Bright Environmental Light Accelerates Rhodopsin Depletion in Retinoid-Deprived Rats

Martin L. Katz, Holly J. Stientjes, Chun-Lan Gao, and Michael Norberg

Purpose. Dietary deficiency in the retinoid precursors of the visual pigment chromophore 11-cis retinal eventually results in selective degeneration of the photoreceptor cells of the vertebrate retina. An early effect of retinoid deficiency is depletion of chromophore from the photoreceptor outer segments. Experiments were conducted to determine whether the rate of chromophore depletion was affected by the intensity of environmental light.

Methods. Rats were maintained on diets either containing or lacking retinoid precursors of 11-cis retinal for up to 30 weeks. Animals in both dietary groups were exposed to either bright (90 lux) or dim (5 lux) cyclic light for the duration of the experiment. At various time intervals retinal rhodopsin content and photoreceptor densities were determined in animals from each treatment group.

Results. Bright light greatly accelerated the depletion of rhodopsin from the retina. Rhodopsin was almost completely depleted from the retinas of the retinoid-deficient animals raised under bright light for 25 weeks, whereas the dim-light-reared animals fed the retinoid-deficient diet still had significant amounts of retinal rhodopsin even after 30 weeks. Bright light alone moderately depressed retinal rhodopsin levels in animals fed the diet containing a vitamin A precursor of 11-cis retinal. Among rats fed the latter diet, retinal rhodopsin content in the animals kept in bright cyclic light was maintained throughout the experiment at about 70% of the amount of rhodopsin in rats housed in dim cyclic light. The light-related rhodopsin depletion in the retinoid-deprived rats was accompanied by photoreceptor cell death. After 30 weeks of treatment, photoreceptor cell densities remained similar in all treatment groups except the retinoid-deprived group housed under bright cyclic light. In the latter group, photoreceptor cell densities in the central retinas were reduced by an average of more than 50% after 30 weeks. Retinoid deficiency and bright light exposure alone each resulted in a reduction in rod outer segment size. An even greater reduction in outer segment size was observed in vitamin A-deprived animals housed under bright cyclic light.

Conclusion. These findings indicate that light accelerates the depletion of retinoids from the retina and the accompanying photoreceptor cell degeneration. Invest Ophthalmol Vis Sci 1993;34:2000-2008.

The discovery that vitamin A deficiency causes night blindness1 served as the basis for subsequent research that has produced a detailed understanding of the role of retinoids in the visual process. Aldehyde forms of retinoids have been shown to act as chromophores for the visual pigment proteins in the photoreceptor cells of all vertebrates and invertebrates studied.2-6 Dietary deficiencies in the retinoids serving as precursors for vitamin A aldehydes lead to substantial reductions in the visual pigment content of the vertebrate retina.7,8 In the early stages of deficiency, this loss of visual pigment is accompanied by a reduction in photoreceptor outer segment size.9 After prolonged vitamin A deprivation, the photoreceptor cells begin to die, resulting in an irreversible loss in visual sensitivity.7,8

Light of the appropriate wavelengths drives the 'visual cycle' in the retina. In the dark-adapted state, there is little turnover of retinoids involved in vision,
other than that resulting from photoreceptor outer-
segment membrane renewal. Most of the retinal vita-
min A in dark-adapted animals is either bound to vi-
sual pigment protein as 11-cis retinaldehyde or stored
in the retinal pigment epithelium as retinol esterified
to fatty acids.\textsuperscript{10-13} Exposure of the retina to light re-
results in the isomerization of visual pigment-bound reti-
inaldehyde to the all-trans configuration, its release
from the visual pigment protein, and its reduction to
the alcohol form.\textsuperscript{2,3} The all-trans retinol is then trans-
ported to the retinal pigment epithelium, where it is
esterified to fatty acids, and isomerized back to the
11-cis configuration.\textsuperscript{2,14,16} After isomerization, the 11-
cis retinol is oxidized to 11-cis retinaldehyde, which is
then transported back to the photoreceptor cells,\textsuperscript{12,17}
where it can recombine with the protein component of
the visual pigment (opsin in rods) to regenerate a
functional visual pigment molecule. The brighter the
light to which the retina is exposed, the more rapidly
the retinoids involved in vision must pass through this
cycle. If both the visual cycle and the recycling of pho-
toreceptor outer segment components during turn-
over were perfectly efficient, no replenishment of the
retinoids involved in vision would be required. How-
ever, it has been shown that in normal circumstances
vitamin A is continually taken up into the retina from
the choroidal circulation.\textsuperscript{18} Thus, retinoids involved in
the visual cycle must be gradually lost from the retina.
If these retinoids cannot be replenished by uptake
from the blood, retinoid levels in the retina will de-
crease. Light appears to promote loss of these com-
ounds from the retina.\textsuperscript{19} If any of the transitional
forms of the retinoids are more susceptible to destruc-
tion or loss from the retina than are the retinyl esters
or protein-bound retinaldehyde, or if light directly de-
stroys the retinoids in the retina, one would expect
that in retinoid-deprived animals retinal vitamin A lev-
els would decrease more quickly under bright-light
than dim-light conditions. Experiments were con-
ducted to evaluate the relationship between light ex-
posure and retinoid depletion from the retina.

\section*{MATERIALS AND METHODS}

\subsection*{Animals and Treatments}

Male Fischer 344 albino rats were obtained at 21 days
of age from Harlan Sprague-Dawley, Inc. (Indianapo-
lis, IN). On arrival, the animals were divided into four
treatment groups. One half of the rats were fed a syn-
thetic diet that contained adequate levels of all nu-
trients known to be required by the rat.\textsuperscript{13} This diet
(+A) contained vitamin A in the form of retinyl palmi-
tate, which can be metabolized to all of the retinoids
involved in the visual process, as well as to the reti-
noids used by other tissues. The remaining rats were
fed an identical diet, except that retinoic acid was sub-
stituted for retinyl palmitate (\textminus A).\textsuperscript{13} Retinoic acid can
satisfy the metabolic requirements of most tissues for
vitamin A, but cannot be converted metabolically into
the retinoids involved in vision.

The animals were all housed in the same room
under 12 hr/12 hr cyclic illumination. Illumination
was provided by 75 watt incandescent bulbs. Light lev-
els in the cages were varied by changing the placement
of the cages relative to the light sources. One half of
the animals in each dietary group were exposed to an
average illuminance of 5 lux (dim-light groups) and
the remaining rats were kept under cyclic light of 90
lux average illuminance (bright-light groups). Light
levels were determined with a Lutron model LX-101
light meter (Markson Science, Tucson, AZ) with the
probe placed face-up on the bottoms of the animal
cages. Total darkness was maintained during each 12-
hour dark cycle. The room in which the animals were
housed was maintained at a relatively constant temper-
ature of 20°C. All investigations were performed in
accordance with the ARVO Statement for the Use of
Animals in Ophthalmic and Vision Research.

\subsection*{Rhodopsin Determinations}

To monitor retinoid depletion from the retina, the
amount of the visual pigment rhodopsin was measured
in eyes of rats that had been fed the diets for various
time intervals. Rhodopsin measurements were per-
formed with a modification of a widely used spectro-
photometric technique.\textsuperscript{8} Rats were placed in total
darkness for a minimum of 24 hr. All subsequent pro-
cedures were performed under dim red light unless
otherwise noted. The animals were killed with CO\textsubscript{2} gas,
and their eyes were immediately enucleated. The lens
and vitreous were removed from each eye through a
slit in the cornea. The remainder of each eye was cut
into several pieces in 700 \textmu l of a homogenization
buffer consisting of 100 mM sodium phosphate, 2%
sucrose, and 1.4% emulphogene BC-720 at pH 7.4.
The tissues were homogenized in glass sleeves with a
motor-driven teflon (Du Pont, Wilmington, DE) pes-
tle, and the homogenates were then centrifugated at
10,000 g for 5 min to remove material that had not
been solubilized. A 500 \textmu l aliquot of the supernatant
was transferred to a quartz cuvette and 50 \textmu l of freshly
prepared 100 mM hydroxyamine in 100 mM sodium
phosphate, pH 7.4 was added with mixing. The absorb-
bances of the resulting solutions were measured at 495
nm before and after bleaching for 5 min with light
from a Sylvania F15T8-D fluorescent lamp (GTÉ, Danvers, MA). A molar extinction of 42,700 for the
difference in absorbance before and after bleaching
was used to calculate the rhodopsin content of each eye.\textsuperscript{20}

\subsection*{Photoreceptor Cell Densities}

Analyses were performed to determine the effects of
light and diet on photoreceptor cell densities. Animals
in each treatment group were killed with CO₂ gas after 30 weeks. Animals were killed between 1 and 1.5 hours after the onset of the daily lighting cycle. The eyes were immediately placed in a cacodylate-buffered aldehyde fixative. The corneas, irides, and lenses were removed and the remaining portions of the eyes were gently agitated in the fixative at room temperature for a minimum of 24 hr. One eye from each animal was cut along a superior–inferior plane approximately 0.5 mm lateral to the optic nerve head. The eyes were then embedded in a methacrylate-based plastic (JB-4, Polysciences, Inc., Warrington, PA). Sections along the superior–inferior meridian passing through the optic nerve head were cut at a thickness of 2 μm. The sections were stained with toluidine blue and were analyzed with a computer-based image analysis system (Analytical Imaging Concepts, Irvine, CA) interfaced to a microscope through a video camera. The lengths of each retina were measured from the edges of the optic nerve head to the inferior and the superior ora serrata. Regions centered at 20 and 70% of the distance from the edge of the optic nerve head to the ora serrata in both the superior and inferior halves of the retinas were selected for photoreceptor density determinations. The numbers of photoreceptor nuclei in 153 μm lengths of retina were determined in each of these regions. Three sections from each retina were analyzed and the average photoreceptor densities obtained from these analyses were determined.

Retinal Ultrastructure and Outer Segment Measurements

The effects of light and retinoid deficiency on photoreceptor ultrastructure were evaluated in rats that had been subjected to the various treatments for 30 weeks. Animals were killed and their eyes were fixed as described earlier. The eyes were then dissected to obtain the regions on either side of the superior–inferior meridian extending approximately 0.5–2 mm superior to the optic nerve head. These tissues were washed in 0.17 M sodium cacodylate (pH 7.4), postfixed in 1% OsO₄, and embedded in epon-araldite resin. Ultrathin sections of the embedded tissues were cut in planes parallel to the long axis of the photoreceptors. The sections were stained with uranyl acetate and lead citrate and were examined with a JEM 1200 EX electron microscope (JEOL Ltd., Tokyo, Japan).

The total area occupied by the outer segments per unit length of the retina was determined in these sections. A series of electron micrographs of the outer segments covering a minimum of 200 μm of retinal length, measured along the base of the retinal pigment epithelium, was obtained. In these micrographs, the areas occupied by the outer segments and the lengths of the regions examined were determined with the assistance of an Image-1 image analysis system (Universal Imaging Corporation, West Chester, PA). Image analysis was performed using the electron micrograph negatives. The negatives were placed on a light box and the images were captured from them by using a video camera. The video images were transferred to a Gateway 2000 computer (North Sioux City, SD) for analysis. With software image enhancements, the outer segments could be specifically recognized by the image analysis software, and their cross-sectional areas determined. Retinal length was determined from these micrographs by measuring the length of a line drawn parallel to the retinal pigment epithelium (RPE) basal lamina. The image analysis system was calibrated using the magnification bar that the electron microscope projects onto each negative. Areas occupied by the outer segments in sections per unit retinal length were measured in retinas from four animals from each treatment group.

Statistical Analyses

Determination of whether light and diet had significant influences on the measured parameters at specific time-points was accomplished using analysis of variance. Comparisons between individual treatment groups were performed using the Newman-Keuls procedure. Only in those cases where analysis of variance indicated a significant treatment effect were the Newman-Keuls analyses performed.

RESULTS

Rhodopsin Depletion

In animals maintained under dim cyclic light, retinoid deficiency resulted in a gradual reduction in retinal rhodopsin content (Fig. 1). After 30 weeks of retinoid deficiency, the mean amount of retinal rhodopsin in dim light-reared rats was reduced to 27% of that present in animals that had been housed under the same lighting conditions but fed a diet containing retinyl palmitate (P < 0.001). Rhodopsin levels declined much more rapidly in retinoid-deprived rats kept under brighter cyclic light (Fig. 1). After only 8 weeks of retinoid deficiency, the amount of rhodopsin per eye in the bright-light-reared animals was an average of only 31% of that in the eyes of rats housed under dim cyclic light and fed retinyl palmitate (P < 0.001). By 25 weeks, rhodopsin was barely detectable in the eyes of retinoid-deprived rats kept under bright cyclic light. Bright cyclic light alone depressed rhodopsin levels in rats fed the diet containing retinyl palmitate (Fig. 1). Among animals fed the latter diet, rhodopsin levels were approximately 30% lower in animals housed under bright cyclic light than in those kept under dimmer lighting (P < 0.003 at all times). This reduction in rhodopsin levels had occurred by 3 weeks, and the relative amount of rhodopsin in the bright and dim
Vitamin A, Light, and Rhodopsin

FIGURE 1. Influence of dietary vitamin A and light on retinal rhodopsin content. The four treatment groups are indicated by the following labels. +A: retinyl palmitate A-containing diet. −A: retinoic acid-containing diet devoid of other forms of vitamin A. L: bright cyclic light. D: dim cyclic light. Each data point represents the mean and SEM of determinations performed on eyes from between 3 and 6 animals.

not significantly different from those observed in the +A rats kept in dim cyclic light.

Retinal Ultrastructure

Even after severe depletion of the retinoids involved in the visual cycle, the photoreceptor cells continued to synthesize outer segments (Figs. 3 and 5). Among the rats maintained under dim cyclic light, retinoid deprivation resulted in a significant decrease in outer segment size; in sections cut parallel to the long axes of the photoreceptors, the area occupied by outer segments per unit retinal length was an average of 27% less in the −A rats than in the +A animals (P < 0.03) (Fig. 6). Among animals fed the +A diet, the mean area occupied by outer segments per unit retinal length was 25% lower in the rats housed under bright cyclic light than in those under the dim light condition (P < 0.04) (Fig. 6). In rats fed the −A diet and housed under bright cyclic light, the mean area occupied by outer segments per unit retinal length was only 27% of that in the animals fed the +A diet and maintained under

light groups fed retinyl palmitate did not change after this time.

Photoreceptor Cell Densities

Photoreceptor cell densities, relative to those of the +A rats maintained under dim cyclic light, were significantly reduced throughout the retinas of the −A animals housed under bright cyclic light (Figs. 2–4). After 30 weeks of treatment, the mean photoreceptor cell density in the latter group was reduced by 56% in the central–superior region of the retina, and by 54% in the central–inferior region (P < 0.001) (Fig. 2). Photoreceptor cell loss was somewhat less in the peripheral retina. After 30 weeks, photoreceptor cell densities in the superior–peripheral retinas of −A rats housed under bright cyclic light were an average of 34% lower than those in the same retinal region of +A animals maintained under dim cyclic light (P < 0.005) (Fig. 4). In the inferior–peripheral region, the mean decrease in photoreceptor density was 37% (P < 0.005) (Fig. 4). No decreases in photoreceptor cell densities were observed in the superior halves of the retinas from either the −A rats housed under dim cyclic light nor the +A animals kept under bright cyclic light (Figs. 2–4). Moderate photoreceptor cell losses were, however, observed in the inferior–central retinas of both the latter treatment groups (Figs. 2 and 4). The photoreceptor nuclei densities in the central inferior retinas of both treatment groups were approximately 14% lower than the cell densities observed in the +A rats housed under dim cyclic light (P < 0.05). However, mean photoreceptor cell densities in the inferior peripheral retinas of the −A dim light reared rats and the +A bright light maintained animals were
FIGURE 3. Light micrographs of representative regions of the superior retinas of rats maintained on the four treatment regimens for 30 weeks. See legend to Fig. 1 for treatment group designations. Photoreceptor nuclei densities are similar in all groups except the −A/L animals. Photoreceptor outer segment size is clearly reduced in the +A/L, −A/D, and −A/L treatment groups.

dim cyclic light ($P < 0.001$) (Fig. 6). The decreased outer segment density in the −A rats housed under bright cyclic light was accompanied by decreased regularity in outer segment orientation (Figs. 3, 5). In the latter treatment group, the decreased area occupied by outer segments per unit retinal length resulted from both a decrease in outer segment size and a loss of photoreceptor cells. As indicated earlier, photoreceptor cell density in the central–superior region of the retina was reduced by 50% after 30 weeks of treatment in the −A rats housed under bright cyclic light. Thus a decrease of 50% in the area occupied by outer segments per unit length in this group was due to photoreceptor cell death. The remaining 23% decrease in this parameter is caused by a decrease in size of the remaining outer segments.

DISCUSSION

In the dark-adapted state, most of the vitamin A in the retina-RPE complex of rats is present as retinaldehyde in rhodopsin. Thus, rhodopsin measurements can be used to evaluate the retinoid content of these tissues. Based on rhodopsin measurements, the depletion of retinoids from the retinas of vitamin A-deprived rats has previously been shown to be almost completely dependent on light. Rats fed a retinoid deficient diet for up to 20 weeks showed no measurable decrease in retinal rhodopsin and very little change in electroretinogram amplitudes if the animals were kept in constant darkness. By contrast, in retinoid-deprived rats kept in cyclic light for the same period, rhodopsin levels and visual sensitivity decreased substantially. Based on these observations, it appears that the efficiency of retinoid recycling within the RPE-photoreceptor complex due to photoreceptor outer-segment renewal is probably close to 100%. Retinoid recycling caused by visual pigment bleaching, conversely, does not appear to be as efficient. In the absence of the normal source of retinoids in the blood, vitamin A is gradually lost from the retinas of rats maintained under cyclic light conditions.

Because low-intensity cyclic light promotes the loss of vitamin A from the retina, it is possible that retinoid depletion is caused merely by daily cycling of the light environment from dark to light. If such were the case, the intensity of light during the daily cycle...
FIGURE 4. Effects of diet and light on photoreceptor nuclei densities in the peripheral retina. The mean ± standard errors of cell densities determined in retinas from five animals per group are indicated.

would not be expected to influence the rate of loss. Alternatively, the loss of vitamin A may be attributable to inefficiency in preservation of vitamin A at one or more steps in the visual cycle, perhaps as a result of light-dependent destruction of one or more of the retinoid intermediates. In the latter case, one would expect that the greater the light intensity (below levels that produce acute damage to the retina), the more rapidly vitamin A would be depleted from the retina. Such a relationship between light intensity and the rate of vitamin A depletion was proposed by Noell and colleagues, but the data to confirm such a relationship have not been presented. In the present study, the decline in retinal rhodopsin content was much more rapid in vitamin A-deprived rats maintained under cyclic light of 90 lux than in rats fed the same diet but housed under cyclic light of only 5 lux intensity. Thus, it appears that the more rapidly vitamin A is driven through the visual cycle, the more rapidly it is lost from the retina-RPE complex.

The light-dependent acceleration of rhodopsin loss from the retina is not primarily the result of direct light damage to the photoreceptors. The higher light intensity used in the present experiments is well below the intensity previously reported to be required to produce retinal damage in albino rats under cyclic light conditions. Retinas of +A rats housed under 90 lux cyclic light displayed no apparent damage to the outer segments and only a slight decrease in photoreceptor cell density in a single retinal region. Thus, most of the loss of retinoids required for rhodopsin synthesis does not appear to be the result of photoreceptor cell damage by light. Light could lead to vitamin A loss from the retina through photosensitized oxidation. It has been shown that retinal can act as a photosensitizer for singlet oxygen production, and the singlet oxygen could in turn destroy the retinal. Although the absorbance maximum of retinal is in the ultraviolet region of the spectrum (373 nm in cyclohexane), it does have considerable absorbance at higher wavelengths that would be expected reach the retina. Thus, it is possible that photosensitized oxidation of retinal is responsible for the light-dependent depletion of retinoids from the retinas of vitamin A-deprived rats. This possibility is consistent with reports that the retina is most sensitive to damage by light in the blue and near-ultraviolet regions of the spectrum, and that the sensitivity of retina to blue light damage is increased when blood oxygen levels are elevated.

The observation that lipofuscin accumulation in the RPE is dependent on dietary vitamin A intake is also consistent with the possibility that retinal vitamin A is susceptible to photooxidation. Lipofuscin is an autofluorescent pigment that accumulates in the RPE during senescence. A similar pigment accumulates in the RPE of rats fed antioxidant-deficient diets, suggesting that formation of these pigments may involve oxidation of some retinal components. The deposition of the antioxidant deficiency pigment in the RPE, like that of lipofuscin, is highly dependent on dietary retinoid intake. It is possible that light promotes the oxidation of retinoids in the retina and their conversion into autofluorescent products that accumulate in RPE lipofuscin. If this is the case, it can be predicted that the deposition of lipofuscin in the RPE during a long period of time could be retarded by reducing the daily exposure of the retina to light of wavelengths that could be absorbed by retinal. Consistent with this prediction is the observation that the RPE of whites contains significantly more lipofuscin than that of blacks of the same age.

The acceleration of vitamin A depletion from the retina may also be attributable to loss of retinoids to the blood in the vitamin A deficient animals. A small fraction of the retinoids that reach the RPE as a result of rhodopsin bleaching are probably lost to the choroidal circulation. The amount of vitamin A lost from the
retina by this route may be greater under higher light intensities, particularly because the retinoids cycle through the RPE more rapidly under bright light than under dim light.

Experiments by St. Jules and colleagues indicated that under normal conditions, retinal is attached to opsin in the photoreceptor inner segment before incorporation of opsin into newly assembled outer segment disc membranes. However, it is not certain that chromophore binding to opsin is a necessary prerequisite for incorporation of the visual pigment protein into new disc membranes. Outer segment renewal continues in retinoid-deprived rats, and opsin content of the disc membranes in these animals greatly exceeds the amount of vitamin A present in the retina. The opsin packing within the disc membranes was not altered by retinoid deficiency. In the present study, the retinoid content of the retina, as measured by rhodopsin levels, was reduced by approximately 96% after 25 weeks and more than 99% after 30 weeks in the -A rats housed under 90 lux cyclic light. Despite the paucity of vitamin A in the retina at these two time points, significant outer segment disc assembly apparently was still occurring (Fig. 5). Thus, it appears that although attachment of retinal to opsin can occur before disc membrane assembly, this is not a necessary step in the incorporation of opsin into new disc membranes. This conclusion is supported by the observation of Penn and Williams that animals fed a retinoid-containing diet and raised under 800 lux cyclic light had no detect-

FIGURE 5. Electron micrographs of the photoreceptor outer segment regions in the central-superior retinas from rats in the four treatment groups. See legend to Fig. 1 for treatment group designations.
result of direct damage to the photoreceptors by light, because the +A rats kept under 90 lux cyclic light showed very little photoreceptor cell loss. It appears that retinal vitamin A levels must drop below a certain threshold before significant photoreceptor death occurs. In the -A animals housed under 90 lux cyclic light, retinoid levels were insufficient to maintain rhodopsin levels at more than 6% of normal after 19 weeks. Conversely, in vitamin A-deprived rats kept under 5 lux cyclic light, retinal vitamin A levels were sufficient to maintain rhodopsin levels at 27% of normal, even after 30 weeks. Thus, the threshold vitamin A level below which photoreceptor cell loss occurs is relatively low. It can be inferred that in humans with significant loss of visual sensitivity caused by retinoid deficiency, complete recovery after vitamin A repletion may be possible.7,38

As reported previously,9 vitamin A deprivation was found to cause a reduction in outer segment size, as measured by area occupied by outer segments per unit retinal length in sections through the retina (Figs. 5 and 6). In the -A rats housed under dim cyclic light, there was no decrease in photoreceptor density in the superior–central retina, but the amount of outer segment membrane per photoreceptor cell was reduced significantly, indicating that outer segment size had been reduced. Bright cyclic light alone also caused the outer segments to decrease in size. The acceleration of vitamin A depletion from the retina by bright cyclic light was accompanied by both a loss of photoreceptor cells and shrinkage of the remaining outer segments. These findings suggest that light may regulate outer segment size by affecting the availability of retinoids to the outer segments.

The results of this study clearly demonstrate that although an individual molecule of vitamin A can pass through the visual cycle many times, there is a finite probability that vitamin A will be lost with each passage through this cycle. The more frequently vitamin A passes through the cycle of rhodopsin bleaching and regeneration, the more likely the retinoids involved will be lost. More study is needed to determine the precise mechanism by which light promotes loss of retinal vitamin A.

**Key Words**

vitamin A, retina, photoreceptors, aging, retinal degeneration

**Acknowledgments**

The authors thank J. S. Christianson and W. S. Stark for assistance with the morphometric analyses and M. J. Kutryb for performing some of the rhodopsin measurements.

**References**

1. McCollum EV, Simmonds N. A biological analysis of pellagra-producing diets. II. the minimum requirements of the two unidentified dietary factors for main-


