BUFF

Safety Assessment of Retinol and Retinyl Palmitate as Used in Cosmetics

CIR EXPERT PANEL MEETING

SEPTEMBER 10-11, 2012

Cosmetic Ingredient Review

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August 16, 2012

Memorandum

To: CIR Expert Panel

From: Wilbur Johnson, Jr.
Manager/Lead Specialist

Subject: Re-review Document on Retinol and Retinyl Palmitate

A final report on the safety assessment of retinol and retinyl palmitate with the following conclusion was published in 1987: On the basis of the available animal and clinical data presented in this report, the CIR Expert Panel concludes that retinyl palmitate and retinol are safe as cosmetic ingredients in the present practices of use and concentration. The Expert Panel confirmed this original conclusion in 2005, after reviewing published and unpublished data that became available since 1987. Since then, a National Toxicology Program (NTP) photocarcinogenicity study on retinyl palmitate and retinoic acid was completed and studies on the photogenotoxicity of retinyl palmitate and retinol have entered the published literature. With support from FDA, CIR staff determined that these new data warranted another re-review of the safety of retinol and retinyl palmitate in cosmetics.

A copy of the re-review document on these two ingredients is included along with the CIR report history, Literature search strategy, Ingredient Data profile, Minutes from the June 13-14 2005 Expert Panel Meeting, 1987 published CIR final report on retinol and retinyl palmitate, and 2012 FDA VCRP data. The following memoranda from the Environmental Working Group (EWG) and Personal Care Products Council (PCPC) relating to the NTP photocarcinogenicity study on retinyl palmitate and retinoic acid are included as well:

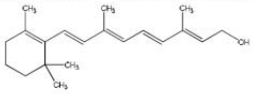
1. Letter from EWG to the NTP, provided by PCPC (pcpc1 pdf file);
2. Letter (study comments) from EWG to the NTP (data 1 pdf file); and
3. Letter (study comments) from the Personal Care Products Council to the NTP (data 2 pdf file)

After reviewing this re-review document and accompanying materials, the Expert Panel needs to determine whether the CIR final report on retinol and retinyl palmitate needs to be reopened.

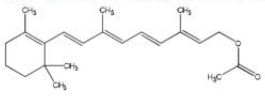
If a decision to reopen is made, it is recommended that the 7 other retinyl esters, namely Retinyl Acetate (a generally recognized as safe food additive), Retinyl Propoinate, Retinyl Linoleate, Retinyl Oleate, Retinyl Rice Branate, Retinyl Soyate, and Retinyl Tallate should be added. Their structures, along with those of retinol and retinyl palmitate for reference, appear on the last page.

If this safety assessment is reopened and these additional 7 ingredients are included as recommended, the Panel should determine what, if any, additional data would be needed to evaluate the safety of these additional ingredients, keeping in mind the likelihood that each ingredient would be metabolized, if absorbed, to retinol and the respective fatty acids. For example, Retinyl Oleate would be cleaved by esterases in the skin to retinol and oleic acid (by the same pathway that vitamin D is released from endogenous retinyl esters). Additionally, these potential add-ons only vary structurally from Retinyl Palmitate by derivations in fatty-acid residue chain length and/or degree of unsaturation.

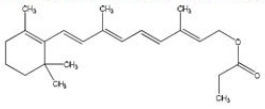
Retinol – CIR safe 2005 – re-open for new data



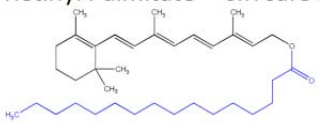
No brainer add-on? Retinyl Acetate – GRAS foods – 27 uses (2012 VCRP)



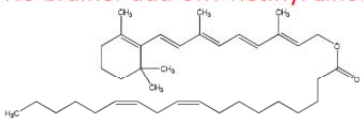
No brainer add-on? Retinyl Propionate – 9 uses (2012 VCRP)



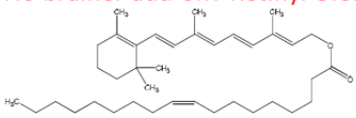
Retinyl Palmitate – CIR safe 2005 – re-open for new data



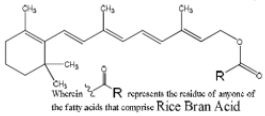
No brainer add-on? Retinyl Linoleate – 30 uses (2012 VCRP)



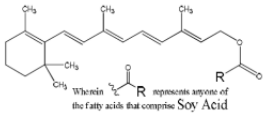
No brainer add-on? Retinyl Oleate



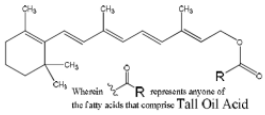
No brainer add? Retinyl Rice Branate



No brainer add-on? Retinyl Soyate



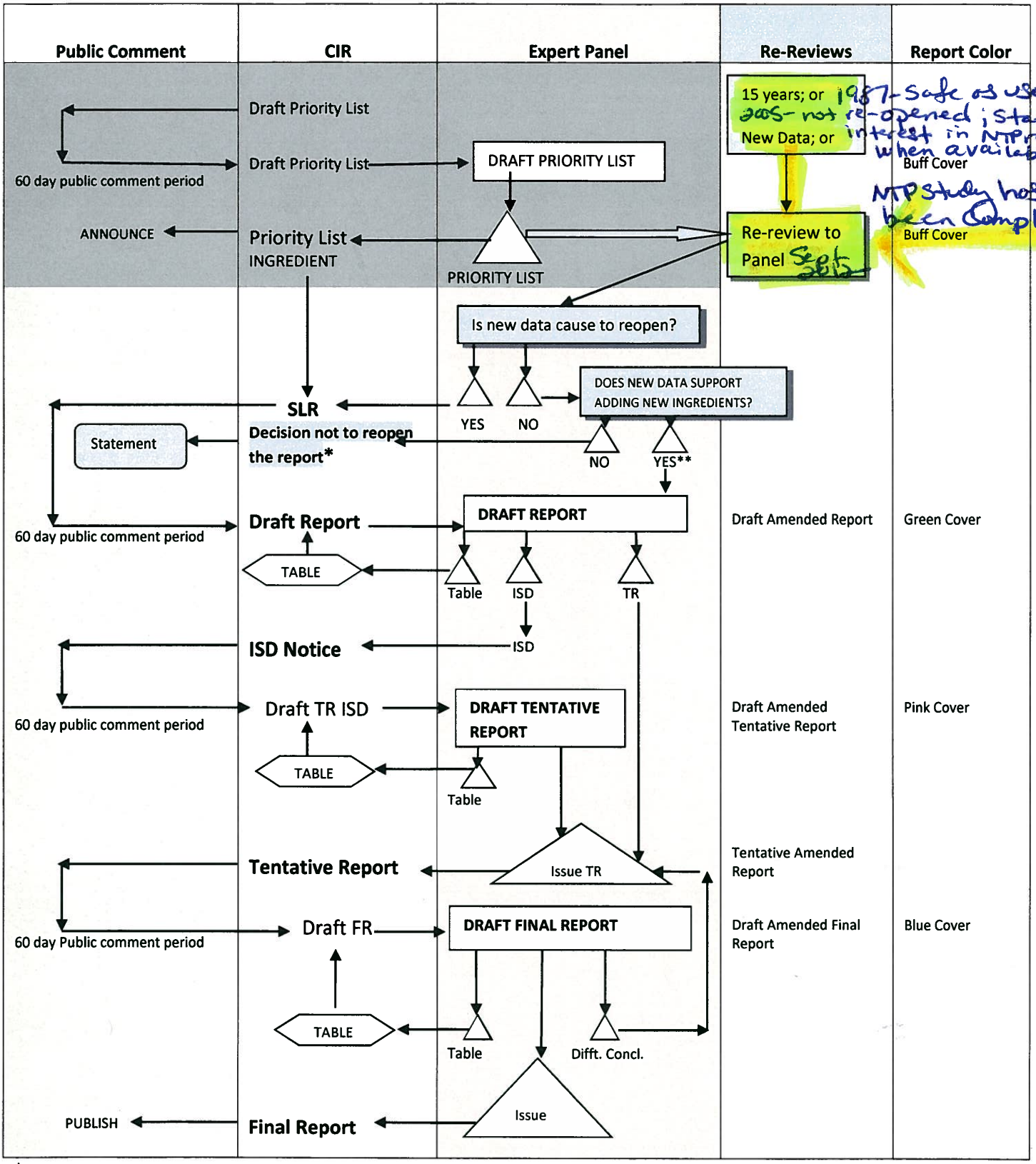
No brainer add-on? Retinyl Tallate



Retinyl Palmitate

SAFETY ASSESSMENT FLOW CHART

Sept 2012



***The CIR Staff notifies of the public of the decision not to re-open the report and prepares a draft statement for review by the Panel. After Panel review, the statement is issued to the Public.**

****If Draft Amended Report (DAR) is available, the Panel may choose to review; if not, CIR staff prepares DAR for Panel Review.**

Expert Panel Decision

Document for Panel Review

Option for Re-review

CIR History of:

Retinol and Retinyl Palmitate

A final report on the safety assessment of retinol and retinyl palmitate with the following conclusion was published in 1987: On the basis of the available animal and clinical data presented in this report, the CIR Expert Panel concludes that retinyl palmitate and retinol are safe as cosmetic ingredients in the present practices of use and concentration.

1st Re-review, Belsito and Marks Teams/Panel: June 13-14, 2005

The Panel confirmed its original conclusion and agreed that the final report on retinyl palmitate and retinol should not be reopened. This decision, published in 2008, was based on a review of published and unpublished data that became available after publication of the final report in 1987. The Panel stated its awareness of an ongoing National Toxicology Program (NTP) photococarcinogenicity study on retinyl palmitate and interest in reviewing the results upon study completion.

2nd Re-review, Belsito and Marks Teams/Panel: September 10-11, 2012

In response to a request from the Food and Drug Administration, a re-review document containing pertinent studies that entered the published literature since the Expert Panel's 2005 decision not to re-open the final report on retinol and retinyl palmitate was prepared for the Panel's review. Prior to development of this document, a letter from the Environmental Working Group (EWG) to the National Toxicology Program on the need to expedite the NTP photocarcinogenicity study on retinyl palmitate that was ongoing was made available by the Personal Care Products Council. The same is true for 2 letters to the editor of *Mutation Research* (summarized in the report text) relating to earlier published studies on the photogenotoxicity of retinyl palmitate. The letter from the EWG is being made available as an attachment for the Panel's review, along with comments on the completed NTP photocarcinogenicity study on retinyl palmitate from the EWG and Personal Care Products Council.

Retinol and Retinyl Palmitate Check List for September, 2012. Analyst – Wilbur Johnson																				
			Acute toxicity					Repeated dose toxicity				Irritation			Sensitization					
	Skin Penetration	Penetration Enhancement	ADME	Oral	Parenteral	Dermal	Inhale	Oral	Parenteral	Dermal	Inhale	Ocular Irritation	Dermal Irr. Animal	Dermal Irr Human	Sensitization Animal	Sensitization Human	Repro/Devel toxicity	Genotoxicity	Carcinogenicity	Phototoxicity
Retinol	X		X	X	X			X		X				X		X	X	X	X	X
Retinyl Palmitate	X		X					X				X	X	X		X	X	X	X	X

Literature Search on Retinol and Retinyl Palmitate*

Ingredients	Toxline &PubMed	ChemIDplus	Multidatabase (See legend*)	DART	SciFinder	RTECS
Retinol	14,895				5,706	
RP	106				1,379	

*Data in Table: Publications found; Multidatabase = HSDB, CCRIS, ITER, IRIS, Gene-Tox, and LacMed

Searches (years 2004-2012) Performed on12/6/2011
Search Updated on 7/24/2012

Ingredients/Search Terms

Retinol
CAS No. 68-26-8
CAS No. 11103-57-4

Retinyl Palmitate **(RP)**
CAS No. 79-81-2

SciFinder Search Terms

68-26-8
11103-57-4
79-81-2

Transcript

June 13-14,2005(95th)CIR Expert Panel Meeting (Full Panel) – Day 2

Retinol and Retinyl Palmitate

Dr. Belsito stated that a Final Report with the following conclusion was published in 1987: On the basis of the available data presented in this report, the Expert Panel concludes that Retinyl Palmitate and Retinol are safe as cosmetic ingredients in the present practices of use and concentration.

After reviewing data on these ingredients that have been made available since the Final Report was issued, Dr. Belsito noted that the only new information relates to the fact that Retinol and Retinyl Palmitate are used in hair sprays and that no inhalation toxicity data are available. He added that the Panel's inhalation toxicity boilerplate could be used to address the absence of these data.

Dr. Belsito also noted that both Retinol and Retinyl Palmitate absorb light in the short-wavelength UVA range, and that no photoirritation or phototoxicity data are included in the original safety assessment. Furthermore, the original safety assessment does not contain an explanation in the discussion as to why these data were not needed.

In light of the preceding concerns, Dr. Belsito stated that Dr. McEwen provided the Panel with recent photoirritation and photoallergy data on sunscreen products containing Retinol at concentrations ranging from 0.04% to 0.09% or 0.01% Retinyl Palmitate at today's meeting. He noted that these concentrations are consistent with the current use concentration data that were provided. The study results were as follows: For products containing Retinol, the photoirritation data on 30 subjects and the photoallergy data on 88 subjects were all negative. The product containing 0.01% Retinyl Palmitate induced neither phototoxicity (10 subjects tested) nor photoallergy (29 subjects tested).

Dr. Belsito said that the preceding data plus consideration of the lack of clinical reports of Retinol-

or Retinyl Palmitate-induced photoirritation/photoallergy in the published literature indicate that the original safety assessment does not need to be reopened, provided that these data/observations are incorporated into the re-review document.

Dr. Marks noted that the following information should also be mentioned in the discussion section: (1) the ongoing NTP photococarcinogenicity study on Retinyl Palmitate, (2) the epoxy photodecomposition products of Retinyl Palmitate are phototoxic, but not photomutagenic, and (3) low rate of percutaneous absorption of Retinyl Palmitate (in acetone vehicle, not a cosmetic vehicle) *in vitro*.

As a point of clarification concerning the photodecomposition of Retinyl Palmitate by UV light, Dr. Slaga noted that epoxy and ethoxy photodecomposition products were formed. He noted that these are generally reactive intermediates, but, in the study by Cherng et al. (2005), they did not bind to DNA nor induce mutagenicity.

Dr. Bergfeld noted that the following points were elaborated upon by the Panel and will be addressed/mentioned in the discussion section: (1) photoirritation/photo-allergy potential of Retinol and Retinyl Palmitate, (2) the ongoing NTP photococarcino-genicity study on Retinyl Palmitate, (3) the epoxy photodecomposition products of Retinyl Palmitate are phototoxic, but not photomutagenic, and (4) low rate of percutaneous absorption of Retinyl Palmitate (in acetone vehicle, not a cosmetic vehicle) *in vitro.*

The Panel unanimously concluded that the CIR Final Report on Retinyl Palmitate and Retinol should not be reopened.

Dr. Bergfeld said that the Panel will have an opportunity to review the final version of each re-review document that is being considered today at the next Panel meeting

Report

Safety Assessment of Retinol and Retinyl Palmitate as Used in Cosmetics

Status: Re-Review for CIR Expert Panel Review
Release Date: August 17, 2012
Panel Meeting Date: September 10-11, 2012

The 2012 Cosmetic Ingredient Review Expert Panel members are: Chair, Wilma F. Bergfeld, M.D., F.A.C.P.; Donald V. Belsito, M.D.; Curtis D. Klaassen, Ph.D.; Daniel C. Liebler, Ph.D.; Ronald A Hill, Ph.D. James G. Marks, Jr., M.D.; Ronald C. Shank, Ph.D.; Thomas J. Slaga, Ph.D.; and Paul W. Snyder, D.V.M., Ph.D. The CIR Director is F. Alan Andersen, Ph.D. This report was prepared by Wilbur Johnson, Jr., M.S., Manager/Lead Specialist and Ivan Boyer, Ph.D., Toxicologist.

1101 17TH STREET, NW, SUITE 412 ◊ WASHINGTON, DC 20036-4702 ◊ PH 202.331.0651 ◊ FAX 202.331.0088 ◊ CIRINFO@CIR-SAFETY.ORG

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INTRODUCTION

A Cosmetic Ingredient Review (CIR) Final Report with the following conclusion was published 1987: On the basis of the available data presented in this report, the Expert Panel concludes that retinyl palmitate and retinol are safe as cosmetic ingredients in the present practices of use and concentration.[1] The summary from this final safety assessment is included at the end of this report.

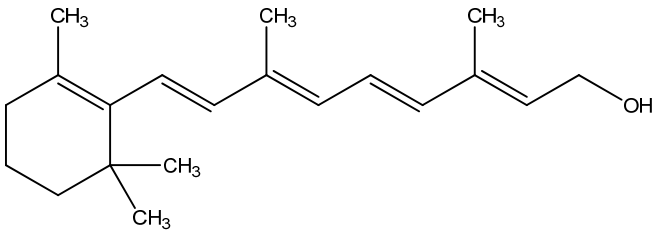
Subsequently, at the June 13-14, 2005 CIR Expert Panel meeting, the Panel confirmed its original conclusion and agreed that the final report on retinyl palmitate and retinol should not be reopened. This decision, published in 2008, was based on a review of published and unpublished data that became available after publication of the final report in 1987.[2] In the discussion section that established the basis for confirming the original conclusion, the Panel stated its awareness of an ongoing National Toxicology Program (NTP) photococarcinogenicity study on retinyl palmitate and interest in reviewing the results upon study completion. This statement as well as others from the published discussion are included at the end of this report for the Panel's review.

The NTP photocarcinogenicity study has been completed and a draft NTP technical report on the photocarcinogenesis study of retinoic acid and retinyl palmitate (scheduled peer review date: January 26, 2011) was made available in 2010. Excerpts of the NTP study abstract are included in the Carcinogenicity section of this CIR report. Other pertinent safety test data that became available since the 2005 decision are also included for the Panel's review. It should be noted that CIR's decision to evaluate the safety of retinol and retinyl palmitate in cosmetics for the third time is based on its commitment to reviewing the NTP data, with the support of the Food and Drug Administration.

CHEMISTRY

Definition and Structure

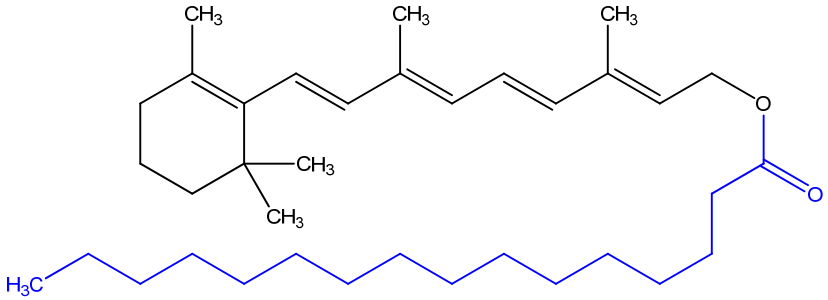
Retinol (CAS Nos. 68-26-8 and 11103-57-4) is the fat-soluble, diterpenoid that conforms to the following structural formula.[3]



RETINOL

Other chemical names include: 3,7-dimethyl-9-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2,4,6,8-nonatetraqen-1-ol; dry formed vitamin A; vitamin A; and vitaminum a.

Retinyl palmitate (CAS No. 79-81-2) is the palmitic acid ester of retinol. Other chemical names include: axerophthol palmitate; retinol, hexadecanoate; retinol palmitate; and vitamin A palmitate.[3] The structural formula for this ester is included below.



RETINYL PALMITATE

USE

Cosmetic

Both retinyl palmitate and retinol function as a skin conditioning agent – miscellaneous in cosmetic products.[3] According to information supplied to the Food and Drug Administration (FDA) by industry as part of the Voluntary Cosmetic Registration Program (VCRP) in 2012 (Table 1), retinyl palmitate was being used in 2,059 cosmetic products and retinol was being used in 186 cosmetic products.[4] In a survey of 29 consumer cosmetic skin products labeled to contain retinoids, most products were found to contain either retinol or retinyl palmitate at concentrations of 2.2% (w/w), while few products contained both ingredients. A number of products also contained cis isomers of retinol that could be quantitatively distinguished from the all-trans compound.[5] These survey results were included in a 2009 FDA publication.

Cosmetic products containing retinyl palmitate and retinol may be applied to the skin and hair, or, incidentally, may come in contact with the eyes and mucous membranes. Products containing these ingredients may be applied as frequently as several times per day and may come in contact with the skin or hair for variable periods following application. Daily or occasional use may extend over many years.

Retinyl palmitate is used in hair sprays, dusting and face powders, and foot powders and sprays, and could possibly be inhaled. In practice, 95% to 99% of the droplets/particles released from cosmetic sprays have aerodynamic equivalent diameters >10 μm.[6,7,8,9] Therefore, most droplets/particles incidentally inhaled from cosmetic sprays would be deposited in the nasopharyngeal and bronchial regions and would not be respirable (i.e., they would not enter the lungs) to any appreciable amount.[6,7]

TOXICOKINETICS

Percutaneous Absorption and Distribution

Retinol

Retinol absorption from cosmetic formulations has been measured through excised human skin in diffusion cell studies.[10] Absorption through skin into the receptor fluid was 0.3% of the applied dose from a gel vehicle and 1.3% from an emulsion vehicle in 24-h studies.

In an FDA publication, it was noted that after reviewing animal and clinical data available in 1987 and 2005, the CIR Expert Panel concluded that retinyl palmitate and retinol were safe as cosmetic ingredients in the then-current practices of use and concentration. However, questions have persisted concerning the safety of exposure to retinoids in cosmetic products.[5] It was also noted that because topically applied retinoids such as retinol and retinyl palmitate readily penetrate the skin, systemic increases in retinoid levels could result from exposure to retinoid-containing cosmetics [CIR's staff

toxicologist, Dr. Ivan Boyer, noted that this statement does not clearly convey the distinction between dermal penetration and absorption into the bloodstream, and may be misleading to some readers. The statement was revised to read as follows: The FDA noted that topically applied retinoids , such as retinol and retinyl palmitate, readily penetrate the skin and, thus, may beabsorbed into the bloodstream to elevate retinoid levels systemically.] This statement is based on the following *in vitro*/*in vivo* study.

The percutaneous absorption of retinol (vitamin A) from cosmetic formulations was studied to predict systemic absorption and to understand the significance of the skin reservoir in *in vitro* absorption studies.[11] Viable skin samples from fuzzy rats or human subjects were assembled in flow-through diffusion cells for *in vitro* absorption studies. In vitro studies using human skin and a gel or emulsion vehicle found 0.3% and 1.3% of the applied retinol, respectively, in the receptor fluid at 24 h. Levels of absorption in the receptor fluid increased over 72 h with the gel and emulsion vehicles. Using the gel vehicle, *in vitro* rat skin studies found 23% in skin and 6% in receptor fluid at 24 h, while 72-h studies found 18% in skin and 13% in receptor fluid. Thus, significant amounts of retinol remained in rat skin at 24 h and these amounts decreased by approximately 5% over 72 h, with proportional increases in the receptor fluid.

In vivo absorption studies using fuzzy rats were performed in glass metabolism cages for collection of urine, feces, and body content. Retinol (0.3%) formulations (hydroalcoholic gel and oil-in-water emulsion) containing ^{3}H-retinol were applied and absorption was measured at 24 h or 72 h. Results were reported as % of applied dose. The *in vivo* rat studies with the gel indicated 4% systemic absorption of retinol after 24 h, which did not increase at 72 h. Retinol remaining in rat skin was 18% and 13% of the applied dermal dose after 24 h and 72 h, respectively. Similar results were obtained using the oil-in-water vehicle.

The studies summarized above indicated that retinol formed a reservoir in rat skin both *in vivo* and *in vitro*. Little additional retinol was bioavailable from the gel or emulsion 24 h after application. Comparison of the *in vitro* and *in vivo* results for rat skin indicates that the fraction of the applied retinol in the receptor fluid after 24 h *in vitro* was comparable to the fraction absorbed systemically through the skin after 24 h *in vivo*. Therefore, the best single estimate of retinol systemic absorption from *in vitro* human skin studies is the 24-h receptor fluid value. However, the receptor fluid value from the 72-h extended study may be used in worst-case exposure estimates. In conclusion, *in vivo* skin absorption studies can be useful in determining whether to include material in the in vitro skin reservoir as absorbable material in estimates of systemic absorption.[11]

Enhanced penetration of retinol was found from the dermal application of retinol in solid lipid nanoparticles incorporated into an oil-in-water cream, when compared to a conventional formulation.[12] Highest retinol concentrations were found in the stratum corneum and the upper viable epidermal layer. The penetration of retinyl palmitate was influenced even more by incorporation into the solid lipid nanoparticles.

Retinyl Palmitate

The accumulation of retinyl palmitate and retinol was evaluated in the skin of SKH-1 mice (8 groups; 12 mice/sex/group) treated tropically with the following concentrations of retinyl palmitate (in cream) for 13 weeks (5 days/week): 0.1%, 0.5%, 1.0%, 5.0%, 10%, and 13%.[13] Additionally, 2 groups were untreated and treated with control cream, respectively. Because products containing retinyl palmitate are frequently applied to sun-exposed skin, and exposure to sunlight and UV light can alter endogenous concentrations of retinoids in the skin, mice in this study were also exposed to simulated solar light 5 days per week.

Retinyl palmitate diffused into the skin and was partially hydrolyzed to retinol. The levels of retinyl palmitate in the skin of mice administered retinyl palmitate cream were greater than control values. The levels of both retinyl palmitate and retinol increased with the application of higher concentrations of retinyl palmitate in the cream (statistically significant linear dose trends). The levels of retinyl palmitate and retinol in the stratum corneum, epidermis, and dermis of the untreated group, control cream-treated group, and 0.5% retinyl palmitate-treated group were determined. When compared to untreated mice and mice treated with the control cream, the levels of retinyl palmitate and retinol in the 0.5% retinyl palmitate group were substantially higher in all layers of the skin. In each of the 3 treatment groups, levels of both retinyl palmitate and retinol were significantly higher in the epidermis, lowest in the dermis, and somewhat intermediate in the stratum corneum ($P < 0.05$). The study results indicated that topically applied retinyl palmitate can elevate the concentrations of retinyl palmitate and retinol in the skin of SKH-1 mice.[13]

Retinol and Retinyl Palmitate

To evaluate the potential use of solid nanoparticles (SLN) in dermatology and cosmetics, glyceryl behenate SLN loaded with vitamin A (retinol and retinyl palmitate) and incorporated in a hydrogel and oil-in-water cream were tested for their influence on the penetration of these substances into porcine skin.[12] Conventional formulations served for comparison. Excised full thickness skin was mounted in Franz diffusion cells and the formulations were applied for 6 h and 24 h. High retinol concentrations were found in the upper skin layers following SLN preparations. The deeper regions showed only very low vitamin A levels. Because of a polymorphic transition of the lipid carrier with subsequent drug expulsion following the application to the skin, the drug localizing action appears to be limited for 6 h to 24 h. Best results were obtained with retinol SLN incorporated in the oil-in-water cream retarding drug expulsion. The penetration of the occlusion-sensitive drug retinyl palmitate was enhanced even more thatn that of retinol by SLN incorporation. Transepidermal water loss and the influence of drug free SLN on retinyl palmitate uptake exclude pronounced occlusive effects. Therefore, enhanced retinyl palmitate uptake probably derives from specific SLN effects and not from non-specific occlusive properties. In summary, enhanced absorption of retinol was found from SLN incorporated into an oil-in-water cream, when compared to a conventional formulation. Highest retinol concentrations were found in the stratum corneum and the upper viable epidermal layer. The penetration of retinyl palmitate was elevated even more than that of retinol by incorporation into the SLN.

Three experiments were performed to determine the time course for accumulation and disappearance of or retinyl palmitate and retinol in the stratified layers of skin from female SKH-1 mice that received single or repeated topical applications of creams containing 0.5% or 2% retinyl palmitate.[14] In the first experiment, 10-week old female SKH-1 mice (3 per group) received topical application of 2% retinyl palmitate cream (~ 75 µl) to the dorsal skin areabetween the base of the neck and the base of the tail. The cream was applied manually with a gloved finger to cover the entire dorsal region of the animal. Each application achieved approximately 2 mg cream/cm^2 of skin. Control animals received the vehicle cream only. At 1, 2, 3, or 6 days after cream application, the animals were killed and skin samples collected. The design of the second experiment was the same as that of the first, except that the animals (3 per goup)received topical application of 0.5% retinyl palmitate cream. The animals were killed at 1, 2, 3, or 6 days after cream application and skin samples collected. In the third experiment, the protocol was similar to that of the first experiment except that the mice received daily topical application of 2% retinyl palmitate cream (~75 µL) to the dorsal skin area, base of the neck and anterior to the base of the tail for 4 consecutive days. The animals were killed at 1, 3, 6, 11, or 18 days after the last cream application, and skin samples were collected. In all 3 experiments, samples of skin were separated into the stratum corneum, epidermis, and dermis.

Study results indicated that retinyl palmitate diffused rapidly into the stratum corneum and epidermal skin layers within 24 h following application of creams containing retinyl palmitate. Of the 3 skin layers, the highest level of retinyl palmitate and retinol per weight unit (ng/ml) at all time points was found in the epidermis. Levels of retinyl palmiate and retinol were lowest in the dermal layer and intermediate in the stratum corneum. The levels of retinyl palmitate and retinol in the separated skin layers and in the intact skin decreased with time, but levels of retinyl palmitate remained higher than control values for a period of up to 18 days. The application of retinyl palmitate to mouse skin elevated the normal physiological levels of retinyl palmitate and retinol in the skin.[14]

Levels of retinyl palmitate and retinol in the skin of female SKH-1 mice were investigated in another study. The animals received a standard HNIH-31 diet.[15] Retinoid levels were evaluated at ages ranging from 10 weeks to 68 weeks of age. An age-related effect on the levels of retinyl palmitate and retinol in the skin and liver of female mice was noted. The levels of retinyl palmitate and retinol were highest in the epidermis of 20-week-old mice, and decreased when the age increased to 60 and 68 weeks. The total amount of retinyl palmitate at 20 weeks of age was found to be 1.52 ng/mg skin, and decreased approximately 4-fold at 60 and 68 weeks of age. A similar trend was found for the effects of age on the levels of retinol.

Physiological Role of Vitamin A and Metabolites

A review article updating the available information on the physiological role of vitamin A and its biologically active metabolites in the skin is available.[16] Many of the studies included in this review are captured in the published CIR final report and re-review summary on retinol and retinyl palmitate.

TOXICOLOGY

Repeated Dose Toxicity

Oral – Animal

Retinol

A study was performed to analyze the vitamin A content of liver and serum from 13 adult female African green vervet monkeys (*Chlorocebus aethiops*).[17] The monkeys were wild-caught and held in captivity for 2 years, during which they consumed a standard primate diet. Monkeys were fed lab diet containing 45 nmol (43 IU) vitamin A/g dry food (as retinyl acetate). Liver vitamin A concentration (mean ± 1 standard deviation) was 14.6 ± 2.3 μmol retinol/g liver. In comparison, subtoxicity in humans is defined as at least 1 μmol/g liver. Retinyl palmitate accounted for most of the hepatic vitamin A (59% ± 2.5%). The serum retinol concentration (0.93 ± 0.21 μM) was not elevated. Hypertrophy and hyperplasia of hepatic stellate cells were observed, which, in conjunction with elevated hepatic vitamin A, are evidence of toxicity.

Subclinical hypervitaminosis A in rats causes fragile bones. A study was performed to investigate possible mechanisms for vitamin A action.[18] Three groups of 15 mature female Sprague-Dawley rats were fed the following, respectively, for 12 weeks: standard diet containing 12 IU vitamin A per g pellet (control, C), or a standard diet supplemented with 120 IU (10 x C), or 600 IU (50 x C) vitamin A/g pellet. At the conclusion of the study, the concentrations of serum retinyl esters were elevated 4- and 20-fold in rats fedc the supplemented diets. Although neither average food intake nor final body weights were significantly different among the groups, a dose-dependent reduction in serum levels of vitamins D and E, but not K, was found. In the 50 x C group, the length of the humerus was the same as in controls, but the diameter was reduced (- 4.1%, $p < 0.05$). Peripheral quantitative computed tomography (pQCT) at the diaphysis showed that bone mineral density (BMD) was unchanged and that periosteal circumference was decreased significantly (- 3.7%, $p < 0.05$). However, ash weight of the humerus was not affected. Because bone volume decreased, volumetric BMD, as measured by the bone ash method, increased (+ 2.5%, $p < 0.05$). It was concluded that vitamin A interference with other fat-soluble vitamins is a possible indirect mechanism of vitamin A action. Moreover, BMD measurements did not reveal early adverse skeletal changes induced by moderate excess vitamin A ingestion in rats.

Retinyl Palmitate

The consequences of acute and chronic vitamin A (retinyl palmitate) supplementation at therapeutic and excessive doses on the redox state of SMP, isolated from adult rat cerebral cortex and cerebellum, were studied.[19] Groups of 5 adult male Wistar rats were used. The animals were treated once a day during 3 different periods: acutely (3 days or 7 days) or chronically (28 days). The 5 groups of animals were gavaged daily with one of the following: vehicle (0.15 M NaCl), and retinyl palmitate at 1000 IU/kg, 2500 IU/kg, 4500 IU/kg, and 9000 IU/kg. The lower 2 doses were described as therapeutic and, the other 2, excessive. All doses induced lipid peroxidation, protein carbonylation, and oxidation of protein thiol groups in cerebral cortex and cerebellum SMP. Furthermore, retinyl palmitate supplementation induced an increase in the superoxide ($O_2^{\bullet -}$) anion production, indicating an uncoupling in the electron transfer chain. In addition, locomotory and exploratory activity, which are associated with the cerebral cortex and cerebellum, were reduced by both acute and chronic retinyl palmitate supplementation. Retinyl palmitate induced a decrease in both locomotory and exploratory behavior. Together, these results show that vitamin A could be toxic at the subcellular level, inducing mitochondrial dysfunction and altering cerebral cortex and/or cerebellum-dependent behavior.

The effect of short-term vitamin A (retinyl palmitate) supplementation on the rat liver was studied using groups of 6 to 7 male Wistar rats (90 days old).[20] Groups were dosed orally (gavage; dose volume = 0.8 ml maximum) with one of the following: 0.15 M saline (control); 1,000 IU/kg/day; 2,500 IU/kg/day; 4,500 IU/kg/day; and 9,000 IU/kg/day. The animnals were dosed once per day for 3 or 7 days. The animals were killed 24 h after the last dose; the liver was removed and homogenized, and liver mitochondria were isolated and studied. Increased liver peroxidation was observed in the liver of rats that received retinyl palmitate supplementation at 2,500; 4,500; or 9,000 IU/kg/day for 3 days (1.4- to 1.7-fold; $p < 0.01$). However, hepatic lipid peroxidation levels did not change after vitamin A supplementation for 7 days.

Increased (1.3- to 1.6-fold; $p < 0.01$) $O_2^{\bullet -}$ production in hepatic submitochondrial particles (SMP) of the rats that were treated with retinyl palmitate at 2,500; 4,500; or 9,000 IU/kg/day for 3 days was observed. Retinyl palmitate supplementation at any dose tested induced a 1.3- to 1.7-fold increase in $O_2^{\bullet -}$ production in hepatic SMP isolated from rats that were treated for 7 days ($p < 0.01$). Mitochondria that were isolated from the liver of rats that received vitamin A supplementation at 2,500; 4,500; or 9,000 IU/kg for 3 days presented higher lipid peroxidation levels when incubated for 10 minutes with buffer ($p < 0.05$). $CaCl_2$ (75 μM) induced a 2.5- to 2.9-fold increase of lipid peroxidation in liver mitochondria

from animals that received retinyl palmitate supplementation at 2,500; 4,500; or 9,000 IU/kg for 3 days, when compared with mitochondria isolated from the liver of animals that received saline for 3 days ($p < 0.01$). Overall, the results of this study showed that mitochondria are a target of vitamin A-associated toxicity *in vivo*.[20]

A study was performed to compare electron flux and oxidative/nitrosative stress parameters on the heart among rats supplemented with vitamin A.[21] Adult male rats (strain not stated; 90 days old) were grouped (7 per group) and treated (by gavage; total volume = 0.8 ml) as follows for 28 days: vehicle (0.9% saline solution), and 1000, 2500, 4500, and 9000 IU retinyl palmitate (in saline)/kg/day. The heart was removed for analysis after 28 days. Electron flux and oxidative/nitrosative stress parameters were evaluated and statistics were conducted using Anova one-way, followed by Dunnet's *post hoc* test of significance. Retinyl palmitate supplementation induced an increase in the oxidation of lipids and proteins, and mitochondrial 3-nitrotyrosine content, an enzymatic imbalance (indicated by the increased superoxide dismutase (SOD)/catalase (CAT) ratio), and a decrease in electron transfer between respiratory chain complexes. These results suggest that vitamin A induces oxidative/nitrosative stress and mitochondrial impairment in the heart.

Oral - Human

Retinyl Palmitate

One hundred and twenty-nine participants with severely sun-damaged skin on their lateral forearms were randomized to receive placebo or 25,000; 50,000; or 75,000 IU/day retinyl palmitate for 12 months.[22] The primary study end points were clinical and laboratory safety of vitamin A (retinyl palmitate). The measurement end points included quantitative karyometric image analysis and assessment of retinoid and rexinoid receptors in sun-damaged skin. There were no significant differences in expected clinical or blood toxicities between the groups of all participants randomized to placebo, 25,000 IU/day, 50,000 IU/day, and 75,000 IU/day. There was no evidence of a dose response for any of the following toxicities: alopecia, cheilitis, conjunctivitis, dry skin, peeling, epistaxis, headache, muscle stiffness, dysuria, exanthema, serum liver function tests (i.e., aspartate aminotransferase, alanine aminotransferase, and serum alkaline phosphatase and triglycerides). Because liver scans were only to be repeated in participants who experienced severe clinical or other signs of toxicity, there were no participants who underwent repeat liver scans during this clinical trial.

Karyometric features were computed from the basal cell layer of skin biopsies, and a total of 22,600 nuclei from 113 participants were examined, showing statistically significant, dose-response effects for retinyl palmitate at the 25,000 and 50,000 IU/day doses. These karyometric changes were associated with increases in retinoic acid receptors αand β, and retinoid X receptor α at the 50,000 IU/day retinyl palmitate dose. It was concluded that the the retinyl palmitate doses of 50,000 and 75,000 IU/day for 1 year proved to be safe and equally more efficacious than the 25,000 IU/day dose and can be recommended for future skin chemoprevention studies.[22]

Oral/Dermal - Human

Retinyl Palmitate and Retinol

Two groups of 14 female volunteers of child-bearing age were maintained on a vitamin A-poor diet and treated topically for 21 days with creams containing 0.30% retinol or 0.55% retinyl palmitate on approximately 3000 cm^2 of their body surface area, amounting to a total of approximately 30,000 IU vitamin A/subject/day.[23] Subsequently, after a 12-day wash-out period, the study groups received single oral doses of 10,000 IU or 30,000 IU retinyl palmitate, corresponding to the maximal European union (EU) allowance during pregnancy or three-times higher, respectively. Blood samples were collected over 24 h on study days -3 (pre-study), 1, 21 (first and last days of topical treatment), and 34 (oral administration) at 0, 1, 2, 4, 6, 12, 14-16 h and 24 h after treatment for determination of plasma concentrations of retinol and retinyl palmitate. Except for transient mild (retinyl palmitate group) to moderate (retinol group) local irritation on treatment sites, no adverse local or systemic effects were noted. Details relating to skin irritation reactions are presented in the following section. On days 1 or 21 of topical treatment, no changes were measured in individual or group mean plasma C_{max}, $AUC_{0\text{-}24\ h}$, or other pharmacokinetic parameters of retinol or retinyl palmitate relative to pre-study data. In contrast, single oral doses of retinyl palmitate at 10,000 IU or 30,000 IU produced dose-related and sustained increases in *Cmax* and $AUC_{0\text{-}24\ h}$ values of plasma retinyl palmitate. The results of this study provide evidence that human topical exposure to retinol- or cosmetic creams containing retinyl palmitate at 30,000 IU/day and maximal use concentrations do not affect plasma levels of retinol or retinyl palmitate , whereas, single oral doses at 10,000 IU or 30,000 IU produce significant increases in plasma retinyl palmitate.

Skin Irritation

Human

Retinyl Palmitate and Retinol

Two groups of 14 female volunteers of child-bearing age were maintained on a vitamin A-poor diet and treated topically for 21 days with creams containing 0.30% retinol or 0.55% retinyl palmitate on approximately 3000 cm^2 of their body surface area, amounting to a total of approximately 30,000 IU vitamin A/subject/day.[23] Subsequently, after a 12-day wash-out period, the study groups received single oral doses of 10,000 IU or 30,000 IU retinyl palmitate, corresponding to the maximal EU allowance during pregnancy or three-timesthe allowance, respectively. Transient mild (retinyl palmitate group) to moderate (retinol group) local irritation reactions on treatment sites were noted, but no adverse local or systemic effects. After approximately 1 week of topical application, 13 of 28 study subjects had skin reactions (rash, itching) on treated sites. Therefore, the regimen was temporally adjusted to partial treatment for 9 subjects, or treatment of affected sites was suspended for one to 4 days.

In the retinol treatment group, skin reactions were observed in 9 or 14 subjects. For one subject with moderate to severe reactions, treatment was discontinued for 4 days. Although 2 of 14 subjects in the retinyl palmitate treatment group experienced itching, treatment was continued as scheduled. In all subjects, skin reactions stabilized and/or subsided after approximately 10 to 12 treatment days. One-hundred nine (total) adverse events in 25 subjects were reported. The severity of adverse events was rated by the medical investigator to be mild for 70 events, moderate for 37 events, and severe for 2 cases. For 44 adverse events, a possible or probable relationship to the treatment was considered, including itch, rash, or other dermal manifestations at the treatment sites. No objective adverse effects or individual complaints were recorded after the oral administration of retinyl palmitate at 10,000 IU or 30,000 IU. Additional study results are included at the end of the preceding section on Repeated Dose Toxicity.[23]

Modification of Allergic Sensitization/Immune Responses

Animal

Retinyl Palmitate

The role of vitamin A on allergic sensitization during lactation and after weaning was investigated using an *in vivo* system for postnatal allergic sensitization in Balb/c mice.[24] Different diets (basal/vitamin A (as retinol equivalents); elimination/vitamin A (as retinyl palmitate supplemented) were fed to the dams throughout lactation and directly to the pups after weaning. The diets were defined as follows: basal diet (4.5 mg vitamin A [i.e., 4500 retinol equivalents]), VA-elimination diet (i.e., prepared using a vitamin A-free 'vitamin mix']), and vitamin A supplemented diet (122,000 retinol equivalents as retinyl palmitate [i.e., 216 mg of retinyl palmitate/kg diet supplemented]). Allergic sensitization was induced with a single peritoneal ovalbumin (OVA) injection at day 28 after weaning. The phenotype of lymphocytes was analyzed by flow cytometry and functional data were obtained by analysis of IL-4/IFN-γ cytokine production and antibody production (OVA-specific IgG1 and IgE) in the offspring.

Vitamin A/retinyl palmitate supplementation during lactation and after weaning decreased CD3+, CD4+, CD8+, and B220+ populations in splenic lymphocytes and significantly enhanced IL-4 production and OVA-specific IgE measured after sensitization. In contrast, mice fed the vitamin A-elimination diet displayed no significant alteration of lymphocyte numbers and a slightly increased IL-4 production after sensitization. Thus, a single allergen injection during postnatal development induced allergic sensitization, the degree of which depended on the vitamin A content of the maternal diet during lactation and the diet of the pups after weaning. This suggests that dietary vitamin A levels can play an important reole determining the severity of the allergic sensitization.[24]

Human

Retinyl Palmitate

A study comparing the effect of vitamin A on cytokine secretion by mononuclear cells of adults and preterm newborns was performed.[25] Mononuclear cells (MC) from individuals of the 2 age groups were incubated with retinyl palmitate (0.5 to 50 μM) in the presence of phytohemagglutinin forassessing IL-2 and IFNγ production or LPS for assessing

IL-1β, IL-1ra, IL-6, and IL-10 secretion. ELISA was used to test the level of cytokines in the supernatants. Retinyl palmitate *in vitro* inhibited the production of the anti-inflammatory cytokine IL-1ra by MC of preterm newborns and adults, but did not affect the secretion of the pro-inflammatory cytokines IL-1β, IL-6, and IL-10. Retinyl palmitate inhibited IL-10 secretion by cells from adults, but it did not significantly affect this function in cells from newborns, except when a supraphysiological concentration (50 μM) was tested. Additionally, retinyl palmitate stimulated the secretion of IL-2 by cells isolated from adults, but had no effect on those derived from premature neonates. The results suggest that retinyl palmitate may affect the immune function of premature infants via inhibition of IL-1ra secretion.

A study was conducted to determine how retinol supplementation modified associations between gut-cytokine immune responses and the resolution of different diarrheal pathogen infections.[26] Stools were collected from 127 children (5 to 15 months old) enrolled in a randomized, placebo-controlled vitamin A supplementation trial. The children were screened for enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), and *Giardia lamblia*. Fecal concentrations of the following were measured using an enzyme-linked immunosorbent assay: interleukin (IL)-6, IL-8, IL-4, IL-5, IL-10, monocyte chemoattractant protein 1 (MCP-1), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ). Retinol-supplemented children with fecal MCP-1 or IL-8 concentrations less than the median of detectable concentrations and IL-10 concentrations of at least median concentrations had longer durations of EPEC infection than did children in the placebo group. In supplemented children, detectable fecal TNF-α or IL-6 concentrations were associated with shorter ETEC infection durations, whereas, MCP-1 concentrations of at least the median were associated with longer infection durations. Children in this group who had Il-4, IL-5, or IFN-γ concentrations of at least median detectable concentrations had shorter durations of *G. lamblia* infections. It was concluded that the effect of supplementation on association between fecal cytokine concentrations and pathogen infection resolution depends on the role of inflammatory immune responses in resolving specific pathogen infections.

Case Reports

Retinol

A 25-year-old male patient had a history of increased vitamin A intake from a natural source, in addition to a high dose of vitamin A supplements.[27] He had supplemented his food intake with high doses of vitamin A (220,000 IU/day) and consumed steroidal anabolic drugs. The patient was diagnosed with chronic liver disease (attributed to increased vitamin A intake) with severe fibrosis, signs of portal hypertension, and marked hyperplasia of Ito cells. It was noted that chronic vitamin A toxicity may produce severe liver damage. The authors also noted that additional toxicity produced by anabolic intake could not be ruled out.

A 60-year-old male presented with symptoms of muscle soreness, alopecia, nail dystrophy, and ascites.[28] His clinical history revealed ingestion of large doses of vitamin A. He had been taking 500,000 units of vitamin A daily for 4 months, then 100,000 units monthly for 6 months. The patient continued to deteriorate with the development of refractory ascites, renal insufficiency, encephalopathy, and failure to thrive. Liver biopsy revealed the presence of Ito cells and vacuolated Kupffer cells, without the presence of cirrhosis. The symptoms after orthotopic liver transplantation.

Facial dermatitis developed in a 54-yer-old female, who had no history of allergy nor atopy, after using an antiwrinkle cream.[29] A use self-test of the product resulted in development of itchy erythema. Subsequent avoidance of the product resulted in completely normal skin. Results of a repeated open application test (ROAT) of the cream indicated a positive reaction after 2 days (i.e., 5 applications). Test results for one of the ingredients, retinyl palmitate (in polycaprolactone [PCL]) were strongly positive (++ reaction). Subsequent patch tests yielded a + reaction for 5% retinyl palmitate in petrolatum, negative results for 5% PCL in petrolatum, and a ++ reaction for retinyl palmitate in PCL. When the patient performed an ROAT on 5% retinyl palmitate in petrolatum, a strongly infiltrated and itchy reaction (spreading to the forearm), appeared within 2 days (3 applications). Retinyl palmitate in PCL and 5% retinyl palmitate in petrolatum were both tested in consecutive controls. Retinyl palmitate in PCL yielded a doubtful reaction (+?) on day 3 in 1 of 25 control patients. Retinyl palmitate (5% in petrolatum) yielded a doubtful reaction (+?) on day 3 in 3 of 27 patients. All were negative on day 7.

The authors noted that PCL may be used to encapsulate the retinyl palmitate in nanoparticles, and thereby increase the bioavailability in the skin. According to the manufacturer's data, the particle size in the antiwrinkle cream is > 100 nm; therefore, they do not satisfy nanotechnology criteria. However, it is suspected that the combination of retinyl palmitate in PCL may have contributed significantly to the development of allergic contact dermatitis to a rare cosmetic allergen (retinyl palmitate). The concentration of retinyl palmitate in PCL is confidential information, but it is much less than the 5% concentration used for patch testing in petrolatum.[29]

In the following case report, intrahepatic cholestasis was caused by vitamin A intoxication. A 46-year-old patient consumed a shake and 2 multivitamin tablets for 12 years.[30] This equated to more than the recommended daily allowance for vitamin A consumption. Deranged liver function tests were consistent with a cholestatic process. Liver biopsy revealed features that were pathognomonic of vitamin A toxicity, without the usual fibrosis. When administration of the supplements ceased, both the jaundice and alkaline phosphatase levels normalized completely. It was noted that this case highlights the importance of health care providers documenting non-prescribed dietary supplements and considering them in the etiology of cholestatic liver disease.

A case of acute hypervitaminosis A was reported.[31] An 18-year-old female presented with complaints of headache, vomiting, back pain, and diplopia after ingesting a high-dose vitamin A capsule (~ 10 million international units). The following signs were reported: bilateral papilloedema, slightly dilated pupils symmetrically (reaction to light noted), visual acuity of 6/60 (left eye) and 6/18 (right eye), and bilateral 6th cranial nerve palsy (more marked on the left side). MRI of brain and orbits were normal.

REPRODUCTIVE AND DEVELOPMENTAL TOXICITY

Oral - Animal

Retinyl Palmitate

The effects of retinyl palmitate ingestion on sexual maturation and mammary gland development were determined in the context of a human food-based diet (whole food diet).[32] Female adolescent Sprague-Dawley rats randomized into 3 dietary groups (6 rats per group) were used. At 20 days of age (postnatal day 20 [p20]), female rats received either a whole-food diet with adequate levels of vitamin A, a diet with a 5.5-fold increase in vitamin A from fruits and vegetables (S diet), or a diet with a 6.2-fold increase in vitamin A provided as retinyl palmitate (RP diet). To determine the effect of dietary intervention on pubertal mammary gland development, rats were fed the experimental diets from p21 to p63 and had free access to food and distilled water. All rats were killed at p63.

To evaluate the effects of diet on early estrous cycles, defined as cycles within the first 2 weeks of sexual maturation, 12 rats per group received vaginal lavages daily from p43 through p50. To evaluate estrous cycles in more mature rats, 24 rats per dietary group were evaluated from p51 to p58. All rats were killed at p63.

The onset of vaginal opening was evaluated as a marker for sexual maturation. The age of vaginal opening onset was significantly delayed in rats fed the S diet ($p < 0.001$), when compared to those fed the adequate diet and the RP diet. Thus, the S diet suppressed the onset of sexual maturation. The S diet also inhibited markers of mammary alveologenesis more than the RP diet. These data demonstrate that the amount and source of vitamin A consumed by adolescent female rats can influence the onset of puberty and mammary gland alveolar development. Effects on mammary carcinogenesis included in this study are found in the Carcinogenicity section of this report.[32]

A study was performed to evaluate the effects of defined doses of retinyl palmitate at the critical time of limb morphogenesis limb morphogenesis in Swiss Webster albino mouse embryos.[33] Pregnant Swiss albino mice were administered retinyl palmitate (10,000 or 15,000 IU/kg, i.p.) on different days of pregnancy. The higher dose produced malformations in the forelimbs, by day 10, in 28.6% of mice and in the hindlimbs, by day 11, in 20.6% of the mice. Limb abnormalities (at both doses) were as follows: unilateral or bilateral micromelia (abnormally short) in the forelimbs and hindlimbs, shorter unilateral anterior and posterior limbs, limb malrotation, absence or malformation of fingers or toes, and an increased cleft between the metacarpal or metatarsal bones. Furthermore, 2 injections in one day with the lower dose resulted in more teratogenic effects than a single 15,000 IU/kg injection. Two injections of either dose on day 10 caused a greater incidence of embryo absorption.

The effects of retinyl palmitate supplementation, during gestation and lactation, on oxidative stress parameters of maternal and offspring tissues of female Wistar rats was studied.[34] Each group of pregnant female rats (except for one group of 7 rats), contained 6 animals. The respective groups received retinyl palmitate during pregnancy and lactation (21 days of gestation and 21 days of lactation) at oral (gavage) doses of 2500, 12,500, or 25,000 IU/kg/day. The maximum dose volume was 0.5 ml. The control group was dosed with saline. An increase in oxidative-damage markers in the reproductive tissues

and plasma of dams was observed. In uteri, lipid peroxidation was increased at all doses ($p < 0.0001$). Also, the liver and kidney had significant alterations in glutathione-S-transferase (GST) activity. It was increased in the liver of dams and decreased in the kidneys of mothers and offspring. In pups, supplementation decreased the total antioxidant potential of the liver, along with decreased superoxide dismutase/catalase activity ratio in the kidney. The levels of lipoperoxidation were increased in male offspring, but decreased in female pups. Collectively, the results suggest that excessive vitamin A intake during gestation and lactation, at oral doses sufficient to produce signs of maternal toxicity, may cause adverse effects in the developing offspring.

A study was performed to investigate the effects of vitamin A supplementation in pregnant and nursing rats on maternal and offspring striatum and hippocampus.[35] Female Wistar rats (7 per group) were orally supplemented with retinyl palmitate (2500; 12,500; and 25,000 IU/kg/day) or saline (control) throughout pregnancy and nursing. A homing test was performed on offspring on postnatal days (PND) 5 and 10, and an open field test (OFT) was carried out on dams and offspring on PND 19 and 20. Redox parameters were evaluated at PND 21 for both dams and offsprings. Supplementing Vitamin A during pregnancy and nursing increased the ratio of superoxide dismutase/catalase (SOD/CAT) ratio and oxidative damage in maternal and offspring striatum and hippocampus. Additionally, these effects were accompanied by behavioral alterations observed through the homing and OFT tests.

Oral - Human

Retinol

A study examining the effects of vitamin A or beta carotene supplementation on pregnancy-related mortality and infant mortality in rural Bangladesh was performed.[36] The study involved pregnant women (13 to 45 years old) and their live-born infants to 12 weeks (84 days) postpartum. Five-hundred ninety-six community clusters (study sectors) were randomized for pregnant women to receive 7000 μg retinol equivalents (as retinyl palmitate), 42 mg of all-*trans* beta carotene, or placebo from the first trimester through 12 weeks postpartum. Married women (n = 125,257 total; 32,180 to 32,719 per group) underwent surveillance for pregnancy, ascertained by a history of amenorrhea and confirmed by a urine test. Groups were comparable across risk factors. For the maternal mortality, neither of the supplemental groups was significantly different from the placebo groups. The numbers and all-cause, pregnancy-related mortality rates (per 100,000 pregnancies) were: 41 and 206 (95% confidence interval [CI] = 140-273) in the placebo group, 47 and 237 (95% CI = 166-309) in the vitamin A group, and 50 and 250 (95% CI = 177-323) in the beta carotene group. Relative risks of maternal mortality in the vitamin A and beta carotene groups were not statistically significantly different from controls (1.15; 95% CI = 0.75-1.76 and 1.21; 95% CI = 0.81-1.81 for the vitamin A and beta carotene groups, respectively).

There were 703 stillbirths in the placebo group, 665 in the vitamin A group, and 766 in the beta carotene group. Rates of stillbirths per 1000 births were: 47.9 (95% CI = 44.3 -51.5 [placebo]), 45.6 (95% CI = 42.1-49.2) [vitamin A]), and 51.8 (95% CI = 48.0-55.6 [beta carotene]). Relative risks of infants being still born were not statistically significantly different in the supplemented groups, compared to the placebo group (0.95; 95% CI = 0.85 -1.06 for the vitamin A group and 1.08; 95% CI = 0.97 to 1.21 for the beta carotene group). Infant mortality rates per 1000 births were: 68.1 (95% CI = 63.7-72.5 [placebo]), 65.0 (95% CI = 60.7-69.4 [vitamin A]), and 69.8 (95% CI = 65.4-72.3 [beta carotene]). It was concluded that, compared to the placebo, weekly vitamin A or beta carotene supplementation in pregnant women in Bangladesh did not reduce all-cause maternal, fetal, or infant mortality.[36]

In Vitro Study

Retinol

The developmental toxicity of retinol was evaluated in the embryonic stem (ES)-D3 cell differentiation assay of the embryonic stem cell test.[37] The murine ES-D3 cell line was used, and this assay was performed to determine the test concentrations affecting ES-D3 cell differentiation into contracting cardiomyocytes. Exposure to the test substance, added from a 400-times concentrated stock solution in DMSO to culture medium, started at day 1 and lasted for 10 days. A test was considered valid when at least 21 out of 24 wells of the blank (non-exposed) cells and the solvent control plate contained contracting cardiomyocytes. Retinol caused a concentration-dependent decrease in ES-D3 cell differentiation in the 10^2 to 10^4 nM concentration range.

GENOTOXICITY

In Vitro

Retinyl Palmitate

Positive photogenotoxic/photoclastogenic effects of retinyl palmitate in mouse lymphoma cells were reported in the following 2 studies.

To evaluate the photomutatgenicity of retinyl palmitate in cells when exposed to ultraviolet A (UVA) light, L5178/$Tk^{+/-}$ mouse lymphoma cells were treated with several concentrations of retinyl palmitate either alone or in the presence of UVA light.[38] The treatment of cells with retinyl palmitate alone at concentrations of 25 to 100 µg/ml did not increase mutant frequencies over the negative control (i.e., no RP or UVA exposure). However, the treatment of cells with retinyl palmitate at concentrations of 1 to 25 µg/ml in the presence of UVA light (82.8 mJ/cm^2/min for 30 min) caused a dose-dependent mutation induction. The mean induced mutant frequency for treatment with 25 µg/ml in the presence of UVA light was approximately threefold higher than that for UVA alone (122 x 10^{-6}), suggesting potentiation by retinyl palmitate of the effects of UVA light exposure.

To elucidate the underlying mechanism of action, the mutants were examined for loss of heterozygosity (LOH) at 4 microsatellite loci spanning the entire chromosome 11, on which the Tk gene is located. The mutational spectrum for the RP + UVA treatment was significantly different from the negative control, but not significantly different from that of UVA exposure alone. Ninety-four percent of the mutants from combined retinyl palmitate + UVA treatment lost the Tk^+ allele, and 91% of the deleted sequences extended across more than 6 cM in chromosome length, indicating clastogenic events affecting a large segment of the chromosome. These results suggest that retinyl palmitate is photomutagenic in combination with UVA exposure in mouse lymphoma cells, with a clastogenic mode-of-action.[38]

In the presence of UVA light, retinyl palmitate (RP) decomposes into multiple products, including anhydroretinol (AR) and 5,6-epoxyretinyl palmitate (5,6-epoxy-RP). The photomutagenicity of AR and 5,6-epoxy-RP in L5178Y/$Tk^{+/-}$ mouse lymphoma cells was evaluated.[39] The treatment of cells with AR or 5,6-epoxy-RP alone at 10 and 25 µg/ml for 4 h did not yield a positive mutagenic response. However, because these concentrations did not induce a sufficient degree of cytotoxicity for the mouse lymphoma assay, it was not possible to determine whether these 2 compounds are mutagenic at concentrations approaching cytotoxic levels.

The treatment of cells with 1 to 25 µg/ml AR or 5,6-epoxy-RP in the presence of UVA light (315 to 400 nm) for 30 min (1.38 mW/cm^2) caused a potentiated photomutagenic effect. At 10 µg/ml (37.3 µM) AR in the presence of UVA light, the mutant frequency was approximately 3-fold higher than for UVA exposure alone. The mutant frequency for 5,6-epoxy-RP at a concentration of 25 µg/ml (46.3 µM) in the presence of UVA light was approximately 2-fold higher than for UVA exposure alone.[39]

To determine the underlying photomutagenic mechanism, the loss of heterozygosity (LOH) at 4 microsatellite loci spanning the entire chromosome 11 was examined for mutants induced by UVA light and either AR or 5,6-epoxy-RP exposure. Most mutants lost the $7k^+$ allele, and more than 70% of the chromosome damage extended across 38 cM of the chromosome. AR + UVA induced approximately twice as many mutants, with all 4 microsatellite markers lost from the chromosome 11 carrying the Tk^+ allele, compared to retinyl palmitate + UVA or 5,6-epoxy-RP + UVA treatments. These results suggest that 2 of retinyl palmitate's photodecomposition products are photomutagenic in mouse lymphoma cells, affecting a large segment of the chromosome. For detailed information on the photodecomposition of retinoids, see 2 review articles on this subject that have been published.[40,41]

In an earlier study, photoirradiation of anhydroretinol with UVA light in the presence of methyl linoleate generated lipid peroxidation products (i.e., methyl linoleate hydroperoxides) in an exposure-dependent manner.[42] In a subsequent study, electron spin resonance (ESR) spin-trap techniques were employed to explore the mechanism of lipid peroxidation initiation in such systems.[43] Irradiation of anhydroretinol with UVA in the presence of 2,2,6,6,-tetramethylpiperidine (TEMP), a specific probe for singlet oxygen, resulted in the formation of TEMP-O, demonstrating the formation of singlet oxygen under these conditions. During photoirradiation in the presence of 5,5-dimethyl N-oxide pyrroline (DMPO), a specific probe for superoxide, ESR signals for DMPO-OOH were formed, and these signals were quenched by superoxide dismutase. The involvement of singlet oxygen in the induction of lipid peroxidation was also evidenced by the inhibition of lipid peroxidation by sodium azide and the enhancement of lipid peroxidation by deuterium oxide. Overall, the results

indicate that irradiation of anhydroretinol with UVA light generates reactive oxygen species, including singlet oxygen and superoxide, which mediate lipid peroxidation.

In another study, the same technique was used to determine whether or not irradiation of retinyl palmitate with UVA light produces reactive oxygen species.[44] Photoirradiation of retinyl palmitate in the presence of TEMP resulted in the formation of TEMPO. Both DMPO and 5-tert-butoxycarbonyl 5-methyl-1-pyrroline N-oxide (BMPO) are specific probes for superoxide. When photoirradiation of retinyl palmitate was performed in the preasence of DMPO or BMPO, ESR signals for DMPO–$^{\bullet}$OOH or BMPO DMPO–$^{\bullet}$OOH were obtained. These results unambiguously confirmed the formation of superoxide radical anion. Consistent with free radical mechanism, there was a near complete and time-dependent photodecomposition of retinyl palmitate and its photodecomposition products. ESR studies on the photoirradiation of 5,6-epoxy-RP and AR indicate that these compounds, as well as RP, can mediate free-radical formation and lipid peroxidation through the light-induced breakdown of RP.

The photogenotoxicity of retinyl palmitate, anhydroretinol (AR), and 5,6-epoxyretinyl palmitate (5,6-epoxy-RP) in human skin Jurkat T-cells was evaluated using the Comet assay.[45] This assay was used to assess light-induced fragmentation of cellular DNA, and was performed with and without UVA irradiation. Initially, a cell viability assay was performed, whereby fluorescein diacetate was added to light-irradiated cell suspensions. Light irradiation for up to 60 minutes did not significantly affect the viability of T cells. The viability of T cells, with and without light irradiation, in the presence of retinyl palmitate, AR, or 5,6-epoxy-RP at concentrations of 0, 50, 100, 150, and 200 μM was then determined. Cell death caused by these compounds was described as low in the absence of light (AR > 5,6-epoxy-RP > retinyl palmitate). With AR (100 μM) and 5,6-epoxy-RP (150 μM), 75% of the cells remained viable. With retinyl palmitate (200 μM), > 80% of the cells remained viable.

Cell death (photocytotoxicity in the presence of retinyl palmitate, AR, or 5,6-epoxy-RP in human skin Jurkat T-cells exposed to UVA (3.5 J/cm^2) and visible (6.3 J/cm^2) was much higher than without UVA or visible light exposure. For all 3 compounds, significant photocytotoxicity was observed for concentrations of 100 μM and greater. With 150 and 200 μM, the relative phototoxic potency of the retinoids conformed to the following order: retinyl palmitate = ARE > 5,6-epoxy-RP. When treated with 100 μM of retinyl palmitate, 5,6-epoxy-RP, or AR, cell death was 39%, 22%, and 45%, respectively. These results indicate that retinyl palmitate, 5,6-epoxy-RP, and AR are photocytotoxic.

In the comet assasys for DNA fragmentation, the retinoid concentrations were 0 to 200 μM and the light exposure was 3.5 J/cm^2 (UVA light) or 6.13 J/cm^2 (visible). Photoirradiation of retinyl palmitate, AR, or 5,6-epoxy-RP, in ethanol, with UVA light produced UVA-induced DNA fragmentation in human skin Jurkat T-cells, but only when accompanied by significant levels of photocytotoxicity. There was a dose-response relationship between the levels of DNA fragmentation and the concentration (50, 100, 150, and 200 μM) of the retinoid used. In the presence of supercoiled ΦX174 phage DNA, retinyl palmitate, AR, and 5,6-epoxy-RP at concentrations of 0.1 and 1.0 mM in 10% ethanol were irradiated with UVA light at light doses of 7, 21, and 50 J/cm^2. Single strand breaks in supercoiled ΦX174 plasmid DNA were observed. At 50 J/cm^2, all 3 compounds yielded DNA-strand cleavage to significantly higher extents when compared to that of the control group. The retinoids in decreasing order of DNA-strand cleavage observed were AR > 5,6-epoxy-RP > retinyl palmitate ≥ control. No DNA cleavage was observed when either retinoid or light was absent. These results indicate that retinyl palmitate, AR, and 5,6-epoxy-RP can damage DNA in a cell free system and can be cytotoxic in cultured mammalian cells that are also exposed to light.[45]

The genotoxicity and photogenotoxicity of retinyl palmitate was evaluated using Chinese hamster ovary (CHO) cells in a standard chromosome-aberration test.[46] The procedure involved pre-irradiation (UVA irradiation followed by treatment with retinyl palmitate) or simultaneous irradiation (irradiation of cells in the presence of retinyl palmitate together). UVA irradiation was 350 or 700 mJ/cm^2, with the high UVA exposure (700 mJ/cm^2) selected to produce a small increase in the incidence of structural chromosome aberrations in cells in the absence of retinyl palmitate. Retinyl palmitate was tested up to concentrations exceeding its limit of solubility in the culture medium (i.d., ranging from 20 to 40 μg/mL).

No overt cytotoxicity was found in the dark or following irradiation. Treatment of the cells with retinyl palmitate in the dark as well as treatment under pre- or simultaneous irradiation conditions failed to produce biologically significant increases in the incidence of structural chromosome aberrations. The positive control substances 4-nitroquinolone and 8-methoxypsoralene caused significantly positive effects in the dark or under simultaneous irradiation, respectively. It was concluded that, under standard conditions for evaluating photo-genotoxicity, retinyl palmitate had no *in vitro* genotoxicity or photogenotoxic potential and, therefore, that it is unlikely that retinyl palmitate would pose a local or systemic genotoxic or photogenotoxic risk.[46]

A letter to the editor addressing the preceding genotoxicity/photogenotoxicity study stated:[47]

"Dufour et al. conclude that application of retinyl palmitate to human skin posed no local or systemic genotoxic or photogenotoxic risk, based on a single *in vitro* genotoxicity test. This conclusion appears overstated; such a conclusion requires a full battery of *in vivo* and *in vitro* genotoxicity tests and perhaps even human studies. The authors inappropriately used results from one genotoxicity assay to refute results from various other genotoxicity assays that measure different genotoxic endpoints with varying sensitivities. We feel that it is important to bring these issues to the attention of the readers of *Mutation Research*."

A response to the aforementioned letter states:[48]

"In summary, our paper[46] and the papers by Mei et al.[38,39] exemplify the dilemmas facing genetic toxicologists in the current age. Different results can be obtained under different conditions, in different cells and using different culture media. This dilemma may be particularly relevant for in vitro photo-genotoxicity tests. For example, a recent concept paper by the European Medicines Agency stated that "oversensitivity and the occurrence of pseudo-effects with in vitro models, in particular the mammalian cell test for photogenotoxicity, has become a major problem" and no longer recommends these tests for regulatory purposes. Nobody wants to see humans exposed to dangerous chemicals, but determining whether the positive results cause unnecessary alarm or whether the negative results cause unjustified complacency will continue to exercise the minds of the scientific community."

In a subsequent study involving many of the authors of the preceding study, results following the photoirradiation of retinyl palmitate (RP) in ethanol using a UV lamp generating approximately equal levels of UVA and UVB light were as follows:[49] The photodecomposition products identified were: 4-keto-RP, 11-ethoxy-12-hydroxy-RP, 13-ethoxy-14-hydroxy-RP, anhydroretinol (AR), and *trans*- and *cis*-15-ethoxy-AR. Photoirradiation of RP in the presence of a lipid, methyl linoleate, resulted in induction of lipid peroxidation. Lipid peroxidation was inhibited when sodium azide was present during photoirradiation, which suggests free radicals were formed. These results demonstrate that RP can mediate the UV light-induced free radical formation and induction of lipid peroxidation through the light-induced breakdown of RP.

Retinol

The photocytotoxicity and photomutagenicity of retinol were investigated using L5178Y/Tk$^{+/-}$ mouse lymphoma cells concomitantly exposed to retinol and UVA light.[50] The cells were treated with retinol at concentrations of 5 or 10 µg/ml for 4 h without UVA exposure, or treated with retinol (0.25 to 4 µg/ml) for 4 h and UVA exposure at 2.48 J/cm^2 during the first 30 minutes of the 4 h incubation period. In cells treated with retinol alone at 5 or 10 µg/ml in the absence of light, there was no increase in the mutant frequency (MF) in the *Tk* gene, and there was minimal cytotoxicity. However, treatment of cells with 1 to 4 µg/ml retinol in the presence of UVA (1.38 mW/cm^2 for 30 minutes) increased the MF in the *Tk* gene in a concentration-responsive manner and increased cytotoxicity. To elucidate the underlying mechanism of action, the mutational types of the *Tk* mutants were examined by determining their loss of heterozygosity (LOH) at four microsatellite loci spanning the entire length of chromosome 11, on which the *Tk* gene is located. The mutational spectrum for the retinol + UVA treatment was significantly different from those of the control and UVA exposure alone. More than 93% of the mutants in the retinol + UVA treated cells lost heterozygosity at the *Tk*1 locus, and the major types (58%) of mutations were LOHs extending to D11*Mit*42, an alternation involving approximately 6 cM of the chromosome. The main types of mutations in the control were non-LOH mutations. These results suggest that retinol is mutagenic in the presence of UVA light in mouse lymphoma cells through a clastogenic mode-of-action.

In Vivo/In Vitro

Retinyl Palmitate and Retinol

Retinyl palmitate, retinol, and retinoic acid, in oil-in-water creams at a concentration of 0.05%, were applied as to the backs of adult Skh:hr-1 albino hairless mice, once per day for 3 days.[51] The application period was followed by exposure of the treated sites to UVB. The animals were then killed and the epidermis was removed for biochemical analysis. A431keratinocytes were incubated with retinol (2 µM) or retinyl palmitate (2 µM) in 1% ethanol for 24 h. Cell cultures were also incubated alone or in 1% ethanol only. At end of the 24-h incubation period, all cultures were exposed to UVB light and then incubated for 24 h. The action of retinyl palmitate and retinol on UVB-induced DNA damage and apoptosis in cultured A431 keratinocytes was analyzed. Topical retinol and retinyl palmitate significantly decreased (≈ 50%) the number of apoptotic cells as well as the formation of thymine dimers in the epidermis of mice exposed to acute UVB. However, neither of the 2 retinoids interfered with the apoptotic process in A431 keratinocytes exposed to UVB, whereas, DNA photodamage was decreased slightly in these cells in the presence of retinoids. It was concluded that the decrease in the numbers of UVB-induced apoptotic cells in hairless mouse epidermis following topical application of retinoids reflects a

protection of DNA, which is a primary target of UVB irradiation, by a mechanism that is independent of the activation of retinoid nuclear receptors and does not involve the direct inhibition of apoptosis.

Modulation of Genotoxicity

Retinol

Irinotecan (CPT-11) is a common chemotherapeutic agent that causes genotoxicity, which damages the DNA of blood cells. A study was performed to evaluate the modulating effect of vitamins A (retinol), C, and E on the genotoxic activity of CPT-11 and to analyze the efficacy of DNA repair in lymphocytes (*in vitro*) of patients with diagnosed colorectal carcinoma and healthy individuals.[52] In healthy donors' cells, CPT-11 did not exert a strong genotoxic effect in the presence or absence of the vitamins. In turn, a statistically significant increase of DNA migration in the comet tails was noted in the patients' lymphocytes exposed to CPT-11. Vitamins A, C, and E in the incubation solutions acted synergistically to increase the level of DNA lesions in the cells exposed to CPT-11 *in vitro*. Analysis of the efficacy of DNA repair, performed after 2 h of post-incubation, showed a decrease in the percentage of DNA in the comet tails in all experimental samples.

CARCINOGENICITY

Oral

Retinol and Retinyl Palmitate

The effects of retinyl palmitate ingestion on carcinogenesis were determined in the context of a human food-based diet (whole-food diet). Female adolescent Sprague-Dawley rats (total = 135), were randomized into 3 dietary groups (45 rats per group).[32] From p21 to p63 (i.e., the period of adolescent mammary-gland development), female rats received either a whole-food diet with adequate levels of vitamin A, a diet with a 5.5-fold increase in vitamin A from fruits and vegetables (S diet), or a diet with a 6.2-fold increase in vitamin A provided as retinyl palmitate (RP diet). Rats were injected with 50 mg 1-methyl-1-nitrosourea/kg body weight on p66. The rats were palpated twice per week for 6 months for the detection of mammary tumors. At 6 months post-carcinogen injection, latency and incidence of mammary tumors did not differ among dietary groups. However, compared with adolescent rats that consumed the adequate diet, consumption of S and RP diets reduced mammary cancer multiplicity (relative risk ~ 0.7, $P \leq 0.002$), and the reductions were associated with reductions in mammary gland alveolar development. More specifically, tumor multiplicity was reduced in rats fed either the S ($P = 0.0002$) or RP ($P = 0.002$) diet during adolescence. These data demonstrate that the amount and source of vitamin A consumed by adolescent female rats can influence breast cancer risk. Effects on sexual maturation and mammary-gland development included in this study are found in the Reproductive and Developmental Toxicity section of this report.

Dermal

Retinyl Palmitate

The following text is from a critical analysis of the safety of retinyl palmitate in sunscreens, which explains the basis for recently publicized concerns about the potential photocarcinogenicity of sunscreens containing this ingredient:[53]

> "In its annual sunscreen report (2010), the Environmental Working Group[54] claimed that 41% of the sunscreens on the market contain retinyl palmitate (RP), an ester form of vitamin A. Based on its internal analysis of the data from the National Toxicology Program (NTP), the Environmental Working Group issued a health warning regarding the photocarcinogenic potential of sunscreens containing RP. This claim has received significant media coverage with many news organizations running stories such as "your sunscreen may give you cancer." In fact, the media frenzy has prompted the senior Senator from New York, Chuck Shumer, to urge the Food and Drug Administration (FDA) to address this issue. This has resulted in many individuals questioning the overall safety of sunscreens, and with some expressing strong reluctance to use sunscreens for photoprotection."

The following are statements relating to the potential toxicity of retinyl palmitate in cosmetics from a 2005 FDA publication:[55]

"Regarding human toxicity, the long-term consequences of using cosmetics containing retinyl palmitate are currently unknown. It has been demonstrated that photoirradiation of retinyl palmitate can result in forming toxic photodecomposition products, generate ROS, induce lipid peroxidation, and cause DNA damage. Also, topically applied retinyl palmitate produces many of the cutaneous changes associated with the use of drug products containing retinyl palmitate, which in some instances can enhance photocarcinogenesis. Thus, a study of the photocarcinogenesis of retinyl palmitate, under conditions relevant to the use of retinyl palmitate in cosmetics, is timely and important."

In 2000, retinyl palmitate was nominated by the U.S. FDA's Center for Food Safety and Applied Nutrition (CFSAN) and selected by the National Toxicology Program (NTP) as a high priority compound for phototoxicity and photocarcinogenicity studies at the National Center for Toxicological Research.[56] The nomination was based on the following:

- Increasing widespread use of retinyl palmitate in cosmetic retail products for use on sun-exposed skin
- The biochemical and histological cutaneous alterations elicited by retinyl palmitate
- The association between topical application of all-trans retinoic acid (tretinoin) and enhancement of photocarcinogenesis

Accordingly, the principal objective of NTP's 1-year photocarcinogenesis study was to investigate the effects of topically applied skin cream containing retinol or retinyl palmitate on the photocarcinogenicity of simulated solar light or UV light in SkH-1 mice. Excerpts from the abstract of the NTP study are presented below (CIE = Commission Internationale de l'Eclairage).

"Groups of 36 male and 36 female Crl:SKH-1 *(hr$^-$/hr$^-$)* hairless mice were irradiated 5 days per week (Monday through Friday) in the morning for 40 weeks with SSL at levels of 0.00, 6.85, or 13.70 mJ•CIE/cm^2 that were emitted from glass-filtered 6.5kW xenon arc lamps.[57] The mice received topical applications of control creams or creams containing 0.001% (w/w) retinoic acid or 0.1%, 0.5%, 1.0%, or 2.0% retinyl palmitate to the dorsal skin region in the afternoon of the same days of irradiance exposures. Separate groups of 36 female Crl:SKH-1 *(hr$^-$/hr$^-$)* hairless mice were irradiated with UV light emitted from fluorescent UVA or UVB lamps at a single level that was equivalent to the amount of UVA or UVB generated by SSL at a level of 13.70 mJ•CIE/cm^2 SSL, and received topical application of control cream or creams containing 1.0% retinyl palmitate or 0.001% retinoic acid. A 12-week observation period followed the 40-week treatment/exposure period. Additional groups of 36 male and 36 female mice received no cream and were exposed to 0.00, 6.85, 13.70, or 20.55 mJ•CIE/cm^2 SSL or to a single level of either UVA or UVB light (females only), equivalent to the amount of UVA or UVB generated by SSL at a level of 13.70 mJ•CIE/cm^2."

"Mice that received no cream treatment and were exposed to increasing levels of SSL showed significant SSL exposure-dependent decreases in survival, earlier in-life onset of skin lesions, and significant SSL exposure-dependent increases in the incidences and multiplicities of in-life skin lesions, as well as in the incidences and multiplicities of histopathology determined squamous cell nonneoplastic skin lesions (hyperplasia and focal atypical hyperplasia) and neoplastic skin lesions (papilloma, carcinoma *in situ*, and/or carcinoma). Female mice that received no cream treatment and were exposed to UVA showed significant increases in survival, later onset of in-life skin lesions, and significantly decreased incidences and multiplicities of in-life skin lesions, when compared to female mice that received SSL at a level of 13.70 mJ•CIE/cm^2. Female mice that received no cream treatment and were exposed to UVB demonstrated significant decreases in survival and significant increases in the multiplicities of in-life skin lesions, when compared to female mice that received SSL at a level of 13.70 mJ•CIE/cm^2."

"The control cream was composed of a base cream (85%, w/w) and diisopropyl adipate (15%, w/w). The topical treatment of mice with the control cream imparted significant effects, when compared with comparable measurements in mice that received no cream treatment and were exposed to the same level of SSL. Specifically, the exposure of mice to control cream resulted in decreased survival rates, earlier times to the onset of skin lesions, and increased incidences and multiplicities of in-life skin lesions and squamous cell neoplasms in both the absence and presence of SSL exposure and increased incidences and multiplicities of in-life skin lesions in female mice exposed to UVA."

"The application of retinoic acid (0.001%, w/w) creams to mice significantly decreased survival, even in the absence of SSL exposure in male mice, when compared to mice that received the control cream and the same level of SSL. Significantly earlier in-life skin lesion onset and significantly increased multiplicities of skin lesions were observed

at each SSL level, including 0.00 mJ•CIE/cm^2, in male mice and in female mice exposed to 6.85 mJ•CIE/cm^2 SSL, UVA, or UVB. No histopathology was conducted on the retinoic acid cream-treated mice."

"Significant dose trend effects and earlier in-life skin lesion onsets were observed in mice that received the retinyl palmitate cream treatments in the presence of SSL, UVA, or UVB, compared with mice that received control cream treatment and the same level of irradiation. In mice exposed to SSL, there were significantly increased multiplicities of in-life skin lesions at retinyl palmitate doses of 0.1% to 1.0%. Significant dose-related trends were observed in the incidences of squamous cell carcinoma and/or squamous cell carcinoma *in situ* in female mice exposed to 6.85 mJ•CIE/cm^2 SSL. Significant retinyl palmitate dose-related increases were also observed in the multiplicities of squamous cell papilloma and in the combination of all squamous cell neoplasms."

The conclusions from this NTP study follow:

- "Under the conditions of these studies, the topical treatment of SKH-1 mice with the control cream resulted in earlier onsets of in-life skin lesions and higher incidences and multiplicities of in-life skin lesions in the absence and presence of SSL or UVA, and higher incidences and multiplicities of squamous cell neoplasms, when compared to untreated controls in the absence and presence of SSL."
- "Compared to the control cream, retinoic acid enhanced the photocarcinogenic activity of SSL and UVB in SKH-1 mice, based upon earlier onsets and increased multiplicities of in-life skin lesions."
- "Compared to the control cream, retinyl palmitate enhanced the photocarcinogenicity activity of SSL and UVB in SKH-1 mice, based upon earlier onsets and increased multiplicities of in-life skin lesions and increased incidences and multiplicities of squamous cell neoplasms."[57]

The composition of the cream vehicles used in NTP study is a critical factor in the evaluation of the results. NTP used:

- 85% base cream plus 15% disiopropyl adipate to prepare the "control cream"
- 85% base cream plus 15% of a retinoic acid diisopropyl adipate solution to prepare a 0.01% retinoic acid "master batch cream"
- 85% base cream plus 2% retinyl palmitate and 13% diisopropyl adipate to prepare the retinyl palmitate "master batch cream"

The control and master batch creams were mixed together to prepare the "dose creams" to which the animals were exposed, except that the 2% retinyl palmitate master batch cream was used as the 2% retinyl palmitate dose cream. The base cream had the following composition:

- 70.02% deionized water
- 3.25% 96% glycerin
- 8.00% 2% Keltrol T solution
- 1.2% Veegum ultra
- 2.5% cetearyl alcohol
- 4.00% Eutanol G
- 0.80% dimethicone DC 200-100
- 2.40% Lipomulse 165
- 2.40% Brij 721 (Steareth-21)
- 4.00% Lipowax D
- 1.00% Germaben II
- 0.43% 10% solution of 85% phosphoric acid (q.s. pH to 3.5)

The NTP found that the control cream, alone, without any retinol or retinyl palmitate, caused substantial adverse effects in the control mice exposed to simulated sunlight, UVA, or UVB, as indicated by markedly reduced survivability and in-life skin lesion onset and elevated in-life skin lesion incidence and multiplicity. The already severe effects of the vehicle (control cream) were further aggravated by the presence of retinoic acid or retinyl palmitate in the dose creams.

The results indicate that the NTP study was significantly confounded and, thereby, seriously compromised by the effects of the "control cream" that was used as the vehicle.
In contrast, no such vehicle effects were found in a similar NTP 1-year photocarcinogenesis study on glycolic and salicylic acids. The composition of the base cream in the study was:

- 59.65% deionized water
- 0.10% disodium EDTA
- 0.10% disodium EDTA
- 2.50% 96% glycerin
- 7.5% 2% Carbopol 981 solution
- 7.50% mineral oil 65/75
- 1.50% BRIJ 721
- 2.00% Stearic Acid XXX
- 0.25% cetearyl alcohol
- 2.50% octyl palmitate
- 1.00% Germaben II
- 0.50% 20% NaOH solution (q.s. pH to 7.0)

In this study, glycolic acid or salicylic acid was added to the base cream, which also served as the control cream, to produce the dose creams; No diisopropyl adipate was used in this study. Otherwise, there were few differences in the protocol of this NTP study compared to that of the retinoids.

Diisopropyl adipate is among the esters of dicarboxylic acids recently evaluated by the CIR Expert Panel and found to be safe in the present practices of use and concentrations. Maximum use concentrations were up to 8% in leave-on products in 2010. That assessment noted studies on human subjects showing that undiluted diisopropyl adipate was not irritating in 4-h patch tests and only moderately irritating in a 21-day cumulative irritancy test patch test. Formulations containing up to 20.75% diisopropyl adipate caused minimal to mild irritation but no sensitization in such tests. A photopatch study demonstrated that formulations containing up to 17.0% diisopropyl adipate were not phototoxic, primary irritants, or sensitizers.

These results indicate that the Crl:SKH-1 *(hr^{-}/hr^{-})* hairless mice used in the NTP study of the retinoids are much more sensitive to the potential phototoxicity of diisopropyl adipate than human subjects. The striking sensitivity of these mice, compared to humans, indicates that the use of a vehicle containing 13% to 15% diisopropyl adipate in the NTP study severely limits the usability of the results to support the assessment of safety and risks associated with dermal exposure to retinol or retinyl palmitate in cosmetic products.

A critical analysis regarding the photocarcinogenic potential of retinyl palmitate has been reviewed by Wang et al.[53] and the findings are summarized as follows:[58]

> "A number of studies regarding retinyl palmitate has been published by the FDA. Of the 8 *in vitro* studies published by the FDA from 2002-2009, 4 demonstrated the generation of reactive oxygen species by retinyl palmitate when exposed to UVA radiation.[43,44,49,42] The generation of free radicals and their subsequent mutagenic potential have garnered understandable concern. However, when considered in the context of the antioxidant milieu found in human skin, the relevance of these findings becomes questionable. The capacity to quench reactive oxygen species is magnified by the complex network of antioxidants found in the normal human biochemical environment. In conjunction with both enzymatic antioxidants (e.g., catalase, peroxidase, superoxide dismutase, and glutathione reductase) and nonenzymatic antioxidants (e.g., vitamins C and E), vitamin A can neutralize harmful free radicals. In the isolative environment of laboratory study, however, cooperative interactions among other antioxidants are absent. As such, the protective properties of a single antioxidant are quickly depleted and may even become pro-oxidative when exposed to harmful stimuli. Furthermore, many antioxidants are inherently unstable if not properly formulated, preventing the full spectrum of enzymatic and nonenzymatic antioxidants from acting to reduce the pro-oxidative effects observed in these *in vitro* experiments."
>
> "To assess the carcinogenic potential of retinyl palmitate, the National Toxicology Program (NTP) conducted a large study using SKH-1 hairless mice. While the results from this study have not yet been published in a peer-reviewed forum, the preliminary data are available for review online.[59] In this study, SKH-1 hairless mice received 2 different concentrations of retinyl palmitate (0.1% and 0.5%), with controls receiving a vehicle control pH 7 cream. The animals were then irradiated with UV doses of 6.75 and 13.7 mJ/cm^2 and subsequently assessed for photocarcinogenesis. In the groups irradiated with low-dose (6.75 mJ/cm^2) UV radiation, retinyl palmitate induced higher incidences of malignant lesions at concentrations of both 0.1% and 0.5%, when compared with the vehicle control pH 7 cream. However, only the group exposed to retinyl palmitate at a concentration of 0.5% showed a statistically significant increase. In the groups exposed to high-dose (13.5 mJ/cm^2) UV radiation, no statistically significant difference in the incidence of malignant lesions was observed between the vehicle control group and the

group exposed to either 0.1% or 0.5% retinyl palmitate. Therefore, the study failed to demonstrate conclusively that the combination of retinyl palmitate and UV is photocarcinogenic. Of worthy note, the thinner epidermis of mice used in these studies allows for increased penetrance of UV radiation. Additionally, these mice are known to have a higher propensity to develop skin cancer. These intrinsic qualities suggest that data generated from these animal studies should be examined in context and caution should be excercised in extrapolating the relevance of these findings to humans."

"While published data on the photocarcinogenic potential of retinyl palmiate in humans are lacking, evidence from 40 years of use in clinical medicine provides a powerful basis from which to question the notion that retinyl palmitate in sunscreens is photocarcinogenic. Clinically, retinoids are used by dermatologists in two major areas of therapy. First, oral retinoids have been used with great success to prevent skin cancers in populations who are at high-risk, such as patients with xeroderma pigmentosum[60] and immunosuppressed patients (e.g. organ transplant)[61]. Second, dermatololgists commonly prescribe topical retinoids in the management of skin disorders such as acne, psoriasis, photoaging, cutaneous T-cell lymphoma, and a variety of other skin conditions. Among patients treated with topical or oral retinoids, no published data exist to date suggesting that these medications increase the risk of skin cancer."

"In conclusion, the available evidence from *in vitro* and animal studies fails to demonstrate convincing evidence indicating that retinyl palmitate imparts an increased risk of skin cancer. Furthermore, while no human data examining this relationship are available, decades of clinical observations support the notion that retinyl palmitate is safe for use in topical applications such as sunscreens."[58]

Anticarcinogenicity

Human

A study was performed to determine whether retinyl palmitate alone or plus beta-carotene (BC) would be as effective as and less toxic than low-dose 13-cis retinoic acid (13cRA) in treating oral premalignant lesions and reducing the risk of oral cancer.[62] Initially, patients (167; ≥ 18 years) were stratified by dysplasia versus hyperplasia and randomly assigned to 13cRA (0.5 mg/kg/day orally for 1 year, followed by 0.25 mg/kg/day orally for 2 years) or beta-carotene (50 mg/kg/day orally) plus retinyl palmitate (25,000 U/day orally) for 3 years, and later (by protocol revision) to 13cRA or retinyl palmitate alone (25,000 U/day orally). However, after other randomized trials suggested an adverse effect of beta-carotene on lung cancer incidence/mortality, beta-carotene was dropped (i.e., the patients were randomly re-assigned to 13cRA or retinyl palmitate alone). The primary endpoint was oral premalignant clinical response at 3 months.

The 3-month clinical response rate of the combined BC plus retinyl palmitate and retinyl palmitate alone arm (32.5%) was not statistically equivalent to that of 13cRA (48.1%). The clinical response rate of retinyl palmitate alone (20%) was significantly lower than that of beta-carotene plus retinyl palmitate (42.9%; $P = 0.03$). Similar oral cancer-free survival rates were observed across all arms. There was no significant association between 3-month oral premalignant lesions response and subsequent oral cancer development ($P = 0.11$). Grades 2 and higher adverse events were more common in the 13cRA than other groups ($P < 0.0001$). It was concluded that this chemoprevention trial did not establish the equivalence of retinyl palmitate plus beta-carotene or retinyl palmitate alone with low dose 13cRA in reducing the long-term risk of oral cancer. Additionally, it was stated that 13cRA, beta-carotene plus retinyl palmitate, and retinyl palmitate alone cannot be recommended for chemoprevention.[62]

SUMMARY FROM 1987 PUBLISHED CIR FINAL REPORT

Retinol is the primary naturally occurring form of vitamin A. Retinyl palmitate is the ester of retinol and palmitic acid, also known as vitamin A palmitate.[1] These compounds are soluble in most organic solvents and insoluble in water. Retinol and retinyl palmitate have ultraviolet absorption maxima in the range of 324-328 nm.

Retinol and retinyl palmitate are produced today largely by commercial methods in which retinyl acetate is the end product. Retinol is also still obtained by concentration from animal fats and fish liver oils.

Retinol oxidizes readily and is inactivated by ultraviolet light, giving off a characteristic green fluorescence. Retinol is relatively heat stable and is more stable in alkaline than acid solution. The acetic and palmitic esters of retinol are commercially important because of their greater degree of stability when compared to the alcohol.

High-pressure liquid chromatography (HPLC) has become the preferred method of retinoid analysis due to the stability of retinoids on most HPLC columns, its high resolution and sensitivity, and the rapidity of most analyses.

Cosmetic uses of retinol and retinyl palmitate are primarily in hair, facial makeup, and skin care preparations. Retinol and retinyl palmitate were used in 138 and 102 formulations, respectively, in 1981. Generally, these ingredients were used at concentrations ≤ 1%.

Retinol and retinyl palmitate are both affirmed as GRAS (generally recognized as safe) food ingredients; their functional use in foods is as nutrient and dietary supplements. They are also used for this purpose in the veterinary field. Retinol sees further use in various pharmaceutical products and in the treatment of dermatoses.

Retinol is essential for the growth, health, and life of higher animals. It is required for vision, reproduction, and for the maintenance of differentiated epithelia and of mucous secretion. The molecular mechanisms for its biological effects are largely unknown, with the exception of its role in the visual process.

The primary natural sources of vitamin A in the diet are certain plant carotenoid pigments, particularly β-carotene, and the long-chain retinyl esters found in animal tissues. β-Carotene is converted (50% maximum) to retinol primarily in the intestinal mucosa, although conversion also is known to occur in the liver and other tissues.

Dietary retinyl esters, retinol, and provitamin A carotenoids are dispersed and emulsified in the stomach during the gastric phase of lipid digestion. The esters are hydrolyzed in the intestinal lumen, and the resulting retinol, as well as that obtained from the diet, is absorbed into the mucosal cell. Here, it is reesterified with long-chain, mainly saturated fatty acids, incorporated into chylomicrons, and transported via the lymph into the general circulation. The chylomicrons are metabolized in extrahepatic tissues and reduced to smaller cholesterol-rich particles containing essentially all of the original retinyl esters. These chylomicron remnants are removed from the circulation almost entirely by the liver.

Upon uptake by the liver, the retinyl esters are hydrolyzed, reesterified, and stored in the liver, primarily as retinyl palmitate. Vitamin A is mobilized from these hepatic stores as retinol bound to a specific plasma transport protein, retinol-binding protein (RBP), in a highly regulated process. A specific intracellular binding protein for retinol has also been identified and designated as cellular retinol-binding protein (CRBP).

In a number of studies, radioactive metabolites of retinol were excreted via urinary and fecal or biliary routes. The amount excreted in the urine depended on the position of the radioactive atom. The urinary metabolites have been only partially characterized; they are mainly water-soluble and contain no detectable free retinol or retinyl esters. The biliary and fecal metabolites have also not been characterized, with the exception of retinol, retinoic acid, and their conjugates. The amount of the administered dose recovered in the bile and feces varies depending on the position of the radioactivity, the mode of administration, and on the quantity administered. Some retinol is metabolized through retinoic acid. Therefore, the well-characterized metabolites of retinoic acid would also be metabolites of retinol.

Retinol has long been known to interact with other micronutrients, including vitamin E, ascorbic acid (vitamin C), iron, and zinc. Vitamin E is generally believed to have a nonspecific antioxidant role. It has been suggested that retinol influences the hepatic synthesis of ascorbic acid, whereas the latter acts as an antioxidant for excess hepatic retinol. Iron may facilitate oxidative destruction of vitamin A active compounds in the intestine, whereas the vitamin may facilitate the mobilization of stored iron and its incorporation into erythrocytes. The hepatic mobilization of retinol may also be impaired by zinc deficiency.

In other interaction studies, gonadal steroid and adrenocortical hormones have increased the hepatic mobilization of retinol, DDT, and other drugs as well as xenobiotics have reduced hepatic stores of retinol. Acute and chronic ingestion of alcohol has resulted in reduced plasma concentrations and hepatic storage (chronic only) of retinol.

The retinoids have modified the growth and differentiation of both neoplastic and nonneoplastic cells in culture. Retinoids have various effects on the activity and synthesis of cellular enzymes and effectors as well as profoundly influencing the biosynthesis of all types of glycoconjugates. Two dominant theories of retinoid mechanism exist: the proposed cofactor role of retinoids in glycosyl transfer and the proposed steroid model for retinoid control of gene expression.

In studies on physiological effects, vitamin A affected multiple parameters of thyroid function. There appears to be an inverse relationship between thyroxine and vitamin A concentrations in the plasma. Retinyl palmitate has decreased glucose tolerance in man.

In acute oral studies, retinol was slightly toxic to mice, whereas retinyl palmitate was practically nontoxic in mice and rats. Large single doses can be lethal. Retinol was considerably more toxic than retinyl palmitate when administered i.p.

Two cosmetic products, each containing 0.1% retinyl palmitate, were evaluated for dermal irritation in rabbits: one was no more irritating than the control product, whereas the second was quite irritating to rabbit skin. These same two products were relatively nonirritating to rabbit eyes.

Multiple low doses (several-fold greater than required intake level) of vitamin A can be toxic to laboratory animals. Characteristic symptoms of hypervitaminosis A include weight loss, erythema, hair loss, internal hemorrhage, and fractures. Many of these effects are reversible upon cessation of administration. The time to first appearance of clinical signs depends on the route of administration, the species and age of the animal, the duration of treatment, the sign in question, and the dose size. In a review of current literature, the lowest reported adverse effect concentration in experimental animals was in the range of 25,000 to 60,000 IU retinol per kg per day for periods of 3-5 weeks. Water-miscible vitamin A was more toxic than oil-soluble vitamin A because it was more readily absorbed.

In specific dermal studies, topical application of retinol to rats for periods of 10-60 days produced acanthosis and approximately doubled the thickness of the epidermis. Subcutaneous injection of retinol for up to 60 days induced no significant effect on the epidermis of rats. Topical application of retinol to the nipples of guinea pigs for 10 days produced an acanthotic response. A drop of bear liver oil applied to the skin of mice daily for 14 days produced signs characteristic of hypervitaminosis A. Two drops of an oily vitamin A solution applied on alternate days to the surface of guinea pig skin wounds promoted healing and produced signs of local hypervitaminosis A. A body lotion containing 0.1% retinyl palmitate produced a mild dermatitis in all rabbits after daily administration of 6 mg/cm^2 for 90 days. No systemic toxicity was observed.

Retinyl palmitate did not produce any adverse effects during 10 months of oral administration to dogs and rats at doses of up to 25,000 and 50,000 IU/kg/day, 5 days per week, respectively.

Vitamin A toxicity occurs when, due to excessive intake of vitamin A, retinol begins to circulate in the plasma in a form other than bound to RBP.

Although it is recognized that retinol is essential for reproduction, high intake of retinol has produced adverse effects on several reproductive functions. These include decreased sperm motility and sperm survival in rabbits as well as testicular changes and inhibition of cyclic ovulatory activity in rats.

Vitamin A has produced more than 70 types of malformations in rats, mice, hamsters, guinea pigs, rabbits, dogs, pigs, and monkeys. The type and incidence of malformations depended on the dose and stage of pregnancy and, to a lesser extent, on species and strain. Abnormalities of the face, ears, eyes, and nervous system were most commonly observed. Minor brain defects, growth disturbances, and behavioral abnormalities have also developed postnatally after in utero exposure to retinol. Appreciable amounts of vitamin A are transferred to suckling offspring through maternal milk.

Vitamin A was nonmutagenic in the Ames test both with and without metabolic activation. Retinol also did not increase the frequency of sister chromatid exchanges or cell cycle delay in Chinese hamster cells either with or without metabolic activation. Retinol and retinyl palmitate have modified the effects of established mutagens, having both an inhibitory and a stimulatory effect (at low doses only).

There is no evidence that vitamin A is carcinogenic. However, the vitamin has both enhanced and inhibited responses to viral or chemical carcinogens. Results of many studies conducted in search of therapeutic roles for retinoids have indicated that retinoids can suppress the process of carcinogenesis in laboratory animals *in vivo* and the development of malignant phenotypes *in vitro*.

Retinoids have inhibited or stimulated the immune system: High doses have effectively inhibited both humoral (antibody-mediated) and cell-mediated immunity, whereas subtoxic doses have been stimulatory. Timing, dose, and mode of administration play a major role in determining the effects of the retinoids on the immune system.

The RDA of vitamin A for humans varies between scientific groups. The NRC recommends 5000 and 4000 IU daily for male and female adults, respectively, lesser amounts for infants and children, and an increased 5000 and 6000 IU daily for pregnant and lactating women, respectively. The FAO/WHO has recommended lower daily intakes for all groups: 2500 IU for adults (including pregnant women), lesser amounts for infants and children, and an increased 4000 IU daily for lactating women. The requirement for vitamin A appears to be proportional to body weight. This proportion is believed to decline rapidly following birth and increase only slightly during the adolescent growth phase. Surveys of representative samples of the United States population (1971-1974) indicated that the daily intake of vitamin A from all food sources was 4774 IU for the overall ages 1-74 years.

In a review of hypervitaminosis A in humans dating from 1850 to 1979, 579 cases were reported. These indicated a wide variability in individual tolerance to retinol. Acute toxicity is frequently reported when single doses of 100,000 IU vitamin A or more are given to infants or 300,000 IU or more to young children. Multiple doses of 200,000 IU orally or 100,000 IU intramuscularly on sequential days also may produce toxicity. Symptoms usually occur within a few hours after consumption and are of a transient nature, causing no permanent or adverse effects. The primary effects are on the central nervous system, and the gastrointestinal tract is secondarily affected.

Chronic hypervitaminosis A generally occurs when high doses, usually greater than 25,000 IU daily, have been administered over long periods of time. The most prominent signs of chronic toxicity are cutaneous, followed by gastrointestinal and central nervous system effects. Most of these signs disappear when the administration of vitamin A is discontinued. However, growth retardation caused by premature epiphyseal closure has been reported in children. The least adverse effect intake in humans appears to be from 700 to 3000 IU retinol/kg/day for several months, with most estimates skewed toward the upper end of this range. Daily intakes at this level would probably be attained only through supplementation, since the mean daily intake from usual dietary sources is of the order of 80 IU/kg for adults and 300 IU/kg for infants.

In repeated insult patch tests, cosmetic products containing 0.1-1% retinyl palmitate were at most slightly irritating and nonsensitizing in a total of 607 subjects. Results of cumulative irritation tests of two products containing 0.1% retinyl palmitate indicated that these products are “probably mild” in normal use and essentially nonirritating and nonsensitizing, respectively. One case of contact allergy to retinyl palmitate has been recorded.

The majority of epidemiology studies, both prospective and retrospective, have related dietary intake of vitamin A as well as serum concentration of retinol to the incidence of cancer. Controversy exists as to whether the observed anticancer effects are due to retinol, β-carotene, some other dietary constituent, or to a combination of these factors. The current recommendation of the National Academy of Sciences states that the epidemiological evidence suggests that foods rich in carotenes or vitamin A are associated with reduced risk of cancer. However, due to the toxicity of vitamin A in doses exceeding those required for optimum nutrition as well as the difficulty in distinguishing the effects of carotene from vitamin A, the NAS does not recommend increasing vitamin A intake by the use of supplements.

Retinol has been used as a treatment (both orally and topically) for malignant tumors since the 1930s. Numerous clinical studies are ongoing in this area, although most are using analogs of vitamin A because of more favorable therapeutic indices.

Several cases have been reported of fetal malformations after maternal ingestion of high doses of vitamin A during pregnancy. These included malformations of the central nervous system, urinary tract, and craniofacial area. Hypervitaminosis A during pregnancy may adversely affect the developing embryo and fetus.[1]

DISCUSSION FROM 2008 CIR RE-REVIEW DECISION

The number of ingredient uses reported for Retinol and Retinyl Palmitate in 1981 were 138 and 102, respectively, and use concentrations were < 0.1% to 5% for both.[2] Data provided by FDA in 2002 indicated 50 and 677 uses for Retinol and Retinyl Palmitate, respectively. Current use concentration data for Retinol are between 0.00006% and 2%, and for Retinyl Palmitate, between 0.000001% and 1.7%.

It was noted that both Retinol and Retinyl Palmitate are used in hair sprays, and that inhalation toxicity data on these ingredients are/were not available. The Expert Panel reasoned that the two ingredients can be used safely in aerosolized products if particulates from those products are not respirable. Because the particle size of anhydrous hair sprays (60 - 80

μm) and pump hair sprays (>80 μm) is large compared to the median aerodynamic diameter of 4.25 ± 1.5 μm for a respirable particulate mass, it was considered unlikely that inhalation would be a route of exposure of lung tissue.

The Panel also noted that Retinol and Retinyl Palmitate absorb light in the low UVA range and that neither photoirritation nor photoallergy data were included in the final safety assessment. However, recent photoirritation and photoallergy data on sunscreen products containing Retinol at concentrations ranging from 0.04% to 0.09% or 0.01% Retinyl Palmitate were provided by the cosmetics industry and these data demonstrated no adverse reactions. These concentrations are consistent with the current use concentration data that were provided. After considering these data and the the lack of clinical reports of Retinol or Retinyl Palmitate-induced photoirritation/photoallergy in the published literature, it was agreed that no concerns relating to the phototoxicity/photoallergenicity potential of Retinol or Retinyl Palmitate in cosmetic products are warranted.

The Panel is aware of an ongoing NTP photococarcinogenicity study on Retinyl Palmitate, and is interested in reviewing the results of this study as soon as they are available. Relative to this ongoing research, the Panel noted that data from the published literature indicate that the epoxy photodecomposition products of Retinyl Palmitate are phototoxic, but not photomutagenic.

After reviewing *in vitro* percutaneous absorption data (human skin) on Retinyl Palmitate that were published after the final safety assessment was issued, the Panel noted that the results of this study demonstrated a very low rate of absorption when acetone (not a cosmetic vehicle) served as the vehicle. It was agreed that no issues relating to the percutaneous absorption of Retinyl Palmitate are apparent.[2]

Table 1. Current Frequency and Concentration of Use According to Duration and Type of Exposure Provided in 2012[4]

	Retinyl Palmitate		Retinol	
	# of Uses	Conc. (%)	# of Uses	Conc. (%)
Exposure Type				
Eye Area	177		17	
Incidental Ingestion	191		12	
Incidental Inhalation-sprays	85		NR	
Incidental Inhalation-powders	51		1	
Dermal Contact	1539		167	
Deodorant (underarm)	1		NR	
Hair - Non-Coloring	277		3	
Hair-Coloring	6		NR	
Nail	30		4	
Mucous Membrane	303		23	
Baby Products	3		1	
Duration of Use				
Leave-On	1657		170	
Rinse off	395		12	
Diluted for (bath) use	7		4	
Totals*/Conc. Range**	2059		186	

NR = Not Reported; Totals = Rinse-off + Leave-on Product Uses
NOTE: Because each ingredient may be used in cosmetics with multiple exposure types, the sum of all exposure types may not be equal to sum total uses.

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trial of 13-cis retinoic acid compared with retinyl palmitate with or without beta-carotene in oral premalignancy. *J Clin.Oncol.* 2009;27(4):599-604.

Data

2012 FDA VCRP Data

Retinyl Palmitate

01B - Baby Lotions, Oils, Powders, and Creams	2
01C - Other Baby Products	1
02A - Bath Oils, Tablets, and Salts	1
02B - Bubble Baths	3
02D - Other Bath Preparations	3
03A - Eyebrow Pencil	12
03B - Eyeliner	15
03C - Eye Shadow	39
03D - Eye Lotion	47
03E - Eye Makeup Remover	2
03F - Mascara	16
03G - Other Eye Makeup Preparations	46
04A - Cologne and Toilet waters	4
04C - Powders (dusting and talcum, excluding aftershave talc)	1
04E - Other Fragrance Preparation	12
05A - Hair Conditioner	75
05B - Hair Spray (aerosol fixatives)	17
05C - Hair Straighteners	2
05E - Rinses (non-coloring)	1
05F - Shampoos (non-coloring)	77
05G - Tonics, Dressings, and Other Hair Grooming Aids	74
05I - Other Hair Preparations	31
06B - Hair Tints	1
06C - Hair Rinses (coloring)	1
06D - Hair Shampoos (coloring)	2
06H - Other Hair Coloring Preparation	2
07A - Blushers (all types)	42
07B - Face Powders	48
07C - Foundations	75
07D - Leg and Body Paints	2
07E - Lipstick	191
07F - Makeup Bases	14
07G - Rouges	3
07H - Makeup Fixatives	2
07I - Other Makeup Preparations	61
08A - Basecoats and Undercoats	4
08B - Cuticle Softeners	8
08C - Nail Creams and Lotions	4
08E - Nail Polish and Enamel	5
08G - Other Manicuring Preparations	9
10A - Bath Soaps and Detergents	69
10B - Deodorants (underarm)	1
10D - Feminine Deodorants	1
10E - Other Personal Cleanliness Products	35
11A - Aftershave Lotion	20

11E - Shaving Cream	4
11G - Other Shaving Preparation Products	7
12A - Cleansing	81
12B - Depilatories	5
12C - Face and Neck (exc shave)	191
12D - Body and Hand (exc shave)	141
12E - Foot Powders and Sprays	1
12F - Moisturizing	304
12G - Night	69
12H - Paste Masks (mud packs)	31
12I - Skin Fresheners	8
12J - Other Skin Care Preps	87
13A - Suntan Gels, Creams, and Liquids	3
13B - Indoor Tanning Preparations	38
13C - Other Suntan Preparations	8
Total	**2,059**

2012 FDA VCRP Data

Retinol

01B - Baby Lotions, Oils, Powders, and Creams	1
02A - Bath Oils, Tablets, and Salts	4
03D - Eye Lotion	10
03G - Other Eye Makeup Preparations	7
05G - Tonics, Dressings, and Other Hair Grooming Aids	2
05I - Other Hair Preparations	1
07C - Foundations	2
07E - Lipstick	12
07F - Makeup Bases	1
07I - Other Makeup Preparations	2
08E - Nail Polish and Enamel	3
08G - Other Manicuring Preparations	1
10A - Bath Soaps and Detergents	6
10E - Other Personal Cleanliness Products	1
12A - Cleansing	3
12C - Face and Neck (exc shave)	39
12D - Body and Hand (exc shave)	16
12F - Moisturizing	19
12G - Night	15
12H - Paste Masks (mud packs)	2
12I - Skin Fresheners	6
12J - Other Skin Care Preps	33
Total	**186**

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2

Final Report on the Safety Assessment of Retinyl Palmitate and Retinol

Retinol is the naturally occurring form of vitamin A; Retinyl Palmitate is the ester of Retinol and Palmitic Acid. In acute oral studies, Retinol was slightly toxic to mice, and Retinyl Palmitate was practically nontoxic in mice and rats. Large single doses can be lethal. It is recognized that Retinol is essential for reproduction; however, high intake of Retinol has produced adverse effects on several reproductive functions. Vitamin A was nonmutagenic in several in vitro tests. There is no evidence that vitamin A is carcinogenic. However, the vitamin has both enhanced and inhibited responses to viral or chemical carcinogens. Cosmetic products containing 0.1–1% Retinyl Palmitate were, at most, slightly irritating and nonsensitizing when tested on a total of 607 subjects. Results of cumulative irritation tests of two products containing 0.1% Retinyl Palmitate indicated that the products were nonirritating and nonsensitizing. On the basis of the available animal and clinical data presented in this report, it is concluded that Retinyl Palmitate and Retinol are safe as cosmetic ingredients in the present practices of use and concentration.

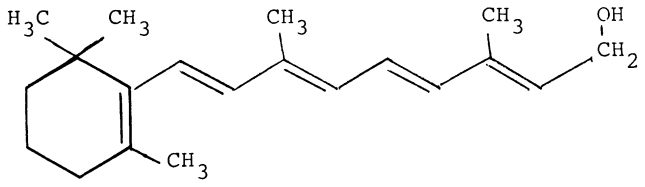
INTRODUCTION

The literature on Retinol and Retinyl Palmitate is voluminous. That literature dating from 1920 to 1980 has been previously reviewed in a series of GRAS (generally recognized as safe) reports and evaluations and is only briefly summarized here.[1–3] More recently, Sporn et al.[4] have reviewed the chemistry and biology of the retinoids. Unpublished cosmetic industry data have also been included.

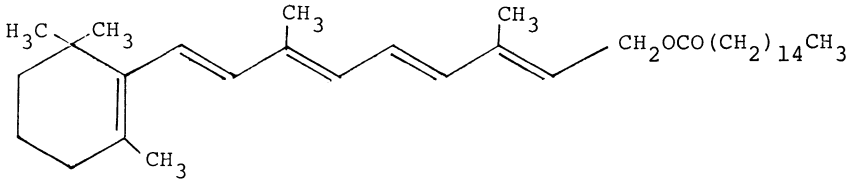
CHEMICAL AND PHYSICAL PROPERTIES

Retinol, chemically known as 3,7-dimethyl-9-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2,4,6,8-nonatetraen-1-ol, is the primary naturally occurring form of vitamin A.[5] Retinyl Palmitate is the ester of Retinol and palmitic acid, also

known as vitamin A palmitate (when used as a nutrient or drug).[6] These two compounds conform to the following structures:



Retinol[6]



Retinyl Palmitate[7]

Retinol occurs as a pale yellow, crystalline compound or viscous liquid, and Retinyl Palmitate is a yellow to yellow-red solid or oily substance. Both compounds have a faint characteristic odor and are soluble in most organic solvents while insoluble in water.[5,7,8–10] Retinol and Retinyl Palmitate have ultraviolet absorption maxima in the range of 324 to 328 nm.[5,9,10] The physicochemical properties of Retinol and Retinyl Palmitate are presented in Table 1.

A large number of processes have been developed for the synthesis of Retinol and its esters. The most important commercial methods today are based on the work of Isler et al.[11] at Hoffmann-La Roche and of Pommer et al.[12–14] at the BASF laboratories in Ludwigshafen, Germany. Retinyl acetate is the end product of both these processes (see Frickel[15] for more details). Retinol can be esterified by various methods, usually under very mild conditions. The imadazolide method is useful because of its nonacidic reaction conditions.[15] Retinol can also be obtained by concentration from animal fats and fish liver oil. This is accomplished by molecular distillation, saponfication, and crystallization of the distillate and conversion to the desired ester.[10]

Retinol oxidizes readily although it is quite stable in oil solutions. Ultraviolet (UV) light inactivates the vitamin and its solutions, which give off a characteristic green fluorescence. Retinol is relatively heat stable and is more stable in alkaline than acid solution. The acetic and palmitic acid esters of Retinol are commercially important due to their considerably greater degree of stability when compared to the alcohol.[5,10,15,18,19]

TABLE 1. Physiochemical Properties.

Property	*Retinol*	*Retinyl Palmitate*	*Reference*
Physical appearance	Yellow viscous liquid/yellow crystals; odorless or with a faint characteristic odor	Light yellow to yellow-red solid or oily substance with faint characteristic odor	5, 7, 8, 10, 16
Molecular formula	$C_{20}H_{30}O$	$C_{36}H_{60}O_2$	5, 9
Molecular weight	286.44	524.87	5
Melting point	62–64°C	27–20°C (all-trans form)	5, 9, 17
Boiling point	137–138°C		8, 16
Distillation point at 0.005 mm Hg	120–125°C		5, 9
Absorption max (UV)	324–325 nm (ethanol) ($E^{1\%}_{1cm}$ = 1835); 328 m	325–328 nm (ethanol) ($E^{1\%}_{1cm}$ = 940–975)	1, 5, 9, 10
Solubility[a]			
Water	I	I; S	5, 8, 9, 17, 18
Glycerol	I	I	5, 18
Alcohol	S	S	5, 8, 18
Methanol	S		5, 8, 18
Ethanol	S		16
Acetone	S		16
Benzene	S		16
Chloroform	S	S	5, 8, 18
Ether	S	S	5, 8, 18
Most organic acids, fats, and oils	S	S (most vegetable oils)	5, 8, 9, 18
Light ptroleum	S		8
Refractive index (22°C)	N_D-1.6410 (calculated from refraction indices of 20–70% solutions in mineral oil)		1
Optical rotation	±0°		1

[a]S, soluble; I, insoluble.

The analysis of these retinoids* depends on certain inherent chemical properties: their intense absorption in the near UV region, their ability to fluoresce, their light sensitivity, and their ready conversion to charged, intensely colored complexes in the presence of certain acids. High-pressure liquid chromatography (HPLC) has become the preferred method in retinoid analysis because of the stability of retinoids on most HPLC columns, its high resolution and sensitivity, and the rapidity of most analyses. HPLC also has the ability to separate a wide range of retinoids that may differ only in their isomeric configuration. Other analytical methods used include conventional column, thin-layer, and gas–liquid chromatographies, fluorescence and the UV-VIS (visible light) absorption of the retinoids themselves or of their colored products. Nuclear magnetic resonance (NMR) spectroscopy is used routinely to establish the chemical structure, whereas mass spectroscopy is useful for determining the molecular weight and structural information.[15,10,20]

*Retinoids, designated term for the natural forms of vitamin A and their synthetic derivatives.

The reader is referred to Frolik and Olson[20] for a detailed presentation of the analytical methods used to identify Retinol and Retinyl Palmitate.

USE

Cosmetic Use

Retinol and Retinyl Palmitate are used primarily in hair, facial makeup, and skin care preparations. They are generally used at concentrations ≤ 1%.[21] Table 2 presents the FDA product formulation data for Retinol and Retinyl Palmitate.[21] These computerized data, made available by the FDA, are compiled through voluntary filing in accordance with Title 21 part 270.4 (d)(1) of the Code of Federal Regulations.[22] Ingredients are listed in prescribed concentration ranges under specific product type categories. Since certain cosmetic ingredients are supplied by the manufacturer at less than 100% concentration, the value reported by the cosmetic formulator may not necessarily reflect the actual concentration found in the finished product; the actual concentration would be a fraction of that reported to the FDA. The fact that data are only submitted within the framework of preset concentration ranges also provides the opportunity for overestimation of the actual concentration of an ingredient in a particular product. An entry at the lowest end of a concentration

TABLE 2. Product Formulation Data.[21]

Product category	*Total no. of formulations in category*	*Total no. containing ingredient*	*No. of product formulations within each concentration range (%)*			
			Unreported concentration	*> 1–5*	*> 0.1–1*	*≤ 0.1*
Retinol						
Baby lotions, oils, powders, and creams	56	1	—	—	1	—
Eye makeup remover	81	1	—	—	—	1
Hair conditioners	478	2	2	—	—	—
Hair sprays (aerosol fixatives)	265	1	—	—	—	1
Hair rinses (noncoloring)	158	1	1	—	—	—
Hair shampoos (noncoloring)	909	4	3	—	—	1
Tonics, dressings, and other hair grooming aids	290	2	—	—	1	1
Wave sets	180	4	—	—	—	4
Other hair preparations (noncoloring)	177	1	—	—	1	—
Blushers (all types)	819	1	1	—	—	—
Face powders	555	1	1	—	—	—
Makeup foundations	740	4	—	—	—	4
Lipstick	3319	5	5	—	—	—
Makeup bases	831	5	2	—	—	3
Nail creams and lotions	25	1	1	—	—	—
Other manicuring preparations	50	1	—	—	—	1
Personal cleanliness products	227	1	1	—	—	—

TABLE 2.

Product category	*Total no. of formulations in category*	*Total no. containing ingredient*	*No. of product formulations within each concentration range (%)*			
			Unreported concentration	*> 1–5*	*> 0.1–1*	*≤ 0.1*
Aftershave lotions	282	1	1	—	—	—
Preshave lotions (all types)	29	1	1	—	—	—
Skin cleansing preparations (cold creams, lotions, liquids, and pads)	680	7	2	—	2	3
Face, body, and hand skin care preparations (excluding shaving preparations)	832	20	10	1	2	7
Hormone skin care preparations	10	3	—	—	3	—
Moisturizing skin care preparations	747	39	13	—	6	20
Night skin care preparations	219	11	4	—	2	5
Paste masks (mud packs)	171	4	2	—	—	2
Skin fresheners	260	4	—	—	—	4
Wrinkle smoothers (removers)	38	1	—	—	—	1
Other skin care preparations	349	8	—	—	1	7
Suntan gels, creams, and liquids	164	3	—	—	1	2
1981 TOTALS		138	50	1	20	67
Retinyl Palmitate						
Bubble baths	475	1	—	—	—	1
Other bath preparations	132	1	—	—	—	1
Eye makeup preparations	230	4	1	—	1	2
Hair conditioners	478	2	1	—	—	1
Tonics, dressings, and other hair grooming aids	290	2	—	—	2	—
Blushers (all types)	819	2	—	—	1	1
Face powders	555	1	—	—	—	1
Makeup foundations	740	7	—	—	—	7
Lipstick	3319	14	—	—	3	11
Makeup bases	831	1	—	—	—	1
Rouges	211	2	—	—	1	1
Other makeup preparations (not eye)	530	1	—	—	—	1
Nail creams and lotions	25	1	—	—	—	1
Nail polish and enamel	767	1	—	—	—	1
Face, body, and hand skin care preparations (excluding shaving preparations)	832	12	1	—	5	6
Moisturizing skin care preparations	747	28	5	—	11	12
Night skin care preparations	219	9	2	1	2	4
Paste masks (mud packs)	171	4	1	—	1	2
Wrinkle smoothers (removers)	38	1	—	—	1	—
Other skin care preparations	349	7	2	—	4	1
Suntan preparations	28	1	—	—	1	—
1981 TOTALS		102	13	1	33	55

range is considered the same as one entered at the highest end of that range, thus introducing the possibility of a 2- to 10-fold error in the assumed ingredient concentration.

In 1981, Retinol reportedly was used in a total of 138 formulations, most of which were hair (noncoloring), makeup (not eye), and skin care preparations. Of these 138, 49% incorporated Retinol at concentrations $\leq 0.1\%$; 14% at concentrations of $> 0.1–1\%$; less than 1% at concentrations of $> 1–5\%$; and 36% at unreported concentrations.[21]

Retinyl Palmitate reportedly was used in a total of 102 formulations in 1981, most of which were makeup (not eye) and skin care preparations. Of these 102, 54% incorporated Retinyl Palmitate at concentrations $\leq 0.1\%$; 32% at concentrations of $> 0.1–1\%$; 1% at concentrations of $> 5–10\%$; and 13% at unreported concentrations.[21]

The formulation data presented in Table 2 indicate that cosmetic products containing Retinol and Retinyl Palmitate may contact all external body surfaces and hair, as well as oral, ocular, and vaginal mucosae. These products may be used daily or occasionally over a period of up to several years. The frequency and length of application could result in a continuous exposure.

Retinol and Retinyl Palmitate are both approved for use in cosmetics in Japan.[23]

Noncosmetic Use

Retinol and Retinyl Palmitate are both affirmed as GRAS (generally recognized as safe) food ingredients when used in accordance with good manufacturing practices.[24,25] Their functional uses in foods are as nutrients and dietary supplements.[18] Retinol is also used as a nutrient and dietary supplement in the veterinary field.[5,15,26]

Vitamin A is used in pharmaceutical products for the treatment of diaper rash and hemorrhoids (internal and external creams), in corn and callus removers and vaginal creams, and as dietary supplements for the prevention of vitamin A deficiency. Retinyl Palmitate is also used in products for the treatment of diaper rash.[27,28]

Retinol administered orally has been used as therapy for a variety of dermatoses for the past 40 years. However, since the hypervitaminosis A syndrome (see section Clinical Assessment of Safety, Hypervitaminosis A) has interferred with chronic therapeutic use of vitamin A, current research is searching for synthetic derivatives that are less toxic and yet as efficacious as vitamin A. Two synthetic retinoids, isotretinoin and etretinate, have been introduced into the clinical practice of dermatology.[29] Some clinicians still advocate the use of oral Retinol in the treatment of severe acne.[30]

BIOLOGY

Retinol is essential for the growth, health, and life of higher animals. It is required for vision, reproduction, and the maintenance of differentiated epithelia and of mucous secretion. Although its role in the visual process is

well understood, little is known about the molecular mechanisms of these other biological effects.[31]

A synopsis of the biology of Retinol and Retinyl Palmitate is presented in the following sections.

Biosynthesis and Absorption

Natural sources of Retinol in the diet include certain plant carotenoid pigments, particularly β-carotene, and the long-chain retinyl esters found in animal tissues. β-Carotene is converted to Retinol primarily in the intestinal mucosa, although conversion also occurs in the liver and other tissues.[31,32] The overall biological efficiency of conversion of β-carotene to Retinol is about 50% at the maximum; this level of efficiency occurs at lower concentrations of carotene intake and declines as intakes rise.[33]

Dietary retinyl esters, Retinol, and provitamin A carotenoids are dispersed and emulsified in the stomach during the gastric phase of lipid digestion. Extensive hydrolysis of the retinyl esters is carried out in the intestinal lumen by an enzyme derived from the pancreas, commonly called the "pancreatic nonspecific lipase or cholesterol esterase." Repeated hydrolysis and reesterification of retinyl ester occur during its digestion and assimilation.[32]

Retinol obtained from the diet and that resulting from the hydrolysis of retinyl esters is solubilized in mixed micelles, transported across the aqueous diffusion barrier (the unstirred water layer), and absorbed by the mucosal cells. Here, it is reesterified with long-chain mainly saturated, fatty acids (predominantly palmitic acid).[32] An acyl-Coenzyme A retinol acyltranferase is believed to be responsible for the intestinal esterification of Retinol.[34] The retinyl esters are then incorporated along with other lipids and apolipoproteins into chylomicrons and transported via the lymph into the general circulation. The chylomicrons are metabolized in extrahepatic tissues by the lipolytic removal of most of the triglyceride, leaving a smaller, cholesterol-rich particle that contains essentially all of the chylomicron retinyl esters. These chylomicron remnants are removed from the circulation almost entirely by the liver.[32]

The reader is referred to Hollander et al. for an in-depth review of the intestinal absorption of Retinol and β-carotene.[35-39]

Lee[40] has studied the disposition of topically applied vitamin A in the cornea, conjunctiva, iris-ciliary body, and aqueous humor of healthy male albino rabbits using radiotracer techniques. Single doses (25 μl volume) of a 0.1% solution of vitamin A (all trans[^{3}H] and nonradioactive) in arachis oil were instilled directly into the cornea of the test rabbits. Both eyes of the test animals were used, but the dosing time was staggered so that the eyes could be evaluated at different time points. Rabbits were sacrificed at various times by intravenous (IV) injection of 30% sodium phenobarbitol solution into a marginal ear vein and the ocular tissues were obtained. Concentrations of vitamin A were studied for up to 120 min after instillation. The highest concentration of vitamin A was found in the cornea and conjunctiva; these tissues had peak times early, suggesting a rapid uptake of vitamin A from the

tear pool. A sustained concentration of vitamin A was evident in all ocular tissues studied beginning at 30 min postinstillation. Lee found that vitamin A was available to the conjunctiva and the cornea from topical dosing, since these two tissues are known to be affected by vitamin A deficiency and dry-eye states.

Hepatic Metabolism and Storage

The uptake of chylomicron remnants by the liver appears to occur primarily by receptor-mediated endocytosis, followed by lysosomal degradation. The retinyl esters are again hydrolyzed, reesterified, and stored in both the parenchymal liver cells and in the nonparenchymal fat-storing cells. The enzyme catalyzing the esterification of Retinol in the liver is found in the microsomal fraction and appears to qualify as a fatty acyl-Co A: retinol acyltransferase.[32,41-43]

The retinyl esters may be stored in several forms, as lipid droplets or granules or as a high molecular weight lipid-protein aggregate in the cytosol of the liver cell. Futterman and Andrews[44] studied the composition of retinyl ester isolated from the livers of a number of vertebrate species (calf, sheep, rabbit, rat, human, frog, trout, and cat). With the exception of the cat, livers of all the species contained substantial amounts of Retinol, of which 95% was present as long-chain retinyl esters. Retinyl Palmitate was the predominant ester in all species, comprising 66% of the retinyl esters in the human. Retinyl stearate and oleate were the next most common esters. These hepatic stores normally represent over 90% of the total body reserves of vitamin A.[32,42]

The hepatic parenchymal cell is the major cell type responsible for the uptake and initial metabolism of newly absorbed Retinol. Recent evidence suggests that Retinol is then transferred from parenchymal cells to nonparenchymal fat-storing cells for storage (as retinyl ester in lipid droplets).[45-47] The hepatic parenchymal cells also plays an essential role in the mobilization of Retinol in that it synthesizes and secretes the specific plasma transport protein retinol-binding protein (RBP).[31,32,42]

The mammalian liver is capable of storing varying quantities of dietary vitamin A. However, on an individual basis, plasma vitamin A concentrations remain remarkably constant over a wide range of dietary intakes and liver stores.[31] The hepatic fat-storing cells appear to act as a reservoir for excessive vitamin A intake and are capable of storing Retinol up to a point, beyond which hypervitaminosis occurs.[48]

McKenna and Bieri[49] have used the total parenteral nutrition (TPN) rat and its sham-operated control as a model to compare the storage and fate of vitamin A (and E) when administered intravenously or orally. Plasma concentrations of Retinol were the same for the TPN rats (infused through the jugular vein) as for the controls (orally administered). However, hepatic storage was much higher in the infused rats. This could be due to the different physical state in which the vitamin was delivered to the liver: in chylomicrons when given orally and in micellar form when given IV.

Mobilization, Transport, and Delivery of Retinol

From the hepatic stores of retinyl esters, vitamin A is mobilized and transported in plasma to peripheral target tissues as Retinol bound to retinol-binding protein (RBP). In humans, RBP is a single polypeptide chain with a molecular weight of approximately 21,000 and a single binding site for one molecule of Retinol. In plasma, RBP normally circulates complexed with Retinol.[31,50]

RBP also interacts with another protein, plasma transthyretin (TTR) (more commonly known as plasma prealbumin) and usually circulates as a 1:1 molar RBP–TTR complex. The formation of this RBP–TTR complex reduces the glomerular filtration and renal catabolism of RBP.[31,50] The reader is referred to Goodman[50] and Rask et al.[51] for an extensive review of the chemistry and biology of RBP.

The mobilization and delivery of Retinol are highly regulated and are particularly controlled by the processes that regulate the hepatic rates of RBP synthesis and secretion (one of which is the nutritional Retinol status). There is also evidence that the delivery of Retinol to peripheral target tissues involves specific cell-surface receptors that recognize RBP.[32,50]

A specific intracellular binding protein for Retinol has been identified and designated as cellular retinol-binding protein (CRBP). CRBP is a single polypeptide chain with a molecular weight of approximately 14,600 and differs from plasma RBP in a number of ways. CRBP may (1) play a direct role in the biological expression of vitamin A activity, (2) facilitate the specific interaction of Retinol with binding sites for Retinol in the cell nucleus, or (3) serve as an intracellular transport protein, transporting Retinol from one locus to another between metabolic events in the liver.[32,52] Information available on CRBP has been summarized in several reviews.[53–56]

Oxidative Metabolism

Retinoic acid is a natural physiological metabolite of Retinol, although it is not known if the acid is at the same time an obligatory metabolite. Most structural studies on retinoid metabolism have been conducted with retinoic acid; therefore, since it is probable that at least some Retinol is metabolized through retinoic acid, many of the metabolites identified for retinoic acid would also be metabolites of Retinol.[41]

The overall metabolism of Retinol has been examined in a number of studies, with emphasis placed on the urinary and fecal or biliary excretion of radioactive metabolites.[41] The results of administration to rats of either [^{14}C] Retinol or [^{3}H] retinyl acetate indicated that 4–15% of the dose was excreted in the urine during the first 24–48 h. The amount of dose excreted in the urine depended on the position of the radioactive atom in the starting retinoid. These data suggest that decarboxylation of retinoic acid, a metabolite of Retinol, is occurring. Some of the urinary Retinol metabolites have been partially characterized by Wolf et al.,[57] although none has been ascribed a chemical structure. These metabolites are mainly water soluble and contain

no detectable free Retinol or retinyl esters. Clark et al.[58] have reported that a protein-bound form of Retinol, or of a metabolite of Retinol that is different from retinoic acid, was excreted in the urine of rats administered a large (23.1 mg) dose of Retinol. A similar substance has been detected in human urine.

The biliary and fecal metabolites of Retinol have not been extensively characterized. With the exception of Retinol, retinoic acid, and their conjugates, the structures of these compounds have not been clarified. The amount of the administered dose recovered varies depending on the position of the radioactivity, the mode of administration, and the quantity administered. Several investigators have reported that 18–25% of a 10–26 μg dose of Retinol can be recovered in the bile of rats 24 h after administration. A small portion of the metabolites has been identified as free or conjugated retinoic acid, whereas almost no free Retinol has been detected. An O-ether derivative of Retinol, retinyl β-glucosiduronate, has been identified in the bile of rats given a 3 mg dose of Retinol. The fecal metabolites are believed to originate from the bile. Recovery values range from 0.3% of a 10 μg dose in 24 h to 26% of a 2 μg dose in 5 days. An initial delay in the fecal excretion of metabolites after injection of Retinol has been noted.[41]

Interactions

Micronutrients

Retinol has long been known to interact with other micronutrients, including vitamin E, ascorbic acid (vitamin C), iron, and zinc. Vitamin E is generally believed to have a nonspecific antioxidant role. Studies have confirmed that the tocopherols (with vitamin E activity) provide protection from oxidation to Retinol. This results in increased hepatic content of Retinol.[33]

The synthesis of ascorbic acid in the liver of rats was impaired during both a deficiency and an excess of Retinol. Feeding ascorbic acid to the Retinol-deficient rats prevented the decrease in hepatic ascorbic acid. However, this had no effect on the rats with an excess of Retinol. Retinol may influence the hepatic synthesis of ascorbic acid, whereas the latter acts as an antioxidant for excess hepatic Retinol.[33]

Iron acts as a prooxidant and may facilitate oxidative destruction of vitamin A-active compounds in the intestine. Other studies in rats have demonstrated that the intestinal absorption of iron is not altered during vitamin A deficiency and that the vitamin appears to facilitate the mobilization of stored iron and its incorporation into erythrocytes.[33]

Zinc is essential for many enzyme systems in the body, some of which are directly or indirectly critical to the metabolism of Retinol. Liver alcohol dehydrogenase catalyzes the reactions for several primary alcohols, including Retinol, and a deficiency in zinc reduces the activity of this enzyme. Some zinc-dependent enzymes are involved in protein synthesis and metabolism and may include the synthesis of RBP and CRBP. In several studies, zinc deficiency has ben accompanied by decreased plasma concentrations of Retinol. The hepatic mobilization of Retinol may be impaired by zinc deficiency.[33] Solomons and Russell[59] have reviewed the impact on human nutrition of vitamin A and zinc interactions.

Hormones — Gonadal Steroid and Adrenocortical

Estradiol exerts a stimulatory effect on the hepatic synthesis of RBP and subsequently increases the mobilization of Retinol as the Retinol–RBP complex. This is supported by results of studies with synthetic estrogen-containing anovulatory hormones: plasma concentrations of Retinol and RBP increased in both animals and humans due not to a variation in dietary vitamin A but to increased mobilization of hepatic reserves.[33]

Adrenocortical hormones have also increased the hepatic mobilization of Retinol in animals, thus increasing plasma Retinol levels.[33]

Drugs, Xenobiotics, and Alcohol

Results of animal studies have indicated that DDT and other chemicals are capable of reducing hepatic stores of Retinol.[33] A decrease in the hepatic content of vitamin A has also been noted after the administration of certain xenobiotics, including polychlorinated biphenyls (PCBs),[60,61] polybrominated biphenyls (PBBs),[62] and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD).[63] Cullum and Zile[64] studied the effects of a single oral dose of 3,3′,4,4′,5,5′-hexabromobiphenyl (HBB), the most potent of the PBB congeners, on the steady-state metabolism of vitamin A in rats. HBB produced an abnormal twofold increase in the metabolic output of degraded vitamin A in the urine and feces for at least 8 days. The investigators suggested that these effects most likely reflect a stimulation of renal vitamin A metabolism and a deregulation of vitamin A metabolism in the liver, possibly leading to an increased dietary vitamin A requirement.

Lower concentrations of vitamin A have been found in the livers of human drug addicts than in the livers of those dying from multiple injuries and asphyxiation.[33]

The effects of acute and chronic ingestion of alcohol on the metabolism of Retinol are complex and not totally defined. Under conditions of acute hepatic toxicity (due to acute ingestion of alcohol), it was reported that plasma levels of Retinol, RBP, and TTR were all reduced, although hepatic stores may not have been affected. Chronic alcohol ingestion associated with all stages of alcoholic liver disease results in lowered hepatic storage and lowered plasma levels of Retinol. Studies conducted with rats have also suggested that ethanol potentiates the hepatotoxicity due to vitamin A supplementation.[33,65,66]

Cellular and Biochemical Effects

The effects of retinoids on cell differentiation and proliferation have been studied extensively in the past 10 years. Much of this study has been conducted in search of therapeutic roles for retinoids in the treatment of cancer. These studies show that the cellular effects of retinoids in the induction and control of various biochemical processes are extremely variable. High and low concentration of retinoids have produced opposite effects even within the same experimental system. Furthermore, results obtained a short time after the administration of the retinoid may be entirely opposite those obtained at a later time.[67]

Retinoids have been shown to modify the differentiation of nonneoplastic cells in culture, both epithelial cells (keratinocytes) and mesenchymal cells. Similar effects have been observed both in vivo and in organ culture in vitro. Only growth is affected in certain other cells.[67]

Retinoids have been shown to have marked effects on the growth and differentiation (in culture) of many neoplastic cell types, including epithelial and mesenchymal cells as well as cells derived from neural and primitive ectoderm. Retinoids have inhibited growth in monolayer and anchorage-independent growth in semisolid medium. They have promoted the terminal differentiation of certain fully neoplastic embryonal carcinoma and promyelocytic leukemia cells to nonneoplastic differentiated cell types. Although many of the effects of retinoids on the growth of neoplastic cells are reversible, the transformation of phenotype from neoplastic to nonneoplastic is fairly stable. These effects on differentiation may be able to induce remission of disease, whereas the inhibitory effects of retinoids on the growth of neoplastic cells could be valuable when used in conjunction with other therapeutic treatments. Retinoic acid and its analogs, in most cases, were considerably more active than Retinol, retinyl esters, or retinaldehyde in modulating the behavior of cultured cells.[67]

The effects of retinoids on the activity and synthesis of cellular enzymes and effectors have also been studied. Various cell responses were seen with respect to orinthine decarboxylase, transglutaminase, cyclic AMP, cyclic AMP-dependent protein kinases, plasminogen activator, collagenase, and prostaglandins. Due to the variety of responses, these particular enzymes and effectors are not believed to play a major role in the mechanism of action of the retinoids.[67]

Retinoids profoundly influence the biosynthesis of all types of glycoconjugates, including glycoproteins, glycolipids, and proteoglycans. Evidence has been provided for a scheme involving direct participation of retinoid phosphate derivatives in glycosyl transfer reactions. Others have suggested that retinoids might affect the biosynthesis of the lipid glycosyl carrier dolichol or the activity of specific glycosyltransferases.[67]

There exist two dominant theories of retinoid mechanism: the proposed cofactor role of retinoids in glycosyl transfer and the proposed steroid model for retinoid control of gene expression. In the first theory, retinoids are believed to participate directly in the transfer of mannose to glycoconjugates, which control cell growth, differentiation, and transformation. However, no evidence has demonstrated conclusively a function for retinoids separate from that of dolichol. The second hypothesis suggests the retinoids act in a way similar to the accepted model for steroid hormone action. Thus, retinoids would bind to a specific intracellular receptor (CRBP for Retinol and CRABP for retinoic acid), be translocated to the nucleus, bind to chromatin, and lead to altered genomic expression. Data exist to support this hypothesis and also suggest that there are differences in the modes of action of Retinol and retinoic acid.[67,31]

For a complete review of the cellular biology and biochemistry of the retinoids, the reader is referred to Roberts and Sporn,[67] Sporn and Roberts,[68] Lotan,[69] Schroder et al.,[70] Wolf,[71,72] and Zile and Cullum.[73]

Physiological Effects

Morley et al.,[74] using both in vivo and in vitro techniques, studied the effects of the administration of pharmacological doses of vitamin A (as Retinyl Palmitate) on multiple parameters of thyroid function. Three groups of 8 rats each were administered vitamin A orally for 2 weeks at doses of 30 mg (100,000 IU) three times weekly, 45 mg (150,000 IU) three times weekly, or 45 mg (150,000 IU) five times weekly. Vitamin A decreased total T_4 (thyroxine) and T_3 (triiodothyronine) levels. The serum-free T_3 and T_4 levels as measured by dialysis were normal in these vitamin A-treated rats. Treatment with vitamin A in vitro (10^{-4}–$10^{-6}M$) resulted in a marked increase in the percentage dialyzable T_3 and T_4. Total T_4 levels were still decreased in thyroidectomized rats (administered 15 mg vitamin A daily for 7 days) maintained on T_4, suggesting that vitamin A produced its effects by increasing peripheral clearance of T_4. Vitamin A did not alter basal thyroid-stimulating hormone (TSH) release or its release in response to thyroid-releasing hormone, indicative of a normal hypothalamic–pituitary–thyroid axis in vitamin A-treated (45 mg three times weekly for 2 weeks) rats. Decreased Na-K-ATPase activity in the livers from vitamin A-treated (15 mg daily for 7 days) rats and decreased growth hormone (GH) response to T_3 in GH_3 pituitary cultures indicated that tissue responsiveness to thyroid hormones may be decreased by vitamin A. Vitamin A significantly decreased thyroid weight and increased ^{125}I thyroidal uptake in vivo. In vitro, vitamin A (10^{-4}–$10^{-6}M$) enhanced T_4 to T_3 conversion in hepatic homogenates. The investigators stated that the results of this study, as well as previous studies, suggest that there is an inverse relationship between plasma thyroxine and vitamin A levels. A different relationship may exist in humans, where the major binding protein is thyroxine-binding globulin compared to rat thyroid hormone-binding prealbumin (TBPA).

Chertow et al.[75] evaluated the effects of Retinyl Palmitate on IV glucose disposition and insulin secretion in man. Intravenous glucose tolerance tests (IVGTT) with 26 g glucose were performed on 10 healthy subjects before and after two intramuscular injections of Retinyl Palmitate (25,000 IU) 18 h apart. Glucose disposition was impaired after treatment with Retinyl Palmitate in 9 of the 10 subjects. In vitro, 10^{-4}–$10^{-6}M$ Retinyl Palmitate did not affect the binding or displacement of insulin ^{125}I from IM-9 cultured human lymphocytes. Other studies have also indicated that Retinyl Palmitate decreases glucose tolerance.[76,77] The effect of vitamin A appears to be mediated through stimulation of adrenal cortisol secretion.[78–82] However, in this study Retinyl Palmitate did not affect serum cortisols. Another proposal is that Retinyl Palmitate has a direct effect on hepatic glucose metabolism, therefore limiting hepatic glucose uptake and the disposition of an IV glucose load.[77,79,80,83]

Ultraviolet Light Effects on Cutaneous Retinol

Barne et al.[84] investigated the effect of UV irradiation on the concentration of the cutaneous retinoids Retinol and 3-dehydroretinol in rabbit skin in vivo and human skin in vitro. The irraditation source consisted of four Philips

SP500 W water-cooled high-pressure mercury lamps with water-cooled filters. Irradiations were performed by applying the light aperture (2.2 cm in diameter) directly to the skin for periods varying between 30 sec and 15 min depending on the wavelength and irradiation dose studied. The ears of the rabbits were irradiated with either 313, 334, 365, or 405 nm at a dose of 3 J/cm^2 (five rabbits), 334 nm at a single dose of 0.5, 1.0, or 3.0 J/cm^2 or 6 repeated doses of 0.5 J/cm^2 (4 rabbits), or 334 nm at a single dose of 3 J/cm^2, with skin samples removed at 1, 24, 72, and 168 h. Fresh human skin was irradiated in vitro with 334 nm at a dose of 3 or 10 J/cm^2, and frozen skin (on dry ice) was irradiated with 313, 334, 365, or 405 nm at a dose of 3 J/cm^2. The irradiated skin was cut out and separated into epidermis and dermis using 1% acetic acid at 4°C for rabbit skin and heat treatment for human skin. Analysis was performed by HPLC.

Dose-dependent reductions of Retinol where noted in the epidermis and dermis of humans and rabbits, with the maximal effect obtained at 334 nm (a wavelength that approximates the absorption maximum of Retinol in organic solutions). Results of pilot studies indicated that irradiation of frozen human skin at 334 nm reduced the concentration of epidermal Retinol to 88, 74, and 51% of the control value at 1, 5, and 10 J/cm^2, respectively. A similar reduction was noted in irradiated fresh skin, suggesting independence of cellular metabolism. 3-Dehydroretinol was not significantly reduced. The photodecomposition of Retinol in human skin was most extensive in the epidermis and progressively less so in the dermis, presumably reflecting the extent to which 334 nm radiation penetrates the skin. Significant reductions in the concentration of Retinol were noted down to the middermis of irradiated human skin, indicating that the effect of 334 nm extends to a depth of about 1 mm. In rabbit ear skin, similar reductions of Retinol were observed in the epidermis and dermis. The investigators suggested that the thin and nonpigmented rabbit epidermis may allow more radiation to penetrate into the underlying dermis.[84] These investigators had previously found that the concentration of epidermal Retinol was significantly reduced in humans treated for uremic pruritis with a series of 12 UV irradiations (UVA and UVB) over a period of 2–3 months.[85] The nutritional and biological significance of these findings has not been established. Berne et al.[84] are interested in the possibility that radiation-induced depletion of cutaneous vitamin A and formation of photosensitizing retinoid intermediates may promote UV carcinogenesis.

ANIMAL TOXICOLOGY

Single high doses and multiple low doses of Retinol are toxic to laboratory animals. Single large doses can be lethal, whereas chronic intoxication has adverse effects on many tissue and organ systems.[86,87] Characteristic effects of chronic hypervitaminosis A in animals include weight loss, erythema, hair loss, internal hemorrhage, and fractures. Many of these effects are reversible upon cessation of administration.[88]

Theory of Retinol Toxicity

Excess Retinol both in vivo and in vitro results in increased lability of biological membranes. This is attributed to its surface-active membranolytic properties. Excess Retinol increases the synthesis and release of lysosomal enzymes, which play a major role in the effects of vitamin A on cartilage and limb–bone rudiments. Retinol bound to RBP as the Retinol–RBP complex does not appear to exert membranolytic effects. Therefore, the mode of transport of Retinol is believed to be the critical factor in the development of the symptoms of hypervitaminosis A.[50]

This theory has been supported by studies in rats and humans. Rats given excessive doses of vitamin A had large increases in serum vitamin A, due to higher circulating concentrations of retinyl esters and decreased concentrations of serum RBP. Practically all of the serum vitamin A and retinyl esters were found in association with serum lipoproteins, thus suggesting that they play an important role in the transport of excess vitamin A. Similarly, 3 patients with chronic hypervitaminosis A had increased plasma concentrations of total vitamin A, particularly of retinyl esters; plasma RBP levels were normal. These data suggest that vitamin A toxicity occurs only when, due to excessive intake of vitamin A, Retinol begins to circulate in the plasma in a form other than bound to RBP.[50]

Acute Toxicity

Oral and Subcutaneous

The reactions of mice and rats to single large cutaneous or oral doses of Retinol have been described by Nieman and Obbink.[86] Death is preceded by convulsions and paralysis, and those animals that survived had signs of malaise, decreased motor activity, stupor, muscular weakness, and occasionally changes in the pelt. The animals that survived recovered with no apparent residual toxic effects.

Retinol had an oral LD_{50} of 2570 mg (8.6 million IU) per kg body weight in mice.[89] Retinyl Palmitate had oral LD_{50}s of 6060 mg (11 million IU) per kg in mice and 7910 mg (14.4 million IU) per kg in rats.[89]

Intraperitoneal

Intraperitoneal (IP) injection of 200–500 mg (667,000–1.6 million IU*) Retinol per kg body weight to 20 adult Sprague-Dawley rats produced 90% mortality in 3 days. Similarly, IP injection of 425 mg (1.4 million IU) Retinol per kg body weight to 11 weanling rats produced 64% mortality. Survival time was inversely related to plasma Retinol and retinyl ester concentrations. However,

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*Vitamin A or Retinol values are commonly expressed in international units (IU) or both as IU and as retinol equivalents (RE).[33]

1 IU = 0.3 μg preformed retinol.

1 RE = 1 μg retinol or 6 μg β-carotene.

no signs of toxicity were observed over a period of 2 months in 7 adult rats injected IP with 370 mg (673,000 IU) Retinyl Palmitate per kg body weight.[3]

Retinol had an IP LD_{50} of 1510 mg (5 million IU) per kg in mice.[89]

Dermal Irritation

Two lots of a moisturizer containing 0.1% Retinyl Palmitate were evaluated for dermal irritation in albino rabbits. A 0.5 g sample of each lot was applied to one side of the shaved back of each of 3 rabbits. A control lot was applied to the other side of the back. Applications were repeated daily for 4 days. Irritation indices for the experimental and control lots were 3.5 and 3.4, respectively, in one group, and 3.3 and 3.1, respectively, in the other group. These differences were considered slight and insignificant, although it was noted that the first experimental lot was slightly more irritating in 2 of the 3 treated rabbits.[90]

A body lotion containing 0.1% Retinyl Palmitate was evaluated for dermal irritation in 3 albino rabbits. A 0.5 ml sample of the body lotion was applied daily for 4 days to the shaved back of each rabbit. A well-defined erythema and edema developed within 48 h and persisted throughout the 7-day study, resulting in subsequent dehydration and desquamation. The body lotion had an irritation index of 3.1 (max = 8.0).[91]

Ocular Irritation

Two cosmetic products, a moisturizer and a body lotion, each containing 0.1% Retinyl Palmitate, were evaluated for ocular irritation in two groups of 6 albino rabbits (12 total). A 0.1 ml sample of each product was instilled into 1 eye of each rabbit. Slight conjunctival redness was noted in all treated eyes at 1 h; those eyes treated with the moisturizer cleared within 24–48 h, whereas those treated with the body lotion cleared within 24 h. Corneal and iridial membranes were unaffected.[90,91]

Short-Term and Subchronic Toxicity

General

The subchronic toxicity of Retinol and vitamin A preparations as reported in the literature up to the early 1950s has been extensively reveiwed by Nieman and Obbink.[86] The rat was the primary test animal, although a variety of animal species were used. Retinol was administered in doses ranging from 3000 to 180,000 RE (10,000–600,000 IU)/day (approximately 2–120 times the recommended dietary allowance) for periods of time from a few days up to several weeks. Hypervitaminosis A was produced by doses as low as 3000 RE (10,000 IU)/day. The time to first appearance of symptoms depended on the route of administration, the species and age of the animal, duration of treatment, the symptom in question, and the dose.[88]

Hypervitaminosis A is characterized by the following,[86] as summarized by Kamm et al.[88].

1. General signs of toxicity: Anorexia, weight loss, emaciation, anemia, cachexia, and death. Spontaneous fractures (particularly in young rats) that

heal and do not recur after treatment is stopped. Extensive subcutaneous and/or intramuscular hemorrhages and sometimes inflammation of nasal passages, gut, and conjunctivae.[86]

2. Dermal effects: Hair loss, localized hyperemia and/or erythema and thickened epithelium. Topical administration of Retinol is irritating and may produce peeling skin, reduced keratinization, and hyperplasia of the dermal papillae and blood vessels.[86]

3. Internal organ effects: Fatty change of the liver, fatty changes in the spleen, heart, and kidney, hemosiderosis of the spleen, and glomerulonephritis and necrotizing nephrosis. Testicular hypertrophy has been noted in adult rats and degenerative testicular changes in weanling rats after prolonged treatment.[86] In addition, degenerative myocardial fibers were noted in rats treated with 3000 or 6000 RE (10,000 or 20,000 IU)/kg/day for 3 months; these were associated with EKG changes.[88]

4. Hematological effects: Hypochromic anemia with hyperplastic bone marrow, decreased hemoglobin concentration, and transient increases in total circulating lipid and serum cholesterol.

5. Skeletal system effects: Bone fractures due to either increased activity of osteoclasts or decreased activity of osteoblasts while osteoclastic activity remains unchanged. Longitudinal bone growth exceeds circumferential bone growth, resulting in a thin, fragile cortex that fractures easily. Young rats are more susceptible than adults. These fractures were observed in mice and rats after 1 week of daily dosing with 10,000 times the amount of Retinol required to sustain growth and in rats after 1 week of daily dosing with 8500–13,600 RE (28,300–45,300 IU)/day (as retinyl acetate). This bone toxicity has also been demonstrated in dogs, cats, calves, and hogs.[88]

6. Other bone effects: Reduced formation of dentine, atrophy of lingual odontoblasts, and degeneration of pulp and odontoblasts accompanied by amorphous calcification of dentine.[88]

The more current literature on subchronic toxicity has been summarized by FASEB.[3] The lowest reported adverse effect level in experimental animals appears to be in the range of 25,000 to 60,000 IU Retinol per kg per day for periods of 3–5 weeks. All observed signs of hypervitaminosis A toxicity were similar to those recorded. Of note was a study in which high doses (500,000 IU/kg/day for 21 days) of Retinol administered as a water-dispersible commercial preparation were exceedingly more toxic to rats than equal doses of Retinol administered as natural esters.[92] It is now recognized that water-miscible vitamin A is more readily absorbed than oil-soluble vitamin A and therefore capable of eliciting toxic effects at lower doses.[3]

Dermal — Specific Studies

Bern et al.[93] studied the influence of Retinol on the epidermis of rats. They divided 61 male Long-Evans rats into 5 groups: (1) 13 untreated rats, (2) 11 rats receiving topical applications of 0.3 ml sesame oil, (3) 17 rats receiving topical applications of Retinol (1000 IU) in 0.3 ml sesame oil, (4) 8 rats receiving subcutaneous injections of 0.3 ml sesame oil, and (5) 12 rats receiving subcutaneous injections of Retinol (1000 IU) in 0.3 ml sesame oil.

Each of the 5 groups was additionally divided into 4 approximately equal sized groups receiving treatment for periods of 10, 20, 30, and 60 days. A 1-inch square felt pad was applied to the shaved dorsal area of each rat (including controls) and taped in place. Topical applications were injected into the pad, and subcutaneous injections were accomplished with a 2-inch 20-gauge hypodermic needle inserted into the skin outside of the pad and extending to a point underneath it. At the end of the treatment periods, the rats were killed, and the skin of the test area was removed and examined. Epidermal thickness also was measured. Topical applications of Retinol resulted in acanthotic responses after all 4 treatment periods. The thickness of the epidermis (including the stratum granulosum) was approximately twice the normal value after 10 and 20 days of treatment. Topical treatment for 30 days with sesame oil alone produced a mild acanthotic response; this response was noticably stronger after 60 days. The investigators, therefore, attributed part of the increased response to Retinol in sesame oil at 30 and 60 days to be a nonspecific response to the vehicle. The acanthotic responses produced by the topical applications of Retinol were maintained even after 60 days, with no signs of adaptation of the epidermis to Retinol. The subcutaneous administration of Retinol in sesame oil produced no significant reactions in the overlying epidermis. Large deposits of the oil solutions were evident in the subcutaneous tissues of these rats. Results of cytochemical tests performed on the skin sections from untreated and topically treated rats indicated no significant differences among these groups. In cytologic observations and electron microscope studies, the tonofibril system appeared unchanged, although a gradual decrease in the number of intercellular bridges to desmosomes was noted.

Bern et al.[93] also studied the effect of vitamin A on the epidermis of the nipple region of male guinea pigs. Twenty guinea pigs were divided into 5 treatment groups: (1) 2 untreated, (2) 3 treated with 0.2 ml sesame oil, (3) 4 treated with estradiol, (4) 5 treated with vitamin A (1500 IU Retinol or retinyl acetate) in 0.2 ml sesame oil, and (5) 6 treated with both estradiol and vitamin A (1500 IU). A bandage was applied to each nipple and taped in place. The test solutions were injected into the bandage once daily for 10 days. The animals were then killed, and the skin of the nipple was examined. Vitamin A induced acanthosis in the nipple epidermis but did not interfere with its response to estrogen.

Sabella et al.[94] conducted a study similar to that of Bern et al.[93] A total of 55 female rats of the Long-Evans S-1 strain were divided into 5 treatment groups: (1) 11 untreated rats, (2) 11 treated with sesame oil, (3) 11 treated with estradiol in sesame oil, (4) 13 treated with Retinol (5000 IU/ml) in sesame oil, and (5) 9 treated with both estradiol and Retinol in sesame oil. All of the rats had been ovariectomized to eliminate any possible effect of cyclic estrogen production. A 1-inch square felt pad was taped onto the shaved back of each rat. Twice daily for 10 days, 0.37 ml of each oil solution was injected into the felt pads. The total daily dose of vitamin A to each of the rats in groups 4 and 5 was 3700 IU. The rats were killed after 10 days, and the treated skin areas were examined. Both the epidermis and the stratum granulosum alone in the Retinol and Retinol–estradiol treated rats were significantly thicker (approxi-

mately twice as thick) than those of rats in the other groups. This was attributed to an increased number of cell layers and an apparent increase in cell size. The investigators also suggested that an increased rate of keratin formulation or an increased rate of keratohyalin formation may have accounted for the increase in extent of the stratum granulosum. These effects were entirely local in nature, and estradiol did not affect the epidermal response to Retinol.

Rodahl[87] applied 1 drop of saponified bear liver oil (approximately 20,000 IU vitamin A) to the shaved back of each of 5 adult mice daily for 14 days. Marked alopecia was observed on and around the test area (approximately 1 cm in diameter) at the end of the treatment period. The mice were observed for an additional 4 weeks. All of the mice had the typical signs and lesions of hypervitaminosis A 2 weeks after the termination of treatment: exophthalmus, alopecia, soreness and swelling of the palpebrae, loss of hair around the eyes, soreness around the mouth and nose, and clinical signs of fractures of the hind legs. One mouse died at about 3 weeks. One month after the termination of treatment, these symptoms were still marked. The mice were then killed and examined. One mouse had an abscess. Similar abscesses were seen in 3/5 mice. At the postmortem examination, lesions were considered as "similar findings in the organs as described for hypervitaminotic rats" (hyperemia, red blood cells in the space of Bowman's capsule, deposits of sudanophil droplets in the liver, and increased deposits of sudanophil droplets in the adrenal cortex). Since these 5 mice had been kept in the same cage during the study, the investigators believed that the mice may have licked the oil off each other, and therefore they repeated the study with 5 mice individually caged. All procedures were the same except that unsaponified bear liver oil was used. These mice had the same signs and histological changes as the first group. At 18 days, 4/5 had died (1 of peritonitis, others unspecified). It was concluded that topical application of bear liver oil produced lesions of hypervitaminosis A in mice, even when no oil was ingested.

Weslaw et al.[95] have reported that the lower limit of toxicity of vitamin A in the rat is 16,000 IU when rubbed daily into the skin of the back, though admitting that losses occur naturally in these circumstances.

Escarras and Paillas[96] observed signs of local hypervitaminosis A in the guinea pig after applying 2 drops of an oily 3.5% solution of vitamin A every other day to the surface of skin wounds. The wounds treated in this manner healed much more quickly than untreated wounds, while showing extensive conjunctival hyperplasia and vascular hyperplasia with a very high degree of capillary and neovessel proliferation.

A body lotion containing 0.1% Retinyl Palmitate was evaluated for dermal toxicity in New Zealand albino rabbits. The 2 test groups, experimental and control, consisted of 5 males and 5 females each. All of the animals were shaved twice weekly. The body lotion was applied daily for 90 days at a dose of 6 mg/cm^2 to the flank skin of each test rabbit. The lotion was applied to over 10% of each animal's total body surface, and the dose was calculated to be three times the anticipated human use of 2 mg/cm^2. No systemic toxicity was observed in any of the parameters studied: individual weight gain, group mean food consumption data, hematology values, clinical chemistry, uri-

nalyses, organ weight/body weight ratios, and microscopic evaluation. One test rabbit and 2 controls died during the study due to causes not considered treatment-related. All of the treated animals developed slight to moderate erythema and edema during the first week of the study. These symptoms persisted throughout the study and were accompanied by slight to moderate desquamation in all animals. These dermal lesions were characterized histologically by mild dermatitis.[97]

Chronic Toxicity

The relatively few reported long-term toxicity studies with retinoids have been conducted by the laboratories of Hoffmann-La Roche and are unpublished except in the form of brief descriptive reports.[88] Randall[98] has described one study on Retinyl Palmitate.

Retinyl Palmitate was administered orally to dogs and rats for 10 months. The high dose of Retinyl Palmitate in the dogs was selected on the basis that it was tolerated by humans and was approximately 250 times higher than the human recommended allowance for Retinyl Palmitate (approximately 100 IU or 0.06 mg/kg/day Retinyl Palmitate). Groups of 3 dogs were given capsules orally containing 0.6, 2.8, or 13.8 mg/kg/day Retinyl Palmitate 5 days a week (doses of ~ 1000 to 25,000 IU kg/day Retinyl Palmitate). Groups of 10 rats were orally intubated with 5.5, 13.8, or 27.5 mg/kg/day Retinyl Palmitate 5 days a week (doses of 10,000 to 50,000 IU/kg/day). Six dogs and 30 male rats were maintained as controls. The animals were observed for general behavior and clinical signs of toxicity; body weights were recorded weekly. Hematological values were measured after 8, 18, 28, and 38 weeks in the dogs and 18 and 35 weeks in the rats. Necropsy was performed only on the rats that died or were killed for humane reasons. No adverse effects were observed in either the dogs or rats; body growth and hematological values were within normal limits in both species.[88,98]

Teratogenicity and Reproductive Effects

Effects on Fertility and Reproduction

Retinol is essential for reproduction. Even so, there are reports suggesting that high intake of Retinol produces adverse effects on several reproductive functions. Decreased sperm motility and decreased sperm survival were noted in male rabbits receiving 60,000–90,000 IU/kg vitamin A intramuscularly.[3,88] Testicular changes were also noted in rats, although these were reversible upon cessation of treatment.[88] High but nontoxic oral doses of Retinyl Palmitate (5000 IU) administered 3 times per week for 9 months to female rats resulted in an inhibition of cyclic ovulatory activity.[3,88]

Teratogenicity

Retinol administered in high oral doses was first demonstrated to be teratogenic in rats in 1953. Retinol produced more than 70 types of malformations in rats, mice, hamsters, guinea pigs, rabbits, dogs, pigs, and monkeys. The type and incidence of malformations depended on the dose (some 100 times the daily requirement) and stage of pregnancy and, to a lesser extent, on

species and strain.[3] Abnormalities of the face, ears, eyes, and nervous system were most commonly observed. Teratological effects have been produced in rat fetuses at doses of Retinol that do not cause overt toxicity in the dam.[88]

FASEB[3] has summarized the available information (as of 1980) on the teratogenicity of vitamin A. Although it has been reported that the stage of pregnancy when the vitamin is administered critically affects the appearance of teratogenicity, there are relatively few studies in which moderate maternal doses of vitamin A have been administered during the most susceptible periods. These indicate that the no-effect level in mice could be as low as 2500 IU vitamin A/kg body weight, 75,000 IU/kg in hamsters, and 135,000 IU/kg in rats. Thus, sensitivity varies with species.

More recently, Kamm[89] briefly described unpublished studies (conducted at Hoffman-La Roche, Inc.) on the teratogenicity of Retinyl Palmitate. Retinyl Palmitate was administered orally to rats on gestational days 6 through 15 at doses of 0, 10, 30, and 90 mg/kg/day (doses of ~ 18,180 to 163,640 IU/kg/day). Mice were similarly administered doses of 0, 5, 15, and 50 mg/kg/day (doses of ~ 9090–90,900 IU/kg/day). Rabbits received oral administrations of 0, 0.5, 2, and 5 mg/kg/day Retinyl Palmitate (doses of ~ 900–9090 IU/kg/day) on gestational days 6 through 18. The highest no-effect levels were 30, 15, and 2 mg/kg/day Retinyl Palmitate in the rat, mouse, and rabbit, respectively. (This corresponds to doses of ~ 54,550, 27,270, and 3,640 IU/kg/day, respectively.) Lower doses, although not teratogenic, were sometimes fetotoxic.

Hayes et al.[99] have studied the teratogenic effects of Retinyl Palmitate in Fischer 344 rats. Groups of 39 or 43 rats were administered 3.2, 32, and 128 mg/kg/day Retinyl Palmitate (in corn oil) by gavage on days 6 through 15 of gestation. (This corresponds to doses of 5820, 58,180, and 232,730 IU/kg/day, respectively.) A control group of 44 rats received an equal volume of corn oil. The high-dose level was maternally toxic, as evidenced by decreased body weight gains and reduced food and water consumption. This dose level was also embryolethal and teratogenic. The incidence of fetal resorptions was significantly increased compared to controls, and 52/60 surviving fetuses had one or more major malformations. Eighty percent of these fetuses (100% of the litters) had malformations of the craniofacial area. The lower doses tested were neither embryolethal or teratogenic, although the middle dose (32 mg/kg/day) produced slight maternal toxicity.

Willhite and Balogh-Nair[100] studied the teratogenicity of all-trans-Retinol and all-trans-retinylidene methyl nitrone (RMN) in Swiss-Webster mice. The retinoids were dissolved in acetone and solubilized in polyoxyethylenesorbitan monolaurate (final acetone concentration of 5%). Single oral doses of 75 mg/kg of Retinol or RMN were administered to groups of approximately 6–10 pregnant mice by gavage on either day 7, 8, 9, 10, or 11 of gestation. The doses were administered at a rate of 1.0 ml/100 g body weight. Similar groups of mice were given oral doses of the vehicle as controls. All of the mice were killed (in excess CO_2) on day 18 of pregnancy, and the fetuses were removed and examined. Maternal weight changes were calculated, and the number of resorptions were counted. All results were analyzed statistically. No signs of retinoid intoxication were noted in any of the dams. Maternal weight gains and average litter fetal body weights of both treated and control groups were

comparable. Retinol significantly increased the number of affected litters (litter with one or more malformed fetuses) on days 7, 8, 9, and 11 of gestation. The total percentage and mean litter frequency of malformed fetuses were greater after treatment with Retinol than with RMN. Treatment on day 8 produced the highest incidence of embryonic death. Both Retinol and RMN induced malformations of the palate, head, eyes, ears, jaw, and ribs. Retinol treatment on day 9 produced malformations of the tail and head. Retinol treatment on day 10 did not significantly increase the number of affected litters. Retinol treatment on day 11 produced forelimb reduction deformities, polydactyly, and oligodactyly. The mean numbers of bipartite/asymmetrical or unossified/reduced sternabrae were normal except after Retinol treatment on day 9, where the numbers of unossified sternabrae were significantly increased compared to controls. No significant differences were seen in the numbers of ossification centers in metacarpals and metatarsals or in the numbers of cervical and sacrocaudal vertebrae. The investigators suggested that the similar teratogenic activity of these two retinoids may be related to their in vivo biotransformation to all-trans-retinoic acid and its subsequent interaction with embryonic cellular retinoic acid-binding protein (CRABP).

Vitamin A has been used as the positive control in two studies on the teratogenicity of *p*-aminophenol and oxidative hair dyes, respectively. In the first study, vitamin A suspended in rape oil was administered orally at a dose of 15 mg/kg/day to 23 pregnant Sprague-Dawley albino rats on days 6–15 of gestation. The rats were killed and examined at day 19. Vitamin A produced a marked teratogenic effect. The majority of the malformed fetuses had an exencephaly.[101] In the second study, vitamin A was administered in a single oral dose of 100,000 IU/rat on day 9 of gestation. The rats were killed and examined on day 20. Vitamin A produced a significant increase in the number of abnormal fetuses. Frequency of anomalies ranged from 28 to 95%, with major anomalies including hydrocephaly, exencephaly, prognathia macroglossia, open eye, microphthalmia, cleft palate, hydronephrosis, and agenesis of skull bones.[102]

Ismadi and Olson[103] and Donoghue et al.[104] have studied the fetal–maternal transport of Retinol in rats and sheep, respectively. They found that the fetal–maternal transfer of vitamin A was appreciable and characterized the interaction as a dynamic steady-state relationship. Donoghue et al.[104] also sugested that the carrier system of Retinol in the blood may not be the same in the fetal lamb as in the adults in that fetal plasma Retinol was complexed with RBP and another protein other than prealbumin (TTR).

The reader is referred to Gellen[105] and FASEB[3] for more in-depth reviews of the teratogenic effects of hypervitaminosis A and possible mechanisms of action.

Effects on Perinatal and Postnatal Development

Minor brain defects, growth disturbances, and behavioral abnormalities have been observed in postnatal life after in utero exposure to Retinol even when no growth defects were noted at birth.[88]

Joshi et al.[106] studied the effects of postnatal oral administration of 1000 IU vitamin A (in groundnut oil) on the 4th, 6th, 8th, and 10th days of age on

brain maturation in rat pups. Administration of vitamin A reduced brain weight, free cholesterol, phosphatidal ethanolamine, and the synthesis of myelin sulfatides from $Na_2^{35}SO_4$.

Appreciable amounts of vitamin A are transferred to suckling offspring through the maternal milk. This has been observed in rats, cows, and monkeys. The maternal hepatic reserves of vitamin A are usually the major contributor to the milk supply. Evidence has been obtained that Retinol is transferred from the blood to the milk in preference to retinyl esters. Most of the Retinol is then reesterified in the mammary gland and occurs as retinyl esters in the milk. Concentrations of vitamin A are substantially higher in colostrum and early milk than in mature milk in all species studied.[107]

MUTAGENICITY AND ANTIMUTAGENIC EFFECTS

Vitamin A (as retinaldehyde) was evaluated for mutagenicity in the Ames test using *Salmonella typhimurium* strains TA-1535, TA-1537, TA-1538, TA-98, and TA-100. Evaluation was made both with and without metabolic activation and at a maximum vitamin A concentration of 2.0 mg/plate. Vitamin A was nonmutagenic in all strains.[89]

Retinol, at concentrations up to 16 μg/ml, did not increase the frequency of sister chromatid exchanges (SCE) or cell cycle delay in Chinese hamster V79 cells either with or without the metabolic activation of S-9 mix. However, Retinol did inhibit SCE frequencies and cell cycle delay in V79 cells induced by the indirect mutagens cyclophosphamide and aflatoxin B_1. The inhibition was dose and time dependent. Retinol may have no direct effect on the genetic materials but instead may inhibit the metabolic activation of an indirect mutagen or carcinogen.[108]

Retinyl Palmitate has produced strong inhibition of the mutagenic effects of 3-methylcholanthrene and benzo[a]pyrene in human epithelial-like cells. In the same cell line, Retinyl Palmitate also reduced 3-methylcholanthrene and 7,12-dimethylbenz[a]anthracene binding to DNA.[109]

Retinol has inhibited the mutagenicity induced by aflatoxin B_1 (0–16 μg Retinol/plate),[110] *ortho*-aminoazotoluene (0–150 μg Retinol/plate),[111] and 2-fluorenamine (0–100 μg Retinol/plate)[112] in the Ames *Salmonella*/mammalian microsome test. Retinyl Palmitate also had an inhibitory effect on the mutagenicity of *ortho*-aminoazotoluene, although its inhibition was approximately 50% that of Retinol.[111] However, in a further study using the Ames test, small amounts of Retinol (2–20 μg/plate) increased the mutagenicity of 2-aminofluorene and 2-acetylaminofluorene, although at higher doses (50–150 μg/plate) the mutagenicity of 2-acetyl-aminofluorene remained unchanged while that of 2-aminofluorene decreased gradually.[113]

CARCINOGENICITY

No long-term carcinogenicity studies in laboratory animals are available on Retinol and Retinyl Palmitate. In the opinion of the Select Committee of GRAS Substances, there is no evidence that vitamin A is carcinogenic.[3]

INHIBITION AND ENHANCEMENT OF CHEMICALLY INDUCED CARCINOGENESIS AND PHOTOCARCINOGENESIS

Vitamin A both enhanced and inhibited responses to viral or chemical carcinogens. Much controversy was engendered by studies in the late 1960s showing that 9,10-dimethyl-1,2-benzanthracene (DMBA)-induced carcinogenesis in the hamster cheek pouch was enhanced by application of a 10% Retinyl Palmitate lotion before, together with, or following application of DMBA.[88] The data actually indicated little effect; the incidence and numbers of carcinomas were the same for the Retinyl Palmitate and non-Retinyl Palmitate treated groups, whereas the only indication of potentiation of DMBA-induced carcinogenesis was the slightly larger tumor size in Retinyl Palmitate-treated hamsters. This potentiation has been attributed to a toxic, irritating effect of Retinyl Palmitate as demonstrated by marked histological changes in the cheek pouches of hamsters repeatedly administered 10% Retinyl Palmitate alone. Subsequent studies have confirmed the toxic effect of large doses of Retinyl Palmitate on the hamster cheek pouch epithelium and strongly suggest that nontoxic topical application of Retinyl Palmitate after the appearance of the first neoplasm (DMBA-induced) results in the regression of these tumors.[114]

Results of many studied have indicated that the retinoids can suppress the process of carcionogenesis in laboratory animals in vivo and the development of malignant phenotypes in vitro. Recently, it has been reported that retinoids have surpressed proliferation and led to terminal differentiation of certain fully neoplastic cells, resulting in a more benign, nonneoplastic phenotype. Most of these studies have been conducted in search of therapeutic roles for retinoids in the treatment of cancer[68] (see also section "Cellular and Biochemical Effects").

The reader is referred to Moon and Itri,[114] Roberts and Sporn,[67] Hill and Grubbs[115] for in-depth reviews of the chemopreventative and anticarcinogenic effects of the retinoids.

Retinol (23 mg/kg/day, or 76,670 IU/kg/day) also was reported to have had no effect on the incidence of squamous cell carcinoma in hairless mice exposed to UV light generated by a solar simulator.[88]

IMMUNOLOGICAL EFFECTS

Retinoids may inhibit or stimulate the immune system. The inhibitory or stimulatory effects of the retinoids on various immune responses are reflected in the histological changes in lymphoid organs. High doses of retinoids may effectively inhibit both humoral (antibody-mediated) and cell-mediated immunity, whereas subtoxic doses have stimulated them. Timing, dose, and mode of administration play a major role in determining the effects of the retinoids in the immune system.[116]

Retinol and Retinyl Palmitate stimulated the humoral immune response to soluble or particulate antigens in experimental animals.[116] Cell-mediated immunity studies have shown that Retinyl Palmitate (optimal dose of 150 μg)

stimulated the delayed-type hypersensitivity reaction (DTH) in mice when injected together with the sensitizing antigen (sheep erythrocytes).[117] Skin grafts from male mice transplanted onto syngeneic females were rejected significantly more quickly when Retinyl Palmitate was administered to the transplanted mice. Moderate subtoxic doses of retinoids have significantly increased thymus weight, stimulated thymic involution, and increased the cellularity of lymph nodes in mice.[116]

No clear hypothesis of how retinoids may regulate the immune system has been formulated. However, retinoids are believed to act in the induction phase of immunity. Retinoids stimulate the induction of T-killer activity in vivo and in vitro to both allogeneic and syngeneic (tumor) cells.[116] Walsh et al.[118] studied the influence of Retinol on human gingival cultures and suggested that low doses of Retinol (5 μg/ml) may alter immune reactions within epithelia via stimulation of both keratinocytes and Langerhans cells. The results of recent studies using tumor models have increasingly indicated that the anticarcinogenic effects of the retinoids are due to immunostimulation.[116,119]

The reader is referred to Dennert[116] for an in-depth review of the immunological effects of the retinoids.

CLINICAL ASSESSMENT OF SAFETY

Recommended Dietary Allowance and Daily Intake

The recommended daily dietary allowance (RDA) of vitamin A varies between scientific groups. In 1974, the National Research Council[120] recommended RDAs of 5000 IU for male and 4000 IU for female adults, 1400–2000 IU for infants, 2000–3300 IU for children up to age 11, 5000 IU for pregnant women, and 6000 IU for lactating women. The NRC reaffirmed these values in 1980.[121] On the other hand, the Food and Agriculture/World Health Organization[122] has recommended lower daily intakes for all groups: 750 μg Retinol (2500 IU) for adults, 300 μg Retinol (1000 IU) for infants, 250–400 μg Retinol (833–1333 IU) for children up to 10, 575–725 μg Retinol (1917–2417 IU) for children 10–15 years of age, 750 μg Retinol (2500 IU) for pregnant women, and 1200 μg Retinol (4000 IU) for lactating women.

The requirement for vitamin A appears to be proportional to body weight. This proportion declines rapidly following birth and increases only slightly during the adolescent growth phase. This is reflected in the RDA on a per kg body weight basis: 100, 85, 65, and 50 μg Retinol/kg at birth, 1, 3, and 5 months of age, respectively, declining to 12 μg Retinol/kg for adults.[122] Due to the wide variability of individual responses to vitamin A and reports from postmortem studies showing a substantial number of people with reduced hepatic reserves (even in developed Western countries), the NRC recommended intake is set considerably above the requirement for normal physiological vitamin A functions.[10,33] The reader is referred to Rodriguez and Irwin[123] for an in-depth review of the literature on human vitamin A requirements, including those for infants and children.

Surveys taken from 1971 to 1974 of representative samples of the United States population (all incomes and both sexes) indicated that the daily intake of vitamin A from all food sources was 4774 IU for ages 1–74 and 4500 IU for ages 20–24 years. Approximately 47% of the latter group was subsequently determined to be consuming less than 3500 IU daily.[3]

Hypervitaminosis A

The International Vitamin A Consultative Group has summarized the literature on hypervitaminosis A dating from 1850 to 1979.[124] During this period, 579 cases were described in 195 separate reports. Great differences were observed in the daily doses given and in the frequency and duration of administration associated with the onset of toxicity, indicative of the wide variability of individual tolerance to Retinol.

Acute Toxicity

Acute hypervitaminosis A frequently occurred in Eskimos and Arctic travelers who ingested polar bear or seal liver because of their high content of Retinol (13,000–18,000 IU/g).[3,88] However, acute hypervitaminosis A is now rarely seen in adults, although it is still fairly common in infants due to accidental ingestion or to unintentional overdosing by parents.[88] Acute toxicity is frequently reported when single doses of 100,000 IU vitamin A or more are given to infants or 300,000 IU or more to young children. Multiple doses of 200,000 IU orally or 100,000 IU intramuscularly on sequential days also may produce acute toxicity.[124] Symptoms usually occur within a few days after consumption and are of a transient nature, causing no permanent adverse effects.[88,124] The most prominent effects are on the central nervous system (increased cerebrospinal fluid pressure, as indicated by pseudotumor cerebri and hyperostosis in children and occipital headaches in adults), followed by gastrointestinal tract effects (anorexia, vomiting, and nausea). Other clinical symptoms have included scaling of skin, dry mucous membranes, cheilitis, hair loss, fever, fatigue, somnolence, vertigo, edema, tenderness of long bones, hepetomegaly, and splenomegaly.[124,125] Laboratory abnormalities reported include elevated plasma concentrations of Retinol and calcium and activities of alkaline phosphatase, serum glutamic oxaloacetic transaminase (SGOT), and serum glutamic pyruvic transaminase (SGPT).[125]

Chronic Toxicity

Chronic hypervitaminosis A generally occurs when high doses, usually greater than 25,000 IU daily (~ 400 IU/kg in adults), have been administered over prolonged periods for the treatment of acne or other dermatological conditions by uninformed parents desirous of healthy children or by health food faddists.[33] The majority of documented cases of chronic hypervitaminosis A have been in children.[88]

The most prominent signs and symptoms of chronic toxicity are cutaneous, including skin scaling, erythema, pruritus, disturbed hair growth, dry mucous membranes, cheilitis, and gingivitis. Headache, nausea, anorexia, vomiting, and bone and joint pain are also consistently noted.[124,125] Other

clinical symptoms have included papillary edema, diplopia, optic atrophy, and blindness in children over long-term administration, edema, fatigue, hemorrhage, hypomenorrhea, psychiatric symptoms, hepatic dysfunction associated with hepatosplenomegaly, ascites, and hypoplastic anemia with leukopenia.[88,125,126] Laboratory abnormalities reported include radiologically detected bone changes in children, increased plasma levels of Retinol (also an elevated ratio of retinyl ester to alcohol form), calcium, alkaline phosphatase, SGOT, and lipids, increased cerebrospinal fluid pressure, and disturbed blood clotting.[33,88,125] Most of these symptoms generally disappear when the administration of vitamin A is discontinued. However, growth retardation caused by premature epiphyseal closure has been reported in children.[88,126]

According to FASEB,[3] the least adverse effect intake in humans appears to be from 700 to 3000 IU Retinol/kg/day for several months, with most estimates skewed toward 3000 IU. Dose comparison is complicated; water-miscible vitamin A, being more readily absorbed, elicits toxic symptoms at much lower doses than oil-soluble vitamin A, and it is not always clear which form of the vitamin was administered. However, daily intakes of 700–3000 IU/kg would probably be attained only through supplementation, as mean daily vitamin A intakes from the usual dietary sources are of the order of 80 IU/kg for adults and 300 IU/kg for infants. The Committee on Drugs and the Committee on Nutrition of the American Academy of Pediatrics have indicated that daily doses of 25,000 IU (400 IU/kg in an adult) or more of vitamin A pose a risk when administered over extended periods of time and should not be used except in cases of severe vitamin A deficiency.

There is some concern in developed countries that the prevalence of chronic hypervitaminosis A may increase in the future. Vitamin A is available without prescription in dosage units of 25,000 to 50,000 IU and is also used to fortify several common foods (such as margarine and milk) in the United States. Therefore, young children who are commonly given concentrated vitamin supplements and who are consuming fortified foods as well may have reserves approaching the toxicity concentration. Furthermore, new possible chemopreventive and therapeutic roles of the retinoids may lead to increased consumption of megadoses.[33,127]

Dermal Irritation and Sensitization

A moisturizer containing 1% Retinyl Palmitate was evaluated for irritation and sensitization using a modified Draize (RIPT). A 0.3 ml sample of the moisturizer was applied to the upper arm of each of 100 subjects using an occlusive patch. Patches remained in place for 24 h and were scored (max = 4) upon removal and 24 h later. Patches were applied on alternate days to the same site for a total of 10 applications. After a 2–3 week rest period, similar 24-h challenge patches were applied to the same as well as untreated sites. Reactions were graded at 24 and 48 h. Ninety-nine subjects had no signs of irritation throughout the study. One subject had well-defined erythema (score, 2 +) after the eighth application and after the ninth application on a different site. The tenth application patch was not applied to this subject, although he

did receive both challenge patches. No reactions were observed at challenge. The investigators concluded that the moisturizer was "most likely" not a primary skin irritant nor a fatiguing agent and that it may possibly be a sensitizer agent.[128] Results of dermal irritation and sensitization tests are summarized in Table 3.

A body lotion containing 0.1% Retinyl Palmitate was evaluated for irritation and sensitization using a Shelanski/Jordan RIPT. Occlusive patches were applied for 24 h to the upper back of each of 210 subjects. Patches were applied on alternate days for a total of 10 applications. Sites were examined upon patch removal (max = 4). After a 10–14 day rest period, a 48-h challenge patch was applied, and the reactions were graded upon removal. The subjects received an additional 48-h challenge patch 7–10 days later. Sites were graded at 48 and 72 h. All but 2 of the subjects had completely negative scores throughout the study. One subject had erythema and papules (score, 2) after induction patches 9 and 10; this was considered to be irritation due to occlusive patching, since the subject had no reactions to the challenge patches. The second subject had all negative scores except for a score of 2 (erythema and papules) at the 72-h reading after the second challenge. This reaction was considered to be irritant in nature, since no edema was observed. The body lotion was neither a strong irritant nor a strong contact sensitizer.[129]

TABLE 3. Dermal Irritation and Sensitization (Clinical).

Ingredient	*Method*	*No. of Subjects*	*Results*	*Reference*
Retinyl Palmitate—1% in a moisturizer	Modified Draize RIPT[a]	100	All negative scores in 99; erythema in one subject after 8th and 9th insults—no reaction at challenge; nonirritating and non-fatiguing, possibly a sensitizer	128
Retinyl Palmitate—0.1% in a body lotion	Shelanski-Jordan RIPT	210	Not a strong irritant or a strong sensitizer	129
Retinyl Palmitate—0.1% in a moisturizer	Modified Draize-Shelanski RIPT	189	Nonirritating and non-sensitizing	130
Retinyl Palmitate—0.1% in a moisturizer	Modified Draize-Shelanski RIPT	108	Nonirritating and non-sensitizing	131
Retinyl Palmitate—0.1% in a body lotion	21-day cumulative irritation test	12	Base score of 58 (max = 630); "probably mild" in normal use; slight potential for mild cumulative irritation under test conditions	132
Retinyl Palmitate—0.1% in a moisturizer	21-day cumulative irritation test with challenge	20	Base score of 0 (max = 630); essentially nonirritating; nonsensitizing	133

[a]RIPT, Repeated Insult Patch Test.

A moisturizer containing 0.1% Retinyl Palmitate was evaluated for irritation and sensitization using a modified Draize-Shelanski RIPT. Occlusive patches were applied to the upper back of each of 189 subjects for 48 h, and sites were graded upon removal (max = 4). Applications were made on alternate days for a total of 10 applications. After a nontreatment period of 10–14 days, similar challenge patches were applied for 48 h and were graded upon removal. All reactions to application and challenge patches were negative. The moisturizer did not demonstrate any potential as a primary irritant or an allergic sensitizer.[130]

Another moisturizer containing 0.1% Retinyl Palmitate was evaluated for irritation and sensitization using a modified Draize-Shelanski RIPT. Occlusive patches containing 0.1 g samples of the moisturizer were applied to the upper backs of 108 subjects. Patches remained in place for 24 h, and the reactions were graded at 48 h (max = 4). Patches were applied on Monday, Wednesday, and Friday for 3 weeks and on Monday of the fourth week for a total of 10 applications. After a 2-week nontreatment period, two challenge patches were applied to each subject: one on the original site and one on a previously unpatched site. These patches remained in place for 48 h, and the reactions were graded upon removal. All but 1 of the subjects had negative scores throughout the study. This 1 subject had all negative scores except for a score of 1 (erythema only) after the challenge patch on a new site. The moisturizer did not appear to be a primary irritant or an allergic contact sensitizer.[131]

A body lotion and a moisturizer, each containing 0.1% Retinyl Palmitate, were evaluated for cumulative irritation using panels of 12 and 20 subjects, respectively. In each test, occlusive patches containing a sample of either product were applied to the backs of the subjects for 23 h. Reactions were graded at 24 h. Patches were applied daily to the same site for 21 consecutive days. The 12 subjects receiving applications of the body lotion had a total score of 70 (max = 756) and a base ($\eta = 10$) score of 58 (max = 630). The investigators considered the body lotion to be "probably mild" in normal use, since there was evidence of a slight potential for very mild cumulative irritation under conditions of the test.[132] The panelists used for testing the moisturizer received an additional challenge patch 2 weeks after the 21st consecutive patch. These patches remained in place for 24 h, and the reactions were graded at 48 and 96 h. The subjects had a total score of 0 (max = 1260) and a base ($\eta = 10$) score of 0 (max = 630) over the 21-day test period; the moisturizer was considered essentially nonirritating. Several panelists reacted to the challenge patch of some samples (more than one product were being simultaneously tested); however, since specific results were not given, it is not known whether any panelist reacted to the challenge patch with the moisturizer containing 0.1% Retinyl Palmitate. Most of these reactions subsided by 96 h and appeared to be due to skin fatigue or primary irritation, rather than contact sensitization.[133]

One case report of contact allergy to Retinyl Palmitate has been reported. A 55-year-old woman had a melanoma on her back removed and replaced by a skin graft. She developed eczematous dermatitis after the use of several topical applications on the donor site. Patch tests with a modification of the European Standard series were positive to nickel only. The patient was already

aware that she reacted to jewelry. A pharmaceutical and cosmetical test series including 20 substances was negative. When tested with the medicaments she had used, a positive reaction was obtained only with a magistral preparation. The individual ingredients of this cream were then tested, and a positive reaction was obtained with vitamin A (Retinyl Palmitate) oily solution Merck 10^6 U/g. Further testing with pure Retinyl Palmitate 10^6 U/g without additives as well as butyl hydroxy anisole and butyl hydroxy toluene, used as antioxidants, was positive only to pure Retinyl Palmitate. The pure Retinyl Palmitate was negative in 20 other patients. The investigator noted that Retinyl Palmitate may contain traces of nickel.[134]

Jordan et al.[135] reported that two male prison volunteers who reacted positively to all-trans-retinoic acid were negative when patch tested with 0.1% Retinol or Retinyl Palmitate in petrolatum. A retest conducted 8 months after the initial testing produced the same results.

Vitamin A and Cancer

Epidemiological Studies

The majority of epidemiological studies indicate that an inverse relationship exists between cancer risk and vitamin A consumption. Most of these studies have related dietary intake of vitamin A (estimated by the frequency of ingestion of foods known to have a high content of β-carotene or preformed Retinol) to the incidence of cancer, whereas others compared serum concentration of Retinol to the incidence of cancer. Both prospective and retrospective studies have been conducted. The reader is referred to Moon and Itri,[114] Kummet et al.,[136] and Peto et al.[137] for comprehensive reviews on the epidemiology of vitamin A.

Kummet et al.[136] reviewed 30 epidemiology studies on vitamin A and cancer. These studies were all encompassing: dietary,[17] serological,[13] prospective,[5] retrospective,[25] worldwide, and for multiple tumor types. They found that only 1/13 serological studies and 2/17 dietary studies did not show an inverse relationship between serum Retinol concentrations and cancer incidence and vitamin A consumption and cancer incidence, respectively. Furthermore, it was noted that the 1 negative serological study did not use controls, and one of the two negative dietary studies reported an inverse association between the intake of carotene-containing vegetables and cancer (although no association was made between total vitamin A intake and cancer). A dose–response effect was detected in many of the studies reviewed. Interestingly, both the prospective and retrospective serological studies indicated that the lower the serum viatmin A concentration, the greater the cancer risk, even for concentration within the normal range. The relative risk of low vitamin A intake or low serum Retinol averaged approximately 2 in these studies.

However, the controversy over this issue continues. Several recent prospective studies have produced no correlation between low serum Retinol and increased incidence of lung[138] and breast cancer.[139] The latter study, however, did show that those women who developed breast cancer generally had low serum concentrations of β-carotene, although this was not statisti-

cally significant. Greenwald et al.[140] noted that the mixed evidence from studies of serum vitamin A is not surprising, since hemostatic control maintains serum Retinol within a narrow range and may not reflect what is actually in the tissues.

Munoz et al.[141] conducted a randomized double-blind intervention trial in the People's Republic of China to determine if a combined treatment with 15 mg Retinol (50,000 IU), 200 mg riboflavin, and 50 mg zinc could lower the prevalence of precancerous lesions of the esophagus. The 610 subjects were randomized to receive either the combined treatment or a placebo once a week for 13.5 months. The combined treatment did not have an effect on the prevalence of esophageal lesions, as the incidence in the treated group was 48.9% and that in the placebo group was 45.3%.

It has been difficult to discern whether the observed anticancer effects are due to Retinol, β-carotene, some other dietary constituent, or a combination of these factors.[114,136] Peto et al.,[137] in reviewing 5 prospective and 15 retrospective dietary studies, suggested that β-carotene was of primary importance. On the other hand, most of the serological studies and in vitro laboratory work suggest that Retinol and its derivatives are primary factors.[136] A number of clinical studies and trials with retinoids are in progress in the field of oncology, including a large prospective study in 20,000 United States physicians to test the hypothesis that supplementary β-carotene reduces the risk of cancer.[31,114]

Stitch et al.[142] used the frequency of micronuclei in cells scraped from inside the human cheek as a measure of chromosome breakage in earlier cell divisions and as an indicator of carcinogenic stimuli (increased chromosome breakage indicates the presence of carcinogenic stimuli). They supplemented the diet of 40 rural Filipino betel nut and tobacco chewers with a sealed capsule of Retinol (100,000 IU/week) and β-carotene (300,000 IU/week) for 3 months and found a threefold decrease in the mean proportion of cells with micronuclei in 37/40 subjects. No large increases were noted in any subject, and the mean proportion of micronuclei did not change in a control group of 11. These data suggest that an increase in the dietary intake of Retinol or carotene may reduce the incidence of oral cancer in this population.

The present recommendation of the National Academy of Sciences[143] regarding vitamin A intake for cancer prevention is as follows:

> The epidemiological evidence is sufficient to suggest that foods rich in carotenes or vitamin A are associated with reduced risk of cancer. The toxicity of vitamin A in doses exceeding those required for optimum nutrition, and the difficulty of epidemiological studies to distinguish the effects of carotenes from those of vitamin A, argue against increasing vitamin A intake by the use of supplements.

Clinical Cancer Studies

Retinol has been used as a treatment (both orally and topically) for malignant tumors since the 1930s. However, most of the early clinical trials are difficult to assess for efficacy because of small patient numbers, lack of control groups, variability in response definitions, and imprecise data reporting. An

evaluation is further complicated by the fact that most of the patients also received other therapy, including surgery, irradiation, or hormones. Even so, evidence of activity is apparent in several malignant conditions. Numerous clinical studies are ongoing in this area, although most are using analogs of vitamin A because of more favorable therapeutic indices.[114]

The reader is referred to Bollag[144] and Moon and Itri[114] for reviews of the early and current literature on clinical cancer studies, respectively.

Teratogenic and Embryotoxic Effects

Numerous incidences have been reported of fetal malformations after maternal ingestion of high concentrations of vitamin A during pregnancy. Multiple fetal malformations of the central nervous system occurred in the child of a woman who had been treated (orally) with 150,000 IU vitamin A during gestation days 19–40.[145] Urinary tract malformations were observed in infants born of two women; the first had ingested daily doses of 25,000 IU vitamin A for the first 3 months and 50,000 IU for the last 6 months of pregnancy, and the second ingested 40,000 IU daily from the 6th to the 10th week of pregnancy.[88,105] Multiple craniofacial malformations occurred in the infant of a woman who ingested a very high dose of vitamin A in the second month of gravidity.[105]

Serum samples drawn 1 week postpartum from women who delivered children with spina bifida showed significantly increased vitamin A concentrations. The livers of fetuses with nervous system malformations also had increased vitamin A concentrations. The significance of the first finding was considered unclear because of the natural variation in serum vitamin A concentrations and the lack of specific information on serum vitamin A concentration in pregnancy. However, based on these data, vitamin A was concluded to be potentially embryotoxic in humans.[145]

Geelen,[105] after comprehensively reviewing hypervitaminosis A-induced teratogenicity, concluded that there is no definite proof of the teratogenic effect of vitamin A in humans. However, the well-documented teratogenic effects of vitamin A in different species of animals and the scarce data in humans suggest that hypervitaminosis A during pregnancy may adversely affect the developing embryo and fetus. FASEB[3] has noted that the lowest estimated no-effect level in animals (2500 IU/kg in mice) is more than 25 times greater than the estimated adult human intake from food sources (approximately 100 IU vitamin A/kg/day).

SUMMARY

Retinol is the primary naturally occurring form of vitamin A. Retinyl Palmitate is the ester of Retinol and palmitic acid, also known as vitamin A palmitate. These compounds are soluble in most organic solvents and insoluble in water. Retinol and Retinyl Palmitate have ultraviolet absorption maxima in the range of 324–328 nm.

Retinol and Retinyl Palmitate are produced today largely by commercial methods in which retinyl acetate is the end product. Retinol is also still obtained by concentration from animal fats and fish liver oils.

Retinol oxidizes readily and is inactivated by ultraviolet light, giving off a characteristic green fluorescence. Retinol is relatively heat stable and is more stable in alkaline than acid solution. The acetic and palmitic esters of Retinol are commercially important because of their greater degree of stability when compared to the alcohol.

High-pressure liquid chromatography (HPLC) has become the preferred method of retinoid analysis due to the stability of retinoids on most HPLC columns, its high resolution and sensitivity, and the rapidity of most analyses.

Cosmetic uses of Retinol and Retinyl Palmitate are primarily in hair, facial makeup, and skin care preparations. Retinol and Retinyl Palmitate were used in 138 and 102 formulations, respectively, in 1981. Generally, these ingredients were used at concentrations $\leq$ 1%.

Retinol and Retinyl Palmitate are both affirmed as GRAS (generally recognized as safe) food ingredients; their functional use in foods is as nutrient and dietary supplements. They are also used for this purpose in the veterinary field. Retinol sees further use in various pharmaceutical products and in the treatment of dermatoses.

Retinol is essential for the growthy, health, and life of higher animals. It is required for vision, reproduction, and for the maintenance of differentiated epithelia and of mucous secretion. The molecular mechanisms for its biological effects are largely unknown, with the exception of its role in the visual process.

The primary natural sources of vitamin A in the diet are certain plant carotenoid pigments, particularly β-carotene, and the long-chain retinyl esters found in animal tissues. β-Carotene is converted (50% maximum) to Retinol primarily in the intestinal mucosa, although conversion also is known to occur in the liver and other tissues.

Dietary retinyl esters, Retinol, and provitamin A carotenoids are dispersed and emulsified in the stomach during the gastric phase of lipid digestion. The esters are hydrolyzed in the intestinal lumen, and the resulting Retinol, as well as that obtained from the diet, is absorbed into the mucosal cell. Here, it is reesterified with long-chain, mainly saturated fatty acids, incorporated into chylomicrons and transported via the lymph into the general circulation. The chylomicrons are metabolized in extrahepatic tissues and reduced to smaller, cholesterol-rich particles containing essentially all of the original retinyl esters. These chylomicron remnants are removed from the circulation almost entirely by the liver.

Upon uptake by the liver, the retinyl esters are hydrolyzed, reesterified, and stored in the liver, primarily as Retinyl Palmitate. Vitamin A is mobilized from these hepatic stores as Retinol bound to a specific plasma transport protein retinol-binding protein (RBP) in a highly regulated process. A specific intracellular binding protein for Retinol has also been identified and designated as cellular retinol-binding protein (CRBP).

In a number of studies, radioactive metabolites of Retinol were excreted via urinary and fecal or biliary routes. The amount excreted in the urine

depended on the position of the radioactive atom. The urinary metabolites have been only partially characterized; they are mainly water-soluble and contain no detectable free Retinol or retinyl esters. The biliary and fecal metabolites have also not been characterized, with the exception of Retinol, retinoic acid, and their conjugates. The amount of the administered dose recovered in the bile and feces varies depending on the position of the radioactivity, the mode of administration, and on the quantity administered. Some Retinol is metabolized through retinoic acid. Therefore, the well-characterized metabolites of retinoic acid would also be metabolites of Retinol.

Retinol has long been known to interact with other micronutrients, including vitamin E, ascorbic acid (vitamin C), iron, and zinc. Vitamin E is generally believed to have a nonspecific antioxidant role. It has been suggested that Retinol influences the hepatic synthesis of ascorbic acid, whereas the latter acts as an antioxidant for excess hepatic Retinol. Iron may facilitate oxidative destruction of vitamin A active compounds in the intestine, whereas the vitamin may facilitate the mobilization of stored iron and its incorporation into erythrocytes. The hepatic mobilization of Retinol may also be impaired by zinc deficiency.

In other interaction studies, gonadal steroid and adrenocortical hormones have increased the hepatic mobilization of Retinol, DDT, and other drugs as well as xenobiotics have reduced hepatic stores of Retinol. Acute and chronic ingestion of alcohol has resulted in reduced plasma concentrations and hepatic storage (chronic only) of Retinol.

The retinoids have modified the growth and differentiation of both neoplastic and nonneoplastic cells in culture. Retinoids have various effects on the activity and synthesis of cellular enzymes and effectors as well as profoundly influencing the biosynthesis of all types of glycoconjugates. Two dominant theories of retinoid mechanism exist: the proposed cofactor role of retinoids in glycosyl transfer and the proposed steroid model for retinoid control of gene expression.

In studies on physiological effects, vitamin A affected multiple parameters of thyroid function. There appears to be an inverse relationship between thyroxine and vitamin A concentrations in the plasma. Retinyl Palmitate has decreased glucose tolerance in man.

In acute oral studies, Retinol was slightly toxic to mice, whereas Retinyl Palmitate was practically nontoxic in mice and rats. Large single doses can be lethal. Retinol was considerably more toxic than Retinyl Palmitate when administered IP.

Two cosmetic products, each containing 0.1% Retinyl Palmitate, were evaluated for dermal irritation in rabbits: one was no more irritating than the control product, whereas the second was quite irritating to rabbit skin. These same two products were relatively nonirritating to rabbit eyes.

Multiple low doses (severalfold greater than required intake level) of viatmin A can be toxic to laboratory animals. Characteristic symptoms of hypervitaminosis A include weight loss, erythema, hair loss, internal hemorrhage, and fractures. Many of these effects are reversible upon cessation of administration. The time to first appearance of clinical signs depends on the route of administration, the species and age of the animal, the duration of

treatment, the sign in question, and the dose size. In a review of current literature, the lowest reported adverse effect concentration in experimental animals was in the range of 25,000 to 60,000 IU Retinol per kg per day for periods of 3–5 weeks. Water-miscible vitamin A was more toxic than oil-soluble vitamin A because it was more readily absorbed.

In specific dermal studies, topical application of Retinol to rats for periods of 10–60 days produced acanthosis and approximately doubled the thickness of the epidermis. Subcutaneous injection of Retinol for up to 60 days induced no significant effect on the epidermis of rats. Topical application of Retinol to the nipples of guinea pigs for 10 days produced an acanthotic response. A drop of bear liver oil applied to the skin of mice daily for 14 days produced signs characteristic of hypervitaminosis A. Two drops of an oily vitamin A solution applied on alternate days to the surface of guinea pig skin wounds promoted healing and produced signs of local hypervitaminosis A. A body lotion containing 0.1% Retinyl Palmitate produced a mild dermatitis in all rabbits after daily administration of 6 mg/cm^2 for 90 days. No systemic toxicity was observed.

Retinyl Palmitate did not produce any adverse effects during 10 months of oral administration to dogs and rats at doses of up to 25,000 and 50,000 IU/kg/day, 5 days per week, respectively.

Vitamin A toxicity occurs when, due to excessive intake of vitamin A, Retinol begins to circulate in the plasma in a form other than bound to RBP.

Although it is recognized that Retinol is essential for reproduction, high intake of Retinol has produced adverse effects on several reproductive functions. These include decreased sperm motility and sperm survival in rabbits as well as testicular changes and inhibition of cyclic ovulatory activity in rats.

Vitamin A has produced more than 70 types of malformations in rats, mice, hamsters, guinea pigs, rabbits, dogs, pigs, and monkeys. The type and incidence of malformations depended on the dose and stage of pregnancy and, to a lesser extent, on species and strain. Abnormalities of the face, ears, eyes, and nervous system were most commonly observed. Minor brain defects, growth disturbances, and behavioral abnormalities have also developed postnatally after in utero exposure to Retinol. Appreciable amounts of vitamin A are transferred to suckling offspring through the maternal milk.

Vitamin A was nonmutagenic in the Ames test both with and without metabolic activation. Retinol also did not increase the frequency of sister chromatid exchanges or cell cycle delay in Chinese hamster cells either with or without metabolic activation. Retinol and Retinyl Palmitate have modified the effects of established mutagens, having both an inhibitory and a stimulatory effect (at low doses only).

There is no evidence that vitamin A is carcinogenic. However, the vitamin has both enhanced and inhibited responses to viral or chemical carcinogens. Results of many studies conducted in search of therapeutic roles for retinoids have indicated that retinoids can suppress the process of carcinogenesis in laboratory animals in vivo and the development of malignant phenotypes in vitro.

Retinoids have inhibited or stimulated the immune system: High doses have effectively inhibited both humoral (antibody-mediated) and cell-medi-

ated immunity, whereas subtoxic doses have been stimulatory. Timing, dose, and mode of administration play a major role in determining the effects of the retinoids on the immune system.

The RDA of vitamin A for humans varies between scientific groups. The NRC recommends 5000 and 4000 IU daily for male and female adults, respectively, lesser amounts for infants and children, and an increased 5000 and 6000 IU daily for pregnant and lactating women, respectively. The FAO/WHO has recommended lower daily intakes for all groups: 2500 IU for adults (including pregnant women), lesser amounts for infants and children, and an increased 4000 IU daily for lactating women. The requirement for vitamin A appears to be proportional to body weight. This proportion is believed to decline rapidly following birth and increase only slightly during the adolescent growth phase. Surveys of representative samples of the United States population (1971–1974) indicated that the daily intake of vitamin A from all food sources was 4774 IU for the overall ages 1–74 years.

In a review of hypervitaminosis A in humans dating from 1850 to 1979, 579 cases were reported. These indicated a wide variability in individual tolerance to Retinol. Acute toxicity is frequently reported when single doses of 100,000 IU vitamin A or more are given to infants or 300,000 IU or more to young children. Multiple doses of 200,000 IU orally or 100,000 IU intramuscularly on sequential days also may produce toxicity. Symptoms usually occur within a few hours after consumption and are of a transient nature, causing no permanent or adverse effects. The primary effects are on the central nervous system, and the gastrointestinal tract is secondarily affected.

Chronic hypervitaminosis A generally occurs when high doses, usually greater than 25,000 IU daily, have been administered over long periods of time. The most prominent signs of chronic toxicity are cutaneous, followed by gastrointestinal and central nervous system effects. Most of these signs disappear when the administration of vitamin A is discontinued. However, growth retardation caused by premature epiphysical closure has been reported in children. The least adverse effect intake in humans appears to be from 700 to 3000 IU Retinol/kg/day for several months, with most estimates skewed toward the upper end of this range. Daily intakes at this level would probably be attained only through supplementation, since the mean daily intake from usual dietary sources are of the order of 80 IU/kg for adults and 300 IU/kg for infants.

In repeated insult patch tests, cosmetic products containing 0.1–1% Retinyl Palmitate were at most slightly irritating and nonsensitizing in a total of 607 subjects. Results of cumulative irritation tests of two products containing 0.1% Retinyl Palmitate indicated that these products are "probably mild" in normal use and essentially nonirritating and nonsensitizing, respectively. One case of contact allergy to Retinyl Palmitate has been recorded.

The majority of epidemiology studies, both prospective and retrospective, have related dietary intake of vitamin A as well as serum concentration of Retinol to the incidence of cancer. Controversy exists as to whether the observed anticancer effects are due to Retinol, β-carotene, some other dietary constituent, or to a combination of these factors. The current recommendation of the National Academy of Sciences states that the epidemiological

evidence suggests that foods rich in carotenes or vitamin A are associated with reduced risk of cancer. However, due to the toxicity of vitamin A in doses exceeding those required for optimum nutrition as well as the difficulty in distinguishing the effects of carotene from vitamin A, the NAS does not recommend increasing vitamin A intake by the use of supplements.

Retinol has been used as a treatment (both orally and topically) for malignant tumors since the 1930s. Numerous clinical studies are ongoing in this area, although most are using analogs of vitamin A because of more favorable therapeutic indices.

Several cases have been reported of fetal malformations after maternal ingestion of high doses of vitamin A during pregnancy. These included malformations of the central nervous system, urinary tract, and craniofacial area. Hypervitaminosis A during pregnancy may adversely affect the developing embryo and fetus.

CONCLUSION

On the basis of the available animal and clinical data presented in this report, the CIR Expert Panel concludes that Retinyl Palmitate and Retinol are safe as cosmetic ingredients in the present practices of use and concentration.

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National Toxicology Program (NTP)
Technical Reports Peer Review Panel

Dr. Lori D. White
NTP Designated Federal Officer
NIEHS/NIH P.O. Box 12233, MD K2-03
Research Triangle Park, NC 27709

Dear members of the Technical Reports Peer Review Panel:

These comments are provided on behalf of the Environmental Working Group (EWG), a non-profit public health research and advocacy organization based in Washington, DC. Our work focuses on the human and environmental health impact of potentially toxic chemicals in consumer products, so we reviewed with great interest the National Toxicology Program (NTP) Draft Technical Report on the Photococarcinogenesis Study of Retinoic Acid and Retinyl Palmitate in SKH-1 Mice (simulated solar light and topical application study), released for public comments in December 2010 (NTP 2010).

As you know, this compound is a common ingredient in skin creams and cosmetics, sunscreens and other personal care products. Your assessment of its potential to increase skin tumor risk in the presence of sunlight will be of great importance to public health.

This meticulous study is a culmination of a long-term research program on retinyl palmitate photogenotoxicity and photocarcinogenecity at the NTP/National Center for Toxicological Research (NCTR) Center for Phototoxicology. NTP initiated the program in 2000 in response to the decision of the Food and Drug Administration (FDA) Center for Food Safety and Applied Nutrition to nominate retinyl palmitate for phototoxicity and photocarcinogenicity testing. The FDA selected retinyl palmitate for further study "*based on the increasingly widespread use of this compound in cosmetic retail products for use on sun-exposed skin, the biochemical and histological cutaneous alterations elicited by retinyl palmitate, and the association between topical application of retinoids and enhancement of photocarcinogenesis*" (NTP 2000).

EWG strongly agrees with the conclusion of the draft NTP report that retinyl palmitate "*enhanced the photocarcinogenicity activity of SSL [solar-simulating light] and UVB in SKH-1 mice based upon earlier onsets and increased multiplicities of in-life skin lesions and increased incidences and multiplicities of squamous cell neoplasms*" (NTP 2010). Our comments focus on three major points:

- The experimental protocol in the NTP/NCTR study was appropriately chosen to answer the question of topical retinyl palmitate toxicity posed by the FDA.
- The overall weight of evidence collected by the study supports the finding of retinyl palmitate photococarcinogenicity.

- The findings of the draft report are in agreement with evidence in the research database on the photogenotoxicity and photocarcinogenicity of retinoid compounds.

Details and rationale for our comments are provided below.

1. The experimental protocol in the NTP/NCTR study was appropriate.

The FDA's decision to nominate retinyl palmitate (RP) for NTP testing specifically sought to address, through the use of a widely accepted animal model, potential human health risks that might be associated with the growing use of this compound in cosmetic and personal care products. The one-year study protocol developed by NTP/NCTR scientists involved SKH-1 hairless mice, the laboratory animal model most commonly used in photocarcinogenesis research because "tumors induced in these mice resemble, both at the morphologic and molecular levels, UVR-induced skin malignancies in man" (Benavides 2009). The NTP approach was based on the study designs reported by P.D. Forbes and colleagues using simulated solar light (Forbes 2003). While a variety of experimental approaches has been developed for studying photocarcinogenicity in the SKH-1 model, the NTP protocol adequately addresses the question of retinyl palmitate photocarcinogenecity. This protocol was proven effective in a 1-year study on glycolic acid and salicylic acid described below (NTP 2007) as well as in a pilot 13-week retinyl palmitate study (Yan 2007).

In the NTP study, groups of 36 male and 36 female SKH-1 mice were irradiated five days per week (Monday through Friday, in the morning) for 40 weeks with solar-simulating light (SSL) with a UVA/UVB ratio of 20.5:1, similar to natural sunlight. Two levels of light intensity were tested, 6.75 and 13.7 mJ CIE/cm2, equivalent to 0.3 and 0.6 of a minimal erythemal (sunburn) dose (MED). According to an FDA publication, 0.6 MED is equivalent to nine minutes of unprotected skin UV exposure on a sunny day with a UV index of 10 on the World Health Organization UV scale (WHO 2002; Yan 2007). The mice received topical applications of control cream or creams containing 0.1%, 0.5%, 1.0%, or 2.0% (w/w) retinyl palmitate on the dorsal skin region in the afternoon on the days of irradiance exposures. These doses of retinyl palmitate correspond to real-life concentrations found in personal care products on the market (Cosmetics Ingredient Review 2009; NTP 2000).

The effectiveness of this study protocol and its ability to distinguish between positive and negative results has been clearly demonstrated in another study carried out by NTP/NCTR scientists, *Photocarcinogenesis Study of Glycolic Acid and Salicylic Acid* (NTP 2007). Taking into consideration the survival data, time-to-tumor data and the pathology results, the NTP concluded that glycolic acid did not alter the photocarcinogenesis of simulated solar light and salicylic acid was photoprotective (NTP 2007).

Furthermore, there is a recognized increase in tumor incidence in SSL-exposed animals treated with control cream compared to SSL-exposed animals that did not receive any cream treatment, a phenomenon observed in the retinyl palmitate study (NTP 2010); the glycolic and salicylic acid study (NTP 2007); as well as other studies in this animal model (Jacobs 2004; Lu 2009). Despite

the elevated background associated with the application of the control cream, NTP/NCTR researchers found enhanced photocarcinogenicity associated with retinyl palmitate or retinoic acid application, but no such effects associated with the application of glycolic acid or salicylic acid. This result clearly demonstrates that the SKH-1 study protocol used by the NTP can reveal compound-specific effects which makes it valid for assessing retinyl palmitate photococarcinogenicity.

2. The weight of evidence from the study supports the finding of retinyl palmitate photococarcinogenicity.

The NTP report analyzed the study data in multiple ways, examining mean survival time among exposed animals; in-life median skin lesion onset; in-life skin lesion incidence; multiplicity of in-life skin lesions and different tumor types; as well as incidence rates of skin lesions and tumors. It reached the following conclusions:

- In both sexes, all dose groups and at all levels of UV exposure, daily application of creams containing RP significantly 1) decreased survival time; 2) sped up the onset of skin lesion development; and 3) increased the number of squamous neoplasms per animal.

- Retinyl palmitate exposure in combination with simulated sunlight increased the number of in-life skin lesions and the number of focal atypical hyperplasias per animal in six of eight dose groups.

- In both male and female animals exposed to retinyl palmitate in combination with the lower dose of simulated sunlight, there were significant dose-related increases in the incidence of squamous cell carcinoma. At the higher dose of UV light exposure, however, NTP determined that the high rate of lesions and tumor incidence in the control cream group appeared to overwhelm any increase in RP-exposed animals and precluded detection of a statistically significant increase, an effect consistent with the high sensitivity of the SKH-1 mouse model to UV photocarcinogenicity (Benavides 2009).

EWG agrees with the NTP interpretation of the study data and finds that the overall weight of evidence strongly supports the NTP's conclusion of retinyl palmitate photococarcinogenicity.

3. The findings of the draft report are in agreement with the research database on the photogenotoxicity and photocarcinogenicity of retinoid compounds.

In earlier research, FDA scientists have shown that UV-exposed retinyl palmitate and other retinoids form free radicals and cause DNA mutations (Cherng 2005; Mei 2006; Mei 2010; Yan 2005). The photomutagenic effects of retinyl palmitate and other retinoids suggest a plausible mechanism by which a combination of RP and sunlight exposure could increase the onset, incidence and multiplicity of skin tumors. Other mechanisms could be also involved, such as increased cell division and hyperplasia which have been detected in the NTP research (NTP

2010) as well as other studies on retinoid compounds (Sorg 2006). Notably, in studies with human volunteers, retinol and retinyl palmitate induced skin hyperplasia (Duell 1997).

The NTP research also agrees with the preponderance of studies conducted by university and industry researchers pointing to the likelihood of photocarcinogenicity risks associated with topically applied retinoids. While there is diversity among the findings in the literature (So 2004), seven studies conducted between 1977 and 2008 found evidence of retinoic acid photocarcinogenicity (Epstein 1977; Forbes 1979; Forbes 1981; Davies 1988; Hartmann 1981; Halliday 2000; Lerche 2008). Overall, as noted by NTP (2000), in studies in which solar-simulating UV radiation was used at dose levels less than the human minimal erythema dose, topically applied retinoic acid generally enhanced photocarcinogenesis. In contrast, studies using UV radiation from unfiltered sources that emit UVC radiation not present in terrestrial sunlight (Brown 2000) at levels exceeding the human minimal erythema dose found that topically applied retinoic acid did not affect or inhibit photocarcinogenesis (Epstein and Grekin, 1981; Kligmann and Kligmann, 1981a; Kligmann and Kligmann, 1981b).

The NTP study employed moderate levels of simulated sunlight that correspond to a mild sunlight exposure scenario for both higher and lower SSL doses. NTP also used realistic retinyl palmitate concentrations similar to those found in personal care products. Finally, NTP utilized a research protocol and statistical methods whose effective performance has been demonstrated by earlier research (Howard 2002; NTP 2007; NTP Statistical Methods Working Group 2004). These three key aspects of the NTP protocol allowed the study to detect clear and unambiguous photococarcinogenic activity of retinyl palmitate in this mouse model.

In conclusion, EWG expresses strong support for the high-quality NTP/NCTR study, which provides the best, most detailed source of data currently available on the phototoxicity of retinyl palmitate. Together with other retinyl palmitate toxicity studies carried out by FDA scientists over the last decade, this research will help fill the long-standing data gap on the safety of retinyl palmitate in personal care products used on sun-exposed skin.

Olga V. Naidenko, PhD
Senior Scientist, Environmental Working Group

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Personal Care Products Council
Committed to Safety, Quality & Innovation

January 20, 2011

Lori D. White, Ph.D., PMP
NTP Designated Federal Officer
NIEHS/NIH
P.O. Box 12233, MD K2-03
Research Triangle Park, NC 27709
whiteld@niehs.nih.gov

Dear Dr. White:

The following comments are submitted on behalf of the Personal Care Products Council[1] in response to the National Toxicology Program (NTP) Board of Scientific Counselors Technical Report Subcommittee's review of "*NTP Technical Report on the Photococarcinogenesis Study of Retinoic Acid and Retinyl Palmitate in SKH-1 Mice", (*TR568*)* scheduled to be peer reviewed on January 26, 2011.

NTP BSC Technical Report Review Panel Charge

Retinyl palmitate is approved by U.S. Food and Drug Administration (FDA) as a food GRAS nutrient and as an over-the-counter (OTC) and prescription drug. To achieve premarket approval, FDA, which is the U.S. regulatory authority for retinyl palmitate when used as a drug, in foods and in cosmetics, required extensive and rigorous pre-market testing. It is important that NTP Technical Report (TR) panels recognize that NTP is not a regulatory authority. We were therefore encouraged to note that NTP's charge to the panel is focused and crisp: **(1) peer review the scientific and technical elements of the study and its presentation; (2) determine whether the study's experimental design and conduct support the NTP's conclusions regarding the carcinogenic activity of the substance tested.**

Retinyl Palmitate Nomination

In November, 2000, the FDA's Center for Food Safety and Applied Nutrition (CFSAN) nominated Retinyl Palmitate (RP) to the National Toxicology Program requesting *"– a*

[1] Based in Washington, D.C., the Council is the leading national trade association representing the $250 billion global cosmetic and personal care products industry. Founded in 1894, the Council's more than 600 member companies manufacture, distribute, and supply the vast majority of finished personal care products marketed in the United States. As the makers of a diverse range of products that millions of consumers rely on everyday, from sunscreens, toothpaste, and shampoo to moisturizer, lipstick, and fragrance, member companies are global leaders committed to product safety, quality, and innovation. The Council was previously known as the Cosmetic, Toiletry, and Fragrance Association (CTFA).

photocarcinogenesis study of retinyl palmitate, under conditions relevant to the use of retinyl palmitate in cosmetics" – and -- "mechanistic studies to establish the relevance of the results obtained in the selected animal model". In the draft TR 568 report, made available in December, 2010**,** the nomination rational and testing request states "--- *for phototoxicity and photocarcinogenicity testing based on the increasingly widespread use of this compound in cosmetic retail products for use on sun-exposed skin, the biochemical and histological cutaneous alterations elicited by RP, and the association between topical application of retinoids and enhancement of photocarcinogenesis".* While the term "sunscreen" is not mentioned, we believe it may be implied. **The NTP RP protocol was not properly constructed to test sunscreens or sun blockers containing RP**.

Time Line

We understand the FDA nominated RP to the NTP in 2000, that the one (1) year photo-cocarcinogenesis study was begun in 2003 and that the on-site pathology was not completed until mid-2006. While it is recognized that delays can occur in any study, we question the two-year delay in pathology completion, since the 1% and 2% RP animals did not have pathology performed, and especially the four-year delay from pathology completion to the availability of the draft RP TR in December 2010. **Because of the reported study flaws in the TR 568 report, we wonder if NTP had concerns about the adequacy of the study or ever considered not bringing the study forward.**

Protocol Design

In the standard UVR SKH-1 protocol designed by Forbes, animals receive test agent followed by UVR exposures on Monday, Wednesday and Friday and receive UVR exposures followed by test agent on Tuesday and Thursday, a routine followed for 40 weeks with an additional 15 week no dose/no UVR monitoring; endpoints are typically time to lesion formation (specified size) and/or lesion multiplicity. The exposure protocol for this study was different in that treatment with UVR was in the morning 5 days a week and treatment with test agents was in the afternoon 5 days a week then, after 40 weeks of treatment, the mice were held without treatment for additional 12 weeks prior to sacrifice. The exposure protocol for the TR 568 report was selected to *"mimic human use where people are exposed to sunlight during the afternoon then use the retinoid-containing creams at night"*. **We wonder how the change in the exposure protocol from the widely accepted Forbes standard protocol could have influenced the outcome of these studies.**

Reasons for Removal

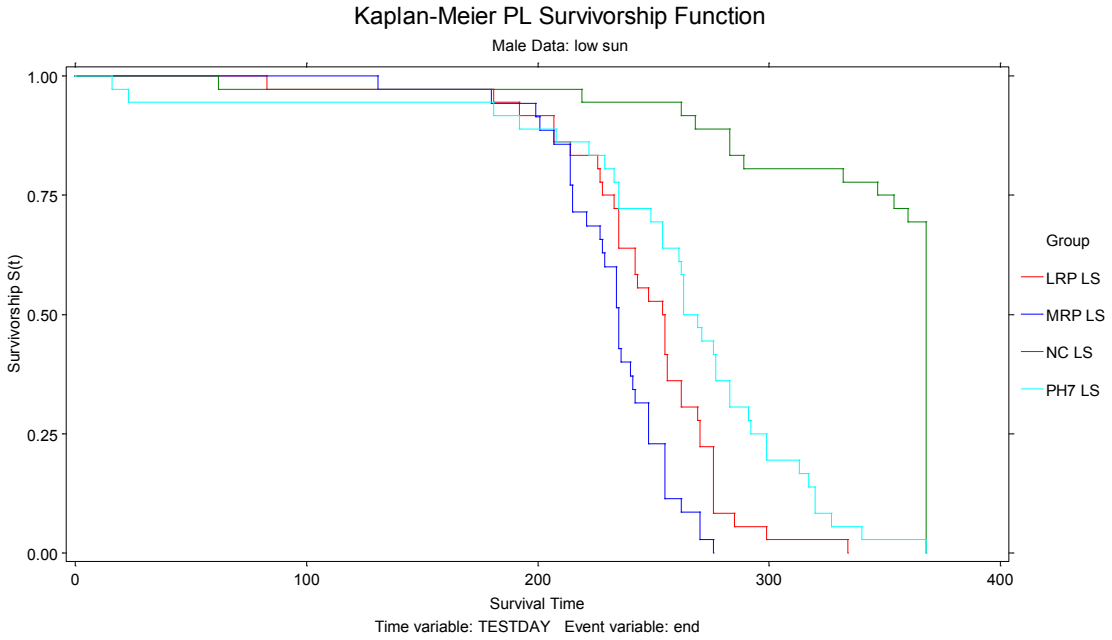
Tables 4 and 5 in TR 568 show that in groups with control cream and RP, the main reason for animals exiting the study was Skin Lesion ≥ 10 mm. However, in the Preliminary Pathology Tables presented on the NTP web site the cause for removal was listed as "harvest." We believe that removal criteria other than tumors ≥10 mm may have been used when determining whether or not to remove animals under the "harvest" terminology (e.g. due to severe toxicity). It would benefit the reader if the Standard Operating Procedure (SOP) for animal removal were included in the appendices of TR568 since the removals were considered to be non-censored animals (known lifetime) and the vast majority of animals exposed to sunlight/cream or sunlight/cream + RP were in the "removed" category. Therefore the criteria for selecting

animals for removal from this experiment were considered non-censored in spite of the fact that many were removed due to toxicity (and thus should have been classified as censored animals).

Note that the issue of censoring is of more than academic interest. For example, one might have comparable numbers of tumors in each of two dose groups but if animals were removed sooner in the first group, it would have a higher tumor rate than the second because the first group would have fewer animal-years. At a more general level, the report notes that the survival analyses presented are in fact survival-removal analyses. So what exactly does removal mean? A full understanding of the data in this study must be accompanied by a detailed discussion of the removal criteria, and the reader would also be assisted by a discussion of the relative numbers of animals removed by removal criterion.

Statistical Analysis and Confounders

Our overall impression is that the statistical analyses applied in TR 568 are appropriate, and that the signals that the test system is generating may appear reasonable to the reader not familiar with the nuances of photo-cocarcinogenesis bioassays. However, we note that the difference between the response of control cream plus UVR compared to UVR only is unacceptably dramatic (see Example 1 this document). We also note that 1% and 2% RP formulations appear toxic even in the absence of UVR (Figures 8 and 9) and assume that this is the reason that neither 1% nor 2% RP animals appear in the pathology evaluations. **To us, this should have resulted in study termination.**



Example 1 *Time on Test (TOT) Data* *Male survival with Low-Sun treatment (6.75 mJ.CIE/cm2) for No Cream (NC - Green); Control Cream (PH7 - Turquoise); Low RP (LRP - Red); and Mid RP (MRP - Blue). Note the magnitude of the shift to the left for the Control Cream (Turquoise line) compared to the No Cream group (Green Line).* **Since the shift to the left is dramatic and unacceptable – can this really be an adequate study?**

A first concern is that there is no way to estimate the effects of RP independent from the effects of the control cream which indeed is a major problem. What would be the effects, if any, of RP administered in a control cream that did not by itself act as a promoter? One can only speculate.

A second issue is the test system itself. TR568 says that 1% and 2% RP levels caused severe skin irritation requiring animal removal, even in the absence of exposure to UVR. However, those levels have reportedly been used without such irritation in other published peer-reviewed studies. So are the 1% and 2% RP levels toxic because too high of a dose was selected, because of a property of the SKH-1 mice used in this study, or because of an effect of the interaction of RP with a component of the control cream, such as diisopropyl adipate? Again, one can only speculate.

Third, we believe it is inappropriate to use time to tumor formation and/or tumor multiplicity data from animals that exhibit toxicity and 1) were removed from the experiment early, and 2) were excluded from pathology examination (1% and 2% RP animals).

Finally, no amount of statistical sophistication or manipulation can legitimately estimate main effects in the presence of large interactions. For example, the Cox Hazard Ratios between cream and various levels of RP do not represent independent RP effects. Rather they represent the effect of the cream, the effect of RP, and the effect of the unknown but also possibly they represent large interaction between the cream and RP. We believe it is simply irresponsible to attempt to present such analyses without caveats concerning the fact that the degree to which such differences exist is unknown and in fact cannot be estimated with the available data.

Control Cream with diisopropyl adipate

A control vehicle must be known not to enhance or prevent a particular biological event; it is only a carrier of the test agent or used to simulate a particular manipulation of the test animal. If it is noted that the control vehicle elicits the same biological response that is to be measured in a study, then reasonable scientists would consider the experiment flawed and the study would be repeated using a non-reactive control vehicle or abandoned.

For this particular study, it is difficult to imagine how, 1) once it was noted that control cream animals were developing comparable numbers of tumors to the test agent animals at the same UVR dosage and/or 2) noted that animals were experiencing severe toxicity reactions requiring removal as “Harvest” (preliminary NTP Pathology Tables), that this study was allowed to proceed for the entire one (1) year duration of the experiment. Indeed, the fact that the 1% and 2% RP dosed animals were in such poor condition as to preclude pathological examination is a strong statement that this experiment was flawed and should have been terminated.

Topically applied vehicle control formulations may include water, emollients, moisturizers, ointments, creams, salves and balms. It is known that, depending on the formulation mixture, all may increase or decrease test agent absorption, change the

optical properties such that UVR penetration is enhanced or reduced or support chemical reactions between the test agent and a control formulation component. This is why it is important to test the vehicle control formulation independently to assure it does not enhance the biological event that the test is measuring. This study suffers from that oversight.

NTP has conducted many properly designed, well managed and accurately reported hazard identification studies over the years that have contributed to public health and found utility by the regulatory community. Unfortunately, for reasons discussed above, the TR 568 study does not measure up to NTP standards. **Therefore, we believe that the only reasonable call that NTP can support for TR 568 is: Inadequate Study of Carcinogenic Activity.**

UVA / UVB Studies

In a separate experiment the NTP tested RP (1.0%) in female SKH-1 mice in the presence and absence of UVA or UVB irradiation. This study utilized the same control cream and thus suffers from the same experimental flaws noted with the UVR study. We believe the results from the UVA / UVB study can only be viewed as "observational" and certainly **cannot be utilized in any capacity to support the NTP call for the one (1) year photo-cocarcinogenesis study.**

Initiation/Promotion/Progression UVR/SKH-1 Animal Model

The SKH-1 / UVR protocol design is a (x) staged initiation-promotion-progression design model. A slope shift to the left could be due to (1) photo-activated production to a bioactive chemical, (2) modulation of UVR-induced genotoxicity, (3) simple enhanced promotion of UVR-initiated cells, (4) test agent acting additively/synergistically with UVR, (5) simple phototoxicity, (6) immune suppression, (7) interaction between control cream and test agent,(8) altered apoptosis, (9) a combination of the above, or (10) other unknown mechanisms. The variability in outcome when testing various substances, including the retinoids, using this animal model is quite large, as even noted in this TR. Study results are more often a consequence of protocol design, test agent purity, exposure times, test agent and UVR application sequence and the type of control vehicle utilized.

Moreover, UVR is, by itself, the initiator and the promoter (it is a "complete" carcinogen) as nicely demonstrated in the UVR dose curves published in this TR; this by itself is a confounder when attempting to interpret study outcomes. Clearly, extrapolation concerns must also exist when considering animal vs. human differences in test agent response, UVR response and/or response to the combination of test agent/UVR. So the question for the FDA becomes: **how does one even begin to understand what a slope shift to the left means in the presence of a test agent and UVR, in a regulatory framework? That is, how does FDA measure the "risk to human health" from such animal studies? Furthermore, can the FDA really regulate an animal photo-cocarcinogen "promoter"?**

Given those questions, we note with interest that the FDA Center for Drug Evaluation and Research (CDER) has recently published, "Guidance for Industry M3(R2) Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing

Authorization for Pharmaceuticals" which in Note 6, states: **"Testing for photocarcinogenicity in rodents using currently available models (e.g., hairless rodent) is not considered useful in support of pharmaceutical development and generally is not recommended**. We wonder if this same policy is also embraced by CFSAN and other FDA product centers.

Summary

There was what we believe an unusual 11-year delay from FDA nomination to NTP reporting the results from this one (1) year photo-cocarcinogenesis study (2000 – 2011) and speculate that the delay may have been driven by NTP questioning the adequacy of the study and debating the merits of bringing this study forward for a public peer review.

The NTP used a protocol design different from the accepted Forbes design which was, we believe, not adequately justified in TR 568 and furthermore was an untested design at the beginning of the RP photo-cocarcinogenesis study. The impact on the outcome of the TR 568 is uncertain.

The UVA and UVB studies suffer from the same confounder's that the UVR study does (active control cream) and can only be viewed as observational in nature and should not be used in any manner in supporting the call for TR 568.

No reasonable scientist would have continued a study so obviously flawed by the presence of a reactive control cream that alone dramatically changed the slope of the response. Moreover, the obvious toxic response to RP dosing in the presence and absence of UVR was another reason to terminate the study.

It is impossible to determine the independent action of RP on the development of skin tumors or tumor multiplicity. Additionally, it is difficult to imagine how any U.S or international regulatory body could use such data in a risk assessment or for formulating any reasonable risk management decision.

Finally, the only reasonable call that the NTP can support for this study is: **Inadequate Study of Carcinogenic Activity.**

Sincerely,

[Redacted]

[Redacted]

John E. Bailey, Ph.D.
Executive Vice President
Science

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May 28, 2010

The Honorable Margaret Hamburg, M.D.
Commissioner
Food and Drug Administration
10903 New Hampshire Ave.
Building 1 Room 2217
Silver Spring, MD 20993-0002

Dr. Linda S. Birnbaum
Director
National Institute of Environmental Health Sciences /
National Institutes of Health, and National Toxicology Program
P.O. Box 12233
Research Triangle Park, NC 27709

Re: Pressing Need to Expedite Photocarcinogenicity Assessment for Sunscreen Ingredient Retinyl Palmitate

Dear Commissioner Hamburg and Dr. Birnbaum:

We are writing to commend the scientists at the Center for Phototoxicology of the National Toxicology Panel (NTP) and Food and Drug Administration (FDA) National Center for Toxicological Research (NCTR) for outstanding research to help illuminate factors driving the rising skin cancer rates in the United States. We urge you to assess rapidly the data generated by the center's investigation into whether retinyl palmitate, a vitamin A derivative and common ingredient in sunscreen products, is toxic and carcinogenic in the presence of sunlight.

Ten years ago FDA nominated retinyl palmitate for testing to determine whether the compound has photocarcinogenetic effects. That possibility was suggested by a series of studies conducted by FDA and academic scientists since 1985. In a document supporting the nomination, the National Toxicology Panel cited FDA's concerns about the use of RP in skin care products (NTP 2000):

> "**Retinyl palmitate was selected** by the [FDA's] Center for Food Safety and Applied Nutrition for phototoxicity and photocarcinogenicity testing **based on the increasingly widespread use of this compound in cosmetic retail products for use on sun-exposed skin, the biochemical and histological cutaneous alterations elicited by retinyl palmitate, and the association between topical application of retinoids and enhancement of photocarcinogenesis**."

Since that nomination, FDA researchers have published 17 studies and science reviews on the toxicity and chemistry of retinyl palmitate on the skin. According to FDA scientists, the study

HEADQUARTERS 1436 U St. NW, Suite 100 Washington, DC 20009 | P: 202.667.6982 F: 202.232.2592
CALIFORNIA OFFICE 2201 Broadway, Suite 308 Oakland, CA 94612 | P: 510.444.0973 F: 510.444.0982

findings suggest that retinyl palmitate breaks down in sunlight to photomutagenic compounds, forms free radicals in the presence of UVA and UVB radiation and "[causes] events that affect a large segment of the chromosome" (e.g., Mei et al. 2005, 2006; Xei et al. 2006, see Addendum).

This research has culminated in the center's completion of a one-year photocarcinogenicity study of retinyl palmitate. The study method, which is based on rodent testing, is currently the state-of-the-art technique for establishing whether a compound is carcinogenic in the presence of sunlight.

Key study data have been published on NTP's website (NTP 2009), but your agencies' final assessment of the work has not yet been made public.

Our review of the publicly available data suggests that your completion of this assessment could not be more urgent. The data show that tumors and lesions developed as much as 21 percent more rapidly in lab animals coated in a retinyl palmitate (RP)-laced cream (at concentrations of 0.1 percent to 0.5 percent), compared to control animals treated with an RP-free cream. Both groups were exposed to the equivalent of nine minutes of bright sunlight each day for up to a year. The differences are statistically significant and dose-dependent (EWG 2010).

The dramatically accelerated development of tumors and lesions in retinyl palmitate-treated animals, compared to untreated animals, has potentially significant implications for public health, which is why EWG raised concerns about the chemical in our 2010 review of sunscreen products (EWG 2010). Sunscreen makers have added retinyl palmitate and related forms of vitamin A to 41 percent of sunscreens on the market this year, according to EWG analysis of ingredient labels for nearly 500 products.

We are concerned that sunscreen industry consultants are attempting to downplay the relevance of the federal study. First, according to recent media reports, they disregard FDA's body of research on retinyl palmitate. As well, they misstate the basic purpose of laboratory toxicity studies that rely on non-human animals. For instance, a dermatologist who consults for a wide range of prominent sunscreen companies was quoted as saying that it was "very premature to even cast doubt about the safety of this chemical," on grounds that rodent studies are not applicable to humans.

As the FDA points out, "testing for photocarcinogenicity in humans is unethical; animal testing has been used as a surrogate." As you well know, FDA, NTP and other scientific institutions are working to develop sorely needed non-animal methods for toxicity testing. Until reliable non-animal models are available, animal tests are established, state-of-the-art methods for evaluating toxicity. FDA acknowledges uncertainties in applying the test results to humans (FDA 2003). But given currently available methods, NTP cancer studies like the RP study conducted by the center are considered the "gold standard" for assessing human carcinogenicity risks (Ball 2009; Bucher 2002). FDA's Guidance for Photosafety recommends the methods and species (hairless mouse) used by the center (FDA 2003). Scientists from the renowned MD Anderson Cancer Center have noted that "SKH1 [hairless] mice are the most widely used in dermatologic

research… tumors induced in these mice resemble, both at the morphologic and molecular levels, UVR-induced skin malignancies in man" (Benavides 2009).

The literature shows that since 2002, FDA scientists have also studied retinyl palmitate with non-animal laboratory assays, including cellular and mechanistic studies, and with short-term animal studies. Your current study of the possible photocarcinogenicity of retinyl palmitate, using rodents, is based on a significant body of science that has deployed a variety of testing methods.

Some industry consultants may not be aware that the center's testing is done in lieu of unethical human testing or that animals susceptible to cancer are selected to reduce the number of animals needed for testing. We are concerned that the broader dermatology community may not be fully aware of the relevance of the center's important work.

As demonstrated by the media response to our report, the public and medical community are expressing immense interest in the safety of retinyl palmitate, especially in suncare products. With this letter we urge you to expedite the final review of the retinyl palmitate study data and provide guidance to consumers, physicians, and the industry about Vitamin A-based products.

Fully 10 years have passed since FDA scientists determined they had sufficient data to initiate research on the possible health hazards of retinyl palmitate, an effort that has culminated in the key photocarcinogenicity study now before us. EWG, like many scientists and health professionals around the country, is eagerly awaiting the final publication of your conclusions. We urge you to place high priority on its timely release. In the meantime, given the public health implications of the data you have published, and the industry's use of RP in hundreds of suncare products *before* the government has completed its safety review, EWG is recommending that consumers avoid sunscreen containing retinyl palmitate.

Sincerely yours,

Kenneth A. Cook
President

Copy: Dr. Paul Howard, Director, NTP/NCTR Center for Phototoxicology

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ADDENDUM

17 FDA/NTP STUDIES AND SCIENCE REVIEWS OF VITAMIN A PHOTOTOXICITY, PHOTOMUTAGENICITY, AND RELATED ISSUES OF ITS CHEMISTRY ON THE SKIN, PUBLISHED SINCE 2002

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Division of Genetic and Reproductive Toxicology, National Center for Toxicological Research, Jefferson, AR 72079, USA. nan.mei@fda.hhs.gov

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> "Vitamin A (all-trans-retinol; retinol) is an essential human nutrient and plays an important role in several biological functions. However, under certain circumstances, retinol treatment can cause free radical generation and induce oxidative stress. In this study, we investigated photocytotoxicity and photomutagenicity of retinol using L5178Y/Tk(+/-) mouse lymphoma cells concomitantly exposed to retinol and ultraviolet A (UVA) light… **[The] results suggest that retinol is mutagenic when exposed to UVA in mouse lymphoma cells through a clastogenic mode-of-action.**"

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> Center for Food Safety and Applied Nutrition, US Food and Drug Administration, College Park, Maryland 20740, USA. junjie.yin@fda.hhs.gov
> "Anhydroretinol is a metabolite of vitamin A (retinol) and a major photodecomposition product of retinyl palmitate and retinyl acetate. Anhydroretinol is biologically active, inducing cell death in lymphoblastoid cells, prevention of N-methyl-N-nitrosourea-induced mammary cancer, and inhibition of cell growth in lymphocytes. In the present study, electron spin resonance (ESR) spin-trap techniques were employed to explore the mechanism of lipid peroxidation initiation… **Our overall results provide evidence that photoirradiation of anhydroretinol with UVA light generates reactive oxygen species**, e.g. singlet oxygen and superoxide, which mediate the induction of lipid peroxidation."

2007 – Yan J, Xia Q, Wamer WG, Boudreau MD, Warbritton A, Howard PC, Fu PP. Levels of retinyl palmitate and retinol in the skin of SKH-1 mice topically treated with retinyl palmitate and concomitant exposure to simulated solar light for thirteen weeks. Toxicol Ind Health. 2007 Nov;23(10):581-9.

> National Center for Toxicological Research, US Food and Drug Administration, Jefferson, Arkansas, USA.
> "Retinyl esters account for more than 70% of the endogenous vitamin A found in human skin, and retinyl palmitate is one of the retinyl esters in this pool. Human skin is also exposed to retinyl palmitate exogenously through the topical application of cosmetic and skin care products that contain retinyl palmitate. In this study, the accumulation of retinyl palmitate and generation of retinol in the skin of male and female SKH-1 mice that received repeated topical applications of creams containing 0.0%, 0.1%, 0.5%, 1.0%, 5.0%, 10%, or 13% of retinyl palmitate 5 days a week for a period of 13 weeks were

studied. Because products containing retinyl palmitate are frequently applied to sun-exposed skin, and because it is well established that exposure to sunlight and UV light can alter cutaneous levels of retinoids, mice in this study were additionally exposed 5 days a week to simulated solar light… **Our results indicate that topically applied retinyl palmitate may alter the normal physiological levels of retinyl palmitate and retinol in the skin of SKH-1 mice and may have a significant impact on vitamin A homeostasis in the skin.**"

2007 – Fu PP, Xia Q, Yin JJ, Cherng SH, Yan J, Mei N, Chen T, Boudreau MD, Howard PC, Wamer WG. Photodecomposition of vitamin A and photobiological implications for the skin. Photochem Photobiol. 2007 Mar-Apr;83(2):409-24.

National Center for Toxicological Research, US Food and Drug Administration, Jefferson, AR, USA. peter.fu@fda.hhs.gov
"Vitamin A (retinol), an essential human nutrient, plays an important role in cellular differentiation, regulation of epidermal cell growth and normal cell maintenance. In addition to these physiological roles, vitamin A has a rich photochemistry. Photoisomerization of vitamin A, involved in signal transduction for vision, has been extensively investigated. The biological effects of light-induced degradation of vitamin A and formation of reactive species are less understood and may be important for light-exposed tissues, such as the skin. Photochemical studies have demonstrated that excitation of retinol or its esters with UV light generates a number of reactive species including singlet oxygen and superoxide radical anion. **These reactive oxygen species have been shown to damage a number of cellular targets, including lipids and DNA. Consistent with the potential for damaging DNA, retinyl palmitate has been shown to be photomutagenic in an in vitro test system. The results of mechanistic studies were consistent with mutagenesis through oxidative damage.** Vitamin A in the skin resides in a complex environment that in many ways is very different from the chemical environment in solution and in in vitro test systems. **Relevant clinical studies or studies in animal models are therefore needed to establish whether the pro-oxidant activity of photoexcited vitamin A is observed in vivo, and to assess the related risks**."

2007 – Fu PP, Xia Q, Boudreau MD, Howard PC, Tolleson WH, Wamer WG. Physiological role of retinyl palmitate in the skin. Vitam Horm. 2007;75:223-56.

National Center for Toxicological Research, Food and Drug Administration, Jefferson, Arkansas 72079, USA.
"The skin is similar to other organs in how it absorbs, stores, and metabolizes vitamin A. However, because of the anatomical location of skin and the specialized physiological roles it plays, there are ways in which the skin is rather unique. The stratified structure of the epidermis results from the orchestration of retinoid-influenced cellular division and differentiation. Similarly, many of the physiological responses of the skin, such as dermal aging, immune defense, and wound healing, are significantly affected by retinoids. While much is known about the molecular events through which retinoids affect the skin's

responses, more remains to be learned. Interest in the effects of retinol, retinyl palmitate, and other retinoids on the skin, fueled in part by the promise of improved dermatologic and cosmetic products, will undoubtedly make the effects of retinoids on skin a subject for continued intense investigation."

2006 – Mei N, Xia Q, Chen L, Moore MM, Chen T, Fu PP. Photomutagenicity of anhydroretinol and 5,6-epoxyretinyl palmitate in mouse lymphoma cells. Chem Res Toxicol. 2006 Nov;19(11):1435-40.

Division of Genetic, National Center for Toxicological Research, U.S. Food and Drug Administration, Jefferson, Arkansas 72079, USA. nan.mei@fda.hhs.gov.
Retinyl palmitate (RP) is frequently used as an ingredient in cosmetics and other retail products. "We previously reported that, under UVA light irradiation, RP is facilely decomposed into multiple products, including anhydroretinol (AR) and 5,6-epoxyretinyl palmitate (5,6-epoxy-RP). We also determined that combined treatment of mouse lymphoma cells with RP and UVA irradiation produced a photomutagenic effect. In this study, we evaluated the photomutagenicity of AR and 5,6-epoxy-RP, in L5178Y/Tk+/- mouse lymphoma cells. Treatment of cells with AR or 5,6-epoxy-RP alone at 10 and 25 microg/mL for 4 h did not show a positive mutagenic response. However, because these doses did not induce the required amount of cytotoxicity for mouse lymphoma assay, we are unable to determine whether or not these two compounds are mutagenic. Treatment of cells with 1-25 microg/mL AR or 5,6-epoxy-RP under UVA light (315-400 nm) for 30 min (1.38 mW/cm2) produced a synergistic photomutagenic effect. At 10 microg/mL (37.3 microM) AR with UVA exposure, the mutant frequency (MF) was about 3-fold higher than that for UVA exposure alone, whereas the MF for 25microg/mL (46.3microM) of 5,6-epoxy-RP + UVA was approximately 2-fold higher than that for UVA exposure alone. Compared with previous results for RP + UVA treatment, the potency of the induced phototoxicity and photomutagenicity was AR > RP > 5,6-epoxy-RP. To elucidate the underlying photomutagenic mechanism, we examined the loss of heterozygosity (LOH) at four microsatellite loci spanning the entire chromosome 11 for mutants induced by AR or 5,6-epoxy-RP. Most mutants lost the Tk+ allele, and more than 70% of the chromosome damage extended to 38 cM in chromosome length. AR + UVA induced about twice as many mutants that lost all four microsatellite markers from the chromosome 11 carrying the Tk+ allele as RP + UVA or 5,6-epoxy-RP + UVA. **These results suggest that two of RP's photodecomposition products are photomutagenic in mouse lymphoma cells, causing events that affect a large segment of the chromosome."**

2006 – Yan J, Xia Q, Webb P, Warbritton AR, Wamer WG, Howard PC, Boudreau M, Fu PP. Levels of retinyl palmitate and retinol in stratum corneum, epidermis and dermis of SKH-1 mice. Toxicol Ind Health. 2006 Apr;22(3):103-12.

National Center for Toxicological Research, U.S. Food and Drug Administration, Jefferson, AR 72079, USA

"Vitamin A (retinol) regulates many biological functions, including epidermal cell growth. Retinyl palmitate (RP) is the major esterified form of retinol and the predominant component of retinoids in the skin; however, how endogenous levels of RP and retinol in the skin are affected by the age of the animal remains unknown. Furthermore, the levels of retinol and RP in the various skin layers – the stratum corneum, epidermis and dermis of skin - have not been reported. In this paper, we report the development of a convenient method for separation of the skin from SKH-1 female mice into the stratum corneum, epidermis, and dermis and the determination of the levels of RP and retinol in the three fractions by HPLC analysis. The total quantities of RP and retinol from the stratum corneum, epidermis, and dermis are comparable to those extracted from the same amount of intact skin from the same mouse. There was an age-related effect on the levels of RP and retinol in the skin and liver of female mice. An age-related effect was also observed in the stratum corneum, epidermis, and dermis. The levels of RP and retinol were highest in the epidermis of 20-week-old mice, and decreased when the age increased to 60- and 68-weeks. The total amount of RP at 20 weeks of age was found to be 1.52 ng/mg skin, and decreased about 4-fold at 60- and 68-weeks of age. A similar trend was found for the effects of age on the levels of retinol."

2006 – Yan J, Wamer WG, Howard PC, Boudreau MD, Fu PP. Levels of retinyl palmitate and retinol in the stratum corneum, epidermis, and dermis of female SKH-1 mice topically treated with retinyl palmitate. Toxicol Ind Health. 2006 May;22(4):181-91.

National Center for Toxicological Research, US Food and Drug Administration, Jefferson, AR 72079, USA.
"Retinyl esters are the storage form of vitamin A in skin, and retinyl palmitate (RP) accounts for the majority of the retinyl esters endogenously formed in skin. RP is also obtained exogenously through the topical application of cosmetic and skin care products that contain RP. There is limited information on the penetration and distribution of RP and vitamin A within the stratified layers of the skin. The purpose of these studies was to determine the time course for accumulation and disappearance of RP and retinol in the stratified layers of skin from female SKH-1 mice that received single or repeated topical applications of creams containing 0.5 or 2% of RP. We developed an HPLC method with detection limits of 5.94 and 1.62 ng, to simultaneously quantify the amount of RP and retinol, respectively, in skin samples. Our results showed that RP rapidly diffuses into the stratum corneum and epidermal skin layers within 24 h following the application of RP-containing creams. Of the three skin layers, the highest level of RP and retinol per weight unit (ng/mg) at all time points was found in the epidermis. Levels of RP and retinol were lowest in the dermal layer and intermediate in the stratum corneum. The levels of RP and retinol in the separated skin layers and in the intact skin decreased with time, but levels of RP remained higher than control values for a period of up to 18 days. **Our results indicate that the application of RP to mouse skin alters the normal physiological levels of RP and retinol in the skin."**

2006 – Xia Q, Yin JJ, Wamer WG, Cherng SH, Boudreau MD, Howard PC, Yu H, Fu PP. Photoirradiation of retinyl palmitate in ethanol with ultraviolet light--formation of photodecomposition products, reactive oxygen species, and lipid peroxides. Int J Environ Res Public Health. 2006 Jun;3(2):185-90.

> National Center for Toxicological Research, U.S. Food and Drug Administration, Jefferson, AR 72079, USA.
> We have previously reported that photoirradiation of retinyl palmitate (RP), a storage and ester form of vitamin A (retinol), with UVA light resulted in the formation of photodecomposition products, generation of reactive oxygen species, and induction of lipid peroxidation. In this paper, we report our results following the photoirradiation of RP in ethanol by an UV lamp with approximately equal UVA and UVB light. The photodecomposition products were separated by reversed-phase HPLC and characterized spectroscopically by comparison with authentic standards. The identified products include: 4-keto-RP, 11-ethoxy-12-hydroxy-RP, 13-ethoxy-14-hydroxy-RP, anhydroretinol (AR), and trans- and cis-15-ethoxy-AR. Photoirradiation of RP in the presence of a lipid, methyl linoleate, resulted in induction of lipid peroxidation. Lipid peroxidation was inhibited when sodium azide was present during photoirradiation which suggests free radicals were formed. **Our results demonstrate that, similar to irradiation with UVA light, RP can act as a photosensitizer leading to free radical formation and induction of lipid peroxidation following irradiation with UVB light.**

2005 – Xia Q, Yin JJ, Cherng SH, Wamer WG, Boudreau M, Howard PC, Fu PP. UVA photoirradiation of retinyl palmitate--formation of singlet oxygen and superoxide, and their role in induction of lipid peroxidation. Toxicol Lett. 2006 May 5;163(1):30-43. Epub 2005 Dec 27.

> National Center for Toxicological Research, U.S. Food and Drug Administration, Department of Biochemical Toxicology, HFT-110, 3900 NCTR Road, Jefferson, AR 72079, USA.
> "We have previously reported that photoirradiation of retinyl palmitate (RP) in ethanol with UVA light results in the formation of photodecomposition products, including 5,6-epoxy-RP and anhydroretinol (AR). Photoirradiation in the presence of a lipid, methyl linoleate, induced lipid peroxidation, suggesting that reactive oxygen species (ROS) are formed. In the present study, we employ an electron spin resonance (ESR) spin trap technique to provide direct evidence as to whether or not photoirradiation of RP by UVA light produces ROS. Photoirradiation of RP by UVA in the presence of 2,2,6,6-tetramethylpiperidine (TEMP), a specific probe for singlet oxygen, resulted in the formation of TEMPO, indicating that singlet oxygen was generated. Both 5,5-dimethyl N-oxide pyrroline (DMPO) and 5-tert-butoxycarbonyl 5-methyl-1-pyrroline N-oxide (BMPO) are specific probes for superoxide. When photoirradiation of RP was conducted in the presence of the DMPO or BMPO, ESR signals for DMPO-*OOH or BMPO-*OOH were obtained. **These results unambiguously confirmed the formation of superoxide radical anion.** Consistent with a free radical mechanism, there was a near complete and time-dependent photodecomposition of RP and its photodecomposition products. ESR

studies on the photoirradiation of 5,6-epoxy-RP and AR indicate that these compounds exhibit similar photosensitizing activities as RP under UVA light."

2005 – Mei N, Xia Q, Chen L, Moore MM, Fu PP, Chen T. Photomutagenicity of retinyl palmitate by ultraviolet a irradiation in mouse lymphoma cells. Toxicol Sci. 2005 Nov;88(1):142-9. Epub 2005 Aug 17.

Division of Genetic and Reproductive Toxicology, National Center for Toxicological Research, FDA, Jefferson, Arkansas 72079, USA.

Retinyl palmitate (RP), a storage form of vitamin A, is frequently used as a cosmetic ingredient, with more than 700 RP-containing cosmetic products on the U.S. market in 2004. There are concerns for the possible genotoxicity and carcinogenicity of RP when it is exposed to sunlight. To evaluate the photomutagenicity of RP in cells when exposed to ultraviolet A (UVA) light, L5178Y/Tk+/- mouse lymphoma cells were treated with different doses of RP alone/or in the presence of UVA light. Treatment of the cells with RP alone at the dose range of 25-100 microg/ml did not increase mutant frequencies (MFs) over the negative control, whereas treatment of cells with 1-25 microg/ml RP under UVA light (82.8 mJ/cm2/min for 30 min) produced a dose-dependent mutation induction. The mean induced MF (392 x 10(-6)) for treatment with 25 microg/ml RP under UVA exposure was about threefold higher than that for UVA alone (122 x 10(-6)), a synergistic effect. To elucidate the underlying mechanism of action, we examined the mutants for loss of heterozygosity (LOH) at four microsatellite loci spanning the entire chromosome 11, on which the Tk gene is located. The mutational spectrum for the RP + UVA treatment was significantly different from the negative control, but not significantly different from UVA exposure alone. Ninety four percent of the mutants from RP + UVA treatment lost the Tk+ allele, and 91% of the deleted sequences extended more than 6 cM in chromosome length, indicating clastogenic events affecting a large segment of the chromosome. **These results suggest that RP is photomutagenic in combination with UVA exposure in mouse lymphoma cells, with a clastogenic mode-of-action.**

2005 – Yan J, Xia Q, Cherng SH, Wamer WG, Howard PC, Yu H, Fu PP. Photo-induced DNA damage and photocytotoxicity of retinyl palmitate and its photodecomposition products. Toxicol Ind Health. 2005 Sep;21(7-8):167-75.

National Center for Toxicological Research, U.S. Food and Drug Administration, Jefferson, AR, USA. yu@ccaix.jsums.edu
"Retinyl palmitate (RP) is an ester of retinol (vitamin A) and the predominant form of retinol found endogenously in the skin. We have previously reported that photoirradiation of RP with UVA light resulted in the formation of anhydroretinol (AR), 5,6-epoxyretinyl palmitate (5,6-epoxy-RP) and other photodecomposition products. While AR was formed through an ionic photodissociation mechanism, 5,6-epoxy-RP was formed through a light-mediated, free radical-initiated chain reaction. In the current study, the phototoxicity of RP, AR and 5,6-epoxy-RP in human skin Jurkat T-cells with and without light

irradiation was determined using a fluorescein diacetate assay. Under similar conditions, the Comet assay was used to assess damage to cellular DNA. Nuclear DNA was not significantly damaged when the cells were irradiated by UVA plus visible light in the absence of a retinoid; however, **when the cells were illuminated with UVA plus visible light in the presence of either RP, 5,6-epoxy-RP or AR (50, 100, 150 and 200 microM), DNA fragmentation was observed.** Cell death was observed for retinoid concentrations of 100 microM or higher. When treated with 150 microM of RP, 5,6-epoxy-RP or AR, cell death was 52, 33 and 52%, respectively. These results suggest that RP and its two photodecomposition products, AR and 5,6-epoxy-RP, induce DNA damage and cytotoxicity when irradiated with UVA plus visible light. We also determined that photoirradiation of RP, AR and 5,6-epoxy-RP causes single strand breaks in supercoiled phi chi 174 plasmid DNA. Using a constant dose of UVA light (50 J/cm2), the level of DNA cleavage was highest in the presence of AR, followed by 5,6-epoxy-RP, then RP. The induced DNA strand cleavage was inhibited by NaN3. **These results suggest that photoirradiation of RP, [and compounds RP breaks down into, in the presence of UV radiation] 5,6-epoxy-RP and AR with UVA light generates free radicals that initiate DNA strand cleavage.**"

2005 – Tolleson WH, Cherng SH, Xia Q, Boudreau M, Yin JJ, Wamer WG, Howard PC, Yu H, Fu PP. Photodecomposition and phototoxicity of natural retinoids. Int J Environ Res Public Health. 2005 Apr;2(1):147-55.

National Center for Toxicological Research, U.S. Food and Drug Administration, Jefferson, AR 72079, USA.
"Sunlight is a known human carcinogen. Many cosmetics contain retinoid-based compounds, such as retinyl palmitate (RP), either to protect the skin or to stimulate skin responses that will correct skin damaged by sunlight. However, **little is known about the photodecomposition of some retinoids and the toxicity of these retinoids and their sunlight-induced photodecomposition products on skin. Thus, studies are required to test whether topical application of retinoids enhances the phototoxicity and photocarcinogenicity of sunlight and UV light.** Mechanistic studies are needed to provide insight into the disposition of retinoids in vitro and on the skin, and to test thoroughly whether genotoxic damage by UV-induced radicals may participate in any toxicity of topically applied retinoids in the presence of UV light. This paper reports the update information and our experimental results on photostability, photoreactions, and phototoxicity of the natural retinoids including retinol (ROH), retinal, retinoid acid (RA), retinyl acetate, and RP (Figure 1)."

2005 – Cherng SH, Xia Q, Blankenship LR, Freeman JP, Wamer WG, Howard PC, Fu PP. Photodecomposition of retinyl palmitate in ethanol by UVA light-formation of photodecomposition products, reactive oxygen species, and lipid peroxides. Chem Res Toxicol. 2005 Feb;18(2):129-38.

National Center for Toxicological Research, U.S. Food and Drug Administration,

Jefferson, Arkansas 72079, USA.
"Photodecomposition of retinyl palmitate (RP), an ester and the storage form of vitamin A (retinol), in ethanol under UVA light irradiation was studied. The resulting photodecomposition products were separated by reversed-phase HPLC and identified by spectral analysis and comparison with the chromatographic and spectral properties of synthetically prepared standards. The identified products include 5,6-epoxy-RP, 4-keto-RP, 11-ethoxy-12-hydroxy-RP, 13-ethoxy-14-hydroxy-RP, anhydroretinol (AR), palmitic acid, ethyl palmitate, and four tentatively assigned cis and trans isomeric 15-ethoxy-ARs. AR was formed as a mixture of all-trans-AR, 6Z-cis-AR, 8Z-cis-AR, and 12Z-cis-AR with all-trans-ARpredominating. 5,6-Epoxy-RP, 4-keto-RP, 11-ethoxy-12-hydroxy-RP, and 13-ethoxy-14-hydroxy-RP were also formed from reaction of RP with alkylperoxy radicals generated by thermal decomposition of 2,2'-azobis(2,4-dimethylvaleronitrile). Formation of these photodecomposition products was inhibited in the presence of sodium azide (NaN3), a free radical inhibitor. These results suggest that formation of 5,6-epoxy-RP, 4-keto-RP, 11-ethoxy-12-hydroxy-RP, and 13-ethoxy-14-hydroxy-RP from photoirradiation of RP is mediated by a light-initiated free radical chain reaction. AR and the isomeric 11-ethoxy-ARs were not formed from reaction of RP with alkylperoxy radicals generated from 2,2'-azobis(2,4-dimethylvaleronitrile), and their formation was not inhibited when NaN3 was present during the photoirradiation of RP. We propose that these products were formed through an ionic photodissociation mechanism, which is similar to the reported formation of AR through ionic photodissociation of retinyl acetate. RP and all its identified photodecomposition products described above (i) were not mutagenic in Salmonella typhimurium tester strains TA98, TA100, TA102, and TA104 in the presence and absence of S9 activation enzymes, (ii) were not photomutagenic in Salmonella typhimurium TA102 upon UVA irradiation, and (iii) did not bind with calf thymus DNA in the presence of microsomal metabolizing enzymes. These results suggest that RP and its decomposition products are not genotoxic; however, photoirradiation of RP, 5,6-epoxy-RP, and AR with UVA light in the presence of methyl linoleate resulted in lipid peroxide (methyl linoleate hydroperoxides) formation. The lipid peroxide formation was inhibited by dithiothreitol (DTT) (free radical scavenger), NaN3 (singlet oxygen and free radical scavenger), and superoxide dismutase (SOD) (superoxide scavenger) but was enhanced by the presence of deuterium oxide (D2O) (enhancement of singlet oxygen lifetime). **These results suggest that photoirradiation of RP, 5,6-epoxy-RP, and AR by UVA light generated reactive oxygen species** resulting in lipid (methyl linoleate) peroxidation.

2003 – Fu PP, Cheng SH, Coop L, Xia Q, Culp SJ, Tolleson WH, Wamer WG, Howard PC. Photoreaction, phototoxicity, and photocarcinogenicity of retinoids. J Environ Sci Health C Environ Carcinog Ecotoxicol Rev. 2003 Nov;21(2):165-97.

National Center for Toxicological Research, US Food and Drug Administration, Jefferson, Arkansas 72079, USA.
"Sunlight is a human carcinogen. Many retinoid-containing cosmetics are used to protect damages caused by sunlight irradiation. Since retinol is thermally unstable and retinyl

palmitate (RP) s relatively more stable, RP is also widely used as an ingredient in cosmetic formulations. **In general, little is known about the photodecomposition of retinoids and the toxicity of retinoids and their photodecomposition products on the skin's responses to sunlight.** This review focuses on the update information on photoreactions, phototoxicity, and photocarcinogenicity of the natural retinoids including retinol, retinal, retinoid acid (RA), retinyl acetate, and RP.

2002 – Fu PP, Howard PC, Culp SG, Xia Q, Webb PJ, Blankenship LR, et al. 2002. Do topically applied skin creams containing retinyl palmitate affect the photocarcinogenecity of simulated solar light? J Food Drug Anal 10: 262-68.

National Center for Toxicological Research, US Food and Drug Administration, Jefferson, Arkansas 72079, USA.
"**Retinyl palmitate (all-*trans*-retinyl palmitate; RP) was nominated in 2001 by the U.S. Food and Drug Administration's Center for Food Safety and Applied Nutrition (CFSAN) to the National Toxicology Program (NTP) as a high priority compound for phototoxicity and photocarcinogenicity studies** at the National Center for Toxicological Research (NCTR). **Studies with SKH-1 hairless mice are required to test whether topical application of RP enhances the phototoxicity and photocarcinogenicity of simulated solar light and UV light.** Mechanistic studies are needed to provide insight into the disposition of RP *in vitro* and on the skin of mice, and to test thoroughly whether genotoxic damage by UV-induced radicals may participate in any toxicity of topically applied RP in the presence of UV light."