EARLY ACTIONS OF CADMIUM IN THE RAT AND DOMESTIC FOWL TESTIS

III. SUBCELLULAR LOCATION OF INJECTED ^{109}Cd MIUM*

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Summary. Mature male rats and domestic fowl were injected subcutaneously with $^{109}\text{CdCl}_2$; some rats were pre-treated 24 or 2 hr previously with Zn or Se, respectively. Testis, muscle and liver samples were taken at 5, 10, 20 or 40 min post-injection. Tissues were homogenized and centrifuged to obtain nuclear, heavy and light mitochondrial, microsomal, and the resultant 103,000 g supernate fractions. This supernate was partitioned by acetone which resulted in non-protein and protein-bound Cd fractions. In the liver nuclear fraction, there was only 10% of the total ^{109}Cd compared to 50 and 30% in the muscle and testis, respectively. These trends were reversed in the 103,000 g supernate. Pre-treatment with Zn or Se reduced the ^{109}Cd in this fraction in the testis and muscle. In these organs, there was more ^{109}Cd in the mitochondrial fractions of the domestic fowl than in the rat. Pre-treatments decreased ^{109}Cd in the testicular microsomes and in the protein of the 103,000 g supernate. After 5 min, there was essentially no free element in the testis of the domestic fowl. If subcellular location is indicative, the domestic fowl and the Zn and Se pre-treated rats appear to have very different mechanisms of defence against Cd.

INTRODUCTION

Recent work has shown that cadmium (Cd) injected into the rat results in immediate reactions, causing oedema, haemorrhage and elimination of the germinal elements of the scrotal testis (reviewed by Pařízek, 1960; Gunn & Gould, 1970). Such damage can be prevented by pre-treatment with Zn or Se. Injected Cd caused a marked increase in testis temperature within 5 min of the injection in the rat (Johnson, Gomes & VanDemark, 1970). This temperature change was prevented by Zn pre-treatment. In the domestic fowl where the testis is not damaged by Cd (Guthrie, 1964; Erickson & Pincus, 1964), testicular temperature was not altered by Cd. Body temperature in the rat was found to drop with Cd injection, suggesting some influence of Cd on

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organs other than the testes. By 30 min after an injection of Cd, a marked inhibition of testicular carbonic anhydrase was observed in the rat (Hodgen, Butler & Gomes, 1969).

Comparison of the tissue distribution of ^{109}Cd at 2.5, 5, 10, 20, 40 and 80 min after injection, revealed no major differences between rats and domestic fowl in arrival time, concentration or removal from the testis or other tissues (Johnson & Miller, 1970). Whether the subcellular location of the ^{109}Cd was different in the testes or other tissues of these two species has not been determined. These early actions of Cd, its rapid movement and the resultant differential effect in the rat and domestic fowl has led to the present study, the objective of which was to determine if there are differences in subcellular location of ^{109}Cd in tissues of these two species and the influence of pre-treatment with Se or Zn. Such differences might explain why testes of certain species are drastically damaged by Cd, while those of other species and other organs of all species are at least tolerant to Cd.

MATERIALS AND METHODS

Experiments were conducted with twenty-four mature male rats and twelve male domestic fowl. The Wistar strain rats weighed from 255 to 478 g and the White Leghorn domestic fowl from 1.8 to 2.5 kg. All animals received an unrestricted commercial ration until injection. Three animals were used in each species or pre-treatment time group.

The $^{109}\text{CdCl}_2$ (specific activity of 5550 $\mu\text{C}/\text{mg}$ of Cd—New England Nuclear Corporation, Boston) was obtained in 0.5 N-HCl. This was diluted with acetate buffer (pH 4.96) in the ratio of 10 parts of buffer to 1 of $^{109}\text{CdCl}_2$: HCl solution to give a concentration of 323.2 μC of $^{109}\text{Cd}/\text{ml}$. Six rats were pre-treated with zinc acetate (1.61 m-moles/kg) 24 hr before the Cd injection. Likewise, there were six rats pre-treated with sodium selenite (0.06 m-moles/kg) 2 hr before the Cd treatment. All animals received subcutaneous (s.c.) injections in the interscapular region and all the rats received 0.25 ml (80.85 μC) and the domestic fowl 0.5 ml (161.6 μC) of the solution.

The domestic fowl and the rats which received no pre-treatment were killed under sodium pentobarbital anaesthesia at 5, 10, 20 or 40 min by removal of the heart to stop circulation at a predetermined time. The pre-treated rats were similarly killed at 10 and 40 min post-injection. The liver, testes and a cross-section sample of muscle from the right posterior limb were removed immediately after the heart was removed. The organs were then frozen at -26°C for later analysis.

When ready for use, the samples were thawed in an ice bath in a refrigerator (4°C). An entire testis minus the tunica albuginea or a sample of muscle or liver weighing about 1.5 g was homogenized in a ground-glass homogenizer in 20 ml of cold phosphate-buffered (pH 7.2) sucrose (0.88 M) solution. After homogenization, a 10-ml sample of the solution was pipetted into a counting tube and ^{109}Cd determined by two 1-min counts in an automatic test tube changer gamma counter with a 7.6-cm NaI (Tl) well crystal detector (Baird Automatic Counter System, Model 709). Another 10-ml sample of the solution

was transferred to a 30-ml Corex tube for cell fractionation by centrifugation and acetone precipitation.

In the development of the centrifugation procedures, the methods of Schneider & Hogeboom (1950), Goswami, Skipper & Williams (1968) and Umbreit, Burris & Stauffer (1951) were tested and the resultant cell fractions were checked for uniformity with phase microscopy. After considering these factors, the modified method as described below was used. The homogenized sample was first centrifuged (Sorvall Model Rc2-B, Rotor Model SS34, 8-placed head) at 1600 g for 10 min. The precipitate was washed with 3 ml of the buffered sucrose solution and centrifuged at 5000 g for 10 min to free trapped micro-particles. The remaining pellet consisted mainly of nuclei, broken cell walls and sperm cells or other intact cells. This was suspended in 10 ml distilled water and ^{109}Cd determined as described above. Likewise, the ^{109}Cd level of each of the subsequent pellets was measured. The combined supernates were centrifuged at 10,000 g for 20 min and the resultant pellet contained heavy mitochondria. The supernate was centrifuged at 29,000 g for 20 min to obtain the light mitochondria. This supernate was then placed in a cellulose nitrate tube and centrifuged in an ultracentrifuge (Beckman Ultracentrifuge Model L2-65, Type 50 Ti Fixed Angle Titanium Rotor) at 103,000 g for 1 hr to sediment the microsomes. The ^{109}Cd in this supernate was determined and was saved for subsequent analyses. The supernate of the 103,000 g centrifugation was mixed with cold acetone (3:7.5, v/v) to precipitate the protein (Pekas, 1966). This was then centrifuged at 480 g for 10 min and the ^{109}Cd content of both the supernate and precipitate determined separately.

The ^{109}Cd counts were corrected for background and the percentage of the dose/g of tissue adjusted for body weight differences as in previous work (Johnson & Miller, 1970). The percentage of the ^{109}Cd in the original homogenate which appeared in each of the cell fractions was calculated and the percentage of the ^{109}Cd in the 103,000 g supernate which appeared in the acetone supernate or precipitate was also calculated. The significance of difference between species, times post-treatment and pre-treatments was determined using Duncan's Multiple Range test (Steel & Torrie, 1960).

RESULTS

Testis

The ^{109}Cd levels in the testis and its various subcellular fractions are illustrated in Text-figs. 1 to 4. As shown in Text-fig. 1, the ^{109}Cd level, as the percentage of the size-adjusted dose, in the testis of the domestic fowl increased stepwise for 20 min and then tended to decrease. The levels in the rat testis increased with each time period through 40 min. In the Zn pre-treated rat, the ^{109}Cd level was reduced ($P < 0.05$) 10 min after the injection and at 40 min, the levels were decreased ($P < 0.01$ and $P < 0.05$, respectively) by both the Zn and Se pre-treatment.

In the nuclear fraction (Text-fig. 1) of the domestic fowl testis, ^{109}Cd decreased ($P < 0.01$) after 5 min while in the rat testis, the levels were constant with time. With both pre-treatments, the ^{109}Cd content was lower ($P < 0.01$)

TEXT-FIG. 1. The size-adjusted percentage of the injected dose in the whole testis homogenate and the percentage of that amount in the nuclear fraction, in the domestic fowl and rat receiving no pre-treatment, and the Zn or Se pre-treated rat at various times after ^{109}Cd injection.

than in the control rats at both 10 and 40 min. In the testicular mitochondrial fractions (Text-fig. 2), there were tendencies for more ^{109}Cd in the domestic fowl. With the domestic fowl, ^{109}Cd decreased ($P < 0.05$) in the light mitochondrial fraction after 5 min while in the heavy mitochondrial fraction, there was a tendency toward an increase with time.

In the 103,000 g pellet (Text-fig. 3), which contained microsomes, there

TEXT-FIG. 2. The percentage of the total amount in the testis which was located in the mitochondrial fractions, in the domestic fowl and rat receiving no pre-treatment, and the Zn or Se pre-treated rat at various times after ^{109}Cd injection.

TEXT-FIG. 3. The percentage of the testis ^{109}Cd in the 103,000 g fractions, in the domestic fowl and rat receiving no pre-treatment, and the Zn or Se pre-treated rat at various times after ^{109}Cd injection. The pellet contained microsomes primarily and the supernate contained protein bound and non-protein bound or free ^{109}Cd .

TEXT-FIG. 4. The portions of the testis ^{109}Cd in the 103,000 g supernate which was either protein bound (precipitate) or non-protein bound (free element), in the domestic fowl and rat receiving no pre-treatment, and the Zn or Se pre-treated rat at various times after ^{109}Cd injection.

was a trend toward a decrease in the domestic fowl testis ^{109}Cd level. At 40 min post-injection in the rat, the pre-treatments resulted in a lower ($P < 0.05$) Cd level than in the rats which received no pre-treatment. In the supernate of the 103,000 g centrifugation, both pre-treatments resulted in increased ^{109}Cd levels at both 10 min ($P < 0.01$) and 40 min ($P < 0.05$) post-treatment, while in the fowl, the ^{109}Cd levels were higher at 40 min ($P < 0.01$) than in the control rat. When this supernate was further sub-divided into protein bound and non-protein bound fractions (Text-fig. 4), the major portion of the increase in the pre-treated group occurred as the free element at 40 min. This was not the case with the fowl, however, since after 5 min, the ^{109}Cd levels as non-protein bound or free element approached zero.

Muscle

The ^{109}Cd levels in the muscle are shown in Text-figs. 5 to 8. The percentage of the size-adjusted dose/g of muscle tissue was smaller than in either the testes

TEXT-FIG. 5. The size-adjusted percentage of the injected dose of ^{109}Cd /g of muscle tissue and its portion in the nuclear fraction, in the domestic fowl and rat receiving no pre-treatment, and the Zn or Se pre-treated rat at various times after ^{109}Cd injection.

or liver. In 10 min, the level in the muscle of both species increased ($P < 0.05$, Text-fig. 5). The pre-treatment with Zn resulted in a reduced ($P < 0.05$) amount of ^{109}Cd at 10 min and this tendency continued at 40 min. Even though total ^{109}Cd was low in muscle, the nuclear fraction of this tissue generally accounted for more than 50% of the total ^{109}Cd therein (Text-fig. 5), while in the liver and testis, this fraction contained about 10 and 30%, respectively. With the domestic fowl, the ^{109}Cd level in this fraction rose ($P < 0.01$) up to

TEXT-FIG. 6. The percentage of the muscle ^{109}Cd in the heavy and light mitochondrial fractions, in the domestic fowl and rat receiving no pre-treatment, and the Zn or Se pre-treated rat at various times after ^{109}Cd injection.

10 min, then decreased ($P < 0.05$) by 20 min. A comparable pattern occurred in the rat but the decrease ($P < 0.05$) did not occur until after 20 min. In the light mitochondrial fraction (Text-fig. 6), ^{109}Cd levels in control rats increased with time. Pre-treatment with either Zn or Se tended to increase the level in this fraction at 10 min, whereas Se alone tended to reduce it at 40 min. As in the testis, the muscle mitochondrial fraction of the domestic fowl contained

TEXT-FIG. 7. The percentage of the ^{109}Cd in the muscle found in the two fractions of the 103,000 g centrifugation, in the domestic fowl and rat receiving no pre-treatment, and the Zn or Se pre-treated rat at various times after ^{109}Cd injection.

more ^{109}Cd than that of the rat. In the heavy mitochondrial fraction of the pre-treated rat, there was a marked decrease with time ($P < 0.01$) to zero. This was also true in the light mitochondrial fraction of the Se pre-treated rat.

In the microsomal fraction of both species (Text-fig. 7), the ^{109}Cd level tended to decrease and then to rise with time while, in the supernate, there were no changes with time, except for the Se pre-treatment group which rose ($P < 0.05$). The rat, however, had a higher percentage ($P < 0.05$) of the total in the supernate at all time periods than the domestic fowl. As seen in Text-fig. 8, there was almost no ^{109}Cd in the domestic fowl protein fraction of the 103,000 g supernate. At 10 min, pre-treatment reduced ($P < 0.01$) the ^{109}Cd level in the protein fraction and tended to increase it in the free-element fraction.

TEXT-FIG. 8. The portion of the 103,000 g supernate muscle ^{109}Cd as protein-bound and non-protein-bound or free Cd, in the domestic fowl and rat receiving no pre-treatment, and the Zn or Se pre-treated rat at various times after ^{109}Cd injection.

Liver

The percentages of the size-adjusted dose of $^{109}\text{Cd}/\text{g}$ of liver tissue (Text-fig. 9) was generally about 10,000-times that in the testis or muscle. The level increased stepwise with time in both species. At 40 min, the ^{109}Cd content in the pre-treatment rat liver was significantly ($P < 0.05$) reduced. Though the amount in the liver was very high, the proportion in the nuclear fraction (Text-fig. 9) was extremely low. The most evident trend in the latter fraction was a somewhat higher ^{109}Cd content in the domestic fowl. In the mitochondrial fractions (Text-fig. 10), there were no notable trends.

TEXT-FIG. 9. The uptake of the ^{109}Cd in the liver as the size-adjusted percentage of the dose, also the percentage of the liver ^{109}Cd in the nuclear fraction, in the domestic fowl and rat receiving no pre-treatment, and the Zn or Se pre-treated rat at various times after ^{109}Cd injection.

In the microsomal fraction (Text-fig. 11), there was a trend towards increasing ^{109}Cd levels with time in both species. The pre-treatment tended to cause a reduction ($P < 0.05$) at 40 min. The bulk of liver cell ^{109}Cd was in the 103,000 g supernate. When this fraction was further broken down (Text-fig. 12), it was evident that in the liver, the ^{109}Cd was protein bound. However, pre-treatment tended to produce a marked increase ($P < 0.01$) in the amount occurring as non-protein bound or free element.

TEXT-FIG. 10. The percentage of the liver ^{109}Cd in the mitochondrial fractions, in the domestic fowl and rat receiving no pre-treatment, and the Zn or Se pre-treated rat at various times after ^{109}Cd injection.

TEXT-FIG. 11. The percentage of the ^{109}Cd in the liver located in the microsomal or 103,000 g supernate fractions, in the domestic fowl and rat receiving no pre-treatment, and the Zn or Se pre-treated rat at various times after ^{109}Cd injection.

TEXT-FIG. 12. The portions of the liver ^{109}Cd of the 103,000 g supernate in the protein-bound or non-protein-bound (free element) fractions, in the domestic fowl and rat receiving no pre-treatment, and the Zn or Se pre-treated rat at various times after ^{109}Cd injection.

DISCUSSION

It is evident from the results of this study that ^{109}Cd enters the testis, muscle and liver at a very fast rate. The uptake pattern, following s.c. injection, in the domestic fowl testis, muscle and liver is very similar to that found in the rat in this study and to that previously reported in the rat by Johnson & Miller (1970). The relative distribution in these tissues in general is in harmony with previous work for later sampling times in the rat (Decker, Byerrum & Hoppert, 1957; Friberg & Odeblad, 1957), mouse (Cotzias, Borg & Selleck, 1961a; Berlin & Ullberg, 1963; Gunn, Gould & Anderson, 1968), dog (Walsh & Burch, 1959), goat (Miller, Blackmon & Martin, 1968) and calf (Powell, Miller, Morton & Clifton, 1964).

Liver and muscle tissues differ considerably from each other and from the testis in the subcellular distribution of ^{109}Cd . In the muscle, the majority of the ^{109}Cd occurred in the nuclear fraction while in the liver, only 10% occurred therein. In the testis, an intermediate level was evident. Part of this difference may have been due to differential homogenization and centrifugation resulting in slightly different cell fractions occurring. This may be particularly true of the nuclear fractions which included nuclei, cell walls and sperm heads or other intact cells. While the centrifugation procedure was developed to make this fraction as uniform as possible in all tissues, there were undoubtedly differences. Previous work (Cotzias, Borg & Selleck, 1961b), involving the subcellular localization of ^{109}Cd in the liver, in general, supports the distribution within the cell found in the present work.

The fractionation of the testicular components revealed that the bulk of the ^{109}Cd in the testis is contained in the nuclear fraction and 103,000 g supernate fraction. The ^{109}Cd in the domestic fowl testis tended to leave the organelle fractions with time and increase in the 103,000 g supernate, primarily in its protein fraction. By contrast, the ^{109}Cd in the rat testis tended to remain fairly constant in the organelles and decrease in the 103,000 g supernate. The amount of non-protein bound ^{109}Cd in the rat testis was far greater than in the domestic fowl.

The pre-treatment with Zn or Se reduced the proportion of the total dose in the testis and also was effective in keeping the percentage of the total in the nuclear fraction low. These pre-treatments resulted in increased ^{109}Cd in the 103,000 g supernate, primarily in the non-protein bound or free element fraction. Selenium and cysteine have been shown to increase the uptake of Cd (Gunn *et al.*, 1968), while Zn decreased Cd uptake by testes. At first (Text-fig. 1), Zn decreased Cd uptake and at 40 min, both Se and Zn had decreased testis uptake of ^{109}Cd . The decrease in ^{109}Cd with these treatments may be due to the early decreased blood flow after Zn pre-treatment which has been suggested previously (Johnson *et al.*, 1970).

The rat suffers testicular damage when injected with Cd. By contrast, testes of the domestic fowl are not damaged by Cd. The greatest differences in the subcellular localization of the ^{109}Cd in the two species seem to be in the decreasing trends in the organelles of the domestic fowl and the near absence of the non-protein bound ^{109}Cd in the domestic fowl. In contrast to these

differences between the rat and domestic fowl, the directions of change in the Zn and Se pre-treated rat are almost the reverse of those in the domestic fowl. It appears that if the protective mechanisms against Cd damage are within the testis, then the domestic fowl and the Zn or Se pre-treated rats have very different mechanisms of defence.

All animals, regardless of species or pre-treatment, had ^{109}Cd in the protein fraction. Whether these are specific enzymes to which Cd is bound or other proteins, cannot be determined from the present study. Simon, Potts & Gerard (1947) suggested that Cd reacts with tissue proteins and causes alterations in protein structure or a decreased solubility sufficient to impair enzyme activity and thus impair cellular function. These workers pre-treated with various thiols, which have been shown to prevent the damaging effects of Cd (Gunn, Gould & Anderson, 1966), and found that Cd and the $-\text{SH}$ groups combined.

Pre-treatment of the rat and the resultant decreases in the ^{109}Cd present in the nuclear fraction and the increases in the protein fraction suggest that the pre-treatment may alter the proteins in the cell or their binding sites and thus increase the protective Cd-protein combinations. Se, being an anti-oxidant may actually act to increase the $-\text{SH}$ groups for binding Cd, while Zn may prevent Cd from entering sites that would be damaging within the cell. Thus, Zn and Se may act within the testis to prevent damaging Cd-protein combinations as suggested by Pařizek (1960). This is further supported by the work which showed that Cd inhibits testicular carbonic anhydrase, a Zn-containing enzyme (Hodgen *et al.*, 1969). By contrast, the domestic fowl testis may be protected by anatomical differences such as the absence of the pampiniform plexus. If this is the case, the relative subcellular location of Cd in the pre-treated rat and the domestic fowl may be of little significance. However, as yet, it is not known whether the Cd is in the same types of cells in the testes of both the rat and domestic fowl. This could be examined using autoradiography.

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REFERENCES

- BERLIN, M. & ULLBERG, S. (1963) The fate of ^{109}Cd in the mouse. *Archs envir. Hth*, **7**, 686.
- COTZIAS, G. C., BORG, D. C. & SELLECK, B. (1961a) Specificity of zinc pathway in the rabbit: zinc-cadmium exchange. *Am. J. Physiol.* **201**, 63.
- COTZIAS, G. C., BORG, D. C. & SELLECK, B. (1961b) Virtual absence of turnover in cadmium metabolism: Cd^{109} studies in the mouse. *Am. J. Physiol.* **201**, 927.
- DECKER, C. F., BYERRUM, R. U. & HOPPERT, C. A. (1957) A study of the distribution and retention of cadmium-115 in the albino rat. *Archs Biochem. Biophys.* **66**, 140.
- ERICKSON, A. E. & PINCUS, G. (1964) Insensitivity of fowl testes to cadmium. *J. Reprod. Fert.* **7**, 379.
- FRIBERG, L. & ODEBLAD, E. (1957) Localization of Cd^{115} in different organs. *Acta path. microbiol. scand.* **41**, 96.
- GOSWAMI, A., SKIPPER, S. K. & WILLIAMS, W. L. (1968) Stimulation of fatty acid synthesis *in vitro* by gonadotrophin-induced testicular ribonucleic acid. *Biochem. J.* **67**, 513.
- GUNN, S. A. & GOULD, T. C. (1970) *Cadmium and other mineral elements*. In: *The Testis*. 3. Influencing Factors. 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. (1966) Protective effect of thiol compounds against cadmium-induced vascular damage of testis. *Proc. Soc. exp. Biol. Med.* **122**, 1036.
- GUNN, S. A., GOULD, T. C. & ANDERSON, W. A. D. (1968) Mechanisms of zinc, cysteine and selenium protection against cadmium-induced vascular injury to mouse testis. *J. Reprod. Fert.* **15**, 65.
- GUTHRIE, J. (1964) Histological effects of intra-testicular injections of cadmium chloride in domestic fowl. *Br. J. Cancer*, **18**, 255.
- HODGEN, G. D., BUTLER, W. R. & GOMES, W. R. (1969) *In vivo* and *in vitro* effects of cadmium chloride on carbonic anhydrase activity. *J. Reprod. Fert.* **18**, 156.
- JOHNSON, A. D., GOMES, W. R. & VANDEMARK, N. L. (1970) Early actions of cadmium in the rat and domestic fowl testis. I. Testis and body temperature changes caused by cadmium and zinc. *J. Reprod. Fert.* **21**, 383.
- JOHNSON, A. D. & MILLER, W. J. (1970) Early actions of cadmium in the rat and domestic fowl testis. II. Distribution of injected ^{109}Cd . *J. Reprod. Fert.* **21**, 395.
- MILLER, W. J., BLACKMON, D. M. & MARTIN, Y. G. (1968) Cadmium absorption, excretion, and tissue distribution following single tracer oral and intravenous doses in young goats. *J. Dairy Sci.* **51**, 1836.
- PAŘÍZEK, J. (1960) Sterilization of the male by cadmium salts. *J. Reprod. Fert.* **1**, 294.
- PEKAS, J. C. (1966) Zinc 65 metabolism: gastrointestinal secretion by the pig. *Am. J. Physiol.* **211**, 330.
- POWELL, G. W., MILLER, W. J., MORTON, J. D. & CLIFTON, C. M. (1964) Influence of dietary cadmium level and supplemental zinc on cadmium toxicity in the bovine. *J. Nutr.* **84**, 205.
- SCHNEIDER, W. & HOGEBOOM, G. H. (1950) Intracellular distribution of enzymes; V. Further studies on the distribution of cytochrome c in rat liver homogenates. *J. biol. Chem.* **183**, 123.
- SIMON, F. P., POTTS, A. M. & GERARD, R. W. (1947) Action of cadmium and thiols on tissues and enzymes. *Archs Biochem.* **12**, 283.
- STEEL, R. G. D. & TORRIE, J. H. (1960) *Principles and procedures of statistics*. McGraw-Hill, New York.
- UMBREIT, W. W., BURRIS, R. H. & STAUFFER, J. F. (1951) *Manometric techniques and tissue metabolism*, pp. 148–155. Burgess, Minneapolis, Minn.
- WALSH, J. J. & BURCH, G. E. (1959) The rate of disappearance from plasma and subsequent distribution of radiocadmium ($\text{Cd}^{115\text{m}}$) in normal dogs. *J. Lab. clin. Med.* **54**, 59.