Zinc Deficiency in Adult Rats Reduces the Relative Abundance of Testis-Specific Angiotensin-Converting Enzyme mRNA$^{1,2,3}$

Lana Stallard and Philip G. Reeves$^4$

Agricultural Research Service, United States Department of Agriculture, Grand Forks Human Nutrition Research Center, Grand Forks, ND 58202-9034

ABSTRACT Zinc deficiency results in reduced testicular angiotensin-converting enzyme (ACE) activity and reduced amounts of ACE protein in the testes of young rats. In the present study, we examined the effect of zinc deficiency on the relative abundance of testicular ACE mRNA and its relationship to ACE activity over time. Forty-five male rats at 7 wk of age were placed on one of three feeding regimens: 1) a diet adequate in zinc, 2) a diet deficient in zinc and 3) a diet adequate in zinc that was fed in an amount equal to that consumed by a paired mate fed the zinc-deficient diet. Rats were killed after 3, 5 and 7 wk. Rats fed the zinc-deficient diet had significantly lower ($P < 0.01$) body weight gain and testis weight at each week sampled than the other groups. They also showed compromised zinc status as evidenced by significantly lower ($P < 0.01$) serum and testis zinc concentrations. At each period, rats fed the zinc-deficient diet had significantly lower ($P < 0.01$) testicular ACE activity than rats fed either of the zinc-adequate diets. Coinciding with low ACE activity, there was a lower ($P < 0.01$) relative abundance of ACE mRNA in the group for the zinc-deficient diet than in either of the zinc-adequate groups. The results suggest that much of the low ACE activity in the testis of rats in the latter stages of zinc deficiency is attributable to a reduction in ACE gene transcription. However, an effect of the deficiency on ACE mRNA turnover is not ruled out. J. Nutr. 127: 25–29, 1997.

KEY WORDS: zinc • angiotensin-converting enzyme • testes • mRNA • rats

Zinc deficiency in males of many species, including humans, causes an impairment of sexual development (Millar et al. 1958 and 1960, Sandstead et al. 1967). Previously, this impairment was shown to manifest itself primarily in young animals and consisted of hypogonadism that resulted in arrested development of secondary sex characteristics (Mason et al. 1982). More recent investigations found that adult animals are affected similarly to young animals in that germ cell maturation is suppressed and the testes begin to atrophy when the animals are fed zinc-deficient diets for up to 5 wk (Reeves and Stallard 1995). The exact role that zinc plays in male sexual maturation and fertility is unknown, but it may be related partly to the enzymes that require zinc for full activity. A zinc-metalloenzyme that is closely related to maturation of the testes, and to its function remains unresolved.

ABSTRACT Zinc deficiency results in reduced testicular angiotensin-converting enzyme (ACE) activity and reduced amounts of ACE protein in the testes of young rats. In the present study, we examined the effect of zinc deficiency on the relative abundance of testicular ACE mRNA and its relationship to ACE activity over time. Forty-five male rats at 7 wk of age were placed on one of three feeding regimens: 1) a diet adequate in zinc, 2) a diet deficient in zinc and 3) a diet adequate in zinc that was fed in an amount equal to that consumed by a paired mate fed the zinc-deficient diet. Rats were killed after 3, 5 and 7 wk. Rats fed the zinc-deficient diet had significantly lower ($P < 0.01$) body weight gain and testis weight at each week sampled than the other groups. They also showed compromised zinc status as evidenced by significantly lower ($P < 0.01$) serum and testis zinc concentrations. At each period, rats fed the zinc-deficient diet had significantly lower ($P < 0.01$) testicular ACE activity than rats fed either of the zinc-adequate diets. Coinciding with low ACE activity, there was a lower ($P < 0.01$) relative abundance of ACE mRNA in the group for the zinc-deficient diet than in either of the zinc-adequate groups. The results suggest that much of the low ACE activity in the testis of rats in the latter stages of zinc deficiency is attributable to a reduction in ACE gene transcription. However, an effect of the deficiency on ACE mRNA turnover is not ruled out. J. Nutr. 127: 25–29, 1997.

KEY WORDS: zinc • angiotensin-converting enzyme • testes • mRNA • rats

Zinc deficiency in males of many species, including humans, causes an impairment of sexual development (Millar et al. 1958 and 1960, Sandstead et al. 1967). Previously, this impairment was shown to manifest itself primarily in young animals and consisted of hypogonadism that resulted in arrested development of secondary sex characteristics (Mason et al. 1982). More recent investigations found that adult animals are affected similarly to young animals in that germ cell maturation is suppressed and the testes begin to atrophy when the animals are fed zinc-deficient diets for up to 5 wk (Reeves and Stallard 1995). The exact role that zinc plays in male sexual maturation and fertility is unknown, but it may be related partly to the enzymes that require zinc for full activity. A zinc-metalloenzyme that is closely related to maturation of the testes, and to its function remains unresolved.

MATERIALS AND METHODS

The Animal Use Committee of the USDA, ARS, Grand Forks Human Nutrition Research Center approved this study, and the study was performed according to the guidelines of the National Institutes of Health on the experimental use of laboratory animals (NRC 1985).


$^2$ Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

$^3$ The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

$^4$ To whom correspondence should be addressed.

$^5$ Abbreviations used: ACE, angiotensin-converting enzyme; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SDS, sodium dodecyl sulfate; SSC, sodium chloride-sodium citrate; +Zn, zinc-adequate diet; −Zn, zinc-deficient diet; +ZnPF, zinc-adequate diet paired fed.

0222-3166/97 $3.00 © 1997 American Society for Nutritional Sciences.

Animals and diet. At wk 7 of age, 45 male Sprague-Dawley rats (Sasco, Madison, WI) were divided into three groups of 15 rats each. One group was fed a basal diet similar to AIN-93G (Reeves et al. 1993) but with the following changes: egg white was substituted for casein, extra biotin (2 mg/kg diet) was added, the mineral mix did not contain a source of zinc, and the amount of phosphorus was increased so that the finished diet provided 3 g phosphorus/kg diet when the mineral mix was used instead of bovine serum albumin (fraction V)/L. The 20 g of polyvinylpyrrolidone and 10 g of bovine serum albumin (fraction V)/L. The 20x SSC was supplemented with 3 mol NaCl and 0.3 mol sodium citrate/L.

Total RNA isolation. All glassware and instruments were properly cleaned and sterilized. Two hundred and twenty-five milligrams of liver was homogenized in a mixture containing 1 mol NH₄OH and 20 g of Triton X-100/L. The 100% ethanol. The tube was set aside at -20°C until used for RNA isolation. Livers were removed, blotted and stored at -20°C until analyzed for zinc content. Testes were removed and immediately frozen in liquid nitrogen and then stored at -80°C until analyzed for RNA isolation. Livers were removed, blotted and stored at -20°C until analyzed for zinc content.

Chemicals and reagents. Unless otherwise noted, chemicals were purchased from Sigma Chemical (St. Louis, MO). The 50x Denhardt's reagent contained 10 g of Ficoll (type 400, Pharmacia Biotech, Uppsala, Sweden), 10 g of polyvinylpyrrolidone, and 10 g of bovine serum albumin (fraction V)/L. The 20x SSC contained 3 mol NaCl and 0.3 mol sodium citrate/L.

Total RNA isolation. All glassware and instruments were properly cleaned and sterilized. Two hundred and twenty-five milligrams of liver was homogenized in a mixture containing 1 mol NH₄OH and 20 g of Triton X-100/L. The 100% ethanol. The tube was set aside at -20°C until used for RNA isolation. Livers were removed, blotted and stored at -20°C until analyzed for zinc content. Testes were removed and immediately frozen in liquid nitrogen and then stored at -80°C until analyzed for RNA isolation. Livers were removed, blotted and stored at -20°C until analyzed for zinc content.

Northern analysis of RNA. The isolated testes RNA (10 μg) was denatured and separated on a 1% agarose gel containing 1.0 mL of buffer (6 mol guanidinium thiocyanate, 40 mmol sodium citrate and 90 mmol 2-mercaptoethanol/L). The homogenate was mixed with 100 μL of 2 mol sodium acetate/L and 1.2 mL of phenol-chloroform (5:1 ratio), pH 4.7. The mixture was placed on ice for 15 min and then centrifuged at 10,000 g for 20 min at 4°C. The upper aqueous phase was mixed with an equal volume of cold isopropanol alcohol and kept at -20°C for at least 1 h. After centrifugation at 10,000 g for 30 min, the pellet was dried, resuspended in 300 μL of TE buffer (10 mmol Tris-HCl and 1 mmol EDTA/L, pH 8). Then 300 μL of phenol-chloroform (5:1), pH 4.7, was added and the mixture centrifuged at 10,000 g for 20 min. The upper aqueous phase was transferred to a fresh tube and 45 μL of 2 mol sodium acetate/L was added followed by 700 μL of cold 100% ethanol. The tube was set aside at -20°C overnight before centrifugation at 13,000 g for 30 min. The supernatant was decanted and the pellet washed with 80% ethanol. The pellet was suspended in 70% ethanol, and RNA was quantitated spectrophotometrically. Both quantity and quality of the RNA sample were verified by gel electrophoresis and staining with ethidium bromide. The RNA samples were stored at -80°C.

RESULTS

At each period, rats in the -Zn group had significantly lower weight gain than rats in either of the zinc-adequate groups (Table 1). Daily gain in body weight was also less (P < 0.01) in the +ZnPF group than in the +Zn group. In rats allowed to eat naturally (groups -Zn and +Zn), the amount gained per day declined between wk 3 and 5 and then stabilized between wk 5 and 7. At wk 5 and 7, rats fed the zinc-deficient diet had significantly lower (P < 0.001) testes weights than either of the zinc-adequate groups (Table 1); however, testes weights were not different between groups fed either of the zinc-adequate diets at any period. Testes weights:body weight ratios showed that the +Zn and -Zn groups were not different, but each was significantly different (P < 0.001) from +ZnPF at wk 5 and 7 only.

Indicators of zinc status were lowered in rats fed the -Zn diet. As early as wk 3, serum zinc concentrations were signifi-
Zinc deficiency affects the body weight gain, testis weight and testis weight:body weight ratio of rats.\(^1,2\)

<table>
<thead>
<tr>
<th></th>
<th>Body weight gain</th>
<th>Testis weight</th>
<th>Testis weight:body weight ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g/d</td>
<td>g</td>
<td>g/100 g body wt</td>
</tr>
<tr>
<td>Week 3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+Zn</td>
<td>6.7 ± 0.3(a)</td>
<td>1.65 ± 0.04</td>
<td>0.543 ± 0.02</td>
</tr>
<tr>
<td>+ZnPF</td>
<td>2.6 ± 0.3(b)</td>
<td>1.55 ± 0.05</td>
<td>0.713 ± 0.03</td>
</tr>
<tr>
<td>-Zn</td>
<td>1.5 ± 0.4(c)</td>
<td>1.38 ± 0.14</td>
<td>0.709 ± 0.08</td>
</tr>
<tr>
<td>Week 5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+Zn</td>
<td>5.5 ± 0.4(a)</td>
<td>1.66 ± 0.16(a)</td>
<td>0.470 ± 0.05(b)</td>
</tr>
<tr>
<td>+ZnPF</td>
<td>1.6 ± 0.2(b)</td>
<td>1.39 ± 0.05(a)</td>
<td>0.630 ± 0.03(a)</td>
</tr>
<tr>
<td>-Zn</td>
<td>0.8 ± 0.1(c)</td>
<td>0.72 ± 0.06(b)</td>
<td>0.380 ± 0.03(b)</td>
</tr>
<tr>
<td>Week 7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+Zn</td>
<td>4.8 ± 0.1(a)</td>
<td>1.83 ± 0.12(a)</td>
<td>0.451 ± 0.02(b)</td>
</tr>
<tr>
<td>+ZnPF</td>
<td>1.8 ± 0.1(b)</td>
<td>1.66 ± 0.06(a)</td>
<td>0.669 ± 0.02(a)</td>
</tr>
<tr>
<td>-Zn</td>
<td>0.6 ± 0.1(c)</td>
<td>0.75 ± 0.16(b)</td>
<td>0.402 ± 0.08(b)</td>
</tr>
</tbody>
</table>

\(^1\) Values are means ± SEM of five rats. Different superscripts within weeks for each variable denote significant differences among treatments, with \(P < 0.001\) for body weight and testis weight and \(P < 0.01\) for testis:body weight ratio.

\(^2\) The +Zn rats were allowed to eat the control diet naturally; +ZnPF rats were only allowed to eat as much food as their paired mates in group -Zn; -Zn rats were allowed to eat the low zinc diet naturally.

FIGURE 1 Zinc deficiency affects testicular angiotensin-converting enzyme (ACE) activity in adult rats. Rats were placed on one of three feeding regimens: 1) a diet adequate in zinc (+Zn), 2) a diet deficient in zinc (-Zn), and 3) a diet adequate in zinc that was paired-fed in an amount equal to that consumed by individuals fed the -Zn diet (+ZnPF). At 3, 5, and 7 wk, testicular ACE activity was determined fluorometrically by using hippuryl-histidyl-leucine as the substrate. Bars represent the means ± SEM of five rats. Different letters within weeks denote significant differences by the Kruskal-Wallis rank means test \(P < 0.05\) for wk 3 and \(P < 0.01\) for wk 5 and 7. The rank means test showed that values for +Zn significantly \((P < 0.004)\) increased with time. There was no change in values for +ZnPF with time, and -Zn decreased \((P < 0.002)\).

TABLE 1

<table>
<thead>
<tr>
<th></th>
<th>Serum zinc</th>
<th>Liver zinc</th>
<th>Testis zinc</th>
<th>Testis DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\mu)mol/L</td>
<td>(\mu)mol/kg</td>
<td>mg/kg</td>
<td></td>
</tr>
<tr>
<td>Week 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+Zn</td>
<td>23.1 ± 0.7(a)</td>
<td>380 ± 10</td>
<td>347 ± 6(a)</td>
<td>6.2 ± 0.8</td>
</tr>
<tr>
<td>+ZnPF</td>
<td>18.2 ± 0.9(b)</td>
<td>400 ± 20</td>
<td>335 ± 17(a)</td>
<td>6.0 ± 0.7</td>
</tr>
<tr>
<td>-Zn</td>
<td>4.9 ± 0.5(c)</td>
<td>360 ± 19</td>
<td>231 ± 32(b)</td>
<td>6.1 ± 0.6</td>
</tr>
<tr>
<td>Week 5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+Zn</td>
<td>22.3 ± 1.7(a)</td>
<td>351 ± 18(b)</td>
<td>346 ± 11(a)</td>
<td>4.9 ± 0.6</td>
</tr>
<tr>
<td>+ZnPF</td>
<td>14.1 ± 0.6(b)</td>
<td>435 ± 9(a)</td>
<td>370 ± 9(a)</td>
<td>4.3 ± 0.7</td>
</tr>
<tr>
<td>-Zn</td>
<td>4.4 ± 0.7(c)</td>
<td>330 ± 16(b)</td>
<td>250 ± 27(b)</td>
<td>4.8 ± 0.7</td>
</tr>
<tr>
<td>Week 7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+Zn</td>
<td>21.4 ± 0.9(a)</td>
<td>386 ± 16(b)</td>
<td>344 ± 8(a)</td>
<td>4.9 ± 0.9</td>
</tr>
<tr>
<td>+ZnPF</td>
<td>16.5 ± 0.4(b)</td>
<td>440 ± 11(a)</td>
<td>340 ± 14(a)</td>
<td>4.9 ± 0.8</td>
</tr>
<tr>
<td>-Zn</td>
<td>5.3 ± 0.4(c)</td>
<td>347 ± 10(c)</td>
<td>205 ± 27(b)</td>
<td>3.6 ± 0.5</td>
</tr>
</tbody>
</table>

\(^1\) Values are means ± SEM of five rats. Different superscripts within weeks for each variable denote significant differences among treatments with \(P < 0.01\). Although there was no significant difference among treatments for testis DNA, there was a significant difference \((P < 0.02)\) among treatment weeks where wk 3 values were higher than values at wk 5 or wk 7.

\(^2\) The +Zn rats were allowed to eat the control diet naturally; +ZnPF rats were only allowed to eat as much food as their paired mates in group -Zn; -Zn rats were allowed to eat the low zinc diet naturally.

TABLE 2

Zinc depletion affects the concentration of zinc in serum, liver, and testis of rats without affecting the amount of testicular DNA.\(^1,2\)

**Zinc Depletion and Testis ACE mRNA**

Zinc concentration was somewhat refractory to low zinc diets. The +Zn and -Zn groups were significantly different \((P < 0.01)\) only after the rats had consumed the diets for 7 wk.

Similar to serum zinc concentrations, testis zinc also was significantly lower \((P < 0.01)\) at each week in rats fed the zinc-deficient diet, compared with each of the zinc-adequate groups (Table 2). Testis zinc was not different between rats in either of the groups fed the zinc-adequate diet.

Dietary zinc did not affect \((P > 0.05)\) the amount of DNA in the testes among groups at any of the sampling periods (Table 2). The amount of DNA significantly \((P < 0.05)\) declined over time, however, and was approximately 25% lower at 5 and 7 wk than at 3 wk.

By the Kruskal-Wallis rank means test, ACE activity at wk 3 was significantly lower \((P < 0.01)\) in the -Zn group than in the +ZnPF group (Fig. 1). The mean of the -Zn group was about 25% lower than that of the +Zn group, but because of the large variation they were not significantly different. Values for two out of five samples in the -Zn group were abnormally low. However, by wk 5 and 7, ACE activity was only 5% \((P < 0.001)\) of that in either zinc-adequate group. The Kruskal-Wallis rank means test showed that values for the +Zn group significantly \((P < 0.004)\) increased with time. There were no changes in ACE activity for the +ZnPF group with time, and those of the -Zn group decreased \((P < 0.002)\).

Autoradiographs prepared for wk 5 and 7 showed reduced intensities in the ACE mRNA bands for the -Zn group compared with either of the zinc-adequate groups. The ACE bands...
produced from the exons identical to those used to assemble the carboxyl terminal half of somatic ACE.

Previously we showed that zinc deficiency in rats before puberty caused a reduction in the activity of testicular ACE (Reeves and O’Dell 1988). Although we also demonstrated that zinc deficiency reduced the activity of somatic ACE in serum (Reeves and O’Dell 1985), it seemed to depend on the type of substrate used in the assay, and it required a sufficient supply of zinc in the assay medium. In addition, we could not find a negative effect of the deficiency on lung ACE (Reeves and O’Dell 1988), which seemed to suggest that rat testicular ACE was unique in its response to zinc deficiency. However, we have since shown that ACE activity in the intestinal mucosa of zinc-deficient young male rats is about 40% less than that in zinc-adequate rats (not published), suggesting that indeed somatic ACE can be affected by zinc deficiency.

Because testicular ACE begins to be expressed only at puberty (Strittmatter and Snyder 1984, Strittmatter et al. 1985), we initially thought that the absence of a sufficient supply of dietary zinc during this period was inhibiting the general development of the testes. However, more recently we showed that zinc deficiency also causes a reduction in testicular ACE activity in adult rats (Reeves and Stallard 1995). In addition, we demonstrated that the reduction in activity was apparently caused in part by a reduction in ACE mRNA in the testes of zinc-deficient rats (Reeves et al. 1995). This observation led us to the present study in which the effect of zinc deficiency on the relative abundance of testes-

FIGURE 2 Northern analysis of angiotensin-converting enzyme (ACE) mRNA from the testes of zinc-adequate (+Zn), paired fed (+ZnPF) and zinc-deficient (-Zn) adult rats. At 3, 5, and 7 wk, 10 μg of testicular RNA was isolated and separated on denaturing gels (one for each week sampled). RNA was transferred to nylon membranes and hybridized with two [α-32P]dCTP-labeled probes, ACE.5 and glutaraldehyde-3-phosphate dehydrogenase. Only examples from wk 5 are shown in this autoradiograph. Testicular ACE mRNA gave a signal at 2.7 kb and the control probe at 1.4 kb. Although all lanes gave near equal intensity for the control probe, the intensity of the ACE.5 probe in lanes containing RNA from -Zn rats was barely detectable.

in four out of five samples were not detectable at wk 5, and three out of five were not detectable at wk 7. At wk 3, ACE bands in all samples were detectable. At each week, the intensity of the control probe was approximately the same in all groups. A representative sample of an autoradiograph from wk 5 is shown in Figure 2.

A statistical analysis of the ACE:GAPDH mRNA ratios was difficult to perform because many of the values in the -Zn group for wk 5 and 7 were zero. However, because of the large variation, we performed the Kruskal-Wallis rank means test, analyzing each week separately. The ACE:GAPDH mRNA ratios during all three periods were significantly (P < 0.01) lower in the zinc-deficient rats than in either of the control groups (Fig. 3). Ratios for the +Zn and +ZnPF groups were not different. Because of the nature of the assay, values between weeks could not be compared.

DISCUSSION

Angiotensin-converting enzyme is a zinc-metalloenzyme that consists of two isozymes. One is the somatic type and is found in vascular endothelium and kidney. This isozyme is thought to function primarily in the regulation of blood pressure by cleaving angiotensin I to form the vasoconstrictor, angiotensin II (Erdos and Skidgel 1987). It also cleaves the vasodilator bradykinin. This isozyme has a molecular mass of approximately 160 kDa and contains 2 mol of zinc per mole of enzyme protein. The other isozyme is found only in the testes, has a molecular mass of approximately 95 kDa, and contains only 1 mol of zinc per mole of enzyme (Ehlers and Riordan 1991). The testicular ACE proteins in mice (Bernstein et al. 1989), humans (Ehlers et al. 1989) and (presumably) rats are unique in that they are
specific ACE mRNA was examined. The results clearly show that zinc deficiency in adult male rats will eventually lead to a reduction in ACE mRNA. In turn, this apparently leads to lower ACE protein, which then causes the reduced activity of ACE as observed in zinc-deficient rats. We are interpreting our results to suggest that ACE mRNA transcription is reduced by zinc deficiency. Because we did not measure mRNA turnover, however, the possibility of increased ACE mRNA degradation by zinc deficiency is not ruled out.

Whether reduced germinal cell maturation during zinc deficiency is directly related to the maintenance of ACE protein and enzyme activity or whether the deficiency is causing a general effect on spermatid maturation is still to be determined. We previously showed that the numbers of germ cells and sperm are reduced in the testes of zinc-deficient rats, but those cells that remain have lower ACE activity than cells from zinc-adequate rats (Reeves and Stallard 1995). Sibony et al. (1994) determined the step-wise expression of germinal ACE mRNA and ACE protein in testes of normal rats and mice as the spermatids matured through various stages. By using in situ hybridization with a testis-specific ACE cDNA probe, they found that ACE mRNA was first expressed in stages four to seven, with maximal expression at stages eight to 12. Expression declined through the remaining stages. Immunolocalization of ACE protein showed a similar progression; however, ACE protein remained elevated throughout the latter stages of maturation. Sibony et al. (1994) concluded that ACE is produced exclusively in haploid germ cells of these species. Because zinc is involved in so many aspects of metabolism, it is possible that spermatid development is arrested in the early stages of zinc deficiency and the spermatids never reach the stage where ACE mRNA is normally expressed.

ACKNOWLEDGMENTS

The authors thank Brenda Skinner for technical assistance, Jim Lindlauf and Karin Tweton for mixing the animal diets, and Denice Schaefer and her staff for care of the animals. We also thank Barry Milavetz, Lauf and Karin Tweton for mixing the animal diets, and Denice Schafer and her staff for care of the animals. We also thank Barry Milavetz for advice concerning RNA isolation and Cold Spring Harbor, NY.

Department of Biochemistry and Molecular Biology, University of North Dakota, Grand Forks, ND, for advice concerning RNA isolation and Northern blotting.

LITERATURE CITED


National Research Council (1985) Guide for the Care and Use of Laboratory Animals. Publication no. 89–23 (rev), National Institutes of Health, Bethesda, MD.


