AFFINITY LABELLING STUDIES WITH ^{109}Cd IN CADMIUM-INDUCED TESTICULAR INJURY IN RATS

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Summary. The technique of affinity labelling was used to investigate the mechanism of Cd-induced testicular injury in rats. Thirty minutes after injecting a minimum threshold damaging dose of $^{109}\text{CdCl}_2$ (0.012 mmol/kg), ^{109}Cd distribution was determined in subcellular fractions of testicular homogenates and in proteins of the soluble phase separated by Sephadex G-75 chromatography. The effect on Cd distribution of three different pretreatments that prevent Cd damage was also studied for comparison. The principal Cd-binding fractions were the crude nuclear fraction, and two peaks (II and III) in the soluble fraction corresponding to proteins having molecular weights of 30,000 and 10,000, respectively. Pretreatments with (1) Se (0.012 mmol $\text{Na}_2^{75}\text{SeO}_3/\text{kg}$ 30 min before Cd), (2) Zn (0.60 mmol ^{65}Zn acetate/kg 24 hr before Cd), or (3) a half-threshold dose of CdCl_2 (0.006 mmol/kg 2 days before Cd) prevented Cd damage and decreased, in all three cases, the uptake of Cd in the crude nuclear fraction and Peak II of the soluble fraction. In the case of Zn, these effects were the results of decreased uptake of Cd by the testes. The other two treatments altered the distribution of Cd between or within the various subcellular fractions. Selenium increased Cd uptake in the testes and the soluble fraction but caused a marked diversion of the Cd in the soluble fraction to the higher molecular weight proteins, the fraction also containing most of the selenium. Pretreatment with Cd increased the relative Cd uptake into the 10,000 mol. wt protein fraction, possibly by inducing the formation of a metallothionein-like protein. These observations suggest that the most plausible target of Cd in Cd-induced testicular injury is the unidentified 30,000 mol. wt Cd-binding protein, although the possibility of the crude nuclear fraction as a target could not be excluded.

INTRODUCTION

Cadmium is known to cause selective injury to the testes following injection of a single subacute dose into animals with scrotal testes (Parizek, 1957). Rats

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treated with as little as 0.012 mmol Cd/kg develop haemorrhagic necrosis of the testes within 48 hr, followed by atrophy and permanent sterility (Gunn & Gould, 1970). Other organs, such as liver and kidney, show no changes in spite of much higher uptakes of Cd. Mason, Brown & Nesbit (1963), Gunn, Gould & Anderson (1963), and Mason, Brown, Young & Nesbit (1964) proposed that the testicular injury resulted from an effect of Cd on the testicular vascular system, rather than on the spermatogenic elements. The extensive evidence supporting this mechanism has been reviewed by Gunn & Gould (1970). Cadmium is generally believed to increase testicular capillary permeability by a direct interaction with the capillary endothelium. Histochemical and autoradiographic studies are consistent with the proposed binding of cadmium to the capillary endothelium (Skold, 1961; Berlin & Ulberg, 1963).

The ability of zinc to prevent cadmium injury, apparently by a competitive mechanism, was noted by Parizek (1957) and confirmed by others. Cysteine and other thiols also protect the testis from cadmium, as does prior administration of a sub-threshold dose of Cd 2 days before the threshold dose. The most efficient protector known is selenium (Kar & Das, 1963). The remarkable effectiveness of selenium is clearly revealed in the experiments of Mason & Young (1967), in which the damage produced by Cd at levels just above the minimal effective dose was prevented by an earlier half-equimolar dose of selenium.

Although much is known about the effect of Cd on the testis, relatively little is known about the biochemical basis of the injury, and its prevention by selenium and other agents. Since the effect of Cd is quite specific for testes, is not duplicated by other metals such as mercury (Parizek, 1960), and is produced by a minimal amount of Cd (about 2 μ g reaches the testes), it seemed to be an attractive system for biochemical investigation. Our approach was to administer ^{109}Cd and use the technique of affinity labelling to identify cadmium-binding moieties in the testes. By studying ^{109}Cd distribution in the presence or absence of protective agents, we have tried to pinpoint the possible biochemical target or targets of Cd related to testicular injury, as well as explore the mechanisms by which protective agents prevent such damage.

METHODS

In preliminary studies, gel filtration chromatography of the soluble fraction of rat testis revealed a 30,000 molecular weight (mol. wt) Cd-binding protein which disappeared in samples stored at 4°C (Chen, Hoekstra & Ganther, 1973). In order to measure Cd distribution in the soluble fraction immediately after killing the rats, the present study was conducted as three small experiments instead of a large experiment. Each experiment consisted of two groups of rats, with six rats per group. Holtzman male rats weighing 280 to 320 g were used. In each experiment, rats were evenly allocated by weight to the control (Group 1) or the protective agent-pretreated (Group 2) group. Each rat was given a threshold damaging dose of Cd, i.e. 0.0036 mmol Cd/rat (about 0.012 mmol/kg) with or without pretreatment with a protective agent. Cadmium in isotonic sodium acetate solution was injected subcutaneously into the intra-

scapular space. Protective agents in the same medium were injected subcutaneously into the right flank.

In Exp. I, Group 2 was pretreated with equimolar Se in the form of $\text{Na}_2^{75}\text{SeO}_3$ (0.012 mmol Se/kg) 30 min before the Cd. Group 2 of Exp. II received $^{65}\text{Zn}^{++}$ in a dose (0.6 mmol/kg) which was fifty times that of Cd and 24 hr before it. In Exp. III, Group 2 was given a half-threshold dose of unlabelled Cd^{++} (0.006 mmol Cd/kg) 48 hr before the damaging dose of Cd. Group 2a was a parallel group pretreated with a half-threshold dose of labelled ^{109}Cd having the same specific activity as the subsequent damaging dose of ^{109}Cd . All isotopes were obtained from New England Nuclear Corporation, Boston. After the addition of carriers, the specific activities were 12,000 to 14,500 ct/min/nmol Cd, 12,760 ct/min/nmol Se, and 154 ct/min/nmol Zn. The results of these experiments are representative of several such studies conducted over a period of 2 to 3 years.

Two control rats and two pretreated rats in each experiment were saved for visual scoring of testicular damage 48 hr after the dose of Cd. The other four rats in each group were killed by decapitation 30 min after the Cd was administered. The testes from each group were immediately removed, chilled in cold isotonic KCl solution, pooled, decapsulated, weighed, and homogenized (eight strokes) with 3 vols isotonic KCl solution in a Potter-Elvehjem homogenizer equipped with a Teflon pestle. The homogenate and successive supernatant fractions were then centrifuged, in order, at 1475 *g* for 10 min, at 10,000 *g* for 10 min, and at 113,000 *g* for 60 min, to obtain crude nuclear, mitochondrial, microsomal, and soluble fractions. All operations were conducted at 4°C.

Pellets of the crude nuclear, mitochondrial, and microsomal fractions were suspended in 10 to 30 ml isotonic KCl solution using four strokes in the homogenizer. Duplicate 2-ml aliquots were taken from each suspension, the original homogenate, and the soluble fraction for measuring radioactivity.

As soon as possible after the soluble fraction was obtained, a 10-ml aliquot of soluble fraction from each of the two groups in each experiment was chromatographed simultaneously on separate Sephadex G-75 columns (2.5 × 95 cm) which were pre-equilibrated and eluted at 4°C with 0.001 M-tris-Cl buffer, pH 8.6, containing 0.05 M-KCl. Chromatography was started 3 to 4 hr after killing the rats. After chromatography, a 2-ml aliquot from alternate eluant fractions was taken for assay of radioactivity. Absorbance at 280 or 250 nm was also measured. Radioactive fractions within each peak were then pooled, triplicate 2-ml aliquots of the pooled peak were assayed for radioactivity, and the total radioactivity of the peak was calculated.

From the radioactivity of subcellular fractions and chromatographic peaks, the absolute amount of each metal was calculated from the specific activity of the injected dose. This is valid for the case of Cd, where the endogenous pool is very low. For Se, the amount injected (0.95 mg Se/kg body weight, or 0.95 parts/10⁶) is approximately ten times the total body pool, much of which would be in a non-exchangeable form, thus the calculation is little affected. In the case of Zn, the amount injected (39 mg/kg) is approximately equal to the total body pool, so calculation of total Zn from the specific activity of the dose only gives an apparent value. A well-type scintillation counter (Nuclear-Chicago)

with a 2×2 in. sodium iodide crystal was used for measuring radioactivity. The counting windows were set so that the energy range was 12.5 to 43.75 keV for ^{109}Cd , 240 to 500 keV for ^{75}Se , and 100 to 1400 keV for ^{65}Zn . The counting efficiency at these settings was approximately 17% for ^{109}Cd , 35% for ^{75}Se and 9% for ^{65}Zn . At these window settings, no significant counts of ^{109}Cd appeared in the counting window of ^{75}Se or in that of ^{65}Zn . However, 2.1% of the ^{75}Se counts and 3.0% of the ^{65}Zn counts appeared in the counting window of ^{109}Cd . Therefore, in cases of double-labelling, the apparent ^{109}Cd counts were corrected for counts contributed by ^{75}Se or by ^{65}Zn present in the same sample to obtain the true ^{109}Cd counts.

Table 1. Subcellular distribution of ^{109}Cd and ^{75}Se in the testes of control and Se-pretreated rats (Experiment I)

Subcellular fraction	^{109}Cd		^{75}Se
	Group 1 (control) (nmol)	Group 2 (+Se) (nmol)	Group 2 (+Se) (nmol)
Testicular uptake	23.5 (100.0)	29.1 (100.0)	45.9 (100.0)
Subcellular fraction			
Crude nuclear	11.1 (47.2)	7.8 (26.8)	16.1 (35.1)
Mitochondrial	0.5 (2.1)	0.6 (2.1)	3.2 (7.0)
Microsomal	0.5 (2.1)	1.5 (5.2)	1.8 (3.9)
Soluble	10.5 (44.7)	18.5 (63.6)	23.1 (50.3)
Peak I	1.4 (6.0)	16.5 (56.7)	13.7 (29.8)
Peak II	4.4 (18.7)	1.8 (6.2)	0.5 (1.1)
Peak III	4.7 (20.0)	1.2 (4.1)	0.5 (1.1)
Peak IV	—	—	9.0 (19.6)
% Recovery: subcellular fractionation	96.1	97.7	96.3
Sephadex G-75 chromatography	100.4	105.3	102.6

Values in parentheses are percentages with testicular uptake as 100%. The soluble fraction was further fractionated into three Cd peaks and four Se peaks as indicated in Text-fig. 1. Soluble fraction (10 ml) was used for this Sephadex G-75 chromatography. Total Cd or Se in the soluble fraction was then calculated for Peak I, II, III or IV, according to its percentage.

RESULTS

Experiment I

Examination of the testes 48 hr after a damaging dose of Cd confirmed that the injection of 0.012 mmol Cd/kg caused marked swelling and haemorrhage. This damage was completely prevented, however, by pretreatment with equimolar Se 30 min before injection of Cd.

At 30 min after the damaging dose of Cd (0.012 mmol Cd/kg), testicular Cd uptake was 0.16% of the administered dose per pair of testes in the control group. Uptake was increased to 0.20% by Se pretreatment, while testicular Se uptake at the same time was 0.32%.

The testicular Cd and Se distributions are shown in Table 1. In control rats, 47% of testicular Cd was found in the crude nuclear fraction and 45% in the

soluble fraction. Little Cd (about 2%) was found in the mitochondrial and the microsomal fractions. Pretreatment with Se decreased Cd uptake in the crude nuclear fraction from 47% to 27%. This decrease was also true in terms of the absolute amount of Cd, despite the fact that total testicular Cd uptake was increased by the pretreatment. Cadmium uptake in the soluble fraction was correspondingly increased by Se pretreatment, from 45% to 64%. Pretreatment with Se also increased Cd in the microsomal fraction from 2% to 5%. This effect on microsomes was consistently observed in other experiments, and in

TEXT-FIG. 1. Sephadex G-75 chromatography of the soluble fraction from the testes of control or Se-pretreated rats (Exp. I). Of the soluble fraction from a 25% homogenate, 10 ml were applied to a 2.5 × 95 cm column and eluted with 0.001 M-tris-Cl buffer, pH 8.6, containing 0.05 M-KCl, at 4°C, 32 ml/hr. The separations were performed simultaneously on two identical columns.

tissues besides testes (data not shown). Testicular ^{75}Se was found primarily in the crude nuclear fraction (35%) and the soluble fraction (50%).

Sephadex G-75 chromatography of the soluble fraction of the testis revealed three major Cd peaks (Text-fig. 1). Peak I had a mol. wt larger than 70,000. Peak II had a mol. wt of 30,500, and Peak III a mol. wt of 10,100 (Text-fig. 2). Although Peak I had a large amount of protein (A_{280}), it contained only 15% of the Cd applied to the Sephadex G-75 column. By contrast, Peak II and

TEXT-FIG. 2. Estimation of molecular weights of cadmium-binding proteins on a 2.5×95 cm Sephadex G-75 column. The column was calibrated with 4 ml buffer containing 40 mg bovine serum albumin (BSA), 40 mg ovalbumin (OA), and 5 mg cytochrome C. The void volume (V_0) was determined with 0.3% blue dextran (Pharmacia) in 4 ml buffer. The elution volume (V_e) was taken as the position on the ascending side of a peak giving half the maximum peak height. The soluble fraction (10 ml) from Cd-treated rats was chromatographed as described in Text-fig. 1. The molecular weight estimated for Cd-BP II was 30,500 and that for Cd-BP III was 10,100.

Table 2. Correlation of changes in Cd distribution in rat testes with protection produced by pretreatments with various protective agents

	% increase (+) or decrease (–) in Cd content, compared with control, by protective agents:		
	Se	Zn	$\frac{1}{2}$ Cd
Testicular Cd uptake	+24	–27	–11
Crude nuclear fraction	–30	–29	–40
Peak II (30,500 mol. wt Cd-BP)	–59	–35	–12
Peak III (10,000 mol. wt Cd-BP)	–75	–3	+58
Protection effectiveness*	++++	+++	++

* The protection effectiveness of various pretreatments was judged by comparing (visually) for purplish coloration (haemorrhage) the protective agent-pretreated testes with the testes of control rats (intensive coloration) and with normal testes (no coloration). + + + + : no coloration, exactly the same as normal testes; + + + : slight coloration; + + : fair amount of coloration but much less intensive than control rat testes.

Peak III lacked a distinct A_{280} peak but contained 41.9% and 45.0% of the Cd, respectively. Interestingly, Se-pretreatment greatly decreased Cd uptake in Peaks II and III, expressed either as a percentage or in terms of the absolute amount of Cd (Table 1), in spite of the fact that the soluble fraction of Se-pretreated rats contained substantially more total Cd than that of control rats. Se distribution within the soluble fraction of the testis was different from that of Cd. About 59% of the Se applied onto the Sephadex G-75 column was found in Peak I and 39% was found in the region of low molecular weight (Peak IV) (Text-fig. 1). Selenium itself was not incorporated into the proteins of Peaks II and III.

Experiment II

As in Exp I, examination revealed that 0.012 mmol Cd/kg induced testicular damage. This damage was prevented by pretreatment with 0.6 mmol Zn/kg as

Table 3. Subcellular distribution of ^{109}Cd and ^{65}Zn in the testes of rats (Experiment II)

Subcellular fraction	^{109}Cd		^{65}Zn
	Group 1 (control) (nmol)	Group 2 (+ Zn) (nmol)	Group 2 (+ Zn) (nmol)
Testicular uptake	18.6 (100.0)	13.5 (100.0)	426.7 (100.0)
Subcellular fraction			
Crude nuclear	9.4 (50.0)	6.7 (49.6)	198.3 (46.5)
Mitochondrial	0.3 (1.6)	0.2 (1.5)	9.7 (2.3)
Microsomal	0.4 (2.2)	0.2 (1.5)	19.9 (4.7)
Soluble	7.7 (41.4)	6.2 (45.9)	189.7 (44.5)
Peak I	1.0 (5.4)	0.8 (5.9)	50.0 (11.7)
Peak II	3.7 (19.9)	2.4 (17.8)	45.2 (10.6)
Peak III	3.1 (16.7)	3.0 (22.2)	80.9 (19.0)
% Recovery:			
subcellular fractionation	(95.2)	(98.5)	(98.0)
Sephadex G-75 chromatography	(101.4)	(100.0)	(92.8)

Values in parentheses are percentages with testicular uptake as 100%.

a single dose given 24 hr before the damaging dose of Cd, although the protection by this pretreatment with a 50-fold excess of Zn (Table 2) was judged less perfect than by pretreatment with equimolar Se.

Cadmium uptake by the testes was considerably decreased by Zn pretreatment (0.09% of the dose versus 0.13%). The testicular Zn uptake was 0.06% of the administered dose per pair of testes.

The testicular Cd and Zn distribution are shown in Table 3. Zinc had little effect on the percentage Cd distribution within the various subcellular fractions. The amount of Cd taken up by the crude nuclear fraction and Peak II, however, was decreased, while Peak III was unaffected. The testicular subcellular Zn distribution was similar to that of Cd, i.e. 46% in the crude nuclear fraction, 44% in the soluble fraction, and little in either the mitochondrial or the

TEXT-FIG. 3. Sephadex G-75 chromatography of the soluble fraction from the testes of control or Zn-pretreated rats (Experiment II). The procedure was the same as that described for Text-fig. 1.

Table 4. Subcellular distribution of ^{109}Cd in the testes of control and Cd-pretreated rats (Experiment III)

Subcellular fraction	^{109}Cd		
	Group 1 (control) (nmol)	Group 2 (+ $\frac{1}{2}\text{Cd}$) (nmol)	Group 2a (+ $\frac{1}{2}^{109}\text{Cd}$) (nmol)
Testicular uptake	20.8 (100.0)	18.5 (100.0)	29.4 (100.0)
Subcellular fraction			
Crude nuclear	11.3 (54.3)	6.8 (36.8)	11.8 (40.0)
Mitochondrial	0.3 (1.4)	0.4 (2.2)	0.7 (2.2)
Microsomal	0.4 (1.9)	0.4 (2.2)	0.6 (2.0)
Soluble	8.6 (41.3)	10.4 (56.2)	15.8 (53.8)
Peak I	1.2 (5.8)	1.6 (8.6)	2.4 (8.2)
Peak II	3.4 (16.3)	3.0 (16.2)	3.6 (12.2)
Peak III	3.8 (18.3)	6.0 (32.4)	10.7 (36.4)
% Recovery: subcellular fractionation	(98.9)	(97.4)	(98.1)
Sephadex G-75 chromatography	(97.8)	(101.8)	(105.3)

Values in parentheses are percentages with testicular uptake as 100%.

microsomal fraction. In the soluble fraction of the testes of rats pretreated with Zn, three Zn peaks were also found after Sephadex G-75 chromatography (Text-fig. 3), corresponding to the three Cd peaks, with Peak I containing 26%, Peak II containing 24%, and Peak III containing 43% of the total Zn applied to the column.

TEXT-FIG. 4. Sephadex G-75 chromatography of the soluble fraction from the testes of control rats (upper) or rats pretreated with unlabelled Cd (middle) or ^{109}Cd (bottom) (Exp. III). The procedure was the same as that described for Text-fig. 1.

Experiment III

Examination again confirmed that 0.012 mmol Cd/kg caused testicular damage, which was partly prevented by pretreatment of 0.006 mmol Cd/kg

given 48 hr before the damaging dose of Cd. The protection by this pretreatment was, however, not as good as that of Zn pretreatment and certainly less than that with Se pretreatment (Table 2).

Pretreatment with the half-threshold dose of Cd (unlabelled) had little effect on testicular ^{109}Cd uptake from the damaging dose of ^{109}Cd , compared with that of the control group (0.13% versus 0.14%). However, pretreatment with the half-threshold dose of Cd decreased ^{109}Cd content in the crude nuclear fraction from 54% to 37% (Table 4). Correspondingly, ^{109}Cd in the soluble fraction was increased from 41% to 56% by this pretreatment. The pretreatment also decreased the percentage of Cd in Peak II from 40 to 29, while Cd in Peak III was increased from 45% to 58% (Text-fig. 4). These percentage changes in ^{109}Cd distribution by pretreatment with a half-threshold dose of Cd were also true in terms of absolute amounts of ^{109}Cd . These data show only the fate of Cd from the damaging dose; however, a parallel study was carried out simultaneously employing the same solution of $^{109}\text{CdCl}_2$ for both the pretreatment and the damaging dose of Cd (Group 2a, Table 4). As expected, total ^{109}Cd in the testes of pretreated rats was increased, and in approximately the same proportion as the increase in administered ^{109}Cd . Although the absolute amount of Cd in a given fraction was generally increased, the crude nuclear fraction was little affected whereas the soluble fraction increased nearly twofold. Within the soluble fraction, Peak III increased nearly threefold in Cd content while Peak II showed only a slight increase. The total Cd in the crude nuclear fraction and in Peak II was about the same in Group-2a animals as in those of Group I, even though they were actually protected from damage.

DISCUSSION

The technique of affinity labelling is based on a stable interaction between a specific compound and a receptor such as a protein, and has been employed for the recognition and isolation of many proteins. Our use of this approach involves two assumptions: (1) that Cd acts directly on the testis, as opposed to binding at some other site to produce an indirect effect on the testes; (2) that cadmium remains bound to its target throughout the isolation process, and does not become redistributed. In regard to the first assumption, no one has proposed that Cd acts other than through a direct effect on the testicular vasculature. The weight of evidence, as reviewed by Gunn & Gould (1970), favours a direct effect of Cd on the permeability of testicular capillaries rather than an indirect one through the release of vasoactive compounds; vasodilators or antihistamines, antiserotonin, or antihyaluronidase drugs have no effect. For the second assumption, it seems reasonable to assume that Cd and other heavy metals form stable complexes with tissue components, as demonstrated by the isolation of metallothionein and a variety of other metalloproteins, as well as nucleic acids and plasma membrane proteins containing firmly bound metals (Wacker & Vallee, 1959; Kagi & Vallee, 1960; Fitzpatrick, Welch & Landon, 1969). Cadmium will bind to a number of substances in the testis, but the circumstances favour a relatively specific interaction. By using a minimum threshold dose such that a half-threshold dose has no effect, the tissue is not

flooded with Cd. It follows that, with threshold doses, the binding of Cd to possible targets must be quantitatively significant. Furthermore, protective treatments might be expected to decrease Cd uptake in the target by as much as 50%, the actual amount depending on a number of factors. Since Cd injection as a single dose in an area remote from the organ will be carried first to sites in the testicular vascular system, localization to the vascular region at early time intervals can be expected. Our selection of 30 min after injection as the time of autopsy was based in part on this idea, which has received support from the autoradiographic studies of Johnson & Sigman (1971). In addition, we wished to avoid non-specific changes at later time intervals that might occur as a secondary result and not directly related to the primary action of Cd.

Probable targets of Cd related to injury

It appears that either the 10,000 or 30,000 mol. wt proteins of the soluble fraction, or some component of the crude nuclear fraction, would be the most likely targets of Cd damage, since these three fractions are the major Cd-binding components in rat testes. We had previously suggested that the 10,000 mol. wt protein might be a target (Chen, Wagner, Ganther & Hoekstra, 1972), but, in view of the discovery of the unstable 30,000 mol. wt Cd-binding protein, this can probably be excluded. Although the Cd content of the 10,000 mol. wt protein was decreased by two protective treatments, it was actually increased by the protective pretreatment with a half-threshold dose of Cd. This metallothionein-like protein more probably protects against Cd damage by diverting Cd from some other target.

As for the 30,000 mol. wt Cd-binding protein and the crude nuclear fraction, it is important to note that the Cd content of these components was substantially decreased by all three protective pretreatments, including that with Se, even though testicular Cd uptake was actually increased in the case of Se. It appears that the correlation between decrease in Cd content and the effectiveness of protection against Cd damage by the various treatments was better for the 30,000 mol. wt Cd-binding protein than for the crude nuclear fraction (Table 2). Furthermore, we have observed a correlation between the presence of this protein in a tissue and susceptibility to Cd injury: the protein is not detectable by Sephadex G-75 chromatography when soluble fractions of rat liver or kidney are treated with ^{109}Cd *in vitro*; moreover, it is not detectable in the domestic fowl, a species whose testes are not susceptible to Cd injury. Such a high degree of correlation at the biochemical level has not previously been observed with regard to Cd and testicular injury; the carbonic anhydrase activity of fowl testes, as of rat testes, was inhibited by Cd (Johnson & Walker, 1970).

The crude nuclear fraction must be regarded as a third possible target of Cd, especially since Cd is thought to bind to capillary endothelial cells, and Cd thus bound might be expected to be present in fragments of the plasma membrane sedimenting in the crude nuclear fraction. In the present studies, the uptake of Cd in the crude nuclear fraction ranged from 47 to 54%. Although they employed a non-damaging tracer dose of ^{109}Cd , Johnson, Sigman & Miller (1970) likewise observed a high uptake (30 to 40%) of testicular Cd in the nuclear

fraction and decreased uptake following pretreatment with Zn or Se. The uptake was equally high, however, in the testes of fowl. Conceivably, the Cd in the nuclear fraction from testis homogenates could represent Cd which formed a complex with the nucleic acids of the relatively voluminous nuclear fraction from this tissue.

The components studied in any particular fraction necessarily reflect the separation method employed. It is possible that further fractionation of Peak I proteins, for example, might reveal one particular protein whose Cd content varied directly with Cd damage, even though Peak I, like the soluble fraction, does not. In this particular case, however, such a possibility is unlikely because of the relatively small amount of Cd associated with this peak after a damaging dose.

Protective mechanisms

The mechanisms of protection against Cd-induced testicular damage with Se, Zn, or a half-threshold dose of Cd appear to be quite different. Pretreatment with Se tended in our experiments to increase Cd uptake in the testes. Similar results were obtained in mice (Gunn, Gould & Anderson, 1968a, b). Johnson *et al.* (1970), using a non-damaging tracer dose of Cd, found little or no effect of Se on Cd uptake. Gunn *et al.* (1968a) observed that Cd and Se each increased the uptake of the other in the testes, and suggested that some non-toxic complex of Cd and Se was formed. Most of the Se later left the testis, however, while the Cd remained, which suggests that Cd was not simply precipitated as cadmium selenide. There is extensive evidence that Se forms complexes with metals to decrease the biological availability of both components (Parizek, Ostadalova, Kalouskova, Babicky & Benes, 1971). We believe that Se protects by diverting Cd from its usual target(s) to some less sensitive site, probably by forming selenoprotein derivatives that have a high affinity for Cd. Selenite is known to react with sulphhydryl groups of proteins, forming S-Se-S linkages (Jenkins, 1968; Ganther & Corcoran, 1969) and tends to follow the sulphur in proteins (Jenkins & Hidiroglou, 1971). Selenium was incorporated into the proteins of soluble fraction Peak I and increased the uptake of Cd in this peak 12-fold, while decreasing Cd uptake in Peaks II and III, as well as the nuclear fraction. We have not as yet fractionated the Peak I proteins further to see if any particular protein is especially high in both Cd and Se. Selenium was not incorporated into Peak II, nor was it present in Peak III, where metallothionein would be eluted. Possibly Cd competes with selenite for binding to sulphhydryl groups in the proteins of these peaks. In the case of metallothionein, the proximity of three sulphhydryl groups (Kagi & Vallee, 1960) may preclude the formation of a stable selenium derivative.

The protective effect of Zn-pretreatment appeared to result from decreased testicular Cd uptake, possibly by inducing the formation of a metallothionein-like protein in liver as proposed by Webb (1972b). Johnson *et al.* (1970) also observed decreased uptake of Cd in Zn-treated rats. Studies in the mouse have shown a slight effect of Zn in decreasing Cd uptake even though this did not appear to be significant in protection (Gunn & Gould, 1970).

Protection by pretreatment with a smaller dose of Cd was first reported by

Ito & Sawauchi (1966). Our results confirm this and show that the prior dose of Cd altered the distribution of the subsequent dose, increasing both the percentage and the amount of Cd taken up in Peak III of the testicular soluble fraction. This effect is consistent with a protective mechanism involving the induced synthesis of a testicular Cd-binding protein of approximately 10,000 mol. wt, such as metallothionein, which binds much of the Cd in the later dose and diverts it from other targets. Webb (1972a, b) observed a similar induction of Cd-binding proteins in the liver and kidney but did not examine the testes, probably because the sensitivity of atomic absorption is not adequate readily to follow Cd metabolism in the testes. The synthesis of new proteins is time-dependent, thus the proposed mechanism can explain the observations reported by Gunn & Gould (1970), who found that protection was not observed if the full dose was administered between 6 and 24 hr after the half-threshold dose, or after 3 weeks, but was observed at 2 to 5 days.

It should be noted that the total amount of Cd in the other major Cd-binding fractions of testes (crude nuclear fraction and Peak II) was as great in rats pretreated with Cd (Group 2a, Exp. III) as it was in the control animals, even though the testes of animals pretreated with Cd were protected from damage. This might be the result of new protein synthesis between the pretreatment and the subsequent dose. Partial inactivation of an essential protein caused by binding of the initial Cd dose could be compensated for by making more of the protein. What is critical is not the amount of Cd bound to the protein, but the amount of Cd-free protein. An example is provided by metallothionein, where more of the metal-free protein (thionein) is apparently made to replace that bound by Cd, so the total metallothionein increases with chronic metal administration and becomes detectable by spectrophotometry (Nordberg, Piscator & Lind, 1971).

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REFERENCES

- BERLIN, M. & ULBERG, S. (1963) The fate of ^{109}Cd in the mouse. *Archs environ. Hlth*, **7**, 686.
- CHEN, R., HOEKSTRA, W. G. & GANTHER, H. E. (1973) An unstable Cd-binding protein in the soluble fraction of rat testes. *Fedn Proc. Fedn Am. Socs exp. Biol.* **32**, 929.
- CHEN, R., WAGNER, P., GANTHER, H. E. & HOEKSTRA, W. G. (1972) A low molecular-weight cadmium-binding protein in testes of rats: possible role in cadmium-induced testicular damage. *Fedn Proc. Fedn Am. Socs exp. Biol.* **31**, 699.
- FITZPATRICK, D. F., WELCH, L. T. & LANDON, E. J. (1969) Characterization of plasma membrane proteins in mammalian kidney. II. The binding of organic mercurials to rat kidney plasma membrane proteins *in vivo*. *J. biol. Chem.* **244**, 3570.
- GANTHER, H. E. & CORCORAN, C. (1969) Selenotrisulfides. II. Cross-linking of reduced pancreatic ribonuclease with selenium. *Biochemistry, N.Y.* **8**, 2557.
- GUNN, S. A. & GOULD, T. C. (1970) Cadmium and other mineral elements. In *The Testis*, Vol. III, p. 377. Eds. A. D. Johnson, W. R. Gomes and N. L. VanDemark. Academic Press, New York.
- 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. (1968a) Mechanisms of zinc, cysteine, and selenium protection against cadmium-induced vascular injury to mouse testis. *J. Reprod. Fert.* **15**, 65.
- GUNN, S. A., GOULD, T. C. & ANDERSON, W. A. D. (1968b) Selectivity of organ response to cadmium injury and various protective measures. *J. Path. Bact.* **96**, 89.
- ITO, T. & SAWAUCHI, K. (1966) Inhibitory effects on cadmium-induced testicular damage by pre-treatment with smaller cadmium dose. *Okajimas Folia anat. jap.* **42**, 107 (cited by Gunn & Gould, 1970).
- JENKINS, K. J. (1968) Evidence for the absence of selenocystine and selenomethionine in the serum proteins of chicks administered selenite. *Can. J. Biochem.* **46**, 1417.
- JENKINS, K. J. & HIDIROGLOU, M. (1971) Comparative uptake of selenium by low cystine and high cystine proteins. *Can. J. Biochem.* **49**, 468.
- JOHNSON, A. D. & SIGMAN, M. B. (1971) Early actions of cadmium in the rat and domestic fowl testis. IV. Autoradiographic location of 115m cadmium. *J. Reprod. Fert.* **24**, 115.
- JOHNSON, A. D., SIGMAN, M. B. & MILLER, W. J. (1970) Early actions of cadmium in the rat and domestic fowl testis. III. Subcellular location of injected 109 cadmium. *J. Reprod. Fert.* **23**, 201.
- JOHNSON, A. D. & WALKER, G. P. (1970) Early actions of cadmium in the rat and domestic fowl testis. V. Inhibition of carbonic anhydrase. *J. Reprod. Fert.* **23**, 463.
- KAGI, J. H. R. & VALLEE, B. L. (1960) Metallothionein: a cadmium- and zinc-containing protein from equine renal cortex. *J. biol. Chem.* **235**, 3460.
- KAR, A. B. & DAS, R. P. (1963) The nature of protective action of selenium on cadmium-induced degeneration of rat testis. *Proc. natn. Inst. Sci. India, B*, **29**, 297.
- MASON, K. E., BROWN, J. A. & NESBIT, R. R. (1963) Cadmium-induced injury of the rat testis. *Anat. Rec.* **145**, 257, Abstr.
- MASON, K. E., BROWN, J. A., YOUNG, J. O. & NESBIT, R. R. (1964) Cadmium-induced injury of the rat testis. *Anat. Rec.* **149**, 135.
- MASON, K. E. & YOUNG, J. O. (1967) Effectiveness of selenium and zinc in protecting against cadmium-induced injury of the rat testis. In *Selenium in Biomedicine*, p. 383. Eds. O. H. Muth, J. E. Oldfield and P. H. Weswig. Avi Publishing Co., Westport, Conn.
- NORDBERG, G. F., PISCATOR, M. & LIND, B. (1971) Distribution of cadmium among protein fractions of mouse liver. *Acta pharmac. tox.* **29**, 456.
- PARIZEK, J. (1957) The destructive effect of cadmium ion on testicular tissue and its prevention by zinc. *J. Endocr.* **15**, 56.
- PARIZEK, J. (1960) Sterilization of the male by cadmium salts. *J. Reprod. Fert.* **1**, 294.
- PARIZEK, J., OSTADALOVA, I., KALOUSKOVA, J., BABICKY, A. & BENES, J. (1971) The detoxifying effects of selenium. Interrelations between compounds of selenium and certain metals. In *Newer Trace Elements in Nutrition*, p. 85. Eds. W. Mertz and W. E. Cornatzer. Marcel Dekker, New York.
- SKOLD, G. (1961) Effect of cadmium poisoning on testes. *Acta path. microbiol. scand.* **53**, 440, Abstr.
- WACKER, W. E. C. & VALLEE, B. L. (1959) Nucleic acids and metals. I. Chromium, manganese, nickel, iron, and other metals in ribonucleic acid from diverse biological sources. *J. biol. Chem.* **234**, 3257.
- WEBB, M. (1972a) Binding of cadmium ions by rat liver and kidney. *Biochem. Pharmac.* **21**, 2751.
- WEBB, M. (1972b) Protection by zinc against cadmium toxicity. *Biochem. Pharmac.* **21**, 2767.