Dose Dependency of Canthaxanthin Crystals in Monkey Retina and Spatial Distribution of its Metabolites

Regina Goralczyk,1 Felix M. Barker,2 Susanne Buser,5 Hans Liechti,1 and Jochen Bausch1

PURPOSE. To establish the threshold level of canthaxanthin crystals in the retina of cynomolgus monkeys. To correlate the spatial distribution of all-trans canthaxanthin and its metabolites with the grade of crystals.

METHODS. Monkeys were orally administered 0, 0.2, 0.6, 1.8, 5.4, 16.2, and 48.6 mg/kg body wt canthaxanthin daily for 2.5 to 3 years. A second group of monkeys were administered 200 and 500 mg/kg body wt/d for 5 years. Ophthalmoscopy, electroretinography (ERG), retina and carotenoid analysis were performed as previously reported.

RESULTS. Crystals in the retina periphery were observed by ophthalmoscopy preterminally only in the extreme high doses of 200 to 500 mg/kg body wt/d. There were no adverse effects on visual functions as measured by ERG. Crystals in the peripheral retina, and/or in the macula, were detected microscopically in all canthaxanthish treated groups except at the lowest dose of 0.2 mg/kg body wt/d. The grade of crystals increased up to a dose of 16.2 mg/kg body wt/d. Dose-dependent increases in canthaxanthin content also were noted in the retina, the liver, and in plasma. All-trans canthaxanthin was the major compound in the peripheral and paracentral retina of very highly dosed animals, where its concentration correlated largely with the grade of inclusions. In the macula, 4'-OH-echinonene was the dominant canthaxanthin metabolite.

CONCLUSIONS. The grade of crystals in monkey retinas was dose dependent with a threshold level at 0.6 mg canthaxanthin/kg body wt/d. It correlated in the retinal periphery with the concentrations of all-trans-canthaxanthin and in the macula with its metabolites. (Invest Ophthalmol Vis Sci. 2000; 41:1513–1522)

It is well established that high dosage ingestion of the carotenoid canthaxanthin for medical purposes or as an artificial skin coloring agent, can cause a crystalline retinopathy in humans. Although, in the end, canthaxanthin retinal crystals were found to be reversible and to not cause any vision loss,1 their presence in the retina of humans ingesting high dosages was of sufficient concern to regulatory bodies that a primate model for crystals was needed to establish a dose relationship and a no effect level (NOEL) for crystals.

Canthaxanthin also has been used at much lower effective dosages for the direct and indirect coloring of human foods for more than 30 years without the development of retinal crystals.

In a recent study with cynomolgus monkeys (Macaca fascicularis),2 it was demonstrated that these animals are a suitable model for canthaxanthin-induced retinal crystal formation similar to that observed in humans after large and long-term intakes. As previously reported, canthaxanthin at doses of 5.4, 16.2, and 48.6 mg/kg body wt/d fed to monkeys over 2.5 years led to the deposition of crystal-like birefringent inclusions in the inner layers of the peripheral retina. The presence of these deposits did not interfere with retinal functions as measured by electroretinography (ERG).

The above previously reported study and the present study were part of a large trial aimed to reproduce canthaxanthin-induced retinopathy and to assess the chronic tolerance to the eye and certain systemic organ systems. In this article, two other aspects of the overall study are reported.

First, the threshold dose level was determined for the induction of the microscopically detectable birefringent, canthaxanthin-induced retinal crystals. This includes a semiquantitative assessment of birefringent particles in the retinas of monkeys on lower doses of canthaxanthin, that is, 0.2 to 1.8 mg/kg body weight/d over 3 years, together with the same analysis of the dose groups from 5.4 to 48.6 mg/kg body weight/d reported earlier. These findings are reported within the context of measured canthaxanthin plasma levels over 3 years and retina and liver canthaxanthin concentrations measured at the time when they were euthanatized.

In the second part of the study, the spatial distributions of canthaxanthin and its main isomers, including possible metabolites, were analyzed in the peripheral and paracentral part of the retina and in the macula after administration of extremely high doses, that is, 200 and 500 mg canthaxanthin/kg body wt/d. The carotenoid concentrations in these parts of the retina were correlated with the presence of crystals in individual animals.

Finally, because lutein and zeaxanthin, both natural constituents of the macula, are considered to be essential for normal visual function,1–5 these carotenoids also were assayed...
to ensure that canthaxanthin did not alter their normal concentration.

**Materials and Methods**

**Chemicals**

A commercial, cold water-soluble carotenoid formulation (Dry Canthaxanthin 10% WS beadlets; F. Hoffmann-La Roche, Basel, Switzerland), containing 10% canthaxanthin (β,β-carotene-4,4′-dione), and filling material were used as reported previously for the 5.4 to 48.6 mg/kg body wt/d, whereas a beadlet distribution part of the study.

These animals were used in the “spatial distribution” part of the study. For the extremely high doses, a 30% crystalline suspension of canthaxanthin in vegetable oil was used (F. Hoffmann-La Roche). Finally, pure, crystalline canthaxanthin, 4′-OH-echinenone, isozeaxanthin, lutein, and zeaxanthin were used as high-performance liquid chromatography (HPLC) standards.

**Study Protocol**

The study was performed at Covance GmbH (Münster, Germany), under Good Laboratory Practice (GLP) conditions and according to the guidelines for animal trials in the European Union (EU) and to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Cynomolgous monkey housing and treatment with canthaxanthine were as previously described.2

Four male and four female animals per group were assigned to doses of 0.2, 0.6, 1.8, 5.4, 16.2, and 48.6 mg/kg body wt/d, and 14 control animals were administered placebo compounds. Eight placebo animals and the 5.4 to 48.6 dose groups were killed after 2.5 years to detect retinal crystals and validate the model as reported previously.2 Because crystals were found in all retinas of the 5.4 to 48.6 mg/kg body wt/d dose groups, the remaining six placebo animals and those subjected to the lower doses of 0.2 to 1.8 mg/kg body wt/d were killed after 3 years of treatment to establish a dose-response relationship and a no-effect level.

The experimental design in search of crystal formation also called for large doses of canthaxanthan, that is, 200 and 500 mg/kg body wt/d. These were administered for 4.5 years as a 30% oily suspension, at which time the formulation was changed to 48.6 mg/kg body wt/d beadlets for the remaining 6 months. Placebo group animals received vegetable oil only during the time period up to the 4.5-year point and placebo beadlets thereafter. These animals were used in the “spatial distribution” part of the study.

**In Vivo Retinal Biomicroscopy and Electroretinography**

The eyes of all animals were examined before the start of treatment, in intervals of approximately 3 months during the trial, and at the point immediately before killing. To test visual function, ERG was performed in accordance with the international standard for clinical ERG on all animals after 12 and 24 months.2 An additional ERG was performed after 36 months in the groups with 0.2, 0.6, 1.8, 200, and 500 mg/kg body wt/d.

Clinical observations of the fundus of both eyes of each animal were performed on frozen sections according to similar principles.1 In some animals of the 5.4 to 48.6 groups, assessment was performed on frozen sections according to similar principles.

**Necropsy**

At the time of killing all animals were examined externally and peripherally. Positive findings of crystals or other retinal anomalies that were noted were documented on a standard clinical indirect ophthalmoscopy retinal diagram. Photographs were taken of each animal’s central retina using a table-mounted Zeiss model RCM 310 fundus camera (Zeiss, Jena, Germany). In addition, selected areas of the retina, especially in the peripheral areas were photodocumented using the Nikon photograph slit lamp system during the course of the examination process.

**Retina Preparation**

Eyes were enucleated and rinsed in phosphate-buffered saline (PBS). Adherent fat and muscle were removed and the retinas prepared as reported previously.1

**Semiquantitative Analysis of Birefringent Retinal Inclusions**

Semiquantitative analysis of canthaxanthin inclusions was performed by screening the flat-mounted retinas of the right eyes under polarized light with low to high magnification. The assessment of the grading took into consideration the density, spatial distribution, and location within the retinal cell layers as well as the size, color, and morphology of the inclusions (Table 1). In some animals of the 5.4 to 48.6 groups, assessment was performed on frozen sections according to similar principles.

**HPLC Analysis of Canthaxanthin and Other Carotenoids in Retinas**

Two methods for preparation of the left retinas were used. One method was used for the whole retina, whereby the tissue was isolated as a whole immediately postmortem and frozen at −70°C for HPLC. In the second method, the spatial distributions of canthaxanthin, its isomers, and derived compounds were analyzed in the macula, paracentral, and periphery of the left retinas of the placebo/oil group and the 200 mg and 500 mg canthaxanthin/kg body wt/d group. Macular samples for HPLC analysis were punched out under a dissecting microscope using a 2-mm trephine (Hans Geuder GmbH, Heidelberg, Germany). A 10-mm trephine was then used to punch out the ring-shaped paracentral sample. The remaining retina was then referred to as the peripheral retinal sample. We noted that the peripheral specimen typically included a residual coating of vitreous humor, which is known to form a strong attachment to the retina near the ora serrata. The fresh weight of each sample was determined, and the retinas were frozen at −70°C for HPLC analysis (see below).

For the chemical analysis of carotenoids, whole retinas were extracted with 10% ethanol in n-hexane, containing 0.05% butylhydroxytoluol. After evaporation under nitrogen at 30°C, the residue was redissolved in n-hexane/dichloromethane (1:1, v:v) and submitted to HPLC. A slightly modified extraction procedure was used for the retina samples designed for spatial distribution analysis. These retina samples were treated with 1 ml ethanol (containing 0.2% butylhydroxytoluene), exhaustively crushed with a glass rod, and
retinas, typical retention times were reported previously.2 Retinoids, respectively. For determinations of carotenoids in whole retina pieces were 15.8, 16.8, 19.8, 22.4, 24.5, and 24.8 minutes for all-trans canthaxanthin, 9-cis, and 13-cis canthaxanthin, 4’OH-echinenone, isozeaxanthin, lutein, and zeaxanthin, respectively. For determinations of carotenoids in whole retinas, typical retention times were reported previously.2 Recoveries determined with reference substances of the above carotenoids, including the whole extraction procedure, were between 85% and 98%. The limit of detection for all carotenoids was approximately 250 pg.

In those cases where whole retinas were analyzed, it was not possible at times to remove all vitreous body. Nonetheless, canthaxanthin concentrations are reported in as nanograms per retina.

Canthaxanthin Analysis in Plasma and Liver
Plasma samples were obtained from all animals before the start of the study and at approximately 3-month intervals thereafter (Fig.1). Canthaxanthin plasma analysis was performed as described previously.2 Photometric detection limit was 20 µg/l canthaxanthin at 470 nm. Canthaxanthin in liver was analyzed by standard methods. The detection limit was 20 µg/kg.

RESULTS
Electroretinography
Routine ophthalmoscopy and ERG did not reveal treatment-related changes when compared to the corresponding control group or to a historical background collective at the study institute (P ≥ 0.05). Thus, there were no electrophysiologically detectable adverse effects on visual function, measured up to a period of 3 years, for all dosages including the highest dose tested of 500 mg canthaxanthin/kg body wt/d.

Retinal Biomicroscopy
Retinal crystals were not detectable in any dose group up to 48.6 mg/kg body wt/d for 2.5 to 3 years using this technique.2

TABLE 1. Semiquantitative Assessment of Canthaxanthin Inclusions in the Retina

<table>
<thead>
<tr>
<th>Periphery</th>
<th>Grade 0</th>
<th>No red, orange, or yellow birefringent material present</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Grade 1</td>
<td>Presence of dichroitic particles, which are in bright field of red, orange, or yellow color. In polarized light they appear as red, orange, yellow but also greenish shining entities, mainly as single microgranules or grouped as nestlike islets in ganglion cells nearby blood vessels, size ~1 µm or smaller</td>
</tr>
<tr>
<td></td>
<td>Grade 2</td>
<td>Presence of red, orange, or yellow colored, birefringent particles, mainly as microgranules, grouped as nestlike islets in ganglion cells nearby blood vessels, but also as single clumps and needles, size ~1-3 µm</td>
</tr>
<tr>
<td></td>
<td>Frequency: regularly scattered along the ora serrata, reaching ~3-5 mm from ora serrata, decreasing to the center. Large areas of the retina free of any particles</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Grade 3</td>
<td>Presence of red, orange, or yellow colored, birefringent particles, both as nestlike islets of microgranules in ganglion cells and the inner plexiform layer and clumps, rods and needles, size ~1-6 µm</td>
</tr>
<tr>
<td></td>
<td>Frequency: high density around the ora serrata, decreasing to the center with particles reaching up to 7-8 mm from the ora serrata</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Grade 4</td>
<td>Presence of red, orange, or yellow colored, birefringent particles, both as nestlike islets of microgranules and clumps, rods and needles, size ~1-6 µm</td>
</tr>
<tr>
<td></td>
<td>Frequency: very high density around the ora serrata, decreasing to the center with particles reaching up to 10-12 mm from the ora serrata</td>
<td></td>
</tr>
</tbody>
</table>
|           | Grade 5 | Like 4, but with ring-shaped, extremely dense accumulations along the ora serrata. Macroscopically visible as intense red ring

<table>
<thead>
<tr>
<th>Macula</th>
<th>Grade 0</th>
<th>No birefringent particles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade 1</td>
<td>Presence of birefringent particles of orange to yellow color in bright field microscopy, in polarized light more golden shining, rodlike structures of ~0.5-1 µm size</td>
<td></td>
</tr>
<tr>
<td>Frequency: only frequently single particles visible</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade 2</td>
<td>Same type of particles</td>
<td></td>
</tr>
<tr>
<td>Frequency: medium density, mainly in the center of the fovea</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade 3</td>
<td>Same type of particles</td>
<td></td>
</tr>
<tr>
<td>Frequency: high density, center of fovea and parafoveal</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Electroretinography
Routine ophthalmoscopy and ERG did not reveal treatment-related changes when compared to the corresponding control group or to a historical background collective at the study institute (P ≥ 0.05). Thus, there were no electrophysiologically detectable adverse effects on visual function, measured up to a period of 3 years, for all dosages including the highest dose tested of 500 mg canthaxanthin/kg body wt/d.

Retinal Biomicroscopy
Retinal crystals were not detectable in any dose group up to 48.6 mg/kg body wt/d for 2.5 to 3 years using this technique.2

FIGURE 1. Time course of plasma canthaxanthin concentrations during treatment of cynomolgous monkeys with canthaxanthin.
In extreme doses of 200 and 500 mg/kg body wt/d, glittering greenish yellow crystals could be seen occasionally in the paramacular area, but more commonly in the periphery close to the ora serrata in those monkeys that demonstrated histologically visible crystals of grade 3 to 5 (see Table 4).

**Canthaxanthin-Induced Crystals**

At the time of preparation of the retinas, the maculas of treated animals, especially of those on extremely high doses more than 5 years, appeared to be stained with an intense yellow color. In contrast, those of the placebo animals were usually pale in color and were hardly distinguishable.

When the monkeys were administered dosages of 0.6 mg canthaxanthin/kg body wt/d or greater, the retinas showed the presence of typical birefringent inclusions within a peripheral, circular zone of the retina, (Table 2). This (0.6 mg/kg body wt/d) was the lowest dose level at which such inclusions are reported. (The grading parameters are shown in Table 1 and the data of all dosage groups from 0.2 to 48.6 mg/kg body wt/d are summarized in Table 2). No retinal inclusions were observed at the lowest dose of 0.2 mg canthaxanthin/kg body wt/d, nor were they observed in the placebo-treated animals. The incidence and grade of birefringent inclusions in periph-

<table>
<thead>
<tr>
<th>Canthaxanthin (mg/kg/d)</th>
<th>Peripheral Retina</th>
<th>Macula</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Incidence</td>
<td>Grade* (range)</td>
</tr>
<tr>
<td>0/placebo</td>
<td>13</td>
<td>0 0</td>
</tr>
<tr>
<td>0.2</td>
<td>8</td>
<td>0 0</td>
</tr>
<tr>
<td>0.6</td>
<td>8</td>
<td>4 1-2</td>
</tr>
<tr>
<td>1.8</td>
<td>7</td>
<td>4 1-2</td>
</tr>
<tr>
<td>5.4</td>
<td>8</td>
<td>8 1-2</td>
</tr>
<tr>
<td>16.2</td>
<td>8</td>
<td>8 2-3</td>
</tr>
<tr>
<td>48.6</td>
<td>8</td>
<td>8 1-4</td>
</tr>
</tbody>
</table>

* Established according to the procedure described in Materials and Methods and Table 1.

**FIGURE 2.** Threshold level and dose-dependent increase of canthaxanthin-induced, crystal-like inclusions in the peripheral retina. In photomicrographs of flat-mounted retina, the crystal-like inclusions are seen as tiny, light reflecting spots. Ora serrata on top of the photographs. (A) Placebo, grade 0; (B) 0.2 mg/kg body wt/d, grade 0; (C) 0.6 mg/kg body wt/d, grade 1; (D) 1.8 mg/kg body wt/d, grade 2; (E) 16.2 mg/kg body wt/d, grade 3; (F) 48.6 mg/kg body wt/d, grade 4. Polarized light; magnification, ×45.
eral retinas and maculas for dosages of 0.6 mg/kg body wt/d or greater were dose dependent (Table 2, Figs. 2, 3). The width of the zone containing particles was from 1 to 5 mm in the groups up to 5.4 mg/kg body wt/d, and 8 to 10 mm or even larger, reaching the nervus opticus, in the groups of 16.2 to 48.6 mg/kg body wt/d. Increasing proportions of larger particles were seen mostly at the high doses.

Grades of canthaxanthin inclusions in the extremely high doses of 200 and 500 mg/kg body wt/d were in the same range as in the 16.2 and 48.6 mg/kg body wt/d groups, except for three animals that exhibited massive accumulations of crystal-line material that was visible macroscopically as a red seam along the ora serrata (Fig. 4). These animals had the highest total canthaxanthin concentrations of up to 2500 pg/mg retina.

Canthaxanthin Plasma and Liver Concentrations
Plasma concentrations of all-trans canthaxanthin were dose related throughout the study ($P = 0.03$; nonlinear). Peak levels were measured at approximately 3 months of treatment (group means of 188-8211 µg/l at 0.2-48.6 mg/kg body wt/d, respectively), followed by a decrease in almost all groups (Fig. 1). Thereafter, concentrations remained at a relatively constant level up to termination after 2.5 or 3 years. Mean plasma concentrations at time of killing were within a range of 160 to 4400 µg/l.

Means of canthaxanthin plasma levels from animals with 200 and 500 mg/kg body wt/d were similar to those of animals with 48.6 mg/kg body wt/d. Only a few individuals of those receiving excessive high doses, had occasionally very high plasma levels, that is, more than 10,000 µg/l (data not shown).

Concentrations of all-trans canthaxanthin in the liver (Table 3) were correlated with the mean plasma levels measured over the last 1.5 to 2 years before killing ($r = 0.784$, $P < 0.001$).

Correlation of All-trans-Canthaxanthin and its Isomers and Metabolites Concentrations within the Retina to the Grades of Birefringent Inclusions
All-trans canthaxanthin, its isomers, as well as its derived compounds are identified in Table 3. In the groups from 0.2 to 48.6 mg/kg body wt/d, all-trans canthaxanthin, its 9-cis/13-cis isomers and the canthaxanthin derived reduction products 4’-OH-echinenone and isozeaxanthin increased with dose (Table 3). This relationship was nonlinear. All-trans canthaxanthin concentrations in the retina were correlated with plasma concentrations over the 1.5 to 2 years before killing ($r = 0.738$, $P < 0.001$) and with semi-quantification of birefringent inclusions.

The macular physiological pigments lutein and zeaxanthin were detected in retinas of all animals. Their relative amounts per retina, as represented by the peak areas, varied considerably and were not affected by canthaxanthin treatment (data not shown).
The existence of any correlations between the presence of canthaxanthin crystals, as determined by the grading scheme, and the canthaxanthin content including the isomers and metabolites, as identified by HPLC, was examined for the three retinal areas, that is, the macula, the paracentral retina surrounding the macula and the peripheral retina from monkeys treated with extremely high doses (200 and 500 mg/kg body wt/d). The main result of this investigation was that there were considerable individual variations in retinas of animals within the same dosage group. This variation was observed both in the density of canthaxanthin-induced inclusions and the respective concentrations of canthaxanthin and its related carotenoids. Nonetheless there was an overall correlation between grades of inclusions and all-trans canthaxanthin concentrations in the periphery. In the macula, this correlation was not as clear.

In the macula, 4'-OH-echinenone was the major canthaxanthin-derived compound and was approximately 1.8-fold higher than all-trans canthaxanthin and 1.6-six higher than isozeaxanthin. The sum of 4'-OH-echinenone and isozeaxanthin concentrations in the macula was 3-fold higher than all-trans canthaxanthin, suggesting that these two canthaxanthin-derived compounds were the major constituents of the macular inclusions. In contrast, in the paracentral and peripheral retina, all-trans canthaxanthin was the predominant carotenoid (Fig. 5).

The physiological carotenoids lutein and zeaxanthin were not affected by the canthaxanthin treatment but showed an area specific ratio (Table 4, Fig. 5). Ratios of lutein to zeaxanthin in the macula were approximately 1:2 to 1:7, whereas in the paracentral part, lutein concentrations were twofold higher than zeaxanthin.

**DISCUSSION**

**Dose–Response Relationship**

This study clearly demonstrated that at the dose of 0.2 mg canthaxanthin/kg body wt/d administered for 3 years in cynomolgus monkeys none of the birefringent inclusions in the retina noted at higher doses were observed. At 0.6 mg/kg body wt/d, 50% of barely detectable positive findings were observed. We, therefore, consider this to be the threshold level. At higher doses, the grade of accumulations was dose related and peaked at 16.2 mg/kg body wt/d, whereas in the very high doses (200 and 500 mg/kg body wt/d), the grading reflected no greater numbers of inclusions. However, the occasional presence of a heretofore unobserved macroscopically reddish, visible ring around the ora serrata was added to the range of observable retinal findings in those animals receiving ultra-high

**Table 3. Canthaxanthin Content in the Retina and Liver**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Canthaxanthin Isomers (ng/retina)</th>
<th>Canthaxanthin-Derived Compounds (% of all-trans)</th>
<th>Liver*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All-trans Canthaxanthin</td>
<td>9-cis/13-cis Canthaxanthin</td>
<td>4'-OH-Echinenone</td>
</tr>
<tr>
<td>0/Placebo</td>
<td>13</td>
<td>&lt;DL†</td>
<td>&lt;DL†</td>
</tr>
<tr>
<td>0.2</td>
<td>8</td>
<td>6.7 ± 2.1</td>
<td>0.7 ± 0.4</td>
</tr>
<tr>
<td>0.6</td>
<td>8</td>
<td>30.7 ± 27.5</td>
<td>4.4 ± 4.2</td>
</tr>
<tr>
<td>1.8</td>
<td>7</td>
<td>87.5 ± 49.2</td>
<td>12.7 ± 6.5</td>
</tr>
<tr>
<td>5.4</td>
<td>8</td>
<td>130.3 ± 100.4</td>
<td>20.0 ± 16.0</td>
</tr>
<tr>
<td>16.2</td>
<td>8</td>
<td>257.2 ± 137.6</td>
<td>38.0 ± 18.7</td>
</tr>
<tr>
<td>48.6</td>
<td>8</td>
<td>173.9 ± 132.4</td>
<td>25.6 ± 22.3</td>
</tr>
</tbody>
</table>

* Mean values from animals sacrificed at weeks 137 and 156.
† Below detection limit (DL) in HPLC retina analysis of 0.25 ng.
doses. The presence of a similar ring has also been reported in a single human postmortem evaluation.6

Our no-effect dose level of canthaxanthin crystals is similar to that reported in humans by biostatistical evaluation of 411 cases.7 In this study, a highly significant dose-response relationship \( P < 0.0001 \) with increasing percentage of cases with crystals was observed both as a function of daily and total dose. The minimum dose where crystals were seen in 9.6% of cases was 30 mg/d, corresponding to \( \frac{1}{0.5} \) mg/kg body wt/d.

The fact that in this study only about half of the animals at the higher doses also had macular inclusions remains unexplained. It may be significant that these macular inclusions differed in their morphology and color from the peripheral type.

It should be emphasized that the observed retinal depositions after long-term high dosage intakes of canthaxanthin had no adverse effects on visual functions neither in humans1 nor in monkeys (this study and Ref. 2).

**Ophthalmological Evaluations**

Clinical retinal biomicroscopic examinations did reveal crystals in the retinas of the study subjects that were administered the highest dosages of 200 and 500 mg/kg body wt/d canthaxanthin. Compared to the crystals seen typically in the human, which appear yellowish, are easily visible and surround the macula,8–10 the in vivo clinical examinations in the cynomolgus monkeys of this study revealed crystals much smaller in size and less densely organized and were therefore much more difficult to detect. Their color was more greenish-yellow in appearance, and their locations in the field of the retina was substantially different. Although macular and paramacular crystals were seen occasionally, these crystals were never found in an organized band surrounding the macula, as with humans. Rather, scattered crystals or isolated groupings were detected within the central retina. In addition, the peripheral retina, adjacent to the ora serrata, was seen to have both scattered crystals, and with the highest doses, a dense band of fine crystals.

Because they were so small and often sparse in their distribution, the crystals were difficult to detect biomicroscopically and virtually impossible to photograph. Table 4 demonstrates that the histologic method was far more sensitive in determining the presence of such fine crystals. Nevertheless, there is nearly perfect correlation (6/7) of the clinical in vivo observation of peripheral crystals with the highest grades of postmortem histologic birefringent crystals. In the macula, although the correlation is less obvious, there were three animals in which the crystals were detected in vivo, compared to the five seen to have crystals by histology.
the macula compared to more peripheral retinal areas has
duction products in a specific manner. Such “special handling”
pecific capability of macular tissue to either reduce this carot-
trans canthaxanthin, was the major fraction where its concentration
alyzed in more detail in monkeys subjected to the extremely
resulted in more detailed information about the physiological
The spatial distribution of putative canthaxanthin metabolites (chemical structure, see Figs. 6A through 6C) was analyzed in more detail in monkeys subjected to the extremely high doses. In the periphery, the mother compound, all-trans canthaxanthin, was the major fraction where its concentration correlated with the grade of peripheral inclusions. In contrast, in the macula the metabolite 4'-OH-echinenone was about twofold higher than all-trans canthaxanthin, with isozeaxanthin being as high as all-trans canthaxanthin, and indicating a specific capability of macular tissue to either reduce this carotenoid or to accumulate these two canthaxanthin-derived reduction products in a specific manner. Such “special handling” of different derivatives and/or isomers of retinal carotenoids by the macula compared to more peripheral retinal areas has already been cited by Bone et al., who found reversal of concentration dominance within the macula favoring (3R,3'R)-zeaxanthin (Fig. 6D) over (3R,3'R,6'R)-lutein (Fig. 6E) as well as an increasing concentration of the (3R,3'S) meso-zeaxanthin isomer (Fig. 6F) in moving toward the macula.
The higher fraction of these canthaxanthin-derived compounds in the macula also suggests that they were major constituents of the more yellowish inclusions noted in the maculae of these monkeys. Notably, the absorbance maxima of 4'-OH-echinenone and isozeaxanthin are approximately 450 nm (in organic solution), which is shifted 12 nm toward the shorter wavelength in peak absorbance from the more red-colored canthaxanthin, thus becoming more similar in color to the physiological yellow macular carotenoids, zeaxanthin and lutein, which are located in the receptor axon layer (Henle’s fiber) and the inner plexiform layer. This might also explain why the macula of treated animals appeared more yellow compared to placebo animals, especially because our monkeys had no external source of zeaxanthin nor lutein over the whole study period. The standard monkey chow contained no carotenoids, and the animals received only low xanthophyll containing fruits (banana, apples) twice a week.

In contrast to our findings in monkey retinas, the relation of 4'-OH-echinenone to all-trans canthaxanthin was approx 1:10 throughout the retina in a single postmortem human canthaxanthin retinopathy case, whereas, in the choroid and pigment epithelium, 4'-OH-echinenone and isozeaxanthin were present as the major carotenoids. Therefore, it may be hypothesized that in primates, various tissues are capable of

### Carotenoid Analysis in the Retina

Chemical analysis demonstrated that canthaxanthin and its isomers, as well as its derived compounds, were present in the retinas of all animals receiving between 0.2 and 48.6 mg/kg body wt/d (i.e., even the 0.2 mg/kg body wt/d dosage, devoid of crystals did demonstrate the presence of some canthaxanthin). For these dosages, all-trans canthaxanthin was the main fraction in whole retina. All-trans canthaxanthin concentrations in the retina increased from approximately 7 ng/retina at the lowest dose to more than 200 ng/retina at the higher doses. Because the grade of inclusions in the retina correlated with the administered canthaxanthin dose, the plasma all-trans canthaxanthin concentrations and the retinal all-trans canthaxanthin concentrations, it can therefore be argued strongly that the retinal inclusions are canthaxanthin-derived compounds.

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reducing canthaxanthin to 4'-OH-echinenone and isozeaxanthin. Unfortunately, our method to analyze plasma and liver canthaxanthin was not designed to detect these two compounds, and we were not able to gain information as to whether 4'-OH-echinenone and isozeaxanthin were present in these tissues as well. Unfortunately, our results also do not provide insight about potential mechanisms nor locations of the interconversion of canthaxanthin and its metabolites. In addition to the possibility of specific enzymatic reduction by the macular tissue, many other scenarios might be possible, including the bioconversion (i.e., reduction of canthaxanthin) as an intermediate step in carotenoid metabolism, which has been reported in several organisms, such as crustaceans, fish and birds, and appears to be a common reaction.

The ratio of zeaxanthin to lutein varied with retinal topographic location. In the macula more zeaxanthin than lutein was found, and in the paracentral and peripheral retina the ratio was reversed. This is consistent with earlier findings in macaque and squirrel monkeys. Zeaxanthin and lutein concentrations in the retina varied considerably as they do in humans, but were not influenced by increasing amounts of canthaxanthin. This is of particular importance, because there is evidence that the presence of these macular carotenoids is linked to a reduced risk for age-related macular degeneration. On the basis of our findings, we have no reason to conclude that canthaxanthin supplementation interfered with absolute amounts or distribution patterns of lutein and zeaxanthin, factors that are normally found within the primate macula and that may have significant physiological and protective value.

Plasma and Liver Analysis

Plasma concentrations of all-trans canthaxanthin in monkeys monitored up to week 131 were dose and time dependent (Fig. 1). Higher plasma concentrations were found at the beginning of the measurements, that is, between weeks 13 to 42 in contrast to the interval of weeks 53 to 131. The mechanism underlying the decreases between week 42 to 52 are as yet unexplained. Canthaxanthin plasma levels of up to 200 µg/l were noted at the no-effect level of 0.2 mg/kg body wt/d. Means of canthaxanthin plasma levels from animals with 200 and 500 mg/kg body wt/d were similar to those of animals with 48.6 mg/kg body wt/d.

CONCLUSIONS

This dose–response relationship study demonstrates for the first time in an animal model that the phenomenon of retinal crystallization of canthaxanthin is associated only with high dosages, that is, more than 0.2 mg/kg body wt/d. Furthermore, the results of this study are consistent with those of Köpcke et al., in his biostatistical analysis of human data for which the minimum dosage canthaxanthin at which crystals were seen was ~0.5 mg/kg body wt/d. His findings are thus in agreement with and validate this monkey model. On the basis in part of the herein reported findings, authorities in several countries (Scientific Committee of Food in Europe, Joint FAO/WHO Expert Committee on Food Additives) established, by applying a safety factor, an allowed daily intake of 0.03 mg/kg body wt/d.

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References