

DNA Damage Action Spectroscopy and DNA Repair in Intact Organisms:  
Alfalfa Seedlings\*

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## INTRODUCTION

Evaluating the effects of increased UVB (290-320 nm) from stratospheric ozone depletion requires knowing the biological effects of the wavelengths which will be increased as well as the effects of other damaging wavelengths. Since UVB radiation is absorbed much more effectively by DNA than UVA (320-400 nm), UVB has long been considered the major biologically damaging radiation in sunlight.

More recently, direct and indirect damaging effects of UVA on DNA have been found, but generally at efficiencies per photon of less than 1% that of UVB. Can such low efficiency processes be biologically significant? The answer lies in two factors: first, the number of DNA lesions induced by a specific wavelength of radiation is the **product** of the efficiency with which photons of that wavelength induce the damage **times** the number of photons of that wavelength which are absorbed by the target DNA. Second, in sunlight at the earth's surface, the number of UVA photons is far greater than the number of UVB photons. Thus the total DNA damage inflicted by UVA--the sum over all UVA wavelengths of the **products** of damage efficiency at given wavelength times the number of photons at that wavelength--is much larger compared to UVB-induced damage than would be anticipated based on the efficiencies alone.

## ACTION SPECTROSCOPY OF DNA DAMAGE

We can measure the distribution of UVA and UVB photons in today's solar spectra, and can predict the spectra for various scenarios of differing degrees of ozone depletion. Thus we require "only" the efficiency measurements (**action spectra**) for DNA lesion induction to calculate the burden of damages inflicted by today's spectrum and by any predicted spectrum

of interest. We have said "only" with regard to obtaining action spectra because of the many pitfalls that must be avoided, and the immense amount of work required for obtaining good, statistically reliable data at a sufficient number of wavelengths for determination of an accurate and useful action spectrum.

What problems make action spectrum measurements so difficult? A complete treatment of action spectroscopy is beyond the scope of this review, and the reader is referred to the chapter by Thomas Coohill in this volume, and to the book of John Jagger (3). However, special problems likely to be encountered in action spectrum measurements on intact organisms are particularly pertinent to problems of ozone depletion, and we shall discuss the most important of them.

#### Action Spectroscopy in Intact Organisms

Three critical issues that must be addressed to measure accurate action spectra are 1. selection of suitable sources of monochromatic radiation covering the desired wavelength range with sufficient intensity over a large enough area to irradiate the desired organism(s), 2. design of the experiments, and 3. analysis of data. Each of these points presents pitfalls to the unwary, especially in studying intact organisms.

We must first distinguish "total effect studies," in which target organisms are irradiated with a broad spectrum source approximating a total environmental condition, from analytical action spectra in which the efficiencies of individual monochromatic or very narrow band radiation are determined for a number of wavelengths. In the latter case, the product of the efficiency at different wavelengths and photon intensity can be obtained for any desired broad

spectrum source, including sunlight, by convolution of the intensity of the spectrum or "spectral irradiance" and the efficiency of damage at each wavelength in that spectrum.

## Radiation

To determine an analytical action spectrum, we must have a light source that can produce sufficient radiation of uniform intensity over the entire specimen area (dish/plant/animal) at each wavelength of interest to induce a measurable effect in a finite time. For most biological systems, this implies a high intensity lamp (Hg or Hg/Xe), grating or prism monochromator and photometer calibrated at each desired wavelength to allow quantitation of the incident photon flux. A particular problem in measuring effects at wavelength regions of low efficiency is that the presence of a very small (1% or less) quantity of shorter wavelength, high damaging efficiency radiation can alter significantly the observed effect. The use of short wavelength cutoff filters and bandpass filters at such wavelengths, for example the UVA, can be used to prevent such problems(3-5).

Interval Between Wavelengths. On first consideration of carrying out a convolution of DNA damage data with a continuous source such as sunlight, one might think that DNA action spectra should be carried out to similar resolution. However, consideration of the absorption spectra of DNA and other biological macromolecules in solution indicates that this is unnecessary. First, consider the absorption of gas atoms: they show very strong absorption lines over tenths of nanometers. Thus, if one were determining the action spectrum for excitation of such a gas, one would have to measure excitation at very closely spaced wavelength intervals or risk missing

large absorptions. However, biological molecules have broad absorption spectra, with smooth envelopes. The absence of fine structure in the UV absorption spectra of biological molecules indicates that the action spectra of these molecules will also be a smooth function. (See pp. 15-17 of Jagger(3) for an excellent discussion.) The properties of biological action spectra which are important to determine are the wavelength maximum of the spectrum, and the magnitudes--the absolute magnitudes if possible, the relative values if the absolute magnitudes cannot be determined--of the effect at several wavelengths in the region of interest.

Light Sources. High pressure mercury arcs offer several useful lines in the ultraviolet, including 275, 280, and 289 nm in the UVC region, 295, 302, and 313 nm in the UVB and 334, 365 and 385 nm in the UVA, as well as 405 nm in the short wavelength visible. Mercury-Xenon arcs supplement the inter-line intervals and allow more flexible choices of wavelength regions. It must be stressed that just because one has set a monochromator to a particular wavelength does not mean that wavelength is the only, or even the principal biologically effective radiation emerging from the system; scattered or stray light, or light from a strong nearby line may comprise the principal biologically effective radiation although its presence is not revealed by the monochromator setting.

## Experimental Design

Reciprocity. In action spectroscopy, preliminary experiments are essential to indicate the light flux and, in practical terms, the time required to obtain a measurable biological effect. Suppose

that the time required is 20 seconds at 275 nm and 200 minutes at 365 nm (in our example, 275 nm is absorbed much more efficiently than 365 nm). How will we know that the biological damage we measure at 365 nm isn't artificially decreased by repair which occurs during the 200 minutes of irradiation? There are two approaches to solving this problem: first, we can establish whether time and fluence reciprocity holds. That is, will the same number of photons administered over the entire range of times to be used in the experiment give the same result? If so, reciprocity holds, and results obtained using the exposure durations within the range we tested are valid. Time-intensity reciprocity is found to hold in many, but not all, purely photochemical reactions. Figure 1 shows a demonstration of reciprocity for pyrimidine dimer formation in alfalfa seedlings. The solid symbols represent dimer yields in plants irradiated at 275 nm at a rate of 0.3 mW/cm<sup>2</sup>, while the open symbols show data for plants irradiated at 1.1 mW/cm<sup>2</sup>, about 3.7 times as high a rate. Clearly, over the time spans required for these irradiations, the exposure-response lines for the two exposure rates are indistinguishable and reciprocity holds for these plants under these experimental conditions(6, 7).

In many biological systems, reciprocity does not hold. For example, if one is measuring DNA photoproduct formation in a DNA repair-proficient organism, repair enzymes might remove many lesions during a 200 minute irradiation at 365 nm (a relatively inefficient wavelength in damaging DNA, thus requiring long irradiation times) but very few during a 20 second irradiation at 275 nm, which is quite efficient in lesion induction. We would thus underestimate the efficiency of 365 nm in damaging DNA. In addition, many organisms carry out photorepair, in which a photoreactivating enzyme uses light to reverse UV-induced cyclobutyl pyrimidine dimers in DNA; during the long 365 nm exposure in our example, the

organism could photoreactivate many dimers, again leading to an underestimate of the damaging effect of 365 nm radiation.

One solution would be to use equal times of irradiation for all wavelengths; this will should allow equal action of all non-light-dependent processes, such as excision repair. Photorepair might be avoided by irradiating chilled organisms (to decrease the rate of formation of enzyme-substrate complexes). However, it is usually desirable to produce comparable damage levels at all wavelengths studied in an action spectrum, and the very great differences in the efficiencies of different wavelengths in producing damage mean that it is usually difficult to produce a given level of damage in the UVA with the same irradiation times as are possible with irradiation in the UVB. Extending the irradiation times in the UVB to match those in the UVA can result in extremely long experiments! Thus, one may simply have to take into account such limitations in interpreting the data.

Fluence-response relations. Once preliminary experiments indicate suitable conditions for obtaining measurable effects, one must determine the effect produced by increasing photon exposures at each wavelength, the "dose-response" or fluence-response lines. For constructing an action spectrum that can be used to predict the level of damage produced by broad spectrum UV radiation, the effect should ideally be a linear function of the exposure of UV at each wavelength. The slopes of these exposure-response lines, in units of effect/photon/area, are then used to construct the action spectrum.

Why is it important to determine fluence-response lines--why not just determine the effect of one exposure at each wavelength, calculate the photon exposure required to induce a constant

effect (e.g., 37% killing; 50% substrate conversion) at each of those wavelengths? The shape of the line is just as important as the slope! If the shapes of the lines are different at the different wavelength regions, this indicates that the biological processes involved in the irradiations at the different wavebands were different, and it is not correct to construct a single "action spectrum" for the biological effect across the entire wavelength range.

What if the fluence-response lines are not linear, but show a sigmoid shape? An example is the sigmoid shape of the exposure-response function of transformation of normal, repair-proficient human cells by UV(9, 10). At low photon exposures, almost no transformants are observed, presumably due to efficient repair of virtually all the lesions. As the exposure increases, however, transformants are detected in increasing frequency. In this case, the use of slope of the fluence-response line is not valid, but the use of a constant level of biological effect (e.g., transformants per survivor/photon/m<sup>2</sup>) allows computation of action spectra for human cell neoplastic transformation(9, 10).

### Calculation of Action Spectra

After obtaining linear fluence-response lines at the different wavelengths, one is ready to calculate an action spectrum. Either the slope of the lines (effect/photon/area) or the reciprocal of the photon flux required to achieve a constant biological effect [ $1/(\text{photon/area/effect})$ , which yields the same effect/photon/area] is plotted as a function of wavelength. Although measurements of photon exposure are sometimes made with instruments whose output reads in units of energy, it is essential, for comparison with absorption spectra, to convert these exposures to number of photons by the relationship  $E = (hc)/\lambda$ , where  $h$  is

Planck's constant,  $c$  is the speed of light, and  $\lambda$  is the wavelength of the incident light. To allow for facile comparison with absorption spectra of suspected target molecules or to allow convolution with broad spectrum sources whose emission at different wavelengths varies over many orders of magnitude, action spectra are most conveniently displayed on a semi-logarithmic scale.

To normalize or not to normalize, that is the question! Frequently one sees comparisons of action spectra in which all the spectra have been normalized to the same value at a particular wavelength. Although this practice allows comparison of spectra for which obtaining absolute cross-sections is not possible (e.g. tumorigenesis), normalization has many pitfalls. Panel A of Figure 2 shows a shallow container of DNA in solution and Panel A of Figure 3 shows the absolute action spectrum for inducing photoproducts in that DNA(6). Panel B of Figure 2 shows the same DNA, but now a filter has been interposed between the light source and the DNA (but the DNA solution is unchanged). The filter is transparent above 320 nm, but absorbs 99% of all radiation less than 320 nm. The action spectrum for damaging the DNA in this case--since we measure the light incident on the entire system, (in this case the filter) not on the DNA surface--is shown in the solid symbols in Figure 3B. The sharp discontinuity in the example results from the sharp change in transmittance of our hypothetical filter.

If these spectra are normalized at a wavelength where there is no shielding by the filter, the relative shapes of the two spectra remain the same, and only the absolute values change. However, if one normalizes at a wavelength where there is significant shielding by cell or tissue components, e.g. 300 nm, the resulting action spectra look very different (remember that the DNA had not changed, only the filter was added)! Panel C of Figure 3 shows the result of

normalizing the spectra in Panel B of Figure 3 at a wavelength where there is significant shielding. One would conclude from these normalized spectrum that the effect in the longer wavelength region was abnormally high, when in fact, it was invariant! Thus, arbitrary sharp can lead to highly misleading comparisons!

A second disadvantage in normalizing action spectra is that quantitative comparisons between different systems, which relate ultimately to differing levels of biological sensitivity, are lost upon normalization. Figure 4 shows the absolute action spectra for inducing pyrimidine dimers DNA in solution(6) and for inducing dimers in the DNA of two intact organisms, human skin *in situ*(2) and intact alfalfa seedlings(6). The absolute action spectra clearly show that DNA in solution is about 100 times more sensitive in the UVB region than DNA in either human skin or in seedlings.

#### Action Spectra in Prediction of Effects of Ozone Depletion

What information does the alfalfa action spectrum provide for evaluating the effects of environmental light on plants? First, the spectrum indicates that radiation throughout the region 270-365 nm induces pyrimidine dimers in alfalfa DNA(6). We could not detect any significant level of dimers over background at either 385 or 405 nm. The absolute efficiency of photons in the UVA region in damaging DNA is approximately  $10^{-4}$  that of photons in the UVB region.

How, then, can such a low efficiency process be of biological significance? The bold solid line in Figure 4 shows a spectrum for solar radiation reaching the surface of the earth under today's conditions of stratospheric ozone at a particular latitude, time of year and time of day(8). The amount of damage inflicted by a broad waveband, such as the UV present in

sunlight, is the product of the efficiency of each wavelength for producing damage within that waveband in damaging the biological system times the number of photons at each wavelength within the waveband, summed over all wavelengths in that waveband. Examination of Figure 4 shows that although UVB is quite effective in damaging DNA in the alfalfa seedlings, there is very little UVB in today's solar spectrum; conversely, although the "per photon" efficiency of UVA radiation is small, sunlight contains very large quantities of UVA radiation. This indicates that both UVA and UVB damage DNA in living organisms.

What will be the impact of ozone depletion on DNA damage to alfalfa? The bold dashed line in Figure 4 shows a spectrum for radiation reaching the earth's surface under conditions of 50% depletion of stratospheric ozone under the same conditions of latitude, *etc.* as the bold solid line(8). More UVB radiation will reach the earth's surface, and thus the plants will suffer increased DNA damage; however, the quantity of UVA is largely unchanged and the amount of damage from this wavelength region will remain constant. These data indicate that ozone depletion would indeed result in more DNA damage in the seedlings. Since a significant portion of today's damage is inflicted by UVA (and that radiation will remain constant), an increase in UVB will not have as large a detrimental effect on living plants as if UVB were the only damaging radiation. In addition, the effect of the increased UVB compared to the constant UVA is modulated by the greater attenuation of UVB by the plant; that is, the cross section for UVB damaging the plant's DNA is reduced compared to the ability of UVB to damage unshielded DNA. In contrast, for most of the UVA the response of DNA in the plant is much closer to that of unshielded DNA.

Many cellular components can be damaged by UV. Why should the level of damage to

DNA be of especial concern? DNA damages can block DNA replication, and if replication does proceed in the presence of damage, mutations may be produced. Even if cells are not actively dividing, DNA damage can have serious consequences: DNA lesions can block transcription, or produce decreased quantities of messenger RNAs for critical cellular proteins. Since some important cellular proteins are thought to be damaged by UV, their replacement by would be essential for cellular function, and UV lesions within their genes would have serious cellular consequences. Finally, even if a messenger RNA coding for an cellular RNA were long lived, some tRNAs are inactivated by UV, and synthesis of their replacements may be essential for continued protein synthesis.

## DNA REPAIR IN INTACT ORGANISMS

Organisms have evolved in the presence of solar radiation, and are coping adequately with stresses inflicted by environmental UV. Most organisms have mechanisms for removing DNA damages, and the overwhelming majority have multiple, at least partially overlapping systems for repairing DNA. Two repair paths that are prominent in most organisms include excision and photorepair.

### DNA Repair Paths

Excision is carried out by a multi-enzyme pathway, and generally can remove many kinds of lesions, especially bulky ones, from DNA. Sequentially or concomitantly, new synthesis using the undamaged strand as template restores the base sequence, and finally, ligation restores the integrity of the DNA backbone. Excision is thus flexible, carrying out repair of many types of

lesions; on the other hand, it requires cellular energy and poses the possibility of errors during resynthesis that can lead to mutation.

Photoreactivation is carried out by a single enzyme pathway, repairs cyclobutyl pyrimidine dimers, and uses longer wavelength UV or visible light as the sole required energy source. It is thus less flexible, with only one class of lesions as substrate, but uses light as an energy source, not cellular energy (and is thus well suited to organisms exposed to sunlight), and is error-free.

#### Repair in Alfalfa

What repair paths does alfalfa use for dealing with UV damages? Figure 5 shows the fate of pyrimidine dimers induced at a low exposure of 280 nm radiation to the seedlings, producing an initial level of about 8 dimers/million bases. (In comparison, 1 J/m<sup>2</sup> of 254 nm radiation produces about 6.5 dimers/million bases in unshielded DNA.) After UV exposure, the seedlings were kept in the dark or exposed to blue light filtered by a yellow filter which excluded wavelengths shorter than about 405 nm (thus preventing DNA damage by the photoreactivating light). Seedlings exposed to this level of UV actively carry out photorepair (▲), but do not seem to remove dimers by excision (●) within the times examined. The relative roles of photorepair and of excision at other exposures, or of damages induced by other waveband sources, remains to be determined.

#### CONCLUSIONS

Understanding the effects of UV, and increased levels of UV, on DNA in living

organisms requires knowledge of both the frequencies of damages induced by the quantities and quality (wavelength composition) of the damaging radiation, and of the capacity of the organism to carry out efficient and accurate repair. The major levels of uncertainty in understanding the responses of intact organisms, both plant and animal, to UV indicates that we cannot assess accurately the impact of stratospheric ozone depletion without major increases in knowledge of DNA damage and repair.

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## Figure Legends

Figure 1. Pyrimidine dimer yields induced in alfalfa seedlings exposed to 275 nm radiation at an exposure rate of 1.1 mW/cm<sup>2</sup> (open symbols) or 0.3 mW/cm<sup>2</sup> (closed symbols). Seedlings were harvested, minced, embedded in agarose and digested with Proteinase K. Companion samples were incubated with or without saturating levels of a pyrimidine-specific endonuclease which makes a single strand nick adjacent to each pyrimidine dimer. the pyrimidine dimer content was determined by an electrophoretic gel assay (1, 7). The DNAs were denatured, separated along with DNAs of known size according to single strand molecular length on alkaline agarose gels; the gel was renatured, stained with ethidium bromide and a quantitative electronic image obtained (11). The number average molecular length of each DNA sample was determined and the pyrimidine dimer content determined as previously described(7). The exposure-response functions for the two exposure rates are indistinguishable, indicating that time-exposure rate reciprocity holds.

Figure 2. Irradiation of DNA in solution without (Panel A) and with (Panel B) an intervening filter. Panel A shows a solution of DNA (hatched lines) exposed to broad spectrum UV radiation from above. Panel B shows the same DNA solution exposed to the same broad spectrum source, but now with an intervening filter which absorbs 99% of all radiation shorter than 320 nm. This situation models the difference between measurements of damage directly in isolated molecules and in the same molecules in intact organisms, in which the molecules are shielded by cellular and tissue components (*e.g.*, skin, cell walls, cell pigments).

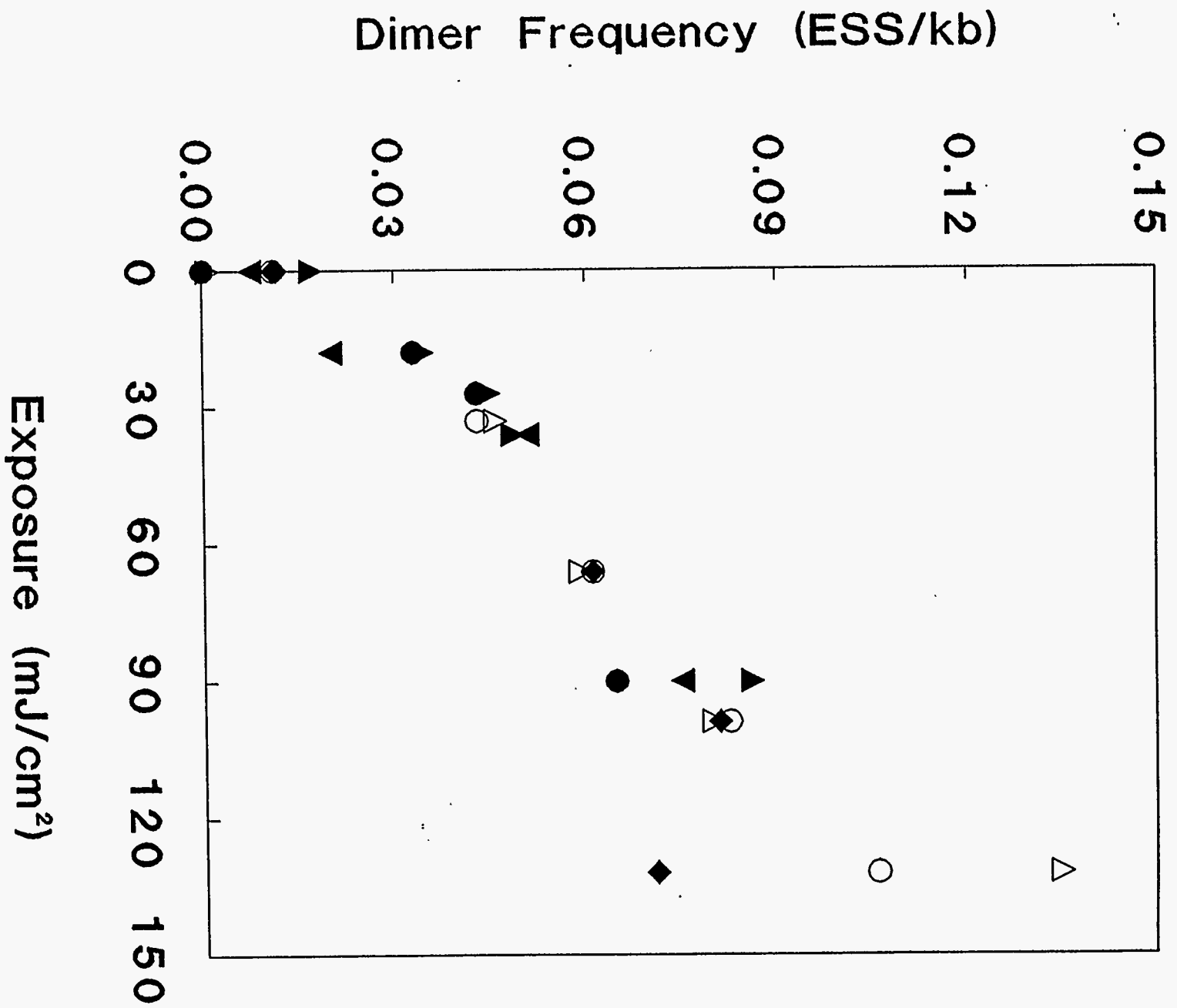
Figure 3. Absolute (non-normalized) and normalized action spectra for damaging DNA irradiated as in Figure 2. Panel A. An absolute action spectrum for damaging DNA irradiated in solution, as shown in Figure 2A. Panel B. Absolute action spectrum calculated for DNA shielded by the filter shown in Figure 2B (●)(6), compared with that for unshielded DNA (□). The filter absorbs 99% of all radiation below 320 nm, thus reducing the action spectrum by two orders of magnitude in the wavelength range 260-320 nm. Panel C. Action spectra for unshielded DNA (smooth curve) and for shielded DNA (dashed curve) normalized at a wavelength less than 320 nm, where the filter shields the DNA. Note that--although the biological system, in this case the DNA, is unchanged-- normalization using a wavelength where there is significant shielding produces a misleading appearance of excessively high values in data which are in fact not altered.

Figure 4. Absolute action spectra for pyrimidine dimer production in unshielded DNA(6) (○), intact alfalfa seedlings(6) (◆), or in skin of healthy human volunteers(2) (□) (left axis). Since these are absolute action spectra, they can be compared directly and quantitatively without normalization. The bold solid line shows solar spectrum for downward global solar flux at the earth's surface for a solar angle of 40° and 0.32 cm stratospheric ozone, compared with that for 0.16 cm ozone (bold dotted line)(8) (right axis).

Figure 5. Repair in alfalfa seedlings exposed to 280 nm radiation producing about 8 pyrimidine dimers/million bases. Seedlings were then kept in the dark (●) or photoreactivated (▲) (exposed to blue photoreactivating light filtered through a yellow filter which removed light below about

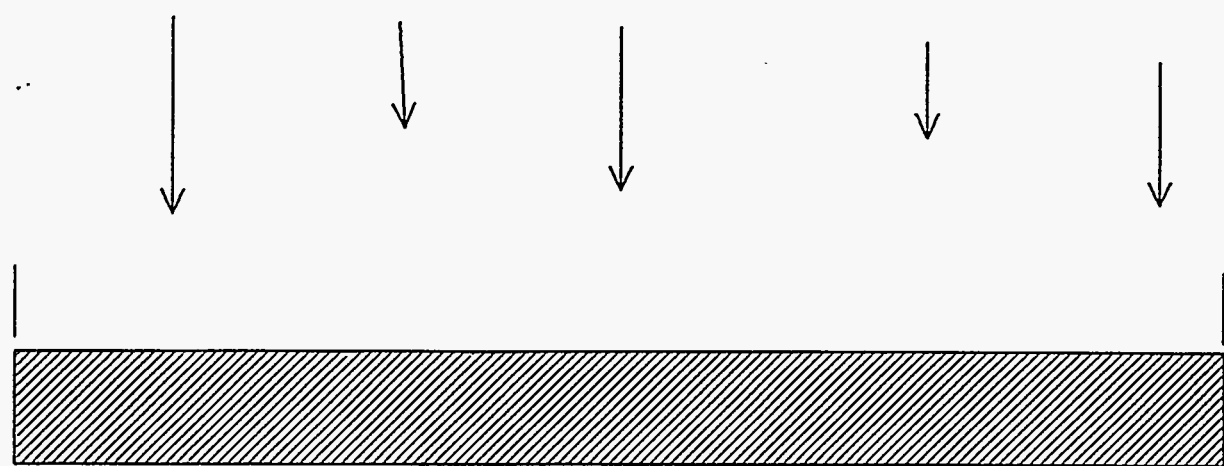
405 nm, thus preventing DNA damage induction during photorepair). Pyrimidine dimer contents were determined as in Figure 1; the lines were fit to the mean of three determinations by least square analysis.

**Fig 1**

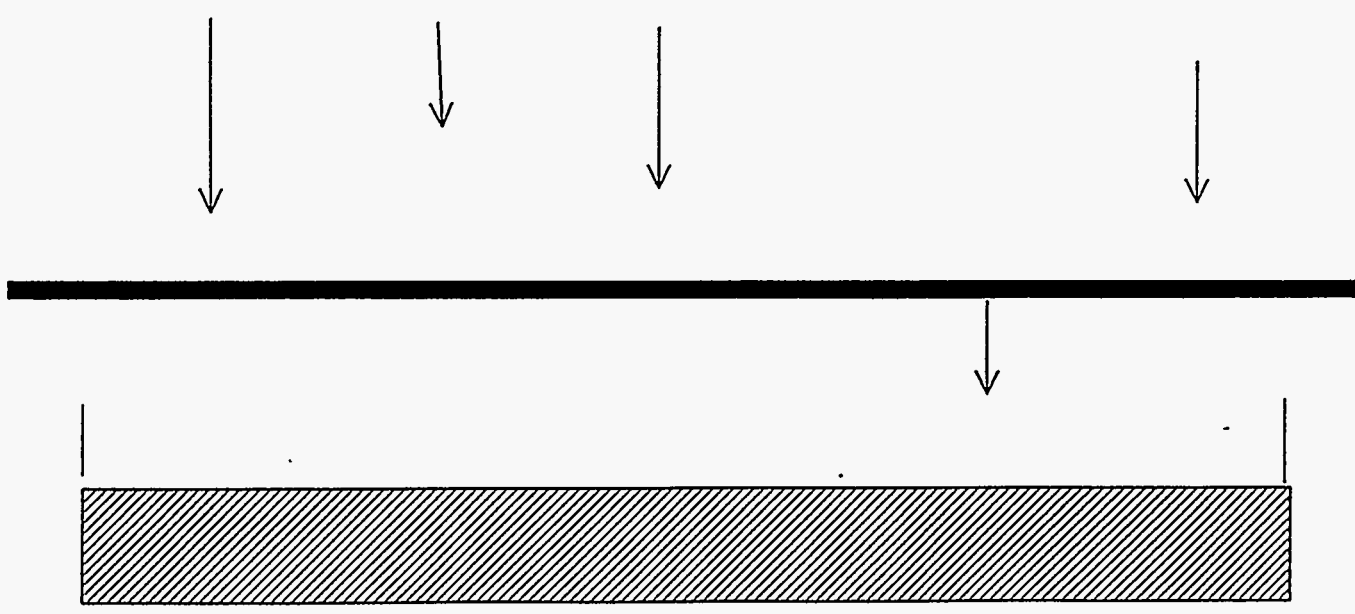


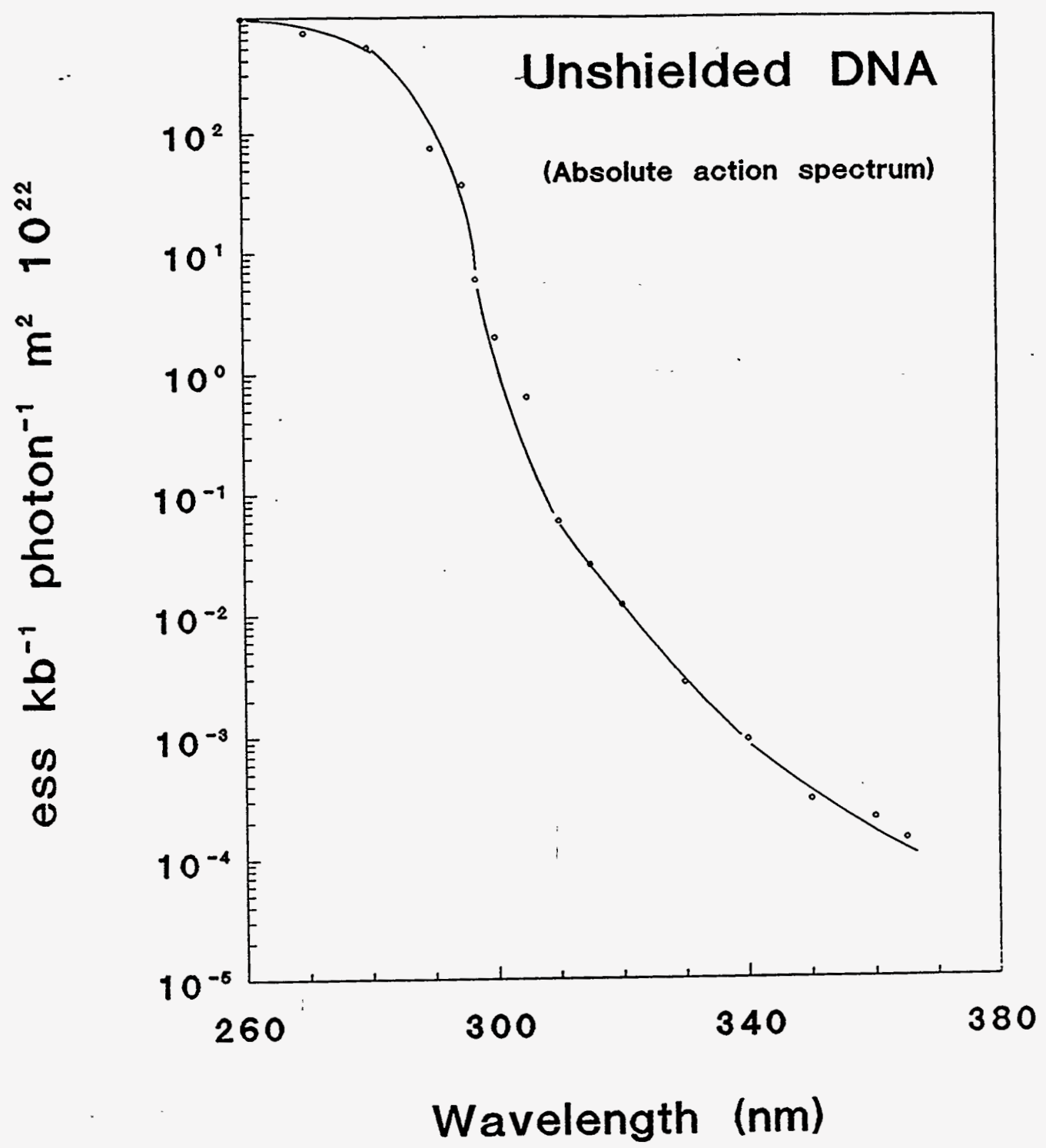
**Fig 2.**

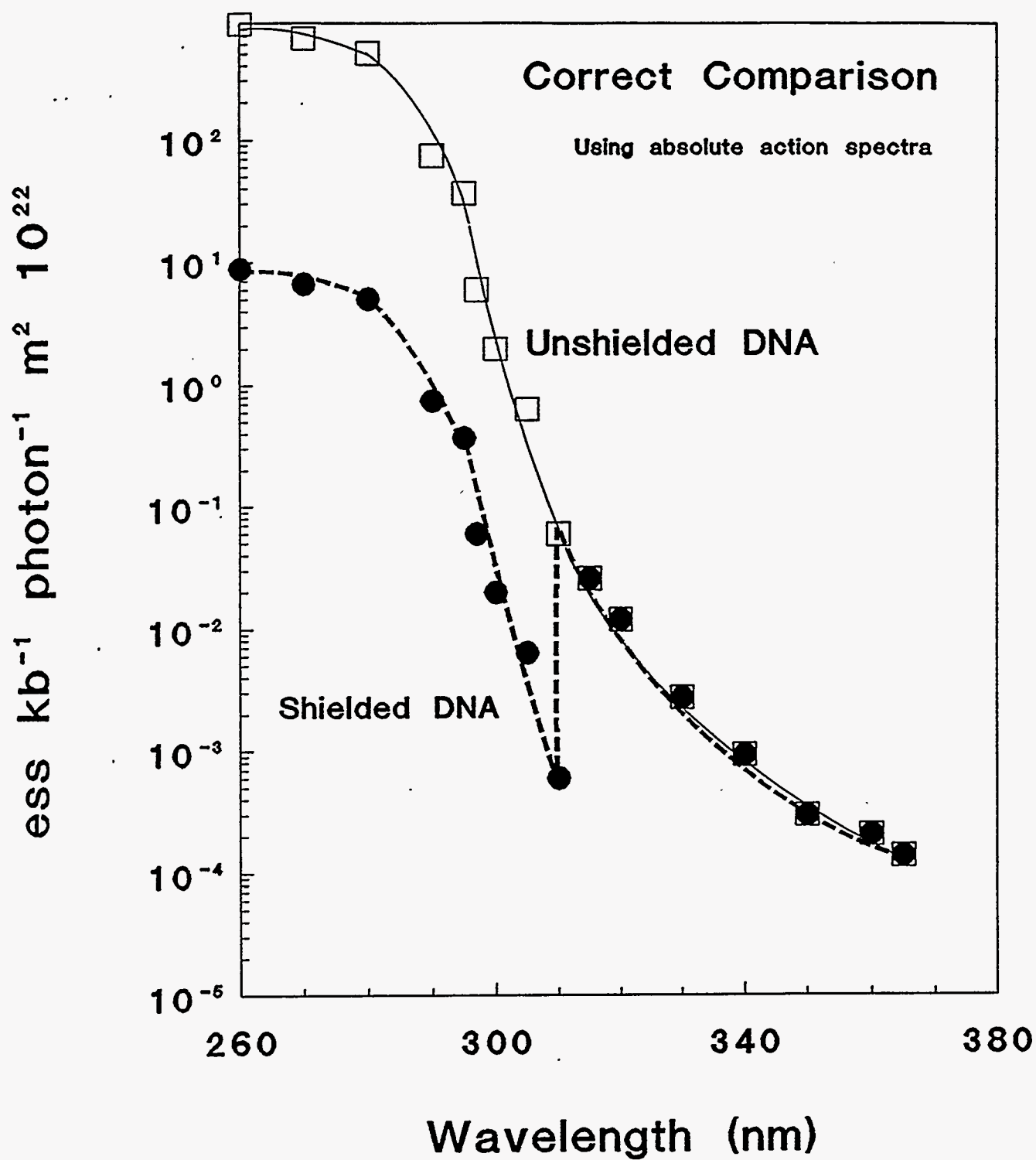
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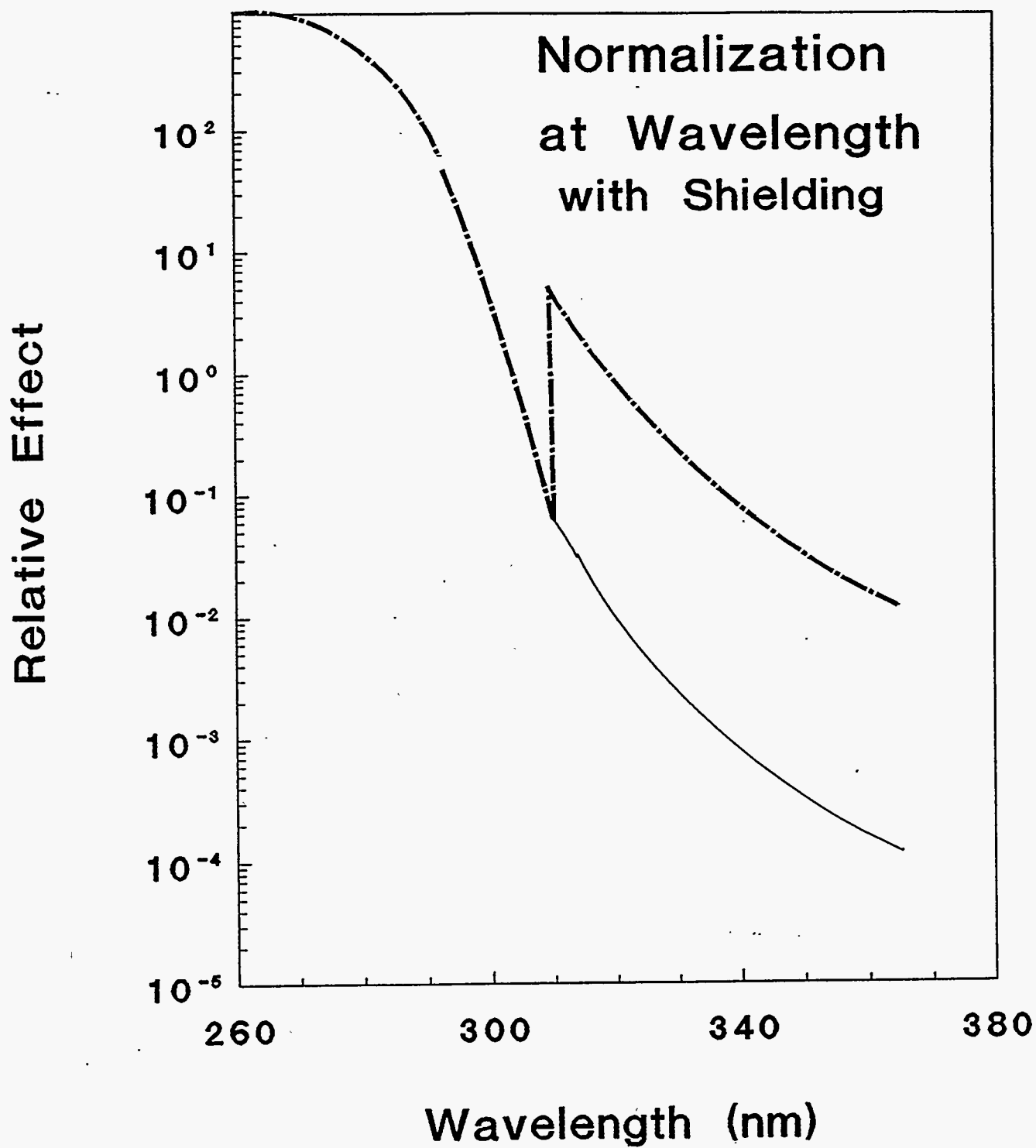


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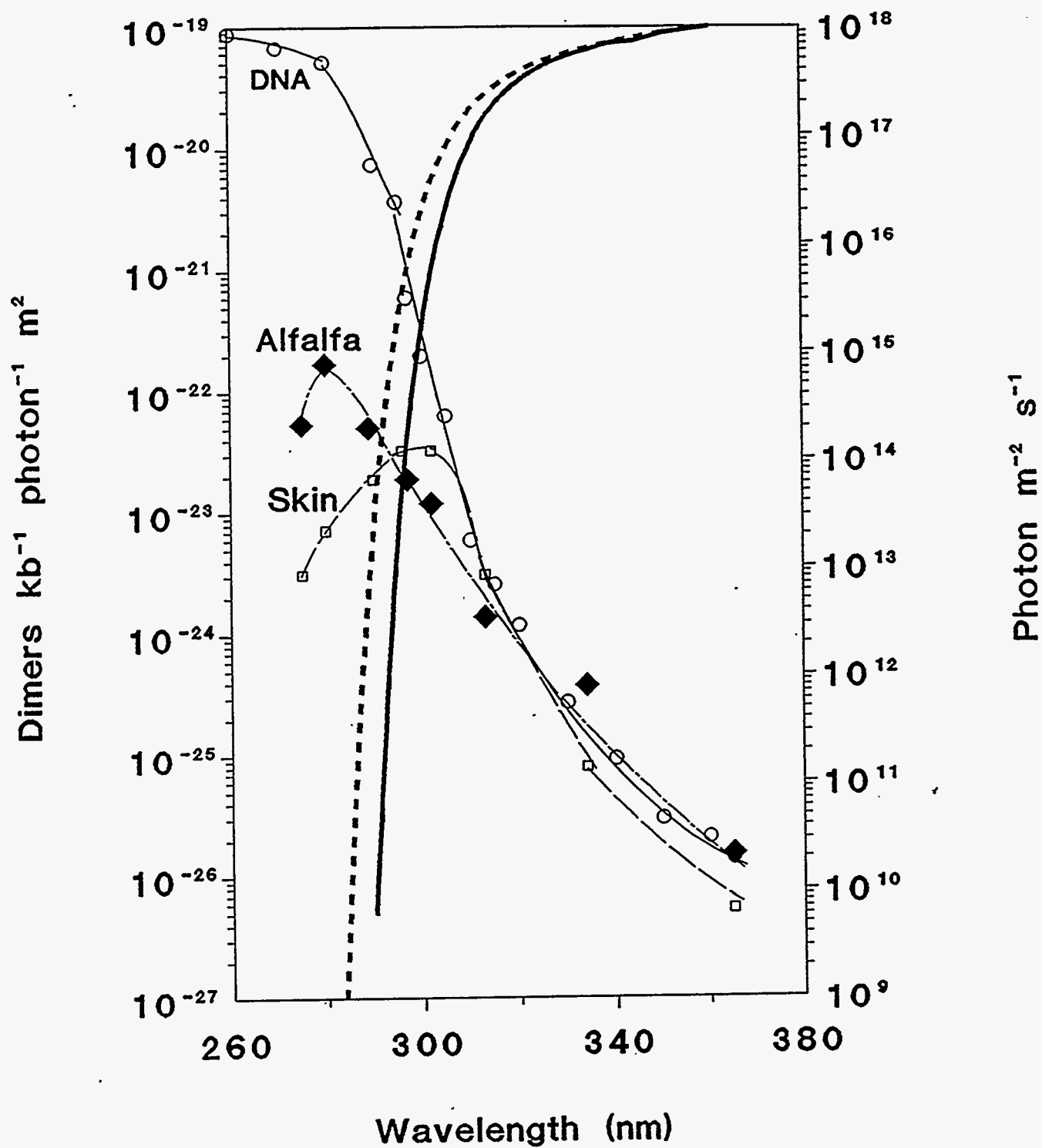


Fig 5

