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PRODUCTION OF LOW MOLECULAR WEIGHT CADMIUM-BINDING PROTEINS IN RABBIT LUNG FOLLOWING EXPOSURE TO CADMIUM CHLORIDE*

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Abstract—Low molecular weight cadmium-binding proteins were studied in lung tissue from rabbits exposed to aerosols of $CdCl_2$. Lungs obtained from animals exposed by inhalation to aerosols of 800 or 1600 $\mu g/m^3$ $CdCl_2$ for 2-hr periods/day, every other day for a 5-day period, were found to contain at least three low molecular weight cadmium-binding proteins, two of which were similar electrophoretically and spectrally to rabbit liver metallothionein. The third protein(s), which accounted for the majority of the cadmium in the soluble fraction of the tissue, did not bind to an anionic exchange gel and did not appear to be a polymerized form of metallothionein. Translocation studies of lung cadmium suggest a long half-life for cadmium in lung tissue following inhalation exposure, due perhaps to the high affinity of cadmium for specific lung cadmium-binding proteins. A small but significant redistribution of lung cadmium did occur to both kidney and liver tissue with time.

Prolonged exposure to cadmium is known to produce a variety of toxic effects which have been reviewed extensively [1]. The main target organ following chronic cadmium exposure is the kidney [1–3], but cadmium has also been shown to cause pulmonary emphysema both in workers with high-dose occupational exposure to cadmium [4] and in experimental animals after acute exposure [5]. There are several published investigations concerning biochemical responses in pulmonary alveolar lavage material after cadmium exposure [6–8], but the mechanism of the damage to the lung is presently unknown.

In organs such as the liver and kidney, the low molecular weight cadmium-binding protein metallothionein (Mt)¶ is produced in response to cadmium exposure. This protein plays a central role in the intracellular metabolism of cadmium [9] by chelating the Cd^{2+} ions in the tissue and, in such a manner, is thought to protect sensitive metabolic functions of the cells.

Cadmium-binding proteins which resemble metallothionein by Sephadex chromatography have also been detected in the lung of Syrian hamsters [10] and several cell types derived from pulmonary tissues, including alveolar macrophages [11], fibroblasts [12] and lymphocytes [13].

Very little attention has been directed towards the characterization of Cd^{2+} binding proteins in the lung in spite of the fact that the lung is one of the major routes of exposure for this highly toxic material. Recent studies [10] have demonstrated a Cd^{2+} binding protein in hamster lungs following an intratracheal administration of $CdCl_2$. The protein had a molecular weight of about 15,000 daltons and was eluted from a column of Sephadex G-75 in a manner similar to that of metallothionein.

The present investigation was undertaken to determine whether acute inhalation exposure to cadmium aerosol would lead to production of low molecular weight Cd^{2+} binding proteins (CdBPs) in the lungs of rabbits and to examine their similarity to metallothionein of the rabbit liver. Studies were also conducted to assess the possible role of CdBPs in the pulmonary retention of Cd^{2+} and its translocation to other organs such as the kidney.

MATERIALS AND METHODS

Inhalation and intratracheal exposure to $CdCl_2$. Male Charles River rabbits weighing 2.4 to 3.0 kg were exposed via nasal inhalation to an aerosol of 800 or 1600 $\mu g/m^3$ $CdCl_2$ for 2 hr/day every other day

* This report has been reviewed and approved for publication. Mention of trade names or commercial projects does not constitute endorsement or recommendation for use.

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¶ Abbreviations: Mt, metallothionein; and CdBP, cadmium-binding protein.

over a 5-day period. The detailed procedure for generation and monitoring the $CdCl_2$ has been described previously [14].

Preparation of animals. Animals were killed by injecting an overdose of pentobarbital into their marginal ear veins. The lungs were carefully removed and lavaged with 175–200 ml of sterile isotonic saline as described previously [15]. The lavage effluent was centrifuged at 680 *g* for 10 min to sediment the cells. The cells were resuspended in isotonic saline and counted using a hemocytometer. Lung damage, as indicated by an inflammatory response, was assessed according to the increase of polymorphonuclear leucocytes found in the lavage fluid.

The cytosolic fractions of non-lavaged lung, liver and kidneys from which cadmium-binding proteins were isolated were prepared as follows. Organs were removed, trimmed free of fat and adhering tissues, minced with scissors and homogenized in 0.25 M sucrose buffered with 0.01 M Tris–HCl (pH 8.6) (2 ml sucrose solution/g organ) using a Polytron homogenizer (model PCU-2-110, Brinkmann Instruments, Westbury, NY). The homogenate was centrifuged at 178,000 *g* for 60 min to remove particulate materials and to isolate the cytosolic fraction.

Gel filtration chromatography. The 178,000 *g* lung supernatant fraction was applied to a 2.8 × 63 cm Sephadex G-75 column for the preparative separation of the cadmium-binding proteins. In some instances where a small amount of sample was applied, a 2.8 × 28 cm column was used. Samples were eluted with a 0.01 M Tris–HCl buffer (pH 8.6) containing 0.02% sodium azide.

Cadmium analyses. Concentrations of Cd^{2+} in lung homogenates and in column fractions were measured by atomic absorption spectrophotometry (AA) in the flame mode using a Perkin–Elmer model 305B atomic absorption spectrophotometer equipped with a hollow cathode Cd^{2+} lamp. The instrument gave an acceptable signal to noise ratio down to 0.2 or 0.3 μg Cd^{2+}/ml. Column effluents were normally monitored using this technique. In cases where the cadmium levels were not detectable by AA, $^{109}Cd^{2+}$ (0.1 μCi; 150 mCi per μmole $CdCl_2$) was added to the cytosolic sample and then passed through a separation column. These column fractions were monitored for $^{109}Cd^{2+}$ activity using a Beckman Biogamma II gamma counter. The use of $^{109}Cd^{2+}$ for the detection of cadmium-binding proteins in these samples was based upon the assumption that the tracer will exchange freely with all fractions of bound cadmium.

Ion exchange chromatography. The cadmium-containing fractions that eluted from a Sephadex G-75 column at an elution volume/void volume (V_e/V_o) of 1.95 were pooled, concentrated to 2 or 3 ml using an Amicon Ultrafiltration Cell with a UM2 membrane, and applied to a 1.6 × 25 cm DEAE Sephadex A25 column equilibrated with 0.01 M Tris–acetate buffer (pH 7.4). The column was washed with 50 ml of 0.05 M Tris–acetate buffer followed by a gradient of 0.05 to 0.2 M Tris–acetate buffer (pH 7.4). The concentration of cadmium and the absorbance at 250 nm were measured in all of the fractions.

Polyacrylamide gel electrophoresis. The cadmium-containing fractions from the ion-exchange column were pooled, concentrated, and applied to 7.5% polyacrylamide disc gels [16]. The samples were run until the front indicator of bromophenol blue had migrated to the end of the rods. The gels were then stained in Coomassie brilliant blue.

Ultraviolet spectrum. The pooled cadmium peaks from the DEAE Sephadex A25 column were concentrated to 100 μg protein/ml. Protein was determined using the difference in absorbance between 215 and 225 nm [17] with bovine serum albumin and bacitracin as the standards (Schwarz/Mann, Orangeburg, NY). A u.v.-spectrum was run between 210 and 300 nm using a Gilford model 250 STET spectrophotometer.

RESULTS

Lung damage. The cells present in pulmonary lavage effluents consisted mainly of alveolar macrophages. Exposure of rabbits to cadmium chloride aerosols at the levels used in this study did not produce any significant increases in the number of polymorphonuclear leuckocytes, lymphocytes, or alveolar macrophages (Table 1), indicating that overt damage to the lungs was probably absent.

Isolation and characterization of cadmium-binding proteins (CdBPs). The lung cytosol fraction from untreated rabbits did not contain any detectable

Table 1. Differential cell count of lung lavage fluid after $CdCl_2$ inhalation*

Exposure dose ($CdCl_2$ concn)	Time after exposure	Cell count		
		AM	PMN	LYMPH
0	Immediate	97	0	3
800 μg/m³	Immediate	97	0	3
0	24 hr	94.5 ± 2.1	0	5.5 ± 2.1
800 μg/m³	24 hr	96.5 ± 1.2	0	3.5 ± 1.3
0	Immediate	97.0 ± 3.0	1.7 ± 1.5	1.3 ± 1.5
1600 μg/m³	Immediate	96.9 ± 1.7	1.1 ± 1.0	2.1 ± 1.7
1600 μg/m³	24 hr	93.2 ± 2.4	0.8 ± 0.9	6.2 ± 3.0
0	96 hr	97.0 ± 1.4	1.5 ± 0.7	1.5 ± 0.7
1600 μg/m³	96 hr	97.2 ± 2.0	1.0 ± 0.7	1.8 ± 1.5

* Two hundred cells were counted using a hemocytometer. Data are expressed as percent of total cells. Mean ± S.D. (N = 4). Abbreviations: AM, alveolar macrophages; PMN, polymorphonuclear leukocytes; and LYMPH, lymphocytes.

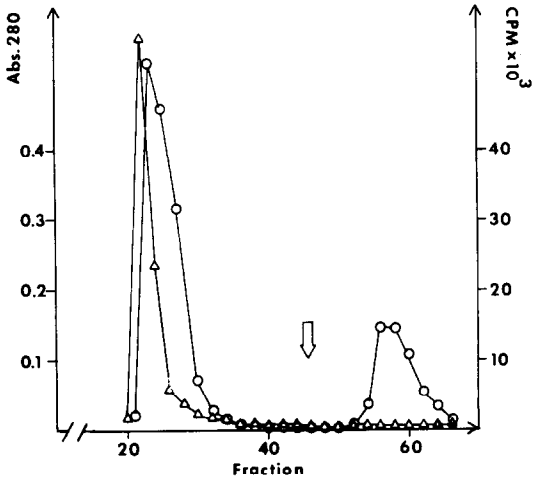

Fig. 1. Sephadex G-75 (2.8 × 63 cm column; 6.8-ml fractions) chromatography of control rabbit pulmonary cytosol. For the detection of cadmium-binding protein, $^{109}CdCl_2$ was added to the cytosol before loading on the column. The arrow indicates the standard elution volume of metallothionein (molecular weight approximately 10,000 daltons). Protein distribution was measured by absorbance of each fraction at 280 nm (○—○). Cadmium-binding proteins are indicated by ^{109}Cd distribution; cpm/fraction (△—△).

amounts of metallothionein-like CdBPs, as indicated by the absence of $^{109}Cd^{2+}$ binding to protein in the expected elution range (Fig. 1). The presence of $^{109}Cd^{2+}$ at or near the void volume peak is normal with most tissues and serum and represents nonspecific binding of the ion to high molecular weight proteins.

Treatment of the animals with cadmium chloride by the intratracheal route resulted in the appearance of low molecular weight CdBPs in the pulmonary cytosol (Fig. 2). Induction of this CdBP was very rapid since it was detectable in the cytosol only 2 hr after the intratracheal instillation of a single dose of cadmium chloride (Fig. 2A). The distribution of cadmium between high and low molecular weight proteins depended upon the time between treatment of the animal and its sacrifice. Animals killed 24 hr after a single intratracheal instillation of cadmium chloride had most of the cadmium bound to the low molecular weight protein(s) (Fig. 2B).

Cadmium-binding proteins could also be induced in lung by the inhalation of cadmium chloride aerosols (Fig. 3). As with the animals receiving cadmium chloride by intratracheal instillation, the distribution of cadmium between high and low molecular weight proteins present in the cytosol fraction of animals exposed to aerosols was dependent upon the time between cessation of inhalation of the cadmium

Table 2. Induction of low molecular weight cadmium-binding proteins in the lungs of rabbits exposed to a cadmium chloride aerosol*

Time after exposure (hr)	Total bound cadmium (μg)	
	High molecular weight proteins	Low molecular weight proteins
1	2.59 ± 0.62	0.27 ± 0.54
24	0.63 ± 0.82	3.51 ± 0.20

* Rabbits were exposed once to 1600 μg cadmium chloride/m³ for 2 hr and killed 1 and 24 hr after exposure. Distribution of cadmium between the high and low molecular weight binding proteins of the rabbit lung cytosol fraction was determined by chromatography on Sephadex G-75 and atomic absorption spectrophotometry. Values are expressed as means ± S.D. (N = 4).

Table 3. Cadmium levels in lung, liver and kidneys at different time intervals after the last $CdCl_2$-aerosol exposure*

Organ	Cadmium (μg/organ)			
	Days after last exposure			
	0	7	17	31
Controls				
Lung	0.2 ± 0.08	0.4 ± 0.2	0.4 ± 0.2	0.4 ± 0.2
Liver	3.0 ± 1.0	3.8 ± 1.8	4.0 ± 1.0	4.3 ± 2.4
Kidneys	1.5 ± 0.4	2.4 ± 0.2	2.8 ± 1.2	3.0 ± 0.4
800 μg/m³ exposure				
Lung	7.0 ± 2.0†	7.5 ± 2.8†	6.8 ± 1.4†	5.8 ± 1.4†
Liver	3.5 ± 1.4	4.6 ± 0.6	5.2 ± 0.8	3.1 ± 0.6
Kidneys	1.5 ± 0.8	2.6 ± 2.4	2.2 ± 0.8	3.8 ± 1.4
1600 μg/m³ exposure				
Lung	9.9 ± 3.2†	14.7 ± 5.8†	16.4 ± 8.0†	11.1 ± 4.4†
Liver	5.0 ± 1.2†	6.9 ± 1.2†	6.4 ± 2.8	8.3 ± 3.0†
Kidneys	1.3 ± 0.4	3.0 ± 1.6	3.2 ± 0.6	5.6 ± 1.8†

* Each value represents mean ± S.D. (N = 4). Organ weights were not significantly affected by the exposure. The mean weights of lungs, livers, and kidneys for exposed (1600 μg/m³) and control animals were as follows: lungs (2) 9.5 ± 0.6 (exposed), 8.8 ± 1.0 (control); liver 94.3 ± 13.3 (exposed), 98.8 ± 22.4 (control); and kidney (2) 18.0 ± 1.6 (exposed), 16.3 ± 2.2 (control).

† Value is significantly different from the control value for the same organ ($P < 0.05$), Mann–Whitney [19].

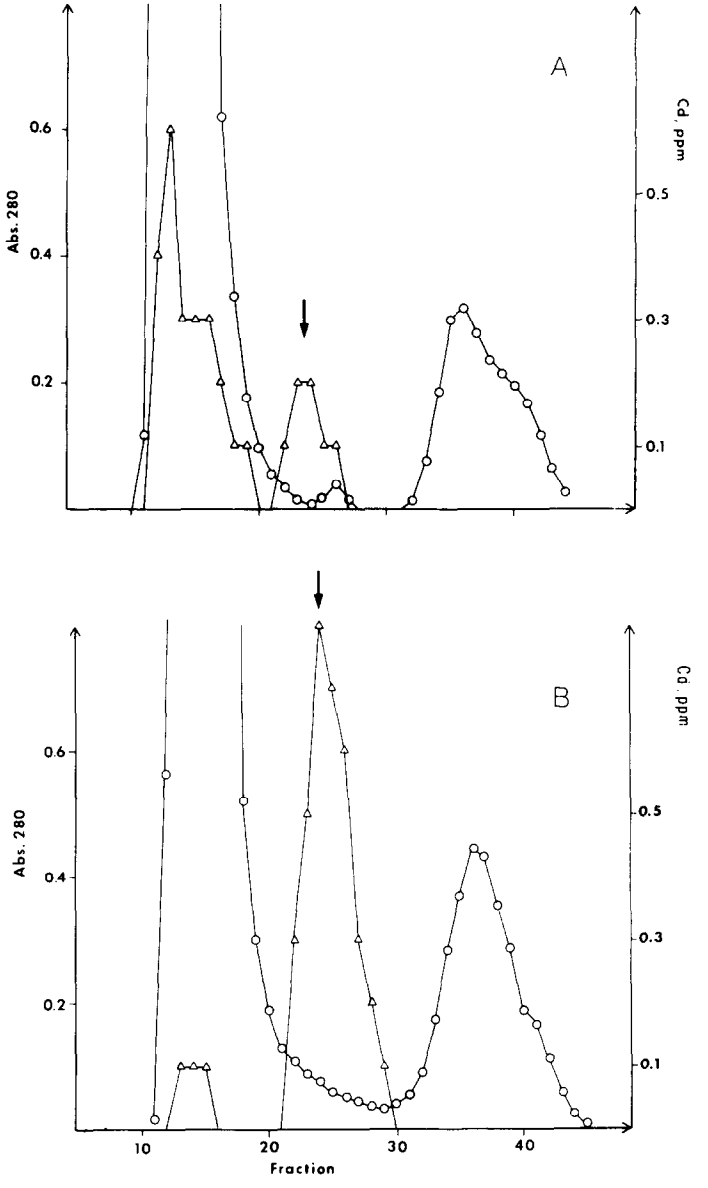

Fig. 2. Sephadex G-75 (2.8 × 28 cm column; 1.8-ml fractions) chromatography of rabbit lung cytosol fractions (A) 2 hr and (B) 24 hr after intratracheal instillation of 0.5 μmole cadmium chloride/kg body weight. Protein was detected by the absorbance of each fraction at 280 nm (○—○). Cadmium-binding protein was detected by the presence of cadmium measured by atomic absorption spectrophotometry (△—△). The arrows indicate the standard elution volume of liver metallothionein.

chloride aerosol and sacrifice of the animal. The low molecular weight CdBPs present in the pulmonary cytosol fraction eluted from Sephadex G-75 with the same elution volume as that of standard liver metallothionein.

The induction of low molecular weight cadmium-binding proteins is shown in Table 2. After 1 hr, the total cadmium present in the pulmonary cytosol was almost entirely bound to high molecular weight proteins. The amount of low molecular weight cadmium binding protein was very low since, in three of the four animals studied, the low molecular weight proteins were undetectable 1 hr after exposure. By 24 hr, however, 85% of the cadmium was bound to low molecular weight proteins.

The low molecular weight CdBPs obtained from gel permeation chromatography on Sephadex G-75 (Fig. 3) were further analyzed by ion exchange chromatography using DEAE Sephadex A25 (Fig. 4). The major portion (75%) of the protein-bound cadmium in the lung did not bind to the column (Fig. 4A); however, other proteins (peaks I and II) did bind and were eluted by increasing concentrations of buffer. Proteins I and II accounted for 15 and 6% of the protein bound cadmium in the lung respectively. The elution characteristics of proteins I and II were very similar to those of metallothionein from the rabbit liver (Fig. 4B). Metallothionein of the rabbit liver exists in two forms designated Mt-I and Mt-II which correspond very closely with proteins

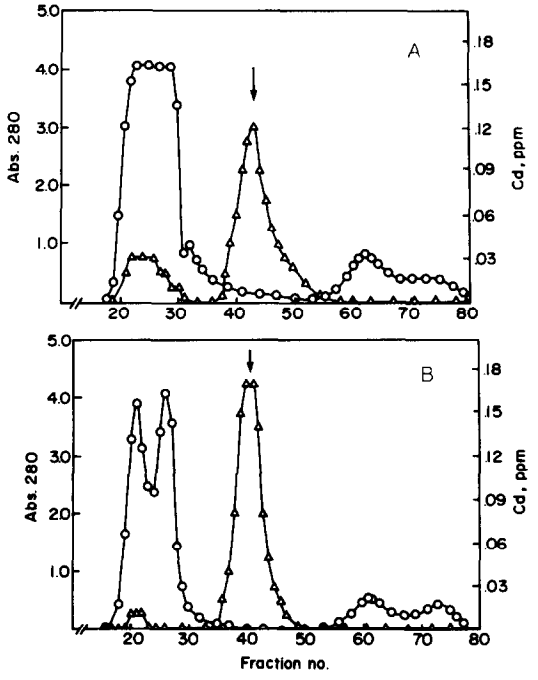

Fig. 3. Sephadex G-75 (2.8 × 63 cm column; 6.8-ml fractions) chromatography of rabbit lung cytosol fractions (A) 2 hr and (B) 96 hr after the last of three inhalation exposures to 1600 $\mu g/m^3$ cadmium chloride. The arrows indicate the standard elution volume of liver metallothionein. Key: absorbance 280 nm (○—○); and cadmium ppm (△—△).

I and II of the lung. In this study, Mt-I and Mt-II accounted for 62 and 37%, respectively, of the total cadmium bound to the low molecular weight protein peak obtained by gel filtration of liver cytosol on Sephadex G-75. Protein-bound cadmium corresponding to protein III from the lung, which did not bind to DEAE Sephadex, was present in only small amounts in the liver.

Protein III from the lungs did not appear to be a polymerized form of the metallothionein-like proteins since treatment of protein III with 1% β-mercaptoethanol did not change its molecular weight or ion-exchange properties. After treatment, protein III did not bind to the DEAE gel, and no evidence for disaggregation into protein I or II was observed. Resistance to treatment with β-mercaptoethanol, which would break disulfide bonds, suggests that protein III is not a polymer of Mt.

As with liver metallothionein [18], the low molecular weight lung CdBP, designated peak I, did not exhibit an absorption maximum at 280 nm (Fig. 5).

The electrophoretic mobilities of proteins I and II on polyacrylamide gels were identical to those of metallothionein I and II isolated from rabbit liver with R_f values of approximately 0.4 and 0.6 (data not shown).

Translocation of cadmium from the lungs to liver and kidneys. The translocation of cadmium from the lungs to other organs was studied in rabbits maintained for up to 1 month after the last exposure to cadmium chloride aerosol. Control rabbits were

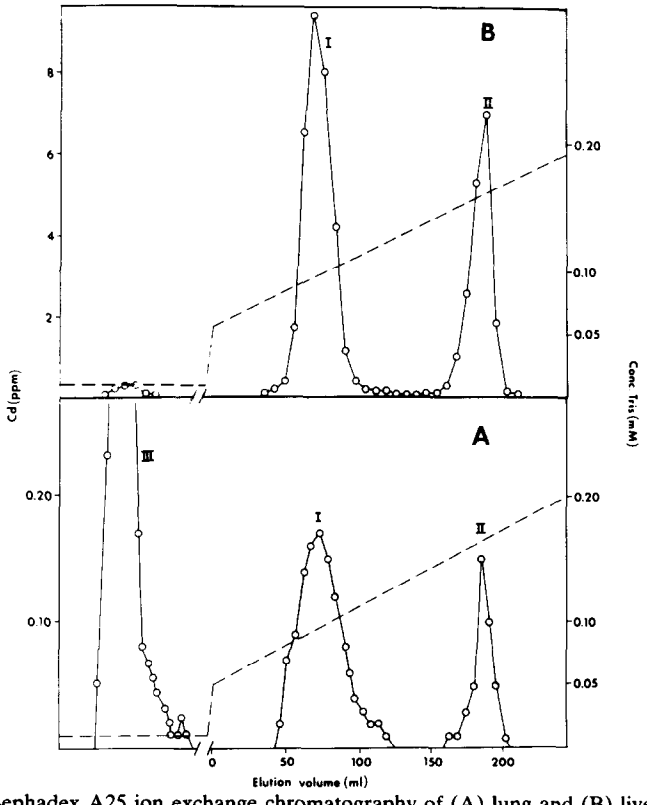

Fig. 4. DEAE Sephadex A25 ion exchange chromatography of (A) lung and (B) liver low molecular weight cadmium-binding proteins isolated by Sephadex G-75 chromatography as indicated in the legend of Fig. 3. Ion exchange chromatography was performed on pooled samples of the cadmium peaks indicated by the arrows in panels A and B of Fig. 3 at 96 hr after the last inhalation exposure. Cadmium-binding proteins were detected by atomic absorption spectrophotometry (cadmium ppm) (○—○). Tris gradient (---).

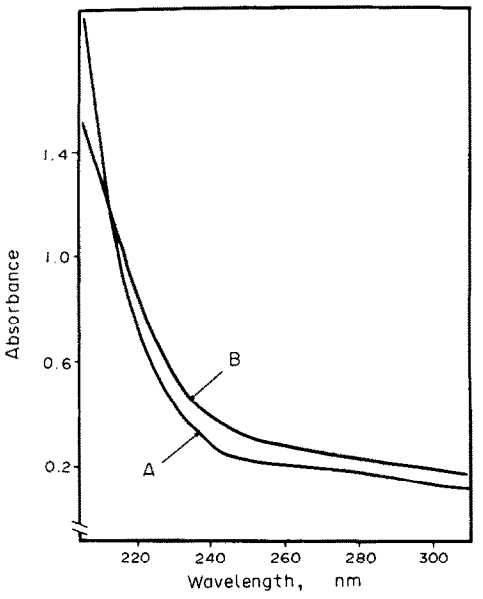

Fig. 5. Ultraviolet spectra obtained from (A) rabbit lung DEAE Sephadex A25 peak I and (B) rabbit liver Mt-I.

found to have cadmium levels ranging from 1.5 to 4.3 μg/organ in the kidney and liver. The 1600 μg/m³ dose group had a statistically significant increase in liver levels of cadmium compared to controls after 0, 7 and 31 days (Table 3). Kidney cadmium levels were significantly elevated above controls after 31 days. Cadmium in the lungs remained elevated throughout the month. Organ weights for lungs, livers and kidneys were not affected by the cadmium exposure regimen at any time point relative to controls.

DISCUSSION

This study has shown that after exposure to cadmium chloride the lungs synthesized low molecular weight cadmium binding proteins similar to metallothionein. In unexposed lungs, the levels of those proteins were below our limits of detection; however, in only 2 hr after exposure, detectable levels were present. Synthesis of these CdBP was induced by inhalation of cadmium as well as by intratracheal instillation of the material.

We have not localized the cellular site of the synthesis of the CdBPs within the lungs although three potential sites have been demonstrated previously. Alveolar macrophages [11], pulmonary fibroblasts [12] and white blood cells such as polymorphonuclear leukocytes [13] have all been shown to synthesize metallothionein-like proteins in response to cadmium exposure. Since inhalation of the cadmium chloride aerosols used in this study did not produce an inflammatory response, it seems unlikely that the synthesis of CdBPs could be accounted for by an influx of polymorphonuclear leukocytes. In a previous study [14], a 2 hr exposure to $CdCl_2$ produced a statistically significant increase in polymorphonuclear leukocytes and lymphocytes in the lavage fluid of rats 24 hr after the cessation of the exposure. There was also a significant reduction in the number of macrophages. This indicates a possible difference in response between the rabbit and the rat to the test substances.

The lavage effluents from the lungs of aerosol-exposed animals contained only about 10% of the total cadmium (unpublished data) and, therefore, alveolar macrophages which are removed by the lavage process are not the major source of CdBPs in the lungs. Interstitial fibroblasts could be responsible as previously demonstrated [12], although other cell types cannot be excluded at this time.

At least three distinct low molecular weight CdBPs were present in the lungs of cadmium-treated animals. Two of these appear to have been similar to metallothionein forms I and II on the basis of their gel filtration, ion exchange, and electrophoretic and spectral properties. The third CdBP which accounted for the major portion of cadmium in the lung, appears to be an entirely new protein form which has not been reported previously. This third protein was present to only a very minor degree, if at all, in liver and kidney and may prove to be unique to the lungs.

A low molecular weight cadmium-binding protein component was reported recently by Benson and Henderson [10] in the lungs of Syrian hamsters. These investigators confined their attention to cadmium-binding proteins isolated by Sephadex G-75 chromatography and did not resolve the protein into its multiple forms. Consequently, the presence of three forms of CdBP is known only for the lungs of rabbits at this time.

The function of CdBPs is not known for certain although considerable speculation has been made concerning their protective role against ingested cadmium. In the lungs CdBPs may play an important role in the retention of cadmium by this organ. Studies measuring the half-life of cadmium in lung tissue are few and difficult to compare due to differences in the form of the cadmium administered (i.e. particulate versus soluble); however, the results of this study, which suggest that cadmium has a long half-life in the lung, are in agreement with other reports such as that of Benson and Henderson [10]. Harrison *et al.* [20] exposed dogs to a cadmium chloride aerosol and found a biphasic pattern in the disappearance of lung cadmium. They reported a relatively rapid clearance during the first 2 weeks with a half-life of approximately 5 days, followed by a half-life which was difficult to quantitate on the basis of the data presented but was much longer than the initial phase. Boisset *et al.* [21] using CdO particles reported a half-life for cadmium of 56 days. The most probable explanation for the long half-life of cadmium observed in these studies and in the present investigation is that cadmium ions are retained in lung tissue because of binding to the CdBPs. Further research is needed to determine the turnover rate for the various CdBPs observed in the present study.

There has been interest lately in determining whether the organ distribution of cadmium varies with different modes of exposure to the ion. Prigge [22] has reported that there is a higher kidney to liver concentration ratio when rats are exposed to cadmium by inhalation compared to those given

cadmium orally. This difference in kidney versus liver uptake of cadmium may reflect a difference in the size of the ligand that carries cadmium in the circulation. There have been several recent reports of studies dealing with the organ deposition pattern of cadmium when the ion is injected as Cd^{2+} alone or with several types of chelating agents. When injected subcutaneously as $CdCl_2$, the metal is mainly deposited in the liver but when given as a CdMt complex, there is a preferential transport of the ion to the kidneys [23]. The pattern of distribution can also be manipulated by injecting cadmium together with other types of chelating agents. When given together with EDTA, relatively more of the cadmium is taken up by the kidneys [24] and the deposition has been shown to be directly correlated to the binding affinity between the chelating agent and cadmium [25]. It appears that preferential renal deposition occurs when circulating cadmium is complexed to low molecular weight biological or non-biological chelators with high affinity for cadmium. In the present investigation, we have shown that the translocation of cadmium from lung tissue after inhalation exposure occurred to both liver and kidneys. This is in agreement with previously reported studies [21, 26] on the distribution of cadmium following inhalation of CdO, although these studies are not strictly comparable to the present investigation due to the different chemical form of cadmium used.

In conclusion, the results of the present study indicate that the lung is capable of synthesizing CdBPs in response to $CdCl_2$-aerosol inhalation. There appear to be three different types of CdBPs of which two are similar to Mt-I and Mt-II. These proteins do not, however, seem to be the dominant lung CdBP. A third, previously unreported protein in the same molecular weight range as Mt, which did not bind to an anion exchange gel, appears to be primarily responsible for intracellular cadmium binding. In addition, this study has verified that inhalation exposure to $CdCl_2$-aerosol results in translocation of cadmium to both liver and kidneys.

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