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INDUCIBLE GOLD-BINDING PROTEINS IN RAT KIDNEYS *

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Abstract—Repeated administration of gold (chloroauric acid) to rats resulted in a marked increase in the level of low molecular weight renal metal binding proteins (RMBP). [^{35}S]Cysteine incorporation studies pointed to an induced biosynthesis of the latter under the influence of gold. The gold-binding proteins (Au-BP) were found predominantly in the kidney cytosol, but also extracts from 12,000 *g* and 1000 *g* sediments (mitochondria and nuclei) contained low molecular weight gold complexes. The Au-BP isolated from the post-mitochondrial supernatant of rat kidneys had an apparent molecular weight of 12,000, and on DEAE-cellulose gave four fractions all containing gold, in amounts 11–33 μg Au/mg protein, and copper, above 18 μg/mg protein. The protein solutions were yellowish and their u.v.-spectra showed extinction maxima at 220–225 nm as well as slight shoulders at 250–280 nm. The Au-BP displayed a tendency for coprecipitation and aggregation. A relation of the renal Au-BP to the mercury- and bismuth-binding proteins is suggested.

Various gold compounds have been applied in the therapy of certain diseases. Many authors reported serious kidney damage due during chrysotherapy of rheumatoid arthritis [1, 2]. It was also shown that gold administered as soluble gold compounds was deposited with high efficiency in the kidneys in animals [3, 4] as well as in man [5]. The mechanism of gold binding in this organ seems to be extremely interesting from the point of view of both subcellular distribution of the metal and binding with the kidney proteins. Few data existing so far on the intracellular distribution of gold in the kidneys point to the mitochondria and peroxysomes or lysosomes as the binding sites [6, 7].

Only preliminary data on the binding of gold by the kidney proteins are available [8]. In the previous report [8] we have shown that gold located in the kidneys is largely bound to low molecular weight proteins (molecular weight about 10,000). The relative share of these proteins in gold binding was much augmented if gold was administered repeatedly. It was not clear, however, whether the increase in contribution of this protein fraction to gold binding was due only to higher saturation of the preexisting proteins by gold or to induction of their biosynthesis.

Investigations on the inducible low molecular weight metal binding proteins in different organs are presently being carried out in many laboratories, mainly in connection with the role of metallothionein in the metabolism of cadmium, zinc and copper (for a review see [9, 10]). Recent data of Winge *et al.* [11] suggest that stimulation of metallothionein biosynthesis in the liver may also be exerted by gold.

In the kidneys induced biosynthesis of low molecular weight metal binding proteins is known to occur under the influence of cadmium, mercury [12], and bismuth [13], and is accompanied by an increase in the level of copper [14].

In the present work we have shown that also gold induces the biosynthesis of low molecular weight metal binding copper-proteins in rat kidneys and we have attempted to provide a preliminary characteristic of these proteins.

Since the identity of the renal metal binding proteins with metallothionein has not been finally dissolved, in the present report the following terms are used: (a) metallothionein (Mt) for proteins the identity of which with Mt has been proved or seems highly likely, (b) renal metal binding proteins (RMBP) for the group of proteins induced in the kidneys by mercury, bismuth and gold and (c) gold-binding proteins (Au-BP) for the low molecular weight inducible copper proteins binding gold.

MATERIALS AND METHODS

Materials. L-[^{35}S]Cysteine hydrochloride, specific activity 20–125 mCi per mM (The Radiochemical Centre, Amersham) or DL-[^{35}S]cysteine hydrochloride, specific activity 4.1 mCi per mM (Zentrallinstitut für Kernforschung, DDR) were used. Mercuric chloride, ^{203}Hg-labelled, activity 300 mCi/g, was obtained from the Instytut Badań Jądrowych, Świerk. Other reagents: chloroauric acid (POCh, Gliwice); Actinomycin D (Merck), Cycloheximide (Sigma), Rhodamine B (The British Drug Houses, Ltd.), Zn-dibenzodithiocarbamate (Zn-DBDTC; Merck). Standard LSM diet was obtained from Wytwórnia Pasz, Łowicz.

Animal experiments. Female rats of the Wistar strain, body weight 200–250 g, fed with standard LSM diet were used. The animals received subcutaneous (s.c.) injections of chloroauric acid solution (adjusted to pH 6) in doses of 10 mg Au/kg, seven doses every other day. Rats were killed 24 hr following the last gold injection. In the case of studies of [^{35}S]cysteine incorporation, only single intravenous (i.v.) doses of 4 mg Au/kg were also applied. In the latter case some rats were also killed 6 hr following metal injection.

[^{35}S]*Cysteine incorporation studies.* Twenty-four rats were used in this experiment. Single i.v. and repeated s.c. gold injections were applied as described under animal experiments. Simultaneously with gold,

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rats were administered intraperitoneally (i.p.) with 20 μCi L-[^{35}S]cysteine or 40 μCi DL-[^{35}S]cysteine. In certain experiments the animals were injected i.p. with Actinomycin D or Cycloheximide, in doses and time intervals suggested by Squibb and Cousins [15]. The solutions of both antibiotics were prepared in 50% propylene glycol. Actinomycin D (0.8 mg per kg of body weight) was injected once, at 4 hr before the last dose of gold. Cycloheximide was administered four times in two-hour intervals (each dose 1 mg per kg), starting at 30 min before the last dose of the metal. Rats were sacrificed by decapitation 24 and 6 hr after the last dose of gold in experiments with Actinomycin D and Cycloheximide, respectively. Control rats were injected only with [^{35}S]cysteine and gold and killed 24 or 6 hr after the injection of gold, to match experiments with both inhibitors. Kidneys of each rat were analysed separately. Homogenates (25 per cent, w/v) were prepared in a solution containing 0.25 M sucrose and 0.01 M Tris–HCl buffer, pH 7.4, at 0–4°, in a glass homogenizer (Universal Laboratory type 309) working at 80–100 V for 3 min. The homogenates were centrifuged at 12,000 *g* for 30 min. The supernatants (3.5–6.0 ml) were subjected to column chromatography on Sephadex G-75 gels.

Differential centrifugation. Homogenates of the kidneys from 8 rats (20 per cent, w/v) were prepared under the conditions described above. Following filtration through gauze the nuclear fraction was obtained at 1000 *g* (10 min), washed with the sucrose solution and recentrifuged. Joint supernatants were spun at 12,000 *g* for 20 min (mitochondrial fraction). The sediment was washed with sucrose solution and recentrifuged. Again, joint supernatants were spun at 100,000 *g* for 1 hr yielding sediment (microsomes) and supernatant (soluble fraction).

Isolation of Au-*BP.* Procedure used routinely in this laboratory for the preparation of metallothionein [16] has been applied. Kidney homogenate of forty rats (30 per cent, w/v) in 0.01 M Tris–HCl buffer, pH 7.4 (other homogenization conditions not controlled in this case) was spun at 12,000 *g*. To the supernatant, acetone fractionation was applied: sediment at 30 per cent saturation was discarded and that at 80 per cent saturation was used for further assay. Up to this step the preparation was carried out at 0–4°. The sediment, solubilized in the buffer, was centrifuged and the supernatant was used for gel chromatography.

Assays. Gel-chromatography was performed on Sephadex G-75, and ion-exchange chromatography on DEAE-cellulose. Column dimensions, buffers and standards are specified in legends to figures. Apparent molecular weight was determined by gel filtration [17].

Absorbance of eluates at 250 and 280 nm was measured with a VSU-2P spectrophotometer. Absorbance spectra were obtained with a Specord UV-VIS recording spectrophotometer.

For the assessment of ^{35}S radioactivity, 5 ml fractions of the eluate were disposed on plates 2.6 cm in diameter and after the samples' evaporation to dryness the beta radioactivity was measured with a Tesla SAD-12 counter using a plastic crystal and PL-5 scaler. The efficiency of counting was about 20 per cent. For the estimation of gold the 5 ml eluate fractions were pooled appropriately to meet the limit of detection of gold.

Gold, following ashing of samples at 650–700°, was solubilized and determined spectrophotometrically with Rhodamine B [18].

Copper, following mineralization of samples with sulphuric and nitric acids was determined spectrophotometrically with Zn-DBDTC [19].

Protein was determined nephelometrically with tannic acid [20].

RMBP were determined by tracing with $^{203}HgCl_2$ as reported for Mt [21]. Internal standards of Mt were applied and results were expressed as metallothionein. The standard Mt was obtained from equine renal cortex [16]. Radioactivity measurements were made with a USB-2 scintillation counter.

RESULTS

The level of low molecular weight metal-binding proteins. Following repeated exposure to gold (7 × 10 mg Au per kg) an approx. five- to six-fold increase of RMBP was found in the kidneys with only a two- to three-fold increase of the low molecular weight metal-binding proteins in the liver (Table 1). For comparison, repeated exposure to copper (7 × 10 mg Cu per kg) resulted in only a two-fold increase in the kidneys of the low molecular weight proteins. The level of copper in the kidneys of non-exposed rats was 10 μg Cu per g wet weight (7.1–12.4). Following exposure to gold it increased to 92 μg Cu per g (43–125) and following exposure to copper, to only about 60 μg per g. Repeated administration of gold resulted in gold levels in the kidneys of 250 μg Au per g wet weight (171–378) on the average.

[^{35}S]*Cysteine incorporation studies.* Figure 1 shows the ^{35}S chromatography of the postmitochondrial supernatants of the kidney tissue from rats: (A) not exposed to gold. (B) given with a single dose of gold. and (C) after repeated injections of gold. The single dose of gold resulted in an almost two-fold increase of radioactivity in the peak of low molecular weight proteins (V_e/V_0 about 2.2-RMBP fraction) whereas the incorporation of ^{35}S into the high molecular weight proteins (V_e/V_0 below 2) and into the amino acid/

Table 1. The level of low molecular weight metal-binding proteins (expressed as metallothionein, mg/g tissue, wet weight) in the liver and kidneys of rats exposed to gold and copper

Exposure	*n*	Liver	Kidney
Control	4	0.11 (0.08–0.13)	0.32 (0.29–0.41)
$HAuCl_4$, 7 × 10 mg Au/kg, s.c.	10	0.29 (0.14–0.47)	1.75 (1.08–3.94)
Control	4	0.13 (0.05–0.15)	0.30 (0.25–0.35)
$CuCl_2$, 7 × 10 mg Cu/kg, s.c.	7	0.94 (0.35–1.14)	0.62 (0.45–0.89)

Random values in parentheses: *n*—number of animals used; s.c.—subcutaneous.

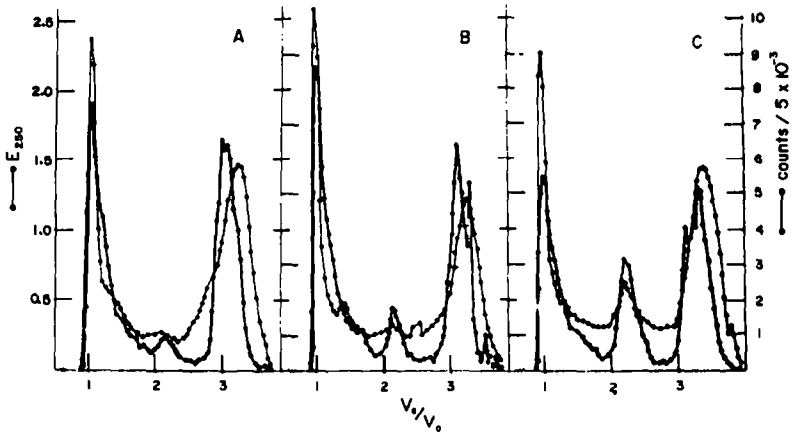

Fig. 1. Chromatography of the kidney 12,000 *g* supernatant. Sephadex G-75, columns 3 × 75 cm, formate buffer pH 8.0, fractions 5 ml each. Markers of the molecular weight: Dextran Blue (m.w. 2 mln), $V_e/V_0 = 1$; ribonuclease (m.wt 12,000), $V_e/V_1 = 2$; K_2CrO_4 (M.wt 129), $V_e/V_0 = 2.8$. (A) control rat, unexposed to gold, (B) after single dose of gold, (C) after repeated doses of gold. Animals were sacrificed 24 hr after (the last) injection of gold and/or DL-[^{35}S]cysteine. Recovery of ^{35}S from columns approximated 70 per cent.

peptide fraction (V_e/V_0 above 2.8) remained unchanged. An approximately three-fold elevation of [^{35}S]activity was found in the RMBP fraction following repeated doses of gold and a simultaneous elevation of the protein content of the said fraction is apparent from the increased E_{250} value. Simultaneously, the activity of ^{35}S decreased in both remaining fractions. The magnitude of ^{35}S incorporation into the RMBP fraction was dependent on gold concentration in the kidney; the latter amounted to approximately 46 μg/g and almost 300 μg/g of tissue after single and repeated gold doses, respectively.

An attempt was made to find out whether the gold-induced biosynthesis of RMBP could be controlled by two typical inhibitors of the transcription (Actinomycin D) and translation (Cycloheximide) processes, respectively. Following injections of Cycloheximide the incorporation of ^{35}S into the RMBP fraction was completely inhibited in rats given both single and repeated doses of gold. Simultaneously, the incorporation of ^{35}S was seriously depressed also in the fraction of high molecular weight proteins, and both processes were accompanied by increase of ^{35}S in the aminoacid/peptide fraction (Fig. 2A). Injections of Actinomycin D had less influence on the incorporation of ^{35}S into the RMBP fraction. Following repeated doses of gold the incorporation of ^{35}S into this fraction was only partly inhibited (Fig. 2B) as compared with rats exposed to repeated gold doses in the absence of antibiotics (Fig. 1C).

Following a single dose of gold the concentrations of the metal in the individual eluate fractions were of the same order, about 0.1 μg/ml (Fig. 3A). Following repeated injections of gold the content of metal considerably increased in the fraction of RMBP and the concentration of gold in the mentioned fraction of eluate amounted to about 1 μg/ml (Fig. 3C).

The inhibitors of protein synthesis seemed to influence the incorporation of gold into proteins only when a single gold dose was concerned (Fig. 3B). In the latter case the presence of gold could be positively stated only within the fraction of high molecular weight proteins

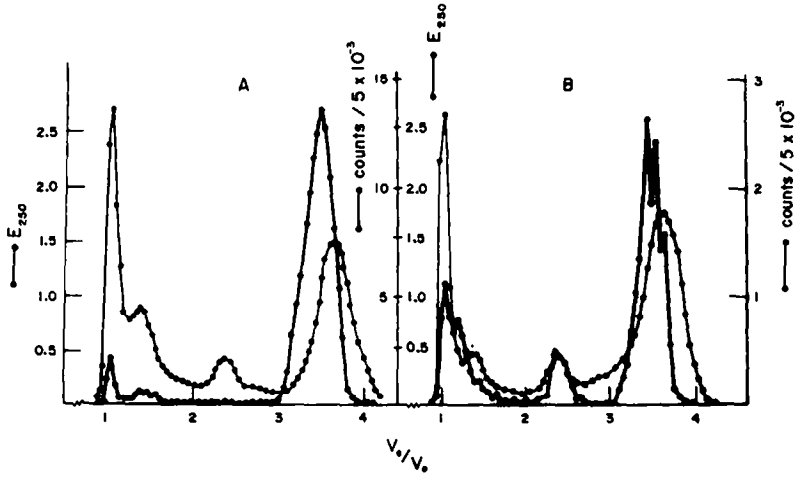

Fig. 2. Chromatography of the kidney 12,000 *g* supernatant. The incorporation of L-[^{35}S]cysteine into renal proteins in rats given with repeated doses of gold and (A) Cycloheximide, and (B) Actinomycin D. Rats killed 6 hr (A) and 24 hr (B) following administration of L-[^{35}S]cysteine. (For details see Fig. 1 and Methods.)

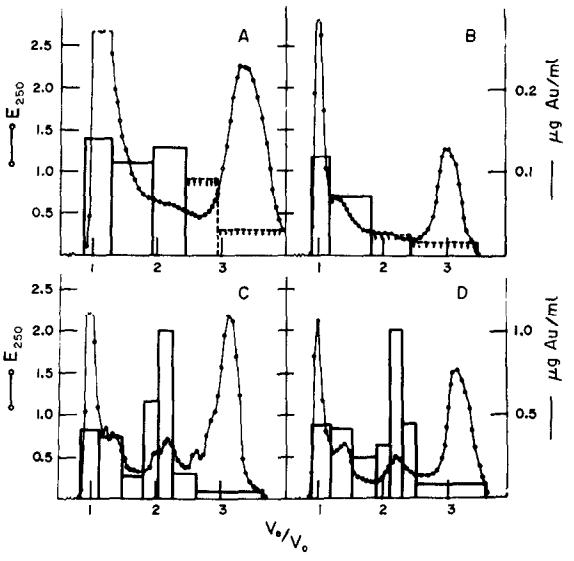

Fig. 3. Chromatography of the kidney 12,000 *g* supernatant. Binding of gold to various protein fractions following administration of (A) single dose of gold; rat sacrificed 6 hr after injection of the metal, (B) as above, rat treated with Cycloheximide, (C) repeated doses of gold; rat sacrificed 24 hr after last injection of metal; (D) as above, rat treated with Actinomycin D. Dotted line—negative results, the upper possible concentrations calculated from the sensitivity of the method. Recovery of gold from columns approximated 100 per cent. Columns: (A) 2 × 60 cm, (B) 3 × 75 cm, (C) and (D) 2.5 × 65 cm. (For other details see Methods.)

(V_e/V_0 below 1.8) whereas its level in the RMBP fraction remained under the limit of detection. In repeated exposure, where gold had been located into the RMBP fraction successively in the time preceding administration of Actinomycin D no change was found in its chromatographic distribution (Fig. 3D) as compared with rat receiving no inhibitor (Fig. 3C). Similar results were obtained for Cycloheximide.

Intracellular distribution of gold and copper. As shown in Table 2 the distribution pattern differed for both metals. Gold was found mainly (67 per cent) in the heaviest fraction (nuclei) as compared with only 30 per cent for copper. In the soluble fraction (100,000 *g* supernatant) only 18 per cent of gold was located, whereas the copper content of this fraction approached 50 per cent. The contribution of the two remaining (mitochondrial and microsomal) fractions to the binding of both metals was comparatively less pronounced.

Gel-filtration of the subcellular fractions. Gel-filtration of the subcellular fractions aimed at localization of the low molecular weight Au-BP. In this study copper was not determined.

The chromatographic patterns of gold distribution are shown in Fig. 4. The postmitochondrial fraction was analyzed jointly. In this fraction, which was supposed to contain most of the low molecular weight gold-binding proteins, the latter constituted the major protein component (Fig. 4C). The mitochondrial fraction was applied to the column following resuspension in water and vigorous rehomogenization. In this fraction the high-molecular weight proteins represented the main binding pool of gold (Fig. 4B). The heaviest nuclear fraction prior to chromatography was resus-

Table 2. The distribution of gold and copper in the subcellular fractions of the kidneys*

μg Au/g tissue†	Gold				μg Cu/g tissue†	Copper			
	N	M	P	S		N	M	P	S
		% Au in fraction					% Cu in fraction		
195 ± 10.4	67.3 ± 1.7	11.3 ± 1.4	2.6 ± 0.2	18.4 ± 0.9	70.9 · 7.7	29.9 ± 3.2	13.2 ± 2.2	9.5 ± 2.3	47.4 ± 3.7
		μg Au per 1 mg protein					μg Cu per 1 mg protein		
	6.9 ± 0.47	1.4 ± 0.16	0.62 ± 0.05	1.18 ± 0.13		0.99 ± 0.16	0.53 ± 0.11	0.91 ± 0.38	1.10 ± 0.10

* Results are expressed as mean ± standard deviation.
† The concentration in the organ calculated as sum of contents of its fractions.
Fractions: N—1000 *g*; M—12,000 *g*; P—100,000 *g*; S—supernatant.

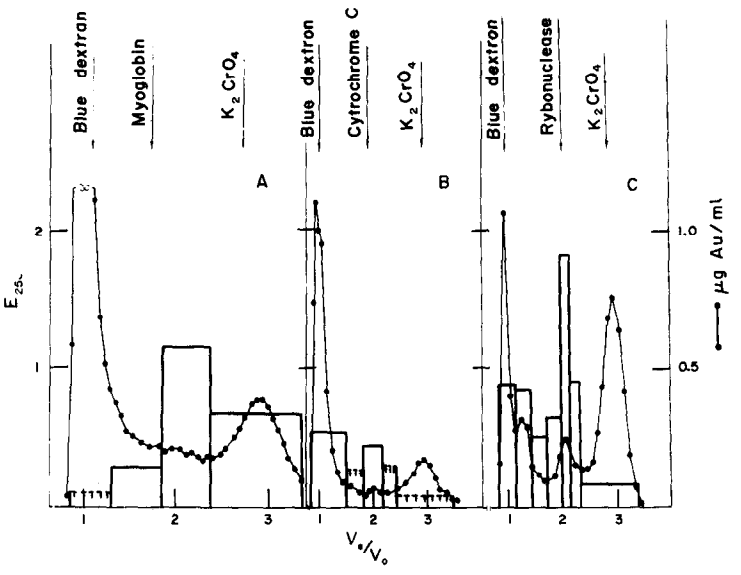

Fig. 4. Sephadex G-75 chromatography of subcellular fractions of the kidneys: (A) soluble extract from 1000 *g* fraction, (B) rehomogenized 12,000 *g* fraction, (C) 12,000 *g* supernatant. Solid bars—gold concentrations, μg Au/ml; dotted lines—negative results, upper possible concentrations calculated from the sensitivity of the method. Recovery of gold from columns approximated 100 per cent. Columns: (A) 2.5 × 70 cm. (B) 2 × 60 cm, (C) 2.5 × 60 cm: formate buffer, 0.01 M, pH 8.

pended in water, vigorously rehomogenized and spun at 12,000 *g* for 10 min. The supernatant contained about 50 per cent of gold of the whole fraction, mainly as complexes of low molecular weight V_e/V_0 about 2 and 3 (Fig. 4A).

Isolation of Au-*BP*. Table 3 shows copper and gold concentrations on subsequent steps of the isolation procedure: the first Sephadex G-75 chromatography was omitted. A progressive impoverishment of the relative gold content at the first two steps of the procedure is evident. The ratio Au:Cu attained a constant value about 0.4 by weight following the second step of acetone fractionation. Figure 5 shows the rechromatography of the Au-BP on Sephadex G-75. By direct comparison to the elution volume of ribonuclease, the apparent molecular weight of the Au-BP is approximately 12,000. An absorbance maximum recorded from peak fraction was apparent at about 220–225 nm. Also, a slight shoulder was seen between 250–280 nm. The solution was yellowish and showed slight absorption in the visible region with maximum at about 410 nm.

Following rechromatography on Sephadex G-75 as discussed above, the fraction of V_e/V_0 in the range of 1.8–2.4 was applied to a DEAE-cellulose column. Four fractions were found eluted at the eluate gradients of 0.01, 0.1, 0.15 and 0.25 M Tris–HCl buffer, pH 8.6 (Fig. 6). These fractions contained gold in concentrations of 15, 33, 24 and 11 μg Au/mg protein, respectively. The copper content of all the fractions was above 18 μg Cu/mg protein. The content of gold in fractions 2 and 3 as compared to the initial value of the joint Sephadex fraction applied to the column (Table 3) points to a further enrichment of Au-BP at this step of procedure. The apparent molecular weight estimated by gel filtration was obtained only for fractions 1 and 4, and amounted to 8,400 and 11,700, respectively.

In parallel attempts carried out to isolate Au-BP by a slightly modified procedure a distinct tendency for aggregation has been noted. This was often observed when protein fractions of the selected apparent molecular weight (about 10,000) were liophylized and preserved in a refrigerator for a few days. On rechromatography they often gave profiles scattered toward lower elution volumes. The same was true for some fractions obtained by ion-exchange chromatography.

Table 3. Gold and copper content on subsequent steps of isolation procedure

Step	μg Me/mg protein*		Au/Cu (w/w)
	Au	Cu	
Homogenate, 30%	1.9	0.9	2.2
12,000 *g* Supernatant	1.0	1.0	1.0
30% Acetone supernatant	0.8	1.3	0.6
80% Acetone precipitate, solubilized	1.6	3.8	0.4
Pooled Au-BP fraction from Sephadex G-75 (rechromatographed)	12	26	0.4

*Me = metal.

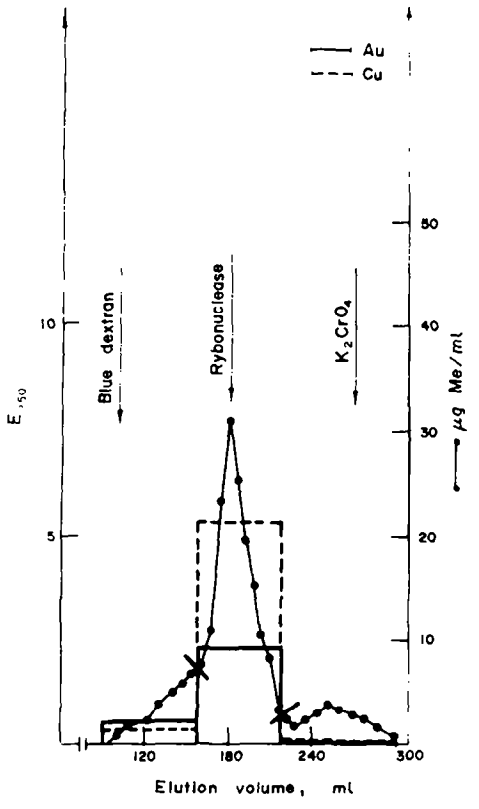

Fig. 5. Rechromatography of the Au-BP on Sephadex G-75. Solid bars—Au concentrations, μg Au/ml (recovery approximately 100 per cent); dotted bars—Cu concentrations, μg Cu/ml (recovery approximately 70 per cent); recovery of protein approximately 90 per cent; column 3 × 70 cm. Formate buffer 0.01 M, pH 8.0, fractions 6 ml each. Pooled fractions for further ion-exchange chromatography are separated with short thick lines.

DISCUSSION

Gold is one of the four metals able to induce the biosynthesis of low molecular weight metal-binding protein in both the liver and kidney. The other three metals Cd, Zn, Cu exert this effect mainly regarding metallothionein in the liver (for a review see [10]). The most recent data of Winge *et al.* [11] point to an elevation of zinc-metallothionein in the liver; however, binding of gold by the gold induced protein has not been established. The interaction of golc with Mt of the liver may be, however, only of subordinate importance: as shown in this report the elevation of Mt level in the liver following repeated gold injections was relatively slight. It seems likely that the main sites of gold binding in the liver are represented by the heavy subcellular fractions (22–24) and in this respect gold resembles silver [25].

In the kidneys, the elevation of the RMBP level following gold exposure is much more pronounced. In this respect gold resembles mercury [12] and bismuth [13]. It seems relevant that the contribution of kidney to the binding of gold is distinctly augmented if gold is administered repeatedly [8]. These two facts, the induction of RMBP and increasing contribution of the kidney to the binding of the metal, seem to be closely interrelated.

The inducing effect of gold on the biosynthesis of RMBP has now been documented using [^{35}S]cysteine incorporation studies. The assay in which the inhibitors of the protein synthesis (Actinomycin D and Cycloheximide) have been used gives rise to the conclusion that the synthesis of Au-BP in the kidneys is dependent upon formation of a specific mRNA; the incorporation of ^{35}S can be abolished by Cycloheximide (inhibitor of translation) independently of the kind of gold exposure. Actinomycin D, on the other hand, was not effective in preventing the synthesis of Au-BP following repeated doses of gold and this may be explained by the presence of mRNA formed prior to administration of Actinomycin D. The overall picture regarding the biosynthesis of Au-BP shows an analogy to induction of Mt in the liver by Cd and Zn [15, 26].

The results of the present study do not allow for an unequivocal conclusion if the Au-BP occur *in vivo* only in the cytosol (like Cd-, Zn-metallothionein in the liver) but also in cellular organellae. Since Au-BP are copper-proteins, the latter possibility should not be excluded: such a phenomenon has already been observed with copper-metallothionein, the polymerized form of which was found in the mitochondrial-lysosomal liver fraction of infants [27]. A similar observation has been found for silver; its complex with the low molecular weight proteins (Mt) has been found partly in the mitochondrial fraction of rat liver [25]. It is surprising, however, that the low molecular weight complexes were mainly responsible for the presence of gold in the soluble extracts from the heaviest fraction of the kidney (nuclei). Except for trace contaminations as found for bismuth [28] this has not been observed for other heavy metals. This may, however, be an artifact emerging during the fractionation procedure. The extraordinary tendency of Au-BP for coprecipitation and aggregation may account for such phenomenon. If a simplified procedure was applied (separation only into 12,000 *g* sediment and supernatant) the supernatant contained

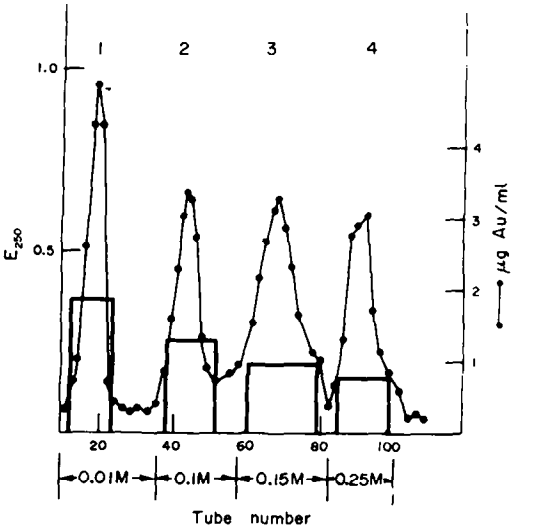

Fig. 6. Ion-exchange chromatography on DEAE-cellulose of the peak fraction from Sephadex G-75. Step-wise concentration gradient, buffer Tris–HCl, pH 8.6, fractions 5 ml each; solid bars—Au concentrations (μg/ml); recovery of gold approximated 100 per cent.

much higher percentage of gold, mainly in the form of Au-BP [8].

The inducible renal gold-binding proteins are characterized by low molecular weight, in the range 8,400–12,000, very close to the apparent molecular weight of metallothionein (10,000). The proteins are heterogenous and may be separated into four fractions by ion-exchange chromatography.

It is worthwhile to mention that in the rat also the other two metals show high affinity to the kidneys; mercury and bismuth also induce copper containing metal-binding proteins in this organ. Except for one fraction of bismuth-binding proteins (chromochelatin) [29] none of these proteins has been definitely identified. It seems likely, however, that—even if not identical—all these proteins may be interrelated.

Further progress in the identification of the renal Au-BP is dependent on improvement of the isolation procedures, in order to prevent aggregation and other physicochemical alterations of the proteins during preparation.

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