THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

IN VIVO CHANGES IN UROCANIC ACID OF GUINEA PIG AND HUMAN EPIDERMIS BY ULTRAVIOLET LIGHT

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

J. HILL ANGLIN, JR.

Oklahoma City, Oklahoma

1962

IN VIVO CHANGES IN UROCANIC ACID OF GUINEA PIG

AND HUMAN EPIDERMIS BY ULTRAVIOLET LIGHT

APPROVED BY

Orley T. Bever

R. Caputto

W R Shetlar

Earl T. Larsen

Josh R. Schutte

DISSERTATION COMMITTEE

ACKNOWLEDGMENT

The author wishes to express his appreciation to Dr. A. T. Bever, Jr. and other members of the faculty of the Department of Biochemistry for their continued interest and assistance in the investigation and in the preparation of the manuscript. He also thanks Dr. M. A. Everett and the Department of Dermatology for making available the necessary facilities for this research.

TABLE OF CONTENTS

Page

LIST OF TABLES v

LIST OF ILLUSTRATIONS. vi

Chapter

I. INTRODUCTION 1

II. EXPERIMENTS. 15

III. DISCUSSION 30

IV. SUMMARY. 36

BIBLIOGRAPHY 37

LIST OF TABLES

Table		Page
1	R_f Values	23
2	R_f Values from Kurogochi et al. . .	26

LIST OF ILLUSTRATIONS

Figure		Page
1.	Absorption Spectra of Acid Soluble Skin Extracts	18
2.	Chromatogram of the 260 mμ Absorption of Guinea Pig Epidermal Extracts	21
3.	Chromatogram of the 260 mμ Absorption of Human Epidermal Extracts	22
4.	Infra-red Spectra of the Urocanic Acids.	27

IN VIVO CHANGES IN UROCANIC ACID OF GUINEA PIG AND HUMAN EPIDERMIS BY UNTRAVIOLET LIGHT

CHAPTER I

INTRODUCTION

Initially it was planned to study biochemical alterations in skin that resulted from pathological responses to ultraviolet light (UV). When attempts to duplicate a process for sensitizing the skin to UV by injection of sulfonamides (1) proved to be unsuccessful, it was decided to embark upon an investigation of the sequence of biochemical events produced in normal skin by UV.

Because the experiments were necessarily exploratory in nature, animal skin was used. Human skin was used later to ascertain whether similar changes were occurring there.

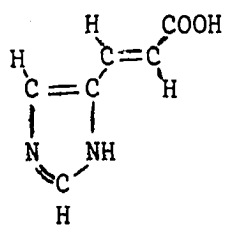
In these exploratory experiments each animal served as its own control. One side of the animal was irradiated while the other side was protected from irradiation. Three minimal erythema doses of UV were used in order to produce a definite sunburn reaction but not sufficient to destroy cells and possible labile intermediate products. Parallel analyses were made on the skin from the control and irradiated sides of each animal. The phosphorus content extractable by several solvents was determined, but a definite difference was not found between control and irradiated sides of the animals. This is attributed to insufficiently

refined sampling technique and the great variety of phosphorus containing substances in each group. Preliminary measurement of hyaluronidase activity and transaminase activity was made but no relationship between enzyme activity and UV irradiation was found.

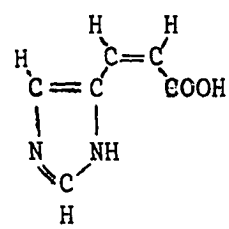
The discovery of a decrease in the specific UV absorbency observable in the dilute acid extract from UV irradiated skin when compared with extracts of control skin led to the investigation to be reported here. This dissertation will be concerned with the isolation and identification of urocanic acid from skin and the determination of its apparent in vivo changes resulting from irradiation of normal skin.

The first report of urocanic acid in the chemical literature was made by Jaffe (2), who in 1874 isolated and characterized the substance from the urine of a dog that had been fed nitrotoluene for a period of time. The next report of urocanic acid was in 1898 by Siegfried (3) who isolated it again from the urine of a dog after feeding sodium telluride. It was constantly present in the urine of both dogs to the extent of 2-3 gm per day. The dogs appeared healthy otherwise.

Urocanic acid (UCA) was shown to be identical with β imidazol acrylic acid (4), that was prepared by the reaction of α chloro β imidazol propionic acid and trimethyl amine (5).



trans UCA



cis UCA

Raistrick (6) isolated urocanic acid from B Paratyphosus A culture medium grown in Ringers Solution with histidine as the sole carbon source.

He also reported that the medium developed an alkaline reaction and that growth was very slow. Several other species of bacteria were reported to produce urocanic acid in a low yield.

In the course of an extensive series of feeding experiments Katake and Konishi (7) found urocanic acid as the only histidine derivative in the urine of rabbits when fed large amounts of histidine. This was in contrast to the results from feeding increased amounts of tyrosine in that the urine from the tyrosine fed animals contained the corresponding α hydroxy- and α keto-acids. No trace of such compounds were found for the imidazole derivatives. Injection of histidine also led to urocanic acid in the urine. However if imidazole lactic acid was injected no urocanic acid was found in the urine.

In 1926 Edlbacher (8) began a series of reports concerned with histidine metabolism. He investigated the possibility that the two nitrogen atoms of the imidazole ring might be intermediate in urea formation and was able to show that liver-brei from the dog, rabbit, guinea pig, duck, chicken and frog would release ammonia but no detectable urea. Only liver preparations were active; while those from kidney, spleen, pancreas, intestinal mucosa, thyroid, testicle, ovary and muscle were unable to break down histidine. Incubation of the liver preparations in the presence of toluene to inhibit bacterial growth, showed that two-thirds of the nitrogen of the histidine was released as ammonia and one-third remained fixed. The following year Edlbacher and Simons (9) reported that arginase was different from histidase.

In a series of observations of histidine metabolism Kauffman and Mislowitzer (10) found that a liver-brei released a little over one-third of the nitrogen as NH_3 from histidine in contrast to about two-thirds found by Edlbacher.

In 1926 Cox and Rose (11) reported, from growth experiments employing rats, that histidine could not be replaced in the diet by purines and that urocanic acid as well as several other imidazole derivatives were not effective substitutes for histidine. Of several imidazole derivatives tested, only imidazole lactic acid was able to replace histidine. A detailed method for preparation of urocanic acid was given.

In the report of an extensive series of experiments Edlbacher and Kraus (13) concluded that 1) histidase can be found only in liver, 2) it is not identical with arginase, 3) splitting one mole of histidine forms two moles of ammonia, one mole of glutamic acid and probably one mole of formic acid, 4) there very probably were no other breakdown products, 5) the splitting probably proceeds by an unknown intermediate product and considered it was probably pyrrolidon carboxylic acid, 6) histidase is a specific enzyme and splits only the naturally occurring histidine and was inactive toward imidazole, imidazole lactic acid, imidazole ethyl amine, methyl histidine, carnosine and histidine ethyl ester, and 7) since no reaction follows enzymatic splitting of histidine as the active (asymmetric) carbon atom, the natural (-) histidine and natural (+) glutamic acid were assigned to the same steric series. On the basis of these observations Edlbacher said that urocanic acid was definitely not a natural intermediate in histidine breakdown.

Kiyzkzwa (14) found that by subcutaneous injection of L-histidine in rabbits UCA could be isolated from the urine. If the same amount of D, L- histidine were injected, UCA was not found but the D-isomer was isolated from urine. He was not able to show the presence of imidazole lactic acid.

In 1931 Edlbacher and Kraus (15) again published an article defending the point that the natural breakdown of histidine proceeded by ring opening with hydrolytic removal of the two nitrogen atoms of the ring. Somewhat later Edlbacher and Neber (16) reported a series of experiments in which no evidence of oxidative deamination was found.

Borghi and Tarantio (17) reported a histidase in skin that removed one atom of nitrogen and quantitatively conserved the imidazole ring. This was different from the known histidase previously described by Edlbacher. These authors thought they were dealing with an oxidative deamination but did not succeed in isolating an αketo acid.

Katake (18) reported an enzyme prepared from cat and rabbit livers that would form D, L-isoglutamine and suggested that UCA was the intermediate product in this pathway of histidine breakdown. Similar enzymatic activity was found in some bacterial preparations.

In 1942 Edlbacher and von Bidder (19) said that, since the natural isomer of glutamic acid could be isolated as a split product of histidine, the first attack by the enzyme could not be by α-deamination but was necessarily ring opening. UCA could not be isolated from splitting of histidine, further UCA could not be isolated from urine by administration of histidine by mouth or injection. They thought this could have been related to a difference in their European animals. However the existence of a possible labile intermediate formed by removing one mole of ammonia was recognized; if hydrolytic breakdown of histidine in the liver was by way of UCA two enzymes would be required.

Again in 1942 Edlbacher (20) reported the separation of a histidase and a urocanase. Histidase activity was isolated by chromatography of the enzyme preparation on lead phosphate by eluting with a citrate buffer,

and urocanase was prepared by adsorption on calcium phosphate. Urocanase activity paralleled the release of ammonia and loss of UV absorption.

In the following paper Edlbacher and Heitz (21) reported results from the experimental injection of urocanic acid and histidine into animals. If histidine was injected subcutaneously into rats, guinea pigs or rabbits, UCA was not detected in the urine; however when the same weight of UCA was injected into these animals not less than 36% and as much as 90% was found in the urine. He believed that these data indicated that UCA could not be an intermediate product in the breakdown of histidine.

It was further reported that there clearly existed two breakdown pathways for histidine. The chief one was by hydrolytic opening of the imidazole ring and the other by α-deamination with the formation of urocanic acid. It was believed that the isolation of natural, L-glutamic acid definitely eliminated the possibility that histidine had undergone α-deamination. In the third (22) paper of the group an improved method for preparation of UCA was reported.

Following the recognition of urocanic acid as a possible intermediate substance in histidine metabolism Edlbacher et al. determined that only one isomer was formed enzymatically. This was designated as the trans isomer. The cis (m.p.=175-6°) and trans (m.p.=218°) configuration being assigned by analogy to the melting points of the cinnamic acids. Enzymatic breakdown of the trans (m.p.=218°) isomer was found to be more rapid than that of the cis (m.p.=175-6°).

In 1943 Darby and Lewis (24) found UCA in the urine of five from a group of eight rabbits after oral administration of histidine but none following subcutaneous injection. In addition it was found that all

rabbits excreting UCA showed signs of toxicity, anorexia, rapid heart rate, difficult respiration, paralysis of the posterior extremities and death in all cases except one.

The pathological findings reported by Darby and Lewis included acute edema of pulmonary tissue, contraction of the pulmonary arteries and smooth muscles of the bronchi, and an abundance of eosinophiles. None of these pathological presentations was found in the animals that did not excrete UCA. The authors concluded that UCA was formed by an abnormal metabolic process and if formed it had a toxic effect on the animal. It should be noted that the animals that did not excrete UCA in the urine did excrete histidine with one excreting both substances. They also found that subcutaneously injected UCA was excreted unchanged in the urine and produced no toxic effects. Darby and Lewis concluded that UCA was probably not in the main pathway for histidine catabolism. In addition it was reported that B paratyphosis A was able to form UCA from histidine, confirming an early report of this by Raistrick.

Walker and Schmidt (25) reported a kinetic study of histidase and found cat liver to be the best source of activity. It was suggested that the same processes did not apply in bacterial systems.

Hall (26) found that UCA could be identified by paper chromatography and that the enzyme capable of forming it was present in aqueous liver extracts. UCA was not found in fresh liver extracts and the possibility of the coexistence of several enzymes participating in the breakdown of histidine by way of UCA was discussed.

Tabor et al. (27), using histidine labeled with N^{15} in the α and γ position and C^{14} at position 2 in the ring, found that the α labeled nitrogen produced 95% N^{15} excess in the NH_3 with a little excess N^{15} in

the glutamic acid. When L-histidine labeled at the γ-nitrogen was used 90% excess N^{15} was found in the glutamic acid formed. If ring 2-C^{14} labeled histidine was used all the C^{14} was in the formic acid.

Mehler and Tabor (28), using a trapping technique in the form of an excess of UCA in the reaction mixture, found an accumulation of tagged UCA in the UCA pool.

Erspomer and Benati (29) were able to show that murexine from the hypobrachial body of Murex trunculus (snail) was the choline ester of UCA. UCA had previously been found only from mammalian and bacterial sources.

Zenisek and Kral (30) found that on each paper chromatogram of human sweat treated with Pauley's diazo reagent an unidentified spot was found. The R_f of this spot did not correspond to any known constituent of sweat that would be able to couple with the diazo reagent. A considerable amount of this substance was found in sweat but not detectable in urine of the same person. It was subsequently shown that the unidentified substance in sweat was UCA. If the sweat was desalted by electrodialysis UCA was reduced to imidizole propionic acid, apparently by electrolytic reduction of the double bond. It should be noted that all of the chromatographic systems reported used were acidic and that many acidic chromatographic media do not separate the cis and trans isomers of UCA.

Vanderbilt and Childs (31) reported the occurrence of a UV absorbing substance in sweat and reported the spectral characteristics. These data were consistent with those reported by Zenisek for UCA. In a note added in proof the identity of the substance with that reported by Zenisek was recognized.

Szakall (32) in an extensive study of the proton donating substances of epidermis was not able to correlate the high UV absorbency of epidermis with the amounts of amino acids isolated or by synthetic mixtures of them. Based on an absorption spectrum with a pH dependent maximum shifting between 260 mμ and 280 mμ it was thought that the UV absorbency was probably due to the presence of purines and pyrimidines of nucleic acid origin.

Goryukhina (33) has reported no difference in the ability of livers from normal and tumorous animals to metabolize UCA.

Tomohiro (34) has reported that histidine irradiated with UV in neutral or alkaline solution was 70-80% decomposed, while in acid solution 12% was decomposed. Decomposition products identified included histamine, imidazole lactic acid, imidazole acetic acid, UCA, asparagine and serine.

Yoshioka (35) has used thioglycolic acid as a protective agent for histidase activity for UCA formation.

Wolf et al. (36) have stated that the metabolism of α-C^{14}-histidine produces carboxyl-labeled imidazole acetic acid in the urine as the principal metabolite. Imidazole acetic acid is generally considered to be an important metabolite of histamine.

Shimomura (37) has isolated 4-oxoglutaramic acid from enzymatic oxidative cleavage of UCA.

Ichihara et al. (38) have isolated hydantoin-acrylic acid formed by the action of a cell free extract from P. aeruginosa on UCA.

Brown and Keis (39) have reported the isolation of a radioactive fraction from urine that has been identified as hydantoin-5-propionic acid from feeding of radioactively tagged UCA.

The cis-trans isomers of UCA were assigned by Edlbacher and von Bidder (22) by analogy to the cinnamic acids. Pasini and Vercellone (40) have arrived at the same designation for the isomers by analogy to the maleic-fumaric pair. It was also reported that murexine is the choline ester of the trans-modification. Cis and trans isomers of UCA give the same product when reacted with ethylene chlorohydrin in the presence of dry HCl. Murexine is formed if this chloroacyl ester is treated with trimethyl amine.

If the trans isomer of UCA is distilled under low pressure (0.1 mm Hg) the 4-vinyl-imidazole dimer is formed. The cis isomer of UCA is reported to isomerize into the more stable trans form on heating. Distillation of the cis isomer forms a small sublimate of the trans isomer with decarboxylation and formation of 4-vinyl-imidazole dimer as the principal product.

Pasini and Vercellone (40) have also determined the ionization constants and report that trans UCA has a pK_1 of 3.5 and pK_2 of 5.9 and the cis-modification has a pK_1 of 3.0 with pK_2 6.7. Imidazole propionic has a pK_1 of 4.0 and pK_2 of 7.5, trans ethyl ester has a pK of 4.8, while murexine has a pK of 4.6.

Miller and Waelsch (41) have reported a study of the properties of urocanase. A mechanism is presented in which the first product is imidazolone propionic acid which is then changed by a second enzyme to L-form-amidinoglutaric acid. They were not successful in demonstrating the presence of easily removable co-factors. The enzyme was inhibited by cyanide, PMB, hydroxylamine, hydrosulfite and bisulfite. Observed inhibition was reduced or removed completely if UCA was added simultaneously with the inhibitor. The active system would catalyze the reduction

of 2,6-dichlorophenolindophenol.

Kurogochi et al. (42) have reported that cis and trans UCA may be separated in alkaline medium by paper chromatography, but the cis and trans isomers both had the same R_f in acid medium and were not resolved. The UV absorbency of both isomers was about the same with maximum absorbency between 265 mμ and 280 mμ depending on the pH, increased acid shifting the maximum absorbency to shorter wave lengths. The melting point of the trans isomer is given 224° (decomp.) and the cis isomer 178-179° (decomp.).

Injection of 500 mg/Kg of cis UCA had no effect on mice; with the same amount of the trans isomer there was a loss of spontaneity and labored breathing. Both isomers decreased the amplitude of heart pulsation followed by a sustained increase. A five fold increase of the cis isomer was required to have as much effect as the trans isomer.

Incubation of rabbit liver extract indicated that trans UCA was readily degraded with the cis showing practically no change. The authors conclude that the biological activity of cis is less than that of trans.

Imidazoleacryl-choline (murexine) and imidazole propionyl choline (dihydroxymurexine) have been shown by I. A. A. Tabachnick and Roth (43) to potentiate the action of histamine on guinea pig ileum, lung and trachea. The author concludes that the observed potentiation results from inhibition of diamine oxidase degradation of histamine.

Acheson et al. (44) have reported the presence of UCA in human urine. The substance was not isolated but the product was identified by R_f values and color formation with diazo reagents.

Kral et al. (45) report a direct relation between pigmentation and concentration of UCA. Urocanic acid has been found to be low in light

colored sensitive skin. It is also reported that UCA does not hinder the tanning reaction.

In a study of the proton contributing substances of epidermis Spier and Pascher (46) continued in the same line of investigation of the UV absorbing substances in human skin that Salzakall (32) had touched on earlier. Strong UV absorbing substances with pH dependent absorption maxima similar to thymine or cytosine were found. It was shown, by paper chromatography, that two strong UV absorbing substances were not any of the known purines or pyrimidines of nucleic acid origin. One of the substances was identified as UCA and the second was very similar but had higher R_f values in alkaline solvent systems. This unknown "Y" substance was not always present and was present in varying amounts, but was always less than the identified UCA. It is interesting to note that the properties of this "Y" substance are consistent with cis UCA, trans UCA apparently being the product identified as UCA.

J. Tabachnic (47) found that an acid soluble extract of guinea pig epidermis contained a substance having a high UV absorbency that was not a purine or pyrimidine as anticipated. It was proved that the substance was UCA. Though the presence of UCA in epidermis was established the effect of light on the substance was not investigated. Later Tabachnic and Weiss (48) reported that about 80% of the UV absorbency of epidermis extracts was due to UCA. Based on this value and 18,800 as the molar absorption coefficient UCA was found to be 0.5%± 10% dry weight of epidermis.

Feinberg and Greenberg (49) describe a preparation of urocanase from liver and show that is able to change UCA to an intermediate, tentatively identified as imidazalone propionic acid. It is postulated

that there is further cleavage of imidazalone propionic acid by a second enzyme to form N-formamino-L-glutamic acid, and a spontaneous non-enzymatic hydrolysis to D,L-formylisoglutamate. It was thought that urocanase was one enzyme of a multiple enzyme system.

Brown and Keis (50) have found that four radioactive substances can be isolated from the in vitro incubation of a liver preparation with C^{14}-UCA. These products are identified as hydantoin propionic acid, formiminoglutamic acid, formylisoglutamine and unchanged UCA. Anaerobic incubation inhibited the formation of hydantoin derivative without affecting the formation of formiminoglutamic acid. Formation of the hydantoin derivative could not be demonstrated except in the presence of urocanase. No cofactor requirement could be demonstrated for urocanase. In the following paper the authors (51) presented further evidence that 4(5)-imidazalone-5(4)-propionic acid was an intermediate in UCA metabolism.

Leif and Herbert (52) were able to demonstrate a marked increase in survival rate of bacteria when irradiated with UV in a medium containing UCA.

Anglin et al. (53) found that UCA present in guinea pig skin was reduced following UV irradiation and that the remaining UCA was a mixture of the cis and trans isomers. Everett et al. (54) reported a similar change in UCA of human skin.

E. Schwarz (55) in an experiment with isolated guinea pig epidermis homogenates, found that histidine 2-C^{14} would be converted to urocanic acid 2-C^{14}. In a series of comparative studies it was concluded that epidermis has the same histidine degrading enzyme that had been previously demonstrated only in liver.

Whitehead and Arnstein (56) have isolated relatively large amounts of UCA from the urine of children suffering from kwashiorkor.

La Du et al. (57) have reported on histidinemia in children. This appears to be an inborn error of metabolism, in which the individuals have a high urinary and plasma level of histidine and apparently are unable to convert histidine to UCA, but are able to metabolize UCA normally if injected. It was also found that the normal epidermis, but not deeper tissue, has a high histidase activity that was not detectable in the epidermis of those having histidinemia.

CHAPTER II

EXPERIMENTS

Preparation and Irradiation of Skin Surface

The first experimental problem was concerned with establishing a standard irradiation procedure to be used. For convenience the minimal erythema dose was taken as the unit for irradiation. It was necessary to remove the hair from the skin of the animal in order for reproducible erythema response to be produced.

Most of the hair was removed with an electric clipper and the remaining stubble removed with an epilating agent. The cosmetic preparation "NEET" has been found to be satisfactory*. It contains thioglycolate which reduces the disulfide bonds of the protein in hair and breaks up the continuity of the fiber. Shaving the animals' hair with a blade or electric shaver appears to cause more damage to the skin than the epilating agent.

NEET was applied to the clipped stubble and allowed to remain for five minutes, gross amounts were then removed with a spatula and the remaining amount removed with a damp cloth or washed off under running water. This procedure produces a smooth non-irritated surface that has been used routinely in this study.

*Manufactured by Whitehall Laboratories, Inc., New York, N. Y.

When the skin of the albino guinea pig was prepared in the above manner the minimal erythema dose of UV was found to be 2 min. ± 15 sec. with a 40 watt hot quartz mercury vapor lamp at 20 inches from the tube. The minimal erythema dose was taken as the amount of radiation that would produce a barely perceptible redness after 20 hours. This is recognized as a qualitative evaluation.

If the prepared skin is irradiated for six minutes a definite redness is produced with some edema in the irradiated areas after 20 hours. These irradiation conditions do not produce a second degree burn with blistering, but some desquamation and scaling may be observed after a few days.

Collection and Preparation of Material for Analysis

The object of the preparatory procedure has been to stop all metabolism as quickly as possible and to analyze the skin with all metabolites present as little changed from that of living skin as attainable. The following methods have been especially developed and employed to reduce the enzymatic and spontaneous changes.

The animals were sacrificed by a blow on the head and the skin was quickly excised, cooled, and placed on the stage of a freezing microtome. It was then frozen to the metal stage by liquid CO_2 released into the expansion chamber of the stage. When frozen the top layers of the skin were removed by the microtome blade; each frozen slice, about 40 micra thick, was collected into a Dewar flask containing dry ice or liquid nitrogen. Occasional sections were examined microscopically to determine that only epidermis was collected. The collected tissue was kept frozen until the water had been removed by lypholization. After the tissues were dried, all subsequent procedures

were carried out at room temperature. The dried, parchment like pieces from the microtome were then ground in a micro Wiley mill with 20 mesh screen*.

The prepared skin powder was then extracted three times with ether to remove much of the lipid which interferes with the subsequent acid extractions. The ether phase was discarded. Following the ether extractions the ether wet residue was broken up and distributed on the tube so that excess ether was allowed to evaporate. The prepared residue was then extracted three times with 0.6 N perchloric acid and the extracts pooled and neutralized with KOH to pH 6.0.

Because the solubility of $KClO_4$ increases with increase of temperature the neutralized extract was placed in the refrigerator or cold room over night to allow more nearly complete precipitation. The prepared extract was not allowed to come to room temperature in the presence of solid $KClO_4$. The clear supernatant was decanted from the precipitate and slowly passed through a prepared resin bed.

Fig. 1 shows the specific absorbency of extracts from control and irradiated guinea pig epidermis. The specific 260 mμ absorbency of the extracts from irradiated skin has been found to be 50-70% of that observed for extracts from control skin.

Preparation of the Ion Exchange Columns

Dowex-1-acetate was prepared from the chloride form by a modification of the procedure described by Hurlburt et al. (58). The chloride form of the resin was put into a large glass chromatographic column. A mixture of 500 ml of glacial acetic acid and 1 pound of sodium acetate

*Product of the Arthur H. Thomas Company of Philadelphia, Pa.

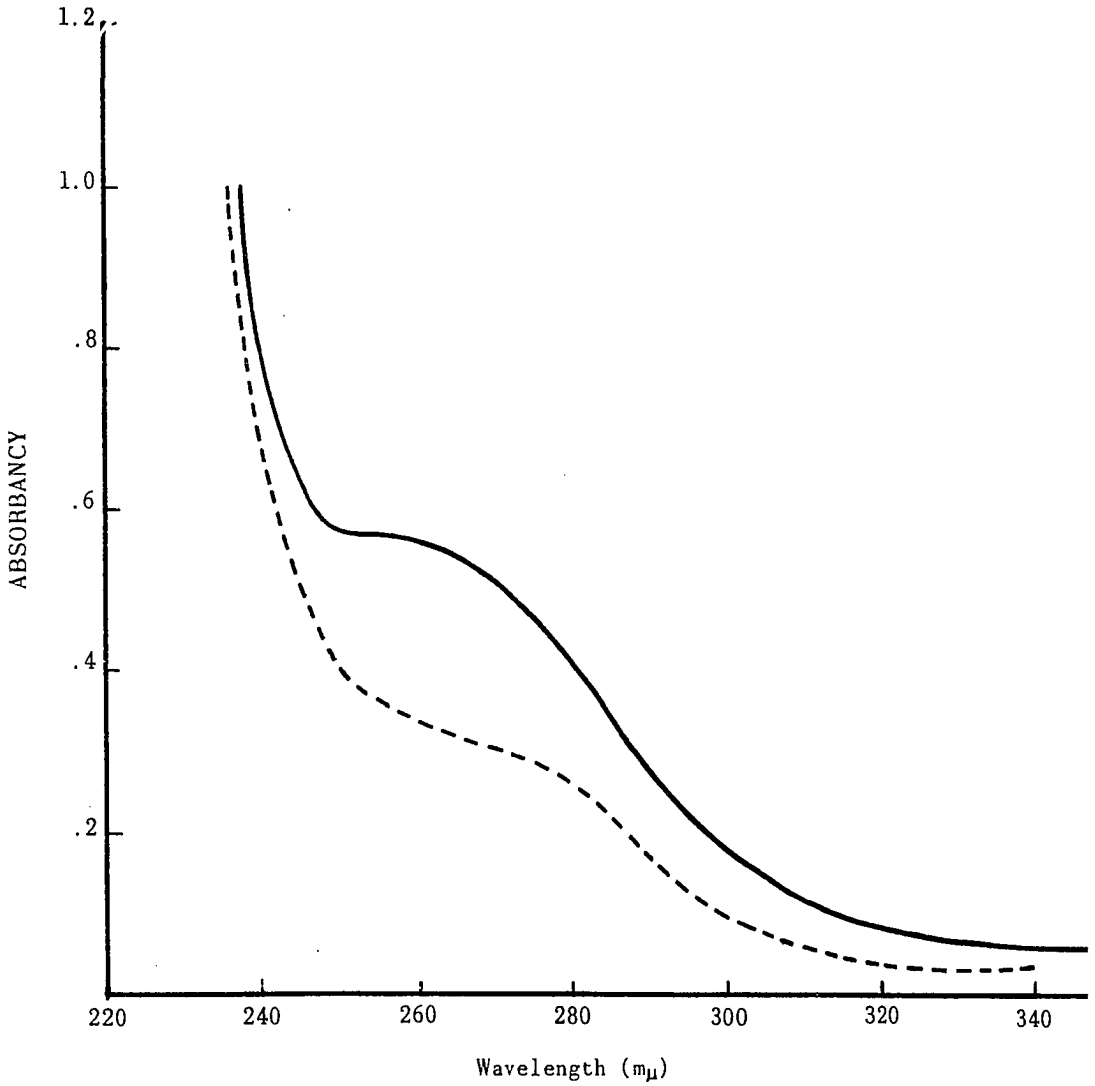


Fig. 1. Spectra of acid-soluble extracts of guinea pig epidermis. ——— , control; ----- , irradiated. 10 mg epidermis/ml, 1-cm light path, water blank.

in a gallon of water was slowly passed through the resin bed until the effluent from the column, when acidified with nitric or sulfuric acid, gave no precipitate with silver nitrate indicating that no more chloride was being displaced from the resin. The prepared resin was stored in an excess of the same medium used for regeneration until used to prepare a column.

If the resin had been used previously with biological extracts it was warmed to about 90° for several hours with 6N HCl to hydrolyze and remove any polymeric substances that may have been held to the resin by adsorption before converting to the acetate form.

The ion exchange columns used for the tissue extracts were prepared by pouring enough of the prepared resin into a 20 mm column to form a bed about 10 times the diameter of the column. The column was washed with distilled water overnight,or until the effluent was the same pH as the distilled water. The required amount of stock prepared resin was poured into the column all at one time and the wash with distilled water started. If the column was vertical this gave a satisfactory, uniform packing in the column.

Application and Elution of Extracts from the Column

The clear acid soluble extract previously described was passed slowly through the washed resin bed followed with an equal volume of distilled water. The loaded column was then put on a fraction collector equipped with a drop counter. Elution was by 0.04 N HCl applied directly to the column without gradiant dilution. It was noted that the top portion of the resin changed appearance as the HCl was added; apparently it was being converted to the chloride form by contact with the chloride ion. In later experiments 0.04 N acetic acid was used

with no change found in the elution pattern for urocanic acid. Chloride ion was apparently exchanged for acetate by the resin so that elution was effected by the acetic acid so released. The 260 mμ absorption of each fraction was recorded and a chromatogram prepared.

Chromatogram of the 260 mμ Absorption of Control and Irradiated Extracts

See Fig. 2 and 3. The molar extinction value of *cis* UCA has been reported to be about the same as that for *trans* (42). In Fig. 2 the specific absorbency of UCA as *cis* and *trans* from the irradiated tissue was 63% of that obtained from the control. While in Fig. 3 the specific absorbency from human tissue extracts of *cis* and *trans* UCA recovered from irradiated skin is about 30% of that found in the control.

Analysis of 260 mμ Absorbing Fractions

The material in the tubes from the absorption peaks was concentrated by freeze drying and taken up in a minimum of water for convenience in preparing the chromatograms. Tubes 40 and 44 as well as tubes 53 and 58 from the extract of irradiated guinea pig skin and tubes 61 and 64 of the extract from the control guinea pig skin as seen in Fig. 2 were used for ascending paper chromatography. The unknowns and a group of known substances were applied to paper and the chromatogram developed by ascending technique. The known substances were uridine, uracil, urocanic acid (*trans*), cytidine, cytosine, guanine, guanosine and inosine. The observed R_f values are in Table 1. It may be seen that solvent system I (n-propanol/ammonia/ water, 60/30/10) indicated that tubes 40 and 44 from the irradiated extract were none of the known substances but possibly were both the same substance, and that tubes 53 and 58 from the irradiated

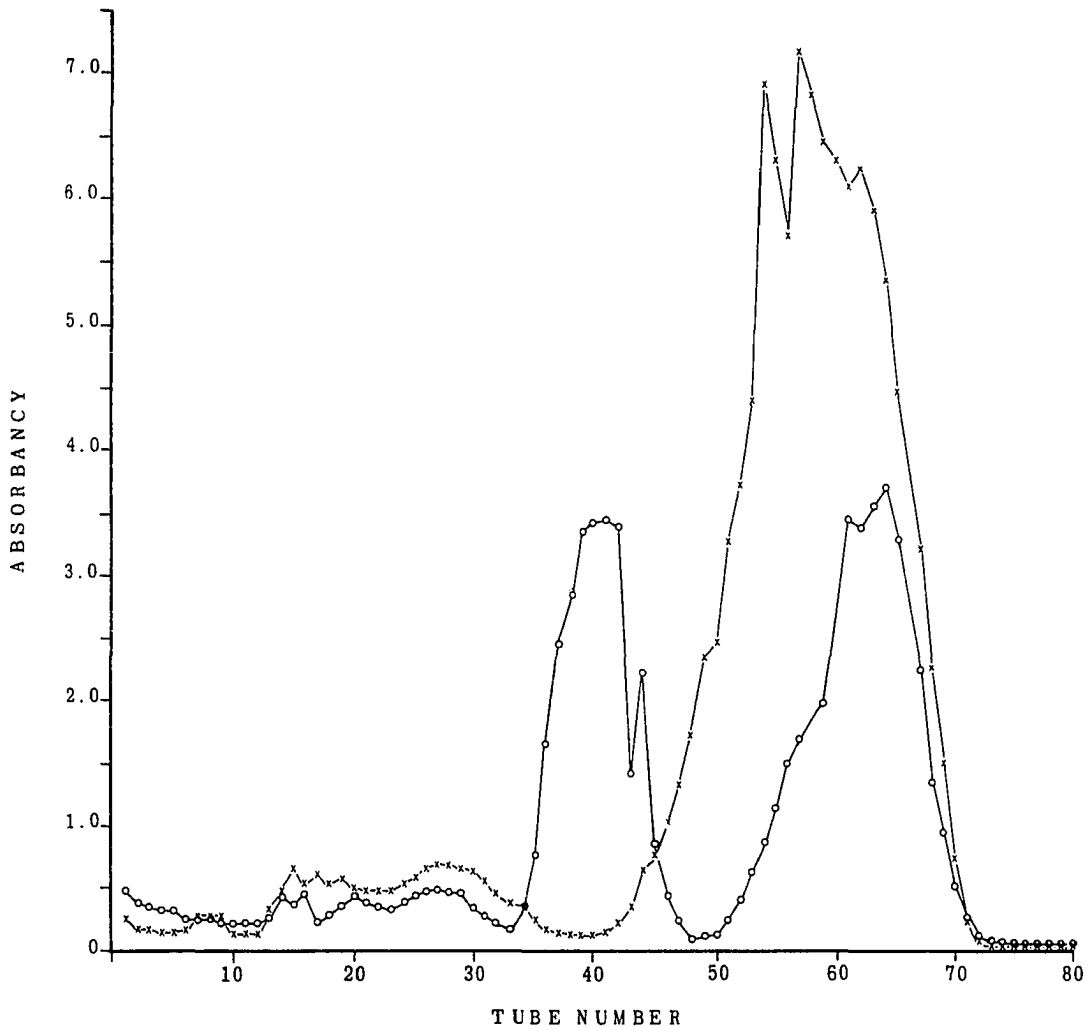


Fig. 2. Chromatogram of 260-mμ absorption. x-x-x-x-x, control (non-irradiated); o—o—o—o—o, irradiated from 3.4 g lypholized guinea pig epidermis, 60 ml resin volume Dowex-I X8 acetate, eluted with 0.04 *N* HCl, 100 drops/tube.

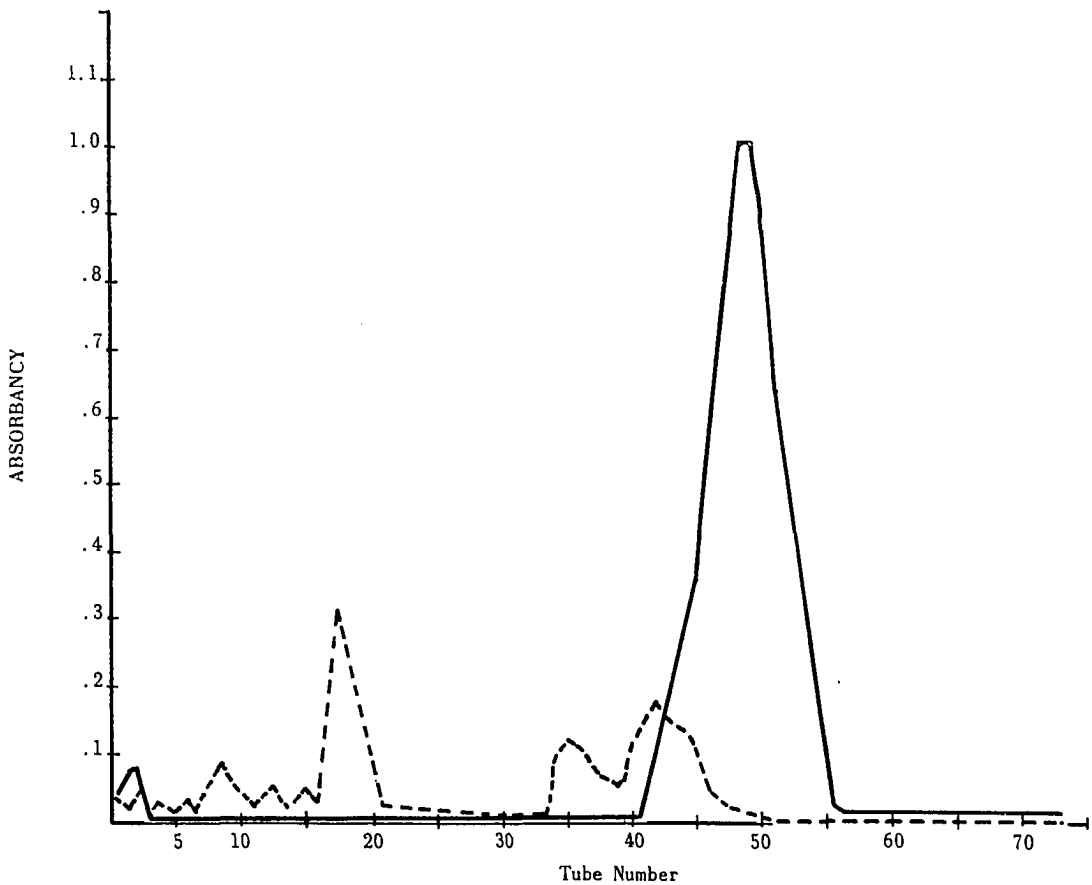


Fig. 3. Chromatogram of 260 m_{μ} absorption. —— control (non-irradiated); ------- irradiated, from 0.8 gm human epidermis, 10ml resin volume Dowex-1 x8 acetate, eluted with 0.04 N HCl, 37 drops/tube.

TABLE 1

R_f VALUES

Tube Number	Solvent I*	Solvent II**	Solvent III***
40I (cis UCA)	.71	.52	.66
44I (cis UCA)	.71	.52	.66
53C (trans UCA)	.58	.52	.66
58C (trans UCA)	.55	.52	.66
61I (trans UCA)	.53	.49	.66
64I (trans UCA)	.55	.52	.68
Known Substance			
Urocanic Acid (trans)	.57	.50	.66
Uracil	.57	.64	.57
Cytidine	.53	.27	.59
Adenine	.52	.24	.90
Adenosine	.60	.23	.76
Cytosine	.58	.32	.66
Guanine	Streak	.32	-
Guanosine	.39 & .48	.18	-
Inosine	.50	.20	-
Uridine	.49	.51	-

* N-Proponal/Ammonia/Water; 60/30/10

** T-Butanol/conc. HCl/Water; 70/14.5/15.5

*** Isobutyric acid/Ammonia/Water; 66/1/33

specimen and tubes 61 and 64 from the control extract could be uracil, urocanic acid (trans), cytidine, adenine, adenosine or cytosine. Application of solvent system II (t-butanol-conc./HCl/water 70/14.5/15.5) indicated that of the possibilities not eliminated in system I the unknowns could not be adenine, adenosine, cytidine, or cytosine. When solvent system III (isobutyric acid/NH_3/H_2O/ 66/1/33) was employed, it was found that the unknown was not uracil. In summary, of the total group of known substances investigated, tubes 40 and 44 of the extract from irradiated skin were shown not to be included and tubes 53 and 58 of the extract of irradiated skin might be identical with urocanic acid. As only one spot was detected in each of the three chromatographic systems from the material in the two tubes from each of the three 260 mμ absorption peaks investigated it was concluded that each peak represented a single substance. Each tube was analyzed for phosphorous by the Fisk-Subbarow method as modified by Dryer (60) and ribose was determined by the orcinol method (61). No phosphorous or pentose was detected.

When commercial urocanic acid was irradiated in vitro it was found to have an extra spot in system I that coincided with the unknown spot from the extract of irradiated tissue. A tentative hypothesis was that normal skin contained one isomer of urocanic acid and irradiation led to a mixture of the two isomers. This hypothesis was supported by the fact that cis-trans isomerization is a typical reaction of substituted ethylenes elicited by UV (62).

Fig. 3 represents a chromatogram of human epidermis extracts. Tube 51 from control extract and tube 42 of irradiated extract gave paper chromatographic spots with the mobility equal to that of trans UCA. Tube 35 from the extract of irradiated human epidermis had the

same paper chromatographic mobility as cis UCA. These substances and authentic samples were developed by ascending paper chromatography in system A described by Kurogochi et al. (42). All three substances isolated from human skin gave a positive Pauley diazo reaction.

Confirmation of the Presence of Trans UCA and Formation of the Cis Isomer

Using chromatographic solvent systems described by Kurogochi et al. (42), Table 2, it was found that material from tubes 40 and 44 from the irradiated guinea pig skin extract corresponded in R_f with that to be expected for cis urocanic acid and that tubes 61 and 64 from the irradiated extract as well as tubes 53 and 58 from the control extract agreed for trans urocanic acid. Considerable variation may be encountered in the actual R_f values but the relative position of the cis and trans isomers is dependable when developed in system A. The wave-length of maximum absorption shifted from 265 mμ in acid to 280 mμ in base to agree with reported data for the two isomers. The substances give a positive Pauley diazo reaction when treated with alkaline diazotised sulfanilic acid and a negative ninhydrin test.

Cis UCA isolated from guinea pig skin melted 168-172° while that prepared by in vitro reactions melted 178-179°. The literature gives the melting point of the cis isomer as 175-176° (22) or 178-179° (42). Trans UCA isolated from skin melted 221-224° and the commercial product melted 224-225°. The literature gives the melting point of trans UCA as 218° (22) or 224° (42).

Fig. 4 shows the infra-red absorption spectrum* of the cis and

*Model 21-Perkin-Elmer recording infra-red spectrophotometer.

TABLE 2

R_f VALUES* FROM KUROGOCHI et al.

cis UCA	Lit.	.66
	found	.60±.08
trans UCA	Lit.	.34
	found	.33±.03

* n-proponal/0.2 N Ammonia; 3/1

Fig. 4. Infrared Spectra

A

Cis UCA
from Skin
M.P. 168-72

B

Cis UCA
Prepared
From Trans

C

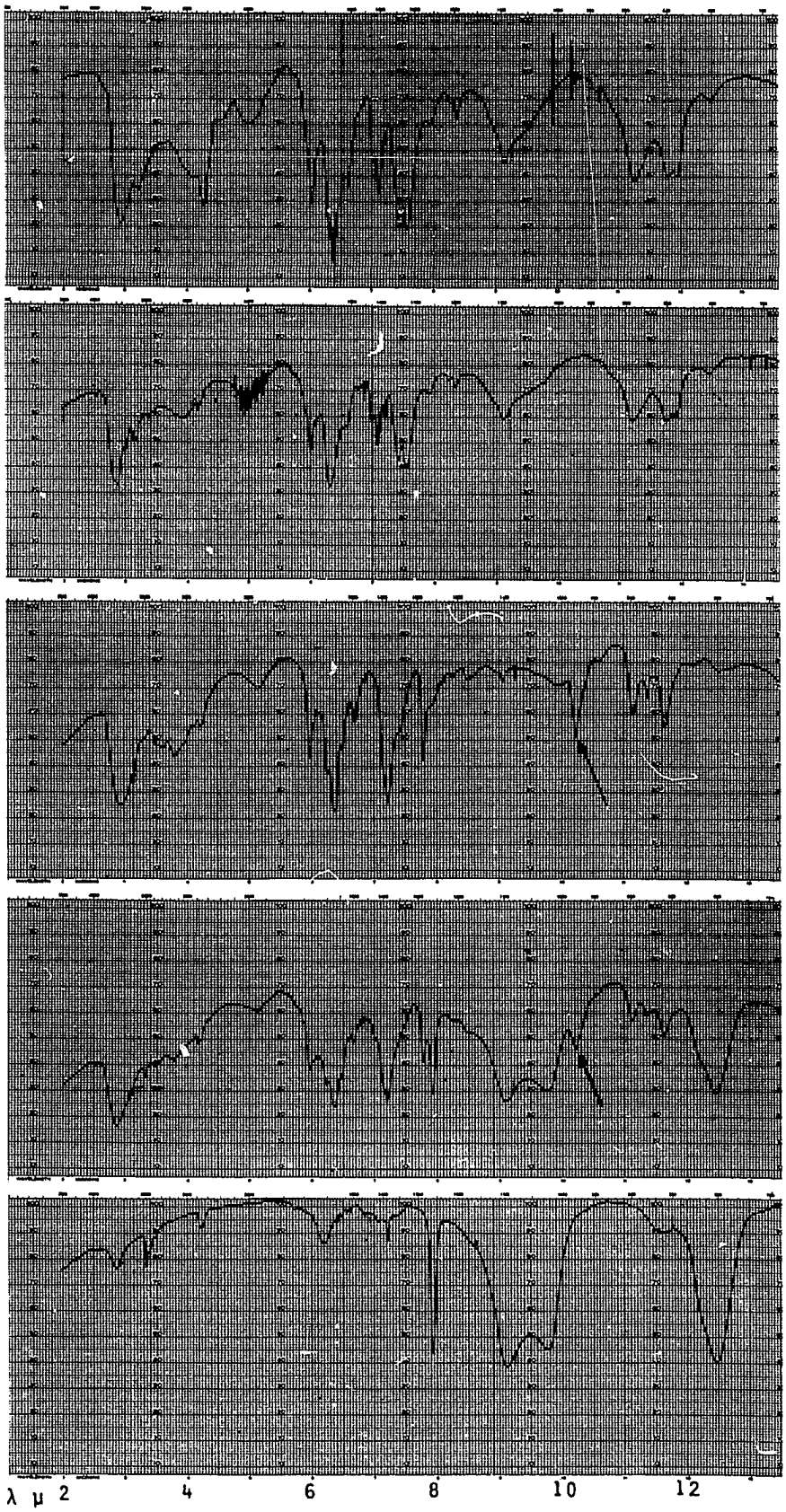
Commercial
Trans UCA

D

from Skin
Trans UCA

E

Dow-Corning
Stopcock
Grease
Silicone
Lubricant



trans isomers of UCA from guinea pig skin and the same substances from in vitro sources.

At the present time many of the infrared absorption maxima cannot be assigned to structural features of the molecules. The absorption at about 2.9 μ (3450 and 3465 cm^{-1}) may be tentatively assigned to a superposition of the absorption due to the carboxylate group and the N-H stretch of the H on the imidazole nitrogen atoms of the ring. The absorption near 6.0 μ (1675 cm^{-1}) is considered characteristic of the -C=C- stretch of the ethylenic bond in disubstituted trans ethylenes while that near (1663 cm^{-1}) is characteristic of the disubstituted cis ethylenes (62).

Another absorption maximum that may be tentatively assigned is the absorption at 10.2 μ (980 cm^{-1}) of the trans UCA. This is considered to be due to the out of plane hydrogen atoms of the disubstituted ethylenes, and is little affected by the mass of the substituents (64). Absorption near this frequency is seen in the trans modifications of other substituted acrylic acids, but not seen in the cis isomers as can be observed in Fig. 4. The urocanic acid previously identified in skin (47) has been recognized as the trans isomer. The trans form of UCA has been shown to undergo isomerization in skin by UV similar to that recognized for in vitro irradiation (22).

The cis isomer has been isolated by ion-exchange chromatography and characterized by mobility in paper chromatographic systems, shifts in wave lengths of maximum UV absorbency with changes of ph, color formation by treatment with alkaline diazo reagents (42), melting points (22,42) and profile of infrared spectrum.

The infrared spectrum of the trans isomer has been found to posses

an absorbency maximum in the range anticipated for the out-of-plane vibrations of the hydrogen atoms [10.2μ] (64). This absorbency maximum is absent in the spectra of the cis isomer isolated from skin and in a separate preparation from in vitro irradiation of the trans isomer.

CHAPTER III

DISCUSSION

At the time it was decided to attempt a series of exploratory experiments on normal and irradiated skin, the hypothesis was that some important chemical differences might be detectable in preparations from the two sources. It seemed reasonable that since the nucleotides have a prominent role in the normal energy transfer processes there might be some energy transfer relationship between some nucleotide UV irradiation. This relationship has not been found. However, it was found that direct correlation of the 260 mμ absorption of these extracts with phosphorous or the ribose content could not be made. Much of the 260 mμ absorbency in the acid soluble extract was found to be due to urocanic acid (47).

When an acid soluble extract of skin at pH 5.0 was applied to Dowex-1-formate columns the UV absorbency was not consistently held on the column. However when the extracts were applied to Dowex-1-acetate columns at pH 6.0 about 95% of the 260 mμ absorbency was held and could be reproducibly eluted.

When Dowex-1-acetate, but not the formate form, was used an additional peak was found in the irradiated extract that was eluted more readily than urocanic acid. This peak proved to be cis urocanic

acid and is the first report of separation of cis-trans isomers of urocanic acid by ion-exchange technique. This peak had light absorbency properties similar to UCA but differed from commercial UCA in mobility in an alkaline system of paper chromatography.

The article by Kurogochi (42) was valuable at this point because it contained information necessary to identify cis and trans UCA in amounts easily obtainable from skin.

Preparation of the skin specimens for analysis was one of the major problems of art necessary to resolve before it was possible to prepare suitable extracts. Ogura (59) has reviewed the methods of separation of epidermis from underlying tissues. They include: 1) enzymatic digestion by proteolytic enzymes, 2) ionic change using acids, bases, or salts, or combinations of them, 3) suction method for introducing water between the epidermis and dermis, 4) heat, and 5) mechanical method. The first three were rejected because of contact with water and possibility of losing water soluble substances, the fourth was considered too violent and apt to destroy labile substances. The fifth method consists in stretching and scraping the epidermis from the deeper lying tissue. The procedure developed is similar to this latter procedure. It is likely that the substances extracted for analysis more closely approximate that to be found in normal viable skin than would be attainable with the previously described procedures. There is also the advantage that the upper dermis may be included without having the entire mass of skin in the analysis.

The ideal method for removal of hair from the animal's skin has not been found. If carefully and consistently done, the application of

an epilating agent gives reproducible results. The total amount of UCA recoverable may be in error. J. Tabachnic claims (private communication) to have removed 60% of the UCA from epidermis by washing with water. Increased erythemal response to UV has been found when areas of an animal's skin have been swabbed with a wet applicator prior to irradiation and compared to adjacent non-swabbed areas. Appreciable amounts of the UCA have been found in the water in which the swab was washed.

Perchloric acid has been used to precipitate proteins and remove water soluble substances from the mass of skin. Perchloric acid is uniquely suited to this purpose if the extract is to be used for ion exchange procedures and ultraviolet absorbency determinations because it has a relatively low absorbency in the UV, and the potassium salt is only slightly soluble. Potassium ion from KOH precipitates the anion and the other reaction product is simply water. An excess of the cation does not precipitate the desired products as would be the case if barium hydroxide were used with sulfuric acid. Trichloroacetic acid was used in initial studies but it absorbs in the shorter UV range and makes spectral determinations difficult or impossible. Removal of excess trichloroacetic acid by ether extraction has been described but this did not prove to be a satisfactory procedure.

It is also important to remove as many inorganic anions as practical from the extract by some means because the ion-exchange resins have only a limited capacity and are converted to the less effective strong acid form if these anions are present.

It was surprising to discover that a large portion of the 260 mμ absorbency of the acid soluble extract was easily eluted from the ion

exchange column and was not associated with ribose or phosphorous. The possibility that purines and pyrimidines might be present in large amounts was the most attractive hypothesis. The observed shift in the maximum UV absorbency with change in pH was suggestive of cytosine. However, when the unknown was compared with authentic cytosine it was shown that the two substances were not identical.

Urocanic acid was a known constituent of sweat and had been reported in guinea pig skin (47). When the unknown UV absorbing substance was compared with authentic UCA it was found that this substance was identical with the UV absorbing substance in normal skin. UCA was one of the UV absorbing substances in irradiated skin but was present in decreased amounts. There was another substance present in the irradiated skin extract that could not be found in extract of normal skin. The possibility of a cis-trans isomerization was recognized. Since the UCA content appeared to be changed after UV irradiation, authentic UCA was irradiated. When UCA was irradiated and examined by suitable paper chromatographic techniques, it was found to contain a second substance similar to the yet unidentified substance in the irradiated skin. This second substance has been found to be the cis isomer of urocanic acid. In the literature urocanic acid usually refers to the trans isomer. No proof of the formation of the cis isomer by a biological process has been reported. In addition the cis modification has not been reported formed in the chemical reactions for the synthesis of UCA. The chemical preparations of UCA cited involve a crystallization from solution. It is possible that some of the more soluble cis isomer may have been present and simply not recognized.

This author has not made even a tentative assignment to many features

seen in the infra-red absorption spectrum of the cis and trans isomers of urocanic acid. Several bands of absorption seem to be characteristic, but have not yet been interpreted. However, the striking similarity of the profile of the isomers reported from different sources constitutes additional evidence for the identity of the substances.

There are several absorption areas that could possibly lead to profitable theoretical investigations of steric and group interaction effects on the infrared spectra of these substances. In the field of infrared spectroscopy, a study of these and related substances could yield information relating environmental effects to shifts in the absorption spectrum.

It is noted that the presence of the 980 cm^{-1} absorbency of the trans isomer and the absence of this band in the cis isomer is further evidence that the present cis-trans assignment is correct.

The infrared spectrum of the stopcock grease used on the freeze-drying containers is included. It is apparently contained in the trans UCA isolated from guinea pig skin.

Cis UCA has been isolated and irradiated with UV. This was found by paper chromatography to give a mixture of both cis and trans isomers indistinguishable from that obtained by irradiation of the trans isomer.

It is probable that an equilibrium mixture of the two isomers is formed. The photo efficiency of this isomerization process has not been determined.

It is also interesting to note that those who have previously (11,22) prepared cis UCA report that the solution becomes brown and the color was removed by charcoal before isolation of the UCA isomers. It has been found that prolonged irradiation of UCA with UV leads to a

brown color and that with sufficient irradiation no UCA, cis or trans, may be detected, but several other substances may be found by paper chromatography of this mixture--some are fluorescent, others quench with short wave UV and still others are not detectable on filter paper with UV light but give a color reaction with alkaline diazo reagents. Only traces of ninhydrin positive substances are obtained from the irradiated mixture. Not less than eight substances are formed by this prolonged irradiation of UCA. Perhaps some of these substances may be important in the response of skin exposed to long term irradiation by intense sunlight. Isolation and characterization of the substances of this mixture resulting from UV irradiation is being actively pursued.

CHAPTER IV

SUMMARY

1) This dissertation presents a procedure for the removal of epidermis for chemical analysis which was designed to reduce to a minimum enzymatic and many spontaneous changes in metabolite content. The procedure further allows complete control of solvents that come into contact with the material to be analyzed.

2) Data are presented to show that there is a decrease in total urocanic acid content after UV irradiation of skin.

3) Evidence has been found that the urocanic acid normally present in guinea pig skin and in human skin is the *trans* isomer, and the *cis* isomer is formed *in vivo* by UV irradiation.

4) A method for separating the *cis* and *trans* isomers of urocanic acid by ion exchange resins has been developed.

5) Preliminary observations indicate that urocanic acid is transformed into several substances other than *cis* urocanic acid by prolonged irradiation with UV *in vitro*. It is suggested that some of the substances may be formed in the skin and have a role in the physiological changes characteristic of chronically sunburned skin.

6) Infrared spectra are presented which support the identification and underwrite the *cis-trans* configurational assignment of urocanic acid.

BIBLIOGRAPHY

(1) Schwarz, K. and Speck, M., Dermatologica, 114, 232 (1957)

(2) Jaffe, M., Ber. Deut. chem. Ges., 7, 1669 (1874)

(3) Seigfreid, M., Hoppe Seylers Z. Physiol. chem., 24, 399 (1898)

(4) Hunter, A., J. Biol. Chem., 11, 537 (1912)

(5) Barger, R. and Ewins, A. J., J. Chem. Soc., 99, 2336 (1911)

(6) Raistrick, H., Biochem. J., 11, 71 (1917)

(7) Kotake, Y. and Konishi, M., Hoppe Seylers Z. physiol. chem., 122, 230 (1922)

(8) Edlbacher, S., ibid., 157, 106 (1926)

(9) Edlbacher, S. and Simons, E., ibid., 167, 76 (1927)

(10) Kauffman, F. and Mislowitzer, E., Biochem. Z., 226, 325 (1930)

(11) Cox, G. J. and Rose, W. C., J. Biol. Chem., 68, 781 (1926)

(12) ibid., 68, 769 (1926)

(13) Edlbacher, S. and Kraus, J., Hoppe Selyers Z. physiol. chem., 191, 225 (1930)

(14) Kiyokawa, M., ibid., 214, 38 (1933)

(15) Edlbacher, S. and Kraus, J., ibid., 195, 267 (1930)

(16) Edlbacher, S. and Neber, M., ibid., 224, 261 (1934)

(17) Borgi, B. and Tarantio, C., Biochem. Z., 305, 101 (1940)

(18) Kotake, Y., Hoppe Seylers Z. physiol. chem., 270, 38 (1941)

(19) Edlbacher, S. and von Bidder, H., ibid., 273, 163 (1942)

(20) Edlbacher, S., ibid., 276, 108 (1942)

(21) Edlbacher, S. and Heitz, Fr., ibid., 276, 126 (1942)

(22) Edlbacher, S. and von Bidder, H., ibid., 276, 126 (1942)

(23) Edlbacher, S., ibid., 276, 63 (1942)

(24) Darby, W. J. and Lewis, B. H., J. Biol. Chem., 146, 225 (1942)

(25) Walker, A. C. and Schmidt, C. L. A., Arch. Biochem., 5, 445 (1944)

(26) Hall, D. A., Biochem. J., 51, 599 (1952)

(27) Tabor, H., Mehler, A. H., Hayaishi, O. and White, J., J. Biol. Chem., 196, 121 (1952)

(28) Mehler, A. H. and Tabor, H., Federation Proc., 11 no. 1, 374 (1952)

(29) Erspomer, V. and Benati, O., Biochem. Z., 324, 66 (1953)

(30) Zenisek, A. and Kral, J. A., Biochem. et Biophys. Acta, 12, 479 (1953)

(31) Vanderbilt, J. M. and Childs, C. E., Science (Lancaster, Pa.), 119, 514 (1954)

(32) Szakall, A., Arch. klin, u exp. Dermatol., 201, 331 (1955)

(33) Goryukhina, T. Z., C. A., 47, 5514 (1953). Doklady. Akad, Nauk. S. S. S. R., 87, 645 (1952)

(34) Tomohiro, Y., C. A., 48, 6842 (1954). Nagasaki Igakki Zassi, 28, 1117 (1953)

(35) Yoshioka, Y., C. A., 50, 12147 (1956). Osaka Diagaku Igaku Zassi, 7, 377 (1955)

(36) Wolf, G., Wu, P. H. L. and Heck, W. W., J. Biol. Chem., 222, 159 (1956)

(37) Shimomura, Y., C. A., 52, 16453 (1958). Osaka Digaku. Igaku. Zassi, 9, 433 (1957)

(38) Ichihara, K., Satani, H., Okada, N., Tagaki, Y. and Sakomoto, Y., Proc. Japan Acad., 33, 105 (1957)

(39) Brown, D. D. and Keis, M. W., J. Am. Chem. Soc., 80, 6147 (1958)

(40) Pasini, C. and Vercellone, A., C. A., 50, 7782 (1956). Gazz. chim. ital., 85, 349 (1955)

(41) Miller, A. and Waelsch, H., J. Biol. Chem., 228, 365 (1957)

(42) Kurogochi, Y., Fukui, Y., Nakagawa, T. and Yammomoto, I., Japan J. Pharmacol, 6, 147 (1957)

(43) Tabachnick, I. I. A. and Roth, F. E., J. Pharmacol. Exp. Therap., 121, 191 (1957)

(44) Acheson, R. M., Paul, R. M. and Tomlinson, R. V., Can. J. Biochem. and Physiol., 36, 295 (1958)

(45) Kral, J., Kutova, M., Zenisek, A. and Hais, I. M., C. A., 54, 1864 (1960). Ceskoslov dermatol., 33, 298 (1958)

(46) Spier, H. W. and Pascher, G., Arch klin. u exp. Dermatol., 209, 181 (1959)

(47) Tabachnic, J., Arch. Biochem. Biophys., 70, 295 (1957)

(48) Tabachnic, J. and Weiss, C., Radiation Research, 11, 684 (1959)

(49) Feinberg, R. H. and Greenberg, David M., J. Biol. Chem., 234, 2670 (1959)

(50) Brown, D. D. and Keis, M. W., ibid., 234, 3182 (1959)

(51) ibid., 3188 (1959)

(52) Leif, W. and Herbert, J., Am. J. Hyg., 71, 285 (1960)

(53) Anglin, J. H., Bever, A. T., Everett, M. A. and Lamb, J. H., Biochem. et Biophys. Acta, 53, 408 (1961)

(54) Everett, M. A., Anglin, J. H. and Bever, A. T., Arch. Dermatol, 84, 717 (1962)

(55) Schwarz, E., Biochem. Z., 334, 415 (1961)

(56) Whitehead, R. G. and Arnstein, H. R. V., Nature, 190, 1105 (1961)

(57) La Du, B. N., Howell, R. R. and Jacoby, G. A., Biochem. Biophys. Research Commun., 7, 398 (1962)

(58) Hurlburt, R. B., Schmitz, H., Brumm, A. F., and Patten, V. R., J. Biol. Chem., 209, 27 (1954)

(59) Ogura, R., Knox, J. M. and Griffin, A. C., J. Invest. Dermatol., 35, 239 (1960)

(60) Dryer, R. L., Tammes, A. R. and Routh, J. I., J. Biol. Chem., 225, 177 (1957)

(61) Hawk, Philip B., Oser, Bernard L. and Summerson, William H., Practical Physiological Chemistry, The Blakiston Co., Philadelphia, 12th Edition, 1947

(62) Finar, I. L., Organic Chemistry, Volume 2, Stereochemistry and the Chemistry of Natural Products, Longmans, Green and Co., New York, 1956, p. 105

(63) Weissberger, A., Technique of Organic Chemistry, Edited by W. West, Interscience Publishers, Inc., New York, 1956

(64) Bellamy, L. J., The Infra-red Spectra of Complex Molecules, John Wiley & Sons, New York, 1958