of total amount injected.

The metallothionein heterogeneity observed in the samples analysed in Figs. 1–3 has two possible origins: (*a*) it could reflect the collection of isoproteins typical of the particular species, and hence observable in kidney or liver of each individual animal within the species; or (*b*) it could be artificially produced through the extraction of the protein from pooled livers and kidneys from animals that contain individually different isoprotein patterns. Considering the reasonable chromatographic yields, sensitivity and resolution observed with the 'crude' rabbit kidney extract (Fig. 3), experiments were undertaken to clarify which of these two possibilities applies.

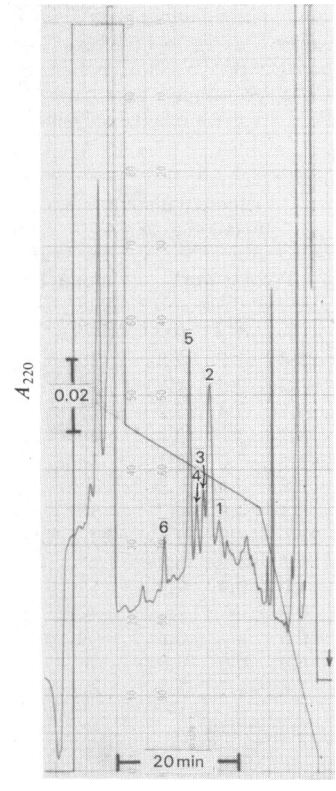

Fig. 3. *H.p.l.c. of 'crude' extract from Zn^{2+}-induced rabbit kidney material in trifluoroacetic acid buffers*
Amount injected was 1300 μg. All other conditions were as given in Fig. 1 legend.

Thus 'crude' liver and kidney extracts from both $CdCl_2$- and $ZnCl_2$-treated rabbits (see the Materials and methods section) were chromatographically compared. Fig. 4 illustrates that an elution pattern similar to that shown in Fig. 3 is observed, i.e. a series of peaks are observed that are eluted at 35–42% of buffer *B*. Peak collection and amino acid analysis confirmed that all of the material within the designated area of each chromatogram was metallothionein (results not given). The elution profiles also suggest that the numbers of metallothionein peaks in liver and kidney extracts are the same between individual animals. [The tissues employed in the present study were obtained from rabbits pretreated with subcutaneous injections of $CdCl_2$ or $ZnCl_2$. Injection schedule and dosage of the metal salt was as described by Kimura *et al.* (1979). For Fig. 4 only representative chromatograms from the h.p.l.c. of the extracts from eight $CdCl_2$-treated or four $ZnCl_2$-treated rabbits are given.] Although the resolution is far from optimal, e.g. with respect to

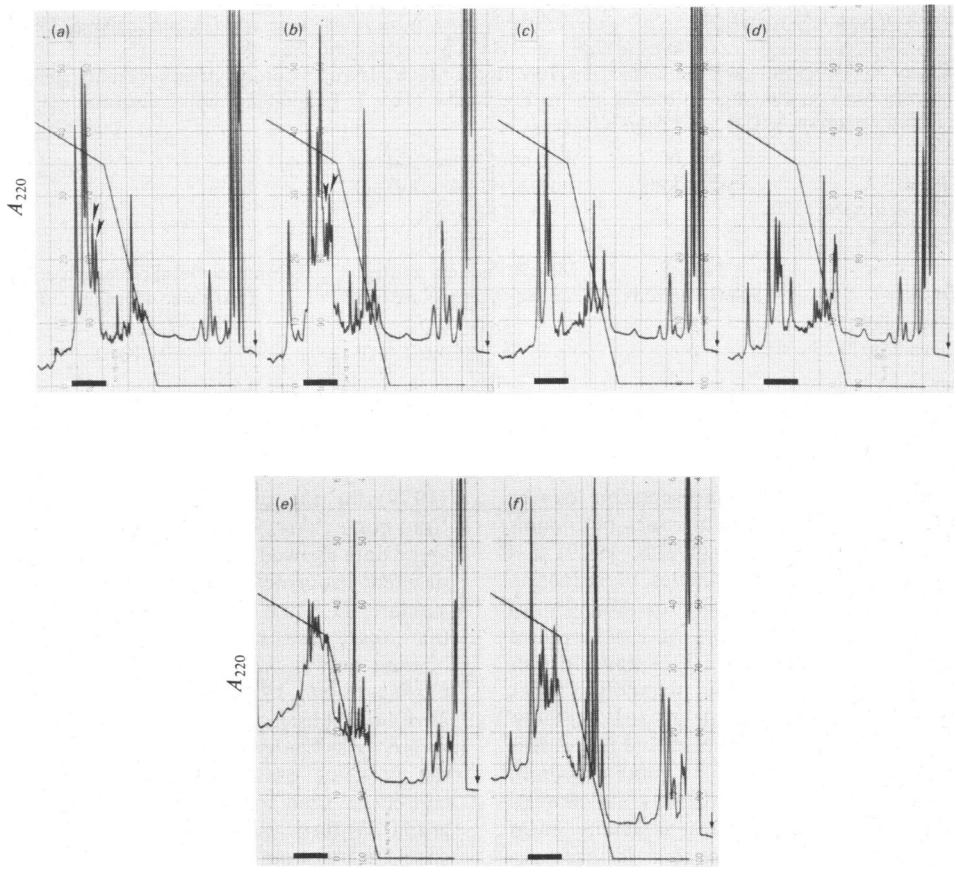

Fig. 4. *Comparison of metallothionein isoprotein h.p.l.c. patterns from rabbit liver (a and b) and kidney (c and d) 'crude' extracts after Cd^{2+}-induction and from liver extracts after Zn^{2+}-induction (e and f)*

Starting amounts for extraction (from two different animals) were 5 g of liver and 2 g of kidney from the $CdCl_2$-treated animals and 10 g of liver from the $ZnCl_2$-treated animals. After freeze-drying and dissolution of extracts in 0.1% trifluoroacetic acid the amounts (μg) injected were 250 (*a*), 300 (*b*) or 500 (*c–f*). Chromatography was in buffer System I (see the text), the horizontal bars indicate the elution areas of the isometallothioneins, and the recorder speed and scale expansion as in Fig. 3.

total separation of all components, it is possible to detect at least six peaks in liver preparations and four in kidney extracts.

Variations exist, however, in the metallothionein peak heights, and presumably concentrations, when extracts from the same tissue of different individual animals are compared. To exclude the possibility that the differences originated from variation between extractions, a series of preparations were made from the same liver and chromatographically compared. The peak heights, as well as numbers, were found to be identical. Thus the comparison of elution patterns between preparations apparently can (does) reflect differences in tissue concentration of the isometallothioneins. A close comparison of the peak profiles originating from liver and kidney extracts indicates that minimally two additional peaks are present in liver preparations (denoted by arrows in Figs. 4*a* and 4*b*). Comparison of the areas of isometallothionein peaks with the amount (μg) injected suggests that on a weight basis the concentrations are about 2-fold higher in liver than in kidney preparations. As indicated in Table 2, the yield of 'crude' extract from liver was approximately twice that from kidney; thus the total concentration of isometallothioneins per g liver is 3–4-fold higher. The yields observed from 'conventional' isolations from these two tissue sources have also indicated a similar metallothionein distribution (Suzuki & Yamamura, 1980).

Table 2. *Yields of 'crude' extract from Cd^{2+}-induced rabbit kidney and liver material (a) and estimated metallothionein contents in Cd^{2+}- and Zn^{2+}-induced rabbit liver (b)*

Preparation of 'crude' extract was performed as given in the Materials and methods section. H.p.l.c., peak collection and hydrolyses were as indicated in the legends to Fig. 3 and Table 1. Estimated amounts of total metallothionein were calculated assuming a 65% yield from h.p.l.c.

(a) Organ	No. of extractions	Weight of 'crude' extract (mg/g wet wt. of tissue)
Kidney (Cd^{2+})	4	12.6 ± 2.8
Liver (Cd^{2+})	4	23.0 ± 6.0

(b) Organ	No. of determinations	Metallothionein content (μg/mg of 'crude' extract)	Estimated metallothionein content (μg/g wet wt. of tissue)
Liver (Cd^{2+})	3	38.0 ± 4.0	600–1200
Liver (Zn^{2+})	2	31.2 ± 4.6	450–1040

The quantities of isometallothioneins that can be isolated are generally higher from tissues of animals pretreated with cadmium salts (Webb, 1979) and suggest differences in (*a*) the kinetics of induction and/or degradation or (*b*) the ease (or difficulty) of isolation from organs from Cd^{2+}- or Zn^{2+}-induced animals. Durnam & Palmiter (1981) have in fact shown that transcription of the metallothionein-1 gene in the mouse is approximately equal for both liver and kidney after Cd^{2+} treatment and more than 4-fold higher in liver after Zn^{2+} administration. These findings, however, do not directly indicate the amounts of isolatable protein present at any given time. The isometallothionein patterns in liver extracts from animals after either Cd^{2+}-or Zn^{2+}-induction are illustrated in Figs. 4(*a*) and 4(*b*) and Figs. 4(*e*) and 4(*f*) respectively. From both liver and kidney sources (results not given) the amounts are about twice as much in the Cd^{2+}-exposed animals. It should be noted, that, although the peak areas for the isometallothioneins are quite similar, a comparison of the weight equivalent chromatographed (see the legend to Fig. 4) shows that roughly twice as much material from the Zn^{2+}-induced animals was injected. When the total concentrations were determined by peak collection and amino acid analysis, the isoprotein content in Cd^{2+}-induced liver preparations was also found to be somewhat higher (Table 2). The estimated metallothionein amounts of 0.5–1.2 mg/g of liver, or 0.05–0.12% on a wet-weight basis, are about 6–8-fold lower than the values reported by Piotrowski *et al.* (1973) for Cd^{2+}-exposed rats. Such variations might well arise from any number of sources: (*a*) Cd^{2+} dosage per kg body wt., length of exposure; (*b*) methods utilized for measuring concentrations; or (*c*) age and species differences.

The metal compositions of metallothioneins have been found to reflect their origin. For example, the following proportions in molar percentages for Zn:Cd:Cu have been reported by Kojima *et al.* (1979) for the two metallothionein-IB isoproteins from horse: liver, 92:5:3, and kidney, 35:63:2. The same data showed that the metal compositions of the different isoproteins from the specific organ are similar. ^1H n.m.r. studies have indicated, however, that there are no subpopulations of molecules binding only a particular metal, i.e. Cd, Zn or Cu (Vašák *et al.*, 1980). The isoprotein patterns that have been illustrated in Figs. 1–4 have not been due to any possible hydrophobic differences induced by the presence of a metal, since, under the acidic conditions used for h.p.l.c., the proteins chromatograph in their metal-free or apo forms (directly checked by atomic-absorption spectroscopy). Our observations that various Ca^{2+}-binding proteins could be isolated by reverse-phase h.p.l.c. with Tris buffers at pH 7.5 (Wilson *et al.*, 1982*a*; Berchtold *et al.*, 1982) prompted us to perform isolations of isometallothioneins under similar conditions.

The h.p.l.c. of fish liver and rabbit liver metallothioneins-1 and -2 are illustrated in Fig. 5. All of the isoproteins are eluted at lower organic-solvent concentrations than when h.p.l.c. was performed in the acidic pH range (compare with Figs. 1 and 2). The isoproteins designated 1 and 2 in Fig. 1(*c*) from the rabbit liver metallothionein-1 preparation were observed to be co-eluted (peak 1, Fig. 5*b*) under neutral pH conditions, however. A comparison of the amounts of material injected (see the legends to Figs. 1 and 5) with the resultant peak areas (note changes in scale expansions) indicates a substantial decrease in the absorbance per μg of protein in the trifluoroacetic acid buffer system and is attributable to disruption of the metal thiolate complexes (molar absorption coefficient approx. $17000\,M^{-1}\cdot cm^{-1}$ at 220 nm; Bühler & Kägi, 1979) and subsequent chromatography of the apo forms of the proteins. Determination of the Cd content of those isometallothioneins subjected to h.p.l.c. in Tris buffer (Fig. 5) indicated that the amount of the metal was not influenced by the chromatography. Because of

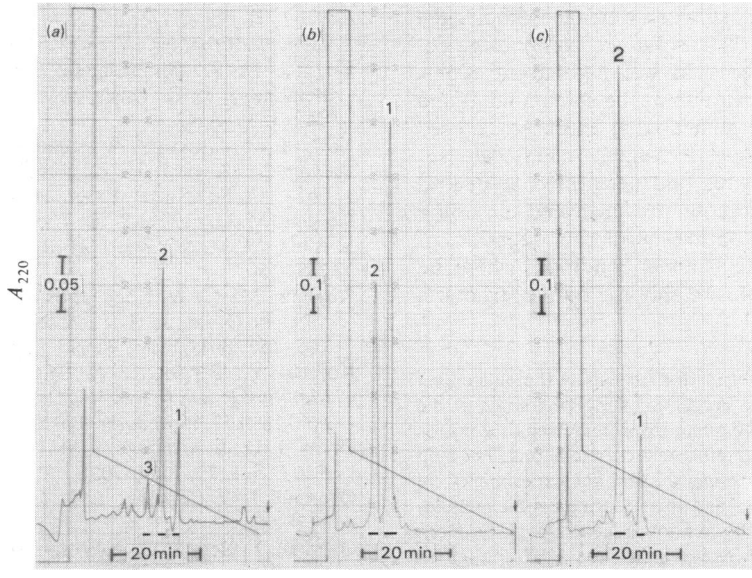

Fig. 5. *H.p.l.c. of isolated plaice liver metallothionein (a) and rabbit liver metallothioneins-1 (b) and -2 (c) in neutral-pH buffers*

The amounts (μg) injected were 30, 143 and 96 for (*a*), (*b*) and (*c*) respectively. Chromatography was in buffer System II (see the text).

Table 3. *Metal/protein ratios of metallothioneins after h.p.l.c. at neutral pH*

All metallothioneins were isolated by 'conventional' methods from Cd^{2+}-induced livers. See Fig. 5 for the respective h.p.l.c. chromatograms and peak identifications. The Zn contents were calculated on the basis of 7 metal binding sites/molecule; Cu content was shown to be negligible.

Protein source	Peak no.	Concn. of protein (μM)	Concn. of Cd^{2+} (μM)	Metal:protein ratio		Recovery of protein (%)
				Measured Cd	Calc. Zn.	
Rabbit liver metallothionein-1	1	19.5	102.2	5.24	1.76	72
	2	12.2	59.0	4.84	2.16	
Rabbit liver metallothionein-2	1	3.8	18.6	4.89	2.11	73
	2	17.6	91.8	5.22	1.78	
Plaice liver metallothionein	1	2.7	18.8	6.96	0.04	67
	2	3.3	22.6	6.77	0.23	

the low amounts of isoproteins that were isolated, only Cd concentrations were determined (Table 3). The Cd values in both rabbit isoproteins are less than the integral value of 7, since the remaining sites are occupied by (presumably) Zn. On the other hand the Cd/protein ratio of approx. 7:1 is exactly that previously noted for plaice liver metallothionein (Overnell & Coombs, 1979). Of the two Cd^{2+}-induced metallothioneins examined, the fish protein is worthy of note in that, unlike those isolated from Cd^{2+}-treated mammals, it contains primarily Cd with only trace amounts of Zn and Cu (Overnell & Coombs, 1979). The differences of approx. 7% and 6% between the measured Cd/protein ratios for peaks 1 and 2 for both rabbit liver metallothioneins are greater than the deviations found for Cd determinations at these concentrations and might indicate isoprotein differences. Similar differences in the metal compositions have been shown by Kojima *et al.* (1979) for the induced horse isometallothioneins and by Suzuki & Yamamura (1980) for the metal-ion-induced rat isometallothioneins.

The h.p.l.c. methodology utilized in the present investigation has proved to be a rapid technique for the isolation of isometallothioneins either directly from tissue extracts or from isoprotein mixtures that had been prepared by 'conventional' techniques. Since the resolution, sensitivity and yields from the

reverse-phase h.p.l.c. support were acceptable, the method proved to be useful in comparing isometallothionein patterns, as well as concentrations, in liver and kidney extracts of the same animal and between tissues from animals that had been exposed to different metal ions. Isolations made with isometallothionein mixtures and performed at neutral pH on analytical-size columns illustrated the utility of the method for preparing microgram quantities of the individual isoproteins under conditions where the bound metals, and presumably the native structure, remained intact.

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