The Rat Electroretinogram in Combined Zinc and Vitamin A Deficiency

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To assess the relative importance and interdependence of zinc and vitamin A in retinal function, a group of 36 male Wistar rats were raised in dim illumination and deprived of both zinc and vitamin A (Z—A—) for 90 days (Phase I) until they lost weight and showed signs of both vitamin A and zinc deficiency. Scotopic electroretinograms (ERGs) showed an average 1.5 log unit elevation in light intensity needed to elicit a 200 nV b-wave (criterion intensity) as compared to a control-pair-fed group of rats. Tissue samples from the Z—A— rats showed undetectable liver vitamin A (<10 μg/g) and a significantly decreased level of femur zinc compared to the control group (P = 0.02). There was photoreceptor outer segment degeneration and loss of cells in the outer nuclear layer of the retina. For Phase II the 24 surviving rats were randomized into three treatment groups—one group remained vitamin A-deficient (A—) but received zinc and retinoic acid, the second remained zinc-deficient (Z—) but received vitamin A and retinoic acid, and the third group was repleted with both zinc and vitamin A (Z+A+) and also received retinoic acid. ERGs were performed 30 and 60 days after randomization. The criterion intensity of the Z+A+ and Z— groups approached normal by 60 days, while that of the A— rats deteriorated a further 1.0 log unit. Histologic studies of the A— rats showed abnormalities in cornea, photoreceptor outer segments, and gut mucosa. All animals had testicular atrophy. Vitamin A repletion significantly reverses retinal degeneration from the Z—A— state even in the presence of moderate zinc deficiency. Zinc supplementation alone does not prevent ongoing retinal degeneration from severe vitamin A deficiency. Invest Ophthalmol Vis Sci 28:975-984, 1987

In recent years increasing attention has been focused upon the roles of trace metals in human and animal physiology and upon the clinical and pathological consequences of deficiencies of these nutrients. There has been a growing interest in the role of zinc in retinal physiology, especially through its effects on vitamin A metabolism.

In humans, vitamin A deficiency causes abnormalities in dark adaptation and the scotopic electroretinogram (ERG), while vitamin A deficiency causes elevated ERG threshold in the rat. Clinical studies have shown that patients with low plasma zinc levels due to cirrhosis, Crohn’s disease, and chronic pancreatitis can have raised dark adaptation thresholds in the presence of normal plasma vitamin A levels. Supplementing these patients with zinc alone causes their thresholds to return to normal. Parker et al showed that rats reared on a zinc-deficient diet from birth developed elevated scotopic ERG thresholds.

Zinc deficiency may affect retinal function through two possible routes: local and systemic (Fig. 1). Reduced zinc levels can affect the enzyme retinal alcohol dehydrogenase, which catalyses a vital step in the rhodopsin pathway. Zinc deficiency can also lead to abnormalities in rat retina cytoarchitecture concurrent with marked reduction in zinc levels in the uveal tract and retina.

A second mechanism by which zinc can affect retinal integrity is through its role in vitamin A mobilization. Zinc is involved in the release of vitamin A from the liver, either by influencing the synthesis of its carrier protein, retinal binding protein (RBP), or the release of the RBP-vitamin A complex from the liver. This study examined the interdependence between vitamin A and zinc in their effect on retinal function. This involved a controlled prospective study in the rat with the use of the scotopic ERG, supplemented by histologic and biochemical data.
Materials and Methods

Male Wistar (pink-eyed) rats aged 21 days were housed in stainless steel cages under 12 hr light (fluorescent light less than 10 ft-candles)-12 hr dark cycles. All equipment used was either stainless steel, plastic or glassware that had been soaked in 5 M hydrochloric acid followed by thorough washing with double-distilled water. All diets were obtained from Teklad Mills (Madison, WI). All procedures involving these rats were performed in compliance with the ARVO Resolution on the Use of Animals in Research.

The 154-day study consisted of two phases (Fig. 2). In Phase I ("depletion phase"; days 0 to 89) 36 rats were fed a Z-A diet (Teklad diet #77403). The drinking water consisted of double-distilled water containing from 5 to 10 parts of zinc (as zinc acetate) per million double-distilled water. These levels produce a moderate zinc deficiency state and prevent fatal severe zinc deficiency in rats.7-26 After 83 days on the diet these rats were supplemented daily with water by oral gastric intubation. To prevent death from severe vitamin A deficiency at this time, they also received a daily intraperitoneal injection of 300 μg retinoic acid dissolved in a 1:4 (v/v) mixture of ethanol and corn oil.27-31 Before the retinoic acid was dissolved, the corn oil was treated for 1 hr with ultraviolet light in order to inactivate vitamin A.26 A control group of rats was pair-fed a diet supplemented with both vitamin A and zinc (Teklad diet #170996).

In Phase II ("repletion phase"; days 90 to 154) the 24 rats surviving from Phase I were computer randomized into 3 equal groups (Fig. 2). Group A— was supplied with a vitamin A-deficient, zinc-supplemented diet (Teklad diet #68436). Group Z— was supplied with a zinc-deficient vitamin A-supplemented diet (Teklad diet #170995) and Group Z+A+ was provided with a diet containing both vitamin A and zinc (Teklad diet #170996).

The water supply of the Z— group was supplemented initially with 5 ppm zinc and then altered to double-distilled water at day 105 to maintain a moderate zinc deficiency state. The A— and Z+A+ groups were provided with 30 ppm of zinc in the water to replenish zinc stores to normal levels.7,14,22,25 In order to sustain growth in the A— rats 1 mg of retinoic acid was added to each food cup of this group of rats three times weekly, and all other rats had similar retinoic acid supplementation.27-31

Rats were weighed at weekly intervals and they were observed for signs of vitamin A and zinc deficiencies.7-26,29,30,32-35 Due to severe double deficiency several rats died between days 90 and 119, leaving five, six, and five rats respectively in groups A—, Z—, and Z+A+ (Fig. 2). Four rats developed corneal scarring and cataracts from ERG needle electrodes which precluded further ERGs in these animals.

Measurement of Electroretinograms (ERGs)

Scotopic ERGs were measured on a randomly selected group of rats at regular intervals during the course of the two phases of the experiment. Prior to the ERGs, rats were dark-adapted for a minimum period of 12 hr. The rats were anesthetized by an intraperitoneal injection of a mixture of Ketamine (4 mg/100 g weight) and Nembutal (2.5 mg/100 g weight), and a subcutaneous injection of Atropine (0.05 mg/100 g weight). The average total amount of zinc injected with these drugs was 0.35 μg.

One drop each of Proparacaine 0.5% (Ophthetic®), 10% Phenylephrine (Neoeynephrine®) and 1% Tropicamide (Mydriacyl®) were instilled into the eyes to be tested. A standard EEG needle electrode was placed
into the anterior chamber of the eye and a reference electrode was placed subcutaneously into the skin of the head near the upper eyelid to record the ERG. The eye was subjected to single stimulus flashes of increasing intensity in half-log unit steps until a linear log V/log I function could be plotted over a range of 2.0 or 2.5 log units. For healthy animals the initial stimulus intensity was 7 log units below full flash intensity (4.5 watt-seconds). The duration of each flash was 20 microseconds and the interval between light flashes was 10 to 15 sec. The ERG was displayed on an oscilloscope and recorded on magnetic tape.

The amplitude of the b-wave was taken from the baseline to the peak of the b-wave, as there was no a-wave recordable at the low stimulus intensities used. The logarithm of the b-wave amplitude in microvolts (log V) was plotted against the log of the stimulus intensity (log I). An example for a single normal rat is shown in Figure 3. A linear fit at low intensities was obtained by the method of least squares, using a computer. An average single linear function for the ERGs for a group of rats was obtained with confidence limits.

We arbitrarily selected the 200 $\mu$V amplitude as criterion voltage. The log of stimulus intensity required to evoke this response, or the criterion intensity (CI), was used in comparing retinal function in the different groups of rats. From the regression equation, 95% confidence limits and standard deviation for the log CI were calculated. The CI for different groups could then be compared by t-test, and $P$-values obtained. By inference, a rise in CI indicated a deterioration in retinal function, and vice versa.

**Biochemical Analyses**

The following tissues were removed under deep Nembutal anesthesia. Cell tissues were stored at $-65^\circ$C until use.

To assess vitamin A stores, in both Phase I and II rats, the liver was removed, rinsed quickly in 0.9% NaCl, and analyzed for vitamin A content by the method of Olson. Because bone stores reflect chronic zinc status in rats, femurs of both Phase I and II rats were removed and adherent tissues cleaned. After the bones were dissolved in a mixture of nitric and perchloric acid and heated, assays were done by atomic absorption spectrophotometry. For the rats in Phase II, acute zinc status was assessed by determining plasma zinc levels. Blood was collected by cardiac puncture with a heparinized (0.095 $\mu$g zinc/ml heparin) plastic syringe. The blood was centrifuged at 1100 g for 20 min. The plasma was removed and analyzed for zinc content by atomic absorption spectrophotometry.

**Histologic Preparation**

The eyes from both Phase I and II testes and pancreas from the Phase II rats in buffered formalin. The duodenum were removed, rinsed quickly in 0.9% NaCl to allow per traction to cease, then spread on beewax each end, and fixed in buffered formalin with a delay of 2000 $\mu$V. Islet cells in the enucleated eyes with H & E stain of vitamin A deficiency. Testes were examined with H & E the testicular function. Islet cells in the enucleated eyes with H & E as zinc is insulin secretion. Duodenum and jejunum were examined with H & E and PAS stains these structures have been observed in vitamin A deficiency. Tissues were normal histologic photomicrographs.

**Results**

**Electroretinograms**

The linear regression log V/log I plots of the Phase I Z-A- rats and pair-fed shown in Figure 4. The data show a :
< 0.001) increase of 1.5 units in the log CI of the Z−A− rats (Fig. 4 and Table 1).

The log V/log I plots from the ERGs of the three groups of Phase II rats performed on days 119 and 146 are displayed in Figure 5. The Z−A− plot (from day 83) and the plot from the pair-fed group of rats are included. Again, the log stimulus intensity needed to evoke a 200 μV response was chosen as the CI for comparison amongst groups. These plots showed definite changes in CI relative to the CI in the Z−A− (prerandomized) group. At day 119 both the Z+A+ and Z− groups had a decrease in CI (i.e., an improvement in function) compared to prerandomization, while the A− group CI did not change. At day 147, the CIs of the Z+A+ and Z− groups remained the same compared to day 119, but the A− group demonstrated a marked increase in CI (i.e., deteriorated function).

The information from Figure 5 is presented in Figure 6 as a bar plot. The number of log units of change in CI relative to the Z−A− (prerandomized) value is illustrated. The relative CI for the pair-fed normal rats is also represented.

By day 119 the A− rats showed no significant change in CI compared to prerandomization (0.2 log unit; P = 0.20). However, 4 weeks later their retinal function deteriorated significantly, as shown by a rise of 1.0 log unit (P < 0.001) in the CI (Fig. 6). On the other hand, the response of the Z− group improved (decreased CI) by 0.8 log unit (P < 0.001) by day 119. The Z+A+ group had the most marked improvement of the three groups compared to the prerandomization level: the CI decreased 1.1 log unit (P

### Table 1. Summary data for Phase I comparing Z−A− rats to normal-fed rats

<table>
<thead>
<tr>
<th></th>
<th>Pair-fed</th>
<th>Z−A−</th>
<th>P* value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of rats</td>
<td>12</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Mean elevation in criterion intensity cf. normal (log units)</td>
<td>0</td>
<td>1.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Liver vitamin A (μg/g tissue)</td>
<td>177.2 ± 31.3*</td>
<td>&lt;10†</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Femur zinc (μg/g tissue)</td>
<td>152.2 ± 17.0*</td>
<td>113.6 ± 20.3*</td>
<td>0.02</td>
</tr>
<tr>
<td>Retina</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Outer segment</td>
<td>—</td>
<td>degeneration in 4/8 (50%) eyes</td>
<td></td>
</tr>
<tr>
<td>Outer nuclear layer</td>
<td>cell loss in 2/8 (25%) eyes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cornea</td>
<td>—</td>
<td>8/8 normal</td>
<td></td>
</tr>
<tr>
<td>Optic nerve</td>
<td>—</td>
<td>4/4 normal</td>
<td></td>
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* Recorded as mean ± 1 standard deviation.
† Less than lower limit of assay.
< 0.001) within 4 weeks. The CI of the Z+A+ group at day 146 was, however, still not within the 0.05 confidence limits of the CI of the control group (Fig. 5).

Changes in Weight

The weight profile of the 36 rats in Phase I is illustrated in Figure 7. The mean weight of the group showed an early rapid rise with 5 ppm zinc in the drinking water. The steady increase stopped with introduction of double-distilled water (days 28–35). Mean weight gain resumed once 10 ppm of zinc was added to the water. The growth rate slowed markedly by day 49. There was a leveling-off (plateau) at a median of 230 gm for 4 weeks, and during the next 14 days a precipitous decrease in median weight occurred.

Weight loss was reversed (Phase II) when these rats were supplemented with zinc and either vitamin A or retinoic acid (Fig. 8). Although retinoic acid supplementation stopped the loss in weight, resumption of growth did not occur if zinc was withheld from the diet (Z− rats). Withdrawal of retinoic acid during the period days 119–126 resulted in loss of weight in the A− rats.

Behavior and Appearance of Animals

The characteristic findings of vitamin A and zinc deficiencies were evident by day 76. The rats showed listlessness, poor feeding, diarrhea, and loss of sense of balance. Thinning of hair, loss of hair luster, and erythema and bleeding of the foot pad were also present. Supplementing the diets (Phase II) with retinoic acid or vitamin A, with or without zinc, led to a recovery of postural balance, alertness and response to sounds, and decrease in erythema of the foot pads.

Fig. 6. Change in CI for three treatment groups compared to prerandomization (Z−A−) level (baseline). Relative CI for normal rats is shown (N). Bars above line indicate improved function (lower CI); below line indicate deterioration (higher CI). Numbers within squares denote number of rats tested. Bars are 1 standard deviation.

Fig. 7. Plot of mean weights for Z−A− rats (Phase I). Zinc levels in the diet varied from 0 to 10 ppm. Retinoic acid (RA) was provided from days 83 to 90. Bars at days 28, 63, and 83 represent range in weights for the group.

Fig. 8. Plot of mean weights for three treatment groups (Phase II). Retinoic acid (RA) was provided to all three groups except 1 week to A− rat group. Zinc was eliminated from Z− diet after day 104. erg = electroretinogram.
The hair regained its luster and texture, except in the Z- rats.

In Phase I rats vital staining of the cornea and conjunctiva with Lissamine Green (10 μl of 1% solution applied to the conjunctival sac) followed by slit-lamp microscopy showed bilateral diffuse punctate staining of the corneas of all rats examined. Some rats had thick mucus plugs in the corneas. Daily administration of vitamin A or retinoic acid (Phase II) reversed these findings.

Biochemical and Histologic Observations

Data in Table 1 show liver vitamin A, femur zinc content, and histology for the tissues of the 12 rats that died in the final stages of the depletion period (Phase I), and for a comparable number of pair-fed control rats. The vitamin A content in the Z-A- livers was negligible, and the mean zinc level in their femurs was significantly below that of the control rats.

Histological examination of the eyes of these rats showed rod outer segment degeneration and loss of photoreceptor cells in a significant number of retinas (Fig. 9). No abnormalities were observed with H & E stain in the optic nerves, corneas or other tissues of any of these rats (Table 1).

Vitamin A and zinc levels in the Phase II groups and the normal control rats are summarized in Table 2. As in the case of the Phase I Z-A- group, the A- group had undetectable vitamin A levels in their livers. There was no significant difference in the mean liver vitamin A levels between the Z- and Z+A+ groups.

The Z- group had femur zinc levels (reflecting chronic zinc status) markedly lower than those of the other three groups (P < 0.001) while no significant differences in femur zinc levels existed among the A-, Z+A+, and normal groups. The femur zinc levels of the Z- group were significantly lower than the prerandomized Phase I rats (P = 0.01), indicating a further decrease in zinc stores. The plasma zinc levels (reflecting acute zinc status) paralleled those found in the femur; there was a highly significant decrease in the plasma zinc levels in the Z- group of rats but no significant difference from control levels in the A- and Z+A+ group of rats.

Histologic examination of eyes obtained from Phase II rats showed degeneration of photoreceptor outer segments and loss of cells in the outer nuclear layer of the retina in 6 of 12 (50%) eyes from the A- rats (Fig. 10). Three of the 12 (25%) eyes had keratinization of the cornea (Fig. 11). There were no abnormalities of the optic nerves. The histology of the retinas, corneas, and optic nerves was normal in the eyes of the Z- and Z+A+ rats.

Three of the six (50%) A- rats showed stunting of the villi (Fig. 12) and decrease in density of goblet cells (Fig. 13) in the jejunum. Two of the five (40%)

Table 2. Vitamin A and zinc data for Phase II for three treatment groups and normal group*

<table>
<thead>
<tr>
<th></th>
<th>A-</th>
<th>Z-</th>
<th>Z+A+</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of rats</td>
<td>6</td>
<td>5</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>Liver vitamin A (μg/g tissue)</td>
<td>&lt;10†</td>
<td>129.4 ± 35.3</td>
<td>119.2 ± 19.4</td>
<td>177.2 ± 31.2</td>
</tr>
<tr>
<td>Femur zinc (μg/g tissue)</td>
<td>151.9 ± 11.7</td>
<td>100.5 ± 10.6</td>
<td>159.7 ± 10.7</td>
<td>152.2 ± 17.0</td>
</tr>
<tr>
<td>Plasma zinc (μg/ml)</td>
<td>1.36 ± 0.06</td>
<td>0.77 ± 0.10</td>
<td>1.57 ± 0.24</td>
<td>1.50 ± 0.28</td>
</tr>
</tbody>
</table>

* Recorded as mean ± 1 standard deviation. † Less than lower limit of assay.
Fig. 10. Retina from A− rat (right) shows outer segment degeneration. The outer nuclear layer is intact but reduced in thickness due to cell loss. Left, normal retina (PAS, ×160).

Z− rats also showed loss of goblet cell density in the jejunum. All rats in all three groups in Phase II had atrophy of seminiferous tubules of the testes, indicating that permanent damage had already occurred during the depletion phase (Phase I) of the study. Pancreatic tissue was normal in all rats.

Discussion

The physiologic role of zinc in human nutrition and the causes of zinc deficiency have been subjects of much research and speculation in recent years. Its effect on vitamin A metabolism is manifest at many levels. 9, 11

Electroretinograms and Eye Histology

The log V/log I plot for the Z−A− group at day 83 showed a significant rise in CI (ie, deteriorated function) compared to a control pair-fed (Fig. 4). This was associated with histologic changes in photoreceptor outer segments (Fig. 9). Such retinal changes have
been seen both in severe vitamin A deficiency\textsuperscript{33,40} and moderate or severe zinc deficiency.\textsuperscript{14}

The A— group in Phase II showed a subsequent large rise in CI, and this was associated with abnormal retina structure at the light microscopic level. However, in spite of the very low levels of liver vitamin A at the time of randomization, the change in CI did not occur until the second month of Phase II. The delayed rise in CI in these A— rats can be explained by a combination of factors. First, the retina is known to be among the last tissues to lose its vitamin A stores in vitamin A deficiency.\textsuperscript{16} Second, supplementation with retinoic acid, which cannot be used for visual function,\textsuperscript{31} “spares” the vitamin A stores of the retina and liver for visual function.\textsuperscript{27-30} Finally, critical plasma zinc levels are required for the release of vitamin A from the liver.\textsuperscript{19,20} The plasma zinc level in the prerandomized (Phase I) rats may have been below this critical level, thus trapping a minimal amount of vitamin A in the liver. The replenishing of the dietary zinc in the A— diet would cause the mobilization of the remaining liver vitamin A, some of which would be used for visual function.

The Z— group were moderately zinc deficient according to their femur and plasma zinc levels,\textsuperscript{10,12,37} and this was confirmed by the fact that their weight gain abruptly stopped when zinc was eliminated from the water supply.\textsuperscript{7,26,37} They did not develop any new mucous membrane or hair abnormalities during Phase II, indicating that severe zinc deficiency was not attained. These rats showed a decrease in CI (ie, improved function). By day 146 their CI was not significantly different from the CI of the doubly-repleted (Z+A+) group. Their eyes had no abnormalities by light microscopy, indicating reversal of changes from the Z—A— state.

The Z+A+ rats showed the greatest functional improvement (reduced CI) of the three groups, but their CI did not reach the 95% confidence limits of the CI of the normal-fed group of rats. The maximal improvement shown by this group was attained by 4
weeks after randomization. Johnson\textsuperscript{22} showed that when retinas were damaged by vitamin A deficiency, the replenishing of this vitamin could induce some repair within a few days. Return to normal status required 3 to 4 weeks. The fact that the Cl of the Z+A+ rats never returned to normal indicates that some irreparable retinal damage had been caused during the Z−A− phase (Phase I). This damage could have been caused by a deficiency of either vitamin A or zinc.\textsuperscript{6,33} Because no light microscopic abnormalities of photoreceptor outer segments or outer nuclear layers were seen in any Z+A+ rat, this damage must have occurred at the electron microscopic or molecular levels.

The fact that the Cl of the Z− group improved almost as much as that of the Z+A+ group supports findings in both rats\textsuperscript{20} and monkeys\textsuperscript{19} that as long as abundant vitamin A is available, animals need only small amounts of circulating zinc to allow the vitamin to serve its normal metabolic functions in the eye. Normal vitamin A metabolism is dependent on an available supply of labile zinc.\textsuperscript{11,18,19,22} Above a low “critical” circulating zinc level vitamin A transport is no longer dependent on plasma zinc concentration.\textsuperscript{19}

Changes in Weight

In Phase I the median weights of the rats followed a pattern typical of rats with vitamin A deficiency.\textsuperscript{32,33} The rapid loss in weight after day 70 was probably due to vitamin A deficiency, as demonstrated by the absence of the vitamin in the liver (Table 1); it is unlikely that zinc deficiency had a significant effect on the weights. This conclusion is based on our findings (Table 1) that although femur zinc levels were significantly below the normal levels they were still higher than those reported for severe zinc deficiency in experimental animals.\textsuperscript{10,12,37}

In a previous study by one of us (Parker JA, unpublished data) it was shown that only with extremely low zinc levels (1 to 2 ppm) do rats demonstrate a plateau in weight profile followed by rapid weight loss. Kirchgesnser et al\textsuperscript{25} showed that weanling rats maintained on zinc levels of 8 ppm or more had normal growth curves, and 5 ppm allowed slower but steady weight gain. Williams\textsuperscript{26} found a plateau after 7 days in young rats fed 1 ppm zinc in the diet, while a diet with 5 ppm allowed steady weight gain. However, the hault in weight gain seen at day 28 with the introduction of double-distilled water suggests that the rats were at least moderately deficient at that stage, as reported in zinc-deficient rats\textsuperscript{12,25} and zinc-deficient mice.\textsuperscript{34} The Z− rats in Phase II showed a similar pattern when double-distilled water was introduced (Fig. 8).

Histologic Findings: Non-Ocular Tissues

The loss of goblet cells, degeneration of gut villus structures, and testicular atrophy seen in A− and Z− rats are consistent with hypovitaminosis A\textsuperscript{27,29,42,43} and the decrease in protein synthesis caused by zinc deficiency.\textsuperscript{12,41,42} However, the finding that Z+A+ rats of Phase II also showed testicular atrophy indicates that permanent damage to the testes had already occurred in Phase I from the Z−A− state. Both vitamin A and zinc are known to affect testicular function, though\textsuperscript{11,42} the mode by which vitamin A deficiency causes testicular atrophy is not known. However, adequate levels of zinc are required for normal spermatogenesis\textsuperscript{11,12,44} and for protein synthesis.\textsuperscript{11,42}

In summary, zinc repletion alone in Z−A− rats is not sufficient to prevent the ongoing deterioration in retinal function due to chronic vitamin A deficiency. Vitamin A repletion in these rats, however, can lead to significant recovery in retinal function as long as a low critical level of zinc is maintained. The recovery with vitamin A repletion is further enhanced when higher levels of zinc are added to the diet.

Key words: electroretinogram, rat, retinoic acid, vitamin A deficiency, zinc deficiency

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References


