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STUDIES TO IDENTIFY THE LOW MOLECULAR WEIGHT BISMUTH-BINDING PROTEINS IN RAT KIDNEY

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Abstract—Low molecular weight bismuth-binding proteins were isolated from kidneys of rats exposed repeatedly to bismuth chloride. The proteins [(Bi,Cu)-BP] isolated by polyacrylamide gel electrophoresis yielded four peaks. The two main fractions [(Bi,Cu)-BP 2 and (Bi,Cu)-BP 3] were characterized by a molecular weight of 7500, bismuth contents of 79 and 90 μg/mg, copper contents of 14 and 23 μg/mg and zinc contents of 3.8 and 3.6 μg/mg respectively, and an A_{250}/A_{280} ratio of 1.5. Their amino acid composition resembled that of the copper chelatin from the liver. Isoelectrofocusing separated the protein into three components of pI values of 3.8, 5.5 and 7.4. The protein differs from metallothionein by its apparent molecular weight, amino acid composition, lower SH group content and a high copper content. Traces of a low molecular weight copper-binding protein of molecular weight of about 7500, of similar electrophoretic properties, were also found in kidneys of non exposed rats. It is suggested that these proteins are related to each other. For the renal bismuth-binding proteins the term 'renal chromochelatin' is proposed.

Previous studies on the metabolism of bismuth have dealt with the distribution and excretion of this metal in humans [1, 2] and in animals [3–7]. In the last few years an increased interest in the toxicology of bismuth has been observed and numerous cases of human intoxication have been reported from France, Germany, Australia and Switzerland [8–11]. The biochemical background of bismuth binding in various tissues and bismuth toxicity remained unexplored. The mechanism of bismuth binding in the kidneys seemed to be of special interest.

Some light was shed on the renal binding of bismuth by our previous work. Thus, it has been found that bismuth elevated the renal level of metallothionein-like proteins [12], and at the same time the metal appeared to be bound to a protein fraction of the same molecular weight as mettalothionein, i.e. about 10,000. The inducible character of the protein in question was confirmed by ^{35}S incorporation studies [13]. Using ^{206}Bi [14], it could be shown that the respective bismuth complex is located in the soluble fraction of rat kidneys [14]. Repeated administration of bismuth to rats brings about an increase of copper content in the kidneys and both bismuth and copper are present in the same fraction of low molecular weight proteins [15].

This study aimed to identify further the bismuth-binding protein from rat kidneys, and in particular to elucidate its relationship to metallothionein.

METHODS

Female rats of the Wistar strain weighing 170–220 g were used in these experiments. The animals received five subcutaneous injections of 3 mg Bi/kg every other day, followed by two more injections (the sixth and seventh injections) of 3 mg Bi/kg to which 0.7 μCi of ^{206}Bi (sp. act. 1 mCi/μg; Amersham) had been added as a tracer. The rats were killed 24 hr after the last dose of metal. The kidneys were removed and processed as indicated in Fig. 1 [16, 17].

The sediment D was dissolved, applied to a Sephadex G-75 column (Pharmacia, Sweden) and eluted with 0.005 M Tris–HCl, pH 8.6. Fractions of 5 cm^3 were collected and their absorptions at 250 and 280 nm were measured in a VSU 2-P spectrophotometer. ^{206}Bi activity was determined in each fraction using an USB-2 scintillation counter with a NaI/Tl crystal.

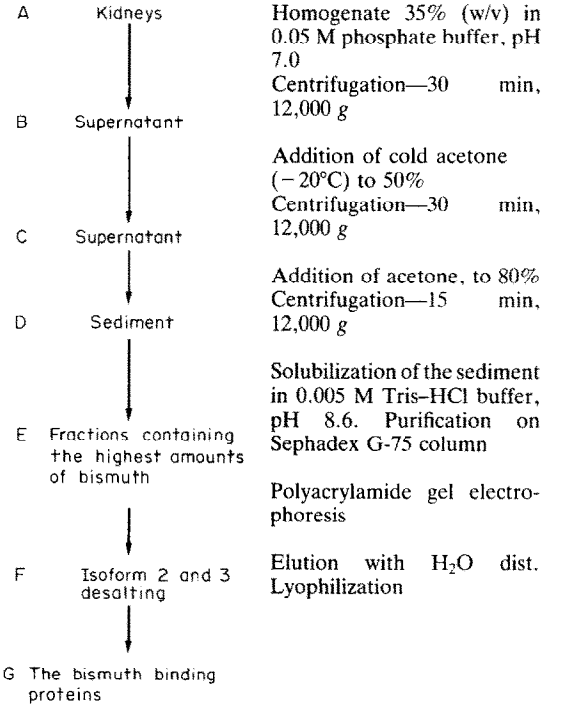

Fig. 1. Scheme of isolation of the bismuth-binding proteins from the kidneys of rats exposed repeatedly to $BiCl_3$.

The low molecular weight protein fractions which bound the highest amounts of bismuth were combined, lyophilized and then purified by polyacrylamide gel electrophoresis [18, 19]. Preparative electrophoresis was run on a column 3 × 10 cm with the following buffers: Tris–HCl, pH 8.1, and Tris–Glycine, pH 8.3, at a current of 50 mA per tube. Analytical electrophoresis was run according to the same method applying a current of 2 mA per tube [19]. The SH groups and copper in the gel were localized by staining with DTNB (5,5'-dithiobis 2-nitrobenzoic acid) and rubeanic acid [20, 21], respectively.

Protein was determined nephelometrically with tannic acid [22] and bismuth and copper by a spectrophotometric method [23] throughout all preparation steps and in the final preparation. For metal determination samples were mineralized in concentrated sulphuric and perchloric acid (1 : 1). The mineralizate was extracted with 10 cm^3 of a 0.03% solution of zinc diphenyldithiocarbamate (DBDTC-Zn) in carbon tetrachloride. Absorbance of bismuth and copper complexes with DPDTC-Zn was measured in a VSU 2-P spectrophotometer at 370 and 440 nm.

In some experiments zinc and copper were estimated in the mineralizates by atomic absorption spectroscopy, using the Pye Unicam SP 192 spectrophotometer, with relative standard deviations of ± 12 and ± 11 per cent, respectively.

Molecular weight of the bismuth-binding protein was determined using a Sephadex G-75 column [24]. Absorption spectra of this protein in the range of 200–600 nm were taken in a SECORD UV-VIS spectrophotometer. Apoproteins of the bismuth-bonding protein was obtained by the method of Weser *et al.* [25].

The amino acid analysis was performed on the JEOL Amino Acid Analyzer. The samples were hydrolysed in 6 N HCl for 24 and 72 hr in 110°. Cysteine was determined as cysteic acid after performic acid oxidation [26, 27].

Isoelectrofocusing [28] was performed in a sucrose concentration gradient, 5–25 per cent (w/v), using 1% ampholine (LKB) of the pH range of 5–8. Fractions of 0.5 cm^3 were collected and pH, absorption at 250 nm and ^{206}Bi activity were determined.

RESULTS

A scheme of isolation of the bismuth-binding proteins is given in Fig. 1. Enrichment of the preparation with respect to the bismuth-binding proteins was accomplished by discarding the nonsoluble components of the homogenate after centrifugation at 12,000 *g*, as it was found from the previous studies that these proteins are contained in the soluble fraction [14]. Further steps involving acetone fractionation were introduced by analogy with the procedure for isolation of equine renal metallothionein [17]. As is documented in Table 1, a 6–7-fold enrichment of the bismuth-binding proteins was achieved in the first two preparation steps. Acetone precipitation of these proteins, which facilitated the procedure, did not improve the efficiency of their purification. Part of the bismuth contained in the acetone precipitate proved to be hardly soluble and remained in the discarded sediment. Bismuth losses took place at every successive isolation step. They were partly due to bismuth bound also to high molecular weight proteins, which were removed in the second separation step, as well as to the withdrawal of samples for analysis at every stage of purification. Sediment D (contained only 4 per cent of original protein content and as much as 25 per cent of bismuth), slightly tawny coloured, was dissolved in Tris–HCl buffer and applied to a Sephadex G-75 column. The separation pattern indicates that bismuth complexes with proteins of different molecular weights are present in the solution examined. A distinct peak is apparent at V_t/V_o of about 2.6, corresponding to a molecular weight below 10,000. Eluate fractions from this region had a characteristic yellow colour which allowed easy identification of successive peaks in later preparations. The combined peak fractions (V_t/V_o = 2.4–2.8) were used for the next step of the preparation. The fraction of the highest peak (tubes No. 53–61) was utilized for analytical purposes. The absorption peak of this fraction was determined without and after 10-fold dilution. The curve obtained without dilution exhibited a slight shoulder in the range of 400 nm, which might be responsible for the colour of the solution. The curve obtained after dilution possessed a distinct shoulder in the range of 240–290 nm.

Table 1. The bismuth and protein content at subsequent preparation steps

Step of the isolation procedure	Total protein (mg)	Total bismuth (μg)	Bismuth (μg/mg protein)
A	7260	6030	0.8
B	2900	4260	1.4
C	468	2500	5.3
D	312	1510	4.8

The combined eluate fractions from the Sephadex G-75 column of V_t/V_o of 2.4–2.8 were applied to a polyacrylamide gel and were separated into four fractions, all of which contained copper and bismuth (Fig. 2). Two major fractions (b,c) of R_m values of

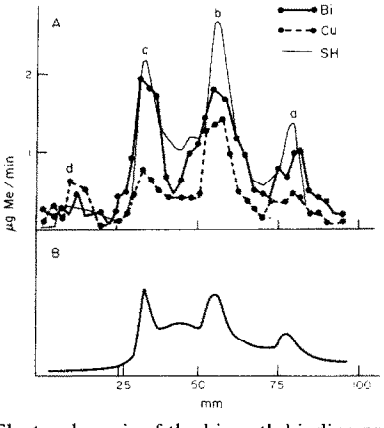

Fig. 2. Electrophoresis of the bismuth-binding proteins on polyacrylamide gel: (A) SH groups, stained in gel: densitogram; Cu and Bi, chemical analysis of 2.5 mm slices; (B) densitogram of the gel without staining (blue filter).

Table 2. The metal content of bismuth-binding proteins [(Bi,Cu)-BP]*

Metal	(Bi, Cu) - BP 2 (μg metal/mg protein)	(Bi, Cu) - BP 2 (mole metal/mole protein)	(Bi, Cu) - BP 3 (μg metal/mole protein)	(Bi, Cu) - BP 3 (mole metal/mole protein)
Bismuth	79.0 ± 6.0	3.8 ± 0.3	90.0 ± 4.5	4.3 ± 0.2
Copper	14.0 ± 3.0	2.2 ± 0.5	23.0 ± 3.5	3.6 ± 0.6
Zinc	3.8 ± 0.5	0.6 ± 0.1	3.6 ± 0.4	0.6 ± 0.1
Sum	96.8	6.6	116.6	8.5

* Results expressed as means ± S.D. from three preparations (mol. wt accepted as 10,000. (Although the molecular weight is 7500, for sake of easy comparison with metallothionein 10,000 was assumed in calculations.)

0.33 and 0.57 [designated (Bi,Cu)-BP 3 and (Bi,Cu)-BP 2] were eluted with distilled water as described by Zelazowski *et al.* [18], concentrated and desalted. Table 2 shows the metal content in pure isoforms (Bi,Cu)-BP 2 and 3, and Fig. 3B shows the absorption spectra of these isoforms.

Table 3 shows the amino acid composition of the (Bi,Cu)-BP 2 and 3 as compared with that of (Cd,Zn)-metallothionein isolated from rat liver [18] and that of copper chelatin [29]. The bismuth-binding proteins contain less cysteine and more leucine and isoleucine than (Cd,Zn)-metallothionein; they also contain histidine and phenylalanine.

Weser *et al.* [25] reported that copper interferes with the determination of amino acid composition, thus explaining the apparently low cysteine content of the respective copper proteins. The explanation does not hold true for the bismuth-binding proteins. The apoproteins of the bismuth-binding proteins obtained according to Weser *et al.* [25] did not display significant differences in amino acid compositions as compared with the respective holoproteins.

In addition, isoelectrofocusing and analytical electrophoresis in polyacrylamide gel were performed, using proteins contained in the peak fraction of the Sephadex G-75 separation. The results of isoelectrofocusing are presented in Fig. 3A. The curve obtained indicated three isoelectric points of pI values of 3.8, 5.5 and 7.4. These values are different

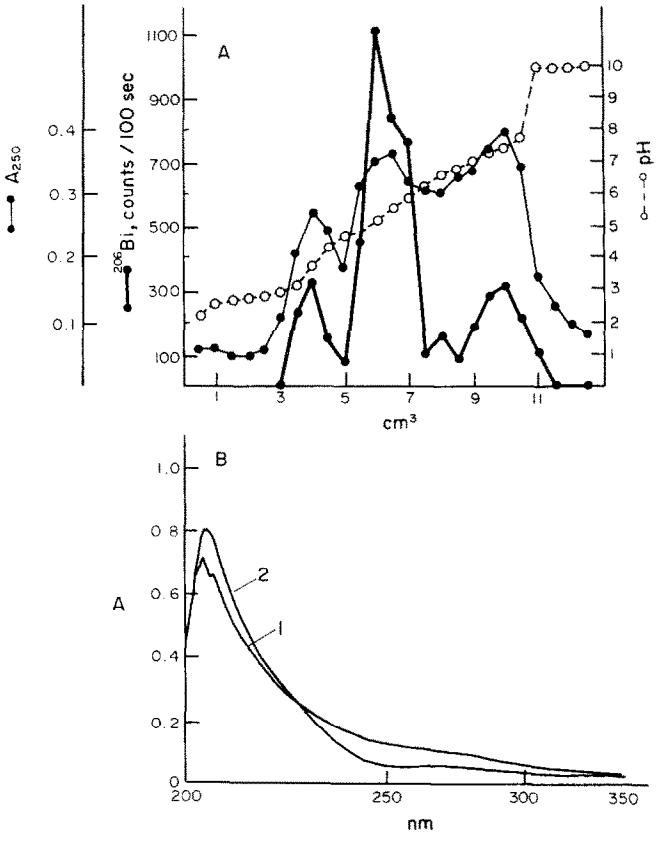

Fig. 3. (A) Isoelectrofocusing of the bismuth-binding proteins. For details see Methods. (B) Absorption spectra: 1, (Bi,Cu) - BP 2; 2, (BiCu) - BP 3.

Table 3. Amino acid composition of (Bi,Cu) - BP 2 and 3

Amino acid	(Bi,Cu)BP 2	(Bi,Cu)BP 3	Copper chelatin [29]	(Cd,Zn)MT 1 [18]	(Cd,Zn)MT 2 [18]
Asp	8.9	8.8	9.8	7.4	6.5
Thr	5.3	7.7	5.1	4.6	5.5
Ser	12.9	17.7	4.2	8.0	16.4
Glu	9.7	6.6	11.1	5.6	3.3
Pro	4.5	5.6	3.6	6.4	4.7
Gly	8.5	10.0	8.0	6.4	9.2
Ala	7.4	6.9	6.0	8.0	4.9
Cys*	17.1	13.7	14.6	31.7	34.2
Val	4.7	4.8	5.2	1.9	3.1
Met†	—	—	1.6	0.6	0.2
Ile	2.6	1.8	4.3	0.7	0.3
Leu	3.2	3.2	6.3	0.9	0.9
Tyr	0.3	0.2	1.3	—	—
Phe	1.7	1.8	2.1	—	—
Lys	12.2	11.7	13.1	16.9	11.5
His	1.1	0.6	1.1	—	—
Arg	—	—	2.7	0.1	—

* Determined as cysteic acid.
† Determined as sulfomethionine.

from those reported for cadmium metallothionein [17, 30, 31].

During analytical electrophoresis, the Sephadex eluate separated into two major (b,c) and two minor (a,d) fractions, all of which contained copper and bismuth (Fig. 2). The intense yellow colour of bismuth-binding proteins was observed in electrophoretic fraction a, b and c, but not in fraction d (Fig. 2B).

To check whether a copper-binding protein of similar molecular weight is present in the rat kidney under physiological conditions, the 12,000 *g* supernatant fraction of the kidney homogenate was subjected to chromatographic separation on a Sephadex G-75 column (Fig. 4A). Trace amounts of a low molecular weight copper-binding protein were found. Peak fractions of the protein in two subsequent separations showed apparent molecular weights of 7400 and 7700. A distinct increase in absorption at 250 nm was found in the same band of V_t/V_o of about 2.3, and a ratio of absorption at 250 and 280 nm amounting to 1.8 in the peak fraction. The respective protein, obtained in the same way as the bismuth-binding protein, i.e. from the Sephadex G-75 chromatography of the sediment D (see Fig. 1) was subject to gel electrophoresis and gave a distribution pattern as shown in Fig. 4B. The R_m values were 0.35, 0.57 and 0.80.

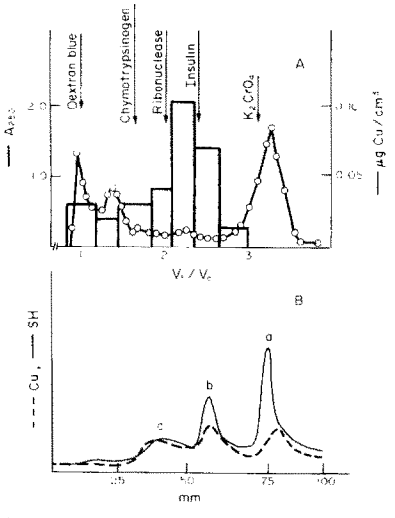

Fig. 4. (A) Chromatographic separation pattern of the supernatant fraction of normal rat kidneys. Sephadex G-75 column, formate buffer, pH 8.0, 3 cm^3 fractions collected. (B) Electrophoresis of the low molecular weight copper-binding proteins from kidneys of nonexposed rats; Cu and SH groups stained in gel: densitogram.

DISCUSSION

The data presented indicate that bismuth, administered repeatedly to rats, is bound in the kidneys to an inducible low molecular weight protein localized in the soluble fraction. This protein appeared to be heterogenous and could be separated into two major fractions—the bismuth-binding proteins 2 and 3 [(Bi,Cu)-BP 2 and 3]. Both fractions, characterized by an intensely yellow colour, represent main sites responsible for binding of bismuth in rat kidneys and are distinguished from the metallothionein by their apparent molecular weight, metal content, amino acid composition, isoelectric points and absorption spectra of the homoproteins.

The apparent molecular weight was varied in different preparations obtained between 6800 and 8100, with mean 7500, as compared with 10,000 for cadmium-induced metallothionein.

Both isoforms differ with respect to metal content (Table 2), but both contain copper as the main endogenous metal, whereas zinc plays this role in the hepatic cadmium-metallothionein [18]. Both isoforms also differ from each other in amino acid composition and are distinguished from the two isoforms of metallothionein by a much lower content

of cysteine and a higher content of leucine, isoleucine and glutamic acid. They are distinguished from copper chelatin at the same time by their higher content of serine and lower contents of leucine, isoleucine and tyrosine, whereas the content of cysteine is similar (Table 3). The isoelectric points of the crude fraction of the bismuth-binding proteins are 3.8, 5.5 and 7.4, as compared with 3.4 and 4.3 for metallothionein [17].

The absorption spectra of the holoprotein of bismuth-binding proteins show an absorption shoulder at 270 nm as compared with 250 nm of metallothionein. At the same time, the bismuth-binding proteins are yellow.

The above findings justify the statement that the low molecular weight renal bismuth-binding proteins are not identical with metallathionein. The term 'renal chromochelatin' is being proposed for these proteins to describe the chelation capability for metals, intense colour and some resemblance to the copper chelatin described by Winge *et al.* [34].

The classification of the 'renal chromochelatin' with respect to low molecular weight copper proteins occuring in the kidneys under physiological conditions is not yet possible because these proteins have not been yet investigated. Our preliminary studies have shown that in physiological conditions the rat kidneys contain a low molecular weight protein of apparent molecular weight about 7500. This protein is heterogenous when subject to gel electrophoresis, yielding three fractions, the R_m of which are close to those observed for the three subfractions (1, 2 and 3) of Bi,CuBP. This physiological copper protein seemed to be colourless. Most likely the colour of the renal chromochelatin depends on the presence of bismuth in the protein molecule. If this is so, it seems most probable that the renal chromochelatin is formed as a result of chelating bismuth by the physiologically occurring low molecular weight renal copper protein, the amount of which increases essentially due to induced biosynthesis [31].

Recent reports seem to suggest the existence of two different classes of low molecular weight metal binding proteins, having many essential properties in common: (a) metallothionein as the basically cadmium, zinc protein occuring in the liver; in the same species the protein is also able to bind copper in the kidney in certain circumstances [13, 32]; (b) copper proteins which have been isolated so far from the liver and intestines of some species, and distinguished from metallothionein by several features, of which the most essential seem to be differences in the amino acid composition, similar to those reported in this work. For one of these proteins Winge *et al.* [34] have proposed the term 'copper chelatin'.

Although the relation between the 'renal chromochelatin' and the 'copper chelatin' isolated from liver has not been definitely established, some of the features discussed in detail in this paper bring the 'renal chromochelatin' closer to the 'copper chelatin' than to the classical metallothionein.

Whether 'renal chromochelatin' and 'copper chelatin' belong to the same family of copper proteins and whether such copper proteins could be classified as a sub-branch of the broader family of metallothioneins remains to be established. Preliminary evaluation based on immunological properties does not exclude the latter possibility, because metallothionein 2 isolated from rat liver does have some determinants in common with the 'renal chromochelatin' isolated from rat kidneys [35].

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