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# CELLULAR ASPECTS OF PHOTOTOXIC REACTIONS INDUCED BY KYNURENIC ACID

## II. *Fine structural analysis and cytochemistry on cultivated cells*

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*Abstract.* A recent report described the establishment of an experimental model for studying photo-oxidative damage inflicted by kynurenic acid on cultivated cells. Preliminary findings by scanning electron microscopy (SEM) were reported. For the present paper supplementary SEM studies were performed, and it was found that maximum cell damage appears 2 to 4 hours post irradiation. With low concentrations of kynurenic acid and brief UVA exposure, repair mechanisms seem to occur. Damage in the form of cellular swelling and plasma membrane alterations is most easily recognized by SEM, which is far more sensitive than ordinary light microscopy. Transmission electron microscopy (TEM) reveals morphologic alterations, consisting of an enhanced formation of endocytotic vacuoles from the plasma membranes, swelling with disruptions of the cell sap continuity, mitochondrial swelling, dilatation of the endoplasmic net, and vesiculation of some of the microvilli. Lysosomal damage, which was not seen until very late post irradiation and only for high concentrations of kynurenic acid and long-term UVA exposure, seems to be a secondary phenomenon. This hypothesis is further supported by the cytochemical analysis. The primary event thus seems to affect the plasma membrane level and originate in the binding of kynurenic acid to the plasma membrane on which subsequent UVA irradiation inflicts secondary intracellular alterations due to increased permeability and swelling.

*Key words:* In vitro cultivated cells, human; kynurenic acid; Photo-oxidative damage; Electron microscopy, scanning and transmission

The tryptophan metabolite, kynurenic acid, proved in earlier work to be a potent photosensitizer with a pronounced phototoxic effect on irradiated erythrocytes and cultivated human cells. Data have recently been published which strongly support the theory that the cellular photodynamic damage induced by kynurenic acid is mediated by single state excited oxygen (14).

The importance of any minor alteration in the tryptophan metabolism has been indicated in several diseases with a concomitant light sensitivity, and this topic was discussed in previous reports (13, 14).

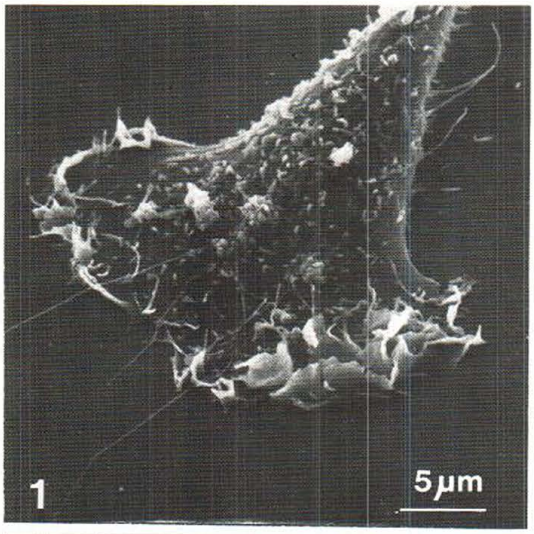
In earlier experiments with red blood cells, photo-oxidative damage of the cellular membranes was demonstrated with kynurenic acid and long-wave ultraviolet light (10, 14). In order to study further the cellular mechanisms involved in the photochemical reactions induced by kynurenic acid, an experimental model using *in vitro* cultivated human glia cells was established and described (17). A pronounced phototoxic effect was registered by the use of cell growth curves, and light and scanning electron microscopy. Photo-oxidative damage to the cellular plasma membranes was demonstrated and seemed to be the primary event in the reaction.

The purpose of the present report is to elucidate further aspects of photosensitization induced by kynurenic acid in different concentrations, to study the importance of varying time periods between irradiation and noticeable cellular damage, and to observe whether or not repair mechanisms can occur. These experiments were performed with transmission electron microscopy (TEM) and scanning electron microscopy (SEM). A light microscopy cytochemical analysis of the distribution of acid phosphatase was also carried out in order to reveal the possible role of the lysosomal vacuome.

## MATERIAL AND METHODS

### *Cell lines and culture conditions*

All the experiments were performed on normal diploid human glia cells in phase II (17, 18). The cells were derived and kept in culture as described by Pontén et al. (11). They were grown in Eagle's minimal essential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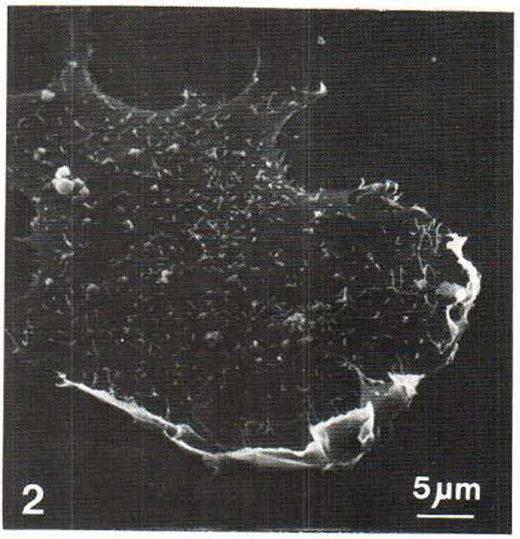
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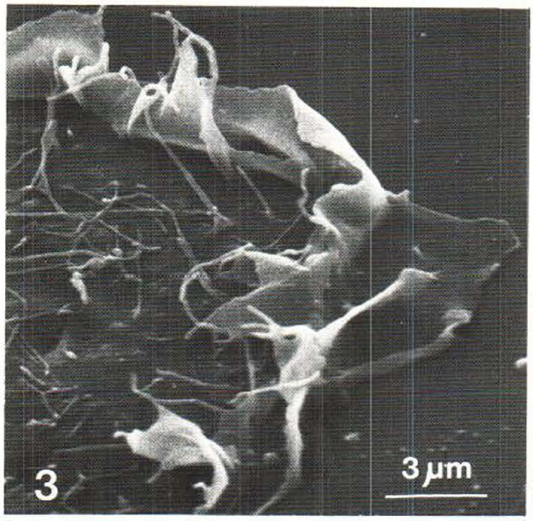
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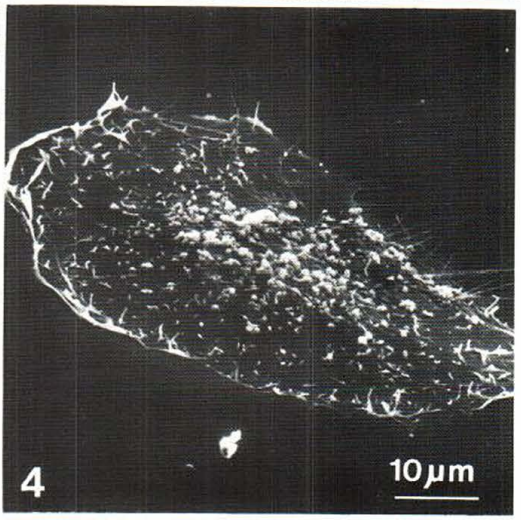
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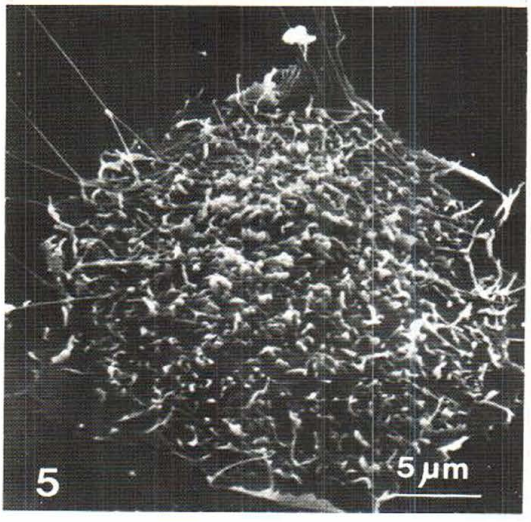
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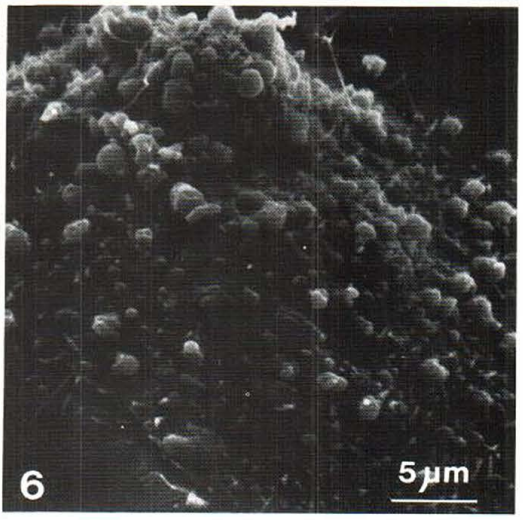
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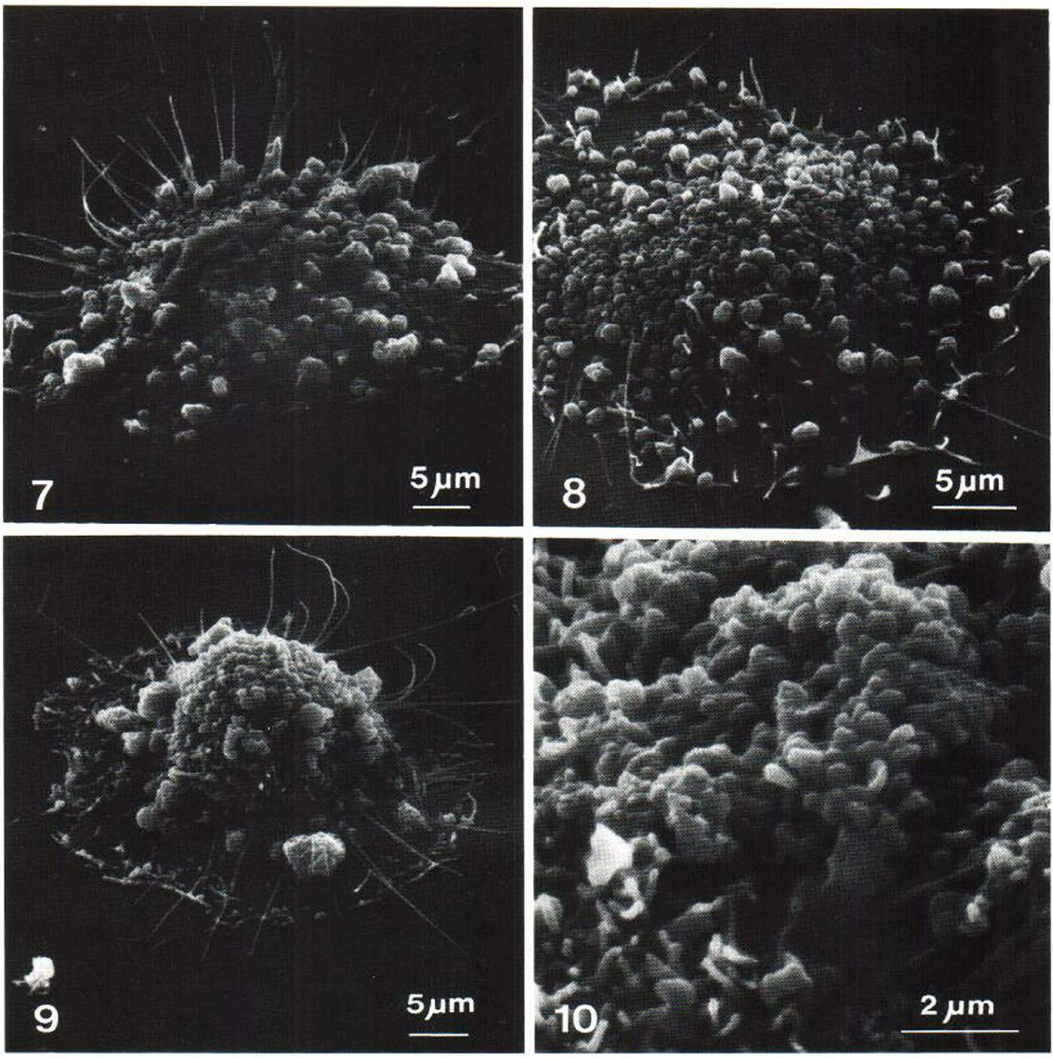



*Figs. 1–3.* Scanning electron microscope pictures of normal human glia cells at various magnifications. Note prominent peripheral ruffling and evenly distributed microvilli along the cells' upper surface.

*Figs. 4–10.* Surface changes induced by kynurenic acid acid and UVA-light in human glia cells as shown in the SEM. The cell surfaces demonstrate varying degree of blebbing and swelling, with retraction fibrils. Increasing cellular damage is seen up to 4 hours post irradiation. Exposure time $0.6\times10^{3}$ sec. *Figs. 4–6* show alterations following treatment with $5.29\times10^{-5}$ M kynurenic acid and UVA-light, 1 min, 60 min and 4 hours after exposure. *Figs. 7–10* show alterations following treatment with $5.29\times10^{-4}$ M kynurenic acid and UVA-light, 30 min, 60 min and 4 hours (Figs. 9, 10) after irradiation.

medium supplemented with 10% calf serum and antibiotics (100 U/ml of penicillin, 50 μg/ml of streptomycin and 1.25 μg/ml of amphotericin B) on the bottom of plastic Petri dishes (Nunclon®), or for scanning electron microscopy on glass coverslips no. 0., 12 mm in diameter, placed on the bottom of the dishes. The cells were harvested 3 days after subcultivation when they were still actively dividing and had not formed a confluent monolayer.

*Ultraviolet light source and conditions of irradiation*

The ultraviolet light source was a Black-Ray B-100 A lamp (Ultraviolet Products Inc.) providing long-wave ultraviolet radiation with an intensity maximum at 365 nm. The intensity of the lamp was 12.5 mW/cm², as measured with a Hewlett & Packard Radiant Flux meter. The cultures were irradiated at a distance of 10 cm from the lamp in accordance with the experimental model

established and described in our previous report (17), which provides an internal dark control. The cells were allowed to interact with kynurenic acid for 2 hours before irradiation. After irradiation the test medium was immediately replaced with fresh, Eagle medium, containing serum. The cells were processed after various intervals following irradiation, up to 24 hours, as described for each experiment.

*Scanning electron microscopy (SEM)*

In the previous study only two concentrations of kynurenic acid were used, $2.65\times10^{-3}$ M and $5.29\times10^{-3}$ M. In the present work, experiments were also performed with the following concentrations, $5.29\times10^{-5}$ M, $5.29\times10^{-4}$ M and $1.32\times10^{-3}$ M. The different exposure times used for each concentration of kynurenic acid were $0.6\times10^{3}$, $1.2\times10^{3}$ and $1.8\times10^{3}$ sec. Cells were harvested for morphologic examination at different intervals post irradiation, namely 1 min, 30 min, 60 min, 2 hours, 4 hours and 24 hours.

Subsequent to fixation for 60 min at +37°C in 2% glutaraldehyde in 0.1 M Na-Cacodylate-HCl buffer with 0.1 M sucrose (pH 7.2: total osmolality=510 mOsmol; vehicle osmolality=300 mOsmol) (2), the cells were postfixed in 2% $OsO_4$ in *s*-collidine buffer (pH 7.2) for 90 min at 22°C. A short rinse in 0.15 M cacodylate buffer (pH 7.2) at 22°C was interposed between the two fixations. Care was taken not to dry the cells during the replacement of medium with the aforementioned fixative, warmed to 37°C. After postfixation, dehydration was performed in a graded series (50%, 70%, 75%, 80%, 85%, 90%, 95%, 100%) of ethanol. The cells were then brought to acetone and critical-point dried from $CO_2$ in a Polaron E 3000 apparatus. After mounting on stubs with silver conductive paint, the specimens were coated with a 300 Å thick layer of gold in a Polaron sputter apparatus. The specimens were studied in a Jeol JSM-SI microscope at 10 kV, and micrographs taken with the specimens tilted 45°.

*Transmission electron microscopy (TEM)*

The concentrations of kynurenic acid used in the TEM studies were $5.29\times10^{-4}$ M and $1.32\times10^{-3}$ M. The exposure times were $06\times10^{3}$, $1.2\times10^{3}$ and $1.8\times10^{3}$ sec. The cells were harvested for examination 2 hours post irradiation.

The cells were initially fixed for 60 min in the glutaraldehyde fixative described above at +4°C, and then postfixed in 2% $OsO_4$ in *s*-collidine buffer (pH 7.2) for 90 min at 22°C. They rinsed briefly in 0.15 cacodylate buffer (pH 7.2) at 22°C between the two fixations. The cells were dehydrated *in situ* in the plastic dishes in a graded series of ethanol solutions, starting at 50%. In the last change, to 100% ethanol, the cells were counterstained with 2% uranyl acetate for 10 min. The cellular layer was then cut with a razor blade into squares about 2 mm by 2 mm and removed by means of propylene oxide according to Biberfeld (3). The squares were washed in propylene oxide several times to completely remove all remnants of plastic from the culture dishes, and embedded in Epon 812. The cell material was finally centrifuged with Epon in Beem capsules at 20 000 *g* for 60 min at room temperature. This procedure is a slight modification of the technique previously described in detail (6). Ultrathin sections were cut on an LKB Ultratome, stained with lead citrate (12), and examined in a Jeol-100 C electron microscopy.

*Enzyme cytochemistry*

Kynurenic acid was used in the concentrations $5.29\times10^{-4}$ M, $1.32\times10^{-3}$ M and $2.65\times10^{-3}$ M. The exposure time was $1.8\times10^{3}$ sec. Irradiated cells were harvested for cytochemical analysis, 1 min, 1 hour, 2 hours and 4 hours post irradiation.

The cell cultures were fixed at 0°C for 60 min in the above glutaraldehyde fixative. The cultures were then washed for 60 min at 0°C in three different changes of 0.2 M sucrose in 0.05 M cacodylate buffer (pH 7.2; 300 mOsm). Acid phosphatase was demonstrated *in situ* in the plastic dishes with a Gomori-type medium (1) containing 0.22 M sucrose and 10% dimethylsulphoxide (DMSO) (4) (pH 5.0, about 1800 mOsm, the DMSO contributing 1500 mOsm). The reaction was run at 37°C in a $N_2$ atmosphere with continuous agitation of the solution for 45 min. The reaction product was converted to visible lead sulphide by treatment of the cultures for 60 sec with 1% ammonium sulphide.

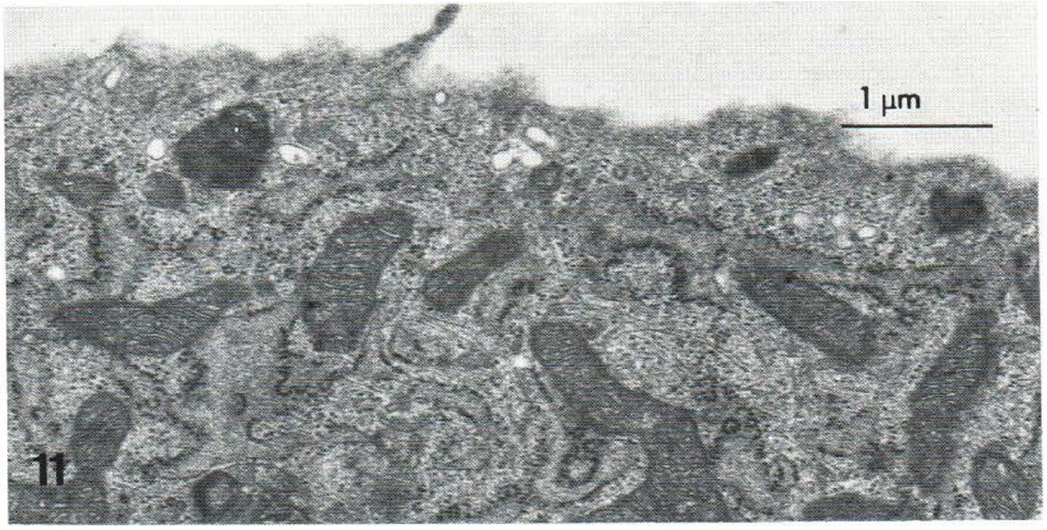
## RESULTS

*Scanning electron microscopy (SEM)*

The findings reported in the previous study (17) following exposure to both kynurenic acid and long-wave ultraviolet light (UVA) were confirmed. Typical cell damage consisted of varying degrees of blebbing, varying from small, discrete and sparsely distributed blebs and microvilli with swollen tips which were noted at the minimum concentration of kynurenic acid ($5.29\times10^{-5}$ M), to an enormous extent of large blebs at the highest concentration ($5.29\times10^{-3}$ M). Another characteristic finding was rounding up of the cells with formation of retraction fibrils around the cell periphery, increasing in tact with the dose of kynurenic acid. Characteristic alterations are depicted in Figs. 4–10.

The cells were not entirely homogeneously dam-

*Figs. 11–13.* Figures demonstrating the fine structure of human glia cells as revealed in the transmission electron microscope. *Fig. 11.* Untreated control cell with a couple of mitochondria, some lysosomes and endoplasmic net studded with ribosomes. Note absence of swollen mitochondria and ruptures in the cell-sap. *Fig. 12.* Swollen cell with ruptures in the cell-sap but no obvious mitochondrial swelling. Note frequent endocytotic vacuoles along the cells' upper and lower surface. Kynurenic acid $5.29\times10^{-4}$ M + UVA-light, $1.8\times10^{3}$ sec 2 h post irradiation. *Fig. 13.* Pronounced swelling with "holes" in the cell sap and condensed mitochondria. Note the still electron-dense and morphologically apparently normal lysosomes. Kynurenic acid $1.32\times10^{-3}$ M + UVA-light, $1.8\times10^{3}$ sec, 2 h post irradiation.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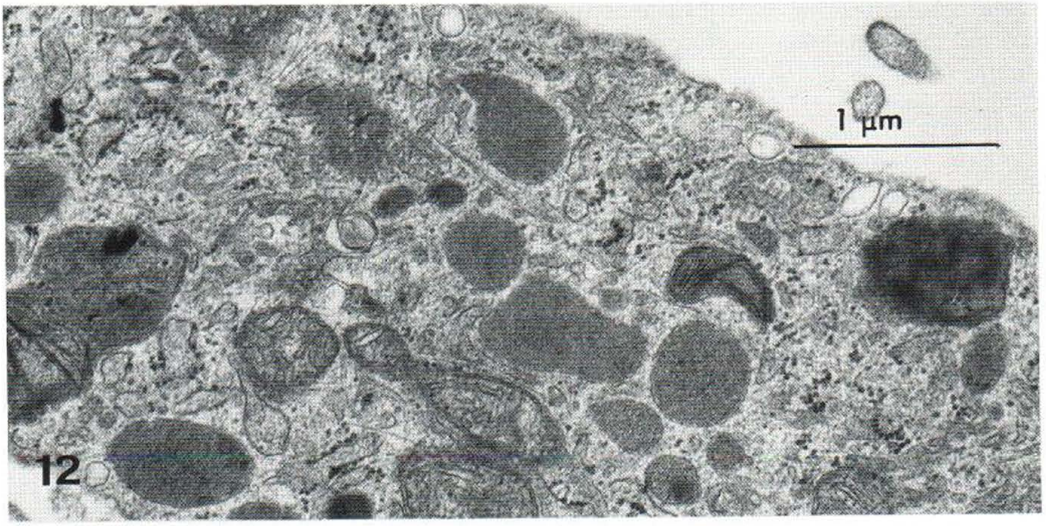
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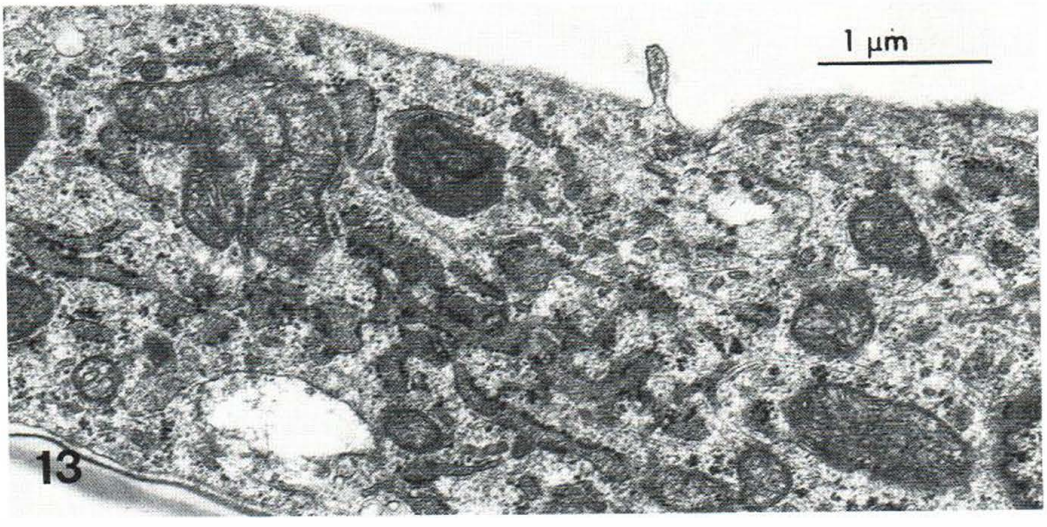
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aged. Typical alterations were already noted in specimens harvested directly after the completed irradiation. The damage increased with increasing concentration of kynurenic acid and exposure to UVA light. Maximum damage was seen after 2 to 4 hours following the termination of the exposure to UVA. When kynurenic acid was added at the lowest concentration ($5.29\times10^{-5}$ M, exposure time $0.6\times10^{3}$ and $1.2\times10^{3}$ sec) and the cells were studied 24 hours post irradiation, they were generally speaking apparently normal. The total number of cells in the irradiated area seemed to be about the same as at 4 hours after the exposure, indicating that some repair can occur when only minimal damage is induced. Kynurenic acid at the concentrations $5.29\times10^{-4}$ and $1.32\times10^{-3}$ M irradiated for $1.2\times10^{3}$ and $1.8\times10^{3}$ sec gave surface changes, with a moderate degree of blebbing noted 2 hours post irradiation, and these conditions were considered suitable for the following studies with transmission electron microscopy.

*Transmission electron microscopy (TEM)*

The ultrastructural features of human diploid glia cells have already been described (6), and the fine structure of control cells in the present experiments was similar (Fig. 11).

Cells treated only with long-wave ultraviolet light (UVA) for $1.8\times10^{3}$ sec did not show any alterations as compared with the controls, and the same was true of cells exposed to kynurenic acid alone at the concentrations investigated.

Cells treated with $5.29\times10^{-4}$ M kynurenic acid and UVA irradiation for $1.2\times10^{3}$ sec showed slight swelling and enhanced formation of endocytotic vacuoles from the plasma membranes. Apart from these findings, no obvious alterations were seen on comparison with the control cells. When the light dose was increased to $1.8\times10^{3}$ sec the cells showed a somewhat more pronounced swelling, with disruptions of the cell sap continuity, swelling of most mitochondria, dilatation of the endoplasmic net and vesiculation of some of the microvilli (Fig. 12). The lysosomal vacuome and the Golgi apparatus were apparently morphologically normal. Endocytotic vacuoles along both the upper and lower plasma membrane surface were a frequent finding. When the dose of kynurenic acid was increased to $1.32\times10^{-3}$ M in combination with the latter light dose, similar alterations were noted but they were still more advanced (Fig. 13).

The TEM studies were performed on cells harvested 2 hours post irradiation, i.e. early during the period (2 to 4 hours) when maximum cellular damage was observed.

*Cytochemistry*

The control cells revealed a distinct pattern of granular enzyme reaction product (Fig. 14). The granulae represent individual secondary lysosomes, primary lysosomes being unresolvable in the light microscope. In many cells there was an empty zone adjacent to the nucleus, probably representing the Golgi area.

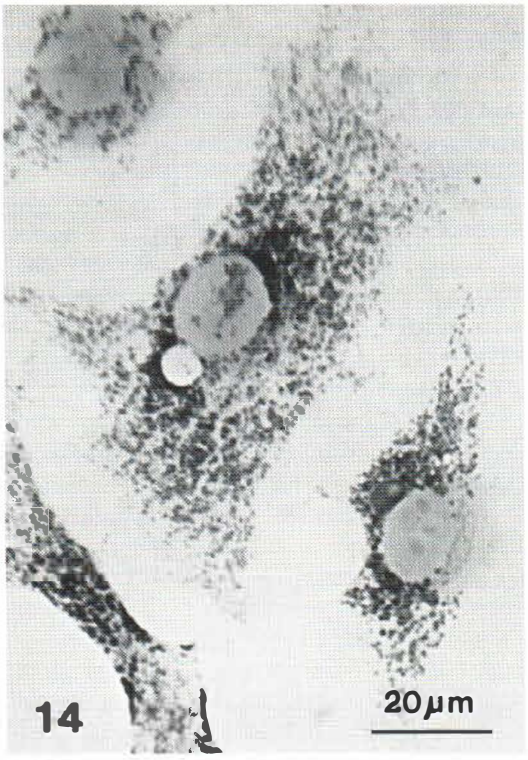
Kynurenic acid at the concentrations $5.29\times10^{-4}$ and $1.32\times10^{-3}$ M, in combination with UVA treatment—which gives pronounced surface alterations on the cellular plasma membrane demonstrable by SEM, and also clearly visible fine structural alterations seen in the TEM—did not induce any changes whatsoever in the enzyme reaction pattern when investigated up to 4 hours post irradiation (Fig 15, 16).

Kynurenic acid at the concentration $2.65\times10^{-3}$ M in combination with UVA treatment caused a change in the granular pattern to a diffuse one with "stained" nuclei 4 hours post irradiation (Fig. 17). Many cells without any reaction product at all, so-called empty cells, were seen. Studies performed 0, and 1 hour post irradiation revealed no alterations, but at 2 hours post irradiation a diffuse enzyme reaction pattern was noted in some of the cells.

## DISCUSSION

Following irradiation with long-wave ultraviolet light (UVA) in the presence of kynurenic acid, cultured human glia cells react with rapid swelling and plasma membrane alterations which are most easily recognized under scanning electron

*Figs. 14–17.* Light micrographs 4 hours post irradiation, illustrating pattern of demonstrable acid phosphatase activity, in glia cells exposed to kynurenic acid and UVA-light and compared with controls subjected to only UVA-light. Exposure time $1.8\times10^{-3}$ sec. *Fig. 14.* Control cell. Note distinct granularity in the cytoplasm and absence of diffuse nuclear or cytoplasmic staining. *Figs. 15, 16* show cells treated with kynurenic acid in the concentrations $5.29\times10^{-4}$ M and $1.32\times10^{-3}$ M respectively and exposed to UVA-light. *Fig. 17* shows severe cellular damage, with nuclear impregnation and diffuse cytoplasmic staining indicating leakage of acid phosphatase out of the lysosomes. Kynurenic acid $2.65\times10^{-3}$ M + UVA-light.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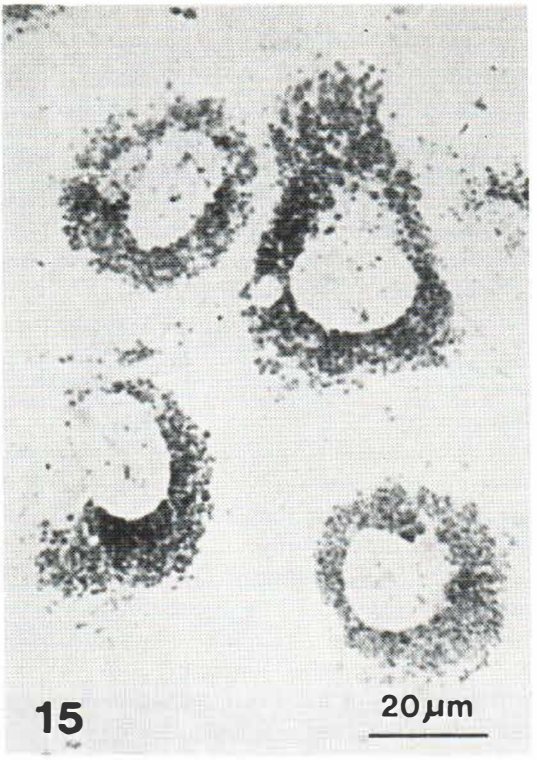
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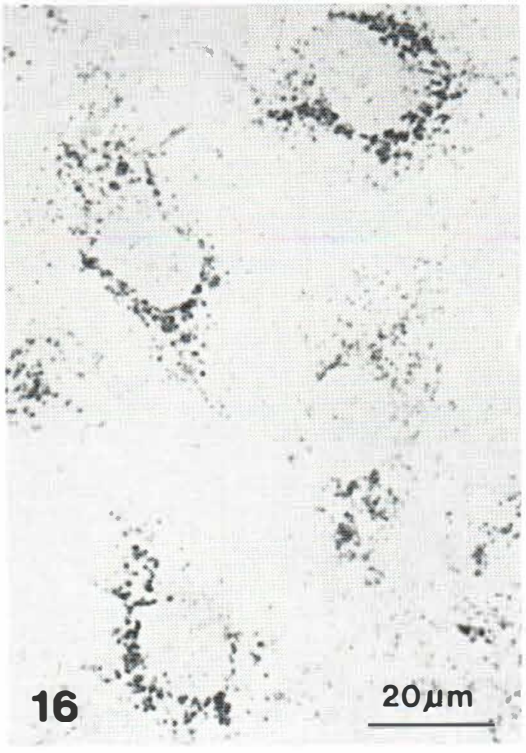
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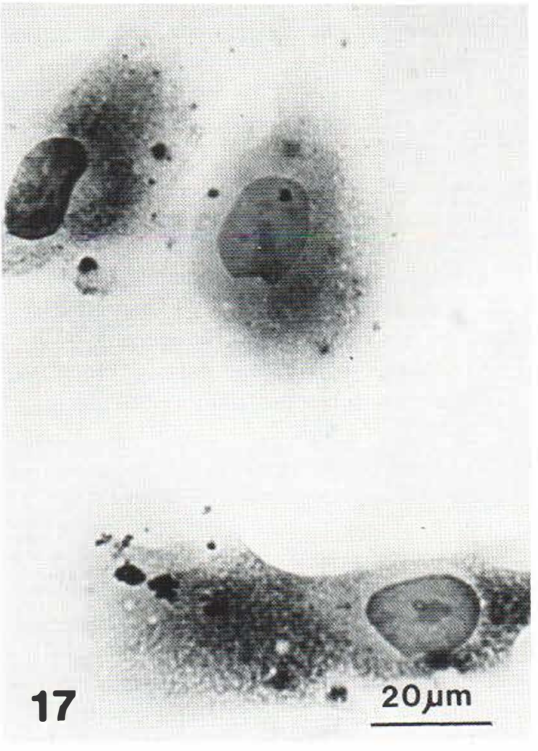
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microscopy (SEM). Such swelling is seen in the SEM following fairly low doses of kynurenic acid and short-term exposure to UVA. The damage is noted directly after the termination of the irradiation, but maximum cellular alterations are observed 2 to 4 hours later. When minimal stress was applied, i.e. the lowest concentrations of kynurenic acid and short-term exposure to UVA light, the cellular damage seemed to be repaired when the cells were studied 24 hours post irradiation.

Following short-term exposure to UVA at low concentrations of kynurenic acid, transmission electron microscopy (TEM) reveals morphologic alterations consisting of enhanced endocytotic activity which would result in internalization of part of the plasma membrane to the lysosomal vacuolar system. This may be indicative of a reparative process, and a sign of the cells' effort to degrade damaged parts of the plasma membrane by autophagocytosis. These findings are similar to those observed when human glia cells are subjected to ionizing irradiation (9). When longer exposure to UVA is combined with higher concentrations of kynurenic acid the changes are more pronounced and consist of increasing swelling and eventual mitochondrial damage. The endoplasmic reticulum was dilated and the cell-sap disrupted, but the lysosomal vacuome did not reveal any major morphological alterations. There was no nuclear degeneration.

The present TEM findings are very similar to those recently reported as induced by cell surface bound porphyrins, namely severe cytoplasmic vacuolization, pyknosis of nuclei and rounding up of the cells with swollen and sometimes disrupted mitochondria (8). Kynurenic acid is thus capable of inducing cell damage in the same way as other known endogenously occuring photosensitizers, viz. porphyrins.

Findings recorded in the cytochemical analysis are indicative of enzyme leakage from the lysosomal vacuome only at the highest concentration of kynurenic acid studied. These findings, however, occurred rather late after termination of the UVA exposure, when there was already a rather pronounced swelling and cytoplasmic disorganization as seen in the SEM and TEM. These findings imply that cellular lysis due to release of lysosomal hydrolytic enzymes must be a secondary phenomenon and not an initial event in the degenerative process. Furthermore, diffusion of kynurenic acid to the lysosomal vacuome is not to be expected, due to the acidity of the substance (7). It is known that cell swelling during hypotonic fixation will lead to leakage of lysosomal enzymes, probably as a consequence of lysosomal swelling (4). The absence of morphological alterations of the lysosomal apparatus is in agreement with findings that acid phosphatase can disappear from morphologically intact lysosomes during hypo-osmotic damage (5).

The primary assault in cellular photo-oxidative damage inflicted by kynurenic acid and UVA irradiation on human glia cells thus appears to originate from the binding of kynurenic acid to the plasma membrane, which is then damaged by subsequent UVA irradiation leading to water transport into the cell, swelling and, subsequently, secondary intracellular alterations (15, 16). This hypothesis is further supported by the fact that cellular damage of similar magnitude can be elicited by UVA irradiation performed either directly after the addition of kynurenic acid to the cell culture, after 2 hours, or after 24 hours of incubation with kynurenic acid before irradiation (17). The active or passive uptake of kynurenic acid by the cell, with subsequent damage to intracellular structures, is therefore probably not of essential importance for the photo-oxidative damage which in the system described seems to be initially operative on the plasma membrane level.

## ACKNOWLEDGEMENT

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