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VACUUM UV EXCITATION OF LIGHT EMISSION FROM AROMATIC AMINO ACIDS AND TRYPSIN

Thesis for the Degree of Ph. D.

MICHIGAN STATE UNIVERSITY

Edward Yeargers

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Vacuum UV Excitation of Light
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ABSTRACT

Vacuum ultraviolet radiation was used to excite light emission from aromatic amino acid and trypsin as powders and in D_2O-glucose glasses at 110°K. This research was an effort to explain the increased ratios of phosphorescence to fluorescence (P/F) observed previously during X ray excitation (compared to UV excitation) in powders. If transitions to higher-lying excited states, as caused by X rays, lead to enhanced intersystem crossing rates, then it might be expected that the P/F ratios of vacuum UV irradiated compounds would increase as the excitation wavelength decreased. Such a mechanism would be important because of the possible role of triplet states in photobiology. When the excitation wavelength was varied from 280 mμ to 120 mμ the P/F values for tryptophan and trypsin powders increased by a factor of ten. For powders of phenylalanine and tyrosine there was no appreciable change in P/F with excitation energy; thus, this work does not explain the X ray data (see above) for tyrosine and phenylalanine. Tyrosine phosphorescence excitation, however, shows an intense band at 200 mμ compared to 280 mμ. Thus, tryptophan, tyrosine, and trypsin as powders can exhibit efficient intersystem crossing between higher states compared to lower states. When these compounds are dissolved in a D_2O-glucose solution their P/F ratios are essentially independent of excitation energy; therefore, enhanced intersystem crossing rates due to high energy excitation must be highly dependent on the molecular environment. For both powders and glasses the total

emission quantum yield decreased for higher energy excitation so that efficient radiationless quenching of higher states must be proposed.

It is concluded that excitation wavelength-dependent effects on P/F ratios may be the result, in part, of differential quenching of higher excited states.

The phosphorescence decay curves for powders can be approximated as the sum of two exponential decay modes whereas that of glasses is best fit by a single exponential. These data plus the dependence of the mean phosphorescence lifetime on excitation wavelength in powders, but not in glasses, imply that aggregation in these compounds leads to more than one triplet emitting species. These species may be completely new emitting centers due to crystallization (such as different positions in a unit cell or photochemically altered molecules) or may simply be the result of a specific interaction of two different chromophores in the same molecule.

The thermal activation energies are, in each case, independent of excitation wavelengths and have the magnitude of vibrational and rotational degrees of freedom. Thus, the activation energy for quenching of the lowest-lying states is probably much larger than for quenching higher states. A consideration of the kinetics of a model absorbing and emitting system show that, in addition to temperature, quantum mechanical factors are also important in determining the rates of quenching different levels. Because the inactivation quantum yield of trypsin is fairly constant for wavelengths between 160 and 280 mμ the rapid quenching of upper states, as

described above, must not necessarily lead to enzyme inactivation.

The observations that the P/F ratios of tyrosine and phenylalanine are essentially independent of wavelength and also that the phosphorescence decay and activation energy data for vacuum UV excitation do not agree with those for X irradiation indicate that ionizations may be important in determining the nature of X ray-induced light emission in the compounds tested here.

VACUUM UV EXCITATION OF LIGHT EMISSION FROM AROMATIC AMINO ACIDS AND TRYPSIN

By

Edward Yeargers

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INTRODUCTION

Because of their long lifetimes and reactive properties, triplet quantum states are of fundamental concern to radiation biologists. Perhaps the sensitivities of organisms to various kinds of radiation depend upon such states. Thus, while this dissertation is nominally a discussion of some structure-directed spectroscopic experiments, I hope that the results will be interpretable in terms of function-directed radiation biology.

Essentially, I have made an investigation of the emissive properties of the biochemicals tryptophan, tyrosine, phenylalanine, and trypsin following excitation with vacuum UV (VUV) and near UV (NUV) radiation. The aim was to examine the relative partitioning of excitation energy between the singlet and triplet manifolds including both radiactive and radiationless processes. The genesis of this research was the observation that the ratios of the phosphorescence to fluorescence quantum yields in these chemicals (as powders) -- their P/F ratios -- at 77°K were about 1-2 orders of magnitude lower for NUV excitation than for X ray excitation (1). Three possible explanations for these data were presented. The increased P/F ratios in the case of X ray excitation could be the result of a.) break-up of collective excitations, b.) direct exchange between orbital electrons and incident slow electrons, and c.) intersystem crossing during energetic relaxation from higher excited states. Possible methods to test these mechanisms are, in order, a.) irradiation with high energy VUV (approximately 25 eV), b.) irradiation with slow electrons (5-20 eV), and c.) irradiation with sub-

ionizing VUV (4-10 eV).

Explanation a.), collective excitations, as proposed by Fano, results from the excitation of a group of oscillators by energies in excess of that necessary for ionization of a single oscillator (2). The similar exciton mechanism for delocalized excitation has been given experimental justification (see, e.g., ref. 3). Mechanism b.) was predicted theoretically and verified experimentally in the cases of atoms and simple organic molecules (4,5). Robinson and Frosch have presented theoretical justification for a mechanism such as c.): Upper states, being rather close together, may be very strongly coupled vibrationally (6). The probability of radiationless transitions, including intersystem crossing, may depend upon the strength of such coupling. Some experimental evidence also indicates that this mechanism is important (7,8).

Although a fair amount of absorption work with hydrocarbons has been performed using VUV (see, for instance, 9, 10 and refs. therein), much less has been done on more complicated organic molecules. Preiss and Setlow measured the VUV absorption of some amino acids, proteins, and other biochemicals in solid films (11). In addition, absorption spectra in this energy region have been carried out on molecules containing lone pair electrons by Tsubomura et al (12) and also by Parkin and Innes (13). An attempt was made by Kimura and Nagakura to correlate the absorption spectra of substituted benzenes with theoretically predicted transitions (14). They proposed as one of the interacting configurations a charge transfer state between the substituted groups and the benzene moiety. Good results

were obtained with phenol, flurobenzene and benzaldehyde. Barnes and Simpson have investigated the VUV absorption of carbonyl and carboxyl groups (15). Other examples of VUV absorption work in more complicated molecules are given in ref. 10.

Little research has been done with the VUV excitation of emission, however. Schoen et al excited fluorescence of certain gases between 50 and 100 mμ. They also refer to some much older but similar work (16). Also, fluorescence of nitrogen was excited between 50 and 100 mμ by R. Huffman et al (17). Furthermore, Lipsky and co-workers and also Laor and Weinreb have reported on the fluorescence efficiency of biochemicals excited with wavelengths between 150 mμ and 280 mμ (18-20). Generally, their results show that the emission efficiency drops to one-half or one-fourth its value when the excitation changes from 280 mμ to 150 mμ.

Since VUV radiation is absorbed by practically all substances, the problem of solvents is very important. To date, the only solvents which can be used even down to 150 mμ are n-heptane and some of the fluorocarbons (see refs. 9, 10). As a consequence, VUV absorption studies almost invariably involve pure gas, liquid, or crystal samples. In those cases where solvents are absolutely necessary, one must also deal with the problem of solubility -- many solvents useful in the VUV region are non-polar. I should add that the amino acids discussed here are solid at room temperature; pure liquid and gas phases could be used under different conditions of temperature and pressure. The use of condensed systems, however, brings on the completely separate problem of interactions with the environment. For instance,

Von Foerster has examined the phosphorescence of certain benzenoid hydrocarbons in crystalline form and in solvents, using NUV for excitation. He found that these compounds in organic solvents exhibited two phosphorescence decay components. In crystals, however, only the shorter of these two decay components was present (21). Using some of the same crystalline compounds, Olness and Sponer reported that at 4^{o} they found non-exponential decays (22). Evenso, crystals have the advantage of being a condensed system and, therefore, these results should be more realistically applied to in vivo systems than data obtained from gases.

Ferguson calculated that the activation energies measured by plotting the intensity of fluorescence against 1/T (T being the temperature) are approximately 150 and 500 calories per mole for tetracene (23). He feels that this indicates that the quenching of fluorescence has the same activation energy as might be exptected if the quenching is due to lattice vibrations (see Discussion). He also reports that the internal conversion from higher electronic states to the electronic state of lowest energy is efficient. However, predissociation and internal conversion can occur.

In spite of the difficulties with solvent absorption, many of the effects of the crystal environment can be removed by using a 0.5% glucose solution of D_2O as a solvent. This matrix transmits down to about 165 mμ.

Spectroscopy in the VUV region is made difficult because of the need to control several environmental parameters such as the pressure and temperature of the sample. Furthermore, continuous, intense VUV

sources are almost impossible to find, gratings are not very efficient in the VUV, and linear photon detectors of high efficiency are not available. A good recent review article on these general topics has been published by Garton (24). A discussion of the so-called "rare gas continua" has been published by Tanaka et al (25). In particular, this article describes several continua which give fairly smooth backgrounds between 60 mμ and 190 mμ. However, these techniques require high lamp pressures and thus a differential pumping system. Okabe has described a lamp and certain gas mixtures which generate very intense VUV light (26). These sources provide up to about 10^{15} quanta per second. Such intensities are sufficient for photochemical work but are available only as line sources.

Another problem in working with VUV radiation is that in order to prevent gas in the source from entering the main chamber, the source must be covered by a lithium fluoride window. Patterson and Vaughn have examined the transmittance behavior of lithium fluoride plates from the cut-off up to the ultraviolet region as a function of the time which they are exposed to air (27). They found that the spectral transmittance of the lithium fluoride at wavelengths shorter than 160 mμ decreased when exposed to atmosphere because of a surface layer being produced on the crystal by reaction with moisture.

The problem of reflectance coatings for VUV is a major field of its own; a recent review is by Madden (28).

In order to make a detector for photons in the VUV, a photomultiplier tube (highly sensitive at 430 mμ) is often covered with

a layer of solid sodium salicylate. The latter compound has a constant quantum yield for absorption between 80 and 300 mμ -- the emission being at 430 mμ (29). In addition, sodium salicylate might also be used as a quantum yield standard. Recently, four papers have appeared, giving what was purported to be the absolute quantum efficiency of sodium salicylate (30-33). These various investigators measured the absolute quantum yield of solid sodium salicylate at 0.50, 0.64, 0.98, and 0.25, respectively. Nygaard, in duscussing this problem (34), reported that the efficiency of the commonly used phototubes (EMI) depends on the angle of incidence of the light to be detected and that if the sodium salicylate layer is too close to the tube, some errors could be introduced. He concluded that the work of Allison et al (ref. 32) was the correct figure (0.98), although it by no means seems to be unequivocal.

Whereas most molecular spectroscopy, particularly for polyatomic molecules, has been concerned with π-orbital excitation in the NUV, when photons in the VUV are used, σ-orbital excitation should also be important. Very little experimental work refers directly to this (e.g., refs. 12, 15, 35) although recently there has been some theoretical work on σ-orbitals by Del Re (36) and also a series of papers by Peters culminating in reference no. 37. (References to the other articles are given therein.) This work provides a basis for predicting σ-bond characteristics through a Hückel-like treatment which may prove to be useful.

MATERIALS AND METHODS[1]

Vacuum UV - The VUV monochromator used in all of these studies was a 0.5-m focal length Seya-Hamioka instrument made by the McPherson Instrument Company of Acton, Massachusetts. The source was a Hinteregger type with AC discharge through hydrogen gas. It was powered by a magnetically regulated power supply which can produce more than 1,000 watts. Prior to entering the monochromator, the radiation was passed through a lithium fluoride window 1/16" thick. This window was necessary to prevent the hydrogen gas from entering the monochromator and raising the pressure. The absolute pressure of the hydrogen gas within the lamp was maintained between 5 and 25 mm. of Hg depending upon the wavelength at which maximal energetic output was desired.

The VUV grating used in these studies was made by Bausch and Lomb with 600 lines per mm., and blazed at 150 mμ. It gave the instrument a dispersion of 3.4 mμ per mm. of slit width.

As detector I used one of two phototubes -- an EMI 9514S with a lime-soda glass end window (S-type spectral response) or an EMI 6255B with a quartz end window (S13-type spectral response). The latter tube was sensitive to light down to 165 mμ, the former was unresponsive below 300 mμ. The phototube was mounted in a thermoelectrically-cooled chamber from Products for Research, Inc., W. Acton, Mass. This chamber can cool the tube down to -25°C. and thus

[1]Except for the differences noted in this section, all techniques discussed are applicable to both powdered and dissolved samples.

can reduce the room temperature dark current by a factor of about 10. At the entrance to the chamber was a plate of either Lucite, glass, or quartz upon which, on the side facing the vacuum, could be a layer of sodium salicylate, the latter having been dried onto the surface from a saturated methanol solution. Sodium salicylate has a constant quantum efficiency throughout the VUV and NUV spectrum and thus this phototube with a sodium salicylate-covered end window had a linear response below the cut-off of whatever material the end window is made; that is, 350 mμ for Lucite, 300 mμ for glass, and 160 mμ for quartz. According to other research, the maximal amount of sodium salicylate for best response is about 4 mg. per square centimeter (32); that amount has been used for my work. The output of the photomultiplier tube was amplified by a Victoreen electrometer and fed into a Bristol strip recorder which could be synchronized with the wavelength scan of the vacuum UV monochromator. For recording spectral distributions, the chart speed was 2" per minute, and for recording phosphorescence lifetimes, the speed was 16" per minute.

The sample (preparation described below) to be examined was placed in an operational vacuum chamber (OVC) fitted between the exit slit of the monochromator and the entrance to the phototube chamber. It had several access ports; one attached to the exit slit of the monochromator, the photomultiplier tube was attached to another, one was used for air exhaust and thermocouple entry, the phosphorescence shutter was installed in another, one was blanked off, and the sample holder was placed in the sixth. The sample was mounted on a

brass coldfinger which in turn contacted a brass, liquid-nitrogen reservoir (see below). This reservoir could be filled from outside the vacuum chamber and thus the temperature of the sample could be regulated. In the back of the coldfinger a small shallow hole could accomodate the sensing tip of the thermocouple. The shutter consisted of the usual rotating (at about 5000 rpm) sectored can coupled by a magnet to a small motor outside of the OVC. Thus, the magnetic coupling eliminated the problem of maintaining a vacuum about the drive shaft. Pressures within the OVC and the main chamber were regulated with a mechanical pump and an oil diffusion pump. The latter was cooled with a continuous flow of water, and a group of baffles in the fore line of the pumps was cooled with liquid nitrogen.

This technique of cooling the chevron baffles essentially eliminated condensation of oil vapors onto the coldfinger and sample holder. In practice, pressures of about 1.0×10^{-6} mm. were obtained routinely. The chevron baffles were cooled with liquid nitrogen for approximately 3-4 hours before the sample itself was cooled. In this way the oil vapors from inside the tank were effectively removed (see below). All O-rings in the vacuum system were made of Viton and were coated with a small amount of silicone grease. Under certain conditions, notably during the measurement of phosphorescence (which is usually much weaker than fluorescence), the second emission monochromator was omitted -- that is, the phosphorescence radiation was collected by a photomultiplier tube completely undispersed. Thus the phosphorescence intensity normally was simply a

measure of the amount of radiation which had a lifetime sufficiently long to get past the rotating phosphorescence shutter. It may include other kinds of radiation if they have sufficiently long lifetimes; it does not, however, contain any scattered light; any delayed fluorescence was removed with filters. (Delayed fluorescence has the same wavelength as normal fluorescence but has a lifetime on the order of msec.)

The slits in the McPherson instrument are variable from about 5-10 μ to 2 mm. Because of the low light levels involved here, the 2 mm. slits were normally used. But in at least one case, tryptophan powder fluorescence, a slit of only 80 μ was used in a search for fine structure of fluorescence excitation; none was found (see Results).

Wavelength calibration was carried out by adjusting the monochromator wavelength indicator to agree with detection of a group of lines from a mercury vapor pen lamp. The central image was aimed at the exit slit by adjusting the grating holder. The wavelength accuracy for a small slit was then about 0.1 mμ.

In order to disperse the emitted radiation where possible, a Bausch and Lomb monochromator was used. This instrument has a dispersion of about 3.0 mμ per mm. and was normally used with a slit opening of 6 mm. It was placed between the output port of the OVC and the photomultiplier detector tube on a sturdy aluminum base to assure stability.

Sample preparation (powders):

The powder samples were prepared by recrystallizing the appro-

priate amino acid (Grade A, chromatographically pure, from Cal Bio Chem) from water several times, drying the crystals, washing them quickly with methanol, drying again, and pulverizing them with mortar and pestle. Twice-crystallized, salt free, lyophilized trypsin from Worthington Chem. Co. was not treated further. Each powder was then filtered through a sieve, normally 100 mesh. Samples of this powder were mixed with a small amount of methanol to make a paste which was smeared onto a slab of brass attached to the coldfinger. After rapid drying the powder sample thickness was about 0.1 mm. The average thickness of a sample on the brass plate was determined by weighing the brass plate before and after application of the amino acid-methanol paste and assuming a density of 1.3 grams per cubic cm. It would be useful to know the crystal structure of the pure amino acids (for interpretation of spectra) but only incomplete data are available. What little information there is, e.g., the unit cell size and the number of molecules therein, has been given by Bernal (38). A more complete crystallographic study of a phenylalanine salt is due to Gurskaya (39).

Sample preparation (glasses):

D_2O solutions (0.5% glucose) were used as a matrix for studies on the amino acids. Thus, down to about 170 mμ the solvent absorption should be small. The amino acids were added to this D_2O solution in a concentration of about 3×10^{-3} M and the solutions were bubbled with helium. (The relatively high concentration assures that most of the incident light is absorbed.) A few ml. of this mixture were placed in the center of an "O" ring on the brass

sample holder and covered securely by a Suprasil window. The window was held in place by a screw-clamp and the pressure resulted in a final sample thickness of about 2 mm.

Techniques:

Measurements described below show that less than 5% of the radiation striking the powder or glass samples was actually reflected. Thus, it is reasonable to assume 100% absorption by most samples and for equal incident excitation light the emission quantum yield is proportional to the amount of emitted radiation (except for phenylalanine in the NUV where absorption is weak. Appropriate corrections were made for absorption in this case). Because of the assumption of total absorption of the incident light, relative quantum yield measurements could be made as follows:

After the proper vacuum was achieved the sample was cooled with liquid nitrogen. The temperature of the coldfinger was measured with a copper-constantan thermocouple, using a Leeds and Northrup potentiometer for potential measurement. (In Appendix I, there is a discussion of the possible variation of the temperature of the sample with its thickness.) An uncorrected excitation spectrum (see Results) was run for either fluorescence or phosphorescence following which the output from the lamp reaching the OVC was measured. The ratio of the first measurement to the second measurement at each wavelength gives the relative excitability per incident quantum for that particular sample. The lamp is sufficiently stable over a period of several hours so that excitation spectra from about 110mμ up to 300 mμ were obtainable. (See Note on errors.) In order to

measure P/F ratios, excitation spectra of fluorescence and of phosphorescence were run and the P/F ratio obtained by computing the ratio of these two, the incident light being approximately the same for both excitation spectra.

Since the lithium fluoride window at the lamp passes 110 mμ radiation, one might expect second order effects to begin around 220 mμ. For this reason, excitation spectra were run up to about 220 mμ as described above. Later the LiF window was in turn covered by Suprasil. This filtered out radiation below 160 mμ so that excitation spectra and P/F ratios could be run from 160 up to 300 mμ. The ratios obtained between 160 and 220 mμ were the same in the two cases.

Corrections were made for the phosphorescence shutter which cuts off two-thirds of the phosphorescence radiation. Corrections were also made for detector response. Since the sensitivity of the phototube is wavelength dependent and the phosphorescence was undisposed, the appropriate mean emission wavelengths (for corrections) were obtained from other work (1). Because the phototube response is constant over large wavelength ranges, wide latitude could be tolerated in wavelength values.

To measure lifetimes, the phosphorescence shutter was set in motion and a flap-valve near the exit slit of the monochromator was closed quickly. In this way only phosphorescence was seen and no corrections for scattered light appearing in the wavelength interval of normal phosphorescence emission were necessary.

Activation energies were measured by noting the emission intensity and the temperature of the coldfinger over a period of time as

liquid nitrogen was poured into the reservoir during irradiation. A gradual increase in the amount of emission was seen as the temperature went down. By not pouring liquid nitrogen into the reservoir for about one minute the temperature could be made to level off and at this point the temperature and intensity were noted. In this way, a plot of relative emission yield vs. the reciprocal of temperature could be made routinely.

Other techniques:

When both the excitation and emission monochromators of an Aminco spectrophosphorimeter (40) were set at the same wavelength the pen deflection was a measure of the light reflected from a solid specimen placed in the sample holder. The light reflected at each wavelength from a dried paste or glass (as described above) was compared to that reflected from a freshly smoked magnesium oxide surface (the latter was taken as 100% reflective). With all four compounds considered here the per cent reflected light changed from 80-90% at wavelengths above 300 mμ to 1-4% between 200 mμ and 300 mμ (i.e., near the absorption bands). This justifies the assumption of 100% absorption by the specimens for λ 300 mμ. (Near the 0-0 band some fluorescence will be detected but this only decreases the amount of reflected light actually measured.)

The Aminco instrument was also used for quantum yield measurements on thick films (made from pastes) of the appropriate sample compounds applied to a glass slide. A similar film was made of sodium salicylate. Assuming, as justified above, that all incident light is absorbed, the quantum yield of the sample with respect to

sodium salicylate is merely the ratio of the areas under their respective emission curves, plotted on an energy scale since this represents the total number of emitted quanta. Appropriate corrections were made for monochromator and phototube efficiencies.

A Note on Errors:

Because of the many technical problems encountered, errors may be fairly large. The brackets in the various excitation spectra for powders represent an estimate of the error induced by instrumental noise, reading of the spectra, and lamp instability over the period during which the measurements were made. Each graph represents a single run and thus the position of one point with respect to another is probably meaningful. When these measurements on powders were repeated completely, the variations between different runs was about +100% to -75% although the curve shapes were the same. Because the lamp output is fairly constant over the spectral range pertinent to glasses, the yield and P/F curves for glasses are not bracketed but have a constant estimated error, for a single run, of about $\pm$ 20%.

For phosphorescence decay curves one source of error is the 0.1 second response time of the tube output amplifier. Further, for wavelengths where lamp output is low, pen noise (from the phototube) is important. I estimate the error in the stated lifetimes to be about $\pm$ 0.2 seconds. This estimate comes from a consideration of my ability to read points from the graphs and also from repeatability tests (which agreed within this error).

In measuring activation energies, the main source of error is lamp instability, although this probably is not due to voltage

fluctuations since the power supply is highly regulated. It is more likely due to clouding of the lithium fluoride window by the lamp. However, if the measurements were made over a period of about 30 minutes, this would not induce an error of more than about 10-15%. I changed the window about every 3-4 running hours. However, there is always a certain amount of pen noise; from considerations of the various errors and repetition of experiments, I have estimated that the activation energies may vary by +40% and -20%. However, to provide greater confidence in comparisons between different excitation wavelengths, during each activation energy run I measured yields at two or three wavelengths. In this way, while there were some errors in the absolute magnitude of the activation energy, I could be more certain that the variation was the same for all excitation wavelengths. The same considerations apply to determinations of the break points in the activation energy curves.

In examining phosphorescence, the so-called integrated position was used; i.e., the phosphorescence radiation was not dispersed but was collected directly by the phototube. To avoid problems with delayed fluorescence, filters which removed fluorescence wavelengths were used to isolate phosphorescence wavelengths. Further, because of the speed of the phosphorescence shutter, any phosphorescence with a lifetime less than about 10 msec. would not have been detected.

Adsorption of oxygen in solutions or onto powdered samples can be a problem in spectroscopy. It is difficult to assess the pertinence of this problem in the present case. As described above, how-

ever, at least four hours of pumping (at 10^{-6} mm. pressure) was used in an effort to outgas the powder samples and the solutions were bubbled with helium. Although the extent of gas remaining in the samples was difficult, or impossible, to assess each sample, through the course of the experiments, was treated identically. Thus, for instance, samples examined on separate days should be comparable in this respect.

In the measurement of quantum yields of powders, even the best sample films showed some inconsistencies from point to point in a single sample. Measurements with the samples in a variety of positions gave yields varying by about ±20%. The yields of powders at 110°K were computed by using Arrhenius plots to extrapolate the yield at 300°K. Thus, the low temperature yields include not only the 20% error described above but also include an error of about ±20% from the Arrhenius plots themselves.

I made no corrections for reabsorption of fluorescence. Actually most light absorption occurs far from the spectral region of absorption and fluorescence overlap. Thus, for λ_{ex} less than 260-280 mμ even the weakest absorber (phenylalanine in this case) has an extinction coefficient on the order of 10^2. But, near the overlap region phenylalanine has an extinction coefficient of less than 10^0. For the crystals, furthermore, most absorption took place in only about one-tenth the depth (in the crystal) that it would take to appreciably absorb the resultant fluorescence.

An estimate of stray light was made as follows: A quartz-faced phototube was mounted at the exit slit of the excitation mono-

chromator. The excitation wavelength was varied between 110 mμ and 155 mμ. If there was no stray light, no pen deflection should have been noted (since the phototube could not respond to the scanned wavelengths). A glass-faced tube was used to extend the usable scanning range (but also the stray light sensitivity cut-off) to 300 mμ. Stray light was, indeed, detected and these measurements gave an estimate of its total magnitude. By putting a Bausch and Lomb monochromator between the excitation monochromator and the detector, I was able to examine the spectral distribution of this stray light. Of course, this spectral distribution showed a large peak when the wavelength setting of the two monochromators were the same but remained fairly flat at other wavelengths.

A second technique for determining stray light was the following: A fluorescence excitation spectrum (of any compound) always showed pen deflection for excitation at 90 mμ. Since the lithium fluoride lamp window does not transmit below about 105 mμ, the pen deflection must represent stray light reflected from the sample. The measurement of stray light (see above) was then normalized to this point and then subtracted away at all wavelengths.

The results of these calculations are: 1. The total intensity of stray light varies with the excitation monochromator wavelength setting -- being about four times greater for a 90 mμ setting than for a 250 mμ setting. 2. The spectral distribution curve of the stray light is fairly flat for all excitation monochromator settings. 3. The stray light output of the excitation monochromator is about 10% of the intensity at the exit slit for a 250 mμ setting and about

1% for a 160 mμ setting (there are intense lines at 160 mμ). Of these fractions, about one-fifth of the intensity is measured at a wavelength which is appreciably absorbed by the compounds of interest here. Thus, absorbed stray light is not a very serious problem and corrections for reflected stray light can be easily made.

Actual spectral curves are described under Results.

RESULTS

Background radiation.

Figure I shows a typical set of raw data before corrections for stray light have been made. The curves have been normalized at 90 mμ prior to subtracting away the scattered light from the monochromator emission and from the sample emission curves. Next the sample emission is divided by the monochromator output to get the relative excitability of the sample at a given wavelength.

Spectra of solids.

Figures II through XIII show plots — as a function of excitation wavelength (λ_{ex}) of the values of fluorescence quantum yield, phosphorescence quantum yield, and P/F ratios computed from the measurements made on the four compounds under consideration. In the case of fluorescence quantum yields (F_{rel}) and phosphorescence quantum yields (P_{rel}) the Y-axis represents the quantum yield in arbitrary units. However, in Table I, I have listed some estimates (accurate to about ±20% of the corrected values) of the fluorescence quantum yields of each of these powders compared to that of similarly treated sodium salicylate. Thus, the Y-axes of excitation spectra for different compounds can be compared with one another (the P/F ratios can then be used to scale phosphorescence excitation spectra). While there are several different estimates of the fluorescence yield of sodium salicylate, it seems plausible that, eventually, more precise work in this area will permit -- using the data of

Table I -- an accurate scaling of the fluorescence and phosphorescence quantum yields of the compounds with which I worked. For the time being, however, the quantum yield of sodium salicylate must be assumed to be between 0.25 and 0.70 (see Table I).

The fluorescence excitation spectrum (F-spectrum) and the phosphorescence excitation spectrum (P-spectrum) of tryptophan show three major excitation band envelopes -- they are centered at about 260 mμ, 200 mμ, and 130 mμ in both cases (Figures II, III). The 200 mμ peak in the P-spectrum is quite strong and leads to some structure at the same place on the P/F curve in Figure IV. While the P/F ratio increases by about a factor of ten when λ_{ex} was changed from 280 mμ to 140 mμ, this ratio starts decreasing for λ_{ex} 140 mμ -- perhaps the result of ionization. One other point seems important: I examined an excitation spectrum of tryptophan fluorescence at 0.1 mμ resolution and found no fine structure.

The F-spectrum of tyrosine (Figure V) shows only two prominent bands -- one at about 270 mμ and another extending from 150 mμ to 240 mμ. The P-spectrum of tyrosine (Figure VI) resembles that for tryptophan although the 130 mμ band is less pronounced for tyrosine. The differences between the F-spectra of tryptophan and tyrosine become more apparent through inspection of Figure VII -- that is, the P/F ratio of tyrosine has a maximum between 170 and 210 mμ but the values do not continue to increase with decreasing excitation wavelengths as is observed with tryptophan.

The F-spectrum of phenylalanine (Figure VIII) bears some resemblance to that of tyrosine, i.e., a band between 240 and 270 mμ

and a large envelope between 230 and 130 mμ. As with tyrosine, the latter envelope is less intense at the shorter excitation wavelengths. However, the P-spectrum of phenylalanine (Figure IX) is quite different from that of tyrosine or tryptophan -- especially near 200 mμ or where there is no sharp band. The net result is that the P/F curve for phenylalanine shows less than a four-fold variation as λ_{ex} is varied between 120 and 270 mμ (Figure X).

The F-spectrum of trypsin shows a single band peaked at about 260 mμ (Figure XI); the efficiency is reduced by an order of magnitude for $\lambda_{ex} \leq 200$ mμ. While the P-spectrum for trypsin also drops off for shorter λ_{ex}, the decrease is not so sharp as for fluorescence; this leads to an increasing P/F ratio for shorter excitation wavelengths (Figures XII, XIII).

Thus, it is only in the case of trypsin and tryptophan that the P/F ratio increases by as much as a factor of ten when λ_{ex} is decreased from 280 mμ to 120 mμ. Of all the excitation spectra, however, only the P-spectrum of phenylalanine showed less than a five-fold change in yield over the same range of λ_{ex}.

Occasionally, in the spectra of powders, there are narrow sharp peaks -- e.g., at 170 mμ in the tryptophan P-spectrum (Figure III) and at 160 mμ in the phenylalanine F-spectrum (Figure VIII). Such narrow "lines" have a band width of about 5-10 mμ (close to the minimum resolution of the instrument. Thus, they probably can be ignored.

Spectra of glasses.

Figure XIV shows the F-spectra, P-spectra and P/F curves com-

puted from the emission data obtained from each of the three amino acids and trypsin in a frozen 0.5% glucose solution of D_2O at 110^oK. In each case it seems apparent that the fluorescence and phosphorescence quantum yields are fairly constant for λ_{ex} 190 mμ but that the yields decrease for λ_{ex} 190 mμ. The sharp excitation bands in the P-spectra of tyrosine and tryptophan powders are not found for excitation in solution. Further, the P/F ratios are almost independent of λ_{ex} (within the limit of the errors).

Phosphorescence lifetimes.

The lifetimes of phosphorescence from powders (for the four compounds in question) as a function of λ_{ex} are given in Table II. All decay curves for powders could be resolved as the sum of two exponential components. For phenylalanine and trypsin powders, but not for tryptophan and tyrosine powders, the total curves can be resolved as the sum of the same two components -- but in relative proportions that depend on λ_{ex}. Phosphorescence of phenylalanine, but not of the other three compounds, shows a dependence on crystal size (Figure XV and Table III). Here again the lifetime changes appear to depend only on varying proportions of the same two components. Figure XVI shows resolved decay curves for phenylalanine phosphorescence. The two decay curves represent two different excitation wavelengths and also two different sizes of crystals. However, the components are, within error, the same.

When dissolved in a glucose -- D_2O glass the aromatic amino acids and trypsin have phosphorescence decay curves which show only a single exponential component. Further, in each case, the decay

time is independent of λ_{ex} (Table IV).

Activation energies.

The thermal activation energies (E_A) for fluorescence and phosphorescence of powders are given in Table V. These data include the activation energy of each straight line component on an Arrhenius plot and the point ("break point") where they meet. Within the stated errors the E_A's for a given compound and type of emission are independent of λ_{ex}. This relationship is demonstrated in Figure XVII.

In Table VI are thermal activation energies for fluorescence and phosphorescence of glucose-D_2O glasses of the aromatic amino acids. As with powders, the E_A's are independent of λ_{ex} but are 5-10 times larger for glasses than for powders.

Comparison with other data.

Some of these comparisons are tabulated in Table VII.

P/F ratios: While my calculations for glasses are in arbitrary units, those for solids can be compared directly with those of Augenstein et al (1). For excitation at 260-280 mμ I measure P/F ratios at 110°K which are about a factor of ten less (in every case) than those of reference no. 1. A sizeable portion of this difference is due to the higher temperatures used in my present work -- if activation energies are used to extrapolate my data to 77°K the difference becomes a factor of 2 to 3. Of greater importance is the observation that both sets of data (mine and reference no. 1) show P/F's for NUV excitation of phenylalanine and trypsin which are similar. Further, in both cases (for NUV) the P/F for tyrosine powder is

greater, and that of tryptophan powder is less, than the P/F's of phenylalanine and trypsin powders. For high energy VUV excitation I observed that only tryptophan and trypsin powders showed greatly increased P/F ratios whereas Augenstein et al reported that X irradiation increased the P/F ratio (over NUV excitation) for all four compounds in question: this is discussed in the next section.

Phosphorescence lifetimes: I found for all excitation wavelengths that phosphorescence decay of the amino acid powders could be resolved into two decay components. Further, for NUV but not VUV excitation, trypsin showed only a single decay component. Both of these results agree with those of reference No. 1. The actual lifetimes are different (by a factor of about 1.5 to 10) but this is not surprising because of the different temperatures used in collecting the two sets of data. Further, the effect of the condition of the crystal on decay time (see Table III) should also lead to differences between the two sets of data.

The lifetimes in glucose-D_2O solutions reported here differ from those of Augenstein and Nag-Chaudhuri (41) by factors of less than about two. However, they used H_2O, not D_2O, and also worked at a lower temperature. In agreement with them is my observation of single-component decay kinetics with each of the four compounds.

Thermal activation energies: Carter et al reported on the E_A's for fluorescence and phosphorescence of powders of the aromatic amino acids and trypsin as excited in a vacuum container

by X rays (42). My data for fluorescence of all the compounds reported and for tryptophan phosphorescence agrees with theirs. For phosphorescence of phenylalanine, tyrosine, and trypsin, however, there are differences ranging between factors of two to ten in comparing the two sets of data. These differences are outside of estimated errors and, as in comparing P/F ratios for VUV and X ray excitation, may represent real differences between non-ionizing and ionizing radiation (see Discussion).

Figure XVIII shows absorption spectra of phenol as a vapor and in a D_2O solution. The entire absorption envelope appears to be red-shifted about 5-10 mμ in the latter compared to the former. Resolution of the vapor absorption envelope into its component bands has been carried out by Kimura et al (14) using SCF techniques.

DISCUSSION

General

It seems useful here to summarize briefly the major conclusions of my work.

While several results of spectroscopical importance were found, the basic purpose was to investigate the greatly increased P/F ratios found in trypsin and the aromatic amino acids due to X irradiation compared to NUV irradiation (1). The present work shows that enhanced intersystem crossing between states of higher energy can occur under certain restrictive physical conditions. For example, tryptophan and trypsin powders have P/F ratios for VUV excitation which are about ten times greater than for NUV excitation. In addition, the phosphorescence excitation spectrum (P-spectrum) of tyrosine powder shows enhanced phosphorescence for 200 mμ excitation compared to either 140 mμ or 280 mμ excitation. None of the glasses containing these compounds nor the crystals of phenylalanine show these properties. The data of reference No. 1 are not, however, completely explained by my work because of two considerations: i) Only the P/F changes of trypsin and tryptophan powders are about the same magnitude as those of reference No. 1. ii) The total quantum yield for all four powders decreases by about a factor of five to ten for VUV excitation compared to NUV excitation. This means that part of the explanation for the P/F changes may involve quenching of upper states -- not necessarily an exchange of energy between singlet and triplet manifolds.

The phosphorescence decay curves can be approximated as the sum

of two exponentials for crystals and one exponential for glasses -- indicating the likely existence of two emitting species in the former and a single one in the latter. Thus, the dependence of phosphorescence lifetime on λ_{ex} in powders may be (as in the case of intersystem crossing) the result of crystalline interactions.

Since the measured activation energies were independent of λ_{ex}, the E_A for quenching the lowest lying states may be much larger than those for quenching higher states. Because the inactivation quantum yield of trypsin is fairly constant for λ_{ex} between 160 and 280 mμ then the rapid quenching of those upper states must not necessarily lead to inactivation in enzymes.

Phosphorescence decay and activation energy data for VUV excitation agree with those for X irradiation only infrequently; thus, as with the spectral data, ionizations may play an important role in determining the nature X ray-induced light emission in these compounds.

The conclusions above are deducible from the following considerations:

Spectra:

The P-spectrum (i.e., the phosphorescence excitation spectrum) of phenylalanine powder has little structure (within the bracketed error) while the P-spectra of tryptophan and tyrosine powders show a very prominent excitation band at about 200 mμ. This band may correspond to a similarly located peak associated with benzene derivatives (14). SCF calculations (43) and experimental absorption studies (14) show that substituted benzene compounds (and even indole) in vapor form have a very intense absorption maximum at

about 180 mμ. (Indole, phenol, and toluene are almost identical with the π-electron systems of tryptophan, tyrosine, and phenylalanine, respectively.) Upon solvation (see Fig. XVIII and reference No. 10) or crystallization this peak moves 5 to 20 mμ toward lower energies. The work of Kimura et al (14) indicates that this benzenoid absorption band probably consists of contributions from several different transitions to states indicated by B, C, and D, in Figure XVIII. If this description is appropriate to the aromatic amino acids the data for tyrosine and tryptophan phosphorescence given just above may be explainable. For instance, light absorption at 200 mμ will preferentially result in state B; if B is strongly coupled to the triplet manifold then 200 mμ excitation should cause phosphorescence with high efficiency. If, however, higher energy radiation is used, state D can result; since D may be more strongly coupled to C than it is to B or to the triplet manifold, rapid energetic relaxation to the ground or first excited singlet may follow -- giving a low phosphorescence yield. Thus, the exact sequence of electronic levels through which relaxation occurs will depend on the strength of the matrix element connecting two sequential states in a perturbation calculation, not upon absorption intensities.

Strong evidence that this effect is dependent on the nature of the environment comes from P-spectra of tryptophan and tyrosine in glasses. That is, the strong phosphorescence excitation band at 200 mμ is not present for the glasses. Thus, aggregation apparently enhances intersystem crossing between certain bands selectively. Phenylalanine powder shows no sharp excitation band at 200 mμ; there-

fore, the effect of crystallization on this band can apparently be modified by molecular structural factors -- perhaps by affecting the nature of the unit cell.

While the P-spectra of tyrosine and tryptophan powders are similar, their F-spectra differ considerably. The net result is that when λ_{ex} is varied from 280 mμ to 110 m2 the P/F of tryptophan powder increases by about an order of magnitude but that of tyrosine powder shows only a peak at 180 mμ with no large overall increase. Additionally, the P/F of trypsin shows an order of magnitude increase for the variation of λ_{ex} from 280 to 110 mμ. The P/F of phenylalanine shows little dependence on λ_{ex}.

I then conclude from the P/F curve of trypsin, the P/F curve and P-spectrum of tryptophan, and the P-spectrum of tyrosine that there is good evidence for enhanced intersystem crossing rates among higher excited states in powders of these compounds. Further, from excitation spectra of the aromatic amino acids and trypsin in glasses it seems likely that these enhanced rates are the result of interactions which occur only in the crystal. For phenylalanine neither the spectral data of powders nor of glasses point to very extensive intersystem crossing among higher excited states.

The F- and P-spectra for trypsin make it clear that as much as 99% of high energy excitations are efficiently quenched in this molecule. In fact, two kinds of evidence indicate that these higher states may be quenched more quickly than energy transfer to other residues of appropriate energy can occur: i) A study of thermal activation energies (discussed later in this section) shows that

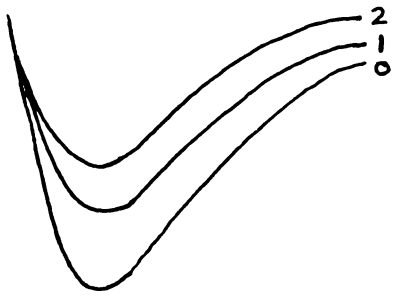
only the lowest excited state in these molecules has an appreciable thermal activation energy for depopulation. ii) Amino acid analyses of X irradiated enzymes have shown a somewhat general, non-specific destruction of residues (44, 45) whereas NUV-irradiated enzymes showed destruction of only tryptophan and cystine residues (46). This is consistent with the idea that high energy states are quenched quickly (leading to destruction of the absorbing molecule) but that low energy states can become involved in energy transfer before quenching can occur.

Another important result of the F and P measurements on trypsin concerns the UV inactivation of enzymes. Setlow has shown that the inactivation quantum yield for dry trypsin varies by less than a factor of four for λ_{ex} between 160 and 280 mμ (47). Since 280 mμ will just excite the lowest levels of amino acids then Setlow's data suggest that the energy quenching act which is pertinent to trypsin inactivation may involve the first excited state of a residue in most cases. As described later in the discussion of activation energies, this important state may be a triplet. Secondly, Setlow's data (47) are in marked contrast to the F- and P- spectra of trypsin Figs. XI and XII which show a decrease of 10-20 times for λ_{ex} decreasing from 280 to 160 mμ. Since only non-radiated energy can lead to enzyme inactivation it is apparent that not all emission quenching (or even a constant fraction thereof) yields inactivation. For instance, these data are consistent with the idea that quenching from higher excited states directly to the ground state of residues (see below) does not lead to inactivation. Such a scheme requires, however,

that, regardless of λ_{ex}, a constant fraction of absorption events lead to a first excited state configuration of a critical residue so as to assure a constant inactivation quantum yield. Thus, protein inactivation by UV light may involve very specific paths of energy transfer and energetic relaxation.

Finally, it is apparent from an inspection of F- and P- spectra for powders that changes in P/F for different λ_{ex} cannot be attributed simply to exchanges of energy between singlet and triplet manifolds. This follows from the observation that the sum of the fluorescence and phosphorescence quantum yields for powders is not constant with λ_{ex}. (The P/F curves for glasses show some structure but, considering the estimated errors in these curves many, but not all, of the variations in these curves probably are not meaningful.) As with powders, however, the sums of fluorescence and phosphorescence quantum yields for glasses are not constant with λ_{ex}. In every case the sum of these yields decreased for VUV excitation compared to NUV excitation. Thus, following excitation to higher states, it must be that energy is being partitioned into non-radiative dissipative modes -- perhaps into environmental or intramolecular degrees of freedom. (The observation that the total relative quantum yield of trypsin, for instance, behaves nearly the same in powders and glasses as a function of λ_{ex} indicates the importance of intramolecular perturbation; the different behavior of P/F curves when trypsin is changed from a crystal to a glass medium, however, indicates that environmental factors may be important, also.) Consequently, the second (and higher) excited states may be coupled to the ground

state by at least some mechanisms which do not go through the first excited states. One such mechanism may involve photolysis of the excited molecule.[1] (In this case, of course, the "ground state" would refer to that of the photoproducts.) A second mechanism would be the result of favorable crossing points of the potential curves of the ground, first and second excited states,[2] as pictured below:



Thus, in this case, high energy excitation to the second excited state might result in rapid radiationless transfer directly to the ground state (provided that the matrix elements for the transition were of the right magnitude). (Alternatively, the appropriate relationships among the potential surfaces to give rapid quenching may

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[1]This can be tested when sufficiently strong VUV sources for photolysis work become available.

[2]Flash photolysis is often helpful in resolving such problems: For a system with N+1 energy levels N independent equations are derivable. Each equation would equate the rate constants for molecules attaining a single energy level to those leaving it. In the present case however, only the rate constants for absorption (from the ground state) and emission (between the first excited and the ground states) are known. By using flash photolysis, more absorption rates between other energy levels can be determined experimentally so as to solve the N equations simultaneously.

be induced through distortion of these surfaces. Such a distortion may be the result of charge transfer (48) or radical production (49), for instance[2,3]; the exact state of vitrification of the glucose-water glass may also influence these surfaces (see references of 41). A third mechanism (to bypass the first excited state) could involve strong coupling of higher vibronic levels to environmental degrees of freedom[4], e.g., lattice vibrations in either the crystal or solvent.

Still another mechanism leading to an apparent decrease in quantum yields for short λ_{ex} would be the following: High energy UV radiation, say 8-10 ev, may cause absorption transitions from occupied molecular orbitals below the highest occupied one. If any of the subsequent emission (due to transitions between any two states) was not detected, perhaps due to instrumental response, F or P would appear to decrease.[5] Such a situation is diagrammed below. The crosses represent electrons occupying the levels. The resultant radiative transition could be between states 3 and 1 or 2 and 1, the former being too high in energy, the latter too low to be detected.

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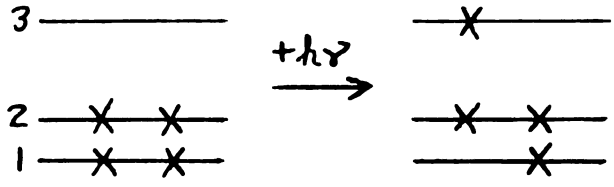
[4]One way to examine this would be to use "non-interacting environments". Vapors would be best but, perhaps, low dielectric constant solvents would also be useful. This experiment would be best run at extremely low temperatures to minimize vibrations.

[5]This can be tested by obtaining accurate emission spectra over a wide range of wavelengths.



Each of the mechanisms described above will probably be complicated, for short λ_{ex}, by excitation to σ-orbitals. Almost all of the work in this area has been concerned mainly with identification of such states in organic molecules (15) and with their participation in VUV photolysis (see reference 10). In particular, the efficiency with which high energy σ-excitations lead to phosphorescence in complex molecules is not known.

The spectra described above suggest that the differences in P/F ratios due to X ray and NUV excitation may not be simply explainable only on the basis of intersystem crossing between higher excited states. Rather, environmental-sensitive mechanisms may lead to efficient quenching of certain of these higher singlet and triplet states <u>in competition</u> with intersystem crossing. The nature of these environmental effects, as suggested by phosphorescence lifetime and thermal activation energy studies, forms the basis for the following discussion.

Phosphorescence lifetimes.

In changing the molecular environment from a crystal to a glass I found that the dependence of phosphorescence lifetime on λ_{ex} was lost. At the same time a good fit to the decay curves changed from the sum of two exponentials for powders to a single exponential line for glasses. Also, for phenylalanine powder, the phosphorescence lifetime was a function of particle-size.

These data suggest several alternative possibilities (no one of which may be equally applicable to all compounds): a. One possibility which seems unlikely is that an excited molecule remains for an appreciable time in upper triplet states. (Upper singlet states are eliminated since a long period of time spent in upper singlet states should lead to singlet emission from those states, and no fluorescence emission was observed in any compound from 200 to 270 mμ. In view of the normally very strong vibronic coupling for radiationless transitions (because of the high density of higher states; see ref. (6)) between upper levels this possibility can probably be excluded, however. This is probably even more rigorously applicable to crystals where molecular states interact strongly (6). b. A second possibility is that excitation to upper levels leads to selective localization of energy in different parts of the unit cell. Thus, by virtue of their orientation and position in the unit cell certain molecules may have somewhat lowered energy. These molecules would then act as traps for energy transferred by the various possible mechanisms. If, in a given unit cell, there are, say, two such traps populated differentially depending on λ_{ex} , then one

might expect certain of the data given in Results. That is, the lifetime dependence on λ_{ex} from powders would not be found in unoriented glasses and the decay curves from glasses could be fitted by only a single exponential -- not the sum of two exponentials as found in powders (the two powder exponentials ostensibly being the result of two "emitting positions" in the unit cell). Since the different "emitting positions" in the unit cell are selectively populated according to λ_{ex} this would probably lead to the same two species (or decay components) regardless of the mean decay lifetime although a different λ_{ex} might excite other locations and so change the components. The data for phenylalanine and trypsin are consistent with the former, those of tryptophan and tyrosine agree with the latter. Likewise, as found for phenylalanine, crystal defects, such as breaks due to pulverizing, could change the relative portioning of energy between the traps without changing the lifetimes of the traps. The nature of such effects would depend upon the position at which the unit cell fractures most easily, i.e., the nature of the crystal forces.[6] c. A third possibility is that separate parts of the same molecule are being excited -- for instance, the benzenoid region and the carboxyl region. If there is no strong interaction between the groups in a rigid crystal two decay components might result -- but if they interact in liquid or frozen (glass) solution only one component could result. The same reasoning could lead to a depen-

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[6] One way of testing these ideas might be to grow crystals by different techniques to give different kinds of unit cells (where possible. If the ideas in (6) are realistic different spectral properties should be found for similar subsequent treatment.

dence of lifetimes in powders on λ_{ex}, i.e., the two groups could absorb separately and/or be coupled differently to other absorbers or the environment. This mechanism probably would not lead to a dependence of lifetime on crystal size and would agree with all of the data except phenylalanine.[7] d. A fourth possibility is that the lifetime dependence on λ_{ex} is the result of charge separation, polymerization or other photochemical reaction. If such a reaction is the result of crystallinity (e.g., by formation of conduction bands, close proximity and proper orientation) then for glasses the lifetime should be independent of λ_{ex}. Furthermore, depending on the individual case, the condition of the crystal (e.g., size, shape, method of crystallization) might affect the phosphorescence lifetime through disruption of the "normal crystallinity". If absorbed radiation leaves some absorbing molecules unaltered (except for being in an excited state) and photochemically alters[1] others according to some rate constants then one might expect that the lifetime of the phosphorescence of the resulting "mixture" would be a function of λ_{ex}. (This phosphorescence could occur in the products themselves or upon recombination.) Further, two or more components should be found in the decay curve. These components should be the same for all mean lifetimes so long as the resultant emitting products are the same.[1] There is, however, no reason to believe that every com-

[7]There is some evidence to support, for instance, an interaction of the benzenoid region and the carboxyl region in glasses and solutions of aromatic amino acids (50, 51). To attempt the same tests in powders would be difficult; crystallographic studies would be necessary to assure similar crystal structure. If considerable electrical charge interaction is involved then magnetic resonance studies might show the perturbation.

pound will form only one or two photoproducts for a given λ_{ex}. In such a case the decay components would change with λ_{ex}. If this is the case, the fact that the mean phosphorescence lifetimes of tryptophan, tyrosine, and phenylalanine powders decrease, but that of trypsin increases, for increasing λ_{ex} is not surprising.[8]

One more point is noteworthy: my data show a smooth transition of lifetimes between those for NUV and X ray excitations reported by Augenstein et al for trypsin (1). Likewise, for NUV excited tryptophan and finely ground phenylalanine there is good agreement between my data and those of reference 1. The rest of the lifetime data, however, are at wide variance between the two studies. This is particularly true, in those cases, comparing my VUV work and the X ray excitation of reference 1. The size of the crystallites may be important but especially the nature of the two radiations should be examined. If these data are correct this provides strong evidence that ionizations may be important in such X ray spectroscopy studies.

Activation energies (E_A):

Perhaps the major point to be made concerning these measurements is that, in both powders and glasses, E_A is essentially inde-

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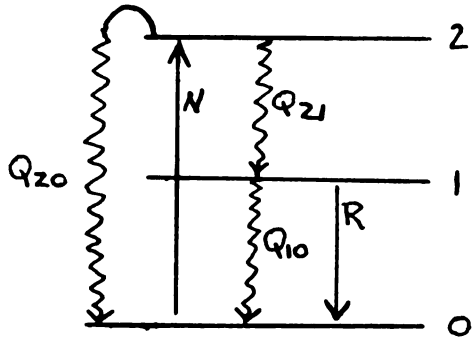
[8]Several ways to test these ideas are possible; they depend on the nature of the products and the phosphorescence that they emit. If phosphorescence occurs upon recombination of two products, say, then a second-order kinetics would be anticipated; this requires very accurate decay data taken over several decades of intensity. If the mechanism involves charge separation then photoconduction would be expected. For a photocatalyzed reaction the resultant products may be isolatable. Accurate emission spectra could show if a product (not the originally irradiated molecule) is emitting.

pendent of λ_{ex}. The implications of this conclusion can be clarified by the following analysis:



Consider a model system with ground state 0 and excited states 1 and 2, all of the same multiplicity. Let Q_{21} be the rate of radiationless quenching between states 2 and 1, R the emission rate (between 1 and 0), N the absorption rate (between 0 and 2 only), and Q_{20} represent the rate of any other process depopulating state 2 which does not result in emission between states 1 and 0. Thus, Q_{20} could include intersystem crossing (where the emissive wavelength would not correspond to that between 1 and 0), photolysis, and direct quenching between 2 and 0. In general, each of the processes shown in the above diagram will have an associated activation energy. The following equations result:

1) $Y = \frac{R}{N}$ where Y is the emission quantum yield.

2) $R = Q_{21} - Q_{10}$

3) $Q_{20} = Ne^{-E_{Q20}/kT}$

4) $Q_{21} = Ne^{-E_{Q21}/kT}$

5) $Q_{10} = Q_{21}e^{-E_{Q10}/kT}$

Combining equations 1)-5) gives

6) $$Y = 1 - e^{-E_{Q20}/kT} - e^{-(E_{Q21}+E_{Q10})/kT}$$

By exciting into the lowest excited state I can determine E_{Q10} (hence, E_R). From eqn. 6) it is apparent that, for Y to be independent of E_{Q20} and E_{Q10}, $E_{Q20} >> kT$ and $E_{Q10} >> E_{Q21}$. (Also if E_{Q20} is very small, one gets the physically realistic result that Y approaches zero.) Since my results, however, demonstrate extensive quenching of higher states (ostensibly represented by rate constants like Qno where $n > 1$) then factors other than activation energies must be considered in the above analysis. Thus, wavelength-dependent factors may be involved in the second and third terms on the right of eqn. 6).

For instance, I can write, as modifications of 3) and 6):

3a) $$Q_{20} = f(\lambda) \cdot N \cdot e^{-E_{Q20}/kT}$$

6a) $$Y = 1 - f(\lambda) \cdot e^{-E_{Q20}/kT} - e^{-E_{Q10}/kT}$$

where $E_{Q10} >> E_{Q21}$ and positive $f(\lambda)$ is small for short λ_{ex}. This can explain the low quantum yields (10^{-1} - 10^{-2}) for high energy excitation: E_{Q20} can now be small but the $f(\lambda)$ factor would keep the second term on the right of eqn. 6a) from getting too large. It is likely that the term involving E_{Q10} will also have some kind of wavelength dependence but it is not so critical as $f(\lambda)$.

This treatment has the effect of building into Y temperature- and wavelength-dependent factors. The latter factor could, for instance, involve perturbation matrix elements for the various radiative and radiationless transitions and would, therefore, be a function of configuration -- and spin -- coordinates as well as tempera-

ture.

This analysis is in agreement with the observation that the total emission quantum yield decreases for excitation to higher states in both glasses and powders. Thus, this decrease in yield may reflect low E_A's for quenching higher excited states as well as indicating strong quantum mechanical coupling factors among the higher states.

Two conclusions follow from these data: First, while the apparently low activation energies for upper state quenching lead to low quantum yields at short λ_{ex}, this quenching may not necessarily lead to enzyme inactivation (as mentioned earlier). This follows from the observations of relatively constant enzyme inactivation quantum yield for trypsin for λ_{ex} longer than about 160 mμ whereas the emission quantum yield for trypsin varies by a factor of ten over the same range of λ_{ex} (47). Secondly, the activation energy for inactivation of trypsin at 300°K is about 10^{-1}ev -- which corresponds to the quenching of triplet, not singlet, emissive states (52). The activation energies for the quenching of emission may correspond to intramolecular vibrations, rotations, or lattice vibrations (53).

Finally, as with phosphorescence lifetimes, there are differences between my E_A data and those of other workers which exceed the estimated errors (42). Thus, the data from spectra, lifetimes, and thermal activation energies indicate that ionizations may be important events in x-ray spectroscopic investigations of amino acids and crystals.

CONCLUSIONS

1. Powders of tryptophan, tyrosine, and trypsin exhibit enhanced intersystem crossing rates among higher excited states compared to lower excited states at 110°K.

2. Phenylalanine, tyrosine, tryptophan, and trypsin as powders and in D_2O-glucose solutions at 110°K show efficient quenching of higher excited states compared to lower states.

3. In powders these compounds probably have at least two separate emitting triplet species but in solutions they have only one. This leads to a dependence of phosphorescence lifetime on excitation wavelength.

4. The thermal activation energy for radiationless transitions from higher states is very small ($< 10^{-3}$ ev); howver, the quenched energy can lead to enzyme inactivation only through specific paths, if at all.

5. Ionizations may be important in determining the nature of X ray-induced emission in aromatic amino acids and trypsin.

6. The nature of NUV and VUV-induced emission in these compounds depends critically upon the molecular environment.

Table I: Fluorescence quantum yields, 250 mμ excitation, 300°K.

Compounds (powders)	Yield relative to sodium salicylate[a]
tryptophan	0.84 ± 0.17
tyrosine	0.09 ± 0.02
phenylalanine	0.70 ± 0.14
trypsin	0.26 ± 0.05

[a]An estimate of the quantum yields at 110°K can be obtained from consideration of activation energy calculations. In such a case, the above figures should be multiplied by about 1.5 (try), 2.5 (tyr), 2.0 (phe), and 2.0 (trypsin). (This apparently limits the quantum yield of sodium salicylate to 0.70 or less.) These factors represent the increase found in quantum yields when the sample temperature was lowered from 300°K to 100°K.

Table II: Phosphorescence decay times for powders.

	Phos. decay, τ_{37} (sec), 110°K		
	λexc. (mμ)	mean (±0.2sec)	components (±0.2sec)
Tryptophan powder	135	2.0	1.2, 3.7
	160	1.6	1.1, 3.6
	205	1.5	0.6, 2.4
	280	1.4	0.7, 2.6
Tyrosine powder	135	1.8	1.5, 4.1
	160	1.8	1.2, 3.7
	205	1.5	1.1, 2.9
	280	1.4	0.6, 3.4
Phenylalanine powder[a]	135	3.6	1.2, 5.0
	160	3.6	1.0, 5.0
	190	2.6	1.0, 4.4
	220	3.4	1.2, 4.5
	260	2.8	1.2, 4.7
Trypsin powder	135	1.9	1.3, 4.3
	160	2.0	0.9, 4.2
	220	2.6	1.1, 5.0
	280	3.6	1.0, 4.4

[a]See Table III for effect of powder size on τ_{37}.

Table III

Decay of phenylalanine phosphorescence, τ_{37}(sec), ±0.2sec, 110°K.

sieve size (μ) given, powders

λ exc. (mμ)	44	44-74	74-149	149-177
160	2.2sec.	1.9sec.	2.8sec.	3.6sec.
185	2.2	1.9	2.8	3.4
200	2.0	1.7	2.4	2.9
225	1.7	1.2	2.6	3.1
260	1.3	1.1	2.6	2.9

Table IV: Phosphorescence lifetimes for solutes in D_2O-glucose glasses at 110°K, excited at 160, 210, and 260 mμ.

	mean $\tau_{3/7}$ (sec)
tryptophan	5.6
tyrosine	2.4
phenylalanine	4.4
trypsin	6.0

Table V: Quenching activation energies for powders.

	Activation Energies (ev)					
	λexc.(mμ)	fluor.Ea	break	λexc.(mμ)	Phos.Ea	break[a]
		$(^{+40\%}_{-20\%})$	±20°K		$(^{+40\%}_{-20\%})$	±20°K
Tryptophan powder	160	0.01,0.004	220	160	0.05,0.01	205
	220	0.01,0.004	240	220	0.05,0.02	180
	280	0.01,0.003	240	280	0.05,0.02	180
Tyrosine powder	160	0.02,0.005	220	160	0.14,0.02	212
	220	0.01,0.005	202	220	0.14,0.02	212
	280	0.01,0.005	202	280	0.14,0.02	197
Phenylalanine powder	160	0.02,0.003	212	160	0.05,0.01	160
	220	0.02,0.003	225	220	0.05,0.01	166
	280	0.02,0.003	220	280	0.05,0.01	166
Trypsin powder	160	0.007,0.003	170	135	0.05,0.01	192
	220	0.007,0.003	170	160	0.05,0.01	192
	280	0.007,0.003	170	220	0.04,0.01	217
				280	0.04,0.01	217

[a]Temperature (°K) at which Arrhenius plot straight-line portions meet.

Table VI: Quenching activation energies (E_A) for solutes in D_2O-glucose glasses.

	fluorescence E_A(ev) ($^{+20}_{-25}$%)	break point (^oK) ($\pm 30^oK$)	phosphorescence E_A(ev) (± 50%)	break point (^oK) ($\pm 30^oK$)
tryptophan	0.05, 0.01	210	0.5, 0.01	230
tyrosine	0.03, 0.02	200	0.1, 0.01	210
phenylalanine	0.03, 0.01	180	0.1, 0.02	160
trypsin	0.04,0.01	200	0.2, 0.02	195

Table VII: Comparison of data with previous work

	P/F, powders (77°K)				Phos. mean τ_{37}(sec), powders			
	NUV[1]	Xray[1]	NUV[2]	VUV[2]	NUV[1]	Xray[1]	NUV[2,3]	VUV[2,3]
tryptophan	0.06	0.6	0.003	0.04	1.5	1.3	1.4	2.0
tyrosine	0.6	100	0.24	0.24	0.4	0.2	1.4	1.8
phenylalanine	0.22	10	0.07	0.60	0.5	3.2	2.8	3.6
trypsin	0.22	2.0	0.07	0.35	4.0	2.2	3.6	1.9

	Phos. mean τ_{37}(sec), glasses		Activation energies (ev) powders			
	NUV[4]	NUV[2]	F[5] (Xray)	P[5] (Xray)	F[2] (UV)	P[2] (UV)
tryptophan	3.9	5.6	0.002,0.012	0.016,0.06	0.004,0.01	0.01,0.05
tyrosine	1.1	2.4	0.005, ?	0.005,0.24	0.005,0.02	0.02, 0.14
phenylalanine	5.5	4.4	0.002,0.01	0.006,0.15	0.003,0.02	0.01,0.05
trypsin	6.5	6.0	? ?	0.007,0.21	0.003,0.007	0.01,0.04

[1]reference 1, [2]present work, [3]200 mesh sieve, [4]reference 41, [5]reference 42.

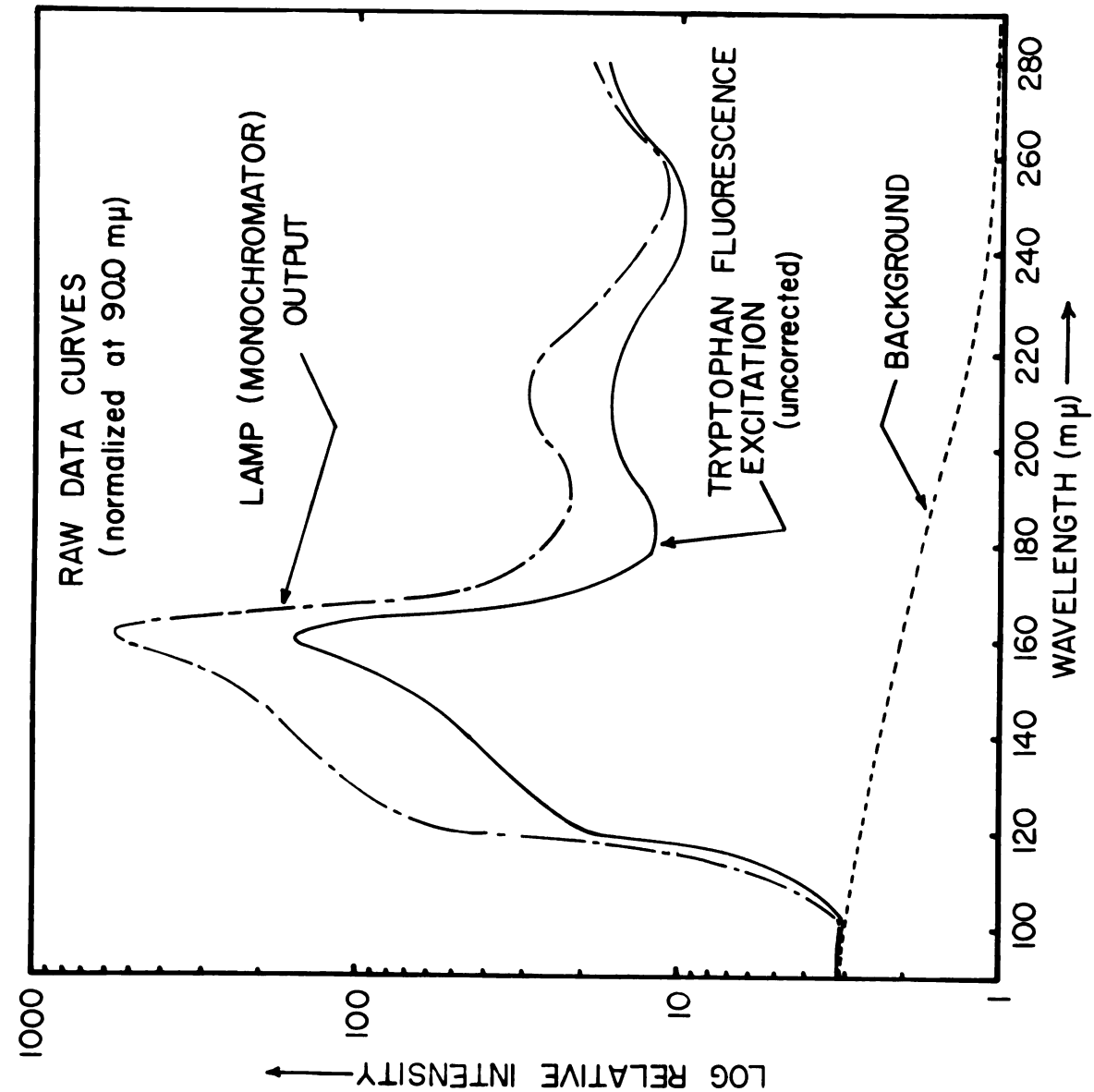

RAW DATA CURVES
(normalized at 900 mμ)
LAMP (MONOCHROMATOR)
OUTPUT
TRYPTOPHAN FLUORESCENCE
EXCITATION
(uncorrected)
BACKGROUND
LOG RELATIVE INTENSITY
1000
100
10
1
100
120
140
160
180
200
220
240
260
280
WAVELENGTH (mμ)

Figure I

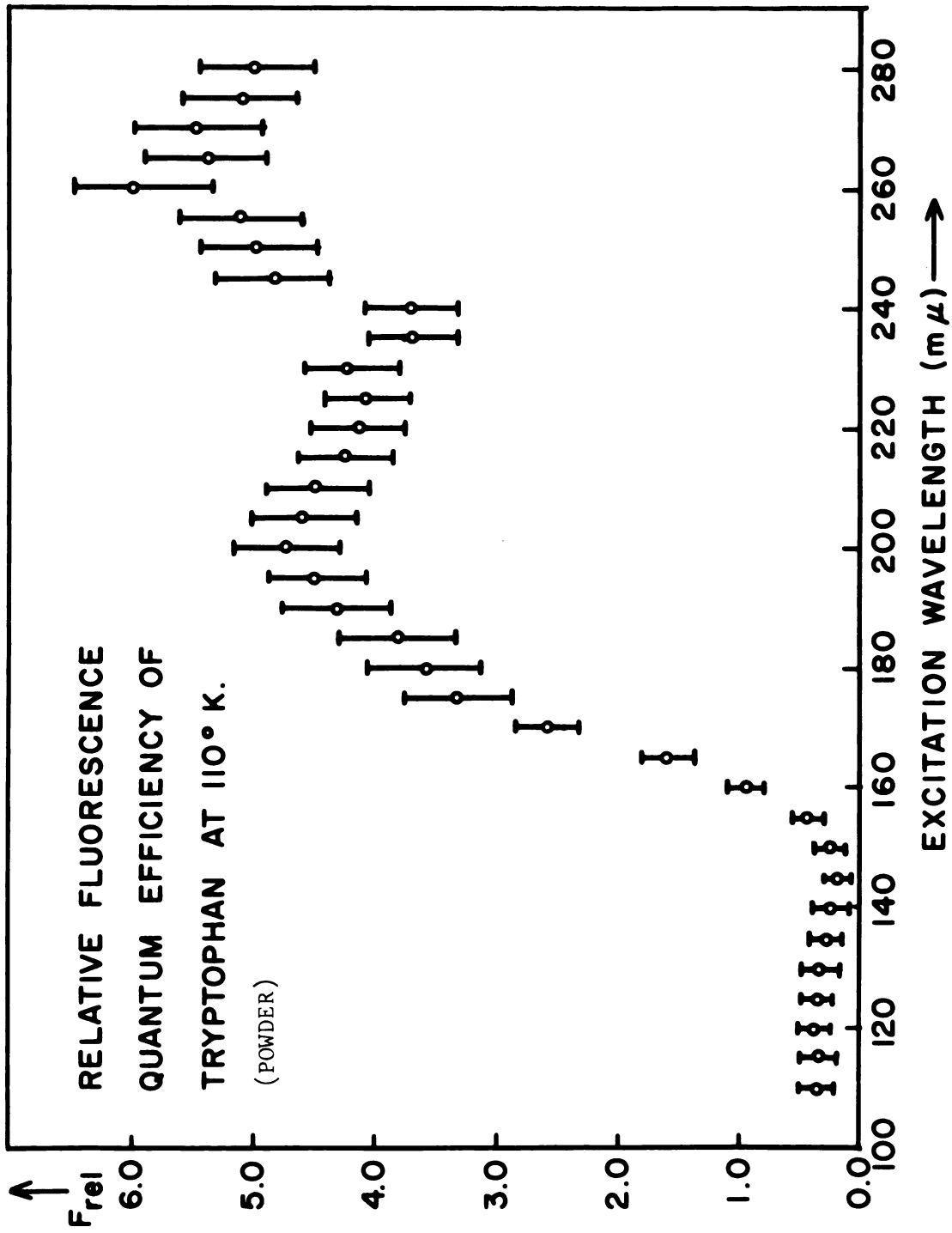

RELATIVE FLUORESCENCE
QUANTUM EFFICIENCY OF
TRYPTOPHAN AT 110° K.
(POWDER)
F_{rel}
6.0
5.0
4.0
3.0
2.0
1.0
0.0
100
120
140
160
180
200
220
240
260
280
EXCITATION WAVELENGTH (mμ)

Figure II

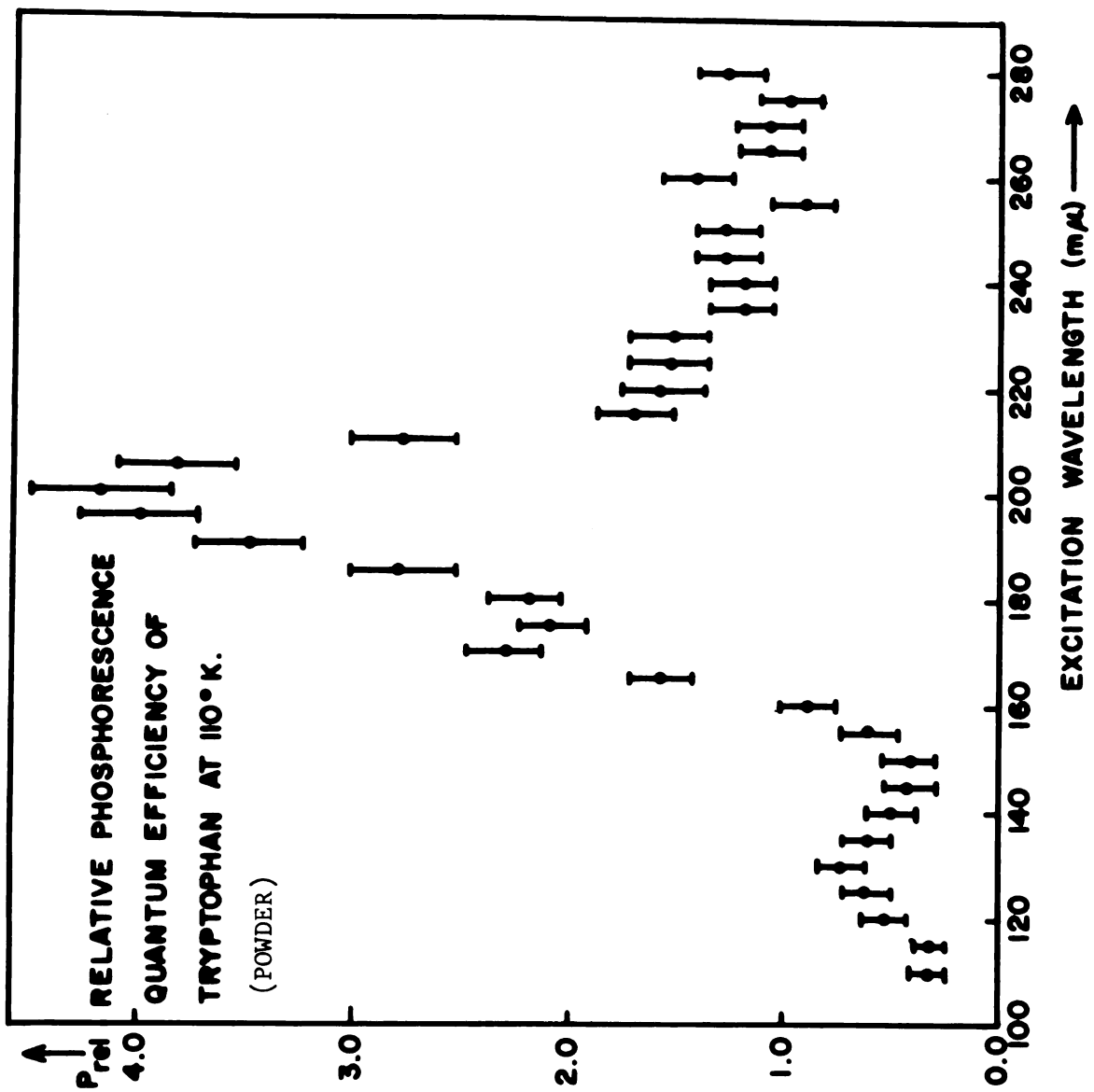

RELATIVE PHOSPHORESCENCE
QUANTUM EFFICIENCY OF
TRYPTOPHAN AT 110° K.
(POWDER)
P_{rel}
4.0
3.0
2.0
1.0
0.0
100
120
140
160
180
200
220
240
260
280
EXCITATION WAVELENGTH (mμ)

Figure III

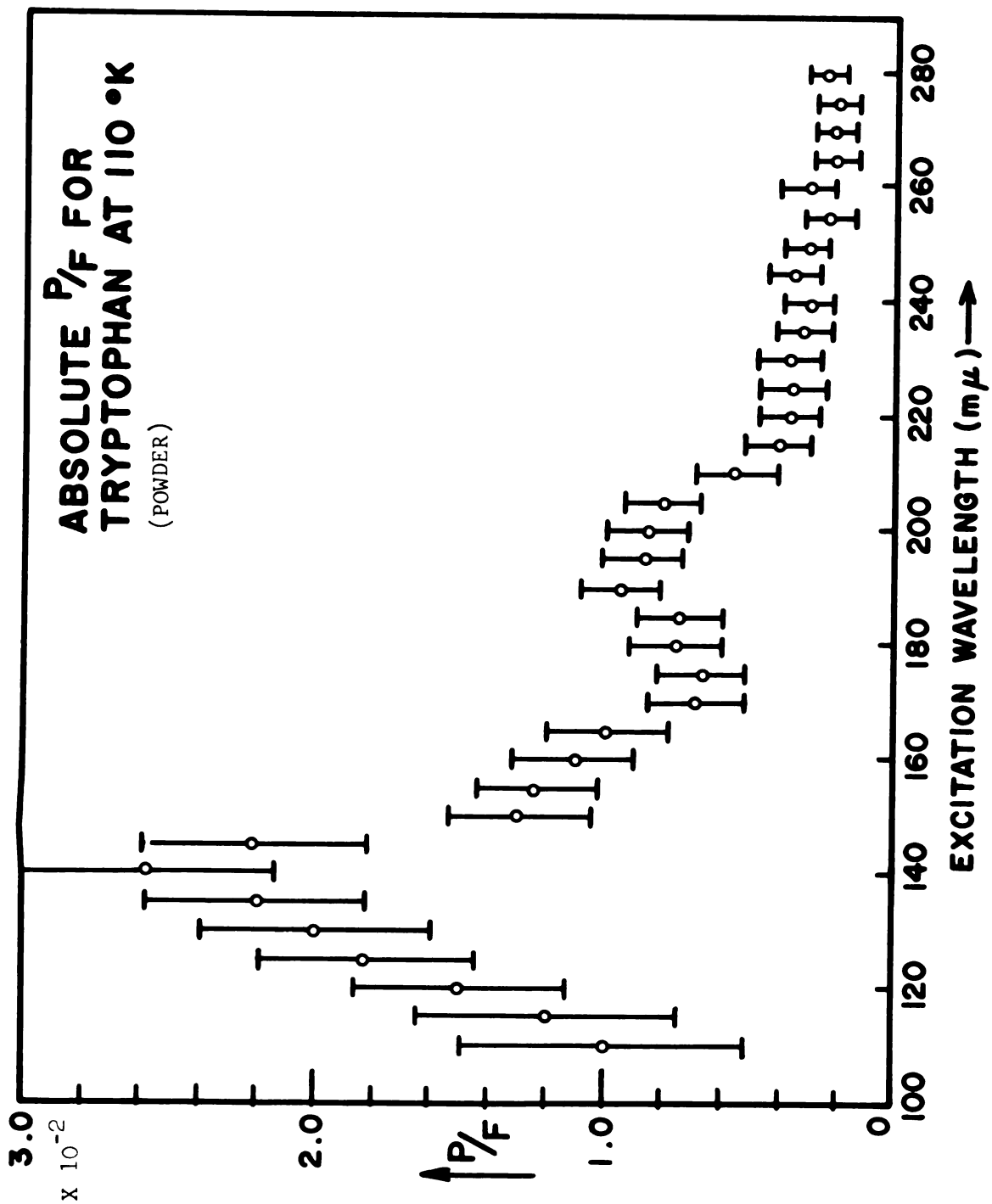

ABSOLUTE P/F FOR
TRYPTOPHAN AT 110 °K
(POWDER)
P/F
X 10⁻²
3.0
2.0
1.0
0
100
120
140
160
180
200
220
240
260
280
EXCITATION WAVELENGTH (mμ)

Figure IV

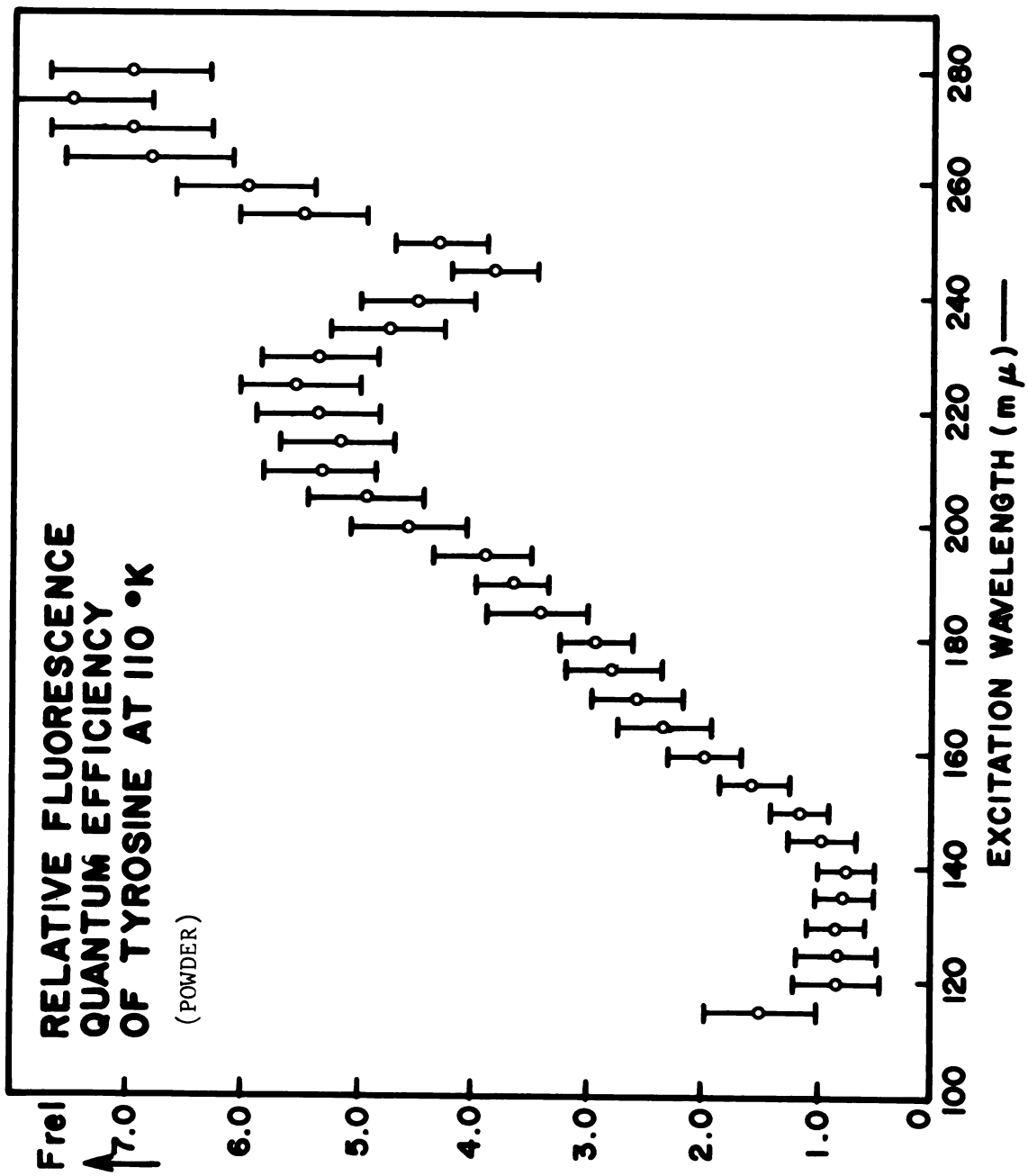

RELATIVE FLUORESCENCE
QUANTUM EFFICIENCY
OF TYROSINE AT 110 °K
(POWDER)
Frel
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
100
120
140
160
180
200
220
240
260
280
EXCITATION WAVELENGTH (mμ)

Figure V

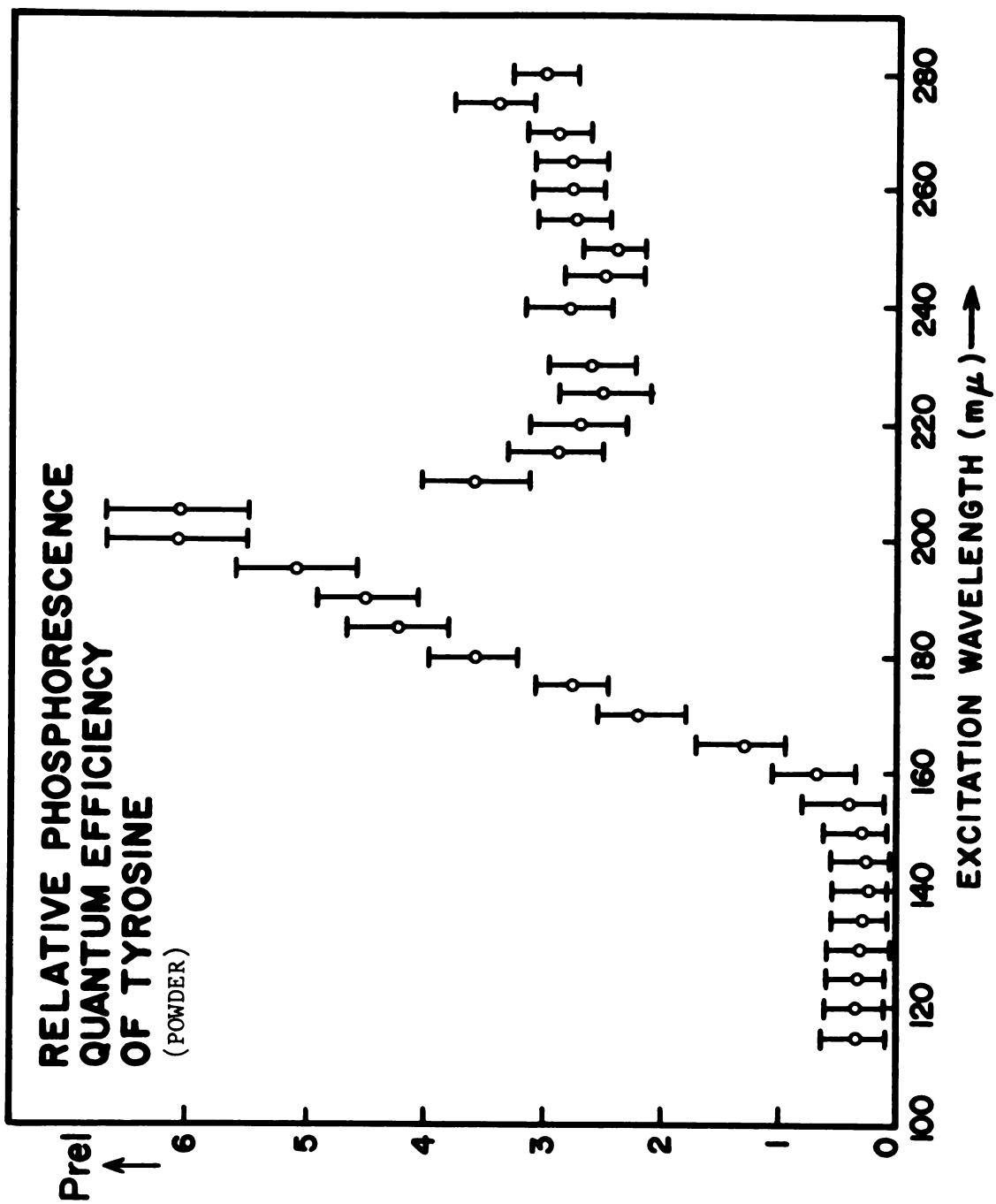

RELATIVE PHOSPHORESCENCE
QUANTUM EFFICIENCY
OF TYROSINE
(POWDER)
Prel
0
1
2
3
4
5
6
100
120
140
160
180
200
220
240
260
280
EXCITATION WAVELENGTH (mμ)

Figure VI

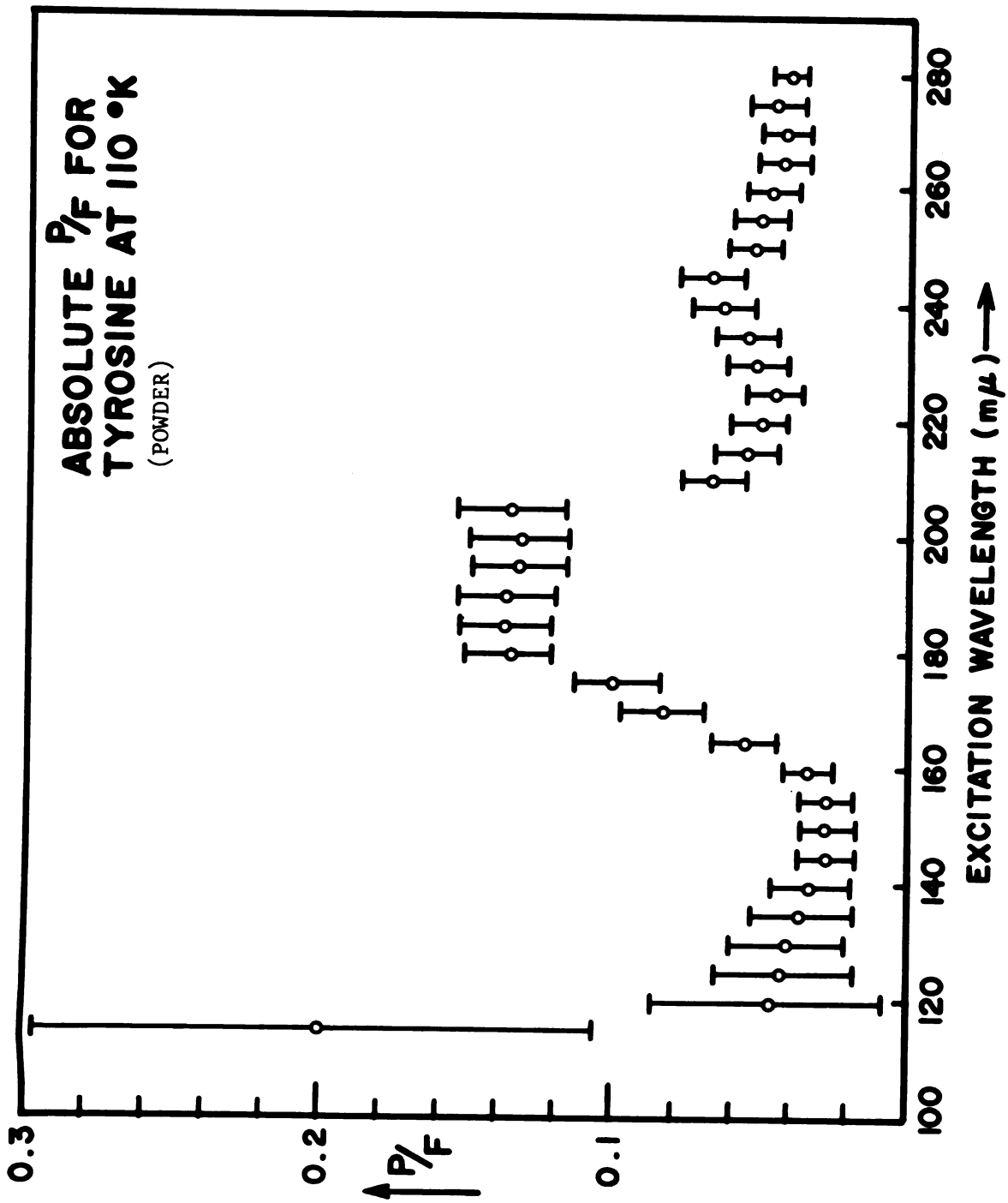

ABSOLUTE P/F FOR
TYROSINE AT 110 °K
(POWDER)
P/F
0.3
0.2
0.1
100
120
140
160
180
200
220
240
260
280
EXCITATION WAVELENGTH (mμ)

Figure VII

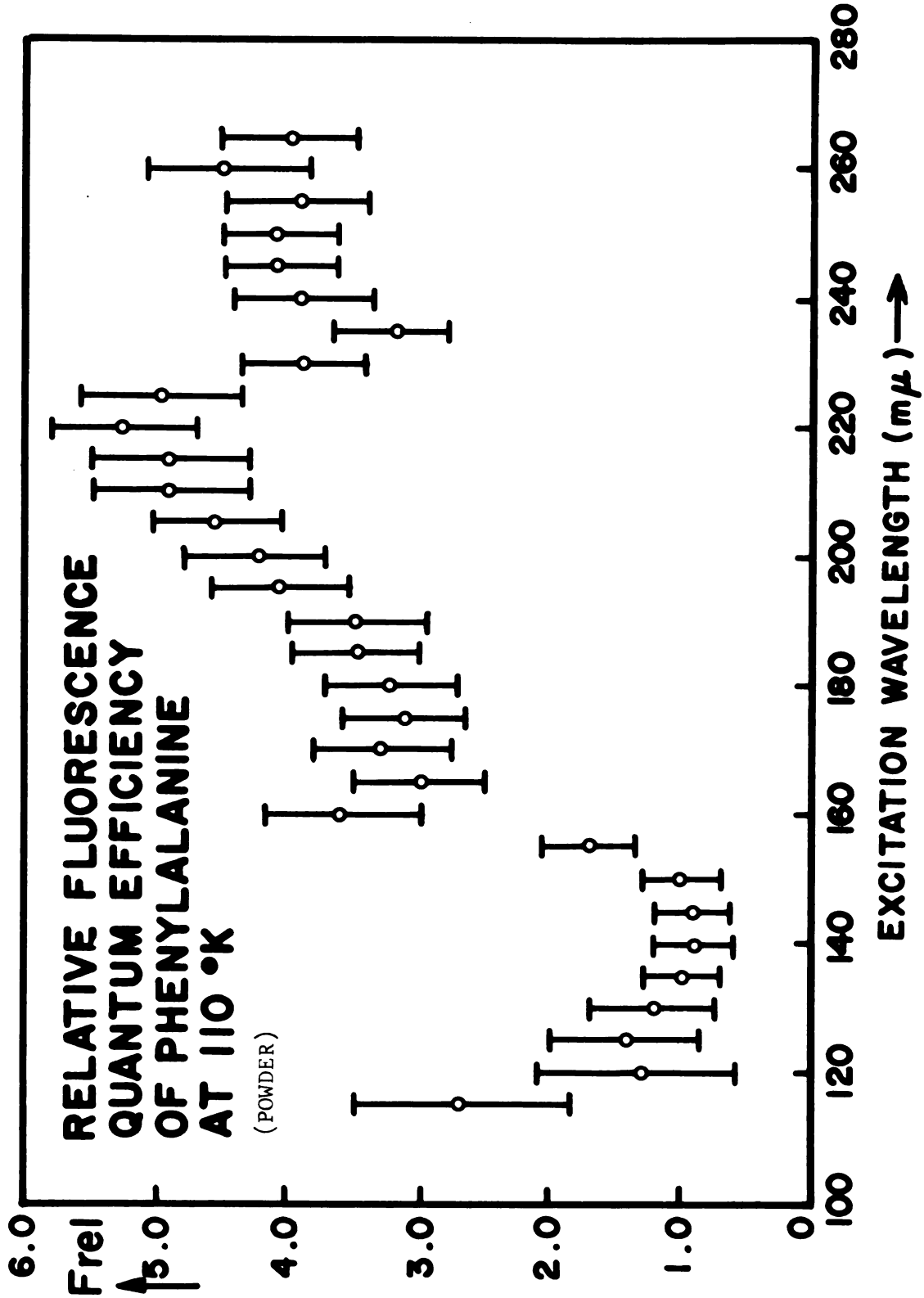

RELATIVE FLUORESCENCE
QUANTUM EFFICIENCY
OF PHENYLALANINE
AT 110 °K
(POWDER)
Frel
6.0
5.0
4.0
3.0
2.0
1.0
0
100
120
140
160
180
200
220
240
260
280
EXCITATION WAVELENGTH (mμ)

Figure VIII

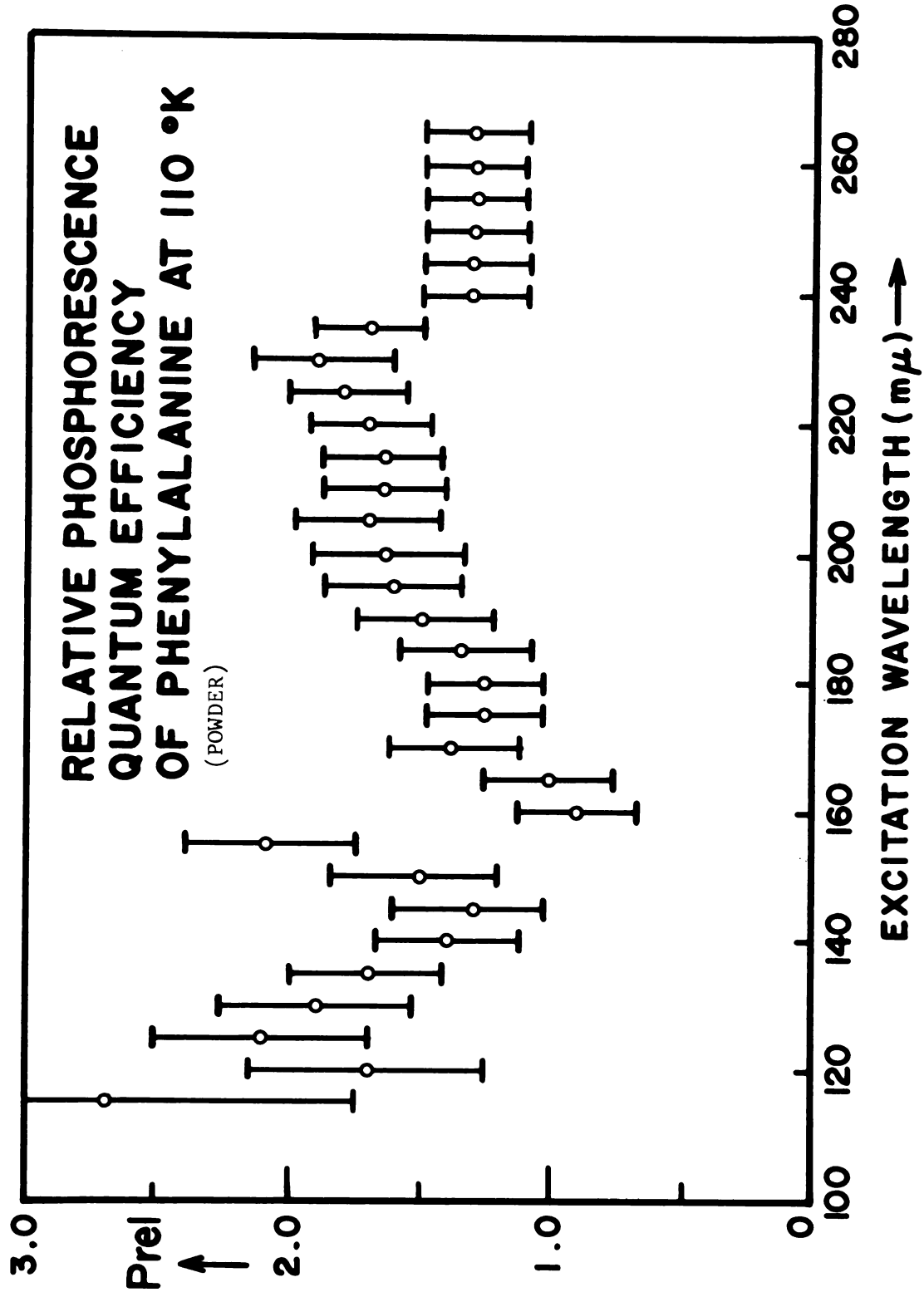

RELATIVE PHOSPHORESCENCE
QUANTUM EFFICIENCY
OF PHENYLALANINE AT 110 °K
(POWDER)
Prel
3.0
2.0
1.0
0
100
120
140
160
180
200
220
240
260
280
EXCITATION WAVELENGTH (mμ)

Figure IX

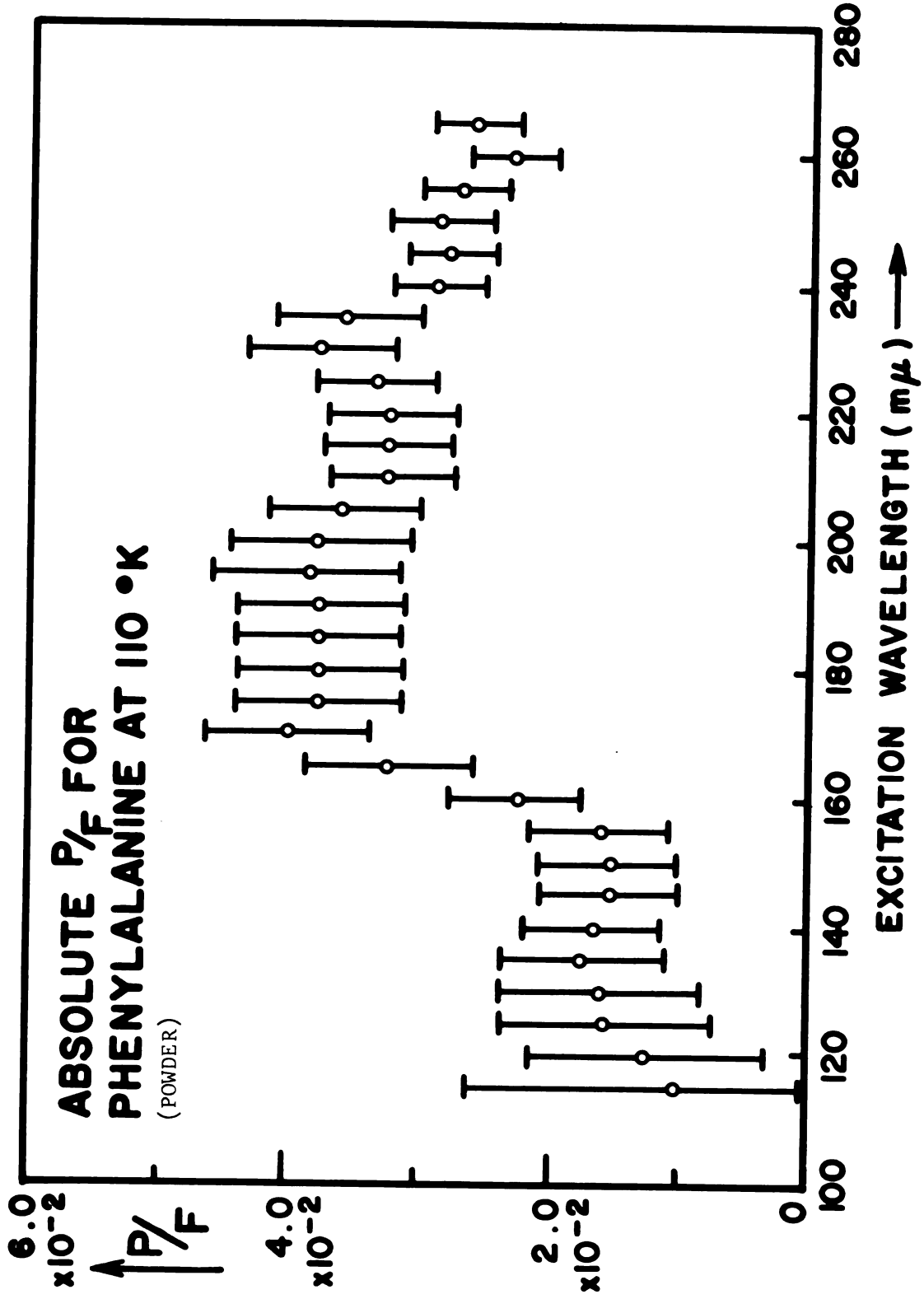

ABSOLUTE P/F FOR
PHENYLALANINE AT 110 °K
(POWDER)
P/F
6.0 x10⁻²
4.0 x10⁻²
2.0 x10⁻²
0
100
120
140
160
180
200
220
240
260
280
EXCITATION WAVELENGTH (mμ)

Figure X

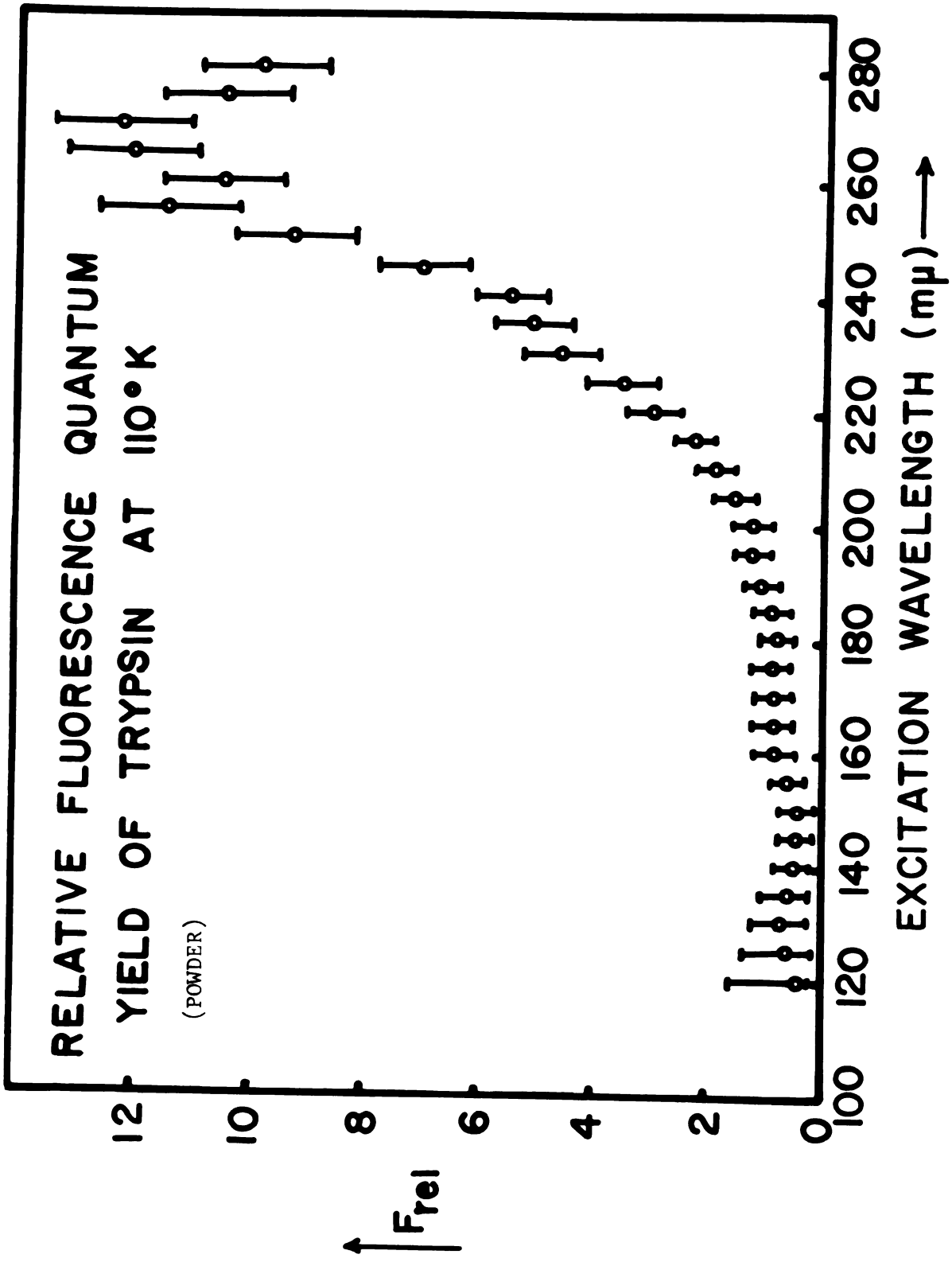

RELATIVE FLUORESCENCE QUANTUM
YIELD OF TRYPSIN AT 110° K
(POWDER)
F_{rel}
0
2
4
6
8
10
12
100
120
140
160
180
200
220
240
260
280
EXCITATION WAVELENGTH (mμ)

Figure XI

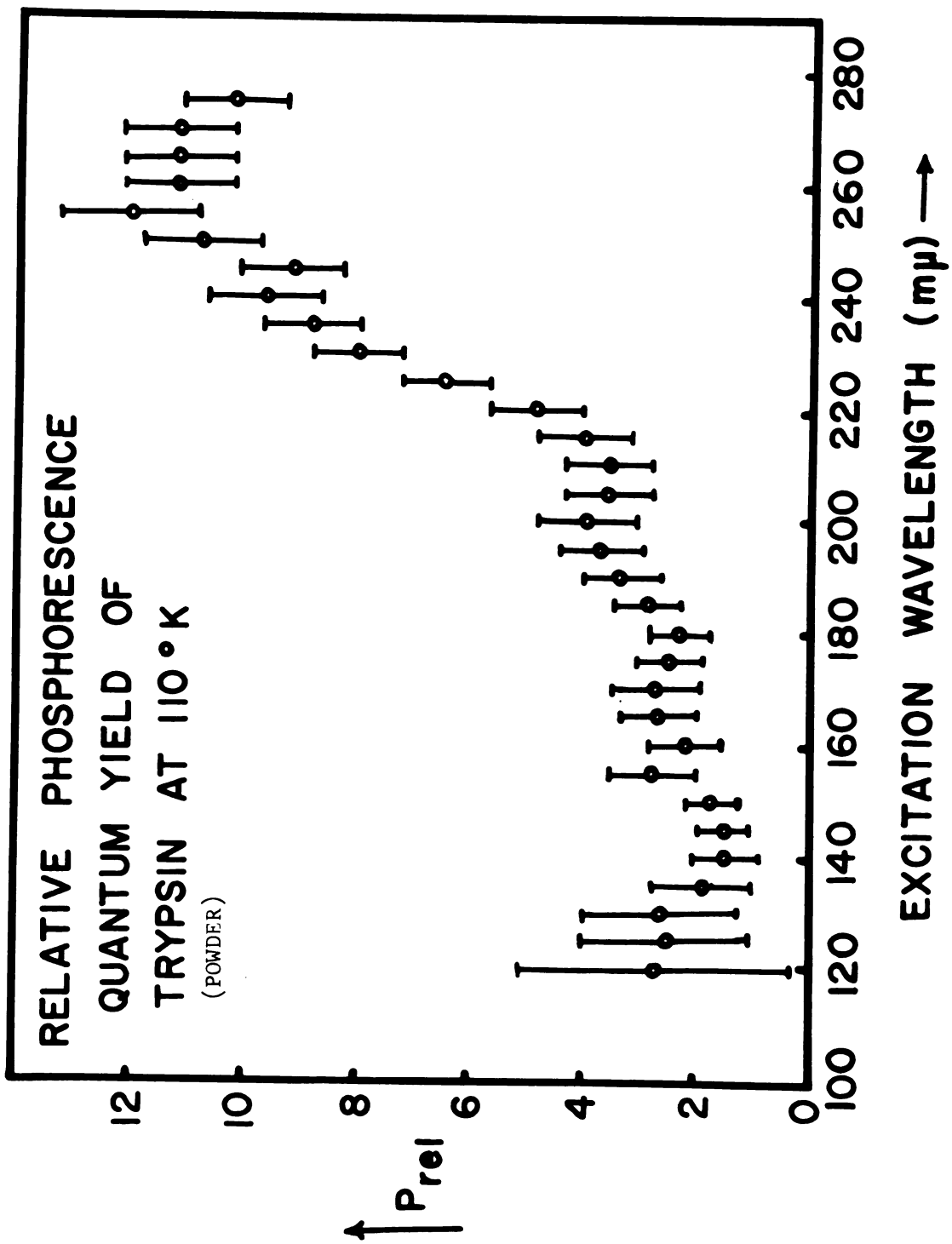

RELATIVE PHOSPHORESCENCE
QUANTUM YIELD OF
TRYPSIN AT 110°K
(POWDER)
P_{rel}
0
2
4
6
8
10
12
100
120
140
160
180
200
220
240
260
280
EXCITATION WAVELENGTH (mμ)

Figure XII

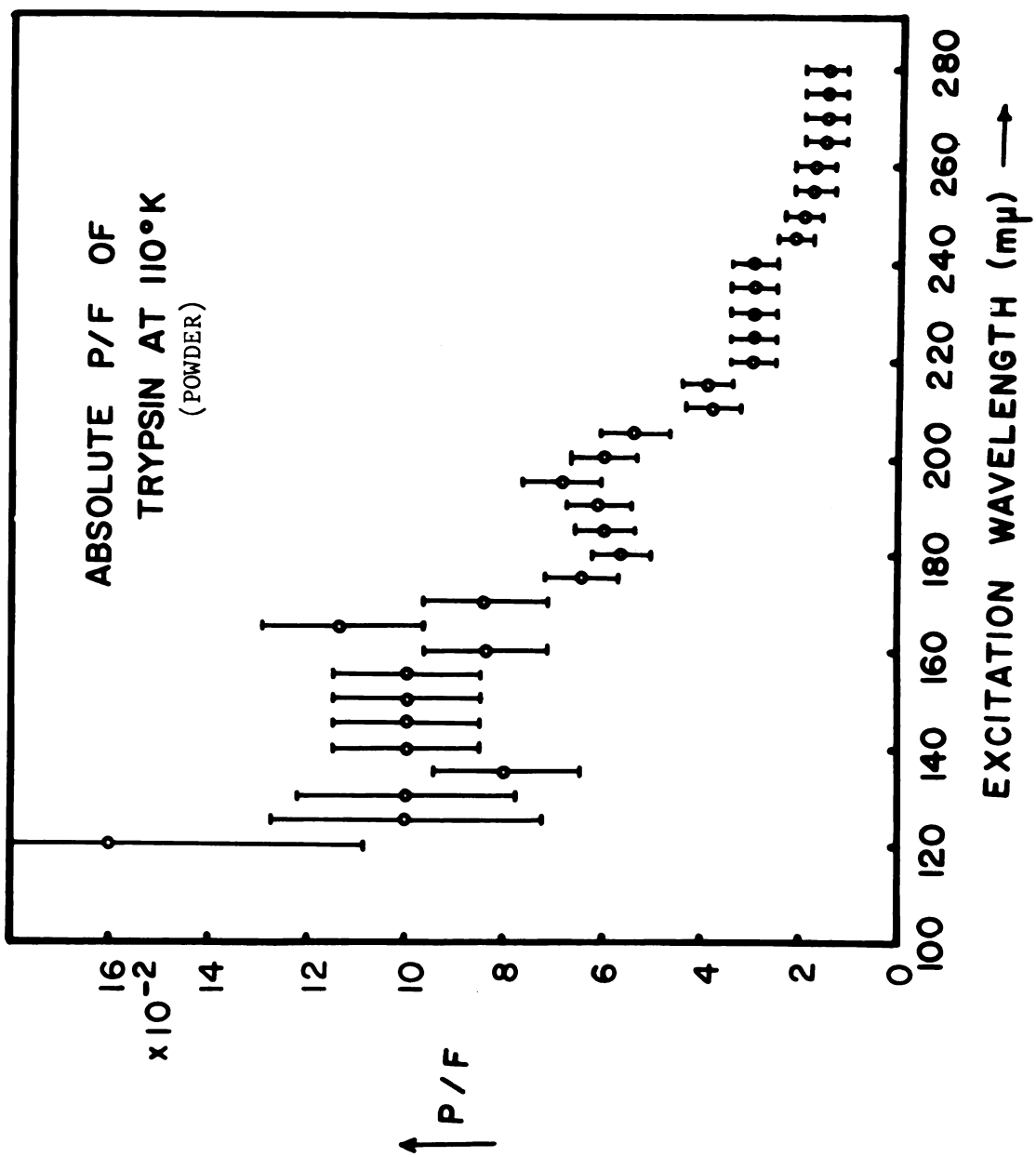

ABSOLUTE P/F OF
TRYPSIN AT 110°K
(POWDER)
P/F
x10⁻²
16
14
12
10
8
6
4
2
0
100 120 140 160 180 200 220 240 260 280
EXCITATION WAVELENGTH (mμ)

Figure XIII

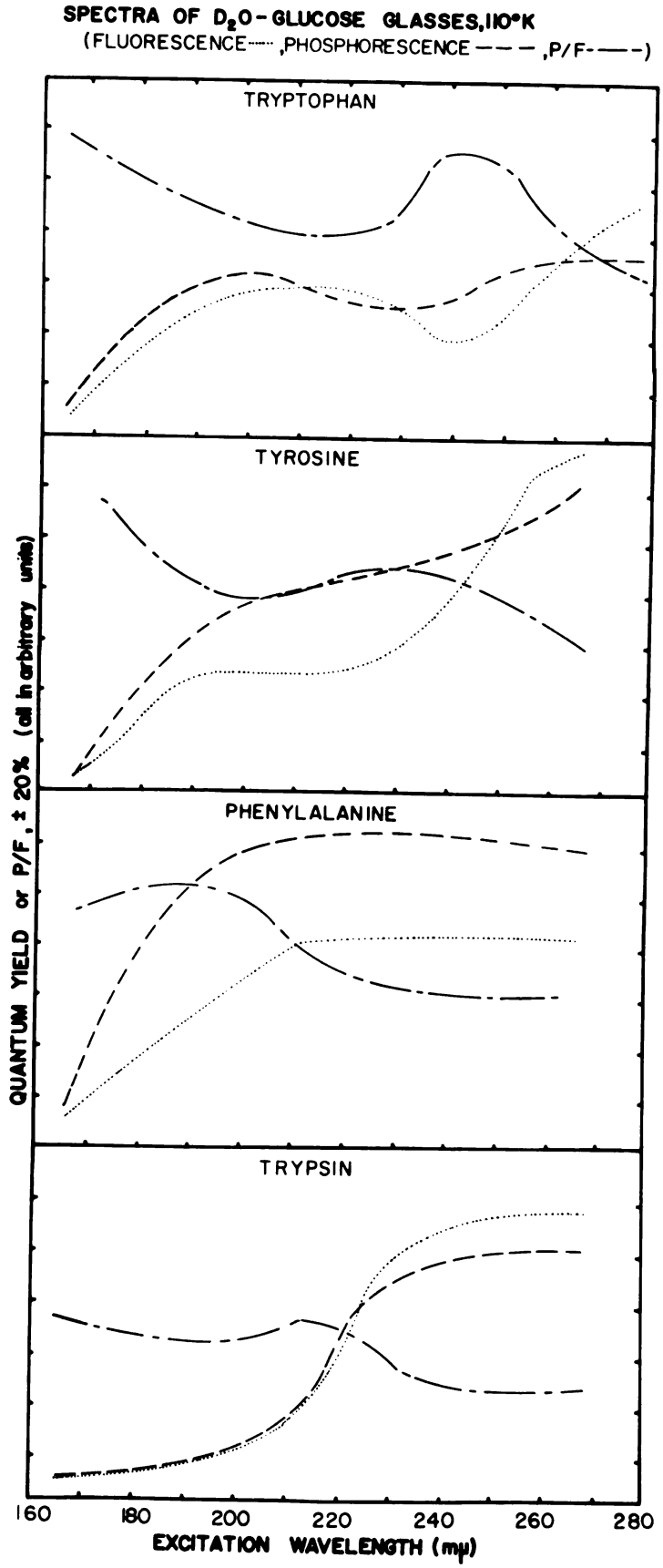

SPECTRA OF D_2O - GLUCOSE GLASSES, 110°K
(FLUORESCENCE ····· , PHOSPHORESCENCE — — — , P/F- ———)
TRYPTOPHAN
TYROSINE
PHENYLALANINE
TRYPSIN
QUANTUM YIELD or P/F, ± 20% (all in arbitrary units)
160
180
200
220
240
260
280
EXCITATION WAVELENGTH (mμ)

Figure XIV

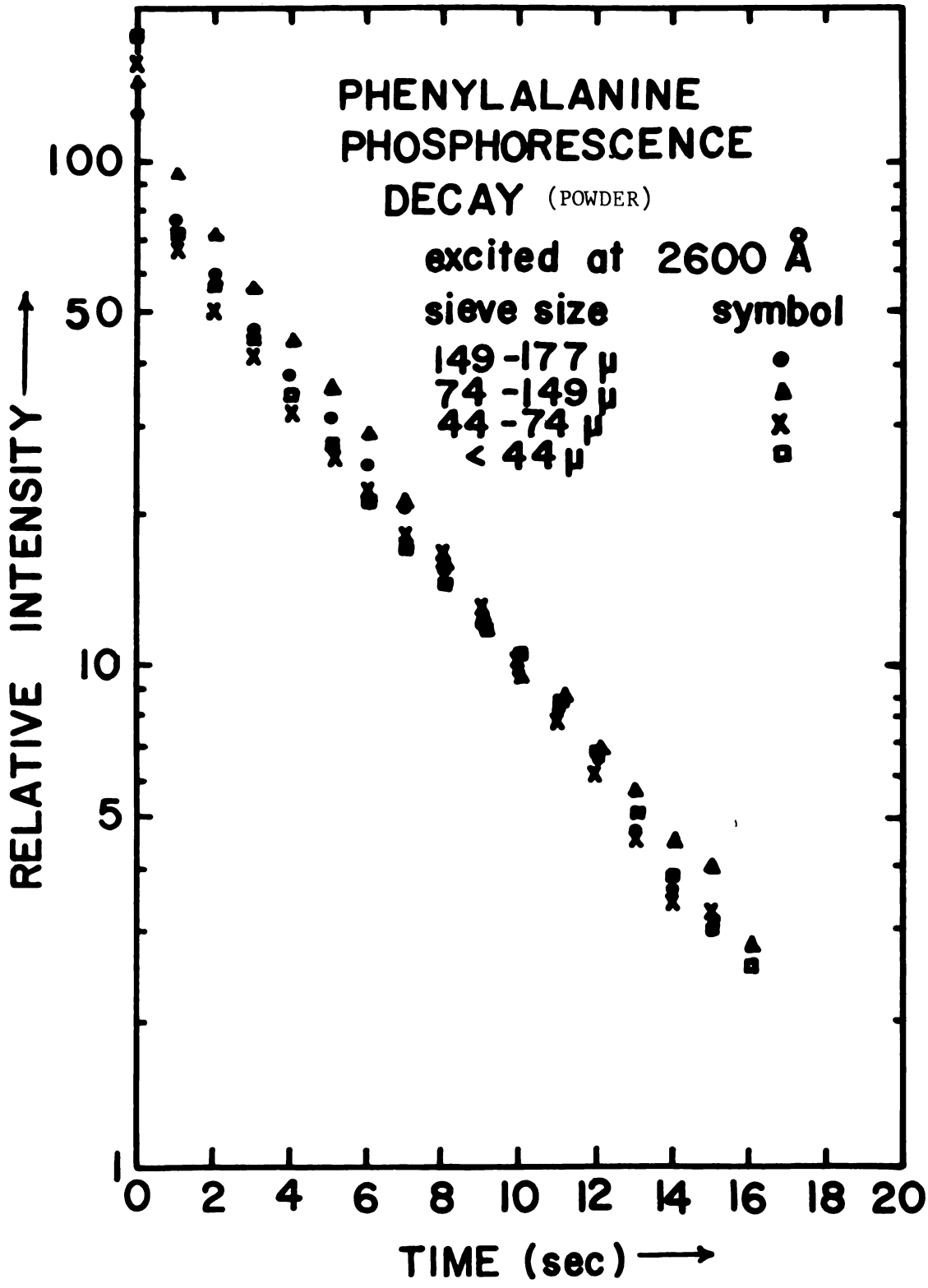

PHENYLALANINE
PHOSPHORESCENCE
DECAY (POWDER)
excited at 2600 Å
sieve size
symbol
149-177 μ
74-149 μ
44-74 μ
< 44 μ
RELATIVE INTENSITY
100
50
10
5
1
0
2
4
6
8
10
12
14
16
18
20
TIME (sec)

Figure XV

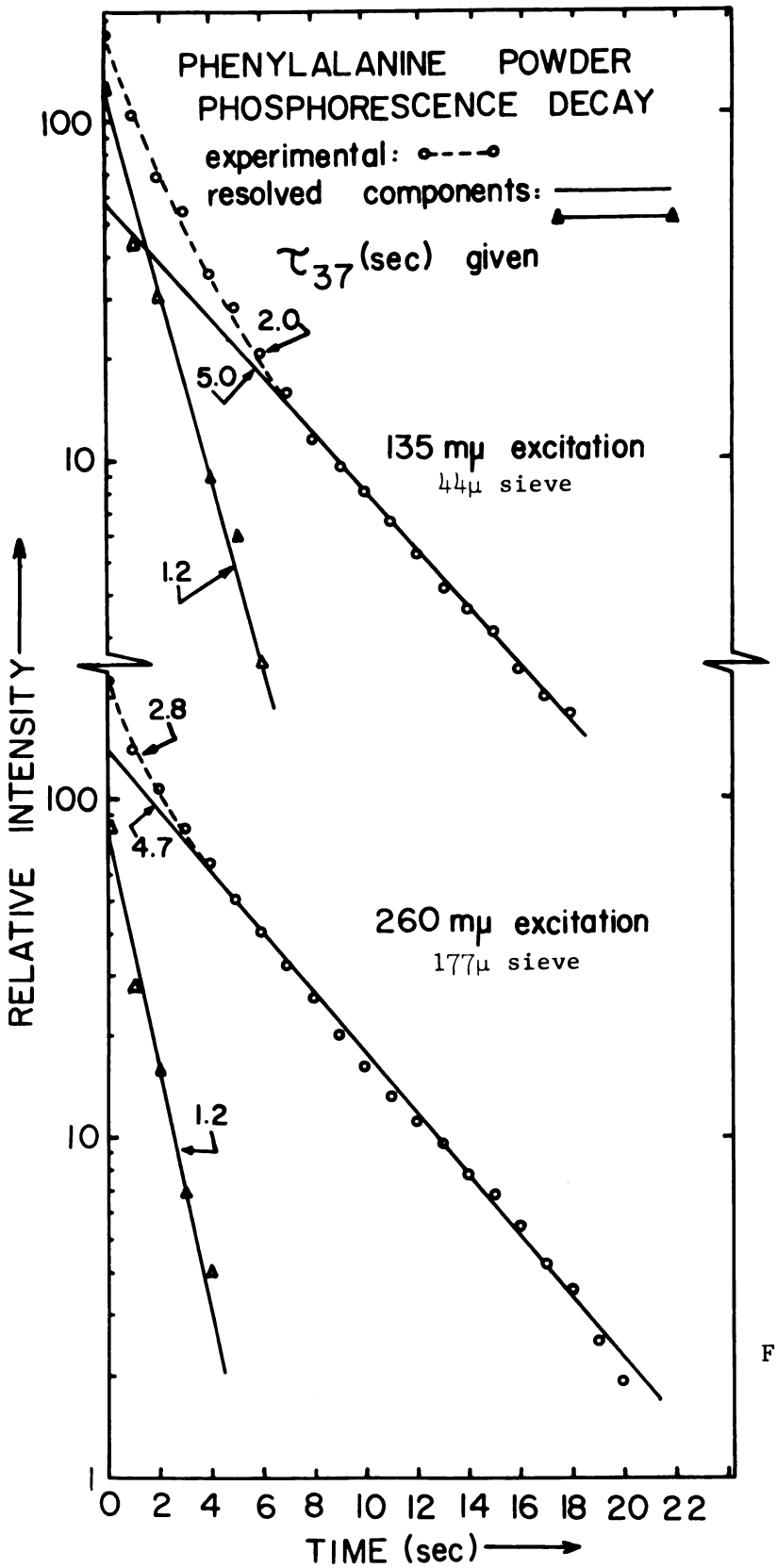

PHENYLALANINE POWDER
PHOSPHORESCENCE DECAY
experimental:
resolved components:
τ_{37} (sec) given
2.0
5.0
135 mμ excitation
44μ sieve
1.2
2.8
4.7
260 mμ excitation
177μ sieve
1.2
RELATIVE INTENSITY
100
10
100
10
1
0 2 4 6 8 10 12 14 16 18 20 22
TIME (sec)

Figure XVI

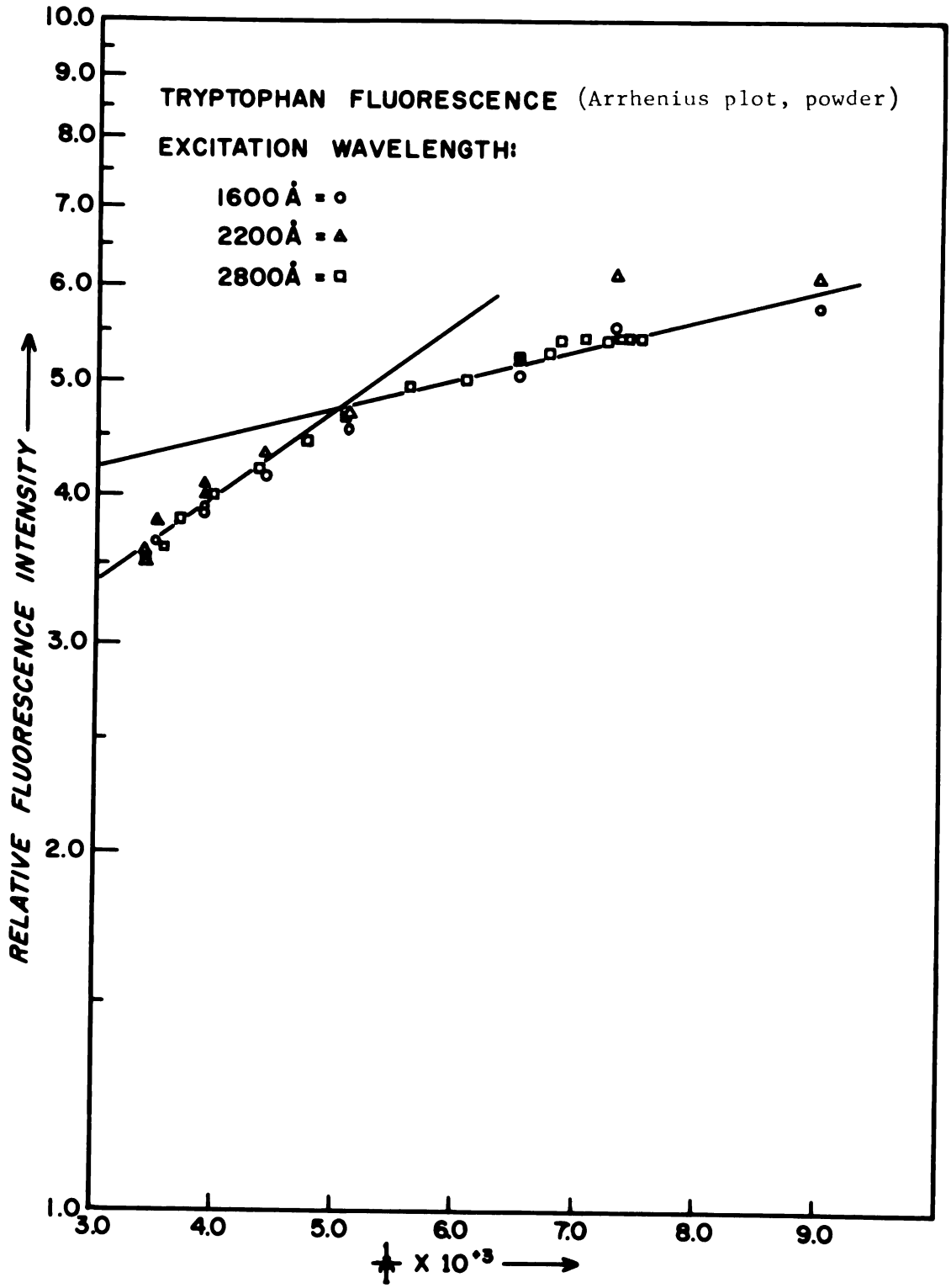

TRYPTOPHAN FLUORESCENCE (Arrhenius plot, powder)
EXCITATION WAVELENGTH:
1600 Å = o
2200Å = ▲
2800Å = □
RELATIVE FLUORESCENCE INTENSITY
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
X 10^-3

Figure XVII

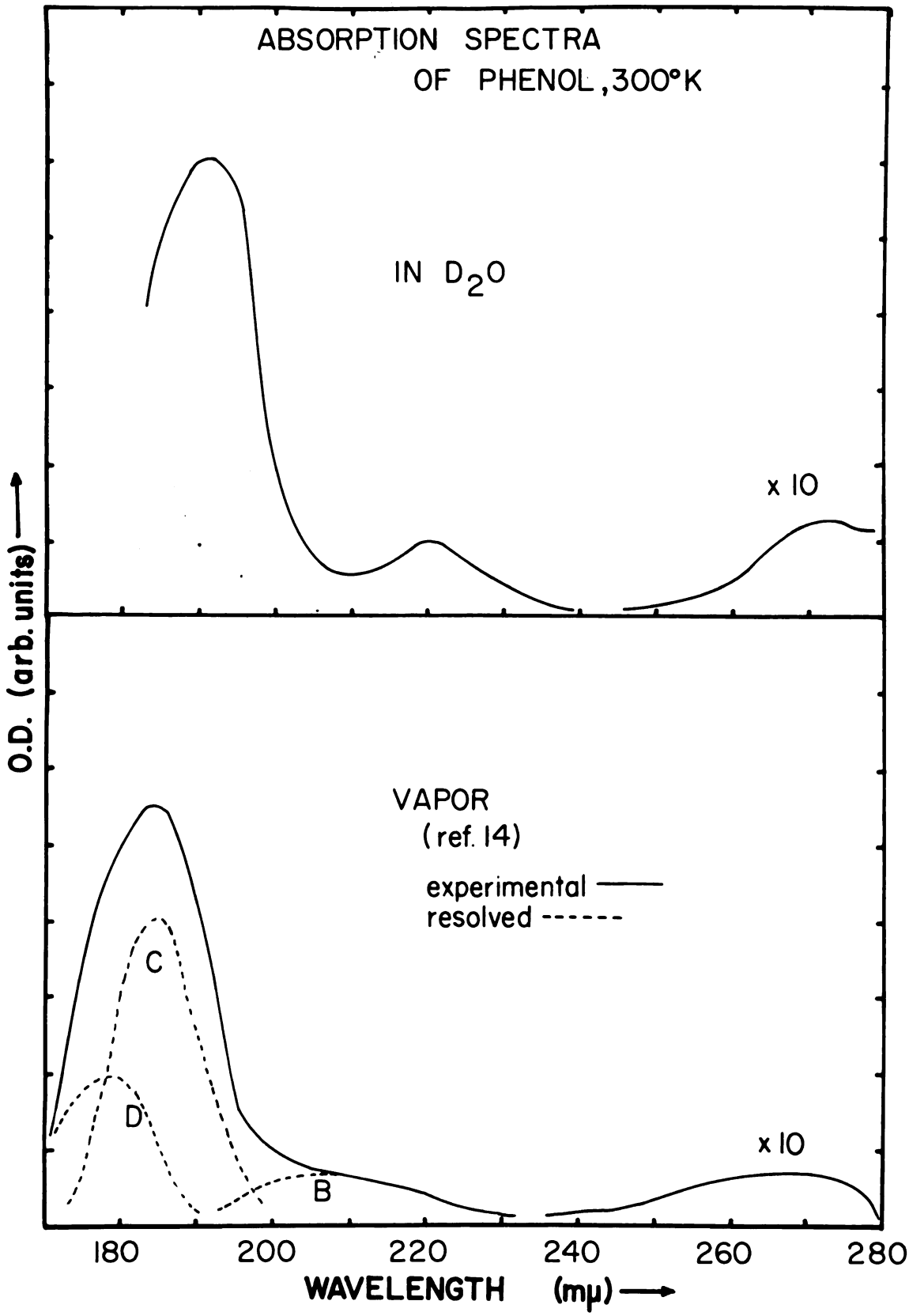


Figure XVIII

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APPENDIX I

The following is an analysis of the surface temperature of a slab of material, one of whose faces is at a fixed low temperature while the other face completely absorbs radiation of flux ϕ (see below).

Let $U(x,t)$ be the temperature as a function of distance x and time t, and K be the thermal conductivity. The heat transfer equation is

$$\frac{\partial U(x,t)}{\partial t} = \frac{K}{C} \cdot \frac{\partial^2 U(x,t)}{\partial x^2}$$

Boundary conditions are

$$U(x_0,t) = U_{x_0}$$

$$\phi = \left[K \cdot \frac{\partial U(x,t)}{\partial x} \right]_{x=x_1}$$

where x_0 and x_1 are the coordinates of the cold surface and irradiated surface respectively, and ϕ has the dimensions of energy per unit area per unit time (54).

After equilibrium is reached, i.e., when $\frac{\partial U}{\partial t}(x,\infty) = 0$, then

$$\frac{\partial^2 U(x,\infty)}{\partial x^2} = 0$$

$$U(x_0,\infty) = U_{x_0}$$

$$\phi = \left[K \cdot \frac{\partial U(x,\infty)}{\partial x} \right]_{x=x_1}$$

If the conduction equation is integrated once and the second boundary conduction is applied, then

$$\frac{\partial U(x,\infty)}{\partial x} = \frac{\phi}{K}$$

Integrating a second time and using the first boundary condition gives

$$U(x,\infty) = \frac{\phi}{K}(x-x_o) + U_{x_o}$$

The thermal conductivity of amino acids is not available, but for practically all organic compounds K is about 4×10^{-4} $\frac{cal \cdot cm}{sec \cdot cm^2 \cdot {}^oC}$ at -80^o C (55).

Now the radiant emittance W for any material is given by

$$W = \epsilon \sigma U^4$$

where ϵ is the emissivity and σ is the Stefan constant.

No value of ϵ for amino acids is available but for some organic materials at 300^oK (i.e., paper and rubber) it is close to unity (55). In addition, for all materials tested ϵ decreases at low temperatures. In the worst instance, the amino acids will behave as black bodies and absorb all heat energy striking the slab. This justifies the original assumption that ϕ is completely absorbed. Sears has shown that if a small black body is isolated within an enclosure the rate of energy gain per unit area is given by $\sigma(U_e^4 - U_{x_1}^4)$ where U_e and U_{x_1} are the temperatures of the enclosure and the surface of the body, respectively (56). The nature of the enclosure is irrelevant. Therefore, the solved heat transfer equation becomes

$$U(x,\infty) = \frac{\sigma(U_e^4 - U_{x_1}^4)}{K}(x-x_o) + U_{x_o}$$

If $U_e > U_{x_1}$ then, as in the present case, $U_e^4 >> U_{x_1}^4$ and U_{x_1} can be

dropped. Because of this, the iterative process that would have been necessary to find $U(x,\infty)$ was not required. Now, $U_e = 300^{\circ}K$ and $\sigma = 1.4 \times 10^{-12}$,

$$\therefore U(x,\infty) = \frac{1.4\times10^{-12}\ \frac{cal}{cm^2\cdot sec}}{4\times10^{-4}\ \frac{cal\cdot cm}{sec\cdot cm^2\cdot {}^{\circ}K}} \cdot .81\times10^{+8}\cdot\Delta x + U_{x_0}$$

$$= 28\ \frac{{}^{\circ}K}{cm}\cdot\Delta x + U_{x_0}$$

In these experiments Δx never exceeds 10^{-2}cm. Therefore, the difference in temperature between the front (irradiated face) and back (in contact with the coldfinger) is about $0.3^{\circ}K$, that is, negligible.