ISOLATION AND CHARACTERIZATION OF CADMIUM-BINDING PROTEIN FROM RAT TESTES

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Summary. A cadmium-binding protein (Cd-BP) has been isolated and characterized using ^{109}Cd to elucidate the nature of the binding of cadmium to rat testis. The protein labelled with ^{109}Cd was isolated using Sephadex gel filtration with G-75 and G-25. The cadmium bound selectively *in vivo* to a low molecular weight protein and ^{109}Cd remained bound to the Cd-BP at pH 7.0, but dissociated from it at pH 2.0. The ultraviolet absorption spectra were characteristically different at the two pH values. The molecular weight of the protein was 15,000 as determined by Sephadex G-75 gel filtration. Disc-gel electrophoresis showed one major and two minor bands. All these observations suggest that rat testis Cd-BP, though similar in some respects, differs from Cd-BP of liver and kidney in absorption spectra, molecular weight and disc-gel electrophoresis.

INTRODUCTION

A single parenteral administration of cadmium has been shown to induce a rapid irreversible degeneration of the germinal epithelium of the testis resulting in permanent loss of fertility and associated with temporary damage to the interstitial tissues of the testis of most mammals including the rat (Pařízek, 1957, 1960; Kar, Das & Mukerji, 1960; Gunn, Gould & Anderson, 1963; Chiquoine, 1964). Gunn, Gould & Anderson (1968) have suggested that the specific site where cadmium produces acute testicular injury is the vascular endothelium. Webb (1972) studied the biochemical effects of cadmium injury on mouse and rat testis with regard to the competitive antagonism between Cd^{2+} and Zn^{2+} and has shown that the antagonism occurs in the epididymis. A cadmium-binding protein (Cd-BP) has been shown to exist in various mammalian tissues, such as the liver and the kidney (Kagi & Vallee, 1960, 1961; Pulido, Kagi & Vallee, 1966; Shaikh & Lucis, 1971a). The administra-

tion of cadmium induces the synthesis of this protein (Piscator, 1964; Shaikh & Lucis, 1970). It has been postulated that this protein in liver and kidney represents a biological system which protects the organs from the toxic effects of cadmium (Shaikh & Lucis, 1971b).

Wisniewska-Knypl & Jablonska (1970) have studied the binding of ^{115}Cd in rat liver and have shown that it binds selectively to metallothionein. Nordberg, Piscator & Lind (1971) studied the distribution of cadmium among protein fractions of mouse liver and concluded that cadmium binds to high molecular weight proteins soon after injection whereas 24 hr later it associates with a low molecular weight protein. Singh & Nath (1972) reported the isolation and identification of Cd-BP from rat testis and showed that cadmium binds to a low molecular weight protein after 24 hr.

Though cadmium in very low dosage damages the testis, its mode of action remains unresolved. To elucidate the nature of the binding of cadmium to rat testis, a detailed study of the isolation and characterization of Cd-BP from this organ has been undertaken.

MATERIALS AND METHODS

Male albino rats weighing 200 to 225 g, which were obtained from the central animal house of this Institute, were used for this study. The animals were maintained on standard diet and had free access to water. Animals were injected intraperitoneally with $20\ \mu\text{Ci}$ ^{109}Cd (carrier-free, obtained from Radiochemical Centre, Amersham, England) and were killed after 24 hr. The scheme used to isolate Cd-BP from the testes is summarized in Text-fig. 1. The testes were removed and 25% (w/v) homogenate was prepared in cold 0.25 M-sucrose, buffered with 0.01 M-tris-HCl at pH 7.4. The homogenate (4.5 ml), containing 7000 ct/min, was centrifuged at 105,000 g in a VAC 60 Z Ultracentrifuge. The supernatant thus obtained, containing high radioactivity (6165 ct/min/4 ml), was used for further studies. The pellet contained only 10% of the radioactivity and was discarded. The Cd-BP labelled with ^{109}Cd was isolated from the supernatant by the modified method of Pulido *et al.* (1966) and Shaikh & Lucis (1971a). The supernatant (4.0 ml), containing 75.0 mg protein, was chromatographed on a Sephadex G-75 column (56×1.4 cm) equilibrated with 0.01 M-tris-HCl buffer and proteins were eluted with the same buffer. The radioactivity and absorbance at 250 and 280 nm were determined. Fractions (17 to 23) from Sephadex G-75 gel filtration (Text-fig. 2), having a total volume of 17.5 ml, were pooled and concentrated in the cold. The concentrate was designated Fraction II and used for further study. All other fractions having low radioactivity (<200 ct/min/fraction) were discarded.

The binding affinity of cadmium was determined as follows: Fraction II was divided into two equal parts. Each portion was chromatographed separately on Sephadex G-25 column (45×1.4 cm). In the first experiment, the column was equilibrated with 0.001 M-tris-HCl buffer, pH 7.0, and proteins were eluted with the same buffer. The fractions numbering 12 to 14 were pooled and named Fraction IIIa (Text-fig. 3). The other portion of Fraction II was acidified to

pH 2.0 with 0.1 N-HCl and chromatographed as above on Sephadex G-25. The protein was eluted with 0.01 N-HCl. The fractions numbering 12 to 14 were named Fraction IIIb (Text-fig. 4). The eluates from the above experiments were assayed for radioactivity and absorbance at 280 and 250 nm. Fractions IIIa and IIIb were subjected to ultraviolet absorption spectra between 225 and 300 nm.

The radioactivity of ^{109}Cd was assayed by a γ -ray medical spectrometer (Atomic Energy Establishment, Trombay, Bombay). The absorbance at 250 and 280 nm and absorption spectra (225 to 300 nm) were measured with a Beckman ODU Spectrophotometer.

TEXT-FIG. 1. Scheme for the isolation of a cadmium-binding protein from rat testis.

The molecular weight of Cd-BP from rat testis isolated as above (Fraction IIIa) was determined according to the method of Andrews (1964), using a Sephadex gel filtration G-75 column (56 × 1.4 cm) equilibrated with 0.01 M-tris-HCl buffer, pH 8.0, at 17°C. A sample containing 4.0 mg of protein in 2 ml was applied to the column. The elution volume (V) of the Cd-BP was estimated by gamma counting of the ^{109}Cd tracer in a 2.5-ml fraction. The void volume (V_0) of the column was determined, using Blue dextran (mol. wt, 2×10^6). The calibration curve was prepared from the elution volumes of three conventional proteins of known molecular weight, i.e. cytochrome C (mol. wt, 12,270),

Table 1. Uptake of ^{109}Cd in rat tissues

Tissues	% administered dose after 24 hr	
	Per g tissue	In total tissue
Liver	11.76	49.39
Kidney	4.10	4.10
Testis	0.11	0.23

lysozyme (mol. wt, 17,500) and ovalbumin (mol. wt, 45,000). Lysozyme and ovalbumin were assayed spectrophotometrically at 280 nm and cytochrome C was measured at 410 nm. The ratio V/V_0 of standard proteins versus the logarithms of their molecular weights gave a linear plot (Text-fig. 6) from which the molecular weight of Cd-BP was determined.

Disc-gel electrophoresis (Davis, 1964) was performed on Fraction IIIa. The polyacrylamide gel (7%) was prepared using tris-glycine buffer of pH 8.9. The current applied was 5 mA/column. The gel was stained with 1% amido schwarz in 7% acetic acid.

RESULTS

The uptake of ^{109}Cd in liver, testis and kidney is shown in Table 1. It is evident from the table that testis accounted for only 0.23% of the total counts injected.

TEXT-FIG. 2. Binding *in vivo* of a single dose of ^{109}Cd to protein of rat testis. Sephadex gel G-75 column of 56×1.4 cm; 0.01 M-tris-HCl buffer, pH 8.0; flow rate, 15 ml/hr; temperature, 17°C ; fractions collected, 2.5 ml each.

TEXT-FIG. 3. Chromatography of cadmium-binding protein on Sephadex G-25 in neutral medium, 0.001 M-tris-HCl, pH 7.0.

The supernatant obtained after centrifugation contained 90% (6165 ct/min/4.0 ml) and the pellet, 10% (700 ct/min), of the radioactivity. The supernatant from rat testis homogenate was fractionated on Sephadex G-75 for the isolation of Cd-BP. The elution pattern (Text-fig. 2) demonstrates that ^{109}Cd binds selectively to a low molecular weight protein. The optical density at 280 nm in this region varied from 0.112 to 0.415. The supernatants of liver and kidney homogenates of these animals when chromatographed on Sephadex G-75 showed similar elution patterns to those obtained for testis (unpublished observation). The elution pattern of one portion of Fraction II chromatographed on Sephadex G-25 in neutral medium is shown in Text-fig. 3. The protein and radioactivity eluted in a single peak, which indicates that the tracer remains bound to the protein at pH 7.0. The other portion of the Fraction II was

TEXT-FIG. 4. Chromatography of cadmium-binding protein on Sephadex G-25 in acidic medium adjusted to pH 2.0 with 0.1 N-HCl.

TEXT-FIG. 5. Absorption spectra of protein fractions IIIa and IIIb.

acidified to pH 2.0 and chromatographed on Sephadex G-25. Here, the protein and radioactivity eluted in different fractions. The elution pattern demonstrated that ^{109}Cd dissociated completely from the apoprotein at acidic pH (Text-fig. 4). No radioactivity could be detected in fractions having protein.

Absorption spectra of Cd-BP at pH 7.0 and pH 2.0 are shown in Text-fig. 5. The absorbance shoulder characteristic for the Cd-BP disappears after the dissociation of cadmium from the apoprotein in acidic medium.

As calculated from Text-fig. 6, the molecular weight of the Cd-BP from rat

TEXT-FIG. 6. Determination of molecular weight of cadmium-binding protein (Cd-BP) from rat testis. V = elution volume, and V_0 = void volume, of particular protein. Sephadex G-75 column of 56×1.4 cm; 0.01 M-tris-HCl buffer, pH 8.0; flow rate, 15 ml/hr; temperature, 17°C ; fractions collected, 2.5 ml each.

PLATE 1

Disc-gel electrophoresis of cadmium-binding protein from rat testis, showing one major and two minor bands: (a) polyacrylamide gel stained with amido schwarz; (b) diagrammatic representation of bands seen in (a).

testis was about 15,000. This is higher than the kidney metallothionein which has a molecular weight around 11,000 (Pulido *et al.*, 1966). The limit of accuracy of the method employed has been estimated to be about 10% (Andrews, 1964). The disc-gel electrophoresis of Fraction IIIa showed one major and two minor bands (Pl. 1, Figs a and b).

DISCUSSION

Cadmium is known to bind selectively to the low molecular weight protein fraction (metallothionein) of the soluble cytoplasmic fraction of liver and kidney (Wisniewska-Knypl & Jablonska, 1970; Shaikh & Lucis, 1971a). The results of this study, taken with those of a previous study (Singh & Nath, 1972), show that a Cd-BP of low molecular weight also exists in the rat testis. The protein is present in the soluble fraction of the testis as shown by the recovery of over 90% of total testis radioactivity in this fraction. It eluted as a single homogeneous peak on gel filtration on Sephadex G-75 at pH 8.0. Lowering the pH to 2.0 caused complete dissociation of the metal from the apoprotein as no radioactivity was detected in the protein peak when chromatographed at pH 2.0 on Sephadex G-25. Liver and kidney homogenates from the same group of rats showed very similar properties when subjected to these procedures (unpublished observation).

The absorption spectra of this protein at pH 7.0 showed high absorption at 225 nm which decreased to 1/3 at 245 nm. The ratio of absorbance at 250 nm to 280 nm was 4.0. The ratio in the case of liver is 5.0 (Nordberg *et al.*, 1971) and for kidney is 2.0 (Pulido *et al.*, 1966). Exposure to low pH resulted in loss of absorption between 225 and 240 nm from 1.14 to 0.13, indicating that this loss of absorption was as a result of dissociation of cadmium from the Cd-BP. In the case of rat kidney protein, the corresponding loss in absorbance was only from 0.18 to 0.075 (Wisniewska, Trojanowska, Piotrowska & Jakubowski, 1970), indicating that the nature and extent of binding of this metal is different for these tissues. Moreover, the absorption spectra of the apoprotein from kidney and testis are different, indicating that the nature of these proteins is characteristic of these organs.

Recently, Chen, Wagner, Ganther & Hoekstra (1972) have reported the isolation of Cd-BP from rat testis using ^{109}Cd . They isolated the protein 30 min after the administration of ^{109}Cd (0.011 mmol/kg). Their data on labelling pattern confirm the above results. They report that the absorbance of this protein is very low in the u.v. range. We have been able to measure the absorbance in the range from 225 to 300 nm (Text-fig. 5) for both the metalloprotein and the apoprotein. This difference may be due to the fact that in this experiment the cadmium was injected 24 hr before the animals were killed. It has been shown by Nordberg *et al.* (1971) that cadmium will only bind to metallothionein in mouse liver 24 hr after its administration. Before that, it is bound only to high molecular weight protein, which is different from the mode of binding of ^{109}Cd to testis (Chen *et al.*, 1972). We have not carried out any experiments at this short interval. The molecular weight reported by Chen *et al.* (1972) is 10,000 for this protein. This is lower than the value reported here. The reason for this

difference may be due to the method used for determination of the molecular weight by these authors (details of the method are not described by them).

The rôle of metallothionein present in liver and kidney as a trap for cadmium and serving as a protecting agent has been postulated (Shaikh & Lucis, 1971b). Chen *et al.* (1972) have shown that Cd-BP from testis has some rôle to play in the testicular damage produced by cadmium. The present study shows that the Cd-BP of rat testis, though similar in some respects, is quite different from the Cd-BP of liver and kidney in absorption spectra, molecular weight and disc-gel electrophoresis. It is possible that cadmium may be dissociating from the apoprotein at a much faster rate than in the case of the liver and kidney, thus producing tissue injury to the testis at a much lower concentration. Further work on the physicochemical properties of this protein is under study to clarify its physiological rôle.

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REFERENCES

- ANDREWS, P. (1964) Estimation of the molecular weights of proteins by Sephadex gel filtration. *Biochem. J.* **91**, 222.
- CHEN, R., WAGNER, P., GANTHER, H. E. & HOEKSTRA, W. G. (1972) A low molecular weight cadmium-binding protein in testis of rats. Possible role in cadmium induced testicular damage. *Fedn Proc.* **31**, 2725, Abstr.
- CHIQUOINE, A. D. (1964) Observations on the early events of cadmium necrosis of the testis. *Anat. Rec.* **149**, 23.
- DAVIS, B. J. (1964) Disc electrophoresis. II. Methods and application to human serum protein. *Ann. N.Y. Acad. Sci.* **121**, 404.
- GUNN, S. A., GOULD, T. C. & ANDERSON, W. A. D. (1963) The selective injurious response of testicular and epididymal blood vessels to cadmium and its prevention by zinc. *Am. J. Path.* **42**, 685.
- GUNN, S. A., GOULD, T. C. & ANDERSON, W. A. D. (1968) Mechanism of zinc, cysteine and selenium protection against cadmium-induced vascular injury to mouse testis. *J. Reprod. Fert.* **15**, 65.
- KAGI, J. R. H. & VALLEE, B. L. (1960) Metallothionein, a cadmium and zinc containing protein from equine renal cortex. *J. biol. Chem.* **235**, 3460.
- KAGI, J. R. H. & VALLEE, B. L. (1961) Metallothionein, a cadmium and zinc containing protein from equine renal cortex. II. Physicochemical properties. *J. biol. Chem.* **236**, 2435.
- KAR, A. B., DAS, R. P. & MUKERJI, B. (1960) Prevention of cadmium induced changes in the gonads of rat by zinc and selenium—a study in antagonism between metals in the biological system. *Proc. natn. Inst. Sci. India*, **B**, **26**, Suppl. **40**.
- NORDBERG, G. F., PISCATOR, M. & LIND, B. (1971) Distribution of cadmium among protein fractions of mouse liver. *Acta pharmac. tox.* **29**, 456.
- PAŘÍZEK, J. (1957) The destructive effect of cadmium ion on testicular tissue and its prevention by zinc. *J. Endocr.* **15**, 56.
- PAŘÍZEK, J. (1960) Sterilization of the male by cadmium salts. *J. Reprod. Fert.* **1**, 294.
- PISCATOR, M. (1964) On cadmium in normal human kidneys together with a report on the isolation of metallothionein from livers of cadmium exposed rabbits. *Nord. hyg. Tidskr.* **45**, 76.
- PULIDO, P., KAGI, J. R. H. & VALLEE, B. L. (1966) Isolation and some properties of human metallothionein. *Biochemistry*, **5**, 1768.
- SHAIKH, Z. A. & LUCIS, O. J. (1970) Induction of cadmium binding protein. *Fedn Proc. Fedn Am. Socs exp. Biol.* **29**, 301, Abstr.
- SHAIKH, Z. A. & LUCIS, O. J. (1971a) Isolation of cadmium binding proteins from rat liver. *Experientia*, **27**, 1024.
- SHAIKH, Z. A. & LUCIS, O. J. (1971b) The nature and biosynthesis of cadmium binding proteins. *Fedn Proc. Fedn Am. Socs exp. Biol.* **30**, 238, Abstr.

- SINGH, K. & NATH, R. (1972) Studies on the identification of cadmium binding protein from rat testes. *Biochem. J.* **128**, 48p.
- WEBB, M. (1972) Biochemical effects of Cd^{2+} injury in the rat and mouse testis. *J. Reprod. Fert.* **30**, 83.
- WISNIEWSKA-KNYPL, J. M. & JABLONSKA, J. (1970) Selective binding of cadmium in vivo on metallothionein in rat's liver. *Bull. Acad. pol. Sci. Cl. II Ser. Sci. biol.* **18**, 321.
- WISNIEWSKA, J. H., TROJANOWSKA, B., PIOTROWSKI, J. & JAKUBOWSKI, M. (1970) Binding of mercury in the rat kidney by metallothionein. *Toxic. appl. Pharmac.* **16**, 754.