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(54) **UTILISATION DE COMPOSES ANTIOXYDANTS LIPOPHILES POUR EMPECHER LES REACTIONS OXYDATIVES INDUITES PAR LES RETINOIDES ET VEHICULEES PAR LES UVA**

(54) **USE OF LIPOPHILIC ANTIOXIDANT COMPOUNDS TO PREVENT RETINOID INDUCED, UVA-MEDIATED OXIDATIVE REACTIONS**

(57) Cette invention se rapporte à des moyens et à des procédés pour appliquer topiquement sur la peau d'un mammifère un rétinoïde qui réduit ou élimine les effets oxydatifs induits par les rétinoïdes et véhiculés par les ultra-violets A. Ce procédé consiste à appliquer topiquement sur la peau une composition de rétinoïde qui contient une quantité efficace de rétinoïde et une quantité efficace d'un antioxydant lipophile.

(57) The invention relates to means and methods of topically applying a retinoid to mammalian skin that reduces or eliminates retinoid induced, ultraviolet A-mediated oxidative effects. The method comprises topically applying to the skin a retinoid composition that contains an effective amount of said retinoid and an effective amount of a lipophilic antioxidant.

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(54) Title: USE OF LIPOPHILIC ANTIOXIDANT COMPOUNDS TO PREVENT RETINOID INDUCED, UVA–MEDIATED OXIDATIVE REACTIONS

(57) Abstract

The invention relates to means and methods of topically applying a retinoid to mammalian skin that reduces or eliminates retinoid induced, ultraviolet A–mediated oxidative effects. The method comprises topically applying to the skin a retinoid composition that contains an effective amount of said retinoid and an effective amount of a lipophilic antioxidant.

USE OF LIPOPHILIC ANTIOXIDANT COMPOUNDS TO PREVENT RETINOID INDUCED, UVA-MEDIATED OXIDATIVE REACTIONS

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to the protection of skin from the oxidative effects of ultraviolet (UV) A radiation that are induced by retinoid use. In particular, the present invention relates to means of topically applying a retinoid to skin that reduces or eliminates skin damage caused by retinoid induced, UVA-mediated oxidative effects.

2. Background Information

Retinoids such as, retinoic acid (also known as vitamin A acid or tretinoin), retinol, and retinol palmitate, are known to have beneficial effects on the skin. For example, they have been used both orally and topically to treat acne.

Conflicting evidence exists concerning the ability of retinoids to act as photoprotectants or agents for photoaging repair. Retinoids have been shown to be able to act as antioxidants at high concentrations, protecting the skin from sunlight and preventing the induction of lipid peroxidation initiated by the use of ascorbic acid and iron [Das et al., *J Neurochem* (1989) 52:585-588; Khettab et al., *Biochimie* (1988) 70:1709-1713; and Geesin et al, *Arch Biochem Biophys* (1990) 278:350-355]. One means by which UV radiation from the sun produces its acute and chronic effects on skin involves the production of reactive oxygen species, which cause pathology by a

number of different mechanisms including lipid peroxidation production [Shea et al., "Nonionizing radiation and the skin." In: *Physiology, Biochemistry, and Molecular Biology of the Skin.* 1991. LA Goldsmith, ed. Oxford University Press:New York. vol. 2, pp. 910-927]. Indeed, the production of free radicals and lipid peroxidation has been associated with characteristic changes associated with aging in many tissues including the skin [Machlin, et al., *FASEB J* (1987) 1:441-445; Emerit, I, "Free radicals and aging in skin." In: *Free Radicals and Aging.* 1992. I Emerit and B Chance, eds. Birkhauser Verlag Base: Switzerland, pp. 328-341; De Quiroga et al., "Relationship between antioxidants, lipid peroxidation and aging." In: *Free Radicals and Aging.* 1992. I Emerit and B Chance, eds. Birkhauser Verlag Base: Switzerland, pp. 109-123; Yagi, K., "Lipid peroxides in the skin." In: *The Biological Role of Reactive Oxygen Species in the Skin.* 1987. O. Hayaishi, S. Imamura, Y. Miyachi, eds. Elsevier: New York, pp. 109-116]. Thus, the ability of retinoids to prevent the induction of lipid peroxidation initiated by ascorbic acid and iron seem to indicate a potential for retinoids to protect against oxidative insult caused by UVA-mediated.

In contrast, retinoids have also been reported to produce undesirable side effects. Retinoids have been reported to reduce the exposure time for individuals to produce UV-mediated erythema resulting in increased sensitivity to sunburn [Szaniawska et al., *Neoplasm* (1988) 35:191-195; Collins et al., *J Am Acad Dermatol* (1986) 14:274; Ferguson et al., *Pharmac Ther* (1989) 40:123-135; and Auffret et al., *J Am Acad Dermatol* (1990) 23:321-322]. A side-effect not all studies have been able to confirm

[Diffey et al., *J Am Acad Dermatol* (1986) 12:119-121; and Wong et al., *J Am Acad Dermatol* (1986) 14:1095-1096]. Additionally, vitamin A has been shown to be able to potentiate the oxidative effects of carbon tetrachloride on hepatotoxicity in rats [Elsisi et al., *Toxicol Appl* Pharmacol (1993) 119:295-301].

Given the contradictory reports surrounding the effects of retinoids on oxidative events, applicants investigated the effects of retinoids on UVA induced lipid peroxidation. This investigation lead to the surprising discovery that retinoids contribute to UVA-mediated oxidative damage but that their effect can be reduced when used in combination with certain lipophilic antioxidants.

SUMMARY OF THE INVENTION

Therefore, it is an object of the present invention to provide means and methods of applying retinoids to the skin while reducing or eliminating retinoid induced, UVA-mediated oxidative damage to the skin including lipid peroxidation.

It is another object of the present invention to provide a topical composition that, when applied to human skin, provides the beneficial effects of retinoids while protecting against retinoid induced, UVA-mediated oxidative effects including lipid peroxidation.

In one embodiment, the present invention relates to a method of topically applying a retinoid to mammalian skin which reduces or eliminates retinoid induced, UVA-mediated oxidative damage to the skin. The method comprises topically applying to the skin a composition comprising a safe and effective amount of a retinoid and a safe and effective amount of a lipophilic antioxidant.

Various other objects and advantages of the present invention will become apparent from the drawings and the following description of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the effect of different culture plates on the production of lipid peroxidation by human dermal fibroblasts exposed to solar simulated light. Cells were exposed to solar simulated light through the covers on Corning 75 cm^2 flasks or through Costar 100 mm tissue culture dishes with or without the covers. Triplicate cultures were exposed to increasing numbers of MED using the solar simulator arrangement of lamps.

Figure 2 shows the spectral dose distribution of light sources with different culture materials. The spectral dose distribution is presented for the three cell culture conditions identified in the lipid peroxidation measurements described previously (see Figure 1) using the solar simulator arrangement of lamps.

Figure 3 shows the spectral irradiance of F40 350BL lamps. The spectral irradiance for the Sylvania F40 350BL lamps (98% UVA, 2% UVB) is presented.

Figure 4 shows the absorbance spectra of Schott filters. The absorbance of various WG filters was determined and is shown.

Figure 5 shows the spectral dose distribution produced with the Schott Filters described in Figure 4.

Figure 6 show the difference spectra produced using the Sylvania F40 350BL fluorescent lamps in combination with the Schott Filters described in Figures 4 and 5. These spectra represent the differences in spectra produced when comparing one filter to the next in the series.

Figure 7 shows lipid peroxidation produced in human dermal fibroblasts (HSF) and Swiss 3T3 cells (S3T3) in the presence or absence of the Schott filters described in Figures 4-6.

Figure 8 shows lipid peroxidation action spectrum for human dermal fibroblasts (HSF) and Swiss 3T3 Mouse Fibroblasts (S3T3). These action spectra were determined using the information from Figures 6 and 7.

Figure 9 shows difference spectra produced using the Sylvania F40 350BL fluorescent lamps as described in Figure 6. This presentation highlights selected wavelengths (290-310nm) from Figure 6.

Figure 10 shows the spectral irradiance of Westinghouse FS40 Sunlamps alone.

Figure 11 shows the effect of exposure to Westinghouse FS40 Sunlamps on the production of lipid peroxidation in human dermal fibroblasts. Triplicate cultures were exposed to increasing number of MED.

Figure 12 shows the effect of retinoids on UVA-induced lipid peroxidation. Swiss 3T3 mouse fibroblasts (S3T3)(A), and human dermal fibroblasts (B) were exposed to 60 joules/cm^2 UVA using Sylvania F40 350BL lamps.

Figure 13 shows dose and wavelength dependence for the effect of retinoic acid on UV-induced lipid peroxidation. Triplicate cultures were irradiated for the indicated number of MED using the solar simulator arrangement of lamps or for the indicated levels of joules/cm^2 UVA in the presence of 100 mM retinoic acid.

Figure 14 shows the effect of retinoids on the levels of lipid peroxidation produced in the presence or absence of the Schott filters.

Figure 15 shows the effect of retinoids on background levels of lipid peroxidation in Swiss 3T3 cells. Triplicate cultures were treated with the indicated concentrations and types of retinoids and maintained either under aluminum foil away from the lamps (NO UV) or under the lamps (FOIL).

Figure 16 shows the lipid peroxidation action spectrum for Swiss 3T3 cells treated with retinoic acid.

Figure 17 shows the absorbance spectrum for a 50 μM solution of all-trans retinoic acid in methanol.

Figure 18 shows the effect of butylated hydroxyanisole, butylated hydroxytoluene, and ascorbic acid (asborbate) on the synergistic effect of retinoic acid and UVA on the production of lipid peroxidation in Swiss 3T3 cells.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to methods of topically applying to the skin a retinoid while reducing or eliminating skin damage caused by retinoid induced, UVA-mediated oxidative effects including UVA-induced lipid peroxidation and products produced by lipid peroxidation. The present invention is also directed to retinoid compositions for topical application to skin of humans and like susceptible animals. The present invention results from the surprising discovery that retinoids contribute to UVA-mediated oxidative damage but that their damaging effect can be reduced by topically applying the retinoids in combination with lipophilic antioxidants.

In the method of the present invention, the skin receives the beneficial effects of retinoids while reducing and/or eliminating retinoid induced, UVA-mediated oxidative damage. This is achieved by topically applying to the skin a composition that contains an effective amount of a retinoid and an effective amount of a lipophilic antioxidant that reduces and/or eliminates retinoid induced, UVA-mediated oxidative damage. Retinoid induced UVA-mediated oxidative effects include, but are not limited to, lipid peroxidation and the products produced by lipid peroxidation such as cross-linking agents.

Lipophilic antioxidants have been found to be effective at preventing skin damage caused by retinoid induced, UVA-mediated oxidative effects. Preferred lipophilic antioxidants include, but are not limited to, ascorbyl-6-palmitate, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT).

Ascorbyl palmitate has previously been shown to be ineffective at preventing UVB induced wrinkling in hairless mice [Bissett et al., *Photodermatol Photoimmunol Photomed* (1990) 7:56-62], however it has been shown to protect endothelial cells from the cytotoxic effects of products of lipid peroxidation [Kaneko et al., *Arch Biochem Biophys* (1993) 304:176-180]. No examination of the effect of ascorbyl-palmitate on UVA-mediated events has been reported.

BHA has also been shown to be effective at preventing UVC-induced lipid peroxidation in liposomes [Pelle et al., *Arch Biochem Biophys* (1990) 283:234-240]. Additionally, BHA has been found to be effective at preventing UVB- or PUVA-induced ornithine decarboxylase activity (associated with tumor formation) [Kono et al., *J Dermatol* (1992) 19:389-392; Black et al., *Photochem Photobiol* (1986) 43:403-408], however, BHA had no effect on UVB-induced photocarcinogenesis [Black et al., *Photochem Photobiol* (1986) 43:403-408]. No results concerning the effects of BHA on other UVA-mediated events have been reported.

Of the three preferred lipophilic antioxidants, BHT has seen the greatest amount of study. A great deal of literature exists concerning the ability of oral ingestion of BHT to provide protection against the acute and chronic effects of UVB exposure [Koone et al., *J Invest Dermatol* (1986) 87:343-347; Black et al., *Photochem Photobiophys* (1980) 1:119-123; Peterson et al., *J Invest Dermatol* (1980) 75:408-410; Black et al., *Photochem Photobiol* (1984) 40:69-75; Black et al., *Photochem Photobiol* (1991) 53:707-716; Black et al. *Photochem Photobiol* (1986) 43:403-408] including photocarcinogenesis, erythema and induction of ornithine decarboxylase activity. In

addition, BHT has been shown to be effective in preventing UVA- [Bose et al., *Radiat Res* (1993) 133:340-344] and UVC- [Pelle et al., *Arch Biochem Biophys* (1990) 283:234-240] induced lipid peroxidation in liposomes. No evidence of activity of BHT against UVA-mediated oxidative effects in animals or cultured cells has been reported.

In the present invention, the retinoid composition containing a lipophilic antioxidant is applied topically to the skin to protect it from retinoid induced, UVA-mediated oxidative effects. The amount of retinoid used in the topical compositions and methods of the present invention can vary within significant limits depending on the therapeutic use for which the retinoid composition will be applied to the skin. Preferably, the retinoid will be present from about 0.001 to about 3.0 percent by weight, more preferably from about 0.025 to about 0.5 percent by weight.

The amount of the lipophilic antioxidant present in the retinoid compositions and applied to the skin cells may vary so long as a sufficient amount of the antioxidant is present to reduce or eliminate retinoid induced, UVA-mediated oxidative damage, including lipid peroxidation, to the skin. Preferably, the antioxidant is present in the composition from about .0001 % to about 10 % (w/w), more preferably from about .01 % to about 1 %, and more preferably still from about .1 % to about .5 %.

The retinoid compositions of the present invention may be made into a variety of product types. The compositions can be in solid, liquid or aerosol form so long as they are suitable for topical administration. For example, the compositions can be formulated into a liposomal formulation, an emollient, a liquid, a cream, a gel, an ointment, a microemulsion, or a solution.

Other typical skin care agents and additives that assist in the purpose of the present invention or that are conventionally used in topical cosmetics or medical compositions may also be included in the retinoid compositions used in the present invention. Various vitamins may also be included in the photoprotective compositions of the present invention. Examples of such vitamins include, but are not limited to, Vitamin A and derivatives thereof, Vitamin B_2, biotin, pantothenic, Vitamin D, Vitamin E and combinations thereof.

The following examples are included to further illustrate the practice of this invention, and are not meant to be limiting in anyway.

EXAMPLES

Cell cultures

Monolayer cultures of mouse Swiss 3T3 (S3T3) and human dermal fibroblasts (HSF) were grown to confluence in the appropriate media as follows. S3T3 cells and HSF were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% calf serum.

Example 1: Effect of culture materials on the production of lipid peroxidation

To determine whether solar simulated light could produce oxidative effects on cells in culture, neonatal human dermal fibroblasts were irradiated using a combination of Sylvania F40 350BL lamps (98% UVA) and Westinghouse FS40 Sunlamps (approximately 50% UVA, 50% UVB) to simulate the normal solar spectrum.

Neonatal human dermal fibroblast cultures were grown as described above in Corning 75 cm^2 tissue culture flasks or in 100 mm Costar culture dishes. Cultures were then irradiated through the use of a solar simulator arrangement of bulbs with a 6:5 mix of Sylvania F40 350BL lamps and Westinghouse FS40 Sunlamps (50% UVA, 50% UVB).

Following irradiation, a lipid peroxidation assay was conducted. Briefly, irradiated plates were scraped with a rubber policeman and cells and solution were homogenized on a dounce homogenizer. An aliquot of the protein extract was taken for Lowry determination of total protein [Lowry et al., *J Biol Chem* (1951) 193:265-275]. The remainder of the extract was precipitated with trichloroacetic acid. The supernatant was assayed for malondialdehyde content in duplicate by combining it with 0.5% thiobarbituric acid solution before boiling for 30 minutes. Samples were measured for their absorbance at 532 nm. Malondialdehyde levels were determined using the reported extinction coefficient [Wilbur et al., *Arch Biochem Biophys* (1949) 23:305-313].

When cultures were grown in Corning 75 cm^2 tissue culture flasks, a dose-dependent increase in the level of malondialdehyde was produced (Figure 1) indicative of an increase in the level of cellular lipid peroxidation. When cells were grown in Costar 100 mm culture dishes the levels of lipid peroxidation were reduced compared to Corning flasks when irradiated at equivalent doses of UV (Figure 1). When cells were irradiated with the lids of the Costar dishes removed, no effect of UV was seen on the levels of lipid peroxidation in irradiated cells at the doses tested (Figure 1).

In order to understand the different results produced by the use of different culture materials, the spectra of

the light penetrating the different culture plates were examined. The spectral power distribution of the Sylvania F40 350BL fluorescent lamps used to irradiate the cells was measured with an Optronics Model 742 Spectroradiometer at 2 nm intervals between 250 and 400 nm. The irradiance was multiplied by the transmission of the Costar lid used to cover the cells at each wavelength to determine the irradiance of the source to the cells. The irradiance at each wavelength was multiplied with a scaler value (representing time) such that the integral equaled 80 J/cm^2.

The spectral dose distribution is presented (Figure 2) for the three cell culture conditions identified in the lipid peroxidation experiments (Figure 1) using the solar simulator arrangement of lamps described above. Under these conditions, the Corning flasks received 40% more UVA than the Costar dishes with the equivalent amounts of UVB. The uncovered dishes received even less UVA (Corning flasks received 140% more UVA than uncovered Costar dishes). These results indicate a likely role for UVA selectively to induce lipid peroxidation in these cultured cells. Consequently, all subsequent experiments (except where noted) were performed using only the Sylvania F40 350Bl lamps (see Figure 3 for spectral irradiance using these lamps) since they produce spectra composed of 98% UVA with little contribution from UVB (2%).

Example 2: Determination of the action spectrum for the production of lipid peroxidation

To determine the portion of the spectra produced by the F40 350BL lamps that was responsible for inducing lipid peroxidation, confluent cultures of HSF and S3T3 cells grown in 60 mm culture dishes were irradiated through the lids using the Sylvania F40 350BL lamps as described above with the addition of a number of Schott filters which absorb varying amounts of energy at varying wavelengths as shown in Figure 4.

Action spectra were determined by using a series of long pass filters to evaluate the differences in dose between two adjacent filters, and attributing the differences in the response being evaluated between the two filters to that waveband in proportion to the total energy difference between the two filters. The spectral dose distributions of adjacent filters were subtracted from each other to determine the difference in spectral dose. This was done for each adjacent pair of filters. These difference dose distributions were integrated to determine the difference dose band (See Figure 6).

The level of lipid peroxidation per unit protein content for each filter pair was determined as described above. The differences in lipid peroxidation for each filter pair was determined by subtracting the levels for the adjacent pair. The peroxidation differences for a filter pair were divided by the difference dose band to determine the level of peroxidation attributable to each unit dose of the difference band, indicating the absolute sensitivity of peroxidation to that wave band. The absolute sensitivities of all wavebands were integrated and each of the individual sensitivities were divided by

the sum to determine the percentage of sensitivity of each waveband. The relative sensitivities were plotted as a function of the difference wavelength band to indicate which portion of the UV spectrum was most effective in causing lipid peroxidation.

Spectral absorbance of each of the Schott long pass filters used for the action spectra determination was measured using a Cary 2300 Spectrophotometer with diffuse reflectance accessory. Absorbances at wavelength between 250 and 400 nm in 2 nm intervals were converted to percent transmission. To determine the spectral dose distribution delivered to the filtered cells, the spectral dose distribution of the source with the Costar lid was multiplied at each wavelength with the transmission of the appropriate Schott filter. Each of these distributions was also integrated to determine the total energy delivered through the filter to the cells. The absorbance of these filters demonstrates the pattern of increased absorbance to higher wavelengths with successive filters. The resultant spectral dose distribution after subtracting the absorbance of each filter from the spectra produced by the lamps is shown in Figure 5. As shown, the use of the filters with increasing wavelength number shifts the absorbance maximum of the resulting spectra to higher wavelengths and eliminates the radiation at lower wavelengths.

The difference in the spectra from one filter to the next (Figure 6) is used to determine the amount of lipid peroxidation involved in each step from one filter to the next.

The amount of lipid peroxidation produced using the filters described in Figures 4-6 is shown in Figure 7. As indicated, the levels of lipid peroxidation decline with

the use of filters with increasing wave number and absorbance into higher wavelengths in the UVA region of the spectrum. To determine the contribution of each filter to the effect on lipid peroxidation, the change in lipid peroxidation per change in filter must be divided by the difference spectra produced by successive filters (Figure 6). The result of this calculation is shown in Figure 8 and represents the contribution of each portion of the spectra to the effect on lipid peroxidation seen using the Sylvania F40 350BL fluorescent lamps.

As indicated, the change in lipid peroxidation produced when the WG280 filter is used compared to no filter represents a region of the spectra which is important for the production of lipid peroxidation in human dermal fibroblasts and Swiss 3T3 cells. Additionally, the change from WG345 filter to the WG360 filter also represents a region which contributes significantly to this effect. The difference spectra for each of these transitions produce a peak with absorbance maximum very close to 345 nm indicating that a chromophore exists which is important for the UV-dependent production of lipid peroxidation which has an absorbance maximum very close to 345 nm. Interestingly, difference spectra with peaks closely adjacent to the two identified peaks are less sensitive and help to specifically identify the important wavelengths in the UVA region of the spectrum.

Human dermal fibroblasts also demonstrate another chromophore in the UVB region of the spectrum. This effect may be produced by the small peaks in the dose distribution of the lamp which correspond with 297 and 303 nm shown best in Figure 9. This effect of UVB on the production of lipid peroxidation in human dermal fibroblasts is further shown by the use of the

Westinghouse FS40 Sunlamps (See Figure 10) for dose distribution produced with this lamp alone. Using the UVB dominant FS40 Sunlamps, lipid peroxidation can still be induced in human dermal fibroblasts in a dose dependent manner as shown in Figure 11.

Example 3: Effect of retinoids on UVA-induced lipid peroxidation

To determine whether or not the antioxidant activities of retinoids would reduce UVA-induced lipid peroxidation, two different cell types were exposed to UVA in the presence or absence of various retinoids. Surprisingly, all three retinoids tested (all-trans retinoic acid, all-trans retinol and retinol acetate) stimulated lipid peroxidation.

Normal human dermal fibroblasts and the mouse fibroblast cell lines Swiss 3T3 grown in 60 mm culture dishes were irradiated through the lids with 60 joules/cm^2 UVA using Sylvania F40 350 BL lamps in the presence or absence of retinoid as described above.

Lipid peroxidation was increased between 2.5- and 4-fold by retinoid treatment in S3T3 cells (Figure 12A). Retinoic acid was the most active in these cells while retinol and retinol acetate required higher concentrations to achieve the same levels of lipid peroxidation. In contrast, in human skin fibroblasts, retinoic acid did not stimulate greater amounts of lipid peroxidation than seen with retinol treatment (Figure 12B).

This effect of retinoids on UV-induced lipid peroxidation appears dose and wavelength dependent as shown in Figure 13. When cultures incubated in 100 µM retinoic acid were irradiated with the solar simulator

collection of lamps for increasing numbers of MED, little or no effect on peroxidation was detected. However, when retinoic acid treated cultures were irradiated with increasing amounts of UVA, a dose dependent increase in the levels of lipid peroxidation was seen.

In order to determine the wavelengths which were responsible for the effect of retinoids on UVA-induced lipid peroxidation, the action spectra for the phenomenon was determined as described above. Briefly, using the Schott filters, the levels of lipid peroxidation were determined in Swiss 3T3 cells in the presence of retinoic acid, retinol or retinol acetate (Figure 14) compared to UVA alone (Figure 14 and Figure 7). Treatment of S3T3 cells with retinoids produced a similar, but not identical, pattern for all the retinoids tested.

The loss in the production of lipid peroxidation with successive filters was quite different than the pattern produced by UVA alone. The use of the WG 280 filter did not produce a decrease in the level of lipid peroxidation in retinoid treated cells. Instead, a consistently noted increase in lipid peroxidation was produced when the WG 280 filter was used, indicating that some wavelength or wavelengths (presumably found in the UVB region of the spectrum) is capable of inhibiting the ability of retinoids to induce the production of oxidative products in the presence of other portions of the emitted spectrum. This effect of the WG280 filter was specific for retinoid treated cells since S3T3 cells in the absence of retinoids did not demonstrate the same increase in lipid peroxidation.

Another effect of some retinoids noted during these experiments was a UV-independent mechanism for producing lipid peroxidation. Control cultures which were

maintained under aluminum foil away from the lamps still produced relatively high levels of lipid peroxidation when treated with 1 mM of either retinol or retinol acetate (See Figure 15). This effect may be caused by the specific retinoid used since retinoic acid did not produce as striking an effect, however it is also likely that the lack of effect is only due to the reduced levels of retinoic acid require to induce lipid peroxidation compared to the other retinoids employed. The increased background levels of lipid peroxidation were even higher in samples which were covered in foil but maintained under the lamps, possibly indicating a temperature dependence for this effect produced by the heat of the lamps.

Due to this UV-independent effect on the production of lipid peroxidation in retinol and retinol acetate treated cultures, only the action spectrum for the retinoic acid-induced effect was determined as described above. As shown in Figure 16, retinoic acid produces a very different action spectrum compared to that produced in untreated S3T3 cells. The filters which block the UVB (WG280 and WG230) wavelengths inhibit the production of lipid peroxidation in these experiments. The slow rise to a peak between 360-375 demonstrated by the greatest effect using the difference between the WG360 and the WG375 filters is similar to the absorbance spectra for retinoic acid (See Figure 17). The large contribution of wavelengths above 400 may be produced by the spikes of absorbance produced in the visible region by the Sylvania lamps used in these studies.

The ability of various antioxidants to prevent the synergistic effect of UVA and retinoids was tested as shown in Figure 18. BHA and BHT (at 100 µM) were quite effective at preventing most, but not all, of the lipid

peroxidation produced when 100 mM retinoic acid was applied to cultures irradiated with 60 joules/CM^2 UVA. Ascorbic acid, however, was ineffective in these experiments.

This UVA and retinoid dependent mechanism apparently involves the production of oxygen-derived free radicals in a hydrophobic environment. This conclusion is based upon the ability of the lipophilic antioxidants, BHA and BHT, to effectively counteract the increase in lipid peroxidation produced in the presence of retinoic acid (Figure 18) while ascorbic acid had no effect. Interestingly, ascorbic acid did not appear to *enhance* the effect that retinoids have on UVA-induced lipid peroxidation as it does with UVA alone. The action spectrum for this combined effect of retinoic acid and UVA on lipid peroxidation (Figure 16) is consistent with, but not proof of, retinoic acid acting as the primary chromophore absorbing the irradiated energy to excite the molecule to a state with is capable of initiating lipid peroxidation. This theory is based upon the similarities between the UV spectrum for retinoic acid (Figure 17) and the action spectrum for this retinoic acid dependent effect (Figure 16) derived from experiments. Another interesting finding from these studies involves the ability of contaminating UVB to inhibit the combined effects of UVA and retinoids on the production of lipid peroxidation. This effect was consistently seen in all experiments performed with all three retinoids examined. Although the mechanism by which UVB inhibits this UVA-dependent phenomenon is unclear, it is possible that UVB alters the structure of the retinoid or some other membrane bound molecule to prevent either the initiation or the propagation of lipid peroxidation in the membrane.

Thus, applicants and their methods have demonstrated a mechanism by which retinoids may cause light-dependent oxidative reactions which may be involved in the reported "retinoid-sensitivity" seen with their pharmacological use. In addition, applicants have discovered a means of reducing or eliminating this "retinoid-sensitivity".

* * * * * * *

The foregoing invention has been described in some detail for purposes of clarity and understanding. It will also be obvious that various combinations in form and detail can be made without departing from the scope of the invention.

All publications mentioned hereinabove are hereby incorporated by reference.

WHAT IS CLAIMED IS:

1. A method of topically applying a retinoid to mammalian skin that reduces or eliminates retinoid induced, ultraviolet A-mediated oxidative effects comprising topically applying to the skin a retinoid composition, said composition comprising an effective amount of said retinoid and an effective amount of a lipophilic antioxidant.

2. The method of claim 1 wherein said retinoid induced, ultraviolet A-mediated oxidative effects are lipid peroxidation.

3. The method of claim 1 wherein said composition comprises from about .0001 % to about 10 % (w/w) of said lipophilic antioxidant.

4. The method of claim 3 wherein said composition comprises from about .01 % to about 1 % (w/w) of said lipophilic antioxidant.

5. The method of claim 4 wherein said composition comprises from about .1 % to about .5 % (w/w) of said lipophilic antioxidant.

6. The method of claim 1 wherein said lipophilic antioxidant is selected from the group consisting of butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and ascorbyl-6-palmitate.

7. The method of claim 1 wherein said composition comprises from about 0.001% to about 3.0% (w/w) of said retinoid.

8. The method of claim 7 wherein said composition comprises from about 0.025% to about 0.5% (w/w) of said retinoid.

9. The method of claim 1 wherein said composition is in a solid, liquid or aerosol form.

10. The method of claim 9 wherein said composition is formulated into a liposomal formulation, an emollient, a liquid, a cream, a gel, an ointment, a microemulsion, or a solution.

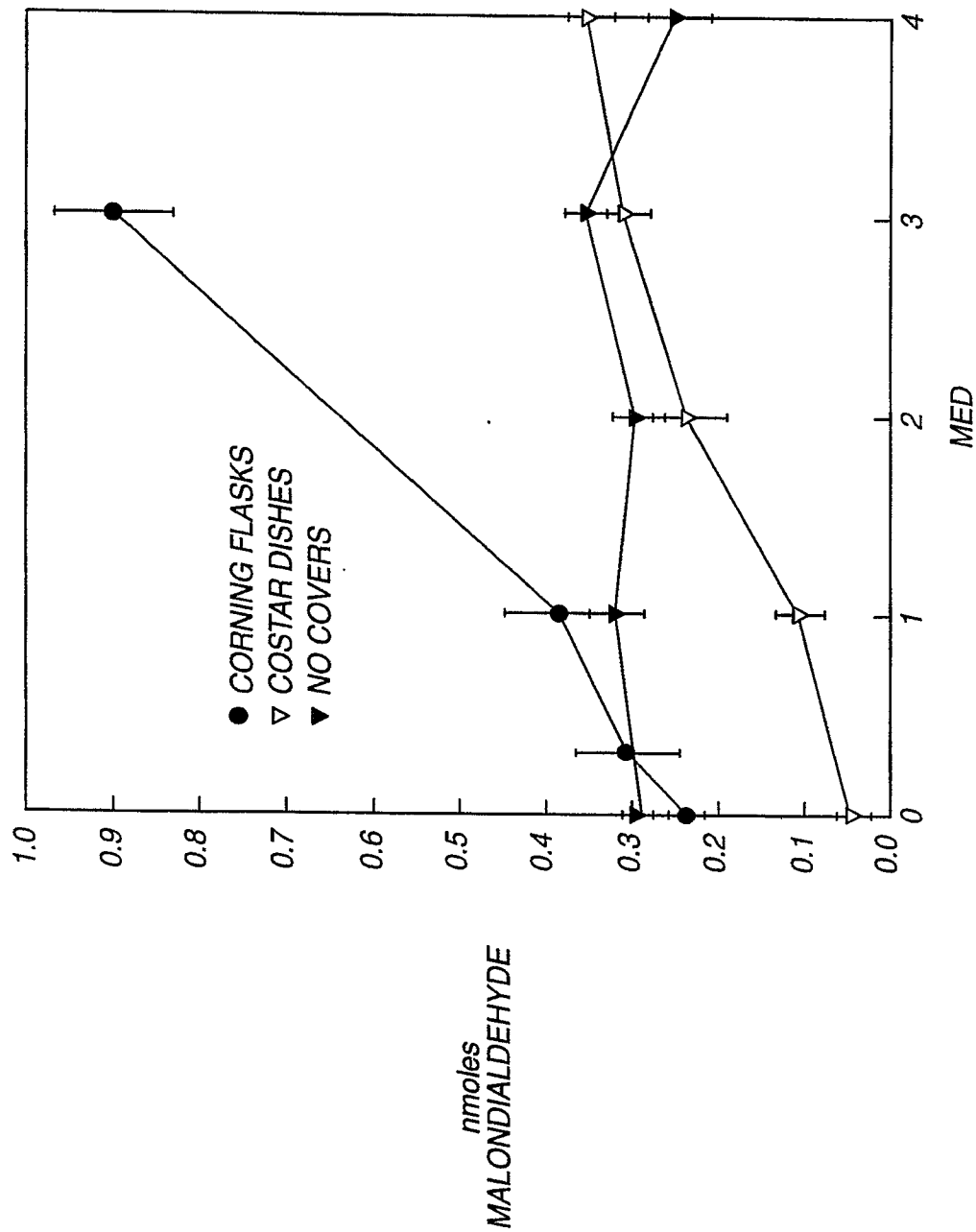

CORNING FLASKS
COSTAR DISHES
NO COVERS
nmoles
MALONDIALDEHYDE
MED
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
0
1
2
3
4

FIG. 1

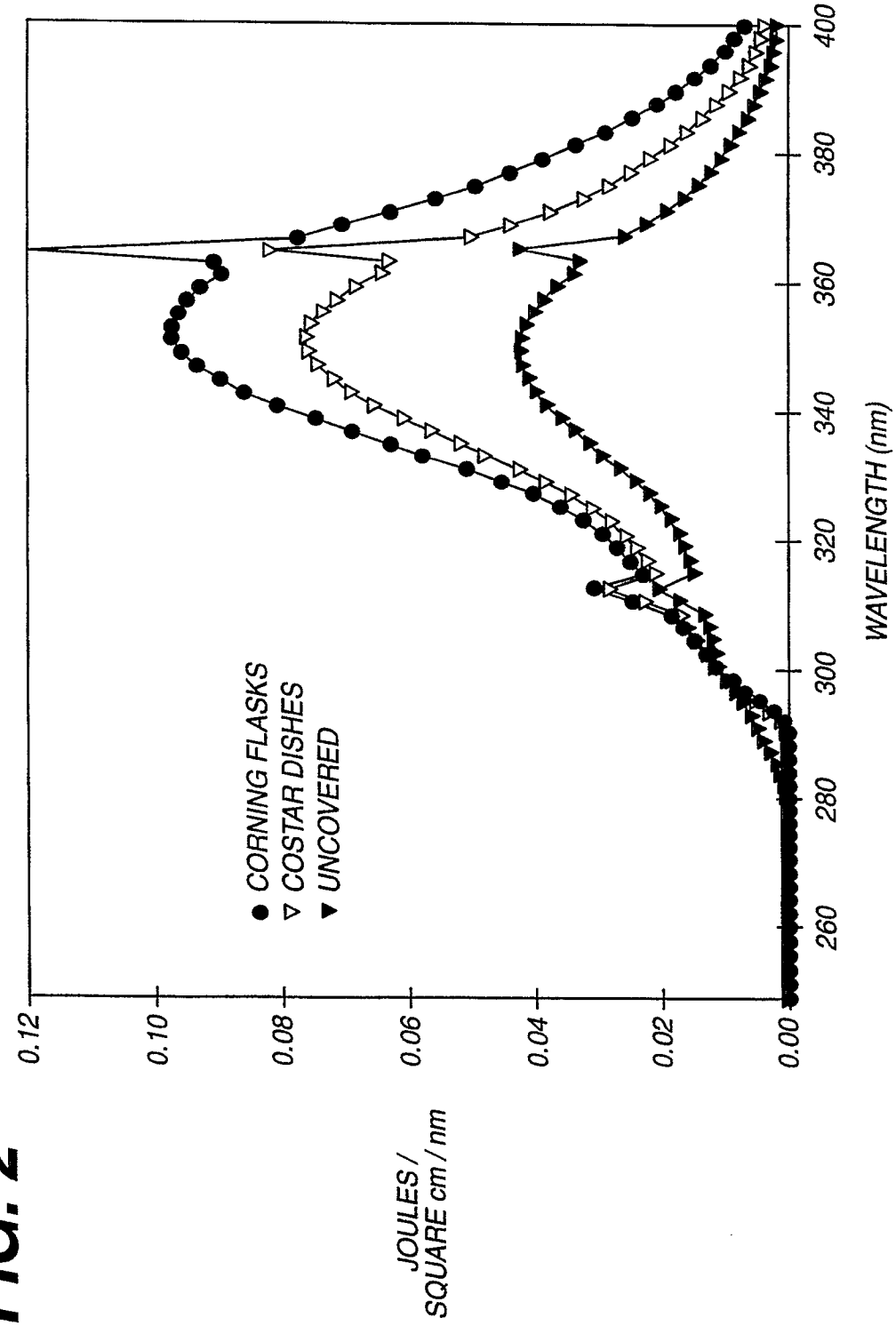

0.12
0.10
0.08
0.06
0.04
0.02
0.00
260
280
300
320
340
360
380
400
WAVELENGTH (nm)
JOULES / SQUARE cm / nm
● CORNING FLASKS
▽ COSTAR DISHES
▼ UNCOVERED

FIG. 2

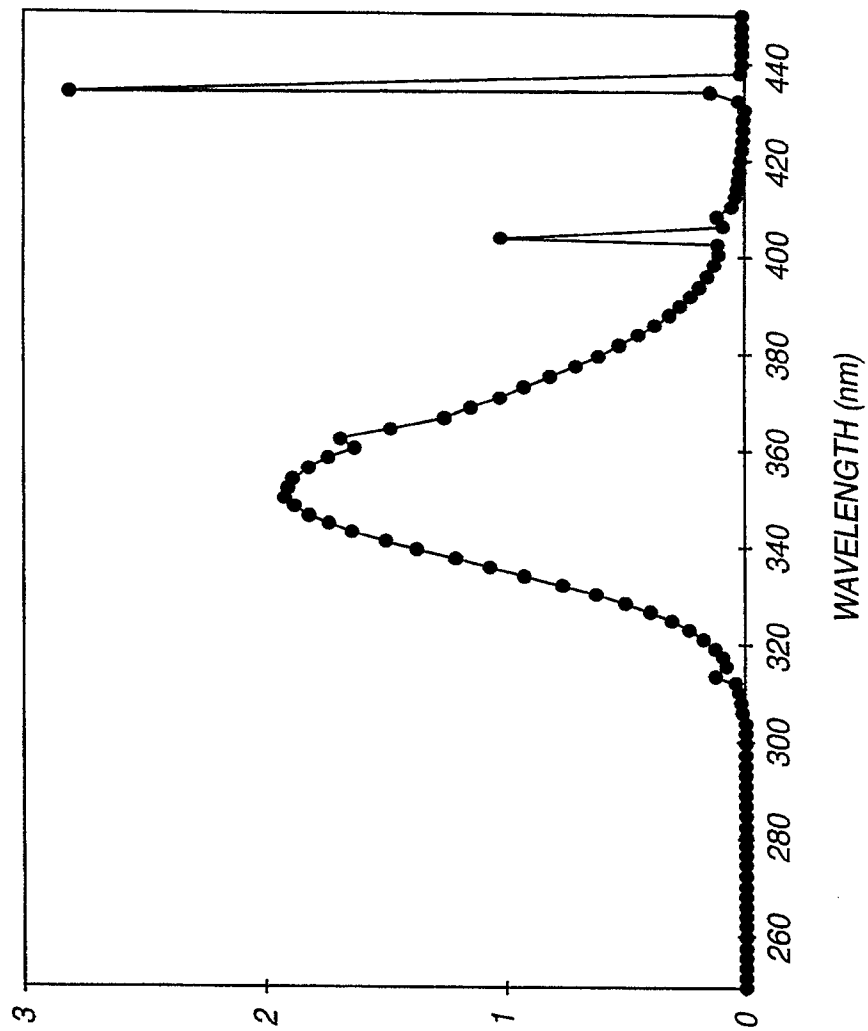

3
2
1
0
JOULES / SQUARE cm
260
280
300
320
340
360
380
400
420
440
WAVELENGTH (nm)

FIG. 3

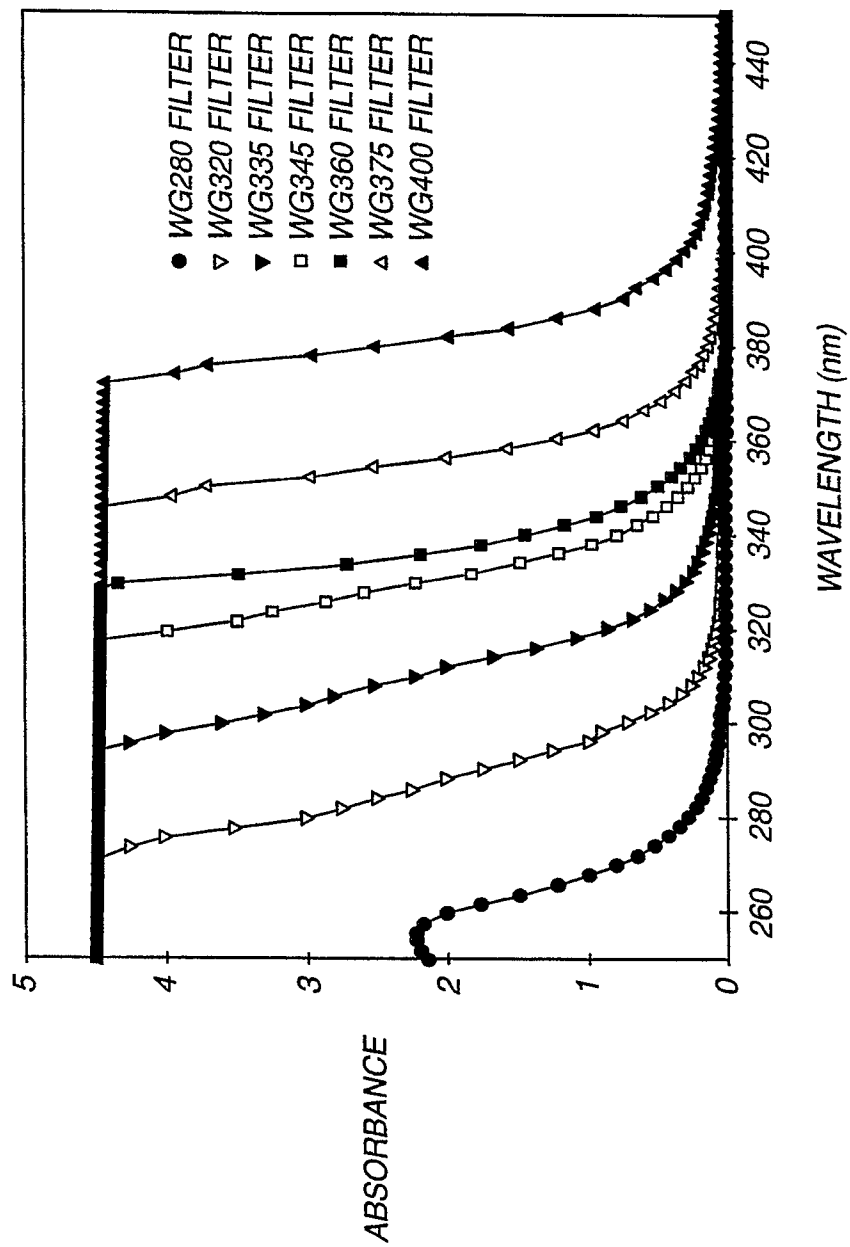

WG280 FILTER
WG320 FILTER
WG335 FILTER
WG345 FILTER
WG360 FILTER
WG375 FILTER
WG400 FILTER
ABSORBANCE
WAVELENGTH (nm)
0
1
2
3
4
5
260
280
300
320
340
360
380
400
420
440

FIG. 4

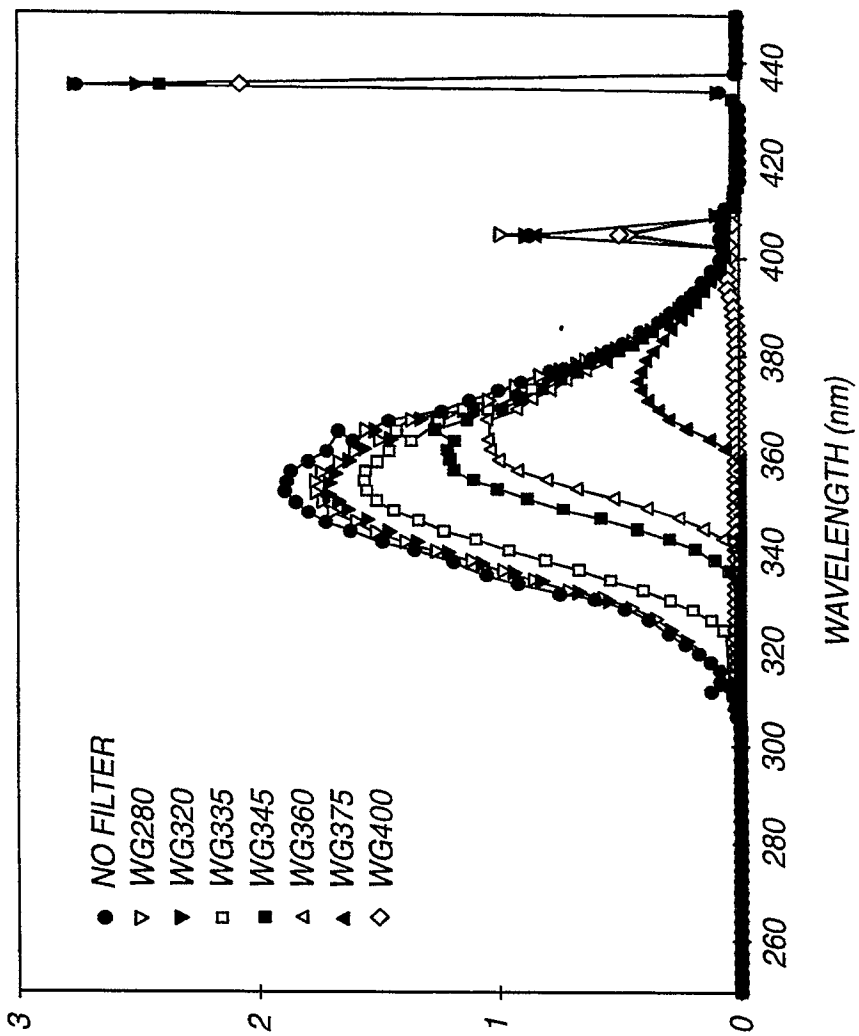
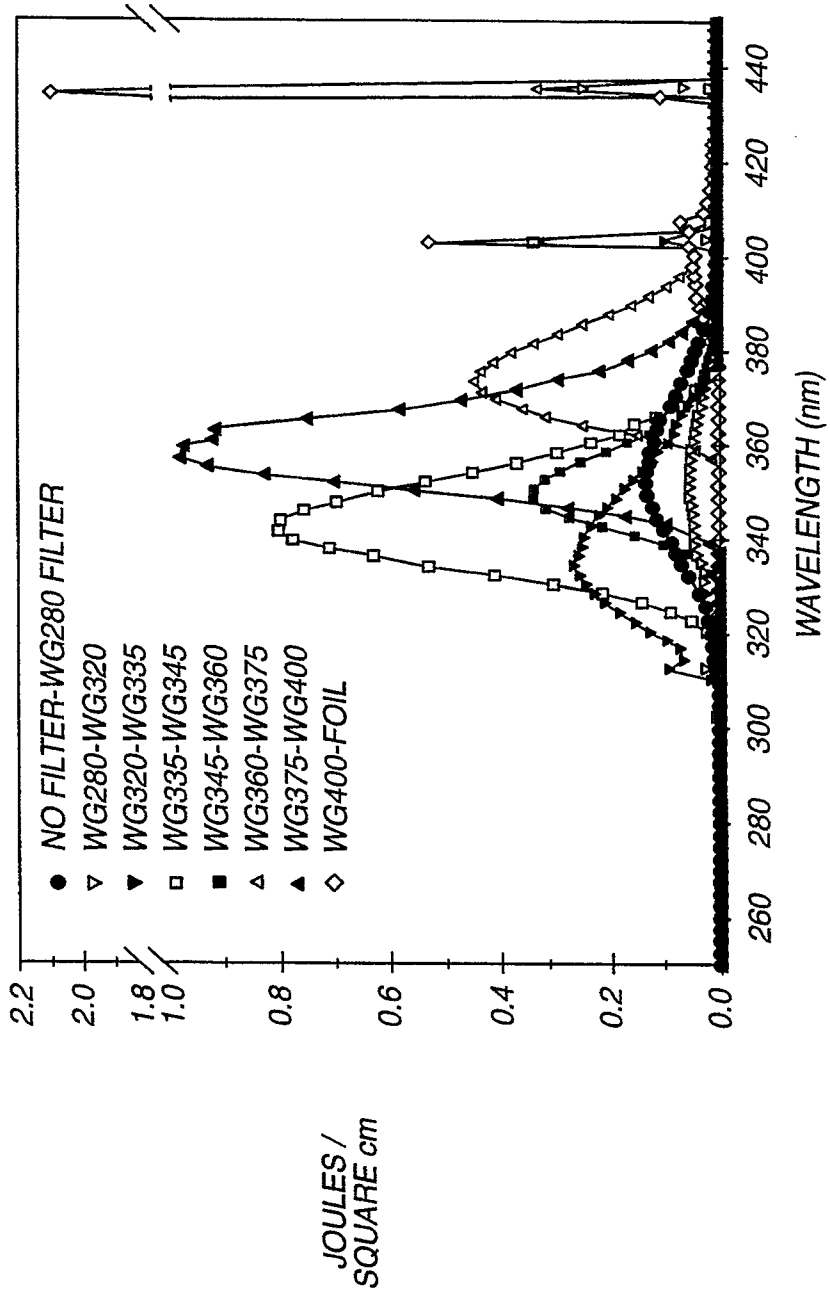

NO FILTER
WG280
WG320
WG335
WG345
WG360
WG375
WG400
0
1
2
3
JOULES / SQUARE cm
260
280
300
320
340
360
380
400
420
440
WAVELENGTH (nm)

FIG. 5

FIG. 6


NO FILTER-WG280 FILTER
WG280-WG320
WG320-WG335
WG335-WG345
WG345-WG360
WG360-WG375
WG375-WG400
WG400-FOIL
JOULES / SQUARE cm
2.2
2.0
1.8
1.0
0.8
0.6
0.4
0.2
0.0
260
280
300
320
340
360
380
400
420
440
WAVELENGTH (nm)

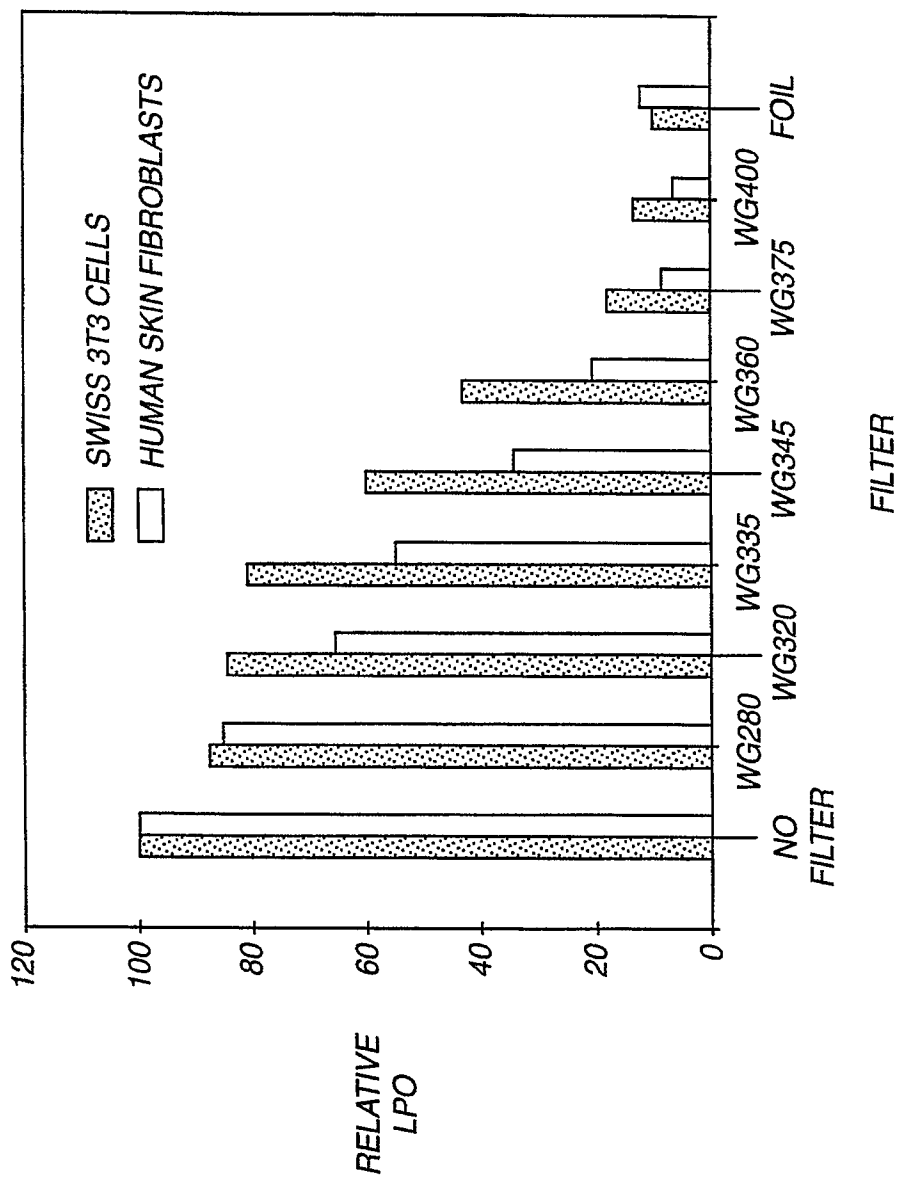

SWISS 3T3 CELLS
HUMAN SKIN FIBROBLASTS
120
100
80
60
40
20
0
RELATIVE LPO
NO FILTER
WG280
WG320
WG335
WG345
WG360
WG375
WG400
FOIL
FILTER

FIG. 7

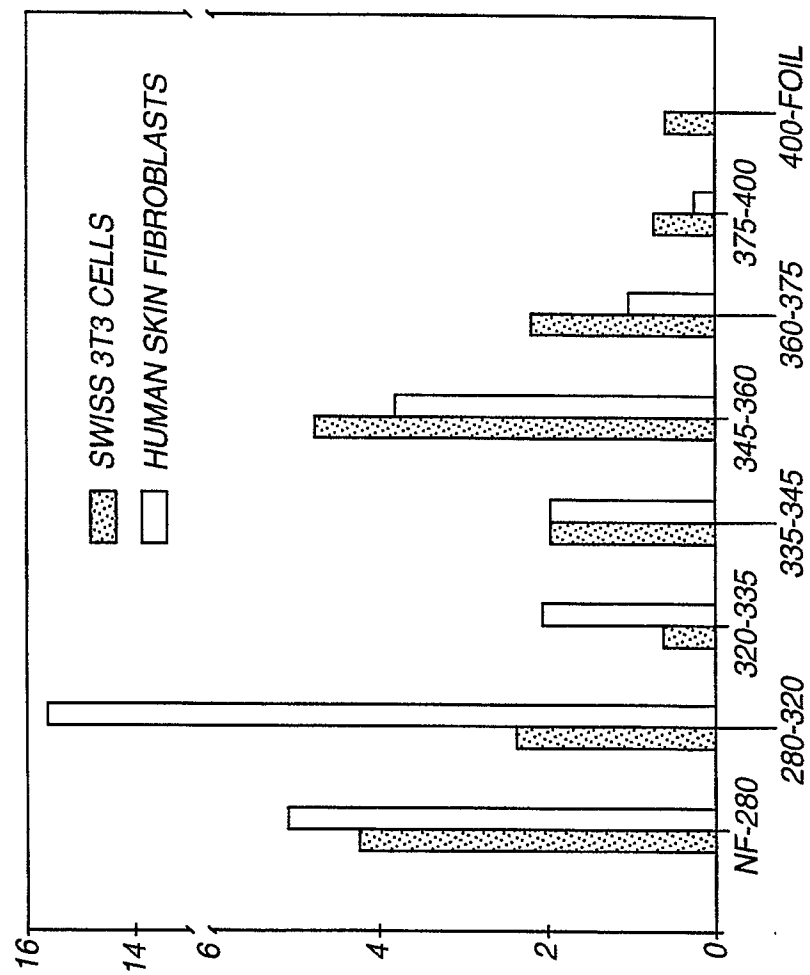

SWISS 3T3 CELLS
HUMAN SKIN FIBROBLASTS
RELATIVE LPO
16
14
6
4
2
0
NF-280
280-320
320-335
335-345
345-360
360-375
375-400
400-FOIL
FILTER

FIG. 8

FIG. 9

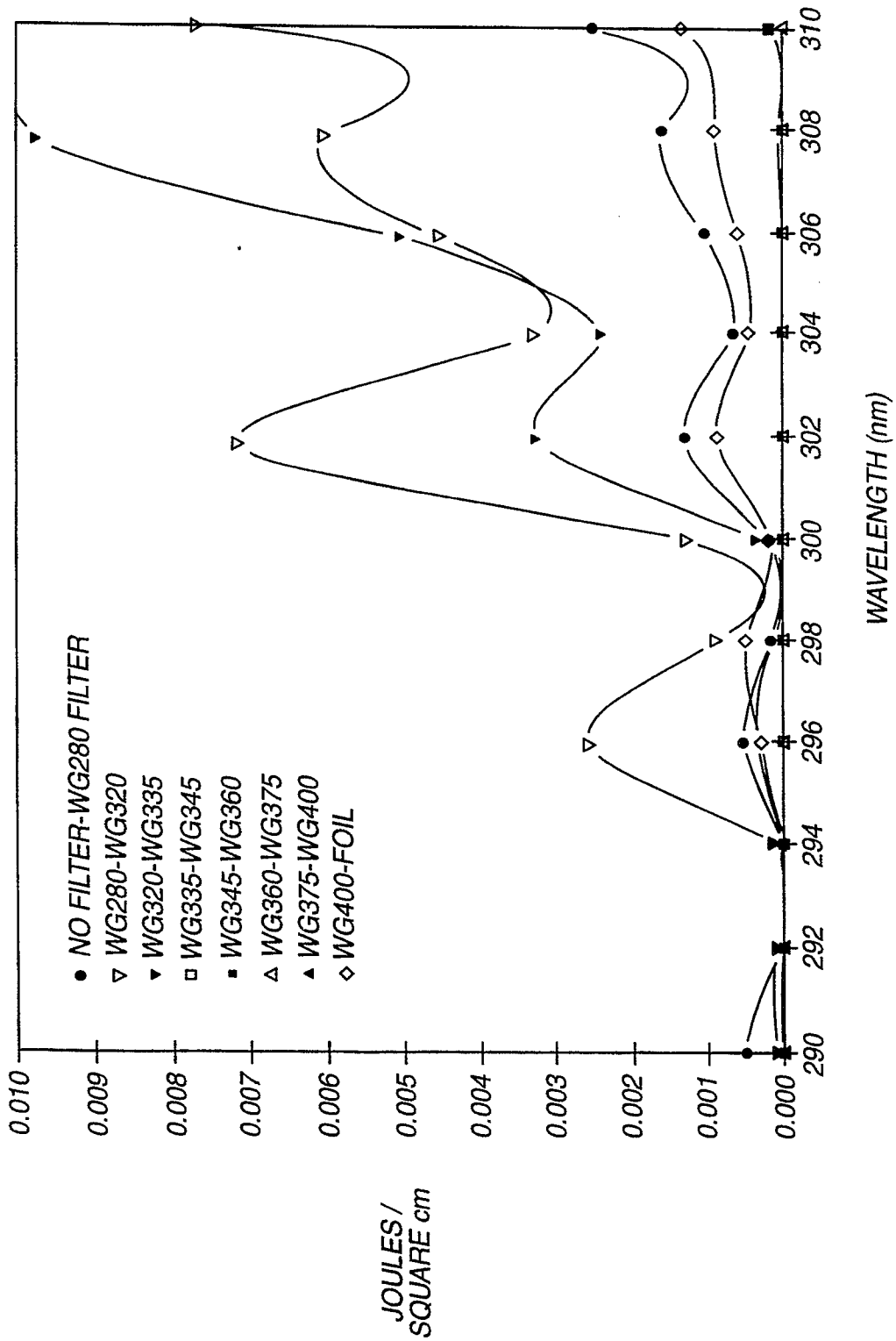

NO FILTER-WG280 FILTER
WG280-WG320
WG320-WG335
WG335-WG345
WG345-WG360
WG360-WG375
WG375-WG400
WG400-FOIL
JOULES / SQUARE cm
0.010
0.009
0.008
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0.000
290
292
294
296
298
300
302
304
306
308
310
WAVELENGTH (nm)

FIG. 10

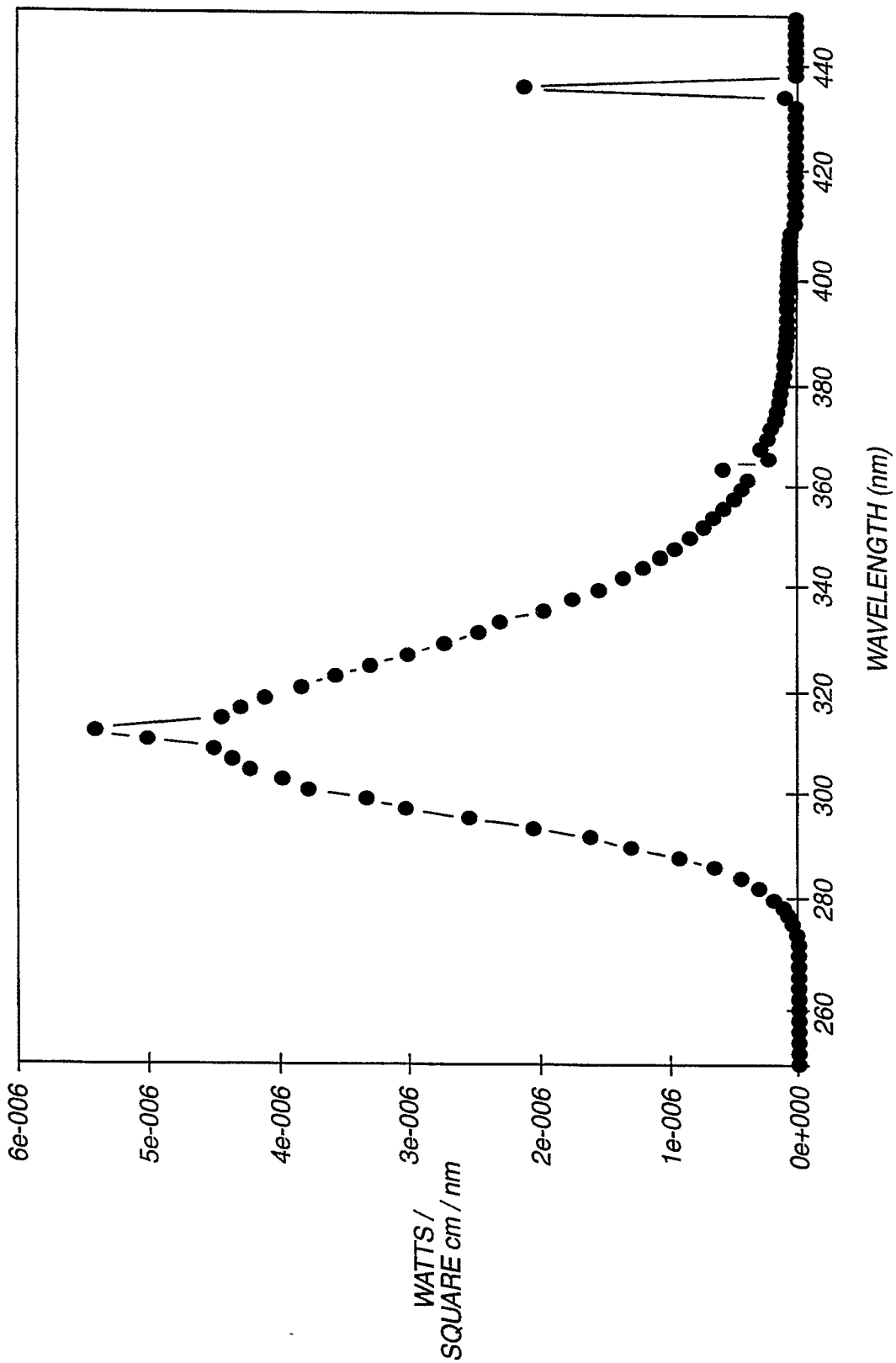
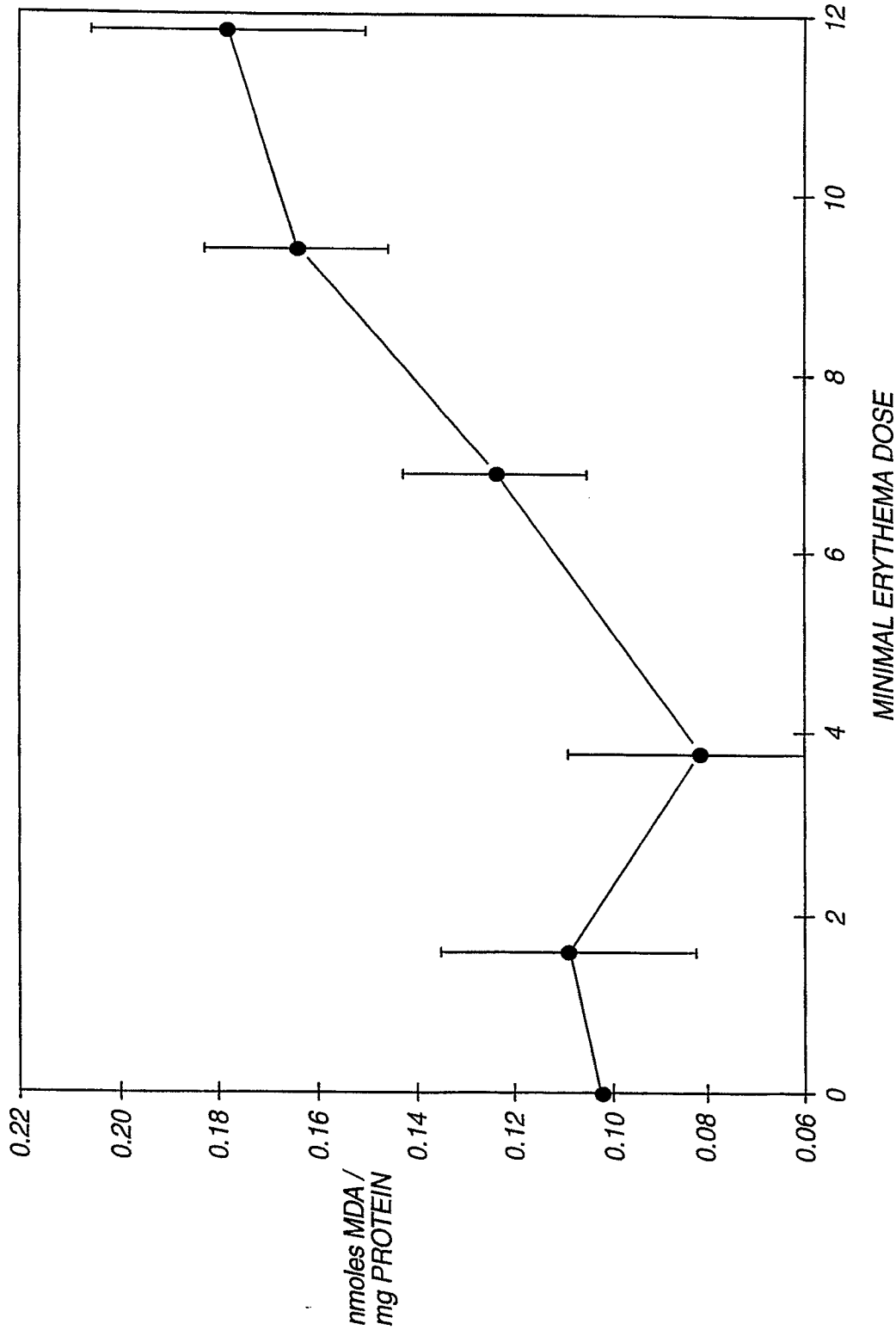

6e-006
5e-006
4e-006
3e-006
2e-006
1e-006
0e+000
WATTS / SQUARE cm / nm
260
280
300
320
340
360
380
400
420
440
WAVELENGTH (nm)

FIG. 11


nmoles MDA / mg PROTEIN
0.22
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0
2
4
6
8
10
12
MINIMAL ERYTHEMA DOSE

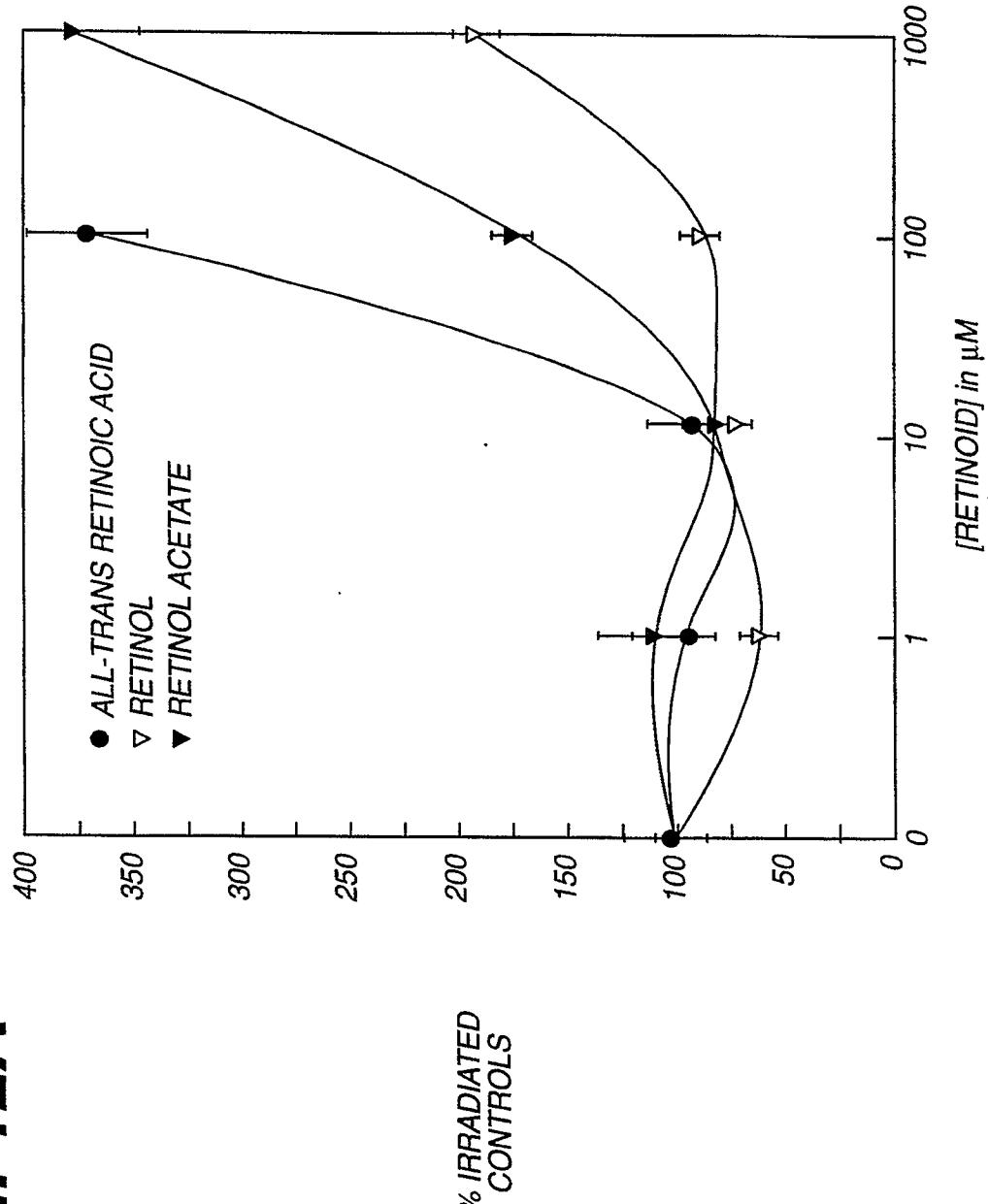

● ALL-TRANS RETINOIC ACID
▽ RETINOL
▼ RETINOL ACETATE
% IRRADIATED CONTROLS
400
350
300
250
200
150
100
50
0
0
1
10
100
1000
[RETINOID] in µM

FIG. 12A

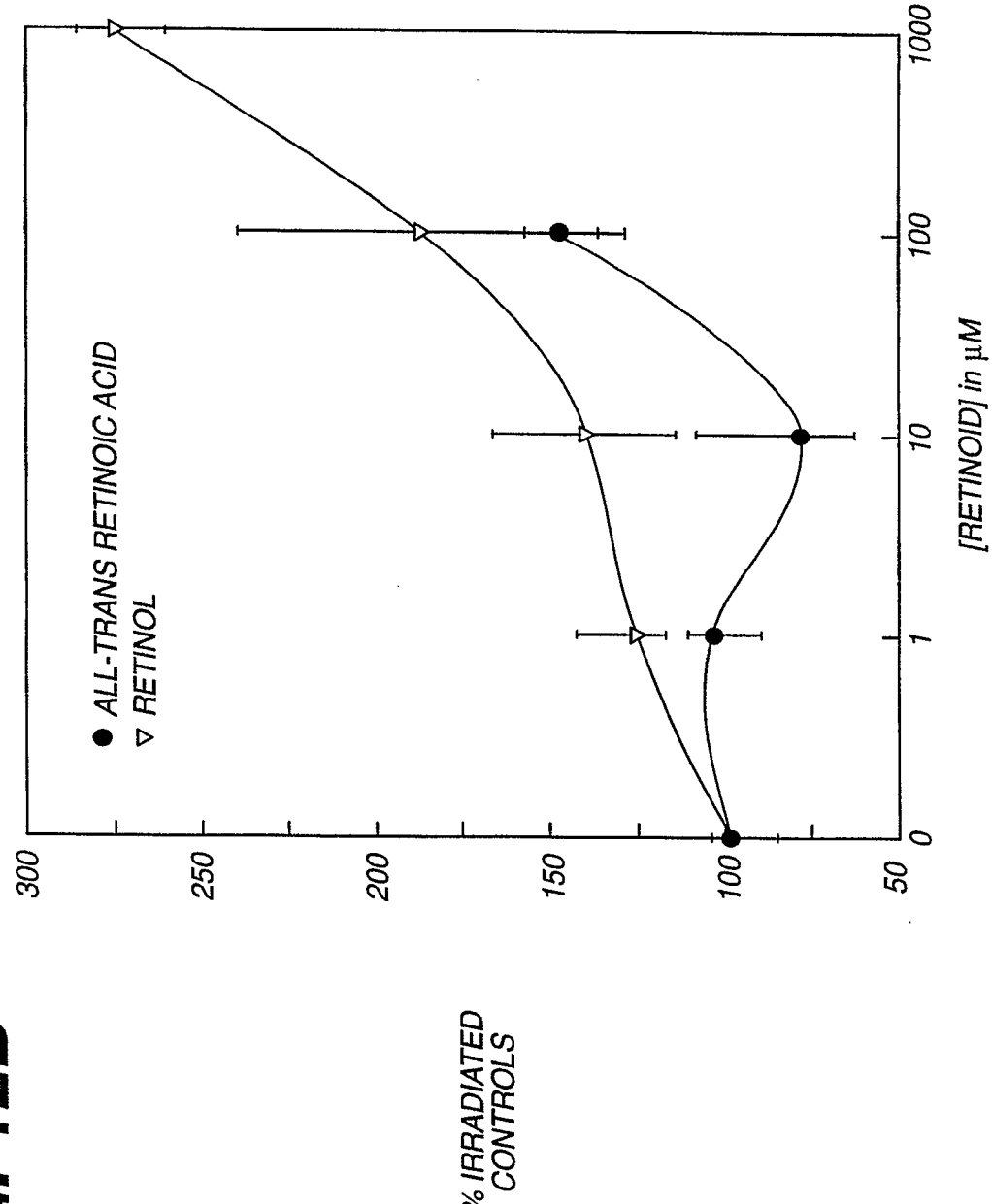
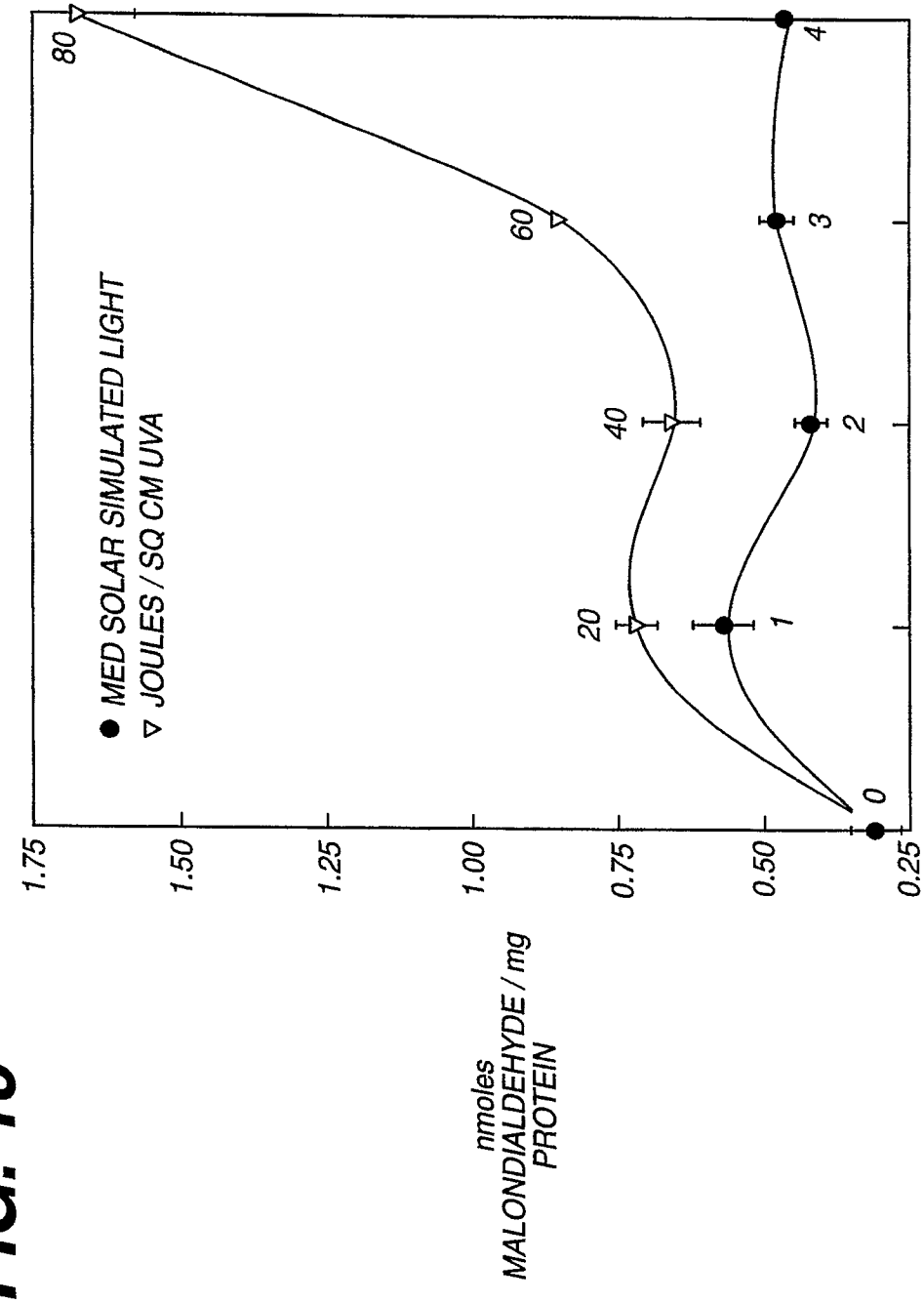

● ALL-TRANS RETINOIC ACID
▽ RETINOL
% IRRADIATED CONTROLS
300
250
200
150
100
50
0
1
10
100
1000
[RETINOID] in µM

FIG. 12B

FIG. 13


MED SOLAR SIMULATED LIGHT
JOULES / SQ CM UVA
nmoles MALONDIALDEHYDE / mg PROTEIN
1.75
1.50
1.25
1.00
0.75
0.50
0.25
80
60
40
20
0
4
3
2
1

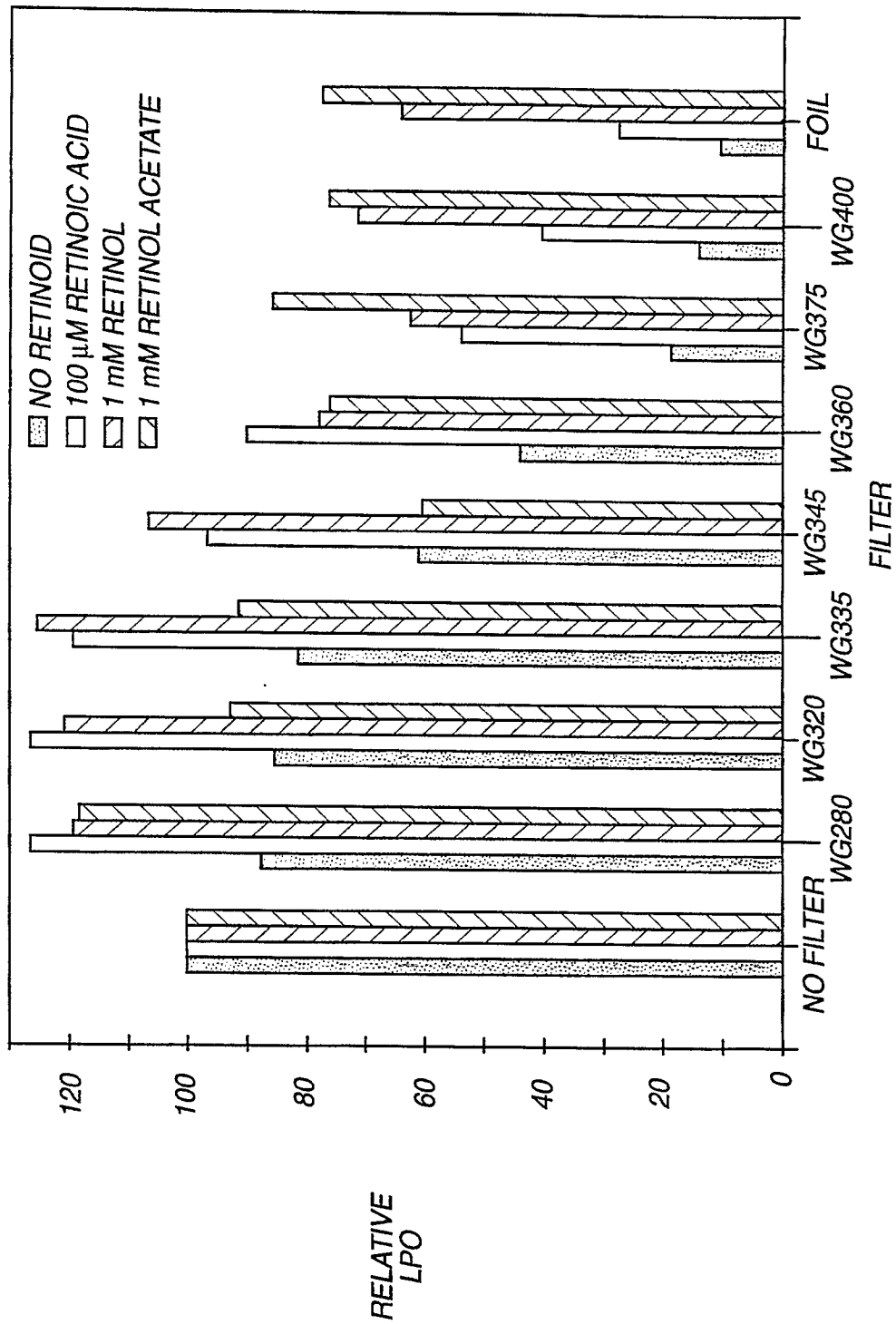

NO RETINOID
100 µM RETINOIC ACID
1 mM RETINOL
1 mM RETINOL ACETATE
RELATIVE LPO
120
100
80
60
40
20
0
NO FILTER
WG280
WG320
WG335
WG345
WG360
WG375
WG400
FOIL
FILTER

FIG. 14

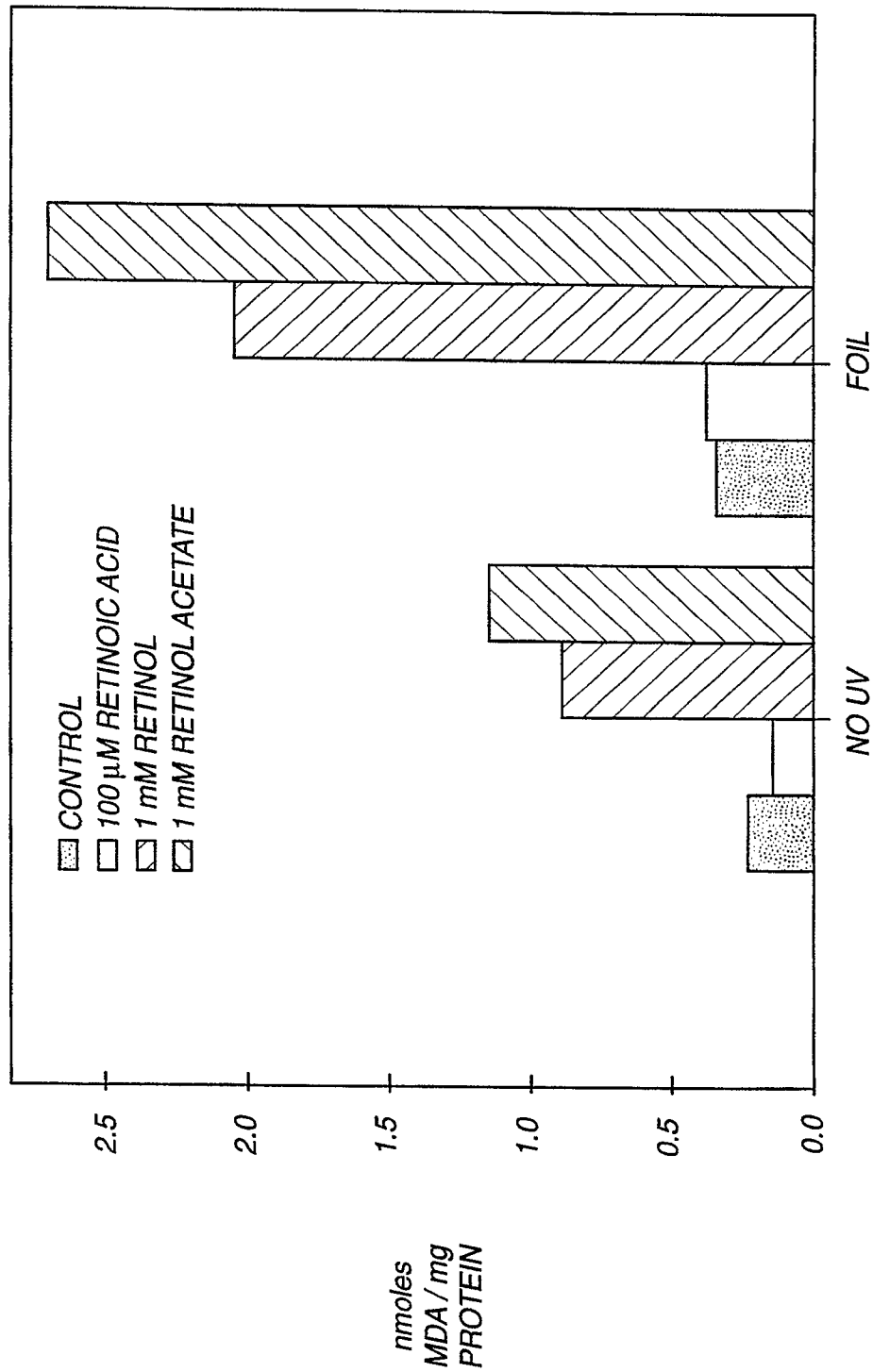

CONTROL
100 µM RETINOIC ACID
1 mM RETINOL
1 mM RETINOL ACETATE
FOIL
NO UV
2.5
2.0
1.5
1.0
0.5
0.0
nmoles MDA / mg PROTEIN

FIG. 15

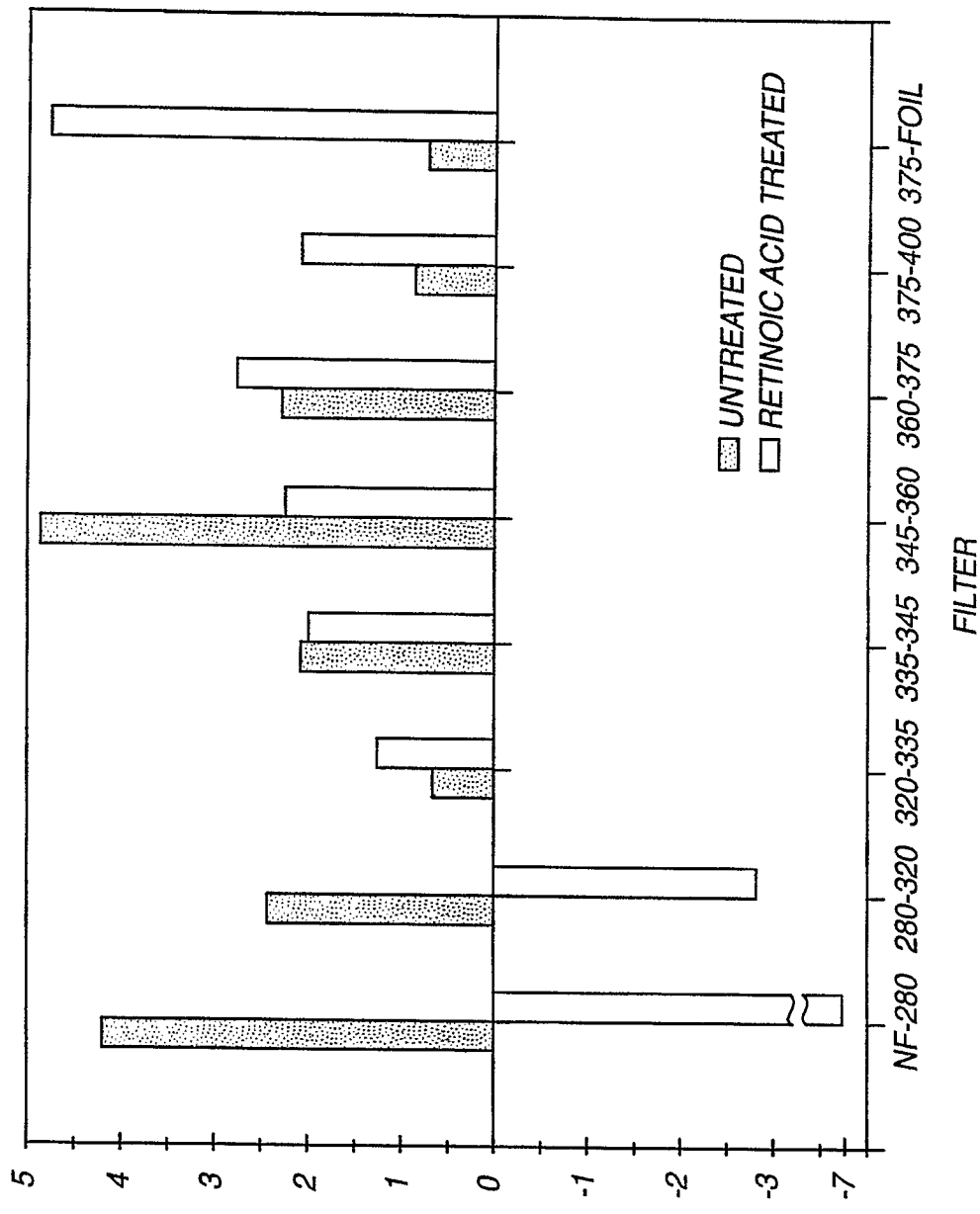

RELATIVE LPO / UNIT ENERGY
5
4
3
2
1
0
-1
-2
-3
-7
UNTREATED
RETINOIC ACID TREATED
NF-280
280-320
320-335
335-345
345-360
360-375
375-400
375-FOIL
FILTER

FIG. 16

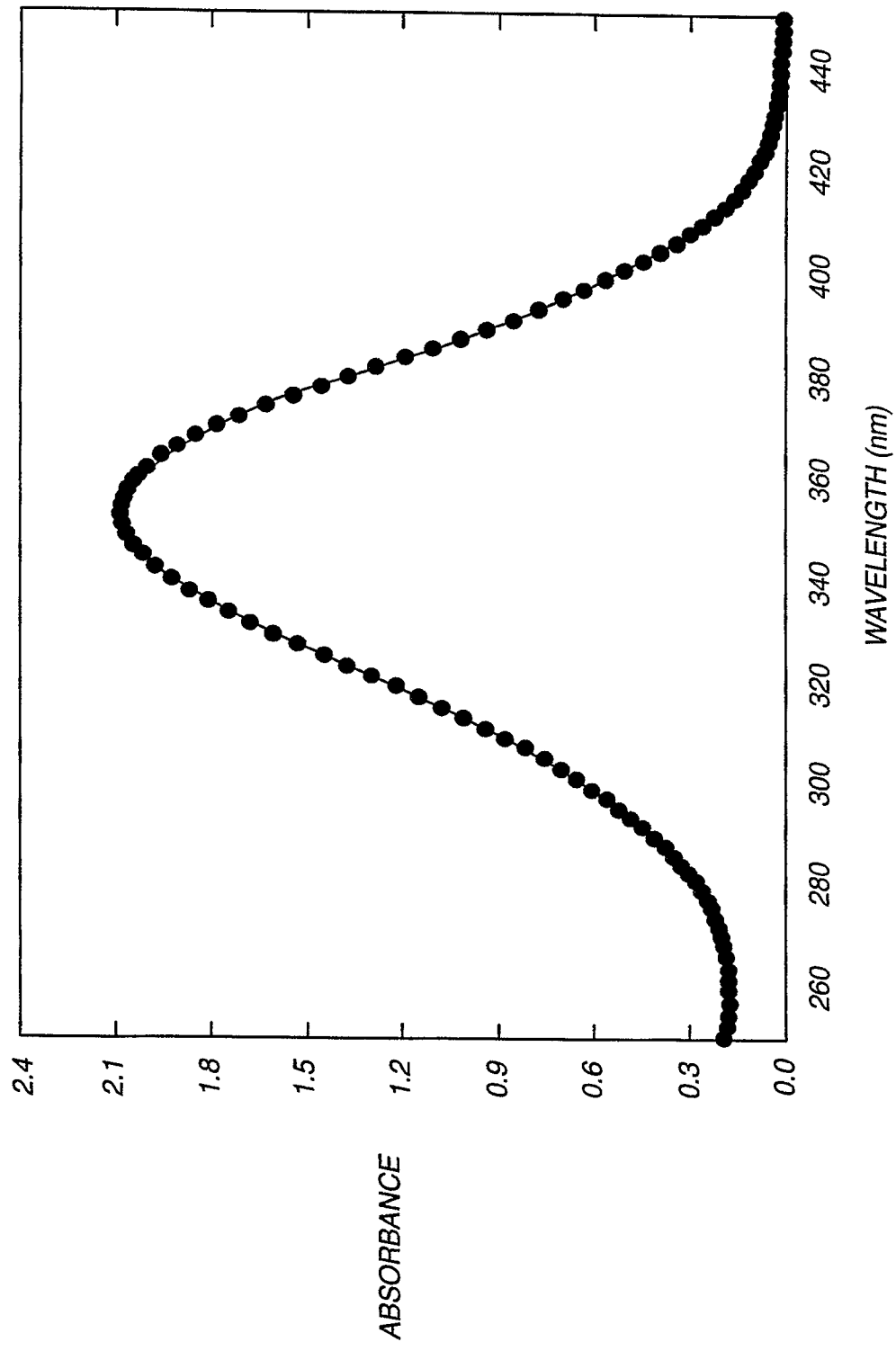

ABSORBANCE
2.4
2.1
1.8
1.5
1.2
0.9
0.6
0.3
0.0
WAVELENGTH (nm)
260
280
300
320
340
360
380
400
420
440

FIG. 17

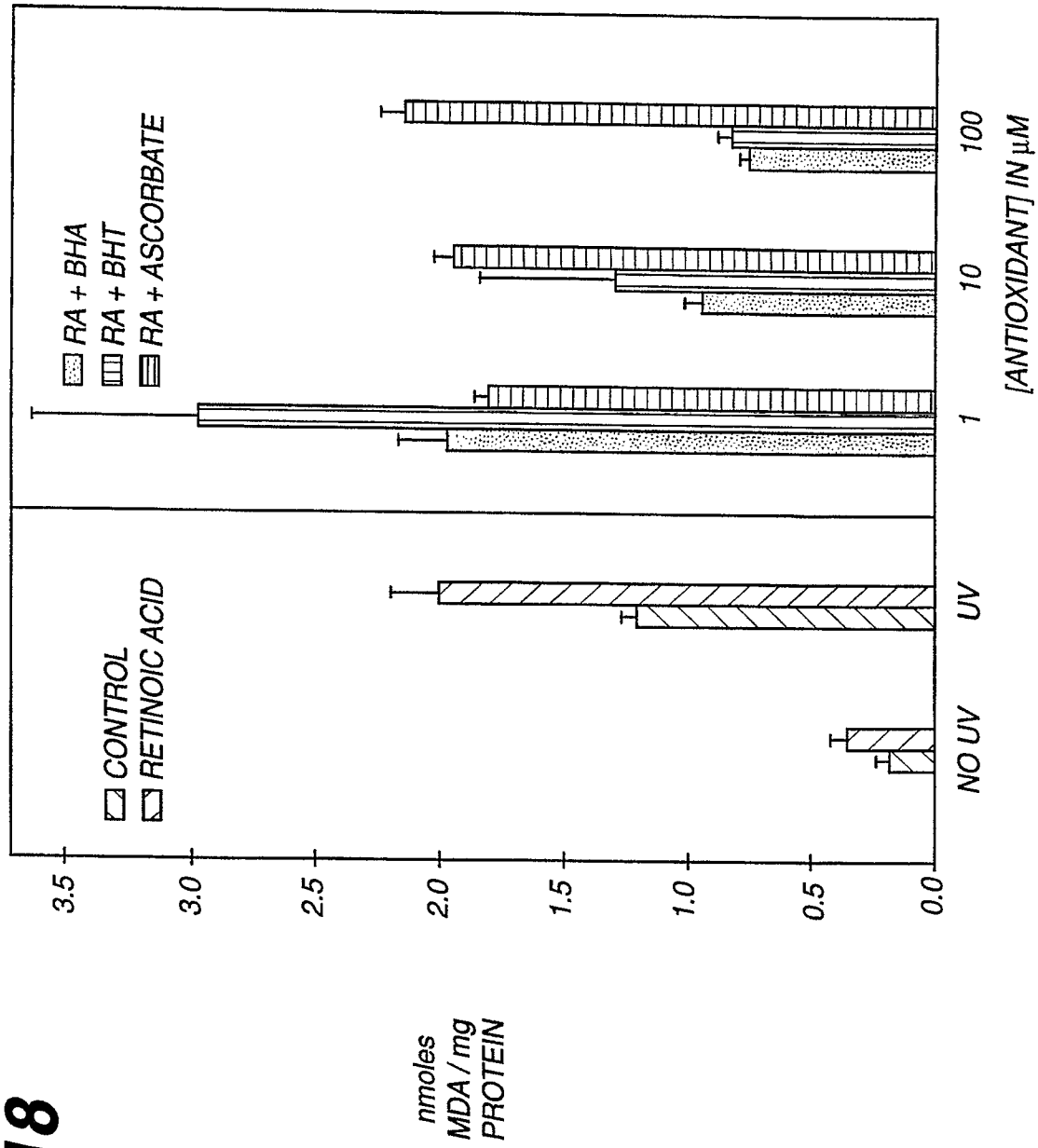

CONTROL
RETINOIC ACID
RA + BHA
RA + BHT
RA + ASCORBATE
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
nmoles MDA / mg PROTEIN
NO UV
UV
1
10
100
[ANTIOXIDANT] IN μM

FIG. 18