Induction of Photohemolysis by Tetrachlorosalicylanilide

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The antibacterial agent 3,3′,4′,5-tetrachlorosalicylanilide (TCSA) enhanced the sensitivity of erythrocytes to long-wave UV light *in vitro*. The exposure of TCSA-treated red cells to UV radiation resulted in photohemolysis; the extent of photohemolysis was related to the duration of irradiation. The photohemolytic reaction appeared to be a consequence of UV excitation of TCSA to the triplet state and subsequent reaction of the latter molecule with the erythrocyte membrane. The interaction of the excited TCSA molecule with the red cell membrane was manifested as increased permeability towards Mg^{2+}, K^+, or Na^+ ions. Photohemolysis was prevented by the UV absorbers urocanic acid and 2-hydroxy-4-methoxybenzophenone-5-sulfonic acid. In addition to TCSA, 3,3′,4′,5- and 2′,3,4′,5-tetrabromosalicylanilides caused photohemolysis. Monobromo-, dibromo-, and tribromosalicylanilides failed to induce significant photohemolysis. The results suggest that some instances of photodermatitis due to TCSA may be related to changes in cell permeability and lysis.

PHOTODERMATITIS resulting from the topical application of the antibacterial agent 3,3′,4′,5-tetrachlorosalicylanilide (TCSA) has been reported by several investigators (1–5). The inflammatory reaction is elicited upon exposure of the skin to long-wave UV light within the range 300 to 425 mμ (6). The manner by which TCSA produces skin damage has not been definitely established. Primary irritant, phototoxic, contact allergic, and contact photoallergic processes have been suggested as possible mechanisms. Recent preliminary work has demonstrated the induction and passive transfer of TCSA photocontact hypersensitivity in guinea pigs (4). These results indicate that photodermatitis due to TCSA may be related to a delayed light-mediated immunological process, although further confirmation is required. However, it is well known that certain factors can contribute to photosensitization. The factors include the concentration of the agent used, the vehicle employed, the nature of the light source, and the length of exposure of the skin to light. Since the latter factors can influence the skin response, it is conceivable that TCSA may also cause photodermatitis by a mechanism other than that responsible for delayed hypersensitivity.

In addition to TCSA, various medicinal agents have been incriminated as photosensitizers (7). With the exception of the furocoumarins (8), it is generally believed that photosensitizing agents produce photodermatitis as a consequence of increased cell permeability and concomitant cell lysis. Increased permeability is the resultant manifestation of a photochemical reaction involving a photosensitizer, oxygen, and an unknown membrane component. Evidence has been presented which indicates that the disruption of lysosomal and mast cell membranes may be the primary event in photosensitization (9).

The possibility that photodermatitis due to TCSA may also be related to changes in cell permeability has not been considered nor substantiated in clinical investigations reported. This report presents the results of *in vitro* studies which demonstrate that the interaction of long-wave UV radiation with TCSA can cause altered membrane permeability, as evidenced by photohemolysis of erythrocytes. The value of erythrocytes as models for assessing photosensitization effects has been documented (9–11).

EXPERIMENTAL

Preparation of Erythrocytes—Adult rats, derived from the Sprague-Dawley strain, were used throughout this investigation. The animals were killed by decapitation and blood was collected from the severed vessels into heparinized tubes. Red cells were sedimented by centrifugation, washed three times with isotonic saline–phosphate buffer of pH 7.4 (0.14 *M* NaCl, 8.8×10^{-3} *M* Na_2HPO_4, 1.4×10^{-3} *M* NaH_2PO_4), and then resuspended in buffer to original volume.

Photohemolysis Assay, General Procedure—The erythrocyte suspension was diluted 1:200 with 10^{-4} *M* TCSA contained in saline–phosphate buffer. Appropriate controls consisted of erythrocytes diluted 1:200 with saline–phosphate buffer lacking TCSA. The samples were dark-incubated (low-actinic test tubes, 18 × 150 mm.) for 30 min. at 25° on a reciprocating shaker, operated at 68 strokes/min. Five milliliters of the suspension was then transferred to an open Stender dish (4.9 cm. diameter) and irradiated on the shaker. All assays were performed in triplicate. The radiation factors employed were: exposure time, 60 min.; target distance, 10 cm.; radiation source, multiray lamp

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fitted with a long-wave UV light having maximum energy output at 366 mμ; intensity of incident radiation, 1.44×10^7 ergs/cm.^{2}, as measured with a YSI model 65 radiometer (Yellow Springs Instrument Co., Ohio). Following the irradiation period, the samples were returned to the dark and stationary incubated for 15 min. The suspensions were then diluted 1:1 with 0.44 *M* $NaNO_2$ to terminate hemolysis.[1] Cellular debris was removed by centrifugation and the hemoglobin content of the supernates determined by measuring the absorbance at 420 mμ with a colorimeter (Klett-Summerson). Samples, similarly prepared, which had been allowed to remain in the dark for 105 min. were used to correct for spontaneous hemolysis[2] (not due to UV light). Results were quantitated in terms of percent photohemolysis with red cells hemolyzed by 5.8×10^{-2} *M* NaCl serving as the standard for 100% hemolysis. The latter standard was also diluted with 0.44 *M* $NaNO_2$ to insure uniform conditions.

Chemicals—3,3′,4′,5 - Tetrachlorosalicylanilide (TCSA) was obtained from Geigy Chemicals, Ardsley, N. Y. 3,3′,4′,5-Tetrabromosalicylanilide was supplied by the Organics Section of Lever Brothers Co., Edgewater, N. J. Other salicylanilides employed were obtained from Fine Organics, Inc., Lodi, N. J. Urocanic acid was purchased from Mann Research Laboratories, Inc., New York, N. Y. (2 - Hydroxy - 4 - methoxybenzophenone - 5 - sulfonic acid) Uvinul MS-40 was obtained from Antara Chemicals, New York, N. Y.

RESULTS AND DISCUSSION

Figure 1 shows the relationship of the length of irradiation to the percent photohemolysis noted in the presence of TCSA. Photohemolysis greater than the control value was observed initially when the TCSA-treated cells were subjected to UV light for 30 min. The amount of photohemolysis increased as the irradiation time was extended, maximum lysis occurring with a 60-min. exposure. The latter irradiation time was therefore chosen for use in the subsequent phases of this investigation.

The exposure of TCSA-treated red cells to long-wave UV light produced marked increases in membrane permeability towards Mg^{2+}, K^+, and Na^+ ions, as compared to irradiated control erythrocytes. Equivalent degrees of photohemolysis (63%) were obtained when the TCSA-treated cells were irradiated in buffer containing 0.14 *M* NaCl, 0.12 *M* KCl, or 0.12 *M* $MgCl_2$. Irradiation of erythrocytes in buffer which contained TCSA and isotonic[3] $CaCl_2$, glucose, or sucrose (0.28 *M*, 0.3 *M*, and 0.25 *M*, respectively) yielded insignificant photohemolysis when compared to irradiated controls. Irradiated TCSA-deficient samples had photohemolytic values of 0% ($CaCl_2$), 12% (glucose), and 10% (sucrose). The results therefore indicate that UV light does not increase permeability of TCSA-treated cells to either

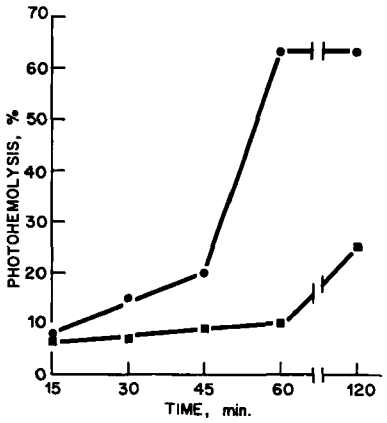

Fig. 1—Photohemolysis versus *time of long-wave UV irradiation for 3,3′,4′,5-tetrachlorosalicylanilide TCSA) at 25°. Intensity of incident radiation, 4 × 10^3 ergs/cm.^{2}/sec.; TCSA, 10^{-4} M in isotonic saline–phosphate buffer, pH 7.4. Key: ●, TCSA; ■, control.*

Ca^{2+}, glucose, or sucrose. The impermeability to Ca^{2+} ions is noteworthy; Ca^{2+} has a role in maintaining the normal low permeability of erythrocyte membranes (12).

Cations leaking across electrochemical gradients of normal erythrocytes are constantly being balanced by cations actively transported against these gradients (13). When the cell membrane is damaged by UV light, there is an isosmotic movement of salts and water into the cell. The accelerated diffusion rates cannot be overcome by active transport mechanisms. The erythrocytes therefore swell and lyse (14). In order to establish that the photohemolysis

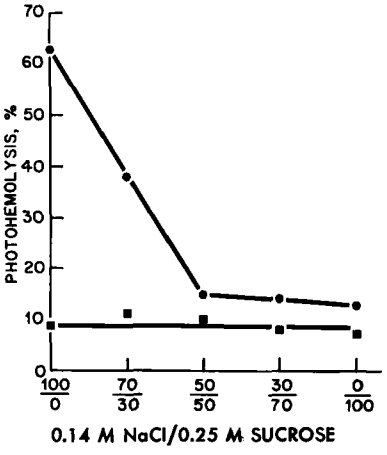

Fig. 2—Effect of NaCl content of the extracellular environment on the induction of photohemolysis by 3,3′,4′,5-tetrachlorosalicylanilide (TCSA). Intensity of incident radiation, 1.44 × 10^7 ergs/cm.^{2}; TCSA (10^{-4} M) and concentrations of NaCl and sucrose shown were contained in phosphate buffer, pH 7.4. Key: ●, TCSA; ■, control.

[1] The hypertonicity of the $NaNO_2$ solution stops hemolysis by causing cell shrinkage and its reducing action prevents further photochemical reactions (10).

[2] Hemolytic values of approximately 35 and 30% were usually noted for the nonirradiated samples with and without TCSA, respectively. These values were subtracted from the hemolytic values of the corresponding irradiated samples to give the degree of hemolysis due to UV light.

[3] Isotonic concentrations of $CaCl_2$, KCl, $MgCl_2$, glucose, and sucrose, respectively, were calculated by determining the amount required to exert approximately the same osmotic pressure as 0.85% NaCl (isotonic to blood).

of TCSA-treated cells, reported here, was due to the facilitated cellular uptake of cations, the red cells were irradiated in various isotonic NaCl + isotonic sucrose mixtures. The results obtained are illustrated in Fig. 2. It is apparent that photohemolysis of TCSA-treated cells was related to the Na^+ content of the external environment; photohemolysis decreased as the NaCl content of the medium was lowered.

Cation leakage associated with irradiated red cells is dependent upon the radiation dose and proceeds after irradiation is terminated (14). Thus, percent hemolysis usually becomes a function of incubation time after irradiation. Higher lytic values are obtained when the postirradiation period is prolonged. An evaluation of the relationship of the length of the postirradiation incubation period to the photohemolysis of TCSA-treated cells, observed here, was therefore of interest. The TCSA-treated cells were irradiated in saline–phosphate buffer and the percent photohemolysis determined 0, 5, 10, and 15 min. after irradiation. At each time interval, a photohemolytic value of 63% was obtained. These results thus indicate that the extent of photohemolysis was independent of the postirradiation sampling time under the conditions employed. Maximal lysis occurred during the exposure of erythrocytes to UV light. These findings suggest that, in this case, hemolysis is probably due to extensive alterations in membrane permeability as a result of the interaction of UV radiation, TCSA, and a membrane component responsible for maintaining cellular integrity.

It is well known that UV light causes the electronic excitation of photosensitive molecules to higher energy states (triplet or singlet). The excited molecules are extremely reactive and can transfer their energy to other molecular species by collision; the latter species then undergo a chemical reaction. Excited molecules can also dissipate their energy by dissociation into either radicals or more stable molecules. The interaction of long-wave UV radiation with TCSA is known to result in free radical production (15, 16). The photoconversion of TCSA to 3′,4′,5-trichlorosalicylanilide has also been reported (16). In the present study, it is conceivable that UV light may have excited TCSA to the long-lived metastable (triplet) state. The reaction of the highly energized TCSA molecule with the red cell membrane could therefore be responsible for the induction of structural alterations and associated permeability changes. Indirect evidence that this may be the case was obtained here through the use of KI and the paramagnetic ions, Cu^{2+} and Mn^{2+}, known quenchers of triplet state molecules (17). Photohemolysis failed to occur if the TCSA-treated cells were irradiated in isotonic KI (0.15 *M*), rather than in isotonic NaCl. When either KI (10^{-3} *M*) or $MnCl_2$ (2.5×10^{-3} *M*) was included in the saline–phosphate buffer, photohemolysis of TCSA-treated cells decreased by approximately 50% in comparison to the value obtained with saline–phosphate buffer alone (Table I). The inclusion of $CuCl_2$ (2.5×10^{-3} *M*) in the buffer prevented the TCSA-induction of photohemolysis, as evidenced by a photohemolytic value identical to that of the corresponding irradiated control. Average photohemolytic values of 10% were obtained when TCSA-deficient cells were irradiated in the presence of KI, $MnCl_2$, or $CuCl_2$. The results thus indicate that the triplet state of TCSA is the reactive species in the photohemolytic reaction. It should be noted that biological photosensitization due to psoralens likewise involves light excitation of the latter agents to the triplet state (18).

Table I—Effect of Various Chemicals on the Induction of Photohemolysis by Tetrachlorosalicylanilide (TCSA)

Chemical[a]	Concn., m*M*	Photohemolysis[b], %
None	—	60
$MnCl_2 \cdot 4H_2O$	2.5	31
KI	1.0	29
$CuCl_2 \cdot 2H_2O$	2.5	10

[a] TCSA, 10^{-4} *M*, and chemical indicated were contained in isotonic saline-phosphate buffer, pH 7.4. [b] Erythrocytes irradiated with long-wave uv light for 60 min. Intensity of incident radiation, 1.44×10^7 ergs/cm.^{2}.

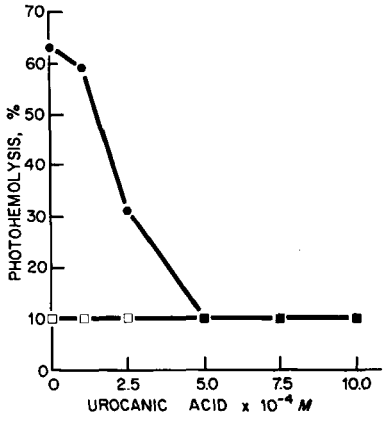

Fig. 3—Effect of urocanic acid on the induction of photohemolysis by 3,3′,4′,5-tetrachlorosalicylanilide (TCSA). Intensity of incident radiation, 1.44 × 10^7 ergs/cm.^{2}; TCSA (10^{-4} M) and urocanic acid concentrations shown were contained in isotonic saline–phosphate buffer, pH 7.4. Key: ●, TCSA; □, control.

Urocanic acid, the principal acid-soluble UV absorber present in the epidermis (19), was found to have an effect on the photohemolytic reaction. The addition of increasing concentrations of urocanic acid to the saline–phosphate buffer produced decreases in the percent photohemolysis of TCSA-treated cells (Fig. 3). Values identical to those of irradiated control cells were noted when urocanic acid was present in the irradiation mixture at a content of 5×10^{-4} *M*. 2-Hydroxy-4-methoxybenzophenone-5-sulfonic acid, a commercial sun screener used in cosmetic preparations, also protected TCSA-treated cells against UV radiation (Fig. 4). The effective concentration (5×10^{-3} *M*) required for inhibition of the photohemolytic reaction was greater than the quantity of urocanic acid needed.

To determine whether the enhanced light sensitivity of erythrocytes was unique to TCSA, red cells were irradiated for 60 min. in saline–phosphate buffer containing various salicylanilide isomers. In addition to TCSA, only 3,3′,4′,5- or 2′,3,4′,5-tetrabromosalicylanilide induced significant photohemolysis (Table II). Although the failure of the other isomers tested to cause photohemolysis significantly greater than the control value may be related to the

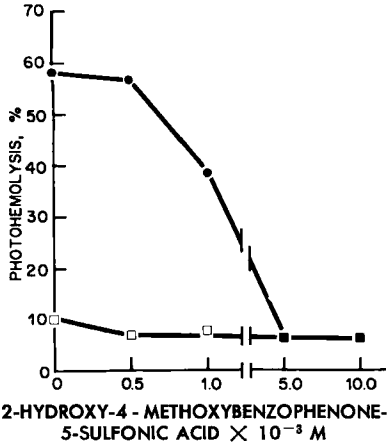

Fig. 4—Effect of 2-hydroxy-4-methoxybenzophenone-5-sulfonic acid on the induction of photohemolysis by 3,3′,4′,5-tetrachlorosalicylanilide (TCSA). Intensity of incident radiation, 1.44 × 10⁷ ergs/cm.²; TCSA (10⁻⁴ M) and 2-hydroxy-4-methoxylbenzophenone-5-sulfonic acid concentrations shown were contained in isotonic saline–phosphate buffer, pH 7.4. Key: ●, TCSA; □, control.

concentration employed (10^{-4} *M*), a study of concentration effects was precluded by limited solubilities of the isomers in aqueous solvents. Similarly, the possibility exists that enhanced photohemolysis may have been observed if a longer irradiation exposure was used. Nevertheless, the monobromo-, dibromo-, and tribromosalicylanilides lack the photohemolytic capabilitity of TCSA since the irradiation time employed was optimal for the TCSA induction of photohemolysis (Fig. 1). Photohemolysis may therefore be of value for comparing the potential photosensitizing effects of select salicylanilides with those of TCSA.

The membrane component damaged by the interaction of long-wave UV radiation with TCSA was not determined in the present investigation. Lipoproteins may be involved, however, since it is well known that lipoproteins have an important role in the structure and permeability characteristics of cell membranes.

The data reported here have shown that TCSA can cause light-mediated permeability changes in cell membranes and that the extent of cell lysis is related to extraneous factors (*i.e.*, exposure time, external cellular environment, UV absorbers). Although the experiments performed were *in vitro* studies, it seems likely that some instances of photodermatitis due to TCSA may be related to increased cell permeability and lysis. The established role of lysosomes in inflammatory processes (20) suggests that lysosomes may be the target organelles *in vivo*. UV light causes increased lysosomal fragility (21); it is therefore conceivable that TCSA may enhance the light sensitivity of lysosomes with concomitant clinical developments (*i.e.*, edema, erythema, wealing, *etc.*). Additional studies are planned to determine if this is actually the case.

TABLE II—INDUCTION OF PHOTOHEMOLYSIS BY SALICYLANILIDE ISOMERS

Isomer[a]	Photohemolysis,[b] %
3,3′,4′,5-Tetrabromosalicylanilide	69
3,3′,4′,5-Tetrachlorosalicylanilide	63
2′,3,4′,5-Tetrabromosalicylanilide	47
3,4′,5-Tribromosalicylanilide	16
3,5-Dibromosalicylanilide	13
5-Monobromosalicylanilide	13
Salicylanilide	12
4′,5-Dibromosalicylanilide	11
None	10

[a] Isomer concentration 10^{-4} *M* contained in isotonic saline–phosphate buffer, pH 7.4. [b] Erythrocytes irradiated with long-wave UV light for 60 min. Intensity of incident radiation, 1.44 × 10^7 ergs/cm.².

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Keyphrases

Photohemolysis induction—tetrachlorosalicylanilide
Erythrocytes—photohemolysis determination
UV irradiation—photohemolysis
Cell permeability, cations—UV light effect