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THE INCREASE OF EPIDERMAL IMIDAZOLEACRYLIC ACID FOLLOWING INSOLATION*

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ABSTRACT

It has been confirmed, by electron microscopy, that suction blisters detach the epidermis at the dermo-epidermal junction. Inter- and intracellular vacuolization was observed in some of the specimens.

On the basis of a study comprising 15 subjects (12 males and 3 females), it was concluded that urocanic acid in the epidermis (suction blister skin) of the upper arm increased 9–11 days following insolation in comparison with specimens situated at an exactly symmetrical site of the control (non-irradiated) arm. This difference was significant in terms of μg urocanic acid per mg dry weight at a 95% level of probability and in terms of μg per cm^2 of blister base at a 99% level (t-test for paired values).

In two of the subjects other time intervals after insolation were also studied and an increase of epidermal urocanic acid level was noted.

Dry weights of epidermis (mg per cm^2) on the irradiated and control side (9–11 days following insolation) did not differ significantly in the group of 15 subjects. Significant increase due to insolation was only demonstrated when the values were divided by control values obtained for the respective arms 2 months before the experiment.

Histidine ammonia-lyase activity was estimated in 8 subjects. The increase on the irradiated side on the 9–11th day after unilateral insolation was not significant.

In 1955, a hypothesis was put forward (1) claiming that urocanic acid (UA)§ acts as a natural sun screen for the skin. This was based on its light-absorbing properties and on the discovery of its presence in human sweat (2, 3). When subsequently UA was shown to be present in the epidermis (4–9) in higher concentrations, the sun-screening role of its content in mammalian epidermis became a more attractive modification of the original hypothesis. Arguments gradually accumulated which supported the suggestion (7) that the presence of UA in sweat most probably reflected its epidermal content and was due to elution from the epidermis before or during the collection of sweat. Thus UA was depressed in the first portion of fractionally collected sweat if a short swim had preceded it (10) and was absent from sweat collected under paraffin (11). UA is present in guinea-pig skin, which has no true sweat glands (4), even if all types of glands have been eliminated by ionizing radiation (12). It does not appear in basal and squamous cell carcinomas (13).

A natural sun screening factor can be defined operationally as a substance which is present on the surface of the body and absorbs ultraviolet light in the 307 nm range. UA exhibits a considerably lower wavelength absorption maximum, which is probably (14) responsible for the minimum of erythrogenic activity occurring at 280 nm (15), but its absorption at 307 nm is considerable and, at normal UA concentrations in the epidermis, markedly higher than that of the proteins (keratins) (14, 16). Protein structures are, of course, responsible for the scattering effect of an optically heterogeneous medium which enhances the absorptive power of any light-absorbing component.

UA thus undoubtedly has the physical properties of a natural sun-screen. In fact, it

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§ Abbreviations: GSH—reduced glutathione, nm—nanometers (millimicrometers), S.D.—standard deviation, TLC—thin-layer chromatography, UA—urocanic acid [imidazole-4(or 5)-acrylic acid, *trans*-form unless stated otherwise], UV—ultraviolet.

has proved to be of value as a component of cosmetic sun-screens (17–18) which resulted in a satisfactory "protective factor" *in vivo* (19) according to Schulze's method (20). The question now arises whether this effect is of physiological importance.

Higher concentrations of UA were found in the sweat of a group of subjects who reported unusually high resistance toward sun than in sweat of other European subjects, irrespective of light or heavy pigment (21–23). With a few exceptions, African Negroes exhibited considerably higher concentrations of UA in the fractionally collected sweat than Europeans (23, 24). Higher concentrations of UA were found in the epidermis (suction blister skins) of African Negroes than in that of Europeans (25).

The hypothesis of the physiological role of UA would be supported if it could be shown that UA rises in response to a UV stimulus. There have already been reports of animal experiments which point in this direction. Thus an increase in histidine ammonia-lyase (18, 27) and urocanic acid (18) has been found following UV irradiation of guinea pigs *in vivo*. Incidentally, UA also increases after ionizing irradiation (11).

Considerable increase in the UA level in the epidermis of abdominal skin following exposure to sun has already been noted in an earlier study (28), but in that case the exposed and covered areas were not contralateral.

The aim of the present study was to ascertain whether the amount of UA per unit area of skin and thus its filtering effect rises after sun exposure of skin if symmetrical areas are compared. Data concerning the first 4 experimental subjects included in the present study have already been published (29–32).

METHODS AND MATERIALS

Basic design. Experimental subjects exposed one of their arms to the sun while the other upper arm remained covered. Left or right arm was chosen for irradiation at random. On Day 9–11 after exposure, suction blisters were formed and epidermal samples analyzed for dry weights and UA. The interval of about 10 days was chosen on the basis of the first experiment (subject 1).

Additional analyses. In subjects 1 to 4 (males aged 25–48) several samples were obtained at additional intervals after unilateral exposure. Irradiated and control samples were not obtained simultaneously, but comparison was based on samples from symmetrical sites. In subjects 5–15 (8 males and 3 females aged 19–21), additional pairs of samples from non-irradiated arms were obtained about 2 months before the day of exposure. This made it possible to explore some relationships to be discussed below. In 8 of the subjects, epidermal histidine ammonia-lyase was also estimated on day 9-11 following unilateral exposure.

Exposure to sun. The duration and intensity of sunshine was variable. The experimental subjects were istructed not to wash their upper arms for 24 hours before the experiment. Subjects 5–15 were allowed to move freely during a skiing excursion on a single day, March 30, at the same place (50°39′ northern latitude, 1150 m above sea level), from 11 a.m. to 1 p.m. The exposure was thus more uniform than in the subjects 1–4, but generally very low (solar erythema was not observed in most cases).

Sampling. Blisters were formed in the middle of the anterior surface of the upper arm by suction, using essentially the method of Kiistale and Mustakallio (33). Negative pressure applied varied between 300 and 400 mm Hg, the time necessary for the production of a blister varied from 1.5 to 4 hours. If a confluent blister was not formed under the whole cylinder of the "angiosterometer" within 4 hours, the area of the blister base was measured using a rectangular grid. The blister skin was then cut with sterilized scissors and adhering droplets of blister fluid removed gently with a piece of gauze. The skin was weighed immediately and then dried to constant weight (30 min. at 105° C). Dry weights varied between 26–43% of the fresh weight, yet neither this percentage nor the fresh weight are reported here, since we consider them fortuitous. For subjects 1–4, they can be found in the preliminary paper (30).

Electron microscopy. Parts of some of the blister skins on the control and insolated upper arm were cut off for electron microscopic examination before weighing. The tissue was prefixed using cold 3% glutaraldehyde in Sörensen buffer of pH 7.4, postfixed by 1% OsO_4 (Caulfield), dehydrated and embedded in Vestopal W. The method of Mäkinen and Arstila (34) was used for correct orientation. Ultra-thin sections cut using a Reichert ultramicrotome were stained with uranyl acetate (Watson) and lead citrate (Reynolds) and examined using a Tesla BS 242B table-type electron microscope.

Thin-layer chromatographic and spectrophotometric estimation of UC. Dry blister skins were ground, using a mechanically rotated glass pestle (rod with a conical end), with 0.1 g of sand in a glass tube, whose end was drawn out in a capillary. The material was then moistened with 1% NH_3 in 70% aqueous ethanol and after 30 min. eluted with the same mixture directly on the origin (10–15 mm wide) of a TL chromatogram.

Thin layers were prepared by spreading 4 g Kieselgel HF_{254} (Merck) in 12 ml distilled water on glass plates 20 cm by 20 cm. After drying at 105° C for 2 hours the layer was 0.2 mm thick (0.01 g per cm^2). Although the UV blank was low

(35), the plates were purified by chromatographic ascent of the solvent system to be used and then heated again. Solvent iPrNH$_3$ consisted of 2-propanol-aqueous ammonia-water (17:1:2 v/v). Solvent BuAc (1-butanol-conc. acetic acid-water 3:1:1 v/v) was employed mainly for two-dimensional chromatograms.

Room temperature and S chambers according to Stahl were used. Representative R_F values were 0.33 for *trans*, 0.47 for *cis* in iPrNH$_3$, 0.48 for *trans* and 0.40 for *cis* in BuAc.

If all operations are carried out in subdued daylight or under an electric bulb or fluorescent lighting, no isomerization occurs. Direct sunlight, even when passed through window-panes, causes appreciable *cis-trans* isomerization.

Although in the case of skin or sweat the TLC zones of UA are practically free of contaminants, the method has been shown to be inadequate for corium or blister fluid, which contain UV absorbing components that are not completely resolved from UA by this method. A combination with further chromatographic methods is indicated (35, 36).

The zones of UA were revealed as dark spots on a fluorescent background using a 254 nm source (37), scraped off, eluted with 3 ml of distilled water, and the eluate subjected to photometry at 277 nm. The results were calculated on the basis of standards (10 μg) which were chromatographed, eluted and measured in parallel (there was linearity between the absorbance and the amount of UA applied). Eluates from chromatograms on which no sample was applied served as blanks.

Histidine ammonia-lyase (EC 4.3.1.3). A modification of the method of Tabor and Mehler (38) was used without the dialysis and perchloric acid deproteinization introduced by Zannoni and La Du (39). An aliquot (2–6 mg) from freshly collected blister skin was added to 0.2 g sand and a small volume of buffer in a test tube and thoroughly ground by mechanical rotation using a loosely fitting glass rod with a conical end.

The material was then suspended in the rest of the buffer (0.01 M pyrophosphate pH 9.2) to make the total buffer volume to 1 ml and the mixture was spun for 20 min. at 5,000 rot. per min.

The incubation mixture contained pyrophosphate buffer pH 9.2 (30 μmoles), L-histidine (2 μmoles), reduced glutathione pH 9.2 (20 μmoles) and the tissue supernatant (0.2 or 0.3 ml). The supernatant was replaced by additional buffer in the control incubation.

The samples were incubated at 37° C in a temperature-controlled cuvette, and using a Zeiss VSU 1 spectrophotometer the absorption at 277 nm was read at frequent intervals. (It was necessary to carry out a blank incubation since a change in absorbance was noted when GSH and histidine were incubated at pH 9.2.) The absorption values were plotted and enzyme activity was calculated from the difference in slope between the enzymic and blank incubations.

Statistical computations. A program was elaborated by the Computer Center of the Faculty of Medicine in Hradec Králové for the estimation of statistical parameters (t-test for unpaired and paired values, correlation analysis etc.). Thanks are due to Dr. T. Husák for his advice. In the following text, unless indicated otherwise, p stands for the probability that the difference between the means of two sets of values is *not* due to random factors. Fiducial limits indicate the range within which the population average is to be found with a probability of 0.95.

RESULTS

Electron Microscopic Control

Electron microscopic control was used to ascertain whether the specimens contained all of the epidermal layers and none of the components of the dermis.

It has been found in the samples from both the control and insolated arm that the epidermis was stripped off at the dermo-epidermal junction, in agreement with the originators of the method (33). None of the preparations contained the connective tissue elements of the dermis, and all of the epidermal layers were present. The basement membrane, which separates epidermis from dermis, could not be demonstrated, whereas the bottle-shaped microvillous cytoplasmic protrusions of the basal cell layer, originally pointing to the dermis, were present. There is no indication that any part of the bodies of the basal cells (and thus of the epidermis as a whole) was missing.

A comparison between the electron microscopic picture of epidermis obtained by the suction blister method and surgical biopsy of the whole skin has been presented elsewhere (41). The main differences consisted in the widening of intercellular spaces and cytoplasmic, especially perinuclear, vacuoles in the prickle cells of some but not all of the suction blister skins.

Epidermal Urocanic Acid of the Irradiated and Control Arm

The results are summarized in Table I. Values referring to 9–11 days after exposure of one arm are italicized. They show that in most cases there was an increase of UA after irradiation, but in male 6 and female 15 the situation was reversed.

For subject 1 it may be gathered that 24 hours following insolation, UA levels were already higher on the insolated side than on the

TABLE I

Urocanic acid in the epidermis of the irradiated and control arm

No. and sex	Time after insolation (days)	Dry weight, mg/cm²		UA, μg/mg dry weight				UA, μg/cm²			
		Control side	Exptl. side	Control side		Exptl. side		Control side		Exptl. side	
				trans	cis	trans	cis	trans	cis	trans	cis
1, m	1	3.16	1.75	0.43	*	0.95	0.62	1.36		1.66	1.08
	3	2.00	2.78	0.74		0.95	0.50	1.48		2.64	1.39
	9	*2.00*	*2.46*	*0.74*		*2.33*	*1.03*	*1.48*		*5.72*	*2.54*
	20	2.19	4.50	1.88	0.16	2.43	0.26	4.13	0.34	10.92	1.17
2, m	*10*	*2.81*	*3.40*	*0.64*	*0.05*	*0.98*	*0.26*	*1.80*	*0.14*	*3.34*	*0.89*
3, m	*11*	*4.18*	*4.11*	*0.98*	*0.07*	*3.93*	*0.57*	*4.10*	*0.29*	*16.20*	*2.35*
4, m	2	4.55	3.59	0.55	0.18	0.70	0.38	2.75	0.80	2.52	1.35
	10	*4.00*	*3.27*	*0.29*		*3.20*	*0.25*	*1.16*		*10.50*	*0.83*
	17	5.00	4.55	0.68		1.40		3.33		6.40	
5, m	0†	2.50	3.81	1.48		0.82		3.70		3.10	
	10	*2.50*	*3.99*	*0.87*		*1.94*		*2.18*		*7.70*	
6, m	0	2.44	1.15	0.23		0.83		0.58		0.96	
	10	*2.66*	*4.33*	*2.37*		*0.60*		*6.33*		*2.62*	
7, m	0	3.30	1.62	0.27		0.38		0.92		0.62	
	10	*3.41*	*4.10*	*0.99*		*2.17*		*3.48*		*8.85*	
8, m	0	3.33	2.42	0.80		1.17		2.66		2.85	
	10	*3.42*	*4.12*	*1.65*		*1.95*		*5.67*		*8.10*	
9, m	0	2.85	3.19	0.90		0.28		2.57		0.88	
	10	*2.93*	*3.10*	*1.08*		*1.00*		*3.16*		*3.10*	
10, m	0	3.44	2.68	0.58		0.25		2.00		0.67	
	10	*3.21*	*2.92*	*1.58*		*2.55*		*5.08*		*7.44*	
11, m	0	5.71	3.30	0.91		1.30		5.22		4.40	
	10	*6.57*	*4.27*	*1.01*		*2.17*		*6.70*		*9.25*	
12, m	0	3.25	2.72	0.80		1.35		2.60		3.69	
	10	*4.46*	*4.39*	*0.90*		*1.18*		*4.06*		*5.21*	
13, f	0	5.50	3.04	1.04		1.60		5.70		4.80	
	10	*3.58*	*5.15*	*1.90*		*3.12*		*6.80*		*16.10*	
14, f	0	3.15	4.20	2.00		2.38		6.31		10.00	
	10	*3.00*	*4.34*	*2.28*		*2.05*		*6.89*		*9.00*	
15, f	0	3.26	2.75	2.97		2.82		9.73		7.77	
	10	*3.47*	*2.82*	*1.73*		*1.45*		*6.03*		*4.10*	

* No entry in the *cis*-UA column means that it was too low for quantitative evaluation or not detectable.

† Day zero refers to samples obtained 2 months before the day of exposure of one arm.

TABLE II

Statistical analysis of the data (15 subjects)

		Symmetrical controls (not exposed to sun)	Exposed to sunlight 9–11 days before production of blisters.
Dry weight, (mg/cm²)	Range	2.00–6.57	2.46–5.15
	Average ± S.D.	3.480 ± 1.075	3.785 ± 0.744
	Fiducial limits	2.885–4.075	3.373–4.197
	Significance of difference	not significant	
UA, (μg/mg dry weight)	Range	0.74–2.37	0.61–4.50
	Average ± S.D.	1.276 ± 0.608	2.183 ± 1.063
	Fiducial limits	0.939–1.613	1.594–2.771
	Significance of difference	$p > 0.95$	
UA, (μg/cm²)	Range	1.16–6.89	2.62–18.55
	Average ± S.D.	4.357 ± 2.039	8.263 ± 4.480
	Fiducial limits	3.228–5.486	5.782–10.744
	Significance of difference	$p > 0.99$	

control side. The increase was still present on day 20 in subject 1 and on day 17 in subject 4.

cis-Urocanic acid showed a conspicuous rise on insolation in subjects 1–4.

No consistent trend was noted in the variations of the dry weights per unit area upon irradiation.

Statistical evaluation of the values for day 9–11 is presented in Table II. A significant difference ($p > 0.95$, according to the *t*-test for paired values) was obtained for UA in terms of μg per unit dry weight of sample. There was a highly significant difference ($p > 0.99$) for UA in terms of μg per unit area of skin surface. Dry weights per unit area showed no significant difference.

Other Relationships Between the Samples

The experimental design in subjects 5–15 allowed the testing of some of the questions which had emerged.

1. *Dominant vs. subordinate arm (both non-irradiated)*. According to Král *et al.* (42) there is a difference between the rate of secretion of pilocarpine-induced sweat on the forearm, which is correlated with the dominance of the hand. We have therefore compared the data for non-irradiated symmetrical upper arm areas and found no significant difference between the dominant and subordinate arm.

2. *Relationship between the first and second collection* on the same side. Ratios of the analytical values of the second and first collections on the same side were calculated and their means (on the irradiated and control side) compared. As shown in Table III, a significant difference of the means was found in the dry weights per unit area. This is the only case in which it was possible to unmask a trend towards thickening of the epidermis and/or the horny layer upon irradiation. This trend, of course, has been generally accepted and needs no confirmation.

3. *The influence of duration of the suction and blister formation*. In a preliminary paper (30) we raised an objection against our own interpretation of the results as constituting evidence for the changes in UA concentration *before* the suction began. Concentration of UA might change during blister formation, e.g. by elution into the blister fluid or enzymic production. In both cases *duration* of blister formation might be relevant. A number of statistical tests were therefore applied to the relationship between the time of blister formation and UA levels and no correlation was found.

In addition, the times of blister formation on the irradiated side (subject 5–15, 9–11 days after exposure) did not differ significantly from those on the contralateral side in the same experiment: 194 ± 43 min. and 187 ± 51 min., resp.

4. Though the subjects were instructed, as already mentioned, not to wash their upper arm for 24 hours before the collection of the

TABLE III

Statistical analysis of ratios between results obtained in April and those in January–February
(Subject 5–15)

		Control arm	Arm irradiated or to be irradiated
Dry weight, (mg/cm²)	Average ± S.D. Fiducial limits Significance of difference	1.025 ± 0.171 0.910–1.140 $p > 0.95$	1.614 ± 0.849 1.043–2.184
UA, (μg/mg)	Average ± S.D. Fiducial limits Difference	2.394 ± 2.783 0.524–4.264 not significant	2.739 ± 2.903 0.789–4.689
UA, (μg/cm²)	Average ± S.D. Fiducial limits Difference	2.445 ± 2.954 0.461–4.429 not significant	4.158 ± 4.413 1.194–7.123

sample, different times since the last washing of the upper arm were reported on interrogation. When the analytical results were plotted against these times, no relationship at all was noted.

Histidine ammonia-lyase. In 8 cases of subgroup B on day 9–11 following unilateral insolation, the means ± S.D. were, for the control arm, $(1.60 \pm 1.07) \times 10^{-4}$ μmole UA/min/mg dry weight and, for the irradiated arm, 1.85 ± 0.67 in the same units. The increase on the irradiated side is not significant (*t*-test for paired values). The correlation of the UA levels and of the enzyme activity was tested, both for values expressed per unit dry weight and per unit skin surface area. The correlation coefficients calculated for the irradiated and control side and for the pooled values (both sides) did not differ significantly from zero.

DISCUSSION

The main objection against the physiological role of epidermal UA as a sunscreen, which has been raised by Zannoni and La Du (39) and quoted by others, is based on the observation that no hypersensitivity towards light has been reported in histidinemia, in which histidine ammonia-lyase and therefore UA are absent from the epidermis. We have also observed one European and one African Negro subject in whom UA was below the detection limit. Neither subject complained of abnormal sensitivity to light. In our opinion, this objection does not rule out the possibility of a physiological protective role for UA, since the individual protective mechanisms may not only be potentiated but, if one of them is genetically blocked, they may substitute for each other. This is well known and is illustrated by the adaptive thickening of the horny layer following exposure to sun of albinos (43).

Our results (Table II) show that UA actually increases following irradiation, even if the latter was comparatively weak in subjects 5–15. Thus UA may, in the same way as pigmentation and the thickening of the horny layer (incidentally, hardly picked up by the dry-weight method in the present study), be considered as a response to the exposure to sun.

It is well known that the time courses of solar erythema, pigmentation and epidermal thickening differ. The increase in UA may also take a time course characteristically different from that of the other responses. Our present results do not allow us to pass a definite judgement about the time course, but the results for subject 1 and 4 (Table I) would suggest that it is much slower than that of erythema and that pigmentation is more persistent.

We ourselves have raised an objection (30) that the different degree of elution of UA into the blister fluid on the irradiated site might affect the result. Results recorded under 3 in the section on other relationships between the samples do not support this possibility. Similarly, the variation in time elapsed since the last washing did not introduce any systematic error.

In keeping with the published data (18, 44–46), *cis*-UA increased upon insolation in subjects 1–4, due to photo-isomerization. We agree with Baden and Pathak (18) who have hinted that UA has one of the rare features of an ideal sun-screen, namely a high degree of practical stability towards light; many of the other UV absorbing compounds are photolysed readily whereas *trans*-UA is converted to its *cis*-isomer until photo-equilibrium is reached. The *cis*-form of UA, unlike that of a number of other related unsaturated substances, does not differ from the *trans*-isomer appreciably as regards its specific absorbance. Other photolytic products, though their formation is well known, are not produced in appreciable quantities at radiation doses compatible with moderate skin damage.

The question arises as to how to explain the increase of UA. The main alternatives are: (a) an increase in the activity of histidine ammonia-lyase, (b) an increased availability of the precursor, namely histidine (c) thickening of the viable layer, provided it contains appreciable amounts of UA.*

(a) An increase of epidermal histidine ammonia-lyase in guinea-pigs irradiated by UV *in vivo* has been noted (18, 27). The analyses reported in the present paper were done too late (day 9–11 post-irradiation) and thus the lack of significance of the increase is not relevant in this respect; the same possibly applies to the lack of significance of the increase of the enzyme activity in guinea-pigs 96 hours after UV irradiation (47).

(b) An increase in amino acids, including histidine, has been noted in a few preliminary paper chromatographic experiments which are insufficient to allow us to pass a definite judgment.

(c) There are many reports on the presence of UA in the horny layer and in the epidermis as a whole, but the presence in the germinative layer is not definitely stated. We have not succeeded in clarifying this question (49) and we intend to throw light upon it using a different technique.†

* We are indebted to a reviewer for this Journal, for his suggestions pointing to the latter possibility.

† UA has been found in the viable layer (the epidermis regenerating after the stripping of the horny layer) by Baden *et al.* (50).

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