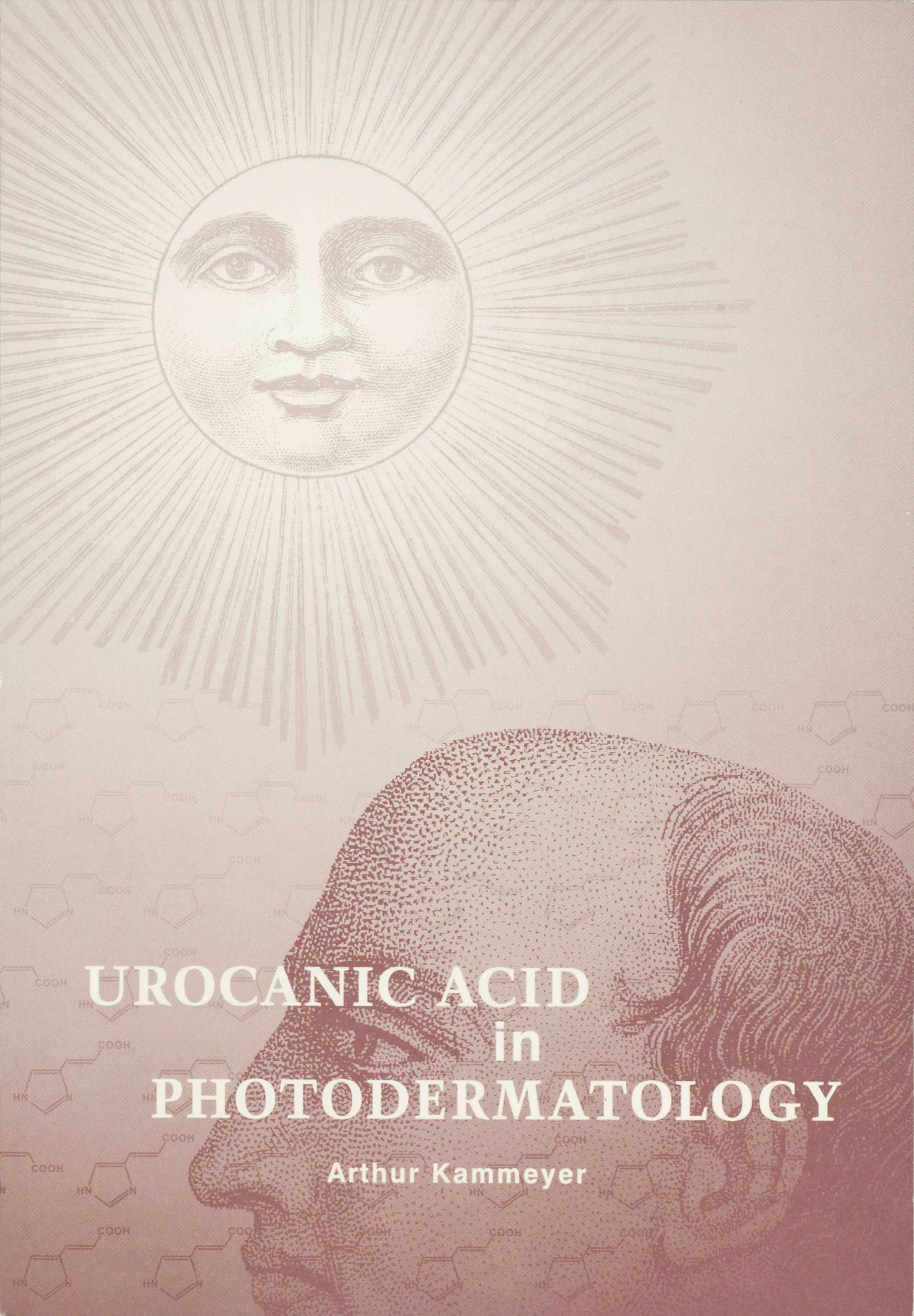

UROCANIC ACID in PHOTODERMATOLOGY

Arthur Kammeyer

Urocanic acid in photodermatology

Stellingen

behorende bij het proefschrift van Arthur Kammeijer

getiteld:

Urocanic acid in photodermatology

Stellingen bij het proefschrift van Arthur Kammeijer

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1 - De oplosbaarheid in water van de *cis*-isomeer van urocaanzuur is vele malen beter dan die van de *trans*-isomeer (dit proefschrift).

2 - *Trans*- en *cis*-urocaanzuur zijn goede hydroxyl-radicaalvangers (dit proefschrift).

3 - Naast DNA mutaties, *cis*-urocaanzuur en lipide peroxidatie is er een vierde weg voor UV-geïnduceerde immuun-suppressie: de urocaanzuur-(foto)oxidatieproducten (dit proefschrift).

4 - UV-geïnduceerde fotoproducten van op de huid aangebrachte cosmetica zijn voor het grootste deel onbekend van aard en vormen dientengevolge een gezondheidsrisico.

5 - Licht is straling die de mens met het visuele systeem kan waarnemen; UV-licht bestaat dus niet.

6 - De grens van het UV-B gebied aan de kortgolvige kant dient 290 nm te zijn, anders dan de door de Commission Internationale de l'Eclairage (CIE) ingestelde 280 nm.

7 - De potentieel schadelijke invloed van de zoetstof Aspartaam™ kan mogelijk vermeden worden door de stof niet als methylester, maar als ethylester op de markt te brengen.

8 - De schematische voorstelling van electromagnetische straling met een in fase lopend electrisch en magnetisch veld, zoals veelal afgebeeld in leerboeken, is onjuist en zou vervangen moeten worden door een figuur, waarin de velden een kwartfase verschillen.

9 - De reclame voor kansspelen zou beperkt moeten worden, net zoals de beperkingen die gelden voor de reclame voor tabak en alcohol.

10 - Wereldburgers maken geen oorlog.

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Urocanic acid in photodermatology

ACADEMISCH PROEFSCHRIFT

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aan de Universiteit van Amsterdam,
op gezag van de Rector Magnificus
prof.dr. J.J.M. Franse

ten overstaan van een door het
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op dinsdag 3 juli, te 12.00 uur

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geboren te Amsterdam

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Voor mijn ouders

Contents

Abbreviations used

BCC	Basal cell carcinoma.
CHS	Contact hypersensitivity.
DTH	Delayed type hypersensitivity.
HPLC	High performance liquid chromatography.
HSV	Herpes simplex virus.
INF-γ	Interferon-gamma.
LPS	Lipopolysacharide.
MED	Minimum erythema dose.
ROS	Reactive oxygen species.
SCC	Squamous cell carcinoma.
SPF	Sunscreen protection factor.
TNF-α	Tumor necrosis factor - alpha.
UCA	Urocanic acid.
UV	Ultraviolet.

Special terms

Spectro-photometry	Analysis methods that involves scanning of UV-VIS spectra.
Spectrometry	Analysis methods that involves scanning of spectra. E.g. mass spectrometry, infra-red spectrometry.
TL-01	Fluorescent tube lamp (TL) of Philips. A narrow-band UV-B emitter. More than 90% of its radiant energy is emitted between 311 and 313 nm.
TL-12	Fluorescent tube lamp (TL) of Philips. A broad-band UV-B emitter. Main emission in the UV-B range. Side-emissions in UV-C, UV-A and VIS range.
UV-A-I	Wavelength range of UV radiation between 340 and 400 nm.
UV-A-II	Wavelength range of UV radiation between 320 and 340 nm.
UV-B	Wavelength range of UV radiation between 290 and 320 nm; sunburn radiation. Commission Internationale d'Eclairage (CIE) defines 280 - 320 nm.
UV-C	Wavelength range of UV radiation between 200 and 290 nm.
VIS	Wavelength range of electromagnetic radiation between 400 and 700 nm; visible radiation.
Zwitter ion	An ion, bearing a positive - and negative electrical charge at the same time in a defined pH range.

CHAPTER 1

General introduction

1. Introduction and scope

Human skin is daily exposed to radiation from several sources, the sun being the principal one. The human skin, hair and eyes are the targets of (solar) radiation. The dermis, and especially the epidermis, are the sites of important photodermatological effects that occur locally (in the skin), or systemically (from the skin towards other parts of the body). The principal inducers of photodermatological effects are the ultraviolet (UV) rays in solar radiation. For instance, an important beneficial effect is the UV-induced formation of cholecalciferol (vitamin D_3), a photoproduct of 7-dehydrocholesterol, and a precursor for the metabolism of calcium and phosphorus to build the bone matrix, which largely consists of calcium phosphate. However, several UV-induced effects in the skin are adverse and should not take place. A direct effect is recognized as sunburn and long-term adverse effects are known as dermatoheliosis, aging of the skin, or its more severe form actinic keratosis. Worldwide, solar UV radiation is the primary cause of skin cancer (1). Solar and artificial UV radiation can cause the outgrowth of skin cancer by causing permanent damage to DNA and by preventing immunological recognition of mutant cells through UV-induced immunosuppression (2). UV-induced malignant states are known as squamous cell carcinoma and basal cell carcinoma. In particular the shortest UV waves from the sun reaching the earth, i.e. the UV-B radiation (290-320 nm), are held responsible for these skin defects. In addition to the direct and long-term effects of UV radiation, a variety of unusual reactions may occur, collectively known as photo(hyper)sensitivity. Skin defects can also be caused by the longer UV-A (320-400 nm) waves. Althoug UV-A is less energetic than UV-B, it penetrates deeper into the skin. Since UV-B radiation cannot penetrate beyond the first few cell layers of the epidermis, there must be one or more photoreceptor(s) in the epidermis that initiate a cascade of reactions leading to the above-mentioned skin defects or even to systemic modulation of the immune system.

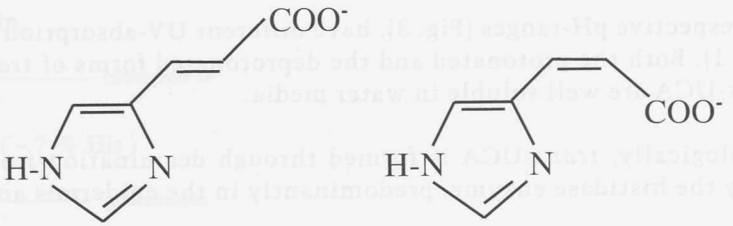
The primary effects are thought to be derived from damage to DNA or from damage to other cellular components by direct photochemical events or by reactive oxygen species induced by UV radiation. The secondary

effect may be suppression of the skin immunity, allowing direct revival of dormant viruses or (in the long term) the outgrowth of tumors. UV-radiation is mutagenic and causes antigenic changes in skin cells. In the course of the evolution, suppression of skin immunity may have been selected to prevent rejection of skin every time sunburn is developed.
UV radiation also causes photoisomerization of one of the major UV-absorbing components of the epidermis: *trans*-urocanic acid (*trans*-UCA). This compound is isomerized into *cis*-urocanic acid (*cis*-UCA), which can mimic suppressive effects of UV radiation on the skin immune system. There are strong indications that this immunosuppression could lead to skin cancer. The skin should therefore be protected against UV-exposure that simultaneously causes formation of *cis*-UCA, with a mode of action that has not been resolved, despite two decades of intensive research by several groups in the world. In this context, the issues of this thesis relate to UV-induced immunosuppression, in which UCA or UCA derivatives are initiators for the reactions leading to immunosuppression.

The studies presented here focussed on the two stereoisomers *trans*-UCA and *cis*-UCA, as well as on their oxidation products. The biokinetics of both isomers (Part I), the scavenging by hydroxyl radicals and the subsequent formation of UCA oxidation products (Part II), and the immunological activity of *cis*-UCA and oxidation products of both UCA isomers (Part III) are described in this thesis. The results of these studies allow another view on *cis*-UCA-induced immunosuppression than had so far been established: newly identified compounds also mediated UV-induced immunosuppression, next to *cis*-UCA.

2. Urocanic acid

Urocanic acid or 3-(imidazol-4-yl)propenoic acid (UCA) is one of the common components of the mammalian and human epidermis. UCA was first described in 1874 as a component in the urine of dogs after administration of high doses of histidine (3). Its name was derived from this discovery: *ouron* (Greek for urine) and *canis* (Latin for dog). UCA, a metabolite of histidine, predominantly occurs in the epidermis but also in the liver of mammals and humans. UCA forms approximately 0.5% of the dry weight of the epidermis (4). It can exist as *trans* and *cis* stereoisomeric forms because it has an α,β-olefinic bond (Fig.1). In the following 'UCA' is written when both *trans*-UCA and *cis*-UCA are referred to. Table 1 shows the differences in some physical properties between the two isomers. Striking differences exist in melting points and in solubilities that may be caused by the unique feature of the *cis*-isomer to form an intramolecular hydrogen bond in its zwitter ionic form (Fig. 2). The ionic forms of *trans*-UCA, with



trans - UCA **cis - UCA**

Fig. 1. The two stereoisomeric forms of urocanic acid.

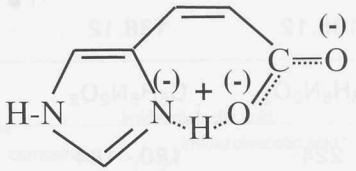


Fig. 2. Intramolecular hydrogen-bridge formation in *cis*-urocanic acid, predominantly in pH range 4-6.

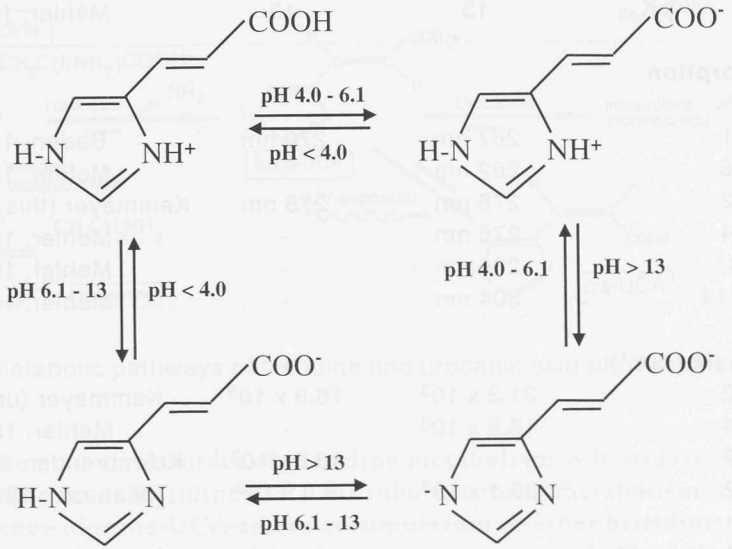


Fig. 3. pH-dependent ionic forms of *trans*-UCA. Borders of pH ranges represent in fact a smooth transition into another ionic form. Mixed ionic forms will exist on both sides of the border across more than one pH unit.

their respective pH-ranges (Fig. 3), have different UV-absorption maxima (Table 1). Both the protonated and the deprotonated forms of *trans*-UCA and *cis*-UCA are well soluble in water media.

Physiologically, *trans*-UCA is formed through deamination from histidine by the histidase enzyme, predominantly in the epidermis and the

Table 1. Physical data of the urocanic acid isomers.

	trans-Urocanic acid	*cis*-Urocanic acid	Reference
Molecular mass	138.12	138.12	
Formula	$C_6H_6N_2O_2$	$C_6H_6N_2O_2$	
Melting points (°C)	224	180 - 184	Baden, 1966 (5)
	218 - 224	175 - 176	Weast, 1982 (6)
pKa values pK_{a1}	4.0	3.3	Roberts, 1982 (7)
pK_{a2}	6.1	7.0	Roberts, 1982 (7)
pK_{a3}	13	13	Mehler, 1953 (8)
UV-absorption maxima			
At pH ~1	267 nm	270 nm	Baden, 1966 (5)
At pH 4.6	262 nm	-	Mehler, 1953 (8)
At pH 7.2	278 nm	278 nm	Kammeyer (this thesis)
At pH 7.4	276 nm	-	Mehler, 1953 (8)
At pH 13	285 nm	-	Mehler, 1953 (8)
At pH > 14	304 nm	-	Mehler, 1953 (8)
(ε-value ($M^{-1}cm^{-1}$)			
At pH 7.2	21.3×10^3	16.3×10^3	Kammeyer (unpubl.)
At pH 7.4	18.8×10^3	-	Mehler, 1953 (7)
At pH 7.5	-	13.6×10^3	Kolenbrander, 1964 (9)
At pH ~2	20.1×10^3	8.3×10^3	Takahashi, 1997 (10)
Solubility in water			
at 0 °C	5 mM=0.7 g/l	195 mM=27 g/l	Kammeyer (this thesis)
at 20 °C	17 mM=2.3 g/l	>460 mM	Kammeyer (this thesis)

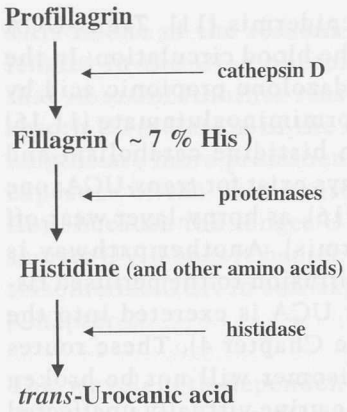


Fig. 4. The formation of urocanic acid from epidermal protein profillagrin.

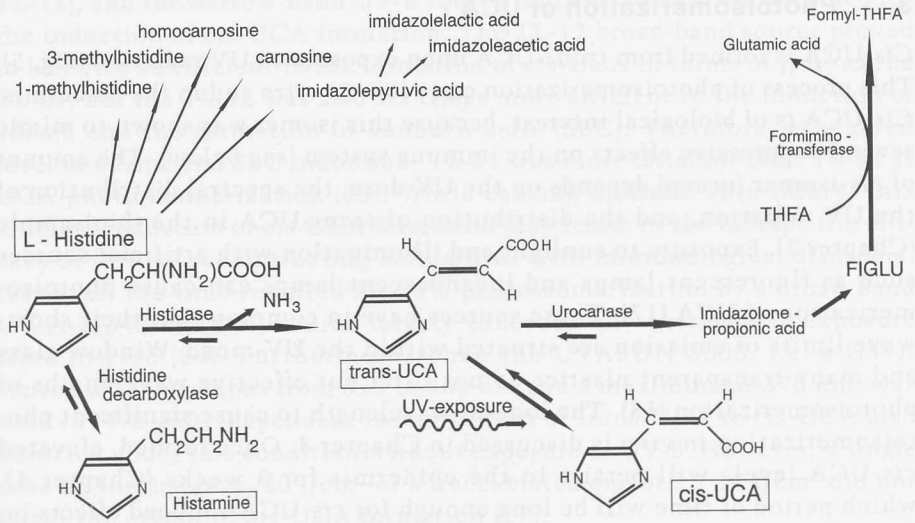


Fig. 5. Metabolic pathways of histidine and urocanic acid photoisomerization.

liver. The primary disorder of histidine metabolism is histidase deficiency, also known as histidinemia. This inborn error of metabolism results in a deficiency of *trans*-UCA and in accumulation of other histidine metabolites (11). In the epidermis, histidine in its turn is catabolized by profillagrin (Fig. 4) in the keratohyaline granules of the stratum granulosum (12,13). *Trans*-UCA accumulates in the epidermis until substantial concentrations are reached (13). It is not broken down due to lack of catabo-

lizing enzyme activity of urocanase in the epidermis (11). *Trans*-UCA may diffuse inwards and will be taken up by the blood circulation. In the liver, *trans*-UCA is partly catabolized to imidazolone propionic acid by liver urocanase (14), and subsequently to N-formiminoglutamate (11,15) which is an important intermediate between histidine catabolism and folate metabolism (Fig. 5). Two removal pathways exist for *trans*-UCA: one to the *milieu exterieure* as excretion of sweat (16), as horny-layer wear-off and as wash-out (extraction from the epidermis). Another pathway is removal to the *milieu interieure*, *i.e.* inward diffusion to the perfused tissues. Finally, part of the epidermal and liver UCA is excreted into the urine, in which fluid it can be quantified (see Chapter 4). These routes apply for *cis*-UCA as well, except that this isomer will not be broken down by liver urocanase and is excreted into the urine virtually unaffected (Chapter 4). The way of *cis*-UCA formation is described below.

3. Photoisomerization of UCA

Cis-UCA is formed from *trans*-UCA upon exposure to UV radiation (Fig. 5). This process of photoisomerization can occur *in vitro* and in the epidermis. *Cis*-UCA is of biological interest, because this isomer was shown to mimic several suppressive effects on the immune system (*see below*). The amount of *cis*-isomer formed depends on the UV-dose, the spectral distribution of the UV radiation, and the distribution of *trans*-UCA in the (bio)sample (Chapter 2). Exposure to sunlight and illumination with artificial sources such as fluorescent lamps and incandescent lamps can cause photoisomerization of UCA (17). These sources have in common that their short-wave limits of emission are situated within the UV-range. Window glass and many transparent plastics do not filter out effective wavelengths of photoisomerization (18). The longest wavelength to cause significant photoisomerization *in vivo* is discussed in Chapter 3. Once formed, elevated *cis*-UCA levels will persist in the epidermis for 3 weeks (Chapter 4), which period of time will be long enough for *cis*-UCA-induced effects on the immune sytem to occur. The extent of *cis*-UCA formation from *trans*-UCA is limited by the so-called photostationary state until a ratio of approximately 65% *cis*-UCA and 35% *trans*-UCA is reached. In this state, there is no net change in the amounts of the two isomers, despite continued irradiation and absorption of radiation. Alternatively, UV-exposure of *cis*-UCA can also yield *trans*-UCA up to 35%. These ratios are influenced by the opaqueness of the *trans*-UCA solution and by the irradiation dose and wavelength. The initial rate of *cis*-UCA formation in the clear, transparant *trans*-UCA samples ($A < 1.0$) often used in studies *in vitro* is higher at 280 nm than at 310 nm, whereas the initial rate in opaque media, such as the stratum corneum, is higher at 310 nm (19) (see also Chapter 2).

This is one of the reasons for the red-shift (shift towards longer wavelength) in the UCA photoisomerization spectrum *in vivo*, compared to that *in vitro*. Another reason is the presence of epidermal proteins that absorb UVradiation in the range 250-290 nm, such that the longer wavelengths are more prominent in *cis*-UCA production (20). Moreover, solar exposure of the skin causes an additional red-shift of photoisomerization, because the longer UV-A waves are much more abundant than the shorter UV-B ones (Chapter 2). As a result, the most effective waves to photoisomerize UCA in sun-exposed skin are between 310 and 326 nm (21-23) (Chapter 2).

The wavelength-dependent photochemistry of *trans*-UCA results from the presence of two distinct, weakly coupled electronic states in UCA molecules absorbing between 264 nm and 310 nm (24). Artificial UV-sources, such as phototherapy lamps, the broad-band UV-B source (Philips TL-12), and the narrow-band UV-B source (Philips TL-01), were studied for the induction of *cis*-UCA formation. The TL-12 broad-band source proved to be twice as efficient in the formation of *cis*-UCA in terms of J/m^2 as the TL-01, but the TL-12 was also six times more efficient in the induction of edema and the formation of sunburn cells (SBC). Therefore, at a given level of edema and SBC induction, TL-01 was more efficient than TL-12 at UCA photo-isomerization (25). While causing minimal skin injury, this efficient induction of *cis*-UCA formation may relate to the therapeutic efficacy of this source in treating dermatoses with immunological disorders. However, the time required for 50% photoisomerization by a broad-band UV-A-I (340-400 nm) source largely exceeded any therapeutic exposure time *in vivo* (26). Furthermore, when the UVASUN 3000, i.e. a UV-A source with an output from 320 to 460 nm and a maximum at 370 nm, was used *in vivo*, dose-dependent isomerization of *trans*-UCA to *cis*-UCA also occurred under this condition without exposure to UV-B. However, a single dose of, for instance, 20 J/cm^2 or a fractionated one of 2 x 10 J/cm^2 did not affect the extent of *cis*-UCA formation (27).

A simple additive wavelength effect of UV-A-I (340-400 nm), UV-A-II (320-340 nm) and UV-A I + II on *cis*-UCA formation in the skin of BALB/c mice was tested (28). Neither complex wavelength interactions, nor the presence of an endogenous photosensitizer of UCA isomerization was observed in this study (28).

Sunscreens were shown to reduce *cis*-UCA formation in the skin. The penetration characteristics of the sunscreens studied did not influence the reduction of *cis*-UCA formation and the sunscreens with comparable SPF (SPF = Sun Protection Factor), but with broad-spectrum protection

instead of small-spectrum protection, offered the largest reduction in *cis*-UCA formation (29). Another study reported a mean formation of 52% of *cis*-UCA in unprotected skin against 7% and 4% of *cis*-UCA in skin protected with sunscreens of SPF 4 and SPF 10, respectively. When the skin was protected with the physical sunscreen agent titanium dioxide in micronized form, a less effective reduction was found (15% of *cis*-UCA) (30). Effective reduction in *cis*-UCA formation was found using low SPF sunscreens, confirming the obvious relationship between decreasing *cis*-UCA formation and increasing SPFs of sunscreens (31). However, the degree of UV-B-induced immunosuppression in the skin of the hairless mouse correlated neither with the amount of *cis*-UCA formed, nor with the extent of edema formation (32).

4. Quantification of the two UCA isomers

Quantification of UCA isomers was reliably carried out with high-performance liquid chromatography (HPLC), a technique for separation, detection and quantification of compounds in complex mixtures. Under distinct

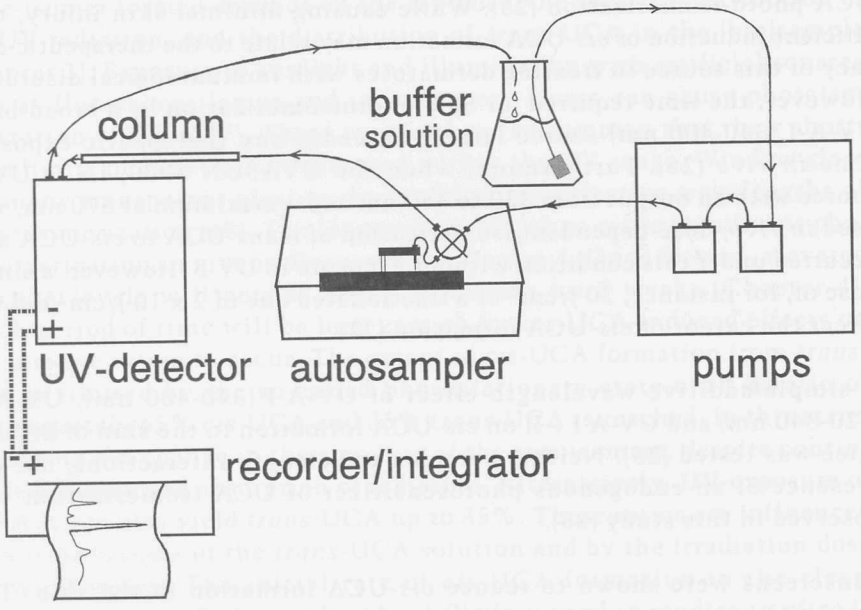


Fig. 6. Schematic representation of the equipment used in high performance liquid chromatography (HPLC).

chromatographic conditions, *trans*-UCA and *cis*-UCA exhibited substantial differences in retention times, especially under reversed-phase conditions with very aqueous eluents containing the ion-pair former tetrabutylammonium(formate) (Chapter 6). The difference might be caused by the ability of *cis*-UCA to form an intramolecular hydrogen bridge (Fig. 2) in the pH range 4-6, which formation does not apply to *trans*-UCA (see section 2). Simultaneous quantification of *trans*-UCA and *cis*-UCA was done in human stratum corneum by the method of tape stripping (33-37), in nails (38), as well as in suction-blister fluid (39). A non-invasive method that was originally called chamber sampling (40) was modified and is described in Chapter 4, together with a method for the quantification of the *trans*-UCA and *cis*-UCA isomers in urine and plasma samples. UCA isomers, together with histidine, were determined according to a recently developed pre-column derivatization method (10); detection limits proved to be dependent on the sample matrix and varied from 5 to 45 pmol. A schematic representation of the HPLC analysis line is shown in Fig. 6.

Besides chromatographic techniques, an enzyme-linked immunosorbent assay (ELISA) was developed for the determination of *cis*-UCA, using a monoclonal anti-*cis*-UCA antibody (41). Cis-UCA levels were demonstrated in UV-B-irradiated mice (either 1 x 2160 or 2 x 1440 J/m^2) as significant competition observed in the ELISA. In serum samples of UV-irradiated mice a *cis*-UCA concentration of 6 µg/ml (43 mM) was indicated, corresponding with 0.1 µg (0.7 nmol)/well. This quantity was regarded as the lowest detectable amount. It was specific for *cis*-UCA, unless *trans*-UCA was present in large quantities (41). In contrast, *cis*-UCA levels in human plasma, obtained after a total-body UV-B exposure of 250 J/m^2 from the same source (Philips TL12), was barely detectable with HPLC and amounted to approximately 0.5 mM (Chapter 4).

5. Epidermal UCA levels

Measurements of skin samples from young healthy individuals of skin type I-IV in Denmark revealed a lower total UCA concentration in July and August than in the rest of the year, irrespective of body site (42). In July, the percentages of *cis*-UCA were close to the maximally obtainable percentage (50-60%) in all body sites except the buttocks. In the three winter months this percentage was < 7% in all body sites. In October and March the mean *cis*-UCA level of the forehead was 18% (42). No consistent relationship was found between UCA isomers and pigmentation or skin type (I-IV) (42), nor was a consistent relationship found between total UCA content and MED, stratum corneum thickness (43), or between sexes (27). The study of skin site variation (44) of UCA content in healthy individuals

revealed the highest levels in the soles of the feet (mean value 29 nmol/cm^2) and the palms of the hands (mean: 20 nmol/cm^2), whereas the lowest level was found in the thighs (mean: 1.5 nmol/cm^2). In the non-UV-exposed controls, the highest levels of *cis*-UCA were found in the hands, both on the dorsum and on the palms (44). When extreme skin types were studied, racial differences were found and expressed as the percentages of UCA in the dry weight of the epidermis, being 0.2% for Caucasoids (skin types II-III) and 0.7% for blacks (skin type VI) (45). No clear-cut differences in total UCA concentration were found between sun-sensitive and sun-resistant skin types (46). However, reduced UCA concentrations were found in lesioned skin of patients with atopic eczema (47), as well as in patients with psoriasis vulgaris (38), ichtyosis vulgaris, hydroa vacciniforme or histidinemia (45) and in patients with discoid lupus erythematosus (48). However, another study (49) reported three times the quantity of UCA in psoriasis patients, compared to that in normal individuals. The *trans*-UCA/histidine ratio, that characterizes the histidase activity in the epidermis, was significantly reduced in atopic eczema and the reduction became larger with increasing age (47). When patients with basal cell carcinoma and malignant melanoma were compared with healthy individuals, a significantly higher level of *cis*-UCA formation was found in both cancer groups, indicating a higher susceptibility to the outgrowth of skin cancer (50). Cumulating suberythemal sun exposure of psoriasis patients who received heliotherapy on the Canary Islands for 4 weeks showed *cis*-UCA levels of 64 to 74% of total UCA. However, the clinical response of psoriasis to heliotherapy is not dependent on UCA isomer levels (51).

6. Photochemical reactions of UCA with possible relevance to photodermatology

Photoisomerization is the most intensely studied photochemical reaction of UCA (see above). However, several other UV-induced conversions were reviewed (52) and worth mentioning here. UCA trans to cis isomerization was shown to occur in the presence of triplet sensitizers, such as biacetyl, naphtalene-2,6-disulfonate, benzophenone and acetone. Apparently, the energy of the UCA triplet state is of the same order as that of these sensitizers (53,54). UV-irradiation of 280-320 nm of UCA in frozen aqueous solution resulted in the formation of the photodimer of UCA (55). When the photodimer was irradiated with 254 nm, monomeric UCA was formed again, among several other products that were not identified. The formation of the dimer could also be demonstrated in guinea pig epidermis after UV-B irradiation; it was extracted with dilute acid and analyzed with thin-layer chromatography (55). Besides dimerization, the *trans*-UCA isomer was capable of covalent binding to bovine serum albu-

min (BSA) *in vitro* during UV-exposure and levels up to 68 nmol were found to bind per mg BSA (56).

Other studies *in vitro* (57,58) show that UV-irradiation ($\lambda > 275$ nm) can transform the UCA molecule to its excited state (UCA*) and evidence was presented that nitro blue tetrazolium (NBT^{2+}) and O_2 can oxidize both the UCA ground state and the excited state. UCA* , NBT^{2+} and O_2 can react to yield superoxide anions (57). UCA was also shown to undergo oxidative cleavage to imidazole-4-carboxaldehyde and glyoxylic acid in the presence of O_2. The reaction was accelerated in the presence of purines, e.g. adenine, and it involved the formation of the UCA radical cation that reacts with O_2. An intermediate dioxetane was proposed, which molecule will yield the aldehydes upon cleavage (58).

If UCA would efficiently bind to DNA under UV-exposure, the resulting product in living cells may obviously initiate important biological reactions. The UV-induced formation of UCA: DNA adducts were indeed reported *in vitro* with UV-doses that also produced *cis*-UCA, and UV-C radiation was found to be more efficient than UV-B (59). However, UV-exposures on cell cultures with the UCA isomers did not provide evidence for direct binding of either UCA isomer to DNA, when the UV-doses were limited to permit cell survival (60). Another demonstration of the inability of UCA to photobind to DNA of living cells was given with mice: immunosuppressive UV-B doses were unable to induce photobinding of UCA to DNA (61).

When DNA in which UCA adducts and pyrimidine dimers were formed was studied (62) with DNA photolyase, release of UCA was not detected, which finding suggests that the reversal of UV-induced immunosuppression by photolyase does not involve repair of UCA-DNA adducts. Apparently, only repair of pyrimidine dimers contributed to the reversal of UV-induced immunosuppression by photolyase (62). Consequently, photobinding of UCA to DNA *in vivo* does virtually not occur and the few possible adducts seems to have no photobiological significance.

Another interaction between UCA and DNA was the induction of pyrimidine dimers and other kinds of DNA damage via a mechanism of photosensitization (63). However, the biological significance of this effect is questionable, since these authors used a 308 nm laser source instead of a solar simulator and the results were obtained *in vitro*.

Upon UV-irradiation, UCA can decompose *in vitro* in the presence of oxygen, which effect can be minimized by the presence of the singlet oxygen

quencher sodium azide (58). This finding indicates that UCA can generate singlet oxygen and also can react with it, at least *in vitro*. For the situation *in vivo* it was speculated that epidermal UCA may be one of the chromophores that induces formation of singlet oxygen when the skin has been irradiated with UV-A, because it was found (64) that the action spectrum *in vitro* for singlet oxygen generation from UV-A-irradiated UCA resembled the UV-A action spectrum *in vivo* for photoaging of murine skin. This speculation remains questionable, since the skin contains several identified UV-A photosensitizers, and most probably many more unidentified ones, that may produce a 'photo-aging-like' action spectrum. To summarize, UCA is an intriguing substance in photochemistry with several photoreactions studied extensively *in vitro*, however, it is not clear how these phenomena relate to immunosuppression *in vivo*, except the lack of relation with UCA: DNA adducts. The interaction of UCA with hydroxyl radicals, the formation of UCA oxidation products and the biological relevance are reported and discussed in Parts II and III of this thesis.

7. Oxidative stress and the skin

Oxidative stress is the exposure of tissue to relatively high concentrations of oxygen radicals and/or other radical species. Oxygen radicals may generate an array of secondary radical reactions, and both kinds of radical can cause tissue damage. Singlet oxygen, superoxide anions, hydrogen peroxide and the very reactive hydroxyl radicals are together named the reactive oxygen species (ROS). An overview of ROS is given in Fig. 7a-d.

The epidermis is in contact with ambient air (21% O_2), and contains the highest O_2 concentration compared to that in other tissues. This circumstance alone already contributes to higher concentrations of ROS. Moreover, the epidermis is exposed to insults caused by the environment, including exposure to solar or artificial UV, SO_2, O_3, air pollution, creams, lotions, detergents, deodorants, waterborne toxins (e.g. chlorine), dissolved metal ions and products of bacteria. Higher epidermal levels of ROS do not only damage the integrity of molecular structures, they induce molecular alterations as well. In this context, a limited amount of research work has been done to elucidate ROS-induced alterations of epidermal components with their eventual biological significance, such as immunomodulation.

Oxidative stress in the skin is a risk factor for skin cancers and a contributor to aging of the skin. The most obvious source is solar UV, of which the UV-component from 290 to 400 nm is accepted as being the most damaging portion of the solar spectrum. Dermal photo-aging comprises wrinkling, loss of elasticity, increased skin fragility and slower wound healing (65).

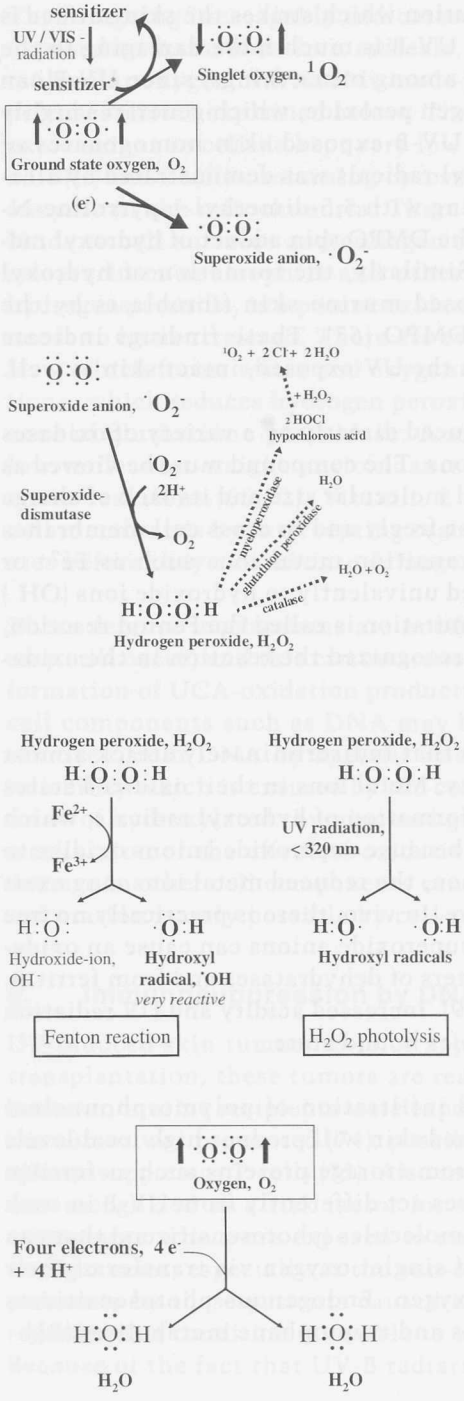


Fig. 7. The reduction of ground state oxygen to water.

a. The formation of singlet oxygen and superoxide anions; the latter by a one electron transfer.

b. The subsequent formation of hydrogen peroxide by dismutation of 2 superoxide anions particles and several ways for removal of hydrogen peroxide.

c. Formation of hydroxyl radicals from hydrogen peroxide: with the transition metal Fe without UV radiation (left) and with UV radiation (right).

d. The overall reduction of one molecule oxygen to two molecules of water, a reduction that requires 4 electrons for transfer.

Approximately 90% of the UV-B radiation which strikes the skin surface is absorbed by epidermal components. UV-B is much more damaging to the skin than UV-A at equal exposures, among other things, since UV-B can cause the homolytic fission of hydrogen peroxide, which generates highly reactive hydroxyl radicals (65). In UV-B-exposed skin homogenates of guinea pig, the appearance of hydroxyl radicals was demonstrated by electron spin resonance (ESR) spintrapping with 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), and the formation of the DMPO spin adduct of hydroxyl radicals (DMPO.OH) was shown (66). Similarly, the formation of hydroxyl radicals was detectable in UV-exposed murine skin fibroblasts by the ESR-spin trapping technique with DMPO (67). These findings indicate the formation of hydroxyl radicals in the UV-exposed, intact skin as well.

Hydrogen peroxide in tissues is produced directly by a variety of oxidases and by dismutation of superoxide anions. The compound must be viewed as a hazardous species, because its small molecular size and its lack of charge at physiological pH allow it to diffuse freely and to cross cell membranes (65). When it encounters reduced transition metal ions such as Fe^{2+} or Cu^{+}, hydrogen peroxide can be reduced univalently to hydroxide ions (OH^{-}) and hydroxyl radicals ($^{\bullet}OH$), This dismutation is called the Fenton reaction, named after H.J.H. Fenton who first recognized the reaction in the oxidation of tartaric acid in 1894 (68).

Hydroxyl radicals are radical species that indiscriminately attack almost every biomolecule in its close vicinity. Metal ions in their oxidized states can also indirectly contribute to the formation of hydroxyl radicals, which can be reduced by superoxide anions because superoxide anions oxidize to normal, ground-state oxygen (O_2). Then, the reduced metal ions may exert the Fenton reaction as described above. In vivo, there is practically no free iron and copper available. However, superoxide anions can cause an oxidative release of Fe^{2+} from [4Fe-4S] clusters of dehydratases and from ferritin, the iron-storage protein of the skin (69). Increased acidity and UV radiation are also capable of releasing Fe from its bound state.

Respiratory bursts of UV-B-induced infiltration of polymorphonuclear leukocytes and macrophages in inflamed skin will produce high local levels of superoxide that can release iron from storage proteins such as ferritin (70). In general, the longer UV-A waves act differently from UV-B in such a way that they can excite certain biomolecules (photosensitizers) that can in their turn induce the formation of singlet oxygen via transfer of their excitation energy to ground state oxygen. Endogenous photosensitizers include porphyrins, flavins, melanins and tryptophane metabolites (71).

The foregoing inescapably indicates that human beings are continuously exposed to hazardous free radicals, derived directly or indirectly from molecular oxygen. Survival is made possible by a multilayered system of defenses that is explained below. The steady-state concentration of superoxide anions in cells is kept very low by two superoxide dismutases (SOD): a Cu(II) and Zn(II)-containing cytosolic enzyme and an Mn(II)-containing enzyme in the mitochondria. There is also an extracellular SOD, which binds to cell surfaces and collagen and which controls superoxide anion release from neutrophils and other cell types. The other type of ROS, hydrogen peroxide, is a potent oxidant (see above; the Fenton reaction) and needs to be neutralized. There are two mechanisms for its removal: catalase, which forms water and oxygen from hydrogen peroxide, and gluthatione, which reduces hydrogen peroxide to water under the catalytic influence of gluthatione peroxidase. Another layer of defense is provided by low-molecular-weight antioxidants that serve to interrupt free-radical chain reactions (65,72). Vitamin E (tocopherol-α) serves such a role in cell membranes by intercepting reactive, lipid-derived radical intermediates before they can initiate damage.

These defense mechanisms are not foolproof, and some oxidative damage may still occur, and this circumstance can lead, among other things, to the formation of UCA-oxidation products (Chapter 6). In other cases, repair of cell components such as DNA may be required. Oxidized DNA bases are removed from DNA by specific endonucleases that initiate repair, the reliability which is ensured by the complementary strand. The excretion in urine of hydroxylated thymine and guanine cleaved from damaged DNA is a reflection of the effect of oxidative stress on DNA (73). The oxidative damage to other cell components cannot be repaired and will be definitive; restoration is only possible by cell renewal.

8. Immunosuppression by DNA or UCA

UV-induced skin tumors in mice appear to be highly immunogenic. Upon transplantation, these tumors are readily rejected by the recipient. When, however, murine recipients were exposed to UV, immunological rejection of the tumor was prevented (74), possibly by recruitment of antigen-specific splenic suppressor T-cells (75). Also in man, UV-induced immunosuppression was recognized as a risk factor for skin cancer (76). Patients with organ transplants who were exposed to sunlight showed a higher incidence of malignancies, especially at the sun-exposed sites. The use of immunosuppressants by these patients caused an immunosuppressive state which is regarded as a condition favorable to outgrowth of malignancies (77).
Because of the fact that UV-B radiation cannot penetrate deeper than the

epidermis (78), it was proposed (79) that UV-B-induced immunosuppression acts via indirect pathways. A number of possible mechanisms were suggested, each including the notion that immunosuppression is initiated by a specific photoreceptor molecule present in the epidermis. One such candidate molecule is DNA (80-82). UV-induced DNA damage in murine keratinocytes caused immunosuppression *in vivo* and the release of cytokines *in vitro* (83).
One type of DNA damage is caused by UV-induced dimerization of the pyrimidine bases thymine and cytosine. The suppressive effect of pyrimidine dimers can be substantially reversed through removal of the dimers by the photoreactivating enzyme (see below) (80,83) or by treatment with T4 endonuclease V (82). As stated before (Section 6), in mice only the repair of pyrimidine dimers reduced the UV-induced immunosuppression and the occurrence and repair of UCA:DNA adducts were not relevant.

Another approach to test the relationship between repair of pyrimidine dimers and UV-induced immunosuppression was obtained by experiments on the Monodelphis domestica (South American opossum) which has a special DNA-repair enzyme for UV-induced pyrimidine dimers that can be activated upon exposure to visible light, the so-called photoreactivating enzyme. When the opossum was irradiated with visible light after but not before UV exposure, the extent of immunosuppression was reduced by the action of the photoreactivating enzyme, and repair of pyrimidine dimers proceeded (84).

Another candidate photoreceptor molecule is epidermal *trans*-UCA that is converted into *cis*-UCA upon UV-irradiation. The first indication came from the observation that *in vitro* the absorption spectrum of *trans*-UCA and the action spectrum of UV-induced immunosuppression of CHS in mice resembled each other (85). Cis-UCA was subsequently shown to mimic at least some of the effects of UV-induced immunosuppression in various experimental systems. For example, *cis*-UCA suppressed CHS responses (86,87) and delayed-type hypersensitivity (DTH) responses (88,89), and delayed the rejection of transplant allografts (90).
Topical application of *trans*-UCA on hairless mice enhanced the UV-induced tumor yield and malignancy (91), because the UV-induced photoisomerization of *trans*-UCA may have exposed the mice to excessive amounts of *cis*-UCA. In other words, topically applied *trans*-UCA, followed by UV-induced isomerization into *cis*-UCA, resulted in augmentation of UV-photocarcinogenesis in mice (91).

In men, UCA isomers were also used cosmetically as skin conditioners and as natural sunscreening agents (92). However, detrimental effects by *cis*-

UCA-induced immunosuppression do not allow its use in sunscreens (93,94). Moreover, the ability of *trans*-UCA to act as a UV-B absorber (UV-B filter) to reduce erythema formation in the skin was weak (95).

9. Immunological aspects of UCA

UV-exposure of the skin is known to cause photochemical alterations in the epidermis with the formation of non-self antigens, the photoantigens. *Cis*-UCA may have been selected in the course of the evolution to exert suppressive effects on the skin immune system, to suppress an uncontrolled autoimmune reaction. The majority of *cis*-UCA studies have been carried out in the last two decades and often used mice as a model system or used cell culture systems. A limited amount of studies were done in human subjects (see below). Studies that focussed on UV-induced immunosuppression but did not include *cis*-UCA-induced suppression were largelyomitted.

9.1. Suppression of contact hypersensitivity

The demonstration of congruence between the absorption spectrum of *trans*-UCA (*in vitro*) and the spectrum of UV-induced suppression of CHS in mice provided a first indication that *trans*-UCA might be the photoreceptor for UV-induced suppression of skin immune functions (85,86). Further studies showed that immunosuppression could be obtained with *cis*-UCA alone, formed from *trans*-UCA by the process of photoisomerization during UV-exposure (see Section 3). Some studies used UV-irradiated *trans*-UCA as test material (UV-UCA) (96,97), while others used chromatographically processed *cis*-UCA with a purity of 98 to 100% (see appendix I) to demonstrate *cis*-UCA-induced suppression of immune functions *in vivo* or in cell culture *in vitro*, and used *trans*-UCA as a control. UV-UCA can contain *cis*-UCA up to at least 60%.

The induction of CHS can be local or systemic; the latter procedure includes the application of the contact sensitizer on another skin test site than the immunosuppressive agent, e.g. UV or *cis*-UCA. CHS was induced in experimental animals using hazardous hapten-forming compounds as contact sensitizers. Frequently used sensitizers are trinitrochlorobenzene (TNCB) (85), its fluoro-analagon DNFB (97), oxazolone (98) and picryl chloride (99). They were applied to the animal skin most often dissolved in acetone. Three to five days later, the same sensitizer was applied to the ear(s), causing ear swelling which was normally recorded one day after this elicitation. When the animal was UV-irradiated prior to sensitization, reduced ear swelling could be demonstrated in the elicitation phase. The suppression was more marked in UV-sensitive strains of mice,

such as C3H/HeN mice. Evidence was collected to attempt to prove that *trans*-UCA was the photoreceptor. First, when the dorsal surface of the mouse skin was removed by four tape-strippings immediately before UV irradiation, no significant reduction in ear swelling could be demonstrated (100). A reciprocal experiment was carried out with histidine-fed mice that showed increased UCA levels after several days on this diet and, indeed, enhanced UV-B- induced immunosuppression in the CHS model was found in these mice (101). Moreover, when a mouse strain that was genetically deficient in histidase, and therefore also deficient in epidermal *trans*-UCA (< 10% of normal wild type), was used in the CHS model, no UV-B induced immunosuppression could be demonstrated (100).

Another study used three separate techniques to remove UCA from the skin of C3H/HeNCR mice and did not find a significant role for *cis*-UCA in immunosuppression. Tape-stripping, washing with water and the use of a depilatory cream reduced the amount of epidermal UCA by 83, 35 and 20%, respectively (102). Similar levels of immunosuppression were obtained after elicitation with TNCB, whether or not UCA was present in the UV-exposed murine skins. It must be noted, however, first that sufficient *trans*-UCA might have been left over to isomerize into *cis*-UCA and, second, that a different type of UV-source was used, emitting 90% of the UV at 254 nm.

Later, a monoclonal anti-*cis*-UCA antibody was developed and used in the CHS model of UV-induced immunosuppression (103), but no significant reduction of immunosuppression could be demonstrated. In contrast, when the antibody was used in the UV-induced DTH, a significant reduction in immunosuppression was observed (see Section 9.2 for DTH) (103).

Generally, it is believed that *cis*-UCA can mimic the effect of UV-B in the CHS model when UV-susceptible mouse strains are used with UV-B radiation sources, such as the Philips TL12 and the Sylvania FS20 or FS40 lamps. In recent years, it became clear that UV-B radiation sources that simultaneously emit high levels of UV-A, such as solar simulator lamps, reduce or abrogate the *cis*-UCA effects on CHS responses, at least in the hairless mouse (104). It was shown that UV-A can protect against immunosuppression, either induced by UV-B or *cis*-UCA (section 9.7). The potential of *cis*-UCA as an anti-inflammatory agent was investigated and both induction and elicitation of contact hypersensitivity to contact allergen oxazolone could be suppressed when particular timing was accounted for. *Cis*-UCA failed to show significant suppression when the respiratory allergen trimellitic anhydride was used. Also croton oil irritation could not be suppressed (105).
Unfortunately, several studies that used the CHS model, have used dif-

ferent regimens within the conditions mentioned above, and comparisons are difficult to make. Optimization and fine-tuning of the CHS model for local immunosuppression was investigated to increase the degree of immunosuppression in C3H/HeN mice (106). Repetitive UV-B doses should be applied in stead of a single dose (e.g. 4 x 250 J/m^2 in stead of 1 x 1000 J/m^2), sensitization should take place 2 days after UV-B, cumulative UV-B doses should have stepwise increments, a decreased amount of sensitizer (DNFB in this study), a larger sensitization area should be used and, last but not least, the optimization requires young mice (< 8 weeks)(106).

The UV-induced suppression of CHS, but not the *cis*-UCA-induced suppression was also studied in human subjects, although, of course, investigational procedures were limited (107-110). UV-induced suppression of CHS to topically applied haptens could be demonstrated (107,109,110). In contrast to the results in mice, in human subjects simulated solar radiation (including UV-B and UV-A) was able to exhibit substantial suppression of the CHS response (110). In this study ultrasound image data were used to evaluate the inflammatory responses after elicitation, but genetic traits for UV-susceptibility and UV-resistance could not be distinguished among the tested individuals (107, 108, 110), in contrast to the findings with mice. Similar to the results of studies in mice, an impairment of the number and function of Langerhans cells (LC) was found in humans after UV-B exposure (107, 109, 110). Cis-UCA must have been formed on the irradiated human test sites, but to prove that *cis*-UCA is mimicking UV in this respect is difficult and might be unethical.

9.2. *Suppression of delayed-type hypersensitivity*

Delayed-type hypersensitivity (DTH) against herpes simplex virus (HSV) in mice was also used as a model to evaluate the effects of the two UCA isomers (88). Mice were sensitized by subcutaneous injection of HSV type 1 and the mice were tested for DTH 8 to 10 days after sensitization. Elicitation was carried out by injection of the UV-inactivated virus into each ear and ear swelling was measured after 24 h. The site at which the *cis*-UCA isomer (pure, or with *trans*-UCA) was administered, was also the site of the sensitizing dose of HSV-1. Smaller amounts of *cis*-UCA, as well as lower UV-irradiation doses, were required to suppress the DTH response to HSV in the C3H/HeN mouse strain than the amount needed for significant suppression in CHS (111). However, the degree of suppression did not correlate with the amount of *cis*-UCA formed upon UV-irradiation (111). As mentioned in the previous paragraph, anti-*cis*-UCA antibodies were able to reduce UV-induced suppression of DTH responses in mice (103). The immunosuppression in the HSV-1 model was associated with the appearance of

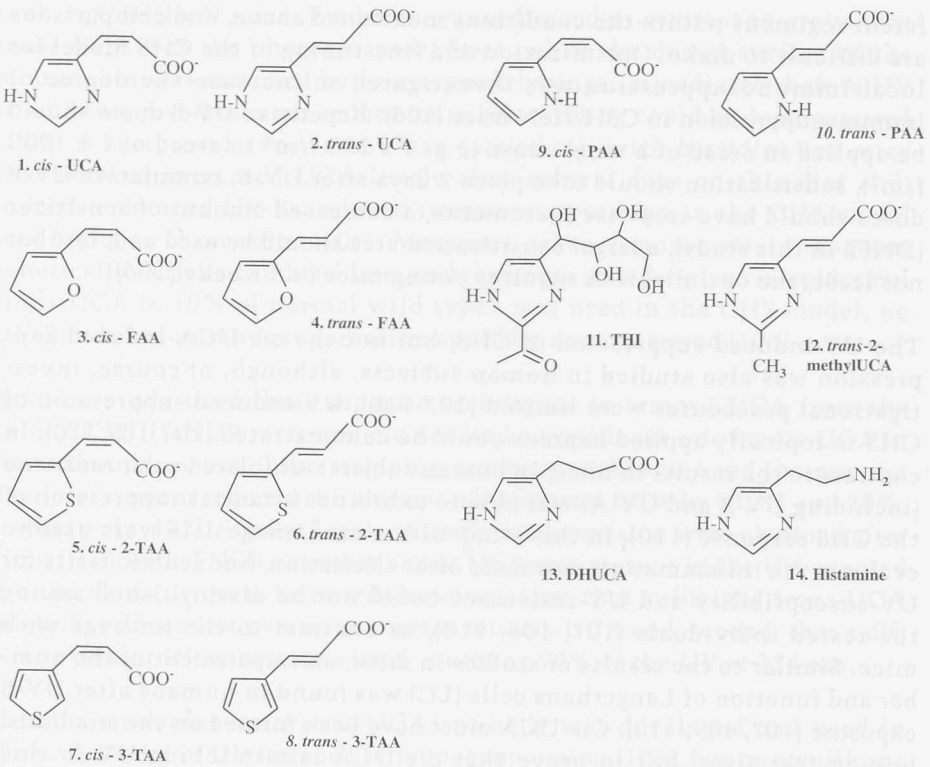


Fig. 8. Structural analogs of UCA, tested for biological activity. 1. *cis*-UCA, 2. 2-acetyl-4-(tetrahydroxybutyl)imidazole (THI), 3. *trans*-2-methylUCA, 4. *trans*-2-furylpropenoic acid, 5. *cis*-2-furylpropenoic acid, 6. *trans*-2-pyrrole propenoic acid, 7. *cis*-2-pyrrole propenoic acid, 8. dihydrourocanic acid (DHUCA), 9. histamine, 10. *trans*-2-thiophene propenoic acid, 11. *cis*-2-thiophene propenoic acid, 12. *trans*-3-thiophene propenoic acid, 13. *cis*-3-thiophene propenoic acid, 14. Histamine.

2 phenotypically distinct T cells ($Thy1^+$, $L3T4^-$, $Ly2^+$) and ($Thy1^+$, $L3T4^+$, $Ly2^-$). This situation parallels the generation of 2 distinct T-suppressor cells for HSV-1 by UV-irradiation in mice and, therefore, provides further evidence for the involvement of UCA in UV-B immunosuppression (112).

The molecular structure of *cis*-UCA is not unique in causing suppression of DTH response to HSV, which finding was proven by testing specially synthesized structural analogs for their ability to suppress DTH to HSV

Table 2. UCA analogs and DTH response.

Compound name (see Fig. 8 for structures) 1 µg/mouse	Abbreviation[1] (as used in Fig. 8)	Biological activity percent suppression of DTH (113)
• *cis*-urocanic acid	*cis*-UCA	72
• *trans*-urocanic acid	*trans*-UCA	Not given
• *cis*-(furan-2-yl)propenoic acid	*cis*-FAA	45
• *trans*-(furan-2-yl)propenoic acid	*trans*-FAA	19
• *cis*-(thiophen-2-yl)propenoic acid	*cis*-2-TAA	29
• *trans*-(thiophen-2-yl)propenoic acid	*trans*-2-TAA	18
• *cis*-(thiophen-3-yl)propenoic acid	*cis*-3-TAA	28
• *trans*-(thiophen-3-yl)propenoic acid	*trans*-3-TAA	53
• *cis*-(pyrrol-2-yl)propenoic acid	*cis*-PAA	68
• *trans*-(pyrrol-2-yl)propenoic acid	*trans*-PAA	46
• *trans*-2-methylUCA	-	21
• dihydrourocanic acid; 3-(imidazol-4-yl)propanoic acid	DHUCA	85
• histamine, 2-(imidazol-4-yl)ethylamine	-	67
• 2-acetyl-4-(tetrahydroxybutyl)-imidazole	THI	58(114)[2]

[1] propenoic acid is current nomenclature for acrylic acid (AA)
[2] percent suppression of CHS response to DNFB.

(113). Their molecular structures are represented in Fig. 8 and their biological activities are summarized in Table 2. Cis-(pyrrol-2-yl)-propenoic acid was as immunosuppressive as *cis*-UCA, but the *trans*-isomer was less effective. The compounds (furan-2-yl)propenoic acid, (pyrrol2-yl)propenoic acid and (thiophen-2-yl)propenoic acid showed appreciable suppression, their *cis*-isomers did so in a more prominent way, whereas the *cis* and *trans*-isomers of (thiophen-3-yl)propenoic acid showed opposite behavior to each other. DihydroUCA was at least as suppressive as *cis*-UCA, and histamine showed appreciable suppression as well. However, the *trans*- and *cis*-isomers with the addition of a methyl group at the two position of the imidazole-ring (2-methyl-UCA) did not show substantial suppressive effects.

One of the constituents of the food colorant ammonia caramel, 2-acetyl-4-tetrahydroxybutylimidazole (THI), which is known to cause lymphopenia in rats, is a potent immunosuppressant of the CHS model (114). A hetero-

cyclic 5-membered molecular ring is a common feature of the compounds listed here, and seems to be essential for immunosuppressive activity. The molecular side-chain at the 4-position may vary considerably. The influence of substituents at the 2-position on the suppressive potential is not clear, when comparing 2-methylUCA (21% suppression of DTH) and THI (58% suppression of CHS). Recently, other 5-membered, heterocyclic compounds were tested in a different way for their biological activities (115) (see Section 9.4).

9.3. Suppression in various models in vivo

UV-B lowered the immune responses to *Trichinella spiralis* after oral infection of rats with the parasitic worm (116). In animals treated with *cis*-UCA the number of T. spiralis larvae in muscle tissue of infected rats was significantly increased and DTH to T. antigen was significantly impaired compared with the rats treated with *trans*-UCA. When the anti-*cis*-UCA monoclonal antibody was injected prior to UV-B exposure, both the UV-B induced suppression of DTH to T. spiralis and the increase in larvae counts were significantly reduced (116).

Treatment of skin grafts with UV-B light (119) or PUVA (118-120), leads to their prolonged survival in allogeneic recipients. Because of its assumed role in mimicking the UV-irradiation effects, *cis*-UCA was studied in murine models of skin transplantation and in murine models of acute graft-versus-host disease. A mix of *cis*-UCA and *trans*-UCA (UV-UCA), but not *trans*-UCA alone, was able to prolong the survival of allogeneic MHC disparate skin grafts and prevented or delayed acute lethal graft-versus-host disease (90). In an another study using rabbits the mean survival time of orthotopic corneal grafts was prolonged from a mean value of 22 days for *trans*-UCA to 29 days for UV-UCA, whereas a salt solution (negative control) had a mean survival time of 20 days (121).

9.4. Involvement of cytokines

UV-B radiation reduces the induction of CHS in mice by converting *trans*-UCA to *cis*-UCA within the epidermis, and *cis*-UCA in turn causes local release of TNF-α. The most pronounced effects were seen in UV-B-susceptible mouse strains (122). The suppressive effect of UV-B on the CHS model was suggested to follow the sequence: UV-B→*trans*-UCA→*cis*-UCA→upregulation of TNF-α→TNF-α-induced changes in the LC cytoskeleton, followed by functional defects. This was, however, questioned by others studying the effects of broad-band and narrow-band UV-B sources. The lack of correlation between *cis*-UCA formation and TNF-α production

was demonstrated by irradiation of mice with broad-band and narrow-band UV-B lamps. Broad-band irradiation causes the formation of *cis*-UCA and TNF-α, whereas narrow-band irradiation (311 nm) only causes the formation of *cis*-UCA and not of TNF-α (123). This finding is in remarkable contrast to the statement at the beginning of this paragraph.

Another discrepancy is the *cis*-UCA-induced reduction in TNF-α levels in LPS-stimulated human monocytes culture (124), but at this stage, it is impossible to draw general conclusions concerning the role of TNF-α in *cis*-UCA induced immunosuppression.

Mouse strains differ from each other in their ability to respond to suppression of CHS induced by *cis*-UCA (and UV). UV-B susceptibility is genetically determined by the polymorphic alleles at the TNF-α and Lps loci (125). Sensitization with DNFB at the same site as *cis*-UCA administration and challenge with DNFB by ear painting 5 days later resulted in a more marked reduction in ear swelling in the *cis*-UCA-sensitive strains (125).

Antibodies directed against TNF-α only abrogated the UV-induced, but not the *cis*-UCA induced suppression of DTH. The latter finding is in contrast to that with *cis*-UCA-induced suppression of CHS, in which model anti-TNF-α antibodies induced abrogation of both the UV-induced and the *cis*-UCA-induced suppression (123). These effects are highly dependent on the mouse strains used, but demonstrate again a possible role for TNF-α in immunosuppression.

IL-10 production by mouse CD4+ T cells from cultured spleen cells was enhanced upon treatment with *cis*-UCA (128). It was suggested by the authors that the elevated production of IL-10 by activated CD4+ T cells may account for the suppressor T-cell phenomena described in UV-irradiated animals that were recipients of highly antigenic UV-induced skin tumors (126). *Cis*-UCA-pretreated spleen cells had a reduced ability to stimulate proliferation of allogeneic cells or to respond by proliferation in a mixed lymphocyte reaction (MLR). These considerations emerged because the immunosuppressive effects could be blocked by neutralizing anti-IL-10 monoclonal antibodies. IL-10 was suggested to be an effector compound, mediating UCA induced immunosuppression, in these murine studies (127).

Recently, another group of UCA analogs was synthesized and tested for their ability to stimulate human blood monocyte PGE_2 levels and to sup press LPS-induced TNF-α levels. Synthesized oxazole and thiazole analogs of UCA (Fig. 9) showed considerable activities *in vitro* (115), as presented in Table 3. Their activities were compared to that of *cis*-UCA. Remarkably,

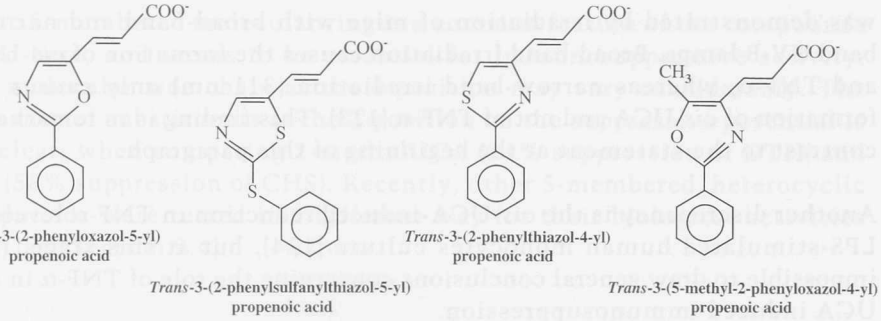


Fig. 9. Oxazole and thiazole analogs of UCA.

the *trans*-isomers of these analogs acted in the same way as *cis*-UCA, although with different strengths, and appeared to be synergistic with *cis*-UCA. In contrast, the *cis*-isomers of the oxazoles and thiazoles were antagonistic to the effect of their *trans*-isomers and to *cis*-UCA. The 2-phenyl-oxazole-analog (Fig. 9) was more potent than the 2-methyl-analog in the two test models *in vitro*. So, it was concluded that the necessary functionality at C-2 of the 5-membered ring was a phenyl-group, suggesting that hydrophobicity plays a role in the activity of each respective compound. The oxazole and thiazole *trans*-isomers, as well as *cis*-UCA, were suggested to be anti-inflammatory agents (115).

9.5. *Histamine receptors*

UCA is metabolically related to histamine: both are formed in one step from histidine (Fig.5). Several structural analogs of UCA have been tested to search for a similar suppressive effect to that of *cis*-UCA and the data obtained indicate involvement of histamine receptors (128,129). In this context, cimetidine (an H2-receptor antagonist) and terfenadine (an H1-receptor antagonist) were tested in the model of DTH response in the mouse. Both pharmaca were able to reduce or abrogate the *cis*-UCA-induced immunosuppression as was measured by ear swelling and number of $ATPase^+$ cells (129). Thioperamide, an H3-receptor antagonist, could not reverse the suppression of the DTH response (129). These results indicate that *cis*-UCA may act through histamine H1 receptors and histamine H2 receptors in the skin. However, in rat cortex membranes, UCA was found to bind to GABA receptors instead of histamine receptors H1 and H2 (130). The relevance of these observations to GABA-like receptors of the skin is presently unknown.

Table 3. UCA analogs, TNF-α and PGE_2.

Compound name (see Fig. 9 for structures)	Biological activity (115) % of control; control = 100 %	
	TNF-α	PGE_2
• *cis*-urocanic acid	62	1105
• *trans*-urocanic acid	102	64
• *trans*-3-(2-phenyloxazol-5-yl) propenoic acid	39	782
• *trans*-3-(2-phenylsulfanylthiazol-5-yl) propenoic acid	83	691
• *trans*-3-(2-phenylthiazol-4 yl) propenoic acid	82	410
• *trans*-3-(5-methyl-2-phenyloxazol-4-yl)propenoic acid	34	265

Alternatively, the release of TNF-α may be regulated via histamine-like receptors in the skin, through which UCA acts. Cis-UCA and histamine were shown to augment the TNF-α- mediated expression of intercellular adhesion molecule-1 (ICAM-1) on keratinocytes (131), comparable to the effect of UV-B on TNF-α-mediated expression. Another keratinocyte study showed a *cis*-UCA-induced reduction of the effects of histamine on intracellular calcium levels and cyclic AMP levels (132).

The relative importance of different mechanistic pathways may be determined by differences in experimental set-up. Differences in mouse strain, contact sensitizer, way of *cis*-UCA administration and the time intervals of experimental steps in CHS induction may explain the different results. Because *cis*-UCA and histamine were described to have differential effects on monocytes (124), keratinocytes (133) and fibroblasts (134), the involvement of histamine-like receptors is less likely. It is too simplistic to expect a single mechanism of immunosuppression through which *cis*-UCA acts in the skin.

9.6. *Suppression of various models in vitro*

It was already shown in 1981 that UV irradiation of murine skin was associated with defective antigen presentation (135). Morphological changes in Langerhans cells (LC) were caused by UV-irradiation, by TNF-α, and by *cis*-UCA as well. All three agents caused a reduction in expression of vimentin within the cytoplasm of LC (136). When these effects were mimicked in the CHS model with vinblastine (an agent that disassembles microtubules) in UV susceptible mice (C3H/HeN), substantial suppression of ear swelling resulted. It was concluded that LC were knocked out to carry out their role

Table 4. *Cis*-UCA effects on different human cells *in vitro*.

Human cell type	Effect of *cis*-UCA	Reference
Peripheral blood lymphoid cells and autologues T-cells	HLA-DR expression ↓ CD4/CD8 ratio ↓ IL-1 production ↓ IL-2 production ↓	Rasanen, 1989 (143)
LPS-stimulated monocytes	TNF-α production ↓ (indirectly) PGE_2 production ↑	Hart, 1993 (124)
Dermal fibroblasts after stimulation with *trans*-UCA or histamine	cAMP production: ↓	Palaszynski, 1992 (144)
Epidermal cell suspension	IL-1 production ↓ HLA-DR expression ↓	Rasanen, 1987 (145)

as antigen-presenting (AP) cells (136). Intradermal injections of *cis*-UCA into C3H/HeN mice led to a substantial reduction in LC 5 hours later. Splenic dendritic cells of mice, taken 7 days after UV irradiation or *cis*-UCA treatment, showed an impaired AP function. However, when taken after 3 days, no effect on AP cell function was observed, indicating a time delay in the generation of the AP defect (137).

Treatment of mice with anti-TNF-α antibodies before *cis*-UCA application resulted only in a small loss of LC numbers (123). However, treatment with a monoclonal antibody to *cis*-UCA prevented the UV-induced changes in LC and DTH responses in mice, but the antibodies did not prevent UV-induced suppression of CHS, as stated before (Section 9.1). Accumulation of dendritic cells in lymph nodes draining the site of irradiation was also not prevented (138).

Cis-UCA has a weak effect on the downregulation of human epidermal antigen-presenting activity. When human epidermal cells in culture were treated with *cis*-UCA before testing their activity as stimulator cells in 'a mixed epidermal cell lymphocyte reaction' (MECLR), 20% suppression was the result (139). Prolonged incubation with *cis*-UCA resulted maximally in a suppression of 27%, but *trans*-UCA had no effect. When *cis*-UCA was used in 'a mixed lymphocyte reaction' (MLR), no effect was seen (139).

Table 5. Overview of studies that showed no effect of *cis*-UCA, while UV irradiation did have an effect.

Although under many different UV irradiation conditions *cis*-UCA can be generated from *trans*-UCA by photoisomerization, this UCA isomer can not mimic every UV-effect on immunomodulation.

Results / Test system	Reference
No inhibition of mitogen-induced lymphocyte transformation in man.	Higaki, 1986 (146)
No inhibition of proliferation of splenic dendritic cells.	Noonan, 1988 (137)
No inhibition *in vitro* of human Langerhans cell function.	Rattis, 1995 (147)
No alteration *in vitro* of development of murine dendritic cells.	Lappin, 1997 (148)
No change in numbers of dendritic cells in draining lymph nodes of mice.	Moodycliffe, 1992 (149)
No effect on cytokine synthesis by keratinocytes.	Redondo, 1996 (150)
No effect on cytokine synthesis by keratinocytes and no induction of expression of the HIV promotor.	Yarosh, 1992 (151)
No inhibition of NK cell activity, in striking contrast to the finding of another study (142) for ***trans*-UCA**.	Uksila, 1994 (141)
No increase in IL-1β and IL-6 levels of cultured monocytes.	Hart, 1993 (124)
No effect on IL-1β production and on TNF-α production by LPS-stimulated monocytes, while *trans*-UCA showed an effect.	Laihia, 1994 (152)
No effect on the expression patterns of CD80 (B7/BB-1) and CD28 costimulatory molecules of LPS or INF-γ- stimulated monocytes.	Laihia, 1996 (153)

Cis-UCA was shown to suppress human natural killer (NK) cell activity in a dose-dependent manner *in vitro*, while *trans*-UCA had little effect (140). In another study *trans*-UCA, and not *cis*-UCA, reduced the cytotoxic function of NK cells (141). The cell-culture conditions of both studies were not comparable, however, and UCA isomers may affect cell types at various body sites in different ways. No correlation was found between the *cis*-UCA concentration in the skin and the degree of suppression in NK cell activity in a group of psoriasis patients treated with narrow-band UV-B (311 nm) (142). More profound effects of *cis*-UCA were seen on the stimulation of prostaglandin E_2 (PGE_2) production of human peripheral blood monocytes (124) and human keratinocytes, although only in synergy with histamine (133). Other effects of *cis*-UCA on human cell types are summarized in Table 4 and Table 5 summarizes the studies that did not show any effect of *cis*-UCA, while UV irradiation did have an effect on various cell types.

9.7. *Various aspects of cis-UCA-induced immunosuppression*

Mast cells were shown to play a critical role in *cis*-UCA-induced immunosuppression, at least in mice. Mast-cell-depleted W^f/W^f mice were not susceptible to *cis*-UCA-induced immunosuppression (154). However, when their skin was reconstituted with bone-marrow-derived mast-cell precursors, given subcutaneously, a *cis*-UCA-induced reduction in ear swelling could be observed. The authors conclude, also based on previous studies with mast cells, that *cis*-UCA directly or indirectly stimulates mast-cell activation that in turn signals downstream immunomodulatory events. Others showed the suppressive effect of both UCA isomers on the activation of human neutrophils *in vitro* and concluded that the function of UCA may be protective, to suppress the activation of human neutrophils in inflamed, sunburned epidermis (155). In their role as hydroxyl radical scavengers, the UCA isomers may act in this way (Chapter 5).

High quantities of *cis*-UCA persist in the human skin during the summer months. To investigate the effect of persistent high levels of *cis*-UCA on the immune system, mice were injected intradermally with *cis*-UCA and *trans*-UCA three times a week for 4 weeks. The weight of their bodies and spleens were unaffected by both UCA isomers, but a decrease in thymus weight accompanied by an increase in lymph-node weight was detected in the mice treated with *cis*-UCA, as well as a net accumulation of lymphocytes and dendritic cells (DC) in the lymph nodes. However, there was no specific migration or proliferation of a particular subset of cells.

The lymphoproliferative response *in vitro* of lymph-node cells to the mitogen concanavalin A was significantly suppressed by *cis*-UCA treat-

ment. The density of LCs in the epidermis of the ears was not altered by chronic *cis*-UCA treatment at a distant site, but it suppressed the mixed skin lymphocyte reaction (MSLR) of epidermal cells from the ears, indicating systemic suppression (156). The reduction in MSLR must therefore be attributable to a reduction in antigen-presenting function of the LCs.

Remarkably, chronic *cis*-UCA treatment did not suppress the CHS response to oxazolone and the DTH response to HSV in the female C3H/HeN mouse (UV-susceptible) strain used in this study (157). The same authors previously showed that *cis*-UCA (20-200 µg/mouse) was unable to suppress the CHS response to oxazolone, irrespective of topical or subcutaneous administration of *cis*-UCA to C3H/HeN mice (157). In contrast, chronic low UV-B irradiation doses for 6 weeks suppressed the CHS response to oxazolone (158).
In comparison, another study reported that an intradermal injection of 100 µg of *cis*-UCA/mouse suppressed the CHS response to 125 µg DNFB in C57BL/6, C57BL/10 and C3H/HeN mice, but not in BALB/c mice (125). The reported discrepancies may be due to differences in the strain of mouse used and/or the route of administration of *cis*-UCA. UV-B irradiation is known to suppress the CHS response and this effect cannot be blocked by injection of a monoclonal antibody to *cis*-UCA prior to the UV-B exposure (138). *Cis*-UCA and UV-B exposure are both known to suppress the DTH response to HSV in C3H/HeN mice and this effect can be abrogated by a monoclonal antibody to *cis*-UCA (138). These findings indicate a role for *cis*-UCA as an initiator of UV-induced suppression of the DTH response. In contrast, the chronic treatment of *cis*-UCA had no significant effect on the DTH response. It was suggested (158) that redundancy of the immune system and adaptation of the mice to persistent high levels of *cis*-UCA had occurred.

Cis-UCA levels in the human skin vary with the season: they are low in the winter, but increase progressively during spring and summer to reach a maximum in the autumn (42). It was suggested that in the human and animal bodies an adaptation mechanism exists for dealing with prolonged exposure to *cis*-UCA. On the other hand, prolonged exposure of mice to topically applied UCA and repeated UV-B exposure over a period of 10 weeks enhanced tumor yield and degree of malignancy (91). The immunomodulations induced by *cis*-UCA are important in this context, although a constant immunosuppressive effect of persistent, substantial *cis*-UCA levels can be questioned, as described above (158).

UV-A radiation (320-400 nm) has different effects on immunosuppression than UV-B (290-320 nm) and immunosuppression induced by UV-B or *cis*-UCA can even be abrogated by UV-A, at least in mice (104). UV-A irradia-

tion of the skin leads to *cis*-UCA formation, but to obtain significant immunosuppression is not possible (see Sections 9.1 and 9.2) (159). It was subsequently postulated that UV-A exposure may induce an immunoprotective photoproduct that can inhibit the activity of *cis*-UCA. At least heme oxygenase (HO; EC 1.14.99.3) was found to mediate photoimmunoprotection (160). HO is activated by UV-A and in its turn it reduces the degree of UV-B-induced immunosuppression or *cis*-UCA induced immunosuppression. However, when activation of heme oxygenase was prevented by the specific inhibitor tin protoporphyrin (SnPP), the immunoprotective effect of UV-A against either UV-B radiation or *cis*-UCA was abrogated (160). It was suggested that UV-A-induced HO activity plays a major role in the skin defenses against UV-B immunosuppression.
Another factor of the photoimmunoprotection by UV-A was indicated to be interferon-gamma (IFN-γ). INF-γ knock-out mice (IFN-γ -/- mice) were compared with C57/BL6 mice (controls) in the model of systemic contact hypersensitivity. After UV-A irradiation both groups showed normal, full responses, but in response to UV-B irradiation or topical *cis*-UCA treatment both groups were immunosuppressed. If, however, UV-A irradiation was given between UV-B irradiation or *cis*-UCA and the sensitization step, the contact hypersensitivity response was totally restored in the control mice, but remained suppressed in the IFN-γ -/- mice. Injection of recombinant IFN-γ into the IFN-γ -/- mice restored the protective effect of UV-A against *cis*-UCA-induced immunosuppression (161). Restoration of UV-B-induced immunosuppression was not reported.

10. Aims of the studies

From the 1980s up to now, the majority of research data have been derived from models, using the mouse as the experimental animal. Although both mouse and man are mammals, there are differences in the function of the skin, as reviewed above. The majority of the data in this thesis refers to quantititave aspects of *trans*-UCA and *cis*-UCA in humans. Because a distinct division can be made between research on the levels of *trans*-UCA and *cis*-UCA, the discovery of the UCA oxidation products and the biomedical aspects of the UCA oxidation products, this thesis was divided into three parts.

In part I, levels of *trans*-UCA and of *cis*-UCA are reported in the human skin and *in vitro* and it is shown how their levels depend on the process of photoisomerization after UV irradiation with defined spectral distributions (Chapter 2). In connection with the basic study of Chapter 2, we wished to know the long-wave limit of *cis*-UCA formation in the human skin and investigated this in volunteers with skin types II-IV. This knowledge pro-

vides information how to protect optically against *cis*-UCA formation in the skin and how to avoid possible immunosuppression resulting in health defects in the longer term (Chapter 3). The last questions in this respect were: does *cis*-UCA, an intriguing compound in photoimmunology, occur as a common constituent of the human epidermis and does it occur in other parts of the human body as well? In Chapter 4, levels of *trans*-UCA and *cis*-UCA are given for the epidermis, plasma and urine, before and after a single UV-exposure.

Because it became gradually known that several questions remained about the immunological action and working mechanism of *cis*-UCA, we focussed on new aspects concerning the UCA isomers. In part II, we postulated the hypothesis that UCA isomers may react with hydroxyl radicals under conditions of oxidative stress and that they may be converted into oxidized derivatives, that may have biological, or more specifically, immunomodulating functions. The analysis of both UCA isomers in skin samples showed considerable variation, due to aspects referred in Section 5. Therefore, an as yet unobserved part of the UCA isomers may have been oxidized to potent immunosuppressive compounds. If so, this would mean that the UCA isomers themselves will be more or less in an 'off-side' position concerning immunosuppression. We studied the interaction of the UCA isomers with the most reactive oxygen species known, the hydroxyl radical (.OH). The presence of these radicals during oxidative stress in epidermal tissue is strongly indicated.

In Chapter 5, our first question in this respect is answered: are UCA isomers poor, moderate, good or excellent hydroxyl radical scavengers? Comparisons were made with other compounds, of which their scavenging behavior was known from literature, and the UCA isomers were shown to be good scavengers. What will happen after the UCA isomers have scavenged hydroxyl radicals? Chapter 6 outlines the formation of several UCA-oxidation products that were identified with a combination of HPLC and spectrometric analyses. Further confirmation was obtained by comparison with reference oxidation products of UCA.

We hypothesized at this stage of the research, that the UCA oxidation products would show potent immunosuppression and that these compounds, as derivatives of the UCA isomers, are the prime cause of immunosuppression in conditions of oxidative stress in the skin. If so, these findings would alter the concept of *cis*-UCA as immunosuppressant, a view that has been settled now for almost two decades. Part III of this thesis is dedicated to the biological effects of *trans*-UCA and *cis*-UCA and last, but not least, the biological effects of the UCA-oxidation products. In Chapter 7,

the effect of *cis*-UCA was studied on the elicitation of contact allergy with four contact allergens, while *trans*-UCA was included as a control. *Trans*-UCA and *cis*-UCA were topically applied in gel vehiculum and epidermal enrichment of both isomers could be demonstrated. A special interaction with the contact allergen nickel sulfate could be demonstrated.

Chapter 8 describes how the hypothesis in chapter 6 led to evidence. The suppressive effects of UCA-oxidation products are demonstrated in the model of contact hypersensitivity in the mouse and comparisons were made with the suppressive effects of *trans*-UCA and *cis*-UCA. Mice were sensitized with picryl chloride and elicitation was monitored by the amount of ear swelling. The extent of the reduction in ear swelling proved to be a measure for immunosuppression.

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PART I

BIOKINETICS OF THE UROCANIC ACID ISOMERS

CHAPTER 2

Photoisomerization spectrum of urocanic acid in human skin and *in vitro*: effects of simulated solar and artifical ultraviolet radiation

Arthur Kammeyer, Marcel B.M. Teunissen, Stanislav Pavel, Menno A. de Rie and Jan D. Bos

Department of Dermatology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

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Abstract

Ultraviolet (UV) irradiation of *trans*-urocanic acid (*trans*-UCA), a major UV-absorbing component of the epidermis, leads to the formation of *cis*-UCA, which mediates immunosuppressive effects. In this study, the net yield of *cis*-UCA was measured after the photoisomerization of urocanic acid by narrow UV-wavebands (spectral range 295-405 nm), with the irradiation doses related to solar irradiance at sea level. The formation of *cis*-UCA in Caucasian skin (*in vivo*), as well as in aqueous solution (*in vitro*) was determined by HPLC analysis. The same irradiation conditions were met in both components of the study. The *in vivo* experiments showed high efficiency of *cis*-UCA formation in the spectral region of 305-341 nm, whereas high efficiency *in vitro* was found at 305 and 326 nm. Remarkably, at 350 and 363 nm *cis*-UCA was only formed *in vivo*, but not in *in vitro*. At longer test wavelengths up to 405 nm, no significant formation of *cis*-UCA was detectable. The established partition between UV-B and UV-A at 320 nm is not relevant for the isomerization pattern of UCA. Additional studies revealed a substantial *cis*-UCA formation in the human skin by UV-A phototherapy lamps. Furthermore, raised levels of 295 nm irradiation dos-

es, a possible effect of stratospheric ozone reduction, were found to increase the *cis*-UCA yield. Our results demonstrate that the formation of *cis*-UCA in the skin with common exposures takes place over a broad spectral range of UV-B and UV-A up to at least 363 nm. These findings emphasize the potency of UV-A to isomerize UCA and they may contribute to further elucidation of the effects of phototherapy and sunbathing.

1. Introduction

The skin is exposed to solar-and artificial UV-radiation on innumerable occasions. At the earth's surface solar UV-radiation intensities increase from approximately 290 nm towards longer wavelengths, reaching a maximum in the visible range. Stratospheric ozone filters out the radiation below 290 nm (UV-C). This property may be impaired by the gradual breakdown of the ozone layer, resulting in an increased intensity of UV-B and UV-C radiation reaching the earth's surface. This environmental change in solar radiation is expected to cause a larger immunosuppressive effect (1) in the skin or elsewhere in the body.

Epidermal layers are rich in UCA which is formed by deamination of histidine by histidase. UCA is initially formed in the *trans*-form, which can be photoisomerized to *cis*-UCA by UV-radiation (2,3). Continued UV-exposures could lead to the photo stationary state with approx imately 70% *cis*-UCA and 30% *trans*-UCA. Evidence has been provided supporting the view that the UV-induced immunosuppressive effect is mediated by the formation of *cis*-UCA in the epidermis (4,5). Exogenous *cis*-UCA was shown to act suppressively in certain immunological test models, such as allograft rejection (6), delayed type hypersensitivity (7,8), and antigen presenting functions (9). In their pioneer work, De Fabo and Noonan (10) constructed an action spectrum from 250-320 nm of the UV-induced suppression of contact hypersensitivity in mouse skin. The lowest irradiation dose required to produce 50% suppression was at 260-270 nm, which also corresponds to the absorption maximum of UCA. Towards longer wavelengths (UV-B, UV-A) higher irradiation levels were required to produce 50% suppression, although UV-radiation up to at least 340 nm caused isomerization of UCA (11).

Solar exposures of the skin comprise a much larger flux of the longer UV-A wavelengths than those of UV-B. In this respect, the contribution of longer wavelengths to *cis*-UCA formation has not been clearly defined. This lack of information prompted us to determine the spectrum of *cis*-UCA formation that would occur in commonly encountered solar UV-exposures in the range of 0.25 to 1 minimum erythema dose(s)(MED). The net yield of *cis*-UCA was measured for each test wavelength, in the human skin, and in

UCA solutions *in vitro*. The results provide a perspective of the distribution of *cis*-UCA yield across the UV-B and UV-A spectrum.
In addition, we studied the yield of *cis*-UCA after an increase of 295 nm irradiation, as a simulation of the effect of stratospheric ozone reduction. We also examined the yield of *cis*-UCA after exposures to a UV-A lamp, from which the UV-B emission was blocked or transmitted by appropriate cut-off filters. Such lamps are widely used in phototherapy and for tanning purposes. Our results may have practical implications for the interpretation of the effects of solar or artificial UV-exposures in relation to photoimmunosuppression.

2. Methods

2.1 Urocanic acid

Trans-3-(4-Imidazolyl)-acrylic acid (*trans*-UCA), was supplied by Aldrich Chemie (Steinheim, Germany). This compound exhibited a single peak after injection in our liquid chromatograph. Melting point determination was in accordance with supplier's data (226-228 °C). Chromatographically purified *cis*-UCA was kindly donated by dr. M. Norval, University of Edinburgh, Scotland.

2.2 High performance liquid chromatography (HPLC)-analysis of trans- and cis-urocanic acid

Both isomers could be determined separately in one run at ambient temperature with an isocratic HPLC-system, equipped with a 25 x 0.4 cm C18 reversed phase column and a UV-detector set at 280 nm (both: Pharmacia, Uppsala, Sweden). Peak areas were assessed by a Spectra Physics integrator model SP 4010. The detector response ratio of equimolar amounts of *trans*-UCA to *cis*-UCA was 1.52. The eluent was a phosphate buffer (50 mM, pH 3.6) with 4% methanol and sodium octanesulphonate (1.0 mM). The *trans*- and *cis*-isomers were eluted after 17 and 23 minutes respectively with a flow rate of 1.0 ml/min. The detection limits of *trans*- and *cis*-UCA were 18 and 29 pmol, respectively.

2.3 UV-irradiation conditions

The narrow band irradiations were performed with a 1000 Watt xenon arc lamp (Oriel, CT, USA). The set-up is a modification of the solar simulator design (12) (Fig. 1). In order to minimize infra-red (heat) radiation, the exit beam was passed through a water filter of 7 cm pathway and the short-wave part (< 500 nm) of the beam was reflected by a dichroic mirror. The UV-emission spectrum was a smooth continuum, from which the desired narrow band was selected by an interference filter (Oriel, CT,

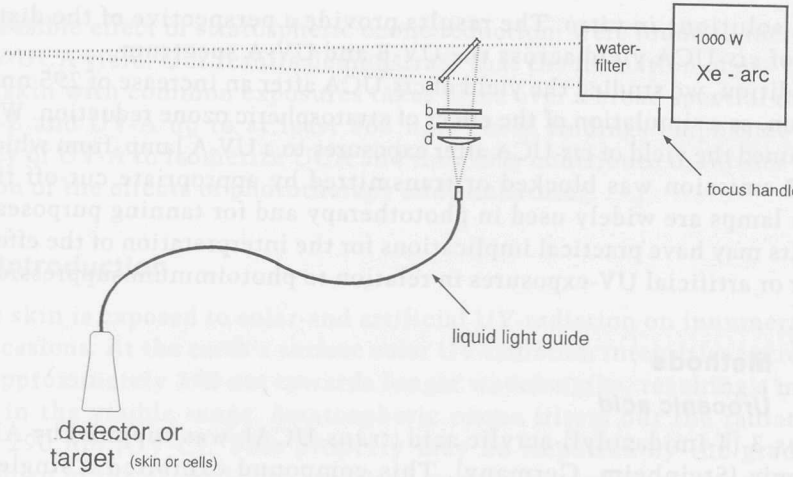

Fig. 1. The equipment for narrow band irradiations. After passage through a 7 cm waterfilter, the xenon arc beam hit a dichroic mirror (a), from which a large part of the UV-spectrum was reflected. Then, the beam passed a narrow band interference filter (b) and a long pass glass filter (c). A positive quartz lens (d) focussed the radiation beam into the entrance aperture of the liquid light guide for transfer to detector or skin target.

USA) and a glass filter (Schott-Jena, Mainz, Germany). Nine narrow wave bands were selected from 295 nm to 405 nm. The half bandwidth of the filter combinations was 5-8 nm. The use of narrow band filters in combination with long pass glass filters provided blocking (transmittance < 10^{-3}), 11-15 nm from the transmission maximum at short-wave side. This spectral property is particularly important for the determination of the ultimate active wavelength of photoisomerization. The blocking at the long-wave side was provided by the narrow band filter itself, at a distance of 17 to 23 nm from the transmission maximum. Transmission spectra of the narrow band filters, long pass glass filters, and their combinations, were recorded on a Perkin Elmer 550-S UV/VIS spectrophotometer.

UV-radiation output was measured by a silicon probe of the EG&G (Salem, Mass., USA) type 550-1 radiometer, fitted with a neutral density filter. The probe sensitivity for 5 nm wavelength intervals was calibrated by the manufacturer using standards traceable to the National Bureau of Standards. The readings were corrected for the probe sensitivity and for the attenuation of the neutral density filter, in order to obtain an output in terms of W/m^2. The irradiation dose of 305 nm was adjusted to 716 J/m^2,

Table 1. Irradiation data based on solar dosimetry.

Center wavelength irradiation wave band (nm)	Relative irradiation doses series (MED)		
	1.0*	0.5	0.25
295	0.009	0.0045	0.0023
305	**1**	**0.5**	**0.25**
326	6.56	3.28	1.64
341	8.47	4.24	2.12
350	9.12	4.56	2.28
363	10.4	5.2	2.6
379	11.9	6.0	3.0
391	13.0	6.5	3.3
405	13.9	7.0	3.5

* The doses were related to the one of 305 nm with a value of 716 J/m^2.

which corresponded to 1-2 MED under our conditions. This wave band has virtually the shape of the erythemal action spectrum, adapted to solar flux (13). This radiation should cause the same degree of erythema as that produced by the complete spectrum of solar UV radiation.

The irradiation doses at other narrow UV-bands were related to the dose of 305 nm by a factor derived from the solar irradiance at sea level (Table 1) and did not appear to be erythemogenic. A clear sky, and the sun at 0° zenith angle were selected as the reference conditions (14). In additional series the irradiation doses were reduced to a half and a quarter to study dose-response behaviour. The series were marked as 0.5 MED and 0.25 MED (Table 1). The range of increased 295 nm UV-doses has been derived from literature (15,16) and the maximum dose included is comparable with approximately 16% stratospheric ozone reduction. Six TL-10R UV-A lamps (Philips, Eindhoven, The Netherlands), commonly used for phototherapy and tanning, were used in this study, after filtering their emission with WG 295 and WG 335 cut-off filters of 3 mm thickness. The latter showed 1% and 0.1% transmission at 316 and 311 nm, respectively. The WG 295 filter did not block the UV-B emission from the lamp. The manufacturer stated that 0.1% of the total emission is UV-B radiation. UV-A levels were monitored with an IL442A radiometer of International Light Newburyport, MA., USA) fitted with a cosine receiving probe.

2.4 Irradiation of human skin and sampling of UCA

Narrow band irradiations were performed with a liquid light guide (Fig.1; Oriel, Stratford, CT, USA) to ensure fixation of the irradiation spot on the skin during the exposure time. This spot was 7 cm from the exit aperture of the light guide. In this way a circular irradiation spot of 2.54 cm^2 was formed. Three volunteers (white Caucasians) were used for each test wavelength and irradiation dose. Irradiations and analyses were performed on duplicate samples derived from both upper arms. The skin had not been exposed to a UV-source for at least two weeks prior to the experiment.

The non-invasive sampling method used was a modification of that of Jansén (17), called chamber sampling. It is based on alkaline extraction of the epidermis. A filter paper of a patch tester (Silverpatch™, van der Bend, Holland) was moistened with 20 µl of 0.1 M potassium hydroxide and attached on the skin test area by elastic adhesive tape. After one hour, the patch testers were removed and the filter papers soaked in 470 µl 0.1 M potassium hydroxide. They were vigorously stirred on a Vortex mixer for one minute and acidified with 10 ml phosphoric acid 87%; followed by stirring for one minute. The epidermal extract was passed through 0.22 µm membrane filter prior to injecting 40-100 µl into the HPLC system. Negative control samples were taken from unirradiated skin in close proximity of the irradiation spots.

2.5. Irradiation of UCA-solutions

Trans-UCA was dissolved in ultrapure water at concentrations of 2, 10 and 50 µM. One and a half ml of these solutions was transferred to quartz cuvettes prior to irradiation at room temperature. The cuvette, with an optical path length of 1 cm, was positioned in the filtered xenon arc beam in front of the light monitoring silicon probe. The UCA-solutions were either magnetically stirred or not, and were uniformly irradiated with the same doses at the same wavelengths as those employed in the *in vivo* experiments. The cuvette of the non-stirred UCA-solution was then turned 180°, and a similar irradiation dose was given to the other side. The quartz window of the cuvette transmitted 93% of the incident UV-radiation.

The applied UV-doses were corrected for this value. Each point on the graphs shown in Fig. 5-7 is an average of four analyzed samples. Negative control values were obtained in a similar way, except that the light path was blocked by a black metal plate of the same dimensions as the optical filters. Photoisomerization was also studied at elevated temperatures (40-45 °C) and in two buffer media with different acidity. One buffer consisted of 0.2 M acetate/acetic acid pH 4.0 and the other one of 0.2 M phosphate pH 5.8.

2.6 Calculations of the net yield of cis-UCA

The relative amounts of both UCA isomers were derived from the peak areas of the HPLC chromatograms. Corrections were made for detector response (trans/cis = 1.52) at 280 nm. In the figures the outcome is marked as % *cis*-UCA (corrected). This value was calculated according the following formula, and it represents the relative net yield of *cis*-UCA formed upon irradiation: in which:

$$\frac{(\%\ cis_{\ irradiated} - \%\ cis_{\ control}) \times 100}{\%\ trans_{\ control}} = \%\ cis\text{-UCA (corrected)}$$

$\%\ cis_{irradiated}$ = relative amount of *cis*-UCA found in irradiated skin,
$\%\ cis_{control}$ = relative amount of *cis*-UCA found in control (non-irradiated) skin,
$\%\ trans_{control}$ = relative amount of *trans*-UCA found in control (non-irradiated) skin.

The relative amount of *trans*-UCA in control skin was considered to be the amount available for photoisomerization.

3. Results

3.1. In vivo experiments

HPLC-analyses of extracts from irradiated human skin showed that net formation of *cis*-UCA was detectable in the wavelength range 295-363 nm (Fig. 2 and 2a) after single exposures of the 1 and 0.5 MED series (Table 1). High ratios were found at 305, 326 and 341 nm, demonstrating a broad spectral range of *cis*-UCA formation (Fig. 2). However, if irradiation doses were reduced four times (0.25 MED), significant net formation of *cis*-UCA occurred in the range 305-350 nm. With the reduction of the maximum irradiation dose at 305 and 326 nm, the photostationary state in the isomerization behaviour became apparent, as *cis*-UCA yield showed a reduction less than proportional. *Cis*-UCA was not significantly formed at 379 nm, nor at longer wavelengths of 391 and 405 nm (data not shown). Each error bar shows the standard deviation of a set of six data derived from the left and right upper arms of the three volunteers. Duplicate samples of non-irradiated skin showed a mean value of 1.4% *cis*-UCA (S.D. ± 0.4%). The total quantity of UCA, in absolute terms, extracted from the epidermis was 23.3 nmol/cm^2 (S.D. 6.6 nmol/cm^2; n = 18). This value is close to previously reported levels (17,20).

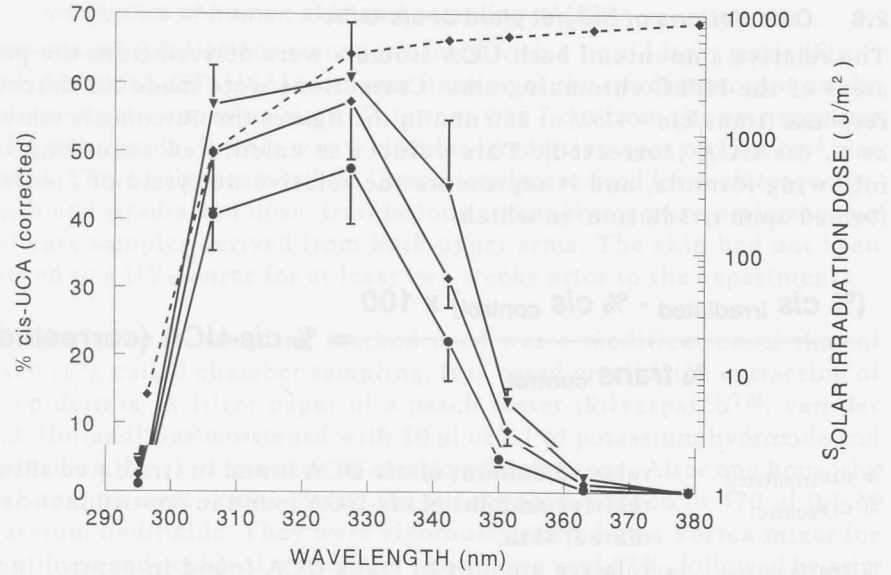

Fig. 2. The relative net yield of *cis*-UCA in human skin in three series of irradiation doses. The correction on the percentage of *cis*-UCA has been explained in the Materials & Methods section. The curve of solar irradiation doses (dashed line) has been included in Fig. 2, 5 and 6 for reference purposes and it is congruent with solar irradiance at sea level. Upper line: irradiations normalized to 1 MED; center line: irradiations normalized to 0.5 MED; lower line: irradiations normalized to 0.25 MED.

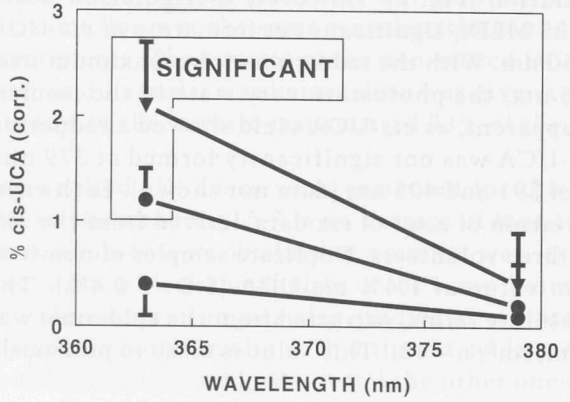

Fig. 2a. Significant *cis*-UCA formation of the 1 and 0.5 MED series at 363 nm.

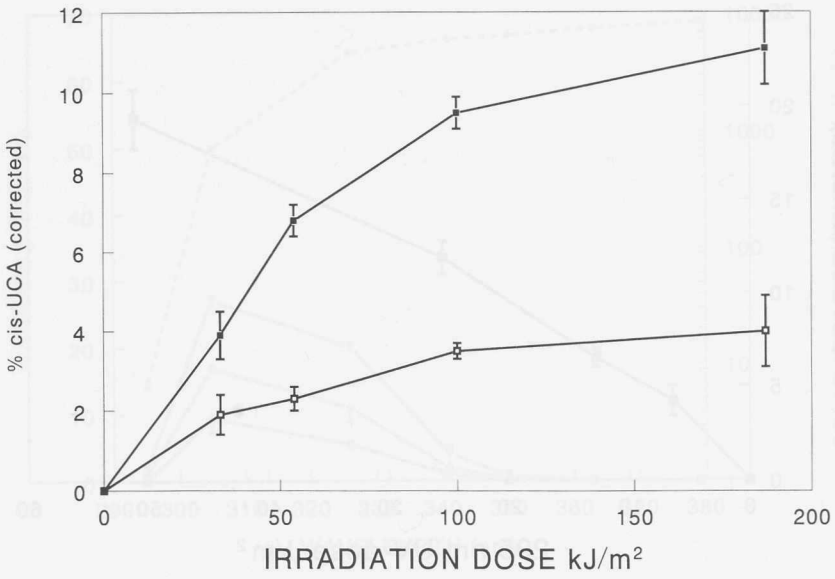

Fig. 3. The relative net yield of *cis*-UCA in the human skin after UV-A doses from Philips phototherapy/tanning lamps type TL-10R. Upper line: the relative net yield of *cis*-UCA after filtering by WG 295. Lower line: the relative net yield of *cis*-UCA after filtering by WG 335.

These *in vivo* findings revealed the potency of UV-A radiation to cause *cis*-UCA formation. Therefore, we studied the effect of a UV-A lamp on UCA isomerization. Fig. 3 shows the relative net yields of *cis*-UCA by two series of graded irradiation doses from UV-A tanning/ phototherapy lamps. The applied doses did not exceed commonly employed tanning regimens, and did not evoke any perceptible tan or erythema on the skins of the volunteers. The UV-A radiation was filtered by a WG 295 cut-off filter, which permitted transmission of UV-B radiation emitted from the UV-A lamp. A WG 335 cut-off filter was used to block UV-B and a part of the UV-A-II (320-340 nm) radiation. This model revealed (Fig. 3) that roughly one-third of the *cis*- UCA yield was due to pure UV-A radiation, whereas the majority of the *cis*-UCA yield was caused by the short waves of the UV-A lamp, including UV-A-II and traces of UV-B radiation.

Another experiment was set up to study the effect of ozone layer reduction on *cis*-UCA formation. Solar UV-B radiation at sea level is largely influenced by the amount of ozone in the earth's atmosphere, and the most pronounced effects of ozone reduction are related to the shortest UV-B wave

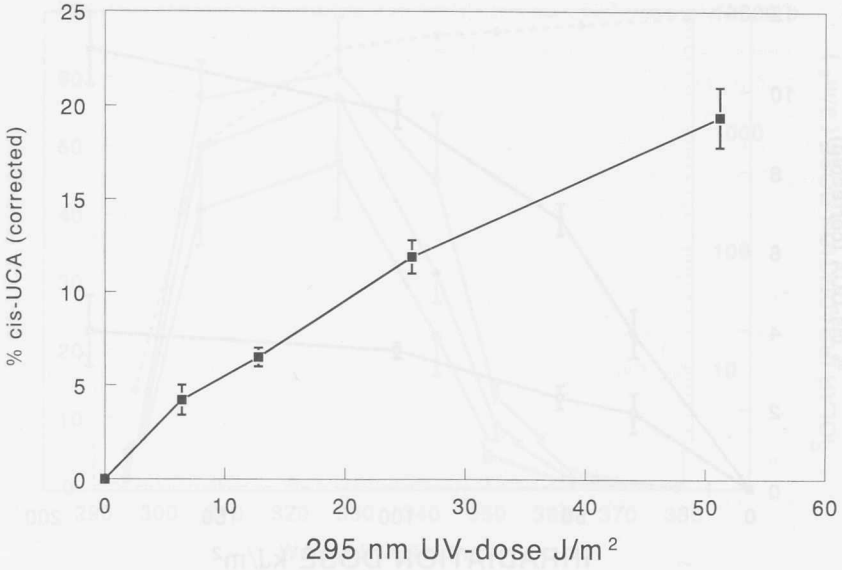

Fig. 4. Increased net yield of *cis*-UCA in the human skin by elevated 295 nm UV-doses. The error bars represent the difference between duplicate measurements.

lengths reaching terrestrial level. Therefore, our shortest test wavelength (295 nm) was chosen to simulate the effect of stratospheric ozone reduction. Increased *cis*-UCA ratios associated with the elevated 295 nm UV-doses are shown in Fig.4. An eightfold increase of the 295 nm UV-dose, equivalent to approximately 16% stratospheric ozone reduction (15,16), caused the formation of 19.4% *cis*-UCA, which is considerably higher than with the original dose of 6.4 J/m^2 (no ozone reduction). The data showed an almost linear relationship increasing by 0.4% *cis*-UCA per J/m^2.

3.2 *In vitro experiments*

UCA-solutions of two, ten and fifty micromol per liter were irradiated using the same conditions at every test wavelength as the *in vivo* component of this study. The dose-response appeared to be linear at every test wavelength from 295 to 341 nm, without a tendency to the photostationary state. The relative net yields of *cis*-UCA appeared to be virtually independent of the UCA concentration, when the solutions were stirred (Fig. 5a,b). However, in another experiment without stirring the amount of the *cis*-UCA yield was dependent on the initial *trans*-UCA concentration. The cuvette was irradiated on both sides with the same dose which

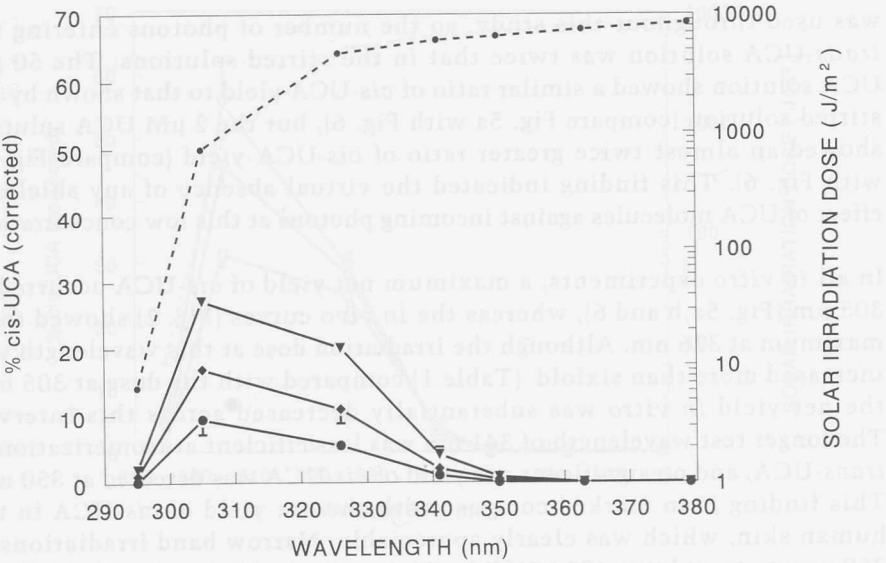

Fig. 5a. The relative net yield of *cis*-UCA *in vitro* in three sets of irradiation doses based on solar irradiance. Initial *trans*-UCA concen-tration is 50 μM. This solution was stirred during exposure.
Upper solid line: irradiations normalized to 1 MED; center solid line: to 0.5 MED; lower solid line: to 0.25 MED. At some data-points the error bars were not larger than the symbols.

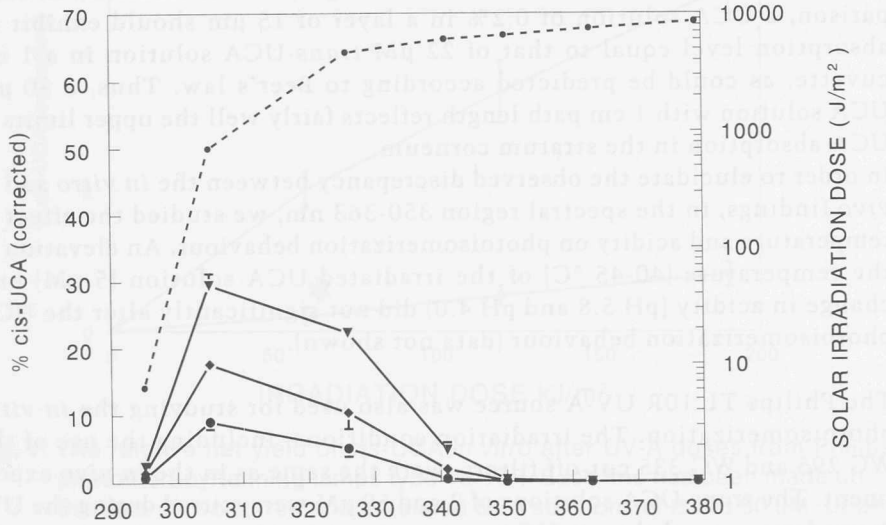

Fig 5b. The same as in (a), except the initial *trans*-UCA concentration is 2 μM.

was used throughout this study, so the number of photons entering the *trans*-UCA solution was twice that in the stirred solutions. The 50 µM UCA solution showed a similar ratio of *cis*-UCA yield to that shown by the stirred solution (compare Fig. 5a with Fig. 6), but the 2 µM UCA solution showed an almost twice greater ratio of *cis*-UCA yield (compare Fig. 5b with Fig. 6). This finding indicated the virtual absence of any shielding effect of UCA molecules against incoming photons at this low concentration.

In all *in vitro* experiments, a maximum net yield of *cis*-UCA occurred at 305 nm (Fig. 5a,b and 6), whereas the *in vivo* curves (Fig. 2) showed their maximum at 326 nm. Although the irradiation dose at this wavelength was increased more than sixfold (Table 1) compared with the dose at 305 nm, the net yield *in vitro* was substantially decreased across this interval. The longer test wavelength of 341 nm was less efficient at isomerization of *trans*-UCA, and no significant net yield of *cis*-UCA was detected at 350 nm. This finding is in marked contrast with the net yield of *cis*-UCA in the human skin, which was clearly appreciable. Narrow band irradiations of 350 nm up to at least 405 nm did not cause a significant net yield of *cis*-UCA *in vitro* (Fig. 5a,b and 6).

The absorbance of a *trans*-UCA solution of 50 µM with 1 cm path length amounted to 0.82 at 268 nm. This level is similar to that (A = 0.71) of stratum corneum at an average thickness of 15 µm (18). UCA is its main UV-absorbing material and is present in a concentration of approximately 0.5% of the dry weight (19) (0.2% of wet weight; own estimation). In comparison, a UCA solution of 0.2% in a layer of 15 µm should exhibit an absorption level equal to that of 22 µM *trans*-UCA solution in a 1 cm cuvette, as could be predicted according to Beer's law. Thus, a 50 µM UCA solution with 1 cm path length reflects fairly well the upper limits of UCA absorption in the stratum corneum.
In order to elucidate the observed discrepancy between the *in vitro* and *in vivo* findings, in the spectral region 350-363 nm, we studied the effect of temperature and acidity on photoisomerization behaviour. An elevation of the temperature (40-45 °C) of the irradiated UCA solution (5 µM) or a change in acidity (pH 5.8 and pH 4.0) did not significantly alter the UCA photoisomerization behaviour (data not shown).

The Philips TL 10R UV-A source was also used for studying the *in vitro* photoisomerization. The irradiation conditions, including the use of the WG 295 and WG 335 cut-off filters, were the same as in the *in vivo* experiment. The *trans*-UCA solutions of 2 and 50 µM were stirred during the UV-A irradiations and the *cis*-UCA ratio appeared to be concentration independent, as was shown above in the results of the narrow band irradiations.

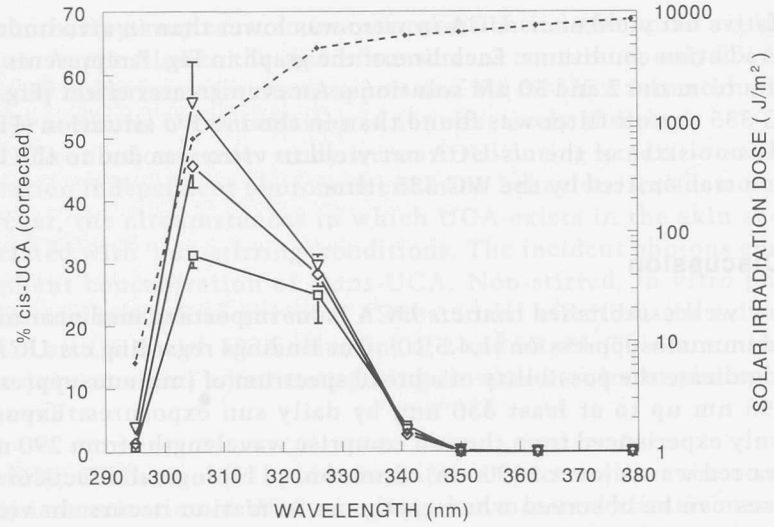

Fig. 6. In vitro photoisomerization at different UCA concentrations without stirring during irradiation. The one MED series of irradiation doses (Table 1), was applied. Both sides of the cuvette were exposed. Upper line: 2 μM. Center line: 10 μM. Lower line: 50 μM.

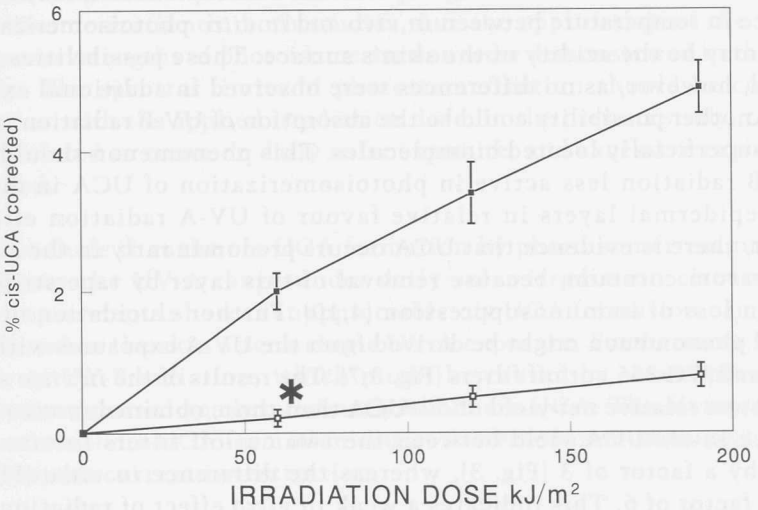

Fig. 7. The relative net yield of *cis*-UCA *in vitro* after UV-A doses from Philips phototherapy/tanning lamps type TL-10R. Each line has been made up of the data of two stirred and irradiated UCA solutions of 2 and 50 μM. Upper line: after filtering by WG 295. Lower line: after filtering by WG 335. No significant *cis*-UCA was formed at the data point marked with *.

The relative net yield of *cis*-UCA *in vitro* was lower than *in vivo* under the same irradiation conditions. Each line of the graph in Fig. 7 represents averaged data from the 2 and 50 µM solutions. An even greater effect (Fig. 7) of the WG 335 cut-off filter was found than in the *in vivo* situation (Fig. 3). Roughly one-sixth of the *cis*-UCA net yield *in vitro* was due to the UV-A radiation transmitted by the WG 335 filter.

4. Discussion

It is now well established that *cis*-UCA is an important mediator of UV-induced immunosuppression (1,4,5,10). Our findings regarding *cis*-UCA formation indicate the possibility of a broad spectrum of immunosuppression, from 295 nm up to at least 350 nm, by daily sun exposures. Exposures commonly experienced from the sun comprise wavelengths from 290 nm to the infra-red waves (over 2400 nm). Pronounced biological effects of solar exposures can be observed when erythema formation occurs. In view of this, we chose a dose of narrow band irradiation at 305 nm, which caused 1-2 MED. The other narrow band irradiation doses did not cause erythema formation in our dosimetric setting.

There is a discrepancy between the *in vitro* and *in vivo* behaviour at the long-wave end of the isomerization spectrum. One explanation may be the difference in temperature between *in vivo* and *in vitro* photoisomerization; another may be the acidity of the skin's surface. These possibilities were excluded, however, as no differences were observed in additional experiments. Another possibility could be the absorption of UV-B radiation in the skin by superficially located biomolecules. This phenomenon should render UV-B radiation less active in photoisomerization of UCA in deeper located epidermal layers in relative favour of UV-A radiation effects. However, there is evidence that UCA occurs predominantly in the superficial stratum corneum, because removal of this layer by tape stripping results in loss of immunosuppression (4,10). Further elucidation of this unsolved phenomenon might be derived from the UV-A exposures with the WG 295 and WG 335 cut-off filters (Fig. 3,7). The results of the *in vitro* study show a lower relative net yield of *cis*-UCA than those obtained *in vivo*. The difference in *cis*-UCA yield between the two cut-off filters *in vivo* was roughly by a factor of 3 (Fig. 3), whereas the difference *in vitro* (Fig. 7) was by a factor of 6. This indicates a weak *in vitro* effect of radiation distributed around 340 nm. The short-wave end of solar UV-A spectrum (mainly UV-A-II, 320 -340 nm) contributed strongly to photoisomerization. This observation is in accordance with the results of Schwarz et al.(20), who found elevated skin levels of *cis*-UCA after UVASUN 3000 exposure. Thus, common exposures to UV-A radiation, such as during sunbathing and UV-

A phototherapy, may cause immunosuppression via substantial *cis*-UCA formation. Application to the skin of sunscreens containing only UV-B filters (18), may not prevent immunosuppression via *cis*-UCA formation, because solar or artificial UV-A radiation is not effectively absorbed.
The UCA solutions under study were stirred during irradiation and a concentration independent photoisomerization behaviour was demonstrated. However, the circumstances in which UCA exists in the skin should be associated with 'non-stirring' conditions. The incident photons encounter a gradient concentration of *trans*-UCA. Non-stirred, *in vitro* photoisomerization conditions revealed a substantial effect on the relative net yield of *cis*-UCA with UCA solutions of 2 and 50 μM. This factor should be taken into account, when transposing *in vitro* results to photochemical skin events.

Recently, Gibbs et al.(11) published action spectra about the unweighed photo-isomerization of UCA, showing that maximal effectiveness of isomerization was found in the spectral range 300-315 nm in mouse skin as well as *in vitro*. However, we found that the range 305-341 nm was maximally active in human skin, with a dosimetry based on solar irradiance. This extension to the long-wave end of the spectrum is probably due to the relatively high levels of UV-A radiation, inherent in solar exposure. The 340nm (11) and 341 nm test wavelengths were found to be active in photoisomerization in both studies, but the application of the ultimate longer wavelengths of photoisomerization were demonstrated in the present study. The spectral limits of photoisomerization should be considered together with the applied irradiation dose. In the present study, the limits were related to common daily solar exposure, and excessive exposure was avoided.

We conclude that the *cis*-UCA formation by photoisomerization extends well into the UV-A range under daily solar irradiation circumstances. Our experiments show that appreciable *cis*-UCA formation can easily be generated in the skin after a single UV-A exposure. Furthermore, an increment of UV-B radiation, which might be caused by stratospheric ozone reduction, caused an elevated net yield of *cis*-UCA. The claim that every induction of *cis*-UCA formation causes increased immunosuppression is a topic under current investigation.

Acknowledgements

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CHAPTER 3

Long-wave limit of the urocanic acid photo-isomerization spectrum in human skin

Arthur Kammeyer, Jan D. Bos and Marcel B.M. Teunissen

Department of Dermatology, Academic Medical Center,
University of Amsterdam, The Netherlands

Abstract

Cis-urocanic acid (*cis*-UCA), a proposed mediator of immunosuppression, is formed from *trans*-UCA in the epidermis upon UV-exposure by photo-isomerization. We determined the long-wave limit of this phenomenon *in vivo* by HPLC analysis of skin extracts of narrow-band UV-irradiated human skin. Volunteers received multiple test irradiations close to unirradiated (control) sites. The long wave limit of UCA photoisomerization in the human skin with a simulated solar UV-exposure causing a barely perceptable erythema was found around 363 nm. Lower simulated solar UV-doses cause a blue shift of the limit towards 350 nm. This information provides ways to protect the human skin against *cis*-UCA formation, which phenomenon is suspected of being carcinogenic *via* immunosuppression.

1. Introduction

The *cis*-isomer of urocanic acid (UCA) has been proposed as an important initiator of UV-induced immunosuppression (1,2). There is evidence that this UCA isomer suppresses hypersensitivity responses (3), promotes UV-induced tumor yield (4) and prolonges allograft acceptance in mice (5). These important biological effects merit the development of knowledge about how to avoid or how to induce *cis*-UCA formation in the skin by manipulation of the UV-exposures. *Trans*-UCA can be isomerized to *cis*-UCA in the skin (*in vivo*) as well as in solutions (*in vitro*) by exposures to UV. The complete absorption spectrum of *trans*-UCA indicates that the longest wavelength at which the photons are capable of interacting with *trans*-UCA molecules is approximately 322 nm. Consequently, 322 nm may be considered as the long wave limit to evoke trans to cis photoisomerization. However, we and five other research groups independently showed

the potency of wavelengths longer than those of the UV-B range (290-320 nm) in isomerizing *trans*-UCA (6-11). The aim of this study was to establish the UCA photoisomerization long-wave limit in the human skin. One of our previous studies (9) already reported a limit of photoisomerization as a part of a broader investigation.
The research, presented here, was carried out with a large number of volunteers, providing new sets of data, to determine the long-wave limit of the UCA photoisomerization spectrum in normal human skin. Knowledge about the exact spectral location of the long-wave limit is important, because it can be used to differentiate between particular UV-exposures to cause *cis*-UCA formation or not.

2. Materials and Methods

2.1. *Narrow band UV-irradiations*

The irradiation equipment and measurements have recently been described in detail elsewhere (9). In brief, we used narrow-band irradiations at 350, 363 and 379 nm, that were filtered out from the emission of a 1000 W xenon-arc lamp, and delivered to the skin with a liquid light guide (Oriel, Stratford, CT, USA). Blocking (T < 0.1%) was obtained at the short-wave side, 11-14 nm from the transmission maximum. This range should be as small as possible, because the short waves are more potent to form *cis*-UCA. Optical conditions are presented in Table 1. The applied dosimetry was based on the solar irradiance spectrum at sea level. With respect to solar exposures, the 307 nm wavelength is the most potent in causing erythema (12). To obtain a dosimetric reference point for erythema formation in the human skin, we used a narrow band filter of 305 nm to define 1.0 MED in terms of J/m^2. The mean irradiation dose to evoke 1.0

Table 1. Optical conditions.

Center wavelength of narrow band (nm)	Optical filter[1] combination used		Blocking range (T<0.1%)		Irradiation
	Narrow-band filter	Short-wave cut-off filter	Short-wave side (nm)	Long-wave side (nm)	intensity of the 1.0 MED related series J/m^2
350	UV-P-IL 349 nm	WG 345	< 339	> 364	6530
363	UV-P-IL 363 nm	WG 360	< 349	> 380	7446
379	UV-P-IL 379 nm	KV 370	< 367	> 395	8520

[1] Optical filters of 2 x 2 inch were from Schott Glaswerke (Mainz, Germany)

minimum erythemal dose (MED) on Caucasian skins was 716 J/m^2 in this setup (9). The irradiation doses of the test wavelengths of this study were related to the mean dose at 305 nm according the definition of the solar irradiance spectrum at sea level (13,14). They are thus proportionally increased and will be further referred to as '1.0 MED related' (Table 1). In two other narrow-band irradiation series the doses were reduced with a factor $1/\sqrt{2}$ ('0.71 MED related') and a factor 1/2 ('0.50 MED related') to estimate the dose-effect relation.

2.2. High Performance Liquid Chromatography

The skin samples were analyzed with high performance liquid chromatography (HPLC). *Trans*-UCA and *cis*-UCA were separated on a 250 x 4.6 mm Luna C_{18} reversed phase column (Phenomenex, Torrence, CA) with a flow of 0.8 ml/minute, delivered by P-3500 HPLC pumps (Pharmacia, Uppsala, Sweden). Fifty microliter samples were taken by a Promis II autosampler (Spark Holland, Emmen, The Netherlands) and chromatographic data were recorded on a SP 4270 integrator (Spectra Physics, San Jose, CA). A model 759A UV detector (Applied Biosystems, Foster City, CA) was set for 268 nm detection. Isocratic elution was performed with 50 mM ammonium formate buffer pH 3.5, containing 0.5 mM sodium octane sulphonate and 2 to 4% acetonitrile. The percentage of both UCA isomers was derived from the peak areas of the HPLC chromatograms. Corrections were made for detector response (trans/cis = 1.48) at 268 nm. The relative amount of *cis*-UCA formed upon UV exposure (% *cis*-UCA) was calculated using the *cis*-UCA and *trans*-UCA values of control (unirradiated) skin as well, according the formula described before (9). Statistical analysis of *cis*-UCA formation was calculated with the Welch's approximate t-test assuming unequal variances of data in the control and UV-exposed group. p-Values < 0.01 (one tail) were considered to differ significantly in *cis*-UCA level.

2.3. Skin sampling

Skin samples were obtained by an extraction method with filter papers attached to the skin surface, originally called chamber sampling (15). The filter papers were moistened with 0.1 M potassium hydroxide solution. After one hour of skin attachment, the filter papers were soaked in 0.1 M potassium hydroxide solution, neutralized with hydrochloric acid and firmly agitated. The solution was filtered through 0.22 µm membrane filter, prior to injection into the liquid chromatograph. Upperarms of 24 volunteers (age 18 – 60) with skin type II to IV served as skin test areas. Control skin samples (3 to 4 per arm) were taken in close proximity of the

irradiated sites. Each arm administered three different irradiation doses ('1.0 MED-, 0.71 MED- and 0.50 MED related') of one test waveband.

Two exclusion criteria were employed. First, skin sample values of total-UCA (= *cis*- plus *trans*-UCA) exceeding 2x the SD range were excluded, because a large variation in the extraction efficacy of the sampling method may affect the analysis of the cis/trans ratio as well. Second, samples of unirradiated (control) skin containing larger levels than 5.3 % *cis*-UCA (= mean value plus one SD of non-irradiated skin) (16) were omitted to ensure similarities in the rate of *cis*-UCA formation.

3. Results

We considered the UCA photoisomerization limit to be dependent on the irradiation dose, i.e. the higher the exposure dose, the more the limit will be red-shifted (towards longer wavelengths). The doses of the narrow-band irradiations in this study were related to natural doses that occur in a solar exposure causing a minimum erythemal dose (1.0 MED) down to 0.5 MED on an average Caucasian skin. The percentages of *cis*-UCA formation upon the UV-irradiations are summarized in Table 2.

350 nm

Very significant amounts of the *cis*-isomer were formed in the human skin at 350 nm, even with the mild irradiation dose ('0.5 MED related') at

Table 2. The long-wave limit of *cis*-UCA formation.

Center wavelength of narrow band	Percentage and significancy of *cis*-urocanic acid (*cis*-UCA) formation[1]								
	1.0 MED related			0.71 MED related			0.50 MED related		
(nm)	Mean	S.D.	p-value[2]	Mean	S.D	p-value[2]	Mean	S.D	p-value[2]
350	**12.6**	2.6	$< 10^{-3}$	**11.7**	3.2	$< 10^{-3}$	**8.7**	3.7	< 10-3
363	**2.6**	1.9	0.009	2.6	2.7	0.080	1.7	1.8	0.037
379	0.42	0.03	0.147	1.2	0.56	0.052	0.17	0.71	0.387

[1] expressed as % *cis*-UCA formed from *trans*-UCA by the process of photo-isomerization and calculated for each skin sample according the formula described before (9).

[2] p-value was calculated according Welch's approximate t-test, assuming unequal variances. Bold printed mean values were considered as very significant.

a rate of 0.0012 % *cis*-UCA per J/m^2. In an action spectrum for UCA photoisomerization in mouse skin the rate was approximately 0.0027% *cis*-UCA per J/m^2 at 340 nm (7).

363 nm

UV-irradiations at 363 nm with the 1.0 MED related irradiation dose yielded very significant mean value ($p = 0.009$) of *cis*-UCA as well. However, when the 363 nm irradiation doses were decreased 0.71 and 0.50 times, *cis*-UCA formation was not significant anymore.

379 nm

No significant *cis*-UCA formation was formed *in vivo* at 379 nm, the next longer test wavelength, at any dose used (Table 2).

Total-UCA

The total-UCA (*trans*-UCA + *cis*-UCA) concentration in the human skin could also be derived from our data. A mean value of 18.4 nmol/cm^2 with an SD of 9.7 nmol/cm^2 was found after analysis of all UCA samples of this study ($n = 198$), of which none was excluded. This value is between previously reported levels (9,15,16), that ranged from 23 down to 12 nmol/cm^2 (9,15).

Conclusion

The long-wave limit of the UCA photoisomerization spectrum appeared to be around 363 nm, when taking a barely perceptable erythema in an average Caucasian skin after a solar exposure as a reference. The limit is blue-shifted towards 350 nm with reduced exposures, comparable with suberythematogenic solar exposures.

4. Discussion

This finding implicates that sunscreens, containing typical UV-B absorbers (e.g. cinnamates), do not protect the human skin against *cis*-UCA formation by UV-A radiation. The same holds true for normal window glass, which transmits UV-A radiation.

It was previously shown that UV-induced elevation of *cis*-UCA levels in the human skin persist for approximately 3 weeks (16). This implies that frequent sun bank use for one to two times a week may lead to maximum *cis*-UCA levels of the photostationary state (approximately 65 % *cis*-UCA). Frequent exposure to all kinds of UV-sources may lead to accumulation of *cis*-UCA in the skin, as can be derived from a previous study (16). In these circumstances formation of *cis*-UCA may go faster than its elimination to the milieu interieur or milieu extérieur. The definition of the long-wave

limit of UCA photoisomerization provides insight to adjust exposure to sunlight by optical filtering or to tune UV-emission sources in such a way that *cis*-UCA formation in the human skin is allowed or avoided.

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CHAPTER 4

Prolonged increase of *cis*-urocanic acid levels in human skin and urine after single total-body ultraviolet exposures

Arthur Kammeyer, Stanislav Pavel#, Syed S. Asghar, Jan D. Bos and Marcel B.M. Teunissen

Department of Dermatology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

Present address: Department of Dermatology, University Hospital of Leiden, Leiden, The Netherlands

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Abstract

Cis-urocanic acid (*cis*-UCA), a mediator of immunosuppression, is formed from *trans*-UCA upon UV-exposure of the skin. This study describes a liquid chromatographic method for the simultaneous quantification of *cis*- and *trans*-UCA in skin, urine and plasma of non-irradiated volunteers. It also describes *cis*- and *trans*-UCA kinetics in UV irradiated volunteers. New procedures to remove interfering substances from urine and plasma are reported. Normal levels of *cis*-UCA in skin, urine and plasma of non-irradiated volunteers were 0.5 nmol/cm^2, 0.03 μmol/mmol creatinine (median 0.00) and undetectable and those of *trans*-UCA were 17.1 nmol/cm^2, 1.36 μmol/mmol creatinine and 0.5 μM, respectively. Upon single total body UV-B (290-320 nm) exposures of 250 J/m^2, epidermal *cis*-UCA levels immediately reached a maximum and returned to basic levels 3 weeks later. *Cis*-UCA levels in urine reached a maximum in 5 to 12 hours post-irradiation and reached base line values in 8 to 12 days. Additionally, a single total body UV-A (320-400 nm) irradiation of 200 kJ/m^2 yielded a similar pattern. The kinetics of *cis*-UCA in plasma could not be followed due to low concentrations, however, that of skin and urine was informative in relation to solar exposures and phototherapy.

1. Introduction

Trans-urocanic acid (*trans*-UCA) is a major UV-absorbing component of the skin. It is formed from histidine by the action of histidase. In the epidermis it accumulates predominantly in the stratum corneum, where it cannot be metabolized due to absence of the *trans*-UCA catabolizing enzyme urocanase (1,2). One route of *trans*-UCA removal is sweat excretion and horny layer slough off; a second route may be the inward diffusion from the epidermis into the circulation. Subsequently it passes the liver where it is partially broken down by urocanase (3,4). *Trans*-UCA can also be formed by histidase in the liver (5) and the final rate of urinary *trans*-UCA excretion is influenced by this organ. When ultraviolet (UV) radiation strikes *trans*-UCA in the epidermis (*in vivo*), *cis*-UCA is formed by photoisomerization (6-8). The most efficient waveband for photoisomerization is 310-320 nm and the longest effective wavelength is 363 nm with a solar exposure of 1 minimum erythema dose (MED) (8). There are indications that *cis*-UCA is not catabolized in the internal body (9).

Cis-urocanic acid (*cis*-UCA) has been shown to mimic the immunosuppressive effects of UV-B radiation in a number of studies, suggesting that this compound is an important mediator of UV-induced immunosuppression (10). Immunomodulating effects were reported on the induction of contact hypersensitivity (11) and delayed type hypersensitivity (12), allograft rejection (13) and the functions of monocytes and T-lymphocytes (14) as well as natural killer cells (15). The mechanism of action of *cis*-UCA is not fully understood, although, its action appears to involve tumor necrosis factor-alpha (TNF-alpha) (16) and histamine-like receptors (17). It is also not clear as to why UV-B is more potent in suppressing the contact hypersensitivity than UV-A (320-400 nm), although similar levels of *cis*-UCA were induced by both types of irradiation (18).

The common knowledge that *cis*-UCA has immunosuppressive properties and that it is easily formed in the epidermis upon solar exposure or upon exposure to artificial UV-radiation, such as during phototherapy, makes it important to obtain information about its formation, persistence and elimination from the human body. This requires a sensitive and reproducible method for the quantification of *cis*- and *trans*-UCA in body samples.

So far, a method for the simultaneous quantification of the UCA-isomers in urine or plasma has not been described. Both UCA isomers have been determined simultaneously by liquid chromatography (HPLC) in other skin specimen, such as homogenized horny layers of the human skin

(19,20) and homogenized murine skin biopsies (21); other studies reported the assay of UCA-isomers in horny layers colllected by tape strippings (22,23). Human suction blister fluids could be analysed by HPLC without pretreatment (24,25). We adapted a non-invasive sampling method (26) with a slight modification to obtain human skin data.

The above mentioned reports were focussed on *cis*- and *trans*-UCA in the epidermis; no correlation was made for UCA isomer levels in internal human body fluids, such as urine and plasma. This report describes the development of an HPLC-analysis for the simultaneous quantification of *cis*- and *trans*-UCA in urine and plasma. A newly developed pretreatment (clean-up) procedure for these specimen was found to be essential. The formation, persistence and elimination of *cis*- and *trans*-UCA in the epidermis, urine and plasma before and after single UV-B exposures or a UV-A exposure were assessed.

2. Materials and Methods

2.1. Chemicals and buffers

Trans-UCA, and 2-amino-4-thiazole acetic acid (ATAA), were purchased from Aldrich Europe (Bornem, Belgium). ATAA was recrystallized from an acetone/hexane mixture. *Cis*-urocanic acid (*cis*-UCA) and *cis*-2-methyl-urocanic acid (*cis*-MUCA) were kindly donated by dr M. Norval of the University of Edinburgh. Two types of washing buffers were used in the solid phase extraction procedure. One consisted of a 0.1 M glycine/ HCl buffer pH 2.2, containing 20% methanol (buffer A). The other consisted of 0.1 M sodium citrate/HCl buffer pH 3.5, containing 0.5 M sodium chloride (buffer B). The elution buffer consisted of 0.2 M sodium acetate buffer pH 4.2, containing 0.5 M sodium chloride and 20% methanol.

2.2. Donors and ultraviolet exposures

Healthy males and females aged 12-58 years participated in this study. Four volunteers received a single total body UV-B exposure of 250 J/m^2 in a cabinet with Philips TL-12 fluorescent tubes (Philips, Eindhoven, The Netherlands). The UV-dose was equivalent to 1.0 to 1.5 MED on skin type II/III volunteers. A single total body UV-A exposure of 200 kJ/m^2, emitted by Philips TL 10R fluorescent lamps, was received by a skin type III volunteer. Other healthy volunteers were not irradiated and served to obtain more data on normal *cis*- and *trans*-UCA levels. None of the donors was exposed to UV-radiation for at least three weeks prior to experimental UV-exposure. Persons that were not exposed to these UV-sources, will be referred to as 'non-irradiated'.

2.3. High Performance Liquid Chromatography (HPLC)

Cis- and *trans*-UCA, *cis*-2-methylUCA, *trans*-2-methylUCA and ATAA were separated on a 4 x 250 mm reversed phase µ-Bondapak column (Waters/Millipore, Milford, MA) with a flow of 1 ml/min, delivered by P-3500 HPLC-pumps (Pharmacia, Uppsala, Sweden). Samples were injected by a Promis II autosampler (Spark Holland, Emmen, The Netherlands) and chromatographic data were recorded on a SP 4270 integrator (Spectra Physics, San Jose, CA). A UV-detector (Applied Biosystems, model 759A, Foster City, CA) was set for 268 nm detection. Isocratic elution was performed with 0.05 M phosphate buffer (pH 3.4-3.6) containing 1 mM sodium octane sulphonate and 4% methanol for skin samples or 3 mM sodium octane sulphonate and 2% methanol for urine and plasma samples.

2.4. Determination of UCA isomers in urine

Urine was collected (see Results) and stored in portions at -20 °C. In contrast to epidermal and plasma sampling, urine was collected from each volunteer at different time-intervals. The creatinine concentration was used to adjust the original urine volume prior to the clean-up procedure following the guideline that a creatinine concentration of approximately 10 mM should correspond with one ml original urine for analysis. This correction was to avoid overload of the solid phase extraction column. The urine samples were cleaned up with strong cation exchange columns of the aromatic sulphonate type using a vacuum manifold SPE 12G (J.T.Baker, Deventer, The Netherlands). One milliliter of buffer A and the internal standard, *cis*-2-methylUCA (20 nmol) were added to the urine samples, followed by acidification with 20 ml 5 M hydrochloric acid prior to the passage through the solid phase extraction columns. The columns were successively washed with 2 ml buffer A, 2 ml buffer B and 2 x 2 ml pure water. UCA-isomers were then eluted with 2 ml elution buffer. The eluate was filtered through a 0.22 µm membrane filter, prior to HPLC injections of 50-100 microliters under the conditions described above. The urinary *cis*- and *trans*-UCA concentrations were either expressed as the percentage of total UCA or as micromoles per millimoles creatinine. An average urinary creatinine excretion is approximately 12 mmol/24 hours (~ 12 mM).

2.5. Determination of UCA isomers in plasma

Blood was collected in lithium heparin containing tubes at planned time-intervals respective to irradiation. After centrifugation (20 minutes at 2000 g) plasma was collected and stored at -20°C. One milliliter of freshly frozen plasma was thawed and known amounts of internal standards,

ATAA and *cis*-2-methylUCA, were added. After standing for 10 minutes at room temperature with occassionally stirring, 0.9 ml of a methanol/ water 1:5 mixture was added, followed by 0.1 ml 40% trichloroacetic acid under agitation. The tubes were kept on ice for 10 minutes with occassionally stirring followed by centrifugation at 3800 rpm for 20 minutes at 4 °C. The supernatants were then centrifuged at 13.000 rpm for 10 minutes. The supernatants were filtered through 0.22 µm membrane filters. HPLC-analysis was then carried out as described above. The plasma values of *cis*- and *trans*-UCA were expressed as micromoles per liter (µM).

2.6. Determination of UCA-isomers in epidermis

Epidermal alkaline extracts or filter samples (originally referred to as 'chamber samples' were collected at planned time-intervals respective to UV-irradiation. Filter samples were taken from the upper arms and from the lumbar spinal region of the volunteers. This non-invasive sampling method was a modification of an earlier reported technique (26), which is based on alkaline extraction of the epidermis. Our modification consisted of the application of patch testers (Silverpatch™, van der Bend, Brielle, The Netherlands), instead of Finn Chambers™. Using the patch testers, we achieved a smaller variation than reported elsewhere (26,27), perhaps because of an improved contact between skin surface and moistened filter paper. A further modification was an enlargement of the filter moistening volume and a reduction of the soaking volume and time. The filter paper (1 cm^2) was covered with a plastic sheet (3 cm diameter) and was moistened with 20 µl potassium hydroxide 0.1 M. It was fixed on the skin test area by elastic adhesive tape. After one hour or after 24 hours the filter papers were collected, put in 472 µl 0.1 M potassium hydroxide and shaken for 30 seconds. The medium was acidified with 8 µl phosphoric acid 87%; followed by stirring for 30 seconds. The epidermal extract was passed through 0.22 µm membrane filter prior to injecting 40-100 µl into the HPLC system. The data were expressed as nmol/cm^2 and as the ratio of *cis*- or *trans*-UCA to total-UCA.

3. Results

3.1. The HPLC-analysis of cis- , trans- and total-UCA levels in human skin

The sampling technique, as described earlier (8), was used to quantify epidermal *cis*- and *trans*-UCA levels before and after the UV-irradiations (vide infra) as well as the epidermal total-UCA (*cis*- plus *trans*-UCA) concentration. In addition, UCA-isomers extracted by 1 hour and 24 hour occlusions were compared, because it has been suggested that the latter method causes the extraction of virtually all UCA (26).

Table 1. *Cis*- and *trans*-urocanic acid levels in skin, urine and plasma in non-irradiated volunteers.

		Skin [a]		Urine [b]		plasma [c]
Urocanic acid isomer	Statistical parameter	nmol/cm^2	% of total UCA	µmol/mmol creatinine	% of total-UCA	µM
	mean	0.50	2.8	0.03	1.9	0
***cis*-urocanic acid**	SD	0.70	2.5	0.05	3.6	0
	median	0.27	1.7	0.00	0.0	0
	mean	17.1	98	1.36	98	0.5
***trans*-urocanic acid**	SD	7.1	2.5	0.74	2.1	0.1
	median	19.0	98	1.26	98	0.5

a. Unimodal skew distribution of data. From each individual 2 to 6 filter samples were taken. In total 32 filter samples from 7 individuals were analysed.
b. Unimodal skew distribution of data; *cis*-UCA level was below detection limit in 40 samples (74%) and was taken as zero in statistics. From each individual 1 to 2 urine samples were obtained. In total 53 urine samples from 44 individuals were analysed.
c. In total 12 plasma samples of 12 individuals were assayed, one sample showed a *trans*-UCA level of 1.9 µM and was not included.

Total-UCA extracted with the 1 h and 24 h occlusion methods had a mean value of 16.5 nmol/cm^2 (± 5.9; 7 individuals, 172 samples) and 50 nmol/cm^2 (± 18; 2 individuals, 12 samples), respectively. A relatively increased *cis*-UCA/total UCA ratio by a factor 1.37 was found using the 1 hour occlusion method.

Because of this finding we chose the 1 h occlusion method to register changes in *cis*-UCA formation by photoisomerization. One hour occlusions also offered more handling convenience. No significant difference was found in total-UCA level between irradiated and non-irradiated skins. Epidermal *cis*- and *trans*-UCA levels of non-irradiated volunteers are summarized in Table 1. Their *cis*-UCA levels in the epidermis were low, though measurable and their *trans*-UCA levels were found comparable to those of total-UCA. The *cis*- and *trans*-UCA detection limits for this analysis were 8 and 5 pmol, respectively.

Table 2. Recoveries of added *cis*- and *trans*-urocanic acid and internal standards.

Standard compound	Final concentration of added standard compound (μM)	Recovery % of added quantities		
		Water	Urine *	
			no UV exposure	UV-exposure
cis-UCA	0	-	*	*
	5	72	90	120
	15	75	89	103
	25	77	81	81
	40	75	86	75
trans-UCA	0	-	*	*
	5	90	88	102
	15	77	72	99
	25	77	68	78
	40	80	67	83
cis-2-methylUCA	5	72	112	107
	15	73	103	103
	25	76	103	87
	40	74	96	87
ATAA	10	87	111	86
	25	87	109	104
	40	86	86	74
	75	82	58	68

* the urine of the non-irradiated volunteer (creatinine: 21.8 mM) contained originally 2.7 μM *cis*-urocanic acid and 25 μM *trans*-urocanic acid. The urine of the same, UV-exposed person (creatinine: 9.8 mM) contained originally 10.1 μM *cis*-urocanic acid and 13.6 μM *trans*-urocanic acid.

3.2. *The HPLC-analysis of cis- and trans-UCA in human urine*

Clean-up procedures were required to facilitate the measurements of *cis*- and *trans*-UCA in human body fluids, as described in Materials and Methods. To assess the reliability and sensitivity of the HPLC-method, including clean-up procedure, we added our reference compounds *cis*- and *trans*-UCA, *cis*-

2-methylUCA and ATAA in nanomole amounts to the urine samples. The recoveries of added UCA-compounds, corrected for original urinary concentrations of *cis*- and *trans*-UCA isomers, are shown in Table 2.

The reproducibility of the method was determined through repeated analysis (18 times) of one ml of urine from a UV-B exposed volunteer supplemented with 20 nmol *cis*-2-methylUCA and *trans*-2-methylUCA and 50 nmol ATAA. The coefficients of variation were 3.5%, 4.9%, 7.1%, 3.6% and 7.3% for *cis*-UCA, *trans*-UCA, *cis*-2-methylUCA, *trans*-2-methylUCA and ATAA, respectively, thus showing an appreciable degree of reproducibility. Cis-2-methylUCA was eluted close to *trans*-UCA. Based on the above results and on similarities in molecular structure, we selected *cis*-2-methylUCA in final concentration of 15 μM or more as the internal standard for regular urine analyses. *Cis*-UCA was eluted considerably faster than its *trans*-isomer. The HPLC-system, including the UV-detector, provided linear results over a range from 45 pmol to 2.5 nmol *cis*- and *trans*-UCA and for *cis*-2-methylUCA. Detection limits of urine samples were as low as 45 pmol for *cis*-UCA and 25 pmol for *trans*-UCA. *Cis*- and *trans*-UCA levels in urine of non-irradiated volunteers are summarized in Table 1. Normal *cis*-UCA levels were extremely low and in a number of analyses its concentration was even below the detection limit. Trans-UCA was measurable in all urine samples. The range of *trans*-UCA concentrations that were not corrected for creatinine clearance, ranged widely from 2.7 to 44.7 μM.

3.3. The HPLC-analysis of cis- and trans-UCA in human plasma

UCA-levels in plasma might reflect photo-induced changes of epidermal UCA. A part of epidermal *cis*-UCA may be distributed via the blood circulation across the internal body, which aspect may be important for systemic immunosuppression. Therefore, we had set up an HPLC analysis of *cis*- and *trans*-UCA in plasma. To avoid interference by high protein concentration in plasma, a deproteinization step with trichloroacetic acid and methanol was introduced, which allowed the recovery of virtually 100% of the added *cis*- and *trans*-UCA and the internal standards *cis*-2-methylUCA and ATAA. The chromatogram of a plasma sample without any addition is shown in Fig. 1a and the one with additions of *cis*-, *trans*-UCA, *cis*-2-methylUCA and ATAA in final concentrations of 2.0, 2.0, 2.4 and 5.0 μM, respectively, is shown in Fig. 1b. These small additions were readily detected, showing that our method was quite sensitive. However, original UCA levels in plasma were extremely low. Cis-UCA concentration in all plasma samples of non-irradiated volunteers were lower than the detection limit. Normal *trans*-UCA levels could be quantified in all plasma samples (Table 1). The detection limits in plasma were 25 pmol for *cis*-UCA and 15 pmol for *trans*-UCA.

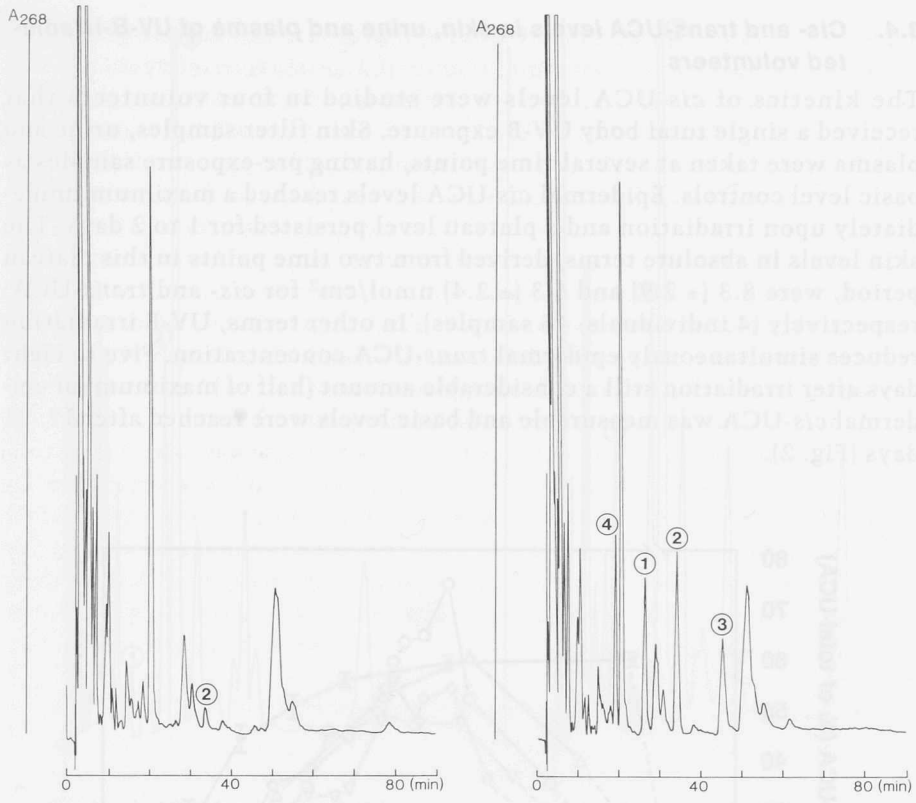

Fig. 1. Chromatograms of cleaned-up plasma samples.
a. (left): a sample of a healthy volunteer without supplements. Peak 2 corresponded to 19 pmol *trans*-UCA.
b. (right): a sample supplemented with *cis*- and *trans*-UCA, *cis*-2-methylUCA and ATAA, followed by clean-up procedure (see text for final concentrations). In both cases the injected volumes were 100 µl.
Peak identity: 1. *cis*-UCA, 2. *trans*-UCA, 3. *cis*-2-methylUCA, 4. ATAA.

When ultrafiltration was used instead of the above mentioned deproteinization procedure, more than 86% binding of these compounds to proteins was observed after mixing of the above standard compounds with 10% bovine serum albumin in phosphate buffered saline. These findings suggest a substantial protein binding of UCA isomers in blood.

3.4. *Cis- and trans-UCA levels in skin, urine and plasma of UV-B-irradiated volunteers*

The kinetics of *cis*-UCA levels were studied in four volunteers that received a single total body UV-B exposure. Skin filter samples, urine and plasma were taken at several time points, having pre-exposure samples as basic level controls. Epidermal *cis*-UCA levels reached a maximum immediately upon irradiation and a plateau level persisted for 1 to 2 days. The skin levels in absolute terms, derived from two time points in this plateau period, were 8.3 (± 2.9) and 6.3 (± 2.4) nmol/cm^2 for *cis*- and *trans*-UCA, respectively (4 individuals; 44 samples). In other terms, UV-B irradiation reduces simultaneously epidermal *trans*-UCA concentration. Five to eight days after irradiation still a considerable amount (half of maximum) of epidermal *cis*-UCA was measurable and basic levels were reached after 17 -21 days (Fig. 2).

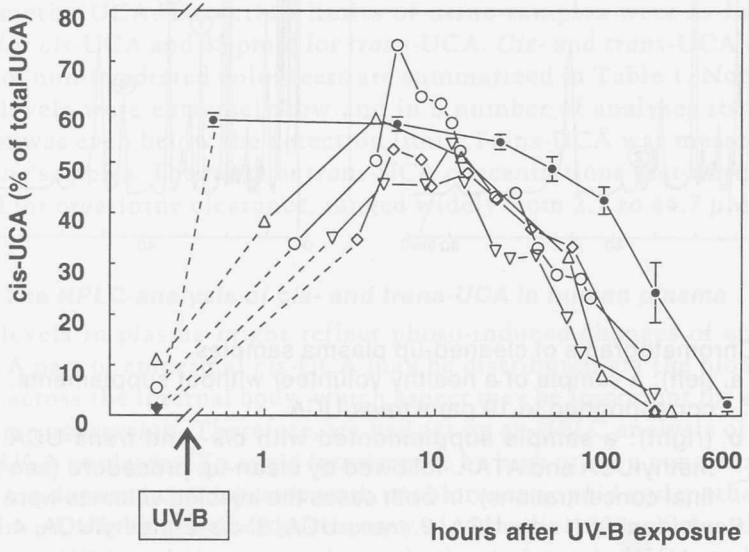

Fig. 2. The effect of UV-B irradiation on *cis*-UCA levels in epidermis (closed; black markers) and in urine (open; white markers). The irradiation dose was 250 J/m^2. Urine sampling did not proceed at same timepoints, therefore the excretion patterns are shown separately for each of the four volunteers. The epidermal *cis*-UCA levels of 4 volunteers are shown as mean value and the error bars represent the standard deviation. Each timepoint of the epidermal pattern represented at least 16 determinations from 4 volunteers. Human epidermis retained increased *cis*-UCA levels for approximately 3 weeks and human urine showed increased *cis*-UCA levels for 8 to 12 days.

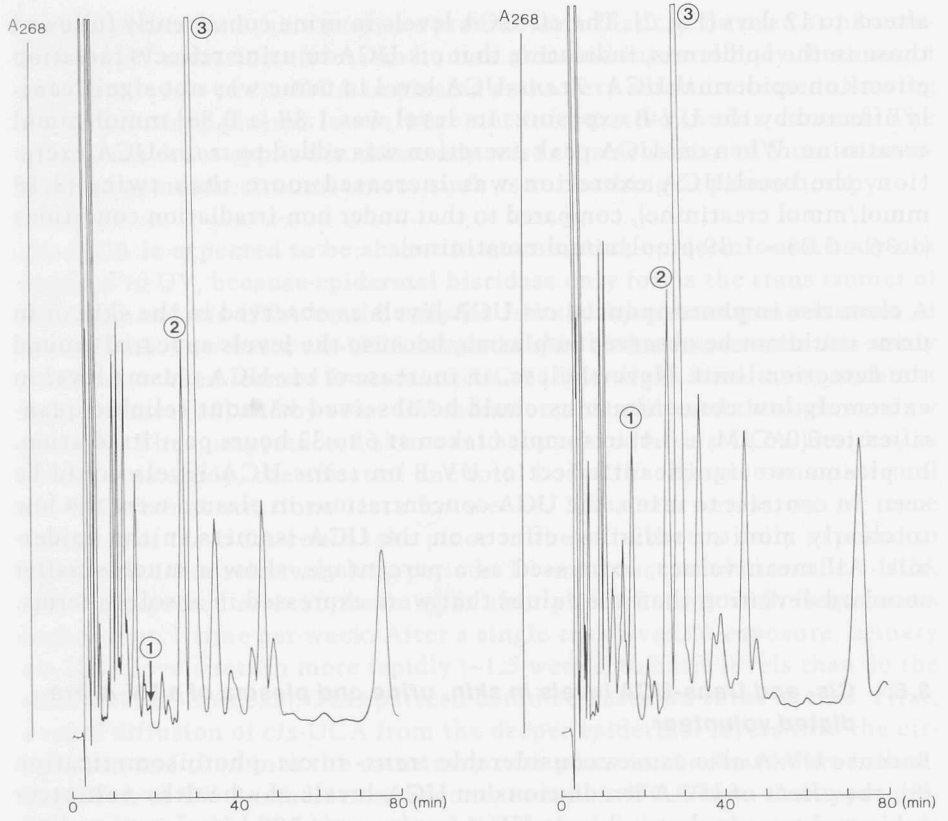

Fig. 3. Chromatograms of cleaned-up urine samples.
a. (left): from a healthy volunteer before irradiation.
b. (right): from the same person 16 hours after a single total body UV-B irradiation of 1 MED. In both cases 50 µl sample was injected into the HPLC-system. See text for further chromatographic conditions.
Peak identity: 1. *cis*-UCA, 2. *trans*-UCA, 3.*cis*-2-methylUCA.
Urinary *cis*-UCA level was increased upon UV-B irradiation.

The HPLC-chromatograms of cleaned-up urine samples, obtained before and 16 hours after a UV-B exposure of 1 MED clearly show the marked increase of *cis*-UCA concentration (Fig. 3a and 3b). The urinary *cis*-UCA concentrations reached a maximum at 5 to 12 hrs after the UV-B exposure. In absolute terms, maximum *cis*-UCA excretion was 1.77 (± 1.05) mmol/mmol creatinine and in relative terms 57% (± 9.1) of total UCA (4 individuals; 8 samples, taken around maximum excretion). After one day the *cis*-UCA level dropped fast, followed by a slow decline. Basic levels were reached

after 8 to 12 days (Fig. 2). The *cis*-UCA levels in urine consistently followed those in the epidermis, indicating that *cis*-UCA in urine reflects radiation effects on epidermal UCA. *Trans*-UCA level in urine was not significantly affected by the UV-B exposure. Its level was 1.38 (± 0.83) mmol/mmol creatinine. When *cis*-UCA peak excretion was added to *trans*-UCA excretion, the total-UCA excretion was increased more than twice (3.15 mmol/mmol creatinine), compared to that under non-irradiation conditions (1.36 + 0.03 = 1.39 µmol/mmol creatinine).

A clear rise in photo-induced *cis*-UCA levels as observed in the skin or in urine could not be observed in plasma, because the levels appeared around the detection limit. Nevertheless, an increase of *cis*-UCA plasma level in extremely low concentrations could be observed without reliable quantification (0.6 µM, ± 0.4) in samples taken at 6 to 32 hours post-irradiation. In plasma no significant effect of UV-B on *trans*-UCA levels could be seen. In contrast to urine, the UCA concentrations in plasma were too low to clearly monitor radiation effects on the UCA-isomers in the epidermis. All mean values, expressed as a percentage, show a much smaller standard deviation than the values that were expressed in absolute terms.

3.5. *Cis- and trans-UCA levels in skin, urine and plasma of a UV-A-irradiated volunteer*

Because UV-A also causes considerable *trans*- to *cis*- photoisomerization (8), the effect of UV-A irradiations on UCA-levels of a healthy volunteer subjected to a single total body UV-A exposure of 200 kJ/m^2 was studied. The results indicated an essentially similar effect as that obtained after a single UV-B irradiation of 250 J/m2.
The initial epidermal plateau level upon UV-A irradiation was 10.8 (± 1.8) and 7.9 (± 1.1) nmol/cm^2 for *cis*- and *trans*-UCA, respectively (1 individual, 11 samples). In relative terms these levels (58% *cis*-UCA and 42% *trans*-UCA) were very close to those obtained after UV-B irradiation. Again, plasma levels of *cis*-UCA were too low to register a clear irradiation effect. In conclusion, the effect of this UV-A irradiation on *cis*-UCA levels in skin, urine and plasma is similar to the effect obtained with UV-B irradiations.

4. Discussion

In this study we found low, but significant, amounts of *cis*-UCA in skin and urine of normal, non-irradiated persons and increased amounts after a single whole body UV-B or UV-A exposure. This increased level of *cis*-UCA was retained in the body for several days. There was a consistent correlation between urinary and epidermal *cis*-UCA kinetics. Urine samples are

easy to obtain and their analyses provide data that can be used as parameters for UV radiation effects on human skin and internal body. Because of very low levels of *cis*-UCA in plasma and a very small increase following UV irradiation, plasma levels were not informative in this context. The irradiation doses applied in this study were representative of those encountered in common circumstances such as sunbathing or phototherapy.

Cis-UCA is expected to be absent in mammalian epidermis and body not exposed to UV, because epidermal histidase only forms the trans isomer of UCA (1) and *cis*-UCA could only be formed by photoisomerization of *trans*-UCA. Low basic *cis*-UCA levels in skin and urine seen in this study could have been formed from *trans*-UCA (7) by light from many ambient light sources (28) and by solar UV radiation, even filtered through window glass (8). Thus, unprotected skin could contain some *cis*-UCA. Besides, as shown this study, the effect of one total body UV-exposure on epidermal *cis*-UCA concentrations lasts for weeks. Thus, accumulation of trace amounts of *cis*-UCA could take place in the skin that is daily exposed to light sources for relatively long periods. Dramatic accumulation of *cis*-UCA may be expected after sunbathing or after phototherapy with frequencies higher than 1 time per week. After a single effective UV-exposure, urinary *cis*-UCA levels return more rapidly (~1.5 weeks) to basic levels than do the skin levels (~3 weeks). This pattern could be based on three events. First, a rapid diffusion of *cis*-UCA from the deeper epidermal layers into the circulation and then into the urine may take place in the first hours following UV-A or UV-B irradiation. Second, some *cis*-UCA from the upper epidermal layers may move inwards while the remaining might move upwards followed by horny layer slough off. Third, some *cis*-UCA may be retained by organs or glands and subsequently released.

Much higher levels of UV-B induced *cis*-UCA were found by a *cis*-UCA specific enzyme linked immunosorbent assay (ELISA) in murine serum (29), following the application of UV-B irradiation doses (2160 J/m^2 or 2 x 1440 J/m^2) to the murine epidermis. Several factors may explain the high *cis*-UCA levels in murine serum. Because of their small size, the ratio of murine skin surface to body volume is larger, therefore a relatively larger quantity of epidermal *cis*-UCA may diffuse into the circulation. Second, this effect might be amplified by a more rapid diffusion from the thinner murine epidermis. Moreover, the larger UV-B doses in this murine study may have caused loss of epidermal integrity with subsequent leakage of soluble components, such as *cis*-UCA, into the circulation. However, the kinetics of UV-induced epidermal *cis*-UCA were rather similar as a plateau level existed for at least 16 hours post-irradiation and seven days later *cis*-UCA level was halved (21).

We did not measure a reported urinary condensation product of UCA (mainly the *cis*-isomer) and L-cysteïne, namely 3-[(carboxymethyl)thio]-3-(1H-imidazol-4-yl) propanoic acid, in normal human urine (30) as its urinary excretion was very small (0.04 to 0.07 mM), less than 1% of the maximum urinary *cis*-UCA excretion found in our investigation.
The role of normal and increased systemic *cis*-UCA levels following UV exposure in relation to immunosuppression remains to be established. Several questions arise that are difficult to answer at this moment; do basic levels of *cis*-UCA exert immunosuppressive effects or is the immunosuppression only restricted to UV-exposed persons whose *cis*-UCA levels crossed a certain threshold? If there is no threshold level, do people live with a certain degree of immunosuppression? Alternatively, it may be hypothesized that a *cis*-UCA derivative mediates the UV-induced immunosuppression.

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PART II

OXIDATION OF UROCANIC ACID BY HYDROXYL RADICALS

CHAPTER 5

Urocanic acid isomers are good hydroxyl radical scavengers: a comparative study with structural analogs and with uric acid

Arthur Kammeyer[a], Teunis A. Eggelte[b], Jan D. Bos[a], Marcel B.M. Teunissen[a]

[a]Department of Dermatology Academic Medical Center, P.O. Box 22660, 1100 DD Amsterdam, Netherlands
[b]Department of Clinical Pharmacology Academic Medical Center, P.O. Box 22660, 1100 DD Amsterdam, Netherlands

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Abstract

UV-exposure of the epidermis leads to the isomerization of *trans*-UCA into *cis*-UCA as well as to the generation of hydroxyl radicals. It was shown by the deoxyribose degradation test that UCA isomers are more powerful hydroxyl radical scavengers than the other 4-(5-) substituted imidazole derivatives, such as histidine, though less powerful than uric acid. UCA, present in relatively high concentrations in the epidermis, may well be a major natural hydroxyl radical scavenger.

Trans-urocanic acid (*trans*-UCA) is a major ultraviolet (UV) absorbing component of the human epidermis. Absorption of UV radiation from the UV-C region (200-290 nm) into the UV-A-I region (340-400 nm) causes photoisomerization of *trans*-UCA into *cis*-UCA *in vivo* as well as *in vitro*

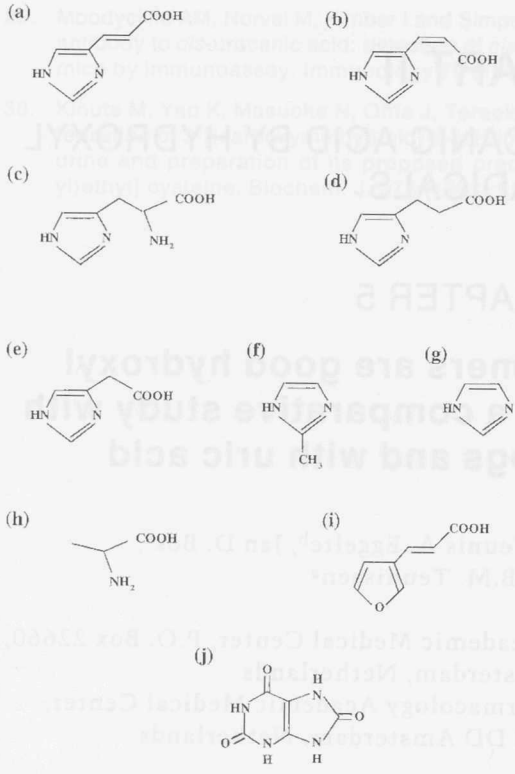

Fig. 1. Compounds tested in this study for hydroxyl radical scavenging ability.

a. *trans*-UCA,
b. *cis*-UCA,
c. L-histidine,
d. dihydro-UCA or 3-(imidazol-4-yl)propionic acid,
e. imidazole acetic acid,
f. 2-methyl-imidazole,
g. imidazole,
h. L-alanine,
i. *trans*-2-furylacrylic acid,
j. uric acid.

[1-3]. Because of this property, *trans*-UCA had been used as natural sunscreen agent [4]. This use had later been minimized since it became clear that photoproduct *cis*-UCA can mimic some of the effects of UV on immunity, suggesting that this compound is an important mediator of UV-induced immunosuppression [5]. UV exposure of the skin causes an increased level of oxidative stress with the inherent formation of reactive hydroxyl radicals [6]. These species can be generated from hydrogen peroxide upon UV irradiation and upon contact with metal ions (e.g. Fe^{2+} and Cu^{2+}), the Fenton reaction. Both types of reaction can occur in the epidermis [7]. Under these conditions, UCA isomers may interact with the randomly produced hydroxyl radicals in situ.

In this study we tested *in vitro* the hydroxyl-radical scavenging ability of both UCA isomers, of chemically related compounds, and of known scavenger uric acid. The results of this comparative study point to certain molecular structures required for good hydroxyl radical scavenging ability and provides a ranking of *trans*-UCA and *cis*-UCA among other (known) scavengers.

Table 1. The hydroxyl radical scavenging ability of urocanic acid isomers and related compounds

Hydroxyl radical scavenger	Second order rate constant x 10^9			Inhibition of deoxyribose degradation [scavenger] = [deoxyribose] = 3 mM
	$M^{-1}.s^{-1}$	S.D.	n [b]	%
Imidazoles				
***trans*-Urocanic acid**	**8.0**	**0.9**	**8**	**67**
***cis*-Urocanic acid**	**7.1**	**0.6**	**6**	**64**
L-Histidine	2.6[c]	0.9	4	34
Dihydrourocanic acid	2.7	0.9	3	34
Imidazole-4-acetic acid	2.2	0.1	3	30
Imidazole	13.0	0.9	5	78
2-Methylimidazole	11.7	2.6	5	76
Other compounds				
L-Alanine	0.1	0.0	3	2
trans-2-furylacrylic acid[a]	< 0.1	-	3	< 2
Uric acid	27.8	3.0	4	91

a. *trans*-2-furylacrylic acid was not tested in concentrations > 8 mM because of poor solubility.
b. n represents the number of slopes from which the rate constant was calculated.
c. 2.3 - 3.0 x 10^9 $M^{-1}.s^{-1}$ in literature [8].

Trans-UCA, *cis*-UCA, L-histidine, dihydrourocanic acid [3-(imidazol-4-yl) propionic acid], imidazole-4-acetic acid (sodium salt), imidazole, 2-methylimidazole, alanine, *trans*-2-furylacrylic acid and uric acid (Fig. 1) were tested on their ability to scavenge hydroxyl radicals by means of the deoxyribose (dR) degradation test. Upon exposure to hydroxyl radicals dR is degraded into malondialdehyde, which reacts with thiobarbituric acid to form a pink chromogen. Hydroxyl radical scavengers will compete with dR, resulting in a reduced amount of malondialdehyde. This dR degradation test was analogous to an earlier described method [8]. Briefly, the reactions were performed in 5 ml screw cap glass tubes in a final volume of 1.0 µl sodium phosphate buffer (50 mM; pH 7.2), containing 3.0 mM 2-deoxy-D-ribose, 0.5 mM hydrogen peroxide and one of the test compounds at graded

concentrations. The reaction was started by the addition of premixed disodium EDTA and ferrous iron solutions (final concentrations 0.5 mM and 0.2 mM, respectively).
Ferrous ammonium sulphate served as source for ferrous ions (Fe^{2+}). Fe^{2+} solutions were freshly prepared each time and were purged with nitrogen. The mixture was left for 15 minutes at room temperature. After addition of 1.0 mL 1% thiobarbituric acid in 50 mM NaOH and 0.75 ml 2.8% trichloroacetic acid, the tubes were heated for 20 minutes in a boiling water bath. The pink color was read at 532 nm and reciprocal absorption values were plotted against the concentration of the test compound after subtraction of appropriate blanks.
A series of six duplicate determinations from test compound dilutions was employed to construct a graph slope for the calculation of a rate constant value. A typical graph with slopes to derive rate constants from is shown in Fig. 2 for both UCA isomers. The mean, SD, number of rate constants and the percentage of inhibition of deoxyribose degradation at equimolar concentrations of scavenger (3 mM) is calculated for each test compound and summarized in Table 1.

Trans-UCA and *cis*-UCA are substantially stronger in scavenging hydroxyl radicals (8.0 and 7.1 x 10^9 $M^{-1}.s^{-1}$, respectively), than the other 4-(5-)-substituted imidazoles, including L-histidine (2.6 x 10^9 $M^{-1}.s^{-1}$). L-histidine, the precursor of UCA, was included as a known scavenger [8-10] with structural similarities to UCA. L-alanine was used as a known poor scavenger [10]. Trans-FAA was tested as a non-imidazole acrylic acid derivative, having a furan ring instead. This substitution yielded a very poor scavenging ability. Other 4-(5-) substituted imidazole analogues, dihydrourocanic acid or 3-(imidazol-4-yl)-propionic acid and imidazole-4-acetic acid, showed moderate scavenging ability, comparable to histidine. However, unsubstituted imidazole and its 2-methyl derivative appeared to be stronger scavengers than the UCA isomers.

The known strong hydroxyl radical scavenger uric acid [11] showed an excellent scavenging ability (27.8 x 10^9 $M^{-1}.s^{-1}$). To summarize, *trans*-UCA and *cis*-UCA, two epidermal compounds, are good hydroxyl radical scavengers. Their scavenging ability is weaker than that of uric acid, but larger than the other 4(-5-) substituted imidazoles, e.g. histidine.
Trans-UCA and *cis*-UCA occur in substantial concentrations in the epidermis, the latter in the UV-exposed skin. There is strong evidence for the occurrence of hydroxyl radicals in the epidermis, especially upon UV irradiation [7]. Normal human skin contains approximately 200 µM iron [12,13], predominantly complexed to ferritin. The release of free ferrous ions by UV irradiation [14] and the presence of hydrogen peroxide [15,16] are pre-

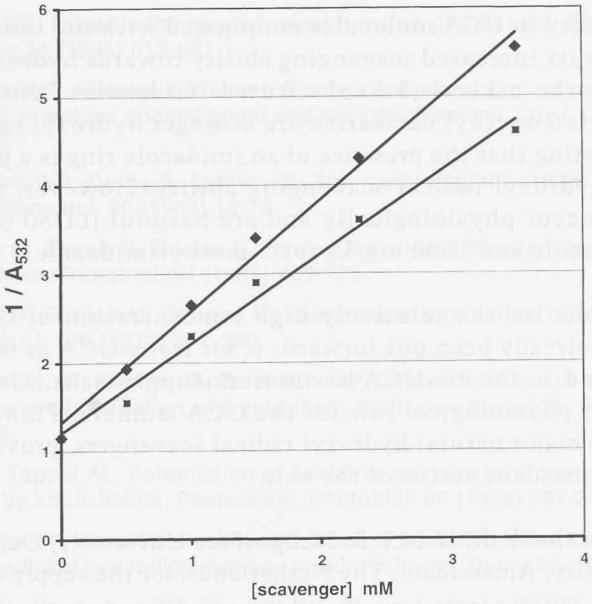

Fig. 2. A determination of the second order rate constants of *trans*-UCA (◆) and of *cis*-UCA (■) with hydroxyl radicals. The rate constant was derived from the slope of the line (k = slope x k_{dR} x [dR] x A_o), where A_o is the absorbance, measured in the absence of hydroxyl radical scavenger. k_{dR} was taken as 3.1×10^9 $M^{-1}s^{-1}$, derived from pulse radiolysis studies [8], and [dR] = 3 mM. The rate constants in this particular set were 8.49 and 7.33×10^9 $M^{-1}s^{-1}$ for *trans*-UCA and *cis*-UCA, respectively. The other scavengers were studied similarly.

requisites for the generation of hydroxyl radicals. Other reports indicate the UV-induced presence of hydroxyl radicals indirectly since their effects on epidermal constituents could be neutralized with antioxidants [17,18]. UCA is an imidazole compound and several other imidazole derivatives have already been shown to be good hydroxyl radical scavengers, e.g. histidine [8-10], histamine [19], histidine containing dipeptides [10,20], cimetidine and other histamine (H2) receptor antagonists [21]. This study reveals that several other imidazoles show similar properties (Table 1). Hydroxyl radicals can react with the imidazole ring to form imidazolone derivatives. Their formation has led to the proposal to use the imidazolones of histidine and histamine as markers for oxidative stress [9,19]. The importance of the imidazole ring in UCA molecules was also demonstrated in our experiments. The poor scavenging ability of *trans*-FAA, having a furan ring instead, was a remarkable contrast. Furthermore, the presence of the

acrylic acid moiety in UCA molecules conjugated with the imidazole ring may account for its increased scavenging ability towards hydroxyl radicals as compared to the other 4-(5-) substituted imidazoles. Unsubstituted imidazole and its 2-methyl derivative are stronger hydroxyl radical scavengers, accentuating that the presence of an imidazole ring is a prerequisite for sufficient hydroxyl radical scavenging ability. However, these compounds do not occur physiologically and are harmful (LD50 oral rat 220 mg/kg for imidazole and 1500 mg/kg for 2-methylimidazole).

Two explanations for the relatively high concentration of UCA in the epidermis have already been put forward: 1. for *trans*-UCA as natural sunscreen agent and 2. for *cis*-UCA as immunosuppressant. Our findings point to another physiological role for the UCA isomers. *Trans*-UCA and *cis*-UCA may be major natural hydroxyl radical scavengers, providing a new view on the antioxidant status of the skin.

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CHAPTER 6

Oxidative breakdown and conversion of urocanic acid isomers by hydroxyl radical generating systems

Arthur Kammeyer[a],* Teunis A. Eggelte[b], Henk Overmars[c], Albert Bootsma[c], Jan D. Bos[a], Marcel B.M. Teunissen[a]

a) Department of Dermatology. b) Clinical Pharmacology and c) Laboratory of Genetic Metabolic Diseases, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

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Abstract

Cis-urocanic acid (*cis*-UCA), formed from *trans*-urocanic acid (*trans*-UCA) by photoisomerization, has been shown to mimic suppressive effects of UV on the immune system. It is our hypothesis that UCA oxidation products in the skin play a role in the process of immunosuppression. Recently, both UCA isomers were found to be good hydroxyl radical scavengers and in this context we investigated the formation of products resulting from the interaction of hydroxyl radicals with UCA. Hydroxyl radicals were generated by 1. UV/H_2O_2 (photooxidation), 2. ferrous ions/H_2O_2 (Fenton oxidation) and 3. cupric ions/ascorbic acid. Oxidation products were identified by spectrometric methods and assessed by reversed phase HPLC analysis. The photooxidation of UCA was induced by UV-B and UV-C, but not by UV-A radiation. Photooxidation and Fenton oxidation of *trans*-UCA, as well as of *cis*-UCA yielded comparable chromatographic patterns of UCA oxidation products. Several of the formed products were identified. The formation of three identified imidazoles was shown in UV-B exposed corneal layer samples, derived from human skin.

1. Introduction

Trans-urocanic acid (*trans*-UCA) is a major ultraviolet (UV)-ab-sorbing component of the human epidermis. Absorption of UV radiation from the UV-C region (200-290 nm) into the UV-AI region (340-400 nm) causes photoisomerization of epidermal *trans*-UCA into *cis*-UCA *in vivo* as well as *in vitro* (1-3). *Cis*-UCA has been shown to mimic some, though not all, of the effects of UV on immunity, suggesting that this compound is an important mediator of UV-induced immunosuppression (4). Although there is experimental evidence for the immunosuppressive potential of *cis*-UCA (4-9), the failure to produce this effect in several models *in vitro* is puzzling (10-13). To seek an explanation for the seemingly contradictory results of *in vivo* and *in vitro* experiments, we suggest the involvement of products formed from UCA isomers upon interaction with reactive oxygen species (ROS). Until now, most attention has been focussed on the photo-isomerization of UCA and only some studies refer to other photochemical aspects (14). Formation of *cis*-UCA from *trans*-UCA may be only part of the initiation of immunosuppression: similar levels of *cis*-UCA can be induced by UV-A and UV-B, whereas only UV-B induces immunosuppression (15). The hypothesis of a possible involvement of ROS is supported by the observations that antioxidants have an abrogatory effect on the *cis*-UCA induced immunosuppression (16,17). There is strong evidence for the generation of hydroxyl radicals in the epidermis. Hydroxyl radicals can be generated under physiological conditions from hydrogen peroxide upon UV irradiation or from hydrogen peroxide with transition metal ions, predominantly ferrous (Fe^{2+}; Fenton reaction) and cuprous (Cu^{+}) ions (18,19). Normal human skin contains approximately 200 µM iron, predominantly complexed to ferritin (20,21). The release of free ferrous ions by UV irradiation (22), the presence of cellular hydrogen peroxide (23,24) and UV itself in combination with hydrogen peroxide (25) are the prerequisites for the generation of hydroxyl radicals. Under conditions of oxidative stress, generated hydroxyl radicals may react with the UCA isomers, which were earlier recognized as good hydroxyl radical scavengers (26). In this paper oxidation products under conditions of photooxidation by UV-B irradiation and through Fenton oxidation. Several of the formed products have been identified and could also be demonstrated in UV-B exposed corneal layer samples of human skin.

2. Materials & methods

2.1. Chemicals

Trans-UCA, L-ascorbic acid, imidazole-4-carboxylic acid, imidazole-4-carboxaldehyde, imidazole-4-acetic acid (sodium salt), 4-(hydroxymethyl)

imidazole-HCl and tetrabutylammonium hydroxide were supplied by Sigma-Aldrich/Fluka Chemie BV (Zwijndrecht, The Netherlands). *Cis*-UCA was kindly supplied by dr. W.M.P.B. Menge of the Free University, Department of Pharmacochemistry, Amsterdam, The Netherlands. Ferrous ammonium sulphate hexahydrate was obtained from Brocades-ACF (Maarssen, The Netherlands) and copper(II) sulphate pentahydrate from BDH Chemicals Ltd. (Poole, England). These salts served as sources for ferrous ions (Fe^{2+}) and cupric ions (Cu^{2+}). Fe^{2+} solutions were freshly prepared each time and were argon purged. The remaining chemicals were obtained from Merck KGaA (Darmstadt, Germany). Ultrapure water was used throughout.

2.2. High Performance Liquid Chromatography (HPLC)

Trans-UCA and *cis*-UCA were separated from each other and from UCA oxidation products on 4.6 x 250 mm reversed-phase columns with a flow of 0.8 ml/min, delivered by P-3500 HPLC-pumps (Pharmacia, Uppsala, Sweden). Isocratic elution was performed with either 10 mM sodium phosphate, containing 1-2 mM tetrabutylammonium hydrogen sulphate and 0-2% acetonitrile (pH 7.2) on an Alltima C_{18} column (Alltech, Deerfield, IL) or with 10 mM ammonium formate buffer, containing 0.4 mM tetrabutylammonium hydroxide and 4% acetonitrile (pH 7.2-7.5) on a Luna C_{18} column (Phenomenex, Torrence, CA). pH was adjusted with 0.1 M formic acid. Samples of 20 to 200 µL were injected by a Promis II autosampler (Spark Holland, Emmen, The Netherlands) and chromatographic data of experiments *in vitro* were recorded on an SP 4270 integrator (Spectra Physics, San Jose, CA) and the data of corneal layer samples were recorded on a computer with software for chromatography (Jasco-Borwin, JMBS Developpements, Le Fontanil, France). Peak area data from samples were only processed under identical HPLC circumstances. A UV-detector (Applied Biosystems, model 759A, Foster City, CA) was set for either 210, 226 and 257 nm detection, depending on the application.

2.3. Perchloric acid extraction of corneal layer of UV-exposed skin

Scrapings of corneal layer were collected from the sole of the foot, micronized with a dismembrator to obtain a powder, vacuum-dried and kept at -20 °C until use. Before UV-irradiation, an amount (~10 mg) of micronized corneal layer powder was moistened with 20 µl water and sandwiched between quartz plates.

For the extraction of UCA oxidation products from irradiated corneal layer material, the samples were first shaken with pure diethylether to remove lipid material. The residue was dried in a mild flow of argon gas. Then, 400

µl of perchloric acid solution (0.6 M) was added and the samples were allowed to stand for 5 minutes with occassional agitation. The perchloric acid solutions were neutralized with approximately 60 µl potassium hydroxide solution (4 M). Potassium perchlorate was allowed to precipitate, followed by centrifugation (3000 rpm for 5 min.). The supernatant was cooled in ice to obtain an additional precipitation. Finally, the sample was centrifuged with higher speed (13000 rpm for 10 min.) and the supernatant was passed through a 0.22 µm membrane filter prior to HPLC analysis.

2.4. *UV-irradiations for the induction of UCA photooxidation*

Photooxidation *in vitro* was performed in a 1-cm quartz cuvette, filled with 2.0 ml sample, which was placed in the parallel beam of a filtered 1000 W xenon arc lamp (Oriel, Stratford, CT). The solutions were magnetically stirred during irradiation. To minimize infrared (heat) radiation, the beam was passed through a water filter (7 cm), reflected by a dichroic mirror and filtered through a 1-mm UG11 filter. Short-wave cut off was achieved by passing the beam through WG280, WG305 or WG335 filters with 3 mm thickness each (Schott-Jena, Mainz, Germany). Xenon lamp emission filtered through WG280 included UV-C, UV-B and UV-A; through WG305 included UV-B and UV-A and through WG335 only UV-A was present. Two narrow bands in the UV-B and UV-A spectral regions were selected to monitor the xenon-arc emission. The probe of a calibrated EG&G 550 radiometer (Salem, MA, USA) was equipped with a neutral density filter, aligned with a narrow band filter type UV-M-IL (Schott-Jena, Mainz, Germany) with a transmission maximum of 21% at 303 nm and a half-width of 11.5 nm to monitor UV-B output. To monitor UV-A output, a filter type UV-PIL (Schott-Jena,) with a transmission maximum of 44% at 363 nm and a half-width of 18 nm was used. Transmission spectra of the optical filters were checked on a Perkin Elmer Lambda 40 UV/VIS spectrometer (Norwalk, CT, USA).

Corneal layer samples were UV-exposed with the use of a liquid light guide added to the solar simulator equipment. A positive quartz lens was placed behind the UG11 filter in such a way that the xenon-arc beam was focussed into the entrance aperture of the liquid light guide. The UV-B output in the UG11-transmitted UV-spectrum with a spectral distribution of approximately 280-400 nm was monitored as described above and a UV-B dose of 9.6 kJ/m^2 was used to induce photooxidation in the corneal layer sample to enable reliable HPLC-determination of the imidazolic UCA oxidation products. A control sample was similarly treated with the exception that the short wave part of the UV-spectrum (< 360 nm) was cut off with a KV 370 filter (Schott-Jena).

Additional UV-irradiations were performed with fluorescent tubes TL12, used as a UV-B source, and with TL10R, used as a UV-A source (Philips, Eindhoven, The Netherlands) on samples *in vitro* that were magnetically stirred in small Petri dishes. The UV-B output was measured with an IL 443 phototherapy radiometer, fitted with a SEE 1240 silicon detector probe and the UV-A output with an IL 442A phototherapy radiometer with a SEE 115 detector probe (International Light, Newburyport, MA, USA).

2.5. Fenton oxidation

Cis-UCA and *trans*-UCA isomers (40-250 µM) were oxidized with a hydroxyl-radical- generating system that consisted of various concentrations of ferrous ammonium sulphate (25-400 µM) with or without EDTA (500 µM) and a fixed hydrogen peroxide concentration of 500 µM (the Fenton reagent), either in sodium phosphate (20 mM) of pH 7.2, or in water. In addition, two hydroxyl radical-generating systems with copper ions (Cu^{2+}) were used, consisting of 50 µM copper(II)sulphate with either 500 µM hydrogen peroxide or 500 µM ascorbic acid. Metal ions were added as a final addition and this was considered to be the start of the oxidation reaction. Fe^{2+} and EDTA were premixed before addition. The reaction volume was most often 2 ml and after the usual reaction time of 10 min. the sample was immediately frozen (-18 °C) until use. Prior to HPLC-analysis the sample was thawed and filtered through 0.22 µm syringe filter.

2.6. UCA photooxidation on a preparative scale

Trans-UCA (8 mM) was photooxidized with hydrogen peroxide (40 mM) in a Petri dish with UV-radiation ($\lambda > 270$ nm) from Philips TL-12 fluorescent tubes. Irradiation was stopped when HPLC-analysis revealed an approximate of 97% breakdown of total-UCA (~16 hours). The final mixture was vacuum dried in a DNA Speedvac® device (Savant Instruments Inc., Farmingdale, NY) to remove volatile components, e.g. excess of hydrogen peroxide. The light-yellow residue was dissolved in water (approximately 1.0 g/l) for chromatographic separation or for other purposes and was referred to as PO-mix (photooxidation mix). PO-mix solutions were injected into the HPLC system and collected fractions were vacuum dried overnight. The removal of tetrabutylammonium ions was required for mass analysis and was performed by solid phase extraction (SPE) on C_{18} silica. The collected fraction was acidified with formic acid up to a concentration of 100 mM. Then, the sample (1-2 ml) was slowly aspirated through the reconstituted SPE column, followed by a wash step with 0.5 ml 100 mM formic acid. After collection, the solution was vacuum dried overnight.

2.7. Spectrometric analyses

UV-spectra were scanned on the spectrophotometer, used for optical filter measurement (see above). Mass spectra were obtained on a Quattro II triple quadrupole mass spectrometer (Micromass, Manchester, U.K.) by the electrospray technique. Capillary voltage was set at 3.5 kV and the cone voltage on 25 V. Samples, dissolved in methanol/water (80/20) were introduced by direct injection. Positive ion scans were made in the mass range of m/z 50-250. 1H NMR (400 MHz) spectra were recorded on a ARX 400 spectrometer (Bruker, Karlsruhe, Germany), using a dual probe.

3. Results

3.1. UCA isomers and photooxidation

The oxygen-oxygen bond of hydrogen peroxide can be cleaved by UV radiation to yield two hydroxyl radicals (25). Upon scavenging these radicals, UCA will be degraded and/or converted into oxidation products. The ability of simulated solar UV radiation to convert *trans*-UCA in the presence of hydrogen peroxide into photooxidation products was tested *in vitro*. The results, obtained by reversed-phase HPLC analysis, are shown in Fig.1 a-d. Under the conditions used, hydrogen peroxide eluted close to void volume and *trans*-UCA and *cis*-UCA eluted with markedly different elution times of 20 and 64 minutes, respectively. The unirradiated control sample showed that no interaction occurred between *trans*-UCA and hydrogen peroxide (Fig.1a). The exposure of 80 µM *trans*-UCA in the absence of hydrogen peroxide at pH 7.2 to WG280-filtered xenon-arc emission (including UV-C and UV-B) resulted only in the formation of *cis*-UCA via the process of photoisomerization (Fig.1b). However, when *trans*-UCA was irradiated in the presence of 500 µM hydrogen peroxide under identical conditions, several additional peaks appeared in the chromatograms and both *trans*-UCA and *cis*-UCA peaks were strongly reduced (Fig.1c). The degree of oxidative breakdown was comparable for both *cis*-UCA and *trans*-UCA, which finding is in accordance with recently published second order rate constants of both UCA isomers for hydroxyl radical scavenging (26).

In contrast, when exposures were performed with simulated solar radiation with UV-C and UV-B blocked out by a WG335 filter, only photoisomerization was seen with virtually no formation of photooxidation products (Fig.1d). Table 1 summarizes the irradiation conditions (condition 1-5) related to the extent of photooxidation, photoisomerization and breakdown of urocanic acid. The most extensive breakdown of UCA was obtained with 'full' UV (UV-C, -B and -A), and showed lowest amount of UCA that was left over. In addition, 'full' UV yielded the largest amount of photooxidation products (condition 1). The virtual absence of photooxidation

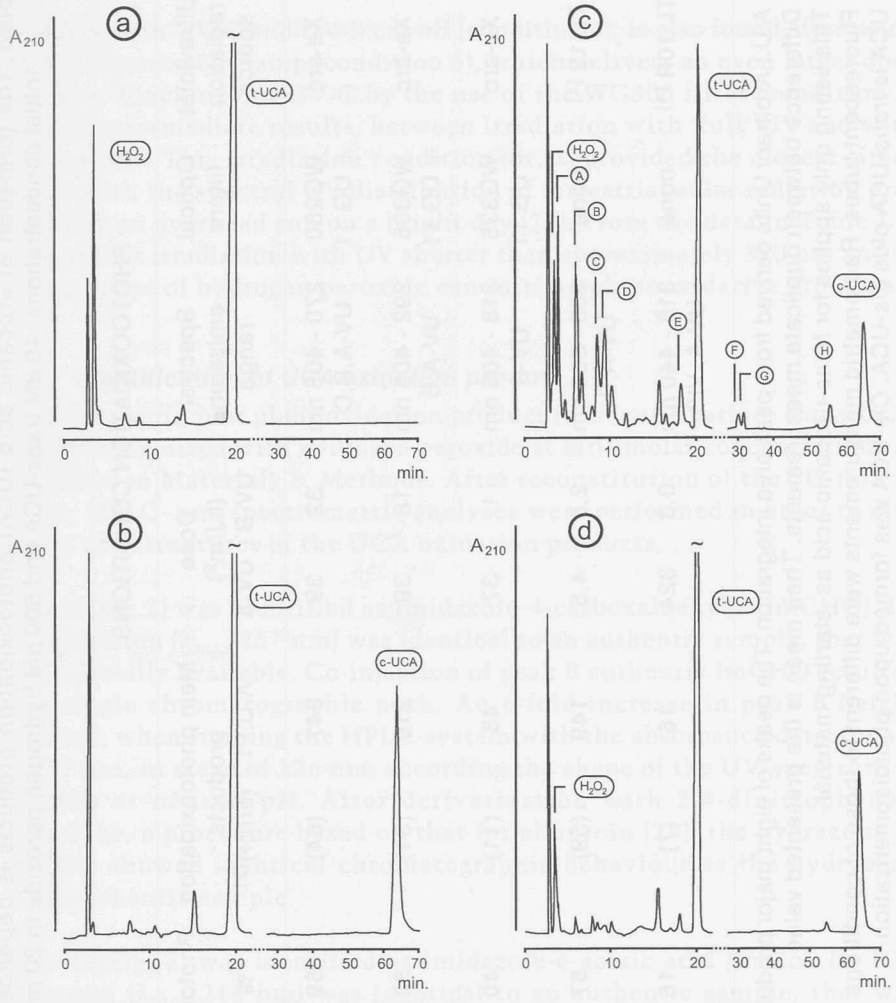

Fig. 1. Chromatograms of 80 μM *trans*-urocanic acid in 20 mM phoshate buffer pH 7.2. The initial concentration of hydrogen peroxide was 500 μM. Injection volume was 80 μL.

a. with hydrogen peroxide; not irradiated,
b. without hydrogen peroxide; irradiated with a WG280 filtered xenon-arc lamp,
c. with hydrogen peroxide and irradiated as 1b,
d. with hydrogen peroxide and irradiated with a WG335 filtered xenon-arc lamp. Peaks assigned with A-H correspond with photooxidation products. Separation was performed on a Alltima C_{18} column with UV detection at 210 nm. The eluent consisted of 10 mM sodium phosphate pH 7.2 with 1.0 mM tetrabutylammonium hydrogen sulphate. Further experimental conditions are described in the text.

Table 1. The formation of urocanic acid (UCA) photooxidation products is dependent on the spectral distribution of UV. Initial concentrations: 40 μM trans-UCA and 500 μM hydrogen peroxide in 20 mM sodium phosphate pH 7.2

	PHOTOOXIDATION CONDITIONS					RESULTS					
	Ultraviolet radiation source	Optical filters	Spectral emission range	Dose (kJ/m^2) UV-B	Dose (kJ/m^2) UV-A	Main photooxidation products A.U.[1]	Main photooxidation products (error)[2]	Photoisomerization[3] *cis*-UCA %	Photoisomerization[3] *cis*-UCA (error)[2]	UCA left[5] over %	UCA left[5] over (error)[2]
1.	Xe-arc	WG280 + UG 11	270 - 405 nm UV-A,B,C	32	39	**347**	(64)	**59**	(2)	**43**	(13)
2.	Xe-arc	WG305 + UG 11	292 - 405 nm UV-A,B	18	39	**219**	(31)	**53**	(4)	**64**	(7)
3.	Xe-arc	WG335 + UG 11	318 - 405 nm UV-A	1	37	**45**	(11)	**40**	(2)	**96**	(6)
4.	TL-12 [4]	none	280 - 350 nm UV-A,B	2,8	4,5	**149**	(59)	**57**	(3)	**90**	(24)
5.	TL 10R [4]	none	318 - 440 nm UV-A, VIS	0,3	324	**16**	(7)	**16**	(7)	**99**	(4)

[1] A.U.: Arbitrary Units derived from peak area integration. The peaks of eight major products, detected at 210 nm, were summed.
[2] Difference between duplicate measurements. Their mean is the presented value.
[3] This listing only applies for *trans*-urocanic acid as starting material.
[4] Fluorescent tubes. Radiometric measurements were different to those of conditions 1 - 3.
[5] UCA is *trans*-UCA plus *cis*-UCA. *Cis*-UCA was formed upon photoisomerization.

products with UV-C and UV-B cut off (condition 3), is also found after using a UV-A fluorescent lamp (condition 5), which delivers an even larger dose of UV-A. Blocking out UV-C by the use of the WG305 filter (condition 2) showed intermediate results, between irradiation with 'full' UV and with UV-A alone. This irradiation condition (nr. 2) provided the closest simulation with the spectral UV distribution of terrestrial solar radiation produced by an overhead sun on a bright day (27). From the data in Table 1 it is clear that irradiation with UV shorter than approximately 320 nm and in the presence of hydrogen peroxide can initiate photooxidative processes.

3.2. *Identification of UCA oxidation product.*

To obtain sufficient photooxidation product for identification, *trans*-UCA was photooxidized with hydrogen peroxide at millimolar concentrations as described in Materials & Methods. After reconstitution of the PO-mix in water, HPLC- and spectrometric analyses were performed in order to elucidate the structures of the UCA oxidation products.

Peak B (Fig.2) was identified as imidazole-4-carboxaldehyde (ImCHO). Its UV-spectrum (λ_{max} 257 nm) was identical to an authentic sample, that was commercially available. Co-injection of peak B authentic ImCHO resulted in a single chromatographic peak. An 6-fold increase in peak B height resulted, when running the HPLC-system with the absorbance detector set at 257 nm, in stead of 226 nm, according the shape of the UV-spectrum of ImCHO at neutral pH. After derivatization with 2,4-dinitrophenylhydrazine, a procedure based on that for allantoin (28), the hydrazone of ImCHO showed identical chromatographic behaviour as the hydrazone of an authentic sample.

Peak C (Fig. 2) was identified as imidazole-4-acetic acid (ImAc). Its UV-spectrum (λ_{max} 213 nm) was identical to an authentic sample, that was commercially available. Co-injection of peak C with authentic ImAc resulted in a single chromatographic peak. The dry sample was treated with methanol/HCl and n-butanol/HCl before mass spectrometric analysis. Peak masses $(M + H)^+$ of 141 and 183 were obtained for the methyl- and butylesters of imidazole-4-acetic acid. Mass spectra were identical to those obtained with the methyl- and butylesters of authentic imidazole-4-acetic acid.

Peak D (Fig. 2) was identified as imidazole-4-carboxylic acid (ImCOOH). Its UV-spectrum (λ_{max} 226 nm at pH values > 5, otherwise λ_{max} 212 nm) was identical to an authentic sample, that was commercially available. Co-injection of peak D with authentic ImCOOH resulted in a single chromatographic peak. Proton resonance (1H-NMR) analysis was done in D_2O, showing vinylic

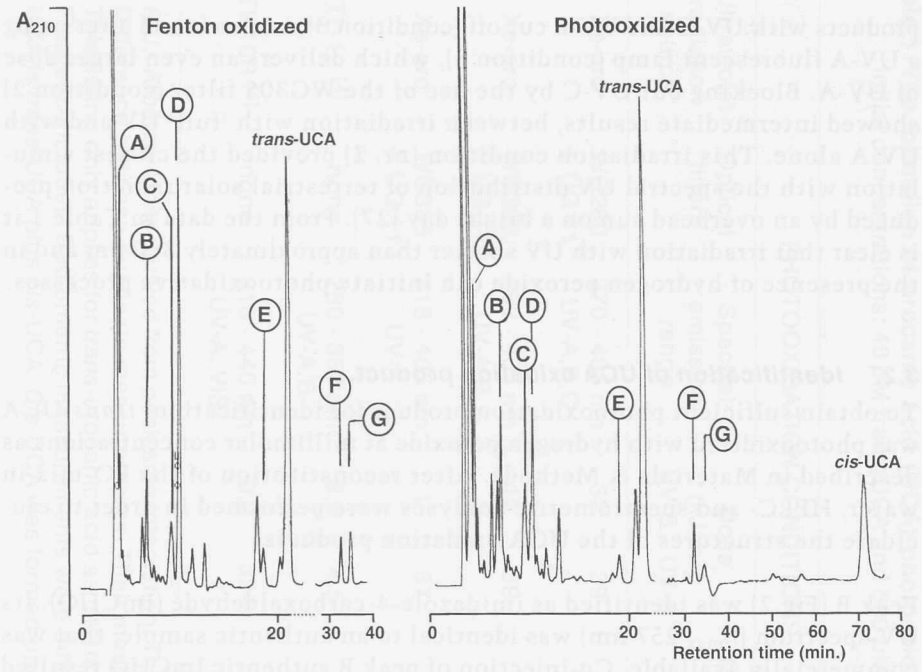

Fig. 2. Comparable chromatographic patterns in the formation of UCA oxidation products from 80 μM *trans*-UCA and 500 μM hydrogen peroxide in water (no buffer). Left: after Fenton oxidation with 250 μM Fe^{2+} and right:. after photooxidation with 'full' UV, containing a UV-B dose of 32 kJ/m^2. The *cis*-UCA peak is missing after Fenton oxidation, due to the absence of photoisomerization. Peak assignation (A-G) was done as in Figure 1c. Peak A is hydrogen peroxide. Peaks B,C and D refer to imidazole-4-carboxaldehyde, imidazole- 4-acetic acid and imidazole-4-carboxylic acid, respectively. Chromatographic conditions were identical to those applied in Figure 1, except the column was renewed.

protons in a ratio 1:1 with shifts of 7.76 and 7.53 ppm, similar to an authentic sample. Mass spectra of the methyl- and butylesters showed $(M + H)^+$ peaks at mass 127 and 169. Similar mass spectra were obtained from an authentic sample of ImCOOH. Checks on co-elution behaviour were carried out with various eluent compositions, to ensure the proposed identities.

Additionally, the formation of glyoxylic acid (GLX) could be demonstrated after derivatization with 2,4-dinitrophenylhydrazine. GLX was not seen in

Table 2. *Trans*-urocanic acid left over in hydroxyl radical generating systems [1]

	% (error) [2]				% (error) [2]
	in phosphate buffer pH 7.2		in water		in phosphate buffer pH 7.2
Fe^{2+} (μM)	- EDTA	+ EDTA[3] 500 μM	initial pH~5.3	Cu^{2+} (μM)	+ ascorbate 500 μM
0	**100** (1)	**100** (5)	**100** (3)	0	**100** (1)
50	**95** (1)	**85** (5)	**77** (11)	25	**19** (9)
100	**92** (6)	**76** (4)	**48** (7)	50	**18** (7)
200	**78** (3)	**52** (7)	**19** (8)	100	**14** (4)
400	**60** (12)	**23** (7)	**< 4**		

[1] All solutions were air saturated, except the iron(II) solution. Initial [hydrogen peroxide] = 500 μM and initial [*trans*-UCA] = 40 μM in all cases.
[2] Difference between duplicate measurements. Their mean value is presented.
[3] Na_2EDTA solution was premixed with iron(II) solution, immediately before the Fenton oxidation.

the chromatogram due to its low UV absorption, but it could be identified by HPLC technique as the 2,4-dinitrophenylhydrazone, based on a procedure for allantoin (28). GLX was detected in all photo- and Fenton oxidized UCA samples, that were derivatized with 2,4-dinitrophenylhydrazine.
Amino acid analysis with ninhydrine derivatization, carried out on the PO-mix, could demonstrate the formation of aspartic acid and glycine. These amino acids were also found on prolonged UV irradiation of UCA without the presence of hydrogen peroxide (29).

3.3. *UCA isomers and Fenton oxidation*

Next, we studied the Fenton oxidation, another natural oxidation process. *Trans*-UCA and *cis*-UCA were oxidized in *vitro* without UV exposure but in the presence of a transition metal ion and hydrogen peroxide. The initial hydrogen peroxide concentration was fixed at 500 μM in all experiments and the ferrous ion concentration was varied from 0 to 400 μM. Four sets of conditions were compared: 1. Fe^{2+} in phosphate buffer pH 7.2 , 2. Fe^{2+} in phosphate buffer plus EDTA , 3. Fe^{2+} without buffer with a initial pH of 5.5-5.3 and 4. Cu^{2+} in phosphate buffer plus ascorbate. Table 2 shows oxidative breakdown of *trans*-UCA with hydrogen peroxide in increasing order: condition 1 < 2 < 4 < 3.

Phosphate buffer of neutral pH was often used in studies that report transition metal driven oxidations of the Fenton type and the buffer was included here. However, the addition of Fe^{2+} at final concentrations of 100-400 µM to phosphate caused a turbid solution of insoluble iron phosphate. Under this condition the smallest degree of breakdown was obtained, probably due to a reduction of free Fe^{2+} in solution. On the other hand, complexation of Fe^{2+} to EDTA prior to addition of phosphate, resulted in a clear solution and a larger breakdown was found (Table 2). The largest breakdown was seen in the absence of phosphate buffer, dependent on the UCA concentration (here: 40, 100 or 250 µM) and the pH value was less defined (5.5-5.3). After the start of the Fenton reaction in water medium, there was a rapid fall of the pH value from 5.1 to 3.4, when 250 µM UCA was used. We attribute this effect to the formation of relatively strong acids, such as glyoxylic acid (GLX).

In another hydroxyl-radical-generating system, based on copper ions (Cu^{2+}), the combination of Cu^{2+}/ascorbic acid/hydrogen peroxide caused a large breakdown of *trans*-UCA (Table 2) and a moderate yield of UCA oxidation products, in favor of ImCOOH. Without ascorbic acid, the system with Cu^{2+} (50 µM) and hydrogen peroxide (500 µM) showed little breakdown (88% *trans*-UCA left; data not shown). For the situation *in vivo*, one must remember that the epidermal copper content is lower than iron (20).

Next, a comparison was made between *trans*-UCA and *cis*-UCA in breakdown and formation of oxidation products and the results are summarized in Table 3. Comparable extents of breakdown, though slightly less pronounced, were obtained with *cis*-UCA. This finding is in accordance with the comparable second order rate constants of *trans*-UCA and *cis*-UCA for hydroxyl radical scavenging (26). Hydrogen peroxide without Fe^{2+} had no effect on the UCA isomers at all; however, Fe^{2+} without hydrogen peroxide resulted in a slow, long-term breakdown of the UCA isomers (data not shown). The primary oxidation products formed are ImCHO and GLX. Additional experi-

[1]. Reaction conditions: 10 minutes at room temperature. Air saturated solutions were used throughout.
[2]. ImCHO = imidazole-4-carboxaldehyde
[3]. ImCOOH = imidazole-4-carboxylic acid
[4]. GLX = glyoxylic acid
[5]. EDTA was also degraded to GLX and may have been a competitor for the oxidative breakdown and conversion of UCA.
[6]. Due to photoisomerization, the breakdown of total-UCA (*trans*-UCA + *cis*-UCA) was calculated.

Table 3. Breakdown of urocanic acid (UCA) and the formation of urocanic acid (UCA) oxidation products in several hydroxyl radical generating systems. Quantities given per liter sample as mean value with its difference between duplicate measurements

Oxidation system[1]	Urocanic acid isomer Initial amount in µmol			UCA breakdown µmol		UCA breakdown %	Formation ImCHO[2] µmol		ImCOOH[3] µmol		GLX[4] µmol	
1. Phosphate pH 7.2 Fe^{2+} / H_2O_2 400 / 500 µM	**1.1.**	*trans*-UCA	40	**16**	(4)	**41**	**1,6**	(0.4)	**0,5**	(0.1)	**0,4**	(0.0)
	1.2.	*cis*-UCA	40	**13**	(0)	**32**	**1,4**	(0.1)	**0,3**	(0.0)	**0,5**	(0.1)
2. Phosphate pH 7.2 Fe^{2+} / EDTA / H_2O_2 400 / 500 / 500 µM	**2.1.**	*trans*-UCA	40	**31**	(8)	**77**	**2,7**	(0.2)	**0,2**	(0.0)	-[5]	
	2.2.	*cis*-UCA	40	**28**	(3)	**70**	**2,6**	(0.4)	**0,3**	(0.0)	-[5]	
3. No phosphate initial Fe^{2+} / H_2O_2 400 / 500 µM	**3.1.**	*trans*-UCA	40	**40**	(0)	**100**	**0,0**	(0.0)	**0,0**	(0.0)	**0,3**	(0.0)
	3.2.	*trans*-UCA	100	**97**	(18)	**97**	**7,9**	(0.5)	**5,1**	(2.5)	**5,0**	(0.7)
	3.3.	*trans*-UCA	250	**195**	(3)	**78**	**22,2**	(5.6)	**32,2**	(5.5)	**11,2**	(0.3)
	3.4.	*cis*-UCA	40	**40**	(0)	**100**	**0,0**	(0.0)	**0,0**	(0.0)	**0,6**	(0.1)
	3.5.	*cis*-UCA	100	**96**	(10)	**96**	**12,5**	(0.5)	**7,4**	(2.0)	**5,7**	(0.2)
	3.6.	*cis*-UCA	250	**188**	(21)	**75**	**46,9**	(5.5)	**31,1**	(1.2)	**11,7**	(0.4)
4. Phosphate pH 7.2 **UV-A,B,C** (Table 1.1) 500 µM H_2O_2	**4.1.**	*trans*-UCA	40	**22**[6]	(2)	**54**	**2,4**	(0.3)	**1,5**	(0.4)	**0,2**	(0.0)
	4.2.	*cis*-UCA	40	**10**[6]	(2)	**24**	**2,8**	(0.2)	**1,0**	(0.0)	**0,4**	(0.1)
5. No phosphate initial pH 5.5 **UV-A,B,C** (Table 1.1) 500 µM H_2O_2	**5.1.**	*trans*-UCA	40	**30**[6]	(5)	**75**	**2,7**	(0.1)	**0,4**	(0.0)	**0,2**	(0.0)
	5.2.	*cis*-UCA	40	**22**[6]	(2)	**56**	**2,6**	(0.5)	**0,4**	(0.0)	**0,5**	(0.0)
6. No phosphate initial pH ~5.5 **UV-A,B** (Table 1.2) 500 µM H_2O_2	**6.1.**	*trans*-UCA	40	**27**[6]	(7)	**67**	**2,3**	(0.0)	**1,2**	(0.4)	**0,1**	(0.0)
	6.2.	cis-UCA	40	**10**[6]	(3)	**25**	**2,2**	(0.3)	not detectable		**0,3**	(0.0)

ments in which ImCHO was used as starting material, a conversion to virtually 100% ImCOOH was obtained after Fenton- or photooxidation, indicating that ImCHO can easily be oxidized into ImCOOH under the conditions used in our experiments. HPLC analysis of a POmix-sample (see Methods) revealed that ImCOOH was the major 226 nm absorbing compound, while ImCHO concentration was largely reduced.

Trans-UCA and *cis*-UCA, in relatively high concentration of 250 µM, were broken down for 78% and 75%, respectively, by the unbuffered Fenton oxidation system. Table 3 section 3 also shows that the yield of oxidation products was proportional with the initial UCA concentration. Remarkably, the yield of ImCHO from *cis*-UCA in this oxidation system was substantially larger than from *trans*-UCA. In the phosphate buffered Fenton system breakdown and yield of oxidation products were low, but comparable, across an initial UCA concentration range from 40 to 250 µM (Table 3, section 1, only results of 40 µM are shown). In the presence of EDTA, a larger breakdown and a higher yield of ImCHO resulted (Table 3, section 2). This yield was increased as higher initial UCA concentrations were used. In the unbuffered Fenton system, the breakdown of UCA and the yield of oxidation products was the largest of all systems tested, if the initial UCA concentration was high (250 µM) (Table 3, section 3).

A close resemblance was observed between the chromatographic patterns of UCA Fenton oxidation products and those of UCA photooxidation products (Fig.2). In photooxidation, the breakdown of *cis*-UCA was substantially reduced in comparison with the *trans* isomer (Table 3, section 4-6). In Fenton oxidation, this effect was less pronounced. The data of Table 3 were given for air saturated solutions. Argon-purging of the solutions, prior to Fenton-or photooxidation, enhanced UCA breakdown as well as the yield of oxidation products, both by a factor 2 to 3. Heating (to 37 °C) of argon-purged solutions slightly enhanced the yield of ImCHO.

3.4. Detection of UCA oxidation products in UV-exposed corneal layer of human skin

The UV-B induced formation of imidazole-4-carboxaldehyde (ImCHO), imidazole-4-acetic acid (ImAc) and imidazole-4-carboxylic acid (ImCOOH), the imidazolic UCA oxidation products, could be demonstrated by HPLC analysis in corneal layer samples after UV-irradiation with a monitored UV-B dose of 9.6 kJ/m^2.

Confirmation of their formation in skin material was based on two observations. First, by comparing the chromatographic retention times of the

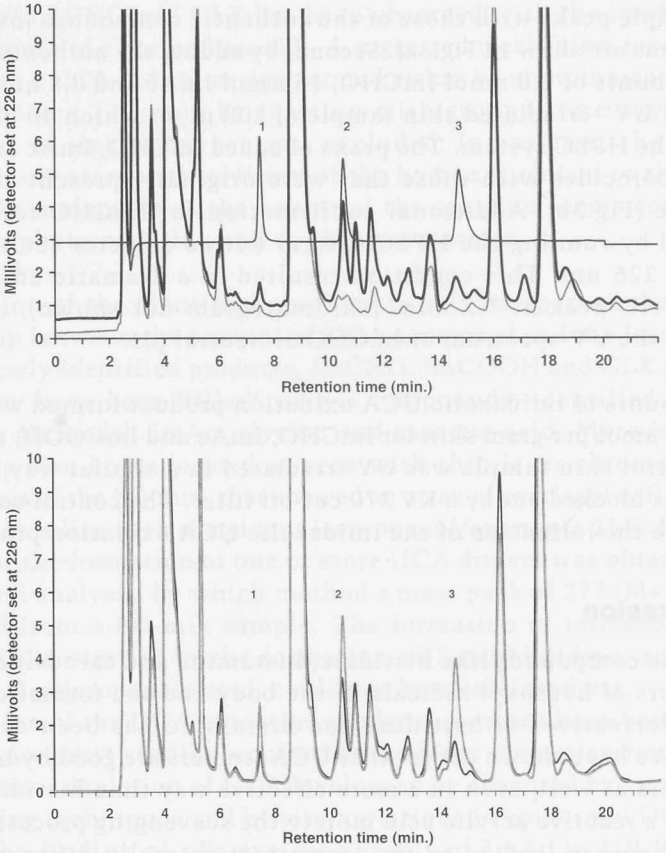

Fig. 3. Overlaid chromatograms showing the formation of the imidazolic UCA oxidation products by UV-B induced photooxidation in corneal layer samples. a. comparison of chromatographic retention times to ensure identity: the lower line was derived from a control skin sample that was exposed to UV with wavelengths > 360 nm; the middle line was derived from a skin sample that was exposed to full 'solar-like' UV (including UV-B). The formation of ImCHO, ImAc and ImCOOH can be seen as indicated by 1,2 and 3, respectively. The upper line was derived from a reference solution, containing the imidazoles in pure form. b. addition of authentic compounds to ensure identity: the lower line was derived from a skin sample irradiated with 'full UV' with a UV-B dose of 9.6 kJ/cm^2 and the upper line was derived from a similar skin sample to which the three authentic imidazoles (ImCHO, ImAc and ImCOOH) were added. A clear increase in peak height with unaffected peak symmetry, as indicated at 1,2 and 3, can be seen, confirming the presence of ImCHO, ImAc and ImCOOH in full UV exposed skin samples.

skin sample peaks with those of the authentic compounds (overlaid chromatograms are show in Fig.3a). Second, by adding the authentic imidazoles with amounts of 1.0 nmol ImCHO, 16 nmol ImAc and 0.8 nmol ImCOOH to a 'full UV'- irradiated skin sample of 200 µl, of which 40 µl was injected into the HPLC system. The peaks of added ImCHO, ImAc and ImCOOH clearly coincides with those that were originally present after full UV exposure (Fig.3b). Additional confirmation for ImCHO formation was obtained by running the HPLC system with a detector set at 257 nm in stead of 226 nm. This condition resulted in a dramatic enlargement of the ImCHO peak at 7.5 min. (chromatogram not shown), conform the shape of the UV-spectrum of ImCHO at neutral pH.

The amounts of imidazolic UCA oxidation product formed were 0.07, 1.4 and 0.04 µmol per gram skin for ImCHO, ImAc and ImCOOH, respectively. The control skin sample was UV-irradiated in a similar way, except that UV-B was blocked out by a KV 370 cut-off filter. The control conditions did not cause the formation of the imidazolic UCA oxidation products.

4. Discussion

Imidazole compounds like histidine, histamine and carnosine are natural scavengers of hydroxyl radicals in the body and the formation of imidazolone derivatives of histidine and histamine has been demonstrated (30,31). We have shown earlier that UCA isomers are good hydroxyl radical scavengers as well, even in a more effective way than histidine. As UCA contains a reactive acrylic acid moiety the scavenging process may take a different course leading to the formation of totally different products. We hypothesized that the formation of such products may contribute to the understanding of the immunomodulating properties of the UCA isomers. HPLC analysis of photooxidation and Fenton oxidation experiments of UCA isomers showed the formation of a number of products (Fig. 1-3).

ImCOOH was the first UCA oxidation product that was identified, because it was prominently formed *in vitro* upon photooxidation or Fenton oxidation having the reactants on preparative scale concentrations. Subsequently it became clear that ImCOOH was a readily derived oxidation product of ImCHO upon scavenging of hydroxyl radicals. The aldehyde was directly formed from UCA, with its counterpart GLX. The formation *in vitro* of ImCHO is more prominent than ImCOOH when using reactant concentrations in the physiological range (Table 3). Most likely GLX may have been further oxidized to oxalic acid and to CO_2. The shift towards lower pH values in the course of UCA oxidation in water without buffer may be indicative for the formation of acidic products.

Formation of ImCHO and GLX has been observed with the irradiation of UCA in presence of a purine base (32). A suggested mechanism for this reaction involved a UCA radical cation formed either by photoionization or by electron transfer, followed by reaction with ground state oxygen. The involvement of singlet oxygen was excluded. In our case, the reaction will most likely start with addition of the hydroxyl radical to the double bond of UCA molecules as the course of the reaction seems comparable between the photooxidation and the Fenton reaction.

The formation of the identified products is only one part of the story as there is a gap between the amount of UCA converted and the formation of the three newly identified products, ImCHO, ImCOOH and GLX (Table 3). The gap may have been 'filled' with 3 compounds, identified from the POmix (see Methods), ImAc, glycine and aspartic acid. Moreover, other products are also being formed as seen with thin-layer chromatography (TLC) analysis of the PO-mix, that showed an array of overlapping fluorescent spots (TLC on silica with the eluent isopropanol/ammonia 25% 4: 1). Evidence for the formation of one or more UCA dimers was obtained from mass spectral analysis, by which method a mass peak of 277 ($M+H^+$) could be recorded from a PO-mix sample. The formation of imidazolon compounds of UCA similar to the formation of 2-oxohistidine, such as (4-imidazolon-2-yl)propenoic acid, could not be established yet.
The formation of the UCA oxidation products may not have been noticed before because most studies focussed only on the isomerization of *trans*-UCA and minor depletion of UCA levels may be obscured by variations in skin sampling techniques and by site-variations in epidermal UCA levels (33, 34). The finding of relative high levels of ImAc in UV-B exposed corneal layer samples (section 3.4) is in contrast with the experiments *in vitro* and may also be derived from other skin precursors than UCA.

As shown in this study, UV-A irradiation only results in UCA photoisomerization and virtually not in UCA photooxidation. The lack of correlation between UV-A-induced *cis*-UCA formation and immunosuppression (15) may suggest a possible role for UCA oxidation products in skin immunity, because it was shown here that UCA oxidation products can only be formed with UV-waves shorter than UV-A (< 320 nm). Another indication for an active role of UCA oxidation products in skin immunity is the finding that antioxidants abrogate UV- or *cis*-UCA-induced immunosuppression. Under these conditions the formation of UCA oxidation products may have been prevented, so that the induction of immunosuppression by UCA oxidation products would not happen.

Preliminary tests with the mouse contact hypersensitivity model showed profound ear swelling reductions when a crude mix of UCA photooxidation products or combinations of the already identified imidazoles were applied (to be published).

For almost two decades UV-induced immunosuppression has been associated with *cis*-UCA. The impact of our findings is the possibility that UCA oxidation products may have a role in the phenomenon of UV-induced immunosuppression. Adopting the view of an active role for UCA oxidation products in skin immunity, we have no explanation yet for the more intense immunosuppressive action of *cis*-UCA over *trans*-UCA e.g. as observed in the mouse contact hypersensitivity model. Our future research will be focussed on the identification of other UCA oxidation products, on their formation in the skin under various conditions of oxidative stress and on tes-ing their effects on the immune system.

In conclusion, both *trans*-UCA and *cis*-UCA can be oxidized in vitro to similar sets of oxidation products by various hydroxyl radical generating systems, which include exposure to UV ($\lambda < 320$ nm) or to the reactive 'Fenton' species. The primary products formed are identified as ImCHO and GLX. ImCHO can be rapidly further oxidized into ImCOOH. These findings *in vitro* may have relevance for the situation *in vivo*, because the formation of the three imidazoles ImCHO, ImAc and ImCOOH was also shown in UV-B exposed corneal layers of human skin.

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PART III

UCA IN IMMUNOLOGICAL MODELS

CHAPTER 7

Cis-urocanic acid is not useful as immunosuppressive agent in the treatment of human allergic contact dermatitis

Arthur Kammeyer, Marcus MHM Meinardi, Jan D. Bos, Marcel BM Teunissen. Department of Dermatology, Academic Medical Center, University of Amsterdam

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Letter to the editors

Sir,

One of the suggested mediators of ultraviolet-induced immunosuppression is *cis*-urocanic acid (*cis*-UCA), that is formed in the epidermis by photoisomerization of *trans*-urocanic acid (*trans*-UCA) [1]. In animal models *cis*-UCA has shown immunosuppressive effects, including the suppression of both sensitization and elicition of contact allergy. Since *cis*-UCA is a low molecular weight compound (enabling it to penetrate into the skin) it might be an interesting topical agent for therapeutic use in a variety of dermatoses where immunosuppression is needed. With great interest we read the contribution of van Strien & Korstanje [2] who demonstrated a suppressive effect of topical UCA in treatment of allergic contact dermatitis in human volunteers.

Coincidently, we completed a preliminary study with comparable experiments, using an extended protocol developed earlier for use in the study of possible efficacy of topical cyclosporin in atopic eczema and allergic contact dermatitis [3]. In brief, patients known to be sensitized to nickel, perubalsam,

quinoline-mix or fragrances were patch tested with the respective compounds according to a modified ICDRG protocol. We studied the possible efficacy of *cis*-UCA in suppressing the contact allergic response, using *trans*-UCA and vehicle as a controls. Patch test sites were pretreated for 48 hours with *cis*- or *trans*-UCA (1% and 0.01% w/v) in 1% carbomer gel (vehicle), prior to the application of allergen, which was present for 72 hours under occlusion without the simultaneous application of a UCA-isomer.

Our results indicated that neither isomers had any effect in preventing elicitation in 4 individuals sensitized for fragrance mix, perubalsum and quinoline mix. In contrast to the findings of van Strien and Korstanje, we found that only in one out of 7 nickel sensitized individuals, did pretreatment with *cis*-UCA moderately suppressed the severity of dermatitis. In three out of 7 individuals both *cis*- and *trans*-UCA showed moderate suppression of elicitation as compared with control patches treated with vehicle alone. In the three remaining individuals no suppression was found with either UCA-isomers. Since the suppressive potential of *trans*-UCA did not substantially differ from *cis*-UCA in this respect, the immunosuppressive capacity ascribed to *cis*-UCA is not very plausible. Unfortunately, van Strien and Korstanje [2] did not include *trans*-UCA as an important control, making it difficult to interpret their results properly. We suggest that both UCA-isomers might have functioned as a chelating agent for nickel under these conditions. Chelation may prevent the availability of free nickel ions to initiate allergen formation with epidermal macromolecules. To that end, we investigated whether the lack of suppressive ability was caused by inefficient penetration of *cis*-UCA, although it was topically applied in excess. We determined the epidermal concentrations *cis*- and *trans*-UCA before and after a load of 48 h under occlusion of 1% *cis*- and *trans*-UCA in carbomer gel. Using a filter sampling method and HPLC-analysis [1], basic epidermal tissue levels of *cis*- and *trans*-UCA were found to be increased (about 8 times) under these conditions, indicating that an effective penetration of UCA-isomers in the epidermis of our volunteers had occurred.

To load the epidermis with increased amounts of UCA isomer requires a certain period of time to elapse. We can assume that the application of UCA isomers immediately before the application of the allergen may have the consequence that the migrating allergen is not surrounded by excess *cis*- or *trans*-UCA in the epidermis. In contrast, in our protocol UCA isomers are present in excess at the time of elicitation and this might favor the study of the possible suppressive effects of *cis*-UCA, and eventually, *trans*-UCA as well.

We conclude that *cis*-UCA, and also *trans*-UCA, could suppress some, but not all of the elicitation of nickel contact allergic reactions by chelation of nickel ions. Neither *cis*-, nor *trans*-UCA were effective in suppressing elicitation responses to other contact allergens. In this respect, neither isomer forms interesting compounds for further development as therapeutic agent in immune-mediated dermatoses.

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CHAPTER 8

Suppression of contact hypersensitivity response by urocanic acid oxidation products

A. Kammeyer[1], J.Garssen[2], H. van Loveren[2], T.A. Eggelte[3], J.D. Bos[1] and M.B.M. Teunissen[1]

1) Laboratory of Experimental Dermatology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands;
2) National Institute of Public Health and the Environment, Laboratory of Pathology and Immunobiology, Bilthoven, The Netherlands;
3) Department of Clinical Pharmacology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

Abstract

Urocanic acid (UCA) can *in vitro* be photooxidized by UV-B irradiation into UCA oxidation products; three of these compounds, the imidazolic UCA oxidation products (the 'imidazoles'), could be detected (*in vivo*) in corneal layers of the epidermis as well. We hypothesized that the imidazoles participate in the UV-induced systemic immunosuppression and tested this in a murine model for contact hypersensitivity (CHS). A crude mixture of photo-oxidized products of UCA (2.0 and 0.2 g/l) significantly inhibited the CHS responses. Two imidazoles, i.e. imidazole-4-carboxylic acid (ImCOOH) at 1.0 g/l and imidazole-4-acetic acid (ImAC) at 1.0 and 0.2 g/l significantly reduced the CHS response with a potency comparable to that of the well-known inhibitor *cis*-UCA. A stronger reduction was observed with the third imidazole, imidazole-4-carboxaldehyde (ImCHO), either at 1.0 and 0.2 g/l. When added in combination, the three imidazoles (each 0.33 g/l) showed strong, very significant inhibition of ear swelling, which effect was more pronounced than the suppression induced by *cis*-UCA. Paired combinations of the three imidazoles showed intermediate, but very significant, reductions in ear swelling. These results indicate that, in addition to *cis*-UCA, the imidazolic UCA oxidation products play a role in UV-induced immunosuppression as well, possibly in concert with each other.

1. Introduction

In the last decade extensive immunological research has been carried out to characterize the immunomodulatory effect of urocanic acid (UCA), predominantly that of the *cis*-UCA isomer. This stereoisomer of UCA is formed upon photoisomerization of *trans*-UCA by UV irradiation of the skin up to a wavelength of approximately 360 nm (1). It was shown that *cis*-UCA can mimic several effects of UV on the immune system such as local and systemic immunosuppression or effects that fit in UV-induced immunosuppressive reaction cascades (2,3). Most convincing evidence for *trans*-UCA, as the photoreceptor, and *cis*-UCA, as the immunosuppressant, was obtained from the mouse model for delayed type hypersensitivity (DTH) and in contact hypersensitivity (CHS) (3). However, in various assays *in vitro* no inhibitory effect of *cis*-UCA could be demonstrated (4-7). The mechanism of *cis*-UCA induced immunosuppression is still a puzzling issue.

In a previous report (8), it was demonstrated that *trans*-UCA and *cis*-UCA are converted to oxidation products in oxidative stress circumstances. There is strong evidence that the epidermis undergoes a relatively high level of oxidative stress during UV-irradiation leading to the formation of reactive hydroxyl radicals (9-11). *Trans*-UCA and UV-induced *cis*-UCA are present at relatively high concentrations in the epidermis and may form potential targets for hydroxyl radicals. UCA isomers are efficient hydroxyl radical scavengers (12) and several oxidation products are formed during scavenging *in vitro* as well as in UV-B exposed corneal skin samples (8).

We suggest that oxidation products of UCA isomers may be involved in the process of UV-induced immunosuppression. This is supported by the following observations. First, similar levels of *cis*-UCA can be induced by UV-A and UV-B, whereas UV-B, but not UV-A, is the principal inducer of immunosuppression (13) and UCA oxidation products are only formed by UV-B (or shorter waves) from both UCA isomers, and not by UV-A (12). Second, antioxidants have an abrogatory effect on the UV-induced and *cis*-UCA induced immunosuppression (14,15).

To demonstrate a possible role for UCA oxidation products in the phenomenon of UV-induced immunosuppression, the suppressive potency of UCA oxidation products was tested in a mouse model and compared with that of the UCA isomers themselves. The inhibition of induction of contact hypersensitivity (CHS) was used as a test, measuring ear swelling reductions after elicitation with the contact sensitizer picryl chloride.

2. Materials and Methods

2.1 Materials

Trans-UCA, imidazole-4-carboxylic acid, imidazole-4-carboxaldehyde, imidazole-4-acetic acid (sodium salt), glyoxylic acid monohydrate and oxalic acid dihydrate were supplied by Sigma-Aldrich/Fluka Chemie BV (Zwijndrecht, The Netherlands). *Cis*-UCA was kindly offered by dr. WMPB Menge of the Free University, Department of Pharmacochemistry, Amsterdam, The Netherlands. Picryl chloride (PCl; Chemotronix, Swannanoa, NC, USA) was used as contact sensitizer in all experiments.

2.2. Animals

Male BALB/c mice (8-10 weeks of age) were obtained from the National Institute of Public Health and the Environment (Bilthoven, The Netherlands). The animals (BALB/c/Rivm) were kept in light-controlled, humidity-controlled and temperature-controlled rooms in the animal facility, already two weeks prior to the experiment. They were fed with water and Hope Farm Chow (SRM-A) ad libitum.

2.3. Contact Hypersensitivity

Picryl chloride (PCl) was recrystallized three times from methanol/water before use and was protected from light and humidity during storage at 4° C. For active contact sensitization, mice were sensitized with PCl by epicutaneous application of 25 µl 5 % PCl in ethanol/acetone (3:1) on each skin location of the abdomen, thorax and each foot (150 µl/mouse). Four days after sensitization, mice were challenged on both sides of each ear by topical application of one drop of 0.8 % PCl in olive oil. Duplicate measurements of ear thickness were made before elicitation and at 24 h after ear challenge with an engineer's micrometer (Mitutoyo model 193-10, Tokyo, Japan). The gain in ear thickness was expressed as the mean ± SEM in micrometers and as a relative expression in percent of the positive control value (= 100 %, derived from non-treated sensitized mice). Nonsensitized (negative) control animals were challenged and measured similarly. These backgrond ear-swelling responses were subtracted from the other measured responses to obtain the net ear-swelling responses.

2.4. Test solutions

Cis-UCA and UCA oxidation products were dissolved in sterile phosphate buffered saline (PBS). 200 µl of freshly dissolved test material was given to each mouse subcutaneously, equally divided in two portions and 1 h prior to sensitization with PCl.

2.5. The UCA photooxidation mixture (PO-mix)

Trans-UCA (8 mmol) was photooxidized in the presence of hydrogen peroxide (40 mmol) by xenon-arc radiation that was tuned by UG11 and WG305 optical filters, affording simulated solar UV with a spectral distribution in the UV-range from 290-400 nm. It was previously shown (12) that UV-radiation < 320 nm induces UCA photo-oxidation. The mixture of photooxidized products to be tested here is referred to as PO-mix. Because the UV-irradiation also contained the effective UV-wavebands to evoke photoisomerization, *cis*-UCA was formed in the mixture as well. However, the PO-mix contained less than 3 % of each UCA isomer, due to breakdown of UCA into known and unknown photooxidation products. The PO-mix, a light-yellow product, is readily soluble in water media and contains the previously identified three imidazolic UCA oxidation products, 'the imidazoles' (12). The imidazoles were tested for their CHS response in pure form, separately and in combination with each other.

2.6. Statistics

Levels of significance were calculated using the one-tailed Student's t-test; $p < 0.05$ and $p < 0.01$ were taken as significant and very significant differences, respectively. Each similarly treated group consisted of three mice, giving 18 ear thickness measurements. The data of the CHS experiment for the separate imidazoles (results of Fig.2) were derived from two sets of 3 mice (total n = 6).

3. Results

3.1. Suppression of CHS by photooxidized products of UCA

A mixture of photooxidized products of UCA (PO-mix), *trans*-UCA and *cis*-UCA were tested for their capacity to suppress CHS responses, using picryl chloride as the contact sensitizer. The test compounds, dissolved in PBS, were subcutaneously injected into the mice one hour prior to sensitization with PCl and the results were compared with those of PBS-treated, sensitized mice (positive controls). The PO-mix at concentrations of 0.2 and 2 g/l strongly decreased the CHS response in a dose-dependent fashion compared with that of the positive control (= 100 % swelling): 29 and 19 % ear swelling, respectively (Fig. 1). Although the CHS response after administration of the PO-mix (2 g/l) was only 19 % of the positive control, the difference with the 'classical' well-known inhibitor *cis*-UCA (31 % of positive control), was not significant.

Although the overall concentration of the PO-mix was twice that of *cis*-UCA, it must be noted that the suppression-inducing compounds in that

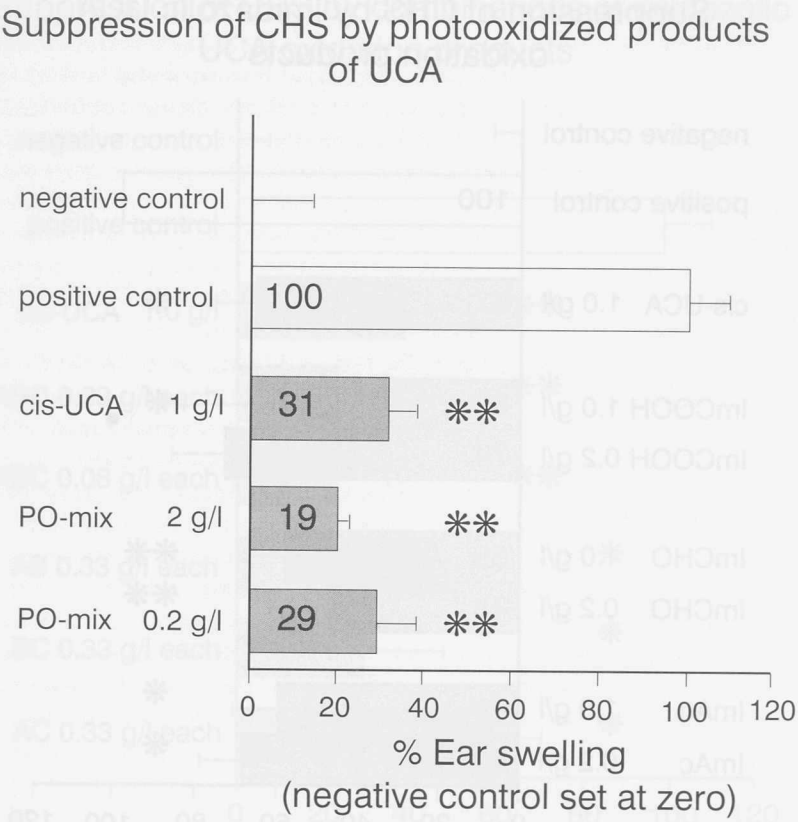

Figure 1. Net (Ag-specific) ear swelling response 24 h after elicitation of the ear pinnea with one drop of 0.8 % picryl chloride (PCl) in olive oil. The background swelling, induced by PCl application of non-sensitized animals (< 10 μm), was subtracted from the swelling in PCl-sensitized animals. The positive control revealed a net ear swelling of 115 μm and was set to 100 %. The other values (dark grey) were converted into percentages by multiplying the particular net ear swelling by 100 and dividing by the net ear swelling of the positive control. The double asterisk means a very significant difference of $p < 0.005$, compared to positive control.

mix each had concentrations lower than that of *cis*-UCA. A tenfold dilution of the PO-mix (0.2 g/l) was still as effective as *cis*-UCA in reducing CHS. *Cis*-UCA and both PO-mix solutions showed very significant CHS reductions ($p < 0.005$) compared to the positive control (Fig. 1).

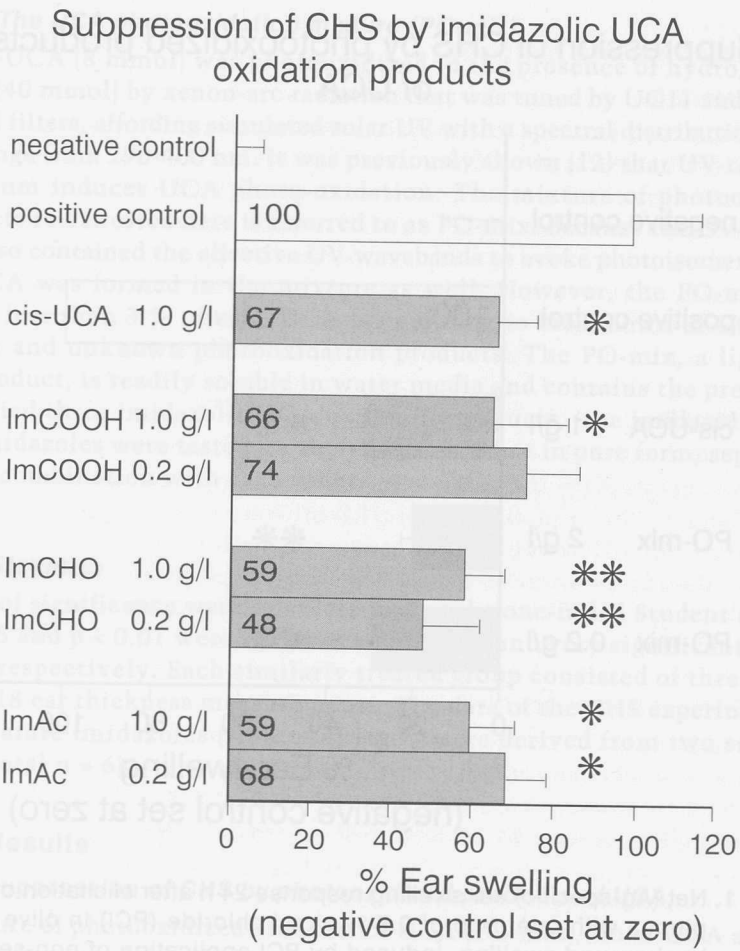

Figure 2. Same conditions as in Figure 1, except that the positive control showed a net ear swelling of 111 μm and that the single and the double asterisks refer to a significant reduction, compared to the positive control, of $p < 0.05$ and $p < 0.01$, respectively.

3.2. Suppression of CHS by imidazolic UCA oxidation products

Within the mix of UCA photooxidation products three imidazolic compounds were identified, i.e. imidazole-4-carboxylic acid (ImCOOH), imidazole-4-carboxaldehyde (ImCHO) and imidazole-4-acetic acid (ImAc), the 'imidazoles'. These three imidazoles were separately tested for the CHS response.

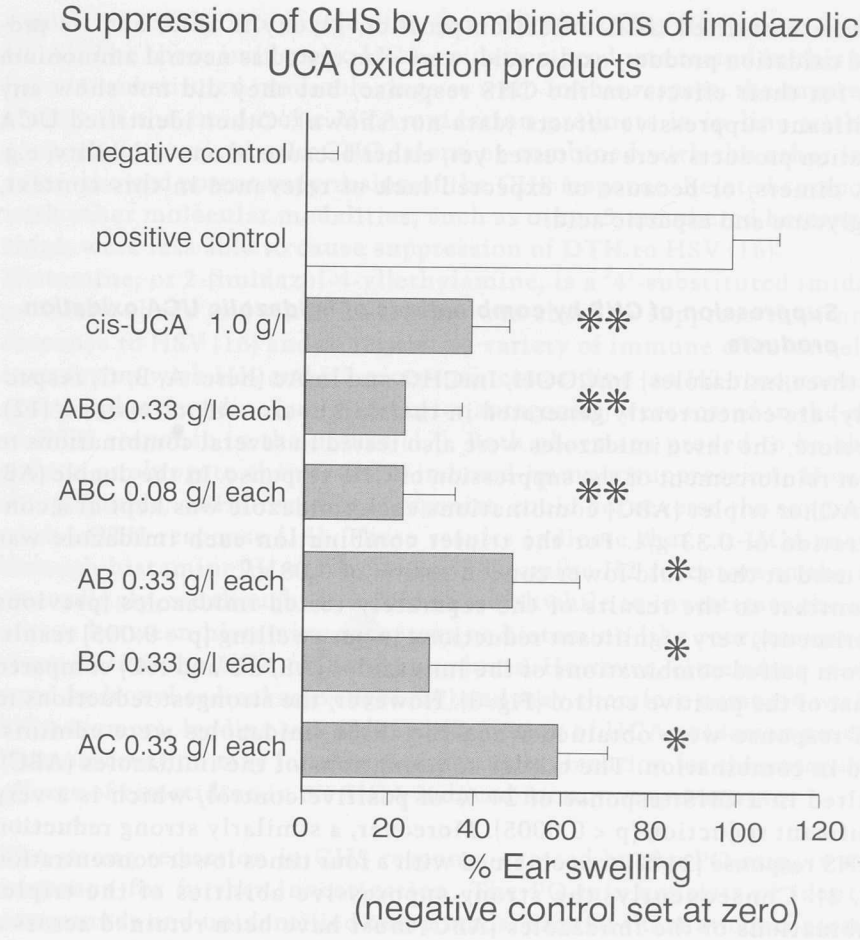

Figure 3. Same conditions as in Figure 1, except that the positive control showed a net ear swelling of 70 μm and that the single and double asterisks refer to very significant differences, compared to positive control, of $p < 0.005$ and $p < 0.0005$, respectively.

ImCOOH 1.0 g/l and ImAc at 1.0 and 0.2 g/l showed significant ($p < 0.05$) reductions in CHS responses, similar to that of *cis*-UCA (Fig. 2). However, ImCOOH at a concentration of 0.2 g/l did not show a significant reduction compared to that of the positive control. In this experiment ImCHO at both 1.0 and 0.2 g/l showed the strongest reductions in CHS response with a very significant level ($p < 0.01$) (Fig. 2).

Another identified UCA oxidation product, glyoxylic acid, and its proposed oxidation product, oxalic acid, were also tested as neutral ammonium salts for their effects on the CHS response, but they did not show any significant suppressive effects (data not shown). Other identified UCA oxidation products were not tested yet, either because of unavailability, e.g. UCA dimers, or because of expected lack of relevance in this context, e.g. glycine and aspartic acid.

3.3. *Suppression of CHS by combinations of imidazolic UCA oxidation products*

The three imidazoles, ImCOOH, ImCHO and ImAc (here: A, B, C, respectively) are concurrently generated in the skin upon oxidative stress (12). Therefore, the three imidazoles were also tested in several combinations to aim at reinforcement of the suppression of CHS response. In the double (AB, BC, AC) or triplet (ABC) combinations each imidazole was kept at a concentration of 0.33 g/l. For the triplet combination each imidazole was also used at the 4-fold lower concentration of 0.08 g/l.
In contrast to the results of the separately tested imidazoles (previous experiment), very significant reductions in ear swelling ($p < 0.005$) resulted from paired combinations of the imidazoles (AB, BC and AC) compared to that of the positive control (Fig. 3). However, the strongest reductions in CHS response were obtained when the three imidazoles were administered in combination. The triplet combinations of the imidazoles (ABC), resulted in a CHS response of 24 % of positive control, which is a very significant reduction ($p < 0.0005$). Moreover, a similarly strong reduction in CHS response (24 %) was obtained with a four times lower concentration (Fig. 3). Consequently, the strong suppressive abilities of the triplet combinations of the imidazoles (ABC) must have been retained across a concentration range, of which the limits are not yet defined.

4. Discussion

Trans-UCA and *cis*-UCA were tested in CHS models many times before during the past two decades and *cis*-UCA was unanimously found to exert the most potent suppression of CHS responses, as compared to *trans*-UCA. Similar results were also obtained in an analogous model, the delayed type hypersensitivity model (DTH), using herpes simplex virus (HSV) as antigen (16). It should be noted that the molecular structures of UCA isomers are not unique in causing suppressive effects in these CHS and DTH models. Other imidazolic compounds have been shown previously to suppress DTH to HSV as well (16,17). It seems that the strongest suppressive effects were found with compounds having a substituent at the

4-position of the imidazole ring (e.g. dihydroUCA, histamine and *cis*-UCA). The three imidazolic UCA oxidation products tested in this study have '4'-substituted imidazole rings as well. In this respect, the suppressive ability of the imidazolic UCA oxidation products is in line with this concept. In particular ImCHO, alone or combined with the other imidazoles, showed potent suppression of the CHS response. Related compounds with other molecular modalities, such as other 5-membered heterocyclic rings, were less able to cause suppression of DTH to HSV (16).
Histamine, or 2-(imidazol-4-yl)ethylamine, is a '4'-substituted imidazole as stated above and since histamine was show to suppress the immune response to HSV (16) and to regulate a variety of immune effector cells by interaction with H1- and H2-receptors, cimetidine (an H2-receptor antagonist) and terfenadine (an H1-receptor antagonist) were tested in the model of DTH response in the mouse (17). Both pharmaca proved to be able to reduce or abrogate the *cis*-UCA-induced immunosuppression. However, thioperamide, an H3-receptor antagonist, could not reverse the suppression of the DTH response (18). These results indicate that *cis*-UCA may act through histamine H1 receptors and histamine H2 receptors in the skin. Accordingly, we thought it would be worthwhile to investigate the interaction between histamine receptors or histamine-like receptors and the three imidazolic UCA oxidation products. However, cimetidine is also a good hydroxyl radical scavenger (19) and may therefore compete with the UCA isomers, leading to a reduced formation of UCA oxidation products. This observation may form an alternative explanation for the antagonistic effects of cimetidine in *cis*-UCA induced immunosuppression.

The strong reduction in CHS response exerted by the PO-mix, is a phenomenon for further investigation. The PO-mix consists of identified compounds and unidentified compounds, each of them at concentrations that must be lower than any test concentration in the performed experiments (< 0.2 g/l), and yet a strong reduction in CHS response resulted, at least as strong as that of *cis*-UCA at the higher concentration of 1 g/l (Fig. 1). The three identified imidazoles may contribute to this suppressive effect, but it is plausible that other, even more powerful immunosuppressants may be present among the unidentified compounds. ImCOOH and ImAc showed moderate suppressive effects on CHS response, while ImCHO was the strongest suppressant (Fig. 2). The reductions in CHS response by the triplet combinations of the imidazoles seem to be stronger than that of *cis*-UCA, which finding is reinforced by a similar large reduction in CHS response, caused by a 4-fold diluted triplet combination (Fig. 3).

In our experiments, testing of the individual imidazoles resulted in smaller reductions in CHS responses than observed with combinations of the

imidazoles at similar or lower concentrations. However, it is too premature to draw conclusions on synergistic effects already, because the results of the separate and combined actions of the imidazoles were derived from different experiments. As yet unanswered questions in this respect are: 1. the degree of suppression that would be observed at lower concentrations of the test compounds, and 2. the possible occurrence of synergy from combinations of the test compounds. The first question is in particular relevant to the expected levels in sun-exposed skin. Our previous study (8) showed that the concentrations of the three imidazolic UCA oxidation products are lower than those of the UCA isomers in the UV-B exposed corneal layer of the skin. Accurate estimations cannot be made because of different routes of gaining the imidazoles (photooxidative induction at a certain skin area versus subcutaneous injection).

Adopting the view of an active role of UCA oxidation products in skin immunity, we have no explanation yet for the more intense immunosuppressive action of *cis*-UCA over *trans*-UCA, as similar chromatographic patterns emerged from oxidized *trans*-UCA and oxidized *cis*-UCA after HPLC-analysis. As with *cis*-UCA, the relation of the immunosuppressive effects of the imidazoles with health hazards such as skin cancer should become known in the future. Our future research will be focussed on the identification of other UCA oxidation products, on their assessment *in vivo* under various conditions of oxidative stress, and on further testing of their immune functions.

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PART IV
MISCELLANEOUS
CHAPTER 9
General discussion and Summary

Trans-UCA is a common constituent of the mammalian epidermis. UV-induced photoisomerization is needed to produce the stereoisomer *cis*-UCA from *trans*-UCA. Epidermal *cis*-UCA levels can exceed those of *trans*-UCA upon a period of sun bathing or upon phototherapy. One may conclude that in opposite cases, with prolonged absence of UV-exposure, the epidermis will not contain *cis*-UCA. Theoretically, this conclusion may be valid. It became clear through our study (Part I, Chapter 2-4) and others (43,44,46*) that *cis*-UCA can be determined in the skin of volunteers who claimed not to be exposed to UV for a prolonged period. *Cis*-UCA levels of so-called non-UV-exposed skin were reported in Chapter 4.

In our view, two circumstances contribute to the presence of *cis*-UCA in so-called non-UV-exposed skin. First, the long wave limit of UCA photoisomerization is 363 nm (Chapter 3) and this wavelength is present in the short-wave emission of common lamp types, e.g. fluorescent tubes. Consequently, exposure to these lamps will commonly cause the formation of *cis*-UCA in the human epidermis, although at a slow photoisomerization rate due to low effectiveness of this UV radiation. Another adventitious contributor can be indirect or window glass-filtered sunlight. Second, we have demonstrated that *cis*-UCA, once formed in the epidermis upon a single exposure, is retained for a period of three weeks (Chapter 4). Therefore, accumulation of trace amounts of *cis*-UCA, induced by repeated low UV doses, may occur in the skin and is referred to as 'background' photoisomerization. This background production of *cis*-UCA may even happen in the winter period, without deliberate exposure to UV sources for tanning purposes. Chapter 4 also presents a new HPLC determination, which enables the simultaneous determination of *trans*-UCA and *cis*-UCA in urine samples. Elevated urinary *cis*-UCA levels could be demonstrated in a

period of a few hours to 8 days following a single total-body UV-exposure. That finding implicates that *cis*-UCA, once formed in the epidermis, spreads across the internal body and is gradually excreted into the urine.

In general, epidermal *cis*-UCA is a persistent factor as is *trans*-UCA. In this respect, *cis*-UCA differs from various cell mediators, such as histamine, cytokines, prostaglandines and hormones, in such a way that the latter compounds exist at temporary, low levels; they act on each other in a fine-tuned tightly-controlled network. From this point of view, it would not be plausible that *cis*-UCA exert suggested immunosuppressive effects by binding to a specific cell receptor, because this will lead to a constant trigger. Cis-UCA can easily enter deeper skin layers in sufficient amounts, simply by diffusion. Through the skin-draining lymph vessels and blood vessels other parts of the body can be reached, where it might suppress immune functions.

In this thesis doubt is raised about *cis*-UCA as the principal inducer for immunosuppression. First, if *cis*-UCA would be the principal inducer, do we live in a state of continuous immunosuppression ? This view does not make much sense, unless there would be a threshold level for *cis*-UCA to exert a cell triggering effect or cells can adapt to substantial *cis*-UCA levels. Second, several studies point to photodamaged DNA as an initiator for immunosuppression, however, its working mechanism is not fully elucidated and final conclusions cannot be made. In a number of experiments *in vitro*, *cis*-UCA failed to exert biological effects (Table 5 of general introduction). Taking these considerations into account, it was speculated in Part II that UCA isomers can be converted into derivatives, which are jointly responsible with *cis*-UCA for the observed immunosuppression.

Chapter 5 describes the recognition of UCA isomers as good hydroxyl radical scavengers. This finding implies at least two aspects: first, another role for epidermal UCA isomers can be envisioned, as they may protect against hydroxyl radical attack on epidermal constitituents during oxidative stress. Second, UCA isomers may be converted into immunological relevant compounds after scavenging hydroxyl radicals. Chapter 6 shows UCA's ability for oxidation into UCA oxidation products by hydroxyl radicals. Similar sets of oxidation products were formed, no matter the hydroxyl radicals were generated via photooxidation or – in the dark – via Fenton oxidation. Chapter 6 outlines the identification of seven UCA oxidation products. One of them, the UCA dimer, can actually comprise a group of several stereoisomers. Three imidazolic UCA oxidation products, imidazole-4-carboxylic acid (ImCOOH), imidazole-4-carboxaldehyde (ImCHO) and imidazole-4-acetic acid (ImAc), glyoxylate and its proposed oxidation product oxalate, were selected for testing immunological

functions. ImCOOH, ImCHO and ImAc are referred to as 'the imidazoles' in the following text. Their molecular structures are related to histamine and UCA, in the way that they are imidazoles substituted at the 4-position.

A new HPLC-method for the simultaneous determination of the three imidazoles in corneal layers of the skin was developed (Chapter 6) and their levels were found to be low, e.g. 0.07 µmol ImCHO/g corneal layer material after a UV-B dose of 9.6 kJ/m^2. The two remaining identified compounds glycine and aspartic acid were considered to be less relevant for the skin immune system and were not tested, unless new views will emerge. In this context, UCA dimers were considered to be relevant and worthwhile to test, however, their availability in sufficient test amounts is a problem. The three above mentioned imidazoles are commercially available, but because imidazole-4-carboxaldehyde is only available very recently, this compound had to be synthesized at our laboratory from 4-(hydroxymethyl)imidazole for the initial studies.

Part III describes immunological tests *in vivo* with the UCA isomers and UCA oxidation products. It is known for quite some time that UV, *cis*-UCA and histamine, can inhibit the induction of contact hypersensitivity (CHS). However, no effect of *cis*-UCA was found on the elicitation reaction to common contact allergens applied on the human skin (Chapter 7), except in case of nickel on nickel-sensitized individuals and *trans*-UCA exerted a suppressive effect as well. It was suggested that both UCA isomers were able to chelate nickel ions with the consequence that nickel was captured and not able to act as a hapten in contact allergy. For the other types of allergy, *cis*-UCA seems to lack any effect, even after loading the human skin with additional amounts of UCA isomer. In this view, it would have been worthwhile to know whether *cis*-UCA is able to suppress the elicitation phase of hypersensitivity in mice, in stead of the commonly tested sensitization phase.

Chapter 8 reports strong reductions of ear swelling by the complete photooxidation mix of UCA, which activity tends to surpass that of *cis*-UCA. The complete photooxidized mix contained the imidazoles as well and the suppressive activity of combinations of the pure imidazoles was of similar degree as that of *cis*-UCA. In Chapter 8 the results on reduction of ear swelling relate to suppression of sensitization, in stead of elicitation of CHS. One report (105*) claims that *cis*-UCA is well able to inhibit sensitization and elicitation responses in BALB/c mice, although different kinds of contact sensitizer were used. Thus far, nobody has confirmed this finding.

Taken the findings of part II and III together and several other reports on the lack of suppressive potential of UCA, one can conclude that epidermal *cis*-UCA may not be the principal initiator of immunosuppression. In several studies *in vivo* observers may not have noticed the concommitant formation of UCA oxidation products in much lower concentrations than the UCA isomers themselves. The (imidazolic) UCA oxidation products may act separately, by the side of the UCA isomers, or they may act in synergy. The issue of synergy requires serious attention, since the combined imidazoles seem to result in a stronger suppression on CHS response than the separate compounds (Chapter 8).

As stated above, the imidazoles were formed *in vivo* in very low levels, and for this particular circumstance (epi-)dermal cells may possess specific receptors for the imidazoles. In contrast to *trans*-UCA and *cis*-UCA, the UCA oxidation products exist in low, temporary epidermal levels that are comparable with those of other cell mediators. Because of the structural relation of the imidazoles with the well-known imidazole histamine, the interaction of the imidazoles with the histamine receptors, as was done for the UCA isomers, would be an interesting topic for further investigation. It was shown in Chapter 6 that the imidazoles can be formed with UV-exposure, as well as without. In the latter case the free availability of iron ions must be somehow induced without UV, e.g. under inflammatory circumstances. Many other analyses and immunobiological tests should be carried out to compare the activities of the newly discovered UCA oxidation products with *cis*-UCA and histamine. The extent of their formation and existence in tissue, in particular the epidermis, should be studied in relation to UV-B exposure rate and in relation to levels of oxidative stress. The future may show a defined role for the oxidation products of UCA in UV-induced immunosuppression.

* citation of reference in Chapter 1, General Introduction.

Algemene discussie en samenvatting

Trans-urocaanzuur (*trans*-UCA) is een algemeen bestanddeel van de huid van zoogdieren. Het proces van fotoisomerisatie, dat geïnduceerd kan worden door ultraviolette bestraling (UV), is nodig om de stereoisomeer *cis*-UCA uit *trans*-UCA te vormen. Epidermale *cis*-UCA spiegels kunnen die van *trans*-UCA overtroeven als gevolg van zonnebaden of van fototherapieën. Het tegenovergestelde zou geconcludeerd kunnen worden bij langdurige afwezigheid van UV: de epidermis zou dan geen *cis*-UCA bevatten. Theoretisch zou dit kunnen, maar het werd duidelijk door ons eigen onderzoek (hoofdstuk 2-4) en dat van anderen (43,44,46*), dat *cis*-UCA ook nog in de huid aangetoond kan worden van personen die beweren een lange tijd niet in de zon gezeten te hebben. Gemiddelde *cis*-UCA spiegels van zogenaamde niet-aan-UV-blootgestelde personen worden genoemd in hoofdstuk 4.

Naar mijn mening zijn er twee omstandigheden die bijdragen aan de aanwezigheid van *cis*-UCA in niet-aan-UV-blootgestelde huid. Ten eerste ligt de langgolvige grens van UCA fotoisomerisatie bij 363 nm (hoofdstuk 3) en dit golflengtegebied komt ook voor in de kortgolvige emissie van algemene lamp typen, zoals de TL. Dientengevolge veroorzaakt blootstelling aan deze lampen toch de vorming van *cis*-UCA in de humane epidermis, ofschoon de snelheid van fotoisomerisatie laag zal zijn door de lage effectiviteit van dit licht (hoofdstuk 3). Een andere, onvoorziene, bijdrage in *cis*-UCA vorming in de huid kan afkomstig zijn van indirect zonlicht of van zonlicht dat door vensterglas valt. Ten tweede is *cis*-UCA, dat reeds gevormd is in de epidermis, voor 3 weken verhoogd aanwezig (hoofdstuk 4). Daarom kan er stapeling van-op zich-lage spiegels *cis*-UCA optreden tot meetbare hoeveelheden. Dit fenomeen wordt hier aangeduid als 'achtergrond' fotoisomerisatie, hetgeen ook in de winter periode zal gebeuren, zonder dat de huid opzettelijk wordt blootgesteld aan UV bronnen, zoals die voor bruiningsdoeleinden. In hoofdstuk 4 wordt ook een nieuwe HPLC-bepaling voor de simultane bepaling van *cis*-UCA en *trans*-UCA in urine gepresenteerd. Verhoogde *cis*-UCA spiegels in de urine konden worden vastgesteld enkele uren tot 8 dagen na een éénmalige UV-blootstelling van het totale lichaamsoppervlak. Deze bevinding impliceert dat *cis*-UCA, gevormd in de epidermis, zich verspreidt

door het lichaam en geleidelijk in de urine uitgescheiden wordt. Algemeen gesteld, epidermaal *cis*-UCA is een persisterende factor, zoals *trans*-UCA dat ook is. Wat dit betreft, verschilt *cis*-UCA dan ook van verschillende celmediatoren, zoals histamine, cytokines, prostaglandines en hormonen, die slechts tijdelijke lage spiegels hebben waarbij zij in een subtiel afgesteld netwerk actief zijn. Vanuit dit gezichtspunt is het niet aannemelijk dat *cis*-UCA de gesuggereerde immunosuppressieve effecten opwekt door zich aan een specifieke celreceptor te binden, omdat dit door het persistente karakter van *cis*-UCA zou leiden tot een constante puls op de betreffende cellen. Cis-UCA kan dieper gelegen huidlagen in voldoende hoeveelheden binnendringen, en wel eenvoudigweg door diffusie. Afgevoerd door de huiddrainerende lymfvaten, of door bloedvaten bereikt *cis*-UCA andere delen van het lichaam, alwaar het ook immuunfuncties zou kunnen onderdrukken.

In dit proefschrift, wordt in twijfel getrokken of *cis*-UCA de belangrijkste veroorzaker van immuunsuppressie is. Ten eerste, als *cis*-UCA de belangrijkste zou zijn, leven we dan onder continue immuunsuppressie ? Een dergelijke conditie zou niet zinvol zijn, tenzij er een drempelspiegel bestaat, waarboven *cis*-UCA op cellen een effect heeft. Of er bestaat een conditie dat cellen zich kunnen aanpassen aan continu hoge *cis*-UCA spiegels. Ten tweede wijzen een aantal studies naar DNA als een initiator voor immuunsuppressie. Het werkingsmechanisme hiervan is echter nog niet opgehelderd en uiteindelijke conclusies kunnen (nog) niet getrokken worden. In een aantal experimenten *in vitro*, blijven biologisch effecten van *cis*-UCA uit. (Tabel 5 van de Algemene Introductie). Tegen de achtergrond van deze bevindingen, wordt in Deel II gespeculeerd dat de UCA isomeren omgezet kunnen worden in derivaten, die gezamenlijk met *cis*-UCA verantwoordelijk zijn voor de waargenomen immuunsuppressie.

Hoofdstuk 5 beschrijft de herkenning van UCA-isomeren als goede hydroxylradicaalvangers. Deze bevinding impliceert minstens twee aspecten: ten eerste kan er nog een andere rol aan de epidermale UCA isomeren worden toegeschreven, wanneer zij kunnen beschermen tegen aanvallen van hydroxylradicalen op epidermale structuren tijdens situaties van verhoogde oxidatieve stress in de epidermis. Ten tweede zouden de UCA isomeren in immunologisch relevante verbindingen omgezet kunnen worden na reactie met hydroxylradicalen. Hoofdstuk 6 toont de eigenschap van UCA isomeren om door hydroxylradicalen in oxidatieproducten omgezet te kunnen worden. Vergelijkbare sets van oxidatieproducten werden gevormd, of de hydroxylradicalen nu gegenereerd werden via fotooxidatie of, zonder UV, via Fenton oxidatie. Hoofdstuk 6 zet ook de identificatie van 7 UCA-oxidatieproducten uiteen. Eén van hen, de dimeer van UCA, kan uit een groep stereoisomeren bestaan. Van de UCA-oxidatie-

producten werden drie imidazol-verbindingen, imidazol-4-carbonzuur (ImCOOH), imidazol-4-carboxaldehyde (ImCHO) en imidazol-4-azijnzuur (ImAc), glyoxylaat en het voorgestelde oxidatieproduct, oxalaat, geselecteerd om hun immunologische functie te testen. In het navolgende worden ImCOOH, ImCHO en ImAc aangeduid met: de imidazolen. Hun moleculaire struktuur is gerelateerd aan histamine en UCA, met dien verstande dat het imidazolen zijn met een substituent op de 4-positie.

Een nieuwe HPLC-methode voor de simultane bepaling van de 3 imidazolen in de opperhuid (stratum corneum) werd ontwikkeld en is ook beschreven in hoofdstuk 6. Hun spiegels waren laag na hun vorming d.m.v. fotooxidatie met 9.6 kJ/m^2 aan UV-B, bijvoorbeeld voor ImCHO: 0.07 µmol/gram hoornhuid. De twee overige oxidatieproducten, glycine en aspartaat werden minder relevant gevonden voor het immuunsysteem van de huid en zijn daarom (nog) niet getest. In deze context werden de UCA dimeren wel relevant gevonden, maar was er het probleem om over voldoende zuivere dimeer te beschikken. De 3 bovengenoemde imidazolen zijn commercieel verkrijgbaar, waarvan ImCHO recent. Deze verbinding is voor de eerste proeven ook nog gesynthetiseerd op het laboratorium uit 4-(hydroxymethyl)imidazol.

Deel III behandelt immunologische tests *in vivo* met de UCA isomeren en de UCA oxidatieproducten. Het is al enige tijd bekend dat o.a. UV, *cis*-UCA en histamine de contact overgevoeligheid (CHS) kunnen remmen. Er werd geen effect van *cis*-UCA waargenomen op de elicitatie reactie van bekende op de menselijke huid aangebrachte contactallergenen (hoofdstuk 7), behalve bij nikkel op nikkel-gesensibiliseerde personen. *Trans*-UCA liet echter ook een suppressief effect zien. Er werd gesuggereerd dat beide UCA isomeren in staat waren nikkelionen te binden d.m.v. complexatie, waarbij nikkelionen niet meer vrij aanwezig zijn om alsnog een hapteen-eiwit complex te vormen voor contactallergie. Voor de overige typen van contactallergie lijkt *cis*-UCA geen enkel effect te sorteren, zelfs niet nadat de menselijke huid was verrijkt met topicaal aangebrachte UCA isomeer. In verband hiermee is het zinvol om te weten of *cis*-UCA in staat is de elicitatie fase van CHS in het muizenmodel te onderdrukken, in plaats van de veel geteste sensibilisatie fase.
Hoofdstuk 8 vermeldt sterke reducties van oorzwelling door het complete fotooxidatiemengsel van UCA, dat de activiteit van *cis*-UCA lijkt te passeren. Het complete fotooxidatiemengsel bevat ook de imidazolen en combinaties van imidazolen, die zijn getest in het CHS-model. Zij vertoonden minstens even sterke suppressie als *cis*-UCA. In hoofdstuk 8 zijn de resultaten van oorzwelreductie gerelateerd aan onderdrukking van de sensibilisatie i.p.v. de elicitatie. Eén artikel claimt dat *cis*-UCA zowel

de sensibilisatie als de elicitatie fase van het CHS-model onderdrukt in BALB/c muizen, met gebruik van verschillende soorten contactsensitizers werden gebruikt. Tot dusver heeft geen ander onderzoek deze resultaten bevestigd.

Wanneer de bevindingen van delen II en III en andere onderzoeksresultaten over het uitblijven van suppressieve effecten van UCA samengenomen worden, kan men concluderen dat *cis*-UCA niet de voornaamste initiator van immunosuppressie zou zijn. Bij verschillende onderzoeken *in vivo* zou de vorming van UCA oxidatieproducten niet opgemerkt zijn vanwege hun lage concentraties. De imidazolen kunnen op zichzelf al immunosuppressief zijn, maar ook in combinatie met de UCA isomeren, of combinaties van de imidazolen onderling kunnen actief zijn. Het gegeven van synergie rechtvaardigt uitgebreide aandacht, omdat combinaties van imidazolen onderling al resulteerden in sterke reductie van de CHS response (hoofdstuk 8).

Zoals vermeld, de imidazolen worden *in vivo* in zeer lage concentraties gevormd en voor deze speciale omstandigheid zouden (epi-)dermale cellen specifieke receptoren kunnen bezitten. In tegenstelling tot *trans*- en *cis*-UCA, zullen de UCA oxidatieproducten, waaronder de imidazolen, lage, tijdelijke spiegels innemen, vergelijkbaar met die van andere celmediatoren. Vanwege de structurele relatie van de imidazolen met de welbekende imidazol histamine, zou het onderzoeken van de interactie van de imidazolen met de histamine-receptors een interessant onderwerp voor verder onderzoek vormen. In hoofdstuk 6 wordt getoond dat de UCA oxidatieproducten, waaronder de imidazolen, met UV blootstelling (fotooxidatie) gevormd kunnen worden, alswel zonder UV. In het laatste geval moet de vrije beschikbaarheid van ijzerionen op de een of andere manier geïnduceerd worden zonder UV, bijvoorbeeld onder inflammatoire omstandigheden. Menige analyse en immunobiologische test zal nog uitgevoerd moeten worden om de activiteiten van de nieuw ontdekte UCA oxidatieproducten, met name de imidazolen, te kunnen vergelijken met *cis*-UCA en histamine. De mate van hun vorming en hun aanwezigheid in weefsel, in het byzonder de epidermis, zal bestudeerd moeten worden in relatie tot de mate van UV-B blootstelling en in relatie tot de graad van oxidatieve stress. In de toekomst zal een definitieve rol voor de UCA oxidatieproducten, zoals de imidazolen, in UV-geïnduceerde immuunsuppressie gevestigd kunnen zijn.

* citatie van referentie in hoofdstuk 1, Algemene Introductie.

Excerpt

The work presented in this thesis is related to the initiation of UV-induced immunosuppression in the skin. This phenomenon is induced by sunburn radiation (UV-B) and it can cause recrudescence of skin infections or skin cancer in the long term. The sunburn radiation is absorbed by many different biomolecules present in the upper skin (epidermis). One of the main epidermal UV-absorbers is trans-urocanic acid (*trans*-UCA) and this UCA-isomer can be photoisomerized by UV-radiation into *cis*-UCA. *Cis*-UCA has been considered for almost two decades as one of the main inducers of UV-induced immunosuppression.

In Part I our results show that *cis*-UCA is a rather persistent compound in the human epidermis. Due to the persistent presence of this putative immunosuppressant, the following question can be asked: do we live in a state of continuous immunosuppression? This view does not make much sense, unless there would be a threshold level to exert a cell triggering effect or cells may be adapted to substantial *cis*-UCA levels. In the course of UCA research through the 80s and 90s it became clear, however, that *cis*-UCA did not mimic every effect of UV on immunity and its working mechanism is still not clarified. This discrepancy had led us to speculate that UCA can be converted by UV-exposure into oxidized derivatives that are jointly responsible with *cis*-UCA for the observed immunosuppression.

In Part II it was shown that both *trans*-UCA and *cis*-UCA are good hydroxyl radical scavengers and that both UCA isomers can be converted into several photooxidation products. Some of these products have been identified, among them are three imidazoles. Moreover, the presence of these imidazoles was also demonstrated in UV-B exposed corneal layer of the human skin. It was shown here that the UCA oxidation products are only formed by sunburn radiation (UV-B) and not by UV radiation that can pass window glass (UV-A). *Cis*-UCA, on the other hand, is formed by UV-B and UV-A from *trans*-UCA.

In Part III the immunobiological activity of cis-UCA, as well as that of the UCA photooxidation products, were tested for their systemic response in a murine model of contact hypersensitivity (CHS). The systemic suppressive effect of three imidazoles on CHS response was at least as strong as that observed with *cis*-UCA when tested in combined form and when having lower concentration than *cis*-UCA.

The above results indicate a role for UCA (photo)oxidation products in UV-B-induced sytemic immunosuppression and these new aspects are fully outlined in this thesis.

Beknopt verslag

Dit proefschrift, "Urocanic acid in Photodermatology", handelt over urocaanzuur en afgeleide verbindingen als veroorzaker(s) van het fenomeen dat ultraviolet (UV) licht de afweer van de huid kan onderdrukken (immuunsuppressie). Vanaf eind jaren 70 is deze immuunsuppressie bekend. De zon is verreweg de voornaamste bron van UV-licht. Immuunsuppressie in de huid kan leiden tot de opleving van sluimerende huidinfecties en tot huidkanker. Vanaf begin jaren 80 werd als veroorzaker *cis*-urocaanzuur (*cis*-UCA) aangewezen, dat onder UV blootsteling uit het in de epidermis (opperhuid) aanwezige *trans*-UCA ontstaat. In de loop van de daarop volgende jaren zijn er door tal van onderzoeken sterke aanwijzingen verzameld dat *cis*-UCA inderdaad de 'initiator' van immuunsuppressie is. Er bleek echter ook dat *cis*-UCA in een aantal celtestproeven geen immuunsuppressief effect kon bewerkstelligen.

In deel I van dit proefschrift en van andere onderzoeken is duidelijk geworden dat *cis*-UCA een algemeen voorkomende stof is in de opperhuid. Eénmaal gevormd, blijft *cis*-UCA in verhoogde concentratie gedurende 3 weken in de epidermis aanwezig en ruim één week verhoogd aantoonbaar in de urine. Dit laatste kan met een speciaal ontwikkelde, in dit proefschrift beschreven, HPLC-methode aangetoond worden (hoofdstuk 4). Vanwege de algemene aanwezigheid van *cis*-UCA in het lichaam kan de vraag gesteld worden of men in een continue staat van immuunsuppressie verkeert. Omdat dit niet erg aannemelijk is, ontstond de gedachte dat naast *cis*-UCA er andere stoffen verantwoordelijk voor immuunsuppressie zouden kunnen zijn. In de delen II en III van het proefschrift worden alternatieven voor *cis*-UCA aangetoond.

In deel II wordt beschreven dat *trans*-UCA en *cis*-UCA goede hydroxylradicaal-vangers zijn, d.w.z. dat zij ontstane hydroxylradicalen (een reactieve zuurstofvorm) goed kunnen neutraliseren. Deze radicalen onstaan bij oxidatieve stress, een situatie die ook in een sterk UV-belichte epidermis voorkomt. Hiermee is er een nieuwe rol voor *trans*-UCA en *cis*-UCA in de epidermis toegekend. Na het neutraliseren van hydroxylradicalen door UCA werd vastgesteld dat uit UCA andere producten gevormd worden en

verscheidene UCA oxidatieproducten zijn geïdentificeerd. Bovendien konden drie oxidatieproducten, de imidazolen, aangetoond worden in humane opperhuid na sterke UV-blootstelling. Onze hypothese was dat deze UCA oxidatieproducten ook een suppressief effect op het immuunsysteem van de huid zouden hebben, vergelijkbaar met dat van *cis*-UCA.
In deel III wordt beschreven hoe de hypothese werkelijkheid wordt. Het complete foto-oxidatiemengsel van UCA en de 3 nieuw ontdekte imidazolen, als UCA oxidatieproducten, blijken een suppressief effect te hebben op de immuunrespons in een gevestigd proefdiermodel. Muizen worden overgevoelig gemaakt tegen een bepaalde stof. Wanneer die stof op de oortjes wordt aangebracht, ontstaat oorzwelling. En wanneer *cis*-UCA of de 3 imidazolen voorafgaande aan het overgevoelig maken toegediend worden, dan meet men een sterk afgenomen oorzwelling. Dit voorlopige onderzoek geeft al aan dat de 3 imidazolen, als UCA oxidatieproducten, minstens zo suppressief op het immuunsysteem werken als *cis*-UCA zelf.

Dit proefschrift beschrijft de ontdekking van een nieuwe set van stoffen, die (mede)verantwoordelijk kunnen zijn voor het fenomeen van UV-geïnduceerde immuunsuppressie. Het mechanisme van de suppressie is met het bestaan van *cis*-UCA alleen niet opgehelderd; mogelijk leidt het voorkomen van de UCA oxidatieproducten in de epidermis wel tot de opheldering van het suppressie-fenomeen en tot de instelling van nieuwe therapieën voor huidaandoeningen met een immunologische oorzaak.

CHAPTER 10

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Curriculum Vitae

De schrijver van dit proefschrift werd geboren op 29 augustus 1950 in Amsterdam. Na de 3-jarige HBS begon hij in 1966 aan de opleiding voor leerling-analist chemische richting die hij in 1968 afrondde. De analisten-opleiding werd voortgezet en afgerond in 1970 met het behalen van het diploma Organisch Chemisch Laboratorium Assistent, welk diploma kort daarop gelijkgesteld werd met HBO-B Organische Chemie. In die periode (1968-1970) was hij als leerling-analist werkzaam in het Laboratorium voor Organische Scheikunde van de Universiteit van Amsterdam. Onvermijdelijk werd hij als goedgekeurde geconfronteerd met de dienstplicht, die na een dienstweigeringsprocedure resulteerde in een vervangende dienstplicht in het Nederlands Kankerinstituut, het Antoni van Leeuwenhoekhuis, op het klinisch chemisch laboratorium, alwaar hij voor het eerst kennis maakte met biomedische vakaspecten, met name die van het kankeronderzoek. Terug bij het Laboratorium voor Organische Scheikunde heeft hij een jaar (1974) gewerkt aan de synthese voor een nucleoside-analogon in de werkgroep potentiële carcinostatica van prof. dr. U.K. Pandit. Na een reis door de Verenigde Staten en Centraal-Amerika begon in oktober 1975 een vast dienstverband als research analist bij het Academisch Ziekenhuis bij de Universiteit van Amsterdam (AZUA), lokatie Binnengasthuis, afdeling Dermatologie met het toenmalig hoofd prof.dr. R. H. Cormane. Tot op de dag van vandaag is zijn onderzoekswerk gerelateerd aan de dermatologie. Vanwege zijn belangstelling voor het onderwijs volgde hij in 1975-1976 de lerarenopleiding bij het Nederlands Genootschap voor Beroepsonderwijs, waarna hij het Pedagogisch Diploma behaalde. Tot op heden geeft hij onderwijs in relevante aspecten uit de natuur- en scheikunde aan opleidingen voor verpleegkundigen en de opleiding tot operatieassistent op part-time basis. Voor één van de te onderrichten vakken schreef hij het boekje 'Medisch Rekenen'.

Het onderzoekswerk betrof eind jaren zeventig en perioden in de jaren tachtig remming van het complement-systeem bij complement gemedieerde aandoeningen, onderzoek geleid door dr. Shafi S. Asghar. Geleidelijk raakte hij in de jaren tachtig betrokken bij de invloed van ultraviolet (UV) licht op de huid, een fotobiologisch aspect dat ook in dit proefschrift bij

meerdere hoofdstukken aan bod komt. Mede door zijn spectrometrische kennis uit de organische chemie, werkte hij voor de afdeling dermatologie fysische aspecten uit van UV-bestralingsprotocollen, de fototherapie en diverse lichttests, en voerde deze ook uit. Deze werkzaamheden stonden in het kader van de diagnose van UV-gemedieerde aandoeningen. Bijkomend - en gerelateerd aan de fotodermatologie – verwierf hij kennis m.b.t. de werking en samenstelling van sunscreens. Deze werden tevens getest op hun beschermingsfactoren en onderzocht op fotochemische stabiliteit.

In de tweede helft van de jaren tachtig betrof zijn onderzoek vooral de melanine-synthese bij pigmentstoornissen en het maligne melanoom. Hierin werkte hij samen met dr. Wiete Westerhof, dr. Stanislav Pavel en promovendus drs. Nico Smit. Met HPLC-techniek werden door hem voorstadia van melanine (indolen) geanalyseerd en ontwikkelde hij later (1990-1992) samen met ing. Lukas Oomen een monoclonaal antilichaam tegen één van deze melanine-voorstadia met de bedoeling een test te ontwikkelen voor de vroege diagnose van maligne melanoom. Plasma en urine componenten bemoeilijkten echter een betrouwbare ELISA test. In deze periode (1990-1992) behaalde hij het HLO-diploma van Biochemisch Ingenieur aan de Hogeschool van Amsterdam.
Begin jaren negentig had de fotoimmunologie als onderzoeksgebied zijn intrede gedaan, geïnitieerd door prof.dr. Jan D. Bos, dermatoloog, en op de werkvloer begeleid door dr. Marcel B.M. Teunissen, immunoloog. Hij kreeg de gelegenheid om aan het werk te gaan met fotoimmunologisch fenomeen urocaanzuur in het kader van een promotie-onderzoek. In 1999 vloeide uit zijn onderzoek een patent voort, dat betrekking heeft op de antioxidant werking van urocaanzuur-isomeren en de immuunsuppressieve werking van urocaanzuuroxidatieproducten. In de huidig periode is hij nog bezig aan de afronding van het patentonderzoek.

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Beste lezer, ik weet dat u meestal een lichte voorkeur heeft het dankwoord eerst te lezen. Dan komen vanzelf de personen onder uw aandacht, die onmisbaar waren voor dit proefschrift.

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Stan Pavel, jij hebt het fenomeen UCA bij dermatologie geïntroduceerd, waarmee ik aan de slag kon gaan in het kader van de fotoimmunologie. Ondanks je nieuwe start als dermatoloog in het LUMC, hebben we in het begin nog vaak contact gehouden en mede daardoor heeft deel I, UCA biokinetics, een goede vorm gekregen, waarvoor ik je zeer erkentelijk ben.

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Appendix

Preparation of pure cis-UCA

Dissolve 276 mg (= 2 mmol) *trans*-UCA in 100 ml ultrapure water under magnetic stirring with gentle warming in a petri dish (Ø 15 cm). Irradiate the uncovered petri dish with TL-12 UV-B bulbs in overhead position (approx. 10 cm distance). The dose was 0.54 kJ/m^2 (1.8 W/m^2; 300 sec.), measured with an International Light IL 443 portable UV-B meter. Follow the photoisomerization process with HPLC by analyzing samples in a 100 fold dilution (approx. 4 hours to irradiate untill 70 % *cis*-UCA has been obtained).
Concentrate *trans*-UCA/*cis*-UCA solution to approx. 2 ml in a evaporator (Rotavapor"). Remove eventually crystallized *trans*-UCA by filtration or centrifugation. Apply the concentrate to a silica gel column (approx. 40 x 2 cm). Use 50 g silica gel with particle size 125 µm or less per gram UCA. Column must be connected to a UV detector set at 280 nm, and it may be run without additional pumps.

Eluent:	ethyl acetate	2 parts
	propanol-1	2 parts
	ammonia 25 %	1 part

Cis-UCA is the first isomer to elute. The combined *cis*-UCA fractions are concentrated to a few milliliters in an evaporator (Rotavapor®). Adjust solution to pH 4 - 5 with 5 M hydrochloric acid and evaporate to dryness. Stirr the residue with 4 ml acetone, and repeat 3 times. The last aceton addition was followed by gentle warming. Centrifuge each suspension in the cold to separate ammoniumchloride from solubilized *cis*-UCA. Collect acetone washings and evaporate to dryness in a Rotavapor®. The residue will be crude *cis*-UCA. Thorough vacuum drying overnight in a Speedvac® with medium heating will cause ammoniumchloride to evaporate.

Specifications *cis*-UCA.
melting point: 178 - 180 °C.
HPLC-analysis: less than 0.2 % *trans*-UCA.

Appendix